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Membranes and Barriers: Targeted Drug Delivery

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Retrometabolic Approaches to Drug Targeting

Nicholas Bodor

INTRODUCTION

Targeted drug delivery is the most important goal of pharmaceutical research and development. In this context drug targeting is defined in the broadest sense, that is, to optimize a drug's therapeutic index by strictly localizing its pharmacological activity to the site or organ of action. This is an important distinction from the basic targeting concept, where the specific drug receptor is the target and the objective is to improve fit, affinity, and binding to the specific receptor that ultimately will trigger the pharmacological activity. This distinction is made since the overall distribution of many drug receptors does not follow the various diseases. Actually, most of the time, drug toxicity is receptor related and receptor mediated; thus, improving intrinsic drug affinity and activity, as well as receptor binding, does not improve the therapeutic index.

In principle, drug targeting can be achieved by physical, biological, or molecular systems that result in high concentrations of the pharmacologically active agent at the pathophysiologically relevant site. If successful, the result of the targeting would be a significant reduction in drug toxicity, reduction of the drug dose, and increased treatment efficacy. All in all, it is evident that with a biologically active agent of reasonable activity at hand, targeting to the site of action should be superior to molecular manipulations aimed at refining the receptor substrate interactions.

Successful drug targeting, however, is a very complicated problem. It involves affecting the various distributional and rate processes, as well as sometimes the drug metabolism and disposition, as will be shown. There are a number of important parameters to be considered in designing drug targeting of any kind. These include the nature of biological and cellular membranes, distribution and presence of drug receptors, as well as the enzymes responsible for drug metabolism, time-plasma concentration profiles, and local blood flow.

A tremendous amount of work has been concentrated worldwide in the past two decades on the research and development of drugs with improved site-specificity, that is, targeted drug delivery systems. Although the concept of a "magic bullet" has been around for almost 100 years, scientists involved in drug research and discovery simply did not have the basic means and concepts to achieve this elusive goal. The advancement of the drug receptor concept and then specific knowledge about the various receptors suggested that this is the direction that should be taken for drug targeting. However, as mentioned before, the receptors alone, due to their distributional properties, cannot be responsible for selectivity and drug targeting.

There were a number of major steps in drug development that activated the field of drug targeting. These approaches can be classified in two major classes: physical-mechanical and biological. In the first class, the concept of controlled and sustained drug release, thus modification of drug concentration-distribution profile by various physical and/or mechanical delivery systems, provided the impetus to attempt drug targeting. The second, the biological type of drug targeting, started with the recognition and the possible use of monoclonal antibodies. Of course, in all these cases of attempted targeting, the active principles were some known drugs. The potential improvement in this direction, modifying drug delivery using the various physical and biological systems, is limited. The selection of the known, marketed drugs in most cases was made considering the best pharmacokinetic (PK)/dynamic profile. Generally, drug researchers selected drugs with an already relatively long duration of action. The improvement, due to controlling drug delivery, thus in general is limited to avoiding high peak concentrations. It should be stressed that research in drug targeting must divert from the classical approaches, that is, to improve some of the known drugs. The design process for drug targeting should be started at a very early stage of the discovery phase. The required properties of a drug that will successfully be involved in the drug targeting, should be different than those of the "classical" drugs. In the foreseeable future, many drugs will be designed with targeting in mind, and the actual new chemical entity will have site specificity and selectivity built into its molecular structure.

The pharmaceutical literature of the past 10 to 15 years is very rich in publications on the topic of drug targeting. There are books and proceedings of scientific meetings discussing all the various aspects of this very general and important problem. For example, one review

(Tomlison 1987) presented a general approach considering, in a combined form, the disease state and the various delivery parameters including the site of interest, drug retention at the site, duration of the drug effect, the responsiveness of the target tissue, and disposition of the drug. This approach to the problem is very important because in many instances the pathogenesis of the disease was simply ignored in designing drug delivery systems. Developing novel delivery methods, without consideration of disease condition, can lead to meaningless results, and the delivery manipulations will not substantially affect the overall selectivity or efficacy of the drug.

The most meaningful classification of drug targeting is mechanism based. Adding one very important class to the two previously mentioned, the three major categories, based on this scheme, can be defined as (1) physical, (2) biological, and (3) chemical (site-specific or targeting systems).

The first, physical delivery systems, was reviewed thoroughly in the literature (Friend and Pangburn 1987; Poznansky and Juliano 1984). It needs to be emphasized, however, that this class of drug targeting actually started with modifying drug PK; the prime objective was to modify delivery without essentially affecting specificity. The various physical delivery forms, such as biodegradable polymers or osmotic pumps, provide a way to achieve a sustained, quasiconstant blood/tissue concentration of a drug. It was further considered that if a physical delivery device is localized at or around the target organ, some differential distribution can be achieved, as the drug is now targeted to the site. This kind of physical targeting, such as the pilocarpine delivery to the eye from a polymeric device or the contraceptive sustained release of progesterone from a vaginal polymeric insert, has achieved only limited success. A more heroic device approach involving surgical implant to the brain of a polymer disc containing the anticancer drug chloroethyl-cyclohexyl-nitrosourea (CCNU) promises very limited use due to the unfavorable concentration gradient that is achieved using this otherwise highly toxic drug.

The biological targeting systems, based on antibodies, in principle, could result in a highly desirable delivery profile. There are, however, a number of problems related to the actual distribution of an antibody-drug conjugate in the body. To name a few: the potential modification of the specificity of an antibody by conjugation to one or more drug molecules; the problem of stoichiometry, that is, the amount of drug the antibodies

can actually carry; and finally, a very important issue of the actual drug delivery, that is, the enzymatic, timely cleavage of the antibody-drug conjugate. In most cases, this critical process, producing pharmacologically active local concentrations of the drug, is the major problem in achieving any success. But the first two drug targeting classes were extensively reviewed in other papers. This chapter will concentrate on the third, the most flexible, and potentially the most rewarding general class: chemical drug targeting.

DRUG TARGETING BY RETROMETABOLIC DRUG DESIGN APPROACHES

The general information and knowledge accumulated on drug metabolism and on the various enzymes systems performing these transformations makes it evident that drug metabolism can be used in the design process of new drugs. Actually, it is proposed to combine the classical structural activity relationships (SARs) based drug discovery approaches with structural metabolism relationships (SMRs), a combination that is termed "retrometabolic drug design approaches."

Essentially, any molecule introduced in the body will be enzymatically modified. This process is the metabolism, where the underlying main physiological purpose is to get rid of this foreign molecule from the body. The chemical enzymatic transformations taking place on a molecule are actually determined by its structure. The structural features have a direct effect on which enzyme will modify the molecule and which part of the molecule will actually be modified. During these enzymatic processes in the body, following structural changes, not only the physical chemical properties of the initial molecule will be modified but also its biological activity. Many times some of the metabolites become biologically more active or will have different types of pharmacological activity than the original compound. Often some of the oxidative processes producing epoxides and radicals will lead to short-lived, highly reactive intermediates. All these will contribute to the apparent drug toxicity. In addition, production of metabolites with a similar type of activity, but different PK properties, will unfavorably affect desired selectivity or PK properties.

Drug metabolism is unquestionably a very important component of drug research and development. In the current general drug development strategies, however, it generally enters into considerations too late. Thus,

only after a drug reaches more advanced stages in development will the metabolism of the drug be studied in some detail. The basic principle of the retrometabolic drug design approach is that drug metabolism considerations should actually be involved at a very early stage of the design process, certainly not as an afterthought, in order to later explain some of the behaviors of the drug.

Chemical drug delivery systems (CDSs) are defined (Bodor and Brewster 1991) as chemical compounds that are produced by synthetic chemical reactions forming covalent bonds between the drug (D) and specifically designed "carrier" and other moieties. At least one chemical bond needs to be broken for the active component (D) to be released. The release of the active component from the CDS takes place enzymatically. Thus, these are the two basic components for a chemical-enzymatic drug delivery or targeting system. Excluded from this definition, however, are "polymeric prodrugs," and in the strictest sense CDSs are referred to as inactive chemical derivatives of a drug, where one or more chemical modifications have been performed to generate an inactive monomer with improved delivery characteristics. The modifiers used are generally comparable in size or smaller than the derivatized target drug molecule.

The chemical modifications applied in general achieve two goals. One is targeting, and the second relates to optimizing molecular properties related to distribution, disposition, elimination, and activation processes. The most important group introduced in the molecule is a "targetor (Tor) moiety," a group that is responsible for the site targeting, site specificity, and site retention. The Tor moiety replaces the formerly used "carrier" in order to avoid misconceptions and confusion concerning the CDSs. The Tor is a general class of modifying groups that include functionalities that produce targeting by changing molecular properties of the overall molecule, as a result of enzymatic conversion, but also involves pharmacophores, groups that are converted by site-specific enzymes to active functions. Accordingly, the distinction between a Tor and a carrier becomes clear. The carrier is a function, molecule, or macromolecule that takes or carries the drug molecule to some desired target. The term "carrier" implies some kind of specific transport or receptor interaction, while the Tor moiety more correctly describes the intent and the result of the process, that is, to concentrate the drug at the site of action by chemical-enzymatic means.

In addition to the Tor moiety, many times other functions will be introduced in the drug, which can be named as "protector functions"

(F) that serve as lipophilicity modifiers or protectors of certain functional groups in the drug molecule. Thus, a CDS can be defined as a drug modified by one Tor and as many F functions as required. This kind of classification differentiates also the classical prodrugs from the CDS, as prodrugs in general contain one or more F moieties, that is, they are derivatized to enhance overall delivery and introduce modified PK properties, but do not contain Tor functions.

This major class, the CDS, represents one end or extreme of the retrometabolic drug design (figure 1). As will be shown, this concept was successfully used for targeting drugs to specific organs like the brain, or to receptors within the eye.

The other extreme of the retrometabolic drug design concept, resulting in very significant improvement in the therapeutic index, can be considered as a specific case of targeted drug delivery. This approach was generally described as the "soft drug" (SD) design. The concept and specific applications were first introduced formally in 1980 and reviewed subsequently several times. Actually, at a special session of an International Union of Pure and Applied Chemistry-International Union of Pharmacology (IUPAC-IUPHAR) joint meeting in 1981, the SD design concept was presented and debated against the zero metabolism drug design (hard drug) concept of Ariens (de Winter 1982). This public debate has dramatically contributed to increased use and consideration of involving drug metabolism into the design of new drugs. The common feature between the SD and the CDS is that both are based on strategic chemical modifications of a lead molecule and on enzymatic conversions to fulfill their therapeutic drug targeting role (Bodor 1992, pp. 35-44). The main difference, however, is that while the CDS by definition is inactive and requires sequential enzymatic reactions to provide for target and/or site activation, the SDs are active biological agents, but they are deactivated in a predictable and controllable way, after they achieve their therapeutic role (Bodor and Kaminski 1980; Bodor et al. 1980a, 1982). In general, SDs behave very differently from the traditional pharmaceuticals (see figure 1). Drugs generally undergo complex, multiple metabolic conversions to analog metabolites $M_1...M_n$ and reactive intermediates $I^*_1...I^*_n$. The main point is that by design SDs simplify the transformation-distribution-activity profile that the specific drug otherwise exhibits. SDs are active as such, and consequently they will produce the desired pharmacological activity at the site of application, but a predicted facile enzymatic process will metabolically deactivate the SD in a one-step process to an inactive species (M,). This

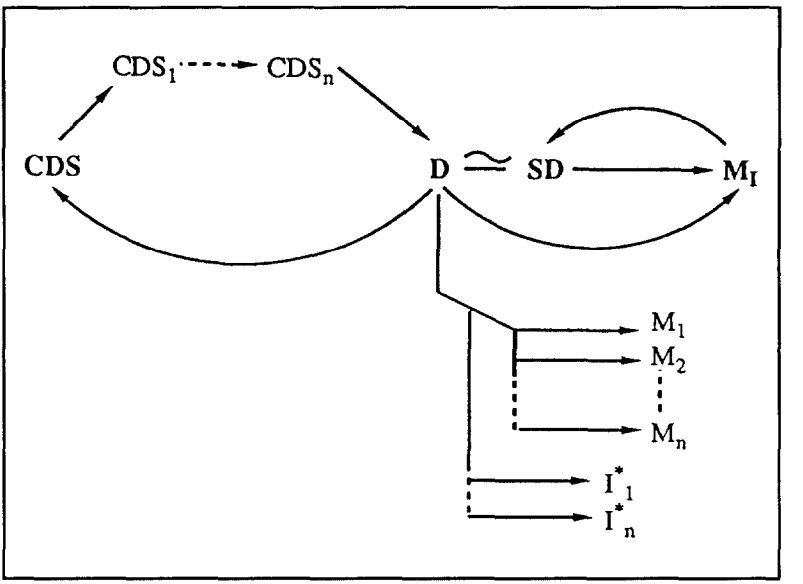

FIGURE 1. *The retrometabolic drug design loop. A drug (D) can be converted to an inactive CDS, which is strategically activated to target the D. Alternately, of the multiple metabolic products M_1-M_n, an inactive metabolite (M_1) is selected, which is the basis for the SD design.*

very property can then be used to enhance drug targeting. Thus, when applied at the desired site of action, be it topical or internal, the SD elicits the desired pharmacological effect locally, but as soon as it is distributed from the site, it is susceptible to the deactivation designed into the structure, thereby preventing unwanted side effects or unwanted characteristic effects at other sites in the body. While the SD, by virtue of its affinity, can interact with the receptors at the site of action, due to its metabolism, it will not be able to activate the same kind of receptors at other parts of the body. Using the SD concept, very significant distributional differences can be generated for a drug by involving drug metabolism in a retroactive manner in the design. This is clearly the opposite of the enzymatically based CDSs, where local concentrations of the active form of a drug are produced by sequential enzymatic conversion of the CDS at the desired site. Ideally, in extreme cases the CDS would be activated to a drug only at the site of action and nowhere else in the body, while in the SD case, the drug will be present as

introduced at the site of action, but will be completely absent from the rest of the body due to the enzymatic deactivation in all nontarget sites.

DRUG TARGETING BY CHEMICAL DELIVERY SYSTEMS

There are a wide variety of CDSs possible both theoretically and in practice. For convenience, the major CDSs can be divided into three classes:

1. Enzymatic physical-chemical-based targeting,
2. Site-specific enzyme-activated targeting, and
3. Receptor-based chemical targeting.

In the enzymatic physical-chemical-based CDS, the target drug (D) is chemically (either directly or indirectly) converted into an inactive analog that is designed based on expected sequential enzymatic conversion of this CDS to the drug. The synthetic modifications of the drug involve coupling it with the strategically selected protective functions (Fs) and the Tor, yielding the CDS. At this stage, the overall physical-chemical properties, as well as solubility and distribution properties, are optimized to allow facile distribution of the CDS throughout the body. In general, the Tor function is rather specific and restricted. However, the modifiers can be used freely to optimize the structure. Some functions serve to protect sensitive groups, or actually the pharmacophore, to ensure the inactive nature of the CDS. After administration and distribution throughout the body, the system can be formally separated into the target side (s) and the rest of the body (r).

Metabolizing enzymes occur in many different tissues and organs; thus, the same kind of metabolic conversions of the CDS will take place to some extent throughout the body. Accordingly, as shown in figure 2, predicted metabolism of the Tor$\rightarrow$T^1 (the appropriately modified Tor structure) and sequential metabolism resulting in removal of the protected functions will take place. The most important metabolic conversion involves modification of the Tor which, in this case, will dramatically alter the solubility and distribution properties of the molecule, thus amplifying the effect of various biological barriers. This stepwise modification of solubility properties in general takes place in the direction of increasing hydrophilicity, that is, an original lipophilic CDS is converted into hydrophilic intermediates that will prevent passage of certain biological barriers. On the other hand, when formed in the periphery, this process will accelerate elimination of this still inactive

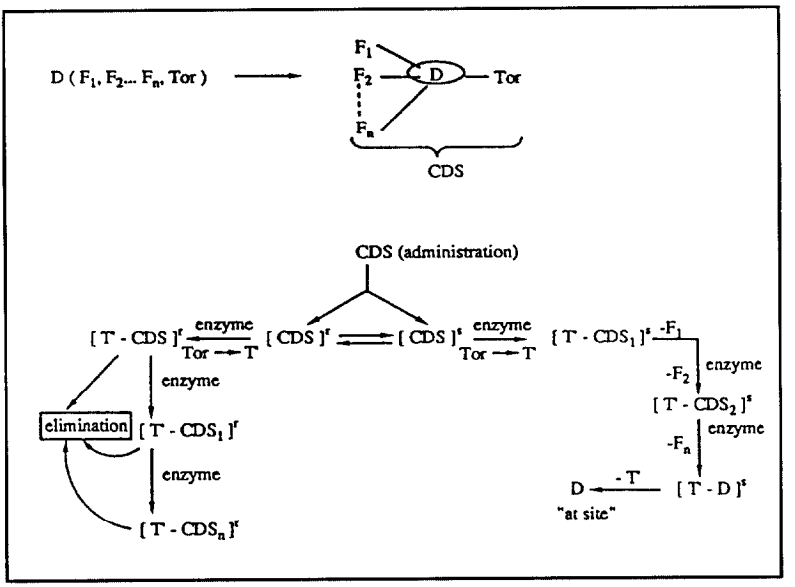

FIGURE 2. *Enzymatic physical-chemical-based CDS.*

drug precursor. The resulting differential distribution, by design, will lead to accumulation of certain drug precursors at the site of drug action.

The final drug-releasing enzymatic activation process completes the function of the CDS. For example, if the blood-brain barrier (BBB) is considered a biological membrane that is permeable to most lipophilic compounds but does not allow hydrophilic molecules to get across, it is logical to assume that these criteria for passive transport apply in most cases to both sides of the barrier. It is well recognized that many times neurotransmitters synthesized in the brain are not going to be easily excreted to the blood, while by introducing the same neurotransmitter in the general circulatory system it will not reach the brain. This is the case for dopamine and other biogenic amines. To go further, if a lipophilic molecule that can pass the BBB would be converted in situ in the brain into a hydrophilic one, it could not come out; it will be locked in. It would be best if this kind of process would take place primarily in the brain. However. the brain is not that different enzymatically from the rest of the body. Fortunately, this kind of specificity is not even desirable. Actually, it is advantageous for this conversion from a lipophilic to hydrophilic molecule to take place everywhere in the body. The original lipophilic inactive CDS, after overall distribution, is converted to a hydrophilic one in the whole body, which will actually accelerate

peripheral elimination and further contribute to brain targeting. A general system of this kind was developed some years ago based on a 1-alkyl-1,4-dihydronicotinate ⇄ quaternary nicotinate system. Accordingly, here the Tor represented as T-T' (see figure 3) is this redox system that has two forms, the lipophilic 1,4-dihydro and the hydrophilic quaternary pyridinium form.

This specific redox targeting system is very close to the ubiquitous NAD^+-NADH system (see figure 3), and thus the conversion involving the hydride transfer will take place everywhere in the body. The resulting charged Tor^+-CDS is locked in the brain while it is easily eliminated from the body due to the acquired positive charge. After a relatively short time, the still inactive locked-in D-Tor^+ is present essentially only in the brain, providing a sustained brain-specific release of the active drug.

Figure 4 shows a simplified version of this process. Since its first use (Bodor and Farag 1983; Bodor et al. 1981). this and analogous systems were used for a wide variety of drug classes (Bodor and Brewster 1981, 1991, pp. 231-284). These include steroid hormones (Bodor et al. 1987; Brewster et al. 1988), anti-infective agents (Pop et al. 1989a, 1989b), anticancer agents (Raghavan et al. 1987; Rand et al. 1986), antiretroviral agents (Gogu et al. 1989; Little et al. 1990; Palomino et al. 1989; Torrence et al. 1988) and many others. Most recently, successful brain delivery of enkephalin using a modified, advanced system called molecular packaging was reported (Bodor et al. 1992). A further extension involving an important role for the modifiers was recently published (Prokai et al. 1994), where a thyrotropic-releasing hormone (TRH) analog was successfully delivered and activated in the brain. The work related to the use of these methods was recently reviewed, including a review in a forthcoming National Institute on Drug Abuse (NIDA) monograph (Bodor 1993).

The second type of CDS is very different. It is based on enzymatic conversions of the strategically designed CDS only at the site of action, a result of significant differential distribution of certain enzymes within the body. If specific enzymes are present only at or around the site of action, their use will lead to high site specificity. Accordingly, a dramatic separation between the desired pharmacological activity and unwanted toxicity can be achieved. These chemical delivery systems are simplistically described in figure 5.

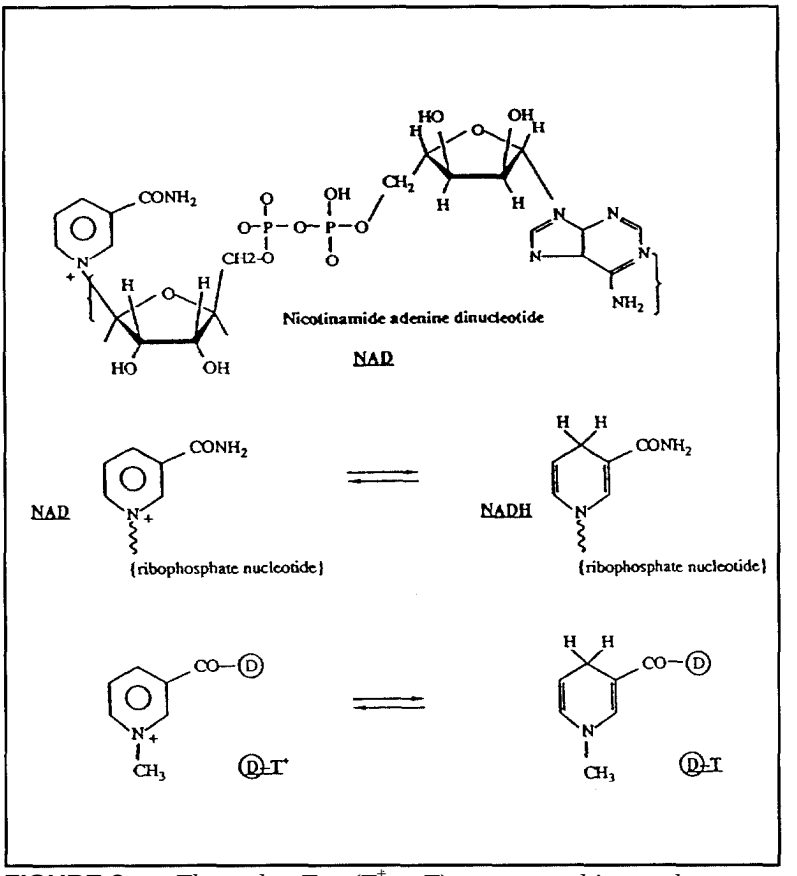

FIGURE 3. *The redox Tor ($T^+ \rightleftarrows T$) system and its analogy to the ubiquitous $NAD^+ \rightleftarrows$ system. The structure of NAD^+ is given and the reduced NADH is illustrated. The same quaternary nicotinic acid derivative (T^+) and the corresponding 1,4-dihydropyridine containing Tare coupled, respectively, to the drug (D).*

The drug, again directly or indirectly, is converted into a CDS, obviously also involving the pharmacophore. When introduced into the general circulatory system, the properly designed CDS will not be activated to the drug. Either directly or indirectly, it will be eliminated without producing any pharmacological effect. After distribution to the site of action, however, it will be converted by the enzymes present there, thus yielding the active drug only at the site. This concept was successfully used within the eye for the site- and stereospecific delivery of intraocular

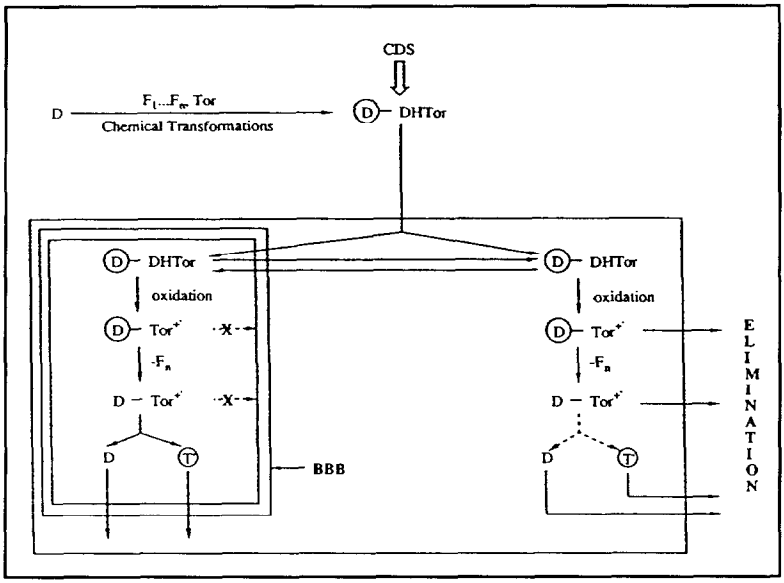

FIGURE 4. *The brain targeting I,4-dihydropyridine (Tor-) quaternary pyridinium (Tor$^+$-) system.*

pressure (IOP)-reducing ß-adrenergic blocking agents (Bodor 1989, pp. 145-164: Bodor et al. 1988; El-Koussi and Bodor 1989). Here, the Tor directly involves the pharmacophore, which is recognized to be the p-amino alcohol part of the molecule. The CDS corresponding to the P-blockers contains a p-amino oxime function. The oxime or alkyloxime derivative was found to be enzymatically hydrolyzed within the eye by enzymes located in the iris-ciliary body, and subsequently reductive enzymes found in the iris-ciliary body will produce only the active stereoisomer (Bodor and Prokai 1990) of the P-blocker.

This was found to be a general approach, and a variety of P-blockers such as oxime analogs of alprenolol, propranolol, betaxolol, and others, were shown to undergo the predicted specific activation within and only within the eye (see figure 6). Even intravenous (IV) administration of these oximes will not produce the active ß-blockers metabolically; hence, they are void of any cardiovascular activity.

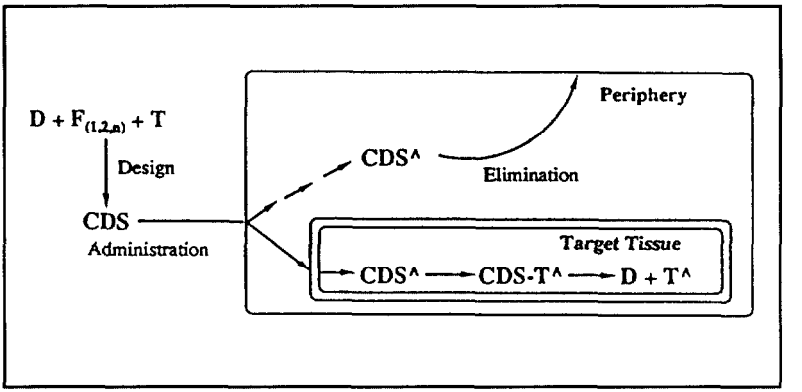

FIGURE 5. *Specific enzymes at the target organ activate the CDS to drug (D) only at the site. In the periphery or rest of the body, D is not formed due to the lack of activating enzymes or unfavorable rate processes.*

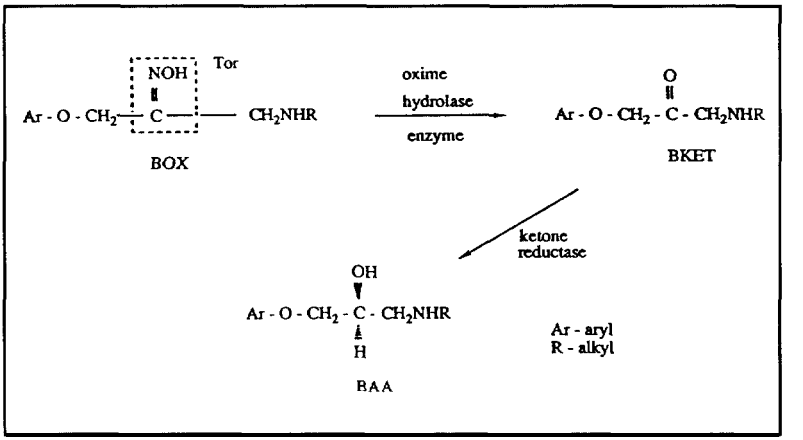

FIGURE 6. *The ß-adrenergic antagonist (BAA) is formed eye- and sterospecifically through a sequence of enzymatic conversions of the corresponding inactive oxime (BOX) and the intermediate inactive ketone (BKET).*

DRUG TARGETING BY SOFT DRUGS

As was shown earlier, SDs are novel active drugs strategically designed to undergo singular metabolic deactivation after they achieve their therapeutic roles. In many instances, these kinds of compounds are ideal for producing specific action at the site of application without affecting the rest of the body. In this reversed targeting method using SDs, very significant distributional differences for a drug can be generated by involving drug metabolism in a retroactive manner in the design. As shown earlier, this method is opposite to the enzymatically based CDSs where local concentration of the active form of the drug is produced by strategic distributional differences produced by the CDS followed by release of the active component based on enzymatic reactions by design.

There are a number of important local sites where application of a drug can be achieved very easily, for example, the eye, the skin, major parts and compartments of the gastrointestinal tract, and the lungs. Local application of a drug to these sites can easily be achieved, and an SD then can produce the desired pharmacological activity at the site of application.

As in the case of CDS technology, a number of SD categories have been defined (Bodor 1982, pp. 137-164; 1983, pp. 217-251; 1984a, pp. 255-331; 1984b) as follows: soft analogs, activated soft compounds, active metabolite types, controlled release of endogenous soft compounds, and the inactive metabolite approach.

Soft analogs are close structural derivatives of known drugs or bioactive compounds that have been designed with a specific metabolic weak spot in their structure. This design allows for one-step deactivation to nontoxic components. A simple example (figure 7) is provided by analogs of the antimicrobial agent cetyl pyridinium chloride (structure **1**), which is used in mouthwash. An isosteric acyloxyalkyl pyridinium salt (structure 2) can readily hydrolyze subsequent to exerting high-contact germicidal activity locally. Unlike the cetyl pyridinium chloride, which needs to undergo oxidative metabolic deactivation, the soft analog will be metabolized easily by esterases, which ultimately destroy in one step both the quaternary head and the long chain that are together responsible for the surface-active properties and antimicrobial activity of cetyl pyridinium chloride. The bottom line is that, while the soft analogs are

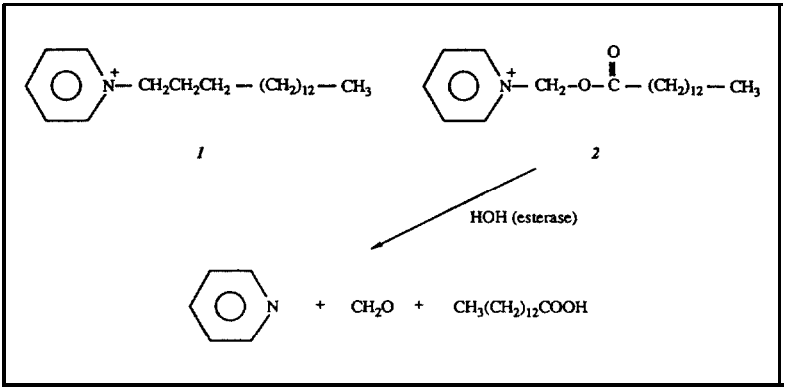

FIGURE 7. *The soft analog 2 of 1 is easily deactivated hydrolically after exerting its antimicrobial role.*

active germicidal agents, they are 10 to 40 times less toxic than cetyl pyridinium chloride as expressed by their relative median lethal dose (LD_{50})

In the second class, the activated soft compounds are not analogs of known drugs but are derived from nontoxic chemical compounds that are activated by introduction of a specific pharmacophore. During expression of activity, the added group subsequently will lose activity while the inactive starting molecule is regenerated. An example of an activated soft compound is provided by N-chloromine antimicrobials. These compounds (figure 8), particularly ones that derive from a-di-substituted amino acid esters and amides (Kaminski et al. 1976a, 1976b), serve as a source of positive chlorine. However, as soon as the chlorine is lost, the nontoxic initial amine is regenerated.

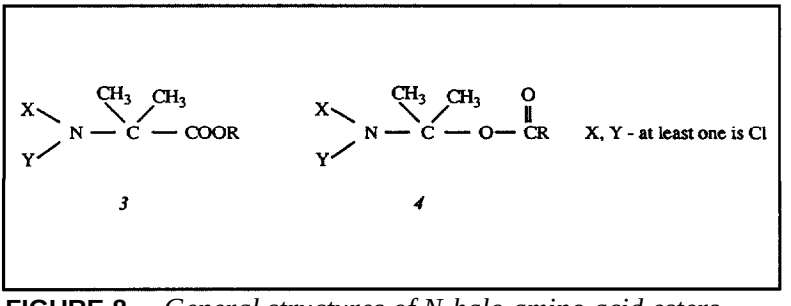

FIGURE 8. *General structures of N-halo amino acid esters (R-alkyl, aryl) (3) and the corresponding N-halo amino alcohol "reverse" esters. 4.*

The third class, active metabolites, refers to compounds or metabolic products of a drug that retain significant activity of the same kind as the parent drug. Judicious selection of an active metabolite can yield a potent drug that will undergo a one-step deactivation process, since it is already at the highest oxidation state. In other words, if sequential oxidative metabolic conversion of a drug takes place, such as the quite common hydroxyalkyl →oxo→carboxy sequence in which the carboxy function is generally the inactive form, some of the previous oxidative metabolites (preferably the one just before deactivation) could be the best choice for a drug. If the various intermediate products are active drugs, they all will have different distributional and PK properties. At any given time, a mixture of active components thus will be present, the relative concentration of which will be changing throughout their presence, providing an almost uncontrollable situation. A case of this kind is provided in figure 9 by the P-blocker bufuralol (structure 5), which undergoes stepwise oxidation in humans, and to corresponding hydroxy (structure 6) and keto (structure 7) intermediates, which have different (interestingly, longer) elimination half-lives, thus providing a mixture of the active components (Bodor 1984a). Applying the principle of active metabolites, the ketone (structure 7), the highest active oxidative metabolite, should be the drug of choice, which will then be deactivated by oxidation to structure 8.

The controlled-release endogenous agents are derivatives of naturally occurring hormones and other biologically active agents like neurotransmitters. These compounds (e.g., hydrocortisone) have a well-developed mechanism for disposition and can therefore be considered natural SDs. As shown in figure 10, applying sustained-release chemistry, such as the spirothiazolidine function, will provide a chemical-sustained release at the site of application of the naturally SD hydrocortisone (HC). The opening of the spirothiazolidine ring in structure 9 (figure 10) will allow trapping the steroid at the site of application, and the following hydrolysis (structure 10) will release the active component HC only locally (Bodor et al. 1982).

The most developed of the SDs are those based on the inactive metabolites. In principle, this approach requires a survey of the metabolic disposition of a drug that is targeted for further design. An inactive metabolite of the drug is selected which is either a known or conceptualized form based on researchers' knowledge of metabolism. This inactive metabolite is then reactivated synthetically by forming an isosteric/isoelectronic analog of the parent drug. Most importantly, this

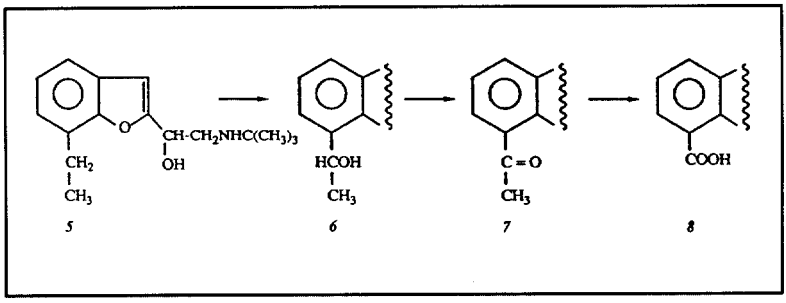

FIGURE 9. *The structures of bufuralol (5) and its stepwise oxidative metabolism to the active metabolites 6 and 7, finally yielding the inactive acid 8.*

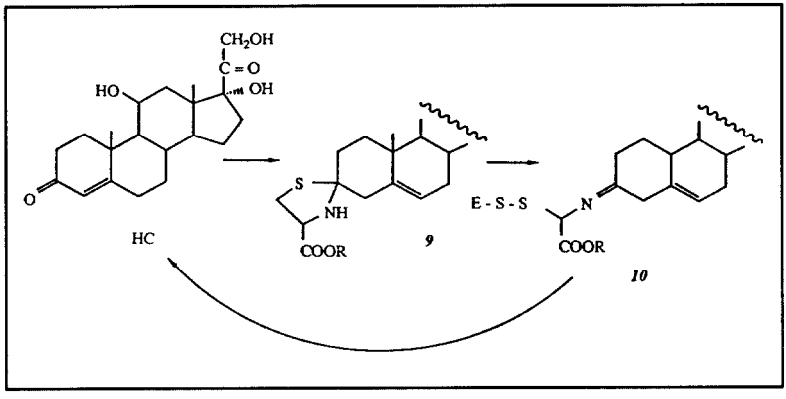

FIGURE 10. *Hydrocortisone (HC) is chemically converted to the inactive spirothiazolidine (9), which after topical activation and opening of the thiazolidine ring will bind to endogenous (E) thiols as a disulfide (10). This protein-bound form serves as a sustained-release form locally for the active hydrocortisone.*

activation step is accomplished so that the new compound can be readily deactivated by a single, predictable step yielding the very starting inactive metabolite. If necessary, other modifications are made to the molecule to optimize transport and receptor-binding properties. The result of this approach is a derivative that interacts with its receptor at the site of application but is readily deactivated, usually by esterases, once outside the place of application. Numerous examples are available, including a number of ophthalmic, dermatological, and other products.

The flexibility and the potential of the SD design is clearly illustrated by the two entirely different classes of soft anticholinergics. As shown in figure 11, certain anticholinergics based on the soft analog concept were first designed (Bodor et al. 1980b), where the basis was not acetylcholine (structure 11), but acetylnorcholine (structure 12). The analogs derived from acetylcholine can be called "hard" anticholinergics, which are generally tertiary or quaternary amino alcohol esters of bulky, hindered carboxylic acids. In the hard anticholinergics of this kind, the amino alcohols used in the structures have at least two and sometimes three carbon atoms separating the alcohol (ester) function from the nitrogen. The corresponding quaternary anticholinergics of this kind would be hydrolyzed to the corresponding quaternary amino alcohol. The chemical and enzymatic hydrolysis of these kinds of compounds in vivo is rather slow, and thus anticholinergics introduced in the body will exert their multiple receptor-based effect for a long time and essentially everywhere in the body. Accordingly, if using an anticholinergic for ophthalmic purposes or as an antiulcer drug, the amount that is absorbed in the general circulatory system will cause systemic anticholinergic or antimuscarinic activity such as blurred vision, dry mouth, and generalized antisecretory activity. Soft analogs of the acetylnorcholine type were designed to have only one carbon atom separating the ester function and the quaternary nitrogen.

These activated, soft anticholinergics (see structure 13, figure 11) undergo facile hydrolytic cleavage where the quaternary head will be converted to a tertiary amine simultaneously with the hydrolysis of the ester function (similar to the soft antimicrobial shown in structure 2, figure 7). Detailed studies of this kind demonstrated that indeed the rate of hydrolysis is at such a level that systemically active concentration of the anticholinergics cannot be achieved when the drug is administered orally or applied to the skin. More recently, a new type of anticholinergic class was developed (Bodor et al. 1990; Kumar et al. 1993a, 1993b, 1993c, 1993d) based on the inactive metabolite approach. Here the lead compound was atropine or the closely related scopolamine. The benzylic hydroxy function in atropine (structure 14, figure 12) can be oxidized to the corresponding carboxylic acid, which is the inactive metabolite (structure 15, figure 12). Esterification of this carboxy function yields the inactive metabolite-based soft anticholinergics (structure 16, figure 12).

Indeed, soft anticholinergics of this kind showed good intrinsic activity, with antispasmodic pA_2 values up to 7.85 as compared with 8.29 for atropine. However, the systemic in vivo activities were found to be much

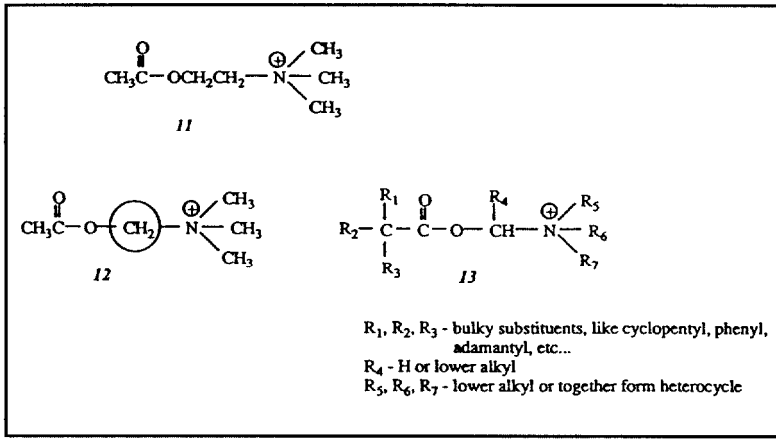

FIGURE 11. *Structures of acetylcholine (II), acetylnorcholine (12), and the soft anticholinergics 13 derived from 12.*

shorter in duration than that of the hard atropine. Accordingly, when equipotent mydriatic concentrations of atropine and the corresponding quaternary soft analog (structure 17, figure 12) were compared, while having the same maximal mydriasis, the area under the curve (mydriasis versus time) was only 11 to 19 percent that of atropine (Hammer et al. 1988, 1991). This is consistent with the facile hydrolytic deactivation of the soft analog. Similarly, the cardiovascular activity of compound 17 (structure 17, figure 12) showed ultrashort duration. The effect of compound 17 on the heart rate and its ability to antagonize the cholinergic cardiac depressant action induced by acetylcholine injection or by electrical vagus stimulation was determined in comparison with atropine methyl nitrate (structure 18, figure 13). It was found that a dose of 1 mg/kg was able to completely abolish the bradycardia induced by acetylcholine injection or by electrical vagus stimulation for more than 2 hours following IV injection. On the other hand, similar doses of compound 17 exerted muscarinic activity for only 1 to 3 minutes following IV injection. Increasing the dose of compound 17 tenfold to 10 mg/kg, which is essentially more than equipotent concentration, did not lead to any significant prolongation of the duration of the anticholinergic activity of this compound. These results provided further support for the ultrashort duration of action and thus the possible safe topical use of these agents as muscarinic drugs. The ultrashort systemic activity provides the basis for their successful SD-based targeting.

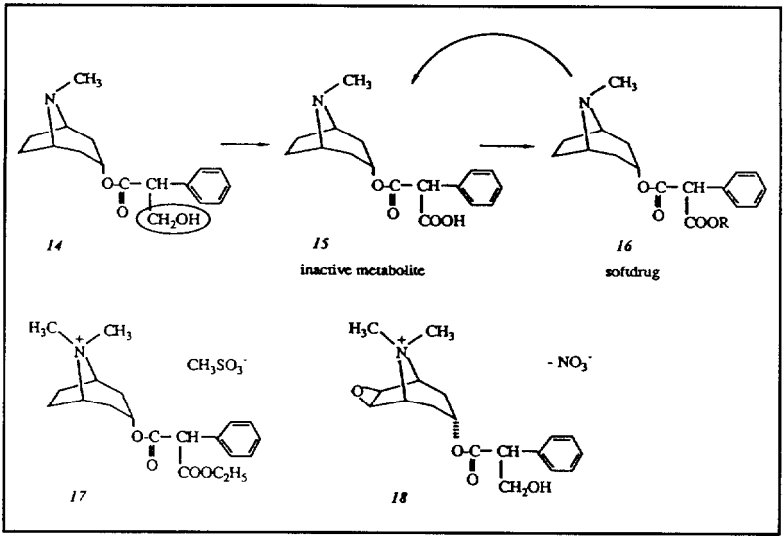

FIGURE 12. *The structures of atropine (14), its assumed inactive acidic metabolite 15, and the corresponding general SD 16 (R-alkyl). The N-quaternary SDs are exemplified by the ether ester 17. Similar SDs were obtained from scopolamine methyl nitrate (18).*

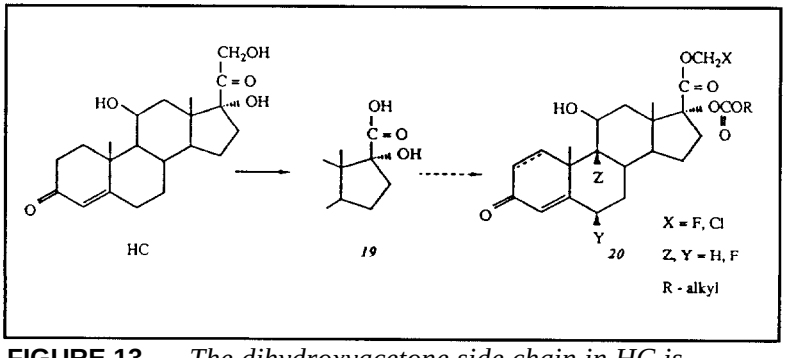

FIGURE 13. *The dihydroxyacetone side chain in HC is oxidatively degraded to the cortienic acid 19, which serves as the lead compound for a class of soft corticosteroids represented by the general structure* ***20.***

Another class of SDs extensively developed involves soft corticosteroids for ophthalmic and other topical uses. Topical corticosteroids are the most commonly used form of anti-inflammatory therapy for the eye. These are used in a variety of inflammatory conditions resulting from surgery, injury, allergy, or infections in the eye and cause severe discomfort to the sufferer. Topical and systemic corticosteroids, however, are associated with a number of problems. Some, like immunosuppression, are due to systemic activity. Others are local effects, like inhibition of wound healing by slowing cell growth replication. One of the most severe side effects of topical and systemic corticosteroids in ophthalmic use in particular is that they cause glaucoma. Corticosteroids cause elevation of the IOP within a relatively short time, and in steroid-sensitive people even short-time usage will cause prohibitive elevation in the IOP.

This author previously reported successful application of the inactive metabolite approach to develop locally potent, but systemically safe soft corticosteroids (Bodor 1982, 1985, pp. 111-127; 1988, pp. 13-25). As shown in figure 13, these are based on the acidic inactive metabolites formed after metabolic degradation of the 17ß-hydroxy ketone side chain, which in the case of hydrocortisone (HC) (figure 13) is the corresponding cortienic acid (structure 19, figure 13). Appropriate substitution of the 17α-OH and 17ß-COOH groups led to highly potent corticosteroids (structure 20, figure 13), which showed orders of magnitude fewer side effects, including thymolytic activity and adrenosuppression, after all routes of application (subcutaneous, oral, or topical). A unique structural feature introduced in these new soft steroids is the use of 17α-carbonate or ether functions. Unlike the 17α-esters, which could undergo formation of mixed anhydrides and consequent reactivity with cellular components, the carbonates or the ethers are resistant to these kinds of transformations. That is, ultimately the 17α-substituted cortienic acids are the inactive metabolites formed after hydrolysis of the ester function in the 17ß-position.

Over 120 of these soft steroids have been made and tested and their relative receptor-binding activity was established (Bodor 1993) using corticosteroid receptors from rat lung cytosol. It was found that even the nonfluorinated derivatives having a fluoromethyl or chloromethyl ester and various carbonates in the 17α-position possess very good receptor-binding activity, comparable to potent steroids such as betamethasone valerate. On the other hand, when comparing the relative potency of anti-inflammatory activity in the antigranuloma test versus thymus inhibition

(toxicity) on subcutaneously introduced drugs, the corresponding therapeutic indices are sigfificantly improved being placed between 20 to even thousandfold as compared with the "hard" corticosteroids. Interestingly, and as expected, the various hard corticosteroids, regardless of being weak, medium, or highly potent compounds, have about the same therapeutic index. That is, an increase in their intrinsic activity is paralleled by an increase in their toxicity. A significant separation of the activity-toxicity could be achieved by the soft corticosteroids. One of these, loteprednol etabonate (LE) (structure 20, figure 13) 20-Δ'; X = Cl; Y, Z = H; R = C_2H_5), was selected for development as an ophthalmic corticosteroid. It was shown in rabbits that after topical application, the IOP was not affected by LE while equipotent doses of dexamethasone did result in significant IOP elevation (Bodor et al. 1992). More importantly, results on a large number of human subjects indicated that even steroid-sensitive people can be treated with this soft corticosteroid for 4 to 6 weeks without any significant elevation in their IOP.

It is evident that the soft steroids offer significant advantages over the conventional steroids, particularly for local topical use.

SD design was successfully applied in several other classes of compounds, including ß-adrenergic blocking agents, prostaglandins, and psychotropic compounds.

CONCLUSION

A number of novel metabolic-based drug design approaches have been described here. The particular advantage of these approaches is to enhance, sometimes very significantly, drug targeting to the site of action. The two major classes reviewed, the CDSs and the SDs, are opposite in terms of how they achieve the drug targeting role. One commonality, of course, is the basic concept of the designed metabolism controlling the drug action and targeting. In the case of CDSs, the drug is designed to be inactive and to undergo strategic enzymatic activation in order to essentially provide the drug only at the site of action. Delivery of this kind can be achieved to the brain, to the eye, and to other organs such as the lungs. On the other side, the SDs are intrinsically potent new drugs that are strategically deactivated after they achieve their therapeutic role. These approaches are general in nature and can essentially be applied to all drug classes. Since these approaches are based on specific design

rules, computerized programs and expert systems were developed for their application.

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Vector-Mediated Delivery of Opioid Peptides to the Brain

Ulrich Bickel and William M. Pardridge

INTRODUCTION

The development of peptide-based opioid analogs toward clinically useful drugs is still impeded by the limited access of these substances to the central nervous system (CNS) following peripheral or systemic administration. Much has been learned about the physiology and pharmacology of opioid-dependent mechanisms of analgesia (Pasternak 1993) from the isolation of a multitude of endogenous opioid peptides as well as from the development of numerous synthetic peptide analogs (Knapp et al. 1990; Schiller 1991, pp. 180-197). However, the importance of pharmacokinetic (PK) aspects in developing clinically applicable peptide pharmaceuticals, particularly the problem of delivery across the blood-brain barrier (BBB) for central nervous action, has only recently been realized (Bickel et al. 1993a). Various strategies are evaluated for overcoming the cerebrovascular endothelium, which represents the morphological substrate of the BBB in vivo (Brightman 1977). Invasive delivery strategies include neurosurgical approaches such as intraventricular or intraparenchymal infusion and temporary BBB disruption by osmotic or chemical mechanisms via the intra-arterial route (i.e., catheterization of the internal carotid artery). Pharmacological strategies include lipidization and encapsulation in liposomes. The purpose of the current chapter is to highlight the progress that has been made recently with a physiological approach to brain drug delivery of peptides and protein drugs, namely vector-mediated transport at the BBB.

DEVELOPMENT OF CHIMERIC PEPTIDES

The principle of the chimeric peptide delivery strategy lies in the coupling of a nontransportable peptide pharmaceutical to a transportable peptide or protein, which undergoes receptor-mediated or absorptive-mediated transcytosis through the BBB (Bickel et al. 1993a). Binding of the vector to its receptor on the lumenal surface of brain capillary endothelial cells initiates endocytosis. Following exocytosis at the ablumenal plasma membrane and release into brain interstitial space, the

pharmacologically active moiety of the chimeric peptide may be released by enzymatic cleavage if a cleavable linkage between the vector and the drug is employed. The free peptide drug would then be able to interact with its specific target receptor on brain cells. A covalent conjugate of cationized albumin and the opioid peptide D-Ala-ß-endorphin (DABE) was the first example of a chimeric opioid peptide to be investigated in vitro (Kumagai et al. 1987) and in vivo (Pardridge et al. 1990) with regard to its transport at the BBB. This chimeric peptide was linked by the disulfide-based cross-linking reagent, N-succinimidyl-3-(2-pyridyl-dithio)propionate (SPDP). In tracer studies where the chimeric peptide was labeled with ^{125}I or [^{3}H] at the ß-endorphin moiety, both the brain uptake in vivo and the presence of disulfide-reducing enzymatic activity in rat brain homogenate could be demonstrated (Pardridge 1992, pp. 153-168).

The model, DABE-cationized albumin, had limitations regarding its usefulness for testing analgesic effects. Among these were the low overall yield of the coupling and purification, which would impede the production of larger amounts for in vivo testing. Moreover, the coupling involved the N-terminal a-amino group of Tyr in DABE, which is known to be crucial for the bioactivity of opioid peptides (Bewley and Li 1983). Therefore, DABE would not be bioactive, even following cleavage from the chimeric peptide, because a mercaptopropionate group remains attached to the N-terminal a-amino group. Recently, substantial progress has been made in the areas of vector development and coupling of the chimeric peptide. This is exemplified by the demonstration of in vivo pharmacologic effects in brain by a chimeric peptide consisting of a vasoactive intestinal peptide analog (VIPa) (nontransportable pharmaceutical) and a covalent conjugate of an antitransferrin receptor monoclonal antibody and avidin vector (see figure 1, upper left panel). The vector used in this study was the mouse monoclonal antibody OX26 (Jefferies et al. 1984). Transferrin receptors are abundant at the mammalian BBB (Jefferies et al. 1984; Pardridge et al. 1987) and probably are involved in the transport of transferrin from plasma into brain tissue (Fishman et al. 1987; Skarlatos and Pardridge 1994). It has been demonstrated that OX26 is transcytosed at the BBB (Friden et al. 1991; Pardridge et al. 1991) and can deliver peptides as large as the 64 kD avidin (Yoshikawa and Pardridge 1992) or the 26 kD nerve growth factor (Friden et al. 1993).

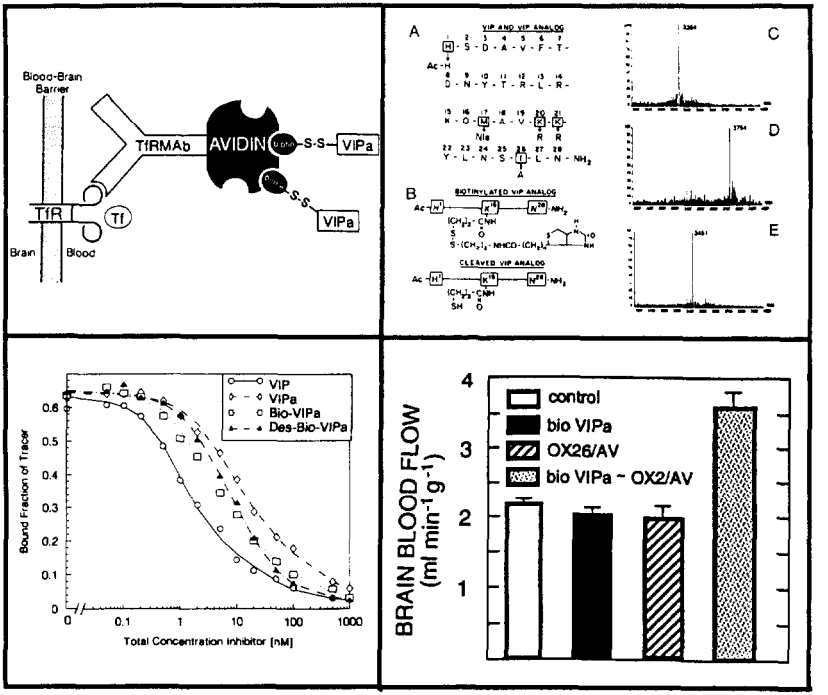

FIGURE 1. *(Upper left) Principle of chimeric drug delivery through the BBB using an antitransferrin receptor monoclonal antibody (TfCRMAb) conjugated to avidin covalently. The antibody targets BBB transferrin receptors at a site distinct from transferrin (Tf) binding and undergoes transcytosis through brain capillary endothelial cells. The disulfide bridge between biotin and the peptide (e.g., VIPa) is cleavable. (Upper right) Sequence of native VIP and amino acid substitutions in the VIPa (arrows in A). (B) shows the structure of the biotinylated and desbiotinylated VIPa. Panels C, D, and E show the mass signals obtained in FAB-MS of the VIPa, biotinylated VIPa, and desbiotinylated VIPa, respectively. (Lower left) Competition curves obtained in a radioreceptor assay with ^{125}I-labeled native VIP. (Lower right) In vivo pharmacologic effect on brain blood flow in anesthetized rats of the biotinylated VIPa. The chimeric peptide (bioVIPa-AV/OX26) was given at a dose of 12 µg/kg as an intracarotid artery infusion (0.18 mL/min over 10 min). Corresponding doses of bioVIPa or AV/OX26 given separately had no effect on control brain blood flow (physiologic buffer infusion).*

SOURCE: Adapted from Bickel et al. (1993b).

Because a good brain delivery vector should be applicable for the delivery of many different peptide pharmaceuticals, it appeared desirable to develop a linker strategy that eliminates the need to modify and optimize the chemical synthesis of the chimeric peptide in each individual case. Instead, a more universal linker strategy should be employed, such as the broadly used avidin-biotin technology. A covalent conjugate of the OX26 antibody and avidin was obtained by mixing the antibody, which was activated with m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and avidin, which was thiolated with Traut's reagent (Yoshikawa and Pardridge 1992). Purification of the conjugate by size exclusion fast protein liquid chromatography yielded a peak with a 1: 1 molar ratio of avidin to OX26. This vector construct can now bind any monobiotinylated drug. Monobiotinylation is required because avidin/OX26 is multivalent for binding biotin (Yoshikawa and Pardridge 1992), and higher degrees of biotinylation would cause the formation of high molecular weight aggregates, which in turn would be rapidly cleared by the reticuloendothelial system in vivo.

The other important point to be addressed in the development of a biotinylated ligand for this transport vector is the preservation of biological activity following biotinylation. The application of these two rules, monobiotinylation and preservation of biological activity, led to the development of the VIPa, as shown in A in the upper right panel of figure 1. The acetylation of the N-terminal cl-amino group and the substitution of arginines for lysines in positions 20 and 21 left only one possible biotinylation site at the e-amino group of lysine in position 15, which is not crucial for receptor binding (Andersson et al. 1991). The other modifications of the native VIP sequence were incorporated to introduce additional stability during radioiodination (norleucine for methionine at position 17) and to increase in vivo metabolic stability (alanine for isoleucine at position 26) (O'Donnell et al. 1991). The obtained VIPa could be efficiently biotinylated and (high-performance liquid chromatography (HPLC) purified (Bickel et al. 1993b). The use of biotinylating reagents with cleavable disulfide linkers such as succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin) typically involves the modification of primary amino groups (N-terminal a-amino group, e-amino group in lysine residues) and leaves behind molecular baggage in the form of a mercaptopropionate group attached to the original primary amino group after cleavage of the disulfide bond (see B in the upper right panel of figure 1). Treatment with the reducing agent dithiothreitol (DTT) converted the biotinylated VIPa quantitatively into the desbiotinylated derivative. The identities of the VIPa and its

biotinylated and desbiotinylated derivative were confirmed by fast atom bombardment mass spectrometty (FAB-MS), as shown in C-E in the upper right panel of figure 1. A radioreceptor assay with rat lung membranes and ^{125}I-labeled native VIP as a tracer was used to evaluate the binding affinity of VIPa, bioVIPa, and desbioVIPa for the VIP receptor (figure 1, lower left panel). The inhibitor constants, K_i, of VIPa and desbioVIPa, were 3.2±0.3 nM and 1.6±0.2 nM, which are within one order of magnitude of the dissociation constant of VIP (K_d = 0.26±0.04 nM). Hence, a synthetic VIPa with retained receptor affinity could be constructed that allows for selective monobiotinylation.

Employing the internal carotid artery perfusion/capillary depletion technique (Triguero et al. 1990) and ^{125}I-labeled VIPa bound to avidin/OX26, transport of the chimeric peptide across the BBB could be confirmed (Bickel et al. 1993b). The ultimate goal of the chimeric peptide delivery strategy is the achievement of pharmacologic effects following systemic administration in vivo. Therefore, the bioVIPa-avidin/OX26 chimeric peptide was given to rats via intracarotid infusion. The effect of this treatment on cerebral blood flow (CBF) was measured. CBF is a suitable biologic indicator of VIP bioactivity in vivo because cerebral blood vessels are densely innervated by VIP-ergic nerve terminals (Itakura et al. 1984; Larsson et al. 1976), and VIP is functionally involved in cerebral vasodilation (Edvinsson 1988, pp. 378-392). Because the VIP receptors responsible for the vasodilation appear to be located on the brain side of the BBB, the demonstration of pharmacologic effects with VIP in vivo typically requires topical application of the peptide (Yaksh et al. 1987).

For the study with the bioVIPa-avidin/OX26 chimeric peptide, an experimental protocol was chosen that allowed for the measurement of CBF in anesthetized rats: The animals were under light anesthesia with nitrous oxide/oxygen and were artificially ventilated to keep arterial pCO_2 and pH within the physiological range. CBF and cerebral blood volume were measured with an external organ technique using [^{14}C]sucrose as a blood volume marker and [^{3}H]diazepam as a flow marker. Diazepam was completely sequestered into brain tissue during a single capillary passage. The tracers were bolus injected into a peripheral vein, and arterial blood was sampled from the cannulated femoral artery for 10 seconds, after which the animal was sacrificed by decapitation to measure brain radioactivity. In this experimental setting, a 10-minute carotid infusion of bioVIPa (dose = 12 µg/kg) coupled to avidin/OX26 caused a 65 percent increase in baseline CBF (figure 1, lower right panel). In contrast,

bioVIPa without the vector or the avidin/OX26 vector alone in corresponding doses did not change CBF compared with control values (infusion of physiological buffer).

OPIOID PEPTIDES SUITABLE FOR AVIDIN/VECTOR CONJUGATES

The strategy demonstrated successfully with the VIPa next was applied to opioid peptides. The tetrapeptide dermorphin analog Tyr-D-Arg-Phe-Lys-NH, (DALDA) (Schiller et al. 1989) was chosen for the following reasons: its single E-amino group provides a suitable target for biotinylating reagents, provided the N-terminal a-amino group of Tyr' is blocked during the reaction; and the substitution of D-Arg in position 2 of the peptide and the carboxyl terminal amidation reduces the susceptibility of the peptide to both aminopeptidase and carboxypeptidase attack. Additionally, DALDA is currently the most u-receptor specific opioid agonist, with a $K_{\delta}\delta$ to $K_{d}\mu$ ratio of 11,400:1 and a μ-receptor affinity in the low nM range (Schiller et al. 1989).

A PK study with DALDA was performed in rats to quantitate the BBB permeability of the peptide (Samii et al. 1994). The results are shown in figure 2. Following intravenous (IV) bolus injection of [^{3}H]DALDA and [^{14}C]sucrose, both tracers displayed an almost identical plasma concentration-time profile (figure 2, upper left panel). Plasma protein binding of [^{3}H]DALDA in rat serum was less than 10 percent as measured by ultrafiltration. Together with the fact that after 30 minutes there was virtually no metabolic degradation detectable in plasma (see HPLC analysis of a 30-minute plasma sample, figure 2, upper right panel), this indicates a systemic clearance of DALDA approaching the clearance of low molecular weight extracellular space markers such as sucrose, which are ret-rally excreted by glomerular filtration. The brain uptake of small solutes, which are not transported by specific carrier systems at the BBB, is generally determined by the lipophilicity of the compounds as long as the molecular weight is not in excess of a threshold of 800-1,000 daltons (Pardridge 1991). The lipophilicity of DALDA and sucrose in terms of the partition coefficient (PC) between octanol and Ringer's solution is depicted in figure 2 (lower left panel). It is evident that the log PC of DALDA is about two orders of magnitude higher than the log PC of sucrose. The higher lipid solubility of DALDA compared with sucrose

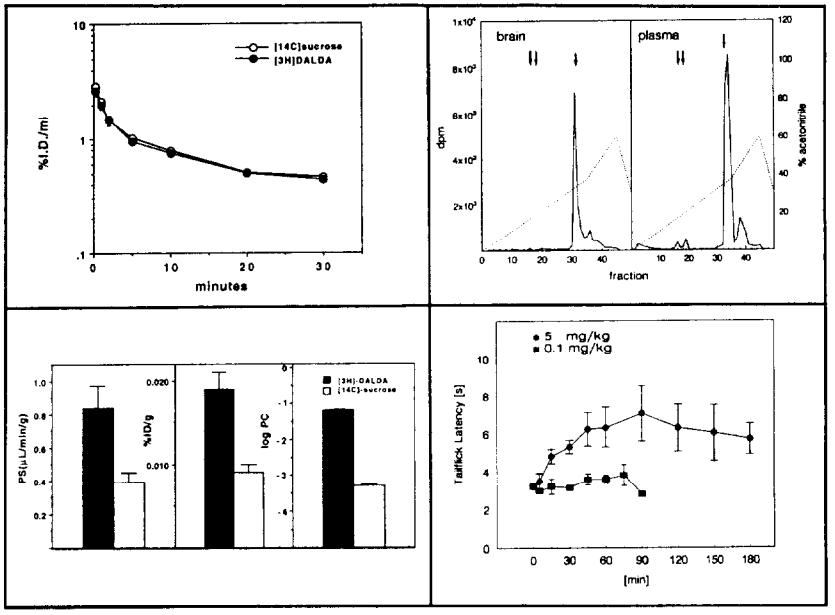

FIGURE 2. *(Upper left) Plasma concentration curves of ([14C]sucrose and [3H]DALDA after IV bolus injection in rats. %ID/mL = percentage injected dose per mL plasma, N = 3 animals per group. (Upper right) Reverse-phase HPLC profiles of extracts of brain (left) or plasma (right) obtained 30 min after IV bolus injection of [3H]DALDA in anesthetized rats. The broken lines indicate the acetonitrile gradient. The arrows indicate the elution volumes of potential metabolic breakdown products, tyrosil-D-arginine and tyrosine, and the DALDA standard, respectively. (Lower left) The BBB PS product, the brain delivery expressed as the percentage injected dose (%ID) per gram brain, and the log octanol/Ringer's PC for [3H]DALDA or [14C]sucrose are shown. Data are mean±SE (N = 3). (Lower right) The tailflick latency after a single IV injection of two different doses of DALDA is plotted versus time after IV administration. Data are mean±SE (N = 3).*

may explain the higher permeability surface area (PS) product, indicating the comparatively higher BBB permeability of DALDA (figure 2, lower left panel).

The concentration in brain tissue at a given time (t) depends on the PS product and the area under the plasma concentration curve (AUC) according to the following equation:

$$\%ID/g(t) = PSxAUC_{0-t}$$

(Bickel et al. 1993a), where %ID/g = % injected dose per gram tissue. Therefore, the brain concentration of DALDA is correspondingly higher than the concentration of sucrose at 30 minutes (figure 2, lower left panel). The brain delivery of DALDA was 0.019±0.002 %ID/g. If linear PK holds, the brain delivery of a peptide on the order of 0.02 %ID/g would yield a brain concentration of 0.25 µg peptide per gram after administration of 5 mg/kg IV to a rat of 0.25 kg body weight. Such a high dose elicited a moderate analgesic effect (increased tailflick latency) as shown in figure 2 (lower right panel). Due to the metabolic stability, relative small size, and relative high lipophilicity (compared with sucrose), the PK parameters of DALDA may serve as a standard against which the brain uptake of peptide analogs and chimeric peptides can be compared.

In order to selectively monobiotinylate DALDA at the E-amino group of Lys^4, the N-terminal a-amino group of Tyr' was protected with the N-9-fluorenyl methoxycarbonyl (Fmoc) group, analogous to an approach described by Goldstein and colleagues (1988) with a t-butoxycarbonyl (Boc)-protected dynorphin analog. Fmoc-DALDA was prepared by a solid-phase peptide synthesis and was HPLC purified. Reaction with NHS-SS-biotin gave the biotinylated derivative, Fmoc-Tyr^1, bioLys^4-DALDA (panel A in figure 3). The Fmoc-protective group was cleaved from the biotinylated peptide to obtain bioLys^4-DALDA (panel B in figure 3). Cleavage of the disulfide bridge yields the desbiotinylated peptide, desbioLys^4-DALDA (panel C in figure 3). The identity of these peptides with the desired structures was confirmed by secondary ion mass spectrometry (SIMS) (panels D-F in figure 3). Starting from Fmoc-DALDA, the overall yield of bioDALDA after biotinylation, deprotection, and purification on reverse-phase HPLC was 50 percent. This demonstrates the gain in efficiency that can be achieved by using the biotin-avidin linker chemistry. Due to the extremely high affinity of the avidin-biotin bond ($K_d = 10^{-15}$M), the biotinylated drug is nearly 100 percent bound by avidin.

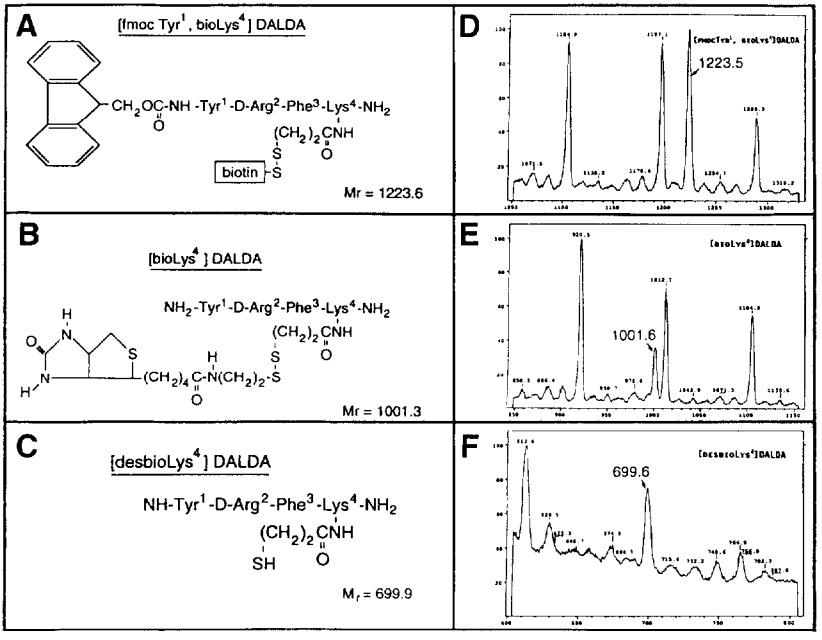

FIGURE 3. *Structural formulas and formula weights of Fmoc-bioDALDA (A), bioDALDA (B), and desbioDALDA (C). Panels D-F show the SIMS obtained with the HPLC-purified peptides. The respective mass peaks are labeled by arrows. Additional peaks represent matrix aggregates. All experimental mass values were within one mass unit of the theoretical values.*

SOURCE: Bickel et al. (1994).

When DALDA and its biotinylated and desbiotinylated derivatives were tested for their bioactivity in a radioreceptor assay, with [^{3}H]DAGO as u-selective ligand, it was evident that there is only a minimal loss of affinity owing to the modification at Lys^4 (see figure 4 and table 1). This is consistent with the finding by Charpentier and colleagues (1991), who reported high u-receptor affinities (K_i in the low nM range) in a series of N-terminal dermorphin tetrapeptide analogs with various amino acid substitutions at position 4. Considering the high u-receptor specificity of the parent peptide DALDA (see table 1), bioDALDA should be the most u-selective monobiotinylated opioid receptor ligand yet described. The K_i of 6.5 nM compares favorably with other described biotinylated opioid analogs like ß-endorphin (Hochhaus et al. 1988) and Leu-enkephalin (Koman and Terenius 1980; Nakayama et al. 1986). Taking into account

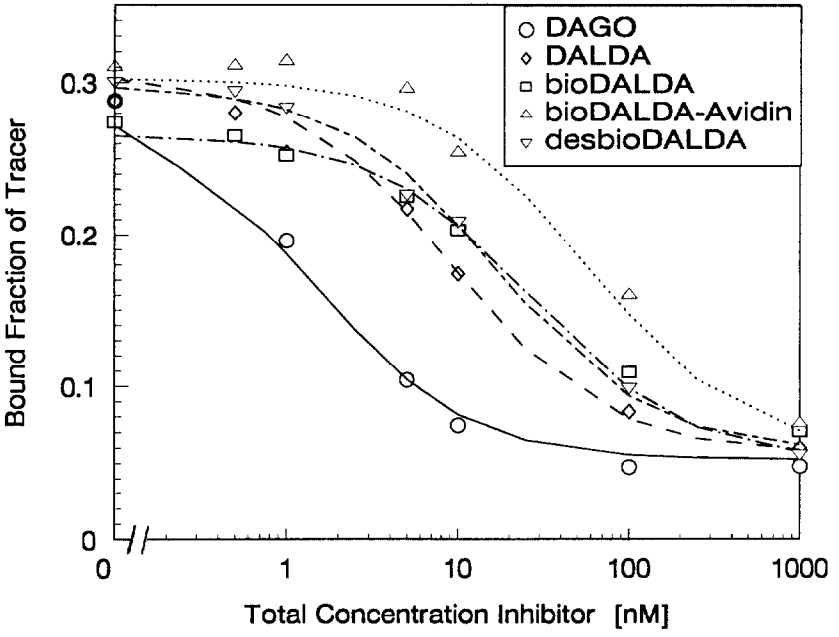

FIGURE 4. *Competition curves obtained in a μ radioreceptor with rat brain synaptosomes and [^{3}H]DAGO. The curves were fitted to the experimental data using least squares nonlinear regression analysis, and the parameters are given in table I. A 2-μM concentration of avidin had no inhibitory effect on [^{3}H]DAG0 binding (data not shown).*

SOURCE: Bickel et al. (1994).

the stability of DALDA and the retained binding affinity in the presence of avidin, bioDALDA should be a useful biotinylated ligand for in vitro studies with μ receptors.

The bioactivities of DALDA and desbioDALDA, as measured in the μ radioreceptor assay, were reflected in a corresponding activity in the in vivo analgesic effect. Owing to the localization adjacent to the ventricular surface of opioid receptors involved in antinociception (central grey) (see Herz et al. 1970), the intracerebroventricular (ICV) route is viable for testing centrally mediated analgesic effects of opioids. ICV application of DALDA to rats demonstrated both the potency and the opioid nature of the analgesic effect in the tailflick paradigm. The left side panel of figure 5 shows that 1 μg DALDA ICV is equipotent to

TABLE 1. *Opioid peptide receptor parameters.*

Receptor	B_{max} (fmol mg_p^{-1})	Ligand	K_D (nM)	K_i (nM)
μ	143±9	[^{3}H]DAGO	0.34±0.05	—
		DALDA	—	2.3±0.4
		desbioDALDA	—	4.0±0.9
		bioDALDA	—	6.5±1.1
		bioDALDA/avidin	—	14.5±2.4
δ	26±5	[^{3}H]DPDPE	0.62±0.16	—
		DALDA	—	> 1000

SOURCE: Bickel et al. (1994).

20 μg morphine ICV, and both responses could be equally antagonized by pretreatment with the opioid antagonist naloxone (10 mg/kg subcutaneously). The dose-response curves on the right side of figure 5 for DALDA and desbioDALDA, which reveal an approximately threefold difference in potency of these two peptides, are also in accordance with the relative u-receptor affinities (see table 1). With respect to the effect of ICV DALDA, it may be pointed out that 0.3 μg ICV elicited a similar analgesic effect compared with 5 mg DALDA IV as described above. This is in accordance with the estimated brain delivery of approximately 0.25 μg/g brain DALDA after 5 mg/kg IV.

OPTIMIZATION OF CHIMERIC PEPTIDE DELIVERY AND POTENCY

The results obtained with the VIPa and DALDA illustrate the potential of the chimeric peptide strategy. As shown in figure 6, the development of an optimized chimeric peptide with good pharmacologic activity following peripheral administration is a complex task. The avidin-biotin linker technology allows for both high-yield coupling and cleavability of vector and drug. With regard to drug development, it could be demonstrated that selective monobiotinylation of an appropriately designed peptide analog can be performed with retention of intrinsic receptor affinity, both after biotinylation and after cleavage of the biotin moiety. There is a need, however, for improvements in the sector of

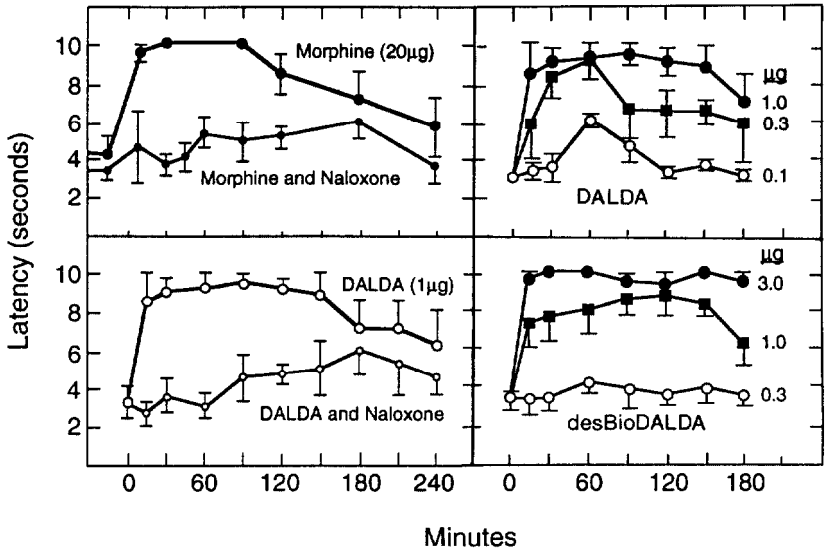

FIGURE 5. *Tailflick analgesia in rats following ZCV injection of morphine (upper left), DALDA (lower left and upper right), and desbioDALDA (lower right). Baseline latencies were taken 30 and 15 min before ZCV injections; cutoff time was IO sec. Naloxone was administered in a subcutaneous dose of 10 mg/kg immediately after the second baseline measurement. Data are means±SE (N = 3).*

SOURCE: Adapted from Bickel et al. (1994).

peptide analogs with increased potency. In the case of DALDA., the initial K, for u-receptor binding is approximately sevenfold higher compared with DAGO (2.3±0.4 nM versus 0.34±0.05 nM); under the assumption of comparable PK in plasma, a correspondingly higher dose of a DALDA-chimeric peptide would have to be peripherally administered. Therefore, the authors are currently evaluating another dermorphin analog. It has features similar to DALDA in terms of suitablility for monobiotinylation. This analog, Lys^7-dermorphin (Negri et al. 1992), however, has a p-receptor affinity in the range of DAGO. This peptide was radiolabeled with ^{125}I and biotinylated as described for DALDA and then coupled to the avidin/OX26 vector. The chimeric peptide tracer was given to rats as an internal carotid artery perfusion over 10 minutes in

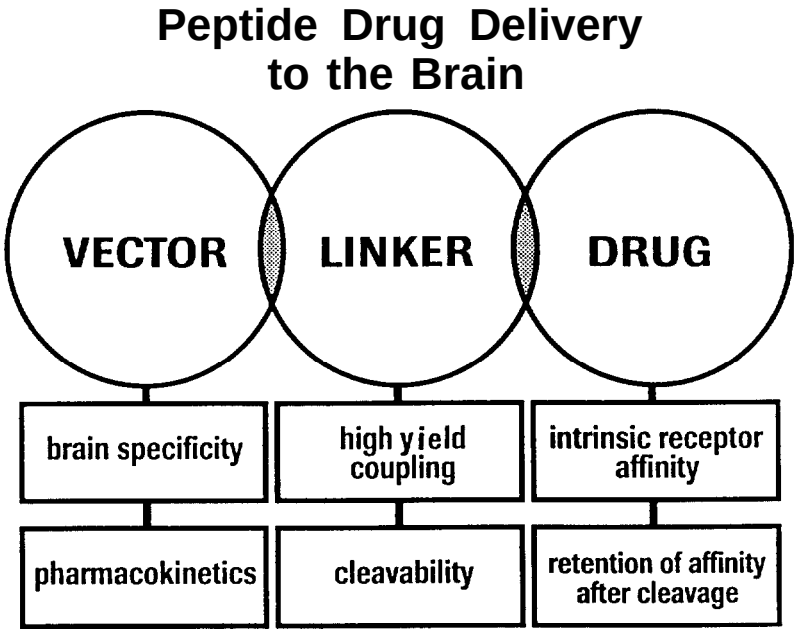

FIGURE 6. *The three interwoven areas of vector, linker, and drug development, with the corresponding criteria for optimization of each segment.*

physiologic buffer. The brain was then homogenized and extracted for gel filtration HPLC analysis (Bickel and Pardridge 1994). The chromatographic analysis of the brain homogenate confirmed the in vivo cleavability of the chimeric peptide in brain since there was a shift of radioactivity from the high molecular weight chimeric peptide peak present in the perfusate to a low molecular weight peak corresponding to the free peptide.

A second area of future improvements resides with the vector part of the chimeric peptides (figure 6). The experience with the avidin/OX26 conjugate highlights the importance of the pharmacokinetic properties of drug delivery vectors. Table 2 gives an overview of the pharmacokinetic parameters of peptides and proteins. It is evident that avidin/OX26 has almost the same BBB permeability (PS product) as the native OX26 monoclonal antibody. However, the coupling to the cationic protein, avidin, decreases the AUC in the plasma of the conjugate significantly. Therefore, the brain delivery of avidin/OX26 is less than 40 percent of the value for OX26. The relatively poor systemic pharmacokinetic behavior of avidin/OX26 had no negative effects in the in vivo

TABLE 2. *Pharmacokinetic parameters of BBB transport **of** peptides and proteins after IV bolus injection in rats.*

Protein	Plasma integral[b] [(%ID)•min mL^{-1}] at t=60 min	BBB PS[c] ($\mu L\ min^{-1}g^{-1}$)	(%ID) g^{-1D} at t=60 min
[^{3}H]OX26	168 ± 76	1.56 ± 0.13	0.262
[^{3}H]biotin/ avidin-OX26	72 ± 18	1.35 ± 0.16	0.100
[^{3}H]catRSA	387 ± 74	0.16 ± 0.02	0.062
[^{3}H]catbIgG[a]	43 ± 14	0.63 ± 0.12	0.027
[^{3}H]rCD4	223 ± 49	0.11 ± 0.01	0.025
[^{125}I]histone[a]	18 ± 10	0.91 ± 0.11	0.016
[^{3}H]biotin/ avidin	13	0.30 ± 0.10	0.004
[^{14}C]nRSA	533 ± 55	0	0
[^{125}I]mIgG$_{2a}$	440 ± 11	0	0

KEY: catRSA = cationized rat serum albumin; catbIgG = cationized bovine IgG; rCD4 = recombinant CD4; nRSA = native rat serum albumin; mIgG = native murine IgG.

NOTES: [a]Estimates based on whole blood analysis; all other studies performed on serum measurements.

[b]Plasma integral = $[A_1(1\text{-}e^{-k1t})/K^1]+[A_2(1\text{-}e^{-K2t})/K_2]$, where t = 60 minutes after single injection.

[c]Permeability surface area (PS) product = $[(V_D - V_O)C_P(T)]$/ plasma integral, where V_O = plasma volume, $C_P(T)$ = plasma concentration at t = 60 minutes after IV injection.

[d]Product of [plasma integral/1000] x PS, where %ID = % of injected dose delivered to brain at 60 minutes after injection.

SOURCE: From Bickel et al. (1993a).

experiments with the VIP analogs as described above because the chimeric peptide was administered via internal carotid artery infusion, thus eliminating systemic clearance. It was not possible to increase the

brain delivery of avidin/OX26 by saturating the systemic clearance with coinjections of the OX26 antibody or avidin (Kang et al. 1994). A solution to this problem, however, was found by the use of a neutralized form of avidin in the vector conjugate (Kang and Pardridge 1994). This neutral avidin/OX26 conjugate has a pharmacokinetic behavior in plasma that is comparable to OX26. After IV injection, the concentrations in brain reached 0.2-0.25 %ID/g tissue.

CONCLUSION

Considerable progress has been made in the development and characterization of chimeric peptides as brain drug delivery systems. With improved pharmacokinetic properties of drug vectors, efficient coupling, and rational design of peptide analogs as described above, the goal to decrease significantly the dose required for a CNS-mediated pharmacologic effect after peripheral IV administration compared with administration of the same peptide without a vector seems achievable. Future research will focus on the discovery and development of new vectors with higher brain specificity and higher BBB PS products. The currently employed chemical coupling procedure for avidin/vector conjugates may be replaced by fusion proteins produced by recombinant DNA technology (Pardridge 1991). Similarly, for use in humans, the immunogenicity of vectors that are monoclonal antibodies of murine origin may be reduced by "humanization" of the antibody using genetic engineering techniques.

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Conformationally Constrained Peptide Drugs Targeted at the Blood-Brain Barrier

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INTRODUCTION

The problem of delivering a drug to the brain has been a frustration that neurologists and other clinicians have had to deal with for many years. The blood-brain barrier (BBB) acts as a selective partition layer between the peripheral and central nervous systems and can limit the passage of many therapeutically important blood-borne substances into the central nervous system (CNS) on the basis of molecular size, charge, and/or hydrophobicity. Certain biologically important nutrients do pass the BBB that are critical for maintaining normal physiological homeostasis such as glucose and some amino acids. What is known of the BBB is that it consists of a series of endothelial microvessels and cells that form tight intracellular functions and lack fenestra (Brightman and Reese 1969; Reese and Kamovsky 1967). The BBB matrix also forms an efficient enzymatic barrier that can degrade small peptides (Brownson et al. 1994; Rapaport et al. 1980) and other biomolecules. However, it is often an advantage to target the BBB-associated proteolytic enzymes with prodrugs of peptides to enhance permeability across the BBB endothelial cells prior to enzymatic cleavage of the prodrug to the biologically active peptide (Brownson et al. 1994; Weber et al. 1993).

To understand the various BBB peptide drug delivery mechanisms, it is necessary to understand what is presently known about the BBB. Next, it is critical to utilize a defined series of peptide drugs and analogs that can help describe how a peptide drug interacts with established (and putative) transport systems in the brain. Finally, it is important to use a logical approach to peptide-drug design to take advantage of transport systems to deliver the peptide drug to the prescribed site of action in the CNS (Banks and Kastin 1985, 1990; Banks et al. 1992).

This chapter describes both in vitro and in vivo methods to study the transport of peptide drugs across the BBB. Several in vivo methods have

already been well described in the literature, such as the brain uptake index, the intravenous (IV) administration method, and the brain perfusion methods (Gjedde 1981; Oldendorf 1970; Takesato et al. 1984; Zlokovic et al. 1985). The advantages to in vivo methods are obvious, but most in vivo preparations are both costly and time consuming and require high concentrations of peptide drugs for analytical detection limit reasons (Davis 1990, pp. 149-177). In vitro techniques offer a good screening method for the first phase of analysis of peptide drug BBB permeability. Originally, only octanol/water partition coefficients were used to describe the predicted ability for a peptide drug to cross the BBB. At the present time the in vitro bovine brain membrane endothelial cell (BMEC) system enables the peptide drug researcher to screen 50 peptide drugs in the same time it takes to screen 10 peptide drugs by classical in vivo methods (Audus and Borchardt 1986, 1987; Banks et al. 1992; Weber et al. 1991, 1992, 1993).

The in vitro BMEC system for studying BBB permeability has been extensively characterized morphologically, biochemically, and immunohistochemically and found to have tight intercellular junctions, attenuated pinocytosis, and no fenestra (see review by Borchardt 1990). Additionally, the BBB enzyme markers gamma-glutamyl transpeptidase and alkaline phosphatase and the endothelial cell markers, Factor VIII antigen and angiotensin-converting enzyme, are present in the system. Continuous cultures of BMECs grown with astrocyte-conditioned media or cocultured with astrocytes have been shown to retain most of the characteristics of the BBB and are free of pericyte contamination (Dehouck et al. 1990; Meresse et al. 1989; Tomaselli and Bard 1991). Both primary and continuous cultures of BMEC are considerably "leakier" than the in vivo BBB, as measured with membrane-impermeant markers (i.e., sucrose) and electrical resistance (Dehouck et al. 1990; Pardridge et al. 1990; Rubin et al. 1991). This leakiness can be attenuated to some extent by the addition of astrocyte-conditioned media or by coculture with astrocytes (Dehouck et al. 1990; Rubin et al. 1991). Recently, the authors' laboratory also showed that the BMEC membranes contain the necessary complement of neuropeptidases (i.e., 3.4.24.11, 3.4.24.15, and 3.4.11.2) capable of degrading several classes of peptides including Met^5-enkephalin (Brownson et al. 1994).

The authors' research group has synthesized several peptide drug analogs modeled after endogenous methionine enkephalin in an attempt to describe transport mechanisms for peptide drugs across the BBB. This chapter describes data from six classes of these analogs, ranging from

conformational constrainment of the peptide drug backbone to para cloro halogenation to improve hydrophobicity and BBB permeability. In each case, data are presented using the in vitro BMEC technique to describe permeability at the BBB and, in a few cases, the in vivo IV distribution data for correlation has been used.

Peptide Structure

The structure of six different classes of peptides is shown in table 1. The authors' laboratory has studied each of these peptide classes, which are all modifications of endogenous methionine-enkephalin. Class II is the well-characterized delta opioid receptor agonist DPDPE. Modification of the D-penicillamine (D-PEN) amino acid in position 5 of DPDPE to L-cysteine yields DPLCE. By substituting both D-PEN amino acids of DPDPE with D-cysteine and adding a carboxy terminal serine and glycine amide, DCDCE was synthesized. Further modification of DCDCE by coupling the serine to D-glucose provided the first glycopeptide to study. This glycopeptide is hypothesized to use the type 1 glucose transporter in the brain (Polt et al., in press).

Effect of Peptide Structure on BBB Permeability

The effect of various structural modifications to enhance the lipophilicity of peptide drugs is shown in table 2. The mechanism employed to enhance lipophilicity and permeability coefficient (PC) values are listed for and compared with the parent peptide classes DPDPE, DPLCE, deltorphins, and biphalin, respectively, whose structures are shown in table 1. The halogenation of DPDPE and biphalin with chlorine on the Phe4 residue was the most effective modification for enhancing permeability across BMEC monolayers. The PC for pC1-Phe4DPDPE was significantly ($p < 0.01$) greater than the parent peptide DPDPE. The acyclic-reduced form of DPDPE was another modification that significantly ($p < 0.01$) enhanced permeability, although this peptide is not enzymatically stable (data not shown). Halogenation with a fluorine and decarboxylation of the C-terminal residue had no effect on the permeability of DPDPE. Other modifications employed for DPDPE resulted in significant decreases in BMEC measured permeability coefficient, Substitution with alanine at the third amino acid position had the greatest negative PC effect by significantly ($p < 0.01$) reducing the permeability of DPDPE. It was interesting to note that the PC of Ala3, pC1 Phe4DPDPE, was not different from that of Ala3, DPDPE

TABLE 1. *Structure of parent peptide for six different classes of peptide drugs.*

Class	Peptide	Structure
I	Met-Enkephalin	H-Tyr-Gly-Gly-Phe-Met-OH
II	DPDPE	H-Tyr-D-Pen-Gly-Phe-D-Pen-OH
III	DPLCE	H-Tyr-D-Pen-Gly-Phe-L-Cys-OH
IV	DCDCE-Glucose	H-Tyr-D-Cys-Gly-Phe-D-Cys-Ser-Gly-C(=O)-NH2 O-D-glucose
V	Deltorphin I	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
	Deltorphin II	H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
VI	Biphalin	H-Tyr-D-Ala-Gly-Phe-C(=O)-NH H-Tyr-D-Ala-Gly-Phe-C(=O)-NH

(i.e., 19.67 versus 20.00). This could be because the pC1 halogen effect was masked or eliminated by the Ala3 substitution.

The permeability of biphalin was significantly ($p < 0.01$) increased by the presence of two chlorine atoms but was significantly ($p < 0.01$) decreased with two fluorines. The conformational changes and amino acid substitutions incorporated into the deltorphin structures all greatly reduced PC values for deltorphin. The PCs for all of the deltorphin compounds synthesized with multiple cationic residues were significantly lower than deltorphin itself.

Effect of Transporters at BBB on Peptide Permeability

The PC values of prodrugs that employ the addition of lipophilic residues or utilize specific transport mechanisms to cross the BBB are shown in table 3. The PC of DPDPE was significantly ($p < 0.01$) enhanced by the

TABLE 2. *Effect of lipophilicity on BMEC passage.*

Class Peptide Analog	Mechanism of Hydrophobicity	Permeability Coefficient (PC x 10^{-4})
I. MET-ENK	Control	14.00±2.50
II. DPDPE	Control	49.24±2.78
[*p*-ClPhe^{4}]DPDPE	Halogenation	82.76±3.46
Acyclic-reduced DPDPE	Conformation	76.22±3.80
[*p*-F Phe^{4}]DPDPE	Halogenation	54.75±11.5
Tri-methyl-TyrDPDPE	Alkylation	35.71±1.22
[Phe^{3}]DPDPE	AA Substitution	34.81±3.91
[D-Ala^{3}]DPDPE	AA Substitution	20.00±2.76
[Ala^{3},p-ClPhe^{4}]DPDPE	AA + Halogenation	19.67±2.70
[L-Ala^{3}]DPDPE	AA Substitution	16.93±2.08
III. DPLCE	AA Substitution	26.00±1.49
Decarboxy DPLCE	H-Bonding N	47.84±1.24
V. Deltorphin II	Control	38.00±6.30
[D-Pen^{2,5}]Deltorphin I	Conformation	25.78±2.46
[D-Phe^{3}]Deltorphin I	AA Substitution	09.00±1.00
[Pro^{6}]Deltorphin II	Conformation	18.88±2.28
VI. Biphalin	Control	55.00±4.98
[*p*-ClPhe$^{4,4'}$]Biphalin	Halogenation	92.00±5.88
[*p*-F Phe$^{4,4'}$]Biphalin	Halogenation	23.21±3.76

incorporation of two cationic lysine residues at the carboxy terminus. The addition of a lipophilic phenylalanine (Phe) residue at the amino terminus was also successful at increasing the permeability of DPDPE ($p < 0.01$). The presence of a Phe at the carboxy terminus of DPLCE significantly (p c 0.01) increased the permeability of its parent peptide DPLCE. The PC for DCDCE-Phe6 was very low, and the incorporation of a glucose molecule had no effect on permeability in the BMEC system. This peptide probably utilizes the type 1 glucose transporter GLUT-l specific carrier protein, localized in the plasma membranes of the

TABLE 3. *Effect of prodrugs on BMEC passage.*

Class Peptide Analog	Mechanism of Transport (Postulated)	Permeability Coefficient (PC x 10^{-4})
II. DPDPE	Control	49.24±2.78
DPDPE-Lys-Lys-Ala	Cationic	74.38±5.28
Phe^{0}-DPDPE	Lipophilic	62.00±4.06
acetylated Phe^{0}-DPDPE	Lipophilic	56.76±3.98
III. DPLCE	Control	26.00±1.49
DPLCE-Phe^{6}	Lipophilic	56.34±2.00
IV. DCDCE Glycosylated DCDCE	Glucose T*	14.34±1.94
DCDCE-Phe^{6}	Lipophilic	11.31±0.65

KEY: * T = type I glucose transporter.

blood-brain endothelial cells and in neurons and glia. However, the in vitro BMEC system does not express the GLUT- 1 transporter to a large degree, and this may explain the very low PC in the BMEC system.

Correlation of In Vitro to In Vivo BBB Techniques

A rank order of PC for a representative set of peptide drugs and endogenous peptides tested to date is shown in table 4. In these studies, in vivo distribution data to in vitro BMEC permeability coefficients were compared, and then three different partition coefficient or hydrophobicity-derived factors were used to provide further comparisons. An excellent correlation coefficient of 0.98 was reached when in vivo data were compared with in vitro BMEC derived data on the four peptides tested (intravenous) to date. The correlation coefficient between PC by BMEC and high-performance liquid chromatography (HPLC) capacity factor was also good at 0.745. However, when the heptane/ethylene glycol partition procedure or H-bonding number process was used, the correlation was very poor (see table 4). In many cases there was no detectable peptide found in the heptane phase.

TABLE 4. *Assessment of the BMEC in vitro BBB model and other methods for their prediction of opioid peptide permeability across the BBB.*

	% of Total IV Dose in Brain	BMEC (PC x 10^{-4}) R = 0.998	Capacity Factor R = 0.745	Log [H / EG] R = 0.032	H Bonding Number
[*p*-ClPhe$^{4,4'}$]Biphalin		92.00±5.88	10.90	-2.12	24
[*p*-ClPhe^{4}]DPDPE	0.178±0.030	82.76±3.46	9.54	*	15
Acyclic-reduced DPDPE		76.22±3.80	11.11	*	15
Phe^{0}-DPDPE		62.00±4.06	10.07	-2.03	17
Acetylated Phe^{0}-DPDPE		56.76±3.98	12.50	*	17
DPLCE - Phe^{6}		56.34±2.00	10.87	*	17
Biphalin	0.089±0.008	55.00±4.98	8.03	-1.86	24
[L-Ala^{3}]DPDPE		54.00±2.76	8.07	*	15
DPDPE	0.064±0.012	49.24±2.78	8.25	-2.87	12
Decarboxy DPLCE		47.84±1.24	8.28	*	
[Met^{5}]enkephalin		46.44±4.28	6.50	-1.51	
Deltorphin II	0.038±0.009	38.00±6.30	8.25	*	
Glycosylated DCDCE		14.34±1.94	3.82	*	High

KEY: * = No detectable amount of peptide in heptane phase; R = Correlation coefficient to IV data.

DISCUSSION

The passage of peptide-based drugs across the BBB has been an area of acute interest to many biomedical researchers. The possibility that these nontoxic peptides (derived from amino acids) can be modified by well-characterized amino acid substitution (or modification) is a significant advantage over present drug-discovery approaches being investigated. Since peptide-based drugs can be enzymatically degraded into biologically available amino acids, structural modifications are necessary to yield an enzymatically stable peptide ligand that will bind to the appropriate receptor site. This chapter provides both in vivo and in vitro data providing evidence that halogenation with chlorine is a good procedure for improving permeability at the BBB for peptide drugs.

Other synthetic approaches studied include conformational change, glycosylation, amino acid substitution, acetylation, and amidation. Of all the modifications studied to date using the BMEC method of screening the BBB permeability, the parachlorination of phenylalanine has proven to be best. However, the glycosylation process yields a very potent analgesic peptide when given peripherally, and glycosylated peptide drugs may be excellent candidates for BBB penetration as well (Polt et al., in press). Since the BMEC lacks a fully developed glucose transporter, it is not a good technique to rank order the BBB permeation of glycosylated peptides. To this end, an in vivo technique is much better. However, if a researcher is to establish chemical rules for synthesizing peptide drugs that can cross the BBB, then a carefully and logically designed set of peptides must be synthesized that can be sequentially tested both in vivo and in vitro using BMECs. By comparing and contrasting the results from both in vivo and in vitro techniques with a set of defined peptide analogs (which have logical substitutions and modifications), then the researcher will be able to advance the field of peptide-drug interaction at the BBB. Now that it is generally accepted that peptides will not only cross the BBB but will cross intact, bind to the appropriate receptor, and elicit a biological response (such as analgesia), researchers are in an excellent position to study the biochemistry and cell biology of the BBB.

This monograph presents only a small sample of the many possible synthetic schemes that can be applied to peptide drugs. Even though the numbers of drugs were limited, exciting data were described on halogenation and glycosylation that will help researchers build a better peptide drug for the laboratory's next series of studies on the BBB.

EXPERIMENTAL DESIGN

Brain Microvessel Endothelial Cells

Bovine BMECs were isolated and purified from gray matter of the cerebral cortex as previously characterized and detailed (Audus and Borchardt 1986, 1987). Isolated BMECs suspended in culture medium with equine serum were seeded onto 25 mm polycarbonate membrane filters (10 µm pore size), that were previously coated with rat-tail collagen first and then with fibronectin immediately prior to seeding. The BMEC were allowed to grow (10-12 days) until a confluent monolayer was formed, then they were used for transendothelial transport studies.

In Vitro BMEC Permeability

The polycarbonate membrane filters with confluent BMEC monolayers were placed in adiffusion cell (0.636 cm^2, 3 mL) maintained at 37 °C. Both the donor and the receptor diffusion cell chambers contained an equal volume (3 mL) of phosphate-buffered saline (122 mM NaCl; 3 mm KCL; 25 mN Na_2PO_4; 1.3 mM K_2HPO_4; 1.4 mM $CaCl_2$; 1.2 mM $MgSO_4$; 10 mM glucose; 10 mM HEPES; pH = 7.4). Membranes were equilibrated for 20 minutes with buffer before the addition of peptide (500 µM) to the donor chamber. The side of the membrane coated with BMEC monolayers faces toward the donor chamber. Radiolabeled sucrose (186,000 dpm/10 µL), a BBB impermeant molecule, was added simultaneously with peptides to test the integrity of the BMEC monolayers and was used in calculating the PC.

A 200 µL aliquot was sampled from the donor chamber at times 0 and 120 minutes and from the receptor chamber at 0, 15, 30, 60, 90, and 120 minutes. An equivalent volume of buffer was replaced in the receptor chambers after the 15 to 90 minute samplings to maintain a constant volume across the chambers. An equal volume of acetonitrile-water (v/v, 50/50) was added to each sample to stop enzyme activity, and a 50 µL aliquot was removed for liquid scintillation counting. The amount of peptide that crossed the BMEC monolayers was determined by quantitative HPLC analysis (Davis 1990, pp. 149-177).

Apparent permeability coefficients were calculated by the following equation:

$$PC = X/(A \times t \times C_D)$$

where PC is the apparent permeability coefficient in cm/min, X is the amount of substance in moles in the receptor chamber after correction for sampling and paracellular passage based on radiolabeled sucrose levels at time (t in minutes), A is the diffusion area (0.636 cm^2), and C_D is the concentration of substance in the donor chamber in mol cm^{-3}(Audus and Borchardt 1986, 1987).

Capacity Factor

Peptide reverse-phase HPLC retention times were previously reported by Weber and colleagues (1993) and were used as a measure of lipophilicity to determine capacity factors (table 4).

$$\text{Capacity factor} = k = (t_r - t_u)/t_u$$

where t_r is the retention time of the retained peak and t_u is the retention time of an unretained peak.

Partition Coefficient

Partition coefficients were determined in N-heptane and ethylene glycol (table 4). Peptides (100 µg) were dissolved in 2 mL ethylene glycol preequilibrated with heptane. The peptide in ethylene glycol was combined with 2 mL of heptane and then continuously shaken horizontally in silanized tubes and Teflon-lined caps for 48 hours at 25 °C. The two phases were separated, the heptane phase dried down to completeness with N_2, and the ethylene glycol phase diluted with an equal volume of water. The heptane fraction was redissolved in 500 µL 0.1M HoAC, and 200 µL of both phases were used to quantitate peptide concentration by HPLC analysis. Standards (12.5. 25, and 50 µg) were injected using a linear gradient of 5 to 35 percent acetonitrile against 0.1 M NaH_2PO_4 buffer (pH = 2.4) to determine the HPLC response factor for each peptide drug studied (Davis 1990, pp. 149-177).

H-Bonding Number

The number of hydrogen bonds provided by each peptide was determined using the rules for hydrogen bonding established by Stein (1967, pp. 65-125) (see table 4).

HPLC Analysis

Samples from all permeability studies were analyzed on a reverse-phase HPLC system consisting of an autoinjector, two solvent delivery pumps, automated gradient controller LC-15 detector (214 nm); integrator, and a 4.6 x 250 mm column as previously described by Davis (1990, pp. 149-177). Samples were eluted using a linear gradient of acetonitrile against 0.1 M NaH_2PO_4 buffer (pH = 2.4). The flow rate was maintained at 1.5 mL/min and the column temperature at 40 °C.

Statistical Analysis

All BMEC permeability experiments were performed at least once in triplicate with five time points per assay. A two-tailed, independent z-test was used to determine significance between PCs for different peptide analogs.

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Passive and Facilitative Transport of Nucleobases, Nucleosides, and Oligonucleotides-Application to Antiviral and Other Therapies

Marcus E. Brewster and Nicholas Bodor

INTRODUCTION

While most mammalian cells biosynthesize nucleosides and nucleotides for anabolic purposes by de novo synthesis, certain cell types, including brain, intestine, and various blood cells, lack the necessary intracellular mechanism for nucleoside fabrication. These cells are therefore dependent on the preformed molecular entities for normal functioning. Importantly, all natural nucleobases and nucleosides are hydrophilic and penetrate plasma membranes only slowly, suggesting that passive diffusion, in and of itself, is inadequate to provide for cellular needs. This deficiency has been compensated for by the occurrence of specialized transporter systems located in various cell types, which provide for the uptake of nucleosides and nucleobases (Franco et al. 1991; Plagemann and Wohlhueter 1980; Plagemann et al. 1988; Vijayalakshmi et al. 1992). In addition to providing for nutritional requirements, these transporters are also important components in mediating the effects of certain nucleosides, such as adenosine, which are now recognized to exert potent neuromodulatory action (Daval et al. 1991; Erion 1993). Thus, nucleoside salvage results from transportation of the extracellular nucleoside into the cell via a nucleoside carrier, which effectively terminates their pharmacological action, followed by phosphorylation of the nucleoside by high-affinity, low-capacity kinases or transphosphoribosylation resulting in nucleotide formation (Plagemann et al. 1988). The addition of a phosphate group to the nucleosides traps these components intracellularly as the nucleotides do not diffuse efficiently through membranes and are not substrates for nucleoside transport.

Nucleoside transport can be subdivided into several categories, including facilitative, concentrative, and passive (table 1).

TABLE 1. *Nucleoside transport.*

1. Facilitative
Erythrocyte
Blood-brain barrier (purine nucleoside)
2. Concentrative [active)
a. Choroid plexus (purine/pyrimidine nucleoside)
3. Passive

As mentioned, passive diffusion is not important for the transport of naturally occurring nucleosides but becomes significant for various nucleoside derivatives and drugs. Facilitative transport refers to nonconcentrative, nonenergy-dependent equilibrative movement of nucleosides down a concentration gradient into and out of cells. For natural nucleosides, such membrane flux occurs much more rapidly than passive diffusion. The transport is mediated by a receptor system, meaning that molecular flux is saturable, selective, and can be characterized by Michaelis K_m constants, which provide information on substrate-receptor affinity. Facilitative transport systems are found in numerous tissue and cell lines including human red blood cells and macrophages in the periphery and at the blood-brain barrier (BBB). In both locations the molecular nature of the carrier is thought to be analogous. The protein transporter is characterized by a molecular weight in the 23,000 range, 12 membrane-spanning domains, and an oligosaccharide N-linked to an asparagine (Asn) (45) residue (Kalaria and Harik 1986; Plagemann et al. 1988).

In the human erythrocyte, the nucleoside transporter functions as a simple carrier displaying directional symmetry. Freshly obtained human red blood cells also exhibit differential mobility with the carrier-laden protein moving faster than the empty transporter through the cell cytosol (Plagemann et al. 1988). The nucleoside carrier in erythrocytes accepts both pyrimidines and purine nucleosides and both deoxy- and ribonucleosides. The transporter does not have significant affinity for ionized substrates. A few examples for substrates of the nucleoside carrier are given in table 2.

TABLE 2. *Michaelis constants for selected nucleoside transport in mammalian cells.*

Nucleoside	K_m (μM)
Adenosine	50-150
5'-deoxyadenosine	115
Thymidine	150-250
Uridine	170-300
Cytidine	2000-4000
Deoxycytidine	500-700
Cytosine arabinoside	250-750

SOURCE: Modified from Plagemann et al. (1988).

The brain is separated from the peripheral circulation by the BBB, a vascular barrier that results from the tight joining of component endothelial cells at the level of the cerebral microvasculature (Bradbury 1992; Neuwelt 1989; Rapaport 1976). The BBB is lipoidal in nature and effectively prevents water-soluble compounds and metabolites from gaining access to the brain parenchyma. However, the cerebral capillary system, the anatomical locus of the BBB, has evolved specialized systems of facilitative carriers to provide for central nervous system (CNS) metabolic needs and to provide an avenue for the elimination of waste products (Pardridge et al. 1975).

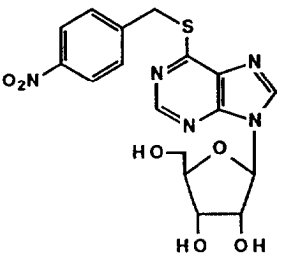
As in the periphery, there is a specialized facilitative carrier system for nucleosides at the BBB (Hertz 1991; Kalaria and Harik 1986, 1988; Spector 1986). The carrier molecule is similar in structure to the erythrocyte proteinaceous transporter but is associated with a higher degree of selectivity. The BBB system transports ribonucleosides more efficiently than deoxyribonucleosides and purine nucleosides more efficiently than pyrimidine systems (Spector 1986). The preference for purines may by related to the importance of adenosine which, as mentioned earlier, is known to act in several neuromodulatory pathways. Adenosine, through its inhibitory effect on adenyl cyclase mediated by A 1 receptor stimulation, or via its stimulatory effect on adenyl cyclase

associated with A2 interaction, is known to reduce neuronal activity, act as a neuroprotectant, and inhibit convulsions (Daval et al. 1991). The antagonistic effect of caffeine on adenosine receptors is thought to be its mechanism of CNS stimulation. Given these effects, the careful regulation of adenosine release and brain uptake are essential to provide an appropriate neuronal environment.

The extent of uptake of a particular nucleoside is a function of both the transporter affinities and the circulating blood levels of the nucleosides (Spector 1986). Thus, for several substrates BBB uptake is poor even in the presence of a functioning carrier due to the relatively high K_m value of the carrier and relatively low circulating blood levels of nucleoside. In several cases, however, the high plasma levels (i.e., as with uridine in humans) make BBB uptake the predominant CNS source of various nucleosides.

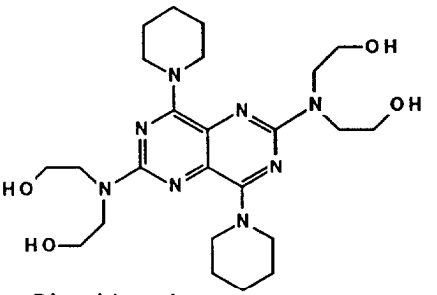
A second mechanism for nucleoside uptake is concentrative. At the choroid plexus and in certain intestinal cells, an Na^+-dependent, energy-dependent, saturable carrier system has been described for nucleosides (Plagemann et al. 1988). This carrier, unlike the facilitative system at the BBB, transports ribonucleosides and deoxyribonucleosides with roughly equal affinity (Spector 1986). The active transport system has been shown to concentrate uridine and thymidine intracellularly to levels more than 10 times plasma concentrations. Influx at the choroid plexus is thought to be the predominant mechanism for CNS uptake for thymidine and deoxycytidine.

Antagonists of nucleoside uptake have been of enormous value in identifying and characterizing the carrier systems (figure 1). The most studied group of antagonists inhibits transport of the facilitative carrier; these antagonists are not effective in inhibiting influx associated with the active (choroid plexus) carrier. The most potent inhibitors are the nitrobenzylthiopurines and, in particular, nitrobenzylthioinosine (NBTI). This nucleoside analog inhibits nucleoside flux in the low nM range and is the defining antagonist for the classical facilitative carrier systems (Plagemann et al. 1988). These analogs are thought to act competitively at the nucleoside binding site. A second important transporter antagonist is the pyrimidopyrimidine, dipyridamole. This compound inhibits facilitative nucleoside transport in the mid to high nM range and is used clinically as a coronary vasodilator as well as to inhibit platelet aggregation. The pharmacological action of this agent is thought to be related to its action on nucleoside transport. Inhibition of adenosine uptake will



Nitrobenzylthioinosine

Nitrobenylthioinosine (NBTI) - potent inhibitors of nucleoside transport and important derivative for defining the transporter.



Dipyridamole

Dipyridamole - clinically used anticoagulant

FIGURE 1. *Antagonists of nucleoside transport.*

increase local concentration of adenosine extracellularly, where the nucleoside can exert its vasodilatory and platelet-aggregating effects. Other possible mechanisms include the inhibitory action of dipyridamole on phosphodiesterase and the possible augmentation of prostacyclin concentrations. Since dipyridamole is structurally distinct from nucleosides, the inhibitory mechanism on nucleoside uptake of this lipophilic derivative may be associated with partitioning into the plasma membrane and interaction with the hydrophobic regions of the transporter (Grem and Fischer 1989). The action of these inhibitors on intracellular nucleotide pools has also been exploited to enhance the action of several anticancer or antiviral agents including 5-fluorouracil, methotrexate, and azidothymidine (Grem and Fischer 1989).

TABLE 3. *Nucleobase transport.*

1. Facilitative
   a. Erythrocyte
      i. Low specificity (cross-transports nucleosides)
      ii. High specificity
   b. Blood-brain barrier
      i. Hypoxanthine
      ii. Others
2. Passive

Nucleobase uptake is also thought to be regulated by a family of proteinaceous carriers that act to facilitate delivery of hypoxanthine, adenine, guanine, and uracil (table 3).

While in some cell lines the nucleobase carrier is thought to be identical with the nucleoside system, in human erythrocytes two systems are likely present (Domin et al. 1988). The nucleobase transporter in these systems conforms to a simple carrier model that exhibits directional symmetry and equal mobility when loaded or empty. This transporter is insensitive to NBTI or dipyridamole. Some suggestions have been posited that adenine and uracil have individual carriers that can be differentiated from the hypoxanthine transporter, but the data are not conclusive.

At the BBB, hypoxanthine, adenine, and uracil appear to be transported on a common carrier that exhibits saturability and is nonconcentrative. The K_m for hypoxanthine in this system is 400 μM, which is 100 times higher than the circulating blood levels of the nucleobase, suggesting that only small amounts of purines gain access to the CNS via this route under normal circumstances (Spector 1987).

Passive uptake of natural nucleosides and nucleobases is limited. The rate of (nonsaturable) passive uptake is highly correlated with molecular lipophilicity as measured by the octanol/water partition coefficient (Balzarini et al. 1989; Lien et al. 1991). In some cases, especially with small, highly water-soluble derivatives, a correction for molecular volume is needed to give adequate correlation with lipophilicity data (Plagemann et al. 1988). Interestingly, diffusion-mediated uptake shows a similar temperature dependence as does facilitative diffusion, meaning

that Arrhenius approaches, especially activation energies, are ill-suited to differentiate between passive and facilitative diffusion.

MECHANISM OF NUCLEOSIDE AND NUCLEOBASE DRUG UPTAKE

A number of drugs have affinity for the nucleoside and nucleobase facilitative carriers and the active nucleoside transporter at the choroid plexus. These include close structural nucleoside analogs such as arabinosides (cytosine arabinoside) as well as nucleobase analogs (6-thioguanine, 6-mercaptopurine, and 5fluorouracil). By contrast, some analogs of natural nucleosides do not have affinities for the carrier systems and gain intracellular access by passive diffusion. The following discussion is directed to the mechanism of cellular and CNS uptake for several recently approved or clinically investigated antiviral agents.

Azidothymidine (zidovudine, AZT) was the first drug approved for the treatment of AIDS. The drug must be taken up into cells where it is converted to its 5'-triphosphate and subsequently inhibits HIV replication at the level of reverse transcriptase. In addition, incorporation of AZT into the growing proviral DNA strand results in chain termination due to the absence of 3'-hydroxy function. The transport of AZT into human erythrocytes does not take place against a concentration gradient; it is a linear function of concentration, meaning that it is not saturable and is insensitive to inhibitors of nucleoside and nucleobase transport. These data suggest that AZT passively diffuses into erythrocytes because of its lipophilicity, which is higher than thymidine (Zimmerman et al. 1987, 1989).

Since AZT is taken up by passive diffusion, and thymidine by facilitative carrier-mediated diffusion, the possibility exists to modulate the action of AZT through modification of intracellular nucleotide pools. Such approaches have been shown to be useful in vitro in that dipyridamole has been demonstrated to increase the effectiveness of AZT in inhibiting HIV replication in monocyte-derived macrophages (Betageri et al. 1990). The mechanism for the action is associated with the uptake of thymidine, which results in the formation of thymidine triphosphate, a competitive inhibitor for the action of AZT-triphosphate at reverse transcriptase. Dipyridamole blocks the uptake of thymidine, which depletes intracellular pools of thymidine triphosphate, which ultimately increases the effectiveness of AZT.

At the BBB, the unidirectional uptake of AZT is low, suggesting that AZT does not have affinity for the BBB nucleoside transporter and is not sufficiently lipophilic to gain access by passive diffusion at this site (Terasaki and Pardridge 1988). This has led to the suggestion that AZT is actively transported at the choroid plexus to the cerebrospinal fluid (CSF) in a manner similar to thymidine (Collins et al. 1988). This supposition is consistent with the significant level of AZT detected in CSF subsequent to oral or intravenous (IV) administration of the antiretroviral agent. If the CSF route represents the predominant CNS source for AZT, the measured levels of AZT in the CSF may severely overestimate the level of AZT at its pathologically relevant site (i.e., in the brain parenchyma). This is suggested by microdialysis experiments that show that the concentration of AZT in CSF is only 15 percent of plasma levels, and brain extracellular fluid concentrations are about one-half the CSF concentrations (Wong et al. 1992, 1993).

3'-Deoxythymidine-2'-ene (d4T) is a potent inhibitor of HIV replication that has been examined in clinical trials. In the H9 human lymphocyte cell line, the uptake of d4T is a linear function of concentration, meaning that it is not saturable. In addition, transport is not inhibited by other nucleosides, inhibitors of nucleoside uptake, or nucleobases. The octanol to water partition coefficient of d4T is intermediate between AZT and thymidine. The data suggest that like AZT, d4T enters cells by passive diffusion (August et al. 1991).

Acyclovir (ACV) is a clinically useful antiherpetic agent with a large therapeutic index. The molecular basis for the safety of this compound derives from the fact that its metabolic anabolism to the active ACV-triphosphate is initiated only in virally infected cells (i.e., only in those cells expressing viral thymidine kinase) (Elion et al. 1977). After the formation of the monophosphate, cellular enzymes convert the acyclic nucleotide to the triphosphate, where it can interact with DNA polymerases.

In human red blood cells, ACV uptake is nonconcentrative and is saturable. Inhibitors of nucleoside influx such as NBTI and other nucleosides with affinity for the nucleoside transporter, however, have little influence on ACV transmembrane movement. On the other hand, hypoxanthine, adenine, and guanine significantly inhibit ACV flux, suggesting that ACV is transported on the nucleobase carrier into erythrocytes (Mahony et al. 1988). Brain microdialysis studies indicate ACV concentrations in the CNS extracellular fluid of approximately

one-third of that found in plasma indicating some propensity for ACV to enter the CNS (Stile and Öberg 1992).

Gancyclovir (DHPG) is also a clinically used acyclic nucleoside that has useful antiviral action. DHPG is especially active against cytomegalo-viral infections, including those of the brain and eye, which have emerged as important opportunistic infections in AIDS (Drew et al. 1988; Quinnan et al. 1984). Like ACV, DHPG inhibits cytomegalovirus (CMV) through the action of its triphosphate, but unlike ACV, DHPG is not selectively phosphorylated in CMV-infected cells as this virus does not code for a viral thymidine kinase. The activation of DHPG is very efficient in CMV-infected cells probably due to the action of virally induced host enzymes (Faulds and Heel 1990; Verheyden 1988).

In human erythrocytes, DHPG uptake is nonconcentrative and is saturable. Inhibitors of nucleoside transport such as NBTI have some inhibitory effect on transport, but only at high concentrations. Inhibitors of nucleobase transport, including adenine, block DHPG uptake at low DHPG levels. These data, as well as the fact that both adenine and dilazep (another inhibitor of nucleoside transport) must be present to completely inhibit DHPG uptake, suggest that DHPG is primarily transported by the nucleobase system and secondarily by the nucleoside carrier (Mahony et al. 1991). Carbovir, a carbocyclic analog of dideoxyguanosine, has a similar uptake pattern and interacts with erythrocytes similarly (Mahony et al. 1992).

6-Methoxypurine arabinoside (Ara-M) and its valerate prodrug exert antivaricella zoster action. The prodrug, in addition to being more lipophilic than the parent compound, is more stable metabolically since it is a poorer substrate for adenosine deaminase, the enzyme responsible for demethoxylation and inactivation. Consistent with the uptake of other arabinosides, Ara-M was found to be transported on the nucleoside carrier into blood cells based on the inhibitory effect of NBTI and other nucleosides (Prus et al. 1992). In contrast, the uptake of the prodrug is not saturable and not inhibited by NBTI or nucleobases, indicating that it passively diffuses through membranes.

These data suggest that saturable carrier-mediated transport of nucleosides or their analogs can be converted to nonsaturable passive diffusion by simple molecular manipulations. Such conversions in mechanism may be useful especially when membrane transport is the rate-limiting step in pharmacological action. From the foregoing

discussion, an increase in the lipophilicity of a nucleoside can improve its movement through biological membranes by passive diffusion, and the higher the lipophilicity (as measured by the log P) the greater the uptake will be into blood cells and the CNS (Levin 1980). Such manipulation can be effected by preparation of lipophilic analogs or prodrugs such as in the case of Ara-M. The drawback to these simplistic approaches is specificity. While extraction of a lipophilic species from the blood may increase the concentration of the conjugate in the tissue of interest, it will likely increase in other tissues as well, leading to a generally increased tissue burden (Stella and Himmelstein 1980). This nonselectivity can result in increased toxicity as a function of the increased efficacy with no resulting change in the therapeutic index of the drug. One proposal to increase tissue specificity, thereby reducing nontarget site toxicities, is the chemical delivery system (CDS) described and developed by Bodor (Bodor 1987; Bodor and Brewster 1991, pp. 231-284; Bodor et al. 1981). While there are several CDSs possible, a method for CNS application has been most thoroughly investigated. This type of CDS utilizes biological barrier properties and physiochemistry to provide for site selectivity. The technology involves the covalent attachment of a redox targetor to the compound of interest, which provides for an increase in brain uptake due to enhanced lipophilicity. Unlike simple prodrugs, however, the targetor is designed to undergo an enzymatically mediated oxidation that converts the membrane-permeable transport system into a hydrophilic, membrane-impermeable conjugate. This polar conjugate is readily eliminated from the systemic circulation but is retained behind the BBB, generating a favorable brain versus blood concentration ratio as a function of time. The locked-in conjugate can then hydrolyze, releasing the parent drug with some selectivity in the CNS. While a variety of targetors have been examined, derivatives of the dihydronicotinate-nicotinate redox couple have proven to be the most successful. The CDS scheme is summarized in figure 2. Application of the approach to a number of drugs has been reported, and clinical trials on an estradiol-based CDS are ongoing.

APPLICATION OF A BRAIN-TARGETING DELIVERY SYSTEM TO NUCLEOSIDE ANALOGS

Brain-enhanced delivery of AZT and other antiretroviral drugs would be beneficial in the management of AIDS encephalopathy and the resulting dementia (Pajeau and Roman 1992; Reinvang et al. 1991). AIDS encephalopathy is caused by brain infection with the AIDS pathogen

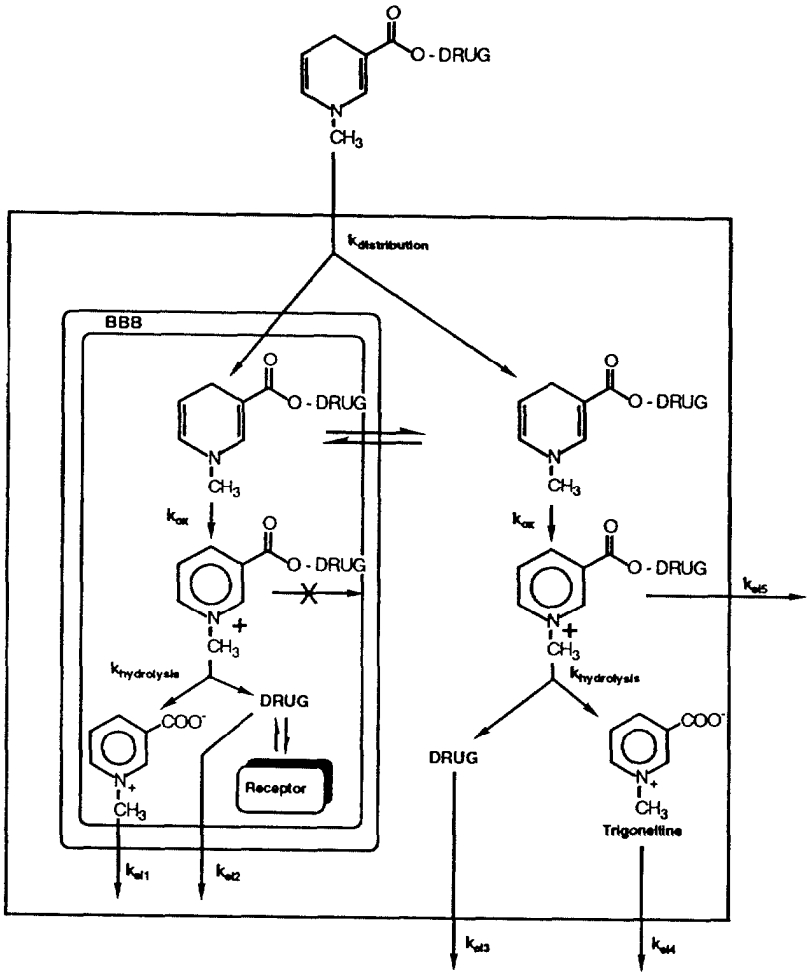


FIGURE 2. *A brain-targeting chemical delivery system (CDS).*

HIV- 1 and is associated with a constellation of debilitating symptoms that affect a large percentage of individuals stricken with the disease (Brew et al. 1988). Treatment of the central components of AIDS is difficult due to the inaccessibility of the infection site, which is protected by the BBB. The CDS approach has been applied by several groups to the CNS delivery of AZT in the hopes of increasing activity and decreasing peripherally mediated toxicity, including suppression of the bone marrow. Animal studies have shown that an AZT-CDS (5'-[(1-methyl-1, 4-dihydropyridin-3-yl) carbonyl]-3'azido-3'-deoxythymidine) can improve delivery and reduce nontarget site concentration of AZT.

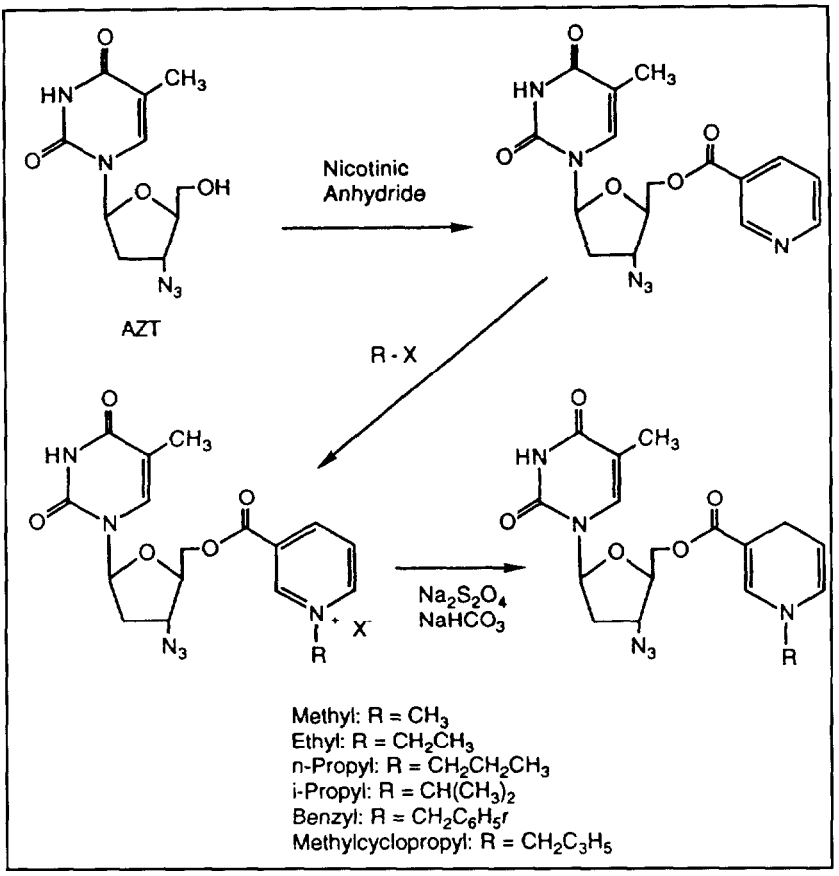


FIGURE 3. *Synthesis of AZT-CDS derivatives. Compound 7 represents the AZT-CDS benchmark.*

Experimental results found, for example, that systemic administration of the AZT-CDS to rats and dogs produced three times as much AZT in brain than did AZT treatment, and less AZT in blood than did AZT dosing (Brewster et al. 1991; Little et al. 1990). Other studies found significantly increased brain levels and improved brain-blood ratios in rabbits and mice (Chu et al. 1990). In addition, in vitro evaluation indicated that not only was the AZT-CDS more effective in inhibiting HIV replication than AZT, but it was also less toxic to the host cells (H9 lymphocytes) than AZT (Aggarwal et al. 1990). Recent studies have examined the effect of molecular manipulation of the effectiveness on the AZT-CDS. In these evaluations, a series of AZT-CDSs were prepared as illustrated in figure 3. The AZT-CDS derivatives were all more lipophilic

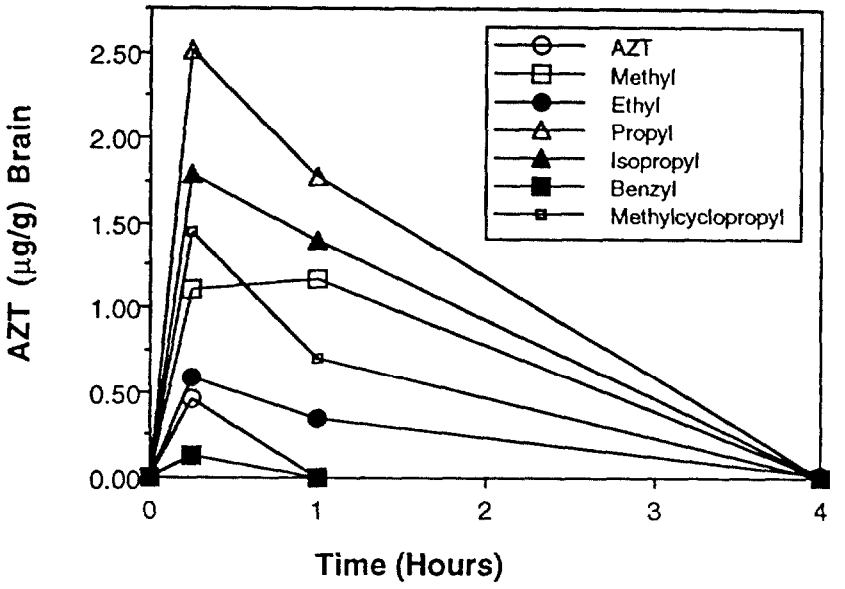


FIGURE 4. *Brain concentration of AZT after IV administration of AZT or various AZT-CDS derivatives at doses of 0.13 mmol/kg.*

as compared with AZT, suggesting facile membrane diffusion (Brewster et al. 1993).

AZT produced from the various AZT-CDS derivatives in brain and blood was determined in a rat model. In brain, AZT was readily released from the locked-in quaternary salt in all cases except for the benzyl derivative. The highest AZT concentrations were produced by the propyl analogs (2.5 µg/g). Brain concentrations of AZT were fairly sustained through 2 hours but fell to undetectable values by 4 hours (figure 4).

In comparing brain and blood levels of AZT subsequent to either AZT dosing or the AZT-CDS analog administration, it is clear that an advantage is seen for all of the AZT-CDS derivatives (figure 5). Blood levels of AZT are significantly lower and brain levels significantly higher after AZT-CDS treatment than after an equimolar dose of AZT. The data indicate that the propyl derivative is the best in this respect with a brain-blood ratio of 1.3 at 15 minutes. The ethyl and isopropyl analogs give a ratio of approximately 0.6 at 15 minutes compared to 0.025 for AZT itself. Importantly, as long as the quaternary salt is present in CNS, the ratio continues to increase. At 1 hour, the AZT brain-blood ratio was 1.6 for the propyl system, and small increases were seen for most of the

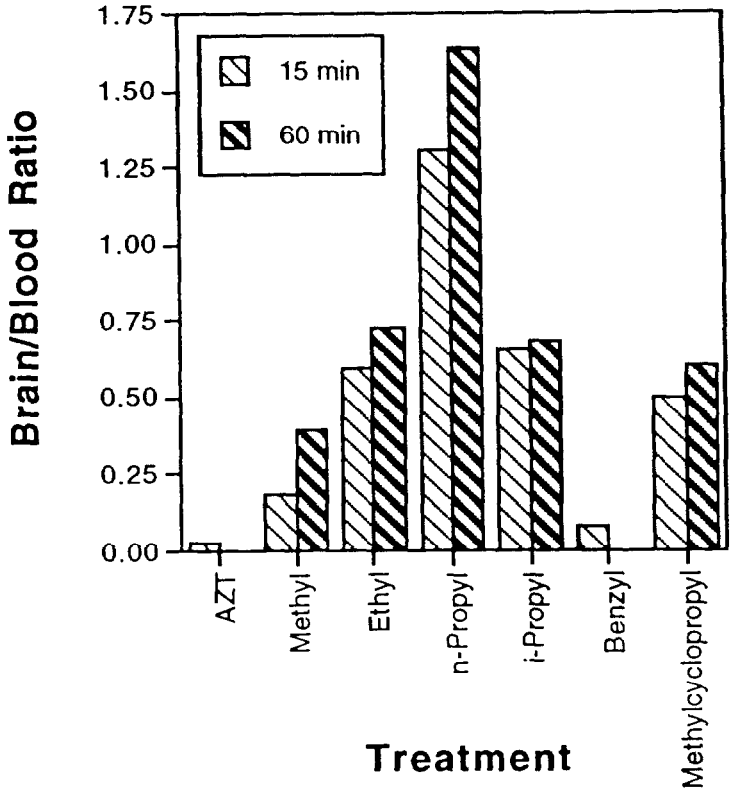


FIGURE 5. *Brain-blood ratios of AZT after treatment with AZT or various AZT-CDS derivatives.*

other systems, especially for the methyl derivative. Of the derivatives examined, the propyl appears to provide the greatest advantage over the prototype, methyl AZT-CDS. The superiority seems to be associated with fine tuning of the lipophilicity of the AZT-CDS as well as its corresponding pyridinum salt.

Brain-enhanced delivery of DHPG was also demonstrated using a redox-based CDS. A DHPG monoester in which a 1-methyl-1, 4-dihydro-nicotinate was covalently attached to one of the hydroxymethyl functions was prepared. The stability of the DHPG-CDS was evaluated in aqueous buffers and organ homogenates (Brewster et al. 1994).

In vivo distribution studies in the rat indicated that while DHPG poorly penetrated into the CNS and was rapidly eliminated, the DHPG-CDS

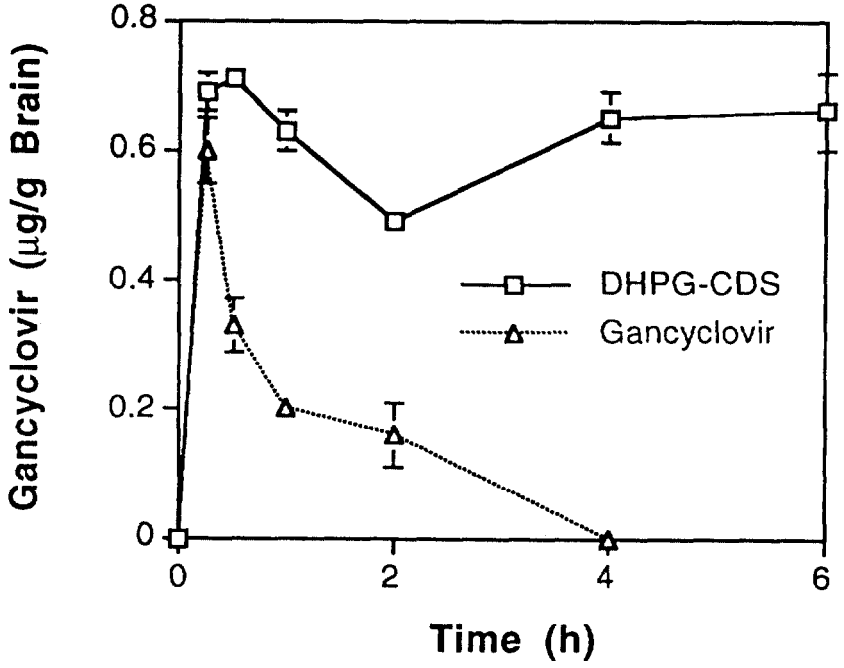


FIGURE 6. *Brain concentrations of gancyclovir after either DHPG-CDS or* gancyclovir.

provided for therapeutically relevant (2.7 μM) and sustained levels of the parent compound through 6 hours. An analysis of the area under the concentration curve (AUC,,) indicated that the CDS delivered more than five times more DHPG than the parent drug (figure 6). The high brain levels and reduced blood gave a 1 -hour brain-to-blood concentration ratio of 1.7 for DHPG when delivered by the CDS as compared to a ratio of 0.06 when the parent drug was administered (figure 7). These data suggest that the DHPG-CDS could be a useful adjunct for the treatment of CMV encephalitis. Similarly encouraging data have been produced for a CDS for ACV, d4T, trifluorothymidine, ribavirin, and several other antiviral nucleosides (Bhagrath et al. 1991; Bodor and Brewster 1991, pp. 231-284; Deyrup et al. 1991; Palomino et al. 1989; Rand et al. 1986).

UPTAKE OF OLIGONUCLEOTIDES

Antisense therapy approaches represent novel possibilities in the antiviral and anticancer therapeutic areas. The basis for these technologies involves the preparation of a complementary oligodeoxynucleotide

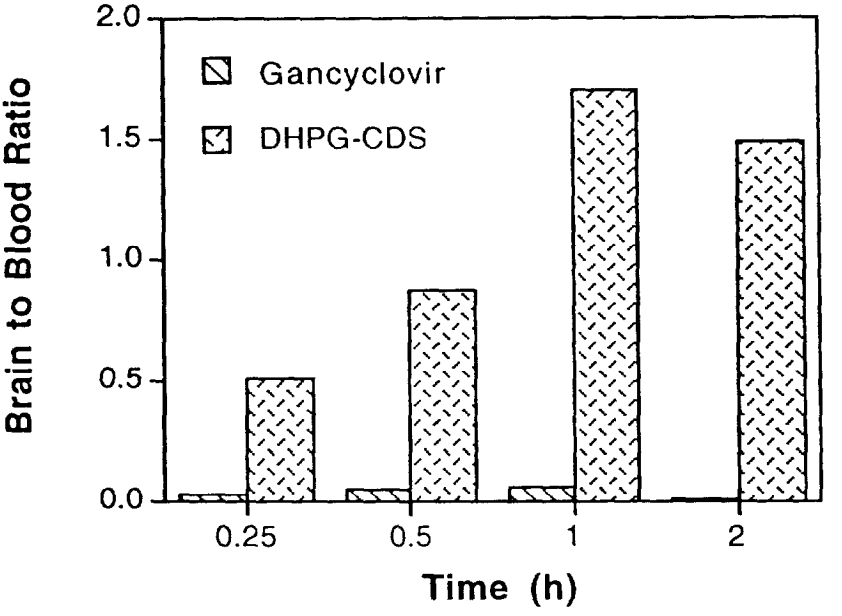


FIGURE 7. *Ratio of gancyclovir in brain and blood after gancyclovir or the DHPG-CDS.*

message to either single-stranded messenger RNA or double-stranded DNA (Milligan et al. 1993; Stein 1992). These fragments can then interact with the targeted mRNA or DNA segments resulting in hybridization through the formation of sequence-specific mRNA duplexes (via Watson-Crick base pairing) or triple helical DNA (e.g., via Hoogsteen base pairing) (Jones et al. 1993). These complexes act to inhibit gene expression by preventing mRNA processing and translation or DNA transcription or replication, the latter at the chromosomal level, thus inhibiting the expression of a particular gene product. The mRNA complexation results in inhibition of translation by several possible mechanisms including the physical blockade of the interon as well as the stimulation of RNase H, an enzyme that degrades the RNA portion of the RNA/DNA duplex rendering it untranslatable. The advantage of such manipulations is that they are exceedingly specific. An oligonucleotide of sufficient length (I 5 nucleotides or longer) will theoretically interact with high selectivity to the targeted complementary mRNA nucleotide sequence and with far lower affinity to other segments of the same or other nucleotide chains (Milligan et al. 1993).

There are several important limitations to the application of antisense drug approaches including in vivo stability of the message, penetration of cell membranes, and ultimately the specific, high-affinity binding of the oligonucleotide with the RNA fragment of interest. Thus, while antisense RNA-DNA interaction occurs endogenously, the hybridization of DNA to mRNA sequences is unnatural. The naturally occurring phosphodiester backbone can be used to form antisense fragments, and this approach has yielded several important examples of in vitro antisense action. It is generally agreed, however, that phosphodiester oligodeoxynucleotides represent poor therapeutic candidates because of their lability. Phosphodiesters are readily degraded by 3'-exonucleases and potentially by endonucleases (Stein 1992). These metabolic problems can be avoided by using backbones that are resistant to the action of nucleases. Such systems include phosphorothiolates, methylphosphonates, and phosphoramidates. These backbone structures lead to the production of a large number of diastereomers since the phosphorus atom is chiral in these derivatives, and this complicated isomeric mixture may reduce RNA-DNA binding.

Of the synthetic "unnatural" phosphorus-containing backbones, the phosphorothiolates have been the most extensively evaluated. These oligomers interact with their RNA-forming complexes with reduced temperature transitions (T,) as compared with phosphodiesters. These oligodeoxynucleotides are known to activate RNase H, a potential advantage over the system that does not stimulate this enzyme. The methylphosphonates backbone is uncharged and, as in the case of phosphonothiolates, chiral (Jones et al. 1993). Unlike the phosphonothiolates, methylphosphonates do not stimulate RNase H activity but form complexes characterized by higher T_m values. Other types of unnatural backbones include the family of achiral, nonionizable systems such as formacetals, 3'-thioformacetals, carbonates, and polyamides. These derivatives are resistant to nuclease cleavage but show variable binding to mRNA, with some derivatives displaying substantially lower affinity for the complementary RNA sequence and other similar or slightly higher complexation affinities.

Interestingly, while many publications have suggested that an antisense mechanism is responsible for the observed antiviral or other effects of various oligodeoxynucleotides, the majority of evidence is indirect (Milligan et al. 1993). This circumstance is even more complicated when the fact that the hydrolyzed oligodeoxynucleotide components can act as inhibitors is considered. It is evident that many of the inhibitory effects

of oligodeoxynucleotides once attributed to an antisense mechanism are now known to be mediated through nonantisense properties such as inhibition of DNA polymerase or inhibition of viral binding to the cell of interest. These nonantisense mechanisms are thought to play a major role in the pharmacology of the phosphorothiolates.

The mechanism by which oligodeoxynucleotides enter cells has been an area of debate. While DNA receptors have been identified on cell surfaces and do act to internalize DNA, these receptors are not thought to be important for the uptake of oligodeoxynucleotides (Milligan et al. 1993; Stein 1992). Other polyanionic receptors have been defined that do have affinity for oligodeoxynucleotides as well as dextran sulfates and other highly negatively charged biopolymers. These receptors may be similar to the family of CD4 receptors. In addition, pinocytosis or fluid-phase endocytosis may be associated with oligomer internalization. While it is clear that oligodeoxynucleotides bind to these anionic receptors and enter the cell by whatever mechanism, it has not been established whether uptake is associated with release of the intact oligodeoxynucleotide within the cytoplasm or nucleus. Fluorescence microscopy indicates that internalized vacuoles containing oligodeoxy-nucleotides form a punctate perinuclear pattern that resembles endosomes and lysosomes, the release from which is usually considered to be inefficient. Recently, similar conclusions were reached in the study of an oligodeoxynucleotide targeted to the gene responsible for expression of the SV40 large T antigen (TAg) and E. coli ß-galactosidase (Wagner et al. 1993). Introduction of the oligomeric antisense message into the culture media was without inhibitory effect even though they localized within the cells. In this case, as in others, the tagged oligodeoxy-nucleotide was found to be confined to endosomes and lysosomes.

Several mechanisms of increasing the effective uptake and intracellular release of oligodeoxynucleotides have been suggested. One important experimental technique for introducing material into the cell is microinjection (Wagner et al. 1993). Introduction of derivatized oligonucleotide into cells by this technique has resulted in clear demonstrations of antisense inhibition of gene product expression. As described in the example above, while an oligodeoxynucleotide designed to inhibit TAg is internalized into a cell via endocytosis, it is retained by the endosome resulting in no pharmacological activity. Microinjection of this same message leads to significant activity. Interestingly, treatment of the endosome-localized oligodeoxynucleotide system with lipofectin, a cell membrane permeabilization reagent, induces release of the oligomer

and provides for inhibition of TAg antigen (Wagner et al. 1993). These data suggest that an appropriately derivatized oligodeoxynucleotide can exert antisense activity if it can gain direct access to the cytosol or nucleus.

The uptake of phosphorothiolate oligomers has been reported to be stimulated by cationic lipids such as N-[1-(2,3-di-oleyloxy)propyl]-N, N, N-trimethylammonium chloride in certain cell types (Felgner et al. 1987). This cationic lipid forms liposomes that serve as delivery canisters. The action of facilitated transfection, termed lipofection, is thought to be associated with the ability of the charged lipid to facilitate fusion of the oligodeoxynucleotide-containing liposome with the plasma membrane. While uptake has been demonstrated in certain endothelial cells, the method appears to be ineffective in other cell types (Stein 1992).

Oligodeoxynucleotides that contain a methylphosphonate backbone are uncharged, and these derivatives may penetrate plasma membranes by passive diffusion. When tagged and followed with a fluorescent label, these compounds appear to be taken up by the same adaptive endocytosis processes responsible for the internalization of other oligodeoxy-nucleotides.

The use of chimeric peptides to deliver antisense messages has been suggested. Chimeric peptides are associated with specific receptors at the BBB and in other tissues that provide for receptor-mediated endocytosis, resulting in the transport of the peptide across the membrane system (Wagner et al. 1990). Such a delivery process was suggested by the attachment of an oligodeoxynucleotide to a chimeric substrate such as transferrin. The resulting conjugate did give improved cellular association. Other examples include a conjugate of an antisense message with asialo-glycoprotein, which increased cellular association and activity when targeted to the hepatitis B virus, and a polymannosylated oligodeoxynucleotide was shown to be internalized in liver cells via the mannose receptor. Such reports are of interest, but the delivery of the antisense message must be to the cytosol and nucleus, not to an impermeable endosome (Milligan et al. 1993). Demonstration of this delivery is a sine qua non to prove an antisense mechanism. Finally, the use of lipophilic modifying groups has been suggested. Thus an oligo-deoxynucleotide linked to cholesterol was found to be taken up more readily by certain cells than the underivatized message (Kreig et al. 1993). The effect could be associated with improved passive diffusion through the cell. On the other hand, the cholesterol function may interact

with low-density lipoprotein (LDL), which may then be further internalized into cells via the LDL receptor (Stein 1992). The delivery of oligonucleotides is a science in its infancy, but is clearly important in exploiting the antisense approach to more practical application.

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Oral Peptide Delivery: Improving the Systemic Availability of Small Peptides and Enkephalin Analogs

Hye J. Lee and Gordon L. Amidon

BACKGROUND

Peptide drug delivery has been of considerable interest over recent decades due in part to rapid developments in biotechnology and peptide synthesis, as well as the unique pharmacological activity of peptide drugs. Many investigators have attempted to develop strategies for peptide delivery. The principal difficulty in the use of these agents as drugs is the requirement for parenteral administration, since peptides generally have very low systemic availability when administered orally. Peptides suffer from a very large first-pass metabolism or hepatic extraction when administered orally (Amidon and Lee 1994), and may also suffer from low membrane permeability, low solubility, or both (Amidon et al. 1988, 1989). Various alternate routes for systemic administration of peptide drugs have also been investigated, including nasal, dermal, pulmonary, buccal, vaginal, ocular, and rectal routes. Furthermore, attempts have been made to use various absorption enhancers to increase the presystemic stability and membrane permeability of peptides and peptide drugs when administered via nonparenteral routes, but these attempts have met with only very limited success (Hussain et al. 1991; Kidron et al. 1982).

This chapter focuses on oral delivery of small peptides, the most preferred route of drug administration. For the purpose of this chapter, a peptide-type drug is defined as a drug composed of amino acids or amino acid analogs whose synthesis is based on some analogy with natural peptides and proteins. Small peptides are peptide drugs containing less than eight amino acids. The most relevant definition of oral drug absorption is drug absorption in the systemic circulation. This view emphasizes that the processes involved in drug transport from the gastrointestinal (GI) lumen to the systemic blood supply must be included to estimate bioavailability of orally administered drugs (Amidon and Lee 1994). Those include metabolism by the enzymes in the GI lumen, brush-border membrane (BBM), cytosol, portal blood, and the hepatic

first-pass metabolism, as well as the intestinal membrane permeation and hepatic uptake.

Among barriers for oral drug absorption, metabolism is generally recognized as particularly significant for peptides and peptide-type drugs. For peptide-type drugs that have no intestinal instability and are dosed below their solubility limit, the fraction of the dose absorbed (not systemic availability) can be correlated with intestinal membrane permeability as shown in figure 1 (Amidon et al. 1988). This positive correlation can be a useful guide in drug design. Generalizations regarding metabolism are more difficult because of the diversity and locations of the specific responsible enzymes. Proteolytic enzymes responsible for peptide metabolism during absorption are listed in tables 1, 2, and 3 (Amidon and Lee 1994). Peptide drugs stable to peptidase activity may still undergo metabolism by other detoxification systems in the body such as cyclosporine (Maurer 1985). Nevertheless, since

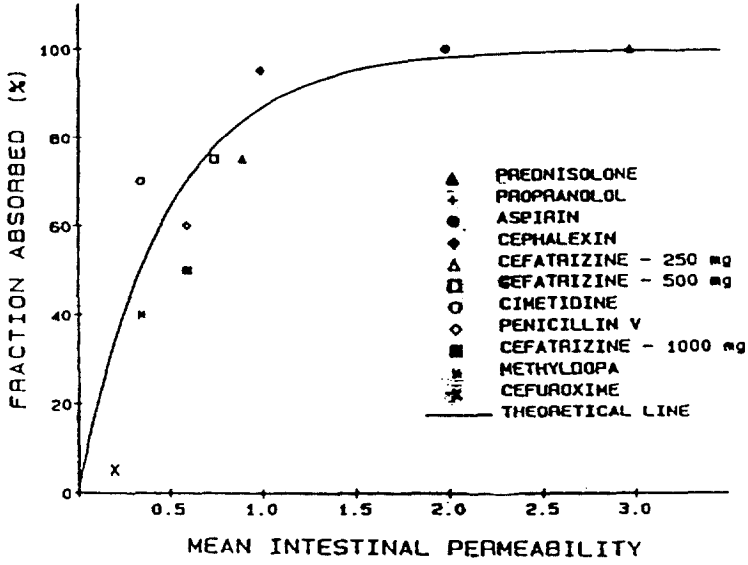

FIGURE 1. *Plot of the fraction of the dose absorbed (%) versus the mean dimensionless intestinal wall permeability Wall permeabilities were calculated from steady-state rat intestinal perfusion experiments.*

SOURCE: Amidon et al. (1988).

TABLE 1. *Luminal proteolytic enzymes and sample substrates.*

Enzyme	Substrate
Pepsin	Z-His-Phe-Phe-OMe
Trypsin	Benzoyl arginine methyl ester (BAEE)
α-chymotrypsin	Benzoyl tryosine ethyl ester (BTEE)
Elastase	Ala-Ala-Ala methyl ester
Carboxypeptidase A	Hippuryl phenylalanine
Carboxypeptidase B	Hippuryl arginine

enzymatic hydrolysis is the dominant cause of metabolism of peptide and peptide-type drugs, the authors attempted to characterize proteolytic enzymes.

This chapter discusses the development of a successful strategy for oral peptide delivery. Presentation of the results of characterization of the permeability and metabolic pathways of enkephalins, a pentapeptide, is followed by the pharmacokinetics (PK) and biopharmaceutics of [D-ala, D-leu]-enkephalin (YdAGFdL). YdAGFdL was chosen for in vivo study because it is a relatively stable enkephalin analog for in vitro metabolic study. The oral peptide delivery strategy, which employs specific enzyme inhibitor(s) and site-directed absorption, was then investigated with YdAGFdL in rats.

CHARACTERIZATION OF THE GI METABOLISM OF ENKEPHALINS

Effect of pH on the Metabolism of YGGFL, YdAGFL, and YdAGFdL

The stability of enkephalins is pH dependent and is greater in an acidic pH, as shown in figure 2 (Tamai et al. 1991). The pH-dependent loss of enkephalins is comparable to the results obtained using the in situ intestinal perfusion method (Friedman and Amidon 1991).

TABLE 2. *Typical intestinal BBM enzymes.*

Type	Specificity	Enzyme
Exopeptidase, NH2 terminus	Many amino acids	Aminopeptidase N
	Asp or Glu	Aminopeptidase A
	Amino acid-Pro	Aminopeptidase P
	Amino acid-Pro, -Ala,	Dipeptidylpeptidase IV
	γ -Glu	y-Glutamyltransferase
Exopeptidase, COOH terminus	Many amino acids	ACE
	Pro, Ala, Gly	Carboxypeptidase P
Exopeptidase dipeptidase	Many amino acids	Microsomal dipeptidase
Endopeptidase	Hydrophobic	Endopeptidase-24.11
	Aromatic	Endopeptidase-24.18

Effect of Peptidase Inhibitors on the Metabolism of YGGFL and YdAGFL

Figure 3 shows the effect of the peptidase inhibitors on leu-enkephalin (YGGFL) and [D-ala-leu]-enkephalin (YdAGFL) metabolism degradation. The peptidase inhibitors amastatin, thiorphan, and captopril were used for aminopeptidase, endopeptidase, and angiotensin converting enzyme (ACE), respectively. The results are expressed as the amount of enkephalin remaining after the reaction with intestinal BBM for 2 minutes. More than 95 percent of YGGFL was metabolized in the absence of peptidase inhibitors. Amastatin at 100 μM reduced the loss of YGGFL sixfold, while captopril and thiorphan were less effective. YdAGFL, which is designed to be resistant to metabolism by substituting ^{2}G to dA, is metabolized to 55 percent of the initial concentration in 2 minutes. These results show greater resistance to peptidase than the unsubstituted peptide YGGFL. Furthermore, amastatin is less effective in preventing YdAGFL metabolism than thiorphan. Captopril did not show a significant effect on YdAGFL metabolism at a concentration of 100 μM.

TABLE 3. *Intestinal brush-border cytosol enzymes with typical substrates,*

Enzymes	Typical Substrate
Dipeptidase	Neutral dipeptides
Aminopeptidase	Tripeptides with N-terminal Pro
Prolidase	lmidodipeptides with C-terminal Pro/Hyp
Prolinase	Imidodipeptides with N-terminal Pro/Hyp
Carnosinase	Camosine (ß -Ala-His)

A combination of amastatin and thiorphan showed an approximately additive effect resulting in a decrease in metabolism compared to amastatin alone. The combinations of amastatin and captopril, and thiorphan and captopril, did not have additive effects. In the case of YdAGFL, the combination of thiorphan with amastatin or captopril showed an additive effect that was not observed with the combination of amastatin and captopril. These results indicate that more than two pathways (enzymes) function simultaneously in the inactivation of the enkephalins. The results further suggest that aminopeptidase and endopeptidase are more important than ACE in the metabolism of YGGFL and YdAGFL.

Timecourse for the Metabolism of YGGFL in the Absence or Presence of Amastatin, Thiorphan, or Captopril

The timecourses for the metabolism of YGGFL, YdAGFL, and YdAGFdL are shown in figure 4. The metabolic pathway of YdAGFL is similar to that of YGGFL. This result suggests that substitution of ^{2}G to ^{2}dA has a comparable efficacy in the prevention of YGGFL hydrolysis with the coadministration of the aminopeptidase inhibitor. In the case of YdAGFdL, the metabolic fragment dAGFdL is quite stable, even after the reaction of YdAGFdL, with four times more membrane protein. No peptide fragments other than Y and dAGFL were found as metabolic products after 60 minutes.

Based on a timecourse metabolism study of enkephalins in the presence and absence of specific enzyme inhibitors using intestinal BBM, schematic pathways for the metabolism are suggested in figure 5. The kinetic analysis of each pathway from the metabolites formation curves

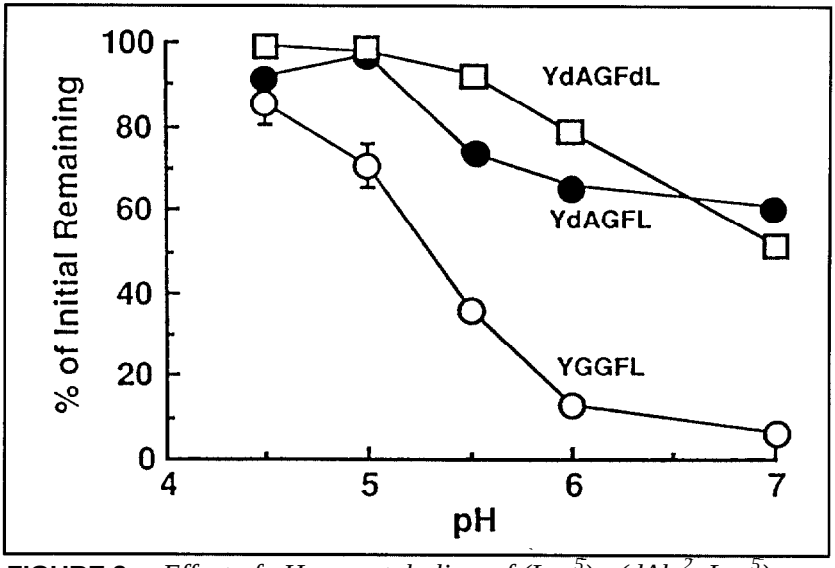

FIGURE 2. *Effect of pH on metabolism of (Leu^5)-, (dAla^2, Leu^5)-, and (dAla^2, dLeu^5)-enkephalins. Intestinal BBM was suspended in 10 mM Hepes/Tris buffer (pH 7.0) containing 100 µM KCl and 100 mM mannitol. In each experiment, 10 µL of the membrane suspension containing 25 µg membrane protein was used. The metabolism of 100 µM enkephalins was studied in 10 mM acetate (pH 4.5 and 5.0), Mes/Hepes (pH 5.5 and 6.0), and Hepes/Tris (pH 6.5 and 7.0) buffers at 25 °C. The ordinate represents the percentage of initial remaining intact peptides after 2 min ((Leu^5)-enkephalin, 0, and (dAla^2, Leu^5)-enkephalin, l) or 60 min ((dAla^2, dLeu^5) enkephalin Cl) reaction. Each point represents the mean SD for three experiments. When SD is not shown, it is less than the symbol.*

based on the assumption of first-order kinetics and the relative stability of three leucine enkephalins are summarized in figure 6. As clearly shown, the stability of enkephalins was significantly increased by the substitution of amino acids and by the addition of appropriate peptidase inhibitors.

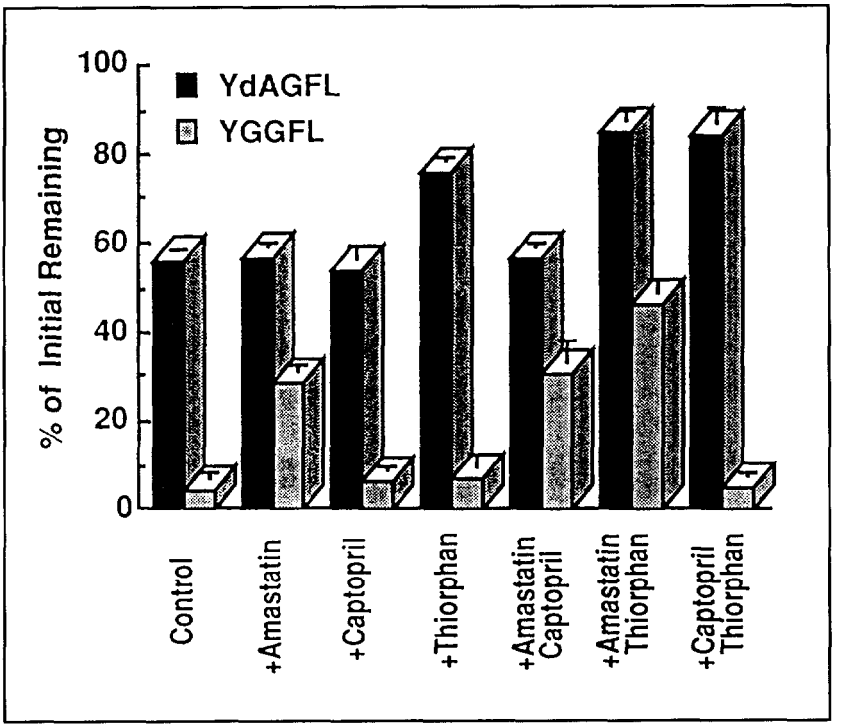

FIGURE 3. *Effect of peptidase inhibitors on the metabolism of (Leu^5)- and (dAla^2, Leu^5)-enkephalin. The metabolism of 100 µM enkephalins was studied in 10 mM Hepes/Tris buffer (pH 7.0) at 25 °C. The ordinate represents percentage of initial remaining of (Leu^5)-enkephalin (dotted bar) or (dAla^2, Leu^5)-enkephalin (closed bar) after 2 min reaction. Concentrations of amostatin, captopril, or thiorphan were 100 µM, 100 µM, or 10 µM, respectively, and used without preincubation. Each bar represents the mean ± SD for three experiments.*

GI PERMEABILITY OF ENKEPHALINS

Peptide and peptide-type molecules are often limited in absorption across the GI membrane due to their relative hydrophilicity and large molecular size. This limitation is known as the permeability barrier. The permeabilities (Pw) of enkephalins were measured for YdAGFL and a cyclic

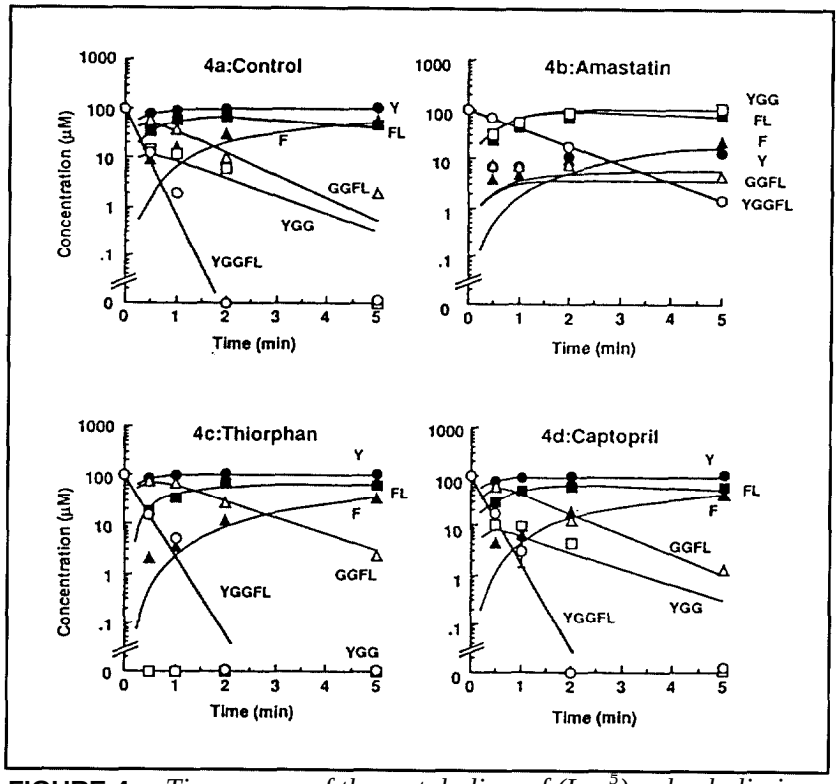

FIGURE 4. *Timecourse of the metabolism of (Leu[5])-enkephalin in the absence (panel a) or presence of amastatin (panel b), thiorphan (panel c), or captopril (panel d). The metabolism of 100 µM of (Leu[5])-enkephalin was studied in 10 mM Hepes/Tris buffer (pH 7.0) at 25 °C. When peptidase inhibitors were used, they were preincubated with BBM for 20 min. The concentrations of amastatin, captopril, and thiorphan were 10 µM, 100 µM, and 10 µM, respectively. Each point represents the mean ±SD for three experiments. When SD is not shown, it is less than the symbol. The peptic fragments shown in the panels are YGGFL (○). GGFL (△), YGG (□), FL (■), F (▲), and Y (●). The solid lines represent theoretical concentrations of each peptide fragment or amino acid calculated from kinetic parameters.*

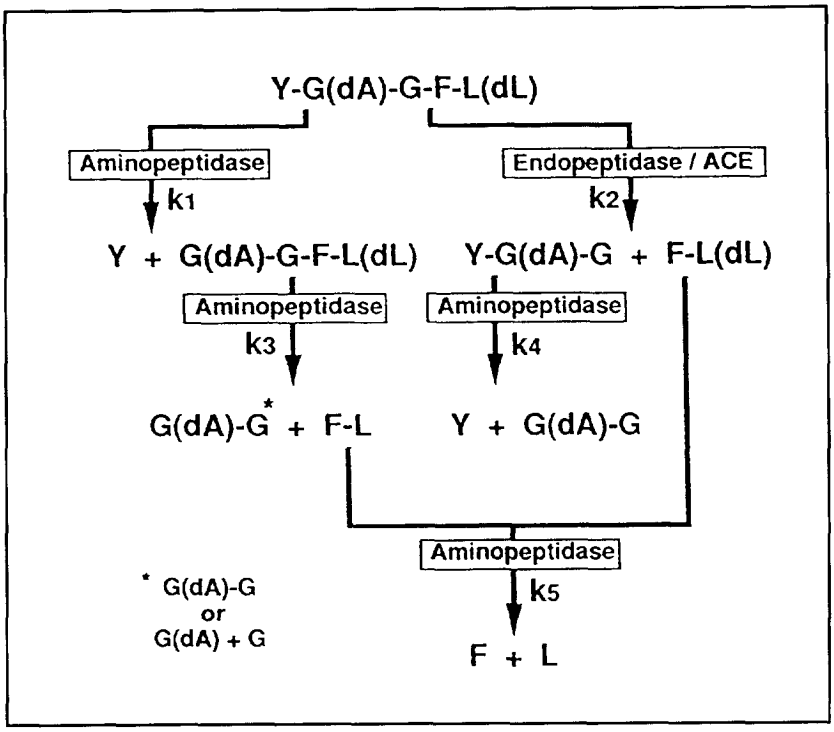

FIGURE 5. *Schematic pathways for the metabolism of enkephalins by intestinal BBM. (Leu^5)-enkephalin: YGGFL; (dAla^2, Leu^5)-enkephalin: YdAGFL; and (dAla^2, dLeu^5)-enkephalin: YdAGFdL.*

KEY: *By the assay method used in the present study, the dipeptides G(dA)-G are not distinguishable from their composing amino acids, G(dA) and G.

pentapeptide derivative, [D-pen^2, D-pen^5]-enkephalin (DPDPE), in the presence of peptidase inhibitors using rat intestinal single-pass perfusion (Sherman and Amidon 1992). As a result, Pw values of 1.40 and 1.45 were found for YdAGFL and DPDPE, respectively. Since compounds with Pw values greater than 1.0 are considered to be well absorbed (Amidon et al. 1989), membrane permeability is not believed to be a limiting factor for poor absorption of enkephalins.

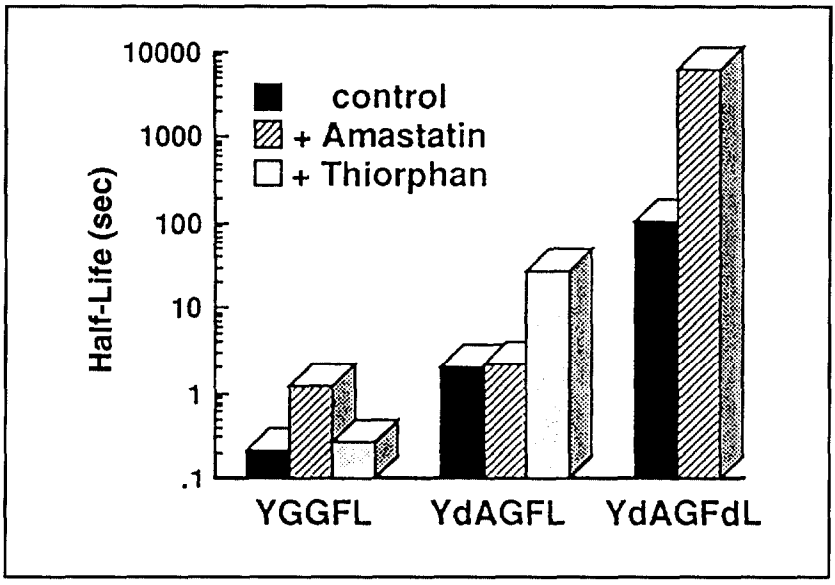

FIGURE 6. *Comparative stabilities of enkephalins and the effect of peptidase inhibitors. The result is expressed by half-life (sec/mg protein), estimated from the kinetic parameters.*

BLOOD STABILITY OF ENKEPHALINS

The blood stability of peptides and peptide-type drugs is not only important to interpreting in vivo data accurately but also to estimating first-pass extraction following oral administration. Mean blood transit time from the GI wall to the systemic blood is about 13 seconds (Lee and Chiou 1989). The mean portal blood transit time is approximately 2 to 3 seconds (Lee and Chiou 1989) and transit time for the liver is about 10 seconds (Lee and Chiou 1989). The existence of proteolytic enzymes in the blood (Lee, in press) and the short half-lives (less than 1 minute) of some peptides in blood suggest the possibility that blood metabolism makes a significant contribution to the first-pass metabolism of peptide drugs.

In studies of serum and plasma stability of enkephalin analogs, half-lives for YGGFL and YGGFM in rats were less than 30 seconds and about 7 minutes in humans (Venturelli et al. 1985). Modified analogs with d-Ala or cyclic bonds increase blood stability of enkephalins (Benovitz and Spatola 1985; Schulteis et al. 1989).

LIVER METABOLISM OF ENKEPHALINS

To achieve successful oral peptide delivery, an understanding of the mechanisms of metabolism and uptake in the liver may be necessary. The rate and extent of metabolism for enkephalins had been studied using rat hepatocytes (Sherman and Amidon 1992). The relative stability of YGGFL, YdAGFL, YdAGFdL, and the cyclic pentapeptide enkephalin derivative DPDPE were found to be 0 percent. 37 percent, 61 percent, and 91 percent, respectively, after 40 minutes in an isolated rat hepatocyte suspension containing 40 mg of protein. After treatment with various enzyme inhibitors such as amastatin, bestatin, and thiorphan, it was determined that the major enzyme involved in liver metabolism is aminopeptidase or like enzymes.

IMPROVING IN VIVO ABSORPTION OF YdAGFdL IN RATS

The PK and biopharmaceutics of the model peptide YdAGFdL were characterized following intravenous (IV), peroral (PO), jejunal, and ileal administration in rats (Lee and Amidon, submitted). The effects of enzyme inhibitors and absorption site on the systemic availability (F) of YdAGFdL were evaluated (Lee and Amidon, submitted), and the results are summarized below.

IV PK of YdAGFdL After Dosing of 0.28 and 500 µg

Figure 7 shows blood concentration-time data for YdAGFdL averaged from six rats after IV administration of 0.28 µg and 500 µg doses. Mean blood concentrations for each sampling point were obtained for two different doses after normalization with a dose of 0.28 pg. The two blood profiles are similar except for the terminal phase. The significance of this difference in the terminal phase is uncertain due to limited assay sensitivity at these low concentrations. As expected for a peptide, YdAGFdL was rapidly eliminated from the blood. The PK parameters including total body clearance (CL) and volume of distribution (Vd) were not significantly different between the two different doses (table 4). The CL values for YdAGFdL at the doses of 0.28 and 500 µg were 42.7±10.7 and 48.0±9.50 (SEM) mL/min, respectively. These estimates are similar to the cardiac output of a rat, which ranges from 15 to 80 mL/min (Van Dongen et al. 1990, pp. 287-289). These results suggest that YdAGFdL may be rapidly metabolized in blood, possibly by membrane-bound

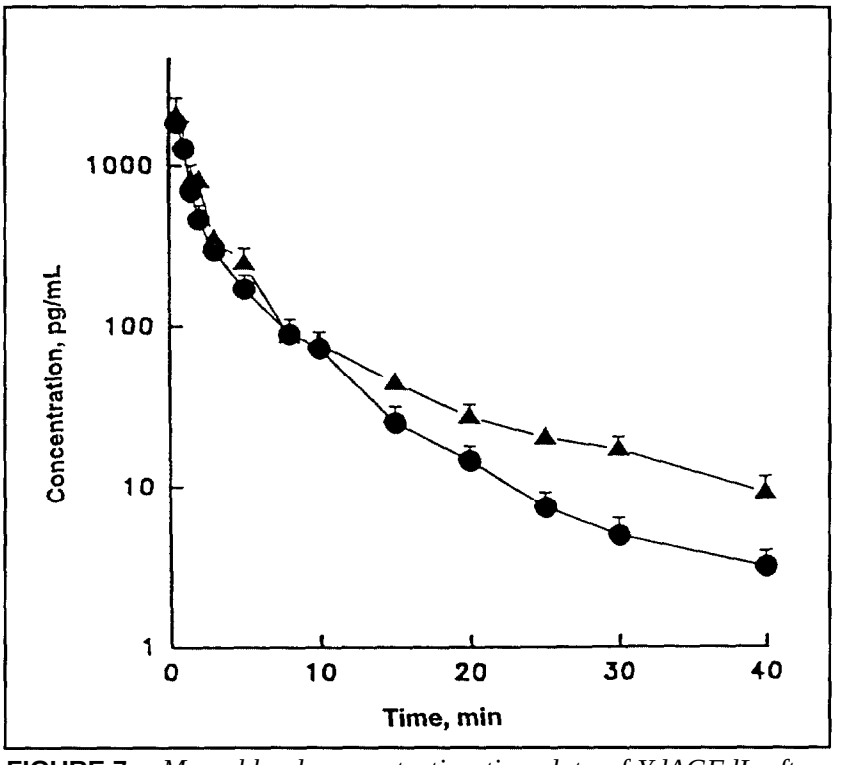

FIGURE 7. *Mean blood concentration-time data of YdAGFdL after IV dosing of 0.28 (●) and 500 µg (▲) to six rats in a crossoverfashion. Mean blood concentrations for each sampling point were obtained from two different doses after normalization by the dose of 0.28 µg. Error bars represent the standard error of the mean (SEM).*

endothelial cell aminopeptidases. Among the three possible radiolabeled tyrosine-containing metabolites, only tyrosine was seen in blood after various routes of administration including IV administration.

PK of YdAGFdL After Various Oral, Jejunal, and Ileal Doses in Rats

The mean YdAGFdL blood profiles after various PO and ileal dosing (1.12, 1,000, and 5,000 µg) in six fistulated rats are shown in figures 8 and 9, respectively. All blood concentrations shown are based on the radiolabeled YdAGFdL. As shown in figure 8, there were no clear

TABLE 4. *PK parameters of YdAGFdL following IV administration.*

Parameters	Dose (µg)	
	500	0.28
CL (mL/min)	48.0 ± 9.50	42.7 ± 10.7
Vd (mL)	71.9 ± 19.8	76.9 ± 20.0
tα (min)	0.588 ± 0.102	0.477 ± 0.069
tß (min)	6.81 ± 1.27	3.98 ± 0.375
a (1 min)	1.38 ± 0.276	1.71 ± 0.323
ß (1 min)	0.13 ± 0.030	0.184 ± 0.016

NOTE: Values are mean±SEM.

KEY: tα = distribution phase half-life
tß = elimination phase half-life
α = distribution rate constant
ß = elimination rate constant

differences in blood concentrations among different doses, indicating dose-independent absorption, distribution, metabolism, and elimination after oral administration within the range of dose tested. However, time for maximum blood concentration (Tmax) after oral dosing appears to vary greatly. This could be due to variation in gastric emptying, enzyme distribution, blood flow, or intestinal contents of the GI tract. The blood concentrations in 6 of 11 PO studies were below the detection limit, indicating negligible absorption of YdAGFdL over the 50-minute experimental period, No significant dose dependency in the PK was observed from ileal administration (figure 9). In contrast to the oral studies, the blood concentration of YdAGFdL was not detectable in only 1 of 16 ileal studies. This finding indicates better and more reproducible absorption of YdAGFdL after ileal administration than after oral administration.

The F values of YdAGFdL after PO administration of 1.12, 1,000, and 5,000 µg of YdAGFdL were 0.39 ± 0.33 (SEM), 0.42±0.46, and 0.38±0.46 percent, respectively, while those after ileal administration were 1.27±0.71, 2.14±0.70, and 1.94±0.86 percent. The bioavailabilities

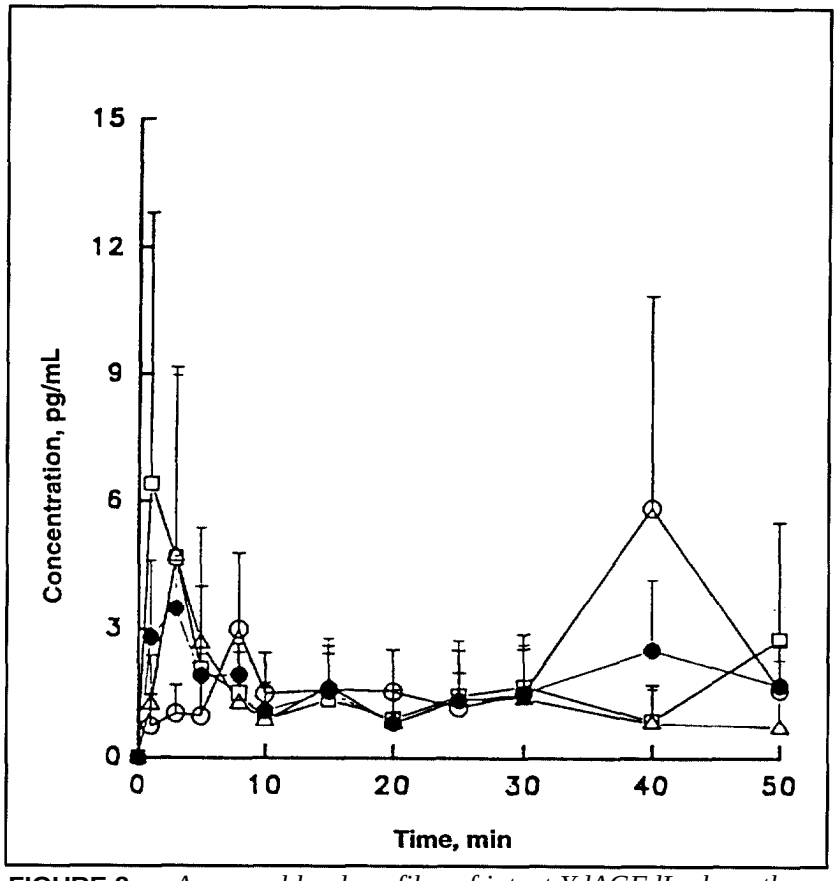

FIGURE 8. *Average blood profiles of intact YdAGFdL along the time after various oral dosing of 1.12 (○), 1,000 (△), and 5,000 µg (□), to six rats, respectively. Values are mean (N = 6)±SE, and closed circles (●) indicate the pooled mean values from three dosing levels after normalization by 1.12 µg dose.*

were not significantly different among doses in either oral or ileal administration. On average the bioavailability of YdAGFdL was improved about fivefold after ileal administration when compared to oral administration.

Blood profiles after jejunal administration of YdAGFdL are shown in figure 10 with the mean blood profiles after IV, PO, and ileal administration. The average blood concentrations of YdAGFdL over 50 minutes were in the following order: ileal > jejunal > PO administration. The

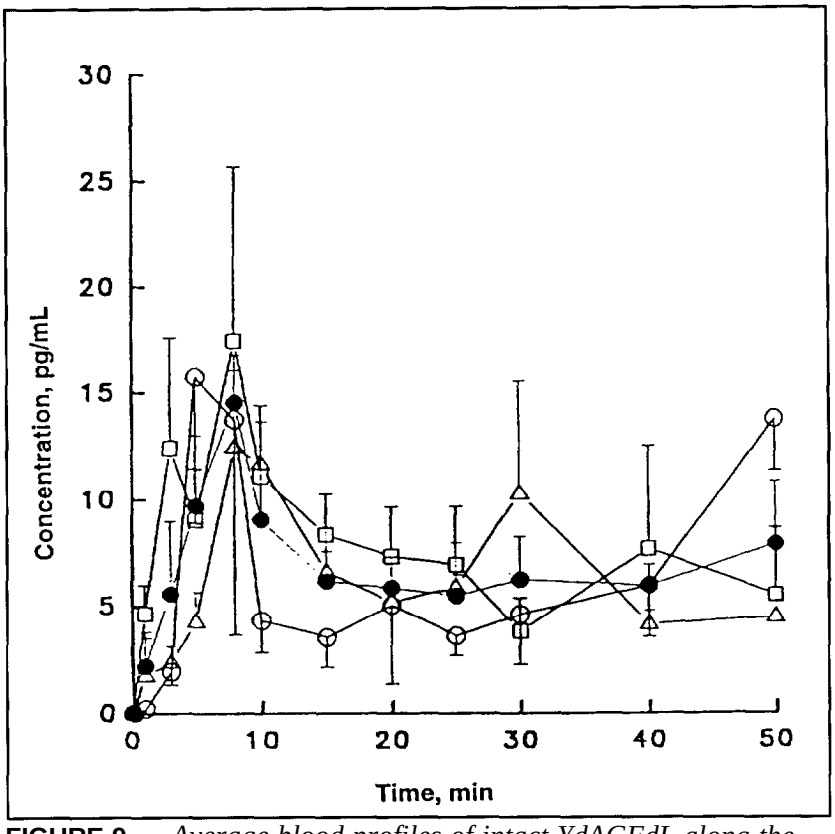

FIGURE 9. *Average blood profiles of intact YdAGFdL along the time after various ileal dosing of 1.12 (○), 1,000 (△), and 5,000 µg (□), to six ruts, respectively. Values are mean (N = 6)±SE, and closed circles (●) indicate the pooled mean values from three dosing levels after normalization by 1.12 µg dose.*

overall mean absolute bioavailabilities were 0.40, 1.25, 1.78, and 8.76 percent for oral, jejunal, ileal, and ileal with inhibitor administration, respectively (table 5).

The Effect of Amastatin on the Ileal Absorption of YdAGFdL

The comparison of YdAGFdL blood profiles after coadministration with amastatin to those without amastatin in individual experiments is shown in figure 12. Amastatin (1 mg) was observed to increase the bioavailability of YdAGFdL to 8.76 ± 4.47 percent (table 5). In separate

TABLE 5. *Absolute bioavailabilities of YdAGFdL after various routes of administration and ileal co-administration of amastatin.*

	Oral	Jejunal	Ileal	Ileal/Inhibitor
No. of exp.	15	6	16	6
F ± S E	0.40 ± 0.24	1.25 ± 0.39	1.78 ± 0.40	8.76 ± 4.47
% in F	Control	213	345	2,090

NOTE: % increase in $F = \frac{F.\ inhibitor - F.\ control}{F,\ control} \times 100$

experiments, simultaneous IV administration of YdAGFdL and PO administration of the inhibitor were performed in two rats (not shown).

No significant changes in the IV PK parameters were observed. These results suggest that the inhibitor, when administered orally, does not affect the systemic clearance and is consistent with its effect on local GI metabolism.

CONCLUSION

The kinetics of enkephalin metabolism in intestinal BBM are rapid relative to passive permeation of even the relatively stable peptide YdAGFdL. The use of metabolic inhibitors is effective in reducing metabolism. Since the wall permeability of enkephalins are all above a value of 1, factors limiting oral absorption for enkephalins seem to be metabolism. In vivo absorption of the stable model peptide YdAGFdL was about 0.4 percent following oral administration. The absolute bioavailability of YdAGFdL was improved about fivefold when delivered to the ileum, and about tenfold in the ileum in the presence of amastatin. The strategy for oral peptide delivery employing a selective enzyme inhibitor and a specific absorption site was successful.

These results demonstrate that a peptide with five amino acids can be absorbed through the GI membrane in a site-dependent manner. Moreover, the present study demonstrates that the systemic availabilities of peptides can be significantly increased by preventing metabolic degradation of peptides by the brush-border enzymes. In addition to the brush-border enzymes, the GI luminal enzymes, cytosolic, portal blood,

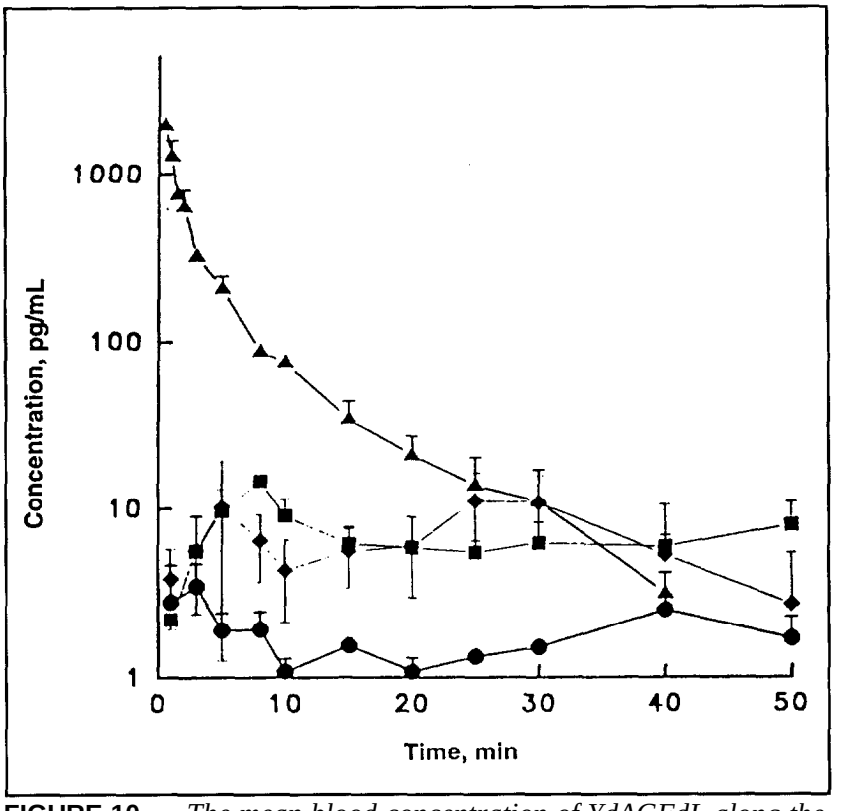

FIGURE 10. *The mean blood concentration of YdAGFdL along the time after intravenous (▲), peroral (●), jejunal (♦), and ileal administration (■) to rats. Values are mean ± SE (N= 15 or 16 for oral and ileal, 4 for jejunal, and 12 for IV)±SE. The doses are 0.28 µg for IV and 1. 12 µg for PO, jejunal, and ileal administrations.*

These results demonstrate that a peptide with five amino acids can be absorbed through the GI membrane in a site-dependent manner. Moreover, the present study demonstrates that the systemic availabilities of peptides can be significantly increased by preventing metabolic degradation of peptides by the brush-border enzymes. In addition to the brush-border enzymes, the GI luminal enzymes, cytosolic, portal blood, and liver enzymes may contribute to the poor systemic availability of peptides. Therefore, it is important to clarify the metabolism of peptides in the GI lumen, portal blood, and the liver, as well as the transport mechanism across BBM and the liver, in order to improve the oral delivery system for peptide and peptide-type drugs.

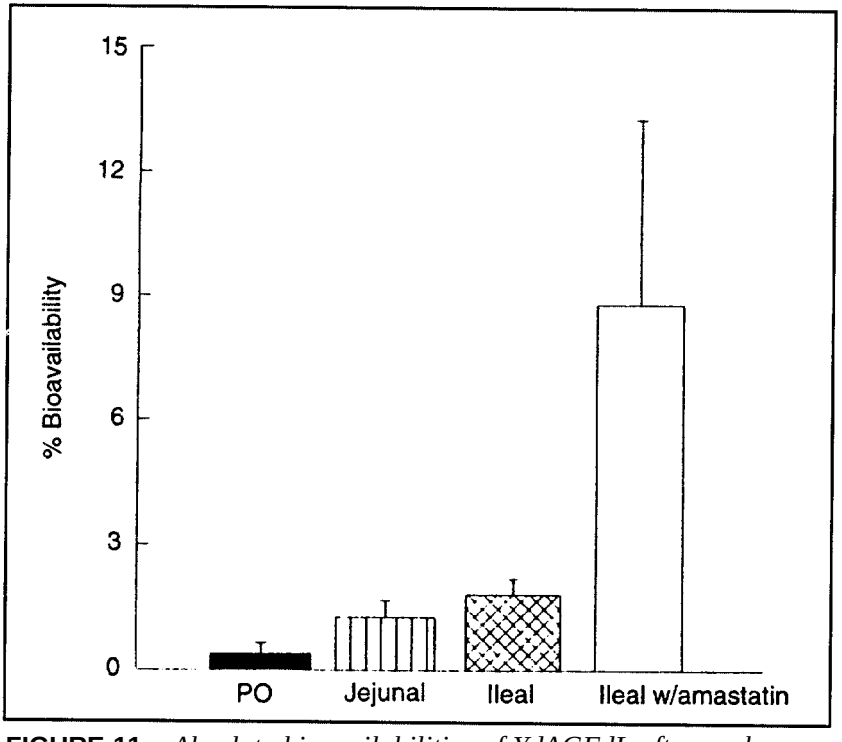

FIGURE 11. *Absolute bioavailabilities of YdAGFdL after oral, jejunal, ileal, and ileal with amastatin administration with SEM bars.*

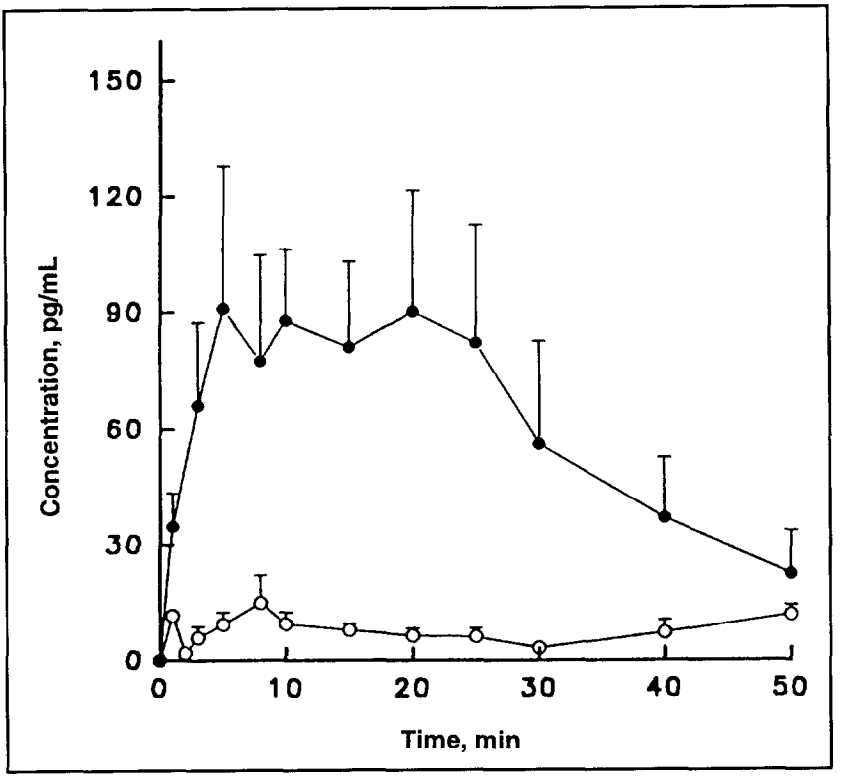

FIGURE 12. *Mean blood concentration-time data of YdAGFdL after ileal administration in the presence of amastatin (●), compared to the those in the absence of amastatin (○) in six chronically fistulated rats.*

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The Use of Polymers in the Construction of Controlled-Release Devices

Jorge Heller

INTRODUCTION

Controlled-release polymeric systems can be classified according to the mechanism that controls the release of the therapeutic agent as shown in table 1. Of these, only diffusion-controlled, water penetration-controlled, and chemically controlled systems are covered in this chapter. Regulated systems, also known as modulated systems, have recently been exhaustively reviewed (Heller 1993a).

This chapter presents a brief theoretical description of each specific methodology followed by representative applications selected, when possible, from commercially available products.

DIFFUSION-CONTROLLED SYSTEMS

These systems can be subdivided into two categories: monolithic devices and membrane-controlled devices. Because the mechanisms of drug release of these two types of devices are fundamentally different, they are discussed separately.

Monolithic Devices

In a monolithic device, the therapeutic agent is dispersed in a polymer matrix, and its release is controlled by diffusion from the matrix into the surrounding environment. Mathematical treatment of diffusion depends on the solubility of the agent in the polymer, and it is necessary to consider two separate cases. In one case, the agent is incorporated into the polymeric matrix below its solubility limit and is completely dissolved. In the other case, the agent is incorporated into the polymeric matrix well above its solubility limit and exists as a dispersion.

TABLE 1. *Classification of controlled-release systems.*

Type of System	Rate-Control Mechanism
Diffusion Controlled	
Reservoir devices	Diffusion through membrane
Monolithic devices	Diffusion through bulk polymer
Water Penetration Controlled	
Osmotic systems	Osmotic transport of water through semipermeable membrane
Swelling systems	Water penetration into glassy polymer
Chemically Controlled	
Monolithic systems	Either pure polymer erosion (surface erosion) or combination of erosion and diffusion (bulk erosion)
Pendant chain systems	Combination of hydrolysis of pendant group and diffusion from bulk polymer
Regulated Systems	
Magnetic or ultrasound	External application of magnetic field or ultrasound to device
Chemical	Use of competitive desorption or enzyme substrate reactions. Rate control is built into device

For an agent that is dissolved in the polymer, release can be calculated by two equations. Equation 1 is known as early-time approximation, and equation 2 is known as late-time approximation (Baker and Lonsdale 1974, pp. 15-71).

$$\frac{dM_t}{dt} = 2M_X \left[\frac{D}{\pi l^2 t}\right]^{1/2} \quad (1)$$

$$\frac{dM_t}{dt} = \frac{8DM_x}{1^2} \exp \frac{\pi^2 Dt}{1^2} \quad (2)$$

These equations predict rate of release from a slab of thickness 1, where D is the diffusion coefficient, M_x is the total amount of agent dissolved in the polymer, and M_t is the amount released at time t. According to equation 1, which is valid over the first 60 percent of release time, the rate decreases as the square root of time. During the latter 40 percent of the release time, the rate decays exponentially as shown by equation 2. Plots of these two approximations are shown in figure 1.

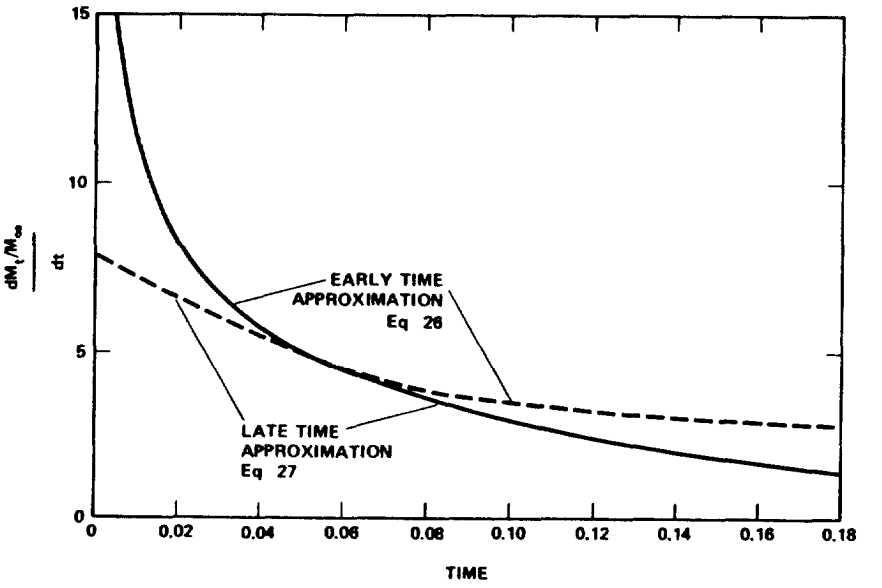

FIGURE 1. *Plot of the release rate of drug initially dissolved in a slab as a function of time, using early-time and late-time approximations. The solid line shows the portion of the curve over which the approximations are valid ($D/1^2=1$).*

SOURCE: Reprinted from Baker and Lonsdale (1974).

When the agent is dispersed in the polymer, release kinetics can be calculated by Higuchi equation 3 (Higuchi 1961).

$$\frac{dM_t}{dt} = \frac{A}{2}\left[\frac{2DC_SC_0}{t}\right]^{1/2} \quad (3)$$

In equation 3, A is the slab area, C_s is the solubility of the agent in the matrix, and C_0 is the total concentration of dissolved and dispersed agent in the matrix. In this case, the release rate decreases as the square root of time over the major portion of the delivery time and deviates only after the concentration of the active agent remaining in the matrix falls below the saturation value, C_s.

Membrane-Controlled Devices

In a membrane-controlled device, the therapeutic agent is contained in a core surrounded by a thin polymer membrane and is released to the surrounding environment by diffusion through the rate-limiting membrane.

When the membrane is nonporous, diffusion can be described by Fick's first law,

$$J = -D\frac{dC_m}{dx} \quad (4)$$

where J is the flux in g/cm^2-sec, C_m is the concentration of the agent in the membrane in g/cm^3, dC_m/dx is the concentration gradient, and D is the diffusion coefficient of the agent in the membrane in cm^2/sec.

Because the concentration of the agent in the membrane cannot be readily determined, equation 4 can be rewritten using partition coefficients that describe the equilibrium ratio of the saturation concentration of the agent in the membrane to that in the surrounding medium.

$$J = \frac{DK\Delta C}{l} \quad (5)$$

where ΔC is the difference in concentration between the solutions on either side of the membrane, K is the partition coefficient, and 1 is the thickness of the membrane. Reservoir devices can also be constructed with microporous membranes that have well-defined pores connecting the two sides of the membrane. Here, diffusion occurs principally through the liquid-filled pores, and the flux is described by equation 6:

$$J = \frac{EDK\Delta C}{\tau 1} \tag{6}$$

where E is the porosity (i.e., number of pores per unit area) of the membrane and τ is the tortuosity (i.e., average length of channel traversing the membrane).

One of the most important differences between monolithic systems and membrane-controlled systems is that in a membrane-controlled system the flux, J, will remain constant provided that the membrane material does not change with time so that D, K, E, and τ remain constant. Most importantly, AC must also remain constant. The practical consequences of this latter requirement are that the concentration of the agent in the core must not change with time and that the agent released from the device must be able to rapidly diffuse away from the device. Constant agent concentration in the core can be achieved by dispersing the agent in a medium in which it has a low solubility so that the solution always remains saturated.

Ensuring that the concentration of an agent does not increase around the device is not always possible, particularly with agents that have a very low water solubility. Deviations from zero-order kinetics are known as boundary layer effects. In an extreme case, the concentration of the agent around the device reaches the concentration of the agent in the core. When this occurs, AC = 0, and J = 0. In this particular case, rate of release is completely determined by the rate at which the agent can diffuse away from the device.

Another factor that contributes to deviations from zero-order release kinetics is migration of the agent from the core into the membrane on storage. Then, when the device is placed in a release medium, initial release is rapid because the agent diffuses from the saturated membrane. This nonlinear portion of the release is known as the burst effect.

Applications

Controlled-release devices that rely on diffusion as the rate-control mechanism are widely used. The decision as to whether monolithic or membrane-controlled devices are used is for the most part dictated by the needs for constant release, control of manufacturing costs, and safety.

Although reservoir devices are capable of achieving very long-term constant-release profiles, their manufacture can be expensive, and the safety of the device could be of some concern because rupture of the membrane can rapidly release the entire contents of the core. Thus, in many applications where device cost is an important factor, inexpensive matrix-type devices are used even though release rates decline with time. The utilization of such devices is very common in the veterinary and agricultural fields, where low cost is of paramount importance.

When cost is not an overriding consideration, such as in human therapeutics, then reservoir-type devices are an excellent choice and a number of such devices are currently available. These include an ocular insert containing pilocarpine, which is inserted in the lower cul-de-sac for control of glaucoma, an intrauterine contraceptive device containing progesterone, and a contraceptive silicone implant. The complete silicone implant consists of six silicone capsules, 20 x 2 mm, which are designed for implantation in the upper arm and contain the contraceptive steroid levonorgestrel. They are capable of maintaining a therapeutically effective concentration of levonorgestrel for as long as 5 years. The excellent control over levonorgestrel blood plasma levels is shown in figure 2 (Diaz et al. 1983, pp. 482-487).

To date, the most commercially successful use of diffusion-controlled systems is in transdermal applications. In these devices, a polymeric delivery system is held on the skin by an adhesive. The device contains the drug either in a reservoir with a rate-controlling membrane or dispersed in a polymer matrix. In less sophisticated devices, the drug is dispersed in the adhesive. A schematic of a membrane-controlled device is shown in figure 3. The drug is released from these devices through the skin and is taken up by the systemic circulation. Because the outer layer of the skin, the stratum comeum, is highly impermeable to most drugs, either drugs that readily traverse the stratum comeum must be used or the flux of the drug through the skin must be augmented by the use of penetration enhancers or electric current, as in iontophoresis or electroporation (Guy 1992).

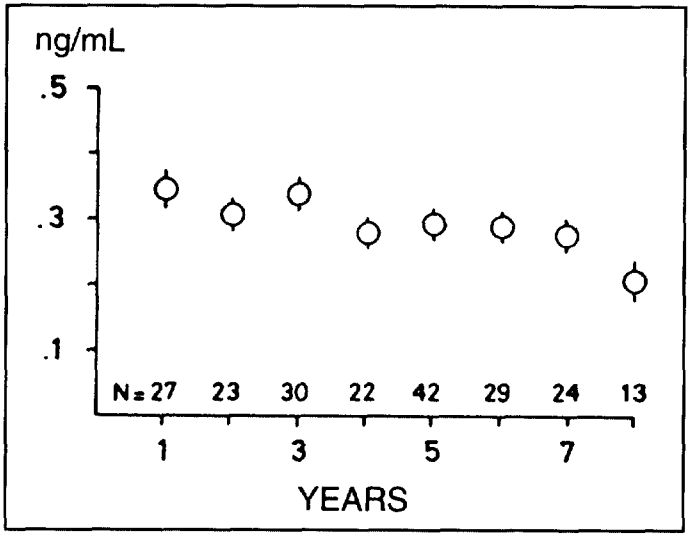

FIGURE 2. *Levonorgestrel plasma levels during long-term use of a contraceptive silicone implant subdermal implant. N=number of samplings per year. Each symbol represents the mean±SE of the averages calculated for the sampling runs of each year. Each sampling consists of 6-13 samples drawn from one subject during 4-6 consecutive weeks.*

SOURCE: Reprinted from Diaz et al. (1983, pp. 482-487).

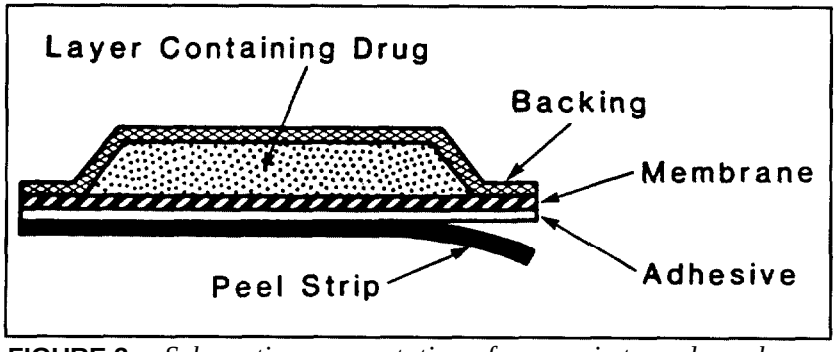

FIGURE 3. *Schematic representation of a generic transdermal device.*

SOURCE: Reprinted from Baker and Heller (1989, pp. 15-71).

WATER PENETRATION-CONTROLLED SYSTEMS

In water penetration-controlled delivery devices, rate control is achieved by the penetration of water into the device. Two general types of this device are in use. In one type, the driving force is provided by osmosis; in the other type, the driving force is provided by swelling.

Osmotically Controlled Devices

The operation of an osmotic device is illustrated in the schematic representation in figure 4 (Theeuwes and Yum 1976). In this device, an osmotic agent is contained within a rigid housing and is separated from the therapeutic agent by a movable partition. One wall of the rigid housing is a semipermeable membrane and, when the device is placed in an aqueous environment, water is osmotically driven across this semipermeable membrane. The resultant increase in volume within the osmotic compartment exerts pressure on the movable partition, which then forces the therapeutic agent out of the device through the delivery orifice.

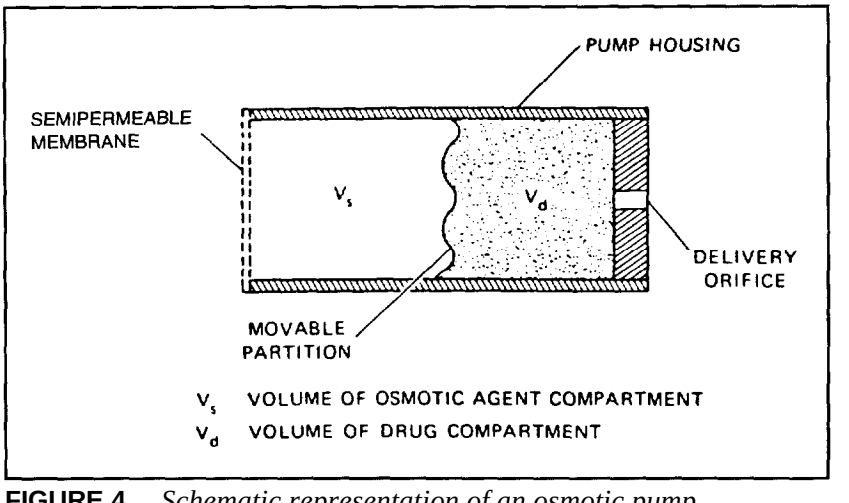

FIGURE 4. *Schematic representation of an osmotic pump.*

SOURCE: Reprinted from Theeuwes and Yum (1976).

The volume flux of water across the semipermeable membrane is expressed as:

$$\frac{dV}{dt} = \frac{A}{1} L_p[\sigma\Delta\pi - \Delta P] \quad (7)$$

where dV/dt is the volume flux, An and AP are, respectively, the osmotic and hydrostatic pressure differences across the semipermeable membrane, L_p is the membrane mechanical permeability coefficient, σ is the reflection coefficient, and A and 1 are, respectively, the membrane area and thickness.

The rate of delivery, dM/dt, of the agent is then expressed as:

$$\frac{dM}{dt} = \frac{dV}{dt} C \quad (8)$$

where C is the concentration of the agent in the solution that is pumped out of the orifice.

Applications. Two types of osmotic devices are currently in use. One device is a capsule approximately 2.5 centimeters long and 0.6 cm in diameter. It is an experimental device that can be implanted in the tissues of animals, where it delivers a chosen therapeutic agent at known, controlled rates. The chosen agent is placed in an impermeable flexible rubber reservoir that is surrounded by an osmotic agent, which in turn is surrounded and sealed within a rigid cellulose acetate membrane (Theeuwes and Yum 1976).

In an aqueous environment, water is osmotically driven across the cellulose acetate membrane, and the resultant pressure on the rubber reservoir forces the agent out of the orifice. The device, shown in figure 5, is sold empty and is filled with the desired therapeutic agent by the user. Because the driving force is osmotic transport of water across the cellulose acetate membrane, the rate of release of the agent from the device is independent of the surrounding environment.

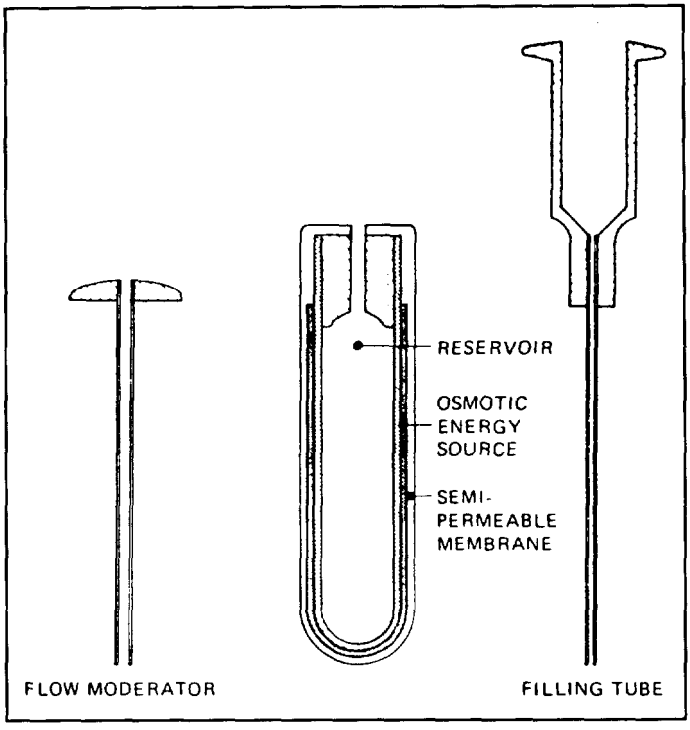

FIGURE 5. *Osmotic pump and components.*

SOURCE: Reprinted from Theeuwes and Yum (1976).

A second type of device is shown in figure 6 (Theeuwes 1975). This device is intended for oral applications and is manufactured by compressing an osmotically active agent into a tablet, coating the tablet with a semipermeable membrane, and drilling a small hole through the coating with a laser. When placed in an aqueous environment, water is driven across the semipermeable membrane, and a solution of the agent is pumped out of the orifice. A major advantage of this device is that a constant rate of release is achieved as it traverses the gastrointestinal tract.

However, such a device can function only with water-soluble drugs that provide the osmotic driving force. A different device, known as a push-pull device, has been developed for excessively water-soluble drugs and water-insoluble drugs (Theeuwes 1979, pp. 157-176). The system consists of two compartments separated by a flexible partition. The top compartment contains the solid drug and has a delivery orifice to the

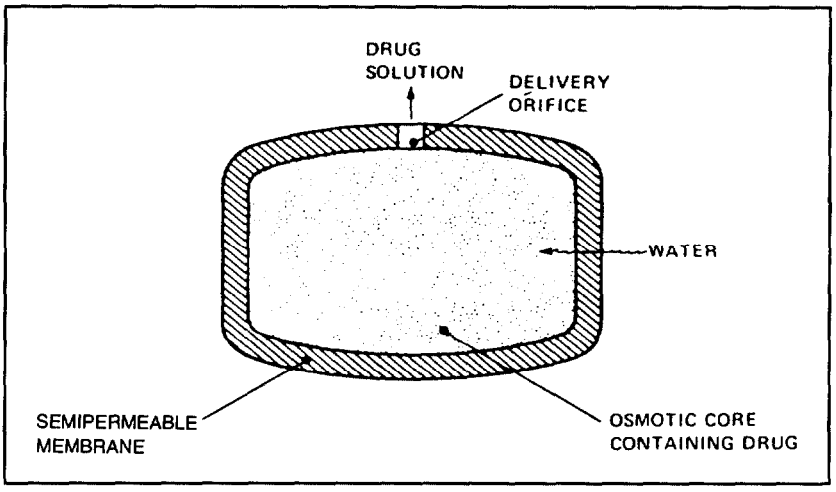

FIGURE 6. *Elementary osmotic pump cross-section.*

SOURCE: Reprinted from Theeuwes (1975).

outside, while the bottom compartment contains a solid osmotic driving agent formulation. A semipermeable membrane surrounds both compartments and separately regulates the influx of water into each. In operation, the drug compartment draws water in at one rate, while the osmotic driving compartment absorbs water at a different rate and, in expanding, exerts pressure against the top compartment. This latter osmotic force is designated as "push," while the former (drug compartment) osmotic force is designated as "pull."

Swelling-Controlled Devices

In this type of delivery system, the agent is dispersed in a hydrophilic polymer that is glassy in the dehydrated state but which swells when placed in an aqueous environment. Because diffusion of molecules in a glassy matrix is extremely slow, negligible release occurs while the polymer is in the glassy state. When such a material is placed in an aqueous environment, however, water penetrates the matrix and, as a consequence of swelling, the glass transition temperature of the polymer drops below the temperature at which the release studies are being carried out and the drug diffuses from the polymer.

The process, shown schematically in figure 7, is characterized by the movement of two fronts (Langer and Peppas 1983). One front, the

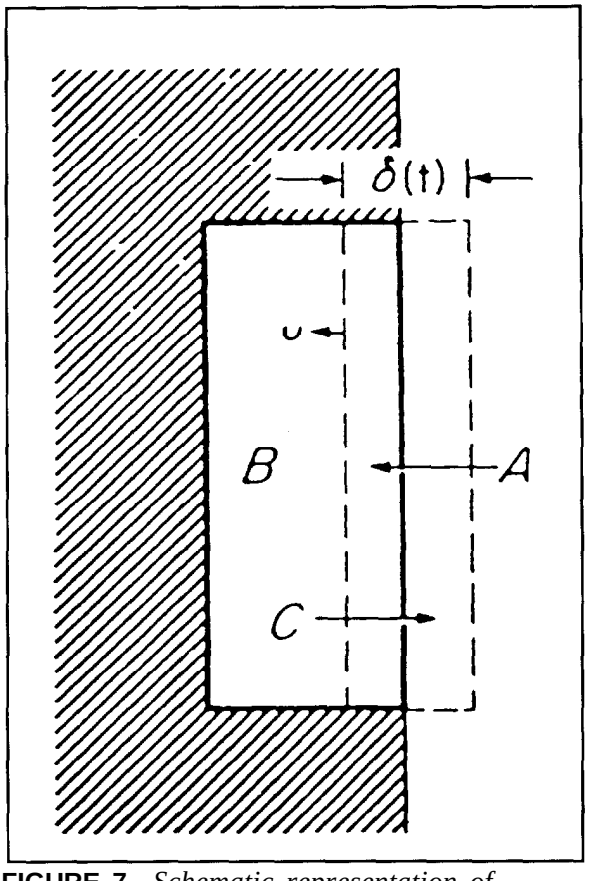

FIGURE 7. *Schematic representation of swelling-controlled systems. As penetrant A enters the glassy polymer B, bioactive agent C is released through the gel phase of thickness $\delta(t)$.*

SOURCE: Reprinted from Langer and Peppas (1983).

swelling interface, separates the glassy polymer from the swollen rubbery polymer and moves inward into the device. The other front, the polymer interface, moves outward and separates the swollen polymer from the pure dissolution medium. In systems where the glassy polymer is

noncrystalline and linear, dissolution takes place. However, when the polymer is highly crystalline or cross-linked, no dissolution takes place.

An interesting system has recently been described (Colombo et al. 1990). In this system, a drug is dispersed in a swellable polymer such as hydroxypropyl methylcellulose, which is compressed into a tablet and two sides coated with a water-impermeable coating such as cellulose acetate propionate. This impermeable coating affects the swelling of the matrix and modifies diffusional release kinetics so that reasonably constant release kinetics are achieved. Such a device is shown schematically in figure 8. This type of oral drug delivery device is currently being commercialized.

CHEMICALLY CONTROLLED SYSTEMS

Drug-Release Mechanisms

Drug release from bioerodible polymers can occur by any one of the three basic mechanisms shown schematically in figure 9 (Heller 1985). In mechanism A, the active agent is covalently attached to the backbone of a biodegradable polymer and is released as its attachment to the polymer backbone cleaves by hydrolysis of bond A. Because it is not desirable to release the drug with polymer fragments still attached for toxicological reasons, the reactivity of bond A should be significantly higher than the reactivity of bond B. In mechanism B, the active agent is contained within a core and is surrounded by a bioerodible rate-controlling membrane. Release of the active agent is controlled by its diffusion across the membrane. In mechanism C, the active agent is dispersed in a bioerodible polymer, and its release is controlled by diffusion, by a combination of diffusion and erosion, or in rare instances by pure erosion.

Drug Covalent/y Attached to Polymer Backbone. This delivery system can be further subdivided into soluble systems and insoluble systems. Insoluble systems are used as a subcutaneous or intramuscular implant for the controlled release of the chemically tethered therapeutic agent. Soluble systems are used in targeting applications. In this case, the polymer with tethered therapeutic agent is water soluble and also contains a chemically tethered targeting moiety so that when it is injected intravenously it concentrates at the target site where the drug is released by cleavage of the labile bond (Duncan 1992).

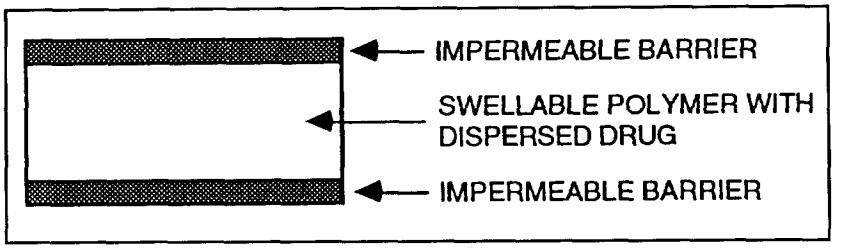

FIGURE 8. *Schematic representation of swelling-controlled delivery system with swelling confined to polymer sandwiched between two impermeable layers.*

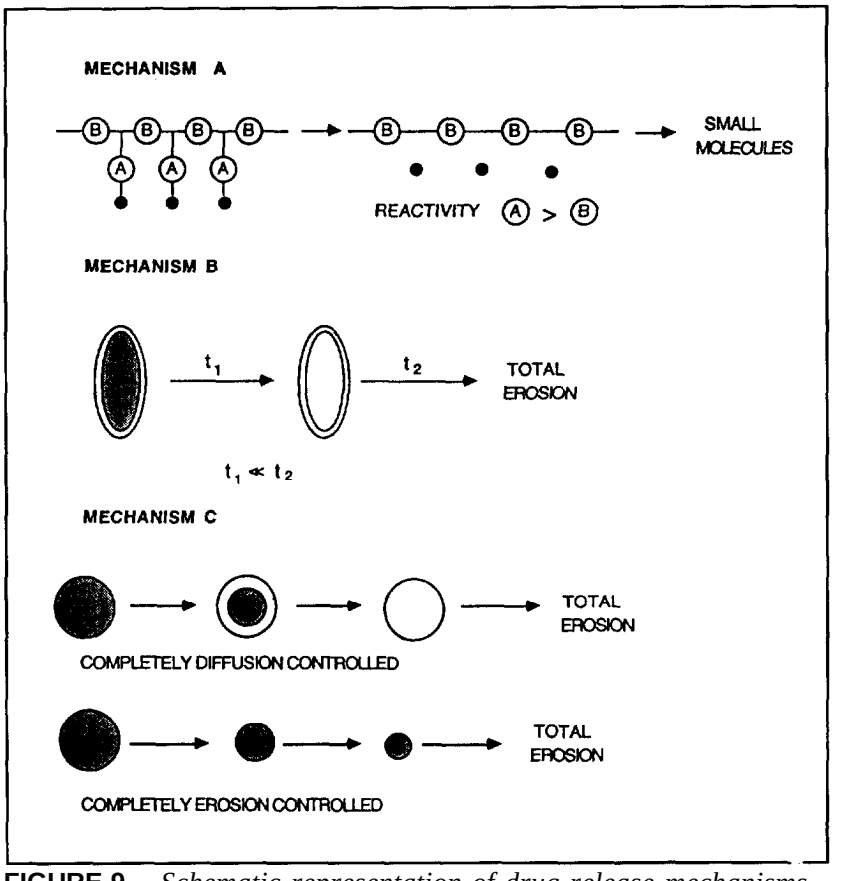

FIGURE 9. *Schematic representation of drug-release mechanisms from bioerodible polymers.*

SOURCE: Reprinted from Heller (1985).

Applications

Water-Insoluble Systems. In one example of a water-insoluble system, the contraceptive steroid norethindrone is covalently attached to poly (N^5-hydroxypropyl-L-glutamate) via a carbonate linkage (Petersen et al. 1980, pp. 45-60). The synthesis and structure of this system are shown in figure 10.

Even though poly(N^5-hydroxypropyl-L-glutamate) is water soluble, attachment of the highly hydrophobic norethindrone results in a water-insoluble product. As the hydrophobic steroid is released by hydrolysis of the carbonate linkage, polymer hydrophilicity increases, and the reaction rate in the hydrophilic region accelerates. As a consequence of

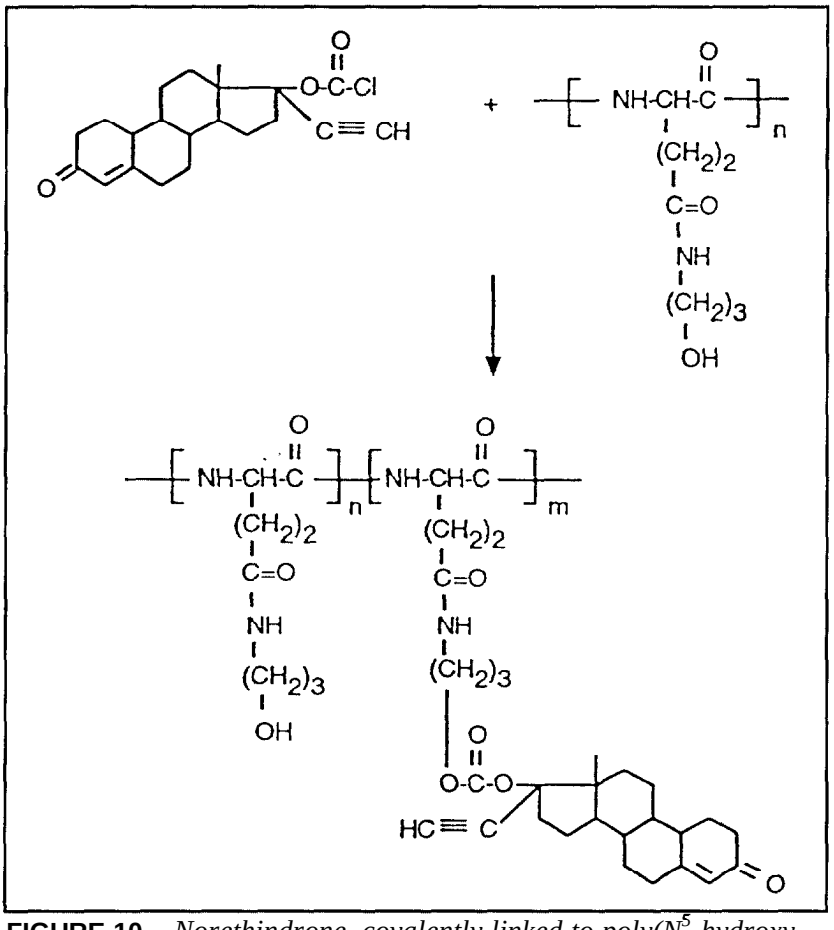

FIGURE 10. *Norethindrone, covalently linked to poly(N^5-hydroxypropyl-L-glutamate).*

this process, a hydrophilic front develops and moves through the solid, hydrophobic polymer (Tani et al. 1981, pp. 79-98). Because diffusion of the steroid through the hydrophilic layer is rapid relative to the movement of the front, the rate of drug release is determined by the rate of movement of this front, and fairly constant release can be achieved. When sufficient norethindrone has been released to solubilize the poly (N^5-hydroxypropyl-L-glutamate) backbone material, enzymatic cleavage takes place to regenerate the α-amino acid constituent.

A system where naltrexone has been covalently attached to a poly (cc-amino acid) biodegradable backbone has also been described (Negishi et al. 1987).

Water-Soluble Systems. One of the more successful approaches utilizes polymers based on N-(2-hydroxypropyl) methacrylamide (HPMA). This polymer, originally developed in Czechoslovakia as a blood plasma expander (Sprincl et al. 1976), is biocompatible, nontoxic, and nonimmunogenic. However, it is nondegradable; to allow excretion, it must be fractionated so that its molecular weight is lower than the renal threshold. The polymer can be modified by the introduction of oligopeptide side-chains for drug attachment and also for the attachment of either carbohydrates or antibodies as targeting moieties. Such materials are synthesized by a two-step process. In the first step, shown in figure 11, a copolymer of HPMA and a p-nitrophenyl ester of N-methacryloylated oligopeptide is prepared.

Because a p-nitrophenyl ester group is a very good leaving group, it can be readily displaced with an amine so that reaction with amino-containing molecules will result in attachment of such molecules to the copolymer. Thus, reaction with an amino group on the drug and an amino group on the targeting moiety results in the macromolecular carrier shown in figure 12.

A key component of this polymer is the oligopeptide side-chain, which can be designed to be stable in blood but to readily degrade in the lysosomal compartment of cells by the action of proteases, glycosidases, and phosphotases residing in that compartment. For example, a glycine-phenylalanine-leucine-glycine (Gly-Phe-Leu-Gly) oligopeptide side-chain is readily degraded in the lysosomal compartment while a glycine-glycine (Gly-Gly) side-chain is nondegradable. Using this approach, copolymers can be prepared with the drug attached via the degradable

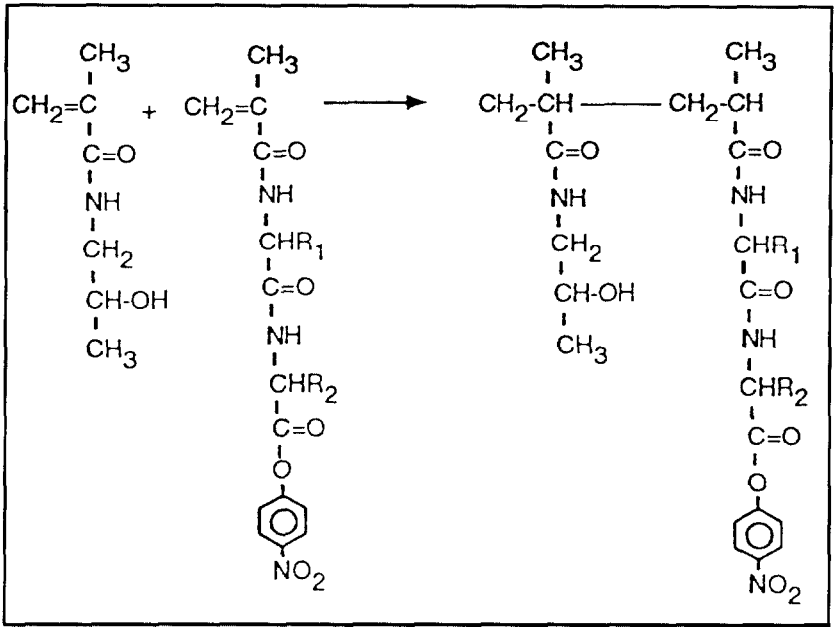

FIGURE 11. *Preparation of copolymer of N (2-hydroxypropyl) methaclylamide and p-nitrophenyl ester of N-methacryloylated oligopeptide.*

Gly-Phe-Leu-Gly oligopeptide side-chain and the targeting moiety attached via the nondegradable Gly-Gly oligopeptide side-chain (Duncan et al. 1980; Rejmanova et al. 1985).

Drug Contained Within a Biodegradable Core. This delivery system is identical to the reservoir system already discussed, with the exception that the membrane surrounding the drug core is bioerodible. Such systems combine the advantage of long-term, zero-order drug release with bioerodibility. However, in this case, polymer hydrolysis is not a factor in determining rate of drug release, and the bioerosion process simply removes the expended device.

Because constancy of drug release requires that the diffusion coefficient D of the agent in the membrane remain constant (equation 5), the bioerodible membrane must remain essentially unchanged during the delivery regime. Most important, the membrane must remain intact as long as there is still a quantity of the drug in the core to prevent the

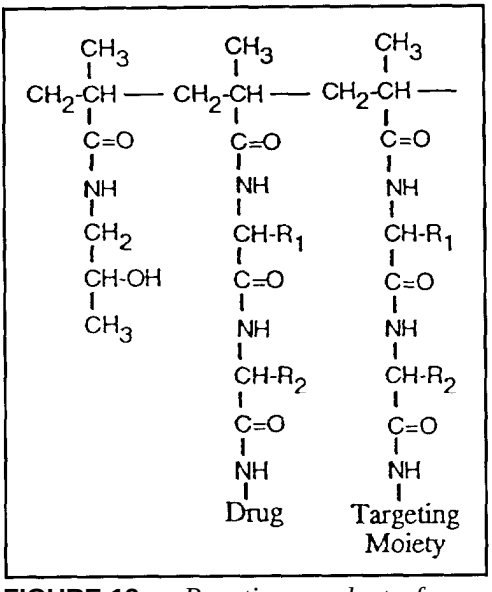

FIGURE 12. *Reaction product of copolymer of N-(2-hydroxypropyl) methacrylamide and p-nitrophenyl ester of N-methacryloylated oiigopeptide with amino groups on drug and targeting moiety.*

drug's abrupt release. For this reason, significant bioerosion must not take place until drug delivery has been completed.

Application. The only system that uses a drug contained in a biodegradable core is a delivery device for contraceptive steroids. A device in advanced stages of development is based on a poly (ε-caprolactone) capsule containing the contraceptive steroid levonorgestrel. This device is a thin cylinder about 2.5 cm in length and 2.35/2.04 mm OD/ID, is designed to release levonorgestrel at constant rates for 1 year and to completely bioerode in about 3 years (Pitt et al. 1980, pp. 19-43). A l-year, phase II clinical trial has recently been completed at the University of California, San Francisco. Additional studies in Europe and Asia are underway. Figure 13 shows blood plasma levels in human volunteers following implantation and removal of the device (Ory et al. 1983).

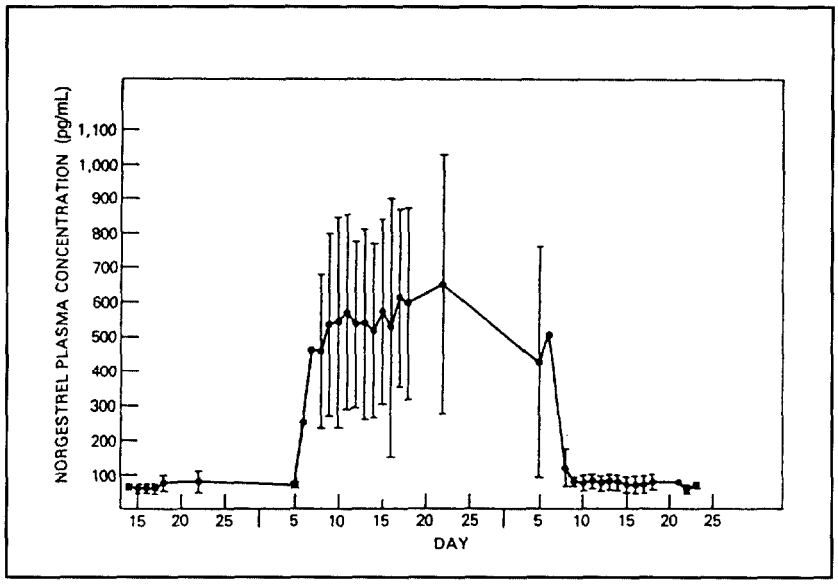

FIGURE 13. *Plasma concentration of levonorgestrel for cycles 3, 4, and 5 of the phase Z clinical evaluation after implant during the fourth cycle. Means and SD for eight women are shown.*

SOURCE: Reprinted from Ory et al. (1983).

Drug Dispersed in a Bioerodible Matrix. This is the most widely investigated system, and its discussion is conveniently divided into systems where drug release is determined predominantly by diffusion and systems where drug release is determined predominantly by polymer erosion. When the polymer undergoes surface erosion, the rate of drug release is completely determined by polymer erosion (Heller and Baker 1980, pp. 1-17).

Drug Release Determined Predominantly by Diffusion. Drug release from polymers where hydrolysis occurs at more or less uniform rates throughout the bulk of the polymer is determined predominantly by Fickian diffusion. When the rate of polymer hydrolysis is slow relative to drug depletion, half-life (t½) kinetics identical to those observed with nondegradable systems are observed. When the rate of polymer hydrolysis is significant before drug depletion, the t½ kinetics are modified by the hydrolysis process (Heller and Baker 1980, pp. 1-17).

The most extensively investigated bulk-eroding polymers are poly (lactic acid) and copolymers of lactic and glycolic acids. These polymers were originally developed as bioerodible sutures and, because they degrade to the natural metabolites lactic and glycolic acids, to this day they occupy a preeminent place among bioerodible drug delivery systems (Heller 1984). Their structure is shown in figure 14.

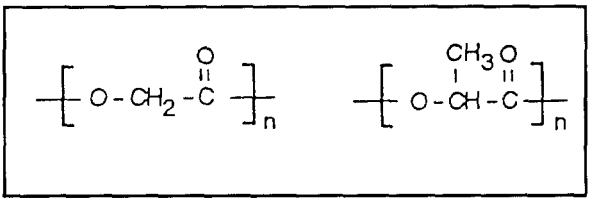

FIGURE 14. *Structure of poly (glycolic acid) and poly (lactic acid).*

Drug Release Determined Predominantly by Erosion. Certain polymers can undergo a hydrolysis reaction at decreasing rates from the surface of a device inward, and under special circumstances the reaction can be largely confined to the outer layers of a solid device. Two such polymers are poly (ortho esters) and polyanhydrides. Because the rates of hydrolysis of these polymers can be varied within very wide limits, considerable control over the rate of drug release can be achieved.

Poly (Ortho Esters). Poly (ortho esters) are highly hydrophobic polymers that contain acid-sensitive linkages in the polymer backbone. Their synthesis and biomedical applications have recently been reviewed (Heller 1993b). Ortho ester linkages undergo a very slow rate of hydrolysis at the physiological pH of 7.4, but as the ambient pH is lowered, hydrolysis rates increase. Thus, the incorporation of small amounts of acidic excipients such as aliphatic dicarboxylic acids into such materials allows precise control over rates of erosion. With highly hydrophobic drugs, surface hydrolysis can take place because, as water intrudes into the polymer, the acidic excipient ionizes, and hydrolysis accelerates due to the decreased pH in the surface layers. As a result of this process, an eroding front develops that moves into the interior of the device. However, when hydrophilic drugs are used, water is rapidly drawn into the polymer and bulk hydrolysis takes place. Three families of poly (ortho ester) have been developed and are shown in figure 15 (Heller 1990).

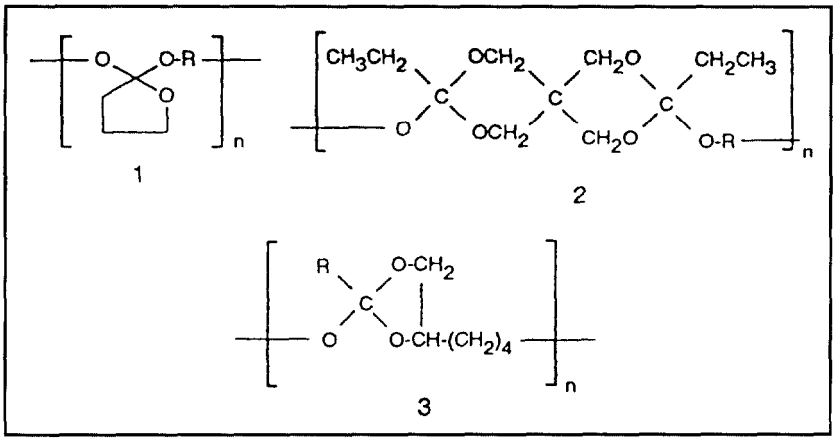

FIGURE 15. *Structure of three families of poly (ortho esters).*

Very long erosion times can be achieved by the incorporation of basic excipients such as $Mg(OH)_2$ into the polymer along with the therapeutic agent. When a hydrophobic drug such as levonorgestrel is used, long-term surface erosion can take place because hydrolysis can occur only in the outer layers where the basic excipient has diffused out of the device and has been neutralized by the external buffer (Heller et al. 1985).

Polyanhydrides. These materials were first prepared in 1909 (Bucher and Slade 1909) and were subsequently investigated as potential textile fibers; they were found unsuitable due to their hydrolytic instability (Conix 1958). Although polyanhydrides based on poly [bis (p-carboxyphenoxy) alkanes] exhibit significantly improved hydrolytic stability, they retain enough hydrolytic instability to prevent commercialization despite their excellent fiber-forming properties.

The use of polyanhydrides as bioerodible matrices for the controlled release of therapeutic agents was first reported in 1983 (Rosen et al. 1983). Because aliphatic polyanhydrides hydrolyze very rapidly while aromatic polyanhydrides hydrolyze very slowly, excellent control over the hydrolysis rate can be achieved by using copolymers of aliphatic and aromatic polyanhydrides. In this way, erosion rates over many days have been demonstrated, and erosions rates measured in years have been projected (Leong et al. 1985, 1986). The structure of a polymer based on bis (p-carboxyphenoxy) propane and sebacic acid is shown in figure 16.

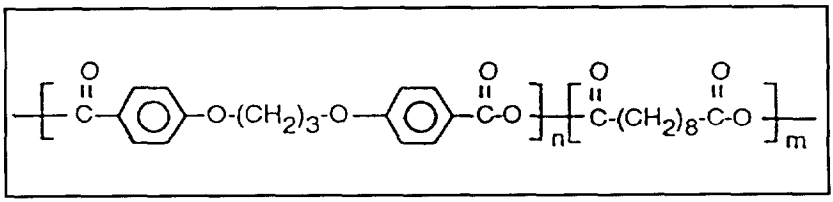

FIGURE 16. *Structure of polyanhydride based on bis (p-carboxyphenoxy) propane and sebacic acid.*

Applications. Lactide/glycolide copolymer systems have been extensively investigated for delivery of the contraceptive steroid norethindrone and levonorgestrel from injectable microspheres (Beck and Tice 1983, pp. 175-199) and for delivery of synthetic analogs of the luteinizing hormone-releasing hormone (LHRH) (Heller 1993c). The contraceptive delivery system is in advanced stages of development, and devices containing norethindrone have completed phase II clinical trials; phase III clinical trials are in the planning stages. An LHRH-releasing system for control of prostate cancer is now commercially available.

Poly (ortho ester) system 2 has been used for the delivery of levonorgestrel, 5-fluorouracil, and naltrexone. Poly (ortho ester) system 3 represents a unique polymer system that is a viscous, hydrophobic, pastelike material at room temperature, even at fairly high molecular weights. The pastelike property allows incorporation of therapeutic agents at room temperature and without the use of solvents, and it is currently under investigation for various topical applications including treatment of periodontitis (Heller 1993b).

Polyanhydrides are currently being explored as a bioerodible implant for the release of BCNU [N,N-bis (2-chloroethyl)-N-nitrosourea] following brain cancer surgery. The polymer has been approved for use in terminally ill cancer patients, and recently completed clinical trials have demonstrated a 30 percent increase in survival times.

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Pharmacokinetics and Pharmacodynamics of Maternal-Fetal Transport of Drugs of Abuse: A Critical Review

Srikumaran Melethil

INTRODUCTION

It is well recognized that maternal intake of a wide variety of drugs, including those of abuse, results in concomitant fetal exposure to these drugs (Mihaly and Morgan 1984; Reynolds and Knott 1989; Szeto 1993, pp. 390-449). A knowledge of the quantitative relationships between the time course of maternal and fetal drug exposure (i.e., pharmacokinetics or PK) and fetal effects (i.e., pharmacodynamics or PD) subsequent to maternal ingestion is essential to a more complete understanding of fetal consequences of drug abuse by the mother. Little direct information is currently available regarding such relationships. A critical analysis of existing information in the area of maternal-fetal PK/PD of drugs of abuse, the primary focus of this chapter, shows that it is reasonable to speculate that fetal PD can be predicted based on maternal PK. As might be expected, moral, ethical, and legal constraints require that all well-designed studies in this area, both past and future, be conducted in animals. The pregnant sheep (though its placental morphology is different from those in humans) is now the most popular species for practical reasons: ready availability; economy; large size, which makes the required surgical manipulations less difficult; and ease of handling. Therefore, potential methods to extrapolate animal data to humans are another key topic of discussion. Areas for future research in the PK/PD area also are identified.

PHARMACOKINETICS

Single-Dose Studies

Detailed maternal-fetal PK data are available only for some popular drugs of abuse such as cocaine (DeVane et al. 1991; Sandberg and Olsen 1992; Woods et al. 1989), meperidine (Szeto et al. 1978), methadone (Szeto

et al. 1981), methamphetamine (Burchfield et al. 1991), and tetrahydrocannabinol (Abrams et al. 1985). These studies collectively show that, following intravenous (IV) administration of these drugs to the mother, their plasma concentration-time profiles can be best characterized by the classical two-compartment model (Gibaldi and Perrier 1982, pp. 45-111). Since many of these drugs of abuse are small, lipid-soluble molecules, passive diffusion is the most common mode of placental exchange of these drugs between the mother and the fetus. As such, major factors that affect maternal-fetal transfer of a drug are its molecular size, lipid solubility, pKa and extent of plasma protein binding, and placental thickness, surface area, and blood flow (Mihaly and Morgan 1984; Reynolds and Knott 1989, pp. 390-449).

These studies also show that elimination half-lives are essentially the same in the mother and the fetus. For example, plasma half-lives of IV cocaine (0.5 to 4 mg/kg, bolus) in pregnant sheep ranged from 4 to 5.6 minutes in the mother and the fetus (DeVane et al. 1991). Similarly, following a 1.2 mg/kg IV bolus to the mother, maternal and fetal mean plasma half-lives for methamphetamine in sheep were 38.8 and 39.5 minutes, respectively (Burchfield et al. 1991). Maternal and fetal plasma half-lives following IV meperidine (2.5 mg/kg) and methadone (0.5 mg/kg) were 20.4 and 22.6 minutes (Szeto et al. 1978), and 57.7 and 58.5 minutes (Szeto et al. 1981), respectively. In studies with Δ-9-tetrahydrocannabinol (Abrams et al. 1985), where pregnant sheep inhaled smoke from a marijuana cigarette, maternal and fetal half-lives both exceeded 10 hours; accurate estimation of half-lives was not possible due to inadequate plasma sampling. The observed similarity in maternal-fetal half-lives, in conjunction with the underdeveloped drug elimination capacity of the fetus, indicate that fetal to maternal transfer is the major route of drug elimination (i.e., removal) from the fetus for small lipid-soluble molecules. (Methods to estimate maternal to fetal and fetal to maternal placental clearances are described later.)

Following IV administration of these drugs to the pregnant sheep, maternal to fetal transfer was rapid (within a few minutes), as might be expected; however, fetal exposure varied considerably. For example, methamphetamine (Burchfield et al. 1991) crossed the sheep placenta within 30 seconds following IV administration of the drug to the mother, and the ratio of maternal to fetal areas under the plasma concentration-time curves (i.e., extent of exposure) ranged from 0.78 to 1.08. From data presented (in graphical form) for meperidine (Szeto et al. 1978), it can be estimated (by visual inspection) that quantitative aspects of

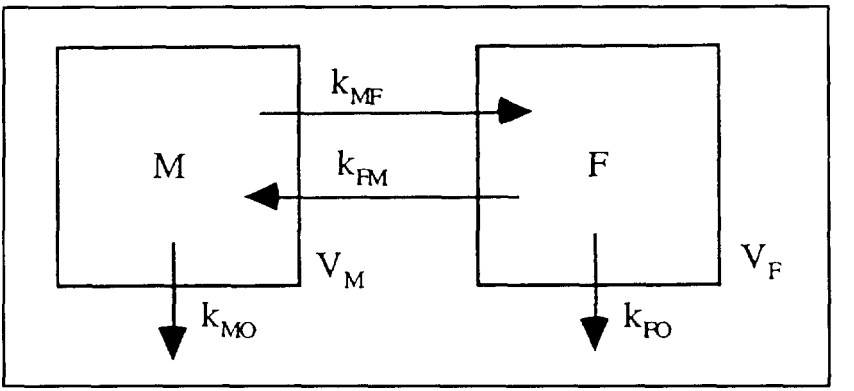

FIGURE 1. *The two-compartment model used to study steady-state maternal-fetal PK.*

KEY: M = maternal compartment
F = fetal compartment
k_{MF} = the first-order maternal to fetal transport rate constant
k_{FM} = the first-order fetal to maternal transport rate constant
k_{MO} = maternal elimination rate constant
k_{FO} = fetal elimination rate constant
V_M = the apparent volume of distribution of the maternal compartment
V_F = the apparent volume of distribution of the fetal compartment

distribution (i.e., rate and extent of exposure) were similar to methamphetamine. While methadone (Szeto et al. 1981) was rapidly transferred to the fetus following maternal administration (like the previous two examples), extent of fetal exposure (again estimated from graphical data) following maternal ingestion was much lower (about 10 to 20 percent).

Steady-State Models

A two-compartment linear system (figure 1), developed by Szeto and coworkers (Szeto 1982, 1989; Szeto et al. 1982a) is the most widely used animal model (near-term pregnant sheep) to describe disposition kinetics of drugs in the conscious mother and fetus; as described, this analysis showed that drug elimination occurred from both central (representing the mother) and peripheral (representing the fetus) compartments. While the model provides extensive information, the animal preparation requires

extensive surgical manipulation, such as placing catheters in the fetal and maternal femoral arteries and veins (for drug administration and blood sampling).

Maternal and fetal clearances of a number of licit [e.g., diphenhydramine (Yoo et al. 1993), metoclopramide (Riggs et al. 1990)], and illicit [morphine (Szeto et al. 1982b), methadone (Szeto et al. 1982b)] drugs have been estimated using this model. Briefly, the experimental protocol involves two steps. The drug is first infused (infusion rate = ko) into the mother, and steady-state plasma concentrations are determined in both the mother (C_{Mss} and fetus (C_{Fss}). In the second phase, the drug is infused into the fetus (infusion rate = ko'), and the corresponding steady-state maternal ($C_{M,ss}$) and fetal ($C_{F,ss}$) concentrations are determined. Based on the infusion rates and steady-state concentrations, several useful maternal and fetal clearances can be estimated, as shown below. In these equations, CL_{MM}, CL_{FF}, CL_{MF}, CL_{FM}, CL_{MO}, and CL_{FO} refer to total maternal (1), total fetal (2), maternal-fetal (3), fetal-maternal (4), nonplacental maternal (5), and nonplacental fetal (6) clearances.

$$CL_{MM} = ko/[C_{Mss} - C_{Fss}(C_{M'ss}/C_{F'ss})] \quad (1)$$
$$CL_{FF} = ko' [C_{F'ss} - C_{M'ss}(C_{Fss}/C_{Mss})] \quad (2)$$
$$CL_{MF} = CL_{FF}(C_{Fss}/C_{Mss}) \quad (3)$$
$$CL_{FM} = CL_{MM}(C_{M'ss}/C_{F'ss}) \quad (4)$$
$$CL_{MO} = CL_{MM} - CL_{MF} \quad (5)$$
$$CL_{FO} = CL_{FF} - CL_{FM} \quad (6)$$

Yoo and colleagues (1993) compared maternal clearance of diphenhydramine (CL_{MM} by model-dependent [i.e., V_M ($k_{MO} + k_{MF}$)] and model-independent (i.e., ko/C_{Mss} methods. Interestingly, they found good agreement between maternal clearance values calculated by both methods. In principle, this agreement is unexpected because

$$ko/C_{Mss} = V_M * [(k_{FM} * K_{MO} + k_{FO} * k_{MF} + k_{FO} * k_{MO})/(k_{FM} + k_{FO})]$$

and can be explained based on equation (1) when the second term in the denominator of equation (1) is either zero or much less than the first term. For diphenhydramine, Yoo and colleagues (1993) found such agreement, because this term (i.e., $C_{Fss} * (C_{M,ss}/C_{F,ss})$) was negligible (2.9 ng/mL) in comparison to C_{Mss} (212.1 ng/mL).

While this model is a valuable tool for assessing maternal-fetal PK, model estimates of maternal and fetal clearances have been independently

validated only for one drug, acetaminophen (Wang et al. 1986). Fetal to maternal clearance was estimated using the extraction ratio method (as follows):

$$ER = [C_{FA} - C_{UmV}]/ C_{FA} \quad (7)$$
$$CL_{FM} = ER \times Q_{Um} \quad (8)$$

where ER is the extraction ratio across the placenta on the fetal side; C_{FA} and C_{UmV} are drug concentrations in the fetal femoral artery and umbilical vein, respectively; and Q_{Um} is the umbilical blood flow. This method is more suitable for drugs with large E values.

Ratios of CL_{FM} (model-based) to CL_{FM} (extraction ratio method) for acetaminophen ranged from 0.903 to 1.06; similarly, CL_{MO}, estimated by the model-independent method, averaged 97.9 percent of corresponding values obtained from the model. However, model-based fetal CL, (i.e., sum of renal and metabolic pathways) was about threefold higher. This partial agreement is certainly encouraging and clearly indicates the urgent need to validate and improve the model, so that accurate estimates of in vivo PK parameters of substances of abuse can be obtained. For the prospective researcher, the complexity of animal preparation required for such validation will require a high degree of surgical skills and experience (J.E. Axelson, personal communication, September 28, 1993). Since direct determination of clearance parameters is needed, a more extensive sampling protocol than that required for estimation of the two-compartment parameters (Szeto et al. 1982a) will be needed. In the acetaminophen study (Wang et al. 1986), additional procedures included catheterization of the fetal and maternal bladders and umbilical and uterine veins for sample collection; the amniotic sac also was exteriorized by means of a catheter to administer antibiotics after surgery, presumably to prevent postsurgical infection.

Model-based indirect estimates of fetal elimination capacity (i.e., CL, values) have been published for several drugs (Riggs et al. 1990; Szeto et al. 1982b; Yoo et al. 1993). However, this remains another area where direct evidence is scarce. In addition to providing direct evidence for fetal conversion of acetaminophen to its glucuronide and sulfate conjugates (Wang et al. 1986), this work also provided information on fetal capacity to metabolize a drug, as compared with the mother; in sheep, the relative abilities of the fetus to convert acetaminophen to its sulfate and glucuronide conjugates were 74 and 16 percent, respectively, and indicated the early maturation of the former pathway. It is interesting

to note that reported in vitro results were in excellent agreement with in vivo findings regarding fetal ability to form acetaminophen glucuronide; in vitro fetal V_{max} for this conjugation was 18 percent of maternal values. If this can be verified for drugs that undergo glucuronide conjugation (e.g., morphine), then the exciting possibility of obtaining in vivo data, which are virtually impossible in humans and quite laborious in animals, from relatively simple in vitro studies would be a reality.

Direct evidence of the ability of fetal lambs to excrete unchanged drug is available for acetaminophen (Wang et al. 1986) and meperidine (Szeto et al. 1979, 1980). Renal clearance of acetaminophen was quite similar in the mother [mean value:(0.31 mL/(min * kg)] and fetus ((mean value: 0.40 mL/(min * kg)). In fetal sheep, renal clearance of meperidine is inversely related to pH, with a mean clearance ratio (meperidine to inulin) of 5 (Szeto et al. 1980). This high ratio has been explained on the basis of ion-trapping, a well-known phenomenon where a basic drug [in this case, meperidine (pKa = 8.6)] passively accumulates on the acidic (i.e., tubular fluid) side of a semipermeable (i.e., tubular wall) membrane separating acidic and basic (i.e., blood, pH: 7.4) regions.

The two-compartment model (Szeto et al. 1982a) has been used to explain the finding that steady-state plasma drug concentrations are lower in the fetus. Experimental data from a number of drugs, where the drug was infused into the mother, show that steady-state fetal (C_{Fss}) to maternal plasma (C_{Mss} concentration ratios (C_{Fss}/C_{Mss}) for a number of drugs, except alcohol, are less than 1. Data summarized by Szeto (1989) are shown in table 1.

It can be shown that elimination of the drug by the fetus is a major contributory reason for obtaining lower plasma drug concentrations in the fetus (Szeto et al. 1982a). According to this model (figure 1):

$$C_{Fss} / C_{Mss} = [CL_{MF} / (CL_{FM} + CL_{FO})] \quad (9)$$

As can be seen from equation (9), steady state can be achieved in the fetus only if fetal drug input rate (i.e., drug transfer rate from mother to fetus $CL_{MF} \times C_{Mss}$) is equal to fetal output rate [i.e., transfer rate from fetus to mother plus rate of loss from fetus $(CL_{FM}+CL_{FO}) \times C_{Fss}$)]. If $CL_{FO} = 0$ (i.e., no fetal drug elimination by processes such as metabolism and renal excretion), rates of mass transfer between the maternal and fetal

TABLE 1. *Steady-state fetal-maternal plasma drug concentration ratios.*

Drug	C_{Fss}/C_{Mss} (Total)	C_{Fss}/C_{Mss} (Unbound)
Methadone	0.15	0.40
Morphine	0.13	0.13
Meperidine	0.30	0.40
Cimetidine	0.04	0.04
Triamterene	0.17	
Acetylsalicylic acid	0.22	0.22
Indomethacin	0.28	0.28
Omeprazole	0.47	0.22
Phenytoin	0.51	----
Dexamethasone	0.67	0.67
Lidocaine	0.76	
Acetaminophen	0.77	0.77
Ethanol	1.00	1.00

SOURCE: Szeto et al. (1989).

compartments become equal, and these compartments also would be in equilibrium, by definition (Riggs 1963, pp. 168-192); then $C_{Fss}/C_{Mss} = 1$. If $CL_{FO} > 0$, steady-state rate of drug transfer from mother to fetus has to be greater than the corresponding transfer rate from fetus to mother; this difference is equal to the rate for loss from the fetus (CL, x C,). Net diffusional drug transfer can occur from the mother to the fetus only when $C_{Mss} > C_{Fss}$. Therefore, when CL, > 0, the ratio $C_{Fss}/C_{Mss} < 1$.

The development of this two-compartment model (Szeto et al. 1982a) has been an important contribution in understanding kinetics of maternal-fetal drug transfer. However, published values for model-based transplacental

clearances are intriguing in two cases. Firstly, CL_{FM} values are consistently greater than CL, for all drugs reported to date. Since C_{Fss}/C_{Mss} is < 1 (table 1), it follows from equation (9) that $CL_{MF} < [CL_{FM} + CL_{FO}]$. If $CL_{FO} << CL_{FM}$ then $(CL_{FM} + CL_{FO}) = CL_{FM}$ and $CL_{MF} < CL_{FM}$. Yoo and colleagues (1993) also found a linear relationship between $(CL_{FM}\text{-}CL_{MF})$ and CL_{FM} based on reported values for diphenhydramine, morphine, methadone, metoclopramide, and acetaminophen. It remains to be determined whether this is due to a potential bias of the model, or merely a coincidence resulting from the limited number of drugs that have been investigated.

Secondly, model-based CL_{FM} and CL_{MF} values were higher in some cases than placental blood flow. For example, transplacental clearances for diphenhydramine in pregnant sheep have been reported (Yoo et al. 1993) to be 661.0 mL/min (CL_{MF}) and 920.2 mL/min (CL_{MF}); the corresponding values for methadone are 390.3 and 504 mL/min. Estimates of placental blood flow (Paulick et al. 1991) in sheep are in the range of 169.4 to 182 mL/(min * kg of fetal weight), and fetal weight is in the range of 2 to 5 kg. Therefore, clearance values higher than 400 to 900 mL/min in the sheep model would indicate unrecognized pathways of drug elimination such as placental metabolism. The role of placenta in the metabolism of methadone and diphenhydramine, a topic on which there is no published information (a thorough literature search did not reveal any reports on the subject), needs to be investigated to validate the model (figure 1).

PHARMACODYNAMICS

Practical methods to quantify fetal effects resulting from maternal drug ingestion are unavailable. The first step toward the development of such methods, which are of great clinical significance, is the identification of quantitative relationships between the time course of exposure to drugs of abuse in the mother (i.e., maternal PK) and the resulting time course of effects on the fetus (i.e., fetal PD). The fact that several drugs of abuse achieve rapid equilibrium between the mother and fetus (see section titled “Single-Dose Studies”) suggest that such relationships can exist, in principle. There also is some experimental evidence for this speculation. For example, following maternal administration of methamphetamine (1.2 mg/kg as IV bolus) Burchfield and colleagues (1991) showed a linear inverse correlation between methamphetamine half-life in fetal sheep and fetal oxyhemoglobin; in addition, a weak (statistically not

significant, p = 0.095) direct relationship between maternal and fetal methamphetamine half-lives also was observed.

PD data presented in their report showed that maximum increases in maternal and fetal mean blood pressures following maternal methamphetamine occurred within 4 to 5 minutes, postinjection; profiles for the time course of changes in mean blood pressure also were similar in the mother and the fetus. While plasma methamphetamine concentrations were determined in mother and fetus, no efforts to integrate maternal PK with fetal PD were attempted.

In studies with morphine, where plasma drug concentrations were not measured, Szeto and colleagues (1988) showed that the dual action (i.e., low-dose stimulation and high-dose suppression) of fetal breathing movements of this drug in fetal sheep can be modeled according to classical receptor theory involving two different receptor systems. These investigators also modeled the bell-shaped dose-response curve for morphine-induced tachycardia; dose-response (percent change in fetal heart rate) data were found to be equally well characterized by either a functional antagonism or a noncompetitive auto-inhibition model (Zhu and Szeto 1989). Animal PK/PD studies with drugs of abuse are needed initially to better understand fetal consequences of drug exposure in pregnant women. If initial expectations (i.e., correlations between fetal effects and maternal plasma concentrations) are confirmed, it will provide a rational basis for the further development of innovative clinical investigations, such as the relationships between fetal drug-effect parameters that can be monitored and maternal plasma drug concentrations. In the long run, results from such studies will enable improved prediction of fetal consequences of maternal drug ingestion.

INTERSPECIES EXTRAPOLATION

It is obvious that one of the formidable challenges faced by investigators in the area of maternal-fetal drug transport and pharmacology is to develop clinically relevant information based on animal data. Therefore, development of new methods (and refinement of existing ones) to extrapolate animal data to humans deserve much attention. This section provides a short discussion of a PK method that is unique in that it offers a solution to the complex problem of interspecies data extrapolation. Theoretical and practical details of this physiological approach to PK modeling, which may be less familiar and mathematically more daunting

than classical or compartmental PK, are explained with the hope that it will encourage initiation of serious efforts to apply these concepts to the area of maternal-fetal PK/PD.

Physiologically Based Pharmacokinetic (PB/PK) Models

Theoretical Aspects. This type of model, originally developed to provide a greater emphasis than that included in classical compartmental models on physiological (e.g., organ blood flow) and physicochemical factors (e.g., blood-to-tissue partitioning) that influence disposition kinetics of a drug, was first applied to methotrexate (Bischoff et al. 1970, 197 1). Since then, the model has been applied for many drugs. While citations are far too many to cite, one exhaustive review (103 references) published by Gerlowski and Jain (1983) summarizes the application of this model to about 40 drugs and chemicals. Recently (starting in the 1980s), toxicologists have begun to apply these concepts to extrapolate animal PK data of toxic substances (which, like drugs of abuse, cannot be deliberately studied in humans) such as methylene chloride (Andersen et al. 1987) and benzene (Medinsky et al. 1989) to humans. Such models (similar to figure 2) also have been developed for two drugs of abuse, namely morphine (Gabrielsson et al. 1983) and methadone (Gabrielsson et al. 1985), using the pregnant rat as the experimental animal. In these reports, the whole fetus was "lumped" (defined later) as a single compartment, in contrast to figure 2 of this review where fetal organs are shown separately as was described for tetracycline (Olanoff and Anderson 1980). While such an approach reduces data gathering efforts and simplifies mathematical formulation of the model, it limits the ability to extrapolate data obtained from these studies to humans, especially the relationship between concentrations in maternal plasma to those in various individual fetal tissues. However, scaled-up models, developed by substitution of human physiological parameters and organ sizes and assuming similarities in tissue distribution between the rat and human (i.e., the same tissue-to-plasma ratios obtained from rats in these two studies), were capable of predicting plasma kinetics of these two drugs in humans. The model-predicted plasma half-lives for morphine (about 2 hours) and methadone (about 20 hours) were in excellent agreement with previously published data in humans (Berkowitz 1976; Nilsson et al. 1982). These results certainly are encouraging and indicate the feasibility of developing more complete PB/PK models for drugs of abuse. A brief, general discussion of this model with the fetus lumped as a single compartment was presented in a recent National Institute on Drug Abuse (NIDA) monograph (DeVane 1991, pp. 18-36).

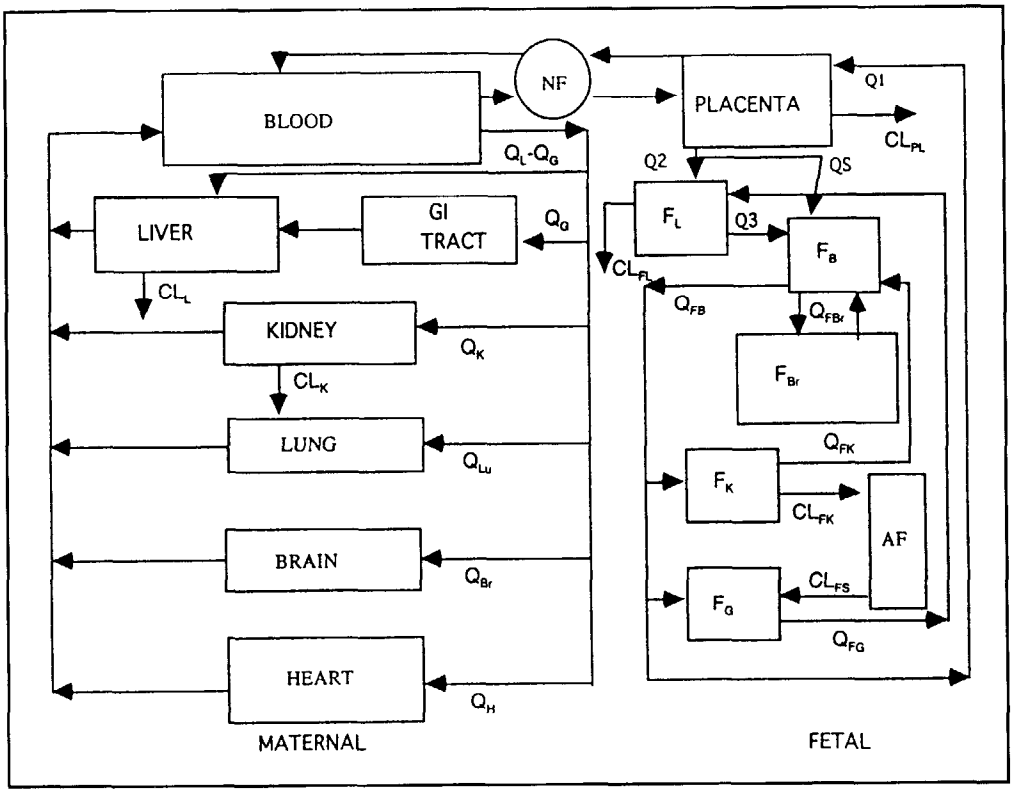

FIGURE 2. *A preliminary PB/PK model for cocaine.*

Conceptual and mathematical details of developing a PK/PB-based model, using cocaine as a specific example (figure 2), are described next to better illustrate the various steps of model development. In this type of model, PK processes of a drug are defined in terms of parameters relevant to physiology, anatomy, and biochemistry (Gibaldi and Perrier 1982, pp. 355-384). When the cell membrane is considered very permeable to a drug, its transfer between capillary blood and interstitial water is very rapid. Therefore, drug transfer from capillary blood to various tissues (i.e., drug disribution is perfusion (i.e., blood flow) limited, and specific tissues can be represented as a single ("lumped") compartment. The emerging (i.e., venous) concentration from a given organ is in equilibrium with the organ in question. This assumption appears reasonable for cocaine, a small, lipophilic molecule (log octanol/water partition coefficient of -2.3) molecule (DeVane 1991, pp. 18-36). Another common and reasonable assumption is that only the unbound drug is transported across membranes and is available for elimination. Since cocaine is not a highly bound drug (Sandberg and Olsen 1992), it is reasonable to express rate equations written using total plasma concentration as a first approximation. Urinary excretion for cocaine is a minor pathway in humans (Ambre 1989, pp. 53-69). Incubation experiments with microsomes derived from human placenta show that it metabolizes cocaine (Roe et al. 1990) to a small extent (-20 percent). Therefore, it is assumed that all organs except the liver, kidney, and placenta are of the noneliminating type in the development of a preliminary model. The validity of these assumptions will become apparent as the model is developed and they (i.e., the assumptions) need to be modified as necessary.

Since information regarding maternal-fetal drug distribution of cocaine is not completely known at present (as is the case with most other drugs of abuse), there is no simple way at the outset to decide which regions should be included. The common practice is to conceptualize a probable model based on the existing knowledge of the physicochemical and PK properties of a given drug and its specific organ toxicities (Bischoff 1975). Therefore, the tentative PK/PB model for cocaine (figure 2) includes those organs that are adversely affected by cocaine such as the heart and brain. (For reasons of page size and clarity of diagram, fetal heart is not shown in figure 2.) Rat studies have shown cocaine uptake by these organs and the placenta (DeVane et al. 1989). Since cocaine is a small, lipid-soluble molecule, organs with high blood flow (e.g., kidney, lung, liver) also are included in the model as they are likely regions of high cocaine uptake.

Based on the above discussion, a schematic representation of a tentative perfusion rate-limited PK/PB model for cocaine is shown in figure 2, where Q_L Q_G, Q_K, Q_{Lu}, Q_{Br}, and Q_H denote maternal blood flow to the liver, gastrointestinal tract, kidneys, lungs, brain, and heart, respectively. Kidney and hepatic clearances are denoted by CL_K and CL_L, respectively. Similarly, in the fetal segment, Q_{FK}, Q_{FG}, Q_{FBr}, and Q_{FB} refer to blood flow to fetal kidneys, gastrointestinal tract and brain, and fetal blood respectively; Ql denotes blood flow to the placenta, Q2 represents liver blood flow derived from the umbilical vein, Q3 represents blood flow shunted via the ductus venosus, and Q3 = Q2+Q_{FG}. Other model abbreviations are as follows: NF = number of fetuses, FL = fetal liver, FB = fetal blood, FBr = fetal brain, FK = fetal kidney, FB = fetal gastrointestinal tract, AF = amniotic fluid, CL_{FK} = fetal urinary drug clearance and CL_{FS} = clearance from amniotic fluid due to fetal swallowing, and CL_{PL} = placental drug clearance.

The mass balance equation for the drug (in this case, cocaine) for any given organ/tissue is:

> Rate of change of drug in organ = rate of entry - rate of exit - rate of elimination (10)

Diffusion of drug between adjacent tissues is generally ignored due to its minor role in drug transport. For flow-rate limited drugs, rate of entry is given by the product of blood flow to the organ and incoming (i.e., arterial) concentration. Similarly, rate of exit is equal to the product of blood flow and outgoing (i.e., venous) concentration. The incoming concentration at any given time is the same for all organs and can be easily determined by monitoring arterial drug concentrations (except the lungs, for which it is the venous blood). On the other hand, venous concentrations emerging from each organ would be different based on differences in drug uptake and elimination by the tissue in question. Hence, it is almost impossible to separately determine emerging drug concentrations from each organ. Venous blood concentrations from a peripheral vein (as is the usual practice due to convenience), is a pooled estimate of drug concentrations leaving the various organs. Fortunately, for perfusion rate-limited (i.e., freely diffusing) drugs like cocaine, emerging (i.e., venous) blood can be assumed to be in equilibrium with the tissue: hence. tissue (C,) and venous (C,) drug concentrations are related by the following relationship:

$$C_{ven} = C_T/K_T \quad (11)$$

where K_T is the equilibrium tissue to blood concentration ratio determined at steady state. Therefore, for noneliminating organs (e.g., brain, heart, lungs, as in figure 2), the mass balance equations can be written as:

$$V_T * dC_T/dt = Q_i (C_{art} - C_T/K_T) \quad (12)$$

where

V_T = Volume of the given organ/tissue
Q_i = Blood flow to the organ/tissue
C_{art} = Cocaine concentration in arterial (incoming) blood
C_T = Tissue concentration of cocaine in a given organ
K_T = Tissue/blood concentration ratio (partition coefficient) for the given organ/tissue

For eliminating organs (e.g., liver, kidney, placenta), equation (12) has to be modified to include drug loss due to elimination as shown below (for the liver):

$$V_L * dC_L/dt = (Q_L-Q_G) * C_{art} - Q_L * (C_L/R_L) - CL_L*C_L/K_L + Q_G * (C_G/K_G) \quad (13)$$

where CL_L represents hepatic clearance.

Similarly, a differential expression representing mass balance can be written for each organ/tissue of consequence in the maternal and fetal compartments. It is clear from the preceding discussion that considerable efforts in generation and analysis of data are needed for model development. The major areas of data acquisition are determination of (a) concentration-time profiles of the drug in maternal and fetal plasma and organs of interest, (b) anatomical/physiological parameters such as organ/tissue volumes (masses) and blood flows (often, these values are available from the literature), (c) partitioning parameters such as tissue to plasma ratios and plasma protein binding, and (d) PK variables such as hepatic and renal clearances. With respect to the last mentioned area, it is encouraging to note that allometric equations have been developed to accurately predict human PK data based exclusively on animal data in several mammalian species. Allometric equations (Adolph 1949) in

general relate a physiological variable (PV) such as liver weight, cardiac output, creatinine clearance, or liver blood flow to body weight (BW) as follows:

$$PV = a * BW^{b} \quad (14)$$

where a and b are dimensionless constants.

This concept has been applied to PK variables. For example, one comprehensive review (Boxenbaum 1984) listed 15 drugs whose interspecies clearance values have been related by allometry. The need to include differences in lifespan to improve interspecies extrapolation has been recognized. Owens and colleagues (1987) showed a significant improvement in the correlation between phencyclidine clearance and body weight in six species (namely mouse, rat, pigeon, monkey, dog, and human) when phencyclidine clearance was adjusted for differences in lifespan.

The final step is the generation of model-predicted concentration-time profiles in various organs of the model based on the required physiological and disposition constants (see equations 11 through 15 and figure 2). This requires a mathematically complex process (i.e., numerical integration) of solving simultaneously the mass-balance (differential) equations representing each of the maternal and fetal organs. Model confirmation requires good agreement between observed and predicted concentrations; lack of such agreement will require appropriate modifications of model assumptions. Development of such a model in at least one (and preferably in several) species will provide the opportunity to generate such drug-concentration profiles in pregnant women based on pertinent physiological information and disposition data (obtained directly, by allometry, or on the assumptions of species similarities). Model validation will require comparison of predicted data with actual data obtained from opportunistic situations that are often encountered in the clinical management of pregnant women who ingest drugs of abuse.

While the model involves considerable effort to develop, the potential to provide insights into the effect of physiological perturbations on drug disposition and action makes such efforts worthwhile. For example, placental abruptio and resulting fetal death have been attributed to cocaine use by pregnant women (Acker et al. 1983; Chasnoff et al. 1985). It has been speculated that fetal hypoxemia caused by cocaine-induced impairment in uterine blood flow is a major factor in this fatal outcome.

Studies in pregnant sheep have shown that fetal pO_2 dropped significantly [maximal drop of about 25 percent (from 23 to 17 mm Hg) at about 5 minutes postadministration] following maternal IV cocaine (1 to 2 mgkg); baseline values were established with 15 to 30 minutes (Woods et al. 1987); maximum reductions in uterine blood flow were dose dependent (plasma cocaine concentrations were not determined) and observed within 5 minutes after cocaine administration in the mother. In a similar sheep study by Moore and coworkers (1986), a log-linear dose-response relationship between cocaine dose and uterine blood flow was observed. In addition, a direct relationship between dose (0.3, 0.5, or 1.0 mg/kg IV, infused over 1 minute) and the corresponding mean plasma concentrations obtained 5 minutes postadministration (229,405, and 746 ng/mL) also was observed. Data are also emerging regarding the integration of such models with drug effects (i.e., PD). Two recent abstracts (Hou et al. 1990, 1991) summarized the development of a PK/PB model-based dosing regimen for patients treated with amiodarone to control atrial fibrillation and flutter with rapid ventricular rates. The complex problem of drug-induced alterations in maternal-fetal hemodynamics and their effects on PK/PD of drugs of abuse remain essentially uninvestigated. Results obtained with cocaine (Moore et al. 1986; Woods et al. 1987) show that the much-needed development of PK/PB-based model is a viable approach to this challenging problem.

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Placental Permeability for Drugs of Abuse and Their Metabolites

George D. Olsen

INTRODUCTION

The ability of a drug to cross the placenta depends upon the properties of the drug and the physiology of the placenta. Physical and chemical features of the drug that are important are lipid solubility and molecular size and charge, all of which should be determined before in vitro and in vivo experiments are undertaken. Maternal and fetal blood flow to the placenta, as well as the structure and permeability characteristics of the placenta, should be understood before animal models are selected for comparison with the human condition. The usual mechanisms for placental transfer are diffusion through the membrane by lipid-soluble substances and diffusion through water-filled pores by hydrophilic compounds. Transfer of the former is blood flow limited, and transfer of the latter is diffusion limited. Active transport may occur if the compound is closely related to an endogenous substance such as an amino acid for which a carrier exists. Lipid-soluble drugs will equilibrate across the placenta rapidly, and hydrophilic compounds will equilibrate slowly during constant maternal drug exposure. Whether fetal blood concentrations equal maternal drug concentrations at steady state during constant-rate maternal infusions depends upon fetal plasma protein binding and blood pH relative to maternal parameters, placental biotransformation, and whether the fetus has the ability to eliminate the drug.

The placenta is a membrane of exchange between mother and fetus and accordingly is supplied with a double perfusion system. Oxygen and other nutrients such as glucose and amino acids are delivered to the placenta by the maternal circulation, whereas carbon dioxide is removed by the same circulation. Most drugs of abuse are lipid soluble and therefore rapidly pass through the placenta. Ethanol is not very lipid soluble (Hansch et al. 1987), but, because of its small molecular weight and high maternal blood levels, it readily reaches the fetus, probably through water-filled pores. Many lipophilic drugs are metabolized in the maternal-fetal unit to hydrophilic compounds, which cross the placenta more slowly than the parent drug. The purpose of this chapter is to

review the principles of placental permeability and to apply these principles to drugs of abuse and their biotransformation products.

PLACENTAL STRUCTURE

The number of tissue layers between fetal blood and maternal blood in animal species commonly used for placental transfer studies varies from two to four (Faber and Thomburg 1983, pp. 79-89). The guinea pig has two layers, the rabbit three, and the rat and sheep each have four layers. Although the rat placenta has the same number of layers as the sheep, it is the more permeable of the two species. The guinea pig placenta is the most permeable, with a hemomonochorial type of placenta similar to the human placenta. The sheep placenta is the least permeable with an epitheliochorial type of placenta that is much different from the human placenta.

STUDY METHODS

In vivo and in vitro methods have been used to study placental drug transfer. In vivo studies are preferred, but caution in interpretation is necessary when general anesthesia is used because of a reduction in placental blood flow and a subsequent decrease in the rate of transfer of lipid-soluble substances. However, this is not a concern with hydrophilic substances (Olsen et al. 1989). In vitro perfusion of the placenta also yields valuable data, but elevated perfusion pressures may artificially increase permeability (Faber and Thomburg 1983, pp. 79-89). The sensitivity and specificity of the analytical method used to quantitate drugs must be determined.

LIPID-SOLUBLE DRUGS

Cocaine, morphine, and nicotine are examples of lipid-soluble compounds that readily cross the placenta. In a perfused cotyledon preparation, cocaine is rapidly transferred across the human placenta (Schenker et al. 1993). It also crosses the placenta of sheep (DeVane et al. 1991; Morishima et al. 1992; Woods et al. 1987), macaques (Binienda et al. 1993), rats (Spear et al. 1989), and guinea pigs (Sandberg and Olsen 1992). Morphine crosses the placenta of many species but has been studied most thoroughly in sheep (Szeto et al. 1982a). In women who

smoke, nicotine passes through the placenta and is found in amniotic fluid and newborn serum (Luck et al. 1985). For these drugs, placental transfer is not limited by diffusion but by reduction in delivery of drug to the placenta (i.e., reduced blood flow will reduce transfer rate).

HYDROPHILIC COMPOUNDS

Metabolites of abused drugs are frequently hydrophilic compounds and cannot pass directly through lipid membranes, but rather they diffuse through the placenta via water-filled channels in the membrane. This passage is termed "diffusion-limited transfer." The size of the channel varies with the species studied, as does the molecular weight cutoff at which drugs can no longer diffuse through the placenta. For the epitheliochorial sheep placenta that cutoff is about 500 daltons (Faber and Thomburg 1983). However, for the hemomonochorial placenta of the guinea pig and human, the permeability exceeds 5,000 and may extend beyond a molecular weight of 50,000 (Challier et al. 1985; Faber and Thomburg 1983, pp. 79-89; Sibley et al. 1983; Thomburg et al. 1988; Willis et al. 1986).

Morphine-3-ß-D-glucuronide, the major metabolite of morphine in man (Boemer et al. 1975) and most mammals including the sheep (Olsen et al. 1988) and the guinea pig (Murphey and Olsen 1993), has a molecular weight of 461. In late-gestation sheep, this metabolite does not cross the placenta in significant amounts even when high maternal levels are sustained for many hours (Olsen et al. 1988). Likewise when the compound is injected into the fetus, it crosses into the maternal circulation very slowly. The 3-ß-D-glucuronide of morphine does cross the guinea pig placenta (Olsen et al. 1989), which has permeability characteristics similar to the human placenta. The permeabilit surface area product for this metabolite in the mature fetus is 3.7×10^{-5} mL/sec/g of placenta and is independent of anesthesia, indicating that diffusion is responsible for its placental passage. The permeability of morphine-3-ß-D-glucuronide increases as the fetus ages during late gestation. This trend has also been noticed for inulin and cyanocobalamin (Adams et al. 1988) and is probably related to structural changes in the placenta (Firth and Farr 1977). The permeability surface area product for this same metabolite in sheep is 2.1×10^{-6} mL/sec/g. By this measure, morphine-3-ß-D-glucuronide is 18 times more permeable in guinea pigs than in sheep.

Benzoylecgonine, a hydrophilic active metabolite of cocaine, has also been studied in the late-gestation guinea pig (Sandberg 1992; Sandberg et al., in press). The permeability surface area product for this compound is estimated to be 3.6×10^{-4} mL/sec/g, indicating greater permeability than morphine glucuronide. This is expected because of its smaller size (molecular weight of 289).

PEPTIDES

Placental permeability of peptides has not been well studied, but their passage should be governed by the same principles as other compounds. Molecular size and lipid solubility are the most important factors. In addition, molecular charge is emerging as a crucial feature for protein permeability of the hemomonochorial placenta. Cationic horseradish peroxidase, a protein with a molecular weight of 40,000 (Maehly 1955), is about seven times more permeable than anionic horseradish peroxidase (Berhe et al. 1987; Sibley et al. 1983). The cationic molecule causes structural changes in the placenta that are associated with the increased permeability. In the human placenta anionic sites have been reported that may be responsible for an increase in the permeability of cationic protein (King 1981, 1985).

If a peptide were small enough, such as a dipeptide or tripeptide, it might be transferred by an active process used for amino acids (Alonso-Torre et al. 1992; Carroll and Young 1983; Pueschel et al. 1983), but there are no published examples for peptides. It is possible that placental transfer of peptides is reduced by peptidases, but this has not been studied. Finally, histamine increases placental permeability to protein in the hemomono-chorial placenta (Berhe et al. 1988).

Oxytocin (Burton et al. 1974) and arginine vasopressin (Forsling and Fenton 1977) are able to pass the guinea pig placenta from mother to fetus, and oxytocin crosses the human placenta (Dawood et al. 1978). There are a number of peptides, however, that do not cross the sheep placenta: oxytocin (Glatz et al. 1980) and arginine vasopressin (Stegner et al. 1984), which are nonapeptides; vasoactive intestinal peptide (Shulkes et al. 1987), which has 28 amino acids; and metkephamid (Frederickson 1986; Frederickson et al. 1983, pp. 150-156), which is a pentapeptide. The peptide studies are summarized in table 1.

TABLE 1. *Peptide transfer across hemomonochorial and epitheliochorial placentas.*

Peptide	M_r	Placenta Type		References
		Hemomonochorial	Epitheliochorial	
Metkephamid	599	Yes (H)	No (S)	Frederickson et al. 1983 C.J. Parli personal communication, September 15 and 23, 1993
Oxytocin	1,007	Yes (GP, H)	No (S)	Burton et al. 1974 Dawood et al. 1978 Glatz et al. 1980
Arginine vasopressin	1,084	Yes (GP)	No (S)	Forsling and Fenton 1977 Stegner et al. 1984
Vasoactive intestinal peptide	3,326	ND	No (S)	Shulkes et al. 1987
Horseradish peroxidase (protein)	40,000	Yes (GP) (Depends on charge)	No (S)	Sibley et al. 1983 Berhe et al. 1987 K. Thornburg personal communication, September 23, 1993

KEY: M_r = molecular weight; Yes = transfer detected within 24 hr; H = human; S = sheep; GP = guinea pig; ND = not done.

Metkephamid (LY127623), an analog of met^5-enkephalin [D-Ala^2-(Me)Met^5-enkephalin amide], was studied in the 1980s for possible use in obstetrical analgesia, but development was stopped when metkephamid produced significant hypotension in obstetric patients during human placental transport studies (Frederickson and Chipkin 1988, pp. 407-417). This peptide, which has a molecular weight of 599, is unlikely to pass through the sheep placenta but should diffuse slowly through the rat and human placenta. The available data support this analysis (Frederickson 1984, pp. 9-68; 1986, pp. 293-301; Frederickson and Chipkin 1988, pp. 407-417; Frederickson et al. 1983, pp. 150-156; C.J. Parli, personal communication, September 15 and 23, 1993). The blood levels of metkephamid in the maternal rat are 60 times the fetal blood level 1 hour after a single subcutaneous injection to the dam, suggesting slow placental transfer to the fetus. Of 19 pregnant human subjects studied, fetal levels were quantifiable in the umbilical vein of 7 newborns following a single intramuscular dose to the mother, except for 1 subject who received 2 doses. The maternal concentrations taken at parturition, about 1 to 2 hours after the injection, were 12 times greater than the umbilical vein concentration taken at birth. It should be emphasized that with one umbilical vein blood sample and one maternal sample taken after a single dose of a slowly transferred substance, the relative placental permeability can only be estimated. The data suggest that, as in the rat, this compound passes slowly through the human placenta and is not actively transported.

FETAL TO MATERNAL CONCENTRATION RATIO

The ratio of fetal to maternal blood drug concentration has often been used in the past to make judgments about rapidity and extent of placental drug passage. Under steady-state conditions the total drug concentrations on both sides of the placenta need not be equal if there are fetal and maternal differences in protein binding and pH (if the drug is a weak acid or base), for it is the unbound and unionized drug that equilibrates across the placenta (Sandberg and Olsen 1992). Placental drug biotransformation would also lower the fetal level relative to maternal concentration, but placenta drug metabolism is negligible for most compounds. In addition, Szeto (Szeto 1982; 1992, pp. 29-45; 1993; Szeto et al. 1982a, b) has clearly demonstrated that even the unbound and unionized drug need not be equal at steady state if the fetus can eliminate the drug, which is the case for morphine in the late-gestation sheep fetus (Olsen et al. 1988). Fetal blood concentration of an unbound, unionized drug is less than

maternal concentration when fetal elimination is present and significant. Using a clearance approach, Szeto (Szeto et al. 1982a; Szeto 1992) estimated that about two-thirds of morphine infused into the fetal lamb was eliminated by the fetus. Later, Olsen and colleagues (1988) demonstrated in a metabolism study that 63 percent of the morphine infused to the fetal lamb was converted to morphine-3-ß-D-glucuronide, which is close to that predicted by Szeto and colleagues (1982a) using the clearance approach. There probably is a small amount of fetal renal elimination of morphine in addition to the substantial fetal biotransformation.

RECOMMENDATIONS FOR EXAMINATION OF PLACENTAL PERMEABILITY FOR NEW COMPOUNDS

The author suggests the following:

- Lipid solubility, molecular size, structure, and charge of the compound should be determined.
- Animal models used should have permeability characteristics similar to the human placenta for compounds that are hydrophilic.
- Fetal ability to eliminate the compound should be determined.
- In vivo studies are preferred to or should be used to validate in vitro placental perfusion studies.
- Nonanesthetized preparations are preferred for lipid-soluble compounds but are not necessary for hydrophilic compounds.

SUMMARY

Placental passage of drugs, including peptides, is determined by the physical and chemical properties of the drug and the physiology of the placenta. Fetal blood concentrations of drugs administered to the mother depend upon time after maternal drug administration, dose, placental blood flow and permeability, plasma protein binding, blood pH, placental biotransformation, and fetal elimination.

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Pharmacodynamics in the Maternal-Fetal-Placental Unit

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Pharmacological agents or drugs administered to pregnant mothers have potential teratogenic effects, as well as possible influences on physiological and biochemical functions in the embryo or fetus. Recently, with advances in the ability to detect not only structured anomalies but also physiological abnormalities in human fetuses by means of ultrasound and Doppler techniques, increasing consideration has been given to treating fetuses by various means, including administration of drugs to the mother. The pregnant sheep model has been used extensively to study the effects of drugs on fetal cardiovascular function, respiratory movements, and brain wave activity, as well as other physiological parameters. Particularly important attributes of this model have been the ability to study the fetus in chronically instrumented preparations without the influence of other drugs such as anesthetics or sedatives and to examine responses at different periods of gestational development.

This chapter addresses some of the factors that may be important in determining the effects on the fetus of drugs administered to the mother. These include placental transfer of the drug and its metabolites; distribution of the drug in the placenta, fetus, and fetal fluids; drug metabolism; elimination of the drug and its metabolites; and pharmacological responses of the fetus.

PLACENTAL TRANSPORT

Drug transfer across the placenta from mother to fetus is dependent on many factors. Large molecules are not transferred, and the size of the molecule that is transferable is greatly influenced by placental morphology and lipid solubility. The rabbit and guinea pig have a hemoendothelial placenta in which maternal and fetal blood is separated only by a single layer, and molecules as large as albumin can pass across the placental membrane. However, the syndesmochorial placenta of the sheep has five layers separating maternal and fetal blood, and this imposes a greater barrier to diffusion.

Lipid-soluble drugs generally diffuse readily across the placenta, but transfer of water-soluble substances is very much dependent on the size of the molecule. Many drugs and substances are transferred by simple diffusion. Under these circumstances, the concentration gradient between maternal and fetal blood determines rate of exchange. This is largely determined by the flow rates in the uterine and umbilical circulations, which determine rate of delivery of drug to the placenta and rate of removal. The possibility also exists that the placenta may actively influence transport. Thus, glucose is transferred from mother to fetus by facilitated diffusion. The placenta is a very active organ metabolically and possibly could metabolize the drug before it enters the fetal circulation. It is estimated that about 30 percent of glucose presented to the placenta in the sheep is metabolized.

Diffusion across the placenta is influenced by the concentration of the physically dissolved substance, not total concentration. Therefore, if the percentage of a drug bound to protein is high in maternal blood, much less would be available for diffusion, thus reducing transfer. Plasma protein concentrations are usually lower in fetal blood than in maternal blood, so that concentration of free drug would be higher at the same total concentration. Many of the physical properties influencing diffusibility of molecules across the placenta have been reviewed by Faber (1973).

FETAL DISTRIBUTION OF DRUG

Drugs traversing the placenta are diverted to the fetus in umbilical venous blood. The course and distribution of blood rationing to the fetal heart from various sites could significantly influence the concentrations of drug affecting different organs. As shown in figure 1, the umbilical vein provides branches to the left lobe of the liver, after which the ductus venosus arises and a transverse branch then continues to the right lobe of the liver, where it is joined by the portal vein. About 50 percent of umbilical venous blood enters the hepatic circulation, and the remainder traverses the ductus venosus, thus bypassing the liver (Edelstone et al. 1978). This pattern of flow thus allows half of all transported drug to bypass the liver, where it potentially could be metabolized during its first pass, and to enter the fetal circulation directly. Also, the highest concentration of drug would be encountered by the left lobe of the liver. The proportion of umbilical venous blood bypassing the liver through the ductus venosus increases during fetal hypoxemia and especially when total umbilical flow is reduced (Edelstone et al. 1980).

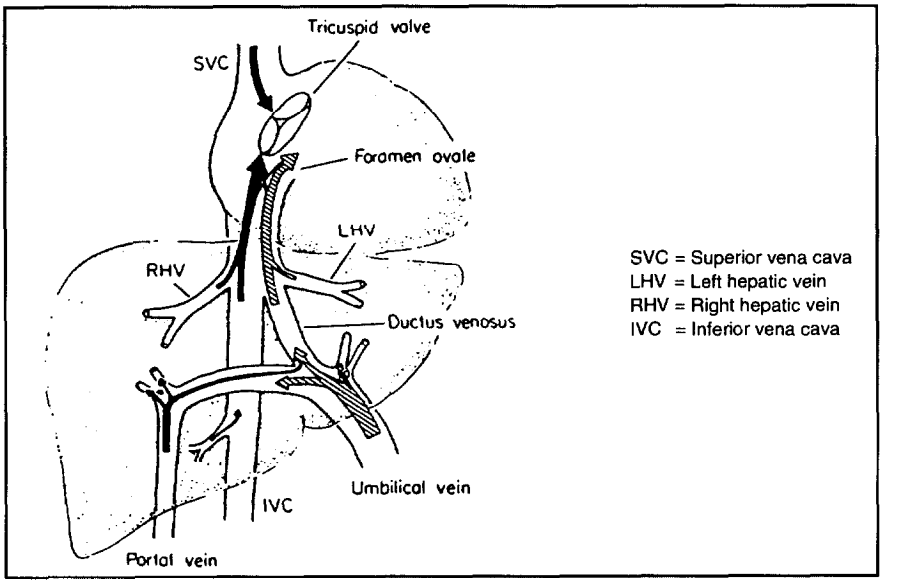

FIGURE 1. *Patterns of venous blood flow in the fetal liver.*

Streaming of blood flow is a prominent feature of fetal venous return (figure 1). The ductus venosus blood entering the inferior vena cava tends to stream through the foramen ovale, to be distributed to the left atrium and ventricle and thus to the ascending aorta to supply the upper body, including the brain and heart (Edelstone and Rudolph 1979; Reuss and Rudolph 1980). Blood from the lower body in the inferior vena cava preferentially flows through the tricuspid valve into the right ventricle and then through the ductus arteriosus to the descending aorta to supply the lower body and placenta. Similarly, all superior vena caval blood passes through the tricuspid valve into the ventricle (Rudolph and Heymann 1967).

These flow patterns are responsible for the higher oxygen content and glucose concentration in ascending aortic blood, as compared with descending aortic blood in the fetus. There is every reason to suspect that drugs transferred across the placenta are delivered in higher concentrations to the brain and heart than to lower body organs. The importance of this phenomenon is yet to be explored. An example of its possible influence on fetal response to drug relates to the action of nicotine on the fetus. The fetus has a well-developed carotid chemoreflex response. Nicotine stimulates peripheral chemoreceptors, and, if it reaches the

carotid artery in high concentrations, it elicits a chemoreflex response, characterized by bradycardia, hypertension, and a fall in cardiac output.

DRUG DISTRIBUTION IN FETUS AND FETAL FLUID COMPARTMENTS

Drugs administered to the mother have been identified in fetal urine and tracheal fluid, and in amniotic and allantoic fluid, in addition to their presence in the fetal blood and tissues. It has been suggested that drug appearance in amniotic and allantoic fluid results from its passage into urine or tracheal fluid, which then is excreted into the amniotic or allantoic sacs, from transfer across the fetal skin, or from direct transport from the mother through the chorioallantoic membranes (Carson et al. 1979; Mellor 1980; Seeds 1981).

To study the disposition of drugs in the maternal-fetal-placental unit, the author developed a model in which the pregnant sheep and her fetus were chronically instrumented. Catheters were placed in a maternal artery and uterine vein, the fetal descending aorta (via a hindlimb artery), an umbilical vein, the fetal and maternal bladder to permit timed urinary collections, and the amniotic and allantoic sacs. Two drugs have thus far been studied extensively-acetaminophen and its glucuronide and sulfate conjugates, and nicotine and its metabolite cotinine. Studies have been conducted after injection of bolus doses of the parent drug or the metabolites into the mother, fetus, allantoic or amniotic fluid, and after continuous infusion of the drug into the maternal and fetal circulations to achieve a steady state.

Acetaminophen

From the bolus injection studies, it is evident that acetaminophen is rapidly transferred reversibly across the placenta (Wang et al. 1990). After a maternal injection, the maternal plasma concentration increases rapidly and then falls with a half-life of 0.89±0.20 hour. The fetal plasma concentration increases instantaneously and peaks in 10 to 20 minutes, with a terminal half-life of 1.20±0.25 hour, which was not significantly different from that in the ewe. However, the acetaminophen concentration in the fetus exceeded that in the mother after about 1 hour. This suggested that acetaminophen was being distributed in a fetal compartment and was then being released into the fetal circulation. Of importance is the fact that although the fetal peak concentration of

acetaminophen was lower than that in the mother, after the first hour, the fetus was exposed to a higher concentration than the mother for several hours.

By examining concentration time profiles of acetaminophen in various sites after different sites of injection, the process of transfer can be assessed after bolus injections (Wang et al., unpublished observations). Thus, injection of acetaminophen into the mother showed a concentration rise in the umbilical vein before the descending aorta due to placental transfer. After fetal injection, the umbilical venous concentration was less than that in the descending aorta, and the uterine venous was higher than the maternal arterial concentration. The appearance of acetaminophen in the amniotic and allantoic fluids was rapid. It could not have entered from urine because all fetal urine was being collected. Although acetaminophen in amniotic fluid could have been due to passage across skin or via tracheal fluid, it most probably was transferred across fetal membranes. When acetaminophen was injected into the amniotic cavity, it appeared in fetal blood before it appeared either in allantoic fluid or maternal plasma. Similarly, when injected into allantoic fluid, acetaminophen first appeared in fetal blood before appearing in amniotic fluid or maternal blood. It also was noted that times for peak concentration in amniotic and allantoic fluids were shorter after fetal than after maternal injection. This indicates that there is a considerable transfer of acetaminophen from fetal blood to the amniotic and allantoic sacs but little from these sites to maternal blood. Also, little if any direct transfer between amniotic and allantoic fluids was evident; the passage from one compartment to the other was largely via the fetal circulation.

The major metabolites of acetaminophen are the sulfate and the glucuronide conjugates. These conjugates were studied in the mother and in the fetal compartments after injection of acetaminophen or of either conjugate into the sites mentioned above. After administration of acetaminophen to the mother, the conjugates were rapidly detectable in maternal plasma, and the concentration of the glucuronide (Wang et al. 1985) was higher than that of the sulfate. In the mother, the terminal half-lives of both conjugates were similar and not significantly different from those of the parent acetaminophen. However, the concentrations of both conjugates in fetal blood increased very slowly, reaching peak levels only after 3 or more hours; the concentration also fell much more slowly, with terminal half-lives of 6.3±1.7 hours for the glucuronide and 9.6±2.0 hours for the sulfate.

When acetaminophen was injected into the fetus, peak concentrations of the glucuronide and sulfate conjugates developed within 60 to 90 minutes. In maternal blood, peak concentration times were longer, and concentrations of the conjugates much lower. As with the maternal injection, the decline in concentration of both conjugates was very prolonged in the fetus as compared with the mother.

Injection of either acetaminophen glucuronide or sulfate into the fetal circulation resulted in their rapid appearance in amniotic fluid (Wang et al., unpublished observations). No difference in fetal arterial and umbilical venous concentrations was noted, and neither conjugate appeared in uterine venous blood. Thus, no transfer of the conjugates from the fetus to the mother was detected. Injection of either conjugate into the amniotic sac resulted in a rapid appearance in fetal plasma, and in the allantoic fluid, followed by a very slow fall in concentration, with high levels still detectable after 30 hours.

In summary, acetaminophen rapidly traverses the placenta in either direction. The glucuronide and sulfate conjugates do not, however, appear to cross the placenta unless they do so very slowly. After maternal administration, acetaminophen enters the fetal circulation and then rapidly distributes into the amniotic and allantoic fluids, which form a reservoir that maintains fetal concentrations at levels higher than those in the mother. Once acetaminophen enters the amniotic or allantoic sac, its passage into the fetal compartments indicates that the sheep fetus, by 0.7 gestation, can metabolize acetaminophen. The conjugates, which are water soluble, are not eliminated across the placenta but do distribute readily between fetal blood and amniotic and allantoic fluid, probably across the membranes.

Nicotine

Studies on the distribution of nicotine and a major metabolite in maternal and fetal blood and amniotic and allantoic fluid were conducted in pregnant sheep in a similar manner to those described for acetaminophen (Inokuchi et al., unpublished observations). When nicotine was infused for short periods (30 minutes) into the mother, fetal blood concentrations increased rapidly, and amniotic fluid concentrations peaked by 20 minutes and exceeded fetal concentrations.

Injection of nicotine into the fetus resulted in rapid appearance of nicotine in the mother’s blood, but concentrations were very low because of the

large maternal pool in which the relatively small amount traversing the placenta was diluted. Amniotic concentration of nicotine increased rapidly, reaching a peak in about 60 minutes, as with maternal injection of nicotine. Whereas fetal blood concentrations fell rapidly to almost undetectable levels within about 2 hours, amniotic fluid concentrations fell very slowly, reaching levels that were just detectable in about 6 hours. Of great interest was the observation that the nicotine metabolite cotinine was not detected in either fetal blood or amniotic fluid after nicotine was administered to the fetus in most of the animals studied. In one animal near term (gestational age 140 days, term 145 days), very low concentrations of cotinine appeared in fetal blood and amniotic fluid. Injection of cotinine into the mother resulted in rapid appearance in the fetal blood and amniotic fluid, and injection of cotinine into the fetus also was associated with a rapid appearance in the amniotic fluid as well as in maternal blood. With both injections, maternal concentration fell most rapidly, followed by fetal, and finally, amniotic fluid concentration.

From these studies, it is evident that both nicotine and cotinine traverse the placenta from mother to fetus, or the reverse, rapidly. The fact that cotinine was not detected after fetal injection of nicotine indicates that the fetal lamb does not significantly metabolize nicotine; the small amount of cotinine appearing in the fetus in the late gestation lamb after nicotine injection suggests that metabolic capability develops close to birth.

The author also infused nicotine into either the mother or fetus for long periods (6 to 8 hours) to achieve steady-state concentrations. Nicotine concentrations rapidly increased in amniotic fluid, achieving concentrations similar to those in fetal blood within 1 hour. However, with continued infusion, nicotine concentrations in amniotic fluid progressively increased to achieve levels three to four times higher than those in fetal blood. This higher concentration in amniotic fluid perhaps could be explained by the fact that nicotine has a relatively high pH (Yamamoto 1960) and amniotic fluid pH is fairly acidic, with a pH of 7.0, whereas fetal blood pH is about 7.4.

As with acetaminophen, the amniotic and, presumably, the allantoic fluid serve as reservoirs that maintain a prolonged increase in the concentration of nicotine in fetal blood, even after brief periods of administration to the mother. It is also likely that, if amniotic fluid concentration of nicotine is high, the development of fetal stress with resulting metabolic acidemia would result in rapid transfer of the drug from amniotic fluid to fetal blood, increasing concentrations significantly.

DRUG METABOLISM

Metabolism of drugs by the liver is accomplished through mechanisms involving a variety of enzyme systems. It is well recognized that the enzymes necessary for various metabolic processes are not fully developed at the time of birth. Evidence is presented based on experience with metabolism of acetaminophen and nicotine that there are variations in the maturation of different enzyme systems during gestational development.

Acetaminophen

As just mentioned, dynamic studies in the sheep suggest that glucuronidation of acetaminophen occurs in the fetus. In those studies, fetal lambs as early as 110 days gestation were capable of glucuronidation of acetaminophen. To further define this activity, hepatic microsomes were prepared from the liver of fetal lambs at 113, 135, and 141 days gestation and from adult sheep. UDP-glucuronosyltransferase activity was determined using acetaminophen as aglycone (Wang et al. 1986). The activity of UDP-glucuronosyltransferase was about five times higher in adult than in fetal liver. Not only were there quantitative differences but also differences in the kinetic properties of the UDP-glucuronosyltransferase. That in the fetal liver had a higher affinity for UDP-glucuronic acid and lower affinity for acetaminophen than the adult liver. Furthermore, when enzyme activity was stimulated with UDP-N-acetylglucosamine, there was not only a marked difference between fetus and adult but also a gestational difference. Thus, in the adult liver, enzyme activity was maximally increased by about 400 percent. In fetal liver it was increased by about 170 percent in the term (14 1 -day) fetus but only by about 30 percent in the 113-day fetus.

Nicotine

In the dynamic studies just discussed, it was suggested that nicotine was not metabolized to cotinine in the fetal lamb, except perhaps in very late gestation. In addition to being metabolized to cotinine, nicotine also is metabolized to nicotine N-oxide. The activities of the enzyme nicotine N-oxidase were measured in microsomal preparations from liver of fetal lambs at 125 to 137 days gestation, in newborn lambs at 1 day, and in adult liver. Negligible enzyme activity was detected in the fetal lamb livers, but the activity increased markedly after birth, reaching a level about 20 to 50 percent of that in the adult liver.

It is thus evident that activity of enzymes utilized in drug metabolism matures at different rates during fetal development; this would greatly influence toxicity of drugs in fetuses of different ages.

DRUG EXCRETION

In the studies in chronically instrumented sheep just discussed, catheters were placed in the fetal and maternal bladder to collect all fetal or maternal urine. Using this procedure, it was thus possible to determine urinary excretion rates of the parent drug and its metabolites and to compare these with total clearance.

When acetaminophen was injected into the mother, less than 1 percent of the total dose was excreted into fetal urine. When injected directly into the fetus, about 20 percent of the total dose was recovered in fetal urine over a 24-hour period as unchanged acetaminophen and its conjugates. Of the total eliminated in the urine, a fraction of 0.25+0.01 was acetaminophen. 0.39±0.07 was acetaminophen glucuronide, and 0.36±0.08 was the sulfate.

It is thus evident that the primary elimination route for acetaminophen from the fetus and amniotic fluid is across the placenta to the maternal circulation (Wang et al. 1990). The glucuronide and sulfate metabolites do not cross the placenta. They are removed from the fetus exclusively by renal clearance; the ratios for renal clearance to total clearance were 1.00±0.06 for the glucuronide and 0.95±0.07 for the sulfate (Wang et al. 1985).

Studies with nicotine showed that only 1.3 percent of the total clearance of nicotine was accounted for by renal clearance, and a smaller percentage of cotinine clearance was due to renal excretion.

These studies confirm, then, that the primary route of elimination for drugs and their metabolites from the fetus and fetal fluid sacs is across the placenta. Therefore, water-soluble metabolites, which do not readily traverse the placenta, remain in fetal blood and fluids for extended periods. The significance of this remains to be assessed.

INTRA-AMNIOTIC DRUG THERAPY

Since it is apparent that there is active exchange between fetal blood and amniotic fluid, the possibility of administering drugs to the fetus via the amniotic sac has been considered (Klein et al. 1978). Because fetal tachyarrhythmias are detectable in utero and therapy by administering drugs to the mother has been attempted, the author has explored the possibility of achieving therapeutic concentrations of digoxin in the fetus by intra-amniotic administration (Hamamoto et al. 1990). Digoxin was injected into the amniotic cavity of chronically instrumented fetal sheep; doses were either high (0.7 to 1.8 nmol/kg fetal body weight) or low (0.1 to 0.6 nmol/kg). Plasma digoxin concentrations increased rapidly and achieved concentrations of 18.2±15.0 nmol/L in fetal plasma by 1 hour in the low-dose group. These concentrations were maintained for at least 6 hours. Maternal concentrations were about 10 percent of those in fetal plasma with the high-dose group, and digoxin could be detected in maternal plasma in the low-dose group. This raises interesting possibilities about the use of intra-amniotic administration of drugs to treat the fetus. It will be necessary, however, to develop information regarding dose requirements and the consistency with which appropriate fetal concentrations can be achieved. An important potential advantage of this therapy is that the mother will not be jeopardized by administering drugs in high dosage to her to achieve fetal therapeutic concentrations.

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New Approaches for Drug and Kinetic Analysis in the Maternal-Fetal Unit

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INTRODUCTION

The possibility of fetal exposure to drugs following maternal administration was postulated by Ginsberg in 1968 (Ginsberg 1968), and several questions followed regarding fetal exposure to drugs consumed by the mother. Later, in a theoretical paper, emphasis was placed on the extent of accumulation and persistence of drug(s) in the maternal-fetal unit and the resulting exposure of the fetus to drugs (Levy and Hayton 1973). Answers to a number of the questions regarding fetal pharmacokinetics (PK) and fetal drug metabolism have been addressed experimentally as more elaborate animal models of pregnancy have been developed. This has been made possible largely due to vast improvements in drug measurement technology. Drug analysis plays an important role in many different disciplines of the biological sciences; however, in PK and pharmacodynamics (PD), the role of drug quantitation is pivotal to success. The soundness of PK and PD parameters normally estimated relies heavily on the sensitivity, precision, and accuracy of the analytical methodology supporting their determination. In fact, it is the reliability of these parameters that often dictates the very type of PK experiments that can be conducted, and thus, the type of data obtained.

This chapter focuses on the authors' 10-year experience examining the PK and PD of drugs in the ovine maternal-placental-fetal unit. In this chapter, a brief overview of the work conducted in the authors' laboratory studying the PK of metoclopramide (MCP), diphenhydramine (DPHM), and labetalol in pregnancy using traditional analytical techniques (i.e., gas chromatography and high-performance liquid chromatography [HPLC]) is presented. With these early techniques, several important issues pertaining to drug disposition in pregnancy could not be readily studied, namely, the study of PK using stable isotope-labeled drugs (SIL) and the

stereoselective disposition of chiral drugs. With the advent of new technology (e.g., economical benchtop mass spectrometers and stereoselective HPLC columns), these fundamental issues have begun to be addressed. Within this chapter, particular reference is paid to the impact that these new developments in analytical technology have had on scientists' ability to investigate drug disposition in pregnancy. This includes the use of simultaneous administration of unlabeled and SIL drugs and their quantitation with mass spectrometry, and the chiral separation and quantitation of optical enantiomers. As a result, scientists now can study the kinetics and disposition of SIL DPHM, valproic acid, and their related metabolites in pregnancy. In addition, scientists now have the capability to study the fetal-maternal stereoselective disposition of labetalol.

THE STUDY OF FETAL-MATERNAL PHARMACOKINETICS: AN OVERVIEW OF THE EARLY YEARS

Metoclopramide

Early investigations concentrated on the PK of the antiemetic drug MCP in pregnancy. MCP has been used to promote gastric emptying, particularly before emergency and elective cesarean section (Chestnut et al. 1987; Cohen et al. 1984; Shaughnessy 1985). Thus, MCP has been shown be an effective preanesthetic medication to prevent aspiration-induced deaths (Chestnut et al. 1987; Cohen et al. 1984). As the use of MCP increased as a preanesthetic, its safety during pregnancy was questioned due to reported central nervous system (CNS) side effects noted in adults, particularly after intravenous (IV) administration (Bylsma-Howell et al. 1983). At the time this issue surfaced, the sensitivity of the packed-column chromatographic method of analysis of MCP was only sufficient to permit single-point plasma concentration determinations as an index of fetal drug exposure. As has been previously suggested, single-point determinations of fetal drug exposure could result in misleading information depending on whether the sample was drawn early or late after drug administration (Levy and Hayton 1973). Thus, it was imperative to increase the sensitivity of the MCP analytical method in order to provide an accurate estimate of the extent of fetal exposure to MCP following maternal administration.

Two technological developments aided attempts to increase the sensitivity of this analytical method to suit the experimental requirements.

Firstly, the development of processor-controlled gas chromatographs and pulsed-mode electron capture detectors (ECDs) provided a greater linear range over which the analyte of interest could be reliably measured. Secondly, the introduction of fused silica capillary gas chromatographic columns in the early 1980s substantially improved the sensitivity of gas chromatography due to improved separation, reduced column reactivity, and improved quality control. With these advances, a selective and sensitive method of analysis for MCP in biological fluids obtained was developed using gas chromatography, fused silica capillary columns, and ECD (Riggs et al. 1983). This method was used in the assessment of the fetal-maternal PK of MCP following bolus administration and infusion in the chronically instrumented pregnant ewes (Riggs et al. 1987, 1988, 1990). As had been predicted earlier (Levy and Hayton 1973), the single-point determination of fetal drug exposure could have yielded three different answers regarding the degree of fetal exposure following maternal administration depending on the time of sampling (figure 1). That is, if the sample were collected at 1 hour the fetal-maternal ratio would be estimated to be roughly 0.5, while if the sample were collected at 2 hours the fetal-maternal ratio would be 1.0. Finally, if the sample were collected at 4 hours after drug administration the fetal-maternal ratio would be 1 SO. The most valid assessment of fetal exposure was obtained when the area under the plasma concentration versus time curve (AUC) ratios were determined. When the AUC ratio was used to determine the fetal exposure (i.e., AUC fetus/AUC mother) the extent of exposure was approximately 0.8. Clearly, single-point determination could lead to highly variable and, in fact, incorrect interpretations of the degree of fetal exposure. The improved sensitivity of this assay method allowed a more accurate determination of the degree of fetal MCP exposure (via AUC ratios). This determination of the AUC ratios was made possible since the concentration of MCP in fetal and maternal plasma could be followed for a longer period of time following maternal administration despite using only small volumes of sample. In addition, MCP was also shown to extensively accumulate in fetal lung fluid. The concentration of MCP in this fluid was roughly fivefold to tenfold greater than the concentration observed in both fetal and maternal plasma (figure 1). The toxicological implications of this accumulation are not yet clear and are the subject of ongoing investigations. MCP was also shown to undergo efficient placental transfer from mother to fetus and from fetus back to the mother, and maternal and fetal nonplacental elimination were also noted.

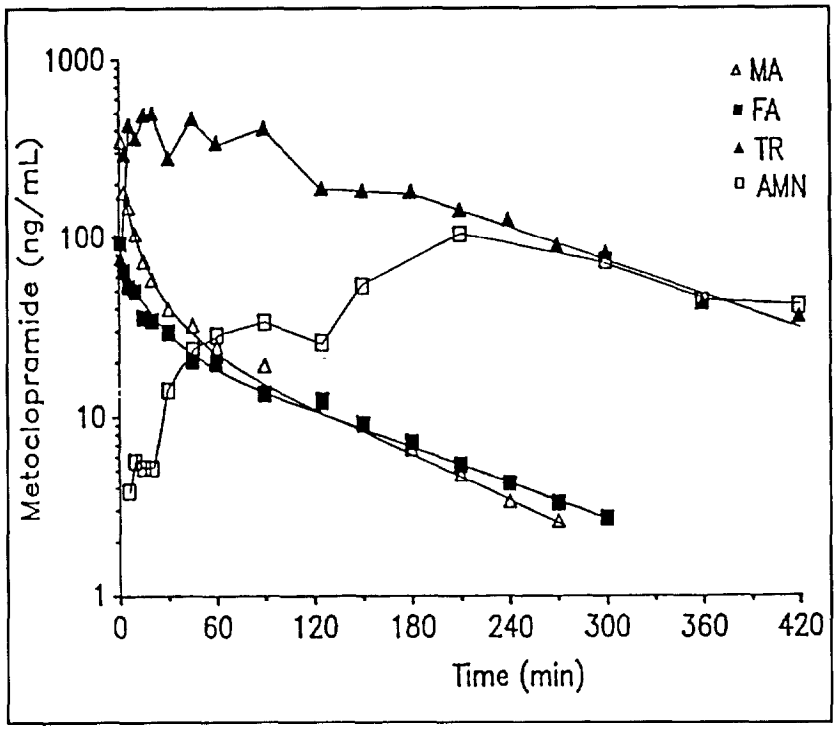

FIGURE 1. *Disposition of metoclopramide in fetal and maternal plasma, amniotic fluid, and fetal lung following a 40 mg IV bolus dose to a pregnant ewe.*

KEY: MA = maternal femoral arterial plasma
FA = fetal femoral arterial plasma
AMN = amniotic fluid
TR = tracheal fluid

Diphenhydramine

The evolution of study from the antiemetic agent to the antihistamine drug DPHM was a natural progression given that DPHM is a classical histamine 1(H_1) receptor antagonist. Antihistamines are used during pregnancy for the symptomatic treatment (usually self-medication) of several pregnancy- and nonpregnancy-related conditions (e.g., insomnia, allergies, urticaria, and coughs) (Piper et al. 1987). Yet little data were available on the disposition of this drug during pregnancy and the effects on the fetus. A selective and sensitive analytical method for the quantitation of DPHM with a lower limit of quantitation of 2.0 ng/mL was

developed in the authors' laboratory using capillary gas chromatography and nitrogen-phosphorus (N/P) selective detection (Yoo et al. 1986a). Using this analytical method, it was possible to thoroughly elaborate the disposition of DPHM in the chronically instrumented pregnant ewe. DPHM was shown to rapidly and readily cross the ovine placenta resulting in significant fetal exposure (AUC fetal/AUC maternal = 0.85±0.40) (Yoo et al. 1986b). Furthermore, like MCP, DPHM was shown to accumulate in fetal lung fluid from three to five times the fetal plasma concentrations (figure 2). The placental and nonplacental clearance values were calculated for both the mother and fetus using the two-compartment open model (Levy and Hayton 1973; Szeto et al. 1982). The experimental design used to calculate the placental and nonplacental clearance of DPHM utilized two separate infusions to steady state (one fetal and one maternal), which were separated by a short 2- to 3-day washout period. The main assumption made in this experimental design is that the calculated PK parameters were not significantly affected by possible time-dependent changes in fetal development and growth. This assumption could not previously be validated using conventional analytical and PK methods (i.e., unlabeled drug). It would be better to utilize simultaneous infusions of an unlabeled and a labeled form of the drug and to have an analytical method that could estimate both forms of the drug when present together in the same sample. The authors' approach was to synthesize a SIL analog of DPHM which then, in turn, could be administered simultaneously, by infusion to steady state to the fetus, with unlabeled drug to the ewe. This method has allowed the authors to examine fetal organ clearance studies in utero. These experiments have been directed toward the determination of the fetal nonplacental clearance of DPHM.

Labetalol

Labetalol has been used, either alone or in combination with diuretics, in the management of systemic hypertension of various etiologies and has been used as the drug of choice in the management preeclampsia (Goa et al. 1989). Although a number of clinical studies have been conducted to assess the efficacy of labetalol in pregnancy, there was no information in the literature regarding the in utero fetal exposure to maternal labetalol as well as its effects on the fetal lamb. Also, labetalol is a chit-al compound with two asymetric carbon atoms. Thus, it exists as four stereoisomers and is marketed as a racemic mixture. There were no data on the stereoselective disposition of the drug in pregnancy.

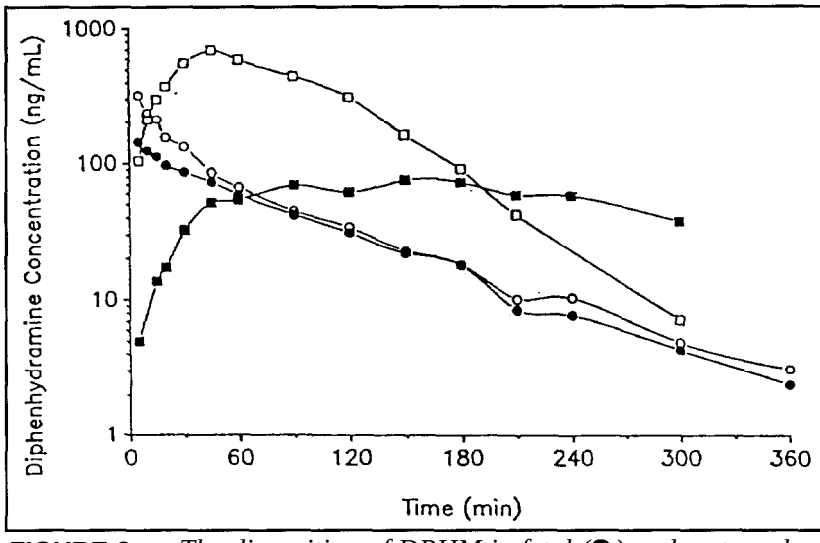

FIGURE 2. *The disposition of DPHM in fetal (●) and maternal (0) plasma, amniotic fluid (■), and fetal lung fluid (Cl) following a 100 mg IV bolus dose to a pregnant ewe.*

Initial work in the authors' laboratory concentrated on the development of an assay for racemic labetalol that was sufficiently sensitive to measure picogram quantities of labetalol in small volumes of biological fluids obtained from the chronically instrumented pregnant ewe (e.g., amniotic fluid, fetal lung fluid, fetal and maternal urine, and fetal and maternal plasma). A sensitive assay, using microbore HPLC and low-dispersion fluorescence detection, was developed for this purpose (Yeleswaram et al. 1991). The method was subsequently used to characterize the PK of labetalol as well as its placental and nonplacental clearances in both nonpregnant and pregnant sheep, as well as in the fetal lamb (Yeleswaram et al. 1993a, b). This method represented a significant improvement over previously published assays (Abemethy et al. 1986; Hidalgo and Muir 1984; Ostrovska et al. 1988; Wang et al. 1985) in terms of sample volume required, precision of quantitation, and the minimum quantitation limit.

NEW TECHNIQUES TO STUDY FETAL-MATERNAL PHARMACOKINETICS

Application of Stable Isotope Techniques to the Investigation of Diphenhydramine and Valproic Acid Disposition During Pregnancy

The past two decades have seen a steady rise in the use of stable isotope techniques to investigate the PK and metabolism of various drugs in both laboratory animals and humans (Browne 1990). In the past, the widespread use of this technique was hampered by numerous factors. Perhaps the most notable factors were the availability and cost of both the SIL drugs (and/or synthetic precursors), and the mass spectrometers required to differentiate between isotope-labeled drugs and their unlabeled counterparts. Although these factors may still be prohibitive in some cases, the advent of smaller and less expensive benchtop mass spectrometers (or mass selective detectors) and a rapidly increasing selection of available SIL drugs and synthetic precursors make this technology accessible to a considerably larger group of investigators.

Stable Isotopes and Mass Spectrometry. Stable isotopes are, as the name implies, stable forms (nonradioactive) of an atom, which differ only in atomic mass due to differing numbers of neutrons in the nucleus. Numerous stable isotopes of elements commonly found in organic molecules have been identified (e.g., ^{13}C, ^{17}O, ^{18}O, ^{15}N, and ^{2}H) (Baillie 1981). When an atom in a molecule or drug of interest has been substituted by its stable isotope, this molecule is referred to as being SIL. In most cases, the SIL analog of the original molecule will have nearly identical physical and chemical properties to the unlabeled molecule. Likely, the only difference between the original molecule and the SIL is the molecular mass. For example, the mass difference between DPHM (molecular weight (MW) 255) and the deuterium (^{2}H)-labeled analog of DPHM [$^{2}H_{10}$]DPHM (MW 265) is 10 mass units (figure 3).

The most widely used analytical methodology used to differentiate between SIL molecules and their unlabeled counterparts was mass spectrometry coupled with gas chromatography (Baillie 1981). However, more recently it is not uncommon to encounter tandem mass spectrometers coupled with high-performance liquid chromatographs. The mass spectrometer is commonly operated in the selective ion monitoring (SIM) mode with attention being given to key fragments characteristic of the labeled and unlabeled drug under investigation. This simply means that

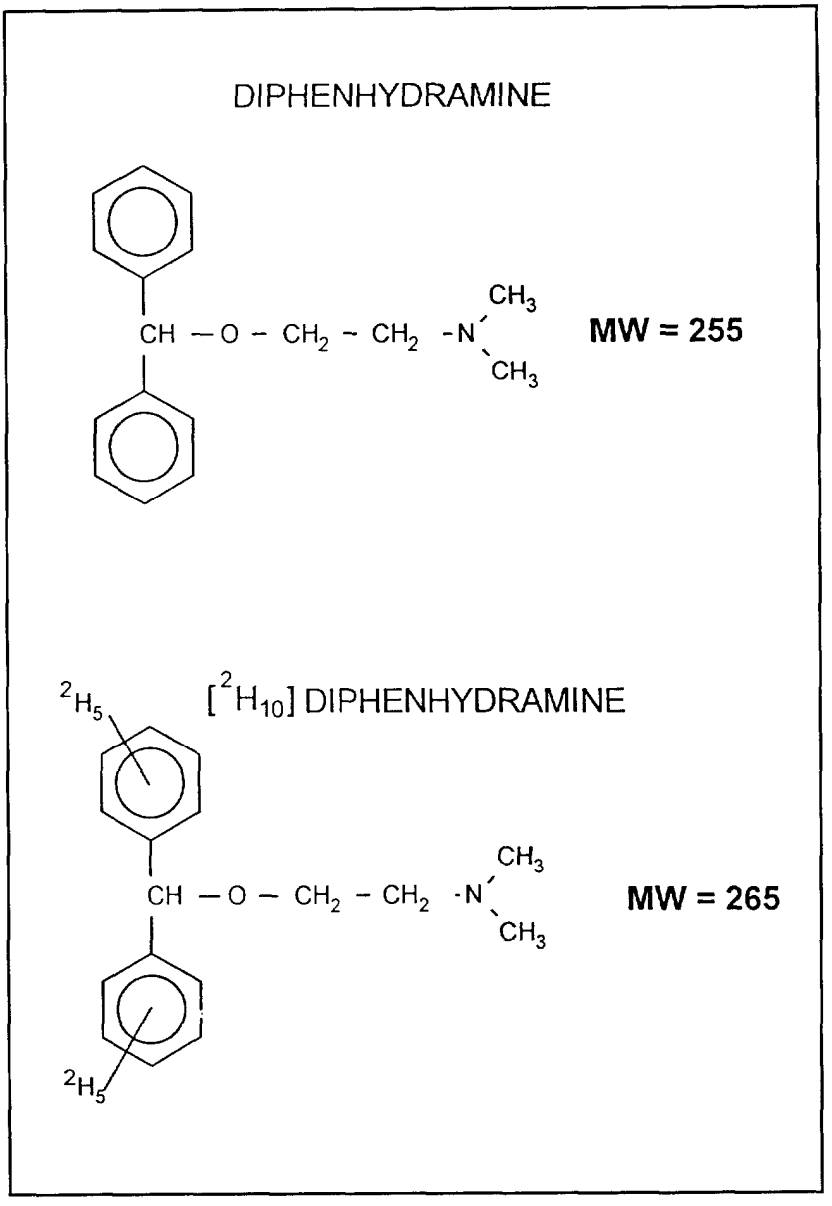

FIGURE 3. *Structures of DPHM and deuterium-labeled DPHM ([$^2H_{10}$]DPHW.*

the mass spectrometer is programmed to focus on individual fragment ions rather than to scan the entire mass spectrum, resulting in a substantial increase in both sensitivity and selectivity. The analytical methodology for the quantitation of DPHM and [$^{2}H_{10}$]DPHM focuses the mass spectrometer to measure only fragment ions with a mass to charge ratio (m/z) of 165 for DPHM and the internal standard, orphenadrine, and 173 m/z for [$^{2}H_{10}$]DPHM (figure 4) (Tonn et al. 1993a). SIM provides both the necessary differentiation between the SIL and unlabeled molecule (selectivity) and the required sensitivity (subnanogram range) (Tonn et al. 1993a).

Stable Isotope-Labeled Drugs: Advantages and Disadvantages. SIL drugs have found great utility in solving analytical, PK, and drug metabolism problems. For a more elaborate discussion of the utility of SIL compounds in PK and drug metabolism, the reader is referred to several indepth reviews on the topic (Baillie 1981; Browne 1990; Eichelbaum et al. 1982; Murphy and Sullivan 1980). The use of SIL compounds in PK experimental design can offer the investigator several advantages over traditional experimental designs where only unlabeled drug is available (Baillie 1981; Browne 1990). SIL compounds are not radioactive and thus do not pose the same degree of risk and handling concerns associated with radioisotopes. The simultaneous co-administration of SIL and the unlabeled counterpart in PK studies (i.e., bioavailability studies) significantly reduces the interday variability and the effects of time-dependent changes in PK parameters (Browne 1990). This is particularly important in the studies using late gestational chronically instrumented pregnant sheep where during the available experimental window there is rapid growth and maturation of the fetus (Battaglia and Meschia 1986). Furthermore, this technique can also reduce the number of exposures to the drug, reduce the number of samples to be analyzed, and reduce the number of experimental days (Browne 1990). With this experimental approach, both the test experiment and the corresponding control experiment can be conducted simultaneously. This essentially translates into a reduction in the number of subjects/animals required for the equivalent degree of statistical power, a potential reduction in cost, and reduced time spent to conduct the work.

There are also numerous limitations and possible disadvantages to using a SIL drug in an experiment. The largest impediment to the routine use of this method is the lack of accessibility to the SIL technology, as alluded

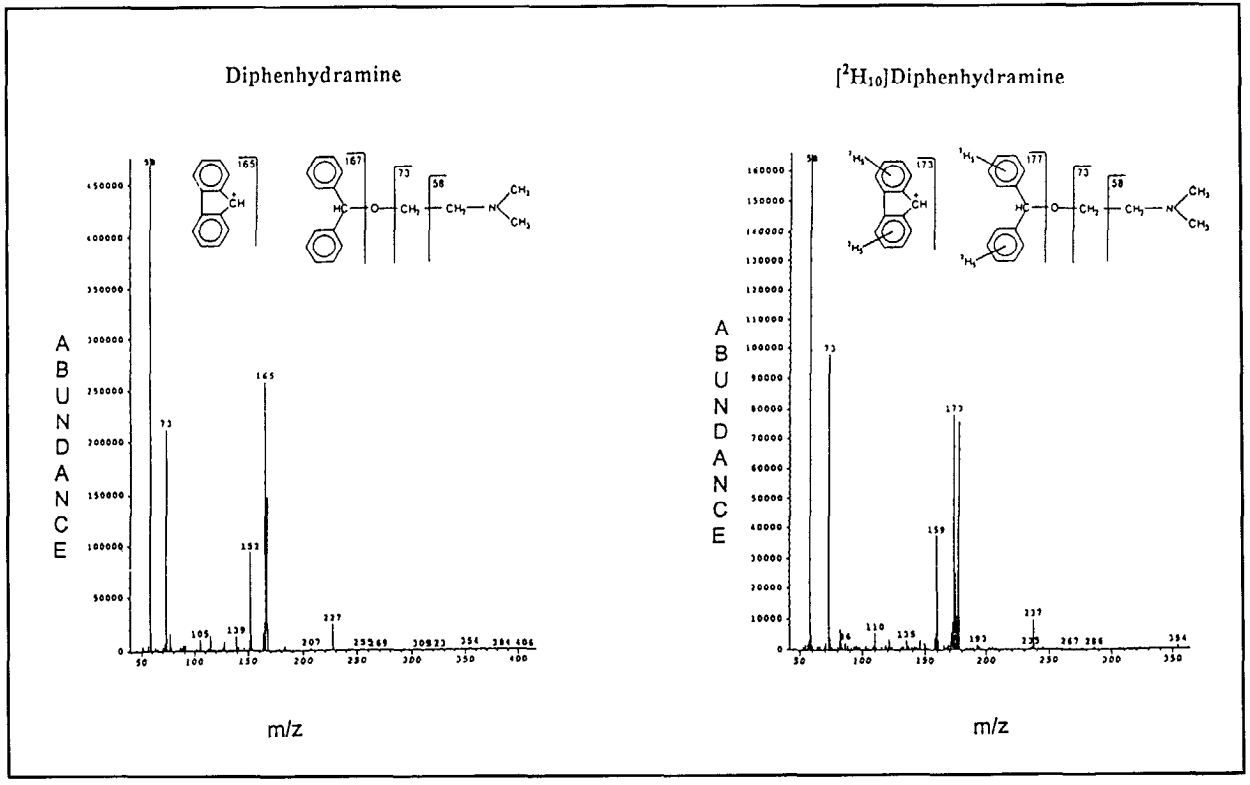

FIGURE 4. *Mass spectra of DPHM and deuterium-labeled DPHM and the mass fragment assignments.*

to earlier. This can be the result of the cost of the analytical equipment required (e.g., gas chromatograph or high-performance liquid chromatograph interfaced with a mass spectrometer), the availability and cost of SIL compounds (or synthetic precursors) of interest, and the lack of available analytical methods able to discern and simultaneously quantitate SIL and unlabeled drug (Browne 1990). Another limitation encountered when using SIL compounds is the possibility of an isotope effect (Van Langenhove 1986). One of the key assumptions that is made following the simultaneous coadministration of the SIL compound and the unlabeled counterpart is that the SIL compound is bioequivalent to the unlabeled compound (Browne 1990; Chasseaud and Hawkins 1990). That is, the SIL compound undergoes the same absorption, distribution, metabolism, and excretion as the unlabeled drug. If this is not the case, then the PK parameters extracted from the data could be artifactual, and the utility of a SIL compound would be limited. Therefore, prior to conducting an experiment using the SIL drug, the presence or absence of an isotope effect must first be investigated. This can be accomplished via the simultaneous coadministration of SIL and unlabeled drug by the same route of administration. If the ratio of SIL and unlabeled drug remain equivalent throughout the experiment, then these two compounds are said to be pharmacokinetically equivalent (Wolen 1986). However, where possible, it is also important to investigate the disposition of the SIL and unlabeled drug metabolites generated following administration of the SIL and unlabeled intact drug to ensure that the observed PK equivalence also corresponds to the metabolites (i.e., to rule out possible metabolic shifting) (Eichelbaum et al. 1982).

Studies Conducted With Stable Isotope-Labeled and Unlabeled DPHM. Following the synthesis of SIL DPHM and the development of an assay in the authors' laboratory capable of measuring SIL DPHM (i.e., [$^{2}H_{10}$]DPHM) in the presence of unlabeled DPHM, the authors were provided with a unique opportunity to apply SIL techniques to study the disposition of DPHM in chronically instrumented pregnant sheep (Tonn et al. 1993a). Previous studies with DPHM have shown that it undergoes both rapid and extensive distribution into the fetal circulation following a maternal IV bolus dose (Yoo et al. 1986b). It has also been shown, using time-separated fetal and maternal infusions to steady state, that the late gestational fetal lamb has the ability to efficiently remove DPHM from its circulation by nonplacental means (Yoo et al. 1993). However, it is difficult to validate the assumption that the time between the maternal and fetal infusions (i.e., 2 to 3 days) does not influence the disposition of DPHM in the rapidly growing fetal lamb. The availability of SIL DPHM

([$^2H_{10}$]DPHM) allows researchers to simulta-neously conduct both fetal and maternal infusions to steady state. The simultaneous infusion of both [$^2H_{10}$]DPHM and DPHM and the simultaneous quantitation of both labeled and unlabeled DPHM allow researchers to partially validate the assumptions made for the PK model for the fetal-placental-maternal unit (Szeto et al. 1982). The fetal nonplacental routes of elimination identified using the two-compartment open model could include excretion of DPHM from fetal lung fluid into the amniotic cavity, fetal renal elimination, fetal hepatic elimination, and/or placental metabolism of DPHM. It is not clear which fetal organ(s) contribute to the fetal nonplacental clearance. It is hoped that with the application of an experimental design incorporating [$^2H_{10}$]DPHM and DPHM, the individual in utero fetal organ clearances can be calculated for the first time,

The simultaneous infusion of DPHM to the mother and [$^2H_{10}$]DPHM to the fetus allows researchers to recalculate and thus in part validate the PK parameters calculated earlier by Yoo and colleagues (1993) for the fetal and maternal unlabeled DPHM clearances (placental and nonplacental clearances). A fetal-maternal simultaneous infusion was conducted for 2 hours in which [$^2H_{10}$]DPHM was infused to the fetus at 170 µg/min and unlabeled DPHM was infused to the mother at 670 µg/min. Serial samples were collected from fetal femoral and maternal femoral arteries, fetal lung fluid, and amniotic fluid. The concentrations of [$2H_{10}$]DPHM and DPHM in these samples were determined, and the fetal and maternal placental and nonplacental clearances parameters were calculated. These results are presented in table 1. As can be seen, the fetal and maternal placental and nonplacental clearances appear to corroborate the earlier values calculated using time-separated infusions of unlabeled DPHM to mother and fetus (Yoo et al. 1993). However, these results are from only one animal and thus more experiments are required before any conclusions can be drawn.

Diphenylmethoxyacetic acid (DPMA) is the deaminated metabolite of DPHM. Currently, a method for the simultaneous measurement of [$^2H_{10}$]DPMA and unlabeled DPMA is under development in the authors' laboratory. This method will be used to follow the PK of this metabolite in both mother and fetus during these simultaneous fetal-maternal infusions. Preliminary results showing the concentrations of [$^2H_{10}$]DPMA and DPMA for the 60-minute sample from the simultaneous infusion of [$^2H_{10}$]DPHM and DPHM are presented in figure 5. These data suggest

TABLE 1. *Transplacental and nonplacental clearance parameters from a previous experiment conducted by Yoo and colleagues (1993) and data from an experiment in which a simultaneous infusion of labeled and unlabeled DPHM was administered to fetus and ewe, respectively. All rates are milliliters per minute.*

Clearance Parameter	Previous Experiment (Yoo et al. 1993)	Simultaneous Infusion of Labeled and Unlabeled DPHM
CL_{MM} (maternal total body clearance)	3426±906	3230±152
CL_{FF} (fetal total body clearance)	473±246	1010±55
CL_{MF} (placental clearance-mother to fetus)	82±41	106±5
CL_{FM} (placental clearance-fetus to mother)	264±139	613±27
CL_{MO} (maternal nonplacental clearance)	3344±891	3130±149
CL_{FO} (fetal nonplacental clearance)	208±80	399±29

that [$^2H_{10}$]DPMA, which is derived from [$^2H_{10}$]DPHM (fetal infusion), is formed on the fetal side of the placenta since the concentration of the [$^2H_{10}$]DPMA in the fetal circulation is much greater than that seen in the maternal circulation. If this metabolite were formed in the maternal circulation and then transported to the fetal circulation by passive diffusion (barring an active transport mechanism), one would expect the concentration of [$^2H_{10}$]DPMA to be higher in the maternal circulation than in the fetal circulation. This does not appear to be the case from these preliminary data. Also of note is the apparent lack of this metabolite in the amniotic fluid (figure 5). It is hoped that with more extensive investigation utilizing DPHM, [$^2H_{10}$]DPHM, DPMA, and [$^2H_{10}$]DPMA the fetal organ responsible for this metabolite, and thus a possible source for the observed fetal nonplacental clearance, can be identified.

One of the possible organs that could be responsible for the fetal elimination of DPHM is the fetal liver. Experiments conducted in adult animals to assess the role of the liver in the elimination of DPHM employed the simultaneous administration of [$^2H_{10}$]DPHM and DPHM in hepatic first-pass metabolism experiments. In nonpregnant adult sheep it

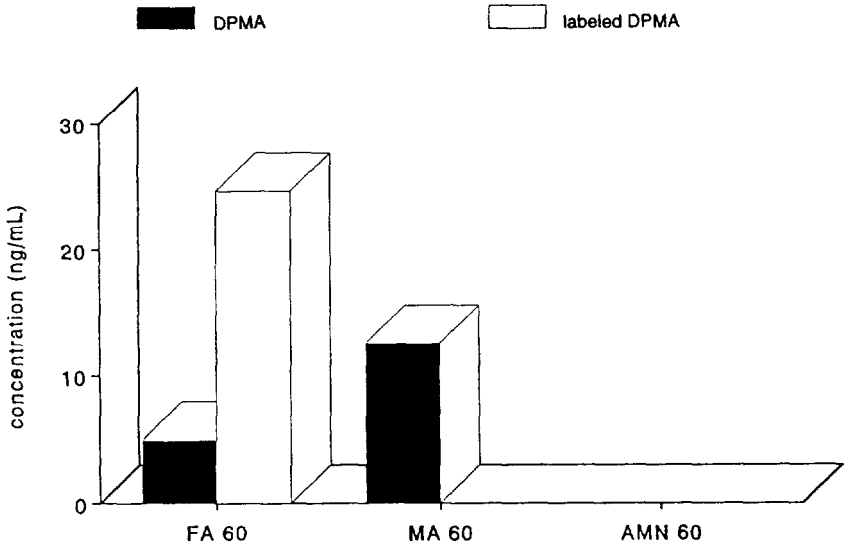

FIGURE 5. *The concentration of the acid metabolite of unlabeled and labeled DPHM, DPMA, and [$^2H_{10}$] DPMA in fetal and maternal plasma, and amniotic fluid at 60 min during a simultaneous constant rate maternal infusion of DPHM and fetal infusion of [$^2H_{10}$]DPHM.*

KEY: FA = fetal femoral arterial plasma
MA = maternal femoral arterial plasma
AMN = amniotic fluid
DPMA = diphenylmethoxyacetic acid

was found that the hepatic first-pass metabolism of DPHM was extensive following mesenteric administration (i.e., ~95 percent), suggesting that the disposition of DPHM in adult sheep mimics a high-clearance drug (Tonn et al. 1993b). Based on these results, it was hypothesized that the fetal liver may also possess the ability to eliminate DPHM, and this might contribute to the observed fetal nonplacental clearance. These fetal hepatic first-pass experiments were conducted using the fetal common umbilical vein (a fetal hepatic route of administration) as the test route and the fetal femoral tarsal vein as the control route (systemic route of administration). The rationale for using the umbilical vein is that approximately 30 to 50 percent of the umbilical venous blood returning to the fetus from the placenta passes through the fetal liver prior to reaching the systemic circulation (Edelstone et al. 1973). Thus, if the

fetal liver were active in the metabolism of DPHM, a portion of the DPHM that has passed through the fetal liver would be extracted by the fetal liver. This would yield a lower systemic concentration of DPHM as compared with [$^2H_{10}$]DPHM given directly via the fetal lateral tarsal vein into the systemic venous circulation.

Testing the hypothesis of fetal first-pass metabolism in utero would have been extremely difficult, if not impossible, using conventional analytical and kinetic techniques. There are two reasons for this. Firstly, one experiment, for example the umbilical administration, must be conducted on one day, followed by a washout period, and then the control experiment (i.e., tarsal venous or systemic administration) must be conducted several days later. This would make if difficult if not impossible to discern time-dependent changes in the observed PK parameters in this dynamic system (i.e., interday variability and developmental differences). Secondly, this protocol would have required significantly more animals to give results with similar statistical power. The use of SIL technology allows researchers to coadminister DPHM via the umbilical vein (fetal liver) while the "biological internal standard" or the [$^2H_{10}$]DPHM is simultaneously administered via the fetal lateral tarsal vein (inferior vena cava or systemic circulation). With this approach, the control and test experiments are accomplished simultaneously, thereby eliminating between-day variability and thus increasing the statistical power of this study.

Prior to conducting this experiment, it must be demonstrated that [$^2H_{10}$]DPHM and DPHM are pharmacokinetically equivalent in the experimental system being investigated. An experiment was performed to rule out any possibility of the existence of an isotope effect. The equivalence of DPHM and [$^2H_{10}$]DPHM was demonstrated by the IV bolus co-administration of equimolar amounts of DPHM and [$^2H_{10}$]DPHM via the same route of administration (i.e., the fetal lateral tarsal vein). Serial plasma samples were collected and the concentrations of DPHM and [$^2H_{10}$]DPHM were measured. As can be seen in figure 6, the plasma concentrations of [$^2H_{10}$]DPHM and DPHM are essentially superimposable. These data suggest that [$^2H_{10}$]DPHM and DPHM exhibit an essentially equivalent PK disposition. Since [$^2H_{10}$]DPHM and DPHM were shown to be equivalent, the fetal umbilical first-pass metabolism experiment was conducted without concern for artifactual data due to isotope effects. An equimolar IV bolus of [$^2H_{10}$]DPHM was given via the fetal lateral tarsal vein (systemic) simultaneously with an IV bolus of

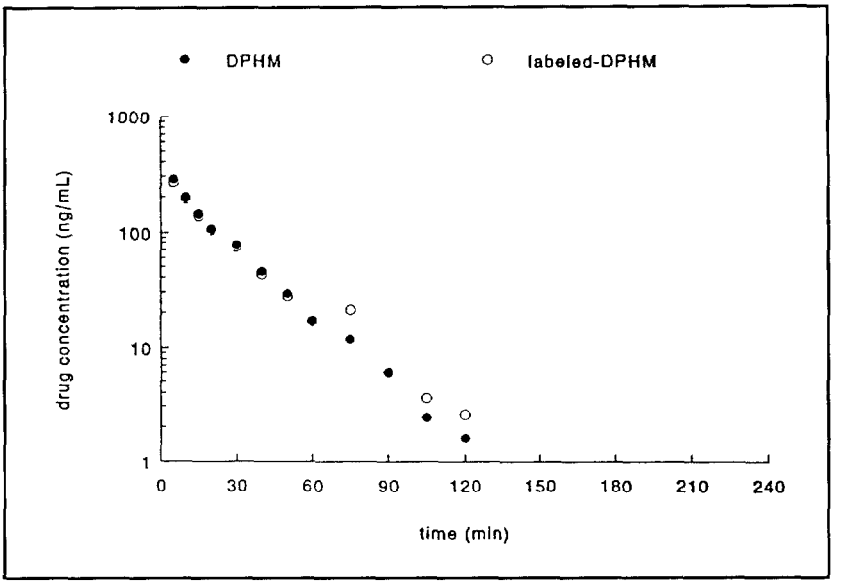

FIGURE 6. *The concentration versus time profile of DPHM and labeled DPHM ([$^2H_{10}$]DPHM) following a simultaneous IV bolus of 4.0 mg of DPHM and 4.2 mg of [$^2H_{10}$]DPHM given via the fetal lateral tarsal vein (isotope effect experiment).*

DPHM via common umbilical vein (fetal hepatic). Serial plasma samples were collected and the concentrations of [$^2H_{10}$]DPHM and DPHM were measured. Figure 7 shows the plasma concentrations of both [$^2H_{10}$]DPHM and DPHM over time. The concentration of [$^2H_{10}$]DPHM and DPHM are again essentially superimposable, demonstrating that the fetus does not have the ability to remove DPHM via first-pass metabolism mechanism following umbilical administration.

Studies Conducted with Stable Isotope-Labeled and Unlabeled Valproic Acid. Another example of the use of SIL technology in studying drug kinetics during pregnancy are studies currently underway with 2-propylpentanoic acid (VPA). VPA is an anticonvulsant agent widely used in me treatment of several types of epileptic seizures. The use of VPA in pregnancy is associated with fetal morphologic abnormalities and may, via its effects on central y-aminobutyric acid (GABA) receptors, also affect fetal behavior in utero. Consequently, studies have been undertaken to assess the PK. placental transfer and fetal CNS,

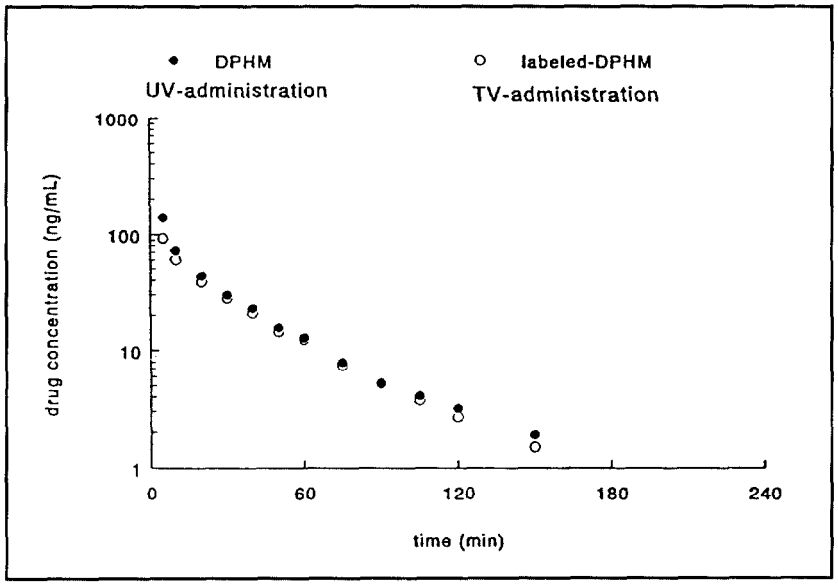

FIGURE 7. *The concentration versus time profile of DPHM and [$^2H_{10}$]DPHM following a simultaneous IV bolus of 4.0 mg of DPHM given via the fetal umbilical vein (fetal hepatic route of administration) and 4.2 mg of [$^2H_{10}$]DPHM given via the fetal lateral tarsal vein (control route of administration) (fetal hepatic first-pass metabolism experiment).*

KEY: UV = umbilical venous
TV = tarsal venous

cardiovascular, and metabolic effects of VPA in pregnant sheep. In addition, the physiochemical properties of VPA differ from those of the basic drugs previously studied (i.e., DPHM, MCP, labetalol, ritodrine, and others). It is important to study a range of drugs with differing physiochemical characteristics if an overall appreciation of drug disposition in pregnancy is to be gained. The use of SIL technology combined with gas chromatography and selected ion monitoring mass spectrometry has permitted the simultaneous determination of both fetal-to-maternal and maternal-to-fetal placental and fetal and maternal nonplacental clearances of VPA. Preliminary results are shown in figure 8. The elimination of VPA is characterized by extensive metabolic biotransformation with at least 16 different metabolites observed consistently in

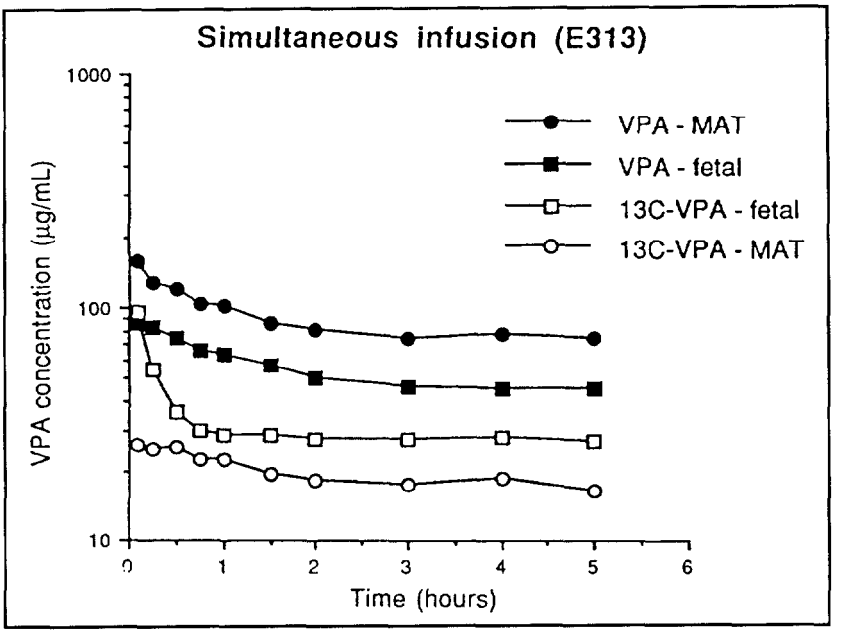

FIGURE 8. *Fetal and maternal serum concentrations of VPA and [$^{13}C_4$]VPA following the simultaneous infusion [$^{13}C_4$]VPA to the fetus and VPA to the mother.*

humans and an apparently similar number in some animal species, including sheep. The application of this analytical technique to separate and quantitate intact drug and these metabolites after extensive sampling of multiple biological fluids will enable the study of the kinetics and metabolism of VPA in the ewe and late-gestation lamb.

Stereoselective Aspects of Labetalol Analysis in Pregnancy

A large number of drugs used in pregnancy contain one or two chiral (asymmetric) carbons. As was discussed earlier, labetalol has two asymmetrical centers resulting in four stereoisomers: RR, SS, SR, and RS. The drug is commercially available as an approximately equipotent mixture of all four isomers. All four stereoisomers of labetalol contribute to its overall pharmacological activity. Of the four stereoisomers or labetalol, dilevalol (the RR stereoisomer) is the most potent ß-adrenergic blocker. In fact, virtually all of the P-receptor blockade and a-receptor mediated vasodilatation attributed to labetalol is produced by dilevalol (Sybertz et al. 1981). The SR isomer is the most potent antagonist of

a, receptors while the RS isomer has α and ß blocking activity, which is intermediate between that of the RR and the SR isomer (Brittain et al. 1982). The SS isomer has very little a and ß blocking property. Therefore, labetalol treatment, in effect, involves the administration of four distinct drugs, given the distinctly different action of each of the specific isomers.

Pharmacokine tic Considerations. Individual enantiomers behave as distinctly different chemicals with respect to their pharmacological and toxicological actions and their fate in the body. In some cases the difference between the pharmacological activity of enantiomers may be of PK as well as PD importance. In most cases the blood concentration and PK parameters of the racemic mixture do not reflect those determined for the individual active enantiomers (Walle 1985). Therefore, the importance of measuring the individual concentrations of enantiomers of racemic drugs is becoming more apparent as differing pharmacological and toxicological actions of the individual isomers are recognized.

Until recently the measurement of drugs in biological fluids has been based on techniques that were unable to differentiate between enantiomers. Therefore, for chiral drugs used in pregnancy, the question of whether the enantioselectivity of processes such as absorption, distribution, metabolism, and excretion is large enough to be of therapeutic significance is unanswered. Given the striking examples of differences in activity, toxicology, and PD of selected enantiomeric drugs, it is imperative that the influence of chirality be determined in pregnancy.

Metabolic Considerations. The metabolism of drugs frequently involves either bioactivation or bioinactivation of the molecule. There are numerous types of enantioselective metabolic conversion of drugs that have been reported in the literature (Jenner 1980, p. 53; Simonyi 1984; Trager and Jones 1987). For example, the enantioselective biochemical conversion may occur through: (1) substrate stereoselectivity, in which one isomer is preferentially metabolized by the enzyme; (2) product stereoselectivity, in which the nonchiral substrate is converted preferentially to one of the possible enantiomers of the product; or (3) enzymatic inversion, in which one of the enantiomers is preferentially inverted to the other enantiomer (Ariëns 1986).

Analytical Methods for Separation of Stereoisomers of Chiral Drugs. Several approaches for the chromatographic separation of

enantiomeric mixtures have been reported in the literature. They can be categorized as either direct or indirect methods.

Indirect methods are based on the reaction of the enantiomeric mixture with chiral reagents to form a pair of diastereomers. The diastereomers have different physicochemical properties that enable their separation on a nonchiral column. While useful in many instances, indirect methods have several disadvantages. They require expensive and optically pure derivatizing agents since enantiomeric contamination of the reagents could lead to false determination. Also, they require further treatment to reclaim the starting enantiomers. Finally, since the diastereomers have different physicochemical properties, the rate of formation may not be the same for each member of the pair.

Direct methods do not require prior derivatization. These methods use chiral stationary phases (CSPs) such as dinitrobenzoyl, protein bonded, cyclodextrin bonded, synthetic polymer, and ligand exchange phases. The CSPs form transient diastereomeric complexes with the solute enantiomers. The diastereomeric complexes have differing stability that causes a difference in retention time and hence separation of the enantiomers.

Proteins can undergo enantioselective interaction with several pharmacologically active compounds (Dappen et al. 1986). Two such protein CSPs, based on bovine serum albumin (BSA) and a,-acid glycoprotein (AGP), are commercially available. A major disadvantage of protein columns results from the sensitivity of the enantiomer separation to such chromatographic conditions as ionic strength, temperature, pH, and concentration of the organic modifier. Despite these disadvantages, these columns provide an excellent means of enantioselective separation.

Separation and Quantitation of Labetalol Stereoisomers in Biological Fluids. A direct chiral HPLC method was developed to study the disposition and conjugative metabolism of individual isomers of labetalol in pregnant sheep (Doroudian et al. 1993). This method has enabled scientists to determine the concentration of individual isomers of labetalol before steady state, during steady state, and following infusion (elimination phase) in biological fluids.

The total concentration of labetalol in numerous fluids collected from pregnant sheep was determined by an achiral method developed in the authors' laboratory (Yeleswaram et al. 1991). For determination of the

individual isomers, aliquots of all of the samples subjected to the achiral assay were once again extracted by the procedure developed (Yeleswaram et al. 1991), but without the addition of the internal standard. Omission of the internal standard was possible since the approach used in the chiral assay was to determine the percentage of each isomer in the racemic mixture and then determine the absolute amount of each enantiomer from knowledge of the total racemate determined by the achiral method. This approach was taken because the supply of pure isomers was insufficient to construct a standard curve for each individual isomer on a routine basis. Thus, the achiral method was used to determine the total concentration of labetalol, after which the concentration of each isomer was determined as described below. The concentration of each isomer was determined from the following relationship:

$$Ci = (\ \%\ \text{isomer} \times [\text{labetalol}])/100$$

where

Ci	Concentration of the individual isomer
% isomer	Percent of the individual isomer determined by the chiral assay
[labetalol]	Concentration of labetalol determined by the achiral assay

Disposition of Labetalol Stereoisomers in Maternal Plasma. The maternal arterial plasma concentration of labetalol and of the SR, SS, RS, and RR stereoisomers in a pregnant sheep during and after the infusion of labetalol is shown in figures 9 and 10, respectively. Plasma concentration of the RR isomer was higher than the other three isomers throughout the infusion. The mean plasma concentration of the SR, SS, RS, and RR isomers at steady state was 99.5, 109.7, 76.5, and 118.0 ng/mL, respectively, while the clearance of the isomers, calculated as the ratio of the infusion rate to steady-state plasma concentration, was 17.9, 16.3, 23.3, and 15.1 mL/min/kg. These results suggest a stereoselective disposition of labetalol isomers in maternal plasma. The concentration of the isomers at steady state and postinfusion is not one-quarter of the concentration of total labetalol, which is what would be expected if nonstereoselective disposition occurred. Therefore, the previously reported PK data for racemic labetalol does not necessarily apply to its individual isomers and the concentrations of the stereoisomers in the other biological fluids obtained in the study (fetal plasma, amniotic fluid, and maternal and fetal

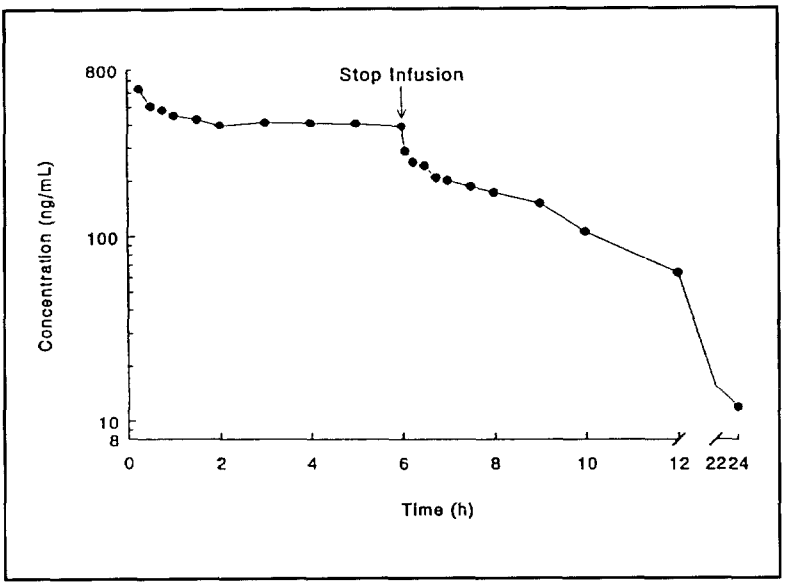

FIGURE 9. *Labetalol concentration in maternal arterial plasma of pregnant sheep following a 100 mg IV bolus and an immediate infusion of 0.5 mg/min. (Ewe# 1118).*

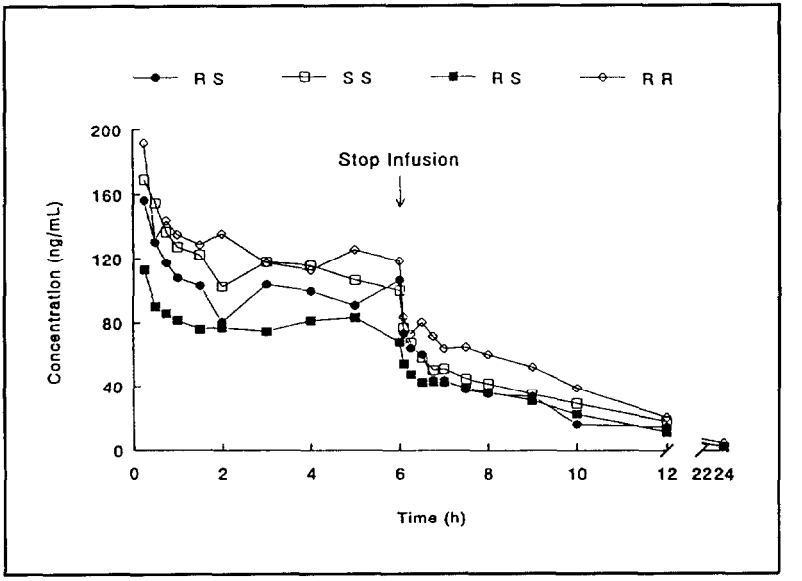

FIGURE 10. *Concentration of labetalol stereoisomers in arterial plasma of pregnant sheep following a 100 mg IV bolus and an immediate infusion of 0.5 mg/min of labetalol. (Ewe# 1118).*

urine) are currently underway. Further investigations regarding the enantioselective disposition of labetalol in pregnancy are also underway.

SUMMARY

The development of more sensitive and selective analytical techniques has greatly improved scientists' ability to study the PK, PD, and metabolism of drugs in pregnancy. This chapter has chronicled the evolution of the study of drug disposition in pregnant sheep in the laboratory. With the advent of more sensitive and selective analytical techniques, researchers have been able to progress significantly beyond measuring the extent of fetal drug exposure by a single-point determination. A number of interesting and significant advances have been made using highly selective and specific analytical techniques (i.e., gas chromatography-electron-capture detection, gas chromatography-nitrogen phosphoros-specific detection, and gas chromatography-mass spectroscopy). Researchers have characterized drug kinetics in multiple fluids from the pregnant sheep (amniotic fluid, fetal and maternal plasma and urine, and fetal tracheal fluid) and have demonstrated extensive accumulation of several basic drugs in the fluid produced by the fetal lung. With the simultaneous administration of labeled and unlabeled drug to the ewe and fetus researchers have, for the first time, examined the assumption that the clearance parameters determined from the model developed by Szeto and coworkers (1982) are not affected by the time interval between maternal and fetal infusions given on separate days. Also using SIL drug, researchers have characterized fetal hepatic first-pass drug clearance in a chronic preparation. With the establishment of a stereoselective assay for labetalol, scientists have begun to examine the fetal exposure to the individual enantiomers of racemic drugs in pregnancy. The availability of newer analytical tools and techniques (i.e., HPLC interfaced with tandem mass spectrometers) will further expand the study of drugs in pregnancy and challenge the creativity of future investigators in this field.

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Maternal-Fetal Pharmacokinetics: Summary and Future Directions

Hazel H. Szeto

INTRODUCTION

Drug use during pregnancy can have serious consequences on fetal development and pregnancy outcome. While some of these adverse effects may be secondary to the effects of these drugs on placental perfusion and fetal oxygenation, others are caused by direct drug action on the fetus. Thus, the intensity of these effects is determined by the extent of fetal drug exposure. Various methods have been used to assess drug exposure in neonates, including examining cord blood, amniotic fluid, neonatal urine, meconium, and hair samples. Although the presence of drugs in any of these biologic samples may indicate intrauterine drug exposure, the absence of any measurable levels or the absolute drug levels detected in these samples may be difficult to interpret without a complete understanding of the pharmacokinetics (PK) of these drugs in the maternal-fetal unit. The understanding of drug disposition in the fetus has increased dramatically during the past 15 years as a result of experimental studies using animal models. Many important findings were presented by speakers at the National Institute on Drug Abuse's (NIDA's) Technical Review on "Membranes and Barriers: Targeted Drug Delivery," and details of those studies can be found in this monograph. Despite the significant progress that has been made in the past decade, many questions remain unanswered.

This chapter provides a brief summary of the current understanding of drug disposition in the maternal-fetal unit. (It must be emphasized that this is not meant to be a comprehensive review of the literature and, because of NIDA's specific interests, most of the examples are limited to drugs of abuse.) Next, the author outlines the areas that need further attention in the coming decade. It is hoped that this information may provide future direction for research on maternal-fetal PK.

MATERNAL-FETAL DRUG DISPOSITION

Much of the understanding of maternal-fetal drug disposition has come from studies using the pregnant sheep model. In this animal model, chronic indwelling catheters are placed in maternal and fetal blood vessels, thereby permitting repeated sampling of maternal and fetal blood after the administration of drugs into either the mother or the fetus (Szeto et al. 1978). This model was first used to study the distribution of drugs in the maternal-fetal unit after single-dose exposure.

Single-Dose Exposure

When drugs are administered by the intravenous (IV) route to the mother, the drug concentration in the fetal plasma initially rises because of a positive maternal-fetal concentration gradient. At one instant in time, the mother and fetus are momentarily in equilibrium with each other; therefore, there is no net diffusion between them. At precisely this time, the fetal concentration is at its peak. As the drug continues to be cleared from the maternal plasma, the diffusion gradient is reversed, and the fetal concentration will begin to fall. This plasma concentration-time profile has been observed for a number of drugs that have been studied in the pregnant sheep, including meperidine (Szeto et al. 1978), methadone (Szeto et al. 1981), morphine (Golub et al. 1986), cocaine (DeVane et al. 1991; Woods et al. 1989), and methamphetamine (Burchfield et al. 1991). The time profile of Δ^9-tetrahydrocannabinol (THC) was similar after inhalational exposure of marijuana smoke (Abrams et al. 1985). There were, however, significant differences in the rate and extent of fetal exposure for the different drugs (figure 1). Peak fetal drug levels were observed as rapidly as 2 minutes after drug administration for meperidine and 5 minutes for methadone, but as long as 2 hours after THC administration. In addition, fetal levels were found to be similar to maternal levels for meperidine but significantly lower than maternal levels for methadone and THC. Currently, it is understood that fetal drug levels depend not only on the rate of placental transfer but also on the rate of drug elimination in the mother. If the placental transfer rate is slow relative to the rate at which the drug is eliminated from the mother, then drug concentrations may never reach high levels in the fetus.

The rate and extent of drug distribution to the fetus are also affected by the route of administration to the mother. The maternal and fetal plasma concentrations of meperidine are illustrated in figure 2 after IV bolus, intramuscular, and constant-rate IV infusion to the mother. Both the rate

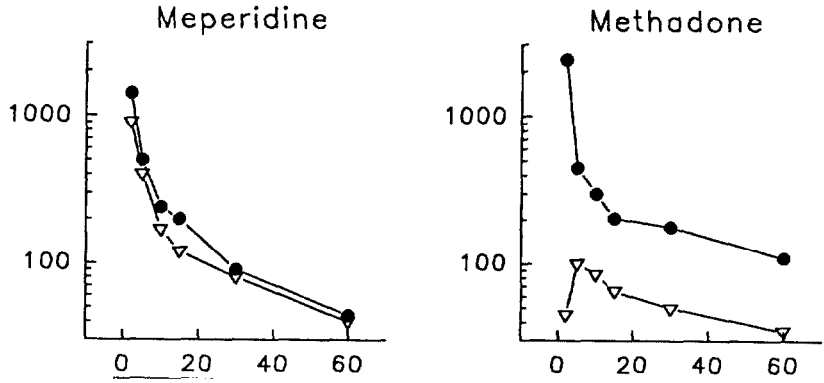

FIGURE 1. *Maternal (closed circles) and fetal (open triangles) plasma levels of meperidine and methadone after an IV bolus to the mother. Plasma levels are shown in ng/mL.*

SOURCE: Szeto et al. (1978, 1981).

and extent of fetal exposure are reduced with an intramuscular injection compared with an IV bolus administration to the mother. It is obvious, then, that interpreting a single maternal and fetal drug concentration obtained at one timepoint after drug administration will be difficult if not impossible.

Repeated Drug Exposure

When drugs are consumed repeatedly by the mother, steady-state plasma drug concentrations should eventually be achieved in both the mother and fetus. This has been demonstrated for several drugs after IV constant-rate infusion to the mother, including meperidine (Szeto et al. 1978), methadone (Szeto et al. 1981, 1982a), morphine (Szeto et al. 1982a), and ethanol (Brien et al. 1985, 1987). However, the ratio of fetal-to-maternal steady-state drug levels varied significantly for the different drugs, ranging from 0.15 for morphine (Szeto et al. 1982a) to 1.0 for ethanol (Brien et al. 1985, 1987).

Under steady-state conditions, factors other than placental permeability and maternal drug elimination become important in determining fetal drug exposure. These include plasma protein binding and fetal drug elimination. The extent of binding to fetal plasma proteins has been reported to be lower than that to maternal plasma proteins for a number of drugs in sheep, including meperidine (Szeto et al. 1978) and methadone

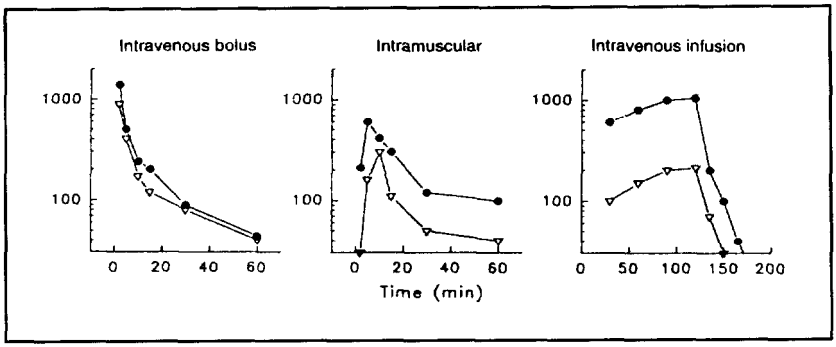

FIGURE 2. *Maternal (closed circles) and fetal (open triangles) plasma levels of meperidine following three different routes of administration to the mother. Plasma levels are shown in ng/mL.*

SOURCE: Szeto et al. (1978).

(Szeto et al. 1982b). Thus, under steady-state conditions, lower drug levels in the fetus do not necessarily imply restricted placental transfer because the concentrations of unbound drug may be the same in the mother and fetus. To complicate the matter further, the extent of drug binding to fetal plasma proteins may change as a function of gestational age; this was shown for methadone throughout the later part of the third trimester in sheep (Szeto et al. 1982b).

Pharmacokinetic Model of the Maternal-Fetal Unit

Even when differences in plasma protein binding had been considered, fetal steady-state drug levels have been found to be lower than maternal levels for all drugs studied in the sheep, with the exception of ethanol (Brien et al. 1985; Szeto et al. 1980). Using PK modeling (figure 3), it was suggested that this might be explained by the elimination of drug from the fetal compartment (Szeto et al. 1982c). Using the method proposed by Szeto and colleagues (1982c), the various clearance values in this model have been determined experimentally for a number of drugs. These data are summarized in table 1. These data clearly show that maternal-to-fetal clearance can vary significantly for these drugs, and the very low clearance of morphine may be explained by the relatively polar nature of this compound. Secondly, these data suggest that placental clearance plays an important role in drug clearance from the

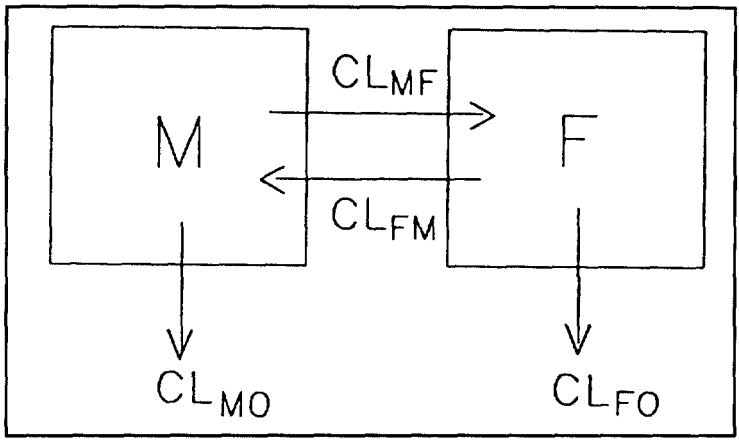

FIGURE 3. *Maternal-fetal pharmacokinetic model. CL,, and CL_{FM} are the transplacental clearances from mother to fetus, and fetus to mother, respectively; CL,, and CL,, are the nonplacental clearances from the mother and fetus, respectively.*

SOURCE: Szeto et al. (1982c).

fetus. Thirdly, there is considerable drug clearance from the fetus by nonplacental pathways. If the fetus had been unable to clear the drug, then fetal plasma drug levels would be expected to equal maternal drug levels at steady state.

Placental Drug Clearance. It has generally been thought that, because of inadequate drug elimination capability, the fetus might act as a sink for drugs. Currently, it seems clear that, for relatively lipid-soluble drugs, the rate of drug elimination from the fetus is dictated largely by maternal elimination characteristics. In all cases studied, the fetal elimination half-life was found to be similar to that of the mother (Abrams et al. 1985; Burchfield et al. 1991; DeVane et al. 1991; Golub et al. 1986; Szeto et al. 1978, 1981), suggesting that placental clearance is the predominant route of drug elimination from the fetus for relatively lipid-soluble compounds. This is confirmed by the data shown in table 1, where transplacental clearance from fetus to mother (CL,) is larger than nonplacental clearance from the fetus (CL,) for all drugs, with the exception of morphine, which is a relatively polar compound. The importance of placental clearance was clearly demonstrated when the elimination half-lives of lidocaine and ethanol were compared between the fetus and the

TABLE 1. *Maternal-placental-fetal pharmacokinetic parameters. $CL_{,}$, and $CL_{,}$ are the trunsplacental clearances for the mother to fetus and fetus to mother, respectively. CL_{MO} and $CL_{,}$ are nonplacental clearances from the mother and fetus, respectively.*

Drug	CL_{MF}	CL_{FM}	CL_{MO}	CL_{FO}	References
Morphine	8	19	40	42	Szeto et al. (1982a)
Methadone	130	168	108	127	Szeto et al. (1982a)
Acetaminophen	31	31	15	11	Wang et al. (1986)
Metoclopramide	72	103	47	28	Riggs et al. (1990)
Diphenhydramine	41	124	43	100	Rurak et al. (1991)

newborn. The elimination half-lives of both drugs were significantly longer in newborn lambs than in fetal lambs with an intact placental circulation (Cummings et al. 1985; Morishima et al. 1979). The half-lives in the fetal lamb were identical to maternal half-lives.

Fetal Drug Elimination. The ability of the fetus to eliminate drugs is supported by the presence of drug-metabolizing enzymes in the fetal liver (Dvorchik et al. 1986). Fetal liver microsomes were found to be capable of catalyzing the N-dealkylation of meperidine and methadone and the glucuronidation of morphine. In addition, alcohol dehydrogenase activity was found in fetal lamb liver during the later part of the third trimester, although the activity was tenfold lower than in the adult sheep (Clarke et al. 1989; Cummings et al. 1985).

The ability of the fetus to metabolize drugs in vivo is suggested by the detection of certain metabolites in fetal plasma. Normeperidine, the N-demethylated metabolite of meperidine, was found in fetal plasma after both maternal and fetal administration of meperidine (Szeto et al. 1978). Acetaldehyde was reported in fetal plasma after ethanol infusion to the pregnant ewe and guinea pig (Clarke et al. 1986, 1988). Both benzoyl-ecgonine and benzoylnorecgonine were detected in fetal guinea pig plasma after chronic maternal cocaine administration (Sandberg and Olsen 1992). However, because these metabolites can be readily transferred across the placenta, it is difficult to prove that they are of fetal origin. Stronger evidence for fetal drug metabolism comes from the finding of conjugated metabolites of acetaminophen (Wang et al. 1986), morphine (Olsen et al. 1988), and labetalol (Yeleswaram et al. 1993).

These conjugates are not expected to distribute across the placenta to any significant extent (Olsen et al. 1988).

The finding of significant levels of metabolites in fetal plasma is important because the metabolites themselves may be pharmacologically active. Furthermore, the more polar metabolites often are cleared more slowly than the parent drug, as was shown for morphine-3-glucuronide (Olsen et al. 1988), and therefore may accumulate in the fetus with repeated drug exposure.

Fetal renal clearance is another important route of drug elimination. Direct evidence of fetal renal clearance has been reported for many drugs, including meperidine (Szeto et al. 1979), ethanol (Clarke et al. 1987), acetaminophen (Wang et al. 1985), cimetidine (Mihaly et al. 1983), omeprazole (Ching et al. 1986), and labetalol (Yeleswaram et al. 1993). Fetal renal clearance of meperidine was found to be greater than creatinine clearance, suggesting renal tubular secretion (Szeto et al. 1980). Renal clearance is particularly important for the polar conjugates, and morphine-3-glucuronide has been detected in fetal urine (Olsen et al. 1988). However, it appears that these conjugates are filtered but not secreted by the fetal lamb kidney.

Excretion of Drugs Into Amniotic Fluid. There are abundant experimental data demonstrating the presence of drugs in amniotic fluid after maternal drug administration (Brien et al. 1985; Olsen et al. 1988; Szeto et al. 1978; Wang et al. 1986). The appearance of drugs in amniotic fluid is usually delayed, but the concentration gradually increases, and the peak concentration usually far exceeds the concurrent concentrations in maternal and fetal plasma. Many metabolites, especially conjugated metabolites, are also detected in amniotic fluid (Brien et al. 1985; Olsen et al. 1988; Wang et al. 1985). The disappearance of drugs and metabolites from amniotic fluid tends to be much slower than from fetal plasma, and significant accumulation may take place with repeated drug exposure. The delay in appearance of a drug in the amniotic fluid suggests that a major source of drugs comes from fetal urine. However, significant accumulation of meperidine (Szeto et al. 1978) and ethanol (Brien et al. 1985) in amniotic fluid was found even with complete diversion of fetal urine, suggesting other sources of drug transfer such as diffusion across the chorioallantoic membranes.

SUMMARY OF IMPORTANT FINDINGS FROM 1973 TO 1993

- *The placenta does not act as a barrier to protect the fetus.* Placental clearance is governed by lipophilicity, size, extent of plasma protein binding, and degree of ionization.

- *The fetus does not act as a sink for drugs.* Placental clearance plays a major role in drug clearance from the fetus.

- *Fetal nonplacental clearance is important in determining the extent of fetal drug exposure.*

- *The fetus has the capacity to eliminate drugs by renal and hepatic clearance.*

- *Amniotic fluid may serve as a drag reservoir.* Most drugs, especially polar metabolites, tend to accumulate in amniotic fluid.

QUESTIONS FOR THE 1990s AND FUTURE DIRECTIONS

1. How does fetal drug exposure differ in early pregnancy versus late pregnancy?

   Almost all of the PK studies to date have been carried out in late pregnancy. Little is known about maternal-fetal PK in early pregnancy. The extent of fetal drug exposure can be expected to change with gestational age due to changes in placental anatomy, plasma protein binding, and maturation of fetal drug elimination systems. There is evidence that the extent of methadone binding to fetal plasma proteins increases as a function of age throughout the third trimester in the fetal lamb (Szeto et al. 1982b). Furthermore, PK analyses have revealed that the contribution of fetal nonplacental clearance to the total clearance of methadone from the fetus also increases throughout the third trimester (Szeto et al. 1982a). Fetal nonplacental clearance can be expected to be much lower in the first and second trimesters, which may result in a higher extent of fetal drug exposure.

2. What is the fate of polar metabolites in the fetus?

   While the placenta plays an important role in clearing lipid-soluble drugs from the fetus, it is unclear how polar metabolites are cleared from the fetus. There is good evidence that the fetus has the ability to form conjugated metabolites (Olsen et al. 1988; Wang et al. 1985, 1986; Yeleswaram et al. 1993), and these tend to accumulate in the fetus because of restricted transfer across the placenta (Olsen et al. 1989). The ultimate fate of these polar metabolites in the fetus is not clear. It is assumed that most are eliminated by renal clearance and eventually end up in amniotic fluid. However, there is still the question of whether these metabolites are secreted into bile and ultimately appear in meconium. In addition, is there glucuronidase activity in the gastrointestinal tract that would result in enterohepatic recirculation? These studies are very important for interpretation of meconium data from human newborns.

3. What are the dynamics of drugs and metabolites in amniotic fluid?

   Much remains to be explored on the source(s) of drugs and metabolites in amniotic fluid and the clearance of drugs from this fluid compartment. Studies from the late-term fetal lamb clearly suggest that there are other sources of drugs and metabolites besides fetal urine (Brien et al. 1985; Szeto et al. 1978). While lipid-soluble drugs may conceivably diffuse across the chorioallantoid membranes, it is unclear whether polar metabolites have access into the fluid compartment other than through urinary excretion. It is also not known whether the contribution of the different sources varies with gestational age of the fetus. Fetal renal excretion can be expected to play a more important role later in gestation. Finally, what is the ultimate fate of drugs and metabolites in amniotic fluid? Lipid-soluble drugs may diffuse back across the chorioallantoic membranes, the umbilical cord, or both into the maternal and fetal circulation. When meperidine was administered directly into the amniotic fluid, a larger fraction of the dose was recovered in maternal plasma than in fetal plasma (Szeto et al. 1978). With polar metabolites, it is thought that recirculation into fetal plasma may occur via fetal swallowing. However, this has never been demonstrated, and it is not known how much of a drug can actually be absorbed by the fetus in this manner. These experimental studies are very important for understanding the impact of amniotic fluid serving as a reservoir of drug for the fetus and to utilizing amniotic

fluid as a potential route of drug administration into the fetus for therapeutic purposes.

4. Does the extent of fetal drug exposure change with chronic drug administration?

   PK analyses of the maternal-fetal unit have suggested that the extent of fetal drug exposure under steady-state conditions is a function of both transplacental clearances and fetal nonplacental clearance (Szeto et al. 1982c). It can be expected, therefore, that the extent of fetal drug exposure may change with chronic drug administration simply because of progressive maturation of the fetal drug elimination systems. In addition, chronic exposure to certain drugs may result in induction of fetal hepatic enzymes and enhanced fetal clearance. However, these issues have not been addressed systematically in an animal model.

5. Are there species differences in maternal-fetal PK?

   While most of the current understanding of maternal-fetal PK has come from studies using the pregnant sheep model, most investigators are well aware of the structural differences between the ovine placenta and the primate placenta. Although the extra layers in the ovine placenta may not present a problem to the diffusion of highly lipid-soluble compounds, it may with less lipid-soluble substances. There have been no systematic comparisons between the maternal-fetal PK of lipophilic versus hydrophilic drugs in different animal species. There is clearly a need for the development of nonhuman primate models. Recent data from a chronically cannulated pregnant baboon model suggest that what has been learned from the pregnant sheep also applies to the baboon. Azidothymidine (AZT) was found to be readily distributed to the fetal baboon, with fetal plasma levels lower than corresponding maternal plasma levels (R.I. Stark, personal communication, 1993). The elimination half-life of AZT was similar in the fetus and the mother. In addition, AZT-glucuronide also was detected in fetal plasma, suggesting the ability of the fetal baboon to form conjugated metabolites in the third trimester. Finally, both AZT and AZT-glucuronide were found in significant levels in amniotic fluid. More studies like these are needed in order to establish whether species differences play a major role in maternal-fetal PK.

6. Can in vitro models be used for studying placental drug transfer?

With the increased understanding of maternal-fetal PK, it may be possible to specifically design therapeutic agents for use in pregnancy that would minimize fetal drug exposure. While in vivo animal models clearly are necessary to fully understand the disposition of a drug in the maternal-fetal unit, it also may be useful to develop in vitro models that would permit a rapid determination of the placental clearance of a drug for screening purposes. The in vitro perfused placenta model has the added advantage that it is possible to use human placentae. The use of these perfused models, however, must be accompanied by rigorous controls over tissue viability and perfusion pressures, as they can become leaky under high perfusion pressure.

Recently, an in vitro model was developed for investigating the transfer of compounds across the blood-brain barrier (BBB) (Audus and Borchardt 1986, 1987). This is a monolayer of bovine brain endothelial cells mounted in a side-by-side chamber. It appears to be reasonably useful for predicting transfer by passive diffusion, but this cell culture may lack functional transporters that exist for certain compounds across the BBB. If a similar cell culture system can be developed for the placenta, it may be useful for screening purposes despite its limitations.

7. What are the differences between the placenta and the BBB?

Basic research into the differences between the placenta and BBB is necessary before attempts can be made toward the rational design and development of a drug that would cross the BBB but not the placenta. Is it possible to design a drug that would not cross the placenta and yet achieve central nervous system actions? Are there specific transport or endocytosis systems that exist in the BBB but not in the placenta? Certain small peptides appear to be able to cross the BBB, but their transfer across the placenta seems to be highly restricted. An example is metkephamid, which clearly has analgesic efficacy when administered systemically but whose distribution to the fetus appears to be very limited (Bloomfield et al. 1983; Frederickson et al. 1981; 1983, pp. 150-156).

8. Can physiologically based PK modeling of the maternal-fetal unit contribute new information?

   Most PK models for the maternal-fetal unit have been based on compartmental modeling. In such models, the various compartments have no anatomical or physiological meaning. In physiologically based PK modeling, the model utilizes real blood flow and tissue distribution values. As a result, these models have the potential for allometric scaling across species so that the PK of a drug in the pregnant human may be predicted from data obtained in another animal model. There have been very few attempts at this type of PK modeling of the maternal-fetal unit, and their predictive values have not been evaluated systematically. These models may hold the future for PK modeling of the maternal-fetal unit.

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Technical Issues Concerning Hair Analysis for Drugs of Abuse

Martha R. Harkey

INTRODUCTION

There has been considerable interest during the past few years in the use of hair analysis for drugs of abuse. Proponents of hair testing suggest that hair is a better specimen than urine because hair may be collected less intrusively, drugs persist in hair longer than in urine, and it would be more difficult to tamper with hair samples. In addition, it is claimed that hair provides a "calendar" or "tape recorder" of an individual's drug use history; that is, the amount as well as the duration of drug use can be determined by *sectional* analysis-analyzing sequential segments of hair and using the location of the drug along the hair shaft and the growth rate of hair (approximately 1 inch per month) to calculate the length of time a person is exposed to drugs. These benefits, along with the fact that hair may be stored indefinitely, make hair analysis a very attractive alternative to urine testing. However, recent studies have shown that the incorporation of drugs into hair is not well understood and that hair testing, like other forms of drug testing, has both strengths and limitations that should be appreciated before hair testing is used or whenever the results from hair tests are interpreted.

HISTORICAL BACKGROUND

Hair analysis has been used for decades as a means of assessing exposure to arsenic, lead, cadmium, and mercury (Chatt et al. 1980, pp. 46-73; International Atomic Energy Agency 1977; Smith et al. 1962), as well as for determining exposure or dietary deficiencies in trace elements such as zinc, copper, and magnesium (Barlow and Kapel 1980, pp. 105-127; Hambridge et al. 1976; Pihl and Parkes 1980, pp. 128-143). At one time hair analysis was suggested as a means of determining nutritional deficiencies; however, it was soon recognized that hair analysis could not be used to reliably determine exposure to trace elements because of the large variations in concentrations reported by laboratories, the lack of agreement on normal concentrations in hair, and the possibility of

external exposure from air, water, and cosmetic treatments (Sorenson et al. 1973).

Drugs were first analyzed in hair over four decades ago during basic pharmacological studies probing the mechanisms of drug-induced dermatitis (Goldblum et al. 1954), alternate pathways for drug elimination (Forrest et al. 1972), and metabolism of drugs by the skin (Harrison et al. 1974). The first studies using hair to detect drugs of abuse did not appear until 1979 when Baumgartner and colleagues reported on the use of hair analysis to evaluate opiate abuse (Baumgartner et al. 1979). These studies were followed shortly thereafter by reports on the use of hair analysis to detect the use of phencyclidine, cocaine, phenobarbital, morphine, amphetamine, methamphetamine, amitriptyline, nortriptyline, and nicotine (Arnold and Püschel 1981; Baumgartner et al. 1981; Ishiyama et al. 1983; Klug 1980; Niwaguchi et al. 1983; Smith and Pomposini 1981; Suzuki et al. 1984; Valente et al. 1981). More recently, it has been suggested that hair analysis might also be used for therapeutic drug monitoring (Sato et al. 1989, 1993).

ANATOMY AND PHYSIOLOGY OF HAIR

Hair appears to be a relatively uniform structure, differing in individuals only in color, texture, or amount. However, hair is quite complex, and the factors that may affect the concentrations of drugs in hair are only partially understood. Figure 1 shows a section of a hair follicle and its surrounding tissues. At the base of the follicle is the hair bulb, the region of active cell division located approximately 3 to 4 mm below the surface of skin. As hair cells are formed they are gradually pushed upward along the follicle into the region of keratinization, where they begin to differentiate, lose water, and coalesce to form the hair shaft. The growing hair follicle may be nourished by the capillary network at the base of the bulb; the cutaneous plexus within the dermis; the sebaceous and apocrine glands, which secrete directly into the hair shaft; and the eccrine gland, which secretes onto the surface of the skin.

The hair shaft consists of three regions: an outer cuticle, cortex, and central medulla, which may be discontinuous along the hair shaft (figure 2). The cuticle consists of flat, overlapping cells and serves to protect the interior fibers of the hair and to anchor the hair shaft in the follicle. Although the cuticle has been described as the armor for hair, it

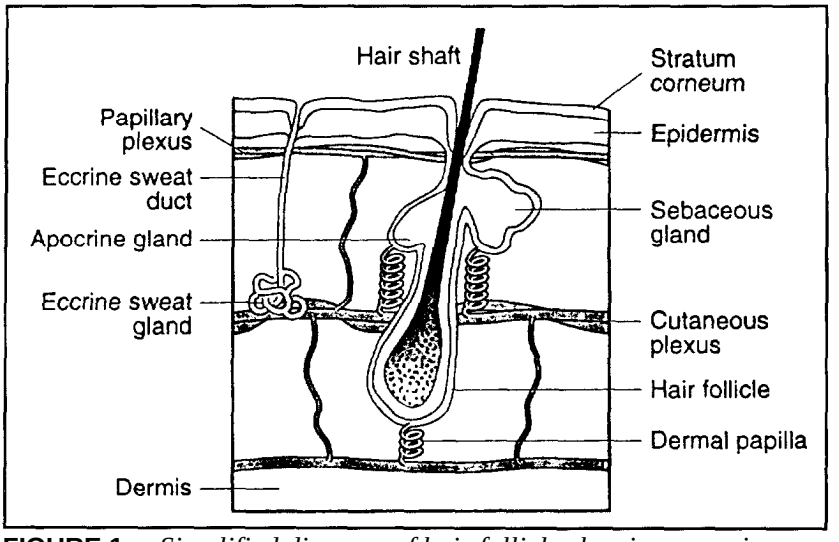

FIGURE 1. *Simplified diagram of hair follicle showing apocrine, sebaceous, and sweat glands, and the multilayered vascular system in the surrounding tissues.*

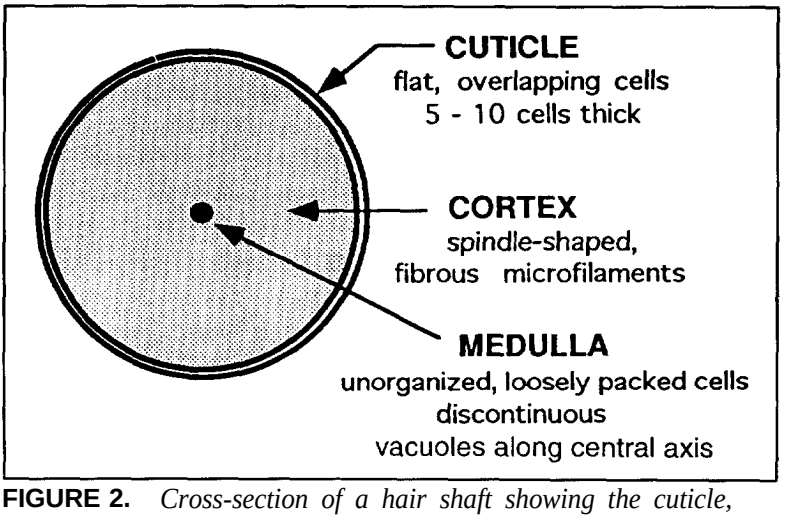

FIGURE 2. *Cross-section of a hair shaft showing the cuticle, cortex, and medulla.*

can be destroyed by heat, ultraviolet light, oxygen, and chemical treatments, and it is easily penetrated by aqueous solutions.

The bulk of the hair shaft is the cortex, which contains long keratinized cells that form fibers approximately 100 μm long separated by small spaces called fusi. Cortical cells contain a variety of chemicals, including melanin and other pigments that give hair its color, as well as proteins, amino acids, water, and lipids.

The medulla consists of loosely packed cells that may dehydrate and shrivel up to leave a series of vacuoles along the hair axis. In general, the number of medullar cells increases as the fiber diameter increases. Medullar cells make up only a small percentage of the mass of human hair and may be discontinuous or absent in some types of hair. It has been suggested that some drugs are selectively incorporated into the medulla. Although this has not been substantiated, it could be an important factor in determining the localization of drugs along the hair shaft.

The structure of hair varies somewhat depending on the type of hair: terminal, intermediate, or vellus. Vellus hair is the very fine, short, nonpigmented hair with a small cross-sectional area that is found on the seemingly hairless parts of the body such as eyelids, forehead, and bald scalp. Intermediate hair is intermediate in length and diameter and is found on arms and legs of adults. Terminal hair is the coarse, long, pigmented hair with large cross-sectional area found in the hairy areas of the body such as the scalp, beard, eyebrows, eyelashes, armpits, and pubic area.

The differences in these three hair types are due to the differences in hair follicles. Intermediate hair follicles, located on the arms and legs, do not change after puberty and are not influenced by hormones. Ambisexual hair follicles, located in the pubic area, axilla, and temple of the scalp, are influenced by hormones and change during puberty from fine, vellus hair to coarse, terminal hair. In addition, there are male hair follicles unique to males and found in the beard area, ears, nose, chest, and abdomen. These follicles respond to high androgen concentrations and change from vellus hair to terminal hair at puberty.

An interesting characteristic of hair is that it does not grow continuously but in cycles, alternating between periods of growth and quiescence. During the anagen phase, there is an increase in metabolic activity in the hair bulb. This is a time of cell division and growth, and it has been suggested that drugs and trace elements are incorporated into hair during this phase of the hair cycle. The catagen phase is a short transitional

phase during which cell division stops, the base of the hair shaft becomes fully keratinized, and the bulb begins to degenerate. The telogen phase is the resting or quiescent period in which there is no hair growth, the follicle is very short, and the hair can easily be removed by pulling. The length of time a follicle is in the resting phase depends on where the follicle is located on the body and the age of the individual. The resting phase lasts approximately 10 weeks for scalp hair, approximately 2 to 6 years in general body hair, and increases somewhat with age.

Although a mean growth rate of 1 cm or 0.5 inches per month is commonly used in segmental analysis calculations, the actual rate of growth varies both within and between individuals and has been found to range from 0.5 to 2 cm per month (Saitoh et al. 1967, pp. 21-34). Hair type and anatomical location are the most important factors determining growth rate; however, race, sex, and age have an effect as well.

The fact that not all hair is growing at the same time and that hair grows at different rates is an important consideration in interpreting results from hair analysis. For drug testing, hair is usually collected from the posterior vertex region of the scalp because this region has the highest percentage of follicles in the anagen phase (approximately 85 percent), as well as the fastest growth rate.

MECHANISMS OF DRUG INCORPORATION INTO HAIR

Although the precise mechanisms by which drugs enter hair are not known, it has been suggested that they enter the growing hair follicle by passive diffusion from the capillaries at the base of the hair follicle (Baumgartner et al. 1989). According to this model, drugs are trapped in the hair cells during early development, are bound in the hair shaft during keratogenesis, and can be detected in the hair shaft as it emerges from the scalp (figure 3). In this model, drug concentration in hair should be proportional to the drug concentration in blood at the time of hair synthesis. The time of drug ingestion also can be calculated from the location of the drug along the hair shaft (assuming a constant hair growth rate of 1 cm per month).

However, more recent studies have suggested that for some drugs this model may be inadequate. First, for some drugs the metabolic profiles in hair are quite different from those found in blood. For example, cocaine is rarely detected in blood or urine, but it is the primary analyte found in

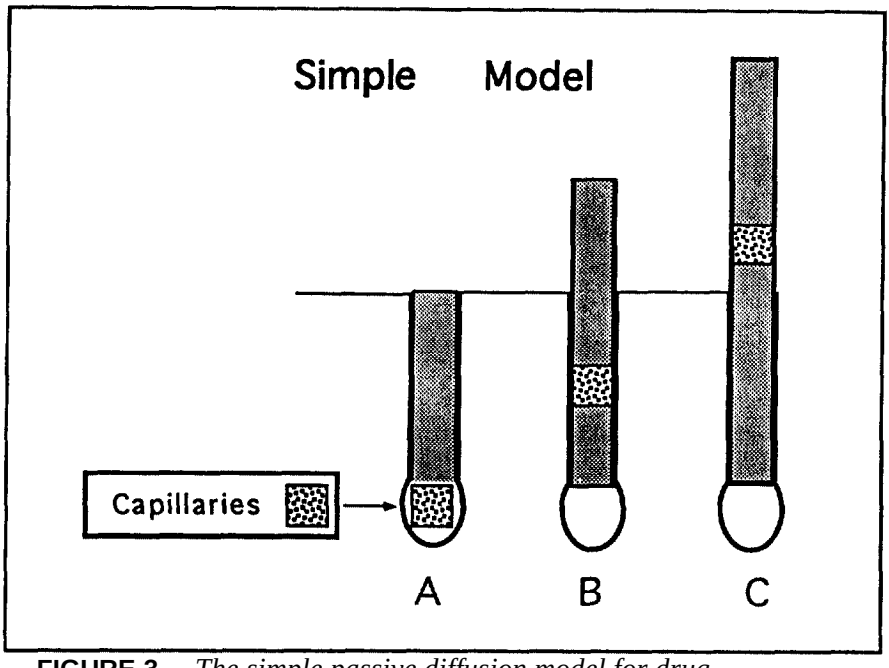

FIGURE 3. *The simple passive diffusion model for drug incorporation into hair. (A) drug absorbed from capillaries into the base of the hair bulb; (B) hair during keratogenesis; and (C) drug detected in hair as it emerges from skin.*

hair; the metabolites of cocaine which are detected in blood, benzoylecgonine (BE) and ecgonine methyl ester (EME), are present in trace, and highly variable, amounts in hair (Cone et al. 1991; Harkey et al. 1991; Henderson et al. 1992; Kidwell 1993; Martz et al. 1991; Möller et al. 1992). Similarly, heroin and 6-mono-acetylmorphine are difficult to detect in blood but easily detected in hair (Goldberger et al. 1991). Finally, the concentrations of drug in hair may be highly variable in subjects receiving the same dose and cannot be explained by individual differences in plasma pharmacokinetics (Henderson 1993). These findings are difficult to explain based on what is known about the plasma pharmacokinetics of these drugs and the simple diffusion model for drug incorporation into hair.

The simple diffusion model is also the pharmacological basis for segmental analysis; that is, the time for appearance of drugs in hair and the subsequent movement along the hair shaft should be determined only by the growth rate of hair. However, controlled dose studies have shown that the time for detection of drugs in hair, as well as the location of drug within the hair shaft, can be quite variable (Cone 1990; Henderson 1993; Martz et al. 1991).

Results from studies using more genetically homogeneous populations (only African-American hair) and with drugs thought to bind preferentially to melanin (quinones and hydrophilic amines) are more consistent with the simple diffusion model (Miyazawa et al. 1991; Nakahara et al. 1992). This has led to speculation that hair type and physiochemical properties of the drug are also important variables affecting the incorporation of drugs into hair.

A more complex model for drug incorporation into hair is shown in figure 4, where drugs may be absorbed into hair from capillaries, sebaceous glands, sweat glands, as well as from the external environment. Using this model, drugs could be incorporated into hair from multiple pools during various times of the hair life cycle (i.e., from blood during growth and differentiation, from sweat and sebum after formation, and from the external environment after formation).

If multiple mechanisms are involved in the incorporation of drugs into hair, then data from hair tests should be interpreted with caution. There may be large intersubject differences in the amount of drug incorporated into hair, and segmental analysis data may not accurately reflect the time or duration of drug intake. For example, drugs secreted in sweat or sebum or absorbed from the environment could be absorbed directly through the cuticle and along the hair shaft. Thus, they would not be found in discrete bands within the hair shaft.

IN VIVO STUDIES USING CONTROLLED DOSES OF d_5-COCAINE

Since 1990, this author's laboratory has been involved in studies on hair analysis for cocaine. Research objectives were to determine the relationship berween dose of drug and amount of drug detected in hair as well as the relationship between time of drug administration and location of drug along the hair shaft. In order to distinguish the cocaine

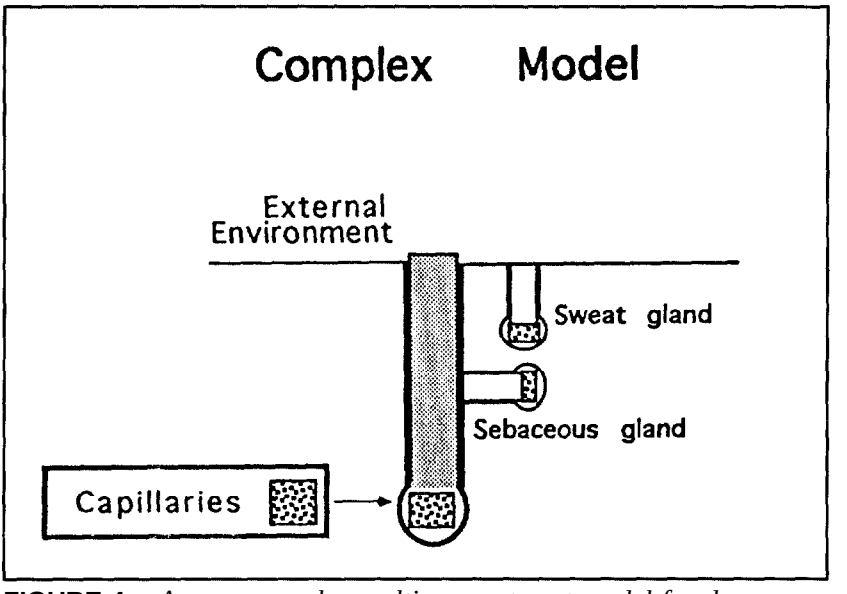

FIGURE 4. *A more complex multicompartment model for drug incorporation into hair showing possible drug entry into hair from capillaries, sebaceous gland, sweat gland, and external environment.*

administered in the study from any residual cocaine in body stores or drug taken surreptitiously by the subject during the several months of the study, researchers administered penta-deuterated cocaine (d_5-cocaine) (i.e., cocaine with five deuterium atoms replacing the five hydrogen atoms on the benzoyl moiety). By using mass spectral analysis for quantitation, the drug administered for the study could easily be distinguished [molecular weight of 308) from any naturally derived cocaine (molecular weight of 303). The specifics of the experimental design, as well as the analytical method, are presented elsewhere (Harkey et al. 1991; Henderson et al. 1992). Briefly, the study was approved by the relevant institutional review boards, and the human subjects were experienced cocaine users. First, the pharmacokinetics of d_5-cocaine in these subjects were studied and found to be identical to that of nonlabeled cocaine. Next, control hair, plasma, and sweat samples were obtained, and then d_5-cocaine was administered intravenously (IV) or intranasally (IN). Researchers used the widest range of doses possible without putting the subjects at risk. Intravenous doses ranged from 0.3 to 1.2 mg/kg (35 to 154 mg per subject) and, in order to achieve higher total doses, a

combined dose of 1.2 mg/kg IV plus 3 mg/kg IN was administered. The highest dose for any subject was 854.5 mg (90.5 mg IV plus 764 mg IN). In all figures and tables, IN doses have been corrected for bioavailability, which was found to be 30 percent (e.g., an IN dose shown as 3 mg/kg represents an administration of 10 mg/kg total drug).

Researchers found that as little as a single dose (approximately 30 mg) could be detected in hair using the laboratory's very sensitive mass spectrometric method. In general, higher doses of cocaine resulted in higher concentrations of parent drug in hair. BE was rarely found and EME, if present, was below the limit of detection (200 pg/mg hair). However, considerable variability was found between subjects receiving the same dose, and no correlation was found between the amount of drug in hair and the dose (figure 5). Thus, data suggest that it would be difficult to determine when or how much cocaine was ingested using hair analysis data alone. Interestingly, the highest amounts of drug in hair were found in the four non-Caucasian subjects who received 1.2 mg/kg cocaine. To date, all "outliers" in the study have been non-Caucasian (African-American, Hispanic, or East Indian), and all non-Caucasians have been outliers. These observations are preliminary, and other factors such as frequency of hair washing could explain these findings. Frequency of hair washing and use of cosmetic hair products were not and probably could not be controlled during the study period, which lasted for up to 9 months for some subjects. The researchers are now conducting a separate study to address the relationship between race or hair type and concentration of drug in hair.

Table 1 shows the amount of d_5-cocaine in hair of the subjects after the administration of a single dose. The amount of drug in hair is expressed as mean amount, maximum amount, or area under the curve (AUC). Mean amount of drug is the value determined from the mean of all positive hair segments from an individual. Expressing the data in this way prevents bias from one or two unusually high segments. Maximum amount is the highest value observed in any hair segment. This amount was usually but not always found 1 to 3 months after drug administration (1 to 3 cm from the root). Drug amounts are also expressed as AUC, a term commonly used in pharmacokinetics that reflects the total amount of drug incorporated into hair over time. Figure 6 shows how the AUC was calculated.

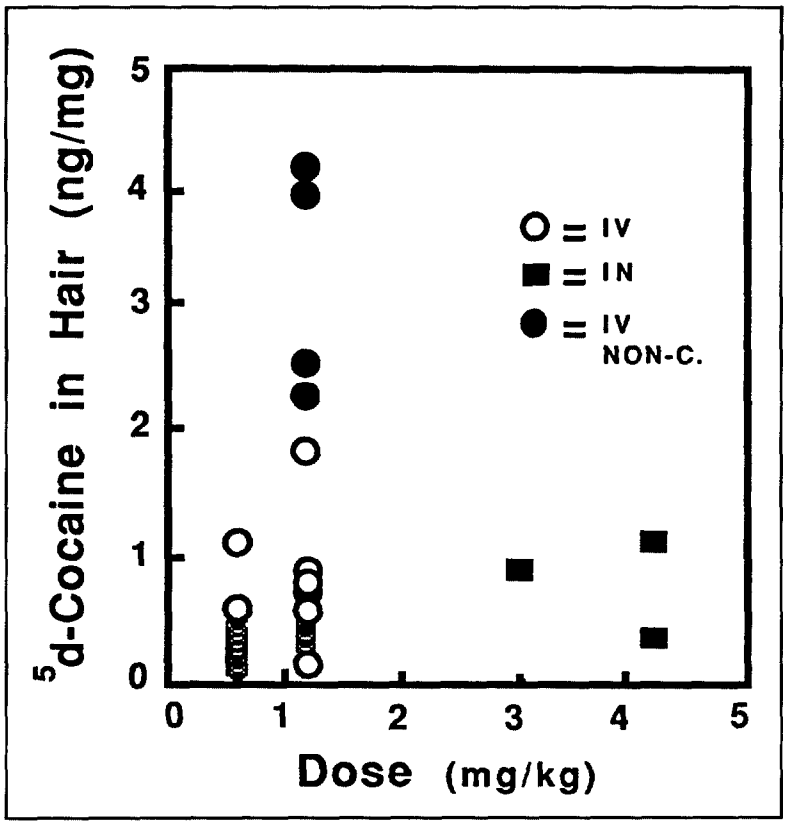

FIGURE 5. *Relationship between dose of d_5-cocaine and amount found in hair.*

As can be seen in table 1, the non-Caucasians in this study had between 2 and 12 times as much drug in their hair as did Caucasians (depending on how the data are presented).

The relationship between time of drug administration and location of drug along the hair shaft was analyzed by segmental analysis. Root ends of a hair sample were aligned and cut sequentially into l-cm segments. Theoretically, each 1 -cm segment should correspond to 1 month's hair growth. For approximately one-half of the subjects, there was a moderate correlation between the location of the drug in hair and the time of administration (e.g., the position of drug along the hair shaft over time corresponded to the time of drug ingestion within an accuracy of 1 to 2 months). For the other half of the subjects, there was little correlation

TABLE 1. *d_5-Cocaine uptake in hair of Caucasian and non-Caucasian subjects, expressed as mean amount, maximum amount, and A UC.*

Race	Dose Regimen	N	Mean Amount ± SD (ng)	Maximum Amount ± SD (ng)	AUC±SC (ng months)
Caucasian	0.6mg/ kg, IV	9	0.21±0.12	0.25±0.14	0.40±0.29
Non-Caucasian	0.6mg/ kg, IV	1	0.48	1.09	3.57
Caucasian	1.2mg/ kg, IV	5	0.40±0.12	0.54±0.20	1.44±1.03
Non-Caucasian	1.2mg/ kg, IV	2	3.36±0.05	4.26±0.52	17.15±3.89
Caucasian	1.2mg/ kg, nasal	1	0.4	0.95	1.69
Non-Caucasian	1.2mg/ kg, nasal	1	2.25	4.01	6.84

between the location of drug along the hair shaft and the time of administration.

For example, cocaine was detected throughout the hair shaft in one subject who received a single dose (shown in table 2). The first few segments of hair continued to test positive 2 and 3 months after dosing. In another subject with longer hair, drug was detected in 14 segments following a sinsle dose of drug. These two cases support a multiple mechanism model for drug entry into hair.

Some subjects' hair tested positive within hours of drug administration, which suggests there is an alternate mechanism, possibly sweating, by which drugs could enter hair. Figure 7 shows that the concentrations of d_5-cocaine in sweat samples are quite high (much higher than d_5-BE) and persist over days. The findings that the metabolic profile of hair (cocaine > BE > EME) is more similar to that of sweat than plasma,

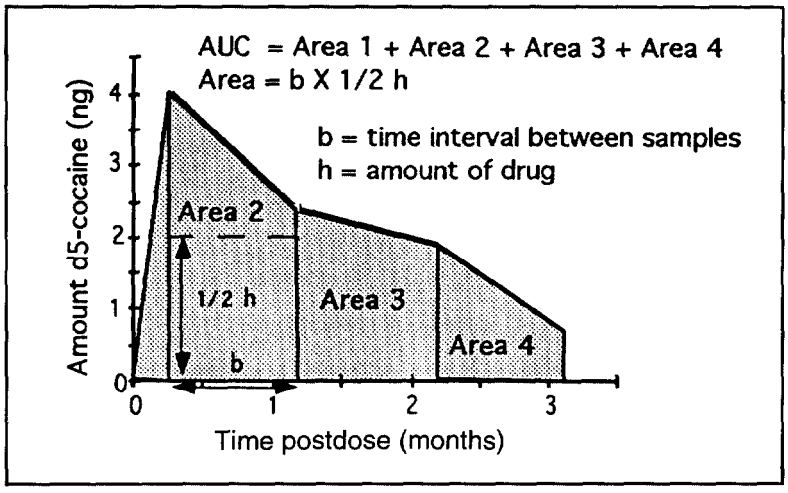

FIGURE 6. *Calculation of A UC by trapezoidal rule.*

TABLE 2. *Segmental analysis of hair samples from subject 88173.*

		Amount of d_5-cocaine in segments (ng/mg hair)				
Hair collected (month postdose)	Amount d_5-cocaine in sample (ng)	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5
0.26	4.01	1.2	2.82			
1.17	2.40	1.26	0.54	0.38	0.22	
2.2	1.89	0.83	0.38	0.25	0.19	0.24
3.13	0.68	0.11	0.23	0.34		

Maximum amount of d_5-cocaine = 4.01 ng.
Mean amount of d_5-cocaine = 2.25 ng.
d_5-cocaine AUC = 6.84 ng.

cocaine is present in sweat as the principle analyte, and cocaine persists in sweat at relatively high concentrations for days suggest that sweat could be a vehicle for cocaine incorporation into hair.

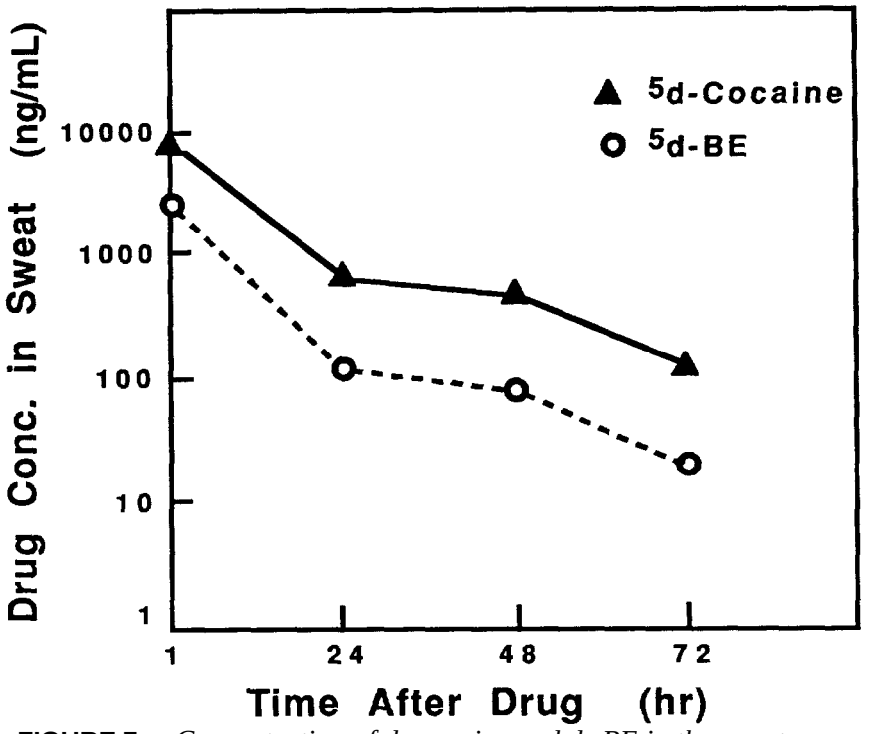

FIGURE 7. *Concentration of d_5-cocaine and d_5-BE in the sweat of a subject who received a single 2 mg/kg dose of d_5-cocaine IN.*

CONCLUSIONS: STATUS OF HAIR ANALYSIS FOR DRUGS OF ABUSE

It is well established that drugs can be detected in hair, and increased doses are generally associated with increased drug concentrations in hair. However, for cocaine considerable differences have been found between individuals in the amount of drug found in hair and the distribution of the drug along the hair shaft. These differences cannot be explained by differences in plasma pharmacokinetics but may be related to a number of factors including other pathways for drug incorporation into hair, differences in the structure of hair (i.e., racial or genetic differences), and personal hygiene (i.e., frequency of washing hair).

Hair is a very complex tissue, and thus researchers must recognize certain considerations when interpreting results from hair analysis. These include:

1. The hair follicle is surrounded by a complex network of blood, lymph vessels, and secretory glands that can provide multiple pathways for drug incorporation into hair.

2. Hair morphology and physiology differ with race, gender, and age, but the impact of these differences on drug incorporation and retention is not known. These differences could lead to large intersubject variability.

3. Skin is known to be a reservoir for many drugs. This could lead to more complicated pharmacokinetics for drugs in hair.

4. Hair is highly porous and hydrates readily. Thus, drugs in the external environment (e.g., smoke or sweat) could be absorbed into the hair shaft and be a source of false positives due to external contamination.

Although hair analysis offers an attractive alternative to urine testing for drugs of abuse, results from hair analysis should be interpreted with caution, for the mechanisms by which drugs are excreted into urine are much better understood that the mechanisms for drug incorporation into hair.

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Models for Studying the Cellular Processes and Barriers to the Incorporation of Drugs Into Hair

Dough E. Rollins, Diana G. Wilkins, and Gerald Krueger

INTRODUCTION

Analysis of hair is being considered for the detection of drugs of abuse in myriad settings because obtaining hair is less invasive than obtaining urine or blood, and hair may provide a record of past drug use that is not available from either urine or blood (Baumgartner 1989). Hair and the hair follicle are complex tissues, and there are numerous questions to be answered before hair can be fully accepted as a tissue for the qualitative or quantitative measurement of drug or chemical exposure; these questions concern the correlation between drug dose and drug concentration in hair; mechanisms of drug incorporation, retention, and loss from hair; and the possibility of exogenous drug contamination. Although human hair is easily obtained, studies to evaluate drug incorporation into human hair in situ are difficult to perform because of limitations in controlling experimental variables and ethical issues involving drug administration. Animal and in vitro models of human hair growth are needed because they allow for control of experimental variables and flexibility of experimental design and because they permit the study of substances that cannot be administered to humans. However, the validity of any experimental model must be established in light of the data obtained and the relevance of the data to understanding the processes involved in the distribution of drugs of abuse into human hair.

Regardless of the mechanism of drug incorporation into hair, the process must involve transfer across several cell membranes. Human hair follicles are composed of cells of epithelial origin (matrix and outer root sheath) and dermal origin (dermal papilla). Cells in the dermal papilla undergo specialized differentiation such that they cause the surrounding epithelial cells to differentiate into hair. As these cells proliferate, keratin filaments in the cells condense, undergo crosslinking, and the hair shaft is formed. The cells that give rise to hair are among the most rapidly dividing cells of the body, undergoing cell division approximately every 24 hours. The growing (anagen) hair follicle, located in the dermis, is

nourished by a rich vascular supply (Montagna and Ellis 1957). Capillary loops penetrate the dermal papilla and a plexus of vessels surround the lower third of the follicle. In the quiescent (telogen) follicle, the dermal papilla contains no capillaries. During the growth phase, the relatively few cells generating the hair display intense metabolic and proliferative activity, producing 0.3 to 0.7 mm of hair per day. As hair grows, it is exposed to the metabolic milieu of the structural and cellular elements of skin, the circulating blood and lymph and, after hair has emerged from the scalp, to the extracellular fluids such as sweat. As hair elongates and approaches the skin surface, the outer layer of hair becomes hardened. This has the potential of locking in the metabolic products accumulated during the period of hair formation, including drugs. Thus, cells that form hair have the potential to contain drugs and their metabolites via the blood flow to each hair follicle. The hair shaft may also be exposed to drugs via sweat. If the predominant route of hair exposure is via sweat, then external contamination may confound the interpretation of hair concentration of drugs. Thus, it is important to fully understand the mechanisms involved in the incorporation of drugs into hair, including the transfer of drugs across the membranes of the hair-forming cells.

The specific mechanisms by which ingested drugs are incorporated into hair and the physiochemical factors that control these processes are poorly understood. The importance played by factors such as molecular size, metabolism, lipophilicity, ionic charge, plasma protein binding, and chemical binding in the incorporation of drugs must be known before meaningful interpretation of hair drug concentrations can be made. Experiments to understand many of these factors are not easily performed in humans. Models that are relevant to in situ human hair growth are needed to design relevant experiments that will address the processes of drug incorporation into human hair, and appropriately designed, well-controlled studies in humans are required to determine if results obtained from the models are applicable to humans.

The purpose of this chapter is to review the available models for the study of drug incorporation into hair and to discuss their relevance to that process in human hair.

ANIMAL MODELS

To test a hypothesis, most areas of research use animal models to obtain experimental data that are often difficult to obtain in human studies. Despite problems with the extrapolation of data from animals to man, the information gained is ultimately useful in designing experiments in humans. In the area of drug analysis in hair, animal models allow the study of drug incorporation into hair that could not be performed in humans, such as dose-response studies and administration of metabolites distinct from the parent drug. However, there are differences between animal and human hair that must be considered when designing experiments and interpreting data. Animal hair generally functions to protect animals from environmental temperature changes, and air spaces remain in the hair shaft. Although such hollow hair provides warmth for the animal, it may result in drug distribution that is distinctly different from drug distribution in solid hair such as human hair. Animals also have different hair growth cycles from humans, and the factors controlling hair growth differ from factors controlling hair growth in humans (Chase 1954; Durward and Rudall 1949). Despite these differences in the physical matrix and growth characteristics of animal hair, there are numerous studies of drug incorporation into animal hair that provide useful information for designing experiments in humans.

Following the administration of cocaine in doses of 5, 10, and 20 mg/kg daily for 28 days, Ferko and colleagues (1992) showed that the concentration of cocaine and benzoylecgonine in white rat hair increased with dose. The ratio of cocaine to benzoylecgonine in rat hair was 10 to 1. Nakahara and Kikura (1994) found a threefold ratio of cocaine to benzoylecgonine in dark-pigmented rat hair despite a threefold greater plasma area under the curve for benzoylecgonine than for cocaine. These studies correspond well with the observations of Cone and colleagues (1991) that the amount of cocaine in human hair predominated over all metabolites by a factor of 5 to 10. Using the male Dark-Agouti rat as a model, Nakahara and Kikura (1994) administered cocaine, benzoylecgonine, and ecgonine methylester independently. They observed that cocaine readily enters the hair whereas the metabolites do not. In further studies they administered cocaine, d_3-benzoylecgonine, and d_3-ecgonine methylester and demonstrated that the incorporation rates of benzoylecgonine and ecgonine methylester into rat hair were very low when compared with cocaine and that most of the benzoylecgonine detected in hair was the hydrolytic product derived from cocaine in hair matrix after incorporation.

These elegant studies illustrate how animals can be used to understand the distribution of drugs and metabolites in human hair. The possibility of cocaine hydrolysis to benzoylecgonine in hair matrix in situ must be studied in humans, but a clear hypothesis can be developed, and the experiments to test this hypothesis should be straightforward. However, as with most model systems, the closer researchers get to the human situation the more relevant the data will be. The use of in vitro culture techniques for human hair and the transplantation of human hair follicles to an animal may provide models that will allow for the flexibility of experimental design and eliminate the differences between animal and human hair that confound experiments with animals.

MODELS OF HUMAN HAIR GROWTH

Models of human hair growth are needed to answer some of the difficult questions surrounding the incorporation of drug of abuse into human hair that cannot be answered by studying animal models. As discussed earlier, animal models are useful, but the fact is that animal hair differs greatly from human hair in physical and growth characteristics. The in vitro culture of human hair and the transplantation of human hair onto athymic mice provides two models in which researchers have the ability, with viable human hair, to study a wide range of drug doses and the distribution of radiolabeled drugs with complete control of experimental variables.

In Vitro Human Hair Growth

The isolation of rat hair follicles and vibrissae follicles has been described (Green et al. 1986; Ibrahim and Wright 1982) and used to demonstrate that minoxidil's effect on hair follicles is direct and includes more than just an increase in blood flow to hair follicles (Buhl et al. 1989). Human hair follicles can be isolated from scalp skin and grown in culture for 4 to 14 days (Knapp et al. 1993; Philpot et al. 1990). Such isolated hair follicles show increased length attributable to the production of a keratinized hair shaft and maintenance of hair follicle morphology. Evidence of viable hair follicles can be demonstrated by the incorporation of ^{3}H-thymidine into the matrix cells of the hair follicle bulb and keratin synthesis as determined by incorporation of ^{35}S-methionine. After 14 days in culture media, isolated human hair follicles showed selective incorporation of ^{35}S-cysteine (Knapp et al. 1993). Philpot and colleagues (1990) have shown that epidermal growth factor (EGF) added to cultures

of human hair grown in vitro mimics its in vivo depilatory action, resulting in the formation of a club-hair-like structure. These investigators have also demonstrated that transforming growth factor (TGF)-ß1 may serve as a negative growth regulatory factor for the hair follicle.

In the authors' laboratory, human hair follicles are isolated from scalp tissue remnants obtained at the time of facelift surgery. To ensure viability, the tissue is immediately placed into culture media such as Dulbecco's Modified Eagle Medium (DMEM) containing 10 percent fetal calf serum, antibiotics, and an antifungal agent. After cutting the scalp tissue into 2-mm thick strips, the follicles are exposed on the cut surface and examined under a dissecting microscope. Follicles that penetrate into the subcutaneous (SC) fat are considered in anagen (growing) phase and are suitable for culture. Using a dissecting microscope, the follicle is removed from SC fat and surrounding adventitia. Released follicles are individually placed into a 24-well tissue culture plate and allowed to float freely in William's Essential Medium (WEM) with insulin, hydrocortisone, and transferrin added. The culture media is removed twice weekly, and the follicle length is measured with the ocular micrometer of a dissecting microscope. Drugs to be studied can be directly added to the culture media in various concentrations, and incorporation into the growing follicle can be measured over time. One limitation of this model is that drugs added to the culture media are exposed to all parts of the growing follicle and hair shaft. Thus, it may be difficult to determine whether drug is incorporated into the rapidly dividing dermal papilla cells or directly into the hair shaft via porous regions. Researchers in the authors' laboratory are currently exploring ways to culture the follicles in a matrix that allows for contact with media only at the hair bulb.

The potential benefits for studying hair biology and the disposition of foreign compounds, including drugs, into cultured human hair follicles are apparent. The advantage of such an in vitro system of hair growth is that drug concentration can be varied greatly without toxicity to the whole organism. Drug incorporation into hair can be studied independent of blood flow, and the incorporation of drugs and their metabolites can be studied separately. Utilizing radioactive drugs and autoradiographic techniques, it may be possible to determine which cells are involved in drug uptake and incorporation into hair structures. However, hair growth in vitro is minimal (as determined by increase in length), and hair follicles can be kept alive and growing for only limited periods of time. Studies

of long-term drug incorporation into hair in vitro are not currently possible. Nevertheless, the model has potential applications for the study of the cellular processes involved in drug uptake and incorporation into hair and, as the techniques for in vitro culture are improved, it will provide useful human hair data.

Transplantation of Human Hair-Bearing Scalp

Nude mice are unique in that they are congenitally athymic and thus are unable to mount a thymus-dependent immune response, such as cell-mediated rejection of a foreign graft and antibody production for most antigens. The transplantation of human tissue to the nude mouse provides a model for the study of the growth and biochemical characteristics of human tissue in a living, nonhuman system. Adult human skin transplanted to nude mice retains donor differentiation characteristics. By transplanting involved psoriatic and nonpsoriatic human skin onto nude mice, Krueger and colleagues (1975) have shown that the psoriatic grafts retain their usual characteristic histological differences and provide a model for the study of psoriasis in a nonhuman living system. Normal human skin transplanted to the nude mouse retains the proliferative and barrier functions of human skin rather than that of the host animal (Krueger et al. 1981). The growth of human hair-bearing scalp on the nude mouse provides a system in which viable human hair is growing in a nonhuman system.

One method for the transplantation of hair-bearing scalp to the nude mouse involves implanting a 6-mm biopsy of the scalp into an incision on the thoracic cage of the mouse. The incision is closed and remains closed for 2 to 3 weeks. After exposure, the graft is covered with a bio-occlusive bandage for 7 to 10 days and then covered with a petroleum jelly gauze for an additional 7 days. The transplantation of human hair-bearing scalp to nude mice has been demonstrated to be of value in understanding the underlying cause of alopecia areata (Gilhar et al. 1986). The authors' laboratory and others have been developing the transplantation of human hair onto nude mice as a model for hair growth and for the purpose of studying the incorporation of drugs and xenobiotics into human hair (Knapp et al. 1993; Zareba et al. 1993). The histological structure of the human hair follicle is maintained after grafting onto nude mice (Van Neste et al. 1989, pp. 117-131). This model has been used to study the underlying cause of alopecia areata (Gilhar et al. 1986), the linear hair growth rates of hair from human androgen-dependent alopecia (Van Neste et al. 1991), and the

morphological and biochemical characteristics of trichothiodystrophy-variant hair (Van Neste et al. 1993). In this later study, the amino acid composition of hair produced by donor scalp follicles was maintained up to 6 months as grafts onto nude mice. Knapp and colleagues (1993) have demonstrated that 35^{S}-cysteine injected into nude mice with hair-bearing human scalp grafts moves in a band from the hair bulb at 3 days postinjection to 2.5 mm from the bulb at 14 days postinjection. The model has also been used to study the incorporation of methyl-mercury into human hair-bearing scalp grafts in nude mice (Zareba et al. 1993). Methyl-mercury was rapidly incorporated into newly growing hair at concentration proportional to that of blood and two orders of magnitude greater than other tissues.

These studies demonstrate the usefulness of human hair-bearing scalp transplanted to the nude mouse as a model that provides human hair growing in a living nonhuman system. Grafting of human fetal scalp may result in even more luxuriant growth than adult scalp (Zareba et al. 1993; Rollins, unpublished observations). Using this model it will be possible to carefully evaluate drug incorporation into human hair by administering a broad range of doses and radiolabeled drugs to the nude mouse. The obvious disadvantage of the model is that the metabolism and distribution in the nude mouse may be different from humans. Nevertheless, this model should provide useful information with which to plan further human studies.

HUMAN STUDIES

It is quite important to address the issue of incorporation of drugs into hair in humans. Studying the hair concentrations of drugs after long-term use will provide useful information regarding the stability of drugs in hair but will unlikely solve the issues surrounding the mechanisms of drug incorporation. The cellular processes by which drugs cross from capillaries into the rapidly dividing cells of the hair bulb with eventual incorporation into the hair matrix are not easily studied by observing the steady-state drug concentrations in hair. These processes can be best studied by simultaneously measuring the concentrations of drugs in the blood or sweat and in the hair bulb. To date, few studies have done this. Researchers at the authors' laboratory have observed that following a single dose of oral codeine, the drug can be quantitated in the human hair bulb within 30 minutes of administration (Rollins et al., in press) in sedentary, resting males. This is not surprising given the high blood flow

to the hair follicle in the scalp. Whether a drug initially detected in the hair bulb is the same as that incorporated in the hair matrix and eventually detected in distal hair remains to be determined. Nevertheless, this observation emphasizes the rapid nature of drug movement into the hair bulb. The presence of rapidly dividing cells and rich blood supply is important in the rapid movement of drug into the hair bulb. The models discussed in this chapter have provided the experimental means to study and solve some of the critical problems involved in drug incorporation into hair.

CONCLUSION

In order to completely describe the mechanisms of drug incorporation into hair it will be necessary to study the process in animal models and models of in vitro hair growth. These models will allow researchers to manipulate the experimental variables to study the route of incorporation (i.e., blood, sweat, sebum, external contamination), the site of incorporation (i.e., hair follicle or hair shaft), the location of drug binding in the hair matrix (i.e., medulla or cuticle), the mechanism of binding (i.e., entrapment, ionic binding, or covalent binding), and the substance to which drugs are bound in hair (i.e., melanin or keratin). Only after these variables are identified and there is a complete understanding of the factors involved in their incorporation can the concentrations of drugs in hair be accurately interpreted.

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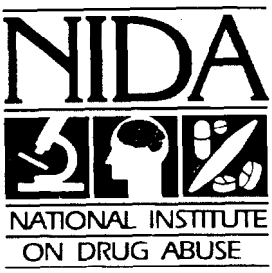

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