Determining the Molar Extinction Coefficient of Antimycin A

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Abstract

Antimycin A is an antibiotic that inhibits the mitochondrial bc1 complex. It has become an important tool for studying the function of the bc1 complex and for modulating respiratory activity in cells. Antimycin A binds extremely tightly to its binding site, with a dissociation constant less than 1 nM, and induces spectral changes in the enzyme. This makes it useful for quantitating the amount of bc1 complex in a sample, and for determining accurate extinction coefficients for the cytochromes of the bc1 complex. Antimycin A is a mixture of compounds with slightly different side chains on the same pharmacophore. Its concentration is usually determined not by weight but by absorbance of the pharmacophore. To date the only determination of the extinction coefficient

is from the chemists who originally characterized it, and is presumably based on weight. We report an more accurate extinction coefficient based on acidometric titration of the phenolic OH in the pharmacaphore, which induces a distinctive spectral change in the compound. The extinction coefficient of antimycin at 320nm is at the range 5.19~5.36.

Introduction and Background

Antimycin A (hereafter antimycin) is an antibiotic produced by *Streptomyces* bacteria that binds with an extremely high afinity to the quinol reduction site (Qi) of the mamalian mitochondrial cytochrome bc1 complex, blocking its action. Perhaps because of the exquisite sensitivity of the human enzyme, antimycin has not found any significant clinical applications, unlike another cytochrome bc1 inhibitor atovaquone which, due to low sensitivity of the human

enzyme, has proven useful in the treatment of malaria, Pneumonia, and AIDS-related secondary infections (Toxoplasmosis).

However, again because of its extremely high afinity binding, antimycin has been a valuable tool in elucidating the mechanism of the bc1 complex function. The use of antimycin in the "double kill" experiment of Deul and Thorne (ref. 1), and "oxidant-induced reduction" (ref. 2) of cytochrome b in the presence of antimycin, were important in development of the Q-cycle mechanism to explain operation of the bc1 complex. Antimycin titration has been proposed as an accurate primary determination of the concentration of a preparation of the bc1 complex, allowing the determination of the extinction coefficients for the complex to be used in secondary determinations. There is however a discrepancy of nearly 10% between the concentration of the bc1 complex determined by antimycin titre and that determined by pyridine hemochrome analysis

of the heme content.

The chemical structure of antimycin can be described as 3-formamido salicylate esterified to the N of threonine which forms a dilactone with a 2-n-alkyl, 3,4-dihydroxy pentanoic acid. The 4-hydroxy group is involved in dilactone formation while the 3-hydroxy is esterified with a branched-chain acyl group. Antimycin is actually a mixture of compounds, with some heterogeneity in both the n-alkyl and the branched acyl sidechains. The major component antimycin A1 apparently has n-pentyl and 2-methylbutanoic acid in these positions. Most of the affinity and specificity of antimycin resides in the aromatic formamido-salicylate group, with the remainder of the molecule serving mainly to affect the partition of the compound between aqueous medium and the hydrophobic environment of the Qo site. Perhaps due to the heterogeneity, or the inconvenience in accurately weighing very small portions, it has been recommended (ref. 3) that

the concentration of antimycin solutions be determined from their absorbance at 320 nm in ethanolic solution, using an extinction coefficient of 4.8 mM^{-1}. Because the two centers of heterogeneity mentioned above are both isolated from the aromatic chromophore by the saturated dilactone ring, it is reasonable to assume that all forms have essentially the same extinction coefficient. The same value, used also in determining the antimycin titre of the bc1 complex, was calculated from $\log E_{320} = 3.68$ determined by chemists working out the structure of antimycin. Details are not given, but it is to be presumed that their main interest was in qualitative analysis of the structure, which does not require a highly precise value. In fact another group of chemists working on the structure reported a value of $\log E_{319} = 3.78$, corresponding to a millimolar extinction coefficient of 6.03.

In order to enhance the usefulness of antimycin as a tool in the study of the bc1 complex, and

in hopes of clearing up the discrepancies between the values for extinction coefficient of cytochrome b based on antimycin titre and heme analysis, we decided to redetermine the molar extinction coefficient of the antimycin. The phenolic hydroxyl of the salicylate moiety is ionizable, in fact it is generally realized that in order to get any meaningful value for absorbance at 320nm it is necessary to acidify the solution, as significant dissociation with accompanying spectral change is observed for dilute solutions in neutral ethanol. We decided to exploit this ionization, and the accompanying spectral change, to perform a spectrally monitored acidometric titration of antimycin in order to determine an accurate extinction coefficient.

Effect of Hydrolysis (Buffering Power) on Titration Curve

An ideal titration curve would be a straight line from ideal acid endpoint and ideal base

endpoint. The ideal acid endpoint (really starting point) is the value of zero of NaOH and the absorbance of the fully protonated form. The ideal basic endpoint is the value of NaOH which is exactly equal to the amount of antimycin and the absorbance of the fully ionized form.

In reality, at the acid endpoint antimycin will be partially dissociated. At the basic endpoint, some of the antimycin will still be protonated. This phenomenon is referred to hydrolysis of a weak acid and its salt. Quantitave expression for the extent can be derived as described in reference (ref. 4). The result of this hydrolysis is that the titration curve does not pass through the ideal endpoints but makes curve which misses them. Figure 2 shows simulating curve based on Nightingale's equation.

Buffering power/hydrolysis. If NaOH or HCl reacted quantitatively to interconvert antimycin and antimycinate until the reaction was complete, then a plot of absorbance vs added

acid or base such as Figures 1 and 2 would give sharp endponts. In fact the endpoints are somewhat rounded,and their position must be estimated by extrapolation from the linear segments before and after.

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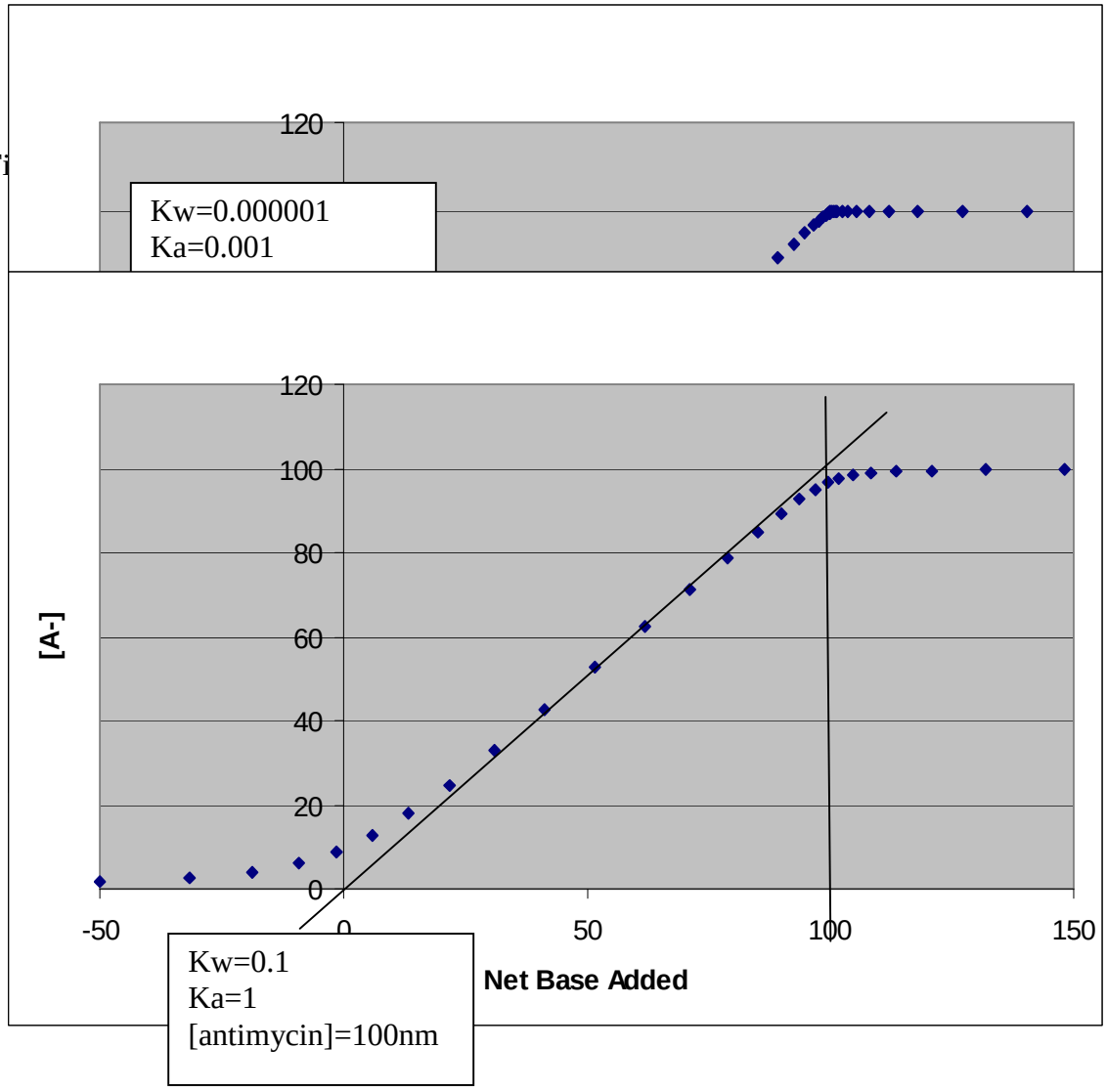

120
Kw=0.000001
Ka=0.001
120
100
80
60
40
20
0
[A-]
-50
0
50
100
150
Net Base Added
Kw=0.1
Ka=1
[antimycin]=100nm

This is due to a phenomenon that can be described either in terms of buffering power of the solvent or what the physical chemists call “hydrolysis” of a weak acid and its salt. These are two equivalent ways to describe the ability of the solvent to absorb and release protons (H^+). The result is that the amount of antimycinate produced is not exactly equal to the amount of NaOH (Na^+) added but loosely coupled by what Nightingale(ref 4) calls the electroneutrality condition:

$$A^- = Na^+ + H^+ - OH^-$$

If the concentration of H+ and OH- are negligible compared to AH + A-, then the titration will be linear with sharp end points. Variation in H+ and OH- provides the slack that allows rounded endpoints.

To understand it in terms of buffering power, consider that to obtain antimycin 99% protonated, the activity of H+ must be 100 times the Ka of antimycin, i.e. pH 2 units below pKa.

Likewise to achieve 99% dissociation, the pKa must be two units above the pKa. Simply adjusting the pH of the solvent over this range would require a certain amount of acid or base. In the midpoint of the titration, i.e. near the pKa, the protonation state of antimycin is changing rapidly with pH, i.e. the buffering power of antimycin is high compared to the solvent and almost all of the base is absorbed by the antimycin reaction. This gives a linear range in which the slope is equal to the extinction coefficient. Near the endpoints, the buffering power of antimycin is low, and most of the acid or base is absorbed by the buffering power of the solvent. This results in rounded shoulders and make estimation of the endpoint less precise.

To understand the phenomenon in terms of hydrolysis, consider that at the acid endpoint you have a solution of pure antimycin, and at the basic endpoint you have a solution of the pure salt of antimycin. If there were no hydrolysis, all the antimycin would be protonated at the acid endpoint,

giving the absorbance of the protonated form. In actuality hydrolysis converts some of the antimycin to the basic form, and additional acid must be added to obtain the fully protonated sample. If the salt of antimycin did not hydrolyse, it would be fully deprotonated. However hydrolysis converts some to the acid form, and additional base must be added to obtain the fully deprotonated form. Exact expressions describing the hydrolysis of a weak acid (like antimycin) and its salt has been treated in detail by Nightingale

Derived extinction coefficients

Beer's law states that the absorbance of a solution is proportional to the concentration of the absorbing species and the length of the path the light follows through the solution.

A simple extinction coefficient for a pure compound at a single wavelength is the proportionality

constant of theBeer-Lambert law applied to that compound at that wavelength:

$$A = C\ E\ L$$

Henceforth we consider the pathlength is 1, and omit L from the equation:

$$A = C\ E$$

Applying the Beer-Lambert law in the following situations leads to the following derived extinction coefficients:

1. Extinction coefficient ΔE for a reaction $\mathbf{\Delta E_{V\text{-}U}}$:

If a reaction converts one absorbing species U into another species V, the change in absorbance accompanying the reaction is due to decrease in the absorbance due to U and increase in the absorbance due to V:

$$\Delta A = \Delta C_U E_U + \Delta C_V E_V$$

but $\Delta C_U = -\Delta C_{V,} == -\Delta C$

$$\Delta A = \Delta C (E_V - E_U) = \Delta C \, \mathbf{\Delta E_{V\text{-}U}}$$

2. Dual wavelength extinction coefficient $\mathbf{\Delta E}^{\lambda 2 - \lambda 1}$.

If the absorbance difference at a pait of wavelengths is measured in order to minimize the effect of baseline drift or maximize the spectral change, applying Beer-Lambert law to each wavelengthand subtracting gives us the dual-wavelength extinction coefficient:

$A_{\lambda 1} = C E_{\lambda 1}$

$A_{\lambda 2} = C E_{\lambda 2}$

$$\Delta A_{\lambda 2 - \lambda 1} = C (E_{\lambda 2} - E_{\lambda 1}) = C \, \mathbf{\Delta E}^{\lambda 2 - \lambda 1}$$

3. Monitoring a chemical change at a wavelength pair leads to the double difference extinction coefficient: $\Delta A = \Delta C \, \Delta\Delta E^{\lambda 2 - \lambda 1}_{V\text{-}U}$

Material and Method

First, we measure a double difference extinction coefficient at a single wavelength pair, by a titration experiment. A 1mM solution of antimycin was titrated with 50mM NaOH. In such a titration experiment, the change of absorbance which is due to loss of absorbance by antimycin and the gain of absorbance by antimycinate, and therefore the absorbance change on adding a certain amount of NaOH is the change of the concentration times the difference in extinction coefficient of antimycinate and antimycin. The titration was actually monitored by the difference in absorbance at 272nm-298nm to cancel out baseline drift and obtain a larger signal. Therefore, the extinction coefficient we obtained is the double difference extinction coefficient (appendix 1),

antimycinate-antimycin and 272nm-298nm. Then in order to have the extinction coefficient at any wavelength, we measure spectra of antimycin in the presence of excess HCl acid and NaOH base, and calculate the difference spectrum. The double difference extinction coefficient is applied to the difference spectrum at the wavelength pair where we measure the extinction coefficient to calculate the concentration using Lambert-Beer Law. Having the concentration, we can calculate the extinction coefficient for antimycin, antimycinate, or the difference spectrum, at any wavelength, from the absorbance at that wavelength, the concentration, and Lambert-Beer Law.

The antimycin A, from Streptomyces species was purchased from Sigma-Aldrich Inc. The standard HCl (10 mmol) was purchased from Sigma-Aldrich Inc, 20mM HCl. The 50mM NaOH and 20mM NaOH was diluted from a 1M NaOH solution which was diluted from a saturated (~14.5M) NaOH solution. The concentration was determined by titrating with standard HCl.

Dimethyl sulfoxide, tetrahydrofuran, dioxane, and water were tried; however, antimycin did not react with NaOH in dioxane or tetrahydrofuran. Thus, we used ethanol as our solvent. Spectra was taken using a UV-visible spectrophotometer UV-1601 Shimadzu, controlled by the “UVPC” program from Shimadzu (details and validation tests are described in Appendix 2). All spectra taken by Shimadzu was at a slow speed (260nm/min). Two black-masked quartz cuvettes with 10mm and 5mm pathlength were used.

Results

1. Spectra of antimycin in ethanol-untreated and with excess acid and base

In order to obtain the antimycin in fully protonated and ionized forms, antimycin from the stock solution was diluted to 200uM in ethanol. A spectrum was taken before and after excess acid and

base(Figure 3).

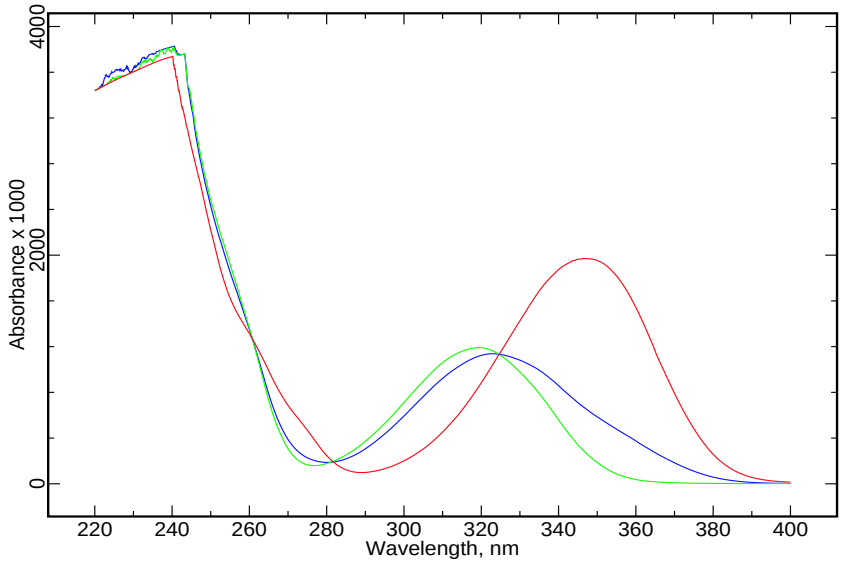


Figure 3. Spectra of antimycin in ethanol untreated and with excess acid and excess base.

Antimycin was diluted to 200uM in 1 mL of ethanol. Spectrum A (blue) was recorded, then 0.5uL 1M HCl was added and spectrum B (green) was reccorded. Then, 2uL 1M NaOH was added and spectrum C (red) was recorded. The absorbance was off scale at the wavelength below 240nm.

Figure 4 shows standard spectra of acid (blue) and base (green) forms. The difference (basic-acid form) is plotted in cyan. There are maximum in the difference spectrum at 270.7 and 351.8 nm and a minimum at 308.3 nm, with isobestic points at 260.8 and 281.4, and 324.7. The spectra will be used to convert extinction coefficients at different wavelengths and to compare with the literature values at 320nm.

Figure 4. Standard spectra of antimycin. Protonated form (blue), ionized form (green), difference (cyan). Nominal concentration was 200 uM based on MW of antimycin A3? and nominal weight in bottle.(, spectra have been scaled to 100 uM (no, unscaled))

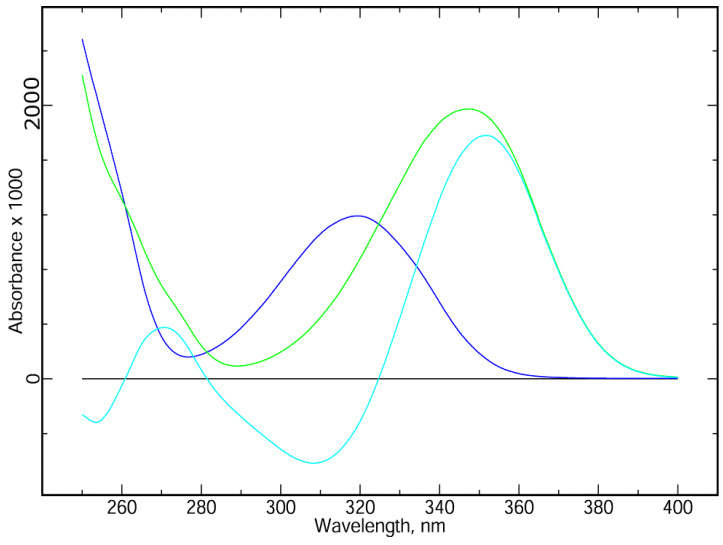


Table 1. Approximate extinction coefficients of antimycin for conversion ratio.

Wavelength	272	272	370	380	390	319	320	347
Vs ref:	298	303	400	400	400	400	400	400
ABS,mAU:								
protonated	-2.056	-3.008	0.036	0.008	0	5.944	5.94	1.352
ionized	4.292	3.392	7.828	2.456	0.416	8.16	8.676	19.604
Difference	6.348	6.396	7.792	2.448	0.416	2.216	2.732	18.248
Relative to 319 peak of protonated form:								
protonated	-0.346	-0.506	0.006	0.001	0.000	1.000	0.999	0.227
ionized	0.722	0.571	1.317	0.413	0.070	1.373	1.460	3.298
Difference	1.068	1.076	1.311	0.412	0.070	0.373	0.460	3.070

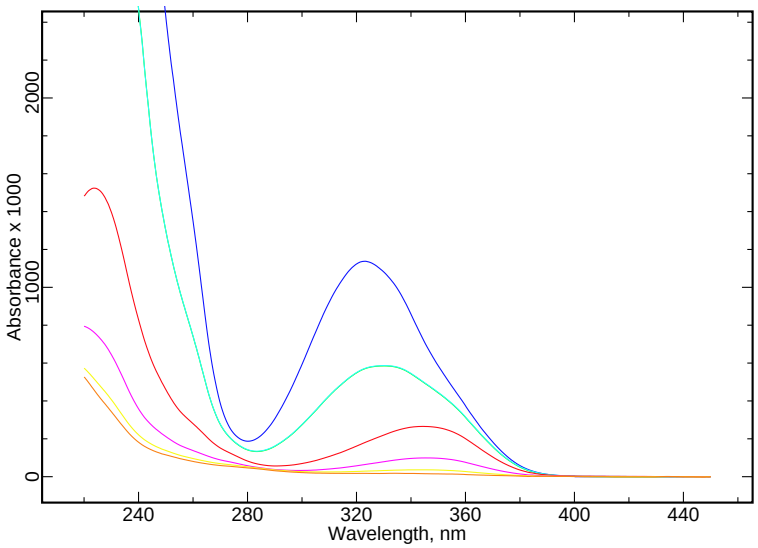
2. Dilution series of antimycin

In order to understand the dissociation of antimycin in ethanol, we wook spectra at different dilusions. As shown in figure 5. The absorption maximum shifted to longer wavelength with

higher dilution indicated it is more ionized. This is expected for the dissociation, however,

attempts to calculate Ka from this data showed the effect of dilution cannot be accounted for

simply based on the dissociation constant Ka.



31.6uM of antimycin (red),

10uM of antimycin (pink),

3.16uM of antimycin (yellow),

1uM of antimycin (orange).

Figure 5. Spectra of antimycin at different dilutions.

200um of antimycin (blue), 100uM of antimycin (cyan),

3a. Acidometric Titration

The first attempt to make extinction coefficient was using 200uM antimycin. Figure 6 shows

spectra taking during the titration. All the curves pass through the isobestic point. These are wavelength pairs where the absorbance of the acid and base form are exactly the same. The same isobestic point can be noticed in Figure 3. Absorbance of selected wavelength was plotted in Figure 7; note the endpoints are somewhere rounded. Although this titration generally confirmed the nominal concentration of antimycin, it cannot be analyzed accurately because of the rounded endpoints.

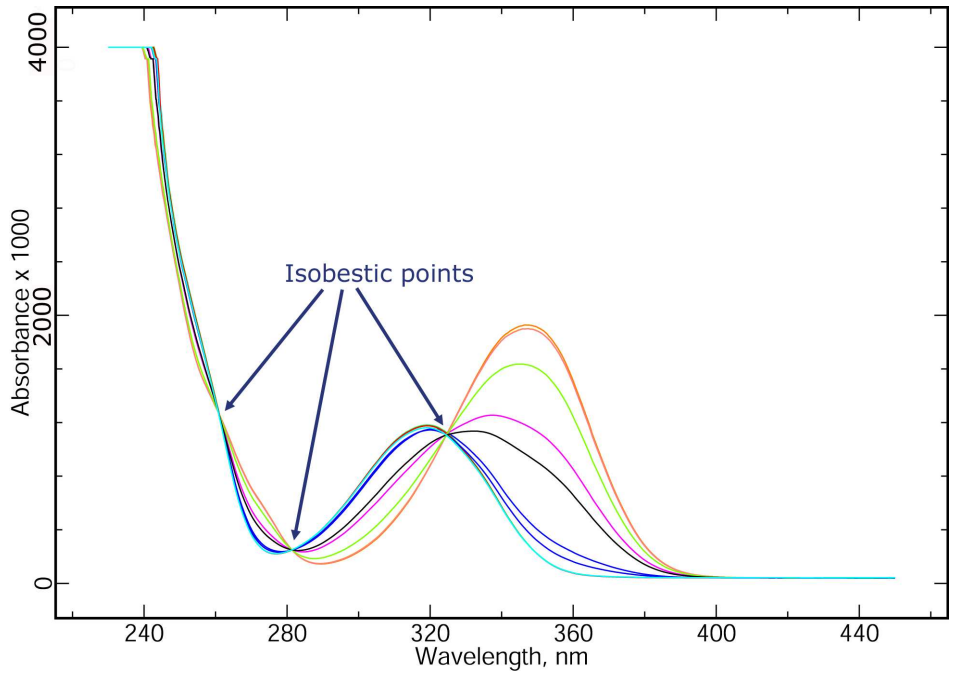


Figure 6. Spectrally monitored acidometric titration of antimycin(032406?). 200 nmol of

antimycin was diluted to 1 ml with ethanol. Two times of 5 ul of 20mM HCl were added until no further spectral change occurred (light blue line) to obtain the spectrum of fully protonated antimycin. Then aliquots of 20 mM NaOH were added to obtain the fully ionized form antimycinate (orange curve). Several spectra at different points in the titration are plotted to show the isobestic points (sidebar) which demonstrate that a single spectral change is taking place between two species whose absorbance is the same at those wavelengths.

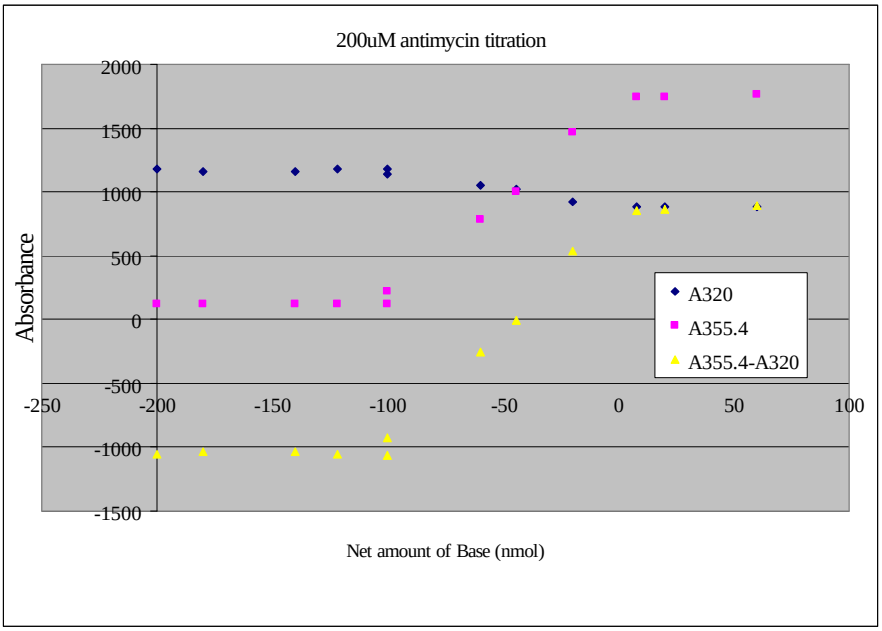


Figure 7, Absorbance vs. net amount of base at specific wavelength(032406?). From Figure 6, absorption at the peak wavelengths for antimycin (320 nm) and antimycinate (355.4 nm), and the difference between them, is plotted against the net base added. The slope of the titration in the linear region should be the extinction coefficient. Under these conditions the endpoints are not sharp due to buffering power of solvent. Also, with this experiment there was a problem with the first portions of acid not getting mixed in, so the zero point on X axis is arbitrary.

In order to minimize the effect of hydrolysis on the endpoints, we repeatedly titrate at a higher concentration (1mM antimycin). As seen in figure 8. This resulted in the spectra being off scale, over most of the range. However, we were able to choose the wavelengths at which the absorbance was on the scale for every spectrum.

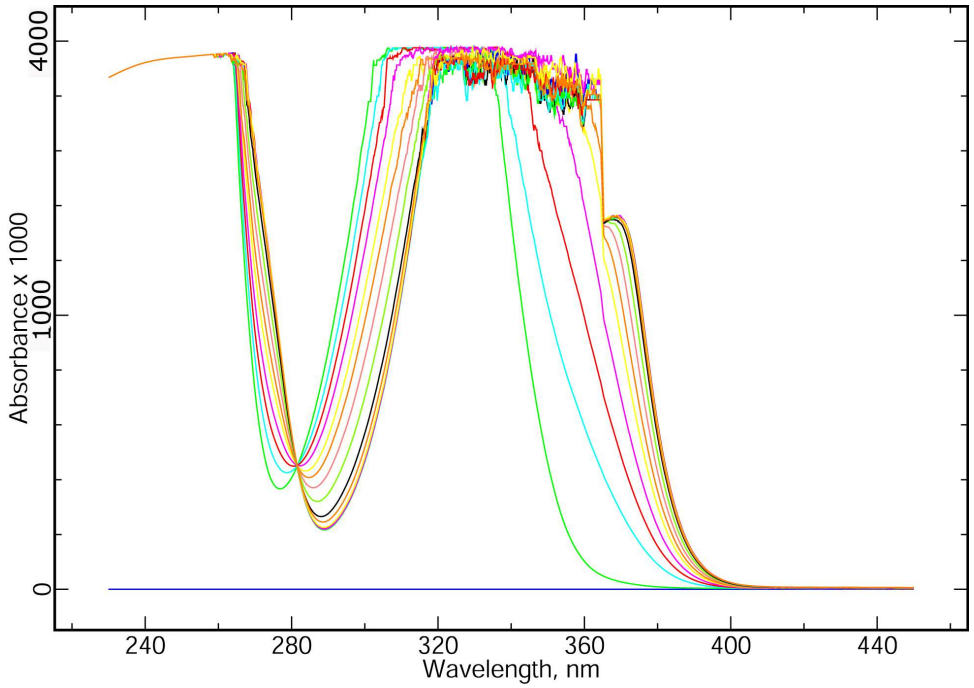


Figure 8 Spectra of antimycin at 1 mM. The peaks are clipped by the absorbance range of the instrument, but spectral data is avaiable at wavelengths where the extinction coefficient is lower.

Figure 9 shows absorbance at selected wavelength, plotted against amount of NaOH added.

In this case, the endpoints are less rounded, and an accurate slope can be determined. The

slope of the curve indicated the extinction coefficient is 4.0 mM^{-1} at wavelength pair

272nm-298nm. Using the standard spectra of figure y, this results the extinction coefficient of

5.37 at 320nm.

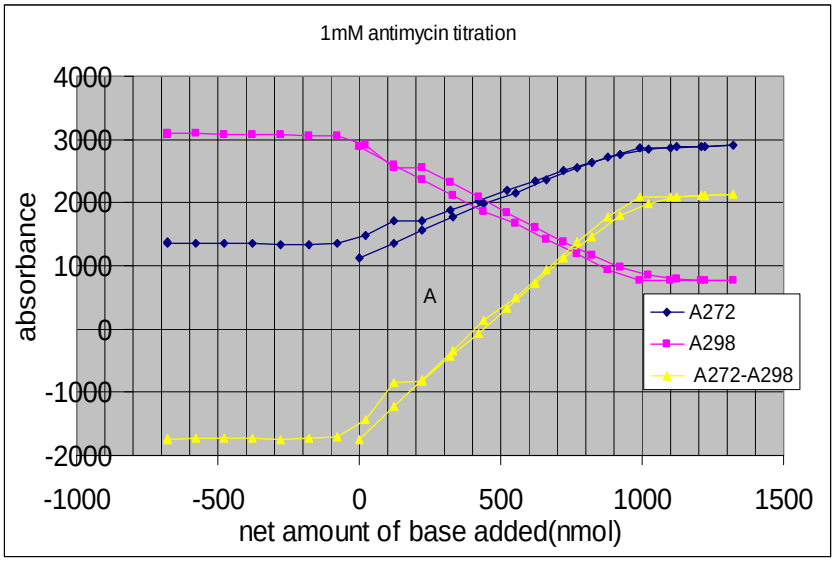


Figure 9 Acidometric titration of 1 mM antimycin. A similar titration was done on a higher

concentration of antimycin solution (figure 8). Fourteen times of two ul of 50mM NaOH was added to get antimycinate (orange curve); then, 19 times of 2 uL of 50mM HCl was adde until no futher change in the spectra (light green curve). A wavelength pair was chosen and its corrsponded absorbance gave the plot 2, the double difference extinction coefficient could be calculated by the slope, which was 4 after titrating the 1mM antimycin with 50mM NaOH and 50mM HCl.

3b. Antimycin titration 1mM with 5 mm pathlength.

This experiement is similar to the previous except 5mm pathlenth cuvet was used to prevent spectra from going off scale. Also, instead of making multiple additions of NaOH to the same sample of antimycin, separate antimycin solutions were made for each titration point with

different amount of NaOH. It can be seen in the figure 10 that the spectra do not exceed 3 unit, except for the basic form in the range around 340nm to 380nm. Therefore, we were able to measure absorbance change at peak-trough of the difference spectrum (figure 4). Specifically the wavelength pair at 270-308nm was used. Absorbance of this wavelength pair was plotted against the amount of NaOH added in figure 11 and figure 12, which show two different ways to analyse the data, depending on the assumption, we make as to the cause of the dissociation of antimycin at the starting point, described under discussion. Depending on which method was used, the extinction coefficient at wavelength pair were 4.462 and 4.257, which could be converted by using the standard spectrum of figure 4 to give the extinction coefficients of 5.36 and 5.11 at 320nm. The correct extinction coefficient probably lies between this range.

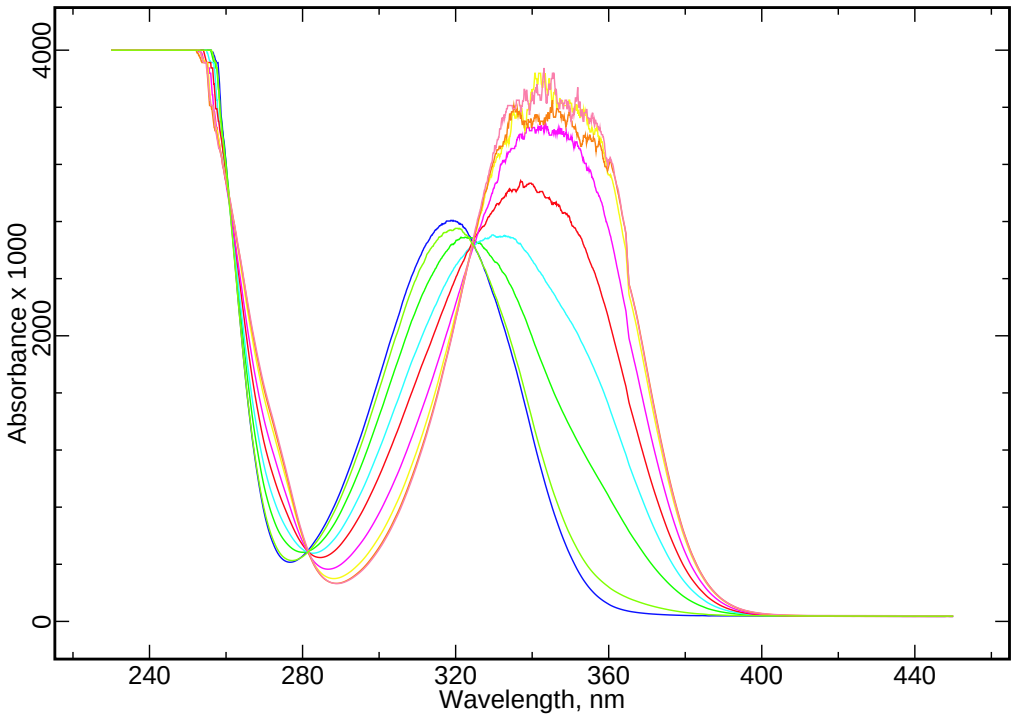
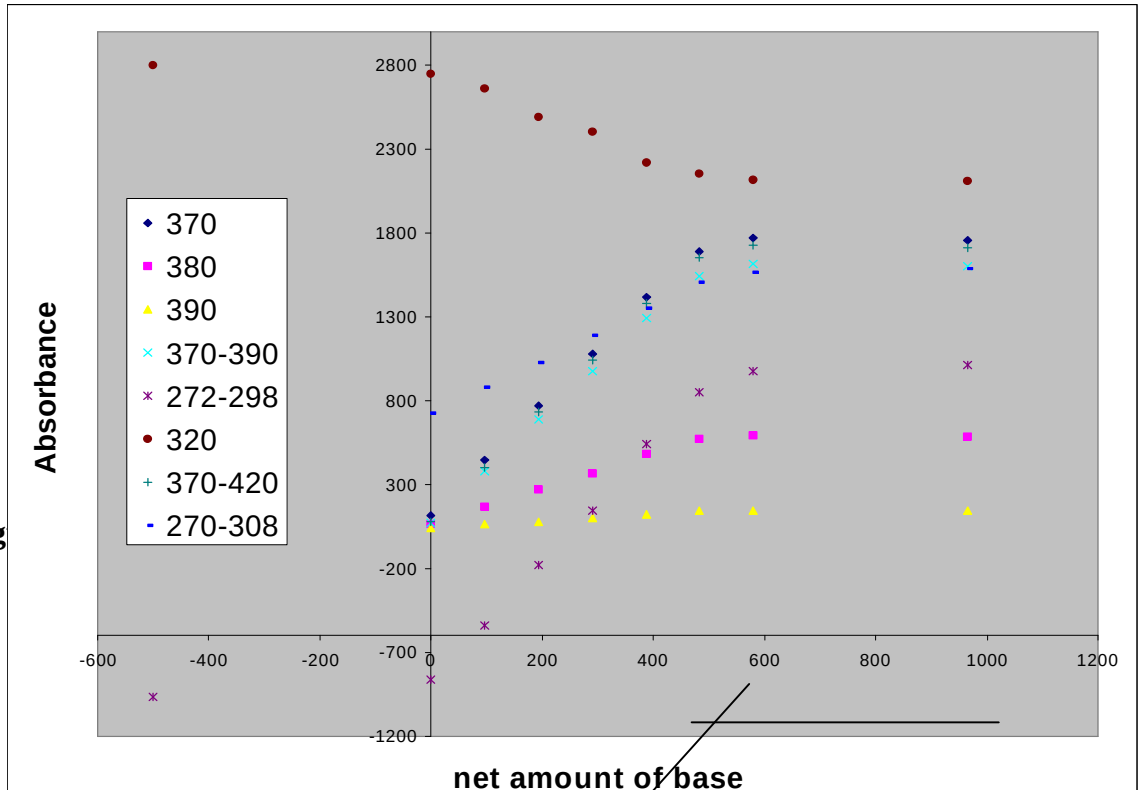


Figure 10. Spectra of 1mM antimycin (5mm pathlength cuvet)



Fig

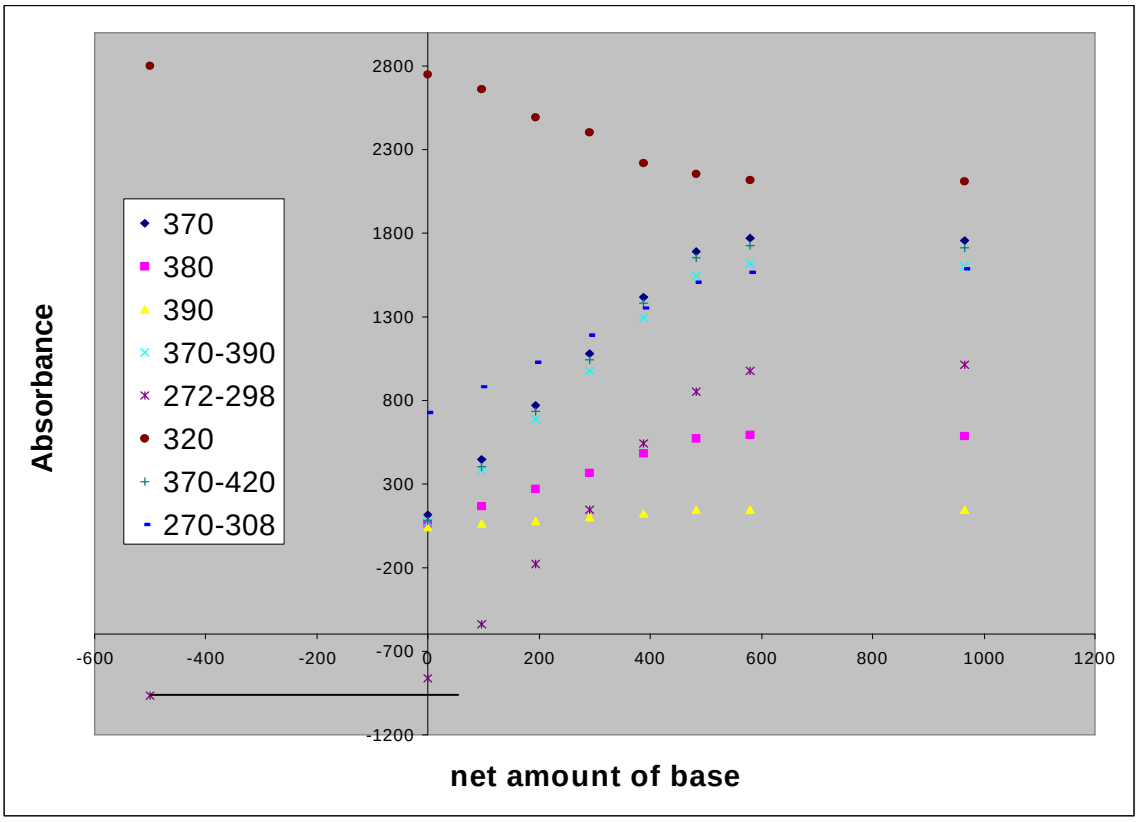


Figure 12. Absorbance of 1mM antimycin vs. net amount of base (analysis 2)

Discussion and Conclusion

The recommended way to determine the concentration of antimycin solutions is by using the extinction coefficient (ref 3). Antimycin titration is one of two methods for determining the concentration of cytochrome bc1. Clearly the results obtained will depend on the value used for the antimycin extinction coefficient. Therefore we want to determine an accurate value for this

number

The results from the titration of Figure 5, using the wavelength pair 272-298, gave a double difference extinction coefficient of 4.0-1. Using this to determine concentration of antimycin in our standard spectrum leads to an extinction coefficient for the protonated form of 5.47 mM-1. In principle the accuracy of our determination is limited by the accuracy of estimation of the slope or endpoints and, ultimately, by the accuracy of the dilutions and titrations relating the NaOH added to the cuvet to the standard HCl solution. The accuracy with which the antimycin solution is prepared and diluted is irrelevant, as we are determining both the number of moles and absorbance change from the same sample in the cuvet.

Literature values for the extinction coefficient of antimycin at the 320nm peak are 4.8 (ref 5) and 6.0 (ref 6). Our values lies between these values. It is significantly larger than the accepted

value of 4.8 mM-1, which may explain a discrepancy in the extinction coefficient of cyt b measured by antimycin binding compared to pyridine hemochrome analysis.

There were clear isobestic points shown on the spectra, showing a single ionization reaction occurs. These isobestic points would be good for measuring concentration of antimycin without the need for acidification. In order to obtain the extinction coefficient of antimycin from the titration curves, we need to understand why antimycin is already partly dissociated when diluted into ethanol. If this is because antimycin obtained fro sigma is already partly in the basic form, or because the ethanol contains traces of base or silicate groups on the cuvet are in the basic form, the we cannot take the starting point as the acid endpoint of the titration. In this case when we draw the line for determining the slope we should force the line to go through the initial point, even though the spectrum is not in the fully acid form. Extrapolating from here down to where the line meets

the absorbance of the fully protonated form gives the true acid endpoint.

The other explanation for the partly ionized spectrum at the initial point is that antimycin is a sufficiently stron acid to dissociate significantly in Ethanol. In this case the starting solution contains H+ equal in amount to [A-], and the net added base is zero at the starting point. In this case we have a rounded curve that misses the acid endpoint, so we should not force the line through the initial point but rather force it through an acid enpoint taken at zero added base but with the absorbance of the fully scidified form.The two possibilities can be distinguished by examinng the shape of the curve in detail around the acid endpoint. If antimycin dissociation is neglible but ther is some base already present in the starting point, then the titratio curve should be linear and follow the extrapolated line back to a sharp endpoint at the true acid endpoint. If the initial point is the true endpoint but antimycin is significantly dissociated, then the line

should curve back and gradually approach the fully protonated form only when considerable excess acid is added. This will be looked at in future experiments.

It was mentioned in the introduction that measuring the extinction coefficient of cytochrome b based on antimycin titre gave a different result than heme analysis. Specifically, Berden & Slater (ref. 7) found extinction coefficients for cytochrome b of 25.6 based on antimycin titre and 28.0 based on heme content. They measured their antimycin concentration using the extinction coefficient of 4.8 at 320 nm reported by Strong et al. If in fact the extinction coefficient of antimycin is greater than that, then they overestimated the concentration of their antimycin, therefor overestimated the amount of bc1 complex and underestimated the extinction coefficient. If the correct extinction coefficient for antimycin is 5.11 mM-1, we can recalculate the extinction coefficient of cyt b as 27.2 mM-1. Likewise an extinction coefficient of 5.36 for antimycin would

give 28.6 mM-1 for cytochrome b. These values are in much better agreement with the value based on heme content (28.0) than the value based on the antimycin extinction coefficient of 4.8 is.

Antimycin dissociates significantly in EtOH or DMSO, resulting in curved endpoints and reducing the accuracy. Aprotic solvents dioxane and tetrahydrofuran prevent dissociation, but we got no reaction with NaOH in these solvents.

Appendix 1. Simulating acid/base titration curves using Nightingale's equations:

We have three relationships:

1. From the definition of Ka:

$[A^-] = Ka[AH]/[H^+]$

if Total anti = C = AH + A-,

[A-] = C Ka/(Ka+[H+])

2. From the definition of Kw = [H+][OH-]

[OH-] = Kw/[H+]

3. Chemical balance: the only reaction consuming or producing protons

are the above dissociation of antimycin and water, and they produce

or consume one A- or one OH- for every H+ produced or concumed.

In the solvent to start with OH- = H+ if we assume neutral solution.

So if we add no NaOH then

$H^+ = OH^- + A^-$

This is what Nightingale calls the electroneutrlity requirement.

If we add some NaOH, $Na^+ = OH$, so we have:

$H^+ + Na = OH^- + A^-$

This is difficult to solve for H^+ given Ka, Kw, C, and Na

But if we take a given H^+ and Ka,Kw,C;

then we can solve for A^- and OH^-

Given H^+, A^-, and OH^-, we can solve for the amount of NaOH

we would have to add to reach this point by electroneutrality.

A- is the extent of the titration, and Na is the total base added,

so repeating this at different H+ and plotting A- vs Na gives

the theoretical titration curves.

Appendix 2. Validation tests of UV-visible spectrophotometer UV-1601 Shimadzu

Table A2. Manufacture and Specification of spectrophotometer UV-1601 Shimadzu

Measurement Wavelength Range	190-1100nm
Spectral Band Width (Resolution)	2nm
Wavelength Display	0.1nm units
Wavelength Setting	0.1nm units (1nm units in scan)
Wavelength Accuracy	±0.5nm On-board automatic wavelength calibration mechanism
Wavelength Repeatability	±0.1nm
Wavelength Scanning Speeds	GOTO WL command: approx. 6000nm/min Very Fast: approx. 3200nm/min Fast: approx. 2200nm/min Medium: approx. 370nm/min Slow: approx. 260nm/min Very Slow: approx. 160nm/min
Stray Light	Less than 0.05%
Photometric System	Double beam optics
Photometric Range	Absorbance: -0.5~3.000Abs (when uncorrected baseline curve is within 0.5 Abs) Transmittance: 0~300%

Recording Range	Absorbance: -3.99~3.99 Abs Transmittance : -399~399%
Photometric Accuracy	±0.004Abs (at 1.0Abs) ±0.002Abs (at 0.5Abs)
Photometric repeatability	±0.002Abs (at 1.0Abs) ±0.001Abs (at 0.5Abs)
Auto Zero Function	[AUTO ZERO] key enables one-touch setting
Baseline Stability	±0.001Abs/h
Baseline Flatness	±0.002Abs
Noise Level	0.002Abs

Linearity Check of spectrophotometer UV-1601 Shimadzu

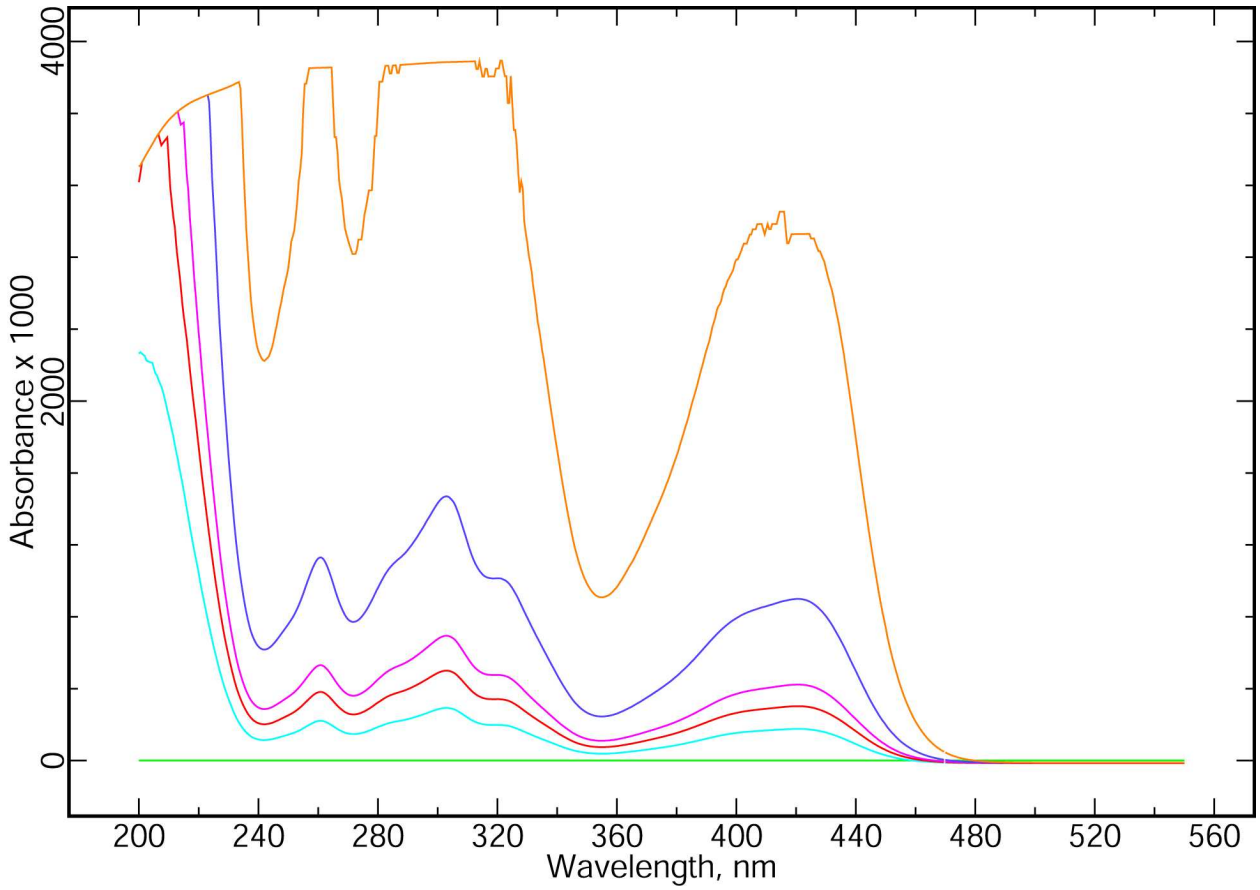


Figure A2a. Spectra of different concentration of FiCN

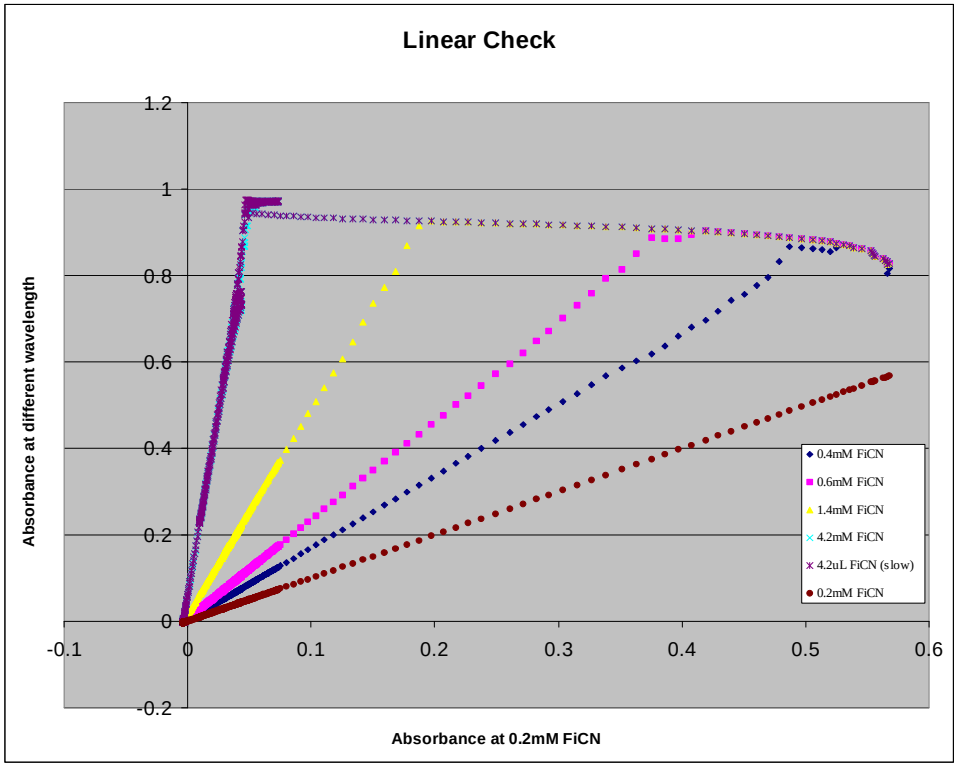


Figure A2b. Each of the spectrum of Figure A2a was plotted against the absorbance of the lowest concentration (cyan curve in Figure A2a) to determine linear range. At the highest concentration, the spectra was scanned twice, once at a lower speed.

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