Molar extinction coefficient of Antimycin A: an acidometric determination

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Abstract:
Antimycin A is an antibiotic which binds to and inhibits cytochrome bc1, a respiratory complex of the mitochondrial electron transfer chain(1). It has been used extensively in studying the mechanism of cytochrome bc1. Because antimycin is a mixture of slightly different compounds, and because it is used in very small amount, it is convenient to determine the concentration of solutions by their absorbance. To do this, it is necessary to know the extinction coefficient of the antimycin A. There are two values in the literature, both determined by organic chemists working out the structure of antimycin

Strong et al. (2) 320 mu log e 3.68 => 4.79 mM^{-1}.

Birch et al. (3) 319 mu log e 3.78 => 6.03 mM^{-1}.

Because antimycin binds stoichiometrically and with extremely high affinity to the bc1 complex, antimycin titration can be used to determine the concentration of bc1 complex. Therefore, it is very important to have an accurate value of the extinction coefficient of antimycin. The phenolic OH group of antimycin dissociates to give antimycinate, with a distinctively different spectrum. Titration of antimycin with sodium hydroxide thus provides a means to determine the extinction coefficient. In this paper, we report a more accurate extinction coefficient based on acidometric titration: 11.57 ± 0.27 $mM^{-1}cm^{-1}$ at 351.8 – 308.2 nm, basic minus acidic; and 5.45 $mM^{-1}cm^{-1}$ at 320 nm in the acidic form. The values based on gravimetric concentration (<MW> =548) were not significantly different.

1. BACKGROUND

(Abstract)

Dual wavelengths and difference extinction coefficient. The extinction coefficient of a compound at a wavelength is the proportionality constant ε of Beer's Law:

$$A = \varepsilon \times l \times C$$

Where A is the absorbance (-log of fraction of light transmitted), l is the path length, and C is the concentration. That is the absorbance is proportional to path length and concentration. So if you know the absorbance and the extinction coefficient and path length, you can calculate the concentration of the compound by:

$$C = A / (\varepsilon \times l)$$

Or if you plot the absorbance against concentration (times path length), the slope is the extinction coefficient.

Often it is convenient to measure the difference in absorbance at two wavelengths. In this case, any interference due to baseline drift will cancel out because it is the same at both wavelengths. We can define a **dual wavelength extinction coefficient Δε** as:

$$A_{\lambda 2} = \varepsilon_{\lambda 2} \times l \times C$$
$$\underline{A_{\lambda 1} = \varepsilon_{\lambda 1} \times l \times C}$$
$$\Delta A_{2\text{-}1} = \Delta\varepsilon \times l \times C$$

From which we can see $\Delta\varepsilon = \varepsilon_{\lambda 2} - \varepsilon_{\lambda 1}$

If a reaction converts one equivalent of X into one equivalent of Y, then the change in concentration of X is equal to the negative of the change in concentration of Y ($\Delta C_Y = -\Delta C_X = \Delta C$).
Therefore, the change in absorbance per mole of reaction will be equal to the increase in absorbance due to generation of ΔC of Y

$\Delta A_Y = \varepsilon_Y \times l \times \Delta C$ plus the change due to removal of X:
$\Delta A_X = \varepsilon_X \times l \times -\Delta C$ so the total change is :
$\Delta A = \varepsilon_Y - \varepsilon_X \times l \times \Delta C$.

We define ($\varepsilon_Y - \varepsilon_X$) as the difference extinction coefficient for the reaction, Δε.

$$\Delta A = \Delta\varepsilon_{(Y - X)} \times l \times \Delta C.$$

If the reaction is monitored at two wavelengths, then we have a dual wavelengths difference extinction coefficients for the reaction:

$$\Delta\Delta\varepsilon_{(y\text{-}x,\lambda 1\text{-}\lambda 2)} = (\varepsilon_{y,\lambda 1} - \varepsilon_{x,\lambda 1}) - (\varepsilon_{y,\lambda 2} - \varepsilon_{x,\lambda 2})$$
$$\Delta A = \Delta\Delta\varepsilon_{(y\text{-}x,\lambda 1\text{-}\lambda 2)} \times l \times \Delta C.$$

2. METHODS

The obvious method to determine the extinction coefficient of a compound is to weigh out a certain amount and dissolve in a certain volume to give a known concentration, then measure the absorbance and calculate extinction coefficient by Beer's Law. In case of antimycin this has some disadvantages. For one thing, the compound is available in small amount of unknown purity. Therefore, in accuracy in weighing the sample and uncertainty about the purity leads to uncertainty of the concentration and therefore, uncertainty in calculated extinction coefficient. Also, antimycin is a mixture of closely related compounds that differ in the alkyl substituent. Since this group is isolated from the aromatic chromophore, all these compounds should have the same molar extinction coefficient, but different molecular weight. What we want is the molar extinction coefficient, so if there is a way to determine it directly without reference to the molecular weight, that will be better.

The phenolic OH group has a pKa in the neutral range, and the spectrum changes when this group ionizes. Therefore, by titrating antimycin by sodium hydroxide we can determine the difference extinction coefficient for ionization of antimycin. This is the method we used, in addition to calculating from the concentration based on the weight. In the process of measuring the spectra of antimycin in the acid and basic forms, it was observed that antimycin was unstable (spectrum was changing) in 100% ethanol in the

presence of HCl. It was much more stable with HCl in 95% ethanol. Therefore, we used 95% ethanol for the experiments.
In order to avoid working with extremely dilute solutions, we used a 0.2 mm path-length cuvet which allowed use of antimycin concentrations around 1 mM without exceeding the linear range of the spectrophotometer.

2@. Stock antimycin solution (090917)
accurate preparation of mM antimycin
50mg Sigma bottle of antimycin
50 mg/548 = .0912 mmol.
For 20 mM, 91.2 umol/(20 uMol/mL)=4.562 ml.
ignore vol of antimycin
Wipe bottle with kimwipe and weigh - 15.0241g
add 3.590g (=4.562ml) EtOH, actual - 3.5905g
total - 18.61g

divide into eppendorf tubes, 1ml each, labe 20 mM antimycin 090917

weigh empty - 14.9917g
later - 14.9703g (21 mg less!)
gross 15.0241 - tare 14.9703 = 53.8 mg (but was final weight stable?)

3.590 g absolute EtOH was added to a bottle nominally containing 50 mg of antimicin to give a 20 mM solution assuming the nominal weight, density of EtOH=?, and using the molecular weight of the predominant form (548 Da).

2a. Spectra of antimycin in the fully protonated and fully ionized states.

2b. Sodium hydroxide titration of antimycin in 95% ethanol.
In order to avoid accumulation of error each point of the titration was prepared separately with a single addition of the correct amount of sodium hydroxide. A dilute solution of antimycin was prepared and divided into 1 ml aliquots. Different amounts of aqueous NaOH or HCl were added to the different aliquots, together with enough water to make the total volume and solvent composition the same in each case. Because of difficulty pipetting ethanolic solutions, the dilutions were based on weight.

After weighing a dry plastic 15 ml conical tube, .33 ml of the 20 mM antimycin (from a solution made on 9/17/09) was added then weighed. Then 10 mL of 95% ethanol was added and the mixture was weighed again. From these three weights and the concentration of the stock solution, the concentration of the dilute antimycin solution was calculated.

Ten eppendorf tubes were labeled one through ten and each was weighed. Using a 1mL volumetric pipette, approximately 1 ml of the mixture was distributed to each of the tubes and each was weighed again. In each of the eppendorf tube, the calculated amount of water was added consecutively. Just before taking each spectrum, 0.1 M NaOH (0 – 10 uL) or 2 uL 0.1 M HCl was added, the solution was mixed and transfered to a 0.2 mM

path length cuvet. In between each of the solutions, the cuvet was drained, washed, and dried. A Hamilton syringe was used to add the water, NaOH and HCl in the mixtures. The syringe was cleaned with ethanol after each use. The spectrum obtained with 10 uL NaOH and 2 uL HCl were of the fully ionized and fully protonated sample, respectively, as shown by no further change on adding more NaOH or HCl.

For each tube the absorbance difference at 351.8, 308.2 (peak minus trough of the difference spectrum) was plotted (Fig.1) against product of path length and concentration of the sodium hydroxide added. The NaOH concentration was calculated based on the volume of standard 0.1 M NaOH added and the volume of antimycin solution, which in turn was calculated from the mass divided by density(0.707). Points lying on a straight line were selected by examination and the slope was determined by linear regression (MS Excel). The difference between the fully basic and fully acidic spectra (total change) was measured at the same wavelengths pair and also at 319 and 320 nm, and the ratio of absorbance of the latter two to the absorbance at 351.8, 308.2 was calculated. These ratios were used to calculate the extinction coefficients at the latter two wavelengths. In addition, the total change at these three wavelengths were divided by the concentration of antimycin to give an alternate estimate of the extinction coefficients at these three wavelengths. For this purpose, concentration of antimycin was calculated based on concentration of stock solution and the gravimetrically determined dilution factor.

3. RESULTS

A. Spectra of antimycin in the fully protonated and fully ionized states, and difference spectrum for ionization of antimycin. As described in methods, in each experiment we took spectra of antimycin in the presence of excess HCl and NaOH. Figure 2 shows typical results as well as the difference spectrum, fully ionized minus protonated. The protonated form of the antimycin has an absorbance maximum at 318.4. The ionized form has a maximum at 347.0. The difference spectrum has maximum at 351.8 and minimum at 308.2 and isobestic at 261.5 and 280.7. Table 1 lists the absorbance differences as selected wavelengths and the extinction coefficient at these wavelengths based on the calculated concentration of antimycin in the experiment.

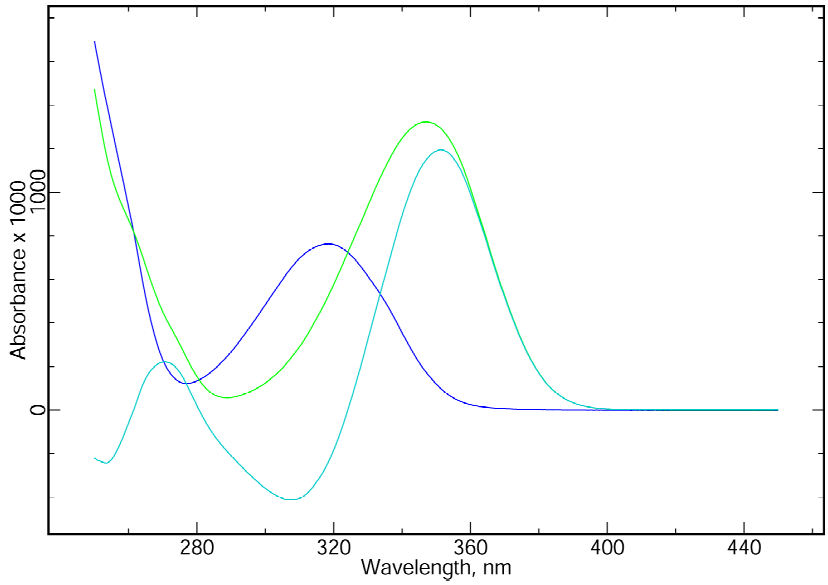

Figure 2. Spectra of antimycin in 95% ethanol Blue-fully protonated form. Green-fully ionized form. Cyan-difference spectrum of ionization of antimycin. Antimycin concentration was 0.658 mM.

Table 1. Absorbance of fully protonated and ionized antimycin from a typical experiment, and the extinction coefficient calculated from the concentration based on weight.

Wavelength	248.6	253.4	261.5	270.5	280.7	307.5	318.4	347.0	351.3	351.8	351.3 -307.5	351.8 -308.2
Max/min/iso												
acid form	1.83	1.42	0.81	0.22	0.14	0.65	0.76^{M}	0.17	0.10	0.09	-0.55	-0.57
basic form	1.62	1.18	0.81	0.44	0.14	0.24	0.52	1.32^{M}	1.30	1.29	1.05	1.03
difference	-0.21^{M}	-0.24^{m}	0.00^{i}	0.22^{M}	0.00^{i}	-0.41^{m}	-0.24	1.15	1.20	1.19^{M}	1.61	1.61
Extinction coefficients:												
acid form	13.90	10.82	6.17	1.65	1.06	4.96	5.80	1.32	0.76	0.69	-4.20	-4.36
basic form	12.30	8.97	6.19	3.33	1.03	1.83	3.97	10.06	9.85	9.78	8.02	7.85
difference	-1.60	-1.85	0.02	1.68	-0.03	-3.13	-1.83	8.73	9.09	9.08	12.22	12.21

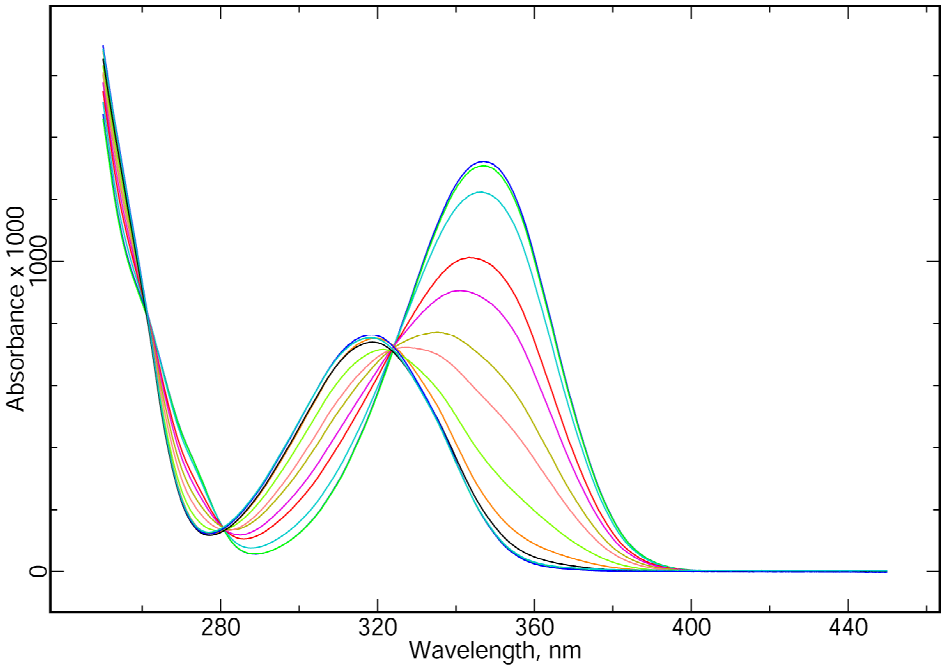

Figure 3. Spectra of antimycin from a sodium hydroxide titration. Spectra at each point in the titration from the experiment of Figure 2.

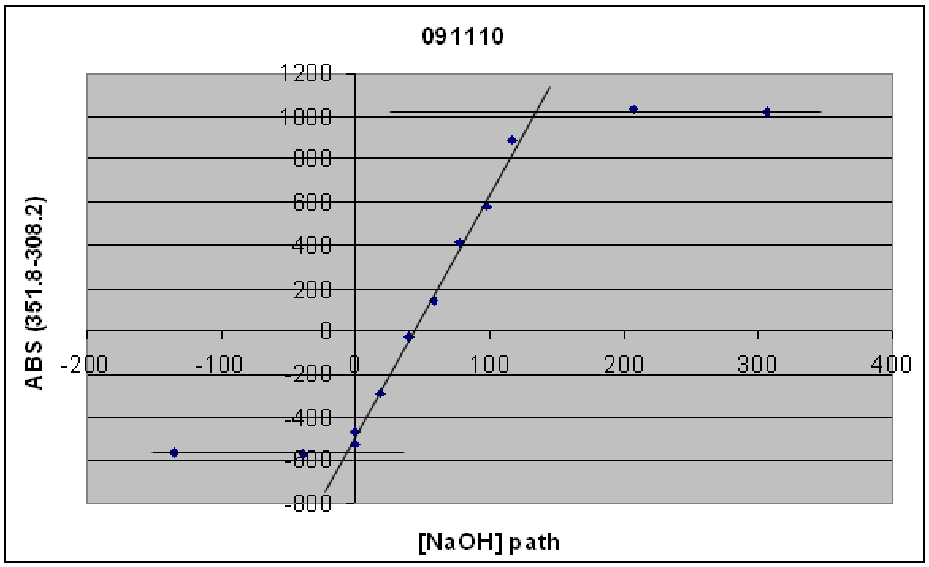

Figure 4. Plotting absorbance vs. concentration to determine the extinction coefficient the best fit line has a slope of 11.48 which is the best estimate of the extinction coefficient from this experiment.

Table 2. Results from four experiments like that of Table 1. Values under "based on slope" are based on the slope of the titration from linear regression. In this case, the value for 319 and 320 are taken from these slopes at 351.8-308.2 and multiplied by a factor determined from the acid and basic absorbances. Values under "based on total concentration" are based on the fully acid and basic absorbances and the concentration calculated by weight.

						based on slope					based on total concentration			
Date	**[anti]**	**(351.8,308.2)A, difference**	**acid A_{319}**	**acid A_{320}**	**base $A_{351.2}$**	**LR slope basic-acid (=$E_{351.8-308.2}$)**	**factor 319**	**factor 320**	**acid E_{319}**	**acid E_{320}**	**basic-acid $E_{351.8-308.2}$**	**acid E_{319}**	**acid E_{320}**	**base $E_{351.2}$**
91008	0.7	1570.3	736.2	734.3	1271.2	11.6	0.47	0.47	5.33	5.32	11.94	5.60	5.58	9.67
91016	0.7	1599.0	747.8	744.9	1290.6	11.9	0.47	0.47	5.59	5.56	12.12	5.67	5.65	9.78
91023	0.7	1385.7	637.2	635.6	1119.0	11.3	0.46	0.46	5.22	5.21	10.55	4.85	4.84	8.52
91110	0.7	1606.2	764.7	762.1	1295.0	11.5	0.48	0.47	5.74	5.72	12.21	5.81	5.80	9.85
average						11.57	0.47	0.47	5.47	5.45	11.71	5.48	5.47	9.45
s.d.						0.2693	0.007	0.006	0.235	0.23	0.781	0.432	0.428	0.630

*In fact [anti] was based on volume dilution (.33 ml to 10 ml)

4. DISCUSSION

From this work we obtained the value for the double difference extinction coefficient 351.8-308.2, basic-acid table value of 11.57 ± 0.27 $mM^{-1}cm^{-1}$. The corresponding value based on a molecular weight of 548 was 11.71 ± 0.78 $mM^{-1}cm^{-1}$, not significantly different, suggesting that the sample was pure. Using this extinction coefficient based on the slope, the extinction coefficient of the acid form is 5.47+0.13 and 5.45+0.13 at 319 and 320 nm, respectively. These values fall in between the values of Strong et al. (4.79) and Birch et al. (6.03). The extinction coefficient for the basic form at 347 nm was 9.63 ± 0.29 $mM^{-1}cm^{-1}$.

The value based on MW is based on the nominal content of 50 mg. Actual measured weight was 53.8 mg which would give double diff ext of 10.88. However this is weighing 15 g bottle after long drying period, may be not that accurate. Or antimycin may not be 100% pure, 50 mg took that into account. Also the ave MW may be quite diff from 548. Remember Deisenhoffer surprise.

On the other hand if the extinction coefficient at 320 is 4.79, gives 10.19 diff, then the 20 mM was really 11.71/10.19 * 20 mM => 115% pure assuming 50 mg, that*50/53.8 = 107% if 53.8 mg.

Difficulties in performing acidometric titrations with very dilute solutions may prevent us from getting better than 2-3% accuracy. Perhaps redox titration of the phenol group, or determination by chromate or Folin-Ciocealtao reaction may be more accurate. Or obtain highly purified antimycin A1 or A2 (single isoform) and determine gravimetrically.

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3. A. J. Birch, D. W. Cameron, Y. Harada and R. W. Rickards (1961) 187. The structure of the antimycin-a complex. J. Chem. Soc. 889 – 895.

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