Analysis of antimycin A by reversed-phase liquid chromatography/nuclear magnetic resonance spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 1989

Impact Factor: 5.64 · DOI: 10.1021/ac00180a005 · Source: PubMed

3 AUTHORS, INCLUDING:

Charles L Wilkins
University of Arkansas
289 PUBLICATIONS **6,825** CITATIONS

Analysis of Antimycin A by Reversed-Phase Liquid Chromatography/Nuclear Magnetic Resonance Spectrometry

Steven T. K. Ha and Charles L. Wilkins*

Department of Chemistry, University of California, Riverside, Riverside, California 92521

Sharon L. Abidi

National Fisheries Research Center, United States Department of Interior, P.O. Box 818, La Crosse, Wisconsin 54602

A mixture of closely related streptomyces fermentation products, antimycin A, is separated, and the components are identified by using reversed-phase high-performance liquid chromatography with directly linked 400-MHz proton nuclear magnetic resonance detection. Analyses of mixtures of three amino acids, alanine, glycine, and valine, are used to determine optimal measurement conditions. Sensitivity increases of as much as a factor of 3 are achieved, at the expense of some loss in chromatographic resolution, by use of an 80-μL NMR cell, instead of a smaller 14-μL cell. Analysis of the antimycin A mixture, using the optimal analytical high-performance liquid chromatography/nuclear magnetic resonance conditions, reveals it to consist of at least 10 closely related components.

A direct-linked stopped-flow high-performance liquid chromatography/nuclear magnetic resonance (HPLC/NMR) system was first demonstrated for mixture analysis by Watanabe and Niki in 1976 (*1*). Subsequently, direct linkage of HPLC flow systems with proton nuclear magnetic resonance spectrometers has been a subject of active research in a number of laboratories (*2–11*). Such an on-line technique provides both chromatographic separation and spectral information simultaneously and can be quite valuable, provided the sensitivity limitations of NMR are tolerable. Fortunately, more sensitive NMR spectrometers have become available during the period since Watanabe's first experiments and current state-of-the-art sample requirements are such that tens to hundreds of microgram sample quantities are adequate for present reversed-phase proton HPLC/NMR detection (*8*). Because residence time within the NMR observation region is a key parameter determining sensitivity, the volume of the region, relative to sample elution volume, is important. Chromatographic constraints must also be considered, as band broadening due to long retention times can decrease the quantity of sample available for NMR detection with good signal-to-noise (S/N) ratio during a predetermined detection period. For chromatographic reconstruction purposes it is desirable, although not mandatory, to use constant time increments between spectral data acquisitions. In previous reports of work using a 300-MHz spectrometer (*6–8*), detection of as little as 50 μg of caffeine, naphthalene, and heptane was achieved by using 20 and 28 μL observe volumes, with limits of detection estimated at 10 μg. However, most reversed-phase HPLC/NMR separations require 300–500 μg of sample for practical analytical purposes.

Several means for improvement of sensitivity exist. Obviously, employing a higher field NMR spectrometer, as used here (400 MHz), can provide some gain, although at relatively great expense. Optimization of probe design by using a microcell probe could also yield better sensitivity if an optimized receiver coil were oriented perpendicular, rather than parallel, to the magnetic field (*12*). An approximate improvement of a factor of 3 in sensitivity might be achieved in this manner. Another alternative, provided the chromatographic performance is not too seriously compromised, is to use a larger observe region (80 μL, here) compatible with standard commercial probe designs. A disadvantage of this approach is the greater demand on digitization dynamic range imposed by the proportional increase in solvent resonance intensities, relative to analytes. However, current NMR spectrometers, such as that used in the present work, are now available with 15-bit analog to digital converters (ADC), which have a factor of 8 better dynamic range than the 12-bit ADC used in the earlier work.

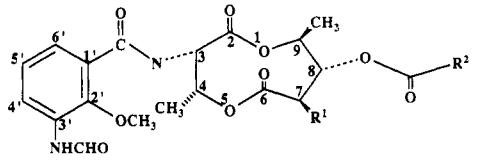
Here, a group of related antibiotics, known collectively as antimycin A, produced from streptomyces by fermentation are investigated. These compounds have long been considered to be a group of very closely related compounds, labeled as A_1, A_2, etc., differing only in the nature of the substituents at the 7- and 8-positions of the dilactone ring (*13–15*) (Figure 1). Recent 2D-NMR experiments established that antimycine A_1 was a mixture of two components and that the previously assigned structure was actually that of the minor component, A_{1b} (*16*). Subsequently, this material was successfully resolved into two components by high efficiency HPLC (*17*). It was inferred from those studies that each of the previously identified antimycins is, in fact, a mixture of two components. Due to the labile nature of these compounds, their isolation is difficult. Thus, on-line HPLC/NMR is an ideal tool for their identification.

For the present analyses of the antimycin A mixture, it was first necessary to investigate the effects of detector volume and sample size on analytical performance, prior to carrying out the actual separation and spectral measurements of the target components. Accordingly, mixtures of alanine, glycine, and valine were used for that purpose.

EXPERIMENTAL SECTION

Instrumentation. HPLC/NMR instrumentation similar to that employed here has been described previously (*6*). For the present work a Varian Model 5060 HPLC pump was connected by transfer lines to either an Alltech 10-μm particle size or a Hibar Lichrob 3 μm particle size 25 cm × 4.6 mm C18 bonded phase column, which was placed within the superconducting solenoid magnet bore of a Varian 400-MHz XL-400 widebore NMR instrument. A standard 5-mm ^1H–^{19}F NMR probe was used for all spectral measurements. The HPLC pump was placed about 3 m from the magnet and connected to the column by using a stainless steel transfer line $^1/_{16}$ in. (o.d) × 0.005 in. (i.d.). The observation cell was constructed, as previously, from capillary tubing (obtained from Wilmad Co.) positioned in the center of the standard probe and connected to the column outlet by a transfer line with a volume of approximately 30 μL. Observe cell volumes of either 14 or 80 μL were used. Separations were carried out with degassed water, deuterium oxide, and methanol-d_1.

0003-2700/89/0361-0404$01.50/0



Antimycin		A_{-a}	A_{-b}
	R^1	R^2	
A_1			
A_2			
A_3			
A_4			
A_5			

Figure 1. Molecular structures for antimycin A. Structures are given for both major (a) and minor (b) components.

NMR Parameters. For the antimycin separations, HPLC/NMR data were collected by using either four (for antimycins A_3, A_4, and A_5) or eight (for antimycins A_1 and A_2) coadded spectral scans for each chromatographic data point. Total data acquisition time for the each free induction decay was 400 ms, with 3200 data points collected. Fourier transformation was done after augmenting the spectral data by zeros to a total of either 8192 or 16 384 data points covering a spectral width of 4000 Hz. With computer data transfer time of about 1.8 s and a pulse delay of 0.5 s, chromatographic time resolution of either 5.4 or 9 s per data point was obtained. A 45° observe pulse was used in all experiments (for the 1-1 hard pulse experiments, the total pulse width was chosen to correspond to 45°). Standard Varian software functions including line broadening, convolution difference, and autophasing were used to process the experimental data.

Solvent Suppression Techniques. Because reversed-phase HPLC separations normally employ protonated solvents, it is difficult to detect small amounts of analytes by proton NMR under the separation conditions. 1-1 hard pulse solvent suppression (1-1HP) has been used successfully for reversed-phase HPLC/NMR with protonated solvents (*7*, *8*, *18*). As indicated previously, the binary solvent mixture, methanol and water, which is used here, displays three distinct solvent resonances when methanol is the major component, posing difficulties for solvent suppression (*8*). One means of solving this problem is to add 0.5 M ammonium acetate, enhancing the methanol hydroxyl–water exchange rate and reducing the number of solvent resonances to two, which can be effectively suppressed by the 1-1HP method. Unfortunately, such a high concentration of salt is incompatible with the chromatographic requirements of the present mixture separation. Accordingly, deuterium oxide and methanol-d_1 were used as solvents in the present work, with 1-1HP used only to suppress methyl protons. Alternatively, 0.005 M potassium hydrogen phosphate can be added with the same results as 0.5 M ammonium acetate. In that case, completely protonated solvents can be used and effective separation is not precluded. Under those conditions, if a 75/25 methanol/water solvent were used, it would be possible to suppress the water resonance simultaneously with the methyl resonance.

Antimycin A. Samples of antimycin A mixtures were prepared from the crude antibiotics obtained from the Aquabiotics Corp. (North Brook, IL), according to procedures described elsewhere (*15*). The sample was a mixture of at least ten antimycin derivatives. It was methylated by treating a solution of the sample in diethyl ether with an ether solution of diazomethane and stirring overnight. Pure samples were obtained from crude materials by thin-layer chromatography, using the detailed procedure described previously (*15*). For analysis, the antimycin mixture was dissolved in methanol-*d* and 50-μL samples were injected for HPLC analysis. Chromatography employed a C18 bonded phase column with a mobile phase comprised of a 75:25 mixture of methanol-*d* and deuterium oxide, using a flow rate of 1 mL/min. The approximate amount of each component was between 200 and 600 μg.

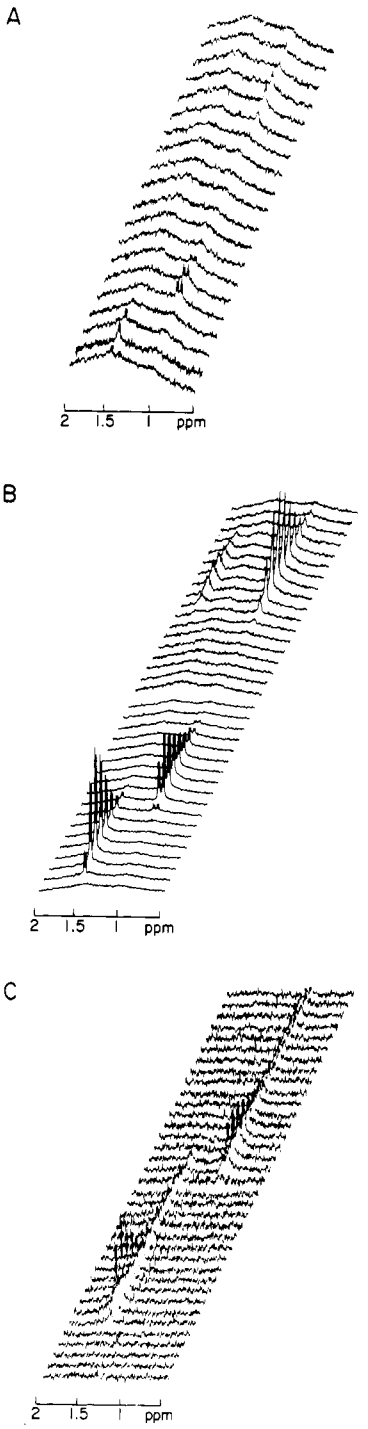


Figure 2. Stacked plots of partial ^{1}H NMR spectra (aliphatic region) for separations of mixtures of alanine, glycine, and valine: (a) 30 μg of each, 80 μL observe volume; (b) 300 μg of each, 80 μL observe volume; (c) 400 μg of each, 14 μL observe volume. Chromatographic conditions are as follows: column, Alltech 10 μm C18 bonded phase; mobile phase water; flow rate, 1 mL/min.

RESULTS AND DISCUSSION

HPLC/NMR Parameters. It has been shown in previous papers that although flow NMR sensitivity depends on a large number of factors, some related to sample properties and some to the parameters of the flow NMR system, it is possible to define experimental protocols adequate for its use as an HPLC detector (*7–9*). Detection of HPLC eluents at the several hundred microgram level for either reversed-phase or normal-phase separations, without serious degradation of chromatographic resolution, is easily accomplished by using the

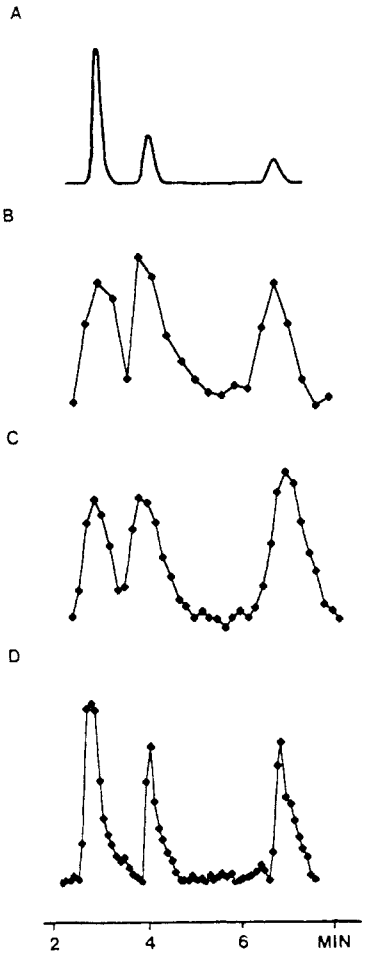


Figure 3. Reconstructed chromatograms for separations of alanine, glycine, and valine: (a) ultraviolet detector; (b) using data from separation plotted in Figure 2a; (c) using data from separation plotted in Figure 2b; (d) using data from separation plotted in Figure 2c.

"in-bore" column placement method introduced several years ago (*7*). As is well-known, it is critical to obtain a sufficient number of chromatographic data points if peaks in reconstructed chromatograms are to be defined; ideally, a minimum of five to seven such points per chromatographic peak are necessary. Time resolution is a function of spectral resolution (i.e. data acquisition time, t_a), data transfer time (t_s), number of spectra coadded to form each data file (N), and delay time between successive coadded spectra (t_d). Thus, sampling time per chromatographic data point (S_t) can be calculated by using eq 1.

$$S_t = N\,(t_d + t_a) + t_s \qquad (1)$$

For the NMR system used in this research, data transfer time was about 1.8 s, necessitating careful attention to selection of optimal N in order to achieve the requisite chromatographic time resolution. This, in turn, is a function of observe cell volume, relative to eluent peak volume and flow rate, because maximum sensitivity depends upon achieving the best match of these parameters. Provided chromatographic resolution remains acceptable, there is a distinct analytical advantage (from the detection standpoint) in use of a larger observe region. This is depicted graphically in the three partial spectral stack plots (methyl protons only) summarized in Figure 2. When an 80 μL observe volume is used and either 30- or 300-μg samples of alanine, glycine, and valine are mixed and then subjected to HPLC/NMR analysis (Figure 2, parts a and b, respectively), the compounds are readily detected at the 30-μg level and easily seen at the higher level. Estimated detection limits of 10 μg or less per component can

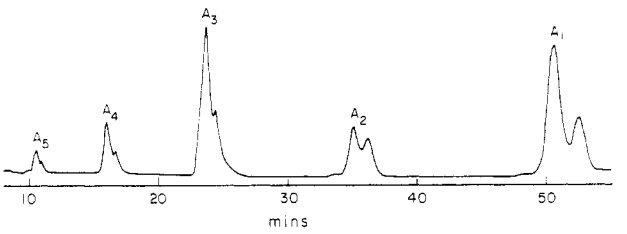


Figure 4. HPLC chromatogram for isocratic separation of antimycin A. Chromatographic conditions are as follows: column, Hibar Lichrob 3-μm C18 bonded phase; mobile phase, methanol-d/D_2O (75/25); flow rate, 1.5 mL/min.

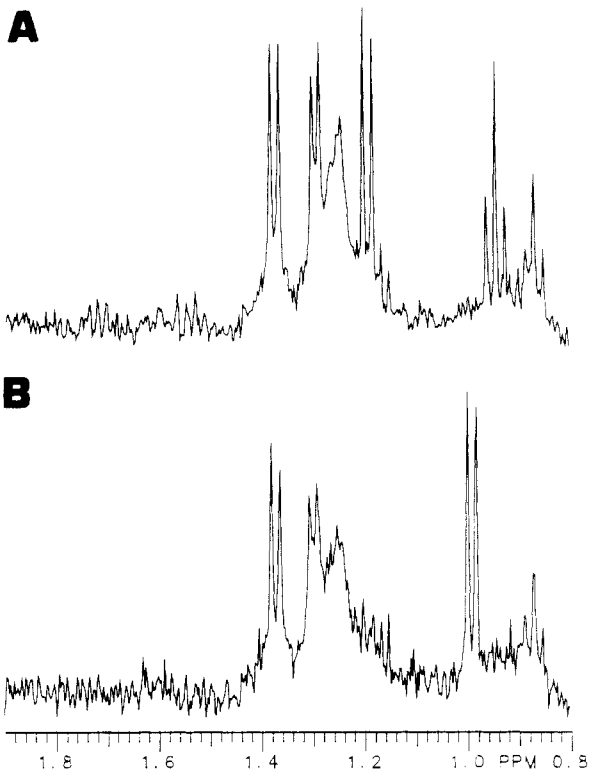


Figure 5. ^{1}H NMR spectra: (a) antimycin A_{1a}; (b) antimycin A_{1b}.

be inferred from Figure 2a. In contrast, Figure 2c shows much worse signal to noise ratio (S/N) for analysis of a mixture of 400 μg of each, using a 14-μL observe region. As the comparative chromatographic reconstructions for these data (Figure 3b–d) and those obtained by using an ultraviolet detector (254 nm) with a 10-μL cell (Figure 3a) show, serious degradation of resolution is not introduced by use of the larger observe volume and, as is clear from Figure 2, it provides superior sensitivity. Thus, the 80-μL configuration was used for antimycin analyses.

Antimycin Separations. The streptomyces antibiotic complex, antimycin A, was originally believed to consist of several closely related compounds differing only in the identity of alkyl substituents at the 7- and 8-portions of the dilactone ring, designated A_1, A_2, A_3, A_4, and A_5 (*13–15*). Their structures were thought to be those designated as the "b-series" in Figure 1. It was later found by 2D NMR that antimycin A_1, thought to have the structure with R^1 as n-hexyl and R^2 as isovaleryl, was actually a mixture of two compounds, with the major component having an isopentyl group as R^2 and the minor component possessing the previously assigned structure (*16*). In the same study, it was confirmed that antimycin A_3 was a mixture of two components, with the structures indicated in Figure 1. Subsequent HPLC separations described elsewhere (*17*) indicate that each of the previously identified antimycins is a two-component mixture. Figure 4 is the chromatogram of the antimycin A sample analyzed here,

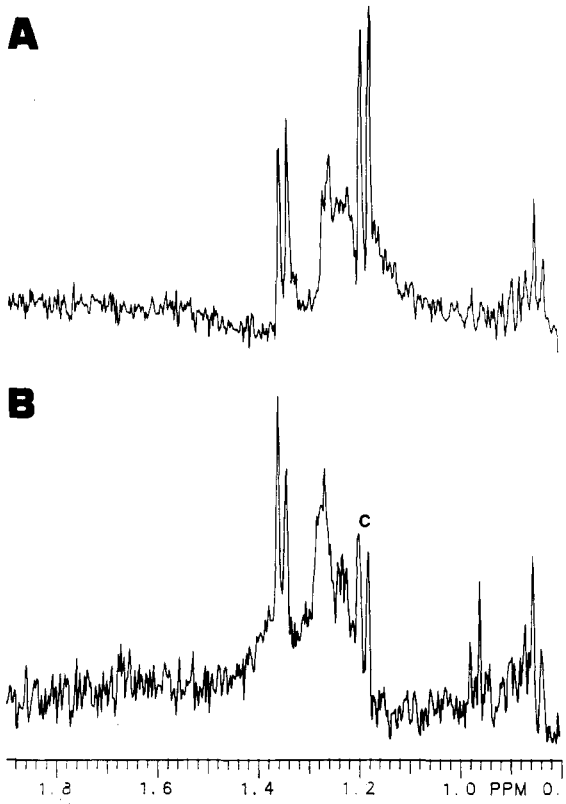


Figure 6. ^{1}H NMR spectra: (a) antimycin A_{2a}; (b) antimycin A_{2b}, "c" is a peak arising from a small amount of unresolved A_{2a}.

Table I. ^{1}H Chemical Shifts[a] (ppm) of Antimycins A_1 and A_2

	chemical shifts			
^{1}H	A_{1a}	A_{1b}	A_{2a}	A_{2b}
7	2.61[b]	2.61[b]	2.59[b]	2.60[b]
4-CH_3	1.39	1.38	1.35	1.35
9-CH_3	1.28	1.32	1.28	1.28
α, α'	1.63, x[c]	1.63, x	1.60, x	1.60, x
β, β'	x	x	x	x
γ, γ'	x	x	x	x
δ	x	x	x	x
ϵ	x	x	x	x
ξ	0.88	0.87	0.87	0.85
2″	2.50[b]	2.34[b]	2.65[b]	2.42[b]
3″	1.54, 1.72	2.12[b]	1.18	1.65
4″	0.95	0.99		0.96
5″	1.18			

[a] Chemical shifts of antimycins in a mixture of 75/25 CH_3OD/D_2O, with TMS as external reference. Only the chemical shifts in the aliphatic region are reported (from 0.5 to 2.6 ppm). [b] Resonances are not shown in figures. [c] x stands for unassigned nuclei of the R^1 alkyl chain, with chemical shifts from approximately 1.10 to 1.30 ppm. Please refer to ref 16 for detailed discussion.

clearly revealing the two-component composition of each of the antimycins (A_1–A_5). From these data, it is obvious that the original mixture contains at least ten individual components.

Figures 5 and 6 are the on-line ^{1}H HPLC/NMR spectra for antimycins A_1 and A_2 (taken from the spectral data file near the chromatographic peak maximum in each case), which are estimated to have been present in the original mixture at the 200–600 μg levels. Chemical shifts and assignments for methylated antimycin A_{1a} and A_{1b} are the same as those of the nonmethylated compounds and confirm the earlier assignments (*16*). Table I summarizes the chemical shifts of the methylated antimycins A_1 and A_2. Because chromatographic resolution was somewhat worse for the earlier-eluting components (A_3, A_4, and A_5), only four scans were coadded for each chromatographic data point, rather than eight scans as for antimycin A_1 and A_2. Spectra of antimycins A_3 (Figure 7) and A_5 are virtually the same as those of antimycin A_1, with the exception that fewer protons are evident in the alkyl portion of the spectrum, supporting the earlier assignment of R^1 for these compounds as *n*-butyl and *n*-ethyl, respectively (*15*). Similarly, antimycin A_2 and A_4 spectra differ only in the alkyl portions of the spectra. Thus, the present HPLC/NMR results permit not only quantitative estimation of the relative amounts of each of the antimycins but also provide qualitative confirmation of the structural assignments summarized in Figure 1. This technique, with its ability to provide such analytical data on submilligram quantities of mixture components, has unique analytical potential, particularly for complex mixture analysis applications, such as the present one, where limited stability of the components to be analyzed may make it difficult to isolate them for conventional NMR analysis.

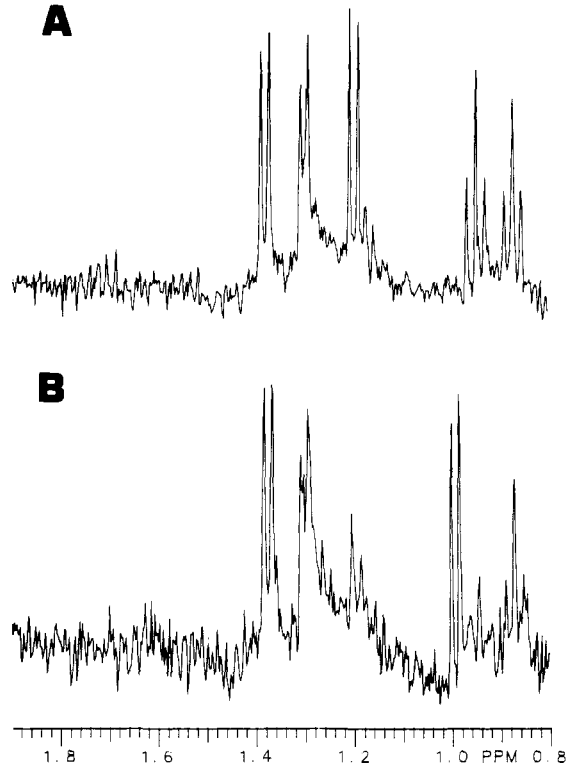


Figure 7. ^{1}H NMR spectra: (a) antimycin A_{3a}; (b) antimycin A_{3b}.

LITERATURE CITED

(1) Watanabe, N.; Niki, E. *Proc. Jpn. Acad., Ser. B* **1978**, *54*, 194–202.
(2) Bayer, E.; Albert, K.; Nieder, M.; Grom, E.; Keller, T. J. *J. Chromatogr.* **1979**, *186*, 497–507.
(3) Buddrus, J.; Herzog, H. *J. Magn. Reson.* **1981**, *42*, 453–459.
(4) Bayer, E.; Albert, K.; Nieder, M.; Grom, E.; Wolff, G.; Rindlisbacher, M. *Anal. Chem.* **1982**, *54*, 1747–1750.
(5) Dorn, H. C. *Anal. Chem.* **1984**, *56*, 747A–758A.
(6) Laude, D. A.; Wilkins, C. L. *Anal. Chem.* **1984**, *56*, 2471–2475.
(7) Laude, D. A.; Lee, R. W. K.; Wilkins, C. L. *Anal. Chem.* **1985**, *57*, 1464–1469.
(8) Laude, D. A.; Wilkins, C. L. *Anal. Chem.* **1987**, *59*, 546–551.
(9) Curran, S. A.; Williams, D. E. *Appl. Spectrosc.* **1987**, *51*, 1450–1454.
(10) Allen, L. A.; Glass, T. E.; Dorn, H. C. *Anal. Chem.* **1988**, *60*, 390–394.
(11) Allen, L. A.; Spratt, M. P.; Glass, T. E.; Dorn, H. C. *Anal. Chem.* **1988**, *60*, 675–679.
(12) Hoult, D. I. *Prog. NMR Spectrosc.* **1978**, *12*, 41–77.
(13) Van Tamelen, E. E.; Dickie, J. P.; Loumans, M. E.; Dewey, R. S.; Strong, F. M. *J. Am. Chem. Soc.* **1961**, *83*, 1639–1646.
(14) Kinoshita, M.; Aburak, S.; Wada, M.; Umezawa, S. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 1279–1287.
(15) Abidi, S. L. *J. Chromatogr.* **1982**, *234*, 187–200.
(16) Abidi, S. L.; Adams, B. R. *Magn. Reson. Chem.* **1987**, *25*, 1078–1080.
(17) Abidi, S. L. *J. Chromatogr.* **1988**, *447*, 65–79.

(18) Hore, P. J. J. Magn. Reson. 1983, 55, 283-300.
(19) Ha, S. T. K.; Lee, R. K. L.; Wilkins, C. L. J. Magn. Reson. 1987, 73, 467-476.

RECEIVED for review September 12, 1988. Accepted December 6, 1988. Partial support for this research provided by the National Science Foundation under Grant CHE-85-19087 to the University of California, Riverside, is gratefully acknowledged.

Fluorometric Measurement of Aqueous Ammonium Ion in a Flow Injection System

Zhang Genfa[1] and Purnendu K. Dasgupta*

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061

An automated flow-injection method for NH_3/NH_4^+ involving the ternary reaction of the analyte with *o*-phthaldialdehyde (OPA) and sulfite is described. The use of malodorous thiol compounds is avoided and the reaction provides much greater sensitivity by either fluorescence (detection limit 300 fmol or 20 nM NH_4^+) or absorption detection compared to the reaction involving 2-mercaptoethanol. The reaction shows considerable selectivity for ammonia over amino acids by a factor of 16 to >500 for 11 common amino acids studied. The throughput rate is 25 samples/h.

Ammonia is the principal atmospheric base responsible for the neutralization of atmospheric acidity (*1*). Ammonium salts, most notably sulfates, are frequently the principal components of the submicrometer fraction of the atmospheric aerosol burden (*2*) and are widely believed to be the primary responsible agents for the degradation of atmospheric visibility (*3*). Indeed, ammonium bisulfate, not organic compounds, was shown to be the dominant species in fine particle aerosol in the Great Smoky Mountains (*4*).

Sensitive determination methods for NH_3/NH_4^+ are needed to improve the time resolution of atmospheric measurements. Classically, ammonia has been determined by the indophenol blue reaction (limit of detection (LOD) 10 μg/L) or Nessler's reaction (LOD 20 μg/L) (*5*). In 1971, Roth discovered the ternary reaction of *o*-phthaldialdehyde (OPA), a "reducing agent" (borohydride or mercaptoethanol (ME)), and ammonia or primary amino acids to produce intensely fluorescent products and described its analytical usefulness (*6*). Detailed studies have shown the generality of the reaction and the products with thiols (which behave as nucleophiles, not reducing agents) have been unequivocally characterized to be 1-(alkylthio)isoindoles (*7*, *8*). Either through precolumn derivatization or postcolumn conversion, this reaction has become the major basis of chromatographic analysis of amino acids, and has appropriately become a textbook example (see, e.g., ref 9). Like many other ternary reactions, the reaction can be used to determine any of the three components involved. Thiols have been the analytes of interest for Nakamura and Tamura (*10*) and Mopper and Delmas (*11*). The latter authors also showed that sulfite and sulfide react in the same fashion as thiols. The (alkylthio)isoindole derivatives are not especially stable and present limitations in the precolumn derivatization method in regard to how long before chromatography the derivatives must be prepared. In looking for more stable derivatives amenable to electrochemical detection, Jacobs (*12*) recently found that, sulfite, in lieu of ME, offers advantages. The reaction can also be used for the sensitive detection of sulfur dioxide (*13*).

Although Taylor et al. (*14*) used the OPA–ME reaction for the determination of ammonia shortly after the discovery by Roth, subsequent exploitation of this reaction has primarily been focused on the determination of amino acids. The measurement of ammonia and ammonium ion by this reaction is enjoying a resurgence in the present decade (*15–19*) culminating in a recent study by Goyal et al. (*20*) which attempts the optimization of all pertinent parameters of the OPA–ME reaction, specifically for determining NH_3/NH_4^+. We have discovered that the spectral characteristics of the OPA–sulfite–NH_3 reaction product are considerably more attractive than the corresponding product with ME, both for absorptiometric or fluorometric detection. Here we present the details of this method, as adapted to a flow-injection system.

EXPERIMENTAL SECTION

Reagents. Standard grade *o*-phthaldialdehyde (P-1378, Sigma Chemical Co., St. Louis, MO) was used in this work without further purification. Experimentation with in-house twice recrystallized OPA or a "specially purified grade" available from the supplier did not show any significant benefits over the standard product. The OPA reagent is prepared by dissolving 268 mg of OPA, in 50 mL of methanol, followed by the addition of 150 mL of water. The solution, 10 mM in OPA, can be stored refrigerated for 1 week. We do *not* recommend inclusion of buffering agents in the OPA preparation, in our experience this decreases useful life of the reagent and leads to variable blanks.

Phosphate buffer (0.1 M) is made by dissolving 26.81 g of analytical reagent grade Na_2HPO_4 in 900 mL of water, adjusting pH to 11.0 with 2 M NaOH and diluting to 1 L. Sodium sulfite solution (3.0 mM, 0.378 g/L) is prepared daily in the phosphate buffer. Carbonate and borate buffers used (see results) were in the form of the sodium salt. Ammonium standards were made from a 0.1000 M NH_4Cl solution. Extreme care is necessary to make low-level standards, freshly deionized water must be used and exposure to ambient air avoided. Such standards must be used immediately after preparation.

Analytical System. The apparatus arrangement is schematically shown in Figure 1. A peristaltic pump P (Minipuls 2, four-channel head, Gilson Medical Electronics, Middleton, WI) is used to pump water (W) as carrier at 50 μL/min through a short column C (55 × 3 mm poly(tetrafluoroethylene) (PTFE) tube packed with monosize H^+-form cation exchange resin (Bio-Rex MSZ 50, Bio-Rad Laboratories, Richmond, CA), glass wool retaining plugs, replaced weekly) to remove traces of NH_3/NH_4^+ and then through an electromechanically actuated six-port rotary

[1] Permanent address: Shanghai Hygiene and Anti-Epidemic Center, 280 Chang Su Rd., Shanghai, People's Republic of China.

0003-2700/89/0361-0408$01.50/0