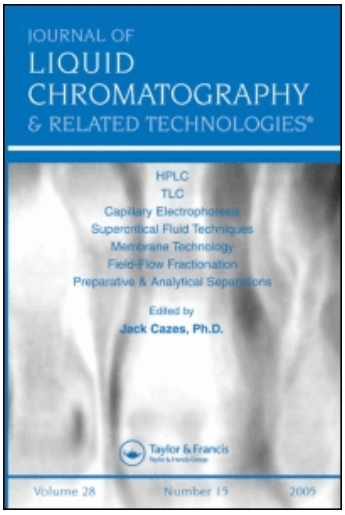

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Analysis of Stibonic Acids by Ion Exchange Chromatography with ESI-MS/Photodiode Array Detection[#]

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ABSTRACT

A method utilizing the counter anion exchange properties of aqueous ammonium acetate at pH 9, increasing in concentration linearly from 0 to 0.1 M NH_4OAc, using a Hamilton PRP-X100 anion exchange column, is

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presented for the resolution of aromatic stibonic acids and their detection by UV and ESI mass spectrometry. Additional phase-bonded silica or polymer backed C_8 and C_{18} column types, eluted with various counter ion solutions ($KClO_4$, NH_4COOH, NaOH, NaH_2PO_4) were evaluated for suitability for stibonic acid analysis.

Key Words: Stibonic acids; Ion exchange chromatography; ESI-MS; Photodiode array detection.

INTRODUCTION

Organic acids containing the element antimony have, for several decades, been of medical interest as chemotherapeutic agents against Schistosomiasis, Leishmaniasis, and Filariasis.[1] Therapeutic treatment of Kala-azar (visceral Leishmaniasis) by sodium 4-acetylamino-3-chlorophenyl stibnate (Van Heyden's 471) was described by Brahmachari in the early 1900s.[3,4] Currently, Stibophen, (T-4)-*Bis*[4,5-dihydroxy-1,3-benzenedisulfonato(4-)-O4,O5]-antimonate(5-)pentasodium heptahydrate; (Fuadin®—Winthrop) is used to treat Schistosomiasis.

Methods for preparing aromatic stibonic acids were reported by Doak, et al.,[5] using the Scheller reaction. This provided the basic chemistry for preparation of arylstibonic derivatives[6] and benzosulfonamide stibonic acids.[7] In the early 1950s, a simplified synthesis of aromatic organo-antimony compounds was found in the reaction of antimony trichloride with arylazoformates.[2]

Purification of these compounds was generally done by recrystallization of the associated pyridinium salt, with identification made by melting point comparison. These methods of purification and qualitative analysis are inadequate for compounds of pharmaceutical interest where both purity and identity need to be rigorously established.

Chromatographic methods of analysis have been reported for antimonal drug chelates (e.g., antimony potassium tartrate and antimony lithium thio-maleate), after extraction from biological specimens into *n*-hexane, utilizing C-18 reverse phase HPLC.[8] Inorganic species of antimony (III) have been separated using cation exchange chromatography with organic solvents in the presence of hydrobromic acid.[9] And Zheng, et al. have investigated anion exchange and size exclusion HPLC–ICP–MS for the analysis and identification of inorganic antimony and methylated species.[10] However, none of these methods was considered applicable for purity and identity confirmation of aromatic stibonic acids.

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Presented here, is a method for ion exchange HPLC resolution of aromatic stibonic acids with photodiode array (PDA) and electrospray ionization mass spectrometric detection (ESI-MS).

EXPERIMENTAL

Apparatus

A Waters Delta 600E Multi-solvent HPLC pump and 2676 Sample Manager were controlled under Waters Micromass MassLynx 3.5 software. Data acquisition from both a Waters Micromass ZQ 2000 Mass Spectrometer in Z-spray configuration, operating in Electrospray ionization (ESI) mode, and a Waters 2996 Photodiode Array detector was with MassLynx 3.5 software (Waters Co., Milford, MA). The chromatographic columns evaluated were FluoroSep Phenyl-HS and AquaSep (ESI, West Berlin, NJ), Cyclobond III (α cyclodextrin, ASTEC Inc., Whippany, NJ), ZirChrom-PBD zirconia-based stationary phases (ZirChrom, Anoka, MI), and a Hamilton PRP-X100 Anion Exchange Column (Hamilton Co., Reno, NV).

Reagents

All reagents used were of HPLC analytical grade unless otherwise noted. Ammonium acetate (NH_4OAc), potassium perchlorate ($KClO_4$), and sodium phosphate (NaH_2PO_4) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ammonium hydroxide (NH_4OH) was purchased from Fisher Scientific Co. (Fall Lawn, NJ). HPLC grade water was from Burdick and Jackson Co. (Muskegon, MI).

Experimental Procedure

Aromatic stibonic acids (Table 1) were obtained from the Developmental Therapeutics Program repository (National Cancer Institute, Rockville, MD) (http://www.dtp.nci.nih.gov/repositories.html). These compounds had been in repository storage for greater than 30 years, and no analyses could be located. Each was solubilized in aqueous 0.05 M NaOH, stirring at 45°C for approximately 30 min, then centrifuged at 1000 rpm for 5 min to pellet any undissolved material. The liquor was syringe filtered through a 0.2 micron Gelman PVDF Acrodisc filter (Pall Corp., Ann Arbor, MI). Injection volume was typically 10 μL at 3.33 mg mL^{-1}.

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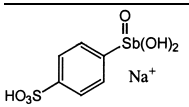
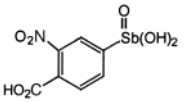
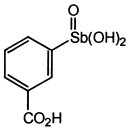
Table 1. Molecular structure, molecular formula, calculated molecular weight, observed weight, observed purity (% PDA peak integration at 254 nm), and corresponding chromatographic retention times of various stibonic acids under our novel chromatographic system.

Structure	NSC #	Molecular formula	Formula weight (calculated)	Observed weight	Observed purity (%)	Retention time (min)
	13746	C6H7O6SSb + Na	328.93 + 22.99	$[M^+ + Na + 2H_2O]$ 388	65.60	30.94
	13755	C7H6NO7Sb	337.88	$[M^- + NH_4OAc]$ 414 $[M^- + H_2O]$ 354	99.33	26.98
	13759	C7H7O5Sb	292.88	$[M^- + NH_4OAc]$ 369	96.77	26.88

	13760	C7H7O5Sb	292.88	$[M^- + NH_4OAc]$ 369 $[M^- + H_2O]$ 310	86.10	24.22
	13765	C7H8NO4Sb	291.90	$[M^+ + OAc]$ 351	41.20	13.15
	13778	C9H9O5Sb	318.92	$[M^- + NH_4OAc]$ 395	98.59	34.03
	13782	C9H12NO5Sb	335.95	$[M^+ + NH_4OAc]$ 413	67.18	16.05

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The mobile phase consisted of an aqueous 0.1 M NH_4OAc solution adjusted with NH_4OH to pH 9.0. A linear gradient was run from 100% water to 100% aqueous 0.1 M NH_4OAc over 30 min, at a flow rate of 2.0 mL min^{-1}. Total run time for an analysis was 60 min.

The Photodiode array detector was set to monitor from 210 to 600 nm at 1.2 nm with a sampling rate of 2.0 spectra per second.

The Mass Detector was configured to receive 20% of LC flow into the Electrospray Ionization probe with the cone voltage @ 20 V, capillary voltage @ 3.00 kV, extractor voltage @ 5.0 V, RF lens @ 0.4 V. The detector source block was set @ 90°C, desolvation gas @ 200°C, flowing 300 L nitrogen gas per hour.

RESULTS AND DISCUSSION

Preliminary trials at the development of a practical method for analyzing stibonic acids, which are relatively water soluble, were unsuccessful using traditional normal stationary phases, even in strongly acid systems. Presumably, this was caused by irreversible adsorption to free silanol sites. C_8 and C_{18} phase bonded silicas treated to suppress silanol groups, proved ineffective even with trifluoroacetic acid (TFA) or triethylamine (TEA) in the mobile phase, and when extremes in pH were employed. Although, polymer backed C_{18} media allowed the detection of desired mass ion complexes, essentially no retention was achieved [Fig. 1(a)].

Recently developed phase-bonded chromatographic materials, such as FluoroSep Phenyl-HS and AquaSep from ESI, and ZirChrom-PBD zirconia-based stationary phases from ZirChrom, and Cyclobond III, a bonded α cyclodextrin from ASTEC Inc., offer improved stability in mobile phases having a high percentage of water and to extremes of pH, and are intended for analysis of highly polar molecules. However, all proved inadequate for the qualitative analysis of stibonic acids due to a lack of on-column retention and component resolution [Fig. 1(b), (c)].

Ion exchange chromatography has a history of applicability for analysis of small water-soluble molecules. An example, provided by the manufacturer, of resolution of a series of small organic acids using the Hamilton PRP-X100 column, a polystyrene divinylbenzene anion exchange resin, suggested its applicability for analysis of stibonic acids. In exploratory trials, a series of counter ions ($KClO_4$, NH_4COOH, NaOH, NaH_2PO_4) were investigated through a broad range of pH (1–11) for their ability to displace stibonic acids from this resin and achieve resolution of components. However, among this group of four, only the perchlorate ion facilitated elution [Fig. 1(d)].

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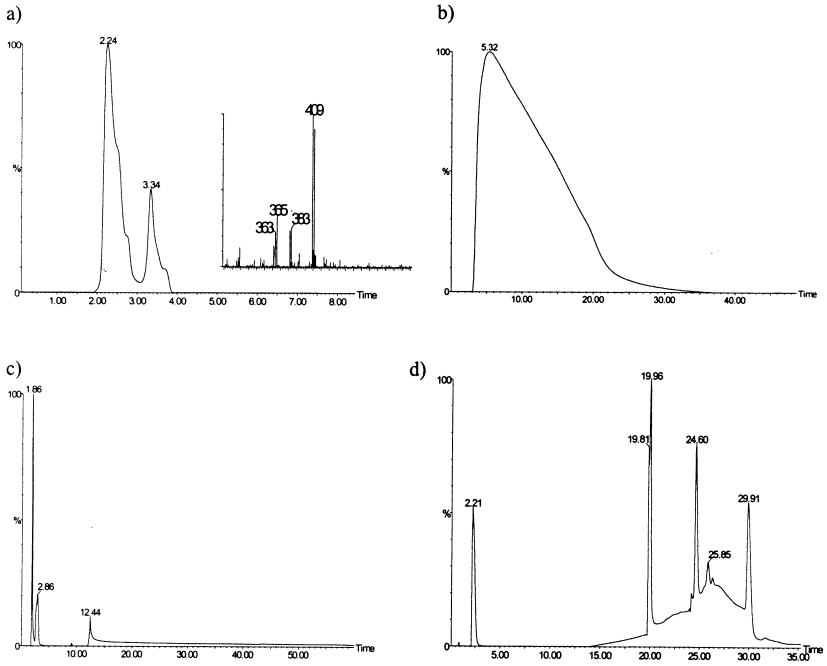

Figure 1. (a) NSC 13778 onYMC-PC-03-6 with 50 mM aq. NH_4COOH. (b) NSC 13778 on ASTEC Cyclobond III with aq. 0.1% TFA. (c) NSC 13778 on ZirChrom PBD with aq. 0.1% acetic acid. (d) NSC 13746 on Hamilton PRP-X100 with aq. 0.1 M $KClO_4$, pH 9.

Difficulties were soon discovered when ClO_4^- was the counter ion. The two stable natural isotopes of antimony, differing by 2 amu, give a characteristic doublet signal when MS is the detection method, and this doublet is very useful for confirmation of the presence of antimony in a compound. But the similar isotopic abundance pattern of chlorine (isotopes 35, 75% and 37, 24%) to that of antimony (isotopes 121, 57% and 123, 42%), and the consequent "drowning out" of target mass signal by ClO_4^- made identification of the antimony signal doublet in the mass spectrum impossible. Therefore, a more mass spectrometer "friendly" system substituting NH_4OAc for $KClO_4$ was developed. At pH 9, the aromatic stibonic acids are well retained on the PRP-X100 column, and when eluted over 30 min with a linear gradient beginning from 100% water and increasing to 0.1 M aqueous NH_4OAc, excellent resolution of sample components was achieved, as is demonstrated for NSC 13782 (Fig. 2). As is seen in Fig. 2, the component with the retention time

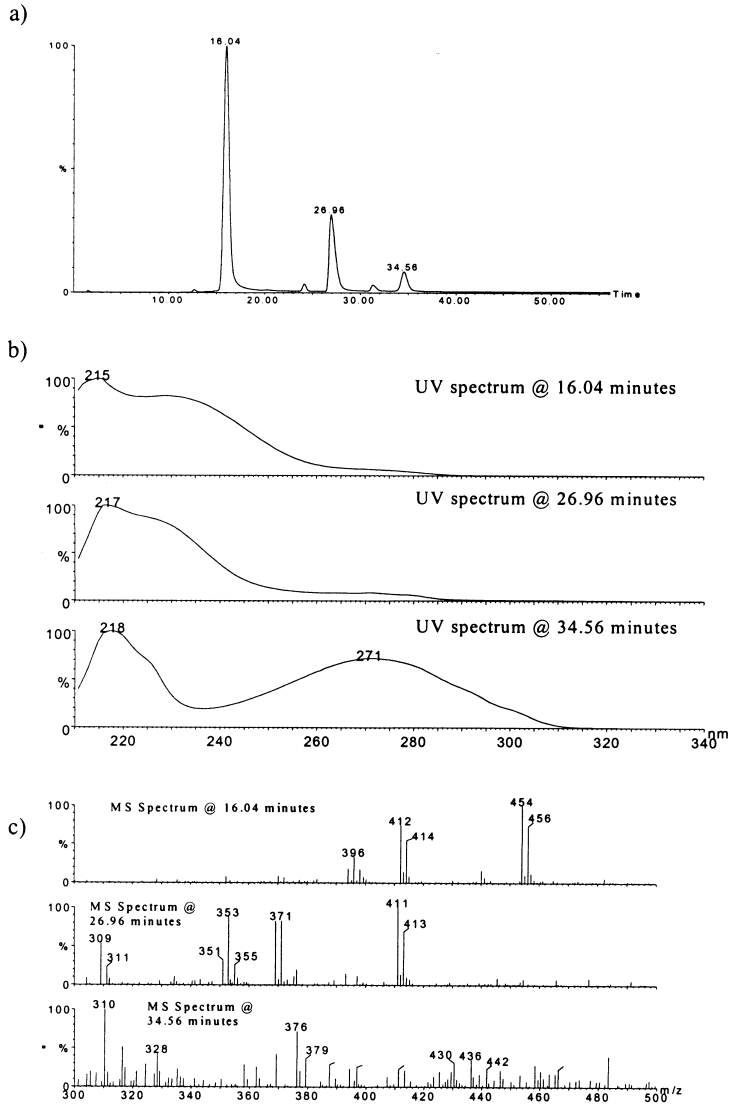

Figure 2. (a) Chromatogram @ UV254 demonstrating resolution of components from NSC 13782 using 0.1 M NH_4OAc, pH 9.0 on a Hamilton PRP-X100 anion exchange column. (b) Extracted UV spectra from 210–340 nM of components of NSC 13782 with retention times @ 16.04, 26.96, and 34.56 min. (c) MS Spectra of components of NSC 13782 with retention times @ 16.04, 26.96, and @ 34.56 min.

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of 16 min, as detected by ESI-MS, has both the doublet diagnostic of antimony and the correct molecular ion for the expected compound, C9H12NO5Sb · NH_4OAc. The component retained for 26.9 min also contains antimony, as evidenced by the mass doublets, but lacks the ion expected for NSC 13782. The component with retention time 34.5 min lacks the doublet

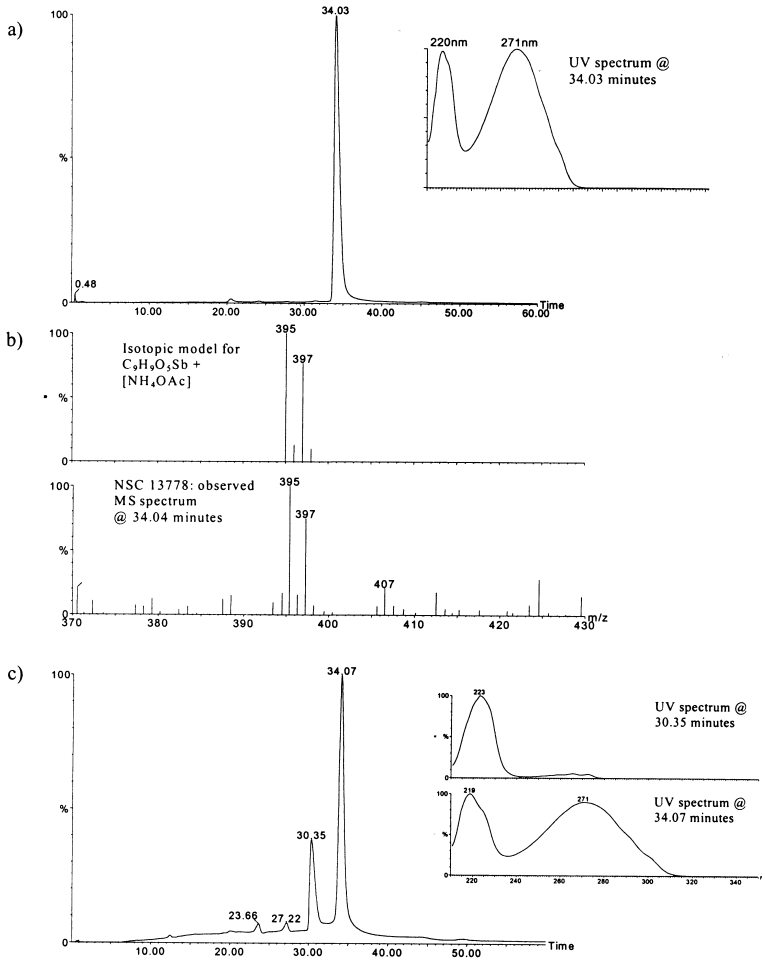

Figure 3. (a) UV Chromatogram @ 254 nm for NSC 13778. (b) ESI-MS spectrum of component @ 34.04 min with isotopic model for $C_9H_9O_5Sb + [NH_4OAc]$. (c) UV spectrum @ 254 nm of NSC 13746 with extracted UV spectra of components @ 30.35 and 34.07 min.

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signal for antimony so it cannot be the desired compound. Thus, this analytical system, using as a mobile phase a gradient of 0.1 M aqueous NH_4OAc at pH 9, is superior to the analogous system containing $KClO_4$ because: (a) non-similar isotopic spectra allow distinction between analyte and counter ion; (b) NH_4OAc is sufficiently volatile that it can be removed under high vacuum, with recovery of pure stibonic acid for preparatory purposes.

A summary of the results of analysis of seven aromatic stibonic acids with a 250×4.1 mm Hamilton PRP-X100 column utilizing the system presented above, is given in Table 1. Examples of these analyses for compounds NSC 13778 and 13746 are given in Fig. 3, with extracted UV and mass spectral results, and an isotopic modeling is shown for NSC 13778. The observed mass ion is detected as a complex with the counter ion [NH_4OAc (+77)] and water [H_2O (+18)]. In all cases, an ion of the mass expected for each NSC-number compound, as a complex, was detected, confirming the presence of the compound in the sample, but purity was variable. The limit of detection is $\sim 0.3\ \mu g\ L^{-1}$.

In conclusion, an anion exchange HPLC method has been devised, which permits resolution of components present in samples of synthetic aromatic stibonic acids without preprocessing or chelation, and in combination with diode array UV and ESI mass spectral detection, provides a powerful tool for detection of these compounds in general; of antimony specifically, and of the identity and purity of this chemotype. It seems probable, that with slight modification, this technique has a wider applicability for purification and analysis of other small, highly polar organic molecules.

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