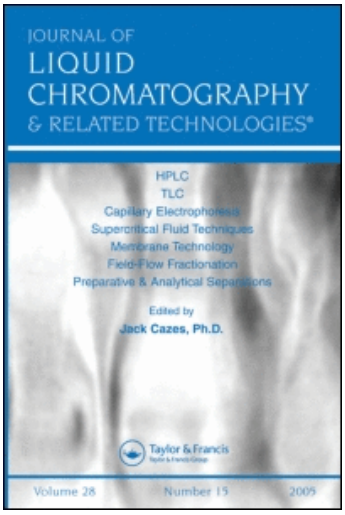

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LIQUID CHROMATOGRAPHIC DETERMINATION OF THE COMPOSITION OF THIACETARSAMIDE SOLUTIONS

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ABSTRACT

Sodium thiacetarsamide in solution is a mixture of p-arsenosobenzamide and thiacetarsamide as determined by two reverse phase liquid chromatographic (LC) procedures with the following mobile phases: Procedure I: methanol:0.25 mM sodium phosphate and 0.0125 mM sodium ethylenediaminetetraacetic acid aqueous buffer, pH 7 (15:85); and procedure II: tetrahydrofuran:2.5 mM sodium metabisulfite and 0.5 mM phosphoric acid aqueous buffer, pH 3 (5:95). Procedure I separated p-arsenosobenzamide from unretained thiacetarsamide and degradation products. Procedure II retained and separated thiacetarsamide, p-arsenosobenzamide, and degradation products. Both procedures inhibited on-column hydrolysis of thiacetarsamide to p-arsenosobenzamide and allowed two independent estimations of the p-arsenosobenzamide content of thiacetarsamide solutions. Only p-arsenosobenzamide and unidentified degradation products were detected when sodium thiacetarsamide-p-arsenosobenzamide solutions were stored for 6 months at room temperature. However, the refrigerated solutions were composed of thiacetarsamide and p-arsenosobenzamide plus trace amounts of unidentified substances. Under strongly basic conditions, thiacetarsamide hydrolyzed to p-arsenosobenzamide, which later degraded to unknown compounds. To stabilize and maintain a relatively constant composition, sodium thiacetarsamide-p-arsenosobenzamide solutions must be refrigerated and protected from light and oxygen.

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INTRODUCTION

Thiacetarsamide sodium is a drug used to treat adult canine heartworm (Dirofilaria immitis) disease (1-3). The reaction of p-arsenosobenzamide with thioglycollic acid (4), the final step in the synthesis of thiacetarsamide, is shown in Fig. 1 along with three potential synthetic contaminants. The reaction is reversible (5, 6) and the As-S bond easily hydrolyzes. As trivalent arsenicals, p-arsenosobenzamide and thiacetarsamide are reactive and oxidize to pentavalent arsenic compounds. Thiacetarsamide is water-soluble, but p-arsenosobenzamide has limited water solubility (1 part in 800) (4, 7). Thiacetarsamide is formulated as a 1% neutral aqueous solution of the dibasic sodium salt by reaction with sodium hydroxide (1).

The instability of thiacetarsamide creates difficulty in liquid chromatographic (LC) analysis. With binary mobile phases composed of water modified with acetonitrile, methanol, or tetrahydrofuran (THF), thiacetarsamide was completely hydrolyzed during LC analysis and eluted as p-arsenosobenzamide (8). The maximum inhibition of on-column hydrolysis of thiacetarsamide was obtained with a mobile phase containing ethylenediaminetetraacetic acid (EDTA), used in procedure I. The chromatographic profile for thiacetarsamide solutions showed a large nonretained peak containing the thiacetarsamide, which tailed into the p-arsenosobenzamide peak, creating a bridge between the two peaks. On-column hydrolysis was confirmed by isolating fractions from the region between the two

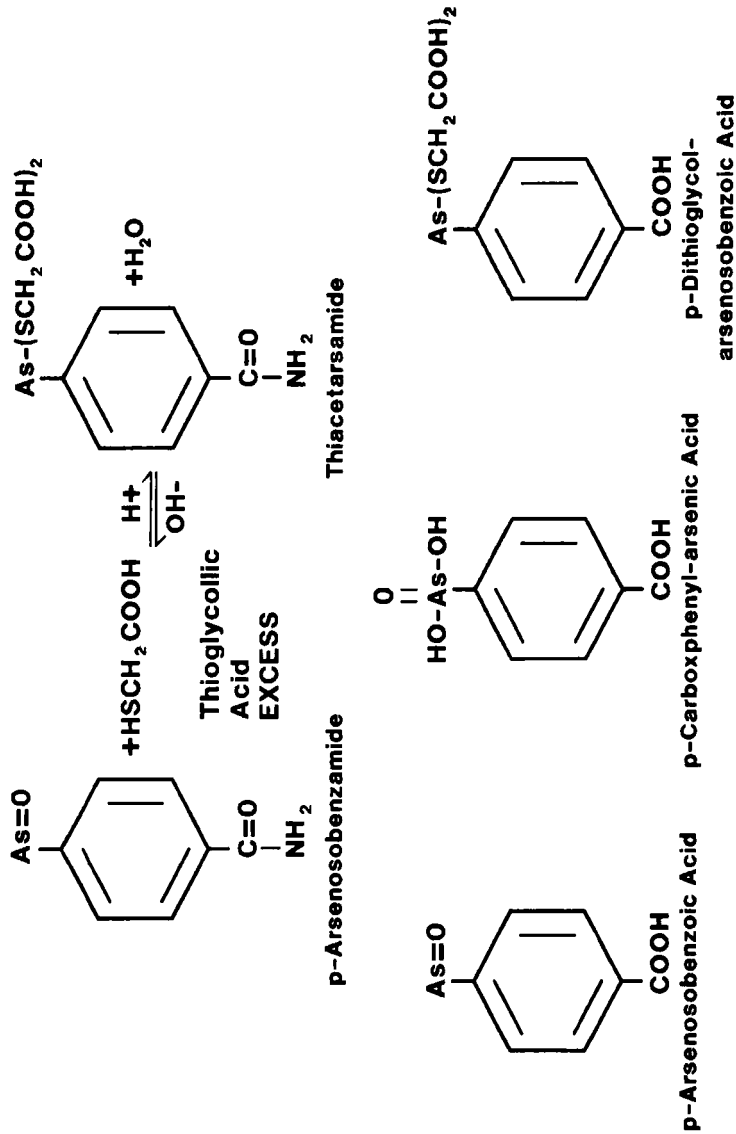

Figure 1. Reaction of p-arsenosobenzamide to form thiacetarsamide and the 3 potential manufacturing contaminants.

peaks. When these fractions were immediately rechromatographed, the late-eluting p-arsenosobenzamide was detected. Procedure II, described in this work, used sodium metabisulfite and phosphoric acid to stabilize and retain thiacetarsamide during analysis. Both metabisulfite and EDTA have been used to increase the stability of compounds in solution (9, 10).

When exposed to oxygen and light, sodium thiacetarsamide solutions become yellow and eventually precipitates form (7). Previous reports (4, 7) indicate that sodium thiacetarsamide solutions are stable at room temperature when protected from light and stored under a nitrogen atmosphere. The data in this paper support the conclusion that thiacetarsamide in solution is a mixture of thiacetarsamide and p-arsenosobenzamide, rather than a solution of thiacetarsamide. When degradation occurs, other unidentified components are detected with procedure II. The sodium thiacetarsamide-p-arsenosobenzamide solutions are shown to degrade more at room temperature than under refrigeration.

EXPERIMENTAL

Apparatus and Materials

LC was performed with a Waters model M-6000A solvent pump, model 440 fixed wavelength detector, and Omni-Scribe 10 mV recorder. Samples were injected with either a CV-6-UHpa-N60 Valco or model 7125 Rheodyne manual injector. Operating conditions for both procedures were: flow rate 2.0

mL/min;detector sensitivity 0.1 absorbance unit full scale; UV light source; ambient temperature; detector wavelength 254 nm; and recorder chart speed 0.1 in./min. A 25 cm X 4.6 mm i.d. Zorbax C-8 6-μm column was used and flushed daily with water.

Methanol, THF, and water were glass distilled. Stock solutions (0.1M) of sodium EDTA were prepared by neutralizing EDTA with 1 N sodium hydroxide. All aqueous components of the mobile phase were filtered through a Millipore 0.45-μm HA filter using all-glass apparatus. The 0.25 M sodium phosphate was prepared by mixing equal molar concentrations of sodium dihydrogen phosphate and sodium monohydrogen phosphate. The mobile phase for procedure I was methanol:0.25 mM sodium phosphate and 0.0125 mM sodium EDTA aqueous buffer, pH 7 (15:85), and for procedure II it was THF:2.5 mM sodium metabisulfite and 0.5 mM phosphoric acid aqueous buffer, pH 3 (5:95). The mobile phases were degassed in an ultrasonic bath under reduced pressure before use.

Spectral data were obtained with a Cary 219 spectrophotometer and 1-cm path length quartz cells. Solvent was used in the reference cell. The settings were: chart speed 10 nm/cm, scan rate 1 nm/cm, gain 0.3, and range 1.0 absorbance. Thiacetarsamide and p-arsenosobenzamide were dissolved in methanol:1% ammonium carbonate, pH 8.0 (90:10) (solution I) or methanol:water (50:50) (solution II). These solutions were stored at room temperature.

Preparation of Reference Solutions

Caparsolate reference standard (thiacetarsamide), caparsolate acid (thiacetarsamide), p-arsenosobenzamide, p-arsenosobenzoic acid, p-dithioglycol-arsenosobenzoic acid, and p-carboxyphenyl-arsenic acid (Abbott Laboratories, North Chicago, IL 60064) were stored at 8°C in the dark and used as received. Solutions of these compounds were prepared in EDTA mobile phase by heating to 60°C on a Temp-Block module heater and frequently mixing with a Vortex-Genie mixer. For preparation of standards, p-arsenosobenzamide was dissolved in methanol:water (15:85) or methanol:0.25 mM sodium phosphate and 0.0125 mM sodium EDTA aqueous buffer, pH 7 (15:85) at a concentration of about 0.06 mg/mL. Five standards of 0.06-0.005 mg/mL or 0.3-0.02 mM were prepared by dilution. Standard curves were prepared by analyzing 10-µL aliquots of standards along with samples. The standard curves produced a coefficient of variation, r, of 0.999. Reference thiacetarsamide solutions, 0.5 mg/mL, were prepared fresh in mobile phase. The solutions were cooled to room temperature, and then filtered through a 0.45-µm Millipore filter and stored at 8°C in the dark.

Preparation of Test Sodium Thiacetarsamide Solution

A 1% sodium thiacetarsamide solution was prepared by neutralizing with sodium hydroxide. Boiled glass-distilled water purged with nitrogen gas was used. Exposure to light during preparation was limited by covering the containers with aluminum foil and reducing room lighting. A sodium hydroxide

solution was prepared by dissolving 0.235 g of sodium hydroxide in 20 mL of water. The sodium hydroxide solution was slowly added to a suspension of thiacetarsamide (0.897 g) in 70 mL of water while mixing with a magnetic stirrer. Temperature was kept below 30°C; however, the pH rose to 10 and declined slowly as thiacetarsamide dissolved. The remaining ingredients (g), 0.068 sodium dihydrogen phosphate, 0.044 sodium phosphate monobasic, 0.9 benzyl alcohol, and 0.4 NaCl, were added before the pH was adjusted to 6.8 with the remaining sodium hydroxide solution. The volume was adjusted to 100 mL with water and mixed; then about 10 mL was removed (sample 1). Darco was added to the thiacetarsamide solution to clarify the solution and mixed; then the mixture was filtered through a 0.45 μm filter, producing sample 2. Sample 1 was also filtered through a 0.45 μm filter. Sample 2 was divided into about 10-mL aliquots and the subsamples were treated and stored as listed in Table 1. The samples were diluted 1:20 in mobile phase, producing a 0.45 mg/mL solution, and filtered through a 0.45 μm filter before LC analysis. Similarly prepared 0.45-mg/mL benzyl alcohol samples, the concentration present in the diluted test sodium thiacetarsamide solutions, did not increase the nonretained peak or produce a retained peak using either procedure in comparison to mobile phase controls.

RESULTS AND DISCUSSION

The chromatographic profiles produced with procedure I alter as the composition of the thiacetarsamide solution changes.

TABLE 1
Storage Conditions for Test Sodium Thiacetarsamide Samples

Sample[a]	Stored at 8°C	Nitrogen Atmosphere	Light Protected
1	yes	yes	yes
2	yes	yes	yes
3	yes	yes	yes
4	yes	yes	no
5	yes	no	yes
6	no[b]	yes	yes
7	no[b]	yes	no

[a]1, test sodium thiacetarsamide; 2-7, test sodium thiacetarsamide treated with Darco-60.
[b]Stored at room temperature.

This provided a qualitative indication of the changing composition of thiacetarsamide solutions. The changing composition was expressed as the ratio of the peak height of the nonretained peak containing thiacetarsamide to the p-arsenosobenzamide peak. This ratio declines as a result of two distinct chemical processes: (1) The nonretained peak decreases and the p-arsenosobenzamide increases when thiacetarsamide hydrolyzes in solution to produce p-arsenosobenzamide. (2) Thiacetarsamide can also degrade in solution, producing other nonretained products that coelute with thiacetarsamide. None of the potential synthetic contaminants were retained (Fig. 1) and only the p-arsenosobenzamide peak was detected in the chromatograms. Therefore, thiacetarsamide and degradation products were separated from the p-arsenosobenzamide.

Spectral data show that degradation products are produced with time in thiacetarsamide solutions. These mixtures absorb less at 254 nm, where the absorbance was monitored during LC analysis, than fresh thiacetarsamide solutions, resulting in lower ratios. Basic conditions increased both the solubility and potential for hydrolysis of thiacetarsamide (5). The UV spectra of the fresh solution I of thiacetarsamide and p-arsenosobenzamide were similar, with maxima between 230 and 232 nm. The solutions were analyzed 3 days later. While the thiacetarsamide spectrum had undergone a hypsochromic shift at the maximum and a bathochromic shift at 254 nm, the p-arsenosobenzamide spectrum was unchanged. The spectral shift at 254 nm for the thiacetarsamide solution resulted in a 40% decrease in absorbance. If thiacetarsamide had hydrolyzed to p-arsenosobenzamide, this shift would not have occurred. In a similar experiment, the absorbance of the thiacetarsamide solution II declined 12% at 254 nm, while p-arsenosobenzamide solution II was unchanged. Thus, thiacetarsamide solutions degraded under both neutral and mildly basic conditions.

A test sodium thiacetarsamide solution was prepared to determine whether conditions similar to those present during preparation of a 1% sodium thiacetarsamide solution would result in a lower ratio (nonretained thiacetarsamide-retained p-arsenosobenzamide). The fresh test sodium thiacetarsamide solution produced a higher ratio than the reference thiacetarsamide solution (Fig. 2 and Table 2). The formulation

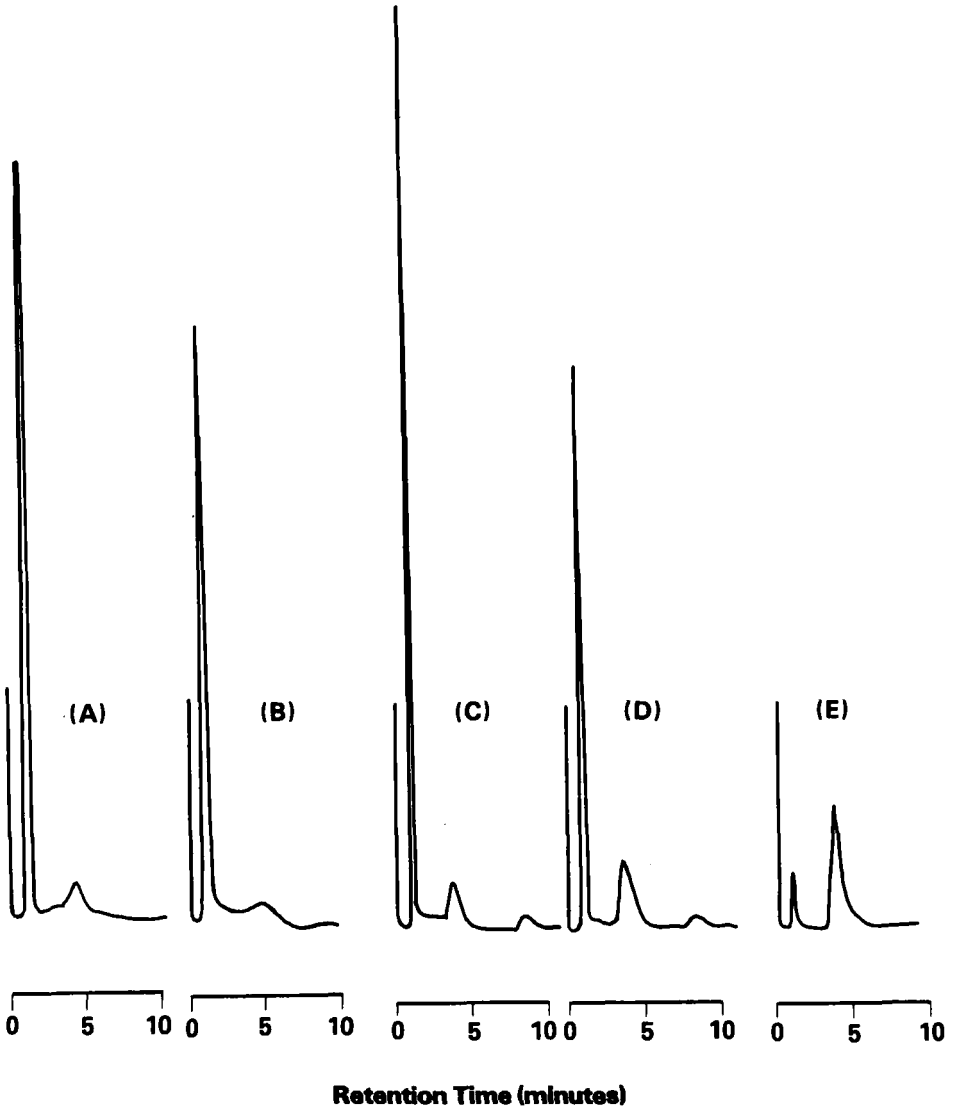

Figure 2. Chromatograms of A, 1.0 µg reference thiacetarsamide; B, 0.9 µg fresh test sodium thiacetarsamide samples; C and D, 2.25 µg test sodium thiacetarsamide samples stored 3 months under nitrogen atmosphere in the dark (C at 8°C and D at room temperature); and E, 0.58 µg p-arsenosobenzamide. Procedure I described in Experimental.

TABLE 2
LC Analysis of Test Sodium Thiacetarsamide Samples and Thiacetarsamide Reference Solutions

Sample[a]	Ratio[b] Fresh	3 Months
1	34	
2	33	22
3		19
4		19
5		18
6		9
7		8
Reference[c]	23	17
Reference[d]	21	11

[a]See Table 1 for storage conditions. Procedure I is described in Experimental; 0.9 µg fresh and 2.25 µg stored samples were assayed; 1.0 µg fresh and 2.0 µg stored reference solutions assayed.
[b]Ratio = nonretained peak height divided by p-arsenoso- benzamide peak height.
[c]Stored 3 months at 8°C.
[d]Stored at 8°C but opened, warmed to room temperature, and exposed to light 2 or more times/week for 3 months.

procedure, which produced basic conditions (pH 10.0), did not result in increased hydrolysis of thiacetarsamide to p-arsenosobenzamide. The test sodium thiacetarsamide solutions and the reference thiacetarsamide solution were divided and stored under the conditions described in Table 1. After these solutions were stored for 3 months, they were analyzed using Procedure I. Storage temperature was the variable which produced the greatest changes in chromatographic profile (Fig. 2) and ratios (Table 2). Test thiacetarsamide samples stored at room temperature were a faint yellow. The additional

manipulation and warming of the reference thiacetarsamide solution also produced increased degradation.

Procedure I was used to estimate the p-arsenosobenzamide content of thiacetarsamide solutions using a p-arsenosobenzamide standard curve (Table 3). To show the reproducibility of the chromatography of reference thiacetarsamide solutions, the p-arsenosobenzamide content of two solutions of thiacetarsamide was determined in duplicate over a 9-day period. Between analyses, the thiacetarsamide solutions were stored at 8°C. Using the analysis of variance (ANOVA) procedure described by Youden and Steiner (11) on the pooled data, a repeatability within a day of 4.5% CV and a reproducibility between days of 9.3% CV were calculated. The reproducibility (9.3%) indicates that the thiacetarsamide solutions are relatively stable over the short term (9 days) if they are refrigerated.

Procedure II retained and partially separated thiacetarsamide, p-arsenosobenzamide, and potential manufacturing contaminants. p-Carboxyphenylarsenic acid is a reaction intermediate in the synthesis of thiacetarsamide. p-Dithioglycol-arsenosobenzoic acid results when the amination step is incomplete. p-Dithioglycol-arsenosobenzoic acid hydrolyzes to p-arsenosobenzoic acid. Ion-suppression chromatography, using sodium metabisulfite and phosphoric acid (procedure II), retained and separated these acid compounds as unionized species.

The resolution with both procedures allowed independent estimation of the p-arsenosobenzamide component of thiacetars-

TABLE 3
Measurement of the p-Arsenosobenzamide Content (mM) of Reference Thiacetarsamide Solutions[a]

	Solution I		Solution II	
Day	1	2	1	2
1	0.212	0.200	0.170	0.165
2	0.224	0.244	0.212	0.214
4	0.240	0.240	0.210	0.210
9	0.210	0.240	0.210	0.210

[a]Solution I, 0.5 mg/mL thiacetarsamide (caparsolate acid), and solution II, 0.5 mg/mL thiacetarsamide (caparsolate reference standard), in procedure I mobile phase and stored at 8°C; 1.0-µg samples were analyzed by procedure I described in the Experimental section.

TABLE 4
LC Estimation of the p-Arsenosobenzamide Content of 6-Month-Old Test Sodium Thiacetarsamide Samples and Percent Conversion of Thiacetarsamide (23.8 mM) to p-Arsenosobenzamide

	Procedure I		Procedure II	
Sample[a]	mM	%	mM	%
1	6	26	5	22
3	7	28	5	22
4	7	28	5	23
5	6	27	5	22
6	9	37	9	37
7	6	27	6	24

[a]See Table 1 for storage conditions. Procedures are described in the Experimental section; 2.24 µg was assayed.

amide solutions using a p-arsenosobenzamide standard curve. The p-arsenosobenzamide content of the thiacetarsamide reference solution prepared in procedure I mobile phase was measured with procedures I and II. The percentage measured as p-arsenosobenzamide with procedures I and II were 17 and 19%, respectively. The p-arsenosobenzamide content of the test thiacetarsamide solutions was measured using both procedures. Since the two independent estimations (Table 4) of the p-arsenosobenzamide content were similar, these results confirm that the on-column hydrolysis of thiacetarsamide to p-arsenosobenzamide was reduced to an insignificant proportion of the thiacetarsamide exposed to the reversed-phase packing.

The test sodium thiacetarsamide solutions were analyzed after 6 months of storage (Figs. 3 and 4). The room temperature samples were yellow-orange, but did not contain visible precipitates, while the refrigerated samples were still clear and colorless. Procedure II produced the most distinctive differences (Fig. 4). The freshly prepared thiacetarsamide reference solution produced a chromatographic profile with a sharp peak of p-arsenosobenzamide at 2.9 min followed by the broad, fused thiacetarsamide peak. Refrigerated test thiacetarsamide samples 1, 3, 4, and 5 produced profiles similar to the freshly prepared reference solution except for a second sharp peak at 14 min. Room temperature samples 6 and 7 produced a chromatographic profile with significant peaks at 1.9, 2.9, 7.7, and 14.0 min, but no broad thiacetarsamide peak. The

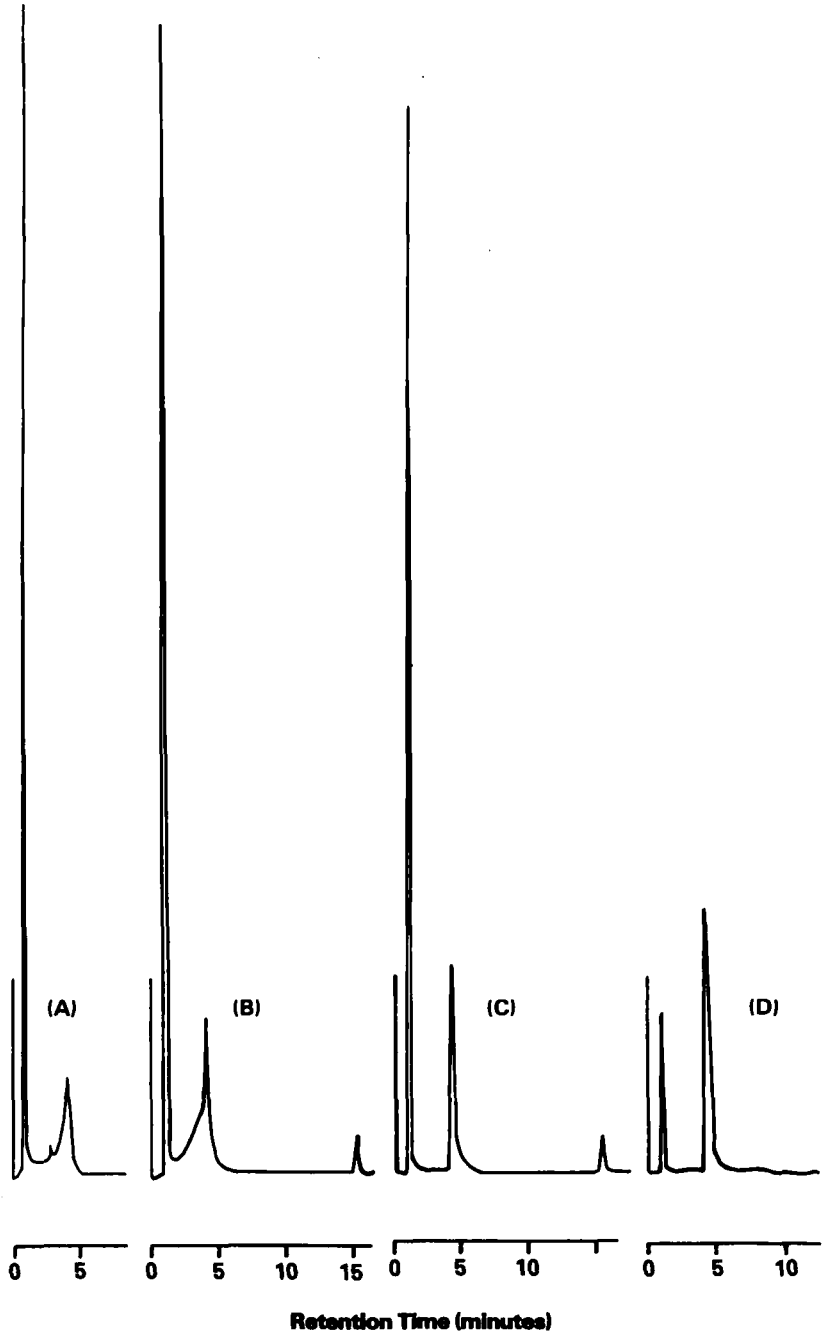

Figure 3. Chromatograms of A, 2.0 µg reference thiacetarsamide; B and C, 2.25 µg test sodium thiacetarsamide samples stored 6 months under nitrogen atmosphere in the dark (B at 8°C and C at room temperature); and D, 0.59 µg p-arsenosobenzamide. Procedure I described in Experimental.

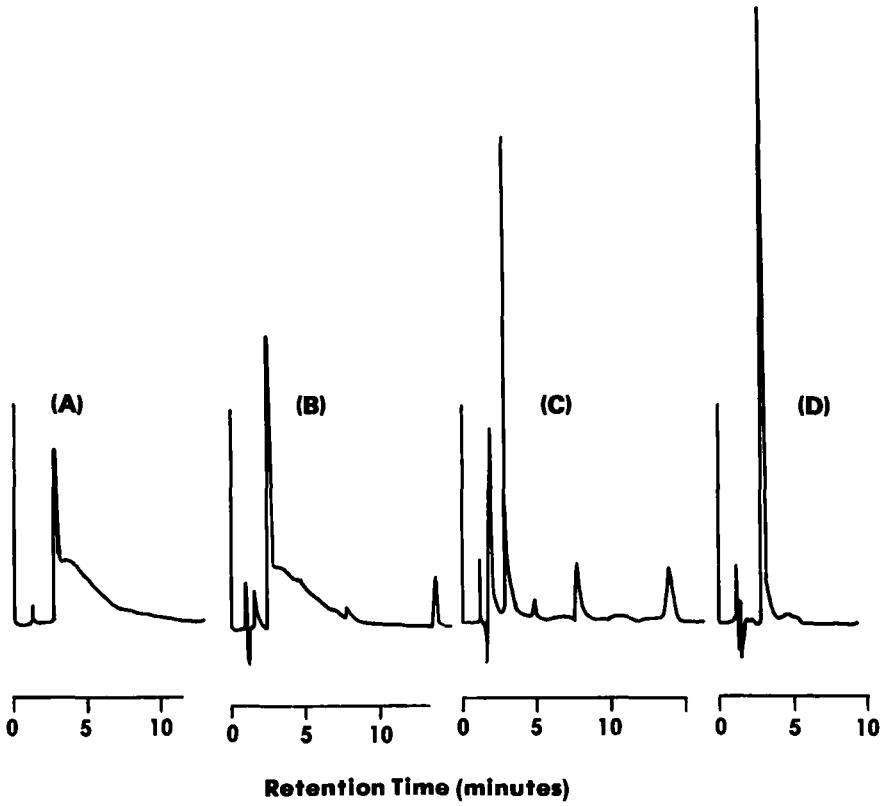

Figure 4. Chromatograms of A, 2.0 µg reference thiacetarsamide; B and C, 2.25 µg test sodium thiacetarsamide samples stored 6 months under nitrogen atmosphere in the dark (B at 8°C and C at room temperature); and D, 0.59 µg p-arsenosobenzamide. Procedure II described in Experimental.

retention times (min) of the three potential contaminants tested (10.1 for p-dithioglycol-arsenosobenzoic acid with a broad tail, 9.8 for p-arsenosobenzoic acid, and 4.3 and 8.8 for p-carboxyphenyl-arsenic acid, which produced two distinct peaks) were all different from the unknown peaks (Fig. 5). Additionally, thiacetarsamide and p-dithioglycol-arsenoso-

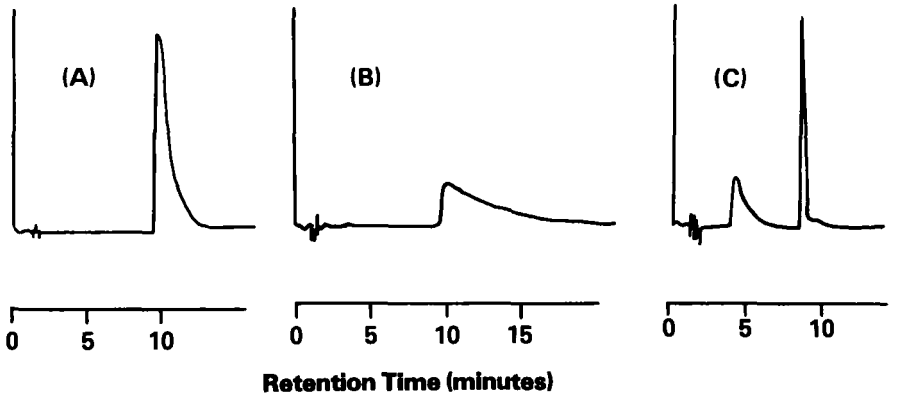

Figure 5. Chromatograms of A, 1 µg p-arsenosobenzoic acid; B, 2.5 µg p-dithioglycol-arsenosobenzoic acid; and C, 2.5 µg p-carboxyphenyl-arsenic acid. Procedure II described in Experimental.

benzoic acid, which are thioglycollic acid derivatives, have similar chromatographic profiles.

The p-arsenosobenzamide content of the test sodium thiacetarsamide samples was calculated (Table 4). The p-arsenosobenzamide concentration in the test sodium thiacetarsamide samples was used to calculate the percent conversion of thiacetarsamide to p-arsenosobenzamide. Samples stored at room temperature contained no detectable thiacetarsamide; therefore, only p-arsenosobenzamide was present. For these degraded test thiacetarsamide samples, unidentified degradation products were the major components. Although protected from light and stored under a nitrogen atmosphere, sample 6 did not maintain composition.

Thiacetarsamide is hydrolyzed to p-arsenosobenzamide under basic conditions (5, 6); therefore, the p-arsenosobenzamide

TABLE 5
Percent conversion of thiacetarsamide to p-arsenosobenzamide when dissolved in different solvents[a]

Solvent	Conversion, %
0.01 N NaOH	27
0.1 N NaOH	65
1.0 N NaOH	74
5 mM Sodium metabisulfite, pH 3	21
2.5 mM Sodium metabisulfite and 0.5 mM phosphoric acid, pH 3	21
2.5 mM Sodium metabisulfite and 0.5 mM sodium phosphate, pH 10	11
THF:water (5:95) with 2.5 mM sodium metabisulfite and 0.5 mM sodium phosphate, pH 10	13
THF:water (5:95) with 2.5 mM sodium metabisulfite and 0.5 mM phosphoric acid, pH 3	14

[a]Samples were analyzed by procedure II described in Experimental; 1.0 µg assayed immediately after preparation.

content of freshly prepared thiacetarsamide solutions (0.5 mg/mL) containing basic and acid modifiers was determined by procedure II. The greatest conversion to p-arsenosobenzamide, 74%, was obtained with 1.0 N NaOH (Table 5). The chromatographic profile of the 1.0 N NaOH solution of thiacetarsamide showed no thiacetarsamide peak; the solution contained 74% p-arsenosobenzamide and 26% other degradation products. When the sample was reassayed the next day, the p-arsenosobenzamide content had decreased to 39% and a new peak appeared at a longer retention time. The 0.1 N NaOH solution of thiacetarsamide assayed the same on both days. Higher concentrations of

base are necessary to degrade p-arsenosobenzamide than to hydrolyze thiacetarsamide. p-Arsenosobenzamide degraded to unidentified products.

CONCLUSIONS

Thiacetarsamide in solution is a mixture whose composition varies with time and the physical and chemical environment. No thiacetarsamide solution contained only thiacetarsamide. Even fresh reference solutions, stabilized with EDTA or metabisulfite, were mixtures; therefore, no suitable thiacetarsamide standards are available. Without pure thiacetarsamide standards, it is difficult to develop a stability-indicating method. Since EDTA and metabisulfite stabilized thiacetarsamide during LC analysis, their inclusion may improve the stability of thiacetarsamide solution.

Thiacetarsamide solutions must be refrigerated to maintain composition during storage. Further work is needed to find other conditions to completely stabilize thiacetarsamide in solution, which would allow the preparation of pure standards and improve the chromatography of thiacetarsamide.

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