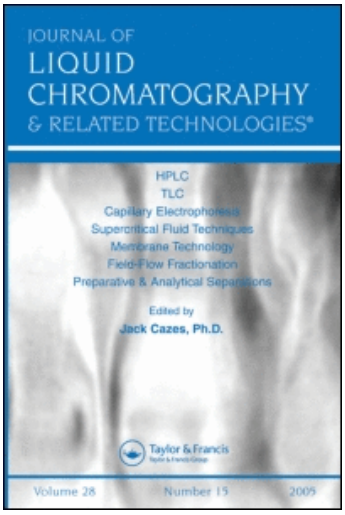

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FACTORS INFLUENCING ON-COLUMN HYDROLYSIS OF THIACETARSAMIDE DURING REVERSED-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

The hydrolytic reaction of thiacetarsamide with reversed-phase packing material produced p-arsenosobenzamide during high-performance liquid chromatography (HPLC). The on-column hydrolysis was inhibited by including compounds in the mobile phase that complex with trace metal contaminants. With ethylenediaminetetraacetic acid (EDTA) and sodium phosphate in the mobile phase at neutral pH, a unique chromatographic profile resulted, with thiacetarsamide not being retained, followed by a bridge to the p-arsenosobenzamide peak. EDTA, bound to the reversed-phase packing material, inhibited on-column hydrolysis of thiacetarsamide when EDTA was not present in the mobile phase. After the HPLC system was cleaned with water to remove the residual EDTA, the bridge disappeared and the p-arsenosobenzamide peak increased, illustrating increased hydrolysis. This occurred whether or not the silica had been treated to remove trace metals. EDTA probably interacted at secondary retention sites containing trace metal contaminants, thus inhibiting the hydrolytic reaction of thiacetarsamide at the silica surface.

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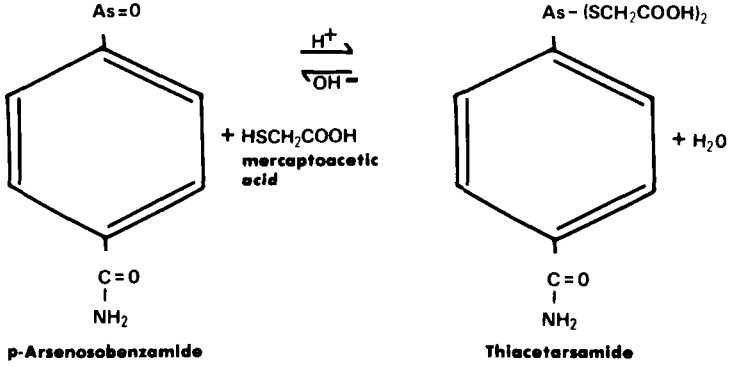
INTRODUCTION

Thiacetarsamide is a mercaptoacetic acid derivative of p-arsenosobenzamide formed in the reversible reaction shown below (1).



Thiacetarsamide, as a 1% neutral aqueous solution (2) is the only drug approved to treat adult canine heartworm (Dirofilaria immitis) disease, and is sold under the tradenames Caparsolate and Filaramide. Thiacetarsamide in aqueous solution produced mixtures of thiacetarsamide and p-arsenosobenzamide with degradation products being detected in strongly basic or room temperature-stored samples (3). The two high-performance liquid chromatographic (HPLC) methods developed to measure the p-arsenosobenzamide content of thiacetarsamide solutions inhibited on-column hydrolysis. The mobile phases contained components previously shown to suppress trace metal activity (4). The adverse effects of trace metals present in HPLC packing material on the chromatography of chelating, basic, or

acidic compounds is often observed as broad or badly tailing peaks and reduced recovery (5). Tetracycline analysis required the presence of ethylenediaminetetraacetic acid (EDTA) in the mobile phase to prevent binding to the reversed-phase packing material (6). Improvement in peak shape, resolution, and recovery of hop bitter acids on reversed-phase packing material required the suppression of trace metal activity or treatment of the packing material to remove trace metals (2,4). This paper presents an analysis of the factors influencing on-column hydrolysis of thiacetarsamide.

EXPERIMENTAL

Reagents and Materials

HPLC grade methanol, acetonitrile, isopropanol, and tetrahydrofuran (Burdick & Jackson Laboratories, Muskegon, MI) were used. All other chemicals were reagent grade. Water was glass-distilled. Aqueous components of the mobile phase were filtered through a Millipore 0.45-um HA filter, using an all-glass apparatus. The mobile phases were degassed in an ultrasonic bath under reduced pressure before use.

Caparsolate acid (thiacetarsamide), caparsolate reference standard, and p-arsenosobenzamide were supplied by Abbott Laboratories, North Chicago, IL, and stored in the dark at 8°C until used.

Preparation of Test Solutions

Thiacetarsamide and p-arsenosobenzamide were dissolved by heating to 60°C on a Temp-Blok module heater and frequently mixing with a Vortex-Genie mixer. The solutions were cooled to room temperature, and then filtered through a 0.45-um Millipore filter before HPLC analysis. The usual solvent was the mobile phase. The solutions were stored at 8°C in the dark. p-Arsenosobenzamide standards were prepared in methanol-water with 0.25 mM sodium phosphate and 0.0125 mM sodium EDTA pH 7 (15:85) at a concentration of approximately 0.06 mg/ml. Solutions of 0.06 to 0.005 mg/ml or 0.3 to 0.02 mM were prepared by dilution. These standards were stable when stored at 8°C in the dark; 10-ul aliquots of the standards were analyzed along with the thiacetarsamide solutions. The coefficient of variation for the standard curves usually exceeded 0.99.

Apparatus

The chromatographic equipment consisted of a Waters M-6000A delivery system, Waters 440 absorbance (254 nm) fixed-wavelength detector, Omni-Scribe 10-mV recorder, Hewlett-Packard Model 3390-A recording integrator, and either a Valco CV-6-UHPa-N60 200-ul or Rheodyne 7125 20-ul loop injection valve. Samples were introduced with the appropriate Hamilton syringe. The analytical columns were 10-um Zorbax C-8 25 cm x 4.6 mm I.D. and R.E. Gourley 10-um Cleer Sil ODS 25 cm x 4.0 mm I.D. The

columns were cleaned each day with water to prevent salt deposition.

RESULTS

The maximum inhibition of on-column hydrolysis of thiacetarsamide was obtained with a mobile phase containing EDTA and sodium phosphate. The chromatographic profile for thiacetarsamide showed a large non-retained peak containing thiacetarsamide, which tailed into the p-arsenosobenzamide peak, forming a bridge between the two peaks (Figure 1). Higher concentrations of the ionic modifiers produced decreased inhibition of the hydrolytic reaction. No improvement in chromatography was observed by including the following antioxidants in the mobile phase: 2,6-di-tert-butyl-p-cresol, 2-mercaptoethanol, sodium bisulfite, and ascorbic acid. The retention time of p-arsenosobenzamide was not affected by the presence of ionic modifiers in the mobile phase. With a mobile phase of water and organic modifiers, thiacetarsamide was completely hydrolyzed and eluted as p-arsenosobenzamide (Figure 1).

To confirm on-column hydrolysis of thiacetarsamide, fractions were collected from a 10-ug injection of thiacetarsamide. Fraction 1, the non-retained peak, contained thiacetarsamide; fractions 2 and 3 were from the bridge; and fraction 4 contained the p-arsenosobenzamide peak. The

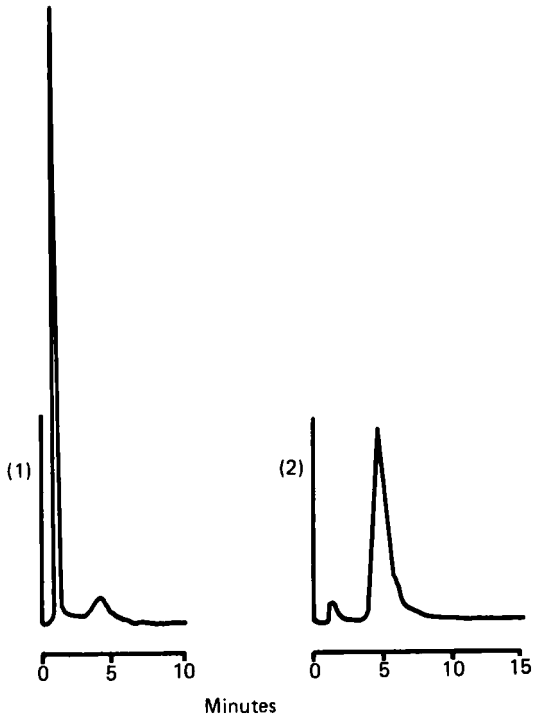


FIGURE 1. Chromatograms of (1) 1 ug of thiacetarsamide, mobile phase methanol-water with 0.25 mM sodium phosphate and 0.0125 mM sodium EDTA pH 6.8 (15:85); (2) 2.5 ug of thiacetarsamide; mobile phase methanol-water (15:85). Column Zorbax C-8, 25 cm x 4.6 mm I.D.; flow rate, 2.0 ml/min; detector wavelength 254 nm at 0.1 absorbance unit full scale (a.u.f.s).

fractions were immediately rechromatographed (Table 1). The chromatographic profile for fractions 1 and 2 showed a significant quantity of non-retained material and p-arsenosobenzamide peaks. This proved that the isolated non-retained material containing thiacetarsamide could further hydrolyze on the column to produce the later-eluting p-arsenosobenzamide.

TABLE 1
Peak Heights of Isolated Thiacetarsamide Fractions[1]

Fraction	Non-retained peak (cm)	p-Arsenosobenzamide peak (cm)
1 Non-retained peak	5.7	1.3
2 First half of bridge	3.6	1.1
3 Second half of bridge	1.4	0.2
4 p-Arsenosobenzamide peak	0.6	1.1

[1]Fractions collected from 10 ug of thiacetarsamide. Chromatographic conditions: Zorbax C-8 column, flow rate 2.0 ml/min, absorbance 0.005 at 254 nm, and mobile phase methanol-water with 0.25 mM sodium phosphate and 0.0125 mM EDTA pH 6.8 (15:85). The non-retained peak is corrected for the mobile phase injection deflection.

The hydrolytic reaction of thiacetarsamide with the reversed-phase packing material can further be demonstrated by reducing the flow rate, thus increasing the contact time of thiacetarsamide with the Zorbax C-8 column. Using the recorded absorbance measurements, the ratio of the non-retained peak height to the p-arsenosobenzamide peak height was calculated. At a 2.0-ml/min flow rate, the calculated ratio was 18. When the flow rate was reduced to 1.0 ml/min and all other conditions were held constant, the calculated ratio declined to 8. Increased exposure to the reversed-phase packing material increased the amount of p-arsenosobenzamide detected.

The role of EDTA and sodium phosphate in the inhibition of on-column hydrolysis was elucidated. The chromatographic profile for thiacetarsamide with sodium EDTA as the only ionic modifier was similar to that observed when both EDTA and sodium

phosphate were present in the mobile phase (Figure 2). When the mobile phase was changed, the column was cleaned with methanol-water (50:50) and then 100% methanol. The column was equilibrated for 1 h before thiacetarsamide was analyzed with the next mobile phase, methanol-water with 0.25 mM sodium phosphate pH 6.8 (15:85). Previous experiments had shown that the magnitude of inhibition of the hydrolytic reaction was greater than could be attributed to the presence of sodium phosphate alone (Figure 2). In an attempt to remove residual EDTA that may have remained bound to the C-8 packing, the column was cleaned with water. After re-equilibration with the same sodium phosphate-containing mobile phase, thiacetarsamide was again analyzed (Figure 2). The chromatographic profile had changed significantly, with the bridge disappearing and the p-arsenosobenzamide peak increasing (Figure 2). The residual EDTA bound to the reversed-phase packing material had inhibited the hydrolysis of thiacetarsamide to p-arsenosobenzamide; water was required to remove the bound EDTA.

In an attempt to further reduce or eliminate on-column hydrolysis of thiacetarsamide, a Cleer Sil ODS column was evaluated. The silica used to manufacture the Cleer Sil ODS column had been treated to remove divalent cations, thus reducing the influence of trace metal contaminants. The response of thiacetarsamide and p-arsenosobenzamide solutions with mobile phases containing 15% methanol and 85% aqueous

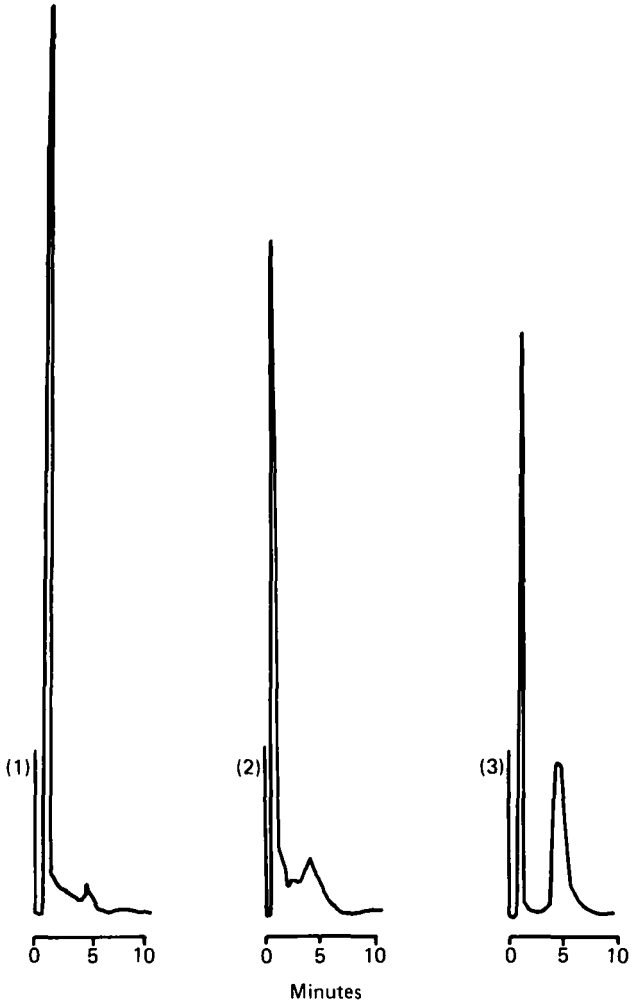


FIGURE 2. Chromatograms of 2.5 ug of thiacetarsamide. Column Zorbax C-8, 25 cm x 4.6 mm I.D.; mobile phase (1) methanol-water with 0.0125 mM sodium EDTA pH 7 (15:85), (2) and (3) methanol-water with 0.25 mM sodium phosphate pH 6.8 (15:85), (2) before and (3) after cleaning column with water; flow rate 2.0 ml/min; detector wavelength 254 nm at 0.1 a.u.f.s.

component (v/v) was determined (Figure 3). p-Arsenosobenzamide standard curves were prepared with each mobile phase tested. The peaks were more uniform for the p-arsenosobenzamide component of thiacetarsamide and p-arsenosobenzamide standards than those produced with the Zorbax C-8 column. The quantity of p-arsenosobenzamide detected in the thiacetarsamide solution was calculated from these standard curves. The percent conversion of thiacetarsamide to p-arsenosobenzamide was then calculated using both peak height and peak area data (Table 2).

TABLE 2
Thiacetarsamide Solutions Analyzed for p-Arsenosobenzamide Recovered on a Cleer Sil ODS Column[1]

Mobile phase	Caparsolate acid[2]		Caparsolate reference standard[3]	
	mM	% Conversion	mM	% Conversion
A[4]	1.4	102 (3)[5]	1.3	98 (3)[5]
B	0.5	37 (3)	0.5	37 (3)
C	0.2	16 (4)	0.2	15 (4)
D	0.3	19 (3)	0.3	20 (3)
E	0.8	57 (4)	0.7	51 (3)
A[6]	1.3	100 (3)	1.2	94 (3)
B	1.2	89 (3)	0.9	68 (3)
C	0.4	31 (4)	0.3	21 (4)
D	0.6	47 (3)	0.6	46 (3)
E	1.3	100 (4)	1.2	90 (3)

[1]1.326 mM solutions of thiacetarsamide were analyzed with a p-arsenosobenzamide standard curve using peak height and area data and different aqueous components 85% v/v with methanol. The aqueous components were (A) water, (B) 0.25 mM sodium phosphate pH 6.5 before the column was exposed to EDTA, (C) 0.25 mM sodium phosphate and 0.0125 mM sodium EDTA pH 6.7, (D) same mobile phase as (B) after the column was exposed to EDTA without cleaning with water, and (E) with cleaning with water.
[2]Active ingredient for the manufacture of the formulated drug product.
[3]Abbott Laboratories house reference standard.
[4]Peak height.
[5]Number of replicate analyses in parentheses.
[6]Peak area.

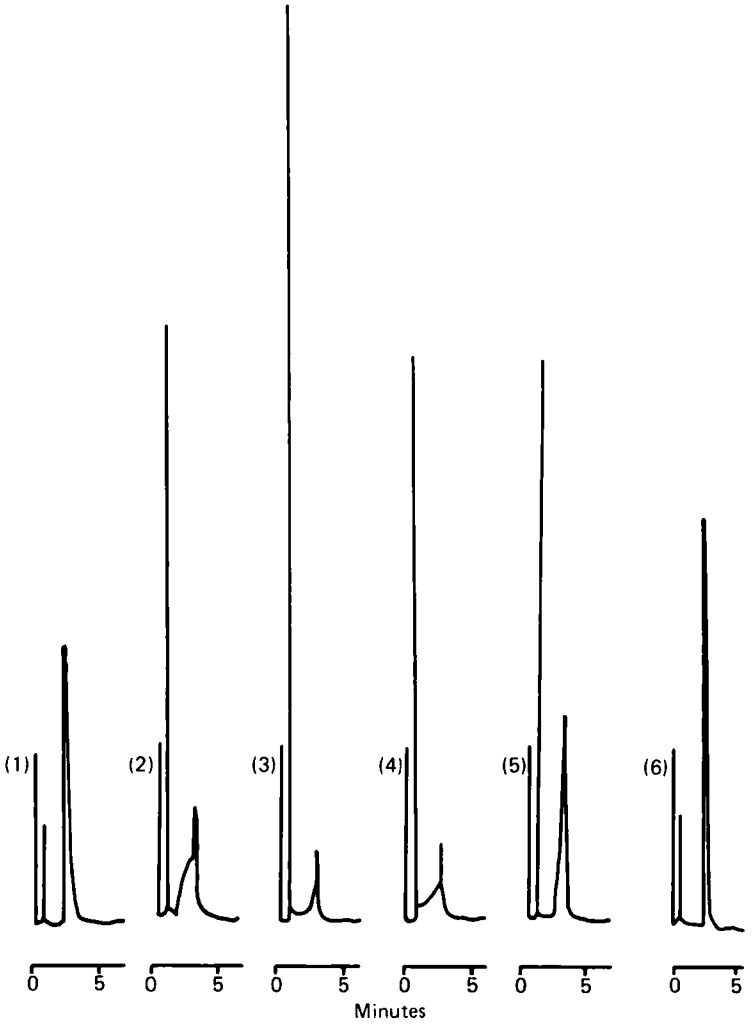


FIGURE 3. Chromatograms of 1 ug of thiacetarsamide (1) to (5) and 0.57 ug of p-arsenosobenzamide (6). Column Cleer Sil ODS 25 cm x 4.0 mm I.D.; mobile phase (1) methanol-water (15:85), (2) methanol-water with 0.25 mM sodium phosphate pH 6.5 (15:85) before column was exposed to EDTA, (3) and (6) methanol-water with 0.25 mM sodium phosphate and 0.0125 mM sodium EDTA pH 6.7 (15:85), (4) and (5) same mobile phase as (2) after column was exposed to EDTA, (4) without cleaning with water and (5) with cleaning with water; flow rate 2.0 ml/min; detector wavelength 254 nm at 0.1 a.u.f.s.

The response of thiacetarsamide to water and sodium phosphate was determined before the Cleer Sil ODS column was exposed to EDTA (Figure 3). Without ionic modifiers in the mobile phase, thiacetarsamide was quantitatively hydrolyzed to p-arsenosobenzamide. Sodium phosphate decreased the hydrolysis; however, the pronounced peculiar skewing on the front of the p-arsenosobenzamide peak distorted this measurement. The maximum inhibition of on-column hydrolysis was observed with both EDTA and sodium phosphate in the mobile phase; this produced the smallest quantity of p-arsenosobenzamide detected. The resulting chromatographic profile showed the largest non-retained peak containing thiacetarsamide and the smallest bridge resulting from inhibition of the hydrolytic reaction. Without cleaning the column with water, the mobile phase was changed to contain sodium phosphate alone. The p-arsenosobenzamide detection was similar to that obtained with EDTA and sodium phosphate in the mobile phase; however, the non-retained peak was smaller and the bridge larger. The column was then cleaned with water and re-equilibrated with the sodium phosphate-containing mobile phase. The detected p-arsenosobenzamide increased approximately two-fold; cleaning the column with water was necessary to remove residual EDTA. Again, the EDTA bound to the reversed-phase packing material was able to inhibit on-column hydrolysis of thiacetarsamide.

DISCUSSION

The inhibition of on-column hydrolysis of thiacetarsamide is very sensitive to mobile phase composition and reversed-phase packing material. The procedure developed to analyze thiacetarsamide solutions for degradation products required that the concentration of both ionic modifiers, sodium metabisulfite and phosphoric acid, be carefully adjusted to avoid fused peaks and achieve partial resolution of thiacetarsamide and p-arsenosobenzamide (3). The best results were obtained with the Zorbax C-8 column. The especially prepared Cleer Sil column produced better peak shapes; however, the hydrolytic reaction was not eliminated (Figure 3). This contrasts with the behavior of hop bitter acids, where both peak shape and recovery were improved by using acid-treated reversed-phase packing material, without the need for trace metal activity suppressors in the mobile phase (4,7). Tetracycline analysis at residue levels, however, required the presence of EDTA in the mobile phase as well as acid-treated reversed-phase packing material to achieve good recovery and peak shapes (6,8). Trace metal contaminants probably are involved in the hydrolytic reaction of thiacetarsamide to produce p-arsenosobenzamide.

In reversed-phase HPLC, the primary retention mechanism is binding to the bonded groups; however, the silica surface is capable of absorbing or reacting with the solute molecules, giving rise to secondary retention mechanisms (9,10). Since the

hydrophobic bonded groups are poorly wetted by the very ionic mobile phases used, they preferentially interact with each other, providing exposure to the silica surface. Very polar or ionic molecules like thiacetarsamide easily penetrate between the bonded groups and interact with secondary binding sites at the silica surface. This interaction with reactive secondary retention sites catalyzed the hydrolysis of thiacetarsamide to p-arsenosobenzamide. Once thiacetarsamide was hydrolyzed to p-arsenosobenzamide, it was retained and eluted with a retention time between the retention times of thiacetarsamide and p-arsenosobenzamide, producing a bridge between the two compounds. Unprotected, thiacetarsamide hydrolyzed instantly at the beginning of the column and eluted with a retention time identical to that of p-arsenosobenzamide.

The measurement of the p-arsenosobenzamide content of thiacetarsamide solutions was influenced by the bridge region produced during analysis. Since the bridge region was more pronounced with the Zorbax C-8 than the Cleer Sil ODS column, larger distortions were observed in the area measurements with the Zorbax C-8 column. The peak width for p-arsenosobenzamide detected in thiacetarsamide solutions was wider than for p-arsenosobenzamide solutions, thus influencing the area and height measurements. Although the p-arsenosobenzamide content calculated from the height and area measurements differs as a result of these distortions, the trends are identical (Table 2). As previously reported, with both ionic modifiers in the

mobile phase, p-arsenosobenzamide content calculated from height data was more consistent than that obtained with area data (3). A significant inhibition of hydrolysis by sodium phosphate alone was observed with the height data but not with the area data. The inhibition of hydrolysis by EDTA, either in the mobile phase or bound to the column packing material, was clearly reflected in both measurements of p-arsenosobenzamide conversion.

The silica used in reversed-phase packing material is variable (10), including the quantity and variety of metals (11). The silica produced pH differences in aqueous solutions (12), and this variability has an influence on solute retention and catalytic reactivity. Zorbax silica produced acid conditions that should reduce reaction with thiacetarsamide. Cleer Sil silica was extensively washed with acid to remove divalent cations. EDTA was tightly bound to both reversed-phase packing materials, being eluted only with 100% water. An EDTA derivative, bound to a silica ion-exchange surface, also required 100% water to remove it (13). The mobile phase, the chromatographic equipment, and the derivatization reagent used to prepare the reversed-phase packing material could all introduce reactive trace metal contaminants to the silica surface (5). Reversed-phase packing material can behave as a cation-exchange resin; the residual silanols bond strongly to trace metal cations, especially iron and magnesium (14). The on-column hydrolytic reaction of thiacetarsamide was inhibited by the presence of trace metal activity suppressors, which

probably interact with secondary retention sites containing trace metal contaminants.

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