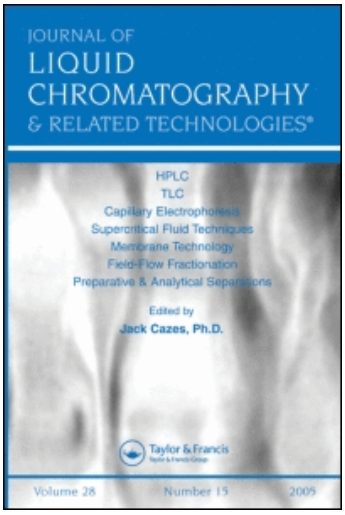

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High-Pressure Liquid Chromatography Separation of Potential Impurities of Altretamine

Alem Ghiorghis[a]; Abdol H. Talebian[a]
[a] Department of Chemistry, Georgetown University, Washington, D.C.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHY SEPARATION OF POTENTIAL IMPURITIES OF ALTRETAMINE

ALEM GHIORGHIS AND ABDOL H. TALEBIAN

Department of Chemistry
Georgetown University
37th & O Street N.W.
Washington, D.C. 20057

ABSTRACT

Two isocratic liquid chromatographic separation methods and UV detection were developed to allow for sensitive and specific analysis of potential impurities in Altretamine using a reversed phase C_{18} column and mixtures of water-acetonitrile as mobile phase. Linear calibration curves for each of the possible contaminants of Altretamine, in the range of 0.25-125 μg/ml, were also obtained. The detection limit for all of the compounds (except cyanuric acid) was less than 0.25 μg/ml. Several Altretamine lots were examined and their impurities identified. Hydrolysis of cyanuric chloride to cyanuric acid was studied and shown to follow 1^{st} order kinetics. Evidence for the formation of chlorohydroxytriazine intermediates during the hydrolysis of cyanuric chloride to cyanuric acid is given.

INTRODUCTION

Altretamine, Hexamethylmelamine (HMM) is an antitumor agent active against several solid human tumors (1-

2). HMM has recently been approved by FDA as a secondary line of treatment against ovarian cancer. It is now being manufactured for clinical use and as such it is desirable that its quality be assured by common standards acceptable to the FDA and all participating countries of the European Pharmacopoeia Convention.

Altretamine is synthesized by reacting cyanuric chloride with dimethylamine in aqueous media. Several potential byproducts, however, could be formed during the reaction as shown in Scheme **1**. Complete and partial hydrolysis of cyanuric chloride produces cyanuric acid and chlorohydroxytriazines (compounds **6** and **7**), respectively. On the other hand, its incomplete reaction with dimethylamine would produce 6-chloro-2,4-bis(dimethylamino)-1,3,5-triazine (compound **1**) and 4,6-dichloro-2-dimethylamino-1,3,5-triazine (compound **2**). Partial hydrolysis of compound **2** leads to 4-chloro-6-dimethylamino-1,3,5-triazin-2(1H)-one (compound **4**). Hydrolysis of **1** or substitution of the chlorines in compounds **4** and **6** with dimethylamine would result in compound **3**. Replacement of the chlorine in compound **7** with dimethylamine or hydrolysis of **4** would give 6-dimethylamino-1,3,5-triazine-2,4(1H,3H)¬dione (compound **5**).

Potential contaminants of Altretamine during its manufacture can be one or a combination of two or more of the above listed byproducts. Since the finished HMM should have, if any, less than 0.1% impurity(ies), it is important that the method of analysis be sensitive and capable of detecting, separating, and quantifying traces of these possible impurities in the presence of a very large excess of HMM.

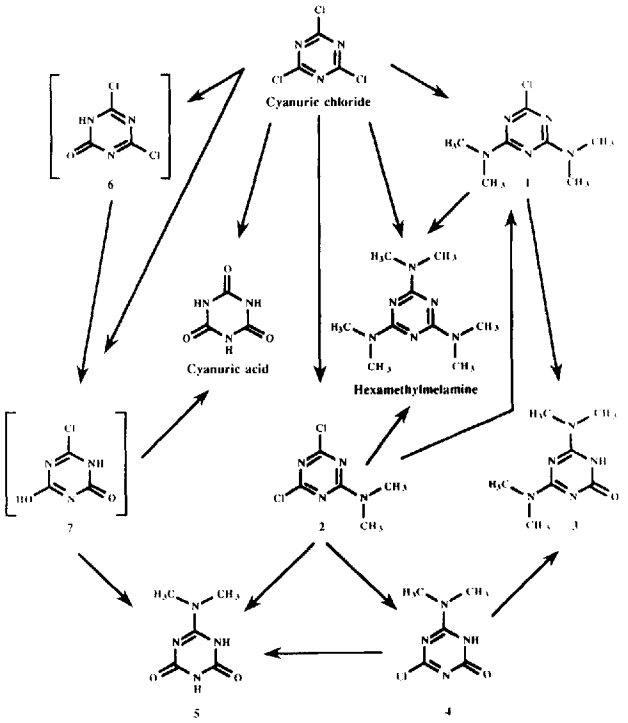
High pressure liquid chromatography has been widely employed in determining the purity of samples, separations, identification of impurities and metabolites, and pharmacological studies of drugs. We have therefore con-

sidered liquid chromatography as the first method of choice for detection and separation of potential contaminants in HMM. Several HPLC methods have previously been developed for determination of HMM and its metabolites in biological fluids (3-7). Because of the herbicidal activity and dye properties of the s-triazine derivatives, a number of papers have been published on their separation and detections (8-14). But, no report was found on the chlorohydroxytriazines and only a few have dealt with hydroxy and aminotriazine derivatives (15-16). Lawrence and Leduc (17) have used normal phase HPLC with organic solvents containing traces of organic acids as mobile phases to separate hydroxytriazines. Although this method gives satisfactory results, it is not entirely suitable for our purpose since the chlorotriazines and the chlorohydroxytriazines are sensitive to acid hydrolysis (18). Furthermore, cyanuric acid and compound **5** are insoluble in organic solvents. Here we report on two isocratic HPLC systems that together separate and detect all of the potential byproducts (0.01% relative to the amount of HMM) of HMM shown in Scheme **1**.

EXPERIMENTAL

Chemicals

The following chemicals were obtained from the source indicated: HPLC grade water (Fisher Scientific Co., Fair Lawn, New Jersey); HPLC grade acetonitrile (J. T. Baker, Phillipsburg, New Jersey); cyanuric acid (98%) and cyanuric chloride (99%) (Aldrich Chemical Co., Milwaukee, Wisconsin); Altretamine (Pharm-Eco, Simi Valley, California, lot#'s 3244, 3244B, 2808, 2623, 3060, 2840 and 799.A.90.1-5; Park-Davis H739646. Other standards, this work (19).



SCHEME 1

Chemical Pathway for the Formation of Potential Impurities of Altretamine

Apparatus

All HPLC assays for the separation of standards were performed at ambient temperature using a chromatographic system consisting of a Waters 6000A pump, a Waters U8K injector and a Waters Lambda-Max 480 detector (Waters Associates, Milford, Massachusetts) connected to a linear 282/MM recorder (Linear Instruments Corp., Wheaton, Maryland) or a Waters Model 730 Data Module. A 5-μm Ultrasphere C_{18} column (Beckman), with dimensions of 250 mm x 4.6 mm, was used.

Liquid Chromatography Conditions

HPLC solvents were filtered through a millipore GVWP 0.5 μm membrane and degassed under vacuum before use. Mobile phases consisted of: water-acetonitrile, 50:50 (system 1) or water-acetonitrile, 95:5 (system 2). Solutions of triazine derivatives were prepared in the mobile phase for injection unless otherwise mentioned in the experimental section. Samples of each standard, blank injection and the mixtures were injected onto the column equilibrated with the mobile phase with a flow rate of 1.5 ml/min. The column eluent was monitored at 226 nm and, depending on the solute concentrations, the detector sensitivity was set between 0.01-1.0 AUFS (absorption unit for full scale).

Preparation of standards

HMM and HMM solutions containing 0.01% and 0.1% (relative to HMM) of cyanuric acid and compounds **1**, **2**, **3**, **4** and **5** were prepared as follows: 50 mg HMM was accurately weighed and transferred into a 100 ml volumetric flask. This was dissolved in the mobile phase and diluted to the 100 ml mark with the same solvent to give HMM stock solution. Separate samples of cyanuric acid and compounds **1**, **2**, **3**, **4** and **5** (5 mg each) were accurately weighed and transferred into a 10 ml volumetric flask. This mixture was then dissolved in the HMM stock solution and further diluted to the 10 ml mark with the HMM stock solution. This provided a solution containing 0.5 mg/ml of HMM, cyanuric acid, and compounds **1**, **2**, **3**, **4** and **5**. Exponential dilution of this solution by 3 and 4 fold with the HMM stock solution provided HMM solutions containing 0.1 and 0.01% of cyanuric acid and compounds **1**, **2**, **3**, **4** and **5**. Cyanuric chloride was not included in the mixture since our preliminary data showed that it hydrolyzes very rapidly when dissolved in a 1:1-water-

acetonitrile (mobile phase 1). Standard solutions of individual compounds were prepared by accurately measuring 10 mg of each compound and dissolving them in 20 ml of the mobile phase. These provided stock solutions of each compound. Serial dilutions of these stock solutions, by the mobile phase, to the desired concentrations gave the standards used for constructing the calibration curves. For the separation of cyanuric acid and compounds **4** and **5**, stock solutions of each compound were prepared by dissolving 10 mg of each compound in 20 ml of water-acetonitrile (95:5). A mixture of the stock solutions of cyanuric acid and compounds **4** and **5** (100: 5: 1), respectively, were used for injections.

RESULTS AND DISCUSSION

The main purpose of this investigation was to develop an HPLC method which would allow the separation and identification of all of the possible impurities of Altretamine, shown in Scheme **1**, in the presence of a large excess of the drug. In pharmacopoeial testings it is thought inappropriate to use a gradient HPLC technique because of possible reproducibility problems in different laboratories. Thus, we decided to focus on developing a simple isocratic system with UV detection that is fast and sensitive.

After testing several C_{18} and silica columns, the best separation was obtained with a 5-μm Beckman Ultrasphere C_{18} column (250 mm x 4.6 mm) using water-acetonitrile (50:50) as mobile phase. As shown in Figure **1**, except for cyanuric acid and compounds **4** and **5**, clean separation with acceptable capacity factors (>1) are observed for the mixture. Cyanuric chloride was not included in this mixture since our preliminary data showed that it decomposes very rapidly in water-acetonitrile (50:50).

A representative liquid chromatogram for a blank injection, HMM (Pharm-Eco 3244 as finished product representative) and an HMM solution containing 0.01 and 0.1% of cyanuric acid and compounds **1**, **2**, **3**, **4** and **5** are shown in Figure **1**. As evident from the chromatogram of the mixture, clean separation of all peaks (except for cyanuric acid and compounds **4** and **5**) with symmetrical peak shape occurred. Chromatogram $\mathbf{1}_c$ (Figure **1**) shows that traces (0.25 μg/ml, 0.01% relative to the amount of HMM) of compounds **1**, **2**, **3**, **4** and **5** can be detected. Examination of several batches of Altretamine lots (crude and finished products) revealed that they contain 2 to 4 impurities ranging from 0.05 to 0.6% of Altretamine (Table **1**). The peaks at about 5.7 and 7.8 minutes correspond to compounds **1** and **2**, respectively, but those at 4.7 and 9.7 minutes do not match the retention times of any of the potential impurities listed in Scheme **1**.

Table **2** shows the capacity factors for Altretamine and its potential impurities. With water-acetonitrile (50:50) as eluent, the capacity factors are greater than 1 except for cyanuric acid and compounds **4** and **5**. Cyanuric acid and compound **5** elute together with compound **4** as an unresolved shoulder (chromatograms $\mathbf{1}_c$ and $\mathbf{1}_d$, Figure **1**).

Polarity of the mobile phase influences solute retention in reversed-phase liquid chromatography. The retention time of most solutes increases as the polarity of the mobile phase is increased. Complete separation of cyanuric acid and compounds **4** and **5** is achieved by increasing the polarity of the mobile phase by raising its water content to 95% (system 2). Figure **2** shows the effect of eluent polarity on separation of cyanuric acid and compounds **4** and **5**. As evident from Figure **2**, there is a clear separation between cyanuric acid and compounds **4**

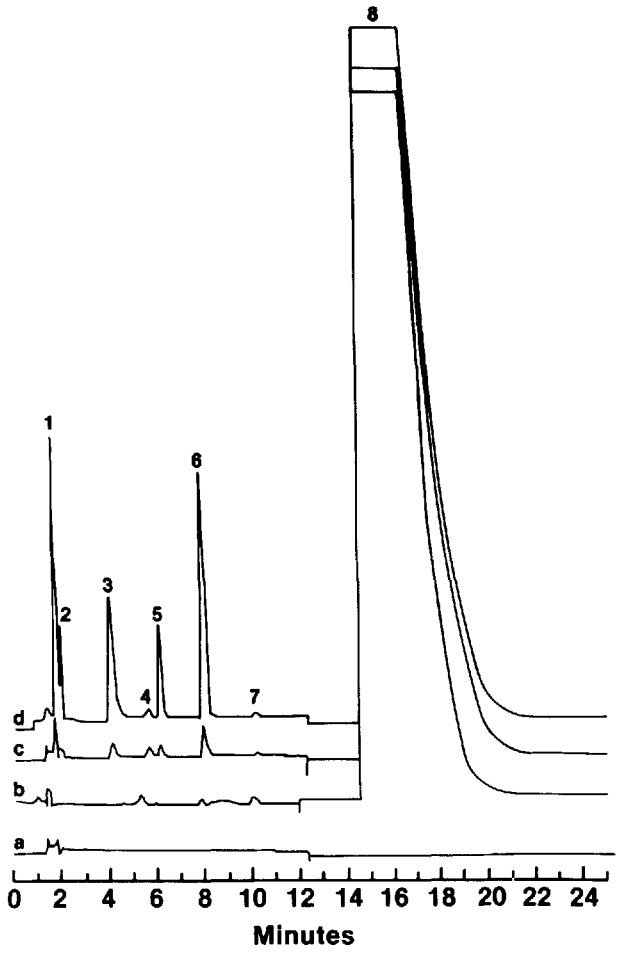


Figure 1. HPLC separation of potential impurities of Altretamine. a) blank injection. b) HMM (Pharm-Eco 3244). c) 0.01% cyanuric acid and compounds **1-4** and **5** in HMM solution. d) 0.1% cyanuric acid and compounds **1-4** and **5** in HMM solution. Peaks: 1= cyanuric acid + compound **5**; 2= compound 4; 3= compound 3; 4= unknown; 5= compound 2; 6= compound 1; 7= unknown; 8= Altretamine.

TABLE 1

HPLC ANALYSIS OF DIFFERENT BATCHES OF ALTRETAMINE

Batch	%Impurities						
	compd.1	compd. 2	compd. 3 & CA[d]	compds. 4, 5	CC[a]	unk. 1[b]	unk. 2[c]
Pharm-Eco.							
3244	<0.01	--	--	<<0.01	--	<0.03	<0.01
3244B	<0.01	--	--	--	--	<0.03	<0.01
A.90.1[e]	<0.04	--	--	--	--	<0.06	<<0.01
A.90.2[e]	<0.04	--	--	--	--	<0.06	<<0.01
A.90.3[e]	<0.04	--	--	--	--	<0.06	<<0.01
A.90.4[e]	<0.04	--	--	--	--	<0.06	<<0.01
A.90.5[e]	<0.04	--	--	--	--	<0.06	<<0.01
2840	--	<0.15	--	<0.01	--	<0.08	<0.05
3060	--	--	--	--	--	<0.06	<0.01
2808	--	<0.15	--	--	--	<0.06	<0.05
2623	--	<0.01	--	--	--	<0.40	<0.15
Park-Davis							
739646	<0.01	--	--	--	--	<0.03	<0.015

Reversed-phase column: 5 μm Beckman Ultrasphere C_{18}, 250 mm X 4.6 mm. Mobile phase: water-acetonitrile (50:50). Determination carried out by peak area. [a] Cyanuric chloride. [b] Unknown with retention time of 4.7 minutes. [c] Unknown with retention time of 9.7 minutes. [d] Cyanuric acid. [e] Crude sample.

TABLE 2

Retention Times and Capacity Factors for Potential Impurities of HMM

Compounds	System 1[a]		System 2[b]	
	t_r[c]	k'[d]	t_r	k'
1	7.80	5.24	--	--
2	5.95	3.76	--	--
3	3.87	2.10	--	--
4	1.81	0.45	12.09	7.76
5	1.61	0.29	2.70	0.96
CA	1.61	0.29	1.95	0.41

Reversed-phase column: 5-μm Beckman Ultrasphere C_{18}, 250 mm x 4.6. Mobile phase: [a] water-acetonitrile (50:50); [b] water-acetonitrile (95:5).[c] retention time. [d] capacity factor.

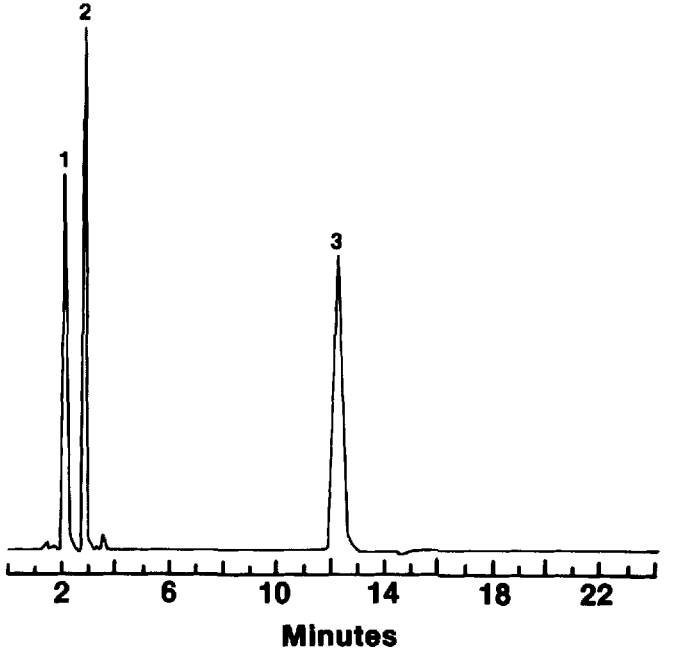


Figure 2. HPLC separation of cyanuric acid and compounds **4** and **5**, system 2. Peaks: 1= cyanuric acid, 2= compound **5**, 3= compound **4**.

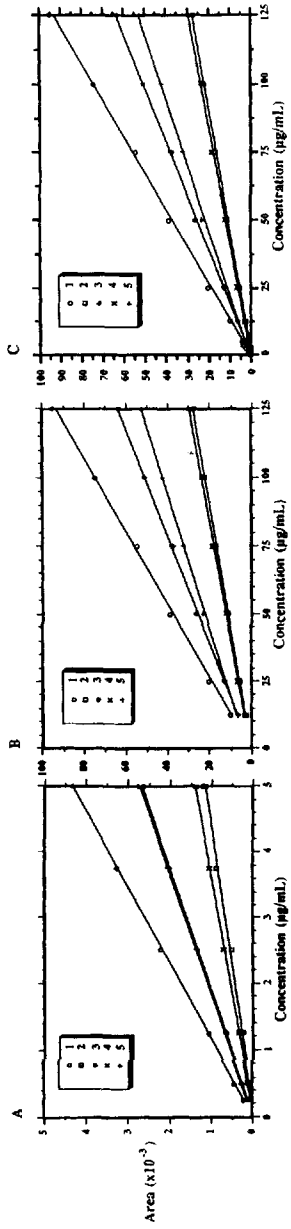
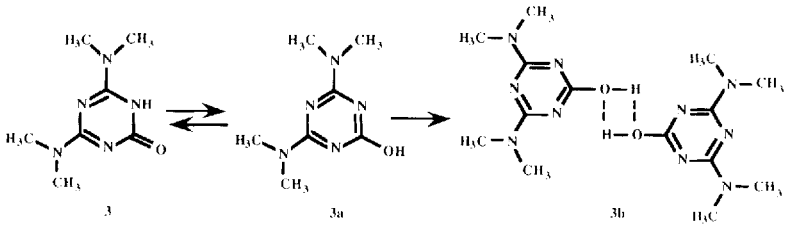


Figure 3. Caliberation curves for compounds **1-5**. A) 0.25-5 µg/ml. B) 12.5-125.0 µg/ml. C) 0.25-125.0 µg/ml.



and **5** with capacity factors of 0.41, 0.96, and 7.76, respectively (Table **1**). Under system **2** conditions compounds **1**, **2**, **3**, and HMM remain on the column but they can be eluted by increasing the acetonitrile content of the mobile phase.

Linearity and detection limit were determined by constructing separate calibration curves for each compound using linear regression. As shown, calibration curves for each compound for the lower range of 0.25-5.0 μg/ml (Figure 3_a) and the higher range of 12.5-125.0 μg/ml (Figure 3_b) and over the entire range of 0.25-125.0 μg/ml (Figure 3_c) are linear with correlation coefficients greater than 0.995. The detection limit for cyanuric acid was 5 μg/ml. This represents a peak about two times the baseline noise at 0.01 AUFS.

An interesting observation was the retention time behavior exhibited by compound **3**. Its retention time changed from experiment to experiment (depending on the sample size) in the region of 1.90-3.95 minutes when system **1** was employed.

Compound **3** can theoretically exist in two tautomeric forms (keto-enol, structures **3** and 3_a). The enolate isomer is known to form a hydrogen-bonded dimer (structure 3_b) (20). Normally, the degree of dimerization increases as the concentration of monomer increases. Since the hydrogen-bonded dimer is expected to be less polar than its monomer, it should elute at a later retention time in

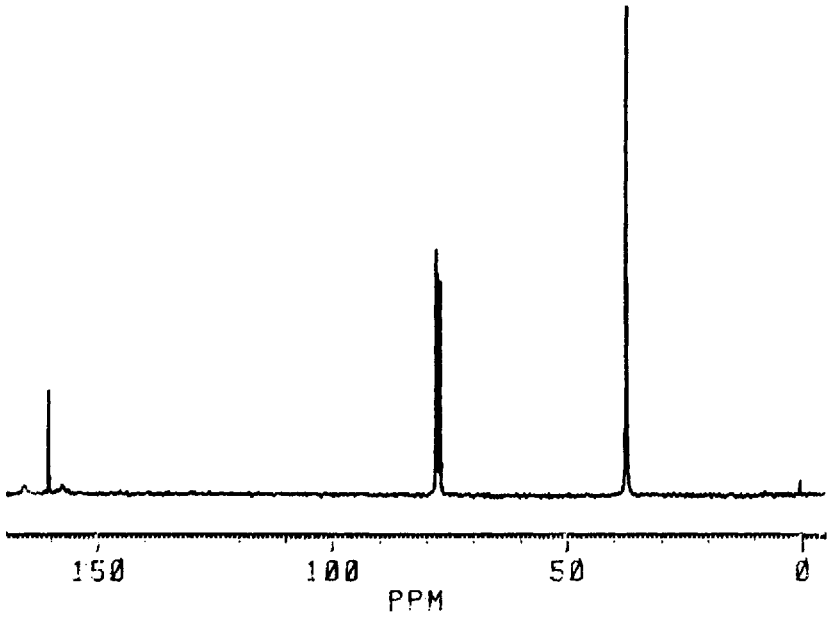


Figure 4. 75.469 MHz ^{13}CNMR spectra of compound **3** in $CDCl_3$. Bruker AM-300. Chemical Shifts are relative to TMS as internal standard.

a reverse phased HPLC mode. However, as the concentration of **3** increases, its retention time decreases. This is opposite to what is expected if its degree of dimerization is to be responsible for the changes in its retention time.

^{13}CNMR spectrum of compound **3** (Figure **4**) shows three different carbon peaks at 165.156, 160.092, and 157.190 ppm. The resonance signal at 160.092 ppm is assigned to carbons 4 and 6. The two broad peaks at 165.516 and 157.190 ppm are assigned to the keto-enol carbon (carbon 2). The broadening of the signals is due to the slow interchange of the tautomers on the NMR time scale.

The decrease in the retention time for the more concentrated solution of **3** together with the manifestation of two different carbonyl resonances in the ^{13}CNMR for carbon 2 suggest that, in solution, compound **3** exists in its two tautomeric forms. Since keto-isomer is more polar than the enolate form, the decrease in retention time for the more concentrated solution of **3** may indicate that, the keto isomer is favored as concentration increases.

Although cyanuric chloride was not included in the mixture its retention time was determined at conditions used for the mixture. Figure **5** shows that cyanuric chloride dissolved in a water-acetonitrile (1:1) decomposes to compounds **6** and **7** in less than two hours and then slowly produces cyanuric acid (24 hours). The rate of hydrolysis of cyanuric chloride to its chlorohydroxytriazine derivatives follows 1st order kinetics (Figures **6**$_a$ and **6**$_b$). Under the HPLC condition 1, cyanuric acid and compounds **6** and **7** are eluted together. Although there has not been a complete study on the hydrolysis of cyanuric chloride, the formation of the chlorohydroxytriazines (compounds **6** and **7**) has been suggested (21,22). Our HPLC data confirms, though could not identify, the formation of such intermediates. This is based on the observed absorption intensity for the peak at 1.21 minutes during the first two hours of hydrolysis and its gradual decrease in the next 24 hours. Cyanuric acid displays a much smaller absorption intensity than that observed for its chlorohydroxy derivatives (Figure **5**$_b$) as evident from the chromatogram of an authentic sample of cyanuric acid (Figure **5**$_d$).

To determine whether or not cyanuric chloride decomposes on the column or in the presence of Altretamine, separate samples of cyanuric chloride were dissolved in pure acetonitrile and pure acetonitrile

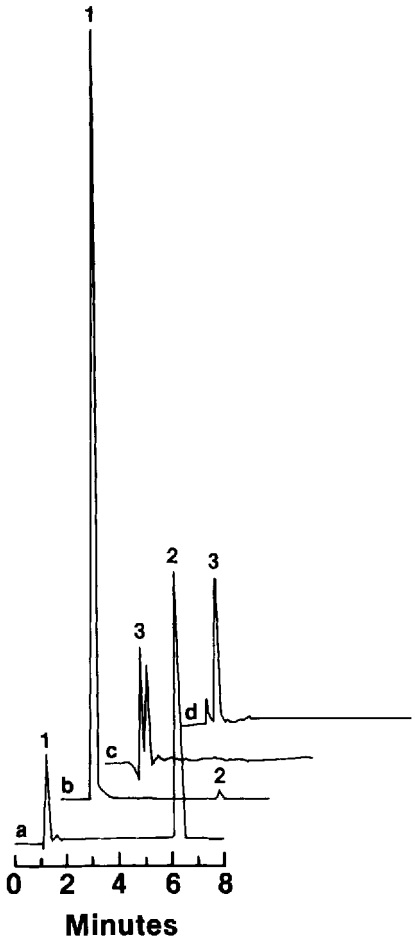


Figure 5. Decomposition study of cyanuric chloride in water-acetonitrile (1:1). a) immediately after dissolution. b) 63 minutes after dissolution. c) 24 hr after dissolution. d) authentic sample of cyanuric acid. Peaks: 1 = chlorohydroxytriazine intermediates (compound **6** and **7**), 2 = cyanuric chloride, 3 = cyanuric acid.

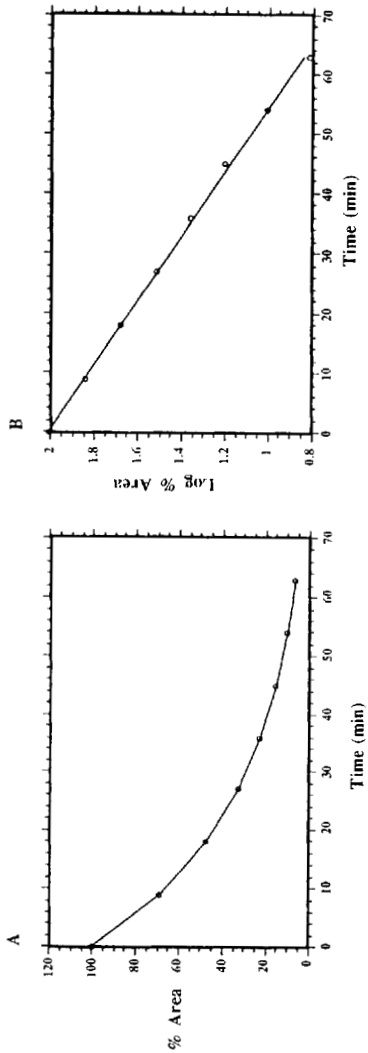
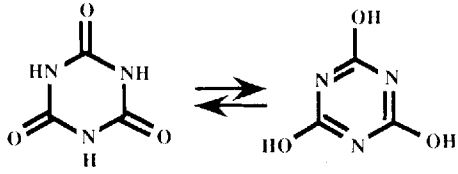


Figure 6. Decomposition of cyanuric chloride in water-acetonitrile (1:1). A) rectilinear graph. B) semi-logarithnic graph.

containing Altretamine and its rate of decomposition was monitored by HPLC over several hours. Minimal decomposition, if any, was observed indicating that it does not decompose in acetonitrile, on the column, or in the presence of HMM.

Hydroxytriazines can exist in two tautomeric (keto-enol) forms in which the enolate isomer has greater double bond character. The lower absorption intensity displayed by cyanuric acid (Figure 5_d), under these conditions, indicates that it exists in its keto-form.



The isocratic separation systems developed here provide a direct and fast method for detection and analysis of the potential byproducts in the finished HMM. System **1** can be used to unequivocally detect and identify compounds **1**, **2**, **3** and the two unknowns, retention times of about 4.7 and 9.7 minutes, in Altretamine samples. If a peak(s) at the region where cyanuric acid and compounds **4** and **5** overlap is observed system **2** can then be employed to separate and identify it(them). This system is particularly advantageous since the hydroxytriazines are detected and separated without derivatization, which has been used previously (16). Furthermore, it does not require addition of acids or bases to the mobile phase which could decompose chlorotriazines.

ACKNOWLEGMENTS

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