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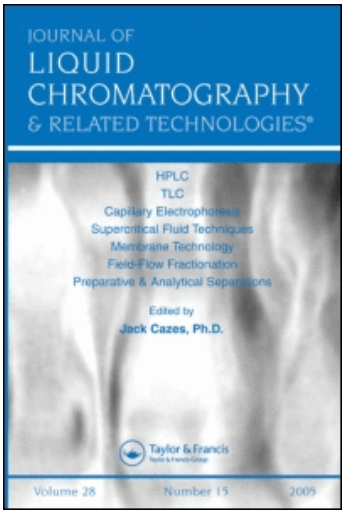

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USES OF ALKALINE DEGRADATION FOR THE ANALYSIS OF DIHYDROTRIAZINES VIA HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC method is described for the determination of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3',4'-dichlorobenzyloxy)-1,3,5-triazine hydrochloride (WR 38839). The procedure required the isomerization of the drug sample by alkaline treatment with sodium hydroxide, as the parent compound was retained by the column. The reaction product of the drug was analyzed by HPLC using a strong cation exchange resin as the stationary phase and glycine buffer, pH 10.4 as the mobile phase. The product was isolated and identified by TLC, UV, IR, mass spectroscopy and elemental analysis. The postulated mechanism indicates that this would be a general analytical method for dihydrotriazine compounds. This technique, developed for the assay of the dihydrotriazine in an aqueous system, was successfully applied to rat urine samples spiked with the drug.

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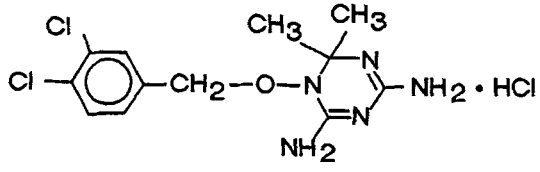
INTRODUCTION

Dihydrotriazine compounds have been shown to exhibit antimalarial (1,2), anti-cancer (3) and anthelmintic activity (4), but often have presented bioavailability problems (5). Studies, which were initiated to improve their bioavailability, required that a highly sensitive assay be first developed as poorly soluble, poorly absorbed compounds generally exhibit low biological concentrations. The compound 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3',4'-dichlorobenzyloxy)-1,3,5-triazine hydrochloride(6) (see Compound I), presented such a problem.

Compound I did not fluoresce in several buffer systems ranging from pH 1 to 12, upon the attempt of a fluorometric technique. This drug has a characteristic UV spectra, but could not be assayed in urine due to the interference of other urinary components absorbing UV light in the same range. Attempts were made to eliminate this intereference by extracting solutions with a variety of solvent systems exhibiting a wide range of polarity, pH, and ionic strength, but were unsuccessful. A high pressure liquid chromatographic technique was then attempted, but found to be unsatisfactory for direct analysis of Compound I. This paper reports the results of our efforts to develop a suitable high pressure liquid chromatographic technique for analysis of I in urine via its reaction product with sufficient sensitivity to detect the drug absorbed from an orally administered dose to rats. The technique should also be applicable to other dihydrotriazine compounds as the alkaline isomerization involved only the dihydrotriazine moiety of the molecule.



4, 6-DIAMINO-1, 2-DIHYDRO-2, 2-DIMETHYL-1-(3', 4'-DICHLOROBENZYLOXY)-1, 3, 5-TRIAZINE HYDROCHLORIDE

COMPOUND I

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EXPERIMENTAL

Reagents

The mobile phases were prepared from reagent grade glycine(7) sodium nitrate(7) distilled methanol(7), sodium hydroxide(7) and distilled water. All other chemicals were of reagent quality.

Preparation of Samples

A stock solution containing 100 µg/ml of I in distilled water was made. Alkaline samples were prepared by mixing the appropriate amounts of I and 1M sodium hydroxide solutions.

Spiked urine samples were prepared by adding the appropriate volume of stock solution of I to rat urine.

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Alkaline Treatment of Samples

When alkaline treatment was necessary, 0.8 ml of sample to be treated was added to 0.2 ml of 1M sodium hydroxide solution and incubated in a water bath at 100°C (boiling temperature) for 1 hour.

Liquid Chromatography

A constant displacement liquid chromatography(8) was used in which the eluent was monitored with a UV detector at 254nm. A constant temperature water bath was used to control the temperature of the column. A strong cation exchange resin(9) was employed as the stationary phase and packed by the dry packing technique(10) in a 100 cm x 2 mm i.d. stainless steel column. The mobile phase was thoroughly degassed before using and passed through the strong cation exchange column. Generally, the flow rate of the solvent was controlled between 0.8 to 1.0 ml/min at 50°C. Initially, the photometer was operated at a maximum attenuation of 0.005 absorbance units full scale to determine the highest sensitivity for the assay. The detector noise at this attenuation was generally less than 1% after the column and detector were stabilized. When the baseline drift occurred after prolonged usage, the column was repacked. The recorder was set at 0.1 in/min. Generally, 5 to 20 µl of the sample to be analyzed

was injected via the injection port. Its concentration was then determined from the resultant peak height.

Thin Layer Chromatography

Ten, twenty and thirty microgram quantities of sample to be analyzed were spotted on silica gel plates(11), Methanol/concentrated ammonium hydroxide (9:1) solvent system was used as a mobile phase. Spots were detected by use of ultraviolet light and/or exposure to iodine vapors.

Solubility Measurement

Solvents were exposed to excess solid powder in a vessel which was shaken and equilibrated in a water bath.

UV Absorptivities

Solutions were made and absorbances measured at different pH buffer systems in a UV spectrophotometer(12) as a function of the concentration. The data was plotted and absorptivities calculated from the slope.

pK_a

The pK_a was determined by UV absorbance method as there was a shift in the peak absorbance of the protonated and deproponated species.

RESULTS AND DISCUSSION

Development of Assay

Since I is ionic with a pK_a of 10.4, we felt that a cation exchange resin might be appropriate as a stationary phase. Several buffer systems were employed in the evaluation of a solvent system most suitable for the elution of this compound. Under the conditions of buffer systems ranging from pH 1 to 12, column temperature from 25°C to 50°C, and varied flow rates, the compound did not elute. This was rather surprising as it was felt that the free base of the drug would interact minimally with the cationic

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exchange resin used as the stationary phase and therefore should have been eluted from the column at pH's above 10.

Since the above were preliminary experiments in which the eluents were employed after minimal conditioning of the column which may have been necessary, it was decided to repeat experiments, but to use overnight conditioning before injection of samples. Initial conditioning was performed at pH of 12 using sodium hydroxide solution in an attempt to remove all traces of previous samples; at this pH over 97% of the drug would exist as the free base which should be the most easily removed form. Since solubility could be a problem at this pH, the column temperature was maintained at the higher temperature, 50°C, in order to maximize the solubility of I. Under these conditions, the residues of previously injected drug appeared to be removed after lengthy conditioning as there was a gradual increase in the base line which then diminished with time.

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Experiments using buffers from pH 9 to 12 as eluents were then repeated, but the drug could not be detected on the chromatogram conclusively showing that the drug in the form of salt or free base is not eluted from the column. This indicated that a transformation of the drug had occurred on the column during the previous overnight conditioning of the column under alkaline conditions. To test this hypothesis, alkaline solutions of I were studied employing ultraviolet spectrophotometry. Such results showed an increased absorbance with a hypsochromic shift in the UV spectra with time. These observations strongly supported the possibility that a reaction product (instead of the free base) was eluted from the column under strong alkaline conditions.

Compound I was therefore reacted in sodium hydroxide solution and the resulting solution injected into the liquid chromatograph using several buffer systems (pH 9 to 12) as eluents. As expected, a chromatographic peak emerged in all samples. These results were consistent with the conclusion that I was transformed to reaction product. It was found that glycine pH 10.4(13) buffer system of the buffers studied was the optimum mobile phase at 50°C with a flow rate of about 1 ml/minute.

Further development of the assay involved the optimization of conditions for the alkaline treatment of I. Samples at pH 12 were boiled and injected into the HPLC as a function of time to determine the efficiency of the method. Figure 1 shows that the reaction product of I

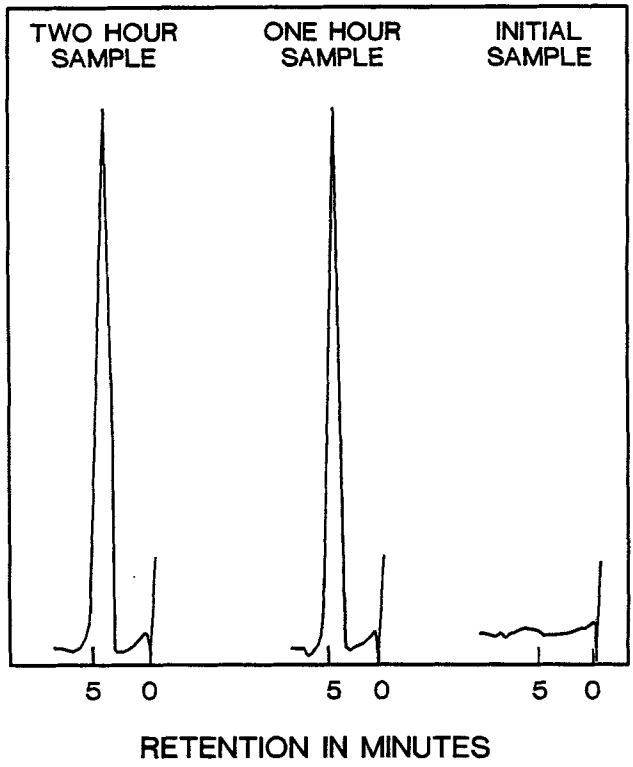

Figure 1 - Chromatograms of I (50 µg/ml) as a function of reaction time with 0.2M NaOH incubated at 100°C. The flow rate was at 0.9 ml/min. UV detection was at 254nm, 0.01 absorbance unit full scale (aufs) and the sample size was 5 µl or 250ng of I.

with sodium hydroxide at boiling temperature tends to be constant after one hour. No peak was observed at the initial time prior to heating. This shows that the method can be used to monitor the reaction and that one hour is sufficient time for the reaction to go to completion. In addition, the results also show that the final absorbance does not deteriorate with further heating of the sample and therefore one does not need to be concerned about the possibility of over-reacting the sample.

These studies were repeated at 25°C and 50°C. The results tabulated in Table 1 show that an incomplete reaction was observed at lower temperatures and reaction times.

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TABLE 1

Completeness of Reaction (as indicated by peak height) of 40 µg/ml of I as a Function of Temperature and Time Reacted Using 0.2 M NaOH

Temperature	Time (hour)	Peak Height (mm)[a]
25°C	1	0
25.C	2	0
50°C	1	24
50°C	2	38.6
100°C	1	95
100°C	2	97

[a] The flow rate was 0.83 ml/min, UV detection was at 254nm at 0.01 aufs., and sample size was 5 µl or 200ng of I.

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Table 2 shows that the peak heights of all the aqueous samples of I with varying amounts of sodium hydroxide appear to have the same magnitude. This indicates that 0.04M sodium hydroxide can be used satisfactorily for analysis of aqueous samples. Samples at lower pH's did not react.

Since the concentration of drug in the biological fluids of the rats was expected to be extremely small, calibration experiments were performed at the highest available instrumental sensitivity (0.005 absorbance units full scale). Table 3 shows the peak heights as a function of concentration of I in aqueous solution and indicates that quantities as low as 0.4 µg/ml drug can be satisfactorily assayed by this technique. (S.D. for 5 replicate injections was 1.2%).

Figure 2 shows the calibration plot for the peak heights as a function of the concentration of I both in aqueous solution as well as in spiked urine samples. A linear relationship appears to be followed. Obviously, there was no interference from urinary components since the aqueous and urinary data are superimposable. The control urine samples in the absence of this compound did not show any peak. The urine assay was found to be simple and reproducible.

TABLE 2

Peak Heights Obtained by Treating Compound I (160 µg/ml) with Varying Amounts of NaOH under Identical Reaction and Chromatographic Conditions

M NaOH	Peak Heights (mm)[a]
0.04	98.6
0.08	98.8
0.12	98.8
0.16	89.7
0.20	95.5

[a] The flow rate was 0.83 ml/min, UV detection was at 254nm at 0.04 aufs., and sample size was 5 µl or 800 ng.

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TABLE 3

Peak Heights Obtained with Varying Concentrations of Compound I Reacted with 0.08M NaOH at 100°C for 1 Hour Under Identical Reaction and Chromatographic Conditions.

Concentration I[b] (µg/ml)		Peak Height (mm)[a]
0.4	(8)	6.10
0.8	(16)	11.68
1.6	(32)	21.84

[a] The flow rate was 0.83 ml/min, UV detection was at 254nm at 0.005 aufs., and the sample size was 20 µl.

[b] Nanograms of Compound I are given in parentheses.

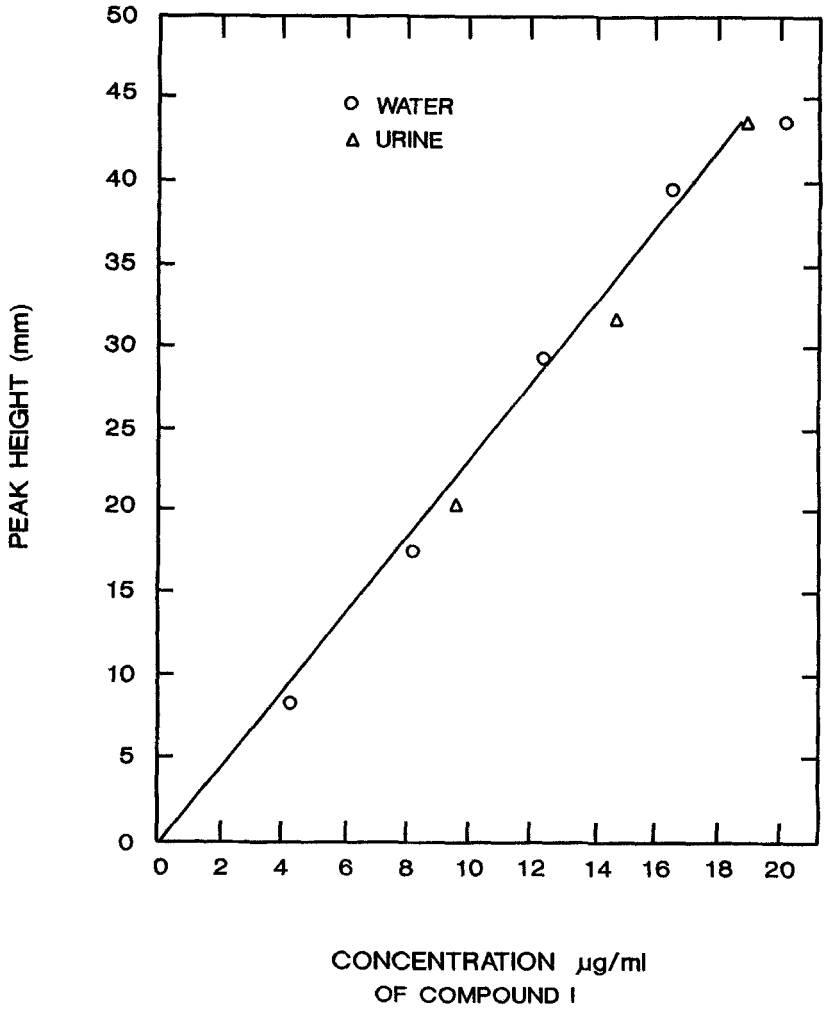

Figure 2 - Calibration plot for peak heights obtained by HPLC analysis of the reaction product of I as a function of the concentration of I used in the reaction mixture in water and in urine. The flow rate was at 1 ml/min. Detection was in the UV range at 254nm, 0.01 aufs and the sample size was 5 µl.

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Further Investigations

Since the conditions imposed during the reaction were severe, it was felt that more definitive information should be obtained to be certain that only one reaction was occurring and to identify the reaction product(s). A thin layer chromatographic analysis was performed in order to detect if one or more products are formed by alkaline treatment of the drug. When silica gel plates with 9:1 methanol/ammonium hydroxide were used as the mobile phase, it was found that one spot was obtained from a completely reacted solution of I. The reaction product had an R_f value of 0.7 as compared to the R_f value of 0.35 observed for I. The reaction product (II) was isolated and found to melt sharply at 215°C as compared to I which melted at 226°C.

Both the ultraviolet and the infrared(14) spectra for I showed a significant shift when treated with sodium hydroxide. The reaction product showed an increase in ultraviolet absorbance and a hypsochromic shift from 247nm to 242nm. The free base of I also showed a small ultraviolet absorbance in that region but was easily differentiated from II. Although the infrared spectra showed a characteristic absorbance due to the amino group, 1, 3, 4 aromatic substitution, and S-triazine for both I and II, a significant spectral shift was observed enabling them to be easily differentiated. This indicated that the above moieties were not destroyed during the reaction process and suggests that a rearrangement has occurred.

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In order to confirm this, elemental analysis was obtained for the isolated reaction product. Table 4 compares the required elemental percentage of various elements for the hydrochloride salt and free base of I and the elemental analysis obtained for the reaction product. This indicated that the observed elemental analysis of the base-treated product agrees with the required percentage of the free base of I.

In addition, a sample of I and II were analyzed by mass spectrophotometry(15). As expected, it was found that the molecular weight of the molecular ion obtained from the reaction product corresponded to that of the free base of I.

In summary, the functional groups present in the infrared spectra, the elemental analysis and the mass spectral data did not show any changes from the original drug; but thin layer chromatography, melting point, infra-

TABLE 4

Elemental Analysis for the Product Formed by NaOH Treatment and its Comparison with Required Percentages of the Salt and Free Base Forms of I

Element	% Element		
	Required*		Found
	Salt	Base	Product
Carbon	40.87	45.58	44.22
Hydrogen	4.57	4.78	4.83
Nitrogen	19.86	22.16	21.80
Halogen	30.16	22.42	22.93
Oxygen	4.54	5.06	—

* Calculated from emperical formulae.

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red and ultraviolet spectral shift data showed definite differences between I and II. This data confirms that isomerization had occurred with alkaline treatment.

Work done in our laboratory(16, 17) on the alkaline treatment of other triazine compounds such as Baker's Antifol and a series of substituted benzene triazine compounds showed that isomerization occurs with alkaline treatment. Although I has an oxygen rather than a nitrogen or carbon between the triazine ring and the rest of the molecule, it is believed that the same mechanism for alkaline treatment transformation shown for the benzene triazines is applicable. See Scheme I.

The possibility that poor solubility and/or UV absorption were responsible for the inability to elute Compound I from the column was investi-

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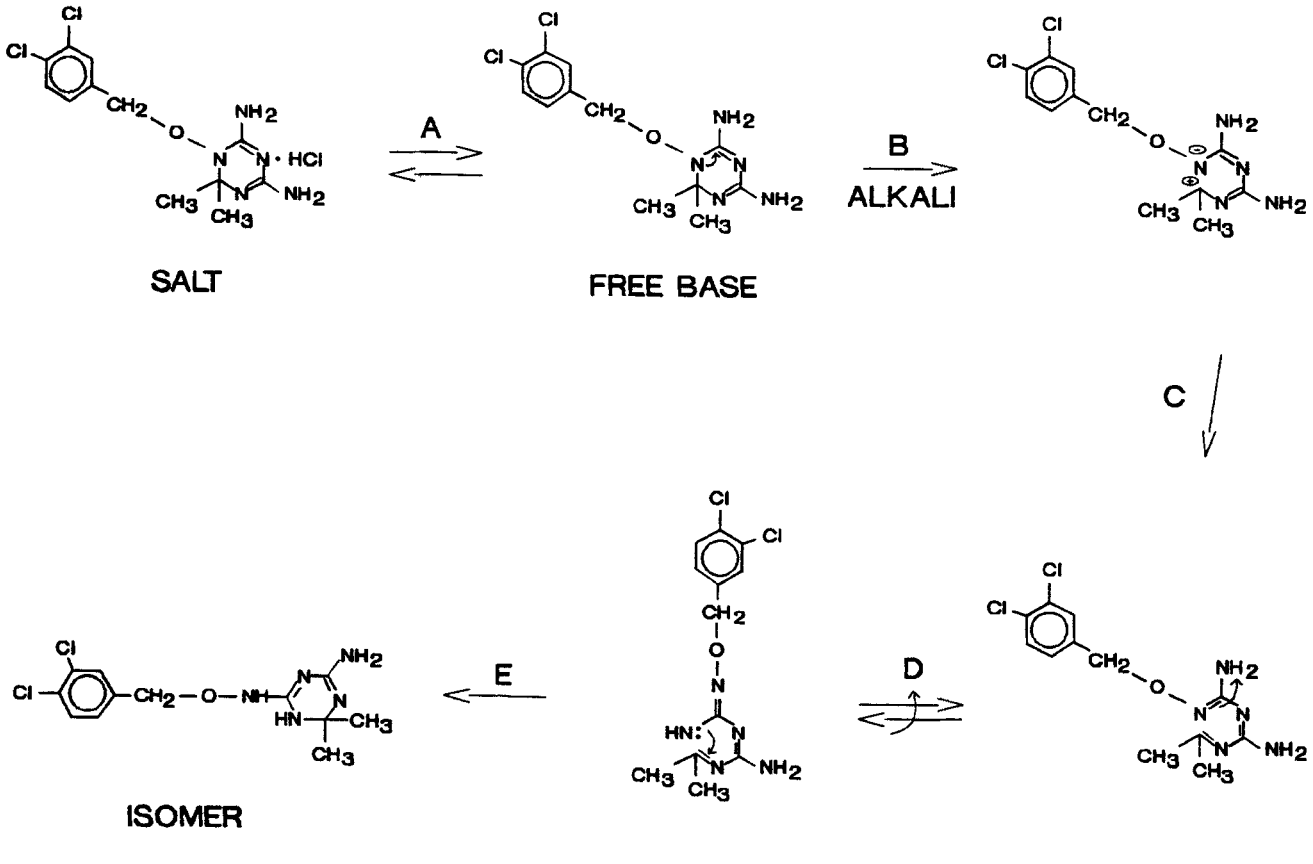

SCHEME I

gated. The results of the study are shown in Tables 5 and 6. Table 5 shows that the absorptivity of I and II cannot be used to explain the lack of detection of Compound I using HPLC as their absorptivities are similar. See absorptivities at 254 nm wavelength. The solubilities are shown in Table 6 and also do not present the reason for the lack of detection of Compound I.

This technique was successfully used to obtain bioavailability data in rats by collecting their urine as a function of time using metabolism cages after oral administration of various formulations of I. This method was found to be sufficiently sensitive as well as reproducible and therefore suitable for our needs.

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TABLE 5

Absorptivities of I and II Each In pH 7 and pH 12 Buffer Systems Measured At 245nm and 254nm.

	Absorptitivities at 245nm		Absorptivities at 254nm	
	pH 7	pH 12	pH 7	pH 12
Compound I	.0242	.0115	.0193	.0110
Compound II	.0277	.0392	.0175	.0353

TABLE 6

Solubilities of I and II Each in pH 7 and 12 Buffer Systems Measured at 25°C.

Conditions	Compound I (mg/ml)	Compound II (mg/ml)
pH 12 at 25°C	0.134	0.244
0.1M NaOH at 50°C	—	0.510

CONCLUSIONS

The results of this paper showed that boiling for one hour in 0.04M sodium hydroxide solution completely converted I to its isomerization product which was readily analyzed via HPLC using a column of strong cation exchange resin and pH 10.4 glycine buffer as the eluent. The parent compound I, on the other hand, could not be analyzed directly by the above chromatographic conditions. The reaction was found to be simple as no other products were isolated via TLC despite the severe conditions of the reaction. A postulated mechanism was proposed based on previous work(16, 17) in our laboratory which clearly shows that this method should be applicable to all dihydrotriazine compounds. The method was shown to be sufficiently sensitive to permit quantitation in the nanogram range of the triazine compound to be able to determine successfully oral bioavailability of I in rats.

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(11) E. Merck Co., Darmastadt, Germany.

(12) Model 15 and 118 UV spectrophotometer, Cary Instruments, Monrovia CA.

(13) One liter solutions contains 561 ml of (0.1M sodium nitrate and glycine), 50 ml distilled methanol, approximately 43 ml of 1M sodium hydroxide and sufficient distilled water.

(14) Model 4240 IR Spectrophotometer, Beckman Instruments, Palo Alto, CA.

(15) Model MS-902, Associated Electrical Industries, Manchester, England.

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