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Note

Liquid chromatography with UV absorbance and polarographic detection of ethylenethiourea and related sulfur compounds

Application to rat urine analysis

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Ethylenethiourea (ETU) is a major degradation product of ethylenebisdithiocarbamate fungicides that has been shown to be teratogenic in the rat[1]. Several urinary metabolites of ETU have been tentatively identified in the rat by thin-layer chromatography with radioscanning[2]. One of the metabolites, identified in this manner was the 4-imidazolone, which may be formed from ethyleneurea, the oxygen analogue of ETU via sulfur replacement. The occurrence of the imidazolone suggests that its sulfur-containing analogue, thioimidazole, might also be formed. The thiohydantoin was also included in this study since it has been reported[3] that the analogous N-methyl compound is a metabolite of methimazole, an anti-thyroid compound structurally related to ETU. High-performance liquid chromatography (HPLC) has much potential for the analysis of traces of organic compounds present in biological fluids and tissues. This report illustrates the use of HPLC for the separation and detection of ETU and related sulfur-containing compounds and application of the technique to the confirmation of the metabolite, thioimidazole, in the urine of rats fed ETU.

EXPERIMENTAL

Reagents

Table I shows the structures of the compounds studied. ETU, methimazole and thiohydantoin were obtained from Aldrich (Montreal, Canada). The thiomidazole and N-methyl ETU were obtained as gifts. Stock solutions of these were prepared at 1.0 mg/ml in methanol and were used directly for spiking purposes and recovery studies. The same solutions were diluted as required with liquid chromatography mobile phase for use as chromatographic standards. All organic solvents were distilled-in-glass grade materials. Distilled deionized water was used throughout.

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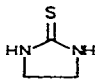
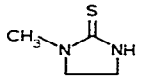
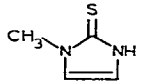
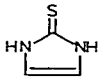
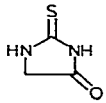
TABLE I

STRUCTURES OF THE COMPOUNDS STUDIED

Compound	*Structure*
ETU	
N-Methyl ETU	
Methimazole	
Thioimidazole	
Thiohydantoin	

High-performance liquid chromatography

Two different chromatographic systems were employed for the analyses. Normal-phase chromatography was carried out with a Waters Model 6000A solvent delivery pump, a Valco loop injector (25-μl loop) and a LiChrosorb Si 60 (10 μm) column (25 cm × 3.2 mm I.D.). The mobile phase was 15% ethanol + 0.5% NH_4OH in hexane at a flow-rate of 1.0 ml/min. Detection was carried out with a Schoeffel Model 770 variable-wavelength detector at 254 or 264 nm. The absorbance range was set to 0.01 absorbance units full scale. Reversed-phase chromatography was carried out with a Waters Model 6000A pump, a Valco loop injector (100-μl loop) and a Supelco LC-18 (5 μm) column (25 cm × 4.6 mm I.D.). The design of the dropping mercury electrode (DME) polarographic detector has been described elsewhere[4]. The mobile phase consisted of 0.1 *M* KNO_3 adjusted to pH 3. The detection mode chosen was sampled current polarography at $E = +200$ mV.

Sample preparation

Urine was obtained from male rats given an oral dose of 5.0 mg/kg ETU[2]. For normal-phase chromatography with UV detection, 1 ml of the urine was added to a 15-ml screw-capped culture tube. Following this about 250 mg of solid NaCl and 5.0 ml of ethyl acetate were added. The mixture was shaken vigorously for *ca.* 30 sec; then the tube was centrifuged at 1000 *g* to aid separation of the phases. A 25-μl aliquot of the organic layer was injected into the HPLC system for analysis.

Less sample preparation was required for reversed-phase chromatography with polarographic detection due to the detector selectivity. A 1-ml volume of urine was passed through a 0.45-μm Millipore filter and 100 μl of the filtrate injected directly into the HPLC system for analysis.

RESULTS AND DISCUSSION

Chromatography and detection

Fig. 1 shows the normal-phase separation of the compounds studied. Methimazole, besides being of interest in the ETU studies, is of some clinical importance since it has therapeutic applications and is the active metabolite of carbimazole. An HPLC method for methimazole in plasma has been reported earlier[5]; however the system described was not suitable for the compounds included herein in samples of rat urine.

Table II lists the detection limits by UV absorbance and polarographic detection and response ratios (254/264 nm) for the sulfur compounds studied. The response ratios were of some help in identifying the compounds in urine. Fig. 2 illustrates the different responses obtained at 264 nm for the same standard solution that was analysed in Fig. 1.

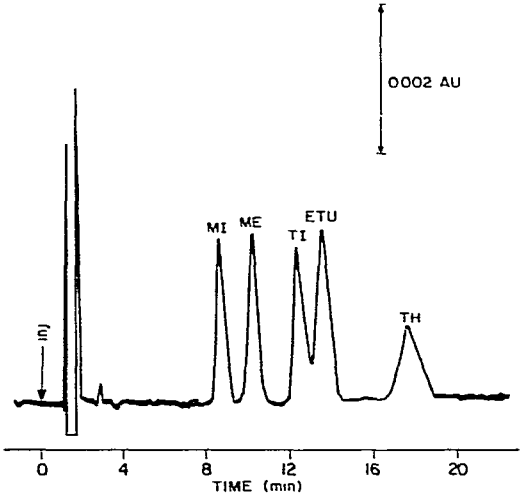


Fig. 1. Normal-phase chromatogram of ETU and related compounds. MI = methimazole (20 ng), ME = N-methyl ETU (5 ng), TI = thioimidazole (10 ng), ETU = ethylenethiourea (20 ng), TH = thiohydantoin (30 ng). Chromatography conditions as described in the text. Detection at 254 nm.

TABLE II

DETECTION LIMITS AND UV ABSORBANCE RESPONSE RATIOS

Compound	*Detection limit (ng)**		*Response ratio 254/264 nm*
	254 nm	*Polarography*	
ETU	2	1	25
N-Methyl ETU	1	—**	2.1
Methimazole	0.5	2	16
Thioimidazole	1	5	2.1
Thiohydantoin	4	15	1.4

* At signal-to-noise ratio of 3:1.

** Not determined.

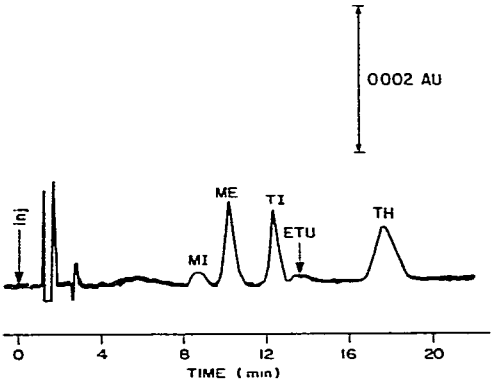


Fig. 2. Normal-phase chromatogram of ETU and related compounds. Conditions as in Fig. 1 except that detection was made at 264 nm.

The polarographic detector was found to be more selective than UV absorbance although detection limits for the compounds studied were similar, being 1–15 ng (Table II). The detection mechanism is based on a complexation between the thiourea and the mercury surface according to the reaction

$$Hg^0 + 2\,TU \rightarrow Hg\,(TU)_2^{2+} + 2\,e$$

where TU = thiourea. No such reaction occurs for the oxygen analogues. Optimum response was obtained under acidic conditions. Half-wave potentials were typically in the range of +50 to +250 mV. The advantage of operating in the positive voltage range reduces oxygen and trace metal interferences thus affording considerable selectivity for the thiourea compounds.

Urine analysis

The recoveries of all of the thiourea compounds from rat urine spiked in the range of 1–15 μg/ml urine were greater than 90% with the ethyl acetate extraction employed for normal-phase chromatography.

Fig. 3 compares UV absorption results (254 and 264 nm) of a 24-h pooled urine sample obtained from ETU-treated rats, compared to a control urine. The ETU is clearly evident. Also present is a peak corresponding to thioimidazole and one near where the thiohydantoin would be expected to elute. The response ratios (254/264 nm) correlate well with ETU and thioimidazole but not for the peak (indicated by an arrow) in the region of the thiohydantoin. Since a peak appears in the control urine in that same region further doubt is placed on any suggestion that the thiohydantoin is present in the treated rat urine.

Fig. 4 shows a reversed-phase chromatogram with polarographic detection of similarly treated urine samples. A peak corresponding to the thioimidazole confirms the findings of the normal-phase system. No thiohydantoin was detected by polarography in the treated samples.

It is interesting to note that in both HPLC systems, the thioimidazole elutes before ETU. It was found that depending on pH of the mobile phase the order of

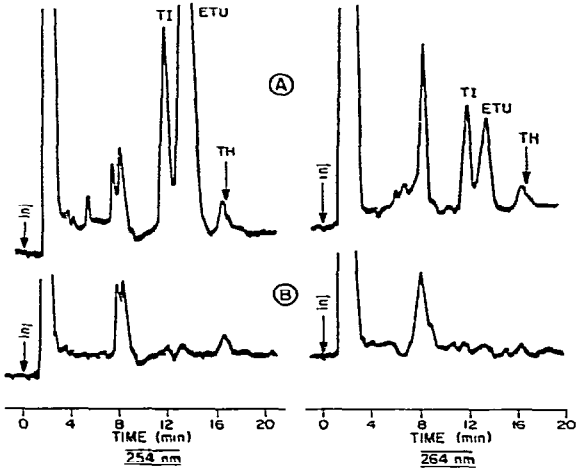


Fig. 3. Comparison of 254 and 264 nm for the analysis of ETU and metabolites in rat urine. A = Urine from treated rats, B = control urine. The arrow indicates the retention time of standard thiohydantoin (TH). Conditions as described in the text.

elution would reverse. The thiohydantoin was also significantly affected by mobile phase pH. These effects appear to be due to the combination of hydrophobicity and base strength of the compounds.

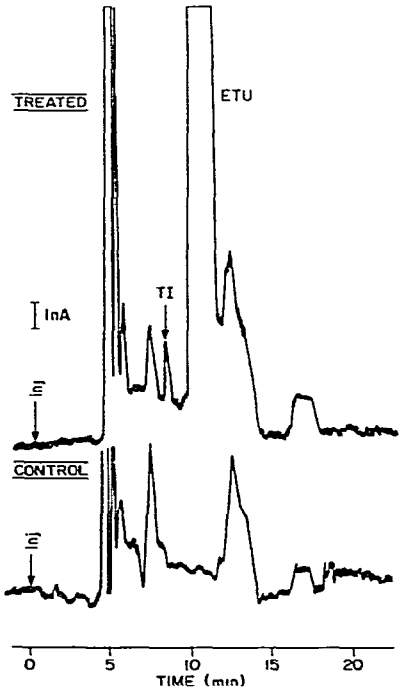


Fig. 4. Reversed-phase chromatogram with polarographic detection of rat urine sample. $E = +200$ mV. Conditions as described in the text.

CONCLUSION

Both normal-phase and reversed-phase HPLC has been found to be useful for the separation of ETU and related thiourea compounds. Both UV absorbance and polarographic detection are suitable for application to urine determinations. Because of the selectivity of the DME it is particularly suited to confirmation as well as rapid analysis with little or no sample preparation. The confirmation of thioimidazole as an ETU urinary metabolite enables the further understanding of its metabolic route in rats and other mammalian systems.

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