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IDENTIFICATION AND QUANTITATION OF A METABOLITE OF ANETHOL DITHIOLTHIONE IN RAT AND MOUSE URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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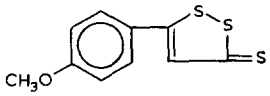
SUMMARY

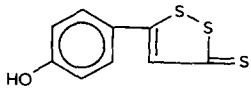
Urine samples from rats and mice fed anethol dithiolthione (ADT) [3-(*p*-methoxyphenyl)-1,2-dithiol-3-thione] were analyzed using reversed-phase high-performance liquid chromatography. Urine was introduced directly on the liquid chromatograph which was modified by replacing the sample loop with a guard column. Highly polar urine components were washed off the guard column prior to chromatography. A major metabolite and the parent compound (ADT) were separated and detected using the chromatographic conditions described in this study. The metabolite was identified as desmethyl ADT. The identification was based on co-chromatography on two columns using two mobile phases and peak height ratios of the metabolite and the reference standard. Data pertaining to the pattern of excretion of ADT and desmethyl ADT in the animals studied are reported.

INTRODUCTION

Anethol dithiolthione (ADT) [3-(*p*-methoxyphenyl)-1,2-dithiol-3-thione] has been reported to stimulate salivary secretion and to be an antidote for the dryness of the mouth produced by antidepressant drugs [1]. More recently it has been found that this and other dithiolthiones have chemo- and radioprotective properties [2]. As a first step towards the study of the metabolism and pharmacodynamics of ADT, we have identified one of its metabolites and developed a method for the determination of both the parent compound and the metabolite in the urine of rats and mice using reversed-phase high-performance liquid chromatography (HPLC).



ADT



desmethyl ADT

0378-4347/83/$03.00

EXPERIMENTAL

Instrumentation

An isocratic HPLC system assembled in our laboratory consisted of a Milton Roy reciprocating minipump, Model 396; a stainless-steel tube, 1 m × 6.4 mm O.D. as a pulsation damper, a 34.5 MPa pressure gauge; a Rheodyne injector Model 7125 and a variable-wavelength ultraviolet (UV) detector, Model Spectro-Monitor III. All parts were obtained from Laboratory Data Control (Riviera Beach, FL, U.S.A.). A Kratos variable-wavelength UV detector Model Spectroflow 769 (Westwood, NJ, U.S.A.) was connected in tandem with the Spectro-Monitor III for the simultaneous detection and identification of the metabolites, a Brownlee (Santa Clara, CA, U.S.A.) RP-18 LiChrosorb 3-cm guard column was connected in place of the sample loop of the injector. Two analytical columns were used in this study; a Whatman (Clifton, NJ, U.S.A.) Partisil PXS 10/25, ODS-2, microparticulate, 10—12 μm, reversed-phase column, and a Waters Assoc. (Milford, MA, U.S.A.) μBondapak Phenyl column. A Brownlee guard column identical to the one mentioned above was connected between the injector and the Whatman reversed-phase column to further protect the column from urine components, since most of the analyses were carried out on that column. No guard column was connected between the injector and the Waters column since very few runs were carried out on that column. A Hewlett-Packard (Avondale, PA, U.S.A.) computing integrator Model 3390A was connected to each detector. The analytical columns were kept inside a Bioanalytical Systems (West Lafayette, IN, U.S.A.) column heater Model LC-23A and column temperature was maintained at 35°C throughout this study.

Chemicals

Anethol dithiolthione (ADT) and desmethyl ADT were obtained from Dr. Baronnet of Laboratoires Therapeutique Moderne (Suresnes, France) and were used without further purification.

Methanol (glass distilled) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled, deionized and demineralized.

Reference standards were dissolved in methanol and all dilutions and mixtures were prepared also in methanol. Solutions were stored in vials provided with PTFE-lined caps and the vials were wrapped with aluminum foil to protect the solutions from light. Standard solutions were gassed with nitrogen and kept refrigerated when not in use.

Animals and treatments

Male Sprague-Dawley rats weighing approximately 200 g and CD-1 female mice weighing 20—25 g were used. The rats were placed in individual metabolism cages and urine was collected in Erlenmeyer flasks and analyzed individually. Six mice were placed in one metabolic cage and urine was collected and analyzed as one sample. A solution containing 25% glycerol and 1% Cremophor EL (BASF, Wyandott, Parsippany, NJ, U.S.A.) was used to suspend ADT prior to feeding. To 1 g of ADT, 10 ml of the above vehicle was added, the mixture was then homogenized and given to the animals by

gastric intubation to provide a dose of 1 g/kg body weight for both rats and mice.

Mobile phases

Two mobile phases containing 70% and 65% methanol in water were used. The mobile phases were degassed under vacuum immediately prior to use and kept at approximately 40°C during chromatography to prevent the introduction of air bubbles into the system. The flow-rate was kept at 1.1 ml/min throughout this study.

Detector settings

The absorption spectra of ADT and desmethyl ADT were studied using a Varian scanning spectrophotometer Model Cary 219. ADT was found to have maxima at 225, 342 and 425 nm and the extinction coefficients (α) were 61.5, 96.0 and 57.0, respectively. Desmethyl ADT was found to have maxima at 228, 350 and 425 nm and the extinction coefficients (α) were 31.0, 50.5 and 35.0, respectively. Based on these data, the variable UV detector Model Spectro-Monitor III was set at 350 nm for the routine determination of ADT and desmethyl ADT since both absorb most at or close to that wavelength. Peak height ratios for desmethyl ADT and the metabolite suspected to be desmethyl ADT were determined simultaneously using the two detectors connected in tandem. The Spectroflow 769 was set at 228 nm while the Spectro-Monitor III was set at 350 nm.

Integrator settings

Each UV detector provided a constant signal to the computing integrator of 1 absorbance unit (AU)/V. The sizes of the peaks on the chromatograms were thus determined by the attenuation of the integrator. During this study two attenuation settings were used, attenuation 3 which provides a full scale of 8 mV or attenuation 4 which provides a full scale of 16 mV. The sensitivity of the system would be 0.008 absorbance units full scale (AUFS) or 0.016 AUFS, respectively.

Sample preparation

Urine was collected in Erlenmeyer flasks which were kept on ice. The samples were collected every morning and centrifuged for 20 min at 12,100 *g* using a refrigerated Sorvall RCB-2 centrifuge set at 5°C to remove suspended particles. One ml of the centrifuged urine was filtered using Bioanalytical Systems centrifugal filters containing membranes of pore size 0.2 μm. The filtrates were directly introduced onto the chromatograph. Prior to the introduction of the sample on the guard column loop, 0.5 ml of 30% methanol was introduced, followed by the sample (5 μl) then 1 ml of 30% methanol was used to flush the highly polar urine components out of the guard column. Then the injector was turned to the inject position for the chromatography of the compounds remaining on the guard column.

The concentration of methanol used to flush the guard column was determined by preliminary experiments using Baker (Phillipsburg, NJ, U.S.A.) C_{18} disposable extraction columns. ADT and desmethyl ADT were added to

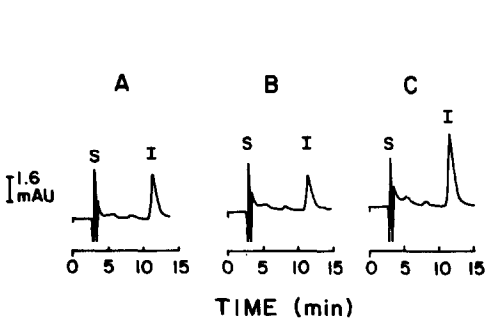
the urines and washed with various concentrations of methanol and the eluates were monitored by HPLC. The use of 30% methanol removed most of the polar urine components and did not elute the compounds of interest.

RESULTS AND DISCUSSION

Identification of the metabolite

Desmethyl ADT was added to rat urine samples and peak shape and peak heights were compared with those without added desmethyl ADT and those of desmethyl ADT standard. This was carried out using the two columns and the two mobile phases described earlier. Figs. 1 and 2 show the chromatograms on the reversed-phase column and on the phenyl column respectively using 65% methanol. As demonstrated from the peak shape and from the peak heights, it is clear that the urine metabolite is co-chromatographing with desmethyl ADT, this results in an increase in peak height without any change in peak shape in both systems. Similar results were obtained using 70% methanol on the reversed-phase column.

To further establish the identity of these compounds, chromatography was carried out at the two UV absorption maxima of desmethyl ADT, 228 nm and 350 nm. The peak height ratio of the signals produced at 228 nm to that produced at 350 nm were calculated for the reference standard and the metabolite using column A and 70% methanol. Ten samples of each were determined over a period of four days to reflect day-to-day variations of peak height ratios. The mean ($\bar{x}$) standard deviation (σ_x) and standard error of the mean ($\sigma_{\bar{x}}$) for the ten samples were determined and are presented in Table I.



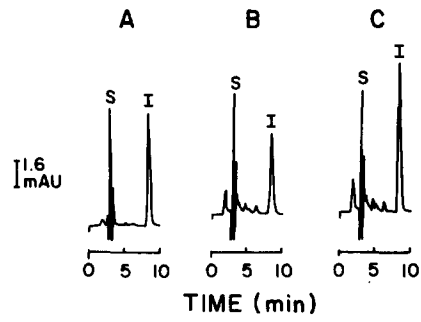


Fig. 1. Chromatograms of (A) 5 μl of 10 μg/ml desmethyl ADT; (B) rat urine of animal fed 1 g/kg of ADT, diluted with an equal volume of methanol, 5 μl injected; (C) same rat urine as in (B) spiked with an equal volume of 20 μg/ml desmethyl ADT in methanol, 5 μl injected. Chromatographic conditions: column, Whatman, Partisil PXS 10/25, ODS-2; mobile phase, 65% methanol; detector, UV 350 nm; sensitivity, 0.016 absorbance units full scale (AUFS); flow-rate, 1.1 ml/min; column temperature, 35°C; chart speed, 0.2 cm/min. Peaks: S = solvent front; I = metabolite identified as desmethyl ADT.

Fig. 2. Chromatograms of (A) 5 μl of 10 μg/ml desmethyl ADT; (B) rat urine of animal fed 1 g/kg of ADT, diluted with an equal volume of methanol, 3 μl injected; (C) same rat urine as in (B) spiked with an equal volume of 20 μg/ml desmethyl ADT in methanol, 3 μl injected. Chromatographic conditions: same as Fig. 1 except that the column used is a Waters μBondapak Phenyl. Peaks as in Fig. 1.

TABLE I

PEAK HEIGHT RATIOS OF DESMETHYL ADT AND METABOLITE

Number of samples = 10 in each case; data collected over a period of four days.

Compound	$\bar{x}^*$	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	0.617	0.0067	0.0021
Metabolite	0.628	0.0265	0.0083

*Peak height ratios of detection responses at 228 nm to those at 350 nm, using column A and 70% methanol.

Accuracy and specificity

To evaluate the accuracy and specificity of the method, retention times (t_R) for desmethyl ADT and ADT were determined several times during the course of the day to determine within-run variations, and over a period of four days to reflect day-to-day variations. The mean ($\bar{x}$), standard deviation (σ_x) and standard error of the mean ($\sigma_{\bar{x}}$) were calculated and are presented in Tables II and III. As expected the σ_x and $\sigma_{\bar{x}}$ values were smaller for the within-run determinations than the day-to-day determinations. The statistical variations were also smaller for desmethyl ADT than for ADT since the former had a shorter t_R value.

Since these compounds are well separated and no other peaks are close enough to cause interference, these variations confirmed the accuracy and specificity of the analytical method.

TABLE II

WITHIN-RUN VARIATIONS OF RETENTION TIMES (min)

Number of samples = 8 in each case; data collected the same day; column A and 70% methanol were used.

Compound	$\bar{x}$	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	8.11	0.072	0.025
ADT	17.58	0.135	0.047

TABLE III

DAY-TO-DAY VARIATIONS OF RETENTION TIMES (min)

Number of samples = 8 in each case; data collected over a period of four days; column A and 70% methanol were used.

Compound	$\bar{x}$	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	8.58	0.102	0.113
ADT	18.77	0.814	0.288

Reproducibility and precision

Desmethyl ADT and ADT were determined in one mouse urine sample containing these compounds several times during the course of one day and over a period of four days. Reference standards were chromatographed each day and detection responses for the reference standards were used to determine the amount present in the urine samples assayed on the same day. To ascertain reproducibility reference standards were chromatographed twice and the mean values were used. The statistical computation of the amounts of these compounds reflecting within-run variations and day-to-day variations are presented in Tables IV and V. The values reflecting the day-to-day variations were very similar to those reflecting the within-run variations. This is expected due to the fact that actual variations due to day-to-day variability in chromatographic conditions is eliminated by the use of detection responses of reference standards produced the same day for the computation of sample contents.

TABLE IV

WITHIN-RUN VARIATIONS OF QUANTITATIVE DETERMINATION OF COMPOUNDS STUDIED IN MOUSE URINE

Number of samples = 4 in each case; data collected the same day.

Compound	Day*	$\bar{x}$**	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	A	26.2	1.979	0.989
	B	25.3	1.319	0.659
ADT	A	8.1	0.639	0.319
	B	8.3	0.547	0.273

*Same data collected on Day A and day B.
**Amount calculated in ng on column, injection volume was 5 μl; column A, 70% methanol and 350 nm were used.

TABLE V

DAY-TO-DAY VARIATIONS OF QUANTITATIVE DETERMINATION OF COMPOUNDS STUDIED IN MOUSE URINE

Number of samples = 10 in each case; data collected over a period of four days.

Compound	$\bar{x}$*	σ_x	$\sigma_{\bar{x}}$
Desmethyl ADT	25.5	1.823	0.576
ADT	8.4	0.783	0.247

*Amount calculated in ng on column, injection volume was 5 μl; column A, 70% methanol and 350 nm were used.

Detection responses and sensitivity

The detection responses for the two compounds studied were determined using Column A, 70% methanol and 350 nm. Eight determinations were carried out over a period of four days. Desmethyl ADT produced a mean

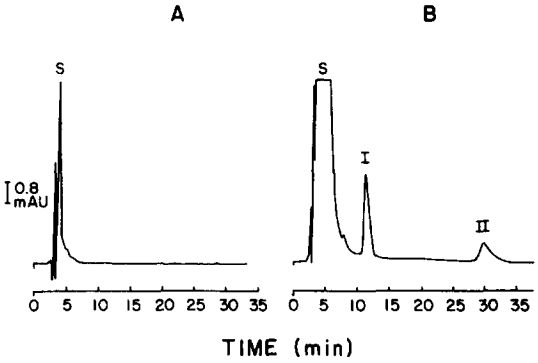


Fig. 3. Chromatograms of (A) 5 μl of control mouse urine collected prior to feeding of ADT; (B) 5 μl of same pool of mouse urine 48 h after feeding with 1 g/kg of ADT. Chromatographic conditions as in Fig. 1 except sensitivity was 0.008 AUFS. Peaks: S = solvent front; I = metabolite identified as desmethyl ADT; II = ADT.

signal size of 94.5 ± 12.9 mAU per μg on column, while ADT produced a mean signal size of 120.4 ± 9.4 mAU per μg on column. When a sensitivity setting of 8 mAUFS was used as in Fig. 3, no detectable noise was observed. Under these conditions a signal size of 5% of the full scale or 0.4 mAU will be easily detected. Such a signal represents less than 5 ng on column of both compounds studied. The sensitivity can be further increased by increasing the sensitivity setting of the detector.

Since the procedure provides both extraction and trace enrichment, as described under sample preparation, sample volumes larger than 5 μl can be introduced onto the system without affecting the baseline or the column performance.

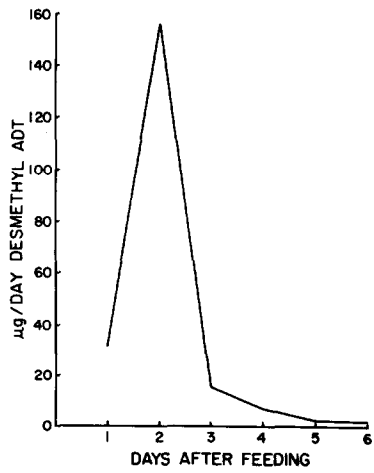


Fig. 4. Graphic representation of amounts of desmethyl ADT present in rat urine fed 1 g/kg of ADT.

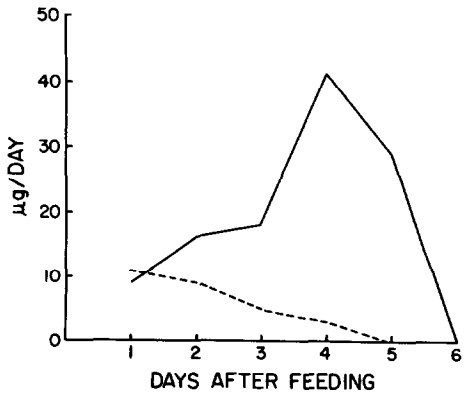


Fig. 5. Graphic representation of amounts of ADT (— — —) and desmethyl ADT (——) present in mouse urine fed 1 g/kg of ADT.

Quantitation of ADT and desmethyl ADT

ADT was not found in detectable quantities in rat urine. It was, however, detected and its quantity determined in mouse urine. Identification of ADT was based on retention characteristics using the three chromatographic conditions described above. The quantities of ADT and desmethyl ADT were determined as described above. Fig. 3 reproduces the chromatograms of urines of mice fed 1 g/kg of ADT and collected 48 h thereafter, on the reversed-phase column. The solvent front representing the polar compounds unretained under these conditions is much larger in the urine of animals fed ADT than in control animals. This suggests that this large unretained peak is due to other polar metabolites of ADT. Fig. 4 represents the amount of desmethyl ADT excreted per day in rat urine. Fig. 5 represents the amounts of ADT and desmethyl ADT excreted per day in mouse urine. ADT is absent from rat urine, while it requires five days to be cleared from the mouse. The metabolite excretion pattern is also different in the rat where the peak is reached after two days while in the mouse the peak is reached after four days.

Chromatography

The use of the RP-18 guard column in place of the injection loop and the removal of undesirable urine constituents by washing with 30% methanol after the introduction of the sample allowed the direct injection of urine on the liquid chromatograph and eliminated tedious and time consuming sample preparation. Also, the use of another guard column between the injector and the analytical column assured the complete protection of the column. The two mobile phases consisting of 65% and 70% methanol provided reasonable retention of the compounds studied. The lower methanol concentration was preferred because some urine samples produced large peaks at the solvent front which were not completely separated from desmethyl ADT when 70% methanol was used. Also, 70% methanol did not produce adequate retention on the phenyl column.

The use of two columns such as the reversed-phase column and the phenyl column for identification of unknown compounds provides stronger evidence than a single column because the physico-chemical basis for separation is different on each column. Previous work in our laboratory, in which sixteen dithiolthiones were studied indicated that the order of elution of these compounds varies on these two columns [3].

Peak height ratios at the two UV maxima of desmethyl ADT served as an additional parameter for the identification of the metabolite as desmethyl ADT.

CONCLUSION

A procedure for the separation, identification and determination of ADT and of desmethyl ADT using HPLC is described. A major metabolite of ADT in rat and mouse urine has been identified as desmethyl ADT using co-chromatography and peak height ratios. Data pertaining to the excretion of desmethyl ADT in the rat and mouse urine are reported. ADT was determined in the mouse urine and was found to decrease steadily and to disappear after five days of ingestion. Unmetabolized ADT was not detected in rat urine.

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