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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 2-MERCAPTOPROPIONYLGLYCINE AND ITS METABOLITE 2-MERCAPTOPROPIONIC ACID IN PLASMA AND URINE AFTER TREATMENT WITH THIOPRONINE

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SUMMARY

2-Mercaptopropionic acid has been identified as a normal metabolite of 2-mercaptopropionylglycine (thiopronine) when this drug was given to humans and dogs. A high-performance liquid chromatographic method was developed to resolve the derivatives of these two thiols and thus enable simultaneous determination of the two compounds in plasma and urine.

INTRODUCTION

2-Mercaptopropionylglycine (2-MPG, Thiola®) has been used for several years as an effective and safe treatment for cystinuria both in humans [1–3] and in dogs [4]. However, the pharmacokinetics and metabolism of the drug remain obscure because of the lack of adequate methods for the determination of the drug in biological fluids. We have recently developed a high-performance liquid chromatographic (HPLC) method for its determination in plasma and urine [5]. In this method N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) was used as a derivatizing reagent for thiols, reacting with 2-MPG to give a fluorescent derivative suitable for HPLC. Applying this method to samples from subjects undergoing treatment with 2-MPG, we have now detected and identified 2-mercaptopropionic acid (2-MPA) as a metabolite from 2-MPG both in

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plasma and urine. The present paper describes the optimization of the method for simultaneous determination of both the metabolite and the parent drug.

EXPERIMENTAL

Clean-up and derivatization

The procedure we used was adapted from earlier work on the determination of thiols in plasma [4,5] and urine [6,7]. Derivatization was thus performed with DACM, which reacts rapidly with thiols to give highly fluorescent compounds. The procedure is given here in brief and details noted only if new or different from the earlier paper on 2-MPG [5], where details regarding chemicals and clean-up procedure can be found.

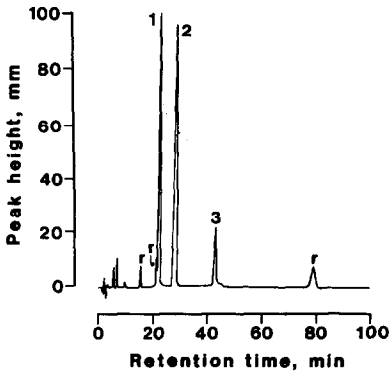
Both 2-MPG and 2-MPA were obtained from two chemical companies: Sigma (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland). As standards we prepared 1 m*M* stock solutions of the thiols in 10 m*M* hydrochloric acid and 2 m*M* disodium EDTA. For derivatization of standards, 0–400 μM working solutions were prepared, and 50 μl were pipetted into tubes containing 5 ml of 50 m*M* carbonate buffer and 10 m*M* disodium EDTA (pH 9.0). Then 0.5 ml of 20 μM DACM dissolved in acetone were added and the tubes were incubated at 37°C as described earlier [5].

EDTA plasma was used without further pretreatment. We mixed 0.25 ml of sample with 0.75 ml of 0.1 *M* Tris buffer (pH 8.0) and added 2.0 ml of 20 m*M* tributylphosphine dissolved in ethanol. After reduction at 50°C for 30 min, and subsequent separation from proteins by centrifugation at 1000 *g*, the thiols were purified by affinity chromatography and ion-exchange chromatography [5]. From the last eluate containing 10 m*M* hydrochloric acid, 1 ml was neutralized with 0.1 ml of 0.1 *M* sodium hydroxide. Then 1.5 ml of the 50 m*M* carbonate buffer with 10 m*M* disodium EDTA was added followed by 0.25 ml of 20 μM DACM.

Urine samples were diluted ten-fold, and 0.25 ml were reduced and further treated in the same way as plasma samples. However, if the concentration of 2-MPG was above the linear range of the method, the amount of DACM had to be increased. In that case we used the remaining eluate and a five-fold stronger DACM solution for derivatization. Standards were derivatized accordingly.

High-performance liquid chromatography

The HPLC equipment was a Model M-45 solvent delivery system from Waters (Milford, MA, U.S.A.), a WISP Model 710B autosampler, a Fluoromonitor III filter fluorometer from Laboratory Data Control (Riviera Beach, FL, U.S.A.) and an SP 4270 integrator from Spectra-Physics (San Jose, CA, U.S.A.). The fluorometer was operated with a mercury lamp, a 360-nm excitation filter and a 418–700 nm emission filter. We used an LC-8-DB column (150×4.6 mm I.D., particle size 3 μm) from Supelco (Bellefonte, PA, U.S.A.). The mobile phase was a water mixture of phosphate, tetramethylammonium (TMA) hydroxide and acetonitrile prepared to contain 0.3 m*M* sodium dihydrogenphosphate, 1.3 m*M* disodium hydrogenphosphate, 10 m*M* TMA and 6% (v/v) acetonitrile. The pH was adjusted to 7.4 with 6 *M* hydrochloric acid before addition of the acetonitrile.



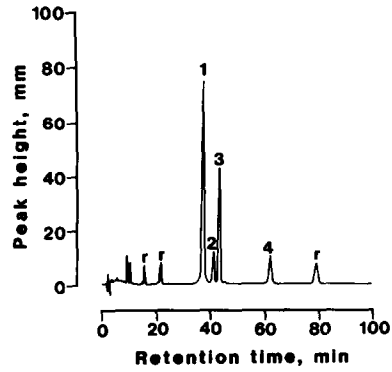


Fig. 1. Chromatogram of 2-MPA derivatized with DACM. The injected amount of derivative corresponded to 4.5 pmol of 2-MPA. Peaks 1, 2, and 3 are from,2-MPA, and r indicates reagent peaks.

Fig. 2. Chromatogram of 2-MPG derivatized with DACM. The injected amount of derivative corresponded to 4.5 pmol of 2-MPG. Peaks 1, 2, 3 and 4 are from 2-MPG, and r indicates reagent peaks.

Chromatography was performed at 30°C, the flow-rate was 0.8 ml/min and the injection volume was 10 μl.

Calculations

The two thiol derivatives were quantified by external calibration based on peak heights. The serum and urine concentrations were then calculated using correction factors to compensate for sample volume changes during clean-up and differences in volumes used for derivatization of standards and samples.

In vivo studies

Venous blood samples taken in EDTA tubes were from a cystinuric dog, a dachshund, given 250 mg of Thiola (Santen Pharm., Osaka, Japan) orally. The urine was collected at the intervals 0–6, 6–12, 12–24, and 24–30 h after intake, and was stored at −20°C until analysis.

RESULTS

Chromatography

Figs. 1 and 2 show chromatograms obtained with derivatized 2-MPA and 2-MPG standards. With the former compound, three peaks appeared with retention times 21.7, 27.5 and 42.0 min, and with the latter compound four peaks were found at 36.1, 39.9, 42.0 and 57.4 min. Small peaks due to reagents were found as verified in blank samples (results not shown), one of them interfering with the first 2-MPA peak. There was no interference with the second main 2-MPA peak (27.5 min). This peak was, therefore, suitable for the determination of 2-MPA in plasma and urine (Figs. 3 and 4). For similar reasons, the first peak from 2-MPG (36.1 min) should be chosen in the determination of the parent drug.

Because of the presence of several peaks from each thiol compound, the standards of each obtained from two different companies (Sigma and Fluka) were

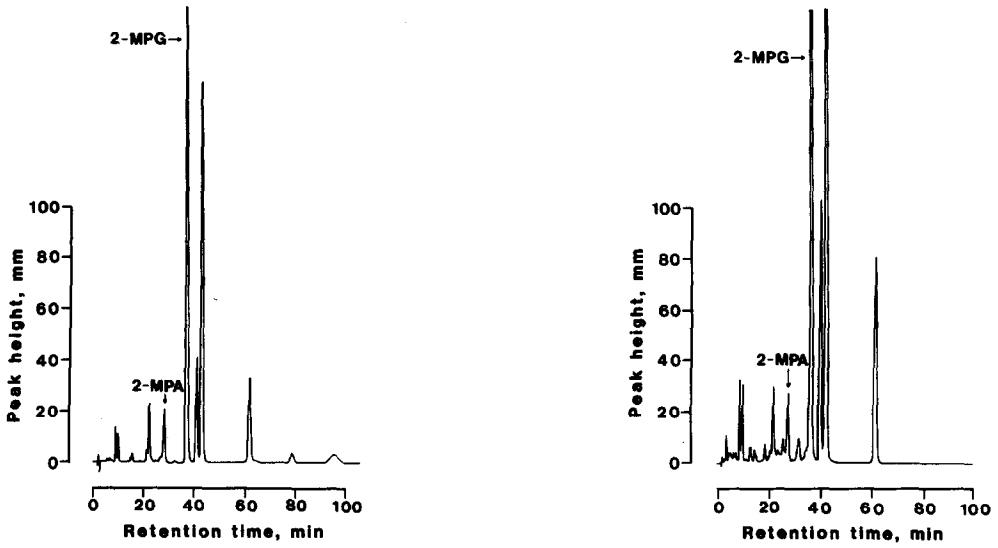


Fig. 3. Chromatogram of plasma from a dog after intake of Thiola. The peaks most suitable for quantitation are indicated. The concentration of 2-MPA in the plasma sample was 4.1 μmol/l and that of 2-MPG was 56 μmol/l.

Fig. 4. Chromatogram of urine from a dog after intake of Thiola. The peaks most suitable for quantitation are indicated. The concentration of 2-MPA in the urinary sample was 53 μmol/l and that of 2-MPG was 1900 μmol/l.

derivatized. 2-MPG from the two sources gave identical chromatograms as did 2-MPA. Furthermore, a 250-mg tablet of Thiola was ground in a mortar, dissolved in 1 l of a 10 m*M* solution of hydrochloric acid containing 2 m*M* EDTA and further diluted 50-fold to a theoretical concentration of 30.7 μ*M*. Analysis of this solution as described in Experimental gave the "fingerprint" chromatogram of 2-MPG, and no contamination of 2-MPA was found. The concentration was found to be 27.1 μmol/l: thus the recovery of 2-MPG from the tablet was 88%. This was similar to the recovery of 87% obtained when pure 2-MPG was carried through the clean-up procedure. A similar figure of 80% recovery was obtained with 2-MPA.

Linearity and dynamic range

Standard curves were obtained by direct derivatization of each of the two thiol compounds. With use of a 200 μ*M* solution of 2-MPG or 2-MPA the amount of thiol per tube (10 nmol) was equivalent to the amount of DACM. If the reaction goes to completion the peak height should be a maximum at this point. This was not the case with either of the thiols, certainly not with 2-MPG, although the peak of 2-MPA leveled out only slightly above this concentration. This phenomenon might indicate that only a portion of the thiol reacts with the maleimide. For practical reasons, the standard curve can be regarded as linear up to an added amount of 5 nmol per tube (i.e. a 100 μ*M* concentration of the standard) corresponding to a plasma concentration of 63 μmol/l and a urine concentration of 630 μmol/l.

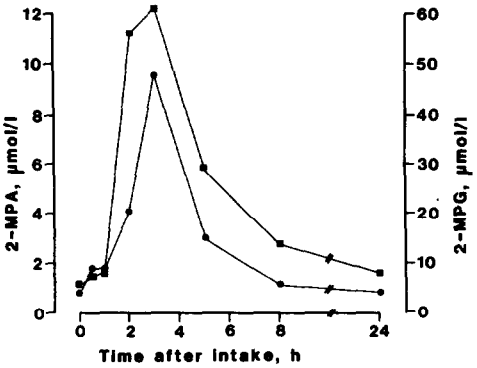


Fig. 5. Plasma concentrations of 2-MPG (■) and 2-MPA (●) in a dog on daily treatment with 250 mg of Thiola.

Precision

Precision studies for 2-MPG analysis have been performed earlier with the same clean-up procedure and shown to be satisfactory [5]. With the present method, which includes determination of 2-MPA, similar precision was obtained. Thus, with a serum pool analysed on six different days, we found the mean ± S.D. concentration to be 0.85 ± 0.076 μmol/l (C.V. = 8.9%) for 2-MPA and 21.9 ± 0.66 μmol/l (C.V. = 3.0%) for 2-MPG. Duplicate analysis of urines showed mean ± S.D. values of 38.8 ± 1.8 μmol/l (C.V. = 4.6%) for 2-MPA ($n = 19$), 33.1 ± 2.5 μmol/l (C.V. 7.6%) for 2-MPG levels below 150 μmol/l ($n = 7$) and 1078 ± 76.3 μmol/l (C.V. = 7.1%) for 2-MPG levels above 150 μmol/l ($n = 10$).

Application to plasma

Fig. 5 shows the total plasma concentrations of 2-MPG and 2-MPA in a cystinuric dog on daily treatment with 250 mg of Thiola. The starting levels originate from treatment 24 h earlier. It can be seen that the parent drug appears earlier after intake than the 2-MPA, and no accumulation of either compound seems to occur, since the 24-h level approached the starting levels.

Application to urine

Table I shows the urinary excretion of the two thiols in ten human subjects given 500 mg of Thiola orally as a single dose. Most of the 2-MPG found in urine was excreted during the first 6-h period, whereas the 2-MPA was mostly excreted during the second 6-h period. This delay becomes more obvious when the ratio between the two compounds is regarded as a function of time after intake.

DISCUSSION

With DACM as reagent, highly fluorescent derivatives of thiols can be obtained which are suitable for HPLC [5–9]. This work shows that this reagent is suitable for the determination of both 2-MPG and 2-MPA. The latter compound has not until now been detected in plasma and urine. For the parent compound we recently developed an HPLC method for plasma and urine and elaborated satisfactory

TABLE I

URINARY EXCRETION OF 2-MPA AND 2-MPG

Oral intake of 500 mg (3.1 mmol) of Thiola by ten healthy subjects.

Collection period after intake (h)	Urinary excretion (mean ± S.D.) (μmol)		Ratio 2-MPA/2-MPG
	2-MPA	2-MPG	
0- 6	7.0 ± 4.1	559 ±213	0.014 ± 0.010
6-12	19.1 ± 10.3	221 ±166	0.23 ±0.28
12-24	9.5 ± 6.1	14.7 ± 9.3	0.77 ±0.46
24-30	1.9 ± 1.3	1.7 ± 0.8	1.24 ±0.90

procedures for its determination [5]. By scrutinizing the chromatograms we detected small peaks in samples from subjects undergoing treatment with 2-MPG. The presence of 2-MPA was suspected, and by changing the chromatographic conditions we now have a method for determination of this compound together with the parent compound both in plasma and urine. We have also verified the compound in urine by gas chromatography–mass spectrometry [10]. Very little is yet known of the pharmacokinetics and metabolism of 2-MPG owing to the lack of suitable methods for its determination. We have found that ca. 30% of a given dose of 2-MPG in both humans [5] and dogs [11] is excreted in the urine when determined as free thiol after reduction. The presence of 2-MPA in plasma and urine indicates that 2-MPG is metabolized by the action of a hitherto undefined dipeptidase or amidase, which splits the amine bond. Preliminary data from dogs (Fig. 5) and human subjects (Table I) given 2-MPG orally show that 2-MPA appears later in plasma and urine than 2-MPG, which strengthens the postulation that 2-MPA is a degradation product of 2-MPG. However, the concentrations of the compound in plasma and urine are much lower than those of the parent drug, and it is therefore not clear that the occurrence of the metabolite is of any great significance for the prohibition of cystine stone formation. Further pharmacokinetic studies have to be performed in order to assess the absorption, bioavailability, metabolism and urinary excretion of 2-MPG.

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