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DETERMINATION OF 2-MERCAPTOPROPIONYLGLYCINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Methods for quantitative analysis of total and non-protein-bound 2-mercaptopropionylglycine (2-MPG) in plasma, and total 2-MPG in urine, have been developed. By reduction of urine, plasma or deproteinized plasma samples with tributylphosphine, 2-MPG is liberated from its disulphides, and after clean-up of the sample, 2-MPG is derivatized with N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM). The 2-MPG—DACM derivative is then quantified by high-performance liquid chromatography (HPLC) with fluorimetric detection. Both ion-suppression and ion-pair HPLC gave satisfactory chromatograms. The precision of the methods was satisfactory (coefficient of variation 3.1—5.8%), analytical recovery was quantitative (85—99%) and the two HPLC techniques were well correlated ($r = 0.99$).

Five healthy subjects receiving 500 mg of 2-MPG showed maximal total plasma concentration of 13.8—26.9 μmol/l at 3—5 h after intake, and their non-protein-bound 2-MPG was, at the same time, 62—77% of the total 2-MPG. The urinary excretion was 27.8 ± 3.8% (mean ± S.D.) of the given dose, most of it excreted within 12 h after intake.

INTRODUCTION

Cystinuria is a common genetically inherited disease characterized by impaired reabsorption of cystine in the renal tubuli [1]. This results in a markedly increased cystine excretion in the urine, and due to the low solubility of cystine the affected subjects often suffer from recurrent renal calculi [2].

Effective prophylactic treatment of cystinuria has been well documented, first with D-penicillamine [3, 4], and later on with 2-mercaptopropionylglycine (2-MPG) [5—8]. These drugs react with the symmetric cysteine disulphide by a thiolate—disulphide interaction, which gives the more soluble mixed drug—

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cysteine disulphide and cysteine as products [7]. The mixed disulphide can be quantified in urine by amino acid analysis, and the decrease in cystine excretion can be measured at the same time [8].

Several side-effects are associated with penicillamine therapy. These are gastrointestinal disturbances, skin lesions, proteinuria and nephrotic syndrome, trombocytopenia, and allergic reactions [9]. Such adverse effects are also observed with 2-MPG treatment, but less frequently and to a lesser degree [2, 4, 6, 7]. These side-effects disappear with treatment withdrawal, and usually do not reappear when the drug is restored [7]. This may indicate that the side-effects are dose dependent, and that the accumulation of the drug in blood may be responsible for the side-effects.

In previous publications [10—12], we dealt with the determination of thiol-containing compounds by high-performance liquid chromatography (HPLC) after derivatization with fluorogenic compounds. The aim of the present work was to develop a quantitative method for determination of 2-MPG. The principles used earlier were found suitable for quantitative analysis of this thiol in plasma and urine.

EXPERIMENTAL

Materials

2-Mercaptopropionylglycine, N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide and tri-*n*-butylphosphine were from Fluka (Buchs, Switzerland), and tetramethylammonium hydroxide (10% in water) was from Merck (Darmstadt, F.R.G.). The organomercurial adsorbent *p*-acetoxy-mercurianiline-Sepharose 4B (PAMAS) was prepared according to Sluyterman and Wijdenes [13], and was stored slurried in ethanol—water (58:42). We prepared 0.5-ml PAMAS columns (1.3 × 0.7 cm I.D.) in Econo-Columns (Bio-Rad Labs., Richmond, CA, U.S.A.) with a capacity of about 6 μmol of thiol per ml of packed gel. Similarly, the resin AG 50W-X8 (H^+ form, 100—200 mesh) obtained from Bio-Rad was packed into 0.5-ml columns (2.5 × 0.5 cm I.D.).

High-performance liquid chromatography

Ion-pair reversed-phase system. The equipment used in this system was a Model M-45 solvent delivery system from Waters Assoc. (Milford, MA, U.S.A.), a Waters Model 710B intelligent sample processor, a Waters automated switching valve, a Fluoromonitor III filter fluorimeter from LDC (Riviera Beach, FL, U.S.A.), and a Model SP 4270 integrator from Spectra-Physics (San Jose, CA, U.S.A.). The fluorimeter was operated with a mercury lamp, a 360-nm excitation filter, and a 418—700-nm emission filter.

The analytical column, a Supelcosil LC-8 column (150 × 4.6 mm I.D., particle size 5 μm) from Supelco (Bellefonte, PA, U.S.A.), was thermostated at 28°C using a circulating water-bath. The mobile phase was a water mixture of phosphate, tetramethylammonium (TMA) hydroxide and methanol prepared to contain 0.3 m*M* NaH_2PO_4, 1.3 m*M* Na_2HPO_4, 10 m*M* TMA and 19% methanol. The pH was adjusted to 7.4 with hydrochloric acid (6 *M*) before addition of the methanol. Mobile phases were filtered through a 0.8-μm cellulose acetate filter, type SM 111 (Sartorius, Göttingen, F.R.G.). An RP-8 (particle size

10 μm) guard column, 30 × 4.6 mm I.D. (RP-GU) from Brownlee (Santa Clara, CA, U.S.A.) was connected to the automated switching valve in such a way that when the peaks of interest had passed this column onto the analytical one, the guard column could be switched out of the stream line. The late-eluting peaks were then eluted from the guard column with mobile phase using a mini HPLC pump (Eldex E-120-S-2, Eldex Labs., Menlo Park, CA, U.S.A.), which made the system ready for the next injection.

Ion-suppression system. For this procedure, we used a Constametric III pump from LDC, an M7110 automatic injector (Magnus Scientific Instrumentation, Aylesbury, U.K.) with a 20-μl sample loop, and a Perkin-Elmer (Beaconsfield, U.K.) Model 3000 spectrofluorimeter with an LC-cell accessory. Excitation wavelength was 400 nm (band width 15 nm) and emission wavelength was 480 nm (band width 20 nm). The analytical column was a Supelcosil LC-8 column (250 × 4.6 mm I.D.), particle size 5 μm. The mobile phase was a mixture of 50 m*M* phosphoric acid and methanol (13:7). The system was operated at 24°C.

Blood and urinary samplings

Venous blood samples were drawn from a suitable fore-arm vein into 10-ml vacuum tubes containing 0.1 ml of 0.34 *M* EDTA (Vacutainer, Becton Dickinson). Immediately after mixing, the tubes were centrifuged at 1000 *g*, and a 1-ml aliquot of plasma was then deproteinized using ethanol (see procedure) for the determination of non-protein-bound 2-MPG. The remaining plasma was stored frozen until the analysis of total 2-MPG. Urine was collected in plastic bottles without additives, and an aliquot was stored at −20°C.

Procedure

Standard solution. A standard solution of 25 μ*M* 2-MPG was prepared in 10 m*M* hydrochloric acid, containing 2 m*M* disodium EDTA. A 50-μl aliquot of this solution was added to PTFE-lined screw-cap tubes containing 5.0 ml of 50 m*M* carbonate buffer and 10 m*M* disodium EDTA (pH 9.0). Then, 0.5 ml of DACM (20 μ*M* dissolved in acetone) was added. After mixing, the tubes were placed in a water-bath at 37°C for ca. 16 h.

Total plasma 2-MPG. Aliquots of 0.25 ml of plasma were added to tubes containing 0.75 ml of 0.1 *M* Tris buffer (pH 8.0), followed by 2.0 ml of 20 m*M* tributylphosphine in ethanol. After incubation at 50°C in a water-bath for 30 min, the tubes were centrifuged at 1000 *g* and 2.0 ml of the supernatant were transferred to a PAMAS column. After washing with 2 ml of 10 m*M* hydrochloric acid, the column was eluted with 3 ml of 10 m*M* cysteine hydrochloride. The eluate was then transferred to a column containing the cation exchanger AG 50W-X8, which retained the cysteine. 2-MPG was quantitatively eluted by an additional 1.0 ml of 10 m*M* hydrochloric acid, and was collected in tubes containing 0.2 ml of 0.18 *M* disodium EDTA. From the combined eluate and the washing, 2.0 ml were used for derivatization, and mixed with 3.0 ml of the carbonate buffer and 0.2 ml of 0.1 *M* sodium hydroxide, which was added to neutralize the hydrochloric acid in the sample. Derivatization was then performed with 0.5 ml of DACM (20 μ*M*), as with standard solutions.

Non-protein-bound plasma 2-MPG. In addition to the existence of 2-MPG as free thiol and as a protein-bound drug, it may exist in plasma as a symmetrical disulphide or in the form of mixed disulphides with other low-molecular thiols such as cysteine. For the determination of such a non-protein-bound low-molecular fraction including the free thiol, protein precipitation was effected as soon as possible after plasma sampling by mixing 1.0 ml of plasma with 2.0 ml of 99.5% ethanol. After centrifugation at 1000 *g*, the supernatant was transferred to a new tube, and stored at −20°C until analysis. For quantification, the supernatant was allowed to attain room temperature, then it was mixed and recentrifuged. Then, 1.0 ml of the supernatant was mixed with 0.5 ml of 0.1 *M* Tris buffer (pH 8.0) and 0.5 ml of 20 m*M* tributylphosphine in ethanol. After reduction for 30 min at 50°C, the mixture was quantitatively transferred to a PAMAS column. Purification and derivatization was then performed as for total 2-MPG.

Urinary 2-MPG. For determination of total 2-MPG in urine, a 0.25-ml sample of 10—100-fold diluted urine was taken for reduction and further processed according to the method for total plasma 2-MPG.

Pharmacokinetic study

We gave a single oral dose of 500 mg of 2-MPG (Thiola®, Santen, Osaka, Japan), as two 250-mg tablets, to three men and two women. They were all healthy, aged 24—37 years, and were fasting overnight before the study. No drugs were allowed, and the renal function of the subjects was normal as evaluated by serum creatinine determinations. Venous blood samples were collected before and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 30 and 48 h after the intake of 2-MPG. Urine was collected for 48 h.

RESULTS

Derivatization and high-performance liquid chromatography

The derivatization of 2-MPG was performed in the same way as with N-acetylcysteine [12]. The formation of the 2-MPG—DACM derivative was completed after ca. 30 min (Fig. 1), and the product obtained was stable for at least seven days.

We had both ion-suppression and ion-pair HPLC techniques available, and both of them gave suitable chromatograms for 2-MPG (Figs. 2 and 3). With ion-suppression HPLC (Fig. 2), 2-MPG gave three peaks, one main peak eluting in 13 min, and two smaller peaks with retention times of 15 and 19 min, one of them with nearly the same retention time as an unknown plasma constituent (cf. Fig. 2B and C). In addition, another small peak appeared in plasma devoid of 2-MPG. The reagent peaks close to the main 2-MPG peak were small, and therefore did not influence the results to any appreciable extent. With ion-pair HPLC (Fig. 3), three peaks appeared from 2-MPG, two of them rather close to each other. They were well separated from reagents and unknown plasma peaks. It should be noted that by determination of the ratio between the two main 2-MPG peaks in ion-pair HPLC, it is possible to check for hidden unrelated peaks from unknown drugs. The occurrence of

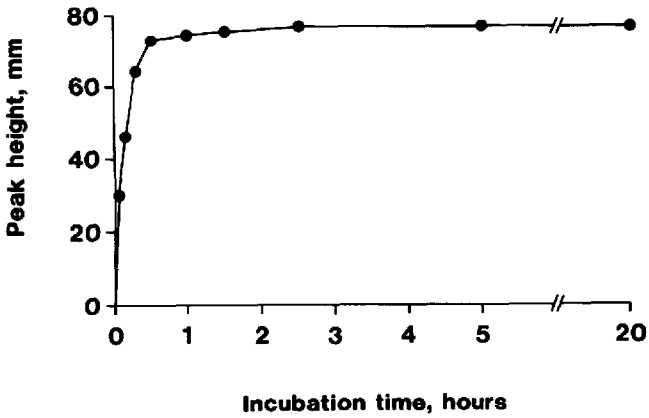

Fig. 1. Development of the fluorescent 2-MPG derivative. A 50-μl sample of 25 μM 2-MPG was added to 5 ml of a 50 mM carbonate buffer containing 10 mM disodium EDTA (pH 9.0), followed by 0.5 ml of 20 μM DACM in acetone.

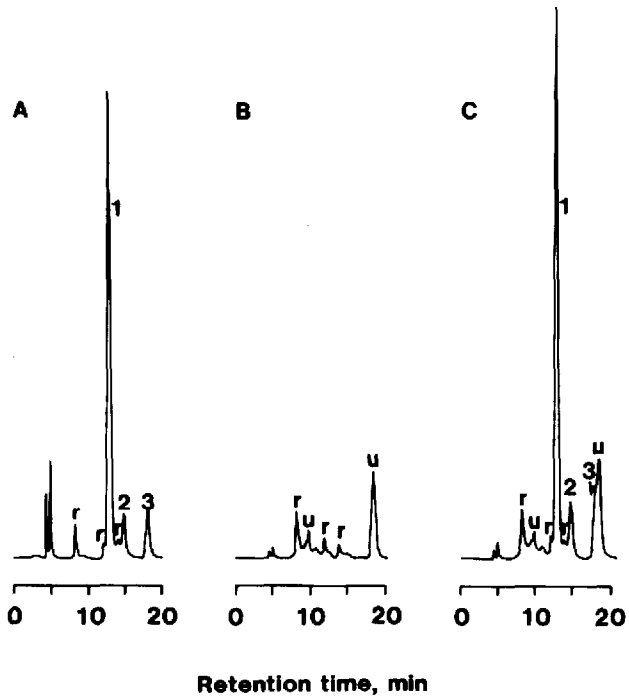

Fig. 2. Ion-suppression HPLC of DACM derivatives with 2-MPG. (A) Standard (injected amount 4.5 pmol), (B) blank patient plasma sample, and (C) plasma sample prepared according to the total method. The sample contained 19.8 μM 2-MPG and the injected amount corresponded to 5.5 pmol of 2-MPG derivative. Symbols: 1, 2 and 3 = peaks from 2-MPG, r = reagent peaks, u = unknown plasma peaks.

more than one peak from the 2-MPG—DACM derivative is probably due to the formation of diastereomers.

Sensitivity and detection limit of high-performance liquid chromatography

The detection limit of the HPLC analysis was estimated for the ion-pair

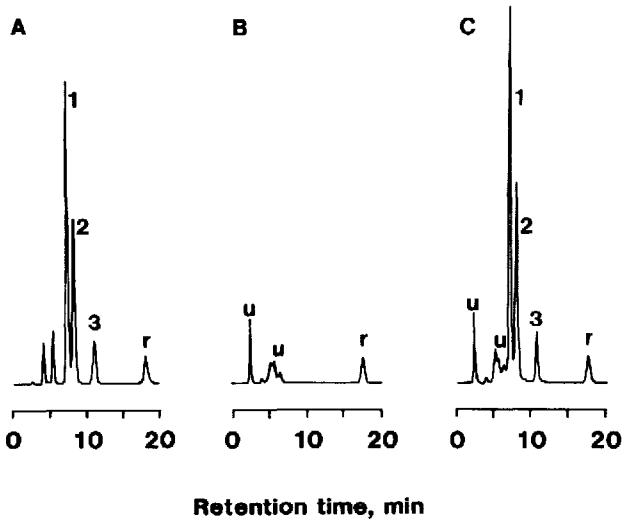

Fig. 3. Ion-pair HPLC. Samples are as in Fig. 2.

system with the injection of 180 fmol of 2-MPG—DACM derivatives. The detection limit was found to be 9.9 fmol when calculated as $A \cdot 2N/S$, where N = noise, S = signal (peak height) and A = amount of substance injected. With repeated injections of this standard sample, the peak response was 2500 ± 23 μV (mean ± S.D.), coefficient of variation (C.V.) = 0.92%, which also signifies a high precision of the HPLC method.

Linearity

We derivatized standard solutions of different concentrations of 2-MPG, ranging from 0 to 125 μM 2-MPG. The standard curve was linear for the range tested. With an injection volume of 10 μl, this corresponded to injected amounts of 0—11 pmol. The injected amount could be increased further, since the dynamic range for the fluorimeter was much higher. The range tested corresponded to plasma concentrations of 0—79 μM 2-MPG according to the procedure for clean-up.

Reduction of plasma and clean-up of sample

After intake, 2-MPG may exist in plasma either as a free thiol, as a low-molecular-weight disulphide, or as a disulphide with proteins. To obtain the total plasma 2-MPG content, including protein-bound 2-MPG, we reduced plasma samples with varying amounts of tributylphosphine. Fig. 4 shows that an added tributylphosphine concentration of 20 mmol/l was suitable. If higher concentrations were used, the samples became turbid. Similar experiments with supernatants obtained after alcohol precipitation of proteins showed that a lower amount of tributylphosphine could be used. In order to obtain conformity with both total and non-protein-bound 2-MPG, we chose a 20 mM reagent concentration.

Precision

Plasma and urine samples obtained after intake of Thiola were used for

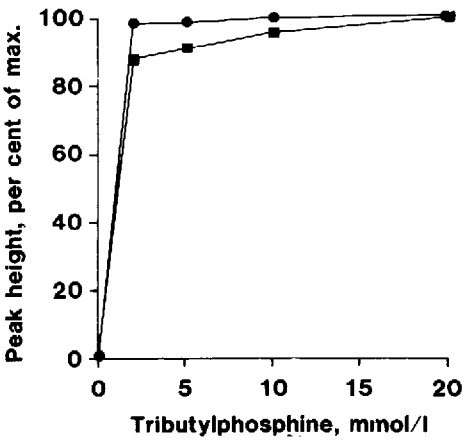

Fig. 4. Reduction of a plasma sample with different reagent concentrations of tributylphosphine, added according to the method for total (■) and non-protein-bound (●) 2-MPG.

TABLE I

PRECISION OF THE METHODS AS DETERMINED FROM DUPLICATE ANALYSIS AND FROM DETERMINATION OF RUNNING CONTROL SAMPLES

	n	Mean (μmol/l)	C.V. (%)
Plasma total 2-MPG			
Duplicate analyses	16	7.7	3.1
Running control	16	25.3	4.3
Plasma non-protein-bound 2-MPG			
Duplicate analyses	6	16.6	4.0
Running control	14	17.2	6.1
Urine 2-MPG			
Duplicate analyses	6	642	5.8
Running control	11	1807	5.6

precision studies. From duplicate determinations (utilizing ion-pair chromatography in the final step), the C.V. was calculated as 3.1—5.8% (Table I). From repeated analysis of pooled urine, plasma and protein-precipitated plasma, similar coefficients of variation were obtained (Table I), indicating satisfactory stability of the method in the long run.

Recovery

We added 2-MPG to plasma to increase the concentration by 9.9 and 19.6 μmol/l. The recovery was found to be quantitative (Table II).

Comparison of high-performance liquid chromatographic procedures

Plasma samples (n = 10) obtained from subjects after intake of 2-MPG were analysed both by ion-suppression HPLC (y) and ion-pair HPLC (x). For total plasma 2-MPG, the regression line was $y = 0.98x + 0.2$ ($r = 0.999$), and for non-

TABLE II

ANALYTICAL RECOVERY OF 2-MPG ADDED TO PLASMA

	Added concentration (μmol/l)	Recovery (%)		Added concentration (μmol/l)	Recovery (%)
Total 2-MPG	9.9	101	Non-protein-bound 2-MPG	19.6	92
	9.9	98		19.6	91
	19.6	99		19.6	78
	19.6	101		19.6	81
	19.6	97		19.6	83
	19.6	97		19.6	80
				19.6	82
	Mean ± S.D.	98.8 ± 1.8		19.6	91
				19.6	80
				19.6	83
				19.6	91
				19.6	86
				Mean ± S.D.	84.8 ± 5.1

protein-bound 2-MPG it was $y = 1.02x + 0.2$ ($r = 0.999$). Thus, an excellent agreement between the two HPLC methods was obtained, indicating that either of the two methods could be used in further studies.

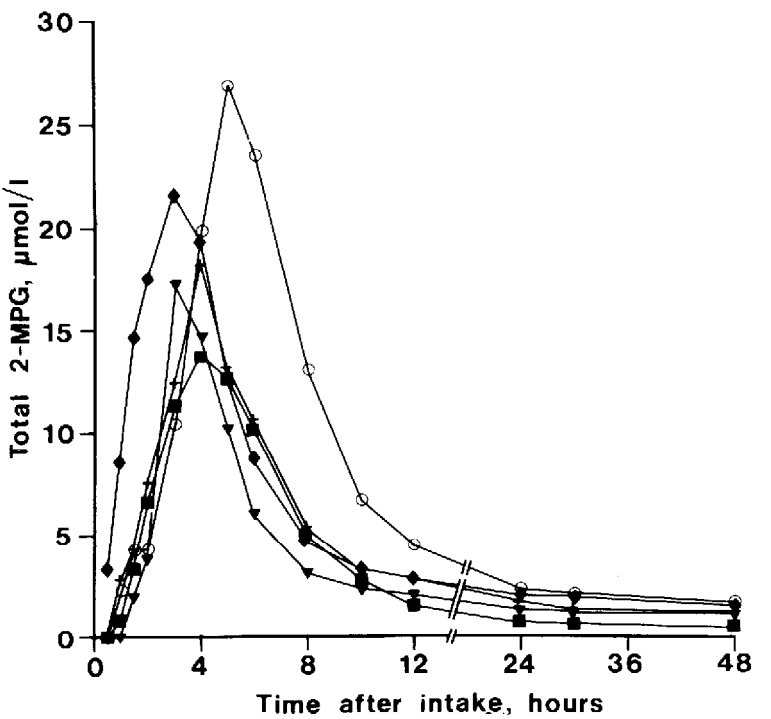

Fig. 5. Plasma total 2-MPG in five healthy subjects after oral intake of 500 mg of Thiola.

Applications to pharmacokinetic studies

Fig. 5 and 6 depict the results for total and non-protein-bound plasma 2-MPG in five fasting, healthy subjects receiving 500 mg of Thiola orally. It should be noted that the non-protein-bound 2-MPG concentration returned to low values earlier than the total concentration. For total plasma 2-MPG, the individual maximum value ranged from 13.8 to 26.9 μmol/l and was obtained at 3—5 h after intake. The maximum for non-protein-bound 2-MPG occurred simultaneously and attained a value of 62—77% of the total concentration. The difference between non-protein-bound and total 2-MPG represented the

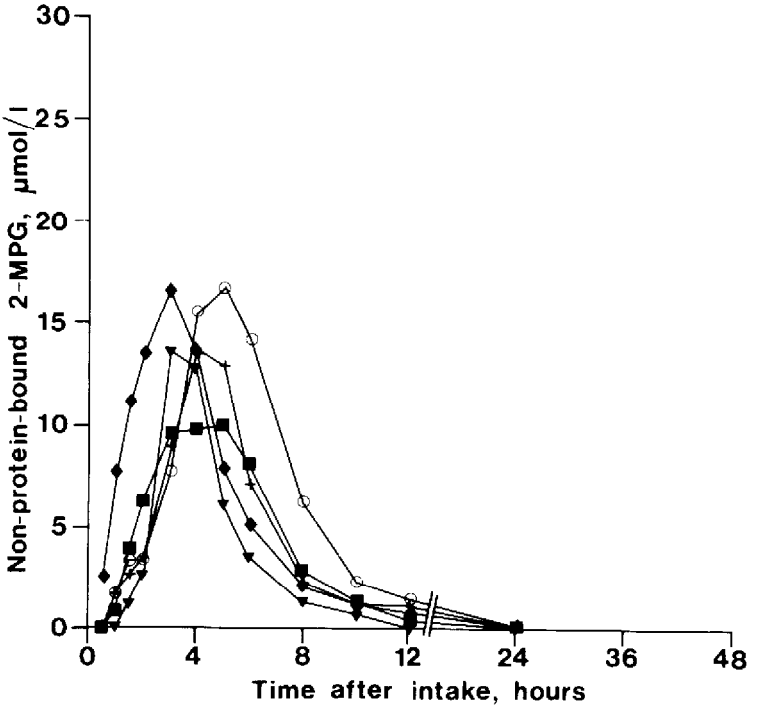

Fig. 6. Plasma non-protein-bound 2-MPG in five healthy subjects after oral intake of 500 mg of Thiola.

TABLE III

URINARY EXCRETION (μmol) OF 2-MPG AFTER ORAL INTAKE OF 500 mg (3.1 mmol) OF THIOLA BY FIVE HEALTHY SUBJECTS

Subject No.	Collection periods (hours after intake)				Recovery of dose (%)
	0—6	6—12	12—24	24—48	
1	314	469	20	0	26.2
2	588	80	7	0	22.0
3	775	184	11	0	31.7
4	745	132	15	0	29.1
5	544	363	17	0	30.2
Mean ± S.D.	593 ± 185	245 ± 164	14 ± 5	0	27.8 ± 3.8

protein-bound fraction. As a percentage of the total, this fraction increased during elimination of the drug. We also measured the urinary excretion of 2-MPG after the intake of Thiola (Table III), and found that it was ca. 28% of the given dose. The main part of this was excreted during the first 6 h (ca. 70% of the amount excreted), and more than 98% was excreted during the first 12 h.

DISCUSSION

Although 2-MPG (Thiola) has been utilized in the treatment of cystinuria [5—8] and rheumatoid arthritis [14] for several years, there is still no direct method available for its determination in blood. The compound was detected in biological fluids by use of the amino group—free thiol method [15] or the radioactive ^{35}S-labelled method [16]. Recently, Springolo et al. [17] reported an HPLC method for the determination of 2-furoyl-thiopronine, which was obtained when 2-furoyl chloride was reacted with 2-MPG. This method was suitable for quantitative determination of 2-MPG in urine. By using an amino acid analyser, a suitable resin for chromatography, and ninhydrin—amino acid quantification at 570 nm, Denneberg et al. [8] determined the free 2-MPG thiol, the symmetric 2-MPG disulphide and the mixed 2-MPG—cystine disulphide in urine together with cystine. Although well suited for urine assays, this method was not applied to plasma. The method described in this paper for plasma and urine offers good sensitivity and precision, and its accuracy is adequate. Thus, it may be a suitable tool for pharmacokinetic studies. With the use of this method, about fifteen samples can be processed during one working day, and on the following day the HPLC is performed. Thus, the analytical capacity is reasonable for such studies.

The data obtained for urine indicate that an appreciable amount of 2-MPG is absorbed after oral intake, since more than 22% of a given amount was excreted in urine within 24 h. The urine excretion was much higher than that described in rats by Springolo et al. [16]. This discrepancy may be explained by species differences. Furthermore, since a reduction step was not used, they measured the free thiol, while our method gives values for total excretion. From the limited application study included here, we conclude that the binding to protein is rather low, ca. 30%, in the post-absorbtive period. This fraction may increase in importance during the late elimination phase.

From the preliminary data discussed in this paper, it seems that the method is suitable for pharmacokinetic studies. Much remains to be investigated in this field, such as determination of bio-availability and evaluation of influence of food intake on absorption of the drug. Such studies are necessary for individual therapeutic control during treatment of cystinuria or rheumatoid arthritis with 2-MPG.

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