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High-performance liquid chromatographic evaluation of 2-(α-thenoylthio)propionylglycine and its two metabolites in biological fluids

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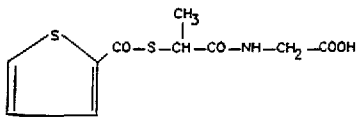
ABSTRACT

A high-performance liquid chromatographic (HPLC) method for determining 2-(α-thenoylthio)propionylglycine (TTPG) and its two main metabolites, thiophenecarboxylic acid and thiopronine, in biological samples was developed. TTPG and its metabolites were extracted by solvent partition and then determined by reversed-phase HPLC with UV detection at 245, 295 and 360 nm. This procedure was validated in order to allow the assay of these compounds in plasma and urine samples with sufficiently low detection limits (50 ng/ml for TTPG and TCA and 100 ng/ml for thiopronine) and with good linearity within the concentration range investigated. It was applied to a comprehensive pharmacokinetic investigation of TTPG in healthy volunteers.

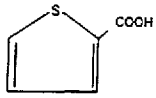
INTRODUCTION

2-(α-Thenoylthio)propionylglycine (TTPG) is a mucolytic agent which modifies bronchial secretion [1,2]. In man, it reduces mucus viscosity. In order to evaluate the pharmacokinetics of TTPG and its two main systemic metabolites, thiophene-2-carboxylic acid (TCA) and 2-mercaptopropionylglycine (thiopronine), a high-performance liquid chromatographic (HPLC) method was developed.

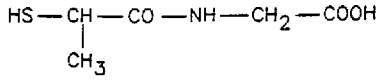
TTPG



TCA



Thiopronine



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Owing to the differences in the chemical and physical properties of the three substances, it is not possible to evaluate all of them by the same method. Two HPLC techniques with UV detection have been described previously, but only for TTPG in biological samples [3,4]. Few methods for the determination of thiol compounds by derivatization for fluorimetric or UV detection have been reported [5–8]. Our technique was based on reversed-phase HPLC, following a two-step extraction process, which enabled both TTPG and TCA to be determined with UV detection at 295 and 245 nm, respectively, and thiopronine to be determined with UV detection at 360 nm after derivatization with 2,4-dinitrofluorobenzene.

EXPERIMENTAL

Materials

TTPG and thiopronine were supplied by Sigma Tau Labs. and trichloroacetic acid (TCA), thiopheneacetic acid (TAA) and N-acetylcysteine by Janssen Chimica (Beerse, Belgium). All chemicals and HPLC-grade solvents were purchased from Merck (Bracco, Milan, Italy). The liquid chromatograph consisted of a Waters Assoc. Model M 590 pump (Millipore, Milford, MA, U.S.A.), an Waters Assoc. Wisp 710 B automatic injector and a Model 2550 variable-wavelength UV detector (Varian, Sunnyvale, CA, U.S.A.). The chromatographic columns were Nucleosil C_{18}, 5 μm (125 × 4.6 mm I.D.), purchased from Bischoff (Lymark, Roissy Charles de Gaulle, France) for TTPG and TCA and Hypersil ODS, 5 μm (250 × 4.6 mm I.D.), purchased from Shandon (Runcorn, U.K.) for thiopronine.

Assay of TTPG and TCA

Extraction procedure. In a 20-ml test-tube, 1 g of sodium chloride, 1 ml of plasma, 50 μl of a 15 μg/ml methanolic solution of TAA, 50 μl of a suitable standard solution of TTPG, TCA (calibration or control) or 100 μl of methanol (plasma samples) and 1 μl of buffer (pH 1.0) were successively introduced. After mixing for 10 s, the extraction solvent (5 ml), consisting of diethyl ether–hexane (50:50), was added. The tube was shaken for 15 min and centrifuged (10 min, 2000 *g*). The organic phase (4 ml) was transferred into a 10-ml tube containing 0.04 *M* disodium hydrogenphosphate solution (300 μl). After shaking for 5 min and centrifuging (5 min, 2000 *g*), the organic phase was discarded. An aliquot of the aqueous layer (200 μl) was transferred into a tube containing 20 μl of 10 *M* hydrochloric acid. The tube was shaken for 10 s and the mixture was ready for injecton into the HPLC system. Extraction from urine was carried out as described for plasma, with the addition of a washing step (3 ml of ethyl acetate) after the back-extraction of TTPG and TCA with the disodium hydrogenphosphate solution.

Preparation of standard solutions and eluent. Stock solutions of TTPG and TCA were prepared by dissolving the analytical standard (50 mg) in methanol (50 ml) in order to obtain a (1000 μg/ml) solution. The internal stock standard solution (TAA) was prepared by dissolving the analytical standard (15 mg) in methanol (100 ml) in order to obtain a 150 μg/ml solution. Mobile phase was prepared by adding acetonitrile (150 ml), potassium dihydrogenphosphate (1.36 g) and orthophosphoric acid (1 ml) and water to give a 1000 ml volume.

HPLC analysis. HPLC was performed under isocratic conditions at a flow-rate

of 1 ml/min and room temperature; a change of the wavelength was used for the detection of TCA and TAA at 245 nm (0–15 min) and TTPG at 295 nm (5–25 min). Under these conditions the retention times of TCA, TAA and TTPG were 8, 9.3 and 25 min, respectively.

Preparation of calibration graphs. Calibration graphs were obtained from drug-free blank plasma or urine. To each 1 ml of blank plasma or urine 50 μl of the appropriate solution were added to achieve final concentrations of TTPG and TCA of 2000, 1000, 500, 100 and 50 ng/ml. The blank sample was obtained by adding 100 μl of methanol to 1 ml of plasma. Reproducibility was assessed using blank plasma samples spiked with concentrations of the analytes (TCA or TTPG) of 2000, 500, 100, 50 and 0 ng/ml.

Assay of thiopronine

Extraction procedure. In a 20-ml test-tube, 1 ml of plasma, 100 μl of a 10 μg/ml solution of N-acetylcysteine containing 50 μg/ml dithiothreitol (DTT), 100 μl of the appropriate solution (calibration or control) or 100 μl of water containing 50 μg/ml DTT (plasma samples), 1 ml of water and 100 μl of 5 *M* hydrochloric acid were successively introduced. After mixing for 10 s, the extraction solvent (4 ml) consisting of diethyl ether–methylene chloride (60:40) was added. The tube was shaken and centrifuged (10 min, 2000 *g*). Then they were kept at −20°C for 10 min. The organic phase was discarded. This operation was repeated once. The aqueous phase (2.2 ml) was transferred to another tube and 0.5 ml of 0.5 *M* sodium carbonate solution was added. After mixing for 10 s, 400 μl of a 5 mg/ml aqueous solution of DTT were added. Then 0.269 *M* 2,4-dinitrofluorobenzene solution (125 μl) was added and the mixture was heated for 40 min at 60°C. The tube was cooled and hexane (5 ml) was added. After shaking for 10 min and centrifuging (5 min, 2000 *g*), the organic phase was discarded. To the aqueous phase 5 *M* hydrochloric acid (100 μl) and the extraction solvent (7 ml) were added. The tube was mixed for 10 min and centrifuged (5 min, 2000 *g*). The organic phase was transferred to a 10-ml tube, then evaporated to dryness under a gentle stream of nitrogen (40°C). The residue was resuspended in the mobile phase (250 μl) and the sample was ready for injection into the HPLC system. Extraction from urine was the same as described for plasma, except that 1 *M* $NaHCO_3$ was used instead of 0.5 *M* Na_2CO_3, the residue being dissolved in 1 ml of mobile phase.

Preparation of standard solutions and eluents. Stock solutions of thiopronine and of N-acetylcysteine were prepared by dissolving analytical standards (50 mg) in water containing 50 μg/ml DTT, in order to obtain two 1000 μg/ml solutions. Mobile phase A for plasma analysis was prepared by adding acetonitrile (70 ml), potassium dihydrogenphosphate (1.36 g) and orthophosphoric acid (1 ml), completed to 1000 ml with distilled water. Mobile phase B for urine analysis was prepared by adding acetonitrile (50 ml), tetrahydrofuran (20 ml), distilled water (950 ml) and potassium dihydrogenphosphate (1.36 g).

HPLC analysis. HPLC was performed at a flow-rate of 1 ml/min and room temperature and with UV detection at 360 nm.

Urine samples were analysed under isocratic conditions (90% B, 10% acetonitrile) and plasma samples according to the following gradient programme: 0–12 min, A–acetonitrile from 80:20 to 70:30; 12–15 min, A–acetonitrile from 70:30 to 65:35; 15–25 min, linear increase to 100% acetonitrile; 25–30 min, 100% acetonitrile; 30–32 min, linear decrease to A–acetonitrile (80:20); 32–35 min, A–acetonitrile (80:20).

Under these conditions, the retention times were thiopronine 14 min and internal standard 11 min. In urine analysis, the retention time of thiopronine was 33 min.

Preparation of calibration graphs. For the preparation of calibration graphs, to each 1 ml of blank plasma (100 μl of blank urine) 100 μl of the appropriate solution were added to achieve final thiopronine concentrations of 2, 1, 0.5, 0.2 and 0.1 μg/ml

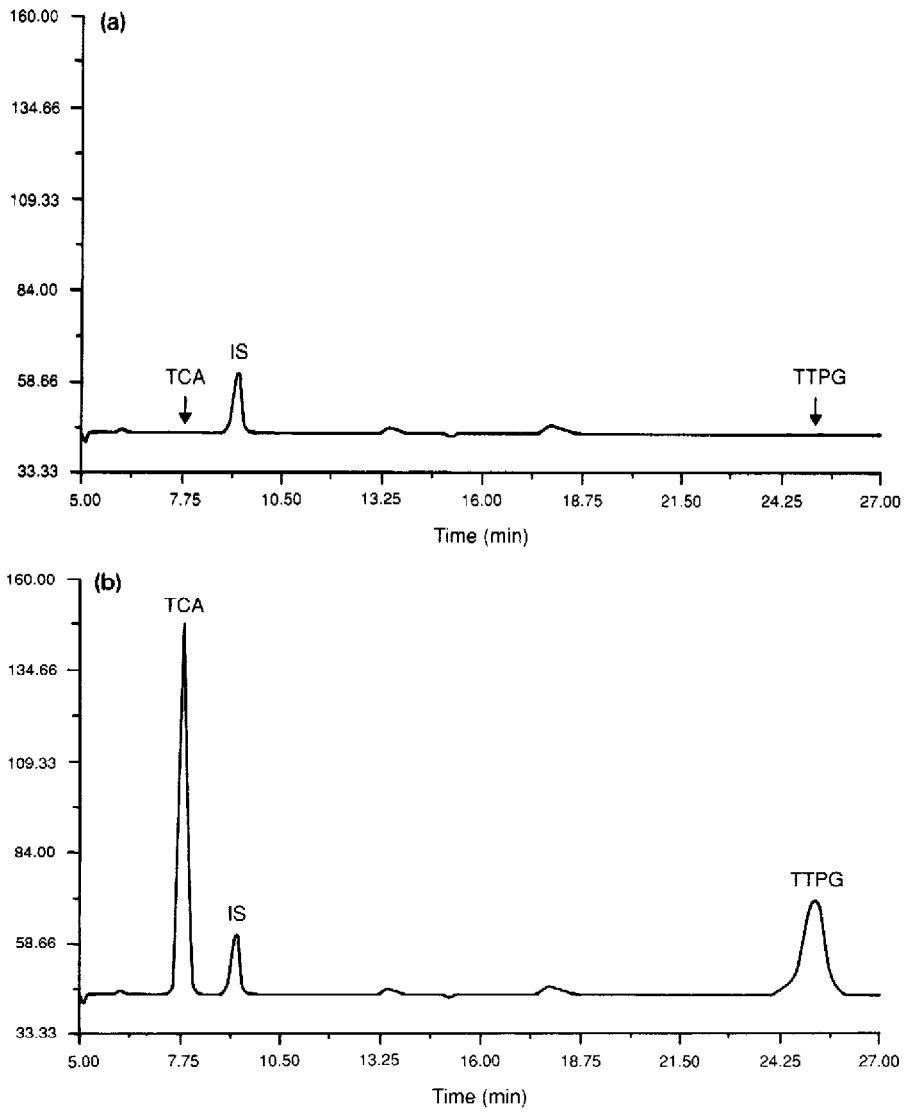

Fig. 1. Representative chromatograms obtained with (a) a blank plasma sample and (b) a blank plasma sample spiked with 2000 ng/ml of TTPG and TCA. The chromatographic column was Nucleosil C_{18}, 5 μm (125 × 4.6 mm I.D.). The eluent was 0.01 *M* potassium dihydrogenphosphate and 1 ml orthophosphoric acid in acetonitrile–water (15:85). The sample aliquot injected was 50 μl. The flow-rate was 1 ml/min. UV detection was performed at 245 nm (0.02 a.u.f.s.) from 0 to 15 min and at 295 nm (0.01 a.u.f.s.) from 5 to 25 min.

for plasma and 200, 100, 50, 20 and 10 μg/ml for urine. The blank plasma (or urine) was obtained by adding a 50 μg/ml aqueous solution of DTT (100 μl) to 1 ml of plasma (or 100 μl of urine). Reproducibility was assessed using blank plasma spiked with concentrations of the analyte of 1.5, 0.5, 0.2 and 0.1 μg/ml.

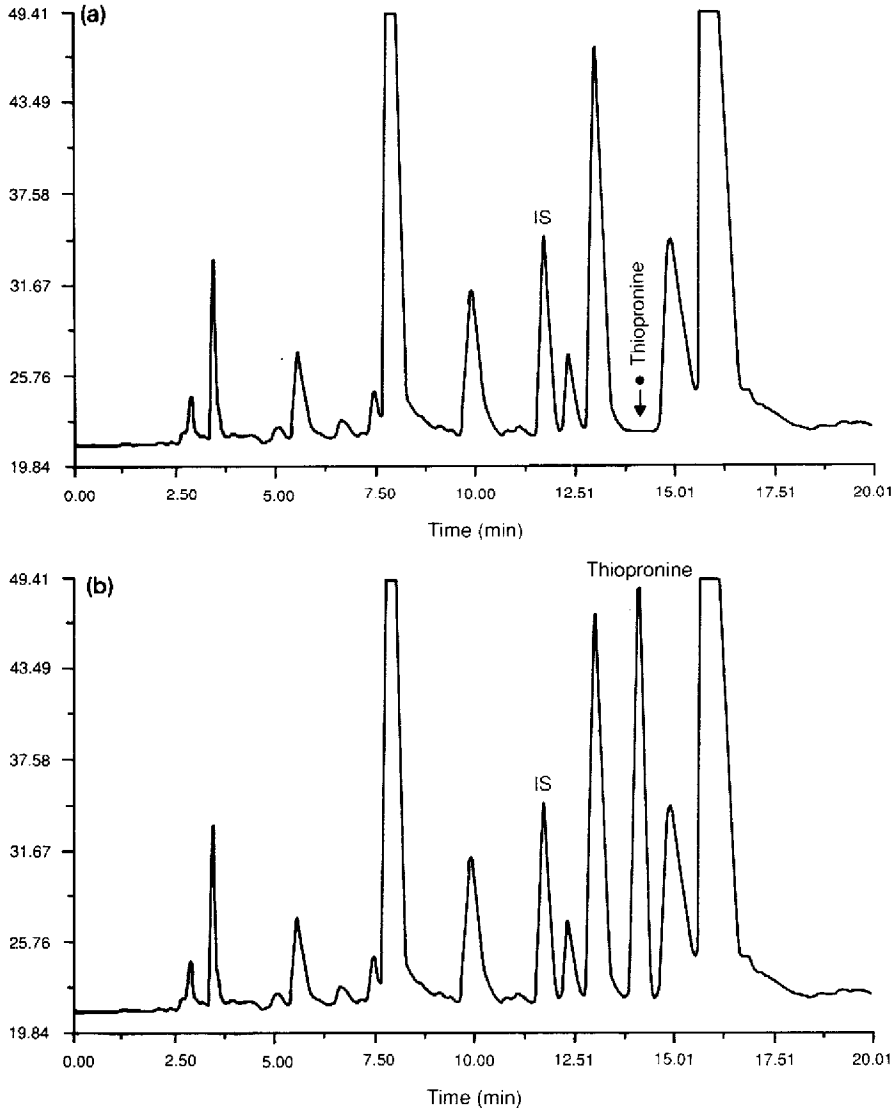

Fig. 2. Representative chromatograms obtained with (a) a blank plasma sample and (b) a blank plasma sample spiked with 2000 ng/ml of thiopronine. The chromatographic column was Hypersil ODS, 5 μm (250 × 4.6 mm I.D.). Eluent A was 0.01 *M* potassium dihydrogenphosphate and 1 ml orthophosphoric acid in acetonitrile–water (7:93). Elution was carried out according to the gradient programme given in the text. The sample aliquot injected was 20 μl. The flow-rate was 1 ml/min. UV detection was performed at 360 nm (0.01 a.u.f.s.).

Pharmacokinetic study with healthy volunteers

Twelve subjects were involved in the pharmacokinetic study; all of them received a single oral dose of 540 mg of TTPG. Blood samples were collected 10, 20, 30 and 45 min and 1, 1.25, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after administration. Urine samples were collected at 0–2, 2–4, 4–6 and 6–12 h. A basal sample of blood and urine was also collected.

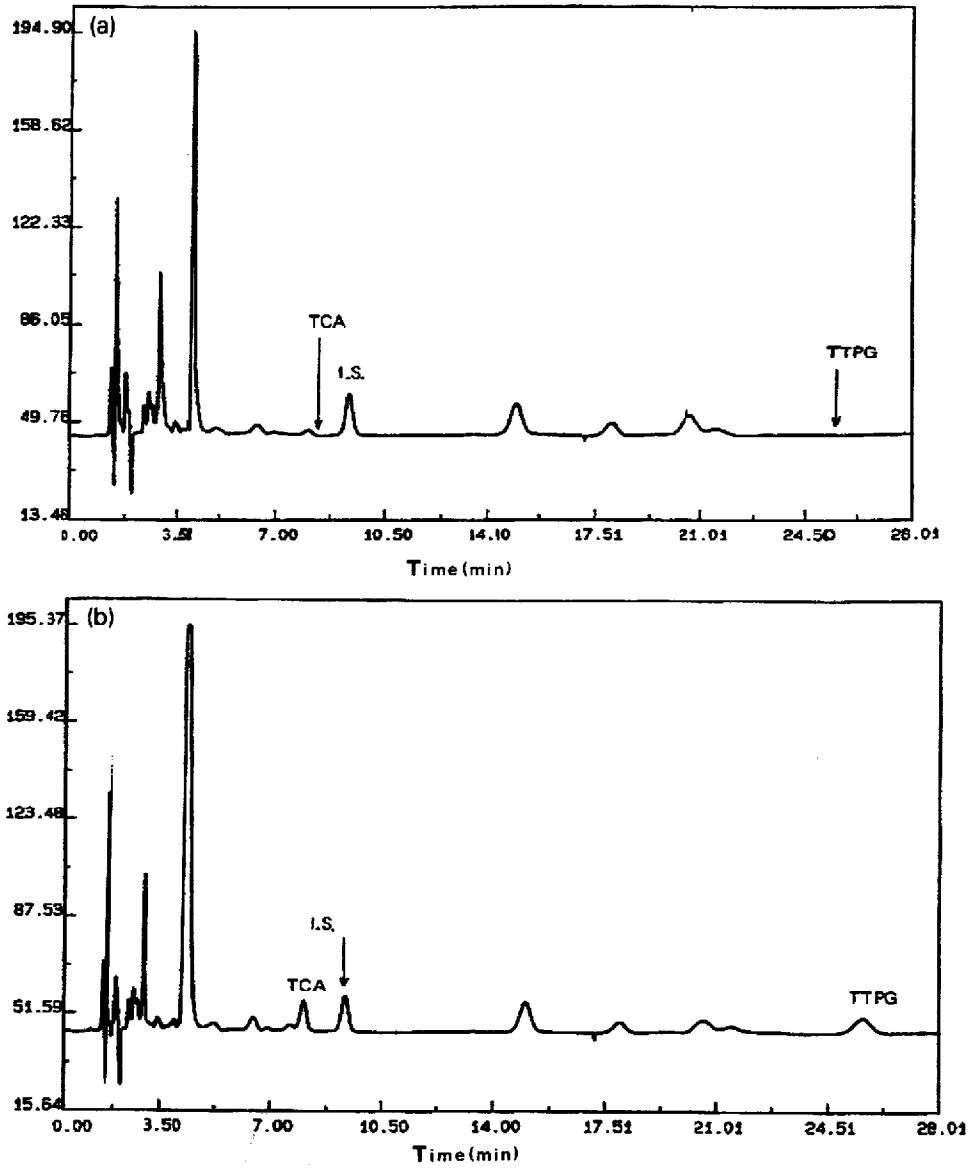

Fig. 3. Representative chromatograms obtained with (a) a blank urine sample and (b) a blank urine sample spiked with 500 ng/ml of TTPG and TCA. For chromatographic conditions, see Fig. 1.

TABLE I

RECOVERY AND LINEARITY IN THE DETERMINATION OF TTPG, TCA AND THIOPRONINE IN PLASMA AND URINE

Sample	Range studied (μg/ml)	n	Linear correlation[a]	r	Mean recovery (%)	R.S.D. (%)
TTPG in plasma	0.05–2.0	31	$y = -7.490 + 1.040x$	0.9995	100	1.72–5.26
TTPG in urine	0.2 –2.0	19	$y = 0.006 + 0.928x$	0.9996	92.7	1.03–7.73
TCA in plasma	0.05–2.0	30	$y = -4.520 + 1.059x$	0.9998	100	0.52–3.13
TCA in urine	0.2 –2.0	17	$y = -0.018 + 1.006x$	0.9937	99.1	5.27–10.88
Thiopronine in plasma	0.1 –1.5	62	$y = 10.844 + 0.9931x$	0.9941	99.3	2.01–5.40
Thiopronine in urine	10 –200	28	$y = -1.608 + 0.998x$	0.9848	97.4	7.42–13.31

[a] y = Drug recovered (μg/ml); x = drug added (μg/ml).

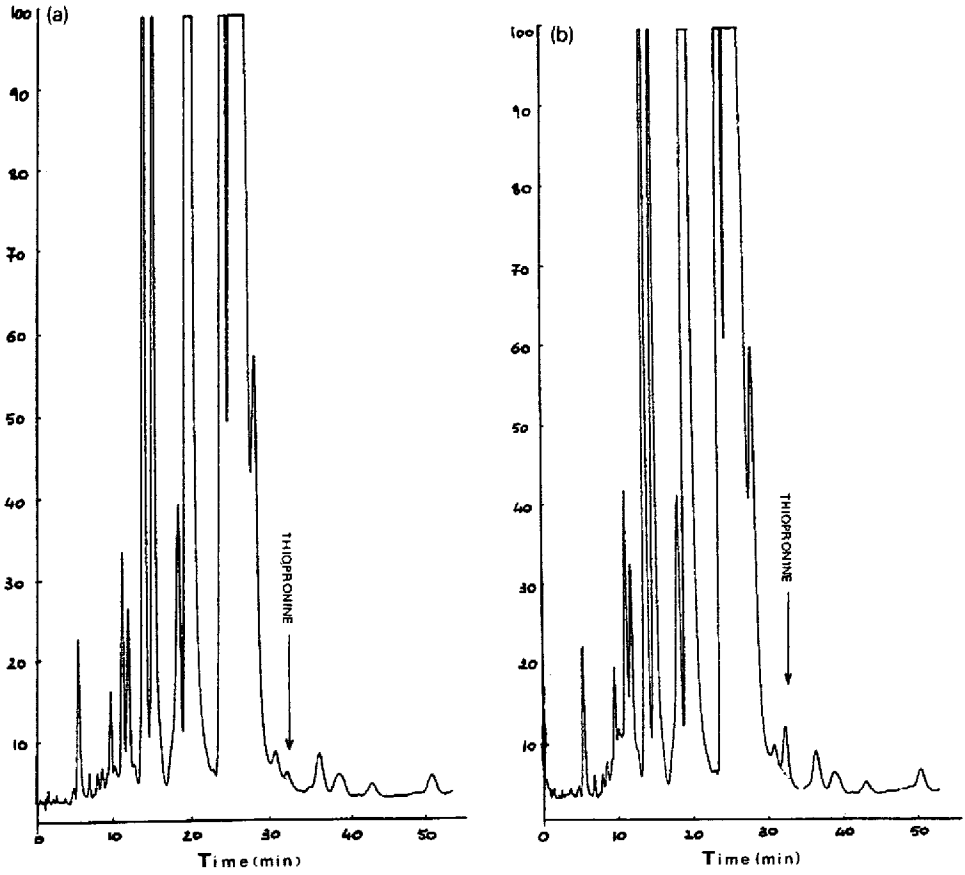

Fig. 4. Representative chromatograms obtained with (a) a blank urine sample and (b) a blank urine sample spiked with 10 μg/ml of thiopronine. The chromatographic column was Hypersil ODS, 5 μm (250 × 4.6 mm I.D.). Eluent B was 0.01 *M* potassium hydrogenphosphate in acetonitrile–tetrahydrofuran–water (5:2:93). Elution was carried out under isocratic conditions using B–acetonitrile (90:10). The sample aliquot injected was 20 μl. The flow-rate was 1 ml/min. UV detection was performed at 360 nm (0.01 a.u.f.s.).

TABLE II

MEAN VALUES ± STANDARD DEVIATION OF TTPG, TCA AND THIOPRONINE PHARMACOKINETIC PARAMETERS AFTER A SINGLE 540-mg ORAL ADMINISTRATION OF TTPG TO HEALTHY VOLUNTEERS

Parameter[a]	TTPG	TCA	Thiopronine
C_{max} (μg ml^{-1})	2.49 ± 0.75	1.22 ± 0.43	8.02 ± 2.27
t_{max} (h)	0.39 ± 0.08	0.53 ± 0.32	0.71 ± 0.23
AUC_{0-t} (μg ml^{-1} h)	2.04 ± 0.66	1.34 ± 0.51	23.16 ± 6.81
$AUC_{0-\infty}$ (μg ml^{-1} h)	2.19 ± 0.71	1.50 ± 0.48	25.06 ± 7.20
$t_{1/2}$ (h)	0.54 ± 0.24	0.91 ± 0.36	3.51 ± 0.64
MRT (h)	0.94 ± 0.31	1.38 ± 0.47	4.04 ± 0.44
Lag time (h)	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.05

[a] C_{max}, peak concentration; t_{max}, time to peak; AUC, area under the plasma concentration–time curve; $t_{1/2}$, dominant half-life; MRT, mean residence time.

RESULTS AND DISCUSSION

TTPG and TCA were extracted from plasma (or urine), then back-extracted into an aqueous phase and separated from endogenous compounds in a reversed-phase chromatographic mode with UV detection at 295 nm (TTPG) and 245 nm (TCA, TAA) (Figs. 1–3). The lowest detectable concentration was 50 ng/ml in plasma and 200 ng/ml in urine. The plasma and urine calibration graphs were linear for concentrations ranging from 50 to 2000 ng/ml of TCA and TTPG (Table I). The relative standard deviations (R.S.D.) were 1.72–5.26% and 1.03–7.73% for TTPG in plasma and urine, respectively, and 0.52–3.13% and 5.27–10.88% for TCA in plasma and urine (Table I).

A method able to measure thiopronine was also developed. A two-step extraction was performed, always using a reducing compound (DTT) in order to avoid the easy oxidation of thiol groups. Then a chromatographic gradient allowed the separation of both thiopronine and N-acetylcysteine from endogenous compounds to be carried out; UV detection was performed at 360 nm after derivatizing thiopronine with 2,4-dinitrofluorobenzene (Figs. 2–4). The lowest detectable concentration was 100 ng/ml. The calibration graphs were linear for concentrations ranging from 0.1 to 2.0 μg/ml of thiopronine for plasma and from 10 to 200 μg/ml of thiopronine for urine (Table I). The R.S.D.s were 2.01–5.40% for plasma and 7.42–13.31% for urine.

TABLE III

URINARY EXCRETION OF TTPG, TCA AND THIOPRONINE FOLLOWING A 540-mg SINGLE ORAL ADMINISTRATION OF TTPG TO HEALTHY VOLUNTEERS

Mean values in mg and as a percentage of the dose administered in the 0–12-h period, ± standard deviation.

Unit	TTPG	TCA	Thiopronine
mg	3.14 ± 2.42	0.125 ± 0.124	170.49 ± 49.42
%	0.58 ± 0.45	0.05 ± 0.05	52.88 ± 15.33

After a preliminary pharmacokinetic study, it was concluded that, following oral administration of a single 540-mg dose, TTPG is rapidly absorbed. As the two main metabolites, TCA and thiopronine, are rapidly detected in plasma, the metabolism of TTPG should be considered as a rapid event (Table II). Moreover considering the area under the curve and cumulative recovery for urine, thiopronine appears to be the main circulating compound (Table III).

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