Journal of Chromatography, 564 (1991) 258–265
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5628

Short Communication

Determination of 2-mercaptopropionylglycine and its metabolite, 2-mercaptopropionic acid, in plasma by ion-pair reversed-phase high-performance liquid chromatography with post-column derivatization

PIERRE LEROY, ALAIN NICOLAS*, CATHERINE GAVRILOFF and MURIEL MATT

Laboratoire de Chimie Analytique, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, B.P. 403, 54001 Nancy Cedex (France)

PATRICK NETTER and BERNARD BANNWARTH

Laboratoire de Pharmacologie Clinique, URA CNRS 1288, Faculté de Médecine, Avenue de la Forêt de Haye, 54500 Vandoeuvre (France)

and

BERNARD HERCELIN and MICHEL MAZZA

Laboratoires Cassenne, 17 Rue de Pontoise, B.P. 31, 95521 Cergy-Pontoise Cedex (France)

(First received May 30th, 1990; revised manuscript received September 3rd, 1990)

ABSTRACT

A simple and fast high-performance liquid chromatographic method was developed for the simultaneous measurement of 2-mercaptopropionylglycine (Tiopronine®) and its metabolite (2-mercaptopropionic acid) in human plasma after the administration of a pharmaceutical dosage form (Acadione®). The sample treatment before high-performance liquid chromatographic analysis consisted of the reduction of the corresponding disulphides by tri-*n*-butylphosphine and protein precipitation with ethanol. Separation was achieved by ion-pair high-performance liquid chromatography on a reversed-phase column (LiChrospher RP 18e) with cetrimonium bromide as counter ion and detection by fluorimetry after post-column derivatization with a selective thiol reagent, *i.e.* pyrenemaleimide. The high frequency of the analyzed samples and validation results make the method suitable for pharmacokinetic studies, and this was demonstrated by the first results obtained after the administration of an oral dose of 500 mg of Tiopronine to two healthy subjects.

INTRODUCTION

N-(2-Mercaptopropionyl)glycine (2-MPG) is a drug used in cystinuria and rheumatoid arthritis treatment [1]. 2-MPG in plasma exists in numerous forms since its free sulphydryl group is sensitive to oxidation. So 2-MPG can be ox-

0378-4347/91/$03.50

idized to disulphides either as a dimer or as mixed forms with endogenous thiols, *i.e.* cysteine and glutathione or proteins such as albumin. Moreover, the 2-MPG amide bond slowly hydrolyzes to release 2-mercaptopropionic acid (2-MPA) and, in turn, various oxidized forms. This complex metabolic pathway prevents the simple measurement of each species. Previous analytical methods reported for the metabolism and pharmacokinetic studies of thiol drugs, *e.g.* D-penicillamine [2], N-acetylcysteine [3,4] and captopril [5], only measured free or total drug concentrations in plasma by high-performance liquid chromatography (HPLC) before and after a reduction step.

Pre-column derivatization for the HPLC analysis of 2-MPG with UV detection has been reported and applied to its determination in urine [6]. More recently, a HPLC technique including a pre-column fluorigenic derivatization has been described for the simultaneous measurement of the free or total 2-MPG and 2-MPA contents in plasma [7,8].

We have developed a simple and fast HPLC method for the measurement of plasma concentrations of 2-MPG and 2-MPA. Chromatographic conditions were optimized to provide good resolution between 2-MPG and 2-MPA with short retention times. Detection was operated after post-column derivatization using a thiol-selective fluorigenic reagent. This HPLC system has been combined to a two-step pretreatment of plasma.

EXPERIMENTAL

Chemicals and reagents

2-MPG (Tiopronine®), its disulphide and Acadione® tablets (250 mg of 2-MPG per unit) were supplied by Cassenne Laboratories (Cergy-Pontoise, France). Brij-35, triethylamine, tetrabutylammonium bromide, hexadecyltrimethylammonium (cetrimonium) bromide, *o*-phthaldialdehyde (OPA), taurine, tri-*n*-butylphosphine, and 2-MPA were obtained from Aldrich (Strasbourg, France). N-(1-Pyrenyl)maleimide was purchased from Fluka (Buchs, Switzerland). All other chemicals and solvents were of analytical-reagent grade and used without further purification.

Apparatus

The HPLC system consisted of a double reciprocal piston pump with a pulse damper (Spectroflow 400, Applied Biosystems, Foster City, CA, U.S.A.), an injection valve (7125, Rheodyne, Cotati, CA, U.S.A.) fitted with a 50-μl sample loop and a column oven (Croco-Cil, Spectra-Physics, Les Ulis, France). The outlet of the analytical column was connected to a three-way post-column reagent delivery pump (Hitachi 655 A-13, Merck-Clevenot, Nogent-sur-Marne, France). A PTFE-knitted open tubular reactor (3 m × 0.5 mm I.D., Supelco, St.-Germain-en-Laye, France) was used to perform the post-column reaction at ambient temperature.

The derivatives were measured either with a fluorescence spectrophotometer (Spectroflow 980, Applied Biosystems) or with an amperometric detector (IBM E 230, Merck-Clevenot) fitted with a thin-layer cell incorporating a glassy carbon working electrode and a saturated calomel reference electrode (SCE).

Chromatogram recordings and peak-area measurements were realized with a data station (Winner, Spectra-Physics).

Sample preparation

Venous blood samples were drawn in chilled EDTA vacutainer tubes (Becton Dickinson, Grenoble, France) and were immediately centrifuged at 1000 *g* for 5 min at 5°C. Plasma samples were stored at −80°C until analysis.

The reduction step was performed as follows: plasma (1 ml) was added with 0.2 *M* phosphate buffer pH 8.0 (0.2 ml) and 10% (v/v) tributylphosphine in chloroform (0.2 ml), vortexed for 15 s and heated at 50°C for 30 min. Protein precipitation was effected after cooling in an ice-bath and the addition of ethanol (2 ml). The samples were vigorously mixed by vortexing for 15 s and immediately centrifuged at 1800 *g* for 10 min at 5°C. An aliquot of supernatant (50 μl) was subjected to HPLC analysis with a delay time before injection which did not exceed 15 min.

Chromatographic conditions

Two chromatographic systems were tested.

(I) A column (250 mm × 4 mm I.D.) prefilled with LiChrospher RP-18 (5 μm particle size, Merck-Clevenot) and eluted with acetonitrile–10^{-2} *M* phosphate buffer pH 5.5 (15:85, v/v) containing 5 m*M* tetrabutylammonium bromide.

(II) A column (125 mm × 4 mm I.D.) prefilled with LiChrospher RP-18e (5 μm) and eluted with acetonitrile–10^{-2} *M* phosphate buffer pH 7.0 (25:75, v/v) containing 5 m*M* cetrimonium bromide.

Analytical columns were protected by a guard column (4 mm × 4 mm I.D.) prefilled with the same material. All mobile phases were filtered through a 0.2-μm Nylon microfilter (Sartorius, Palaiseau, France), degassed under vacuum and used at a flow-rate of 1 ml min^{-1}. Chromatography was performed at 35°C.

Post-column derivatization and detection conditions

Two thiol post-column reactions were compared to obtain selective and sensitive detection of 2-MPG and 2-MPA.

(I) OPA–taurine reaction [9,10] including the successive addition of 25 m*M* taurine in 0.4 *M* borate buffer pH 9.5 and 25 m*M* OPA in methanol–water (50:50, v/v), both at a flow-rate of 0.1 ml min^{-1}. Derivatives were monitored either by amperometry (+0.7 V *vs* SCE) or by fluorimetry (excitation at 360 nm; emission selected by a long-pass wavelength filter at 389 nm).

(II) Pyrenemaleimide reaction [3,4] after alkalinization of the mobile phase with a 2% triethylamine and 1% Brij-35 solution in acetonitrile–water (30:70,

v/v) and then the addition of $5 \cdot 10^{-5}$ *M* pyrenemaleimide solution in anhydrous acetonitrile, both at a flow-rate of 0.35 ml min^{-1}. Derivatives were monitored by fluorimetry (excitation at 260 nm, emission selected by a long-pass filter at 370 nm).

Optimization and validation procedure

The elaboration of the different steps of plasma treatment, the development of the HPLC method and its validation were performed on several plasma pools spiked with 2-MPG and 2-MPA standard solutions. Stock solutions (0.1 mg ml^{-1}) and dilutions were prepared daily in 10^{-2} *M* phosphate buffer added with 10^{-2} *M* EDTA and adjusted to a final pH value of 7.5. They were stored at 5°C.

Different delay periods ranging from 2 to 30 min between the addition of thiols to the plasma and the beginning of the reduction step were tested either in an ice-bath or at room temperature. Since no significant difference was observed, a 2-min waiting time at room temperature was retained.

Pharmacokinetic study

A single oral dose of 500 mg of 2-MPG (Acadione) was given to two healthy men aged 19 and 22 years, respectively. No drugs were allowed and the renal and the hepatic functions of the subjects were normal as evaluated by biological determinations. Venous blood samples were collected before and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 24 and 48 h after the intake of 2-MPG.

RESULTS AND DISCUSSION

Elaboration of HPLC method

2-MPG and 2-MPA lack a chromophore, hence sensitive UV detection is precluded and other modes have to be considered. D-Penicillamine, a major drug in the same therapeutic class, and other thiols have been detected by amperometry on gold/mercury amalgamed electrodes at low potential values or on glassy carbon at higher potentials [2]. Electrochemical detection on a gold/mercury amalgamed electrode offers high selectivity and sensitivity but the unstability of the response still remains a drawback for routine analysis [11]. A glassy carbon electrode does not have this disadvantage but the high potential values needed to detect sulphydryl compounds give poor selectivity. Numerous pre- or post-column derivatization procedures have also been developed coupled with various detection modes: electrochemistry [11,12], UV–visible spectrophotometry [5,6,13] or fluorimetry [3,4,7–10,14].

Previously reported HPLC techniques for 2-MPG determination in biological fluids rely upon pre-column derivatization reactions [6–8].This approach is characterized by multi-step sample preparation and late eluting peaks during the chromatographic run. Post-column derivatization generally allows the automation of the analytical procedure and we have investigated two different reactions.

The use of OPA and taurine, also called "OPA reversed reaction", has already been applied to various thiols [9,10] but in our case, a fluorescent derivative was obtained only with 2-MPA. However electrochemical detection gave a signal with a ratio of molar response 2-MPA/2-MPG close to 4. The reaction with pyrene-maleimide has been recently reported for the determination of N-acetylcysteine and other endogenous thiols in plasma [3,4]. We successfully applied it to the detection of 2-MPG and 2-MPA. A triethylamine–Brij-35 solution was preferred as an alkaline reagent to a borate buffer in order to prevent the blocking of the post-column module and the detector flow cell. The reaction medium resulting from mixing the mobile phase with the post-column reagents presents an apparent pH value of 11 which allows a complete reaction in few seconds [3]. Selectivity *versus* endogenous compounds was better than in the case of electrochemical detection and the technique was chosen for plasma monitoring of 2-MPG and 2-MPA.

Chromatographic optimization relied upon suitable buffering of the mobile phase. A pH value higher than pK_a of the analytes carboxylic group provided:
— their full dissociation and hence an ion-pairing technique was possible. In preference to tetrabutylammonium, a structurally "bulky ion", the chosen counter ion was cetrimonium since, having a long alkyl chain, it gave better resolution (2.1) between 2-MPG and 2-MPA, both structurally linear;
— a better selectivity *versus* thiols owing an amino group (*e.g.* cysteine) which were protonated and eluted near the void volume of the column.

Plasma treatment procedure

Detection relies upon the properties of the free sulphydryl groups, hence a reduction step was required prior to analysis. In this study, tributylphosphine was chosen rather than dithiothreitol [3,4] or borohydride natrium [15] since no reagent peak appeared on the chromatogram and it gave complete reaction under milder pH conditions. Chloroform was chosen as a solvent because other solvents including dimethylformamide, ethanol or acetonitrile induced gelification during heating and prevented the complete reduction of the disulphides forms. Optimum time and temperature conditions (*i.e.* 30 min at 50°C) were found to achieve full liberation of both 2-MPG and 2-MPA from their respective disulphides.

2-MPG and 2-MPA are too polar solutes to permit the use of an efficient partition technique (liquid–liquid or solid-phase) for extraction. Protein precipitation remains the main approach for the preparation of plasma samples. Ethanol appeared to be better than acetonitrile or trichloroacetic acid in spite of the greater sample dilution [16]. Indeed, trichloroacetic acid lowered the pH of the supernatant such that 2-MPG became unstable, and variations in retention time were observed for both analytes. The better stability of the solutes in the supernatant obtained with ethanol can be explained by medium conditions (the presence of reductant, slightly alkaline pH) similar to those of the reduction step. However, a slow decrease of the 2-MPG and 2-MPA amounts was observed with

TABLE I

VALIDATION PARAMETERS OF THE ASSAY OF 2-MPG AND 2-MPA IN PLASMA

Parameter	2-MPG	2-MPA
Capacity factor	5.5	6.3
Linearity	0.2–10 $\mu g\ ml^{-1}$ ($r=0.997$)	0.2–5 $\mu g\ ml^{-1}$ ($r=0.999$)
Precision ($n=5$)	6.2% at 0.2 mg ml^{-1}	9.9% at 0.2 $\mu g\ ml^{-1}$
	1.5% at 5.0 $\mu g\ ml^{-1}$	5.1% at 5.0 $\mu g\ ml^{-1}$
Recovery	95% at 1.0 $\mu g\ ml^{-1}$	95% at 1.0 $\mu g\ ml^{-1}$
Detection limit	0.05 $\mu g\ ml^{-1}$	0.05 $\mu g\ ml^{-1}$

time: the delay between the end of centrifugation and the injection in the HPLC system must not exceed 15 min. Among the multiple and time-consuming pre-column operations previously reported [7,8], only the reduction and protein precipitation steps have to be performed and so the analysis frequency can be increased up to 5–6 samples per hour.

Results from the validation of the technique are given in Table I and typical chromatograms from spiked samples and human plasmas, after drug administration, are shown in Fig. 1.

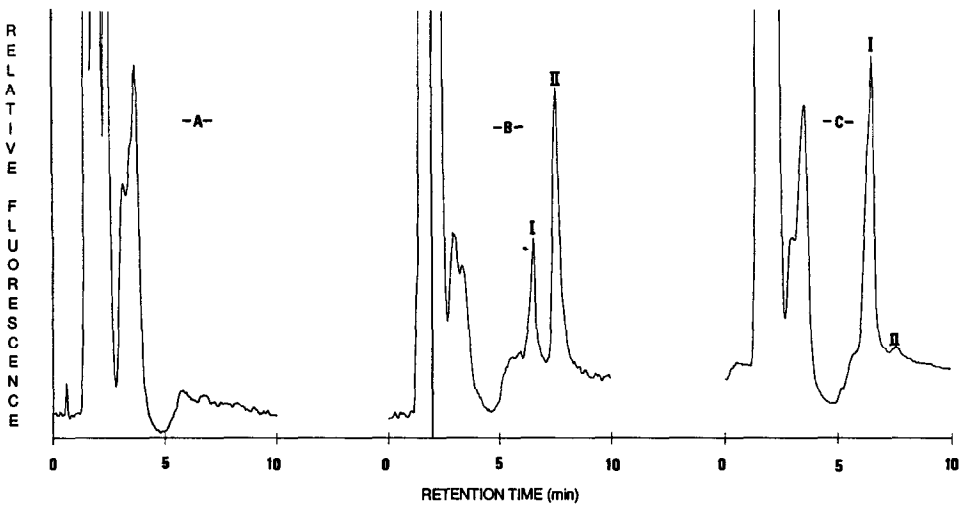

Fig. 1. Typical chromatograms obtained with (A) blank plasma, (B) plasma spiked with 1 $\mu g\ ml^{-1}$ 2-MPG (I) and 2-MPA (II) and (C) plasma from a healthy subject 6 h after intake of 500 mg of 2-MPG. Chromatographic conditions were: column: LiChrospher 100 RP-18e (125 mm × 4 mm I.D.; particle size 5 μm); mobile phase: acetonitrile–10^{-2} *M* phosphate buffer pH 7.0 (25:75; v/v) added with $5 \cdot 10^{-3}$ *M* cetrimonium bromide. Flow-rate was 1 ml min^{-1}. Detection was fluorimetry after pyrenemaleimide post-column derivatization.

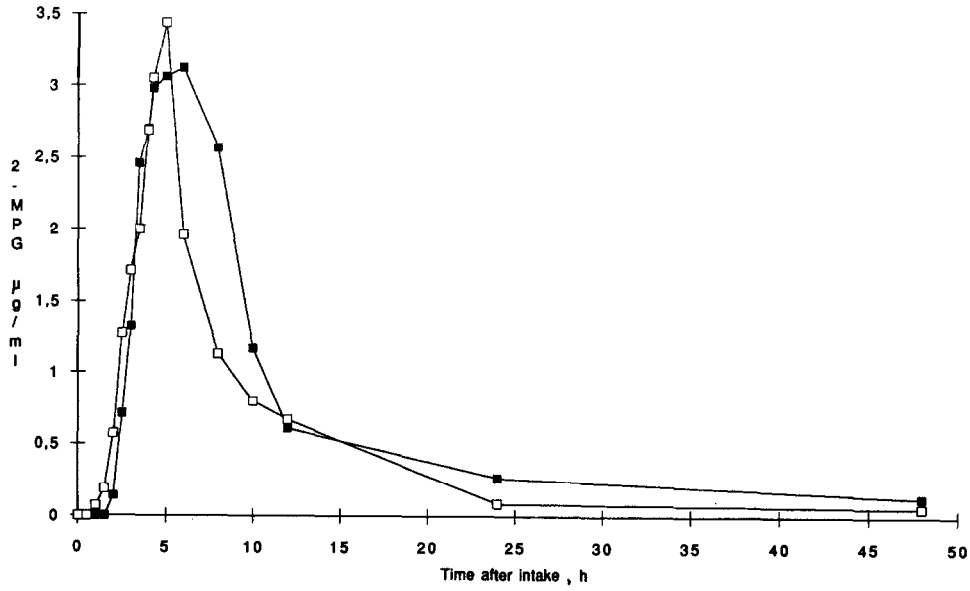

Fig. 2. Plasma concentrations of 2-MPG in two healthy subjects after a 500-mg single oral intake.

Pharmacokinetic study

Fig. 2 depicts the results for 2-MPG in two healthy subjects after administration of a 500-mg single oral dose. The parent compound was detectable 1–2 h after intake. The maximal concentrations ranged from 3.12 to 3.44 $\mu g\ ml^{-1}$ and were observed at 5–6 h. Metabolite 2-MPA appeared much later (6–8 h) and its concentration was under the limit of quantitation in both cases (0.2 $\mu g\ ml^{-1}$). These first results are close to those previously reported [7].

The method which has been developed meets the requirements for pharmacokinetic studies and further developments are under investigation in order to produce measurements for other fluids such as in the liquid synovial.

ACKNOWLEDGEMENT

We are grateful to Cassenne Laboratories for their financial support.

REFERENCES

1 T. Denneberg, J. O. Jeppson and P. Stenberg, *Proc. Eur. Dial. Transplant Assoc.*, 20 (1983) 427.
2 P. Netter, B. Bannwarth, P. Pere and A. Nicolas, *Clin. Pharmacokin.*, 13 (1987) 317.
3 M. Johansson and D. Westerlund, *J. Chromatogr.*, 385 (1987) 343.
4 M. Johansson and S. Lenngren, *J. Chromatogr.*, 432 (1988) 65.
5 P. Colin and E. Scherer, *J. Liq. Chromatogr.*, 12 (1989) 629.
6 V. Springolo, W. Bertani and G. Coppi, *J. Chromatogr.*, 232 (1982) 456.

7 B. Kagedal, M. Carlson and T. Denneberg, *J. Chromatogr.*, 380 (1986) 301.
8 B. Kagedal, T. Andersson, M. Carlson, T. Denneberg and A. Hoppe, *J. Chromatogr.*, 417 (1987) 261.
9 H. Nakamura and Z. Tamura, *Anal. Chem.*, 53 (1981) 2190.
10 H. Nakamura and Z. Tamura, *Bunseki Kagaku*, 37 (1988) 35.
11 W. Th. Kok, J. J. Halvax and R. W. Frei, *J. Chromatogr.*, 352 (1986) 27.
12 K. Shimada, T. Oe and T. Nambara, *J. Chromatogr.*, 419 (1987) 17.
13 D. Beales, R. Finch, A. E. M. McLean, M. Smith and I. D. Wilson, *J. Chromatogr.*, 226 (1981) 498.
14 T. Toyo'Oka, S. Uchiyama, Y. Saito and K. Imai, *Anal. Chim. Acta*, 205 (1988) 29.
15 D. Dupuy and S. Szabo, *J. Liq. Chromatogr.*, 10 (1987) 107.
16 J. Blanchard, *J. Chromatogr.*, 226 (1981) 455.