Journal of Chromatography, 426 (1988) 305–314
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4077

SIMULTANEOUS DETERMINATION OF *R*- AND *S*-PRENYLAMINE IN PLASMA AND URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(First received June 8th, 1987; revised manuscript received December 4th, 1987)

SUMMARY

A high-performance liquid chromatographic method for the determination of *R*- and *S*-prenylamine in human plasma and urine is described. It involves a two-step liquid–liquid extraction of prenylamine from biological material and preparation of diastereomeric urea derivatives with *R*-(−)-naphthylethyl isocyanate, a chiral fluorescence marker. Separation and quantitation of the diastereomeric prenylamine derivatives are carried out by a reversed-phase high-performance liquid chromatographic system with fluorimetric detection. The limit of determination is less than 2 ng of enantiomer per ml of urine and less than 1 ng of enantiomer per ml of plasma. A preliminary kinetic study on one healthy volunteer who had received a single oral dose of racemic prenylamine (100-mg film tablet) showed distinctly higher plasma and urine concentrations of the *R*-enantiomer.

INTRODUCTION

R,S-Prenylamine (Segontin®), N-(3,3-diphenylpropyl)-N-(α-methylphenethylamine) (Fig. 1), an unspecific calcium antagonist, was first introduced in 1960 for the treatment of angina pectoris. Although prenylamine has been applied to the treatment of cardiac disorders for nearly 30 years now, some pharmacological and pharmacokinetic aspects of this drug are still left to be investigated. Owing to the poor chromophoric properties of prenylamine, assay methods for pharmacokinetic studies have relied on radioactive labelling [1–8], gas chromatography [8–11] and radioreceptor assay [12]. Kinetic data referring to the maximum plasma levels of racemic prenylamine are highly contradictory, ranging from 90 ng/ml to 1 μg/ml after oral administration of 120 mg of prenylamine [1,2] or averaging 981 ng/ml after administration of 30 mg of prenylamine [10].

*This paper forms part of the Ph.D. thesis of Yvonne Gietl.

0378-4347/88/$03.50

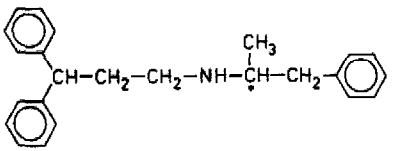

Fig. 1. Structure of prenylamine.

In addition, quantitative estimation of the enantiomers of prenylamine in investigations on their pharmacokinetic disposition have not been undertaken. This is especially interesting, since Rodenkirchen et al. [13] discovered stereospecific differences concerning the effects of *R*- and *S*-prenylamine on the contractility of the heart of the cat.

In order to investigate an enantioselective drug disposition, we developed an assay method that enables us to simultaneously determine even low concentrations of prenylamine enantiomers in human plasma and urine, avoiding special requirements such as isotope labelling or sophisticated equipment. The method is based on the formation of diastereomers with the chiral fluorescence marker *R*-(−)-1-(1-naphthyl)ethyl isocyanate (NEIC) [14,15], followed by the separation of the derivatives on a reversed-phase column and fluorimetric detection. The applicability of this method was tested on one healthy volunteer who had received a single oral dose of 100 mg of racemic prenylamine.

EXPERIMENTAL

Chemicals and materials

Racemic prenylamine lactate and *R*- and *S*-prenylamine glycolate were kindly supplied by Hoechst (Frankfurt, F.R.G.). Toluene-4-sulphonate sodium, chloroform LiChrosolv-grade, *n*-heptane LiChrosolv-grade and solvents of analytical grade were supplied by E. Merck (Darmstadt, F.R.G.). Methanol p.a. was purchased from J.T. Baker (Gross-Gerau, F.R.G.). Ethanolamine was supplied by Fluka (Buchs, Switzerland). NEIC was obtained from Aldrich (Steinheim, F.R.G.). Stock solutions of NEIC (1 mg/ml) in chloroform–N,N-dimethylformamide (8:2, v/v) were prepared and stored at room temperature, shielded from light. Under these conditions NEIC remained sufficiently reactive for ca. two weeks. Film tablets of racemic prenylamine lactate (100 mg) were kindly supplied by Albert Roussel Pharma (Wiesbaden, F.R.G.).

Equipment

The high-performance liquid chromatographic (HPLC) system consisted of a Knauer 64 HPLC pump (Knauer, Berlin, F.R.G.), a Shimadzu RF-530 fluorescence HPLC monitor (Shimadzu, Düsseldorf, F.R.G.), a Waters 712 WISP automatic injection system (Waters Millipore, Eschborn, F.R.G.) and a Knauer TY recorder. The vacuum centrifuge used was a Speed Vac concentrator (Bachofer, Reutlingen, F.R.G.).

Preparation of plasma samples

Alkaline extraction. In a screw-capped glass centrifuge tube, 2 ml plasma were extracted with 4.5 ml of *n*-heptane–propan-2-ol (99.5:0.5, v/v) after addition of 0.1 ml of 1 *M* potassium hydroxide solution. After shaking (20 min) and centrifugation (20 min, 3000 *g*), 4 ml of the organic layer were transferred to another centrifuge tube. The extraction was repeated with 4 ml of heptane–isopropanol, and the combined organic phases (8 ml) were evaporated to dryness in a vacuum centrifuge at 100°C.

Ion-pair extraction. In an additional extraction step, the residue was redissolved in 1 ml of 1 *M* hydrochloric acid; 0.2 ml of 0.01 *M* aqueous solution of toluene-4-sulphonate sodium and 5.5 ml of chloroform were added. After shaking (20 min) and centrifugation (15 min, 3000 *g*), the aqueous layer was aspirated and 5 ml of the chloroform phase were transferred to another centrifuge tube and evaporated to dryness in the vacuum centrifuge, when 0.1 ml of toluene was added for complete removal of water traces.

Preparation of urine samples

Alkaline extraction. To 1 ml of urine, 0.1 ml of 1 *M* potassium hydroxide solution was added. As in the preparation of plasma samples, the urine samples were extracted twice with 3.5 and 3 ml of *n*-heptane, and 6 ml of the combined organic phases were evaporated to dryness in the vacuum centrifuge.

Ion-pair extraction. To the residue were added 1 ml of 1 *M* hydrochloric acid, 0.1 ml of 0.01 *M* toluene-4-sulphonate and 5.5 ml of chloroform. The subsequent extraction procedure is identical with the one described for plasma.

Derivatization procedure

Volumes of 10 and 20 µl of NEIC stock solution were added to the residues of urine and plasma samples, respectively. The samples were briefly vortexed and heated to 80°C for 10 min, then 100 µl of dry chloroform (LiChrosolv grade) were added and the preparation was allowed to react at room temperature overnight (ca. 15 h). Excess reagent was destroyed by addition of 100 µl of ethanolamine–chloroform (1:100, v/v) before, 20 min later the reaction mixture was evaporated to dryness. To the residue of the plasma samples 1 ml of methanol was added. The mixture was briefly vortexed and 3 ml of *n*-heptane were added. After shaking (15 min) and centrifugation (10 min, 3000 *g*), the heptane layer was aspirated and 1 ml of the methanolic phase was transferred to another centrifuge tube and evaporated to dryness. The samples were dissolved in 150 µl of the mobile phase and 110 µl were injected onto the column.

High-performance liquid chromatography

Resolution of the diastereomers was achieved on a Waters Nova Pak Resolve C_{18} column (150 mm×3.9 mm I.D., 4 µm particle size) attached to a Waters Guard Pak µBondapak C_{18} cartridge precolumn with phosphate buffer ($8.4 \cdot 10^{-3}$ *M* disodium hydrogenphosphate dihydrate) in methanol (20:80, v/v) as mobile phase. The following conditions were used: injection volumes, 110 µl, WISP automatic injection system; flow-rate, 1 ml/min; pressure, 10 MPa; temperature,

ambient; detection wavelengths, excitation 285 nm, emission 333 nm; light source, xenon lamp with two monochromators.
The retention times were: *R*-(−)-prenylamine derivative, 15.0 min; *S*-(+)-prenylamine derivative, 16.6 min.

Reproducibility studies

Reproducibility studies were performed at three different concentrations (5, 10 and 20 ng of enantiomer per ml of plasma and 5, 25 and 50 ng of enantiomer per ml of urine) with nine samples at a time according to the described procedure. Coefficients of variation (C.V.) were calculated.

Standard curves

The linearity of the standard curves was tested with spiked plasma samples containing 5, 10, 20 and 40 ng enantiomer per ml and spiked urine samples containing 3, 5, 10, 20, 25, 50, 75, 100 and 125 ng enantiomer per ml. Peak areas were measured and plotted against the concentration of *R*- and *S*-prenylamine.

Relative recovery

Relative recovery was determined at three different concentrations by analysing spiked plasma (5, 20 and 40 ng enantiomer per ml) and urine samples (5, 25 and 50 ng enantiomer per ml) and comparing the peak areas with those of non-extracted standards.

Optical purity

The optical purity of *R*-(−)-NEIC was determined by derivatization with *S*-(−)- and *R*-(+)-metoprolol of defined enantiomeric purity. Derivatization was achieved by treating the single metoprolol enantiomers with a two-fold molar excess of the NEIC stock solution at room temperature for 15 h. Excess reagent was afterwards destroyed by addition of ethanolamine, and the reaction mixture was evaporated to dryness and redissolved in 150 μl of the mobile phase. The diastereomers were resolved using a Waters Nova Pak Resolve C_{18} column with methanol–water (70:30, v/v) as mobile phase. The same conditions as described for the resolution and detection of the prenylamine derivatives were used.

Racemization test

To ensure that no racemization occurred during the derivatization process, *R*- and *S*-prenylamine were derivatized separately according to the described procedure. Stock solutions of both enantiomers (0.01% in chloroform LiChrosolv grade) were stored at room temperature, shielded from light, for five months, before undergoing a repeated racemization test. The enantiomeric stability of NEIC was also tested by treating the prenylamine enantiomers with freshly prepared NEIC stock solution as well as stock solution stored for two weeks.

Pharmacokinetics

In order to test the applicability of this method for studies in patients, one healthy female volunteer (27 years old) received a film tablet containing 100 mg of racemic prenylamine. At timed intervals venous blood was drawn into hepa-

rinized screw-capped glass centrifuge tubes according to a protocol approved by our Ethical Committee. Plasma was separated immediately after collection and stored at −20 °C until analysed. The erythrocyte fraction of several samples was briefly washed with physiological sodium chloride solution, centrifuged and also frozen until analysed. Additionally, urine was fractionally collected for 28.5 h.

RESULTS AND DISCUSSION

Derivatization of amines and alcohols with isocyanates to ureas or carbamates with enhanced fluorescence or UV absorbance properties has already been widely used in HPLC and thin-layer chromatography (TLC). Reaction of *R*-(−)-NEIC with racemic prenylamine leads to diastereomeric naphthylethyl ureas (Fig. 2), which can be separated by HPLC on reversed-phase C_{18} stationary phases with mobile phases containing water or phosphate buffer in methanol.

Extraction procedure and relative recovery

Since the single extraction left traces of plasma and urine impurities in the sample, which had similar retention times as the prenylamine derivatives, a second clean-up step (ion-pair extraction) had to be introduced. Additionally, the relatively lipophilic extraction mode led to an accumulation of highly hydrophobic plasma impurities with long chromatographic retention times, which disturbed the chromatographic separation of consecutively injected samples. Thus, a third extraction after derivatization had to be added for plasma samples. Despite the numerous extractive steps, the relative recovery in urine samples was still above 95% (Table I). In contrast, the relatively low recovery yields from plasma samples (Table II) did not result from the additional extraction step, since comparison of the peak heights of plasma samples with and without the post-derivatizational extraction indicate only slight losses during extraction. Neither addition of sodium chloride nor dilution of the plasma with water led to increased recovery yields.

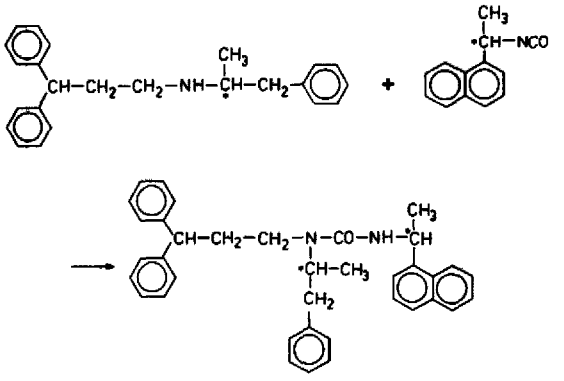

Fig. 2. Reaction scheme of prenylamine with NEIC.

TABLE I

RECOVERIES OF *R*- AND *S*-PRENYLAMINE FROM URINE ($n=3$)

Compound	Amount added (ng/ml)	Mean recovery (%)
R-Prenylamine	50	96.9
	25	95.3
	5	98.7
S-Prenylamine	50	97.9
	25	98.2
	5	97.7

TABLE II

RECOVERIES OF *R*- AND *S*-PRENYLAMINE FROM PLASMA ($n=3$)

Compound	Amount added (ng/ml)	Mean recovery (%)
R-Prenylamine	20	69.7
	10	69.5
	5	69.4
S-Prenylamine	20	68.1
	10	67.4
	5	64.0

TABLE III

CORRELATION COEFFICIENTS

	R-Prenylamine	*S*-Prenylamine
Plasma, 5–40 ng/ml	0.99978	0.99969
Urine, 3–20 ng/ml	0.99992	0.99943
Urine, 25–125 ng/ml	0.99999	0.99980

Standard curves and limit of determination

Calibration graphs obtained for both enantiomers were linear over the investigated concentration range, which covers the clinically relevant concentration range. Correlation coefficients were in all cases better than 0.999 (Table III). The limit of determination is below 1 ng of enantiomer per ml of plasma and below 2 ng of enantiomer per ml of urine, owing to the strong fluorescence intensity of the NEIC derivatives with excitation and emission maxima at 285 and 333 nm, respectively.

Derivatization procedure and chromatography

In contrast to the derivatization of β-adrenoceptor blockers [12], the reaction of prenylamine with NEIC requires higher temperatures and greater excess of derivatization reagent. A reaction procedure of heating the mixture to 80°C for

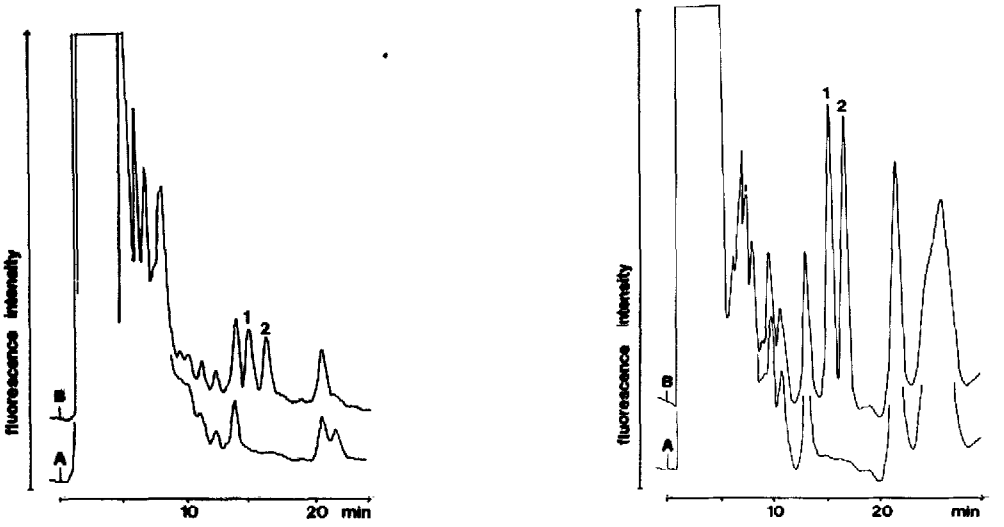

Fig. 3. Representative chromatograms of (A) blank plasma sample and (B) plasma sample containing 4 ng/ml racemic prenylamine. Peaks: 1 = derivative of *R*-(−)-enantiomer; 2 = derivative of *S*-(+)-enantiomer.

Fig. 4. Representative chromatograms of (A) blank urine sample and (B) urine sample containing 50 ng/ml racemic prenylamine. Peaks: 1 = derivative of *R*-(−)-enantiomer; 2 = derivative of *S*-(+)-enantiomer.

1 h leads to maximum yields, but the described process avoids the formation of excessive by-products, provoked by the high temperature, while almost maintaining the reaction yield. Addition of basic agents, such as triethylamine, depresses the reaction yield of both salt and free base. The use of chloroform–dimethylamide (8:2, v/v) as reaction medium leads to higher yields than reaction in pure chloroform. However, addition of more than 20% dimethylformamide (DMF) leads to poor peak resolution since DMF cannot be completely removed in the vacuum centrifuge within a reasonable time. Another important factor to be considered is the volume of the initial reaction mixture. While addition of 10–20 μl of the 0.1% stock solution of NEIC shows satisfactory reaction yields, nearly no reaction takes place if prenylamine is heated in 100 μl of a 0.01% solution of NEIC. Destruction of excess NEIC at the end of the procedure is achieved with ethanolamine by formation of hydrophilic products, which are rapidly eluted from the HPLC column. Addition of disodium hydrogenphosphate to the mobile phase improved separation of the diastereomers from impurities that stem from plasma and urine traces or degradation processes of NEIC. Increased water or buffer contents of the mobile phase lead to higher α-values of the prenylamine derivatives in connection with (unnecessarily) prolonged retention times, which is of disadvantage for routine analyses in pharmacokinetic studies. With the described chromatographic system a resolution factor α of 1.11 with a resolution $R_s = 1.28$ was achieved. *R*-(−)-prenylamine elutes before the *S*-(+)-enantiomer with retention times of 15.0 and 16.6 min, respectively. Representative chromatograms of plasma and urine samples are given in Figs. 3 and 4.

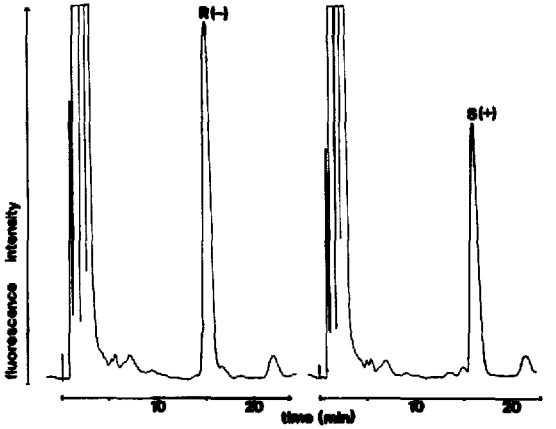

Fig. 5. Chromatograms after derivatization of the separate enantiomers.

TABLE IV

RESULTS OF REPRODUCIBILITY STUDIES IN PLASMA ($n=9$)

Compound	Concentration (ng/ml)	C.V. (%)
R-Prenylamine	20	3.7
	10	3.9
	5	8.5
S-Prenylamine	20	3.5
	10	4.4
	5	9.4

Optical purity

The enantiomeric purity of *R*-(−)-NEIC was found to be greater than 99.4%.

Racemization test

During the derivatization no racemization was observed (Fig. 5). Storage of the stock solutions of NEIC (two weeks) and the prenylamine enantiomers (five months) showed no significant alterations of the optical purity.

Traces of a second peak shown in the chromatograms of Fig. 5 result from the poorer optical purity of the prenylamine enantiomers (greater than 97%).

Reproducibility studies

The reproducibility of this assay method was determined as described under Experimental. Results are given in Tables IV and V.

Pharmacokinetics

The plasma concentrations of *R*- and *S*-prenylamine determined in one healthy volunteer who had received a 100-mg film tablet of racemic prenylamine are shown in Fig. 6. Maximum plasma concentrations were 25 ng/ml for the *R*-enantiomer

TABLE V

RESULTS OF REPRODUCIBILITY STUDIES IN URINE ($n=9$)

Compound	Concentration (ng/ml)	C.V. (%)
R-Prenylamine	50	3.5
	25	6.7
	5	8.5
S-Prenylamine	50	4.2
	25	5.2
	5	6.4

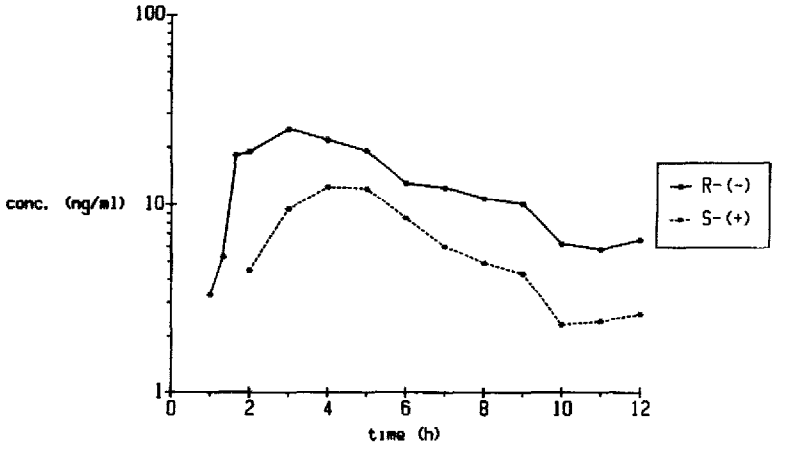

Fig. 6. Plasma concentrations of *R*- and *S*-prenylamine in one subject after oral administration of 100 mg of racemic prenylamine.

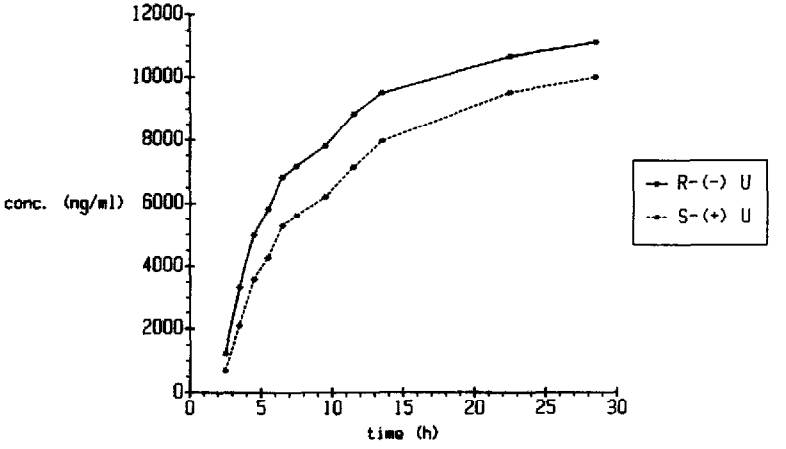

Fig. 7. Cumulative urinary excretion of *R*- and *S*-prenylamine in one subject after oral administration of 100 mg of racemic prenylamine.

and 13 ng/ml for the *S*-enantiomer, which were reached 3 and 4 h after administration. The half-lives were calculated to be 4.2 h for the *R*-enantiomer and 3 h for the *S*-enantiomer. These values differ from those stated in literature for racemic prenylamine, where plasma levels were reported to be 90–1000 ng/ml racemate after oral administration of 120 mg of prenylamine [1,2]. In the erythrocyte fraction of selected samples no prenylamine was detectable. The cumulative urinary excretion of *R*- and *S*-prenylamine is shown in Fig. 7. In accordance with the plasma concentrations, the urine concentrations of *R*-prenylamine are also higher than those of the *S*-enantiomer. Within 2.5 h after oral administration, 1.2 μg of *R*-prenylamine, but only 0.68 μg of the *S*-enantiomer, were excreted into the urine. After 28.5 h 0.022% of the dose was excreted as *R*-prenylamine, 0.020% as *S*-prenylamine.

In conclusion, this method allows the simultaneous determination of *R*- and *S*-prenylamine in clinically relevant concentrations in human plasma and urine. Neither complicated techniques nor isotope labelling of the drug are required. The results indicate that the enantiomers do have different kinetics. In order to gain more representative data on the kinetics of the prenylamine enantiomers, we plan to use this method in future clinical studies.

ACKNOWLEDGEMENTS

This work was supported by the Graduiertenförderung of the state Hessia, F.R.G. The authors thank the Dr. Robert Pfleger-Stiftung (Bamberg, F.R.G.) for partial support. They also thank Dr. Irene Spahn, MD, for medical help with the kinetic study.

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