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Enantioselective determination of DN-2327, a novel non-benzodiazepine anxiolytic, and/or its active metabolite in human plasma and urine using high-performance liquid chromatography

Ziad Hussein*, Sou-yie Chu and G. Richard Granneman

Drug Metabolism Department, D-463, AP9/2, Abbott Laboratories, One Abbott Park Road, Abbott Park, IL 60064-3500 (USA)

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ABSTRACT

A new rapid, specific and sensitive reversed-phase HPLC method has been developed for simultaneous measurement of the *R*- and *S*-enantiomers of DN-2327 (I), a novel non-benzodiazepine anxiolytic, and those of its pharmacologically active metabolite, M_{II} (II), in human plasma or urine. Extraction of all the enantiomers and internal standard was achieved using solid-phase extraction on C_8 columns. Resolution was achieved using a Chiral-AGP column with mobile phase comprising 6.5% (v/v) acetonitrile in 50 m*M* potassium acetate buffer, pH *q3 3.10, at typical flow-rates of 0.35 ml/min for plasma and 1.0 ml/min for urine assays. Fluorescence detection was employed using excitation and emission maxima of 328 and 367 nm, respectively. Analytes were well resolved and no interfering endogenous peaks were observed either from plasma or urine. Standard curves for urine were linear for concentrations up to 500 ng/ml for *R*- and *S*-II, with correlation coefficients higher than 0.994 and limit of quantitation (LOQ) of 1 ng/ml for each enantiomer. The LOQ in plasma was 0.1 ng/ml for each of the four enantiomers. The precision and accuracy of the method for the enantiomers of both I and II were good for plasma and urine with coefficients of variations typically within 10%. The stability of the *R*- and *S*-enantiomers of II and I in plasma and those of II in urine was excellent, with no evidence of degradation or interconversion during storage and handling.

INTRODUCTION

Racemic DN-2327, (±)-2-(7-chloro-1,8-naphthyridine-2-yl)-3-[(1,4-dioxa-8-azaspiro[4,5]dec-8-yl)carbonyl-methyl]isoindolin-1-one (I), is a new non-benzodiazepine anxiolytic compound (Fig. 1). Metabolic and pharmacokinetic studies in animals and humans showed that I is extensively metabolized to M_{II} (II; Fig. 1), a predominant pharmacologically active circulating metabolite. The results also showed that no intact parent drug was excreted unchanged in human urine, and II was excreted mainly as the glucuronide. Recently, a sensitive and rapid achiral high-performance liquid chromatographic (HPLC) method was developed for the determination of I and II in human plasma and II and its glucuronide in urine [1]. However, the pharmacological activity of parent drug and metabolite is associated with the *S*-enantiomer. Thus, a chiral analytical method is needed for characterization of the pharmacokinetics and associated pharmacodynamics of the *R*- and *S*-enantiomers of I and II in human plasma and urine after administration of clinical doses of racemic I. Described herein is a very sensitive, precise, and rapid procedure for the determination of the *R*- and *S*-enantiomers of

* Corresponding author.

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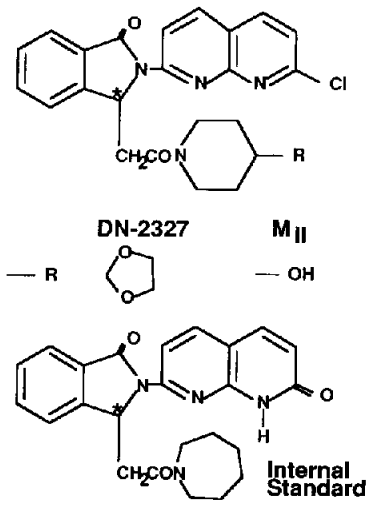

Fig. 1. Structures of DN-2327 (I), M_{II} (II), and the internal standard (I.S.); the asterisk identifies the chiral center.

both I and II in human plasma and those of II in urine.

EXPERIMENTAL

Chemicals

Reference standards of *R*-(+)-II (purity: 99.8%), *S*-(−)-II (purity: 100.0%), *R*-(+)-I (purity: >99%), *S*-(−)-I (purity: >99%), and the internal standard (I.S.; Fig. 1) were obtained from Takeda Chemical Industries (Osaka, Japan). HPLC-grade acetonitrile, methanol, and acetone were obtained from Fisher Scientific (Fairlawn, NJ, USA). Glacial acetic acid (Reagent ACS) was also obtained from Fisher Scientific and potassium acetate crystals (99.4% purity) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Water was deionized which was further purified by Milli-Q Ultra Pure water system. Normal human plasma and urine samples were obtained from healthy subjects.

Chromatographic system

The HPLC system was exactly that described previously [1], except that a Chiral-AGP column (5 μm particle size, 100 mm × 4.0 mm I.D.; ChromTech, Norsborg, Sweden) replaced the Adsorbosphere HS C_{18} column used in the achiral analysis.

Preparation of standard solutions

For each enantiomer, a separate initial stock solution (100 μg/ml) was prepared. Due to a very low aqueous solubility of the *R*- and *S*-enantiomers of I and II, 1 mg of each compound was accurately weighed into a 10-ml volumetric flask and dissolved in 5 ml of acetonitrile by sonication and then diluted to mark with acetonitrile. Each stock solution was further diluted 1:10 with 50% acetonitrile in water to obtain a working standard solution at a concentration of 10 μg/ml. A working I.S. solution (150 ng/ml) was obtained by diluting 1.5:100 a stock I.S. solution (10 μg/ml in acetonitrile) with 10% acetonitrile. Since photodegradation of the enantiomers is a potential source of analytical error, it is important that all solutions be protected from laboratory light by using brown volumetric flasks or covering them with aluminum foil. All solutions were stored refrigerated at 4°C unless when used.

Preparation of quality controls in human plasma

The quality control (QC) samples were prepared in drug-free human plasma at three different ratios of the *R*- and *S*-enantiomers each for I and II. Initially, appropriate aliquots of the working standard solutions (10 μg/ml) were combined and diluted to a final volume of 50 ml with drug-free human plasma. The following quality controls (QC) were prepared: the first at concentrations of 32 ng/ml for *R*-II and *S*-I and 8 ng/ml for *S*-II and *R*-I, the second at a concentration of 20 ng/ml each, and the third at concentrations of 8 ng/ml for *R*-II and *S*-I and 32 ng/ml for *S*-II and *R*-I. Each of these QC samples was further diluted 4:10 and 1:16 with drug-free plasma to provide QC samples at medium and low range of concentrations. All samples were promptly covered with aluminum foil and stored at −20°C until analysis.

Preparation of calibration curves and quality controls in human urine

An initial stock solution consisting of *R*- and *S*-II (100 μg/ml each in acetonitrile) was diluted 1:200 in drug-free human urine followed by serial dilutions with urine to provide calibrators of *R*-

and *S*-II. The set of calibrators used for the method validation in urine had nominal concentrations of *ca.* 5, 15, 30, 60, 125, 250, and 500 ng/ml of each analyte. Similarly, a quality control at *ca.* 400 ng/ml each was prepared by diluting a separately prepared stock standard solution consisting of *R*- and *S*-II (100 μg/ml each) into drug-free human urine. This was followed by serial dilutions with urine to provide QC samples in the medium (QC-Medium: *ca.* 160 ng/ml) and low (QC-Low: *ca.* 10 ng/ml) range of calibration curves. Handling and storage conditions for urine samples were identical to those described for plasma.

Extraction procedure for human plasma and urine

Aliquots of plasma (0.5 ml) and urine (0.1 ml supplemented with I.S.) were processed and extracted as described previously in the determination of racemic I and II in plasma and II in urine [1].

Recovery

The recovery of *R*- and *S*-II from human urine was assessed at three different concentrations of each enantiomer (10, 160, and 400 ng/ml). Aliquots of 0.1 ml of urine were diluted to 2.5 ml with water and processed according to the extraction procedure previously described for urine [1], and eluates were collected into tubes containing I.S. Recovery was calculated as the percentage of the added concentration present in the extraction residue. Evaluation of recovery was performed through comparison of the peak-height ratios obtained by direct injection of a reference solution of *R*- and *S*-II and the I.S. with the peak-height ratios from chromatography extracts.

Chromatography

The same mobile phase was used in the chromatography of plasma and urine extracts; it consisted of 6.5% (v/v) acetonitrile in 50 m*M* potassium acetate buffer, pH $\cong$3.10. It was delivered normally at 0.35 ml/min for plasma extracts and at 1.0 ml/min for urine extracts. The residues of extracts from plasma and urine samples were each reconstituted in 0.2 ml of 10% acetonitrile, and aliquots (normally 40–80 μl) were injected onto the HPLC system. Fluorescence detection was performed at excitation and emission maxima of 328 and 367 nm, respectively. A guard filter was positioned between the injection valve and the Chiral-AGP column.

Stability

The stability of a stock standard solution of each individual enantiomer and that of the I.S., stored at 4°C for four months, was examined monthly against a freshly prepared solution. The stability of the working standard and I.S. solutions, each prepared in a brown glass, was also tested at various times, against a fresh solution, following room temperature storage for four days. Finally, the stability of the *R*- and *S*-enantiomers of I and II in plasma and those of II in urine was examined following several freeze–thaw cycles of frozen plasma and urine stored at −20°C.

Calculations

For plasma, the peak-area ratios between *R*- and *S*-II and between *R*- and *S*-I were calculated for the different quality controls. Intra- and inter-assay precision of the assay procedure was assessed based on the calculated *R*/*S* ratio for I and II after the analysis of replicates of the various quality controls. The inter-assay variability was based on the analysis of the quality controls over three different days. Accuracy error is expressed as the percentage deviation (+ or −) of the calculated *R*/*S* ratio for either I or II from the theoretical ratio of the sample.

For urine, the analyte concentrations were quantified by calculating the peak-area ratios of *R*- and *S*-II with respect to the I.S. These ratios were subjected to linear regression, with a weighing factor of 1/(concentration), to construct the calibration curves for each enantiomer. Concentrations of the *R*- and *S*-enantiomers in unknown and QC samples were computed based on peak-area ratios and the regression results of the calibration curve. Intra- and inter-assay precisions of the assay procedure for urine were assessed at three concentrations for *R*- and *S*-II. The intra-

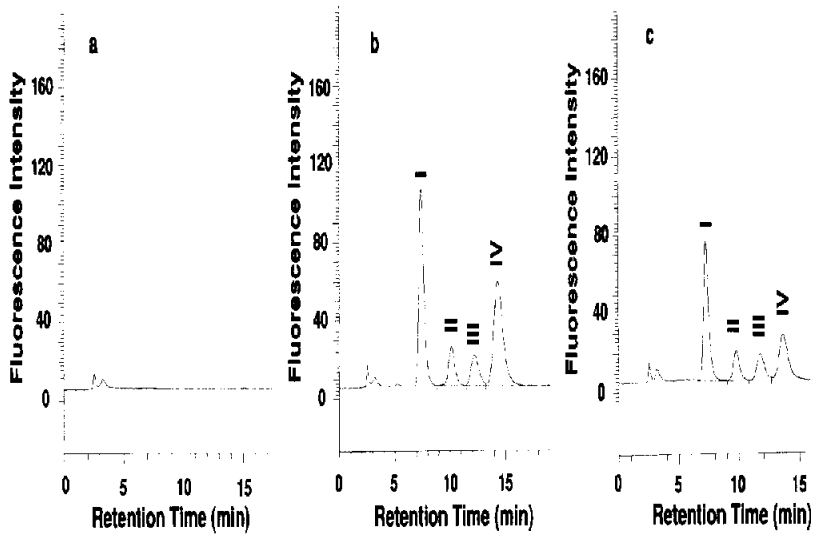

Fig. 2. Representative chromatograms of (a) drug-free human plasma, (b), drug-free human plasma fortified with 12.8 ng/ml *R*-M_{II} (I) and *S*-I (IV), 3.2 ng/ml *S*-M_{II} (II) and *R*-I (III), and (c) plasma sample collected from a healthy subject 16 h following a twice daily dosing of 4 mg of racemic I.

assay precision was based on the analysis of replicates of low, medium, and high concentrations of each enantiomer in the quality controls. The inter-assay variability was based on the analysis of the quality controls over four different days.

RESULTS AND DISCUSSION

Chromatography

Figs. 2 and 3 are representative chromatograms from human plasma and urine samples,

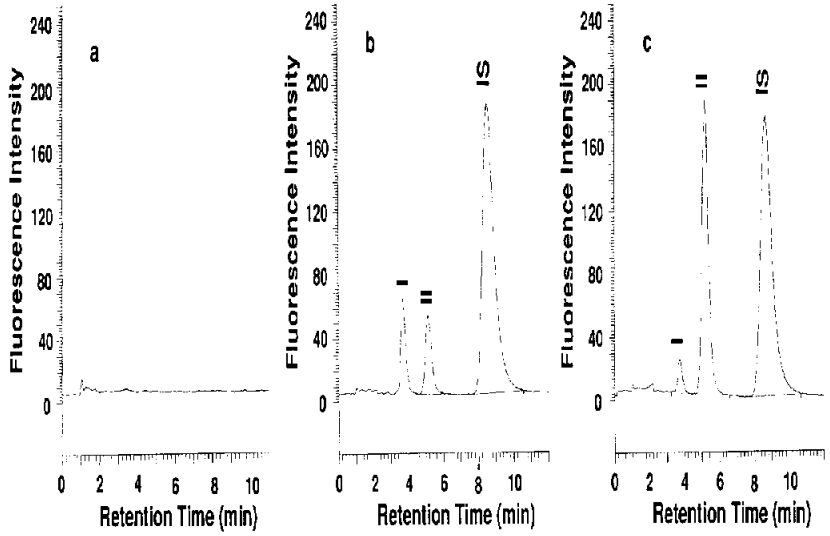

Fig. 3. Representative chromatograms of (a) drug-free human urine. (b) drug-free human urine fortified with 65 and 62 ng/ml *R*-II (I) and *S*-II (II), respectively, and with I.S., and (c) hydrolyzed pooled urine (0–24 h) collected from a healthy subject after a single 4-mg dose.

respectively, processed using the analytical method described. For the two biological matrices, chromatographic peaks due to the analytes of interest were all well resolved. For plasma extracts, at a flow-rate of 0.35 ml/min, *R*- and *S*-II eluted at *ca.* 8.8 and 12.1 min, respectively, and *R*- and *S*-I eluted at *ca.* 14.5 and 17.1 min, respectively (Fig. 2b). For urine extracts, at a flow-rate of 1.0 ml/min, *R*-II, *S*-II and the I.S. eluted at *ca.* 3.7, 5.2, and 8.5 min, respectively (Fig. 3b). Drug-free plasma (Fig. 2a) and urine (Fig. 3a) samples obtained from normal subjects resulted in chromatograms free of interfering endogenous peaks.

After oral administration of racemic I to a healthy subject, the *R*- and *S*-enantiomers of I and those of II were detected in plasma (Fig. 2c); however, only *R*- and *S*-II were recovered in urine (Fig. 3c), largely in their conjugated form. A late-eluting peak from the *S*-enantiomer of the racemic I.S. was observed; therefore, an injection interval of 20 min was required for urine extracts to ensure that this peak did not interfere with subsequent chromatograms. Using the *R*-enantiomer of the I.S., an injection interval of 12 min was found to be adequate.

It should also be noted that during method development we experienced a carry-over effect from previous injections. Among several flushing solutions tested, the mixture of acetonitrile–acetone–water (4:4:2) eliminated the carry-over.

Recovery

The work-up procedure recovered more than 84% of *R*- and *S*-II from urine; the recovery was reproducible for each enantiomer with a coefficient of variation (C.V.) of less than 5% for the analysis of five replicates at each concentration. The recoveries of the *R*- and *S*-enantiomers of I and II from plasma was not examined; however, the recoveries of racemic I and II were approximately 92% for each [1]. At a nominal *R*/*S* ratio of 1:1 for II and I (20 ng/ml each enantiomer), the peak-area ratios were within 7% of the target, indicating similar recoveries for *R*- and *S*-II and for *R*- and *S*-I.

Attention to certain details was found to be important in assuring reproducible recovery of all four enantiomers from either plasma or urine using the described extraction procedure. First, the extraction column must not be allowed to dry out between the pre-conditioning step and the addition of the plasma or urine mixture; if so, the column should be rinsed with an additional 0.5 ml of water and kept wet. Second, a gentle vacuum must be used during the application of the plasma or urine mixture, with subsequent rinses through the column, and in the final elution step with acetone.

Linearity

Previous analyses of clinical urine samples using an achiral HPLC method [1] suggested that intact I was not recovered unchanged in urine and that II was excreted both as the free and glucuronidated forms. Thus, assay validation was performed only for *R*- and *S*-II and over a low and high concentration range for measurement of free and total levels of each enantiomer, respectively. The standard curves for *R*- and *S*-II were highly linear between 5 and 500 ng/ml, using aliquots of 0.1 ml, with correlation coefficients exceeding 0.994. For each enantiomer, all mean recalculated values were accurate within 8% of the target concentrations. Inter-assay precision was also good, with C.V. values being below 9%. The low standard curve was highly linear in the concentration range 0.5–50 ng/ml, with correlation coefficients exceeding 0.998, and the recalculated concentrations of the calibrators were generally within 7.5% of the nominal concentrations.

Due to initial difficulties in finding an appropriate I.S. for plasma and since a validated achiral HPLC method was available for the determination of I and II in plasma [1], the chiral method validation was based on *R*/*S* ratios for a wide range of concentrations of quality controls rather than on standard curve with fixed concentrations of each enantiomer. The various *R*/*S* ratios of the examined concentrations cover those obtained in a clinical pharmacokinetic study employing twice a day dosing of 4 and 8 mg of I.

Precision and accuracy

As indicated previously, validation of the analytical method in plasma was based on low, medium and high QC samples, each with three different *R*/*S* ratios for both I and II. Mean calculated ratios for all nine quality controls and the associated intra- and inter-assay precision characteristics are listed in Table I. For I, the C.V. for the analysis of replicates for the low, medium and high QC samples, at *R*/*S* concentration ratios of 1:4, 1:1, and 4:1, ranged from 0.0 to 7.7%, with most values below 5%. The inter-assay precision for the same QC samples, calculated from the overall measurements over three different days, was good with C.V. ranging between 1.8 and 4.4%. For different *R*/*S*-ratio of concentration for II, the C.V. values also ranged between 0.0 and 7.7%, with most values below 5%. The inter-assay precision for the same QC samples was excellent, with all C.V. values below 5%.

TABLE I

PRECISION OF THE PROCEDURE FOR SIMULTANEOUS MEASUREMENT OF CONCENTRATIONS OF *R*- AND *S*-I AND *R*- AND *S*-II IN HUMAN PLASMA

Added concentration[a] (ng/ml)	Coefficient of variation (%)			
	Intra-day variability			Inter-day variability ($n=12$)
	Day 1 ($n=6$)	Day 2 ($n=3$)	Day 3 ($n=3$)	
R-II/S-II				
0.5:2.0	3.7	7.7	0.0	3.8
1.25:1.25	2.9	3.0	1.9	2.9
2.0:0.5	2.8	5.3	1.0	3.2
3.2:12.8	0.0	3.9	0.0	3.8
8:8	1.9	1.0	0.0	1.9
12.8:3.2	4.4	3.2	5.4	4.2
8:32	0.0	3.9	0.0	0.0
20:20	1.0	0.0	2.0	1.0
32:8	3.7	0.7	1.4	3.1
R-I/S-I				
2.0:0.5	4.6	4.9	2.0	4.1
1.25:1.25	2.8	2.7	4.5	3.7
0.5:2.0	3.8	7.7	0.0	3.8
12.8:3.2	4.1	2.3	0.8	4.4
8:8	0.9	2.8	1.9	1.8
3.2:12.8	3.8	4.0	3.7	3.8
32:8	3.1	4.2	1.2	3.6
20:20	0.9	0.9	1.9	3.6
8:32	3.8	3.8	3.8	3.8

[a] Pooled drug-free human plasma fortified with *R*- and *S*-II and *R*- and *S*-I.

The data in Table I also demonstrate the accuracy of the analytical method for plasma based on the good agreement between nominal and calculated *R*/*S* concentration ratios for both I and II. For I, deviations of calculated *R*/*S* ratios from the nominal ratios were relatively low, ranging from +7.9% to −8.2%, with most values typically within ±5%. Deviations were smaller for the different *R*/*S* ratios for II, ranging from +3.4% to −4.8%.

For urine, mean concentrations for the low, medium and high QC samples calculated using the standard curves for *R*- and *S*-II and the associated intra- and inter-assay precision and accuracy are presented in Table II. The precision of the method for urine was good, with C.V. values ranging from 0.9 to 6.9% for *R*-II and from 1.0 to 6.6% for *S*-II. The inter-assay C.V. values for the same QC samples were 6.9% or below. The accuracy was relatively high for the QC samples; calculated concentrations were within ±9.6% of the theoretical concentrations for *R*-II and within ± 12.1% for *S*-II.

Stability

The *R*- and *S*-enantiomers of I and II are light-sensitive. Photodegradation was prevented by storing all solutions, calibrators, and/or QC samples in brown glass containers or transparent containers covered with aluminum foil. Stock and working solutions for each enantiomer were stable for more than four months when stored at 4°C. Additionally, extracts of human plasma and urine reconstituted in 10% acetonitrile were stable at room temperature standing in the autosampler for more than four days.

The stability of *R*- and *S*-I in human plasma and those of II in human plasma and urine, while frozen at −20°C, was examined after several freeze thaw cycles. For plasma, after almost three months of frozen storage, the deviation of calculated *R*/*S* ratios from nominal ratios was

TABLE II

PRECISION OF THE PROCEDURE FOR SIMULTANEOUS MEASUREMENT OF CONCENTRATIONS OF *R*- AND *S*-II IN HUMAN URINE

Added concentration[a] (ng/ml)	Coefficient of variation (%)				
	Intra-day variability				Inter-day variability ($n=14$)
	Day 1 ($n=5$)	Day 2 ($n=3$)	Day 3 ($n=3$)	Day 4 ($n=3$)	
R-II					
10.4	3.1	3.0	4.0	6.0	4.0
166	2.5	1.7	6.9	6.0	5.6
416	5.4	3.3	0.9	4.0	6.9
S-II					
9.9	4.6	3.3	6.2	6.3	6.5
158	2.6	1.9	6.6	6.6	4.5
396	5.3	4.2	1.0	3.6	5.4

[a] Pooled drug-free urine fortified with *R*- and *S*-II.

±6.4% or less for I and ±8.0% or less for II. For urine, the calculated concentrations of *R*- and *S*-II in QC samples, stored frozen at least for three months, were generally within 100 ± 8.5 and 100 ± 7.9%, respectively, of the targeted concentrations. Thus, the overall results clearly indicate excellent stability of all four enantiomers since no degradation and interconversion of enantiomers had occurred during storage and handling.

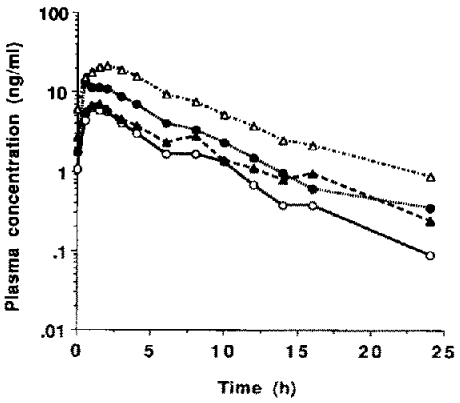

Fig. 4. Representative plasma concentration–time profiles for *R*-I (○) and *S*-I (●) and *R*-II (△) and *S*-II (▲) in a healthy subject after the final dose of a 4-mg twice daily dosing regimen of racemic I.

Clinical pharmacokinetics

The described analytical procedure was used to determine the *R*/*S* ratios for I and II in more than 500 plasma samples, and to quantitate urinary concentrations of *R*- and *S*-II in approximately 200 samples. Samples were obtained during a multiple-dose safety study in healthy males to whom 4- and 8-mg doses of I were administered twice daily. The same QC samples prepared in plasma and urine during method validation were used for routine analysis of clinical plasma and urine samples.

For plasma, the intra- and inter-assay precision was comparable to that obtained during the assay validation. The C.V. values ranged from 0.4 to 6.4% for *R*/*S* ratios for I and generally between 0.2 and 3.6% for *R*/*S* ratios for II. The accuracy was also comparable to that obtained during assay validation with good agreement between theoretical and calculated ratios; deviations in the *R*/*S* ratios were within the acceptance criteria of ±15%. The *R*/*S* ratios for I and II

were calculated for unknown clinical samples, concentrations of the respective enantiomers were computed by applying those *R*/*S* ratios to the concentrations of racemic I and II determined using a previously described method [1]. Fig. 4 presents representative plasma concentration–time profiles of the *R*- and *S*-enantiomers of I and those of II following a multiple twice a day 4-mg dose of racemic I to one of the subjects. As demonstrated in Fig. 4, the method was capable of measuring plasma levels of the four enantiomers up to 24 h after dosing, with a detection limit of 0.1 ng/ml for each enantiomer. The maximum plasma concentrations for *R*- and *S*-I of 6.2 and 13.2 ng/ml occurred at the 1- and 0.5-h sampling times, respectively, and the corresponding half-lives were 4.5 and 7.7 h. Maximum plasma concentrations for *R*- and *S*-II of 21.3 and 6.9 ng/ml occurred at the 2- and 1.5-h sampling times, respectively, and the corresponding half-lives were 6.6 and 5.2 h.

For urine, the C.V. for intra-assay precision for the analysis of replicate QC samples at *ca.* 10, 160, and 400 ng/ml ranged from 4.1 to 6.3% for *R*-II and from 3.7 to 6.1% for *S*-II. The two enantiomers were present mainly in the conjugated forms and those of *S*-II were predominant at an *S*/*R* ratio of approximately 4:1.

CONCLUSION

The described analytical method is simple, highly sensitive, precise, and accurate; thus it is very suitable for the determination of the *R*- and *S*-enantiomers of I and II in the plasma and urine samples collected during clinical pharmacokinetic trials in the development of this novel anxiolytic agent.

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