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High-performance liquid chromatographic method for determination of DN-2327, a novel non-benzodiazepine anxiolytic, and/or its active metabolite in human plasma and urine

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ABSTRACT

A sensitive and precise high-performance liquid chromatographic procedure has been developed for the determination of a new non-benzodiazepine anxiolytic agent, DN-2327 (I), and its pharmacologically active metabolite, M_{II} (II), in human plasma and urine. Extraction of I, II, and the internal standard from plasma and urine samples were achieved using solid-phase extraction. Separation of the analytes was performed on a reversed-phase C_{18} column. The effluent was monitored with fluorescence detection at excitation and emission maxima of 328 and 367 nm, respectively. The work-up procedure was reproducible and recovered more than 92% of I and II from either plasma or urine. The chromatographic system for plasma and urine extracts allowed complete resolution of I and II from the internal standard with excellent selectivity. For each analyte, the lower detection limits were 0.1 and 1 ng/ml in plasma and urine, respectively. For each analyte, standard curves were linear in the ranges of 0.1–50 and 1–500 ng/ml in plasma and urine, respectively. The method was highly precise, with coefficients of variation for each analyte in quality controls that were generally below 7 and 5% for plasma and urine samples, respectively. The accuracy of the method was good with the deviations between added and calculated concentrations of each analyte being typically within ± 10% and ± 5.6% for plasma and urine samples, respectively. The stability of I and II in standard solutions, plasma and urine samples protected from laboratory light was excellent, with no evidence of degradation after 72 h at room temperature, five months at 4°C, or three months at −20°C.

INTRODUCTION

DN-2327, (±) 2-(7-chloro-1,8 -naphthyridine-2-yl)-3-[(1,4-dioxa-8-azaspiro[4,5]dec-8-yl)carbonylmethyl]isoindolin-1-one] (I), is a new non-benzodiazepine anxiolytic compound (Fig. 1), which possesses a very potent binding affinity for benzodiazepine receptors, exceeding that of diazepam by a factor of twenty [1]. The specificity of mediation of the pharmacological activity of I via benzodiazepine receptors suggested that I acts as an agonist with respect to the anti-conflict and anti-convulsant effects but as an antagonist to the muscle-relaxant and sedative effects of diazepam [2]. The anxiolytic effects of oral administration of both I (5 mg/kg) and diazepam (10 mg/kg) to rats were significantly antagonized by the benzodiazepine receptor antagonist flumazenil (20 mg/kg, intraperitoneally) [3]. Compound I does not impair acquisition or working memory, both of which are impaired by diazepam, and tends to act as an antagonist on the acquisition impairment caused by diazepam in the passive avoidance task [4]. In animals and humans, I is hydrolyzed to an intermediate metabolite which is instantaneously reduced to a pharmacologically ac-

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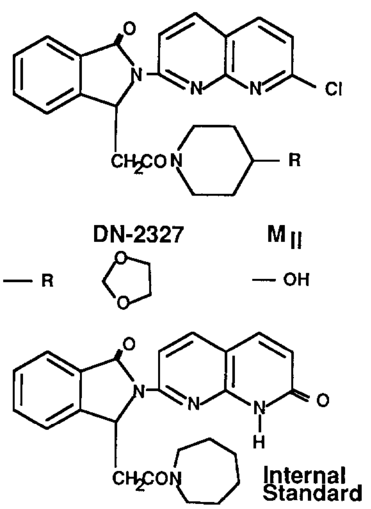

Fig. 1. Structures of parent drug (DN-2327, I), metabolite (M$_{II}$, II) and the internal standard (I.S.).

tive metabolite, M$_{II}$ (II; Fig. 1), that is predominant in blood. The intermediate metabolite neither circulates in blood nor is excreted in urine.

Currently, no analytical method is available to determine I and II either in human plasma or urine. Due to the high potency of I, clinical pharmacokinetic studies during the development of I will involve low clinical doses. Thus, a very sensitive analytical method is needed for the measurement of I and II in human plasma and urine to adequately characterize their pharmacokinetics. Described herein is a very sensitive, precise and rapid procedure for simultaneous determination of I and II in human plasma and II in urine after clinical doses, which is also applicable for rat and dog plasma.

EXPERIMENTAL

Chemicals

All organic solvents were high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Fairlawn, NJ, USA). The enzyme β-glucuronidase (1000 U per vial) and D-saccharic acid 1,4-lactone were purchased from Sigma (St. Louis, MO, USA). Reference standards of I (purity: 99.9%), its II metabolite (purity: 98.8%), and the internal standard (I.S.) were provided by Takeda Chemical Industries (Osaka, Japan). Structures of I, II and I.S. are shown in Fig. 1. Water used in the assay development and validation was deionized water 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 consisted of a Model SP8800 solvent-delivery system (Spectra-Physics, San Jose, CA, USA), a pulse dampener (Lo-Pulse, SSI, State College, PA, USA), an Adsorbosphere HS C_{18} (5 μm particle size, 150 mm × 4.6 mm I.D.) reversed-phase HPLC column (Alltech Assoc., Deerfield, IL, USA), and a Model RF-551 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan). Samples were injected onto the chromatographic system using a Model SP8880 autosampler, and the detector signal output was monitored on a Model SP-4270 computing integrator (Spectra-Physics) or TurboChrom data acquisition system (Nelson Analytical, Cupertino, CA, USA).

Preparation of standard solutions

A stock solution consisting of I and II (100 μg/ml) was prepared. Due to low solubility of the two compounds, 10 mg of each were first dissolved together in 50 ml of acetonitrile by sonication and then diluted to a final volume of 100 ml with acetonitrile. This was further diluted 1:10 with 50% acetonitrile to obtain a working standard solution (10 μg/ml). A working I.S. solution (10 ng/ml) was obtained by diluting 1:1000 a stock solution (10 μg/ml in acetonitrile) into 10% acetonitrile. Since photodegradation of the above compounds is a potential source of analytical error, it is important that all solutions be prepared in brown volumetric flasks or covered with aluminum foil. All solutions were refrigerated at 4°C when not used.

Preparation of calibration curves and quality controls in human plasma

The working standard solution (10 μg/ml each) was diluted 1:200 in drug-free human plasma followed by serial dilution with plasma to provide combined calibrators for the two compounds, between 0.1 and 50 ng/ml. Similarly, a quality control (QC) containing 45 ng/ml (QC-High) of each analyte was prepared by diluting a separately prepared working standard solution (10 μg/ml each) with drug-free human plasma; this was serially diluted to provide QC samples at 10 ng/ml (QC-Medium) and 2.5 ng/ml (QC-Low). Samples were covered with aluminum foil and stored frozen at −20°C until analysis.

Preparation of calibration curves and quality controls in human urine

A stock solution of I and II (*ca.* 100 μg/ml each) was diluted initially 1:200 with drug-free human urine, followed by serial dilution to provide a set of calibration standards in human urine with nominal concentrations of *ca.* 1, 5, 16, 32, 65, 130, 260, and 520 ng/ml of each analyte. Similarly, a QC sample was prepared at 430 ng/ml for each analyte (QC-High) by diluting separately prepared stock solution of both analytes with urine; this was serially diluted to provide QC samples at 170 ng/ml (QC-Medium) and 8.5 ng/ml (QC-Low). Handling and storage conditions for urine samples were identical to those described for plasma.

Extraction procedure for human plasma

A 0.5-ml aliquot of plasma was mixed with 0.5 ml of working I.S. solution and 1.5 ml of water, and the mixture was centrifuged at *ca.* 1000 *g* for 10 min. Bond Elut C_8 solid-phase extraction columns, 3 ml (Varian, Harbor City, CA, USA), were preconditioned by sequential elutions with 1 ml acetone, 1 ml methanol, and 2 ml water; the C_8 matrix was kept wet. The plasma mixture supernatant was decanted onto the C_8 column and drawn through the column under gentle vacuum. The extraction column was rinsed with two 3-ml portions of water followed by two 3-ml portions of 40% methanol under gentle vacuum. After columns were suctioned to near dryness under vacuum, the analytes were eluted with 1.5 ml of acetone under a very gentle vacuum, and the eluent was evaporated to dryness under a stream of nitrogen at 40–50°C.

Extraction procedure for human urine

Concentrations of I and II in human urine were determined before and after hydrolysis with β-glucuronidase. For the pre-hydrolysis analysis, an aliquot of 0.5 ml of urine was diluted with 0.5 ml working I.S. solution and 1.5 ml of water. For the post-hydrolysis determination, the contents of one vial containing lyophilized β-glucuronidase was freshly reconstituted with 10 ml of water. Thereafter, 0.1 ml of the β-glucuronidase solution was added to an aliquot of 0.1 ml urine, diluted with 0.3 ml of triple-distilled water and incubated for more than 1 h in a water bath preheated to 37°C. This was immediately followed by the addition of 0.5 ml of I.S. solution and dilution with 1.5 ml of water. All urine samples were then processed according to the extraction procedure described for plasma.

Recovery

The recovery of I and II from human plasma and urine was assessed at three different concentrations of each analyte in samples consisting of both analytes. Plasma was spiked with 1, 10, and 40 ng/ml of each analyte, whereas urine was fortified with *ca.* 16, 130, and 520 ng/ml of each compound. The spiked plasma and urine samples were processed according to the extraction procedure described for plasma, and eluates were collected into tubes containing I.S. The 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 I, II, and the I.S. with the peak height ratios from chromatography extracts.

Chromatography

The mobile phase for plasma extracts consisted of 7.5% (v/v) isopropanol and 45% (v/v) acetonitrile in water, and was delivered to the C_{18} column at a flow-rate of 1.2 ml/min. The mobile phase for urine extracts consisted of 7.5% (v/v) isopropanol and 38% (v/v) acetonitrile in water, and was delivered at 1.0 ml/min. The residues of the extracts from plasma and urine samples were reconstituted each in 0.2 ml of the appropriate mobile phase, centrifuged at 1000 *g* for 10 min, and portions of the clear supernatant were injected onto the HPLC system. Fluorescence detection was accomplished at excitation and emission maxima of 328 and 367 nm, respectively. A guard filter was positioned between the injection valve and analytical column.

Standard curve and calculations

Daily standard curves were constructed for each analyte by weighted [1/(concentration)^2] linear regression of the observed peak-height ratios *versus* concentration. The peak height ratios of I and II for the quality controls and unknown samples were used to compute concentrations based on the respective standard curves.

Intra- and inter-day precision of the assay procedures was assessed at concentrations of I and II in the concentration range 0.1–50 ng/ml in plasma and 1–500 ng/ml in urine. For plasma and urine, intra-day precision was based on the analysis of six replicates of low, medium and high concentrations of quality controls; eighteen samples from each matrix, analyzed six samples per day, were used for the assessment of the inter-day variability. For each analyte and biological matrix, the accuracy was expressed as the percentage deviation (+ or −) from the theoretical concentration of the sample.

Stability

The stability of a stock standard solution of a mixture of I and II and that of the I.S., stored at 4°C for five months was examined monthly. The stability of the working standard and I.S. solutions prepared in 10% acetonitrile in a brown glass was also tested at various times following room temperature storage for 72 h. The stability of the older stock or working solution was compared with a freshly prepared solution. Finally, the stability of I and II in plasma and urine was examined following several freeze–thaw cycles of frozen plasma and urine stored at −20°C for up to three months.

RESULTS AND DISCUSSION

Work-up procedure

Attention to certain specific details was found to be important in assuring specificity and reproducible high recovery of I and II from either plasma or urine using the described solid-phase ex-

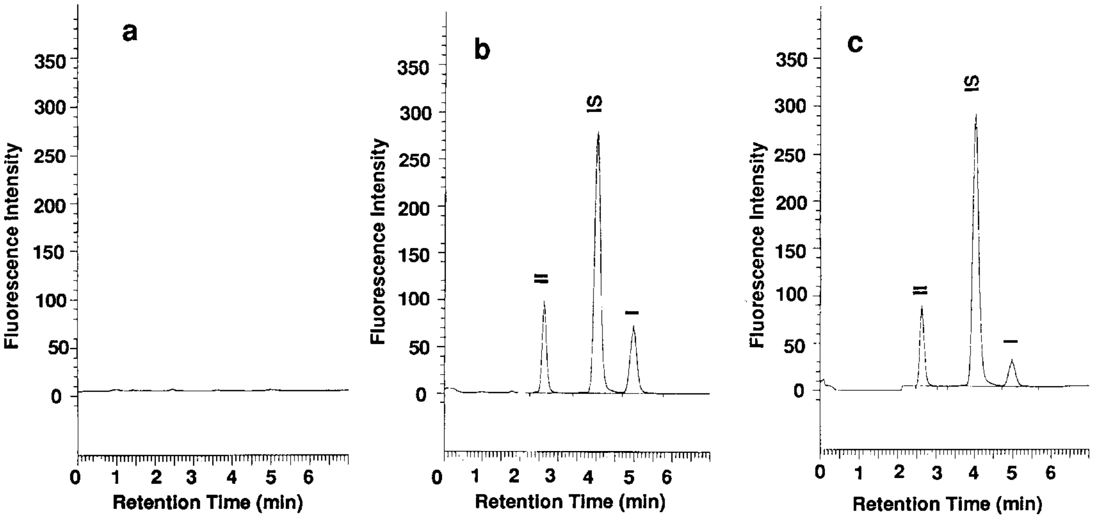

Fig. 2. Representative chromatograms of (a) drug-free human plasma, (b) drug-free human plasma fortified with 6.25 ng/ml M_{II} (II) and DN-2327 (I) and with I.S., and (c) plasma sample collected from a healthy subject 14 h after a single 4-mg dose.

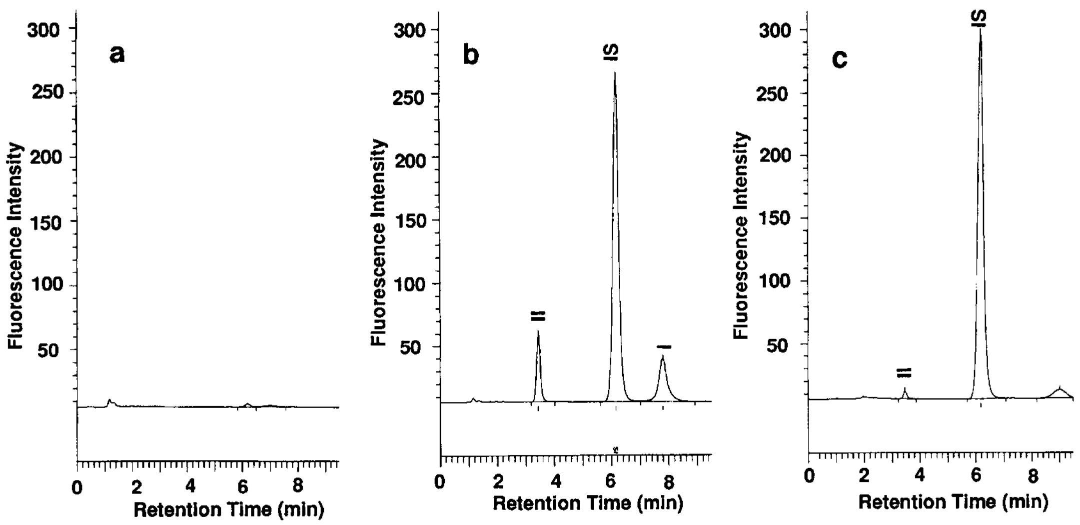

Fig. 3. Representative chromatograms of (a) drug-free human urine, (b) drug-free human urine fortified with *ca.* 31.25 ng/ml of M_{II} (II) and DN-2327 (I) and with I.S., and (c) pooled urine (0–24 h) collected from a healthy subject after a single 4-mg dose.

traction procedure. First, acetone was a critical solvent in the pre-conditioning of the extraction column; otherwise contaminants on the packing material from some batches of Bond Elut C_8 column might be eluted later with acetone and interfere with the compounds of interest. Second, the extraction column must not be allowed to dry out between pre-conditioning 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. Finally, a gentle vacuum must be used to draw the plasma or urine mixture, with subsequent rinses through the column, and the final elution step with acetone.

Chromatography

Figs. 2 and 3 present representative chromatograms from human plasma and urine samples, respectively, processed using the described analytical method. For each biological matrix, chromatographic peaks due to II, the I.S., and I were all well resolved. For plasma extracts, II, the I.S., and I eluted at *ca.* 2.6, 4.1, and 5.1 min, respectively (Fig. 2b) at a flow-rate of 1.2 ml/min. For urine extracts, the components eluted at *ca.* 3.5, 6.2, and 7.8 min, respectively, at a flow-rate of 1.0 ml/min (Fig. 3b). Drug-free plasma (Fig. 2a) and urine (Fig. 3a) samples obtained from normal subjects resulted in chromatograms free of endogenous peaks. After oral administration of I to a healthy subject, both I and II were present in plasma (Fig. 2c); however, no unchanged I was recovered in urine and II was the only measurable metabolite under the applied chromatographic conditions (Fig. 3c). A late-eluting peak related to an impurity in the I.S. was observed. Injection intervals of 10.5 and 15 min were usually employed for plasma and urine extracts, respectively, to ensure that this late-eluting peak did not interfere with subsequent chromatograms.

Under the same chromatographic conditions, I, II and the I.S. were also resolved on an Adsorbosphere HS C_8 column (150 mm × 4.6 mm I.D.) with similar selectivity, but as expected, the analytes were slightly less retained with the C_8 column than with C_{18}. Therefore, for baseline resolution between the I.S. and I a slight reduction in the concentration of acetonitrile in the mobile phase was needed.

Finally, the fluorescent compounds quinine, quinidine sulfate, terazosin, prazosin, temafloxacin, and pefloxacin showed no interference with the compounds of interest under the applied chromatographic procedure.

Recovery

The work-up procedure recovered *ca.* 92% from either plasma or urine, for tested concentrations up to 500 ng/ml. The recovery was very reproducible with a coefficient of variation (C.V.) for the analysis of replicate samples of $\leq 7.2\%$ and 6.0% for I and II, respectively, from plasma and $\leq 2.1\%$ and 3.0% from urine. Such high extraction efficiencies suggest that adsorption of the analytes to possible extractable endogenous substances, if any, during sample processing and chromatography was very minimal and even non-existent.

Linearity

Daily calibration curves were generated for each batch analysis for I and II in plasma and urine. To evaluate the curves, the observed responses for the individual calibrators were substituted back into the equation to calculate the predicted concentrations based on the calibration curve. For I and II in plasma, the mean recalculated concentrations (0.1–50 ng/ml) were all within 92–116 and 97–103% of theory, respectively, and the coefficients of determination (r^2) for the calibration curves (n = 6) exceeded 0.996. For urine, the mean recalculated concentrations of II (1–500 ng/ml) were within 98–104% of theory and the r^2 of the calibration curves were ≥ 0.9999.

TABLE I

PRECISION OF THE PROCEDURE FOR MEASURING CONCENTRATIONS OF I AND II IN HUMAN PLASMA

Added concentration[a] (ng/ml)	Coefficient of variation (%)			
	Intra-day variability ($n = 6$)			Inter-day variability ($n = 18$)
	Day 1	Day 2	Day 3	
Compound I				
2.5	7.0	1.7	5.0	6.1
10.0	2.7	4.6	6.7	6.5
45.0	4.8	3.5	1.8	5.5
Compound II				
2.5	4.5	1.8	6.2	5.5
10.0	4.5	4.6	6.7	5.6
45.0	4.7	3.6	1.7	3.2

[a] Pooled drug-free human plasma fortified with both I and II.

TABLE II

PRECISION OF THE PROCEDURE FOR MEASURING CONCENTRATIONS OF I AND II IN HUMAN URINE

Added concentration[a] (ng/ml)	Coefficient of variation (%)			
	Intra-day variability ($n = 6$)			Inter-day variability ($n = 18$)
	Day 1	Day 2	Day 3	
Compound I				
8.9	1.1	1.1	2.2	2.2
179	1.5	1.5	0.7	2.7
446	1.5	1.1	2.3	3.0
Compound II				
8.3	1.2	1.2	1.2	2.4
167	2.5	1.6	0.9	3.5
416	4.5	2.3	5.7	5.4

[a] Pooled drug-free human urine fortified with both I and II.

Precision and accuracy

Intra- and inter-day precision and accuracy data of the analytical procedure are presented in Tables I and II for plasma and urine, respectively. For plasma, the C.V. for the analysis of replicate samples at concentrations of 2.5, 10, and 45 ng/ml ranged from 1.7 to 7.0% for I and from 1.7 to 6.7% for II. The inter-day variability presented in Table I for the same quality control samples was below 6.5 and 5.6% for I and II, respectively. For urine, the C.V. for the analysis of replicate samples at concentrations of 8.9, 179, and 446 ng/ml ranged from 0.7 to 3.0% for I. For II, the C.V. ranged from 0.9 to 5.7% at concentrations of 8.3, 167, and 416 ng/ml, with most values below 5%. The inter-day variability determined from the overall precision data presented in Table II was below 3.0 and 5.4% for I and II, respectively. These results indicate good precision of the analytical method for the measurement of plasma concentrations of I and II between 0.1 and 50 ng/ml each, and those in urine between 1 and 500 ng/ml with C.V. values less than 7.0%.

The same data presented in Tables I and II also

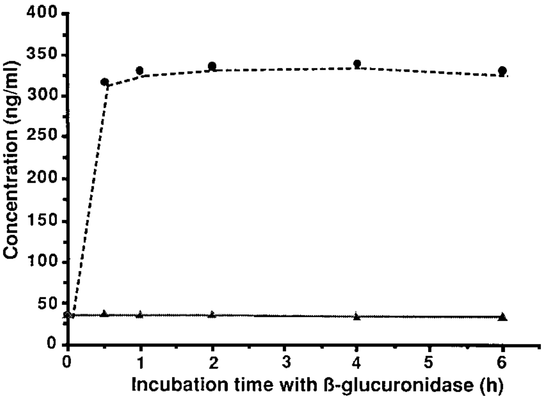

Fig. 4. Concentrations of M_{II} in urine, collected from a healthy subject after a single 8-mg dose, prior and after hydrolysis with β-glucuronidase in the presence (▲) and absence (●) of D-saccharolactone.

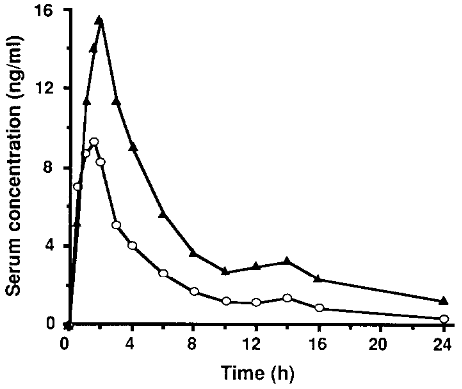

Fig. 5. Representative plasma concentration–time profiles for I (○) and II (▲) in a healthy subject following a single 4-mg dose of I.

demonstrate the good accuracy of the method based on the good agreement between added and calculated concentrations of the two analytes both in plasma and urine. For I and II, deviations were generally within ± 10% for each analyte in plasma, with values ranging from − 10.4% to 13%, and within ± 5.6% for each analyte in urine, with values ranging from − 1.3% to 5.6%.

Stability

I and II are light-sensitive compounds and photodegradation was prevented by storing all solutions, calibrators, and quality controls in brown glass containers or transparent containers covered with aluminum foil. Stock standard solutions of I, II and the I.S. prepared in acetonitrile were stable up to four months stored at 4°C. Working standard solutions prepared in mobile phase or 10% acetonitrile were stable at room temperature for more than 72 h standing on the laboratory bench or in the autosampler. I and II were stable in plasma and urine following several freeze–thaw cycles or three months of frozen storage at −20°C.

Hydrolysis of II-glucuronide

As seen in Fig. 4, the metabolite (II) is excreted in human urine both as free and conjugated form. The results might suggest that conjugated II was completely hydrolyzed with β-glucuronidase at 37°C within 30 min of incubation of a urine sample from a healthy subject who received a single 8-mg dose of I. Indeed, samples from *in vitro* metabolism studies by liver slices were analyzed under different chromatographic conditions. The chromatography confirmed the disappearance of a peak in the chromatogram identified as the glucuronide of II after samples were hydrolyzed with β-glucuronidase under the above conditions. Urine levels of the conjugated II were almost nine times those of the free II. As also demonstrated in Fig. 4, the presence of D-saccharic acid 1,4-lactone, a specific inhibitor of β-glucuronidase, prevented the hydrolysis of conjugated II, which strongly indicates that the conjugate of II is present in human urine as a glucuronide.

Application for rat and dog plasma

The described analytical method was adapted for the determination of I and II in rat and dog plasma and complete validation was performed using a 0.1-ml plasma aliquot. The method was specific and the recovery for each analyte was comparable to that obtained from human plasma. For each analyte, the standard curves were highly linear (r^2 = 0.993) in the concentration range 1–500 ng/ml, and all mean recalculated calibrators were within ± 7% of the theoretical concentrations. For each analyte and biological matrix, the intra- and inter-day variabilities were similar to those found for human plasma.

Clinical pharmacokinetics

Approximately 960 plasma samples and 220 urine samples, obtained during a multiple-dose safety and pharmacokinetic study in healthy male subjects, were analyzed for the determination of I and II levels using the described analytical procedure. The overall C.V. values for quality controls (0.1, 4, 16, and 40 ng/ml) during the course of serum sample analysis for I and II were generally within 8.8 and 7.5%, respectively. For both I and II, individual quality control results generally ranged from 80 to 120% of theory. Fig. 5 presents representative plasma concentration–time profiles of I and II following a single 4-mg dose of I to one of the subjects. As demonstrated in Fig. 5, the method was capable of measuring plasma levels of the two analytes up to 24 h after a single-dose administration. The maximum plasma concentrations of 9.3 and 14.1 ng/ml for I and II, respectively, occurred at the 1.5- and 2-h sampling times.

CONCLUSION

The described analytical method is simple, highly sensitive, precise, and accurate; thus, it is very suitable for the determination of I and II in the plasma and urine samples collected for clinical pharmacokinetic trials at the low dose anticipated for this novel anxiolytic agent. The method is also applicable for rat and dog plasma from toxicokinetic studies, using plasma and urine aliquots of only 50–100 μl.

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