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Enantioselective determination of *R*- and *S*-(α-bromoisovaleryl)urea in plasma using high-performance liquid chromatography after solid-phase extraction

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ABSTRACT

A stereoselective method has been developed for the determination of *R*- and *S*-(α-bromoisovaleryl)urea in plasma and saliva after oral administration. The chiral separation was carried out on Chiralcel OJ or OD columns with hexane–2-propanol as the mobile phase. The poor detection properties of the analyte required the development of an effective sample pretreatment procedure to enable ultraviolet detection at 210 nm. Solid-phase extraction using hydrophobic Amberlite XAD-2 in combination with washing steps at alkaline and acidic pH completely removed interfering components of the biological matrix and allowed the detection of the optical isomers at concentrations down to 10 ng/ml (0.05 μ*M*). The method was validated by determining the recovery, linearity, accuracy and within-day and between-day repeatability at 50, 200 and 2000 ng/ml. Application to the analysis of plasma and saliva samples is demonstrated.

INTRODUCTION

Glutathione (GSH) conjugation is an important detoxification reaction for many xenobiotics, including drugs and environmental chemicals [1,2]. In general, when there is a deficiency in GSH conjugation in humans, drugs and other compounds might be expected to show toxic effects after low-dose exposure. In spite of this important role of GSH conjugation, and the fact that glutathione S-transferase isoenzymes have been purified from several human tissues [2,3], the pharmacokinetics of drugs that are metabolized by GSH conjugation in humans have only rarely been studied. Recently, it was proposed that the (obsolete) racemic hypnotic drug (α-bromoisovaleryl)urea (BIU, Fig. 1) might be a good model substrate for studies on GSH conjugation, which is an important primary metabolic pathway *in vivo* [4]. Pronounced stereoselectivity in urinary excretion of the mercapturates derived from the BIU enantiomers was found in healthy subjects after oral administration of racemic BIU. The mercapturate formed from *R*-BIU was excreted two to three times faster than that formed from *S*-BIU [5]. These results are in agreement with those obtained for excretion in bile (BIU-GSH conjugates) and urine (BIU mercapturates) in rats [6].

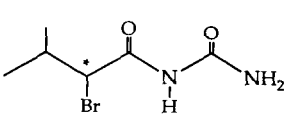

Fig. 1. Structural formula of BIU (the chiral centre is marked with an asterisk).

The diastereomeric *R*- and *S*-BIU mercapturates can be analysed by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection [7]. However, to study a possible correlation between the pharmacoki-

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netics of the separate BIU enantiomers in plasma and the excretion of their corresponding mercapturates in urine, an enantioselective assay for BIU in plasma is required. Few assays have been described for the determination of racemic BIU in plasma. Existing methods are based on colorimetry [8], HPLC [9–12] or gas chromatography (GC) [13]. Okamoto *et al.* [9] reported a reversed-phase HPLC method for the serum analysis of BIU with a detection limit of 10 μg/ml. The determination of BIU in biological matrices has been described by Matsubara *et al.* [12], who monitored BIU in plasma or brain tissue and reported a good linearity in the concentration range 0.5–50 μg/ml using reversed-phase HPLC with UV detection. No separation of BIU enantiomers, however, has been reported so far.

This paper describes a method for the determination of BIU enantiomers in human plasma and saliva with a limit of quantitation of 40 ng/ml. The method therefore allows pharmacokinetic studies in humans after oral administration of the racemic drug. The analytical method includes an off-line solid-phase extraction step in combination with a chiral HPLC separation and UV detection.

EXPERIMENTAL

Chemicals

Methanol and 2-propanol (Baker, Deventer, Netherlands) were of HPLC grade. All other organic solvents, sodium hydroxide, anhydrous sodium sulphate and perchloric acid were analytical grade purchased from Baker. Racemic BIU was obtained from Onderlinge Pharmaceutische Groothandel (Utrecht, Netherlands, Ph. Ned. VI quality). (*R*)-(+)-BIU and (*S*)-(−)-BIU were synthesized as described previously [14]. Millipore (Bedford, MA, USA) water was used throughout all the experiments. Anhydrous sodium sulphate was purified by washing with methanol and 2-propanol, and dried at 80°C for 12 h.

HPLC apparatus and chromatographic system

The HPLC system consisted of a Kratos-ABI (Ramsey, NJ, USA) Spectroflow 400 pump, a Rheodyne (Berkeley, CA, USA) 7010 six-port injection valve with a 100-μl injection loop, and a Kratos 773 UV detector operated at 210 nm. Enantiomer separation was carried out on a 250 mm × 4.6 mm I.D. stainless-steel Chiralcel OJ or OD (Daicel Industries, Tokyo, Japan) analytical column. A 50 mm × 4.6 mm I.D. column packed with the same stationary phase was used as a guard column. BIU enantiomers were eluted from the column with a mobile phase of hexane–2-propanol (90:10, v/v), which was degassed ultrasonically before use. The flow-rate was maintained at 1.2 ml/min.

Sample pretreatment

Solid-phase extraction columns were prepared by filling 55 mg of Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, USA) with a particle size range of 15–20 μm into empty Bond Elut (Analytichem International, Harbor City, CA, USA) cartridges. The extraction column was washed with 5 ml of hexane, 2 ml of 2-propanol, 3 ml of acetone and 2 ml of methanol, and conditioned with 2 ml of aqueous sodium hydroxide (pH 11.5).

Saliva and plasma samples spiked with racemic BIU, blanks and samples collected after oral administration of racemic BIU were stored at −30°C. After thawing at room temperature, 2 ml of plasma were vortex-mixed for 30 s and centrifuged for 30 min at 2000 *g*. The supernatant was applied to the extraction column, which was subsequently washed with 2 ml of aqueous sodium hydroxide (pH 11.5), 1 ml of water, 2 ml of aqueous perchloric acid (pH 2.5) and 2 ml of water. Residual water was removed by flushing the extraction column with nitrogen for 10 min. The compounds adsorbed were eluted with 4 ml of 2-propanol. In order to remove traces of water, the eluate was flushed through another Bond Elut column filled with anhydrous sodium sulphate. The eluate was evaporated to dryness in a water-bath at 80°C under a nitrogen stream. The residue was redissolved in 300 μl of mobile phase, being kept on ice, and vortex-mixed for 1 min. A 100-μl volume of the residue was injected into the HPLC system.

Assay validation

The method was validated the Chiralcel OJ analytical column.

Determination of the limits of detection (LOD) and quantitation (LOQ). A standard solution (7 μg/ml) was made of BIU in *n*-hexane–2-propanol (90:10) in order to determine the chromatographic parameters of the chiral separation system. The LOD was defined as the sample concentration of BIU resulting in a peak height of three times S_N, and the LOQ was estimated as ten times S_N. To determine the LOD and LOQ for the assay, spiked plasma was used. Six measurements were made at five concentrations, and the standard deviations of the measurements were plotted against the BIU concentration. The estimate of S_N was determined by extrapolation to zero.

Linearity. Plasma was spiked with 0.05, 0.10, 0.20, 1.00 and 2.00 μg/ml BIU to generate a calibration curve. Six measurements were made at each concentration. Using the computer program SAS/STAT (SAS Institute, Cary, NC, USA) following the REG procedure, a weighted linear regression equation was calculated for BIU. This incorporated the peak-height standard deviation at each concentration and produced a linear relationship between peak height and concentration. The corresponding confidence limits were also determined using this program, from which confidence intervals for sample data could be determined. Interpolation of the sample peak height on the upper and lower curves thus gave the uncertainty range at a 95% confidence level.

Accuracy and repeatability. Between-day repeatability was determined by assaying spiked plasma at various concentrations and measuring the subsequent peak heights. The procedure was repeated for five more days on the same spiked plasma standards. Within-day repeatability was determined by assaying the same spiked plasma standard six times in one day. Three different concentrations were used: 50, 200 and 2000 ng/ml. The coefficient of variation (C.V. %) was calculated as the ratio of S.D. (defined as the standard deviation divided by the square root of the number of samples measured) to the mean peak height at a given concentration multiplied by one hundred.

RESULTS

Chiral separation and detection

The separation of BIU enantiomers was carried out on a Chiralcel OJ or OD column, which contain modified cellulose coated 10-μm silica particles [15]. The capacity ratio, selectivity factor and resolution for *R*- and *S*-BIU are shown in Table I. Unfortunately, no reversed-phase-type chiral column, which would allow the use of aqueous mobile phases, was found suitable for the separation of the BIU enantiomers [16]. This assay also seemed to be valid for the enantioselective separation of BIU analogues, as was demonstrated for (α-bromovaleryl)urea (BVU), where the retention times of the *R*- and *S*-isomers were 13.0 and 16.4 min, respectively, resulting in a selectivity of 1.26 on the Chiralcel OJ column.

The solvent composition of the sample solution injected is important for the quality of the separation. The solution to be injected should be identical with the mobile phase. A slightly higher content of 2-propanol, for example, resulted in a complete loss of resolution. Furthermore, the presence of traces of water caused a negative peak that interfered with the *R*- and *S*-BIU peaks. Therefore, water should be removed as completely as possible, *e.g.*, by treatment of the final extract (see below) with anhydrous sodium sulphate.

Since BIU possesses only poor UV absorbance, the post-column conversion into a fluorescent or electroactive derivative would be desirable. However, the absence of reactive functional groups in BIU severely limits the application of

TABLE I

CAPACITY RATIO (k'), SELECTIVITY FACTOR (α) AND RESOLUTION (R) OF *R*- AND *S*-BIU ON CHIRALCEL OJ AND OD ANALYTICAL COLUMNS

Column	k'		α	R
	R-BIU	*S*-BIU		
Chiralcel OD	2.1	2.5	1.19	1.06
Chiralcel OJ	3.1	3.6	1.12	0.78

selective and sensitive detection methods, such as fluorescence or electrochemical detection. Therefore, UV detection at 210 nm, although both unselective and insensitive, was chosen in combination with a selective sample handling procedure.

Sample pretreatment

The sample pretreatment procedure developed is based on the fact that BIU (pK_a 10.8) is relatively non-polar and non-ionizable over a wide pH range. It can therefore be extracted from aqueous solutions using non-polar supports, such as C_{18} bonded silica or polymeric XAD-2. Because of the washing steps in alkaline and acidic media, the latter material was chosen because of its pH stability. The breakthrough volume of BIU for XAD-2 packed columns was higher than 10 ml between pH 2 and 12. In order to remove proteins and other low-molecular-mass acidic and basic compounds, the XAD-2 column (after being loaded with 2 ml of plasma) was flushed successively with aqueous sodium hydroxide (pH 11.5) and aqueous perchloric acid (pH 2.5). Before elution of the remaining components with 2-propanol, residual water, which strongly interferes with the separation of *R*- and *S*-BIU, was removed by flushing the column with nitrogen for 10 min at ambient temperature.

Fig. 2. shows chromatograms obtained from blank plasma, water spiked with 50 ng/ml *R*-BIU and 250 ng/ml *S*-BIU, and plasma spiked with 100 ng/ml *R*-BIU and 500 ng/ml *S*-BIU. The chromatograms for plasma and water samples were almost identical, which demonstrates the efficiency of the clean-up procedure with respect to the removal of proteins and low-molecular-mass plasma components, since no interferences were encountered despite the low detection wavelength. The major peaks at the beginning of the chromatograms can be attributed to the presence of impurities in the solvents used. Of all the solvents tested (methanol, acetone, ethanol, diethyl ether, 2-propanol, hexane and dichloromethane), 2-propanol possessed the highest purity.

Assay validation

A comparison between the peak heights of a BIU standard and those of a spiked plasma sample that had undergone the assay procedure yielded a mean (± S.D.) recovery of 88.5 ± 3.5% ($n = 6$). The LOD and LOQ for both isomers in spiked plasma were 12 and 40 ng/ml (pretreatment of 2 ml of plasma), respectively).

The weighted linear regression equations for

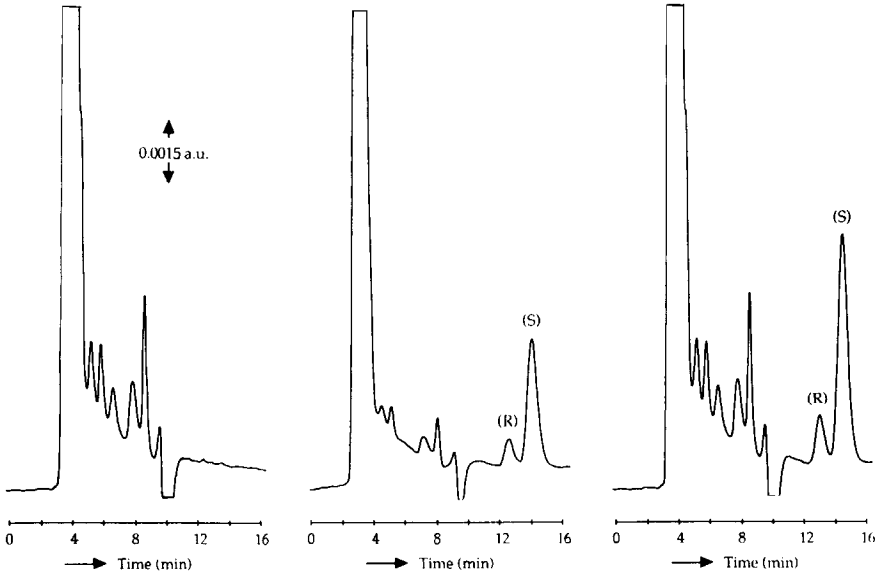

Fig. 2. Chromatograms obtained from (left) blank plasma, (middle) water spiked with 50 ng/ml *R*-BIU and 250 ng/ml *S*-BIU and (right) plasma spiked with 100 ng/ml *R*-BIU and 500 ng/ml *S*-BIU after sample pretreatment. Peaks: (R) = *R*-BIU; (S) = *S*-BIU. Chromatographic conditions: analytical column, Chiralcel OJ; other conditions, see Experimental.

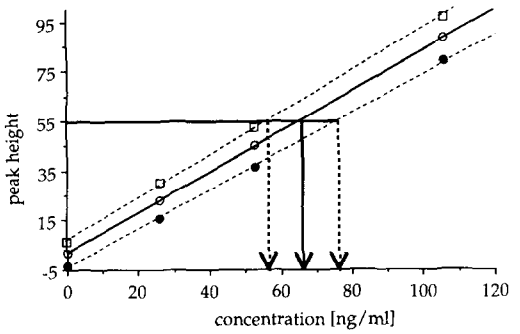

Fig. 3. Calculation of the confidence intervals for *R*-BIU in plasma. The solid line is given by the linear regression equation and is used to calculate the concentration of the analyte. The dashed lines are the upper and lower confidence limits on which direct interpolation for a given peak height will give the corresponding confidence intervals for the determined concentration.

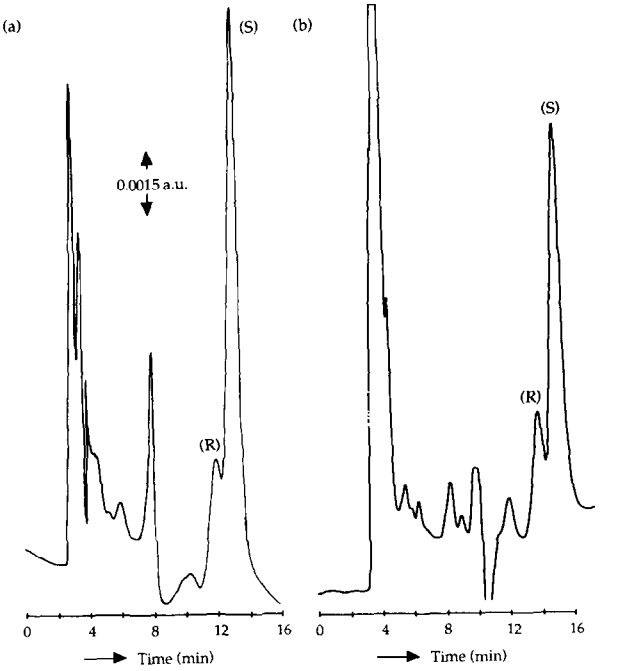

Fig. 4. Chromatograms obtained and used for the determination of *R*- and *S*-BIU in (a) plasma and (b) saliva 3 h after administration of 600 mg of racemic BIU. Peaks: (R) = *R*-BIU; (S) = *S*-BIU. Chromatographic conditions: analytical column, Chiralcel OJ; other conditions, see Experimental.

R- and *S*-BIU in plasma were calculated as $y = (813.2 \pm 45.7)x + (1.6 \pm 4.8)$ and $y = 815.5 \pm 82.7)x + (-0.7 \pm 6.8)$, respectively, y being the peak height and x the concentration of *R*- and *S*-BIU in μg/ml. The corresponding correlation coefficients were 0.9995 and 0.9985, respectively. The upper and lower confidence limits were determined as shown in Fig. 3 for *R*-BIU. (This statistical method was also used to calculate the confidence intervals shown in Fig. 5a and b for plasma and saliva samples, respectively).

The accuracy of the method was determined by spiking plasma samples with 50, 200 and 2000 ng/ml. The data for accuracy and within-day and between-day repeatability are presented in Table II. The relatively high C.V. for the 50 ng/ml sam-

TABLE II

ACCURACY AND WITHIN-DAY AND BETWEEN-DAY REPEATABILITY AND COEFFICIENTS OF VARIATION FOR THE DETERMINATION OF *R*- AND *S*-BIU IN PLASMA

Added concentration[a] (ng/ml)	Within-day repeatability		Between-day repeatability	
	Concentration found (mean ± S.D., $n=6$) (ng/ml)	C.V. (%)	Concentration found (mean ± S.D., $n=6$) (ng/ml)	C.V. (%)
R-BIU				
25	25 ± 8	32.0	28 ± 3	10.7
100	107 ± 4	3.8	107 ± 5	4.7
1000	1049 ± 24	2.3	1043 ± 47	4.5
S-BIU				
25	23 ± 5	21.7	28 ± 7	25.0
100	109 ± 13	11.9	99 ± 8	8.1
1000	990 ± 43	4.3	930 ± 44	4.7

[a] BIU added as racemic mixture.

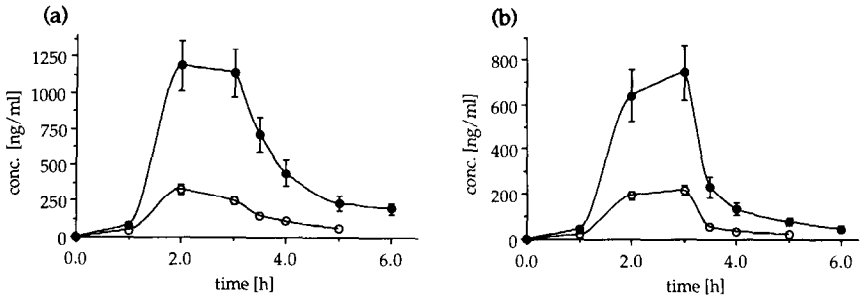

Fig. 5. Concentration *versus* time curves for (a) plasma and (b) saliva samples after oral administration of 600 mg of racemic BIU to a healthy subject.

ple (32% for the within-day repeatability of *R*-BIU) was caused by one suspect outlier which, however, could not be rejected using Dixon's Q-test. Excluding this suspect value, the C.V. would be 12%, which is of the same order of magnitude as the value for the between-day repeatability.

Drug monitoring

Typical chromatograms are shown in Fig. 4 for plasma (Fig. 4a) and saliva (Fig. 4b) samples measured 3 h after BIU administration. Examples of concentration *versus* time curves for both plasma and saliva samples after oral administration of 600 mg of racemic BIU to a healthy subject are presented in Fig. 5. A pronounced stereoselectivity is observed. It seems that for the *S*-enantiomer the area under the curve is three to four times higher than for the *R*-enantiomer, whereas the elimination half-lives for the two isomers are more or less similar. BIU thus seems to be a suitable compound for *in vivo* assessment of stereoselective GSH conjugation in humans.

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