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# Quantitative assay of lorazepam and its metabolite glucuronide by reverse-phase liquid chromatography-tandem mass spectrometry in human plasma and urine samples

Olga Papini [a], Carlo Bertucci [b,*], Sergio Pereira da Cunha [c], Neife Aparecida Guinaim dos Santos [a], Vera Lucia Lanchote [a,**]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP, Universidade de São Paulo, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Avenida do Café s/n, Campus da USP, 14040-903 Ribeirão Preto, Brazil*
[b] *Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[c] *Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil*

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**Abstract**

A LC/MS/MS method for the quantitative determination of lorazepam in human plasma and urine samples was developed and validated. The enantioselective assay allowed to separate the enantiomers and to verify the stereochemical instability of lorazepam. The linearity assessed for lorazepam unchanged was 0.2–20 ng of each enantiomer/ml plasma and 0.2–15 ng of each enantiomer/ml urine. The linearity assessed for total lorazepam (after enzymatic hydrolysis) was 1–30 ng of each enantiomer/ml plasma and 10–150 ng of each enantiomer/ml urine. The coefficients of variation obtained for the intra- and interassay precision were less than 15%. The method was applied to the investigation of the kinetic disposition and metabolism of racemic lorazepam administered as a single oral dose of 2 mg to a parturient. The occurrence of racemization required the calculation of the pharmacokinetic parameters as enantiomeric mixtures of lorazepam ($t_{1/2a}$ 3.5 h; $K_a$ 0.198 ng $h^{-1}$; $t_{1/2}$ 11.5 h; $\beta$ 0.060 $h^{-1}$; $AUC^{0-\infty}$ 192.1 ng h/ml; CLt/f 2.41 ml/min kg; Vd/f 173.5 l; Fel 0.41%, and $Cl_R$ 0.0099 ml/min kg) and its metabolite lorazepam–glucuronide ($t_{1/2f}$ 1.2 h; $K_f$ 0.578 $h^{-1}$; $t_{1/2}$ 16.6 h; $\beta$ 0.042 $h^{-1}$; $AUC^{0-\infty}$ 207.6 ng h/ml; Fel 51.80%, and $Cl_R$ 98.32 ml/min kg). However, the determined confidence limits make the method suitable for application to clinical pharmacokinetic studies, even if the quantification of both the enantiomers is required.


*Keywords:* Lorazepam; Glucuronide; Enantiomers; LC-MS/MS; Pharmacokinetics; Metabolism; Parturient

## 1. Introduction

Lorazepam is a benzodiazepine clinically employed for the treatment of anxiety associated or not with depressive symptoms, as preoperative medication, and also as an anticonvulsant in neonates [1–5].

Lorazepam (7-chloro-5-(2-chlorophenyl)-1,3dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one) is commercially sold as a racemic mixture of *S* and *R* enantiomers. In vivo studies have demonstrated stereoselectivity in binding to the receptors of benzodiazepines presenting a chiral C3 atom. The apparent affinity for the binding site of the (+)-(*S*) enantiomer is 100–200 times higher than for (−)-(*R*)-lorazepam [6,7].

Lorazepam is essentially eliminated by phase II metabolism by conjugation with UDP–glucuronic acid (Fig. 1). Approximately 75% of the lorazepam dose is excreted into 72 h urine as a glucuronic acid conjugate [8]. Other metabolites include the hydroxylated products in the ring and the quinazoline derivatives [9]. The elimination half-life of lorazepam is 11–16 h and its total clearance were approximately 1.1 ml/min/kg [8,10–13].

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* Corresponding author. Tel.: +39 051 2099742; fax: +39 051 2099734.
** Corresponding author.
*E-mail addresses:* carlo.bertucci@unibo.it (C. Bertucci), lanchote@fcfrp.usp.br (V.L. Lanchote).

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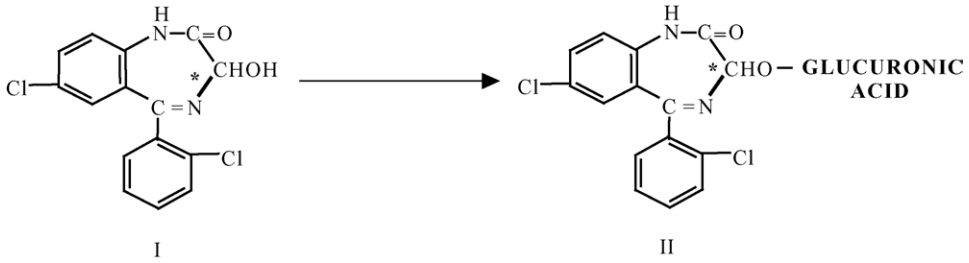


Fig. 1. Chemical structure of lorazepam (I), its metabolite lorazepam–glucuronide (II) [(*) denotes the chiral center].

The enantioselective determination of lorazepam in plasma was reported by Kanazawa et al. [14] using the LC-MS technique and enantiomer separation on an AS (amylose *S*-methylbenzylcarbamate) chiral phase column with a mobile phase consisting of a 5:5:1 mixture of hexane:2-propanol:ethanol. The authors did not mention the validation of the analytical method or its application to studies of kinetic disposition.

Kanazawa et al. [15] reported the stereoselective analysis of lorazepam in plasma by HPLC with an ultraviolet detector and circular dichroism, and enantiomer separation on a chiral phase CDBS-453 (silica gel chemically bonded by a derivative of β-cyclodextrin) column with an aqueous mobile phase with 13% acetonitrile. The authors reported the analysis of one plasma sample from a patient orally treated with racemic lorazepam, detecting only the enantiomer (+)-(*S*)-lorazepam. These data are not in agreement with other papers reporting a low stereochemical stability of 3-hydroxybenzodiazepines in aqueous solution. As an example, Pham-Huy et al. [7] reported the enantioselective HPLC analysis of oxazepam, lorazepam and temazepam. The enantiomers were separated on a Cyclobond I-2000 RSP (β-cyclodextrin derivatized with *R*,*S*-hydroxypropyl ether) column with a mobile phase consisting of 19% acetonitrile. However, the experimental conditions used by the authors resulted in in vitro racemization of lorazepam, which prevented the application of the method to biological material.

Racemization of the lorazepam enantiomers in different solvents was first reported by Lu and Yang [16], who observed that the rate of lorazepam racemization increased progressively in solvents of higher polarity. The authors inferred a racemization half-life of 9.2 min in methanol, 14.3 min in ethanol, 33 min in 2-propanol, and more than 5000 min in dichloroethane and acetonitrile. Thus, the enantiomers of lorazepam are more stable in an aprotic environment. The authors observed that a reduction in temperature also stabilized the enantiomers of lorazepam. The mechanism of lorazepam racemization appears to be similar to that of oxazepam and of other 3-hydroxy-1,4-benzodiazepines and has been proposed as the equilibrium between the tautomeric form and the open aldehyde form.

On-column enantiomerization of lorazepam was also reported by Nishikawa et al. [17] during chiral liquid chromatography with optical rotation detection. The cited authors separated the enantiomers of lorazepam on a CDB-453 HQ (β-cyclodextrin derivative chemically bonded to a polymer gel) column using a mobile phase consisting of 30% de acetonitrile.

Wang and Porter [18] used eletrochemically modulated liquid chromatography for the enantiomeric separation of oxazepam, lorazepam and temazepam using a porous graphitic carbon stationary phase and β-cyclodextrin as a chiral mobile phase additive. The mobile phase consisted of a mixture of 75% water (0.1 M $LiCLO_4$, 6 mM β-CD, 20 mM phosphate buffer, pH ∼ 1.8) and 25% acetonitrile (0.1 M $LiCLO_4$). The authors observed apparent enantiomerization of lorazepam and oxazepam.

We report here the quantitative assay of lorazepam in plasma and urine samples from a parturient using for the first time LC-MS/MS with an OD-R column and a mobile phase consisting of 80% acetonitrile, one of the solvents allowing higher stereochemical stability to the chiral center of lorazepam. Furthermore the racemization process of the drug has been investigated in the experimental conditions adopted for the extraction procedure, in order to better define the controversial aspect of the enantioselective distribution and excretion of lorazepam [7,15,16].

## 2. Methods and subjects

### 2.1. Drugs and chemicals

A stock standard solution of *rac*-lorazepam (99.8% Wyeth, São Paulo, SP, Brazil) was prepared in methanol at the concentration of 1 mg/ml. Working solutions were prepared at concentrations of 8 ng–6 μg/ml methanol of each enantiomer. The internal standard solution (nitrazepam) was prepared in methanol at the concentration of 25 ng/ml.

Methanol, acetonitrile (chromatography grade) and *tert*-butyl methyl ether (analytical grade) were obtained from Merck (Darmstadt, Germany). Water was purified in a Milli-Q Plus System (Millipore, Bedford, MA, USA).

β-Glucuronidase type HP2: *Helix pomatia* (Sigma St. Louis, MO) was used for enzymatic hydrolysis.

### 2.2. Instruments and conditions

The liquid chromatographic system consisted of an LC10AD pump and a CTO-10AS column oven from Shimadzu (Kyoto, Japan). The chiral column Chiralpak® OD-R

0.46 cm × 25 cm, 10 μm particle size (Chiral Technologies, Inc., Exton, PA, USA), was used for the resolution of the lorazepam enantiomers. A LiChrospher® 100 RP-18 column (4 mm × 4 mm i.d., 5 μm) from Merck was used as guard column. The mobile phase consisted of a mixture of acetonitrile–water–acid acetic (80:20:0.1%, v/v/v). The column was kept in an oven set at 25(±1) °C and a flow-rate of 1.0 ml/min was used.

The effluent from the chromatographic column was split (0.2 ml/min) by a Micro-splitter valve (Upchurch Scientific, WA, USA) and attached to the inlet of a Quattro Micro LC triple-stage quadruple mass spectrometer (Micromass, Manchester, UK) fitted with a Z-electrospray interface (ESI) operated in the positive ion mode.

The capillary voltage in the ESI probe was 3.5 kV. The source block and desolvation temperatures were set at 100 and 200 °C, respectively. Nitrogen was used as nebulizing gas at 350 l/h and argon was used as collision gas at a pressure of approximately $3.5 \times 10^{-3}$ mbar. The cone voltage was set at 40 V and collision energy of 38.0 and 30.0 eV was used for lorazepam and IS, respectively.

Optimization of MS conditions was obtained by direct infusion of the standard solution (10 μg/ml of the mobile phase) into the ion spray at a flow-rate of 10 μl/min. Quantitation was performed by MRM (dwell time of 1 s) of the protonated molecule $[MH]^+$ and its corresponding product ion using two functions, 321 > 275 for lorazepam and 282 > 236 for the IS.

A MassLynx (Micromass, Manchester, UK) data sampling system, version 3.5 was used for sample acquisition and quantitation.

### 2.3. Enzymatic hydrolysis

Human plasma samples from healthy volunteers were supplied by the Blood Center of the University Hospital, Faculty of Medicine of Ribeirão Preto (University of São Paulo, Brazil).

Urine samples from healthy volunteers were supplied by the Department of Clinical, Toxicological and Bromatological Analyses, Faculty of Pharmaceutical Sciences of Ribeirão Preto (University of São Paulo, Brazil).

Urine samples (100 μl) were spiked with 25 μl of nitrazepam solution (internal standard, 25 ng/ml), 1.0 ml 0.25 M aqueous acetate buffer, pH 5.0, and 50 μl of β-glucuronidase type HP2: *Helix pomatia* (Sigma, St. Louis, MO). Plasma samples (500 μl) were spiked with 25 μl of nitrazepam solution (internal standard, 25 ng/ml), 0.5 ml 0.75 M aqueous acetate buffer, pH 5.0, and 25 μl of β-glucuronidase type HP2: *Helix pomatia* (Sigma, St. Louis, MO). After homogenisation, all samples were shaken continuously for 16 h at 37 °C.

### 2.4. Extraction procedure

Eighty microliters of an aqueous 1 M sodium hydroxide (previously washed with methyl *tert*-buthyl ether) were added to the urine hydrolysed samples and 20 μl of the same solution were added to the plasma-hydrolysed samples to give pH 10. After shaking in a mixer for 30 s, the hydrolysates were extracted with 5.0 ml of methyl *tert*-buthyl ether.

Plasma or urine samples of 1.0 ml do not submitted to the enzymatic hydrolysis were spiked with 25 μl of nitrazepam solution (internal standard, 25 ng/ml) and with 1.0 ml 50 mM aqueous carbonate buffer. Lorazepam enantiomers were extracted from plasma and urine samples with 5.0 ml of methyl *tert*-buthyl ether.

All tubes were shaken horizontally for 30 min and then centrifuged for 5 min at 1800 × *g*. The organic phases were collected and evaporated to dryness in a centrifugal evaporator vacuum system (RCT90 & RC10.22 model) from Jouan AS (St. Herblain, France), set at 25 °C. The residues were reconstituted in 50 μl mobile phase, vortexed for 15 s and 20 μl were injected into the analytical column.

### 2.5. Method validation

Calibration curves for unchanged lorazepam analysis were prepared by analysing 1.0 ml drug-free plasma or urine samples spiked with 25 μl of each working *rac*-lorazepam solution, in duplicate, resulting in plasma concentrations of 0.2–20 ng/ml of each enantiomer and urine concentrations of 0.2–15 ng/ml of each enantiomer. Calibration curves for lorazepam–glucuronide analysis were constructed in urine concentrations of 10–150 ng/ml of each enantiomer and in plasma concentrations of 1–30 /ml of each enantiomer. Plots of concentrations versus peak area ratios, lorazepam/internal standard, were constructed and the linear regression lines were used for the determination of enantiomer concentrations in plasma and urine samples.

The efficiency of the extraction procedure for unchanged lorazepam was assessed by analysing drug-free plasma or urine aliquots of 1.0 ml in quintuplicate, spiked with three different concentrations of *rac*-lorazepam in plasma (0.2, 2 and 10 ng/ml for each enantiomer) and in urine (0.2, 1 and 5 ng/ml for each enantiomer). The efficiency of the extraction procedure after enzymatic hydrolysis (total lorazepam) was evaluated in plasma (2, 10 and 20 ng/ml for each enantiomer) and urine samples (5, 20 and 100 ng/ml for each enantiomer). The samples were submitted to the extraction procedure and the internal standard was added to the extracts. Peak area ratios were compared with those obtained by the direct injection of lorazepam and internal standard into the mobile phase.

The precision and accuracy of the assay were determined by analysing the enantiomers of lorazepam in plasma and urine samples spiked with three concentrations, 0.2, 2, 10 and 0.2, 1, 5 ng/ml, of each enantiomer, respectively. The precision and accuracy of the assay after enzymatic hydrolysis (total lorazepam) were determined by analysing plasma samples at concentrations 1, 2 and 10 ng/ml of each enantiomer and urine samples at concentrations 10, 40 and 100 ng/ml of each enantiomer. Within-day precision and accuracy were

determined by analysing 10 aliquots of spiked human plasma or urine and between-day precision and accuracy were determined for five consecutive days. The precision and accuracy of the method were calculated as the relative standard deviation (coefficient of variation) and the percent deviation of observed concentration from theoretical concentration, respectively.

The quantitation limit (LOQ) of the assay without enzymatic hydrolysis (unchanged lorazepam) was obtained by the analysis in quintuplicate of plasma or urine samples spiked with *rac*-lorazepam at concentrations as low as 0.2 ng/ml of each enantiomer. The LOQ of the assay after enzymatic hydrolysis (total lorazepam) were determined in plasma or urine samples at concentrations as low as 1 and 10 ng/ml of each enantiomer, respectively. Criteria for the LOQ were established as the lowest concentration for which the variation in precision and accuracy was less than 20%.

### 2.6. Elution order

The order of elution of lorazepam enantiomers was established by HPLC using simultaneous ultraviolet detection and circular dichroism (Jasco, Japan, model 1595) operating at 265 nm wavelength. The lorazepam enantiomers were eluted through the same column and mobile phase as described in item 2.2. A 25 μl aliquot of a racemic solution of lorazepam diluted in methanol at the concentration of 4.0 μg/ml was evaporated dry at room temperature. The residue was reconstituted with 50 μl of the mobile phase and 20 μl were submitted to chromatographic analysis.

### 2.7. Racemization test

Methanol solutions of 1, 0.1 and 0.01 mg of *rac*-lorazepam/ml were injected into the HPLC system as described above using a 20-μl loop and an ultraviolet detector operating at a wavelength of 220 nm. The individual enantiomers were collected as peak 1 (retention time = 4.8 min) and peak 2 (retention time = 6.5 min). The enantiomers collected were injected separately into the LC-MS/MS system. The racemization test was also performed after the liquid–liquid extraction procedure in plasma and urine. Aliquots of 1 ml plasma and urine were spiked with 25 μl of each *rac*-lorazepam solution and with 25 μl of 50 mM carbonate buffer, pH 10. After mixing for 30 s, 5 ml *tert*-butyl methyl ether were added. The samples were shaken horizontally for 30 min, followed by centrifugation at 1800 × *g* for 5 min. The organic phases were collected and evaporated to dryness in a centrifugal evaporator vacuum system (RCT90 & RC10.22 model) from Jouan AS (St. Herblain, France), set at 25 °C. The residues were reconstituted with 35 μl of the mobile phase immediately prior to analysis. A 20 μl aliquot was assayed by HPLC. The enantiomers collected as peaks 1 and 2 were injected separately into the LC-MS/MS system.

### 2.8. Subjects and clinical protocol

The method developed was used for analysis of samples obtained from a healthy parturient (M.F.L., a 37-year-old, 72 kg, 1.63 cm tall woman with a gestational age of 39 weeks) during labor. The protocol was approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil. The parturient received detailed information about the study and gave written informed consent to participate. The parturient was admitted to the Airport Complex Maternity Hospital (Mater), Ribeirão Preto, SP, Brazil. After clinical examination and biochemical tests for the confirmation of normal hepatic, renal and cardiac functions, the parturient received one tablet of 2 mg *rac*-lorazepam (Lorax® Wyeth, São Paulo, SP, Brazil).

Maternal blood samples were collected through an intravenous catheter at times zero, 0.5, 1, 2, 3, 4, 6, 8, 12, 30 and 48 h and urine samples were obtained at 12 h intervals up to 48 h after lorazepam administration. Heparin was used as anticoagulant (Liquemine® 25000 IU, Roche, São Paulo, SP, Brazil). Plasma samples were separated by centrifugation at 1800 × *g* for 10 min and stored at −70 °C until chromatographic analysis.

## 3. Pharmacokinetic analyses

The kinetic disposition of the enantiomeric mixture of lorazepam after the oral administration of a 2 mg dose to a term parturient during labor was evaluated using a two-compartment model and first-order kinetics. The half-life distribution ($t_{1/2}\alpha$) was determined after correction of the respective phase by the residue method. The elimination half-life ($t_{1/2}\beta$) was directly determined by the graphic method ($\log c$ versus $t$). The distribution ($\alpha$) and elimination ($\beta$) rate constants were calculated using the $0.693/t_{1/2}$ equation. The maximum plasma concentration ($C_{max}$) and the time to reach $C_{max}$ ($t_{max}$) were directly calculated from the plasma concentrations of the enantiomers obtained. The area under the plasma concentration time curve ($AUC^{0-\infty}$) was calculated by the trapezoidal method with infinite extrapolation by dividing the last plasma concentration by the elimination rate constant ($\beta$). The apparent total clearance (CLt/f) of each enantiomer was derived from the dose/$AUC^{0-\infty}$ and the apparent volume of distribution (Vd/f) from CL/$\beta$. The amount of enantiomeric mixture of lorazepam excreted into urine (Ae) was determined by multiplying the urinary concentrations (Cu) by the respective urine volumes measured at each collection time (Vu). The total amount excreted into 48 h urine was calculated by summing the values obtained at each collection time. The Ae parameter was used to calculate the fraction of lorazepam dose excreted into urine in an unchanged manner according to the equation Fel = Ae/dose and also to calculated renal clearance ($CL_R$) according to the equation $CL_R = Ae/AUC^{0-\infty}$. The elimination half-life

of the enantiomer mixture of lorazepam was calculated by the curve for log urinary excretion rate ($\Delta Ae/\Delta t$) versus mid-point of the urine collection interval ($t_m$). The elimination rate constant (kel) was calculated by the equation $kel = 0.693/t_{1/2}$ [8,10–13].

## 4. Results and discussion

The present study reports for the first time the analysis of lorazepam and its metabolite glucuronide in plasma and urine by LC-MS/MS with the report of racemization, despite the high percentage of acetonitrile used in the mobile phase. The method was applied to the investigation of the kinetic disposition and metabolism of lorazepam administered as a single dose to a parturient.

The lorazepam enantiomers were separated on an OD-R column with a mobile phase consisting of 80% acetonitrile (Figs. 2 and 3). Kanazawa et al. [14,15] used mobile phases consisting of 50% or more ethanol, 2-propanol or a mixture of ethanol and 2-propanol even though Lu and Yang [16] reported the rapid racemization of lorazepam in ethanol and 2-propanol. Other studies [6,7,17] used aqueous mobile phases consisting of low percentages of acetonitrile (15–30%) for the separation of the lorazepam enantiomers. Acetonitrile, one of the solvents of higher stability for the chiral center of lorazepam [16], used in the present study at the 80% proportion, was not sufficient to prevent the enantiomerization of lorazepam.

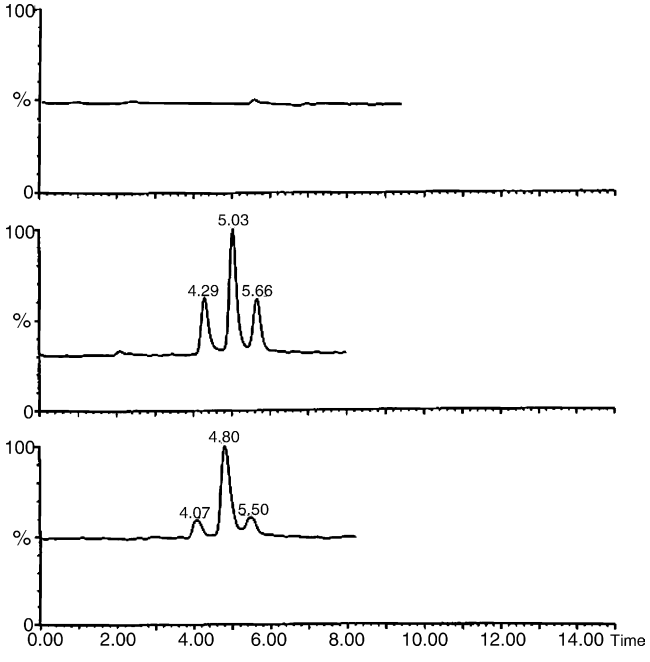


Fig. 2. Total ion chromatogram of (A) blank human plasma, (B) blank human plasma spiked with *rac*-lorazepam and the internal standard and (C) plasma sample collected from a parturient 12 h after administration of lorazepam. Elution order of the peaks: (+)-(*S*)-lorazepam, internal standard (nitrazepam) and (−)-(*R*)-lorazepam.

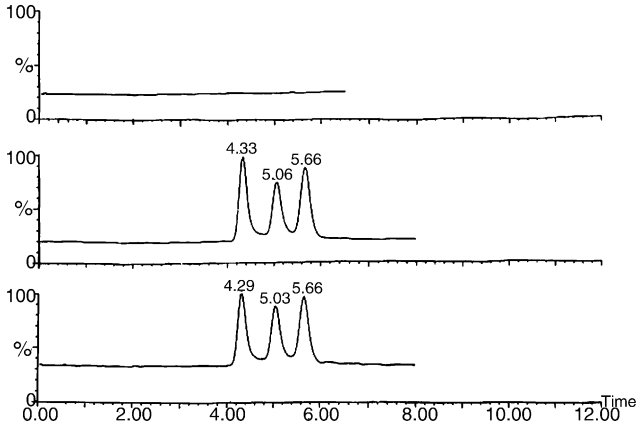


Fig. 3. Total ion chromatogram of (A) blank human urine, (B) blank human urine spiked with *rac*-lorazepam and the internal standard and (C) a urine sample collected from a parturient 12–24 h after administration of lorazepam. Elution order of the peaks: (+)-(*S*)-lorazepam, Internal Standard (nitrazepam) and (−)-(*R*)-lorazepam.

The elution order of the lorazepam enantiomers through the OD-R column was established by the simultaneous detection of ultraviolet absorption and circular dichroism (265 nm). Sequential elution of the two enantiomeric fractions was observed with respective retention times of 4 and 5.8 min (Fig. 4). The (+)-(*S*)-lorazepam enantiomer resulted the less retained one, under the adopted experimental conditions, on the basis of the observed positive circular dichroism signal at 265 nm [19]. Kanazawa et al. [15] observed changes in the order of elution of the lorazepam enantiomers when using different chiral columns. The first column was CDBS-453 (β-cyclodextrin immobilized on silica gel) with a mobile phase consisting of a mixture of acetonitrile–NaCL 0.2 M-acetic acid (13:87:1%, v/v/v) that resulted in elution order (−)-*R* and (+)-*S*-lorazepam. Then, a Chiralcel OD column was used with a mobile phase consisting of a mixture of *n*-hexane-2-propanol-ethanol (50:50:10, v/v/v), which resulted in the elution order (+)-(*S*) and (−)-(*R*)-lorazepam.

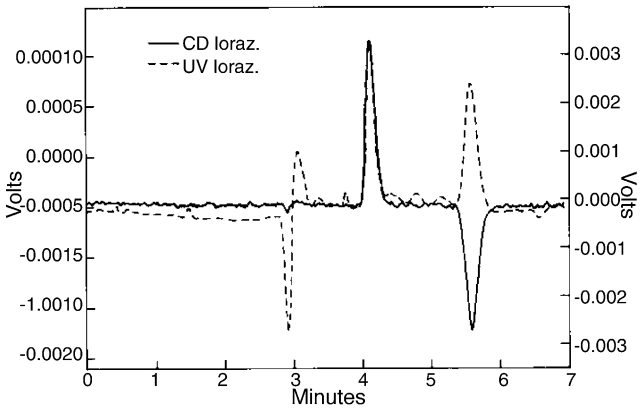


Fig. 4. Racemic lorazepam resolved on a Chiralpak OD-R column. UV and CD monitoring at 265 nm.

Table 1
Confidence limits for the enantioselective analysis of unchanged (without enzymatic hydrolysis) and total lorazepam (after enzymatic hydrolysis) in plasma samples

| | (+)-(*S*) unchanged lorazepam | (−)-(*R*) unchanged lorazepam | (+)-(*S*) total lorazepam | (−)-(*R*) total lorazepam |
|---|---|---|---|---|
| Recovery (%) | | | | |
| 0.2, 2 and 10 ng/ml | 86 | 83 | | |
| 2, 10 and 20 ng/ml | | | 84 | 87 |
| Linearity (ng/ml) | 0.2–20.0 | 0.2–20.0 | 1.0–30.0 | 1.0–30.0 |
| *r* | 0.9962 | 0.9962 | 0.9913 | 0.9951 |
| Quantification limit (ng/ml) | 0.20 | 0.20 | 1.0 | 1.0 |
| Precision (CV%) | 16.4 | 19.3 | 5.6 | 18.0 |
| Accuracy (bias%) | 11.1 | 1.1 | 10.0 | 3.0 |
| Intra-assay precision (CV%) | | | | |
| 0.2, 2 and 10 ng/ml ($n = 10$) | 8.2 | 9.6 | | |
| 1, 2 and 10 ng/ml ($n = 10$) | | | 11.6 | 9.4 |
| Interassay precision (CV%) | | | | |
| 0.2, 2 and 10 ng/ml ($n = 5$) | 8.5 | 7.3 | | |
| 1, 2 and 10 ng/ml ($n = 5$) | | | 6.8 | 9.2 |
| Intra-assay accuracy (bias%) | | | | |
| 0.2, 2 and 10 ng/ml ($n = 10$) | 4.2 | 4.1 | | |
| 1, 2 and 10 ng/ml ($n = 10$) | | | 7.0 | 8.0 |
| Interassay accuracy (bias%) | | | | |
| 0.2, 2 and 10 ng/ml ($n = 5$) | −1.9 | −4.4 | | |
| 1, 2 and 10 ng/ml ($n = 5$) | | | 1.8 | 10.3 |

Lorazepam was extracted from human biological fluids (plasma and urine) in alkaline medium (pH ∼ 10) using *tert*-butyl methyl ether as the extraction solvent. Recovery was more than 80% in plasma and urine and was independent of concentration for both enantiomers of lorazepam. The extraction of lorazepam enantiomers from human plasma described by Kanazawa et al. [14,15] was performed by solid phase extraction after a step of protein precipitation. However, the authors did not mention the recoveries of the enantiomers.

The calibration curves for unchanged lorazepam in plasma and urine, respectively constructed for the intervals of 0.2–20 ng and 0.2–15 ng of each enantiomer/ml, are linear and encompass the concentrations observed in the clinical study of kinetic disposition of racemic lorazepam administered as a single dose (2 mg) (Tables 1 and 2).

Kanazawa et al. [15] reported a quantitation limit of 3 ng of each enantiomer/ml plasma (HPLC with ultraviolet detection) and analysis of a plasma sample from a patient treated orally with racemic lorazepam, and observed 32 ng of (+)-(*S*)-lorazepam/ml plasma and no detection of the antipode (−)-(*R*)-lorazepam. In the present study the quantitation limit was 0.2 ng for each enantiomer/ml plasma or urine (Tables 1 and 2), a fact that permits us to infer that the method is more sensitive than that reported by Kanazawa at al. [15].

The precision and accuracy of the method for the analysis of unchanged lorazepam were evaluated at the concentrations of 0.2, 2 and 10 ng of each enantiomer/ml plasma and at concentrations of 0.2, 1 and 5 ng of each enantiomer/ml urine in intra-assay ($n = 10$) and interassay ($n = 5$) runs, with deviations of less than 15% in relation to the respective nominal values (Tables 1 and 2).

The plasma and urine concentrations of lorazepam–glucuronide were determined in an indirect manner. The concentrations of lorazepam–glucuronide were determined by subtracting the value obtained in the extraction without hydrolysis (unchanged lorazepam) from the value obtained in the extraction preceded by hydrolysis (total lorazepam). Tables 1 and 2 summarize the data obtained in the validation of the method for the analysis of total lorazepam in plasma and urine samples, respectively.

There are no data about stereoselectivity in the kinetic disposition and metabolism of lorazepam. Kanazawa et al. [15] only reported the enantioselective concentrations of unchanged lorazepam in a plasma sample from a patient treated with a single oral dose of racemic lorazepam. The authors stated that the method was sensitive and accurate but did not report data concerning the study of lorazepam racemization under the analytical conditions used.

Pham-Huy et al. [7] observed in vitro racemization of lorazepam and reported the impossibility of applying the enantioselective method to biological material. Lu and Yang [16] and Nichikawa et al. [17] reported that the enantiomers of lorazepam racemize easily in a variety of solvents used as mobile phase mixtures in chiral chromatography. The authors emphasize that acetonitrile is one of the solvents of highest stability of the chiral center of lorazepam. The method developed in the present study using a high percentage of acetonitrile in the mobile phase (80%), despite

Table 2
Confidence limits for the enantioselective analysis of unchanged lorazepam (without enzymatic hydrolysis) and total lorazepam (after enzymatic hydrolysis) in urine samples

| | (+)-(*S*) unchanged Loraz lorazepam | (−)-(*R*) unchanged Loraz lorazepam | (+)-(*S*) total lorazepam | (−)-(*R*) total lorazepam |
|---|---|---|---|---|
| Recovery (%) | | | | |
| 0.2, 1 and 5 ng/ml | 86.7 | 90.6 | | |
| 5, 20 and 100 ng/ml | | | 84.1 | 85.4 |
| Linearity (ng/ml) | 0.2–15.0 | 0.2–15.0 | 10.0–150.0 | 10.0–150.0 |
| *r* | 0.9922 | 0.9922 | 0.9982 | 0.9971 |
| Quantification limit (ng/ml) | 0.20 | 0.20 | 10.0 | 10.0 |
| Precision (CV%) | 12.4 | 15.3 | 15.5 | 6.9 |
| Accuracy (bias%) | 0.5 | −3.5 | 7.5 | 13.0 |
| Intra-assay precision (CV%) | | | | |
| 0.2, 1 and 5 ng/ml ($n = 10$) | 7.4 | 7.8 | | |
| 10, 40 and 100 ng/ml ($n = 10$) | | | 7.7 | 8.3 |
| Interassay precision (CV%) | | | | |
| 0.2, 1 and 5 ng/ml ($n = 5$) | 7.4 | 8.4 | | |
| 10, 40 and 100 ng/ml ($n = 5$) | | | 7.5 | 7.0 |
| Intra-assay accuracy (bias%) | | | | |
| 0.2, 1 and 5 ng/ml ($n = 10$) | −4.9 | −2.6 | | |
| 10, 40 and 100 ng/ml ($n = 10$) | | | −2.9 | 4.0 |
| Interassay accuracy (bias%) | | | | |
| 0.2, 1 and 5 ng/ml ($n = 5$) | −6.2 | −7.8 | | |
| 10, 40 and 100 ng/ml ($n = 5$) | | | −3.3 | 2.7 |

a high sensitivity, specificity and accuracy, demonstrated racemization both in the direct injection of standard solutions of the isolated enantiomers and in the injection of the enantiomers isolated after the extraction procedure (Table 3). Consequently, the pharmacokinetic parameters of unchanged lorazepam and lorazepam–glucuronide were obtained with isomeric mixtures through the sum of the plasma and urinary concentrations of the two isomers; Tables 4 and 5 and Figs. 5 and 6.

Table 3
Racemization test of lorazepam in plasma and urine samples

| | Fraction | (+)-(*S*) peak area % | (−)-(*R*) peak area % |
|---|---|---|---|
| Standard solution | | | |
| 1 mg/ml | 1 | 50.94 | 49.06 |
| | 2 | 50.99 | 49.01 |
| 0.1 mg/ml | 1 | 51.28 | 48.72 |
| | 2 | 49.14 | 50.86 |
| 0.01 mg/ml | 1 | 51.55 | 48.45 |
| | 2 | 52.96 | 47.04 |
| Plasma | | | |
| 1 mg/ml | 1 | 50.97 | 49.03 |
| | 2 | 52.58 | 47.42 |
| 0.1 mg/ml | 1 | 51.12 | 48.88 |
| | 2 | 51.82 | 48.18 |
| 0.01 mg/ml | 1 | 51.62 | 48.38 |
| | 2 | 51.35 | 48.65 |
| Urine | | | |
| 1 mg/ml | 1 | 48.21 | 51.79 |
| | 2 | 50.61 | 49.39 |
| 0.1 mg/ml | 1 | 51.98 | 48.02 |
| | 2 | 58.55 | 41.45 |
| 0.01 mg/ml | 1 | 54.84 | 45.16 |
| | 2 | 49.14 | 50.86 |

Fraction 1 = collected fraction from the HPLC system with a retention time of 4.1 min; fraction 2 = collected fraction from the HPLC system with a retention time of 5.5 min.

Table 4
Kinetic disposition and metabolism of lorazepam as an isomeric mixture in plasma samples from a parturient treated with a single dose (2 mg) of racemic lorazepam

| Parameters | Lorazepam | Lorazepam–glucuronide |
|---|---|---|
| $C_{max}$ (ng/ml) | 11.6 | 12.4 |
| $t_{max}$ (h) | 3.0 | 4.0 |
| $t_{1/2a}$ (h) | 3.5 | 1.2 |
| $K_a$ ($h^{-1}$) | 0.198 | 0.578 |
| $t_{1/2}$ (h) | 11.5 | 16.6 |
| $\beta$ ($h^{-1}$) | 0.060 | 0.042 |
| $AUC^{0-\infty}$ (ng h/ml) | 192.1 | 207.6 |
| CLt/f (ml/min kg) | 2.41 | nd |
| Vd/f (l) | 173.5 | nd |

nd: not determined.

Table 5
Kinetic disposition and metabolism of lorazepam as an isomeric mixture in urine samples from a parturient treated with a single dose (2 mg) of racemic lorazepam

| Parameters | Lorazepam | Lorazepam–glucuronide |
|---|---|---|
| $Ae_{total}$ (μg) | 8.2 | 1036.4 |
| Fel (%) | 0.41 | 51.80 |
| $CL_R$ (ml/min kg) | 0.0099 | 98.32 |
| $t_{1/2}$ (h) | 16.00 | 18.5 |
| kel ($h^{-1}$) | 0.043 | 0.037 |

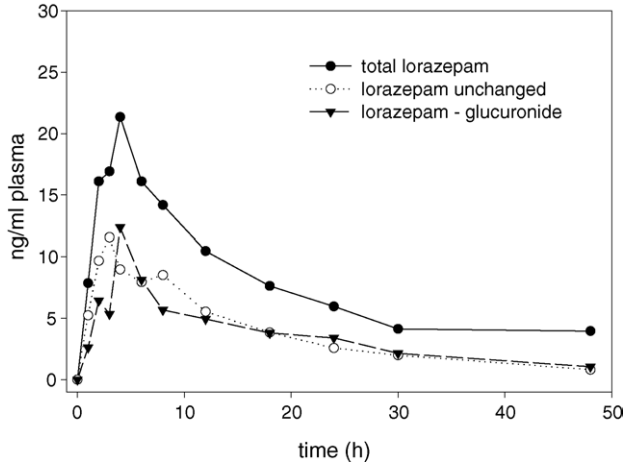


Fig. 5. Plasma concentration vs. time of the isomeric mixture of unchanged lorazepam, total lorazepam and lorazepam–glucuronide. Plasma obtained from a parturient treated with a single dose (2 mg) of racemic lorazepam.

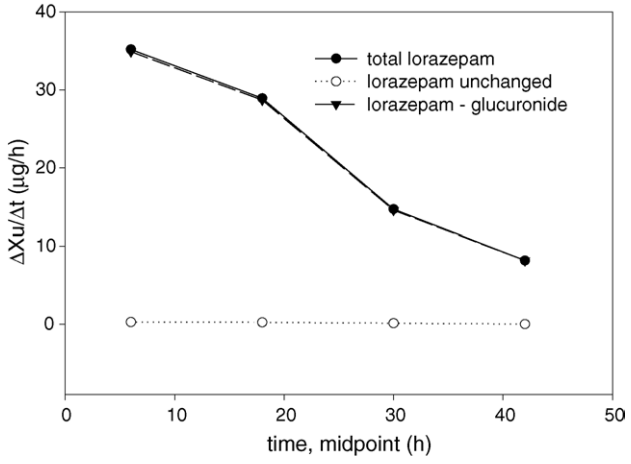


Fig. 6. Urinary excretion rate vs. time of isomeric mixture of unchanged lorazepam, total lorazepam and lorazepam–glucuronide. Urine obtained from a parturient treated with a single dose (2 mg) of racemic lorazepam.

## 5. Conclusion

The in vitro racemization of lorazepam does not permit the determination of the enantiomers in human plasma or urine, and consequently the determination of the implications of stereoselectivity in the kinetic disposition and metabolism. However, the confidence limits of the analytical methods represented by recovery, quantitation limit, linearity, precision and accuracy, obtained with the racemic drug standard proved to be compatible with application to clinical pharmacokinetic studies. A lower quantitation limit of lorazepam was obtained with respect to literature data. The application of the method to biological material required the sum of the concentrations of the unchanged lorazepam and lorazepam–glucuronide isomers, with the possibility of calculating the pharmacokinetic parameters as isomeric mixtures. The method has been applied to study the kinetic disposition and metabolism of lorazepam as an enantiomeric mixture in one parturient.

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