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Simple high-performance liquid chromatographic separation of oxazepam and its diastereoisomeric glucuronides in serum Applications in a pharmacokinetic study in sheep

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Abstract

This paper describes a highly specific and sensitive method for quantifying oxazepam and its diastereoisomeric glucuronides in serum. The method involves sample clean-up by solid-phase extraction on C_{18} cartridge followed by quantitation on a reversed-phase HPLC column. Diazepam is used as internal standard. Extraction recovery from serum proved to be more than 86%. Precision, expressed as C.V., was in the range 1.2–9.5%. The limits of quantification were 40, 400, and 200 nmol/l for oxazepam, *S*-(+)- and *R*-(−)-glucuronides, respectively. This method was applied to the determination of oxazepam and its diastereoisomeric glucuronides in serum collected during a pharmacokinetic study performed in sheep after oral administration of racemic oxazepam. *S*-(+)/*R*-(−) ratios were measured all along the sampling time collection and the pharmacokinetic parameters were determined.

Keywords: Oxazepam; Oxazepam glucuronide; Benzodiazepines

1. Introduction

Oxazepam is the terminal pharmacologically active metabolite of a wide range of benzodiazepines. Its pharmacodynamic and pharmacokinetic properties differ from those of the parent drug. The presence of a chiral centre on the C_3 position of oxazepam gives rise to the existence of two hydroxylated enantiomers, *S*-(+) and *R*-(−), known to have, in aqueous solution, a very short half-life of racemisation (15 min). This phenomenon prevents the utilisation, for pharmacokinetic studies, of common analytical methods like HPLC. Indeed, HPLC separation of enantiomers requires use of tedious and expensive chiral stationary phases.

In a previous paper [1], we overcame such difficulties by taking advantage of the stabilising effect of β-D-glucuronic acid conjugation of oxazepam

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enantiomers. Indeed, *S*-(+) and *R*-(−)-glucuronides are very stable diastereoisomeric metabolites and, unlike the enantiomers, are devoid of racemisation. Moreover, their separations can easily be achieved on common HPLC stationary phases.

Our previously published analytical method [1] allows simultaneous separation of oxazepam and its *S*-(+)- and *R*-(−)-glucuronides in a single chromatographic run. This method was applied to the determination of these three compounds in sheep urine after a simple dilution step of the samples.

In the present paper, we present an HPLC method to quantify these compounds in serum after solid-phase extraction using C_{18} cartridges. This method was validated according to good laboratory practice guidelines [2,3], and was successfully used to determine the pharmacokinetic profiles of oxazepam and its two diastereoisomeric glucuronides in sheep after oral administration of oxazepam.

2. Experimental

2.1. Materials and reagents

Stock solutions of oxazepam and diazepam (internal standard) were prepared in ethanol at concentrations of 25 and 40 μmol/l, respectively.

Double-distilled water, obtained by passing it through a Milli-Q reagent water system (Millipore, Saint Quentin Yvelines, France), was used. Acetonitrile, methanol, ethanol and isopropanol were of Chromasol grade (Merck, Nogent-sur-Marne, France) and used without further purification. Phosphoric acid and sulphuric acid were of analytical grade. Orthophosphoric acid (25%) and sulphuric acid (0.25 *M*) were prepared in distilled water.

The buffer (pH 5.2) used for glucuronide enzymatic hydrolysis consisted of 0.2 *M* sodium acetate in distilled water. *Helix pomatia* juice (100 000 Fishman units/ml, β-glucuronidase activity) was obtained from IBF (Biotechnics, Paris, France).

2.2. Instrumentation and chromatographic conditions

The HPLC system (Hewlett Packard, Evry, France) consisted of the following components: a quaternary gradient pump (HP 1050) equipped with a Rheodyne loading valve (Model 7010) fitted with a 20-μl sample loop, an automatic sample injection system (Model HP 79855A) and a diode array detector (Model HP G1306A). Isocratic separation was achieved according to the previously described method [1] but the pH of the mobile phase must be exactly adjusted to 2.05 to ensure a stable retention time for the internal standard.

2.3. Sample treatment

Urine samples (100 μl) were spiked with internal standard (50 μl) and diluted to 1 ml with distilled water. A 20-μl aliquot of this solution was injected into the column.

The serum sample preparation procedure consists of a solid-phase extraction. C_{18} Bond Elut cartridges (Varian, Les Ulis, France) were previously activated with 2 ml of methanol followed by 30 ml of distilled water. Serum sample (1 ml) was transferred into a 15-ml polypropylene tube; 4 ml of distilled water followed by the internal standard (10 μl) were added. The mixture was adjusted to pH 4.0 (controlled with a pH meter) with 0.25 *M* sulphuric acid and loaded onto the cartridge. The interfering substances of the biological matrix were removed with 10 ml of distilled water and the cartridge was dried under vacuum. Elution was then performed using 5 ml of methanol, then the eluate was evaporated to dryness under a nitrogen stream. The residue was dissolved in 200 μl of methanol and a 20-μl aliquot of this solution was injected into the column.

2.4. Enzymatic hydrolysis

As the glucuronide metabolites are not available, their quantitation in serum and urine involved a specific enzymatic hydrolysis using *Helix pomatia* juice. Acetate buffer was added to the urine samples and the diastereoisomeric glucuronides were hydrolysed into oxazepam using *Helix pomatia* juice (18 h at 37°C) according to our published method [1].

To 1 ml of serum, 10 μl of internal standard, 4 ml of distilled water, 100 μl of acetate buffer and 100 μl of *Helix pomatia* juice were added. The sample was homogenised and the mixture was incubated for 18 h at 37°C. The incubation medium was adjusted

to pH 4.0 then poured onto a C_{18} Bond Elut cartridge using the procedure described above.

2.5. Determination of oxazepam and its diastereoisomeric glucuronides

In a previous study [1], composition of the mobile phase was optimised in order to separate on the same chromatogram *S*-(+)- and *R*-(−)-glucuronides, diazepam (internal standard) and oxazepam with a maximum 30 min elution time. This analysis permits to quantify oxazepam and to compute the *S*-(+)/*R*-(−) ratio from the peak area ratio, considering that absorbances of both glucuronides are equal. Two analyses are necessary: the first one before the hydrolysis to determine oxazepam in the sample and the second one after the hydrolysis to determine the two glucuronides as free oxazepam using the *S*-(+)/*R*-(−) ratio.

2.6. Instrument calibration

Calibration standards were obtained by spiking serum with appropriate concentrations of oxazepam, *S*-(+)- and *R*-(−)-glucuronides. These concentrations are shown in Table 1. The volume added was always smaller than or equal to 2% of total volume of the sample, so that the integrity of the sample was maintained.

These standards were treated concurrently and in the same manner as the unknown samples to be analysed.

2.7. Data analysis

The ratio of the peak area of oxazepam, *S*-(+)- and *R*-(−)-glucuronides, to that of internal standard was used as the assay parameter. Unweighted least-squares linear regression of the peak-area ratios as a function of the theoretical concentrations was applied to each standard curve.

The linearity of the method was confirmed using the classical statistical tests, i.e. comparison of intercept with zero and correlation coefficients.

Concentrations of oxazepam, *S*-(+)- and *R*-(−)-glucuronides, in unknown samples, were determined using the corresponding standard curve.

2.8. Precision and accuracy

Inter-day and intra-day repeatabilities in serum were assessed by performing replicate analysis of spiked serum with oxazepam (0.125, 0.75, 1.75 μmol/l), *S*-(+)-glucuronide (0.66, 3.86, 8.50 μmol/l) and *R*-(−)-glucuronide (0.057, 0.360, 0.835 μmol/l) against calibration curves. The procedure was repeated at different days ($n = 6$) using the same spiked standards to determine inter-day repeatability.

Table 1
Reproducibility of the HPLC analysis

Oxazepam		*S*-(+)-Glucuronide		*R*-(−)-Glucuronide	
Theoretical concentration (μmol/l)	Found concentration (mean±S.D.) (μmol/l)	Theoretical concentration (μmol/l)	Found concentration (mean±S.D.) (μmol/l)	Theoretical concentration (μmol/l)	Found concentration (mean±S.D.) (μmol/l)
0		0		0	
0.25	0.252±0.026 (10.3%)	1.29	1.33±0.047 (3.53%)	0.127	0.133±0.011 (8.27%)
0.50	0.493±0.029 (5.88%)	2.57	2.63±0.078 (2.97%)	0.254	0.261±0.017 (6.51%)
1	0.923±0.036 (3.9%)	5.14	4.99±0.150 (3.0%)	0.508	0.487±0.026 (5.34%)
1.50	1.46±0.040 (2.74%)	7.72	7.64±0.240 (3.14%)	0.762	0.750±0.043 (5.73%)
2.00	2.01±0.060 (2.99%)	10.3	10.4±0.340 (3.27%)	1.06	1.03±0.060 (5.83%)

The values between parentheses are the coefficients of variation.

The intra-day repeatability was determined by treating spiked samples in replicate ($n = 6$) on the same day. The accuracy, expressed as percent deviation of observed concentration from theoretical concentration, with the relative error, was evaluated.

2.9. Recovery

The extraction efficiency (recovery) was determined for all compounds by comparing peak areas from drug-free serum spiked with known amounts of drugs, in the range of concentrations of the calibration curves, assayed accordingly, versus peak areas of the same concentrations prepared in the mobile phase and injected directly into the analytical column. Each sample was determined in duplicate.

The extraction efficiency was also determined for the internal standard.

2.10. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQs were determined from the peak of each compound and the standard deviation of the noise level (S_N). The LOQs were defined as the sample concentrations of oxazepam, *S*-(+)- and *R*-(−)-glucuronides, resulting in a peak area of eight times S_N.

The LODs were defined as the sample concentrations of oxazepam, *S*-(+)- and *R*-(−)-glucuronides, resulting in a peak area of twice S_N.

2.11. Pharmacokinetic study

A 12-month-old, 62 kg weight Texel female sheep received 1000 mg (3487.5 μmol) of racemic oxazepam (Seresta) by the oral route. Urines were collected from a vesical catheter at the following intervals: pre-dose, 0–6, 6–18, 18–24, 24–30, 30–42, 42–48 and 48–72 h. Blood samples (10–20 ml) were collected in polypropylene tubes through an indwelling catheter inserted into a jugular vein immediately before and 3, 12, 21, 27, and 36 h after administration. Blood was allowed to clot at room temperature and serum was immediately separated by centrifugation.

Serum and urine samples were frozen immediately after collection and stored at −20°C until assay.

3. Results

3.1. Retention times

The observed retention times were 12.2 and 13.3 min for *R*-(−)- and *S*-(+)-glucuronides, respectively, 21.7 min for diazepam and 27.9 min for oxazepam. The capacity factors were 9.2, 10.0, 17.0 and 22.2, respectively (Fig. 1) The resolution between the two diastereoisomeric glucuronides was 5.5.

3.2. Specificity

Representative chromatograms are shown in Fig. 1. There were no significant interfering peaks in the control serum at the retention times of the respective analytes.

3.3. Linearity

In serum, the peak-area ratios of oxazepam, *S*-(+)- and *R*-(−)-glucuronides, to the internal standard varied linearly with concentration over the range used (0.04–14 μmol/l, 0.4–10 μmol/l and 0.20–10 μmol/l, respectively). The correlation coefficients (r) for calibration curves were equal to or better than 0.9978. Intra-assay reproducibility was determined for calibration curves prepared in replicate on the same day ($n = 6$) using the same stock solutions. The intra-day average slope of the fitted straight lines, the mean correlation coefficient and the mean intercept are presented in Table 2. Table 2 also contains these data obtained on different days.

For each point of calibration, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations) and the percent relative standard deviations (C.V., %) were computed. Results are presented in Table 1. The small percentage differences between nominal and found concentrations of the standards in the standard curves confirmed that the assays were linear over the concentration ranges investigated.

3.4. Precision and accuracy

For concentrations of calibration standards, the precision around the mean values did not exceed

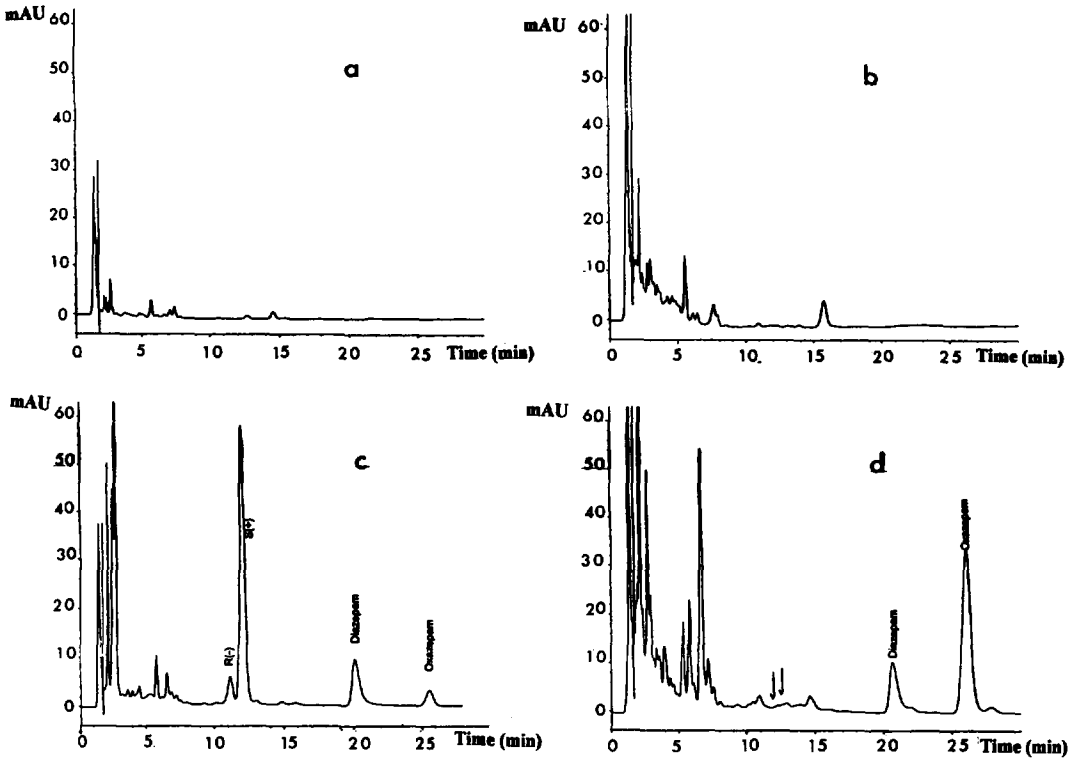

Fig. 1. Chromatogram of blank serum without hydrolysis (a); chromatogram of blank serum after hydrolysis (b); chromatogram of serum containing 1.0 μmol/l oxazepam, 5.14 μmol/l *S*-(+) enantiomer and 0.508 μmol/l *R*-(−) enantiomer (c); chromatogram of the same serum after hydrolysis (d). Chromatographic conditions: column: LiChrospher 100 RP 18 (250 × 4.6 mm I.D., 5 μm); mobile phase: 25% orthophosphoric acid, acetonitrile, 2-propanol (12:180:75, v/v/v) diluted to 1000 ml with distilled water; detection: 230 nm.

10.3, 3.5, and 8.3% coefficient of variation for oxazepam, *S*-(+)- and *R*-(−)-glucuronides, respectively (Table 1).

The intra-day and inter-day precision, and the accuracy of the method are shown in Table 3.

3.5. Recovery

In serum, the recovery varied from 93 to 105% for oxazepam, from 86 to 101% for *S*-(+)-glucuronide and from 88 to 92% for *R*-(−)-glucuronide. The

Table 2
Intra- and inter-assay linearity

	Oxazepam	*S*-(+)-Glucuronide	*R*-(−)-Glucuronide
Intra-assay linearity			
Slope (mean±S.D.)	0.333±0.006	0.73±0.015	0.68±0.038
Intercept (mean±S.D.)	−0.0032±0.009	−0.04±0.052	0.0003±0.014
r (mean±S.D.)	0.9993±0.0007	0.9994±0.0008	0.9975±0.0036
Inter-assay linearity			
Slope (mean±S.D.)	0.361±0.0094	0.78±0.027	0.65±0.036
Intercept (mean±S.D.)	−0.013±0.012	−0.01±0.03	0.001±0.0045
r (mean±S.D.)	0.9974±0.002	0.9992±0.0004	0.998±0.001

r: Coefficient of the linear regression analysis.

Table 3
Precision and accuracy of the HPLC method

	Oxazepam	S-(+)-Glucuronide	R-(−)-Glucuronide
Intra-day (n = 6)			
Low concentration	0.125±0.0076	0.670±0.0080	0.057±0.0018
	(6.1%)	(1.2%)	(3.2%)
Medium concentration	0.742±0.030	3.76±0.0865	0.349±0.0105
	(4.0%)	(2.3%)	(3.0%)
High concentration	1.50±0.143	8.37±0.435	0.809±0.059
	(9.5%)	(5.2%)	(7.3%)
Inter-day (n = 6)			
Low concentration	0.125±0.012	0.703±0.0296	0.054±0.0034
	(9.6%)	(4.2%)	(6.3%)
Medium concentration	0.751±0.018	3.94±0.298	0.383±0.023
	(2.4%)	(7.6%)	(6.0%)
High concentration	1.58±0.054	8.71±0.154	0.864±0.062
	(3.4%)	(1.76%)	(7.2%)

mean recovery of the internal standard averaged 94.1% ($n = 34$)

3.6. Limit of detection and limit of quantification

The LODs and LOQs determined as previously defined were 10 and 40 nmol/l for oxazepam, 100 and 400 nmol/l for S-(+)-glucuronide and 50 and 200 nmol/l for R-(−)-glucuronide. At these levels, precision is always below 8.5%.

3.7. Pharmacokinetic study

Data for each sampling time after oral administration of oxazepam are given in Table 4. Serum concentrations and urinary excretion are approximately ten times higher for the S-(+)-glucuronide than for the R-(−)-isomer. The S-(+)/R-(−) ratios averaged 15.6 in serum and 9.5 in urine.

For the S-(+)- and R-(−)-glucuronide metabolites, pharmacokinetic parameters were evaluated, using a one-compartment open model, after a simultaneous fitting of the serum and urine data, which allowed a better estimation of renal excretion kinetic properties [4,5]. The serum concentration-versus-time profiles with urine data are shown in Fig. 2 and Fig. 3 for the S-(+)- and R-(−)-glucuronides, respectively. The pharmacokinetic parameters are given in Table 5.

Unconjugated oxazepam was detected in serum but not in urine. The serum concentration–time

Table 4
Concentrations of oxazepam and of its glucuronide metabolites in serum and urine

Time intervals (h)	Volume (ml)	S-(+)-Glucuronide (μmol/l)		R-(−)-Glucuronide (μmol/l)		S-(+)/R-(−) ratio		Oxazepam (μmol/l)	
		Urine	Serum[a]	Urine	Serum[a]	Urine	Serum[a]	Urine	Serum[a]
0–6	665	103	22.5	13.1	2.5	7.9	9	<LOQ	16
6–18	1825	128.6	22.2	12.7	1.7	10.1	13	<LOQ	16
18–24	645	118.9	18.7	11.4	1.1	10.4	17	<LOQ	7.5
24–30	460	101.4	12.3	9.7	0.7	10.4	17.6	<LOQ	4.7
30–42	655	65.3	6.4	6.4	0.3	10.2	21.3	<LOQ	2.1
42–48	510	29	<LOQ	3.7	<LOQ	7.8	–	<LOQ	<LOQ
48–72	2000	0.72	<LOQ	<LOQ	<LOQ	–	–	<LOQ	<LOQ

[a]Serum drug concentrations at the midpoint of the drug-excretion intervals.
LOQ, limit of quantification.

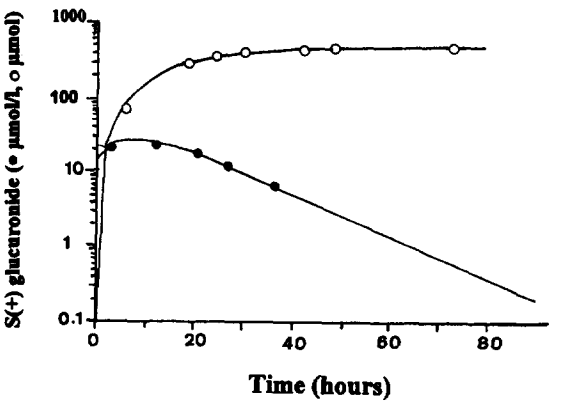

Fig. 2. Simultaneous fit of serum and urine data for the *S*-(+)-glucuronide. ●, Plasma; ○, urine.

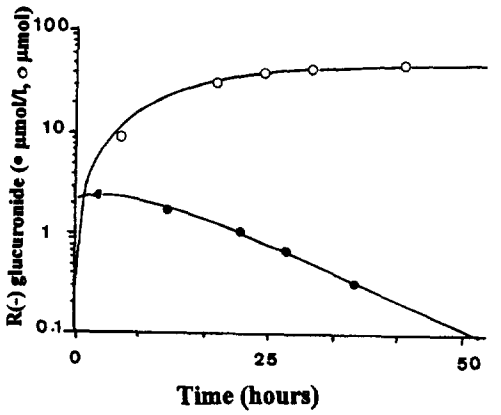

Fig. 3. Simultaneous fit of serum and urine data for the *R*-(−)-glucuronide. ●, Plasma; ○, urine.

curve was modelled using a one-compartment open model with first-order input and output rates.

Pharmacokinetic parameters were estimated by use of the SIPHAR software [4].

4. Discussion and conclusion

Most of published methods to quantify oxazepam and its glucuronides in body fluids involved two to three chromatographic runs [6] or the use of ion-exchange chromatography [7,8] and tedious extraction and purification steps. As described in a previous paper for urine [1], the present HPLC method allows separation and quantitation of oxazepam and its diastereoisomeric glucuronides in a single chromatographic step. These compounds were directly quantified in urine after a simple dilution of the samples. In the present study, they were extracted from serum using solid-phase C_{18} cartridges. The separation between oxazepam, its glucuronides and endogenous substances was satisfactory provided that the pH was exactly controlled (i.e. the retention time of diazepam (IS) appeared very dependent on the pH value). This method has a good reproducibility, accuracy and precision. The limit of quantification was found to be 40, 400 and 200 nmol/l for oxazepam, *S*-(+)- and *R*-(−)-glucuronides, respectively, thus allowing its use in clinical pharmacokinetic studies after oral administration.

Total glucuronide excretion is about three [1] to six times (this study) lower than in humans [6,8], probably due to intense biliary excretion in sheep. As previously reported [1], it is thus confirmed that

Table 5
Pharmacokinetic parameters of oxazepam and its glucuronide metabolites in sheep

	Oxazepam	*S*-(+)-Glucuronide	*R*-(−)-Glucuronide
A_u (µmol)	–	514.4	49.5
A_u (%)	–	14.8	1.42
AUC (µmol/l/h)	365.6	741.6	53.3
Elimination half-life (h)	8.13	10.2	8.41
MRT (h)	15.5	20.0	15.5
Cl_R (l/h)	–	0.70	0.91

A_u, cumulative amount in urine; AUC, area under the curve computed by linear trapezoidal rule; MRT, mean residence time, determined by the ratio of AUMC to AUC, where AUMC is defined as the area under the first moment curve [9]; Cl_R, renal clearance estimated from the slope of the plot of excretion rate versus serum drug concentration at the midpoint of the drug excretion intervals.

S-(+)/*R*-(−) ratios are higher in sheep than in humans [6,8]. In both species, stereoselective glucuronidation is in favour of the *S*-(+)-isomer. Two levels of stereoselectivity can be considered: the hepatic glucuronidation and the microbial glucuronide hydrolysis by the digestive flora during the entero-hepatic cycle. The observed inter-species variations of *R*-(+)/*S*-(−) ratios could find an explanation in one or both of these transformations. The density of the microbial flora and the intensity of the entero-hepatic cycle, which varies between species, are important factors to consider. However, these results must be considered with caution due to the small number of individuals both in man and animal.

Renal clearance of glucuronides (*S*-(+): 0.7 l/h; *R*-(−): 0.9 l/h) are lower than creatinine clearance (7.2 l/h), thus indicating an intense tubular reabsorption in sheep.

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