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SEPARATION, ISOLATION AND IDENTIFICATION OF OPTICAL ISOMERS OF 1,4-BENZODIAZEPINE GLUCURONIDES FROM BIOLOGICAL FLUIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of four separate 1,4-benzodiazepine glucuronides in urine, plasma and bile is presented. We succeeded not only in determining the single glucuronides but also in separating the enantiomers (optical isomers) of the 1,4-benzodiazepine glucuronides. The optical isomers of the glucuronides of oxazepam and cinolazepam and of two other glucuronides of benzodiazepine metabolites could be well separated. The ratio of the isomers could be evaluated. An octadecyl reversed phase was used with a mobile phase of acetonitrile and 0.01 *M* orthophosphoric acid. After the initial separation, the isomers were fractionated by HPLC. After treatment with β-glucuronidase to yield the aglycone, the separated fractions were hydrolysed to the corresponding benzophenones whose identity was confirmed by HPLC. Gas chromatography and gas chromatography—mass spectrometry demonstrated that the separated glucuronides corresponded to the enantiomeric benzodiazepines. Human urine and plasma as well as rabbit urine, plasma and bile were examined.

INTRODUCTION

The determination of glucuronides has been extensively described in the literature [1—7]. Glucuronides of drugs [1—3] as well as glucuronides of steroids [4, 5] have been determined. Baker [6] gave indices to some drugs and their glucuronides depending on the capacity factors of 2-ketoalkanes. This method can be helpful for finding the unknown glucuronide in the chromatogram of a known drug.

Hydrolysis of glucuronides under either acidic or enzymatic conditions does not always lead to the desired result. Goenechea et al. [7] found that under the usual acidic conditions the hydrolysis of codeine glucuronide was either incomplete or that codeine was destroyed to the extent of 8—83%.

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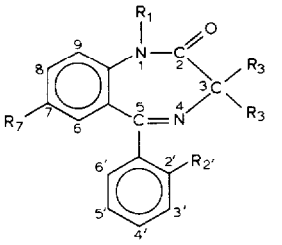
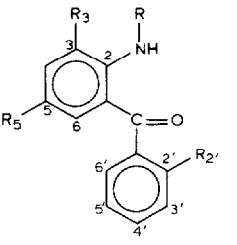


Ring nomenclature for 1,4-benzodiazepines



Benzophenones (all structures verified by MS)

		R_1	$R_{2'}$	R_3	R_7	R	$R_{2'}$	R_3	R_5
Oxazepam	I	H	H	H,OH	Cl	H	H	H	Cl
Cinolazepam	II	CH_2CH_2CN	H	H,OH	Cl	CH_2CH_2CN	H	H	Cl
N-1-Desalkyl--flurazepam	III	H	F	H,OH	Cl	H	F	H	Cl
SAS 643 [17]	IV	CH_2CH_2OH	F	H,OH	Cl	CH_2CH_2OH	F	H	Cl

Fig. 1. Structural formulae of the benzodiazepines and the corresponding benzophenones.

Oxazepam (Fig. 1, I) and cinolazepam (Fig. 1, II) and several other benzodiazepines too have an optically active center. These drugs also have a hydroxyl group in position 3 for glucuronidation. Glucuronidation takes place in vivo and a similar procedure involving chiral reagents was used by some authors [8—11] for the determination of enantiomeric drugs through precolumn derivatization. The separation of the diastereoisomeric glucuronides of oxazepam without chemical derivatization, involving preparative-scale ion-exchange chromatography for isolation and stereoselective enzymatic hydrolysis to yield the pure isomers of oxazepam, was first described by Ruelius et al. [12].

In this study we describe a procedure for the separation and identification of optical isomers of 1,4-benzodiazepines using a mobile phase in which the glucuronides had a capacity factor of 5—10. We analyzed both human plasma and urine, and rabbit plasma, urine and bile after oral administration of I or II.

MATERIALS AND CHEMICALS

The high-performance liquid chromatographic (HPLC) system was either a Spectra Physics 8000 system or was built up by an LDC Constametric III pump in connection with a Rheodyne 7126 injector. Detection was performed by an LDC Spectromonitor III at 230 nm or 380 nm. We used loops of 10, 20, 50 and 500 μl. The integration and plotting of the chromatograms were done by the SP 8000 or by a Shimadzu C-R1A integrator. We usually used Knauer columns (250 × 4 mm or 40 × 4 mm) filled normally with LiChrosorb RP-18 (7 μm) or Polygosil C 18 (7.5 μm). We also used LiChrosorb RP-18 cartridges (125 × 4 mm). For the gas chromatographic (GC) analysis of the benzophenones we used a Dani 6800 gas chromatograph with an SE 52 glass capillary column (25 m × 0.32 mm I.D.), and a Varian CH7 for the mass spectrometric (MS) analysis.

Additional reagents used were: buffer pH 7 (1.82 g of $Na_2HPO_4 \cdot 2H_2O$ + 0.88 g of KH_2PO_4 per litre of water); LiChrosorb RP-18, RP-8, NH_2, each 7 μm

particle size; RP-18 cartridges; acetonitrile p.a.; chloroform p.a.; phosphate buffer (Merck, Darmstadt, F.R.G.); methanol p.a.; extraction columns ODS, 1 ml (Baker, Deventer, The Netherlands); PRP-1 (10 μm particle size) column 150 × 4.1 mm (Hamilton, Bonaduz, Switzerland); Partisil 10 ODS-2 (Whatman, Maidstone, U.K.); HPX 87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Vienna, Austria); Polygosil 60-7 C 18 (Macherey-Nagel, Düren, F.R.G.); β-glucuronidase from *Helix pomatia* and *Escherichia coli* (Boehringer, Mannheim, F.R.G.); β-glucuronidase from marine mollusc and beef liver (P-L Biochemicals, Milwaukee, WI, U.S.A.).

EXPERIMENTAL

Separation of the optical isomeric benzodiazepine glucuronides

For separation we usually used LiChrosorb RP-18 or Polygosil C_{18} packings with ultraviolet (UV) detection at 230 nm. To achieve good separation from other endogenous material it was necessary to keep the capacity factor of the glucuronides greater than 5.

The composition of the mobile phase for the separation of I, II, III and IV glucuronides was acetonitrile—0.01 *M* orthophosphoric acid (20:80). The mobile phase was changed to acetonitrile—0.01 *M* orthophosphoric acid (30:70) for the separation of the optical isomeric glucuronides together with the corresponding free benzodiazepines of I and II.

Identification of the optical isomeric benzodiazepine glucuronides

Although we tried to obtain a glucuronide through a chemical derivatization step we were not successful in obtaining a reference standard.

Identification of the optical isomeric benzodiazepine glucuronides was performed according the following procedure. Several 500-μl aliquots of human or animal urine were injected onto the HPLC system and the optical isomeric glucuronides were separated by fractionation. The single fractions were checked by HPLC. After evaporating the acetonitrile, the benzophenones of the benzodiazepine glucuronides were made by treating the samples with 1 *M* hydrochloric acid for 30 min at 100°C. The resulting peaks are identical with the corresponding benzophenones by HPLC; showed from the fractions only one benzophenone was found, verifying the separation of the optical isomers.

The same samples were made alkaline and extracted with chloroform. After preparation and evaporation of the solvent the residue was redissolved in a small volume of ethanol and 2 μl were injected in the capillary gas chromatograph. As with HPLC the corresponding benzophenones are identical.

In addition, mass spectra of the above samples also confirmed the presence of the corresponding benzophenones of the two optical isomeric benzodiazepine glucuronides.

The fractions were also examined after treatment with β-glucuronidase on a HPX 87H column with a mobile phase of 0.005 *M* sulphuric acid and UV detection at 195 nm. Glucuronic acid was found only after treatment with β-glucuronidase. We used a LiChrosorb RP-18 (7 μm) column with a mobile phase of methanol—buffer, pH 7 (70:30) to separate the benzophenones with UV detection at 380 nm.

Routine determination of glucuronides

Determination of I and II as their optical isomeric glucuronides was performed as follows. Usually urine was injected following centrifugation without further purification. Plasma and bile were pretreated either on a Baker extraction column C_{18} or on-line on a precolumn as described by Roth et al. [13] or Voelter et al. [14]. Detection limits for benzodiazepine glucuronides were better than 50 ng/ml in urine, plasma or bile.

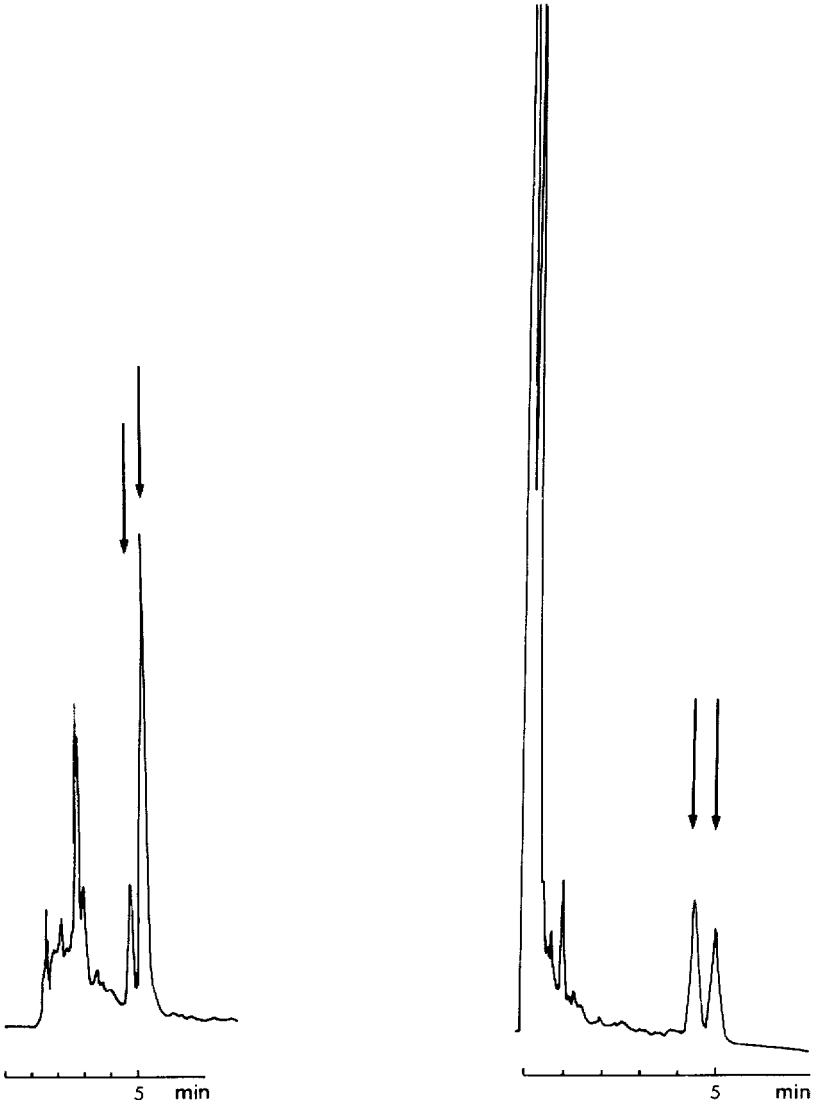


Fig. 2. Separation (Polygosil ODS) of optical isomeric 1,4-benzodiazepine glucuronides in human urine 2 h after oral administration of 20 mg of I. The arrows indicate the two glucuronides.

Fig. 3. Separation (LiChrosorb RP-18) of optical isomeric 1,4-benzodiazepine glucuronides in human urine 4 h after oral administration of 30 mg of II. The arrows indicate the two glucuronides.

For quantitation of I or II glucuronides some of the samples were determined as glucuronides and after hydrolysis with β-glucuronidase as free benzodiazepines. Since sulphate conjugates are not hydrolyzed by β-glucuronidase, showing also a different chromatographic behaviour compared to the glucuronides, any sulphate conjugate present can be excluded. We also checked the efficiency of the hydrolysis by determining the residual glucuronides. Hydrolysis was usually complete in all samples. A conversion factor for the intact glucuronides to aglycone was determined for quantitative analysis. Detection was at 230 nm.

RESULTS

Fig. 2 shows the separation of I glucuronides in human urine after oral administration of 20 mg of I. The separation of II glucuronides in human urine after oral administration of 30 mg of II is shown in Fig. 3. In Fig. 4 the

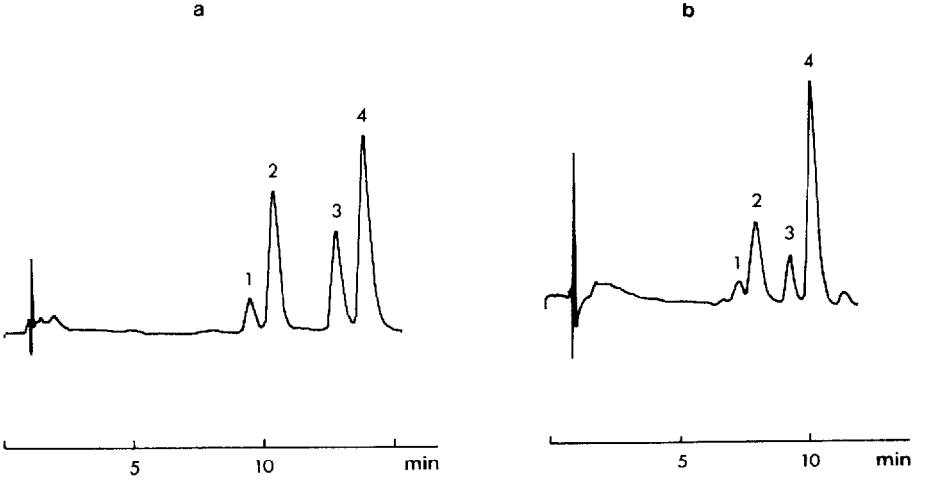


Fig. 4. Separation (Polygosil ODS) of optical isomeric glucuronides isolated from urine. Peaks: (a) 1 and 2 = I glucuronides; 3 and 4 = II glucuronides. (b) 1 and 2 = IV glucuronides; 3 and 4 = III glucuronides.

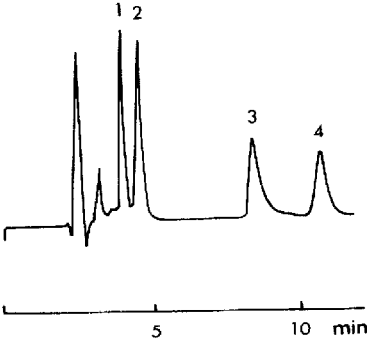


Fig. 5. Chromatogram of the unseparated glucuronides, extracted from human urine, together with the corresponding free benzodiazepines. Peaks: 1 = I glucuronides, 2 = II glucuronides, 3 = I, 4 = II.

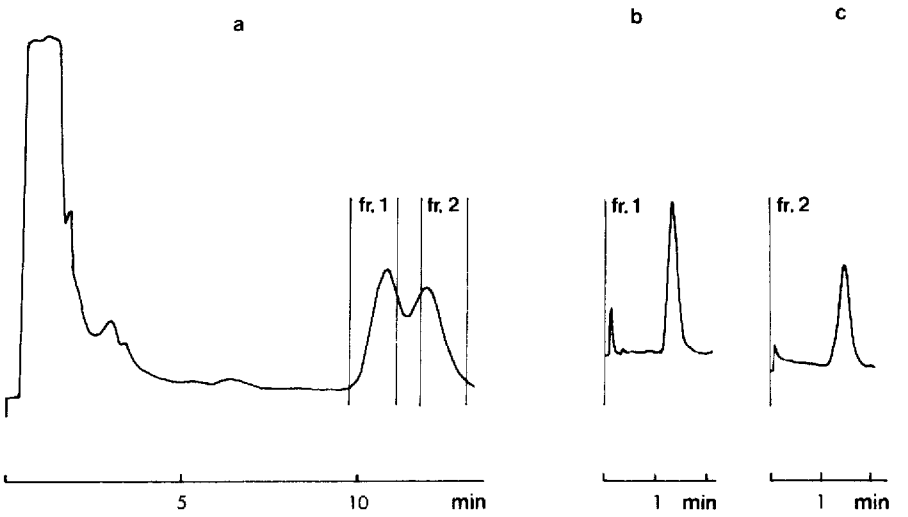


Fig. 6. (a) Chromatogram of the separated II glucuronides indicating which parts of the peaks were fractionated. (b, c) Separate chromatograms of the isolated fractions of the two diastereoisomeric II glucuronides (retention times: fr. 1, 1.32 min; fr. 2, 1.44 min).

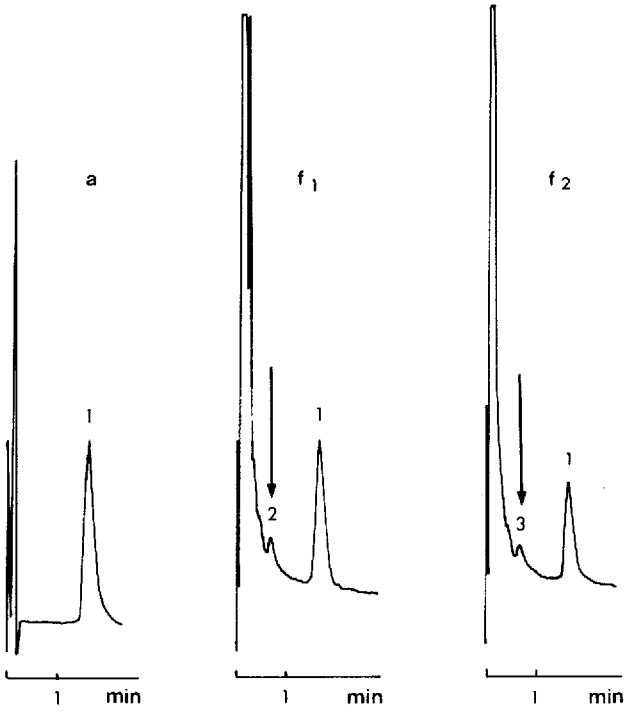


Fig. 7. Chromatograms of the two fractions of II glucuronides (f_1 and f_2) after incomplete hydrolysis with β-glucuronidase as well as the corresponding free benzodiazepine (a). Peaks: 1 = free benzodiazepine (II); 2 = II glucuronide fraction 1; 3 = II glucuronide fraction 2.

chromatographic separation of a mixture of I, II, III and IV glucuronides can be seen. Fig. 5 shows a chromatogram where the optical isomeric glucuronides are not separated together with the corresponding benzodiazepines of I and II.

Separation of II glucuronides before and after fractionation can be seen in Fig. 6. Chromatograms of the fractions of II glucuronides after hydrolysis with β-glucuronidase as well as the reference benzodiazepine are shown in Fig. 7. Results of the mass spectra of the II benzophenones of the two fractions are shown in Table I.

The data suggest that the glucuronides of these benzodiazepines (I, II, III, IV) are mixtures of the enantiomeric glucuronides of these drugs.

TABLE I

m/e VALUES OF THE BENZOPHENONE OF II (BP) AND THE BENZOPHENONES AFTER ACIDIC HYDROLYSIS OF THE TWO FRACTIONS OF THE II GLUCURONIDES (f_1, f_2)

The relative intensity of the mass peaks are indicated by +, ++, +++.

m/e	BP	f_1	f_2
302	+	+	+
262	+++	++	+++
166	+++	++	+++
109	+++	+	+++

DISCUSSION

There are only a few papers published on the determination of glucuronides in vivo (e.g. refs. 1—5). It seems that GC is more difficult to perform than HPLC because quantitative derivatization of the polyhydroxyl groups of the glucuronic acid needs special precautions.

1,4-Benzodiazepines with a hydroxyl group on C-3 undergo rapid glucuronidation. We developed an HPLC method for the direct determination of benzodiazepine glucuronides to circumvent the need for enzymatic hydrolysis. β-Glucuronidase from different sources yielded different results mainly due to the efficiency of hydrolysis of glucuronides. β-Glucuronidase from *Escherichia coli* hydrolysed only 1—5% of the benzodiazepine glucuronides. However, β-glucuronidase from *Helix pomatia*, marine mollusc or beef liver yielded complete hydrolysis. Hydrolysis in acid yields the corresponding benzophenone instead of the parent benzodiazepine. To exclude the formation of benzophenones from other unresolved metabolites, e.g. quinazolines/quinazolone conjugates we compared the retention time of both fractions of the separated glucuronides after enzymatic hydrolysis with the retention time of the aglycone (Fig. 7). Similar results were obtained on different columns.

Benzodiazepine glucuronides under usual separation conditions gave two poorly resolved peaks. Changing the mobile phase such that the glucuronides had a capacity factor of greater than 5, yielded well separated optical isomeric glucuronide peaks. Also at a capacity factor (k') of lower than 5 the ratio of the two diastereoisomeric compounds remains stable; additionally, analysis was performed on columns with different lengths (4, 12.5 and 25 cm; 4—4.6 mm I.D.) with no change in the ratio.

We found that PRP-1 (copolymer of styrene and divinylbenzene) and Li-

Chrosorb NH_2 could not separate the optical isomeric glucuronides, while Partisil ODS 2 gave poorly resolved peaks. However, LiChrosorb RP-8, LiChrosorb RP-18, Polygosil ODS and Sperisorb ODS could be used for complete separation.

To give some explanation of how the separation is effected, we assume that different parts of the molecule, caused by the different steric structure, are getting in contact with the stationary phase. Blaschke and Markgraf [15] showed that the pure enantiomers of I were difficult to handle because of their tendency to racemize. For this reason it was impossible to isolate pure enantiomeric benzodiazepines to verify our results.

Glucuronidation is an important pathway in the biotransformation of 1,4-benzodiazepines and their metabolites [16]. Therefore the advantage of determining the intact glucuronides is obvious. Oxazepam glucuronide is a common excretion product of several marketed 1,4-benzodiazepines, viz. diazepam, medazepam, chlorazepate, prazepam and ketazolam [16].

The proposed method or that of Baker [6] can be used for analyzing unknown benzodiazepine glucuronides. When using Baker's [6] method one has to take in account that there are different k' ratios for each benzodiazepine and its glucuronide under different pH conditions.

Screening of the drugs and their metabolites would be much easier and is independent on the extent of hydrolysis. The only non-benzodiazepine glucuronide we analyzed was codeine glucuronide, and as expected we found only a single peak due to the fact that codeine has no optically active carbon atom.

An interesting observation is the varying degree of optical isomeric benzodiazepine glucuronides in urine. Differences in the concentration of enantiomeric drugs in plasma are known to occur with β-blocking drugs such as propranolol [8], alprenolol and metoprolol [9], or also with naproxen [11].

Human plasma and urine showed a I glucuronide peak area ratio of 19:81 for the optical isomeric glucuronides. Similarly, III glucuronide showed a ratio of 50:50 in human urine, while IV glucuronide showed a ratio of 30:70 in rabbit urine, although presumably a racemic mixture of these benzodiazepines was initially administered. The urine of one man showed a constant ratio during 48 h for II glucuronide of 54.2:45.8 ($\pm$ 3.0; n = 8). In 48-h pooled urines of five men we found II glucuronide ratios of 53.6:46.4 ($\pm$ 3.0). In rabbit urine found in the bladder 24 h after II glucuronide, ratios of 41.1:58.9 ($\pm$ 5.0; n = 9) were noted.

ACKNOWLEDGEMENTS

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