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Short Communication

Identification and quantitation of intact diastereoisomeric benzodiazepine glucuronides in biological samples by high-performance liquid chromatography

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ABSTRACT

A rapid and simple high-performance liquid chromatographic method for the simultaneous detection of intact glucuronides of different benzodiazepines is described. Separation of the diastereomers of the following benzodiazepine glucuronides can be achieved on a reversed-phase column (octadecyl or select B): oxazepam, nordiazepam, temazepam, lorazepam and 3-hydroxyprazepam. If the sample contains both *S*-lorazepam and *R*-temazepam glucuronides, or both *S*-temazepam and nordiazepam glucuronides, further separation on a β-cyclodextrin column is required. The detection limit ranges between 5 and 10 ng of glucuronides per ml of plasma or urine, respectively.

INTRODUCTION

Owing to their relatively low toxicity and few side-effects, benzodiazepines nowadays are among the most "popular" drugs. Depending on the molecular structure of the benzodiazepine in question, dealkylation and/or hydroxylation at positions N-1 and C-3, respectively, is followed by glucuronidation in one or both of these positions. As a hydroxy group in the C-3 position leads to an asymmetric carbon atom, the corresponding benzodiazepine glucuronides exist as pairs of diastereomers.

Numerous analytical methods for the routine analysis of benzodiazepines in biological samples have already been published [1,2]. The drawback of most of these procedures is that the corresponding glucuronides have to be hydrolysed prior to analysis [3–6]. Although enzymic hydrolysis may yield inexact results owing to inhibitors in the sample, acid hydrolysis often produces identical products from different benzodiazepines. For instance, diazepam and temazepam both lead to the same benzophenone, *i.e.* 2-methylamino-5-chlorobenzophenone.

As yet, little attention has been paid to the analysis of intact benzodiazepine glucuronides. Although the detection of oxazepam glucuronides has been described repeatedly [7–11], routine analysis of benzodiazepine glucuronides in

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biological samples still presents a major problem. It has thus been our aim to develop a suitable high-performance liquid chromatographic (HPLC) method.

EXPERIMENTAL

Chemicals

Oxazepam (Adumbran) was purchased from Dr. Karl Thomae (Biberach an der Riss, Germany), nordiazepam from Hoffmann-La Roche (Grenzach-Wyhlen, Germany), prazepam (Demetrin) from Gödecke (Berlin, Germany), and temazepam (Remestan) from Wyeth-Pharma (Münster/Westfalen, Germany).

Standard biochemicals were obtained from Merck (Darmstadt, Germany) in pro analysi quality. Water for the HPLC was provided by J.T. Baker (Deventer, Netherlands), β-glucuronidase from *Escherichia coli* (EC 3.2.1.31) and β-glucuronidase/arylsulphatase (EC 3.2.1.31/EC 3.1.6.1) by Boehringer Mannheim (Mannheim, Germany).

Chromatographic system

Extraction of the benzodiazepine glucuronides was performed on a Baker-10 extraction system (J. T. Baker, Gross-Gerau, Germany) using phenyl and quaternary amine columns (3 ml, 500 mg).

For HPLC analysis the instrument HP 1084 B from Hewlett-Packard (Böblingen, Germany) was used. Most separations were carried out on a 60 RP-select B cartridge (5 μm, 250 mm × 4 mm I.D.) from Merck, held in the corresponding cartridge holder (Hibar LiChroCART manu-fix). The two other columns used were a LiChrospher 100 RP 8 column (8–5 μm, 200 mm × 4 mm I.D.) from Klaus Ziemer (Mannheim, Germany) and a Grom-Chiral-Beta-CD column from Grom (Herrenberg, Kayh, Germany).

The mobile phase, sodium phosphate buffer (0.01 *M*, pH 7)–acetonitrile, was delivered at a flow-rate of 1 ml/min. From 0 to 15 min the concentration of acetonitrile was increased linearly from 12.5 to 16.5%. Over the next 8.5 min the concentration was further increased to 20%, maintained at 20% for 3 min, then again increased linearly to 25% over the next minute and maintained there for another 15 min (Fig. 1).

When a Select B cartridge was used instead of the RP 8 column, for the first 15 min the gradient was as described above, then the acetonitrile concentration was increased to 20% over the next 8.5 min and maintained there for 15 min (Fig. 2).

Separation was achieved at 40°C. Peaks were detected at 230 nm, and the reference wavelength was set at 430 nm.

Identification and quantitation of the R- and S-benzodiazepine glucuronides

The different benzodiazepine glucuronides were isolated from urine by reversed-phase HPLC on a Select B or RP 8 column as described above. The purity of the isolated glucuronides was confirmed by chromatography on a second HPLC column (Beta-CD).

For identification and quantitation, the purified benzodiazepine glucuronides were incubated with 20 μl of β-glucuronidase/arylsulphatase from *Helix pomatia* in 2 ml of 0.2 *M* sodium acetate buffer (pH 4.6) for 4 h at 37°C for complete hydrolysis. As a control, 50 μl of the incubate were mixed with 50 μl of acetonitrile, centrifuged and analysed by HPLC. After 4 h of incubation the benzodiazepine glucuronide peaks had completely disappeared.

Prior to extraction with diethyl ether, 1 ml of water and 1 ml of saturated sodium carbonate–bicarbonate solution (31:100) were added to the samples. Identification of the extracted free benzodiazepines by HPLC was performed according to Gill *et al.* [12]. For quantitation, calibration curves of the pure benzodiazepines were used.

In this way, stock solutions of the different benzodiazepine glucuronides for the direct calibration of glucuronide concentrations were obtained.

As described by Ruelius *et al.* [13], β-glucuronidase from *E. coli* hydrolyses the *S*-diastereoisomer of oxazepam glucuronide 400 times faster than the *R*-diastereoisomer. This stereoselectivity was used to identify the *R*- and *S*-diastereoisomers of the isolated benzodiazepine glucuro-

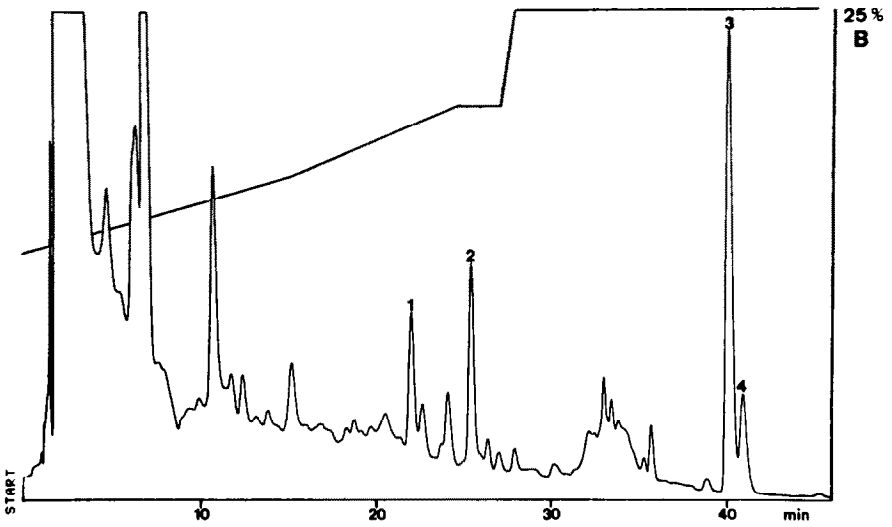

Fig. 1. HPLC of a urine sample after uptake of 20 mg of prazepam. The urine was collected over a period of 7 h and pretreated by SPE. Column, LiChrospher 100 RP 8 (5 μm, 200 mm × 4 mm I.D.); eluent A, 0.01 *M* sodium phosphate buffer (pH 7); eluent B, acetonitrile; gradient, 0 min 12.5% B, 15 min 16.5% B, 25 min 20% B, 28 min 20% B, 29 min 25% B; flow-rate, 1 ml/min; temperature, 40°C; detection wavelength, 230 nm/430 nm; injection volume, 30 μl (equivalent to 300 μl of urine). Peaks: 1 = *R*-oxazepam glucuronide; 2 = *S*-oxazepam glucuronide; 3 = *R*-3-hydroxyprazepam glucuronide; 4 = *S*-3-hydroxyprazepam glucuronide.

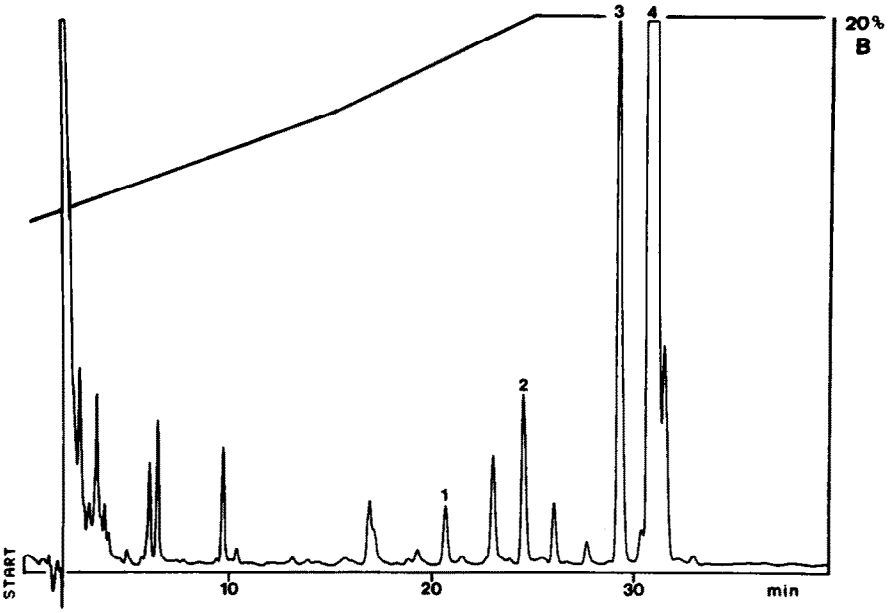

Fig. 2. Analysis of a urine sample after an overdose of diazepam. Column, Select B (5 μm, 250 mm × 4 mm I.D.); gradient, 0 min 12.5% B, 15 min 16.5% B, 25 min 20% B; injection volume, 10 μl (equivalent to 10 μl of urine). Eluent A, eluent B, flow-rate and detection wavelength as in Fig. 1. Peaks: 1 = *R*-oxazepam glucuronide; 2 = *S*-oxazepam glucuronide; 3 = *R*-temazepam glucuronide; 4 = *S*-temazepam glucuronide and nordiazepam glucuronide. Under these conditions the lorazepam glucuronide eluted after 26 and 29 min, respectively.

nides. A 200-μl volume of purified glucuronides (10 μg/ml) in 0.2 *M* sodium phosphate buffer (pH 7) were incubated with 10 μl of β-glucuronidase from *E. coli* (diluted 1:40) and incubated for 2 and 4 h at 37°C. The amount of free benzodiazepines in the digested samples was determined as described above and used to calculate the rate of hydrolysis.

RESULTS AND DISCUSSION

Pretreatment of the biological samples

As direct injection of the centrifuged blood or urine samples into the HPLC column is advisable only if the concentrations of the benzodiazepine glucuronides are rather high, in general we had to purify the samples by solid-phase extraction (SPE) prior to injection.

Best results were obtained by the following approach. Phenyl columns were equilibrated with 6 ml of methanol followed by the same volume of 4 m*M* sodium phosphate buffer (pH 9). After equilibration, 5 ml of urine were applied to the column, washed with 4 ml of buffer and eluted with 4 ml of methanol. Usually this pretreatment was sufficient, but at very low concentrations of glucuronides a second purification step was necessary. Quaternary amine columns were washed with hexane, dried and equilibrated with methanol and buffer as described above, in this case, however, with a molarity of 0.04 and a pH of 8. The purified urine sample (200 μl at the most, corresponding to 2 ml of urine) was then applied to the column, washed with buffer and eluted with 2 ml of 0.4 *M* sodium phosphate buffer (pH 9) saturated with acetonitrile. Recovery was between 90 and 95%.

This method offers the possibility of concentrating the urine samples by a factor of 10, thus lowering the limit of detection to *ca.* 5 ng of glucuronide per ml (injection volume 50 μl).

The pretreatment of plasma samples by SPE differs a little from that of urine samples. A 1-ml volume of plasma was treated as described above, then the eluate of the phenyl column was brought nearly to dryness, resuspended in 100 μl of methanol and centrifuged at 4000 *g*. The supernatant could be injected directly onto the HPLC column.

HPLC of the pretreated samples

Separation of the intact benzodiazepine glucuronides by HPLC was best when a LiChrospher 100 RP 8 or a Select B column was used, the former giving better results if 3-hydroxyprazepam glucuronides had to be separated, the latter in the case of oxazepam glucuronides (Figs. 1 and 2). For concentrations greater than 30 ng/ml, the accuracy of the assay was ± 10% and better.

Under the described conditions, *S*-lorazepam glucuronide coeluted with *R*-temazepam glucuronide and *S*-temazepam glucuronide with nordiazepam glucuronide. After sampling, further separation of these glucuronides was achieved on a β-cyclodextrin column with 1.93 μ*M* H_3PO_4–acetonitrile (50:50, v/v) at a flow-rate of 2 ml/min and a temperature of 40°C. The chromatogram was monitored at 230 and 430 nm. The *R*-glucuronides eluted between 9 and 10 min, the *S*-glucuronides between 10 and 11 min. For routine analysis of these glucuronides a column-switching heart-cut technique would be best. However, we could not perform this in our laboratory.

Ratio of R- and S-glucuronides and time of ingestion

To investigate whether or not the ratio of *R*- and *S*-glucuronides in urine and/or plasma offers any information concerning the time of ingestion, three volunteers were given single 20-mg oral doses of prazepam. Urine samples were collected for the following intervals after ingestion: 0–1, 1–5, 5–10 and 10–20 h. Blood samples were taken at the times indicated in Fig. 3.

Concentrations of 3-hydroxyprazepam glucuronides and oxazepam glucuronides were determined as described above. Although the ratio of *R*- and *S*-glucuronides of oxazepam did not change with time (data not shown), the percentage of the *S*-diastereomer of 3-hydroxyprazepam glucuronide in urine declined from an average of 41% to 13% during the first 15 h after ingestion of prazepam (Table I).

Analysis of the plasma samples yielded similar

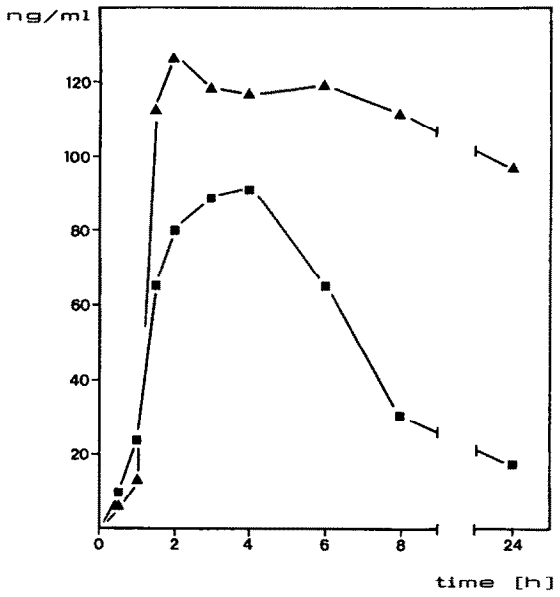

Fig. 3. Plasma concentration–time profiles of 3-hydroxyprazepam glucuronides (■) and nordiazepam (▲). Each point represents the mean for three subjects who received a 20-mg oral dose of prazepam.

results, although the effect was less pronounced than in urine. The percentage of 3-hydroxyprazepam *S*-glucuronide decreased from an average of 26% to less than 10% during the first 4 h after ingestion; quantitation of 3-hydroxyprazepam *S*-glucuronide proved to be difficult though, because the concentrations were too close to the detection limit. However, 3-hydroxyprazepam *R*-glucuronide could still be detected in plasma 24 h after ingestion (Fig. 3).

TABLE I

TIME PROFILE OF 3-HYDROXYPRAZEPAM GLUCURONIDES IN URINE

Three volunteers (V1–V3) each received 20 mg of prazepam.

Time after ingestion (h)	3-Hydroxyprazepam *S*-glucuronide in total 3-hydroxyprazepam glucuronides (%)			Rate of excretion (μg/min)		
	V1	V2	V3	V1	V2	V3
0–1	37	39	46	1.0	1.5	0.7
1–5	27	23	24	3.0	3.3	2.5
5–10	17	16	12	3.9	2.3	1.7
10–15	15	13	10	1.9	1.7	0.9

CONCLUSIONS

The method described permits the quantitation of intact benzodiazepine glucuronides in blood and urine. Besides being less time-consuming than the standard methods employing acid or enzymic hydrolysis, analysis of the intact glucuronides offers direct proof that a benzodiazepine has really been ingested and not been added to the sample afterwards (sometimes a problem in forensic science). Also, knowledge of the metabolic pattern may help to identify the benzodiazepine that was ingested. This information is lost to a great extent if acid hydrolysis is used.

Because the concentrations of drugs and their metabolites in urine naturally depend on the volume of urine excreted, analysis of a single-spot urine sample offers only limited information about the amount of drug ingested. However, cases of overdosage are recognized if the sample was not taken too long after ingestion. Additionally, kinetic studies with prazepam suggest that analysis of the ratio of *R*- and *S*-glucuronides in a urine sample may offer an indication as to when the drug was ingested.

The method presented can be used for the analysis of the blood and urine of patients (for instance in cases of suspected drug abuse) as well as for kinetic studies. It may be modified to include the analysis of benzodiazepines other than those presented in this paper if their main metabolites are glucuronides.

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