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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF INTACT C_3-HYDROXYLATED BENZODIAZEPINE GLUCURONIDES IN URINE

F. MARCUCCI, R. BIANCHI, L. AIROLDI, M. SALMONA, R. FANELLI, C. CHIABRANDO, A. FRIGERIO, E. MUSSINI and S. GARATTINI

Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea, 62-20157 Milan (Italy)

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SUMMARY

A method is described for the determination of C_3-hydroxylated benzodiazepine glucuronides in biological samples. Oxazepam and lorazepam glucuronides are measured as methyl esters and trimethylsilyl derivatives by a gas chromatographic procedure. The applicability of the method has been tested on the urine of rats, guinea pigs, rabbits and man, receiving oxazepam orally. Oxazepam glucuronide was not found in rat urine but it was present in the urine of rabbits (5.7% of the administered dose), guinea pigs (9.5%) and man (13.4–26.9%).

INTRODUCTION

Diazepam belongs to the benzodiazepines, which are powerful anticonvulsant, muscle relaxant and tranquillizing agents[1]. It is known that diazepam (I) is metabolized through at least two different pathways, namely N_1-demethylation and C_3-hydroxylation[2–4]. The two metabolites, N-demethyldiazepam (II) and N-methyloxazepam (III), are further C_3-hydroxylated and N_1-demethylated to form a common metabolite, oxazepam (IV)[2,5,6], as shown in Fig. 1.

The hydroxylated metabolites are excreted in the bile[7] and urine[8] as conjugated glucuronides. The glucuronides excreted in bile are partly re-absorbed from the intestine, thus establishing an enterohepatic circulation that is important as one of the factors affecting the duration of drug action[8,9]. The identification of this conjugated pathway has so far been based mainly upon enzymatic or acid hydrolysis of the glucuronide, followed by analysis of the hydroxylated benzodiazepine[10]. This method is, however, open to criticism with regard to both its qualitative and quantitative accuracy, because acid hydrolysis causes degradation of the drug with the formation of benzophenone derivatives[11]. Further, if β-glucuronidase is used in enzymatic hydrolysis, it may not be entirely specific for the hydrolysis of glucuronides[12], and may produce errors resulting from variations in the substrate affinity and the presence of inhibitors.

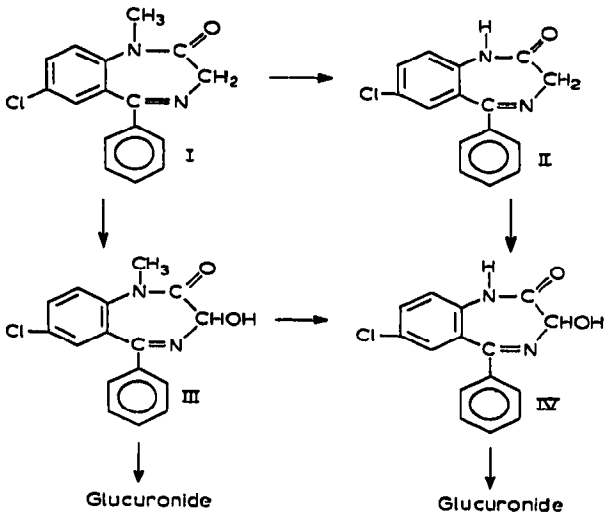

Fig. 1. Metabolic fate of diazepam (I), N-demethyldiazepam (II), N-methyloxazepam (III) and oxazepam (IV).

It was therefore decided to develop a specific method for the determination of intact benzodiazepine glucuronides.

There are only a few known examples of trimethylsilyl derivatives of urinary glucuronides, which can be separated by gas chromatography[13–16]. For this reason, we decided to explore the potential offered by the combination of mass spectrometry with gas chromatography as a simple method for separating and directly identifying drugs in biological and clinical research, and we attempted to form derivatives of C_3-hydroxylated benzodiazepine glucuronides. In order to study the reaction of silylation and the gas chromatographic behaviour of oxazepam glucuronide, a sufficient amount of this metabolite was required, of sufficiently high purity to allow the development of a quantitative method for the gas chromatographic determination of the compound in question.

As synthetic oxazepam glucuronide is not available, it was isolated from the urine of rabbits treated orally with oxazepam.

In order to test the applicability of the isolation procedure to other glucuronides and for comparative analytical purposes, this study was also extended to lorazepam, a benzodiazepine which differs from oxazepam only by the presence of a chlorine atom instead of a hydrogen atom in the *ortho*-position on the aromatic ring (Fig. 2).

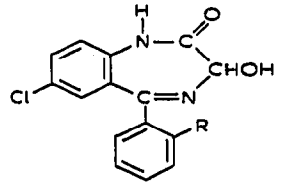

Fig. 2. Chemical structures of oxazepam (R = H) and lorazepam (R = Cl).

EXPERIMENTAL

Isolation of urinary glucuronides for preparative purposes

Urinary glucuronides were isolated by first processing, on a charcoal column, urine from rabbits that had been dosed orally with 250 mg/kg of oxazepam or lorazepam. Elution from charcoal was carried out with 80% aqueous acetone. After the evaporation of acetone, the sample was further purified by a second absorption on a charcoal–Celite (1:1, w/w) column and subsequent separation and elution with a linear gradient (from 0 to 97%) of water–ethanol. The absorbance of the effluent was monitored at 280 nm with a Uvicord I LKB absorptiometer, equipped with a flow cell. Fractions containing glucuronides were dried under vacuum. The residues were dissolved in acetone and streaked across plates coated with 2 mm of an absorbent (preparative TLC plates, silica gel F_{254}, Merck, Darmstadt, G.F.R.). The chromatograms were developed with butanol–ethanol–water (17:3:20). From the zones containing oxazepam and lorazepam glucuronides, the absorbent was scraped off the plates and eluted with 80% aqueous acetone. The final acetone solution containing the conjugates was lyophilized.

The glucuronic acid content of the final preparations was determined, after enzymatic hydrolysis, by a modified carbazole method[17] and the benzodiazepine moiety by gas–liquid chromatography (GLC)[18]. The molar ratio of oxazepam and lorazepam to glucuronide acid was 1:1 for both isolated glucuronides. Moreover, the quantitative determination of the benzodiazepine moieties indicated that the preparations were at least 85% pure. The purity of the isolated compounds was further checked by means of chromatographic techniques. In thin-layer silica gel chromatography, using a range of solvent systems, both isolated compounds gave a single spot. In ion-exchange chromatography, on a cellulose Whatman DE-52 column, they showed a single peak. By using thin-layer chromatography on polyamide 11 (Merck) and the solvent system butanol–ethanol–water (17:3:20), both isolated compounds gave a major and a minor spot, visible only when the amount spotted was increased. Comparison of the size and the degree of fluorescence of the two spots indicated that the second, minor spot was about 10 times smaller than the major spot. These chromatographic results confirm the preceding gas chromatographic data indicating a purity of at least 85%. The contaminating material, which is probably the same for both isolated glucuronides, is still unidentified.

Derivatization of oxazepam and lorazepam glucuronides for gas chromatography

The glucuronides isolated by the above method were considered to be sufficiently pure for analytical studies of their derivatization and subsequent gas chromatography to be undertaken.

The preparation of trimethylsilyl derivatives of glucuronides is generally a two-step process, initially requiring that the uronic acid carboxyl group be esterified with diazomethane. An ethereal solution of diazomethane was added to a methanolic solution of urinary glucuronides in order to obtain the methyl esters. In this step, a methyl group also replaces the hydrogen at the N_1-position.

After methylation of oxazepam and lorazepam glucuronides, the ether, the excess of diazomethane and the methanol were removed under vacuum and the residue was dissolved in 0.1 ml of pyridine. After the addition of 0.05 ml of hexa-

methyldisilazane and 0.01 ml of trifluoroacetic anhydride for 10–100 μg of both glucuronides, the tube was stoppered and the contents mixed gently. The derivatization reaction was completed after 15 min and the sample was then dried under nitrogen. The residue was re-dissolved in a suitable amount of the internal standard solution and 1–2 μl were injected into the gas chromatograph.

Gas chromatographic analysis

A Fractovap Model G I gas chromatograph, equipped with a hydrogen flame ionization detector, was used. The column was a 1-m glass tube packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh. The oven temperature was 320°. The carrier gas was nitrogen, at a flow-rate of 30 ml/min. When the separation is used for quantitative purposes, the internal standard technique is employed. A benzodiazepine previously studied in our laboratory, 7-chloro-1-methyl-5-phenyl-3-dimethylcarbamoyloxy-1,2-dihydro-[3H]-1,4-benzodiazepin-2-one (B 5833) was chosen as the internal standard because of its suitable retention time.

Fig. 3 shows a GLC separation of the glucuronides of oxazepam and lorazepam from the internal standard. Calculations were performed automatically by using a digital read-out system (Infotronics, Model CRS-104).

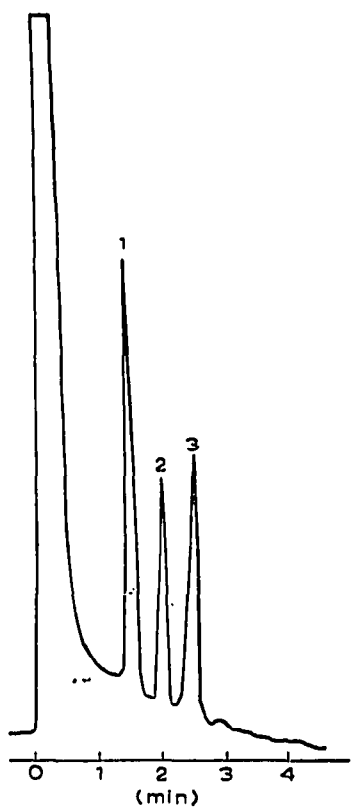

Fig. 3. Gas chromatographic separation of oxazepam glucuronide (2) and lorazepam glucuronide (3) derivatives from the internal standard (1).

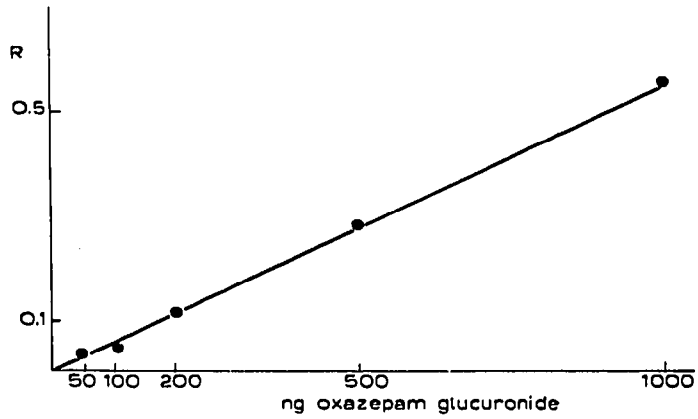

Fig. 4. Calibration graph for oxazepam glucuronide derivative. Abscissa: amount of oxazepam glucuronide derivative in the sample. Ordinate: ratio between the peak area of oxazepam glucuronide derivative and the peak area of internal standard.

Fig. 4 shows a calibration graph for oxazepam glucuronide, where the ratio between the peak areas for the drug and internal standard is plotted on the ordinate and the drug concentration on the abscissa. There is a linear relationship between the two parameters from 50 ng to 1 μg. Urine blanks collected before oxazepam administration and processed through the entire analytical procedure showed no interfering peaks in the gas chromatogram in the region of oxazepam glucuronide. Studies on the recovery of oxazepam glucuronide added to animal and human urine showed a recovery of about 80%. The sensitivity of the method is about 0.3 μg/ml of the drug in urine.

Mass spectrometry

The identity and homogeneity of glucuronide derivatives were established by means of gas chromatography combined with mass spectrometry. The instrument used was an LKB Model 9000 gas chromatograph–mass spectrometer; all spectra were measured at 12 eV.

Mass spectrometric analyses were carried out both with original samples obtained directly from urine and with samples obtained by the preparative technique previously described.

Biological experiments

Male Charles River rats, male albino guinea pigs and male Fulvi di Borgogna rabbits were given 50 mg/kg of oxazepam orally. The animals were placed in individual metabolic cages and urine samples were collected 24, 48 and 64 h after treatment. Samples of urine from animals as well as samples from humans receiving 60 mg/day of oxazepam orally for therapeutic reasons were processed in the following manner before undergoing derivatization and GLC analysis.

Procedure for quantitative analysis of oxazepam or lorazepam glucuronide present in urine

Volumes of 1–5 ml of urine were passed through a 1-g pre-packed Amberlite XAD-2 polypropylene column (Bio-Rad Labs., Richmond, Calif., U.S.A.). The

column was washed with 10 ml of 0.01 *N* formic acid in order to improve the glucuronide recovery. The resin was again washed with 20 ml of water, then oxazepam glucuronide was eluted with 10 ml of 80% aqueous acetone and the eluate was dried under vacuum.

RESULTS AND DISCUSSION

The mass spectrum of the oxazepam glucuronide derivative (Fig. 5) shows a molecular ion at *m/e* 706. Other fragments at *m/e* 691, 616 and 601 arise from molecular ions by typical fragmentations of silyl derivatives[19]. Fragments at *m/e* 423, 407, 333, 317, 217 and 204 are related to the glucuronic acid moiety and have been reported as being typical for glucuronides[19,20].

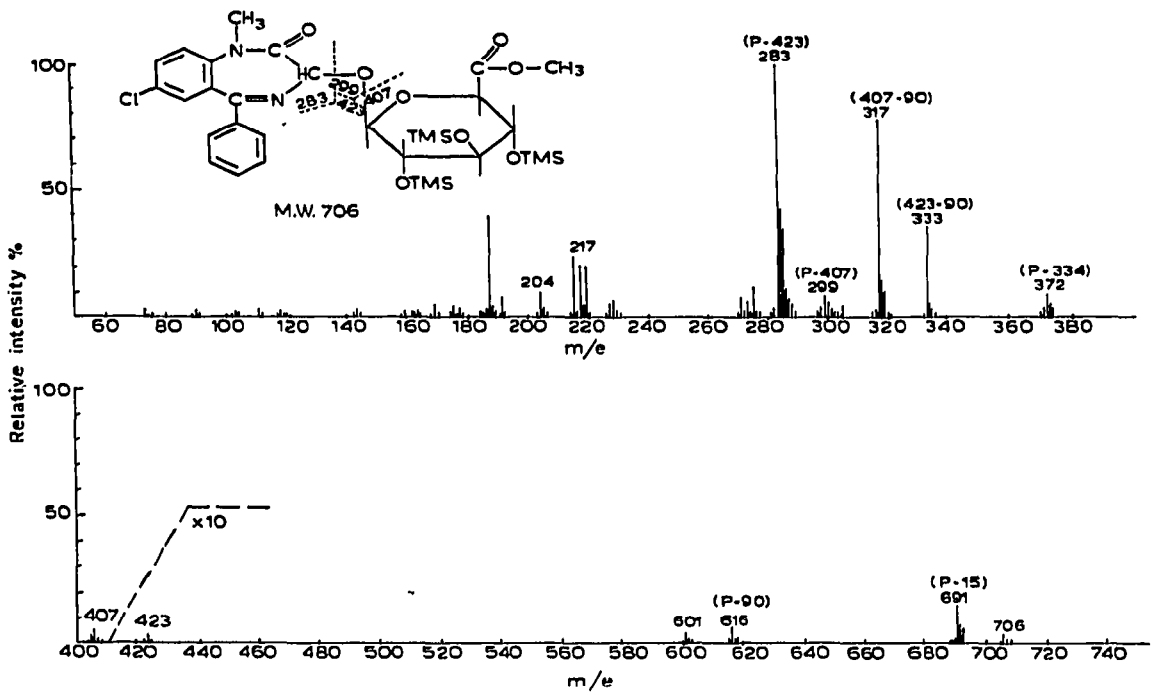

Fig. 5. Mass spectrum of oxazepam glucuronide derivative. Oxazepam glucuronide is methylated in the N_1-position of the benzodiazepine moiety and in the carboxyl group of the glucuronic acid moiety, and is trimethylsilylated in the three alcoholic groups of glucuronic acid.

The fragment at *m/e* 283 consists of the oxazepam portion with a methyl group added, but without the acetal oxygen atom at C_3. The methyl group is probably located at the N_1-position, which indicates that this position is not linked to the glucuronic acid portion. This fragment proves that the link is formed through the C_3-acetal oxygen, rather than through the N_1 atom.

For comparative analytical purposes, lorazepam glucuronide, obtained from rabbit urine and processed by the same isolation method as had been used for the

oxazepam conjugate, was analyzed by GLC–MS. The mass spectrum of lorazepam glucuronide is very similar to that of oxazepam glucuronide. The only difference (Fig. 6) is that the fragments related to the benzodiazepine moiety are shifted by 34 mass units with respect to the corresponding fragments of oxazepam glucuronide.

No major difference in the mass spectra could be found by direct (DIS) injection of the glucuronides in the mass spectrometer, or by analyzing the GLC peaks obtained both by the preparative or the analytical procedure for extracting oxazepam and lorazepam glucuronides.

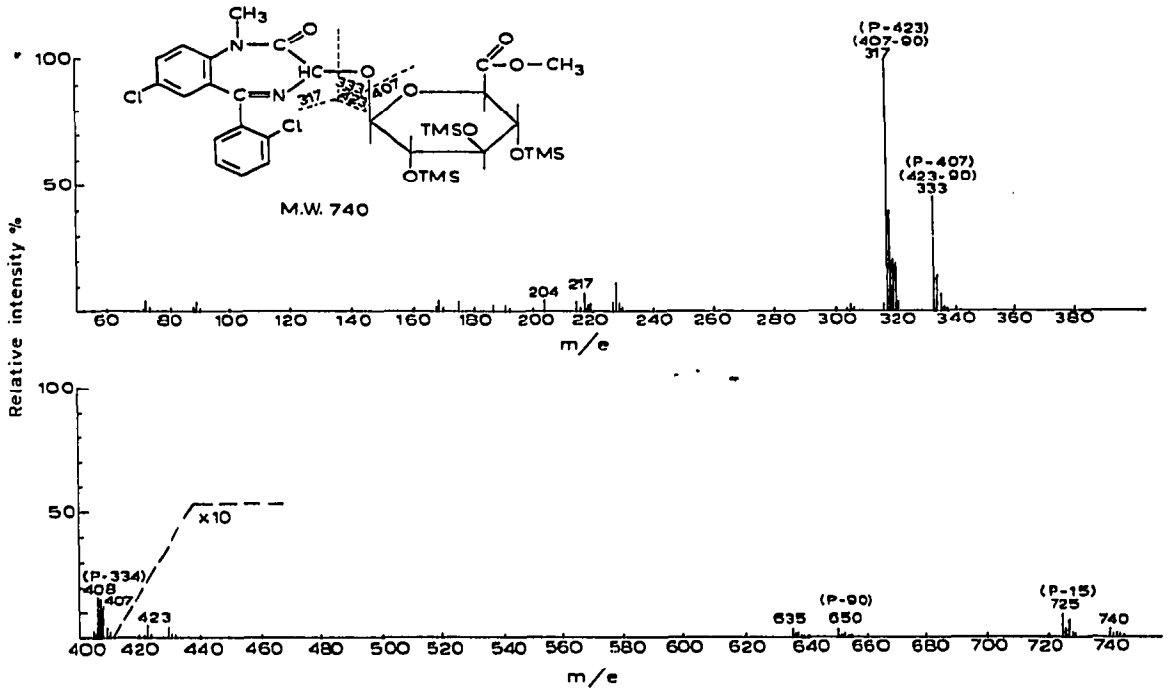

Fig. 6. Mass spectrum of lorazepam glucuronide derivative. Lorazepam glucuronide is methylated in the N_1-position of the benzodiazepine moiety and in the carboxyl group of the glucuronic acid moiety, and is trimethylsilylated in the three alcoholic groups of glucuronic acid.

In order to demonstrate the applicability of the method to the quantitative analysis of urine, a study on oxazepam glucuronide was carried out.

Table I reports the 24-h urinary elimination of oxazepam glucuronide in different animal species dosed with 50 mg/kg of oxazepam. Oxazepam glucuronide is eliminated in the urine of rabbits and guinea pigs. In the subsequent collection periods (24–48 h and 48–64 h), no further oxazepam glucuronide was excreted; no oxazepam glucuronide was found in rat urine. These findings agree with previous results showing a low oxazepam glucuronide elimination in rat bile[7] and with data on the metabolic degradation of oxazepam in rats[21].

Table II shows the results of urinary elimination of oxazepam glucuronide in humans after an oral dose (60 mg) of oxazepam. Its elimination is extensive in the

TABLE I

URINARY ELIMINATION OF OXAZEPAM GLUCURONIDE (mg ± S.D.) IN DIFFERENT ANIMAL SPECIES

No detectable amounts of oxazepam glucuronide were present in the urine of the three animal species during the periods 24–48 h and 48–64 h after oxazepam administration (50 mg/kg, orally).

Species	*0–24 h elimination*	*% of administered dose*
Rabbit	11.15 ± 2.5	5.7 ± 1.27
Guinea pig	6.53 ± 1.2	9.5 ± 1.74
Rat	0.5 μg/ml	<0.01

TABLE II

URINARY EXCRETION OF OXAZEPAM GLUCURONIDE (OX-GLU) IN MAN AFTER AN ORAL DOSE OF OXAZEPAM (60 mg)

Initials of subject	*OX-GLU (mg in 24 h)*	*OX-GLU (mg in 48 h)*	*% of the administered drug**
B.F.	10.71	13.03	13.4
B.L.	18.04	19.88	20.5
F.A.	23.48	25.72	26.5
M.A.	25.04	26.03	26.9
G.M.	14.84	18.92	19.5
C.M.	16.07	20.49	21.1

* Expressed as oxazepam.

first 24 h after its administration, while in the following 24 h the amount eliminated is greatly decreased. The drug eliminated as a glucuronide is about 20% of the dose administered.

The silylation reaction described here is not applicable when N_1-methyl-C_3-hydroxylated benzodiazepine metabolites (*e.g.*, N-methyloxazepam glucuronide) are present in the urine together with N_1-desmethyl-C_3-hydroxylated benzodiazepine metabolites (*e.g.*, oxazepam glucuronide) because the derivatives formed are indistinguishable. In order to overcome this difficulty, the methylation reaction may be replaced by a propylation reaction by utilizing diazopropane[22].

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