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DETERMINATION OF DENZIMOL, A NEW ANTICONVULSANT AGENT, AND ITS MAIN METABOLITE IN BIOLOGICAL MATERIAL BY GAS CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two methods, using gas chromatography (GC) and high-performance liquid chromatography (HPLC), were developed in order to investigate the pharmacokinetics of denzimol hydrochloride, N-{β-[4-(β-phenylethyl)phenyl]-β-hydroxyethyl}imidazole hydrochloride, which is a new anticonvulsant drug, and of its main metabolite, N-{β-[4-(β-phenyl-β(α)-hydroxyethyl)phenyl]-β-hydroxyethyl}imidazole (referred to as M_2), in humans. Both methods involve the use of a homologue of denzimol as an internal standard. The GC method is more sensitive (sensitivity limit 2.5 ng/ml for denzimol and 15 ng/ml for M_2) and was utilized for the determination of denzimol and M_2 in plasma. The GC method is specific, precise (relative standard deviations are 3.26, 2.12 and 1.72% at 10, 100 and 1000 ng/ml for denzimol and 6.45, 4.17 and 3.38% at 50, 500 and 1000 ng/ml for M_2) and accurate (mean recovery ± S.D. is 102.58 ± 4.10% for denzimol and 99.72 ± 7.75% for M_2). The HPLC method is very simple and quick to perform. This method has a sensitivity limit of 0.5 μg/ml for denzimol and 1 μg/ml for M_2, and allows the determination of both compounds in urine with high selectivity, reproducibility (relative standard deviations are 2.05, 3.50 and 1.02% for denzimol and 2.78, 2.80 and 1.73% for M_2, at concentrations of 15, 35 and 70 μg/ml) and accuracy (mean recovery ± S.D. is 103.57 ± 2.97% for denzimol and 95.91 ± 1.59% for M_2). The common anticonvulsants, when present in plasma, do not interfere with the monitoring of denzimol levels.

INTRODUCTION

Denzimol (I; Fig. 1), N-{β-[4-(β-phenylethyl)phenyl]-β-hydroxyethyl}imidazole, is a new anticonvulsant drug developed by the Recordati Research Labs. [1—4]. Denzimol has been selected as one of the most potentially interesting drugs submitted to the screening project of the Antiepileptic Drug

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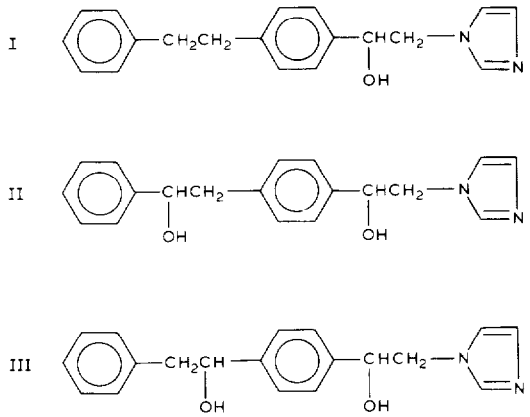

Fig. 1. Chemical structures for the compounds referred to in the text.

Development Program [5] due to its high activity. This compound is currently undergoing phase II clinical studies. Therefore, highly sensitive and specific methods for the determination of denzimol in biological material had to be developed for pharmacokinetic purposes.

Preliminary studies carried out on laboratory animals provided evidence for the presence of denzimol and of a metabolite in the urine of all the species examined (i.e. rat, guinea pig, rabbit, dog, cynomolgus monkey). Both components were conjugated in the form of glucuronides and sulphates [6]. The metabolite was isolated from dog urine and identified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectrometry and by high-resolution mass spectrometry as a mixture (M_2) of N-{β-[4-(β-phenyl-β-hydroxyethyl)phenyl]-β-hydroxyethyl}imidazole (II; Fig. 1) and N-{β-[4-(β-phenyl-α-hydroxyethyl)-phenyl]-β-hydroxyethyl}imidazole (III, Fig. 1) in a 9:1 ratio.

The structure of the M_2 components was confirmed by comparing two authentic samples synthetically prepared [7]. However, using standard analytical separation techniques such as gas chromatography (GC) with packed or capillary columns, high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) we were not able to separate the components of M_2. An "M_2-type" mixture was later found to be the main metabolite of denzimol in human urine, where it is present in a conjugated form.

Analytical methods were thus developed to determine both denzimol and M_2. One method, the more sensitive, is based on a GC technique and allows the determination of denzimol and of M_2 in plasma with detection limits of 2.5 and 15 ng/ml, respectively. The other method was HPLC and allows the determination of denzimol and M_2 in urine with detection limits of 0.5 and 1 μg/ml, respectively. At present, both methods are successfully applied to pharmacokinetic studies in humans.

EXPERIMENTAL

Reagents and chemicals

Denzimol hydrochloride (Lot No. N-5101 B), its superior homologue N-{γ-

[4-(β-phenylethyl)phenyl]-γ-hydroxypropyl}imidazole hydrochloride (Rec 15-1624) (used as the internal standard) and the M_2 metabolite were prepared by the Recordati Research Labs. (Milan, Italy).

Methanol, diethyl ether, acetic acid, sodium acetate and potassium dihydrogen phosphate (all analytical grade), acetonitrile (HPLC grade) and β-glucuronidase-arylsulphatase (12 000 U Fishman) were obtained from Merck (Darmstadt, F.R.G.).

Instrumentation and operating conditions

Gas chromatography. Analyses were carried out on a Hewlett-Packard 5830 A instrument equipped with a nitrogen—phosphorus selective detector and a fused-silica HP Series 530 μm column (10 m × 0.53 mm I.D.), which was coated with 50% phenylmethyl silicone (Hewlett-Packard). The column temperature was 270°C, the injector port and detector temperatures were 300°C and the flow-rate of the carrier gas (helium) was 20 ml/min.

High-performance liquid chromatography. The liquid chromatograph (Model 1084 B, Hewlett-Packard) was equipped with an automatic injection valve and a variable-wavelength UV absorption detector. The analytical Hypersil MOS column (10 cm × 4.6 mm I.D.), packed with octadecylsilane-bonded silica, spherical particles, size 5 μm, was obtained from Hewlett-Packard. The column was protected by a pre-column (2 cm × 4.6 mm I.D.), which was dry-packed by hand with C_{18} reversed-phase material (Kieselgel 60 HPLC 0.01 mm, from Riedel-De Haën, Seelze, Hannover, F.R.G.).

The mobile phase, which was a mixture of acetonitrile and 0.01 *M* potassium dihydrogen phosphate (pH 4.6), was filtered and degassed before use. The column was maintained at 35°C and the eluting mixture was pumped through the column at a flow-rate of 2.2 ml/min. Elution was carried out with a linear gradient from 35 to 70% acetonitrile in 5 min, then isocratically for 10 min. The column effluent was monitored at 214 nm.

Assay procedures

Gas chromatographic determination of denzimol and M_2 metabolite in plasma. To 1 ml of human plasma in a 10-ml glass tube, 100 μl of a working solution of the internal standard (either 500 or 100 ng), 1 ml of water, and 3 ml of 0.01 *M* potassium dihydrogen phosphate (pH 4.6) were added. The mixture was briefly vortexed and applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) using a disposable hypodermic syringe, and the eluate was discarded.

The cartridge had been previously conditioned by passing through it 5 ml of methanol, followed by 20 ml of water. The cartridge was then overturned and washed by flushing with 30 ml of water. The drug and the internal standard retained were eluted with 5 ml of methanol and collected into a glass conical tube. The methanolic solution was evaporated to dryness under a gentle stream of nitrogen in a water bath at 60°C. The sides of the tubes were washed with 100 μl of methanol in order to concentrate the extract in the bottom. The residue was dissolved with 0.1 or 0.5 ml of a mixture of methanol—diethyl ether (1:1). Aliquots of 1—2 μl of this solution were injected into the gas chromatograph.

HPLC determination of denzimol and M_2 in urine. A 100-μl aliquot of β-glucuronidase-arylsulphatase was added to 0.5 ml of human urine and the pH was adjusted to 5.5 with 100 μl of 2 *M* acetate buffer. The mixture was incubated at 45°C for 16 h. Finally, 0.5 ml of methanol and 100 μl (2.5 μg) of an aqueous solution of the internal standard were added. The mixture was vortexed for 30 s and then centrifuged for 5 min at 800 *g*. An aliquot (20 μl) of the supernatant was directly injected into the liquid chromatograph.

Calibration curves

In the GC method, two calibration curves were employed for denzimol: the first one in the concentration range of 10—100 ng/ml with a fixed concentration of the internal standard of 100 ng/ml, and the second curve in the concentration range of 50—2000 ng/ml, with a fixed concentration of the internal standard of 500 ng/ml. Only one calibration curve was employed for M_2. This curve was in the concentration range of 50—2000 ng/ml, with a fixed concentration of the internal standard of 500 ng/ml.

Four aqueous working solutions containing 10, 25, 50 and 100 ng of denzimol, respectively, and 100 ng of the internal standard per 0.1 ml, were prepared by diluting stock solutions of denzimol and of internal standard, each at a concentration of 100 μg/ml. Five aqueous working solutions containing 50, 250, 500, 1000 and 2000 ng of denzimol and M_2, respectively, and 500 ng of the internal standard per 0.1 ml, were prepared by diluting stock solutions of the products, each at a concentration of 100 μg/ml. Samples of blank plasma (1 ml) were spiked with 0.1 ml of the standard solutions. Four aliquots of each concentration were prepared and treated as described under *Assay procedures.*

In the HPLC method, stock solutions of 1 mg/ml denzimol and M_2, and 50 μg/ml internal standard were prepared by dissolving the samples in water. Samples of blank urine (0.5 ml) were spiked with denzimol and its metabolite at concentrations of 1, 10, 25 and 100 μg/ml and with the internal standard at a fixed concentration of 5 μg/ml.

Peak areas were calculated by the integrator of the chromatographs. The peak-area ratios of denzimol or M_2 over the internal standard were measured. A straight-line fit of the data was calculated by linear regression analysis and the correlation was determined. In the GC procedure, the equations were $y = 0.036 + 0.0023x$ and $y = -0.015 + 0.0104x$ for denzimol and $y = -0.058 + 0.0019x$ for M_2. The equations in the HPLC procedure were $y = -0.057 + 0.2071x$ for denzimol and $y = 0.1235 + 0.2028x$ for M_2.

Accuracy and precision

Blank plasma samples (1 ml) were spiked with denzimol to yield mixtures with concentrations of 10, 25, 50 and 100 ng/ml, and with the internal standard at a fixed concentration of 100 ng/ml. Other samples were spiked with denzimol and M_2 to yield mixtures with concentrations of 50, 250, 500, 1000 and 2000 ng/ml, and with the internal standard at a fixed concentration of 500 ng/ml. Blank urine samples (0.5 ml) were spiked with denzimol and its metabolite at concentrations of 3, 15, 35 and 70 μg/ml, and with the internal standard at a fixed concentration of 5 μg/ml. The samples were processed ap-

propriately and analysed under blind conditions as described under *Assay procedures*. Quantitation was achieved by means of the calibration curves. Analysis and measurement at each concentration was repeated on ten occasions both for plasma and urine. The accuracy of the methods was estimated by the recovery of the drug and M_2. The precision of the methods was evaluated by the relative standard deviation of the results obtained at each concentration.

RESULTS AND DISCUSSION

Two internally standardized methods for the determination of denzimol and its main metabolite M_2 were developed. One method utilizes the GC technique and allows the determination of denzimol and M_2 in plasma. The other method utilizes the HPLC technique, and allows the determination of denzimol and M_2 in urine.

The linearity of the methods is demonstrated by the high correlation coefficients (r = 0.999) of peak-area ratio versus denzimol and metabolite concentrations (Tables I and II).

TABLE I

CALIBRATION CURVE DATA OF DENZIMOL AND ITS METABOLITE: GC PLASMA ASSAY

For denzimol two calibration curves were employed: the first one (curve 1) in the low concentration range (10, 25, 50 and 100 ng/ml) and the second one (curve 2) in the high concentration range (50, 250, 500, 1000 and 2000 ng/ml). For metabolite M_2 only one calibration curve was employed (50, 250, 500, 1000 and 2000 ng/ml). For all concentrations, n = 4.

Linear regression parameter	Denzimol		M_2
	Curve 1	Curve 2	
Number of points	16	20	20
Slope	0.0104 ± 0.00015*	0.0023 ± 0.00005*	0.0019 ± 0.00004*
y-Intercept	−0.015 ± 0.009*	0.036 ± 0.049*	−0.058 ± 0.042*
r	0.999	0.999	0.999

*95% Confidence interval.

TABLE II

CALIBRATION CURVE DATA OF DENZIMOL AND ITS METABOLITE: HPLC URINE ASSAY

Samples of blank urine were spiked with denzimol and its metabolite (M_2) at concentrations of 1, 10, 25 and 100 μg/ml. For all concentrations, n = 5.

Linear regression parameter	Denzimol	M_2
Number of points	20	20
Slope	0.2071 ± 0.0030*	0.2028 ± 0.0037*
y-Intercept	−0.0570 ± 0.1538*	0.1235 ± 0.1934*
r	0.999	0.999

*95% Confidence interval.

TABLE III

ACCURACY AND PRECISION OF DENZIMOL AND ITS METABOLITE (M_2): GC PLASMA ASSAY (n = 10)

Added (ng/ml)	Found (ng/ml)	Recovery (%)	R.S.D.* (%)
Denzimol			
10	11.08	110.80	3.26
25	26.19	104.76	2.29
50	50.75	101.50	2.02
100	103.64	103.64	2.12
250	256.81	102.72	3.27
500	505.43	101.09	4.31
1000	983.26	98.33	1.72
2000	1956.26	97.81	3.26
M_2			
50	56.05	112.10	6.45
250	254.05	101.62	4.51
500	460.31	92.06	4.17
1000	952.74	95.27	3.38
2000	1951.11	97.55	2.49

*R.S.D. = Relative standard deviation.

TABLE IV

ACCURACY AND PRECISION OF DENZIMOL AND ITS METABOLITE (M_2): HPLC ASSAY (n = 10)

Added (μg/ml)	Found (μg/ml)	Recovery (%)	R.S.D.* (%)
Denzimol			
3	3.07	102.26	11.58
15	16.18	107.88	2.05
35	36.04	102.97	3.50
70	70.81	101.16	1.02
M_2			
3	2.81	93.81	8.62
15	14.33	95.57	2.78
35	33.96	97.03	2.80
70	68.07	97.25	1.73

*R.S.D. = Relative standard deviation.

The minimum detectable concentrations were 2.5 and 15 ng/ml with the GC method, and 0.5 and 1 μg/ml with the HPLC method, for denzimol and M_2, respectively. At these concentrations, the signal-to-noise ratio was 3:1 for each compound. Both methods were accurate and precise (Tables III and IV).

In the GC method, the average recoveries for denzimol in the range 10—2000 ng/ml and for M_2 in the range 50—2000 ng/ml were 102.58 ± 4.10% and 99.72 ± 7.75%, respectively. In the HPLC method, recovery was 103.57 ± 2.97% for denzimol and 95.91 ± 1.59% for M_2, in the range 3—70 μg/ml. Rela-

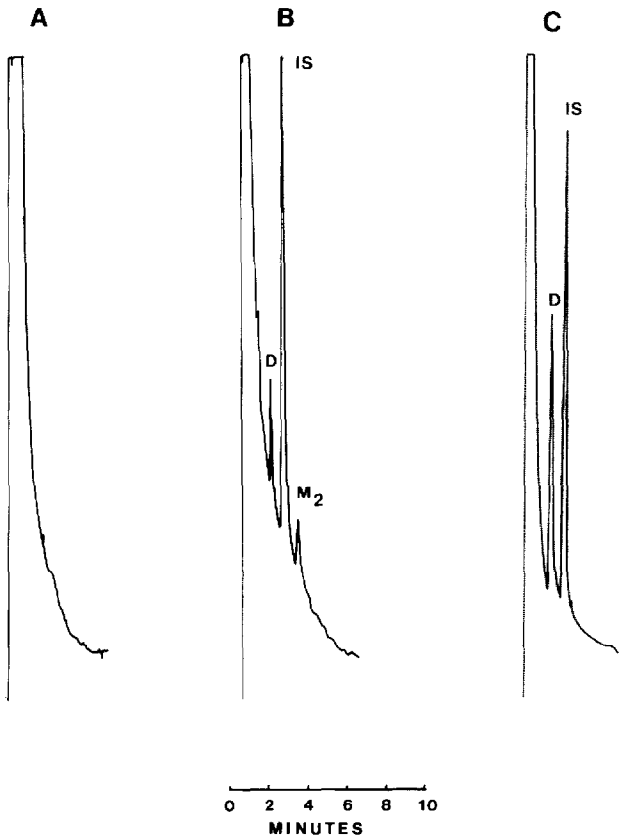

Fig. 2. Gas chromatograms of (A) human blank plasma; (B) human plasma spiked with 0.05 μg/ml denzimol and M_2, and 0.5 μg/ml internal standard; (C) plasma of a human volunteer, withdrawn 12 h after oral administration of 200 mg of denzimol and spiked with 0.5 μg/ml internal standard. Peaks: D = denzimol; IS = internal standard; M_2 = metabolite.

tive standard deviation ranged from 1.7 to 4.3% for denzimol and from 2.5 to 6.4% for M_2 for the GC method; from 1 to 11.62% for denzimol and from 1.7 to 8.6% for M_2 for the HPLC method.

The specificity of the methods was established by the characteristic GC and HPLC retention times of the reference compound, its metabolite and internal standard, and the lack of interfering peaks in the plasma and urine samples from subjects treated with denzimol (Figs. 2 and 3). Approximate retention times under the described conditions were 1.43 min for denzimol, 2.79 min for M_2 and 2.03 min for the internal standard (GC method); 4.8 min for denzimol, 1.9 min for M_2 and 5.5 min for the internal standard (HPLC method). Phenytoin, carbamazepine, valproate, phenobarbital, diazepam and oxazepam were also tested. These drugs do not interfere with denzimol and M_2 monitoring; under the GC conditions used, they are eluted very rapidly because of the high oven temperature; under the HPLC conditions used, they are readily distinguished from their retention times.

Stability of denzimol and M_2 in plasma and urine under the storage conditions used (−20°C) were also tested. No decomposition was detected over a one-month period.

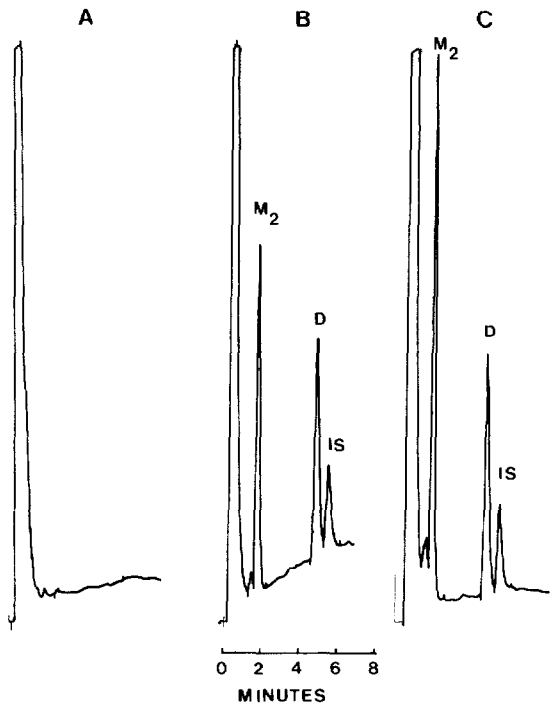

Fig. 3. HPLC profiles of (A) human blank urine; (B) human urine spiked with 10 μg/ml denzimol, 10 μg/ml metabolite and 5 μg/ml internal standard; (C) urine of a human volunteer, collected between 12 and 18 h after oral administration of 200 mg of denzimol, enzymatically hydrolyzed and spiked with 5 μg/ml internal standard. Peaks: M_2 = metabolite; D = denzimol; IS = internal standard.

The methods described here are applicable in routine analysis because of their speed, simplicity, accuracy and precision.

REFERENCES

1 D. Nardi, A. Tajana, A. Leonardi, P. Pennini, F. Portioli, M.J. Magistretti and A. Subissi, J. Med. Chem., 24 (1981) 727.
2 G. Graziani, F. Tirone, E. Barbadoro and R. Testa, Arzneim.-Forsch., 33 (1983) 1155.
3 G. Graziani, P. Cazzulani, C. Luca, G. Nava and R. Testa, Arzneim.-Forsch., 33 (1983) 1161.
4 G. Graziani, E. Barbadoro and R. Testa, Arzneim.-Forsch., 33 (1983) 1168.
5 Antiepileptic Drug Development Program, U.S. Department of Health, Education and Welfare, Public Health Service, National Institute of Health, Bethesda, MD 20014, DHEW Publication No. (NIH) 76-1093.
6 A. Tajana, Recordati, Milan, unpublished data on file.
7 A. Guenzi, Recordati, Milan, unpublished data on file.