Journal of Chromatography, 226 (1981) 413—422
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1040

ANALYSIS OF THE METABOLITES OF ETHYL LOFLAZEPATE BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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(First received March 31st, 1981; revised manuscript received July 22nd, 1981)

SUMMARY

A gas chromatographic assay with electron-capture detection (GC—EC) is described for the metabolites of ethyl loflazepate (Victan), a new benzodiazepine with a potent anti-anxiety activity, in biological fluids. Since the parent drug undergoes a first-pass effect, pharmacokinetic data may only be obtained by measuring the total levels of two of the major metabolites. Accurate data can not be obtained for the metabolites separately since one of them (M1) is chemically transformed to the other (M2) during plasma sampling, storage and extraction.

A sensitive, specific and accurate GC—EC assay is developed using a synthetic analogue of M2 as an internal standard. The limit of detection in plasma is approximately 2 ng/ml and the precision about 3% (within-run and between-run).

The method is applied to plasma samples collected after oral administration of 2 mg and 4 mg of the drug in tablet form to human volunteers. The results obtained are correlated with those from an existing gas chromatographic—mass spectrometric assay. A very good correlation between the results (inter-laboratory comparison) is obtained, validating both techniques.

INTRODUCTION

Ethyl loflazepate (CM 6912, Victan) is a novel benzodiazepine characterized by its potent anti-anxiety activity, clearly demonstrated in animal models as well as in patients [1]. Acute toxicity studies established the very low toxicity of the drug, the LD_0 in rat and mouse being higher than 4 g/kg, whereas human therapeutic active doses are scheduled at lower than 0.1 mg/kg. No significant toxic effects were observed during the subacute and chronic toxicity studies performed in rat and baboon [1].

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The chemical structures of the parent drug and its identified metabolites are shown in Fig. 1. Metabolites M1 and M2 are common metabolites for the rat, the baboon and the dog. A marked species difference was observed between the rat and the other species in the formation of hydroxylated metabolites. The monkey and the dog form predominantly 2'-fluorooxazepam whereas the rat forms the 4'-hydroxylated compound. A comparable difference has been described in the literature on benzodiazepines [2].

Preliminary in vitro studies revealed the high esterase activity of intestinal wall and liver homogenates and plasma when ethyl loflazepate was introduced as a substrate.

Considering the presence of an extensive first-pass effect, it seemed unlikely that the unchanged drug would be detected after oral administration. Never-

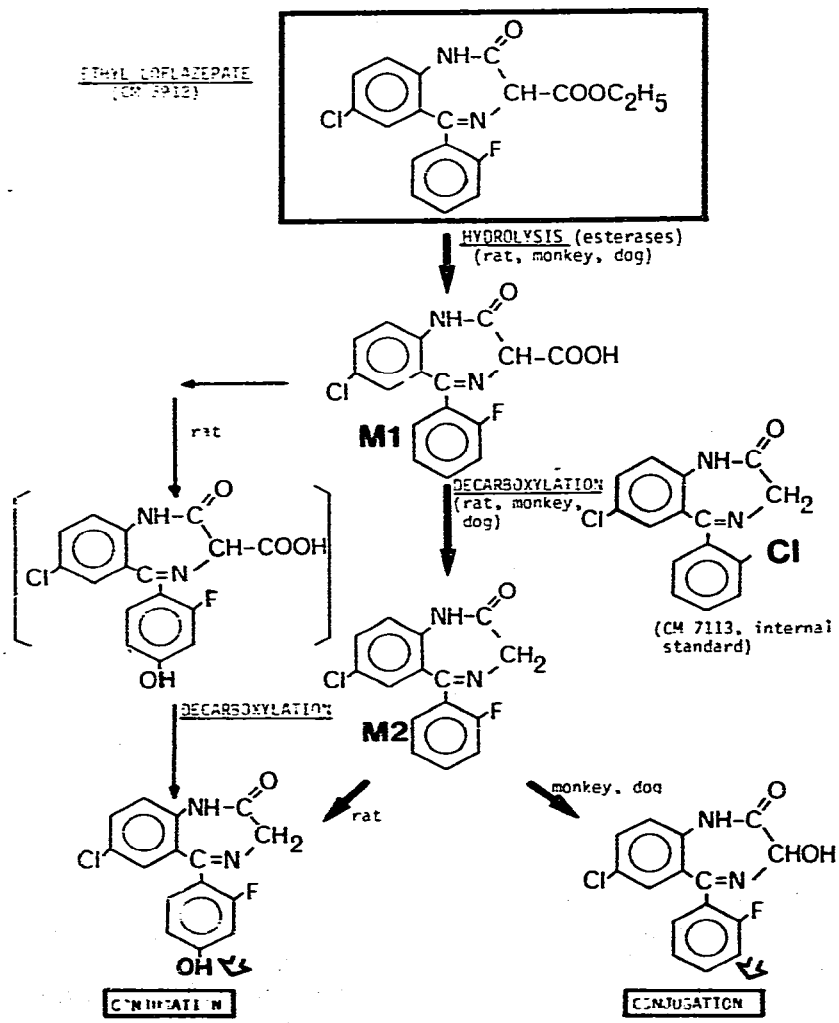

Fig. 1. Tentative metabolic pathway of ethyl loflazepare (rat, monkey and dog). M1 and M2 are monitored for pharmacokinetics. CM 7113, the chlorinated homologue of M2, is used as internal standard.

theless, this surmise had to be confirmed. Thus, the assay of three compounds — parent drug, M1 and M2 — was investigated. The carboxylic acid metabolite M1 is chemically speaking very unstable and is decarboxylated rapidly to form M2 during plasma sampling, storage and extraction.

It is well known [3—7] that dipotassium clorazepate can not be directly analyzed by gas chromatography and is rapidly transformed in an acid or neutral aqueous medium to N-desmethyldiazepam. For that reason, M1 was converted quantitatively to M2 before the analysis and total (M1 + M2) levels were measured.

A previous paper [8] reported the development of gas chromatographic—mass spectrometric (GC—MS) method for the drug and its metabolites. The absence of the parent drug in human plasma after oral administration was clearly demonstrated. Preliminary pharmacokinetic data obtained on human volunteers were described in the same paper.

This present paper deals with the development of a GC method with electron-capture (EC) detection for the monitoring of total (M1 + M2) levels in biological samples. This technique will be developed and used for field pharmacokinetic studies. The GC—EC and GC—MS techniques will be evaluated for precision and reproducibility and their correlation determined.

EXPERIMENTAL

Apparatus and chromatographic conditions

A Pye-Unicam gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The all-glass column (1.80 m × 0.4 cm I.D.) was packed with 3% OV-1—OV-17 (1 : 3) on Gas-Chrom Q (80—100 mesh). The temperature settings were: oven, 270°C; injection port, 300°C; detector, 300°C. Nitrogen was used as carrier gas with a flow-rate of 60 ml/min. The column was conditioned as described elsewhere [9]. A Hewlett-Packard 3352 B laboratory data system, a 18652 A/D convertor and a Teletype teleprinter were connected to the chromatograph.

All GC—MS analyses were run on a Ribermag 1010B gas chromatograph—mass spectrometer system coupled on-line to the Ribermag SIDAR data system (Rueil Malmaison, France). GC separations were performed on a packed column (1.5 m × 0.18 cm I.D.) filled with 3% OV-1 on Chromosorb W AW (80—100 mesh). Helium was used as carrier gas at a flow-rate of 25 ml/min. The oven temperature was 245°C. The mass spectrometer was operated in the chemical ionization (CI) mode using ammonia as the reagent gas. Quantitative analyses were performed in the "switched" single-ion detection mode focusing the respective MH^+ ions as described previously [8]. Quantification was performed automatically after data acquisition using the SIDAR software.

Reagents

All reagents were of analytical grade. The inorganic reagents were all prepared in double-distilled water.

Concentrated sulfuric acid "Ultrex" was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Sodium hydroxide, potassium bicarbonate, potassium hydroxide, dipotassium hydrogen phosphate and a buffer solution at pH 10

were obtained from Merck (Darmstadt, G.F.R.). The aqueous solution of 1 *N* potassium hydroxide—1 *M* dipotassium hydrogen phosphate was washed with diethyl ether before its use in the clean-up procedure.

Organic solvents used were acetone, diethyl ether, *n*-hexane, methanol, *n*-heptane and toluene. All solvents had the label "pestipur" and were obtained from Solvent Documentation Synthèse (Peypin, France).

A chlorinated homologue of M2 was used as internal standard. The structure of this compound, code number CM 7113, is shown in Fig. 1.

Derivatization procedure

The analytes were derivatized before GC—MS analysis. GC—EC analysis did not involve a derivatization procedure. All details of the derivatization procedure used were as described in detail previously [8, 10]. It involves butylation of the NH moiety in the 1-position of the 1,4-diazepine cycle, using 1-iodobutane in the presence of methanol, N,N-dimethylacetamide and tetrabutylammonium hydroxide.

Standard solutions

Standard solutions of M2 and CM 7113 (internal standard) were prepared as follows. An exactly weighed 10-mg amount of the compound was dissolved in 2.5 ml of acetone and 2.5 ml of methanol in a 10-ml volumetric flask and diluted with acetone—hexane (20 : 80, v/v). The dilutions required were prepared with the acetone—hexane mixture.

Standard solutions of M1 were made up in double-distilled water buffered to pH 10 (0.1 *M* potassium bicarbonate adjusted with potassium hydroxide). They were freshly prepared every day and stored at 4°C.

Biological sampling

Blood samples were collected in 10-ml vacutainer tubes containing 10 mg of ammonium oxalate and 8 mg of potassium oxalate. The samples collected were shaken and immediately centrifuged at 5000 *g* for 10 min; the plasma was stored at —20°C pending analysis.

Determination of M2

Into a 30-ml glass centrifuge tube containing 2.5—10 ng of internal standard, 0.5—2 ml of plasma and 2 ml of pH 10 buffer solution were added. The mixture was shaken for 5 min with 8 ml of diethyl ether and centrifuged for 5 min at 3000 *g*. The organic layer was transferred to a fresh centrifuge tube. The extraction was repeated with 8 ml of ether. The ether phases were combined and evaporated to dryness under vacuum with a nitrogen leak. To the residue were added 2.5 ml of 1 *N* sulfuric acid and 10 ml of hexane. The tube was shaken for 10 min and centrifuged for 5 min. The hexane layer was carefully removed by aspiration without removing any of the acid phase. This aqueous phase was adjusted to pH 10 by means of 1 *N* sodium hydroxide—1 *M* potassium hydrogen phosphate solution and extracted twice with 8 ml of ether as described before but with 10 min of centrifugation. The ether phases were pooled and evaporated to dryness. To simplify the extraction, both first and final steps of extraction can be performed once using 10—

14 ml of ether for each. The residue was reconstituted with 50 μl of toluene and 2—3-μl aliquots were injected for analysis.

Determination of (M1 + M2) levels

Into a centrifuge tube containing 2.5—10 ng of internal standard, 0.5—2 ml of plasma were added. The mixture was adjusted to pH 2 by means of 0.25 ml of 1 *N* sulfuric acid and then incubated in a 37°C water-bath for 30 min with continuous agitation (in this step M1 is transformed to M2). The pH was adjusted to 10 with 1 ml of 1 *M* potassium hydrogen phosphate and a sufficient amount of 1 *N* sodium hydroxide—1 *M* potassium hydrogen phosphate. The M2 formed was then extracted together with the M2 initially present as described above.

The concentrations were calculated from the calibration curves after simultaneous determination of specimens of control, blank, plasma to which known amounts of M2 or/and M1 (ranging from 2 to 40 ng/ml of plasma) were added. The curves were constructed by calculating the peak area ratio of M2 to that of internal standard, and plotting the ratio against the amount of (M1 + M2) spiked.

RESULTS AND DISCUSSION

A previous paper [8] confirmed the absence of the parent drug in human plasma after oral administration of the drug. A GC—MS technique was used for that study. The same paper reported preliminary pharmacokinetic data measuring total (M1 + M2) levels. The sensitivity and accuracy of the technique were discussed in detail before. The GC—EC technique developed in this present paper will be used for the complete pharmacokinetic study of the drug after single oral and intravenous administrations and after chronic oral treatment. This technique will therefore be discussed in detail and correlated for sensitivity, specificity and accuracy with the existing GC—MS technique.

Specificity

M1 is not soluble in organic solvents and would thus remain in the aqueous phase during extraction of M2. Hence, it would not interfere in the determination of M2. As explained further in this paper, it is impossible to avoid degradation of M1. Anyway, neither during the analysis of M2 nor of (M1 + M2), were interfering peaks observed in the GC—EC trace (Fig. 2). These chromatograms reveal a complete separation between M2 and its internal standard. Moreover, none of the endogenous substances interfere during the assay. Thus, the method described can be considered to be specific enough for pharmacokinetic studies.

Recoveries and limits of detection

Using diethyl ether for extraction and by performing double extractions for each step, M2 and its internal standard were quantitatively recovered. Normal sulfuric acid, used in the clean-up procedure, back-extracted quantitatively the components from the hexane layer.

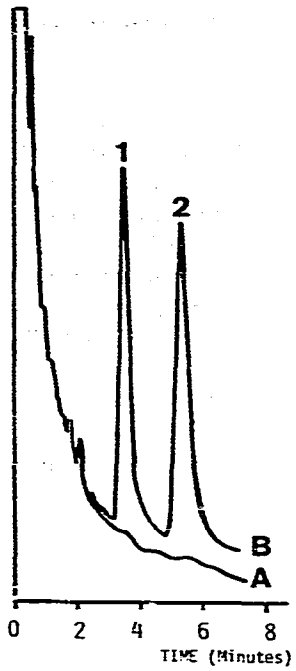

Fig. 2. GC—EC trace obtained from a subject treated with an oral dose of 2 mg of the drug (A) before administration, (B) 1 h after dosing. Peak 1 represents (M1 + M2); peak 2, 15 ng/ml of the internal standard.

The limits of detection were about 1 ng/ml of plasma for M2 and about 2 ng/ml of plasma for total (M1 + M2) levels. With single extractions the recoveries were slightly less quantitative but did not considerably influence the detection limits. Moreover, single extractions were less time-consuming.

Precision

The precision of the assay was determined within run (WR) and between runs (BR) and was found to be excellent. The coefficients of variation (C.V.) with $p = 0.05$ are summarized in Table I.

TABLE I

PRECISION OF THE GC—EC METHOD

Compounds analyzed	Concentration (ng/ml)	*N*	C.V. (%) $p = 0.05$	Note*
M2	2	5	2.8	WR
M2	10	5	1.7	WR
M2	17	8	1.7	WR
M1	50	10	1.4	BR
(M1 + M2)	8.7 (as M2)	6	2.7	WR

*WR = within run; BR = between run.

In vitro stability kinetics of M1 and M2 in plasma

An amount of M1 or M2 was added to about 10 ml of plasma buffered at pH 10 (final concentrations were about 15 ng/ml for M2 and 75 ng/ml for M1). Aliquots of spiked plasma were then distributed to ten fresh tubes, after which they were stored in a 4°C refrigerator or a −20°C freezer. The concentrations of free M2 were determined at set time intervals (one tube for each determination). In the case of M1, concentrations of total (M1 + M2) were also analyzed after decarboxylation of M1 as described above.

The results of this study are presented in Table II. M2 was found to be stable at −20°C as well as at 4°C. As expected, M1 was not stable in plasma. When it was stored at 4°C, after 4–6 days it was quantitatively transformed to M2. At −20°C it seemed to be stable during conservation but there was an instantaneous degradation. This transformation may have occurred during the sample preparation during which the mixture was shaken and the working conditions were at room temperature. We assume that such a transformation may also have occurred during the extraction procedure and during biological sampling. As a consequence of these observations, M1 levels can not be measured precisely and separately from M2. Therefore, pharmacokinetic parameters must be based upon total (M1 + M2) levels in plasma and urine. For preparation of standard curves best results were obtained by spiking control plasma with M1 as reference compound. An example of such a calibration curve is reproduced in Fig. 3.

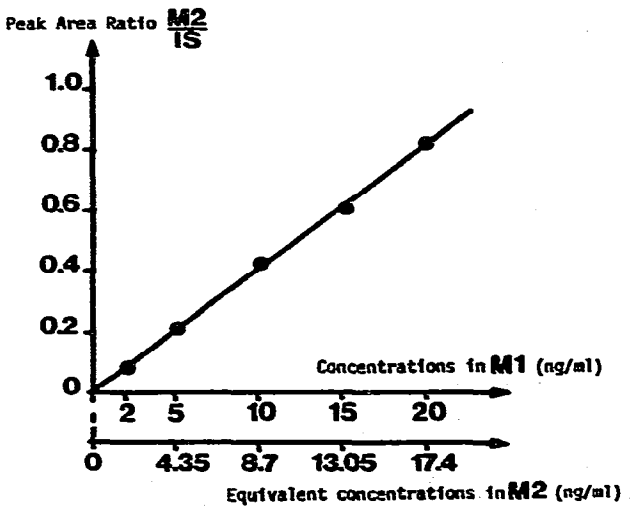

Fig. 3. Calibration curve obtained after spiking control plasma with M1 and decarboxylation–extraction.

Application of the GC–EC method to a human pharmacokinetic study of ethyl loflazepate

The GC–EC method was applied to human plasma samples collected after an oral administration of 2 mg or 4 mg of the drug. Plasma levels of total (M1 + M2) were determined at the following times: 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, 72, 96 and 144 h after drug intake. Fig. 4 reproduces the

TABLE II

STABILITY KINETICS OF M1 AND M2 IN PLASMA

Results are expressed as percentage of compound recovered relative to the initial concentration.

Compound added to control plasma	Temperature of conservation (°C)	Time of conservation (days)															
		0	1	2	3	4	5	6	8	10	13	15	16	20	22	25	30
M1	4	68.2	43.2	31.1	N.A.*	8.1	N.A.	3.5	N.A.	5.4	N.A.	0.7					
	−20	N.A.	65.7	68.3	67.3	N.A.	N.A.	65.1	67.5	65.1	67.5	N.A.	65.1	65.1	65.1		
M2	4	103	102.4	102	N.A.	97.6	N.A.	95.8	N.A.	97	N.A.	101	N.A.	101	N.A.	102	
	−20	102	101	N.A.	N.A.	N.A.	N.A.	96.5	N.A.	101	N.A.	99.4	N.A.	100	N.A.	102	97

*N.A. = not analyzed.

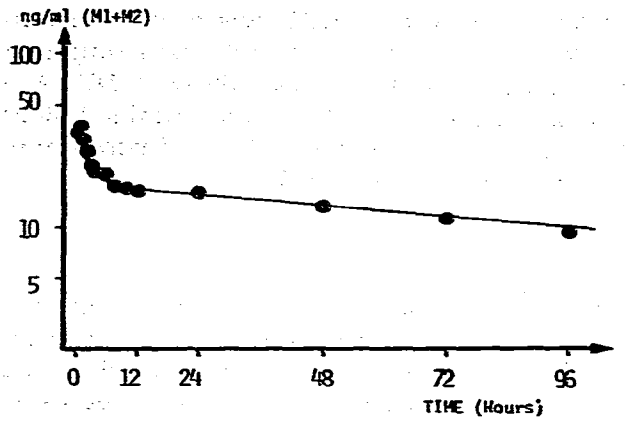

Fig. 4. Plasma concentration vs. time curve for a subject treated orally with 2 mg of the drug in tablet form.

plasma level vs. time curve obtained after a 2-mg administration. A maximum plasma level of about 40 ng/ml was attained 1 h after administration, showing a rapid absorption of the drug from the gastrointestinal tract. The active metabolites monitored were slowly eliminated from the body. The half-life of the terminal phase was found to be 122 h for this subject. These values correlate well with the values of the same parameters, reported previously, after GC—MS analysis of (M1 + M2) levels in four volunteers [8].

Comparison of the results obtained by GC—EC and GC—MS

Since a GC—MS assay technique existed, the plasma samples collected were run simultaneously at the two different facilities using the techniques available (GC—EC and GC—MS). A very good correlation was obtained between the two techniques over the entire concentration range studied. Fig. 5 summarizes the results obtained for the samples collected after the 2-mg administration. The data from Fig. 5 were fitted to a linear regression. The

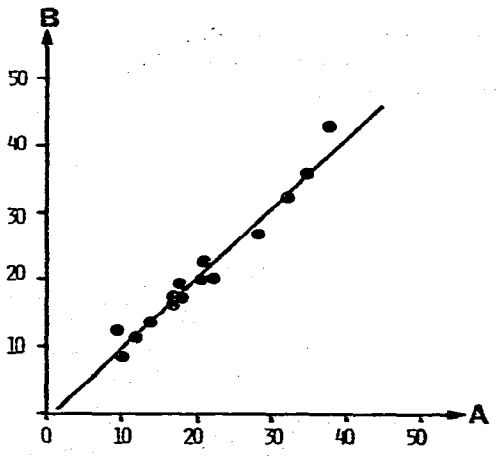

Fig. 5. Correlation of total (M1 + M2) levels assayed by GC—EC and GC—MS (concentrations in ng/ml). A = results obtained by GC—MS; B = results obtained by GC—EC.

best-fit corresponds with the equation $y = 1.06\ x - 1.21$, where y = concentration obtained after the GC—MS analysis and x = concentration obtained after GC—EC analysis. The correlation coefficient r was 0.989 ($n = 16$). Another linear regression was made on a total of 43 data points obtained after analysis of one subject treated with 2 mg and two subjects treated with 4 mg. The equation obtained was $y = 1.06\ x + 1.24$ with $r = 0.947$.

CONCLUSIONS

The inter-laboratory comparison of total (M1 + M2) plasma levels was very satisfactory considering the fact that two different techniques were used on a relatively large number of samples. The sensitivity, precision and reproducibility of the GC—EC assay were at least as good as those described previously for the GC—MS technique.

The GC—EC technique will be used to study the pharmacokinetic profile of ethyl loflazepate in man and animals.

The contribution of M1 to the total (M1 + M2) levels will be investigated. This study will probably need the administration of radiolabeled drug, chemical stabilization of the carboxylic acid moiety of M1, and analysis by high-performance liquid chromatography with a radioactivity detector.

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