Journal of Chromatography, 494 (1989) 247-256
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4869

SIMULTANEOUS DETERMINATION OF FLURAZEPAM AND ITS METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received January 31st, 1989, revised manuscript received May 10th, 1989)

SUMMARY

A sensitive isocratic high-performance liquid chromatographic method is described, which allows the precise and accurate quantification of flurazepam and four metabolites with a single determination A pharmacokinetic study was performed on nine volunteers and the main pharmacokinetic data are reported The method was used to demonstrate that monodesethylflurazepam and didesethylflurazepam are major metabolites in men One more unidentified flurazepam metabolite was detected

INTRODUCTION

Flurazepam, a benzodiazepine derivative, is very effective in the treatment of insomnia Synthesized by Sternbach et al [1], flurazepam has gained a lot of scientific interest due to its hypnotic properties and extensive metabolism [2] The parent drug disappears very quickly from the human body, producing a number of metabolites monodesethylflurazepam (I), didesethylflurazepam (II), N-1-hydroxyethylflurazepam (III), N-1-hydroxyethyl-3-hydroxyflurazepam, N-1-desalkylflurazepam (IV), N-1-desalkyl-3-hydroxyflurazepam, flurazepam-N-1-acetaldehyde and flurazepam N-1-acetic acid A number of intermediates have been suggested and uncharacterised metabolites detected by several authors [3–5]

Over the years, the attention of researchers was mainly focused on the par-

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ent drug, IV and III As a result, there is a significant body of data concerning pharmacokinetic parameters of these three substances, whereas much less is known about the concentration and pharmacokinetics of the other metabolites

Another major metabolite, flurazepam aldehyde, was discovered by Garland et al in 1983 [6] Recently, Miller et al [7] provided some data on this metabolite in man and Lau et al [3] provided data on I and II in rats

Extensive research on the drug and its metabolism was initiated by a group of scientists at Hoffmann La Roche, with the most fundamental contribution by De Silva et al (see references in ref 2) Several analytical techniques were employed to measure flurazepam and its metabolites in physiological fluids· selective extraction followed by a spectrofluorometric [8] or polarographic [2] assay, differential pulse chromatography [7], a broad spectrum of gas chromatographic [9–15] and high-performance liquid chromatographic (HPLC) methods [3, 16–20] The immense problems with the simultaneous assay of the parent drug and its metabolites stem from the necessity to measure subnanogram concentrations of flurazepam (suited best by radioimmunoassays [21] or gas chromatography–mass spectroscopy [22]), together with the need to separate a large number of metabolites of different polarity This has required derivatization in gas chromatography (9, 13, 15) or resulted in several disadvantages of HPLC methods, such as the use of more than one chromatographic system, quantification of only the parent drug and one or two metabolites and lack of sensitivity

The goal of this study was to develop a simple, isocratic HPLC method to measure flurazepam, III, IV, I and II while separating all other metabolites which would be suitable for pharmacokinetic and/or relative bioavailability studies in humans following a 30-mg oral administration of flurazepam

EXPERIMENTAL

Materials

Flurazepam·2HCl, IV and III were obtained from Hoffman La Roche (Basel, Switzerland) I·2HCl, II·2HCl and 3-hydroxydesalkylflurazepam (V) were synthesized by Dr George Just at McGill University (Montreal, Canada) Pronethalol hydrochloride, used as an internal standard, was obtained from Ayerst Labs (Rouses Point, NY, U S A) All the other reagents and solvents were of HPLC grade and used without further purification

Chromatographic conditions

Chromatography was performed on a system consisting of a Model 590 solvent delivery module, Model 481 UV detector and WISP 710B autosampler, all from Waters (Milford, MA, U S A) The column was 12 5 cm × 0 46 cm I D , in-house packed with Spherisorb C_8, 5 μm particle size, obtained from Phase Separations (Norfolk, CT, U S A) Data were collected, processed and

the regressions run on a Spectra Physics Chrom Station (San Jose, CA, U S A) The detector was set at 230 nm, 0 01 a u f s The flow-rate was 1 5 ml/min with a resulting back-pressure of 12 MPa at a room temperature of 22 ± 3 °C Total run time was 15 min

Mobile phase

Mobile phase was prepared by adding 370 ml of acetonitrile, 370 ml of methanol and 200 ml of 1 *M* sodium phosphate monobasic (pH 4 1) to a 2-l volumetric flask The flask was then brought to volume with distilled water, the mixture stirred and filtered through a 0 45-μm Nylon filter (The pH of the mobile phase was not adjusted after mixing)

Standard preparation

The standard samples in plasma were prepared by adding appropriate volumes of methanolic spiking solution to human plasma containing EDTA (0.15%, w/v) as an anti-coagulant The volume added was always less than or equal to 2% of the total volume of the sample so that the integrity of the plasma was maintained After aliquoting, 2-ml samples were stored at −15 °C until required

Clinical pharmacokinetic study

Nine healthy male volunteers, 18–45 years of age, weighing at least 60 kg and not receiving any medication for the seven days preceding the study, participated in the project after giving a written informed consent. After overnight fasting, a single capsule containing flurazepam·2HCl (30 mg) was administered orally with 200 ml of water, and blood samples were collected at 0 0, 0.5, 0 75, 1 0, 1 5, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 264 h After centrifuging for 15 min at 1000 *g* at 20 °C, plasma was collected and stored at −15 °C until analyzed. Ethical aspects of this study were considered and approved by the Institutional Review Board.

Extraction procedure

To a standard, quality control plasma or clinical sample (2.0 ml each), the internal standard was added (500 μl of pronethalol hydrochloride, 120 ng/ml in 2 *M* sodium carbonate, prepared fresh daily) After mixing, 10 ml of the extraction solution was added (dichloromethane–pentane–propan-2-ol, 500 500 10, v/v) and the tube shaken for 10 min on a reciprocating shaker at 220 rpm

After shaking, the samples were centrifuged at 1000 *g* for 10 min at 20 °C and the upper organic layer transferred into a conical tube containing 150 μl of 2 *M* hydrochloric acid The tubes were then shaken again and centrifuged under the same conditions as described above. The upper organic phase was aspirated off and 100 μl of the acidic phase were put into a 400-μl Eppendorf

micro test tube A 175-μl volume of 2 *M* acetic buffer pH 5 was added to the tube and mixed After mixing, 200 μl of the solution were injected into the chromatographic system

RESULTS AND DISCUSSION

Precision and accuracy

A set of six calibration standards, a zero and a drug-free plasma sample were analyzed with every batch of clinical samples The inter-assay precision and accuracy, concentration range and correlation coefficients are shown in Table I A linear regression analysis using a least-squares fit was performed with the reciprocal of the drug concentration as weight Precision of the method, expressed as coefficient of variation (C V), for all five analytes was 8 7–6 4% at the concentration of 1 ng/ml and improved to 1 4–3 9% at the upper limits of the calibration curve One of the analytes, flurazepam, required higher sensitivity due to its extensive metabolism and low concentration in plasma A limit of detection of 0 2 ng/ml was necessary to obtain valid pharmacokinetic data At a concentration of 0 42 and 1 04 ng/ml the C V was 16 6 and 7 4%, respectively The accuracy of the method at concentrations higher than 1 ng/ml is

TABLE I

CALIBRATION CURVE RANGES, CORRELATION COEFFICIENTS AND INTER-ASSAY PRECISION AND ACCURACY OF THE METHOD

Drug/ metabolite	Range of calibration curve (ng/ml)	Correlation coefficient	Nominal concentration (ng/ml)	Concentration found (mean ± S D) (ng/ml)	C V [a] (%)	Percentage of nominal concentration[b]	*n*
Flurazepam	0 21- 7 31	⩾0 9943	0 42	0 500 ± 0 0831	16 6	119 0	5
			1 04	0 940 ± 0 0693	7 4	90 4	4
			7 31	7 588 ± 0 2019	2 7	103 8	5
I	0 99-34 8	⩾0 9974	0 99	0 916 ± 0 0602	6 6	92 5	5
			4 97	4 940 ± 0 3411	6 9	99 4	5
			34 8	33 87 ± 1 062	3 1	97 3	5
II	1 12-39 2	⩾0 9959	1 12	1 058 ± 0 0858	8 1	94 5	5
			5 60	5 542 ± 0 3684	6 6	99 0	5
			39 2	37 93 ± 1 492	3 9	96 8	5
III	0 99-34 7	⩾0 9981	0 99	0 944 ± 0 0607	6 4	95 4	5
			11 90	11 992 ± 0 3796	3 2	100 8	5
			34 7	34 40 ± 0 489	1 4	99 1	5
IV	1 08-37 8	⩾0 9985	1 08	1 133 ± 0 0988	8 7	104 9	4
			5 40	5 058 ± 0 1690	3 3	93 7	5
			37 8	38 24 ± 0 566	1 5	101 2	5

[a]Inter-assay precisions
[b]Inter-assay accuracy

90 4–104 9% of the nominal value At sub-ng/ml concentrations of flurazepam, the deviation reached 19 0%

Recovery

Recovery was calculated by comparing extracted quality control samples with an additionally prepared calibration curve which represented 100% recovery and correcting for the losses in volume due to sample transfer The mean recovery of flurazepam was 87 2% (C V of 14 3% at 0 60 ng/ml and 4 4% at 12 0 ng/ml) The mean recoveries of I, II, III and IV were 73.5, 73 3, 32 9 and 36 6%, respectively The C V ranged from 9 2% at lower concentrations to 2 7% at higher ones

Pronethalol, α-[(isopropylamino)methyl]-2-naphtalene methanol, chosen as the internal standard, does not belong to the benzodiazepine group However, it has an appropriate retention time under the described conditions, strong UV absorbance at 230 nm, chemical stability and good extraction recovery (79 0%, C V =2 5%), which rendered it suitable for our purpose

Back-extraction recovery of IV and III was a limiting factor in obtaining a greater extraction efficiency for these two analytes In order to achieve this, the concentration of hydrochloric acid used for back-extraction should be as high as possible On the other hand, it is well known [2] that flurazepam and its metabolites are hydrolized to a benzophenone derivative in acidic conditions This reaction is quite fast and fortunately reversible above pH 4 5 This is the rationale behind the addition of concentrated acetate buffer pH 5 to the back-extracts before injection As a result, the stability of the analytes is good and the life-time of the analytical column is increased, as injections of high volumes (100 μl) of 2 *M* hydrochloric acid would be harmful to the column in the long run

Chromatography

Fig 1 shows a drug-free plasma sample from a volunteer before flurazepam administration (A) This is followed by a sample obtained from the volunteer 1 5 h after oral administration (B) Finally, there is a chromatogram representing a standard containing 1 04, 4 97, 5 60, 4 96 and 5 40 ng/ml of flurazepam, I, II, III and IV, respectively

The chromatographic conditions were appropriate for running big batches of samples due to the low pressure on the column and the stability of the analytes in the conditions of pH and temperature All these factors contributed to the consistency and robustness of the assay

One of the critical factors in maintaining the good quality of the assay was the purity of the acetate buffer, as the addition of the buffer may introduce interfering peaks In such a case, the buffer might be purified by repeated extraction with the extracting solvent used for the assay

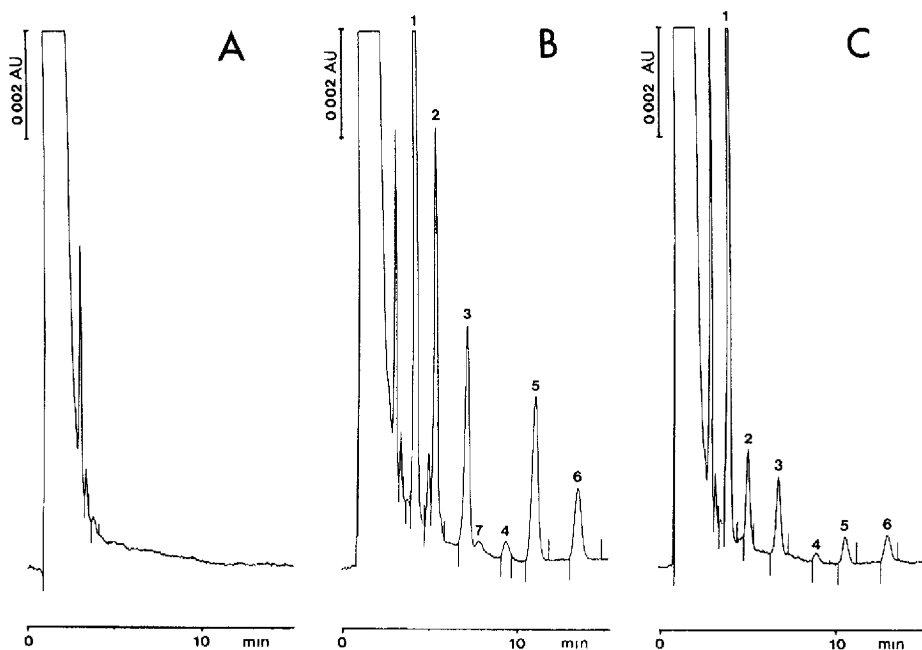

Fig 1 (A) Chromatograms of drug-free human plasma (B) Plasma taken from a volunteer 1 5 h after oral administration of 30 mg flurazepam (C) Plasma spiked at concentrations of 1 04, 4 97, 5 60, 4 96 and 5 40 ng/ml of flurazepam, I, II, III and IV, respectively Chromatographic conditions column, C_8, 12 5 cm×0 46 cm I D , mobile phase, 18 5% acetonitrile, 18 5% methanol in 0 1 *M* sodium phosphate monobasic (pH 4 1), flow-rate, 1 5 ml/min, detector wavelength, 230 nm, 0 01 a u f s Peaks 1=pronethalol (internal standard), 2=didesethylflurazepam, 3=monodesethylflurazepam, 4=flurazepam, 5=hydroxyethylflurazepam, 6=desalkylflurazepam, 7=unknown Vertical bars represent integration marks

Pharmacokinetic data

Plots of the logarithm of plasma concentration versus time were constructed for intact flurazepam and each of its metabolites The slope of the log-linear terminal phase was determined by least-squares regression analysis The apparent elimination half-life was calculated directly from elimination rate constant (K_{el}) The trapezoidal method was used to calculate the area under the curve (AUC) until the final detectable plasma concentration. To this area, the residual area, extrapolated to infinity, was added, calculated by dividing the final concentration by K_{el}

The main pharmacokinetic data are listed in Table II The pharmacokinetic profiles, log C–f (time) are shown in Figs 2–4 Our values for flurazepam, IV and III are in agreement with those published by other authors The flurazepam half-life was reported to be 1 8–3 h [7, 21, 22] as compared to our 1 34 h, IV was 71 4 and 74 h in young men [4, 23] as compared to our 68 3 h and III was 1 3 and 3 [4, 8] against our 1 6 h Remarkable similarities exist between

TABLE II

MEAN PHARMACOKINETIC PARAMETERS OF FLURAZEPAM AND ITS FOUR METABOLITES ($n=9$)

Drug/ metabolite	Peak plasma level (ng/ml)	Time of peak after dose (h)	Elimination half-life (h)	Total AUC (ng h/ml)
Flurazepam	2 10	1 0	1 34	5 10
I	11 6	1 5	3 03	70 4
II	18 7	1 75	18 0	466
III	17 9	1 0	1 60	55 3
IV	22 9	12 0	68 3	3035

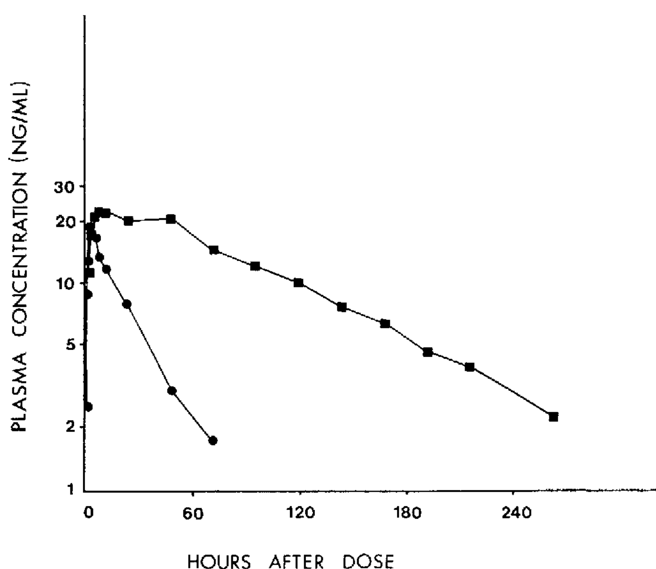

Fig 2 Mean plasma concentration-time profiles of desalkylflurazepam (■) and didesethylflurazepam (●) after a single oral dose of 30 mg flurazepam ($n=9$)

our values and those reported by Miller et al [7] in terms of half-lives, peak plasma concentrations, time of the peak and most AUC

The extensive metabolism of flurazepam presents several interesting issues The parent drug is usually so quickly metabolized and to such an extent, that quite often no flurazepam can be detected On the other hand, some subjects have maximum concentrations at 7–10 ng/ml, while half-life remains 1–1 5 h

To our knowledge, there are no published pharmacokinetic data concerning I and II in human plasma At least from a quantitative point of view, they should no longer be considered minor metabolites The peak plasma concentration of II is similar to that of IV and III The AUC for this metabolite is the

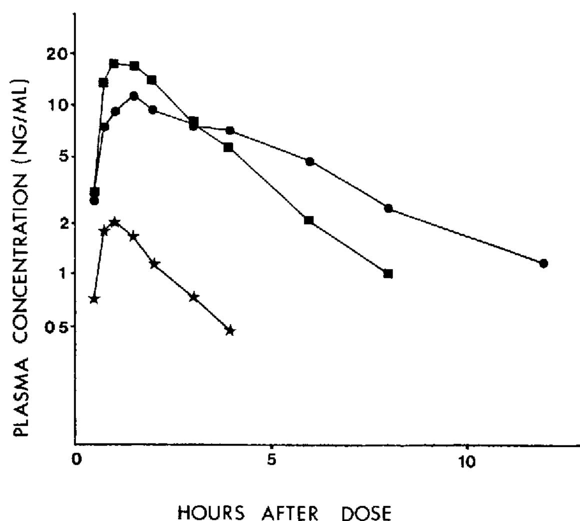

Fig 3 Mean plasma concentration–time profiles of hydroxyethylflurazepam (■), monodesethylflurazepam (●) and flurazepam (★) ($n=9$)

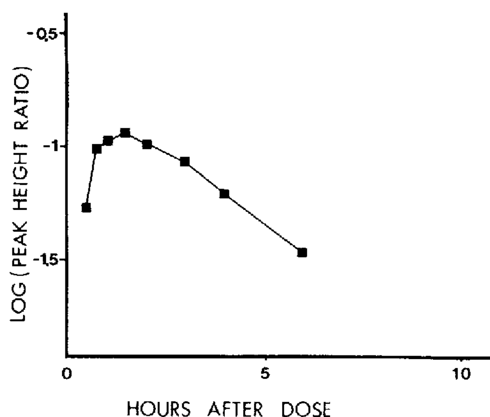

Fig 4 Plasma profile of the unknown flurazepam metabolite ($n=9$)

second highest after IV and it has a half-life much longer than other known metabolites, IV excluded The AUC and peak plasma concentration for I is similar to those of III and flurazepam aldehyde [7] and it has a half-life in between that of the above-mentioned metabolites

Out of other tentative flurazepam metabolites, N-1-desalkyl-3-hydroxyflurazepam was not found, although this metabolite is well separated from other analytes Lau et al [3] made similar observations in rats It should be noted that in the described procedure, recovery of the above-mentioned metabolite is very low and it would therefore only be detected in very high concentrations.

There is one more flurazepam-related peak seen in chromatograms of clinical samples, with a retention time of 7 8 min While this minor metabolite remains unidentified for the moment, its profile might be drawn using peak-height ratio (drug/internal standard) instead of concentration, since concentration is simply a function of this ratio (Fig 4) It has a half-life of 2 2 h and the peak plasma levels are achieved 1 5 h after the dose It does not appear that this metabolite is flurazepam aldehyde, since the latter has a much longer half-life (6 9 h) and cannot be detected using back-extraction with acid [6]

CONCLUSIONS

The described method is simple, quick and sensitive enough to be used in pharmacokinetic, bioavailability and bioequivalency studies in man It allows for quantitation of flurazepam and its four metabolites and detection of one more unidentified metabolite The pharmacokinetic study indicates that I and II should be considered major metabolites, at least from a quantitative point of view

ACKNOWLEDGEMENTS

The authors are much obliged to Carmen Discenza and Michael MacDonald for their technical assistance and Joanne Kinnear for her editorial work on the manuscript

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