Journal of Chromatography, 338 (1985) 347—355
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2428

PLASMA DETERMINATION OF 3-METHYLCLONAZEPAM BY CAPILLARY GAS CHROMATOGRAPHY

PHILIPPE COASSOLO, CLAUDE AUBERT and JEAN-PAUL CANO*

Pharmacy Faculty, INSERM SC No. 16, F-13385 Marseille Cédex 5 (France)

(Received August 9th, 1984)

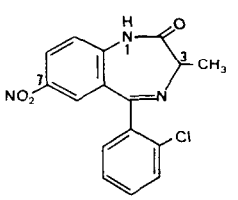
SUMMARY

A capillary gas—liquid chromatographic method for the determination of 3-methylclonazepam in plasma was developed. This method involved a single extraction by butyl acetate followed by analysis of the organic extract on a CP-Sil 5 glass capillary column with detection by electron capture. The detection limit was about 0.1 ng/ml, and the inter- and intra-assay precision did not exceed 8% for the concentration range 0.1—6.0 ng/ml. Specificity towards some of the possible metabolites in human plasma was demonstrated. This method was used for the measurement of the pharmacokinetic parameters of 3-methylclonazepam in healthy volunteers after a single intravenous administration of 1 mg, and oral administrations of 1 and 4 mg.

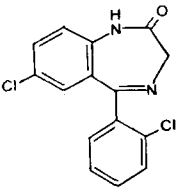
INTRODUCTION

3-Methylclonazepam (Ro 11-3128, compound I) (Fig. 1) is a new benzodiazepine which exhibits very interesting anxiolytic [1] and antiparasitic properties [2]. The anxiolytic effect is reached with low doses of 1 or 2 mg per os. Therefore, the pharmacokinetic characterization of such a compound requires very sensitive and reproducible methods for its quantification in plasma or urine samples. Due to the electronegative character of some of the substituent groups of I, only two techniques were considered suitable for this purpose: gas—liquid chromatography (GLC) with electron-capture detection (ECD), and GLC coupled with mass spectrometry (GC—MS) operating in the negative chemical ionization mode [3—6]. The GC—MS procedures are, undoubtedly, the most specific and sensitive, but routine determinations could not be performed with these methods. Thus, a rapid and highly sensitive GLC procedure had to be developed, adapted to the analysis of large number of samples. This assay needed to be sensitive enough to measure precisely the

0378-4347/85/$03.30



compound I



compound II

Fig. 1. Chemical structure of 3-methylclonazepam (I) and its internal standard (II).

small concentrations of unchanged I in plasma after oral administration of 1 mg of this compound.

MATERIALS AND METHOD

Materials

Capillary columns. Capillary wall-coated superior-capacity open tubular (WSCOT) glass columns (25 m × 0.5 mm I.D.), with an apolar polydimethylsiloxane stationary phase (CP-Sil 5), supplied by Chrompack (Orsay, France), were used for the determination of I. These columns were primed each day with an injection of 1 μl of a methanolic solution of cholesterol (1%) and slow temperature programming (2°C/min) from 280°C to 300°C.

Chromatographic analysis. GLC analysis was performed on a GLC—ECD system from Girdel (Suresnes, France), equipped with a solid injection system (moving glass needle injector). The carrier gas was helium N55 at a head column pressure of 0.75 bar; the auxiliary gas was argon—methane (95:5) at a flow-rate of 10 ml/min. The temperature settings for injection port and detector were 300°C. Respective retention times for compound I and its

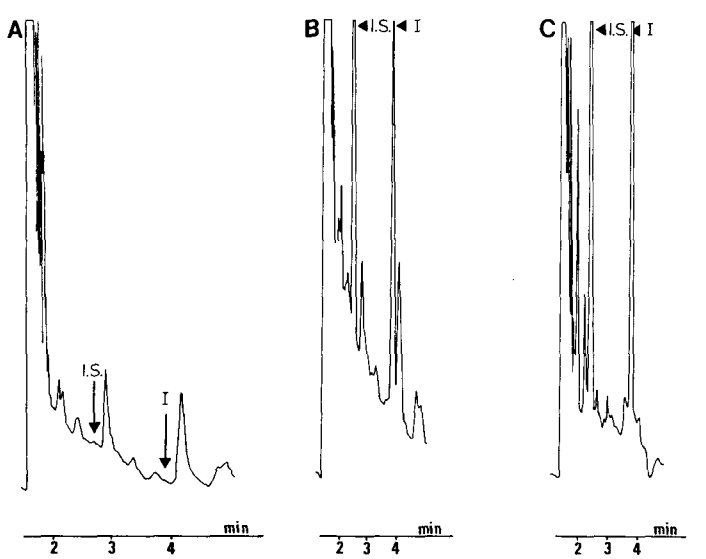


Fig. 2. Chromatograms obtained after extraction of (A) control plasma, (B) control plasma spiked with 2.5 ng/ml I, and (C) subject's plasma 0.5 h (6.7 ng/ml I) following a 1-mg intravenous bolus injection (I.S. 1.38 ng).

internal standard at an oven temperature of 280°C were 3.9 and 2.6 min (Fig. 2).

Standard solutions. The standard solutions of compound I and its internal standard, a desalkylated benzodiazepine (compound II) (Fig. 1), were prepared by carefully weighing about 10 mg into a 10-ml volumetric flask, and dissolving in methanol. Dilutions were also prepared in methanol.

Reagent. Only butyl acetate, Purex brand, supplied from S.D.S. (Peypin, France), was used for the extraction of I from plasma.

Method

Extraction procedure. An adequate volume of a methanolic solution of the internal standard was concentrated to a volume of about 20—25 μl in tapered 10-ml tubes under a gentle stream of pure nitrogen. Then 0.5 ml of plasma to be analysed was added, and extracted for 3 min with 0.3 ml of butyl acetate; the tubes were gently shaken on a vortex system to avoid serious emulsion formation. After centrifugation for 15 min at 4500 *g*, the organic phase was transferred to a 1.0-ml minivial (Pierce, Rockford, IL, U.S.A.), and then concentrated to a volume of 20—100 μl, depending on the range of expected concentrations, prior to GLC analysis.

Quantification of unknown samples. Data relating to plasma concentrations of I were obtained from least-square linear regression curves, established daily from four or five calibration points. Peak height ratios were computed by means of a SP 4270 system (Spectra Physics, Orsay, France). Quality control samples were also analysed together with the unknowns to confirm the assay accuracy.

RESULTS AND DISCUSSION

Reproducibility

The intra-day reproducibility of the method was evaluated over a concentration range of 0.6 to about 6 ng of I per 0.5 ml of plasma. The data presented in Table I indicate that the precision (given by the relative standard deviation) and the accuracy (defined by the difference between found and expected concentrations) were acceptable over this concentration range.

TABLE I

INTRA-ASSAY REPRODUCIBILITY AND ACCURACY

I · HCl concentration (ng per 0.5 ml of plasma)		*n*	Relative standard deviation* (%)	Difference between found and added concentration (%)
Added	Found			
0.63	0.62	7	8.0	−1.6
1.25	1.23	7	3.3	−1.6
3.12	3.15	7	3.2	+1.0
4.68	4.63	9	3.0	−1.1
6.25	6.14	5	2.1	−1.8

*95% Confidence.

TABLE II

INTER-ASSAY REPRODUCIBILITY AND ACCURACY

I • HCl concentration (ng per 0.5 ml of plasma)		*n*	Relative standard deviation* (%)	Difference between found and added concentration (%)
Added	Found			
0.61	0.52	13	7.4	−14.7
1.10	1.08	11	10.4	−1.8
2.49	2.35	25	3.8	−5.6
2.80	2.62	16	3.8	−6.4

*95% Confidence.

The inter-assay reproducibility was estimated from quality control samples analysed during routine determinations of the unknowns (about 10% of the analysed samples were quality controls). The results of these determinations, reported in Table II, were acceptable, except the inter-assay accuracy at 1.2 ng/ml which showed a "significant" loss of about 15%.

Limit of detection

The limit of detection, defined by a signal-to-noise ratio of 4—5, was about 0.1 ng of compound I per ml of plasma (Fig. 3), corresponding to an absolute amount of 4—5 pg per injection. The intra-assay reproducibility near this detection limit was better than 5% (Table III).

Fig. 3. Chromatograms illustrating the detection limit of I. (A) Control plasma; (B) control plasma spiked with 0.125 ng/ml I • HCl (I.S. 0.55 ng).

TABLE III

INTRA-ASSAY REPRODUCIBILITY NEAR THE DETECTION LIMIT

I • HCl plasma concentration (ng/ml)	*n*	Relative standard deviation* (%)
0.13	5	4.8
0.25	7	3.3

*95% Confidence.

Linearity

The linearity of the method was checked for concentrations of I in the range of 0.3 to about 9.4 ng per 0.5 ml of plasma. Some linearity test results are presented in Table IV. The difference, at each concentration, between the actual amounts used and the amounts found by the linear regression did not exceed 5—6%; the correlation coefficients were in the range 0.9986—0.9995, and the intercepts of the calibration curves did not differ significantly from zero.

TABLE IV

LINEARITY OF PLASMA DETERMINATION OF 3-METHYLCLONAZEPAM

I • HCl concentrations added (ng per 0.5 ml of plasma)	Equation of the non-weighted linear regression curve	Correlation coefficient	Found/added concentration ratio (mean value)
0.312, 0.625, 1.250, 1.875	$0.4368x - 0.012$	0.9986	0.994
0.625, 1.56, 3.12, 6.25	$0.3382x - 0.003$	0.9993	0.980
3.12, 6.25, 9.36	$0.1867x - 0.009$	0.9995	0.986

Recovery

The extraction of compound I from 0.5 or 1.0 ml plasma using 0.3 ml of butyl acetate was nearly quantitative up to the maximum investigated plasma concentration of about 10 ng per 0.5 ml. But, due to the formation of an emulsion during plasma extraction, it was not possible to obtain more than

TABLE V

EXTRACTION RECOVERY OF 3-METHYLCLONAZEPAM

I • HCl concentration (ng per 0.5 ml of plasma)	*n*	Extraction recovery (%)	Relative standard deviation* (%)
11.3	6	56.8	9.3
5.65	6	64.0	8.2
2.82	6	54.7	4.5
1.41	6	60.0	6.4

*95% Confidence.

0.2 ml of the organic phase. Under these conditions, the recovery of I from plasma was about 60—70%, independent of the concentration or of the plasma volume (Table V).

Specificity

Possible metabolites in man [1] would be the 7-amino derivative (Ro 12-5520, III), the 3-hydroxymethylclonazepam (Ro 11-5564, IV) and the 7-amino-3-hydroxymethylclonazepam (Ro 12-8063, V). In relation to underivatized III, the two peaks (t_R = 2.2 and 3.75 min) observed before extraction by butyl acetate were identified by GC—MS as non-chlorinated compounds; therefore, this amino metabolite could not be chromatographed in the unchanged form. Moreover, no interference from the unchanged hydroxylated compounds, IV and V, was seen under the analytical conditions described.

Stability in human plasma

Compound I was added to blank plasma at different concentrations (0.6, 2.4 and 5.6 ng per 0.5 ml plasma), and stored at different temperatures for different periods of time (one day at 20° C, three months at —20° C). Then, a set of six to seven freshly prepared control samples was analysed together with the same number of stored samples of the same concentration. The results are presented in Table VI. For plasma concentrations above 2 ng per 0.5 ml, no significant difference was detectable between the control plasmas and the plasmas stored in glass tubes. But the plasma samples at concentrations below 1 ng per 0.5 ml, stored for either three months at —20° C in glass tubes, or one day at 20° C in glass or in polypropylene tubes, showed that a "significant" decrease occurred during the storage period. That did not seem to be the case for storage during three months at —20° C in polypropylene tubes, even for very low plasma levels.

TABLE VI

STABILITY OF 3-METHYLCLONAZEPAM IN HUMAN PLASMA

Sample	Storage tube	Experimental I · HCl concentration* (ng per 0.5 ml of plasma)	n	Difference from control (%)	Confidence limits** (%)
1. Freshly spiked		2.42 ± 0.07 (2.54)***	6		
Stored one day at 20° C	Glass	2.33 ± 0.10 (2.54)	6	—3.9	—6.7 to —1.0
2. Freshly spiked		0.61 ± 0.04 (0.61)	7		
Stored one day at 20° C	Glass	0.51 ± 0.04 (0.61)	7	—15.8	—21.1 to —10.1
3. Freshly spiked		0.55 ± 0.03 (0.56)	7		
Stored one day at 20° C	Polypropylene	0.48 ± 0.06 (0.56)	6	—14.0	—22.7 to —4.4
4. Freshly spiked		5.59 ± 0.18 (5.50)	7		
Stored three months at —20° C	Glass	5.36 ± 0.12 (5.50)	6	—4.0	—0.7 to —7.2
5. Freshly spiked		0.63 ± 0.06 (0.55)	6		
Stored three months at —20° C	Glass	0.48 ± 0.04 (0.55)	5	—24.4	—31.6 to —16.4
6. Freshly spiked		0.55 ± 0.02 (0.55)	7		
Stored three months at —20° C	Polypropylene	0.59 ± 0.05 (0.55)	6	+7.4	+0.5 to +14.7

*95% Confidence.
**90% Confidence.
***Theoretical value.

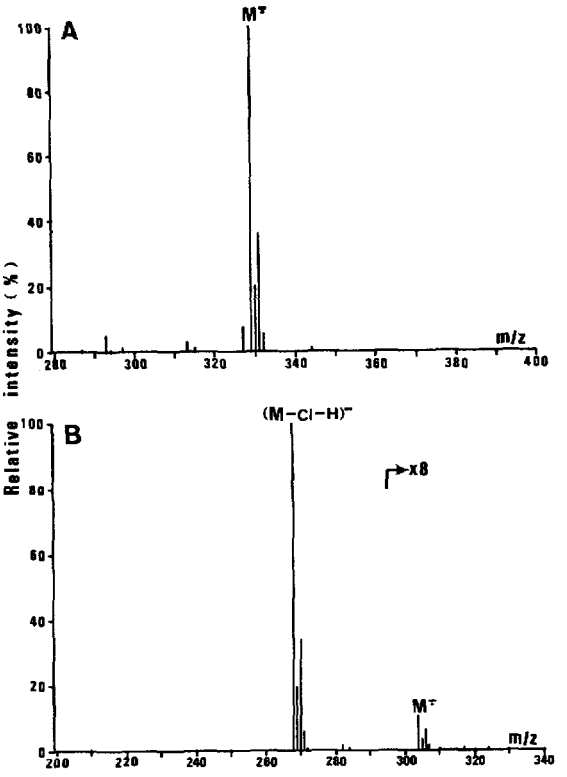


Fig. 4. Mass spectra of compound I (A) and compound II (B).

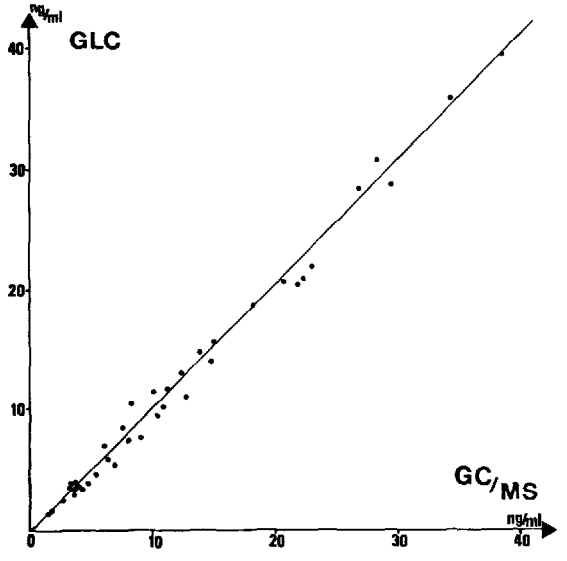


Fig. 5. Correlation between GLC and GC—MS for determination of I in plasma.

Gas chromatographic—mass spectrometric analysis

An assessment of the assay quality was realized by GLC coupled with mass spectrometry (GC—MS). The experiments were performed on a Hewlett-Packard apparatus (HP 5985 connected to a data system HP 1000) operating in the negative chemical ionization (NCI) mode. GLC analysis were carried out using a CP-Sil 5 glass (WSCOT) capillary column (8 m × 0.5 mm I.D.)

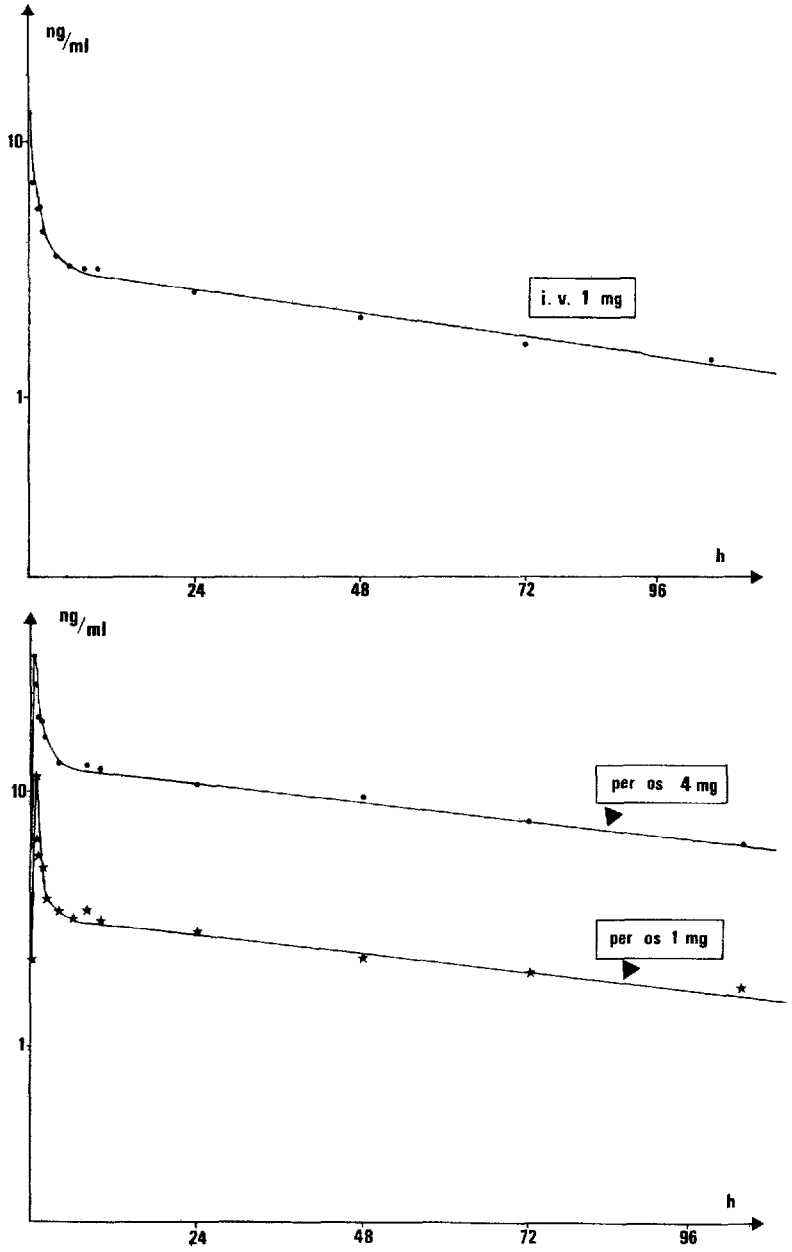


Fig. 6. Concentration—time course of I after intravenous (i.v.) administration of 1 mg and oral administration of 1 and 4 mg of this compound to a healthy volunteer.

programmed from 240°C to 280°C in steps of 15°C/min. The solid injector, ion source and GC—MS interface (transfer platinum line) temperatures were 280°C, 150°C and 285°C, respectively. Helium and methane were used as carrier gas (head column pressure of 0.8 bar) and reagent gas (ion source pressure of 1 Torr), respectively. The mass spectrometer was operated with an emission current of 300 μA and an electron energy of 230 eV.

Under these conditions, NCI mass spectra of compound I and its internal standard, presented in Fig. 4, were characterized principally by the molecular ions M^- for compound I and $(M - Cl - H)^-$ for compound II. These ions, the most intense, were used for plasma measurements by mass fragmentography in selective ion monitoring mode.

Plasma extracts of the same subject were simultaneously analysed by GLC—ECD and GC—MS: correlations between these two techniques, for about 40 plasma concentrations ranging from 1.5 to 40 ng per ml of plasma, are reported in Fig. 5 (regression curve $y = 1.029x - 0.050$, $r = 0.9929$). The mean value of the differences between each GLC and GC—MS result was 9.0 ± 1.8% (95% confidence). This validates the GLC—ECD methodology previously described.

APPLICATION

The described method was used for the analysis of plasma from healthy volunteers who had received compound I doses of 1 mg intravenously (i.v.) and of 1 and 4 mg per os in a cross-over design. Fig. 6 shows a typical plasma concentration—time course after each of these different administrations in a representative subject. Extensive treatment of these experimental data will be the subject of a further publication.

REFERENCES

1 E. Eschenhof, G. Oesterhelt and P. Fink, Internal Research Report, 1983.
2 E. Eschenhof, G. Oesterhelt and J. Ackermann, Internal Research Report, 1978.
3 Ph. Coassolo, C. Aubert and J.P. Cano, J. High Resolut. Chromatogr. Chromatogr. Commun., 5 (1982) 31.
4 Ph. Coassolo, C. Aubert and J.P. Cano, J. Chromatogr., 274 (1983) 161.
5 H. Bun, B. Ba, Ph. Coassolo, C. Aubert and J.P. Cano, Anal. Lett., 16 (1983) 327.
6 Ph. Coassolo, C. Aubert and J.P. Cano, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 258.