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SIMULTANEOUS DETERMINATION OF BLOOD CONCENTRATIONS OF METHOHEXITAL AND ITS HYDROXY METABOLITE BY GAS CHROMATOGRAPHY AND IDENTIFICATION OF 4'-HYDROXY-METHOHEXITAL BY COMBINED GAS—LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A simple, sensitive and selective method is described for the simultaneous determination of low concentrations (less than 50 ng/ml) of underivatized methohexital and its hydroxy metabolite in small (0.1 ml) samples of human and rat plasma or whole blood by gas chromatography with nitrogen-selective detection.

Moreover, the main metabolite in rat and man was identified as 4'-hydroxymethohexital by comparison of chromatograms from gas—liquid chromatography (GLC) with data obtained from GLC—mass spectrometry and ^{1}H-nuclear magnetic resonance spectrometry of this metabolite, produced both by incubating methohexital with isolated rat liver microsomes and by isolating this metabolite from rat urine.

INTRODUCTION

Methohexital [α-*dl*-1-methyl-5-allyl-5(1'-methylpentyn-2'-yl) barbituric

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acid], Brevimytal®*, is an ultra-short-acting barbiturate that has found widespread use in clinical situations in which rapid and complete recovery from a short anaesthesia is desired [1].

Several authors have described methods for the determination of methohexital and its metabolites in biological fluids. Brand et al. [2] studied plasma and adipose tissue concentrations of methohexital in man. The lack of sensitivity of their ultraviolet assay method required the administration of very high doses of at least 1200 mg to measure concentrations for up to 5 h after intravenous administration. Bush et al. [3] measured whole blood concentrations of methohexital following oral administration of 10 mg/kg by an optical density differences method. This assay method needs 10-ml samples of blood.

Sunshine et al. [4] developed a gas chromatographic method to measure blood concentrations after clinical doses (1.5—2.0 mg/kg). However, the sensitivity of their procedure only allowed the measurement of unchanged drug concentrations for 10 min following intravenous injection (5 μg/ml).

Using gas chromatography with a nitrogen-selective detector Breimer [5] succeeded in measuring human plasma concentrations down to 50 ng/ml, which made it possible to study the pharmacokinetics after therapeutic doses in man for longer time periods.

Distribution and metabolism studies with methohexital have been performed in rat [6], dog [6] and man [2, 4]. Radioactively labelled methohexital was used in such studies. In the rat and dog 4'-hydroxymethohexital was identified as the main metabolite and assayed by thin-layer chromatography (TLC) and paper chromatography.

In this paper we described a simple and sensitive gas chromatographic method for the simultaneous measurement of methohexital and its hydroxy metabolite in small plasma or whole blood samples of man and rat. The main metabolite, 1-methyl-5-allyl-5(1'-methyl-4'-hydroxypentyn-2'-yl)barbituric acid (Fig. 1), formed by allylic oxidation in rat and man was identified by combined gas—liquid chromatography—mass spectrometry (GLC—MS) and ^{1}H-nuclear magnetic resonance spectrometry (^{1}H-NMR).

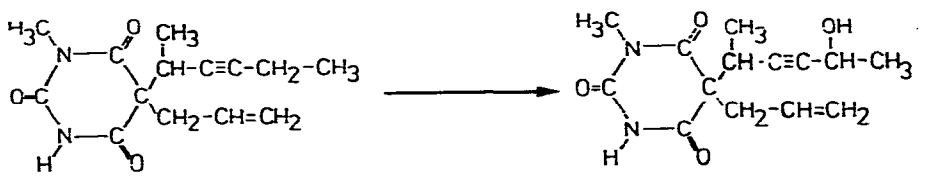


Fig. 1. Structural formulae of methohexital and 4'-hydroxymethohexital.

*Eli Lilly GmbH, Lahn Giessen, Reg. No. B 156-1, as methohexital (sodium salt).

MATERIALS AND METHODS

Chemicals and drugs

Methohexital sodium (Brevimytal®-Natrium) was purchased from Eli Lilly (Lahn Griessen, G.F.R.) and hexobarbital-sodium (Evipan®-Na) was purchased from Bayer (Leverkusen, G.F.R.).

The organic solvents light petroleum (b.p. 50—70°C), diethyl ether, propanol-2, benzene, chloroform, acetone and the chemicals for the in vitro reaction, NADPH tetrasodium salt, tris(hydroxymethyl)aminomethane for the preparation of Tris—HCl buffer and concentrated hydrochloric acid were obtained from E. Merck (Darmstadt, G.F.R.).

Absolute ethanol was purchased from C. Roth (Karlsruhe, G.F.R.).

OV-17 (phenylmethyl silicone, 50 : 50) and PPE-21 (poly-*m*-phenyl ether high polymer) were from Chrompack (Middelburg, The Netherlands).

Apparatus

A Hewlett-Packard Model 5710 gas chromatograph, equipped with a nitrogen-selective detector (HP 18789A) was used. The column (1.8 m × 2 mm I.D., borosilicate glass, silanized with 10% dimethyldichlorosilane) was supported with 3% OV-17 on resilanized Gas-Chrom Q (100—120 mesh). Temperatures were: injection port, 250°C; column, 220°C; detector, 350°C. Gas flow-rates were: air, 60 ml/min; hydrogen, 3 ml/min; carrier gas, helium, 30 ml/min.

In routine measurements plasma or whole blood concentrations of the drug and its metabolite were calculated with a Hewlett-Packard automation system (Model 3385A), which was calibrated with samples containing known amounts of methohexital and internal standard.

An LKB 2091—2130 gas chromatograph—mass spectrometer—computer system was used for the unambiguous identification of the compounds eluted from the gas chromatograph. It was equipped with a capillary SCOT column (8 m × 0.5 mm I.D. Duran 50 glass, Cab-O-Sil, coated with the mixed stationary phase 1.6% PPE-21—2.6% OV-17) and a modified pyrolysis sluice system (Becker, Model 767) [7]. Details of the solid injector and the preparation of the column have been previously reported [8]. Temperatures were: injection port, 240°C; column, 170—220°C; ion source, 200°C; separator, 210°C. The carrier gas (helium) flow-rate through the column was 5 ml/min; electron energy, 70 eV; accelerating voltage, 3.5 kV; and trap current, 50 μA.

The proton magnetic resonance (^{1}H-NMR) spectra were recorded on a 100-MHz Jeal INM-PS-100 instrument in deuterated chloroform with tetramethylsilane as internal standard.

Assay of methohexital and 4'-hydroxymethohexital

Extraction procedure for plasma. To 1.0 ml plasma were added 1.0 ml of distilled water and 0.05 ml of ethanol containing 0.5 μg of the internal standard hexobarbital. Subsequently the mixture was extracted twice for 15 sec with 5 ml of a mixture of light petroleum (b.p. 50—70°C)—diethyl ether—propanol-2 (50 : 50 : 2) on a Cenco whirlmixer.

The organic solvent layers were transferred to a conical tube and evap-

orated to dryness at 40°C under a light stream of nitrogen. The residue was dissolved in 0.05 ml of absolute ethanol and 2—3 µl of this solution were injected into the gas chromatograph.

Extraction procedure for whole blood. To 0.1 ml of whole blood 0.3 ml of distilled water and 0.1 ml of standard solution containing 0.5 µg of hexobarbital were added. After homogenization and extraction for three times with 3-ml portions of light petroleum—diethyl ether—propanol-2 (50 : 50 : 2) on the whirlmixer, the organic solvent layers were treated as described above.

Preparation of calibration graphs. The concentrations of methohexital and 4'-hydroxymethohexital were calculated with the aid of calibration graphs, which were prepared by adding known amounts of methohexital and 4'-hydroxymethohexital to 1.0 ml of blank plasma or 0.1 ml of blank whole blood. The samples were analysed by the same procedure described above and the ratios of the peak areas of methohexital and its metabolite to the internal standard were plotted against the known concentrations.

Determination of recovery. For the determination of the extraction yields of methohexital and 4'-hydroxymethohexital from plasma at different concentrations, various amounts of methohexital and the metabolite were added to 1-ml portions of blank human plasma and carried through the extraction procedure described above, except that hexobarbital was used as an external standard. The relative peak area ratios were calculated and compared with the ratios obtained by GLC of standard amounts of methohexital and 4'-hydroxymethohexital.

Incubation of methohexital. The standard incubation mixture contained 6 µmol of methohexital, 4 µmol of NADPH and a suitable amount of enzyme solution, mostly 0.2 ml of the purified microsomal fraction of rat liver homogenate [9]. To this mixture Tris—HCl buffer (pH 7.4) was added to give a total volume of 2 ml. The reaction was carried out in a Warburg shaking water-bath at 37°C for 15 min. The incubation was stopped by deep-freezing the reaction mixture. After adjusting the pH to 4—5 by adding 0.1 *N* HCl, extraction was carried out four times as described before. Further details for this reaction will be reported in a following paper [10].

Isolation of 4'-hydroxymethohexital from rat urine. To accumulate sufficient metabolite for characterization three male Wistar rats each received 1 g of methohexital sodium in 1 l of drinking water during three days. During this time urine was collected. Portions of 200 ml of the pooled urine were adjusted to pH 3—4 with 4 *N* HCl and extracted three times with 200 ml of diethyl ether. The combined organic layers were dried over sodium sulfate and evaporated in vacuo. The brown—yellow viscous residue was redissolved in 2 ml of diethyl ether and applied to the column or the TLC plate.

Column chromatography. The ether extract was passed through a column of silica gel 60 (Merck; 44 × 3.7 cm) conditioned with the mobile phase diethyl ether—light petroleum (50 : 50, v/v). The effluent fractions were checked by GLC. The fractions containing the largest amounts of metabolite were combined and evaporated. Recrystallisation of the yellow residue with diethyl ether—light petroleum yielded 4'-hydroxymethohexital as colourless needles (m.p. 100—102°C).

Thin-layer chromatography. Portions (500 µl) of the ether extract were placed as a band on a PSC-plate of silica gel 60 (Merck; 20 × 20 cm) precoated for preparative-layer chromatography (layer thickness 2 mm); the plate was developed three times with benzene—chloroform—acetone—ethanol (80 : 10 : 5 : 5, v/v). 4′-Hydroxymethohexital (R_F = 0.34) was extracted from the silica gel with diethyl ether.

RESULTS AND DISCUSSION

Gas chromatographic sensitivity and selectivity

The sensitivity and the selectivity of the alkali-flame ionization detector (nitrogen detector) for nitrogen-containing compounds allows the relatively simple and rapid determination of low concentrations of methohexital and 4′-hydroxymethohexital in small plasma and whole blood samples. The detection limit is about 1 ng per single injection. Typical gas chromatograms obtained after extraction of 1 ml of human blank plasma and plasma containing 1.06 µg of methohexital and 1.12 µg of 4′-hydroxymethohexital are shown in Fig. 2. In Fig. 3 the corresponding gas chromatograms obtained after extraction of 0.1 ml of rat blank whole blood containing 2.85 µg/ml methohexital and 21.56 µg/ml 4′-hydroxymethohexital are shown. There is no interference with endogenous constituents. A derivatization procedure is not required. In patients with liver cirrhosis in some cases an interfering peak with a retention time of about 4.27 min can be observed, which may prohibit an accurate determination of the metabolite. In these cases a decrease of the column temperature to 210°C yields better results.

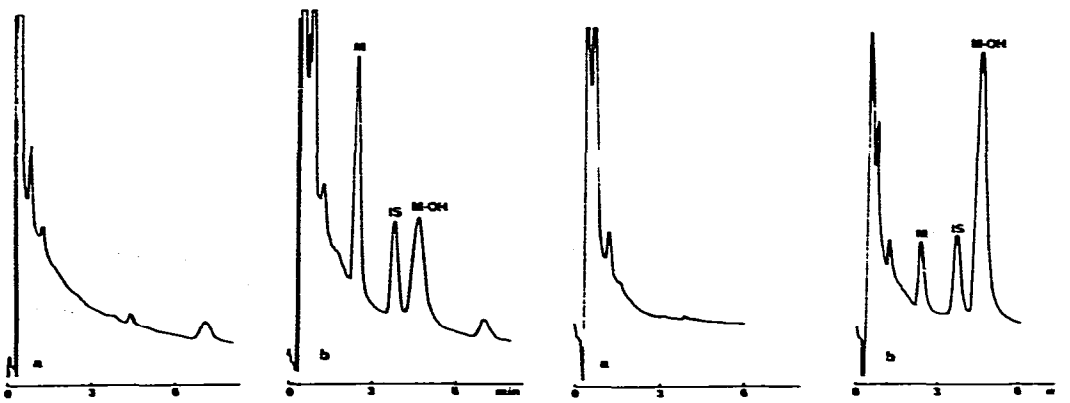


Fig. 2. Gas chromatograms of (a) a 1.0-ml extract of human blank plasma and of (b) human plasma containing 1.06 µg/ml methohexital (M) and 1.12 µg/ml 4′-hydroxymethohexital (M-OH). IS = internal standard, hexobarbital, 0.5 µg/ml.

Fig. 3. Gas chromatograms of (a) a 0.1-ml extract of rat blank whole blood and (b) of rat whole blood containing 2.85 µg/ml methohexital (M) and 21.56 µg/ml 4′-hydroxymethohexital (M-OH). IS = internal standard, hexobarbital, 5.0 µg/ml.

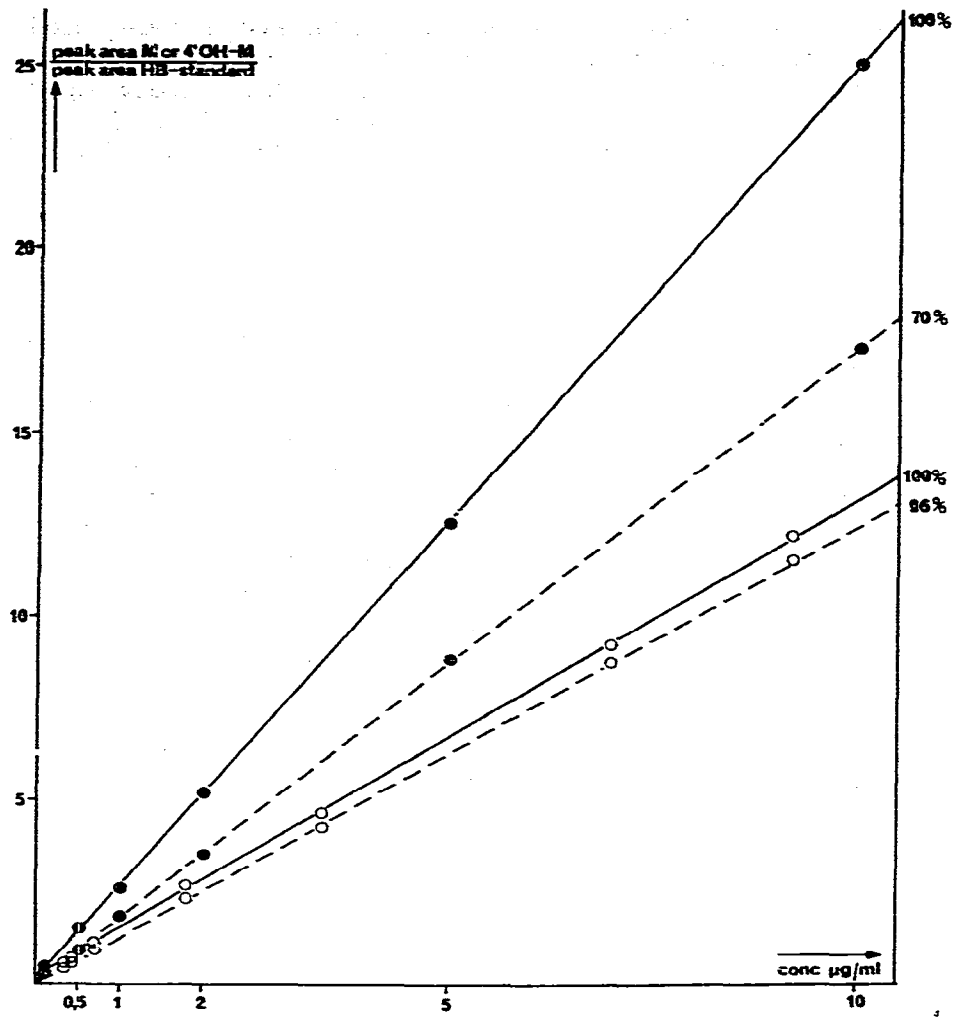


Fig. 4. Peak area ratios of methohexital (M, •) and 4'-hydroxymethohexital (4'-OH-M, ○) as a function of known concentrations for the determination of the recovery from human plasma. The human plasma curves (— — —) were obtained in the same way as described for the preparation of the calibration graphs, except that 0.5 µg of hexobarbital (HB) was added after extraction. The standard curves (——) were obtained by comparing known standard amounts of M and 4'-OH-M with 0.5 µg of HB.

Extraction procedure and precision

The extraction of methohexital and 4'-hydroxymethohexital with a mixture of light petroleum (b.p. 50—70° C)—diethyl ether—propanol-2 (50 : 50 : 2, v/v) proved to be quite suitable. The extraction yields are satisfactory and constant over a large concentration range. For plasma samples, good linearity is obtained for concentrations of methohexital and the hydroxy metabolite between 50 ng/ml and 5 µg/ml. In whole blood samples the calibration curve is linear between 100 ng/ml and 20 µg/ml. The corresponding correlation coefficients were in all cases better than 0.998.

In addition, the mean recoveries were determined for the same concentration range using hexobarbital as an external standard. For plasma, extraction yields of methohexital and its hydroxy metabolite are 70% and 96%, respectively, with standard deviations in the range 5% or less (n = 5) (Fig. 4).

Identification of the hydroxy metabolite

Although there are several reports on enzymic oxidation at allylic and benzylic positions [11], examples of enzymic oxidation at a position alpha to an acetylenic bond are rather scarce. Lindeke et al. [12] have reported enzymic oxidation alpha to a triple bond in the metabolism of N-(5-pyrrolidinopent-3-ynyl)succinimide in vitro. Propynylic oxidation in the metabolism of 7-alkynyl-substituted theophyllins [13] and the occurrence of N-methylbenzylamine as a metabolite in vivo of pargyline (N-benzyl-N-methyl-propynyl-2-amine) [14] must also implicate α-acetylenic oxidation.

Welles et al. [6] subjected pooled urine extracts from dogs, treated intravenously with [^{14}C]methohexital, to paper chromatography. They succeeded in isolating the main metabolite and characterising it as 4'-hydroxymethohexital by melting point, ultraviolet spectrometry, catalytic hydrogenation, iodoform formation of the reduced metabolite, and elemental analysis. We can, furthermore, report the mass and ^{1}H-NMR spectrum of this metabolite obtained by incubation of methohexital with rat liver microsomes and by isolating this metabolite from rat urine.

The analysis of these samples by means of GLC (Figs. 2 and 3) and a GLC—MS—computer system (Fig. 5) showed that methohexital and 4'-hydroxymethohexital are eluted from the gas chromatograph without chemical altera-

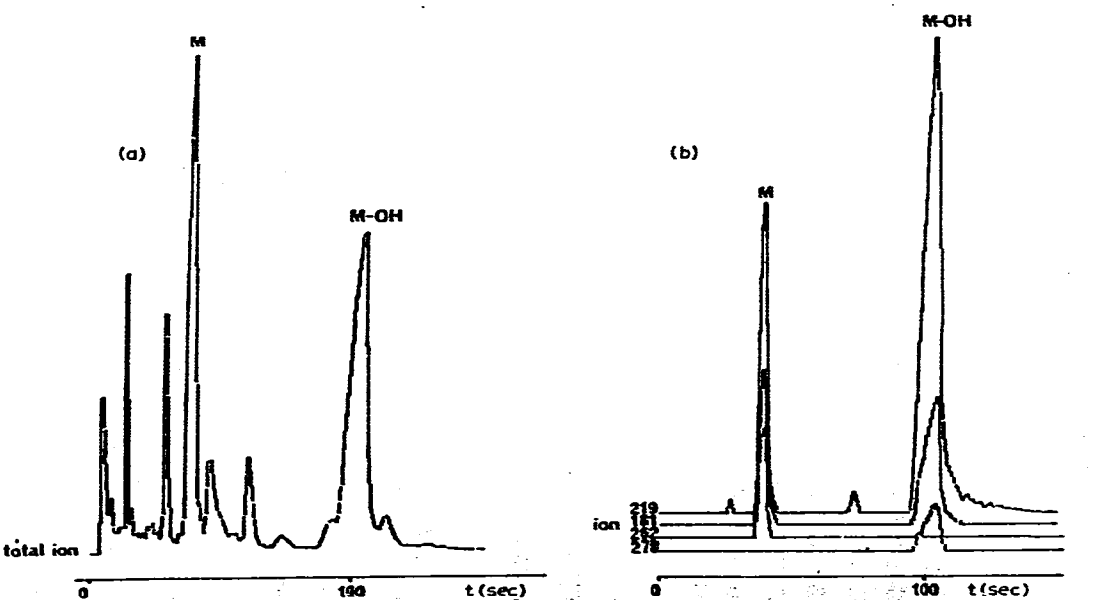


Fig. 5. (a) Total ion current and (b) mass chromatogram for four selected m/e values characteristic of methohexital (M) and its 4'-hydroxy metabolite (M-OH): m/e = 181, m/e = 219 and m/e = 262 for M and M-OH, and m/e = 278 for M-OH only.

tion under the conditions described. The mass spectra of the eluted compounds (peaks M and 4'-OH-M in the obtained chromatograms) and those after direct sample introduction, contain the molecular ions *m/e* = 262 for methohexital (M) and *m/e* = 278 for 4'-hydroxymethohexital (4'-OH-M), as shown in Fig. 6.

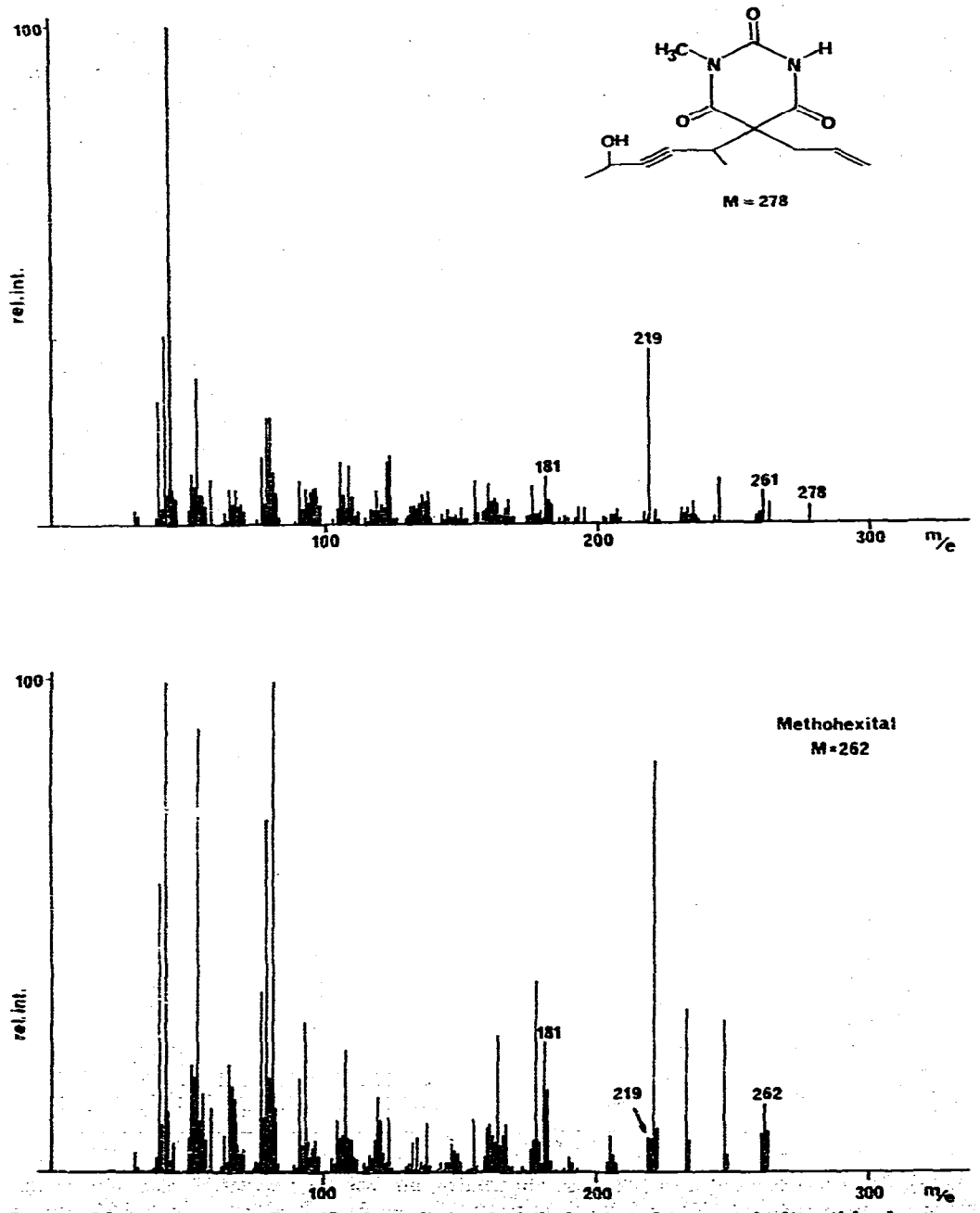


Fig. 6. Mass spectra at 70 eV of methohexital (below) and its metabolite 4'-hydroxymethohexital (above).

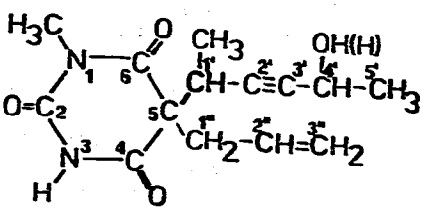
Fig. 5b shows a mass chromatogram of four characteristic *m/e* values: the molecular ions, *m/e* = 262 for methohexital and *m/e* = 278 for the metabolite; *m/e* = 181 for both formed by the loss of the 2-pentynyl chain as a radical from methohexital and the hydroxylated pentynyl chain in hydroxymethohexital; and *m/e* = 219 due to a fragment produced by the loss of an allyl radical and a molecule of water in the metabolite. In methohexital the loss of isocyanic acid, HCNO, followed by ring contraction of the barbiturate ring leads to this *m/e* value.

Further evidence for the proposed structure of 4′-hydroxymethohexital was obtained from the ^{1}H-NMR spectrum of the isolated metabolite. Comparison of the ^{1}H-NMR spectrum of the metabolite 4′-OH-M with that of the parent drug M (Table I) showed that hydroxylation has occurred in the 4′-position. In 4′-OH-M an OH signal becomes evident at δ = 4.40 ppm. The C(4′)H_2 signal in M (δ = 2.05 ppm) is lost upon hydroxylation. A C(4′)H signal appears in 4′-OH-M at δ = 2.72 ppm. The triplet from C(5′)H_3 from M disappears and shifts as a doublet under the doublet from C(1′)-CH_3 in 4′-OH-M.

TABLE I

CHEMICAL SHIFTS FOR THE ^{1}H-NMR SPECTRA OF METHOHEXITAL AND 4′-HYDROXYMETHOHEXITAL IN DEUTERATED CHLOROFORM

Tetramethylchlorosilane was used as internal standard.



	δ (ppm) methohexital	δ (ppm) 4′-hydroxymethohexital
C(5′)H_3	1.00 (t, 3H)*	
C(1′)-CH_3	1.24 (d, 3H)	1.30 (t, 6H)*
C(4′)H_2	2.05 (q, 2H)	
C(4′)H		2.72 (m, 3H)
C(1″)H_2	2.70 (m, 2H)	
C(1′)H	3.00 (q, 1H)	3.10 (q, 1H)
N(1)-CH_3	3.22 (s, 3H)	3.22 (s, 3H)
C(4′)-OH		4.40 (broad m, 1H)
C(3″)H_2	5.08 (m, 2H)	5.08 (m, 2H)
C(2″)H	5.42 (m, 1H)	5.42 (m, 1H)
N(3)H	8.78 (broad m, 1H)	9.24 (broad m, 1H)

*s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet.

CONCLUSIONS

The assay described is simple and rapid and allows the simultaneous quantitative determination of methohexital and 4'-hydroxymethohexital in plasma or whole blood. Endogenous substances do not interfere with the method and, with the exception of a short extraction procedure, time-consuming clean-up or derivatisation procedures are not necessary. This method also proves quite suitable to determine the drug and its metabolite in other biological fluids such as bile and urine, and it has been successfully applied to a study of the pharmacokinetics of methohexital and 4'-hydroxymethohexital in humans and rats [15, 16].

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REFERENCES

1 I.G. Whitwam, Anaesthesiologie und Wiederbelebung, Vol. 57, Springer Verlag, Berlin, Heidelberg, New York, 1972.
2 L. Brand, L.C. Mark, M.M. Snell, P. Vrindten and P.G. Dayton, Anesthesiology, 24 (1963) 331.
3 M.T. Bush, G. Berry and A. Hume, Clin. Pharmacol. Ther., 7 (1966) 373.
4 I. Sunshine, J.G. Whitwam, W.W. Fike, B. Finkle and J. Le Bean, Brit. J. Anaesth., 38 (1966) 23.
5 D.D. Breimer, Brit. J. Anaesth., 48 (1976) 643.
6 J.S. Welles, R.R. Mahan and W.J. Doran, J. Pharmacol. Exp. Ther., 139 (1962) 166.
7 O. Driessen and A. Edmonds, Kon. Ned. Akad. Wet. Versl. Gewone Vergad. Afd. Natuurk., Ser. c, 77 (1974) 171.
8 N.P.E. Vermeulen, M.W.E. Teunissen and D.D. Breimer, J. Chromatogr., 157 (1978) 133.
9 H. Gallenkamp and E. Richter, Biochem. Pharmacol., 23 (1974) 2431.
10 J. Epping, H. Heusler and E. Richter, in preparation.
11 J. Daly, in O. von Eichler (Editor), Handbuch der Experimentellen Pharmakologie, Vol. 28, Part II, Springer Verlag, Berlin, Heidelberg, New York, 1972, p. 286.
12 B. Lindeke, G. Hallström, E. Anderson and B. Karlén, Xenobiotica, 8 (1978) 341.
13 J. Reich and U. Seeger, Arch. Pharm. (Weinheim), 310 (1977) 888.
14 E. Diehl, S. Najm, R.E. Wolff and J.D. Ehrhardt, J. Pharmacol., 7 (1976) 563.
15 E. Richter, J. Epping, M. Fuchshofen-Röckel, H. Heusler and W. Zilly, Leber Magen Darm, 10 (1980) 234.
16 J. Epping, H. Heusler, D. Brachtel and E. Richter, in N. Rietbrock, B.G. Woodcock and G. Neuhaus (Editors), Methods in Clinical Pharmacology, Vieweg Verlag, Braunschweig, Wiesbaden, 1980, p. 203.