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RAPID AND SENSITIVE GAS CHROMATOGRAPHIC DETERMINATION OF HEXOBARBITAL IN PLASMA OF MAN USING A NITROGEN DETECTOR

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SUMMARY

The use of a nitrogen detector (alkali flame ionization detector) in a gas chromatographic method for the assay of hexobarbital in plasma is described. After extraction of the samples with light petroleum (boiling range 40–60°)–amyl alcohol (100 : 2), using methohexital as the internal standard, direct analysis is carried out. The high sensitivity and selectivity of the nitrogen detector permit the determination of hexobarbital in plasma at levels down to 50 ng/ml. The sensitivity and selectivity are compared with those obtained in normal flame ionization detection. A procedure is given for the purification of plasma samples of hepatitis patients with a high bilirubin content. Some pharmacokinetic applications of the method are reported.

INTRODUCTION

Hexobarbital is an ultra-short-acting barbiturate and has been widely used as an intravenous anaesthetic agent[1]. It can also be of great value in rational hypnotic drug therapy, provided that pharmaceutical preparations with an appropriate absorption rate and bioavailability are used[2].

In many species and also in man, hexobarbital is extensively metabolized by the liver[3], which is the reason why this substance is often used in drug metabolism investigations, in studies of metabolic interactions with other compounds (induction, inhibition, etc.).

Very little information is available, however, on the pharmacokinetics of hexobarbital, especially in man. Data such as the half-life, metabolic clearance and apparent volume of distribution are important for the evaluation of drug interactions and in the *in vivo* evaluation of pharmaceutical preparations that contain this drug. Also, changes in metabolic capacity, *e.g.* in cases of liver disease[4], can be followed by studying the pharmacokinetics of this drug in individual patients. A sensitive plasma assay is a prerequisite for such studies.

Although several gas chromatographic methods for the quantitative determination of barbiturates in biological fluids have been described[5–9], in general they

lack sensitivity for measuring therapeutic levels in man. An exception is phenobarbital, which is used as an antiepileptic agent, the therapeutic levels of which are much higher than those encountered in sedative and hypnotic drug therapy[10,11]. For amobarbital and pentobarbital, sensitive methods are available, but they are laborious[12] or they require the formation of derivatives[13]. Williams *et al.*[14] described a rapid and sensitive method for the determination of barbiturates in serum by adding formic acid to the carrier gas.

The use of a nitrogen detector or an alkali flame ionization detector (AFID), a nitrogen-specific and nitrogen-selective version of the thermionic detector (FID), seems to offer new possibilities in the sensitive determination of nitrogen-containing drugs in biological fluids[15]. Goudie and Burnett[16] described the simultaneous determination of phenobarbital, primidone and phenytoin in serum using a nitrogen detector. Other nitrogen-containing drugs such as amphetamines and narcotic analgesics have been analysed with the use of such a detector[17,18].

This paper describes the use of a nitrogen detector in the determination of hexobarbital in human plasma. After 18 months of experience, it can be stated that the method described is highly suitable for the routine measurement of therapeutic hexobarbital levels in plasma.

MATERIALS AND METHODS

Reagents

Hexobarbital (5-cyclohexen-1-yl-1,5-dimethylbarbituric acid) was obtained from OPG, Utrecht, The Netherlands. Methohexital (α-DL-5-allyl-1-methyl-5(1-methyl-2-pentenyl)barbituric acid) was obtained from Eli Lilly, U.S.A. (a gift of methohexital for reference purposes is gratefully acknowledged). Light petroleum, boiling range 40–60°, and amyl alcohol, were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R.). Standard hexobarbital solution was prepared at a concentration of 1 mg per 100 ml of absolute ethanol (analytical-reagent grade, E. Merck). Standard methohexital solution was prepared at a concentration of 3 mg per 100 ml of absolute ethanol (analytical-reagent grade). Silica gel (Kieselgel 60 für Säulenchromatographie, 70–230 mesh) was obtained from E. Merck.

Extraction procedure

Normal plasma. To 2.0 ml of plasma in a conical tube are added 2 ml of distilled water and 0.1 ml of internal standard solution, containing 3 μg of methohexital. The mixture is extracted twice with 10 ml of light petroleum–amyl alcohol (100:2) on a Cenco whirlmixer. Mixing is carried out vigorously for 5–10 sec (twice). The upper organic layer is removed each time with a Pasteur pipette and transferred into a conical evaporation tube. The solvent is evaporated to dryness at 40° in a stream of dry air. The residue is dissolved in 0.1 ml of absolute ethanol and 2–5 μl of this solution is injected into the gas chromatograph using a Hamilton syringe.

Hepatitis plasma. Plasma or serum samples from hepatitis patients sometimes show strong interference with the hexobarbital and methohexital peaks in the gas chromatogram (Fig. 3). Simple purification is obtained by transferring the light petroleum–amyl alcohol extract in 0.2–0.4 ml of absolute ethanol into a silica gel column (I.D. 8 mm, height 4 cm) and eluting with absolute ethanol. The first 3 ml of eluate

are collected and then concentrated to a volume of 0.1 ml, and this solution is analysed by gas chromatography. With the aid of calibration graphs, it was verified that the peak area ratio of hexobarbital to internal standard was not changed by the purification procedure.

Gas chromatography

Apparatus. A Hewlett-Packard (Avondale, Pa., U.S.A.) Model 5750 gas chromatograph, equipped with a dual nitrogen detector (rubidium bromide; Hewlett-Packard, Model 15161A), was used. For comparison of sensitivity and selectivity with a normal flame ionization detection (FID) system, a Hewlett-Packard Model 402 gas chromatograph was used.

Column. A glass column (1.8 m × 4 mm I.D.), packed with 3% OV-17 on Gas-Chrom Q, 60–80 mesh (Applied Science Labs., State College, Pa., U.S.A.), was used.

Temperatures. The temperature of the column was maintained at 230° (isothermal), the injection port at 280° and the detector at 380° (FID 280°).

Gas flow-rates. The carrier gas was helium at a flow-rate of 25 ml/min; in order to obtain optimal performance of the nitrogen detector, an auxiliary stream of carrier gas at a flow-rate of 25 ml/min was led straight into the detection system. The hydrogen flow-rate was 30 ± 0.5 ml/min (FID 40 ml/min), and the air flow-rate was 200 ± 10 ml/min (FID 400 ml/min).

Operation of the nitrogen detector. The distance between the collector, containing the rubidium bromide crystal, and the flame is very important with respect to the achievement of high sensitivity and selectivity with nitrogen-containing compounds. At the beginning of each day, the collector was moved step by step in the direction which gave the maximum ionization current (maximum recorder deflection, range 10^3, attenuation 32). Twice a week the crystal was cleaned by carefully wiping it with a soft brush.

Preparation of calibration graphs

The concentration of hexobarbital in plasma samples was calculated with the aid of calibration graphs, which were prepared by adding known amounts of hexobarbital to 2.0 ml of blank plasma. The samples were run through the extraction procedure described above and the ratio of the peak areas of hexobarbital to internal standard was plotted against known concentrations of hexobarbital.

Recovery studies

Recoveries of hexobarbital at different concentrations were determined by adding known amounts of hexobarbital to 2.0 ml of blank plasma. After extraction, 3.0 μg of methohexital were added and the relative peak area ratio (R_I) was calculated. This value was compared with the ratio (R_{II}) obtained by GC of the same standard amount of hexobarbital with 3.0 μg of methohexital:

$$\text{Recovery } (\%) = \frac{R_I}{R_{II}} \times 100.$$

RESULTS AND DISCUSSION

Gas chromatographic sensitivity and selectivity

In Fig. 1, typical gas chromatograms are shown of hexobarbital and methohexital standards, obtained both by normal flame ionization detection and by nitrogen-selective detection. A large increase in sensitivity when using the nitrogen detector is evident. Barbiturates contain two nitrogen atoms, and as the detector signal is proportional to the nitrogen equivalent in the molecule[19], the presence of two nitrogen atoms favours high sensitivity. The position of the rubidium bromide crystal with respect to the flame and the gas flow-rates are important factors in obtaining a

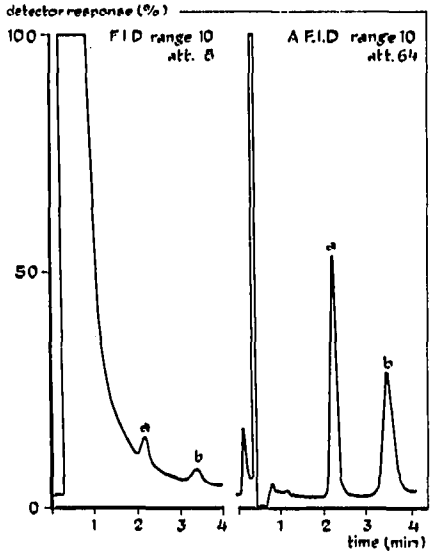

Fig. 1. Difference in response of FID and nitrogen detector (AFID) to methohexital (a) and hexobarbital (b) after injection of 20 ng of each.

high N:C ratio and the influence of these factors has been extensively studied[15,20]. In order to obtain optimal performance, the collector must be adjusted in the correct position each day, which is easily carried out by rotating the adjusting nut. The hydrogen flow-rate should be constant because of its great influence on sensitivity[19]. This constancy is achieved by using a differential flow controller before the normal flow control of the gas chromatograph. A relatively high carrier gas flow-rate is required for high sensitivity and "make up" gas is therefore added at the auxiliary inlet. This addition, of course, is necessary only if a carrier gas flow-rate through the column of less than 50–60 ml/min is required. The negative detector response shortly after injection has been observed by several investigators using a nitrogen detector[21].

The detection limit is about 0.5 ng per single injection for the two barbiturates under investigation.

In Fig. 2, typical gas chromatograms are shown of a plasma extract containing hexobarbital and methohexital. With the normal FID, there is interference of a plasma peak with the hexobarbital. However, when using the nitrogen detector there is a large

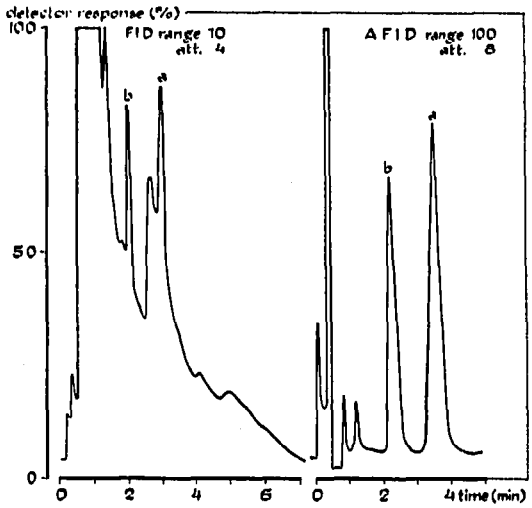

Fig. 2. Gas chromatograms of a 2-ml plasma extract (FID and AFID) obtained from a subject 4 h after receiving 600 mg of hexobarbital orally in a hard gelatin capsule. Injection volume 2 μl. a, hexobarbital (concentration in plasma 2.1 μg/ml); b, methohexital (internal standard; concentration in plasma 1.5 μg/ml).

increase in selectivity towards the nitrogen-containing compounds. There is hardly any solvent peak and the base line has become horizontal. Time-consuming purification would have been necessary if normal FID had been used, whereas the extract can be analysed directly by using the nitrogen detector. Purification is required only in some cases of hepatitis patients with very high plasma bilirubin contents. This can be achieved very satisfactorily by running the extracts through a silica gel column (Fig. 3).

Concentrations in plasma down to 50 ng/ml can be determined.

Extraction procedure and precision

The extraction by light petroleum–amyl alcohol (100:2) was chosen arbitrarily after trying several solvent systems, as it is suitable for the highly lipophilic barbiturates hexobarbital and methohexital (both N-methylated). This solvent system is not suitable, however, for more polar barbiturates as the extraction yields are too low. No interfering plasma constituents are extracted by this solvent system and the formation of an emulsion never occurred. Only amyl alcohol appears in the gas chromatogram shortly after elution of hexobarbital. Vigorous extraction for 5–10 sec (twice) gives highly reproducible results. From the calibration curve in Fig. 4, which is composed of at least six individual calibration curves prepared on different occasions over a period of 1 year, it can be deduced that the procedure has a very good precision over a large concentration range. Standard deviations did not exceed $\pm$ 5%, except at the 2.0 μg/ml level ($\pm$ 7%).

The extraction yields (recoveries) were determined in the same concentration range as those encountered in practice and they appeared to be constant, with a mean value of 65% (Fig. 5). Standard deviations were in the range 3–5% ($n = 4$). Although in general an extraction should give a high yield, in this particular instance the low

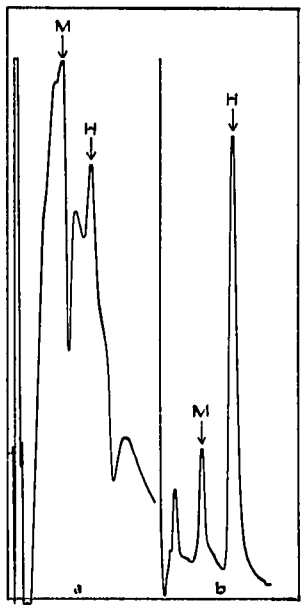

Fig. 3. Gas chromatography of hexobarbital in serum of hepatitis patients with a high bilirubin content: a, gas chromatogram of the crude light petroleum extract; b, gas chromatogram of the same extract after purification through a silica gel column. H = hexobarbital; M = methohexital (internal standard).

detection limit and the good reproducibility permit the use of light petroleum–amyl alcohol as the extraction solvent.

The size of the plasma samples was 2.0 ml throughout the investigation, but it is reasonable to assume that smaller samples can also be analysed satisfactorily by this method. This could be of advantage in clinical situations where the sample size is a limiting factor.

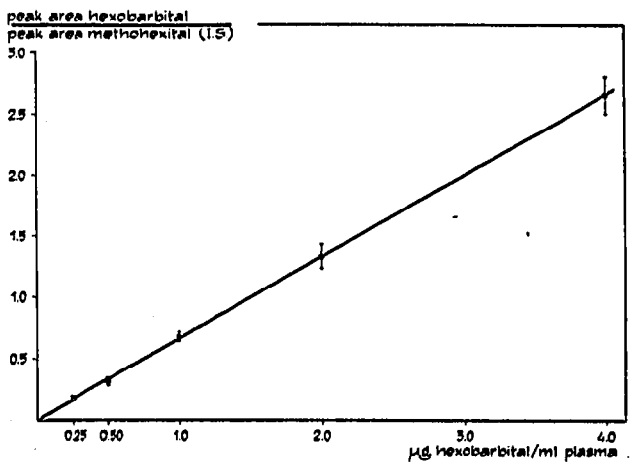

Fig. 4. Calibration curve for the determination of hexobarbital in plasma, using methohexital as internal standard (1.5 µg/ml). Mean values and standard deviations of at least six determinations.

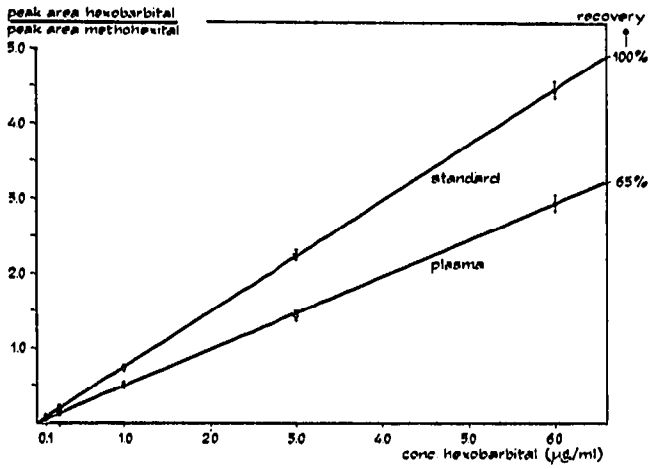

Fig. 5. Peak area ratio of hexobarbital to methohexital as a function of known hexobarbital concentration for the determination of recovery. Mean values and standard deviations ($n = 4$).

Applications

This assay procedure has been used for the study of hexobarbital pharmacokinetics in man after oral, rectal and intravenous administration[2]. An example of a curve obtained after oral intake of hexobarbital is shown in Fig. 6. Absorption and elimination both seem to occur according to a first-order process (linear on logarithmic scale) and the plasma half-life can be calculated from the descending part of the curve. In healthy subjects, the half-life of hexobarbital is in the range of 3–5 h, which is very short in comparison with those of many other barbiturates such as amobarbital (20–25 h)[22] and pentobarbital (50 h)[23]. It was found that the rate of absorption of hexobarbital is slow, whereas its sodium salt is absorbed much more rapidly.

The study of hexobarbital kinetics in patients with liver disease is also possible by this assay, sometimes including purification over silica gel. In many cases, the

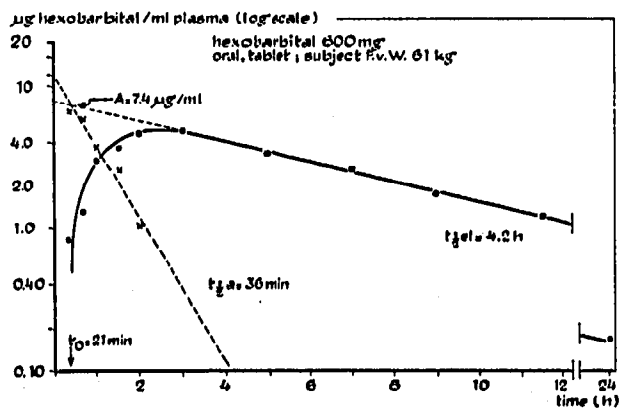

Fig. 6. Plasma concentration curve on a semilogarithmic scale following oral administration of 600 mg of hexobarbital to a healthy human subject. The dotted line represents the absorption phase. $t_{\frac{1}{2}el}$ is the half-life of elimination, $t_{\frac{1}{2}a}$ is the half-life of absorption and t_0 is the lag time.

capacity of the liver to metabolize hexobarbital is strongly reduced during the diseased state, which results in a longer half-life owing to a reduced metabolic clearance[24]. In hepatitis patients, central tolerance to hexobarbital is also significantly decreased[4].

Although very little hexobarbital is excreted into the urine unchanged[3], the method described is sufficiently sensitive to measure its renal excretion rate and cumulative excretion. Urine samples (10 ml) are treated in the same manner as plasma, except that extraction is carried out twice with 20 ml of solvent and cyclobarbital is used as the external standard. Less than 0.5 % of the dose administered is excreted unchanged during the first 24 h.

CONCLUSION

It can be concluded that this method for the assay of hexobarbital in plasma is rapid, sensitive and of good precision. It is therefore suitable for routine analyses serving different purposes. In principle, the use of the nitrogen detector in the determination of therapeutic levels of other barbiturates is very promising and satisfactory results have already been obtained in some of these determinations.

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