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DETERMINATION OF BARBITURATES AND THEIR METABOLITES IN SMALL PLASMA SAMPLES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

AMYLOBARBITONE AND 3'-HYDROXYAMYLOBARBITONE

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SUMMARY

Techniques for the measurement of amylobarbitone and its principal metabolite, 3'-hydroxyamylobarbitone in 100-μl plasma samples are described. The methods which employ a mass spectrometer as a gas chromatographic detector may be used to determine 0.01 μg/ml of the drug and 0.05 μg/ml of the metabolite and were developed initially to permit pharmacodynamic studies in human newborn. With minor modification, they are applicable to other barbiturates including butobarbitone, pentobarbitone and the metabolites 3'-hydroxybutobarbitone and 3'-hydroxypentobarbitone providing scope for more general pharmacodynamic studies with barbiturates where sample size precludes conventional assay.

INTRODUCTION

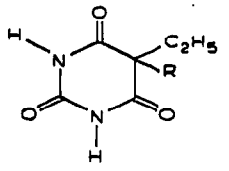
Investigation of drug metabolism in newborn infants is in general hindered by analytical problems in the quantitation of drugs and metabolites in limited volumes of blood. In a study of drug oxidation in the newborn we required to measure the elimination half-life of amylobarbitone (I) and the concentration of its principal metabolite 3'-hydroxyamylobarbitone (IV) in neonatal plasma during several days after exposure of the child to the drug. Published assays for I and IV[1,2] were not sufficiently sensitive for such measurements in the 100–200 μl plasma samples available by serial capillary blood sampling. The required sensitivity and precision of measurement was obtainable using mass spectrometry (MS).

More commonly an instrument for structural identification, a mass spectrometer may, by monitoring one or more abundant fragment ions, become a highly sensitive and specific detector, especially when coupled to a gas chromatograph[3,4]. Quantitative assay from biological material normally requires that response to sample should be measured relative to that of a co-extracted reference compound. This may be the same compound labelled with stable isotopes which can act also as a carrier in the separation procedure and in minimising adsorptive loss of sample in gas chromatography (GC)[5–7]. Alternatively, the reference may be a close analogue,

distinctly eluted by the gas chromatograph[8,9], in which case if adsorptive loss of sample in the column or GC–MS separator is encountered, this must now be eliminated by conventional protective derivatisation.

The barbiturates are particularly amenable to quantitation by the second, more general approach. GC behaviour is optimised by methylation[10] and the N,N'-dimethyl derivatives of barbiturates with an aliphatic side-chain R (I–VI) have abundant fragment ions in common in their mass spectra[11]. Further, since extraction conditions within this group are comparable, one member may provide the internal reference for another.

In this communication we report the development of assays based on single ion monitoring of fragment ions in the mass spectra of methylated barbiturates as they elute from the gas chromatograph. The methods have lower limits in determination of 1 ng for amylobarbitone and 5 ng for the metabolite in 100-μl aliquots of plasma and have been used in monitoring blood levels in newborn children after single therapeutic doses of the drug to the mothers prior to birth.



I-VII

I	= amylobarbitone	$-CH_2CH_2CH(CH_3)_2$
II	= butobarbitone	$-CH_2CH_2CH_2CH_3$
III	= pentobarbitone	$-CH(CH_3)CH_2CH_2CH_3$
IV	= 3'-hydroxyamylobarbitone	$-CH_2CH_2C(OH)(CH_3)_2$
V	= 3'-hydroxybutobarbitone	$-CH_2CH_2CH(OH)CH_3$
VI	= 3'-hydroxypentobarbitone	$-CH(CH_3)CH_2CH(OH)CH_3$
VII	= phenobarbitone	$-C_6H_5$

MATERIALS AND METHODS

Combined GC–MS was carried out using a Varian 1400 gas chromatograph coupled to an AEI MS 12 mass spectrometer. A 6 ft. × 1/8 in. Pyrex column packed with 3 % OV-1 on Gas-Chrom Q (100–120 mesh) was used with on-column injection (block temperature, 250°) and helium as carrier gas. A silicone membrane[12] separator was employed at 240°. At a helium flow-rate of 40 ml/min the mass spectrometer source ionisation gauge indication was 5×10^{-6} torr and analyser 1×10^{-7} torr. The mass spectrometer was operated at R.P. 800 (source slit, 0.005 in.; collector slit, 0.010 in.); source temperature, 250°; accelerating voltage, 8 kV, unless used with an accelerating voltage alternator (AVA)[3]; trap current, 250 μA; ionising voltage, 23 eV and electron multiplier, 1.8 to 2.5 kV. In ion monitoring modes, masses for detection were initially selected using a perfluorokerosene (PFK) marker bleed. After withdrawal of the PFK, fine refocussing was effected during the elution of standard barbiturate derivatives from the gas chromatograph. Output from the head amplifier, modified to incorporate a 1000 MΩ grid resistor, via the bandwith filter at 1 C/sec and main signal amplifier, was taken to a multi-range potentiometric

recorder. Reference spectra were recorded on 1-μg samples at 4 sec/decade magnet scan using a Digital Equipment Corporation PDP 8/I computer for data reduction and column bleed background subtraction.

Tetramethylammonium hydroxide (TMAH) obtained as a 24 % aqueous solution was evaporated to dryness and reconstituted in methanol (0.1 *M*). Dimethyl sulphate-$^2H_6$ (99.5 % $^2H_6$) was used in preparing N-C^2H_3 barbiturate derivatives[13]. Solvents were analytical grade and were not redistilled.

The purity of barbiturate hydroxy metabolite reference samples was established by thin-layer chromatography (TLC) (silica gel; chloroform–acetone, 9:1) and by GC after methylation. [^{14}C]hydroxyamylobarbitone and [^{14}C]hydroxypentobarbitone were prepared from the parent drugs ([^{14}C]amylobarbitone 6.2 μCi/mg, and [^{14}C]pentobarbitone 14.3 μCi/mg) by incubation with rat liver microsomal preparations[14]. The products were separated by differential solvent extraction. Their purity was confirmed by TLC, radio-GLC and GC–MS after methylation.

Plasma assay

Plasma samples were stored at −20 ° until analysis.

(*i*) *Amylobarbitone* (I). To an aliquot of plasma (40 to 100 μl) in a 3-ml capacity tapered vial, internal standard, sodium butobarbitone in water (25 ng in 10 μl) was added and the pH adjusted to 5–6 by addition of 4 *M* NaH_2PO_4 (10 μl). Ether (water saturated, 1.0 ml) was added, mixed (1 min, "Vortex"), centrifuged (2000 r.p.m, 5 min) and 0.8 ml of the ether layer transferred to a further tapered vial and evaporated under nitrogen at room temperature. The residue was finally reconstituted in 0.1 *M* TMAH in methanol (10 μl). 1 μl was injected on to the gas chromatograph (140 °, 40 ml/min) forming the N,N'-dimethyl derivatives on the column[10]. The mass spectrometer response was monitored at *m/e* 169. Calibration for amylobarbitone added to plasma against a constant level of butobarbitone was achieved as a plot of amylobarbitone/butobarbitone derivative, peak height ratio at *m/e* 169 against amylobarbitone concentration.

Alternative procedures (*ii*)*a* and *b* were evalutated for 3'-hydroxyamylobarbitone (IV).

(*ii*)*a*. To an aliquot of plasma (100 μl) was added sodium phenobarbitone in water (40 ng in 10 μl) as internal standard, NaH_2PO_4 (10 μl, 4 *M*) and ether (1.0 ml). A concentrated extract was made as for amylobarbitone and then reconstituted in diazomethane, in ether–methanol, 10:1 (200 μl) left at room temperature for 30 min, evaporated (nitrogen) and finally taken up in methanol (10 μl). 1 μl was injected on to the gas chromatograph (170 °, 40 ml/min). After elution of dimethylhydroxyamylobarbitone, recorded at *m/e* 169, the accelerating voltage was switched to a preset lower value using the AVA in its manual mode, to record the response to dimethylphenobarbitone at *m/e* 175. Calibration was effected as response ratio at *m/e* 169 to 175 against hydroxyamylobarbitone concentration.

(*ii*)*b*. To plasma, buffered to pH 5 as in (*ii*)*a*, hydroxypentobarbitone (V) was added (60 ng in 10 μl of methanol). The aqueous phase was extracted twice with heptane/1.5 % isoamyl alcohol (1 ml). An ether extract was then made and methylated as in (*ii*)*a*. *m/e* 169 was monitored for both sample and reference. A column temperature of 160 ° at a helium flow-rate of 40 ml/min was used.

GC with flame ionisation detection was effected on extracts of large plasma

samples (2 ml) using a Pye 104 gas chromatograph with a 6 ft. × 1/4 in. O.D. Pyrex column packed with 3 % OV-1/Gas-Chrom Q (100–120).

RESULTS AND DISCUSSION

Gas chromatography and mass spectrometry

The tendency of barbiturates to adsorb on to GC columns preventing quantitation at lower levels is widely recognised and is conveniently overcome by methylation. Use of the on-column methylating reagent TMAH[10] resulted in quantitative conversion of the parent compounds I, II and III to their N,N'-dimethyl derivatives. The hydroxylated barbiturates IV–VI did not react reproducibly with this reagent or with the alternative trimethylanilinium hydroxide; they could, however, be reproducibly methylated with diazomethane[11]. Column conditions and retention times of the dimethyl derivatives are given in Table I.

TABLE I

RETENTION TIMES OF METHYLATED BARBITURATES AND FRACTION OF THE TOTAL ION CURRENT (% Σ) AT THE FRAGMENT IONS MONITORED

N,N'-dimethyl compound of	% Σ_{40} *at 23 eV*		*Retention time (min)*	
	m/e 169	*m/e 175*[a]	*140 °*	*170 °*
I	33.4		3.6	
II	39.5		2.9	
III	37.2		3.7	
IV	28.5			2.4
V	22.0			2.2
VI	19.2			3.0
VII		9.3		3.4

[a] *m/e* 175 in the spectrum of N,N'-dimethyl VII is a singlet at a resolving power of 10,000 with the composition $C_{10}H_9NO_2$[17].

The mass spectra of the dimethyl derivatives of I–III and V–VII have been reported[11]. The spectrum of the N,N'-dimethyl derivative of hydroxyamylobarbitone (IV) (Fig. 1) conforms to the pattern for barbiturates with an alkyl side-chain, R, which have fragment ions at *m/e* 169 and 184 in common[11], carrying a substantial fraction of the total ion current (Table I). Monitoring *m/e* 169, 0.1 ng of dimethylamylobarbitone and 0.5 ng of dimethylhydroxyamylobarbitone injected

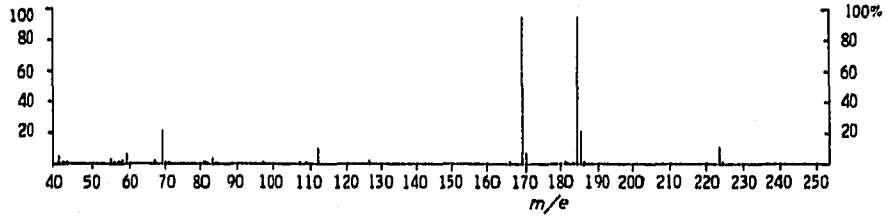
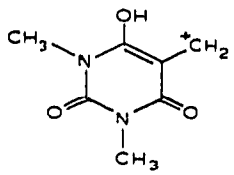
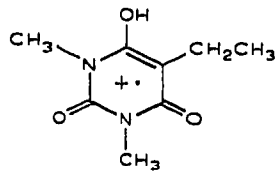


Fig. 1. Mass spectrum of N,N'-dimethyl-3'-hydroxyamylobarbitone at 23 eV.

on to the gas chromatograph were detectable with a signal-to-noise ratio of 6:1. Response in each case was linear to 10 ng.



m/e 169



m/e 184

Losses of dimethylamylobarbitone were apparently negligible through column and GC–MS separator. The detection limit was 0.02 ng and response at 0.1 ng was not significantly enhanced in the presence of a 100-fold excess of the deuterated $(C^2H_3)_2$ derivative as carrier. Losses of the dimethyl derivative of the metabolite, IV, were significant; the linear regression for concentration against response indicated an absolute loss of about 0.1 ng. However, this proved not to be the limiting factor in making the measurements required in plasma. The assays described are based on a carrier-free approach with separately eluted internal references.

Estimation in plasma

The extraction yield of amylobarbitone (pK_a 7.86) (ref. 15) from 100 μl of plasma at pH 5 into ether was determined using the ^{14}C-labelled compound and found to be 95 ± 11.2 % at 10 ng (S.D., $n = 6$) and 105 ± 14.1 % at 100 ng (S.D., $n = 6$). The micro-extraction technique adopted is essentially as described for phenobarbitone (pK_a 7.36) where recovery was judged to be complete[16]. Butobarbitone (pK_a 7.86) provided the internal reference for amylobarbitone. These compounds have similar extraction properties, a suitable relative retention time of their dimethyl derivatives and a comparable detector response per mole at *m/e* 169 (Table I). Calibration for amylobarbitone in plasma (100 μl) against added butobarbitone was linear in the range 4 to 100 ng (Fig. 2), one tenth of the final extract being injected on to the gas chromatograph. Reproducibility of measurement from plasma at 20 ng was ± 2.2 % (S.D., $n = 6$) and at 10 ng it was ± 5.2 %. Below this level the standard deviation rises and at 1 ng it was ± 20 %, an uncertainty discussed in the following section.

Recovery of hydroxyamylobarbitone (IV) from 100 μl of plasma at pH 5 into ether was 85 ± 2.0 % (S.D., $n = 6$) at 100 ng and 83 ± 4.0 % (S.D., $n = 6$) at 10 ng as determined with the ^{14}C-labelled compound. Disparity in retention times between the dimethyl derivatives of I and IV together with artefact formation in the TMAH methylation of IV prevented drug and metabolite measurement in a single plasma extract. A separate reference was required for IV. In early work this was co-extracted phenobarbitone (VII) recording *m/e* 169 for dimethyl IV and *m/e* 175 (9.3 % Σ) for dimethyl VII after diazomethane methylation of plasma extracts. Calibration for hydroxyamylobarbitone in plasma from 5 to 100 ng per 100-μl aliquot was linear as a plot of peak height ratio *m/e* 169 to 175 against plasma concentration (Fig. 3). Reproducibility of estimation at 30 ng was ± 4.7 % (S.D., $n = 6$) and at 15 ng ± 7.0 % (S.D., $n = 6$).

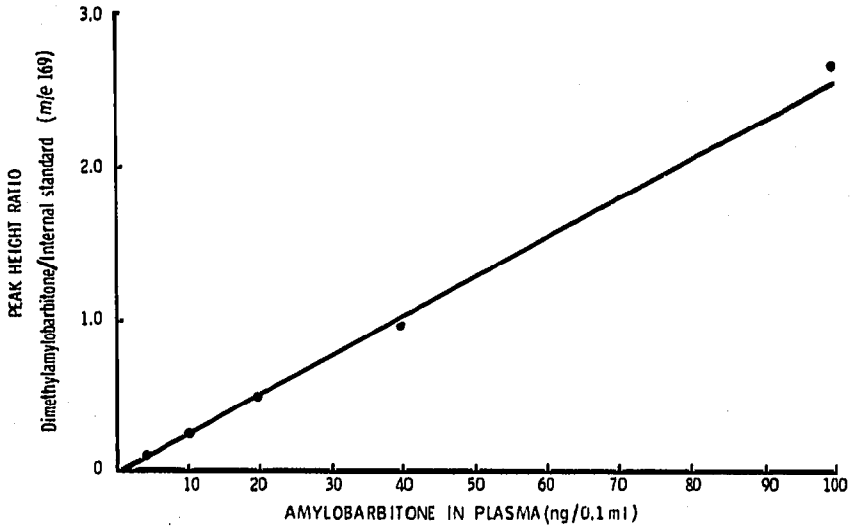


Fig. 2. Calibration for amylobarbitone recovered from 100 μl of plasma against butobarbitone as the reference ($R = 0.995$). Each value is the mean of duplicate estimations. Reproducibility is discussed in the text.

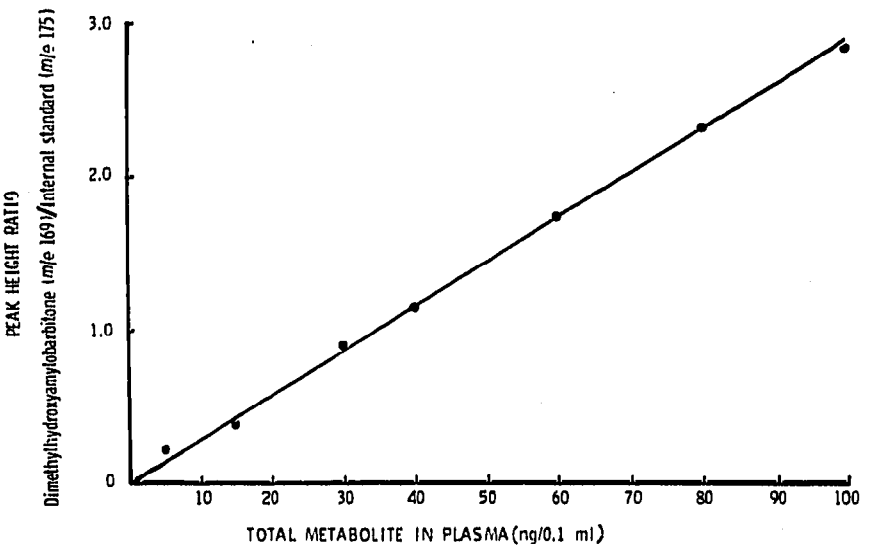


Fig. 3. Calibration for 3'-hydroxyamylobarbitone recovered from 100 μl of plasma against phenobarbitone as the reference ($R = 0.999$). Each value is the mean of duplicate estimations. Reproducibility is discussed in the text.

Satisfactory results were obtained with phenobarbitone as the reference for hydroxyamylobarbitone, however, a subsequent requirement for metabolite measurement in cases where phenobarbitone had also been used therapeutically led to a modified procedure and the adoption of hydroxypentobarbitone (VI) as the reference. A prior heptane/isoamyl alcohol extraction step (METHODS *(ii)b*) was introduced, substantially removing free drug followed by recovery of IV and VI

into ether. Recovery of VI, checked with the ^{14}C-labelled material was 89 ± 3 % (S.D., $n = 6$). Reverting to single ion monitoring at *m/e* 169 (Table I), calibration for IV against VI was linear in the range 5 to 100 ng per 100-µl aliquot of plasma ($R = 0.995$).

Background interference

Figs. 5b and 5d show control plasma extracts with recording of *m/e* 169 in the region of elution of the derivatives of I and II (Fig. 5b) and *m/e* 169 and 175 for the derivatives of IV and VII (Fig. 5d). Interference from co-chromatographing impurities contributing to response at these mass-to-charge ratios is minimal for measurement made within the calibrated range for I and IV. At high electron multiplier and recorder gain, distinction between electronic instability and interfering ionisation becomes equivocal. Repeated injection of 0.1 ng of dimethyl-I and 1.0 ng of dimethyl-II results in a standard deviation of ± 5.1 % in the measurement of I reflecting instrument instability, variance in background ionisation and simple errors in peak height measurement at high amplification. However, recovery from 100 µl of plasma containing 1 ng of I and 10 ng of II with injection of one tenth of the final methylated extract results in a standard deviation of ± 20 % in the measurement of I. A principal contribution to this uncertainty

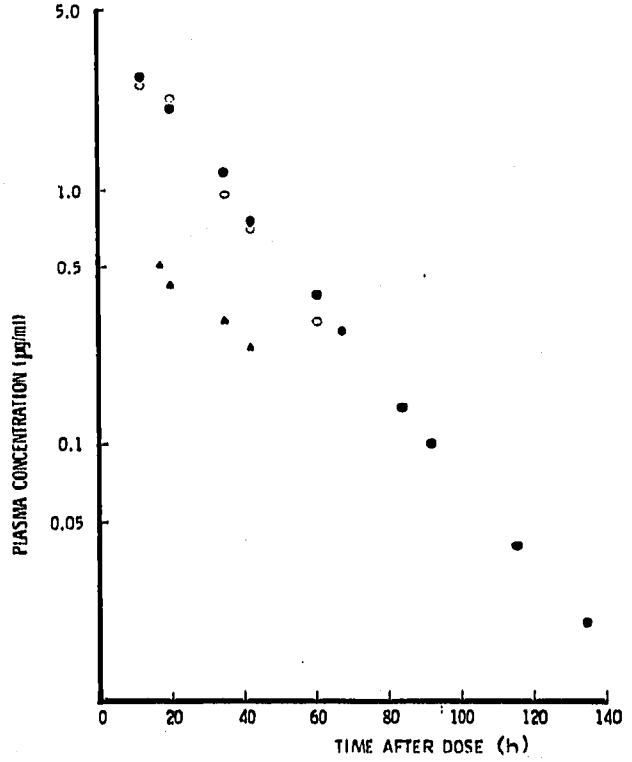


Fig. 4. Plasma concentrations in an adult after an oral dose of sodium amylobarbitone (200 mg). ●, Amylobarbitone ($t^1/_2$ = 16.4 h ± 3.1); ▲, 3′-hydroxyamylobarbitone by GC–MS each measured in 100-µl plasma samples; ○, amylobarbitone by GC with flame ionisation detection in 2.0-ml plasma samples ($t^1/_2$ = 14.9 h ± 4.6).

is inferred to be ionisation of random co-chromatographing material from solvents and plasma which becomes significant at high gain.

Refinement of separation procedures with the possible inclusion of carrier might well permit greater accuracy in measurement of I below 1 ng in plasma.

Clinical application

Plasma levels of amylobarbitone were measured in an adult at intervals during 5 days after a 200-mg oral dose. The half-life of 16.4 ± 3.1 h determined using 100-μl aliquots of plasma was in good agreement with a value of 14.9 ± 4.6 h obtained by GC with flame ionisation detection after extraction of 2.0 ml of plasma samples (Fig. 4). Levels of metabolite were measured in four further 100-μl samples; the concentration fell from 0.5 μg/ml at 16 h to 0.25 μg/ml at 43 h after the dose. Plasma assays with conventional GC detection have limits in the order of 0.2 μg/ml for I and IV after recovery from at least 2.0-ml samples[1,2].

The methods described have been used in a comparison of amylobarbitone plasma half-lives in mothers and newborn infants after administration of the drug to the mothers prior to birth. A more than twice prolonged plasma half-life was observed in the children following placental transfer of the drug from the mother. The results of this study will be published in detail elsewhere[18]. Fig. 5a shows the

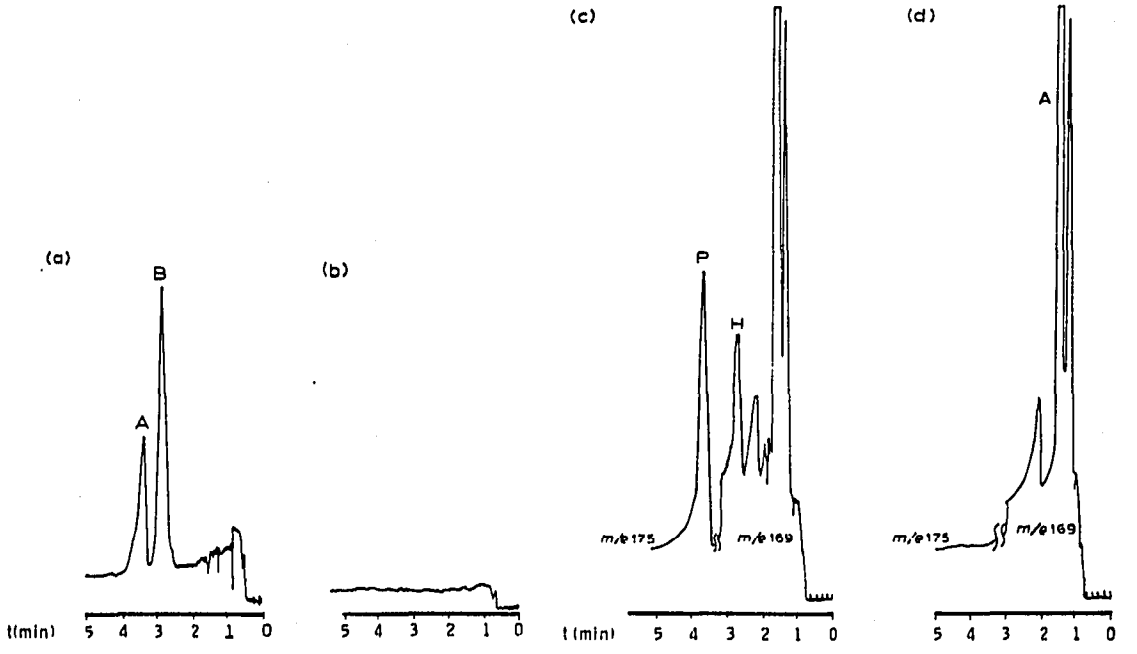


Fig. 5. Chromatograms of control plasma extracts and of plasma extracts from a child 60 h after birth following a 200 mg i.m. dose of sodium amylobarbitone to the mother before delivery. (a) Dimethylamylobarbitone (peak A) and the reference dimethylbutobarbitone (peak B) at *m/e* 169 from 50 μl of the child's plasma. The amylobarbitone concentration was 0.35 μg/ml. (b) Control for Fig. 5a. (c) Dimethylhydroxyamylobarbitone (peak H) at *m/e* 169 and the reference dimethylphenobarbitone (peak P) at *m/e* 175 from 100 μl of the child's plasma. The concentration of metabolite was 0.20 μg/ml. (d) Control for Fig. 5c with the addition of dimethylamylobarbitone (peak A).

chromatogram at *m/e* 169 from an extract of 50 μl of plasma taken from a child 60 h after birth. The level of amylobarbitone was 0.35 μg/ml. The concentration of metabolite (Fig. 5c) measured at the same time after the dose in a further 100 μl of plasma was 0.20 μg/ml.

CONCLUSIONS

By using a mass spectrometer as a GC detector, high sensitivity and specificity in barbiturate and hydroxy metabolite measurements are obtainable after N-methylation. After therapeutic dosage, determinations may be made through several biological half-lives in the limited volumes of plasma obtainable by capillary blood sampling. Satisfactory precision is attained without refinement of separation procedures or recourse to a stable isotope carrier assay. While fully evaluated and in use only for amylobarbitone and its metabolite, the methods are in principle applicable to other barbiturates including butobarbitone, pentobarbitone and their 3'-hydroxy metabolites.

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