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Plasma concentrations of alphaxalone by gas chromatography: comparison with other gas chromatographic methods and gas chromatography-mass spectrometry

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Existing methods for the measurement of plasma or tissue alphaxalone concentrations by gas chromatography are not wholly satisfactory because of the problems of interfering peaks and detector contamination by the silylation reagent. This paper describes an alternative gas chromatographic method using the nitrogen selective alkali flame ionization detector. Plasma samples from patients given Althesin (alphaxalone–alphadolone acetate) were extracted with light petroleum. *O*-Methyl oximes were formed using methoxyamine hydrochloride, and the *O*-methyloxime-3-acetate prepared by heating the extract with acetic anhydride–pyridine. Gas liquid chromatography was carried out on a column of 3% OV 17 on Celite 545 (mesh 80–100), initial temperature 235 °C and increasing by 1 °C min^{-1}. Comparison has been made of the *O*-methyl oxime acetates with trimethylsilyl ethers using both gas chromatography and gas chromatography-mass spectrometry. The results of the two methods were not significantly different. The coefficient of variation for the *O*-methyloxime-3-acetates at a plasma concentration of 1·09 μl^{-1} was 6·9%. This method has an improved sensitivity and selectivity over the existing method, and allows determination of the plasma alphaxalone concentrations found in patients receiving Althesin-supplemented anaesthesia.

Alphaxalone (3α-hydroxy-5α-pregnane-11,20 dione) is the major and active constituent of the anaesthetic agent, Althesin (Glaxo Laboratories Ltd). Procedures developed so far for the measurement of alphaxalone in plasma are not entirely satisfactory, as problems have occurred with interfering peaks or with the silylating reagents which have caused loss of sensitivity by clogging the flame ionization detector jet (Child et al 1972; Dubois et al 1975; Simpson 1978). To measure the plasma concentrations of alphaxalone in patients anaesthetized with Althesin, we have devised a gas chromatographic method that uses the alkali flame ionization (selective nitrogen) detector, chromatographing the drug as the 20-*O*-methyloxime-3-acetate (OMO-acetate).

MATERIALS AND METHODS

Reagents were Analytical Reagent grade (BDH, Poole, Dorset) and were used without purification unless otherwise specified. Light petroleum (b.p. 80–100 °C) was redistilled before use and Pyridine was shaken with activated charcoal (Sigma Chemical Co, Poole, Dorset) and redistilled. Methoxyamine hydrochloride was obtained from Eastman-Kodak Ltd, Kirkby, Liverpool. Steroids were from Steraloids Ltd, Croydon, Surrey. Alphaxalone and alphadolone acetate (21-Acetoxy-3α-hydroxy-5α-pregnane 11, 20-dione) and 3β-Δ^{16}-alphaxalone (3β-hydroxy-5α-preg-16-ene-11,20-dione) were generous gifts from Glaxo Research Laboratories.

Gas chromatography was on 2·13 m columns (i.d. 0·4 cm) of OV1, OV17, QF1 and Silar 10C (Applied Science Europe BV, Holland) coated onto acid-washed, dimethyldichlorosilane-treated Celite 545 (80/100 mesh, PhaseSep Ltd, Queensferry, Flintshire). Carrier gas (oxygen-free nitrogen) flow rate 40 ml min^{-1}. For FID work, hydrogen flow rate was 40 ml min^{-1}, air 500 ml min^{-1}. The alkali FID flow rates were optimized using an AFIDO needle (Pye-Unicam Ltd). Gas chromatography-mass spectrometry (g.c.-m.s.) was with an LKB Model 2091 (LKB Clinicon Systems Ltd, Lewes, East Sussex) using helium as the carrier gas. Mass fragmentography was carried out using a four channel multiple ion detector (LKB 2091–710), focusing on the lowest mass using the magnet current and varying the accelerating voltage to focus alternately on the

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higher masses. Dwell time on each mass was 60 ms, trap current 50 μA and electron energy 20 eV.

Formation of O-methyloxime acetates (OMO-acetates): Plasma (0·5 ml), to which 200 μl M NaOH and 50 μl of a pyridine solution of the internal standard pregnenolone (3β-hydroxy-pregn-5-en-20-one, approx 1 μg/50 μl) had been added was extracted with 4 ml of light petroleum by shaking for 4 min. The organic layer was removed and evaporated to dryness at 40 °C. OMO-derivatives were formed by the addition of 50 μl of pyridine saturated with methoxyamine hydrochloride. The derivatives were incubated at 50 °C for 5 min, then 1 ml 5% (v/v) HCl saturated with ammonium sulphate was added and the mixture extracted with 2 ml light petroleum and the extract evaporated to dryness. Acetates were formed by heating the extract with acetyl chloride or acetic anhydride–pyridine (1 : 1, v/v) at 50 °C for 1 h. Reagents were then removed under vacuum. Light petroleum (20 μl) was added and 10 μl injected on to a gas chromatograph containing a column of 3% OV17, initial temperature 235 °C increasing by 1 °C min^{-1}. For g.c.-m.s., only 1 μl was injected. Trimethylsilyl ethers were formed as described by Sear & Prys-Roberts (1979), using bis-trimethylsilyl acetamide (BSA)–pyridine (2 : 1, v/v) or Simpson (1978) using bis-trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane.

RESULTS

Fig. 1 shows the gas chromatographic tracings from a plasma extract containing approximately 1 μg ml^{-1} alphaxalone as the trimethylsilyl ether on OV1 and OV17 using the FID and as the *O*-methyl oxime and OMO-acetate on OV17 using the nitrogen detector. On OV1, there are many interfering peaks, and the pregnenolone and alphaxalone are not completely resolved. (3β-alphaxalone and 3β-Δ^{16}-alphaxalone have been used as internal standards here and are separated from alphaxalone). On OV17, the separation is much improved, but mass spectrometric analysis of the peaks indicated contamination (arrows). It was originally considered that *o*-methyl oxime derivatives could be used, but an interfering peak was detected which could not be separated from OMO-alphaxalone. There was no interference when OMO-acetates and the nitrogen detector were used, and the sensitivity was high.

Plasma samples from patients given Althesin either by bolus injection or by continuous infusion were analysed as trimethylsilyl esters by g.c., and as OMO-acetates on OV17 using the nitrogen detector and by g.c.-m.s. focusing on the molecular ion minus 31 (M^+-31). For the OMO-acetates measured by g.c.-m.s. and g.c., least squares regression analysis gave the equation $y = 0{\cdot}92x + 0{\cdot}08$ ($n = 25$, $r = 0{\cdot}945$, $P < 0{\cdot}01$). For comparison of trimethylsilyl ethers and OMO-acetates by g.c., regression analysis

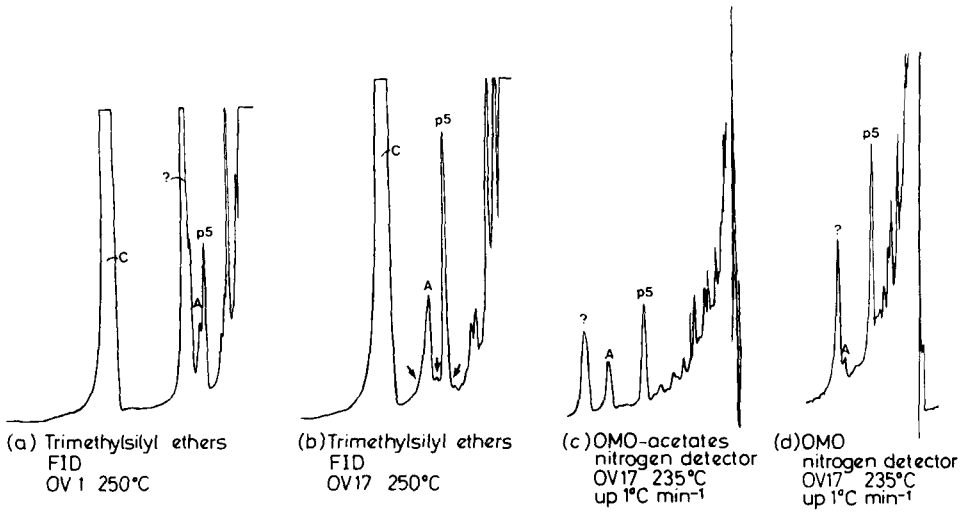

FIG. 1. Gas chromatographic analysis of alphaxalone (A) in human plasma. Pregnenolone (p^5) was added to plasma containing approximately 1 μg ml^{-1} alphaxalone. The plasma was extracted as described in the text. Extracts were analysed by g.l.c. as trimethylsilyl ethers (a) on OV1 at 250 °C and (b) on OV17 at 250 °C using FID, as OMO-acetates on OV17 using the nitrogen detector (c) and as the *O*-methyloxime also on OV17. (d) For the latter two derivatives, the initial column temperature was 235 °C increasing at 1 °C min^{-1}. C indicates the cholesterol-3-trimethylsilyl ether peak and ? indicates an unknown peak. The arrows in (b) indicate areas examined by g.c.-m.s.

gave $y = 1{\cdot}09x + 0{\cdot}22$ ($n = 10$, $r = 0{\cdot}942$, $P < 0{\cdot}01$).

Reproducibility, using OMO-acetates and the nitrogen detector, was apparently slightly worse ($1{\cdot}09\,\mu g\,ml^{-1} \pm 0{\cdot}08$ (s.e.m.); coefficient of variation 6·9%, n = 10) than that reported by Simpson (1978), who reported a coefficient of variation of 4% at this concentration. In the work reported here, pregnenolone has been used as the internal standard and OV17 as the stationary phase for g.c. analysis. Other internal standards have been used (3β-alphaxalone, Child et al 1972, and 3β-Δ^{16}-alphaxalone, personal communication from Glaxo Research Laboratories). Table 1 shows some relative retention times for alphaxalone and alphadolone acetate, and these other possible internal standards run as various derivatives on some stationary phases of differing polarity, ranging from the relative non-selective OV1 to the highly polar Silar 10C.

Table 1. Relative retention times of alphaxalone, alphadolone acetate and related steroids in several different g.l.c. systems.

Approximate retention time for 5α-cholestane (min)	Retention times relative to 5α-cholestane				
	OV17	QF1	Silar 10C		OV1
	280°	260°	240°	260°	240°
	4·5	3·5	2·0	1·5	6·0
Pregnenolone					
free	1·18	2·32		4·16	0·67
3-trimethylsilyl ether	1·00	1·59	1·28		0·80
20-OMO*	1·44	1·42		2·00	0·80
20-OMO-3-acetate	1·74	2·06		1·85	1·10
3α-Alphaxalone					
free	1·81	4·76		12·16	0·90
3-trimethylsilyl ether	1·18	2·88	2·28		0·89
20-OMO	2·18	3·00		4·77	1·14
20-OMO-3-acetate	2·41	4·24		3·85	1·57
3β-Alphaxalone					
free	1·92	5·46		14·62	0·94
3-trimethylsilyl ether	1·52	3·70	3·46		1·14
20-OMO	2·18	3·36		5·54	1·14
20-OMO-3-acetate	2·92	4·65		4·31	1·57
3β-Δ^{16}-Alphaxalone					
free	1·88	5·53		10·12	0·90
3-trimethylsilyl ether	1·54	3·67	2·85		1·18
20-OMO	1·88	3·00		3·89	1·11
20-OMO-3-acetate	2·38	4·14		2·89	1·56
Alphadolone-21-acetate					
free	4·59	12·30		43·08	2·34
3-trimethylsilyl ether	2·92	7·42	8·19		2·15
20-OMO	4·52	8·18		13·39	2·22
20-OMO-3-acetate	5·04	11·65		9·70	2·58
Cholesterol					
free	2·11	2·38		2·47	1·84
3-trimethylsilyl ether	1·74	1·65	2·09		2·30
3-acetate	2·67	3·59	3·19		2·73

* OMO = *O*-methyloxime

Comparison of two different extracting solvents, light-petroleum (80–100 °C) alone and with 10% dichloromethane, was made with direct derivatization of alphaxalone and pregnenolone. Recovery of alphaxalone was found to be greater than 100% with the two solvent systems, indicating a presumed higher partition coefficient for alphaxalone than for pregnenolone when extracting plasma samples.

DISCUSSION

The results of this study indicate that alphaxalone can be assayed satisfactorily as the OMO-acetate derivative, and that the sensitivity and specificity of the method allow measurement of drug concentrations found in the plasma of patients during Althesin supplemented anaesthesia.

The formation of *o*-methyloxime derivatives (Fales & Luukkainen 1965) under the conditions used depends upon the presence of a 20-oxo group in the steroid (11-oxo groups are sterically hindered and do not react). Naturally-occurring steroids with other derivativable oxo groups (notably at positions 3 and 17) are not present in human plasma, under normal conditions, in sufficient amounts to interfere. The major steroid component of plasma, cholesterol, does not form an OMO derivative and is therefore not detected. Analysis times can therefore be shortened. Formation of OMO-acetate derivatives and use of the nitrogen detector confers an extra degree of specificity.

We have found that the OMO-acetate procedure gives, in general, cleaner chromatograms and avoids the problem of detector contamination by the silylating reagent used in the FID systems so far described (Child et al 1972; Simpson 1978). Formation of a 20-OMO derivative also prevents the enolization of the 20-oxo group which has occasionally been observed when preparing trimethylsilyl ethers. In addition, no epimerization of the C17 side chain has been noted when using OMO-acetates. Values obtained by the OMO-acetate procedure compared with values obtained on the same plasma samples using trimethylsilyl ethers and FID gave a good correlation ($r = 0{\cdot}942$) but the OMO-acetates gave the lower values. Both procedures have similar precision. Comparison of values obtained from the OMO-acetate procedure with those estimated by g.c.-m.s., generally accepted as the most specific and sensitive assay for many plasma steroids (Riad-Fahmy et al 1979), gave a good correlation ($r = 0{\cdot}945$) and the slope 0·92 was not significantly different from 1·00. The use of g.c.-m.s. allows detection of alphaxalone in plasma down to 5–10 ng ml^{-1}. Thus the OMO-acetate procedure is an alternative, which although not as good as g.c.-m.s., is more specific and sensitive than earlier methods.

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REFERENCES

Child, K. J., Gibson, W., Harnby, G., Hart, J. W. (1972) Postgrad. Med. J. (Suppl. 2) 48: 37–43

Dubois, M., Allison, J., Geddes, I. (1975) Br. J. Anaesth. 47: 902

Fales, H. M., Luukkainen, T. (1965) Anal. Chem. 37: 955–957

Riad-Fahmy, D., Gaskell, S. J., Dyas, J., Hindawi, R. (1979) Clin. Chem. 25: 665–668

Sear, J. W., Prys-Roberts, C. (1979) Br. J. Anaesth. 51: 861–864

Simpson, M. E. (1978) Ibid. 50: 1231–1236