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Note

Sensitive gas chromatographic method for the determination of alphadolone in plasma

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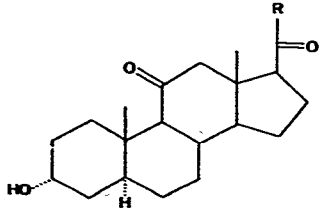
Alphadolone acetate (21-acetoxy-3α-hydroxy-5α-pregnane-11,20-dione; ADA) is one of the two active ingredients of Althesin, a steroidal intravenous anaesthetic [1]. While a number of workers have published assays for alphaxalone (AX) [2—7] an assay for alphadolone (AD) has not been described previously. The aim of the present work was to develop an assay for AD in the plasma of experimental animals as a preliminary to obtaining pharmacokinetic data. It will be shown later that it is important to assay AD rather than its 21-acetate because ADA is rapidly hydrolysed to AD in rat plasma.



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ADA: $-CH_2OCOCH_3$
AD : $-CH_2OH$
AX : $-CH_3$

EXPERIMENTAL

Standards and reagents

Alphadolone (3α,21-dihydroxy-5α-pregnane-11,20-dione), alphadolone 21-acetate, and 3α-hydroxy-2β-*n*-butoxy-5α-pregnane-11,20-dione, the internal standard (IS), were obtained from the Organic Chemistry Department of Glaxo Group Research Ltd. Heptafluorobutyrylimidazole (HFBI) was obtained from Pierce (Chester, Great Britain). A solution (2%, v/v) was prepared in SLR grade toluene (Fisons, Loughborough, Great Britain). Diethyl ether

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(anaesthetic ether, Macfarlan Smith, Edinburgh, Great Britain) was used without further treatment.

Ammonium chloride buffer was prepared by the dropwise addition of concentrated hydrochloric acid (BDH, Poole, Great Britain) to 0.5 *M* aqueous ammonia (Fisons) until pH 9 was attained.

Phosphate buffer was prepared by adding potassium dihydrogen orthophosphate solution (0.5 *M*) to disodium hydrogen orthophosphate solution (0.5 *M*; BDH), until pH 7.4 was attained. The mixture was then diluted to 0.1 *M* total phosphate with water.

Stock solutions of steroids were prepared in ethanol to give concentrations of 10 μg/ml (AD), 50 μg/ml (IS) and 500 μg/ml (ADA).

Apparatus

A Perkin-Elmer Model F33 gas chromatograph equiped with a ^{63}Ni electron-capture detector was used. The glass column (2 m × 4 mm I.D.), packed with 2% Dexsil 300 on Gas-Chrom Q (100—200 mesh), had been conditioned for 24 h at 270°C. For subsequent analytical operation the column temperature was 240°C, the injection port and detector temperatures were 275°C, the carrier-gas was a mixture of argon—methane (9:1) (flow-rate 65 ml/min), the detector pulse setting was 5, and the amplifier attenuation setting was ×256—×64. Peak areas were measured using a Trivector Triton 3 computing integrator (Trivector Systems, Sandy, Bedfordshire, Great Britain).

The temperature of the column was raised to 265°C and that of the detector to 300°C each night to maintain detector sensitivity and minimise adsorption.

For mass spectrometry a Varian Aerograph series 2740 gas chromatograph combined with a Varian MAT 311A mass spectrometer were used. The electron energy was 70 eV and the temperature of the ion source 200°C. The carrier gas was helium (flow-rate 40 ml/min), but all other conditions were the same as those used for the conventional gas chromatography (GC) described previously.

Extraction and derivatisation

Internal standard solution (25 μl = 1.25 μg) was added to 1 ml of plasma in a 50-ml glass-stoppered test-tube, then 1 ml of ammonium chloride buffer and 10 ml of diethyl ether were added. The mixture was shaken by hand for 1 min. The organic layer was transferred by pipette to a conical test-tube, an anti-bumping granule was then added, and the solution was evaporated to dryness at 45°C.

The residue was dissolved in HFBI solution (100 μl), the tube was stoppered, and the mixture was incubated for 10 min in a 55°C water-bath. Phosphate buffer (0.5 ml) was then added and mixed by vortexing for 15 sec and the mixture was then centrifuged at 300 *g* for 5 min at room temperature. The aqueous phase was discarded, the organic layer was diluted with 700 μl toluene and 1 μl of this solution was injected into the GC column.

Calibration graph

Using the procedure described above, a calibration graph was obtained by

running plasma samples spiked with AD at concentrations varying from 10—600 ng/ml and with IS at a fixed concentration of 1.25 μg/ml.

RESULTS AND DISCUSSION

Typical chromatograms obtained from blank rat plasma, and plasma spiked with AD (300 ng/ml) and IS (1.25 μg/ml) are shown in Fig. 1. Similar chromatograms obtained using human plasma are shown in Fig. 2. The retention times of AD and IS were 6.5 and 8.5 min respectively. ADA had a retention time of 12 min but is very unstable in rat plasma. The success of the derivatisation of AD and IS with HFBI was confirmed by gas chromatography—mass spectrometry (GC—MS, Fig. 3).

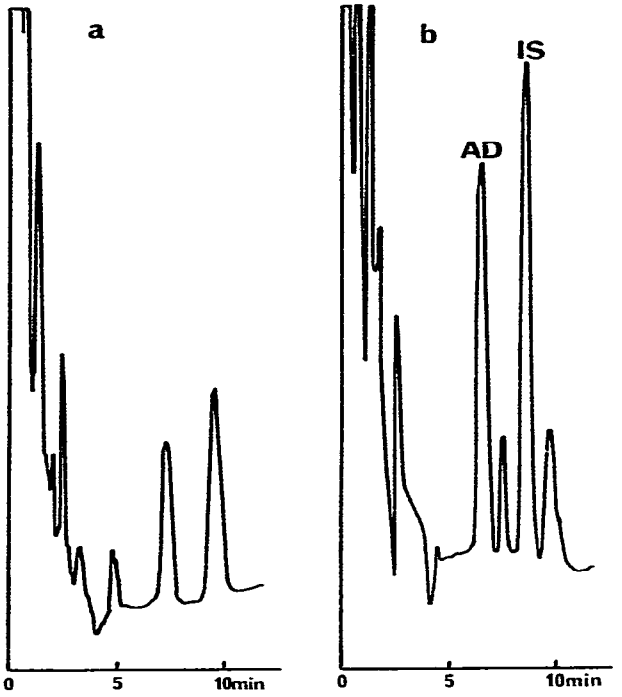

Fig. 1. Gas chromatograms of extracts from (a) normal rat plasma and (b) rat plasma spiked with 300 ng/ml of AD and 1.25 μg/ml of IS.

A calibration graph for extracted AD obtained by plotting the ratio of the peak area of AD to that of IS against the concentration of AD in rat plasma is shown in Fig. 4. The graph was slightly sigmoidal in the range studied, and the minimum measurable concentration (as determined by inspection) was 10 ng/ml. The corresponding value in human plasma is likely to be somewhat higher than this due to the less favourable blank chromatogram.

The accuracy and precision of the assay were determined by analysing samples of plasma spiked with several concentrations of AD. The results are presented in Table I.

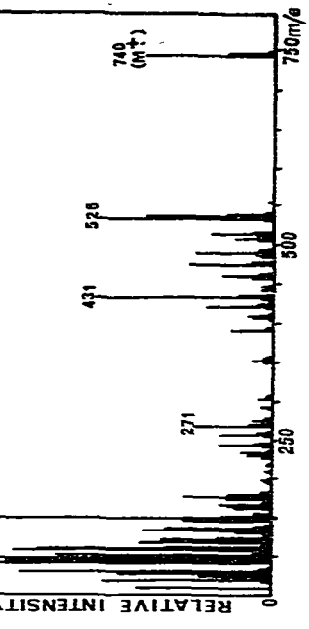

Fig. 2. Gas chromatograms of extracts from (a) normal human plasma and (b) human plasma spiked with 300 ng/ml of AD and 1.25 μg/ml of IS.

Fig. 3. Mass spectra obtained during GC–MS analysis for (a) AD-diHFB and (b) IS-monoHFB.

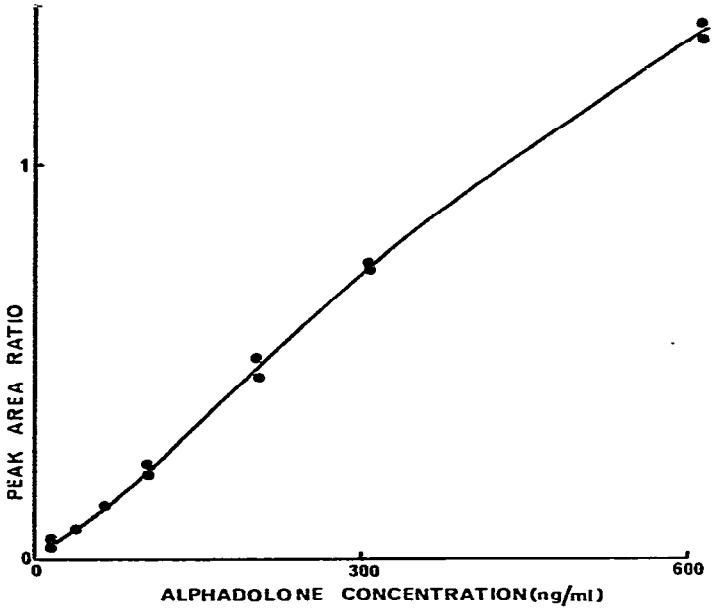

Fig. 4. Calibration graph for AD with IS.

TABLE I

RECOVERY OF ADDED ALPHADOLONE FROM PLASMA

Alphadolone (ng/ml)	
Added	Found (± S.E.M.)*
50	47 ± 1
200	181 ± 4
600	584 ± 18

*Each value is the mean of 5 estimations; S.E.M. = standard error of the mean.

When rat plasma, to which ADA had been added at a concentration of 1 μg/ml, was assayed it was found that within 5 min the amount of AD formed corresponded to complete hydrolysis of the ester. This indicates that AD, and not ADA, is the appropriate steroid to assay after the administration of Althesin to rats.

Fig. 5 shows that the method allowed the determination of the plasma concentration—time curve after a single intravenous anaesthetic dose of Althesin (= 3 mg/kg ADA) to rats. The plasma concentration of AD 2 min after dosing was 1.8 μg/ml, and at this time the concentration of ADA was less than 0.05 μg/ml, the lowest concentration of ADA that could be detected under these conditions. The half-life of AD, when measured between 2 and 50 min after dosing, was 7 min. This is very similar to the half-life of AX in rats [2]. Beyond 50 min the plasma concentration rose slightly from 0.015 μg/ml to 0.028 μg/ml and then resumed its fall. This disturbance in the plasma level—time curve has been observed for other steroidal anaesthetics (un-

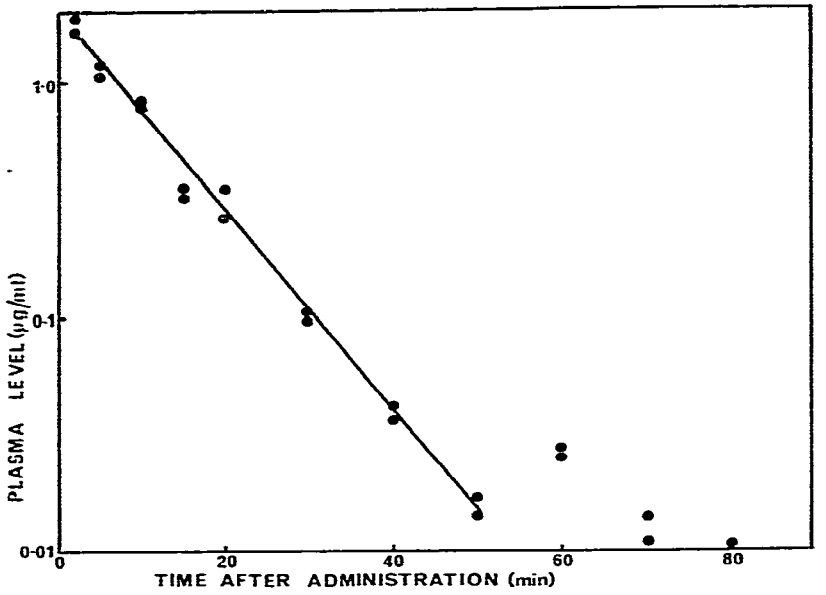

Fig. 5. Plasma levels of alphadolone in rats after a single intravenous dose of Althesin of 1 ml/kg. A separate animal was used for each time point. Samples were assayed in duplicate, and both values are shown.

published observations) and is probably not spurious. It could reflect the release of AD from a tissue depot into the blood stream or be a result of biliary recirculation.

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