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Note

Simple high-performance liquid chromatographic method for the determination of medroxyprogesterone acetate in human plasma

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Medroxyprogesterone acetate (MPA) is widely used in the treatment of breast and endometrial cancer [1]. Plasma concentrations of MPA in the literature have been determined mainly by radioimmunoassay (RIA) [2] and gas—liquid chromatography (GLC) [3]. RIA has the advantage of sensitivity (100 pg MPA) but its limited selectivity can lead to overestimation of MPA [4], whereas GLC methods are selective but require derivatisation. A simple high-performance liquid chromatographic (HPLC) method using Sep-Pak extraction cartridges has previously been reported [5].

We propose an alternative HPLC method which employs solvent extraction and a more conventional HPLC column. The method has been successfully automated and is therefore suitable for the rapid and inexpensive analysis of large batches of samples.

EXPERIMENTAL

Materials

All chemicals were of AnalaR grade and obtained from commercial sources. The solvents, methanol and hexane, were of HPLC grade. The steroids were

obtained from Sigma (Poole, U.K.). Pooled human plasma was collected from healthy subjects; EDTA was the anticoagulant.

Standards

Medroxyprogesterone acetate (6α-methyl-17α-hydroxyprogesterone acetate) was obtained from Sigma. Stock standards (1 mg/ml) and working standards (1 and 10 μg/ml) of MPA were prepared in methanol and stored at 4°C for up to one month.

The internal standard 16β-methylprogesterone, was obtained from Sigma and stock solutions (100 μg/ml) were prepared in methanol. The internal standard working solution (0.5 μg/ml) was prepared daily by dilution of the stock solution in 0.2 *M* phosphate buffer, pH 7.0.

Extraction procedure

Plasma (2.0 ml) was transferred to a disposable screw-capped glass tube and 1.0 ml of the internal standard working solution was added. After brief vortex-mixing (3 sec), 7.0 ml hexane were added and the tube was sealed with a PTFE-lined cap. After gentle mixing on a rolling mixer (Luckham, Burgess Hill, U.K.) for 30 min, the hexane layer was transferred to a clean glass tube and evaporated to dryness under nitrogen at 30°C. The residue was dissolved in 200 μl of the HPLC mobile phase and 150 μl were injected onto the HPLC column.

Chromatography

The stainless-steel HPLC column, 25 cm × 4.6 mm I.D., contained 5-μm reversed-phase particles of Spherisorb 5-ODS2 (Hichrom). The mobile phase comprised methanol—0.02 *M* acetate buffer, pH 4 (79:21), and was pumped at a flow-rate of 1.5 ml/min (Beckman pump, Model 110A). A variable-wavelength Pye Unicam LC-UV detector was used in conjunction with a pen recorder (J.J. Instruments) to monitor the eluent at 240 nm (the λ_{max} of MPA) at a sensitivity of 0.05 a.u.f.s. Injections were made using a Rheodyne 7125 manual loop injector or a Waters WISP automatic sampler with limited-volume inserts.

Calibration

Calibration standards were prepared by the addition of 10—50 μl of an MPA working standard to 2.0-ml aliquots of pooled blank plasma. At least six calibration standards containing between 5 and 250 ng/ml MPA were analysed with each batch of samples.

Recovery

Duplicate spiked plasma samples containing 100 ng/ml were analysed as described except that exactly 5.0 ml of the 7.0-ml hexane extract were evaporated under nitrogen. A 1 μg/ml solution of MPA in mobile phase was injected to give a peak height equivalent to 100% recovery. The recovery of MPA from the spiked plasma was calculated as follows:

$$\text{Recovery} = \frac{\text{mean sample peak height}}{1\ \mu\text{g/ml MPA peak height}} \times \frac{7}{5} \times 100\%$$

Reproducibility and stability

Pooled plasma was spiked with MPA at 25.8, 103 and 258 ng/ml, and aliquots were stored at −20°C.

Intra-assay variation was determined on two occasions by analysis of five samples at each concentration, spread randomly throughout the batch of samples. Inter-assay variation was assessed by analysis of one sample at each concentration on five separate occasions.

The stability of MPA at −20°C was assessed by comparing the assayed concentration with the original spiked concentration of MPA over a period of four months.

RESULTS

Selection of chromatographic conditions

Reversed-phase packing materials and a methanol—aqueous mobile phase were suitable for the chromatography of MPA. The retention of MPA by various types of column packing was examined by comparison of their phase capacity ratios (k') for MPA (see Table I). Since a 25-cm Spherisorb 5-ODS2 column gave the greatest retention of MPA, this was the column of choice. Although the retention of MPA was not affected by changes in pH or buffer molarity of the mobile phase, we preferred to control the pH and a 0.02 *M* acetate buffer at pH 4 was used. The methanol content of the mobile phase was optimised to achieve the best separation of low levels of MPA from endogenous peaks, in the shortest time. Under the conditions described, retention times of MPA and 16β-methylprogesterone, the internal standard, were 5.3 and 9.0 min respectively.

TABLE I

EFFECT OF COLUMN PACKING MATERIAL ON THE RETENTION OF MEDROXYPROGESTERONE ACETATE

HPLC conditions as described in text.

Column packing	Column length (cm)	Phase capacity ratio (k')* of MPA
Spherisorb 5-C_8	25	1.6
μBondapak C_{18}	30	1.8
Hypersil ODS	25	2.5
Spherisorb 5-ODS	25	2.8
Spherisorb 5-ODS2	25	3.5

*$k' = \frac{t_R - t_0}{t_0}$ where t_R = retention time of drug and t_0 = retention time of an unretained solute.

Chromatograms

Typical chromatograms of pooled plasma (A), a calibration standard (B), and a patient's sample (C) are shown in Fig. 1. The pooled plasma chromatogram shows no interference at the retention time of MPA, and is typical of many of the individual blank plasma chromatograms investigated. However, small inter-

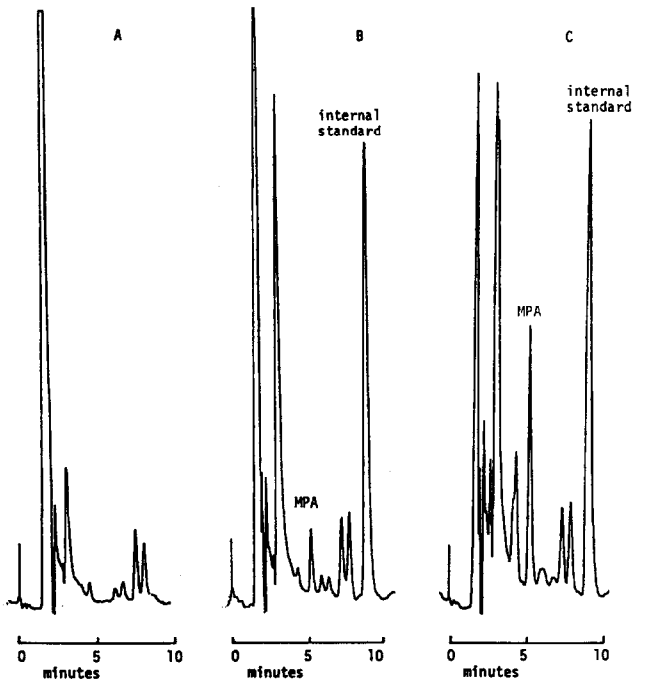

Fig. 1. Typical chromatograms of plasma samples. (A) Pooled plasma; (B) calibration standard containing 25 ng/ml medroxyprogesterone acetate (MPA) and 16β-methylprogesterone (internal standard), 500 ng; (C) patient sample following oral administration of Provera; MPA concentration = 115 ng/ml.

fering peaks equivalent to approximately 5 ng/ml MPA were occasionally found in plasma samples from healthy female subjects. A broad peak eluting after 50—60 min was also present in some plasma samples. The use of lignocaine as a local anaesthetic during blood collection, produced a large interfering peak in the resulting plasma chromatogram. The peak was found to be lignocaine itself, which should therefore not be used in conjunction with this method.

TABLE II

RETENTION TIMES OF ENDOGENOUS STEROIDS

HPLC conditions as described in text.

Compound	Retention time (min)
6α-Methyl-17α-hydroxyprogesterone acetate (MPA)	5.3
16β-Methylprogesterone (internal standard)	9.0
Progesterone	6.8
17-α-Hydroxyprogesterone	4.0
Cortisone	2.5
Corticosterone	3.0
Aldosterone	2.4
Testosterone	4.0
Androstenedione	3.8
Oestradiol	3.5
Cholesterol	Not detected

The similarity between plasma chromatograms from patients receiving MPA therapy and those of healthy subjects, suggests that MPA metabolites were not detected by this method.

Retention times of a selection of endogenous steroids are shown in Table II. None had the same retention times as MPA or 16β-methylprogesterone.

Sensitivity

The absorption spectrum of a methanolic solution of MPA showed the λ_{max} to be at 240 nm (see Fig. 2). The assay sensitivity was therefore optimised by UV monitoring at this wavelength.

Although 5 ng/ml MPA could be reliably detected in spiked pooled plasma, individual plasma samples occasionally contained an interfering peak equivalent to 5 ng/ml MPA, as described above, therefore the sensitivity of the method was limited to 10 ng/ml MPA.

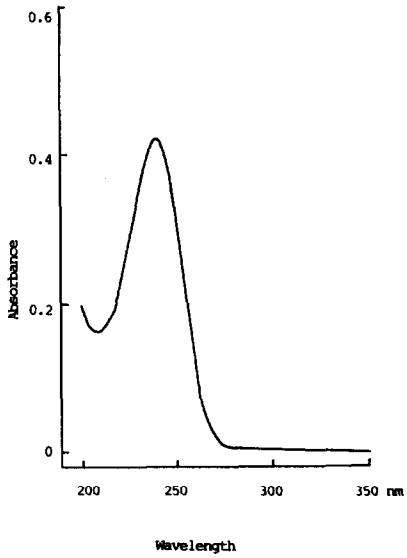

Fig. 2. Absorption spectrum of medroxyprogesterone acetate (MPA), a 10 μg/ml solution in methanol; λ_{max} = 240 nm.

TABLE III

RECOVERY OF MEDROXYPROGESTERONE ACETATE (MPA) IN SOLVENTS OF VARYING POLARITY

Plasma concentration of MPA = 100 ng/ml.

Extraction solvent	Recovery (%)
Dichloromethane	10
Ethyl acetate	37
Cyclohexane	66
n-Pentane	84
Hexane	87
Diethyl ether	91

Recovery

The mean recovery of 100 ng/ml MPA from plasma, at pH 7, was 86 ± 1.5% S.D., n = 5. The effect of extraction pH on MPA recovery was investigated by addition of 1.0 ml of 0.2 *M* buffers at pH 2, 5, 7, 9 or 12, prior to extraction of 100 ng/ml MPA from plasma. Recoveries (79—86%) were not affected by the pH of extraction over the range studied. Extraction of plasma containing 100 ng/ml MPA with solvents of varying polarity, resulted in recoveries ranging from 10% to 91%, as shown in Table III. MPA was efficiently extracted by pentane, hexane and diethyl ether but hexane was the solvent of choice since fewer endogenous background materials appeared in the chromatograms.

Reproducibility and linearity

Reproducibility data at 25.8, 103 and 258 ng/ml MPA are shown in Table IV. Intra- and inter-assay coefficients of variation (C.V.) were 1.1—7.3% and 1.2—3.5%, respectively.

Calibration curves were linear between 5 and 250 ng/ml, and the mean coefficient of correlation (r) was 0.999 (n = 8).

TABLE IV

INTRA- AND INTER-ASSAY VARIATION

MPA concentration (ng/ml)	Intra-assay C.V.* (%)	Inter-assay C.V.* (%)
25.8	7.3, 6.6	3.1
103	3.8, 1.6	3.5
258	1.2, 1.1	1.2
Mean	3.6	2.6

*Coefficient of variation (C.V.) = 100% × standard deviation/mean (n = 5).

Sample stability

Spiked plasma samples stored at −20°C showed no deterioration over a period of four months.

Drug monitoring in patients

Twenty patient's samples have been analysed using this method. The patients had been receiving oral MPA therapy at 1 g per day, for at least 21 days, for the treatment of breast cancer, and samples were taken 2—4 h after the morning dose of 500 mg MPA. The plasma concentrations of MPA were between 12.6 and 270 ng/ml with a mean value of 104 ng/ml (± 68.9 S.D.).

The plasma profile of MPA was studied in one patient who had been taking oral MPA, 500 mg, once a day, for at least 21 days previously. Plasma concentrations were monitored at 0, 1, 2, 3, 4, 6 and 8 h after dosing (500 mg MPA) and are shown in Fig. 3.

The MPA concentration before dosing (i.e. 24 h after the previous dose) was 5 ng/ml, and the peak plasma concentration, 118 ng/ml, occurred at 3 h after dosing.

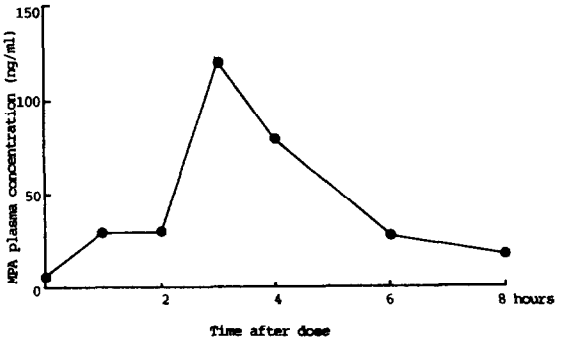

Fig. 3. Plasma profile of medroxyprogesterone acetate (MPA) following a 500-mg oral dose, in a patient who had been taking 500 mg once a day for at least 21 days previously.

DISCUSSION

This HPLC method was developed as a simple, reliable and selective alternative to the current methods [2, 3, 5] available for measuring plasma concentrations of MPA. A solvent extraction followed by HPLC separation and analysis appeared to be the most straightforward approach.

MPA was efficiently extracted, at all pH values, by non-polar solvents (Table III). Hexane extraction at pH 7 gave the cleanest chromatogram and the use of HPLC-grade solvents considerably reduced the background noise. Emulsion formation was avoided by a gentle mixing technique, and contamination was minimised by the use of disposable glassware.

The Spherisorb 5-ODS2 HPLC column gave excellent separation of MPA from the endogenous steroids of similar structure and from the extraneous plasma blank peaks, many of which originated from the hexane.

The main advantage of this method is its rapid yet inexpensive sample preparation. A batch of 30—40 samples can be prepared for HPLC analysis in 2—3 h, and the chromatographic run time is 10 min per sample. Sample extracts are stable for at least 24 h at +4°C, if storage is required before injection.

Calibration curves between 5 and 250 ng/ml show a good correlation (r = 0.999) between peak height ratio and MPA concentration, even at low levels. The reliability of the method is shown in the low C.V. values (3.6% and 2.6%) of the intra- and inter-assay analyses (Table IV).

Plasma concentrations of MPA in patients receiving 1 g per day were between 12.6 and 270 ng/ml in this study, suggesting that the sensitivity of the method, 10 ng/ml, is sufficient for monitoring therapeutic concentrations of MPA. These results show a wide individual variation in plasma concentrations following similar dosing schedules, as reported by other workers [6]. The plasma profile following once daily dosing at 500 mg MPA (Fig. 3) covered a wide range of MPA concentrations (5—118 ng/ml) during the 24-h dosing interval. Since the response of the drug has been related to the plasma concentration [7], routine monitoring would be helpful in the use of this drug.

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