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SPECIFIC AND SENSITIVE QUANTITATION OF MEDROXYPROGESTERONE ACETATE IN HUMAN SERUM BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A procedure for the quantitation of medroxyprogesterone acetate in serum using gas chromatography–quadrupole mass spectrometry is described Medroxyprogesterone propionate, synthesized from medroxyprogesterone, was used as the internal standard The serum samples were extracted on Bond Elut C_{18} cartridges, and the acetate and propionate were determined as their 3-enol heptafluorobutyrate esters by selected-ion monitoring technique The linear range of the standard curve in serum was 0 5–30 ng/ml with a lower limit of quantitation of 0 5 ng/ml The coefficient of variation of the method was 3 1% at 10 ng/ml and 5 5% at 1 ng/ml The method is very rapid, and it has been applied for routine measurements of medroxyprogesterone acetate levels in human serum after oral administration of 10 or 20 mg

INTRODUCTION

Medroxyprogesterone acetate (6α-methyl-17α-acetoxyprogesterone, MPA) is a synthetic steroid derived from progesterone MPA has a strong progestional effect, unlike progesterone, when administered orally MPA is used in the control of fertility [1], treatment of breast cancer [2], prostatic carcinoma [3] and endometrial carcinoma [4]

Several analytical methods for the quantitation of blood MPA levels have

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been developed, such as gas chromatography (GC) with electron-capture detection [5, 6], high-performance liquid chromatography (HPLC) [7, 8], gas chromatography–mass spectrometry (GC–MS) [9, 10] and radioimmunoassay (RIA) [11, 12] The sensitivity and the selectivity of GC and HPLC methods limit their use in the quantitation of MPA Dikkeschei et al [9] reported a specific GC–MS method for MPA as a trifluoroacetate (TFA) derivative in serum after oral administration (400 mg of MPA daily) RIA is most commonly used in the analysis of MPA in clinical chemistry because of its simplicity and commercial availability, but the cross-reactivity of MPA with other steroids [13] limits its use in pharmacokinetic studies

This paper describes a GC–MS method using a selected-ion technique for the determination of MPA in human serum after a single oral dose (10 or 20 mg) We measured MPA as a heptafluorobutyrate (HFB) derivative, which improved the sensitivity of the method by producing a better response and a clean baseline at focused channels monitored during the assay Preparation of a sample with the Bond Elut cartridge was very rapid, and the method is suitable for routine measurements of MPA

EXPERIMENTAL

Reagents

MPA was kindly supplied by Leiras (Turku, Finland) Heptafluorobutyric anhydride (HFBA) and medroxyprogesterone (MP) were from Sigma (St Louis, MO, U.S.A). Trifluoroacetic anhydride (TFAA) and chloroform were from Merck (Darmstadt, F R G) and propionic acid from Merck (Munich, F R G). Methanol and hexane were from Baker (Deventer, The Netherlands) The Bond Elut C_{18} cartridges were from Analytichem International (Harbor City, CA, U S.A)

Preparation of the internal standard

The internal standard, medroxyprogesterone propionate (MPP), was synthesized from MP The esterification was modified from previous reports [14, 15] MP (ca 50 mg) was added to a pre-heated mixture of propionic acid (0 5 ml) and TFAA (0 5 ml), and the mixture was incubated at 60 °C for 30 min The mixture was poured into 100 ml of a 5% sodium bicarbonate solution, and the MPP was extracted with chloroform (2×50 ml) The solvent was evaporated under vacuum, and the residue was dissolved in methanol to a concentration of ca 12 5 ng/ml This concentration was derived from comparison of the GC response of an aliquot of this solution with the response of a known concentration of MPA, after derivatization

Preparation of the standard curve

MPA (5 mg) was dissolved in methanol (200 ml) and diluted with methanol to final standard concentrations of 300, 100, 50, 10 and 5 ng/ml The standard curve was prepared by addition to 1 ml of serum of 100 μl of each final standard

solution 300 ng/ml (30 ng), 100 ng/ml (10 ng), 50 ng/ml (5 ng), 10 ng/ml (1 ng) and 5 ng/ml (0 5 ng) A 100-μl volume of the internal standard solution was added to each standard sample The samples were shaken on a multi-tube vortex for 60 s, allowed to stand at room temperature for 30 min, and extracted as described in the extraction procedure

Extraction procedure

Methanol (100 μl) was added to a serum sample (1 ml) followed by 100 μl of the internal standard solution (MPP) After mixing in a multi-tube vortex for 60 s, the sample was allowed to stand at room temperature for 30 min The mixture was extracted with a Bond Elut (C_{18}, 3 ml) cartridge in a Vac Elut system The cartridge was pre-washed with 2 ml of methanol and 2 ml of distilled water During the washing a small amount of solution was left on the column A 1-ml serum sample with 100 μl of methanol and 100 μl of the internal standard were applied to the cartridge, and the cartridge was washed three times with 2 ml of distilled water, then allowed to dry under vacuum for 10 s Elution of the cartridge was performed with 1 ml of methanol The eluate was evaporated to dryness under a stream of air

Derivatization

The residue of the eluate was dissolved in 200 μl of acetone–acetonitrile (1 1), and 50 μl of HFBA were added The silanized tube was closed with a PTFE-lined cap The tube was shaken on a multi-tube vortex for 30 s After 60 min at room temperature the solution was evaporated to dryness under a stream of air, 5 ml of hexane were added, and the solution was washed once with 4 ml of water The hexane phase was evaporated to dryness under a stream of air, and the residue was dissolved in 50 μl of hexane on a multi-tube vortex for 60 s

Gas chromatography–mass spectrometry

A Hewlett-Packard 5890 gas chromatograph interfaced with a VG TRIO-2 quadrupole mass spectrometer was used The samples were introduced by splitless injection at 270°C on OV-1 fused silica capillary column (25 m$\times$0 32 mm I D , Nordion, Helsinki, Finland) The column was initially at 200°C for 1 min, and the temperature was then raised at 25°C/min to 300°C, which was maintained for 2 min Helium was used as a carrier gas with a flow-rate of 1 ml/min The mass spectrometer operating conditions were the following ion source, 250°C, electron energy, 70 eV, ionization current, 100 μA, interface, 270°C The mass spectrometer was set to monitor m/z 582 2 and m/z 596 2$\pm$0 5

RESULTS

Selection of MPA derivative

For quantitation purposes we evaluated the TFA, PFP and HFB derivatives The selection of the reagent was based on the minimum formation of the enol isomer, the recovery of the derivatization step, the simplicity of the derivati-

zation, and the possibility of using the most selective monitoring ion for quantitation Use of the TFAA reagent in the derivatization procedure created a problem in the enolization of MPA. the TFA derivative and its enol isomer had identical molecular ions and similar fragmentation patterns Studies of the derivatization also indicated that the response of an equal amount of the PFP derivative or the HFB derivative was double that of the TFA derivative Be-

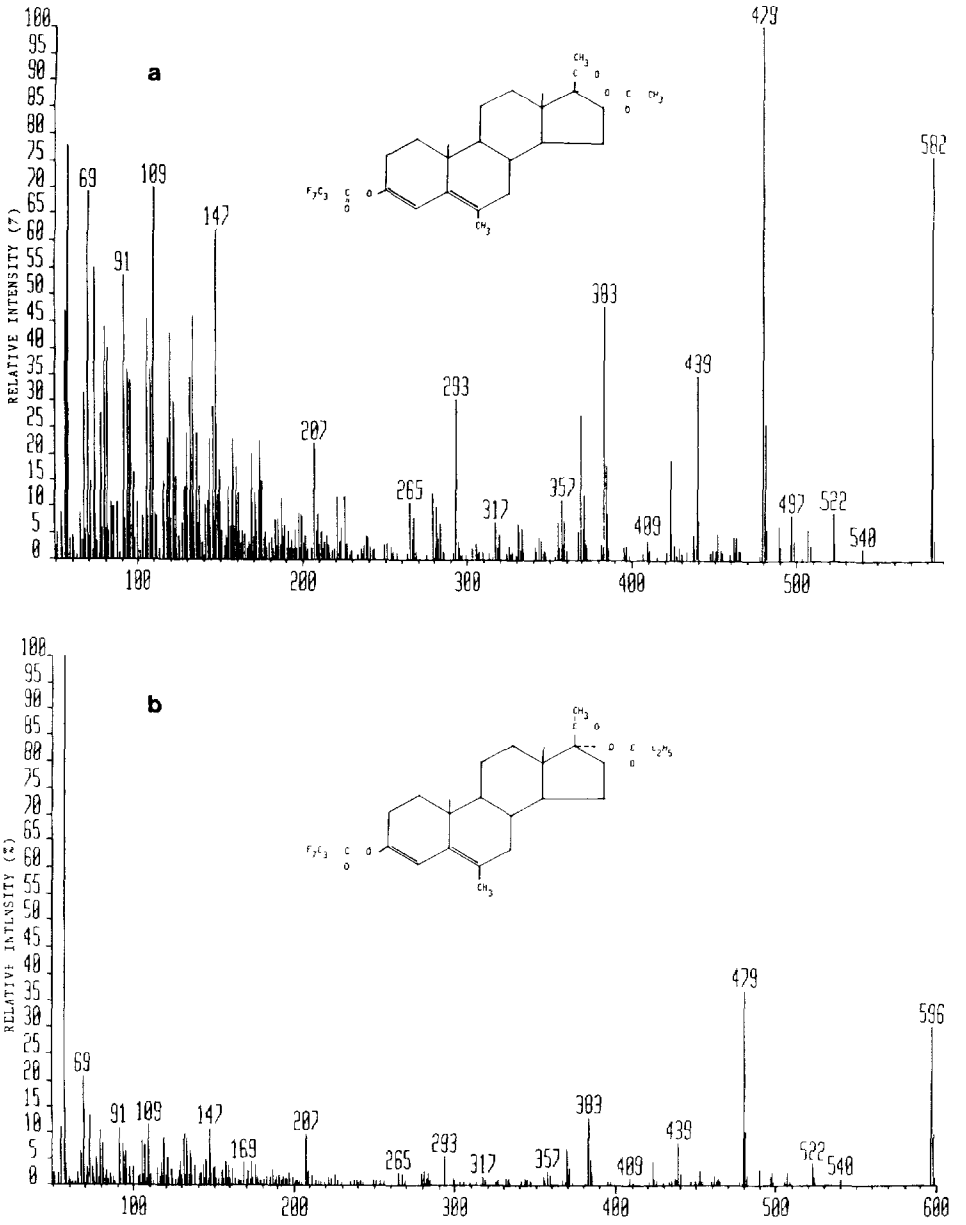

Fig 1 Mass spectra of the HFB derivative of (a) medroxyprogesterone acetate and (b) medroxyprogesterone propionate

cause the HFB derivative had more selective molecular ions of MPA and MPP [m/z 582 (MPA) and 596 (MPP)] than did the PFP derivative [m/z 532 (MPA) and 546 (MPP)] we decided to use the HFB derivative

Mass spectra

Electron-impact spectra of the HFB derivatives of MPA and MPP are shown in Fig 1 Ion m/z 582 and ion m/z 596 are the molecular ions of the MPA HFB derivative and the MPP HFB derivative, respectively Both compounds have a base peak at m/z 479 formed by loss of the 17-acetate group and the C-20–C-21 group [10] The intensity of both molecular ions was found to be at a maximum when the temperature of ion source was 250 °C

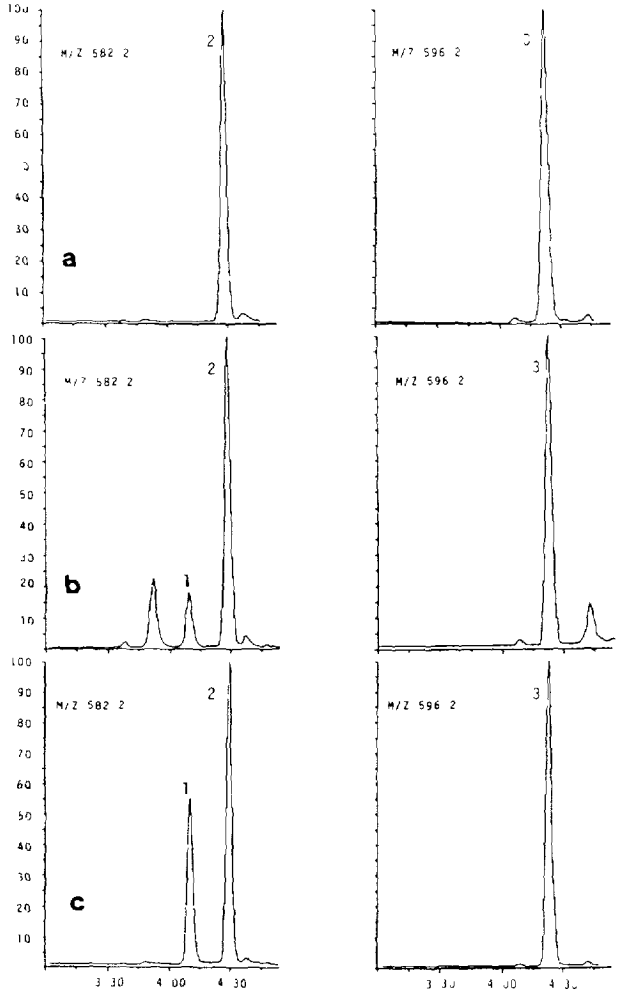

Fig 2 Selected-ion current profiles at m/z 582 2 and m/z 596 2 of (a) a blank serum sample incorporating MPP as internal standard, (b) a serum sample (3 h after administration) of a person receiving MPA (20 mg) orally and (c) a spiked serum sample (20 ng/ml) Peaks (corresponding to HFB derivatives) 1 = MPA, 2 = cholesterol, 3 = MPP

Typical selected-ion current profiles obtained from (a) a blank serum sample incorporating MPP as internal standard, (b) a serum sample (3 h after administration) of a patient receiving MPA (20 mg) orally and (c) a serum sample spiked with 20 ng/ml MPA are shown in Fig 2

Standard curve and sensitivity

The standard curve was obtained by plotting the peak-area ratio of the MPA HFB derivative (m/z 582 2) and the MPP HFB derivative (m/z 596 2) versus the serum concentration of MPA over the range 0.5–30 ng/ml The standard curve was prepared daily The equation of a typical standard curve was $y=0\ 04146x-0\ 01178$ with a correlation coefficient of 0 9994 The limit of quantitation of the method was 0 5 ng/ml MPA in serum at a signal-to-noise ratio better than 5 1

Precision

The precision of the method was tested by spiking a sample of serum with 10 ng and 1 ng MPA per ml After extraction and derivatization, five identical standard serum samples were measured in the same series and gave coefficients of variation (C V) of 3 1% at 10 ng/ml and 5 5% at 1 ng/ml

Serum levels of MPA in human serum

We evaluated the effects of four different MPA formulations on the MPA concentration in serum after oral administration An oral dose (10–20 mg) of each formulation was given to 36 patients Blood samples were taken at 0, 1, 3, 5, 24 h following oral administration The blood samples were centrifuged, and the serum obtained was frozen and stored at $-20\,^{\circ}C$ until analysis Table I shows MPA concentrations in serum of nine typical patients after oral administration of 20 mg of MPA The results indicate that there are large individual differences between patients Full details of the study will be published later

TABLE I

SERUM MPA LEVELS IN PATIENTS RECEIVING AN ORAL 20-mg DOSE OF MPA

Patient	MPA level (ng/ml)				
	0 h	1 h	3 h	5 h	24 h
1	<0 5	1 0	1 0	1 0	<0 5
2	<0 5	5 4	2 1	1 1	<0 5
3	0 6	4 2	3 5	2 3	<0 5
4	<0 5	0 6	2 3	0 9	0 6
5	<0 5	2 9	1 8	0 9	0 5
6	<0 5	<0 5	9 1	6 5	<0 5
7	<0 5	<0 5	9 2	2 4	0 6
8	<0 5	<0 5	11 2	2 1	<0 5
9	<0 5	15 3	10 7	5 9	1 0

DISCUSSION

MPA was determined by GC–MS with selected-ion monitoring after MPA had been converted into the HFB derivative The method was applied for routine measurements of MPA in human serum after oral administration of MPA (10 or 20 mg)

The advantages of the HFB derivative of MPA are the simple derivatization procedure (incubation at room temperature), the high molecular mass (which shifts the monitored ions to a cleaner background) and the analytical process (which does not form an enol isomer of MPA) Using the HFB derivative the C V of the method was 3 1 and 5 5% at 10 and 1 ng/ml, respectively Using the TFA derivative for quantitation of MPA, a similar C V has been achieved at higher serum concentrations of MPA [9] In pharmacokinetic studies, however, it is important that the quantitation of the compound in serum be reproducible at low serum concentrations After a single oral dose of 20 mg of MPA the serum MPA was found to be very low (Table I)

The most prominent ions, m/z 596 (MPP), m/z 582 (MPA) and m/z 479 (MPA, MPP) (Fig 1), can be monitored to quantitate the concentration of MPA Impurities in samples that had the same retention time and m/z fragments as MPA prevented the use of the base peak of MPA. Therefore monitoring the intensity of the characteristic molecular ions at m/z 582 2 (MPA) and m/z 596 2 (MPP) gave more precise results than use of the ion pair m/z 479 1 versus m/z 479 1 for quantitation of MPA The C V of this ion pair was 8 8% at 10 ng/ml and 10 8% at 1 ng/ml Typical selected-ion current profiles from the analysis of human serum are shown in Fig 2 Fig 2a shows that the background to the channel m/z 596 2 is very low, and there is only one extra peak at the channel for m/z 582 2 This peak was verified to be the HFB derivative of cholesterol, which eluted on OV-1 phase after the MPA derivative and so did not interfere with the quantitation of MPA Because there were no other peaks at m/z 596 2 and m/z 582 2, it was possible to use a very rapid temperature programme in GC The retention time of MPA HFB and the MPP HFB derivatives ranged from ca 4 to 4 5 min, and the total cycle time for the assay was ca 8 min This makes it possible to measure ca 60 samples a day

One GC–MS method for quantitation of MPA has been described in which MPP was used as an internal standard [9] We prepared MPP from MP This modification reduced the amount of impurities in the internal standard, especially as no trace amount of MPA was found

MPA has no specific binding sites in plasma and it binds only to serum albumin [16] A mild denaturation of serum proteins with methanol is sufficient to destroy the weak protein–steroid interactions [9] Using Bond Elut C_{18} cartridges, the extraction procedure is simple, rapid and reproducible, and liquid–liquid extraction problems (low recovery levels, formation of emulsion, several wash steps) can be prevented The re-usability of the extraction cartridge was studied by repeated extractions on the same extraction cartridges We conclude that, after regeneration, a Bond Elut cartridge can be used for serum extraction at least five times without loss of efficiency Moreover, after

the extraction and derivatization, the samples are extremely pure, so it is possible to analyse hundreds of samples on the same column without loss of sensitivity We have evaluated our method for more than six months and analysed over 1000 samples with a minimum of problems

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