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A MASS FRAGMENTOGRAPHIC METHOD FOR THE DETERMINATION OF MEGESTROL ACETATE IN PLASMA AND ITS APPLICATION TO STUDIES ON THE PLASMA LEVELS AFTER ADMINISTRATION OF THE PROGESTIN TO PATIENTS WITH CARCINOMA CORPORIS UTERI

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SUMMARY

A mass fragmentographic method for the determination of megestrol acetate in plasma is described. The plasma extract is purified on a silica gel column and the steroid is then converted to its 3-monomethoxime derivative and quantitated by mass fragmentography using medroxyprogesterone acetate as internal standard. The method was shown to fulfil adequate reliability criteria. The precision of mass fragmentographic assays with the accelerating voltage alternator of the LKB 9000 gas chromatograph—mass spectrometer is discussed. Determinations in plasma following oral administration of megestrol acetate demonstrated great inter-individual variations. In two subjects the plasma levels were followed during the 24 h after oral administration of 50 mg of megestrol acetate and the peak level (35 and 50 nmol/l) was found at 2–3 h.

INTRODUCTION

Quantitative plasma determinations of synthetic progestational compounds and their metabolites have been carried out to a rather limited extent and because of this our knowledge of the metabolism of these compounds is relatively scanty [1]. With regard to megestrol acetate (3,20-dioxo-6-methyl-4,6-pregnadien-17-yl acetate) a method involving three thin-layer chromatographies, conversion of the extracted megestrol acetate to megestrol and further to 6-methyl-4,6-androstadiene-3,17-dione and final determination by gas–liquid chromatography with electron capture detection has been published [2], but to our knowledge it has never been used for actual plasma concentration measurements. After administration of 10 μCi of megestrol acetate with a mass of 4 mg to one male volunteer a peak plasma level of the compound (or its metabolites) of 1 μg/100 ml of plasma was demonstrated after 1–3 h. The value fell to 350 ng/100 ml after 24 h [3].

However, measurement of radioactivity in plasma following administration of labelled progestins without chromatographic purification of the samples can only give an approximate view of the situation. Radioimmunological assays (RIA) for steroidal drugs are gaining in popularity and technical improvements in the combined gas chromatography–mass spectrometry (GC/MS) instruments have made it possible to measure steroids with a sensitivity close to that of RIA methods and with a very high degree of specificity. This latter technique, called mass fragmentography, requires only reasonably short purification of the sample prior to analysis [4, 5]. In this paper such a method is described for the determination of megestrol acetate in plasma. The intestinal absorption and plasma levels of this progestin was investigated following its administration to some hospitalized patients with carcinoma corporis uteri.

EXPERIMENTAL

Patients

All ten patients were female subjects hospitalized for treatment of carcinoma corporis uteri. Their age varied from 53 to 78 yr. Eight of the subjects including the two used for the absorption studies did not receive any other medicaments than megestrol acetate. Two of the subjects (Patients Nos. 3 and 4) received medication

Table 1. Blood sampling time in eight subjects receiving 50 mg megestrol acetate twice daily at 08:00 and 17:00 h. Administration of the drug was started on day 1 immediately after drawing the control sample

Case No.	Day 1	Day 2		Day 3	
1.	08:00 (control)		17:00	09:00	17:00
2.	08:00 (control)	09:00	18:00	09:00	18:00
3.	08:00 (control)	08:30	17:30	08:30	18:00
4.	08:00 (control)	—	17:30	08:30	17:00
5.	08:00 (control)	09:00	18:00	09:00	18:00
6.	07:00 (control)	09:00		08:30	—
7.	08:00 (control)	08:45	—	09:00	
8.	08:00 (control)	08:30	—	08:40	—

for cardiac insufficiency or angina pectoris but no diuretics were given.

Patients Nos. 1–8 received 50 mg of megestrol acetate orally at 8 a.m. and 5 p.m. Blood samples were drawn into heparin tubes before the drug was given (control samples) and then at the same time as the steroid was administered or within one h of administration 2 to 4 times during the therapy. The exact sampling times are shown in Table 1. Two subjects who had not received any medication prior to investigation were given 50 mg of megestrol acetate orally in the morning and blood samples were drawn before and at 1/2 to 2 h intervals during the day and one sample was taken the next morning. The blood was immediately centrifuged and the plasma stored at −20°C until analysed. Most determinations were carried out in duplicate.

Materials

Reference standards. Crystalline megestrol acetate (MA) (British Drug House, Chemicals Ltd, Poole, England) and medroxyprogesterone acetate (MPA) (3,20-dioxo-6-methyl-4-pregnen-17-yl acetate) (The Upjohn Co., Kalamazoo, Michigan, U.S.A.) were kindly donated by the two companies. The purity of the steroids was found to be good as judged by gas chromatography and thin-layer chromatography (see later).

Solvents and reagents. The following solvents were redistilled using all-glass fractionating columns: diethyl ether (Orion-yhtymä Oy, Helsinki, Finland) chloroform (Merck AG- Darmstadt, Germany), ethyl acetate (Merck), benzene (Merck), toluene (Merck). Methanol (Merck) was not redistilled and pyridine (Merck) was distilled twice. The water was distilled twice, the second time in a quartz-glass distillation apparatus. Other reagents used: 140/200 mesh Silica gel (Adsorbosil-CAB, Applied Science Laboratories, Inc., State College, Pennsylvania, U.S.A.) and methoxyaminehydrochloride (Eastman Kodak Co., Rochester, N.Y., U.S.A.). NaOH p.a. (Merck), KOH p.a. (Merck), Na_2CO_3 p.a. (Merck), K_2HPO_4 p.a. (Merck), KH_2PO_4 p.a. (Merck) and $NaHCO_3$ p.a. (Merck). 3α,20β-hydroxysteroid dehydrogenase 5 mg/ml (Boehringer, Mannheim, Germany), reduced nicotinamide adenine dinucleotide (NADH) (Boehringer).

Stationary phases for gas chromatography. 1% SE-30, 3% OV-210 (coated in this laboratory) and 3% XE-60 (all from Applied Science Laboratories, Inc.).

Methods

Extraction and purification. Usually 1–2 ml of plasma was extracted with diethyl ether–chloroform (3:1 v/v) (3 × 10 ml). The extract was washed with 0·5 ml of distilled water and evaporated to dryness in a stream of nitrogen on a sand bath heated to 45°C. A 2 g silica gel (Adsorbosil-CAB) column (0·5 × 15 cm glass column with reservoir) was prepared and washed 3 times with 5 ml of ethyl acetate–benzene (5:95 v/v) and the sample transferred to the column in 3 × 1·5 ml of the same solvent. The column was first eluted with 30 ml of this solvent to remove cholesterol. The MA fraction was then eluted with 20 ml of ethanol–benzene (5:95 v/v) and the solvents evaporated to dryness as described above.

Formation of methoximes. The 3-monomethoxime derivative of the steroid was formed by adding 1 ml of saturated methoxyaminehydrochloride in pyridine (twice distilled) to the dry sample [6]. After standing overnight the pyridine was evaporated, 2 ml of distilled water added and the steroid derivatives extracted with 1 × 5 ml and 2 × 2·5 ml portions of toluene. The toluene extract was concentrated and a known amount of MPA 3-monomethoxime derivative (internal standard) in toluene added and the fraction transferred to a graduated microtube for mass fragmentography.

Gas chromatography–mass spectrometry (GC/MS) and mass fragmentography (mf). In most experiments the LKB model 9000 gas chromatograph–mass spectrometer equipped with an accelerating voltage alternator (AVA) was used. Of the three signals that can be monitored with the AVA-unit two were recorded using a single pen potentiometric recorder. In order to decrease the noise level the following modifications of the instrument were made: the frequency of the AVA was altered by increasing both capacitances of the multivibrator (C_3, C_6; integrated circuit N 2) to about 60 μF, resulting in a change in the voltage of the AVA every 4th s. The signal from the preamplifier of the multiplier to the potentiometric recorder is taken through a passive filter with a time constant of about 3 to 3·5 s.

For recording most of the mass spectra a Hewlett-Packard model 2100 A computer coupled on-line to the mass spectrometer was used. The computer has a

8 K core memory, a disc memory, a Hewlett–Packard 7210 A X/Y plotter and the dynamic range of the system is 1 to 8192. The programme, developed by Mr Esa Soini in this laboratory, has been found to be very reliable and accurate, even for isotope measurements.

The stationary phases used in mf were 3% OV-210 or 1% SE-30. The OV-210 column bleeds less than the SE-30 column and therefore provides for more sensitive mf measurements. However, because the monomethoxime derivatives of MA and MPA do not separate on this column one of the isotope peaks of the MA fragment ions interfered with the measurement of the corresponding fragment ions of MPA. This interference was taken into account when calculating the results. Thus, in later studies only the SE-30 column was used for mf measurements as sufficient separation of the two steroid derivatives was obtained using this phase. The temperature of the SE-30 column was maintained at 230°C, the flash heater at 240°C, the separator at 250°C and the ion source at 290°C.

The two very abundant base peaks (m/e 310 and 312 in the spectra of the 3-monomethoxime derivatives of MA and MPA are used for the mf measurements (see mass spectra in Figs. 1 and 2). The electron energy is kept at 45 eV, which is the mean value for the optima for both compounds (MA = 40 eV, MPA = 50 eV). The entrance slit is 0·1 and the collector slit 1·0 mm. The monomethoxime derivatives of MA and MPA reference standards were analysed at least after every 4th to 5th sample, some 6 times/day.

Calculation of results was done as described previously [5]. With every set of samples two recovery experiments were carried out and the analytical values were corrected for losses during the procedure on the basis of the results of these recovery experiments. The difference in recovery values was not permitted to be more than 25% calculated from the higher value. If the difference was greater the samples were re-analysed. If duplicate determinations differed more than 25% calculated on the higher value the analysis was repeated.

Thin-layer chromatography (t.l.c.)

For this purpose Eastman chromatogram sheets 6060 silica gel with fluorescence indicator (0·1 mm) (Eastman Kodak Co.) were used. The sheet was washed once with methanol and reactivated for 1 h at 110°C. The chromatogram was developed with chloroform and the compounds were eluted with methanol. The R_F-value of both MA and MPA was 0·42 in this system. The spots were vizualized under U.V.-light.

Hydrolysis of megestrol acetate

This was carried out according to the method of Elce *et al.*[2] or in the following way: to the dry sample in a tube 0·5 ml of methanol is added, the contents of the tube are mixed with a Vortex mixer and left standing at room temperature for 10 min. Then 1 ml of 0·25 M NaOH in 70% methanol (v/v) is added and the contents of the tube mixed with the Vortex mixer. Nitrogen is blown into the solution, through a Pasteur pipette, for 1 min, the tube is closed and left standing at room temperature overnight. The solvents are evaporated to a volume of about 0·3 ml and 1 ml of distilled water is added and the steroid is extracted with 3 × 5 ml of ethyl ether. The ether is washed with 1 ml of 8% $NaHCO_3$ and 0·5 ml of distilled water and evaporated to dryness.

Reduction of megestrol with 3α,20β-hydroxysteroid dehydrogenase

To the dry sample 0·4 ml of methanol is added and the tube is mixed with a Vortex mixer. Thereafter 5 ml

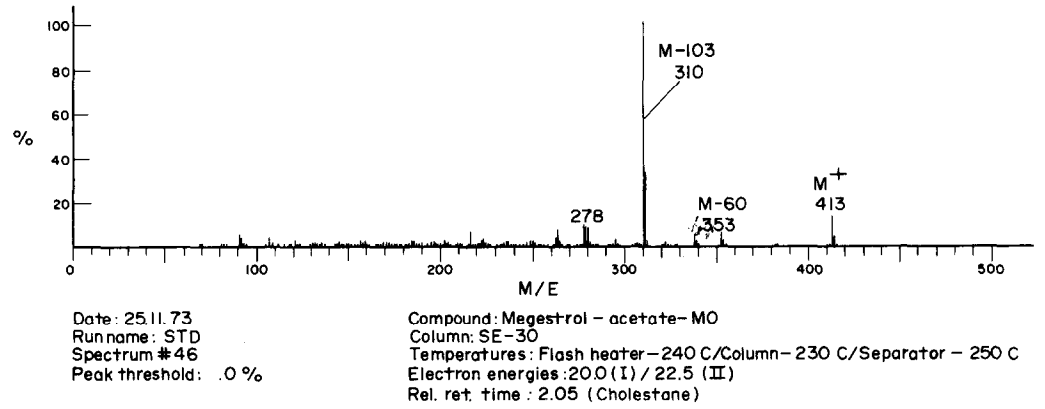

Fig. 1. Mass spectrum of megestrol acetate 3-monomethoxime obtained using an electron energy of 22·5 eV and the LKB 9000 GC-MS instrument coupled on-line to a Hewlett-Packard 2100 A computer with a HP 7210 A X/Y plotter.

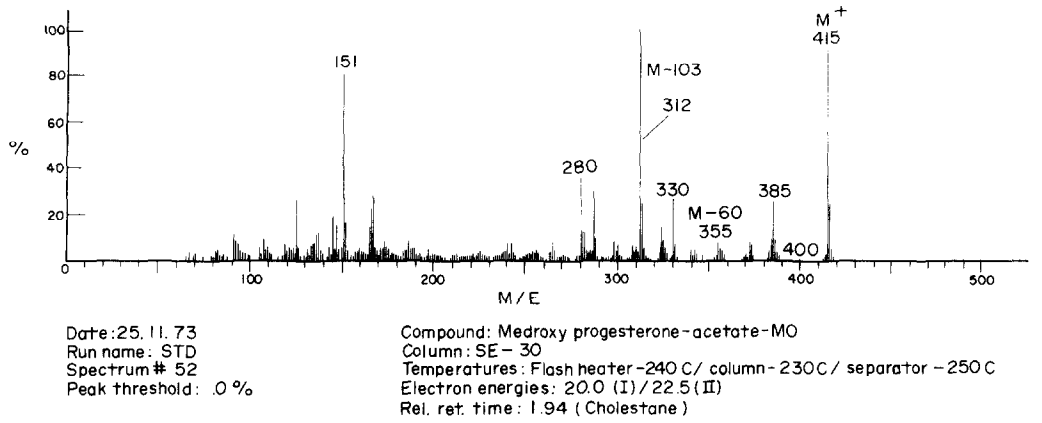

Fig. 2. Mass spectrum of medroxyprogesterone acetate 3-monomethoxime using the same conditions and instruments as in Fig. 1.

of 0·1 M potassium phosphate buffer pH 6·8 and 0·6 ml of prewarmed (15 min at 60°C in order to destroy contaminating NAD^+) NADH solution (5 mM NADH in 0·1 M sodium carbonate buffer pH 10·6) (final pH = 7·0) and 25 μl of the 3α,20β-hydroxysteroid dehydrogenase solution are added. The tube is shaken for 1 h at room temperature (23°C) in a mechanical shaker and the contents thereafter extracted 3 times with 10 + 10 + 5 ml of ethyl ether–ethyl acetate (1:1 v/v). For each extraction the tubes are shaken mechanically for 10 min. The extract is evaporated to dryness and silated in 0·2 ml of a mixture containing pyridine, hexamethyldisilazane and trimethylchlorosilane 9:3:1 v/v (the 2 latter reagents from Fluka AG, Buchs, Switzerland) for 30 min and the sample is ready for gc.

This method results in the quantitative reduction of megestrol to its 20β-hydroxy derivative. However, if long silation times, e.g. overnight, are employed more than one peak will be obtained on GC.

Gas chromatography (GC). For this purpose two F & M model 400 and 402 gas chromatographs with flame ionization detectors and 3 m U-shaped glass columns were used.

RESULTS

Reliability of the procedure

Accuracy. The extraction and silica gel chromatography steps were tested by adding 0·2 to 5 μg of megestrol acetate per ml of plasma and measuring the recovery by GC. The whole method was tested by adding 10 to 100 ng of the steroid per ml of plasma the final measurement being carried out by mf. The following results were obtained:

Extraction: recovery = 96 ± 4% (SD) (8 experiments)
Extraction + silica gel chromatography: recovery = 85 ± 5% (SD) (5 experiments)
Complete method: recovery = 82 ± 11% (SD) (19 experiments).

Precision. The precision is very much dependent on the mass fragmentographic step in the method. Repeated calibrations and injection of standards result in a reasonable precision of the method. The coefficient of variation is ± 13·7% in the range 11–40 nmol/l of plasma (8 determinations) and ± 31·6% in the range 1·5–10 nmol/l of plasma (9 determinations). The precision was calculated from duplicate determinations carried out on different days and thus includes also the day to day variation. When the precision was calculated from the values obtained for 10 determinations of a pooled sample (mean value 114 nmol/l) the coefficient of variation was found to be 5·3%.

Sensitivity. The ultimate sensitivity reached with the instrument analysing megestrol acetate standard was 26 fmol (10 pg) per injection giving a 1·0 cm. high peak. In practice it is not possible to have the instrument kept at its maximal sensitivity (e.g. different optimums for MA and MPA) and the lowest concentration of the MA standard used for calibration was 520 fmol (200 pg) at which point the most linear part of the standard curve starts. In the present investigation the plasma values in most instances were rather high and it was not necessary to work at the limit of sensitivity.

Specificity. The specificity of the method was tested by taking several mass spectra of extracts of plasma with high megestrol acetate concentrations following the normal purification procedure. These spectra were identical with that obtained for the reference standard and did not contain any extra significant peaks in the higher mass range. Several pooled plasma samples were also extracted in the normal way and chromatographed on silica gel and were then submitted to thin-layer chromatography (see Methods). The t.l.c. plate

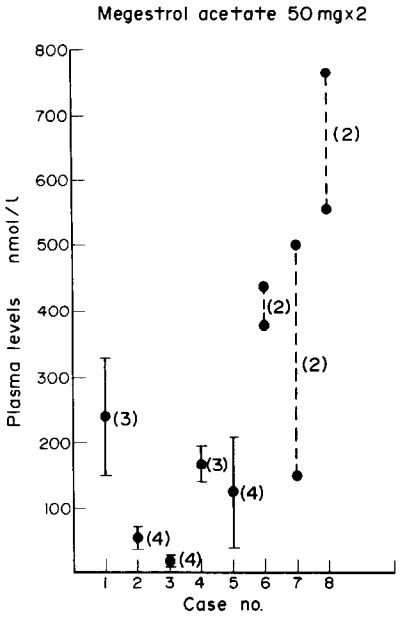

Fig. 3. Plasma levels of megestrol acetate following oral administration of 50 mg of the progestin twice daily to women with carcinoma corporis uteri. The numbers in brackets indicate number of different plasma samples analysed. When more than 2 samples were analysed the mean value is shown. For all subjects the range of values is presented.

was divided into six zones (zones 1–6) which were removed and extracted with methanol, zone 1 having the mobility of the megestrol acetate standard. All extracts were submitted to derivatization with methoxyaminehydrochloride and were subsequently analysed by GC–MS. None of the fractions other than zone 1 contained any significant amounts of megestrol acetate or its metabolites or any other compounds which had similar retention times as the 3-monomethoxime derivative of megestrol acetate and interfering at m/e 310 and 312. Zone 1 showed strong absorption of U.V.-light and the retention time and mass spectrum of its contents were identical with the corresponding derivative of the reference standard. In several experiments the zone was eluted and submitted to mild hydrolysis in alkali. Under this treatment both the compound from plasma and the reference compound yielded two compounds namely megestrol and megestrol with the loss of the 17-hydroxyl in the form of water. Both compounds separated well in gc as their monomethoxime derivatives on a 3% XE-60 column (relative retention time to cholestane were 0·70 and 0·55, respectively). It was also observed that the main compound, megestrol, could be reduced to its 20-hydroxylated derivative with 3α,20β-hydroxysteroid dehydrogenase but the dehydrated megestrol could not. This was true for both the substances derived from the standard and the compounds isolated from plasma after an oral load of megestrol acetate. Thus it may be concluded that the method developed is specific for megestrol acetate.

Quantitative results

The plasma concentrations of megestrol acetate in 8 patients after oral administration of megestrol acetate 50 mg twice daily are shown in Fig. 3. For those patients in whose case three or more determinations were made a mean value was calculated. As can be seen very wide inter-individual variation in plasma levels were found indicating great individual differences in absorption or metabolism or both.

In Fig. 4 the results obtained with two patients after one single oral administration of 50 mg of megestrol acetate are shown. The peak concentrations (about 35 and 50 nmol/l) were seen 2–3 h after administration and the elimination was rather slow because measurable amounts could easily be found after 24 h. In one of the patients a second peak occurred at about 7·5 h. The absorption was more rapid in this subject and the second peak may be due to enterohepatic circulation of the steroid.

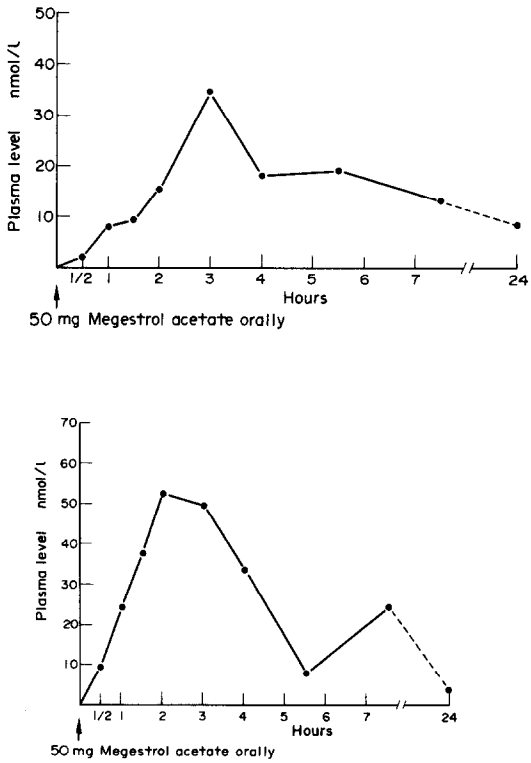

Fig. 4. Plasma levels of megestrol acetate following oral administration of single 50 mg dose of megestrol acetate to two women with carcinoma corporis uteri.

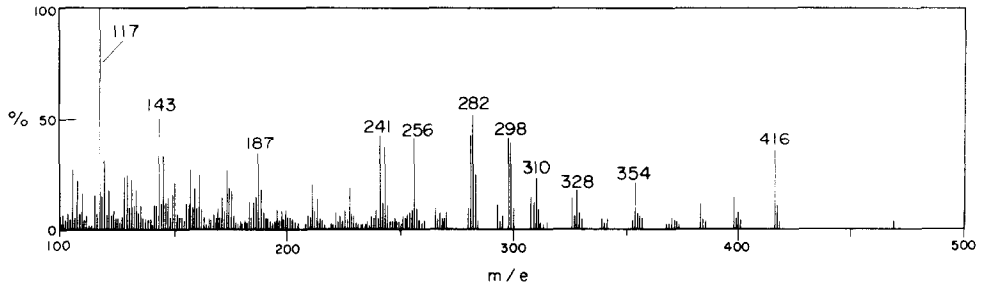

Fig. 5. Mass spectrum of the 20β-monotrimethylsilyl ether derivative of megestrol reduced with the 3α,20β-hydroxysteroid dehydrogenase. The mass spectrum was obtained using an electron energy of 70 eV and the LKB 9000 GC–MS instrument.

DISCUSSION

Methodological studies

Before the final method was adopted several other methods were tested. The primary aim was to develop a very sensitive method which would allow plasma measurements after oral administration of the compound in dosages corresponding to those used for contraception. Because it was known from previous studies that the silylated secondary alcohol side chain of pregnanediol gives a very intense fragment at m/e 117 [7] attempts were first made to hydrolyse the acetate group and then reduce the 20-oxo group with 20β-hydroxysteroid dehydrogenase in order to get the 20β-reduced megestrol. However, it was found that despite trying to use many various concentrations of KOH and NaOH, different temperatures and reaction times it was never possible to obtain a single compound, a significant amount (more than 15%) of the megestrol obtained was always dehydrated with loss of the 17α-hydroxyl group. In the methodological work of Elce *et al.*[2] this was obviously not observed. Following reduction with 3α,20β-hydroxysteroid dehydrogenase and silylation the spectrum seen in Fig. 5 was obtained. As can be seen the fragment at m/e 117 is the base peak, but not very abundant in comparison with many of the other fragments. A great disadvantage was that there were fragments of every mass close to m/e 117 and no internal standard could be used. There was therefore no special advantage of measuring m/e 117. In addition the mean recovery in the method was 67% (11 experiments). In view of the complicated procedure and several other disadvantages the method was used only for investigation of the specificity of the final method developed.

The greatest difficulties in the method are encountered in the mass fragmentographic step. The primary idea was the MPA could be used as internal standard throughout the method. However, for a very long time we believed that other compounds with fragmentation pattern similar to MPA occurred in the extracts of plasma containing unconjugated steroids and therefore the internal standard could not be added in the beginning, but the sample was divided in two before mf and to one of them the MPA internal standard was added. Later on it was found that MA and MPA were sometimes absorbed on to the gc column or septum material after injection of standards or samples. It was found absolutely necessary in order to ensure that such absorption did not occur to inject pure toluene after samples containing high concentration of steroids. After this discovery the MPA internal standard was added directly to the plasma, but because all measurements presented here have been made with the original method and the experience with the modified procedure is too small, these results cannot be presented.

It is rather difficult to arrive at a reasonable precision for the method using the LKB 9000 and the AVA system. The main difficulties are: (1) The obstruction of the first jet of the separator, which decreases the sensitivity of the measurement and can change significantly during the day; (2) The valve between the separator and the ion source cannot be opened exactly to the same degree every time which results in variation in the amount of the compounds entering the mass spectrometer between injections; (3) The stability of the mass marker is not good, during the 2–3 first hours of work, which is due to change in the temperature of the magnet and frequent calibration during the day is necessary. Therefore at present we usually start the analysis after first keeping the mass marker setting for 3 h at the highest mass to be measured. Sometimes the setting is left overnight, a method which is not recommended by the manufacturer of the instrument. There seem to be other factors (adsorption to the column? instability of the ion source?) which influence the precision of analysis, which are more difficult to establish. The cumulative result of all these factors is that despite internal standardization great differences in values may be obtained for two injections of the same sample made out during the same day. Therefore

the injections of sample must sometimes be made several times with standard injections between the samples. Other workers have also noticed that frequent calibration is necessary [8]. The coefficients of variation at various concentration levels obtained in the present investigation must be regarded as the best possible for this method because extreme care was taken to obtain reliable results.

Using 1–2 ml of plasma the sensitivity of the method would theoretically be good enough to measure plasma concentrations of MA after oral administration of 4 mg of the steroid, which was the amount primarily used for contraception. However, in practice, this would be very difficult and it seems that for such purposes more starting material would have to be used and a t.l.c. step included. The lower recovery and precision caused by the inclusion of the additional step would be compensated for by adding the internal standard, MPA, directly to the plasma. The method may in principle also be used for the determination of MPA using MA as the internal standard. However, specificity studies are needed before such a method could be used.

Metabolic studies

Specific measurements of plasma levels of megestrol acetate following administration of the compound has to our knowledge not been carried out previously. When the labelled compound was administered the metabolites were excreted slowly in the urine and 20% of the dose was found in the faeces [9]. This would suggest significant biliary excretion of megestrol acetate or its metabolites and it seems possible that unchanged megestrol acetate may be secreted into the bile because of the detection of a second peak in plasma corresponding to a similar circulation time as found for estrogens [10].

Previously it has been demonstrated that the human organism hydroxylates megestrol acetate at C-2 and at the methyl-group at C-6 [9]. In the unconjugated fraction of plasma no mass spectra of metabolites could be obtained in the present investigation but no more than 30 ml of plasma was ever processed. However, in the glucuronide fraction of plasma after MA administration metabolites have been detected and in urine several mono- and dihydroxylated and probably also methoxylated metabolites have been found by GC–MS (F. Martin and H. Adlercreutz, unpublished). This work is still in progress.

The inter-individual variation in the plasma level after administration of the same dose of megestrol acetate is considerable as can be seen in Fig. 3. The reason for this is unknown. Patients No. 3 and 4 received other medicaments which may have influenced the metabolism of MA. It must also be remembered that all patients were more than 50 yr old and that absorption of the compound therefore may be less efficient than in younger individuals. Because of individual variations in absorption and metabolism contraceptive failures are more likely to occur when the dosage is low as has been recognized in connection with low dose progestin contraception.

Recently it has been observed that antibiotics like ampicillin have pronounced effects on estrogen metabolism [11, 12] and also to some extent affect neutral steroid metabolism (13 and unpublished results from this laboratory). Contraceptive failures may therefore be due to interference of other drugs with the metabolism of the progestin or oestrogen used and this may be especially true in subjects receiving low doses of progestins or progestins and oestrogens. It would therefore be of interest to study the effect of antibiotics on plasma levels of progestins.

MPA is widely used in the treatment of carcinoma corporis uteri. The mf determination of MPA is less sensitive than mf determination of MA due to the m/e 312 fragment being of lower intensity than that of m/e 310. This is however of no disadvantage if the method is used for evaluating the effectiveness of therapy by assaying the plasma levels of free steroid, because the dosage is high. MPA may also be determined by RIA [14].

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