Journal of Chromatography, 432 (1988) 21–28
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4379

DETERMINATION OF 17-HYDROXYPROGESTERONE IN PLASMA BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY WITH HIGH-RESOLUTION SELECTED-ION MONITORING

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(First received April 27th, 1988; revised manuscript received July 4th, 1988)

SUMMARY

A method for determining 17-hydroxyprogesterone in plasma by isotope dilution–mass spectrometry is described. For the internal standard 17-hydroxy[$^2H_4$]progesterone is used. Extraction of plasma is followed by conversion into the 3,20-dienol,17-tristrimethylsilyl ether derivative and analysis by capillary gas chromatography–mass spectrometry with selected-ion monitoring, at a resolution of 6000. The lower limit of quantitation was 1 pg, judged from a criterion of a signal-to-noise ratio of 10. The precision and accuracy of the method were satisfactory.

INTRODUCTION

It is well known that 17-hydroxyprogesterone is present in extremely high concentrations in the blood of patients with a 21-hydroxylase deficiency, compared with normal subjects [1,2] and that the determination of 17-hydroxyprogesterone in blood provides the most valuable parameter for the diagnosis of the disease [3]. Several methods for the determination of 17-hydroxyprogesterone by radioimmunoassay (RIA) or enzyme immunoassay (EIA) have been reported [4–7]. Although these methods are suitable for routine analyses, they usually give values that are influenced by cross-reactions with other steroids or other interfering compounds. Therefore, a specific, accurate and sensitive method for determination of 17-hydroxyprogesterone was needed.

Gas chromatography–mass spectrometry (GC–MS) using stable isotope dilu-

0378-4347/88/$03.50

tions and selected-ion monitoring or isotope dilution–mass spectrometry (ID–MS) is increasingly accepted as a sensitive and highly specific method for quantitative assays of many biomedical substances, including hormonal steroids [8–13]. This paper describes a method of plasma 17-hydroxprogesterone determination by ID–MS using 17-hydroxy[$^2H_4$]progesterone as internal standard.

EXPERIMENTAL

Chemicals and reagents

17-Hydroxy[11,11,12,12-^2H]progesterone was synthesized in our laboratory as described elsewhere [14]. The ^2H content was: $^2H_0$, 0.3%; $^2H_1$, 1.1%; $^2H_2$, 8.6%; $^2H_3$, 37.1%; $^2H_4$, 52.1%; $^2H_5$, 0.8%. Tritium-labelled 17-hydroxyprogesterone was obtained from the Radiochemical Centre (Amersham, U.K.). All other chemicals and solvents were analytical grade and used without further purification.

Derivatization procedure

The trimethylsilyl (TMS) ether derivatives were prepared as follows. Reactions were carried out in 1-ml micro reaction vials with PTFE-lined screw-cap seals (Pierce, Rockford, IL, U.S.A.). A solution of 1 mg of sodium formate in 100 μl of water was added to the vials, and the water was evaporated under a stream of nitrogen followed by heating for 30 min at 120°C. After cooling, the steroid to be derivatized was added in methanol or ethanol. The solvent was evaporated under a stream of nitrogen, and 100 μl of heptane and 20 μl of trimethylsilylimidazole (TMSI) were added. The vials were purged with nitrogen, closed and placed in a reaction block at 100°C. After 3 h, 20 μl of N,O-bis-(trimethylsilyl)acetamide (BSA) were added, and heating was continued at 90°C for another 3 h. After cooling, the reaction mixture was filtered through a column (16 mm × 7 mm I.D.) of Sephadex LH-20 swollen in heptane, and the vial was washed three times with a total of 1 ml of heptane, each wash being filtered through the column. The filtrates were collected in a tube. The tube was stoppered and stood at room temperature for 2 h, during which a flocculent precipitate separated out. The precipitate was filtered through a cotton plug placed in a disposable pipette and washed twice with 1 ml of heptane. The combined filtrate and the washings, which contained the trisTMS derivative, were evaporated to dryness under a stream of nitrogen, and the residue was dissolved in hexane.

Blood samples

To 100 μl of plasma were added 0.5 ml of water and 10 μl of ethanol containing 1.0 ng of 17-hydroxy[$^2H_4$]progesterone. The mixture was allowed to stand for 1 h at room temperature and then extracted with two 4-ml portions of diethyl ether. The ether phase was collected, washed with two 1-ml portions of water, dried over sodium sulphate and evaporated under a stream of nitrogen. The residue was derivatized using conditions described above.

Gas chromatography–mass spectrometry

Mass spectra were obtained with a Jeol JMS-D 300 gas chromatograph–mass spectrometer and a computerized data system JMA-2000. The chromatographic column was a glass tube (2 m×2 mm I.D.) packed with 1% OV-1 on 100–120 mesh GasChrom Q. The separations were carried out by temperature programming at 2°C/min from 270°C. The ion source temperature was 200°C; the ionizing current was 300 μA; the ionizing voltage was 24 eV.

High-resolution selected-ion monitoring (SIM) was performed with a Jeol JMS-HX110 gas chromatograph–mass spectrometer and a JMA-DA5000 computerized data system. The gas chromatograph was equipped with a 30 m×0.32 mm I.D. 0.1-μm bonded-phase DB-1 fused-silica capillary column and splitless injector. The column was programmed from 100 to 200°C at 50°C/min, from 200 to 290°C at 30 or 40°C/min and from 290 to 320°C at 25°C/min. The last part of the programme allows the chromatographic separation of 17-hydroxyprogesterone. The ion source temperature was 265°C. The ionizing voltage was 120 eV and the ionizing current was 300 μA. The MS resolution was 6000 and the instrument was focused on molecular ions of the derivatives of 17-hydroxyprogesterone (m/z 546.338) and 17-hydroxy[$^2H_4$]progesterone (m/z 550.363).

RESULTS

Treatment of 17-hydroxyprogesterone with TMSI in heptane using sodium formate as a basic catalyst yielded the 3-enol,17-bisTMS derivative as the major product [15,17]. The other minor product was 3,20-dienol,17-trisTMS derivative, the yield of which was not increased appreciably even after prolonged heating. The bisTMS derivative shows intense ions at m/z 474 (M^+) and at m/z 431; the latter arises by elimination of the CH_3CO group at C-17 (Fig. 1). Gleispach [18] reported that BSA combined with a basic catalyst reacts quantitatively with the 17-hydroxy-20-keto group to yield the 17,20-enol-TMS derivatives. Therefore, BSA was added to the reaction mixture at a time when the 3-keto-4-ene and the 17-hydroxy groups had been converted into the TMS derivatives. The conditions led to an excellent yield (more than 90%) of the 3,20-dienol,17-trisTMS derivative. The mass spectra of the trisTMS derivatives of 17-hydroxyprogesterone and 17-hydroxy[$^2H_4$]progesterone are shown in Fig. 1. The molecular ions at m/z 546 and 550 are the base peaks of the unlabelled and the labelled compounds, respectively. It has been reported that the 3-enol-TMS derivative of corticosteroids with a 3-keto-4-ene structure is rapidly hydrolysed to the free 3-keto group during evaporation and redissolution in the absence of silylating reagent [17]. However, the trisTMS derivative of 17-hydroxyprogesterone could be evaporated and redissolved in hexane and it was stable in hexane for at least three days at room temperature.

It has been reported that preparation of the enol-TMS derivatives of 3-keto-4-ene steroids usually leads to a mixture of isomers to which 2,4-diene and 3,5-diene structures have been assigned [19]. In the course of this work, little evidence of isomer formation was encountered, the trisTMS derivative of 17-hydroxyprogesterone giving only one GC peak either on a packed OV-1 column or on a capillary

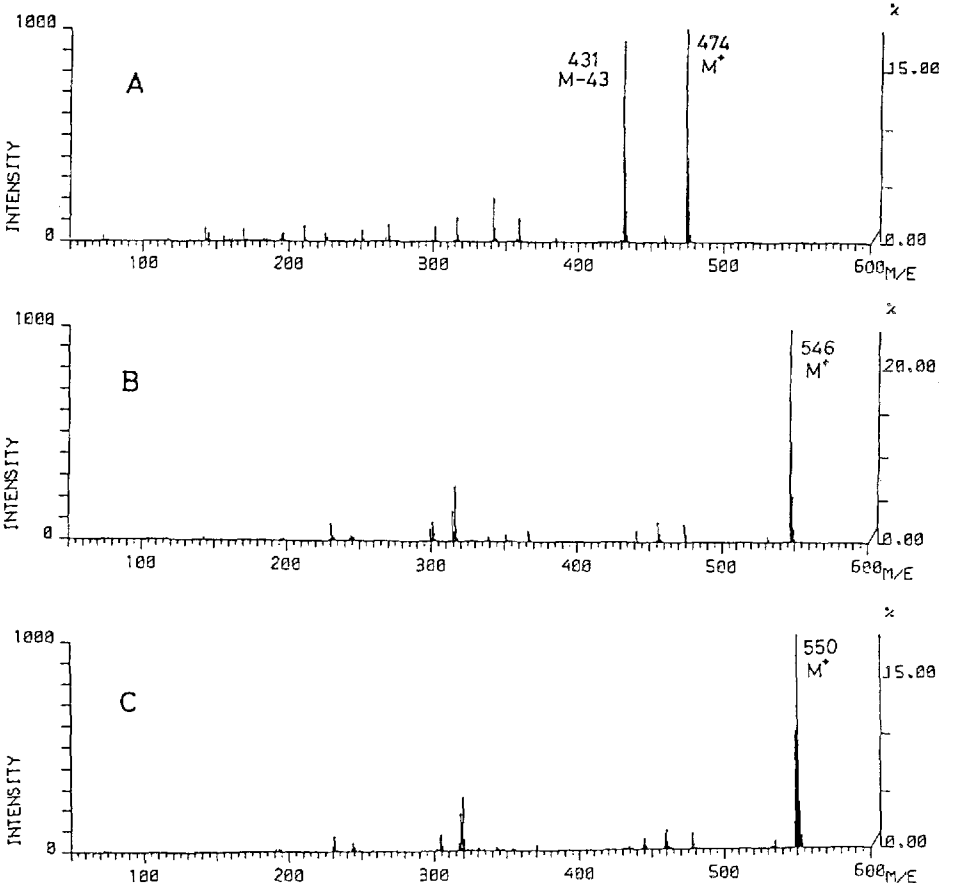

Fig. 1. Mass spectra of (A) 3-enol,17-bisTMS ether derivative of 17-hydroxyprogesterone, (B) 3,20-dienol,17-trisTMS ether derivative of 17-hydroxyprogesterone and (C) 3,20-dienol,17-trisTMS ether derivative of 17-hydroxy[$^2H_4$]progesterone.

DB-1 column. A single peak has been observed in the formation of 3-enol-*tert.*-butyl dimethylsilyl ether derivatives from several 3-keto-4-ene steroids [15] and of the 3-enol,17-bisTMS derivative from norethisterone [20]. The 3,5(6)-diene structure has been assigned to the norethisterone enol-TMS derivative.

Recoveries of 17-hydroxyprogesterone from plasma achieved in extraction through derivatization were determined by supplementing plasma with 17-hydroxy[^3H]progesterone. The overall recovery following the present procedure was $88.2 \pm 1.4\%$ ($n=5$).

High-resolution SIM of the molecular ions of the trisTMS derivatives of 17-hydroxyprogesterone and 17-hydroxy[$^2H_4$]progesterone provided excellent sensitivity. Fig. 2 shows results of analysis of a 1/20 aliquot of a mixture of 50 pg of 17-hydroxyprogesterone and 1 ng of 17-hydroxy[$^2H_4$]progesterone. The figure indicates that 2.5 pg of 17-hydroxyprogesterone could readily be detected with a signal-to-noise ratio of ca. 20. The lower limit of quantitation, based on a criterion of a signal-to-noise ratio of 10, is ca. 1 pg of 17-hydroxyprogesterone.

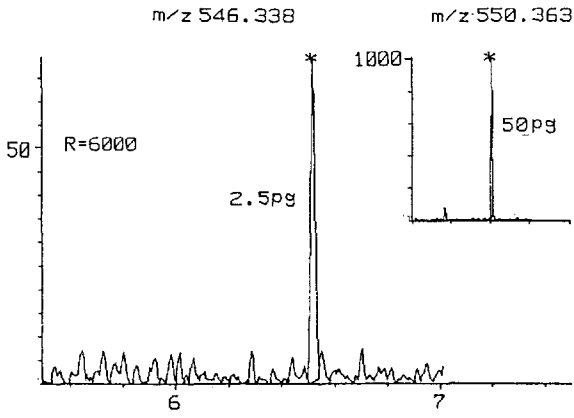

Fig. 2. GC–MS analysis with high-resolution selected-ion monitoring of a 1/20 aliquot of a mixture of 50 pg of 17-hydroxyprogesterone and 1 ng of 17-hydroxy[$^2H_4$]progesterone as the trisTMS derivative. The chromatographic column was programmed from 200 to 290°C at 30°C/min. The peak-area ratio (m/z 546.338/550.363) was determined to be 0.0621.

Known mixtures of 17-hydroxyprogesterone and 17-hydroxy[$^2H_4$]progesterone were prepared so that the sample size injected into the gas chromatograph–mass spectrometer covered the 17-hydroxyprogesterone range 1–320 pg with a fixed amount (50 pg) of 17-hydroxy[$^2H_4$]progesterone. Each mixture was then analysed as the trisTMS derivative, focusing on the molecular ions at m/z 546.338 for 17-hydroxyprogesterone and m/z 550.363 for 17-hydroxy[$^2H_4$]progesterone. When the peak-area ratios (y) were plotted against the mass ratios (x) of 17-hydroxyprogesterone to 17-hydroxy[$^2H_4$]progesterone, there was a good correlation between the observed peak-area ratios and the mass ratios. The regression equation for the standard curve is $y=0.9847x+0.0080$, with a correlation coefficient of 0.992.

Fig. 3 shows an example of the GC–MS analysis of a plasma pool. The technique allowed analysis of the total extract, following derivatization, without further purification. Two parameters, capillary GC retention time and high-resolution molecular ion detection, were sufficient to detect and quantify 17-hydroxyprogesterone regardless of the complexity of the mixture. The isomeric C_{21} steroids, deoxycorticosterone and 6β-hydroxyprogesterone, yielded, after trimethylsilylation by the present procedure, trisTMS derivatives with molecular ions at m/z 546.338. Another isomeric steroid, 16α-hydroxyprogesterone, yielded a product giving no ion at m/z 546 but an intense ion at m/z 456 (M−trimethylsilanol). The TMS derivatives of deoxycorticosterone ($I^{DB\text{-}1}_{270°C}=3216$) and 6$\beta$-hydroxyprogesterone ($I^{DB\text{-}1}_{270°C}=3087$, 3109) were completely separated from the trisTMS derivative of 17-hydroxyprogesterone ($I^{DB\text{-}1}_{270°C}=3064$) on the gas chromatograph.

To assess the accuracy and precision of the procedure, 1 ml of a plasma pool was spiked with 16 ng of 17-hydroxyprogesterone and analysed by the present methods. As seen from Table I, the values for percentage recovery and intra-assay coefficients of variation (C.V.) were satisfactory. Portions of another two plasma

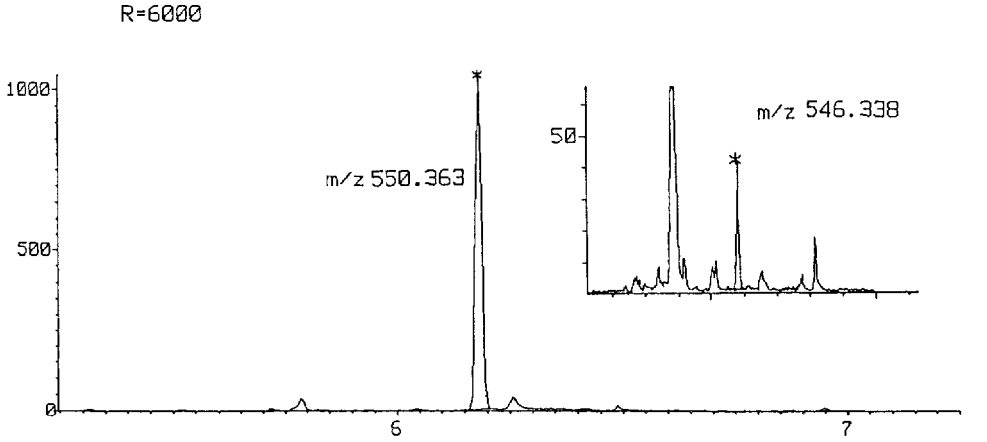

Fig. 3. GC–MS analysis with high-resolution selected-ion monitoring of a 1/20 aliquot of an extract of 100 μl of plasma, as the trisTMS derivative. The chromatographic column was programmed from 200 to 290 °C at 40 °C/min. The peak-area ratio (*m/z* 546.338/550.363) was determined to be 0.0420, corresponding to a plasma concentration of 0.35 ng/ml.

TABLE I

VALUES FOR 17-HYDROXYPROGESTERONE IN NORMAL AND SUPPLEMENTED PLASMA, AS MEASURED BY ID-MS

Added (ng/ml)	Found (mean ± S.D., $n=5$) (ng/ml)	C.V. (%)	Relative error (%)
0	1.10 ± 0.01	0.9	–
16	17.16 ± 0.14	0.8	+0.4

pools were analysed for 17-hydroxyprogesterone by the present method of five occasions. The mean values and their standard deviations were 0.41 ± 0.02 and 0.89 ± 0.04 ng/ml, the intra-assay C.V. being 4.9 and 4.5%, respectively.

The specificity of this method is excellent, because the mass spectrometer is used at high mass resolution as a very sensitive and specific detector of compounds separated by capillary GC. The chromatographic column does not resolve the labelled from the non-labelled 17-hydroxyprogesterone, but causes a minute time-shift (1 s) in the maxima of both peaks, which, together with the expected retention time, serves as additional evidence for the identities.

DISCUSSION

The present method provides a sensitive and reliable technique for determining plasma levels of 17-hydroxyprogesterone with good accuracy and precision.

The difference in mass number between the non-labelled and the labelled 17-hydroxyprogesterone is 4, and this reduces the interference with the natural isotope contributions at the selected mass of the internal standard. The interference

of the internal standard at the selected mass of the compound being measured may be neglected, because the isotope ratio $^2H_0/^2H_4$ of the internal standard is only 0.6% and a constant amount of the internal standard was used throughout all the assay samples. These properties of the internal standard would enhance the accuracy and sensitivity in the isotope ratio measurements in ID–MS.

The lower limit of quantitation, based on a criterion of signal-to-noise ratio of 10, is 1 pg of 17-hydroxyprogesterone [21]. The main disadvantage in GC–MS analysis is that a large portion of the sample is often unused, and with the present technique only a 1/20 aliquot of the extract from 100 μl of plasma is required. This may be unacceptable when only limited amounts of blood samples are available. In these circumstances, an injection device such as that of Van den Berg and Cox [23], which permits larger aliquots of the extract to be applied to capillary columns, would reduce the amounts of blood samples necessary for analysis.

It is generally accepted that, in the measurement of 17-hydroxyprogesterone in plasma, the specificity of RIA or EIA is enhanced by extraction and chromatographic isolation of the steroid prior to quantification. The reported plasma levels of 17-hydroxyprogesterone obtained by RIA with prior chromatographic purification of sample extracts are in the range 0.33–1.49 ng/ml in males and follicular phase women [24] and 0.74 ± 0.13 and 0.66 ± 0.14 ng/ml in adult male and female, respectively [25]. The values are broadly similar to those obtained by the present ID–MS method, which gave values for the concentrations of 17-hydroxyprogesterone in four pooled plasma samples of 0.35, 0.41, 0.89 and 1.10 ng/ml, respectively.

We believe that the present ID–MS assay for 17-hydroxyprogesterone will be useful as a reference method for checking RIA or EIA methods and will also provide an independent assay in cases of doubt about RIA or EIA methods. We also believe that the present method should be considered as useful in its own right when only a limited number of assays have to be performed for research purposes.

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