Journal of Chromatography, 345 (1985) 1—10
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2771

SPECIFIC AND SENSITIVE DETERMINATION OF MEDROXYPROGESTERONE ACETATE IN HUMAN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received April 24th, 1985; revised manuscript received June 26th, 1985)

SUMMARY

The mass fragmentographic determination of medroxyprogesterone acetate (MPA) in serum, using as internal standard medroxyprogesterone propionate (MPP) synthesized from MPA, is described. After addition of MPP, the sera are extracted on Sep-Pak C_{18} cartridges

0378-4347/85/$03.30

and MPA and MPP are detected as their respective 3-enol trifluoroacyl esters. Serum samples from 84 patients with breast cancer, daily receiving MPA orally, were determined showing a large variation in MPA concentrations (4—349 ng/ml). Our proposed gas chromatographic—mass spectrometric (GC—MS) method, which can be considered as a reference, was compared with a radioimmunoassay (RIA) method showing a correlation coefficient of 0.73 ($n = 69; p < 0.001$). The assay was also used to determine sequential serum levels of patients receiving a single oral dose of MPA. With only minor adjustments, the GC—MS method allows the determination of serum concentrations of related steroids such as megestrol acetate and cyproterone acetate.

INTRODUCTION

Medroxyprogesterone acetate (MPA), synthesized for the first time in 1958 [1], has proved to be a strong oral progestagen and has therefore been used as a contraceptive. The more recent introduction of MPA in oncology followed on from the discovery of its effect on several hormone-dependent carcinomas, i.e. endometrial and breast carcinomas [2—4].

Studies on the effect of MPA administration led to the development of several analytical methods for the determination of blood MPA levels. Most of these methods use radioimmunoassay (RIA) [5—8]. In addition, other techniques have been developed such as gas chromatography (GC) with electron-capture detection [4, 7, 9], high-performance liquid chromatography (HPLC) [10] and gas chromatography—mass spectrometry (GC—MS) [11].

RIA is most commonly used because of its simplicity and commercial availability. However, large differences in serum levels have been reported [5, 6], indicating a lack of specificity in some of the reported RIA methods, possibly due to cross-reactivity of MPA metabolites [8]. In a study on the effect of MPA treatment on patients with disseminated breast carcinomas, performed in our hospital, MPA levels of serum samples of these patients were routinely determined elsewhere by RIA [12, 13]. The large variations in MPA concentrations raised the question of whether they were solely due to existing differences in MPA levels and/or due to a lack of specificity of the employed RIA technique. To solve this question, this RIA technique should be compared with a method of high selectivity. Often, GC—MS has been advocated as a reference method for a large number of substances, e.g. to check the reliability of serum steroid concentrations obtained by RIA techniques [14, 15]. However, to date, publications on GC—MS determinations of serum MPA concentrations have been scarce and give incomplete descriptions of the analytical details. In 1980, Phillipou and Frith [11] described a GC—MS method in which d_3-MPA (the deuterium atoms being present in the 17-acetoxy moiety) was used as the internal standard. We decided to apply the described GC—MS procedure on our patient samples, to compare the results with those obtained by RIA. However, our attempts to synthesize d_3-MPA failed, possibly owing to a rapid exchange of the deuterium atoms by hydrogen atoms. To obtain a stable internal standard, we replaced d_3-MPA with medroxyprogesterone propionate (MPP), which can be easily synthesized from MPA. The analytical procedure was also improved. Thus, a very sensitive and selective method for determining MPA in serum was developed, which was suitable for

comparison with RIA methods. We studied the bioavailability of MPA by measuring sequential serum samples from patients receiving a single oral dose of MPA and samples from patients who received the drug regularly. In addition, we compared our results with those obtained by a RIA technique [12, 13].

EXPERIMENTAL

Materials

The reference substance, MPA, was obtained from Sigma (St. Louis, MO, U.S.A.), and MPA tablets (Provera) from Upjohn (Kalamazoo, MI, U.S.A.). The Sep-Pak C_{18} cartridges were from Waters Assoc. (Milford, MA, U.S.A.). All other reagents (analytical grade) were from Merck (Darmstadt, F.R.G.) and used as supplied, except for hexane, which was distilled before use.

Preparation of the internal standard

Since the intermediate, medroxyprogesterone (MP), was not commercially available, it was prepared by hydrolysing MPA. A 100-mg amount of MPA was dissolved in 10 ml of acetone and 100 ml of a saturated solution of sodium methylate in methanol were added and the mixture was gently shaken in a waterbath at 45°C for 48 h. The hydrolysis reaction was ended by slowly adding 100 ml of water. The solution was extracted twice with 50 ml of chloroform. The chloroform extract was evaporated under vacuum. To convert MP into its propionate, an esterification procedure was developed, based on previous reports [1, 16]. After resolving the residue (MP) in 2 ml of propionic acid and allowing to stand at 37°C for 30 min, 2 ml of trifluoroacetic anhydride (TFAA) were added and again incubated for 30 min. The mixture was poured into 100 ml of a 2% sodium bicarbonate solution in water and neutralized with solid sodium bicarbonate. The MPP formed was extracted from the emulsion with chloroform, (2 × 50 ml). After evaporating the solvent in vacuo, the residue was dissolved in methanol to a final concentration of approximately 1000 ng/ml. This concentration was derived from comparison of the GC response of an aliquot of this solution with the response of a known quantity of MPA, after derivatization, as described below.

Calibration curve

MPA (10 mg) was dissolved in acetone (50 ml) and diluted with methanol to a final concentration of 100 ng/ml. Different volumes covering the range 10—150 ng were pipetted into glass-stoppered 10-ml tubes followed by the addition of 100 μl of the internal standard solution. After mixing, the samples were evaporated to dryness under a gentle stream of nitrogen at room temperature.

Extraction procedure

The sera were extracted on Sep-Pak C_{18} cartridges as described by Hofreiter et al. [17]. A 100-μl volume of methanol and 100 μl of internal standard solution were added to 1.0 ml of serum. After mixing, the samples were allowed to stand for approximately 1 h and applied to pre-washed Sep-Pak C_{18} cartridges. A 5-ml volume of water was passed through the cartridges,

and eluted with 2 ml of methanol. The eluate was reduced to about 0.5 ml by evaporation under a gentle stream of nitrogen at room temperature. Then 5 ml of cyclohexane were added. The samples were shaken for 10 min with 5 ml of a solution containing 0.9% (w/v) sodium chloride and 0.1 mol/l sodium hydroxide, and then with 5 ml of water. The cyclohexane layer was evaporated under a gentle stream of nitrogen at room temperature.

Derivatization

Standards and samples were dissolved in 100 μl of TFAA and the closed tubes were placed in a dry block at 45°C for 45 min. After cooling to room temperature, 5 ml of hexane were added and the solution washed with 5 ml of 0.1 mol/l hydrochloric acid and 5 ml of water. The solvent was evaporated under a gentle stream of nitrogen at room temperature and the residue dissolved in 50 μl of hexane.

Gas chromatography—mass spectrometry

A Varian 3700 gas chromatograph directly coupled to a Finnigan Mat 212 mass spectrometer and equipped with an SS 200 data system was used. The samples were introduced by split/splitless injection at 250°C on a CP-Sil-5 fused-silica capillary column (25 m × 0.32 mm I.D.) (Chrompack, Middelburg, The Netherlands). The initial oven temperature (200°C) was raised at a rate of 20°C/min to a final temperature of 280°C, maintained for 2 min. Helium was used as a carrier gas (flow-rate, 1.9 ml/min). The mass spectrometer operating conditions were: ion source 250°C, electron energy 70 eV, emission current 0.5 mA and multiplier at 2000 V. The interface was kept at 250°C. The mass spectrometer was focused on the ions at *m/e* = 482.2 (M^+ of the MPA—TFAA derivative) and *m/e* = 496.2 (M^+ of the MPP—TFAA derivative). Mass spectra were recorded by scanning at a speed of 1.5 s/decade and storing the mass spectra by means of the SS 200 data system. Peak area ratios of 482.2/496.2 were calculated for both standard and serum samples. Also by means of the data system, a linear regression calibration plot was constructed from the standards, followed by calculation of the serum MPA concentration.

RESULTS

Evaluation of the assay

In Fig. 1a, the mass spectrum of the MPA derivative (3-enol ester) is depicted showing significant ions at *m/e* 482 (M^+) and *m/e* 379, formed by the removal of the C_{20}/C_{21} moiety and the 17-acetate group. The synthesized MPP was adequate for use as the internal standard as was proven by GC—MS analysis. An MPA peak could not be detected, whereas a large MPP peak was noted. Its mass spectrum (Fig. 1b) shows the expected molecular ion at *m/e* 496 (14 mass units higher than the molecular ion of MPA) and a peak with *m/e* 379, similar to MPA.

Fig. 2 shows the mass fragmentograms of a standard sample (Fig. 2a), a blank serum (Fig. 2b) and a serum sample from a patient receiving MPA (Fig. 2c). Linear calibration plots were obtained up to MPA concentrations equivalent to 150 ng/ml, with correlation coefficients ranging from 0.9975

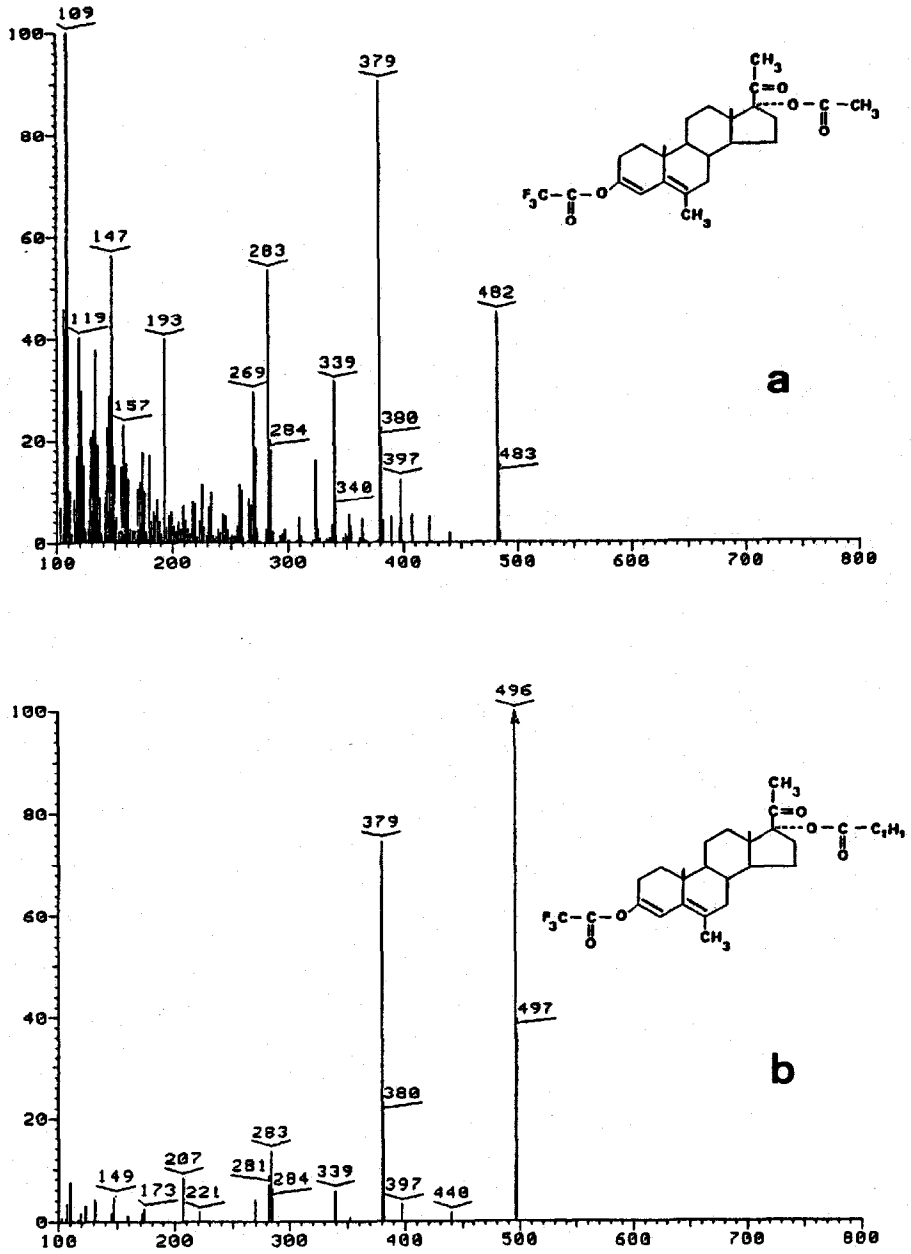

Fig. 1. Mass spectra of the TFAA derivatives of (a) medroxyprogesterone acetate and (b) medroxyprogesterone propionate.

to 0.9997. The method enables serum MPA concentrations as low as 0.5 ng/ml to be determined accurately. To test the analytical merits of the method, three different experiments were devised.

First, we tested the reproducibility by spiking an MPA-free serum pool with 31.5 ng MPA per ml and assayed the samples five times on three different days. The calculated results (n = 15) were: mean 32.8 ng/ml (104% recovery);

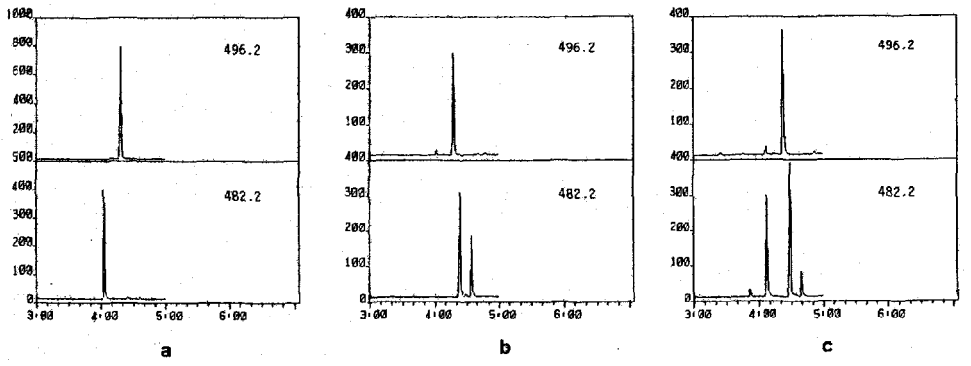

Fig. 2. The mass fragmentograms of a standard and two serum samples. The TFAA derivative of MPA is monitored at *m/e* 482.2 and the TFAA derivative of MPP (internal standard) at *m/e* = 496.2. Examples are shown of (a) standard sample, (b) serum sample containing no MPA and (c) serum sample of a patient receiving MPA orally.

standard deviation 1.6 ng/ml, corresponding to a coefficient of variation (C.V.) of 5.0%. Typical results of a five-fold determination on one of these days were: mean 33.2 ng/ml (105% recovery); standard deviation 1.8 ng/ml, corresponding to a C.V. of 5.3%.

Secondly, we prepared calibration standards as described in Experimental and also added the same amounts of MPA to 1.0-ml aliquots of a pool serum. Standards and spiked sera were determined on one day as usual. The regression plots obtained were compared. The equation of the standard calibration curve was $y = 0.0111x + 0.0279$, with a correlation coefficient of 0.9970; for the serum samples $y = 0.0116x + 0.0285$, with a correlation coefficient of 0.9978, y being the peak area ratio and x the MPA concentration.

Thirdly, in order to test the linearity of the assay, patient sera containing high levels of MPA were pooled and different volumes ranging from 0.10 ml to 2.00 ml were pipetted into glass-stoppered tubes. The samples were made up to a final volume of 2.00 ml with blank serum and determined. The results obtained were plotted, yielding a straight line which could be expressed by the equation $y = 0.1762x + 0.0008$, with a correlation coefficient of 0.9988 (n =

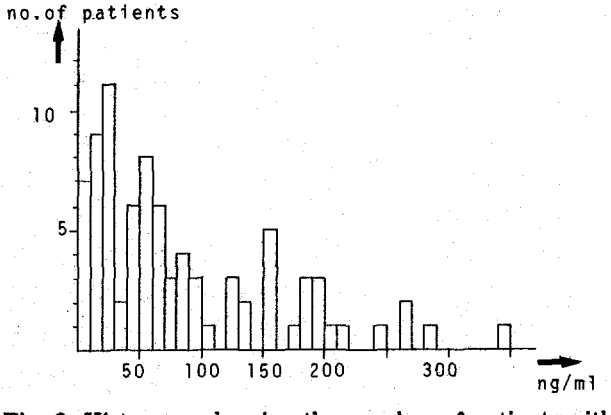

Fig. 3. Histogram showing the number of patients with a certain serum MPA concentration. Patients orally received 400 mg of MPA daily.

8), y being the peak area ratio and x the volume of pool serum. Thus the results of the three experiments demonstrate good linearity, accuracy and precision of the proposed assay

Patient samples

Samples of 84 patients with metastatic breast cancer, undergoing MPA treatment (400 mg per day orally), were investigated. The MPA concentrations

TABLE I

SEQUENTIAL SERUM MPA LEVELS IN PATIENTS RECEIVING ONE ORAL DOSE OF THE DRUG AT $t = 0$

Patients 1—8 received a 600-mg dose; patients 9—14 received a 300-mg dose.

Patient	MPA level (ng/ml)					
	0 min	30 min	60 min	120 min	180 min	240 min
1	4.1	4.8	27.8	29.8	46.5	32.3
2	4.5	6.5	7.0	8.5	23.8	20.8
3	4.5	4.7	5.2	7.0	8.6	8.1
4	9.1	13.0	10.4	13.2	15.6	12.1
5	0.4	4.7		5.8	8.0	12.1
6	30.7	46.7	55.9	101.7	55.2	61.9
7	5.9	9.6	27.0	21.9	15.9	8.5
8	4.4	21.4	31.6	24.9	27.4	44.3
9	2.3	4.3	4.0	5.4	6.1	5.9
10	9.3	10.1	9.6	9.0	9.1	9.0
11	2.3		3.2	5.8	4.6	4.1
12	17.5	20.0	33.1	46.1	60.3	71.7
13	6.1	2.6	9.7	21.8	19.0	23.1
14	4.7	5.2	2.7	12.8		9.1

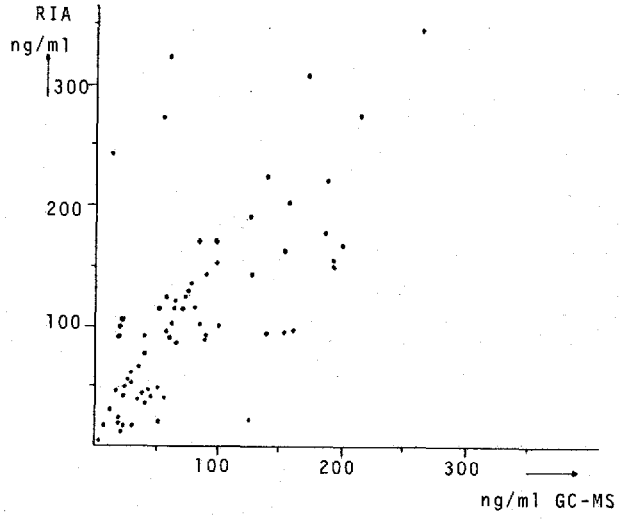

Fig. 4. Correlation graph comparing serum MPA concentrations determined by RIA, with those determined by GC—MS.

of the samples are shown in Fig. 3. It is evident that these patients showed a wide range of serum MPA levels (4—349 ng/ml), owing to large differences in uptake and metabolism. To obtain an impression of the uptake of orally administered MPA, we selected fourteen patients who temporarily interrupted their medication for two weeks and then received an oral dose of MPA. Eight patients received 600 mg and the other six patients 300 mg. Blood samples were taken just before receiving medication and then at 30, 60, 120, 180 and 240 min. The results are shown in Table I. As expected, large differences in serum levels and in the kinetics of uptake were observed.

Subsequently, we compared the present GC—MS method with a published RIA [12], and the results of this correlation study are shown in Fig. 4. Finally, a pilot study was undertaken, testing the possibility of determining megestrol acetate (MA) and cyproterone acetate (CPA) with the described assay. Preliminary results showed the feasibility of such an assay when focusing on the ions at m/e = 480.2 (MA) and m/e = 626.2 (CPA), respectively.

DISCUSSION

Publications dealing with the determination of MPA in serum by GC—MS are scarce. To our knowledge, only one method has been described in some detail, in which d_3-MPA was used as the internal standard, the deuterium atoms being located in the acetate moiety [11]. In trying to introduce the same GC—MS method in our laboratory, we attempted to synthesize the internal standard as described [11]. Apart from the low yield, we obtained a product, which, by GC—MS analysis, proved to be composed of a variety of differently labelled MPA molecules and a considerable quantity of unlabelled MPA instead of the expected triple-deuterated MPA. Most can probably be ascribed to a rapid exchange of deuterium atoms by hydrogen atoms. Subsequently, we tried to synthesize d_3-MPA by other means, resulting in a similar mixture of differently labelled products. Since deuterium atoms adjacent to a carbonyl group are known to exchange easily, our findings were not too surprising. Obviously, the d_3-MPA is not suitable for use as an internal standard and therefore we looked for another. MPP, which is very similar in structure to MPA, was chosen and synthesized as described. MP required for this synthesis was prepared from MPA via a mild hydrolysis with sodium methylate and subsequently esterified with propionic acid in the presence of TFAA with a yield of 60%. This internal standard meets the necessary demands. As can be concluded from comparison of the slopes of the two plots (one obtained from the standards and the other from the serum pool spiked with these same standards and analysed on the same day), MPP recovers only about 4% less than MPA during the analytical procedure, which is a negligible difference. This is also in accordance with the 104% recovery found in the fifteen-fold determination of a spiked pool serum.

By using Sep-Pak C_{18} cartridges, we could reduce extraction time and avoid liquid—liquid extraction problems [18]. Since MPA has no specific plasma carrier [19], a mild denaturation of serum proteins with methanol, just before extraction, is sufficient to abolish the weak protein—steroid interactions [7]. The wide inter-individual range of serum MPA concentrations, reported by

others [7, 10, 11], which could partially or entirely be ascribed to non-selectivity of the used techniques, was also found with our GC—MS method (Fig. 3). This validates the true nature of these differences, caused by inter-individual differences in uptake, storage and metabolism of MPA. Contemplating the results shown in Fig. 3 and Table I, the following comments can be made.

First, the range of serum levels found (Fig. 3) is in accordance with the findings of Laatikainen et al. [8], using a RIA technique. In addition, Fig. 4 shows that our results correlated rather well with those obtained from a RIA procedure [12] ($r = 0.73$, $p \ll 0.001$, $n = 71$). This proves that, in principle, RIA can be applied adequately, provided that the antibody does not cross-react with MPA metabolites or endogenous steroids, and a prior extraction step is introduced.

Secondly, Table I shows that the serum levels after two weeks of withdrawal are still measurable and rather variable. This indicates that MPA, once resorbed, circulates for a prolonged time after withdrawal, in accordance with the reported variable serum half-life of MPA [8]. We found that the maximum serum level was achieved 2—3 h after intake, its height depending mainly on baseline values.

In conclusion, the described method can be successfully used as a reference method. Moreover, since it is possible to determine about 40 samples a day by one technician, the GC—MS technique can be considered applicable for routine purposes.

It has the additional advantage that other related steroids in pharmaceutical use can be analysed by introducing only minor changes. Pilot studies showed the possibility of detecting megestrol acetate and cyproterone acetate as TFAA derivatives, also with MPP as internal standard.

ACKNOWLEDGEMENT

We are grateful to Mrs. A.W. de Ruyter-Buitenhuis for her technical assistance.

REFERENCES

1 J.C. Babcock, E.S. Gutsell, M.E. Herr, J.C. Stucki, L.E. Barnes and W.E. Dubin, J. Am. Chem. Soc., 80 (1958) 2904—2905.
2 J. Bonte, J.M. Decoster, P. Ide and G. Billiet, Gynecol. Oncol., 6 (1978) 60—75.
3 F. Pannuti, C.M. Camaggi, E. Stochi, M. Giovannini, A.R. di Marco and B. Costanti, Cancer Treat. Rep., 66 (1982) 2043—2049.
4 F. Pannuti, A. Martoni and G.R. Lenaz, Cancer Treat. Rep., 62 (1978) 499—504.
5 J.C. Cornette, K.T. Kirton and G.W. Duncan, J. Clin. Endocrinol. Metab., 33 (1971) 459—466.
6 M. Hiroi, F.Z. Stanczyk, U. Goebelssmann, P.F. Brenner, M.E. Lumkin and D.R. Mishell, Steroids, 26 (1975) 373—386.
7 D.G. Kaiser, R.G. Carlson and K.T. Kirton, J. Pharm. Sci., 403 (1974) 420—424.
8 T. Laatikainen, U. Nieminen and M. Adlercreutz, Acta Obstet. Gynecol. Scand., 58 (1979) 95—99.
9 E. Rossi, E. de Pascale, P. Negrini, A. Frigerio and E. Castegnaro, J. Chromatogr., 169 (1979) 416—421.
10 G. Milano, G. Carle, N. Renée, J.L. Boublil and M. Namer, J. Chromatogr., 232 (1982) 413—417.

11 G. Phillipou and R.G. Frith, Clin. Chim. Acta., 103 (1980) 129—133.
12 K. Shrimankar, B.N. Saxena and K. Fotherby, J. Steroid Biochem., 9 (1978) 359—363.
13 H. van Veelen, P.H.B. Willemse, D.T. Sleijfer, W.J. Sluiter and H. Doorenbos, Cancer Treat. Rep., in press.
14 I. Bjorkhem, A. Bergman, O. Falk, O. Kallner, L. Svensson, E. Akerloff and R. Blomstrand, Clin. Chem., 27 (1981) 733—735.
15 H. Breuer and L. Siekmann, J. Steroid Biochem., 6 (1975) 685—688.
16 E.J. Bourne, M. Stacey, J.C. Tatlow and J.M. Tedder, J. Chem. Soc., 7 (1949) 2976—2979.
17 B.T. Hofreiter, A.C. Mizera, J.P. Allen, A.M. Masi and W.C. Hicok, Clin. Chem., 29 (1983) 1808—1809.
18 R. Heikkinen, T. Fotsis and H. Adlercreutz, Clin. Chem., 27 (1981) 1186—1189.
19 M. Mathrubutham and K. Fotherby, J. Steroid Biochem., 14 (1981) 783—786.