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A chemiluminescence enzyme immunoassay (CLEIA) for the determination of medroxyprogesterone acetate in human serum

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Abstract

An enzyme immunoassay with chemiluminescene detection (CLEIA) for measuring serum levels of medroxyprogesterone acetate (MPA), a synthetic progestational agent currently used in fertility control and hormonal cancer, is reported. The polyclonal antiserum was obtained by immunizing rabbits with the synthetized 17-hemisuccinate derivative of medroxyprogesterone (MPS) coupled to serum albumin. This antiserum does not display any cross reactivity with extracted metabolites or with corticosteroid analogs with modifications at positions 11 and 16. The same MPS coupled to alkaline phosphatase is used as tracer. For the chemiluminescent detection system, adamantyl-1,2-dioxetane phosphate is selected as substrate. The typical standard curve ranges from 18.5 to 1182 pg per well and displays a slope factor of 0.74, with an ED_{50} of 143.8 pg of MPA per well and a minimum detectable and maximal level of 0.83 and 12 400 pg per well respectively. The assay has been validated on spiked serum samples in terms of precision (intra- and interassay coefficient variations of less than 8% and 13%, respectively), and of accuracy (mean recovery 105%). The validation on clinical samples demonstrates a good correlation of MPA serum values obtained both by radioimmunoassay and CLEIA. This specific and sensitive CLEIA, which requires less than 100 μl of serum sample, appears to be an interesting alternative for the monitoring of serum levels of MPA in humans.

Keywords: Adamantyl-1,2-dioxetane phosphate derivative; Drug analysis; Enzyme immunoassay; Medroxyprogesterone acetate

1. Introduction

Medroxyprogesterone acetate (MPA; 17α-acetoxy-6α-methyl-4-pregnene-3,20-dione) is a synthetic progestational agent shown to be effective in fertility control and hormonal cancer therapy such as endometrial and breast carcinomas [1,2]. Bioavailability and pharmacokinetic studies for MPA in humans have been carried out by measuring MPA serum levels using various analytical approaches, as reviewed by Sturm et al. [3]: gas chromatography (GC), gas chromatography coupled to mass fragmentation (GC/MF), high-performance liquid chromatography (HPLC) with UV detection, enzymatic assay with fluorescence detection, and radioimmunoassay (RIA). The latter methods are largely applied to the monitoring of MPA involved polyclonal antisera developed against MPA-3-carboxymethyloxime or MPA-11-hydroxysuccinyl derivatives coupled to bovine serum albumin (BSA) [4,5]. The antisera display cross reactivities with the MPA metabolites, especially those with alterations in the A-ring or at position C_6, resulting

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in up to ten-fold overestimation of MPA levels compared with those obtained in direct RIA [6,7]. To increase the specificity of the antibody oriented towards the A ring and position C_6 of the B ring, we proposed the development of a hemisuccinate derivative of medroxyprogesterone at position C_{17} based on the study by Stockdale and Rostom [8]. Using the antibody thus generated, an enzyme immunoassay using chemiluminescence detection (CLEIA) was developed and validated by assessing the serum MPA levels following a 20 mg oral dose of MPA to a female volunteer.

2. Experimental

2.1. Materials

Medroxyprogesterone (MP), MPA, trizma base, trizma HCl, polyoxyethylene sorbitan monolaureate (Tween 20), Freund's complete and incomplete adjuvants, BSA, and *p*-nitrophenyl phosphate (pNPP) 20 mg tablets were purchased from Sigma (St. Louis, MO). All the other steroids used were supplied by Steraloids (Wilton, NH). ^{3}[H]-MPA was from DuPont (Boston, MA). 4-Dimethylaminopyridine, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline were from Aldrich (Milwaukee, WI). Semi-preparative Silica gel GF plates were from Analtech (Newark, DE). Protein G Sepharose (Mab Trap G), Sephadex G-50 medium gel and dextran T-70 were obtained from Pharmacia (Uppsala, Sweden). Alkaline phosphatase/EIA grade, Lumigen (4-methoxy-4-(3-phosphate-phenyl) - spiro (1, 2 - dioxetane - 3, 2′ - adam - antane)disodium salt) with cetyltrimethyl ammonium bromide and 5-N-tetradecanoylamino-fluorescein (Lumi-Phos^{R}530) were purchased from Boehringer Mannheim (Gmbh, Germany). Di-sodium 3-(4-methoxyspiro (1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3.7}$]decan)-4-yl)phenyl phosphate (CSPD^R), disodium 3-(4-methoxyspiro(1, 2 - dioxetane - 3, 2′ - tricyclo[3.3.1.1.$^{3.7}$]decan)-4-yl) phenyl phosphate (AMPPD^R), and EMERALD^R luminescent amplifier were from Tropix (Bedford, MA). Other chemicals were of analytical grade otherwise specified and were provided by Fisher (Montreal, Que.). Maxisorp U-16, 96-well polystyrene plates were from NUNC (Danemark). MicroFLUOR "w", flat bottom plates from Dynatech (Chantilly, VA) were used for chemiluminescence assay. Behring ELISA processor (Behring Diagnostics Marburg, Germany) and the ML-1000 luminescent reader (Dynatech) were used as plate readers. The HPLC system used was model 600, from Waters (Milford, MA). The HPLC column was from CSC (St-Laurent, Que.). Female rabbits weighing 2–2.5 kg were purchased from Charles River (Saint-Constant, Que.).

2.2. Methods

Synthesis and characterization of medroxyprogesterone-17-hemisuccinate derivative

MP (2 mmol) was refluxed with succinic anhydride (14 mmol) in pyridine in the presence of 4-dimethylaminopyridine (6 mmol) at 140 °C for 24 h. Pyridine was then removed under vacuum and the residue dissolved in 40 ml of an ether–dichloromethane mixture (3:1, v/v). The organic phase was washed with 20 ml of 10% HCl followed by 2 × 20 ml of distilled water. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was then dissolved in 0.5 ml of hot methanol and the resulting solution was streaked onto a preparative silica gel GF plate. The thin layer chromatography conditions using an ethylacetate–cyclohexane–acetic acid mixture (70:30:5, v/v/v) allowed the separation of a medroxyprogesterone-17-hemisuccinate derivative (MPS) from MP with R_f values of 0.327 and 0.631. The band corresponding to the R_f of the MPS derivative was scraped off and extracted with a mixture of dichloromethane–ether (3:1, v/v). This organic phase was then evaporated to dryness under vacuum and the residue obtained was further purified by HPLC using an ODS-2 column (5 μm, 25 × 0.46 cm) and a methanol–water mixture (80:20, v/v) as mobile phase at a flow rate of 1 ml min^{-1}. The elution volume corresponding to that of MPS was collected and evaporated to dryness, giving a tan–yellow residue which was characterized by fast atom bombardment (FAB) mass spectrometry, infrared spectroscopy and proton nuclear magnetic resonance (PNMR).

Coupling of medroxyprogesterone-17-hemisuccinate to BSA

MPS (11 μmol) was dissolved in dioxane (100 μl) alkalinized with TEA (30 μl), and was further activated with 10% isobutylchloroformate solution in triethylamine (15 μl). This solution was dropped into the BSA solution

consisting of 6.44 mmol of BSA dissolved in distilled water (200 µl) under constant stirring and the coupling reaction continued for 3 h at 4 °C. The antigen complex was then purified by gel exclusion on Sephadex G50. The efficiency of the coupling procedure assessed by the ratios of absorbances at 245/280 nm on the excluded fraction relative to the MPS reference standard gave a yield of 5 haptens per mol of BSA.

Production of antisera against medroxyprogesterone-17-hemisuccinate

Rabbits were immunized by subcutaneous injection of 150 µg antigen complex emulsified in 1 ml complete Freund adjuvant using a multisite approach. Six weeks after the primary immunization, boosters were given at 2 week intervals and the blood samples collected for the titer monitoring. Polyclonal IgGs were purified from antisera by affinity chromatography on a Mab Trap G.

Assessment of the specifity of the antisera

The specificity of the antisera was assessed by cross reactivity studies with corticosteroid analogs and with a pool of MPA metabolites generated in vivo. The cross reactivity with corticosteroids was determined by the dose of MPA at 50% displacement of [^{3}H]-MPA relative to that of MP, progesterone, pregnenolone, 17α-OH-progesterone, 16α-CH_3-progesterone, 11β-OH-progesterone, deoxycorticosterone and 18-OH-deoxycorticosterone.

The cross reactivity with MPA metabolites generated in vivo was assessed using a pool of serum samples collected from 0.25 up to 5 h after a 20 mg oral dose of MPA from a female subject. The serum extracts prepared as described later were chromatographed by HPLC using an ODS reversed-phase column (5 µm, 25 × 0.46 cm) and a mobile phase consisting of a methanol–ammonium acetate mixture (20 mM; pH 4) (78:22, v/v) at a flow rate of 1 ml min^{-1}. The eluted fractions (1 ml) were collected for 15 min after the sample injection, evaporated to dryness and the immunograms set on these fractions.

Sample preparation procedure for standard curve

Standards of MPA in serum were prepared from a stock solution of 1.15 mg ml^{-1} in methanol and diluted in human serum to give final concentrations of 1.08–69 ng ml^{-1}. For the calibration curve, 60 µl of standards of MPA in serum were diluted with 240 µl of drug free serum and submitted to the extraction procedure using 2.5 ml petroleum ether (35–60 °C). The organic phase was then collected and evaporated to dryness under vacuum. The residue was dissolved in acetonitrile (500 µl) and partitioned with hexane (500 µl). The hexane layer was discarded by aspiration and the acetonitrile phase was evaporated to dryness. The residue was resuspended in 175 µl of incubation buffer which consisted of Tris-HCl buffer–CH_3OH (85:15, v/v) (50 mM, pH 7.4) containing NaCl (100 mM), $ZnSO_4$ (0.1 mM), Tween 20 (0.5 ml l^{-1}) and 175 µl of an MPS–alkaline phosphate conjugate at an initial dilution of 1/640. Aliquots of 100 µl were transferred in each well in triplicate for the incubation step of the CLEIA procedure.

For the serum samples obtained from the pharmacokinetic profile, the extraction of MPA and the reconstitution of the sample extracts were carried out as described above for the standard curve.

CLEIA procedure

MPS–alkaline phosphatase conjugate used as tracer was obtained by coupling MPS (6.5 µmol) preactivated with 1-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline (18.2 µmol) to calf intestine alkaline phosphatase (1 mg) in carbonate buffer (100 mM, pH 9.5) containing $ZnSO_4$ (0.1 mM) for 18 h at room temperature, according to the method of Belleau and Maleck [9]. The MPS–alkaline phosphatase conjugate was then dialyzed against Tris-HCl buffer (50 mM, pH 7.4) containing $ZnSO_4$ (0.1 mM).

Microfluor 96-well immunoplates were coated with purified polyclonal IgGs (62.3 ng per 100 µl) in carbonate buffer (100 mM, pH 9.5) for 24 h at 4 °C. Following the removal of the coating buffer and five washing cycles, 100-µl aliquots of the mixture of MPA standards or serum extracts and the MPS–alkaline phosphatase as tracer were added to each well and incubated for 16 h at 4 °C under rotational agitation. After five washing cycles, the activity of bound alkaline phosphatase was revealed in the presence of CSPD^R (100 µl) for 20 min at 37 °C. The intensity of light emission was measured at 540 nm and the results expressed in relative light units (RLU).

Kinetic profile of MPA

A kinetic profile of MPA was set following an oral dose of 20 mg of Provera® given to a

female subject. Blood samples (2 ml) were collected at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 7, 8, 24, 48 and 72 h after drug administration, and serum samples were collected for the MPA extraction procedure as described above.

3. Results

3.1. Characterization of medroxyprogesterone-17-hemisuccinate derivative

The characterization was performed by FAB mass spectrometry giving molecular ions at 444 ($M.H^+$) and 467 ($M.Na^+$). The PMNR spectrum of the synthetized derivative was characterized by signals at δ0.5 ppm (S, 3H, $C_{19}H$), 1.1 ppm (S, 3H, C_6H), 1.7 ppm (m, 6H, $C_{11}H$, $C_{12}-H$, C_1-H), 2.04 ppm (S, 3H, $C_{21}-H$), 2.4 ppm (m, 2H, $O=C-CH_2$), 2.7 ppm (m, 4H, $O-CH_2-C=O$, CH_2-C-OH), and 5.8 ppm (S, 1H, C_4-H). The IR spectrum of this derivative recorded in a KBr pellet displayed broad bands at 2600–3300 cm^{-1} (O–H stretch), and bands at 1750–1770 cm^{-1} (C=O ester stretch) and 1300–1000 cm^{-1} (C–O ester stretch).

3.2. Assessment of the specificity of the antisera generated

The cross reactivity of the antisera generated with progesterone and corticosterone analogs was carried out by radioimmunoassay according to Ong et al. [10]. The ratio of the dose at 50% displacement of [^{3}H]-MPA for MPA relative to the steroid analogs tested is presented in Table 1. In order to document the potential cross reactivity with MPA metabolites, HPLC-CLEIA was set for the extract of the pool of serum reconstituted with samples collected at 0.25–72 h after a 20 mg oral dose of MPA. As shown in Fig. 1, a unique peak of immunoreactivity was detected and coeluted with that of MPA spiked in blank serum. The specificity of the antisera thus generated was confirmed by the lack of cross reactivity with any extracted metabolite.

Table 1
Cross reactivity of MPA antisera with corticosteroid analogs

Steroid analog	Cross reactivity (%)
Medroxyprogesterone	5.90
Progesterone (PG)	0.18
16α-CH_3-PG	0.09
17α-OH-PG	0.08
Deoxycorticosterone (DOC)	0.03
18-OH-DOC	<0.001
11β-OH-PG	<0.001
Pregnenolone	<0.001

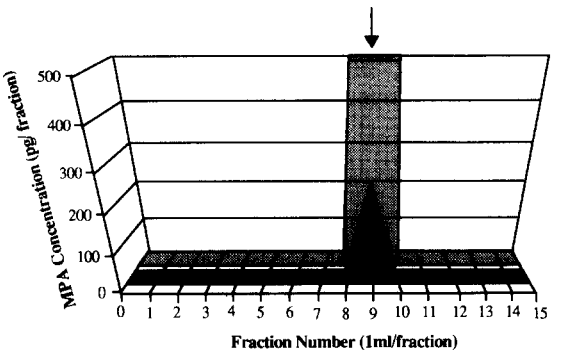

Fig. 1. Immunoreactive profiles of blank serum (□), pooled serum samples (■) from a patient after 20 mg oral dose of MPA, and a blank serum spiked with 5.7 ng ml^{-1} of MPA (□) after HPLC separation. The unique immunoreactive signal detected for the pooled serum sample corresponds to that of MPA. The arrow indicates the position of elution of MPA standard.

3.3. CLEIA

In order to select an appropriate chemiluminescent substrate for the assay, three substrates, Lumi-phos^R 530, AMPPD^R and CSPD^R, were tested according to the manufacturers' specifications [11]. The ratios of RLU detected relative to that of the zero concentrations obtained for MPA calibration curves in the presence of the three luminescent substrates and *p*-nitrophenylphosphate are shown in Fig. 2. It appears that the light signals recorded with CSPD^R and AMPPD^R as substrates were 100-fold that obtained with colorimetric reaction using *p*-nitrophenylphosphate. The CSPD^R was selected as a chemiluminescent substrate throughout the study. A typical competitive displacement binding curve of the MPS– alkaline phosphatase complex as tracer by increasing concentrations of MPA from 18.5 to 1182 pg per well is shown in Fig. 3. The binding curves analyzed by non-linear regression using a four-parameter logistic equation [12] were characterized by a slope factor of 0.74, and an ED_{50} of 143.8 pg per well, with a minimum detectabel and maximum concentration of 0.83 pg per well and 12 400 pg per well respectively.

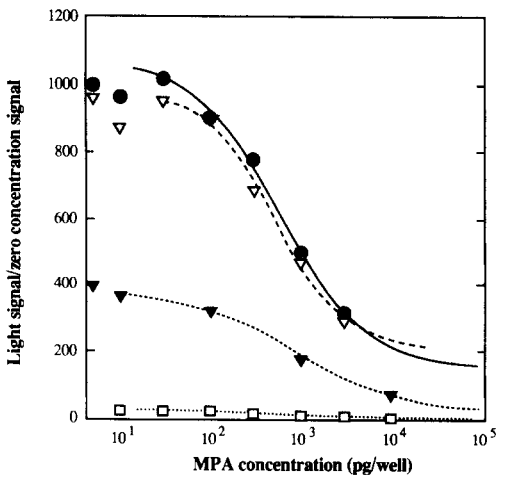

Fig. 2. MPA calibration curves using different chemiluminescent substrates. The ratio of the signal of the standard to the signal of the zero value in the presence of increasing MPA concentrations from 18 to 1182 pg per well is plotted. The chemiluminescent light output was measured in RLU after the addition of CSPS^R (0.4 mM) (●), AMPPD^R (0.4 mM) (▽), Lumi PHOS^R 530 (750 mM) (▼) and pNPP absorbance 1 mg ml^{-1} (□).

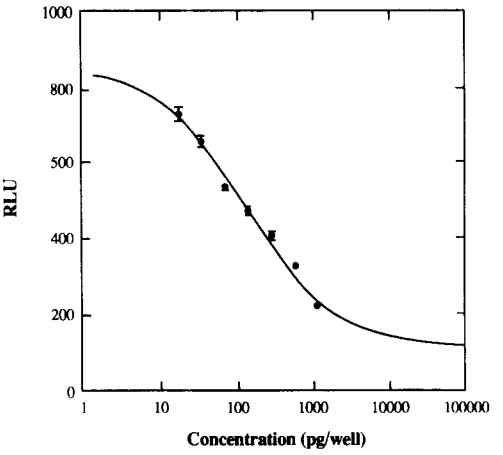

Fig. 3. Standard curve of MPA by CLEIA. MPA serum extracts were prepared as described in the sample preparation procedure for standard curve and analyzed in triplicate corresponding to MPA concentrations from 18 to 1182 pg per assay. Data were presented as means ± SE.

3.4. Precision and accuracy

To assess the precision and accuracy of the assay, spiked MPA in serum at low, medium and high concentrations corresponding to 3.6, 7.19 and 14.38 ng ml^{-1} was studied. As shown in Table 2, the intra-assay precision (measured as % RSD) was 8% at both 3.6 and 7.19 ng ml^{-1} and 3.9% at 14.38 ng ml^{-1}. By assaying the same group of spiked samples on four different days, the inter-assay was found to be 7% for both 7.19 and 14.38 ng ml^{-1}, and 13% for 3.6 ng ml^{-1}. The accuracy ranged from 99.7 to 110.9% for MPA concentrations from 3.6 to 14.38 ng ml^{-1}.

3.5. Validation with clinical samples

Clinical samples ($n = 30$) from patients receiving Provera® have been analyzed by both RIA [10] and CLEIA. The correlation between these data could be described by the following equation $\hat{Y} = 1.066X + 0.512$ ($R = 0.98$), as shown in Fig. 4.

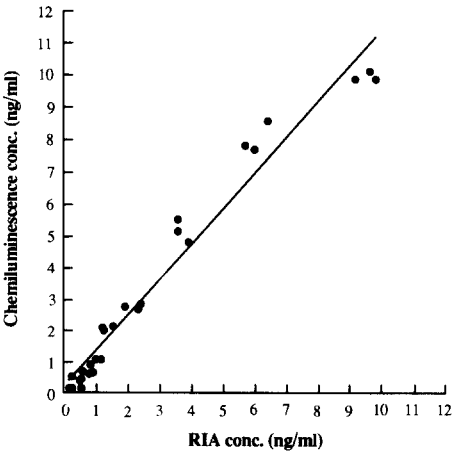

Fig. 4. Correlation between the data from clinical samples ($n = 30$) analyzed by both RIA and CLEIA.

3.6. Application

The application of the CLEIA method to the determination of the kinetic profile of MPA

Table 2
Intra-assay and inter-assay accuracy and precision of CLEIA for MPA determination in serum

Spiked concentration (ng ml^{-1})	Intra-assay ($n = 6$)			Inter-assay ($n = 4$)		
	Mean	RSD (%)	Accuracy (%)	Mean	RSD (%)	Accuracy (%)
3.60	3.72	8.29	103.33	3.59	13.07	99.72
7.19	7.65	8.46	106.40	7.98	7.26	110.99
14.38	14.88	3.93	103.48	15.79	6.64	109.81

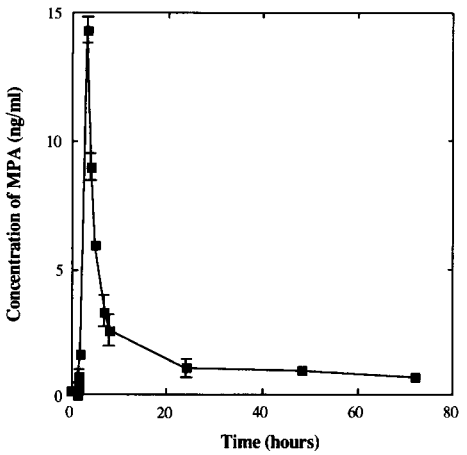

Fig. 5. MPA serum levels (ng ml^{-1}) in a female volunteer following the administration of a 20 mg oral dose of MPA. Serum samples were collected at 0, 0.25, 1, 1.5, 2, 3, 4, 5, 7, 8, 24, 48 and 72 h after the dose.

from a female subject following a 20 mg oral dose of Provera® is shown in Fig. 5. The mean serum concentration of MPA peaked at 14.25 ng ml^{-1} (C_{max}) after about 3 h (T_{max}). The monitoring of MPA could be performed up to 72 h after the oral dose, as a serum level of 0.65 ng ml^{-1} could be detected. These results clearly demonstrate that CLEIA is suitable for the evaluation of the pharmacokinetics of MPA in humans.

4. Discussion

Among the immunological methods reported for MPA measurement, the specificity of the assays was often a major issue. MPA is known to undergo extensive metabolism, from which the reduction of the A-ring or the side chain followed by hydroxylation at positions 2, 6 or 21 have been considered as major pathways [13]. Effectively, 2α-hydroxy- and 21α-hydroxy-MPA, as well as 17α-acetoxy-2α,3β-dehydroxy-6α-methylpregn-1,4-diene-20-one and 6α-hydroxymethyl-21-hydroxy-MPA, have recently been identified [3]. Taking into consideration these metabolic pathways, we have developed antisera against the 17-hemisuccinate derivative of MPA. The antisera thus generated do not display any cross reactivity with the extracted metabolites or with substituted analogs of progesterone at positions 11 and 16.

Using this specific antisera, we proposed to develop the enzyme immunoassay for MPA, which has emerged as a widely used technique to avoid the problems of radioactive disposal and limited shelf-life of radiolabeled tracers. To enhance the sensitivity of the detection system, chemiluminescence using adamantyl substituted 1,2-dioxetanes as substrates for alkaline phosphatase, was first applied to the detection of a hormonal steroid. CSPD^R in the presence of Emerald^R enhancer appeared to be the most promising chemiluminescent substrate; a 100-fold increase in the zero signal ratio could be observed for this derivative compared to that of *p*-nitrophenylphosphate.

Taking into account the high sensitivity of the detection system, serum samples of less than 100 µl only were required for the assay. The assay was validated on spiked serum samples, giving intra- and inter-assay coefficient of variations of less than 8 and 13% respectively, and an accuracy of 105% for all the concentrations tested. It was also validated on clinical samples as a good correlation of the MPA serum values obtained by both RIA and CLEIA.

5. Conclusion

A competitive enzyme immunoassay with chemiluminescence detection was developed and validated for the determination of MPA concentrations in human serum samples. This assay is a specific and sensitive non-isotopic alternative to measuring MPA at subnanogram levels, suitable for clinical monitoring and pharmacokinetic studies of this drug hormone.

References

[1] F. Pannuti, A. Martoni, E. Pollutri, P. Camera and G.R. Lenaz, IRCS, 2 (1974) 1605.
[2] T. Laatikainen, U. Nieminen and H. Adlercreutz, Acta Obstet. Gynecol. Scand., 58 (1979) 95–99.
[3] G. Sturm, A. Haberlein, T. Bauer, T. Plaum and D.J. Stalker, J. Chrom. Biomed. Appl., 562 (1991) 351–362.
[4] M. Mathrubuthan and K. Fortherby, J. Steroid Chem., 14 (1981) 783–786.
[5] J.C. Cornette, K.T. Kirton and G.W. Duncan, J. Clin. Endocrinol., 33 (1971) 459–466.
[6] G. Sturm and K.D. Schulz, in A. Pelligrini et al. (Eds.), MPA Assays: Measurement of plasma MPA levels in high dose MPA-treated patients, Raven Press, New York, 1984, pp. 23–42.
[7] H. Adlercreutz, P.B. Eriksen and M.S. Christensen, J. Pharm. Biomed. Anal., 1 (1983) 153–162.

[8] A.D. Stockdale and A.Y. Rostom, Clin. Pharm., 16 (1989) 129–133.
[9] B. Belleau and G.J. Malek, J. Am. Chem. Soc., 90 (1968) 1651–1652.
[10] H. Ong, A. Adam, M. Bellemare, P. Delahaut and J.C.K. Loo, Drug Safety, 5 (Suppl. 1) (1990) 164–165.
[11] I. Bronstein, K. Van Dyke and R. Van Dyke (Eds.), in Luminescence Immunoassay and Molecular Applications, CRC Press, Boca Raton, FL, 1990, pp. 255–274.
[12] A. De Lean, P.J. Munson and D. Rodbard, Am. J. Physiol., 235 (1978) E97–102.
[13] A. Ortiz, M. Hiroi, F.Z. Stanczyk, U. Geobelsmann and D.R. Mishell, J. Clin. Endocrinol. Metab., 44 (1977) 32–38.