RADIOIMMUNOASSAY METHOD FOR SIX STEROIDS IN HUMAN PLASMA[a]

P.F.Brenner[b], R.Guerrero[c], Z.Cekan and E.Diczfalusy

Swedish Medical Research Council, Reproductive Endocrinology Research Unit, Karolinska sjukhuset, 104 01 Stockholm 60, Sweden

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ABSTRACT

A radioimmunoassay method has been developed for the simultaneous determination of progesterone, 3β-hydroxy-5-pregnen-20-one, 3β-hydroxy-5-androsten-17-one, testosterone, estrone and estradiol in 2 ml plasma samples. The first three steroids are isolated on a celite:propylene glycol column (1:1 w/v), the latter three steroids on a celite: ethylene glycol column (2:1 w/v). Steroids obtained from individual chromatographic fractions are incubated for 40 min. with the appropriate antisera and labelled steroids and the bound and free fractions are separated by charcoal.

Precision and accuracy studies have shown that the assays of all steroids are reproducible and accurate. Specificity studies carried out with 57 steroids, in conjunction with the accuracy experiments, indicate that a very high degree of specificity has been achieved. One possible exception may be the assay of 3β-hydroxy-5-pregnen-20-one. In this assay 3β-hydroxy-5α-pregnan-20-one, if present in plasma in appreciable amounts, would lead to an overestimation.

INTRODUCTION

By increasing the number of individual steroids assayed more information can be obtained on hormonal patterns and correlations in normal and pathological conditions.

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[a] A preliminary report has been presented at the IX Acta Endocrinologica Congress, Oslo, 17th - 21st June, 1973[1].

[b] Ford Foundation Fellow in Reproductive Endocrinology.

[c] Fellow of the Swedish International Development Authority (SIDA).

There is,however, a paucity of radioimmunoassay methods which would make possible simultaneous measurements of a number of steroids in a small volume of plasma.

In a reliable assay the steroids have to be separated from the majority of other compounds prior to the radioimmunoassay,even if the antisera used have a high degree of specificity. It was found that celite chromatography is very suitable for this purpose. It gives very low blank values and it allows a high versatility for the separation of a number of steroids.

In this paper we wish to describe a simultaneous assay of the six steroids mentioned. For the separation of the steroids we utilized the principles of the celite chromatography described by Siiteri[2] and further developed by Korenman et al.[3] and Abraham et al.[4]

MATERIAL AND METHODS

Solvents

Anhydrous ethyl ether (Mallinckrodt,St.Louis,U.S.A.) stored at -20°C was used, each time from a freshly opened can. Isooctane (Kebo,Stockholm,Sweden),cyclohexane,benzene and ethyl acetate (all purchased from Merck,Darmstadt,Germany) were redistilled once. Toluene (Merck) and absolute ethanol were used without further purification,as were propylene glycol (Kebo) and ethylene glycol (Merck).

Phosphate buffer system (PBS)

The phosphate buffer system consisted of phosphate buffer[5] (pH 7.0,0.1 M), sodium chloride (0.9 % w/v),gelatin (0.1 % w/v) and sodium azide (0.1 % w/v).

Charcoal

Norit A (Serva,Heidelberg,Germany),particle size 4 - 5 μ, was washed three times with distilled water.The fine particles were decanted and the residue dried at 100°C overnight.A 1 per cent (w/v) suspension in PBS was prepared every 3 days.

Celite

Celite (Analytical Filter Aid,Johns-Manville,Denver,U.S.A.) was kept in an oven at 540°C overnight.Having reached room temperature,it was mixed with propylene glycol (1:1 w/v) or ethylene glycol (2:1 w/v) for 15 min.

Non-radioactive steroids

Progesterone[a], pregnenolone, dehydroepiandrosterone, testosterone, estrone and estradiol were purchased from Steraloids (Pawling, U.S.A.). The purity was checked by chromatography and melting point determinations. The compounds were purified by crystallization, if necessary. Each steroid was diluted with absolute ethanol to a concentration of 20 - 80 ng per 100 µl. This was the stock solution. An aliquot of the stock solution (50 - 100 µl) was increased in volume with PBS to 10 ml and then placed in a 30°C water bath for 10 min. and vortexed for 30 sec. Further dilutions were prepared by mixing this solution with appropriate amounts of PBS. All buffer solutions were prepared every two weeks.

Other steroidal compounds (cf. Table 9) were purified and diluted in a similar fashion.

Radioactive steroids

[1,2,6,7-^{3}H]Progesterone (110 Ci/mmol), [7-^{3}H]pregnenolone (25 Ci/mmol), [7-^{3}H]dehydroepiandrosterone (16 Ci/mmol), [1,2,6,7-^{3}H]testosterone (100Ci/mmol), [2,4,6,7-^{3}H]estrone (85 Ci/mmol) and [2,4,6,7-^{3}H]estradiol (100 Ci/mmol) obtained from New England Nuclear Corp. (Dreieichenhain-Frankfurt/Main, Germany) were tested for purity by Sephadex LH-20 column chromatography using the solvent system toluene:methanol 85:15. An aliquot was then crystallized with non-radioactive steroid to constant specific radioactivity. Each radioactive steroid was diluted with toluene:methanol 85:15 to a concentration of approximately 10^6 dpm per 100 µl. This was used as the stock solution. Depending on the volume (50 or 100 µl) of labelled steroid solution used in the assay, 50 or 100 µl of this stock solution was evaporated under nitrogen. PBS (10 ml) was added, the vial was placed in a 30°C water bath for 10 min. and then vortexed for 30 sec.

The radioactive steroids were purified at regular intervals; tetralabelled compounds monthly, the other compounds every two months.

Synthesis of antigens

The haptens were prepared according to Erlanger et al.[6] with one modification. This involved using milder reaction conditions in the synthesis of (O-carboxymethyl) oximes as shown in the following example:

Testosterone (1.0 g), (O-carboxymethyl)hydroxylamine hydrochloride (1.0 g ; Aldrich Chemical Co., Milwaukee, U.S.A.), 25% ammonia solution (2.5 ml) and ethanol (250 ml) were refluxed for 5 h. The solution was evaporated in vacuo and the residue was dissolved in water made alkaline with sodium hydroxide. The aqueous solution was extracted with ether and acidified with hydrochloric acid. The precipitate was collected, washed with water, dried and crystallized to analytical purity. This modification resulted in higher yields with less impurities.

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[a] For systematic names see Table 9.

The following haptens pertinent to this study were synthesized: 11α-Hydroxy-4-pregnene-3,20-dione hemisuccinate, pregnenolone hemisuccinate, testosterone (O-carboxymethyl)oxime and dehydroepiandrosterone hemisuccinate.

The antigens were synthesized from the haptens and bovine serum albumin (Sigma, St.Louis,U.S.A.) by the mixed anhydride procedure[6]. The number of hapten residues was measured by the differences in ultraviolet spectra of haptens and their conjugates with bovine serum albumin[7],and/or by differences in specific radioactivity of the haptens and conjugates. The antigens contained 18 - 32 hapten residues per bovine serum albumin molecule.

Antisera

The antibodies were raised in rabbits (3 months old) by injecting the antigens mentioned above in aqueous Freund adjuvant emulsion at 3 - 4 monthly intervals. The procedure of Hurn and Landon[8] was adopted.

For estrone and estradiol assays antisera supplied by Dr.J.-P.Raynaud, Roussel Uclaf Research Centre,France,were used. These antisera were raised against bovine serum albumin conjugated with 3-hydroxy-1,3,5(10)-estratriene-7,17-dione 7-(O-carboxymethyl)oxime and 3,17β-dihydroxy-1,3,5(10)-estratrien-7-one (O-carboxymethyl)oxime, respectively.

The properties of the antisera are described in Table 1 in terms of titres and association constants. The specificity is apparent from Tables 10 - 15.

TABLE 1

Titres and association constants of the antisera

Antiserum	Final dilution (in 0.3 ml)	Association constant[a] x 10^9 (litres/mole)
Progesterone	1:2100	2.5
Pregnenolone	1:24000	3.5
Dehydroepiandrosterone	1:24000	1.8
Testosterone	1:45000	7.0
Estrone	1:60000	14.9
Estradiol	1:72000	10.4

[a] The association constants were estimated from Scatchard plots calculated on the basis of standard curves.

Separation of plasma steroids

Extraction: A plasma sample (1.0 ml) was placed in a counting vial by means of an Oxford pipette with a disposable tip (Oxford Labs.,Foster City,Calif.,U.S.A.), mixed with a known amount of radioactive steroid(s)(approx.2000 dpm each) and vortexed with ethyl ether (10 ml) for 45 sec.The aqueous layer was frozen in an ethanol: dry ice mixture and the ether solution was decanted into a conical tube(11 x 1.5 cm).

TABLE 2

Chromatographic separation of steroids - elution pattern

Solvent	Volume (ml)	Eluate
Celite:propylene glycol column		
Sample	1	discarded
Isooctane	2	discarded
	4	PROGESTERONE
Cyclohexane:benzene 9:1	2	discarded
	4	PREGNENOLONE
	3	discarded
	4	DEHYDROEPIANDROSTERONE
Celite:ethylene glycol column		
Sample	1	discarded
Isooctane	10	discarded
Cyclohexane:benzene 97:3	4	TESTOSTERONE
	1	discarded
Isooctane:ethyl acetate 85:15	4	ESTRONE
Isooctane:ethyl acetate 70:30	3	ESTRADIOL

The ether was evaporated under nitrogen in a 40°C water bath. The residue was dissolved in isooctane (1.0 ml) and transferred to one of the columns.

Preparation of columns: Disposable glass columns (length 350 mm, inner diameter 5 mm) were washed by submerging in isooctane overnight. They were fitted with a teflon disc (dipped in isooctane overnight) and tightly packed with a celite:propylene glycol or celite:ethylene glycol mixture to a height of 50 mm. Nitrogen pressure was used to percolate all solvents through the columns. The flow-rate was about 1 drop per 2 sec. The chromatography was performed in a constant temperature room at 18°C. Isooctane (10 ml) was percolated through the columns before the sample was applied.

Chromatography: The elution pattern is shown in Table 2. The steroid fractions collected were evaporated under nitrogen in a 40°C water bath. The residues were dissolved in 1.0 ml of PBS (0.3 ml for estrone and estradiol) by heating in a 60°C water bath for 5 min. and vortexing for 15 sec. The solution was divided for recovery measurement and for the assay. Then, 0.5 ml (0.1 ml for estrone and estradiol) of the solution was transferred to a counting vial and counted (see below). Recoveries of radioactive steroids which were below the lower limit of the range shown in Table 3 indicated that the chromatographic separation should be re-examined. In such a case changes in the elution pattern amounting to $\pm$ 0.5 ml were allowed.

Radioimmunoassay

In order to obtain a standard curve, 100 µl of the PBS solution (200 µl for progesterone and pregnenolone) of the non-radioactive steroid in increasing doses

TABLE 3

Recovery of radioactive steroids following the chromatographic separation

Steroid	Mean recovery[a] (%)	Range (%)
Progesterone	87	69 - 96
Pregnenolone	65	52 - 82
Dehydroepiandrosterone	70	52 - 81
Testosterone	77	65 - 83
Estrone	83	71 - 87
Estradiol	76	60 - 83

[a] Established on 20 replicates.

(for the first and following doses added cf. Table 4) was pipetted in replicates of four into assay tubes.

For every plasma sample 100 µl of the eluate PBS solution (200 µl for progesterone and pregnenolone) was pipetted into an assay tube.

In all instances the volume was then made up to 300 µl with a mixture of equal volumes of the corresponding antiserum dilution and radioactive steroid solution. Usually about 10^4 dpm was used. The test tubes were incubated at 60°C for 10 min. followed by 30°C for 30 min. The tubes were then cooled down in an ice-water bath and the ice-cold charcoal suspension (0.5 ml) was delivered into them. The amount of test tubes in one experiment was chosen such that the time required for adding the charcoal to all tubes did not exceed 3 min. The tubes were allowed to remain in ice for 10 min. They were agitated frequently. The contents were then centrifuged at 1000 x g at 4°C for 5 min. The supernatant was decanted into counting vials and the radioactivity was measured. The above incubation and separation conditions are as described previously[9].

Radioactivity measurement

To counting vials containing the aqueous supernatant 10 ml of scintillation liquid (5 g PPO, 0.5 g dimethyl-POPOP per 1 l toluene) was added. The vials were closed and placed in a 60°C water bath for 5 min. Afterwards they were vortexed for 5 sec. and after cooling down placed in the β-counter.

The radioactivity was measured on Nuclear Chicago β-counters with efficiencies for tritium ranging from 48 to 58 per cent.

Calculations

All computations were performed on a Wang programmable electronic calculator, model 700 A (Wang Labs., Tewksbury, Mass., U.S.A.). For every standard curve a best-fit straight line in logit-log co-ordinates was computed and its linearity was statistically tested by means of an analysis of variance[10]. For the calculation of unknowns from the equation of the best-fit straight line a correction was used for

TABLE 4

Least detectable doses of steroids in the standard curves and in plasma

Steroid	Least detectable dose	
	Standard curve[a] (= First dose)[c] (pg)	Plasma[b] (1 ml) (pg/ml)
Progesterone	20	125
Pregnenolone	25	200
Dehydroepiandrosterone	50	625
Testosterone	12.5	160
Estrone	12.5	50
Estradiol	12.5	50

[a] The least detectable dose was computed for every standard curve as the intercept of the linearized standard curve with the lower confidence limit of the error in the test tubes containing no non-radioactive hormone[10]. In all instances this computed dose was lower than the first dose of non-radioactive hormone. Therefore, the first dose was considered as the least detectable dose.

[b] The least detectable dose in plasma is an estimate calculated from the least detectable dose in the standard curve by multiplying by factors for aliquoting and estimated procedural losses.

[c] Five doses of non-radioactive hormone were used for each standard curve. The second and following doses were always obtained by doubling the previous one.

the mass of the radioactive steroid added to plasma to monitor the procedural losses[10].

RESULTS

Within-assay and between-assay variation

The results of within-assay and between-assay variation measurements are demonstrated in Table 5. The values of coefficient of variation in both studies indicate a satisfactory precision.

Water blank and plasma blank

Water or plasma exhaustively extracted with ether was assayed in 20 replicates. The results are shown in Table 6. It has to be noted that the means indicated in the table are approximate values only. Due to the variation of individual measurements

TABLE 5

Within-assay and between-assay variation

Steroid	Within-assay variation[a]				Between-assay variation[b]	
	Male plasma pool		Female plasma pool			
	Mean (pg)	CV[c] (%)	Mean (pg)	CV (%)	Mean (pg)	CV (%)
Progesterone	402	9.5	503[d]	10.6	9330	15.1
Pregnenolone	921	7.6	2500[e]	7.4	1630	14.0
Dehydroepiandrosterone	4440	5.5	5230[e]	5.1	6120	5.0
Testosterone	4070	6.6	357[d]	12.0	306	11.4
Estrone	55	10.1	94[d]	5.0	97	15.7
Estradiol	33[f]	24.4[f]	155[d]	5.2	165	4.8

[a] The pools were assayed in 20 replicates. [b] A pool of female luteal plasma was assayed six times for each steroid, on different days and by different investigators. [c] CV = coefficient of variation (S.D./mean x 100). [d] Follicular phase. [e] Luteal phase. [f] The values found in the assay (prior to corrections for recovery and aliquoting) were mostly below the least detectable dose; the numbers indicated in the table are, therefore, approximate estimates only.

TABLE 6

Water blank and plasma blank values

Steroid	Water blank[a]	Plasma blank[a]
Progesterone	0.6	7.8
Pregnenolone	6.6	1.3
Dehydroepiandrosterone	3.7	2.5
Testosterone	7.1	8.0
Estrone	2.5	3.4
Estradiol	0.4	0.3

[a] Water or plasma exhaustively extracted with ether was assayed in 20 replicates. The values indicated in the table represent estimated means (pg/0.3 ml).

in the region close to 0 pg, negative values were sometimes obtained. Such values were necessarily considered as zeros in the calculation of the means. Therefore, the means indicated in Table 6 represent overestimates. Furthermore, the majority of positive individual observations was found between zero and the least detectable dose (cf. Table 4), i.e., outside the range of reliability of the linearized standard curve.

The blanks arising from plasma and from the extraction and separation procedures were so low that they could not be exactly measured and mathematically treated as valid values, i.e., corrected for procedural losses and aliquoting. For this reason, in none of the steroid assays described were they taken into consideration.

Recovery of non-radioactive steroids added to plasma

Various doses of standard hormone, approximately corresponding to endogenous levels, were added to plasma. Each dose was added to 1 ml of male plasma, either genuine or pre-extracted with ether, and measured in replicates of five. A regression analysis for the relationship between the amount added and measured was computed for each steroid (Table 7). As can be seen from the F-values, the relationship is statistically linear in all instances. The best-fit straight lines have slopes which are not significantly different from the theoretically expected ones ($b = 1$). This fact indicates that the amounts present in plasma correspond to the

TABLE 7

Relationship between the added and measured amounts of steroids in plasma following the addition of standard hormones

Steroid	Doses of standard hormone added (pg)	Slope[a] b	Confidence limits of the slope[b]	Correlation coefficient	F-value found[c]
Progesterone	80,160,320,640	0.952	0.808 - 1.097	0.956	0.460
Pregnenolone	400,800,1600,3200	1.016	0.965 - 1.067	0.995	0.226
Dehydroepiandrosterone	800,1600,3200,6400	1.039	0.939 - 1.139	0.982	1.978
Testosterone	100,200,400,800	1.009	0.900 - 1.118	0.979	0.213
Estrone	100,200,400,800	0.978	0.933 - 1.023	0.996	0.296
Estradiol	100,200,400,800	0.982	0.922 - 1.042	0.993	0.134

[a] Theoretical slope b = 1. [b] $b \pm s_b \cdot t_{.95\,(n-2)}$; n = 20. [c] Tabulated $F_{.95\,(2,16)} = 3.63$.

TABLE 8

Parallelism between increasing amounts of endogenous hormone and standard hormone

Steroid	F-value[a]	
	Parallelism	Linearity
Progesterone	2.96	0.77
Pregnenolone	1.00	0.42
Dehydroepiandrosterone	0.05	0.01
Testosterone	0.87	0.11
Estrone	0.00	4.30
Estradiol	1.10	1.29

[a] Tabulated $F_{.95(1,17)} = 4.45$

amounts measured, at least in the range of the doses investigated.

Assay of steroids in various volumes of plasma

Different amounts of pooled female plasma (0.5, 1.0 and 2.0 ml) were assayed. Plasma (0.5 and 1.0 ml) was made up to 2.0 ml with the assay buffer. All samples were extracted with 10 ml ethyl ether. The ether solutions were processed as described above.

A comparison was made between the logits of five replicate measurements of each of the three plasma volumes and the logits of four replicate measurements of each of three doses of non-radioactive, standard hormone. A complete analysis of variance was carried out using orthogonal contrasts[11] for all the assays under investigation. As can be seen from Table 8, the relationship between various doses of endogenous and standard hormone was parallel in all cases. This suggests that in none of the plasma assays were there appreciable amounts of interfering compounds present.

Furthermore, the data presented in Table 8 indicate that the dose-response relationships were linear in all cases.

Specificity of the assays

The overall specificity of all steroid assays described in this paper is an expression of the specificity of the antibody together with that of the chromatographic separation. The cross-reactions of 57 steroids (Table 9) with all antisera were tested. The elution pattern was investigated in all steroids having a cross-reaction higher than 0.1 per cent (1.0 per cent for dehydroepiandrosterone). The presence of these steroids in individual chromatographic fractions was assayed either by using labelled compounds, or, if these were not available, by radioimmunoassay. In the latter case, non-radioactive steroid standards in nanogram amounts were chromatographed. Individual chromatographic fractions were then assayed using antisera which could detect the presence of the particular steroid under investigation.

From Tables 10 - 15 it can be seen which compounds were present in the steroid fractions collected for the radioimmunoassay of the six steroids. All these compounds exhibited a low cross-reaction. The only exception was 3β-hydroxy-5α-pregnan-20-one in the pregnenolone assay. The cross-reaction of this compound was about

TABLE 9

Compounds used in the specificity studies

Systematic name	Trivial name or abbreviation
5-Cholesten-3β-ol	Cholesterol
4-Pregnene-3,20-dione	Progesterone
3β-Hydroxy-5-pregnen-20-one	Pregnenolone
5α-Pregnane-3,20-dione	
5β-Pregnane-3,20-dione	
3α-Hydroxy-5α-pregnan-20-one	
3β-Hydroxy-5α-pregnan-20-one	
3α-Hydroxy-5β-pregnan-20-one	
20α-Hydroxy-4-pregnen-3-one	20α-Dihydroprogesterone
20β-Hydroxy-4-pregnen-3-one	20β-Dihydroprogesterone
17-Hydroxy-4-pregnene-3,20-dione	17-OH-Progesterone
16α-Hydroxy-4-pregnene-3,20-dione	16α-OH-Pregesterone
5β-Pregnane-3α,20α-diol	Pregnanediol
5α-Pregnane-3α,20α-diol	
5α-Pregnane-3β,20α-diol	
5-Pregnene-3β,20α-diol	
17,20α-Dihydroxy-4-pregnen-3-one	
3β,17-Dihydroxy-5-pregnen-20-one	17-OH-Pregnenolone
3β,16α-Dihydroxy-5-pregnen-20-one	16α-OH-Pregnenolone
5β-Pregnane-3α,17,20α-triol	
5-Pregnene-3β,17,20α-triol	
21-Hydroxy-4-pregnene-3,20-dione	Deoxycorticosterone
11β,21-Dihydroxy-4-pregnene-3,20-dione	Corticosterone
21-Hydroxy-4-pregnene-3,11,20-trione	
17,21-Dihydroxy-4-pregnene-3,20-dione	11-Deoxycortisol
17,21-Dihydroxy-4-pregnene-3,11,20-trione	Cortisone
11β,17,21-Trihydroxy-4-pregnene-3,20-dione	Cortisol
18,11-Hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al	Aldosterone
17β-Hydroxy-4-androsten-3-one	Testosterone
3β-Hydroxy-5-androsten-17-one	Dehydroepiandrosterone
3α-Hydroxy-5α-androstan-17-one	Androsterone
3β-Hydroxy-5α-androstan-17-one	Epiandrosterone
3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
4-Androstene-3,17-dione	Androstenedione
5α-Androst-1-ene-3,17-dione	
17β-Hydroxy-5α-androstan-3-one	Dihydrotestosterone
4-Androstene-3,11,17-trione	Adrenosterone
17α-Hydroxy-4-androsten-3-one	
5α-Androstane-3α,17β-diol	

TABLE 9 (cont.)

5-Androstene-3β,17β-diol	
5-Androstene-3β,17α-diol	
4-Androstene-3β,17β-diol	
3α-Hydroxy-5β-androstane-11,17-dione	
11β-Hydroxy-4-androstene-3,17-dione	11β-OH-Androstenedione
3α,11β-Dihydroxy-5α-androstan-17-one	
3α,11β-Dihydroxy-5β-androstan-17-one	
3β,17β-Dihydroxy-5-androsten-16-one	
3β,16α-Dihydroxy-5-androsten-17-one	
17β-Hydroxy-4-estren-3-one	19-Nortestosterone
3-Hydroxy-1,3,5(10)-estratrien-17-one	Estrone
1,3,5(10)-Estratriene-3,17β-diol	Estradiol
1,3,5(10)-Estratriene-3,17α-diol	17α-Estradiol
1,3,5(10)-Estratriene-3,16α,17β-triol	Estriol
3,16α-Dihydroxy-1,3,5(10)-estratrien-17-one	16α-OH-Estrone
17α-Ethinyl-17β-hydroxy-4-estren-3-one	
17α-Ethinyl-1,3,5(10)-estratriene-3,17β-diol	Ethinylestradiol
3-Methoxy-1,3,5(10)-estratriene-3,17β-diol	Estradiol 3-methyl ether

50 per cent.

In Tables 10 - 15, all compounds are indicated which have a cross-reaction higher than 0.1 per cent (1.0 per cent for dehydroepiandrosterone). These data demonstrate the specificity of each antiserum.

DISCUSSION

The reliability of an analytical method is given by its precision, accuracy, sensitivity and specificity. In this study the precision was established by repeated measurements of the same plasma pool. The coefficients of variation in both the within-assay and between-assay variation experiments (Table 5) indicated that the precision was satisfactory. Similar coefficient of variation values have been reported by other authors for the radioimmunoassay of various individual steroids[12-17]. Also, the steroid levels found in the plasma pools are in good agreement with the results of many other authors. A detailed comparison of hormonal levels measured by the

TABLE 10

Specificity of the progesterone assay

Steroid	In the chromatographic fraction[a]	Outside the fraction[b]
Progesterone	100	
3α-Hydroxy-5α-pregnan-20-one	0.6[c]	
16α-OH-Progesterone	1.5	
5α-Androst-1-ene-3,17-dione	< 0.1	
5α-Pregnane-3,20-dione		57.0
5β-Pregnane-3,20-dione		96.0
3α-Hydroxy-5β-pregnan-20-one		1.1
3β-Hydroxy-5α-pregnan-20-one		3.2
Pregnenolone		1.7
20α-Dihydroprogesterone		3.3
20β-Dihydroprogesterone		1.0
17-OH-Progesterone		3.2
Testosterone		0.2
Corticosterone		1.0
Deoxycorticosterone		17.0

[a] In this column all steroids are included which were found in the chromatographic fraction.

[b] In this column only those steroids are mentioned which exhibited a cross-reaction higher than 0.1 per cent.

[c] The percentage of cross-reaction was estimated as a ratio of the mass of the steroid assayed (times 100) to the mass of the cross-reacting steroid when either mass brought about the same per cent binding. In the case of steroids having a cross-reaction higher than 0.1 per cent, multiple point dose - per cent binding curves were constructed and the cross-reaction was estimated at 50 per cent binding.

TABLE 11

Specificity of the pregnenolone assay

Steroid	In the chromatographic fraction[a]	Outside the fraction[b]
Pregnenolone	100	
3β-Hydroxy-5α-pregnan-20-one	48.0[c]	
3α-Hydroxy-5β-pregnan-20-one	0.2	
5-Pregnene-3β,20α-diol	2.4	
16α-OH-Pregnenolone	1.1	
Etiocholanolone	< 0.1	
Androstenedione	< 0.1	
Dihydrotestosterone	< 0.1	
Androsterone	< 0.1	
3α-Hydroxy-5α-pregnan-20-one		28.0
5α-Pregnane-3,20-dione		28.0
5β-Pregnane-3,20-dione		14.0
17-OH-Pregnenolone		1.2
16α-OH-Progesterone		0.5
Progesterone		23.0
17-OH-Progesterone		0.2
Deoxycorticosterone		0.5

[a,b,c] cf. Table 10

TABLE 12

Specificity of the dehydroepiandrosterone assay

Steroid	In the chromatographic fraction[a]	Outside the fraction[b]
Dehydroepiandrosterone	100	
Adrenosterone	1.0[c]	
11β-OH-Androstenedione	< 1.0	
3β,17β-Dihydroxy-5-androsten-16-one	< 1.0	
20α-Dihydroprogesterone	< 1.0	
Deoxycorticosterone	< 1.0	
Androstenedione		32.0
5α-Androst-1-ene-3,17-dione		54.0
Androsterone		77.0
Epiandrosterone		67.0
Estrone		4.8

[a] cf. Table 10. [b] Only those steroids are included which exhibited a cross-reaction higher than 1.0 per cent. [c] Same as in Table 10, except that the multiple point curves were constructed for steroids having a cross-reaction higher than 1.0 per cent.

TABLE 13

Specificity of the testosterone assay

Steroid	In the chromatographic fraction[a]	Outside the fraction[b]
Testosterone	100	
17-OH-Progesterone	< 0.01 [c]	
Dihydrotestosterone		133.0
19-Nortestosterone		6.9
Androstenedione		1.4
5α-Androstane-3α,17β-diol		9.1
4-Androstene-3β,17β-diol		11.0

[a,b,c] cf. Table 10.

TABLE 14

Specificity of the estrone assay

Steroid	In the chromatographic fraction[a]	Outside the fraction[b]
Estrone	100	
5α-Androstane-3α,17β-diol	0.2[c]	
5-Androstene-3β-17β-diol	< 0.01	
17-OH-Pregnenolone	< 0.01	
5β-Pregnane-3α,20α-diol	< 0.01	
Corticosterone	< 0.1	
Estradiol		5.6
17α-Estradiol		0.5
Ethinylestradiol		0.6
Estriol		0.2
16α-OH-Estrone		3.0

[a,b,c] cf. Table 10.

TABLE 15

Specificity of the estradiol assay

Steroid	In the chromatographic fraction[a]	Outside the fraction[b]
Estradiol	100	
17α-Estradiol	0.2[c]	
Ethinylestradiol	< 0.1	
4-Androstene-3β,17β-diol	< 0.01	
5-Androstene-3β,17β-diol	< 0.01	
11-Deoxycortisol	< 0.01	
Estrone		0.8
Estradiol 3-methylether		0.7
Estriol		9.3
19-Nortestosterone		0.2
16α-OH-Estrone		0.3

[a,b,c] cf. Table 10.

present method and those of other authors will be presented in a forthcoming publication[18].

One part of the accuracy study is the assessment of blanks. In the present assay, all blanks had values below the least detectable dose, and could not be exactly measured. They were, therefore, neglected.

Another criterion of accuracy is the relationship between the amounts of standard hormone added to plasma and the amounts detected by the assay. In the assays described in this communication the relationship corresponded to the expected one, i.e., the various amounts tested lay on a straight line which was identical with the theoretical relationship. Therefore, the amounts of hormones measured by the assay can be expected to be in agreement with the amounts present in the plasma.

The assay of increasing volumes of plasma is of great importance for the assessment of accuracy. If the steroid amounts measured in increasing volumes of plasma correspond to the amounts expected, it can be assumed that there are no appreciable quantities of interfering compounds present. A statistical evaluation of the experimental data has shown that this is the case in all assays.

The specificity of the present assays is a combined result of the chromatographic purification and the specificity of antisera used. The chromatographic fractions used for the assay of individual steroids contain only a few compounds which significantly cross-react with the corresponding antisera. In the progesterone assay (Table 10) it is 16α-OH-progesterone and 3α-hydroxy-5α-pregnan-20-one. The former was reported in human placental blood and in corpora lutea[19], and the latter has been found in pregnancy plasma as the monosulfate[20]. Whether the latter compound occurs in appreciable amounts in the unconjugated form in human plasma remains to be shown.

In the pregnenolone fraction 3β-hydroxy-5α-pregnan-20-one gave the highest cross-reaction (48 per cent: see Table 11). This compound has been described in the plasma of pregnant women as the monosulfate[20], as was 3α-hydroxy-5β-pregnan-20-one[20]. 5-Pregnene-3β,20α-diol has been reported to occur as both the mono- and disulfate in human male and female plasma[21]. 16α-OH-Pregnenolone was found as the monosulfate in human fetal blood at early and mid-pregnancy[22] and in the umbilical cord blood[23].

Among the steroids eluted in the dehydroepiandrosterone fraction (Table 12) only adrenosterone showed a significant cross-reaction. This steroid was described in human cord blood[24].

The testosterone fraction does not contain significantly cross-reacting steroids (Table 13).

Concerning the estrone fraction, it can be seen from the data of Table 14 that 5α-androstane-3α,17β-diol exhibits 0.2 per cent cross-reaction. The highest individual plasma value of this compound, so far described, is 670 pg/ml[25]. This amount would behave in the assay as approximately 1.3 pg of estrone. This would represent an error of about 3 per cent in the estimation of estrone in male plasma, and about 1.5 per cent in female plasma. Thus, the influence of this cross-reaction on the assay results appears to be negligible.

In the estradiol fraction (Table 15) 17α-estradiol gives a significant but slight (0.2 per cent) cross-reaction. This steroid was found[26] in minute quantities in human pregnancy plasma, urine and cord blood in a conjugated form.

In view of the data mentioned above the specificity of the assays can be consid-

ered to be very high, with one possible exception. If non-conjugated 3β-hydroxy-5α-pregnan-20-one occured in non-pregnancy plasma in amounts comparable to those of pregnenolone, then the pregnenolone content would be overestimated.

The conclusions concerning the specificity of the assays are based on the study of the chromatographic behavior and cross-reacting properties of 57 steroids studied up to date. Therefore, the possibility cannot be excluded that there are other compounds, not tested or detected so far, which may significantly influence the specificity of the assays.

Experiments are now in progress to increase the number of steroids simultaneously assayed and at the same time to decrease the volume of plasma needed. It can be expected that in some cases the simultaneous analysis of a number of steroids will be used to follow multiple hormonal correlations. In other cases a selective use of the method can be envisaged, restricting the number of steroids assayed to those most likely to provide the information needed.

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