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QUANTITATION OF PREGNENOLONE AND 17-HYDROXYPREGNENOLONE IN HUMAN SERUM BY AUTOMATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT RADIOIMMUNOASSAY

M. SCHÖNESHÖFER*, J. MAXEINER and A. FENNER

Institute of Clinical Chemistry and Clinical Biochemistry, Free University of Berlin, Klinikum Steglitz, Hindenburgdamm 30, 1000 Berlin 45 (G.F.R.)

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SUMMARY

A method for the determination of pregnenolone and 17-hydroxypregnenolone in human serum is described which uses high-performance liquid chromatography as a prepurification step followed by radioimmunological quantitation. As to specificity and practicability, the present technique is superior to previously reported methods. Chromatographic assessment of unspecific pregnenolone and 17-hydroxypregnenolone immunoreactivities arising in the ether extracts of normal serum samples clearly emphasizes the necessity of efficient chromatographic isolation of the steroids prior to immunoassay, if specific estimation is to be made. Normal values and physiological changes of serum pregnenolone and 17-hydroxypregnenolone accord well with the data already published in literature.

INTRODUCTION

Pregnenolone and 17-hydroxypregnenolone are precursors of gonadal and adrenal steroid hormones. Their estimation in serum is of special importance for the chemical diagnosis of the different forms of the adrenogenital syndrome [1] and is of a complementary nature in the diagnosis of other adrenal disorders [2]. Due to insufficient specificity of the corresponding antisera, the immunological methods described hitherto require cumbersome chromatographic purification preceding immunological quantitation [3—7]. On the other hand, estimation solely by chromatography with photometric detection is scarcely feasible, particularly as these steroids lack UV-absorbing configurations in the molecule.

The present paper describes an automatic high-performance liquid chromatographic (HPLC) procedure with subsequent quantitation of the purified steroid fractions by radioimmunoassay (RIA).

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EXPERIMENTAL

Materials and reagents

Extrelut® was purchased from Merck (Darmstadt, G.F.R.); plastic syringes (20 ml) used as extraction columns were from Pharmaseal Laboratories (Glendale, CA, U.S.A.). Solvents, reagents and other accessories used for the RIA were as previously described [8].

Radioactive steroids: [7-^{3}H]pregnenolone (17 Ci/mmol) and 17-OH-[7-^{3}H]-pregnenolone (14 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.).

Non-radioactive steroids: progesterone, 17-OH-progesterone, androstendione, 11-deoxycorticosterone, 11-deoxycortisol, 18-OH-11-deoxycorticosterone, corticosterone, aldosterone, cortisone, cortisol, testosterone, dehydroepiandrosterone and estriol were from Merck; pregnenolone and 17-OH-pregnenolone were from Steraloids (Pawling, NJ, U.S.A.).

Antisera: antisera against pregnenolone and 17-OH-pregnenolone were raised in rabbits immunized with the corresponding 3-hemisuccinate bovine serum albumin conjugates synthesized according to the procedure described by Erlanger et al. [9]. The technique of immunization followed the protocol outlined by Vaitukaitis et al. [10]. The final dilutions of antisera used in this method were 1:60,000 for pregnenolone and 1:70,000 for 17-OH-pregnenolone. Cross-reactivities of the antisera with related steroids (Table I) were studied by the method of Abraham [11].

TABLE I

SPECIFICITY OF ANTISERA USED

Steroid	Cross-reactivity (%) of steroids with antisera against	
	pregnenolone	17-OH-pregnenolone
Progesterone	45	0.02
17-OH-Progesterone	0.03	16.4
11-Deoxycorticosterone	1.3	0.02
11-Deoxycortisol	<0.02	0.44
Corticosterone	<0.02	<0.01
Aldosterone	<0.02	<0.01
Cortisol	<0.02	<0.01
Cortisone	<0.02	<0.01
Pregnenolone	100	0.19
17-OH-Pregnenolone	11.0	100
Testosterone	<0.02	<0.01
Androstendione	<0.02	<0.01
Dehydroepiandrosterone	<0.02	<0.01
Estriol	<0.02	<0.01

Instrumentation

A Hewlett-Packard high-performance liquid chromatograph (Model 1084 B), equipped with a fixed-wavelength UV detector at 254 nm, two solvent and two pump systems, a variable-volume injector and a plot/print terminal were used for chromatography. All operating parameters were regulated and controlled by

microprocessors. For the chromatography of steroids applied in the present method, a stepwise gradient elution technique was used. Automatic injection of up to 60 samples was provided by an autosampler. Automatic collection of individual fractions was achieved by a fraction collector (LKB, UltroRac 7000) triggered by the microprocessors of the chromatograph itself.

^{3}H-Radioactivity was measured in a liquid scintillation spectrometer (Packard Instruments, Model 2480).

An IBM 1800 computer was used for computing of assay data.

Samples: blood was drawn between 8 and 9 a.m. except when evening samples were required for studies of diurnal rhythm. After clotting and centrifugation, serum was stored at −20°C until analysis.

Procedures

The protocol of the total assay procedure is outlined in Table II.

TABLE II

FLOW SHEET OF THE TOTAL ASSAY

I. Extraction
- 1 ml serum + [^{3}H]pregnenolone and 17-OH[^{3}H]pregnenolone (each dissolved in 500 μl of water)
- Transfer to Extrelut column
- Elute with 20 ml of carbon tetrachloride
- Evaporate
- Redissolve in 150 μl of *n*-hexane—isopropanol (95 : 5)

II. High-performance liquid chromatography
- Transfer to autosampler
- Automatic injection and chromatography
- Automatic collection of individual fractions
- Evaporate individual fractions
- Redissolve in 180 μl of ethanol—water (50 : 50)
- Count 50 μl for recovery

III. Radioimmunoassay
- 50 μl of sample (duplicate) or standard (1600—6.25 pg) (triplicate) + 800 μl of γ-globulin buffer containing ^{3}H-labelled steroid and antiserum
- Incubation for at least 2 h at 4 °C
- Add 100 μl of charcoal suspension
- Shake, centrifuge and decant
- Count supernatant for ^{3}H-radioactivity
- Calculate results

Extraction. The extraction procedure followed the technique recently described [12]. In brief, tracer amounts of [^{3}H]pregnenolone and 17-OH-[^{3}H]-pregnenolone, each dissolved in 500 μl of distilled water, were added to 1 ml of serum. After equilibration for about 30 min, the mixture was transferred on to an Extrelut column and eluted with 20 ml of carbon tetrachloride. Concomitantly with elution, the organic phase was evaporated under a stream of air in a 30°C water-bath. The residue was redissolved in 150 μl of a mixture of *n*-hexane—isopropanol (95 : 5).

Automatic high-performance liquid chromatography. For HPLC, a column of polar-bonded phase packing (DIOL®, Knauer, Berlin, G.F.R.), *n*-hexane—isopropanol as mobile phase and gradient elution was used [13]. Further conditions of HPLC were as follows: flow-rate 1.3 ml/min, column pressure 62 bar, temperature of solvents and oven 40°C, attenuation $64 \cdot 10^{-4}$ a.u./cm, slope sense 0.5, column length 25 cm, diameter of column 4.6 mm, diameter of particles 5 μm, solvent A *n*-hexane, solvent B *n*-hexane—isopropanol (85:15). The gradient was run from 15% B to 100% B within 41 min. The organic solution (150 μl) of the serum extracts (see above) was transferred to microvials, then positioned in the autosampler and automatically subjected to HPLC. The specific fractions containing pregnenolone and 17-OH-pregnenolone were automatically collected by time-triggering of the chromatograph dictated by the retention times of the steroids. Before each batch, these retention times were established by a calibrating run of a ^{3}H-labelled steroid mixture containing about 10 nCi of each steroid. In this run, ^{3}H-radioactivity was measured in 1-min fractions eluted by HPLC.

Radioimmunoassay. The organic fractions were evaporated under a stream of air and redissolved in 180 μl of ethanol—water (50:50). A 50-μl aliquot of the solution was measured for recovery, and 50 μl aliquots in duplicates were subjected to RIA. Evaluation of RIA data was done by a computer program using the "spline approximation" technique as standard curve model [14].

RESULTS

Extraction

The percentage recoveries of [^{3}H]pregnenolone and 17-OH-[^{3}H]pregnenolone added to a serum sample were found to be more than 95% after carbon tetrachloride extraction.

High-performance liquid chromatography

Fig. 1a demonstrates the chromatogram with UV detection of a steroid

TABLE III

CHROMATOGRAPHIC PARAMETERS OF THE HPLC SYSTEM APPLIED TO THE SEPARATION OF ADRENAL STEROIDS

Steroid	Retention time (min)	Capacity ratio	Resolution
Progesterone	7.23	1.78	
			1.27
Androstendione	9.88	2.80	
			1.40
Pregnenolone	12.80	3.90	
			2.52
Deoxycorticosterone	18.06	5.94	
			0.70
Testosterone	19.50	6.50	
			1.50
17-OH-Progesterone	22.64	7.70	
			3.16
17-OH-Pregnenolone	29.20	10.23	
			1.85
11-Deoxycortisol	33.07	11.71	
			0.32
18-OH-Deoxycorticosterone	33.73	11.97	
			0.63
Corticosterone	35.05	12.48	
			1.87
Aldosterone	38.97	13.98	

mixture chromatographed by the HPLC system applied. The non-UV-absorbing steroids pregnenolone and 17-OH-pregnenolone were located by ^{3}H-radioactivity measurement in 1-min fractions. The chromatographic parameters, such as retention time, capacity ratio and resolution between adjacent steroids, are

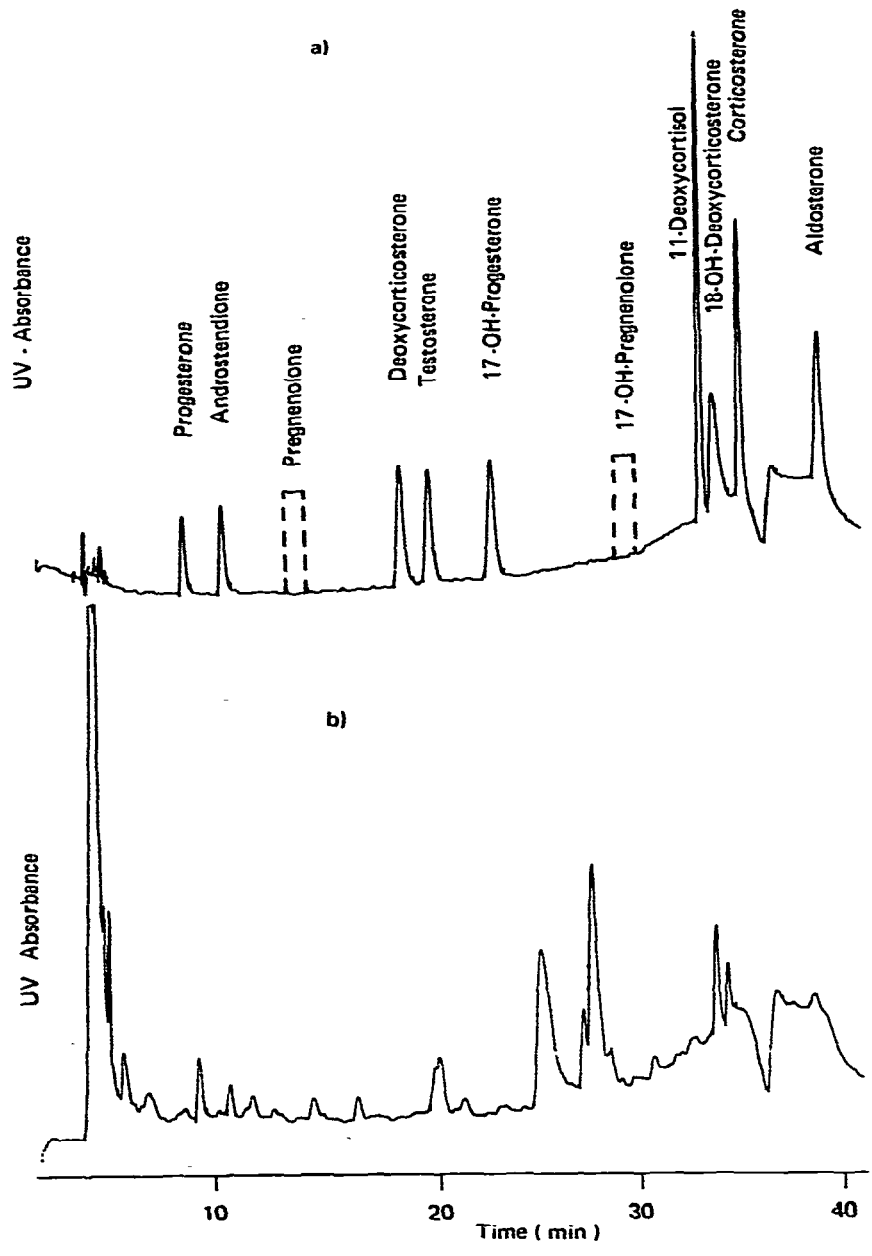

Fig. 1. Chromatogram of a mixture of steroid standards (a) and of the ether extract of a normal serum sample (b). HPLC parameters used are outlined in Methods. Amounts of steroids injected for chromatogram a were about 500 ng of each. The non-UV-absorbing steroids pregnenolone and 17-OH-pregnenolone were localized by chromatography of the tritiated steroids.

summarized in Table III. The reproducibility of retention times of UV-absorbing steroids was studied in 20 runs and found to be less than 0.6% (C.V.). The chromatogram with UV detection of a normal serum sample is shown in Fig. 1b, thus demonstrating that considerable amounts of non-specific, UV-absorbing compounds occur in the areas of the steroids of interest.

The percentage recovery of [^{3}H] pregnenolone and 17-OH-[^{3}H] pregnenolone after HPLC was found to be 62.5 ± 8.5% (S.D.) and 65.3 ± 14.4% (S.D.), respectively. Due to the considerable variation in recoveries, the individual losses of each sample were adjusted according to the radioactive count of 50 μl of the final HPLC fraction.

Analytical variables

Sensitivity. The detection limit of the total assay is mainly dictated by the affinity of the antisera used, i.e. the sensitivities of standard curves, the procedural losses and the blank levels. The sensitivity of a standard curve was defined as the mass of steroid necessary to suppress the ^{3}H-radioactivity bound to the antibody at zero conditions by two standard deviations of the radioactive count. The corresponding values were found to be 28.3 ± 16.3 pg (S.D.) for pregnenolone and 7.7 ± 3.8 pg (S.D.) for 17-OH-pregnenolone. Blank levels arising in water were found to be lower than the sensitivities of the standard curves. Thus, the total detection limits of the total assays, deriving from standard curve sensitivity and procedural losses, amounted to 0.69 nmol/l for pregnenolone and 0.21 nmol/l for 17-OH-pregnenolone.

Specificity. The cross-reactivities of the antisera used against known steroids of related structure are shown in Table I. In addition to this documentation of assay specificity, we studied unknown interferences potentially arising in serum. Thus, the pregnenolone and 17-OH-pregnenolone immunoreactivities were estimated in 1-min fractions eluted by HPLC of carbon tetrachloride extracts of normal serum samples. The profiles outlined in Fig. 2 demonstrate that — although assay specificity checked by cross-reactivity (Table I) seems quite satisfactory — there arise considerable unspecific immunoreactivities which are not attributable to steroids commonly studied for specificity.

Precision and accuracy. Within-batch and between-batch variability was studied in replicates of a normal serum sample. Within-batch variation (C.V.) was found to be 7.5% for pregnenolone and 5.7% for 17-OH-pregnenolone; between-batch variation was 12.5% and 8.7%, respectively. Accuracy was studied by recovery measurement of three different amounts of each steroid added to a charcoal-stripped serum sample. Amounts found ranged from 89.4% to 102.1% for pregnenolone and between 92.3% and 98.2% for 17-OH-pregnenolone.

Practicability. The automatic mode of HPLC, which eliminates the time-consuming handling of the manual sequential technique, renders the present method suitable for processing large batches of samples within a reasonable time, particularly if the facility of overnight operation is utilized.

Normal values

Pregnenolone and 17-OH-pregnenolone concentrations were estimated from 28 normal males, aged between 22 and 42 years. The mean concentration as

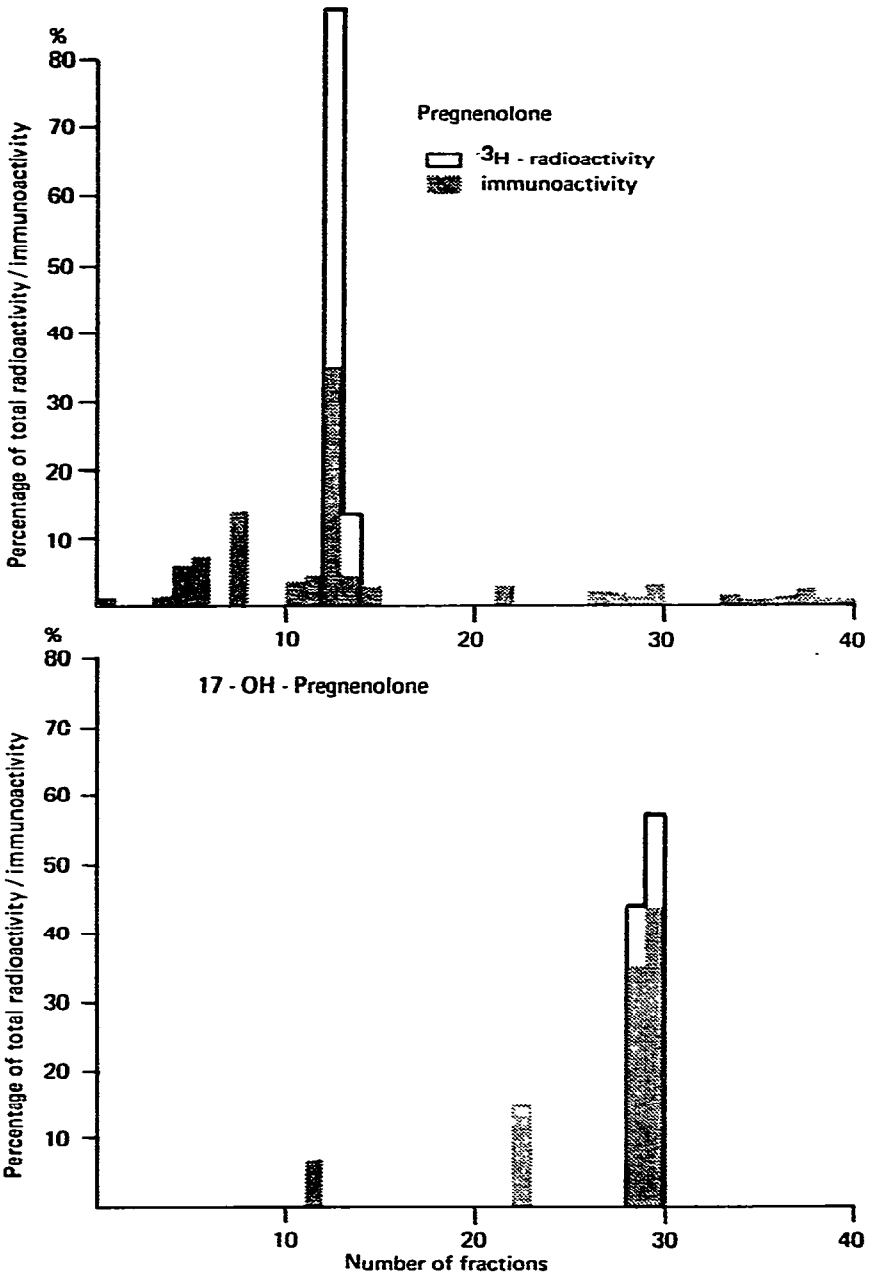

Fig. 2. Profiles of immunoreactivities (hatched areas) and of the specific ^{3}H-radioactivities (open areas) as measured in fractions eluted by the HPLC system described. Volume of each fraction was 1.3 ml. ^{3}H-Radioactivities added to the serum samples were 2 nCi.

well as the upper and lower limits established according to a logarithmic distribution are listed in Table IV.

Physiological validation

Serum concentrations of pregnenolone and 17-OH-pregnenolone were as-

sessed before and after adrenal stimulation and suppression, respectively. Diurnal variation was monitored by steroid estimation at 8 a.m. and 8 p.m. The corresponding data evaluated in four subjects are listed in Table V.

TABLE IV

SERUM CONCENTRATIONS OF PREGNENOLONE AND 17-OH-PREGNENOLONE IN NORMAL MALES

Values were evaluated according to a logarithmic distribution.

	Lower limit (nmol/l)	Median (nmol/l)	Upper limit (nmol/l)
Pregnenolone	2.34	3.34	4.75
17-OH-Pregnenolone	4.25	5.52	7.16

TABLE V

PHYSIOLOGICAL CHANGES OF SERUM PREGNENOLONE AND 17-OH-PREGNENOLONE

	Pregnenolone (mean ± S.D., nmol/l)	17-OH-Pregnenolone (mean ± S.D., nmol/l)
Adrenal stimulation with 250 μg Synacthen® i.m.		
before	1.28 ± 0.36	2.31 ± 1.78
30 min after	2.38 ± 0.34	10.95 ± 1.52
Adrenal suppression with 2 mg of dexamethasone at midnight		
8 a.m. before	2.24 ± 0.9	2.80 ± 1.4
8 a.m. after	1.71 ± 0.27	0.41 ± 0.13
Diurnal variation		
8 a.m.	4.37 ± 1.02	9.7 ± 3.08
8 p.m.	2.4 ± 1.49	2.05 ± 0.95

DISCUSSION

The present data on pregnenolone and 17-OH-pregnenolone immunoreactivities arising in the ether extract from normal serum samples (Fig. 2) clearly demonstrate that assessment of crude organic extracts would overestimate specific steroid concentration due to considerable amounts of unspecific immunoreactivities. Furthermore, it is well documented by these data that exclusive monitoring of cross-reactivity of antisera against some steroids related to that of interest is not sufficient for validation of assay specificity at all and that only efficient chromatographic prepurification would provide specific steroid estimation by immunoassay.

In comparison to other chromatographic techniques, the present HPLC system, which is related to that featured previously [13], provides, besides the chromatographic qualities outlined elsewhere [15], the great advantage that it is well suited for automation. Thus, running the HPLC system automatically and overnight, the tedious and time-consuming operations well known from the

chromatographic techniques applied hitherto [4—6], are almost completely eliminated.

The validation of assay parameters and of physiological changes in serum pregnenolone and 17-OH-pregnenolone accord well with the data published in the literature [3—6].

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