RAPID IMMUNOASSAY FOR PREGNENOLONE SULFATE AND ITS APPLICATIONS IN ENDOCRINOLOGY

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Received October 10, 2001
Accepted January 9, 2002

Dedicated to the memory of Dr Václav Černý.

The importance of pregnenolone sulfate (PregS) in human physiology has increased in the last decade in connection with its neuroactivating effect *via* positive modification of *N*-methyl-D-aspartate receptors and negative modulation of GABA receptors. Therefore, a novel rapid radioimmunoassay was developed and evaluated for measurement of PregS in body fluids. Given the differences in concentrations of cross-reacting substances in various biological materials, several modifications of the method were elaborated and used. Circulating levels of PregS were measured in serum of normal subjects. In both sexes, the age dependences exhibited maximum values before 30th year of age. For the first time, circulating levels of the hormone were measured in patients with a mixed anxio-depressive disorder, where they significantly exceeded those in controls. Further, the levels of PregS were evaluated in time profiles of women around parturition and compared with those in umbilical blood at delivery. A significantly decreasing time profile of PregS was found in maternal blood. No correlation between maternal and umbilical blood was found indicating its autonomous production in mother and in fetus. In addition, concentrations of PregS were measured in breast cystic fluid where they exceeded those in circulation more than by two orders of magnitude.

Keywords: Pregnenolone sulfate; RIA; Neurosteroids; Steroids; Serum; Parturition; Breast cyst fluid; Analytical methods.

Biological effects of neuroactive steroids became the subject of increased interest of endocrinologists in the past decade. Steroid sulfates represent a considerable portion of total steroids in the human organism. In addition to an important role that they play in steroid metabolism as precursors or metabolites of nonconjugated steroids, they act primarily as neuro-

modulators[1–5]. Thanks to good solubility both in polar and in nonpolar solvents caused by their dipolar character, the sulfates are present in body fluids in concentrations frequently exceeding their nonconjugated analogues by several orders of magnitude[6–10]. Steroid sulfates are the most abundant steroids in human blood. Pregnenolone sulfate (PregS) is a highly efficient positive modulator of membrane neuronal *N*-methyl-D-aspartate (NMDA) receptors influencing permeability of calcium channels. The steroid conjugate acts on extracellular binding sites where it supports an influx of calcium ions into neurons[11–17] and, consequently, in a dose-dependent manner, its rapid activation. Inorganic sulfates do not possess such property[15]. The influence of unconjugated pregnenolone is more than by an order of magnitude lower in comparison with PregS (ref.[18]). Besides in the central nervous system (CNS), NMDA receptors are present also in periphery[19–22]. In contrast to some reduced metabolites of progesterone, the sulfates of pregnenolone and dehydroepiandrosterone also negatively modulate membrane receptors of 4-aminobutanoic acid (GABA), which are responsible for attenuation of neuronal activity[1,23–25].

The neuroactive effect of PregS was studied in detail on animals. In humans, the extensive diagnostics of PregS still remains to be utilized. The aim of the work was to develop a rapid, simple, sensitive and, at the same time, sufficiently specific method for evaluation of steroid conjugates in serum and optionally in other body fluids. This could enable evaluation of its diagnostic informative value in connection with disturbances afflicting both the central and potentially also peripheral nervous system, which could be also connected with various types of autoimmune diseases. Our aspiration was to develop an optimum method for determination of PregS in serum and optionally in other body fluids as regards not only an analytical precision and accuracy but also work expenditure with intention of routine laboratory use. Determination of PregS could be of interest from the several viewpoints. The evaluation of the steroid conjugate in patients suffering from various diseases of CNS could result in finding a connection between the etiology of the disease and the levels of PregS in body fluids or connections with some endocrinopathies such as adrenal hypo- or hyperactivity or with various enzyme systems. As indicated by the results obtained from patients suffering from anxio-depressive syndrome, the enhancement PregS levels could be in relation with the patient psychic condition[8].

Another consequence of positive modulation of the calcium ion influx into neurons[12–15,26–28] is the activation of phospholipases[29–32] associated with degradation of cell membranes, which results in release of free arachidonic acid, the well-known precursor of prostanoids, as well as toxic

intermediates and free radicals. Such substances are involved in the mechanism of inflammation. Therefore, the determination of PregS could be of interest even in connection with autoimmune diseases such as the systemic lupus erythematosus, rheumatoid arthritis, *etc.* In such diseases, decreased levels of steroid sulfates in body fluids were found[10,33,34].

Finally, PregS position in the steroid metabolic pathway together with its unconjugated analogue close to common precursors of all steroids, cholesterol and its sulfate (Fig. 1), is an important component of steroidogenesis

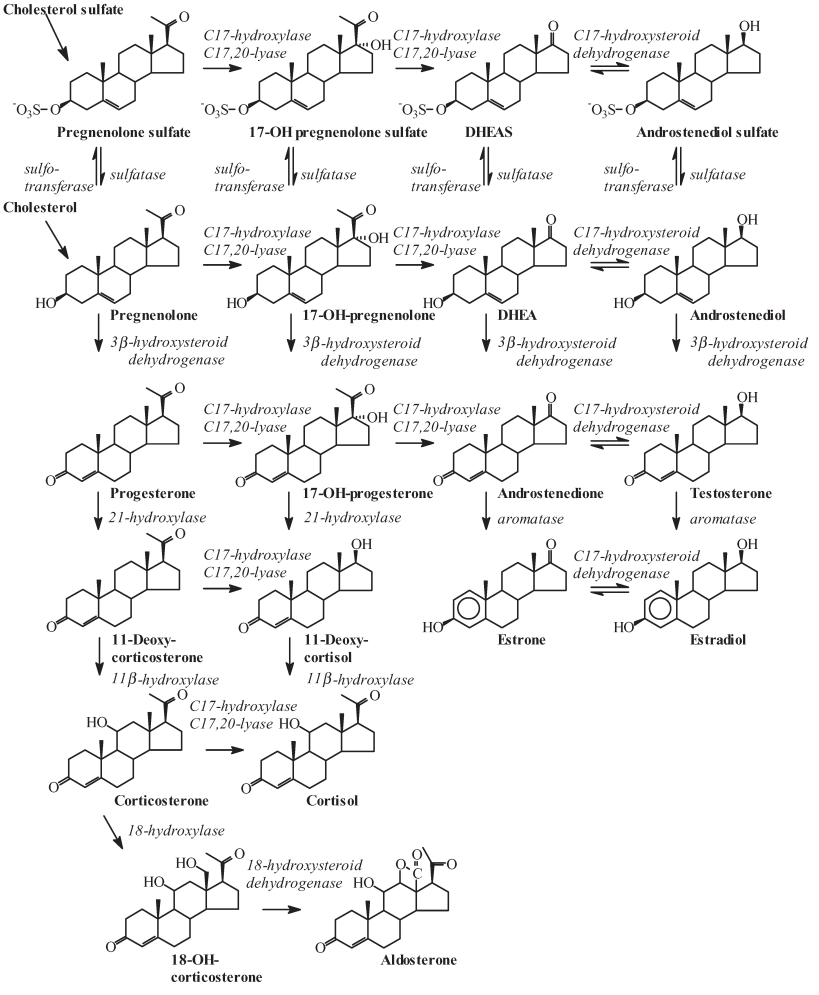

FIG. 1
Simplified scheme of steroidogenesis

and its determination is useful for diagnostics of some endocrine disturbances. Some authors recommend the determination of PregS in functional tests of children considering the fact that, in contrast to DHEAS, PregS well responds to ACTH stimulation[35].

The most common method for determination of PregS is radioimmunoassay (RIA) with antiserum raised against pregnenolone hapten linked to a high molecular-weight carrier in position 3 of the steroid. Antisera prepared using such approach are nonspecific to steroid ring A, which enables the use of an antiserum against pregnenolone hapten in determination of pregnenolone derivatives modified in ring A such as PregS (ref.[6]). The contribution of unconjugated serum pregnenolone could be mostly neglected[6] by virtue of its serum levels being by at least one order of magnitude lower. However, a certain difficulty could be cross-reactivity with free progesterone. Nevertheless, the extraction of unconjugated steroids by an appropriate nonpolar solvent avoids this problem. Older methods involved solvolysis of PregS followed by the determination of released unconjugated pregnenolone[36]. Besides the mentioned approaches, chromatographic separation of the analyte could be used[36]. Then a high selectivity could be expected, however, on the expense of labor consumption. Besides RIA, other immunoanalytical methods are available, however, the distance between epitopes characteristic of both ring D and ring A (sulfate) is a difficulty in preparation of an antiserum specific for both steroid rings. The use of immunogens linked to a high molecular carrier in positions 6, 7, 8, 11, 12 or 14 could be considered. In such cases, the distance to both epitopes is comparable and the chance of reaching sufficient selectivity is higher.

Considering the experience obtained in the course of anti-pregnenolone and anti-17-hydroxypregnenolone antisera development[37,38], the low specificity of the antisera raised against immunogens with haptens linked in position 19 to ring A may be employed (Fig. 2). Thus an antiserum that was recently raised against pregnenolone-*O*-(carboxymethyloxime) conjugated with bovine serum albumin could be further utilized for the determination of PregS. Regarding pregnenolone sulfate/pregnenolone ratio in human serum, it could be expected that for the screening of PregS even the non-extraction variant of the method could be feasible. In pregnant women as well as in fetal sera, we presumed the elimination of cross-reacting unconjugated steroids by extraction with a nonpolar solvent followed by determination of PregS in the polar residue after extraction.

EXPERIMENTAL

Materials

Steroids and Chemicals

Non-radioactive steroids and methyl tyrosinate (TME) were purchased from Sigma (St. Louis, U.S.A.). Synthesis of carboxy derivative for immunogen 3β-hydroxypregn-5-en-20-one-19-*O*-(carboxymethyl)oxime (Preg-19-CMO) was described elsewhere[39]. Complete Freund's adjuvant was from Difco (Detroit, U.S.A.), Norit A from Serva (Heidelberg, Germany) and dextran T-70 from Pharmacia (Uppsala, Sweden). The other chemicals of analytical grade were from Lachema (Brno, Czech Republic). Methanol, acetonitrile and water for HPLC Lichrosolv® were from Merck (Darmstadt, Germany). [7-^{3}H]Pregnenolone (specific activity 832.5 Gbq/mmol) was from NEN Product (Boston, U.S.A.). Scintillation cocktail Optiphase "HiSafe 2" used in measurement of β-activity in analyses with tritiated tracers was obtained from Fischer Chemicals (Loughborough, U.K.).

Iodinated tracer. Iodinated tracer was prepared by iodination of pregnenolone-19-*O*-(carboxymethyl)oxime-methyl tyrosinate ester (Preg-19-CMO-TME) using the method described elsewhere[40].

Antiserum. Preg-19-CMO was conjugated with BSA using the method of mixed anhydride[41]. The immunogen obtained was emulsified in the mixture of Freund's complete adjuvant/saline (1 : 1, v/v) and further used for immunization of rabbits. Initial molar ratio steroid/BSA was 50, and the final ratio hapten/BSA of the immunogen was 22 as measured by titration of free aminogroups by 2,4,6-trinitrobenzene-1-sulfonic acid[42].

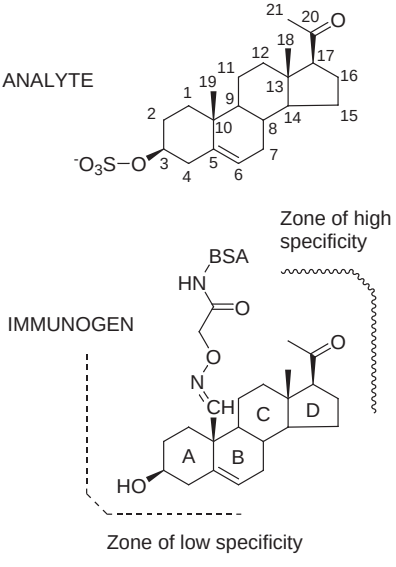

FIG. 2
Specificity patterns in antiserum against pregnenolone-(*O*-carboxymethyl)oxime-BSA

Steroid Free Serum

A mixed pool of male and female serum was treated by repeated adsorption on adsorbent carbon (50 mg of Norit A per 1 ml of serum). The mixture was centrifuged (8 000*g*, 30 min) and filtered using a Milipore filter (0.45 μm).

Samples

To obtain pooled sera with different levels of the analyte, human sera of different concentration of the analyte were mixed. In addition, the reliability performance of the method was checked even in individual sera from healthy persons (77 women and 96 men). The samples were obtained in the framework of studies on iodine deficiency in various regions of the Czech Republic. Further, female sera collected at different stages around parturition were used for measurement as the corresponding umbilical sera of newborns at labor. In all cases, a written informed permission was obtained from the subjects.

Devices

HPLC system. HPLC system was from Gilson (Villiers Le Bel, France). It contained a master pump 305 with manometric module 805, slave pump 306, dynamic mixer 811 C, autoinjector 234 and fraction collector FC 203 B. UV detector LCD 2082 and column oven LCO 100 was from ECOM (Prague, Czech Republic). Reverse-phase column ET 250/4 Nucleosil® 100-5 C_{18} was purchased from Macherey–Nägel (Düren, Germany). The data obtained were treated using a software CSW Apex from DataApex (Prague, Czech Republic).

Scintillation spectrophotometer. The scintillation spectrophotometer used for measurement of β-activity, LS 65000, was purchased from Beckman Instruments, Inc. (Fulerton, U.S.A.).

γ-*Counter*. γ-Activity was measured using a twelve-channel γ-counter from Berthold (Wildbad, Germany).

Methods

RIA with [^{3}H]Tracer

Non-extraction method. Phosphate buffer (90 μl, pH ≈ 7.1) was added to 10 μl of serum, 100 μl of antiserum (diluted 1 : 2 500) and 100 μl of tracer (7-[^{3}H]pregnenolone) with β-activity 5 000 cpm/tube). The mixture was incubated at 37 °C for 45 min and further at 5 °C for 60 min. Free analyte and tracer was adsorbed on dextran-coated adsorbent carbon (500 μl of suspension containing 0.025 g dextran T-70 and 0.25 g Norit A per 100 ml of neutral phosphate buffer). After mixing and incubation at 5 °C for 15 min, the mixture was centrifuged (8 000*g*, 10 min). Scintillation cocktail (4 ml) was added to the supernatant and the β-activity of the mixture was measured.

Extraction method. Phosphate buffer (90 μl, pH ≈ 7.1) was added to 10 μl of serum, 1 ml of nonpolar solvent (modification with ether or with hexane) was added and agitated for 1 min. The nonpolar layer containing cross-reacting progesterone and unconjugated pregnenolone was removed and the polar layer was lyophilized in vacuum centrifuge. The dry residue was reconstituted with 100 μl of the buffer and underwent the process described above. The modification with hexane allowed direct processing without drying.

RIA with [^{125}I]Tracer

In this case, only a non-extraction modification of the method was tested and the remaining steps were the same as in the method using tritiated tracer except the measurement of γ- instead of β-activity.

Calibration Curve

Calibration curve was constructed from 8 standard solutions of PregS in concentrations corresponding to 3.87, 1.93, 0.97, 0.48, 0.24, 0.12, 0.06 and 0.03 nmol/l of serum PregS.

Data Analysis

Calculations

For the calculation of analyte concentrations, personal software was used. The calibration curve was evaluated using of the linearized regression between the activity of supernatant and concentration of the analyte using log–logit transformation.

Statistical Analysis of the Results

Age dependences were evaluated using polynomial regression as follows: The optimum degree of polynomial was determined using the minimum of mean error prediction. Then the high leverage points and outliers were detected using diagnostic plots such as Williams plot, normal probability plot, histogram, box plot and symmetry plot of studentized residues. Power transformation was applied to avoid skewed residual distribution and heteroscedasticity. Optimum transformation was determined by the minimum value of skewness of studentized residues with absolute value less than 2. The limit was used to avoid troublesome effect of outliers. The significance of the regression model was evaluated using the *F*-test. The variance inflation factor was used to check that the effect of multicollinearity was within the tolerance limit.

Besides regression, the age relationships and sex differences in PregS were evaluated using the two-way analysis of variance with sex as the first and the subject as the second factor. As previously, analysis of variance was followed by the least significant difference (LSD) test for evaluation of differences between individual groups. For the same reasons as in regression, transformation and residual analysis with the use of diagnostic plots was carried out except the analysis of influential points. Instead of even more appropriate studentized residues, normalized residues were used regarding the scope of the statistical software used.

For evaluation of time profiles of PregS in maternal serum around parturition, two-way analysis of variance with the stage of parturition as the first and the type of analgesia as the second factor followed by LSD test for evaluation of differences between individual groups were used. For comparison of differences between the levels of PregS in umbilical and maternal serum at labor, the non-parametric Mann Whitney test was used considering the asymmetric and, at the same time, flat data distribution which could not be improved by transformation.

RESULTS

Analytical Criteria

Selectivity

Cross-Reactions

Cross-reactions in anti-pregnenolone-*O*-(carboxymethyl)oxime-bovine serum albumin antiserum were measured for steroids with similar structures as PregS (Table I). Specifically, the cross-reactions were measured for 17-hydroxypregnenolone, pregnenolone sulfate, pregnenolone glucuronide, progesterone, 17-hydroxyprogesterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, allopregnanolone (3α-hydroxy-5α-pregnan-20-one), allopregnanolone sulfate, pregnanolone (3β-hydroxy-5α-pregnan-20-one), isopregnanolone (3α-hydroxy-5β-pregnan-20-one) and epipregnanolone (3β-hydroxy-5β-pregnan-20-one).

The antiserum was non-specific in steroid ring A. Cross-reactivity of PregS was 42.1% when compared with pregnenolone. Regarding about 50 times higher levels of the conjugate in comparison with unconjugated steroid, a non-extraction variant of the method for screening could be developed when accepting limited demands on analytical quality of results in human serum samples with the exception of pregnancy where high concentrations

TABLE I
Cross-reactions of different steroids in anti-pregnenolone-*O*-(carboxymethyl)oxime-BSA antiserum

Steroid	Cross-reaction %	Steroid	Cross-reaction %
Pregnenolone	100	DHEA	0.016
Pregnenolone sulfate	42.05	DHEAS	<0.001
Pregnenolone glucuronide	2.04	Allopregnanolone	0.63
Progesterone	5.98	Allopregnanolone sulfate	0.31
17-Hydroxypregnenolone	0.69	3β-Hydroxy-5α-pregnan-20-one	5.18
17-Hydroxyprogesterone	<0.001	3α-Hydroxy-5β-pregnan-20-one	0.11
Androstenedione	<0.001	Epipregnanolone	0.53

of progesterone could considerably influence the results. For this reason, it is necessary to involve an extraction step for elimination of unconjugated cross-reacting steroids.

Examination of Selectivity by the Immunochromatogram Method

For examination of the presence and influence of all cross-reacting substances in the matrix, the method of immunochromatogram was used. The fundamental of the method is a chromatographic non-destructive fractionation and measurement of immunoreactivity in individual fractions. The record thus obtained is confronted with the chromatogram of known standards made under identical separation conditions. A comparison of both records results with high probability the identification of cross-reacting substances. Using this technique, it was proved that besides PregS and pregnenolone, the method could be sensitive even to progesterone particularly in samples of maternal and fetal serum around parturition (Fig. 3). Chromatographic conditions of sample fractionation are shown in the Table II.

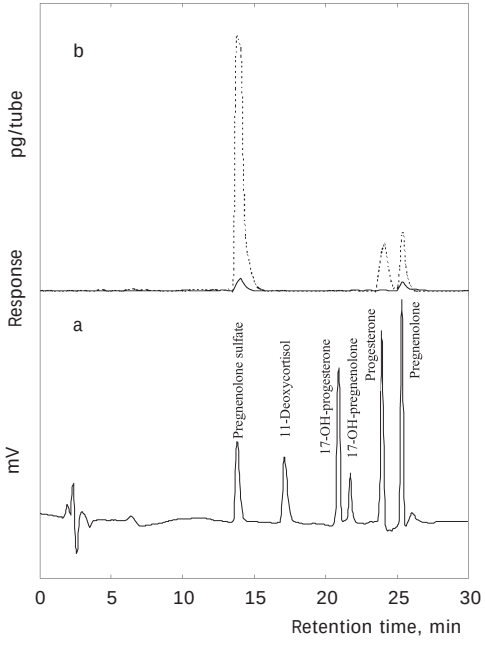

FIG. 3

Comparison of chromatogram of concentrated standards (a) with immunochromatogram (b) of pooled female serum with higher concentration of analyte (full line) and pooled sample of umbilical serum of newborns at labor (dashed line)

Choice of the Solvent for Elimination of Uunconjugated Steroids

Four extraction solvents were tested for separation of steroids from biological matrix – ethyl acetate, diethyl ether, hexane and dichloromethane. The test involved extraction of 20 μg pregnenolone sulfate, progesterone or pregnenolone dissolved in 200 μl of neutral phosphate buffer with 1 ml of organic solvent, drying of the extract aliquot (800 μl), its dissolution in 100 μl of methanol and analysis of steroids followed by HPLC. The extraction efficiencies are demonstrated in Table III. It is obvious that the diethyl ether is

TABLE II
Chromatographic conditions for evaluation of the influence of cross-reacting substances in human serum

Column	ET 100-5 C_{18} from Macherey–Nägel						
Gradient	Eluent A: 15% acetonitrile in water						
	Eluent B: methanol						
	Time, min	0	3	6	20	23	30
	Eluent B, %	0	0	40	80	100	0
Flow rate of mobile phase	1 ml/min						
Detection of standards	UV 205 nm						
Temperature of column	40 °C						
Column pressure	22 MPa						

TABLE III
Extraction efficiency (in %) for pregnenolone sulfate, progesterone and pregnenolone of various extraction solvents

Solvent	Steroid[a]		
	pregnenolone sulfate	progesterone	pregnenolone
Diethyl ether	<0.1	79.0 ± 3.1	87.7 ± 3.3
Hexane	<0.1	70.1 ± 6.2	71.3 ± 9.5
Dichloromethane	6.3 ± 0.9	72.1 ± 5.7	83.4 ± 5.8
Ethyl acetate	71.6 ± 5.4	92.4 ± 3.1	99.7 ± 3.7

[a] Steroids were partitioned between 200 μl of neutral phosphate buffer and 1 000 μl of extraction solvent.

the most suitable solvent due to better extraction efficiency than in hexane at no losses in the organic phase as in ethyl acetate or dichloromethane.

Non-Extraction RIA with [^{3}H]Tracer

Sensitivity of non-extraction RIA with [^{3}H]tracer was determined at 95% probability level from the variance of activity of 8 blanks (Table IV). Intra-assay was evaluated using 8 parallel measurements in one batch while inter-assay was determined from 7 measurements in different batches (Table V). Four pooled serum samples with increasing concentration of the analyte were used. The dilution test was carried out using the medium pool diluted 1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32 and 1 : 64. The 100 µl initial volume of

TABLE IV
Sensitivity of two variants of the method for determination of PregS

Tracer	Antiserum dilution	Sensitivity	
		nmol/l	pg/tube
[^{3}H]	1 : 2 500	7.8	32
[^{125}I]	1 : 80 000	6.4	26

TABLE V
Reproducibility of non-extraction method with [^{3}H]tracer

Sample		Intra-assay n = 8	Inter-assay n = 7
Low pool	mean, nmol/l	141	155
	CV, %[a]	4.5	12.2
Medium pool	mean, nmol/l	221	228
	CV, %[a]	3.9	11.1
High pool	mean, nmol/l	383	410
	CV, %[a]	3.7	9.85
Extreme pool	mean, nmol/l	575	534
	CV, %[a]	5.1	13.9

[a] CV is the coefficient of variance.

the medium pool was adjusted to 200 μl with neutral phosphate buffer (pH ≈ 7.1) and further serially diluted (Table VI). The recovery was determined in the low pool after spiking with analyte (4 ng/tube). The results are shown in Table VII.

Non-Extraction RIA with Iodinated Tracer

Intra- and inter-assay coefficients of variance are shown in Table VIII. Dilution test was carried out using the medium pool diluted 1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 16 and 1 : 32. The 50 μl initial volume of the medium pool was adjusted to 200 μl with neutral phosphate buffer (pH ≈ 7.1) and further serially diluted (Table IX). The recovery was determined in the low pool after spiking with PregS (80 ng/tube). The results are shown in Table X.

TABLE VI
Dilution test (non-extraction RIA with [^{3}H]tracer)

Dilution	Concentration		Relative to initial dilution
	nmol/l	pg/tube	%
1 : 1	210	4 342	100
1 : 2	122	2 522	117
1 : 4	47	972	89
1 : 8	30	620	105
1 : 16	15	310	111
1 : 32	5	103	81
1 : 64	3	62	80

TABLE VII
Recovery test (non-extraction RIA with [^{3}H]tracer)

Parameter	Without spiking	Addition	Spiked sample	Yield
nmol/l	164	967	1 410	
pg/tube	678	4 000	5 830	5 152
Recovery, %				125[a]

[a] Recovery (%) = $\frac{\text{found in the spiked sample}}{\text{found in the sample without spiking + spiking}} 100$.

TABLE VIII
Reproducibility of non-extraction method with [^{125}I]tracer

Sample		Intra-assay $n = 8$	Inter-assay $n = 7$
Low pool	mean, nmol/l	197	180
	CV, %[a]	4.6	14.6
Medium pool	mean, nmol/l	231	233
	CV, %[a]	8	19.6
High pool	mean, nmol/l	497	410
	CV, %[a]	7.1	14.7

[a] CV is the coefficient of variance.

TABLE IX
Dilution test (non-extraction RIA with [^{125}I]tracer)

Dilution	Concentration		Relative to initial dilution
	nmol/l	pg/tube	%
1 : 1	104	1 075	100
1 : 2	51	527	98
1 : 4	25	258	96
1 : 8	11	114	84
1 : 16	4	41	61

TABLE X
Recovery test (non-extraction RIA with [^{125}I]tracer)

Parameter	Without spiking	Addition	Spiked sample	Yield
nmol/l	231	19 350	19 655	
pg/tube	955	80 000	81 273	80 318
Recovery, %				100[a]

[a] Recovery (%) = $\frac{\text{found in the spiked sample}}{\text{found in the sample without spiking + spiking}}\,100$.

Extraction RIA with [^{3}H]Tracer

Reproducibility

Intra- and inter-assay coefficients of variance determined using the low pool are shown in Table XI.

Dilution Test

Dilution test was carried out using the low pool diluted 1 : 1, 1 : 2, 1 : 4, 1 : 8 and 1 : 16 with steroid-free plasma (Table XII). The 100 µl initial volume of medium pool was adjusted to 200 µl with steroid-free plasma (pH ≈ 7.1) and further serially diluted. The samples were further processed using RIA following extraction elimination of free steroids with diethyl ether or with hexane.

Recovery

The recovery was determined in the low pool after spiking with PregS (4 ng/tube). The results are shown in Table XIII.

TABLE XI
Reproducibility of extraction method with [^{3}H]tracer

Sample		Intra-assay n = 8	Inter-assay n = 7
Low pool	mean, nmol/l	141	155
	CV, %[a]	4.5	12.2
Medium pool	mean, nmol/l	221	228
	CV, %[a]	3.9	11.1
High pool	mean, nmol/l	383	410
	CV, %[a]	3.7	9.85
Extreme pool	mean, nmol/l	575	534
	CV, %[a]	5.1	13.9

[a] CV is the coefficient of variance.

TABLE XII
Dilution test (RIA with [^{3}H]tracer for extraction elimination of cross-reactants)

Extraction solvent	Dilution	Concentration		Relative to initial dilution
		nmol/l	pg/tube	%
Diethyl ether	1 : 1	104	1 075	100
	1 : 2	51	527	98
	1 : 4	25	258	96
	1 : 8	10	114	85
	1 : 16	4	41	62
Hexane	1 : 1	78	806	100
	1 : 2	49	507	126
	1 : 4	21	217	105
	1 : 8	8	83	86
	1 : 16	3	31	71

TABLE XIII
Recovery (extraction RIA with [^{3}H]tracer)

Extraction solvent	Parameter	Without spiking	Addition	Spiked sample	Yield
Diethyl ether	nmol/l	185	967	1 264	
	pg/tube	765	4 000	5 227	4 462
	Recovery, %				110[a]
Hexane	nmol/l	132	967	1 287	
	pg/tube	546	4 000	5 321	4 775
	Recovery, %				117[a]

[a] Recovery (%) = $\frac{\text{found in the spiked sample}}{\text{found in the sample without spiking + spiking}}\,100$.

APPLICATION OF THE METHOD

Age Relations and Sex Differences in Circulating Pregnenolone Sulfate

Modification of the method involving non-extraction radioimmunoassay with [^{3}H]tracer was used for evaluation of age relations and sex differences in humans.

The significant age dependence of PregS was found with a steep rise in puberty and adolescence, maximum values before 30th year of age and gradual decrease in adulthood and senescence (Fig. 4). In men, the effect was less pronounced.

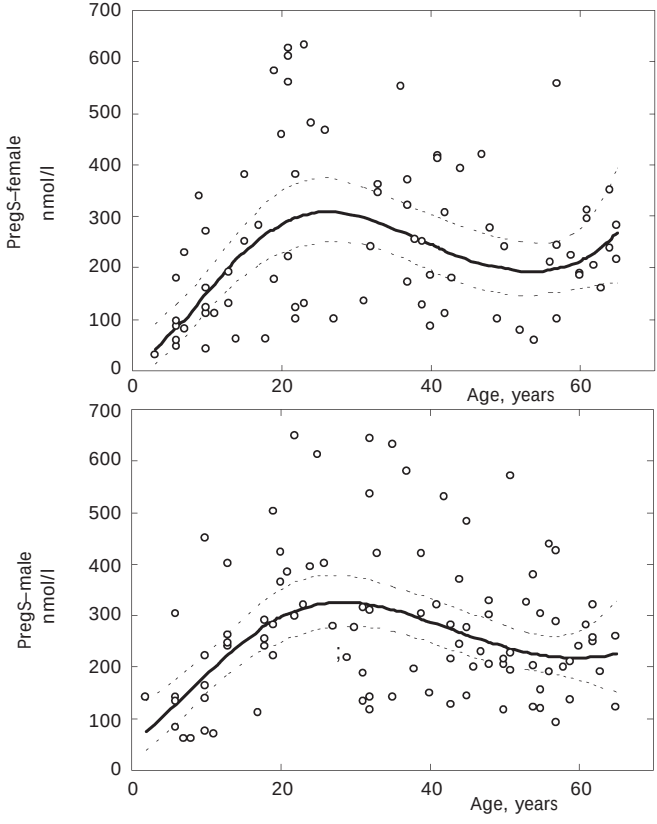

FIG. 4

Age dependence of PregS in men and in women. Full lines represent re-transformed mean predictions of polynomial regression of the third degree calculated from the data with the dependent variable transformed by power transformation to minimum skewness of studentized residues. Dashed lines depict re-retransformed 95% confidence interval of the fit. Circles represent the experimental points

Besides regression analysis, the data were subdivided into 8 age groups and two-way analysis of variance was applied with sex and age groups as the first and the second factor, respectively. The analysis of variance was followed by multiple comparisons using the least significant difference (LSD) test (Fig. 5). In the sex factor, no significant difference was found ($p < 0.053$). Highly significant differences were found ($p < 0.0001$) between age groups. Specifically, the difference between the 21–30 (maximum) and 16–20 years of age groups was significant as well as between the maximum and 41–50 years of age.

Time Profiles of Pregnenolone Sulfate in Maternal Serum in Epidural and Subarachnoidal Analgesia Around Rarturition

PregS was followed in sera of 21 women in epidural ($n = 6$) and in subarachnoidal ($n = 15$) analgesia around parturition (Fig. 6) and in maternal and umbilical serum at delivery (Fig. 7). Due to high concentration of cross-reacting substances in both body fluids, particularly progesterone and free pregnenolone, the variant of the method was chosen with extraction elimination of free steroids with diethyl ether followed by radioimmunoassay of PregS in polar residue after extraction. The residue was first lyophilized and then reconstituted to original value with neutral phos-

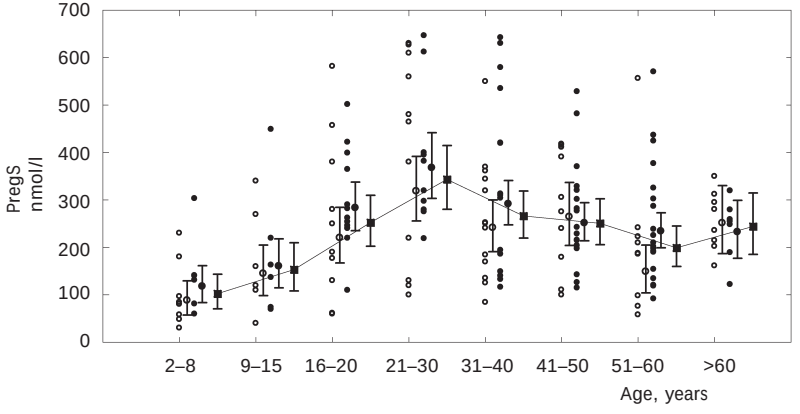

FIG. 5
Levels of PregS in age groups. Small full and open circles represent individual levels in men and in women, respectively. The circles with error bars depict mean values in individual age groups with their 95% confidence intervals while squares represent mean values in groups involving both sexes

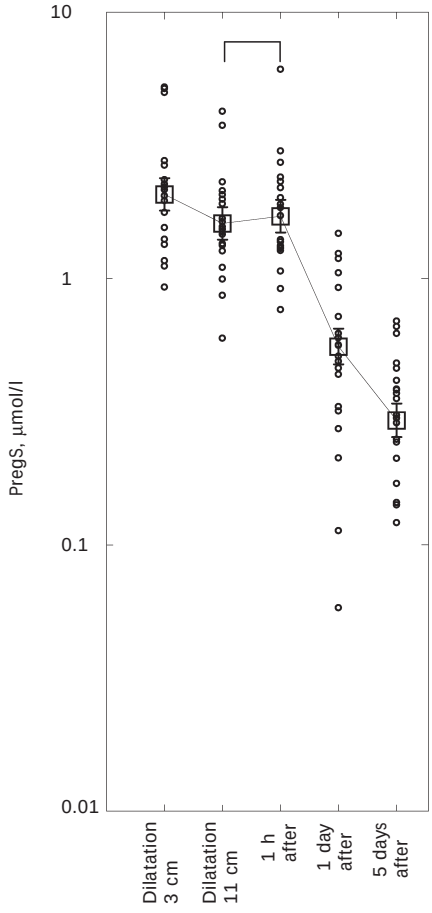

FIG. 6

Time profile of PregS in women in epidural and subarachnoidal analgesia. Open circles represent the levels of the steroid in 21 women with epidural (n = 6) and subarachnoidal analgesia (n = 15). Squares with error bars depict means with their 95% confidence intervals at individual stages of parturition. The clamp denotes the homogeneous groups. The data (n = 21) were analysed by the ratio (F) explained by Anova model and the random variance, and by the level of statistical significance (p) either for the factor stage (F = 138, $p < 0.0001$) or the factor subject (F = 8.3, $p < 0.0001$)

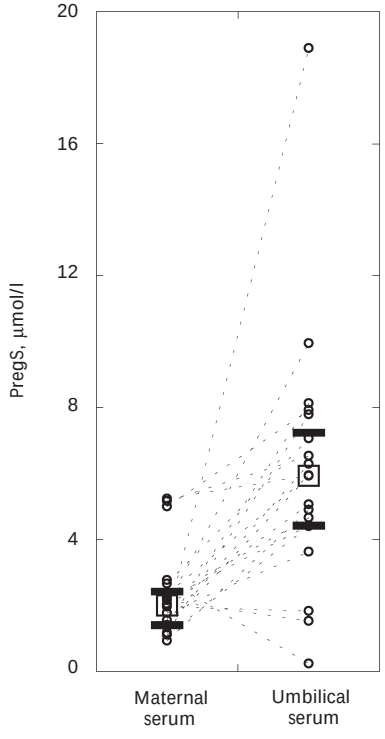

FIG. 7

Differences between maternal and umbilical serum in the levels of PregS. The open circles represent the levels of PregS in maternal and umbilical serum at delivery. The open squares and the short bold lines represent medians and quartiles, respectively. The dashed lines express the paired values in maternal and umbilical serum. The data (n = 19) were analysed by Student's paired t-test ($p < 0.0001$) and Wilcoxon's paired test ($p < 0.001$)

phate buffer. The tritiated pregnenolone was used as a tracer. The experiment samples were collected at the following stages: (i) cervical dilatation 3 cm, (ii) cervical dilatation 11 cm, (iii) 1 h after labor, (iv) 1 day after labor and 5 days after labor. The concentrations of PregS were determined in corresponding umbilical sera immediately after delivery. No correlation was found between PregS in maternal and umbilical serum.

Pronounced differences were found between the stages of parturition ($p < 0.0001$, Anova). After a moderate decrease up to the third stage (1 h after delivery), a pronounced fall followed within the first day after delivery. No significant change was observed in the following period between the first and fifth days. As expected, higher levels of PregS at delivery were found in fetuses (Fig. 7).

DISCUSSION

Determination of PregS is a helpful tool in the diagnostics of classical endocrinopathies[35] such as various forms of congenital adrenal hyperplasia, hypercortisolemia or disturbances of sulfatase activity[43–45], which could also influence the initiation of parturition[43–45], and possibly even in diagnostics of neuronal disturbances such as premenstrual syndrome, catamenial epilepsy, depression, Alzheimer disease or parkinsonism. The method may be beneficial also in the diagnostics of some autoimmune diseases such as the systemic lupus erythematosus, rheumatoid arthritis or some thyreopathies[10,33,46,47].

For the purposes mentioned above, various modifications of PregS determination were developed at different concentrations of cross-reactants in body fluids under different physiological conditions regarding time and labor expenditure.

Considering cross-reactivities of the antiserum, concentrations of cross-reactants in body fluids and immunochromatograms, it is obvious that out of the endogenous substances, only pregnenolone sulfate, unconjugated pregnenolone and progesterone could influence the results. Fortunately, pregnenolone and progesterone can be easily eliminated by extraction with an appropriate nonpolar solvent and the remaining PregS can be determined in the polar residue after extraction. Further improvement of the method with regard to selectivity could be primary extraction of both free steroids and most of the PregS from polar matrix with ethyl acetate, and subsequent partitioning of free steroids between phosphate buffer and a nonpolar solvent eliminating free steroids. However, such approach brings

an additional source of variability as well as time and labor expenditure and therefore it was omitted.

The use of radioiodination for preparation of the tracer was helpful respecting instant analytical expediency and material cost. On the other hand, the necessity to synthesize the tracer with limited durability and the decreased analytical criteria in the modified method as well as the manipulation with γ-activity resulted in the choice of non-extraction modification with tritiated tracer, which was used in evaluation of age and sex relations of the steroid conjugate in humans from childhood to senescence. Up to date, no such study covering the all lifespan has been published. The results obtained here were in accordance with the uncompleted studies published before[6].

A decrease in PregS in senescence was up to 65% of the maximum value found before 30th year of age. In comparison with free pregnenolone, the circulating levels of PregS are more than one order of magnitude higher[6,10,33]. In this connection, its capability of transport across the blood-brain barrier[48] should be mentioned, as well as its activating effect on the membrane neuronal receptors that are responsible for calcium ion transport into the neuron, resulting in improvement of perception and memory. Higher levels of the conjugate in women suffering with mixed anxiety-depressive syndrome should be also mentioned[8].

Further application of the method for measurement of PregS was the evaluation of time profiles of the conjugate in circulation of women with epidural and subarachnoidal analgesia around parturition. The levels of PregS were exceedingly elevated in fetal and maternal serum. However, no correlation was found between them. A steep fall within the first hour and day after delivery was found in maternal serum. These finding are of interest with respect to possible influencing the onset and/or course of parturition by PregS. In the application mentioned above, the method involving an extraction step for elimination of cross-reactants was introduced due to excessive concentrations of progesterone and unconjugated pregnenolone in fetal and maternal serum during pregnancy and around parturition.

The methodical variant involving extraction step for elimination of cross-reactants was used also for determination of PregS in two types of breast cystic fluids where the extreme concentrations of the conjugate were found[9]. The finding was in agreement with others[6] who found excessive amounts of other steroid sulfates in breast cyst fluid.

The rapid non-extraction variant was used for evaluation of age and sex relations of the conjugate in humans, where a strong age relationship and borderline sex differences were found.

Pronounced time changes of the conjugate around parturition in maternal serum and concurrent independence of maternal PregS on fetal levels indicating autonomous production of maternal PregS, negligible transport of the conjugate across the blood-brain barrier as well as possible influencing the onset and course of parturition by PregS were found.

Exceedingly elevated levels of PregS were found in breast cystic fluid, which could indicate autonomous production of the steroid by the cyst and/or hyperactivity of steroid sulfotransferase as well as a possible accumulation of the steroid in breast cysts. The latter effect allows speculating about deactivation of free steroids by sulfatation, which could be alternatively metabolized to estrogens, thus increasing the risk of cancer development.

CONCLUSION

A novel, simple and rapid radioimmunoassay of PregS was evaluated in several variants for different matrixes and at different concentration of cross-reactants. The variants of the method enabled both rapid screening measurements of the conjugate in human serum and specific determination in matrixes with high concentrations of cross-reactants such as serum of pregnant women, fetal serum or breast cyst fluid.

The study was supported by the Internal Grant Agency of the Czech Ministry of Health (grant No. 5398-3). We wish to express our sincere thanks to Prof. V. Zamrazil, Dr A. Šimečková and Dr J. Kvíčala for the samples of biological material from their screening studies of iodine deficiency. The excellent technical assistance of Miss I. Karlíková is gratefully acknowledged.

REFERENCES

1. Majewska M. D., Ford-Rice F., Falkay G.: *Brain Res.* **1989**, *482*, 397.
2. Turner J. P., Simmonds M. A.: *Br. J. Pharmacol.* **1989**, *96*, 409.
3. Purdy R. H., Morrow A. L., Blinn J. R., Paul S. M.: *J. Med. Chem.* **1990**, *33*, 1572.
4. Prince R. J., Simmonds M. A.: *Neurosci. Lett.* **1992**, *135*, 273.
5. Zhang S. J., Jackson M. B.: *J. Neuroendocrinol.* **1994**, *6*, 533.
6. de Peretti E., Mappus E.: *J. Clin. Endocrinol. Metab.* **1983**, *57*, 550.
7. Mathur R. S., Landgrebe S., Moody L. O., Powell S., Williamson H. O.: *J. Clin. Endocrinol. Metab.* **1980**, *51*, 1235.
8. Bičíková M., Tallová J., Hill M., Krausová Z., Hampl R.: *Neurochem. Res.* **2000**, *25*, 1623.
9. Bičíková M., Szamel I., Hill M., Tallová J., Stárka L.: *Steroids* **2001**, 66, 55.
10. Hedman M., Nilsson E., de la Torre B.: *Clin. Exp. Rheumatol.* **1989**, *7*, 583.
11. Hauser C. A., Wetzel C. H., Rupprecht R., Holsboer F.: *Biochem. Biophys. Res. Commun.* **1996**, *219*, 531.

12. Mathis C., Vogel E., Cagniard B., Criscuolo F., Ungerer A.: *Neuropharmacology* **1996**, *35*, 1057.
13. Fahey J. M., Lindquist D. G., Pritchard G. A., Miller L. G.: *Brain Res.* **1995**, *669*, 183.
14. Wakerley J. B., Richardson C. M.: *Neurosci. Lett.* **1997**, *226*, 123.
15. Irwin R. P., Maragakis N. J., Rogawski M. A., Purdy R. H., Farb D. H., Paul S. M.: *Neurosci. Lett.* **1992**, *141*, 30.
16. Bergeron R., de Montigny C., Debonnel G.: *J. Neurosci.* **1996**, *16*, 1193.
17. Flood J. F., Morley J. E., Roberts E.: *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10806.
18. Bukusoglu C., Sarlak F.: *Eur. J. Pharmacol.* **1996**, *298*, 79.
19. Maurice T., Junien J. L., Privat A.: *Behav. Brain Res.* **1997**, *83*, 159.
20. Jezova D., Tokarev D., Rusnak M.: *Neuroendocrinology* **1995**, *62*, 326.
21. Yu X. M., Sessle B. J., Haas D. A., Izzo A., Vernon H., Hu J. W.: *Pain* **1996**, *68*, 169.
22. Davidson E. M., Coggeshall R. E., Carlton S. M.: *NeuroReport* **1997**, *8*, 941.
23. Gee K. W., Bolger M. B., Brinton R. E., Coirini H., McEwen B. S.: *J. Pharmacol. Exp. Ther.* **1988**, *246*, 803.
24. Majewska M. D., Harrison N. L., Schwartz R. D., Barker J. L., Paul S. M.: *Science* **1986**, *232*, 1004.
25. Paul S. M., Purdy R. H.: *FASEB J.* **1992**, *6*, 2311.
26. Irwin R. P., Lin S. Z., Rogawski M. A., Purdy R. H., Paul S. M.: *J. Pharmacol. Exp. Ther.* **1994**, *271*, 677.
27. Sun P., Rane S. G., Gunasekar P. G., Borowitz J. L., Isom G. E.: *J. Pharmacol. Exp. Ther.* **1997**, *280*, 1341.
28. Park-Chung M., Wu F. S., Purdy R. H., Malayev A. A., Gibbs T. T., Farb D. H.: *Mol. Pharmacol.* **1997**, *52*, 1113.
29. Saitoh H., Hirato K., Tahara R., Ogawa K., Noguchi Y., Yanaihara T., Nakayama T.: *Acta Endocrinol. (Copenhagen)* **1984**, *107*, 420.
30. Bonney R. C., Franks S.: *J. Steroid Biochem.* **1987**, *26*, 467.
31. Bonney R. C., Qizilbash S. T., Franks S.: *J. Steroid Biochem.* **1987**, *27*, 1057.
32. Noguchi Y., Hirato K., Saito H., Takasaki K., Yanaihara T., Nakayama T.: *Nippon Sanka Fujinka Gakkai Zasshi* **1988**, *40*, 359.
33. Hedman M., Nilsson E., de la Torre B.: *Clin. Exp. Rheumatol.* **1992**, *10*, 25.
34. Foldes J., Lakatos P., Zsadanyi J., Horvath C.: *Eur. J. Endocrinol.* **1997**, *136*, 277.
35. de Peretti E., Forest M. G., Loras B., Morel Y., David M., Francois R., Bertrand J.: *Acta Endocrinol. Suppl.* **1986**, *279*, 259.
36. Magyar D. M., Elsner C. W., Eliot J., Glatz T., Nathanielsz P. W., Buster J. E.: *Steroids* **1981**, *37*, 423.
37. Hill M., Lukáč D., Lapčík O., Šulcová J., Hampl R., Pouzar V., Stárka L.: *Clin. Chem. Lab. Med.* **1999**, *37*, 439.
38. Hill M., Hampl R., Lukáč D., Lapčík O., Pouzar V., Šulcová J.: *Steroids* **1999**, *64*, 341.
39. Pouzar V., J. Fajkoš: *Steroids* **1994**, *59*, 696.
40. Lapčík O., Hampl R., Hill M., Stárka L., Kasal A., Pouzar V., Putz Z.: *Collect. Czech. Chem. Commun.* **1996**, *61*, 799.
41. Cook B., Beastall G. H. in: *Steroid Hormones: A Practical Approach* (B. Green and R. E. Leake, Eds), p. 1. IRL Press, Oxford and Washington D.C. 1987.
42. Fields R.: *Biochem. J.* **1971**, *124*, 581.
43. Lykkesfeldt G., Nielsen M. D., Lykkesfeldt A. E.: *Obstet. Gynecol. (N. Y.)* **1984**, *64*, 49.

44. Osathanondh R., Canick J., Ryan K. J., Tulchinsky D.: *J. Clin. Endocrinol. Metab.* **1976**, *43*, 208.
45. France J. T., Seddon R. J., Liggins G. C.: *J. Clin. Endocrinol. Metab.* **1973**, *36*, 1.
46. de la Torre B., Hedman M., Nilsson E., Olesen O., Thorner A.: *Clin. Exp. Rheumatol.* **1993**, *11*, 597.
47. Nilsson E., de la Torre B., Hedman M., Goobar J., Thorner A.: *Clin. Exp. Rheumatol.* **1994**, *12*, 415.
48. Wang M. D., Wahlstrom G., Backstrom T.: *J. Steroid Biochem. Mol. Biol.* **1997**, *62*, 299.