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Automated chromatographic system for the simultaneous measurement of plasma pregnenolone and 17-hydroxypregnenolone by radioimmunoassay

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Abstract

A new, simple, rapid and highly practicable automated chromatographic system for the separation, and a sensitive radioimmunoassay system for the subsequent measurement of pregnenolone and 17-hydroxypregnenolone has been developed. Pregnenolone and 17-hydroxypregnenolone were extracted with methylene chloride and separated from cross-reacting steroids by mechanised Sephadex-LH20 multi-column chromatography. Anti-pregnenolone and anti-17-hydroxy-pregnenolone were obtained by immunising rabbits with pregnenolone-20-oxime-BSA and 17-hydroxypregnenolone-20-oxime-BSA. The lower detection limit of the assay is 0.15 and 0.28 nmol/l for pregnenolone and 17-hydroxypregnenolone, respectively. Normal values for this assay in young male adults, in adult females, and in prepubertal boys and girls were established as a basis for the functional diagnosis of androgen excess syndromes/steroidogenesis defects.

Keywords: Steroids; Pregnenolone; 17-Hydroxypregnenolone

1. Introduction

Pregnenolone (Preg) (3β-hydroxypregn-5-en-20-one) and 17-hydroxypregnenolone (17OH-Preg) (3β,17-dihydroxypregn-5-en-20-one) are early precursors in the biosynthesis of corticosteroids, estrogens and androgens. There is an increasing demand to measure these steroids to rule out adrenal and/or gonadal enzymatic deficiencies in paediatric and adolescent patients with accelerated growth, premature pubarche or hirsutism [1] and in adult women with polycystic ovaries, hirsutism, acne or amenorrhoea [2]. For the paediatric endocrinologist it is of special interest to determine Preg and 17OH-Preg in order to rule out or confirm the diagnosis of 3β-hydroxysteroid dehydrogenase (3βHSD) deficiency. This is a form of congenital adrenal hyperplasia that impairs steroidogenesis in both adrenals and gonads resulting from mutations in the HSD3B2 gene [3] and causing various degrees of salt-wasting in both sexes and incomplete masculinisation of the external genitalia in genetic males. 3βHSD catalyses the 3β-hydroxysteroid dehydrogenation and Δ5- to Δ4-isomerisation of the Δ5-steroid

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precursors Preg and 17OH-Preg. With mutant 3βHSD these less metabolised substrates show an abnormal elevation and thus are key diagnostic markers.

However, the measurement of plasma Preg and 17OH-Preg is not a standard procedure in endocrinological laboratories. There are various reports concerning methods for the detection of Preg and 17OH-Preg [4–10]. All systems use an ether extraction to remove water-soluble fractions. The steroid separation is done either by thin-layer chromatography [8,11] or microcolumn chromatography [4–7,9,10]. All these systems are very time consuming, as they are not automated. Methods using gas chromatography–mass spectrometry (GC–MS) are established for Preg [12–14] but not for 17OH-Preg. They are not used in clinical routine. Direct radioimmunoassays (RIA) of 17OH-progesterone are highly unreliable due to the presence of large amounts of cross-reacting progestational steroids [15–18]. It is most likely that the same problems exist for direct Preg and 17OH-Preg measurements. We therefore developed a new, simple, rapid and highly practicable automated chromatographic system for the separation, and a sensitive RIA system for the measurement of plasma Preg and 17OH-Preg. Normal reference values for this method in young male adults, in adult females during the follicular phase of the menstrual cycle, and in prepubertal boys and girls were established.

2. Experimental data

2.1. Materials

2.1.1. Radioactive steroids

[7(n)-^{3}H] Pregnenolone (NET 039) with a specific radioactivity of 10–25 Ci/mmol and 17α-hydroxy-[7(n)-^{3}H] pregnenolone (TRQ 6586) with a specific radioactivity of 15 Ci/mmol were purchased from NEN Life Science Products (Boston, MA, USA) and AEA Technology QSA GmbH (Otterfing, Germany), respectively. The radiochemical purity was 98%. The amount to be used in a 3-month period was repurified by chromatography on a 40 cm Sephadex LH-20 column using methylene chloride–methanol (98:2) as solvent. Purified radioactive steroids were kept in benzene–ethanol (9:1) at 4°C.

2.1.2. Chemicals

Methylene chloride p.a. (Merck, Darmstadt, Germany), methanol p.a. (Merck), and ethanol p.a. (Merck) were used without further purification. Nonlabelled steroids were obtained from Sigma (Munich, Germany). The suppliers of the following chemicals were: Sephadex LH20, Dextran T70, Pharmacia+Upjohn (Uppsala, Sweden); Human Gamma Globulin, Sigma; Charcoal Norit A, Serva (Heidelberg, Germany); OPTI-Fluor, Packard (Dreieich, Germany). Borate buffer (50 m*M*) was prepared by dissolving 3.092 g boric acid p.a. (Merck) and 3.728 g KCl p.a. (Merck) in a final volume of 1000 ml of bidistilled water, after adjusting the pH to 7.8 with approximately 39 ml of 0.1 *M* NaOH. Gamma globulin buffer (0.06%, w/v) was prepared by dissolving 600 mg gamma globulin plus 325 mg sodium azide (Merck) in a final volume of 500 ml of the borate buffer. It was stored at 4°C for up to 3 months. Dextran (0.25 g) and 2.5 g Norit A were mixed with gamma globulin buffer to a final volume of 100 ml.

2.1.3. Instruments

Standard borosilicate glass columns (750×90 mm) were packed uniformly with Sephadex LH-20 suspended in a solvent of methylene chloride–methanol (98:2, v/v). For elution of the 10 parallel Sephadex LH-20 columns, a 10-channel pulsation free, double-piston precision pump (pmp 10/10, Ismatec, Zurich, Switzerland) was used in the reversed flow mode. For tubing, connectors and fittings, long-term solvent-resistant materials (PTFE) were used. Fractions were collected automatically according to preset elution limits by a gravimetric 10-channel linear fraction collector (Bender & Hobein, Munich, Germany) controlled by a programmed 10-channel processor. All glassware used was made steroid free by previous heating to 500°C for at least 3–5 h as previously described [19]. Hamilton syringes (1 ml, Eydam, Kiel, Germany) were used for plasma injection. Disposable 12×55 mm round-bottom polystyrene tubes were used for the RIA (Sarstedt, Nümbrecht, Langenfeld, Germany). Incubations

were carried out in changeable tube racks each holding 90 RIA tubes in a rotary water bath (Julabo, Seelbach, Germany). For centrifugations, a refrigerated Cryofuge C-4 laboratory centrifuge (Heraeus, Osterrode, Germany) with a swing-out head was used. Radioactivity was counted in a LSC TRI-CARB 2300 TR liquid scintillation spectrometer (Packard, Dreieich, Germany) with an efficiency of 65% for tritium.

2.2. Methods

2.2.1. Extraction of plasma samples

To 0.1–1.0 ml of peripheral plasma, 1500 cpm of Δ5[7(n)-^{3}H] pregnenolone and 17α-hydroxy[7(n)-^{3}H] pregnenolone dissolved in 100 μl of gamma globulin buffer were added and incubated at 4°C for 1–2 h. The steroid concentration per milliliter was recalculated from the used plasma volume after the RIA. After thorough mixing the plasma was extracted twice with 5 ml of ice-cold methylene chloride and then the extract washed once with 3 ml of sterile distilled water. After gentle centrifugation (2000 $g\times$20 min) at 4°C, separation of the upper (aqueous) from the lower (methylene chloride) phase was performed by suction followed by freezing at −20°C overnight. The final extracts were then evaporated to dryness under a gentle stream of nitrogen at 37°C.

2.2.2. Automated Sephadex LH20 multi-column chromatography

An automated Sephadex LH20 chromatography system using ten 750×90 mm columns running in parallel was used for steroid separation as described in detail elsewhere [20,23]. Briefly, plasma extracts were redissolved in 300 μl of the solvent system and then injected using Hamilton syringes into each of 10 parallel Sephadex LH20 packed chromatography columns. Elution was performed against gravity by a central precision pump at a constant flow of 40 ml/h. Preg and 17OH-Preg fractions were collected automatically from 49 to 57 and from 86 to 98 ml, respectively. Thus, a complete separation of Preg and 17OH-Preg could be obtained (Fig. 1).

The Preg and 17OH-Preg fractions were evaporated to dryness and redissolved in 2.0 ml of absolute ethanol (15°C). They were then divided into two different aliquots at a constant temperature of 15°C. For determination of internal tracer recovery, one aliquot (400 μl) was transferred into scintillation vials containing 9 ml counting fluid (OPTI-Fluor). After thorough mixing, vials were allowed to stand for at least 10 min before counting radioactivity up to 10 000 counts. The other aliquot was used in duplicate for steroid quantification by RIA.

2.2.3. Steroid radioimmunoassay (RIA)

Of the 2.0 ml ethanolic Preg and 17OH-Preg fractions, 400 μl (=20%) was taken for recovery and 750 μl (=37.5%) in duplicate for RIA. Standards were prepared in duplicate (zero samples in quadruplicate) by evaporating 0, 32, 79, 158, 316, 790, 1580, 3160 and 9489 pmol of Preg and 0, 30, 75, 150, 301, 752, 1503, 3006 and 9018 pmol of 17OH-Preg in ethanolic solution using a vacuum oven at 50°C.

To unknowns and standards, 100 μl of gamma globulin buffer, containing 6000 cpm of radioactive steroids and 500 μl antiserum to the steroids were added. The Preg antiserum (AK39a/1/12) was raised in rabbits after immunisation with pregnenolone-20-oxime linked covalently to bovine serum albumin (BSA). The 17OH-Preg antiserum (AK50a/1/11) was raised in rabbits after immunisation with 17-hydroxypregnenolone-20-oxime linked covalently to BSA. Both antibodies were kindly supplied by Prof. P. Vecsei (Heidelberg, Germany).

All tubes were incubated with gentle shaking at 37°C for 30 min and then allowed to stand in an ice bath for 2 h. Then 100 μl of stirred dextran-coated charcoal suspension was added to each tube. After horizontal shaking for exactly 15 s, the tube rack was replaced in the ice bath and bound and free fractions were separated exactly 10 min later by centrifugation (2000 $g\times$15 min) at 4°C. The supernatants (containing the bound fraction) were decanted directly into scintillation vials containing 9 ml counting fluid (OPTI-Fluor). The scintillation vials were allowed to stand for 10 min. Radioactivity was then counted.

Standard curves were constructed by computer, using a modified Gaussian regression of third order for reciprocal standard values [23]. Unknown sample

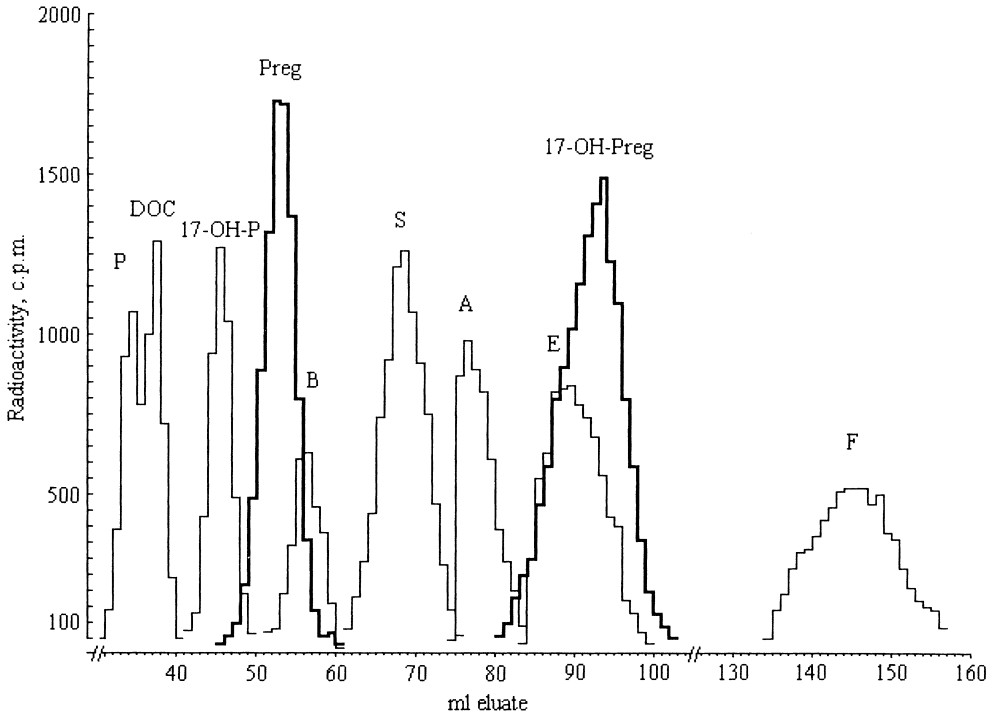

Fig. 1. Chromatogram of tritiated steroids eluted from mechanized 75-cm Sephadex LH-20 columns using 40 ml/h of methylene chloride–methanol (98:2) as solvent. Peaks eluted: (P) progesterone, (DOC) 11-deoxycorticosterone, (17-OH-P) 17-hydroxyprogesterone, (Preg) pregnenolone, (B) corticosterone, (S) 11-deoxycortisol, (A) aldosterone, (E) cortisone, (17-OH-Preg) 17-hydroxypregnenolone and (F) cortisol.

concentrations were then obtained using the parameters of the calculated standard curve.

3. Results

3.1. Recovery

The average recovery of radioactive steroids added to plasma was 67.6±11.3% (mean±SD, n=50) for Preg and 67.8±13.5% (mean±SD, n=50) for 17OH-Preg after extraction and automated Sephadex LH-20 chromatography.

3.2. Evaluation of the method

3.2.1. Standard curves

In Table 1, the mean displacement of the labelled steroid from the antibody by known amounts of added steroid is expressed as the percentage of radioactivity of the zero samples in which no unlabelled steroid was present. The data are listed as the mean percentage of bound radioactivity±SD together with the coefficients of variation of duplicate standards from six typical standard curves.

3.2.2. Specificity

Percent cross-reactions, calculated according to Abraham, between the antisera used for each RIA and major plasma steroids are listed in Table 2 [22]. Preg was eluted on the 75-cm columns from 49 to 57 ml. Steroid cross-reactions within this chromatographic fraction were observed in the case of 17-hydroxyprogesterone and corticosterone, which were eluted to 5 and 20% together with Preg, respectively. The cross-reactions of the antiserum used in the Preg RIA with 17-hydroxyprogesteron and corticosterone were 0.04 and 0.02%, respectively.

17OH-Preg was eluted on the 75-cm columns from 86 to 98 ml. This resulted in an overlapping of

Table 1
Standard curves for pregnenolone and 17-hydroxypregnenolone; mean values±SD and coefficients of variation ($n=6$)

	Amount of steroid added (pmol)	% of bound radio-activity	±SD	Coefficient of variation
Pregnenolone	0	100.00	6.86	4.28
	32	85.72	5.98	4.08
	79	77.59	6.32	4.19
	158	72.52	5.57	3.78
	316	66.53	6.02	4.15
	790	50.13	5.16	3.53
	1580	33.28	3.34	3.09
	3160	21.53	2.60	2.59
	9489	11.23	1.35	1.31
17-Hydroxy-pregnenolone	0	100.00	10.62	5.83
	30	95.27	9.72	5.53
	75	88.79	11.64	6.20
	150	78.99	9.51	5.24
	301	73.04	8.31	4.02
	752	60.58	6.86	3.59
	1503	44.09	4.60	2.42
	3006	28.73	2.44	1.98
	9018	13.96	1.81	1.19

the peak by about 5% with aldosterone and by 75% with cortisone. Cross-reactions with these steroids were 0.02 and 0.04%, respectively. The overall cross-reactivity did not exceed 0.04%. Table 2 also includes the antiserum titre used in the RIA at which approximately 50% of the labelled steroids are bound.

Table 2
Steroid cross-reactions (%) with antisera used in radioimmunoassays

	Anti-pregnenolone	Anti-17OH-pregnenolone
Pregnenolone	100.00	12.30
17OH-Pregnenolone	3.45	100.00
Progesterone	0.21	1.07
11-Deoxycorticosterone	2.10	0.60
17OH-Progesterone	0.04	4.68
Corticosterone	0.02	0.02
11-Deoxycortisol	0.30	33.20
Aldosterone	0.02	0.02
Cortisone	0.02	0.04
Cortisol	0.02	0.06
Testosterone	0.50	0.09
Androstenedione	0.30	0.05
Titer used	1:25 000	1:45 000

3.2.3. Lower detection limits and blanks

The lower detection limit of the standard curve is by convention defined as the smallest amount of steroid standard that is significantly different from zero at the 95% confidence limit [22]. As shown in Table 3, these lower detection limits were 39.5 pmol for Preg and 70.5 pmol for 17OH-Preg.

One milliliter water blanks, when carried through the entire procedure, were found to be below the lower detection limit of the standard curve, i.e. they were undetectable. The lower detection limits of the assay (Table 3) were therefore calculated from the lower detection limits of the standard curves using the mean percent recovery and the fraction of each recovered steroid used in the RIA.

3.2.4. Precision

To evaluate the precision of our method, the steroid content of a total of 10 plasma aliquots from a plasma pool of healthy pregnant women was measured individually in eight different runs each including six plasma samples carried in parallel through extraction, chromatography and RIA. Coefficients of variation within and between complete assays were calculated for Preg and 17OH-Preg. Intra-assay coefficients of variation and inter-assay

Table 3
Lower detection limits and blank values

Steroid	Lower detection limit of the standard curve (pmol)	1 ml water blanks (mean±SD) (pmol)	Lower detection limit of the assay (nmol/l)
Pregnenolone	39.5	0.47±0.82	0.15
17OH-Pregnenolone	70.5	0.06±0.09	0.28

coefficients of variation at a mean concentration of 3.76 nmol/l were 8.4 and 11.6% for Preg and 7.0 and 10.9% for 17OH-Preg, respectively.

3.2.5. Accuracy

The accuracy of the assay was examined by replicate analyses of increasing amounts of steroid added in physiological concentration to 1 ml of steroid-free plasma, which was carried through the entire procedure. Throughout the concentration ranges examined, there was a linear relationship with coefficients of correlation of 0.9978 for Preg and 0.9989 for 17OH-Preg (Fig. 2). Accuracy was further tested by measuring the steroid content of external quality control samples (RSL medium HB23, DRG Instruments, Marburg, Germany) with defined amounts of steroids in physiological concentrations. The given mean±SD (range) concentration for Preg and 17OH-Preg in these samples was 2.31±0.32 nmol/l (1.71–3.48) and 1.83±0.39 nmol/l (1.23–2.56), respectively. The quality control samples were carried through the entire procedure as for the unknown plasma samples. All measurements found steroid concentrations within the given range (Table 4).

3.2.6. Practicability

Since many steps in our procedure have been mechanised, e.g. automation of evaporation after extraction, the Sephadex LH-20 column chromatography [21,23] for separation of the steroids and standardised RIA procedures, one technician is able to determine Preg and 17OH-Preg concentrations in a total of 50 samples during five working days.

3.3. Normal values

The mean values, standard deviations and ranges for basal 9–10 a.m. plasma levels in 10 young healthy male adults (age 20–26 years), in 13 adult

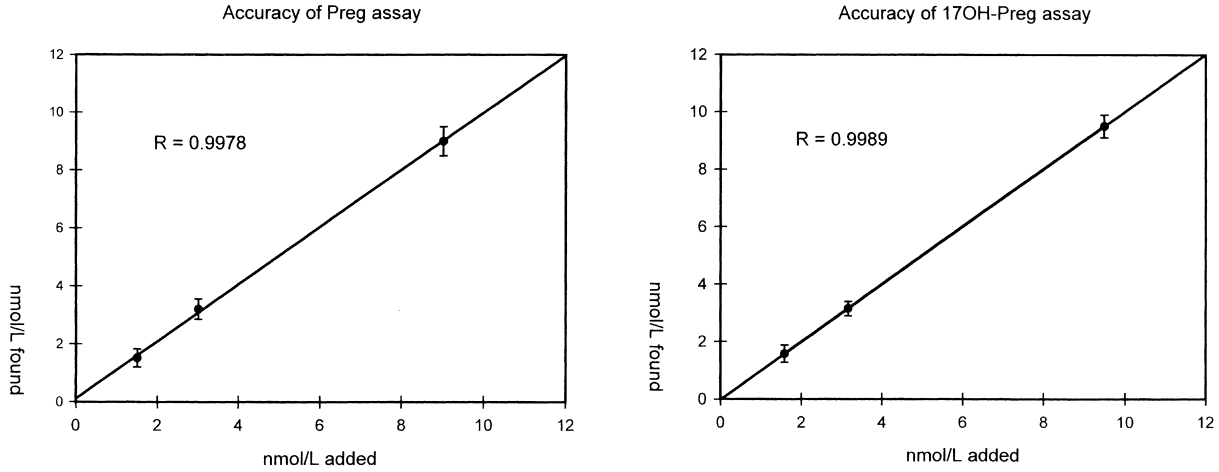

Fig. 2. Accuracy of steroid assays as determined by replicate analyses ($n=10$) of increasing amounts of steroid added to 1 ml steroid-free plasma (mean±SD).

Table 4
Accuracy of pregnenolone and 17OH-pregnenolone assay ($n=13$)[a]

Steroid	Known plasma conc. (ng/ml)	Measured plasma conc. (ng/ml)	Coefficient of variation
Pregnenolone	0.73±0.10 (0.54–1.10)	0.84±0.16 (0.61–1.09)	6.57
17OH-Pregnenolone	0.61±0.13 (0.41–0.85)	0.64±0.17 (0.42–0.82)	3.85

[a] Steroid levels as mean±SD with range in parentheses.

healthy females (age 28–40 years) during the follicular phase of the menstrual cycle, in 12 prepubertal boys (age 4–12 years) and in eight prepubertal girls (age 4–12 years) are summarised in Table 5.

All subjects were on a random diet and had no signs of endocrine or systemic diseases. Peripheral venous blood was drawn by rapid venipuncture in the supine (children) or sitting (adults) position only a few seconds after the subjects' normal upright activity. Samples from subjects showing signs of stress (e.g. crying children) were not included.

Significant differences of Preg and 17OH-Preg levels between males, female subjects or children were not observed. Sex differences for Preg and 17OH-Preg in the group of prepubertal children were not seen. The plasma levels for children are therefore shown under one column (Table 5).

4. Discussion

A system for the simultaneous measurement of plasma Preg and 17OH-Preg has been demonstrated. Possible interference with water-soluble components was avoided by a methylene chloride extraction. Liquid gel column chromatography on Sephadex LH-20 with a solvent system of low polarity used in this study separates plasma steroids according to their polarity. Preg and 17OH-Preg can be completely and simultaneously separated from each other in our system, despite their very similar polarity and molecular size. Chromatography is a necessary step to avoid overestimations of Preg and 17OH-Preg due to cross-reactions of the antibodies with other extracted steroids. Preg was eluted to 5 and 20% with 17-hydroxyprogesterone and corticosterone, respectively. Overestimation of Preg levels can be ignored because of the minimal cross-reaction of the antiserum for Preg, 17OH-progesterone and corticosterone. 17OH-Preg eluted with aldosterone and cortisone in significant amounts. However, the overestimation is negligible because of the small cross-reactivity of the antisera for these three steroids. As the antisera showed a very high specificity, addition of an excess of cross-reactant as mentioned by Hill et al. was not necessary [24].

Our basal values for Preg in females in the follicular phase and prepubertal children are comparable to those in the few existing reports [4,5]. As other authors have reported, sex differences in prepubertal children (age 4–12 years) could not be found. In agreement with Nishida et al., we did not find differences in Preg levels in adult males compared to females [6]. Along with the report of Apter

Table 5
Normal 9–10 a.m. plasma levels (nmol/l) determined simultaneously[a]

	Males ($n=10$)	Females (follicular phase) ($n=13$)	Children (prepubertal) ($n=20$)
Pregnenolone	7.87±4.04 (1.55–12.73)	3.82±2.72 (1.17–10.49)	2.84±1.74 (0.28–7.84)
17OH-Pregnenolone	8.09±4.63 (2.04–15.30)	3.82±2.98 (1.08–11.72)	3.67±1.77 (1.20–7.21)

[a] Steroid levels as mean±SD with range in parentheses.

et al., our values for plasma Preg in male adults are somewhat higher than those of other authors [7]. This is probably due to the relatively young age of our adult male group.

The plasma levels of 17OH-Preg are comparable to those of McKenna et al., Shimozawa et al. and Lashansky et al. [8–10]. As for Preg, sex differences in prepubertal children (age 4–12 years) could not be found. Plasma 17OH-Preg levels of adult males and adult females in the follicular phase were comparable. The male levels were also among the highest reported. Because of the wide range, there seems to be a high inter-individual variation.

In general, the quality and reliability of the assay described in this paper as expressed by specificity, precision, accuracy and practicability are comparable to or superior to other studies [9,10]. The combination of the assay with our automated multi-column chromatography system, which in our hands has proven its reliability for over 25 years, offers a very rapid and economic approach [23]. The advantages of the technique over other chromatographic procedures such as paper and thin-layer chromatography are well established and have been described in detail by several workers [25–27]. GC–MS methods for Preg are available but not established for clinical routine in humans [12–14]. To our knowledge there is no GC–MS system for 17OH-Preg available. Non-extractive steroid assay methods using competitive protein-binding assays should not be used, particularly in the perinatal period, during infancy and under pathological conditions, as they are less specific and sensitive than our procedure [28,29]. Direct RIAs lead to overestimation of steroid levels, as already shown for 17OH-progesterone in neonates [15–18]. The measured plasma levels of Preg and 17OH-Preg in our system are comparable to reported values.

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References

[1] Y. Nishi, T. Tezuka, Eur. J. Pediatr. 151 (1992) 19.
[2] C. Moran, H.D. Potter, R. Reyna, L.R. Boots, R. Azziz, Am. J. Obstet. Gynecol. 181 (1999) 596.
[3] A.M. Moisan, M.L. Ricketts, V. Tardy, M. Desrochers, F. Mebarki, J.L. Chaussain, S. Cabrol, M.C. Raux-Demay, M.G. Forest, W.G. Sippell, M. Peter, Y. Morel, J. Simard, J. Clin. Endocrinol. Metab. 84 (1999) 4410.
[4] G.E. Abraham, J.E. Buster, F.W. Kyle, P.C. Corrales, R.C. Teller, J. Clin. Endocrinol. Metab. 37 (1973) 140.
[5] D. Apter, R. Vihko, J. Clin. Endocrinol. Metab. 45 (1977) 1039.
[6] S. Nishida, S. Matsumura, M. Horino, H. Oyama, A. Tenku, Kawasaki Med. J. 3 (1977) 219.
[7] D. Apter, O. Jänne, P. Karvonen, R. Vihko, Clin. Chem. 22 (1976) 32.
[8] T.J. McKenna, D.L. DiPietro, R.D. Brown, C.A. Strott, G.W. Liddle, J. Clin. Endocrinol. Metab. 39 (1974) 833.
[9] K. Shimozawa, S. Saisho, J. Yata, A. Kambegawa, Endocrinol. Jpn. 35 (1988) 189.
[10] G. Lashansky, P. Saenger, K. Fishman, T. Gautier, D. Mayes, G. Berg, J. Di Martino-Nardi, E. Reiter, J. Clin. Endocrinol. Metab. 73 (1991) 674.
[11] C.A. Strott, J.A. Bermudez, M.B. Lipsett, J. Clin. Invest. 49 (1970) 1999.
[12] P. Liere, Y. Akwa, S. Weill-Engerer, B. Eychenne, A. Pianos, P. Robel, J. Sjovall, M. Schumacher, E.E. Baulieu, J. Chromatogr. B 739 (2000) 301.
[13] K. Shimada, K. Yago, J. Chromatogr. Sci. 38 (2000) 6.
[14] M.H. Choi, Y.S. Yoo, B.C. Chung, J. Chromatogr. B 745 (2001) 495.
[15] S.K. Makela, G. Ellis, Clin. Chem. 34 (1988) 2070.
[16] T. Wong, C.H. Shackleton, T.R. Covey, G. Ellis, Clin. Chem. 38 (1992) 1830.
[17] M.S. Lo, M.L. Ng, L.L. Wu, B.S. Azmy, B.A. Khalid, Malays. J. Pathol. 18 (1996) 43.
[18] S. al Saedi, H. Dean, W. Dent, E. Stockl, C. Cronin, Pediatr. 97 (1996) 100.
[19] D. Knorr, Z. Klin. Chem. Klin. Biochem. 9 (1971) 175.
[20] B.E.P. Murphy, Nat. New Biol. 232 (1971) 21.
[21] W.G. Sippell, P. Lehmann, G. Hollmann, J. Chromatogr. 108 (1975) 305.
[22] G.E. Abraham, Acta Endocrinol. Suppl. 183 (1974) 7.
[23] W.G. Sippell, F. Bidlingmaier, H. Becker, T. Brünig, H. Dörr, H. Hahn, W. Golder, G. Hollmann, D. Knorr, J. Steroid Biochem. 9 (1978) 63.
[24] M. Hill, R. Hampl, D. Lukac, O. Lapcik, V. Pouzar, J. Sulcova, Steroids 64 (1999) 341.
[25] B.E.P. Murphy, Acta Endocrinol. Suppl. 147 (1970) 37.
[26] B.H. Shapiro, F.G. Peron, J. Chromatogr. 65 (1972) 568.
[27] T. Ito, J. Woo, R. Haning, R. Horton, J. Clin. Endocrinol. 34 (1972) 106.
[28] D.L. Loriaux, M.B. Lipsett, Steroids 19 (1972) 681.
[29] G.E. Abraham, P.C. Corrales, R.C. Teller, Anal. Lett. 5 (1972) 915.