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Chromatographic system for the simultaneous measurement of plasma 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone by radioimmunoassay: reference data for neonates and infants and its application in aldosterone-synthase deficiency

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Abstract

A new chromatographic system for the steroid precursor separation and a sensitive radioimmunoassay system for the subsequent measurement of 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone has been developed. 18-Hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone were extracted with methylene chloride and separated from cross-reacting steroids by Sephadex LH-20 column chromatography. Anti-18-hydroxy-11-deoxycorticosterone and anti-18-hydroxycorticosterone antibodies raised in rabbits were used. The lower detection limit of the assay is 0.03 nmol/l and 0.128 nmol/l for 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone, respectively. Normal values for this assay in 128 healthy neonates and infants aged 0–5 months were established as a basis for the early hormonal diagnosis of aldosterone synthase deficiency types I and II. Its application for the diagnosis of aldosterone synthase deficiency is demonstrated in two patients with homozygous mutation/deletion in the encoding *CYP11B2* gene.

Keywords: Aldosterone-synthase deficiency; 18-Hydroxy-11-deoxycorticosterone; 18-Hydroxycorticosterone

1. Introduction

Aldosterone is the most important mineralocorticoid of the human adrenal gland. Aldosterone is synthesised from 11-deoxycorticosterone (DOC) in the mitochondria of zona glomerulosa cells. The biosynthesis of aldosterone requires 11β-hydroxylation of DOC for conversion to corticosterone (B) followed by hydroxylation at position C_{18} to form 18-hydroxycorticosterone (18OH-B). Finally, oxidation at position C_{18} is required for conversion to aldosterone. The last two steps of the biosynthetic pathway are catalyzed by a single cytochrome P450 enzyme (P450aldo) with both hydroxylating and oxidative activity. The gene encoding this enzyme is called *CYP11B2* and was isolated by Mornet and

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White [1] and further characterized by Kawamoto et al. [2]. 18-Hydroxy-11-deoxycorticosterone (18OH-DOC) is synthesized in very small amounts under physiologic conditions from DOC by P450aldo in the adrenal zona glomerulosa [3].

The diagnosis of a selective aldosterone deficiency was established by improving laboratory methods using urinary steroid metabolite determinations as early as the 1960s [4,5]. Visser and Cost were the first to suggest an 18-oxidation defect [6]. Aldosterone deficiency is an autosomal inherited disorder caused by various mutations of the *CYP11B2* gene (see OMIM 124080). Based on biochemical findings, aldosterone synthase deficiency can be categorised in two types, type I with a lack of 18-hydroxylase activity and type II with a lack of 18-oxidase activity [7]. All affected children present with frequent vomiting, failure to thrive, and severe, life-threatening salt loss in the first weeks of life. The clinical spectrum of aldosterone synthase deficiency was reviewed recently [8–11].

The measurement of plasma 18OH-B is performed in a small number of specialised endocrinological laboratories. The determination of plasma 18OH-DOC is not a standard procedure. Reference data for 18OH-B were reported in one group of children for the first year of life [12]. To our knowledge, to date no data are available describing the postnatal course of 18OH-DOC and 18OH-B in newborns and infants over the first months of life. As it can be postulated that, as with all other adrenal steroids, 18OH-DOC and 18OH-B show a significant decline from birth to early infancy, normative data are crucial for the early diagnosis of aldosterone synthase deficiency in this age range. We therefore developed a chromatographic system for the separation and a sensitive RIA system for the measurement of plasma 18OH-DOC and 18OH-B. Reference values for this method in young newborns and infants (range 0–5 months) were established and their applicability was tested in two patients with genetically confirmed aldosterone synthase deficiency.

2. Experimental data

2.1. Materials

2.1.1. Radioactive steroids

18-Hydroxy-11-deoxy-[1,2-^{3}H]corticosterone (TRQ 2405) with a specific radioactivity of 30 Ci/mmol and 18-hydroxy[^{3}H]corticosterone (TRQ 9001) with a specific radioactivity of 33 Ci/mmol were purchased from AEA Technology QSA (Otterfing, Germany). The radiochemical purity was 98%. 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 acetone p.a. (Merck) were used without further purification. Non-labelled steroids were obtained from Makor Chemical (Jerusalem, Israel). Suppliers of the following chemicals were: Sephadex LH-20, Dextran T70: Pharmacia+Upjohn (Uppsala, Sweden); human gamma globulin: Sigma (Munich, Germany); Charcoal Norit A: Serva (Heidelberg, Germany); OPTI-Fluor: Packard (Dreieich, Germany); Freund's adjuvant: BD Diagnostic Systems (Sparks, MD, USA). 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 pH to 7.8 with ~39 ml of 0.1 *M* NaOH. Gamma globulin buffer (0.06%, w/v) was made 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 (450×10 mm) were packed uniformly with Sephadex LH-20 suspended in methylene chloride–acetone (1:2, v/v). Long-term solvent resistant materials (PTFE) were used for tubing, connectors and fittings. Fractions were collected in calibrated 10-ml glass tubes (Assistant, Sondheim, Germany). All glassware used was rendered steroid-free by heating to 500 °C for at least 3–5 h as previously described [13]. Disposable 12×55 mm round bottom polystyrene tubes were used for the RIA (Sarstedt, Nümbrecht, Germany). Incubations were carried out in changeable tube-racks each holding 90 RIA-tubes in a rotary water bath (Julabo, Seelbach, Germany). A refrigerated Cryofuge C-4 lab centrifuge (Heraeus, Osterrode, Germany) with a swing-out head was used for

centrifugation. Radioactivity was counted in an LSC TRI-CARB 2300 TR liquid scintillation spectrometer (Packard) with an efficiency of 65% for tritium.

2.2. Methods

2.2.1. Extraction of plasma samples

18-Hydroxy-11-deoxy-[1,2-^{3}H]corticosterone and 18-hydroxy[^{3}H]corticosterone (1500 cpm) dissolved in 100 μl of gamma globulin buffer were added to 0.2–0.5 ml of peripheral plasma and incubated at 4 °C for 1–2 h. The steroid concentration per ml 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 the extract was then washed once with 3 ml of sterile distilled water. After gentle centrifugation (2000 $g\times20$ 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. Sephadex LH-20 chromatography

A Sephadex LH-20 chromatography system with 450×10 mm columns was used for steroid separation. Plasma extracts were redissolved in 1000 μl of the solvent system and injected into Sephadex LH-20 packed chromatography columns. Elution was performed following gravity. 18OH-DOC and 18OH-B fractions were collected from 16–21 ml and 23–33 ml, respectively. Thus, 18OH-DOC and 18OH-B were completely separated from each other (Fig. 1).

The 18OH-DOC and 18OH-B fractions were evaporated to dryness and redissolved in 2.0 ml 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.

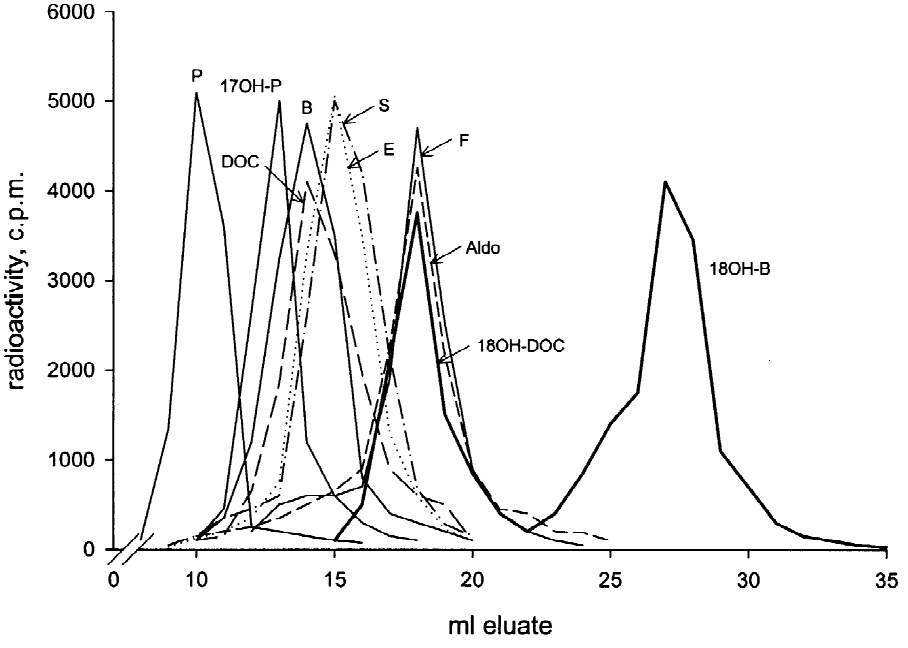

Fig. 1. Chromatogram of tritiated steroids eluted from 45-cm Sephadex LH-20 columns using methylene chloride–acetone (1:2, v/v) as solvent. Peaks eluted: P, progesterone; 17-OH-P, 17-hydroxyprogesterone; B, corticosterone; DOC, 11-deoxycorticosterone; E, cortisone; S, 11-deoxycortisol; F, cortisol; Aldo, aldosterone; 18OH-DOC, 18OH-deoxycorticosterone; 18OH-B, 18OH-corticosterone.

2.2.3. Steroid radioimmunoassay (RIA)

Of the 2.0 ml ethanolic 18OH-DOC and 18OH-B fractions, 400 μl (20%) were taken for recovery and 750 μl (37.5%) in duplicate for RIA. Standards were prepared in duplicate (zero samples in quadruplicate) with 29, 72, 144, 289, 722, 1443, 2886 and 8658 pmol 18OH-DOC and 28, 69, 138, 276, 690, 1380, 2759 and 8277 pmol 18OH-B in ethanolic solution.

One hundred μl of gamma globulin buffer, containing 6000 cpm of radioactive steroids and 500 μl antiserum to the steroids were added to unknowns and standards. The 18OH-DOC antiserum and 18OH-B antiserum were raised in rabbits after immunisation with 18OH-DOC-20-oxime and 18-OH-B-3-oxime linked covalently to bovine serum albumin. Female New Zealand albino rabbits ($n=5$) were immunized i.m. at intervals of 4 weeks with 1 mg of the immunogen dissolved in 0.5 ml saline. Complete Freund's adjuvant (0.5 ml) was used with the first (priming) antigenic dose. Ten to 14 days after booster injections, blood samples were taken to determine titres. Final antibody titres used were 1:5000 for 18OH-DOC and 1:3000 for 18OH-B. Both antibodies were kindly supplied by Prof. P. Vecsei, Heidelberg, Germany.

All tubes were incubated under gentle shaking at 37 °C for 30 min and then allowed to stand in the ice bath for 2 h. One hundred μl of stirred dextran-coated charcoal suspension were then added to each tube. After horizontal shaking for 15 s, the tube rack was replaced into the ice bath and bound fractions were separated from free fractions 10 min later by centrifugation (2000 $g\times15$ 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 after which radioactivity was counted.

Standard curves were constructed by computer, using a modified Gaussian regression of the third order for reciprocal standard values [14]. Unknown sample concentrations were then obtained by using the parameters of the calculated standard curve.

2.2.4. Multisteroid hormone measurements, DNA preparation and CYP11B2 sequencing

Hormone measurements by multisteroid analysis (progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17-hydroxyprogesterone, 11-deoxycortisol, cortisol) were performed as previously described [14]. Genomic DNA preparation was carried out from peripheral venous blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). *CYP11B2* amplification and sequencing was performed as previously described [15,16].

2.2.5. Normal values

Peripheral venous blood was drawn from 128 healthy neonates and infants (age 1 day–5 months) by rapid venipuncture in the supine position. Left-over samples from routine blood checks were used, after informed consent by the parents, in order to set up normative data for 18OH-DOC and 18OH-B. All subjects were breast-fed or on a standard formula and had no signs of endocrine or systemic diseases. Samples from subjects showing signs of stress were not included. Kruskal–Wallis one-way analysis of variance on ranks and Dunn's test as post hoc procedure were used for statistic comparisons between different age groups. Results are expressed in nmol/l; to convert to ng/ml divide by the following factors: aldosterone, 2.774; 18OH-DOC, 2.886; DOC, 3.026; 18OH-B, 2.759; B, 2.886.

2.2.6. Patients

Patient 1 is a girl born after an uneventful pregnancy. She presented with failure to thrive and mild dehydration at the age of 4 months. Plasma sodium was decreased (125 m*M*). Potassium (6.5 m*M*) and plasma renin activity (46 ng/ml/h) were markedly elevated. Patient 2's medical history has been reported previously [16]. In brief, she showed signs of salt loss at the age of 6 weeks with decreased plasma sodium, increased plasma potassium and increased plasma renin activity. Both patients thrived well following initiation of fludrocortisone treatment. Plasma samples were taken at an age of 4 months after a 5-day discontinuation of fludrocortisone treatment during close in-patient monitoring.

3. Results

3.1. Recovery

The average recovery of radioactive steroids added

Table 1
Standard curves for 18OH-DOC and 18OH-B; mean values±SD and coefficients of variation (n=6)

	Amount of steroid added (pmol)	% of bound radioactivity	±SD	Coefficients of variation
18-Hydroxydeoxycorticosterone	0	100.0	1.10	1.10
	29	99.4	0.32	0.32
	72	97.1	1.55	1.60
	144	93.9	2.44	2.60
	289	88.4	0.72	0.81
	722	63.6	2.64	4.15
	1443	49.1	1.26	2.56
	2886	32.7	0.02	0.06
	8658	19.7	0.56	2.85
18-Hydroxycorticosterone	0	100.0	2.16	2.16
	28	94.5	4.70	4.97
	69	92.7	3.51	3.79
	138	85.0	3.24	3.81
	276	78.2	1.15	1.47
	690	59.3	2.61	4.40
	1380	43.8	3.20	5.95
	2759	32.7	1.05	3.21
	8277	28.5	1.33	4.67

to plasma was 68.1±6.0% (mean±SD, n=40) for 18OH-DOC and 61.9±6.3% (mean±SD, n=30) for 18OH-B after extraction and 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 figures are listed as mean percentage of bound radioactivity±SD together with the coefficients of variation of the duplicate standards from six typical standard curves.

3.2.2. Specificity

Steroid cross-reactions, calculated according to Abraham between the antisera used for each RIA and major plasma steroids are listed in Table 2 [17]. 18OH-DOC was eluted on the 45-cm columns from 16 to 21 ml. Steroid cross-reactions within this chromatographic fraction were estimated in the case of 11-deoxycortisol, cortisol, cortisone, 11-deoxycorticosterone, corticosterone and aldosterone which

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

	Anti-18-Hydroxy-deoxycorticosterone	Anti-18-Hydroxycorticosterone
18-Hydroxydeoxycorticosterone	100.00	n.a.
18-Hydroxycorticosterone	n.a.	100.00
11-Deoxycorticosterone	2.32	26.0
Corticosterone	0.31	19.5
11-Deoxycortisol	0.48	3.25
Aldosterone	1.05	2.89
Cortisone	0.03	1.34
Cortisol	0.009	1.44
Titer used	1:5000	1:3000

n.a., not assessed.

Table 3
Lower detection limits

Steroid	Lower detection limit of the standard curve (pmol, mean±SD)	Lower detection limit of the assay (nmol, mean±SD)
18-Hydroxy-deoxycorticosterone	20.4±0.99	0.04±0.002
18-Hydroxycorticosterone	79.2±1.55	0.15±0.003

were eluted to ~33%, 100%, 33%, 40%, 20% and 100% together with 18OH-DOC, respectively. The cross-reactions of the antiserum used in the 18OH-DOC RIA with adrenal steroid hormone precursors are given in Table 2. Table 2 also includes the antiserum titre used in the RIA, at which ~50% of the labelled steroids were bound. 18OH-B was eluted on the 45-cm columns from 23 to 33 ml. This resulted in no overlapping of the peak with any other steroid analysed.

3.2.3. Lower detection limits and blanks

The lower detection limit of the standard curve is defined by convention as the smallest amount of steroid standard that is significantly different from zero at the 95% confidence limit [17]. These lower detection limits are shown in Table 3. When carried through the entire procedure, 1-ml water blanks were found to be below the lower detection limit of the standard curve, i.e. they were undetectable (data not shown). The lower detection limits of the assay 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 (Table 3).

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 men 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 18OH-DOC and 18OH-B. Intra- and inter-assay coefficients of variation were 5.03% and 9.04% for 18OH-DOC (mean concentration 1.48 nmol/l) and 5.53% and 9.56% for 18OH-B (mean concentration 4.34 nmol/l), respectively.

3.2.5. Accuracy

The accuracy of the assay was examined by replicate analysis of increasing amounts of steroid added in physiological concentration to 1 ml of steroid-free plasma. There was a linear relationship with coefficients of correlation of 0.982 for 18OH-

Table 4
Accuracy of 18OH-DOC and 18OH-B assays as determined by replicate analysis ($n=10$) of increasing amounts of steroid added to steroid-free plasma (mean±SD)

Added steroid (nmol/l)	Measured steroid 18OH-DOC (nmol/l, mean±SD)	Measured steroid 18OH-B (nmol/l, mean±SD)
0.75	0.73±0.25	–
3.0	2.98±0.13	3.00±0.18
14.0	13.93±0.15	14.03±0.18
28.0	–	27.87±0.35
Linear equation	$y = 0.996x - 0.0117$	$y = 0.994x + 0.0511$
R^2	0.98	0.99

Table 5
Normal 08:00–09:00 h plasma levels (nmol/l) determined simultaneously

nmol/l	Day 1 $n=29$	Day 2–3 $n=20$	Day 4–7 $n=21$	Day 8–14 $n=13$	Day 15–30 $n=11$	2 months $n=18$	3–5 months $n=14$
18OH-DOC	2.02±1.17 (0.86–6.14)	3.27±1.20 (1.76–4.41)	1.35±1.19 (0.11–3.14)	2.19±0.92 (0.86–3.29)	1.47±0.74 (0.54–2.51)	1.29±0.75 (0.34–3.26)	1.18±0.74 (0.05–2.30)
18OH-B	14.60±5.98[a,b] (4.60–28.69)	18.79±10.29[c,d] (7.44–40.00)	8.74±5.38 (3.86–21.13)	9.34±5.51 (3.44–22.43)	8.08±4.74 (4.58–16.03)	5.84±1.56[a,c] (3.17–7.86)	5.20±2.05[b,d] (3.14–10.01)

Steroid levels as mean±SD with range in parentheses. [a,b,c,d]Matching letters indicate significant differences in steroid levels between age groups; $P<0.05$.

DOC and 0.987 for 18OH-B throughout the concentration ranges examined (Table 4).

3.2.6. Practicability

The Sephadex LH-20 column chromatography for separation of the steroids and the standardised RIA procedure allows one technician to determine 18OH-DOC and 18OH-B concentrations in a total of eight plasma samples during 3 working days using 10 chromatography columns in parallel. The actual working time is ~12 h.

3.3. Normal values

The mean values, standard deviations and ranges for basal 08:00–09:00 h plasma levels in 128 healthy neonates and infants (age 1 day–5 months) are summarised in Table 5. Significant differences of 18OH-DOC and 18OH-B levels between male and female infants were not noticed. Plasma levels are therefore shown as one group.

3.3.1. 18OH-DOC

There was a minor rise of plasma 18OH-DOC from day 1 to day 2–3 of postnatal life. Thereafter the mean plasma 18OH-DOC level decreased and remained in the lower range. After 1 month of postnatal life, plasma 18OH-DOC levels were slightly lower than at birth and stayed in this range. No significant differences were found between the different age groups.

3.3.2. 18OH-B

There was a rise of mean plasma 18OH-B from day 1 to day 2–3 after delivery. Plasma 18OH-B levels showed a significant drop to levels below day 2–3 after 2 and 3–5 months of postnatal life ($P<0.05$). Plasma 18OH-B levels were also significantly lower after 2 and 3–5 months of postnatal life when compared to day 1.

3.4. Patients

The diagnosis of isolated aldosterone synthase deficiency was proven by direct sequencing of the *CYP11B2* gene. Patient 1 showed a homozygous T185I substitution in exon 3, as shown in another such patient diagnosed by our group [15]. The multisteroid measurements are given in Table 6. All precursors of aldosterone were elevated. Patient 2 showed a homozygous deletion of arginine in codon 173 (delR173) in exon 3 of the *CYP11B2* gene [16]. All mineralocorticoid precursors were elevated. The hormonal diagnosis of aldosterone synthase deficiency type II was established in both patients on the basis of the B/18OH-B and 18OH-B/aldosterone ratios [18].

4. Discussion

A practicable system for the simultaneous measurement of plasma 18OH-DOC and 18OH-B was established. A possible interference with water-soluble components was avoided by using a methylene chloride extraction procedure. Liquid gel column chromatography on Sephadex LH-20 with a solvent system of low polarity was used in this study to separate plasma steroids according to their polarity. 18OH-DOC and 18OH-B can be completely and simultaneously separated from each other in our system, despite their similar polarity and molecular size. Chromatography is a necessary step to avoid

Table 6
Electrolytes and PRA at diagnosis, basal plasma steroids in untreated patients with aldosterone synthase deficiency type II at age 4 months and *CYP11B2* genotype

	Unit	Patient 1	Patient 2	Normal range
Na	mmol/l	125	127	132–147
K	mmol/l	6.5	7.0	3.6–5.8
Plasma renin activity (PRA)	ng/ml/h	46	135	4.0–23.8
17OH-progesterone	nmol/l	0.64	0.12	0.09–4.69
11-Deoxycortisol	nmol/l	0.52	6.03	0.43–5.66
Cortisol	nmol/l	168.30	220.72	58.49–477.31
Progesterone	nmol/l	0.73	0.29	0.10–4.01
11-Deoxycorticosterone (DOC)	nmol/l	0.79	101.98	0.09–1.00
Corticosterone (B)	nmol/l	80.52	68.11	0.14–15.9
18OH-corticosterone (18OH-B)	nmol/l	15.84	33.10	3.14–10.01
18OH-deoxycorticosterone (18OH-DOC)	nmol/l	6.64	9.18	0.05–2.30
Aldosterone	nmol/l	<0.06	<0.06	0.17–2.55
B/18OH-B		5.08	2.06	
18OH-B/aldosterone		264	551.7	
CYP11B2 genotype		T185I	R173del	

overestimation of 18OH-DOC and 18OH-B plasma levels as a result of cross-reactions of the antibodies with other extracted steroids. Relevant steroid co-elution of 18OH-DOC within this chromatographic fraction was observed with 11-deoxycortisol, cortisol, cortisone, 11-deoxycorticosterone, corticosterone and aldosterone. Overestimation of 18OH-DOC levels can be ignored because of the minimal cross-reactions of the antiserum with these steroids. On our chromatographic system, 18OH-B showed no co-elution at all with other adrenal steroids.

Normal values for 18OH-DOC in healthy adults are in the range of 0.03 to 0.52 nmol/l [19,20]. It is not surprising that the neonatal range and the range in infants shows considerably higher 18OH-DOC levels up to 6.14 nmol/l. This is due to the still persisting activity of the fetocortex of the adrenal gland during the first postnatal months. Data for older prepubertal children measured with our system (18OH-DOC range 0.13–0.72, 18OH-B 0.56–1.46) match well with the ranges reported by Kayes-Wandover [19] and Aupetit-Faisant [20].

The first reference values for 18OH-B in adults were published as early as 1975 [21]. More reference data with different methods for the adult population were set up during the following years [19,20,22]. The results match with data for older children [10]. There is one report on 18OH-B levels in childhood [12]. These authors found a mean level of 2.35 nmol/l$\pm$2.88 (mean$\pm$SD) (range 0.14–8.56 nmol/l) for children younger than 1 year. No subgroups were shown for different ages within that age period. The median age was 5 months. 18OH-B was measured by RIA after purification with paper chromatography. Our basal values for 18OH-B are comparable to those already published.

In our earlier work, we showed a postnatal decline for several adrenal steroid levels in full-term and preterm infants [23–26], which in preterm infants was prolonged over several days [25,26]. The fall of adrenal steroids is caused by the rapid involution of the highly active fetal zone (fetocortex) of the adrenal gland after birth [27]. Similarly, in this cross-sectional study we provide evidence for a postnatal decline of 18OH-B after an early postnatal rise on day 2–3. This increase can be interpreted as the response to postnatal salt loss due to renal tubular immaturity in neonates who have to autonomously regulate the balance of fluids and electrolytes after the transition from an intra- to an extra-uterine environment. For 18OH-DOC a postnatal decline of plasma levels could not be demonstrated.

In the present study, we were able to demonstrate the clinical application of our assay method by the hormonal diagnosis of aldosterone synthase deficiency in two patients aged 4 months. Both 18OH-DOC and 18OH-B were elevated. Using Ulick's classification [18], the diagnosis of aldosterone synthase (formerly: corticosterone methyl oxidase, CMO) deficiency type II was established and the

diagnosis was confirmed by demonstrating a homozygous mutation/deletion in the *CYP11B2* gene.

In general, the quality and reliability of the assay described in this paper are of equal or superior quality in regard to specificity, precision, accuracy and practicability compared with other assay systems. The advantages of this technique over other chromatographic procedures, such as paper or thin layer chromatography, are well established and have been described in detail by several groups [28–30]. To our knowledge, GC–MS methods for 18OH-DOC and 18OH-B are not published. Steroid assays without prior extraction methods using competitive protein-binding assays should not be used during infancy and under pathological conditions, particularly in the perinatal period, as they are of insufficient specificity and sensitivity due to cross reacting adrenal steroids from the adrenal fetocortex [31,32]. For this reason direct RIAs lead to overestimation of steroid levels, as already shown for 17OH-progesterone in neonates [33–36]. Our assay has demonstrated its reliability and practicability in the diagnosis of the rare but life-threatening disorder aldosterone synthase deficiency, based on new and valid reference data for the early neonatal period and infancy.

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