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Fast and direct quantification of adrenal steroids by tandem mass spectrometry in serum and dried blood spots

Nils Janzen *, Stefanie Sander, Michael Terhardt, Michael Peter, Johannes Sander

Screening-Laboratory Hannover, c/o Box 91 10 09, Hannover D-30430, Germany

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Abstract

We present a fast and reproducible method for steroid analysis (corticosterone, deoxycorticosterone, progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone, dihydrotestosterone and cortisol) in small volumes of serum and in dried blood spot samples by LC-MS/MS. No derivatisation was needed. LC separation was achieved by using an Atlantis® C_{18} column and water–methanol–formic acid gradient as a mobile phase and a flow rate of 250 μL/min over a run time of 6 min. Steroids were measured in MRM mode with electrospray interface (positive ion mode). Validation showed excellent precision, sensitivity, recovery and linearity with coefficients of determination $r^2 > 0.992$.

Keywords: Steroid profile; Tandem mass spectrometry; Adrenal cortex disorders

1. Introduction

Steroids belong to a large group of substances derived from isoprene. This group comprises steroid hormones, vitamin D, bile acids and cholesterol, a precursor of several steroids formed in the gonads and the adrenal cortex [1]. Steroid hormones regulate glucose metabolism, function as mineralocorticoids and sex hormones. Genetic or transient functional disturbances of adrenal hormone synthesis form an important part of pediatric and especially of neonatal diseases. Analytical procedures for the identification and differentiation of endocrine disorders should provide results in very short time requiring only small amounts of blood or serum. Immuno assays, which are still in use, have the disadvantage of significant cross-reactions [2,3] while GC-MS separations require time-consuming complex sample preparation [4–7].

Our intention was to develop and validate a sensitive and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with electrospray ionisation (ESI) for measurement of steroids without derivatisation, which could be used for detection of several pediatric endocrine disorders.

2. Experimental

2.1. Chemicals and reagents

The steroids corticosterone, deoxycorticosterone, progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone, dihydrotestosterone and cortisol were from Sigma–Aldrich, the deuterated substances d_8-17α-hydroxyprogesterone and d_2-cortisol were from Cambridge Isotope Laboratories Inc. (LCG Euriso-top, Saarbruecken, Germany). Acetone, acetonitrile, formic acid, methanol and HPLC grade water of highest purity grades were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of internal deuterated standards

Internal standards d_8-17α-hydroxyprogesterone (300 μmol/L) and d_2-cortisol (100 μmol/L) were dissolved in methanol and stored at −18 °C. Before use the stock solutions were diluted with methanol to give final concentrations of

* Corresponding author. Fax: +49 5108 9216319.
E-mail address: n.janzen@metabscreen.de (N. Janzen).

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15 nmol/L for d_8-17α-hydroxyprogesterone and 100 nmol/L for d_2-cortisol.

2.3. Preparation of dried blood and serum material

Dried blood calibrators and controls were prepared from venous blood using a modified method reported by Lacey et al. [8]: whole blood was centrifuged at 1.900 U/min and, red blood cells were washed three times with saline, the supernatant was discarded. Red blood cells were then diluted with steroid-free serum (MP BIOMEDICALS, Eschwege, Germany) to obtain a haematocrit of 55%. Cortisol and the remaining steroids were dissolved in methanol with a final concentration of 10 and 4 μmol/L, respectively. These stock solutions were added to the "whole blood pool" to give calibrators for corticosterone, deoxycorticosterone, progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone, dihydrotestosterone at final concentrations of 0 (blank), 5, 25, 50, 75, 100, 125, 150, 200 nmol/L and for cortisol at concentrations of 0, 12.5, 62.5, 125, 187.5, 250, 312.5, 375, 437.5 und 500 nmol/L.

To ensure complete haemolysis, samples were twice frozen at −18 °C and thawed again. Subsequently, calibrators were spotted on filter cards (Schleicher & Schuell 903) with each spot containing 25 μL. These were dried for at least 24 h at room temperature and stored at −18 °C.

Serum calibrators and controls were prepared from steroid-free serum plus aliquots of the stock solutions described earlier. The calibrator concentrations were the same as in the whole blood samples. These sera were aliquoted into small Eppendorf cups and stored at −18 °C.

2.4. HPLC and MS/MS apparatus

For HPLC analysis an Atlantis® d_{18} 3 μm, 2.1 mm × 20 mm IS column (Waters, Eschborn, Germany) was used. The precolumn was a C_{18} ODS 4 mm × 2 mm (Phenomenex, Aschaffenburg, Germany).

The mobile phase consisted of two solvents: HPLC-grade water (solvent A) containing 32 mM formic acid, and methanol (solvent B). The initial solvent mixture was 45% A and 55% B. The gradient increased up to 78% B within 1.9 min, then up to 100% B within 0.1 min, held for 0.5 min, returning via a step gradient to the initial state after 2.42 min. The total run time was 6 min. Chromatography was done at ambient temperature (23 °C).

The LC-MS/MS system consisted of a Waters 1525 μ HPLC pump with internal solvent degasser unit connected to a Ultima ESI triple quadrupole mass spectrometer obtained from Waters Corp. (Eschborn, Germany). The autosampler and injection system was a CTC Pal (Axel Semrau, Sprockhoefel, Germany) with microtitre stacks. All parts were controlled by the MassLynx Software 4.0 and the integration and quantitation was done by QuanLynx. The settings on the instrument were optimized for maximum ion yield. For all steroid and calibrators the optimal settings and fragments were defined after injection of aqueous single samples.

The following settings were used: capillary voltage was 3.5 kV, cone voltage 20–64 V, collision energy 11–24 eV depending on the steroid, source temperature 120 °C, desolvation temperature 300 °C, dwell time 0.05 s and argon gas cell pressure 2.2×10^{-3} mbar. During HPLC-MS/MS performance the ambient temperature was kept at 23 °C by air conditioning.

2.5. Assay procedure

Six millimeters blood spots equaling 14 μL whole blood each were punched out (DBS-puncher, Perkin-Elmer, Rottgau, Germany) from filter cards. The spots were placed into microtitre plates (ABGENE, Hamburg, Germany). Two hundred microliters acetone/acetonitrile 50:50 (v/v) and 20 μL each of the internal standards d_8-17α-hydroxyprogesterone (15 nmol/L) and d_2-cortisol (100 nmol/L) were added to each sample. The plate was sealed and shaken for 50 min at ambient temperature. The supernatant was transferred to a polypropylene microtitre plate and carefully dried at 60–70 °C. Dried extracts may be stored in a refrigerator or freezer for several weeks. For LC-MS/MS analysis the extracts were reconstituted in 80 μL methanol/water 50:50 (v/v) and vigorously shaken for 30 min. The eluates were then transferred to a 384 well microtitre plate (Greiner, Frickenhausen, Germany) and centrifuged for 10 min at 2.000 U/min to remove residues of the filter paper and to herewith prevent blockage of the capillary.

Fifteen microliters serum were mixed with 200 μL acetone/acetonitrile 50:50 (v/v) and 20 μL of each of the internal standard solutions in a well of a microtitre plate, gently shaken for 10 min and centrifuged for 10 min at 2.000 U/min to separate precipitated proteins. Further processing of the supernatant was done as described for the of dried blood spot eluate.

Sera of children with various adrenal disorders were also analysed. The results were compared with those from the paediatric hospital, University of Kiel (Germany) [9,10].

2.6. Quantification

Quantitative results were obtained by dividing the peak area of each steroid (except cortisol) by the peak area of the internal standard d_8-17α-hydroxyprogesterone. For quantification of cortisol the internal standard d_2-cortisol was used. Concentrations were calculated using the QuanLynx-Software (Waters) via linear regression with reciprocal fit weighting.

3. Results and discussion

The ionisation efficiency of all steroids in mobile phase was tested in electro spray ionisation (ESI) positive and negative mode. In both modes of cortisol and 17α-hydroxyprogesterone detectable signals were found. However, the positive mode produced far higher intensities compared to the negative mode. For all remaining steroids detectable ions were received in positive ion mode only. Steroids were injected separately and the cone voltage und collision energy for $[M+H]^+$ was optimized (Table 1). Fig. 1 gives a typical chromatogram, depicting the total

Table 1
Optimal MRM functions and MS/MS parameters for detecting steroids (Fig. 2)

Function	Time (min)	Name	Parent (Da)	Daughter (Da)	Cone voltage (V)	Collision energy (eV)
1	0–2.55	Cortisol	363.3	121.1	50	24
		d_2-Cortisol	365.3	122.1	50	22
2	2.10–2.95	Corticosterone	347.3	329.3	40	24
3	2.10–2.95	11-Deoxycortisol	347.3	109.1	25	30
		21-Deoxycortisol	347.4	311.2	50	16
4	2.50–3.35	Androstenedione	287.3	97.0	60	22
		Testosterone	289.5	109.1	60	24
		Deoxycorticosterone	331.5	109.1	50	30
5	2.55–6.00	17α-Hydroxyprogesterone	331.3	109.0	64	28
		d_8-17α-Hydroxyprogesterone	339.4	113.2	60	32
6	3.05–6.00	Dihydrotestosterone	291.4	255.3	30	16
		Progesterone	315.4	109.2	50	25

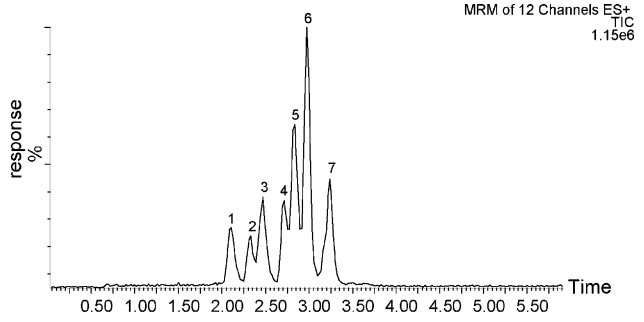

Fig. 1. Total ion chromatogram (TIC) of steroids including all MRM functions. All functions shown in Table 1 are superimposed in this diagram. (1) Cortisol, d_2-cortisol; (2) 21-deoxycortisol; (3) corticosterone, 11-deoxycortisol; (4) androstenedione; (5) deoxycorticosterone; (6) testosterone, 17α-hydroxyprogesterone, d_8-17α-hydroxyprogesterone; and (7) dihydrotestosterone, progesterone.

of all acquired multiple reaction monitoring (MRM) functions listed in Table 1.

3.1. Linearity

The linearity of response was tested by analysing the steroid standard solutions (serum) prepared at eight different concentrations (cortisol: nine different concentrations) on three consecutive days. Excellent linearity was found within a range of 5–200 nmol/L (cortisol 12.5–500 nmol/L) (Table 2). Coefficients of determination (r^2) were greater than 0.992 for all steroids and cortisol.

3.2. Precision, sensitivity and recovery

Precision and accuracy were measured at three different concentration ranges (Table 3). The coefficient of variation for serum samples was 4.6–11.8% (between-run) and 3.3–9.8% (within-run). Due to matrix effects, however, it was higher in dried blood spot samples for both within-run and between-run. Due to the fact that both parameters were quantified using a specific internal standard, the best precision in serum and dried blood was retrieved for 17α-hydroxyprogesterone and cortisol in both within-run and between-run. Recovery data of serum and dried blood at three different concentrations are listed in Table 4. Recovery was lower in dried blood spots. Of all steroids, dihydrotestosterone showed the lowest recovery over the total concentration range.

Performance data for all steroids are depicted in Table 5. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on criteria of maintaining accuracy within 80–120% and imprecision <20%, each sample was analysed six times. The ionisation efficiency [11] was determined analysing a diluted sample containing 1 nmol/L of each steroid (cortisol: 2.5 nmol/L).

Table 2
Concentration range, regression equation and coefficient of determination

Steroid	Concentration range (nmol/L)	Regression equation	r^2
Corticosterone	5–200	$y = 0.173x + 0.015$	0.996
Deoxycorticosterone	5–200	$y = 0.363x + 0.042$	0.997
Progesterone	5–200	$y = 0.922x + 0.049$	0.997
17-Hydroxyprogesterone	5–200	$y = 0.486x + 0.017$	0.992
11-Deoxycortisol	5–200	$y = 0.278x + 0.009$	0.996
21-Deoxycortisol	5–200	$y = 0.175x + 0.002$	0.996
Androstenedione	5–200	$y = 0.608x + 0.011$	0.993
Testosterone	5–200	$y = 0.533x + 0.075$	0.997
Dihydrotestosterone	5–200	$y = 0.146x + 0.015$	0.995
Cortisol	12.5–500	$y = 0.111x + 0.010$	0.998

Table 3
Precision data from serum and dried blood spot material

Steroid	Serum						Dried blood spots					
	CV (%) within-run ($n = 10$)			CV (%) between-run ($n = 10$)			CV (%) within-run ($n = 10$)			CV (%) between-run ($n = 10$)		
	25 nM	75 nM	175 nM	25 nM	75 nM	175 nM	25 nM	75 nM	175 nM	25 nM	75 nM	175 nM
Corticosterone	9.8	6.7	6.1	10.8	7.2	6.9	11.8	6.7	6.1	13.1	8.6	7.1
Deoxycorticosterone	8.3	8.0	5.7	8.5	8.4	6.3	11.0	8.0	5.7	11.8	10.2	9.0
Progesterone	9.7	6.8	6.6	9.9	9.8	7.0	17.0	9.8	6.6	17.9	10.6	8.2
17-Hydroxyprogesterone	6.3	4.9	3.3	6.8	5.8	6.3	9.3	8.9	6.3	8.5	7.7	5.7
11-Deoxycortisol	9.1	6.9	5.7	10.9	7.2	6.5	14.4	9.9	3.7	10.3	10.6	5.3
21-Deoxycortisol	8.2	7.9	5.0	10.1	9.2	5.1	8.3	7.9	5.0	11.2	9.9	6.9
Androstenedione	7.5	6.5	6.2	11.1	8.4	7.9	8.0	6.5	4.2	12.7	10.6	9.6
Testosterone	8.6	9.3	6.8	10.7	9.8	8.6	10.6	9.3	6.8	10.8	9.8	8.0
Dihydrotestosterone	9.8	8.2	5.4	11.8	9.8	7.6	19.8	8.2	5.4	12.4	9.5	5.7
	63 nM	188 nM	438 nM	63 nM	188 nM	438 nM	63 nM	188 nM	438 nM	63 nM	188 nM	438 nM
Cortisol	7.1	4.7	3.5	7.7	5.1	4.6	7.9	5.7	3.5	9.1	6.3	5.5

Table 4
Recovery data of serum and dried blood material at three different concentrations

Steroid	Serum with added concentrations (%)			Blood spots with added concentrations (%)		
	25 nM	100 nM	175 nM	25 nM	100 nM	175 nM
Corticosterone	91.2	106.7	99.2	78.2	72.9	77.1
Deoxycorticosterone	104.4	102.6	97.8	69.3	72.8	74.7
Progesterone	102.0	101.0	99.2	80.3	77.9	79.9
17-Hydroxyprogesterone	95.4	92.6	93.7	81.3	83.7	82.6
11-Deoxycortisol	100.4	102.3	98.3	79.3	74.8	75.9
21-Deoxycortisol	86.9	96.3	89.2	82.0	79.2	75.6
Androstenedione	96.8	101.4	97.2	68.7	72.6	70.9
Testosterone	92.8	96.7	93.9	92.8	96.7	93.9
Dihydrotestosterone	82.0	86.7	85.2	66.7	68.0	65.1
Cortisol	92.8	92.6	94.6	92.6	90.5	88.9

Cortisol showed the lowest LOQ (0.75 nmol/L), progesterone showed the highest (6.3 nmol/L) LOQ. The higher the ionisation efficiency the lower the LOQ. Therefore, the LOD and LOQ appeared to be dependent on the degree of ionisation efficiency.

3.3. Ion suppression

Ion suppression was evaluated by analysing extracted samples with steroid concentrations of 25 nmol/L injected into a flow of adrenal steroids prepared at a concentration of 5 nmol/L inserted by a syringe pump at a flow rate of 10 μL/min. Decreased intensity of the baseline in the mass transition of the steroids at a flow rate of 250 μL/min was shown between 0.7 and 1.2 min. There was no ion suppression at any later point of time. With the first peak eluting at 2.17 min none of the steroids was affected by ion suppression (Fig. 2).

Table 5
Performance data (ionisation efficiency, IE; limit of detection, LOD; limit of quantification, LOQ) of steroids

Steroid	IE (%)	LOD (nM)	LOQ (nM)
Corticosterone	8.60	1.10	3.30
Deoxycorticosterone	11.70	0.77	2.31
Progesterone	4.40	2.10	6.30
17-Hydroxyprogesterone	26.90	0.33	0.99
11-Deoxycortisol	16.20	0.55	1.65
21-Deoxycortisol	30.10	0.31	0.93
Androstenedione	19.60	0.47	1.41
Testosterone	13.00	0.69	2.07
Dihydrotestosterone	9.20	0.98	2.94
Cortisol	36.20	0.25	0.75

3.4. Quantitative analysis of steroids in blood and serum samples

Steroid concentrations in serum of healthy children aged 3–8 years as well as concentrations in dried blood samples of healthy newborns contrast well with steroid levels of 5 serum samples of children with defined adrenal hormone deficiencies under therapy (Table 6).

Steroid concentrations (except cortisol) of healthy children were generally fairly low. All but one of the children with homozygous 21-hydroxylase deficient congenital adrenal hyperplasia (21-CAH) under therapy showed only elevated 21-deoxycortisol; patient 2 also showed elevated levels of 17α-hydroxyprogesterone, while cortisol was within the reference range due to hydrocortisone therapy. In contrast, patients

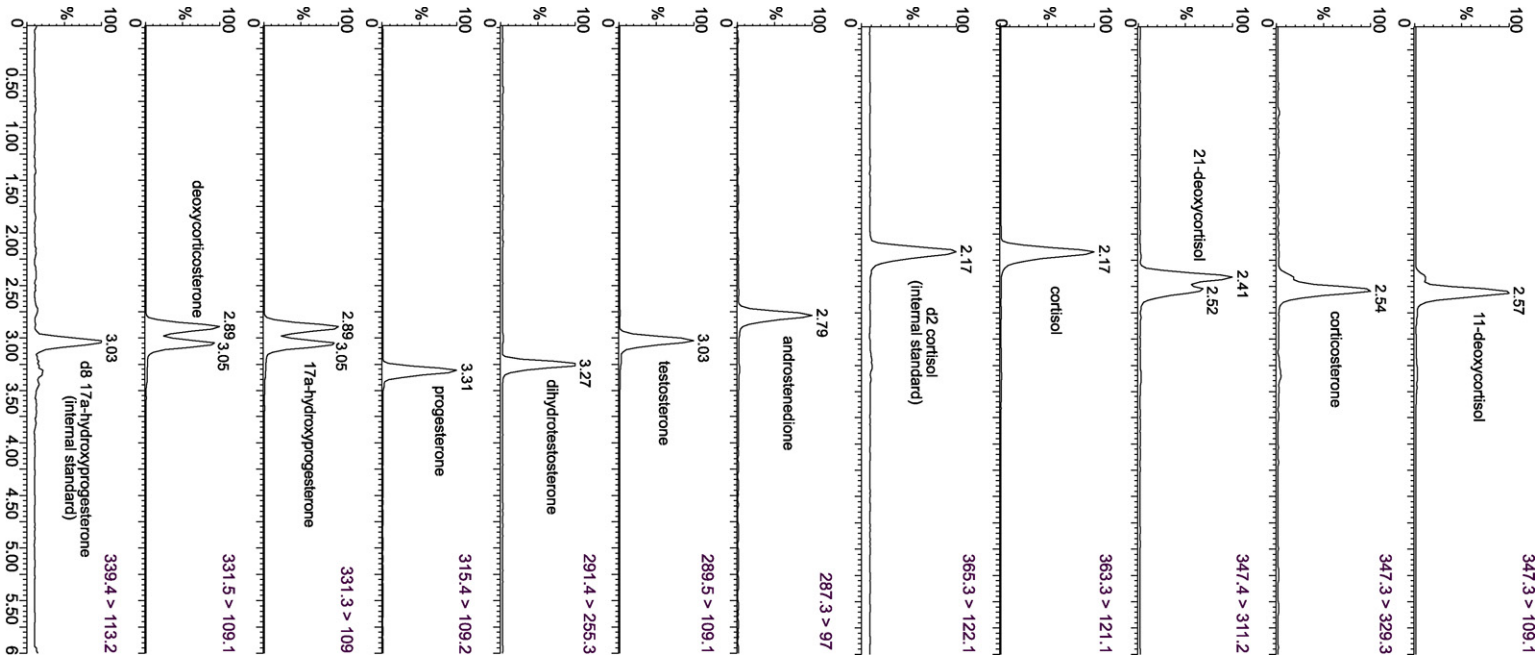

Fig. 2. MRM transitions of all steroids and deuterated calibrators.

Table 6
Steroid analysis in blood samples of healthy children and neonates (dried blood spots) and of five children with congenital adrenal hyperplasia (under therapy) (nmol/L)

	Corticosterone	Deoxycorticosterone	Progesterone	17-Hydroxyprogesterone	11-Deoxycortisol	21-Deoxycortisol	Cortisol	Androstenedione	Testosterone	Dihydrotestosterone
Normal group[a] (serum sample ($n = 5$))	1.9–8.1	0.7–1.9	0.0–10.2	3.6–6.5	2.5–8.5	0.0–0.7	143.3–423.1	0.0–1.1	0.0–16.4	0.0–3.7
Normal group[a] (dried blood spots ($n = 5$))	2.1–10.3	0.8–4.2	0.0–11.0	4.7–8.1	5.3–7.7	0.0–2.1	143.9–339.2	0.0–3.2	0.0–10.9	2.0–5.3
Patient 1 (homozygeous 21-CAH)	0.0	0.4	5.2	5.8	0.2	28.9	192.5	3.5	1.6	4.4
Patient 2 (homozygeous 21-CAH)	2.2	1.2	6.6	172.1	0.4	19.8	185.6	42.9	10.0	42.2
Patient 3 (11-hydroxylase-deficency)	142.0	101.6	3.7	23.0	771.1[b]	0.0	17.4	122.4	5.1	11.6
Patient 4 (11-hydroxylase-deficency)	96.9	15.0	9.6	20.1	511.9[b]	0.0	64.3	48.2	4.2	7.8
Patient 5 (insufficent adrenal cortex)	0.0	4.8	5.9	3.6	0.0	0.0	0.0	1.5	4.8	2.8

[a] Values expressed as range in nmol/L.
[b] Extrapolated value.

detected at newborn screening showed the typical steroid profile with elevated 17α-hydroxyprogesterone and 21-deoxycortisol, and decreased cortisol [12].

The typical metabolite 11-deoxycortisol was extremely increased in patients with 11β-hydroxylase deficiency. In addition, corticosterone, deoxycorticosterone and androstenedione were significantly elevated, while cortisol was decreased despite therapy. The patient suffering from insufficient adrenal cortex showed low concentrations for all steroids due to defective synthesis.

4. Conclusion

For the particular challenge of judging elevated levels of 17α-hydroxyprogesterone in neonatal screening for 21-CAH we were already able to provide a LC-MS/MS procedure generating a profile of 4 steroids using material from the original neonatal blood sample [12]. For a more detailed differentiation of neonatal adrenal cortex diseases, however, a broader spectrum of steroids is necessary.

Several authors have reported the use of atmospheric pressure chemical ionisation (APCI-) MS/MS [13–15] and atmospheric pressure photo ionisation (APPI-) MS/MS [16,17] in order to increase the sensitivity of the method. However, neither of these have been adopted as a routine measure for steroid analysis. Higashi et al. [15] described several methods of derivatisation, all designed to increase ionisation efficiency and therefore to improve sensitivity.

Our method for steroid quantification is equally applicable to serum and dried blood spots. However, due to matrix effects the recovery is lower in the latter material.

Linearity of the method is excellent for all steroids, and even low physiological concentrations can be quantified [12]. Main advantages of our method compared to conventional GC-MS analysis are simple sample preparation and rapid direct analysis of steroids. Only small sample volumes are needed, derivatisation or time-consuming concentration steps are not required. The method is especially suited for small blood volumes and dried blood samples which are used in clinical laboratories and screening centres for fast detection of endocrine disorders. It should also be useful for experimental studies in small laboratory animals or cell cultures.

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References

[1] R.I. Kelley, G.E. Herman, Annu. Rev. Genomics Hum. Genet. 2 (2001) 299.
[2] F.Z. Stanczyk, M.M. Cho, D.B. Endres, J.L. Morrison, S. Patel, R.J. Paulson, Steroids 68 (2003) 1173.
[3] T. Torresani, A. Grüters, R. Scherz, J.J. Burckhardt, A.M.Z. Harras, M. Zachmann, Screening 3 (1994) 77.
[4] K. Homma, T. Hasegawa, M. Masumoto, E. Takeshita, K. Watanabe, H. Chiba, T. Kurosawa, T. Takahashi, N. Matsuo, Endocr. J. 50 (2003) 783.
[5] J.W. Honour, C.G. Brook, Ann. Clin. Biochem. 34 (Pt 1) (1997) 45.
[6] J.W. Honour, Ann. Clin. Biochem. 34 (Pt 1) (1997) 32.
[7] S.A. Wudy, U.A. Wachter, J. Homoki, W.M. Teller, Pediatr. Res. 38 (1995) 76.
[8] J.M. Lacey, C.Z. Minutti, M.J. Magera, A.L. Tauscher, B. Casetta, M. McCann, J. Lymp, S.H. Hahn, P. Rinaldo, D. Matern, Clin. Chem. 50 (2004) 621.
[9] M. Peter, C.J. Partsch, W.G. Sippell, J. Clin. Endocrinol. Metab. 80 (1995) 1622.
[10] W.G. Sippell, F. Bidlingmaier, H. Becker, T. Brunig, H. Dorr, H. Hahn, W. Golder, G. Hollmann, D. Knorr, J. Steroid Biochem. 9 (1978) 63.
[11] D.H. Chace, J.R. Barr, M.W. Duncan, D. Matern, M.R. Morris, D.E. Palmer-Toy, A.L. Rockwood, G. Siuzdak, A. Urbani, A.L. Yergey, Y.M. Chan, CLSI C50-P, 27 (2007) 1.
[12] N. Janzen, M. Peter, S. Sander, U. Steuerwald, M. Terhardt, U. Holtkamp, J. Sander, J. Clin. Endocrinol. Metab. 92 (2007) 2581.
[13] S. Cristoni, D. Cuccato, M. Sciannamblo, L.R. Bernardi, I. Biunno, P. Gerthoux, G. Russo, G. Weber, S. Mora, Rapid Commun. Mass Spectrom. 18 (2004) 77.
[14] V.F. Fredline, P.J. Taylor, H.M. Dodds, A.G. Johnson, Anal. Biochem. 252 (1997) 308.
[15] T. Higashi, N. Takido, A. Yamauchi, K. Shimada, Anal. Sci. 18 (2002) 1301.
[16] T. Guo, M. Chan, S.J. Soldin, Arch. Pathol. Lab. Med. 128 (2004) 469.
[17] M.M. Kushnir, R. Neilson, W.L. Roberts, A.L. Rockwood, Clin. Biochem. 37 (2004) 357.