Clinica Chimica Acta 415 (2013) 302–305

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Measurement of reference intervals for urinary free adrenal steroid levels in Japanese newborn infants by using stable isotope dilution gas chromatography/mass spectrometry

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ARTICLE INFO

Article history:
Received 24 April 2012
Received in revised form 23 October 2012
Accepted 7 November 2012
Available online 15 November 2012

Keywords:
Urinary free steroid
SID-GC/MS
Newborn infant
Reference interval

ABSTRACT

Background: In newborn infants, there are no reference intervals for urinary free steroids, which are thought to reflect the bioavailable fraction of steroids in the blood. We establish a method for simultaneous measurement of urinary free adrenal steroids such as pregnenolone, progesterone, 16α-hydroxyprogesterone, 17α-hydroxyprogesterone, 21-deoxycortisone, 21-deoxycortisol, dehydroepiandrosterone, androstenedione, and 11β-hydroxyandrostenedione by using stable isotope dilution gas chromatography/mass spectrometry (SID-GC/MS) and determined the reference intervals for urinary levels of free adrenal steroids in Japanese newborn infants.

Methods: Newborn pooled urine was used for validation. Spot urine samples were collected from 67 full-term Japanese newborn infants (34 male and 33 female infants) at 3–4 days of age to determine reference intervals. The extracted and purified free steroids were delivered with heptafluorobutyric anhydride and analyzed by SID-GC/MS.

Results: We validated a SID-GC/MS method with good repeatability and recovery rate. The preliminary reference intervals (median [range], μmol/mol creatinine) were as follows: pregnenolone, 4.2 (0.7–31.6); progesterone, 0.5 (not detected (n.d.)–0.6); 16α-hydroxyprogesterone, 1.4 (n.d.–10.3); 17α-hydroxyprogesterone, 1.1 (n.d.–1.9); 21-deoxycortisone, n.d. (n.d.–n.d.); 21-deoxycortisol, n.d. (n.d.–n.d.); dehydroepiandrosterone, 2.2 (0.6–27.3); androstenedione, 0.7 (n.d.–5.2); and 11β-hydroxyandrostenedione, 2.9 (n.d.–26.7).

Conclusions: We established a reliable SID-GC/MS method and were able to determine preliminary reference intervals for 9 urinary free adrenal steroids in newborn infants.

1. Introduction

Urinary free cortisol is thought to reflect the bioavailable fraction of cortisol in the blood. Clinically, this measurement has been widely used for diagnosing Cushing's syndrome [1,2]. In newborn infants, urinary free cortisol and its metabolites have been measured [3,4], but little is known about the dynamics of other urinary free adrenal steroids.

Simultaneous measurement of urinary adrenal steroids and metabolites using gas chromatography/mass spectrometry (GC/MS) has been reported to be useful in the diagnosis of abnormal steroidogenesis in adults and newborn infants [5–7]. However, in these previous reports, urinary steroids were measured after enzymatic hydrolysis; in other words, not only the free forms of steroids but also glucuronide conjugates and sulfate conjugates were measured. Moreover, as far as we know, there is no report on the reference intervals of urinary free steroids in newborn infants.

2. Materials and methods

2.1. Chemicals and reagents

Standards for P5, P4, 16OHP4, 17OHP4, 21DOE, 21DOF, DHEA, AD4, and 11OHAD4 were from Sigma-Aldrich (St. Louis, MO). [17, 21, 21, 21-d]-P5 (d4-P5), [2, 2, 4, 6, 6, 17α, 21, 21, 21-d]-P4 (d9-P4), [2, 2, 4, 6, 6, 9, 12, 12, 21, 21, 21-d]-21DOE (d11-21DOE), and [2, 2, 4, 6, 6, 16, 16-d]-11OHAD4 (d7-11OHAD4) were from Medical Isotopes Inc. (Pelham, NH). [19, 19, 19-d]-AD4 (d3-AD4) was generously provided by the Australian Government Analytical Laboratories (Canberra, ACT, Australia). [11, 11, 12, 12-d]-17OHP4 (d4-17OHP4) was provided by

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http://dx.doi.org/10.1016/j.cca.2012.11.006

Dr. Yamaga (Tottri University, Japan). Heptafluoro-n-butyric anhydride was from GL Science (Tokyo, Japan). All ethyl acetate, acetonitrile, toluene, cyclohexane, and ethanol used were analytical reagent grade. Toluene and cyclohexane were dehydrated by 4A 1/8 molecular sieve pellets ($Na_{12}[(AlO_2)_{12}(SiO_2)_{12}]\cdot 27H_2O$, diameter: 1/8 in. [3.2 mm]) (Wako, Osaka, Japan). Bond-Elute C18 cartridges (silica sorbent mass: 100 mg) for solid phase extraction were purchased from Varian Inc. (Lake Forest, CA).

2.2. Subjects

Newborn pooled urine (n = 5) was spiked with standards of P5, P4, 16OHP4, 17OHP4, 21DOE, 21DOF, DHEA, AD4, and 11OHAD4 to validate the following method. We then recruited 67 full term newborn infants (34 males and 33 females, gestational age 37–41 weeks, birth weight 2562–3788 g) without neurological and endocrinological abnormalities. None of the subjects received antenatal or perinatal glucocorticoids before urine sampling. A spot urine sample was collected at 3–4 days of age. This study was approved by the institutional review board committee at Keio University Hospital, and all legal guardian(s) gave written informed consent.

2.3. SID-GC/MS selected ion monitoring (SIM) analysis

SID-GC/MS SIM analysis was performed using a previously described method [8,9] with partial modification. The sample preparations used to analyze free steroids were made as follows. (1) 6 internal standards (20 μg/ml [final 40 ng/injection] d4-P5 and d11-21DOE, 10 μg/ml [final 20 ng/injection] d9-P4, d4-17OHP4, d3-AD4, and d7-11OHAD4) were added to 1 ml of urine or standard solutions (P5, P4, 16OHP4, 17OHP4, 21DOE, 21DOF, DHEA, AD4, and 11OHAD4). (2) Free steroids were extracted using 2 ml ethyl acetate. (3) The mixture was evaporated at room temperature under nitrogen gas. (4) The residue was dissolved in 100 μl methanol, and then added to 2 ml purified water. (5) The solution was applied to a Bond-Elute C18 cartridge (pre-conditioned with 3 ml acetonitrile and 5 ml purified water). (6) The cartridge was washed with 1 ml purified water and then with 2 ml acetonitrile-purified water (10:90 v/v) and eluted with 3-ml acetonitrile-purified water (70:30 v/v). (7) The eluate was evaporated at 50 °C under nitrogen gas. (8) The residue was dissolved in 100 μl dehydrated toluene. (9) The mixture was added to 25 μl of heptafluoro-n-butyric anhydride and incubated for 60 min at 50 °C. (10) The mixture was evaporated at 50 °C under nitrogen gas. (11) The residue was dissolved in 20 μl dehydrated cyclohexane. (12) Finally, a 4 μl sample solution was analyzed using GC/MS.

GC/MS analysis was performed on a HP5890II GC system with a HP-ULTRA1 fused silica column (25 m × 0.2 mm, d_f 0.33 μm; Agilent Technologies, Palo Alto, CA) coupled to a HP5973 or HP5975 MS (Agilent). Helium was used as a carrier gas at a flow rate of 1 ml/min. The temperature was initially maintained at 51 °C for 2 min, increased by 10 °C/min to 200 °C, and then further increased by 5 °C/min to 290 °C, which was maintained for 15 min. SIM analysis was performed with 2 characteristic mass ions selected for each steroid (Table 1). The ratio of the first ion peak area of each steroid to that of each internal standard was used for quantification. For 16OHP4, 21DOF, and DHEA, we used d9-P4, d11-21DOE, and d3-AD4 as the internal standard for quantification, respectively. A calibration curve was calculated using 5 standard solutions (0.1, 1, 5, 10, and 20 ng/ml [final 0.02, 0.2, 1, 2, and 4 ng/injection, respectively]). The turnaround time of the assay was 6.5 h.

Urinary creatinine was measured by IATRO-LQ CRE (A) II (Mitsubishi Chemical Medience Co., Tokyo, Japan). Urinary steroid concentration was expressed relative to urinary creatinine (μmol/mol creatinine). Relative urinary steroid concentration was defined as "not detected" (n.d.) in newborn infants in whom urinary concentration was under the detection limit.

Table 1
The essentials of SID-GC/MS SIM analysis.

Internal standard		Steroid	Retention	1st ion	2nd ion
			Time (min)	(m/z)	
d4-P5			32.5	302	516
	→	P5	32.5	298	512
d9-P4			32.4	518	503
	→	P4	32.5	510	495
	→	16OHP4	32.0	508	493
d4-17OHP4			34.0	530	469
	→	17OHP4	34.1	526	465
d11-21DOE			35.1	549	459
	→	21DOE	35.1	540	453
	→	21DOF	36.9	542	463
d3-AD4			29.8	485	467
	→	DHEA	29.9	270	255
	→	AD4	29.8	482	467
d7-11OHAD4			32.6	504	471
	→	11OHAD4	32.7	498	465

2.4. Validation of method performance

The intra-assay accuracy and precision were evaluated by measuring 5 replicates of standard solution (0.1, 0.5, 5, and 10 ng/ml) on the same day. The inter-assay accuracy and precision were evaluated by measuring standard solution (0.1, 0.5, 5, and 20 ng/ml) on 5 different days. Analyte recovery rates were determined by measuring urine aliquots spiked with standard solution (1, 5, and 10 ng/ml). The sensitivity was determined by diluting standard solution (final concentration of each steroid 0.1, 0.3, 0.5, 1, and 5 ng/ml) with ethanol and measuring these diluted samples in 5 assays on the same day. Sensitivity was defined as the minimum concentration with an intra-assay CV of <10%.

2.5. Statistical analysis

Reference intervals were determined as ranges (minimum–maximum). Statistical analysis between males and females was performed using Mann–Whitney *U* test. A $p<0.05$ was considered statistically significant.

3. Results

3.1. Validation of method performance

The total ion mass chromatogram and first ion mass chromatogram of each steroid are shown in Fig. 1. Validation results are summarized in Table 2. All values for inter-assay CV were under 10.6% and those for intra-assay CV were <24.3%. The recovery rate was between 85.7% and 118.3%. Sensitivity is indicated in Table 2.

3.2. Preliminary reference intervals

Preliminary reference intervals for urinary free adrenal steroids in newborn infants are shown in Table 3, both as urinary concentration and relative to creatinine values. The number of patients in whom urinary free steroids were under the detection limit was as follows: P5, n = 0; P4, n = 55; 16OHP4, n = 7; 17OHP4, n = 55; 21DOE, n = 67; 21DOF, n = 67; DHEA, n = 0; AD4, n = 3; and 11OHAD4, n = 17. None of the steroid levels were significantly different between males and females.

4. Discussion

In this study, we validated a modified GC/MS method for simultaneous measurement of 9 urinary free adrenal steroids and demonstrated good accuracy and precision. Using this method, we successfully determined preliminary reference intervals for these urinary free adrenal