Journal of Chromatography B, 879 (2011) 1565–1572

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Simultaneous quantification of 17α-OH progesterone, 11-deoxycortisol, Δ4-androstenedione, cortisol and cortisone in newborn blood spots using liquid chromatography–tandem mass spectrometry

P. Magnisali[a], M.-B. Chalioti[a], T. Livadara[a], M. Mataragas[b], S. Paliatsiou[c], A. Malamitsi-Puchner[c], P. Moutsatsou[a,*]

[a] Department of Biological Chemistry, Medical School, University of Athens, 75 Mikras Asias Str, Goudi 11527, Athens, Greece
[b] Department of Food Science and Technology, Laboratory of Food Microbiology and Biotechnology, Agricultural University of Athens, 75 Iera Odos, Votanikos 11855, Athens, Greece
[c] Neonatal Division, 2nd Department of Obstetrics and Gynecology, Athens University Medical School, 76 Vas.Sofias Str., Athens 11528, Greece

ARTICLE INFO

Article history:
Received 19 November 2010
Accepted 25 March 2011
Available online 4 April 2011

Keywords:
Tandem mass spectrometry
Blood spots
17α-OH progesterone
11-Deoxycortisol
Δ4-Androstenedione
Cortisol
Cortisone

ABSTRACT

Adrenal steroid profiling, including 17α-OH progesterone (17OHP), 11-deoxycortisol (S), Δ4-androstenedione (Δ4-A) and cortisol (F) in blood spots by tandem mass spectrometry, is used for newborn screening to detect congenital adrenal hyperplasia (CAH). Pre-analytical sample processing is critical for assay specificity and accuracy; however, it is laborious and time-consuming. This study describes the development and validation of a new Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS) method for the simultaneous quantification of five steroids: 17OHP, S, Δ4-A, F and cortisone (E) in blood spots from newborns. Whole blood was eluted from a 5.00 mm dried blood spot by an aqueous solution containing the deuterium-labeled internal standards d8-17OHP and d4-cortisol. The steroids extracted from blood spot into aqueous solution were subsequently purified via Extelut mini NT1 column using diethylether. The extracts were evaporated and quantified using LC–MS/MS. The detection limit was 0.25 ng/mL for 17OHP and S, 0.4 ng/mL for Δ4-A and 0.5 ng/mL for F and E. The limit of quantification was 0.5 ng/mL for 17OHP, S and Δ4-A and 1 ng/mL for F and E. Precision for 17OHP, S, Δ4-A at concentrations of 0.5, 2, and 8 ng/mL ($n = 5$) in fortified steroid free serum samples was 1.3–3.5% (intra-assay CV) and 7–14.8% (inter-assay CV). Precision for F and E at concentrations of 5 and 20 ng/mL was 1.5–4.8% (intra-assay, CV%) and 6–15% (inter-assay, CV%). Accuracy was calculated at concentrations of 0.5, 2, and 8 ng/mL for 17OHP, S and Δ4-A and ranged from −0.3 to 0.2%, while for F and E it ranged from −3.2 to 0.2%. Relative recoveries at concentration 2 ng/mL and 8 ng/mL for 17OHP, S, Δ4-A and at 5 ng/mL and 20 ng/mL for F and E ranged from 55% to 80%. Reference intervals were estimated for all steroids in newborns (on day 3). The steroid profile assay herein described is sensitive, specific and accurate and involves a simple pre-analytical sample manipulation; it is therefore suitable for routine analysis and provides data for samples within normal range as well as those with elevated levels. For the first time to our knowledge, cortisone levels are reported in dried blood spots from newborns.

1. Introduction

The simultaneous measurement of multiple steroids provides more valuable clinical information than single steroid assays, this rendering steroid profiling in steroid diagnostics highly desirable [1,2]. Steroid profiling is highly effective for accurate diagnosis of congenital adrenal hyperplasia (CAH), a disease characterized by inherited enzyme deficiencies in steroidogenesis. The classical form of CAH, due to 21-hydroxylase deficiency, is the most frequent, accounting for almost 95% of all cases, whereas 11β-hydroxylase deficiency accounts for 5% of all cases [3,4]. Other enzyme deficiencies are rarely found. These enzyme defects lead to reduced cortisol biosynthesis, which in turn, because of hypothalamus–pituitary–adrenal axis negative feedback, results in the accumulation of steroid precursors such as 17α-OH progesterone and 11-deoxycortisol as well as the androgens Δ4-androstenedione, dehydroepiandrosterone and testosterone. When the enzyme defects are also present in the mineralocorticoid biosynthetic pathway, this may lead to salt-wasting crisis.

Neonatal screening for diagnosis of CAH requires the specific and accurate determination of adrenal steroids; however, the lack of specificity in immunoassays due to interferences from other

* Corresponding author. Tel.: +30 210 7462682; fax: +30 210 7462682.
E-mail address: pmoutsatsou@med.uoa.gr (P. Moutsatsou).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.03.048

endogenous steroids and lipids as well as matrix effects results in poor diagnosis and a high rate of false positive results [2]. Moreover, newborns under stress as well as pre-term neonates characterized by delayed maturation of 11β-hydroxylase, although they are not CAH cases, they exhibit high 17α-OH progesterone levels [5]. Several groups have developed single steroid assays or steroid profiles using tandem mass spectrometry to minimize the high number of false positive results in neonatal screening produced by immunoassays [6–8]. Of note, Janzen et al. [9] improved the specificity of steroid profiling in blood spots by using LC–MS/MS thus allowing the accurate detection of 17α-OH progesterone, cortisol, 11-deoxycortisol, Δ4-androstenedione and 21-deoxycortisol for CAH diagnosis.

The sample preparation is an important aspect of steroid determination by tandem mass spectrometry. Various techniques have been employed, including liquid–liquid extraction and solid phase extraction, while derivatisation, protein precipitation and/or centrifugation step may take place beforehand in most procedures [5,6,8,9]. Among the several approaches is on-line extraction coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS), which yields high-speed, precise and sensitive assays. However, on-line extraction procedures are recommended in high throughput laboratories. In medium-size laboratories, sample preparation is carried out manually and the application of rapid, simple pre-analytical sample processing enabling a sensitive, precise and specific assay is highly desirable. In the present study, we developed a simple, fast LC–MS/MS method with minimal sample manipulation which provides accurate detection of 17α-OH progesterone (17OHP), 11-deoxycortisol (S), cortisol (F) and Δ4-androstenedione (Δ4-A) in blood spots suitable for the screening and diagnosis of CAH in neonates.

Recent studies suggest that alterations in 11β-hydroxysteroid dehydrogenase type II [2] (11β-HSD2) and in the cortisol/cortisone ratio may be involved in "foetal programming" of adult disease such as hypertension and diabetes type II [2]. There are, to the best of our knowledge, no studies so far which determine cortisone in neonates; we therefore included cortisone (E) in the steroid profile and determined all aforementioned five steroids in blood spots from 50 healthy neonates.

2. Experimental

2.1. Chemicals and steroids

Standards of 17OHP, Δ4-A, F, E and S (all purity 99%) were purchased from Sigma (St. Louis, MO, USA), d4-cortisol (98% purity) and d8-17α-hydroxyprogesterone were obtained from Cambridge Isotope Laboratories. Methanol, distilled water and formic acid of high analytical grade were purchased from Merck (Darmstadt, Germany). Extrelut NT1 mini columns, filled with granular Kieselguhr were also obtained from Merck (Darmstadt, Germany). Sterile glass tubes for blood collection were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). The dried blood spot cards were purchased from Whatman S&S 903.

2.2. Filter paper blood specimen collection

Filter paper blood spot samples were obtained between 8.00 and 10.00 a.m. from 50 healthy full-term infants (on third day of life), born at the Department of Obstetrics and Gynaecology in "Aretaieio" hospital (Athens, Greece) during Guthrie test screening. All the parents signed informed consent and the study was approved from the local ethic medical committee. The blood samples were collected into dried blood spot cards and stored at −20 °C until analysis.

2.3. Instrumentation and analytical conditions

The LC chromatograph was a Model 1200 series (Agilent Technologies, Boeblingen, Germany) which consisted of a binary pump equipped with an on-line solvent degasser unit and an autosampler. HPLC analysis was performed on a 5-μm particle size C18 column [Hypersil Gold; 50 mm × 2.1 mm i.d; Thermohypercil] at a flow rate of 0.3 mL/min at 30 °C. The injection volume was 40 μL. Chromatography was performed by isocratic reversed phase separation and the mobile phase consisted of water/acetonitrile (54:46, v/v) containing 0.1% formic acid. The total run time was 3.00 min.

An API 5000 triple-quadropole mass spectrometer (Applied Biosystems/MDS Sciex) was used, operated in positive ion mode with atmospheric pressure chemical ionization (APCI) interface coupled to the HPLC system. The following instrument settings were used: collision gas (GAD): 5 L/min, curtain gas flow (nitrogen):15 psi, ion source gas 1 (GS1): 55 L/min, nebulizer current (NC): 5.0 μAm, source temperature: 400 °C. At these settings, analytes and internal standards yielded protonated molecules $[M+H]^+$ and were optimized for maximum ion yield by infusion of 50 ng/mL of 50% aqueous methanolic solution containing 0.1% formic acid, for each individual compound. Sample analysis was performed in the multiple reaction monitoring (MRM) mode using a dwell time of 150 ms per transition to detect the daughter ions. System control and data acquisition was performed with Analyst™ 1.5 software (Applied Biosystem/MDS SCIEX).

2.4. Analytical procedure

2.4.1. Preparation of glassware

All glassware was siliconized with 1% dimethylchlorosilane in toluene to avoid possible losses owing to sorption on glassware [10].

2.4.2. Preparation of serum free of steroids

The preparation of steroid – free serum was carried out using activated charcoal as described by Aburuz et al. [11]. Four grams (4 g) of activated charcoal were added to 100 mL serum, and mixed using a magnetic stirrer for 2 h. The mixture was then centrifuged for 3 h at 3000 rpm (1610 × *g*). After centrifugation the supernatant serum was filtered using sintered glass filter (grade 4). A pool of serum, free of major medical problems or medication known to affect the steroid metabolism, was kindly supplied from the medical diagnostic laboratories Biomedicine S.A (Athens, Greece). A pool of steroid-free serum was used for the preparation of calibrator and control samples.

2.4.3. Preparation of dried blood spot (DBS) calibrators and controls and blank samples

Dried blood spot calibrators and controls were prepared from venus blood using a method reported by Higashi et al. [12]: whole blood (10 mL) of a healthy male volunteer was collected in a heparinized tube and centrifuged at 1500 × *g* at room temperature (15 min) to be separated into plasma and blood red cells. The obtained plasma (4 mL) was discarded. The red blood cells were washed with saline (30 mL) and centrifuged at 1500 × *g* at room temperature for 15 min. This procedure was repeated four times. The washed cells were combined with charcoal-treated serum at a ratio of 55:45 (v/v) (artificial blood).

Artificial blood (1 mL) was placed in glass tubes and was spiked with an appropriate amount of stock methanolic solution of 17OHP and S (2 ng/mL and 250 ng/mL, respectively) to yield calibrators of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 ng/mL and controls of 0.5, 2 and 8 ng/mL. Another stock methanolic solution was prepared containing a mixture of F, E and Δ4-A (10.0, 10.0 and 1.0 μg/mL, respectively). An appropriate amount of this stock methanolic solu-

tion was also added to artificial blood to yield calibrators of 5.0, 10.0, 20.0, 40.0 and 80.0 ng/mL for F and E and 0.5, 1.0, 2.0, 4.0, 8.0 ng/mL for Δ4-A. Blood control samples were also prepared at concentrations of 5.0 and 20 ng/mL for F and E and 0.5, 2.0 and 8.0 ng/mL for Δ4-A. It is important to say that for blood controls new methanolic stock solutions of steroid mixture at the above concentrations for each substance were prepared, different than those used for blood calibrators. Blood calibrators, controls and blank samples were placed in an ultrasonic waterbath for 60 min. An aliquot (56 μL) of each blood calibrator, control and blank was spotted on a Whatman 903 card and left to dry overnight. For blank samples artificial blood (56 μL) was spotted unspiked on a Whatman 903 card. The blood spot cards were kept at −20 °C until analysis.

2.4.4. Dried blood spot sample preparation

A 5 mm disk (equal to 8.2 μL of whole blood) was punched out of each dried blood spot calibrator, control and sample into a disposable glass tube. A methanolic solution (3 μL) of the internal standards d8-17α-hydroxyprogesterone (IS1) and d4-cortisol (IS2) (0.5 and 5 ng, respectively) was added onto punched discs along with 1 mL distilled water. The punched disks were allowed to elute at room temperature in an ultrasonic waterbath for 60 min. The blood spot eluate was then loaded onto an Extrelut® NT1 mini column. After distribution of the aqueous phase on the column, extraction of steroids from the blood spot eluate was performed with 2× 3 mL diethylether inside silanized glass tubes with a conical end. The eluate was evaporated at 40 °C under stream of nitrogen. Methanol–water solution (50:50, v/v, 100 μL) was added to reconstitute the dry residue and this solution was transferred to LC autosampler vials.

2.4.5. Quantification

The standard curves were constructed by plotting peak area ratios (17OHP/d8-17α-hydroxyprogesterone, S/d4-cortisol, F/d4-cortisol, E/d4-cortisol and Δ4-A/d8-17α-hydroxyprogesterone) versus the concentrations of the steroids. 1/× weighting was used to ensure maximum accuracy at the lower concentrations.

2.5. Statistical evaluation of data

Means and standard deviations as well as the standard regression curves were determined using EXCEL® software (Micrososft Corporation, USA). The mean values and the reference intervals for each steroid hormone were estimated by using the @Risk 4.5 software (Pallisade Corp., New York, USA).

2.6. Method validation

2.6.1. Matrix effect

The matrix effect (ME) was calculated according to Matuszewski et al. [13] and expressed as the ratio of the mean peak area of the analyte spiked postextraction in the blood spot extract (B) to the mean peak area of the same analyte standards in methanolic solution (50%, v/v) (A) multiplied by 100, i.e. (B/A × 100). The ME was evaluated at 0.5, 2.0, 4.0 8.0, 32.0 ng/mL for 17OHP, S and Δ4-A and at 5.0, 10.0, 20.0, 40.0, 80.0, for F and E. The analyses were repeated five times at each concentration. A value of 100% indicates that the response in the methanolic solution and in the blood spot extract are the same and no matrix response is observed. A value of >100% indicates ion enhancement and a value <100% indicates an ion suppression. Ion suppression was also evaluated qualitatively according to Janzen et al. [14] by postcolumn infusion. Extracted blood spot samples at 5 ng/mL for each steroid were injected under the chromatographic conditions used. An infusion pump is used to deliver a constant flow of steroids (prepared at 1 ng/mL) at a flow rate of 10 μL/min into the extracted blood spot samples (HPLC eluent) at a point after the chromatographic column and before the mass spectrometer ionization source.

2.6.2. Recovery

Blood spots (5 mm, equal to 8.2 μL of whole blood) fortified with standard amounts of the analytes at levels of 2.0 and 8.0 ng/mL for 17OHP, S, Δ4-A and at 5.0 and 20 ng/mL for F and E were submitted to extraction procedure and the dry eluate was spiked with ISs at 0.5 and 5.0 ng/mL (for d8-17α-hydroxyprogesterone and d4-cortisol respectively) (procedure I).

Blank blood spots (5 mm, equal to 8.2 μL of whole blood) were also submitted to extraction procedure and then the dry eluate was spiked with standard amounts of the analytes at levels of 2.0 and 8.0 ng/mL for 17OHP, S, Δ4-A and at 5.0 and 20 ng/mL for F and E. The dry eluate was also spiked with ISs at 0.5 and 5.0 ng/mL (for d8-17α-hydroxyprogesterone and d4-cortisol respectively) (procedure II). The calculation for the relative recoveries of each analyte was based on the formula: relative recovery = peak area ratio for extracted analyte (procedure I)/peak area ratio for unextracted analyte (procedure II) × 100.

2.6.3. Accuracy and precision

The accuracy and the precision (intra-day and inter-day) were calculated at three concentrations (0.5, 2 and 8 ng/mL) for 17OHP, S and Δ4-A and at two concentrations (5 and 20 ng/mL) for F and E over a 5-day period. Artificial blood was spiked at above concentrations (control samples), were spotted onto filter card and were analyzed on each day of the 5-day validation ($n = 5$) at each concentration. Accuracy was estimated as the mean relative error (RE%) and precision (intra- and inter-) as the coefficient of variation (CV%).

2.6.4. Lower limit of quantification (LLOQ) and limit of detection (LOD)

In general, the observed LC–MS/MS response for any analyte at the LLOQ should be at least five times greater than any response detected from the blank dried blood spot extract. The acceptable LLOQ is defined as the lowest analyte concentration that gives accuracy within ±20% (bias) of the nominal value and inter-assay imprecision (CV) ≤20% [6]. In addition, according to signal-to-noise (S/N) criteria, the LLOQ is determined as the lowest analyte concentration giving a minimum signal-to-noise ratio (S/N) >8:1 in replicate analyses ($n = 10$) [15]. The limit of detection (LOD) is determined as the lowest analyte concentration giving a minimum signal-to-noise ratio (S/N) >3:1 in replicate analyses ($n = 10$) [16].

The LLOQ and LOD of the proposed method were calculated according to these criteria.

3. Results and discussion

3.1. LC–MS/MS analysis

For the MS/MS operation, the ionization efficiency of the analytes and internal standards in the mobile phase were tested in electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) positive mode. However, detectable signals of the most effective intensities were observed only with APCI positive mode for all steroids. Steroids were injected separately in a continuous flow of 10 μL/min and the MS/MS conditions were optimized. Table 1 shows the optimized MS/MS parameters for each steroid and internal standard..

Table 1
Optimal MRM functions and MS/MS parameters for the analysis of steroids and internal standards.

Name	Mass transition (Da)	Declustering potential (V)	Collision energy (eV)	Entrance potential (V)	Collision cell exit potential (V)
17OHP	331.2 → 97.0	90	34	10	11
S	347.3 → 109.0	111	43	10	16
Δ4-A	287.3 → 97.0	105	35	10	12
F	363.3 → 121.1	111	43	10	14
E	361.1 → 163.0	200	40	10	14
d8-17α-hydroxyprogesterone	339.2 → 100.0	90	34	10	11
d4-cortisol	366.9 → 121.2	111	43	10	14

3.2. Method validation

3.2.1. Matrix effect

According to Taylor [17] the two main techniques used to determine the degree of matrix effects on an LC–MS/MS method are postextraction addition and postcolumn infusion. In present method the matrix effect was assessed with both techniques.

The postextraction technique provides information about matrix effects at the point of elution of the analyte of interest [17]. From the postextraction addition experiment the mean matrix effect was calculated for each steroid and for 17OHP was 109.10%, for S was 90.50%, for Δ4-A was 105.34%, for F was 95.47% and for E was 92.47%. According to Matuszewski et al. [13] under the same chromatographic and extraction conditions a very small enhancement was observed for 17OHP and Δ4-A and a very small ion suppression was observed for S, F and E.

According to Taylor [17] the postinfusion technique enables the influence of the matrix on analyte response to be investigated over the entire chromatographic run. From the results obtained decreased intensity of the baseline in the mass transition of the steroids at a flow rate 300 μL/min was not observed at any of the retention time region of the steroids, and therefore none of the steroids was affected by ion suppression.

Both techniques confirm that the matrix effect in present study is not significant. This can be explained by the fact that atmospheric pressure chemical ionization is less prone to matrix effect than electrospray [13,17].

3.2.2. Recovery

The efficiency of the extraction technique is determined as relative recovery and is shown in Table 2. The relative recovery for the steroids ranged from 55 to 80%. Of all steroids Δ4-A showed the lowest recovery. However, the addition of internal standards in blood spots before extraction compensates for any inherent loss during the whole procedure. Our results are in agreement with previous reports [9] in which the recovery for 17OHP, S, Δ4-A and F ranged from 60.8% to 89.4%, with Δ4-A showing the lowest recovery (60.8%). The use of internal standards compensates for losses in recovery or change in response due to matrix interference [15].

Table 2
Relative Recoveries of steroids in fortified blood spots at 2.0 and 8.0 ng/mL for 17OHP, S and Δ4-A and at 5.0 and 20.0 ng/mL for F and E, respectively. Relative recovery was determined based on calculation of peak area ratio for extracted analyte/peak area ratio for unextracted analyte × 100%.

Steroid name	Relative recovery (%) (2 or 5 ng/mL)	Relative recovery (%) (8 or 20 ng/mL)
17OHP	57	71
S	68	79
Δ4-A	55	64
F	78	80
E	71	71

3.2.3. Specificity

The specificity of the method was demonstrated using blank blood spot samples ($n = 3$), fortified blood spot samples at 0.5 ng/mL (LLOQ) for 17OHP, S and Δ4-A and at 1 ng/mL (LLOQ) for F and E and real biological blood spot samples (from a female newborn). The expectation is that the chromatogram obtained from fortified blood spot samples and real biological blood spot samples should be free from interfering peaks at the retention time region of the analytes or ISs. In addition, none of the representative peaks generated from the analytes will appear in the chromatogram obtained from blank blood spot samples.

According to our results in the blank blood spot samples, there are no responses of interfering peaks at the corresponding retention times of the ions being monitored for each of the analytes, including those of both internal standards (Fig. 1A).

Fig. 1B shows a normalized extracted ion chromatogram (XIC) for a fortified blood spot sample at the LLOQ level of all analytes. We observed that the representative peaks of the analytes and ISs at their corresponding retention time generated in a fortified blood spot sample or in female newborn blood spot sample (Fig. 2) do not appear at the corresponding retention time region of analytes in a blank blood spot sample (Fig. 1A). The comparison between Fig. 1A and B as well as between Figs. 1A and 2 indicated that our LC–MS/MS method is of high specificity for the analytes of interest at the LLOQ level and that there was no response of interferences from endogenous compounds at the retention time region of the steroids of interest in real biological samples.

3.2.4. Linearity

The linearity of the method was tested by inter-assay analysis of the standard curves of blood spot calibrators ranging from 0.5 to 32.0 ng/mL for 17OHP, S and from 0.5 to 8.0 ng/mL for Δ4-A. For F and E, the standard curve ranged from 5.0 to 80 ng/mL. It should be mentioned that the concentration ranges were chosen after consideration of the normal reference values for steroids in good-health full-term neonates [14]. Peak area ratios (analyte/internal standard) were plotted against concentration. The slopes, the intercepts and the correlation coefficients were determined for a 5-day validation by linear regression analysis. The slope, the correlation coefficient and the intercept of the standard curves are given for each of the steroid in Table 3. The determination coefficient (R^2) was greater than 0.992 for all steroids.

3.2.5. Accuracy and precision

According to the acceptable limits for precision and accuracy, both CV% and RE% must be ≤15% except at the detection limit. The results obtained for accuracy and precision (intra- and inter-) are summarized in Table 4.

3.2.6. Lower limit of quantification (LLOQ) and limit of detection (LOD)

The LLOQ was calculated to be at the level of 0.5 ng/mL for 17OHP, S and Δ4-A and at the level of 1 ng/mL for F and E in a fortified blank blood spot. Fig. 1B shows a normalized extracted

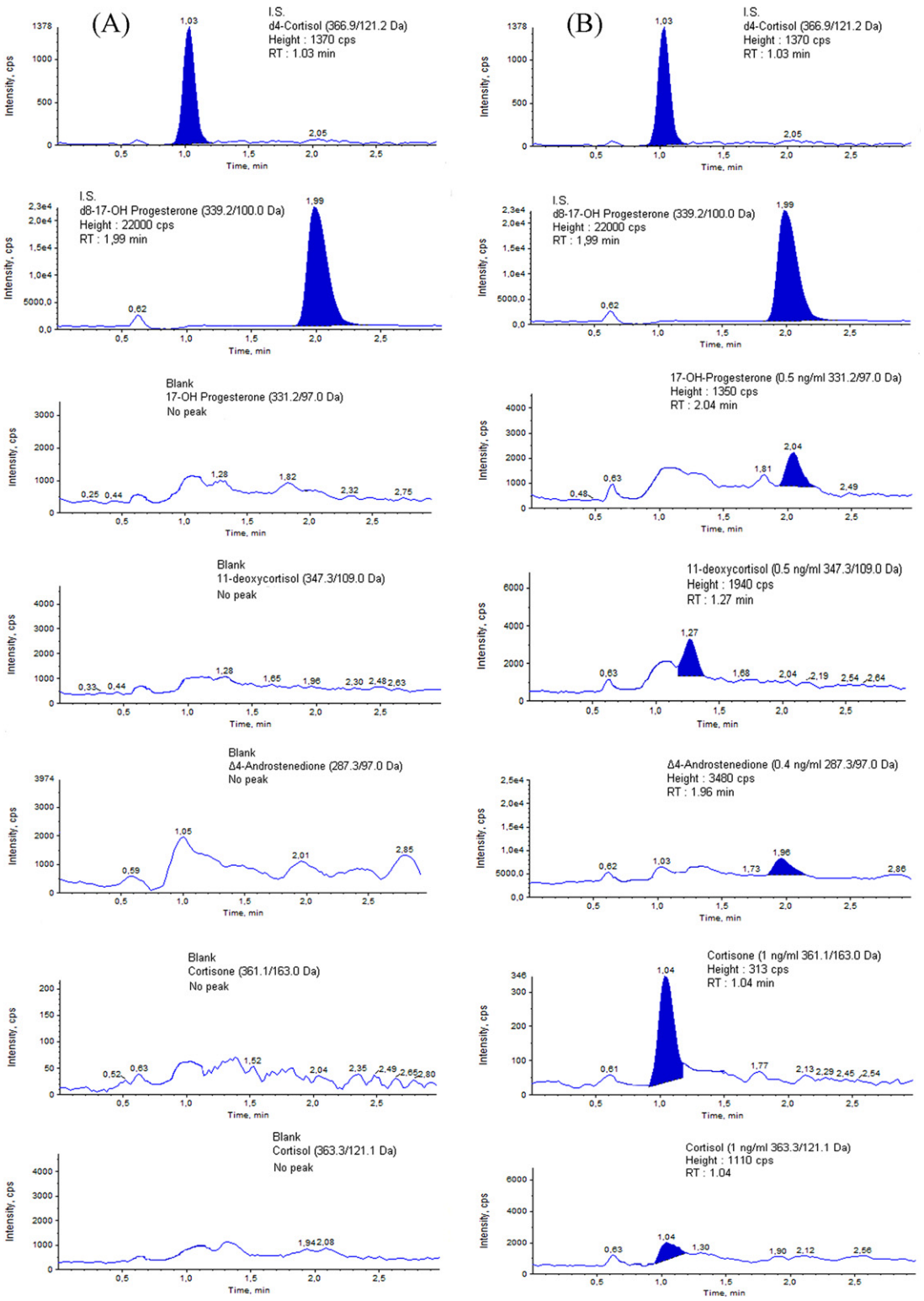

Fig. 1. MRM chromatogram of a blank blood spot sample fortified with d8-17α-hydroxyprogetserone and d4-cortisol (A) in comparison with the MRM chromatogram of a blood spot sample fortified at 0.5 ng/mL (LLOQ) for 17OHP and S, at 0.5 ng/mL (LLOQ) for Δ4-A and at 1 ng/mL (LLOQ) for F and E (B). Peak heights are given for better evaluation of the background noise. In the blank blood spot sample (A) there are none of the representative peaks generated from the steroids in the LLOQ blood spot sample.

ion chromatogram (XIC) of a spiked blank blood spot at 0.5 ng/mL for 17OHP, S and Δ4-A and at 1 ng/mL for F and E.

Under the chromatographic and detection conditions used the LOD for 17OHP, S was calculated to be at the level of 0.25 ng/mL, for Δ4-A at the level of 0.4 ng/mL and for F and E at 0.5 ng/mL in fortified blank blood spots.

The LOD and LLOQ values of the proposed method are considered adequate for monitoring steroid concentration in newborn samples. Moreover, the LOD and LLOQ are lower than or in agreement with the previously published LC–MS/MS methods in newborn screening [9,12,18].

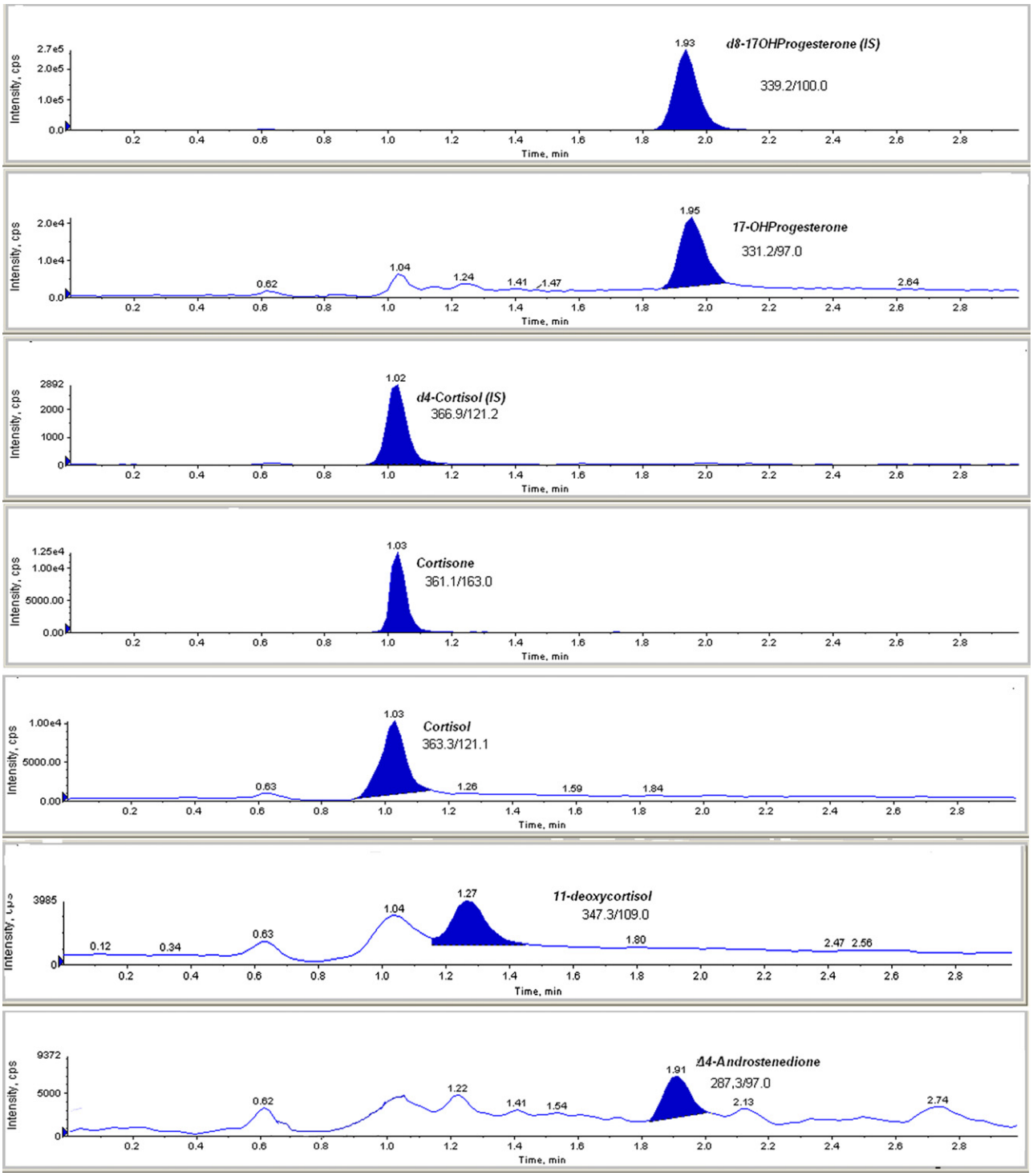

Fig. 2. MRM chromatogram of a blood spot from a healthy full-term female newborn. The levels of steroids were found to be at: 1.39 ng/mL for 17OHP, 0.69 ng/mL for S, 70.0 ng/mL for F, 2.0 ng/mL for Δ4-A and 39.2 ng/mL for E.

Table 3
Linearity equation parameters ($y = ax + b$).

Compound	Slope (a)	Intercept (b)	Correlation coefficient (R^2)
17OHP[b]	0.031	0.003	0.9995
S[b]	0.816	0.300	0.9964
Δ4-A[c]	0.088	0.029	0.9995
F[a]	0.179	0.396	0.9997
E[a]	0.085	0.022	0.9999

[a] Calibration standard curve for F and E ranges from 5 to 80 ng/mL.
[b] Calibration standard curve for 17OHP and S ranges from 0.5 to 32 ng/mL.
[c] Calibration standard curve for Δ4-A ranges from 0.5 to 8 ng/mL.

Table 4
Intra-day and inter-day precision and accuracy (relative error, RE%) ($n = 5$).

Compound	Precision		Accuracy	Precision		Accuracy	Precision		Accuracy
	0.5 ng/mL			2.0 ng/mL			8.0 ng/mL		
	Intra-day (CV%)	Inter-day (CV%)	(RE%)	Intra-day (CV%)	Inter-day (CV%)	(RE%)	Intra-day (CV%)	Inter-day (CV%)	(RE%)
17OHP	2.5	12.3	2.4	2.3	9.4	0.5	1.4	12.4	0.5
S	3.4	11.2	2.9	1.3	13.0	0.7	1.4	6.8	−0.3
Δ4-A	2.9	14.5	3.2	1.9	11.7	0.3	2.2	11.0	0.6
		5.0 ng/mL			20 ng/mL				
F	1.5	14.8	−2.0	1.3	5.7	0.2			
E	4.8	12.6	−3.2	3.7	11.8	−0.1			

Table 5
Steroid reference intervals (mean ± SD) in healthy full-term male newborns ($n = 23$) and in healthy full-term female newborns ($n = 27$) on third day of life.

Sex	17OHP (ng/mL)	S (ng/mL)	F (ng/mL)	Δ4-A (ng/mL)	E (ng/mL)	(17OHP+Δ4-A)/F	F/E
Male ($n = 23$)	<LLOQ-1.00 (0.48 ± 0.21)	<LLOQ-1.15 (0.50 ± 0.27)	1.80–68.20 (26.90 ± 17.10)	<LLOQ-0.78 (0.50 ± 0.12)	8.90–61.20 (28.70 ± 13.0)	<0.01–0.15 (0.06 ± 0.04)	0.29–1.99 (0.93 ± 0.44)
Female ($n = 27$)	<LLOQ-1.36 (0.60 ± 0.31)	<LLOQ-1.09 (0.52 ± 0.24)	5.80–76.50 (28.30 ± 20.0)	<LLOQ-0.66 (0.45 ± 0.09)	6.80–69.10 (30.30 ± 16.0)	<0.01–0.15 (0.06 ± 0.04)	0.13–2.34 (0.97 ± 0.58)

Table 6
Values of blood spot samples from six healthy neonates aged 3 days determined by LC–MS/MS (Mayo) and LC–MS/MS (our method).

	17OHP (ng/mL)		S (ng/mL)		F[a] (ng/mL)		Δ4-A[b] (ng/mL)		Ratio 17OHP + Δ4-A F	
	LC–MS/MS (Mayo)	LC–MS/MS (our method)	LC–MS/MS (Mayo)	LC–MS/MS (our method)	LC–MS/MS (Mayo)	LC–MS/MS (our method)	LC–MS/MS (Mayo)	LC–MS/MS (our method)	LC–MS/MS (Mayo)	LC–MS/MS (our method)
Neonate 1	0.30	0.50	0.20	<0.50	–	3.00	–	<0.50	0.25	0.33
Neonate 2	0.80	0.50	0.70	<0.50	–	8.44	–	<0.50	0.04	0.06
Neonate 3	1.90	1.60	1.60	1.20	–	20.0	–	0.70	0.08	0.11
Neonate 4	<1.40	<0.50	0.70	0.83	17.5	18.9	0.20	<0.50	0.07	0.05
Neonate 5	1.50	0.97	<0.20	<0.50	9.3	13.9	0.67	<0.50	0.24	0.07
Neonate 6	2.00	1.69	0.20	<0.5	13.9	14.5	1.30	<0.50	0.24	0.15

[a] Mayo Medical Laboratories did not provide individual values for F, but only values for the ratio (17OHP + Δ4-A)/F: (abnormal >2.50).
[b] Mayo Medical Laboratories did not provide individual values for Δ4-A, but only values for the ratio (17OHP + Δ4-A)/F: (abnormal >2.50).

3.3. Quantitative analysis of steroids in blood spots of neonates

All samples from 50 healthy neonates were analyzed by our LC–MS/MS method. A representative MRM chromatogram of a healthy female newborn is depicted in Fig. 2. It is obvious that the relevant steroids are chromatographically well separated from each other and that the endogenous steroids do not interfere with the steroids of the present analysis.

Reference intervals were estimated for all steroids for both genders (Table 5). The results are in agreement with reference interval values reported in previous studies in which steroid analysis was performed by LC–MS/MS [5,8,9,14]. The number of false positive results has been shown to be further reduced when the ratio 17OHP plus Δ4-A divided by F [(17OHP + Δ4-A)/F] is considered in the interpretation [5,19]. This is largely attributed to the fact that newborns not affected with CAH but under stress (e.g. prolonged birth, illness) will show increased F in addition to high 17OHP levels and therefore a low peak-area ratio, thus resulting in normalised 17OHP levels. In view of the above, we also calculated this ratio for all newborns (Table 5). The values obtained (mean ± SD, 0.06 ± 0.04) are comparable with previous reports which suggest a cut-off response ratio of either <3.75 [5] or <2.5 [19].

Our study provides, to the best of our knowledge, the first LC–MS/MS data on measured concentration of cortisone in blood spots from healthy full-term newborns. Because F/E ratio may be an index of 11β-HSD2 activity during the neonatal period, we calculated this ratio in the fifty neonates and provided the respective reference interval values (Table 5).

For comparison purposes, the levels of 17OHP, S, Δ4-A and F were determined in six newborn samples by LC–MS/MS method in Mayo Medical Laboratories (Test Code: 84113, Rochester, Minnesota, USA) [20]. Table 6 shows the comparison between the values for respective steroids obtained from Mayo Medical Laboratories and from our evaluated LC–MS/MS method. The comparison revealed that there is good agreement between the results obtained by both LC–MS/MS methods and that the differences achieved especially in the ratio (17OHP + Δ4A)/F are unlikely to be of diagnostic significance (Mayo's reference range for 17OHP: male <7.0 ng/mL, female <4.1 ng/mL, for S: < 10.1 ng/mL, for F: >2.5 ng/mL).

4. Conclusion

The method herein presented provides accurate quantitative analysis of 17OHP, S, F and Δ4-A in blood spots and is therefore suitable for newborn screening to detect CAH which is caused by deficiency of the most common enzymes, namely, 21-hydroxylase and 11β-hydroxylase.

Our procedure is characterized by good linearity ($R^2 > 0.992$) and accuracy, acceptable recoveries and precision (intra- and inter-assay CV values were less than 12%, while at the LLOQ level CV was <20%) for all steroids studied (Tables 3 and 4). Of note, the values obtained for the analytical characteristics of the present assay were comparable to those reported by similar LC–MS/MS approaches in the literature, thus indicating that the performance of our assay is satisfactory [5,9]. The sensitivity achieved for all steroids was adequate for the diagnosis of CAH in neonates and in some cases it was better than those mentioned in previous LC–MS/MS new-

born screening reports [18,21]. Although derivatisation is one of the common approaches for enhanced MS/MS detection sensitivity for trace analytes [12,22], it is liable to be a source of assay error due to unwanted reactions, e.g. hydrolysis [23]. Therefore, in our study the use of atmospheric pressure chemical ionization (APCI)-MS/MS was adopted in order to increase the sensitivity of the method, as several authors have also reported [12,14,24,25]. Our detailed interference study indicated that the present LC–MS/MS method is very specific for the steroids of interest even at the LLOQ level; there was no peak of interferences from analytes in the blank blood spot sample at the corresponding retention time region of steroids in the fortified blood spot sample at the LLOQ level (Fig. 1B). Consequently, our method is capable of providing meaningful data for low steroid concentration samples (within normal range) as well as for those with elevated levels.

The sample manipulation prior to chromatographic separation is a critical step in LC–MS/MS analysis of steroids for newborn screening for CAH. Compared to previously published reports (which include a manual sample preparation step), our method is advantageous because it uses less laborious pre-analytical sample processing. We omitted a derivatisation step and an extensive clean-up step, i.e. multiple solvent washing and transferring [5,8,9,12,14,21]. The simplified pre-analytical step, the instrumental analysis time of 3 min and the small sample volume (8.2 μL) renders the method suitable for routine application.

A major problem in steroid diagnostics is that reference intervals are often method-specific; therefore, in immunoassays the different degree of interferences results in different reference intervals which are not interchangeable. As a consequence, the follow-up of patients between different laboratories or over time is extremely difficult and may result in misdiagnosis. Obviously, correct reference ranges using accurate assays are urgently needed. We therefore collected such reference data for 17OHP, Δ4-A, S, F and E in blood spots in 50 full-term healthy neonates. The number of neonates included in our study is relatively small; however, the results are indicative of normal values in this group of neonates and the reference intervals determined and herein reported are similar to those described in previous reports [9,14].

Our study provides, to the best of our knowledge, the first data on cortisone levels in neonates. The potential of our assay to measure simultaneously F and E makes it particularly useful for the investigation of the role of the F/E ratio (as a marker of 11β HSD2 activity) in intrauterine growth retardation (IUGR) and in "foetal programming of adult disease", such as hypertension and type II diabetes [2]. Furthermore, the method may be used for the determination of cortisol and cortisone in older children and adults for the purpose of psychobiological and endocrinological studies which require serial measurements of cortisol and cortisone.

In conclusion, we herein report a simple, sensitive, specific and accurate low-cost steroid profile suitable for newborn screening for CAH in routine analysis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank BIOMEDICINE S.A. (Medical Diagnostic Laboratory, Greece) for providing LC–MS/MS laboratory facilities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.048.

References

[1] M.M. Kushnir, A.L. Rockwood, W.L. Roberts, B. Yue, J. Bergquist, A.W. Meikle, Clin. Biochem. 44 (2011) 77.
[2] M. Rauh, Mol. Cell. Endocrinol. 301 (2009) 272.
[3] A. Wedell, Endocrinol. Dev. 20 (2011) 80.
[4] S. Parajes, L. Loidi, N. Reisch, V. Duir, I.T. Rose, R. Hampel, J. Clin. Endocrinol. Metab. 95 (2010) 779.
[5] J.M. Lacey, C.Z. Minutti, M.J. Magera, A.L. Tauscher, B. Casetta, M.M. Cann, J. Lymp, S.H. Hahn, P. Rinaldo, D. Matern, Clin. Chem. 50 (2004) 621.
[6] W. Li, F.L.S. Tse, Biomed. Chromatogr. 24 (2010) 49.
[7] M.M. Kushnir, A.L. Rockwood, W.L. Roberts, E.G. Pattison, A.M. Bunker, R.L. Fitzgerald, A.W. Meikle, Clin. Chem. 52 (2006) 120.
[8] C.Z. Minutti, J.M. Lacey, M.J. Magera, S.H. Hahn, M. McCann, A. Schulze, D. Cheillan, C. Dorche, D.H. Chace, J.F. Lymp, D. Zimmerman, P. Rimaldo, D. Matern, J. Clin. Endocrinol. Metab. 89 (2004) 3687.
[9] N. Janzen, M. Peter, S. Sander, U. Steuerwald, M. Terhardt, U. Holtkamp, J. Sander, J. Clin. Endocrinol. Metab. 92 (2007) 2581.
[10] C.P. Petroff, P.P. Nair, D.A. Turner, Int. J. Appl. Radiat. Isot. 15 (1964) 491.
[11] S. AbuRuz, J. Millership, L. Heaney, J. McElnay, J. Chromatogr. B 798 (2003) 193.
[12] T. Higashi, T. Nishio, S. Uchida, K. Shimada, M. Fukushi, M. Maeda, J. Pharma. Biomed. Anal. 48 (2008) 177.
[13] M.L. Matuszewski, Costanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[14] N. Janzen, S. Sander, M. Terhardt, M. Peter, J. Sander, J. Chromatogr. B 861 (2008) 117.
[15] C. Rossi, L. Calton, G. Hammond, H.A. Brown, A.M. Wallace, P. Sacchetta, M. Morris, Clin. Chim. Acta 411 (2010) 222.
[16] P. Magnisali, M. Dracopoulou, M. Mataragas, A. Dacou-Voutetakis, P. Moutsatsou, J. Chromatogr. A 1206 (2008) 166.
[17] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[18] C.C. Lai, C.H. Tsai, F.J. Tsai, J.Y. Wu, W.D. Lin, C.C. Lee, J. Clin. Lab. Anal. 16 (2002) 20.
[19] D. Matern, S. Tortorelli, D. Oglesbee, D. Gavrilov, P. Rinaldo, J. Inherit. Metab. Dis. 30 (2007) 585.
[20] http://www.MayoMedicalLaboratories.com. Rochester, Minnesota, USA.
[21] C.C. Lai, C.H. Tsai, F.J. Tsai, C.C. Lee, W.D. Lin, Rapid Commun. Mass Spectrom. 15 (2001) 2145.
[22] D. Eyles, C. Anderson, P. Ko, A. Jones, A. Thomas, T. Burne, P.B. Mortensen, B.N. Pedersen, D.M. Hougaard, J. McGrath, Clin. Chim. Acta 403 (2009) 145.
[23] A. Schulze, C. Schmidt, D. Kohlmuller, G.F. Hoffman, E. Mayatepek, Clin. Chim. Acta 335 (2003) 137.
[24] 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.
[25] V.F. Fredline, P.J. Taylor, H.M. Dodds, A.G. Johnson, Anal. Biochem. 252 (1997) 308.