

Improved Specificity of Newborn Screening for Congenital Adrenal Hyperplasia by Second-Tier Steroid Profiling Using Tandem Mass Spectrometry

JEAN M. LACEY,¹ CARLA Z. MINUTTI,^{1,2} MARK J. MAGERA,¹ ANGELA L. TAUSCHER,¹
BRUNO CASSETTA,⁶ MARK McCANN,⁷ JAMES LYMP,⁵ SI HOUN HAHN,^{1,3,4} PIERO RINALDO,^{1,3,4}
and DIETRICH MATERN^{1,3,4*}

Background: Newborn screening for congenital adrenal hyperplasia (CAH) involves measurement of 17 α -hydroxyprogesterone (17-OHP), usually by immunoassay. Because this testing has been characterized by high false-positive rates, we developed a steroid profiling method that uses liquid chromatography–tandem mass spectrometry (LC-MS/MS) to measure 17-OHP, androstenedione, and cortisol simultaneously in blood spots. **Methods:** Whole blood was eluted from a 4.8-mm (3/16-inch) dried-blood spot by an aqueous solution containing the deuterium-labeled internal standard d₈-17-OHP. 17-OHP, androstenedione, and cortisol were extracted into diethyl ether, which was subsequently evaporated and the residue dissolved in LC mobile phase. This extract was injected into a LC-MS/MS equipped with pneumatically assisted electrospray. The steroids were quantified in the selected-reaction monitoring mode by use of peak areas in reference to the stable-isotope-labeled internal standard. We analyzed 857 newborn blood spots, including 14 blood spots of confirmed CAH cases and 101 of false-positive cases by conventional screening.

Results: Intra- and interassay CVs for 17-OHP were 7.2–20% and 3.9–18%, respectively, at concentrations of

2, 30, and 50 $\mu\text{g/L}$. At a cutoff for 17-OHP of 12.5 $\mu\text{g/L}$ and a cutoff of 3.75 for the sum of peak areas for 17-OHP and androstenedione divided by the peak area for cortisol, 86 of the 101 false-positive samples were within reference values by LC-MS/MS, whereas the 742 normal and 14 true-positive results obtained by conventional screening were correctly classified.

Conclusion: Steroid profiling in blood spots can identify false-positive results obtained by conventional newborn screening for CAH.

© 2004 American Association for Clinical Chemistry

Congenital adrenal hyperplasia (CAH)⁸ is caused by inherited defects in steroid biosynthesis, in particular 21-hydroxylase deficiency. The resulting hormone imbalances with decreased glucocorticoids and mineralocorticoids and increased 17 α -hydroxyprogesterone (17-OHP) and androgens can lead to life-threatening salt-wasting crises in the newborn period and incorrect gender assignment of virilized females (1, 2). Hormone replacement therapy when initiated early enables a substantial reduction in morbidity and mortality, making newborn screening for CAH highly desirable. Since 1977, when immunoassays for 17-OHP became available, 35 states and several countries have included CAH in their newborn-screening programs (3). The main reason for an incomplete implementation of CAH newborn screening is the large number of false-positive results associated with traditional immunoassays (1). Premature newborns, for example, are often subjected to unnecessary follow-up investigations for secondary 17-OHP increases that could be attributable to

Departments of ¹ Laboratory Medicine and Pathology, ³ Medical Genetics, ⁴ Pediatric and Adolescent Medicine, and ⁵ Biostatistics, Mayo Clinic College of Medicine, Rochester, MN.

² Department of Pediatrics, John Stroger Jr. Hospital of Cook County, Chicago, IL.

⁶ Applied Biosystems, Monza, Italy.

⁷ Minnesota Department of Health, Minneapolis, MN.

*Address correspondence to this author at: Biochemical Genetics Laboratory-Hilton 330, Department of Laboratory Medicine and Pathology, Mayo Clinic and Foundation, 200 First St. SW, Rochester, MN 55905. Fax 507-266-2888; e-mail matern@mayo.edu.

Received September 15, 2003; accepted November 11, 2003.

Previously published online at DOI: 10.1373/clinchem.2003.027193

⁸ Nonstandard abbreviations: CAH, congenital adrenal hyperplasia; 17-OHP, 17 α -hydroxyprogesterone; LC-MS/MS, liquid chromatography–tandem mass spectrometry; SRM, selected reaction monitoring; and CI, confidence interval.

stress or physiologically delayed expression of 11- β -hydroxylase (4, 5). Furthermore, the specificity of immunoassays is compromised by cross-reactivity of antibodies with other steroids (6). Adjustments of cutoff values for 17-OHP based on birth weight and gestational age also do not significantly reduce the false-positive rate (5, 7).

To improve this situation, we developed and validated a new method based on the simultaneous determination of 17-OHP, cortisol, and androstenedione to achieve a steroid profile better suited to reduce the rate of false-positive results than direct measurement of 17-OHP alone, as described recently by others for plasma and blood spots (8–10).

Materials and Methods

SAMPLES

A total of 857 newborn-screening blood spot samples were provided by the newborn-screening laboratory at the Minnesota Department of Health and Mayo Clinic Biochemical Genetics Laboratory after removal of all personal identifying information. The samples were also blinded with respect to the outcome of routine newborn screening for CAH until testing by liquid chromatography–tandem mass spectrometry (LC-MS/MS) was completed. The analysis of these specimens was approved by the Mayo Clinic's Institutional Review Board.

MATERIALS

17-OHP, androstenedione, and cortisol were purchased from Sigma, and d_8 -17-OHP was purchased from CDN Isotopes Inc. Working solutions were established by diluting stock solutions (1 g/L) prepared in methanol with reverse osmosis water. Dried blood spots for calibration and quality-control monitoring were obtained from the Newborn Screening Quality Assurance Program at the CDC (Atlanta, GA). All other chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

ASSAY PROCEDURES

Additional dried-blood spots for calibration, recovery, precision, and stability studies were prepared as follows. Red blood cells were washed repeatedly with equal volumes of saline and centrifuged, and the supernatant and buffy coat were aspirated and discarded. The washed cells were combined with serum in volumes to obtain a whole-blood pool with a final hematocrit of 55%. A stock solution of 17-OHP was prepared by dissolving 100 mg in 500 mL of ethanol. Calibrators were prepared by diluting the stock solution with saline, which was then added to a whole blood pool to achieve concentrations of 0 (no added 17-OHP), 10, 20, 40, 60, 80, 100, and 160 μ g/L. To hemolyze the blood, it was frozen (-20°C) and thawed; it was then spotted on filter paper (S&S 903; Schleicher & Schuell). After the blood spots were dried overnight at room temperature, they were transferred to zip-lock bags with desiccant and stored at -20°C until use.

SAMPLE PREPARATION AND ANALYSIS

A 4.8-mm (3/16-inch) disc was punched from each dried-blood-spot calibrator, control, and sample and placed in a snap-cap disposable centrifuge tube. A solution of the internal standard (d_8 -17-OHP in water; 500 μ L; 0.375 ng) was added, and the discs were allowed to elute at room temperature on an orbital shaker for 30 min. Diethyl ether (3 mL) was added, and the aqueous phase was extracted by a combination of shaking and vortex-mixing for 1 min. The diethyl ether phase was then transferred by Pasteur pipette to a clean glass tube. Another 3 mL of diethyl ether was added, extracted for 1 min, transferred to tubes, and evaporated to dryness in a 40°C water bath under a gentle stream of nitrogen. We then added 50 μ L of methanol–water (50:50 by volume) to reconstitute the remaining residue and transferred this solution to LC autosampler vials.

METHODS

An API 3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) operated in positive ion mode (source voltage, 5500 V) was used. The system also included a Perkin-Elmer Series 200 autosampler and a Perkin-Elmer Micro LC pump. Separation of 17-OHP, d_8 -17-OHP, androstenedione, and cortisol from the bulk of the specimen matrix was achieved by use of a narrow-bore LC column [Symmetry C_{18} ; 50×2.1 mm (i.d.); Waters]. Autosampler injections of 20 μ L were made, and a gradient elution program was implemented with a reversed-phase mobile phase composed of water (solvent A) and methanol (solvent B). The LC program consisted of a linear gradient from 50% to 100% B in 5 min followed by a washing step at 100% B for 3 min and a subsequent 4-min reequilibration step at initial conditions. The column flow of 250 μ L/min directly entered the TurboIon-Spray ionization source, which was operated with the turbo gas on (6 L/min; sensor temperature, 400°C). 17-OHP, d_8 -17-OHP, cortisol, and androstenedione eluted at times from 2 to 4 min. A 12-min total run was required to elute an unrelated, water-soluble compound released by the filter paper with a retention time of 9 min (data not shown).

All results were generated in positive ion mode with a declustering potential of 36 V. Mass calibration and resolution of both resolving quadrupoles were automatically optimized by use of a poly(propylene)glycol solution introduced by an infusion pump. Unit mass resolution (0.7 atomic mass units full-width at half height) was specified for both Q_1 and Q_3 . Collision-activated dissociation MS/MS was performed through the closed-design Q_2 collision cell, operating with nitrogen as collision gas. The optimum collision energy of 6 V (laboratory frame) was determined automatically by the AutoTune algorithm.

MS/MS product-ion scans of calibrators were acquired in continuous flow mode by connecting an infusion pump directly to the TurboIonSpray probe. For MS/MS optimi-

zation, a 10 $\mu\text{mol/L}$ solution was prepared in methanol-water (50:50 by volume) containing 0.25 g/L formic acid and infused at a flow rate of 10 $\mu\text{L}/\text{min}$. For the selected-reaction monitoring (SRM) mode, the instrument was optimized automatically by the built-in algorithm to monitor the transitions m/z 331.2 to 97.0, m/z 339.2 to 100.0, m/z 287.2 to 97.0, and m/z 363.3 to 121.2 for 17-OHP, d_8 -17-OHP, androstenedione, and cortisol, respectively.

CALCULATIONS AND DEFINITION OF CUTOFF VALUES

The concentration of 17-OHP was calculated by the TurboQuan software (Applied Biosystems/MDS Sciex) based on the area under the curves for 17-OHP and the internal standard (d_8 -17-OHP). To characterize the diagnostic accuracy of the method, we selected various cutoff values for 17-OHP and for another index, the sum of the peak areas for 17-OHP and androstenedione divided by the peak area for cortisol. For each cutoff value, sensitivity and specificity were examined. Exact 95% binomial confidence intervals were computed for each of these characteristics. We considered a test positive only when two indices were above the respective cutoff value.

Results

The positive-mode SRM transitions for 17-OHP, androstenedione, cortisol, and d_8 -17-OHP were determined by infusion of pure calibrators (10 $\mu\text{mol/L}$). Collisionally activated dissociation of 17-OHP yielding products at m/z 97.0 and m/z 109.1 has been reported (9); our instrument tuning algorithm favored the m/z 331.2 to 97.0 transition. Likewise, the internal standard d_8 -17-OHP was optimized for the transition m/z 339.2 to 100.0, reflecting the mass difference between unlabeled and labeled 17-OHP. Androstenedione and cortisol exhibited similar results with the transitions m/z 287.2 to 97.0 and m/z 363.3 to 121.2, respectively.

Shown in Fig. 1 are the steroid profiles for blood spots from a healthy control (Fig. 1A), a case who was subsequently ruled to be a false-positive event (Fig. 1B), and a newborn with CAH (Fig. 1C). As illustrated in Fig. 1B, a large majority of the false-positive cases had a 17-OHP concentration $<10 \mu\text{g/L}$ by LC-MS/MS, accompanied by a substantial cortisol peak. Samples from confirmed cases of CAH characteristically lacked cortisol, whereas 17-OHP and androstenedione were increased. Gradient LC elution was necessary to provide the separation of the steroids, most importantly, from a late eluting unrelated component that gave a strong signal in the SRM transition for the internal standard. Attempts at eliminating this compound were unsuccessful, and its elution before subsequent injections remained important because signal contamination by "ghosting" was evident.

LINEARITY

The linearity of the method for 17-OHP was evaluated by interassay analysis of the blood-spot calibrators from 0 to 160 $\mu\text{g/L}$ over a period of 7 days. The mean slope,

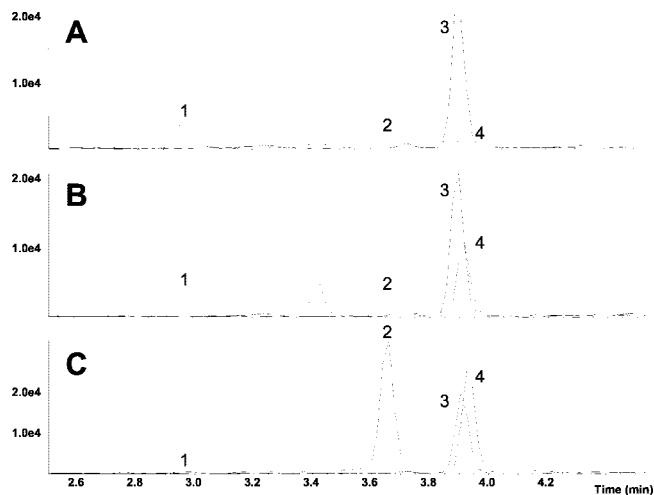


Fig. 1. Steroid profiling by MS/MS in blood spots.

(A), healthy control; (B), false positive by fluorescence immunoassay; (C), confirmed case with CAH. Peak identification is as follows: 1, cortisol; 2, androstenedione; 3, d_8 -17-OHP (internal standard); 4, 17-OHP. The total run time is 12 min to allow for the elution of an unidentified, water-soluble compound released by the filter paper (not shown).

y -intercept, and coefficient of linear regression (r^2) were 0.707 [95% confidence interval (CI), 0.667–0.749], 0.088 $\mu\text{g/L}$ (95% CI, 0.054–0.121 $\mu\text{g/L}$), and 0.995 (95% CI, 0.990–0.999), respectively.

PRECISION, STABILITY, AND SENSITIVITY STUDIES

We determined method precision by analyzing six replicates per day of blood-spot samples containing 2, 30, and 50 $\mu\text{g/L}$ 17-OHP over a 6-day period. The intraassay CVs were 20%, 18%, and 7.2%, respectively. Interassay CVs were 18%, 9.6%, and 3.9%, respectively, for the same samples (Table 1). The stability of prepared specimens was investigated by repeat injection of five blood-spot samples (selected over a concentration range of 2.1–114.7 $\mu\text{g/L}$) with interim storage at 4 °C. The data indicated stability of 17-OHP and d_8 -17-OHP stored up to 72 h after sample preparation. We assessed analytic sensitivity by determining the signal-to-noise ratio for extracted SRM chromatograms, using as a criterion a minimum of signal-to-noise ratio $>3:1$.

METHOD COMPARISON AND CUTOFF VALUES

To compare our LC-MS/MS-based method with currently available immunoassays, we measured 17-OHP, andro-

Table 1. Precision of LC-MS/MS method.

No. of aliquots ^a	Measured 17-OHP, ^b $\mu\text{g/L}$	CV, % (n = 6)	
		Intraassay	Interassay
6	1.9 (0.4)	20	18
6	37.0 (3.6)	18	9.6
6	51.0 (2.0)	7.2	3.9

^a Samples selected from blood spots provided by the CDC Newborn Screening Quality Assurance Program.

^b Mean (SD) of all values.

stenedione, and cortisol in 857 blood spots that were previously analyzed by an immunoassay as part of the Minnesota newborn-screening program. All identifiers were removed from these samples, and results were compared after our analysis. Of the 857 blood spots, 115 tested abnormal by immunoassay, necessitating follow-up evaluations of the newborns. Only 14 were eventually diagnosed with CAH deficiency.

As shown in Fig. 2, the LC-MS/MS method yielded consistently lower values for 17-OHP than the immunoassay. This is not surprising, considering the known limitations of the immunoassay and the ability of MS/MS to detect a specific analyte without interference. Cutoff values were established after the code indicating the final diagnosis for each blood spot was broken. The cutoff for 17-OHP was set at 12.5 $\mu\text{g/L}$ [100% sensitivity (95% CI, 76.8–100%); 91.2% specificity (95% CI, 89.1–93.1%)], and that for the ratio of the sum of 17-OHP and androstenedione divided by cortisol was set at 3.75 [100% sensitivity (95% CI, 76.8–100%); 94.4% specificity (95% CI, 92.7–95.9%)]. Use of both cutoff values together gave 100% sensitivity (95% CI, 76.8–100%) and 98.1% specificity (95% CI, 96.9–98.9%). When we used the 17-OHP cutoff value alone, only 41 of the 101 blood spots that gave false-positive results by the immunoassay were abnormal by LC-MS/MS. The number of false-positive results was further reduced (for a total reduction of 85%) when we included the peak-area ratio of 17-OHP plus androstenedione divided by cortisol in the interpretation (Fig. 3).

Discussion

MS/MS allows rapid, highly sensitive, and specific determination of analytes in complex mixtures, giving higher accuracy and efficiency than other, more traditional analytical platforms such as HPLC and immunoassays (11). Over the last decade, MS/MS has also been incorporated into a growing number of newborn-screening laboratories. In this arena, MS/MS is typically used for the

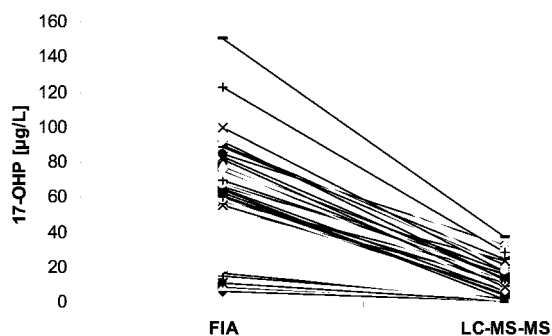


Fig. 2. Comparison of 17-OHP concentrations in newborn-screening blood spots as determined by fluorescence immunoassay (FIA) and LC-MS/MS.

All 54 blood spots were from newborns not affected by CAH, but 42 of these had abnormal screening results by fluorescence immunoassay. Note the consistently lower 17-OHP concentrations measured by LC-MS/MS, demonstrating the high specificity of this technology, which is not affected by cross-reactivity.

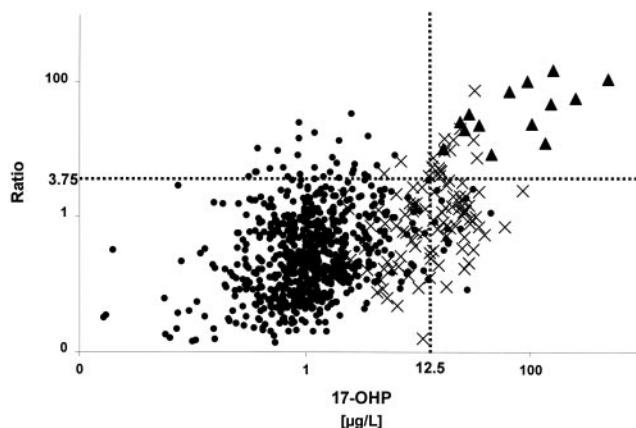


Fig. 3. Distribution of the 17-OHP concentration and the ratio of 17-OHP plus androstenedione divided by cortisol in the study population ($n = 857$).

Data are shown based on immunoassay results: \blacktriangle , newborns with CAH ($n = 14$); \times , false-positive results ($n = 101$); \bullet , normal results ($n = 742$). Note that by applying cutoffs of 12.5 $\mu\text{g/L}$ for 17-OHP and 3.75 for the analyte ratio (dotted lines), the LC-MS/MS method detects all neonates affected with CAH while reducing the number of false positives from 101 to 15 (upper right quadrant).

diagnosis of more than 30 inborn errors of amino acid, organic acid, and fatty acid metabolism by analysis of amino acids and acylcarnitines (12, 13). These analyses are performed simultaneously on the same blood-spot sample and give metabolite profiles that allow more accurate diagnoses by the added value of metabolite ratios (14–17).

CAH is caused by defects in the steroid synthesis pathway, primarily 21-hydroxylase deficiency. The phenotype is dependent on the amount of residual enzyme activity, but most patients with classic 21-hydroxylase deficiency present with the severe form characterized by salt-wasting attributable to secondary aldosterone deficiency and virilization of females attributable to secondary overproduction of testosterone. Salt-wasting and adrenal crises typically do not present until the second or third week of life, with nonspecific symptoms such as poor feeding, vomiting, and diarrhea, which are often erroneously considered of gastrointestinal origin, particularly in male patients whose genitalia are not apparently abnormal. However, because such crises are life threatening, correct diagnosis followed by rapid initiation of treatment is crucial. Electrolyte and hormone replacement are effective and life saving, and are the primary argument for universal newborn screening for CAH. However, 15 state newborn-screening programs, corresponding to approximately one-third of all births in the US, do not provide screening for this disorder because of the poor specificity of existing assays and because the follow-up costs of false-positive results are considered to outweigh the benefit of population screening (1).

The significant reduction in false-positive results we have achieved by use of LC-MS/MS is largely attributable to the fact that newborns not affected with CAH but under stress (e.g., because of prolonged birth or an

infection) will have increased cortisol in addition to secondary accumulation of 17-OHP and, consequently, a low peak-area ratio. A larger multicenter study evaluating the applicability of our approach to blood spots collected up to 5 days of age, as is customary outside of the US, will be reported separately.

We believe that this assay is well suited to become a second-tier screening test. Although confirmatory testing based on molecular genetic analysis of the *CYP21* gene has been advocated, >50 mutations are known and a minimum of 10 different mutations must be screened to identify at least 90% of patients with CAH (18). The biochemical approach would not encounter such problems and potentially could even identify patients with CAH attributable to 11- β -hydroxylase deficiency.

In summary, we developed a new LC-MS/MS-based method for the analysis of 17-OHP and other steroids in newborn-screening blood spots. This assay is more accurate than the conventional immunoassays for the screening for CAH, but the analytical time required for this MS/MS method is currently not compatible with its use as a primary screening test of hundreds of samples per day. Nevertheless, it could significantly reduce the false-positive rate of the conventional immunoassays as a second-tier test when newborn blood spots that yield increased 17-OHP concentrations are reanalyzed by LC-MS/MS to determine the final screening result. Consequently, expensive follow-up investigations of abnormal results can be avoided, and the patient's family can be spared the anxiety associated with a positive screening test.

References

1. Donohue PA, Parker KL, Migeon CJ. Congenital adrenal hyperplasia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, eds. *The metabolic and molecular bases of inherited disease*, 8th ed., Vol. 3. New York: McGraw-Hill, 2001: 4077–115.
2. Speiser PW, White PC. Congenital adrenal hyperplasia. *N Engl J Med* 2003;349:776–88.
3. Pang S, Hotchkiss J, Drash AL, Levine LS, New MI. Microfilter paper method for 17 α -hydroxyprogesterone radioimmunoassay: its application for rapid screening for congenital adrenal hyperplasia. *J Clin Endocrinol Metab* 1977;45:1003–8.
4. Hingre RV, Gross SJ, Hingre KS, Mayes DM, Richman RA. Adrenal steroidogenesis in very low birth weight preterm infants. *J Clin Endocrinol Metab* 1994;78:266–70.
5. Nordenstrom A, Wedell A, Hagenfeldt L, Marcus C, Larsson A. Neonatal screening for congenital adrenal hyperplasia: 17-hydroxyprogesterone levels and CYP21 genotypes in preterm infants. *Pediatrics* 2001;108:E68.
6. Wong T, Shackleton CH, Covey TR, Ellis G. Identification of the steroids in neonatal plasma that interfere with 17 α -hydroxyprogesterone radioimmunoassays. *Clin Chem* 1992;38:1830–7.
7. Allen DB, Hoffman GL, Fitzpatrick P, Laessig R, Maby S, Slyper A. Improved precision of newborn screening for congenital adrenal hyperplasia using weight-adjusted criteria for 17-hydroxyprogesterone levels. *J Pediatr* 1997;130:128–33.
8. Wudy SA, Hartmann M, Svoboda M. Determination of 17-hydroxyprogesterone in plasma by stable isotope dilution/benchtop liquid chromatography-tandem mass spectrometry. *Horm Res* 2000;53:68–71.
9. Lai CC, Tsai CH, Tsai FJ, Wu JY, Lin WD, Lee CC. Rapid screening assay of congenital adrenal hyperplasia by measuring 17 α -hydroxyprogesterone with high-performance liquid chromatography/electrospray ionization tandem mass spectrometry from dried blood spots. *J Clin Lab Anal* 2002;16:20–5.
10. Kao PC, Machacek DA, Magera MJ, Lacey JM, Rinaldo P. Diagnosis of adrenal cortical dysfunction by liquid chromatography-tandem mass spectrometry. *Ann Clin Lab Sci* 2001;31:199–204.
11. Matern D, Magera MJ. Mass spectrometry methods for metabolic and health assessment. *J Nutr* 2001;131:1615S–20S.
12. Chace DH, Kalas TA, Naylor EW. The application of tandem mass spectrometry to neonatal screening for inherited disorders of intermediary metabolism. *Annu Rev Genomics Hum Genet* 2002; 3:17–45.
13. Matern D. Tandem mass spectrometry in newborn screening. *Endocrinologist* 2002;12:50–7.
14. Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF. Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin Chem* 1993;39:66–71.
15. Van Hove JL, Zhang W, Kahler SG, Roe CR, Chen YT, Terada N, et al. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood. *Am J Hum Genet* 1993;52:958–66.
16. Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW. Rapid diagnosis of maple syrup urine disease in blood spots from newborns by tandem mass spectrometry. *Clin Chem* 1995; 41:62–8.
17. Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL. Rapid diagnosis of homocystinuria and other hypermethioninurias from newborns' blood spots by tandem mass spectrometry. *Clin Chem* 1996;42:349–55.
18. Hughes I. Congenital adrenal hyperplasia: phenotype and genotype. *J Pediatr Endocrinol Metab* 2002;15:1329–40.