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

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A rapid, simple, and specific method was developed for the diagnosis of congenital adrenal hyperplasia (CAH) from dried blood spots on newborn screening cards based on high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS). The usefulness of 17 α -hydroxyprogesterone (17OH-P) determination on dried filter-paper blood samples from patients with CAH caused by 21-hydroxylase deficiency was evaluated. The LC/MS/MS detection of 17OH-P was rapid, <4 min. The intra- and interday accuracy and precision of the method were <7%. Our procedure maintained good linearities ($R^2 > 0.992$) and recovery rate (>83%). We used this new method to directly determine the 17OH-P levels in dried blood specimens from abnormal children of various ages, with a detection limit of 20 ng/ml (~240 pg), to

avoid the time-consuming derivatization steps required by the gas-chromatography/mass spectrometry (GC/MS) method. Four dried filter-paper blood samples of CAH patients (three girls and one boy, 1–14 years old) were all quantified in an LC/MS/MS study and revealed high 17OH-P levels (>90 ng/ml). After treatment, all of the elevated 17OH-P levels either decreased or disappeared. Compared with CAH patients, 17OH-P was nearly undetectable (<20 ng/ml) in the normal infants by LC/MS/MS. This LC/MS/MS assay is not only useful for both diagnosis and monitoring of treatment of CAH in all other age groups, it also can be used as a screening test for CAH infants. In this study, we provided the first data on 17OH-P in dried blood specimens affected with CAH using HPLC/ESI-MS/MS. *J. Clin. Lab. Anal.* 16:20–25, 2002. © 2002 Wiley-Liss, Inc.

Key words: newborn screening; corticosteroid; HPLC/MS/MS; electrospray; MS/MS; metabolic disorders; enzymatic defect

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is a group of diseases whose common feature is an enzymatic defect in the steroidogenic pathway leading to the biosynthesis of cortisol. Screening for CAH in newborns has become a routine part of many programs by measuring levels of 17 α -hydroxyprogesterone (17OH-P) (1,2). Currently, neonatal screening procedures for CAH are based on immunoassays (3,4). This approach, while practical, lacks some degree of specificity because of cross-reacting congeners that cannot be separated from 17OH-P in the direct assay (4–6). These direct immunological procedures are advantageous but are likely to produce false-positive elevated results.

Analytical methods based on mass spectrometry (MS) present the most specific quantitative methods for steroid determination. Analyses of 17OH-P by gas chromatography

(GC) and GC/MS methods, while highly sensitive and specific, require derivatization prior to injection because the compounds are thermally labile and their volatility is too low for direct GC analysis (7–9).

Because of its low detection limit, high specificity, soft ionization, and, more importantly, abundant structural information, electrospray ionization (ESI)-MS and its related techniques have been considered an ideal device for drug and

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screening analysis. Today, tandem MS (MS/MS) has emerged as a powerful analytical tool in clinical biochemical genetics (10). MS/MS was developed as a technique for expanding the scope and efficiency of newborn screening for inherited metabolic disorders (11–13). Liquid chromatography coupled with multidimensional MS (LC/MSⁿ) (14–16) represents a powerful alternative, combining rapidity, easy process, high throughput, accuracy, specificity, and sensitivity. One advantage of using MS instead of ultraviolet (UV) spectroscopy as the detector is that baseline separation may not be needed for a clear identification, and the relative concentration of each compound can be calculated based on its peak area. Unlike GC/MS analysis, LC/MS/MS offers the advantage that the 17OH-P can be analyzed without derivatization. Several publications have proposed the detection of several corticosteroids using LC/MS/MS (17–21). However, large sample volumes of blood or urea were needed in these studies.

For this study, we designed an LC/MS/MS coupled with microtiter plate technology to perform lower-sample volume screening for CAH in the newborn filter-paper blood specimens used in routine newborn screening. Our aim was to develop the first alternative method for the determination of 17OH-P that takes advantage of the analytical versatility, specificity, and high throughput unique to the combination of HPLC and MS/MS. The results of the analysis, and the cost and efficiency of these techniques are discussed.

MATERIALS AND METHODS

Chemicals

Glacial acetic acid, 17 α -hydroxyprogesterone (17OH-P), 6 α -methylprednisolone (6M-P), and related compounds were purchased from Sigma (St. Louis, MO). HPLC-grade methanol was obtained from LAB-SCAN Analytical Science (Labscan Ltd., Dublin, Ireland). Deionized (18 M Ω) water (Milli-Q water system; Millipore Inc., Bedford, MA) was used in the preparation of the samples and buffer solution. Prior to use, the mobile phase was filtered through a 0.45- μ m membrane filter (Gelman Sciences, MI).

Standard Solutions

Standard solutions were prepared at a concentration of 1 mg/ml in methanol. Working solutions were prepared monthly and obtained by successive dilutions from 500 ng/ml to 20 ng/ml. All these solutions were stored in the dark at –20°C.

Collection of Filter-Paper Blood Specimens

Standardized filter-paper forms (standardized Schleicher & Schull filter-paper S&S 903; Dassel, Germany) impregnated with whole capillary blood from CAH patients or 2–5-day-old infants were collected from the Department of Genetics, China Medical College Hospital, Taichung, Taiwan. The patients (three girls and one boy) with confirmed CAH

were 1–14 years old. All patients had the salt wasting form of the disorder, while a few had simple virilization. We collected blood spot samples from these CAH patients to establish corresponding abnormal 17OH-P profiles. All filter-papers containing blood samples were dried at room temperature for at least 3 hr and then stored in polypropylene bags at room temperature until analyzed.

Sample Extraction

The preparation of samples from blood spots consisted of a simple solvent extraction. Four 1/8-inch circles from each blood spot (equivalent to 11.5 μ l of whole blood) were excised automatically from a 0.5-inch (12.7 mm)-diameter dried blood spot into a flat-bottomed 96-well block (individual 250- μ l wells; Corning Inc., Corning, NY) by a Wallac DELFIA DBS puncher (Turku, Finland). An extracted solvent stock solution containing a known concentration of internal standard (50 ng/ml, 6M-P) was prepared and added to each vial (200 μ l). The wells were capped and shaken using a Vibromix 203E flatbed shaker (Tehtnica Co., Zelezniki, Slovenia) for 50 min. Subsequently, using a multichannel pipette, the extracts were transferred into a clean V-bottomed 96-well microplate (individual 220- μ l wells; Corning Inc.). The samples were then placed in an evaporator (Techne Ltd., Cambridge, U.K.) and evaporated to dryness under a gentle stream of dry nitrogen. The residue was reconstituted in 20 μ l of 50% methanol solution, and the plate was covered with aluminum foil and placed in an autosampler tray for HPLC/ESI-MS/MS analysis.

HPLC System

The HPLC system consisted of two Perkin-Elmer Series 200 micropumps (PE-Sciex, Foster City, CA). HPLC analysis was performed on a reversed-phase Polaris C₁₈ column (2 mm i.d. $\times$ 5 cm; particle size, 3 μ m) (MetaChem Technologies Inc.). A guard column (Polaris C₁₈ cartridge; MetaChem Technologies Inc.) was used to prolong the life of the HPLC column. The mobile phase was 1% acetic acid/methanol = 30:70 (v/v), and the flow rate was 200 μ l/min. Separation was performed at ambient temperature. The autosampler was a Perkin-Elmer Series 200 autosampler fitted with a 10- μ l loop (PE-Sciex) and equipped with a 96-well sample plate stack. The HPLC and autosampler systems were all synchronized via a Power Macintosh G3 workstation (sample control 1.4 software).

Electrospray and MS

A bench-top triple quadrupole mass spectrometer API 2000 (PE-Sciex) operated in ion evaporation mode with a TurboIonSpray ionization probe source (operated at 5 kV) was used. The TurboIonSpray ionization probe was operated with the turbo gas on (6 L/min; sensor temperature, 400°C). Nitrogen was used as the curtain, nebulizing, turbo, and collision gas.

For all MS/MS experiments, mass calibration and resolution adjustments (at 0.7 amu full width at half height) on both the resolving quadrupoles were automatically optimized using a poly(propylene)glycol 1×10^{-4} mol/L solution introduced via the built-in infusion pump on the API 2000.

Collision-activated decomposition MS/MS was performed through the closed-design Q_2 collision cell. The collision energy (Q0-RO2) was varied from -20 to -40 V. The orifice (OR) and ring (RNG) voltages were set at 70 and 150 V, respectively. MS/MS spectra were collected in continuous flow mode by connecting the built-in infusion pump directly to the TurboIonSpray probe. For MS/MS optimization, a 10- μ g/ml 17OH-P solution (MeOH/H₂O = 1/1, 0.5% formic acid) was prepared and infused at a flow rate of 5 μ l/min.

For LC/MS/MS, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The instrument was optimized automatically by the built-in algorithm to monitor the 331.2–109.1 m/z and 375.2–357.2 m/z transitions for 17OH-P and the internal standard 6M-P, respectively. Following HPLC separation, the peak areas corresponding to the MRM reaction (dwell time = 400 msec) for 17OH-P were measured relative to that of the MRM reaction (dwell time = 200 msec) of the internal standard 6M-P. A power Macintosh G3 workstation was utilized for data acquisition and processing. Sample control (version 1.4), TurboQuant (version 1.0), and Microsoft Excel (version 6.0) were used for data processing and statistical analysis. Background subtraction and a three-point smoothing algorithm were applied to all ion-chromatograms and viewed using MultiView (version 1.4) software.

Preparation of Calibration Curves, Recoveries, and Method Validation

For the standard curve, the concentration of 17OH-P prepared in plasma was as follows: 500, 300, 250, 200, 150, 100, and 50 ng/ml. Standardized filter-paper forms impregnated with the standard plasma were prepared and analyzed on each validation day. The calibration curve was generated using the results of the calibration samples by linear least-squares regression analysis according to the equation $y = a + b \cdot x$, where y is the peak-area ratio of substance to the internal standard, x is the concentration ratio of substance to the internal standard, a is the intercept, and b is the slope of the regression line.

Absolute recoveries of 17OH-P from filter-paper samples were calculated by comparing the peak areas of analytes extracted from spiked filter-paper samples with those of nonextracted standards.

Method validation was carried out using a set of spiked filter-paper standards (50–500 ng/ml) with 50, 150, 250, and 500 ng/ml quality control samples analyzed in triplicate.

RADIOIMMUNOASSAY (RIA) FOR 17OH-P

The RIA of 17OH-P was carried out with an IMMULITE analyzer (DPC); preparation, setup, dilutions, adjustment,

assay, and quality control procedures were performed according to the IMMULITE operator's manual (22).

RESULTS

MS/MS Analysis

In MS/MS experiments, 17OH-P and 6M-P were directly infused and ESI mass spectra were acquired in MS/MS mode. For 17OH-P and 6M-P, the $[M + H]^+$ (m/z 331 and 375) ion was the most abundant ion in full scan mode, and was selected as the parent ion in MS/MS analysis. For 17OH-P, the collision-induced dissociation of the $[M + H]^+$ parent ion in MS/MS produced two major daughter ions: m/z 97 and 109 (Fig. 1). The latter is probably formed by fission of 9-10 and 5-6 bonds (23) and was used in following MRM analysis. According to a recent study on testosterone, it seems that the absence of substitution in position 11 generates these two fragments, but the mechanism is still unclear (24).

At normal collision energy, spontaneous loss of water (one H₂O for each hydroxyl group fixed on the molecule) and some fragment ions for 6M-P (internal standard) was observed (Fig. 1). The ion at m/z 135 can be explained by cleavages in the B ring (Fig. 1). The ion at m/z 161 for 6M-P contained a 6 α -methyl group, which appears to be specific for Δ^1 -corticosteroids. Unfortunately, no mechanism has been found to

Fig. 1. Positive ion ESI product ion mass spectra of 17OH-P and 6M-P.

explain this fragmentation (21). The major daughter ion, $[M + H - H_2O]^+$ (m/z 357) was used in the MRM analysis.

LC/MS/MS Analysis

To avoid the influence of salts and matrix from the filter-paper specimens on the analytes, and to provide higher specificity for compound identification, each component was separated by HPLC before MS/MS analysis. 17OH-P was extracted from the calibration filter-paper specimens (500 ng/ml) for the LC/MS/MS tests. The applied method was MRM, with Q1 transmitting the $[M + H]^+$ parent ions, and Q3 monitoring the daughter ion signals. It is known that ESI sensitivity can be improved if a mobile phase contains a low concentration of volatile buffer and has a high percentage of organic modifiers. Under optimal conditions (1% acetic acid, 30:70, v/v methanol), 17OH-P and 6M-P were observed by LC/MS/MS (Fig. 2). The monitored reactions and MRM chromatograms for 17OH-P and 6M-P in dried blood specimens are shown in Fig. 2.

Linearity, Sensitivity, and Recovery of the Method

For evaluation of the MRM detection method, standard plasmas containing the 17OH-P were prepared at concentrations of

Fig. 2. Mass chromatograms of 17OH-P and 6M-P obtained from dried blood specimens (containing 500 ng/ml 17OH-P) under MRM operation. Mobile phase: 1% HOAc/methanol 30:70 (v/v).

500, 300, 250, 200, 150, 100, and 50 ng/ml, and 30 μ l of a blank sample (mobile phase) and of each plasma standard were loaded on newborn screening cards. Pooled filter-paper samples were analyzed to determine the precision and accuracy of the employed LC/ESI-MS/MS-MRM. The calibration curve of 17OH-P is shown in Fig. 3. Linearity was evaluated via the R^2 regression coefficient of determination, and values varied from 0.992 to 0.996. The limit of detection (LOD) was 20 ng/ml ($\sim$ 240 pg) based on a signal-to-noise ratio of 3. The efficiency of the extraction technique, determined as recovery, is shown in Table 1. Several kinds of elution buffers and solvents were tested, and an optimal figure of about 85% was obtained with methanol. The coefficients of variation (CV) of recovery of 17OH-P from dried blood spots of uniform size under these conditions were below 4% (Table 1). This suggests that methanol is a suitable eluant for removing 17OH-P from blood spots. The volume of methanol used for extraction was 200 μ l, with no significant increase in the percentage of recovery by increasing the volume. Increasing extraction times did not enhance 17OH-P recovery in this study.

Precision and Accuracy

The accuracy of the method was measured by determining the mean concentration at various concentrations of analyte. Both the intra- and interday accuracy and the precision of the method were determined by triplicate analysis of blank filter-paper spiked with plasma containing 17OH-P at the concentrations used to construct the calibration curves. Accuracy was calculated as percentage error of theoretical vs. measured concentrations. The interday accuracy was 2.38–4.42%, and the intraday accuracy was 3.14–6.63% for 17OH-P (Table 2). Precision was estimated as the CV of the analyses. Intra- and interday CVs are summarized in Table 2. The interday CV of 17OH-P ranged from 4.07% to 5.11%, while the intraday CV was smaller than 6%.

Fig. 3. Calibration curves for 17OH-P in dried blood specimens by LC/MS/MS-MRM.

TABLE 1. Recovery of 17OH-P determinations in filter paper

	500 (ng/mL) (%)	250 (ng/mL) (%)	150 (ng/mL) (%)	50 (ng/mL) (%)
Recovery	83.8	84.6	84.9	85.0
CV (<i>n</i> = 10)	3.9	4.2	3.7	4.4

Infant and Patient Blood Spot Analyses of 17OH-P

All samples from the 200 infants (control groups) and four patients (CAH groups) affected with CAH were analyzed by HPLC/MS/MS. Table 3 shows the results of the quantitation of 17OH-P from dried blood spots in the control and CAH groups through LC/MS/MS and RIA. 17OH-P was undetectable (<20 ng/ml) in the control group by LC/MS/MS, although the internal standard (6M-P) was readily detected in all cases. In the CAH group, however, 17OH-P was readily detectable and could be quantified. The concentration of 17OH-P was >90 ng/ml in all CAH patients, suggesting that quantitation of 17OH-P may be the best discriminant for the detection of CAH.

DISCUSSION

Measuring levels of 17OH-P in newborn filter-paper blood specimens has become a routine part of screening for CAH in newborns. LC/MS/MS analysis in the MRM mode is the most specific and sensitive, as it monitors only selected specific transitions (Figs. 1 and 2). When the quantification of 17OH-P in filter-paper was performed by LC/MS/MS using 6M-P as the internal standard, it was found that this method was successful in achieving accurate quantification. Our procedure maintained good linearities under dilution of 17OH-P on filter-paper ($R^2 > 0.992$). The estimated amounts of 17OH-P were in agreement with the expected amounts, the intra- and interday accuracy and precision were less than 7% (Table 2), and the recovery rate was greater than 83%. The measurements of 17OH-P in dried blood on filter-paper using LC/MS/MS have been shown to be reliable and simple to carry out. The level of 17OH-P in blood specimens of CAH patients can be used to screen the 21-hydroxylase deficiency.

TABLE 2. Accuracy and precision of 17OH-P determinations in filter paper

	Concentration added (ng/mL)			
	500	250	150	50
Inter-day ^a				
Error (%)	3.65	3.06	2.38	4.42
CV (%)	4.60	4.91	4.07	5.11
Intra-day ^b				
Error (%)	4.70	3.81	3.14	6.63
CV (%)	4.86	4.70	4.62	5.80

^a*n* = 9 on three days.

^b*n* = 3 within-day.

TABLE 3. 17OH-P concentration in dried blood spots in normal control and CAH patients by LC/MS/MS

	17OH-P concentrations in ng/mL			
	Sex	Year of life	LC/MS/MS	RIA
Control group (<i>n</i> = 200)		3~5 days	<20	<20
Patients with CAH				
1	XX	7	102 (<20) ^a	128(<20) ^a
2	XX	14	93 (<50) ^a	103(<58) ^a
3	XX	1	120 (<60) ^a	139(<66) ^a
4	XY	8	125	130

^aDried blood spots were obtained after therapy with hydrocortisone.

The sensitivity obtained by the present LC/MS/MS method was inferior to that obtained by the RIA method. Nevertheless, the sensitivity achieved by LC/MS/MS was adequate for determining 17OH-P in CAH patients (17OH-P > 90 ng/ml). According to the preliminary results of neonatal screening for CAH in Taiwan (established by the National Taiwan University Hospital) (25), high-risk babies with 17OH-P levels > 90 ng/ml (as assessed by the RIA method) should be recalled immediately. In our study, the minimum concentration for calibration was 50 ng/ml (detection limit for 20 ng/ml). A cutoff of 50 ng/ml will be used for continued testing for CAH newborn screening in our study, and improvement of the method sensitivity will be tried. By comparing the results from the LC/MS/MS screening with those from traditional screening methods (such as RIA), no false positives or negatives were found in the studied samples (Table 3). In Table 3, the concentrations of 17OH-P in the CAH group are all higher in the RIA analysis. In fact, comparison of our values from the calibrated dried blood samples with those obtained by direct immunologic assays shows that immunoassays, in general, overestimate 17OH-P concentrations (data not shown). Therefore, the RIA is likely to produce false-positive elevated results. Our results prove that the accuracy of LC/MS/MS screening in detecting newborn metabolic disorders (such as CAH) is comparable to that of traditional methods.

Ideally, a sample preparation method should be fast, easy to perform and/or to automate, and solvent-free, providing valuable and reliable screening information. The approach should be economically sound, relatively noninvasive, have minimal interference, and should be sufficiently specific for routine application. The cost-effectiveness of this newborn screening procedure using LC/MS/MS was estimated. In our laboratory, one technician, using a semiautomatic procedure, can prepare two 96-well microplates (a total of 192 samples) in a regular working day. The maximum number of samples that can be analyzed by our tandem mass spectrometer is 384 samples per day. The cost of analysis for each sample is about \$1 (U.S.), depending on the life of the HPLC columns and the kind of machine used in the laboratory. Sample preparation for LC/MS/MS is very easy and requires only 2–2.5 hr. This method was found to be useful for screening infants

with 21-hydroxylase deficiency. This LC/MS/MS system is capable of analyzing additional corticosteroids, which are responsible for at least five CAH diseases, in one analysis: CYP21 (21-hydroxylase) deficiency, CYP11B1 (11 β -hydroxylase) deficiency, CYP17 deficiency, 3 β -HSD deficiency, and CYP11A deficiency. The present method will be applied in the future to other corticosteroids related to CAH, as it is far more effective than any other kind of screening method. Because of these advantages, LC/MS/MS may be a powerful tool for CAH screening in newborns. In addition, we have also been using this method to monitor the treatment of some CAH patients (Table 3). Repeated blood sampling on filter-paper can help improve the monitoring of, and compliance with CAH treatment.

A successful CAH screening method based on LC/MS/MS has been established in our laboratory. As indicated by our study, LC/MS/MS is very suitable for newborn screening of 21-hydroxylase deficiency. Furthermore, it is possible to detect other corticosteroid hormones in blood, and we plan to develop more screening methods for other CAH diseases using LC/MS/MS. Work is in progress to quantify all the corticosteroids related to CAH (e.g., deoxycorticosterone, and 11-deoxycortisol for 11 β -hydroxylase deficiency) in one analysis, and to investigate the use of LC/MS/MS analysis in multisteroid monitoring and the possibility of improving its sensitivity.

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