RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2001; **15**: 2145–2151

RCM

Rapid monitoring assay of congenital adrenal hyperplasia with microbore high-performance liquid chromatography/electrospray ionization tandem mass spectrometry from dried blood spots

Chien-Chen Lai, Chang-Hai Tsai, Fuu-Jen Tsai*, Cheng-Chun Lee and Wei-De Lin
Department of Medical Genetics and Medical Research, China Medical College Hospital, Taichung, Taiwan

Received 31 July 2001; Revised 17 September 2001; Accepted 18 September 2001

17-Hydroxyprogesterone (17OHP) is the most important plasma parameter for diagnosing and monitoring congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency. A rapid, simple, and specific method based on microbore high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (μ-HPLC/ESI-MS/MS) was developed to determine the presence of 17OHP on dried filter-paper blood samples from patients with CAH caused by 21-hydroxylase deficiency. 17OHP from dried blood spots formed by the action of Girard reagent P (GirP) turned out to be a water-soluble hydrazone complex. Derivatization with GirP led to higher ESI sensitivity for 17OHP. The LC/MS/MS detection of GirP-derivatized 17OHP (GirP-17OHP) was rapid ($<$3 min). The method is repeatable and reproducible, with CVs $<$7% and 12%, respectively. This new method was used for direct determination of 17OHP in dried blood specimens obtained from abnormal children and infants of various ages with a detection limit of 10 ng/mL ($\sim$12 μL blood). The method described allows for rapid and reliable measurements of 17OHP in dried blood specimens from patients affected by CAH. Copyright © 2001 John Wiley & Sons, Ltd.

Congenital adrenal hyperplasia (CAH), a disorder caused by a deficiency of the 21-hydroxylase enzyme, is the most common inborn error of the adrenal steroid pathways. Early diagnosis and monitoring of CAH can be lifesaving. Monitoring and screening for CAH patients by measuring levels of 17α-hydroxyprogesterone (17OHP) or other steroids has become a routine part of many programmes.[1,2] Numerous methods have been described to determine these steroid hormones, such as fluorimetry,[3,4] radioimmunoassay (RIA),[5–8] and high-performance liquid chromatography (HPLC).[4,9,10] Most of these, however, are affected by a degree of interference or cross-reactivity with other steroids. Currently, monitoring for CAH or neonatal screening procedures are based on immunoassays.[3,4] This approach, while practical, lacks some degree of specificity due to cross-reacting congeners that are inseparable from 17OHP in the direct assay.[4,11–13]

In recent years, electrospray ionization (ESI) has become an important method for the generation of gas-phase ions from biomolecules for mass spectrometric analysis. In particular, ESI mass spectrometry is highly useful for the analysis of biopolymers such as proteins and nucleotides with readily ionizable functional groups. Due to the low proton affinity of neutral steroids, the ESI sensitivity is poor in comparison to that of these peptides and proteins. Neutral steroids have been less well studied, partly because of the expected lower ESI yields. Thus, in order to improve the ESI sensitivity, derivatization methods designed to form a covalent bond to a moiety containing a permanent positively charged nitrogen atom was applied in this study. The carbonyl group in 17OHP was derivatized with a quaternary ammonium salt, 1-(carboxymethyl)pyridium chloride hydrazide (Girard reagent P), to form a water-soluble hydrazone with a permanently charged pyridine moiety. The main use of Girard reagent P (GirP) or Girard reagent T (GirT, the analog of GirP which replaces the pyridinium fixed-charge moiety with trimethylammonium) is in the separation of mixtures of natural products (mainly steroids) into ketone and non-ketone fractions. Their analytical applications in ESI,[14] spectrophotometric[15] and capillary electrophoresis (CE)[16] analysis of ketosteroids is also well documented. This derivative was selected for its ease of introduction of a permanent positive charge into the molecule of the ketosteroid 17OHP. The positively charged derivative GirP-17OHP is expected to lead to higher ESI sensitivity and to be suitable for high-performance liquid chromatography/mass spectrometry (LC/MS).

Tandem mass spectrometry (MS/MS) has emerged as a powerful analytical tool in clinical biochemical screening,[17] and is about to revolutionize neonatal metabolic screening.[18–21] Liquid chromatography coupled with multidimensional mass spectrometry (LC/MS^n) represents a highly

---

**Correspondence to*: F.-J. Tsai, Department of Pediatrics, Medical Genetics and Medical Research, China Medical College Hospital, 2 Yuh-Der Road, Taichung, 404 Taiwan
E-mail: d0704@www.cmch.org.tw

Contract/grant sponsor: China Medical College Hospital; Contract/grant number: DMR-90-122.

DOI:10.1002/rcm.493

Copyright © 2001 John Wiley & Sons, Ltd.

sensitive, specific, simple, accurate and rapid diagnostic tool for the clinical laboratory.[22] A few publications have proposed the detection of several corticosteroids using LC/MS/MS.[22–27] However, large sample volumes of blood or urine were needed in these studies.

Dried blood spots on filter paper have potential as a collection method due to ease of handling and the small blood sample volumes required. This collection method has been applied in various newborn screening programs.[18–21] Therefore, the purpose of this study was to evaluate the applicability of LC/MS/MS to clinical 17OHP analysis of dried filter-paper blood samples from patients with congenital adrenal hyperplasia caused by 21-hydroxylase deficiency. The results of the analysis, and the relative merits and pitfalls of these techniques, are discussed.

EXPERIMENTAL

Chemicals

Glacial acetic acid, 17α-hydroxyprogesterone (17OHP), 6α-methylprednisolone (6MP), Girard reagent P (GirP) and related compounds were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were obtained from LAB-SCAN Analytical Science (Labscan Ltd. Dublin, Ireland). Deionized (18 mΩ) water (Milli-Q water system; Millipore Inc., Bedford, MA, USA) was used in the preparation of the samples and buffer solution. Prior to use, the mobile phase was filtered through a 0.45-μm membrane filter (Gelman Sciences, Michigan, USA). Blank human whole blood samples were obtained from China Medical College Hospital, Taichung, Taiwan.

Standard solutions

Standard stock solutions were prepared at a concentration of 1 mg/mL in methanol and kept in the dark at −20 °C when not in use. For the calibration, standard human whole blood samples containing 17OHP were prepared at 500, 300, 250, 200, 150, 100, 50 and 30 ng/mL. 30 μL of a blank sample (mobile phase), and of each whole blood spiked standard, were loaded on to newborn screening cards. All newborn screening cards for calibration were dried at room temperature for at least 3 h, and then stored in polypropylene bags at room temperature until analyzed.

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. Patients with confirmed CAH were between 1 and 14 years of age (three girls and one boy). We collected blood spot samples from these CAH patients in order to establish corresponding abnormal 17OHP profiles. Five dried filter-paper blood samples of CAH infants were kindly donated by the National Taiwan University Hosipital. All filter papers containing blood samples were dried at room temperature for at least 3 h, 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 μL of whole blood) were excised from a 0.5-inch (12.7 mm)-diameter dried blood spot and placed into a flat-bottom 96-well block (individual 250-μL wells; Corning Incorporated, USA) using an automated Wallac DELFIA DBS puncher (Turku, Finland). A stock solution containing a known concentration of internal standard (50 ng/mL, 6MP) in methanol was prepared and added to each vial (200 μL). Internal standard 6MP was only used in this extraction step. The wells were capped and shaken using a Vibromix 203E flatbed shaker (Tehtnica Co., Slovenia) for 50 min. Subsequently, using a multi-channel pipette, the extracts were transferred into a clean V-bottomed 96-well microplate (individual 220-μL wells; Corning Inc., USA). Each 96-well microplate was placed in an evaporator (Techne (Cambridge) Ltd, UK) and the solutions evaporated to dryness under a gentle stream of dry nitrogen.

Derivatization of 17OHP

The residue in each well was derivatized with 160 μL of GirP solution (10 mg/mL in ethanol, 0.1% trichloroacetic acid as catalyst), incubated at 65 °C for 50 min, and evaporated to dryness under a gentle stream of dry nitrogen. The GirP-derivatized 17OHP (GirP-17OHP) and 6MP (GirP-6MP) were reconstituted in 30 μL of 50% aqueous acetonitrile. The plate was covered with aluminum foil and placed on an autosampler tray for analysis by μ-HPLC/ESI-MS/MS.

μ-HPLC system

The HPLC system consisted of two Perkin-Elmer series 200 micropumps (PE-Sciex, Foster City, CA, USA). HPLC analysis was performed on a 5-μm C_4 microbore (Vydac) column (50 × 1.0 mm i.d.), operated at ambient temperature. A guard column (C_4 cartridge, Vydac) was used to prolong the life of the HPLC column. The mobile phase was water/acetonitrile (50:50, v/v) and the flow rate was 50 μL/min. At the end of each series, the column was thoroughly rinsed with a mixture of acetonitrile/deionized water (30:70, v/v) at a flow rate of 50 μL/min for 2 h, and stored. The autosampler was a Perkin-Elmer series 200 autosampler fitted with a 10-μL loop (PE-Sciex, Foster City, CA, USA) 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 mass spectrometry

We used a bench-top triple quadrupole mass spectrometer, API 2000 (Perkin-Elmer Sciex, Foster City, CA, USA), operated in ion evaporation mode with a TurboIonSpray ionization probe source (operated at 5 kV). The TurboIonSpray ionization probe was operated with the turbo gas on (5 L/min; sensor temperature, 300 °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 Th full peak width at half height) on both the resolving quadrupoles were automatically opti-

Copyright © 2001 John Wiley & Sons, Ltd.

mized 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 using the closed-design Q_2 collision cell. The collision energy (Q0-RO2) was varied from 30 to 40 eV. The orifice (OR) and ring (RNG) voltages were set at 50 and 360 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 μg/mL steroid derivative solution (MeOH/H_2O, 1:1) was prepared and infused at a flow rate of 5 μL/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 transitions *m/z* 299.4 to 80.1 and *m/z* 321.4 to 80.1 for GirP-17OHP and the internal standard GirP-6MP, respectively. Following HPLC separation, the peak area corresponding to the MRM reaction (dwell time 300 ms) for GirP-17OHP was measured relative to that of the MRM reaction (dwell time 200 ms) of the internal standard GirP-6MP. 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 3-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 concentrations of 17OHP prepared in whole blood were as follows: 500, 300, 250, 200, 150, 100 and 30 ng/mL. Standardized filter-paper forms impregnated with the standard whole blood were prepared and analyzed on each validation day. The calibration curve was generated using the results of the calibration samples by unweighted linear least-squares regression analysis.

Absolute recoveries of 17OHP from filter-paper samples were calculated by comparison of peak areas of analytes extracted from spiked filter-paper samples with those of non-extracted 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 17OHP

The RIA of 17OHP was carried out with the IMMULITE analyzer (DPC, USA), using the procedure listed in the IMMULITE operator's manual for: preparation, setup, dilutions, adjustment, assay and quality control procedures.[28]

RESULTS AND DISCUSSION

MS/MS analysis

For Girard reagent P (GirP)-derivatized 17OHP and 6MP (GirP-17OHP and GirP-6MP), the $[M]^{+2}$ (*m/z* 299 and 321) ion was the most abundant in full scan mode and was selected as the precursor ion in MS/MS analysis. Figure 1 shows the MS/MS spectra of GirP-17OHP and GirP-6MP as obtained by infusion in positive ion mode. The derivatized steroids provided 10 times the ESI-MS/MS sensitivity of the underivatized steroids due to the pre-charged property of GirP-derivatized steroids (data not shown). This observation indicates that derivatization of ketosteroids to their hydrazones will be highly advantageous for the analysis of 17OHP by LC/MS/MS. For GirP-17OHP, the collision-induced dissociation of the $[M]^{+2}$ precursor ion in MS/MS produced several product ions, *m/z* 80, 93, 121, 239, 253 and 260 (Fig. 1(a)). Mass losses observed for GirP-17OHP were 79, 93 and 121 Da. The M-79 (*m/z* 260) fragment is readily explained by the loss of $(C_5H_5)N$ from the Girard derivative moiety, and a corresponding abundant fragment ion, $(C_5H_5)NH^+$, is observed at *m/z* 80. The remainder of the neutral losses, yielding fragment ions at *m/z* 93, 121, 239 and 253, can be explained similarly, as proposed in Fig. 1(a). The internal standard GirP-6MP showed similar fragmentations but the precursor ion $[M]^{+2}$ for 6MP (*m/z* 321.4) was cleaved to product ions (e.g. *m/z* 93 and 121) more effectively (Fig. 1(b)). This lack of surviving precursor ion (*m/z* 321.4) is shown in Fig. 1(b). In fact, the response of the *m/z* 321.4-80.2 MS/MS transition was similar to that of the *m/z* 299.4-80.2 MS/MS transition for the same concentration of 6MP and 17OHP.

LC/MS/MS analysis

In the present LC/MS/MS analysis, 17OHP was extracted from the calibration filter-paper specimens (500 ng/mL) and derivatized with GirP. In order to avoid the influence of salts and matrix from the filter-paper specimens on the analytes, each component was separated by μ-HPLC before MS/MS analysis. The use of a microbore column allowed us to avoid post-column splitting, and 50 μL/min were analyzed by MS directly. The reduction of solvent consumption was an advantage, and the complete sample introduction into the mass spectrometer without splitting made it easier to interface μ-HPLC with MS. The detection method was MRM, to provide optimum sensitivity with good selectivity. Under optimal isocratic conditions (50:50, v/v acetonitrile), GirP-17OHP and GirP-6MP were readily observed by LC/MS/MS. The MRM chromatograms for 17OHP and 6MP in a dried blood specimen are shown in Fig. 2. No other steroids and interferences were observed in this LC/MS/MS analysis.

Since no buffers were used in the mobile phase, approximately 300 samples could be analyzed without interruption for instrument cleaning. Each run required 3 min, and an inter-sample time delay was not needed. The 3-min run-time was sufficient for the autosampler to be ready for the next injection. In this study, the needle and injection port rinses of our autosampler were effective in avoiding carry-over, commonly observed in the sensitive detection that MRM-LC/MS/MS provides. The retention times of the analytes were highly reproducible, indicating that chromatographic stability was not sacrificed by either the lack of buffer or the short column equilibration time. Column pressure was continually monitored and noted at the beginning of the analyses. When the first two plates (192 samples) were removed from the autosamplers, and prior to the installation of the next two plates, the microbore-HPLC column was thoroughly flushed with a mixture of acetonitrile/deionized

Copyright © 2001 John Wiley & Sons, Ltd.

water (30:70, v/v) at a flow rate of 50 µL/min for 2 h. The column was equilibrated with mobile phase, whereupon analysis of the next two plates commenced. The guard column should be replaced after the analysis of 2–3 plates (200–300 samples). The µ-HPLC column pressure remained normal in the present continued study (400–500 samples).

Linearity, sensitivity and recovery of the method

For evaluation of the analytical method, standard human whole bloods containing 17OHP were prepared at 500, 300, 250, 200, 150, 100, 50 and 30 ng/mL, and 30 µL of a blank sample (mobile phase) and each whole blood sample were loaded on to a newborn screening card. These filter-paper standard samples were analyzed as described above, to determine the precision and accuracy of the LC/ESI-MS/MS-MRM method. It was found that the quantification of 17OHP on filter paper performed by LC/MS/MS, using 6MP as internal standard, was of acceptable accuracy and precision. The calibration curve for 17OHP is shown in Fig. 3. Linearity was evaluated via the R^2 regression coefficient of determination, and values varied from 0.994 to 0.997 (median 0.996). The limit of detection (LOD) was 10 ng/mL (~12 µL whole blood) based on a signal-to-noise ratio of 3. The efficiency of this technique was 81–84% for quantities of 17OHP corresponding to the spike concentration range. The coefficients of variation (CV) of recovery of 17OHP from dried blood spots of uniform size under these conditions were below 7%. This suggests that methanol is a suitable eluant for removing 17OHP from blood spots.

Precision and accuracy

The accuracy of the method was measured by determining the mean concentration at various concentrations of analyte. Both the intra- and inter-day accuracy and precision of the

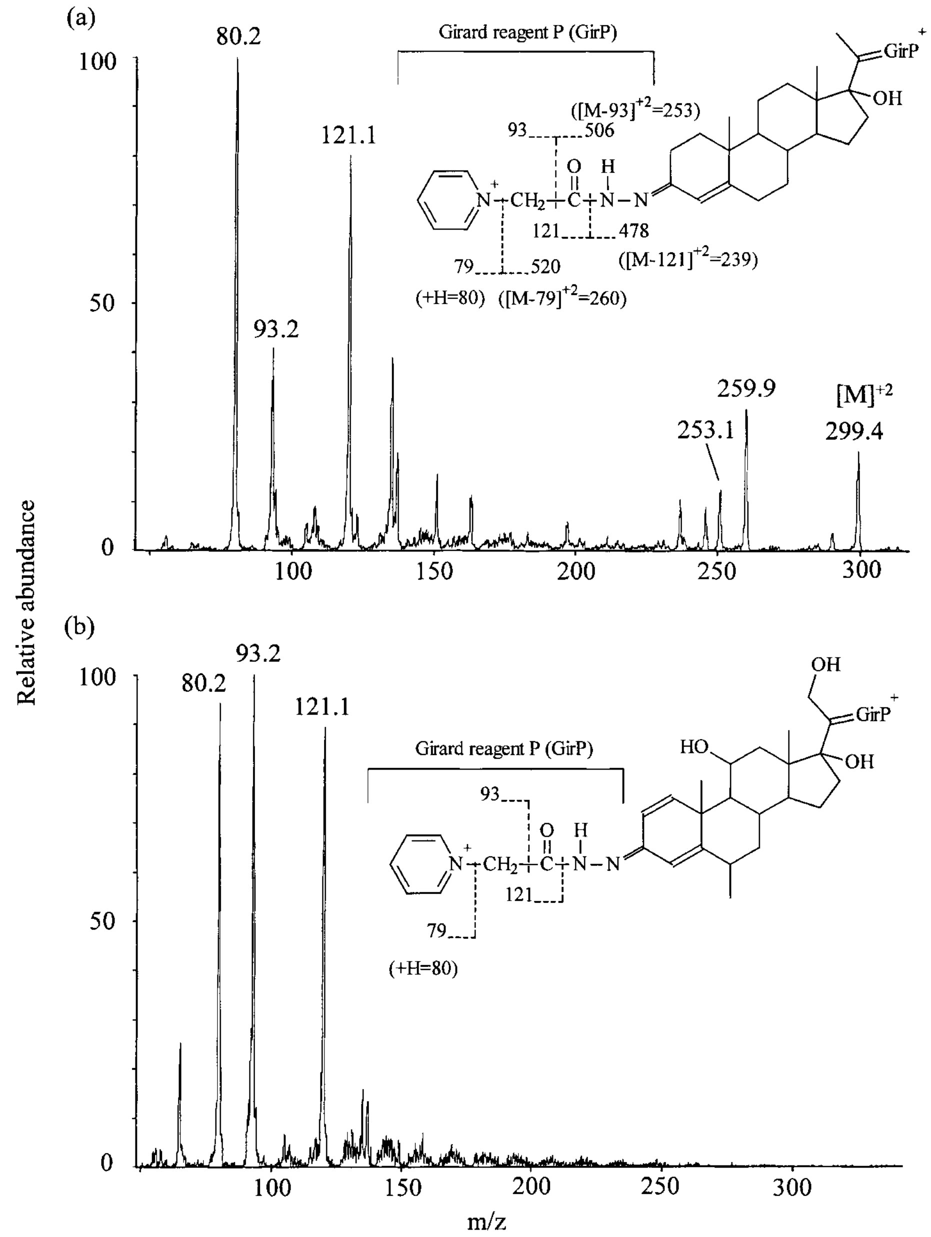

Figure 1. Positive ESI product ion mass spectra of (a) GirP-17OHP and (b) GirP-6MP.

Copyright © 2001 John Wiley & Sons, Ltd.

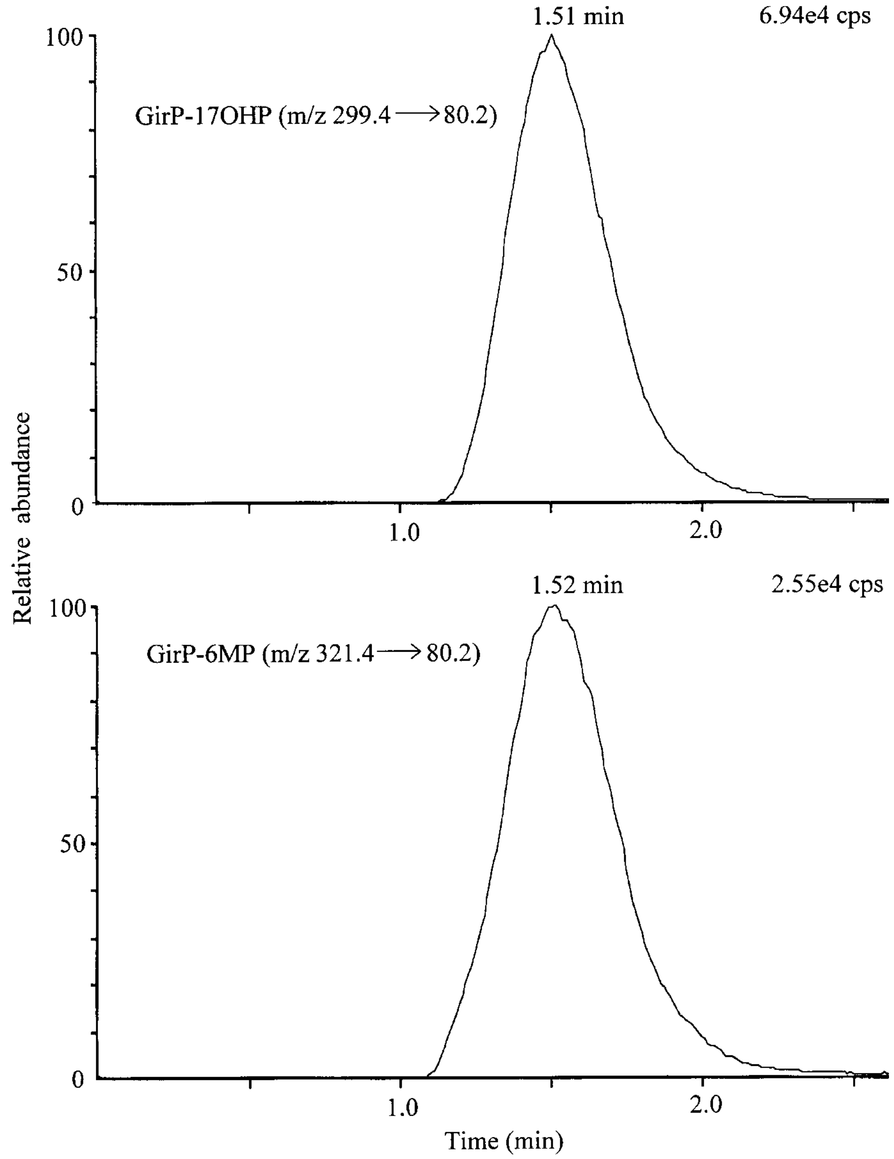

Figure 2. Mass chromatograms of GirP-17OHP and internal standard GirP-6MP (10 ng in 30 μL mobile phase) obtained from dried blood specimen (containing 500 ng/mL 17OHP) under MRM operation. Mobile phase: H_2O/acetonitrile 50:50 (v/v).

method were determined by triplicate analysis of blank filter paper spiked with whole blood containing 17OHP at the concentrations used to construct the calibration curves. Precision was estimated as the CV of the analyses and the accuracy was calculated as percentage error of theoretical versus measured concentrations. The estimated amounts of 17OHP were in good agreement with the expected amounts, the intra- and inter-assay precision were less than 12% (Table 1). The inter-day accuracy was 4.65–6.23% and the intra-day accuracy was 4.17–7.11% for 17OHP. The inter-assay CV of 17OHP ranged from 4.34% to 10.06%. The intra-assay CV of 17OHP was less than 12%.

Patient blood spot analyses of 17OHP

In order to test the clinical applicability of our assay, dried blood filter-paper samples collected from subjects with 21-hydroxylase deficiency were analyzed independently by RIA and LC/MS/MS. Table 2 shows the results of the quantitation of 17OHP from dried blood spots in CAH groups by LC/MS/MS and RIA. The two methods yielded results in reasonable agreement. We have been using LC/MS/MS to monitor the treatment of four CAH patients (numbers 1–4 in Table 2). Repeated blood sampling on filter paper can assist in improving the monitoring of CAH treatment in this study. After treatment, all of the elevated 17OHP levels (Table 2, numbers 1–4) either decreased or disappeared. In addition, five dried filter-paper blood samples of CAH infants (numbers 5–9) were also all quantified in this study and revealed high 17OHP levels. In the preliminary results of neonatal screening of CAH in Taiwan, established by the National Taiwan University Hospital using the RIA method,[29] high-risk babies with 17OHP levels higher than 90 ng/mL will be recalled immediately. In this study, 17OHP levels in five CAH babies were all higher than 40 ng/mL and a cut-off of 30 ng/mL might be suitable for continued testing for CAH newborn

Copyright © 2001 John Wiley & Sons, Ltd.

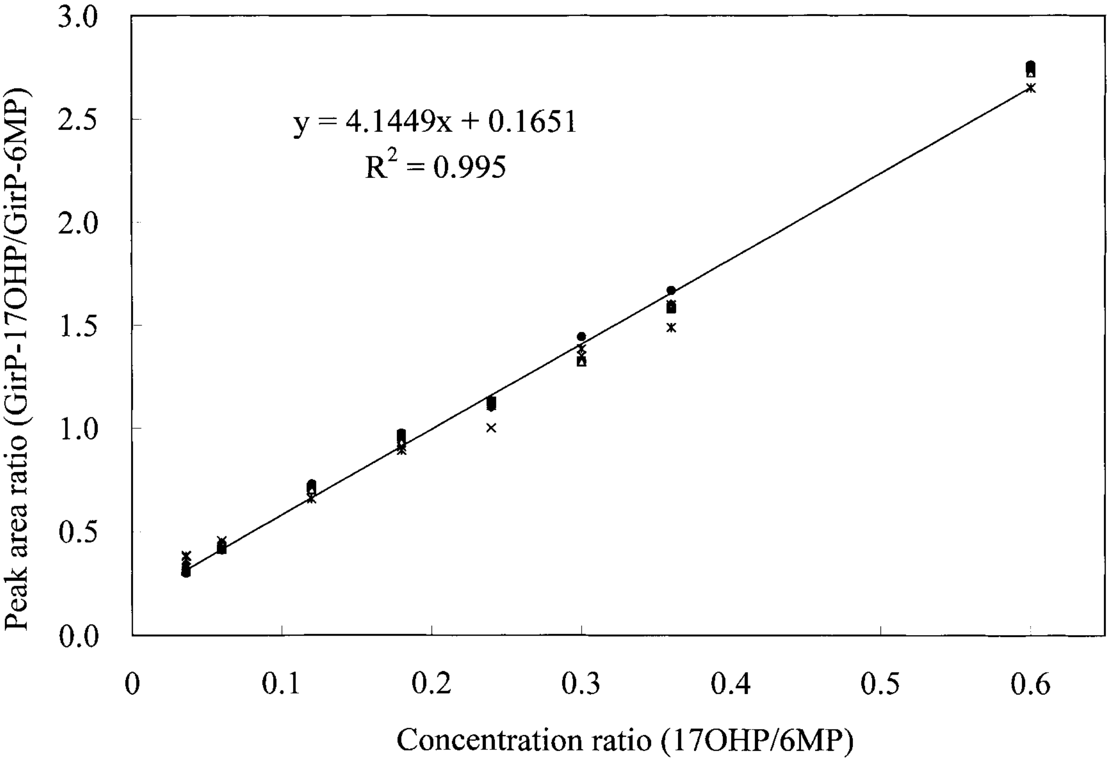

Figure 3. Calibration curves for 17OHP in dried blood specimens by LC/MS/MS-MRM (six calibration results were collected and combined).

screening. In our laboratory, one technician can prepare two 96-well microplates, a total of 192 samples, using our semi-automated procedure, in a regular working day. The maximum number of samples that can be analyzed by LC/MS/MS is 192 samples per day, which is comparable with the RIA method employed in our hospital.

CONCLUSIONS

In conclusion, the high-throughput LC/MS/MS method described in this report, although it includes a derivatization step, does not require long and complicated preparation of samples. Compared with RIA, specificity is the advantage of LC/MS/MS analysis. A further advantage of our method is the elimination of the handling of radioactive-labeled materials. The measurements of 17OHP in dried blood on filter paper using LC/MS/MS has been shown to be reliable and simple to carry out. Monitoring of 17OHP is a reliable and practical approach for assessing adrenal steroid activity in patients with CAH. The major aim of this study is to examine whether repeated blood sampling on filter paper can assist in improving the monitoring of the effectiveness of the CAH treatment, and of the patient's compliance with it. This LC/MS/MS assay is not only useful for both diagnosis and monitoring of treatment of CAH in all other age groups, but may also be used as a screening test for CAH babies.

Table 1. Accuracy and precision of 17OHP determinations on filter paper

	Concentrations added (ng/mL)			
	250	150	100	30
Inter-day[a]				
Error (%)	4.65	4.26	3.74	6.23
CV (%)	4.34	5.69	5.89	10.06
Intra-day[b]				
Error (%)	4.17	3.23	4.56	7.11
CV (%)	4.11	6.32	5.63	11.63

[a] n = 9 on 3 days.
[b] n = 3 within-day.

Table 2. 17OHP concentration in dried blood spots in CAH patients by LC/MS/MS

	17OHP concentrations (ng/mL)			
	Sex	Age	LC/MS/MS	RIA
Patients with CAH				
1	female	7-year-old	132 (32*)	128 (35*)
2	female	14-year-old	97 (50*)	103 (47*)
3	female	1-year-old	123 (60*)	139 (67*)
4	male	8-year-old	141 (47*)	130 (53*)
5	female	4-day-old	52	62
6	female	5-day-old	136	186
7	female	2-day-old	174	182
8	male	3-day-old	190	173
9	male	4-day-old	47	44

* Dried blood spots were obtained after therapy with hydrocortisone

Copyright © 2001 John Wiley & Sons, Ltd.

Work is in progress to investigate the possibility of CAH screening of newborns by this LC/MS/MS analysis method.

Acknowledgements

The study was funded by a grant from the China Medical College Hospital (DMR-90-122).

REFERENCES

1. Pang S, Wallace MA, Hofman L, Thuline HC, Dorche C, Lyon ICT et al. *Pediatrics* 1998; **81**: 866.
2. Mitchell ML, Hermos RJ. *Clin. Endocrinol.* 1998; **48**: 757.
3. Boudi A, Giton F, Galons H, Eulry B, Villette JM, Soliman H et al. *Steroids* 2000; **65**: 103.
4. Katayama M, Nakane R, Matsuda Y, Kaneko S, Hara I, Sato H. *Analyst* 1998; **123**: 2339.
5. Castracane VD, Gimpel T. *J. Clin. Lab. Anal.* 1997; **11**: 179.
6. Nahoul K. *J. Steroid Biochem. Mol. Biol.* 1994; **50**: 197.
7. Sainio EL, Lehtola T, Roininen P. *Steroids* 1988; **51**: 609.
8. Erhardt E, Solyom J, Homoki J, Juricskay S, Soltesz Gy. *J. Pediatr. Endocrinol. Metab.* 2000; **13**: 205.
9. Saisho S, Shimozawa K, Yata J. *Horm. Res.* 1990; **33**: 27.
10. Hariharan M, Naga S, VanNoord T, Kindt EK. *Clin. Chem.* 1992; **38**: 346.
11. Wang T, Shakleton CHL, Covey TR, Ellis G. *Clin. Chem.* 1992; **38**: 1830.
12. Honour JW, Rumsby G. *J. Steroid Biochem. Mol. Biol.* 1993; **45**: 69.
13. Lo MS, Ng ML, Wu LL, Azmy BS, Khalid BA. *Malays. J. Pathol.* 1996; **18**: 43.
14. Shackleton CHL, Chuang H, Kim J, de la Torre X, Segura J. *Steroids* 1997; **62**: 523.
15. Toya K. *Nippon Naibunpi Gakkai Zasshi* 1988; **64**: 310.
16. Gorog S, Gazdag M, Kemenes-Bakos P. *J. Pharm. Biomed. Anal.* 1996; **14**: 1115.
17. Nyhan WL, Ozand PT. *Atlas of Metabolic Disease*, Chapman and Hall: London, 1998; 680.
18. Millington DS, Kodo N, Norwood DL, Roe CR. *J. Inherit. Metab. Dis.* 1990; **13**: 321.
19. Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL. *Clin. Chem.* 1997; **42**: 349.
20. Rashed MS, Rahbeeni Z, Ozand PT. *Semin. Perinatol.* 1999; **23**: 183.
21. Levy H. *Clin. Chem.* 1998; **44**: 2401.
22. Millington DM, Chace DH, Hillman SL, Kodo N, Terada N. ***Diagnosis of Metabolic Disease***. In *Biological Mass Spectrometry: Present and Future*, Matsuo T, Caprioli RM, Gross ML, Seyama Y (eds). McGraw-Hill: New York, 1995; 1239–1277.
23. Volmer DA, Hui JPM. *Rapid Commun. Mass Spectrom.* 1997; **11**: 1926.
24. Dodds HM, Taylor PJ, Cannell GR, Pond SM. *Anal. Biochem.* 1997; **247**: 342.
25. Fiori M, Pierdominici E, Longo F, Brambilla G. *J. Chromatogr. A* 1998; **807**: 219.
26. Savu SR, Silvestro L, Haag A, Sorgel F. *J. Mass Spectrom.* 1996; **31**: 1351.
27. Antignac JP, Bizec BL, Monteau F, Poulain F, Andre F. *Rapid Commun. Mass Spectrom.* 2000; **14**: 33.
28. Burtis CA, Ashwood ER (eds). *Tietz Textbook of Clinical Chemistry* (2nd edn). W. B. Saunders: Philadelphia, 1994.
29. Chu SY, Hwu WL, Chien YH, Tsao PN, Tsai WY. *Preliminary Result of Neonatal Screening of Congenital Adrenal Hyperplasia in Taiwan*; 164th Scientific Meeting of the Taiwan Pediatric Association, 2000; 212.

Copyright © 2001 John Wiley & Sons, Ltd.