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Quantitative Analysis of Porphyrins in Dried Blood Spots Using Liquid Chromatography-tandem Mass Spectrometry with Pre-column Derivatization

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Porphyrins are intermediates of the heme biosynthetic pathway in mammalian tissues. The porphyrin molecules consist of cyclic tetrapyrrole. Porphyrin metabolism may be disordered or impaired among some Autism Spectrum Disorder (ASD) children; therefore porphyrin levels might have potential clinical utility as a biomarker for ASD identification and/or classification.[1] ASD, which may develop in infancy or during the first three years, is a neurodevelopmental disorder characterized by impaired social relatedness, communication, repetitive behaviors, abnormal movement patterns, and sensory dysfunction.[2-4] In accordance with the most recent studies, the prevalence of ASD in the United States is 1.47% on average.[5] The incidence of ASD in the Ilsan area of South Korea is reportedly 2.64%.[6] Thus, early detection, which leads earlier treatment of the ASD population is important. Genetic factors of ASD have been proposed;[7] however, genetic studies carried out in a specific genetic background have not yielded an early diagnostic test yet. A number of techniques have been developed for determining urinary porphyrins, including spectrofluorometry,[8] high performance liquid chromatography,[9,10] thin-layer chromatography,[11] ion-pair high performance liquid chromatography,[12] capillary electrophoresis with fluorescence,[13] matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF),[14] and fast atom bombardment mass spectrometry (FAB-MS).[15] Recently, liquid chromatography-mass spectrometry (LC-MS),[16,17] and tandem mass spectrometry (LC-MS/MS)[18-21] have been used to determine porphyrins in biological matrices (blood, urine and feces) because of their high sensitivity and specificity. However, there have been no publications regarding the measurement of porphyrin metabolites in whole blood because the metabolites are endogenous and found in very low concentrations.

Considering both the necessity of early treatment and the availability of dried blood spot (DBS) newborn screening tests in clinics, we began to develop a method validation of porphyrin metabolites using LC-MS/MS. A few drops of whole blood were collected on filter paper from a finger prick. We conducted the esterification process of porphyrin to improve the sensitivity of the porphyrin metabolites using LC-MS/MS. Finally, we measured infinitesimal porphyrin metabolite, as the potential biomarker of ASD, in DBS samples with esterification. Here, we report the development and validation of the use of LC-MS/MS to quantify porphyrin metabolites in DBS.

Experimental

Coproporphyrin (CP, ≤ 97%), pentacarboxylporphyrin (PP, ≤ 90%), hexacarboxylporphyrin (HexaP, ≤ 90%), heptacarboxylporphyrin (HeptaP, ≤ 90%), and uroporphyrin (UP, ≤ 98.9%) were obtained from Frontier Scientific Inc. (Logan, UT, USA). Coproporphyrin I-$^{15}N_4$ (IS, ≤ 98%), as an internal standard, was purchased from TRC Inc. (Toronto Research Chemicals, North York, Canada). HPLC grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA). HPLC grade chloroform, formic acid, and sulfuric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were analytical grade except those for HPLC.

Chromatography was performed with a Shimadzu (Shimadzu Corporation, Tokyo, Japan) LC-20 series binary pump (LC-20AD), which was used to inject 5 μL aliquots of the processed samples onto a Unisol C18 column (150 mm × 2.0 mm, 3 μm, Agela, Wilmington, DE, USA) equilibrated at 30 °C. The gradient mobile phase, with solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v) were set at a flow rate of 300 μL/min. The gradient program started the mobile phase A-B (95:5, v/v) with a linear increase of the organic phase (5:95, v/v) from 0.5 to 2.5 min. Mobile phase B was held at 95% for another 7.0 min (from 2.5 to 9.5 min) and then decreased linearly to the start condition (95:5, v/v). The total run time was 12.0 min. MS/MS analysis was performed on an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA). It was operated using an electrospray ionization source in the positive ion mode. Multiple reaction monitoring (MRM) measurements with the transitions were *m/z* 655.3→596.4 for coproporphyrin, *m/z* 699.3→595.4 for pentacarboxylporphyrin, *m/z* 743.3 →639.4 for hexacarboxylporphyrin, *m/z* 787.4→683.3 for

heptacarboxylporphyrin, *m/z* 831.4→727.4 for uroporphyrin, *m/z* 659.2→600.4 for coproporphyrin I-$^{15}N_4$, *m/z* 711.5 →565.4 for coproporphyrin tetramethyl ester, *m/z* 769.5→ 637.4 for pentacarboxylporphyrin pentamethyl ester, *m/z* 827.4→695.4 for hexacarboxylporphyrin hexamethyl ester, *m/z* 885.4→753.4 for heptacarboxylporphyrin heptamethyl ester, *m/z* 943.4→811.3 for uroporphyrin octamethyl ester, and *m/z* 715.5→569.4 for an coproporphyrin I-$^{15}N_4$ tetramethyl ester (Figure 1). Analyst software (version 1.4.2) was used to control the HPLC, the mass spectrometer, and to process the data.

The stock solutions of five porphyrins (10 μmol/L) and an internal standard (10 μmol/L) were prepared in 6 M formic acid and kept at 4 °C. A series of working standard solutions of the five porphyrins with a concentration range of 1–1000 nmol/L was prepared by diluting the stock solution with 1 M formic acid. The internal standard solution was prepared with 50% methanol in 6 M formic acid to obtain a 50 nmol/L concentration. Standard DBS samples were prepared by spiking 10% of the working standard solutions of the analyte to 90% of the human whole blood control. Finally, standard DBS concentrations for the calibration curve were 0.2, 0.5, 1, 5, 10, 30, and 50 nmol/L. Quality control samples of 0.2, 0.6, 20, and 40 nmol/L were used to determine the test's precision and accuracy.

Collected DBS samples on filter paper cards were hole-punched (6 mm diameter, containing approximately 10 μL blood), a 10 μL internal standard solution (50 nmol/L) was added to the collected samples, and then they were reconstituted with 200 μL of 0.5 M formic acid in water. For derivatization, a mixture solution of MeOH/H_2SO_4 was used. Samples were vortexed for 60 min and 1000 μL of chloroform was added. After vortexing for 30 min and a subsequent centrifugation at 13,000 rpm for 10 min, the supernatant was transferred into a 13 mL glass tube. After the organic layer was evaporated in a stream of nitrogen at 40 °C in a dark room, the residue was reconstituted in 100 μL of methanol and a 5 μL aliquot was injected into the LC-MS/MS system.

The method was validated by carrying out a test of matrix effect, selectivity, linearity, limit of quantification (LOQ), precision, accuracy, and recovery. Matrix effect and selectivity were each assessed by different lots of matrix. Three sets were also prepared to a final concentration of 0.6 and 40 nmol/L. The matrix effect was estimated by measuring the peak area ratio of extraction of the working standard (A) and the post-extraction spiked blank DBS (B). Three replicates of each set were used for determination of the matrix effect (ME). An internal standard was not added in this procedure.

$$\text{ME (\%)} = \text{B/A} \times 100$$

The LOQ was defined as the minimum concentration of the signal-to-noise ratio (S/N). Calibration curves were determined by the peak area ratio of each porphyrin compared to the internal standard against the analyte concentration. The acceptance criteria for each back-calculated

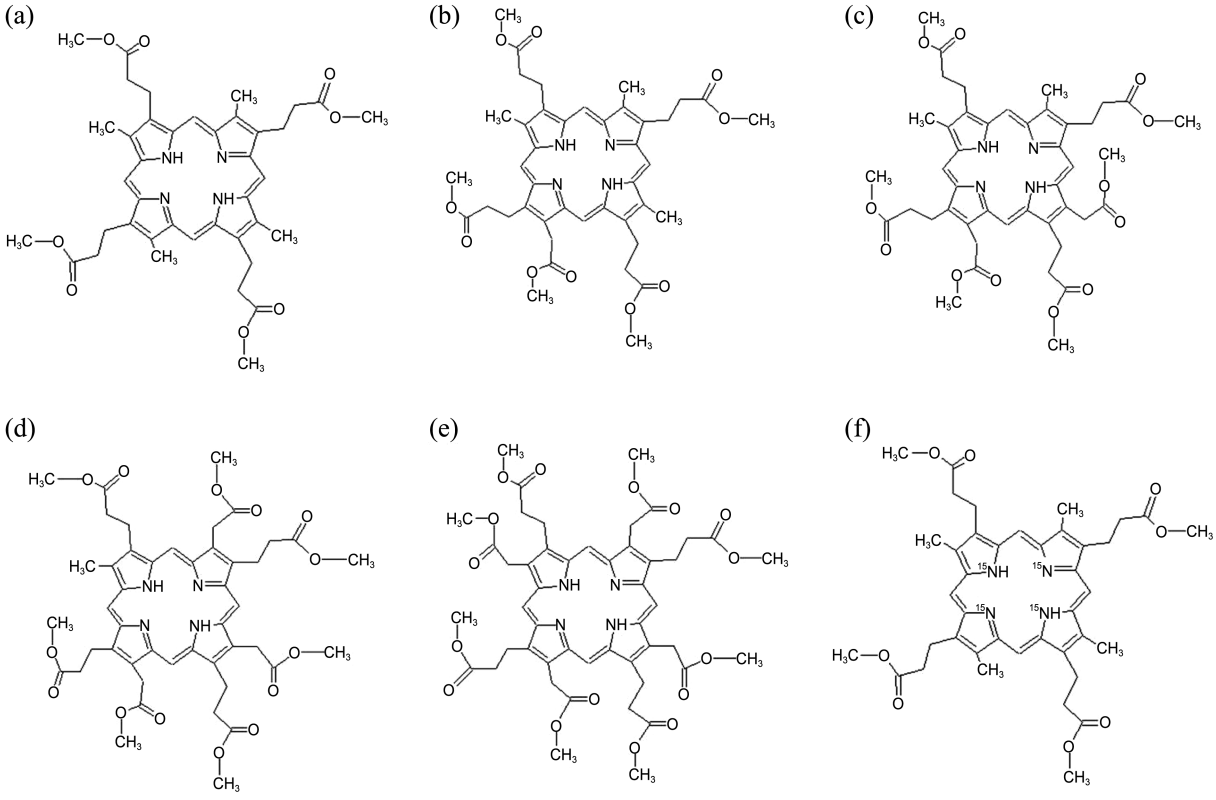

Figure 1. Chemical structure of coproporphyrin tetramethyl ester (a), pentacarboxylporphyrin pentamethyl ester (b), hexacarboxylporphyrin hexamethyl ester (c), heptacarboxylporphyrin heptamethyl ester (d), uroporphyrin octamethyl ester (e) and its internal standard, coproporphyrin I-$^{15}N_4$ tetramethyl ester (f).

standard concentration were ± 15% standard deviation (SD) from the nominal value. LOQ had to be ± 20%. The intra-day precision and accuracy were estimated by analyzing five replicates at four different concentration levels, 0.2, 0.6, 20, and 40 nmol/L. The inter-day precision was determined by analyzing four different concentration level samples. Accuracy was expressed as the percentage of observed value to true value, and precision was expressed as the relative standard deviation (RSD). The criteria for acceptability of the data included accuracy below ± 15% deviation from the nominal values, and precision below ± 15% RSD. Additionally, LOQ had to be ± 20%.

Results and Discussion

We focused on manipulating the chromatographic parameters to increase the intensity and achieve the fast separation of porphyrins with high chromatographic resolution. Additionally, we found it was possible to develop an ESI-MS/MS method for the rapid simultaneous analysis of five porphyrins. The five porphyrins were further characterized by their MS/MS product ion spectra. Previous studies have shown the major fragmentation pathway of porphyrins. Figure 2 shows the reconstructed LC-ESI-MS/MS MRM chromatogram of a standard mixture of the five porphyrin methyl esters. In the present study, several columns, including Synergi Fusion RP column (150 mm × 2.0 mm, 4 μm, Phenomenex, USA) and Unisol (150 mm × 2.1 mm, 3 um, Agela, USA), were compared based on peak separation, sensitivity, and column lifetime in order to evaluate column performance (data not shown). The suitable mobile phase for porphyrins was formic acid solution with an organic solvent (reversed-phase mode). The results showed that the Unisol C18 column had best performance, (*i.e.*, a better resolution sensitivity and shorter analysis time); however, it was hard to optimize an analytic condition for the simultaneous analysis of porphyrins because the reduction of analysis time requires an increase in the pressure of the column. To obtain complete separation and a shorter retention time of the porphyrin metabolites, we used solvent A (0.1%, v/v, formic acid in water) and solvent B (0.1%, v/v, formic acid in acetonitrile) was delivered to the column at a flow rate of 0.3 mL/min in the optimal gradient mobile phase. Finally, to obtain the optimal mobile phase, which improved peak shape and separation, we used 5% of mobile phase B, followed by a linear increase in B to 95% from 0.5 to 2.5 min. Mobile phase B was held at 95% for another 7.0 min (from 2.5 to 9.5 min) and then reduced linearly to the start condition (5% of mobile phase B).

Optimization of the Derivatization. Alkylation of the carboxyl groups increased the sensitivity of the mass spectrometry. The carboxyl group of porphyrin was a derivatized methyl ester group, considering both the steric hindrance effect of porphyrins and that the mass range of the triple quadrupole mass spectrometry detector (API 5000^{TM}) is up to 1250. In this study, strong acid such as sulfuric acid, hydrochloric acid and nitric acid, were compared based on peak area, sensitivity, and reproducibility in order to evaluate acid performance (data not shown).

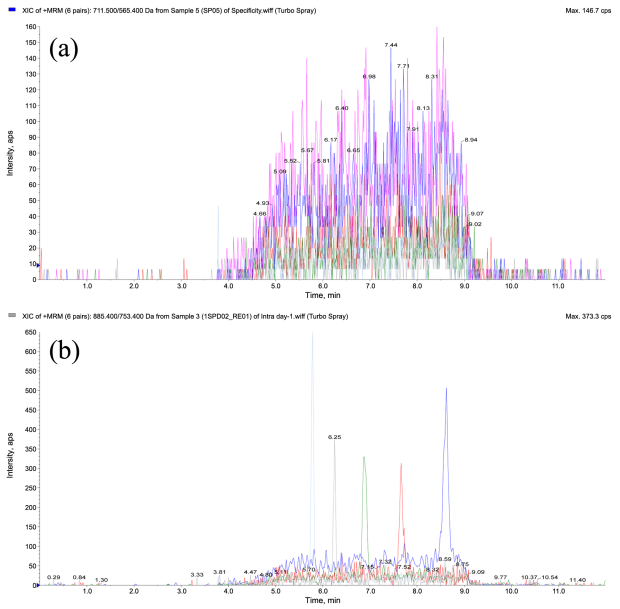

Figure 2. LC-MS/MS chromatogram of (a) blank and (b) LOQ standard mixture of the five porphyrin methyl esters in dried blood spots.

A nylon membrane filter was used to remove the endogenous porphyrins from the pooled human DBS. No interfering peaks from endogenous components were detected at the retention times of analytes and IS. The matrix effect and post-column infusion methods were used in this validation. There was negligible ion suppression in the region for retention time of analytes and IS. Precision and accuracy were assessed from four different concentrations of QC samples. The limit of quantification (LOQ) was determined as the concentration at which the signal-to-noise (S/N) ratio was greater than 10, precision of < 20%, and accuracy between 80–120% for both intra- and inter-day assays. Other QC concentration precision was < 15% and accuracy was in between 85–115% for both intra- and inter-day assays. The determination of porphyrins for precision and accuracy in DBS are shown in Table 1. In this study, the LOQ for five porphyrins was 0.2 nmol/L, which was slightly better than earlier reports using tandem mass spectrometry. The standard calibration curve for the five porphyrin methyl esters was constructed using seven points of calibration standard solutions (0.2–50 nmol/L). The total chromatographic run time was 12 min. The linearity of the calibration curve was evaluated by determining the best fit of the peak area ratios (peak area of analyte/peak area of IS) versus concentration using weighing factor ($1/X$) because of the calibration range. The coefficient of determination (r^2) was greater than 0.99. The percent accuracy observed for the mean of back-calculated concentrations of calibration curves was within 15%. The study subjects included 102 healthy volunteers and 101 subjects suffering from ASD.

The method was successfully applied to porphyrins in DBS, therefore the DBS samples from the 203 subjects were

Table 1. Precision and accuracy for the determination of porphyrins in DBS (n=5)

Porphyrins	Conc. (nM)	Intra-Day/ Inter-Day	
		Precision (%)	Accuracy (%)
CPTE	0.2	4.35 ± 0.01/ 4.80 ± 0.01	114.63 ± 5.34/ 114.72 ± 4.39
	0.6	4.76 ± 0.02/ 12.11 ± 0.07	109.10 ± 6.03/ 103.08 ± 8.61
	20	9.20 ± 0.81/ 12.71 ± 2.48	88.06 ± 8.09/ 97.49 ± 12.39
	40	6.48 ± 1.38/ 7.14 ± 3.11	106.51 ± 6.90/ 109.05 ± 7.77
PPPE	0.2	4.14 ± 0.01/ 3.99 ± 0.01	108.69 ± 4.49/ 111.49 ± 3.65
	0.6	7.58 ± 0.05/ 10.61 ± 0.06	103.64 ± 8.13/ 102.53 ± 10.77
	20	5.93 ± 1.10/ 6.46 ± 1.31	92.87 ± 5.51/ 101.14 ± 6.53
	40	5.39 ± 2.01/ 8.85 ± 3.63	93.32 ± 5.03/ 102.60 ± 9.08
HexaPHE	0.2	11.53 ± 0.02/ 8.30 ± 0.02	94.13 ± 10.69/ 99.32 ± 8.06
	0.6	4.88 ± 0.03/ 6.91 ± 0.04	86.06 ± 3.99/ 94.33 ± 6.63
	20	2.63 ± 0.46/ 10.91 ± 2.20	86.74 ± 2.27/ 100.69 ± 10.99
	40	2.28 ± 0.78/ 12.62 ± 5.23	85.20 ± 1.94/ 103.69 ± 13.08
HeptaPHE	0.2	17.79 ± 0.04/ 9.11 ± 0.02	104.07 ± 18.08/ 106.66 ± 9.13
	0.6	7.57 ± 0.02/ 12.37 ± 0.07	101.50 ± 7.54/ 104.24 ± 8.42
	20	14.27 ± 1.33/ 13.37 ± 2.71	93.47 ± 13.34/ 100.13 ± 14.68
	40	13.15 ± 2.72/ 9.99 ± 3.99	103.24 ± 13.59/ 99.97 ± 11.38
UPOE	0.2	4.35 ± 0.01/ 12.90 ± 0.03	114.63 ± 5.34/ 105.35 ± 13.71
	0.6	4.76 ± 0.02/ 13.09 ± 0.08	109.10 ± 6.03/ 105.42 ± 7.50
	20	9.20 ± 0.81/ 9.87 ± 2.05	88.06 ± 8.09/ 101.94 ± 13.80
	40	6.48 ± 1.38/ 10.08 ± 4.11	106.51 ± 6.90/ 101.99 ± 10.28

Table 2. Porphyrins concentrations in DBS samples from 203 Korean children (mean ± SD)

Porphyrins	Group	
	ASD	Control
CPTE	8.90 ± 15.78	5.74 ± 5.84
PPPE	0.56 ± 0.50	0.38 ± 0.26
HexaPHE	1.31 ± 1.42	0.58 ± 0.77
HeptaPHE	0.68 ± 0.35	0.55 ± 0.27
UPOE	2.67 ± 1.45	2.26 ± 1.52

analyzed. The measured concentration of porphyrins in the DBS of our study participants are shown in Table 2. Porphyrins concentration in the autistic group was higher than in the control group. The results of this analysis show that the porphyrins content of the DBS, can provide useful information as a potential biomarker for the early diagnosis of autism.

Conclusions

We have developed, validated, and applied the analysis of five porphyrins in DBS. The advantages of this novel method were high sensitivity, specificity, reproducibility, rapid sample throughput, and minimal sample volume due to the derivatization by using the HPLC-MS/MS. For improving the sensitivity in LC-MS/MS, we have developed derivatization methods. The method was successfully applied to determine the level of porphyrins in DBS from clinical samples as well. Further investigation of the applicability of the method to the quantification of newborn porphyrin levels should be performed.

Acknowledgments. ISS has submitted a patent application for this method of analysis (Application No.: 10-2014-0033713).

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