Development, Validation and Application of Tandem Mass Spectrometry for Screening of Inborn Metabolic Disorders in Kuwaiti Infants

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Key Words

Inborn metabolic disorders · Tandem mass spectrometry · Validation study

Abstract

Objective: The aim of this work was to develop a specific and validated tandem mass spectrometric (MS/MS) method for screening of amino acidopathies, organic acidurias, urea cycle disorders and fatty acid oxidation defects in Kuwaiti newborns and sick infants. **Materials and Methods:** A total of 1,520 blood samples were tested for inborn metabolic disorders in Kuwaiti newborns and sick infants. Positive electrospray MS/MS was used to measure diagnostic acylcarnitines and amino acids in blood spots after simple extraction and derivatization procedures. Validation and stability studies were conducted using control blood samples supplemented with known concentrations of the diagnostic amino acids or acylcarnitines. Reference and cutoff levels of the diagnostic metabolites were determined in a group of 500 normal Kuwaiti babies for quantitative evaluation. **Results:** Of the 1,520 samples, 32 were positive newborn cases and 27 positive symptomatic infants. For the validation studies, the range of relative standard deviation was 2.6–14.7%, whereas the range of the percent deviation from nominal concentrations was –23.0 to +25.0 of the diagnostic metabolites. Sta-

bility studies indicated appropriate stability of the diagnostic amino acids and acylcarnitines in dried blood spots stored at $22 \pm 1^\circ\text{C}$ and relative humidity of 50–60%. **Conclusions:** Tandem mass spectrometry can significantly contribute to a newborn screening program as a fast and highly specific diagnostic technique for screening of a broad range of inborn metabolic disorders.

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Introduction

Inborn metabolic disorders (IMDs) are rare types of genetic disorders that cause irreversible mental and/or physical disability or death in affected children. Early diagnosis of IMDs can dramatically reduce the risk of death and can prevent long-term neurological complications [1, 2]. Recently, electrospray tandem mass spectrometry (MS/MS) has been described as a powerful technique for screening of IMDs. The technique permits the detection and quantification of many IMDs in a single blood spot for most fatty acid oxidation, amino acid, organic acid and urea cycle disorders. At present, MS/MS is being used as the screening technique for the diagnosis of IMDs in newborns and sick infants in many clinical biochemical laboratories in the USA, Europe, Australia and Japan [3–

6]. The outcomes and implications of tandem mass spectrometry in metabolic screening in comparison to other screening techniques have been reported [5]. In the Arab region, with the exception of the Kingdom of Saudi Arabia, the use of MS/MS in metabolic screening programs is at its developmental stage [7]. Presumably, this may be attributed to the high costs of equipment and consumables and lack of technical expertise. The recent development in tandem mass technology has significantly contributed to the development of automated and routinely applied methods for IMD screening [8–9]. Clinical studies have shown a high incidence rate of IMDs among the Kuwaiti population [5, 9]. Comparable high frequencies of IMDs have been reported in the Kingdom of Saudi Arabia, Egypt and other Gulf countries [10, 11]. The high prevalence of metabolic disorders is most likely due to the widespread tradition of consanguineous marriages in these regions [12].

Recently, the Kuwait Medical Genetic Center has introduced the neonatal screening service for phenylketonuria and congenital hypothyroidism in Kuwait using fluorescence immunoassay technique. This screening procedure has several limitations, e.g. a narrow range of metabolic diseases detected, non-specificity and cross-reactivity of analytes, which may lead to false-positive tests.

To avoid this, only samples of severely affected infants are sent abroad for metabolic screening by MS/MS. However, this outsourcing is costly and time-consuming, particularly when the child is hospitalized in the intensive care unit. Accordingly, it was decided to use MS/MS available at the Faculty of Pharmacy, Kuwait University, for screening IMDs. Hence the objective of this study was to develop a specific and validated MS/MS method screen for IMDs in newborns and sick infants in Kuwait.

Materials and Methods

Materials

Acylcarnitines and amino acids purchased from Sigma-Aldrich (St. Louis, Mo., USA) were used as supplied. Isotopically labeled internal standards of amino acids (Set A) and acylcarnitines (Set B) were supplied by Cambridge Isotope Laboratories (Woburn, Mass., USA). Butanolic HCl was obtained from Regis Technologies (Morton Grove, Ill., USA). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific, Auburn, USA. Water was purified by Milli-Q device (Millipore, Bedford, Mass., USA). Guthrie filter paper cards (S&S 903; Schleicher and Schull, Dassel, Germany) were used to collect blood samples. For validation and stability studies, certified human blood samples were donated by the Kuwait Central Blood Bank, Kuwait.

Methods

A total of 1,520 blood samples were collected between May 2004 and March 2006 from 1,158 newborns and 362 sick infants. All newborns and infants tested were cared for in maternity wards and pediatric clinics of the seven major governmental maternity and pediatrics hospitals in Kuwait. The blood samples taken by heel stick were spotted on Guthrie filter paper cards, left to dry at room temperature for at least 24 h, placed inside plastic envelopes and then sent to the Faculty of Pharmacy, Kuwait University, for screening by MS/MS. The blood spots were analyzed for acylcarnitines and amino acids by in-house triple-quadrupole tandem mass spectrometer (Quattro LC; Micromass, Manchester, UK) with a positive electrospray ionization probe. Using a BSD 400 Puncher, a single 3-mm disk ($\sim 3 \mu\text{l}$ blood) was punched in the center of the dried blood spot. The disk was placed in a capped glass for extraction of the blood with 200 μl of methanol, containing known concentrations of a mixture of isotopically labeled internal standards of diagnostic acylcarnitines and amino acids. After ~ 20 min of shaking, the solvent was gently evaporated and the residue was mixed with $\sim 80 \mu\text{l}$ of butanolic HCl and heated at 65°C for 15 min in a capped glass tube. The solvent was evaporated again and the residue was finally reconstituted in 80 μl of the mobile phase (acetonitrile/water, 80/20 v/v). A 20- μl aliquot of each sample was directly injected into the mobile phase flowing to the ionization probe of the tandem mass spectrometer, at a flow rate of 0.1 ml/min. A Waters 2690 HPLC with an autosampler (Waters, Milford, Mass., USA) was used for automatic injection. The run cycle time for each sample was 2–3 min from injection to injection. A designed MS/MS program for automatic profiling of acylcarnitines and amino acids was used. The NeoLynx program for Neonatal Screening (Micromass) was used for automatic detection of abnormal samples. The cutoff levels and reference values were based on the mean concentration or peak ratio $\pm$ standard deviation values of selected metabolites measured in 500 normal Kuwaiti babies. The values obtained were compared with those reported in other studies. The discriminative power of the NeoLynx program for automatic detection of abnormalities was tested by analyzing samples of normal and known sick patients in a blind manner. All sample results together with newborn or infant demographic data were stored in the database.

Validation and Stability Studies

Stock solutions of the diagnostic amino acids (phenylalanine, tyrosine, methionine, leucine and citrulline) and acylcarnitines (octanoylcarnitine and palmitoylcarnitine) were separately prepared in methanol/water (1:1) at concentrations of 1 mg/ml. Blood samples supplemented with known concentrations of amino acids or acylcarnitines, which simulate normal and abnormal sick samples, were prepared. Blood samples containing phenylalanine at a concentration of 60–600 $\mu\text{mol/l}$, tyrosine at 55–330 $\mu\text{mol/l}$, methionine at 65–585 $\mu\text{mol/l}$, leucine at 300–900 $\mu\text{mol/l}$, citrulline at 55–165 $\mu\text{mol/l}$, octanoylcarnitine at 7–14 $\mu\text{mol/l}$ and palmitoylcarnitine at 10–20 $\mu\text{mol/l}$ were used. A 50- μl aliquot of each blood sample was spotted on the circle of a Guthrie card and left to dry. For validation and stability studies, five Guthrie cards of each compound were prepared, dried, stored in plastic envelopes and kept at room temperature for 10 weeks. The extraction and derivatization processes were done as previously mentioned. The concentrations of the diagnostic amino acids and acylcarnitines

Fig. 1. Stability profiles of selected diagnostic amino acids in dried blood spots determined by MS/MS. ■ = Leucine; ● = phenylalanine; ▲ = methionine; ◆ = tyrosine; □ = citrulline.

were measured and compared to the supplemented concentration values (table 1). The NeoLynx program for neonatal screening was used to determine the concentration of the diagnostic metabolites in blood spots ($\mu\text{mol/l}$) using an isotopically labeled mixture of the diagnostic acylcarnitines and amino acids as internal standard.

Results

The percent relative standard deviation and percent-age deviation from the nominal concentration ranges were 2.6–14.7 and –23.0 to +25.0%, respectively, as shown in table 1. Stability studies indicated that the analytes were stable in the dried blood spots over a 10-week period when stored in plastic envelopes at $22 \pm 1^\circ\text{C}$ and relative humidity of 50–60% (fig. 1, 2). Of 1,158 tested newborns, 32 samples showed metabolic abnormalities, while out of 362 tested sick infants, 27 samples demonstrated positive results (table 2). Out of the total 1,520 samples screened, a total of 21 cases were previously diagnosed by another screening technique and were confirmed by MS/MS, whereas 38 positive cases (32 newborns and 6 sick infants) were considered undiagnosed cases and were detected by the developed MS/MS. The detected individual metabolic disorders are listed in table 2. Fatty acid oxidation defects such as very-long-chain acyl CoA dehydrogenase deficiency, long-chain hydroxyacyl CoA dehydrogenase deficiency and carnitine palmitoyl transferase type II deficiency (carnitine-acylcarnitine translocase deficiency) were the most frequently detected disorders

Fig. 2. Stability profiles of the diagnostic acylcarnitines in dried blood spots determined by MS/MS. ■ = Palmitoylecarnitine; ● = octanoylcarnitine.

Table 1. Precision and accuracy studies for the determination of some diagnostic amino acids and acylcarnitines in quality control blood spots by MS/MS

Amino acid/ acylcarnitine	Nominal concentra- tion, $\mu\text{mol/l}$	Means $\pm$ SD*	%RSD	DEV%
Phenylalanine	60	68.0 ± 4.4	6.4	+2 to +25
	600	598.0 ± 28.9	4.8	–18 to +18
Tyrosine	55	53.0 ± 7.8	14.7	–23 to +20
	330	340 ± 8.9	2.6	–3 to +6
Methionine	65	69 ± 8.7	12.6	–15 to +11
	585	568 ± 63.1	11.1	–23 to +12
Leucine	150	141 ± 10.1	7.2	–12 to +7.5
	900	926 ± 25.9	2.8	–2.0 to +8
Citrulline	55	57 ± 4.8	8.7	–11 to +15
	165	150 ± 9.1	6.1	–1.5 to –19
Octanoyl- carnitine	7.0	6.8 ± 0.7	9.5	–17 to +13
	14	15 ± 0.8	5.5	–2.9 to +16.5
Palmitoyl- carnitine	10	12.4 ± 1.1	8.9	–11 to +20
	20	19.6 ± 1.2	5.9	–7.5 to +10

%RSD = Percent relative standard deviation; DEV% = percent-age deviation from nominal concentration. * n = 10.

among newborns and symptomatic infants. The MS/MS profile of a newborn with a significantly high concentration of palmitoylecarnitine (C16) at m/z 456 is shown in figure 3. Organic acid disorders such as propionic and

Table 2. Screening for IMDs by tandem mass spectrometry in 1,520 samples collected from newborns and symptomatic infants

Metabolic disorder	Diagnosis prior to MS/MS		Screening (1,520)	
	yes	no	symptomatic infants (362)	newborns (1,158)
<i>Amino acid disorders</i>				
Transient tyrosinemia		x		7
Tyrosinemia type I		x		1 (1 ND)
Phenylketonuria	x		1	
Hyperphenylalaninemia		x		3
Maple syrup urine disease		x		1
Homocystinuria	x		1	
Non-ketotic hyperglycinemia		x		1
Hyperalaninemia secondary to pyruvate carboxylase deficiency		x		3 (1 ND)
<i>Organic acid disorders</i>				
Propionic acidemia	x	x	3 ¹	
Methylmalonic acidemia	x	x	2 ¹	
Isovaleric acidemia	x		1	
Glutaric acidemia type I	x	x	1 ¹	
Hydroxymethyl glutaric acidemia	x		1	
<i>Fatty acid oxidation defects</i>				
Very-long-chain acyl CoA dehydrogenase deficiency		x	3	5 (1 ND)
Long-chain hydroxyacyl CoA dehydrogenase deficiency		x		3 (1 ND)
Medium-chain acyl CoA dehydrogenase deficiency		x	2	2
Multiple acyl CoA dehydrogenase deficiency		x		1 (1 ND)
Carnitine palmitoyl transferase Type I deficiency		x	1	
Type II deficiency		x		3 (2 ND)
<i>Urea cycle disorders</i>				
Citrullinemia		x	2	2
Argininosuccinic acidurias	x		2	
<i>Others</i>				
Canavan's disorder	x		1	
Cases			27	32
Previously diagnosed			21	0
Diagnosed by MS/MS only			6	32
Newborn deaths (ND)				7

¹ Infants were previously undiagnosed and only detected by MS/MS.

methylmalonic acidemia were common among symptomatic infants. A similar MS/MS profile of a sick infant with a distinct high level of propionylcarnitine (C3) at m/z 275 is shown in figure 4. A very rare case of Canavan's disorder was detected in the urine sample of 1 sick infant (fig. 5). The profile shows a significantly increased concentration of N-acetylaspartate at m/z 288. Generally,

the obtained concentrations of the metabolites in sick infants were much higher than the normal values. Seven newborn deaths were reported due to fatty acid oxidation defects, pyruvate carboxylase deficiency and tyrosinemia type I.

Fig. 3. Blood spot of a newborn with carnitine palmitoyltransferase type-II deficiency determined by MS/MS. C16: 11.3 $\mu\text{mol/l}$ (normal range 1.5–2.5 $\mu\text{mol/l}$).

Fig. 4. Blood spot of a sick infant with propionic acidemia indicated by MS/MS. C3: 35.5 $\mu\text{mol/l}$ (normal range 1.0–1.5 $\mu\text{mol/l}$).

Fig. 5. Urine sample of a sick girl with Canavan's disorder detected by MS/MS. N-acetylaspartate (NAASP; a marker of Canavan's disease): 235 $\mu\text{mol/l}$ (normal range 20–25 $\mu\text{mol/l}$).

Discussion

In pediatric practice, early diagnosis of IMDs in newborns and young infants is significantly important to avoid serious consequences such as mental retardation and developmental delay. In clinical metabolic screening laboratories, many screening techniques are applied such as ion-exchange chromatography [13], thin-layer chromatography, paper electrophoresis [14] and gas chromatographic methods [15, 16]. The drawbacks of these techniques are: low sensitivity and specificity, narrow spectrum of diseases detected, extensive sample preparation, too long chromatographic run time and a high rate of false-positive results. Furthermore, these techniques are not capable of detecting serious fatty acid oxidation disorders. Metabolic screening using tandem mass spectrometry permits a fast, sensitive and specific screening for IMDs. Equally important, the technique has high sample throughput which permits analysis of a large number of samples (>100 samples) in few hours. Consequently MS/MS enables the detection of diagnostic levels of amino acids and acylcarnitines in blood

spots; in addition, more than 20 IMDs can be detected with a high degree of specificity and sensitivity in a single run. Optimization of the experimental and instrumental conditions is necessary to achieve optimum measurement of metabolites in blood samples. Prior to the application of the developed MS/MS to patient samples, the validity of the method for analysis of the target metabolites was evaluated. Quality control blood spots containing the diagnostic metabolites at normal and abnormal concentrations were analyzed by the developed MS/MS procedure using an isotopically labeled internal standard mixture. The calculated percent relative standard deviation and percentage deviation from nominal concentration values were appropriate and proved the reproducibility and accuracy of the method. Further, the stability of the diagnostic metabolites in blood spots, during sample collection and storage, was assessed by replicate analysis of quality control blood samples over a 10-week period. The calculated concentrations fluctuated within 10–15% concentration ranges. Application of the developed MS/MS to the analysis of blood samples collected from Kuwaiti newborns and symptomatic

infants from different governmental maternity and pediatric hospitals in Kuwait indicated high rates of IMDs (table 2). Fatty acid oxidation defects such as very-long-chain acyl CoA dehydrogenase deficiency and carnitine-palmitoylcarnitine transferase deficiency and organic acid disorders such as propionic and methylmalonic acidemias were clearly recognized. The robustness of MS/MS technology enables the detection of these metabolic abnormalities with high certainty. The obtained results suggest the justification of the establishment of a national newborn screening program in Kuwait using tandem mass spectrometry to avoid the burden of neurometabolic diseases and the associated long-term care on the health system. Although newborn screening by tandem mass spectrometry is more expensive than other screening techniques, the screening program is beneficial and is likely cost saving to society in the long term [17].

Conclusion

Tandem mass spectrometry is a powerful technology that facilitates an early diagnosis of more than twenty IMDs in newborns and sick infants. The utility of the MS/MS technique in a newborn screening program and as a high-quality clinical service for metabolic screening in maternity and pediatric hospitals in Kuwait is highly recommended.

Acknowledgments

The authors are grateful to the Research Administration, Kuwait University, for the financial support through Research Grant PC02/02. They are also thankful to the Faculty of Pharmacy, Kuwait University, for providing the facility of MS/MS used throughout this work. The authors highly appreciate the cooperation of metabolic specialists and pediatricians in the Kuwait Medical Genetic Center, and maternity and pediatric hospitals in Kuwait. They are also indebted to the Secretary General of the Kuwait University for his kind approval to provide the necessary funds to continue the clinical service of metabolic screening at the Faculty of Pharmacy, Kuwait University.

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