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Analysis of Acylcarnitine Ester for Neonatal Screening of Inborn Errors of Metabolism Using Tandem Mass-spectrometry

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Summary. Tandem mass-spectrometry has been introduced worldwide into neonatal screening programs for the quantitative analysis of acylcarnitine species and amino acids for the diagnosis of organic acidurias, defects of fatty acid oxidation, and amino acidopathies, respectively. Since April 2002 more than 200000 newborn infants have been screened in Austria using tandem mass-spectrometry. In this cohort 37 infants with amino acidopathies and 38 infants with fatty acid oxidation defects or organic acidurias have been diagnosed. The overall incidence of these disorders is 0.036%. The analysis of acylcarnitine species using tandem mass-spectrometry has enabled the diagnosis of infants with inborn errors of metabolism prior to clinical presentation and to initiate therapy early enough to prevent long-term sequelae and to reduce mortality in these disorders.

Keywords. Acylcarnitine; Butylation; Fatty acid oxidation; Inborn error of metabolism; Tandem mass spectrometry.

Introduction

During the last decade electrospray tandem mass spectrometry (MS/MS) has been introduced into many newborn screening programs for the detection of amino acidopathies, fatty acid oxidation disorders (FAO), and organic acidurias [1–3]. This diagnostic strategy with it's almost 100% sensitivity and specificity is based on the analysis of amino acids and acylcarnitine ester that are characteristic for each of the disease categories [1–3]. A diagnosis is suspected when amino acids and acylcarnitine ester and their respective ratios exceed upper or lower cut-off limits. These cut-off limits are usually set at the 99.5th or 0.5th percentile which has led to very few false negative results in patients until now [4, 5].

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Table 1. Diagnostic yield of inborn errors of metabolism among 209000 infants screened from April 2002 until November 2004 using tandem mass-spectrometry

n = 209000 infants	Recall	Diagnoses	False negative
Amino acids	121 (0.05%)	37	0
Acylcarnitine ester	960 (0.46%)	38	3
Total	1081 (0.52%)	75 (0.036%)	3

In most "classic" inborn errors of metabolism the levels of characteristic metabolites are significantly higher than the respective cut-off limits. However as metabolite levels may vary depending on the metabolic status, dietary intake, and maturity of the infants levels may exceed upper cut-off limits only marginally requiring confirmatory testing such as measurement of enzyme activity in fibroblasts and/or mutation analysis [1].

Acylcarnitine profiling by MS/MS has been first accomplished by *Millington* and co-workers who applied chemical derivatisation to biological samples in order to generate positively charged large acylcarnitine butylester [6]. These butylester were semi-quantitatively analysed using electrospray ionisation and positive precursor ion scan of $m/z = 85$ [6]. Technological advances in the fields of mass-spectrometry and electronics have further improved sensitivity and accuracy. The detection limit for many acylcarnitine species has been brought to the nanomolar range using as little as $3\,mm^3$ of whole blood [1–3]. MS/MS has not only been used for neonatal screening but also for selective diagnosis of patients presenting clinically at an older age and for assessment of the physiological evolution of acylcarnitine concentrations during childhood and adulthood [7–9]. Herein we present our experience with MS/MS in the Austrian Neonatal Screening Program.

Results

From April 2002 until November 2004 209000 infants have been screened by the Austrian Neonatal Screening Program at the University Children's Hospital using MS/MS. Seventy-one diagnoses (0.034%) could be confirmed through additional biochemical and molecular genetics methods. Thirty-six diagnoses were based solely on the analysis of acylcarnitine species, while 35 diagnoses were based on abnormal amino acid concentrations. Total recall was 0.5% (Table 1).

Discussion

Neonatal screening by MS/MS has been the mainstay of many screening programs throughout the world [1–3]. It has significantly impacted on early diagnosis and treatment of many different inborn errors of metabolism including amino acidopathies, defects of fatty acid oxidation, and organic acidurias, thus reducing disease associated morbidity and mortality [1, 3]. In particular, analysis of butylated acylcarnitine species as their respective ester allow the assessment of organic acidurias and defects of fatty acid oxidation [6]. MS/MS has been introduced into the

Austrian Neonatal Screening Program in April 2002 following an evaluation phase of 6 months [3]. Since then more than 209000 newborn infants have been screened for 17 different disorders. In this cohort 37 infants with different amino acidopathies and 38 infants with different fatty acid oxidation defects or organic acidurias, respectively, have been diagnosed. The overall incidence of these disorders and the recall in our screening program is comparable to others [1, 2]. As the detection of inborn errors of metabolism is based on acylcarnitine concentrations exceeding set cut-off limits, some disease variants may not be diagnosed due to borderline concentrations [1]. Secondly, metabolism of fatty acids may be suppressed due to intravenous glucose infusions and consequently lower disease specific acylcarnitine concentrations. In our experience false negative diagnoses occur rarely. The development and initiation of tandem mass-spectrometry in neonatal screening programs has significantly changed the natural course of many of the inborn errors of metabolism screened, reducing the risk of metabolic decompensation and thus the rates of morbidity and mortality. Additional technological advancements will further broaden the spectrum of disorders evaluated in neonatal screening programs.

Materials and Methods

Acylcarnitine Analysis

From each dried blood filter card a 3 mm spot containing 3.2 mm^3 blood was punched and automatically transferred to a 96 well plate. Each spot was eluted in 100 mm^3 methanol containing deuterated acylcarnitine standards. The concentrations of the internal standards were as follows: $^2H_9$-carnitine 0.76 $nmol/cm^3$, $^2H_3$-acetylcarnitine 0.19 $nmol/cm^3$, $^2H_3$-propionylcarnitine, $^2H_3$-butyrylcarnitine, $^2H_9$-isovalerylcarnitine, $^2H_3$-octanoylcarnitine, and $^2H_9$-myristoylcarnitine 0.04 $nmol/cm^3$, $^2H_3$-palmitoylcarnitine 0.08 $nmol/cm^3$ (stable isotope standards, CIL, Andover, MA, USA).

The samples were dried during centrifugation under vacuum and butylated. Butylester of free carnitine and various acylcarnitine species were detected by tandem mass spectrometry (MS^2 Wallac, Turku, Finland) in positive electrospray mode. Source block temperature was 140°C. Nebuliser gas was set at 106 dm^3/h and desolvation gas was set at 518 dm^3/h. Capillary voltage, cone voltage, and extractor voltage were 2.17 kV, 28 V, and 4 V, respectively. Total acquisition time was 1.5 minutes. Acylcarnitines were measured by positive precursor ion scan of $m/z = 85$. Individual acylcarnitine species were quantified by comparing their yield to the respective deuterated standard.

Total carnitine was calculated as the sum of all acylcarnitines and free carnitine. The following metabolites were analysed: free carnitine (FC), acetylcarnitine (C2), propionylcarnitine (C3), butylcarnitine (C4), methylmalonylcarnitine (C4DC), 3-hydroxyisovalerylcarnitine (C5OH), hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), dodecanoylcarnitine (C12), tetradecanoylcarnitine (C14); 3-hydroxytetradecanoylcarnitine (C14OH), palmitoylcarnitine (C16), hexadecenoylcarnitine (C16:1), 3-hydroxyhexadecanoylcarnitine (C16OH), stearoylcarnitine (C18), oleylcarnitine (C18:1), linolenylcarnitine (C18:2), 3-hydroxyoctadecanoylcarnitine (C18OH), 3-hydroxyoctadecenoylcarnitine (C18:1OH), 3-hydroxyoctadecadienoylcarnitine (C18:2OH) [3, 5].

The concentrations are automatically transferred into a database and flagged when they exceed the respective cut-off value, which had been set on approximately the 99.5^{th} percentile based on measurements from 200000 newborn infants. Cut-off values are continuously updated. A diagnosis is indicated only when all the respective specific acylcarnitine concentrations and ratios are flagged. The results are then evaluated by a metabolic specialist and the infant is referred to the nearest metabolic center (Table 2).

Table 2. Diagnostic decision criteria based on different acylcarnitine concentrations; PA-propionic acidemia, MMA-methyl-malonic acidemia, IVA-isovaleric acidemia, GA I-glutaric acidemia type I, GA II-glutaric acidemia type II, b-KT-β-ketothiolase deficiency, HMG-HMG-CoA lyase deficiency, 3-MCC-3-methylcrotonyl carboxylase deficiency, MCAD-medium chain acyl CoA dehydrogenase deficiency, LCHAD-long chain acyl CoA dehydrogenase deficiency, VLCAD-very long chain acyl CoA dehydrogenase deficiency, CPT I-carnitine palmitoyl transferase deficiency, CPT II-carnitine palmitoyl transferase deficiency, CT-carnitine transporter deficiency

Disorder	Acylcarnitine (μmol/dm^3)					
PA/MMA	C3 > 3.5	C3/C2 > 0.25				
IVA	C5 > 0.85	C5/C2 > 0.09				
GA I	C5DC > 0.15	C5DC/C2 > 0.03				
GA II	C5DC > 0.15	C5DC/C2 > 0.03	C8 > 0.35	C8/C2 > 0.04		
b-KT	C5OH > 0.50	C5OH/C2 > 0.05	C5:1 > 0.20	C5:1/C2 > 0.05		
HMG	C5OH > 0.50	C5OH/C2 > 0.05	C6DC > 0.25	C6DC/C2 > 0.03		
3-MCC	C5OH > 0.50	C5OH/C2 > 0.05				
MCAD	C6 > 0.3	C6/C2 > 0.05	C8 > 0.35	C8/C2 > 0.04		
LCHAD	C16OH > 0.10	C16OH/C2 > 0.01	C18:1OH > 0.16	C18:1OH/C2 > 0.02		
VLCAD	C14 > 0.5	C14/C2 > 0.05	C14:1 > 0.35	C14:1/C2 > 0.03	C14/C16 > 0.05	
CPT I	C0/(C16 + C18) > 100					
CPT II	C16 > 6	C16/C2 > 0.03	C18:1 > 3	C18:1/C2 > 0.12	C18:2 > 0.35	C18:2/C2 > 0.06
CT	C0 < 7					

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