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Short-incubation mass spectrometry assay for lysosomal storage disorders in newborn and high-risk population screening

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ABSTRACT

The interest in early detection strategies for lysosomal storage disorders (LSDs) in newborns and high-risk population has increased in the last years due to the availability of novel treatment strategies coupled with the development of diagnostic techniques. We report the development of a short-incubation mass spectrometry-based protocol that allows the detection of Gaucher, Niemann-Pick A/B, Pompe, Fabry and mucopolysaccharidosis type I disease within 4 h including sample preparation from dried blood spots. Optimized sample handling without the need of time-consuming offline preparations, such as liquid–liquid and solid-phase extraction, allows the simultaneous quantification of five lysosomal enzyme activities using a cassette of substrates and deuterated internal standards. Applying incubation times of 3 h revealed in intra-day CV% values ranging from 4% to 11% for all five enzyme activities, respectively. In a first clinical evaluation, we tested 825 unaffected newborns and 16 patients with LSDs using a multiplexed, turbulent flow chromatography–ultra high performance liquid chromatography–tandem mass spectrometer assay. All affected patients were identified accurately and could be differentiated from non-affected newborns. In comparison to previously published two-day assays, which included an overnight incubation, this protocol enabled the detection of lysosomal enzyme activities from sample to first result within half a day.

1. Introduction

Lysosomal storage disorders (LSDs) result in the accumulation of macromolecular substrates that would normally be degraded by enzymes involved in lysosomal metabolism. These diseases have a progressive course, and might occur at any age affecting a number of different tissues and organ systems [1]. New impetus for the development of diagnostic techniques was acquired by the availability of novel treatment strategies including enzyme replacement, stem cell transplantation and substrate reduction [2]. However, high throughput screening for LSDs is still a technological challenge due to laborious sample preparation and the need for additional resources. In addition to fluorescent methods using for example 4-methylumbelliferone, efforts have been made to use tandem mass spectrometry (MS/MS) as the method of choice particularly for high-throughput analysis in routine newborn screening laboratories [3]. In this context it is mandatory to achieve high laboratory standards in terms of technical proficiency and reproducibility of results; hence quality control materials provided by the Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC, Atlanta, GA) are available [4].

Protocols for analyzing lysosomal enzyme activities continuously evolved. Procedures were refined and optimized, but the complexity of sample preparation prior to mass spectrometry still remains. Drawbacks of these protocols were the need of liquid–liquid extraction (LLE), solid phase extraction (SPE), and the handling with hazardous organic compounds such as ethyl acetate [5,6]. Novel aspects such as online multi-dimensional chromatography prior to flow injection analysis facilitate ease-of-use sample

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introduction and increased speed of analysis [5,6]. Our research group previously reported the use of turbulent flow chromatography (TFC or TurboFlow®) for online sample clean-up to remove matrix interferences such as salts, proteins and detergents for the analysis of lysosomal enzyme activities in DBS [7]. Subsequently, purified analytes of interest that were removed from potential matrix interferences were transferred from a TFC-column to an analytical column for ultra high performance liquid chromatography (UHPLC) separation prior to MS/MS analysis in order to separate enzymatic products from residual substrate. This simplified protocol has recently been evaluated in a comprehensive pilot screening of more than 8500 newborns to demonstrate the technical feasibility and robustness [8].

Nonetheless for future implementation of high-throughput LSD assays in routine clinical diagnostics, sample handling and mass spectrometric analysis has to be simplified; specifically, sample pretreatment, speed of analysis and finally detection must become more integrated [9]. We have recently introduced a multiplexed TFC–UHPLC–MS/MS based assay for simplified analysis of LSDs [7,8]. The aim of the study was to optimize this protocol in terms of sample handling and workflow, and to reduce the previously required incubation times from 16–20 h to 3 h to provide rapid sample analysis in a daily clinical laboratory.

2. Experimental

2.1. Chemicals and reagents

Substrates and internal standards were provided by the Newborn Screening Translation Research Initiative, Centers for Disease Control and Prevention, Atlanta, GA, USA. Deionized water (18 MΩ) produced by a Millipore Milli-Q Reference A+ System, HPLC-grade acetonitrile, isopropanol, acetone were purchased by Merck Chemicals; all other reagents were purchased from Sigma–Aldrich Co. LLC, St. Louis, MO, USA; Cyclone-P™ (0.5 mm × 50 mm) TurboFlow-columns and Hypersil Gold C8 (1.9 μm, 50 mm × 2.1 mm) columns were purchased from Thermo Fisher Scientific Inc., MA, USA; and 96-well/F bottom microplates and deep well plates by Eppendorf AG, Hamburg, Germany.

2.2. Preparation of the reagent cocktails

A modified previously used incubation system first published by Li et al. was used for incubation [3]. In brief, the enzyme specific substrates and associated internal standards were reconstituted as follows: ABG cocktail; add 15.6 ml of ABG buffer (0.715 mol/L phosphate and 0.358 mol/L citrate buffer, pH 5.1) plus 2.4 ml of a 120 g/L sodium taucholate solution/ASM cocktail; add 17.85 ml of ASM buffer (0.930 mol/L sodium acetate and 0.604 mmol/L zinc chloride, pH 5.7) and 0.15 ml of a 120 g/L sodium taucholate solution/GAA cocktail; add 1.8 ml of a 100 g/L CHAPS, 15.9 ml of GAA buffer (0.340 mol/L phosphate plus 0.170 mol/L citrate, pH 4.0) and 0.3 ml of 0.8 mmol/L acarbose solution/GLA cocktail; add 14.67 ml of GLA buffer (0.174 mol/L sodium acetate, pH 4.6), 0.45 ml of a 120 g/L sodium taucholate solution and 2.88 ml of a 1 mol/L N-acetylgalactosamine solution/IDUA cocktail; add 17.5 ml of IDUA buffer (0.112 mol/L sodium formate, pH 3.6) and 0.5 ml of a 3.0 mmol/L saccharolactone solution; GALC cocktail; add 1.8 ml of a detergent solution (96 g/L sodium taucholate plus 1.6 g/L oleic acid) and 16.2 ml of GALC buffer (0.2 mol/L phosphate plus 0.1 mol/L citrate, pH 4.4) to the corresponding vials containing substrates and internal standards. All buffers and detergent solutions were prepared with HPLC grade water. The total dilution volume was 18 ml.

2.3. Optimization of sample preparation and workflow

For the detection of five lysosomal enzyme activities two punches from a DBS card in two separate 96-well microplates were needed (Fig. 1). Punch 1 was used for the determination of enzyme activities of acid β-glucocerebrosidase (ABG; Gaucher disease), acid sphingomyelinase (ASM; Niemann-Pick A/B disease), α-glucosidase (GAA; Pompe disease) and α-galactosidase (GLA; Fabry disease), respectively. Punch 2 was used for α-L-iduronidase (IDUA; mucopolysaccharidosis type I) analysis. The first punch was diluted with 60 μL extraction buffer (20 mmol/L sodium phosphate; pH 7.1) and the 96-well microplate was sealed with silicon cover. For enzyme extraction the 96-well microplates were centrifuged for 5 min at 2000 × *g* at room temperature and subsequently shaken for 15 min at 37 °C at 750 rpm on a heated orbital shaker.

Aliquots of 10 μL were used to perform separate incubations for ABG, ASM, GAA and GLA in separate 96-well microplates with specific aqueous cocktails containing a cassette of enzyme specific substrates and internal standards. The second punch was directly incubated by adding 30 μL specific IDUA cocktail and centrifuged for 3 min at 2000 × *g* at room temperature prior to incubation. Incubation for all reactions was performed at 37 °C shaking at 250 rpm. Aliquots of the reactions were stopped at 1 h, 3 h, 6 h and 20 h by adding 100 μL stopping solution (80:20 acetonitrile:water plus 0.2% formic acid). The reaction mixtures of all five corresponding reactions were transferred to one new deep-well plate, covered with aluminum foil and subsequently centrifuged at 3000 × *g* for 15 min prior to mass spectrometry analysis.

2.4. TFC–UHPLC–MS/MS analysis

The chromatographic separation was performed with a previously described online-sample clean-up TFC–UHPLC–MS/MS method [7,8]. In short: A Transcend™ TLX-2 TFC–UHPLC system with quaternary pumps was used (Thermo Fisher Scientific). The mobile phases were: A = 0.1% formic acid, 0.01% TFA in water, B = 0.1% formic acid, 0.01% TFA in acetonitrile, and C = 45:45:10 isopropanol/acetonitrile/acetone. The sample injection volume was 10 μL. The Transcend system employs two TurboFlow® chromatography channels, each with two six-port valves configured in focusing mode (recovered analytes from the TFC column were transferred and focused on a subsequent UHPLC column for further separation) [10]. Samples were separated from matrix components during the loading step by TFC using a Cyclone-P column with mobile phase A with a flow rate of 1.5 ml/min. After buffer-salts, proteins and DBS residuals were rinsed away, the valves were switched and the extracted analytes were back-flushed off the TurboFlow column by the contents of a 200 μL eluting loop filled with 20:80 mobile phase A/mobile phase B and focused on a Hypersil Gold C8 UHPLC-column. All analytes were separated using a linear gradient from 0% to 100% B in 40 s with a flow rate of 0.7 ml/min [8]. The columns were then washed with buffer C and re-equilibrated for the next injection. The system was operated by Aria™ Software V 1.6.3 (Thermo Fisher Scientific). The total run time for one TurboFlow/UHPLC experiment including online sample clean-up, transfer step, separation and re-equilibration was 4 min per channel. MS/MS data acquisition started 2.15 min after injection and continued for 90 s until all analyte signals were recorded. MS/MS analysis was performed on a TSQ Quantum Ultra™ (Thermo Fisher Scientific) equipped with an HESI-II heated electrospray probe and operated by Xcalibur™ V 2.1.0.1139 (Thermo Fisher Scientific). The MS settings for all compounds including selected reaction monitoring (SRMs) and electrospray parameters were used according to Metz et al. [8]. The amount of product was calculated from the ion abundance ratio (peak area) of the product to internal

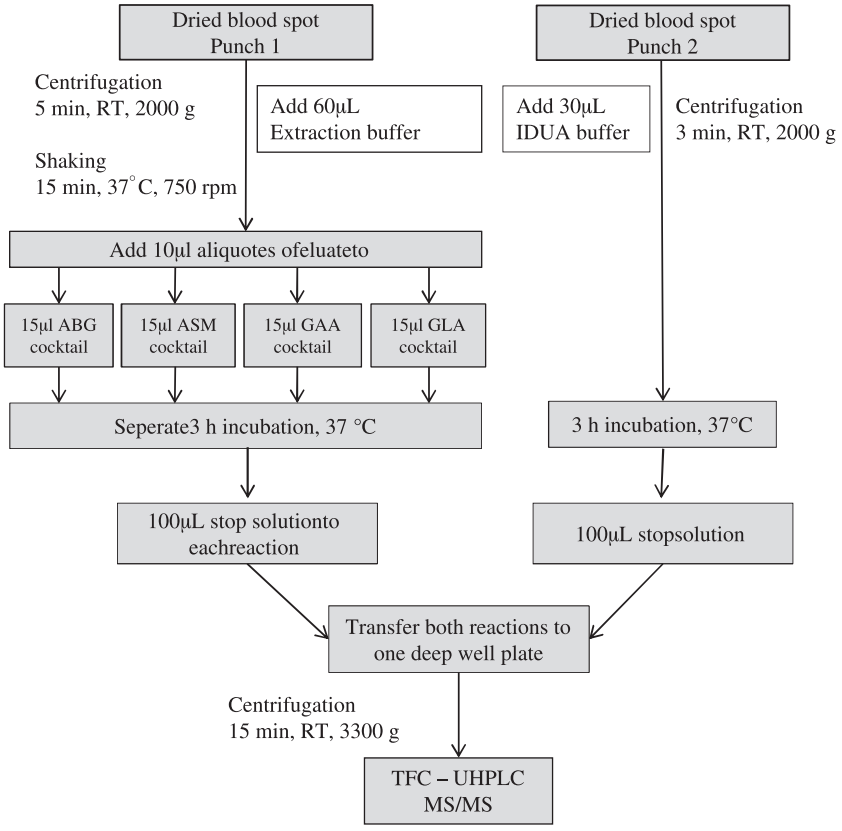

Fig. 1. Optimized workflow for the analyses of up to five lysosomal enzyme activities simultaneously from dried blood spots within 4 h.

standard for a sample multiplied by the amount of added internal standard, divided by the incubation time and the added blood volume (~3.1 µL per 3.2 mm DBS punch).

2.5. Clinical evaluation

Three 3.2-mm (1/8-in.) punches from DBS of 16 known patients with LSDs and 825 single punches of non-affected newborns were tested in parallel with a new modified DBS extraction and short (3 h) incubation protocol and compared to a previously published protocol with an overnight incubation of 20 h [8].

2.6. Statistical analysis

All mass spectrometry data were analyzed with LCquan™ 2.6.0.1128 (Thermo Fisher Scientific). We used SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) for data analysis.

3. Results and discussion

3.1. Time-optimization of DBS extraction and incubation time

Compared to the protocol published by Li et al., Zhang et al. [3,5] and our previous published work the extraction time for DBS could by shortened from 60 min shaking at 750 rpm to a total time of 20 min by adding a 5 min centrifugation step prior to shaking for 15 min at 750 rpm [10]. Using these optimizations, sample preparation time before incubation could be reduced to approximately 30 min each (for up to 90 samples plus quality control materials) including preparation time. Incubation times of 1, 3, 6 and 20 h were tested. We observed that the enzyme kinetics of ABG, ASM, GAA, and GLA were linear over time and revealed an intra CV% between 4% and 11% even after 3 h of incubation time (Table 1 and Fig. 2A–E). The GALC activity assay revealed MS signals for low normal enzyme activities after at least 16 h of incubation time (data not shown). In comparison to our previously published protocol [10], an additional DBS punch was needed for the analysis of IDUA activity. Representative data of the enzyme kinetics of GAA are displayed in Fig. 2A, including different incubation times and their respective peak intensities of product versus product/internal-standard ratios.

3.2. Method validation

We performed a first method validation using a TFC–UHPLC–MS/MS assay to reduce incubation time for five lysosomal enzymes. For selectivity, masses of the substrates of all five LSDs were additionally analyzed by mass spectrometry using SRMs. The separation between in-source fragmented substrates and enzymatic derived products was evaluated. As shown in Fig. 3A and B all product peaks were separated accurately from peaks derived from in-source fragmentation.

For precision and accuracy, we measured all three levels of CDC QC samples in 10 replicates on five different days. The reference values for calculating accuracy have been determined by measuring 10 CDC QC DBS with the method previous described by Metz et al. [8]. The results showed that the intra-day accuracy ranged from 82% to 125%, and the precision from 4% to 11%, respectively. Inter-day accuracy was between 70% and 109%, and inter-day precision between 7% and 20% (Table 1).

For recovery experiments, we compared signal intensities (cps) of 10 µL of stopped and precipitated QC samples (low, medium, high) with and without the use of online extraction using a Turboflow-column. Hereby sample material was injected directly to the Hypersil Gold C8 UHPLC-column and subsequently 10 µL of the same sample was injected to the TFC-column prior to UHPLC

Table 1
Intra-day and inter-day accuracy and precision of the short enzyme incubation assay of 5 lysosomal enzymes at 3 different levels measured by online sample clean up TFC–UHPLC–MS/MS.

Analyte (level)	[a]Reference values (n = 10) (μmol/L/h)	3 h protocol values (n = 10) (μmol/L/h)	Intra-day (n = 10)		Inter-day (n = 50)	
	Mean	Mean	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
ABG						
QCL	1.12	1.40	125	7	106	16
QCM	7.32	6.89	94	9	99	11
QCH	13.58	13.86	102	10	106	8
ASM						
QCL	0.21	0.18	93	10	104	20
QCM	1.63	1.59	99	8	81	14
QCH	3.42	3.41	100	8	89	8
GLA						
QCL	0.58	0.49	84	11	81	17
QCM	4.90	5.54	113	6	109	7
QCH	11.60	13.36	115	8	101	10
GAA						
QCL	0.88	0.90	102	9	100	10
QCM	7.00	7.31	104	5	105	5
QCH	13.98	15.25	109	4	101	8
IDUA						
QCL	0.68	0.56	82	9	70	18
QCM	5.81	5.89	102	9	106	9
QCH	11.72	10.34	88	7	93	9

[a] Values are determined by measuring 10 spots of CDC Quality control DBS on 5 separate days: the reference values for calculating accuracy have been determined by measuring 10 CDC QC DBS with the method previously published by Metz et al. ABG, acid β-glucocerebrosidase; ASM, acid-sphingomyelinase; GAA, α-glucosidase; GLA, α-galactosidase; IDUA, α-L-iduronidase; QCL, low, QCM, medium and QCH high quality control material of the CDC Newborn screening quality assurance program for lysosomal storage disorders.

analysis. The results on recoveries for all compounds were between 33% and 111% (shown in detail in Table 2).

Carryover experiments have been carried out according to the protocol of EP Evaluator® (EP Evaluator, Release 10, Data Innovations, LLC: VT, US). Five dried blood spots of CDC QCL following a QCL sample and 5 CDC QCL samples following a QCH sample were measured by the novel protocol. Carryovers of products and internal standards were considered as acceptable when its

Table 2
Assessment of product and corresponding internal standards recovery of ABG, ASM, GLA, GAA and IDUA using TFC–UHPLC–MS/MS.

Analyte	Level[a]	Mean peak area (n = 10)		Recovery[b] (%)	Ratio (P/IS) unextracted[d]
		Unextracted[b]	Extracted reaction[c]		Ratio (P/IS) extracted
ABG P (ABG IS)	QCL	4.53E10^4 (7.94E10^5)	1.61E10^4 (2.64E10^5)	36 (33)	1.09
	QCM	1.78E10^5 (8.99E10^5)	1.14E10^5 (5.18E10^5)	64 (58)	1.10
	QCH	3.44E10^5 (9.26E10^5)	2.04E10^5 (5.20E10^5)	59 (57)	1.04
ASM P (ASM IS)	QCL	1.79E10^4 (7.17E10^6)	1.10E10^4 (4.66E10^6)	61 (65)	0.94
	QCM	1.20E10^5 (6.90E10^6)	8.32E10^4 (5.04E10^6)	69 (73)	0.95
	QCH	2.28E10^5 (8.09E10^6)	1.73E10^5 (5.90E10^6)	76 (73)	1.04
GLA P (GLA IS)	QCL	2.71E10^4 (3.68E10^5)	2.63E10^4 (3.60E10^5)	97 (98)	0.99
	QCM	1.08E10^5 (3.60E10^5)	9.56E10^4 (3.34E10^5)	89 (93)	0.96
	QCH	2.40E10^5 (4.07E10^5)	2.02E10^5 (3.45E10^5)	84 (85)	0.99
GAA P (GAA IS)	QCL	2.54E10^4 (6.12E10^5)	2.65E10^4 (6.77E10^5)	105 (111)	0.95
	QCM	1.35E10^5 (7.44E10^5)	1.03E10^5 (5.92E10^5)	76 (80)	0.95
	QCH	3.00E10^5 (8.3E10^5)	2.61E10^5 (7.67E10^5)	87 (92)	0.95
IDUA P (IDUA IS)	QCL	9.59E10^3 (7.79E10^4)	1.03E10^4 (7.76E10^4)	107 (100)	1.08
	QCM	5.36E10^4 (8.65E10^4)	5.97E10^4 (9.62E10^4)	111 (110)	1.01
	QCH	1.25E10^5 (8.55E10^4)	1.26E10^5 (8.96E10^4)	100 (105)	0.95

[a] CDC QC samples incubated for 3 h prior to analysis.
[b] Directly injected to UHPLC-column.
[c] TFC online extraction prior to UHPLC analysis.
[d] Ratio (P/IS) of direct injection on UHPLC-column divided by the ratio (P/IS) derived by samples injected to online extraction TFC-column prior to UHPLC analysis.

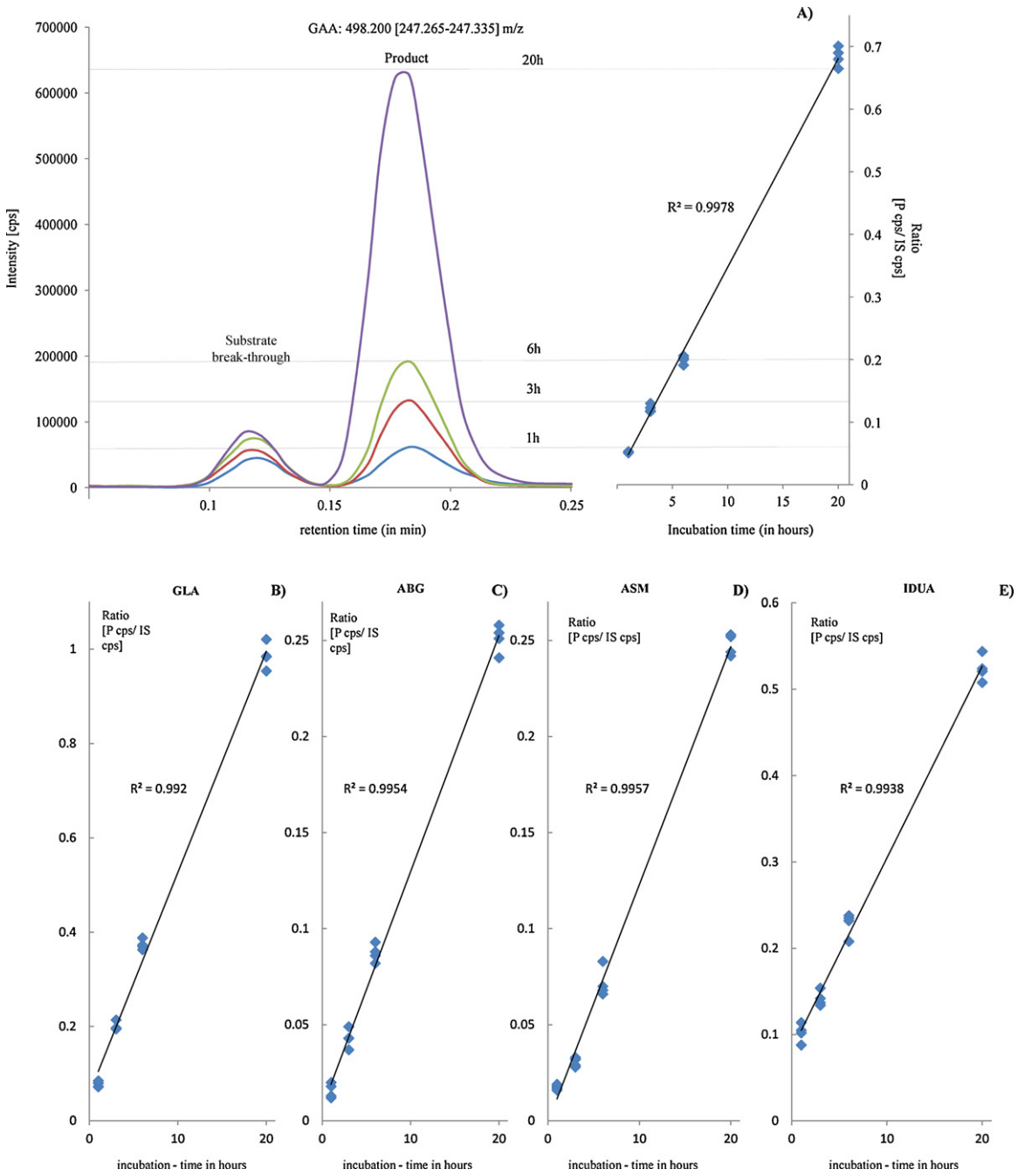

Fig. 2. (A–E) Exemplary data including different incubation times and their respective peak intensities of product versus product to internal standard ratios for lysosomal enzyme activities.

influence on enzyme activities were lower than three times the standard deviation of a series of five QCL samples (Table 3).

3.3. Stability

The DBS of known LSD patients were stored at −80 °C until analysis. The storage time of samples was between two and 14 months. The 825 control samples were analyzed in parallel to the NBS routine. For the evaluation of post-processing stability, triplicates of three patient samples (two Gaucher, one Fabry patient) and 10 NBS DBS samples have been determined directly after stopping the incubation and after 24 h stored at room temperature. The signal intensities and overall product to internal standard ratio were in the range of the assay variation after retesting at 24 h compared to the results of the immediate analysis.

3.4. High-throughput analysis

The workflow of sample preparation for all five lysosomal storage disorders was optimized thus results were available within one working day (Fig. 1). In summary, pre-sample treatment (approximately 30 min), short-incubation (3 h), preparation for MS-analysis (approximately 20 min) and TFC–UHPLC–MS/MS (2 min per sample), provided the first results within 4 h. Using this protocol, 90 samples including quality controls could be analyzed within 7 h.

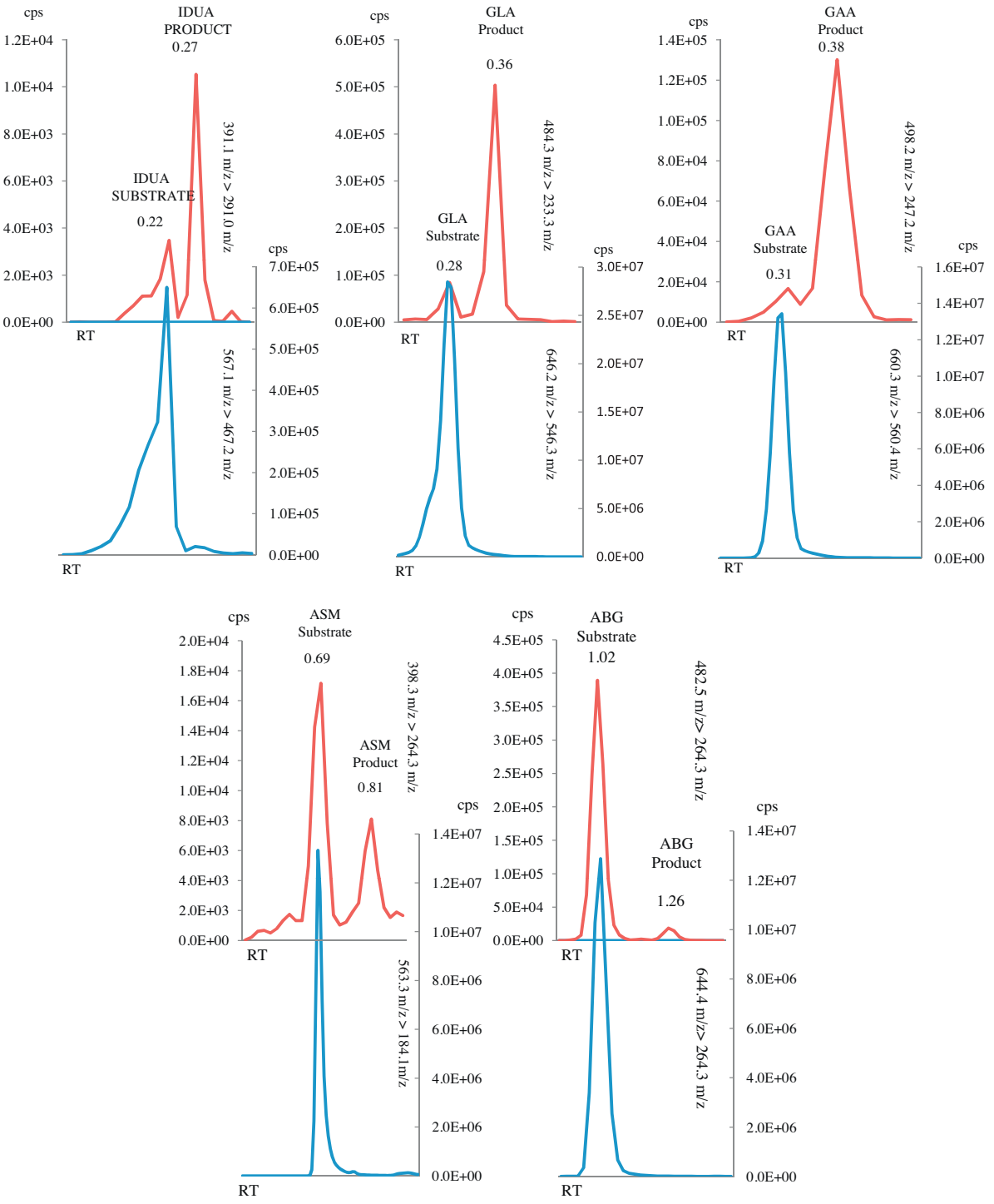

Fig. 3. Example chromatogram showing the separation of all 5 enzyme products to their corresponding substrates.

3.5. *Clinical evaluation*

We analyzed a total of 16 known patients with LSDs (four patients with Pompe, five with Gaucher, five with Fabry, one with Niemann-Pick A/B, and one with MPS I disease) for clinical evaluation. We compared the short-incubation with our previous developed reference protocol (16–20 h incubation) [8]. In Table 4, a detailed overview of all single enzyme activities using short versus the long incubation time is displayed, and we did not observe any statistical difference between both protocols in this first clinical setup. In addition, we analyzed all five lysosomal enzyme activities in 825 normal non-affected newborns (Table 5). All patients presented clinically with late onset phenotypes [1,11] except Pompe Patient 3, Niemann Pick Patient I and MPS I Patient 1 (Table 4). All affected patients could be differentiated from normal newborns in this first pilot study by using the 0.5th percentile of lysosomal enzyme activities from normal newborns as a preliminary cut-off value. Certainly, further studies with a larger number of newborns

Table 3
Carryover of the short enzyme incubation assay of 5 lysosomal enzymes measured by TFC–UHPLC–MS/MS.

Analyte	Mean ($n = 5$) (μmol/L/h)	SD (μmol/L/h)	Error limit[a] (μmol/L/h)	Carryover (μmol/L/h)
ABG				
Low–low	1.43	0.18	0.55	0.26
High–low	1.70	0.17		
High	13.86	1.36		
ASM				
Low–low	0.17	0.01	0.02	0.01
High–low	0.18	0.02		
High	3.50	0.31		
GLA				
Low–low	1.08	0.11	0.33	0.22
High–low	1.3	0.07		
High	11.10	0.69		
GAA				
Low–low	0.72	0.08	0.25	0.02
High–low	0.74	0.10		
High	13.68	1.19		
IDUA				
Low–low	0.56	0.05	0.14	0.05
High–low	0.61	0.03		
High	12.12	0.66		

[a] Error-limit was set to 3 times the standard deviation of low samples following a low sample ($n = 5$).

Table 4
Enzyme activities of affected patients according to their incubation time.

	Storage time of DBS at −80 °C before analysis in months	Enzyme activity 20 h incubation (μmol/L/h)	Enzyme activity 3 h incubation (μmol/L/h)	Patient statistics 20 h incubation (μmol/L/h)	Patient statistics 3 h incubation (μmol/L/h)
Gaucher Patient 1	4	1.2	1.5	Mean: 0.8 Std.: 0.4 Max: 1.2 Min: 0.2	Mean: 1.1 Std.: 0.4 Max: 1.5 Min: 0.5
Gaucher Patient 2	4	0.8	1.3		
Gaucher Patient 3	6	0.9	1.1		
Gaucher Patient 4	8	0.7	1.0		
Gaucher Patient 5	5	0.2	0.5		
Fabry Patient 1 (m)	2	0.3	0.5	Mean: 1.1 Std.: 0.9 Max: 2.2 Min: 0.3	Mean: 1.0 Std.: 0.8 Max: 1.9 Min: 0.4
Fabry Patient 2 (m)	4	0.7	0.5		
Fabry Patient 3 (m)	4	0.4	0.4		
Fabry Patient 4 (f)	5	2.2	1.8		
Fabry Patient 5 (f)	8	2.0	1.9		
Pompe Patient 1	6	0.6	0.2	Mean: 0.5 Std.: 0.2 Max: 0.6 Min: 0.2	Mean: 0.4 Std.: 0.3 Max: 0.7 Min: 0.1
Pompe Patient 2	3	0.2	0.1		
Pompe Patient 3	3	0.6	0.7		
Pompe Patient 4	3	0.4	0.4		
Niemann Pick A/B Patient	14	0.1	0.5	–	–
Mucopolysaccharidosis type I Patient	2	0.1	0.1	–	–

m, male; f, female.

and also adults for cut-off determination are needed. We concluded that this time saving protocol could be used for different clinical areas including selective metabolic screening for suspected patients at risk in a hospital, as well as for newborn or high-risk population screening.

3.6. Implication for early detection strategies for lysosomal storage disorders

Currently, routine newborn screening for LSDs has been introduced for Pompe disease in Taiwan [12] and for Krabbe disease in

Table 5
Enzymatic activities (μmol/L/h) of 825 neonates determined by 3-h incubation and TFC–UHPLC–MS/MS.

$n = 825$	ABG n	ASM n	GAA n	GLA n	IDUA n
Mean	19.6	4.4	18.6	6.4	12.4
Percentile 0.5%	6.97	1.06	5.63	2.60	3.27
Percentile 1.0%	7.99	1.12	6.90	2.68	3.87
Percentile 25%	15.41	2.34	13.55	4.35	9.21
Median	18.78	3.80	17.17	5.52	11.73
Percentile 75%	23.06	5.67	22.49	7.38	14.77
Percentile 99%	39.30	13.41	43.52	18.88	27.18
Percentile 99.5%	41.49	13.73	50.31	22.24	31.12
Min	5.19	1.06	4.75	2.54	2.67
Max	44.02	21.34	69.25	60.02	62.72

ABG, acid β-glucocerebrosidase; ASM, acid-sphingomyelinase; GAA, α-glucosidase; GLA, α-galactosidase; IDUA, α-L-iduronidase.

the State of New York [13]. The Austrian Newborn Screening center [14] and others, e.g. in Washington State [15], have successfully started pilot studies using multiplexed MS/MS screening assays [16–18]. The aim of this study was to develop a mass spectrometry based protocol for the rapid and accurate detection of lysosomal enzyme activities. Previously published protocols used 16–20 h incubation time [3,8,19]. We report the simplification and optimization of pre-analytical sample preparation to decrease the total analysis time to approximately 4 h.

Most metabolic screening laboratories do not have a seven-days a week service. One drawback of previously published two-day working protocols for newborn screening or high-risk population screening for LSDs is that the analysis cannot be performed in parallel to the routine NBS for amino acids and acylcarnitines. Early diagnosis is of importance for e.g. infants suspected to infantile Pompe disease. In daily clinical routine, the use of this rapid and reliable assay would also offer the opportunity to accelerate differential diagnosis for juveniles and adults submitted to the hospital with symptoms similar to LSDs.

We used TurboFlow technology for sample clean-up prior to flow injection MS/MS analysis. The proof of concept using this technology was published recently by our research group [7]. Moreover, in a first comprehensive clinical evaluation we could demonstrate that the technology is robust and accurate to detect patients with diminished lysosomal enzyme activity and to differentiate them from normal newborns [8]. We took advantage of online sample clean-up using multidimensional chromatography that eliminates time-consuming and laborious protocol steps such as LLE and SPE, the use of toxic and volatile organic solvents such as ethyl acetate [3,19,20], and reduce the need for large amounts of consumables. Benefits of using TFC were described in a large number of varied analytical environments, drug discovery and pharmacokinetics, metabolite profiling, and clinical applications [10,21]. The combination of both a TFC-column and an analytical UHPLC column, improved the multiplexed enzymatic assay by eliminating interfering compounds and by separating enzymatic products from residual substrates. The accurate separation of product and residual substrate is of importance because previously published LSD assays reported the potential interference of the enzyme product signal from excess substrate due to in-source fragmentation [5]. The higher resolving power of UHPLC, completely eliminates such interferences while keeping total analysis time to 2 min per sample and facilitates the expansion of the screening panel.

However, one drawback of using MS/MS-based assays for LSDs was the long enzyme incubation time of more than 16 h that required two-day protocols (with an overnight incubation). We optimized and adapted our previously published protocol and modified the work-flow (Fig. 1). This allows the reduction of DBS incubation time with a cassette of substrates and deuterated internal standards from 16 to 3 h. Sista et al. reported the use of a digital microfluidic platform to perform multiplexed enzymatic analysis using fluorescence with 4-methylumbelliferone within 2 h on a small set of samples. However, it was restricted to two LSDs (Pompe and Fabry disease), and still needed evaluation [22]. Conventional fluorescence methods usually include incubation times of more than three to 20 h depending on the respective lysosomal enzyme and sensitivity of the assay [23].

One limitation of the current protocol is the use of several buffer systems for different enzymes, and the requirement of a second DBS punch for IDUA due to loss of sensitivity, and poor separation of IDUA product and substrate. In addition, the lysosomal enzyme activity for galactocerebrosidase (GALC; Krabbe disease) was also reported to be very low [7,24], and thus it was not possible to include this LSD in the current assay. However, novel buffer systems for the combined incubation of more than 6 or 9 enzymes simultaneously are on the horizon including substrates for mucopolysaccharidosis type II, IVA and VI [25–28], and were presented recently by Gelb and his research group [29]. These new buffer systems might allow the incubation of several enzymes in one reaction vial, and help to reduce costs for personnel, consumables and reagents.

Our results using the short-incubation assay for Gaucher, Niemann-Pick A/B, Pompe, Fabry and mucopolysaccharidosis type I disease from DBS were in close agreement with previously published standard incubation time (16–20 h) in our clinical reference laboratory. The mean activities for ABG, ASM, GAA, GLA and IDUA using short-incubation protocol with 3 h time were similar to those obtained using 16–20 h incubation time. There was clear separation between normal non-affected newborn samples and confirmed affected samples for all five LSDs despite the much lower incubation time.

4. Conclusion

In conclusion, we successfully demonstrated and evaluated the performance of a multiplexed mass spectrometry-based assay to screen for Pompe, Fabry, Niemann-Pick A/B and Gaucher and mucopolysaccharidosis type I diseases using a short-incubation of 3 h. After careful clinical and method validation and evaluation, this protocol could be used for selective metabolic screening for patients who are suspected to LSDs, and for newborn and high-risk population screening in future routine and research studies.

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References

[1] M. Beck, J. Inherit. Metab. Dis. 24 (Suppl. 2) (2001) 47.
[2] M. Beck, IUBMB Life 62 (2010) 33.
[3] Y. Li, C.R. Scott, N.A. Chamoles, A. Ghavami, B.M. Pinto, F. Turecek, M.H. Gelb, Clin. Chem. 50 (2004) 1785.
[4] V.R. De Jesus, X.K. Zhang, J. Keutzer, O.A. Bodamer, A. Muhl, J.J. Orsini, M. Caggana, R.F. Vogt, W.H. Hannon, Clin. Chem. 55 (2009) 158.
[5] B. Shushan, Mass Spectrom. Rev. 29 (2010) 930.
[6] G. la Marca, B. Casetta, S. Malvagia, R. Guerrini, E. Zammarchi, Anal. Chem. 81 (2009) 6113.
[7] D.C. Kasper, J. Herman, V.R. De Jesus, T.P. Mechtler, T.F. Metz, B. Shushan, Rapid Commun. Mass Spectrom. 24 (2010) 986.
[8] T.F. Metz, T.P. Mechtler, J.J. Orsini, M. Martin, B. Shushan, J.L. Herman, R. Ratschmann, C.B. Item, B. Streubel, K.R. Herkner, D.C. Kasper, Clin. Chem. 57 (2011) 1286.
[9] T. Annesley, J. Majzoub, A. Hsing, A. Wu, A. Rockwood, D. Mason, Clin. Chem. 55 (2009) 1236.
[10] R.P. Grant, C. Cameron, S. Mackenzie-McMurter, Rapid Commun. Mass Spectrom. 16 (2002) 1785.
[11] C. Desnuelle, L. Salviati, Curr. Opin. Neurol. 24 (2011) 443.
[12] Y.H. Chien, S.C. Chiang, X.K. Zhang, J. Keutzer, N.C. Lee, A.C. Huang, C.A. Chen, M.H. Wu, P.H. Huang, F.J. Tsai, Y.T. Chen, W.L. Hwu, Pediatrics 122 (2008) e39.
[13] J.J. Orsini, M.A. Morrissey, L.N. Slavin, M. Wojcik, C. Biski, M. Martin, J. Keutzer, X.K. Zhang, W.L. Chuang, C. Elbin, M. Caggana, Clin. Biochem. 42 (2009) 877.
[14] T.P. Mechtler, S. Stary, T.F. Metz, V.R. De Jesus, S. Greber-Platzer, A. Pollak, K.R. Herkner, B. Streubel, D.C. Kasper, Lancet 379 (2012) 335.
[15] T.A. Duffey, G. Bellamy, S. Elliott, A.C. Fox, M. Glass, F. Turecek, M.H. Gelb, C.R. Scott, Clin. Chem. 56 (2010) 1854.
[16] A. Dajnoki, G. Fekete, J. Keutzer, J.J. Orsini, V.R. De Jesus, Y.H. Chien, W.L. Hwu, Z. Lukacs, A. Muhl, X.K. Zhang, O. Bodamer, Clin. Chim. Acta 411 (2010) 1428.
[17] A. Dajnoki, A. Muhl, G. Fekete, J. Keutzer, J. Orsini, V. Dejesus, X.K. Zhang, O.A. Bodamer, Clin. Chem. 54 (2008) 1624.
[18] E. Legnini, J.J. Orsini, C. Hung, M. Martin, A. Showers, M. Scarpa, X.K. Zhang, J. Keutzer, A. Muhl, O.A. Bodamer, Clin. Chim. Acta 412 (2011) 343.
[19] X.K. Zhang, C.S. Elbin, W.L. Chuang, S.K. Cooper, C.A. Marashio, C. Beauregard, J.M. Keutzer, Clin. Chem. 54 (2008) 1725.
[20] T.A. Duffey, G. Bellamy, S. Elliott, A.C. Fox, M. Glass, F. Turecek, M.H. Gelb, C.R. Scott, Clin. Chem. 56 (12) (2010) 1854.

[21] R. Harlan, W. Clarke, J.M. Di Bussolo, M. Kozak, J. Straseski, D.L. Meany, Clin. Chim. Acta 411 (2010) 1728.
[22] R.S. Sista, A.E. Eckhardt, T. Wang, C. Graham, J.L. Rouse, S.M. Norton, V. Srinivasan, M.G. Pollack, A.A. Tolun, D. Bali, D.S. Millington, V.K. Pamula, Clin. Chem. 57 (2011) 1444.
[23] G. Civallero, K. Michelin, J. de Mari, M. Viapiana, M. Burin, J.C. Coelho, R. Giugliani, Clin. Chim. Acta 372 (2006) 98.
[24] Y. Li, K. Brockmann, F. Turecek, C.R. Scott, M.H. Gelb, Clin. Chem. 50 (2004) 638.
[25] T.A. Duffey, M. Sadilek, C.R. Scott, F. Turecek, M.H. Gelb, Anal. Chem. 82 (2010) 9587.
[26] T. Khaliq, M. Sadilek, C.R. Scott, F. Turecek, M.H. Gelb, Clin. Chem. 57 (2010) 128.
[27] D. Wang, B. Eadala, M. Sadilek, N.A. Chamoles, F. Turecek, C.R. Scott, M.H. Gelb, Clin. Chem. 51 (2005) 898.
[28] D. Wang, T. Wood, M. Sadilek, C.R. Scott, F. Turecek, M.H. Gelb, Clin. Chem. 53 (2007) 137.
[29] M.H. Gelb, C.R. Scott, APHL Newborn Screening and Genetics Testing Symposium, 2011.