Development of a fast LC-MS/MS protocol for combined measurement of six LSDs on dried blood spot in a newborn screening program

Journal of Pharmaceutical and Biomedical Analysis 165 (2019) 135–140

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of a fast LC-MS/MS protocol for combined measurement of six LSDs on dried blood spot in a newborn screening program

Emanuela Scolamiero[a,1], Bruno Casetta[a,1], Sabrina Malvagia[a], Tetsuo Tanigawa[b], Giulia Forni[a], Silvia Funghini[a], Massimo Mura[a], Francesca Raspini[a], Sara Poggiali[a], Giancarlo la Marca[a,c,*]

[a] Newborn Screening, Biochemistry and Pharmacology Laboratory, Pediatric Neurology, Unit and Laboratories, Meyer Children's University Hospital, Florence, Italy
[b] Analytical & Measuring Instruments Division Shimadzu Corporation, Kyoto, Japan
[c] Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

ARTICLE INFO

Article history:
Received 30 August 2018
Received in revised form 9 November 2018
Accepted 2 December 2018
Available online 3 December 2018

Keywords:
Lysosomal storage disorders
Newborn screening
Dried blood spot
Mass spectrometry

ABSTRACT

New treatment options and improved strategies for Lysosomal Storage Disorders (LSDs) diagnosis on dried blood spot (DBS) have led to the development of several pilot newborn screening programs.

Building on a previously published protocol, we devised a new 6-plex assay based on a single DBS punch incubated into a buffer containing a combination of substrates (GAA, GLA, ASM, GALC, ABG and IDUA). This new protocol incorporates a new trapping and clean-up procedure using perfusion chromatography connected on-line with an analytical column for analyte separation, after enzymatic reaction. Results are available after 4.5 min.

Several incubation times were tested in order to reduce sample preparation times and to improve accuracy and reproducibility, also regarding the quenching of the reaction within the time window of linear product accumulation. The collected data demonstrate that an incubation time of 4 h is enough to achieve good reaction efficiency without any impact on sensitivity.

The method proved versatile and robust for various instrument configurations. The fast sample preparation and running times allow a high sample throughput; an advantage in newborn screening procedures. This method can also be used for diagnostic purposes, allowing a rapid diagnosis in a few hours.

1. Introduction

Lysosomal Storage Disorders (LSDs) are a group of more than 50 diseases sharing an impairment of lysosomal function. Over recent years, many of these conditions, which can be more successfully treated if diagnosed early, have been added to newborn screening panels as pilot projects [1–3].

Since 2001, approaches for identifying LSD patients have been developed in research settings. Chamoles and colleagues described a fluorometric assay for diagnosing LSDs based on the use of dried blood spots (DBS) [4–7]. Subsequently, Gelb and colleagues provided appropriate substrates and isotopically-labelled internal standards for performing multiple enzymatic assays by LC–MS/MS on DBS [8]. Currently, LC–MS/MS is the most commonly used technique for identifying LSDs during the neonatal period before clinical symptoms appear [2,3,9].

In 2009, there was a significant breakthrough when a specific LC configuration was devised [10] for skipping the purification steps that were required before instrumental measurement in the original protocol. This configuration led to a more routine application of the method as the relatively short analytical measurement time (around 4 min, injection-to-injection), meant that it was possible to achieve higher sample throughputs. Moreover, the protocol relied on a single high-pressure binary LC pump and switching valve,

Abbreviations: ABG, ß-Glucocerebrosidase; ASM, Acid sphingomyelinase; CDC, Centre for Disease Control and Prevention; CV, Coefficient of variation; DBS, Dried Blood Spot; DL, Desolvation Line; ESI, Electrospray ionization; GAA, Acid α-glucosidase; GALC, Galactocerebrosidase; GLA, α-galactosidase A; HPLC, High Performance Liquid Chromatography; IDUA, α-L-iduronidase; IS, Internal standard; LC–MS/MS, Tandem mass spectrometry coupled with liquid chromatography; LSDs, Lysosomal Storage Disorders; MPS-I, Mucopolysaccharidosis-I; MRMMultiple, reaction monitoring; P, Product; Q1, First Quadrupole; Q3, Third Quadrupole; QC, Quality Control; RF, Response Factor; S, Substrate.

* Corresponding author at: Department of Experimental and Clinical Biomedical Sciences, University of Florence, Newborn Screening, Clinical Chemistry and Pharmacology Lab, Meyer Children's Hospital, viale Pieraccini 24, Florence, Italy.
E-mail address: giancarlo.lamarca@meyer.it (G. la Marca).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jpba.2018.12.002
0731-7085/

making the hardware configuration easily affordable for any Newborn Screening Center.

Since this time, Mucopolysaccharidosis type I (MPS-I) [α-L-iduronidase (IDUA) deficiency] has also been included in screening protocols, alongside the five classical disorders, Pompe [acid α-glucosidase (GAA) deficiency], Fabry [acid α-galactosidase A (GLA) deficiency], Niemann-Pick [acid sphingomyelinase (ASM) deficiency], Krabbe [galactocerebrosidase (GALC) deficiency] and Gaucher [acid ß-glucocerebrosidase (ABG) deficiency] [11]. This was possible thanks to some modifications on the chromatographic run which made the related IDUA IS and IDUA enzymatic product measurable.

Many protocols for lysosomal enzyme activation and measurement have been developed to allow the simultaneous detection of several enzymes on one or two DBS punches [12–15].

To ensure complete substrate digestion all the previously described protocols require long incubation times (at least 16–22 hours). However, this study demonstrates that more accurate product quantification could be achieved when the enzymatic reaction is still in its linearity window where product formation yield is directly proportional to incubation time.

Shortening incubation times makes large scale newborn screening more affordable and considerably reduces times for recall, diagnosis and therapeutic intervention in the case of severe neonatal onset forms for which very early treatment contributes to better outcomes [16].

2. Material and methods

2.1. Chemicals and reagents

Substrate-Internal standard mixtures were obtained from the Centre for Disease Control and Prevention (CDC, Atlanta, Georgia).

Methanol, Isopropanol (LC–MS grade) and Formic acid (purity 98–100%) were purchased from Sigma-Aldrich (Riedel de Haën, Germany). Acetonitrile (LC–MS grade) and water (LC–MS grade) were procured from Biosolve Chemicals, (Valkensward, The Netherlands). In vitro diagnostic Whatman 903® grade blood spot cards were purchased from Whatman (Dassel, Germany). All the chemicals and reagents were used as commercially supplied without further purification.

2.2. Assay cocktail preparation

Each vial containing substrate-IS mixture was dissolved in methanol following the protocol developed by Spacil et al. [12]. A 1 mL aliquot from each vial was mixed together, dried under nitrogen flow and hydrated in 10 mL of 6-plex reaction buffer, prepared according to the procedure reported by Elliott et al. [17].

2.3. Sample preparation

A 3.2 mm diameter disc, equivalent to approximately 3.4 μL and 2.8 μL of whole blood for newborns and adults respectively, was punched out directly into a 96-well plate. Samples were extracted and incubated in 30 μL of cocktail buffer at 37 °C; different incubation times up to 12 h were tested. Four samples were prepared and analysed at each time point (2, 4, 6, 8, 10 and 12 h). After incubation, enzymatic reactions were quenched by 250 μL of methanol + 0.1% formic acid and the plate was centrifuged at 2500 *g* for 5 min. The supernatant was transferred to a new 96-well plate and a 2 μL sample was injected into the LC–MS/MS system through the autosampler kept at 8 °C.

2.4. Mass spectrometry and HPLC parameters

All measurements were performed using an LCMS-8050 Triple-Quad Mass Spectrometer (Shimadzu, Japan) equipped with an ESI source and working in MRM mode. System control and data analysis were performed by LabSolutions software, version 5.65. MRM transitions for each compound were optimized by flow injection and are listed in Supplementary Information (Table S1).

Other operating parameters were as follows: Nebulizing Gas Flow of 3.0 L/min; Heating Gas Flow of 5.0 L/min; Interface Temperature of 100 °C; DL Temperature of 100 °C; Heat Block Temperature of 100 °C; Drying Flow of 15 L/min.

The HPLC system (Shimadzu, Japan) consisted of a Degassing Unit DGU-20A5R, controller CBM-20 A, two pump modules Nexera X2 LC-30AD, autosampler Nexera X2 SIL-30AC and column oven CTO-20AC equipped with a six-port valve. The LC modules were plumbed as shown in Fig. 1.

Trapping and elution procedures were performed, as a modification of the described protocol [12] with a perfusion column POROS R1/20 2 × 30 mm (Applied Biosystems, FosterCity, CA, USA). For the chromatographic elution different columns were tested, including a Synergi Fusion RP-80 2 × 50 mm, 4 μm (Phenomenex, Torrance USA), a ShimPack XR-ODS column 2 × 50 mm, 2.2 μm (Shimadzu, Japan), and a Zorbax SB-CN 2,1 × 50 mm, 5 μm (Agilent, Italy). The Zorbax SB-CN was chosen for routine use. The temperature of the column oven was set at 45 °C.

Pump A module was fed by an aqueous solution of 0.05% formic acid containing 10 mmol/L of ammonium formate while pump B module was fed by a mixture of acetonitrile-methanol-isopropanol (50:25:25, v/v/v) containing formic acid at 0.1%.

The entire workflow was programmed in three steps managed by the switching valve: sample loading and clean-up (1 min); analyte elution (2.5 min); for the final columns reconditioning (1 min) (Table 1).

In the first step, the injected sample is moved to the trapping column with 1.6 mL/min of eluent A for the cleanup. After 1 min, the valve is switched, and both the eluents A and B flows are merged for providing, after a 0.5 min-delay at 0%, a gradient of 50% eluent B moving to 100% in 2.0 min at a total flow rate of 400 μL/min over the trapping and eluting columns now connected in series. With the analytes moved from the trapping column to the elution column, the valve is switched back to disconnect the two pumps and enable both the completion of chromatographic separation of the analytes with the full solvent B at 400 μL/min over the chromatographic column, and the simultaneous reconditioning of the trapping column for the following injection. This last step lasts 1 min for a total run of 4.5 min.

2.5. Calculation of enzyme activity

Enzyme activity was reported as μmol/L/h and calculated by using the following formula:

$$\text{Enzymeactivity} = RF \times (P_a/IS_a) \text{x} ([IS_c] \text{x} V_a/V_{dbs})/4\,h$$

V_a = Assay cocktail volume = 30 μL
P_a = Peak area Product
IS_a = Peak area Internal Standard
IS_c = Internal Standard concentration
RF = Response Factor: ratio between the Internal Standard response and the Product response.
V_{dbs} = 3.4 or 2.8 μL of whole blood contained into a 3.2 mm punch for newborn and adults, respectively
4h = incubation time

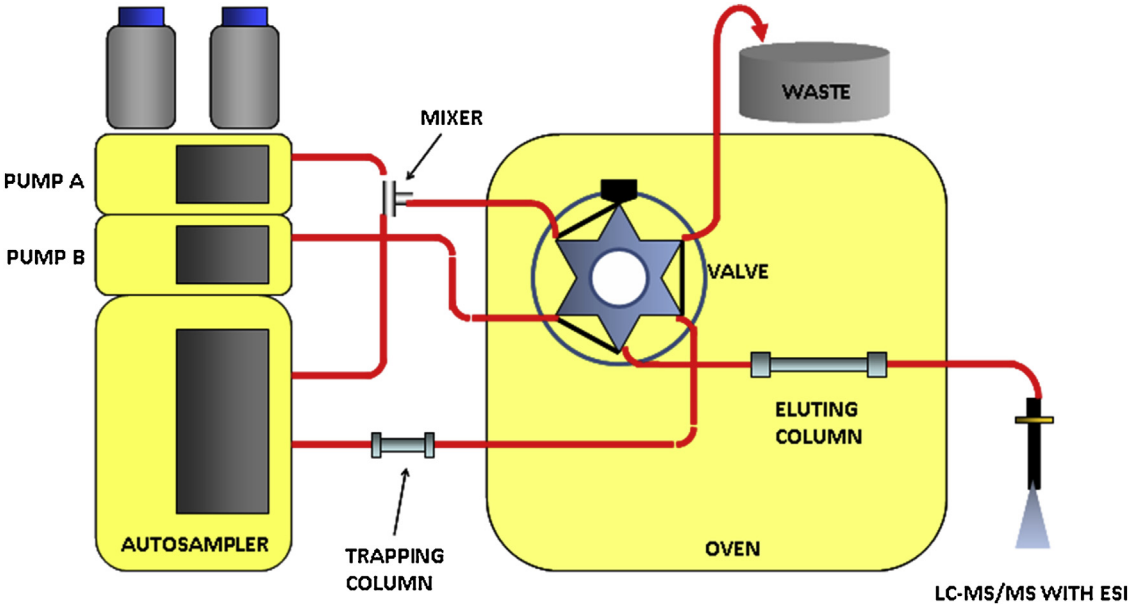

Fig. 1. Layout of the new plumbing. Position of depicted valve is A.

Table 1
LC parameters.

Step	Valve Position	Time	Pumps	Total Flow	% B	Pump A Flow	Pump B Flow
1	A	0.0 min	Disconnected			1.6 mL/min	0.4 mL/min
2	B	1.0 min	Connected	0.4 mL/min	0		
		1.5 min			50		
		3.5 min			100		
3	A	3.5 min	Disconnected			1.6 mL/min	0.4 mL/min
End		4.5 min					

Table 2
Reference value of LSD enzyme activity in newborn and adult population.

ENZYME	CUT OFF (μmol/L/h)	
	NEWBORN (n = 1000)	ADULTS (n = 200)
Galactocerebrosidase (GALC)	≥ 0.4	≥ 0.4
α-Glucosidase (GAA)	≥ 6.0	≥ 6.7
α -L-Iduronidase (IDUA)	≥ 3.4	≥ 6.6
α -Galactosidase (GLA)	≥ 1.9	≥ 2.2
ß-Glucocerebrosidase (ABG)	≥ 2.9	≥ 2.9
Sphingomyelinase (ASM)	≥ 2.6	≥ 2.7

2.6. Interday and intraday variability

Imprecision was determined in six replicates of four CDC control samples on the same day (intra-day imprecision) and duplicates for 5 times over a period of one week (inter-day imprecision). The imprecisions of the assay were expressed as a percentage coefficient of variation (CV%).

2.7. Cutoff determination

Reference values for the six lysosomal enzymes were determined both for newborn and adults by processing DBS of 1000 randomly chosen newborns and 200 adults. Quality controls provided by CDC and true positive samples were analysed to test the performance of the method and its ability to distinguish healthy individuals from affected individuals.

The lower limit of normal was set at the level of the 0.2nd percentile (Table 2)

3. Results and discussion

3.1. Enzyme reaction parameters

Compared to similar studies, we totally revised the sample preparation process including the incubation time which is pivotal for a timely diagnosis. Long incubation times are often used to allow complete substrate digestion. However, this means that the calculated time-based enzyme activity may be underestimated after the reaction plateau is reached. A short assay time should lead to a more accurate product quantification since the reaction is still in the linear range.

Several incubation times of up to 12 h were tested for the six enzymatic reactions. The relationship between product yield and time was different for the six enzymes but commonly linear up to 6 h (Fig. 2).

Within the time window of linear product accumulation, we chose an incubation time of 4 h because this proved long enough to produce a good signal of products and is short enough to be incorporated into a routine lab procedure. An incubation time of less than 4 h would risk compromising accuracy and an incubation time of more than 4 h would be incompatible with the time demands and routines of many laboratories."

This shortened sample preparation time satisfies the need for a more rapid analytical strategy which leads to faster diagnoses meaning that ERT can be introduced earlier and patient outcomes improved [16].

DBS from healthy newborns and adults were analysed for cut-off determination and the method was validated by means of both CDC controls and true positive samples. Although, it is often very difficult to find sufficient numbers of DBS from true newborn posi-

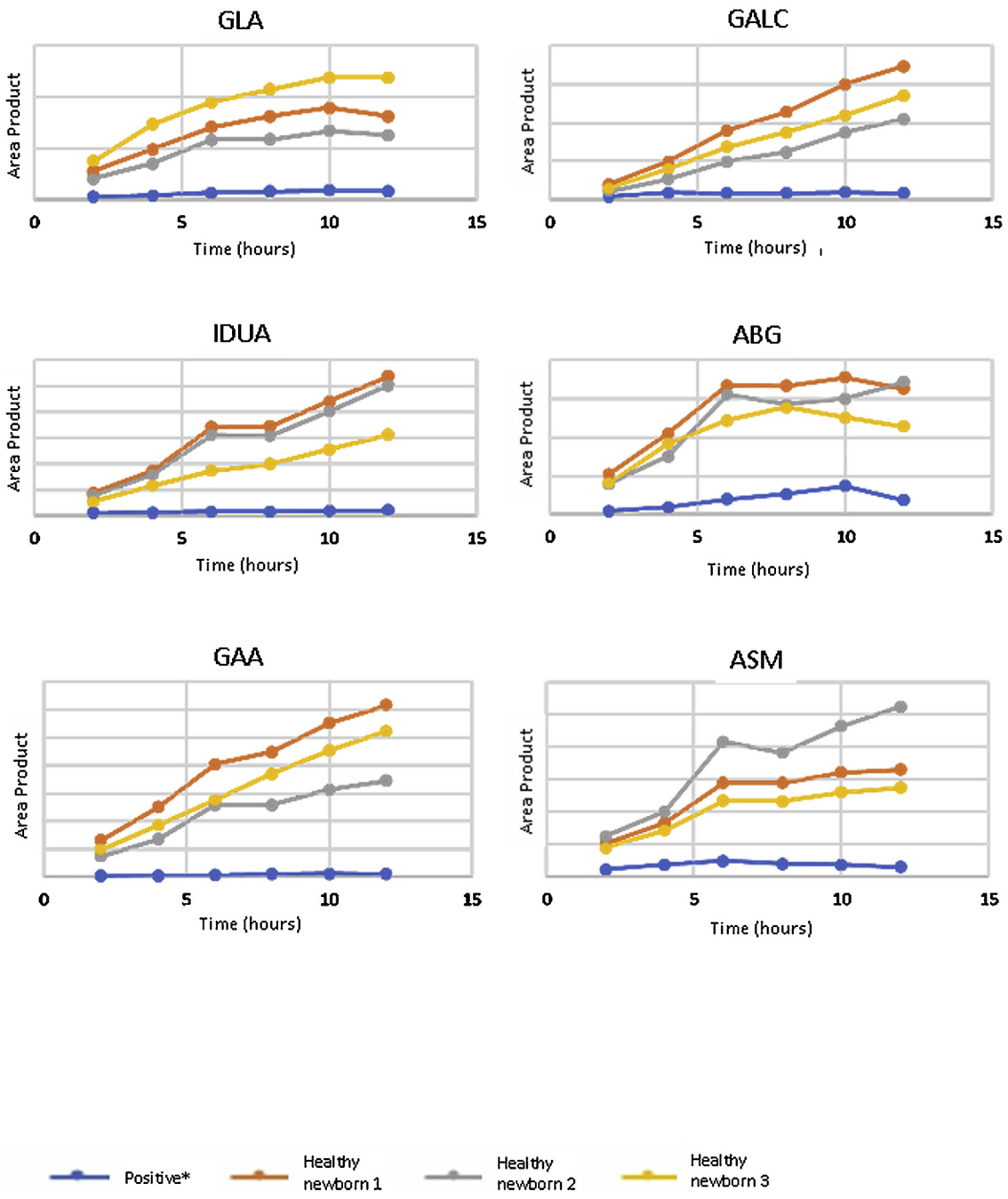

Fig. 2. The graph above shows the enzymatic reactions stopped at different times. The results are plotted as area product against incubation time in order to identify the window time of linearity. The curves appear to have a linear portion up to 6 h. The enzymatic activity can be accurately quantified if the reaction is stopped in the linear phase when the product formation still increases in a given unit of time.

tive patients, we were able to test the assay effectiveness on 8 truly affected patients of whom 4 were newborns (1 Pompe, 2 Fabry, 1 MPS I) and 4 adults (1 Pompe, 2 Gaucher and 1 Krabbe). Since no patient with Niemann-Pick A/B disease was available, the assay was tested on CDC samples. All positive samples were correctly identified.

3.2. MS/MS conditions and LC configuration

In 2009 a first approach for a five LSD enzyme assay based on an on-line cleanup followed by chromatographic separation was developed in order to simplify and speed up sample preparation [10]. Subsequently a multiplex assay was implemented by adding IDUA measurement to the other five enzymes [11].

In the new protocol described in this work, specific mass spectrometric parameters have been slightly modified, while LC configuration and optimization have been profoundly revised. This was necessary because of discrepancies concerning the accuracy of IDUA results after running several thousands of samples. After checking all the steps of the protocol, concerns arose about the peak shape of both the IDUA IS and IDUA product. Their peak shapes denoted as the signals were significantly distorted by residual matrix components supposedly eluting at the same retention time (RT). IDUA IS is a homologous compound of the investigated IDUA product and not an isotopologue. It is likely that some minor

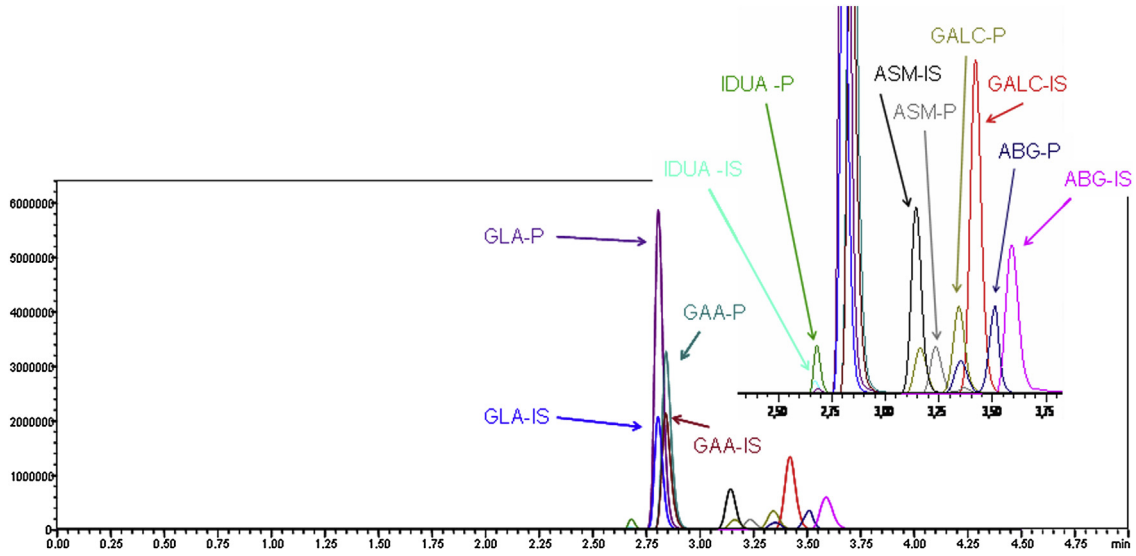

Fig. 3. Extracted ion chromatograms related to the six products and their relative internal standards by running a CDC control sample. Chromatograms of ABG and GALC product result in two peaks. The first peak (ghost) is due to the product generated by in-source breakdown of substrate. While the RT of the ghost peak is the same as the substrate, the RT of the second peak corresponds to the product of the enzymatic reaction.

matrix components released by the trapping column at the valve switching were making the ion-suppression impact slightly different on both analyte and internal standard.

To address this problem without elongating the running time, plumbing supporting the previous protocol was redesigned. The layout shown in Fig. 1 and the LC operating parameters (Table 1) provided a gradient elution at the time the valve was switched for a shifted release of the analytes from the trapping column. With this shift the ion-suppression impact mitigated and was similar for both the IDUA IS and the IDUA product. On the other side, eluting solvent B composition was reconsidered in order, to run the entire chromatographic process in less than 4.5 min, and to guarantee the cleanliness of both trapping and eluting columns, injection after injection. The eluting column was chosen to permit large-scale use.

For the chromatographic separation different columns were tested, including a Synergi Fusion RP-80 2 × 50 mm, 4 μm (Phenomenex, Torrance USA), a ShimPack XR-ODS column 2 × 50 mm, 2.2 μm (Shimadzu, Japan), and a Zorbax SB-CN 2,1 × 50 mm 5 μm (Agilent, Italy). Besides, different organic solvent mixtures of acetonitrile and methanol, with or without isopropanol, were tested in order to evaluate the most appropriate composition of mobile phase B. Experimental data showed that a concentration of acetonitrile higher than 50% was not suitable since it extended the chromatographic run up to six minutes. A mixture of acetonitrile/ methanol/ isopropanol in the following ratio 50:25:25 (v/v/v) provided the best results in terms of chromatographic separation, robustness and short run time.

In terms of efficiency and resolution associated with high long-term robustness the Zorbax SB-CN column performed better than the other tested columns. A representative chromatogram is shown in Fig. 3.

The final choice of the Zorbax SB-CN column instead of the previously used Synergi Fusion column, together with the aforementioned organic mixture for mobile phase B and the new plumbing led to an efficient and rapid chromatographic separation while maintaining a low pressure in the chromatographic plumbing.

This new configuration retained the advantages of a fast sample injection for a direct enzyme activity measurement without any other pretreatment.

Table 3
Intraday and interday imprecisions (expressed as CV%).

INTRADAY					INTERADAY				
QC CDC	0	Low	Med	High	QC CDC	0	Low	Med	High
IDUA	9.3	3.5	6.9	9.3	**IDUA**	16.6	13.9	16.9	15.8
GLA	4.2	5.8	5.3	5.3	**GLA**	5.8	11.2	8.2	7.6
ABG	8.7	9.6	9.2	6.4	**ABG**	28.8	13.8	13.0	7.9
GALC	7.0	9.0	7.4	8.5	**GALC**	15.4	17.2	10.7	11.3
ASM	4.6	1.7	5.9	9.9	**ASM**	9.4	10.1	12.5	10.4
GAA	6.9	9.5	6.1	8.2	**GAA**	7.6	18.8	11.5	10.8

3.3. Method validation

The results for intra-day and inter-day imprecision (Table 3) were acceptable considering many factors contributing to variability, included matrix inhomogeneity. The intra-day and inter-day imprecision studies were less than 20% CVs for all 6 enzyme activities.

4. Conclusions

At present enzymatic activity assays performed on DBS, for newborn screening purposes or for confirming a clinical diagnosis, have an incubation time of at least 16 h. This study demonstrates that this time can be safely reduced to permit greater workloads and faster response times. We developed a protocol with a 4 h-incubation time.

The major concern regarding an enzyme activity assay with a short time is that the product formation may be insufficient to allow a clear distinction between healthy and affected individuals. However, this protocol was extensively tested not only on CDC but also on true positive samples to confirm that the four hour time interval, though short, is sufficient to convert the substrate into a quantifiable product when supported by the described LC–MS/MS measurement setting.

A fast diagnosis of severe neonatal-onset forms of LSDs is essential for effective pre-symptomatic intervention. A rapid screening procedure can reduce recall times for newborns who stand to benefit from the prompt introduction of treatment. The shorter

incubation time adopted in our protocol reduces the time needed to make a diagnosis and introduce treatment by two days.

Any analytical method devised for clinical application must satisfy requirements of sensitivity, accuracy and precision as well as taking into consideration the reality of routine lab testing, which demands speed and robustness. The concept of robustness is often a little vague, but can be defined as the capacity of an analytical method to process a significant number of samples without compromising accuracy or sensitivity. In an LC–MS or LC–MS/MS system there are two potentially weak links; the ionization source and the chromatographic system. If chromatographic columns are not properly flushed, residues of the previous matrix may remain and contaminate the next sample injected. Residues that accumulate in an unexpected way generate unpredictable effects of ion suppression of the targeted components

An optimized method must be conducted in a time frame compatible with a large-scale routine.

As outlined above, testing in our laboratory proved that the method we describe in this paper fulfils requirements of accuracy, sensitivity, precision, speed and robustness over a very large scale for three different instrumental settings.

Declaration of interest

Tetsuo Tanigawa is an employer of Shimadzu corporation. The other authors declare no conflicts of interest.

Funding

This research did not receive any specific grant from funding agencies in the public or commercial sphere. The work was partially supported by AMMeC Onlus (Associazione Malattie Metaboliche Congenite ONLUS).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.12.002.

References

[1] P.C.J.I. Schielen, E.A. Kemper, M.H. Gelb, Newborn screening for lysosomal storage diseases: A concise review of the literature on screening methods, therapeutic possibilities and regional programs, Int. J. Neonatal Screen. 3 (2017) 1–15, http://dx.doi.org/10.3390/ijns3020006.

[2] D. Matern, D. Gavrilov, D. Oglesbee, K. Raymond, P. Rinaldo, S. Tortorelli, Newborn screening for lysosomal storage disorders, Semin. Perinatol. 39 (2015) 206–216, http://dx.doi.org/10.1053/j.semperi.2015.03.005.

[3] M.H. Gelb, C.R. Scott, F. Turecek, Newborn screening for lysosomal storage diseases, Clin. Chem. 61 (2015) 335–346, http://dx.doi.org/10.1373/clinchem.2014.225771.

[4] N.A. Chamoles, M. Blanco, D. Gaggioli, Fabry disease: enzymatic diagnosis in dried blood spots on filter paper, Clin. Chim. Acta. 38 (2001) 195–196, http://dx.doi.org/10.1016/S0009-8981(01)00478-8.

[5] N.A. Chamoles, M.B. Blanco, D. Gaggioli, C. Casentini, Hurler-like phenotype: enzymatic diagnosis in dried blood spots on filter paper, Clin. Chem. 47 (2001) 2098–2102.

[6] N.A. Chamoles, M. Blanco, D. Gaggioli, Diagnosis of alpha-L-iduronidase deficiency in dried blood spots on filter paper: the possibility of newborn diagnosis, Clin. Chem. 47 (2001) 780–781.

[7] N.A. Chamoles, M. Blanco, D. Gaggioli, C. Casentini, Gaucher and niemann-pick diseases–enzymatic diagnosis in dried blood spots on filter paper: retrospective diagnoses in newborn-screening cards, Clin. Chim. Acta 317 (2002) 191–197, http://dx.doi.org/10.1016/S0009-8981(01)00798-7.

[8] Y. Li, C.R. Scott, N.A. Chamoles, A. Ghavami, B.M. Pinto, F. Turecek, M.H. Gelb, Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening, Clin. Chem. 50 (2004) 1785–1796, http://dx.doi.org/10.1373/clinchem.2004.035907.

[9] D. Ombrone, E. Giocaliere, G. Forni, S. Malvagia, G. la Marca, Expanded newborn screening by mass spectrometry: New tests, future perspectives, Mass Spectrom. Rev. 35 (2016) 71–84, http://dx.doi.org/10.1002/mas.21463.

[10] G. la Marca, B. Casetta, S. Malvagia, R. Guerrini, E. Zammarchi, New strategy for the screening of lysosomal storage disorders: the use of the online trapping-and-cleanup liquid chromatography/mass spectrometry, Anal. Chem. 81 (2009) 6113–6121, http://dx.doi.org/10.1021/ac900504s.

[11] D. Ombrone, S. Malvagia, S. Funghini, E. Giocoliere, M.L. Della Bona, G. Forni, A. De Luca, F. Villanelli, B. Casetta, R. Guerrini, G. la Marca, Screening of lysosomal storage disorders: application of the online trapping-and-cleanup liquid chromatography/mass spectrometry method for mucopolysaccharidosis I, Eur. J. Mass. Spectrom. 19 (2013) 497–503, http://dx.doi.org/10.1255/ejms.1257.

[12] Z. Spacil, H. Tatipaka, M. Barcenas, C.R. Scott, F. Turecek, M.H. Gelb, High-throughput assay of 9 lysosomal enzymes for newborn screening, Clin. Chem. 59 (2013) 502–511, http://dx.doi.org/10.1373/clinchem.2012.189936.

[13] S. Tortorelli, C.T. Turgeon, D.K. Gavrilov, D. Oglesbee, K.M. Raymond, P. Rinaldo, D. Matern, Simultaneous testing for 6 lysosomal storage disorders and X-adrenoleukodystrophy in dried blood spots by tandem mass spectrometry, Clin. Chem. 62 (2016) 1248–1254, http://dx.doi.org/10.1373/clinchem.2016.256255.

[14] R. Mashima, E. Sakai, M. Kosuga, T. Okuyama, Levels of enzyme activities in six lysosomal storage diseases in Japanese neonates determined by liquid chromatography-tandem mass spectrometry, Mol. Genet. Metab. Rep. 9 (2016) 6–11, http://dx.doi.org/10.1016/j.ymgmr.2016.08.007.

[15] G.S. Ribas, J.F. De Mari, G. Civallero, H.M. de Souza, M.G. Burin, C.R. Vargas, R. Giugliani, Validation of a multiplex tandem mass spectrometry method for the detection of selected lysosomal storage diseases in dried blood spots, JIEMS 5 (2017) 1–7, http://dx.doi.org/10.1177/2326409817692360.

[16] C.F. Yang, C.C. Yang, H.C. Liao, L.Y. Huang, C.C. Chiang, H.C. Ho, C.J. Lai, T.H. Chu, T.F. Yang, T.R. Hsu, W.J. Soong, D.M. Niu, Very early treatment for infantile-onset pompe disease contributes to better outcomes, J. Pediatr. 169 (2016) 174–180, http://dx.doi.org/10.1016/j.jpeds.2015.10.078, e1.

[17] S. Elliott, N. Buroker, J.J. Cournoyer, A.M. Potier, J.D. Trometer, C. Elbin, M.J. Schermer, J. Kantola, A. Boyce, F. Turecek, M.H. Gelb, C.R. Scott, Dataset and standard operating procedure for newborn screening of six lysosomal storage diseases: By tandem mass spectrometry, Data Brief. 8 (2016) 915–924, http://dx.doi.org/10.1016/j.dib.2016.06.052.