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Easy method for newborn screening of six lysosomal storage disorders using online solid-phase extraction with mass spectrometry

Altı lizozomal depo hastağının yenidoğan taramasında, online katı faz eksraksiyon kullanılarak kütle spektrometresi ile ölçümünde kolay bir metod

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Abstract: Objective: A modified method for screening of six lysosomal storage disorders (LSDs) by tandem mass spectrometry was presented.

Methods: The enzyme activities for six LSDs (Gaucher, Pompe, Krabbe, Fabry, Niemann–Pick A/B and Mucopolysaccharidosis Type I) was measured by using ultra-HPLC and mass spectrometry. After overnight incubation of dried blood spots with three distinct reaction cocktails containing substrates and internal standards, reactions were stopped and online trapping was performed with ultra-HPLC preceding to mass spectrometry. Ultra-HPLC was equipped with online solid phase extraction and Hypersil Gold C8 analytical columns and coupled with TSQ Quantum Access Max mass spectrometry.

Results: Activities of acid-ß-glucocerebrosidase (ABG), acid glucosidase (GAA), galactocerebroside-ß-galactosidase (GALC), acid-galactosidase A (GLA), acid sphingomyelinase (ASM), and α-L-iduronidase (IDU) were obtained from DBSs of patients and healthy individuals. The intra- and inter-assay precisions were <20% (CV).

Conclusion: Our modified method, needing less DBS punches and only three reaction coctails, with the online trapping methodology, accurately differentiates newborns with LSDs from healthy newborns.

Keywords: Lysosomal storage disorders, Tandem mass spectrometry, Newborn screening

Özet: Amaç: Altı lizozomal depo hastalığının (LSD) taranmasında modifiye bir tandem kütle spektrometrisi metodu sunulmaktadır.

Metod: Altı LSD (Gaucher, Pompe, Krabbe, Fabry, Niemann-Pick A/B ve Mukopolisakkaridoz Tip I) için enzim aktiviteleri ultra-HPLC ve kütle spektrometresi kullanılarak ölçüldü. Kuru kan örneklerinin, substrat ve iç standartları içeren üç farklı reaksiyon kokteyl ile bir gece inkübe edilmesinden sonra, reaksiyonlar durduruldu. Kütle spektrometri analizi öncesi online ektraksiyon ultra-HPLC ile gerçekleştirildi. Ultra-HPLC analizinde katı faz ekstraksiyon ve

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Hypersil Gold C8 analitik kolonlar kullanıldı. Ultra-HPLC, TSQ Quantum Access Max kütle spektrometresi ile arayüz bağlantılı olarak kuruldu.

Bulgular: Hasta ve sağlıklı kuru kan örneklerinde asit-ß-glukoserebrozidaz (ABG), asit glukozidaz (GAA), galaktoserebrozit-ß-galaktozidaz (GALC), asit-galaktozidaz A (GLA), asit sfingomyelinaz (ASM) ve α-L-iduronidaz (IDU) aktiviteleri elde edildi. Çalışma içi ve çalışmalar arası tekrarlanabilirlik <%20 (CV) olarak tespit edildi.

Sonuç: Daha az kuru kan örneği, sadece 3 reaksiyon kokteyli gerektiren ve online ektraksiyon metodolojisi içeren modifiye metod, LSD'na sahip yenidoğanları sağlıklılardan başarılı şekilde ayırt edebilmektedir.

Anahtar Kelimeler: Lizozomal depo hastalığı, Tandem kütle spektrometresi, Yenidoğan taraması

1 Introduction

Tandem mass spectrometry screening of lysosomal storage disorders (LSDs) has been developed in the past 12 years. First, screening methods for Pompe, Gaucher, Niemann-Pick A/B, Fabry and Krabbe diseases were introduced and followed by optimized and high performance analytical methods through the subsequent years. [1–3]. Recently, analyses for Mucopolysaccharidosis (MPS) I, II, IVA, and VI were included, expanding the coverage for lysosomal diseases [4–7].

In the present study, we aimed to apply a simple and practical LSD screening method for Gaucher, Pompe, Krabbe, Fabry, Niemann–Pick A/B and Mucopolysaccharidosis I.

2 Materials and Methods

Reaction cocktails: Three distinct reactive mixes were prepared as shown in Table 1. ABG, GAA, GALC, and GLA were prepared in the same mix, where ASM and IDU were prepared separately.

Vials of pre-mixed substrates (S) and internal standards (IS) for each enzyme and quality control dried blood samples (DBS) were provided by Dr. Hui Zhou (Centers for Disease Control and Prevention, Atlanta, Georgia). DBS

Table 1: Preparation of reaction cocktails.

Quadruplex Solution (ABG, GAA, GALC, GLA)	Quadruplex Buffer (0.2 M sodium phosphate +0.10 M citrate, pH 4.4): 6.9 g sodium phosphate monobasic monohydrate and 7.35 g sodium citrate tribasic dehydrate are dissolve in~200 mL deionized water and adjusted to pH 4.40(±0.05) using 6N HCL and filled to 250 mL with deionized water. Store at 2–8°C for up to 6 months.
	GAA Inhibitor (0.8 mM acarbose in water): 12.9 mg acarbose is dissolve in~20 mL and filled to 25 ml with deionized water. Prepare on day of use, shelf life has not yet been determined.
	Detergent (96 g/L sodium taurocholate in water): 9.6 g sodium taurocholate is dissolve in 100 mL deionized water. Store at ≤-20°C for up to 1 year.
	Quadruplex Solution (ABG, GAA, GALC, GLA): Using 1 vial each of ABG, GAA, GALC and GLA S/IS are transferred contents of ABG and GAA vials into GALC vial using MeOH, removed MeOH by evaporation and transfered contents of GLA vial into GALC/ ABG/ GAA vial using 1.8 mL detergent. Vortex until completely dissolved. Heat in water bath if necessary. Add 0.3 mL GAA inhibitor. Add 15.9 mL Quadruplex Buffer. Vortex to homogenize. Store at ≤-20°C for up to 1 month.
ASM Solution	ASM Buffer (0.840 M sodium acetate +0.604 mM zinc chloride, pH 5.7): 17.14 g sodium acetate is dissolve in~ 200 mL deionized water, added 1.34 mL glacial acetic acid and 1.51 mL 0.1 mol/L zinc chloride and adjust pH to 5.7 using sodium hydroxide solution. This reactive is filled to 250 mL with deionized water. Store at 2–8°C for up to 6 months.
	ASM Solution: 0.15 mL detergent and 17.85 mL ASM Buffer were added to 1 vial of ASM S/IS. Store at ≤-20°C for up to 1 month.
IDUA Solution	IDUA Buffer (0.112 mol/L sodium formate + 0.158 mol/L formic acid, pH 3.6): 1.904 g sodium formate was dissolved in~200 mL deionized water, added 1.0 mL concentrated formic acid and adjusted to pH 3.6±0.05 with diluted formic acid or NaOH. Filled to 250 ml with deionized water. Store at 2–8°C for up to 12 months.
	IDUA Inhibitor (3.0 mmol/L D-saccharic acid 1, 4 lactone monohydrate): 0.0315 g D-saccharic acid 1,4 lactone monohydrate was dissolve in~40 mL deionized water and filled to 50 ml with deionized water. Store at -20°C for up to 6 months.
	IDUA Solution: 17.5 mL IDUA Buffer and 0.5 mL IDUA inhibitor were added to vial of IDUA S/IS. IDUA solution may be slightly hazy. Do not sonicate or warm the IDUA solution to clarify it. The solution should be clear after undergoing a freeze-thaw cycle. Store at -20°C for up to 6 months. We used Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Inc.).

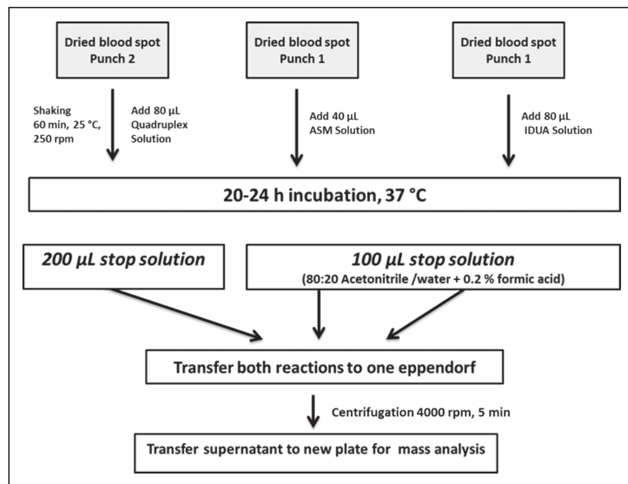

Figure 1: Optimized workflow for the simultaneous analyses of up to six lysosomal enzyme activities from dried blood spots.

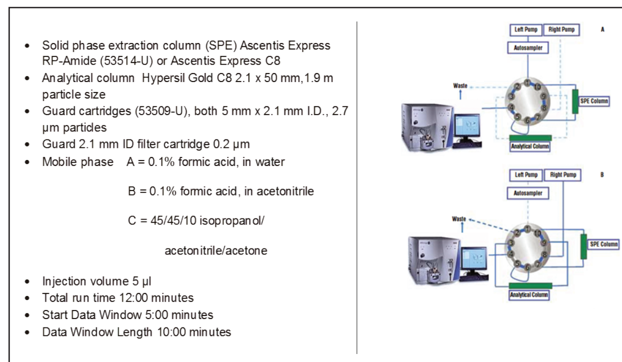

Figure 2: Ultra-HPLC conditions.

spots were punched into duplicate 96-well microtiter plates using a Wallac DBS. The workflow applied for the 6 lysosomal enzymes were provided in detail at Figure 1.

Ultra-HPLC - Tandem Mass Spectrometry (LC-MS/MS) conditions: Ultra-HPLC conditions were shown in Figure 2. Additionally, online solid phase extraction (SPE) and analytical column flow conditions were provided in Table 2. The chromatographic system was coupled to a triple quadrupole TSQ Quantum Access Max Mass Spectrometry (ThermoFisher Scientific, Inc. USA) equipped with an electrospray ionization (ESI) interface and operated with XCalibur software. Mass spectrometry conditions were shown Table 3.

The reference range values for ABG, GAA, GALC, GLA, ASM, and IDUA were measured in 122 newborn DBSs along with values for disease-positive DBSs. The enzyme activities for each specimen were calculated in units of µmol/L/h, [(P/ IS) blood µL)*[IS] *(volume of IS µL/ volume (1/incubation time h)] assuming as a 3.2-mm DBS contains 3.4 µL of blood [8,9].

Table 2: On-Line SPE and analytical column conditions.

PUMP 1	On-line SPE	Analytical column					
Step	**Start**	**Flow**	**Grade 1**	**A%**	**B%**	**C%**	**Valve 1**
1	0.000	1.500	Step	100	0	0	Load
2	0.000	1.500	Step	100	0	0	Load
3	0.200	1.500	Step	100	0	0	Load
4	0.205	0.100	Step	100	0	0	Inject
5	9.000	0.100	Step	100	0	0	Inject
6	9.000	1.500	Step	0	80	0	Inject
7	9.500	1.500	Step	0	80	0	Inject
8	9.500	0.100	Step	100	0	0	Load
9	13.00	0.100	Step	100	0	0	Load
PUMP 2	**Analytic column**	**Flow**	**Grade 1**	**A%**	**B%**	**C%**	**Valve 2**
1	0.000	0.700	Step	100	0	0	out
2	0.000	0.700	Step	100	0	0	out
3	1.000	0.700	Ramp	100	0	0	out
4	1.000	0.200	Ramp	100	0	0	in
5	6.500	0.800	Ramp	0	80	0	in
6	8.000	1.200	Step	0	80	0	in
7	8.000	1.200	Step	0	0	80	in
8	9.000	1.200	Step	0	0	80	in
9	9.000	0.700	Step	100	0	0	in
10	12.000	0.700	Step	100	0	0	out

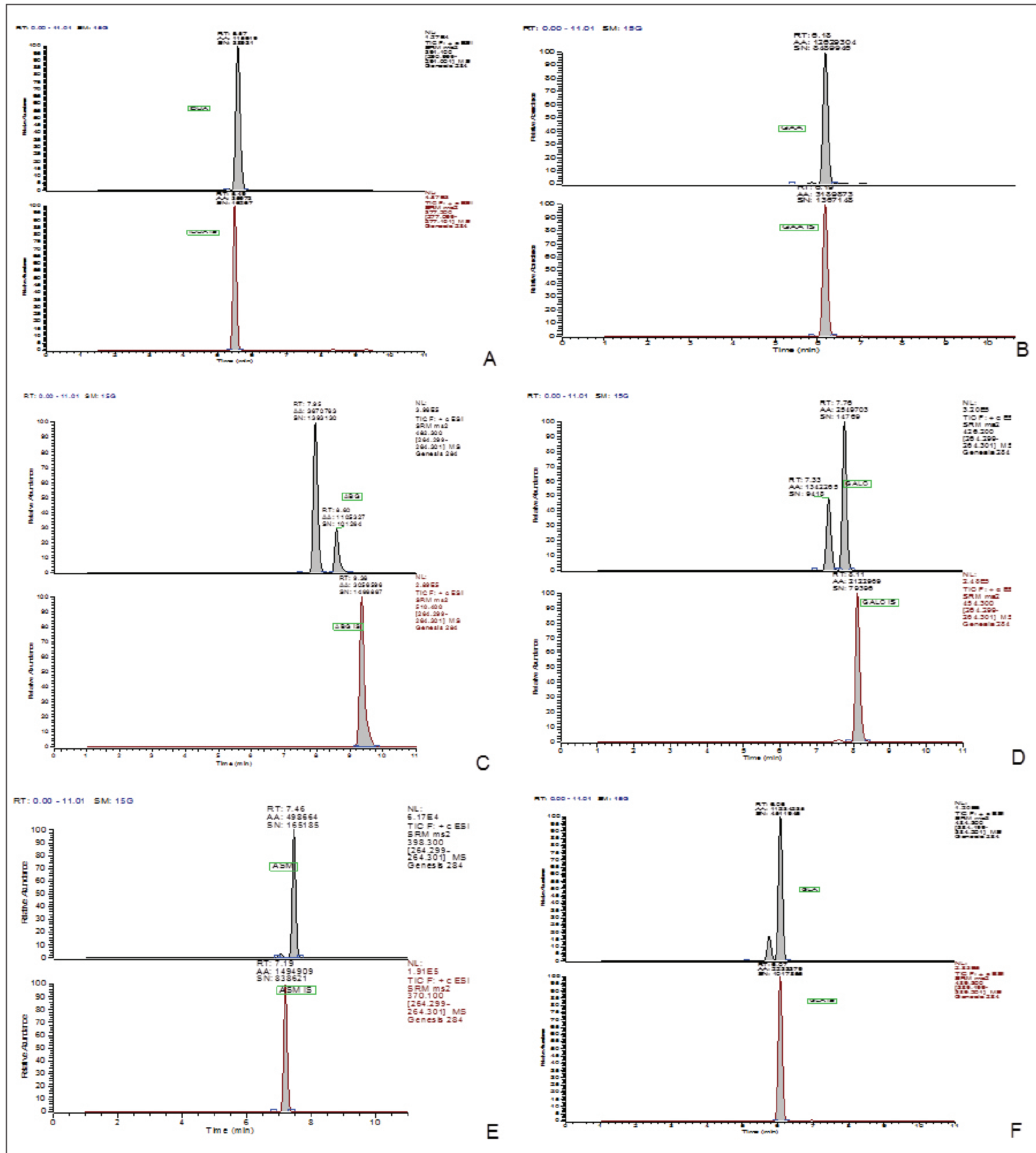

Figure 3: Ion chromatograms for all enzyme activities. (a) IDUA and IDUA internal standar, (b) GAA and GAA internal standard, (c) ABG and ABG internal standard, (d) GALC and GALC internal standar, (e) ASM and ASM internal standard, (f) GLA and GLA internal standard.

3 Results

Figure 3 suggested that the ion chromatograms for the internal standards associated with the ion chromatograms of the six enzymes. Ion chromatograms for Gaucher and Krabbe diseases were also provided in Figure 4. LoDs for each test ranged between 0.05 and 0.25 μmol/h/L in our study. The intra- and inter-assay precisions, measured in normal and abnormal samples, ranged between 5% and 20%. Results of CDC quality

Table 3: Mass spectrometry parameters and SRM values.

Enzyme	Parent ion	Product ion	Collision energy	Tube lens
GLA	484.3	384.2	17	103
GLA-IS	489.3	389.2	17	104
GAA	498.3	398.3	14	96
GAA-IS	503.3	403.3	15	97
ASM	398.3	264.3	18	103
ASM-IS	370.1	264.3	18	51
GALC	426.2	264.3	20	55
GALC-IS	454.3	264.3	21	100
ABG	482.3	264.3	25	96
ABG-IS	510.4	264.3	20	95
IDUA	377.2	277.1	12	80
IDUA-IS	391.1	291.0	12	80
Ion spray voltage			3500 V	
Sheath gas pressure			60 Arb	
Aux gas			35 Arb	
Skimmer offset			0 V	
Peak width (FWHM)			Q1 0.7	
Vaporizer temperature			350°C	
Ion sweep gas pressure			2.0 Arb	
Capillary temperature			250°C	
Collison gas pressure			0.4 m torr	
Scan width			0.08	

control materials and regression equations were provided in Table 4.

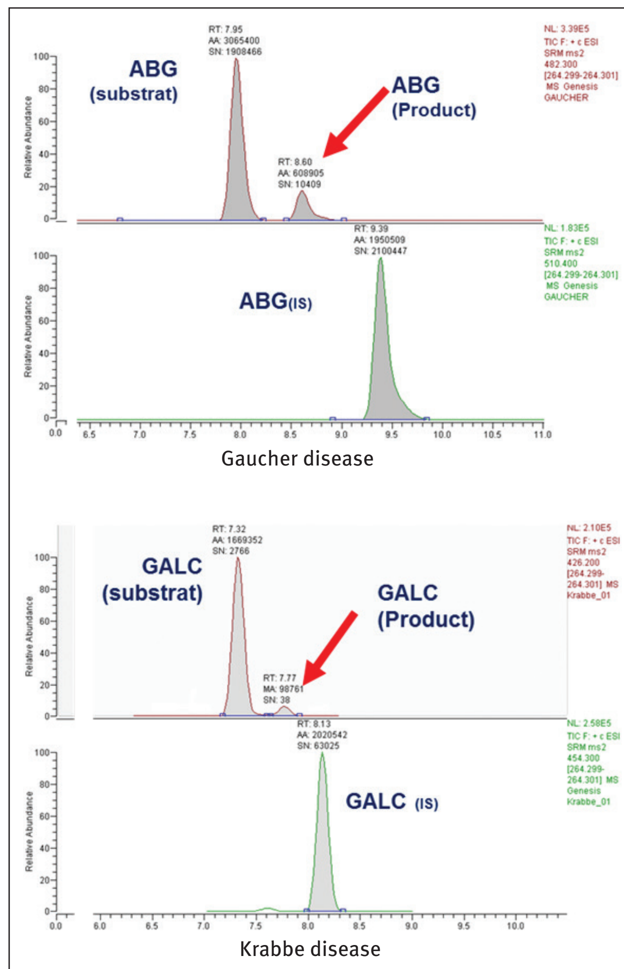

Figure 4: Ion chromatograms from Gaucher and Krabbe patients.

4 Discussion

Despite the fact that applicability of screening programs is expanding each day, LSD screening is currently limited throughout the world due to both the methodological difficulties, and the limited success in the treatments of LSDs. Nevertheless, it is foreseeable that, together withthe advances in high throughput technologies and the development of new therapeutic approaches in LSDs each year, screening of LSDs would come into prominence. A recent report optimized the sample preparation stagesand measured 9 lysosomal enzyme activities in a rather short period of time (approximately 1.8 min) with turbo flow methodology [4,10].

Our study did not involve turbo flow system, and thus the methodology took approximately 12 min to apply. This is the most practical method for screening laboratories without turbo systems, which perform the analyses on a rather smaller populations. Given that the duration of incubation stage was long, 20 to 24 hours, the analysis time of 12 min was considered to be acceptable. In addition, the 4+1 multiplex assay as introduced by Orsini *et al*. was modified as 4+1+1 multiplex assay to screen 6 LSDs [9]. It was acceptable with a <%20 (CV) repeatability. Liquid-liquid extraction (LLE) and online SPE are being used in certain centers for sample preparation procedure. We preferred online SPE since LLE is difficult to apply, required longer durations, and comprised more exposure to toxic materials.

In conclusion, screening of LSDs by mass spectrometry based on the appropriate SPE clean up procedures on line with the available facilities, is in progress and improving each day. We conclude that based on our modifications, LSD patients can be discriminated and screened simultaneously for Gaucher, Pompe, Krabbe, Fabry, Niemann–Pick A/B, and Mucopolysaccharidosis I by an easy and inexpensive screening method.

Conflict of interest: None declared.

Table 4: CDC quality control sample results and regression equations with R^2.

Analyte	CDC values (µmol/L/h)	Measured average (µmol/L/h)	Regression equations	R^2
ABG	0.28	0.18	y=0.9929x + 0.0815	0.9983
(Gaucher)	0.92	1.00		
	7.19	7.55		
	15.0	14.8		
ASM	0.00	0.00	y=0.9874x + 0.0277	0.9893
(Niemann–Pick A/B)	0.16	0.10		
	1.53	1.75		
	3.16	3.05		
GAA	0.04	0.02	y=0.9864x + 0.2177	0.9963
(Pompe)	0.97	1.07		
	9.92	10.7		
	19.9	19.6		
GALC	0.09	0.01	y=0.9989x + 0.0002	0.9979
(Krabbe)	0.39	0.38		
	3.14	3.32		
	6.04	5.94		
GLA	0.20	0.15	y=1.0002x - 0.0008	0.9995
(Fabry)	0.61	0.73		
	4.76	4.64		
	10.4	10.5		
IDUA	0.13	0.23	y=0.9999x - 0.0668	0.9849
(Mucopolisaccaridosis I)	0.87	0.77		
	8.31	8.00		
	18.6	18.6		

5 References

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