Original Article

Development of a novel approach for the second-tier estimation of homocysteine in dried blood spot using tandem mass spectrometry

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ABSTRACT

Background: Tandem mass spectrometry (TMS) -based screening for homocystinuria in the routine newborn screening (NBS) primarily relies on the elevated levels of methionine (Met) in the dried blood spot (DBS) of affected neonates. However, due to the lack of required specificity of Met as a marker for homocystinuria, an additional re-sampling and follow-up analytical testing for a definitive diagnosis, this emphasis us to develop a second-tier testing for a more specific marker, that is, homocysteine (HCY) in the same residual blood spot without any new sample. **Objective:** The objective of the study was to develop a novel approach for the second-tier estimation of total HCY in residual blood spot using TMS. **Materials and Methods:** Retrospective analysis of DBS from all neonates with deranged levels of Met and Met to phenylalanine in primary NBS was included in the study. The study duration was for a period of 24 months (November 2015-October 2017). Heel prick samples were collected within 24–72 h of birth and after first feed. Further, the samples with abnormal Met was analyzed for total HCY by isotopic dilution approach using multiple reaction monitoring mode for mass transition m/z 136→90 for HCY, m/z 140→94 for d4-homocystine, and m/z 269.2→134.4 for d8-HCY. **Results:** The newly developed methodology demonstrated a sensitive method for the quantification of total HCY over a linear range of 2 µmol/L–100 µmol/L. Assay validation parameters such as intraday and interday assay precision, accuracy, and recovery were also within acceptable range. **Conclusion:** In the present study, an in-house tandem mass spectrometric-based assay for the quantification of total HCY was developed which can be employed as an important strategy for the differential diagnosis of homocystinuria in neonates.

Key words: Inborn error of metabolism, Liquid-chromatography/Mass spectrometry, Methionine, Newborn screening

Newborn screening (NBS) is recognized as the largest and one of the most successful health promotion and disease prevention approach in the world. It primarily aims at the testing of all neonates for certain harmful or potentially fatal conditions that are not otherwise apparent at birth and whose prompt detection and timely medical intervention can prevent death or severe lifelong disabilities [1].

The introduction of tandem mass spectrometry (TMS) technology in the neonatal screening has greatly increased the coverage for many inborn metabolic disorders and emerged as a powerful platform for expanded NBS [2-4]. The TMS provided many advantages such as a simple sample preparation, rapid analysis time, and accurate quantitative analysis using small sample volume [5]. Furthermore, the incorporation of TMS in NBS has revolutionized many aspects of the process beyond the most obvious of allowing for testing of many analytes on the same sample at the same time [6,7].

Homocystinuria is an inherited metabolic disorder resulting from a defect in the methionine (Met) metabolism usually characterized by an aberrant buildup of total homocysteine (tHCY) and its cognates such as HCY, HCY-cysteine complex, and others in blood and urine of the affected neonate. Clinical presentation for homocystinuria is often accompanied by symptoms such as mental retardation, thromboembolism, psychiatric disorders, ectopia lentis, and skeletal abnormalities [8-10]. However, the re-methylation disorder is often characterized by diverse clinical presentations such as developmental delay, seizure, hypotonia, microcephaly, feeding difficulty, neurological deterioration,

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stomatitis, and megaloblastic anemia [11-13]. Early diagnosis is the key to effective management and favorable outcome.

NBS for homocystinuria in the routine NBS relies primarily on the detection of elevated levels of Met by TMS [14]. However, earlier studies provided a strong evidence for the poor sensitivity and specificity of this approach [15]. Furthermore, to resolve this issue, a few published studies showed that measured total HCY in dried blood spot (DBS) samples can be used as a second-tier screening for homocystinuria [16,17]. However, there is a research gap in the application of such approach in the Indian population and often requires additional purchase of commercially available kits and instrumentations, resulting in an added economic burden on the screening machinery. Hence, in this paper, we seek to remedy this paucity in literature by proposing a new in-house developed methodology for the measurement of tHCY in DBS using TMS.

MATERIALS AND METHODS

This retrospective analysis was conducted in a tertiary care center in north India for a period of 24 months (November 2015 to October 2017). The study group consisted of 150261 DBSs obtained as part of NBS initiative by Department of Science and Technology-Science and Engineering Research Board (DST-SERB). DBS from all neonates with deranged levels of Met and Met to phenylalanine in primary NBS was included in the study. The subjects were recruited among the newborns born in 23 hospitals that were part of the DST-SERB initiative.

Heel prick samples were collected from term babies, without any pre-confirmed metabolic disorders, within 24–72 h of birth and after first feed and who were not on blood transfusion or any parenteral nutrition. The subjects were enrolled in the study after obtaining a written consent from the participants, parents, or legal guardians and ethical approval from the Institutional Ethics Committee. For the determination of reference ranges, leftover DBSs from control samples (n=2000) were analyzed. These DBSs were negative for all screened conditions. The cutoff value for Met and Met to Phenylalanine (Met:Phe) were 45 μmol/L and 20 μmol/L, respectively.

Reagents

Stable isotope labeled HCY, that is, d_8-HCY and d_4-HCY were purchased from Cambridge Isotope Laboratories (USA) and used as Internal Standard (IS). DL-HCY and dithiothreitol were purchased from Sigma Aldrich (St. Louis, MO, USA). Mass spectrometric grade acetonitrile, methanol, and water were procured from JT Baker (Avantor Performance Materials, PA, USA), formic acid (analytical grade), and 96 well V- bottomed and truncated Nunc brand microplates were purchased from Thermo Fischer Scientific (PA, USA). All chemicals were of the highest purity commercially available.

Instrumentation

Mass spectrometric analysis was performed using AB Sciex 3200MD QTRAP-LC/MS/MS System (USA) with PerkinElmer Flexar Liquid Chromatography separation module. The system was controlled using Analyst MD software (1.6.1) that runs under windows 10 operating system. Analyte separation was based on flow injection analysis method. The mobile phase consisted of (Acetonitrile:Water:Formic acid; 30:70:0.1;v/v/ %).

Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode by isotopic dilution method. Before the analysis, all the compound dependent and source parameters were standardized using syringe-pump direct infusion method. The optimized parameter is as illustrated in Table 1.

Preparation of Control and Standards

The DBS controls and calibrators were prepared in-house by spiking HCY of known concentration to achieve the concentration covering the analytical range of tHCY, that is, (2 μmol/L–100 μmol/L). DBS control containing 5 μmol/L–20 μmol/L of HCY was used as low quality control (LQC) and high quality control (HQC), respectively.

Standard stock solution of HCY and HCY IS was prepared in diluents containing (acetonitrile:water:formic acid, 50:50:0.5,v/v/ %). DL-HCY was soluble only in 1% 0.1N sodium hydroxide solution. Stock concentration was 1 mg/mL. The working concentration of IS was 2 nmol. Multiple levels of quality control and calibrator sample were prepared by spotting 70 μL of the spiked blood onto filter paper and dried at room temperature (22°C–30°C) for 3 h and upon drying it was stored in a zip-lock bag with silica gel desiccators and humidity indicator and long time storage at −20°C until analysis.

Sample Extraction

During sample preparation a single blood spots of 3.2 mm diameter was punched out from the HCY spiked blood spot using a Panthera Puncher and was extracted with 150 μL of extraction solution containing a mixture of acetonitrile, water, and formic acid (70/30/0.5, v/v/%), 30 mmol/L dithiothreitol and IS. The samples were incubated for 30 min at 45°C followed by 10 min centrifugation at 2500 rpm. The supernatant (100 μL) was transferred into a fresh V- bottomed microtiter plate and submitted for injection and analysis. All the sample preparation procedure was performed in a bio-safety cabinet at ideal room temperature of 22°C–30°C. The representative MRM chromatogram for ion spectra after optimization of method is as displayed in Fig. 1.

Assay Validation

Various assay validation studies were performed in accordance with the Clinical and Laboratory Standards guidelines. The validation study included linearity, intra-, and inter-day assay precision, accuracy, stability analysis of the samples at different storage conditions, and its recovery. Linearity of the method was estimated in by analysis of six non-zero concentration ranging from (2 μmol/L to 100 μmol/L). The interday and intraday precision and accuracy were determined by replicated analysis

Table 1: Compound optimization

Compound dependent parameters							
Analyte	**Polarity**	**MRM**	**DP**	**EP**	**CE**	**CEP**	**CXP**
HCY	(+)	136-90	26	3	15	12	3
d_4-HCY	(+)	140-94	30	5	11	15	4
d_8-HCY	(+)	277-140	23	4	14	18	5
Source parameters							
Analyte	**CUR**	**CAD**	**IS**	**Temp**	**GS1**	**GS2**	
HCY	30	LOW	5500	500	30	30	
d_4-HCY	30	LOW	5500	500	30	30	
d_8-HCY	30	LOW	5500	500	30	30	

HCY: Homocysteine; MRM: Multiple reaction monitoring; DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CEP: Cell entrance potential; CXP: Cell exit potential; CUR: Curtain gas; GS-1: Gas 1; GS2: Gas 2; CAD: Collision associated dissociation

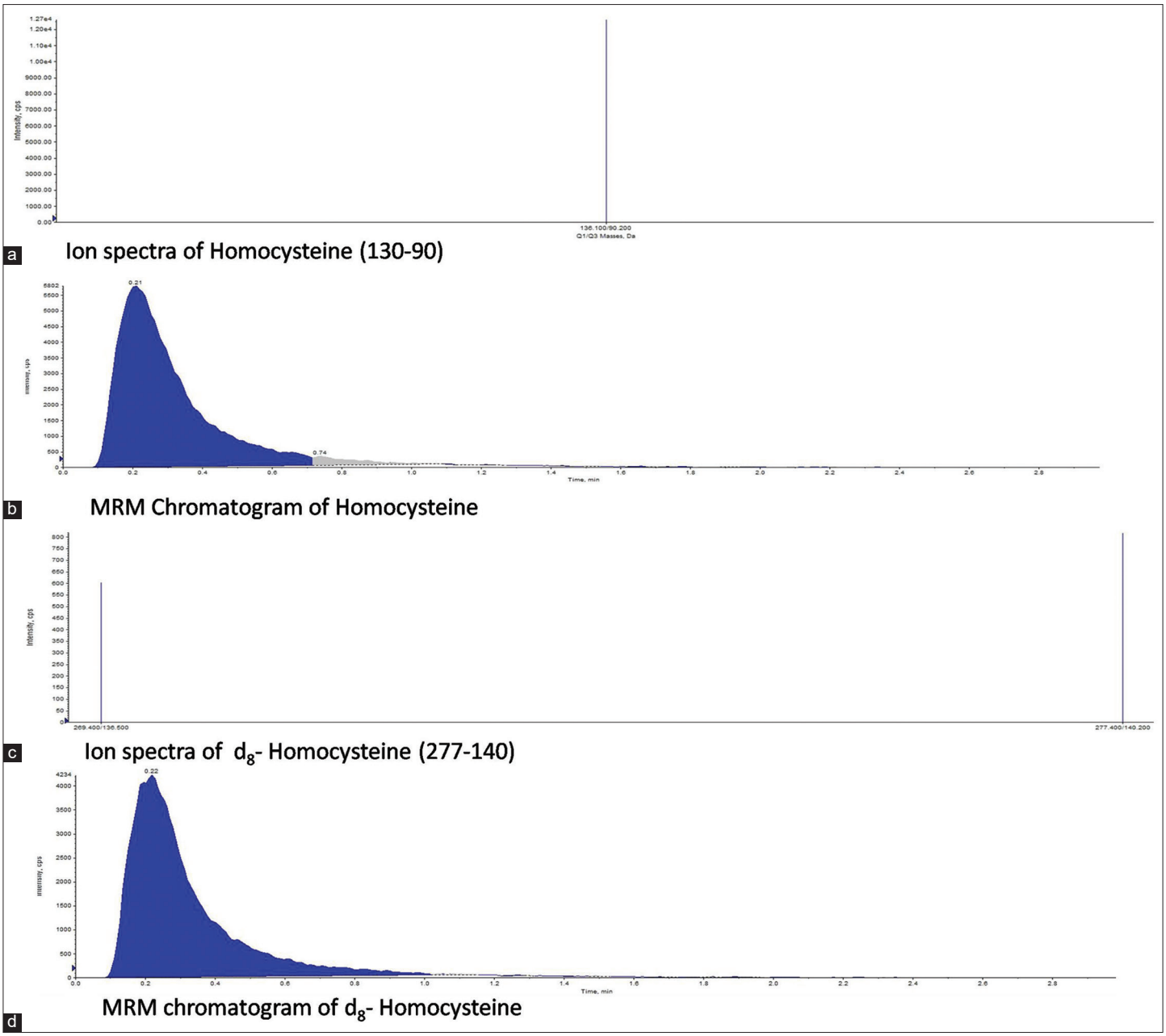

Figure 1: (a-d) Ion spectra MRM chromatogram using appropriate mass transitions

of two quality control levels (LQC and HQC) on three different batches, two on the same day, and one on the next day. For intraday (n=12) and for interday (n=18) replicates were studied over a period of 2 days and the standard deviation, accuracy, precision,

Table 2: Linearity study

Linearity level	1	2	3	4	5	6	Regression
Nominal Conc. (µmol/L)	2.015	5.004	10.009	20.067	50.044	100.089	-
Batch-1	1.898	5.124	9.998	20.224	52.256	103.562	0.9950
Batch-2	2.115	5.221	11.215	20.154	51.698	101.235	0.9985
Batch-3	2.114	5.201	10.114	20.223	50.236	100.254	0.9952
Average	2.042	5.182	10.442	20.200	51.397	101.684	
% Accuracy	101.4	103.6	104.3	100.7	102.7	101.6	

Table 3: Intraday and interday precision and accuracy study

Storage conditions	Fresh samples		2–8°C		-20°C	
Parameters	LQC	HQC	LQC	HQC	LQC	HQC
Mean (µmol/L)	5.09	19.94	4.70	18.80	4.80	19.05
Standard deviation (±)	0.12	0.32	0.28	0.58	0.29	0.48
CV (%)	2.38	1.65	6.13	3.13	6.23	2.57
Avg. Accuracy (%)	101.5	99.1	94.1	93.7	96.0	94.9

LQC: Low quality control; HQC: High quality control; CV: Coefficient of variation

Table 4: Stability at different temperature

(Quality control)	Intraday precision		Interday precision	
Parameters	Lower limit	Higher limit	Lower limit	Higher limit
Avg. Conc. (µmol/L)	5.06	20.23	4.90	19.66
Standard deviation (±)	0.24	1.36	0.30	1.43
CV (%)	4.91	6.74	6.29	7.29
Avg. Accuracy (%)	101.1	100.8	98.1	98.0

CV: Coefficient of variation

Table 5: Recovery studies

Quality Control	LQC response		HQC response	
Parameter	DBS	AQS	DBS	AQS
Mean.(cps)	82444.83	131141.67	350603.50	557980.83
Standard deviation (±)	3513.50	9196.008	13490.958	32642.822
CV (%)	4.26	7.01	3.85	5.85
Recovery (%)	62.87		62.83	

LQC: Low quality control; HQC: High quality control; DBS: Dried blood spot; CV: Coefficient of variation

and coefficient of variation (CV) was calculated. Stability of the prepared controls was determined at different temperature (freshly prepared, 2–8°C and −20°C) at an interval of 10 days for over 2 months. The extraction efficiency of analytical method was determined at two QC concentrations was also calculated by comparing the peak area of extracted samples with area of aqueous samples.

RESULTS

Linearity

The linearity was tested from 2 µmol/L to 100 µmol/L. The calibration plot showed that with increasing amount of standard spiked DBS displayed a linear relationship with the analyte response detected and with all slopes and linear regression coefficient values are very close to 1, that is, (>0.995). DBS calibrators of HCY at six different concentrations showed detectable and reproducible signals with a linear response and R^2 of 0.9985 (Table 2).

Precision and Accuracy

The intra-day and inter-day precision and accuracy are as tabulated in Table 3. The 12 replicates of intra-day and 18 replicates of the inter-day for two quality control levels of 5 µmol/L and 100 µmol/L showed a acceptable %CV below 20% with an average accuracy (%) within the acceptable limits for the analytical runs.

Stability

The result of the stability is illustrated in Table 4. The sample group stored at −20°C and freshly prepared samples reported significantly more stability as compared with the sample group stored at 2–8°C making it most preferred option for long time sample storage.

Recovery

Recovery analysis of the sample is done by comparing the intensity response of the six extracted LQC and six HQC samples with the area response of aqueous sample as per the procedure mentioned in the section above. The average recovery of HCY at LQC level was 62.87 % and at HQC level it was 62.83% (Table 5).

DISCUSSION

Considering the increasing applicability of TMS technology, previous studies have noted the emergence of TMS as a powerful analytical tool in the clinical biochemical genetics [18]. TMS has gradually replaced the earlier methods used in the screening of inborn errors of metabolisms (IEMs) in neonates. Hence, we emphasized to find a new method that enables quantitative determination of tHCY, thereby facilitates in the diagnosis of homocystinuria. Conventionally in clinical practice high-performance liquid chromatography-based procedure was the most widely applied [19,20]. However, this approach often resulted inconclusive results because of the interfering peaks or incomplete separation of the samples. Non-availability of specific IS and the long analytical time often resulted in a limited utility.

Our results indicate that TMS-based quantification of tHCY in DBS provided an easy to adopt, an high throughput method of choice not only in the diagnosis but also in the monitoring of patients with homocystinuria. Furthermore, our study incorporated a stable isotope-labeled IS with similar chromatographic behavior, served as internal control which boost the analytical reliability in corroboration with earlier study by Casetta *et al*., Yuan *et al*., and Chace *et al*. [21-23].

Our study results are in concurrence with the previous studies by McCann *et al*. [24], who also developed and validated method for the quantification of tHCY in DBS samples, which was linear over the range of 10–100mmol/L of tHCY and provided an excellent precision with intra-batch CV of 4% and inter-batch precision of 6.5%. Our results also demonstrated a comparable value of intra-batch and inter-batch precisions CV ranging between (2.4% and 5.3%). However, since the assay was aimed purely for second-tier NBS testing, no endeavor was made to DBS comparable results with plasma. The range for calibration determined in the present NBS sample was 5 µmol/L–20 µmol/L which were similar with the findings of Josef *et al*. [25].

Although, our method demonstrated a sturdy and easy to adopt technology in a resource constrained setup, there are few limitations, which is worth discussing. Our observation was supported by the usage of the elevation in Met as primary marker in routine NBS for classical homocystinuria. However, this could lead to a miss in patients with other causes of homocystinuria and has normal or low levels of Met.

In accordance of study published in 2005 by Bowrona *et al*. [26], we also observed a small but consistent reduction in HCY in DBS stored at room temperature and a decrease in HCY concentration between day 1 and day 30 could be attributed to bacterial breakdown of amino acid in the sample as demonstrated. However, more research on this topic need to be undertaken before the association between tHCY and biochemical changes during storage is more clearly understood.

CONCLUSION

A new TMS-based method for the quantitative determination of total HCY has been developed which can be employed as an important tool to detect IEM. The developed method has been thoroughly validated and showed a good correlation with earlier published methodology. All the results of the validation procedure showed a new reliable, sensitive and selective method with the potential to detect and quantify total HCY and could be used as a second-tier testing for the all the neonates who showed deranged Met in the routine primary NBS.

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