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Newborn Screening for Inborn Metabolic Disorders in Iranian Infants: Comprehensive Evaluation of the Paya Hamsan Derivatized Technique for Tandem Mass Spectrometry

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Abstract

Background: Screening newborns for inborn metabolic disorders (IMDs) is important for early diagnosis and prevention of serious health complications. Tandem mass spectrometry (MS/MS) analysis of metabolites in dried blood spots (DBSs) has improved the accuracy and scope of neonatal screening. This study evaluated the performance of the Paya Hamsan Newborn Screening Derivatized (PH-NBS-D) MS/MS method for detecting and quantifying amino acids and acylcarnitines in newborns.

Methods: The PH-NBS-D MS/MS assay was validated using DBS samples analyzed on an AB SCIEX 3200 MS/MS system. Its performance was compared with two commercially available newborn screening kits, MassChrom® and ClinSpot®, using clinical DBS specimens. Analytical performance was assessed according to relevant Clinical and Laboratory Standards Institute (CLSI) guidelines, including accuracy, precision, and linearity.

Results: The PH-NBS-D assay showed good analytical performance, with recovery rates ranging from 91% to 115%. Repeatability CVs ranged from 4.1% to 12.9%, between-day variation from 0.0% to 8.6%, and total precision CVs from 7.1% to 25.7%. Linearity was excellent across the evaluated concentration ranges, with R^2 values above 0.99. The PHNBS-D assay showed comparable performance to the two commercial kits. In clinical samples, percentage deviations remained within the predefined acceptance limits (±30%), and Bland–Altman analysis showed no significant systematic bias, indicating good agreement between the methods.

Conclusions: The PH-NBS-D MS/MS assay showed satisfactory analytical performance and good agreement with the evaluated commercial newborn screening assays. These findings support its potential use for measuring amino acids and acylcarnitines in DBS specimens and for further evaluation in routine newborn screening.

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Introduction

Inborn Metabolic Disorders (IMDs) can be detected early through the Newborn Bloodspot Screening (NBS) program, a crucial part of the public health prevention system. These diseases occur due to genetic defects that disrupt metabolic processes. If they are not promptly identified and addressed, they can lead to signif-

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icant health issues such as cerebral damage, developmental impairments, and perhaps fatal outcomes. Early diagnosis with NBS enables immediate therapies, which can dramatically improve the health outcomes and quality of life of afflicted newborns. Tandem mass spectrometry (MS/MS) can quantitatively evaluate several metabolites from a single Dried Blood Spot (DBS), establishing it as a cornerstone of NBS programs globally [1,2]. Modern technology provides comprehensive and accurate testing, leading to improved rates of early diagnosis. While metabolites can sometimes be easily detected and quantified in their natural state in the newborn, chemical derivatives often provide an efficacious alternative that can significantly enhance detection accuracy in NBS. Analysis of derivatives can result in better reproducibility and reliability through increased ionization efficiency, higher signal-to-noise ratios, and superior chromatographic separation. By matrix effects that can influence accuracy and ionization efficiency, particularly in Electrospray Ionization (ESI)-MS, derivatized methods offer a more sensitive and specific analytical approach in NBS for detecting Inborn Errors of Metabolism (IEM) [3-5].

The Paya Hamsan (PH) NBS derivatized (PH-NBS-D) kit (Paya Hamsan Technologies Co., Arak, Iran) is a newly developed, locally produced derivatized MS/MS assay designed for the semi-quantitative assessment of amino acids and acylcarnitines in DBS specimens for NBS. The use of a derivatization-based approach is intended to improve ionization efficiency and analytical sensitivity while providing reliable measurement of a broad range of metabolites relevant to IEM screening. Although derivatized MS/MS methods are widely established in NBS, comprehensive analytical validation of newly developed, locally produced assays, together with their direct comparison with established commercial screening methods, remains limited. Therefore, analytical evidence is required to establish whether such assays can provide reliable and comparable results under routine NBS conditions. This kit was engineered to meet the accuracy, precision, and dependability demands of NBS programs and to comply with Clinical and Laboratory Standards Institute (CLSI) international MS/MS procedural guidelines [CLSI NBS04] [6] and patient sample measurement comparisons and bias estimations published by the [CLSI EP09] [7].

The authors assessed the usability and preciseness of the PH-NBS-D kit for detecting IMDs as a part of routine NBS in Iranian newborns. Here the authors reported results of the assay's performance in terms of linearity, stability, accuracy, and precision using CLSI methods for evaluating the precision of quantitative measurement procedures [CLSI EP05] [8] and the detection capability of clinical laboratory measurement procedures [CLSI EP17] [9]. In addition, the assay was compared with two commercially available newborn screening kits (MassChrom® and ClinSpot®) using clinical samples to assess method agreement and demonstrate its suitability for routine newborn screening in Iran. To the best of our knowledge, this study provides the first comprehensive analytical validation of the PH-NBS-D MS/MS assay combined with direct comparison with established commercial newborn screening assays. This approach addresses the current need for analytical and comparative evidence supporting the potential use of a locally developed derivatized MS/MS assay in routine newborn screening in Iran [10].

Materials and Methods

Study design

The study was designed to thoroughly assess the efficacy of the PH NBS-D assay in NBS using DBS specimens. The kit-recommended sample preparation techniques for derivatization were evaluated to determine their appropriateness for semi-quantitative analysis of acylcarnitines and amino acids using DBSs. Methodological rigor was ensured by adherence to CLSI NBS04 [6], an accepted international guideline for the use of MS/MS in NBS. Assay performance was assessed by repeat analyses of known concentration standards and control samples at two concentrations, Levels I and II (see 2.9 below). To validate its capabilities versus current technologies, comparative analyses were conducted with two commercial kits already in use for IMD screening of newborns in Iran (see 2.10 below).

Ethical considerations

Research methodology complied with ethical norms consistent with ISO 15189 [10], which highlights laboratory quality, safety, and data protection. Clearance was obtained from the local Ethics Committee of the National Health Institute (approval number: IR. ACECR. AVICENNA.REC.1403.006). Blood samples from neonates were obtained in accordance with rigorous informed consent protocols, which guaranteed that parents or legal guardians were thoroughly informed and consented to participation. The research upheld maximum secrecy by anonymizing all samples to safeguard the identity and privacy of the participants.

Chemicals and reagents

Complete PH-NBS-D kits contained all necessary chemicals and reagents for the derivatized analyses. Each kit contained derivatization reagent for butyric esterification of the amino acids and acylcarnitines, lyophilized internal standards, an extraction buffer, a mobile phase, and a washing solution for cleaning the injector needle. To facilitate rapid sample processing and assay integrity, reconstitution buffer, 96-well plates, and protective sheets were also included.

Analytical equipment details

A SCIEX Triple Quad 3200 MS/MS (SCIEX, Concord, Ontario Canada) in combination with an Agilent 1200 Liquid Chromatography (LC) system (Agilent, Tokyo, Japan) served as the analytical platform. LC-

MS/MS methodology was chosen for its sensitivity and selectivity. Various derivatization strategies were evaluated for their effectiveness in detecting the target analytes using in-house prepared control samples. All analytical processes followed stringent protocols to ensure accuracy and comparative data reliability.

Kit quality assurance and performance evaluation

Assay quality assurance was consistent with CLSI NBS04 [6] and ISO 15189 [11] recommendations and requirements. This included the routine use of validated internal controls and assessment of intra- and inter-day reproducibility.

Clinical specimens

DBS specimens used for method comparison were obtained from the routine Iranian NBS program. Blood samples were collected between 24 and 72 *hr* after birth according to the national newborn screening protocol. A total of 40 actual clinical specimens were included in this study, comprising 40 specimens analyzed using the MassChrom® kit and 9 clinical specimens exhibiting metabolic disorders specimens analyzed using the ClinSpot® kit for method comparison.

Specimen collection and preparation

DBS specimens consisted of newborn heel-prick blood collected onto pre-labeled Whatman 903 filter paper cards. Blood samples were collected between 24 and 72 *hr* after birth according to the national newborn screening protocol. DBS specimens were stored under controlled conditions, with short-term storage at ambient temperature and long-term storage below -18°*C* until analysis.

For analysis, DBS specimens were processed according to the sample preparation procedure specified for the PH-NBS-D kit. The required DBS punch was obtained from each specimen and extracted using the extraction buffer supplied with the kit. Following extraction, the specified volume of the derivatization reagent was pipetted directly onto the prepared sample. The 96-well plate was covered with a protective sealing film and incubated at 60°*C* for 20 *min*. This step enabled the derivatization of amino acids and acylcarnitines to their corresponding butyl ester derivatives. After incubation, the protective film was removed and the derivatized samples were evaporated to complete dryness at 40°*C* for 30 *min*. The dried samples were subsequently reconstituted using the reconstitution solution supplied with the kit and prepared for LC-MS/MS analysis.

Control samples at two concentration levels (Levels I and II) were processed using the same extraction, derivatization, evaporation, and reconstitution procedures as the DBS specimens. These control materials were used throughout the analytical performance evaluation, including accuracy, precision, and other assay performance assessments. Clinical specimens used for method comparison were processed using the same standardized procedure to ensure consistency between analytical methods.

LC-MS/MS parameters

The LC-MS/MS analysis was performed using an Agilent 1200 LC system coupled to a SCIEX Triple Quad 3200 mass spectrometer. The LC was operated under isocratic conditions using injection quantities of 10 to 20 μl and a two-minute run time for high throughput. In addition to isotope-labeled internal standards, individual analytes were identified by Multiple Reaction Monitoring (MRM) and Electrospray Ionization (ESI) in positive mode. Best chromatographic performance necessitated precise control over the mobile phase and flow rate. In order to reduce sample carryover and maintain assay integrity, injection needles were cleaned with a rinse solution between injections. Precise conditions were required for repeated and accurate analyte detection. Table 1 lists the parameters evaluated, including both precursor and product ion masses, Collision Energy (CE), and Declustering Potential (DP) for each analyte.

Accuracy and precision measurements

Assay recovery and accuracy were tested using control samples at two concentrations (Levels I and II). Data were obtained over multiple days and with different instrument operators. Intra-assay precision was assessed using ten clean-ups of the same sample at different concentrations while inter-assay precision was evaluated over 20 days with two series per day and two repetitions per series following CLSI guidelines [CLSI EP05] [8]. Precision was measured by Coefficient of Variation (CV) comparisons and two-way nested ANOVA using Microsoft Excel 2019 Analysis-it.

Intra-laboratory precision and accuracy were determined using control sample preparations at Levels I and II, with measurements taken on different days by different operators. Following CLSI criteria [CLSI EP05 8], precision was assessed by CV comparison and accuracy was confirmed by comparing measured values to target concentrations. A low CV and accurate results demonstrated intra-laboratory reliability.

Precision and accuracy were assessed in different labs to validate assay uniformity and resilience under varying testing conditions. Accredited screening facilities, using the same sample preparation and analysis techniques, examined standardized control samples. Accuracy was evaluated by comparing the measured values to the target concentrations, while inter-laboratory precision was assessed using CVs from aggregated findings across laboratories. To examine site variations, the assessors applied statistical methods, such as two-way Nested ANOVA and CLSI guidance criteria. High precision and accuracy across laboratories were considered indicative of clinical and diagnostic utility was validated by its high precision and accuracy across laboratories.

Table 1. LC-MS/MS parameters for derivatized amino acids and acylcarnitines analysis [Time (*msec*):50]

Amino Acids-Derivatization				Acylcarnitines and Free Carnitine-Derivatization			
Analyte	**MRM (*m/z*)**	**DP (Volts)**	**CE (Volts)**	**Analyte**	**MRM (*m/z*)**	**DP (Volts)**	**CE (Volts)**
Alanine	146→44	27	23	Carnitine	218→85	27	36
Alanine-d4	150→48	27	23	Carnitine-D9	227→85	27	36
Arginine	231→70	40	42	Acetylcarnitine	260→85	27	33
Arginine-d7	238→77	40	42	Acetylcarnitine-D3	263→85	27	33
Aspartic acid	246→144	25	20	Propionylcarnitine	274→85	43	35
Aspartic acid-d3	249→147	25	20	Propionylcarnitine-D3	277→85	43	35
Citrulline	232→113	23	27	Butyrylcarnitine	288→85	33	30
Citrulline-d2	234→115	23	27	Butyrylcarnitine-D3	291→85	33	30
Glutamic acid	260→158	40	22	Isovalerylcarnitine	302→85	50	34
Glutamic acid-d5	265→163	26	22	Isovalerylcarnitine-D9	311→85	44	34
Glycine	132→76	28	15	Glutarylcarnitine	388→85	44	34
Glycine-13C2,15N	135→79	25	15	Glutarylcarnitine-D9	397→85	44	34
Leucine	188→86	35	16	Hexanoylcarnitine	316→85	55	46
Leucine-d3	191→89	20	16	Hexanoylcarnitine-D3	319→85	50	46
Methionine	206→104	35	23	Octanoylcarnitine	344→85	48	40
Methionine-d3	209→107	40	23	Octanoylcarnitine-D3	347→85	48	40
Ornithine	189→70	25	40	Decanoylcarnitine	372→85	48	40
Ornithine-d6	195→76	25	40	Decanoylcarnitine-D3	375→85	48	40
Phenylalanine	222→120	35	23	Dodecanoylcarnitine	400→85	56	47
Phenylalanine-d5	227→125	25	23	Dodecanoylcarnitine-D3	403→85	56	47
Proline	172→116	21	25	Tetradecanoylcarnitine	428→85	49	58
Proline-d7	179→123	21	25	Tetradecanoylcarnitine-D3	431→85	49	58
Tyrosine	238→136	31	22	Hexadecanoylcarnitine	456→85	58	57
Tyrosine-d4	242→140	31	22	Hexadecanoylcarnitine-D3	459→85	58	57
Valine	174→72	32	21	Octadecanoylcarnitine	484→85	80	50
Valine-d8	182→80	21	21	Octadecanoylcarnitine-D3	487→85	80	50

The Limit of Detection (LOD) and Lower Limit of Detection (LLOQ) were used as indicators of the kit's ability to accurately quantify analytes at low concentrations. The LOD represents the lowest concentration at which an analyte can be detected and the LLOQ is the lowest concentration at which accurate quantification can be achieved. Calibration curves and blank sample analysis were used to evaluate the signal-to-noise ratio.

To evaluate the kit's analytical sensitivity, blank filter papers were prepared using the same process as patient samples. These blanks were analyzed twelve times by LC-MS/MS. The measured concentrations were compared to the LLOQ for each analyte to verify that the procedure met the required sensitivity threshold. Blank filter paper samples were also analyzed immediately after injecting a high-concentration sample to evaluate potential carryover effects between analyses. Blank samples were injected five times following two high-concentration control samples (Level II) to validate whether detected quantities remained below the LLOQ.

To evaluate linearity, calibrators were tested at increasing concentrations of the target analyte. The method's linearity was assessed by MS/MS over five different days, with two sample preparations at nine concentration levels. By visualizing the results and calculating the correlation coefficient (R^2), the linear range was determined.

Method comparison

A comparison study of the PH-NBS-D kit with the MassChrom® kit (ChromSystems, Munich, Germany), widely used for screening newborns in Iran, used DBS control samples at two concentration levels and an additional 40 actual specimens. The same LC-MS/MS equipment was used to quantify all amino acids and acylcarnitines for both kits.

The PH-NBS-D kit and the ClinSpot® kit (RECIPE Chemicals and Instruments, München, Germany), also used in Iran, were evaluated for their ability to identify problematic and borderline analytes utilizing clinical specimens. This investigation encompassed nine clinical specimens exhibiting metabolic disorders such as Tyrosinemia (TYR), methionine metabolism disorder (MET), Phenylketonuria (PKU), Maple Syrup Urine Disease (MSUD), Nonketotic Hyperglycinemia (NKH), Carnitine Palmitoyl Transferase 1A (CPT1A) deficien-

cy, Propionic Acidemia (PA), and Medium-chain Acyl-CoA dehydrogenase Deficiency (MCAD).

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2019 with the Analyse-it statistical add-in. Precision was expressed as the coefficient of variation (%CV). Repeatability, between-day, within-instrument, between-instrument, and total precision components were estimated using variance-component analysis and two-way nested ANOVA, where applicable, in accordance with CLSI EP05. Accuracy was evaluated by comparing measured values with the assigned target concentrations and expressed as percentage recovery. Linearity was assessed by regression analysis across the evaluated concentration range, with R^2 used to describe the strength of the relationship between measured and assigned concentrations. Method comparison was evaluated using percentage deviation and Bland–Altman analysis. The predefined acceptance criterion for percentage deviation was ±30%, and 95% limits of agreement were calculated for Bland–Altman analysis. Statistical results were interpreted according to the predefined acceptance criteria and the applicable CLSI recommendations.

Results

The ability of the PH-NBS-D assay to reliably detect and quantify amino acids and acylcarnitines in newborn DBS specimens was extensively evaluated. Analytical results confirmed the kit's precision, accuracy, and recovery, and demonstrated its usefulness in early disease detection through NBS. Low CVs and minimal deviation from target values, confirmed the assay's robustness for routine use in detecting metabolic disorders.

Accuracy and precision

Box plots showing the recovery (%) of the 26 analytes at concentration levels I and II are shown in figure 1, with each box illustrating the distribution of 15 replicate measurements (5 days×3 analysts). Detailed numerical results are provided in table 2. Strong intra- and inter-laboratory precision with low %CV and clinically acceptable limits across multiple locations validated the accuracy of the PH-NBS-D assay. High recovery rates, reflecting both precision and dependability, demonstrated assay reliability. Recovery was consistently within the acceptable range of 40 to 140% for all tested analytes at both concentration levels. Compared to the non-derivatized method, the recovery percentage for certain analytes, such as C5DC, was increased [12]. These consistent and reproducible results support the assay's use in detecting metabolic disorders.

The intra-lab precision and accuracy of the PH-NBS-D assay were evaluated using control samples at two concentration levels (Level I and II) over 20 days, with two series per day in duplicate (Figure 2). We assessed key precision metrics such as repeatability, variation between runs, and within-lab variation, expressed as %CV. Most analytes demonstrated strong precision, with %CV values below the clinical guideline limit of 25%. Amino acids like alanine, arginine, and aspartic acid, along with acylcarnitines such as C0, C2, and C18, showed reliable performance. While some analytes (*e.g*., C5DC and C6 carnitine) had higher %CVs, they remained within acceptable ranges. These findings confirm the assay's reliability, reproducibility, and suitability for routine clinical use in newborn metabolic disorder screening.

Inter-laboratory precision was assessed to validate kit consistency in different laboratory settings. This evaluation used DBS control samples at two concentration levels over five days, with five series per day. Two different LC-MS/MS instruments (AB SCIEX 3200 and MDS SCIEX 3200) were used, operated by different personnel in separate labs. Repeatability, between-day precision, within-instrument precision, between-instrument variation, and total %CV were evaluated. Most analytes (Figure 3) maintained %CVs within the CLSI suggested acceptable clinical limit of 35% for reliability across sites [CLSI NBS04-A] [6]. For instance, alanine showed total %CVs of 10.2% at Level I and 7.6% at Level II, while glycine showed 9.3% at Level I and 8.0% at Level II. While some analytes (methionine, ornithine, and C5 carnitine) exhibited higher variability (especially between instruments) with %CVs up to 25.7%), most analytes met the precision stand-

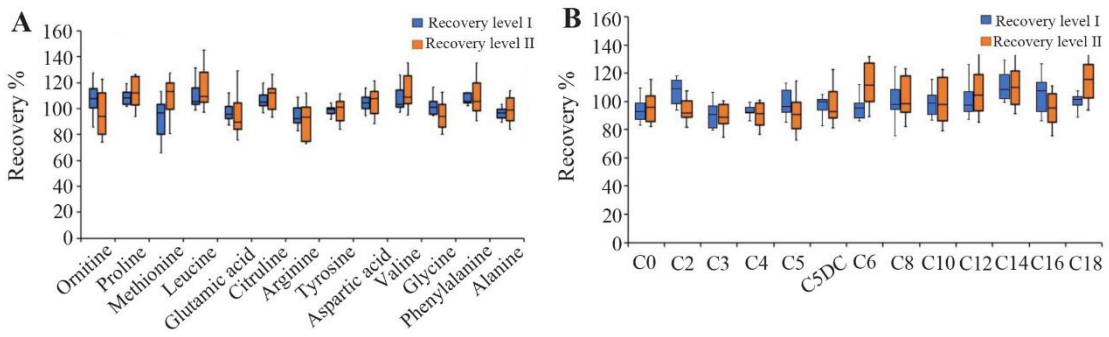

Figure 1. Box plots showing the recovery (%) of (A) amino acids and (B) acylcarnitines at levels I and II.

Table 2. Recovery rates for analytes in control samples (Level I and II) using the PH NBS-D derivatized MS/MS Assay

Analyte	PH NBS-D (pH=2002)							
	Control Sample (Level I)				Control Sample (Level II)			
	Target [*µmol/L*]	Range [*µmol/L*]	Mean [*µmol/L*]	Recovery %	Target [*µmol/L*]	Range [*µmol/L*]	Mean [*µmol/L*]	Recovery %
Amino Acids								
Ala	332	152-511	320.94	96.67	645	283-1007	641.91	99.52
Arg	118	44-193	111.97	94.89	317	163-472	288.67	91.06
Asp	96	62-131	100.20	104.38	255	169-342	268.68	105.36
Cit	65	46-84	69.04	106.21	261	192-330	286.10	109.62
Glu	466	300-633	464.16	99.61	834	574-1094	797.77	95.66
Gly	303	220-386	312.67	103.19	883	621-1146	840.91	95.23
Leu	245	135-355	271.55	110.84	505	335-674	580.38	114.93
Met	51	16-85	47.15	92.45	202	81-324	220.50	109.16
Orn	170	100-239	183.44	107.91	422	265-579	409.52	97.04
Phe	102	65-139	112.17	109.97	403	248-555	439.36	109.02
Pro	272	200-344	295.02	108.46	729	447-1033	817.68	112.16
Tyr	203	137-268	200.17	98.61	585	401-769	581.11	99.33
Val	247	155-339	264.22	106.97	463	317-649	519.12	112.12
Acylcarnitines and free carnitine								
C0	42.8	23.9-61.8	40.20	93.92	92.00	57.3-127	88.38	96.07
C2	22.4	15.4-29.5	23.90	106.70	63.00	39.0-87.0	59.35	94.20
C3	4.76	2.75-6.77	4.32	90.74	13.60	8.80-18.4	12.30	90.46
C4	0.81	0.38-1.25	0.76	94.38	3.56	2.08-5.04	3.23	90.76
C5	0.45	0.23-0.67	0.44	98.52	2.04	1.15-2.93	1.86	91.35
C5DC	0.5	0.12-0.88	0.50	99.15	2.02	0.93-3.11	1.95	96.44
C6	0.41	0.24-0.58	0.40	97.07	1.76	1.12-2.40	1.97	111.87
C8	0.41	0.22-0.6	0.41	101.01	1.90	1.17-2.64	1.95	102.62
C10	0.33	0.2-0.46	0.33	98.71	1.44	0.79-2.09	1.44	99.74
C12	0.47	0.21-0.73	0.48	102.03	2.03	1.33-2.73	2.16	106.19
C14	0.46	0.24-0.69	0.51	111.03	1.97	1.17-2.77	2.19	111.25
C16	5.03	3.05-7.01	5.25	104.35	15.10	9.26-21.0	14.21	94.14
C18	2.28	1.27-3.29	2.31	101.28	7.27	3.93-10.6	8.38	115.22

ards confirming the assay's reliability across different labs. These results highlight the importance of standardized protocols and operator training in reduce variability. Overall, the PH-NBS-D MS/MS assay demonstrated strong interlaboratory precision, supporting its use for newborn metabolic disorder screening on a broader scale.

Comparative analyses of real samples processed by PH NBS-D and MassChrom® kits

The comparative analyses of 40 real samples processed using the PH NBS-D and MassChrom® kits are summarized in figure 4. The deviation percentages for the PH NBS-D kit were calculated for each analyte, with the MassChrom® kit serving as the reference method. Overall, the deviation percentages fell within the acceptable range of -30% to +30% (95% confidence level), confirming the comparable accuracy of both kits in real sample analysis. Notably, analytes such as alanine showed a deviation of 8.744%, while aspartic acid exhibited a deviation of -15.715%. The full breakdown of analytes and their respective deviations demonstrate the suitability of both kits for accurate IMD screening in clinical settings. Regression analysis was performed to compare the performance of the two kits using 40 clinical samples from newborns. The regression equations, and 95% Confidence Intervals (CIs) for the slopes are presented in table 3. Overall, Passing–Bablok regression indicated acceptable agreement between the two kits for most analytes, while evidence of proportional and/or constant bias was observed for a limited number of analytes such as alanine.

Bland-Altman analysis for method comparability (Figure 5) revealed negligible to moderate bias for all paired measurements (95% limits of agreement and no proportional bias).

Comparison of the PH-NBS-D and ClinSpot® kits in identifying metabolic disorders

The comparative analysis of the PH-NBS-D and ClinSpot® kits on nine clinical samples demonstrated

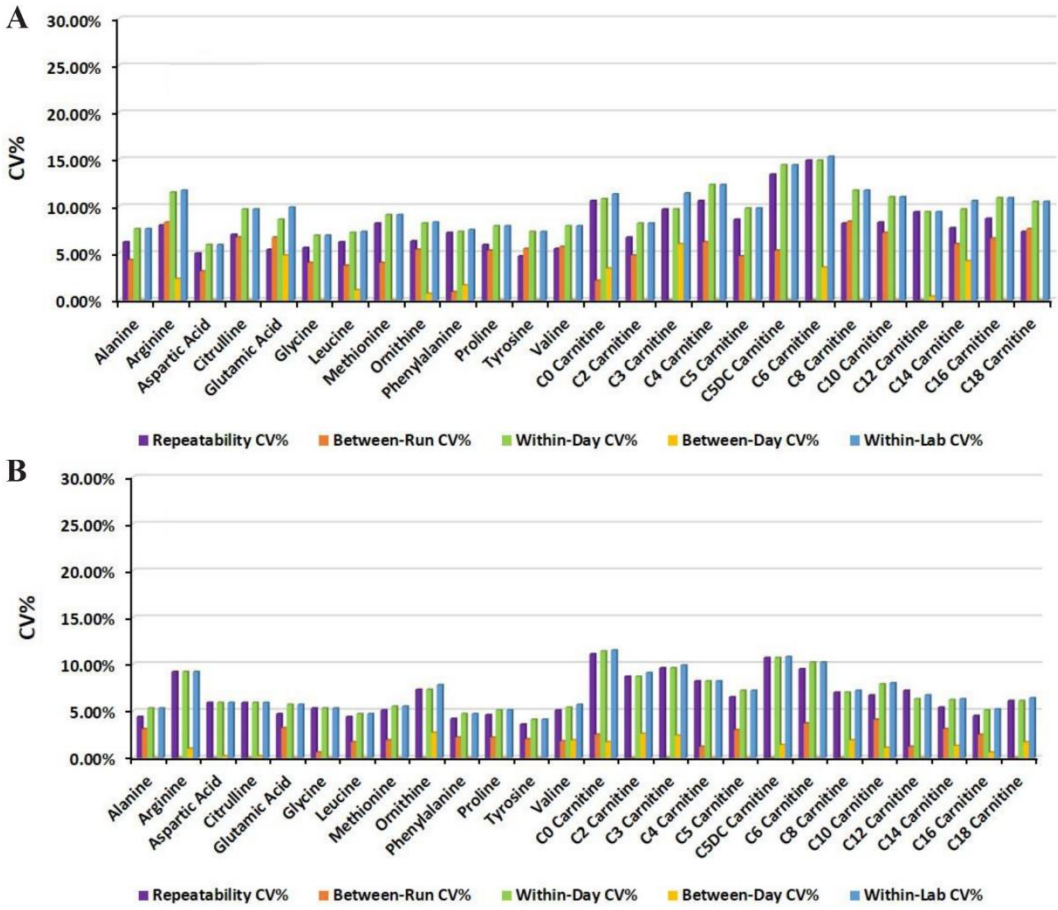

Figure 2. Intra-lab precision (%CV) of amino acids and acylcarnitines at a) level I and b) level II.

the effectiveness of both kits in identifying key markers associated with various metabolic diseases (Table 4). The relative closeness of assay results is evident in comparisons between the two columns of results on the right side of the table. Minimal deviation between the two kits was present, confirming the reliability of both for detecting abnormal levels of analytes indicating possible metabolic disorders.

Analytical sensitivity assessment (Limit of Detection and Lower Limit of Quantification)

The evaluation of analytical sensitivity for the PH NBS-D kit assessed the LOD and LLOQ of various analytes to ensure its effectiveness in newborn screening (Table 5). The concentrations where the %CV met the 25% threshold for acceptability are indicated. For example, alanine had an LOD of 3.50 *µmol/L* and an LLOQ of 10.50 *µmol/L*, while acylcarnitine C0 had an LOD of 0.20 *µmol/L* and an LLOQ of 0.60 *µmol/L*. These results confirm the PH NBS-D kit's high sensitivity, precision, and reliability for detecting and quantifying metabolic markers in NBS. Analyte concentrations were measured in diluted Level I and Level II control samples, ranging from undiluted (1:0) to highly diluted (1:1000), to evaluate the kit's precision across the different dilution stages.

Blank test analysis

For assay accuracy and reliability, the measurements of the blank filter paper samples should be below the LLOQ. The analysis of blank filter papers showed that the concentrations of all analytes were below their respective LLOQ values, confirming the robust sensitivity of the PH-NBS-D kit. As seen in table 6, analytes like alanine, glutamic acid, and glycine had mean concentrations consistently below their LLOQ thresholds. This consistency demonstrates the kit's ability to accurately detect low-level analytes, necessary for reliable NBS. The results also confirm acceptable analytical sensitivity.

Carry-over

Investigation of potential carryover confirmed the reliability of the PH NBS-D kit, with no significant residual impact from high-concentration samples. All analytes in blank samples remained well below their LLOQ thresholds. As shown in table 7, key amino acids like alanine and glycine, and acylcarnitines such as C0 and C3, had concentrations significantly below their LLOQ values, highlighting the kit's ability to prevent carryover and maintain result integrity in high-throughput metabolic screening.

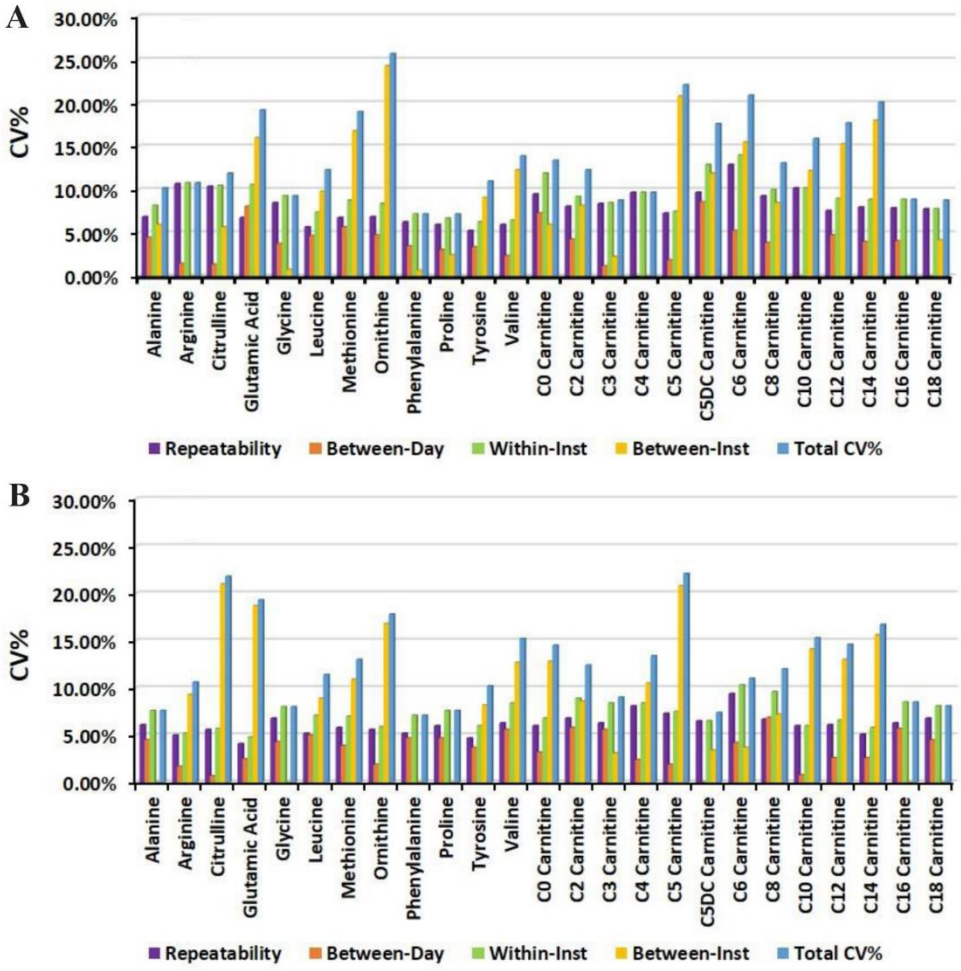

Figure 3. Interlaboratory precision (%CV) of amino acids and acylcarnitines at A) level I and B) level II.

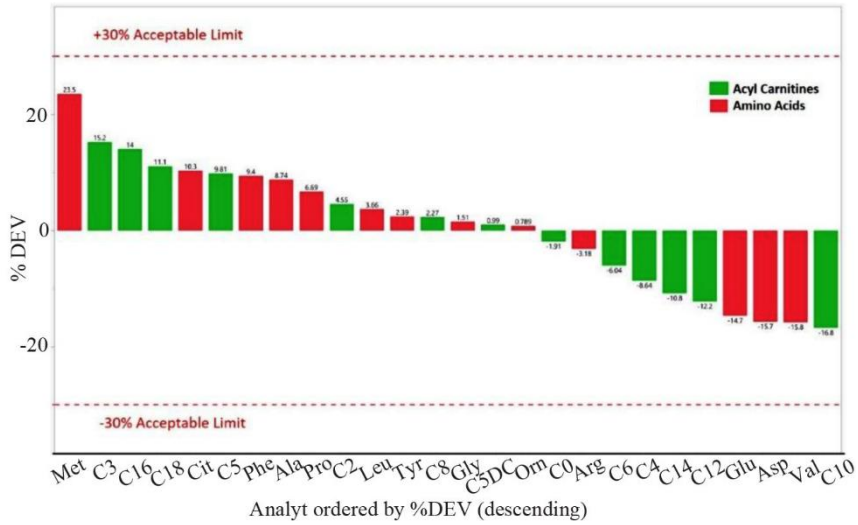

Figure 4. Deviation percentages for 40 real samples analyzed using PH NBS-D and MassChrom® kits (n=40).

Linearity

The assessment of linearity and ULOQ for the PH NBS-D kit included assessment of a full clinical concentration range of analytes, with nine concentration levels (L0-L8) tested using diluted and enriched dried blood spot control samples. This study was conducted

Table 3. Method comparison results between PH-NBS-D and MassChrom® using Passing–Bablok regression for the analyzed metabolites and acylcarnitines (n=40)

Analyte	Equation	Slope (95%CI)	Intercept (95%CI)	Analyte	Equation	Slope (95% CI)	Intercept (95%CI)
C0	Y=-2.697+1.102 X	0.9746 to 1.361	-7.091 to -0.2459	Ala	Y= -14.38+1.173 X	1.016 to 1.339	-43.63 to 8.806
C2	Y= -1.238+1.1 X	0.9382 to 1.270	-4.810 to 2.045	Arg	Y=-0.376+0.9972 X	0.8672 to 1.178	-3.212 to 1.981
C3	Y= -0.279+1.419 X	1.213 to 1.637	-0.7373 to -0.08741	Asp	Y= 0.0669+0.8577 X	0.5823 to 1.233	-22.20 to 14.83
C4	Y=0.02885+0.8046 X	0.6512 to 0.9259	0.0061 to 0.0599	Cit	Y=1.150+1.150 X	0.8829 to 1.444	-2.622 to 2.373
C5	Y=0.0024+1.111 X	0.8718 to 1.364	-0.0270 to 0.02778	Glu	Y=-3.483+0.8690 X	0.6724 to 1.126	-96.62 to 59.51
C5DC	Y=0045+1.00 X	0.6786 to 1.500	-0.0290 to 0.02033	Gly	Y= 9.137+0.9995 X	0.7121 to 1.249	-48.16 to 70.76
C6	Y=-0.007+1.097 X	0.9000 to 1.333	-0.0173 to 0.001316	Leu	Y=-5.755+1.148 X	0.9372 to 1.398	-32.81 to 10.87
C8	Y=-0.0005+1.00 X	0.8158 to 1.600	-0.0167 to 0.005375	Met	Y=1.337+1.204 X	0.9897 to 1.455	-4.444 to 7.265
C10	Y=0.01716+0.5517 X	0.2857 to 0.8889	3.3E-04 to 0.032	Orn	Y=-12.78+1.144 X	0.9461 to 1.413	-37.53 to 9.009
C12	Y=0.01079+0.7143 X	0.5000 to 1.000	-0.0043 to 0.02747	Phe	Y= -4.339+1.184 X	0.9572 to 1.517	-19.93 to 7.345
C14	Y=0.02365+0.7665 X	0.6111 to 0.9055	0.0020 to 0.041	Pro	Y=1.848+1.062 X	0.8487 to 1.285	-35.69 to 38.75
C16	Y=0.0081+1.142 X	1.050 to 1.269	-0.1618 to 0.1892	Tyr	Y= -7.86+1.119 X	1.006 to 1.265	-25.30 to 4.107
C18	Y=0.06670+1.065 X	0.8670 to 1.358	-0.1003 to 0.1650	Val	Y= 17.21+0.6176 X	0.4574 to 0.9835	-14.51 to 34.14

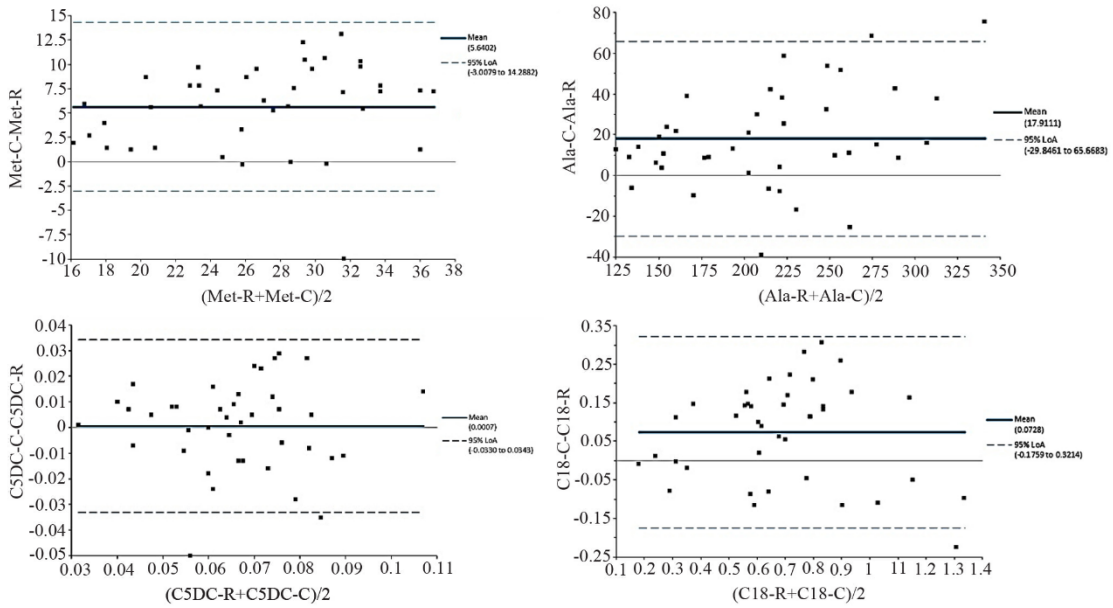

Figure 5. Bland–Altman plots of Met, Ala, C5DC and C18. R: Reference kit (MassChrom), C: Candid kit (PH NBS-D).

over five days, using duplicate sample preparations and the AB SCIEX 3200 instrument. Results in table 8 showed that the %CV remained under 25% at all concentration levels, meeting NBS04-A [5] standards. The linear range was confirmed by comparing the predicted values from fit equations, with acceptable deviations of -15% to +15%. The kit demonstrated consistent linearity and precise quantification for both amino acids and acylcarnitines within the specified range.

Discussion

The results obtained in this study indicate that the PH-NBS-D MS/MS assay provides reliable analytical performance for the measurement of amino acids and acylcarnitines in DBS specimens. The use of derivatization with formation of butyl esters is an important feature of the method, as it can improve ionization efficiency and signal response during electrospray ionization MS/MS. This is particularly useful for metabolites present at relatively low concentrations in newborn DBS samples. Derivatization may also improve the detection of structurally related acylcarnitines and some low-mass amino acids, although non-derivatized methods have the advantage of a simpler sample preparation procedure.

Table 4. Comparative results of clinical samples analyzed using ClinSpot® and PH-NBS-D kits, including pathologic assessments and deviations

Patient ID	Metabolite	Expected clinical condition	Reference interval	Pathologic borders	Results from ClinSpot®	Results from PH NBS-D
2923	Tyr	TYR	<264	>303	499	443.21
4076	Phe	PKU	<69	>112	375.0	340.2
131	Met	MET	9.0-32	>36, <9	916	907.4
0606	Leu	MSUD	<170	>191	1850.00	1740.13
	Val		<159	>166	550.00	648.84
4439	Leu	MSUD	<170	>191	217.00	213.21
	Val		<159	>166	189.00	210.10
4439	Gly	NKH	<417	>486	512.00	593.23
0831	Gly	NKH	<417	>486	808.00	746.87
3700	C2, C3, C6, C8, C10:1, C16, C18, C18:1	MCAD	Varies by component	Varies by component	Multiple values	Multiple values
4060	C3	PA/MMA	0.3-4.6	>5.0, <0.3	13.30	15.89
4047	C0	CPT1A	8-40	>45, <6.5	288.00	188.95
	C16		0.55-7.08	>8.68, <0.55	0.24	0.17
	C18		0.22-1.67	>1.9, <0.2	0.16	0.12
	C18:1		0.35-2.5	>2.76, <0.2	0.16	0.11

Table 5. Limit of detection (LOD) and lower limit of quantification (LLOQ) for analytes using the PH NBS-D kit [*µmol/L*]

PH NBS-D kit (PH 2002)					
Analytes	**LOD**	**LLOQ**	**Analytes**	**LOD**	**LLOQ**
Alanine	3.50	10.50	C0	0.20	0.60
Arginine	1.10	3.30	C2	0.10	0.30
Aspartic acid	2.20	6.60	C3	0.03	0.09
Citrulline	1.20	3.60	C4	0.04	0.12
Glutamic acid	4.60	13.80	C5	0.01	0.03
Glycine	6.90	20.70	C5DC	0.01	0.03
Methionine	2.10	6.30	C6	0.02	0.06
Leucine	1.30	3.90	C8	0.03	0.09
Ornithine	4.10	12.30	C10	0.01	0.03
Phenylalanine	1.20	3.60	C12	0.02	0.06
Proline	1.30	3.90	C14	0.01	0.03
Tyrosine	1.10	3.30	C16	0.01	0.03
Valine	4.40	13.20	C18	0.01	0.03

Table 6. Measured concentrations of analytes in blank filter papers for sensitivity assessment [*µmol/L*]

PH NBS-D kit (PH 2002)					
Analyte	**Mean**	**LLOQ**	**Analyte**	**Mean**	**LLOQ**
Alanine	5.71	10.50	C0	0.098	0.60
Aspartic acid	6.61	6.60	C2	0.120	0.30
Arginine	2.39	3.30	C3	0.013	0.09
Citrulline	1.61	3.60	C4	0.005	0.12
Glutamic acid	6.31	13.80	C5	0.004	0.03
Glycine	13.26	20.70	C5DC	0.006	0.03
Leucine	3.49	3.90	C6	0.006	0.06
Methionine	3.10	6.30	C8	0.003	0.09
Ornithine	10.43	12.30	C10	0.002	0.03
Phenylalanine	1.46	3.60	C12	0.006	0.06
Proline	3.27	3.90	C14	0.006	0.03
Tyrosine	2.38	3.30	C16	0.015	0.03
Valine	8.64	13.20	C18	0.010	0.03

Lower Limit of Detection (LLOQ).

The recovery values obtained for the evaluated analytes ranged from 91 to 115%, indicating good agreement between the measured and expected concentrations. Repeatability CVs ranged from 4.1 to 12.9%, while between-day variation ranged from 0.0 to 8.6% and total precision CVs from 7.1 to 25.7%. The higher variability observed for some analytes, particularly in between-instrument comparisons, indicates that instrument-related variation may contribute to the overall imprecision of some measurements. Nevertheless, the precision observed under the evaluated conditions was considered suitable for the intended screening application. The assay also showed good linearity over the evaluated concentration ranges, with R^2 values generally exceeding 0.99 and non-linearity ranging from −14.8% to +14.7%. These results indicate that the

Table 7. Analyte concentrations in blank samples for carryover effect evaluation using the PH NBS-D kit (*µmol/L*)

PH NBS-D kit (PH 2002)					
Analyte	**Mean**	**LLOQ**	**Analyte**	**Mean**	**LLOQ**
Alanine	6.61	10.50	C0	0.112	0.60
Arginine	2.34	3.30	C2	0.131	0.30
Aspartic acid	6.11	6.60	C3	0.015	0.09
Citrulline	1.87	3.60	C4	0.007	0.12
Glutamic acid	6.21	13.80	C5	0.005	0.03
Glycine	12.54	20.70	C5DC	0.005	0.03
Leucine	3.39	3.90	C6	0.008	0.06
Methionine	4.01	6.30	C8	0.004	0.09
Ornithine	9.63	12.30	C10	0.003	0.03
Phenylalanine	1.55	3.60	C12	0.006	0.06
Proline	3.69	3.90	C14	0.006	0.03
Tyrosine	2.51	3.30	C16	0.020	0.03
Valine	7.81	13.20	C18	0.016	0.03

Lower Limit of Detection (LLOQ).

Table 8. Evaluation of linearity, repeatability (CV%), non-linearity, and linearity range for analytes using the PH-NBS-D kit (PH 2002)

Analyte	Repeatability (CV%)	Non-linearity (%)	Linear Range (*µmol/L*)	Correlation coefficient (R^2)	Analyte	Repeatability (CV%)	Non-linearity (%)	Linear Range (*µmol/L*)	Correlation coefficient (R^2)
Ala	12.82	-14.16-2.88	50.5-3235.5	**0.999**	C0	11.23	-14.28-2.38	5.3-337.5	**0.995**
Arg	2.54	-14.32-5.08	14.86-475	**0.998**	C2	9.20	-13.70-7.70	4.11-131.4	**0.995**
Asp	8.99	-12.35-3.45	7.52-481.5	**0.992**	C3	15.31	-13.39-2.68	0.358-22.95	**0.998**
Cit	10.61	-8.02-3.93	16.8-537.75	**0.991**	C4	11.12	-6.18-2.82	0.55-17.48	**0.998**
Glu	15.48	-6.56-3.17	64.125-2052	**0.997**	C5	14.76	-14.78-3.02	0.078-4.973	**0.992**
Gly	14.51	-14.69-1.94	24.27-3107.25	**0.992**	C5DC	8.79	-3.93-14.74	0.478-7.65	**0.993**
Leu	19.43	-10.17-5.28	46.48-1487.25	**0.997**	C6	6.71	-5.49-4.36	0.165-2.64	**0.999**
Met	27.86	-2.51-7.58	27.7-886.5	**0.998**	C8	11.76	-8.04-3.69	0.13-4.16	**0.999**
Orn	16.82	-14.48-4.31	15.94-1001.25	**0.998**	C10	12.99	-10.41-5.28	0.08-2.56	**0.990**
Phe	17.51	-10.58-5.65	52.38-1676.25	**0.998**	C12	11.52	-9.03-4.23	0.426-13.63	**0.992**
Pro	11.6	-6.60-3.23	37.76-1208.25	**0.995**	C14	13.09	-11.88-5.58	0.223-7.15	**0.991**
Tyr	12.05	-11.49-1.72	19.55-1251	**0.999**	C16	14.07	-11.53-0.73	0.206-26.325	**0.997**
Val	19.59	-8.02-4.09	38.67-1237.5	**0.996**	C18	11.66	-8.14-1.31	0.127-8.145	**0.999**

method provides a consistent response over the concentration ranges relevant to the evaluated NBS analytes.

The comparison with established commercial assays provides additional support for the analytical performance of PH-NBS-D. Results obtained with PH-NBS-D were compared with those obtained using the MassChrom® and ClinSpot® kits. For the MassChrom ® comparison, the observed percentage deviations were within the predefined acceptance limits of ±30%, and Bland–Altman analysis did not show significant systematic bias. The ClinSpot® comparison also showed close agreement in the identification of abnormal metabolic profiles. These findings suggest that the developed assay produces results comparable to those obtained using commercially available methods and provide a practical benchmark for evaluating its performance in clinical DBS specimens.

To the best of our knowledge, this is the first comprehensive analytical validation of the PH-NBS-D MS/MS assay using DBS specimens for the simultaneous analysis of amino acids and acylcarnitines. However, the clinical comparison was performed using a relatively limited number of specimens, particularly for the ClinSpot® evaluation. The number of confirmed metabolic disorder cases was also insufficient to determine the clinical sensitivity and specificity of the assay for individual IEMs. Therefore, the clinical findings should be interpreted primarily as evidence of method comparability rather than as an assessment of diagnostic accuracy. Studies involving larger numbers of confirmed positive cases and a wider range of metabolic disorders would be useful for establishing the clinical performance of the assay.

Conclusion

The PH-NBS-D derivatized LC-MS/MS assay met CLSI requirements for accuracy, precision, linearity, analytical sensitivity, and carryover, with robust intra- and inter-laboratory reproducibility. Its performance was comparable to two established commercial newborn screening kits in both control materials and clinical specimens, including samples from infants with confirmed inborn metabolic disorders. These results support PH-NBS-D as a reliable, sensitive, locally developed assay suitable for routine use in Iranian newborn screening programs, with further multicenter and outcome-based studies warranted to refine cut-offs and confirm long-term clinical utility.

Institutional review board statement

The study was approved by the Research Ethics Committees of Avicenna Research Institute under registration code IR.ACECR.AVICENNA.REC.1403.006.

Informed consent statement

Informed consent was obtained from all the parents or legal guardians of all subjects involved in the study.

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Conflict of Interest

The authors declare no conflict of interest.

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