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Development, Validation, and Application of the Paya Hamsan Technolgies Underivatized Newborn Screening Assay (PHUNSA) for Inborn Metabolic Disorders in Dried Blood Spot Samples from Iranian Infants

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Abstract: Screening for inborn metabolic disorders (IMDs) in newborns is an important way to prevent serious metabolic and developmental difficulties that can result in lasting disabilities or even death. Electrospray ionization tandem mass spectrometry (MS/MS) provides an efficacious newborn blood spot screening (NBS) mechanism for analyzing dried blood spot specimens (DBSs) for biochemical markers for these conditions. Where possible, elimination of derivatization in specimen preparation can simplify and streamline analysis. The Paya Hamsan Technologies Underivatized Newborn Screening Assay (PHUNSA) is an innovative underivatized MS/MS test kit for IMD NBS. Validation of accuracy, precision, linearity, and stability was based on the ISO 15189 Standard and the CLSI NBS04 Guideline. The PHUNSA kit demonstrated superior performance along with excellent recovery rates and negligible bias for many IMD analytes. Assay sensitivity was demonstrated through acceptable limits of detection (LOD) and lower limits of quantification (LLOQ). Specimen preparation times were decreased, coefficients of variation were consistently below 10%, and accuracy and stability were demonstrated under various testing conditions, including prolonged storage and transportation. The PHUNSA kit provides a simplified, efficient, and reliable approach to IMD NBS with the potential to enhance NBS in Iran and other locations by providing a scalable, cost-effective and streamlized option for early IMD detection and management.

Keywords: inborn metabolic disorders; newborn screening; tandem mass spectrometry; dried blood spot; non-derivatized assay

1. Introduction

Untreated inborn metabolic disorders (IMDs) can cause neurological damage, physical disability, and even death due to the abnormal levels of metabolites involved in important physiological processes [1,2]. A combination of genetic factors and high consanguinity increases the risk for IMDs in Iran and underscores the need to develop effective and efficient screening procedures. Newborn blood spot screening (NBS) allows for the prevention of serious metabolic and developmental problems that can result from IMDs and may cause permanent impairment or even death [3,4]. Through early detection and disease management, newborns and their families can

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experience improved quality of life and correspondingly reduce the strain on national healthcare resources.

Consistent with the growth of NBS, the emergence of modern technologies like electrospray ionization tandem mass spectrometry (MS/MS), with its enhanced diagnostic capacity, has significantly contributed to expanded NBS for IMDs, thus improving newborn healthcare worldwide [5,6]. Using MS/MS technology, it is possible to simultaneously quantify a wide range of metabolites using only a single dried blood spot (DBS). Consequently, screening and diagnostic processes are accelerated and disease management can begin earlier. Specimen preparation for MS/MS can be streamlined by using non-derivatized specimens, which eliminates a sample preparation step in the analysis protocol used in conventional derivatization MS/MS methodologies. This improved laboratory efficiency reduces the risk of errors during sample handling and assay preparation, which increases assay reliability [4,7–10].

This report describes the development and validation of the Paya Hamsan Technologies Underivatized Newborn Screening Assay (PHUNSA) for IMDs in DBS specimens (Paya Hamsan Technologies Co., Arak, Iran). This novel non-derivatized MS/MS assay targets amino acids and acylcarnitines critical for identifying various IMDs. To document that the screening test is reliable and robust, results have been validated using well-established international criteria for accuracy, precision, linearity, and stability. Beyond improving Iran's NBS system, the PHUNSA kit provides a model IMD NBS process for use in other locations with similar genomic and socioeconomic profiles. Incorporation of this assay into NBS systems is a scalable, efficient, and cost-effective way to solve a pressing public health problem [11,12]. Correct application of this screening test has the potential to impact metabolic disease management globally. Better understanding the prevalence and types of metabolic disorders in Iranian infants will also inform metabolic disease research, advance early treatments and improve disease outcomes [13,14].

2. Materials and Methods

2.1. Study Design

The objective of this study was to utilize the newly developed PHUNSA kit (PH NBS complete kit, order no. PH 2001) to analyze DBSs from Iranian newborns for the presence of indicators of various IMDs. The assay's capacity to detect IMD-related biomarkers was validated using specimens prepared without the use of derivatization techniques.

2.2. Ethical Considerations

The study was approved by the Research Ethics Committees of Avicenna Research Institute under registration code IR.ACECR.AVICENNA.REC.1403.006. Prior to specimen collection, informed consent was obtained from the parents or legal guardians of all participating infants. All were informed of the study's objectives, procedures, risks, and advantages. In order to ensure that all participants could understand and provide meaningful consent, the permission documents were translated into the local language. Consent was concluded prior to the commencement of any protocols associated with the study.

2.3. Chemicals and Reagents

A non-derivatized reagent kit specifically intended for the analysis of amino acids and acylcarnitines by liquid chromatography tandem mass spectrometry (LC-MS/MS) was used in the study. By eliminating the derivatization step, the analytical procedure is simplified and the potential for specimen handling errors is minimized. Essential components, such as mobile phase, calibration standards, and quality control samples, were included in the kit for direct DBS analysis on our analytical platform.

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2.4. Analytical Equipment Details

Quantitative analysis was performed using an AB Sciex 3200 Mass Spectrometer (SCIEX, Concord, Ontario Canada) equipped with an electrospray ionization (ESI) source. This system's precision and capacity for simultaneous multi-analyte measurement provided the required suitability for high-throughput screening. Data acquisition and analysis were managed using SCIEX Analyst Software version 1.6.3, which gave the robust data processing capabilities required for accurate quantification and reliability.

All laboratory equipment was routinely calibrated and serviced in compliance with manufacturer's specifications and industry standards to guarantee consistent performance throughout the study.

2.5. Kit Performance Comparison and Quality Assurance

A side-by-side comparison was performed versus CE-certified MS/MS kits currently being used nationwide in Iran, ChromSystems (Munich, Germany). The performance comparison adhered recommendations published in both the CLSI NBS04 Guideline for Newborn Screening by Tandem Mass Spectrometry (Clinical Laboratory Standards Institute, Wayne, Pennsylvania USA) [15] and the ISO 15189 Standard for Medical Laboratories (International Organization for Standardization, Geneva, Switzerland) [16] quality management standards. To conform to the stringent validation standards of the Ministry of Health and Medical Education (MOHME), assays were carried out by specialized laboratory centers using an the MS/MS system and in accordance with directive issued by the Health Deputy of the Ministry of Health and Medical Education of Iran (HD-IMD-00-MN-SD-006-001).

2.6. Sample Collection and Preparation

Newborn blood was obtained by heel prick and absorbed onto pre-labled Whatman 903 filter paper cards in compliance with the CLSI NBS01 Standard, Dried Blood Spot Specimen Collection for Newborn Screening [17]. In order to maintain the metabolites during drying, the DBS cards were dried for 3 hrs at room temperature and kept at -20°C until analysis [18]. Each card was given a unique laboratory identification number for traceability.

2.7. Analytical Methods

A 3.2 mm disc was punched from each collection card and inserted into a 96-well flat bottom plates. Then, 100 µl internal standard (reconstituted with extraction buffer (Methanol/Water)) was added to each sample. The analytes were extracted by shaking for 30 minutes at 700 rpm, and the supernatant was transferred to a 96-well conical bottom plates for MS/MS analysis. Extensive literature review coupled with experimental data were used to predetermine the metabolite transitions to be selectively detected. Table 1 (amino acids) and Table 2 (acylcaintines), list the analytes and their associated quantitative parameters along with details for each transition. Analyte concentrations were determined using standards in similar matrices. To ensure accuracy and reliability, quality control (QC) samples were analyzed with each batch. Assay validation included evaluation of calibration and recovery data for standards and QC samples, as shown in the tables.

Table 1. LC-MS/MS Parameters for Underivatized Amino Acids.

Amino Acids - Non-Derivatization					
Analyte	**Precursor (Q1 Mass (Da))**	**Product (Q3 Mass (Da))**	**Time (msec)**	**DP (Volts)**	**CE (Volts)**
Alanine	90	44	50	7	18
Alanine-d4	94	48	50	7	18
Arginine	175	70	50	7	35
Arginine-d7	182	77	50	7	35
Aspartic acid	134	116	50	7	11
Aspartic acid-d3	137	119	50	7	11

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Citrulline	176	113	200	7	22
Citrulline-d2	178	115	200	7	22
Glutamic acid	148	130	50	7	14
Glutamic acid-d5	153	135	50	7	14
Glycine	76	30	50	7	16
Glycine-13C2,15N	79	32	50	7	16
Leucine	132	86	50	7	14
Leucine-d3	135	89	100	7	14
Methionine	150	133	50	7	14
Methionine-d3	153	136	50	7	14
Ornithine	133	70	50	7	25
Ornithine-d6	139	76	50	7	25
Phenylalanine	166	120	50	7	17
Phenylalanine-d5	171	125	50	7	17
Proline	116	70	50	7	22
Proline-d7	123	77	50	7	22
Tyrosine	182	136	50	7	17
Tyrosine-d4	186	140	50	7	17
Valine	118	72	50	7	16
Valine-d8	126	80	50	7	16

Table 2. LC-MS/MS Parameters for Underivatized Acylcarnitines and Free Carnitine.

Acylcarnitines and Free Carnitine - Non-Derivatization					
Analyte	**Precursor (Q1 Mass (Da))**	**Product (Q3 Mass (Da))**	**Time (msec)**	**DP (Volts)**	**CE (Volts)**
Carnitine	162	85	50	7	16
Carnitine-d9	171	85	50	7	16
Acetylcarnitine	204	85	50	7	27
Acetylcarnitine-d3	207	85	50	7	27
Propionylcarnitine	218	85	50	7	27
Propionylcarnitine-d3	221	85	50	7	27
Butyrylcarnitine	232	85	50	7	31
Butyrylcarnitine-d3	235	85	50	7	31
Isovalerylcarnitine	246	85	100	7	32
Isovalerylcarnitine-d9	255	85	100	7	32
Glutarylcarnitine	276	85	50	10	32
Glutarylcarnitine-d9	285	85	50	7	32
Hexanoylcarnitine	260	85	50	7	34
Hexanoylcarnitine-d3	263	85	50	7	34
Octanoylcarnitine	288	85	50	7	35
Octanoylcarnitine-d3	291	85	50	7	35
Decanoylcarnitine	316	85	50	7	40
Decanoylcarnitine-d3	319	85	50	7	40
Dodecanoylcarnitine	344	85	50	7	40
Dodecanoylcarnitine-d3	347	85	50	7	40
Tetradecanoylcarnitine	372	85	50	7	46
Tetradecanoylcarnitine-d3	375	85	60	7	46
Hexadecanoylcarnitine	400	85	60	7	48
Hexadecanoylcarnitine-d3	403	85	50	7	48
Octadecanoylcarnitine	428	85	50	7	52
Octadecanoylcarnitine-d3	431	85	50	7	52

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2.8. Recovery and Accuracy Evaluation

Recovery analyses were conducted adhering to CLSI NBS04. Intra-assay variability was assessed by performing each test in duplicate across five independent working sites over several days. To simulate routine screening conditions, two concentration levels (Level I and Level II) of DBS were used as control samples. Acceptable recovery rates, defined as 40-140%, were established based on control sample guidelines.

2.9. Accuracy and Precision Measurements

Accuracy was assessed at individual and multiple testing locations following the recommendations in CLSI EP05-A3 [19]. Testing was performed over 20 days using two levels of DBS controls (ChromSystems, Munich, Germany) to assess and ensure kit precision. Tests were performed twice daily using a SHIMADZU 8045 LC-MS/MS instrument (Shimadzu, Kyoto, Japan). Measurements were taken to evaluate consistency, variances between runs, variations within a single day, and variations across days. Data analysis was conducted using a two-way nested ANOVA process. Over the course of five days, various operators from different laboratories used two separate LC-MS/MS machines to assess the inter-laboratory precision across more than one testing site. A two-way nested ANOVA was used to assess repeatability and consistency between different instruments, using two different concentration levels of controls from Paya Hamsan Technologies. This two-pronged technique not only confirmed the assay's high performance in a variety of laboratory circumstances but also ensured that the detection and quantification of metabolic analytes were accurate.

2.10. Intra-Lab Precision and Accuracy

To demonstrate diagnostic efficacy of the non-derivatized test versus traditional derivatized procedures, analyses using the PHUNSA kit were compared to the MassChrom kit to validate its reliability, accuracy, and effectiveness. Both kits were used to examine control samples and assess any measurement variations while maintaining kit consistency. Primary focus was on each assay's ability to precisely detect and measure pertinent metabolites. The intra-lab precision and reproducibility were evaluated by obtaining multiple measurements of control samples over a period of 20 days. Limits of detection (LOD) and the lower limits of quantification (LLOQ) were determined by dilution of prepared dried blood samples with extraction buffer/internal standard at various ratios (1:10, 1:20, 1:50, 1:100, 1:200, 1:500, and 1:1000) and evaluated using the AB SCIEX 3200 apparatus on five separate occasions. The %CV was computed for each analyte. LOD was determined as the concentration level at which the %CV reached 25%. The LLOQ was determined by multiplying LOD by a factor of three.

The degree of linearity, the capacity to endure changes in concentration levels, and the ability to analyze stored specimens (including freeze-thawed and specimens stored long-term) were also determined along with testing reliability and accuracy and precision across different laboratories. Multiple repetitions of the tests were conducted across different variables (times, instruments and operators) to evaluate the consistency within each study and to ensure accurate recovery rates. Statistical analyses were made using Microsoft Office Excel 2019 with a p-value less than 0.05 defining statistical significance.

3. Results

3.1. Recovery

The recovery data in Table 3 demonstrate the efficacy of the PHUNSA kit. With one exception, all recoveries equalled or exceeded 75%, well within the 40%-140% noted as suitable in CLSI NBS04. Several recoveries exceeded 100% indicating increased sensitivity for these indicators, further confirming the clinical viability of the underivatized assay.

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Table 3. Recovery of Amino Acids and Acylcarnitines in PHUNSA MS/MS Kit.

Analyte	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								
Alanine	354	163-545	378.87	107.03	736.00	323-1149	671.15	91.19
Arginine	97	36-158	109.27	112.65	225.00	115-335	260.23	115.66
Aspartic Acid	110	71-149	102.47	93.16	261.00	173-350	259.58	99.46
Citrulline	68	48-88	64.76	95.23	300.00	221-379	262.21	87.40
Glutamic Acid	464	298-630	507.39	109.35	730.00	502-958	855.76	117.23
Glycine	257	187-327	252.83	98.38	649.00	456-842	627.52	96.69
Leucine	278	153-403	304.51	109.54	504.00	335-673	568.36	112.77
Methionine	49	15-83	51.74	105.59	191.00	76-306	209.00	109.42
Ornithine	230	136-324	237.39	103.21	547.00	343-751	521.64	95.36
Phenylalanine	121	77-165	132.93	109.86	436.00	269-603	523.65	120.10
Proline	299	220-378	310.63	103.89	774.00	475-1074	806.95	104.26
Tyrosine	192	130-254	212.36	110.61	565.00	381-731	626.41	110.87
Valine	244	153-335	267.61	109.68	424.00	278-570	508.09	119.83
Acylcarnitines and Free Carnitine								
Carnitine (C0)	49.7	27.8-71.7	58.28	117.26	101.00	63.0-139	121.69	120.49
Acetylcarnitine (C2)	21	14.4-27.6	21.63	102.99	60.10	37.2-83.0	58.90	98.01
Propionylcarnitine (C3)	4.37	2.52-6.22	4.50	102.90	13.00	8.44-17.6	12.49	96.09
Butyrylcarnitine (C4)	0.83	0.38-1.28	0.84	101.53	3.29	1.92-4.66	3.58	108.89
Isovalerylcarnitine (C5)	0.49	0.25-0.73	0.54	110.79	2.17	1.22-3.12	2.23	102.59
Glutarylcarnitine (C5DC)	0.6	0.15-1.05	0.54	90.79	2.60	1.20-4.0	1.16	44.72
Hexanoylcarnitine (C6)	0.45	0.26-0.64	0.41	91.44	2.12	1.35-2.89	1.89	89.11
Octanoylcarnitine (C8)	0.49	0.26-0.72	0.41	84.00	2.17	1.33-3.01	1.82	83.64
Decanoylcarnitine (C10)	0.48	0.29-0.67	0.36	74.97	1.96	1.08-2.84	1.48	75.43
Dodecanoylcarnitine (C12)	0.46	0.2-0.72	0.52	112.25	2.09	1.37-2.61	2.27	108.50
Tetradecanoylcarnitine (C14)	0.48	0.25-0.71	0.44	92.24	2.09	1.24-2.94	1.85	88.53
Hexadecanoylcarnitine (C16)	4.72	2.86-6.58	4.48	94.81	13.20	8.08-18.3	11.72	88.82
Octadecanoylcarnitine (C18)	2.47	1.38-3.56	2.59	104.72	8.28	4.47-12.3	8.70	105.02

3.2. Accuracy and Precision

Accuracy assessments revealed minimal bias across all analytes as shown in Table 4. Exceptional reproducibility was observed both within and between assays, with coefficient of variations (%CV) consistently below 10%. Intra-lab precision was consistent throughout the 20-day assessment period demonstrating a high degree of reliability.

Table 4. Intra-labortory precision of the PHUNSA kit for selected analytes at two concentration levels.

Analyte	Level I					Level II				
	Repeat-ability CV%	Between Run CV%	Within Day CV%	BetweenDay CV%	WithinLab CV%	Repeat-ability CV%	BetweenRun CV%	WithinDay CV%	BetweenDay CV%	WithinLab CV%
Alanine	4.1%	4.7%	6.2%	4.7%	7.8%	1.6%	5.0%	5.2%	2.0%	5.6%
Arginine	2.6%	4.9%	5.6%	3.7%	6.7%	1.5%	4.3%	4.5%	3.7%	5.9%
Aspartic Acid	4.1%	3.6%	5.5%	2.7%	6.1%	3.9%	2.5%	4.6%	5.0%	6.8%
Citrulline	3.7%	5.4%	6.5%	3.5%	7.4%	3.0%	5.5%	6.3%	1.2%	6.4%
Glutamic Acid	5.6%	4.5%	7.1%	3.9%	8.1%	3.2%	2.7%	4.2%	2.7%	5.0%
Glycine	3.0%	4.2%	5.2%	4.0%	6.5%	2.6%	4.8%	5.4%	1.8%	5.7%
Leucine	1.5%	4.8%	5.0%	0.5%	5.1%	0.8%	4.4%	4.5%	2.9%	5.3%
Methionine	3.9%	6.7%	7.8%	2.6%	8.2%	3.7%	4.6%	5.9%	2.8%	6.5%
Ornithine	3.4%	3.7%	5.0%	3.9%	6.4%	1.4%	4.5%	4.7%	5.5%	7.2%
Phenylalanine	3.1%	4.6%	5.6%	2.2%	6.0%	0.7%	4.8%	4.8%	4.2%	6.4%

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Proline	2.1%	4.5%	5.0%	1.2%	5.1%	1.5%	5.0%	5.2%	3.5%	6.3%
Tyrosine	3.4%	5.4%	6.4%	3.1%	7.2%	1.4%	4.4%	4.6%	0.0%	4.6%
Valine	3.3%	4.6%	5.7%	2.6%	6.2%	1.2%	4.8%	4.9%	6.7%	8.3%
Carnitine (C0)	5.4%	4.4%	6.9%	2.9%	7.5%	2.2%	5.1%	5.5%	6.3%	8.4%
Acetylcarnitine (C2)	2.3%	5.6%	6.1%	4.8%	7.8%	1.2%	4.5%	4.6%	0.8%	4.7%
Propionylcarnitine (C3)	2.1%	4.7%	5.1%	4.1%	6.5%	1.7%	4.6%	4.9%	0.0%	4.9%
Butyrylcarnitine (C4)	2.8%	4.4%	5.2%	4.6%	7.0%	2.5%	4.3%	5.0%	0.0%	5.0%
Isovalerylcarnitine (C5)	2.4%	5.5%	6.0%	1.6%	6.3%	1.5%	4.8%	5.1%	2.5%	5.6%
Glutarylcarnitine (C5DC)	10.3%	0.0%	10.3%	3.0%	10.7%	8.2%	0.0%	8.2%	2.8%	8.7%
Hexanoylcarnitine (C6)	2.4%	4.6%	5.2%	5.7%	7.7%	1.7%	3.2%	3.6%	2.7%	4.5%
Octanoylcarnitine (C8)	1.9%	4.8%	5.1%	3.7%	6.3%	1.1%	4.5%	4.7%	0.0%	4.7%
Decanoylcarnitine (C10)	1.9%	4.7%	5.0%	4.6%	6.8%	1.0%	4.3%	4.5%	0.0%	4.5%
Dodecanoylcarnitine (C12)	1.8%	5.2%	5.5%	3.3%	6.4%	1.0%	4.3%	4.4%	0.0%	4.4%
Tetradecanoylcarnitine (C14)	1.5%	5.2%	5.4%	3.6%	6.5%	0.9%	4.6%	4.7%	0.0%	4.7%
Hexadecanoylcarnitine (C16)	0.8%	5.0%	5.0%	2.9%	5.8%	0.6%	5.1%	5.1%	0.0%	5.1%
Octadecanoylcarnitine (C18)	0.9%	5.0%	5.1%	2.6%	5.7%	0.6%	5.6%	5.7%	0.4%	5.7%

Multi-site precision was evaluated by analyzing DBS controls at two different concentratios (supplied by Paya Hamsan Technologies) (see Table 5). Two different instruments (Each one an operator), were used in different laboratories over a period of 5 days with 5 replicates per day. The data provides a comprehensive overview of the repeatability and variability metrics for the relevant analytes. The low coefficients of variation imply high precision. The inter-day and intra-instrument data indicate analytical stability and consistency over time.

Table 5. Multi-site precision of the PHUNSA kit for various analytes across two concentration levels.

Analyte	Level I					Level II				
	Repeat-ability	BetweenDay	Within Instru-ment	Between Instu-ment	Total CV%	Repeata-bility	BetweenDay	Within Instru-ment	Between Instru-ment	Total CV%
Alanine	6.8%	6.0%	9.0%	0.0%	9.0%	6.3%	5.0%	8.1%	6.2%	10.2%
Arginine	7.3%	3.9%	8.3%	1.5%	8.4%	5.6%	2.6%	6.2%	9.5%	11.3%
Citrulline	10.6%	0.0%	10.6%	9.4%	14.2%	6.0%	2.7%	6.5%	8.0%	10.3%
Glutamic Acid	6.3%	0.0%	6.3%	12.7%	14.2%	5.7%	1.3%	5.9%	16.9%	17.9%
Glycine	8.2%	3.3%	8.8%	0.0%	8.8%	7.9%	4.5%	9.1%	0.0%	9.1%
Leucine	7.0%	0.0%	7.0%	11.9%	13.8%	6.1%	4.9%	7.9%	14.3%	16.3%
Methionine	8.8%	2.4%	9.1%	14.7%	17.3%	5.6%	3.8%	6.8%	15.8%	17.2%
Ornithine	5.9%	4.5%	7.5%	21.3%	22.6%	5.9%	1.9%	6.2%	18.7%	19.7%
Phenylalanine	7.2%	2.7%	7.7%	0.0%	7.7%	6.0%	4.0%	7.2%	6.1%	9.5%
Proline	7.3%	1.4%	7.4%	0.0%	7.4%	5.9%	4.8%	7.7%	2.1%	7.9%
Tyrosine	6.2%	2.4%	6.6%	4.4%	8.0%	5.4%	3.3%	6.3%	5.3%	8.2%
Valine	7.6%	0.0%	7.6%	10.1%	12.7%	6.3%	6.1%	8.8%	7.3%	11.5%
Carnitine (C0)	9.6%	1.8%	9.8%	10.4%	14.3%	6.8%	6.3%	9.3%	7.0%	11.6%
Acetylcarnitine (C2)	-	-	-	-	-	7.5%	4.2%	8.6%	7.0%	11.1%
Propionylcarnitine (C3)	7.9%	0.9%	8.0%	6.3%	10.2%	7.0%	4.6%	8.4%	8.3%	11.8%
Butyrylcarnitine (C4)	9.9%	0.0%	9.9%	5.4%	11.3%	7.2%	4.5%	8.5%	7.7%	11.5%
Isovalerylcarnitine (C5)	6.8%	6.4%	9.4%	24.6%	26.4%	5.5%	4.0%	6.8%	19.2%	20.4%
Glutarylcarnitine (C5DC)	7.4%	3.8%	8.3%	11.4%	14.1%	8.9%	3.0%	9.4%	18.4%	20.7%
Hexanoylcarnitine (C6)	11.2%	0.5%	11.2%	20.1%	23.0%	6.8%	5.8%	9.0%	2.1%	9.2%
Octanoylcarnitine (C8)	7.6%	0.0%	7.6%	6.0%	9.7%	7.5%	6.4%	9.9%	2.9%	10.3%
Decanoylcarnitine (C10)	8.6%	0.0%	8.6%	10.9%	13.8%	8.4%	6.7%	10.7%	7.6%	13.1%
Dodecanoylcarnitine (C12)	8.6%	0.0%	8.6%	12.3%	15.0%	8.0%	6.6%	10.4%	10.3%	14.6%
Tetradecanoylcarnitine (C14)	8.5%	0.0%	8.5%	15.2%	17.4%	8.5%	6.8%	10.9%	11.3%	15.6%
Hexadecanoylcarnitine (C16)	7.8%	0.0%	7.8%	3.6%	8.6%	7.8%	5.1%	9.3%	6.3%	11.2%
Octadecanoylcarnitine (C18)	7.8%	2.4%	8.1%	1.0%	8.2%	8.2%	3.4%	8.9%	2.3%	9.2%

* Level I C2 Carnitine concentration was below the detection limit of the AB SCIEX 3200 instrument and is not reported.

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3.3. Method Comparison Using Control Samples

Comparative data between the PHUNSA kit and the MassChrom kit are shown in Tables 6 and 7. Analyses of the control samples were performed using a Shimadzu 8045 instrument. Mean values, %CV, and percentage deviation from target concentrations were determined for a comprehensive range of amino acids and acylcarnitines. PHUNSA kit values were found to be in good agreement with the MassChrom kit indicating satisfactory comparative performance.

Table 6. Comparison of PHUNSA and MassChrom kits for analyzing control sample I (n=40).

Analyte	Target [µmol/L]	Range [µmol/L]	PHUNSA			MassChrom		
			Mean [µmol/L]	%CV	Deviation%	Mean [µmol/L]	%CV	Deviation%
Amino Acids								
Alanine	705	324-1086	580.56	7.43	-17.65	609.80	5.94	-13.50
Arginine	66	24-108	59.41	6.44	-9.98	58.69	5.26	-11.07
Aspartic Acid	184	118-250	206.89	5.91	12.44	197.84	6.38	7.52
Citrulline	73	51-94	75.48	7.15	3.40	65.33	7.07	-10.51
Glutamic Acid	856	550-1162	836.83	7.87	-2.24	828.70	6.24	-3.19
Glycine	448	325-570	497.35	6.22	11.02	454.32	5.13	1.41
Leucine	471	259-682	441.77	4.99	-6.21	396.12	5.58	-15.90
Methionine	102	32-173	93.52	8.02	-8.32	88.85	5.75	-12.89
Ornithine	526	310-742	531.04	6.07	0.96	514.96	7.86	-2.10
Phenylalanine	297	189-404	241.50	5.85	-18.69	238.65	5.53	-19.65
Proline	480	353-606	452.15	5.03	-5.80	410.69	6.90	-14.44
Tyrosine	235	159-311	208.18	6.94	-11.41	215.28	5.26	-8.39
Valine	367	230-504	356.70	6.06	-2.81	309.81	7.83	-15.58
Acylcarnitines and Free Carnitine								
Carnitine (C0)	55.5	31-80	49.61	7.35	-10.61	42.58	7.57	-23.28
Acetylcarnitine (C2)	21.3	14.6-28	19.82	7.36	-6.95	18.58	5.17	-12.76
Propionylcarnitine (C3)	4.39	2.54-6.24	4.32	6.19	-1.64	3.84	5.84	-12.44
Butyrylcarnitine (C4)	0.93	0.43-1.43	0.98	6.56	5.05	0.82	7.35	-11.63
Isovalerylcarnitine (C5)	0.54	0.27-0.80	0.49	6.15	-8.62	0.47	5.91	-12.89
Glutarylcarnitine (C5DC)	0.53	0.13-0.93	0.66	10.55	24.05	0.59	7.59	11.78
Hexanoylcarnitine (C6)	0.46	0.27-0.65	0.47	7.16	1.89	0.42	5.58	-8.18
Octanoylcarnitine (C8)	0.55	0.29-0.81	0.53	5.99	-4.47	0.48	5.61	-13.13
Decanoylcarnitine (C10)	0.49	0.29-0.68	0.47	6.39	-3.33	0.44	6.53	-9.30
Dodecanoylcarnitine (C12)	0.42	0.18-0.66	0.41	6.18	-1.62	0.39	6.31	-8.18
Tetradecanoylcarnitine (C14)	0.46	0.23-0.68	0.45	6.19	-2.94	0.41	6.13	-11.10
Hexadecanoylcarnitine (C16)	4.34	2.63-6.05	4.25	5.60	-2.07	3.81	6.25	-12.30
Octadecanoylcarnitine (C18)	2.51	1.40-3.62	2.37	5.50	-5.76	2.72	7.08	8.35

Table 7. Comparison of PHUNSA and MassChrom kits for analyzing control sample II (n=40).

Analyte	Target [µmol/L]	Range [µmol/L]	PH NBS			MassChrom		
			Mean [µmol/L]	%CV	Deviation%	Mean [µmol/L]	%CV	Deviation%
Amino Acids								
Alanine	813	357-1269	655.32	5.43	-19.39	660.86	6.20	-18.71
Arginine	196	100-291	180.98	5.54	-7.66	174.68	5.40	-10.88
Aspartic Acid	416	275-557	422.59	6.34	1.58	379.41	8.51	-8.80
Citrulline	238	175-301	254.52	6.31	6.94	216.20	6.14	-9.16
Glutamic Acid	921	634-1208	918.97	4.79	-0.22	872.96	7.00	-5.22
Glycine	678	476-879	694.00	5.58	2.36	608.86	5.94	-10.20
Leucine	598	397-798	626.71	5.11	4.80	541.91	6.00	-9.38
Methionine	243	97-389	237.42	6.35	-2.30	214.39	5.66	-11.77
Ornithine	793	498-1088	726.36	6.69	-8.40	699.58	7.94	-11.78
Phenylalanine	549	338-760	517.22	5.99	-5.79	489.75	6.35	-10.79
Proline	821	503-1138	823.95	5.98	0.36	729.15	6.68	-11.19
Tyrosine	508	348-668	474.93	4.26	-6.51	469.15	5.37	-7.65
Valine	550	360-740	543.99	7.66	-1.09	453.49	7.86	-17.55
Acylcarnitines and Free Carnitine								
Carnitine (C0)	101	62.6-139	97.83	7.78	-3.14	80.41	7.06	-20.39
Acetylcarnitine (C2)	53.6	33.2-74	53.88	4.64	0.53	49.41	5.87	-7.82

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Propionylcarnitine (C3)	12.1	7.83-16.3	12.12	4.69	0.19	10.57	6.19	-12.62
Butyrylcarnitine (C4)	4.27	2.49-6.05	4.49	4.75	5.23	3.68	6.18	-13.93
Isovalerylcarnitine (C5)	2.19	1.24-3.14	2.10	5.44	-4.20	1.97	6.67	-9.86
Glutarylcarnitine (C5DC)	2.08	0.95-3.20	1.70	8.01	-18.25	2.03	6.91	-2.23
Hexanoylcarnitine (C6)	1.98	1.26-2.70	2.13	4.27	7.79	1.85	5.76	-6.73
Octanoylcarnitine (C8)	2.11	1.29-2.92	2.18	4.38	3.21	1.89	5.58	-10.21
Decanoylcarnitine (C10)	1.95	1.07-2.83	2.03	4.26	3.89	1.80	5.44	-7.70
Dodecanoylcarnitine (C12)	1.93	1.26-2.59	1.95	4.26	1.23	1.75	5.11	-9.14
Tetradecanoylcarnitine (C14)	1.95	1.16-2.74	1.96	4.37	0.51	1.71	5.88	-12.31
Hexadecanoylcarnitine (C16)	11.3	6.91-15.6	11.66	4.81	3.23	9.95	6.33	-11.90
Octadecanoylcarnitine (C18)	8.49	4.59-12.4	8.04	5.59	-5.30	8.90	6.87	4.86

3.4. Analysis of Control Sample Deviations Using PH NBS and MassChrom Kits

Precision of the PHUNSA kit versus the MassChrom kit was evaluated by analyzing variations from desired concentrations for a wide range of analytes. With the ChromeSystems kit values as a reference, the percentage deviations of mean values were compared (see Table 8). Most analytes displayed deviations within the acceptable range, reinforcing the assays' capability to deliver precise and consistent results.

Table 8. Comparative deviation analysis of PHUNSA and MassChrom kits (n=40).

Analyte	Level I			Level II		
	MassChrom	PH NBS	%Deviation	MassChrom	PH NBS	%Deviation
Amino Acids						
Alanine	609.804	580.558	-4.796	660.857	655.324	-0.837
Arginine	58.694	59.410	1.220	174.684	180.980	3.604
Aspartic acid	197.836	206.891	4.577	379.413	422.585	11.379
Citrulline	65.331	75.484	15.542	216.204	254.518	17.721
Glutamic acid	828.699	836.826	0.981	872.958	918.972	5.271
Glycine	454.322	497.350	9.471	608.857	693.996	13.983
Leucine	396.118	441.772	11.525	541.907	626.707	15.648
Methionine	88.850	93.516	5.251	214.392	237.415	10.739
Ornithine	514.958	531.038	3.123	699.585	726.364	3.828
Phenylalanine	238.650	241.500	1.194	489.750	517.221	5.609
Proline	410.694	452.146	10.093	729.149	823.946	13.001
Tyrosine	215.284	208.184	-3.298	469.148	474.932	1.233
Valine	309.810	356.704	15.136	453.495	543.986	19.954
Acylcarnitines and Free Carnitine						
Carnitine (C0)	42.582	49.610	16.505	80.407	97.826	21.663
Acetylcarnitine (C2)	18.581	19.820	6.669	49.406	53.883	9.062
Propionylcarnitine (C3)	3.844	4.318	12.333	10.573	12.123	14.655
Butyrylcarnitine (C4)	0.822	0.977	18.865	3.675	4.493	22.256
Isovalerylcarnitine (C5)	0.470	0.493	4.902	1.974	2.098	6.284
Glutarylcarnitine (C5DC)	0.592	0.657	10.981	2.034	1.700	-16.377
Hexanoylcarnitine (C6)	0.422	0.469	10.972	1.847	2.134	15.566
Octanoylcarnitine (C8)	0.478	0.525	9.978	1.895	2.178	14.951
Decanoylcarnitine (C10)	0.444	0.474	6.578	1.800	2.026	12.560
Dodecanoylcarnitine (C12)	0.386	0.413	7.146	1.754	1.954	11.423
Tetradecanoylcarnitine (C14)	0.409	0.446	9.168	1.710	1.960	14.619
Hexadecanoylcarnitine (C16)	3.806	4.250	11.665	9.955	11.664	17.173
Octadecanoylcarnitine (C18)	2.720	2.365	-13.022	8.903	8.040	-9.693

3.5. Comparative Analysis Using Real Samples with Non-Derivatized Preparation Method

Forty real specimens from Iran newborns were evaluated using both the PHUNSA kit and the MassChrom kit. Analyses were performed using the SHIMADZU 8045 LC-MS/MS instrument. The percentage deviation was computed for each analyte, with the Chrome Systems kit serving as the reference standard. A wide range of analytes was analyzed including both normal specimens and specimens from newborns suspected of having a metabolic disorder and the data are displayed in Table 9. As shown in the percent deviation column, all values were within the range of +30% to -30%, indicating statiscally significant results.

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Table 9. Kit comparison using 40 real samples for PHUNSA and MassChrom kits (n=40).

Analyte	MassChrom Kit	PHUNSA Kit	%Deviation	Analyte	MassChrom Kit	PHUNSA Kit	%Deviation
Amino Acids				**Acylcarnitines and Free Carnitine**			
Alanine	141.57	133.93	-5.39	Carnitine (C0)	20.86	17.79	-14.71
Arginine	22.04	23.66	7.39	Acetylcarnitine (C2)	11.35	9.76	-14.01
Aspartic acid	118.30	107.09	-9.48	Propionylcarnitine (C3)	1.14	1.02	-11.03
Citrulline	14.80	16.38	10.67	Butyrylcarnitine (C4)	0.18	0.15	-16.01
Glutamic acid	172.03	188.49	9.57	Isovalerylcarnitine (C5)	0.12	0.13	5.47
Glycine	114.66	101.45	-11.52	Glutarylcarnitine (C5DC)	0.19	0.24	25.88
Leucine	125.16	119.15	-4.80	Hexanoylcarnitine (C6)	0.06	0.06	-4.89
Methionine	13.10	16.52	26.12	Octanoylcarnitine (C8)	0.07	0.06	-16.94
Ornithine	65.48	70.26	7.30	Decanoylcarnitine (C10)	0.09	0.07	-19.47
Phenylalanine	30.38	26.21	-13.74	Dodecanoylcarnitine (C12)	0.05	0.04	-9.18
Proline	116.05	103.45	-10.85	Tetradecanoylcarnitine (C14)	0.08	0.06	-20.33
Tyrosine	46.64	40.18	-13.84	Hexadecanoylcarnitine (C16)	0.73	0.72	-1.91
Valine	66.86	56.36	-15.71	Octadecanoylcarnitine (C18)	0.44	0.44	0.66

3.6. Comparatison of the Identification of Clinical IMDs

Immediately following validation of the PHUNSA kit, a comparison study was carried out on 9 clinical specimens from patients suspected to have a metabolic condition. A number of disorders were identified, including TYR (Tyrosinemia), MET (Hypermethioninemia), PKU (Phenylketonuria), MSUD (Maple syrup urine disease), NKH (Nonketotic hyperglycinemia), CPT1A (Carnitine palmitoyltransferase 1 deficiency), PA (Propionic acidemia), and MCAD (Medium-chain acyl-CoA dehydrogenase deficiency). Both kits performed equally at detecting disease-specific markers in the true clinical specimens. The analytical results from both kits are shown in Table 10.

Table 10. Comparative analysis of the PHUNSA and MassChrom kits across selected metabolic conditions.

Patient ID	Metabolite	Expected Clinical Condition	Reference Interval	Pathologic Borders	Results from MassChrom	Results from PHUNSA	Comments
2923	Tyrosine	TYR	< 292.74	> 336.58	467.00	535.63	Out of range
4428	Tyrosine	TYR	< 292.74	> 336.58	382.32	423.91	Out of range
4063	Methionine	MET	6.97-24.8	> 28.5, <6.34	193.10	230.33	Out of range
4060	C3	PA	0.37-4.30	> 5.0, <0.31	10.41	15.31	Out of range
4047	Multiple (C0, C16, C18, C18:1)	MCAD	Varies by component	Varies by component	Multiple values	Multiple values	Check individually
2691	Phe	PKU	< 68	> 109	480.76	694.23	Out of range
3700	Multiple (C6, C8, C16, C18, C10:1, C8/C2)	CPT1A	Varies by component	Varies by component	Multiple values	Multiple values	Check individually
606	Multiple (Leu, Val)	MSUD	Varies by component	Varies by component	Multiple values	Multiple values	Check individually
831	Gly	NKH	< 308.46	> 336.58	470.49	520.01	Out of range

3.7. Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

To assess the PHUNSA kit's sensitivity, the LOD and LLOQ were determined for each sample. The LOD is the smallest concentration that the method can identify with a certain level of trust and the LLOQ is the lowest concentration that can be quantified with a specific level of precision and accuracy (see Table 11).

Table 11. LOD and LLOQ results for the PHUNSA kit (µmol/L).

Analyte	LOD	LLOQ	Analyte	LOD	LLOQ
Amino Acids			**Acylcarnitines and Free Carnitine**		
Alanine	3.54	10.61	Carnitine (C0)	0.41	1.22
Arginine	1.11	3.33	Acetylcarnitine (C2)	0.29	0.88
Aspartic Acid	2.14	6.42	Propionylcarnitine (C3)	0.03	0.09
Citrulline	2.39	7.17	Butyrylcarnitine (C4)	0.04	0.12

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Glutamic Acid	2.97	8.91	Isovalerylcarnitine (C5)	0.01	0.03
Glycine	6.91	20.72	Glutarylcarnitine (C5DC)	0.02	0.06
Methionine	0.77	2.30	Hexanoylcarnitine (C6)	0.01	0.02
Leucine	1.32	3.97	Octanoylcarnitine (C8)	0.00	0.01
Ornithine	4.16	12.48	Decanoylcarnitine (C10)	0.01	0.03
Phenylalanine	1.17	3.51	Dodecanoylcarnitine (C12)	0.02	0.07
Proline	2.69	8.06	Tetradecanoylcarnitine (C14)	0.01	0.02
Tyrosine	1.12	3.35	Hexadecanoylcarnitine (C16)	0.03	0.08
Valine	2.23	6.69	Octadecanoylcarnitine (C18)	0.01	0.03

3.8. Blank Test Analysis

Blank filter papers were prepared like patient samples, undergoing 12 rounds of processing. Blank filter paper samples must have measurements below the LLOQ to ensure assay accuracy and reliability. The blank samples were analyzed 12 times using the PHUNSA kit and the AB SCIEX 3200. Analytical results using blank filter paper are shown in Table 12. Mean values of blank filter paper collection cards were consistently below the LLOQ.

Table 12. Measured values of blank filter paper samples [µmol/L].

Amino Acids			Acylcarnitines and Free Carnitine		
Analyte	**Mean**	**LLOQ**	**Analyte**	**Mean**	**LLOQ**
Alanine	8.73	10.61	Carnitine (C0)	0.72	1.22
Aspartic acid	5.39	6.42	Acetylcarnitine (C2)	0.17	0.88
Arginine	1.73	3.33	Propionylcarnitine (C3)	0.03	0.09
Citrulline	1.90	7.17	Butyrylcarnitine (C4)	0.02	0.12
Glutamic acid	3.56	8.91	Isovalerylcarnitine (C5)	0.01	0.03
Glycine	12.31	20.72	Glutarylcarnitine (C5DC)	0.02	0.06
Leucine	2.95	3.97	Hexanoylcarnitine (C6)	0.01	0.02
Methionine	0.52	2.30	Octanoylcarnitine (C8)	0.00	0.01
Ornithine	8.91	12.48	Decanoylcarnitine (C10)	0.01	0.03
Phenylalanine	2.13	3.51	Dodecanoylcarnitine (C12)	0.01	0.07
Proline	4.81	8.06	Tetradecanoylcarnitine (C14)	0.01	0.02
Tyrosine	3.48	3.35	Hexadecanoylcarnitine (C16)	0.04	0.08
Valine	3.38	6.69	Octadecanoylcarnitine (C18)	0.01	0.03

3.9. Carry-Over

The influence of specimens with high analyte concentrations on the analysis of a following specimen (carry over) was investigated by testing the 'memory' effect. Carry-over is mostly caused by the autosampler injection port contamination or contamination of the tubing that leads to the electrospray ionization source. To measure the carryover effect, samples of blank filter paper were analyzed immediately following a containing a very high concentration of the analyte, e.g. a high-concentration control. Analysis was completed five times with the PHUNSA kit and the AB SCIEX 3200 (Table 13).

Table 13. Measured Values for Memory Effect [µmol/L].

Amino Acids			Acylcarnitines and free Carnitine		
Analyte	**Mean**	**LLOQ**	**Analyte**	**Mean**	**LLOQ**
Alanine	8.78	10.61	Carnitine (C0)	0.63	1.22
Arginine	1.65	3.33	Acetylcarnitine (C2)	0.15	0.88
Aspartic acid	5.25	6.42	Propionylcarnitine (C3)	0.03	0.09
Citrulline	1.25	7.17	Butyrylcarnitine (C4)	0.01	0.12
Glutamic acid	3.33	8.91	Isovalerylcarnitine (C5)	0.01	0.03
Glycine	11.77	20.72	Glutarylcarnitine (C5DC)	0.02	0.06
Leucine	2.75	3.97	Hexanoylcarnitine (C6)	0.01	0.02
Methionine	0.48	2.30	Octanoylcarnitine (C8)	0.00	0.01
Ornithine	9.43	12.48	Decanoylcarnitine (C10)	0.01	0.03
Phenylalanine	1.92	3.51	Dodecanoylcarnitine (C12)	0.01	0.07
Proline	4.76	8.06	Tetradecanoylcarnitine (C14)	0.01	0.02
Tyrosine	3.23	3.35	Hexadecanoylcarnitine (C16)	0.04	0.08

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Valine	3.18	6.69	Octadecanoylcarnitine (C18)	0.01	0.03

3.10. Linearity and Method Robustness

The PHUNSA kit was evaluated for linearity and robustness. Typically, the correlation values exceed 0.99, indicating a strong ability to accurately measure changing analyte concentrations. The repeatability results consistently demonstrated a coefficient of variation (%CV) of less than 10% for all investigated analytes, indicating technical reliability (Table 14).

Table 14. Linearity and performance data for the PHUNSA kit.

Analyte	Repeatability (CV%)	Non-linearity (%)	Linear Range (µmol/L)	Analyte	Repeatability (CV%)	Non-linearity (%)	Linear Range (µmol/L)
Amino Acids				**Acylcarnitines and Free Carnitine**			
Alanine	7.02	-11.75-2.62	50.55-3235.5	Carnitine (C0)	8.36	-9.15-0.77	2.837-337.5
Arginine	8.91	-3.12-12.19	15.61-249.75	Acetylcarnitine (C2)	9.93	-0.46-11.52	0.513-131.4
Aspartic acid	7.74	-3.17-14.85	30.1-481.5	Propionylcarnitine (C3)	6.92	-12.94-0.95	0.179-22.95
Citrulline	8.02	-7.05-3.36	16.8-537.75	Butyrylcarnitine (C4)	7.77	-1.10-7.64	0.273-17.48
Glutamic acid	10.42	-9.65-5.45	64.125-2052	Isovalerylcarnitine (C5)	9.11	-11.32-0.57	0.019-4.973
Glycine	13.92	-1.72-7.91	24.27-3107.25	Glutarylcarnitine (C5DC)	13.21	-4.01-12.56	0.119-0.956
Leucine	7.00	-8.55-1.61	23.24-1487.25	Hexanoylcarnitine (C6)	9.01	-11.95-6.47	0.072-2.295
Methionine	10.77	-5.76-2.46	27.7-886.5	Octanoylcarnitine (C8)	7.80	-6.76-1.17	0.065-4.1625
Ornithine	10.15	-10.66-6.89	31.29-1001.25	Decanoylcarnitine (C10)	8.51	-1.33-8.43	0.04-2.565
Phenylalanine	9.13	-7.5-3.49	52.38-1676.25	Dodecanoylcarnitine (C12)	7.64	-5.78-2.59	0.426-13.635
Proline	6.62	-9.03-4.78	37.76-1208.25	Tetradecanoylcarnitine (C14)	9.79	-0.38-10.71	0.028-7.155
Tyrosine	6.99	-5.95-1.92	39.1-1251	Hexadecanoylcarnitine (C16)	7.13	-13.41-2.20	0.411-26.325
Valine	8.57	-2.76-1.26	38.67-1237.5	Octadecanoylcarnitine (C18)	7.65	-13.76-2.23	0.127-8.145

3.11. Stability

An evaluation of the specimen and stability was carried out in a variety of conditions in order to ensure consistent behavior throughout the analytical process. Specimens that were stored at a -18°C remained unaltered for a period of 12 months. After being subjected to simulated transit conditions to evaluate their transportation stability, the specimens were found not to have undergone any significant degradation. During the course of their use, stability testing revealed that specimens that were allowed to remain at room temperature for a maximum of twenty-four hours maintained their reliability and uniformity. By evaluating specimen stability under a variety of conditions, we were able to ensure reliable and consistent performance across the many stages of analysis, including handling. Researchers assessed the transport stability of the materials by subjecting them to simulated transport conditions and observed no noticeable degradation. The stability data, including the results from accelerated stability testing and other related evaluations, are provided in the Supplementary Materials with the article (Tables S1-S4).

4. Discussion

This study introduces and validates the PHUNSA MS/MS kit, which offers a significant improvement compared to conventional derivatized procedures. The PHUNSA kit simplifies NBS laboratory workflows by eliminating the time-consuming derivatization step thus improvin operational efficiency and lowering costs. This is particularly appealing and beneficial in high-throughput screening environments where speed and accuracy are crucial.

While traditional MS/MS procedures using derivatization methods have been in use for a long period of time, they continue to be laborious and result in chemical alteration of the analytes as part of specimen preparation. This adds an uncertainty factor to the process and may decrease analyte recovery and accuracy. Unnecessary speed and accuracy issues are avoided an underivatized protocol such as that present with PHUNSA kit, which is used with ESI-MS/MS to directly measure acylcarnitines and amino acids in DBS samples [20,21]. This avoidance of derivatization streamlines the sample preparation technique, minimizes errors, and expedites the time to obtain a diagnosis, all goals of NBS.

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The PHUNSA kit has successfully undergone a thorough validation usully internationally recognized protocols and satisfies the rigorous criteria for accuracy, precision, linearity, and stability. Low CVs during intra-assay, inter-assay, and inter-laboratory studies have demonstrated that the assay consistently exhibits high recovery rates with little bias for amino acids and acylcarnitines. Assay repeatability and accuracy data confirm its suitability for routine clinical usage, especially in NBS programs where precise and rapid diagnosis are crucial [10].

Focusing on its relevance in Iran, we investigated PHUNSA in a real-life, large-scale screening environment in comparison to a comparable, non-derivatized, commercially available MS/MS assay. As a resulty of unique genetic and socioeconomic characteristics, the Iranian population is marked by high consanguinity and increased IMDs, which presents major opportunities for NBS for IMDs. The PHUNSA kit was designed to meet the analytical challenges of high throughput, accurate NBS. Our study findings provide robust evidence that the PHUNSA kit is both accurate and precise. However, our study goes beyond simply confirming the technical performance of the kit and provides evidence of its importance, ability to grow, and cost-effectiveness in a real-world setting. The PHUNSA kit can be used in other NBS programs constrained by limited resources. The the method is easy to understand and is technically less complex than traditional derivatized methods making it a more cost effective choice for large-scale screening. This is especially important in poor countries, where money for health care is often tight and diagnostic tools that are both cheap and accurate are needed.

Earlier diagnosis using faster, less expensdive screening techniques lowers the oveall treatment costs and reduces healthcare financial concerns. Early disease identification and treatment usually leads to notable improvements in medical outcomes, which improves family and societal well-being [22]. Successful implementation of the PHUNSA kit in Iran may have implications for public health globally, if it is utilized in other similar settings [23,24].

The successful implementation of the assay depends on accurate calibration and maintenance of the MS/MS equipment, and skilled staff to conduct the analyses. Future investigations should focus on improving the efficiency of the assay for use in different clinical situations, as well as examining its ability to detect a wider range of metabolic diseases [25]. Expansion of the assay's biomarker coverage coupled withs increased sensitivity and specificity could improve its usefulness in the global setting.Further research should also explore the long-term impacts on newborns identified by NBS with the PHUNSA kit including provision of medical care and overall health outcomes.

5. Conclusions

Successful production and validation of the PHUNSA kit marks a major development in NBS technologies in Iran. Its simplified technique, speed, precision, and economy make it a viable alternative to derivatized kits currently in use for high-throughput NBS. By addressing the problems characteristic of conventional derivatized MS/MS NBS approaches, the PHUNSA kit produces a more streamlined, dependable, and effective method for IMD detection in newborns. It’s use in NBS programs has the potential to significantly improve health outcomes by providing faster and precise diagnoses of IMDs. Furthermore, its scalability and potential global applicability make it a valuable tool for enhancing public health outcomee and cutting healthcare costs in both developed and developing nations. Its effective implementation in Iran can serve as a model for other NBS programs.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Table S1: % Deviation from the target for Level I control sample of amino acids for the accelerated stability; Table S2: % Deviation from the target for Level I control sample of acylcarnitines for the accelerated stability; Table S3: % Deviation from the target for Level II control sample of amino acids for the accelerated stability; Table S4: % Deviation from the target for Level II control sample of acylcarnitines for the accelerated stability.

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