Liquid–Liquid Extraction and Solid Phase Extraction for Urinary Organic Acids: A Comparative Study from a Resource Constraint Setting

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Abstract Pre analytical process of extraction for accurate detection of organic acids is a crucial step in diagnosis of organic acidemias by GCMS analysis. This process is accomplished either by solid phase extraction (SPE) or by liquid–liquid extraction (LLE). Both extraction procedures are used in different metabolic laboratories all over the world. In this study we compared these two extraction procedures in respect of precision, accuracy, percent recovery of metabolites, number of metabolites isolated, time and cost in a resource constraint setup. We observed that the mean recovery from SPE was 84.1 % and by LLE it was 77.4 % (p value <0.05). Moreover, the average number of metabolites isolated by SPE and LLE was 161.8 ± 18.6 and 140.1 ± 20.4 respectively. The processing cost of LLE was economical. In a cost constraint setting using LLE may be the practical option if used for organic acid analysis.

Keywords Solid phase extraction · Liquid–liquid extraction · Organic acidemia · Urinary organic acids · Gas chromatography mass spectrometry

Introduction

Urine contains a number of substances representing intermediates and end products of metabolic pathways of both endogenous and exogenous compounds. Though considered a waste product of the body, it has great value as a diagnostic biofluid [1–3]. Organic acidemias are a heterogeneous group of Inborn errors of metabolism in which a specific enzyme is defective or absent in metabolic pathway [4–8]. In most organic acidemias certain metabolites are accumulated in the body fluid like urine, blood and CSF. These accumulated metabolites are toxic to various body organs.

The qualitative and quantitative analyses of urinary organic acids are used as diagnostic tools for organic acidemias. The diagnosis is based on the detection of excretion of excess amount of urinary organic acid which are normally found to a certain extent in urine, or the appearance of organic acids that are not normally present in the urine. The composition of urine sample may vary considerably and require sample preparation procedure prior to GCMS analyses. This is accomplished either by LLE or SPE [9–14]. In this article we discuss our experiences with sample preparation by both LLE and SPE and discuss the silent advantages and disadvantages of the same in a resource constrain settings.

Materials and Methods

The organic solvents ethylacetate, methanol, *n*-butanol, Formic acid and SPE columns (ENVI-Carb/NH₂) were purchased from Sigma Aldrich and all acylglycines were purchased from Toronto Research chemicals, Canada. Other reagents including Sulphuric acid, Hydrochloric

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acid, Acetic acid, Barium hydroxide, Sodium sulphate, Sodium chloride and Hydroxylamine hydrochloride were purchased from Merck.

Collection and Storage of Study Sample

Urine specimens were obtained from 500 healthy pediatric volunteers (350 male, 2–12 years +150 female, 2–4 years), with no dietary restriction and 300 suspected cases of IEM were also included. Confirmed cases of IEM were used as positive control to compare the recoveries of organic acids and for the evaluation of extraction procedures. All samples were stored at -20°C until analysis.

Sample Preparation

Liquid–Liquid Extraction

The organic acids were extracted by method described by Greter and Jacobson [14] with slight modification. Six ml of ethyl acetate was added to the urine sample equivalent to 1 mg of creatinine in a 10 ml test tube and 40 μl of internal standard (100 μM /l of tropic acid in methanol) was added. This mixture was supplemented with 1 gm NaCl, 500 ml of a 50 g/l aqueous hydroxylamine hydrochloride solution. The pH of the mixture was adjusted to 14 with 7.5 mol/l NaOH and incubated for 30 min at 60°C . After cooling, the mixture was acidified with 6 mol/l HCl and extracted thrice with ethyl acetate.

Solid Phase Extraction

SPE was carried out using SPE columns. The columns were activated by washing them twice with 2 ml methanol, 2 ml distilled water, and 2 ml of 1 mol/l acetic acid. Excess acetic acid was washed away by rinsing the column with distilled water, at the rate of 4 ml/min until the pH was neutral. Urine samples were prepared by adding an equal volume of 0.01 mol/l BaOH to an amount of urine containing 1 mg of creatinine and 100 μM of internal standard. Mixing and centrifugation was done; half of the supernatant was taken and diluted with three volumes of distilled water and pH adjusted to 8–8.5. These were applied onto the activated column at a reduced flow rate (2 ml/min) and the column was washed four times with 2 ml distilled water to eliminate neutral and basic compounds. The excess water was removed by centrifugation and dehydrated by rinsing with 1 ml of methanol. Column was eluted consecutively with 1 ml of *n*-butanol/formic acid/concentrated sulphuric acid (80/20/0.5 by vol), 1 ml of ethyl acetate/formic acid/concentrated sulphuric acid (80/20/0.5 by vol), and 1 ml of pure methanol.

Derivatization

The elutes were collected in glass tubes and evaporated to dryness under gentle stream of nitrogen at 50°C . The dried residue was converted into trimethylsilyl derivatives by adding 100 μl of BSTFA + TMCS (99:1) and pyridine in a ratio of 1:1. The capped vials containing organic acids and derivatizing reagent were incubated at 80°C for 30 min. One micro liter of the derivatized organic acid extract was injected into the GCMS manually.

GCMS Analysis

Analysis were carried out using 7890A GC system interfaced to a 5975C inert XL/CI MSD with Triple Axis Detector (Agilent technologies). The injector is operated in a split mode with split ratio of 1:10. The chromatographic separation were achieved on HP-5 column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness). Data acquisition was performed using ChemStation software (TMAgilent).

One micro liter sample was injected in split mode with helium as the carrier gas. The oven temperature was held at 50°C for 3 min, increased to 140°C at $10^{\circ}\text{C}/\text{min}$, and further increased to 280°C at a rate of $20^{\circ}\text{C}/\text{min}$, held for 3 min. The total run time was 23 min. The injector and transfer line temperature was 250°C respectively. The ion source and quadrupole temperature were set at 200 and 150°C respectively.

Standard Curve for Method Comparison

A derivatized standard mixture of all organic acids was run prior to patient samples to determine the absolute and relative retention times. A standard curve was prepared from a stock solution of 20 mM concentrations of each compound having tropic acid (100 $\mu\text{mol}/\text{l}$) as internal standard. From this, a master mix of 200 μM was prepared containing all organic acids. Dilutions of different concentrations (25, 50, 75 and 100 $\mu\text{mol}/\text{l}$) were prepared from this master mix and extracted from an aqueous calibration mixture through the procedure described for urine samples.

One micro liter sample was then injected into the GCMS and the product ion spectra were monitored. Total ion chromatograms (m/z 30–550 amu) was acquired and processed with MSD ChemStation software. The identities of the peaks with reference spectra were confirmed by computerized comparison of the mass spectra underlying the peaks with the reference spectra of NIST mass spectra library. For data analysis one product ion plot was extracted for each of the marker compounds and the presence of a peak with the expected retention time was determined. Quantification of the organic acids was based

on the specific ion masses. The ratio of response factor of the target ion and qualifier ion for a compound was used to quantify analytes. The urinary concentrations of organic acids in relation to creatinine (mmol/mol creatinine) were calculated from peak area ratios of the unknown vs the internal standard.

Results

There is limited literature available that has compared both extraction procedures [10, 11, 15]. Within day precision and accuracy was obtained from six replicate extraction of same standard mixture by both extraction procedures in a day. For day to day precision and accuracy, same standard mixture was extracted by both procedures over a period of 3 weeks. Within day and total imprecision data (Table 1) were quite acceptable for both liquid–liquid extraction and solid-phase extraction. Most of the CVs are within limit i.e., <10 %. Only tiglylglycine, 3-methylcrotonylglycine, propionylglycine and succinylacetone showed higher variability.

Recovery data of organic acids from urine calibrator is presented in Table 2. Recovery of organic acids extracted by LLE ranged from 44.82 to 105.75 % and by SPE ranged from 53.7 to 111.0 %. However, for most organic acids, the yield is acceptable with relative recoveries of >60 %. Lui et al. and Duez et al. found similar result in their studies [13, 16]. The total number of metabolites extracted by each method was determined using library search report of the NIST mass spectrum library. Each peak was labeled and identified so as not to miss a small peak.

The number of metabolites isolated by LLE and SPE is illustrated in Fig. 1. The number of metabolites isolated by SPE is comparably higher than the LLE i.e. the mean number of metabolites isolated by LLE was 140.1 ± 20.4 with a CV of 13 % and the mean number of metabolites isolated by SPE was 161.8 ± 18.1 with a CV of 11.4 % (Table 3).

Even though LLE is the simplest method to perform, SPE takes shorter time. LLE of 12 urine samples takes a mean time of about 4 h 20 min in comparison to SPE which take a mean time of about 2½ h in our set up. In accordance to a previous study done by Bart J. Verhaeghe et al., SPE was less time consuming. S.Lakshmana Prabu and T.N.K. Suriyaprakash in their review mentioned that LLE is more time consuming however their study is on drug extraction [11, 17]. On cost effective front LLE was much more cost-effective than SPE. The processing cost of one urine sample by LLE was approximately 251 INR and by SPE it costs approximately 509.1 INR. Nikolaos Raikos et al., in their study mentioned that LLE is more cost effective [18].

In samples extracted with SPE we found the overlapping of peaks, this could be due to large number of possible compounds isolated by this method [16]. The metabolites of carbohydrates, inositol, myoinositol, sorbitol, glucuronides and sugar alcohols are easily isolated with a quite good response. This is of value when analyzing urine as a snapshot. For example Fumaric acid has two isomeric forms, by LLE only one form i.e. fumaric acid (E) was extracted but by SPE both isomers of fumaric acid were isolated. Similarly Polyhydroxy compounds such as glycerol and glyceric acid were poorly extracted by LLE [11]. Phosphate was present in high quantities in LLE that masks the nearby peaks.

Acylglycines are detected in significant amount only in urine from patients with inborn errors of metabolism and their identification as well as quantification is essential for the diagnosis of these diseases. Urine samples from confirmed cases of IEM were also extracted simultaneously by two procedures and analyzed the difference between their extraction recoveries of different classes of compounds. Table 4 shows the mean excretion of some clinically important metabolites obtained in pathological condition extracted by both methods.

Discussion

Eight hundred urine samples (500 controls and 300 suspected IEM) were extracted simultaneously by both extraction procedures and analysed by GCMS.

We obtained within day imprecision ranging from 2.9 to 14.5 % for LLE and 1.12 to 14.2 % for SPE respectively, with a total imprecision ranging from 2.5 to 15.7 % for LLE and 2.2 to 13.3 % for SPE. Within day and total imprecision data are quite acceptable for both LLE and SPE. Similar precisions are obtained from analysis of normal urine sample as well as patients specimen (data not given). Comparison of total imprecision obtained from SPE and LLE shows statistically significant difference with p value <0.05. But it was observed that SPE has higher variation in comparison to LLE (Table 1). This variation in recovery could be occurred due to the slight variation in pH of the SPE cartridge. Most of the organic acids examined showed the linear line of regression with a coefficients of correlation (r^2) >0.99. Adipic acid and Suberic acid show curvilinear relationships between signal response and concentrations. These acids at higher concentration present a proportionally decreased response. Apart from these, hexonylglycine presented decreased response for whole range of tested concentrations. Methylmalonic acid, 3-methylcrotonylglycine, tiglylglycine and isovaleroylglycine shows a linear relationship for whole range of

Table 1 Inter and intra day precision and accuracy data of liquid–liquid extraction and solid phase extraction procedure

Compound	Added amount $\mu\text{mol/l}$	Liquid–liquid extraction				Solid phase extraction			
		Within day		Day-to-day		Within day		Day- to- day	
		Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)
Propionyl glycine	Blank	nd	0	nd	0	nd	0	nd	0
	20	10.4 $\pm$ 0.92	8.8	10.6 $\pm$ 0.7	7.0	11.7 $\pm$ 1.6	14.2	12.2 $\pm$ 1.4	11.7
	50	25.4 $\pm$ 2.9	11.7	26.0 $\pm$ 2.3	8.8	30.5 $\pm$ 3.5	11.7	30.6 $\pm$ 2.7	9.0
	100	52.0 $\pm$ 4.6	8.8	52.4 $\pm$ 5.1	9.8	61.2 $\pm$ 5.5	9.0	61.2 $\pm$ 7.0	11.5
Butryl glycine	Blank	nd	0	nd	0	nd	0	nd	0
	20	16.2 $\pm$ 1.0	6.2	16.2 $\pm$ 1.0	6.2	16.2 $\pm$ 0.9	6.1	16.3 $\pm$ 1.2	7.6
	50	39.6 $\pm$ 3.4	8.7	40.6 $\pm$ 2.5	6.2	40.6 $\pm$ 3.0	7.5	41.0 $\pm$ 2.4	5.8
	100	81.2 $\pm$ 5.0	6.2	79.4 $\pm$ 6.8	8.5	82.1 $\pm$ 4.8	5.8	81.6 $\pm$ 5.8	7.2
Isovaleryl glycine	Blank	nd	0	nd	0	nd	0	nd	0
	20	12.7 $\pm$ 1.5	11.8	12.7 $\pm$ 1.5	11.8	12.9 $\pm$ 0.8	6.2	13.2 $\pm$ 0.9	7.1
	50	32.8 $\pm$ 5.2	10.8	31.9 $\pm$ 3.7	11.8	33 $\pm$ 2.4	7.4	33.1 $\pm$ 2.1	6.4
	100	63.8 $\pm$ 7.5	11.8	64.8 $\pm$ 6.3	9.7	66.2 $\pm$ 4.2	6.4	66.3 $\pm$ 5.1	7.8
3-Methyl crotonyl glycine	Blank	nd	0	nd	0	nd	0	nd	0
	20	15 $\pm$ 1.8	12.4	13.0 $\pm$ 2.0	15.7	15.1 $\pm$ 1.6	10.6	16.2 $\pm$ 1.9	11.8
	50	36.8 $\pm$ 5.5	10.1	37.5 $\pm$ 4.6	12.4	40.5 $\pm$ 4.7	11.7	40.6 $\pm$ 3.7	9.2
	100	75 $\pm$ 9.3	12.4	74.2 $\pm$ 9.1	12.2	81.2 $\pm$ 7.5	9.2	81.2 $\pm$ 9.2	11.4
Tiglyl glycine	Blank	nd	0	nd	0	nd	0	nd	0
	20	13.1 $\pm$ 1.9	14.5	9.2 $\pm$ 0.57	6.2	15.4 $\pm$ 1.3	8.9	16.3 $\pm$ 0.93	5.7
	50	33.1 $\pm$ 4.2	12.8	32.4 $\pm$ 4.7	14.6	40.7 $\pm$ 2.3	5.8	40.8 $\pm$ 2.2	5.6
	100	65.0 $\pm$ 10.2	15.7	64.4 $\pm$ 7.8	12.2	81.6 $\pm$ 4.5	5.6	81.2 $\pm$ 4.0	4.9
Hexanoyl glycine	Blank	nd	0	nd	0	nd	0	nd	0
	20	8.9 $\pm$ 0.80	8.9	14.5 $\pm$ 1.2	8.8	10.7 $\pm$ 0.9	8.9	11.1 $\pm$ 0.8	7.9
	50	22.8 $\pm$ 4.2	3.3	23.7 $\pm$ 3.0	12.9	27.5 $\pm$ 2.3	8.3	26.9 $\pm$ 3.0	11.1
	100	46.3 $\pm$ 2.8	6.2	45.9 $\pm$ 5.7	12.6	53.9 $\pm$ 6.0	11.1	55.1 $\pm$ 4.6	8.4
Methyl malonic acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	19.8 $\pm$ 1.7	9.0	16.6 $\pm$ 1.1	7.1	19.9 $\pm$ 0.5	2.7	19.1 $\pm$ 1.1	5.9
	50	47.7 $\pm$ 5.0	10.5	49.5 $\pm$ 4.4	9.0	47.8 $\pm$ 2.9	6.2	48.1 $\pm$ 2.6	5.4
	100	98.1 $\pm$ 8.5	8.7	98.7 $\pm$ 8.3	8.4	96.3 $\pm$ 5.2	5.4	96.2 $\pm$ 5.5	5.7
Fumaric acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	15.0 $\pm$ 0.94	6.2	19.3 $\pm$ 0.8	4.4	19.2 $\pm$ 3.5	10.5	18.8 $\pm$ 1.1	6.2
	50	36.7 $\pm$ 3.4	9.3	37.6 $\pm$ 2.3	6.2	47.3 $\pm$ 3.0	6.4	47.2 $\pm$ 3.1	6.7
	100	74.8 $\pm$ 5.0	6.7	75.7 $\pm$ 4.2	5.6	94.4 $\pm$ 6.3	6.7	94.5 $\pm$ 6.0	6.3
Succinyl acetone	Blank	nd	0	nd	0	nd	0	nd	0
	20	13.5 $\pm$ 1.4	10.6	20.1 $\pm$ 1.3	6.8	23.5 $\pm$ 4.8	10.6	24.4 $\pm$ 3.0	12.6
	50	34.6 $\pm$ 4.2	12.3	33.8 $\pm$ 3.5	10.6	55.5 $\pm$ 5.5	9.9	61.1 $\pm$ 8.1	13.3
	100	67.7 $\pm$ 7.1	10.6	67.6 $\pm$ 6.2	9.2	122.2 $\pm$ 16	13.3	123.5 $\pm$ 15	12.6
Glutaric acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	20.1 $\pm$ 1.3	6.8	16.5 $\pm$ 0.9	5.9	19.3 $\pm$ 1.2	6.2	19.4 $\pm$ 0.8	4.1
	50	48.6 $\pm$ 4.6	9.5	50.2 $\pm$ 3.4	6.8	27.7 $\pm$ 2.7	9.9	48.7 $\pm$ 2.3	4.7
	100	100 $\pm$ 6.9	6.8	100.1 $\pm$ 6	6.2	97.4 $\pm$ 4.6	4.7	98.3 $\pm$ 4.6	4.7
3Methyl glutaric acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	16.5 $\pm$ 0.98	5.9	16.6 $\pm$ 1.0	6.4	15 $\pm$ 1.9	13.0	15.9 $\pm$ 1.3	8.5
	50	40.3 $\pm$ 1.6	4.0	41.2 $\pm$ 2.4	5.9	40.3 $\pm$ 3.6	8.9	40.1 $\pm$ 3.9	9.8
	100	82.5 $\pm$ 4.9	5.9	82.9 $\pm$ 4.2	5.1	80.3 $\pm$ 7.8	9.8	81.1 $\pm$ 7.7	9.6

Table 1 continued

Compound	Added amount $\mu\text{mol/l}$	Liquid–liquid extraction				Solid phase extraction			
		Within day		Day-to-day		Within day		Day- to- day	
		Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)
Adipic acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	16.6 $\pm$ 1.0	6.4	16.6 $\pm$ 1.0	6.1	37.4 $\pm$ 7.1	9.0	25.7 $\pm$ 2.6	10.4
	50	40.9 $\pm$ 2.1	5.3	41.6 $\pm$ 2.6	6.4	47 $\pm$ 5.0	10.6	44 $\pm$ 5.8	13.1
	100	83.2 $\pm$ 5.3	6.4	83.1 $\pm$ 5.1	6.1	99 $\pm$ 13.0	13.1	98 $\pm$ 12.0	12.2
Sebacic acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	19.6 $\pm$ 0.72	3.6	19.4 $\pm$ 0.6	3.5	18.7 $\pm$ 3.1	7.0	19.0 $\pm$ 1.5	8.1
	50	47.1 $\pm$ 2.5	5.4	49.1 $\pm$ 1.8	3.6	47.9 $\pm$ 3.2	6.8	47.7 $\pm$ 3.1	6.6
	100	97.4 $\pm$ 3.4	3.5	97.8 $\pm$ 2.5	2.5	95.4 $\pm$ 6.3	6.6	93.7 $\pm$ 6.7	7.2
Suberic acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	21.1 $\pm$ 1.1	5.4	21.0 $\pm$ 1.1	5.4	42.4 $\pm$ 5.1	1.12	41.3 $\pm$ 2.6	6.3
	50	47.8 $\pm$ 2.8	5.8	49.1 $\pm$ 2.8	5.7	49.8 $\pm$ 6.6	12.6	47.9 $\pm$ 4.0	8.3
	100	95 $\pm$ 5.6	5.8	94.7 $\pm$ 5	5.2	98 $\pm$ 8	8.1	97.1 $\pm$ 10	10.2
2-Ketoiso caproic acid	Blank	nd	0	nd	0	nd	0	nd	0
	20	26.8 $\pm$ 0.78	2.9	26.7 $\pm$ 0.8	3.3	18.9 $\pm$ 1.8	9.9	19.3 $\pm$ 1.5	8.2
	50	64.7 $\pm$ 6.7	10.3	62.2 $\pm$ 3.6	5.8	48.2 $\pm$ 4.1	8.5	48.2 $\pm$ 3.9	8.0
	100	73.8 $\pm$ 4.4	5.9	72.6 $\pm$ 5	6.8	66.5 $\pm$ 7.9	11.8	64.9 $\pm$ 5.5	8.4

Table 2 Analytical recoveries of organic acids from spiked urine sample extracted by liquid–liquid extraction and solid phase extraction

Compound	Liquid–liquid extraction		Solid phase extraction	
	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
Propionylglycine	52.08	8.8	61.12	10.6
Butrylglycine	81.26	6.2	81.46	6.2
Isovaleryl glycine	63.86	11.8	65.06	6.2
3-Methyl crotonylglycine	75	12.4	75.96	10.6
Tiglylglycine	65.9	14.5	77.16	16.2
Hexanoylglycine	44.82	8.9	53.7	8.3
Methylmalonic acid	99.0	9.0	99.6	2.7
Fumaric acid	75.25	7.9	96.25	18.5
Succinylacetone	67.75	10.6	93.4	16.2
Glutaric acid	100.5	6.8	99.6	8.3
Adipic acid	83.25	6.4	101	18.4
Sebacic acid	98.25	3.6	93.4	16.6
Suberic acid	105.75	5.4	111.0	11.5
2-Ketoisocaproic acid	70.6	9.8	68.9	10.9
Mean	77.4	8.7	84.1	11.5

tested concentrations. Our findings regarding the imprecision were slightly different from the findings of previous study [16].

SPE seems to be better with a mean relative recovery of 84.1 % in comparison to LLE which has a mean relative recovery of 77.4 %. The mean CV for LLE was 8.7 % while for SPE it was 11.5 %. The difference in extraction

recoveries by two procedures was significant as the p value obtained was <0.05 . A few metabolites showed relative recovery of more than 100 %; this could be due to matrix effect, giving an enhancement in the signal for these compounds (Table 2). In some previous studies, the recovery of methylmalonic acid by LLE was reported between 20 and 55 % which was much less than what we

Fig. 1 Chromatogram of a normal urine sample extracted by (a) liquid–liquid extraction 1 Lactic acid, 2 Ethanedioic acid, 3 3Hydroxybutyric acid, 4 3Hydroxyisovaleric acid, 5 Methylmalonic acid, 6 2Methyl 3hydroxybutanoic acid, 7 Phosphate, 8 Succinic acid, 9 Fumaric acid, 10 Adipic acid, 11 4Hydroxy benzoic acid, 12 4Hydroxybenzene acetic acid, 13 Acotinic acid, 14 citric acid, 15 4Hydroxybenzene lactic acid and (b) solid phase extraction 1 Lactic

acid, 2 2Hydroxy acetic acid, 3 3Hydroxybutyric acid, 4 3Hydroxypropionic acid, 5 3Hydroxyisovaleric acid, 6 Methylmalonic acid, 7 Phosphate, 8 Succinic acid, 9 Fumaric acid, 10 Glyceric acid, 11 Methylmalic acid, 12 Threitol, 13 Adipic acid, 14 4Hydroxy benzoic acid, 15 4Hydroxybenzene acetic acid, 16 Acotinic acid, 17 Citric acid, 18 Galactonic acid, 19 Glunic acid, 20 Mannonic acid, 21 Galactitol, 22 Myoinositol

Table 3 Number of metabolites isolated, time consumed and total cost of both procedures

Method	Number of metabolites isolated (Mean $\pm$ SD) (CV)	Approx time utilized (min)	Total cost (INR)
Liquid–liquid extraction	140.1 $\pm$ 20.4 (13.4)	257	251
Solid phase extraction	161.8 $\pm$ 18.6 (11.4)	160	509.1

have observed in our study [16, 19]. In our study both extraction procedures showed a mean recovery of $>98\%$ for methylmalonic acid. The recovery of glutaric acid in our study agrees with the recovery observed by some other studies with slightly higher recovery of suberic acid [19,

20]. In accordance to a previous study, Adipic acid showed a recovery of 83.2 % by LLE, whereas by SPE a recovery of 101 % was obtained [21]. Another study showed that LLE was less selective towards methylmalonic acid due to the interference caused by an unidentified compound

Table 4 Mean excretion of marker compounds of clinical interest extracted simultaneously from liquid–liquid extraction and solid phase extraction in all IEM positive samples

Disorder	No. of patients (N = 102)	Marker compound	Mean excretion (mmol/mol of creatinine)	
			Liquid–liquid extraction	Solid phase extraction ^a
Methylmalonic acidemia	27	Methylmalonic acid	13,061.13	15,026.07
Glutaric aciduria	21	Glutaric acid	6622.74	6227.78
		Glutaconic acid	287.4	389.3
		3Hydroxyglutaric acid	213	267
Biotinidase deficiency	05	3Hydroxyisovaleric acid	564.2	432.2
		3Hydroxypropionic acid	132.2	99.78
		Isovaleryl glycine	31.1	42.1
		3Methylcrotonyl glycine	54.4	50.9
3Methylcrotonyl CoA deficiency	03	3Hydroxyisovaleric acid	234.1	335.4
		2-methyl-3-hydroxybutyric acid	112.5	212.1
β Ketothiolase deficiency	01	2-methylacetoacetic acid	23.1	34.5
		Tiglylglycine	31.7	40.8
		Fumaric acid	1126.8	1899.8
Fumarase deficiency	03	4Hydroxybutyric acid	324.2	278.2
Ketosis	07	3Hydroxybutyric acid	123.7	156.7
		3hydroxy2methylbutyric acid	98.9	121.1
		3Hydroxyisovaleric acid	255.1	205.1
		Acetoacetic acid	39.8	34.1
		2ketoisocaproic acid	129.9	120.0
Maple syrup urine disease (MSUD)	03	2Ketoisovaleric acid	325.8	301.2
		2Hydroxyisovaleric acid	299.8	278.9
		2Hydroxy3Methyl valeric acid	192.6	190.0
		Phenyl acetic acid	250.9	300.9
		Phenyl lactic acid	99.8	112.1
Phenylketone urea (PKU)	03	Phenyl pyruvic acid	198.1	220.1
		Isovaleryl glycine	552.0	600.2
		3hydroxyisovaleric acid	132.9	190.4
Isovaleric acidemia	01	Propionyl glycine	13.7	20.2
		Methylcitric acid	132.2	150.9
		3hydroxypropionic acid	89.08	90.8
Propionic acidemia	04	Lactic acid	3363.0	15996.7
		Lactic acid dimer	150.2	212.1
Lactic acidemia	10	Methylmalonic acid	1049.36	1459.2
B12 deficiency	12	Succinic acid	1231	1006.1
Riboflavine deficiency	03	Parahydroxyphenylacetic acid	113.2	100.0
Tyrosinemia type II	02	Parahydroxyphenyl lactic acid	134.2	102.1
		Parahydroxyphenyl pyruvic acid	92.3	150.9
		N-acetyltyrosine	99.0	212.6
		Acotinic acid	132.2	156.1
Glutathione depletion	01	Citric acid	118.1	134.2

Table 4 continued

Disorder	No. of patients (N = 102)	Marker compound	Mean excretion (mmol/mol of creatinine)	
			Liquid–liquid extraction	Solid phase extraction ^a
2MethylbutyrylCoA dehydrogenase deficiency	01	2Methylbutyrylglycine	7.08	14.11
Dicarboxylic aciduria	02	Adipic acid	652.77	1078.1
		Suberic acid	303.68	921.1
		Sebacic acid	444.62	765.2
Neuroblastoma	01	Homovanillic acid	113.5	145.1
		Vanillylmandelic acid	2400	2675.2
4-Hydroxybutyric aciduria	05	4-Hydroxybutyric acid	1187.89	1562.1

^a The final result is calculated using dilution factor

extracted by LLE. However such interference was completely absent with SPE. In our study, no such co-extraction occurred but significant background interference was observed in sample prepared by LLE [22].

Urine samples extracted by SPE showed overlapping of peaks, which could be due to large number of possible compounds isolated by this procedure in comparison of LLE (Fig. 1). In other words SPE may extract compounds that might contain information of disorders in patients that were not extracted by the LLE. The extraction of urea and creatinine were better in SPE. Particularly the ill shaped peak of urea with large area, masks peak of benzoic acid as its retention time was near the retention time of urea. This finding of our contradicts with various previous studies which stated LLE isolates many unwanted neutral compound like urea highly from urine samples [11, 15, 23, 24]. This could be averted by urease pretreatment of samples.

Samples extracted with SPE were devoid of phosphoric acid. This could be due to the treatment of urine samples with Ba(OH)₂ prior to SPE that precipitates all phosphates and sulphates. It also lowers the recovery of oxalic acid, citric acid and aconitic acid [11].

The peak area of phosphate was large in LLE that masks the metabolites near to its retention time [25]. This phenomenon could be the reason for poor recovery of glycerol by LLE as the retention time of phosphate was 12.494 min and that of glycerol was 12.440 min which was very close to phosphate. This phenomenon also works in case of fumaric acid that has two structural isomers; the retention time of cis form was at 13.189 min while the retention time of trans form was at 13.400 min. The peaks of these isomeric forms are clearly separated in SPE but in LLE only one isomer of fumaric acid i.e. cis form is isolated.

The total number of metabolites isolated by the extraction procedures was also important factor for deciding which method was better. The mean number of metabolites isolated by SPE was higher than the LLE. As LLE isolates

less number of metabolites it may miss some important metabolites. The SPE may be a choice of extraction for the planer analysis for simultaneous analysis of multiple categories of compounds.

The total processing time taken for 12 samples by LLE was about 4 h 20 min in comparison to SPE which take a mean time of about 2½ h. This processing time was an important factor, because in many of these disorders early diagnosis and treatment are essential to prevent life threatening episodes [11]. In our resource constraint setup, cost effectiveness of extraction procedure was also an important parameter for selecting the method of extraction. The processing cost of one urine sample by LLE was approximately 251 INR and by SPE it costs approximately 509.1 INR [18].

The recovery of keto acids was better in LLE in comparison to solid phase extraction (Table 3). One possible reason for this could be the long time taken by the elutes of solid phase extraction to get dried totally. In LLE only oximated samples recovered the ketoacids while non oximated process does not yield any keto-acids. Regarding keto acids our results contradicts with the results of a study that reported that keto acids are completely lost in SPE [10].

LLE happens to be a widely used sample preparation technique for many years. However it was time consuming and uses large volume of organic solvents. Production of phase emulsions and wet extracts are the major difficulties in LLE. Besides, SPE uses less organic solvents and avoids emulsions formation it offers clean extracts and superior recoveries [11]. The main problem in the SPE procedure was optimization of the method, especially the choice of appropriate eluents for the conditioning, washing, and elution steps. But, once optimized, this technique was less time demanding, quick, sensitive and efficient. It may be used for the planar analysis for simultaneous analysis of multiple classes of compounds. From this analysis very comprehensive diagnosis and metabolic studies are possible [26].

In summary SPE offers an effortless method for quickly obtaining extracts with better recoveries and may be a precise analytical test for the checking organic acids concentrations in urine sample during metabolic studies [11]. It also consumes less volume of organic solvents.

Conclusion

Each extraction procedures had some advantages and disadvantages. When few numbers of samples needs to be processed, LLE was the suitable method, while for more samples SPE may be significantly better procedure. Samples with a presumptive diagnosis of organic acidemia may be processed by LLE and for samples referred with an index of suspicion of an inborn error of metabolism; SPE could be used as the extraction procedure.

LLE was cost effective but it takes a mean time of 4 h 20 min which is twice the time taken by SPE technique. In our limited resource setup LLE represents a convenient method for obtaining extracts with optimum recoveries.

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