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Short Communication

Comparison of two extraction procedures for urinary organic acids prior to gas chromatography–mass spectrometry

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ABSTRACT

We have compared a new isolation procedure for urinary organic acids using strong anion-exchange columns with a solvent partition (ethyl acetate) method. Urinary samples from two healthy children and from nine children with organic acidurias were analysed by both procedures. Although the solid-phase extraction is more efficient for polyhydroxy acids, some polar acids, and some glycine derivatives, clinically important compounds such as oxalic, methylcitric, pyruvic, glyoxylic and 2-ketoglutaric acids, are not recovered or are only poorly recovered. However, both procedures may be used as a routine method for the diagnosis of the organic acidurias included in this study.

INTRODUCTION

Analysing urinary organic acids and derivatives such as glycine conjugates is a powerful tool for the diagnosis of numerous inborn errors of metabolism known as organic acidurias. This analysis relies on the recognition of characteristic urinary patterns obtained by gas chromatography or gas chromatography–mass spectrometry [1,2].

For sample preparation, two extraction methods are in current use: weak anion exchange on diethylaminoethyl-Sephadex [3,4] and solvent partition [5,6]. Although the first method yields higher recoveries for the more polar acids and gives more reproducible results [7,8], the solvent extraction is more frequently used for reasons of speed and simplicity. A third method claimed to combine the advantages of both previous procedures has been described by Verhaeghe *et al.* [9]. These authors perform a strong anion-exchange (SAX) extraction on disposable columns filled with a trimethylaminopropyl phase.

The purpose of the present work was to compare this new isolation procedure with a classical ethyl acetate extraction method [10]. With this aim, we analysed, using both methods, urine specimens from healthy children and from patients with organic acidurias.

EXPERIMENTAL

Chemicals

Hydroxylamine hydrochloride was purchased

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from Serva (Heidelberg, Germany). 2-Phenylbutyric acid was obtained from Janssen Chimica (Beerse, Belgium). N,O-Bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (99:1) (BSTFA–TMCS) was from Pierce Europe (Oud-Beijerland, Netherlands). SAX columns (500 mg solid phase, 2.8 ml) were manufactured by Alltech (Laarne, Belgium). All other chemicals were from Merck (Darmstadt, Germany) and were of analytical grade purity.

Samples

Urine specimens from an infant aged six months and a child aged eight years were included in the study. Nine pathological specimens, kindly provided by Dr. M. Duran (Wilhelmina Kinderziekenhuis, Utrecht, Netherlands), were also analysed. These were obtained from patients with the following disorders: isovaleric acidaemia, propionic acidaemia, 2-ketoadipic aciduria, 2-methylacetoacetyl-CoA thiolase deficiency, 3-hydroxy-3-methylglutaric aciduria, 3-methylcrotonyl-CoA carboxylase deficiency, methylmalonic acidaemia, glutaric acidaemia (type I) and mevalonic aciduria.

Ethyl acetate extraction

We slightly modified the method we have described elsewhere [10]. In a 10-ml glass-stoppered tube, 200 μl of aqueous internal standard solution (2-phenylbutyric acid, 1 g/l) were added to 2 ml of urine. Ketoacids were converted into stable oxime derivatives by incubating for 30 min at 60°C with 0.5 ml of a 50 g/l aqueous hydroxylamine hydrochloride solution, the pH of the mixture being adjusted to 14 with 7.5 mol/l sodium hydroxide. After cooling, the mixture was adjusted to pH 1 with 6 mol/l hydrochloric acid, supplemented with 1 g of sodium chloride and extracted three times with 6-ml ethyl acetate portions. The combined organic phases were dried on 0.5 g of anhydrous sodium sulphate and evaporated to dryness under nitrogen at 50°C in a 1-ml PTFE-capped glass vial. To convert the compounds into trimethylsilyl derivatives, 100 μl of (BSTFA–TMCS)–pyridine (99:1:20, v/v) were added to the residue and the capped vial was left overnight at room temperature. A 1-μl aliquot of the final solution was injected into the chromatograph.

SAX extraction

The samples were treated as described by Verhaeghe *et al.* [9] with the following modifications: 200 μl of the internal standard solution (2-phenylbutyric acid, 1 g/l) were added to 2 ml of urine; after precipitation with barium hydroxide, the total alkalinized supernatant was applied onto the SAX column; for all the samples analysed, the oxime derivatives were formed after extraction; trimethylsilylation was performed as for the solvent extraction.

Gas chromatography–mass spectrometry

The analyses were performed on a Hewlett-Packard HP5890A gas chromatograph coupled to an HP5970B mass-selective detector and an HP59940A ChemStation.

A polydimethylsiloxane stationary phase, 0.33 μm film thickness, chemically bonded on a 12 m × 0.2 mm I.D. fused-silica capillary column (Ultra I, Hewlett-Packard) was used. The column was directly introduced into the ion source. Helium was used as carrier gas at a linear velocity of 27 cm/s. Samples were injected using a split ratio of 1:40. Gas chromatography was performed with a temperature programme from 60 to 260°C at a rate of 5°C/min after an initial delay of 0.5 min at 60°C. The final temperature was kept for 2 min. The injector and interface temperatures were 260 and 280°C, respectively.

The mass spectrometer was operated in the electron-impact mode (70 eV). Mass chromatograms are total ion profiles, the scan range being 40–600 a.m.u.

For peak identification, we relied on methylene unit values and on mass spectra which were compared with a user-built library and with the National Bureau of Standards libraries.

RESULTS AND DISCUSSION

Fig. 1 shows an example of an organic acid profile obtained after solvent (A) and SAX (B)

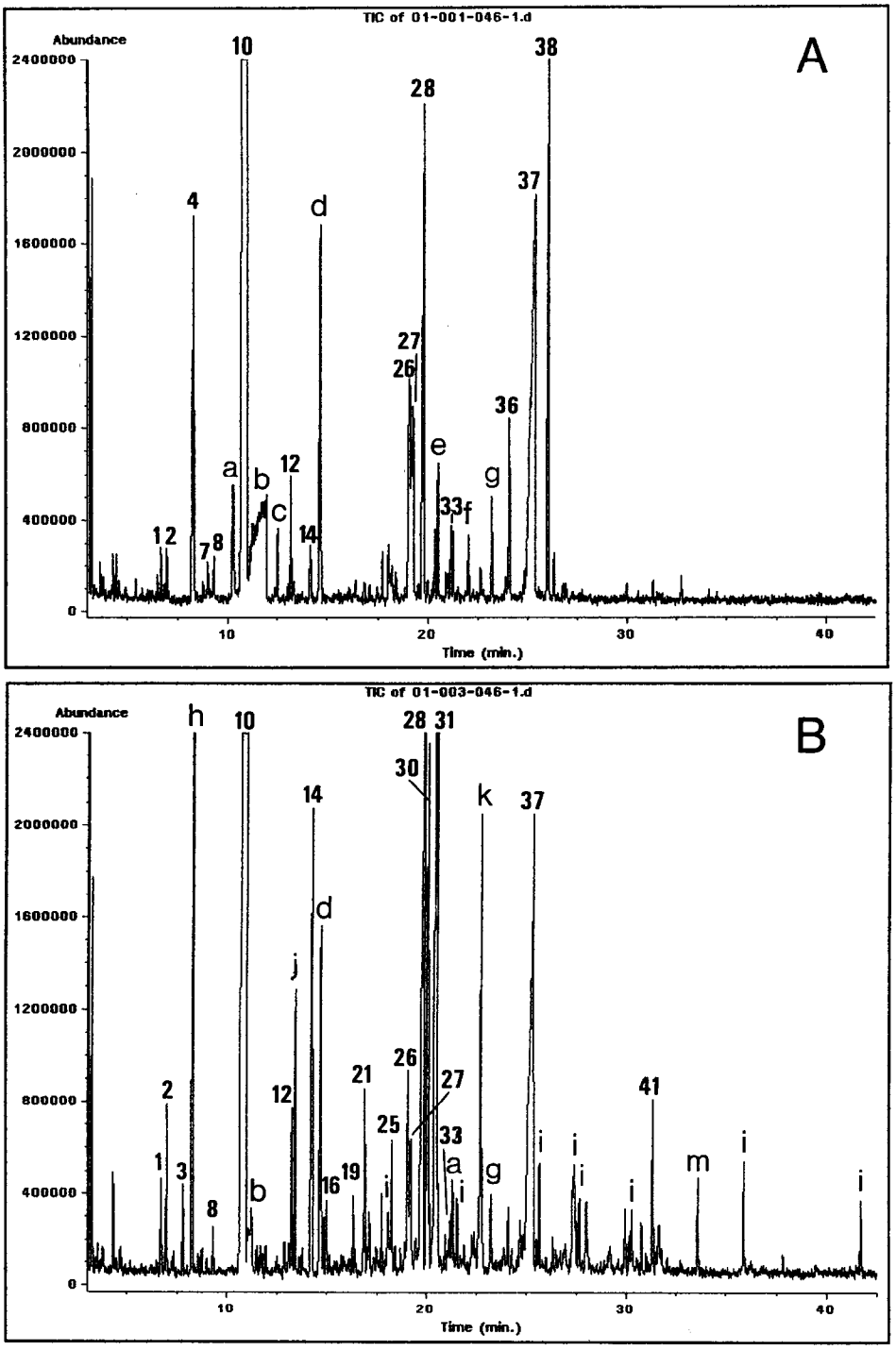

Fig. 1. Total ion chromatograms of a urine from a patient with 3-methylcrotonyl-CoA carboxylase deficiency, extracted by ethyl acetate (A) and by the SAX column procedure (B). For peak identification, see numbers in Table I. Lettered peaks, not included in Table I, are: a = unknown; b = urea; c = phosphoric acid; d = internal standard (2-phenylbutyric acid); e = 3-hydroxyphenylacetic acid; f = tartaric acid; g = 3-hydroxyphenylpropionic acid; h = impurity; i = "sugar acid"; j = glycine; k = a deoxypentonic acid; m = stearic acid.

TABLE I

PEAK AREAS, RELATIVE TO THAT OF 2-PHENYLBUTYRIC ACID (INTERNAL STANDARD), OF SELECTED METABOLITES FOUND IN AT LEAST ONE OF THE ANALYSED URINES

Compound	Peak-area ratio (compound/internal standard)[a]		Urine specimen's identification[b] or number of urine specimens from which the figures are calculated
	Ethyl acetate extraction	SAX extraction	
1 Lactic acid	0.153 ± 0.144	0.193 ± 0.110	9
2 Glycolic acid	0.131 ± 0.171	0.273 ± 0.213	10
3 N-Methylglycine	N.D.	0.109 ± 0.050	3
4 Oxalic acid	0.343 ± 0.311	N.D.	7
5 Glyoxylic acid	0.127 ± 0.123	N.D.	5
6 3-Hydroxypropionic acid	0.508	1.128	MMA
	0.266	0.690	PA
7 Pyruvic acid	0.223 ± 0.268	0.014 ± 0.035	6
8 3-Hydroxyisobutyric and 3-hydroxybutyric acids	0.143 ± 0.126	0.107 ± 0.085	10
9 2-Methyl-3-hydroxybutyric acid	1.93	1.95	MAATD
10 3-Hydroxyisovaleric acid	9.58	9.12	MCCD
	0.420 ± 0.338	0.360 ± 0.429	6
11 Methylmalonic acid	14.2	9.01	MMA
12 Succinic acid	0.294 ± 0.280	0.499 ± 0.401	8
13 Propionylglycine	0.099	0.209	MMA
	0.167	0.423	PA
14 Glyceric acid	0.106	1.055	MCCD
15 Mevalonic acid	Very high level	High level[c]	MA
16 4-Deoxytetronic acid	N.D.	0.112 ± 0.043	6
17 Glutaric acid	24.8	31.0	GA I
	1.52	1.82	KAA
18 3-Methylglutaric acid	0.687	0.719	HMGA
19 3-Deoxytetronic acid	N.D.	0.096 ± 0.041	6
20 3-Methylglutaconic acid (2TMS)[d]	2.944	3.56	HMGA
21 2-Deoxytetronic acid	0.027 ± 0.057	0.596 ± 0.612	9
22 Isovalerylglycine (1TMS)[e]	0.788	0.887	IVA
23 3-Hydroxy-3-methylglutaric acid	5.12	7.50	HMGA
	0.075 ± 0.062	0.110 ± 0.106	4
24 Adipic acid	0.341 ± 0.390	0.285 ± 0.428	5
25 Pyroglutamic acid	0.397 ± 0.726	0.494 ± 0.668	7
26 5-Hydroxymethyl-2-furoic acid	0.099 ± 0.031	0.046 ± 0.003	2
27 3-Methylcrotonylglycine (1TMS)[e]	0.95	0.42	MCCD
28 3-Methylcrotonylglycine (2TMS)[d]	1.47	2.19	MCCD
29 Tiglylglycine	0.707	1.158	MAATD
30 Erythronic acid	N.D.	1.034 ± 0.774	10
31 Threonic acid	N.D.	0.584 ± 0.297	10
32 2-Hydroxyglutaric acid	0.095	0.234	KAA
33 4-Hydroxyphenylacetic acid	0.295 ± 0.324	0.162 ± 0.267	9
34 2-Ketoglutaric acid	0.214 ± 0.103	0.013 ± 0.015	3
35 Suberic acid	0.043	0.010	GA I
	0.038	N.D.	Six-month-old infant
36 Aconitic acid	0.193 ± 0.096	N.D.	8
37 Benzoylglycine (1TMS)[e]	0.896 ± 1.038	0.966 ± 1.083	10

TABLE I *(continued)*

Compound	Peak-area ratio (compound/internal standard)[a]		Urine specimen's identification[b] or number of urine specimens from which the figures are calculated
	Ethyl acetate extraction	SAX extraction	
38 Citric acid	1.375 ± 2.016	0.086 ± 0.184	10
39 Methylcitric acid	0.058	N.D.	MMA
	1.073	0.161	PA
40 3-Hydroxysebacic acid	0.035	N.D.	Six-month-old infant
41 Uric acid	0.014 ± 0.032	0.687 ± 0.715	5

[a] Values are mean or mean ± S.D.; N.D. = not detected.

[b] IVA = isovaleric acidaemia; PA = propionic acidaemia; KAA = 2-ketoadipic aciduria; MAATD = 2-methylacetoacetyl-CoA thiolase deficiency; HMGA = 3-hydroxy-3-methylglutaric aciduria; MCCD = 3-methylcrotonyl-CoA carboxylase deficiency; MMA = methylmalonic acidaemia, GA I = glutaric acidaemia (type I); MA = mevalonic aciduria.

[c] The large peak of mevalonic acid obscures the internal standard peak.

[d] Ditrimethylsilyl derivative.

[e] Monotrimethylsilyl derivative.

extraction of a urine specimen from a patient suffering from 3-methylcrotonyl-CoA carboxylase deficiency.

The comparison of peak area of selected metabolites relative to that of the internal standard (Table I) shows that the polyhydroxy compounds (glyceric, 2-, 3- and 4-deoxytetronic, erythronic, threonic acids) are hardly extracted by ethyl acetate, unlike with the SAX column. Other polar compounds (glycolic, 3-hydroxypropionic, succinic, 3-hydroxy-3-methylglutaric and 2-hydroxyglutaric acids), as well as N-methylglycine, propionylglycine and tiglylglycine, are also more recovered by the second procedure. Uric acid is mainly found in the SAX extract.

On the other hand, other substances are almost completely lost during this solid-phase extraction: some polar acids (oxalic, suberic, aconitic, citric, methylcitric and 3-hydroxysebacic acids) and the oxime derivatives of the keto acids (pyruvic, glyoxylic and 2-ketoglutaric acids). Methylmalonic, mevalonic, 5-hydroxymethyl-2-furoic and 4-hydroxyphenylacetic acids are also more extracted by ethyl acetate. The recovery of the internal standard was similar with both extraction methods.

These results illustrate that both isolation procedures present advantages and disadvantages. A serious drawback of the SAX method is, at least in our hands, the low recovery achieved for clinically significant substances such as oxalic, methylcitric, pyruvic, glyoxylic and 2-ketoglutaric acids. With regard to oxalic, pyruvic and 2-ketoglutaric acids, our results are in contrast to those of Verhaeghe *et al.* [9], who obtained better recoveries by SAX than by solvent extraction for these three acids. The five compounds of clinical interest that we did not extract on SAX columns, or with a lower recovery, could have been partly eliminated during the barium hydroxide precipitation. Our observation that phosphoric acid is no longer detectable on the chromatograms obtained after column extraction supports this hypothesis. As already stated by Verhaeghe *et al.* [9], these observations stress the importance of the amount of this precipitating salt added, an excess of which eliminates clinically important compounds. The amount of barium hydroxide should therefore be further optimized.

The higher recovery observed with the SAX method for glycolic, 3-hydroxypropionic, 3-hydroxy-3-methylglutaric and 2-hydroxyglutaric acids, as well as for some glycine derivatives, is not an outstanding advantage since their extraction yield by ethyl acetate is still sufficient. The polyhydroxy acids, which are hardly extracted by

the solvent, are generally considered to be of no clinical importance, with the significant exception of glyceric acid. However, if their study should prove to be useful, the column extraction will be the method of choice.

Ethyl acetate is expected to extract not only acidic but also unwanted neutral compounds [7,8]. In fact, the only non-acidic substance we observed with this extraction procedure was urea, which gives a large ill-shaped peak, potentially obscuring compounds of interest. Other impurities or artefacts observed by Verhaeghe *et al.* [9] were not noted on our chromatograms. Thus, with the exception of urea, ethyl acetate yields an extract as clean as do the SAX columns.

Liquid–liquid extraction is a rapid and simple procedure and remains a convenient approach when no more than six samples are to be treated at a time. When more samples are to be processed, the solid-phase extraction may be a better procedure in terms of speed, although its cost may limit its use.

Although ethyl acetate extraction yields higher recoveries for a greater number of compounds of clinical interest, the two sample preparation procedures may be used for a rapid diagnosis. Indeed, at least for the disorders included in this study, the main substances of interest for the biochemical diagnosis are often obviously present in both chromatograms. However, for more detailed studies of organic acid profile the two methods are, in our opinion, complementary.

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REFERENCES

1 R. A. Chalmers and A. M. Lawson, *Organic Acids in Man*, Chapman & Hall, London, New York, 1982.
2 T. Niwa, *J. Chromatogr.*, 379 (1986) 313.
3 P. Sims, R. Truscott and B. Halpern, *J. Chromatogr.*, 222 (1981) 337.
4 R. A. Chalmers and R. W. Watts, *Analyst*, 97 (1972) 958.
5 K. Tanaka, A. West-Dull, D. G. Hine, T. B. Lynn and T. Lowe, *Clin. Chem.*, 26 (1980) 1847.
6 C. Jakobs, M. Bojasch, E. Monch, D. Rating, H. Siemes and F. Hanefeld, *Clin. Chim. Acta*, 111 (1981) 169.
7 J. A. Thompson and S. P. Markey, *Anal. Chem.*, 47 (1975) 1313.
8 W. L. Fitch, P. J. Anderson and D. H. Smith, *J. Chromatogr.*, 162 (1979) 249.
9 B. J. Verhaeghe, M. F. Lefevere and A. P. De Leenheer, *Clin. Chem.*, 34 (1988) 1077.
10 C. Wurth, A. Kumps and Y. Mardens, *J. Chromatogr.*, 491 (1989) 186.