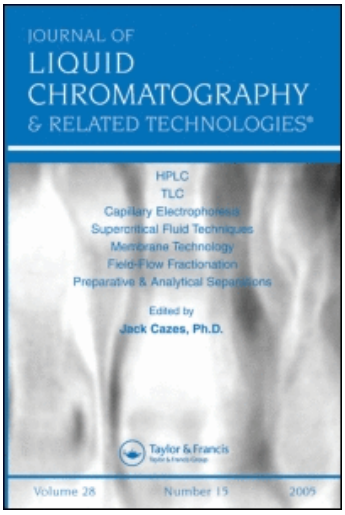

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ANALYSIS OF ORGANIC ACIDS IN PHYSIOLOGICAL FLUIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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I INTRODUCTION

(i) The Clinical Need for Organic Acid Analysis.

Most organic acids in physiological fluids are carboxylic acids or their glycine or glucuronide conjugates. Also included are hydroxyl compounds, such as phenols or cresols. By definition, compounds with a primary amino group, detectable by conventional amino acid analysers, are excluded. Organic acids are produced continuously in the body as intermediates in the metabolism of amino acids, carbohydrates and fats, and of drugs and food additives. They do not accumulate in the body, since they are rapidly converted to non-acidic end products or excreted as water soluble metabolites in urine. However, if they are produced in excess, or if their metabolism is prevented by an inherited enzyme defect, concentrations increase in the tissues, blood and urine. Examples of excessive production are during fasting ketosis, in which acetoacetic and

3-hydroxybutyric acids and a range of dicarboxylic acids derived from breakdown of fatty acids accumulate, and lactic acidosis, secondary to hypoxia, when increases in lactic, pyruvic and 2-hydroxybutyric acids are common.

Inherited defects were first recognised in 1966, when Tanaka *et al* (1) reported two children with an illness characterised by excretion of large amounts of isovaleric acid in their urine, identified by gas chromatography-mass spectrometry. They postulated, and later proved, (2) that this was due to deficiency of the enzyme, isovaleryl-CoA dehydrogenase (E.C. 1.3.99.10). Since then, a large number of other inherited defects have been identified. Today, more than 45 are recognised, many with variant forms (3). These are clinically important conditions. The most severe, for example propionic acidaemia (McKusick (4) 23200 and 23205) and methylmalonic acidaemia (McKusick 25100, 25110, and 27741), often cause a fulminating and fatal illness in the first days of life. Others, such as the congenital lactic acidoses (5,6), fumarase deficiency (McKusick 13686 (7)), and glutaric aciduria type I (McKusick 23167 (8)) produce irreversible and/or progressive neurological damage. Since first reported in 1976 (9), a further group of disorders has been identified in which defects of fatty acid oxidation cause a life-threatening *Reye's Syndrome*-like illness in apparently normal children and have, rarely, caused sudden death in infancy (10). From neonatal screening programmes in West Germany (11) and Harrow, England (12), the collective incidence of organic acid defects was estimated at 1 in 10,000 to 1 in 15,000 live births, but this is an underestimate. Not all conditions are detectable neonatally, and 'new' disorders have been identified since these studies. The incidence of fat oxidation defects, alone, may be as high as 1 in 5,000 births (13). Some defects are treatable, provided that a diagnosis is established quickly. For those that are not, prenatal diagnosis is now available in most cases (14,15).

Diagnosis of these serious disorders depends entirely on biochemical analyses, generally by demonstrating an abnormal organic acid *profile* in urine, and sometimes plasma, followed by confirmation of the enzyme defect in leukocytes or cultured skin fibroblasts. Although the disorders may be suspected clinically, their presentation resembles that of many much commoner non-inherited illnesses of infants and children. It is important that paediatricians have ready access to organic acid analyses, if these conditions are not to be missed.

(ii) Organic Acid Analysis

Detection of the full range of diagnostic organic acids, which have a wide diversity of chemical structures, is analytically demanding. Only a small number of relevant acids are detectable by thin layer chromatographic procedures. Except for specific screening for orotic aciduria, phenolic acid disorders and, arguably, methylmalonic aciduria, they have no place in diagnosis. Unquestionably, the best available profiling methods use capillary gas chromatography-mass spectrometry (GC-MS) (3,16). However, as this technique needs expensive equipment, is time-consuming and requires a high degree of operator skill, its availability is restricted to specialised centres and to those samples where an inherited defect is considered a strong possibility. Children not presenting 'classically' may therefore go undetected. Some organic acid disorders can be detected by high resolution proton nuclear magnetic resonance (NMR) spectroscopy (17-20). However, this method is inferior to GC-MS because of its insensitivity, and the capital costs are high.

There is a need for a simple screening method to investigate samples from a wide range of patients, to select those needing more comprehensive analysis. The lower costs associated with high performance liquid chromatography (HPLC), and its ease of use, would make it an attractive screening technique.

II ANALYSIS OF ORGANIC ACIDS BY HPLC

(i) Columns

Successful separation of a variety of organic acids in biological fluids has been achieved with reverse phase, ion exchange and, particularly, ion moderated partition chromatography, used alone or in combination.

Reverse phase: Using an octadecylsilane (ODS) column (5μm LiChrosorb at 70°C), and gradient elution with acetonitrile in aqueous phosphoric acid, Molnar and Horvath (21) obtained good resolution of over one hundred acidic urinary constituents in less than 30 minutes. Benzoic and hippuric acids were analysed in deproteinised plasma with an ODS column (7μm particles) at 30°C and acetonitrile-water-acetic acid used as mobile phase (22). Oxo-acids in deproteinised serum and urine, derivatised to fluorescent quinoxalines, were resolved on a Zorbax-ODS column with methanol-water as mobile phase (23).

Disadvantages of reverse phase columns are stripping of the bonded stationary phase by basic or acidic buffer solutions or by oxidising agents (24), tailing and irreversible adsorption because of interaction between residual silanol groups in the stationary phase and the solute, and poor reproducibility because of slow solute mass transfer (25). Problems with gradient elution methods have been poor reproducibility due to changes in the viscosity and compressibility of the eluent with the solvent composition, fluctuations in ambient temperature if the column is not thermostatically controlled, and the appearance of 'ghost' peaks due to solvent impurities (26).

Ion exchange: As early as 1968, Scott (27) reported the separation of 140 ultraviolet-absorbing constituents in deproteinised urine by anion exchange HPLC (Dowex 1, 5 to 10μm particles) and gradient elution for 40 hours with sodium acetate buffer. Analysis time was later halved and the method automated (28). Poor reproducibility and difficulties in obtaining high quality resin were problems. Good resolution of hydroxamic derivatives of short chain aliphatic acids and some of the acids of the citric acid cycle in urine and plasma was obtained in two to three hours with an anion exchange column (29). Traditional cation exchange HPLC has not been widely used for organic acid analysis of biological fluids. Turkelson and Richards (30) separated tricarboxylic acid cycle acids in neat, filtered, human and rat urine, using an Aminex 50W-X4 (30-35μm particles) cation exchange resin.

Ion Moderated Partition Liquid Chromatography: This technique utilises porous cross-linked ion exchange styrene divinylbenzene resins with acidic or basic groups introduced before or after polymerisation (25,31). Porosity depends upon hydration of the matrix and this, in turn, on hydration of the functional groups. Substances are separated by a combination of ion exchange, ion exclusion and ligand exchange gel permeation (32,33), and thus a high resolution may be possible.

A sulphonated cation exchange resin is used in the Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, U.K.) introduced in 1980 for organic acid analysis of wines and foodstuffs. Because it is cross-linked (8%), the resin is relatively impermeable to high molecular mass compounds. Several groups have used HPX-87H columns with isocratic elution with 5mmol/L sulphuric acid to analyse organic acids in aqueous solution, urine and plasma (34-41). Like Rumsby et al (40) we obtained good resolution, with good precision, of most of

the biologically important organic acids in 45 minutes with a 300mm x 7.8mm column at 50°C (Table I), or in 18 minutes with a Bio-Rad short fermentation monitoring HPX-85H column (41). However, some clinically important acids co-eluted, and this might cause diagnostic problems (*eg.* citric, orotic and 2-oxoglutaric acids; uric, acetoacetic and fumaric acids). Increasing the column temperature from 20°C to 60°C shortened the retention time of the aromatic acids and of the dicarboxylic acids, hexanedioic and octanedioic acids (41). Working at room temperature, Bennett and Bradey (36) observed long retention times for the phenolic acids, which had broad flattened peaks. A maximum operating temperature of 65°C is advised by the column manufacturers. Addition of the solvent modifier, acetonitrile, to the mobile phase reduced the retention times of standard organic acids at 20°C, 40°C and 60°C, but at 60°C this was at the expense of resolution (41). It was concluded that acetonitrile added little to the advantage gained by high column temperature alone and, for simplicity, it is generally not used. Most groups use a mobile phase flow rate of around 0.7-1.0mL/min.

Recently a highly cross-linked polydivinylbenzene resin based column has been introduced (Alltech Associates, Carnforth, U.K.) specifically for organic acid analysis. The manufacturers claim that the column is stable up to 150°C and will tolerate very high concentrations of organic modifier without causing problems of resin swelling. No published results for its use for the analysis of physiological fluids are yet available.

Two-Dimensional HPLC: In order to improve resolution, two HPLC columns have been used in sequence. With a C_{18} reverse phase column followed by an anion exchange column, Mattiuz et al (42) resolved over 100 urinary acidic components. Buchanan and Thoene (43) found that coupling a C_{18} reverse phase column to an Aminex HPX-87H column, achieved better resolution of organic acids in urine than either column alone. Coupled anion and cation exchange columns have also been used (44). Haas et al (45) used two linked Aminex HPX-87H columns to analyse citric acid cycle intermediates in plasma, and this improved reproducibility. Buchanan and Thoene (34) used a different approach. Unknown compounds eluting from an Aminex HPX-87H column were collected by the method of 'heart cutting' (46) and then re-chromatographed on an

TABLE I

Relative Retention Times and Relative Response Factors, of Thirty Clinically Important Organic Acids.

Acid	Relative Retention Time*	Relative Response Factor 210nm*
orotic	0.162	1.545
2-oxoglutaric	0.162	0.183
pyruvic	0.186	0.115
3-hydroxy-3-methylglutaric	0.207	0.017
methylmalonic	0.213	0.020
lactic	0.253	0.009
2-oxoisocaproic	0.260	0.180
glutaric	0.272	0.013
3-hydroxybutyric	0.273	0.012
acetoacetic	0.281	0.012
fumaric	0.282	2.160
uric	0.286	0.049
hexanedioic	0.321	0.013
propionylglycine	0.351	0.109
2-oxoproline	0.358	0.161
propionic	0.359	0.007
4-hydroxy-3-methoxy-mandelic	0.400	3.484
isovalerylglycine	0.466	0.096
4-hydroxyphenyllactic	0.484	0.883
isovaleric	0.505	0.041
2-hydroxyphenylacetic	0.600	1.176
octanedioic	0.617	0.001
4-hydroxyphenylacetic	0.665	1.282
phenyllactic	0.717	1.195
4-hydroxy-3--methoxyphenylacetic	0.771	2.899
phenylacetyglutamine-	0.772	0.742
hippuric	0.825	1.368
I.S.	1.000	1.000
decanedioic	1.583	0.003
benzoic	1.727	0.716
2-hydroxybenzoic	1.980	3.922

Column: Aminex HPX-87H

HPLC Conditions: temperature, 50°C; eluent, 5mmol/L sulphuric acid; flow rate, 0.8mL/min; attenuation, 32; chart speed, 0.5cm/min; detector wavelength, 210nm.

* relative to internal standard, 3-(4-hydroxyphenyl) propionic acid (I.S.), retention time 37.84 ± 0.19 min (mean ±1SD,n=10)

analytical C_{18} column. The cost of improved resolution with two dimensional HPLC is a longer and more complex analysis, making it unsuitable for rapid screening.

(ii) Detectors:

Ultraviolet Absorbance: This has been used frequently for organic acid analyses (36,40,41). At 210nm, most diagnostically useful organic acids have some measurable absorbance. Aromatic acids absorb well, as shown by their relative response factors (RRF's) when compared to the internal standard 3-(4-hydroxyphenyl) propionic acid (Table I). With the exception of fumaric acid, however, aliphatic organic acids generally have low absorbances. This is true, particularly, of the medium chain dicarboxylic acids, hexanedioic (adipic), octanedioic (suberic) and decanedioic (sebacic) acids. The glycine conjugates of propionic, isovaleric and benzoic acids had higher absorbances than the respective unconjugated acids (41). At 210nm and with an HPX-87H column maintained at 50°C, we found that detection limits in urine varied widely, being a function of response and capacity factors. For example, the minimum detection limits in urine were: 4-hydroxyphenylacetic acid 4μmol/L, 4-hydroxyphenyllactic 4μmol/L, orotic 4μmol/L, pyruvic 15μmol/L, lactic 25μmol/L, methylmalonic 42μmol/L, 3-hydroxybutyric 75 μmol/L. These figures are similar to those obtained for a short HPX-85H column held at 60°C (41).

Most aromatic organic acids absorb reasonably well at 280nm, but aliphatic acids have a low absorbance. This observation has a useful application when investigating for possible urea cycle defects. The diagnostic metabolite orotic acid co-elutes from the HPX-87H column with citric and 2-oxoglutaric acids, and all three absorb at 210nm. At 280nm, however, only orotic acid is detectable. 280nm is *not* suitable for screening for organic acidurias, since the vast majority of diagnostic metabolites are aliphatic acids. Monitoring at different wavelengths does, however, allow determination of peak height ratios to help in positive identification, but requires the sample to pass through the HPLC system several times (43).

UV absorbance of some acids has been improved by preliminary derivatisation procedures. These include benzylation with 1-benzyl-3-p-tolutriazine (47) and o-p-nitrobenzyl-N,N'-diisopropylurea (48),

formation of 2-naphthacyl bromide esters (49) phenacyl esters (50), aryldiazoalkanes (51), diazomethylanthracene derivatives (52) and 2, 4-dinitrophenylhydrazones (53).

Even with improved sensitivity, a major problem with UV absorbance is its poor specificity for organic acids. *Diode-array detectors* are rapid scanning spectrophotometers which monitor the UV spectra over a range of wavelengths, *eg.* 190-350nm, of compounds as they elute from the column (54,55). This technique has been used to detect abnormal metabolites in urine in several organic acid disorders: methylmalonic acidaemia, branched chain ketoaciduria and lactic acidaemia (37) and 3-hydroxy-3-methylglutaric aciduria (39,56). However, co-eluting acids with similar UV spectra cannot be distinguished by this method (37).

Electrochemical detection has found a limited application for the determination of uric, ascorbic and some phenolic acids in urine and serum (57,58). The majority of diagnostically useful organic acids lack a readily oxidisable/reducible group and cannot be detected by this method. However, a UV detector with a glassy carbon electrochemical detector placed downstream, in series, was found to facilitate peak identification in urinary organic acid profiling (34). The technique was particularly useful for the identification of 2-oxo carboxylic acids (35).

Fluorescence detection: Few organic acids fluoresce, but some can be converted to fluorescent derivatives. Fatty acids have been analysed after converting to 4-bromomethyl-7-methoxycoumarin derivatives (59) phenanthrimidazoles (60) and 9-anthryldiazomethane (ADAM) derivatives (61), and 2-oxoacids to quinoxaline derivatives (23). The problems of forming derivatives of a vast range of diagnostically useful organic acids seem insurmountable, and this approach is therefore not useful for routine profiling. A urinary profiling study in which derivatives were *not* formed, detected naturally fluorescent tryptophan, catecholamines and hydroxyphenyl metabolites (21).

Liquid Chromatography-Mass Spectrometry (LC-MS): Technological developments are occurring rapidly in this area and, hopefully, in the future definitive identification of organic acids will be possible. *Thermospray* interfaces suffer the disadvantage of not generating electron impact ionisation-type spectra, and therefore of providing only limited structural information. In addition, buffers must be added to the mobile phase (62,63). *Plasmaspray* interfaces can

operate with aqueous or non-aqueous mobile phases. The mass spectral profiles are smoother than with thermospray, and the degree of fragmentation can be controlled by altering the probe temperature.

Mills et al (64) reported a preliminary investigation of LC-MS with a plasmaspray interface for organic acid profiling. Acids, eluted from an Aminex HPX-87H column with 25mmol/L formic acid, were passed via a UV detector to a double focusing mass spectrometer. Negative ion spectra of 15 standard organic acids exhibited abundant $[M-H]^-$ ions with very few fragment ions, enabling their molecular mass to be determined readily. Analysis of urine from healthy neonates was not informative because of poor sensitivity. However, methylmalonic acid was readily detected by single ion recording in urine from a patient with methylmalonic acidaemia. Subsequent modifications to the interface have improved the sensitivity (65).

(iii) Sample Preparation

Filtration of urine through 0.3μm (34,43) or 0.22 μm filters (36) has been used as a rapid, simple, preparative procedure, followed by direct application to an HPLC system. However, this does not remove UV interfering substances, the chromatograms are generally of poor quality, and problems of high back pressure and contamination of the HPLC column are likely (66). Ultrafiltration through semipermeable membranes (27,67) may be less deleterious to the columns. Protein precipitation procedures used have included addition of perchloric acid (29), acetonitrile (45), methanol (23,68) and tungstic acid (23). Recoveries were better with perchloric acid than acetonitrile, but interfering peaks in the chromatogram were a problem (45).

Solvent extraction with ethyl acetate (21) or ethyl acetate and diethyl ether (69) has been used for organic acid profiling, and to extract derivatised acids. Disadvantages are, firstly, that non-acidic substances, including urea, some sugars and alcohols, are also extracted and, secondly, that recoveries of polyhydroxy acids are poor. In general, however, these acids are not diagnostically important.

Recently, solid phase extraction procedures have become popular. Disposable octadecylsilane cartridges (Sep-Pak C_{18} cartridges, Waters, U.K.) have been used to remove non-polar substances prior to urinary oxalate analysis (70). Cation exchange, followed by extraction with ether and phosphate buffer, has

been used for analysis of branched chain oxo-acids in plasma (71). However, for organic acid profiling, Rehman et al (69) found that anion exchange chromatography, with pyridinium acetate as the eluting solvent, was better than two other preparative procedures evaluated (manual solvent extraction with ethyl acetate and diethyl ether, and anion exchange with hydrochloric acid as the eluting solvent), and had a similar performance to continuous solvent extraction in terms of reproducibility and recoveries of various acids.

Disposable anion exchange preparative columns are commercially available (*eg.* Bond Elut strong anion exchange (SAX) columns, Analytichem International) and these have been applied successfully to plasma and urinary profiling (38,40,41). Using a vacuum manifold, multiple samples can be prepared in one batch. The SAX columns are re-usable for urine for up to 20 times, but should not be re-used for plasma due to protein contamination (40,41). By adjusting the sample to pH 7.0 before extraction, interference from urate is minimised (40). Pre-dilution of urine before extraction according to creatinine concentration allows for variation in urine strength (41). For routine profiling, 3-(4-hydroxyphenyl) propionic acid is a satisfactory internal standard (40,41).

Using a SAX column extraction procedure (40,41), followed by HPLC on an Aminex HPX-87H column at 50°C with UV detection at 210nm, recoveries of 16 clinically important acids from spiked urine (60-138%, median 94%) and plasma (58-142%, median 88%) in our laboratory, were generally acceptable. However, recoveries of 3-hydroxybutyric (20%) and propionic (47%) acids were poor. As both these acids have a very low absorbance at 210nm, integration errors probably account for the findings. Volatility of propionic acid may have been contributory.

III CLINICAL APPLICATIONS OF ORGANIC ACID ANALYSIS BY HPLC

(i) Screening For Inherited Organic Acid Defects.

Ideally, a screening procedure should be 100% sensitive - *ie.* detect all affected individuals tested. It should also have an acceptably low false positive rate, so that the number of samples from normal individuals requiring further investigation by GC-MS is kept to a minimum. How well do available procedures meet these criteria?

There is, unfortunately, no published data to provide the answers. As a first step, several groups examined samples from healthy individuals, and concluded that a normal HPLC profile could be recognised, even though many of the peaks were not positively identified (34,36,40,41). Using an HPX-87H column, we found that for babies and children, the major identifiable peaks in urine were pyruvic, lactic, hippuric and 4-hydroxyphenylacetic acids, and in plasma, pyruvic and lactic acids, with small amounts of urate not removed by the pre-extraction stage. Unidentified peaks were recognised as a constant feature (41). In addition, samples from patients with confirmed inherited organic acids defects have been analysed. However, the data is fragmentary, in some reports limited to only one or two examples to indicate the potential value of HPLC.

In urine, abnormalities were found for methylmalonic acidaemia, propionic acidaemia, branched chain ketoaciduria, isovaleric acidaemia, 3-ketothiolase deficiency, glutaric aciduria type II, orotic aciduria (urea cycle defects), tyrosinaemia, and 3-hydroxy-3-methyglutaric aciduria (23,34,36,37,39,40,41,43,56,72). *In plasma*, diagnostic abnormalities were found for methylmalonic acidaemia, propionic acidaemia, branched chain ketoaciduria, isovaleric acidaemia, 3-hydroxy-3-methylglutaric aciduria, pyruvate dehydrogenase deficiency and fumarase deficiency (23,38,41). In only one disorder, gross dicarboxylic aciduria, was no abnormality detected (36). Most of the studies used HPX-87H columns alone or, in the case of Buchanan and Thoene (39,43,56), as part of a dual system with a reverse phase column. There is general agreement that the urinary HPLC profiles were simpler than GC-MS profiles of the same samples, in which a much wider range of diagnostic metabolites could be identified.

Using an Aminex HPX-87H column at 50°C, we have analysed 22 samples of urine and 6 of plasma from 22 patients with 15 different inherited organic acid defects (Table II). In 13 of the defects, at least one abnormal peak was present in the chromatogram which was identified as a diagnostic metabolite of the condition, either by comparison with authentic standards or with published work (38,40). Chromatograms for normal urine, 5-oxoprolinuria, fumarase deficiency, and branched chain ketoaciduria are shown in Figure 1. A worrying observation, however, was that in two disorders, glutaric aciduria type I and medium chain acyl-CoA dehydrogenase (MCAD) deficiency, the chromatograms looked normal. On GC-MS, the glutaric aciduria sample contained glutaric acid

TABLE II

Abnormalities Detected by HPLC in Urine and Plasma from Patients with Proven Organic Acid Disorders.

Organic acid disorder	Diagnostic metabolites identified
Urine and plasma	
Branched chain ketoaciduria (maple syrup urine disease)	2-hydroxyisovaleric acid 2-oxoisovaleric acid* 2-oxo-3-methylvaleric acid* 2-oxoisocaproic acid
Fumarase deficiency	fumaric acid
Methylmalonic acidaemia	methylmalonic acid
5-Oxoprolinuria	5-oxoproline
Urea cycle defects:	
a) Citrullinaemia	orotic acid
b) Ornithine transcarbamylase (OTC) deficiency	orotic acid
Urine	
Glutaric aciduria Type II	none
3-Hydroxy-3-methylglutaric aciduria	3-hydroxy-3-methylglutaric acid 3-methylcrotonylglycine*
Isovaleric acidaemia	isovalerylglycine
β-Ketothiolase deficiency	tiglylglycine*
MCAD deficiency	none
3-Methylcrotonylglycinuria	3-methylcrotonylglycine* tiglylglycine*
Primary lactic acidosis	raised lactic and pyruvic acids
Propionic acidaemia	propionylglycine tiglylglycine*
Tyrosinaemia Type I	4-hydroxyphenylacetic acid 4-hydroxyphenyllactic acid 4-hydroxyphenylpyruvic acid

* Identified tentatively (Rumsby et al 1987, reference 40.)

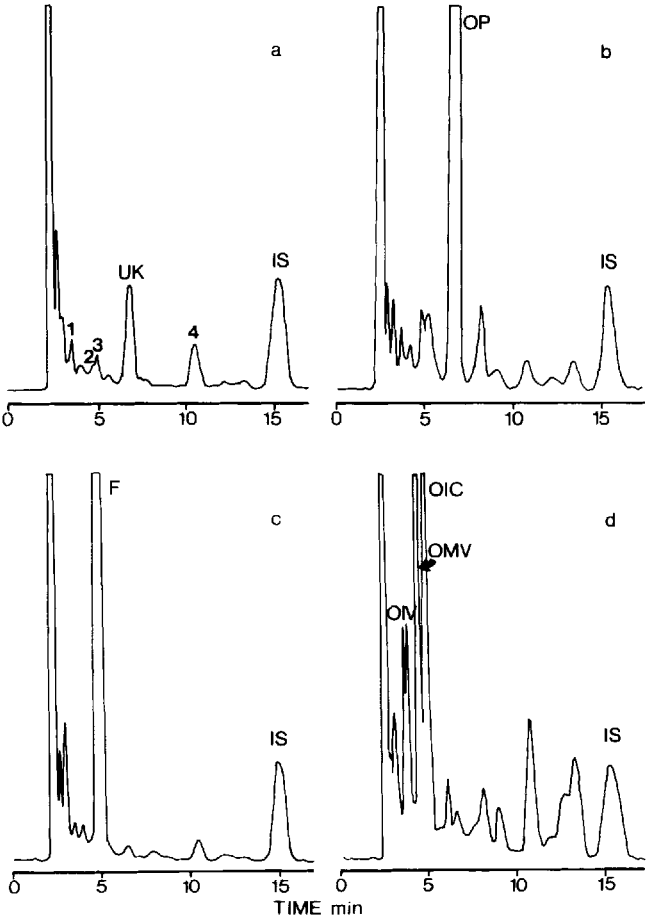

FIGURE 1. Urinary organic acid profiles of neonates using Bio-Rad fermentation monitoring column, 150mm x 7.8mm. Temperature, 60°C; eluent, 5mmol/L sulphuric acid; flow-rate 0.8mL/min, detector wavelength, 210nm. (a) Normal profile. Identifiable acids: 1, pyruvic; 2, lactic; 3, uric; 4, 4-hydroxyphenylacetic; UK= common unknown (b). With 5-oxoprolinuria; OP= 5-oxoproline. (c) With fumaric aciduria; F= fumaric acid. (d) With branched chain ketoaciduria; OIV= 2-oxoisovaleric acid; OMV= 2-oxo-3-methylvaleric acid; OIC= 2-oxoisocaproic acid.

and hexanedioic acid, and both MCAD samples analysed had high concentrations of the dicarboxylic acids hexanedioic, octanedioic and decanedioic acids and the corresponding unsaturated acids. The very poor UV absorbance of dicarboxylic acids accounts for the findings. Other fat oxidation defects, for example long chain acyl-CoA dehydrogenase deficiency and 3-hydroxyacyl-CoA dehydrogenase deficiency, are characterised by dicarboxylic aciduria, and it is clear that HPLC procedures using UV detection of underivatised acids *must not be* used when these disorders are suspected. In chromatograms from sick babies who did not have inherited defects, we sometimes observed unknown peaks which we attributed to drug therapy. Peaks due to sodium valproate, paracetamol and salicylate and/or their metabolites have been identified (40).

(ii) Other Clinical Applications of HPLC Organic Acid Analysis

MCAD deficiency is a disorder of β-oxidation of fatty acids which can lead to a *Reye's Syndrome*-like illness in young children after fasting or during minor infections. Typically, excretion of diagnostically important metabolites occurs only when the patient is unwell. Seakins and Rumsby (73) recently reported a 3-phenylpropionic acid loading test which will detect the disorder in asymptomatic babies and children, who can then be treated by dietary manipulation. After an oral dose of the acid, affected individuals excrete 3-phenylpropionyglycine and hippuric acid whereas normal subjects excrete only hippuric acid. Due to their high response factors, both compounds are readily detected by HPLC using either ion moderated partition chromatography (HPX-87H columns) or reverse phase columns (5μm ODS). We found the Bio-Rad HPX-85H fermentation column (41) convenient for rapid analyses, and have obtained positive results in affected babies aged only 7 and 18 days, respectively (74,75).

Another useful application of HPLC has been to monitor patients with inherited defects of the urea cycle, treated with sodium benzoate and phenylacetic acid to lower plasma ammonia. These drugs, after conjugation to hippuric acid and phenylacetylglutamine, are rapidly excreted in the urine and thereby remove waste nitrogen from the body (76). We have found 2-phenylpropionic acid a satisfactory internal standard for analysis of these compounds. 3-(4-Hydroxyphenyl) propionic acid is unsuitable since it co-elutes with phenylacetic acid.

One further application which we have found useful is for monitoring urinary excretion of 4-hydroxyphenyllactic as a measure of tyrosyluria in very premature babies being fed intravenously (77). These babies do not have inherited disorders.

IV SUMMARY

At present, HPLC-MS cannot compete with GC-MS as a definitive *diagnostic* procedure for inherited organic acid defects. HPLC by ion moderated partition chromatography with UV detection is an attractive and potentially valuable *screening* method for these disorders, but it still needs careful evaluation in clinical studies. Any samples producing abnormal results must be investigated in detail by GC-MS. HPLC must *not* be used as a first-line procedure for suspected fat oxidation defects. Although, generally, a normal HPLC chromatogram, considered in conjuction with the clinical features, will exclude an inherited disorder, when the clinical suspicion is very strong it would be wise to proceed to GC-MS. The 3-phenylpropionate loading test for MCAD deficiency is becoming established as an invaluable application of HPLC. Other potentially useful clinical applications are in managing patients with urea cycle defects and monitoring protein tolerance of premature babies.

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