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Note

High-performance liquid chromatographic method for the rapid profiling of plasma and urinary organic acids

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The organic acidurias are inherited metabolic defects characterised by an abnormal excretion of carboxylic acids [1,2], with an estimated incidence of around one in 10 000 live births [3]. Generally they are associated with serious clinical disease of variable presentation, often in the neonatal period. These conditions are diagnosed by demonstrating an unusual urinary organic acid profile by combined capillary gas chromatography–mass spectrometry (GC–MS) [2,4]. As this technique is expensive, time-consuming and requires a high degree of operator skill, its availability is restricted to specialised centres, and then only to samples where an inherited defect is considered a strong possibility on clinical grounds. Children not presenting 'classically' may therefore go undetected [5]. There is a need for a simple screening method to investigate samples from a wider range of patients, to select those needing more comprehensive analysis. As urine is often unavailable from babies who are acutely ill, the method should be applicable to plasma as well as urine.

Recently, high-performance liquid chromatography (HPLC) using polymeric strong cation-exchange columns (300 mm × 7.8 mm, Aminex HPX-87H) eluted isocratically with dilute acid, combined with ultraviolet (UV) detection, has been proposed as a screening procedure for organic acidurias using neat [5] or prefractionated urine [6] or plasma samples [6,7]. Unlike GC, this technique also has the ability to detect compounds of high polarity and glucuronide conjugates [6]. By maintaining the HPLC column at 50°C, elution of the diagnostically important acids was effected within 45 min [6]. We have been able to reduce this time to 18 min by using a short HPLC column of similar material, without sig-

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nificant loss of resolution, thus enabling the screening of up to twenty samples a day.

EXPERIMENTAL

Patients and materials

Random urine and plasma samples from healthy su jects and patients were stored at −20°C without preservative. Urinary creatin e was determined by an alkaline picrate method [8]. Standard organic acids w e obtained from Sigma (Poole, U.K.) or Aldrich (Gillingham, U.K.). Commer lly unavailable glycine conjugates were gifts from other laboratories. Stock or nic acid solutions, prepared in 5 m*M* sulphuric acid, were diluted as required. Water was distilled, deionised and filtered to 0.22 μm. All other chemicals were of Analar grade. Supelchem 1-ml LC-SAX disposable anion-exchange columns were from R.B. Radley (Sawbridgeworth, U.K.).

HPLC instrumentation

The chromatographic system consisted of a Bio-Rad Model 1350 pump (Bio-Rad Labs., Watford, U.K.), equipped with a Rheodyne Model 7125 injector and an LDC Spectromonitor III (LDC UK, Stone, U.K.) variable-wavelength UV monitor. Data handling was performed by a Trio 2+computing integrator (Trivector Systems International, Sandy, U.K.). The organic acids were separated on a 150 mm×7.8 mm Bio-Rad fermentation monitoring column (8% cross-linked sulphonated divinyl benzene–styrene copolymer in the hydrogen form) which was protected upstream by an Aminex HPX-85H guard cartridge (40 mm×4.6 mm). Both were installed in a Bio-Rad column heater and maintained at 60±0.1°C. The mobile phase was 5 m*M* sulphuric acid, degassed with helium and pumped at 0.8 ml/min; UV absorbance was measured routinely at 210 nm.

Sample preparation

Urine or plasma was prepared for HPLC as described by Rumsby et al. [6], with the modification that all urine samples were diluted with water to a creatinine concentration of 0.1 mmol/l before fractionation. The disposable LC-SAX clean-up columns could be re-used up to twenty times for urine samples if they were regenerated carefully. The columns were not reused for plasma because of protein and lipid contamination.

RESULTS

Effects of column temperature and flow-rate

The effects of column temperature over the range 20–60°C was investigated on the elution of twenty standard acids found in plasma and urine. Changes in temperature influenced the retention times of aromatic and medium-chain dicarboxylic acids but had little effect on short-chain aliphatic acids (Fig. 1). Since urine contains aromatic acids, running at higher temperatures considerably reduced analysis time. The retention time of the internal standard (I.S.) was halved

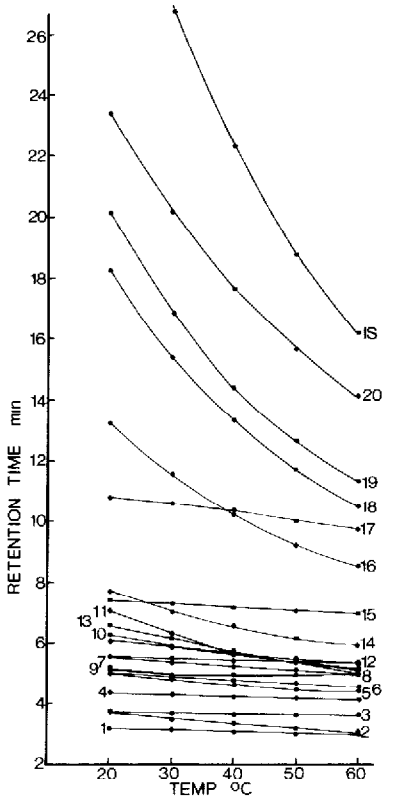

Fig. 1. Effect of column temperature on retention times of twenty clinically important acids and the internal standard 3-(4-hydroxyphenyl)propionic acid. Eluent, 5 m*M* sulphuric acid; flow-rate, 0.8 ml/min. Key to acids: 1, citric; 2, orotic; 3, pyruvic; 4, methylmalonic; 5, succinic; 6, ethylmalonic; 7, lactic; 8, 2-oxoisocaproic; 9, 4-hydroxyphenylpyruvic; 10, glutaric; 11, uric; 12, 3-hydroxybutyric; 13, acetoacetic; 14, adipic; 15, propionic; 16, 4-hydroxyphenyllactic; 17, isovaleric; 18, suberic; 19, 4-hydroxyphenylacetic; 20, hippuric; IS, internal standard.

from 32.90 min at 20°C to 16.08 min at 60°C. The maximum column operating temperature advised by the manufacturer is 65°C. Operation at 60°C did not decrease resolution appreciably, although elution order changed in some cases. Altering the flow-rate from 0.8 to 0.4 ml/min had no effect on resolution, but doubled the retention times. The maximum flow-rate advised for the resin is 1.2 ml/min.

Effect of acetonitrile in the mobile phase

The effect of the solvent modifier acetonitrile added to the mobile phase at three concentrations, 1, 2 and 5%, at column temperatures of 20, 40 and 60°C, was investigated on the elution and resolution of twenty acids. At 20 and 40°C, acetonitrile at a concentration of 5% was most effective in decreasing the retention times of the acids, the I.S. eluting at 19.54 and 14.34 min, respectively. Resolution showed little change. At 60°C, the I.S. retention time was reduced to 10.99 min, but at the expense of resolution. Acetonitrile therefore added little to the

advantage gained by higher column temperature alone and for simplicity it was not used.

Retention times and response factors of organic acids

Three mixtures of standard organic acids were analysed twenty times, and retention times and response factors related to the I.S. (Table I). Precision was good, ranging from 0.5 to 3.2% for relative retention times and 1.3 to 7.8% for relative response factors. A further twenty-one clinically relevant acids and glycine conjugates were also analysed. Conjugation with glycine enhanced the response factor at 210 nm, for example relative response factors of propionic acid and propionyl glycine were 0.006 and 0.109, of isovaleric acid and isovalerylglycine 0.008 and 0.129 and of benzoic acid and hippuric acid 0.432 and 1.148, respectively.

Recoveries, linearity and detection limits

The recovery of twelve clinically important acids from spiked plasma and urine was measured (Table II). The method was shown to be linear for nine acids tested.

TABLE I

MEAN RELATIVE RETENTION TIMES AND RELATIVE RESPONSE FACTORS OF ORGANIC ACIDS

Column conditions: temperature, 60 °C; eluent, 5 m*M* sulphuric acid; flow-rate, 0.8 ml/min.

Acid	RRT*	C.V.** (%)	RRF***	C.V.** (%)
Citric	0.192	2.8	0.031	2.3
Orotic	0.199	1.9	1.272	7.8
Pyruvic	0.229	1.0	0.095	2.0
Methylmalonic	0.257	2.8	0.021	2.5
Succinic	0.278	2.3	0.010	5.8
Ethylmalonic	0.283	0.7	0.024	1.3
Lactic	0.308	3.2	0.009	3.1
2-Oxoisocaproic	0.314	2.3	0.179	2.5
4-Hydroxyphenylpyruvic	0.314	1.6	0.869	1.6
Glutaric	0.315	0.8	0.012	1.3
Uric	0.326	1.5	0.655	7.0
3-Hydroxybutyric	0.333	0.9	0.007	1.5
Acetoacetic	0.336	2.6	0.015	7.1
Adipic	0.363	1.5	0.014	7 5
Propionic	0.430	0.9	0.006	5.5
4-Hydroxyphenyllactic	0.525	0.9	0.992	4.8
Isovaleric	0.600	3.0	0.008	4.9
Suberic	0.646	0.7	0.014	6.3
4-Hydroxyphenylacetic	0.699	0.5	0.935	7.3
Hippuric	0.871	1.0	1.148	5.3
I.S.	1.000		1.000	

*Retention time relative to 3-(4-hydroxyphenyl)propionic acid (internal standard, I.S.).

**Coefficient of variation; $n=20$.

***Response factor at 210 nm relative to I.S.

TABLE II

RECOVERY OF ORGANIC ACIDS

Acid	Spiked urine		Spiked plasma	
	Recovery (%)	C.V. (n=6) (%)	Recovery (%)	C.V. (n=6) (%)
Orotic	92	5.2	67	20.9
Pyruvic	119	9.5	107	14.9
Methylmalonic	109	4.8	133	13.4
Lactic	102	26.7	106	22.0
3-Hydroxybutyric	–*	–*	61	28.7
Propionic	66	6.6	74	14.1
4-Hydroxyphenyllactic	104	7.1	119	8.9
Isovalerylglycine	109	5.2	106	7.7
2-Hydroxyphenylacetic	84	7.2	104	13.1
4-Hydroxyphenylacetic	110	2.6	115	5.1
Homovanillic	95	3.1	105	9.8
Hippuric	111	5.8	127	10.0

*Interfering substance in urine blanks.

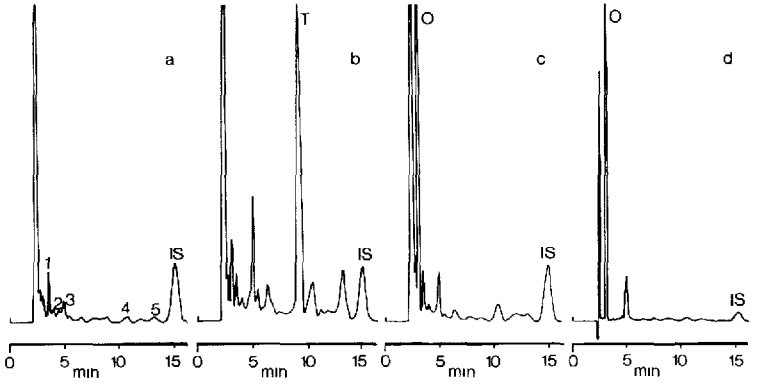

Fig. 2. Urinary organic acid profiles. Column temperature, 60°C; eluent, 5 mM sulphuric acid; flow-rate, 0.8 ml/min; detector wavelength, 210 nm (a–c), 280 nm (d). (a) Normal profile from a neonate aged five days. Identifiable acids: 1, pyruvic; 2, lactic; 3, uric; 4, 4-hydroxyphenylacetic; 5, hippuric. (b) Profile from a twenty-day-old neonate with propionic acidaemia. The abnormal peak T was identified tentatively as tiglylglycine. (c) and (d) Profiles from an eight-year-old girl with untreated ornithine transcarbamylase deficiency. A large peak of orotic acid, O, was detected at 210 nm (c) and 280 nm (d).

Detection limits in urine varied among the acids and were a function of their response and capacity factors, e.g. 4-hydroxyphenylacetic 2 μmol/l, 4-hydroxyphenyllactic 3 μmol/l, orotic 4 μmol/l, pyruvic 33 μmol/l, lactic 100 μmol/l, methylmalonic 167 μmol/l and 3-hydroxybutyric 300 μmol/l.

Clinical studies

Normal urinary and plasma profiles were established for babies and young 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 pre-extraction. Other unidentified peaks were a constant feature and were recognised as part of the normal 'pattern'. Conditions were adjusted to reduce the signal from normal acids occurring at low concentration, thus simplifying the profile (Fig. 2a). Abnormal peaks were seen in urine from patients with eleven different inherited organic acidurias previously confirmed by GC–MS. In the case of isovaleric acidaemia, the abnormal peak was the glycine conjugate of the parent acid. In samples from two children with propionic acidaemia, and a third with 3-ketothiolase deficiency, the major abnormal peak was identified tentatively as tiglylglycine (Fig. 2b), by comparison with GC–MS findings. Orotic acid was clearly increased in urine from three patients with urea cycle defects (Fig. 2c) and was further characterised by its absorbance at 280 nm (Fig. 2d). Plasma profiles were grossly abnormal for babies with fumarase deficiency, maple syrup urine disease and methylmalonic aciduria.

DISCUSSION

Using the short fermentation monitoring column (150 mm × 7.8 mm) at 60 °C, analysis of organic acids was achieved with a resolution equal to that of Rumsby et al. [6] with the Aminex HPX-87H column (300 mm × 7.8 mm) at 50 °C, but in a shorter time. The retention times of 3-(4-hydroxyphenyl) propionic acid (I.S.) were 16.08 ± 0.03 min and 39.2 ± 1.01 min (mean ± 1S.D.), respectively. The elution order of the acids was similar. Analytical performance of the column was satisfactory with respect to linearity over the ranges tested, and the relative retention times of the compounds showed little day-to-day variation. Recoveries were acceptable with the exceptions of 3-hydroxybutyric and propionic acids. As both these acids have a very low absorbance at 210 nm, integration errors probably account for the findings. Volatility of propionic acid may have been contributory. Failure to adjust urine pH to 7.0 before fractionation on the LC-SAX columns [6] was not the cause.

Improved resolution of organic acids can be achieved by dual-column HPLC [9–13], and sensitivity improved by forming fluorescent organic acid derivatives [14]. These complex modifications, however, are unsuitable for routine use in hospital laboratories. Inclusion of an additional detector system, such as amperometric [15] or photodiode array [11,16], assists in the identification of unknown compounds, but is unnecessary if abnormal samples are subsequently analysed by GC–MS.

The method described is potentially useful as a screening procedure for inherited organic acid disorders and its applicability to plasma is advantageous when investigating acutely ill neonates. Deviations from a normal profile occurred in samples from patients with a range of diagnosed organic acidurias, where detection of both aromatic and aliphatic metabolites would have prompted further investigation. GC–MS, however, should be the first line of investigation for suspected defects of fatty acid oxidation, in view of the poor response for diagnostically important dicarboxylic acids. The low recovery of propionic acid is less important in propionic acidaemia since other UV-absorbing compounds, notably

tiglyglycine, are excreted in increased amounts. Drugs, for example paracetamol, salicylate and valproate, can cause interferences during profiling [6], but improvements in HPLC–MS techniques should help to overcome some of these problems [17].

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