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Sample preparation with an automated robotic workstation for organic acid analysis by gas chromatography–mass spectrometry

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Abstract

We attempted to automate sample preparation for analysis of organic acids by gas chromatography–mass spectrometry using a computer-controlled, automated robotic workstation that is integrated and connected to the gas chromatography–mass spectrometry (HP-5890/5971) system. Of the two methods developed, one employed solvent extraction, while the other utilized a silica, solid-phase extraction cartridge. Both automated methods were compared to a manual, solvent extraction procedure used routinely in our laboratory. Normal, spiked urine, and urine from patients with a variety of metabolic abnormalities were analyzed. The robotic workstation did not meet all our requirements for a rapid, reliable, laboratory device. Recoveries with the automated procedures were less than with the manual method, and some organic acids important in the diagnosis of inborn errors of metabolism were not detected. Additionally, the robotic device had mechanical and design problems that made it slower and less reliable than the manual procedure.

Keywords: Automated robotic workstation; Robotic workstation; Organic acids

1. Introduction

Analysis of urinary organic acids is used for the diagnosis of a group of hereditary diseases known as the organic acidurias [1]. Gas chromatography–mass spectroscopy is the method of choice for the determination of trace levels of organic acids in urine samples. However, extensive sample preparation prior to GC–MS analysis is required. Currently several manual sample preparation methods are employed including solvent extraction (SE) [2], ion-exchange chromatography [3], and solid phase extraction (SPE) [4]. All of these methods are time consuming and labour intensive. Analysis and interpretation of organic acids have recently been reviewed [1].

There are several advantages to be gained by automating analytical methods. In automated procedures all samples are processed in an identical manner whereas, analytical variability is usually greater with manual methods, and results may vary with the analyst. Automation allows decreased exposure to toxic chemicals, cost savings in staff salaries, and the analyst is able to spend more time on other duties, increasing the efficiency of the laboratory.

Sample preparation is often the most time consuming part of any analysis, particularly where complex biological matrices need to be tested. It has been automated in a wide range of methods, including analysis of CGS 10787B (a non-steroidal anti-inflammatory drug) in plasma [5], opiates in plasma

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[6], glycohemoglobin in plasma [7], the antiparasitic agent ivermectin in animal plasma [8], and fungal mycotoxin ochratoxin A in cereal [9]. The use of robotic instruments for dilution, mixing, vortex-mixing etc. to reduce the amount of on-hand manipulation before the analysis has also been described. These include Solid phase Micro Extraction (SPME) [10], Gilson Automatic Sample Preparation with Extraction Column system (ASPEC) system [11], Automated Sequential Trace Enrichment of Dialysate (ASTED) system [6], Hamilton Microlab 2200 Automated Pipetting Cartesian Robot [7], and Zymate Laboratory Automation Systems [8].

Until recently, only simple tasks in the pre-analytic work-up of a sample have been automated. A combination of steps involving a variety of purification, concentration and separation techniques have not been automated to date, presumably because of the complexity of the procedures and the problems involved in the integration and programming of the various steps. We recently investigated the performance of an automated robotic workstation to determine if a complex manual procedure could be automated without loss of sensitivity, specificity and flexibility. The robotic device, the Hewlett–Packard 7686 Prepstation, is designed to automate the preparation and analysis of samples for GC–MS. It is connected to the HP-5890 Series II gas chromatograph. The complete operation of sample preparation, analysis, data acquisition, and processing is computer controlled. A detailed description of the hardware has been published [12].

The aims of our experiments were to automate the sample preparation for urinary organic acid analysis that would be as efficient and reliable as the manual solvent extraction currently employed in our laboratory, but would also allow operation of the system in an unattended 24 h a day fashion.

2. Experimental

2.1. Materials

Hexane, chloroform, 2-methyl-2-butanol, sodium chloride, and sodium sulphate were purchased from BDH Chemicals (Toronto, Canada). Ethyl acetate, ethyl ether, and short range pH paper were acquired from Fisher Scientific (Toronto, Canada). Lactic acid, adipic acid, 2-ketoglutaric acid, citric acid, and pentadecanoic acid were obtained from Sigma–Aldrich Chemical (Mississauga, Canada). Glutaric acid was from Aldrich (Milwaukee, WI, USA), and *n*-tetracosane (C_{24}) from Alltech Associates (Deerfield, IL, USA). *N,O*-Bis(trimethylsilyl)trifluoro-acetamide-trimethylchlorosilane (BSTFA-TMCS) was purchased from Chromatographic Specialties (Brockville, Canada). Cartridges containing 300 mg silica (G1204-62103) were obtained from Hewlett–Packard (Mississauga, Canada).

2.2. Samples

Urine specimens were collected from a normal volunteer and from several patients with inborn errors of metabolism. The normal urine specimen was spiked with 500 nmol each of lactic acid, adipic acid, glutaric acid, 2-ketoglutaric acid, and citric acid in a volume of urine containing 0.25 μmol of creatinine. Abnormal urinary organic acids analyzed were from patients suffering from medium chain acyl-CoA dehydrogenase (MCAD) deficiency, ornithine transcarbamylase (OTC) deficiency, and lactic acidemia. Urine from a patient suffering with seizures who was being treated with valproic acid (VPA) was also studied.

2.3. Automated sample preparation

We developed two different types of automated sample preparation methods. One was based on solvent extraction (SE) while the other was a solid phase extraction (SPE) procedure. Both of the automated sample preparation methods were compared to the manual solvent extraction method. Following the sample preparation, the organic acids were analyzed by an HP 5890/5971 GC–MS system in a completely integrated, automatic mode, linked to the HP-7686 Prepstation (Hewlett–Packard, Wilmington, DE, USA).

2.4. Set-up for automated sample preparation

For both the manual and automated sample preparation methods, the steps listed below were done by hand. The creatinine level was estimated by a

refractometer by measuring specific gravity. Pentadecanoic acid (PDA) (500 nmol) was added to each sample as the internal standard. Keto acids were oximated by adding 0.05 ml of 1.0 mol/l hydroxylamine–HCl to 1.0 ml of urine and heated at 60°C for 30 min prior to extraction. Because of sample vial limitations, prior to automated extraction and derivatization, the pH of each sample was adjusted to 8.0 with 3 *M* NaOH. The sample was lyophilized overnight, redissolved in 0.200 ml of 2 *M* HCl, transferred into a 1.8-ml sample vial, and placed in the automatic sampler tray of the HP-7686 Prepstation.

2.5. Manual solvent extraction

A volume of urine containing 0.25 μmol creatinine (anywhere from 0.1 to 1.0 ml of sample) was diluted, if necessary, to 1.0 ml with water, acidified with 1 *M* HCl to a pH of about 1.0, saturated with NaCl, then extracted with 3 ml of ethyl acetate and 3 ml of diethyl ether. The organic phases were combined and evaporated to dryness under nitrogen as described by Tanaka et al. [2]. The sample was derivatized with 0.100 ml of BSTFA-TMCS at 65°C for 10 min, diluted with 0.400 ml of hexane/ethyl acetate (50%, v/v), and 1 μl was injected into the GC–MS and analyzed.

2.6. Automated solvent extraction sample preparation

Ethyl acetate (0.700 ml) was dispensed by the Prepstation into the sample vial, mixed by the bar code reader/mixer, then the device paused for a 1–2 min to allow the phases to separate. The organic layer was then aspirated and transferred into an empty vial. This cycle was repeated twice with ethyl acetate and once with diethyl ether. After the last extraction, 500 nmol of tetracosane in 0.100 ml of hexane was added to the organic phase and the combined organic phase was evaporated to dryness under nitrogen. The sample was derivatized with 0.100 ml of BSTFA–TMCS at 65°C for 10 min, diluted with 0.400 ml of hexane–ethyl acetate (50:50, v/v), and 1 μl was injected into the GC–MS and analyzed.

2.7. Automated solid-phase extraction sample preparation

The sample was aspirated into the sample loop of the Prepstation and applied to the top of a 300-mg silica cartridge. Air was blown through the cartridge to remove excess solvent and the sample was allowed to equilibrate for 15 min. The organic acids were eluted from the cartridge with 2 ml of 45% (v/v) 2-methyl-2-butanol in chloroform. The solvent was applied from the bottom of the cartridge to create a backflush pathway. By applying the solvent through the bottom of the cartridge, the elution path of the organic acids was reduced: hence, less solvent was required for the elution. After the elution was completed, 500 nmol of C_{24} in 0.100 ml of hexane was added, the sample was evaporated to dryness, derivatized in 0.100 ml of BSTFA–TMCS at 65°C for 10 min, diluted with 0.400 ml of hexane–ethyl acetate (50:50), then 1 μl was injected into the GC–MS in splitless mode and analyzed. This method using a silicic acid SPE column was modeled on the method of Hoffmann et al. [13].

2.8. Gas chromatographic, mass spectral and data analysis

Samples were analyzed by GC–MS by injecting 1 μl of the sample in splitless mode onto an open tubular glass capillary column (SPB-1, 30 m, 0.25 mm I.D., 0.25 μm coating, made by Supelco and purchased from Sigma–Aldrich, Mississauga, Ont.), and the injector was kept at 250°C. The carrier gas was helium, with a flow-rate of 1 ml/min. The GC oven was held at 90°C for 4 min, then raised at 8°C/min to 300°C, and maintained at that temperature for 4 min. The peaks were identified by reference to a mass spectral library. Each sample was analyzed repeatedly to allow calculation of the reproducibility of each method. Absolute recoveries were calculated from the sum of ion abundances, or total ion current (TIC) divided by the TIC of the external standard, tetracosane. Relative recovery was estimated by dividing the TIC of each compound with the TIC of the internal standard, PDA. Methods for identification and quantitation or organic acids from biological samples exist. These usually employ selected ion monitoring (SIM mode), standard curves

where compounds are identified and quantitated by spectra and retention time. This was not necessary in this study, since the performance of the Prepstation and the automated procedures could be evaluated and compared with the existing method by simply using area ratios.

3. Results

Specimens of either normal urine spiked with lactic acid, adipic acid, glutaric acid, 2-ketoglutaric acid, and citric acid, or pathological urine samples, were analyzed for organic acid content. Three different methods of sample preparation were used: manual solvent extraction, automated solvent extraction, and automated solid-phase extraction (SPE). The prepared samples were analyzed by HP 5890/5971 GC–MS. A representative chromatogram of the same sample analyzed by the three different procedures is shown in Fig. 1. The relative recoveries of selected compounds are listed in Table 1. The numerical value given is a ratio of the area of a particular organic acid divided by the area of the internal standard, PDA.

A total of 18 representative organic acids were detected and are shown in Table 1. Some of these compounds were present in the spiked normal urine (citrate, glutarate, 2-ketoglutarate, and lactate). Others were detected in abnormal urines. These were: 5-hydroxyhexanoate, hexanoylglycine, phenylpropionylglycine, and suberylglycine from the MCAD urine; valproate, 3-hydroxydipropylacetate, 3-hydroxyphenylacetate and 2-propylglutarate from the patient on valproate therapy; orotic acid from the patient with OTC, and 4-hydroxyphenylacetate and 3-phenyllactate from the patient with lactic acidemia.

The absolute recovery of the external standard, tetracosane (C_{24}), was very similar for each sample indicating that the performance of the GC–MS part of the analysis and the injection did not contribute to the variability found between the manual and the different automated extraction procedures (data not shown).

With the automated solvent extraction procedure, absolute recoveries of all compounds including the internal standard, pentadecanoic acid, ranged from 25–75% of the amounts detected with the manual procedure. This estimate was based on the ratio of the various compounds to C_{24}, which was constant in all three samples. The relative proportions of the various compounds were similar to those observed in the manual method.

Absolute recoveries with the automated SPE method were even less than with the SE procedures (ranging from 11 to 45%), and the compounds had a different distribution from the manual procedure: the relative recovery of polar compounds was higher. Highest relative recoveries were observed with citrate, urea, and phosphate. These observations are consistent with the data of Hoffmann et al. [13], who studied the elution characteristics of organic acids from silica columns in detail.

4. Discussion

The usefulness of organic acid analysis depends on its ability to diagnose inborn errors of metabolism. To do this, the method must be reliable, reproducible, fast, and it must be able detect changes in the concentration of organic acids that are markers for the many diseases one is attempting to diagnose. The use of the HP-7686 Prepstation for automated preparation of urine samples for organic acid analysis did not meet all of these requirements. While the sensitivity of detection of some organic acids was equal to the manual procedure, the detection of specific compounds important in the diagnosis of fatty acid oxidation defects (5-hydroxyhexanoic acid, hexanoylglycine, etc) was less reliable, or the sensitivity was reduced.

The inability to detect 5-hydroxyhexanoic acid or other similar compounds by either one of the automated sample preparation methods may be due to losses during the sample work-up prior to the automated part of the procedure, poor solubility or extractability, or losses during lyophilization. Extraction efficiency from an aqueous phase into a non-polar solvent depends on the partition coefficient of the compound in question, the relative volume of each phase used for extraction, and the number of extractions performed. We used the same solvents both in the manual and automated solvent extraction procedures. Differences in extraction efficiency between the two methods therefore depended on the

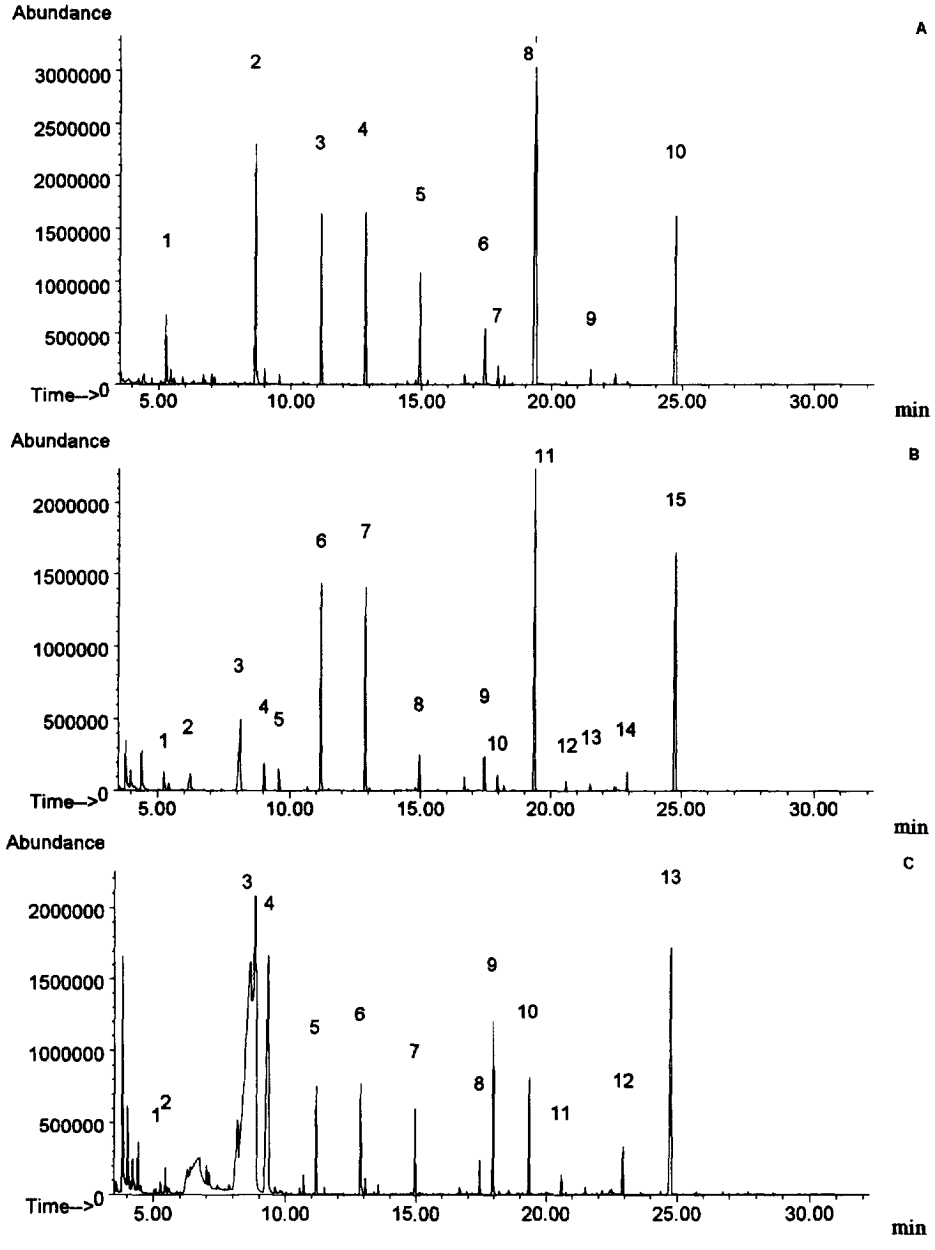

Fig. 1. (A) Spiked normal urine analyzed following manual solvent extraction. The numbered peaks correspond to the following compounds: 1, lactic acid; 2, 2-ketovaleric acid (added as a second I.S. in some runs); 3, glutaric acid; 4, adipic acid; 5, 2-ketoglutaric acid; 6, hippuric acid; 7, citric acid; 8, pentadecanoic acid (I.S.); 9, 4-hydroxyhippuric acid; 10, tetracosane (C_{24}). (B) Spiked normal urine analyzed following automated solvent extraction. 1, lactic acid; 2, unidentified peak; 3, urea; 4, phosphate; 5, succinic acid; 6, glutaric acid; 7, adipic acid; 8, 2-ketoglutaric acid; 9, hippuric acid; 10, citric acid; 11, pentadecanoic acid (I.S.); 12, palmitic acid; 13, 4-hydroxyhippuric acid; 14, stearic acid; 15, tetracosane (C_{24}). (C) Spiked normal urine analyzed following automated SPE extraction. 1, lactic acid; 2, glycolic acid; 3, urea; 4, phosphate; 5, glutaric acid; 6, adipic acid; 7, 2-ketoglutaric acid; 8, hippuric acid; 9, citric acid; 10, pentadecanoic acid (I.S.); 11, palmitic acid; 12, stearic acid; 13, tetracosane (C_{24}).

Table 1
Recoveries of selected organic acids prepared by manual and automated extraction procedures

Compound	Manual solvent extraction (*n*)	Automated solvent extraction (*n*)	Automated solid phase extraction (*n*)
5-Hydroxyhexanoic acid	1.58 (2)	N.D. (4)	N.D. (3)
Valproic acid	0.16 (2)	0.10±0.01 (4)	0.23±0.096 (3)
3-Hydroxydipropyl acetic acid	0.37 (2)	0.20±0.02 (4)	N.D. (4)
3-Hydroxyphenyl acetic acid	0.13 (2)	0.08±0.004 (4)	N.D. (4)
4-Hydroxybenzoic acid	0.08 (2)	0.06±0.006 (4)	N.D. (4)
Hexanoylglycine	0.21 (2)	0.09±0.01 (4)	0.10±0.001 (4)
Phenyl propionyl glycine	0.64 (2)	0.26±0.02 (4)	0.21±0.02 (4)
Suberylglycine	2.43 (2)	0.90±0.10 (4)	1.00±0.09 (4)
Lactic acid	0.10±0.014 (3)	0.04±0.01 (5)	0.11±0.02 (5)
2-Propylglutaric acid	0.08 (2)	0.15±0.01 (4)	0.14±0.02 (3)
3-Hydroxyadipic lactone	0.03 (2)	0.08±0.014 (3)	N.A.
4-Hydroxyphenyl acetic acid	1.21 (2)	1.77±0.18 (3)	N.A.
3-Phenyllactic acid	1.30 (2)	1.66±0.25 (3)	N.A.
Orotic acid	0.19 (2)	N.A.	0.28 (2)
Citric acid	0.04±0.002 (3)	0.09±0.05 (5)	1.90±0.26 (5)
2-Ketoglutaric acid	0.05±0.006 (3)	0.13±0.05 (5)	0.76±0.06 (5)
Glutaric acid	0.35±0.04 (3)	0.54±0.04 (5)	1.00±0.06 (5)
Adipic acid	0.37±0.04 (3)	0.55±0.06 (5)	1.08±0.07 (5)

Peak areas relative to that of pentadecanoic acid (internal standard), of selected metabolites found in urine.Samples were analysed and relative recoveries calculated based on the ratio of each compound to the I.S., pentadecanoic acid. Resullts are given as mean±S.D., and each sample was run *n* times to allow estimation of reproducibility. The compounds presented in this table were from different urine specimens. N.D.=not detected; N.A.=not analyzed.

respective volumes of solvent and aqueous phase used, and the number of extractions performed. In the automated solvent extraction procedure we were limited by the size of the vial that the Prepstation was able to handle (total volume of only 1.8 ml). Although we reduced the size of the aqueous phase to 0.2 ml to improve the extraction efficiency in the automated procedure, some compounds were not detected. This may have been due to losses during lyophilization (less likely), or to lesser amounts of a poorly soluble compound dissolving in a smaller volume of sample, leading to smaller amounts being present during analysis by GC–MS. Since the detection limits of the analytical part of the procedure were the same for all three methods, the lesser amounts extracted in the automated solvent extraction procedure resulted in lack of detection of some compounds.

The manual and automated solvent extraction worked best for less polar organic acids. In general, these compounds had only one acid functional group and a relatively large non-polar section of the molecule. Examples of acids well extracted by solvent extraction were 4-hydroxyphenylacetic acid, 3-phenyllactic acid, 3-hydroxydipropylacetic acid, and 3-hydroxyphenylacetic acid. These acids were poorly extracted by the SPE sample preparation, or not extracted at all.

The glycine-containing compounds (hexanylglycine, phenylpropionylglycine, suberylglycine) detected in the MCAD urine sample had relative recoveries by automated methods that were approximately half those for the manual sample preparation. This may be due to losses during lyophilization or to difficulty in redissolving these compounds in a small volume of acid.

Polar acids such as lactate, citric acid, orotic acid, glutaric acid and 2-ketoglutaric acid had equal or better recoveries with the SPE sample preparation when compared with either manual and automated solvent extraction methods. The recovery of compounds from the automated SPE procedure depends on how well each substance adsorbs to the silica, and how efficiently the solvents elute the compounds from the SPE column. The Prepstation could only use SPE columns supplied by the manufacturer, Hewlett-Packard. The fixed size (only 100 mg or 300 mg sizes were available) of the SPE columns was

also a limiting factor in making the procedure flexible. The original manual, silicic acid procedure of Hoffmann et al. [13] used larger amounts of silica for adsorbing the compounds and larger volumes of solvent for elution, which probably contributed to better recoveries by their method.

The detection of large amounts of urea and phosphate may create problems, since they elute at a region of the chromatogram that sometimes contains diagnostically important compounds. Others have circumvented this type of interference by enzymatic pretreatment of the sample with urease to remove urea [14], or by stopping the elution of organic acids from silica before urea and phosphate elute [13]. Given the limitations of the system we were using, this last option did not seem feasible. Pretreatment of the sample with urease is obviously a possibility, and Shoemaker and Elliott has published a manual method utilizing this approach [14].

The reproducibility of the automated sample preparation procedure was similar to that of the manual method, hence, considered to be satisfactory. The reliability of the Prepstation was less than desirable due to a variety of design features.

After prolonged use of the SPE backflush sample preparation method with a silica cartridge and 45% (v/v) 2-methyl-2-butanol in chloroform as solvent, small particles of dissolved silica precipitated in the flow lines, preventing the instrument from functioning. This occurred despite the fact that the flow lines were being rinsed with methanol between each sample.

The manual solvent extraction was faster per sample than either of the two automated sample preparation methods, mainly because the robotic device was capable of processing only one sample at a time, whereas the manual method allowed batch processing of several samples simultaneously. Also, each sample had to be evaporated twice due to the small sample size of the Prepstation vials (1.8 ml). Preparation of one sample by either of the two automated processes required 3 h, whereas one technician could process 5–10 samples in 1 h (not including the GC–MS runs) using the manual solvent extraction method. The Prepstation was not able to start processing a second sample till the first sample was completely finished, including GC–MS analysis. The inability to handle several samples at once made the use of the automated robotic analyzer much slower that the manual procedure for the analysis of organic acids.

In spite of its limitations, we were able to automate the sample preparation of urinary organic acid analysis using the HP 7686 Prepstation. Improvements in speed, reliability and better design might make such an automated robotic workstation an attractive and feasible alternative to manual sample preparation of urinary organic acids.

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