Profiles of Urinary Volatiles from Metabolic Disorders Characterized by Unusual Odors

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The profiles of urinary volatiles from patients with phenylketonuria, maple syrup urine disease, isovaleric acidemia, or trimethylaminuria (fish-odor syndrome) were in each case vastly different from the normal urinary volatiles profile. In the maple syrup urine disease case, metabolites that occur distal to the block were found and a mechanism for their formation is suggested. A new major metabolite in isovaleric acidemia was also found. As well as providing a reliable diagnostic tool for diseases characterized by odors, the analysis of urinary volatiles may provide information to help our understanding of still unexplained aspects of the diseases.

Additional Keyphrases: inborn errors of metabolism, phenylketonuria, maple syrup urine disease, isovaleric acidemia, trimethylaminuria, chromatography, gas-liquid volatile metabolites, headspace analysis, pediatric chemistry

Clinicians frequently associate peculiar body odors with a disease state; for several disorders, the odors are distinctive enough to be diagnostic (1–3). The first phenylketonuric (PKU) patients were recognized because the children's mother complained of a musty or mousey odor, now known to be due to the presence of phenylacetic acid in the urine. Maple syrup urine disease (MSUD) can be readily distinguished from other disorders because of the characteristic odor of the urine, and isovaleric acidemia is unmistakable because of the odor of "sweaty feet" (4).

Despite the apparent utility of an assessment of patient odor, little work has been done to define these diagnostic criteria, either qualitatively or quantitatively. In some cases the identity of the compound or compounds responsible for the odor is unknown, the relevant technology for isolating and identifying volatile compounds having been described only in the early 1970s.

Despite considerable variation in volatiles profiles among individuals, the normal urinary profile is well characterized (5, 6). Metabolites present can include ketones, alcohols, furans, pyrroles, and sulfur compounds. Diagnostic patterns are known for several disease states despite the wide reference interval, but these patterns are evident only after considerable computer processing of the chromatographic data (7).

We used the analytical system developed by Murray (8) to profile the volatile metabolites in the urine of patients with MSUD, PKU, isovaleric acidemia, and trimethylaminuria, each of these disorders being associated with a characteristic odor. We made no attempt to correlate odor of the patient with the volatiles profile; rather, the odor merely served to direct attention to the possibility of obtaining some useful information from the volatiles complement.

Materials and Methods

The volatiles trap, the method of extraction of volatile compounds from aqueous solutions, and their subsequent transfer to the gas chromatograph (GC) are described in detail by Murray (8).

As the technique was developed, we found that representative profiles could be obtained using less urine than we had thought; thus the Figure legends show that 40–50 mL of urine was used for early work, but only 2–5 mL for later profiles.

A Varian 1800 GC (Varian Associates Inc., Walnut Creek, CA 94598) was modified to incorporate the thermal desorption and cryogenic recondensation apparatus and was interfaced through a single-stage glass jet separator to a mass spectrometer (Quadrupole 300D, Electronics Associates, West Long Branch, NJ 07764). Though we manufactured specialized equipment ourselves, similar apparatus can now be obtained through Alltech Associates Inc., Deerfield, IL 60015, or S.G.E. Inc., Austin, TX 78758.

A Carbowax-support-coated open tubular column (S.G.E., Melbourne), 1.0 mm × 0.5 mm × 50 m with an efficiency of 36 800 (effective theoretical plate no.), was used in all profiling work. The carrier gas was helium (99.5% purity grade) at a flow rate of 5 mL/min.

The GC temperature program was slightly adjusted in each case to obtain the most compact profile consistent with adequate resolution. In each case, however, we first tried a general temperature program of 70°C for 8 min, then increasing by 3°C/min to 200°C, which was adequate to reveal a diagnostic profile of urinary volatiles.

Results and Discussion

The metabolic and genetic backgrounds underlying PKU, MSUD, and isovaleric acidemia are detailed in texts on inherited metabolic disease (4, 9, 10). Trimethylaminuria is discussed in the literature (11–13). Metabolic disorders are characterized by the accumulation of a small number of metabolites in body fluids, generally because a deficiency in enzymatic activity blocks the normal biochemical pathway. The disorders are usually diagnosed by the detection of increased concentrations of these metabolites.

Some of these diseases are so rare that few cases are available for study. MSUD, for example, has an incidence of only 1 in 200 000 live births; only one diagnosed case of this disease was available to us. Thus we do not claim that our volatiles profile is necessarily characteristic of all sufferers of the disorder, merely that for the sample we analyzed the profile was distinct enough to be classifiable as abnormal.

A typical profile of volatiles from urine of a healthy

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5 Nonstandard abbreviations: PKU, phenylketonuria; MSUD, maple syrup urine disease; GC, gas chromatography; MS-MS, gas chromatography–mass spectroscopy.

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individual is shown in Figure 1. This profile, obtained in our laboratory, was highly reproducible, 20 control runs on different urine samples taken from the same subject over a period of three years showing only minimal variation. Because our aim was to investigate only profile differences between normals and individuals with inborn errors of metabolism, it was not essential to establish the identity of each GC peak. Some large peaks in this profile, however, were readily matched with library spectra (14) and are listed in Figure 1. As can be seen, therefore, our procedure gives results comparable with previously published work on normal subjects (5–7).

Profiles of Urinary Volatiles from Patients with PKU

Urine samples from untreated phenylketonurics were obtained from patients (ages 16–25 years) in an institution for the mentally retarded. The disorders were detected during a screen of such institutions for inborn errors of metabolism and the diagnosis was based on high concentrations of phenylalanine in blood.

Figure 2 is typical of the urinary volatiles profiles, at acid pH, of four PKU patients. There were only four major peaks—4-heptanone, benzaldehyde, p-cresol, and phenol—and a minor peak for benzoic acid. The chromatogram of the neutral (pH = 5) urinary volatiles (Figure 3) showed only a single major peak, the mass spectrum and retention time of which were the same as for an authentic sample of benzaldehyde.

The excretion rate of 4-heptanone, a normal constituent of urinary volatiles, is between 10 and 30 \( \mu \)g per 24 h, although the amount detected increases on heating the urine sample (15). The amounts we detected in PKU patients were not abnormal.

Benzaldehyde has not been reported as a characteristic urinary metabolite in PKU but is a normal constituent of the urinary volatiles (5). Normal reference intervals have not been established; however, the amounts detected in our cases (about \( 10^{-4} \) mol/L) were at least an order of magnitude greater than the amounts we detected for normal individuals. It is possible that free benzaldehyde arises from benzoyl CoA, an intermediate in the enzymatic formation of hippuric acid from benzoic acid and glycine. The increased concentration of benzoic acid in phenylketonurics may lead to an increased concentration of urinary benzaldehyde.

![Fig. 1. Profile of urinary volatiles from a healthy subject](image1)

![Fig. 2. Acidic urinary volatiles profile from an untreated PKU patient](image2)

![Fig. 3. Neutral urinary volatiles profile from an untreated PKU patient](image3)

Urinary phenol and p-cresol have been reported as bacterial degradation products of unabsorbed tyrosine and are normally excreted at a combined rate of about 50–100 mg/day (16, 17). Their occurrence in the urine of PKU patients is to be expected, the amount of unabsorbed tyrosine in the intestine of these patients being comparable with that in normals.

Benzoic acid has been reported in the urine of patients having high serum and urine concentrations of phenylalanine. Its main source has been suggested to be from bacterial degradation of unabsorbed phenylalanine in the intestinal lumen (18, 19).

The commonly observed urinary metabolites of PKU patients (phenylpyruvic and phenylactic acids) were not detected by our headspace analysis.

The distinctive feature of the volatiles profiles of PKU patients was the predominance of a small number of large
peaks: benzaldehyde in neutral urine (pH 5), and the phenolics in acidic urine (pH 1). This contrasts sharply with the large number of smaller peaks in the volatiles profiles of normal urines.

Urinary Volatiles from the MSUD Patient

At the age of eight days, patient S.W. was diagnosed as suffering from MSUD by clinicians at the Oliver Latham Laboratory, North Ryde, Australia. She had increased concentrations of plasma leucine and valine and of urinary organic acids, but no unusual odor was reported. Dietary treatment gave some control over the disease, but she occasionally presented with acidic traumas. We obtained urine samples for volatiles analysis on such occasions, when she was between 19 and 25 months old.

A typical profile of urinary volatiles from this patient is shown in Figure 4. The difference between this chromatogram and that from a normal control of the same age is striking. The normal "volatiles finger print" is characterized by numerous small peaks, whereas her profile is dominated by a few large peaks eluting at the higher temperatures of the chromatogram.

Preparative capillary GC followed by GC-MS analysis of the collected fractions showed the identities listed in Figure 4. The presence of isovaleric and 2-methylbutyric acids is most surprising because these compounds are products of the enzymatic activity that is defective in this disorder. Similar profiles were obtained from samples of this patient's urine on two other occasions (once after treatment for a urinary-tract infection), and control experiments to test whether these compounds could be produced as artefacts of the experimental procedures were negative. In addition, a separate method for the analysis of short-chain volatile organic acids developed in this laboratory and published separately (20), also showed the presence of these two acids in the urine. The currently accepted method for the screening of urinary organic acids for inborn errors of metabolism (21) does not detect these two volatile acids.

Despite the numerous MSUD case reports in the literature, only our case and one reported by Harkness et al. (22) have confirmed the presence of short-chain fatty acids in the urine. The formation of these compounds could be through bacteria in the gut acting on the increased branched-chain amino and keto acids. The production of volatile fatty acids and the degradation of tyrosine to p-cresol (19) by colonic bacterial species is well known (23). p-Cresol having been found in these urine samples, intestinal flora could also be responsible for degrading unabsorbed branched-chain amino acids to the volatile fatty acids we observed.

The presence of these neurotoxic (24) volatile acids may help to explain the damage to the central nervous system symptomatic of this disease. The difficulty in detecting the volatile acids has been responsible for a misdiagnosis before (25) and could account for their not having been observed in other earlier cases.

Owing to the rarity of this disorder, we were only able to study a single case; confirmation of these two proposals, therefore, must await further case investigations.

Urinary Volatiles from a Patient with Isovaleric Acidemia

This patient's volatiles profile (Figure 5) was dominated by two large peaks. We found that peak 2 corresponded to isovaleric acid; peak 1, the major peak in the chromatogram, we confirmed to be 3-methylbutyrolactone by its synthesis in our laboratory (26). We propose that the lactone is formed in the body fluids from an hydroxy acid precursor, but the proportion of 4-hydroxyisovaleric acid existing in the lactone form in the child's urine is not known, because lactone formation increases on acidification.

Two more cases of isovaleric acidemia have since presented at the Royal Children's Hospital, Melbourne, and their urine samples were analyzed in this laboratory. Both displayed the same volatiles profile as the patient described above, but in addition had a large peak for 4-hydroxyisovaleric acid. We have discussed the origin and significance of this compound in a separate publication (26), but have not attempted to establish any link between this compound and the odor observed in isovaleric acidemia.

The discovery of the new metabolite has lead to a greater understanding of the metabolic pathways involved in leucine catabolism, and we can now state that the presence of both isovaleric acid and 4-hydroxyisovaleric acid or its lactone in the urine is diagnostic of isovaleric acidemia.

Urinary Volatiles Associated with Trimethylaminuria

The volatiles profiles of patients suffering from this disorder were strikingly simple. A single large peak, usually the

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Fig. 4. Profile of urinary volatiles from an MSUD patient
The acid volatiles were extracted from a 25-ml urine sample with use of a 24-L headspace. Temperature program: 70°C for 5 min, then increased 1.5°C/min to 179.5°C, then 179.5°C for 5 min. 1 = breakthrough of isovaleric and 2-methylbutyric acids, 2 = unknown, 3 = mixture of isovaleric and 2-methylbutyric acids, 4 = 2-keto-3-methylvaleric acid, 5 = p-cresol

Fig. 5. Profile of urinary volatiles from a patient with isovaleric acidemia
The volatiles were extracted from a 5-ml urine sample (pH = 1) with use of an 83-L headspace. Temperature program: 70°C for 5 min, 3°C/min to 200°C. 1 = 3-methylbutyrolactone, 2 = isovaleric acid
of the large increase in concentration of blocked metabolites in the body fluids of such patients. When these metabolites are volatile, their concentration in the vapor above the urine sample increases, leading to a headspace dominated by few major components. These compounds then saturate the adsorption trap (27), and the resulting profile is grossly distorted because minor components are prevented from adsorbing or are masked on GC.

Because the adsorbent in this procedure is nonselective (8), this process is applicable over a range of compounds, thus allowing the investigation of many other metabolic disorders involving volatile compounds. As we have shown, using this technique, one can find new and unexpected metabolites that may extend our understanding of the biochemistry of disease states.

References

Fig. 6. Profile of urinary volatiles from a patient with trimethylaminuria. The large peak was identified by GC-MS as trimethylamine. The volatiles were extracted from a 2-mL urine sample (pH 4) with use of a 36-L headspace. Temperature program: 70 °C for 4 min, then increased 2.5 °C/min to 180 °C.
Optimizing Reference Values for the Measurement of Alpha$_1$-Antitrypsin in Serum: Comparison of Three Methods

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We studied three methods (rate nephelometry, radial immunodiffusion, and trypsin-inhibitory capacity) for their ability to detect those individuals with a deficiency of alpha$_1$-antitrypsin. The phenotype represented in 170 serum samples was determined by isoelectric focusing as the reference method. All three methods correctly identified Pi Z, Pi S, and Pi SZ phenotypes but varied in their ability to detect Pi MZ and Pi MS phenotypes. The rate-nephelometric method was the least sensitive in detecting Pi MZ and Pi MS variants because of the inappropriately low reference interval suggested by the manufacturer. We found that the three screening methods are comparable when the limiting values are properly selected. We suggest that the reference value for the rate-nephelometric method be increased from 0.85 g/L to 1.40 g/L to improve the sensitivity of the test.

Additional Keyphrases: rate nephelometry, radial immunodiffusion, trypsin-inhibitory capacity, heritable disorders

Normal reference intervals for clinically important analytes are generally established by measuring the substance in the serum of a sufficient number of normal subjects to obtain statistically representative values. This method may be satisfactory for compounds whose concentration becomes abnormal during a disease process and returns to normal upon recovery. However, when establishing reference intervals for substances related to the presence of a genetic variant, the usual method of identifying the apparently healthy population becomes less meaningful. Rather, it is necessary that the test detect the genetic variants underlying the disease state. A representative of this class of compounds is alpha$_1$-antitrypsin (AAT). In this case the protein concentration in serum is not used as a marker for clinical disease, but rather to screen for those individuals who have inherited a protein variant associated with a deficiency. Therefore, any method for measuring AAT in serum must be tested for its ability to detect those variants related to AAT deficiency, which in turn may predispose the individual to pulmonary and hepatic disease.

The biochemical investigation of suspected AAT deficiency is commonly approached in two stages. The first step, quantitative estimation of the AAT concentration in serum by immunoprecipitin or functional activity methods, is followed by confirmation of the suspected deficiency variant by use of isoelectric focusing to determine the phenotype. Clearly, the screening methods play an important role in sample pre-selection for phenotype determination. Manufacturers of the reagent kits used for the screening methods usually suggest a reference interval that has been established in a population of healthy subjects. Using these suggested reference intervals, we tested the ability of three commercially available screening methods to detect AAT deficiency variants. The methods we studied were rate nephelometry (ICS), radial immunodiffusion (RID), and trypsin-inhibitory capacity assay (TIC).

Materials and Methods

Serum from patients' specimens was sent to us for phenotyping and measurement of AAT concentration or activity. They arrived frozen, and were kept at $-20^\circ$C until analyzed, usually within a week.

The Immunochemistry System (Beckman Instruments Inc., Fullerton, CA 92634) was used to measure AAT by rate nephelometry according to the procedure supplied by the manufacturer. Duplicate determinations were made on all specimens with values lower than 1 g/L. The suggested reference interval of 0.85 to 2.13 g/L was used for detection of deficiency states.

The "M-Partigen" kit (Calbiochem-Behring Corp., La Jolla, CA 92037) was used to measure AAT in serum by the RID endpoint technique, again according to the procedure supplied by the manufacturer. The reference interval of 2.0 to 4.0 g/L was used to classify the samples as normal or deficient.

Serum trypsin-inhibitory capacity was measured spectrophotometrically (1, 2). The reference interval of $>0.7$ g of