Determination of Total Organic Acids in Urine by Extraction with Organic Solvents

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Metabolic carboxylic acids are extracted, with a mixture of equal volumes of t-amyl alcohol–chloroform, from an acidified sample of as little as 200 μl of urine that has been adsorbed to a short column of silica gel. Total acid in the extract is measured by titration with tetramethylammonium hydroxide (10 mmol/liter) to a phenolphthalein end point. There is no interference from protein, creatinine, creatine, bicarbonate, or amino acids, and no pretreatment of the sample is required. Results compared well (r = 0.94) with those obtained by the Palmer–Van Slyke method for total organic acids. This procedure should prove useful as a simple, rapid screening test for those metabolic disturbances and inborn errors of metabolism characterized by increased excretion of organic acids.

Additional Keyphrases: adsorption of urinary acids on, and their elution from, silica gel • titration in nonaqueous medium • inborn errors of metabolism • screening test

The Van Slyke and Palmer method (1, 2), and some of its more recent modifications (3–6) have been used extensively to determine total organic acids in urine and other physiological fluids. These techniques require that interfering substances be removed before titrimetric estimation of acid content: protein is denatured by heat, bicarbonate released as carbon dioxide by acidification, and phosphate precipitated with calcium hydroxide. Greenwald (3) removed other interfering substances, such as creatinine and amino acids, by absorbing them on Lloyd's reagent; other investigators have used a correction factor for these substances (1).

After the interfering substances are removed, the sample is brought to either physiological pH (4) or to a phenolphthalein end point (1), a pH at which most physiological acids are assumed to be completely ionized. The weak organic acids are then measured by back-titrating the solution to a tropaeoline 00 end point (pH 2.7).

Our purpose was to develop a simpler micromethod for estimating organic acids, by selective, quantitative extraction with organic solvents and titration in this nonaqueous medium.

Materials and Methods

Reagents

Silica gel, Woelm, 0.05–0.2 mm particle diameter (Waters Assoc., Framingham, Mass. 01701). This is dried at 110°C for 24 h and stored in a tightly closed jar.

Chloroform, AR, (J. T. Baker Co., Phillipsburg, N. J. 08865; Cat. No. 9180) is used directly.

Tertiary amyl alcohol, “practical” grade t-pentyl alcohol, 100°–103°C (Distillation Products Industries, Eastman Kodak Co., Rochester, N. Y. 14650). Once a month, this solvent should be redistilled over pellets of sodium hydroxide.

Extracting solvent. This consists of equal volumes of t-amyl alcohol and chloroform.

Standard acid, potassium hydrogen phthalate, primary standard, 10 mmol/liter.

Base, tetramethylammonium hydroxide, 10 mmol/liter, prepared by diluting 1 ml of a 10% aqueous solution supplied by Matheson Coleman and Bell (Cat. No. 9930) to 100 ml with 95% ethanol that has been redistilled over sodium hydroxide pellets. This titrant should be standardized frequently with the standard acid.

Indicator, phenolphthalein, 500 mg/liter of 95% ethanol.

Sulfuric acid, 3 molar, aqueous.

Citric acid standard, 50 mmol/liter in water.

Apparatus

Extraction columns. The catalog numbers in the following are those of the Fisher-Porter Co., Warrington, Pa. 18974: 0.9 × 75 cm and 0.5 mm × 25 cm columns (274–738), Teflon seals (571–158), clamps (275–347), end pieces (274–761), and fritted disks (275–263). It is necessary to specify whether these parts are for the 0.9-cm or 0.5-cm column. The description of the pump and its high-pressure connectors may be found in reference 8.

Vibrator. “Home Vibrator,” Model E, Wall Clipper Corp., Sterling, Ill. The tip must be covered with

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one of the plastic ends supplied, to avoid shattering the column.

**Microtitration apparatus.** The organic solvent extract is easily titrated with an apparatus similar to that described by Dole and Meinertz (7). A carbon dioxide-free atmosphere, with continuous agitation of the sample, is produced by use of a fine stream of nitrogen bubbles delivered to the bottom of the sample vessel through a short piece of 18-gauge Teflon tubing. The tubing is held in position by a rubber band attached to the tip of the microburet. The gas is passed through sodium hydroxide (1 mol/liter) before use in the titration assembly, to remove any traces of carbon dioxide. The rate of bubbling should be carefully adjusted to provide rapid mixing without splattering. The microburet (2–5 ml capacity) should be calibrated in 1-μl divisions. Two drops of indicator solution are used for each titration, and the persistence of a pink color for 15 s indicates that the end point has been reached.

Urine should be collected without preservative in an ice bath when a 24-h collection is required, and representative samples should be frozen until analysis.

**Procedures**

Two milliliters of urine is partially frozen in a 100-ml beaker so as to prevent loss of volatile acids, during the following steps. To this is added 100 μl of 3 molar sulfuric acid, followed by 4 g of the oven-dried (110°C) silica gel (a spatula, previously calibrated to deliver 4 g to within 200 mg, is satisfactory and convenient). The mixture is then stirred with a glass rod until a free-flowing powder is formed that does not cling to the walls of the beaker.

The mixture is transferred quantitatively to a 0.9-cm extraction column by touching the lip of the beaker to the top of the column while vibrating the column with the plastic-coated tip of the vibrator. The sample falls into the column and comes to a stable height after less than 3 min of vibration. The column above the silica gel is filled with 50 ml of the extracting solvent and the extract is collected in a 50-ml volumetric flask. Under 2 atm (200 kN/m²) pressure (the usual laboratory air-line pressure), this takes about 10 min. Figure 1 shows how a number of samples may be run simultaneously. The volumetric flask is filled to the mark with extracting solvent. After thorough mixing, 5-ml aliquots of this extract are titrated to a phenolphthalein end point with the 10 millimolar tetramethylammonium hydroxide in ethanol.

The procedure outlined above can be scaled down as follows, to accommodate a sample volume of 0.2 ml. To 200 μl of sample add 10 μl of 3 molar sulfuric acid and 0.5 g of dry silica gel. Mix with a glass rod and, by vibration, transfer the free-flowing powder into a 0.5 × 25 cm extraction column. Extract with 5 ml of the solvent mixture and titrate 2-ml aliquots in duplicate. This micro-scale system requires only a tenth the solvent used for the larger column, and is thus more economical.

Individual samples may be assayed in 2–3 min by use of a single chamber and a high-pressure pump that is available as part of the Automatic Organic Acid Analyzer (8). This configuration is shown in Figure 2.

A blank, in which 2 ml of distilled water is used in place of the sample, must be run with each series of assays, because a column containing 600 microequivalents of sulfuric acid in 2.0 ml of water will yield about 5 microequivalents of acid in the extract. The blank value observed when only sulfuric acid is present in the extraction column may be the result of slow leaching of small amounts of sulfuric acid or titratable impurities from the silica gel, extracting solvent, and (or) sulfuric acid solution.

**Calculation.** The total organic acid concentration of the urine is calculated as follows:

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\text{Total organic acid (mEq/liter) concentration in urine} = \frac{5,000 \times \text{titrant concentration (mEq/liter)} \times \text{[volume (ml) of titrant for 5.0 ml of sample extract] - volume (ml) of titrant for 5.0 ml of blank extract]}{50 \times \text{[volume (ml) of titrant for 2.0 ml of sample extract] - volume (ml) of titrant for 2.0 ml of blank extract}}
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**Results**

The volume of extracting solution used is the volume that extracts the most organic acid without extracting significant portions of the sulfuric acid. Two milliliters of the citric acid standard (50 mEq/liter) was adsorbed onto the silica gel and a 0.9-cm column prepared as described above. The column was then extracted (eluted) with 100 ml of the extracting solvent; the extract was collected in 11 fractions of 5 to 10 ml each. Figure 3 shows the pattern of citric acid
Selective extraction with immiscible solvents has been successfully used for many years to determine long-chain nonesterified free fatty acids in plasma (7). In the procedure described here, the aqueous phase is immobilized on silica gel and selectively extracted (i.e., eluted) by passing a mixture of chloroform and t-amyl alcohol through a short column of silica gel containing the sample. t-Amyl alcohol esterifies relatively slowly with carboxylic acids, is less acidic than other alcohols such as methanol and ethanol, and is incompletely miscible with water, which makes it very useful for partition systems.

Previous experience with the Automatic Organic Acid Analyzer (8) shows that most common organic acids of metabolic origin can be separated and quantitated by use of a partition chromatographic system involving an increasing gradient of t-amyl alcohol in chloroform. The most polar organic acid detectable by this method, citric acid, was not eluted from the hydrated silica gel column until the proportion of alcohol approached 1:1 by volume. Alcohol proportions greater than 1:1 begin to elute the sulfuric acid. Thus, by using the extracting solvent described here, we could extract both polar and nonpolar acids with a small volume of solvent and titrate them as a group. The volume we chose is sufficient to extract the organic acids quantitatively, but excludes most of the sulfuric acid (see "Results").

Most organic acids are soluble in organic solvents in their un-ionized form. Therefore, sulfuric acid is added to the sample in sufficient quantity to decrease the pH below 2, and most charged substances such as amino acids are not eluted from the column. Thus, when 10 μmol each of alanine, arginine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, ornithine, phenylalanine, proline, serine, threonine, and valine were extracted individually in 5-mm columns, less than 0.3 microequivalent could be titrated in the extract. This was confirmed by analyzing the extract by the quantitative ninhydrin method of Rosen (9). When 100 μmol of creatinine was extracted in a 9-mm column, only 1.3 microequivalent was titrated in the extracting solvent.

We find that the purity of many commercial biochemicals is such that the extraction of small amounts of acid from substances being tested for interference may be due to contaminating acids rather than partial extraction. Protein is firmly adsorbed to the silica gel and need not be removed before the extraction. Acids that are loosely bound to proteins such as plasma albumin can also be eluted by this technique.

If it is unnecessary to know the amounts of individual acids, but only the total organic acid content of a physiological fluid such as urine, this method is
useful. For example, urines can be rapidly screened by this method for acids such as methylmalonic, orotic, propionic, isovaleric, or phenylpyruvic acids, which are present in large amounts in certain inborn errors of metabolism. It is particularly appropriate to examine urine from children in metabolic acidosis that is not associated with ketonemia or elevated blood urea nitrogen.

Two major sources of error with the Palmer–Van Slyke total organic acid method (1, 2) are obviated by the present technique.

The first is that the Palmer–Van Slyke method will detect more than 90% of only those organic acids that have pK's between 3.7 and 6.0. Many common carboxylic acids—such as citric, fumaric and methylmalonic acids—have pK's near 3 or less, and so would be considerably underestimated by the Palmer–Van Slyke titration. Also, human urine contains varying amounts of other compounds that will titrate between pH 2.7 and 7.0, such as amino acids, organic phosphates, and creatinine. Thus, the nonspecificity of titrating unextracted urine leads to considerable uncertainty concerning the reliability of results obtained by the Palmer–Van Slyke procedure.

The second major source of error in the Palmer–Van Slyke method is that if Ca(OH)₂ is used to remove phosphorus before titration, some organic acids—notably citric acid (5), a major urinary organic acid—will also be removed.

The net effect of all of these errors is difficult to assess, so we compared values obtained by the Palmer–Van Slyke method with those obtained by the extraction method for 100 different urines. The results (Figure 4) show that while results of the two methods were highly correlated (r = 0.94), the extraction method gives, on average, slightly lower results than does the Palmer–Van Slyke method. This was particularly true at values greater than 10 mEq/liter, so that the slope of the line was 0.74. Two possible explanations for this discrepancy were not excluded. One is that there are other compounds in urine that titrate between pH 2.7 and 7.0 (see above), and the other is that there are carboxylic acids in the urine that are more polar than citric acids—for example, ascorbic acid and β-glucuronides—that were not extracted. If the urine is likely to contain these acids, then methods specific for these acids will have to be used.

The major advantages of our extractive procedure are: greater specificity for carboxylic acids, direct use of urine without preliminary removal of interfering agents, less destruction of citric acid, miniaturization of technique, and increased speed of analysis.

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References