Quantitative determination of hippuric and m-methylhippuric acids in urine by high-speed liquid chromatography

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ABSTRACT High-speed liquid chromatography employing an ultraviolet photometric detector has been applied to the simultaneous determination of hippuric and m-methylhippuric acids in urine. Reversed-phase partition chromatography is carried out on a μ Bondapak C₁₈ column with methanol-water as the eluent system. This method obviates the necessity for isolation or reaction of these acids before assay. The only pretreatment necessary is extraction of samples with ethyl acetate. A linear relationship is obtained between the peak heights and the hippuric or m-methylhippuric acid concentrations. Mean recovery of hippuric and m-methylhippuric acids in urine is 99.8% and 99.3% respectively. The determination of hippuric acid by this method gives lower concentrations in normal urine than does the colorimetric method of Umberger and Fiorese (1963).

It has been observed that the concentration of hippuric and m-methylhippuric acids in urine correlates well with exposure of the subject to toluene and m-xylene (Pagnotto and Lieberman, 1967; Ikeda and Ohtsuji, 1969; Ogata *et al.*, 1970).

Many methods have been used for the determination of these metabolites using colorimetry (Gaffney *et al.*, 1954; Umberger and Fiorese, 1963), fluorometry (Ellman *et al.*, 1961), and ultraviolet spectrophotometry (Pagnotto and Lieberman, 1967). However, some constituents in the urine interfere with the assay and have to be removed by liquid/liquid extraction and chromatography (Ogata *et al.*, 1962). Where the urine contains both hippuric and m-methylhippuric acids, they can be separated using paper or thin-layer chromatography, with subsequent conversion to azlactones and assay of each constituent (Ogata *et al.*, 1969). Recently, a gas-liquid chromatographic method (Buchet and Lauwerys, 1973) has been reported based on the conversion of hippuric and m-methylhippuric acids for their specific determination.

This report describes a high-speed liquid chromatographic method which obviates the necessity for isolating these acids or for converting them before assay.

Materials and methods

CHROMATOGRAPHIC SYSTEM AND OPERATING CONDITIONS

A Waters ALC 202 high-speed liquid chromatograph equipped with a low-pressure mercury (254 nm) photometric detector was used in this study. Samples were introduced on to the column through a Waters U6K septumless injector. A μ Bondapak C₁₈ pre-packed column (Waters Assoc.), which is a reversed-phase column (30 cm $\times$ 4 mm I.D.) containing small-diameter porous silica particles chemically bonded to aliphatic hydrocarbon groups, was used. The column was eluted at 1 ml/min with methanol and 0.01 mol/l KH₂PO₄ containing 0.5% acetic acid (20/80 by volume) at room temperature. Injections were performed with a 10 μ l syringe (Hamilton 701N).

URINE TREATMENT

Extraction of hippuric and m-methylhippuric acids from urine was carried out according to the method described by Ogata *et al.* (1969). One millilitre of urine, 0.04 ml of concentrated HCl, 0.3 g of NaCl and 4.0 ml of ethyl acetate were shaken vigorously in a glass-stoppered tube for two minutes. After centrifugation for five minutes at 2500 rpm, 0.2 ml of the supernatant ethyl acetate was transferred to a test tube and evaporated to dryness on a water bath

at 70°C. The residue was dissolved in 0.2 ml distilled water and 4 μ l aliquots were injected into the chromatograph.

CALIBRATION CURVES

Standard solutions containing hippuric acid (Tokyo Kasei, Japan) and *m*-methylhippuric acid (Tokyo Kasei, Japan) were made up in distilled water to give a range of concentrations of 2-10 mg/ml. One volume of each solution was mixed with nine volumes of water or urine, and these were treated as described above. Peak heights were plotted against the concentrations of hippuric or *m*-methylhippuric acid.

COLORIMETRIC DETERMINATION OF HIPPURIC ACID IN URINE

Urinary hippuric acid was estimated by the procedure of Umberger and Fiorese (1963) with slight modifications. The samples were treated as indicated under 'urine treatment'. The residue was mixed with 0.5 ml of pyridine and 0.2 ml of benzenesulphonyl chloride. After the mixture had stood for 30 minutes it was diluted with 3.0 ml of ethanol and the absorbance was measured at 410 nm against a pyridine-benzenesulphonyl chloride blank on a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. Blank and hippuric acid standards were treated by the same procedure.

Results

CHROMATOGRAM

The chromatograms obtained for sample extracts are shown in Figs. 1a-c, and hippuric and *m*-methylhippuric acids were found to be separated in the same run. Hippuric acid but not *m*-methylhippuric acid was shown to be present in the urine of a person who had not been exposed to solvent (Fig. 1c).

Several mobile phases were evaluated for the liquid chromatographic development of these acids in water and in urine. Satisfactory development was observed with mixtures of methanol and water (0.01 mol/l KH_2PO_4 + 0.5% (v/v) acetic acid).

LINEARITY OF CALIBRATION CURVES

As the peaks of the acids were well resolved and symmetrical, peak heights were used as measures of concentration. As Fig. 2 indicates, the peak heights showed a linear relationship with the hippuric or *m*-methylhippuric acid concentrations. Furthermore, the linearity and the slope of calibration curves were not influenced by the addition of urine to the aqueous standard solutions. Recoveries for hippuric and *m*-methylhippuric acids were $99.8\% \pm 6.6\%$ (mean $\pm$ standard deviation) and $99.3\% \pm 6.2\%$, respectively.

Fig. 1 Chromatograms of sample extracts. a) standard aqueous solution; b) urine supplemented with standard acids; c) control urine. 1 = hippuric acid; 2 = *m*-methylhippuric acid.

COMPARISON OF COLORIMETRIC AND LIQUID CHROMATOGRAPHIC METHODS FOR URINARY HIPPURIC ACID

The urine of 39 subjects with no occupational exposure to solvents was analysed both by liquid

chromatography and by the colorimetric method of Umberger and Fiorese (1963). The results are compared in Fig. 3. The values obtained by liquid chromatography were lower than those found by the colorimetric method. This suggests that in the colorimetric determination of hippuric acid, other urinary constituents may interfere. However, there was a good correlation between the concentration of hippuric acid in urine as measured by these two methods. The regression line is: $y = 0.4068x - 0.0290$, where y is hippuric acid concentration (mg/ml) as obtained by liquid chromatography and x is that obtained by the colorimetric method. The correlation coefficient, r , is 0.939 ($n = 39$).

Discussion

Several methods using spectrophotometric procedures have been described for the determination of hippuric and m-methylhippuric acids in human urine. However, in most of these methods there is interference by various compounds present in normal urine and by metabolites of certain drugs. Furthermore, when the urine contains both hippuric acid and m-methylhippuric acid, the acids must be separated before spectrophotometric measurement.

In the present investigation using high-speed liquid chromatography, simultaneous determination of hippuric and m-methylhippuric acids in urine

Fig. 3 Comparison of results for hippuric acid in urine: liquid chromatographic vs. colorimetric methods.

can be accomplished. This method does not require any isolation or conversion of these acids before assay.

The study presented here suggests that hippuric acid in urine is determined with specificity by high-speed liquid chromatography. On the other hand, the colorimetric method of Umberger and Fiorese (1963) which is not specific for hippuric acid gives higher concentrations than those by liquid chromatography. These data are similar to those reported by Porter *et al.* (1975). In their study, hippuric acid in normal urine determined by gas-liquid chromatography accounted for approximately one-third of that measured by the fluorescent method (Ellman *et al.*, 1961) which lacks specificity.

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Fig. 2 Calibration curves for hippuric acid (solid line) and m-methylhippuric acid (dotted line). ● = aqueous standard solution; × and ▲ = urine supplemented with standard acids.

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