Journal of Chromatography, 145 (1978) 231—236
Biomedical Applications

CHROMBIO. 110

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HIPPURIC ACID IN HUMAN URINE

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(Received June 13th, 1977)

SUMMARY

A method is described for the determination of urinary hippuric acid by high-performance liquid chromatography. The method used ethyl acetate extraction for partial clean-up of the urine. The separation was carried out on a reversed-phase column using 20% methanol in 0.01 *M* aqueous potassium phosphate containing 0.5% acetic acid as a mobile phase. The column effluent was monitored with a UV detector at 254 nm. Hippuric acid was separated from other normal urine constituents in less than 10 min. Metabolites of xylene and styrene did not interfere with the assay. Analytical recoveries from urine were excellent and peak height and concentration were linearly related.

INTRODUCTION

Hippuric acid is normally present in human urine as a metabolite of dietary components. After exposure to toluene, large quantities of hippuric acid are excreted in the urine and quantities of it are correlated to toluene exposure. Therefore, the determination of hippuric acid in urine provides an exposure test [1—3]. Methods for the determination of hippuric acid in urine depending upon spectrophotometry [1, 4] and fluorometry [5] have been described. These methods, although simple and sensitive, suffer from the lack of specificity. Ogata et al. [6] have described a specific colorimetric method, which requires clean-up of the urine by paper chromatography or thin-layer chromatography before the determination step. A gas chromatographic method has been described by Buchet and Lauwerys [7]. However, this method requires derivatization. The present report describes a high-performance liquid chromatographic method for the accurate determination of hippuric acid in human urine. This method is simple and does not require the formation of volatile derivatives.

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EXPERIMENTAL

Materials

Hippuric acid, *o*-, *m*- and *p*-methylhippuric acids and mandelic acid were obtained from Tokyo Chemical Industry (Tokyo, Japan). Phenylglyoxylic acid and nicotinuric acid were from Aldrich (Milwaukee, Wisc., U.S.A.). Salicyluric acid was from Sigma (St. Louis, Mo., U.S.A.). The other reagents and solvents used were of reagent grade.

Standard solutions

A standard solution of hippuric acid in distilled water was prepared at a concentration of 1 g/100 ml. Hippuric acid was first dissolved in about 10 ml of 0.01 *M* NaOH before dilution with water. This solution was further diluted with water to produce solutions of the desired concentration.

Extraction procedures

Urinary hippuric acid was isolated as described by Ogata et al. [6]. To 1.0 ml of urine in a glass-stoppered tube were added 0.04 ml of concentrated HCl and 0.3 g of NaCl. This mixture was extracted with 4.0 ml of ethyl acetate by shaking vigorously for 2 min. The tube was centrifuged for 5 min at 1000 *g*, then 200 μl of the supernatant organic phase were transferred into a test tube. The ethyl acetate was evaporated to dryness in a water-bath at 70°. The residue was dissolved in 200 μl of water, and 4 μl was injected into the high-performance liquid chromatograph.

Apparatus and chromatographic conditions

A Waters Assoc. Model ALC/GPC 202 liquid chromatograph equipped with a μBondapak C_{18} prepacked column (30 cm × 4 mm I.D., Waters) at ambient temperature and a UV detector set at 254 nm, was employed for chromatographic analysis. The detector was operated at 0.05 absorbance units full scale for most samples.

The eluting solvent was 20% (v/v) methanol in 0.01 *M* potassium phosphate containing 0.5% (v/v) acetic acid. The flow-rate was 1.0 ml/min at a pressure of 2000 p.s.i. Sample injections were made on-column through a Waters U6K septumless injector with a 10-μl syringe (Hamilton 701 N).

RESULTS

Separation of hippuric acid in urine

Fig. 1 illustrates typical chromatograms obtained with samples prepared from (a) a urine control, (b) a urine sample to which synthetic hippuric acid has been added, and (c) a urine sample from a person exposed for two hours to 15—25 ppm of toluene in air. Samples prepared from the control urine gave a peak corresponding to endogenous hippuric acid. To illustrate the specificity of the method, the peak fractions were collected, extracted with ethyl acetate which was evaporated to dryness, and the residue obtained was examined by infrared spectroscopy. Infrared spectra were measured with a Hitachi Perkin-Elmer 225 grating infrared spectrophotometer. Fig. 2 shows the infrared

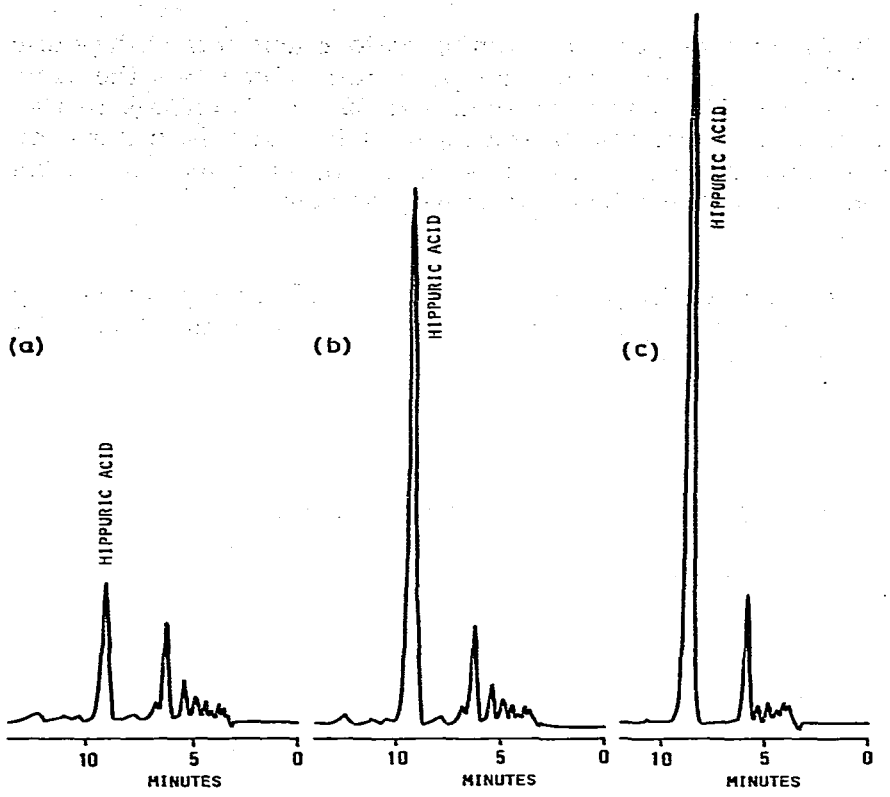

Fig. 1. Liquid chromatograms of extracts from (a) normal urine, (b) normal urine containing added hippuric acid, and (c) urine from a person exposed to toluene.

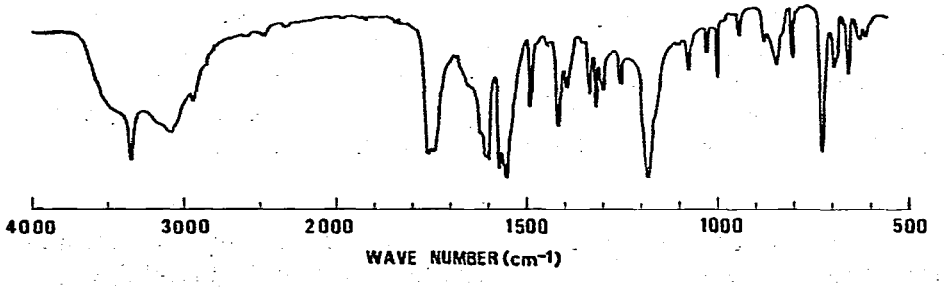

Fig. 2. Infrared spectrum of a peak fraction corresponding to hippuric acid in urine, which was separated by liquid chromatography. Spectrum was measured for KBr disc (200 mg of KBr and 0.8 mg of sample).

spectrum of the fraction. The spectrum was the same as that of synthetic hippuric acid. This result suggests that hippuric acid is separated well from other normal component(s) in urine.

Fig. 1c shows that an increased level of hippuric acid was found after exposure to toluene.

Calibration

Standard solutions were prepared containing various amounts of hippuric acid and analysed by the above extraction procedure which was the same as that for urine samples. The detector response at 254 nm is linearly related to the concentration of hippuric acid over a range of 0 to at least 5.0 mg/ml. By using the calibration curve the concentration of hippuric acid in a urine sample is easily determined after calculation of its peak height.

Recovery

The analytical recovery of hippuric acid from urine was determined by adding known quantities of hippuric acid to urine and analysing. Recoveries (Table I) ranged between 98 and 102%.

TABLE I

ANALYTICAL RECOVERY OF HIPPURIC ACID ADDED TO URINE

Hippuric acid (mg/l)		Mean recovery (%)
Added	Recovered*	
200	196	98
500	504	101
1000	1016	102

*Mean of 10 assays.

Precision

Within-run precision of the method was obtained by processing 10 aliquots of pooled urine. The concentration of hippuric acid was established at 868 ± 9.3 mg/l ± S.D. (coefficient of variation, 1.1%). Day-to-day precision was calculated from values for a single sample assayed on 10 consecutive days. The mean was 877 ± 11.9 mg/l ± S.D. (coefficient of variation, 1.4%).

Interfering substances

Other compounds for possible interference were studied by chromatographing aqueous solutions of them (Fig. 3). Mandelic acid and phenylglyoxylic acid, and methylhippuric acid, which are the known biotransformation products of the organic solvents styrene and xylene, respectively, were well separated from hippuric acid. Salicyluric acid and nicotinuric acid, which interfere with the determination of hippuric acid in Umberger's colorimetric method, did not interfere in this analysis.

Comparison with the paper chromatographic procedure

Thirty-two urine samples from persons not exposed to solvents were assayed by the liquid chromatographic method and the paper chromatographic method of Ogata et al. [6] in which hippuric acid is separated by paper chro-

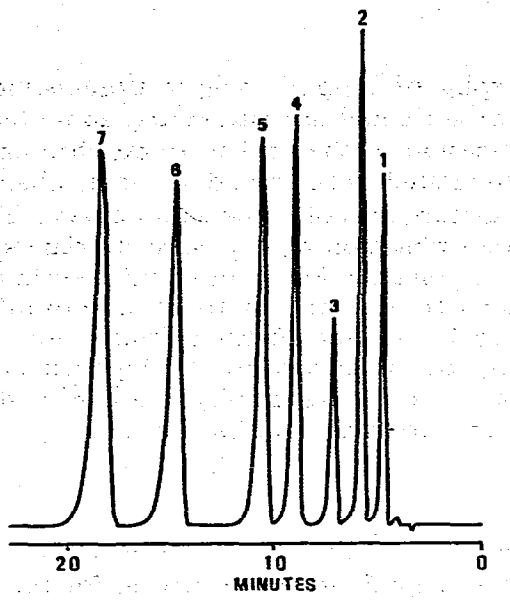

Fig. 3. Separation of a mixture of standards. 1 = Nicotinuric acid; 2 = phenylglyoxylic acid; 3 = mandelic acid; 4 = hippuric acid; 5 = *o*-methylhippuric acid; 6 = salicyluric acid; and 7 = *m*- (*p*-)methylhippuric acid.

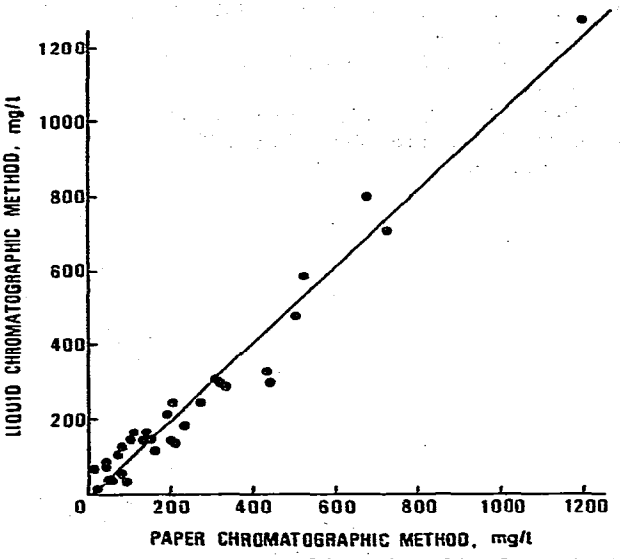

Fig. 4. Relation between hippuric acid values obtained by the paper chromatographic technique of Ogata et al. [6] and the present method.

matography, converted to azlactone and measured colorimetrically. Fig. 4 shows the results obtained with the two methods. The linear regression parameters for these results are: correlation coefficient, 0.959; *y* intercept, —0.0003; slope, 0.965.

DISCUSSION

High-performance liquid chromatography of hippuric acid in urine is more rapid and simpler than are spectrophotometric and gas chromatographic techniques, and does not necessitate derivatization of the acid as do gas chromatographic methods. Hippuric acid was separated from mandelic acid, phenylglyoxylic acid and methylhippuric acid, metabolites of styrene and xylene. This procedure can therefore be used for the estimation of exposure to toluene in cases of workers exposed to a mixture of solvents. Salicyluric and nicotinuric acids, interfering substances in Umberger's colorimetric method, did not interfere in the determination of hippuric acid. The specificity of this method is confirmed by infrared spectrophotometry of the eluted urinary hippuric acid. Results obtained by high-performance liquid chromatography and paper chromatography on the same urine sample correlated well for hippuric acid.

ACKNOWLEDGEMENT

The authors thank Dr. Masayuki Ito of the Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, for the infrared spectrophotometric data.

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