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Note

High-performance liquid chromatographic determination of hippuric acid in human urine

Preliminary results for normal urine levels

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Hippuric acid (HA) is the glycine conjugate of benzoic acid. It is the normal excretory metabolite of benzoic acid and is a common constituent of human urine. After exposure to toluene, which is oxidized in the body to benzoic acid, drastically increased levels of HA are found and the determination of HA in urine is thus important for estimating the degree of exposure [1]. In the Lesch-Nyhan syndrome [2] and in liver dysfunction [3], Ha is decreased or absent; it has been found in large quantities in hypertense females [3].

Routine determination of HA is normally hampered by the low sensivity and poor specificity of known methods. Photometric methods [4, 5] are unspecific and salicyluric acid (a metabolite of salicylic acid) and *m*- or *p*-methylhippuric acid (metabolites of *m*- or *p*-xylene) also react.

The isotachophoretic method [6] is complex and gas chromatography [7, 8] is time consuming and laborious (derivatization with diazomethane). Thin-layer chromatography [9] is simple, but only for screening, and is lengthy. A high-performance liquid chromatographic (HPLC) procedure for measurements of the urine concentration of HA has been published recently by Matsui et al. [10].

In a search for a more satisfactory and less difficult technique, HPLC was used with the expectation that it would offer decisive advantages over existing methods. This paper reports a liquid chromatographic method using a simplified procedure as compared to the above mentioned method of Matsui et al. [10] i.e. no extractions are performed. This technique is simple, rapid and specific. The present study is an application of this method to investigate the normal distribution of HA in human urine.

EXPERIMENTAL

Reagents

Hippuric acid, acetonitrile (Licrosolv^{D} grade) and interfering substances used were purchased from Merck (Darmstadt, G.F.R.).

Apparatus

A high-performance liquid chromatograph (LC3, Pye Unicam, Cambridge, Great Britain) was equipped with a variable-wavelength UV detector (Pye Unicam). The effluent stream was monitored at 250 nm.

A 250 mm × 4.6 mm I.D. column packed with C_{18}-bonded silica gel (particle size 10 µm: Partisil ODS-2; Whatman, Clifton, N.J., U.S.A.), a septumless syringe loading sample injector (20-µl injector, Rheodyne, Berkeley, Calif., U.S.A.) and a chart speed of 5 cm/min were employed.

Mobile phase

An amount of 500 ml of mobile phase was prepared fresh daily by thoroughly mixing 400 ml of 1% aqueous acetic acid with 100 ml of acetonitrile. The mobile phase was filtered through a 5-µm glass filter prior to use, degassed by a helium stream during the determination and pumped at a constant flow-rate of 1.35 ± 0.05 ml/min.

Urine samples

Normal 24-h urine specimens were obtained from laboratory personnel and from hospital patients with normal renal and hepatic functions. The samples were filtered (0.22 µm, Millipore, Bedford, Mass., U.S.A.) and stored at −24° if not analyzed immediately.

Calibration curves

A calibration curve was prepared daily by appropriate dilutions of a stock solution of HA (10 g/l in water) with 1 *N* acetic acid. The concentration range was 0.1–1.5 g/l (0.56–8.4 m*M*).

Urine determinations

Aliquots of 100 µl of 24-h urine specimens were diluted with 900 µl of 1 *N* acetic acid. Exactly 20 µl of this solution were then injected into the chromatograph. The peak height of HA was measured and the unknown concentration was calculated from the calibration curve.

RESULTS AND DISCUSSION

The separation of HA was satisfactory and retention time was 250 ± 10 sec (mean ± S.D.). A typical chromatogram is shown in Fig. 1. Endogenous and exogenous constituents such as creatinine, urea, oxalic acid, glycolic acid, glyoxylic acid, benzoic acid, salicylic acid, salicyluric acid, malonic acid and acetaminophen do not coelute with HA.

The influence of acidity on the mobile phase was studied. The effect of pH on linearity (Fig. 2) shows that the best results were obtained with 1% aqueous

acetic acid. Decreasing the pH of the diluent used for urinary determinations increases sensitivity. The optimal pH of 3.1 was obtained by diluting samples with 1 N acetic acid.

Good linearity was observed in the range of 0–1.5 g/l (0–8.4 mM) (r = 0.9999, four determinations for each point). An additional sample dilution was necessary at concentrations greater than 1.5 g/l.

The precision of this method was determined by first assaying 16 samples of pooled normal urine, followed by assaying samples of this pooled urine to

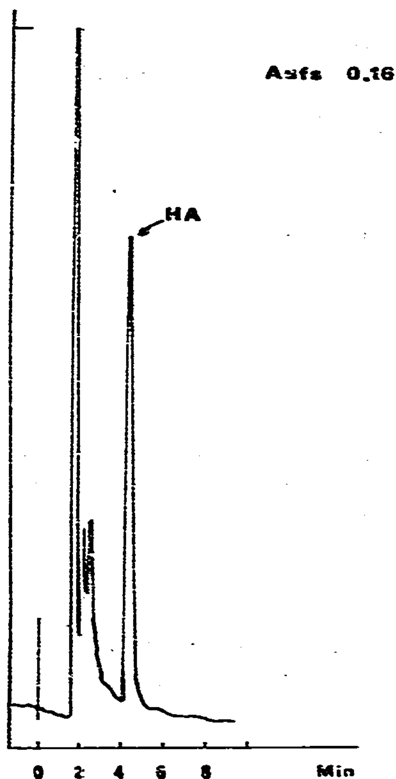

Fig. 1. HPLC separation of HA from normal human urine. Concentration of HA 0.82 g/l (4.56 mM).

TABLE I

PRECISION AND ACCURACY OF HA DETERMINATION

	Mean (n = 16) HA (mg/l)	Standard deviation (%)	Standard error (%)
Pooled normal urine	253	6.2	1.6
Pooled normal urine spiked with 50 mg/l HA	301	4.5	1.1
HA found	48 (96% recovery)		

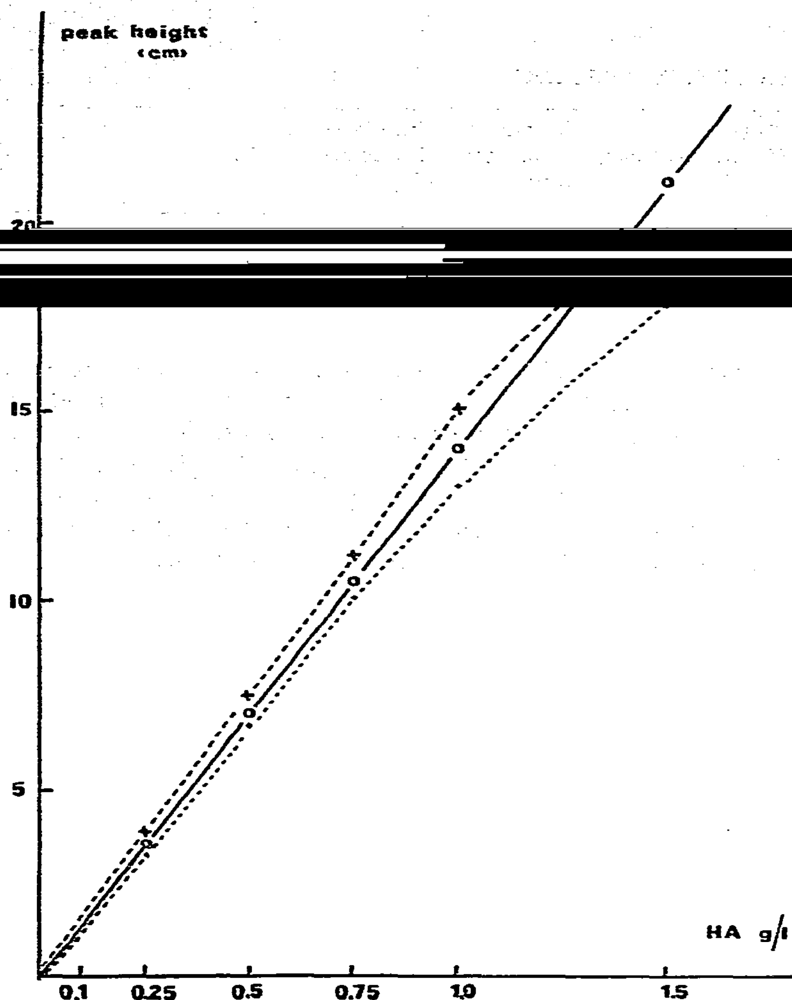

Fig. 2. Calibration curves for various concentrations of acetic acid in the mobile phase. (x——x) 0.1% acetic acid, (+· · · · ·+) 0.2% acetic acid and (O——O) 1% acetic acid ($y = 139.9\ X - 0.38$; $r = 0.9999$).

which 50 mg/l of HA (280 μM) were added. The accuracy was evaluated from recovery data (difference between mean values of pooled normal urine and urine containing 50 mg/l HA) (Table I).

Our HPLC method showed good day to day reproducibility. The results of the analysis of pooled normal urine and HA spiked urine (2 g/l, 11.2 mM) (3 replicate analyses of each on 30 consecutive days) are shown in Table II.

Biological results

Preliminary results for normal urine levels of HA were obtained. The reference range for hippuric acid content has been reported to be 2.55 ± 1.19 mmoles/24 h [9].

TABLE II

REPRODUCIBILITY OF HA DETERMINATION

	Mean HA found (mg/l)	Standard deviation (mg/l)	Standard deviation (%)
Normal urine (pooled)	273	15	5.5
HA-spiked urine (2 g/l)	2077	125	6.0

Our results obtained with a control group (no exposure to toluene and normal renal and hepatic functions) (n = 69) are in agreement with this value but we found evidence for a bimodal distribution of HA excretion in normal subjects. Fig. 3 shows the frequency distribution histogram for HA excretion. A bimodality is seen with a mean for one subpopulation at 1.07 ± 0.51 mmoles/24 h (66%) and a mean for the other subpopulation at 4.4 ± 1.1 mmoles/24 h (33%) (t-test: $p < 0.01$). Sex is not an interfering factor.

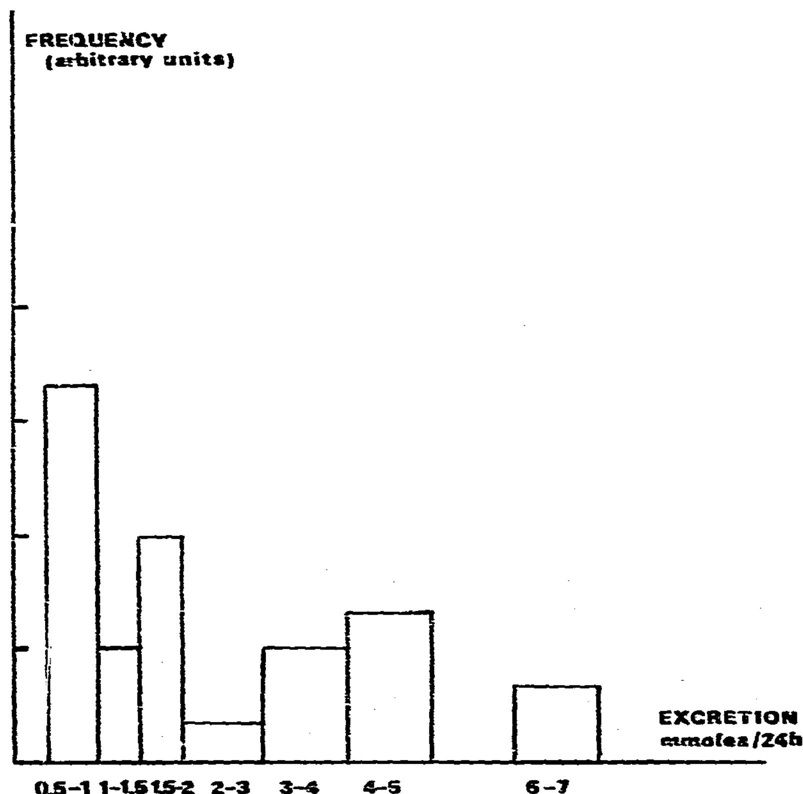

Fig. 3. Frequency distribution histogram for hippuric acid in normal human urine (n = 69). First subpopulation (66%: 1.07 ± 0.51 mmoles/24 h); other subpopulation (33%: 4.4 ±1.1 mmoles/24 h).

CONCLUSION

The HPLC method described for the determination of hippuric acid in human urine is specific, accurate, reproducible, rapid and simple. It promises to be of great help in cases of toluene exposure as well as for biochemical studies. Further work is in progress [11].

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