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Note

Rapid method for the determination of hippurate in biological fluids by high-performance liquid chromatography

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The determination of hippurate has a long tradition in clinical toxicology. Hippurate accumulates in large concentrations in the plasma of patients with renal failure [1,2] and is considered to be a potential marker of uremic solute retention [3]. Hippurate is not an inert metabolic end-product, and may account for some biological effects: (i) hippurate binds to serum albumin and competes with various drugs and metabolites increasing their free/bound ratio [4]; (ii) it competes with the excretion of organic anions in proximal tubular cells and prolongs their biological half-life [5]; (iii) it interacts with the tubular γ-glutamyl transpeptidase (GMT) activity, influencing the resorption of amino acids [6]; (iv) it inhibits glucose utilization in striated muscle and kidney [7,8].

Hippurate has been measured by various methods. The colorimetric method [9] and gas chromatography [10–12] are time-consuming. Thin-layer chromatography [13] is simple, but suitable only for semiquantitative determination. A number of high-performance liquid chromatographic (HPLC) methods have been reported for hippurate determination in urine. Most HPLC analyses are developed on reversed-phase columns [14–16] or anion-exchange resins [17,18]. Recently, an HPLC procedure for the measurement of serum hippurate concentration by gradient elution has been published [19]. This paper describes a rapid, precise and useful isocratic HPLC method for the determination of a wide range of concentrations of hippurate in serum and urine.

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EXPERIMENTAL

Materials

All chemicals and reagents were of the highest quality available commercially. Hippuric acid was obtained from Lachema (Brno, Czechoslovakia).

Instrumentation

A modular high-performance liquid chromatograph (Gilson, Middleton, WI, U.S.A.) consisted of a pump (Model 302), an auto-sampling injector (Model 231), a variable-wavelength UV spectrophotometer (Model 116), a recorder (Epson FX-80, Nagano, Japan) and controller (Model 502 Contact Module). A computing integrator (Model 620) controlled by an Apple computer was used for the identification and quantification of chromatographic peaks by the external standard method. The chromatograph was equipped with a commercially available reversed-phase column (150 mm × 3.3 mm I.D.) filled with Separon SGX C_{18} (7 μm particle size, Laboratorní přístroje, Prague, Czechoslovakia). A rapid spectral detector (LKB Chromatography Instruments, Bromma, Sweden) was used for spectral analyses. A Vortex-Genie rotary mixer (Scientific Industries, Bohemia, NY, U.S.A.) and a centrifuge (Type 320a, Mechanika Precyzyjna, Warsaw, Poland) were used to process samples in 1.5-ml plastic centrifuge tubes.

Reagents

The mobile phase was 0.2 *M* acetic acid–methanol (8:2, v/v). The pH of the solution was adjusted to 6.5 by the addition of concentrated ammonia. The eluent was degassed with helium before use.

A stock standard solution was prepared by dissolving 179 mg of hippuric acid in 100 ml of deionized water (10 mmol/l). This stock solution was diluted with deionized water to prepare 10 and 100 μmol/l standard solutions for low and high hippurate concentration samples.

Analytical procedure

A 200-μl sample of control or 50 μl of uremic serum was applied on a commercially available reversed-phase cartridge, Sample Container Presep 1 (1 ml volume of packing) filled with Separon SGX C_{18} (60 μm particle size, Laboratorní přístroje). After the cartridge had been washed with 2 ml of 0.2 *M* acetic acid, hippurate was eluted with 1 ml of methanol. The samples were dried under a stream of nitrogen at 70°C. The residue was dissolved in 200 μl of the mobile phase and vortex-mixed for 10 s. The samples were centrifuged at 12 000 *g* for 10 min, then transferred to sample vials and inserted for automatic injection into a chromatograph.

Urine samples were diluted 20 or 100 times with mobile phase without methanol, and 20-μl aliquots were injected. For the isocratic chromatographic separation, a flow-rate of 0.8 ml/min was used with automatic injection of 20 μl of samples. The sensitivity setting was 0.05 a.u.f.s. for low and 0.1 a.u.f.s. for high hippurate concentrations at 10 mV full-scale deflection. The separation was car-

ried out at room temperature and required less than 5 min . Hippurate was detected at 240 nm.

Recovery, precision and reproducibility

Recovery studies were carried out in plasma by adding a standard solution of hippurate. The within-day variation was determined by assaying standard serum samples with low and high plasma levels of hippurate (10 and 100 μmol/l) ten times each. Moreover, we assayed these samples on different days, in different assays, to determine the between-day variation.

Quantification

The linearity of the detector response was evaluated by measuring two standard curves: the low standard curve (0–10 μmol/l) for measuring plasma hippurate levels in healthy subjects and the high standard curve (0–100 μmol/l) for measuring samples from patients with renal insufficiency and urine samples.

Spectral analyses

To check the identification of serum and urine hippurate, the UV spectra were compared with that of the hippurate standard. The exact chemical structure was confirmed by mass spectrometry and NMR spectroscopy (unpublished data).

RESULTS AND DISCUSSION

The primary requirement of the chromatographic system was rapid and precise hippurate separation. Sufficient separation was obtained within 5 min by isocratic elution with acetate–methanol buffer (pH 6.5). Typical chromatograms of control and uremic plasma and urine are shown in Fig. 1.

The mean analytical recovery was 100.33% [coefficient of variation (C.V.) = 5.3%]. The within-day C.V. for 10 and 100 μmol/l concentrations of hippurate were 5.34 and 2.66% ($n=10$), respectively. Between-day C.V. for the same concentrations were 6.0 and 2.94% ($n=10$), respectively.

The low-concentration standard curve (range 0–10 μmol/l) and the high-concentration standard curve (range 0–100 μmol/l) were linear with $r=0.999$ ($y=6.17x-3.56$ μmol/l and $y=5.34x-12.67$ μmol/l).

The plasma hippurate concentration measured in healthy subjects was 5.38 ± 0.54 μmol/l ($n=42$). Plasma levels in patients with decreased renal function closely correlated with the clearance of endogenous creatinine ($y=12.7x^{-1.42}$, $r=-0.845$, $p<0.001$) [2]. This explained the great variability (more than 100-fold increase) of hippurate concentrations.

Fig. 2 illustrates the UV spectra of the hippurate standard and the hippurate peaks from serum and urine, and the similarities indicate that the serum and urine peaks under investigation were indeed hippurate. Eight spectra of each peak were overlaid and compared. No endogenous substances in plasma and urine of healthy subjects interfered with this method. In studies of renal failure, no interferences with other drugs or substances accumulating in this disease were observed.

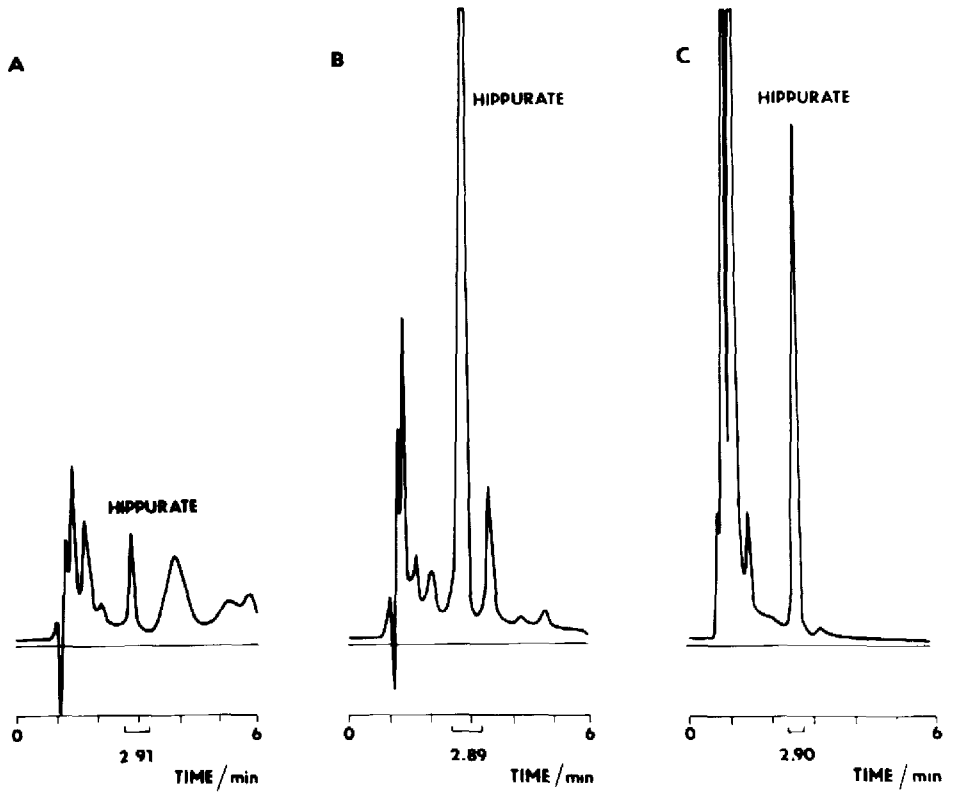

Fig. 1. Representative chromatograms of hippurate determination, under the chromatographic conditions described in *Analytical procedure*: (A) normal serum (5.4 μmol/l hippurate); (B) uremic serum (510 μmol/l hippurate); (C) diluted urine (680 μmol/l hippurate).

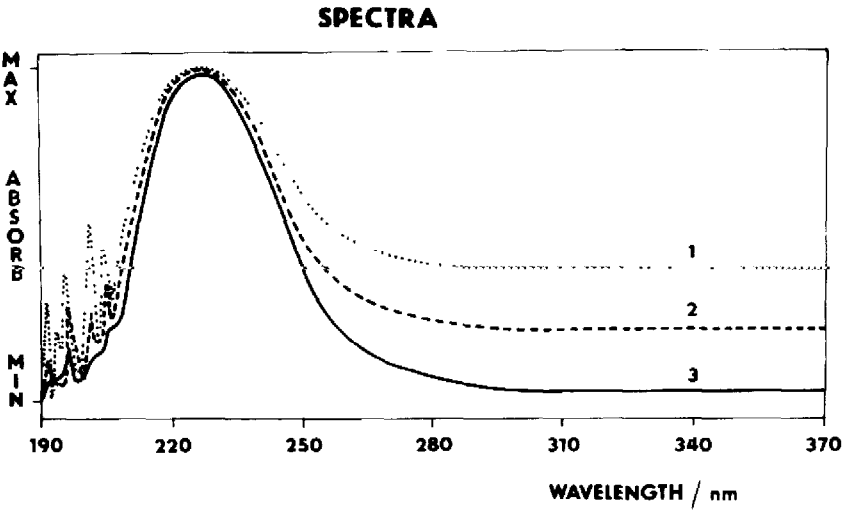

Fig. 2. UV spectra of HPLC hippurate peaks Curves: 1 = hippurate standard; 2 = serum hippurate; 3 = urine hippurate.

CONCLUSION

A reversed-phase chromatographic system has been developed for the separation of hippurate in plasma and urine, in which no time-consuming extraction procedure is necessary, a simple mobile phase is used and a good reproducibility is achieved. The method developed can further be used to study the synthesis and metabolism of hippurate in large-scale routine analyses and to elucidate its biological effects in patients with decreased renal function.

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