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Note

Analysis of homogentisic acid in body fluids by high-performance liquid chromatography

B. ÅKESSON*

Department of Clinical Chemistry, University Hospital, S-221 85 Lund (Sweden)

and

K. FORSLIND and F. WOLLHEIM

Department of Rheumatology, University Hospital, Lund S-221 85 (Sweden)

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Patients with alcaptonuria lack homogentisate 1,2-dioxygenase, and up to 5–10 g of homogentisic acid is excreted daily in the urine. Several analytical methods have been used to demonstrate the presence of homogentisic acid in urine, including a simple filter paper strip test [1], spectrophotometry [2], paper chromatography [3], an enzymic method [4] and gas chromatography—mass spectrometry [5]. The last two techniques are also sensitive enough for the quantification of homogentisic acid in plasma, which in alcaptonuric patients amounts to 5–10 mg/l. These methods require purification of an enzyme [4] or use of a deuterated standard in a derivatisation procedure [5]. We therefore attempted to devise a simple high-performance liquid chromatographic (HPLC) method for the analysis of homogentisic acid both in urine and plasma.

EXPERIMENTAL

Homogentisic acid (2,5-dihydroxyphenylacetic acid) and 3,4-dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma (St. Louis, MO, U.S.A.). Urine and plasma samples were obtained from patients with alcaptonuria. Clinical data on the patients will be described elsewhere [6]. The 24-h urine samples were collected in 2-l plastic flasks containing 30 ml of acetic acid. Blood samples were obtained using EDTA as anticoagulant. Plasma (1 ml) was mixed with 0.2 ml of

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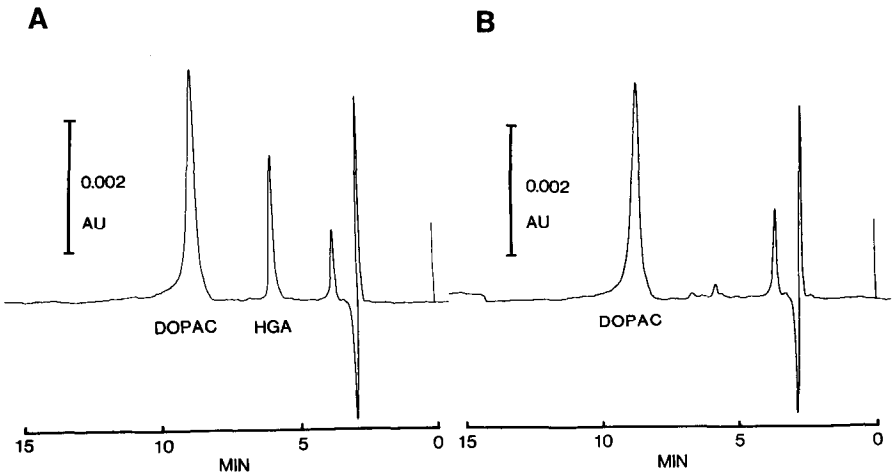

Fig. 1. Chromatograms of urine from (A) a patient with alcaptonuria and (B) a healthy subject. Urine was diluted 1000-fold with 10 m*M* acetic acid, and 20 μl were injected using DOPAC as internal standard. HGA = homogentisic acid. The experimental conditions are described in the text. The concentration of HGA in the patient's urine sample was 8.6 mmol/l.

50% trichloroacetic acid, and the supernatant was recovered after centrifugation. All samples were stored at $-70\,^{\circ}$C until analysis.

Urine was diluted 1000-fold with 10 m*M* acetic acid, and DOPAC was added as an internal standard (10 mg/l). The sample (20 μl) was injected, using a 7125 Rheodyne injector, onto a 200 mm $\times$ 4 mm I.D. Nucleosil C_{18} column (5 μm). The mobile phase was methanol—10 m*M* acetic acid (85:15, v/v), and the flow-rate was 1 ml/min. A Constametric III pump and a variable-wavelength UV detector (Spectromonitor III) both from LDC (Riviera Beach, FL, U.S.A.) were used, and the wavelength was set at 292 nm. Quantification was by peak-height measurement. Standard solutions of homogentisic acid in 10 m*M* acetic acid were included in all runs. The standard curve was linear for concentrations of 1–10 mg/l (5.9–59 μmol/l). The detection limit was 2 ng of homogentisic acid at a signal-to-noise ratio of 2.

The trichloroacetic acid supernatant from plasma samples was diluted with four volumes of 10 m*M* acetic acid containing DOPAC as internal standard, and analysed as described above.

RESULTS AND DISCUSSION

When urine samples were chromatographed, homogentisic acid was well separated from the internal standard and other UV-absorbing peaks (Fig. 1). In three patients with alcaptonuria the urinary excretion of homogentisic acid was 2–10 g/day (12–60 mmol/day), which agrees with previous data [2,4]. The coefficient of variation (C.V.) when duplicate runs were made was 2.8% ($n = 62$). In samples from healthy subjects only small amounts of homogentisic acid were observed under the conditions used, and no quantification was made.

The concentration of homogentisic acid in plasma of alcaptonuric patients is

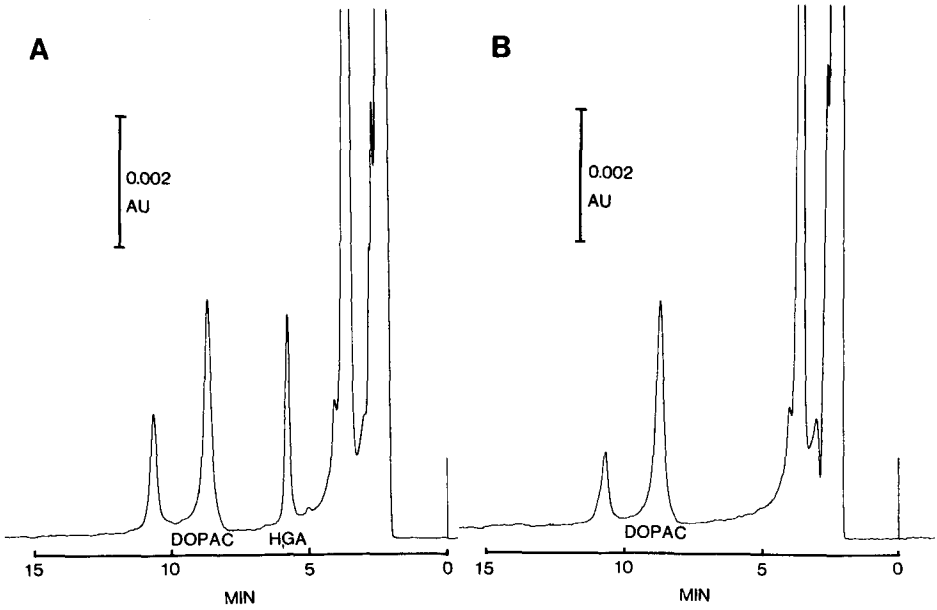

Fig. 2. Chromatograms of plasma from (A) a patient with alcaptonuria and (B) a healthy subject. The concentration of homogentisic acid (HGA) in the plasma sample shown in (A) was 54 μmol/l.

much lower than that in urine. Therefore the samples were not diluted so much, and plasma homogentisic acid could then be quantified (Fig. 2). The homogentisic acid concentration in the plasma of three patients with alcaptonuria was 26–60 μmol/l, which agrees with previous data [4]. The C.V. when duplicate runs were made was 2.4% ($n=12$). No accurate determination of homogentisic acid in the plasma of healthy subjects was possible with this method.

Homogentisic acid is very unstable at alkaline pH, but little information is available on its stability in acidic solutions at different temperatures. We therefore stored urine collected with the addition of acetic acid and urine diluted with 10 m*M* acetic acid at −70°C, −20°C, +4°C and room temperature for up to five months and then analysed for homogentisic acid. The concentration of homogentisic acid in undiluted urine remained stable at −20°C and −70°C, and decreased by 25% after five months at room temperature. In samples diluted with 10 m*M* acetic acid, homogentisic acid was less stable. It could not be detected in samples stored for one month at room temperature or for five months at +4°C but was essentially unchanged at −20°C and −70°C. Thus storage at −20°C or −70°C of undiluted urine after the addition of glacial acetic acid is recommended for the preservation of homogentisic acid.

In conclusion, this communication describes a rapid and simple HPLC procedure for the analysis of homogentisic acid in body fluids. It can be used for the diagnosis of alcaptonuria and also for studies of factors affecting the concentration of homogentisic acid in body fluids.

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