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Note

Improved high-performance liquid chromatographic determination of urinary glycolic acid

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Ascorbic (ASC), glyoxylic (GLX) and glycolic (GLY) acids are the only known precursors of oxalate (OX) in humans [1]. The concentration of GLY in biological fluids has been shown to be a suitable index for the differential diagnosis of the hyperoxaluria syndromes [1].

In type I primary hyperoxaluria, vitamin B_6 deficiency and intake of some toxic metabolic precursors, high OX concentrations are observed together with increased levels of GLY [1,2–6]. Conversely, normal rates of urinary excretion of GLY are observed in type II primary hyperoxaluria, increased intake of ASC or OX and in a variety of malabsorptive syndromes [1,7–9].

In a previous paper, we described a high-performance liquid chromatographic (HPLC) method for determining GLY in urine as the phenylhydrazone derivative [10]; that method was based on the enzymic conversion of GLY into GLX, coupled with the derivatization of the α-keto acid with phenylhydrazine (PH). The phenylhydrazone formed was separated and determined by HPLC. Afterwards, in spite of using cleaned glass vials and deionized and distilled water for dilution, sometimes the observed "blank" peak was not negligible. In this paper we propose some modifications of the original method, which have been made in order to improve the chromatographic separation and to avoid any "blank" and unspecific contribution to the GLY peak.

EXPERIMENTAL

Chemicals

Analytical-grade reagents were used and water was deionized and distilled. Glycolate oxidase (GAO) from spinach (glycolate oxygen oxidoreductase, EC 1.1.3.15, lyophilized powder, 4.2 U/mg protein), GLY and PH · HCl were obtained from Sigma (St. Louis, MO, U.S.A.); HPLC-grade ethanol, L-cysteine hydrochloride, phosphoric acid, dipotassium hydrogenphosphate and potassium

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dihydrogenphosphate were obtained from Merck (Darmstadt, F.R.G.). Amino acid calibration standards, types P-B and P-AN, were obtained from LKB Biochrom (Cambridge, U.K.). A 0.1 *M* L-cysteine solution was prepared weekly by dissolving 439 mg of L-cysteine hydrochloride in 25 ml of water and stored at 4°C. PH · HCl was recrystallized twice from water, dried overnight at 37°C and stored in the dark. The 500 m*M* working solution was prepared daily by dissolving 289 mg of the salt in 4.0 ml of water.

Phosphate buffer (0.1 *M*) was prepared weekly by dissolving 5.70 g of $K_2HPO_4 \cdot 3H_2O$ in 250 ml of water and adjusting the pH to 8.3 with H_3PO_4. Portions of 25 U of enzyme were reconstituted with 1.0 ml of 0.1 *M* potassium phosphate buffer by gentle shaking. The lyophilized powder is stable for at least four months when stored at 4°C, but the reconstituted portions must be used within a week.

HPLC-grade ethanol (mobile phase A) and potassium phosphate solution (0.226 g of $K_2HPO_4 \cdot 3H_2O$ and 1.773 g of KH_2PO_4 dissolved in 1.0 l of water, pH 6.0, mobile phase B) were used for the chromatographic separation

Stock solutions of 0.5 *M* GLY were prepared in water and stored at −20°C. The working standard solutions, containing 200 or 800 μM, were prepared daily by diluting the stock solution.

Derivatization and sample handling

Samples from 24-h urines with 2.0 ml of chlorhexidine gluconate added as a preservative were obtained from twenty healthy adults (normals) and five patients with type I primary hyperoxaluria (type I HOx) on an unrestricted diet. Urine samples were stored at −20°C and analysed within a month. Before analysis, the samples were filtered through 0.22-μm cellulose filters (Millipore, Segrate MI, Italy).

Before analysis, the phosphate buffer was preincubated with GAO by mixing, into 10-ml glass vials, 1.0 ml of 0.1 *M* phosphate buffer with 25 μl of GAO and 50 μl of cysteine solution. The mixture was left at room temperature for 2 h. Derivatization was performed by adding 50 μl of PH and 50 μl of standard or sample to the preincubated mixture. After mixing on a vortex-mixer, 25 μl of enzyme were added and the mixture was vortex-mixed again. After a 20-min reaction the solution was injected into the chromatographic column.

Chromatography

A Varian (Palo Alto, CA, U.S.A.) 5500 liquid chromatograph, equipped with a UV–VIS variable-wavelength detector and a Rheodyne (Berkeley, CA, U.S.A.) 7126 automatic injection valve with a 50-μl loop, was used. An octadecylsilyl LiChrospher RP-18 (10 μm) column (250 mm × 4.0 mm I D.) (Merck) was used throughout, connected to a Perisorb RP-18 (30–40 μm) (Merck) guard column (30 mm × 4.0 mm I.D.). GLY was eluted using a binary gradient at a flow-rate of 2.0 ml/min starting with 3% (v/v) mobile phase A (ethanol) and 97% (v/v) mobile

phase B (potassium phosphate buffer as described previously). After 5 min of elution at these initial conditions, the gradient was increased linearly over 3 min to a final concentration of 54% mobile phase A and 46% mobile phase B. After 3 min with this mixture, the proportion of mobile phase A was decreased over 2 min to the original starting conditions. The system was allowed to re-equilibrate for 7 min prior to the next injection.

Detection was performed at 324 nm at 0.002 a.u.f.s The buffer composition in the mobile phase was accurately controlled, and the column was periodically cleaned by flushing with methanol and acetonitrile. Peak heights were measured using a Shimadzu (Kyoto, Japan) C-R1A recorder–integrator, set at an attenuation of 8 mV at full-scale.

RESULTS AND DISCUSSION

Twelve "blank" solutions were analysed on different days, with and without preincubation with GAO, using freshly prepared deionized and distilled water for the buffer solution. The mean GLY equivalent concentration measured without preincubation was 24.4 ± 29.1 μM (four samples were less than 10 μM), whereas in preincubated buffers GLY was always less than 1.5 μM. This can be taken as proof that the GAO preincubation of the buffer solution completely eliminates any "blank" contribution to the GLY peak.

The kinetics of the enzymic conversion have been detailed in a previous paper [10] and apply also to this method, in which only slight changes have been made to the derivatization procedure. Quantitative conversion is achieved within 20 min of reaction time, and samples with a GLY content of up to 1000 μM might be diluted before analysis. Linearity is satisfactory in the concentration range 0–1000 μM, and the minimum detectable concentration is 1.5 μM at a signal-to-noise ratio of 10:1. Urine concentrations have been calculated with respect to the heights of GLY peaks from standard aqueous solutions containing 200 or 800 μM GLY. As detailed previously [10,11], the urine GLX contribution to the GLY peak had been considered negligible, compared with urinary GLY, and was not taken into account.

Under the above chromatographic conditions the derivative of GLY elutes in 6.5 min. Chromatograms of a standard solution, of a sample from a healthy subject and from a patient with type I primary hyperoxaluria are shown in Fig. 1.

Two urinary samples containing 196 and 843 μM GLY were analysed six times, yielding intra-assay coefficients of variation (C.V.) of 2.6 and 4.3%, respectively, and inter-assay C.V. of 4.0 and 8.2%, respectively. The urinary GLY stability was studied [10] and it was found that urinary GLY, in the presence of 0.04% chlorhexidine gluconate, is stable for at least a month when stored at −20°C.

The accuracy was tested by spiking ten urine samples with 100 μM GLY. A mean recovery yield of 98.1 ± 6.4% indicated that there was no appreciable enzymic inhibition due to the matrix effect.

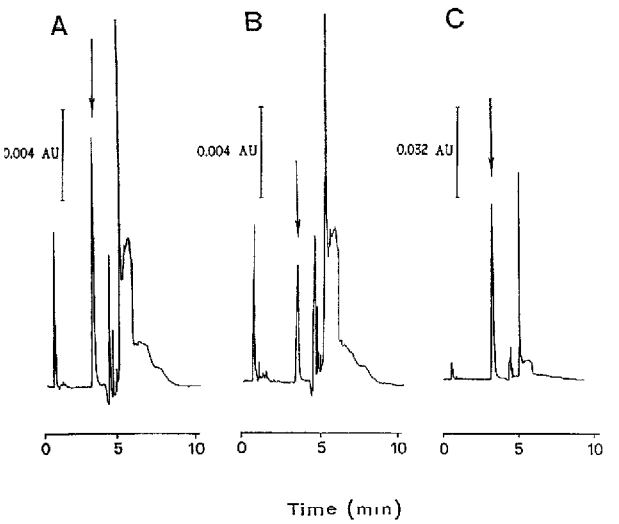

Fig. 1. HPLC profiles for the determination of glycolate under the conditions described in the text. (A) Aqueous glycolate standard, 800 μM; (B) urine sample from a healthy subject; (C) urine sample from a patient with primary type I hyperoxaluria. The arrows indicate the glyoxylate phenylhydrazone peak

The peak specificity was evaluated as described previously [10], and no interfering substances were found. Urinary excretion levels determined in normal subjects and patients with type I primary hyperoxaluria are listed in Table I. As expected, the means of the hyperoxaluric patients were markedly higher than those of controls; a complete separation between controls and patients could be obtained if GLY excretion was corrected for urine creatinine (Table I). The rates of excretion observed in our normal subjects are similar to those reported previously [10]. Thus, in comparison with our earlier methodology, the modifications proposed do not appear to contribute additional analytical variation to the assessment of GLY excretion. However, the complete elimination of any blank

TABLE I

GLY EXCRETION IN HEALTHY SUBJECTS AND IN TYPE I PRIMARY HYPEROXALURIA PATIENTS

Subjects	n	GLY excretion			
		μmol per 24 h		μmol per mmol creatinine	
		Mean ± S D	Range	Mean ± S D	Range
Normals	14	468.8 ± 124.5	313.3–761.2	41.6 ± 11.9	30 0–76.1
Type I HOx	5	3722 ± 4951	121–12 420	535.8 ± 218 8	291 2–863 6

contribution to the peak, which has been achieved with this method, represents an improvement in the accuracy of the procedure. It can also be stressed that the use of gradient elution was effective in eluting without delay the more retained peaks, which otherwise could be detected only non-specifically in subsequent chromatograms; this represents an additional factor contributing to improved reliability and suitability of the procedure.

The method proposed here is simple, accurate and not time-consuming. Its reproducibility makes unnecessary the use of an internal standard. Also it enables urinary GLY to be determined within 20 min, making it suitable for routine purposes even in emergencies such as ethylene glycol poisoning.

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