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# DERIVATIZATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF URINARY GLYCOLIC ACID

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## SUMMARY

A high-performance liquid chromatographic method for the determination of urinary glycolic acid is proposed, based on pre-column derivatization with phenylhydrazine coupled with the enzymatic oxidation of glycolate to glyoxylate. The phenylhydrazone formed is separated by liquid chromatography and detected at 324 nm. The minimum detectable concentration of glycolate was 10.0 μmol/l. The recovery of glycolate added to urine averaged 96.1%. The day-to-day coefficients of variation calculated by analysis of two urine samples with normal and high glycolate contents were 4.6 and 7.5%, respectively. Results of analyses of urine samples from healthy persons, idiopathic calcium stone formers and Type I primary hyperoxaluria patients are reported.

## INTRODUCTION

Apart from ascorbate (ASC), glyoxylic (GLX) and glycolic (GLY) acids are the only known precursors of oxalate (OX) in man. In mammalians, the endogenous non-enzymic transformation of ASC into OX accounts for significant amounts of urinary OX[1]. GLY and GLX are connected with the metabolism of glycolaldehyde and serine and are enzymatically interconvertible and easily transformable into OX. The endogenous production of GLX is slower than its oxidation to OX, which is catalysed by lactate dehydrogenase (LDH) and glycolic acid oxidase (GAO), and consequently GLX serum and urine levels are extremely low[2].

An increase in GLX, GLY and OX in either serum or urine occurs in cases of Type I primary hyperoxaluria, vitamin $B_6$ deficiency and intake of toxic metabolic precursors[1,3–7]. Further, it has been reported that an increase in aromatic amino acid intake produces a mild increase in OX synthesis via GLY and GLX[8]. On the other hand, increases in OX levels may be coupled with normal urinary excretion of GLX and GLY in cases of Type II primary hyperoxaluria, with increased intake of ASC or OX and in a variety of malabsorptive syndromes[1,9–11].

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In a previous paper[2], we described a high-performance liquid chromatographic (HPLC) method for determining GLX in urine as the phenylhydrazone derivative, but the clinical use of the GLX determination in screening for hyperoxaluria syndromes gave partly meaningless results on account of the poor stability of GLX in biological fluids. Therefore, we have developed a new HPLC method for the determination of GLY acid in urine.

Simple colorimetric determination is one of the most widely used methods, in which a purification step with ion exchangers is followed by a colour reaction with chromotropic acid[12]. The use of glycolate oxidase (E.C. 1.1.3.3) for the determination of GLY in urine and plasma was described previously[13]. The method requires the treatment of the sample with charcoal and the determination of lactate for an indirect evaluation of GLY. Some gas chromatographic methods are also available, but they often require complex and lengthy sample processing[14–17].

In this paper we propose a new HPLC procedure for the determination of GLY in urine, based on the enzymatic conversion of GLY to GLX, coupled with the derivatization of the α-keto acid with phenylhydrazine (PH). The phenylhydrazone formed is then isolated and determined by HPLC.

## EXPERIMENTAL

### *Materials*

Analytical-reagent grade chemicals were used and water was deionized and distilled. Sodium glyoxylate monohydrate and oxalacetic acid were purchased from Fluka (Buchs, Switzerland) and GAO from spinach (glycolate oxygen oxidoreductase, E.C. 1.1.3.15, lyophilized powder, 3.8 U/mg protein), glycolic acid and phenylhydrazine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol, L-cysteine hydrochloride, concentrated hydrochloric acid, concentrated acetic acid, 30% ammonia solution, phosphoric acid and dipotassium hydrogenphosphate were obtained from Merck (Darmstadt, F.R.G.). Amino acid calibration standards, Type P-B and P-AN were purchased 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. Phenylhydrazine hydrochoride was recrystallized twice from water, dried overnight at 37°C and stored in the dark until used. A 494 mmol/l working solution was prepared daily by dissolving 286 mg of the salt in 4.0 ml of water. Portions of 25 U of enzyme were reconstituted with 1.0 ml of 0.1 *M* potassium phosphate buffer (pH 8.3) by gentle shaking. The lyophilized powder is stable for at least 2 months when stored at 4°C, but the reconstituted portions must be used within 1 week.

Stock solutions of 1.0 *M* glycolic acid were prepared in water and stored in a refrigerator. Working standard solutions, containing 200 and 800 μmol/l, were prepared daily by diluting 50 μl and 200 μl, respectively, of the stock solution with 250 ml of water. An aqueous solution containing approximately 600 μmol/l of oxaloacetate was prepared daily and used as an internal standard.

### *Methods*

Samples from 24-h urines, collected in the presence of 2.0 ml of chlorhexidine

gluconate, were obtained from twenty healthy persons (normals), 60 idiopathic calcium stone formers (ICaSF) and two patients with Type I primary hyperoxaluria (Type I HOx) while on an *ad libitum* diet. The urine samples were stored frozen and analysed within 1 month. Before analysis, the samples were filtered through 0.22-μm cellulose filters (Millipore, Segrate, Milan, Italy).

The derivatives were prepared by adding to 10-ml glass vials 50 μl of standard solution or urine, 50 μl of internal standard solution, 2.0 ml of phosphate buffer, 100 μl of cysteine and 100 μl of 494 mmol/l PH. After vortex mixing, 50 μl of the suspended enzyme were added and the solutions were mixed. The mixtures were left to react for 10 min at room temperature; the light yellow solutions were injected into the liquid chromatograph.

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. Peak heights were measured with a Shimadzu (Kyoto, Japan) R1A recorder–integrator. The detector output was set at 0.002 a.u.f.s. and the recording sensitivity was 8 mV at full scale.

An octadecylsilyl LiChrospher RP-18 (10 μm) column (250 × 4 mm I.D.) (Merck) was used throughout, connected with a Perisorb RP-18 (30–40 μm) (Merck) guard column (30 × 4 mm I.D.). Isocratic elution was performed at a flow-rate of 2.0 ml/min with methanol–0.15 *M* acetic acid (12:88, v/v) adjusted to pH 6.80 with ammonia solution as the mobile phase. Detection was performed at 324 nm. The buffer composition in the mobile phase was accurately controlled and the column was periodically cleaned by flushing with methanol and acetonitrile.

## RESULTS AND DISCUSSION

The reactivity of GLX towards PH was detailed in a previous paper[2], in which the respective *syn*- and *anti*-phenylhydrazones were shown to be produced rapidly and quantitatively under the conditions described. The determination of GLY was performed with a reaction system based on the above derivatization. GAO catalyses the oxidation of GLY to GLX by molecular oxygen, producing hydrogen peroxide; oxidation appears to proceed further, making the disappearance of GLX very rapid. GLX is a reaction intermediate which, in the presence of a suitable PH concentration, is converted to the corresponding hydrazone, as described above. The conversion is performed at pH 8.3, where the enzyme activity is highest, and in the presence of an excess of cysteine as a reducing substrate to protect the GLX from the hydrogen peroxide formed:

$$CH_2OHCOOH + O_2 \xrightarrow{GAO} H_2O_2 + CHOCOOH \xrightarrow{PH} C_6H_5NHN{=}CHCOOH$$

$$\begin{array}{ll} \text{cysteine} \downarrow & \downarrow \text{GAO}, \text{O}_2 \\ \text{cystine} & \text{unknown} \end{array}$$

The enzymatic conversion requires at least 0.6 U of enzyme per millilitre of reaction mixture to be complete within 10 min for GLY concentrations lower than 1000 μmol/l. Under these conditions, the enzymatic conversion represents the rate-determining step. Longer reaction times are required for the analysis of samples with a high GLY content. The quantitative conversion of GLY at concentrations between 1000 and 3000 μmol/l is achieved within 1 h, but appropriate dilution of the sample is advisable for concentrations up to 1000 μmol/l (see Fig. 1).

Under the chromatographic conditions described, derivatives of GLY and of the less retained internal standard are eluted in *ca.* 5 and 3 min, respectively. Chromatograms of a standard solution and a urinary sample are shown in Fig. 2.

Chromatograms from blanks show the presence of a small peak, eluted at the same retention time of that of the GLX phenylhydrazone. It corresponds to a GLY concentration of approximately 10 μmol/l. Further, PH reacts with other α-keto acids to produce the corresponding hydrazones. The retention times of the derivatives of some α-keto acids of biological relevance are as follows: oxaloacetate, 2.95; α-ketoglutarate, 4.57; glyoxylate, 5.15; and pyruvate, 5.92 min.

Urinary GLY stability was studied by storing two pools of urine, in the presence of 0.1 *M* hydrochloric acid, 0.04% of chlorhexidine gluconate and without stabilizers, either at room temperature or at −20°C. GLY was measured immediately and on subsequent days. The results showed that GLY is stable, irrespective of the means of preservation, for at least 1 month when stored in the frozen state.

Oxaloacetate (600 μmol/l) was used as an internal standard in the determination of GLY in order to minimize the injection error. Its natural concentration in urines was found to be insignificant in comparison with the amount added. Its phenylhydrazone was eluted in a blank zone of the chromatogram. The rate of the reaction between oxaloacetate and PH, under the above conditions, is so rapid that optimum conditions for its use as an internal standard are fulfilled. The instability of the 600 μmol/l oxaloacetate solutions was not considered to cause problems in this context.

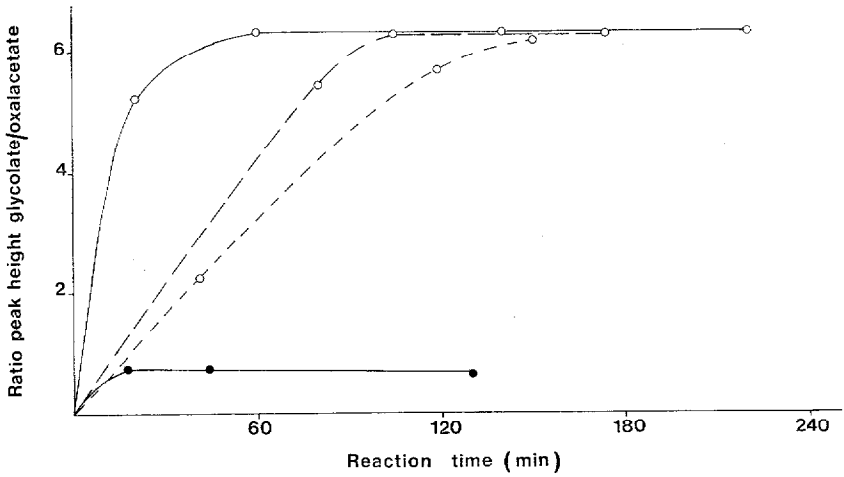


Fig. 1. Urinary glycolate conversion as a function of enzyme activity and glycolate concentration. The reaction was carried out as described in the text. Urine samples containing (i) 3356 μmol/l (○) and (ii) 363 μmol/l (●) of GLY reacted in the presence of 1.25 (———), 0.75 (— —) and 0.25 (– – –) U of enzyme.

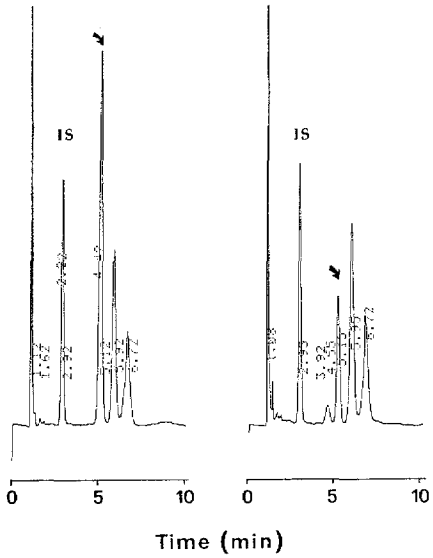


Fig. 2. Typical HPLC traces for the determination of glycolate under the conditions described in the text. Left, glycolate standard, 800 μmol/l in water; right, urine sample. The arrows show the glyoxylate phenylhydrazone peak.

The concentrations of GLY in urine samples were calculated according to the equation

$$\text{glycolate concentration} = \frac{(c_{gly})_{st}}{(h_{gly}/h_{is})_{st} - (h_{bl}/h_{is})_{bl}}\left[(h_{gly}/h_{is})_{sa} - (h_{bl}/h_{is})_{bl}\right]$$

where $h_{gly}$ and $h_{is}$ represent the peak heights of GLY and the internal standard (IS), respectively, in the standard solution (st), in urine samples (sa) and in blanks (bl); $(c_{gly})_{st}$ is the GLY concentration of the 800 μmol/l standard solution.

In each batch of samples, a 200 μmol/l standard and one sample, spiked with 200 μmol/l of GLY, were included as a check on the enzyme activity. The natural GLX contribution to the peak was found to be negligible[2] and was therefore not taken into account.

The calibration graph for the determination of GLY, obtained by plotting the GLY and IS peak-height ratios at various GLY concentrations was linear in the range 10.0–1000 μmol/l, and the minimum detectable concentration was 10.0 μmol/l at a signal-to-noise ratio of 10:1.

Two urine samples containing 166 and 643 μmol/l of GLY were analysed five times, yielding an intra-assay coefficient of variation (C.V.) of 3.8% and 4.6%, respectively, and a day-to-day between-run C.V. of 4.6% and 7.5%, respectively. The accuracy was tested by adding known amounts of GLY, ranging from 200 to 1000 μmol/l, to twenty urine samples. A mean recovery of 96.1 ± 7.5% shows the absence of any appreciable enzymatic inhibition or matrix effect.

The addition to urine samples of 1.0 mmol/l of oxaloacetate, α-ketoglutarate, glutarate, L-lactate, pyruvate, oxalate, mesoxalate, L-citrate, L-glucose, L-ascorbate,

L-tartrate, tartronate, malonate, maleate, malate, succinate and the common physiological L-amino acids did not produce any significant interference in the determination of GLY. The same also holds for the addition of 1.0 mmol/l of β-phenylpyruvic and *p*-hydroxyphenylpyruvic acids.

Chromatograms of reaction mixtures without addition of enzyme show the absence of potentially interfering extraneous peaks. Similar results were obtained on adding PH 1 h after the start of the enzymatic reaction, demonstrating the absence from the end products of the enzymatic conversion of non-specific peaks.

Urinary excretion levels determined on normals, ICaSF and Type I HOx patients are reported in Table I. Mean values and standard deviations are shown. The significance of differences was assessed by means of the Mann–Whitney rank-sum test.

The following considerations support the specificity of the proposed enzyme–HPLC method; first, it makes use of the enzymatic and selective transformation of an α-hydroxy acid to the corresponding α-keto acid. Second, only this class of urine carbonyl compounds show a relevant reactivity toward PH, with the rapid production of the corresponding phenylhydrazones.

In the reaction mixture, in the absence of pH, GLX has been found to disappear rapidly. This could be due to a certain affinity of the anzyme for GLX, but in our hands, in the presence of cysteine, in agreement with previous reports[13], neither oxalate nor formate could be detected among the end-products of the oxidation.

The lack of selectivity of α-keto acid oxidases for a homologous series of substrates has already been reported[13,18]. This could represent a drawback to the analytical use of glycolate oxidase in colorimetric determinations. In our work, the derivatives of the homologous compounds of GLY are easily resolved by HPLC, and this obviates the lack of enzymic specificity.

The low detection limits allow the determination of GLY in both normal and pathological urines. The normal ranges of urinary GLY so far reported are conflicting. Our results are in agreement with those obtained by both isotope dilution[19,20] and colorimetric[12] methods and are slightly higher than those found by an earlier enzymatic method[13].

Preliminary results on GLY excretion confirm sharp increases in patients with Type I HOx. In the present series ICaSF also show slightly higher values than healthy individuals. This could be ascribed to the fact that most of the patients considered here had mild hyperoxaluria, which has been reported to be either intestinal[10] or endogenous[8] in origin.

TABLE I

24-h URINARY GLY LEVELS IN NORMALS, ICaSF AND TYPE I HOx

| *Parameter* | *Normals* | *ICaSF* | *Type I HOx* |
|---|---|---|---|
| *n* | 25 | 60 | 6[a] |
| Mean (μmol/l) | 450 | 629[b] | 6167[c] |
| S.D. (μmol/l) | 139.9 | 354.9 | 4616–0 |
| Range (μmol/l) | 207–732 | 162–2120 | 1180–10950 |

[a] Means from three separate urine collections from two patients.
[b] $p < 0.024$ *vs.* normals.
[c] $p < 0.001$ *vs.* normals.

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