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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GLYOXYLIC ACID IN URINE

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the determination of urinary glyoxylic acid is proposed. The system is based on the precolumn derivatization of α-keto acids by means of phenylhydrazine, separation of the phenylhydrazone formed by HPLC and spectrophotometric detection at 324 nm. The method is precise and allows the determination of 0.5 μmol/l glyoxylate. The poor stability of glyoxylate under all conventional preservation conditions requires the analysis to be carried out as soon as possible after urine collection. Results of determinations on urine samples from healthy controls and from patients with idiopathic calcium stone disease and type I primary hyperoxaluria are reported.

INTRODUCTION

Apart from ascorbate (ASC), glyoxylic (GLX) and glycolic (GLY) acids are the only known precursors of oxalate (OX) in man. Exogenous ASC contributes to oxaluria via its endogenous non-enzymic transformation to OX and it is widely agreed that in healthy subjects about 30% of urinary OX can be ascribed to ASC [1].

GLY and GLX are involved in the metabolism of glycolaldehyde and serine and are enzymatically interconvertible and easily transformed into OX. The rate of endogenous GLX production is lower than its LDH (lactate dehydrogenase) and GAO (glycolic acid oxidase) catalysed oxidation, so that both serum and urine GLX levels are usually low. In contrast, endogenous GLY, which is a more

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stable molecule, has higher serum and urine levels. Both acids are considered to be useful parameters in metabolic studies of hyperoxaluric syndromes.

The absence of the soluble thiamine pyrophosphate-dependent carboligase enzyme is the pathogenetic cause of type I primary hyperoxaluria; the GLX consumption through this metabolic route is hindered, providing an increase in GLX and also GLY and OX biosynthesis [1]. Similar trends have been reported in cases of vitamin B_6 deficiency, owing to the reduced transamination of GLX to glycine [2]. Also it has been proposed that in some instances the mild hyperoxaluria of idiopathic stone disease, when endogenous in origin, could be detected by an increase in the excretion of the above acids [3].

A spectrophotofluorimetric method for the determination of GLX in biological materials has been proposed but the complex sample processing and the lengthy treatment procedure make it unsuitable for routine analysis [4]. More recently, different chromatographic methods have been proposed for determining acids or keto acids in physiological fluids. The gas chromatographic procedures include matrix extraction by means of ion exchangers, lyophilization of the extracts and silylation of the residues to produce volatile and detectable derivatives [5–8]. These techniques are tedious and time-consuming but allow information about the total acidic content of the biological sample to be obtained.

Some other techniques have been developed involving liquid chromatography (LC) with precolumn derivatization. Phenacyl bromide [9] 2-nitrophenylhydrazine [10] and 4-bromomethyl-6,7-dimethoxycoumarin [11] have been proposed as derivatizing agents for carboxyl groups to give UV-absorbing or fluorescent products. However, the chromatographic resolution of urinary compounds at low concentrations may be difficult. The well known reaction between carbonyl groups and phenylhydrazine derivatives can be used to obtain highly UV-absorbing products. This reaction has been used for the colorimetric determination of keto acids [12,13]; it can be made quantitative and produces stable UV-absorbing hydrazones. Moreover, the derivatives can be resolved and detected by LC procedures, enhancing the specificity of the determination [14].

In this paper we propose a high-performance liquid chromatographic (HPLC) procedure for the determination of glyoxylate in urine, which involves the precolumn derivatization of glyoxylate with phenylhydrazine (PH). The stable hydrazone formed can be resolved by HPLC. The reaction is rapid and does not require heating. This procedure allows any further decomposition and/or overproduction of GLX by parallel decomposition steps possibly enhanced by higher temperatures, to be minimized. The results of studies on the stability of GLX in urine samples were also obtained, with particular reference to the rapid decomposition of GLX.

EXPERIMENTAL

Reagents

Analytical-reagent grade chemicals and deionized water were used. Sodium glyoxylate monohydrate, sodium pyruvate, oxaloacetic acid and sodium α-ketoglutarate were purchased from Fluka (Buchs, Switzerland), concentrated hydro-

chloric acid, glacial acetic acid, ammonia solution (30%), phosphoric acid, sulphosalicylic acid and dipotassium hydrogenphosphate from Merck (Darmstadt, F.R.G.) and chlorhexidine gluconate (20% aqueous solution) from ICI (Macclesfield, U.K.) phosphate buffer (0.1 mol/l) was prepared by dissolving 4.35 g of dipotassium hydrogenphosphate in 250 ml of water and adjusting the pH to 8.3 with phosphoric acid.

HPLC-grade methanol and acetate buffer solution (0.15 mol/l acetic acid adjusted to pH 6.80 with ammonia solution) were used for the mobile phase.

Glyoxylate stock standard solution was prepared daily by dissolving 114.0 mg of sodium glyoxylate monohydrate in 100 ml of water; 50 and 100 μl of this solution were diluted to 100 ml with water to obtain 5.0 and 10.0 μmol/l working standard solutions.

Urine collection

Urine samples (24 h), collected with 1 ml of 20% chlorhexidine gluconate as preservative, were obtained from 10 healthy volunteers (controls), 29 idiopathic calcium stone formers (ICaSF) and 2 patients with type I primary hyperoxaluria (Type I HOx) while on their free home diet. All the subjects were invited to bring samples just when the collection was terminated. GLX analysis was performed immediately after the samples were provided. Before analysis the samples were filtered through 0.22-μm cellulose filters (Millipore).

Derivatization procedure

The PH derivatization was carried out by mixing, in 10-ml screw-capped glass vials, 30 μl of standard or of urine, 2.0 ml of phospate buffer and 100 μl of 5% aqueous PH solution. The mixtures were shaken and left to react for 10 min at room temperature; the light yellow solutions were then injected into the HPLC apparatus. The mild conditions under which derivatization occurs ensures a very limited stress of the samples.

Chromatography

A Varian 5500 liquid chromatograph equipped with a UV–Vis detector and a Rheodyne 7126 automatic injection valve with a 50-μl loop was used. Peak heights were measured with a Shimadzu R1A recorder–integrator. An octadecylsilyl LiChrospher RP-18 (10μm) column (250 mm$\times$4 mm I.D.) (Merck) was used throughout, connected with a 30 mm$\times$4 mm I.D. Perisorb RP-18 (30–40 μm) (Merck) guard column. Elution was performed isocratically at a flow-rate of 1.0 ml/min with methanol–acetate buffer (see above) [11:89 (v/v)] as the mobile phase. Detection was carried out at 324 nm, where the GLX–PH derivative shows an absorption maximum.

In order to achieve optimum resolution and reproducibility in the separation, the buffer composition in the mobile phase has to be accurately controlled and the column has to be periodically cleaned by flushing with pure methanol and acetonitrile.

RESULTS

Kinetics

PH reacts with activated carbonyl compounds at neutral pH to produce the corresponding hydrazones. The reaction between GLX and PH produces two (*syn* and *anti*) stereoisomers. The less polar, under the described chromatographic conditions, is very strongly retained. The mixture can be resolved by increasing the methanol concentration to 40%.

Experiments showed that in aqueous buffered solution at pH 6–8.5 and in the presence of GLX (50 μmol/l) and PH (20 mmol/l) the *syn*/*anti* ratio is constant. Moreover, in aqueous solutions buffered at pH 8.3 (the present conditions) the amount of each isomer formed is proportional to the total GLX at concentrations up to 50 μmol/l.

The method was developed for the less retained isomer, which is formed in higher yield. In the pH range 6–8.5, the glyoxylate phenylhydrazone is produced very rapidly. In a 2.3 μmol/l GLX aqueous buffer solution at pH 8.3, in the presence of 20 mmol/l PH, the reaction reaches equilibrium within 10 min and the hydrazone produced is stable for at least 8 h. At lower PH concentrations, the decomposition of the derivative seems to become significant to such an extent that complete conversion is not achieved (see Fig. 1).

The reactivity of urinary GLX was investigated by adding known amounts of GLX to urine samples and following the rate of formation of the product. The kinetic behaviour was essentially the same as that observed with standard aqueous solutions, indicating the absence of appreciable matrix effects (see Fig. 2). Nevertheless, if the reaction is allowed to proceed for more than 10 min, the GLX peak begins to increase slowly owing to degradation reactions, probably induced by excess of PH, resulting in an increased GLX level. Extrapolation to time zero allows such decomposition to be avoided. However, if the chromatographic analysis is carried out from 10 to 30 min after the start of the reaction, the interference

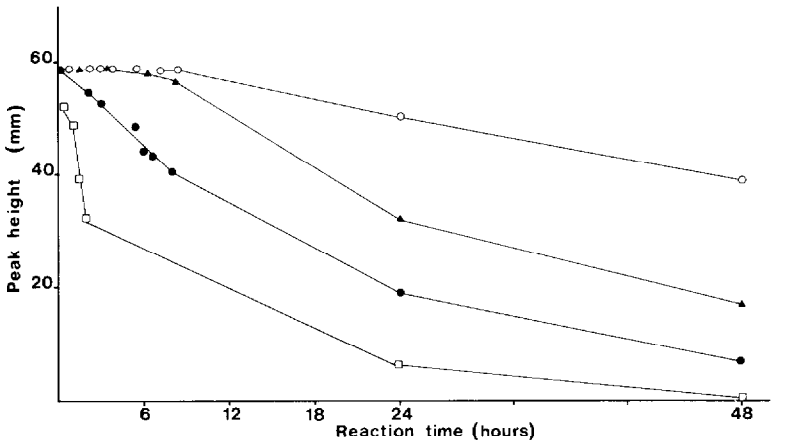

Fig. 1. Time dependence of the stability of glyoxylate phenylhydrazone in solution containing 100 μl of 50 μmol/l glyoxylate, 2 ml of pH 8.3 phosphate buffer and 100 μl of (○) 10%, (▲) 5%, (●) 1% and (□) 0.1% phenylhydrazine.

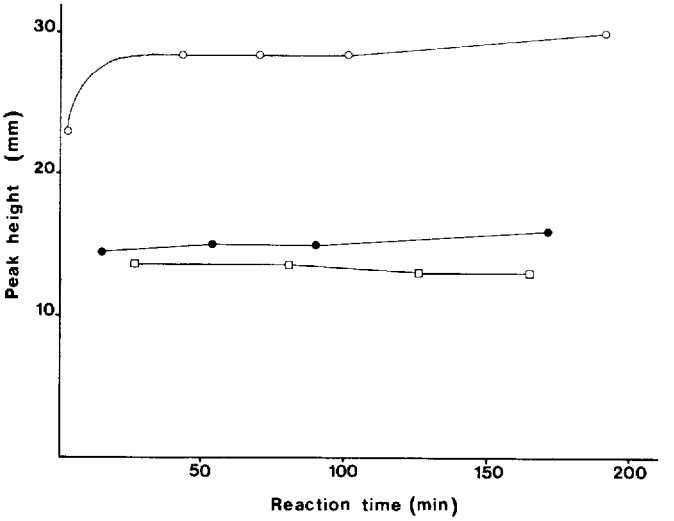

Fig. 2. Reactivity of urinary glyoxylate. (●) Unspiked urine, (○) urine spiked with 10 μmol/l glyoxylate and (□) 10 μmol/l aqueous solution of glyoxylate. The analysis was performed as outlined under Experimental.

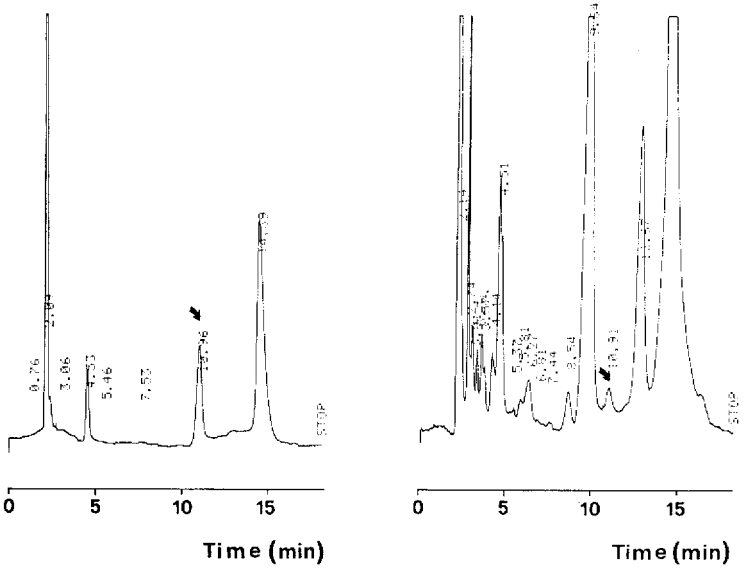

Fig. 3. Typical chromatograms for the determination of glyoxylate (arbitrary units at 324 nm). Left: 10 μmol/l glyoxylate aqueous standard. Right: urine sample. The arrows indicate the glyoxylate phenylhydrazone peak.

appears to be negligible (Fig. 2). Typical chromatograms of a GLX standard solution and a urine sample are reported in Fig. 3.

By heating the buffered (pH 8.3) sample, before PH addition at 100°C for 30 min, GLX is decreased but the slow increase of the peak still occurs. Heating of

TABLE I

EFFECT OF TEMPERATURE ON NATIVE GLYOXYLATE IN BUFFERED SAMPLES AND ON THE YIELD OF THE REACTION BETWEEN PHENYLHYDRAZINE AND GLYOXYLATE

Parameter	Proposed procedure	Heating after PH addition		Heating before PH addition	
Temperature (°C)	25	100	100	100	100
Heating time (min)		10	30	10	30
Standard (5 μmol/l)	5.0	6.3*	22.9*	2.9*	1.7*
Urine A	0.7	12.9	54.3	1.3	0.4
Urine A + 5 μmol/l	5.4	14.3	52.9	2.0	0.6
Absolute recovery (%)*	94.0	28.0	<0	14.0	4.0
Relative recovery (%)**		22.8	<0	24.1	11.8
Urine B	1.7	20.0	44.0	3.1	1.1
Urine B + 5 μmol/l	6.9	25.7	45.1	2.9	0.9
Absolute recovery (%)*	104.0	114.0	22.0	<0	<0
Relative recovery (%)**		90.5	4.8	<0	<0

*Evaluated with reference to 5.0 μmol/l GLX aqueous solution at 25°C.
**Evaluated with reference to 5.0 μmol/l GLX aqueous solution in the same treatment series.

the final reaction mixture at 100°C for 30 min causes an overestimation of endogenous GLX (see Table I).

Stability

Glyoxylate (200 μmol/l) was added to two urinary pools, which were then stored either at room temperature or at −20°C under the following conditions: (i) with addition of 0.1 mol/l hydrochloric acid, (ii) with addition of 0.04% of chlorhexidine gluconate and (iii) without stabilizers. GLX analyses were carried out immediately after sample collection and on the following days. Very rapid decomposition, independent of the preservation procedure, was observed. Preservation by addition of chlorhexidine gluconate was perferred as the decomposition with time was much reduced compared with the use of hydrochloric acid (Fig. 4). Native urinary GLX showed similar trends when analyses were delayed (Fig. 5).

A deproteinization procedure with sulphosalicylic acid, which had been carried out in preliminary tests, was discarded as no improvement in GLX stability was observed.

Calibration graph

The calibration graph is linear in the range 0.5–50 μmol/l and the minimum detectable concentration in urine is 0.5 μmol/l (signal-to-noise ratio 10:1).

Accuracy and precision

Three urine samples containing 2, 12 and 52 μmol/l GLX were analysed five times, giving intra-assay coefficients of variation (C.V.) of 15.8, 11.0 and 4.8%, respectively. The accuracy was tested by adding 2, 5 and 10 μmol/l GLX to urine samples. The results are given in Table II.

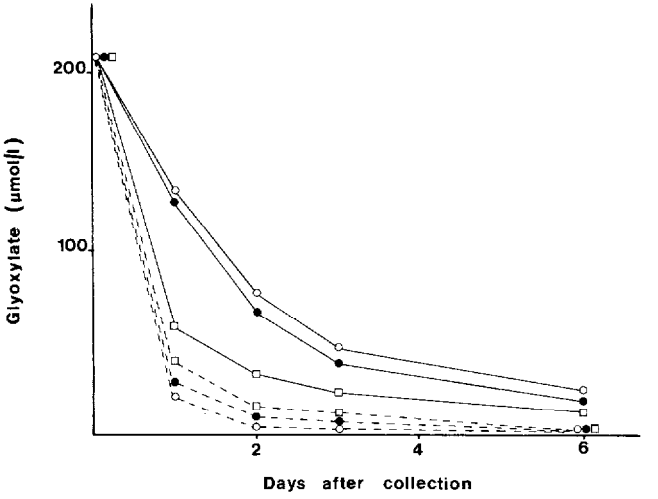

Fig. 4. Stability of urine glyoxylate. Analysis performed on a urine pool spiked with 200 µmol/l glyoxylate and stored in the presence of (i) 0.1 mol/l hydrochloric acid (□), (ii) 0.04% chlorhexidine gluconate (○) and (iii) without stabilizers (●), at −20°C (solid lines) and at room temperature (broken lines).

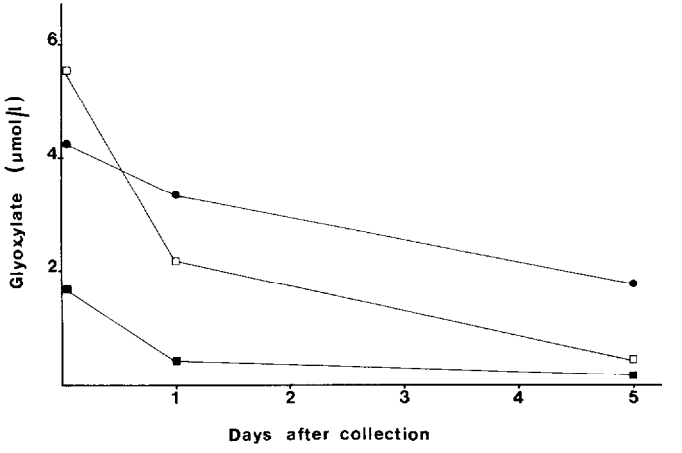

Fig. 5. Stability of native glyoxylate in three urine samples stored at −20°C in the presence of 0.04% chlorhexidine gluconate.

The addition to urine samples of 1.0 mmol/l oxaloacetate, α-ketoglutarate, glutarate, pyruvate, oxalate, mesoxalate, glycolate, glucose, ascorbate, tartrate, tartronate, malonate, maleate, malate, succinate and the common physiological L-amino acids did not lead to significant interferences in the GLX determination.

It should be mentioned that other keto acids can be easily determined by the described procedure. Fig. 6 shows an example of the chromatogram obtained with a mixture of oxaloacetic, α-ketoglutaric, glyoxylic and pyruvic acids.

TABLE II

RECOVERY OF ADDED GLYOXYLATE FROM URINE USING THE PROPOSED METHOD

Initially present (μmol/l)	Added (μmol/l)	Found (μmol/l)	Recovered (μmol/l)	Recovery (%)
0.5	2.0	2.9	2.4	120
1.3	2.0	3.5	2.2	110
0.4	5.0	4.3	3.9	78
0.7	5.0	5.8	5.1	102
0.7	5.0	5.4	4.7	94
1.7	5.0	6.9	5.2	104
1.3	5.0	6.6	5.3	106
2.3	5.0	7.1	4.8	96
5.4	5.0	10.6	5.2	104
2.8	10.0	12.6	9.8	98
9.7	10.0	19.6	9.9	99
1.3	20.0	20.7	19.4	97
7.3	20.0	24.7	17.4	87
Mean				99.6
Coefficient of variation (%)				10.4

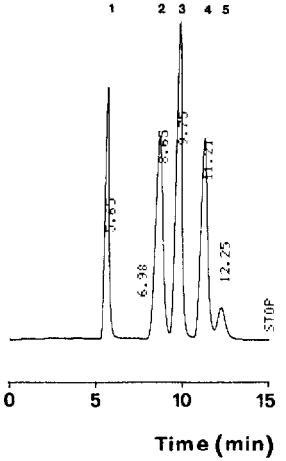

Fig. 6. HPLC of a mixture of α-keto acids in aqueous solution. Peaks: 1 = oxaloacetate; 2 = α-ketoglutarate; 3 = glyoxylate; 4 = pyruvate; 5 = phenylhydrazine.

Clinical results

Urinary excretion levels determined on controls, ICaSF and Type I HOx patients are reported in Table III.

CONCLUSIONS

The proposed method was shown to be suitable for use in clinical practice owing to its speed and simplicity. Its sensitivity is such that even very low levels of GLX,

TABLE III

24-h URINARY GLYOXYLATE LEVELS IN NORMAL SUBJECTS, ICaSF AND TYPE I HOx

Subject	Urinary glyoxylate level (μmol per 24 h)			*n*
	Mean	S.D.	Range	
Normal	2.18	1.64	0.2–5.4	10
ICaSF	5.82	5.49	0.2–21.0	29
Type I HOx	30.10	18.50	11.0–46.4	4*

*Means from two separate urine collections from each patient.

such as those found in normal urine samples, could be easily detected. Indeed, the urine GLX excretion for normal subjects found here was lower than that given by previous methods [4,14]; however, the results of accuracy determinations indicate that no significant underestimation of the actual urine GLX occurred. In fact, complete recoveries of added amounts of GLX prove that the conversion is rapid and quantitative also for native urinary GLX.

We conclude that our low values for normal subjects are due to decomposition of GLX, which proceeds over the collection period, rather than to an analytical underestimation of GLX. Our experimental data indicate that excessive sample manipulation should be avoided in order to obviate overestimation of GLX. GLX excretion by hyperoxaluric patients was confirmed to be higher than that of control subjects. However, a certain overlap was observed among the three groups of subjects studied, namely between ICaSF and Type I HOx. Therefore, although urine GLX determined by the proposed method is a fairly good index of the source of endogenous oxalate, its poor stability in biological fluids deserves further study. At present metabolic studies of hyperoxaluria syndromes must include, in addition to urine GLX, also the determination of the more stable glycolate in plasma and urine.

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