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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GLYOXYLIC ACID AND OTHER CARBONYL COMPOUNDS IN URINE

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SUMMARY

A high-performance liquid chromatographic method for the determination of carbonyl compounds, namely aldehydes, ketones and keto acids in urine, has been developed. Pre-column derivatization with 2,4-dinitrophenylhydrazine offers sufficient sensitivity for the determination of glyoxylic acid in urine with a detection limit of 0.5 mg/l. The separation is performed on a C_{18} 10-μm column and with an acetonitrile–aqueous buffer mobile phase, which also allows the resolution of the *syn* and *anti* geometric isomers. Matrix effects, precision, accuracy, limits of detection and structural selectivity of the method are discussed.

INTRODUCTION

The massive increase in urine oxalate that occurs in the course of type I primary hyperoxaluria is known to be associated with relevant derangements in glyoxylic acid excretion [1]. Lower increases in oxalic acid excretion are often also found in the case of idiopathic calcium oxalate stone disease, and they are believed to depend on either intestinal hyperabsorption [2] or an increase in endogenous production [3]. An accurate determination of glyoxylic acid could, therefore, be a very useful tool for investigation of the pathogenesis of this "mild hyperoxaluria". The determination of glyoxylic acid in urine presents several difficulties, mainly because of its low concentrations and the interferences from the matrix. The methods actually available are very lengthy owing to tedious pretreatments and therefore cannot be adopted for routine assays. The main procedures described are based on enzymatic methods [4], chromatographic meth-

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ods [5,6], fluorimetric methods [7], colorimetric methods [8,9] and high-performance liquid chromatography (HPLC) with fluorimetric detection [10–15] or UV detection [16,17].

In this paper we present an analytical method for the determination of carbonyl compounds (glyoxylic and keto acids) in urine by reversed-phase HPLC. Analysis times are short and reduced sample pretreatment is required. The method is based on pre-column derivatization of the carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH) [18–25], chromatographic separation on a reversed-phase C_{18} column and UV detection at 360 nm of the corresponding hydrazones.

EXPERIMENTAL

Chemicals

Reagent-grade chemicals and high-purity water W (Millipore Milli-Q, filtered through 0.45-μm membrane filters) were used. Organic solvents used in mobile phases and in the derivatization procedure (acetonitrile and methanol) were HPLC grade (Merck, Darmstadt, F.R.G.). DNPH was purchased from Fluka (Buchs, Switzerland). Glyoxylic acid and all other carbonyl compounds were analytical-grade reagents (Merck or Fluka).

Instrumentation

A Varian (Palo Alto, CA, U.S.A.) 5500 HPLC instrument equipped with a variable UV–Vis detector was used. Chromatographic data were processed with a C-R1A integrator/printer from Shimadzu (Tokyo, Japan). The injection valve (Rheodyne 7126 automated sample injector) had a 10-μl sample loop. The chromatographic column used throughout was a μBondapak (250 mm $\times$ 4.6 mm I.D.; 10 μm) from Waters Assoc. (Milford, MA, U.S.A.). The mobile phase was acetonitrile–aqueous 0.01 *M* acetate buffer (pH 6.1), with a linear gradient from 20% acetonitrile.

Derivatization

The derivatization was carried out in a 5-ml Pyrex vial with a threaded cap, with 1 ml of urine sample (24-h urine was collected on 10 ml of 37% hydrochloric acid filtered through a 0.2-μm filter and stored) and 1 ml of derivatizing agent (DNPH, 300 mg/l in 2.0 *M* hydrochloric acid). The vial was heated in a water-bath at 50 °C for 30 min and stored in the dark. During the reaction, a slight colour increase appeared, and at the end the solution was perfectly clear and it might be injected directly into the HPLC apparatus.

RESULTS AND DISCUSSION

Derivatization

The derivatizing agent employed, DNPH, gives rise to highly UV-absorbing hydrazones, which are amenable to reversed-phase separation. The reaction occurs with acid catalysis, at pH ca. 0 according to the following mechanism (for glyoxylic acid):

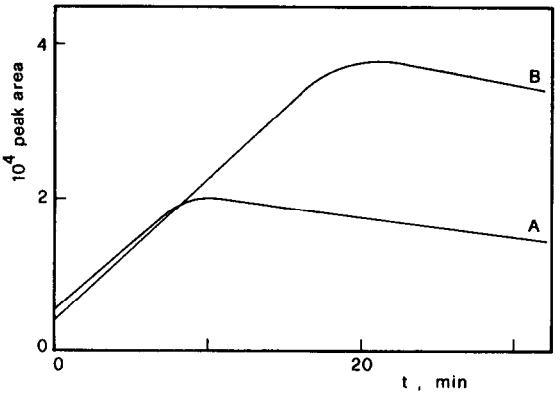

Fig. 1. Derivatization yield as a function of time as determined from the peak area of glyoxylic acid in (A) a standard solution (1 mg/l) and (B) a urine sample.

$$HOOC\text{-}CHO + H_2N\text{-}NH\text{-}C_6H_3(NO_2)_2 \rightarrow$$

$$HOOC\text{-}CH{=}N\text{-}NH\text{-}C_6H_3(NO_2)_2 \qquad (1)$$

An acidic medium is necessary for the derivatization, especially for ketocarboxylic acids, in order to avoid the deactivating effect of the dissociated $-COO^-$ moiety.

It is worth mentioning that the formation of hydrazones may give rise, for some compounds, to two distinct geometric *syn* and *anti* isomers, which in some cases may be resolved into two distinct peaks [26].

The conditions for the derivatization reaction were optimized using a standard solution of glyoxylic acid containing 1.0 mg/l in the presence of an excess of DNPH (300 mg/l). At higher concentrations the derivatization is almost instantaneous, but at 1.0 ppm the reaction goes to completion in ca. 10 min at 50°C (Fig. 1). The derivatization of a urine sample, however, takes place more slowly, and ca. 20 min are necessary (Fig. 1). The procedure of heating at 50°C for 30 min was adopted in order to ensure complete reaction. Longer times and higher temperatures resulted in partial decomposition of the product and concomitant appearance of additional peaks in the chromatograms. Therefore, cooling and keeping the final solution in the dark are recommended.

In order to make the reaction quantitative, the analyte must be present in undissociated form (where dissociable acid groups are present). This may be achieved using a non-polar solvent or an acidic medium. The latter alternative was adopted, and 2.0 *M* hydrochloric acid was added to the reaction medium. Under these conditions, the extent of formation of the two *syn/anti* isomers (when resolved) is similar and either of the two peaks can be quantified.

No variation in the peak area of glyoxylic acid was observed using a different excess of DNPH, thus showing that the reaction is almost quantitative. A concentration of 300 mg/l of the derivatizing agent solution was used in order to ensure suitable conditions for all the carbonyl compounds present in a sample of unknown composition.

Chromatographic separation

The optimum separation conditions and the pH of the mobile phase were determined using a mixture of carbonyl compounds usually present in urine, namely α-ketoglutaric acid, glyoxylic acid, and pyruvic acid, and others such as formaldehyde, acetaldehyde, acetone, crotonaldehyde, acetophenone and benzophenone. With a mobile phase of acetonitrile–acetate buffer, the products showed a remarkable increase of retention time with increasing number of CH_3–units in the molecule, while carboxylic groups significantly reduced the elution time. The eluent pH that gave the best separation was 6.10. At this pH, dicarboxylic keto acids are eluted first, followed by monocarboxylic keto acids, and finally by aldehydes and ketones. Lower pH values resulted in increased retention times for dicarboxylic acids with reduced resolution from the monocarboxylic acids; at higher pH values, all the keto acids were eluted with retention times that were too short. The chromatographic behaviour of carbonyl compounds that do not bear carboxylic groups is independent of mobile phase pH.

Fig. 2 shows a chromatogram of the above mixture, and a typical chromatogram of a urine sample is shown in Fig. 3. Table I reports the retention times, together with an estimate of the coefficient of variation (C.V.) and the detection limit for the compounds investigated (in the case of resolved *syn/anti* isomers, i.e. glyoxylic and pyruvic acids, values reported in Table I refer to the major peak). Each compound identified in the urine chromatogram (Fig. 3) was confirmed on the basis of comparison with the standards.

It must be pointed out that the conditions employed in derivative formation likely cause conversion of β-keto acids into the neutral decarboxylation products; thus acetone may have been, in part, acetoacetic acid in the urine. However, quantification of acetone with the present method gives values that compare well with its known urinary levels [27].

Spectrophotometric detection was carried out at 360 nm, where DNPH and the derivatized hydrazones show maximum molar absorptivities. At this wavelength, the molar absorptivities of all the 2,4-dinitrophenylhydrazones investigated were nearly the same and independent of the structure of the carbonyl compounds. This is an advantage, since the correlation between peak area and concentration was the same for all the compounds. A second advantage is the low interference at this wavelength from non-carbonyl compounds. However, the use of a calibration plot does not ensure the absence of matrix effects, therefore such interference was controlled by using urine samples spiked with known amounts of glyoxylic acid. Table II reports the results. The relative error (E) in the determination is represented by the difference of the mean values of glyoxylic acid concentration in the two series: $E=100(X_2-X_1)/A$, where X_1 and X_2 are the concentrations before and after the additions and A is the amount added.

Comparison of the results for the two series enabled us to detect and account for systematic effects. As it can be seen, such matrix effects are reasonably low, and the repeatability is also satisfactory. Also, the retention times for the investigated compounds alone or in mixtures were reproducible.

The above control indicates the presence of a small matrix effect, revealed by the reported error. Such error is, however, very low for urine samples at such low

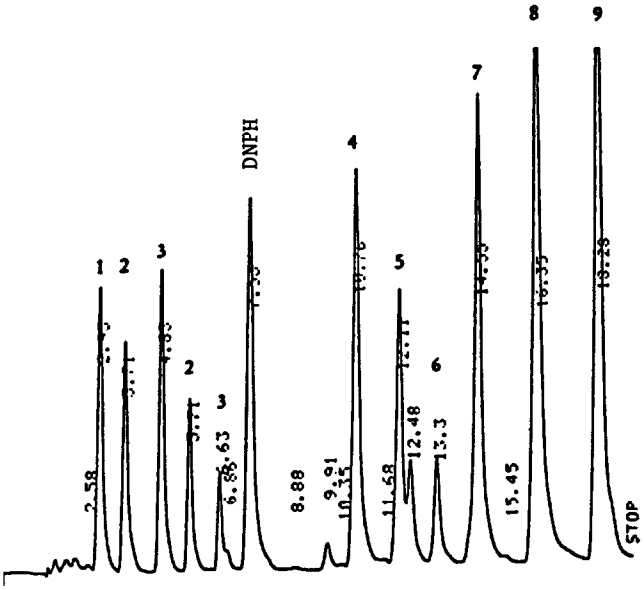

Fig. 2. Chromatogram of a mixture of standards derivatized according to the described procedure. Peak numbers are as in Table I. The amounts injected were 75 ng acetaldehyde and acetone, and 100 ng for the other compounds. Column, Waters μBondapak RP C_{18}, 10 μm; mobile phase, acetonitrile–aqueous acetate buffer (0.01 M) at pH 6.1; linear gradient, 3.5% units/min from 20% acetonitrile at $t=0$; flow-rate 2.0 ml/min; detection, UV at 360 nm.

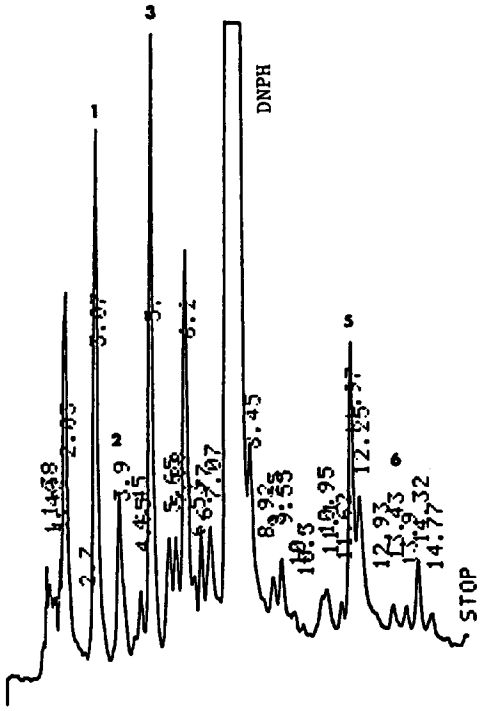

Fig. 3. Chromatogram of a urine sample. Conditions as in Fig. 2. Peak numbers are as in Table I. Glyoxylic acid determined: 1.22 mg/l.

TABLE I

RETENTION TIMES AND DETECTION LIMITS FOR THE CARBONYL COMPOUNDS INVESTIGATED

Compound	Retention time (min)	Detection limit (mg/l)
1 α-Ketoglutaric acid	3.0±0.1	0.5
2 Glyoxylic acid	3.8±0.1	0.5
3 Pyruvic acid	4.9±0.1	0.5
4 Formaldehyde	10.8±0.2	0.1
5 Acetaldehyde	12.1±0.2	0.1
6 Acetone	13.3±0.3	0.3
7 Crotonaldehyde	14.6±0.3	0.2
8 Acetophenone	16.3±0.4	0.5
9 Benzophenone	18.3±0.4	0.5

concentrations, so that the method is satisfactorily accurate even if an external standard calibration method is used.

The precision of the method was tested by the determination of glyoxylic acid in urine. In a series of determinations, five urine samples were derivatized independently at the same time, and the analysis was replicated; five additional sam-

TABLE II

DETERMINATION OF GLYOXYLIC ACID IN UNSPIKED AND SPIKED (1 mg/l) URINE

Sample	Glyoxylic acid (mg/l)		$100(X_2-X_1)/A$ (%)
	Initial	Final	
1	1.13	2.09	96
2	3.20	4.08	88
3	1.22	2.08	86
4	1.01	1.93	92
5	3.17	4.07	90
6	1.80	2.96	116
7	1.28	2.25	97
Mean			95±10
C.V. (%)			10.6

TABLE III

REPLICATE ANALYSES OF GLYOXYLIC ACID IN URINE

	n	Glyoxylic acid (mean) (mg/l)	C.V. (%)
Intra-assay	5	1.31	6.9
Inter-assay	5	1.44	10.4

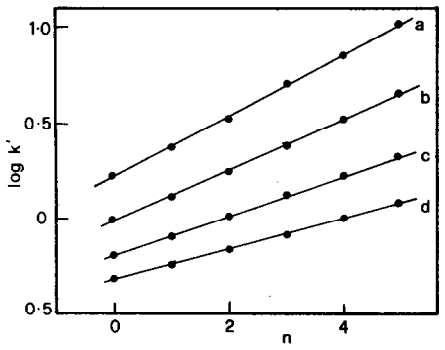

Fig. 4. Variation of log k' as a function of the number of carbon atoms in the series $H(CH_2)_nCHO$, and with different amounts of acetonitrile in the mobile phase: (a) 60%, (b) 70%, (c) 80% and (d) 90% acetonitrile.

ples were analysed for glyoxylic acid on successive days. Table III shows the intra- and inter-assay precision.

The total amount of glyoxylic acid excreted daily by a series of fourteen normal patients, determined with the present method, was 1.50 ± 1.10 mg per day, which compares well with the normal ranges reported in the literature [27].

In order to characterize the interaction conditions of the separated solutes with the mobile and stationary phases, the chromatographic behaviour of the series of aldehydes $H-(CH_2)_n-CHO$ ($n=0$, 1, 2, 3, 4 and 5) was investigated under different isocratic conditions. The linearity of plots of the logarithm of the capacity factor as a function of n is shown in Fig. 4. The linearity shows the increasing effect of the chain length and points out the possibility of predicting chromatographic behaviour from the structure of a member of the series. A similar linear behaviour was also observed within the keto acid series $H-(CH_2)_n-CO-COOH$ ($n=0$, 1 and 2).

A linear trend within the investigated concentration range was observed for glyoxylic acid as well as for α-ketoglutaric and pyruvic acid. The concentration of each compound may therefore be evaluated by using a transformation factor from peak area to concentration determined with the standard solutions.

In conclusion, the present method allows the determination of carbonyl compounds in complex samples such as urines, with particular reference to glyoxylic acid.

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