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Short communication

Fast method for the simultaneous determination of 2-oxo acids in biological fluids by high-performance liquid chromatography

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Abstract

A fast and sensitive method for the single-run quantification of various 2-oxo acids including 2-ketoglutarate, glyoxylate and pyruvate is described. It ensures good separation of peaks with minor interference by other substances. The 2-oxo acid derivatives are measured photometrically at 324 nm after derivatization with phenylhydrazine and subsequent isocratic separation with ethanolic phosphate buffer on a C_{18} reversed-phase HPLC column. Recovery was found to be complete [range: $96.3 \pm 5.6\%$ (pyruvate) to $104.8 \pm 5.2\%$ (2-ketoglutarate)]. Detection limits ranged from less than 0.1 μmol/l (glyoxylate) to 0.25 μmol/l (2-ketoglutarate and pyruvate). Results for all substances examined showed good linearity with correlations (r^2) of equal to or better than 0.998.

1. Introduction

2-Oxo acids are important substrates in vertebrates. They take part in several metabolic processes (gluconeogenesis, amino acid metabolism, fatty acid oxidation, maintenance of acid–base balance). Determination of plasma or tissue concentrations of 2-oxo acids may be a useful diagnostic tool, e.g. in evaluating disorders of the glyoxylate cycle with subsequent hyperoxaluria [1–3] or in cases of impaired acid–base balance with altered turnover of 2-ketoglutarate [4,5].

The majority of the methods used for quantification of 2-oxo acids in clinical or scientific laboratories is based on enzymatic, NAD(P)H-dependent reactions with consecutive registration of the absorbance change [6–9]. Reactions of this type are easy to perform and are sufficiently reliable, at least the single-step types. Multiple-step analytic procedures using indicator reactions are less advantageous; they often require additional substances and enzymes and are therefore inherently more expensive and less reliable. Moreover, most enzymatic tests are suitable for the quantification of only one substance at a time.

The above difficulties in the detection and quantification of 2-oxo acids can be avoided (at least theoretically) by the use of analytical HPLC instead of enzymatic reactions. Indeed, several methods have already been described for the analysis of 2-oxo acids by HPLC [2,3,10–13]. While they are optimized for the determination of the particular oxo acid they were developed for, no verified method exists for the simulta-

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neous quantification of several of the biologically important 2-oxo acids in a single run.

It was therefore our aim to adapt HPLC analysis to the simultaneous determination of several 2-oxo acids. The method evaluated below was developed for the analysis of small amounts of 2-oxovalerate, which may be formed in vitro by renal tissue of the rat during the production of ammonia from hippurate in the presence of propionaldehyde [14]. Additionally, the concentration of pyruvate (PYR), 2-ketoglutarate (KG) and glyoxylate (GLX) was measured with the same set up. The method was supplemented by adding an internal standard to the medium prior to the analysis. For this purpose 2-oxobutyric acid was used. Significant amounts of 2-oxobutyrate which might lead to underestimation of resolved 2-oxo acid peaks were never found either in the medium or in human plasma samples (unpublished results).

In most of the published HPLC procedures for the analysis of 2-oxo acids, derivatization is performed either with *o*-phenylenediamine with subsequent fluorimetric detection, or with phenylhydrazine (PH) and spectrophotometric analysis. The methods utilizing *o*-phenylenediamine are reported to be sensitive but require complex and time-consuming sample processing [10,12]. The use of PH, a reagent widely used in carbohydrate analysis [2,3,13] allows simple and fast sample preparation. Moreover, it ensures the advantages of photometry. PH forms hydrazones with carbohydrates, which can be detected at 324 nm. The simplified method described below is based on derivatization with PH. It allows a high throughput of samples with satisfactory sensitivity and precision.

2. Experimental

2.1. Apparatus

A standard high-performance liquid chromatography unit consisting of a Model 300 high precision solvent delivery system (Gynkotek, Germering, Germany), a C-R6A Chromatopac data module (Shimadzu, Kyoto, Japan) and a LiChroGraph L-4200 UV-Vis variable-wavelength spectrophotometer (Merck, Darmstadt, Germany) was used. A 250 × 2 mm I.D. Spherisorb ODS 2 C_{18} steel column (CS, Langerwehe, Germany) with a spheric particle size of 5 μm served as chromatography column.

2.2. Reagents

The mobile phase was 15 mmol/l phosphate buffer (pH 6.0)–ethanol (95:5, v/v). The reaction buffer (RB) was 150 mmol/l phosphate buffer adjusted to pH 8.0. The internal standard solution (I.S.) was a 100 μmol/l solution of 2-oxobutyrate in RB which was prepared weekly and stored refrigerated. PH solution was prepared by dissolving 50 μl of PH with 1000 μl of RB. It was freshly prepared each day. Ethanol was of chromatography grade, and all other reagents of analytical grade. Chemicals were obtained from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany) and Sigma (Deisenhofen, Germany). The amino acid standard solution P-ANB containing 34 different amino acids (100 μmol/l) was purchased from Benson Polymeric (Reno, NV, USA).

2.3. Preparation and analysis of samples

A 100-μl volume of incubation medium was mixed with 100 μl of RB, 5 μl of PH reagent and 10 μl of I.S.. Since the incubation medium in our experiments contained only a small amount of protein (0.5% albumine) no deproteinization procedures were needed prior to analysis.

Plasma and urine samples were prepared as follows. Urine was obtained by bladder puncture and centrifuged at 3000 *g* for 5 min. The supernatant was transferred into an Eppendorf microtube and stored frozen until analysis. Whole blood was obtained from healthy individuals by puncture of the cubital vein using a common syringe without additives. The blood was kept cool and centrifuged at 1000 *g* as soon as possible.

The plasma or urine supernatant, respectively, was transferred into an Eppendorf microtube

and stored frozen until analysis. In experiments where the recovery of the substances was tested fresh plasma samples were enriched with an aqueous mixture of PYR, KG and GLX (25 μmol/l, 2.5 μmol/l and 0.5 μmol/l, respectively) and stored as described above. Analysis was carried out on 100 μl of plasma or urine samples. These were mixed with 10 μl of trichloroacetic acid (TCA 50%), 10 μl of sodium hydroxyde solution (3 mol/l), 100 μl of RB and 10 μl of I.S.. The mixture was vigourously shaken and the protein was separated from the sample by centrifugation at 3000 *g* for 2 min. A 100-μl volume of the supernatant was transferred into another microtube where derivatization was initiated by adding 5 μl of PH reagent.

After a reaction period of 15 min at a temperature of 37°C, 20-μl aliquots of the final sample were injected onto the HPLC system. Analysis was performed isocratically at a flow-rate of 0.4 ml/min. Separation of the 2-oxo acid phenylhydrazones formed in plasma or urine samples was completed 20 min after injection of the sample. Due to the isocratic chromatography conditions, the column was ready for the next injection immediately after completion of the previous run. The spectrophotometer was operated at 324 nm. After each batch of 30 samples the column was flushed with methanol in order to remove possible hydrophobic derivatization byproducts which might have interfered with later runs.

3. Results and discussion

3.1. General considerations

The derivatization of 2-oxo acids with phenylhydrazine is a simple and rapid procedure. The same applies to the spectrophotometric quantification of the phenylhydrazones formed. However, phenylhydrazine reacts not only with 2-oxo acids, but with a variety of substances containing oxo-groups. Among these, only sugars are present in significant concentrations in biological fluids. The presence or absence of sugar derivatives on the chromatogram depends on the reaction conditions chosen: at pH > 7.0 and at a temperature of 37°C [15] (conditions of the present experiments) sugars do not show chromatographic peaks that interfere with the quantification of 2-oxo acids. Similar to the findings of Petrarulo et al. [13], the final concentration of PH in our samples is sufficient to ensure a stable peak-height of the hydrazones formed up to 18 h after derivatization (Fig. 1).

Some additional technical points should be mentioned. Thus, the mobile phase should be prepared with special care: even small changes in the concentration of ethanol will result in a distinct shift of the retention times of the more hydrophobic 2-oxo acid peaks like 2-oxo valerate (OVA) which usually elutes 12 min after injection. The deproteinization procedure as described above using small amounts of TCA has no influence on the recovery of the substances tested and ensures satisfactory resolution of peaks in the course of the entire chromatogram (Table 1, Fig. 2). Resolution of the oxo-acid hydrazones can be greatly improved by heating the analytical column to a temperature of 40°C during separation. Heating is not an absolute prerequisite, but is useful in analysis of small amounts (< 1 μmol/l) of GLX or KG. Should no column heater be available, adequately warmed mobile phase can be used for a batch of samples with satisfactory results.

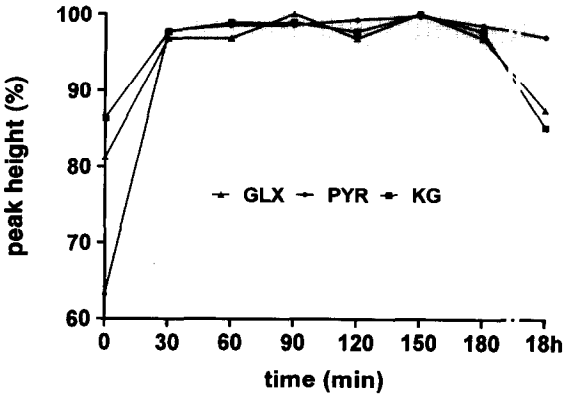

Fig. 1. Peak heights of 2-oxo acid hydrazones (KG, GLX and PYR, 3.0 μmol/l each) in an aqueous sample depending on time after derivatization. The grey bar indicates the range from 95 to 100% peak height.

Table 1
Recovery of KG, GLX and PYR in plasma samples after deproteinization with TCA

Substance tested	Concentration (μmol/l)			Recovery (%)
	initial	added	found	
2-Oxo glutarate (KG)	8.45 ± 2.8	2.5	11.01 ± 3.9	104.78 ± 5.2
Glyoxylate (GLX)	1.12 ± 0.4	0.5	1.67 ± 0.59	104.25 ± 6.86
Pyruvate (PYR)	43.33 ± 6.56	25	67.89 ± 8.47	96.29 ± 5.58

n = 10. Values are mean ± S.D.

3.2. Recovery

The recovery of the substances tested was evaluated adding known quantities of substance to previously analyzed plasma samples obtained from ten healthy individuals. The calculated results are presented in Table 1.

3.3. Accuracy and precision

The linearity of the method proposed and the detection limits were checked by examining independently diluted aqueous samples containing equimolar amounts of GLX, KG and PYR in the range from 0.17 to 166.5 μmol/l (Table 2).

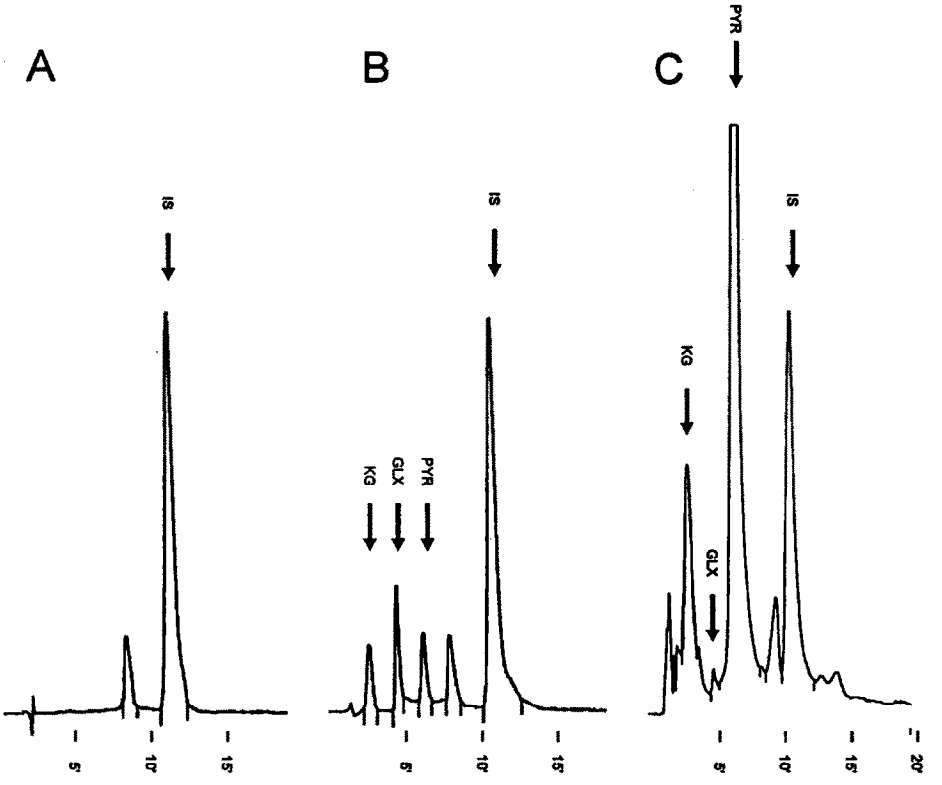

Fig. 2. Typical chromatograms (0.01 AUFS) of an aqueous blank (A), an artificial sample containing KG, GLX and PYR (1.3 μmol/l each) (B) and a TCA-deproteinized plasma sample (C). KG, GLX, PYR and I.S. are indicated with arrows.

Table 2
Detection limit, retention time and linear regression analysis for KG, GLX and PYR

Substance tested	Detection limit (μmol/l)	Retention time (min)	r^2	Equation
2-Oxo glutarate (KG)	<0.25	2.31 ± 0.06 (2.79%)	0.998	$y = 0.98x + 0.062$
Glyoxylate (GLX)	<0.1	4.55 ± 0.12 (2.61%)	1.0	$y = 0.98x + 0.051$
Pyruvate (PYR)	<0.25	6.33 ± 0.14 (2.15%)	0.998	$y = 1.01x - 0.045$
Internal standard (I.S.)	n.t.	11.12 ± 0.2 (1.81%)	n.t.	n.t.

Concentration range tested: 0.1–100 μmol/l ($n = 15$).
Values for retention time are mean ± S.D., n.t. = not tested.

The samples were (i) repeatedly injected (test for within-run precision) and (ii) independently prepared samples of identical concentration of GLX, KG and PYR (between-batch precision) were analyzed at least ten times. Coefficients of variation (C.V.) were calculated for samples containing 10.4 and 83.25 μmol/l substance respectively. The results obtained are shown in Table 3. Additionally, between-batch precision was determined by analyzing the same plasma sample on 5 subsequent days. The calculated C.V.s were 7.8% (KG), 7.0% (GLX) and 5.9% (PYR).

Addition of various substances (glucose, fructose, amino acids, lactate, oxalate) to samples prior to derivatization gave no significant interference with the analysis of 2-oxo acids (Fig. 3). Moreover, several 2-oxo acids which may occur in plasma and urine of subjects suffering from disorders in branched-chain amino acid metabolism (e.g. maple sirup disease) were tested with regard to possible chromatographic interference. The corresponding phenylhydrazones of 2-oxomalonate, -3-methylvalerate, -isovalerate and -isocaproate did not interfere with the quantification of GLX, PYR or KG (unpublished results) since their peaks occurred later in the chromatogram (t_R: 13–19 min).

Under the conditions applied, the entire quantification procedure of 2-oxo acids in bio-

Table 3
Within-run and between-batch coefficients of variation for all substances tested

Substance tested	Coefficient of variation (%)			
	Within-run		Between-batch	
	10.4 μmol/l	83.25 μmol/l	10.4 μmol/l	83.25 μmol/l
2-Oxoglutarate (KG)	4.81 (11.39 ± 0.48)	6.35 (83.66 ± 5.31)	7.46 (10.69 ± 0.8)	3.23 (86.45 ± 2.79)
Glyoxylate (GLX)	5.44 (9.88 ± 0.54)	2.96 (87.74 ± 2.6)	6.9 (10.05 ± 0.69)	4.59 (87.53 ± 4.02)
Pyruvate (PYR)	5.68 (11.62 ± 0.66)	3.7 (84.22 ± 3.11)	7.19 (11.21 ± 0.81)	5.59 (84.61 ± 4.73)

Values are %, [mean ± S.D. (μmol/l)].

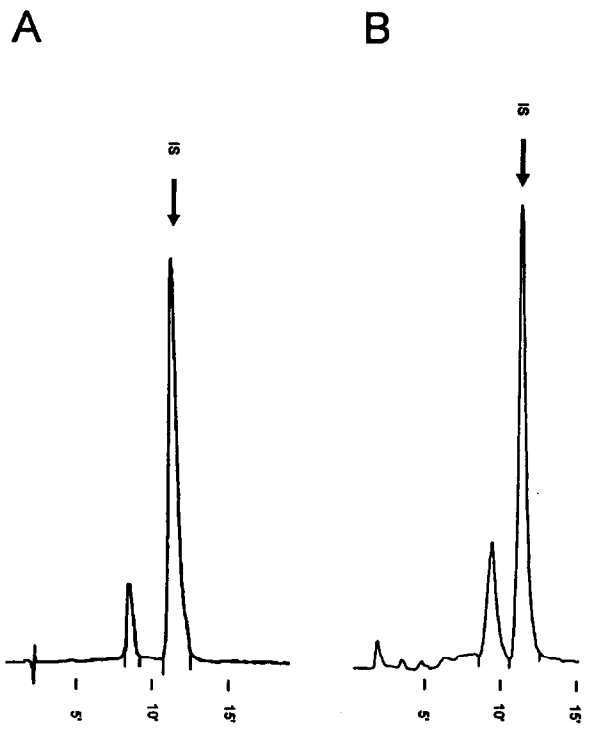

Fig. 3. Chromatograms (0.01 AUFS) of an aqueous blank (A) and an artificial aqueous sample containing fructose (0.5 mmol/l), glucose (5 mmol/l), oxalate, lactate (1 mmol/l each) (B) and 34 different L-amino acids (100 μmol/l each).

logical fluids including all steps (collecting the sample until receiving the results) could be performed in less than 45 min.

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