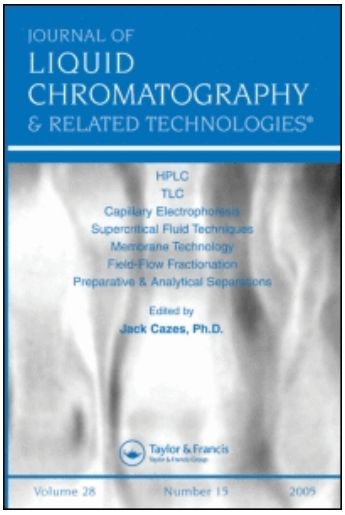

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IMPROVED HPLC DETERMINATION OF PLASMA AND URINE OXALATE IN THE CLINICAL DIAGNOSTIC LABORATORY

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ABSTRACT

An improved HPLC method for the measurement of oxalate as 2.3-dihydroxyquinoxaline derivative in plasma and urine has been developed. A reversed phase C18 column with isocratic elution and UV detection at 314 nm was used. Plasma samples immediately were deproteinized by acetonitrile with addition of phosphate buffer pH 7, to avoid the conversion of blood constituents to oxalate; acetonitrile, present in the supernatant, was dried. No pretreatment was necessary for urine. The derivatizing procedure with 1.2-diaminobenzene was performed by heating at 140° C for 25 min. The detection limits at a signal/noise $\geq$ 3 ranged from 0.15 mg/L for plasma to 0.5mg/L for urine. The within run reproducibility (n=20) evaluated for urine at 12 mg/L) was 3.4% $\pm$ 0.3%; for plasma at 1.5 mg/L was 5.6 $\pm$ 0.5%; the between run reproducibility (n=10) was 5.0 $\pm$ 0.4% for urine and 7.5

± 0.5% for plasma. The reference ranges (n=20) were: 10 to 30 mg/L for urine: 0.9 to 1.8 for plasma. Oxalate levels were measured in urine of exposed workers (n=200, mean 56.0 ± 5 mg/L) and in plasma of patients with renal damage (n=10, mean 4.5 ± 0.5 mg/L). The procedure is accurate, rapid and allows the analysis of samples collected at different times, without pre-analycal errors.

INTRODUCTION

Oxalate measurements provide important clinical insight in primary hyperoxaluria, in gastrointestinal disorders favoring oxalate absorption and in biological monitoring of exposed workers (e.g., in antifreeze preparations).

Calcium oxalate stone formation cause chronic renal failure, but if hyperoxaluria is recognized early, the onset of renal complications can be avoided [1-5].

Several analytical methods have been published [6-17]. However, there are difficulties in the determination of plasma oxalate because of its low concentrations and the rapid in vitro conversion of plasma constituents to oxalate [18-22]. The variation of results between laboratories is acceptable if HPLC procedures or more sophisticated approaches are used [23].In this report,the 2.3 dihydroxyquinoxaline oxalate derivative was separated and tested by an isocratic procedure with a reversed phase C18 column.

The derivatizing procedure, already adopted by B.C.McWhinney [10] for measuring urinary oxalate, has

been improved and used to analyse urine and (for the first time) plasma samples.

MATERIALS

Chemicals and Reagents

HPLC-grade methanol; KH_2PO_4, Na_2HPO_4, ammonium acetate, hydrochloric acid, sulphuric acid, potassium oxalate, oxalic acid, sodium hydroxide, were purchased as AR-grade reagents from Merck (Darmstad, Germany); 1.2-diaminobenzene (DAB) was obtained from Fluka (Buchs Switzerland).

METHODS

Preparation of the solutions

A stock standard solution was made up of 4.6 g oxalic acid dissolved in 1 L of distilled water and frozen in 2 mL aliquots at -20° C.

Working solutions were obtained by diluting with urine to final concentrations of 48, 24, 12 mg/L or with plasma to final concentrations of 12, 6, 3, 1.5 mg/L oxalic acid.

DAB (50 mg/L; 0.46 M) was prepared fresh prior to use in 2 M hydrochloric acid.

Phosphate buffer (0.35 mol/L pH 7.0) was obtained by dissolving 19.04 g KH_2PO_4 and 37.4 g $Na_2HPO_4.H_2O$ in 1 L of distilled water.

Treatment of samples

Urine samples (24 h) for analysis were collected over 5 mL of concentrated hydrochloric acid.

Heparinised blood was centrifuged for 10 min at 2000xg at 4° C immediately after collection. Plasma (1 mL) was deproteinized by adding an equal volume (1 mL) of acetonitrile and 50 uL of phosphate buffer, pH 7.0. After vortexing and centrifugat on (10 min at 2000xg at 4°C), the acetonitrile of the supernatant was vaporized by blowing in air at 37° C.

The derivatizing procedure was performed using 2 mL urine samples or 0.5 mL plasma supernatant.

The samples were placed in glass test tubes; 5 uL concentrated hydrochloric acid and 0.5 mL DAB reagent were added to each samples. The test tubes were then capped and heated at 140° C for 25 min. The tubes were removed and allowed to cool to room temperature. After the pH of each sample was adjusted to 5-6 by addition of 10 M sodium hydroxide solution, the samples were centrifuged for 10 min at 2000xg and the supernatants were placed in vials with limited-volume inserts prior to chromatography by C18 column.

HPLC instrumentation and chromatographic conditions

The Perkin Elmer high performance liquid chromatographic system included a Series 410 Liquid Chromatograph and a detector LC 90 UV (Perkin Elmer, Norwalk, Conn.) with a Data Station of SPE, Sistemi e Progetti Elettronici (Brescia, Italy).The analytical column was a reversed phase Pecosphere 3x3 C18, 3 um packing, 3x3 cm x 4.6 mm by Perkin Elmer.

20 uL of urine and 50 uL of plasma samples after derivatizing procedure were injected for isocratic determination; the mobile phase was a solution of ammonium acetate (27 g) and methanol (150 mL), made up to 1 L with distilled water; the flow rate was 2 mL/min at ambient temperature. The mobile phase was degassed before use, by filtering through a 0.45 um PTFE filter (Millipore, Australia) under vacuum (10).

The UV absorbance was read at 314 nm.

RESULTS

Figures 1 and 2 illustrate the HPLC elution profile of oxalate-quinoxaline derivative in normal and pathological plasma and urine samples.

The standard curve obtained by the derivatizing procedure showed good linear correlation between peak height and analite concentration for urine in the range 12 to 96 mg/L and was represented by the regression equation $y=2.7 + 20.3x$; for plasma in the range 1.5 to 12 mg/L and the regression equation was $y=5.5 + 4.1x$.

The recovery was studied only for plasma, because it was pretreated by a deproteinization process; the value ($85.2 \pm 5.1\%$) was determined by analyzing plasma samples spiked with standard (n=10).

The detection limits, at a signal/noise ≥ 3, ranged from 0.15 mg/L for plasma to 0.5 mg/L for urine.

The within run reproducibility (n=20), evaluated for urine at 12 mg/L, was $3.4 \pm 0.3\%$;for plasma,at 1.5

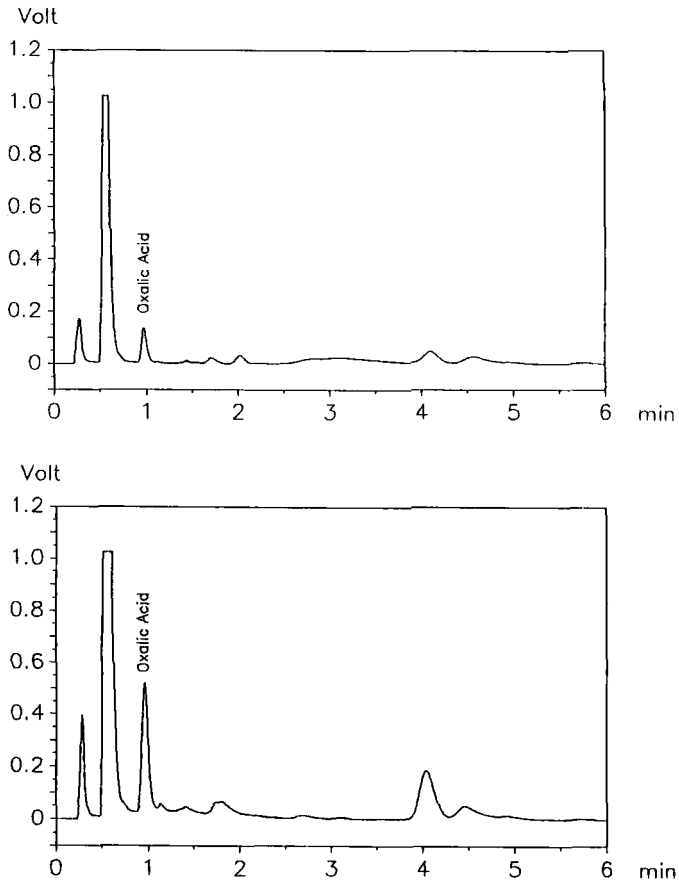

Figure 1. Chromatograms (at 314 nm) of oxalate-quinoxaline derivative (retention time 0.58 min): above,normal plasma (1.5 mg/L); below, plasma of patient with renal damage (5.5 mg/L). The profiles were obtained by a reversed phase Pecosphere 3x3 C18, 3 um packing, 3x3 cm x 4.6 mm by Perkin Elmer: 50 uL of sample were injected; flow rate was 2 mL/min at ambient temperature. The mobile phase was a solution of ammonium acetate (27 g) and methanol (150 mL), made up to 1 L with distilled water.

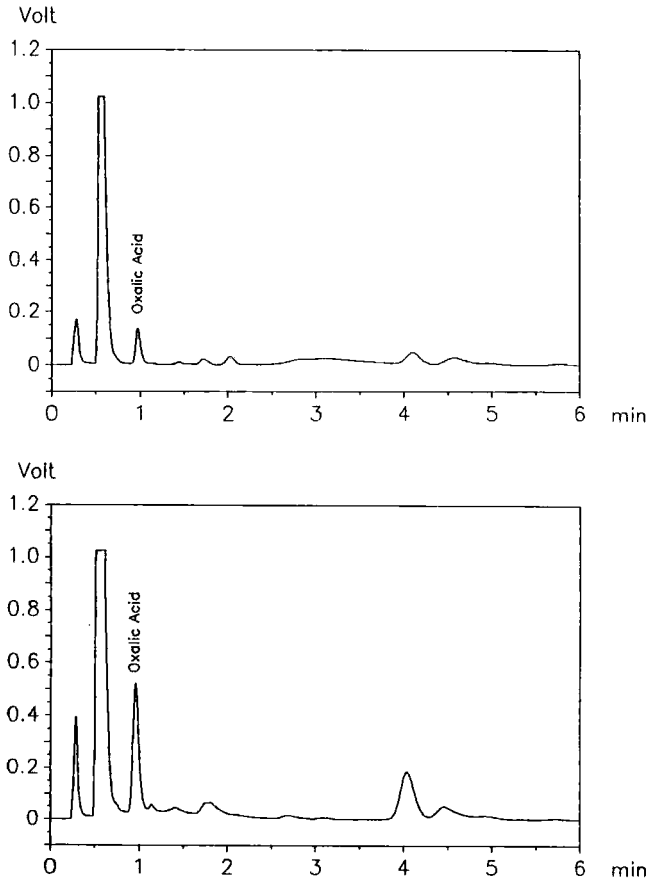

Figure 2. Chromatograms (at 314 nm) of oxalate-quinoxaline derivative(retention time 0.58 min): above,normal urine (17 mg/L); below, urine of workers exposed to ethylene glycol (90 mg/L), end of shift and end of workweek. For conditions see Figure 1.

mg/L, was 5.6 ± 0.5%; the between run reproducibility (n=10) was 5.0±0.4% for urine and 7.5±0.5% for plasma.

The reference range (n=20) was : 10 to 38 mg/L with a mean of 30 ± 1 mg/L for urine;0.9 to 1.8 with a mean of 1.5 ± 0.2 mg/L for plasma.

Urine samples taken from workers exposed to ethylene glycol,end of shift and end of workweek, resulted in high oxalic acid levels up to 95 mg/L, with a mean of 56.0 ± 5mg/L (n=200). Plasma samples taken from patients with renal damage also resulted in high levels up to 8 mg/L with a mean of 4.5 ± 0.5 mg/L (n=10).

DISCUSSION

Numerous HPLC procedures have described the determination of human urine oxalic acid concentration [6,8,10,14] by use of derivatization; much work has been done in the search for a method suitable to reveal the "real" plasma oxalate concentration [18-23].

The derivatizing procedure of B.C. McWhinney for measuring urinary oxalate (10) was modified to improve the between run reproducibility. The temperature of 2.3 -dihydroxyquinoxaline derivative formation was 140° C (vs. 125° C of McWhinney procedure) and the heating time 25 min (vs. 15 min), without the intermediate step at 70-80° C, performed by above-mentioned author. In our condition, the complessive heating-cooling step, took about 35 min vs.50 min of McWhinney procedure. The final

pH of each sample was adjusted to a higher value (5-6 vs. 3-4), with a better preservation of derivatized compound (2 days vs. 6 hours). Under our chromatographic conditions, the retention time of oxalic acid was about 1 min (vs.11 min) and the time run between the injection of one urinary sample to another was 5 min (vs.13 min). The spectrophotometer was set at 314 nm, since the peak height of DAB at 312 nm (10) was so high that it could not be successfully used as the internal standard.

The same analytical procedure was used for the first time on plasma samples. The method was sufficiently sensitive to detect the much lower concentrations of normal plasma with fair precision and reproducibility.

In vitro conversion of blood constituents to oxalate were avoided by the immediate deproteinization of plasma and conservation of supernatant at 4°C. The viability of the specimen was still demonstrated after 5 days.

The reference range obtained by this method correlated well with the data reported in other studies [10, 20,23,24].

The present HPLC procedure is suitable to detect plasma and urine oxalate for the routine clinical determination; it is accurate, rapid (6 min/sample for plasma; 5 min/sample for urine) and allows the examination of many samples taken at different times, without preanalytical errors.

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