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Note

Simplified column liquid chromatographic method for measuring urinary oxalate

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Calcium oxalate calculi represent about 70% of all renal stones and an increased 24-h urinary oxalate excretion is a major risk factor predisposing to calcium nephrolithiasis [1—3]. Currently several methods for measuring the concentration of urinary oxalate are available and include chromatographic [4—7], spectrophotometric [8, 9] and enzymatic methods [10—16]. Many of these methods correlate poorly and are tedious and non-specific [17]. Perhaps the most convenient and specific methods are enzymatic with the exception of the oxalate oxidase method which appears to give higher results than most other methods [17]. The enzymatic method which uses oxalate decarboxylase coupled with formate dehydrogenase is now automated and has been shown to correlate with a specific gas chromatographic method [13, 14]. A major drawback with the enzymatic methods are the cost and secondly the current poor availability of the enzymes, perhaps reflecting the demand for these methods.

Some of the high-performance liquid chromatographic (HPLC) methods which do not use derivatization were found unsuitable for urine analysis since urine contains many substances which absorb in the low UV region (< 220 nm) [5, 18]. We recently tried the method of Hughes et al. [7] which is based on the quantitative reaction of oxalate with 1,2-diaminobenzene to yield the 2,3-dihydroxyquinoxaline derivative which is then separated and quantified by HPLC. The main disadvantages with their method was the long analysis time and the use of a complex solvent programming cycle. We have simplified this method considerably and the results of these modifications are described in this manuscript and compared with a specific enzymatic method [14].

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EXPERIMENTAL

Reagents

HPLC-grade methanol (Waters-Millipore, Sydney, Australia) and AR-grade ammonium acetate (Ajax Chemicals, Australia) were used in the HPLC mobile phase. 1,2-Diaminobenzene (DAB) (Sigma, St. Louis, MO, U.S.A.), potassium oxalate (BDH, Australia), sodium thimerosal (BDH), sodium hydroxide (BDH) and disodium ethylenediaminetetraacetate (EDTA; BDH) were purchased as AR-grade reagents.

Standard and quality-control preparation

Dissolve 4.6 g potassium oxalate in 1 l of 0.2 *M* hydrochloric acid to give a 25 m*M* stock standard. The stock standard was diluted 100-fold with water to give a working standard (250 μM) which was stored frozen (−20°C) in 2-ml aliquots. A control urine containing 480 μM oxalate was prepared from pooled hydrochloric acid-acidified urine. This urine was diluted two- and four-fold in 0.2 *M* hydrochloric acid to give three ranges of urine oxalate values. The pooled urine control was initially analyzed ten times to determine the oxalate concentration. The three quality-control urines were frozen in 2-ml aliquots at −20°C. With each batch analysis of urines, the working standard plus three quality-control urines were analysed concurrently.

Reagent preparation

Sodium hydroxide (400 g/l; 10 *M*) and DAB (50 mg/ml; 0.46 *M*) were prepared in water and 2 *M* hydrochloric acid, respectively. The DAB derivatizing reagent was prepared fresh prior to use.

Derivatizing procedure

Urines (24 h) for analysis were collected either over 50 ml of 5 *M* hydrochloric acid or both 0.2—0.6 mmol sodium thimerosal and 20—25 mmol disodium EDTA to prevent ascorbate instability and oxalate precipitation and generation [19]. A 2-ml urine sample and 2 ml DAB reagent were mixed in a 15-ml polystyrene tube, capped and heated in a domestic pressure cooker at 125°C for 15 min. The pressure cooker was allowed to cool to about 70−80°C, and the tubes were removed and allowed to cool to room temperature. The pH of each sample was adjusted to 3—4 by addition of 0.3 ml of 10 *M* sodium hydroxide solution (the reaction mixture usually changes from green to brown on alkali addition). Urines were then centrifuged for 10 min at 1000 *g*. The supernatants were placed in vials with limited-volume inserts (Waters-Millipore) prior to chromatography.

Chromatographic system

The HPLC system used (Waters-Millipore) consisted of a Model 6000A pump, a 740 data module/printer, a WISP 710B automatic sample injector, a 480 variable-wavelength spectrophotometer and a Rad-Pak Z module containing a Rad-Pak C_{18} reversed-phase column (5 μm particle size, 100 mm × 8 mm I.D.).

Mobile phase

Ammonium acetate (27 g) and methanol (150 ml) were made to 1 l with water. This solution was degassed by filtering through a 0.45-μm PTFE filter (Millipore Australia) under vacuum before use.

HPLC conditions

The chromatography was isocratic with a flow-rate maintained at 2 ml/min and the time set between the injection of one sample to another was 13 min. A 20-μl sample was injected for each analysis. The spectrophotometer was set at 312 nm. After approximately 50 urine analyses, the column was washed with 100 ml of methanol.

Quantitation

The oxalate-DAB quinoxaline derivative was identified by its retention time. The unreacted DAB reagent was used as an internal standard and the peak-area ratio of the quinoxaline to DAB was compared to that obtained from a single standard of potassium oxalate (250 μM).

$$\text{Urine oxalate concentration } (\mu M) = \frac{\text{area ratio (urine)} \times 250}{\text{area ratio (standard)}}$$

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC elution profile of oxalate-quinoxaline derivative (retention time 10.0 min) and the DAB internal standard (6.0 min). The standard curve obtained for oxalate was linear from 0.1 to 2.5 mM. The internal standard for this curve was constant [202 ± 3 (S.D.) area units] confirming that the excess DAB used in the assay was unaffected, even at high oxalate concentrations. By serially diluting the standard and pooled urine control, the lower limit of detection of oxalate was found to be 10 μM (peak height-to-noise ratio of 10:1). The DAB concentration used in the assay was approximately half that used by Hughes et al. [7]. Compared with the assay concentration of 50 mg/ml, concentrations of DAB of 100, 75, 25, 10 and 5 mg/ml resulted in 101.4, 100.5, 91.8, 45.6 and 24.0% relative conversion, respectively, of oxalate to its quinoxaline derivative.

The inter- and intra-assay coefficients of variation (C.V.) obtained on the three control urines are shown in Table I, indicating excellent precision (< 2.2%) of this method. Ten different urine samples with oxalate concentrations ranging from 80 to 600 μM and spiked with 250 μM oxalate gave a recovery of 101.8 ± 2.3% (mean ± S.D.). To test the specificity of the method the following oxalate precursors, ascorbate, glycollate, glyoxalate and glucose, were used at a concentration of 10 mM in acid and EDTA—thimerosal collected urines. None of these compounds gave any interference with the HPLC method. Ten different urine samples analyzed by the HPLC method correlated well with a specific enzymatic method [r = 0.98, $p < 0.01$; mean ± S.D. (HPLC) = 0.23 ± 0.15 mM versus mean ± S.D. (enzyme) = 0.20 ± 0.11 mM].

The reference range for the HPLC oxalate method using eleven urines from normal individuals (eight females, three males) was 0.17 to 0.42 with a mean

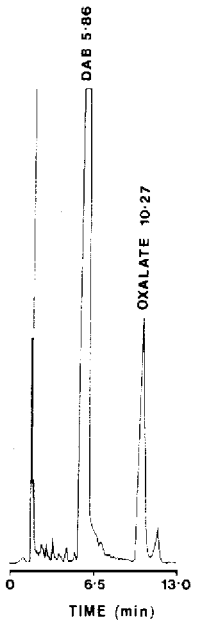

Fig. 1. The chromatographic profile of a urine sample containing 180 μM oxalate. The DAB and oxalate-quinoxaline peaks are indicated.

TABLE I

INTRA- AND INTER-ASSAY VARIABILITY ON THREE QUALITY-CONTROL URINES

The inter-assay variability was determined over a two-month period.

	Intra-assay (n = 10)		Inter-assay (n = 10)	
	Mean ± S.D. (μM)	C.V. (%)	Mean ± S.D. (μM)	C.V. (%)
Normal	118 ± 1.6	1.4	116 ± 2.5	2.2
Medium	237 ± 2.9	1.2	235 ± 3.1	1.3
High	483 ± 9.2	1.9	475 ± 8.6	1.8

of 0.30 mmol per day and agrees well with the more specific methods for measuring urinary oxalate [8, 10—13].

The cost of reagents and disposables (including the HPLC column) per assay for this method was less than 80 cents per assay compared to $2.20 (Australian) per test by an enzymatic method [10]. The stand alone capabilities of our HPLC system allowed us to run and report about 50 samples within a 24-h period. The work-up time for these specimens was about 2 h. The life of each Rad-Pak column used as specified in this manuscript was in excess of 800 samples.

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