Determination of Urinary Oxalate by Reversed-Phase Ion-Pair "High-Performance" Liquid Chromatography

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In this liquid-chromatographic method for determination of urinary oxalate, interfering compounds in the urinary samples were eliminated before chromatography by passage through a preparative C₁₈ mini-column (Sep-pak cartridge, Waters Associates). In the reversed-phase system tetrabutyl ammonium was included as a counter ion to enhance the retention of oxalate. The pH of the mobile phase was kept low (2.00) to avoid precipitation of calcium oxalate. This method is faster and significantly more precise than the colorimetric method we currently use (Clin. Chim. Acta 36:127-132, 1972).

Most reported studies on the content of oxalic acid in urine are insufficiently precise to satisfactorily measure the low concentration of oxalate normally present in human urine. Published methods are based on a preliminary concentration of oxalate by either direct precipitation (1, 2), solvent extraction (3), or ion-exchange chromatography (4), followed by colorimetry of oxalic acid. Indirect measurements of oxalic acid in urine by atomic absorption spectroscopy have also been described (5), but they also present difficulties in measuring these low concentrations.

Enzymatic methods (recently reviewed in ref. 6) tend to underestimate oxalate by 10 to 20%, as compared with results by isotopic dilution, the technique considered the most nearly accurate for analyzing oxalate in urine. This latter technique, however, requires an extra step of adding and measuring [¹⁴C]oxalate, which makes it less suitable for routine use.

Recently, a method involving high-performance liquid chromatography (HPLC) was published (7), which included both derivatization and gradient elution.

Although the problem of measuring urinary oxalate is essentially considered solved by the oxalate oxidase method (8), no comparisons between this enzymatic and colorimetric and gas-liquid chromatographic procedures are reported.

The method currently used in our laboratory is that of Hodgkinson and Williams (2). Although considered to be accurate, it is tedious and time consuming.

Here, we describe a rapid and precise method for determination of urinary oxalate, based on a reversed-phase ion-pair HPLC technique as described previously (9).

Materials and Methods

Apparatus. The HPLC apparatus consisted of a Cons-tametric II G pump (Laboratory Data Control, Riviera Beach, FL 33404), a Model 7125 sample injector with a 50-μL sample loop (Rheodyne, Inc., Berkeley, CA 94710), a Spectro Monitor III Spectrophotometer (Laboratory Data Control), and a Servogor S Type RE 542 recorder (Goerz Electro, Vienna, Austria). The analytical column was a LiChrosorb RP-8, 10 μm, 250 x 4.0 mm (E. Merck, Darmstadt, F.R.G.).

Chemicals and reagents. Oxalic acid, orthophosphoric acid (H₃PO₄), sodium chloride, and potassium dihydrogen phosphate, all of analytical grade, were from E. Merck. Methanol was HPLC-grade (Rathburn Chemicals L., Walkerburn, Peebleshire, Scotland). Tetrabutyl ammonium hydrogen sulfate (TBA) was supplied by E. Merck.

We prepared a stock solution of oxalic acid (100 mmol/L) in water adjusted to pH 2.00 with orthophosphoric acid. Working standard solutions were then prepared with the same solvent to concentrations of 1000, 750, 500, 400, 300, 200, 100, and 50 μmol/L. Addition of 90 mg of NaCl to 10 mL of the respective working standard solutions resulted in improved oxalate peaks. The NaCl addition was prepared daily.

The mobile phase was prepared from a mixture of KH₂PO₄ (5 g/L) and TBA (1.7 g/L) in water buffered to pH 2.00 with orthophosphoric acid. The mobile phase was filtered through a 0.5-μm cellulose acetate filter (type EH; Millipore Corp., Bedford, MA 01730) and could be stored at room temperature for two weeks.

Procedure. From 31 patients, we collected 24-h urine specimens in plastic bottles containing 15 mL of 6 mol/L hydrochloric acid. Before analysis we adjusted the pH to 2.00 ± 0.1 with orthophosphoric acid. A 2-mL aliquot of this urine was injected into an octadecyl-silane bonded-phase packing (Sep-pak C₁₈ cartridge; Waters Associates, Milford, MA 01757) that had been pre-treated by successively passing through it 2 mL of methanol and 5 mL of water. The first 0.5 mL of urine that passed through the cartridge was discarded and the next 1 mL was used for analysis. The standard solutions were treated in the same way. This procedure removes non-polar substances, and the pretreatment with methanol and water is important if semi-polar substances also are to be removed efficiently. We injected a 50-μL aliquot of this "cleaned up" sample into the HPLC system and determined oxalate in the effluent by measuring the absorbance at 220 nm. The chromatographic conditions were: flow rate, 2.0 mL/min; pressure, 7.25-7.45 MPa (1050-1080 psi); full-scale absorbance of the recorder, 0.05 A; and chart speed, 1.0 cm/min.

After each day's analysis, we washed the column with aqueous methanol (500 mL/L) at a flow rate of 0.5 mL/min for at least 30 min and with pure methanol once a week.

Results and Discussion

An important feature of the present method is the preparative treatment of urine samples with the Sep-pak C₁₈ cartridges, to eliminate interfering substances in the sample. Figure 1 shows a chromatogram of urine before and after Sep-pak treatment. This treatment clearly yields a well-re-
solved peak of oxalic acid, as is evident in Figure 1 (bottom).

Oxalic acid, a strong organic acid, is dissociated even at pH 2.00 (pKa₁ = 1.23, pKa₂ = 4.19). In contrast to other, weaker acids, oxalic acid can thus form ion-pairs with TBA in the reversed-phase system, which enhances its retention as a highly polar compound. We kept the pH of the mobile phase low, to avoid precipitation of calcium oxalate.

The curve representing the relation between peak height and oxalic acid concentration (Figure 2) was slightly sigmoid, but it was linear between 150 and 750 μmol/L, which includes most normal and hyper-oxaluric specimens. Oxalic acid concentrations as low as 15 μmol/L could be measured.

Overall analytical recovery for three different oxalate concentrations (50, 100, and 250 μmol/L) added to a urine containing 200 μmol of oxalate per liter ranged from 90.3 to 104.6%, with a mean of 95.9% (n = 6 to 9 for each concentration).

Addition of glucose up to 15 mmol/L and citric acid up to 5 mmol/L to a urine with an oxalic acid concentration of 215 μmol/L gave no interference. Ascorbic acid concentrations exceeding 1.4 mmol/L gave a negative interference of 10%.

Figure 3 shows a comparison between results by the present HPLC method and the colorimetric method (2), as applied to 31 consecutive urine samples sent to our laboratory for routine analysis for oxalate. The within-assay standard deviation, calculated from the duplicate determinations with analysis of variance (10), was 12.7 and 4.2 μmol/L for the colorimetric and HPLC procedures, respectively, corresponding to a CV of 5.1 and 1.7% at 250 μmol/L. The precision of the HPLC method was significantly better (F = 9.12, df = 30/30, p < 0.01).

The between-assay precision of the colorimetric procedure was estimated from duplicate analyses of the assay control, a pooled urine. The mean value was 398 (SD 30.6) μmol/L (CV 7.7%). For the HPLC procedure the between-assay precision was characterized from duplicate analyses of the same pooled urine analyzed at the beginning of a six-sample series; the mean value was 372 (SD 12.2) μmol/L (CV 3.3%).

The HPLC method described by Hughes et al. (7), based on the quantitative reaction of oxalic acid with 1,2-diaminobenzene and subsequent determination of quinooxaline, also includes gradient elution, which makes the technique more complicated than our proposed method. The sample “clean-up” procedure before HPLC analysis we describe is easy and fast. The chromatography time for each sample is 20 min and the injection procedure can be automated. Therefore, this method is readily adapted to routine clinical use, and can accommodate a greater workload.
Liquid-Chromatographic Procedure for Simultaneous Analysis for Eight Benzodiazepines in Serum

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We describe an efficient extraction and liquid-chromatographic method for separating commonly encountered benzodiazepine drugs and their pharmacologically active metabolites. After a single extraction of the drugs from serum, chlordiazepoxide, demoxepam, N-desmethyl-chlordiazepoxide, diazepam, N-desmethyl-diazepam, N-desalkylflurazepam, oxazepam, and prazepam can be resolved and quantified by using a C_{18} reversed-phase "high-performance" column and a ternary-solvent gradient system. Three separate solutions [60 mmol/L ammonium acetate (pH 7.69), 60 mmol/L acetic acid (pH 2.8), and acetonitrile] were incorporated into a gradient mobile phase such that changes in pH and solvent composition occur. Complete chromatographic resolution of the benzodiazepines resulted, permitting quantification of all within 15 min. The standard curve is linear to at least 8 mg/L for each drug, and the detection limit for each was 0.05–0.10 mg/L. The day-to-day precision for both high and low concentrations yielded CVs of 5 to 9%. Extraction of each drug from serum was 95 to 100% complete. Exogenous and endogenous interferences are minimal. Finally, we circumvented the instability problem of benzodiazepine standards in solution by using a simple reduced-pressure drying process that produces a working standard that is stable for at least nine months.

Additional Keyphrases: effects of column aging on separation • drug assay • chromatography, reversed-phase

The benzodiazepines are the most widely-prescribed drugs in the United States today and are encountered in our hospital service laboratory in 70–90% of samples for which a "drug screen" is requested.

Measurement of these compounds has been either by ultraviolet spectrophotometry (1, 2) or gas chromatography (3–9). The ultraviolet procedures have low specificity and low sensitivity. A sensitivity near 0.1 mg/L is often necessary; when specificity in the presence of multiple drugs is required, the ultraviolet and gas chromatographic methods are usually adequate. Analytical interference of a parent drug with its metabolite(s) and vice versa has often not been adequately evaluated in the gas chromatographic method. Gas chromatography can effectively resolve certain drugs, but thermal instability of chlordiazepoxide and its metabolites is a problem.

"High-performance" liquid-chromatographic (HPLC) instrumentation and reversed-phase column technology has made possible effective resolution of the benzodiazepines and their pharmacologically active metabolites (9–11).

Some of the benzodiazepines are unstable in solution (7, 8), an obstacle in developing adequate procedures for quantifying these drugs. Accordingly, we used a composite benzodiazepine standard, dried under reduced pressure, which is stable for at least nine months.

Our method involves a one-step liquid-extraction procedure and a small sample in which a non-drug internal standard is incorporated. The commonly prescribed benzodiazepines and their active metabolites are resolved on an octadecylsilane (ODS) reversed-phase HPLC column.

Materials and Methods

**Chemicals.** All chemicals used were ACS reagent grade or better. They included concentrated ammonium hydroxide and glacial acetic acid obtained from Hi Pure Chemicals, Nazareth, PA 18064. Acetonitrile (HPLC grade), chloroform (99 mol %), and methanol (HPLC grade), were purchased from Fisher Scientific, Fair Lawn, NJ 07410; isopropanol (AR grade) was...