We describe a liquid-chromatographic procedure for detection in urine of all thiazide drugs currently used clinically. Urine is treated initially with NaBH₄ to convert any chlorothiazide present to hydrochlorothiazide. The urine is acidified with NaH₂PO₄ (1.0 mol/L, pH 5), and thiazides are extracted with ethyl acetate. Interfering substances are then removed in two washes with 0.1 mol/L Na₂HPO₄ at pH 8. The ethyl acetate is evaporated and the residue redissolved in mobile phase. Thiazides are assayed by reversed-phase chromatography, with detection by ultraviolet absorption. Analytical recovery of thiazides ranged from 53 to 93%. Urines from 48 patients were studied, and the results were compared with results by the currently used spectrophotometric method. The two methods agreed for 56% of samples. Evaluation of the discrepancies by review of patients' histories clearly showed liquid chromatography to have correctly identified seven of eight positive urines that the spectrophotometric method failed to detect. Ultraviolet scanning incorrectly identified as positive two samples, whereas liquid chromatography did not falsely identify any urines as positive. Our method was more sensitive and more specific than the spectrophotometric method.

Additional Keyphrases: chromatography, reversed-phase - hypokalemia - screening

The benzothiadiazide ("thiazide") diuretics are widely used for the treatment of hypertension and conditions of fluid retention such as congestive heart failure. All thiazides augment excretion of potassium in the distal tubule and can cause hypokalemia in a patient taking them for a prolonged period (7). Thus, investigation of the cause of hypokalemia may include screening the urine for the presence of thiazides when reliable information cannot be obtained by history.

Several methods have been developed to measure qualitatively or quantitatively thiazide diuretics in biological fluids (2-12). An early method involving colorimetric determination of the sulfonamides produced by hydrolysis lacked sensitivity and specificity (5, 12). A simpler spectrophotometric procedure was developed later by Pilsbury and Jackson (3). Although this procedure was in use by our laboratory, it lacked specificity, and the ultraviolet spectra were often difficult to interpret. Alternative methods based on spectrofluorometry (6), thin-layer chromatography (7), paper chromatography (3, 5), and gas-liquid chromatography (8-10) have been developed for specific thiazides. Although these methods may give improved specificity, they have not been applied to the measurement of the wide range of thiazides required for a screening test. Liquid chromatographic methods for hydrochlorothiazide (2, 4), betamethazide (11), and chlorothiazide (12) have also been reported. Our attempts to use these methods to screen urines for the presence of thiazides failed, principally because of interfering substances in the extracts.

Here we report our development of a liquid-chromatographic method for screening urines for thiazide diuretics.

Materials and Methods

All chemicals and reagents were of analytical grade or better purity and are commercially available. Water was de-ionized and distilled. Standard solutions of the following drugs were made from pure compounds kindly provided by the manufacturer listed: hydrochlorothiazide and chlorothiazide, Merck Sharp & Dohme, West Point, PA 19486; hydroflumethiazide, Bristol Laboratories, Syracuse, NY 13201; polythiazide, Pfizer Labs., New York, NY 10017; trichlorothiazide, Schering Corp., Kenilworth, NJ 07033; bendroflumethiazide, Squibb and Sons, Princeton, NJ 08540; methyclothiazide, Abbott Laboratories, North Chicago, IL 60064; cyclothiazide, Eli Lilly and Co., Indianapolis, IN 46225; and benzthiazide, A. H. Robins Co., Richmond, VA 23220. Stock solutions of standards were prepared, in water alkalized to pH 8, to contain 10 mg/L and were stored at 4 °C until use.

Apparatus. A CV-6-UHPA-N60 valve (Valco Instruments Co., Houston, TX 77055) and a 25-μL sample loop were used for injection. A μBondapak C18 column (30 cm x 3.9 mm, i.d.; Waters Associates, Milford, MA 01757) with an inlet filter (Model 7302; Rheodyne, Berkeley, CA 94710) was used for separation. A Model 990 pump with pulse damper (Tracor, Austin, TX 78721) was used to deliver mobile phase at a rate of 2.0 mL/min. An ultraviolet detector (Model SP770; Schoeffel Instrument Corp., Westwood, NJ 07675) was used with a wavelength setting of 271 nm at a sensitivity of 0.1 A full scale. Detector signal was plotted with a 10-mV recorder and a chart speed of 0.68 cm/min. Quantitation was based on peak height.

Procedures

Mobile phases. Analysis for all thiazides required two mobile phases. These were prepared by diluting a 10 mL/L solution of acetic acid in water with acetonitrile to give final concentrations of acetonitrile of 80 and 350 mL/L. Each mobile phase was filtered (0.5-μm pore FHUP filter; Millipore Corp., Bedford, MA 01730) before use.

Urine extraction. Centrifuge turbid urine to remove particulates before testing. Place 2 mL of urine, 2 mL of 1.0 mol/L NaH₂PO₄ at pH 5.0, and about 50 mg of NaBH₄ powder in a 50-mL beaker. After 3 to 4 min, add 4 mL of ethyl acetate to the beaker. Transfer the contents of the beaker to a 17 × 100-mm polypropylene tube with cap (Falcon, Oxnard, CA 93030). Mix the tube contents for 5 min and then centrifuge to eliminate any emulsion. Transfer the organic phase to a second tube, containing 6 mL of 0.1 mol/L Na₂HPO₄ at pH 8.0. Mix the tube contents for 3 min, then transfer the organic phase to a third tube, containing 6 mL of 0.1 mol/L Na₂HPO₄ buffer at pH 8.0. After mixing for 3 min, transfer the organic phase to a fourth tube and evaporate in a water bath at 60 °C under a stream of nitrogen. Redissolve the residue in 0.2 mL of the mobile phase containing 350 mL of acetonitrile per liter. Make separate 25-μL injections with each mobile phase. A "positive" chromatogram of an extract must have a peak that co-chromatographs with a thiazide standard and is twice as high as any background peak occurring after the peaks at the void volume.
Confirmation of hydrochlorothiazide. Urine specimens demonstrating a hydrochlorothiazide peak were re-extracted as described, except that NaBH₄ was omitted. Disappearance of the hydrochlorothiazide peak in the extract when NaBH₄ was not used indicated the presence of chlorothiazide in the original specimen. A peak due to hydrochlorothiazide in the specimen was unaffected by the omission of NaBH₄.

Extraction recovery. Recovery was determined by analysis of urine supplemented with 10 mg of each drug per liter. The peak height for each drug after extraction was compared with the peak height obtained by injection of an aqueous solution containing 10 mg of the same drug per liter. In the case of chlorothiazide, the peak height was compared with that for hydrochlorothiazide (reduced chlorothiazide).

Human Testing
Presumably healthy volunteers, on no other medications, consumed a single tablet of the lowest dose of each drug commercially available. Urine samples taken at 2 to 4, 12, and 24 h after ingestion were analyzed for thiazide by both methods. Urine samples from two volunteers who took a mixture of triamterene and hydrochlorothiazide (Dyazide; Smith Kline & French, Philadelphia, PA 19101) and from a third volunteer who took acetaminophen were similarly studied.

Comparison Studies
Aliquots from 48 urine specimens submitted for qualitative thiazide screens were analyzed by the liquid-chromatographic method and by the spectrophotometric method of Pillsbury and Jackson (3). For the spectrophotometric procedure, two 10-mL aliquots of urine were adjusted to pH 4.0 by titration with hydrochloric acid. One aliquot was labeled "specimen," and the other, to which 0.2 mg of hydrochlorothiazide was added, was labeled "control." Each sample was saturated with ammonium sulfate, filtered, and extracted with two 10-mL portions of diethyl ether. The ether phase from each sample was dehydrated by addition of anhydrous sodium sulfate and passed through activated charcoal to remove interfering substances. An ultraviolet scan from 320 to 240 nm of the specimen extract was compared with that from the control extract. If the scans showed matching peaks, thiazides were assumed to be present. If the scans did not show similar characteristics at or near 270 nm, the specimen was considered to contain no thiazides (Figure 1). For thiazide-containing specimens, the ether phase was extracted twice with a fresh solution of saturated sodium bicarbonate and again dehydrated over sodium sulfate. The ultraviolet scans of the ether phases were again compared. If matching peaks were still seen, polythiazide was presumed to be present. If the peaks no longer matched, chlorothiazide, hydrochlorothiazide, or furosemide was presumed to be present. Interpretations for each method were made without knowledge of results from the other method.

Results

Chromatographic Characteristics
Representative chromatograms of thiazide mixtures obtained with each mobile phase are shown in Figures 2a and 3a. Figures 2b and 3b are representative of chromatograms of the same drugs obtained after treatment with NaBH₄ and extraction from urine. Figure 4 shows typical chromatograms of an extract from thiazide-free urine after treatment with NaBH₄. Chromatograms of an extract from the same urine without NaBH₄ treatment were identical. Studies with pure drugs showed that chlorothiazide was converted to hydrochlorothiazide by treatment with NaBH₄. Conversion was
Fig. 4. Typical chromatograms of an extract from thiazide-free urine after treatment with NaBH₄.
Mobile phase contains acetonitrile (a) 80 mL/L or (b) 350 mL/L.

Essentially complete, as shown by disappearance of the peak due to chlorothiazide, appearance of a peak of approximately equal size corresponding to hydrochlorothiazide, and the fact that analytical recovery of chlorothiazide, when calculated on the basis of hydrochlorothiazide, was identical to the recovery of hydrochlorothiazide (Table 1).

In contrast, benzthiazide was only partly converted to a compound that we presumed to be hydrobenzthiazide (Figure 3). We could not obtain pure hydrobenzthiazide for a standard.

The chromatogram of cyclothiazide (Figure 3) consistently showed evidence of at least three components. Because several stereoisomers of cyclothiazide are possible, we presume that these isomers account for the chromatographic characteristics. Pure samples of each isomer could not be obtained for study. Lastly, thiazides can hydrolyze to give a 6-substituted 4-amino-m-benzene disulfonamide (DSA). Both aqueous standards and extracts from thiazide-containing urine usually showed a little of the corresponding DSA when chromatographed with a mobile phase containing 80 mL of acetonitrile per liter. The DSA was most evident in extracts from urine containing hydrochlorothiazide or methyclothiazide. In all cases, the DSA derivatives were well resolved from the parent thiazides (Figure 2).

Extraction Recovery

Table 1 shows the analytical recovery of each thiazide from urine. Approximately a 30% full-scale response was obtained for each thiazide with a concentration of 10 mg/L of urine.

Human Testing

All but one of the thiazides were detected by both methods in urine collected 2 to 4 h or 12 h after ingestion, but were undetectable by 24 h. The exception to this was polythiazide, which was detected by the liquid-chromatographic method at 12 h, and only after ingestion of 2 mg, twice the smallest dosage tablet commercially available. This finding is compatible with the slow renal clearance of polythiazide and should not limit the clinical usefulness of liquid chromatography as a screening test. Although we have no substantiating evidence, it seems unlikely that a person taking the minimum dose of this drug would attain the degree of hypokalemia that would cause the test to be ordered in the first place.

Comparison Studies

Table 2 compares the results of the chromatographic assay with those of the spectrophotometric assay. There are marked differences between results by these methods. There was complete agreement in only 27 of 48 specimens. Interfering substances prevented interpretation of the ultraviolet scan in three cases and of the chromatogram in two cases. The two chromatograms that could not be interpreted were obtained from urines that contained many hemolyzed erythrocytes. The two methods gave conflicting results for 17 urines from 12 subjects (Table 2). Patients' records were examined, when possible, to resolve these discrepancies (see below).

Discussion

Methodology

The procedure we describe here, if applied as a screening test for thiazides in urine, is complicated by the need to use two mobile phases and to reduce the chlorothiazide in urine to hydrochlorothiazide before extraction. To avoid the use of two mobile phases, we performed preliminary experiments in which the thiazides were converted to their DSA derivatives. All of these derivatives could be chromatographed with a single mobile phase similar to that used for hydrochlorothiazide. We found that the thiazides in urine could be converted quantitatively to their DSA derivatives by adding an equal volume of 2 mol/L NaOH, then heating the mixture to 120 °C for 15 min. The mixture was acidified, and the DSA derivatives were extracted into ethyl acetate, then back-extracted into a solution containing 0.1 mol of NaOH per liter, and subsequently analyzed. Although this procedure allowed use of a single mobile phase, the specific identification of various thiazides, which was deemed desirable by several clinicians, was not possible. Direct extraction of thiazides into ethyl acetate, followed by a second extraction with dilute NaOH, failed for the parent thiazides because the dilute NaOH did not extract polythiazide from the organic phase. Subsequently, we found that extraction of the organic phase with phosphate buffer at pH 8.0, followed by evaporation of the organic phase and analysis of the residue, removed interferences and allowed detection of polythiazide. Unfortu-
nately, chlorothiazide extracted into the mildly alkaline phosphate buffer, presumably because the unsaturated compound was more acidic than hydrochlorothiazide. Therefore, we used a reduction with NaBH₄ as an initial step, to convert chlorothiazide to hydrochlorothiazide. This final modification resulted in the procedure that we compared with a colorimetric method.

Specificity

We tested common medications that might interfere in the chromatographic procedure by injecting extracts prepared from dilute (e.g., 100 mg/L) aqueous solutions. Triamterene, salicylic acid, ethacrynic acid, and furosemide gave no detectable peaks. An extract of a 2 g/L solution of furosemide gave a small peak with a retention time of 4.5 min with the mobile phase containing 350 mL of acetonitrile per liter. Acetaminophen eluted with the 80 mL/L acetonitrile mobile phase at a retention time of 3.6 min. Although acetaminophen was detected in the urine of individuals who took this drug, it does not interfere with detection of any of the thiazides. The failure to observe furosemide in extracts was due probably to extraction by the pH 8 phosphate buffer. Furosemide can be detected at clinically relevant concentrations by the liquid-chromatographic method of Carr et al. (13), which does not incorporate the wash step with phosphate buffer.

Comparison Studies

There were 17 of 48 urine samples for which the liquid-chromatographic and spectrophotometric methods gave conflicting results (Table 2). Our resolutions of these discrepancies, based on a review of the patients’ records, are summarized as follows.

For eight specimens the spectrophotometric method failed to demonstrate thiazides that were evident by liquid chromatography. In six specimens from three patients, the liquid-chromatographic result was proven correct by the patients’ admission of thiazide ingestion. The liquid-chromatographic result was proven correct in another situation when review of the spectrophotometric data showed an incorrect original interpretation of a specimen that was actually positive. We could not determine the actual situation for one specimen from a 37-year-old woman who had been extensively investigated for hypokalemia without a proven etiology. She had a personality disorder, with a history of multiple drug abuse, including documented salicylate and laxative abuse. No history of thiazide ingestion was obtained. Thus, for seven of eight specimens, the liquid-chromatographic method gave a result that was deemed correct and we could make no conclusion regarding the eighth specimen.

Resolution of the nine discrepancies in which the liquid-chromatographic method failed to detect thiazides evident by the spectrophotometric method proved more difficult. In the absence of a patient’s statement of thiazide ingestion, there was no means of demonstrating thiazide in the urine other than with the methods themselves.

Two of the seven patients’ histories were consistent with the assumption that the spectrophotometric method was falsely positive. One case was a 56-year-old man with metastatic bronchogenic carcinoma. His serum potassium concentration varied between 2.9 and 3.1 mmol/L during a six-month period. After the spectrophotometric method results suggested the ingestion of thiazides, his medications were examined by his wife and by the physician twice without any evidence to implicate the use of thiazides. The patient himself repeatedly affirmed that he was ingesting only the medications prescribed. The second patient, a 21-year-old woman physical-education student, was noted incidentally to have a potassium of 2.8 mmol/L on a routine screening of electrolytes before orthopedic surgery. Further investigation revealed no detectable underlying abnormality and the patient repeatedly denied taking any medication at all. Multiple absorbance peaks other than at 270 nm were noted on repeated examination of the ultraviolet scan. Although the clinical situation of this last case does not disprove thiazide ingestion, the unusual ultraviolet scan and the patient’s apparent psychological stability suggest that this etiology was unlikely.

A third discrepancy was resolved when the patient admitted taking furosemide. The spectrophotometric method detected this drug, giving a positive test. The chromatographic method does not detect furosemide and thus was interpreted correctly as negative for thiazides.

The remaining six specimens were from four patients who did not have histories that would permit a decision as to the likelihood of thiazide ingestion. One of the four patients was a 28-year-old woman being evaluated for fatigue, who was discovered to have a potassium value of 1.6 to 1.8 mmol/L. An extensive evaluation failed to reveal any underlying abnormality. The patient was the wife of a pharmacist but denied the use of diuretic medication. She had a previous diagnosis of anorexia nervosa. Another patient was a 40-year-old woman under investigation for recurrent vomiting and hypokalemia. No organic pathology had been found after her work-up. Although she had access to furosemide and thiazides through her psychiatrist husband, she denied the use of either drug. The third case was a reference specimen from outside our facility. The specimen records had been lost, and therefore no history was obtained. The fourth patient was a 20-year-old woman being evaluated for weakness and emotional difficulties. When hypokalemic alkalosis was discovered, she underwent extensive evaluation without uncovering definite pathology. She denied knowingly taking diuretics, but admitted taking street drugs called “black beauties”; these were not available for analysis.

Evaluation of these discrepancies gave substantial evidence that the liquid–chromatographic method was more sensitive than the spectrophotometric method. Liquid chromatography identified seven urines with thiazides that had been incorrectly called negative by ultraviolet scan. The relative specificity of the two methods was more difficult to assess. Results from the spectrophotometric method were interpreted as showing thiazides in urine from two patients with histories not suggestive of thiazide ingestion. It is impossible to tell, in those specimens that were positive only by the spectrophotometric method, whether that method was detecting furosemide that the chromatographic method would not. In no case did the liquid-chromatographic method fail to detect thiazides when they were known to be present.

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References