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Note

The detection of thiazide diuretics in urine

Column extraction and thin-layer chromatography*

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Thiazide diuretics are prescribed in treatment of congestive heart failure and of hypertension. They may be administered in cases of barbiturate overdosage to accelerate excretion of the drug. If thin-layer chromatography (TLC) of such a patient's urine is employed to follow the patient's clinical course, it is essential that these drugs can be distinguished, particularly since thiazides react with spray reagents used to identify barbiturates.

Most oral diuretics have a thiazide structure (substituted 1,3-benzene-disulfonamide) with Chlorthiazide, 6-chlorobenzo-1,2,4-thiadiazine-7-sulfonamide-1,1-dioxide, as the parent compound.

Methods for identification include (a) ultraviolet (UV) absorption spectra between 230 and 350 nm at acid and at alkaline pH (ref. 1); (b) paper chromatography with localization with UV light at a wavelength of 250 nm (ref. 2) or 253.4 nm and spraying with sodium 1,1-naphthaquinone-4 sulfonate (NQS) in alkaline solution (thiazides produce stable orange-red colors) or with mercurous nitrate, a relatively non-specific reagent[1]; and (c) by TLC with UV localization and identification with NQS[3]; by hydrolysis in solution followed by TLC of the dried hydrolyzed extracts and the Bratton–Marshall reaction (BMR)[4,5], or by "on-plate" hydrolysis followed by the BMR[6]. Interfering substances may occur: barbiturates may distort the UV spectra[1] and sulfonamides may give a positive BMR[6].

Screening of thiazides with *p*-dimethylaminobenzaldehyde (DMAB) will be particularly discussed. Bradshaw employed DMAB to detect unsubstituted sulfonamides[7].

These methodologies ulitize liquid–liquid extractions with organic solvents, particularly ethyl acetate. A lead acetate wash may be employed to remove urinary pigments[6]. Our procedure entails extraction by non-ionic resin column at alkaline pH, ethyl acetate elution, TLC with silica gel G on glass plates, UV localization at

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254 nm, and identification with DMAB or the BMR. DMAB is used to screen both thiazides and sulfonamides.

Osborne[6] reported that acidic DMAB reacted, prior to hydrolysis, with sulfonamides but not with thiazides. He utilized concomitant TLC of unhydrolyzed and hydrolyzed urine extracts to distinguish them. The R_F values of the thiazide hydrolysis products were different from the original compound. With *in situ* hydrolysis, the R_F values are the same.

The parameters to be considered here are:

(1) The effects of changes of pH of extraction for liquid–liquid and for column extraction, as they influence the presence of extraneous pigments and of "tailing" and smearing of developed material on TLC;

(2) The contrast on TLC, both before and after hydrolysis, of spraying with DMAB, with the BMR reagents, or with mercuric sulfate;

(3) The comparison of spray reactions with acid and with alkaline DMAB.

MATERIALS AND METHODS

Reagents

Aliquots of urine, which were analyzed and found to be drug-free, had one of the following compounds added: *Thiazides* (as pure compounds): Bendroflumethiazide (Bristol Labs., Division of Bristol-Meyers, Syracuse, N.Y., U.S.A.), Cyclothiazide (Eli Lilly & Co., Indianapolis, Ind., U.S.A.). Furosemide (Hoechst Pharmaceuticals, Cincinnati, Ohio, U.S.A.), Hydrochlorthiazide (Merck, Sharp & Dohme, West Point, Pa., U.S.A.), Hydroflumethiazide (Bristol Labs.); *Sulfonamides* (U.S.P.): Sulfisoxazole (Hoffmann-La Roche, Nutley, N.J., U.S.A.), Sulfasuxidine (Merck, Sharp & Dohme).

The thiazides were weighed and added to the urines to achieve final concentrations of 10 μg/ml.

The Bratton–Marshall reagents (BMR)[4] used were: (a) 1% (w/v) sodium nitrite in 1% sulfuric acid, freshly prepared; (b) 5% (w/v) ammonium sulfamate, stored in refrigerator; (c) coupling reagent: 2% (w/v) N-(1-Naphthyl)-ethylenediamine dihydrochloride (Sigma, St. Louis, Mo., U.S.A.) in 95% (v/v) ethanol, stored in brown bottle in refrigerator.

DMAB (J. T. Baker, Phillipsburg, Pa., U.S.A.): Acidic[6]: 1 g DMAB in 90 ml of ethanol and 10 ml concentrated hydrochloric acid; Alkaline[7]: 2 g DMAB in 75 ml of 80% acetone and 25 ml of concentrated ammonium hydroxide.

Diphenylcarbazone–mercuric sulfate (DMS): diphenylcarbazone, 0.01% in equal parts of acetone and water; 0.25% mercuric sulfate in 10% sulfuric acid[8].

Thin-layer chromatography

Plates: 250 μm silica gel G on glass; extracting solvent: ethyl acetate; developing solvents: (a) benzene–ethyl acetate (2:8) and (b) ethyl acetate–methanol–ammonium hydroxide (85:10:5) (ref. 8).

When pH was adjusted, concentrated hydrochloric acid was added dropwise to attain an acid pH of 3, or concentrated ammonium hydroxide was added dropwise to reach an alkaline pH of 8.

TABLE I

REACTION OF THIAZIDES AND SULFONAMIDES WITH SELECTED SPRAY REAGENTS

R = Reaction with specific visualizing agent; 0 = no reaction.

Drugs	R_F	*Appearance at 254 nm*	*BMR*		*DMAB*				*DMS*	
			Before hydrolysis	*After hydrolysis*	*Acid*		*Alkaline*		*Before hydrolysis*	*After hydrolysis*
					Before hydrolysis	*After hydrolysis*	*Before hydrolysis*	*Plate previously sprayed with acid DMAB*		
Thiazides										
Bendroflumethiazide	82	Blue	0	R	0	R	R	0	R	0
Cyclothiazide	61	Orange	0	R	0	R	R	0	R	0
Furosemide	26	Blue	R	R	R	R	R	R	R	0
Hydrochlorthiazide	19	Absorption	0	R	0	R	R	0	R	0
Hydrofluthiazide	46	Blue	0	R	0	R	R	0	R	0
Sulfonamides										
Sulfisoxazole	74, 60	Absorption	R	R	R	R	R	R	0	0
Sulfasuxidine	0	0	R	R	R	R	R	R	0	0

Columns

Resin columns for extraction (Drug Extraction Columns, Bio-Rad Labs., Richmond, Calif., U.S.A.).

Procedure

All extractions employed 10-ml aliquots of urine. With each test sample, blank urine specimens underwent simultaneous identical extraction and processing. The following procedure was used.

(1) Extraction at acid or alkaline pH of a blank and of urines containing one of each of the 5 thiazides.

(a) Liquid–liquid extraction: 20 ml ethyl acetate at pH 3 and pH 8, the phases were separated, the organic phase was dried with anhydrous sodium sulfate.

(b) Column extraction: Samples were adjusted to pH 3 and to pH 8, before pouring through the columns. Elution with 20 ml of ethyl acetate, drying with anhydrous sodium sulfate.

Ethyl acetate was evaporated. Residues were redissolved in 50 μl of acetone. Portions of 25 μl of the redissolved residue were used for TLC. TLC developing was stopped when the solvent front had traveled a minimum of 10 cm (about 15 min). The plates were examined by UV at 254 nm.

(2) Thiazides and sulfonamides pre- and post-hydrolysis: Companion plates with thiazides and sulfonamides were developed. To hydrolyze, one plate in each set was sprayed with 12 *N* hydrochloric acid and heated at 100° in an oven for 10 min. Following spraying some plates were covered with aluminum foil to delay the dissipation of the hydrochloric acid. Both sets of plates (hydrolyzed and unhydrolyzed) were: (a) examined under UV; (b) sprayed with sodium nitrite, dried with warm air, sprayed with ammonium sulfamate, then sprayed with the BMR coupling reagent; (c) sprayed with ammoniated (alkaline) DMAB; (d) sprayed with mercuric sulfate, then with diphenylcarbazone.

Sequences 1 and 2 were performed for urines at both acid and alkaline pH and by both liquid–liquid and column extraction (4 variables).

(3) Comparison of spray reactions to acid and to alkaline DMAB: Companion plates were sprayed with acid DMAB, then oversprayed with alkaline DMAB only; or sprayed with acid DMAB then hydrolyzed on the plate.

RESULTS

(1) pH changes

(a) (Liquid–liquid extraction), under UV, thiazides all fluoresced and visually all were of equal density. Acid extracts had more urinary pigments and greater smearing. Alkaline extracts were much clearer.

(b) (Column extraction), under UV, no smearing of either extract. Visual density of each compound was equal. Fewer pigments were present in the alkaline extract.

(2) Pre- and post-hydrolysis, various spray reagents (Table I)

(a) Under UV, pre-hydrolysis, the thiazides, except hydrochlorthiazide, all fluoresced. Sulfasuxidine remained at the origin. Hydrochlorthiazide and sulfisoxazole

showed absorption. After *in situ* hydrolysis, all of the thiazides except hydrochlorthiazide had an orange fluorescence. Sulfisoxazole no longer appeared. There was no discernable difference between the plates hydrolyzed while covered with aluminium foil and the uncovered plates. The same remarks as in (1) apply to the thiazides for liquid–liquid and column extractions at acid and alkaline pH. For the sulfonamides, acid extraction yielded visibly greater recovery.

(b) With BMR, only furosemide and sulfonamides reacted before hydrolysis. After hydrolysis, thiazides and sulfonamides all reacted to yield violaceous spots.

(c) Alkaline DMAB, both pre- and post-hydrolysis produced deep yellow spots which deepened with time, for the thiazides and the sulfonamides. Each thiazide appeared in both acid and alkaline extracts. Alkaline extracts provided somewhat better differentiation.

(d) DMS reacted only with thiazides and only before hydrolysis.

(3) Comparison of acid and alkaline DMAB (Table I)

With acid DMAB only the sulfonamides and furosemide reacted, there was no change following spraying with alkaline DMAB. All reacted with alkaline DMAB or with acid DMAB following hydrolysis.

DISCUSSION

(1) pH changes

Acid pH introduced more urinary pigments and metabolites. Some smearing occurred in the liquid–liquid acid extract. Except for additional pigment with acid pH, pH changes did not effect the columns. Columns yielded cleaner, more sharply demarcated patterns (particularly with an alkaline extract), without mechanical shaking.

(2) Pre- and post-hydrolysis, various spray reagents

Ad (a). Increased post-hydrolysis thiazide fluorescence may represent cumulative effects of hydrolysis products. That aluminum foil did not appear to influence hydrolysis suggests that hydrolysis is rapid.

Ad (b). The BMR identifies free sulfonamides which react both before and after hydrolysis. Hydrolysis of thiazides converts them to reacting mono- and disulfonamides. Furosemide, here, reacted prior to hydrolysis. This provides means for distinguishing the thiazides from the sulfonamides since only the sulfonamides (and furosemide) react prior to hydrolysis and all react after hydrolysis[6].

Ad (c). Alkaline DMAB provides apparently sensitive screening for the thiazides. All of those tested reacted. This reaction did not require formation of sulfonamides. Since DMAB reacts with sulfonamides, the BMR may be employed to exclude them *i.e.* sulfonamides would react with DMAB and with BMR without hydrolysis. Thiazides would react with DMAB but not with BMR before hydrolysis.

Ad (d). DMS is a common spray reagent for barbiturates[8], which react neither with BMR nor with DMAB. DMS in conjunction with alkaline DMAB may distinguish the thiazides from sulfonamides without the necessity of hydrolysis. Thiazides react with both alkaline DMAB and DMS, sulfonamides only with alkaline DMAB.

(3) Comparison of acid and of alkaline DMAB

With acid DMAB, sulfonamides react, and thiazides do not. We differ with Osborne[6] in that we found that furosemide reacts with acid DMAB.

CONCLUSIONS

Alkaline DMAB appears effective as a reagent to identify thiazides. Acid DMAB can distinguish the sulfonamides. Furosemide will have to be distinguished. This can be done by spraying with DMS with which it, but not the sulfonamides, will react; or by UV; furosemide appears as a blue fluorescent smear, the sulfonamides do not fluoresce. Alternatively compounds fluorescing under UV (except for hydrochlorthiazide which gives an absorption spot) react with alkaline DMAB. Characteristic R_F values permit identification as thiazides. For confirmation, BMR or DMS may be used. Column extractions eliminate the need for mechanical shaking and phase separations and yield clear extracts free of extraneous pigment. Their use with alkaline DMAB and UV visualization provides for rapid screening of thiazide diuretics without requiring hydrolysis or diazotisation.

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