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IDENTIFICATION OF SELECTED ANTIHYPERTENSIVE DRUGS BY THIN-LAYER CHROMATOGRAPHY

JACK E. WALLACE, HORACE E. HAMILTON, HAROLD SKRDLANT, LARRY L. BURKETT and HARVEY SCHWERTNER

Department of Pathology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284 (U.S.A.)

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SUMMARY

A thin-layer chromatographic procedure is described for the qualitative identification of several antihypertensive drugs including certain thiazide diuretics, spironolactone, triamterene, methyldopa and their metabolites. Utilization of new solvent developing systems and spray detecting reagents provides a method useful for the identification of these compounds in biologic fluids at low therapeutic concentrations. Sensitivity limits for these antihypertensive drugs are given, and alternate techniques to provide confirmatory analyses are also presented.

INTRODUCTION

A need exists for an analytical procedure that will identify a number of different antihypertensive agents in urine. A technique of this type would be useful for establishing compliance by patients and for detecting misuse by unscrupulous individuals. Presently no general method for this purpose has been described in the scientific literature. In a recent report Stohs and Scratchley describe a thin-layer chromatographic (TLC) technique for separation of several thiazide diuretics[1]; yet, no attempt was made to extract and determine the drugs in biologic specimens. Sohn *et al.*[2] reported on an excellent and extensive study for the separation of several thiazide compounds by TLC but the procedure has no applicability to the identification of other types of antihypertensive drugs. Cooper *et al.*[3] developed a high-pressure liquid chromatographic determination of hydrochlorothiazide in serum and urine but unfortunately the procedure has little applicability for the screening of a number of antihypertensive drugs simultaneously. To our knowledge no TLC methods for identification in urine exist in the literature for the antihypertensive drugs reserpine, spironolactone, triamterene and methyldopa. In fact, no comprehensive method has been presented for the TLC screening and identification in urine for a group of antihypertensive drugs in a single assay after therapeutic treatment. This report describes an analytical procedure for qualitatively analyzing several types of antihypertensive drugs. They are separated and identified by TLC using several

solvent systems and a variety of chromogenic or fluorogenic reagents. The multiple screening technique has sufficient sensitivity and specificity to detect the compounds or their metabolites in the urine of individuals who have taken low therapeutic amounts of the pharmacologically active agents.

EXPERIMENTAL

Reagents

All chemicals were ACS certified grade from Fisher Scientific (Fair Lawn, N.J., U.S.A.), unless otherwise noted. The antihypertensive agents were generously provided by the following pharmaceutical firms: Merck, Sharp & Dohme, West Point, Pa., U.S.A. (chlorothiazide and methyldopa); Parke, Davis & Co., Detroit, Mich., U.S.A. (hydrochlorthiazide); A. H. Robins, Richmond, Va., U.S.A. (reserpine); Searle & Co., Chicago, Ill., U.S.A. (spironolactone and canrenone); and Smith, Kline & French Lab., Philadelphia, Pa., U.S.A. (triamterene). The anion-exchange resin used in this procedure was AG-1-X2, 50–100 or 200–400 mesh, obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Chromatography

The glass chromatographic tanks used were 9.0 × 25 cm at the base and 24 cm deep with a glass plate lid sealed with vacuum grease. A paper lining placed in the interior of the tank significantly reduced the migration time required for development of the chromatograms. The TLC chromatographic plates (Uniplate®) were prescored silica gel G, 250 μm, 20 × 20 cm, obtained from Analtech (Newark, Del., U.S.A.). Solvent system A consisted of *n*-butanol–water–glacial acetic acid (40:20:10). The *n*-butanol and water are mixed separately, allowed to equilibrate for 2–3 min before the glacial acetic acid is added. Mixing the solvent system in that sequence minimizes ester formation. Solvent system B consisted of chloroform–glacial acetic acid–methanol (80:20:15). The chloroform and methanol are mixed initially followed by addition of the glacial acetic acid.

Detection reagents

Iodoplatinate spray reagent was prepared by mixing 1.0 ml of a 5% (w/v) platinic chloride solution (Fisher; No. So–A-118), 1.5 g potassium iodide, 3 ml conc hydrochloric acid and 11 ml water. The napthoquinone spray reagent consisted of a saturated solution of 1,2-naphthoquinone-4-sulfonate (Aldrich, Milwaukee, Wisc., U.S.A.) in ethanol–water (1:1). Ethanol was obtzined from Commercial Solvents (Terre Haute, Ind., U.S.A.). The ceric sulfate spray reagent consisted of a 5% (w/v) solution of ceric sulfate in a 10% aqueous sulfuric acid solution (Fisher; No. A-300). The sodium hydroxide reagent used was an aqueous one normal solution.

General extraction

Two 5-ml aliquots of urine from each patient are placed into separate 50-ml screw-cap extraction tubes, a and b. Anhydrous potassium carbonate (2 g) is added to tube a, and 10 ml of ethylacetate are added separately to tubes a and b. The tubes are subsequently mixed for 3–5 min by vortexing, centrifuged briefly, and the organic phases combined from both tubes (a+b). The aqueous fraction from tube a (only)

can be discarded at this point. If analysis for α-methyldopa is not required, tube b can be eliminated, significantly reducing the time of analysis for the other antihypertensive drugs.

Extraction of thiazides, spironolactone, triameterene and reserpine

The combined organic extracts (ethylacetate) from the general extraction are evaporated under a slow stream of dry, filtered air at 35°–40° to near dryness, and the residue dissolved in 200 μl of methanol. The reconstituted residue will contain the thiazides, spironolactone (metabolite), triamterene or reserpine if present in the original urine sample. The entire amount of the concentrated extract is chromatographed as described under *Thin-layer chromatography* (below).

Extraction of methyldopa

Two grams of the anion-exchange resin are added to the aqueous fraction from tube b (no potassium carbonate added), mixed vigorously and centrifuged. The supernatant portion is removed from the centrifuge tube and discarded. Water (5 ml) is added to the residue (primarily resin), the suspension mixed, centrifuged and the resulting supernatant again discarded. The remaining resin is subsequently mixed with 5 ml of conc. formic acid, the suspension mixed, centrifuged and the liquid formic acid layer transferred with care by filtration (Whatman filter paper No. 1) into a 50-ml evaporating flask. The formic acid elution is repeated and the filtrates combined. The combined formic acid solutions are evaporated to a near residue by means of a rotary evaporator suspended in a water bath at 40°. The residual solution is transferred with small portions of methanol (3 × 3 ml) to a small conical tube and evaporated to approximately 0.5 ml under a stream of filtered air at 35°–40°. The tube and contents are centrifuged and the clear methanol supernatant is streaked on TLC plates as described below.

Thin-layer chromatography

Thiazides, spironolactone (and metabolite), triamterene and reserpine. The TLC plate is divided into 5-cm segments (perpendicular to the solvent migration front) for each of the four antihypertensive drug classes being assayed in this part of the procedure. Prescored plates (20 × 20 cm) are very desirable to use as the different sections (5 cm in width) can be broken off separately for specific spraying and identification of each antihypertensive drug. Solvent standards (10 μl of a methanolic 1 mg/ml solution; 10 μg total) for the drugs are spotted separately 1 cm from both the bottom and the scored line dividing each 5-cm segment. Subsequently 40–50 μl of the urine extract is streaked on a line approximately 3 cm in length next to each of the standards so that one part of the sample extract is superimposed upon the area corresponding to the drug standard. Spotting the samples in this manner minimizes differences in the migration of the drug in the sample and the standard. After the chromatographic plate has been spotted and streaked for spironolactone, thiazides, reserpine and triamterene, it is placed in a developing tank containing chloroform–acetic acid–methanol (80:20:15) and the chromatogram is allowed to develop for approximately 1 h. The chromatographic plate is removed and air dried until the acetic acid is completely removed. The elimination of acetic acid can be expedited by utilization of a hot-air blower at low heat (50°–75°). The plate is then examined under

long-wavelength ultraviolet (UV) light (300 nm). Reserpine appears as a pale, olive-colored fluorescent spot with an R_F value of 0.90–0.95. Triamterene is identified as an intense, medium-blue fluorescent area at an R_F of 0.64–0.65 with a tendency to tail.

For spironolactone identification, the section of the prescored plate containing the spironolactone standard is broken off (if not prescored, the sections of the plate not to be sprayed for spironolactone are covered up) and moderately sprayed with the ceric sulfate reagent. The sprayed plate is heated at 110° for 5 min and the sprayed area observed under long-wavelength UV light. Spironolactone (and metabolite) appears as a bright yellow-green fluorescent area at an R_F of 0.95–1.0. Although spironolactone and reserpine are each detected by fluorescence at similar, though non-idential R_F values, the presence of one does not interfere with the detection of the other. The colors observed differ, but more important, the fluorescence observed for reserpine is native whereas the fluorescence observed for spironolactone is chemically induced. Further the ceric sulfate reagent utilized in the detection of spironolactone destroys the natural fluorescence of reserpine. Thus even at high concentrations of one antihypertensive and low concentrations of the other, neither false positives nor false negatives are encountered. The section spotted with the thiazide standard is sprayed lightly with the iodoplatinate reagent, then slightly dampened with the sodium hydroxide reagent and finally sprayed with the naphthoquinone reagent. The three reagents should be used in the above sequence as rapidly as possible. The chromatogram is subsequently heated at 110° for 5–10 min. Chlorothiazide or hydrochlorothiazide appear as a pink colored area at an R_F of 0.55–0.57. If further identification of the thiazide compound is desired it can be effectively accomplished by the method of Sohn *et al.*[2].

Methyldopa. A TLC plate of 5 × 20 cm is prescored at 5-cm intervals for each sample to be analyzed. A 10-μl aliquot of solvent standard (methyldopa, 1 mg/ml in methanol) is applied as a spot approximately 1.0 cm from both the bottom and the scored line or edge of the chromatographic plate. Then 100 μl of the urine extract are streaked on a line approximately 3 cm in length. It is important that one end of the sample streak be superimposed upon the standard spot to assure that the methyldopa in the sample and in the standard migrate in the same environment. The plate is then placed in a solvent system of *n*-butanol–water–glacial acetic acid (40:20:10) for approximately 4 h. Afterwards the plate is removed and allowed to air dry. Then, as rapidly as possible, the plate is lightly sprayed with the iodoplatinate spray, then until damp (not soaking) with 1 *N* NaOH, and finally with the naphthoquinone spray. The chromatogram is immediately placed into a drying oven at 110° for 5–10 min. Methyldopa in the urine sample is confirmed by the presence of a dark, black streak extending from the standard spot across the width of the migrated sample. Care must be taken not to confuse a light brown area (line) resulting from normal urine constituents as a positive for methyldopa, as they both have similar R_F values, 0.73.

Confirmatory analysis

Positive indication for any antihypertensive drug for which this method provides detection can be confirmed by the following specific methods.

Hydrochlorothiazide. Hydrochlorothiazide can be determined by gas–liquid chromatography (GLC) after conversion to its tetramethyl derivative according to the technique of Lindström *et al.*[4].

Spironolactone. Confirmation of spironolactone in urine is most effectively accomplished by measuring its primary metabolite, canrenone, which can be isolated by the extraction techniques of Sadée *et al.*[5] or Karim *et al.* utilizing electron-capture GLC[6]. The carbonyl conjugation in rings A and B of that particular metabolite of spironolactone make it conducive to analysis by electron-capture GLC techniques.

Triamterene. The suspected triamterene can be removed from the respective chromatograms by careful transfer of the silica gel corresponding to the fluorescent spot. The residue is placed into ethylacetate, centrifuged to separate the silica gel, and the clear supernatant determined spectrofluorometrically for triamterene (exicitation wavelength 360 nm, emission wavelength 435 nm). The confirmatory test has been effectively utilized in our laboratory for several suspected positive urines.

Reserpine. Reserpine can be alternately determined in the urine as the metabolite trimethoxybenzoic acid[7] since less than 1% of ingested reserpine is excreted as the unchanged form[8]. The procedure described in this report identifies unchanged reserpine. Alternately the fluorescent area that is indicative of reserpine can be extracted from the plate, dissolved in ethanol and determined spectrofluorometrically. The wavelengths for maximum excitation and emission of reserpine are 300 and 380 nm, respectively. An alternate fluorometric procedure[9] can be accomplished after oxidation of the drug with vanadium pentoxide.

Methyldopa. For confirmation, α-methyldopa and its primary metabolite can be oxidized to highly fluorescent derivatives according to the procedures of Bertler, Carlsson and Rosengren[10] and Euler and Floding[11]. The area on the chromatogram relative to methyldopa migration is removed from an unsprayed plate, dissolved, centrifuged, and assayed spectrofluorometrically after ferricyanide oxidation[12]. The fluorescent derivative of α-methyldopa has excitation and emission maxima of 400 and 515 nm, respectively.

Clinical evaluation

Table I summarizes the results of a screening program performed in this laboratory to detect users of antihypertensive drugs. The specimens analyzed in this study did not represent a random sampling of the examined population; rather, specimens for analysis were selected on the basis of clinical suspicions, *i.e.*, abnormal

TABLE I

RESULTS OF AN EMPLOYEE SCREENING PROGRAM FOR THE DETECTION OF ANTIHYPERTENSIVE DRUGS

Drug(s)	*Number of specimens*
No antihypertensives detected	11
Thiazides only	3
Triamterene only	1
Reserpine only	0
Spironolactone only	0
Methyldopa only	0
Thiazides and triamterene	1
Thiazides and methyldopa	1
Thiazides, spironolactone and methyldopa	2
Total	19

electrocardiograms *etc.* No false positives or false negatives were reported for spiked urine control specimens provided as part of a quality control program accompanying the employee screening. Of 19 specimens analyzed, 8 were positive for one or more antihypertensive compounds; of these 8 patients, 4 were positive for only one drug, 2 were positive for two drugs and 2 were positive for three compounds.

RESULTS AND DISCUSSION

The procedures utilized and results obtained in this study are summarized in Table II.

TABLE II

DETERMINATION OF ANTIHYPERTENSIVE DRUGS

Compound	*Extraction*	*Solvent system*	*Detection (color)*	R_F	*Limit of detection (μg/ml, 5-ml specimen)*	*Confirmatory method(s)*
Chlorothiazide, hydrochlorothiazide	Ethyl acetate	Chloroform–acetic acid–methanol (80:20:15)	Iodoplatinate, NaOH, naphthoquinone (pink)	0.55	5.0	GLC
Spironolactone (canrenone)			Ceric sulfate, fluorescence (bright yellow-green)	0.95–1.0	0.1	GLC
Triamterene			Fluorescence: (intense medium blue)	0.65 (tail)	0.5	Fluometry
Reserpine			Fluorescence: (pale olive)	0.90–0.95	0.1	Fluorometry or assay for metabolite
Methyldopa	Resin, formic acid	Butanol–water–acetic acid (40:20:10)	Iodoplatinate, NaOH, naphthoquinone (dark black)	0.73	2–5	Fluorometry or oxidation–spectro fluorometry

Thiazides

Concentrations of hydrochlorothiazide and chlorothiazide in urine generally exceed 10 μg/ml during the first 48-h period after ingestion of a single therapeutic dose[13,14]. The sensitivity of the proposed screening procedure for the thiazides is approximately 5 μg/ml; consequently utilizing a 5–10-ml urine sample it is possible to identify these compounds for up to 3–4 days in urine from patients who have received a single 50-mg dose of hydrochlorothiazide or a single 100-mg dose of chlorothiazide.

Spironolactone

Spironolactone is converted very rapidly by dethioacetylation to its primary

metabolite, canrenone. Approximately 80% of the spironolactone is present in plasma as canrenone. In urine, canrenone accounts for approximately 36% of the total spironolactone metabolites and corresponds to 7.4% of the total administered dose. Unmetabolized spironolactone is not detectable in urine; therefore, in urine screening the metabolite must be the compound identified. Both spironolactone and canrenone give identical R_F values and color intensities with the solvent systems and spray reagents utilized in the proposed procedure. In one study performed in this laboratory, 8–10 mg of canrenone were present in a 24-h urine after the subjects had been administered 400 mg of the active drug. This would correspond to 7–10 μg/ml based on an average 24-h urine of 1000–1200 ml. Since sensitivity of the proposed method is 0.1 μg/ml, spironolactone therapy can be monitored effectively and a single therapeutic dose under most situations would yield a positive urine for 3–4 days. Our studies show that the metabolite is also extremely sensitive to electron-capture (^{63}Ni) GLC analysis[15].

Triamterene

Sensitivity for triamterene in urine by the proposed system is 0.5 μg/ml. 10–80% of triamterene can appear in the urine within 24 h in the unchanged form[16]. A normal dose regimen for the drug is 100 mg taken twice daily which usually yields estimated urine levels of 20–176 μg/ml; consequently, the current procedure gives more than sufficient sensitivity for identifying triamterene at concentrations in urine well below that associated with therapeutic dosing.

Reserpine

Only 0.29% of this drug is excreted unchanged in urine. With a normal 0.5-mg dose and a 24-h urine volume of 1 l, a concentration of 0.002 μg/ml needs to be detected for identifying therapeutic use of this antihypertensive agent. The sensitivity of our screening test is 0.1 μg/ml; therefore, the method is capable of identifying only those urines associated with patients receiving toxic amounts of reserpine. Maronde *et al.*[17] using a photofluorometric method observed positive urines for unchanged reserpine in only 2 of 31 patients taking oral reserpine in therapeutic amounts. The present TLC method is approximately 10 times less sensitive than the fluorometric method if one considers that the sensitivity of the TLC procedure is 0.1 μg/ml compared to the photofluorometric sensitivity of 0.01 μg/ml.

Methyldopa

The analysis of methyldopa is the more time-consuming part of the proposed procedure. About 8 h are required; however, several samples (10–12) can be assayed simultaneously, and only approximately 1½ h actual working time is required. Extraction recovery for α-methyldopa is approximately 20%, due probably to the inability of the drug to be released quantitatively from the resin bed. Attempts to improve the extraction efficiency were not explored as the method as described provides adequate sensitivity for detecting α-methyldopa in urine after therapeutic treatments. For example, considering a dose of 1.0 g/day distributed into 1200 ml urine and based on the reported excretion rate per day of 13.4% of unchanged drug[12], the procedure needs to detect 110 μg/ml of drug using a 5-ml specimen. Sensitivity of the proposed method is 12–15 μg/ml providing ample detectability of α-methyldopa even at sub-therapeutic concentrations in urine.

CONCLUSION

A method for routine screening of antihypertensive agents in urine has been described. The procedure should be useful to ascertain patient compliance and to detect covert use of these drugs. With the exception of reserpine, sensitivity of detection is well below that required to detect these antihypertensive agents for several days in urine after therapeutic dosing.

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