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High-Performance Liquid Chromatographic Analysis of Triamterene and *p*-Hydroxytriamterene in Plasma

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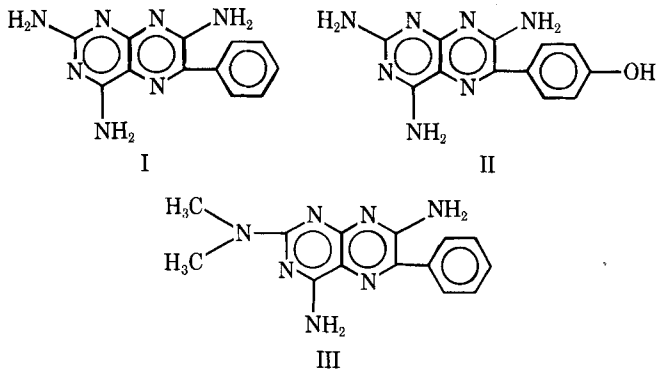
Abstract □ A rapid and sensitive high-performance liquid chromatographic assay was developed for triamterene and 6-*p*-hydroxytriamterene in plasma. Plasma (0.5 ml), after addition of the internal standard, was extracted with 10 ml of ether–isopropanol (95:5). After thorough mixing and separation of phases, the organic layer was evaporated to dryness under nitrogen. The residue was reconstituted with 500 μl of mobile phase [acetonitrile–water–acetic acid (60:39.5:0.5)], and 100 μl was injected into the chromatograph. Chromatographic separation was carried out on a C_{18} μBondapak column at a flow rate of 1 ml/min. Detection of compounds in the column eluent was by UV absorption at 365 nm. The retention times for 6-*p*-hydroxytriamterene, triamterene, and the internal standard were 7.5, 9.0, and 12.0 min, respectively. The lower limit of detection for each compound was 20 ng/ml. Recoveries of triamterene and 6-*p*-hydroxytriamterene were 91–99 and 82–95%, respectively, over a 40–240-ng/ml range.

Keyphrases □ Triamterene—high-performance liquid chromatographic analysis, plasma □ *p*-Hydroxytriamterene—high-performance liquid chromatographic analysis, plasma □ Diuretics—high-performance liquid chromatographic analysis of triamterene and *p*-hydroxytriamterene, plasma □ High-performance liquid chromatographic analysis—triamterene and *p*-hydroxytriamterene, plasma

Triamterene, 2,4,7-triamino-6-phenylpteridine (I), is a natriuretic and a potassium-sparing diuretic. It is used mainly in combination with hydrochlorothiazide in the treatment of edema associated with congestive heart failure, cirrhosis of the liver, and the nephrotic syndrome.



I

II

III

BACKGROUND

Studies of plasma and urine concentrations of triamterene and its major metabolite, 2,4,7-triamino-6-*p*-hydroxyphenylpteridine (II), in humans and animals have been performed either with nonspecific fluorescence methods or more specific methods that require time-consuming and involved separation procedures (1–5). The separation techniques utilized have included paper chromatography (4), TLC (5), and liquid–liquid extraction (2, 6). One method (5) detected nanogram levels of the compounds, but the other methods lacked the sensitivity to measure nanogram concentrations of triamterene in plasma. A sensitive and specific assay is particularly desirable for study of triamterene pharmacokinetics since concentrations of the metabolite may be 10-fold those of the parent compound 30 min following oral administration (2).

Recently, two high-performance liquid chromatographic (HPLC) assays for triamterene in plasma were reported. One method (7) involved extraction of triamterene as its perchlorate ion-pair and subsequent normal-phase HPLC utilizing a fluorescence detector. Triamterene concentrations as low as 2 ng/ml could be detected, although the investigators did not employ an internal standard. The other method (8) involved extraction with ethyl acetate and subsequent reversed-phase HPLC with fluorescence detection; it had a sensitivity to 1 ng/ml. Neither research group attempted to quantitate metabolites.

This article describes a rapid, sensitive, and selective assay for triamterene and its major metabolite in plasma. The method involves a simple extraction of plasma with organic solvent and reversed-phase HPLC of the extract using UV detection.

EXPERIMENTAL

Reagents—Acetonitrile[1] and methanol[1] were chromatography grade. Anhydrous ether[2], acetic acid[2], and isopropanol[2] were spectranalytical grade.

Equipment—A high-performance liquid chromatograph[3] with a syringe-loading sample injector[4], a recorder[5], and a variable-wavelength

[1] MCB Manufacturing Chemists Inc., Cincinnati, Ohio.
[2] Fisher Scientific Co., Fair Lawn, N.J.
[3] Tracor 950 chromatographic pump, Tracor Instruments, Austin, Tex.
[4] Model 7120, Rheodyne, Berkeley, Calif.
[5] OmniScribe, Houston Instruments, Austin, Tex.

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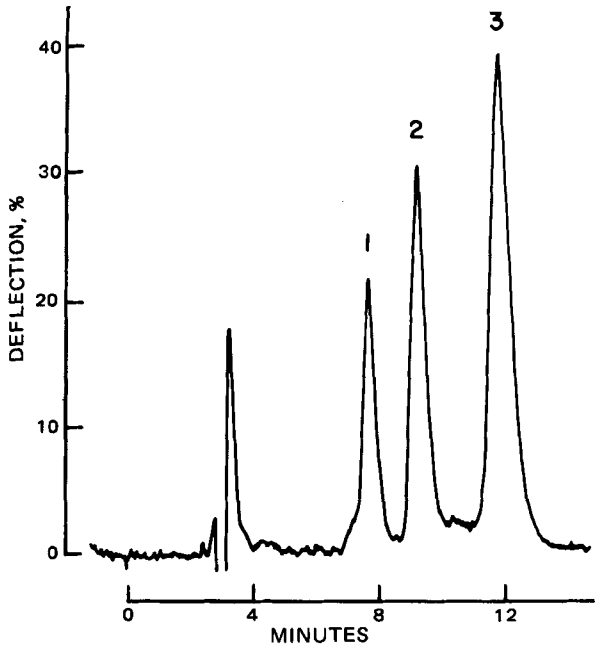

Figure 1—*Chromatogram of the extract of 0.5 ml of plasma containing 100 ng/ml each of 6-p-hydroxytriamterene (1) and triamterene (2) and 200 ng/ml of the internal standard (3). The extract was reconstituted with 500 μl of mobile phase, and 100 μl was injected.*

UV detector[6] were used. Chromatography was done on a μBondapak C_{18} column[7].

Assay Standards—Stock methanol solutions of triamterene[8] (1 mg/100 ml), 6-*p*-hydroxytriamterene[9] (1 mg/100 ml), and the internal standard[8], 4,7-diamino-2-dimethylamino-6-phenylpteridine (1 mg/100 ml), were prepared and stored in light-resistant flasks to prevent photodecomposition. Plasma standards were prepared by adding appropriate amounts of methanol stock solutions to drug-free plasma to give final concentrations of 40–240 ng/ml of triamterene and 6-*p*-hydroxytriamterene and 200 ng/ml of the internal standard.

Extraction—To 0.5 ml of plasma in a silylated tube, 10 ml of anhydrous ether–isopropanol (95:5) was added. The tube contents were mixed with gentle agitation in an aliquot mixer[10] for 10 min and then centrifuged[11] for 20 min at 1300×*g* to separate the phases. The ether–isopropanol layer (top layer) was transferred to a sample concentrator[12] with a disposable Pasteur pipet and evaporated to dryness under nitrogen at ambient temperature. The dried extract was reconstituted with 500 μl of mobile phase, and 100 μl was injected into the chromatograph.

Chromatography—All chromatography was done at ambient temperature. The mobile phase was acetonitrile–distilled water–acetic acid (60:39.5:0.5). Prior to use, the mobile phase was filtered through a 10-μm polypropylene filter[13]. The flow rate was set at 1 ml/min, and the UV detector was set at 365 nm. The λ_{max} values for triamterene, 6-*p*-hydroxytriamterene, and 4,7-diamino-2-dimethylamino-6-phenylpteridine were 365, 368, and 380 nm, respectively. The recorder was run at a chart speed of 0.635 cm/min.

Quantitation—Peak height ratios of triamterene or 6-*p*-hydroxytriamterene to the internal standard were obtained from the chromatograms and plotted against concentration or amount of drug to obtain calibration curves for each compound.

RESULTS AND DISCUSSION

6-*p*-Hydroxytriamterene, triamterene, and the internal standard were separated and eluted in ~12 min; the retention times were 7.5, 9.0, and 12.0 min, respectively (Fig. 1). Extracts from blank plasma gave no interference from endogenous plasma components.

[6] Tracor 970, Tracor Instruments, Austin, Tex.
[7] Waters Associates, Milford, Mass.
[8] Smith Kline and French Laboratories, Philadelphia, Pa.
[9] Rohm & Haas, GmblH., Darmstadt, West Germany.
[10] Model 4651, Ames, Division of Miles Laboratories, Elkhart, Ind.
[11] Model TJ-6 centrifuge, Beckman Instruments, Palo Alto, Calif.
[12] Model SC/27R, Brinkmann Instruments, Westbury, N.Y.
[13] Gelman Filtration Products, Ann Arbor, Mich.

Table I—Reproducibility of HPLC Assay of Triamterene and *p*-Hydroxytriamterene in Plasma

Concentration, ng/ml	Mean Peak Height Ratio	Mean Peak Height Ratio / Concentration	*RSD*, %
	Triamterene (n = 5)		
40	0.1413	0.0035	6.44
80	0.2914	0.0036	8.44
120	0.4071	0.0034	7.30
200	0.6175	0.0031	7.37
240	0.8044	0.0034	2.86
	6-*p*-Hydroxytriamterene (n = 4)		
40	0.0972	0.0024	18.88
80	0.2124	0.0026	5.84
120	0.2902	0.0024	4.56
200	0.4539	0.0023	7.45
240	0.5895	0.0025	5.45

Standard curves for both triamterene and 6-*p*-hydroxytriamterene were linear over the 40–240-ng/ml range. At least 20 ng/ml of each component could be detected when the dried plasma extract was reconstituted with 100 μl of mobile phase and most of the extract was injected into the chromatograph. The least-squares linear regression curves did not go through the origin, but the mean y intercepts were not significantly different from zero (0.0099 for 6-*p*-hydroxytriamterene and 0.0235 for triamterene). Table I shows standard curve reproducibility for both triamterene and 6-*p*-hydroxytriamterene from analyses of plasma standards done on different days. By using the equations obtained from linear regression analyses of the data, triamterene and 6-*p*-hydroxytriamterene concentrations were calculated for each observed peak height ratio. The relative standard deviations were between 2 and 9% for both compounds with the exception of the metabolite at the 40-ng/ml level.

Recoveries of triamterene and 6-*p*-hydroxytriamterene from plasma extractions were assessed by comparing the peak height ratios of spiked plasma extracts with those of standard methanol solutions. The recoveries ranged from 82 to 95% for the metabolite and from 91 to 99% for triamterene over the concentration range studied.

4,7-Diamino-2-dimethylamino-6-phenylpteridine was chosen as the internal standard because it exhibited similar chromatographic behavior to triamterene. It eluted near the triamterene peak but was completely separated from triamterene and 6-*p*-hydroxytriamterene. Its retention time did not add significantly to the total analysis time, and its recovery from plasma by extraction was quantitative. This internal standard probably would not be present in the plasma of patients treated with triamterene and will not alter assay specificity.

The described HPLC procedure was developed to study triamterene and 6-*p*-hydroxytriamterene pharmacokinetics in dogs and humans. Unlike recently published procedures (7, 8), this assay quantitates both triamterene and the metabolite after direct extraction of plasma samples with organic solvent. Its simplicity and the short assay time are advantageous in the analyses of the large number of samples often encountered in pharmacokinetic and bioavailability studies. The small plasma volume needed for analysis is also an important consideration in such studies. These factors, coupled with the sensitivity and selectivity of the procedure, should provide an excellent technique for the study of the pharmacokinetics of triamterene and 6-*p*-hydroxytriamterene.

The assay described is not as sensitive as the recently published procedures (7, 8) since the UV absorption of the compounds is determined. An increase in sensitivity is expected by using fluorescence determination, and preliminary data suggest that the detection limit can be lowered to 1 ng/ml for each compound should such sensitivity be required.

Triamterene is often administered in combination with hydrochlorothiazide and other antihypertensives. The potential interference of these drugs was not evaluated since the procedure was developed to study the pharmacokinetics of triamterene and 6-*p*-hydroxytriamterene in dogs and humans not on antihypertensive therapy. However, the monitored UV wavelength is in a region that precludes interferences from the thiazides and many other compounds in the unlikely event that compounds of such widely differing structures are not adequately separated in the system described.

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Antimicrobial Activity of Phenolic Constituents of *Magnolia grandiflora* L.

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Received December 8, 1980, from the *Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677.* Accepted for publication January 14, 1981. *Present address: Taiwan Provincial Pingtung Institute of Agriculture, Pingtung, Taiwan, Republic of China.

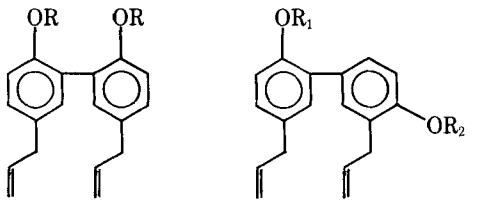
Abstract □ Three phenolic constituents of *Magnolia grandiflora* L. were shown to possess significant antimicrobial activity using an agar well diffusion assay. Magnolol, honokiol, and 3,5′-diallyl-2′-hydroxy-4-methoxybiphenyl exhibited significant activity against Gram-positive and acid-fast bacteria and fungi. The minimum inhibitory concentrations were determined for each compound using a twofold serial dilution assay.

Keyphrases □ *Magnolia grandiflora* L.—antimicrobial activity of phenolic constituents, agar well diffusion assay □ Magnolol—phenolic constituent of *Magnolia grandiflora* L., antimicrobial activity □ Honokiol—phenolic constituent of *Magnolia grandiflora* L., antimicrobial activity □ 3,5′-Diallyl-2′-hydroxy-4-methoxybiphenyl—phenolic constituent of *Magnolia grandiflora* L., antimicrobial activity □ Antimicrobial activity—phenolic constituents of *Magnolia grandiflora* L., agar well diffusion assay

The search for new antibiotics is no longer restricted primarily to microbial products. Recently, constituents of higher plants have exhibited significant antimicrobial activity (1–4). The isolation and identification of magnolol (I), honokiol (II), and 3,5′-diallyl-2′-hydroxy-4-methoxybiphenyl (III) as phenolic constituents of the seeds of *Magnolia grandiflora* L. were reported (5). All three compounds have significant antifungal and antibacterial activity, and the details of this antimicrobial activity are described here.

RESULTS AND DISCUSSION

Magnolol (I), honokiol (II), and 3,5′-diallyl-2′-hydroxy-4-methoxybiphenyl (III), phenolic constituents of *M. grandiflora* L. (5), showed significant antimicrobial activity against Gram-positive bacteria, an acid-fast bacterium, and yeast-like and filamentous fungi. All three compounds were tested qualitatively for activity using an agar well diffusion assay (Table I). The corresponding dimethyl ethers, IV and V, showed no significant activity.



I: R = H
IV: R = CH_3

II: $R_1 = R_2 = H$
III: $R_1 = H$, $R_2 = CH_3$
V: $R_1 = R_2 = CH_3$

Streptomycin sulfate and amphotericin B also were tested to serve as standards for comparison. All three compounds (I–III) had activity comparable to the standards under the same test conditions.

The minimum inhibitory concentration (MIC) of each active compound was determined using a twofold serial dilution assay (Table II). All three compounds apparently were considerably more active than amphotericin B against *Trichophyton mentagrophytes* but not as active against *Candida albicans* and *Saccharomyces cerevisiae*. Methylation of one phenolic group of II to give III resulted in the loss of activity against *Aspergillus niger* and *C. albicans* but not against other fungi or bacteria. In fact, III was somewhat more active against *Staphylococcus aureus*, *Bacillus subtilis*, and *Mycobacterium smegmatis* than the dihydroxy compounds I and II. All three compounds possessed activity comparable to, or better than, streptomycin sulfate against *B. subtilis*, *S. aureus*, and *M. smegmatis*. Compounds I–III were also tested for activity against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), but none showed any activity.

EXPERIMENTAL

Qualitative Antimicrobial Screening—All compounds were tested for activity against the following microorganisms: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 15442), *Mycobacterium smegmatis* (ATCC 607), *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 9763), *Aspergillus niger* (ATCC 16888), and *Trichophyton mentagrophytes* (ATCC 9972). Routine qualitative screening of compounds for antimicrobial activity was accomplished as previously described (1) except for the following modifications: plates for the assay were prepared by dispensing 25 ml of sterile agar medium into 100 × 15-mm sterile petri dishes; and using the quadrant streak method, the sterile agar plates were streaked with a dilution of the test organism (1 ml of broth culture in 9 ml of sterile water).

Antimicrobial activity was recorded as the width (in millimeters) of the inhibition zone measured from the edge of the agar well to the edge of the inhibition zone.

Quantitative Antimicrobial Assay—For compounds that showed significant activity in the qualitative screen, the MIC values were determined using the twofold serial dilution technique previously described (1). All compounds were initially tested using a concentration of 100 μg/ml in the first tube. After preliminary evaluation to determine the range of the MIC value, the concentrations in the first tube were de-

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