High-Pressure Liquid Chromatographic Analysis of Ticrynafen and One of Its Metabolites in Urine and Serum

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Abstract □ A method is described for the extraction of ticrynafen, a new hypotensive agent, and its reduced metabolite from serum and urine. Drug-related material is extracted from biological fluids with ether under strongly acidic conditions and then back-extracted into an alkaline aqueous phase, which is subjected to high-pressure liquid chromatographic analysis. Separations are performed on a reversed-phase column with a mobile phase consisting of phosphate buffer–acetonitrile. This accurate and reproducible method measures serum concentrations of ticrynafen and its reduced metabolite as low as 1.0 and 0.4 μg/ml, respectively.

Keyphrases □ Ticrynafen—analysis, extraction, high-pressure liquid chromatography, reduced metabolite, urine and serum □ Hypotensive agents—ticrynafen, reduced metabolite, extraction, high-pressure liquid chromatography, urine and serum □ High-pressure liquid chromatography—ticrynafen, reduced metabolite

Ticrynafen, [2,3-dichloro-4-(2-thienylcarbonyl)phenoxy]acetic acid (I), is a new hypotensive agent which combines thiazide-like diuretic activity with uricosuric properties (1, 2). The drug is metabolized by reduction to the alcohol (II) or by oxidative cleavage to the dicarboxylic acid (III) (3). Maurer *et al.* (4) described a spectrophotometric analysis of I and II in which plasma and urine samples were extracted and purified by TLC. The material eluted from the scrapings was measured by UV absorption at 290 nm. GLC also has been described for measurement of I (5) and I–III (6) in plasma and urine following extraction and derivatization of the drug and its metabolites.

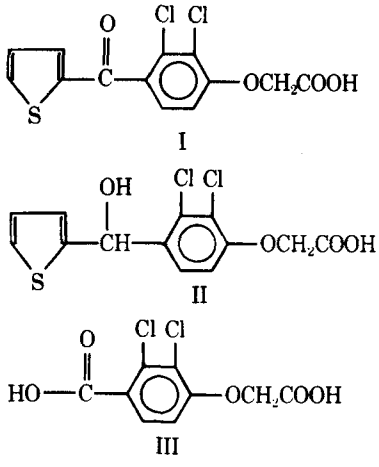
This paper describes a high-pressure liquid chromatographic (HPLC) procedure for measuring I and II in serum and urine. The method is more rapid than either the spectrophotometric or GLC procedure, and its usefulness in providing bioavailability and clearance data has been proven in studies in dogs and humans. Although III is not measured by the HPLC analysis, this component constitutes a very small portion of drug-related material, both in dogs (3) and humans (6).

EXPERIMENTAL

Instrumentation—A constant-flow pumping system[1] was coupled with a variable-wavelength detector[2] set at 210 nm. The column, 2.6-mm i.d. × 25 cm, was obtained prepacked with reversed-phase material[3]; a rotary injection valve[4] equipped with a 15-μl loop was used to introduce samples into the system at the column inlet.

Reagents—Analytical reagent grade ether[5] was used for the extraction of I and II from biological fluids. The HPLC mobile phase was 0.05 *M* phosphate buffer (pH 7.0)–glass-distilled acetonitrile[6] (78:22).

Standard Solutions—Appropriate concentration ranges of I and II[7] were prepared in serum or urine for each set of experimental samples, and these standards were extracted and analyzed concurrently with the unknowns. Following a 250-mg oral dose in humans, suitable concentration ranges were: serum, 1.0–25 μg of I/ml and 0.4–2.5 μg of II/ml; and urine, 20–125 μg of I/ml and 8–50 μg of II/ml. The internal standard in the assay was prepared by dissolving 40 mg of ethacrynic acid in 100 ml of methanol.



Extraction—*Serum*—Serum aliquots, 0.5 ml, were pipetted into 12-ml conical centrifuge tubes, and 10 μl of the internal standard solution, 0.5 ml of 3 *N* HCl, and 1.5 ml of water were added sequentially with vortexing after each addition. Diethyl ether, 5 ml, was added. The tubes were tightly stoppered, placed horizontally in a reciprocating shaker, and shaken slowly (about 60 oscillations/min) for 30 min.

After shaking, samples were centrifuged to separate the layers sharply, and 4.0 ml of the organic layer was transferred to a second 12-ml centrifuge tube containing 0.3 ml of 0.035 *M* Na_2HPO_4. The tubes were shaken

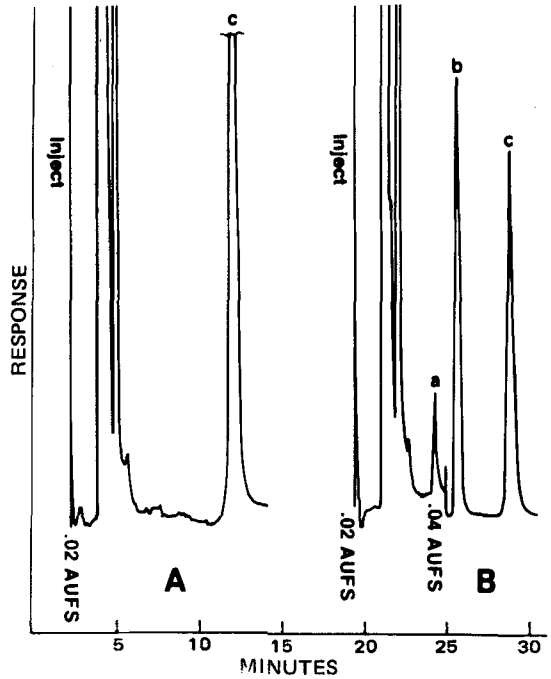


Figure 1—*Chromatograms of extracts from control human serum (A) and serum containing 5 μg of I/ml plus 0.5 μg of II/ml (B). Key: a, II; b, I; and c, ethacrynic acid (internal standard).*

[1] Perkin-Elmer 601.
[2] Perkin-Elmer LC-55.
[3] Perkin-Elmer ODS-HC SIL-X-1.
[4] Valco Instruments Co., Houston, Tex.
[5] Mallinckrodt.
[6] Burdick & Jackson.
[7] Smith Kline & French Laboratories.

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Table I—Recovery from Serum Based on Peak Height Ratios

Sample	Extracted I, 20 μg/ml	Extracted II, 2 μg/ml	Unextracted[a] I, 33.4 μg/ml	Unextracted[a] II, 3.34 μg/ml
1	1.84	1.16	1.98	1.25
2	1.78	1.18	1.98	1.27
3	1.84	1.20	2.02	1.28
4	1.80	1.16	2.02	1.25
5	1.78	1.13	1.97	1.25
$\bar{x}$	1.81	1.17	1.99	1.26
$SD_{\bar{x}}$	±0.03	±0.03	±0.02	±0.01
Recovery[b], %	104	107		

[a] Concentration that would represent 100% recovery, 0.5 ml of serum → 0.3-ml final volume. [b] Recovery = $\bar{x}$ extracted/$\bar{x}$ unextracted, corrected for volume loss in which 4.0 ml was taken from a total 4.6-ml organic layer.

Table II—Recovery from Urine Based on Peak Height Ratios

Sample	Extracted I, 80 μg/ml	Extracted II, 32 μg/ml	Unextracted[a] I, 6.67 μg/ml	Unextracted[a] II, 2.67 μg/ml
1	0.63	0.28	0.78	0.39
2	0.64	0.29	0.80	0.39
3	0.64	0.29	0.80	0.38
4	0.61	0.27	0.78	0.39
5	0.62	0.27	0.77	0.39
$\bar{x}$	0.63	0.28	0.79	0.39
$SD_{\bar{x}}$	±0.01	±0.01	±0.01	±0.004
Recovery[b], %	98	88		

[a] Concentration that would represent 100% recovery, 0.025 ml of urine → 0.3-ml final volume. [b] Recovery = $\bar{x}$ extracted/$\bar{x}$ unextracted, corrected for volume loss in which 4.0 ml was taken from a total 4.9-ml organic layer.

horizontally at high speed (about 120 oscillations/min) for 15 min. The phases were separated by centrifugation, and the organic layer was aspirated and discarded. The unstoppered tubes were placed in a warm water bath for 10 min to remove traces of ether, and the remaining aqueous portion was retained for HPLC analysis.

Urine—Urine samples were diluted (1:10) with distilled water. Aliquots of diluted urine samples, 0.25 ml, were pipetted into 12-ml conical centrifuge tubes and 10 μl of the internal standard solution, 0.25 ml of 3 *N* HCl, and 1.75 ml of water were added with vortexing after each addition. Diethyl ether, 5 ml, was added and the procedure was completed as with serum.

Chromatography—New columns were conditioned with the mobile phase at a flow rate of 0.75 ml/min for several hours. This same flow rate was then used for sample analysis. Column life may be extended by the use of a precolumn packed with pellicular reversed-phase material. Retention times in this system were 4.7 min for the reduced metabolite (II), 6.3 min for ticrynafen (I), and 9.7 min for the internal standard. Because of large differences between the concentrations of I and II in the same sample, it is often necessary to attenuate between peaks and, therefore, to employ a detector that provides linear response at different attenuations.

Quantitation—The ratios of the I and II peak heights to the internal standard peak height were calculated for each standard. Each of these ratios was plotted *versus* concentration, and the resulting curves were used to convert peak height ratios of experimental samples to concentrations.

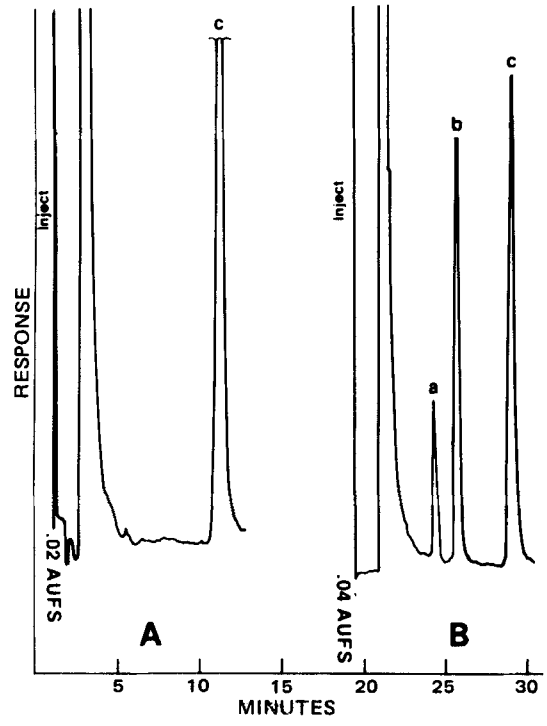

Figure 2—*Chromatograms of extracts from control human urine (A) and urine containing 40 μg of I/ml plus 16 μg of II/ml (B). Key: a, II; b, I; and c, ethacrynic acid (internal standard).*

RESULTS AND DISCUSSION

Recoveries of I and II were determined by adding known amounts of each to blank human serum and urine and extracting as described under *Experimental*. However, for recovery purposes only, the internal standard was not added initially to the samples but was contained in the 0.035 *M* Na_2HPO_4 used for back-extraction. The apparent recoveries of I and II would then not be influenced if the ethacrynic acid extraction was less than 100%. Corresponding unextracted samples were prepared in 0.035 *M* Na_2HPO_4 (also containing internal standard) so that their concentrations would be equal to 100% recovery from the extracted group. Peak height ratios obtained with extracted serum samples and corresponding unextracted standards are shown in Table I; the same information for urine is shown in Table II. These results show good reproducibility and complete recovery of I and II from both serum and urine.

The relationship between peak height ratio and concentration was linear in serum over the ranges of 1.0–50 μg of I/ml and 0.4–5.0 μg of II/ml. Likewise, linear relationships were obtained in urine for concentration ranges of 20–125 μg of I/ml and 8–50 μg of II/ml. The lower detection limits for the described method were 1.0 μg of I/ml and 0.4 μg of II/ml in serum and 20 μg of I/ml and 8 μg of II/ml in urine. Lower levels could be measured by increasing the injection volume for serum samples and using a smaller dilution factor with urine. The 1:10 dilution of urine containing high concentrations of drug prevented unnecessary loading of columns.

Typical chromatograms obtained with blank human serum and serum to which I and II had been added are shown in Fig. 1; chromatograms from control urine and urine with added I and II are shown in Fig. 2. The method is specific for ticrynafen and its reduced metabolite since no endogenous materials interfering with the analysis of either component have been seen in the analysis of numerous control serum and urine samples from dogs and humans. Although UV absorption maxima for both I and II have been reported to be about 290 nm (3), the response was increased threefold for I and 25-fold for II when the column effluents were monitored at 210 nm compared to the response at 290 nm.

Two groups of samples, 18 replicates each, were prepared in blank human serum to test the accuracy of the method. One group contained 4.0 μg of I/ml and 0.5 μg of II/ml; the other contained 12.0 μg of I/ml and 1.2 μg of II/ml. Six samples from each group were analyzed on each of 3 days as described under *Experimental*. For the 36 serum samples analyzed, the averages of actual content measured were: ticrynafen (I), 100.2 ± 4.2%; and reduced metabolite (II), 102.9 ± 8.0%. Similar sets of replicates in blank human urine, containing 40 and 125 μg of I/ml and 16 and

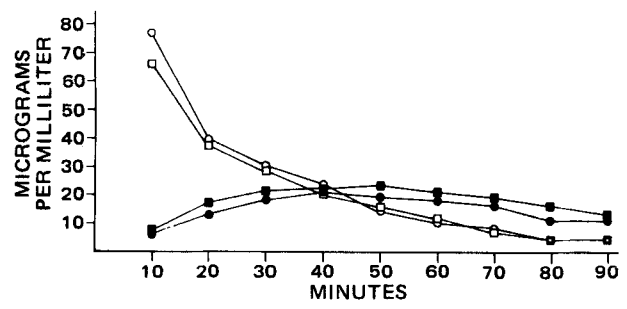

Figure 3—*Serum levels of I and II in a dog following intravenous administration of 10 mg of I/kg, as measured by HPLC and GLC. Key: ○, I (HPLC); □, I (GLC); ●, II (HPLC); and ■, II (GLC).*

50 μg of II/ml, showed averages of actual content measured in 36 samples to be 98.7 ± 4.2% (I) and 97.1 ± 4.1% (II).

Following intravenous administration of 10 mg of I/kg to a dog, serum levels of I and II, measured by this HPLC method and a GLC method (6), were compared (Fig. 3). The two methods differed by an average of 9% in the measurement of total drug-related material (I plus II) in the nine serum samples compared.

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NOTES

Frozen Conformers of Clotrimazole: Reaction of Imidazole with 1-Chloro-9-hydroxy-9-phenylxanthene

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Received March 1, 1979, from the *Department of Medicinal Chemistry, Westwood Pharmaceuticals Inc., Buffalo, NY 14213.* Accepted for publication May 3, 1979.

Abstract □ 1-Chloro-9-hydroxy-9-phenylxanthene reacts with imidazole at 180° to form a 5:1 mixture of the 9-(imidazo-1-yl)- and 9-(imidazo-2-yl)-1-chloro-9-phenylxanthenes. These products lack significant antifungal activity.

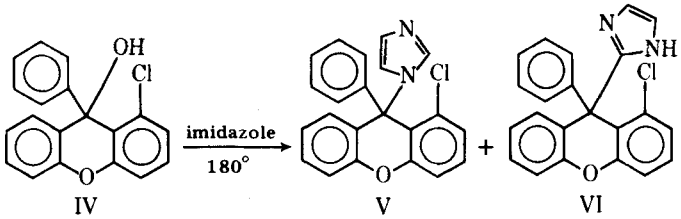
Keyphrases □ Clotrimazole—analogs, frozen conformers, imidazole substitution, antifungal activity □ Antifungal agents—clotrimazole analogs, frozen conformers, imidazole substitution, antifungal activity

The advent of clotrimazole (I) (1, 2) as a clinically useful, broad spectrum antifungal agent has elicited considerable research on the synthesis and testing of analogs of this molecule including the preparation of "frozen conformers." The fluorene analogs II and III were prepared and exhibited good antifungal activity (3).

DISCUSSION

Various other compounds in which the covalent bridge between two phenyl rings (as in II and III) is replaced by sulfur, oxygen, ethylene, or polymethylene have been reported (4–7). The usual synthetic method for these compounds has been displacement of the appropriate tertiary carbinol with thionyl bis-*N,N'*-imidazole (4, 5) or displacement of the appropriate tertiary chloride with imidazole by refluxing in benzene (3) or acetonitrile (6, 7). In all cases, the expected N_1-imidazole derivative was obtained with no mention of other isomers.

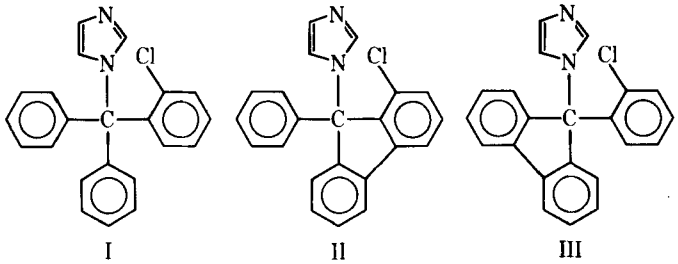
To elucidate the pharmacophoric conformation of clotrimazole, 1-chloro-9-(imidazo-1-yl)-9-phenylxanthene (V) was prepared. When 1-chloro-9-hydroxy-9-phenylxanthene (IV) was fused with imidazole at 180°, the expected V was isolated in a 46% yield along with a 9% yield of the unexpected 1-chloro-9-(imidazo-2-yl)-9-phenylxanthene (VI) (Scheme I).



Scheme I

Structures V and VI were assigned to the two reaction products based on their identical elemental analysis and their physical and spectral properties. Compound V displayed considerably greater mobility on silica gel in accord with its expected lower basicity. The IR spectrum of VI contained an absorption at 2850 cm^{-1} corresponding to the imidazole NH bond, which was absent from V. In the 100-MHz NMR spectrum, both V and VI showed a complex aromatic splitting pattern from δ 6.8 to 7.4. Compound V displayed a pair of overlapping triplets at δ 7.5 and 7.6 corresponding to two imidazole ring CH protons, each individually coupled to the two ring protons (the third proton apparently was concealed under the major aromatic peaks). Compound VI showed two distorted doublets at δ 6.4 and 7.55, each a single proton. These doublets sharpened on deuterium oxide exchange, indicating that a free NH was present but also concealed under the main aromatic peak.

Neither V nor VI displayed significant antifungal activity compared to clotrimazole (Table I).



EXPERIMENTAL[1]

Chemistry—*1-Chloro-9-hydroxy-9-phenylxanthene (IV)*—To a stirred suspension of 4.4 g (0.019 mole) of 1-chloroxanthone (8) in 100 ml of anhydrous ether was added dropwise, over 10 min, 22 ml (0.039

[1] NMR spectra were obtained on a Varian XL-100. IR spectra were taken on a Perkin-Elmer model 281 spectrophotometer. UV spectra were taken in absolute ethanol on a Beckman DB-G grating spectrophotometer. Elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn. Melting points were taken on a Mel-Temp melting-point apparatus and are uncorrected.

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