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Note

Quantitative thin-layer chromatographic determination of ticrynafen in plasma of the dog

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Ticrynafen (I) is a potent diuretic agent with uricosuric properties [1]. It is metabolized by two pathways as shown in Fig. 1 [2]. The first pathway gives a secondary alcohol (II) produced by reduction. This metabolite is then partially transformed into the corresponding methoxy derivative (III). The second metabolic pathway consists of oxidative cleavage at the level of the ketone function and produces a dicarboxylic acid (IV).

Ticrynafen and its metabolites have been determined in biological fluids by gas chromatographic (GC) methods [3, 4] and by measurement of the radioactivity of the ^{14}C-labelled drug in animals [2]. Also a thin-layer chromatographic (TLC) method for the determination of ticrynafen has been reported [5].

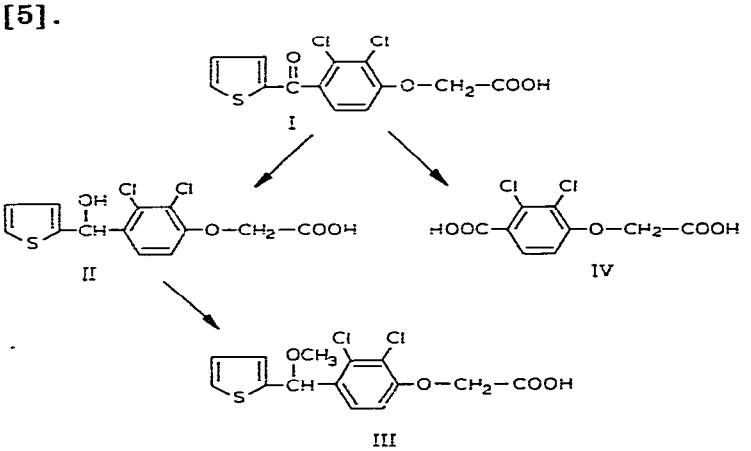

Fig. 1. Proposed pathway of the major metabolites of ticrynafen [2].

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This report describes a TLC method for the determination of tricrynafen in plasma of the dog. The method is characterized by a short analysis time, and good accuracy, sensitivity and specificity.

EXPERIMENTAL

Reagents and materials

Ticrynafen and its pharmaceutical preparation, Diflurex tablets, were obtained from Anphar (Chilly-Mazarin, France). The other chemicals were of analytical reagent grade (Merck, Darmstadt, G.F.R.). Commercial silica-gel K6F TLC plates 20 cm × 20 cm (Whatman, Clifton, NJ, U.S.A.), layer thickness 0.25 mm, were used. Solutions were spotted on to the TLC plates using micropipettes.

Apparatus

The shaker was an M4020 from Köttermann (Hanigsen, G.F.R.), the centrifuge a "Superspeed" from Sorvall (Newtown, CN, U.S.A.) and the block thermostat a Grant BT3 (Cambridge, Great Britain).

Preparation of plasma standards

Three plasma standards were extracted for every six unknown samples. First, 66.7 mg of ticrynafen were weighed out into a 100-ml volumetric flask and dissolved in 100 ml of methanol. Into 30-ml centrifuge tubes were placed 10, 50 and 75 μl of this solution. The solutions were then evaporated to dryness in a 50°C water-bath under a stream of nitrogen, whereupon 2 ml of control plasma were added to the residues. The three plasma standards prepared in this way contained 3.3, 16.7 and 25.0 μg/ml ticrynafen.

Extraction procedure

To 30-ml centrifuge tubes, each containing 2 ml of plasma sample or standard, were added 1 ml of 23% hydrochloric acid and 20.0 ml of chloroform. The tubes were stoppered with glass stoppers and shaken mechanically for 15 min at 80 cycles/min. After centrifuging for 5 min at 2611 *g*, 15.0 ml of the chloroform layers were transferred to 25-ml conical test-tubes and evaporated to dryness at 50°C in a block thermostat under a stream of nitrogen. The residues were dissolved in 100 μl of absolute methanol.

For TLC, 20 μl of the solutions were applied to the origin of the TLC plate. Nine samples (six unknown and three standards) were spotted on the same TLC plate. The TLC tank was lined with filter paper saturated with the solvent mixture ethyl acetate—acetic acid (95:5) and the system was allowed to equilibrate for 60 min. After placing the TLC plate into the tank the solvent front was allowed to migrate 16 cm from the origin (ca. 50 min). The developed plate was dried for 15 min in a stream of warm air, then heated in an oven for 10 min at 120°C. The spots were located under short-wavelength UV light.

Densitometry

The TLC plate was scanned at 20 mm/min in a direction perpendicular to the direction of development using a Shimadzu dual-wavelength TLC scanner,

Model CS-910, with dual-pen recorder (Philips, Model PM 8222), using the following operating conditions: photometric mode, dual wavelength, λ_s = 300 nm, λ_r = 400 nm; detection mode, reflection; measuring mode, absorbance; stage scanning mode, zigzag; working curve linearizer, channel 1. The speed of the recorder was 20 mm/min. The profile and integration curves were recorded for each spot on the TLC plate.

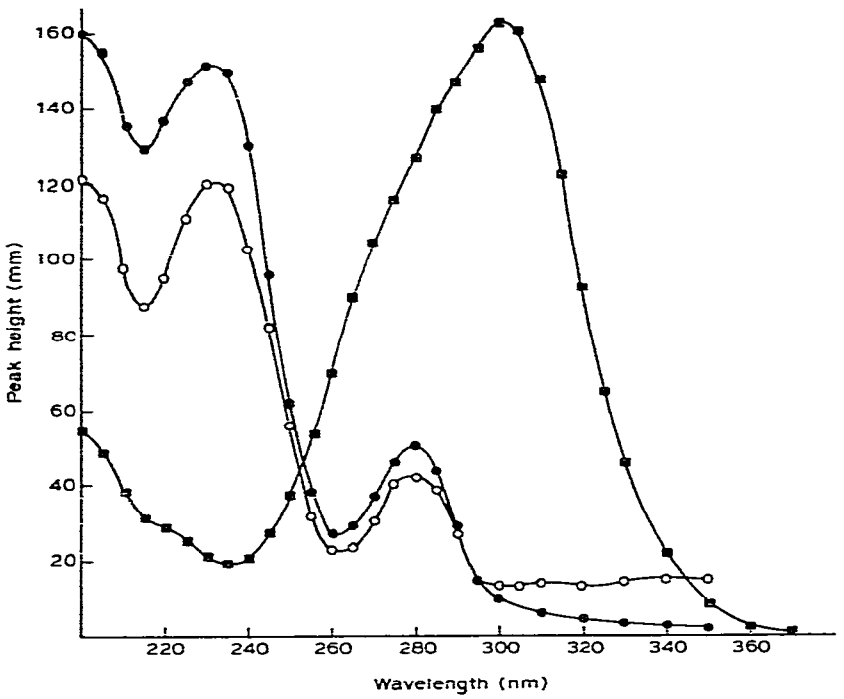

Fig. 2. Ultraviolet absorption spectra of ticrynafen (■), metabolite II (●) and metabolite III (○) obtained by scanning the TLC plate after separation of dog plasma extract.

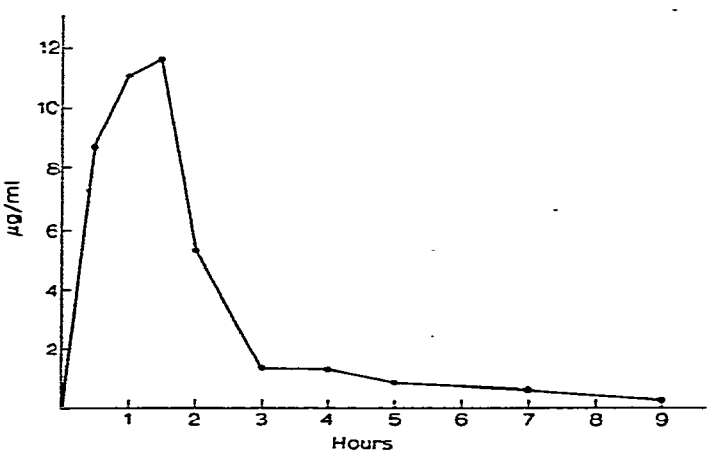

Fig. 3. Plasma level of unchanged drug after oral administration of 46 mg/kg ticrynafen to dog.

RESULTS AND DISCUSSION

The absorption spectrum for the ticrynafen spot was constructed by plotting absorbance at different wavelengths (Fig. 2). The maximum absorbance was at 300 nm. This wavelength was used for the sample side while 400 nm was used for the reference side.

The quantitatively lowest recordable amount is about 0.1 μg/spot (0.33 μg/ml of plasma). Linear responses were obtained up to 7.5 μg/spot.

The relative standard deviations for plasma samples from 3.3 to 25.0 μg/ml in a quintuplicate study on five TLC plates ranged from 3.6 to 13.6%,

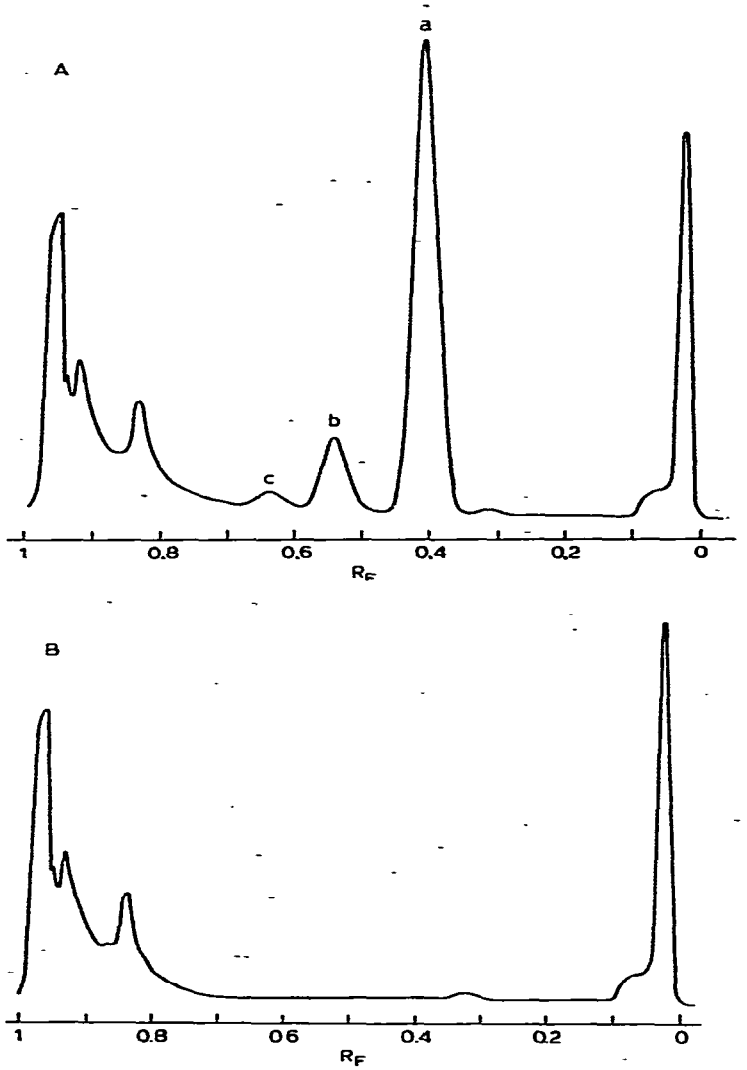

Fig. 4. Typical chromatograms obtained from dog plasma extracts by linear scanning of the TLC plate. (A) Plasma extract from dog treated with ticrynafen. Peaks: a = ticrynafen, b = metabolite II, c = metabolite III. (B) Control plasma.

indicating variabilities among the plates. However, the plasma standards had to be extracted and spotted along with the unknowns on each TLC plate.

Recoveries were 75.3 ± 6.3% (mean ± S.D.) for triplicate plasma standards at 3.3 μg/ml, and 82.3 ± 1.1% for triplicate plasma standards at 16.7 μg/ml.

The accuracy of the assay was tested by determining six unknown spiked plasma samples. The average percentage difference between the observed and the theoretical concentrations for plasma samples from 1.0 to 7.5 μg/spot was 5.2%, indicating a good accuracy.

The method was used for the measurement of plasma ticrynafen concentrations in dog. Prior to dosing the dog was fasted for 12 h. The peak plasma of the ticrynafen concentration, 11.7 μg/ml, occurred at 90 min (Fig. 3). These values agree with the ranges reported earlier [5].

In addition to the spot corresponding to ticrynafen, spots which might be metabolites II and III were also detected (Fig. 4); this assumption is based on data in the literature [2].

The assay of control plasma samples showed no background peak in the area of ticrynafen and metabolites. Using the solvent mixture ethyl acetate—acetic acid (95:5) a good separation of ticrynafen and metabolites was achieved, while the interferences of biological origin proved to be present at the solvent front as well as in the region of lower R_F values (Fig. 4).

Advantages of our method when compared with the TLC method [5] are rapid determination, higher sensitivity and the possible determination of metabolites. A major advantage over the gas chromatographic methods [3, 4] is the possibility of scanning the spots on the TLC plate directly in the UV range. Thus the absorption spectrum and comparison with the known absorption spectra for a positive identification could be achieved. Also extracted substances can be chromatographed without any derivatization.

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