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Note

Determination of prothionamide and its sulphoxide metabolite in physiological fluids by quantitative thin-layer chromatography*

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A specific method for the quantitative analysis of the antituberculoticum prothionamide and its metabolite prothionamide sulphoxide has not previously been available. Prothionamide and its sulphoxide metabolite have been determined quantitatively by UV photometry[1–3], *e.g.*, after separation by thin-layer chromatography (TLC)[4,5]. This paper describes a quantitative and sensitive method for the determination of prothionamide and its sulphoxide metabolite in physiological fluids using UV absorbance photometry on thin-layer plates. The method may be a useful technique in pharmacokinetic analysis.

EXPERIMENTAL

Chemicals

Prothionamide and prothionamide sulphoxide were gifts from Saarstickstoff-Fatol, Schiffweiler, G.F.R. All other substances were of analytical-reagent grade and obtained from Merck, Darmstadt, G.F.R. Glass plates (20 × 20 cm) coated with silica gel (60F-254, 0.5 mm layer; Merck) were used for chromatography.

Methods

Samples of 2 ml of plasma or urine were extracted with 6 ml of ethyl acetate by shaking for 5 min with a shaking apparatus, then centrifuged for 3 min at 1000 *g*. A 3-ml volume of the upper phase was evaporated to dryness at 94° using a Rotavapor (Type KRvr 65/45, Büchi, Flavil, Switzerland). The residues were re-dissolved in 20 μl of ethyl acetate and the solutions applied on the thin-layer plates, which were prepared by pre-chromatography with acetic acid–acetone–methanol–benzene (5:5:20:70) and dried at 60° for 10 min. Three samples (20 μl) and three standard solutions of prothionamide (3.125, 6.25 and 12.5 μg per 20 μl) were applied on a single thin-layer plate by the use of 20-μl capillary pipettes. The diameters of the spots did not exceed 5 mm. Ascending chromatography was performed (15 cm, 45 min) with acetic acid–acetone–methanol–benzene (5:5:20:70). The plates were dried at 60° for 10 min and scanned at 254 nm with a VIS-UV chromatogram analyzer (Farrand, New York,

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U.S.A.), using a 30 × 1 mm slit and a scanning speed of 1 in./min. The intensity of the light reflected from the spots was integrated by the VIS-UV chromatogram analyzer and expressed in arbitrary digital units (area units). The curves were recorded at a chart paper speed of 40 mm/min. The sensitivity was chosen to give a reading of about 90 for the most absorbing spot on the TLC plate.

Recovery experiments

Amounts of 1.56, 3.12, 6.25, 12.5 and 25 μg of prothionamide and its sulphoxide metabolite dissolved in 20 μl of methylideneglycerin (Sericosol N, Österreichische Stickstoff, Linz, Austria) were diluted with 2 ml of plasma and urine, respectively. The quench values (area units) were plotted against the corresponding concentrations of prothionamide and sulphoxide metabolite. The experiments were performed in sextuplicate for prothionamide, prothionamide sulphoxide and a mixture of both. These experiments were repeated four times within 5 months.

The fluorescence quenching of prothionamide and prothionamide sulphoxide was recorded in 10 samples of urine, the pH value of which varied from 4.0 to 9.5 after the addition of sodium hydroxide or hydrogen chloride to urine.

The stability of prothionamide and its sulphoxide metabolite in plasma and urine frozen at —20° in sealed tubes was also demonstrated.

Pharmacokinetic studies

The pharmacokinetics of prothionamide and prothionamide sulphoxide were investigated in the plasma and urine of three patients (one woman and two men, each without nephropathy or hepatopathy). Any medication was withdrawn 72 h before the 2-h infusion of 500 mg of prothionamide (Peteha Spritzampulle, Saarstickstoff-Fatol, diluted in 500 ml of a solution of 138.2 mequiv. of Na^+, 1.3 mequiv. of K^+, 1.8 mequiv. of Ca^{2+}, 140.1 mequiv. of Cl^- and 1.2 mequiv. of HCO_3^- per 1000 ml). Constant infusion was performed by the use of an infusion pump (Ivac Infusions-regler 201). Blood samples of 4 ml were collected hourly from the contralateral vena brachialis of the patients in heparinized plastic tubes and immediately centrifuged at 2700 *g* for 5 min. A 2-ml volume of the plasma was frozen and stored in sealed tubes at —20°. The urine was collected quantitatively in 2-h portions using a bladder catheter. The volumes of the samples were measured and the urine was stored at —20°. The plasma and urine were analyzed as described above.

RESULTS AND DISCUSSION

The TLC of prothionamide followed by UV photometry revealed a single peak at R_F 0.7 while the sulphoxide metabolite gave a peak at R_F 0.65. No impurities were detected at 254 nm in aqueous standard solutes. Fig. 1 shows an absorbance trace for a plasma sample with an unidentified metabolite and about 7.0 μg/ml of prothionamide sulphoxide and 12.6 μg/ml of prothionamide. The two peaks at R_F 0.65 and R_F 0.70 are well separated by zero absorbance over a range from 0.75 to 25 μg/ml in plasma. Prothionamide and prothionamide sulphoxide extracted with ethylacetate from plasma and urine yielded the expected peaks at R_F 0.7 and 0.65, respectively.

For quantification, peak areas of the standard solutions were plotted against the logarithm of the amounts of prothionamide and prothionamide sulphoxide. The calibration graphs were identical for both substances (Fig. 2). Linearity of the semilogarithmic plots was found from 0.75 to 25 μg of each substance. The relative

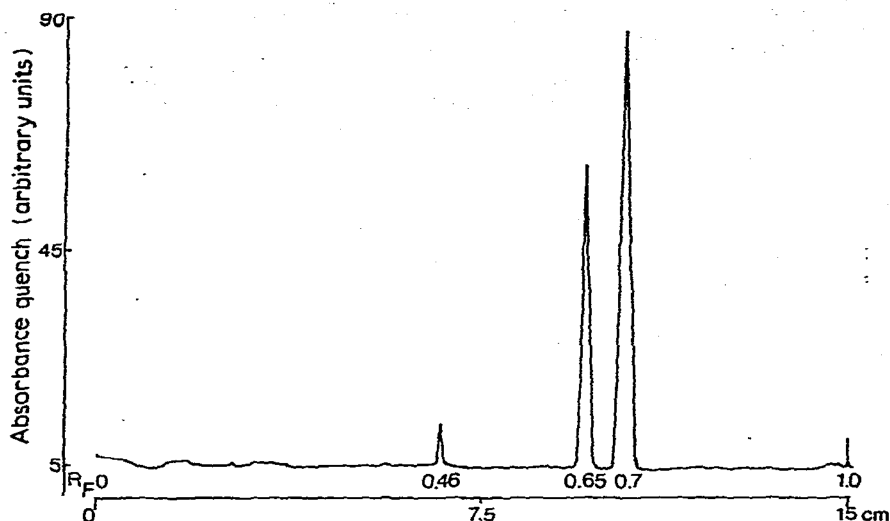

Fig. 1. UV scan (254 nm) in the analysis of a plasma sample. The sensitivity was chosen to give a reading of about 90 for the most absorbing spot on the TLC plate. Peaks: R_F 0.46 = unidentified metabolite; R_F 0.65 = prothionamide sulphoxide (about 7.0 μg/ml); R_F 0.70 = prothionamide (about 12.6 μg/ml).

standard deviation for both was less than 3.0% and the long-time reproducibility was within 4.5%; the standard deviation increased rapidly below 0.75 μg (s = 20% for 0.375 μg).

Other peaks, derived from components of the plasma, were found in the R_F range 0–0.25. With plasma, the R_F range from 0.25 to 0.95 showed only a single microheterogeneity at R_F 0.46. With urine, some impurities were found at the solvent front and some spots were recorded at R_F 0.4, 0.46, 0.53 and 0.58.

The recovery of prothionamide and prothionamide sulphoxide from both plasma and urine was about 98 ± 1.4% ($\bar{x} \pm s$, n = 82, 1.56–25 μg of each substance per millilitre of plasma or urine). The recovery from urine samples with various pH values was about 98 ± 1.2% ($\bar{x} \pm s$, n = 10, 20 μg of prothionamide and prothionamide sulphoxide per 2 ml). We found no correlation between the recovery from

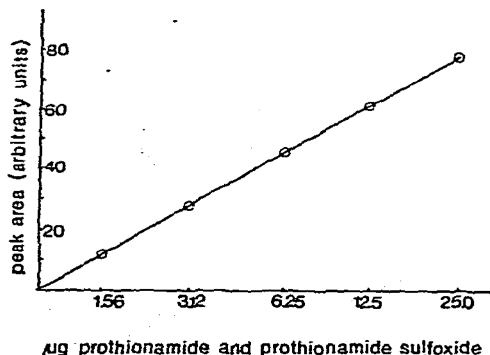

Fig. 2. Plot of peak area *versus* logarithm of amounts of prothionamide and prothionamide sulphoxide. The equation of the calibration graph was calculated by the method of least squares to be $y = 0.625\,x$ for both substances. $\bar{x} \pm s$ = 3.0 ± 4.5% (n = 36).

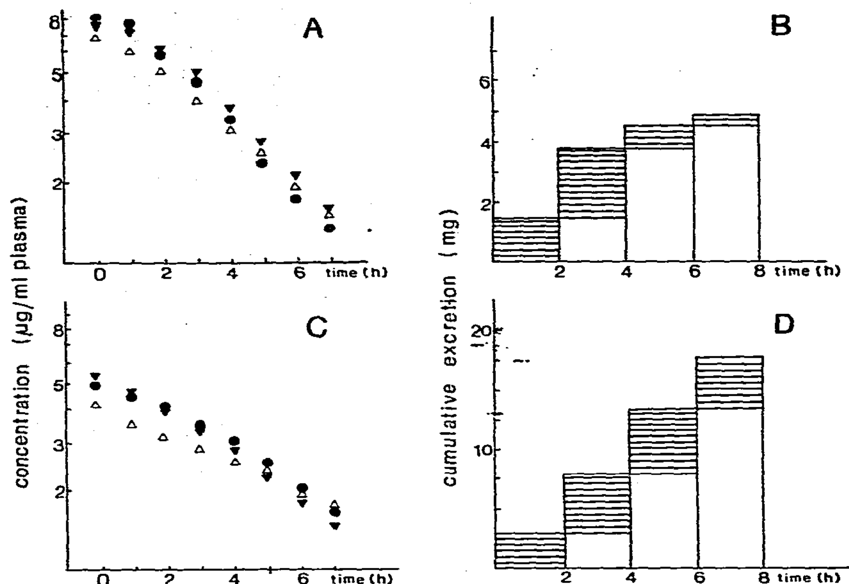

Fig. 3. Plasma levels and cumulative renal excretion of prothionamide (A) and prothionamide sulphoxide (C). Plasma levels were obtained from three patients (▼, △, ●), measured after a 2-h infusion of 500 mg of prothionamide. The cumulative renal excretion of prothionamide (B) for the first 8 h after starting the infusion was determined for one patient (△). The ordinate represents the amount of prothionamide (B) and prothionamide sulphoxide (D) excreted by the kidneys.

these samples and their pH.

Both substances were found to be completely stable for up to 12 months upon storage of frozen plasma or urine.

Therapeutic levels of prothionamide in plasma have been reported[1,4] to be about 3–12 µg/ml. With our method, these levels are measurable; we estimate the limit of detection using the method described here to be 0.75 µg.

Some preliminary results of our pharmacokinetic studies are shown in Fig. 3. Plasma levels of a therapeutic dose of prothionamide (Fig. 3A) and prothionamide sulphoxide (Fig. 3C) were followed for up to 7 h in three patients. Fig. 3B and 3D demonstrate the cumulative renal excretion of prothionamide and prothionamide sulphoxide for the first 8 h after starting the infusion.

Three determinations were performed on one thin-layer plate within 2 h. The pharmacokinetic data for one therepeutic experiment were obtained within 1 day.

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