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Note

High-performance liquid chromatographic determination of ethionamide and prothionamide in body fluids

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Ethionamide (2-ethyl thioisonicotinamide) was first used in the treatment of tuberculosis in the early fifties. Some ten years later the propyl analogue, prothionamide, was introduced since it was shown to be at least as active as ethionamide against *Mycobacterium tuberculosis* in vitro and against experimental tuberculosis in the mouse, but appeared to be better tolerated by patients. Recent experimental studies undertaken using the mouse foot-pad model have shown that both thioamides possess powerful antileprosy activity [1–3]. Both drugs display similar marked bactericidal action in the mouse and the minimal inhibition concentration (M.I.C.) of each drug against *M. leprae* was estimated to be about 0.05 μg/ml. These studies have encouraged considerable interest in the potential clinical use of ethionamide or prothionamide in combination with other established antileprosy drugs for the treatment of lepromatous leprosy.

Simple ultraviolet methods have been described [4, 5] that are capable of determining ethionamide and prothionamide concentrations of down to 0.3 μg/ml in 4—5 ml serum, while polarographic [6], quantitative thin-layer chromatographic [7] and gas—liquid chromatographic [2] methods have been reported for measuring concentrations of down to 0.2 μg/ml of the thioamides in 1 or 2 ml serum. However none of these methods is sufficiently sensitive to permit the measurement of serum levels approaching their M.I.C. against *M. leprae*. Such sensitivity is needed if the crucial pharmacological studies required to assess the role that ethionamide and prothionamide might eventual-

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ly play in the clinical treatment of leprosy are to be undertaken. Recently a radiochemical method of this sensitivity was described for determining serum concentrations of the two drugs in mice [2], but this method depends on the availability of radioactively-labelled ethionamide and prothionamide and is unsuitable for studies of their pharmacology in man.

In this paper, we describe a sensitive and specific high-performance liquid chromatographic (HPLC) method for the determination of ethionamide and prothionamide in plasma and urine and its application to pharmacokinetic studies of the two drugs in man.

EXPERIMENTAL

Chemicals

Ethionamide and prothionamide together with the ethionamide metabolite 2-ethyl-isonicotinamide were kindly donated by May and Baker (Dagenham, Great Britain) while the sulphoxide metabolites of the two drugs and 2-propyl-isonicotinamide were gifts from Dr. J.H. Peters and Professor J.K. Seydel. Stock solutions (1 mg/ml) of ethionamide and prothionamide were prepared by dissolving the drugs in methanol and could be stored at 4°C for many months without appreciable decomposition. The stock solutions were diluted with distilled water to give concentrations of either 15 or 60 μg/ml immediately prior to their use as internal standards, prothionamide being used as an internal standard for the determination of ethionamide and vice versa.

Collection of urine and plasma samples

In the first two studies after obtaining a pre-treatment urine sample, 500-mg doses of either ethionamide or prothionamide (Trescatyl and Trevintix, respectively; May and Baker) were ingested. Complete hourly urine collections were then obtained from 0—3 h, 2-hourly collections from 3—13 h while a pooled collection was made for the period from 13—24 h after dosage. In the third and fourth studies plasma samples were obtained pre-treatment, at hourly intervals up to 7 h, and 24 h after the ingestion of 500-mg doses of each drug, while urine collections were made from 0—0.5 h, at hourly intervals up to 7.5 h, from 7.5—23.5 h and from 23.5—24.5 h. The doses of ethionamide and prothionamide were taken on an empty stomach by a healthy volunteer (G.A.E.) weighing 65 kg. Each of the four doses was separated by an interval of at least a week. Urine samples were stored at —20°C and plasma samples over liquid nitrogen until analysis.

Extraction procedure

Aliquots (3 ml) of plasma/serum or urine were pipetted into stoppered centrifuge tubes together with 0.1 ml of a solution containing either 1.5 or 6 μg of the appropriate internal standard and extracted by shaking with 6 ml diethyl ether on a vortex mixer for 15 sec. After centrifugation, the organic phase was decanted and extracted with 1 ml 0.1 *M* hydrochloric acid. The acid extract was transferred to another centrifuge tube, 0.1 ml 1 *M* ammonium phosphate added and the pH adjusted to between 7 and 8 by the dropwise addition of 10% aqueous ammonia and the thioamides extracted by shaking with 2 ml ethyl acetate. The ethyl acetate extract was transferred to a 10-ml tapered test tube, evaporated to dryness at 50°C under nitrogen, the residue dissolved in 1 ml dichloromethane, transferred to a 2-ml tapered vial and the solvent removed under nitrogen. The dried residue could then be kept at 4°C prior to chromatography.

Liquid chromatography

Analyses were performed using a Waters Assoc. (Northwich, Great Britain) Model M6000A pump, a Cecil CE 212 variable wavelength UV detector (Cambridge, Great Britain) set at 295 nm and a Waters U6K septumless universal injector. A normal-phase system was used consisting of a Waters μPorasil silica column (30 cm × 3.9 mm I.D., particle size 10 μm), which was eluted with a degassed glass microfibre filtered (GF/F, Whatman, Maidstone, Great Britain) mobile phase of diethyl ether—methanol (96:4) delivered at a flow-rate of 1.3 ml/min (ca. 6.2 MPa). The column was periodically purged with acetonitrile (BDH, Poole, Great Britain) that had been dried with magnesium sulphate and stored over a 3-Å potassium alumino-silicate molecular sieve, and re-activated with chloroform that had been dried over calcium hydride. The dried plasma and urine extracts were dissolved in 100 μl of the mobile phase, duplicate 25-μl aliquots injected and the mean ratio of the peak heights for the drug to that of the internal standard calculated.

Calibration curves

Calibration curves designed to encompass the highest levels of the thioamides expected in body fluids after the ingestion of therapeutic doses of the two drugs were prepared by spiking blank urine and horse serum (Gibco Bio-cult, Glasgow, Great Britain) with ethionamide (or prothionamide) to give concentrations of 0, 0.5, 1, 2.5 and 5 μg/ml, respectively. Duplicate 3-ml aliquots were then extracted and chromatographed as described above after the addition of 6 μg of the interal standard (prothionamide or ethionamide, respectively). Standard curves to cover lower thioamide concentrations were prepared using aliquots of urine and serum spiked with a tenth of the above concentrations and 1.5 μg of the internal standard. Calibration curves relating mean peak height ratio of duplicate injections to concentration of thioamide were shown to be linear, and the best straight lines and standard errors of slopes and intercepts were calculated by the least-squares regression method. Overall recoveries of both thioamides, calculated by comparing the peak heights of extracted samples with those when the drugs were injected directly, were between 70 and 80%.

Selectivity

The selectivity of the method with respect to the most widely used anti-tuberculosis and antileprosy drugs and some of their principal metabolites was evaluated by applying the analytical procedure to solutions containing either 100 or 1000 μg/ml of the isonicotinamide and sulphoxide metabolites of ethionamide and prothionamide; clofazimine, dapsone, ethambutol, isoniazid and its metabolites acetylisoniazid and isonicotinic acid, *p*-aminosalicylic acid, pyrazinamide, rifampicin, streptomycin and thiacetazone in water or aqueous ethanol (9:1).

RESULTS

Analytical procedure

A representative chromatogram of the extract from the plasma sample obtained 2 h after the ingestion of 500 mg ethionamide is shown in Fig. 1. For this analysis 6 μg prothionamide was added as the internal standard. The retention times of prothionamide and ethionamide were 4.3 and 4.8 min respectively, giving near baseline separation with a resolution factor (R_S) of 1.21. For peaks with a resolution factor of greater than 1.0, peak height measurements should be accurate to within 3% [8]. Accordingly the concentrations of ethionamide or prothionamide in the samples were calculated from the peak-height ratio of the drug to that of the internal standard. The equations of the series of linear calibration curves for both drugs covering the two concentration ranges in urine and plasma/serum are given in Table I together with the standard errors of the slopes and intercepts. None of the intercepts was significantly different from zero and, as might have been expected, the slopes of the lines were inversely proportional to the amount of internal standard added. Replicate errors were similar for both drugs and averaged 2.2% and 3.6% for the higher and lower concentration ranges, respectively, whether determinations were carried out in serum or urine.

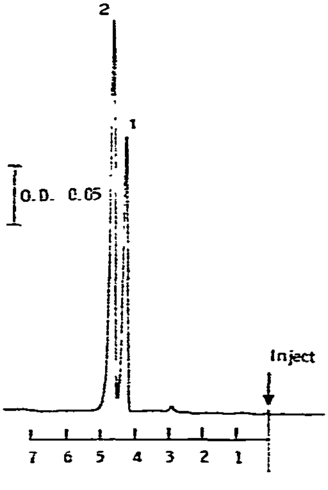

Fig. 1. Chromatogram of an extract of plasma from a volunteer 2 h after the ingestion of 500 mg ethionamide. Peaks: (1) prothionamide (the internal standard); and (2) ethionamide.

The response of the detector to both drugs was linear over a 500-fold range and injections of less than 5 ng could be quantitated giving a practical limit of detection of about 0.01 μg/ml of each thioamide in urine or plasma. Of the antituberculosis and antileprosy drugs and their metabolites that were tested, only dapsone and pyrazinamide interfered. Dapsone, whose retention time was similar to that of ethionamide, contributed about 50% to the method on a weight for weight basis, while pyrazinamide, which eluted with prothionamide, contributed to the extent of about 7%. Concomitant treatment with these

TABLE I

EQUATIONS OF CALIBRATION CURVES

Equation $y = mx + c$ where y is the ratio of the peak height of the drug to that of the internal standard, m the slope, x the concentration of ethionamide or prothionamide and c the intercept.

Drug	Biological fluid	Concentration range (μg/ml)	Slope ± S.E.*	Intercept ± S.E.
Ethionamide	Urine	0.5—5.0	0.435 ± 0.003	0.007 ± 0.012
	Urine	0.05—0.5	1.843 ± 0.009	0.015 ± 0.017
	Serum	0.5—5.0	0.450 ± 0.005	0.043 ± 0.026
	Serum	0.05—0.5	1.827 ± 0.049	0.018 ± 0.011
Prothionamide	Urine	0.5—5.0	0.526 ± 0.00[illegible]	0.015 ± 0.006
	Urine	0.05—0.5	2.069 ± 0.074	0.064 ± 0.038
	Serum	0.5—5.0	0.523 ± 0.003	0.014 ± 0.009
	Serum	0.05—0.5	2.019 ± 0.031	0.014 ± 0.008

*Standard error.

drugs should therefore be avoided when determining ethionamide or prothionamide by this method.

Ethionamide and prothionamide plasma concentrations and urinary excretion after oral dosage in man

The plasma and urinary concentrations of ethionamide and prothionamide after the ingestion of 500-mg doses of each drug are illustrated in Figs. 2 and 3, respectively. The urinary concentrations of ethionamide or prothionamide correlated closely with the concomitant plasma concentrations of the two drugs ($r = 0.94$ for ethionamide from 2—7 h, and 0.99 for prothionamide from

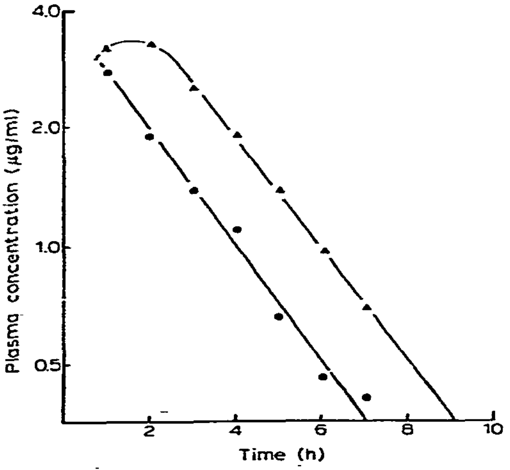

Fig. 2. Plasma concentrations of thioamides after oral dosage with 500 mg ethionamide (▲) or prothionamide (●).

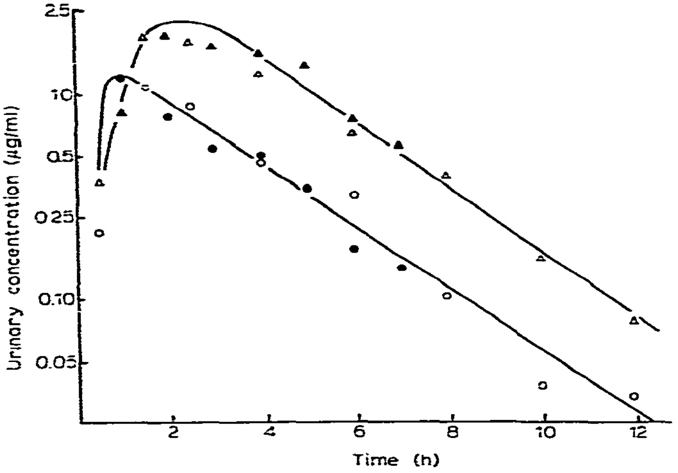

Fig. 3. Urinary concentrations of thioamides after oral dosage with 500 mg ethionamide (△ study I, ▲ study III) or prothionamide (○ study II, ● study IV).

1—7 h). During these time periods the ratio of urinary to plasma concentration averaged 0.75 for ethionamide and 0.42 for prothionamide. The urinary data for the pairs of studies on each drug have been combined in Fig. 3 since the results obtained on each occasion did not differ significantly. Both drugs were rapidly absorbed and maximal plasma and urinary concentrations were achieved within 2 h. Thereafter concentrations of both drugs in each body fluid fell exponentially at rates equivalent to half-lives of 2.23 ± 0.05 and 1.91 ± 0.06 h for ethionamide in plasma and urine, and 2.03 ± 0.07 and 1.94 ± 0.08 h for prothionamide. Although the peak plasma concentrations of both drugs were similar, from 2 h, ethionamide levels were nearly double those of prothionamide. By 24 h, the plasma concentrations of the two drugs had fallen to 0.06 and 0.03 μg/ml, respectively. The cumulative urinary excretion of ethionamide and prothionamide in the two pairs of studies were equivalent on average to 0.15% and 0.05%, respectively, of the administered doses.

DISCUSSION

The HPLC method described for the determination of ethionamide and prothionamide in plasma and urine is far more specific than the ultraviolet methods that were originally devised for their quantitation [4, 5] and some 40 times more sensitive. It is approximately ten times more sensitive than the polarographic [6], quantitative thin-layer chromatographic [7] and gas—liquid chromatographic [2] methods described for estimating the two drugs. Its sensitivity is similar to that of the method reported by Davidson and Smyth [9] using cathodic stripping voltammetry, but it is almost certainly more specific. As well as being sensitive and specific, this HPLC method is reasonably rapid and robust. Thus the average sample time is about 20 min, including extraction and chromatography, while the use of the internal standards that are closely related to the drug being measured and possess similar partition coefficients in the extraction systems employed, means that strict control over the volumes of organic and aqueous extracts recovered at each stage of the extraction procedure is unnecessary.

The ethionamide plasma concentrations determined after oral dosage with 500 mg of the drug (Fig. 2) confirm evidence from previous investigations utilising less sensitive polarographic or ultraviolet analytical methods that indicated that one might expect peak plasma/serum concentrations of about 3 μg/ml to be achieved within about 2 h of giving such a dose followed by an exponential decline equivalent to a half-life of about 2 h [10—12]. The pharmacokinetic studies have also shown that the rates of elimination of prothionamide and ethionamide from the body are virtually identical while the increased sensitivity of the HPLC method has enabled 24-h ethionamide and prothionamide plasma concentrations to be determined for the first time.

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