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Note

High-performance liquid chromatographic determination of thiacetazone in body fluids

P.J. JENNER

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (Great Britain)

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Thiacetazone (TB1, thioacetazone, *p*-acetylaminobenzaldehyde-thiosemicarbazone) is the only oral companion drug to isoniazid that is commonly available for the treatment of tuberculosis in most Third World countries. Although TB1 was shown to have significant antileprosy activity as long ago as 1954 [1], it has been little used, primarily because when given as monotherapy the appearance of TB1-resistant *Mycobacterium leprae* caused many patients to relapse. However, in view of the problems posed by the wide-spread emergence of dapsone-resistant leprosy bacilli, there has been interest recently in the possibility of using TB1 as a cheap companion drug in the multi-drug treatment of lepromatous leprosy [2, 3]. TB1's minimal inhibitory concentration against *M. leprae*, calculated from experimental studies in the mouse footpad model, is approximately 0.2 μg/ml [2, 4]. Its antileprosy activity, like that against *M. tuberculosis*, is purely bacteriostatic [2, 4].

Since TB1 has been shown to be irregularly self-administered by both leprosy and tuberculosis patients [5, 6], it is probable that poor compliance may seriously limit its therapeutic efficacy when it is used as out-patient treatment of both diseases. To assess the likely importance of compliance, it is essential to determine the period for which inhibitory concentrations are maintained after the ingestion of standard daily doses of the drug. Few studies of the human pharmacology of TB1 have, however, been conducted, primarily because of the lack of sufficiently sensitive and specific methods to accurately measure blood levels of the drug. Methods based on the acid hydrolysis of TB1 followed by colorimetric determination of liberated *p*-aminobenzaldehyde by the Bratton and Marshall procedure [7—10] were sensitive to no better than 0.5 μg/ml and inevitably lacked specificity. More satisfactory UV and fluorimetric

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methods have been described but they are only capable of estimating concentrations of down to about 0.3 μg/ml TB1. Although peak TB1 serum concentrations after daily dosage with 150 mg of the drug could be measured with reasonable confidence by these methods, estimates of trough (24 h) concentrations were imprecise indicating the need for improved analytical methods for its estimation [11, 12].

This paper describes a sensitive and selective high-performance liquid chromatographic (HPLC) method for determining TB1 in plasma and urine. A preliminary study of the pharmacokinetics of TB1 in man using the method is also reported.

EXPERIMENTAL

Chemicals

All solvents and chemicals were of analytical grade. Distilled water of high purity was from a Fi-Stream glass still (Fisons, Loughborough, Great Britain). TB1 was donated by Smith and Nephew (Harlow, Great Britain) and was recrystallised thrice from ethanol (m.p. 227–231°C).

4-Propionylaminobenzaldehyde-thiosemicarbazone (PBT), the propionyl analogue of TB1, was synthesised for use as the internal standard. For this purpose *p*-propionylaminobenzaldehyde was first prepared following a procedure described for *p*-acetylaminobenzaldehyde [13]. Sodium sulphide nonahydrate (3 g) from a freshly opened bottle, 1.5 g flowers of sulphur and 2.6 g sodium hydroxide were dissolved by heating with 60 ml water. The solution was transferred to a round-bottomed flask containing a hot solution of *p*-nitrotoluene (5 g) in 30 ml ethanol and the mixture refluxed for 3 h. The product was rapidly steam distilled to leave about 50 ml of a reddish-coloured residue, which on cooling deposited *p*-aminobenzaldehyde as a mass of golden yellow crystals. These were rapidly filtered and immediately dissolved in 5 ml boiling propionic anhydride. After adding 5 ml water and boiling to reduce the volume, the solution was cooled in ice. The dark purple crystals obtained were purified by forming an adduct on boiling with 30% aqueous sodium bisulphite and then decomposed with 2 *M* sodium hydroxide to give a dense white precipitate. The *p*-propionylaminobenzaldehyde was filtered off, washed with water, dissolved in 75% aqueous ethanol and reacted by adding excess thiosemicarbazide in hot aqueous acetic acid. After cooling PBT precipitated out as a white solid which was washed with water and twice recrystallised from ethanol (m.p. 219—221°C). Its purity was confirmed by thin-layer chromatography on silica gel with ethyl acetate as the solvent and by HPLC. Stock solutions (1 mg/ml) of TB1 and PBT were prepared by dissolving the drugs in methanol and could be stored at 4°C for many months without appreciable decomposition. The stock solution of PBT was diluted with distilled water to give concentrations of either 15 or 60 μg/ml immediately prior to use as the internal standard.

Collection of urine, plasma and faecal samples and initial thiacetazone estimations

A preliminary study of the pharmacokinetics of TB1 was undertaken with

the help of a healthy male volunteer (G.A.E.) weighing 65 kg who ingested six consecutive daily doses of 150 mg of the drug on an empty stomach. Plasma samples were obtained immediately before and 2, 4, 6, 24 and 72 h after the final dose was swallowed. Complete pooled urine collections were made for each of the first four days and from 0—23 h on the fifth day. Two-hour collections were then made from 1 h before the last TB1 dose was ingested until 11 h afterwards. Further collections were then made from 11—23 h, 23—25 h, 25—47 h, 47—49 h, 49—71 h and 71—73 h, followed by 2-h collections at 24-h intervals from 95—97 h to 239—241 h, that is ten days after the ingestion of the final TB1 dose. Complete faecal collections were made up to ten days after the final TB1 dose. To arrive at suitable dilutions for the HPLC assays and to obtain evidence concerning the specificity of the formerly employed UV method [12], urinary concentrations of TB1 were first estimated by extracting 3-ml samples in a small separating funnel with 8 ml chloroform—propan-2-ol (4:1) after the addition of 1 ml of 1 *M* K_2HPO_4, drying the lower phase by filtration through anhydrous sodium sulphate and measuring the UV absorption at 333 nm.

Extraction procedure

Aliquots (3 ml) of plasma or appropriately diluted urine samples were pipetted into stoppered centrifuge tubes together with 0.1 ml of an aqueous solution containing 1.5 or 6 μg of PBT and 1 ml of 1 *M* phosphate buffer, pH 7.0, and extracted by shaking with 6 ml ethyl acetate on a vortex mixer for 15 sec. After centrifugation, the organic phase was decanted and washed by shaking with 0.5 ml of 0.1 *M* sodium hydroxide. Daily faecal collections were extracted by homogenisation with acetone (400 ml). After centrifugation and filtration, the extracts were diluted 50-fold with water, a 3-ml sample was taken and 6 μg PBT added. The extraction was carried out as above but with two washes of 0.5 ml of 0.1 *M* sodium hydroxide. The washed extracts were then transferred to a 10-ml tapered test tube and evaporated to dryness at 50°C under nitrogen. The dried residues 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 detector (Cambridge, Great Britain) set at 328 nm and a Waters U6K septumless universal injector. A reversed-phase system was used consisting of a Waters μBondapak column (30 cm × 4 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 acetonitrile (Cambrian Chemicals, Croydon, Great Britain)—water (3:7) delivered at a flow-rate of 1.5 ml/min (ca. 12 MPa). The column was protected with a guard column (5 cm × 0.5 mm I.D.) containing dry-packed pellicular reversed-phase material (CO:Pell ODS, Whatman Reeve Angel, Maidstone, Great Britain). The dried 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 TB1 to that of the internal standard was calculated. The method was modified for the estimation of TB1 plasma concen-

trations to minimize the injection of extractable lipophilic components that might otherwise result in a rapid deterioration of the analytical column. Dried plasma residues were extracted by shaking with 100 μl of the mobile phase together with 100 μl of 2% ethanol in *n*-hexane. After centrifugation, 25-μl aliquots of the lower aqueous phase were then injected onto the column. Dried faecal extracts were treated in the same way.

Calibration curves

A calibration curve for estimating the maximal plasma TB1 concentrations expected after the ingestion of therapeutic doses of the drug was prepared by spiking blank plasma with TB1 to give concentrations of 0, 0.2, 0.5, 1 and 2 μg/ml. A similar calibration curve was prepared employing these concentrations of TB1 in normal urine, while a calibration curve was prepared with TB1 plasma concentrations of 0, 0.05, 0.1, 0.2 and 0.5 μg/ml, in order to determine levels near to its minimal inhibitory concentration against *M. leprae*. Duplicate 3-ml aliquots were then extracted and chromatographed as described above after the addition of the appropriate amount of PBT (6 μg or 1.5 μg). Calibration curves relating mean peak height ratio of duplicate injections to concentration to TB1 were shown to be linear, and the best straight lines and standard errors of slopes and intercepts were calculated by the least-squares method.

Selectivity

The selectivity of the method with respect to the most widely used antituberculosis and antileprosy drugs was evaluated by applying the analytical procedure to solutions containing 100 μg/ml ethambutol, isoniazid, *p*-aminosalicylic acid, pyrazinamide, rifampicin, streptomycin, clofazimine, dapsone, ethionamide and prothionamide in water or aqueous ethanol (9:1).

RESULTS

Analytical procedure

Representative chromatograms of urine and plasma extracts of samples obtained 72 h after the ingestion of the final 150-mg dose of TB1 are shown in Fig. 1a and b. Pretreatment urine and plasma samples contained no interfering peaks (Fig. 1c and d). For these analyses PBT was added as internal standard (6, 1.5 or 0.3 μg as appropriate). The retention times of TB1 and PBT were 3.2 and 4.1 min, respectively, giving baseline separation with a resolution factor (R_s) of 3.2. The TB1 concentrations were calculated from the peak height ratios of TB1 to that of the internal standard (PBT) and by reference to the equations of the calibration curves for the estimation of the drug in plasma and urine. These are given in Table I together with the standard errors of their 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 averaged about 1.5% at the higher concentrations and increased to about 7% when concentrations of 0.05–0.1 μg/ml TB1 were being determined in plasma. The overall recovery of TB1 in the extraction procedure was in excess of 90%.

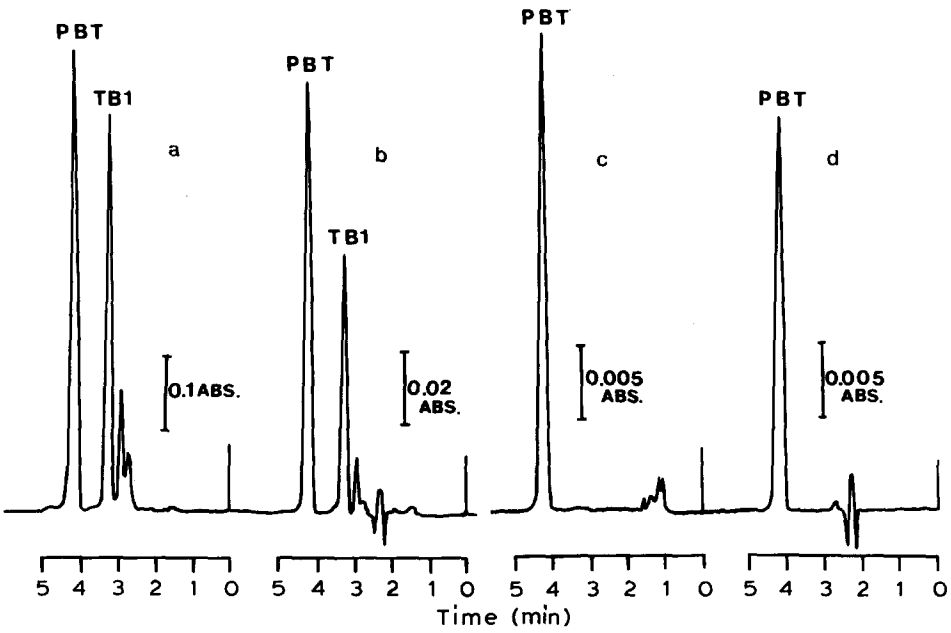

Fig. 1. (a) Chromatogram of an extract of a urine sample from a volunteer obtained 71—73 h after the ingestion of the final (sixth) daily dose of 150 mg TB1; 6 μg PBT was added as internal standard. (b) Chromatogram of an extract of a plasma sample obtained 72 h after the ingestion of the final daily dose of 150 mg TB1; 1.5 μg PBT was added as internal standard. (c) Chromatogram of an extract of a pretreatment urine sample; 0.3 μg PBT had been added to the sample. (d) Chromatogram of an extract of a pretreatment plasma sample; 0.3 μg PBT had been added as internal standard.

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 TB1, and c the intercept.

Biological fluid	Concentration range (μg/ml)	Slope ± S.E.*	Intercept ± S.E.
Plasma	0.05—0.5	2.704 ± 0.024	0.014 ± 0.006
Plasma	0.2 —2.0	0.672 ± 0.005	0.006 ± 0.006
Urine	0.2 —2.0	0.684 ± 0.002	0.002 ± 0.002

*Standard error.

Detector response was linear over a wide range, direct injections of as little as 10 ng TB1 being easily measured. Calculations based on a signal-to-noise ratio of 3 indicated that the method could probably measure TB1 concentrations of down to about 3 ng/ml. None of the antituberculosis or antileprosy drugs tested was found to interfere with the method.

The urinary concentrations of TB1 estimated by the previous UV method [12] were consistently higher than those estimated by HPLC. While the ratios

of the two sets of measurements remained virtually constant (1.25 ± 0.08) during the first eight days of the pharmacokinetic study (up to three days after the administration of the final dose), they increased to about 2.2 from days 10—12. This finding indicated that extractable urinary metabolites of the drug had been estimated by the UV method, and two such metabolites were clearly visible in the HPLC scans with retention times of 2.7 and 2.9 min, respectively. Furthermore the ratio of their peak heights to that of TB1 remained virtually constant during the first eight days (0.12 ± 0.02 and 0.15 ± 0.04, respectively) but then increased to about 0.2 and 0.5 from days 10—12. Their retention times indicated that they were slightly more polar than TB1, but their nature is unknown. A previous study [12] showed that significant amounts of the potential metabolite *p*-aminobenzaldehyde-thiosemicarbazone (retention time 3.3 min) are not excreted after dosage with TB1. Another possible metabolite, *p*-acetylaminobenzaldehyde has a retention time of 3.7 min in the system used and was not found. This accords with the previous failure to detect its acid derivative, *p*-acetylaminobenzoic acid as a terminal metabolite of the drug [12].

Thiacetazone plasma concentrations, urinary and faecal excretion after oral dosage in man

The plasma concentrations and urinary excretion of TB1 are illustrated in Fig. 2. Immediately before the final 150-mg dose of TB1 was ingested, the plasma concentration of the drug was 0.64 μg/ml. Peak plasma TB1 concentrations were achieved after about 4 h (1.2 μg/ml) and thereafter fell to 0.62 μg/ml by 24 h and 0.22 μg/ml by 72 h. There was a strong suggestion that the elimination of the drug was biphasic with apparent half-lives for the decline from 6—24 h and 24—48 h of about 22 h and 33 h, respectively. The sensitivity

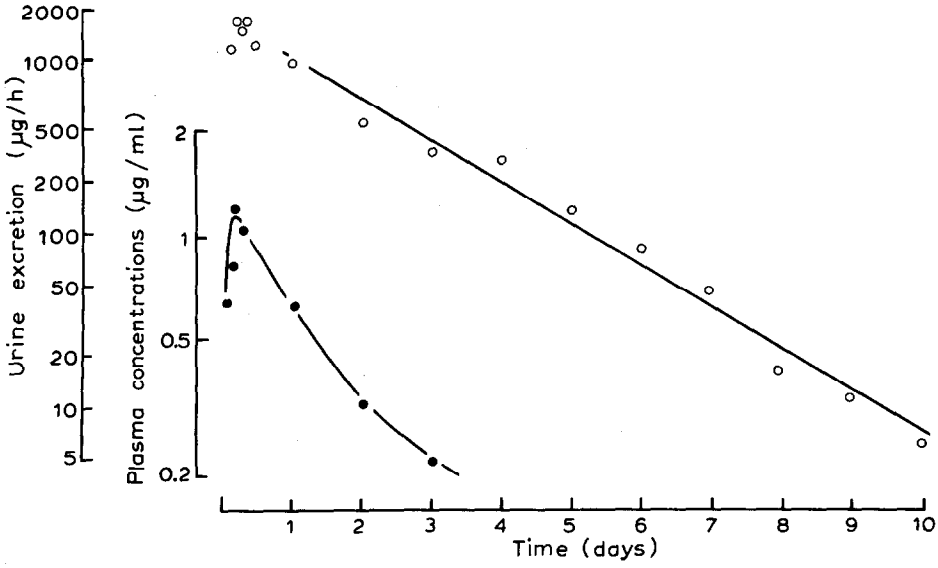

Fig. 2. Urinary excretion (○) and plasma concentration (●) of TB1 after the ingestion of the final (sixth) daily dose of 150 mg TB1.

of the HPLC method, together with the slow rate of elimination of TB1, was such that the urinary excretion could be accurately determined for at least ten days (Fig. 2). Over this period the urinary excretion of TB1 fell at a rate equivalent to a half-life of 29.8 ± 1.5 h. The urinary excretion rates correlated closely with the concomitant TB1 plasma concentrations ($r = 0.993$, $p < 0.001$) and gave a calculated renal clearance of 23 ml/min. The total cumulative excretion of unchanged TB1 in the urine over the whole period was equivalent to 15.4% of the dose. Significant amounts of TB1 were eliminated in the faeces up to six days after the ingestion of the final oral dose and totalled 28 mg or 3.1% of the administered doses.

DISCUSSION

The HPLC method described for the determination of TB1 in plasma and urine is sensitive, selective, precise and simple. Its sensitivity is about 50 times that of previous UV, colorimetric and fluorimetric procedures [7—12]. Furthermore, parallel estimations of urinary TB1 concentrations by a UV method which had been previously shown to be more specific than alternative colorimetric and fluorimetric procedures [12], provided evidence of the improved selectivity of the HPLC method. This was confirmed by the demonstration of extractable UV-absorbing urinary metabolites of TB1 in the HPLC traces. The accuracy of the HPLC method was such that replicate errors in the concentration ranges expected in the plasma of patients receiving standard treatment with the drug were equivalent to about ± 0.015 μg/ml TB1 in contrast to errors of ± 0.15 to 0.3 μg/ml encountered when fluorimetric or UV methods are used [12]. An additional advantage of the HPLC method is its rapidity, extraction and chromatography taking only about 15 min per sample.

The small proportion of the TB1 dose (3.1%) eliminated unchanged in the faeces confirms the previous conclusion, based on urinary excretion of the drug when single doses of 150—600 mg were ingested, that TB1 is well absorbed in man [12]. The peak and trough TB1 plasma concentrations determined in the volunteer after the administration of six consecutive daily doses of 150 mg of the drug were similar to those determined previously using UV and fluorimetric methods among tuberculosis patients from Kenya and Singapore [12].

The results of this preliminary pharmacokinetic study emphasize the inherent weakness of TB1, an essentially bacteriostatic drug, for potential use in the combined treatment of lepromatous leprosy [2—5]. Thus after the administration of standard daily doses of TB1, peak plasma concentrations of the drug only exceeded its minimal inhibitory concentration against *M. leprae* by about 6-fold and such inhibitory concentrations were only maintained for about three days after the final dose was ingested. Other more extensive studies of the blood levels and urinary excretion of TB1 in groups of healthy volunteers and tuberculosis patients using the HPLC method described in this paper will be reported elsewhere [14].

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