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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PYRAZINAMIDE IN CEREBROSPINAL FLUID AND PLASMA IN THE RABBIT

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SUMMARY

A simple procedure for the determination of pyrazinamide in plasma and cerebrospinal fluid in the rabbit is described. The assay involves a preliminary extraction of the drug and an internal standard, paracetamol, from the acidified sample (pH 4.2). The extract is evaporated to dryness at 45°C and the residue is redissolved in methanol (50 μl). A 25-μl aliquot is injected into the liquid chromatograph and eluted with acetonitrile—10 m*M* phosphate buffer of pH 3.5 (10:90, v/v) on a 30-μm C_8 pre-column linked to a 5-μm C_8 reversed-phase column at ambient temperature (25 ± 1°C). The eluate is detected at 215 nm. The method has been used to investigate the disposition of pyrazinamide in plasma and cerebrospinal fluid in six rabbits.

INTRODUCTION

Pyrazinamide (PZA), pyrazine-carboxyamide, is a tuberculostatic agent that is often used in combinations with rifampicin and other antituberculous (anti-TB) drugs for the treatment of tuberculosis (TB). TB is most serious when the meninges and brain are involved, as irreversible brain damage may result [1]. Rational chemotherapy of TB infections of the central nervous system is restricted by grossly limited data on the penetration of anti-TB drugs into the brain [2]. To achieve adequate minimum inhibitory concentrations (MIC) or minimum bactericidal concentrations (MBC) in the cerebrospinal fluid (CSF), larger doses than those for treating systemic TB infections are often used. As unwanted toxic effects, both neurotoxicity and hepatotoxicity, are mostly dose-related, larger doses will produce an increase in these adverse effects [3].

A knowledge of the concentration of the drug in the CSF and plasma during chronic chemotherapy is desirable for adjusting dosage regimen with reference

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to the MIC and/or MBC of anti-TB drugs. Previously published methods on the determination of pyrazinamide involve tedious extraction followed by colour formation, which is measured spectroscopically [4]. Recently, high-performance liquid chromatographic (HPLC) methods have been reported for measuring rifampicin [5], isoniazid and metabolites [6] in plasma samples. The present paper describes a HPLC assay for pyrazinamide in CSF and plasma samples. The method has been used to study the disposition of the drug in the rabbit after oral administration.

EXPERIMENTAL

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of analytical-reagent grade (Merck, Darmstadt, F.R.G.), were freshly distilled before use; 2 *M* hydrochloric acid; 10 m*M* phosphate buffer at pH 3.5; water was double-distilled in a glass apparatus; paracetamol BP; pyrazinamide (Sigma, St. Louis, MO, U.S.A.); 2-pyrazine-carboxylic acid (Aldrich, Gillingham, U.K.).

Apparatus

The liquid chromatograph consisted of a Waters 6000A pump, a U6K injector with a 25-μl loop (Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength Hitachi 220-S UV detector with a chart recorder (Hitachi, Tokyo, Japan). Analyses were performed on a reversed-phase C_8 column (Hibar, LiChroCart RP-8, 250 mm × 4.6 mm I.D., 5 μm, Merck) linked to a C_8 pre-column (10 μm, 50 mm × 4.6 mm I.D., Merck). The operating conditions for the HPLC system were: a mobile phase of a freshly prepared mixture of acetonitrile and 10 m*M* phosphate buffer at pH 3.5 (1:9, v/v); flow-rate 1.5 ml/min; temperature, ambient (25 ± 1°C); UV detector wavelength, 215 nm; sensitivity scale, 0—0.01 a.u.f.s.

Other apparatus included 10-ml and 15-ml centrifuge tubes with well fitting screw caps (Sovirel, Levallois-Perret, France), and 15-ml stoppered evaporation tubes with finely tapered bases (50-μl capacity). All glassware was cleaned by soaking overnight in a 5% solution of Extran (Merck) in water, then rinsed thoroughly with methanol and hot tap-water followed by distilled water. These tubes were subsequently silanized by rinsing with a 1% solution of Prosil-28 silanizing agent (PRC, Gainesville, FL, U.S.A.) followed by rinsing with distilled water and dried at 150°C overnight. This treatment of glassware was necessary to eliminate possible loss of drug owing to adsorption onto the glass walls [7]. Hamilton 10- and 25-μl syringes were used.

Preparation of reagents and standards

Standard solutions calculated as mg of drug per ml in distilled methanol were made of paracetamol (internal standard), pyrazinamide and 2-pyrazine-carboxylic acid and diluted to cover the calibration range for PZA of 0—40 μg ml^{-1}. Dilutions were made up in drug-free plasma and CSF (final volume, 0.2 ml) and to each were added 8 μl (12.5 μg ml^{-1}) of paracetamol as internal standard.

The mobile phase was filtered before use through a Millipore filter type AA (pore size, 0.5 μm; Waters Assoc.). Further degassing was not found to be necessary immediately after filtration.

Extraction of PZA from CSF and plasma samples

Preliminary studies on direct injection of supernatants, after precipitation of proteins in CSF or plasma samples, into the HPLC system indicated that interfering peaks that occurred in chromatograms would mask analytical peaks of PZA. Hence, organic solvent extraction was necessary for subsequent analysis. PZA can be extracted into organic solvent from aqueous acidic or alkaline solutions while the internal standard, paracetamol, is preferably extracted from acidic solution. After a series of experiments, at pH 4.2 both PZA and paracetamol could be extracted optimally into a solvent mixture of diethyl ether and dichloromethane (3:2), which were chosen because of their low boiling points for easy evaporation. Two extractions with 7 ml of organic solvent mixture gave a better recovery.

General procedure

Into a 15-ml glass centrifuge tube, paracetamol (the internal standard) was added to the drug-containing CSF or plasma (0.2 ml) for assay. To precipitate the plasma proteins, methanol (200 μl) was added, followed by 1 *M* potassium dihydrogen phosphate in 0.2% ascorbic acid (1 ml) to adjust the pH to 4.2. The acidic solution was extracted twice with organic solvent (7 ml of a mixture of dichloromethane—diethyl ether, 2:3) by mixing with the aid of an automatic shaker for 15 min. After centrifugation for 10 min at 2500 *g* to break the emulsion, the organic extract was transferred into a 15-ml evaporation tube; the combined extract was then evaporated to dryness at 45°C in a water bath. The residue was dissolved in distilled methanol (50 μl) and vortexed for 30 s. An aliquot (25 μl) was injected into the liquid chromatograph.

Recovery and selectivity

To assess the recovery of PZA from CSF and plasma samples by the extraction procedure, the drug was added to drug-free plasma or CSF (10 μg ml^{-1}) and assayed with the internal standard as described. For comparison, the same concentrations of PZA and internal standard were prepared in a diethyl ether—dichloromethane solution, evaporated and assayed, but with the extraction step omitted. The corresponding peak-height ratios from the CSF and plasma extractions and from the organic solvent solutions were compared.

Samples of plasma spiked with 2-pyrazine-carboxylic acid (metabolite of PZA) and other anti-TB drugs such as isoniazid, *p*-aminosalicylic acid and rifampicin, were analysed to find out if these compounds produced peaks after chromatography that interfered with those of PZA and paracetamol (internal standard).

Quantitation, precision and stability on storage

Calibration graphs were constructed by plotting the peak-height ratio of PZA to the internal standard, against the known concentrations of PZA added to drug-free plasma or CSF to cover the range 0—40 μg ml^{-1}. PZA was quan-

titated by relating the respective peak-height ratio to obtain the concentration from the calibration graph.

Six replicate samples of PZA in plasma or CSF at each of the calibration points were assayed as described under *General procedure*, and the peak-height ratios of the drug to the standard were calculated. For each batch of samples assayed (usually up to 20 samples a day), two standard samples were run, one at the beginning and the other at the end of assaying the batch; the peak-height ratios of these two standard samples were compared with the corresponding concentration on the calibration graph.

Samples of plasma and CSF spiked with drugs or samples obtained from the rabbits were assayed immediately and after storage at —20°C for seven days and three months.

Disposition of PZA in the rabbit

New Zealand white rabbits (fasted overnight), with their dorsal cervical area and occipital area of the skulls shaved, were cannulated with polythene cannulae (Portex, Kent, U.K.) at their ear arteries (size 0.58 mm I.D., 1.96 mm O.D.) and veins (size 0.4 mm I.D., 0.8 mm O.D.). CSF samples and simultaneous blood samples, at various intervals, were obtained while the animal was lightly anaesthetized with intravenous thiopentone (40 mg kg^{-1}); blood samples at other intervals were taken while the animal was conscious. PZA (30 mg kg^{-1} in saline) was administered orally into the rabbit's stomach via a polythene tube (under thiopentone anaesthesia), which was then washed down with distilled water (10 ml). Blood samples (0.5 ml) were taken at 0, 0.25, 0.5, 1, 2, 4, 5, 6 and 24 h and CSF (0.2 ml) at 0, 0.5, 1, 2, 4, 5, 6 and 24 h. Plasma samples was removed as soon as possible (usually not more than 0.5 h after samplings) by centrifugation. All biological samples were stored at once after collection at —20°C before analysis by the HPLC procedure.

RESULTS AND DISCUSSION

Performance of the high-performance liquid chromatographic system

For optimal UV detection, the wavelength was set at 215 nm, which was approximately the λ_{max} for PZA and paracetamol. Fig. 1 illustrates chromatograms of extracts from drug-free plasma and CSF samples and biological samples from rabbit after oral administration of PZA. The analytical peaks of both PZA and the internal standard (paracetamol) are well resolved. The retention times of PZA and paracetamol were 2.9 and 3.9 min, respectively, while their corresponding symmetry factors were 0.98 and 1.01 and the resolution factor was > 1.0 (1.42). These are well within the British Pharmacopoeia limits [8], hence the peak-height ratio technique for quantitation is considered adequate.

Precision, selectivity and calibration

Precipitation of plasma proteins with methanol in the extraction procedure improved recovery of the drug from biological fluids, as shown previously [9]. The precision of between-batch assay was satisfactory (see batch standard variation in Table I). The extraction procedure did take up other anti-TB drugs such as *p*-aminosalicylic acid and rifampicin, but not isoniazid. However, these

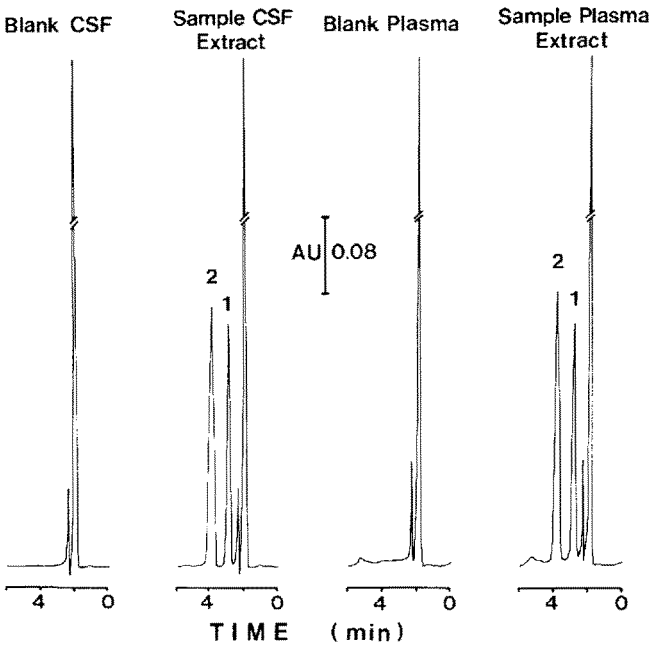

Fig. 1. Chromatograms of extracts (25 μl each) from drug-free CSF and plasma samples, and simultaneous CSF and plasma samples at 0.5 h after oral PZA from rabbit: (1) pyrazinamide (20.5 μg ml^{-1} in CSF; 19.8 μg ml^{-1} in plasma); (2) paracetamol (internal standard, 12.5 μg ml^{-1}).

TABLE I

CALIBRATION AND PRECISION OF THE ASSAY

Concentration (μg ml^{-1})	Pyrazinamide/paracetamol peak-height ratio ± S.D.	Coefficient of variation (%) (n = 6)
2.50	0.116 ± 0.010	8.60
5.00	0.228 ± 0.016	7.00
10.00	0.459 ± 0.024	5.20
20.00	0.910 ± 0.016	1.80
30.00	1.352 ± 0.024	1.80
40.00	1.782 ± 0.024	1.30
Batch standards* at 20 μg ml^{-1} (n = 35)	0.858 ± 0.039	4.50

Calibration graph: $y = 0.045x - 0.0099$; $r = 0.9999$

*An indication of between-day precision.

drugs did not interfere with the analytical peaks of PZA and paracetamol. The use of a solvent mixture (diethylether—dichloromethane in the ratio of 3:2) improves recovery after two extractions, from 55% (50.5—62.0%) to 69% (64.7—74.0%) at a concentration of 20 μg ml^{-1} for CSF or plasma samples. The extraction procedure may possibly reduce the uptake of endogenous con-

taminants as no interfering peak appears in the chromatograms of the drug-free samples (Fig. 1).

Repeated assays of CSF or plasma samples spiked with PZA indicated that the reproducibility of the procedure was satisfactory over the calibration range (Table I). It was observed that the concentrations of PZA in plasma were similar to those in the CSF after oral administration of the drug; thus, the calibration ranges in both CSF and plasma samples were identical. The calibration graphs relating the peak-height ratios and concentrations of PZA added to CSF or plasma blanks were linear (Table I).

Stability on storage

Samples of CSF and plasma, whether fresh or stored at −20°C for one week and three months, did not give peaks that would interfere with the measurement of peaks corresponding to PZA and internal standard. There was no appreciable loss of the drug from the samples stored at −20°C over a period of three months.

Application

The HPLC procedure was used to study the disposition of PZA in CSF and plasma of six rabbits after oral administration of 30 mg kg^{-1} PZA. The absorption of PZA was very rapid, with the highest concentration, 20.9 $\mu g\ ml^{-1}$, attained at 0.25 h (Table II). After absorption, PZA declined bi-exponentially. It is interesting to note that after a single oral dose, PZA was still detectable at 24 h, suggesting that the drug is widely distributed in body tissues. A better protocol for blood sampling, especially between the 6th and the 24th hour after dosing, is necessary to obtain pharmacokinetic parameters. Nevertheless, it is observed that the CSF concentrations of PZA rapidly reached levels similar or higher than those in the plasma (Table II). Thus, PZA traverses the blood/brain barriers with no difficulty, possibly by the passive transfer mechanism. An extensive programme has been planned for the study of bioavailability, single-dose and multiple-dose pharmacokinetics of PZA in the rabbit. The information obtained may shed some light on the use of PZA in the chemotherapy of TB meningitis.

TABLE II

CSF AND PLASMA CONCENTRATIONS AND THEIR RATIOS OF PYRAZINAMIDE IN SIX RABBITS AFTER ORAL ADMINISTRATION (30 mg kg^{-1})

Time (h)	CSF concentration ($\mu g\ ml^{-1}$; mean ± S.D.)	Plasma concentration ($\mu g\ ml^{-1}$; mean ± S.D.)	CSF/plasma ratio
0	0	0	0
0.25	—	20.9 ± 3.9	—
0.50	22.7 ± 1.9	18.4 ± 5.2	1.23
1.00	17.1 ± 4.3	15.2 ± 3.5	1.13
2.00	—	10.0 ± 3.0	—
4.00	4.1 ± 1.6	3.9 ± 1.8	1.05
5.00	—	2.4 ± 1.1	—
6.00	1.9 ± 0.9	1.6 ± 0.8	1.19
24.00	0.3 ± 0.1	0.4 ± 0.1	0.75

The present HPLC method has several advantages over the previously reported colorimetric method [4]. First, the extraction procedure is simple and adequate for biological assay. Second, the HPLC analysis is sensitive and selective such that other co-extracted anti-TB drugs do not interfere with the measurement of analytical peaks. Third, the assay only uses a small amount of biological fluid (0.2—0.5 ml). Thus, the assay can be adapted and modified for study in patients with neural TB infections such that a more rational approach, after monitoring plasma PZA concentrations, for dosage regimen may be obtained. With lower but optimal doses of PZA, hypersensitivity and hepatotoxicity of PZA may be more tolerable.

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