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LIQUID CHROMATOGRAPHIC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF PYRAZINAMIDE AND RIFAMPICIN IN SERUM SAMPLES FROM PATIENTS WITH TUBERCULOUS MENINGITIS

JEAN WOO

Department of Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong)

C.L. WONG

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong)

R. TEOH

Department of Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong)

and

K. CHAN*

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong)

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SUMMARY

A simple high-performance liquid chromatographic assay for the simultaneous determination of pyrazinamide and rifampicin in serum from patients with tuberculous meningitis is presented. The drugs and internal standard, *p*-acetamidobenzoic acid, were extracted from the acidified sample containing 2% ascorbic acid at pH 4.2 into dichloromethane–diethyl ether (2:3). The solvent extract was evaporated to dryness with the aid of nitrogen and the residue redissolved in methanol (75 μl). The concentrate was analysed by a liquid chromatograph using a reversed-phase 30-μm C_8 pre-column linked to a 5-μm C_8 analytical column with a gradient solvent programme, which delivered 6% to 48% (v/v) acetonitrile in phosphate buffer (10 m*M* potassium dihydrogenphosphate, pH 3.5) in 10 min at 1.5 ml/min. The eluate was detected at 215 nm. Twelve patients with tuberculous meningitis were given daily chemotherapy, and their serum samples were assayed for pyrazinamide and rifampicin.

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INTRODUCTION

The determination of concentrations of antituberculous (anti-TB) drugs in plasma or serum during chronic chemotherapy is desirable for adjusting dosage regimens with reference to the minimum inhibition concentrations or minimum bactericidal concentrations of these drugs. Unnecessarily high doses with adverse effects may also be avoided. Analytical methods previously reported for measurements of rifampicin (RFP) often involved microbiological assays, which were non-specific and time-consuming. More recently high-performance liquid chromatographic (HPLC) assays were reported for RFP and its metabolites in plasma and urine [1, 2]. Some of these methods used normal-phase columns and complex mobile phases, consisting of several organic solvents, at fairly high flow-rates. Recently we have developed an HPLC procedure for pyrazinamide (PZA) in biological fluids in rabbits [3]. The present paper describes the simultaneous measurement of PZA and RFP in serum from patients with TB meningitis.

EXPERIMENTAL

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of Analar grade (E. Merck, Darmstadt, F.R.G.), which were freshly distilled before use; 10 m*M* phosphate (potassium dihydrogenphosphate) buffer at pH 3.5; ascorbic acid buffer, which was prepared by dissolving ascorbic acid (2%, w/v) in 1 *M* phosphate buffer and the pH was adjusted to 4.2 with 1 *M* hydrochloric acid; water was double-distilled in glass apparatus; rifampicin (a gift from Ciba-Geigy, Basle, Switzerland); pyrazinamide, *p*-acetamidobenzoic acid (PADB) (Sigma, St. Louis, MO, U.S.A.); propyl-*p*-hydroxybenzoate (propyl-paraben) B.P.; 2-pyrazinecarboxylic acid (Aldrich, Gillingham, U.K.); *p*-aminosalicylic acid and isoniazid, B.P.

Apparatus and operating conditions

A Waters dual-pump liquid chromatograph system was used. It consisted of a 6000A pump and an M45 pump, which was controlled by a 660 solvent programmer and linked to a U6K injector with a 25–200 μl loop (Waters Assoc., Milford, MA,U.S.A.) and a variable-wavelength Hitachi 220-S UV detector with built-in chart recorder (Hitachi, Tokyo, Japan). Analyses were performed at ambient temperature (25°C) on a reversed-phase C_8 column (Hibar, LiChrosorb RP-8, 250 mm$\times$4.6 mm I.D., 5 μm; Merck) linked to a C_8 pre-column (30 μm, 50 mm$\times$4.6 mm I.D.). The mobile phase consisted of 10 m*M* phosphate buffer (pH 3.5) as solvent A and 60% (v/v) acetonitrile in 10 m*M* phosphate buffer (pH 3.5) as solvent B. The elution programme was set at a rate of 10 to 80% of solvent B within 5 min and subsequently running at 80% of solvent B up to 10 min (using curve 9 set by the solvent programmer) at 1.5 ml/min. The solvents were filtered before use through a Millipore Type AA filter (Waters Assoc.). Degassing was not necessary immediately after filtration.

Other apparatus used 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 on the glass walls [6]. Hamilton syringes, 25 and 200 μl, were used.

Reagents and standards

Standard methanolic solutions (1 mg/ml) of PZA, RFP, isoniazid, *p*-aminosalicylic acid, 2-pyrazinecarboxylic acid, PADB and propyl-paraben were prepared. Further dilution was made with methanol (1 μg/ml), and aliquots (25 μl) were injected into the liquid chromatograph for screening conditions. Subsequently, dilutions of PZA and RFP were made up in drug-free serum (final volume, 0.5 ml), and to each sample 10 μl (1 μg/ml) of PADB were added as the internal standard.

General assay procedure

The internal standard, PADB (10 μg), was added to a 15-ml glass centrifuge tube containing 0.5 ml of serum for assay. Methanol (200 μl) was then added to precipitate the serum proteins, followed by 1 *M* phosphate buffer containing 2% ascorbic acid (1 ml). The pH of the mixture was adjusted to pH 4.2 with 1 *M* hydrochloric acid. The acidic solution was extracted twice with organic solvent (7 ml of dichloromethane–diethyl ether, 2:3) by mixing with the aid of an automatic shaker for 15 min. After centrifugation to break the emulsion formed during mixing, the organic extracts were transferred to a 15-ml evaporation tube with a tapered base; the bulked organic extract was then evaporated to dryness at 45 °C in a water-bath, with the aid of nitrogen. The residue was dissolved in distilled methanol (75 μl), which was added round the side of the tube, and vortexed for 30 s. The tube was stoppered and kept in ice to condense the methanol. The contents were injected into the liquid chromatograph.

Recovery and selectivity

The recovery of PZA and RFP from serum using the extraction procedure described was assessed by adding the drugs to drug-free plasma at concentrations of 10 and 40 μg/ml, respectively, and assayed with the internal standard, PADB, as described. For comparison, the same concentrations of PZA, RFP and PADB were prepared in diethyl ether–dichloromethane, evaporated and assayed, but with the extraction step omitted. The corresponding peak-height ratios of PZA and RFP to PADB from plasma extractions and those from the organic solvent solutions were compared.

Methanolic solutions of the other anti-TB drugs, isoniazid and *p*-aminosalicylic acid, were injected into the chromatograph, and serum samples spiked with

these drugs were also assayed to investigate if the assay procedure could be adapted for these compounds.

Quantitation, precision and storage conditions

Calibration graphs were constructed by plotting the peak-height ratios of PZA and of RFP, to the internal standard, against the known concentrations of PZA and RFP added to the drug-free serum, to cover the values 5, 10, 20, 30, 40 and 50 μg/ml for PZA, and 10, 20, 40, 60, 80 and 100 μg/ml for RFP. Quantitation was achieved by relating the respective peak-height ratios to obtain the concentrations from the calibration graphs.

Analysis and measurement at each concentration point of the calibration was repeated six times as described. For interbatch assay of samples, standard serum samples (at 20 and 40 μg/ml, respectively, for PZA and RFP) were run at the beginning and at the end of the batch assay. The peak-height ratios of these two standard samples were compared with the concentration points on the respective calibration graphs. The precision of the assay was evaluated by the coefficient of variation of the peak-height ratios obtained at each concentration.

Samples of drug-free plasma (obtained from expired blood in the blood bank) spiked with drugs or serum samples from patients on anti-TB chemotherapy were assayed immediately, after storage at −20°C for seven days and after six months.

Application

Serum samples from patients in hospital treated with PZA, RFP, isoniazid, ethambutol or streptomycin were analysed as described.

RESULTS AND DISCUSSION

Gradient elution of anti-TB drugs

During the development of HPLC assays for PZA and RFP [3, 6], it was noticed that compounds such as PZA, isoniazid and *p*-aminosalicylic acid could be resolved satisfactorily with acetonitrile in phosphate buffer (10–20%, v/v) as the mobile phase. RFP, being a large molecule with low hydrophilic solubility, required for its measurement 40–50% (v/v) acetonitrile to give a reasonable peak shape and retention time for measurement. The present procedure can determine simultaneously PZA and RFP from a 0.5-ml serum sample after preliminary extraction into organic solvent. The gradient elution could separate five compounds with good resolution (Fig. 1). During the initial 5 min when 6% (v/v) acetonitrile in phosphate buffer was maintained isocratically by the programmer, isoniazid, PZA and *p*-aminosalicylic acid were resolved (Fig. 1). Acetonitrile was increased to 48% (v/v) and maintained for up to 10 min, then PADB and RFP were resolved.

Isoniazid could not be extracted into the organic phase under the conditions described for PZA and RFP. Direct injection of supernatant after precipitation of serum proteins produced interfering peaks, which masked the analytical peaks. *p*-Aminosalicylic acid is infrequently prescribed locally, therefore only PZA and RFP were assayed with the present method.

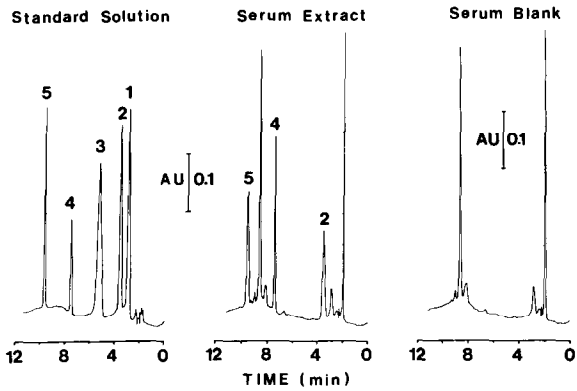

Fig. 1. Chromatograms of isoniazid (1), PZA (2), *p*-aminosalicylic acid (3), PADB (4) and RFP (5). (Left) A standard solution (25 μg of each drug in a 25-μl sample); (middle) serum extract from a patient (PZA, 41.2 μg/ml; RFP, 15.5 μg/ml); (right) drug-free serum extract.

Performance of the HPLC system

The wavelength of the UV detector was set at 215 nm for optimal absorbance of the compounds. Fig. 1 shows chromatograms of isoniazid, PZA, *p*-aminosalicylic acid, PADB and RFP, and those from serum extracts. The analytical peaks of PZA, PADB (internal standard) and RFP were well resolved and no interfering peaks form drug-free serum were found masking the analytical peaks (Fig. 1). The retention time, peak symmetry and resolution factor for each compound are summarized in Table I. These are within the British Pharmacopoeia limits [7], hence the peak-height ratio technique for quantitation is considered satisfactory.

Recovery, precision, selectivity and calibration

The addition of methanol to the serum to precipitate serum proteins prior to extraction improved recovery of drugs from biological fluids [5]. The use of diethyl ether–dichloromethane mixture has two advantages. Firstly, the organic solvent mixture forms the top layer of the extraction mixture, thus making transfer easy for the second extraction. Secondly, the low boiling point of the organic

TABLE I

PERFORMANCE OF THE HPLC SYSTEM

Drug	Retention time (min)	Symmetry factor (0.95–1.05*)	Resolution between PZA, RIF and internal marker (>1.0*)
Isoniazid	2.6	0.96	–
Pyrazinamide	3.3	0.98	2.4
p-Aminosalicylic acid	5.0	0.94	–
p-Acetamidobenzoic acid	7.4	1.02	–
Rifampicin	9.3	1.01	1.58

*Limits defined by the British Pharmacopoeia [7].

TABLE II

CORRELATION BETWEEN PEAK-HEIGHT RATIO AND CONCENTRATION OF PZA AND RFP

Concentration (μg/ml)	PZA peak-height ratio		C.V.* (%)	RFP peak-height ratio		C.V.* (%)
	Mean	S.D.		Mean	S.D.	
5	0.122	0.008	6.5	–	–	–
10	0.228	0.012	5.3	0.170	0.013	7.7
20	0.453	0.020	4.4	0.398	0.022	5.5
30	0.720	0.029	4.0	–	–	–
40	0.955	0.044	4.6	0.770	0.053	6.9
50	1.201	0.063	5.2	–	–	–
60				1.129	0.077	6.8
80				1.434	0.084	5.9
100				1.819	0.099	5.4
Batch standard**	0.461	0.036	7.81	0.776	0.048	6.2
Calibration graphs	$y=0.024x-0.011$; $r=0.9997$			$y=0.018x+0.025$; $r=0.9991$		

*$n=6$.

**Batch standard for PZA and RFP were at 20 and 40 μg/ml, respectively ($n=16$).

solvents makes evaporation more efficient at a lower temperature, thus preventing oxidation of RFP. The two extraction steps increase recovery of PZA from 55% (50.5–62.0%) to 69% (64.7–74.0%) at 10 μg/ml and RFP from 65% (60.5–72.0%) to 95.4 (87.7–99.4%) at 40 μg/ml [3, 6]. The extraction procedure may possibly reduce the uptake of endogenous compounds; although a peak is detected at 8.6 min from an extract of drug-free serum, it does not interfere with other analytical peaks.

Repeated assays of serum samples spiked with PZA and RFP showed that the procedure was reproducible and acccurate over the calibration range (Table II). The linearity of the method is demonstrated by the high correlation coefficients of peak-height ratio versus concentrations of PZA and RFP (Table II).

Stability on storage

Samples of serum or plasma, whether fresh or stored at −20°C for seven days or six months, did not give peaks that interfered with the analytical peaks corresponding to PZA, RFP and the internal standard. As RFP is unstable at room temperature, ascorbic acid was added to the aqueous phase to improve the stability [6]. Further evaporation at low temperature (38°C) with nitrogen was used to minimize loss. There was no appreciable loss of PZA and RFP from samples stored at −20°C over a period of six months or from samples that had been extracted and stored overnight at −20°C before HPLC.

Application of the HPLC assay

As a preliminary study, the assay procedure was used to determine serum concentrations of PZA and RFP in twelve patients suffering from TB meningitis who were treated with various combinations of anti-TB drugs. Aliquots of blood

TABLE III

SERUM CONCENTRATIONS OF PZA AND RFP IN PATIENTS WITH TUBERCULOUS MENINGITIS AT VARIOUS TIME INTERVALS

n = number of serum samples obtained at different occasions from twelve patients.

Drug	Dose (mg/kg body weight)	Concentration (mean ± S.D.) (μg/ml)			
		2 h	5 h	6 h	8 h
PZA	25-35	42.96 (9.9) (n=18)	39.3 (16.6) (n=10)	36.5 (24.6) (n=4)	27.4 ± 10.0 (n=15)
RFP	9-12	10.90 (6.2) (n=19)	9.3 (4.8) (n=10)	6.7 (1.0) (n=4)	5.3 (3.5) (n=16)

from these patients were obtained for clinical reasons to monitor their liver function as part of their management. Portions of these (usually 1–5 ml) were used for assay of anti-TB drugs. Thus, blood samples from different patients at separate occasions were taken at 2, 5, 6 and 8 h after drug administration. PZA and RFP concentrations in sera are summarized in Table III. These results were similar to data published by other workers. The peak plasma concentration of RFP in one study after an oral dose of 600 mg was 8 μg/ml at 3 h and declined to 3.8 μg/ml over 12 h [1], and in another from 15 μg/ml at 2 h to 1 μg/ml in the same period [2]. Both studies used an HPLC technique for the determination of RFP. The first reported cerebrospinal fluid (CFS) and plasma concentrations of PZA, measured by the well established colorimetric method [8], of a patient receiving empirical anti-TB chemotherapy were identical, being 50 μg/ml [9].

There is a lack of information on the penetration of anti-TB drugs from the general circulation into the CSF in patients with TB meningitis [10]. About 1% of plasma RFP concentration was detected in the CSF of healthy rabbits with normal meninges, and the ratio of CSF to plasma concentration of PZA was near unity [11]. We are in the process of monitoring the CSF concentrations of these drugs in patients with TB meningitis. As these patients received various combinations of anti-TB drugs, some of which are enzyme inhibitors (isoniazid) and some are enzyme inducers (RFP), it is likely that drug–drug interactions may occur within this group of antibiotics. A rabbit model has been established for investigation of this aspect [11]. Subsequent studies in human subjects or in clinical management of TB are being planned in our laboratories.

The present HPLC assay procedure offers several advantages. It can determine simultaneously PZA and RFP, and possibly also isoniazid and *p*-aminosalicylate after further refinement of the extraction procedure, from a 0.5-ml sample. The HPLC analysis is sensitive and selective and does not involve complex combinations of organic solvents in the mobile phase. The well established colorimetric assay [8] requires several solvent extractions for separation of PZA from its metabolites. One drawback of the present assay is that it requires two HPLC pumps and a solvent programmer for gradient elution.

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