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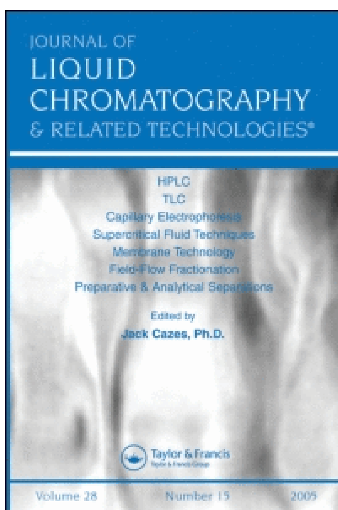

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A Systematic Approach for the Simultaneous Assay of Some Antituberculous Drugs in CSF and Plasma by Liquid Chromatography

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A SYSTEMATIC APPROACH FOR THE SIMULTANEOUS ASSAY OF SOME ANTI-TUBERCULOUS DRUGS IN CSF AND PLASMA BY LIQUID CHROMATOGRAPHY

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ABSTRACT

The rationale behind the measurement of anti-tuberculous (Anti-TB) drugs in biological fluids, especially in the management of tuberculous meningitis (TBM), has been briefly discussed. A short review on methods available for laboratory use has been presented. Finally a systematic approach using liquid chromatography (LC) is presented for consideration. This approach involves a preliminary organic solvent extraction of pyrazinamide (PZA) and rifampicin (RIF) and its 25-desacetyl metabolite, 25-DRIF, from cerebrospinal fluid (CSF) or plasma samples. The drugs and their internal standards are separated on a RP8 precolumn linked to a µBondapak cartridge in a Z-

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module by a gradient solvent program which delivers 6% to 48% v/v of acetonitrile in a phosphate buffer (10 mM KH_2PO_4, pH3.5) in 10 min at 1.5 ml/min. The eluate is detected at 215 nm. This LC system can also resolve 2 other anti-TB drugs (isoniazid and p-aminosalicylic acid). Patients with TBM were treated with various combinations of anti-TB drugs and their CSF and serum samples were assayed.

INTRODUCTION

The measurement of anti-TB drugs in TBM

Infections caused by _Mycobacterium tuberculosis_ are, nowadays, no longer life threatening. Most combinations of anti-TB drugs can eradicate tubercles in the circulation. TBM, though universally not fatal, is most serious because irreversible brain damage, such as mental retardation, paralysis, epilepsy and involuatary movements may result if chemotherapy is not adequate (1). Up to 5 anti-TB drugs are empirically prescribed in larger than conventional doses for systemic infections to ensure adequate penetration into the central nervous system in TBM. Moreover, in view of the serious consequence of TBM, chemotherapy is often prescribed for longer periods and in a daily rather than thrice weekly regimen. Therefore, there is an increase in dose-related unwanted adverse effects (2) and patient compliance is poor on prolonged treatment (3). Such an empirical approach is not entirely satisfactory. Yet available information on anti-TB drug penetration into the CSF in TBM is scanty. Prediction of CSF

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concentrations of anti-TB drugs after multiple chemotherapy is difficult. It is uncertain whether the degree of meningeal inflamation, the use of corticosteroids, the duration of the disease, or the co-administration of anti-TB drugs (isoniazid is an enzyme inhibitor while rifampicin an enzyme inducer) would affect drug disposition and penetration into the CSF. The determination of concentration of anti-TB drugs in serum and CSF during chemotherapy is desirable for adjusting dosage regimens with reference to the minimum inhibition concentrations (MIC) or minimum bacterial concentrations (MBC) of these drugs. Thus, optimal dosage can be estimated in order to minimize toxic effects.

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A brief review on analytical methods

Previously, most antibiotics in bilogical fluids were measured by microbiological assays. The methods are based on a direct relationship between the inhibitory effect of the body fluid samples containing the antibiotic on microbial growth tested in vitro and the concentration of antibioties in the sample (4). In general, these procedures require highly trained and skilful laboratory personnel and some of the assays require 10 days for completion (5). Furthermore, when chemotherapy is given with two or more anti-TB drugs concomitantly, modifications in the procedures are needed to prevent interference by other antimicrobials or active metabolites in the sample. Therefore spectrophotometric and fluorometric methods have been developed but they require tedious extraction and derivatisation. Radioimmunoassays and radio enzymatic assays have been reported in the literature (6,7,8). These methods, though rapid and sensitive, are only available for a few antibiotics and not routinely used.

Several chromatographic procedures are available for the measurement of anti-TB drugs in human biological samples (9) such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), and gas chromatography - mass spectroscopy (GC-MS). Limits of detection range from g to pg levels depending on the detection sensitivity.

Isoniazid (INH) and its major metabolite, N-acetylisoniazid, in plamsa were measured by HPLC with a sensitivity limit of 0.1 µg/ml using UV detection (10, 11). Recently, a HPLC assay for the determination of INH and its metabolites (acetylisoniazid, acetylhydrazine and diacetylhydrazine) in plasma and urine was described (12). Although it was sensitive but pre-column reactions with 3-chlorobenzoyl and 3-fluorobenzyol chloride to form their respective derivatives was required. Another HPLC procedure also required formation of propionyl derivatives prior to HPLC analysis (13). Furthermore, 1-dodecylsulphate was used as ion-pair for better resolution of analytical peaks.

Ethambutol was determined by GLC with electron capture detection and GC-MS (14, 15). Again derivatisation was needed.

RIF and its 3 metabolites (3-formylrifampicin SV, 25-desacetylrifampicin and 3-formyl-25-desacetyl-rifampicin SV) in urine and plasma were assayed by HPLC using a normal phase column and complicated mobile phase consisting of 5 solvent components (16). Recently, a simpler procedure was reported using a reversed phase

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column (17). We have modified these procedures by using a reversed phase column and simple mobile phase (acetronitrile - phosphate buffer at pH 3.5) for measuring rifampicin in plasma (18) and simultaneously rifampicin and 25-desacetylrifampicin (19) in CSF and plasma of rabbits.

PZA in plasma was previously determined by a spectrophotometric procedure which involved complicated and tedious counter-current extraction followed by colour formation (20). A HPLC assay, using reversed phase and simple methanol-buffer mobile phase at pH 7.4, was reported. But, it did not include an internal standard because sample preparation only involved protein precipitation with extraction. Precision of the assay was not reported (21). We have developed a simple procedure for measuring PZA in plasma and CSF of rabbits. The assay involves a preliminary extraction of the drug and paracetamol (internal standard) from acidified sample followed by elution on a C_8 reversed phase column with acetonitrile-buffer (10:90) at pH 3.5 at 1.5 ml/min. The eluate is detected at 215 nm (22).

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Ion-pairing HPLC techniques have been reported for measuring p-aminosalicylic acid (PAS) in plasma (23) and streptomycin (24). Other less commonly used anti-TB drugs such as ethionamide (25), kanamycin (26) and thiacetazone (27) can also be measured by HPLC. A comprehensive review on these is available (9).

Physico-chemical properties of anti-TB drugs

Ethambutol, INH, PAS, PZA, RIF and and streptomycin are often prescribed in combinations of 2 to 3 for treatment of TB meningitis.

These compounds have different physico-chemical properties which present problems in sample preparation and, to a lesser extent, in chromatographic separation. INH and streptomycin, both polar and fairly water soluble, are difficult to extract into organic solvents. Ethambutol, a weakly basic molecule, can easily be extracted into chloroform under alkaline condition. PAS, PZA (a nearly neutral compound), and RIF can be taken up from a slightly acidic (pH 4) medium into organic solvent. RIF is unstable if left exposure to air during extraction step and ascorbic acid is included in the extraction mixture.

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Preliminary experiments showed that the supernatants of plasma samples after direct protein precipitation with methanol or acetonitrile was not suitable for direct HPLC separation because the solvent front was too large and interfering peaks from biological samples often masked analytical peaks. Therefore extraction and subsequent concentration of organic solvent extracts were necessary for better sensitivity and selectivity (22).

EXPERIMENTAL

Apparatus

A Waters Associates (Milford, MA U.S.A.) dual-pump liquid chromatography system consisting of a M6000A pump and a M45 pump which are controlled by a Gradient Programmer and a U6K injector linked to a Hitachi variable wavelength UV detector 220-S (Hitachi, Tokyo, Japan)

was used. Analyses were performed at ambient temperature ($25^{o}C \pm 1^{o}C$) on a reversed phase C_8 column (Hibar, Lichrosorb RP-8, 250 mm x 4.6 mm I.D., 5 µm; Merck, Darmstadt, F.R.G.) or a Z-module fitted with µBondapak C_{18} Radial-Pak cartridge (Waters Associates) linked to a C_8 pre-column (30 µm, 50 mm x 4.6 mm I.D.).

Glasswares used included : 10-ml and 15-ml capacity 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 3% solution of hexamethyldisilazane (HMDS) in distilled chloroform. They were dried at $150^{o}C$ in an oven overnight. This treatment of glassware was necessary to eliminate possible loss of drug owing to adsorption onto the glass walls (28). Hamilton syringes, 25 µl and 200 µl, were used for accurate sampling of standard solutions and sample injection.

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Materials

All reagents used were of Analar or equivalent grade. These included : dichloromethane, diethyl ether, methanol (E. Merck Darmstadt, F.R.G.) which were freshly distilled before use; 1 M hydrochloric acid; 10mM phosphate buffer (KH_2PO_4) at pH 3.5, adjusted by orthophosphoric acid; ascorbic acid buffer for extraction was prepared by dissolving ascorbic acid (2% w/v) in 1M KH_2PO_4 buffer and

the pH was adjusted to 4.2 with 1M HCl; water was double-distilled using a glass apparatus.

The following chemicals and drugs were used : p-aminosalicylic acid B.P., isoniazid B.P. paracetamol B.P. (Universal Pharmaceutical Laboratories, H.K.); pyrazinamide, p-acetamidobenzoic acid, and dimethylaminobenzoic acid (Sigma Chemical Co., St. Louis, MO, USA); rifampicin and 25-desacetylrifampicin (gifts from Ciba Geigy, Switzerland); and butyrylaminophenol (R.B. Laboratories, Inc. Calif. U.S.A).

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Preparation of reagents and standards

Standard solutions, calculated as mg drug per ml in distilled methanol, were made of all drugs and chemicals. Dilutions were made up in drug free plasma and CSF (final volume of sample, 0.5 ml).

Chromatographic conditions

Analyses were performed in air-conditioned laboratory at $25^{o}C \pm 1^{o}C$. The mobile phase consisted of a 10 mM KH_2PO_4 buffer at pH 3.5 as solvent A, and 60% v/v acetonitrile in 10 mM KH_2PO_4 buffer at 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 8 set by the Solvent Programmer) at 1.5 ml/min. The mobile phases were filtered before use through a Millipore type AA filter (Waters Assoc.). Degassing was not necessary

immediately after filtration. Detection of eluates was set out at 215 nm.

Sample preparations

For the assay of anti-TB drugs in plasma or serum, the internal standard, PADB (10 µg), was added to a 15 ml glass centrifuge tube containing 0.5 ml serum. Methanol (200 µl) was then added to precipitate the serum proteins, followed by 1 M KH_2PO_4 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 a mixture 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 into a 15-ml evaporation tube with 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 30s. The tube was stoppered and kept in ice to condense the methanol. The whole content was injected into the liquid chromatograph for analysis.

For the determination of anti-TB drugs in CSF samples, two internal standards, paracetamol for compounds with shorter retention time (ie pyrazinamide) and butyrylaminophenol for drugs with longer retention times (e.g. rifampicin and metabolites), were added to the

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0.5 ml of CSF. This is because a change in the attenuation of recorder is necessary to increase the peak heights of analytical peaks. The whole extraction procedure was identical to that for plasma determination.

Application

As a preliminary study, CSF and serum samples were obtained from 12 patients with TB meningitis. These patients were treated with daily doses of oral isoniazid, pyrazinamide and rifampicin, some received ethambutol or streptomycin and some were also given oral steroids. Lumbar punctures to obtain CSF were performed on clinical grounds and not specifically for anti-TB drug monitoring; thus, only small volume (0.5 ml) aliquots were saved for assay.

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RESULTS AND DISCUSSION

Extraction of anti-TB drugs

Due to the diversity of physico-chemical properties of anti-TB drugs, several extraction methods from plasma were investigated. Simple extraction by protein precipitation using several precipitants (trichloroacetic acid, acetonitrile and methanol) was proven unsuccessful because of lacking adequate sensitivity and selectivity (18, 22). The use of Extrelut (or equivalent) disposible columns for preliminary separation is expensive. Thus, the conventional liquid-liquid extraction, subsequent evaporation and reconstitution procedures were adopted. Procedures reported in the literature often

include the use of organic solvents with high boiling points (ethylacetate, hexane, isoamyl alcohol, n-butanol) which makes evaporation difficult and renders loss of heat labile compounds. We have found a mixture of dichloromethane - diethyl ether (2 : 3) most useful. This solvent mixture stays as the top layer of the extraction mixture, thus, enabling easy transfer of extract. Its low boiling point makes evaporation easy at 40-45°C without the use of nitrogen. The disadvantage is that its extracting capacity may not be as high. Sometimes two extractions are necessary to improve recovery of drugs from biological fluids. Of all 5 anti-TB drugs, extraction of INH from biological fluids was not satisfactory, perhaps, due to its polar nature and high water solubility. Derivatisation before extraction was necessary (11). Streptomycin cannot be extracted by solvent-solvent. Ethambutol can be extracted under alkaline condition while PZA, PAS and RIF can be extracted under acidic conditions.

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A gradient elution program for anti-TB drugs

In the development of chromatographic conditions for separating various anti-TB drugs and metabolites (18, 19, 22), it was noticed that PZA, INH and PAS could be separated satisfactorily using 10% v/v of acetonitrile-KH_2PO_4 buffer at pH 3.5 as the mobile phase whilst RIF and 25 DRIF, being larger molecules with low hydrophilic solubility, required 40 to 50% acetonitrile to give reasonable retention time and peak symmetry for measurement. The present gradient program could separate 8 compounds with good resolution (Table 1). The concave curve 8 on the Gradient Programmer was selected to deliver increasing amount of acetonitrile in the mobile phase from 6% to 48% in 5 min and

Table 1 Performance of the separation program (see text for detail)

Drug	Retention time (min)	Symmetry factor (0.95 - 1.05)*
Isoniazid	2.6	0.96
Pyrazinamide	3.3	0.98
Paracetamol**	5.3	0.97
p-aminosalicylic acid	5.9	0.94
p-acetamidobenzoic acid**	7.4	1.02
butyrylaminophenol**	8.2	1.00
25-desacetyl rifampicin	10.9	1.01
rifampicin	12.9	1.02

* Limits defined by the British Pharmacopoeia, 1980 (ref. 29)

** Internal standards

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subsequently running at 80% of solvent B. During the initial period INH, PZA, paracetamol (internal standard), and PAS are resolved. When the amount of acetonitrile increases to 40% during subsequent period, PADB and butyrylaminophenol (internal standards), 25 DRIF, and RIF are separated.

Performance of the HPLC system

Figure 1 shows representative chromatograms of PZA, PABD and RIF standard solutions and those from plasma extracts. Figure 2 presents chromatograms of PZA, paracetamol (internal standard 1),

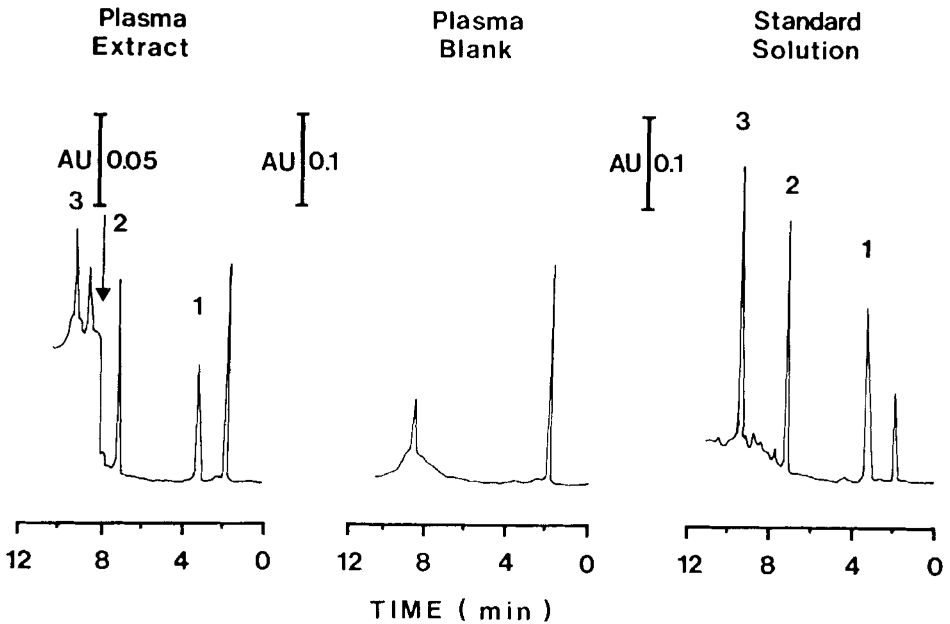

Figure 1 Representative chromatograms of standard solution (right), drug-free plasma extract (middle) and plasma extract from a patient (left). 1 = PZA, 2 = p-acetamidobenzoic acid (internal standard), and 3 = RIF; using LiChrosorb C_8 column.

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butyrylaminophenol (internal standard 2), 25 DRIF and RIF, and those of CSF extracts. The present extraction procedure did not take up INH and streptomycin. Further development work is needed. PAS is infrequently included in the local chemotherapeutic regimens. Ethambutol will be assayed by GLC. Thus the present assay can determine PZA, RIF and its metabolites by HPLC in one sample. The retention time and peak symmetry factor for each compound are within

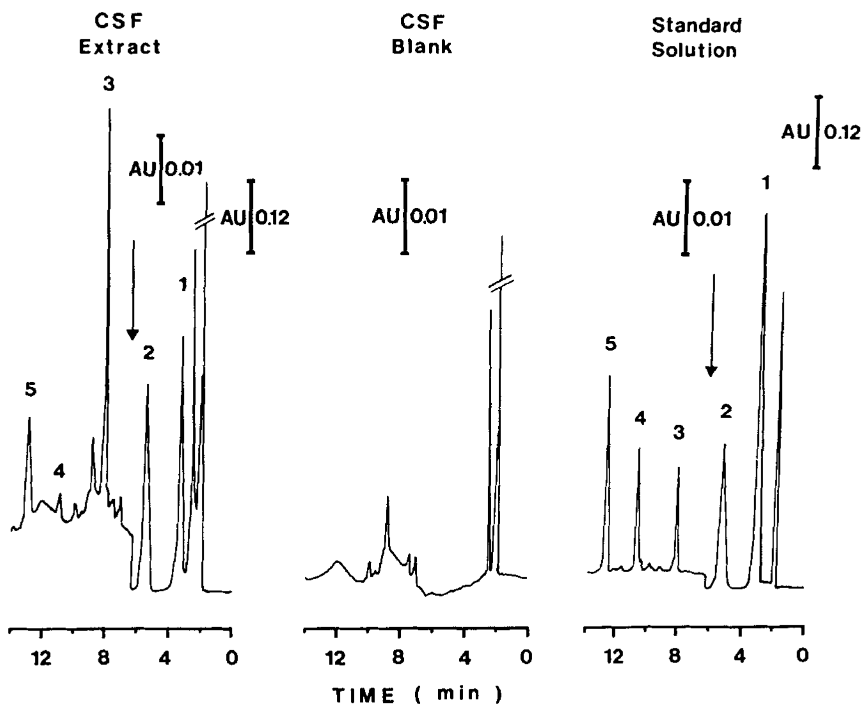

Figure 2 Representative chromatograms of standard solution (right), drug-free CSF extract (middle) and CSF extract from a patient (left). 1 = PZA, 2 = paracetamol (internal standard 1), 3 = butyrylaminophenol (internal standard 2), 4 = 25 DRIF, and 5 = RIF; using μBondapak C_{18} Radial-Pak cartridge in Z-module.

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the British Pharmacopoeia limits (29), hence the peak height ratio technique for quantitation is considered satisfactory.

Both LiChrosorb C_8 column and μBondapak C_{18} cartridge in Z-module can resolve analytical peaks well (Figures 1 & 2). The back pressure resulted from the cartridge in Z-module at 1.5 ml/min was very much lower than that produced by the LiChrosorb column.

Recovery, Precision and Calibration

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Previous experiments on the recovery studies indicate that two extractions, using the dichloromethane - diethyl ether mixture improved the recovery of PZA and RFP from 55% (50.2 - 62.0%) to 69% (64.7 - 74.0%) at) 10μg/ml and from 65% (60.5 - 72.0%) to 95.4 (87.7 - 99.4%) at 40 μg/ml respectively (18, 19, 22). The addition of methanol to the plasma samples precipitate proteins prior to extraction improved recovery of drugs from biological fluid (30). The extraction procedure may, possibly, reduce the uptake of endogenous compounds, although a peak is detected at 8.6 min from extracts of drug-free CSF or plasma (Figures 1 & 2).

Repeated assays of CSF or plasma samples spikes with PZA, RIF and 25 DRIF indicated that the reproducibility of the procedure was satisfactory over the calibration ranges (Tables 2 and 3). As the concentrations of PZA in plasma were similar to those in the CSF, only one calibration was performed. Two different calibrations were constructed for analysis of RIF in CSF and plasma (Tables 2 and 3)

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Table 2 Regression data for calibration graphs of PZA, and RIF for measurement of plasma samples (n = 6) using a LiChrosorb C_8 column

Drug	Concentration range (µg/ml)	Internal Standard	Internal Standard concentration added (µg)	Equation y=a+bx	Regression coefficient, r	Coefficient of Variation, % (range)
PZA	5 - 50	p-acetamido-benzoic acid	10	y=0.024x - 0.011	0.9997	5.3 to 6.5
RIF	1 - 20	p-acetamido-benzoic acid	10	y=0.018x - 0.025	0.9991	5.4 to 7.7

Table 3 Regression data for calibration graphs of PZA, 2 DRIF, and RIF for measurement of CSF samples (n = 6) using µBondapak C_{18} Radial-Pak cartridge in Z-module

Drug	Concentration range (µg/ml)	Internal standard	Internal standard concentration added	Regression equation y=a+bx	Regression coefficient, r	Coefficient of variation, % (range)
PZA	5 - 50	paracetamol	7.5 µg	y=0.045x - 0.028	0.9991	3.6 - 6.6
25 DRIF	0.25 - 5.0	butyrylaminophenol	0.24 µg	y=0.49x + 0.0036	0.9973	5.2 - 7.5
RIF	0.5 - 5.0	butyrylaminophenol	0.24 µg	y=0.335x + 0.017	0.9883	2.5 - 6.9

Two standard samples were run, one at the beginning and the other at the end of assaying the batch (usually 16 samples per day). The coefficients of variation for the between-batch standards at 20 µg/ml for PZA, 10 µg/ml for plasma RIF, 2 µg/ml for CSF RIF, and 1 µg/ml for 25 DRIF were 4.5%, 5.9%, 4.8% and 6.1% respectively. The linearity of the calibration graphs is demonstrated by the high correlation coefficients of peak-height ratios versus concentrations of PZA, RIF and 25 DRIF (Tables 2 and 3).

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Storage of biological samples

Samples of plasma and CSF, whether fresh or stored at -20°C for 6 months, and dried extracts stored at -20°C overnight did not give peaks that interfered with the analytical peaks corresponding to the drugs and internal standards. As RIF was unstable when exposed to air, ascorbic acid was added to the aqueous phase during extraction procedure and successfully improved stability (17, 18). Evaporation of organic solvent at low temperature (40°C) with nitrogen minimized loss. No appreciable loss of the drugs from biological samples was detected after storage at -20°C over 6 months or from samples which had been extracted and stored overnight at -20°C before analysis by HPLC.

Application

The assay procedure was used to determine CSF and plasma concentrations of PZA and RIF in 12 patients who had TB meningitis and were treated with oral PZA (1.5 to 2.5 g daily) and RIF (600 mg

daily). Paired CSF and plasma samples were obtained for clinical reasons to monitor their liver function and culture purposes as part of their management. Some of these were saved for HPLC analysis of PZA and RIF. Thus, biological samples from different patients at separate occasions were available at 2 and 8 hours after drug administration. Table 4 summarises the assay results. Only a very small amount of 25 DRIF was detected in the plasma samples and none in the CSF.

That PZA penetrates into CSF readily confirms previous study in TBM patients (31) and in our rabbit model (32). The CSF concentrations of PZA are above the reported minimal inhibitory concentrations (MIC) of 20 μg ml^{-1} (2) from 2 to 8 hr after a single oral dose. On the other hand, RIF has a poor penetration into the CSF. The present results also confirm previous studies on RIF using a microbiological assay (33) and are in agreement with observations from our animal study (18). The mean CSF concentrations of RIF are all at the lower range of the recommended MIC values, 0.005 - 2μg/ml (2). Thus, this preliminary study suggests that the dosage regimen for PZA is satisfactory for treatment of TBM, but further studies are necessary to determine optimum RIF dosages to attain adequate CSF levels.

In conclusion, a systematic approach has been developed for the simultaneous determination of some commonly used anti-TB drugs. Further refinement in extraction procedure is necessary for INH and streptomycin. The assay uses a small sample (0.5 ml). The HPLC

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Table 4 CSF and plasma concentrations of PZA and RIF in patients with TB meningitis

Time (hr)	Number of samples	CSF concentration µg/ml		Plasma concentration µg/ml		CSF/Plasma Ratio	
		PZA	RIF	PZA	RIF	PZA	RIF
2	18	26.0 ± 1.6	0.19 ± 0.03	42.9 ± 2.3	10.90 ± 1.43	0.62 ± 0.03	0.03 ± 0.01
8	14	27.8 ± 3.0	0.30 ± 0.03	27.4 ± 2.6	5.30 ± 0.88	1.05 ± 0.08	0.10 ± 0.02

analysis is selective and sensitive and does not involve complicated combinations of organic solvent as mobile phase. It should be useful for monitoring anti-TB drugs in TB meningitis.

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