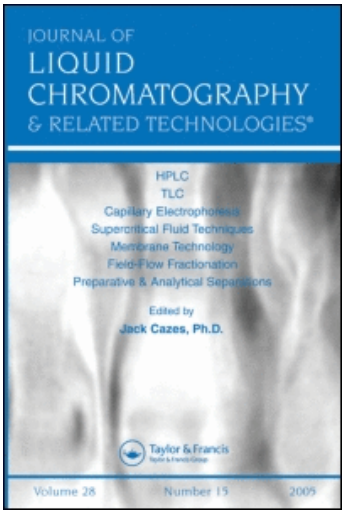

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTI-TUBERCULOSIS DRUGS IN HUMAN BODY FLUIDS

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ABSTRACT

A rapid, simple and sensitive high performance liquid chromatographic (HPLC) assay for the quantification of pyrazinamidum (PZA), rimifon (INH) and rifapentine (RFT) in human serum, urine and cerebrospinal fluid (CSF) has been developed. After acidification and removal of protein, liquid samples were injected into the HPLC system directly. Separation was achieved using a μBondapak phenyl reversed phase column for RFT and ion-pair chromatography on Nova-pak C_{18} for INH and PZA. The retention times of PZA and INH on the -C_{18} column were 2.66 and 5.33 min, respectively. Retention time of RFT was 9.10 min. on the -phenyl column. The PZA, INH and RFT standard plots were highly linear ($r>0.99$) over the concentration range of 0.1 to 10 μg/mL, 0.1 to 10 μg/mL and 0.5 to 10 μg/mL, respectively. PZA mean recovery was 96.5%±4.2, INH 97.4%±3.7 and RFT 92.1%±6.2. The method was applied for the control of the drug doses on treating tuberculous meningitis and for investigating whether RFT passes through human hematoencephalic barrier.

INTRODUCTION

Pyrazinamidum (PZA), Rimifon (INH), Rifampicin (RFP) and p-aminosalycilic acid (PAS), etc. have been used as anti-tuberculosis drugs for many years.[1,2] PAS has been regarded as a necessary drug for tuberculous meningitis treatment. Although other drugs such as RFP, PZA, EMB, etc. have been recently used and the PAS intravenous (i.v.) treatment in long-term is very troublesome, expensive and has side-effects, the PAS has not been abolished. But PAS is only a bacterium inhibitor and not a sterilization drug. Therefore, PAS should may be theoretically abolished. Rifapentine (RFT) is a new antibiotic. It has been used to treat pulmonary tuberculosis, etc., and has many merits such as long-term treating activity, high anti-bacterium activity, etc. In our work, RFT was used as the main treatment drug, with PZA and INH as auxiliary drugs to treat tuberculous meningitis. Although PZA was abandoned, the treatment was very effective.

To understand the actions of the drugs, the concentrations of the drugs in serum, urine and cerebrospinal fluid (CSF) must be investigated. In view of their wide clinical use, different analytical methods have been developed for the quantification of the drugs in blood, plasma, urine and other body fluids. These methods include spectrophotometry,[3,4] high performance liquid chromatography (HPLC),[5,6] gas chromatography,[7] etc. But analysis of RFT in CSF has not been reported.

In this report, we describe the analysis of INH, PZA and RFT in serum, urine and CSF by HPLC. By monitoring the drug levels, doses can be controlled. Also investigated was whether RFT passes through the hematoencephalic barrier.

EXPERIMENTAL

Chemicals

All the reagents used were of analytical grade; organic solvents were of high purity grade for HPLC; water was Milli-Q (Millipore-Waters) deionized. Standard reagents of anti-tuberculosis drugs were purchased from Merck and the Institute of Medicine Identification, Chinese Academy of Preventive Medicine. The paired ion chromatographic (PIC) reagent used was 1-heptane sulfonic acid (PIC-B7, Millipore-Waters); its UV cutoff wavelength was 200 nm.

Apparatus

The Waters Liquid Chromatography System (Waters Associates, Milford, MA, U.S.A.) used consists of a solvent deliver pump (Model 590), a manual injector (Model U6K), and a UV-VIS programmable detector (Model 490) operated at 230 nm, and a Baseline 810 chromatographic station.

Chromatographic Conditions

The column used for the RFT analysis was μBondapak phenyl packing (300mm x 3.9mm I.D., 5μm particle size) purchased from Waters Division (U.S.A). The eluent was methanol:water (35:60) at a flow rate of 1.0 mL/min.

The column used for the INH and PZA analysis was Nova-pak C_{18} packing (150mm x 3.9mm I.D., 5μm particle size) (Waters). The eluent was 5mM PIC-B7 aqueous solution:methanol (90:10) at a flow rate of 1.0 mL/min.

Standards Preparation

Stock aqueous solutions (1.0mg/mL) of INH and PZA were prepared, respectively. Five mixed standards of INH and PZA were prepared by pipetting 10, 20, 40, 80, 100 μL of INH and PZA stock solutions into separated 10mL volumetric flasks and diluting to volume with water. The concentrations of standard series were: INH and PZA 1, 2, 4, 8, 10 ppm. Stock solution (1.0mg/mL) of RFT was prepared with methanol. Five working standards of the drug were prepared by pipetting 10, 20, 40, 80, 100 μL of RFT stock solution into separated 10 mL volumetric flasks and diluting to volume with water. The concentrations of standard series was 1, 2, 4, 8, 10 ppm.

Sample Treatment

A dose of INH was administered to tuberculous meningitis patient via intravenous injection and PZA, RFT orally in a gelatin capsule. Serum, urine and CSF samples were collected at the interval of 10 hrs after administration. An aliquot of 1 mL serum was mixed with 1 mL 10% H_3PO_4 for 15min. followed by extracting in ultrasonator for about 15min. 1 mL extract was then mixed with 1 mL methanol and centrifuged at 3000 G for about 15 min. The supernatant was filtered through a 0.45 μm filter. The filtrate was injected into the HPLC system.

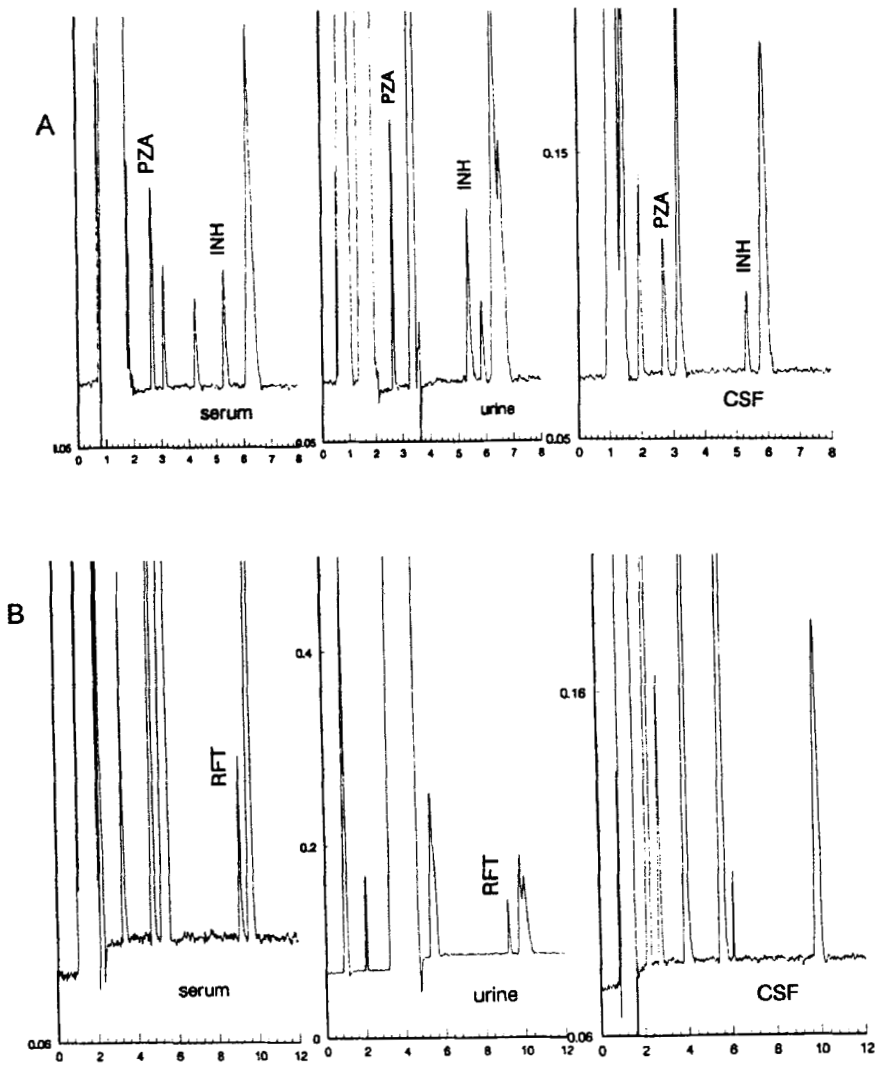

Figure 1. Chromatograms of sample analyses. (A) Serum, urine and CSF for PZA and INH analysis; (B) Serum, urine and CSF for RFT analysis.

An aliquot of 0.2 mL CSF was mixed with 0.2 mL 10% H_3PO_4 for 15min., followed by extracting in an ultrasonator, then filtered through 0.45 μm filter. The filtrate was injected into the HPLC.

An aliquot of 1 mL urine was mixed with 1 mL 10% H_3PO_4 for 15min. followed by extracting in an ultrasonator for about 0.5 hr. The extract was then filtered through a 0.45 μm filter. The filtrate was injected into the HPLC.

RESULTS AND DISCUSSION

Chromatographic Specificity and Sensitivity

Typical chromatograms of (a) INH and PZA analysis of serum, urine and CSF (b) RFT analysis of serum, urine and CSF after administration are shown in Figure 1. The specificity of the method was demonstrated by the lack of interference at the retention times of INH (5.33 min.) and PZA (2.66 min.) on the -C_{18} column and RFT (9.10 min.) on the -phenyl column. The sensitivity of the assay, defined as the minimum concentration that can be quantitated with a statistically acceptable coefficient of variation (10%) in the peak area was 40 ng/mL for INH (CV=7.7%), 45 ng/mL for PZA (CV=9.3%) and 85 ng/mL for RFT (CV=9.6%) (see Table 1). The minimum detectable amounts, defined as the amount, in nanograms, that gives a peak height of the drugs equal to twice the background noise at the most sensitive instrument setting used in the study (0.01 AUFS, time constant: 2.5, injection volume: 50 μL) were 4.0 ng, 4.5 ng and 8.5 ng for INH, PZA and RFT, respectively.

Selection of the Separation Conditions

A mobile phase consisting of water:methanol (35:60) on the reverse phase phenyl column gave the optimum resolution of RFT and other interference components in the samples. The ratio of water with methanol in the mobile phase drastically affected the retention times of the drug; the retention time decreased with increasing percentage of methanol. But, under this condition, INH and PZA could not be separated, and the separation could not be improved by changing the percentage of MeOH (see Figure 2). However, INH and PZA could be separated completely by ion-pair chromatography with PIC-B6 on the Nova-pak C_{18} column, and other components in samples did not interfere with the drugs. But RFT could not be analyzed under this condition. When the ratio of methanol with water was less than 10:90, elution of RFT was very difficult (retention time >18min.), but when the ratio was increased to 15:85, INH and RFT could not be separated. And when the ratio of methanol with water was maintained within 10:90 - 15:85, other components in the samples interfered with the drug.

Table 1

Sensitivity of the Assay (μV·sec)

Repetition	Response of INH (40 ng/mL)	Response of PZA (45 ng/mL)	Response of RFT (85 ng/mL)
1	7432	6821	9627
2	7931	6014	10550
3	8724	5432	10897
4	7855	5243	12843
5	7128	5127	10021
6	8127	6354	9427
7	7043	6742	11456
8	7444	5934	10729
9	8247	5733	9742
10	8931	6217	11737
Mean:	7886	5962	10703
Response (C.V. %):	7.7	9.3	9.6

Additivity standard in blank serum (Injection 50 μL).

Sample Treatment and Recovery of the Drugs

In general, the drug analyses have been completed using a multi-step extraction procedure with different solvents (ethyl acetate, acetonitrile, n-heptane) followed by HPLC.[8,9] The procedures were complex. The drugs exist in body in combined states with other bio-components. Determination of drugs originalating in the body must employ acidification decomposition to free the drugs from combined state. In this paper, 10% H_3PO_4 was used to free the drugs. To investigate effectiveness of treatment, different concentrations of H_3PO_4 and treatment times were tested. The results are shown in Table 2. The results tended to be too low when the hydrolytic decomposition time was less than 10 min.

Absolute recoveries of the three drugs from serum, urine and CSF were determined with samples of known amounts standard addition. Mean recoveries (n=5) of INH, PZA and RFT were, in all cases, not less than 93%, 92% and 85%, respectively.

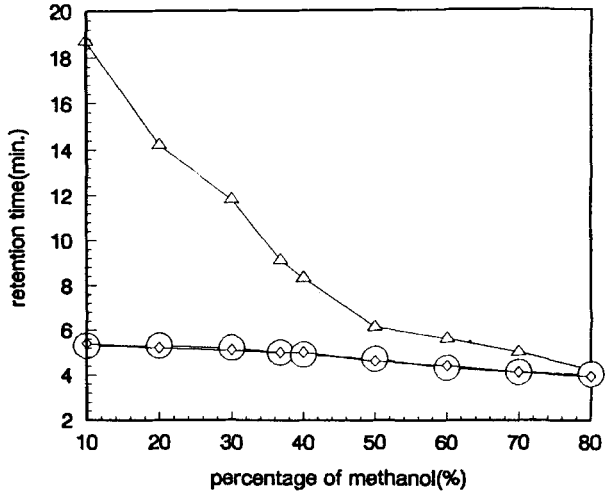

Figure 2. Relationship of drugs' retention time with percentage of mobile phase on phenyl column. —Δ— RFT; ◊ — INH; —○— PZA.

Table 2

Effectiveness of Different Treating Time and H_3PO_4 Conc. (Serum)

		INH (ppm)	PZA (ppm)	RFT (ppm)
Time (min) (with 10% H_3PO_4)	2	4.3	3.1	4.6
	5	5.7	5.2	4.8
	10	6.4	5.4	5.3
	20	6.4	5.4	5.4
	30	6.3	5.3	5.3
	45	6.5	5.4	5.3
H_3PO_4 Conc. (%) (with 30 min)	1	5.1	4.7	5.0
	2	5.3	5.0	5.3
	5	6.2	5.1	5.2
	10	6.4	5.4	5.3
	20	6.3	5.3	5.4
	30	6.4	5.4	5.3

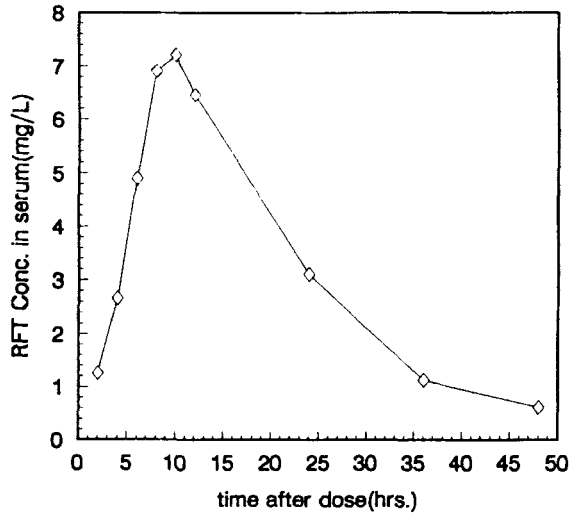

Figure 3. Serum time-concentration profile of RFT to a patient via oral route.

Table 3

Assay Reproducibility (Serum)

Repetition	INH (ppm)	PZA (ppm)	RFT (ppm)
1	13.2	7.4	6.1
2	13.0	7.2	6.4
3	13.2	7.4	6.1
4	13.1	7.3	6.2
5	13.3	7.2	6.6
6	13.6	7.6	6.3
7	13.7	7.1	6.0
8	13.2	7.3	6.3
9	13.4	7.5	6.0
10	13.5	7.6	6.5
Mean	13.32	7.36	6.25
C.V. (%)	1.60	2.20	3.14

Table 4

Sample Analysis Results

Patient No.		INH (ppm)	PZA (ppm)	RFT (ppm)
	Serum	7.8	8.4	6.5
1	Urine	11.3	9.2	7.6
	CSF	5.6	4.1	<0.15
	Serum	11.2	7.5	8.6
2	Urine	12.4	7.8	9.2
	CSF	7.3	3.2	<0.15
	Serum	9.7	5.6	5.4
3	Urine	13.1	7.2	5.2
	CSF	5.2	4.3	<0.15
	Serum	7.4	8.6	7.2
4	Urine	7.7	8.9	9.3
	CSF	4.3	5.3	<0.15
	Serum	12.8	6.8	8.2
5	Urine	14.5	8.3	9.7
	CSF	7.3	4.1	<0.15

Linearity of the Calibration Plots

Least-squares analysis of the calibration curves gave excellent linear responses within the tested concentration range of INH (1-10 ppm), PZA (1-10 ppm) and RFT (1-10 ppm). The correlation coefficients were greater than 0.99.

Reproducibility of the Assay

The intra-day reproducibility of the assay was evaluated by comparing the analysis of the same samples in the same day (Table 3). The Coefficients of variation (CV%) of less than 3.5, indicate excellent intra-day reproducibility.

Analysis Results of Samples and Investigation of RFT Passage through the Human Blood-Brain Barrier

Table 4 shows the analysis results of the three drugs in difference samples. From the results, RFT concentration in CSF is low when the concentration is high in serum. Figure 3 shows the serum concentration-time profile of RFT of a patient via oral route. The peak concentration in serum is at about 7-10 hr. after administration. But, at the time of peak concentration in serum, the concentration in CSF is still less than 0.5 ppm. This indicates that it is difficult for RFT to pass through human blood-brain barrier.

CONCLUSIONS

The HPLC method described herein has sufficient sensitivity to determine INH, PZA and RFT in human serum, urine and CSF following oral and iv bolus dose in the usual therapeutic range. The method is simple, rapid, accurate, and reproducible. Because the drugs have side-effects for humans, this method can be applied to control doses of the drugs to maintain the highest anti-bacterium activity and the lowest concentrations levels of the drugs.

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