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A simultaneous determination of rifampin and 25-desacetylrifampin in cerebrospinal fluid and plasma of rabbit by liquid chromatography

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ABSTRACT A liquid chromatographic assay was modified for the simultaneous determination of rifampin (R) and its major metabolite, 25-desacetylrifampin (25-DR) in cerebrospinal fluid (CSF) and plasma in the rabbit. R, 25-DR and the internal standard, *p*-methylaminobenzoic acid (PMAB), were in an acidified sample at pH 4.2 containing 2% ascorbic acid as anti-oxidant and extracted into organic solvent (diethylether: dichloromethane, 3:2). The residue of the solvent extract was dissolved in 75 μl methanol. The concentration was analysed by a liquid chromatograph consisting of a LDC pump using a reversed phase pre-column (30 μm C_8) linked to a radial pak column (10 μm C_8). The compounds were eluted with acetonitrile-10 mmol/L phosphate buffer, KH_2PO_4, at pH 3.5 (40:60, vol: vol). The eluates are detected at 254 nm. The assay has been used to investigate the disposition of R and 25-DR in CSF and plasma of the rabbits.

KEY WORDS rifampin; 25-desacetylrifampin; cerebrospinal fluid; plasma; rabbits; high pressure liquid chromatography

Rifampin is prescribed as a first line drug in the chemotherapy of tuberculosis (TB). Recent reports described HPLC assays for R and its metabolites in plasma and urine[1,2]. Some of these methods required mobile phases consisting of several organic solvents with fairly high flow rates. We have recently modified a rabbit model to study the penetration of anti-TB drugs into the CSF[3] and a simple HPLC procedure for the assay of R in CSF and plasma in rabbit[4]. The present paper describes the modification of this assay for the simultaneous measurement of R and its major metabolite, 25-DR, in biological fluids of the rabbit.

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MATERIALS AND METHODS

Materials Analar grade organic solvents (dichloromethane, diethylether and methanol) were freshly distilled before use. Water was double-distilled. Ascorbic acid buffer (2%, wt/vol) was prepared by dissolving ascorbic acid (AR) in KH_2PO_4 buffer 1 mol/L and the pH was adjusted to 4.2 with HCl 1 mol/L. Rifampin and 25-desacetylrifampicin were gifts from Ciba Geigy, Switzerland. *p*-methylaminobenzoic acid was purchased from Sigma Chemical Co. Other apparatus used included: 15-ml centrifuge tubes with well fitted screw caps (Sovirel, Levallois-Perret, France) and 15-ml stoppered evaporation tubes with finely tapered bases of 50 μl capacity (Quick-fit tubes modified in own laboratory). All glasswares were cleaned and silanized by a procedure in order to minimise possible loss of drug adsorbed onto glass walls[5].

Operating conditions of HPLC The liquid chromatographic system consisted of a LCD/Milton Roy pump linked to a Rheodyne 7125 injector with a 25-200 μl loop and a Perkin-Elmer LC 55 detector fixed at 254 nm. Chromatograms were recorded on a flat-bed Cole-Palmer recorder. Analyses were performed at ambient temperature (25±1℃) on a radialpak column housed inside a Waters Z-module and packed with C_8 Bondapak (10 μm) packing linked to a C_8 pre-column (30 μm, 50

mm × 4.6 mm I. D.) purchased from Merck. The mobile phase consisted of a freshly prepared mixture of acetonitrile (HPLC grade from Merck) and KH_2PO_4 10 mmol/L at pH 3.5 (4:6, vol:vol) which was filtered before use through a Millipore type AA filter and was run at a flow rate of 1.5 ml/min. Further degassing of the solvent mixture was not found necessary if used immediately after filtration.

Preparation of reagents and standards Standard methanolic solutions of R, 25-DR and some other anti-TB drugs (pyrazinamide, isoniazid and *p*-aminosalicylic acid) and prospective internal standards (*p*-acetamidobenzoic acid, *p*-methylaminobenzoic acid, dimethylaminobenzoic acid and propyl-paraben) were prepared at 1 mg/ml concentration. Subsequent dilutions to 1 μg/ml were made and aliquots of 25 μl were injected into the liquid chromatograph to obtain optimal conditions for analysis. Eventually, p-methylaminobenzoic acid (PMAB) was chosen as the suitable internal standard. Dilutions of R and 25-DR were made up in drug-free CSF or plasma (0.2 ml) to cover the calibration range 1, 2, 3, 4 and 5 μg/ml for DR and 2, 4, 6, 8 and 10 μg/ml for R.

General procedure Into a 15 ml glass centrifuge tube containing 0.2 ml plasma or CSF, the internal standard, PMAB (1.5 μg, 15 μl of 100 μg/ml solution) was added. Methanol (200 μl) was then added to precipitate the proteins in the biofluid, followed by 1 ml of KH_2PO_4 buffer 1 mol/L containing 2% ascorbic acid at pH 4.2. The acidic mixture was extracted twice with 8.5 ml of a solvent mixture (diethylether : dichloromethane, 3:2) 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 an evaporation tube: the combined extract was then evaporated to dryness at 38℃ with the aid of nitrogen. The residue was dissolved in methanol (75 μl) which was added round the side of the tube and vortexed for 30 s. The tube was then stoppered and kept in ice to condense the methanol. The whole content was analysed by the HPLC system.

Recovery, selectivity and precision studies The recovery of R and 25-DR from plasma using the general procedure was assessed by adding the drugs to drug-free plasma samples at 5 and 10 μg/ml concentrations for 25-DR and R respectively. These samples were assayed as described under General Procedure. For comparison, the same concentration of R, 25-DR and PMAB (internal standard) were prepared in evaporation tubes topped up with the extracting solvent mixture (same volume as that for extraction). All samples were assayed with the extraction step omitted. The corresponding peak-height ratios of R and 25-DR to PMAB from plasma extractions and those from the samples without extractions were compared. The selectivity of the HPLC separation was assessed by screening methanolic solutions of various anti-TB drugs which were usually co-administered.

For interbatch measurement of samples, two standard plasma samples (at 3 μg and 6 μg/ml respectively for 25-DR and R) were run, one at the beginning and the other at the end of assaying the batch. The peak height ratios of these two standards were compared with those at concentration points on the respective calibration graphs.

Quantitation and storage studies Drug-free plasma samples were spiked with R and 25-DR to cover the calibration ranges of 2, 4, 6, 8 and 10 μg/ml and 1, 2, 3, 4 and 5 μg/ml, respectively. Calibration graphs were constructed by plotting the peak-height ratios of R & 25-DR to PMAB respectively against the concentrations. Analysis and measurement at each concentration point of the calibration was repeated 6 times as described under the General Procedure. Quantitation of unknown samples was

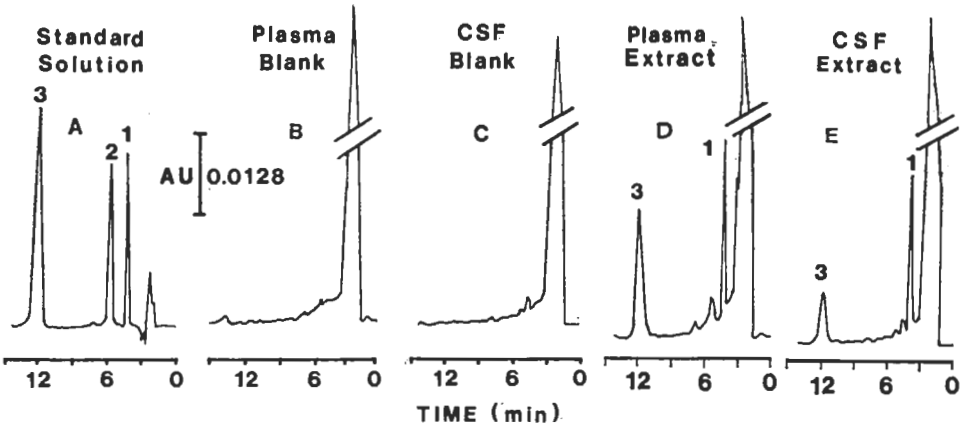

Fig 1. Chromatograms of 1) *p*-methylaminobenzoic acid 1.5 μg, as internal standard, 2) 25-desacetylrifampin 2 μg, and 3) rifampin (R) 6 μg. A) Standard solution, B) Extracts of drug-free plasma, C) Extracts of cerebrospinal fluid, D) Plasma extract of R 3.8 μg/ml, and E) CSF extract of R 1.5 μg/ml

achieved by relating the respective peak-height ratios to obtain the concentrations from the calibration graph.

Samples of drug-free plasma/CSF spiked with R and 25-DR or samples from rabbits after R administration were assayed immediately and after storage at -20℃ for 7 d and 6 months. Peak height ratios were compared.

Application The assay procedure was used to study the concentrations of R and 25-DR in plasma and CSF of the rabbit after iv administration (10 mg/kg) using a rabbit model previously described[3]. Blood samples (0.5 ml) were obtained at 0, 2.5, 5, 10, 15, 30 min and at 1, 2, 4, 5, 6, 8, 12 and 24 h, CSF samples at 0.5, 1, 4, 6 and 24 h after drug administration. Plasma and CSF concentrations of R and 25-DR were measured.

RESULTS AND DISCUSSION

Internal Standard In the process of screening compounds for internal standard for rifampin[4] and *p*-aminobenzoic acid and its metabolites[8], it was established that compounds such as PMAB and dimethylaminobenzoic acid (DMAB) were suitable candidates. Under the present HPLC conditions PMAB was preferred as DMAB could not be resolved from 25-DR. Thus the choice of internal standard was from a chromatographic approach as PMAB and R were quite different structurally.

Fig 1 shows chromatograms of standard solutions of 25-DR, R, and PMAB extracts from drug-free plasma and CSF samples and biological samples from rabbit after iv administration of R. The analytical peaks of R, 25-DR and PMAB are well resolved and their peak symmetry factors are within the BP limits[6]. The use of peak-height ratios for quantitations is considered acceptable. PMAB was chosen as the internal standard because of its better resolution than DMAB from the analytical peaks of 25-DR and R. The respective retention times of PMAB, DMAB, 25-DR and R were 4.5, 6.0, 6.4 and 11.25 min.

Recovery of R and 25-DR The use of diethylether-dichloromethane mixture has two advantages: the mixture forms the top layer of the extraction system thus making transfer easy for second extraction. The organic solvents boil at low temperature and evaporation is more efficient with the aid of nitrogen, hence, oxidation of R or 25-DR is

Tab 1. Correlation between peak-height ratios and concentrations of rifampin (R) and 25-desacetylrifampin (25-DR). Batch standard = a measure of between-day precision. PMAB = *p*-methylaminobenzoic acid, as internal standard. n = 6, $\bar{x} \pm SD$

Concentration μg ml^{-1}	Peak-height ratio R or 25-DR/PMAB	Coefficient of variation (%)
	Rifampin (R)	
2	0.418±0.012	2.9
4	0.813±0.037	4.6
6	1.213±0.049	4.0
8	1.547±0.075	4.8
10	1.962±0.085	4.3
Batch standard at 6 μg/ml (n = 8)		
	1.209±0.076	6.3
Calibration graph:	$y = 5.144x - 0.1036$; $r = 0.9996$	
	25-desacetylrifampin (25-DR)	
1	0.408±0.014	3.4
2	0.814±0.030	3.7
3	1.223±0.084	6.9
4	1.501±0.090	6.0
5	1.897±0.103	5.4
Batch standard at 3 μg/ml (n = 8)		
	1.230±0.076	6.2
Calibration graph:	$y = 2.650x - 0.0798$; $r = 0.9985$	

minimized. The addition of methanol to biological fluids to precipitate proteins prior to extraction improves the recovery of drugs[7]. Two extractions increased the recovery of R from 69% to 95.4% at 40 μg/ml[4]. In the present study, the recovery of R and 25-DR at 5 and 10 μg/ml was 86 (83 to 95%) and 97 (88 to 99.8%), respectively.

Accuracy of the assay The reproducibility and accuracy of the procedure was considered satisfactory over the calibration ranges for R and 25-DR, as shown by the acceptable values of coefficients of variation at each concentration point. The linearity of the calibration is demonstrated by the high correlation coefficients of peak-height ratios against R and 25-DR concentrations (Tab 1).

Stability of R and 25-DR on storage Biological samples containing R and 25-DR, whether fresh or stored at −20℃ for 7 d and 6 months did not show perceptible loss of the drugs. Extracted residues, containing R, 25-DR and PMAB, stored overnight at −20℃ were found to be stable. During the extraction procedure R and 25-DR were unstable on standing at 25±1℃. The addition of ascorbic acid to the aqueous phase improved stability as previously shown[1,2,4].

Tab 2. Plasma and cerebrospinal fluid (CSF) concentrations (μg/ml) of R (n = 4) and 25-DR (n = 1) and their ratios in rabbits after iv of R 10 mg/kg. $\bar{x} \pm SD$

Time (h)	Plasma concn R	Plasma concn 25-DR	CSF concn of R	CSF/Plasma ratio of R
0	—	—	—	—
0.04	57.7±12.1	—	—	—
0.08	35.0±10.2	—	—	—
0.16	27.4±3.2	—	—	—
0.25	24.1±1.9	—	—	—
0.50	15.8±6.1	—	0.68±0.5	0.045±0.029
1.0	14.9±4.3	—	0.87±0.2	0.068±0.047
2.0	12.7±3.1	0.16	—	—
4.0	9.6±4.0	0.10	0.54±0.4	0.111±0.177
5.0	7.4±3.9	0.14	—	—
6.0	6.3±3.4	0.12	0.43±0.4	0.048±0.038
8.0	5.1±3.9	0.23	—	—
12.0	3.5±1.7	0.13	—	—
24.0	1.1±0.6	0.24	—	—

Excessive heat during evaporation also rendered loss of R. Hence, evaporation at low temperature with the aid of nitrogen is essential.

Disposition of R and 25-DR in rabbit The assay was used to determine concentrations of R and 25-DR in the CSF and plasma of 4 rabbits after iv R (10 mg/kg). (Tab 2). The higher concentrations during the early distribution phase were obtained by relating the peak height ratios to the equation derived from the calibration graph, $y = 5.144x - 0.1036$. Between 4 to 10% of plasma concentration of R got across the blood brain barrier. A cross-over study between iv and oral routes is being conducted. This preliminary study suggests that the HPLC assay can be used to follow the disposition and metabolism of R in the rabbit. The method has several advantages over microbiological assays and previously published chromatographic methods. It uses 0.2 ml of biological fluids only. The analysis is sensitive and selective. The inclusion of ascorbic acid helps to stabilize the drugs during extraction. One drawback of the procedure is that two organic solvent extractions are required for better recovery.

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用高压液相色谱法同时测定脑脊液及血浆中利福平及其代谢产物25-脱乙酰利福平

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提要 在含有 2 %抗坏血酸的酸性样品 (pH 4.2)中，将利福平(R)，25-脱乙酰利福平(25-DR)及内标物 *p*-甲氨基苯甲酸(PMAB) 提取到二甲基乙醚:二氯甲烷(3:2)的有机溶剂中.残渣溶于 75 ml 甲醇中.浓缩物在装有带预柱(30 μmC_8)的 μ-Bondapak C_8 径向柱的高压液相色谱系统上，用乙腈 10 mmol/L 磷酸缓冲液，pH 3.5(40:60, vol:vol)冲洗，以 254 nm 检测. 在兔所得数据相关系数分别为 0.9996 (R) 和 0.9985 (25-DR).回收率分别为 86%(R)和 97%(25-DR).

关键词 利福平; 25-脱乙酰利福平; 脑脊髓液; 血浆; 兔; 高压液相色谱法