ELSEVIER

Journal of Chromatography B, 658 (1994) 391–396

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Comprehensive assay for pyrazinamide, rifampicin and isoniazid with its hydrazine metabolites in human plasma by column liquid chromatography

A. Walubo*, P. Smith, P.I. Folb

Department of Pharmacology, University of Cape Town, Medical School, Observatory 7925, South Africa

First received 3 January 1994; revised manuscript received 25 April 1994

Abstract

A comprehensive assay for determination of pyrazinamide (PZA), rifampicin (RIF), isoniazid (INH) and hydrazine metabolites is described. The method involves organic solvent extraction of PZA and RIF, followed by derivatization of INH, monoacetylhydrazine (mHYD) and hydrazine (HYD) with salicylaldehyde and extraction with diethyl ether. Acetylisoniazid (acINH) and diacetylhydrazine (dHYD) were hydrolyzed to INH and mHYD, respectively, and processed as above. Using a gradient solvent programmer, PZA and RIF were analyzed on a C_8 (5 μm) column at 248 nm, while INH and metabolites were analyzed on a C_{18} (5 μm) ODS2 column at 280 nm.

1. Introduction

Since isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA) form one of the most effective antituberculous (anti-TB) regimens used in many countries, considerable effort has been spent on improving the efficacy of this regimen. Combined formulations like Rifater were introduced to improve acceptability and compliance, while intermittent short course therapy was used to reduce adverse reactions and improve the quality of life. In spite of these efforts, the problem of drug toxicity during treatment with these agents still exists, especially in patients with human immunodeficiency virus (HIV) infection [1], and there has been a global increase in the prevalence of drug-resistant tuberculosis (TB) [2,3]. Complicated TB, e.g. multidrug-resistant (MDR) TB or TB with HIV, does not conform to the original TB syndrome for which the present regimens were prescribed. The effect of complicated TB on the disposition of anti-TB drugs is not yet clear and adjustments in treatment regimens during these complications are still empirical [2]. Knowledge of the drug concentrations on plasma is important in the case of drug resistance and toxicity, and monitoring of the concentrations of toxic metabolites may be beneficial in the case of toxicity [4]. Therefore, drug monitoring in patients during anti-TB therapy has become necessary and a methodology for use in therapeutic monitoring had to be developed.

Unfortunately, none of the existing procedures could be used for this purpose since the assay conditions were favorable for only one [5,6] or

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00230-3

two drugs [7] and no metabolites were measured. Furthermore, the use of separate procedures would require large sample volumes which may not be available when blood samples are also necessary for other laboratory evaluations. Therefore, a procedure had to be developed that would be more useful in clinical studies and which would allow optimum use of the sample volume, thereby minimising both interference with other investigations as well as the inconvenience caused to the patient.

Using a modification of previously reported procedures [8–11], we describe here a comprehensive methodology for determination of RIF, PZA and INH with its hydrazine metabolites [acetylisoniazid (acINH), hydrazine (HYD), monoacetylhydrazine (mHYD) and diacetylhydrazine (dHYD)] in human plasma by high-performance liquid chromatography (HPLC) for use in drug monitoring during treatment of complicated TB.

2. Experimental

2.1. Materials

INH, RIF, PZA, hydrazine hydrate, phenelzine (PHEN), N-butarylaminophenol (NBAP), heptane sulfonic acid (HSA), and acetic acid (AA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). AcINH, mHYD and dHYD were synthesised in the laboratory according to the procedures described by Turner [12], and were characterized by HPLC, UV absorption and mass spectrometry, and thin-layer chromatography (TLC: system T1) [13]. Briefly, the compounds, at 1 mg/ml in methanolic solutions, were applied on a silica plate (coated with G 60, 0.2 mm thick; Sigma) 1.5 cm from the bottom. The plate was placed in a tank containing ammonia–methanol (1.5:100, v/v) to a depth of 0.5–1 cm, and the tank was kept air-tight with a lid. After 30 min the plate was air-dried at room temperature and the compounds were visualized with potassium-iodoplatinate (Sigma). The salicylhydrazones (HDZ) of INH (INH-HDZ), PHEN (PHEN-HDZ), mHYD (mHYD-HDZ) and HYD (Azine) were synthesised and identified as described earlier [10,11,14].

2.2. HPLC apparatus

HPLC assays were performed with SpectraPhysics (San Jose, CA, USA) instruments which consisted of a pump Model SP 8800, a degasser SCM 400, autosampler SP 8780 and integrator SP 4290, connected to a variable-wavelength UV detector Model 0200-4000, Linear Corp. (Reno, NV, USA).

2.3. Mobile phase systems

System 1

Mobile phase A consisted of acetonitrile (100%) in bottle A and 5 m*M* phosphate buffer pH 3.5 in bottle B. The flow-rate was 2 ml/min with a gradient from 6% A to 90% A in 5 min. Subsequently this concentration (90% A) was held for 12 min. A reversed-phase C_8 analytical column (Spherisorb, 250 × 4.6 mm I.D., 5 μm; Phase Sep., USA) linked to a C_8 pre-column (50 × 4.6 mm I.D., 30 μm) was used. Maximum pressure was 124 bar.

System 2

Mobile phase B consisted of 100% acetonitrile in bottle A, 5 m*M* HSA in acetonitrile–water–triethylamine (70:30:0.4, v/v) (pH 6) in bottle B and 100% water in bottle C. Composition of the mobile was B–C (75:25, v/v) for the first 5 min and then B–A (85:15, v/v) for 12 min. The flow-rate was 1 ml/min. A reversed-phase C_{18} analytical column (Spherisorb, 250 × 4.6 mm I.D., S5 ODS2; Phase Sep.) linked to a C_8 pre-column (50 × 4.6 mm I.D., 30 μm) was used. Maximum pressure was 103 bar.

2.4. Sample preparation

(a) Determination of RIF and PZA

After addition of internal standards [50 μl of NBAP (50 μg/ml) and 50 μl of PHEN (50 μg/ml)] to 2 ml of plasma in a 15-ml test-tube, 0.4 ml of 10% (v/v) aqueous AA was added to

adjust the pH to 4.2. RIF and PZA were extracted by shaking with 7 ml of diethyl ether–dichloromethane (2:1, v/v) which, after centrifugation for 10 min at 2059 *g*, was transferred to a tapered test-tube and evaporated under nitrogen at 40°C. The residue was dissolved in 200 μl of methanol of which a 20-μl aliquot was injected onto the HPLC column and eluted with system 1. The eluate was detected at 248 nm.

(b) Determination of INH, mHYD and HYD

To the aqueous fraction obtained after extraction of RIF and PZA, further 10% aq. AA (0.6 ml) is added to lower the pH to 3.2. A 0.3-ml volume of an ethanolic solution of 0.1% (v/v) salicylaldehyde was added and derivatization was completed by incubation in a water bath at 60°C for 30 min. The mixture was then cooled to room temperature (25 ± 1°C), 1 ml of 1 *M* K_2PO_4 was added and the mixture was extracted twice with 7 ml of diethyl ether which, after centrifugation for 10 min at 2059 *g*, was transferred to a tapered test-tube and evaporated under nitrogen at 40°C. The residue was dissolved in 200 μl of mobile phase B (system 2) of which a 20-μl aliquot was injected onto the HPLC column and eluted with system 2. The eluate was detected at 280 nm.

(c) Determination of acINH and dHYD

The aqueous fraction obtained after the organic extraction described in (b) was transferred to a clean 15-ml test-tube and 0.5 ml of 5 *M* HCL was added. AcINH and dHYD were hydrolysed to INH and mHYD respectively by incubating in a water bath at 60°C for 45 min. The mixture was then cooled to room temperature (25°C) and 0.45 ml of 5 *M* NaOH was added to adjust the pH to 2.2. Then internal standard PHEN (50 μl of 50 μg/ml) and 0.3 ml of ethanolic solution of 0.1% (v/v) salicylaldehyde were added. Derivatization was completed by incubation in a water bath at 60°C for 30 min. The mixture was then cooled to room temperature (25 ± 1°C), 1 ml of 1 *M* K_2PO_4 was added, and the mixture was extracted with 7 ml of diethyl ether which, after centrifugation for 10 min at 2059 *g*, were transferred to a tapered test-tube and evaporated under nitrogen at 40°C. The residue was dissolved in 200 μl of mobile phase B (system 2) and a 20-μl aliquot was injected onto the HPLC column and eluted with system 2. The eluate was detected at 280 nm.

3. Results and discussion

3.1. Characterisation of compounds

The TLC R_F values were: INH = 0.55 and INH-HDZ = 0.68, acINH = 0.55, mHYD = 0.65 and mHYD-HDZ = 0.71, dHYD = 0.45, and HYD = 0.09 and Azine = 0.76, PHEN = 0.60 and 0.78, and PHEN-HDZ = 0.78 and salicylaldehyde = 0.64.

3.2. Performance of HPLC system

Figs. 1 and 2 show representative chromatograms of standard solutions, blank plasma and a plasma extract from a patient 6 h after dosing, while Table 1 illustrates the standardisation data of the assay. Retention times in min were: PZA = 2.91; RIF = 11.9; mHYD = 3.75; INH = 4.68; HYD = 13.77; NBAP = 4.22; PHEN = 11.09, and excess salicylaldehyde = 5.78 (Fig. 2). The use of one internal standard did not affect linearity in the concentration ranges used. There was a great variation in the washing out of excess salicylaldehyde with 1 *M* K_2PO_4 (Fig. 2B versus 2C and D). This was most probably due to difficulties in attaining the optimum conditions for this process (e.g. pH). However, this had no effect on the resolution or recovery of the relevant compounds.

The run time for PZA and RIF was 15 min while that for INH and metabolites was 17 min. The complete extraction and derivatization procedure takes approximately 4 h, a time uncomparable to the time needed when the drugs were extracted and run separately using different procedures.

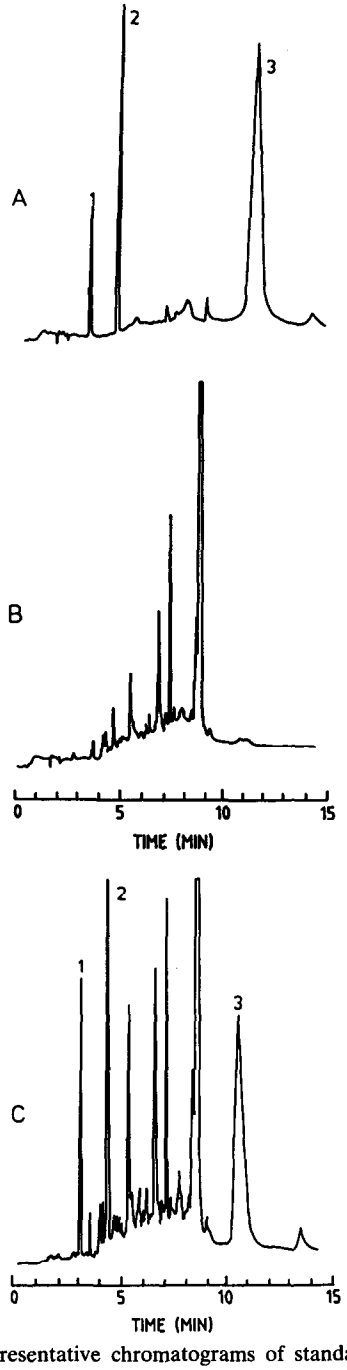

Fig. 1. Representative chromatograms of standard solution (A), drug free plasma (B), and a plasma extract (C) from a patient 6 h after dosing, with PZA, RIF and INH at 25.9, 10.3 and 5.2 mg/kg, respectively. Peaks: 1 = PZA (2.91 min); 2 = NBAP (internal standard, 4.22 min); 3 = RIF (11.9 min). (A) PZA = 0.4 μg and RIF = 0.25 μg was injected. (C) PZA = 24.3 μg/ml and RIF = 4.28 μg/ml.

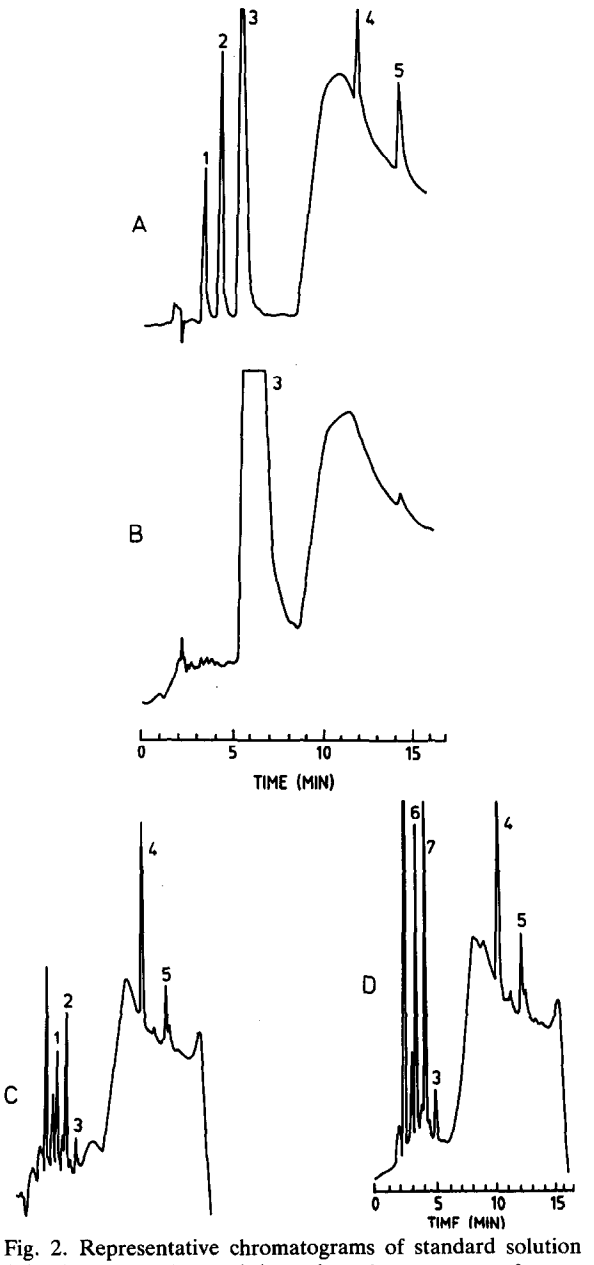

Fig. 2. Representative chromatograms of standard solution (A), drug free plasma (B), and a plasma extract from a patient 6 h after dosing as in Fig 1, before (C) and after hydrolysis (D). Peaks: 1 = mHYD (3.75 min); 2 = INH (4.68 min); 3 = salicylaldehyde (5.78 min); 4 = PHEN (internal standard, 11.09 min); 5 = HYD (13.77 min); 6 = dHYD (3.75 min); 7 = acINH (4.68 min). (A) Salicylhdrazones equivalent to 0.25 μg of INH, 0.1 μg of mHYD and 0.02 μg of HYD were injected. (C and D) mHYD = 1.2 μg/ml, INH = 3.75 μg/ml, HYD = 0.28 μg/ml, dHYD = 0.41 μg/ml and acINH = 4.24 μg/ml.

Table 1
Summary of standardisation data on recovery, linearity, and limit of detection

Drug	Linear regression (r) ($n = 5$)	Inter-assay C.V. (at 5 μg/ml) (%)	Recovery (at 5μg/ml) (%)	Detection limit (μg/ml)
Pyrazinamide	0.997	2.3[a]	52.6 ± 3.2[a]	0.5
Rifampicin	0.996	9.8	54.4 ± 3.7	0.2
Isoniazid	0.998	9.8	46.3 ± 5.2	0.25
Acetylisoniazid	0.997	7.6	32.8 ± 3.5	0.25
Monoacetylhydrazine	0.999	6.2	49.2 ± 4.3	0.2
Diacetylhydrazine	0.999	12.5	34.6 ± 2.8	0.2
Hydrazine	0.999	6.2[b]	48.5 ± 3.5[b]	0.05

[a,b] Evaluation for C.V. and recovery was done at 15 and 0.2 μg/ml, respectively.

3.3. Recovery, precision, linearity and limit of detection

Recovery was assessed by comparing peak heights of the directly injected hydrazones (for INH and metabolites) or drug (for PZA and RIF) with those obtained after derivatization and extraction. Peak heights were evaluated automatically by the built-in integrator and peak-height ratios of the drugs to internal standard were used for deriving concentrations.

Concentration ranges of: 0.5, 1, 2, 5, and 8 μg/ml for RIF, INH, acINH, mHYD, and dHYD; 0.1, 0.2, 0.5 and 0.8 μg/ml for HYD; and 5, 10, 15, 20, and 30 μg/ml for PZA were used for standardisation and regression analysis. Calibration equations of five standard curves were as follows: $y = 0.034x - 0.024$ for PZA; $y = 0.143x + 0.012$ for RIF; $y = 0.209x - 0.034$ for INH; $y = 0.352x - 0.025$ for acINH; $y = 0.463x + 0.009$ for mHYD and $y = 1.435x + 0.022$ for HYD. The mean regression coefficient (r) of the five standard curves and inter-assay coefficient of variation (C.V.%) at mid-point (5 μg/ml) of the curves are shown in Table 1. For PZA and HYD the midpoint was 15 and 0.2 μg/ml, respectively.

Overall recovery at the mid-point of the standard curves is shown in Table 1. These recoveries, although lower, do not significantly differ from the values obtained with the original procedures. In the case of PZA and RIF, the poor recovery could have occurred due to the use of only a single organic extraction step, failure to precipitate proteins before extraction and, for RIF, loss of drug due to auto-oxidation in the absence of vitamin C. Lower recovery for INH and metabolites could have arisen from solubility limitations of the salicylhydrazones in the organic phase and adsorption to glassware.

Minimum reliable concentrations assayed are shown in Table 1. At concentrations lower than the limits stated, the coefficient of variation is rather high and the original procedures are preferred.

Drugs such as paracetamol and PAS were tested and did not interfere with the assay.

3.4. Application

The assay has been used to monitor patients with treatment problems. One of the cases is reported here. Briefly, it concerned a 54-year-old female patient who had been on anti-TB therapy (RIF 8.8 mg/kg, INH 8.8 mg/kg, ethambutol [not assayed] and streptomycin [for the first two months only]) for four months without improvement, in spite of the organism's sensitivity to the drugs. She was found to have moderate gastric outlet obstruction which was causing vomiting and it was not clear whether adequate plasma concentrations were being achieved. Five hours after dosing the plasma concentrations found were (μg/ml): RIF = 2.09, INH = 4.77, acINH = 6.89, mHYD = 1.8, dHYD = 0.56 and HYD 0.35. Except for RIF these levels were

similar to those found in other patients in this setting where other assay procedures have been used and the values did not differ from the usual concentration ranges reported elsewhere [2,12]. Medication of the patient was adjusted to syrup preparations to improve absorption and compliance, and PZA was added to the regimen.

4. Discussion

The assay described here is beneficial in terms of sample utilisation and the limited amount of reagents used to analyse many compounds in a few hours. It saves the burden of running different assays for each drug, and helps the clinician to evaluate all drugs and the relevant metabolites in a short time. The method is reproducible and can be adopted in any laboratory with a gradient programmer. For instance, system 2 is effectively run isocratically at 52.5% acetonitrile for the first five minutes, and then 74.5% acetonitrile for the next 12 min. It was also found that the column in system 2 can be used in system 1 without affecting the resolution of the compounds. The retention time for INH-HDZ was pH sensitive whereby at a pH <4 it co-eluted with the salicylaldehyde. The low sensitivity of this procedure does not preclude its usefulness in the circumstances for which it has been developed. It is a useful clinical and research tool for quantitative assay of the compounds studied.

Acknowledgements

This study was sponsored by the University Research Committee, the Medical Research Council and Department of Pharmacology. The authors are thankful to the technical staff in the Department of Pharmacology for their assistance and Prof. K. Chan of the School of Pharmacy at Liverpool, John Moores University for reviewing the article.

References

[1] P.F. Barnes and S.A. Barrows, *Ann. Int. Med.*, 119 (1993) 400.
[2] M.D. Iseman, *N. Eng. J. Med.*, 329 (1993) 784.
[3] J. Woo, C.H.S. Chan, A. Walubo and K. Chan, *J. Med. (N.Y.)*, 23 (1992) 51-59.
[4] J.H. Grosset, *Tubercle Lung Dis.*, 73 (1992) 378.
[5] A.B.M. Jamaluddin, G. Sarwar and M.K. Rahman, *J. Chromatogr.*, 529 (1990) 595.
[6] K.J. Swart and M. Papgis, *J. Chromatogr.*, 593 (1992) 21.
[7] C.D. Gaitonde and P.V. Pathak, *J. Chromatogr.*, 532 (1990) 418.
[8] K. Chan, *J. Liq. Chromatogr.*, 11 (1988) 1051.
[9] W. Von Sassen, M. Castro-Parra, E. Mush and M. Eichelbaum, *J. Chromatogr.*, 338 (1985) 113.
[10] P.J. Jenner and G.A. Ellard, *J. Chromatogr.*, 415 (1987) 188.
[11] A. Walubo, K. Chan and C.L. Wong, *J. Chromatogr.*, 567 (1991) 261.
[12] R.A. Turner, *J. Am. Chem. Soc.*, 69 (1947) 875.
[13] A.S. Curry and E.G. Clarke (Editors), *Clarke's Isolation and Identification of Drugs, Vol. 1,* The Pharmaceutical Press, London, 1975, p. 43.
[14] H.L. Yale, K. Losee, J. Martins, M. Holsing, F.M. Perry and J. Berstein, *J. Am. Chem. Soc.*, 75 (1953) 1933.
[15] L. Heifets, *Am. Rev. Respir. Dis.*, 137 (1988) 1217.