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Note

Rapid liquid chromatographic method for the estimation of isoniazid and pyrazinamide in plasma and urine

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Three main antitubercular drugs, isoniazid, rifampicin and pyrazinamide, have been classified as first- or second-line drugs. The importance of these drugs is due to their popularity in the treatment of pulmonary tuberculosis, imparting bacteriostatic capabilities and therapeutic value.

Non-chromatographic assays developed for isoniazid in serum and plasma include spectrophotometric [1] and spectrofluorometric [2] procedures. Pyrazinamide and its metabolites in biological fluids can be determined by paper chromatography [3], thin-layer chromatography [4] and an anion-exchange classical column technique [5]. These procedures are often time-consuming and tedious. High-performance liquid chromatographic (HPLC) methods for estimation of isoniazid [6–9] involving derivatization and for pyrazinamide [10,11] in biological fluids are non-specific and laborious. Brouard *et al.* [12] developed an HPLC method for pyrazinamide in plasma samples, but it lacks sensitivity with low recovery rates hindered by inadequacies in the technique used to prepare the plasma samples.

This paper describes a method for the extraction and separation of isoniazid and pyrazinamide from human plasma and urine in a single step. Plasma and urine samples were subjected to a solid-phase extraction clean-up procedure. Quantitation was accomplished with rifampicin as an internal standard. The complete process of extraction and separation took only 12 min, with high recovery rates from biological samples.

EXPERIMENTAL

Reagents

Analytical-reagent-grade tetra-*n*-butylammonium hydroxide (0.1 *M* in aqueous medium; Sisco Research Labs., Bombay, India), chloroform, phosphoric

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acid, 2-propanol (E. Merck India, Bombay, India), HPLC-grade methanol (S.D. Fine Chemicals, Tarapur, India) and distilled, deionized water prepared in our laboratory were used for the analysis.

Reference standards of isoniazid, pyrazinamide and rifampicin were obtained from the Central Drug Laboratory (Calcutta, India).

Equipment

All the chromatograms were obtained with an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) ConstaMetric III dual-piston pump, SpectroMonitor III 1204 UV–visible detector, and a computing integrator 10 (CI-10) with Sekonics (Sekonics, Tokyo, Japan) printer plotter. Sample introduction was with a Rheodyne Model 7125 valve injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 20-μl loop. The column, Excalibar C_{18}-CN (Alltech Assoc., Deerfield, IL, U.S.A.), 250 mm × 4.6 mm I.D. and 5 μm particle size, was used at ambient temperature. The detection wavelength was 265 nm at a sensitivity of 0.005 a.u.f.s.

The mobile phase consisted of methanol–0.005 *M* tetra-*n*-butylammonium hydroxide (80:20, v/v), adjusted to pH 3.0 with phosphoric acid. A flow-rate of 1.5 ml/min (inlet pressure 82.7 bars) was maintained constant throughout the analysis. The mobile phase was filtered through a 0 45-μm Millipore filter (Waters Assoc., Milford, MA, U.S.A.).

Extraction procedure

A solid-phase extraction (SPE) CN-bonded maxi-clean cartridge (Alltech Assoc.) was conditioned with 5 ml of methanol followed by 2 ml of 1% acetic acid in water. Samples of plasma (2 ml) were added to 1.0 ml of 2-propanol–chloroform (1:1, v/v) contained in a culture tube. The internal standard, rifampicin (500 ng/ml) was then added and the tube was shaken for 30 s. The contents were then transferred to a disposable 3.0-ml SPE cartridge with rinses. The eluate was then collected in a sample vial using a vacuum manifold and used directly for chromatographic analysis. The same procedure was used for urine samples. Control plasma and urine samples were treated in the same manner, except for the addition of the rifampicin internal standard.

RESULTS AND DISCUSSION

The samples of plasma and urine were obtained from a patient volunteer receiving an oral dose of pyrazinamide (750 mg) and isoniazid (300 mg) tablets, 3 h after drug administration. Control samples were obtained from the same patient 3 h before drug administration.

Representative chromatograms obtained from plasma and urine extracts are shown in Fig. 1. The retention times for pyrazinamide, isoniazid and rifampicin were 1.4, 2 7 and 4.3 min, respectively. The first peak in all the chromatograms

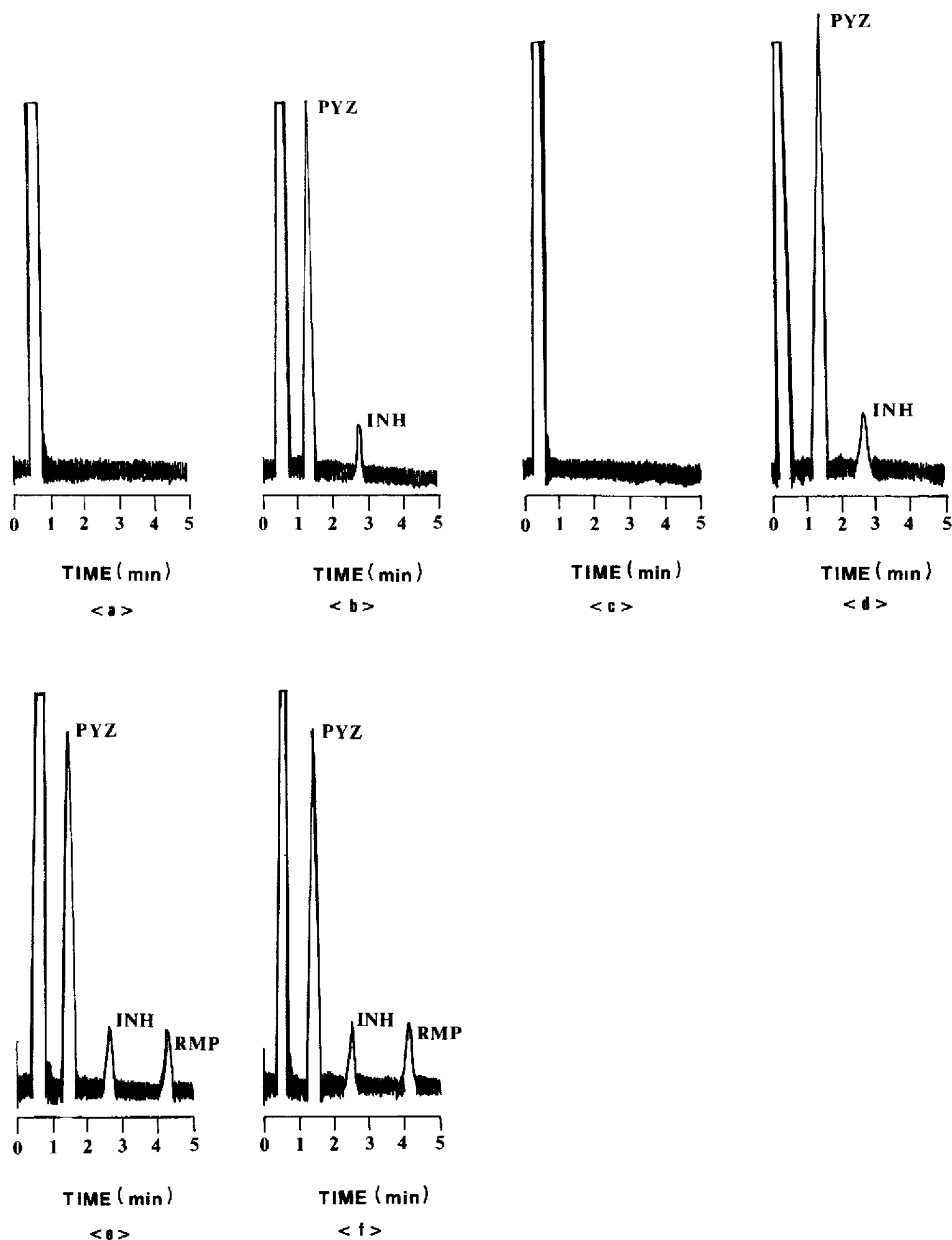

Fig. 1. Chromatograms of extracts obtained from (a) control plasma sample, (b) plasma sample containing 700 mg/ml pyrazinamide (PYZ) and 300 ng/ml isoniazid (INH), (c) control urine sample, (d) urine sample containing 800 ng/ml PYZ and 280 ng/ml INH, (e) plasma sample containing 700 ng/ml PYZ, 300 ng/ml INH and 500 ng/ml rifampicin (RMP, internal standard) added prior to extraction, (f) urine sample containing 800 ng/ml PYZ, 280 ng/ml INH and 500 ng/ml RMP (internal standard) added prior to extraction All analyses were performed using 2-ml samples, monitored at 265 nm

was from interference due to endogenous compounds in plasma and urine. However, the analyses were not affected by these interferences.

The recoveries of the two analytes from plasma and urine were assessed by comparing peak heights obtained from the standard stock solutions of the drugs and drug-free plasma or urine spiked with the respective drugs. The extraction recoveries from plasma and urine averaged 99% for pyrazinamide and isoniazid at concentrations of 200, 400, 600 and 800 ng/ml (Tables I and II). To assess the precision of the procedure, reproducibilities for within-day and day-to-day variations were determined. As shown in Table I, the coefficients of variation (C.V.) for four different concentrations of pyrazinamide in plasma and urine in the within-day study varied between 0.36 and 0.42%, whereas those in the day-to-day study varied between 0.34 and 0.41%. The C.V. for isoniazid in plasma and urine were found to be 0.17–0.33 and 0.16–0.33% for within-day and day-to-day studies, respectively.

TABLE I

RECOVERY OF PYRAZINAMIDE FROM HUMAN PLASMA AND URINE AFTER EXTRACTION

Concentration added (ng/ml)	Concentration found (ng/ml)	Recovery (mean ± S.D., $n = 8$) (%)	Coefficient of variation (%)
Plasma			
Within-day variation			
200	198	99.0 ± 0.42	0.42
400	399	99.7 ± 0.39	0.39
600	601	100.1 ± 0.41	0.40
800	799	99.8 ± 0.37	0.37
Day-to-day variation			
200	199	99.5 ± 0.41	0.41
400	398	99.5 ± 0.40	0.40
800	800	100.0 ± 0.38	0.38
Urine			
Within-day variation			
200	201	100.5 ± 0.36	0.35
400	398	99.5 ± 0.38	0.38
600	600	100.0 ± 0.40	0.40
800	798	99.7 ± 0.41	0.41
Day-to-day variation			
200	200	100.0 ± 0.34	0.34
400	399	99.7 ± 0.37	0.37
800	799	99.8 ± 0.40	0.40

TABLE II

RECOVERY OF ISONIAZID FROM HUMAN PLASMA AND URINE AFTER EXTRACTION

Concentration added (ng/ml)	Concentration found (ng/ml)	Recovery (mean ± S.D., n = 8) (%)	Coefficient of variation (%)
Plasma			
Within-day variation			
200	200	100.0 ± 0.18	0.18
400	401	100.2 ± 0.21	0.20
600	599	99.8 ± 0.20	0.20
800	800	100.0 ± 0.17	0.17
Day-to-day variation			
200	198	99.0 ± 0.16	0.16
400	399	99.7 ± 0.22	0.20
800	801	100.1 ± 0.19	0.18
Urine			
Within day variation			
200	199	99.5 ± 0.28	0.28
400	398	99.5 ± 0.32	0.32
600	598	99.6 ± 0.33	0.33
800	801	100.1 ± 0.29	0.28
Day-to-day variation			
200	198	99.0 ± 0.30	0.30
400	399	99.7 ± 0.33	0.33
800	800	100.0 ± 0.31	0.31

The calibration curves were obtained by plotting the peak-height ratios of pyrazinamide and isoniazid to rifampicin for four different concentrations expected from the dose administration. The response of the UV detector was found to be linear over the concentration range 200–800 ng/ml for pyrazinamide and isoniazid. The regression equations of the calibration curves were found to be $y = 1.053 + 0.0661x$ and $y = 0.8125 + 0.0138x$ for pyrazinamide and isoniazid, respectively. Each point on the calibration curves was repeated at least five times. The correlation coefficients of the calibration curves for pyrazinamide and isoniazid were 0.9998 and 0.9989, respectively. Limits of detection in human plasma and urine samples were estimated to be 250 ng/ml pyrazinamide and 200 ng/ml isoniazid, and 225 ng/ml pyrazinamide and 250 ng/ml isoniazid, respectively, monitored at 265 nm (0.005 a.u.f.s.).

CONCLUSION

The method described here for the extraction and estimation of pyrazinamide

and isoniazid from plasma and urine in a single step is rapid and precise. An internal standard was used to correct for possible errors in handling pipettes and syringes. The high recovery rates (>99%) can be attributed to the SPE procedure used for plasma and urine samples.

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REFERENCES

1 L. Eidus and A. M. T Harananasingh, *Clin. Chem* , 17 (1971) 492
2 W A Olsen, P. G Dayton, Z. H. Israili and A. W. Pruitt, *Clin Chem* , 23 (1977) 745
3 I. M. Weiner and J P Tinker, *J. Pharmacol Exp Ther.*, 180 (1972) 411
4 J. K McClatchy, A. Y Tsang and M. S. Cernich, *Antimicrob. Agents Chemother.*, 20 (1981) 556.
5 C. Auscher, C. Pasquier, P Pehuet and F. Delbarre, *Biomedicine,* 28 (1978) 129.
6 M. A. Moulin, F Albessard, J Lacotte and R Camsonne, *J Chromatogr* , 226 (1981) 250
7 S. J. Saxena, J T. Stewart, I. L. Honigberg, J. G Washington and G R Keene, *J. Pharm Sci* , 66 (1977) 813.
8 M R Holdiness, *J. Liq. Chromatogr.*, 5 (1982) 707.
9 W. Von Sassen, M. Castro-Parra, E. Musch and M. Eichelbaum, *J. Chromatogr.*, 388 (1985) 113
10 B Ratti, A. Toselli, F. Beretta and A. Bernarggi, *Farmaco Ed. Sci.*, 37 (1982) 226.
11 K Chan, C L Wong and S. Lok, *J Chromatogr.*, 380 (1986) 367.
12 A. Brouard, H. Barreteau, H. Merdjan, M Paillet, G. Fredj and M Micoud, *J Chromatogr* , 345 (1985) 453.