TABLE 3: ACCURACY AND PRECISION OF THE METHOD – MEAN VALUES FOR SIMULTANEOUS ASSAY OF TWO DRUGS.

	Initial conc. (mg)	Conc. added (mg)	Total conc. (mg)	Conc. found (mg)	Error (%)	Recovery (%)
Lamivudine	12	0	12	11.9	0.83	99.16
	12	3	15	14.9	0.66	100.6
	12	6	18	17.9	0.55	99.44
Zidovudine	24	0	24	23.9	0.41	99.58
	24	6	30	29.9	0.33	99.66
	24	12	36	36.2	0.55	100.5

% Error=[Difference in conc. added and conc. found/conc. added]x100

drugs incorporating these excipients.

A simple, sensitive method has been developed for the isocratic separation and simultaneous estimation of lamivudine and zidovudine in bulk and tablet dosage form. Hence it can be conveniently adopted for the routine quality control analysis.

REFERENCES

1. Parfitt, K., In; Martindale, The Extra Pharmacopoeia, 32nd Edn., The Pharmaceutical Press, London, 1999, 1.
2. Coates, J.A., Cammack, N., Jenkinson, H.J., Mutton, I.M., Pearson, B. A., Storer, R., Cameron-J.M. and Penn, C.R., **Antimicrob. Agents Chemother.,** 1992, 36, 202
3. Kim, D.D. and Chien,Y.W., **J. Pharm. Sci.,** 1996, 85, 214.
4. Ayrton, J., Evans, M.B., Harris, A.J. and Plumb, R.S., **J. Chromatogr. B.,** 1995, 667, 173.

Simultaneous HPTLC Determination of Rifampicin and Isoniazid in Rat Plasma

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A method enabling the precise and quick simultaneous assessment of rifampicin and isoniazid in rat plasma by HPTLC has been presented. Extracted samples of rifampicin and isoniazid in organic solvents were separated on a plate coated with silica gel 60 F_{254}, chromatograms were developed using a mixture of chloroform:methanol and quantification was carried out by the use of densitometer absorbance mode at 475 nm and 280 nm respectively. Under the operating conditions, the lower limit of detection was found to be 0.3 μg/spot for rifampicin and 0.1 μg/spot for isoniazid. The method has a linearity range of 10 to 80 μg/ml for both the drugs and recovery was found to be 97.5% for both rifampicin and isoniazid. The pharmacokinetic parameters obtained after intravenous bolus injection at a dose of 10 mg/kg has also been presented.

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Combination of rifampicin (RIF) and isoniazid (INH) form one of the most effective antitubercular regimens used in many countries and considerable effort has been spent on improving the efficacy of this regimen. Since therapy requires medication for a prolonged period of time ranging between 3 to 9 months, knowledge of drug concentration in plasma could serve to evaluate the efficacy and adjust the regimen within the therapeutic window. Unfortunately, none of the existing procedures could be used for this purpose since the assay conditions are favorable to only one of these two drugs[1-3]. Furthermore, the use of separate procedure would require large sample volumes and prove to be very tedious process. Therefore, an analytical method that could be executed within minimum time and with less use of organic solvents would serve as an important tool in a clinical setup and research in the development of better dosage regimen using advanced drug delivery technology. Hence the objective of the study was to develop a simple, sensitive and rapid analytical procedure, employing high performance thin layer chromatography (HPTLC), for estimation of plasma concentration of RIF and INH together in rats, which could also be used for analysis of plasma concentration of drugs in human subjects.

RIF and INH were obtained as gift samples from M/s Cadila Pharmaceuticals, Ahmedabad. Pre-coated HPTLC plates (Silica gel G60 F_{254}), methanol and diethyl ether were procured from E. Merck India Ltd., Mumbai. HPTLC system (CAMAG, Switzerland) equipped with application (Camag Linomat IV), twin trough TLC chamber, micro syringe (Linomat syringe 695.0074, 100 μl), PC based TLC scanner 3 and data station with CATS 4 software program were used in the study. All other chemicals and reagents used in the study were of analytical grade.

Standard plots for RIF and INH were prepared by withdrawing 25 ml of blood from 12 normal untreated healthy rats by puncturing the retro-orbital plexus using heparinized capillaries and transferred to sterile heparinized centrifuge tubes taking aseptic precautions. The plasma obtained was used for the standard plot of RIF and INH.

Stock solution of 1 mg/ml of both RIF and INH were prepared in phosphate buffered saline (PBS) (pH 7.4). From this stock solution, different concentrations that include, 40, 80, 120, 160, 200, 240, 280 and 320 μg/ml were prepared in PBS. Then 0.5 ml each of these standard solutions was spiked in 1.75 ml of rat plasma in different test tubes and to all 0.4 ml of 10% aqueous acetic acid was added to adjust the pH to 4.2. Solutions were vortexed in a cyclo-mixer. RIF was extracted thrice by shaking these solutions with 7 ml of diethyl ether:dichloromethane (2:1 v/v). After centrifuging for 10 min at 2000 rpm, organic layer was transferred to a tapered test tube and evaporated under nitrogen at 40°. The residue was reconstituted in 2 ml of methanol to obtain final concentrations of 10, 20, 30, 40, 50, 60, 70 and 80 μg/ml of RIF[4]. Thirty microliters of these samples were applied on the HPTLC plates using Linomat IV micro syringe. HPTLC plates were developed in twin trough chamber saturated with the mobile phase, chloroform: methanol (9:1 v/v). Developed plates were then dried and scanned using a CAMAG scanner 3 in absorbance mode at wavelength of 475 nm. Standard plot was obtained by plotting peak height versus concentration of RIF.

To the aqueous fraction obtained after RIF extraction, 10% aqueous acetic acid (0.6 ml) was added to further lower the pH to 3.2. The solution was vortexed and to this ethanolic solution of salicylaldehyde (0.1 % v/v; 0.4 ml) was added and derivatization was allowed to complete by incubation in water bath at 60° for 30 min. The mixture was then cooled to room temperature. One milliliter of 1M K_3PO_4 was added and mixed thoroughly. INH was extracted thrice with 7 ml of diethyl ether. The procedure followed after this was same as that of RIF except the developed plates here were scanned at wavelength of 280 nm. Analytical method validation was performed according to ICH guidelines[5].

Chromatographic estimations were performed using precoated HPTLC plates of 10x10 cm dimension. Spots were applied as 6 mm bands with a space of 6 mm between two spots using Linomat IV and Camag syringe. The application rate was fixed for 15 s/μl. Plates were then placed in twin trough chambers containing the solvent phase saturated for 20 min at 25±1° and 55±5 % relative humidity. Developed plates were then dried and subjected to scanning using a TLC scanner 3 at wavelengths 475 nm for rifampicin and 280 nm for isoniazid, respectively. Recovery experiments were conducted for both RIF and INH by spiking known concentration of drug in plasma and analyzing the drug concentration after processing the plasma sample as described earlier.

Pharmacokinetic evaluation was performed in male Wistar rats weighing 180 to 200 g. Approval was obtained from Institutional Animal Ethics Committee prior to the commencement of the study. Animals were kept fasting overnight prior to the study. Water was allowed *ad libitum*. Animals were administered 10 mg/kg of free RIF and INH in PBS intravenously through the tail vein[6]. Blood samples were

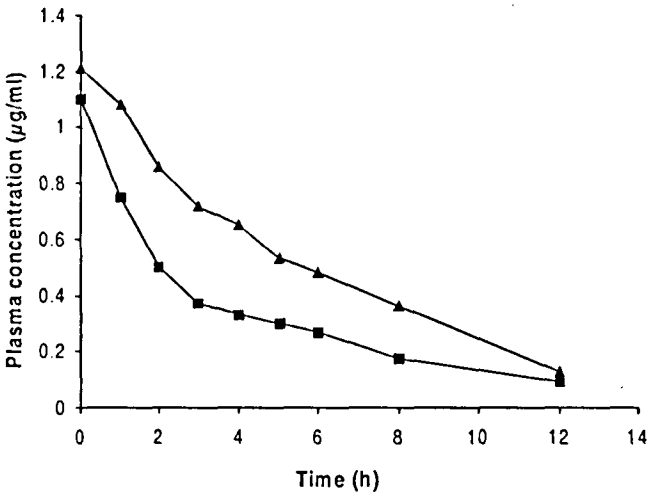

Fig. 1: *In vivo* profile of rifampicin (RIF) and isoniazid (INH) in rat plasma when administered intravenously at a dose of 10 mg kg^{-1} body weight.

Pharmacokinetic study was carried out in male albino rats by administering 10 mg/kg body weight of rifampicin (-▲-) and isoniazid (-■-) intravenously. Blood samples were withdrawn at regular intervals of time and analyzed using the proposed HPTLC method.

collected periodically at 0, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h post treatment in heparinized tubes. Samples obtained were centrifuged at 3000 rpm for 3 min and supernatant i.e. plasma was collected in separate tubes. RIF and INH were extracted and processed as described earlier. Concentrations were obtained from the respective standard plots.

Several chromatographic methods using HPLC have been reported for INH and RIF alone[7] but there is no chromatographic method reported in literature for comprehensive analysis of both the drugs in plasma. Moreover, HPLC requires large volume of organic solvents and is a tedious process. On the other hand HPTLC is a versatile chromatographic tool that could be used with minimum organic solvents with short processing time. This prompted us to develop a new method for RIF and INH estimation in plasma employing HPTLC. Standard plots of both the drugs were linear for the concentrations studied with linear regression coefficient (R^2) of 0.9911 and 0.9887 for RIF and INH respectively. Under the operating condition mentioned above the lower limit of detection were 0.3 μg/spot for RIF and 0.1 μg/spot for INH. Accuracy was determined by calculations of the percentage recovery of the drugs at each level and the average recovery was found to be 97.5% for both rifampicin and isoniazid. The intermediate precision was determined by repeated analysis of the homogeneous samples over a period of 3 d with same instruments and chemicals. The relative standard deviation ranged from 2.02 to 1.81% for intra assay precision and 1.71 to 1.96 for inter assay precision. Fig. 1 depicts the plasma concentration versus time curve for RIF and INH after administration of 10 mg/kg intravenously. Pharmacokinetic parameters obtained are tabulated in Table 1. The proposed HPTLC method is found to be precise, quick and simple. Therefore, this method can easily and conveniently be employed for routine bioequivalence studies of pharmaceutical dosage forms containing RIF and INH in combination.

TABLE 1: PHARMACOKINETIC PARAMETERS OF RIF AND ISONIAZID INH.

Pharmacokinetic parameters	Drugs	
	Rifampicin Mean ± S.D*	Isoniazid Mean ± S.D*
K_e (h^{-1})	0.18±0.013	0.19±0.021
$t_{1/2}$ (h)	3.82±1.18	3.62±0.89
C_{max}	1.21±0.27	1.10±0.16
AUC_0^t	6.51±0.49	3.92±0.31
AUC_0^∞	6.68±0.35	4.01±0.27

*Each value is the mean±standard deviation of six observations. Pharmacokinetic study was carried out in male albino rats by administering 10 mg/kg body weight of rifampicin (RIF) and isoniazid (INH)) intravenously. Blood samples were withdrawn at regular intervals of time and analyzed using the proposed HPTLC method.

REFERENCES

1. Mika, I. and Hiroyasu, O., **J. Chromatogr. Biomed. Appl.**, 1988, 426, 412.
2. Jamaluddin, A.B.M., Sarwar, G. and Rahman, M.K., **J. Chromatogr.**, 1990, 525, 495.
3. Swart, K.J. and Papgis, M., **J. Chromatogr.**, 1992, 593, 21.
4. Walubo, A., Smith, P. and Folb, P. I., **J. Chromatogr. Biomed. Appl.**, 1994, 658, 391.
5. ICH topic Q2B, Validation of Analytical Procedures: Methodology, step 4, Consensus Guideline, 1996
6. Gurevich, G.L., Berezovskaìa, L.N. and Manuilov, K.K., **Antibiot. Khimioter.**, 1992, 37, 25.
7. Eugene, B.H., Kenneth, L.D. and Harold, C.T., **J. Chromatogr. Biomed. Appl.**, 1995, 670, 259.