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Correlation of plasma anti-tuberculosis drug levels with subsequent development of hepatotoxicity

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SUMMARY

OBJECTIVES: To compare the free and total plasma drug concentrations of rifampicin (RMP), isoniazid and pyrazinamide in subjects with or without anti-tuberculosis drug-induced hepatotoxicity (DIH).

METHODS: A total of 110 tuberculosis (TB) patients were administered daily anti-tuberculosis treatment and were prospectively followed for the development of DIH. Plasma drug levels were measured at 0, 1, 2 and 4 h on days 1, 7 and 14 of treatment. Plasma drug levels in 15 patients who developed DIH (cases) were compared with 95 patients who did not (controls).

RESULTS: Female sex, body mass index < 17 kg/m^2 and baseline serum albumin < 4 g/dl predicted risk of DIH on univariate analyses. Free and total plasma RMP levels (C_{max} and $AUC_{0–4}$) on days 1, 7 and 14 were significantly higher in cases compared to controls and predicted development of DIH. Day 7 total RMP C_{max} and $AUC_{0–4}$ were higher in cases (mean 26.73, standard deviation [SD] 5.72 and 47.58, SD 33.10) than in controls (7.87, SD 10.95 and 14.01, SD 10.69, respectively).

CONCLUSIONS: Plasma RMP levels were higher in cases than in controls and independently predicted subsequent development of DIH. The C_{max} of Day 7 total RMP level (cut-off 12.50 mg/l) predicted subsequent development of DIH in 93.3% of the patients.

KEY WORDS: anti-tuberculosis drugs; plasma drug levels; drug induced hepatotoxicity; rifampicin

ANTI-TUBERCULOSIS TREATMENT may cause drug-induced hepatotoxicity (DIH), which is responsible for significant morbidity and mortality (6–12%, particularly if these drugs are continued after symptoms of hepatotoxicity develop).[1–3] DIH is defined as significant abnormality in liver function tests (LFT), with elevation of serum aminotransferases or bilirubin, along with clinical features such as anorexia, nausea, vomiting and jaundice during treatment.[4] DIH usually occurs within 3–135 days after the initiation of anti-tuberculosis treatment.[5] A meta-analysis by Steele et al. revealed a higher incidence of hepatotoxicity with concurrent administration of isoniazid (INH) and rifampicin (RMP) than with RMP or INH alone.[6] The pathogenesis of DIH caused by these offending drugs is still enigmatic, and various mechanisms have been postulated.[7] Although dose-related toxicity is an attractive hypothesis, a direct correlation between serum drug concentrations of anti-tuberculosis drugs and hepatotoxicity has not been well studied.[8,9]

This prospective study was carried out to measure plasma drug levels (free and total) of these anti-tuberculosis drugs and compare levels in cases (subjects who developed DIH) and controls (subjects who did not develop DIH). We also assessed the ability of these plasma drug levels to predict the subsequent development of DIH, which may prevent the occurrence of DIH, avert treatment interruption and increase the success of anti-tuberculosis treatment. Several ongoing trials are currently in progress assessing high doses of RMP in the treatment of tuberculosis (TB). Keeping this in mind, the present study is thus important and will help in the better management of TB.

PATIENTS, MATERIALS AND METHODS

Patients

The present study was carried out at the All India Institute of Medical Sciences (AIIMS) Hospital, New Delhi, India, between August 2010 and June 2012. All patients presenting to the clinic who fulfilled the inclusion/exclusion criteria were recruited. The daily

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drug treatment regimen was administered under the direct supervision of a health worker. Treatment-naïve, newly diagnosed TB patients of either sex aged 18–65 years were enrolled for the study. Patients with human immunodeficiency virus infection, on hepatotoxic drugs and abnormal baseline serum transaminase > 50 international units (IU) were excluded (Appendix A).*

The Institutional Ethics Committee of AIIMS approved the study. Written informed consent was obtained from each participant enrolled in the study.

Study design

In this prospective longitudinal study, eligible patients were enrolled after establishing the diagnosis of TB (pulmonary or extra-pulmonary) according to previously described criteria (Appendix B).[4,10–12] The site of TB, method of establishing the diagnosis, amount of alcohol intake and nutritional status (body mass index [BMI] and mid-upper arm circumference [MUAC]) were recorded.

Patients enrolled in the study received a daily anti-tuberculosis regimen according to body weight, consisting of RMP 10 mg/kg (450 mg for weight <50 kg, 600 mg for weight >50 kg), INH 5 mg/kg (maximum 300 mg), pyrazinamide (PZA) 25–30 mg/kg/day (maximum 2000 mg/day), ethambutol (EMB) 15–20 mg/kg/day. All drugs were manufactured by a single pharmaceutical company. Patients were initially followed up twice weekly for 2 weeks, fortnightly until the end of month 2 and then at the end of month 3 or earlier for symptoms suggestive of DIH. LFTs were performed at every follow-up visit. The duration of follow-up was 3 months.

Estimation of drug levels

Enrolled patients presented at the hospital, after having fasted, in the morning of days 1, 7 and 14 after the initiation of anti-tuberculosis treatment. After a baseline venous blood sample was drawn, they were administered their daily dose of medication with water; 2 ml of venous blood was then drawn at 1, 2 and 4 h after the administration of the anti-tuberculosis drugs. These time points were chosen because it has been documented that the pharmacokinetics of drugs and plasma drug levels tend to stabilise 1 week after starting anti-tuberculosis drugs following induction by hepatic microsomal enzymes.[13] Food intake was allowed 2 h after drug ingestion. Further processing of samples and quantification of the drugs was performed according to previously described criteria[13,14] (Appendix C). Maximum plasma concentration of drugs at 0, 1, 2 and 4 h (C_{max}) and the area under the concentration curve from 0 to 4 h ($AUC_{0–4}$) were used to assess the association between a particular day's drug concentration and subsequent development of DIH.

DIH was diagnosed according to the following criteria: 1) serum aminotransferase levels >5 times the upper limit of normal (ULN; 50 IU/l) on one occasion or >3× ULN on three consecutive occasions, with clinical symptoms as described; 2) serum bilirubin >2 mg/dl with significant elevation in serum aminotransferases (>3 times); 3) increase in serum aminotransferase level above pre-treatment values with the presence of all the following symptoms: anorexia, nausea, vomiting, abdominal pain and jaundice[4,15] (Appendix D).

Markers of acute viral hepatitis (A, B, C and E) were performed in all patients who developed features suggestive of DIH while on anti-tuberculosis treatment.[15] Patients with evidence of acute viral hepatitis or a history of alcohol consumption at the time of DIH were excluded. Patients who developed DIH were classified as cases, while the remainder were classified as controls. Free and total plasma drug levels of RMP, INH and PZA were compared between these two groups.

All patients recovered from DIH with a modified drug regimen consisting of EMB, streptomycin and one of the fluoroquinolones. No mortality was observed. Once LFT had normalised, all three drugs (RMP, INH and PZA) were reintroduced simultaneously.[16] Fourteen patients tolerated all drugs well on challenge with no recurrence of DIH. One patient who developed DIH again with the above regimen received the drugs sequentially.[7]

Statistical analysis

The literature review did not reveal any prospective correlation between plasma drug levels of anti-tuberculosis drugs and DIH. Our earlier study (unpublished) indicated that those who develop DIH later had a plasma RMP concentration (C_{max}) of 9.9 mg/l compared to 3.2 mg/l among those without DIH on day 14, 4 h after drug administration, with an SD of 6 mg/l. Based on this information, it was estimated that eight patients with and 80 patients without DIH would be required to detect this difference of plasma concentration of RMP as significant in a two-sided *t*-test with an α error of 5% and 80% power. We therefore decided to recruit at least 10 patients with DIH and 100 patients without DIH.

Student's *t*-test or Wilcoxon's rank-sum test (Mann-Whitney test) were used to find the difference in C_{max} and $AUC_{0–4}$ of free and total plasma drug levels of RMP, INH and PZA between cases and controls. C_{max} and $AUC_{0–4}$ of plasma drug levels of RMP, INH and PZA were analysed using receiver operating characteristic (ROC) curve analysis to assess the discriminating ability of these plasma drug levels for subsequent development of DIH. Using Cox's regression,

*The Appendices are available in the online version of this article at http://www.ingentaconnect.com/content/iuatld/ijtld/2014/00000018/00000002/art00011

univariate and adjusted (for sex, MUAC and baseline albumin) hazard ratios for the development of DIH with 95% confidence intervals (CIs) were calculated for different study variables. Analysis was performed using Stata 11.2 for Windows (Stata Corp, College Station, TX, USA).

RESULTS

A total of 110 patients diagnosed by different methods were enrolled in the study (Appendix Table A.1). Of these, 15 patients developed DIH. None of the patients with DIH had laboratory or clinical evidence of acute viral hepatitis or chronic liver disease, or a history of intake of hepatotoxic drugs. The median latent period for developing DIH was 22 days (range 10–35). As one patient had developed DIH on day 10 after the initiation of anti-tuberculosis drugs, his treatment was modified and his plasma drug levels were not estimated at the end of week 2.

Table 1 Baseline clinical and laboratory characteristics of study patients

	Proportion of patients developing DIH (n = 15)			
Parameter	n/N	Cumulative survival %	P value	HR (95%CI)
Age, years				
<30	8/68	88.2	0.48	1.00
⩾30	7/42	83.3		1.44 (0.52–3.97)
Sex				
Female	9/40	77.5	0.04	1.00
Male	6/70	91.4		0.36 (0.13–1.00)
Type of TB				
EPTB	5/66	92.4	0.02	1.00
PTB	10/44	77.3		3.24 (1.11–9.50)
Weight, kg				
<48	8/39	79.5	0.12	1.00
⩾48	7/71	90.1		0.46 (0.17–1.26)
BMI, kg/m^2				
<17	11/35	68.6	<0.001	1.00
⩾17	4/75	94.7		0.15 (0.05–0.46)
MUAC, cm				
<26	15/50	70.0	<0.001	1.00
⩾26	0/60	100.0		0.03
Serum albumin, g/dl				
<4	13/25	48.0	<0.001	1.00
⩾4	2/85	97.7		0.03 (0.01–0.15)
Serum total bilirubin, mg/dl				
<0.6	3/18	83.3	0.73	1.00
⩾0.6	12/92	87		0.80 (0.23–2.83)
AST, IU/l				
<30	8/53	84.9	0.67	1.00
⩾30	7/57	87.7		0.80 (0.29–2.21)
ALT, IU/l				
<33	9/52	82.7	0.33	1.00
⩾33	6/58	89.7		0.60 (0.21–1.69)
ALP, IU/l				
<192	9/51	82.4	0.24	1.00
⩾192	6/59	89.8		0.55 (0.19–1.54)

DIH = drug-induced hepatotoxicity; HR = hazard ratio; CI = confidence interval; TB = tuberculosis; EPTB = extra-pulmonary TB; PTB = pulmonary TB; BMI = body mass index; MUAC = mid-upper arm circumference; AST = aspartate transaminase; IU = international unit; ALT = alanine transaminase; ALP = alkaline phosphatase.

Table 2 Comparison of maximum abnormality in liver function tests at the time of hepatotoxicity between subjects with and those without DIH

Parameter	DIH (n = 15) mean ± SD	No DIH (n = 95) mean ± SD	P value
Total protein, g/dl	8.2 ± 0.3	8.5 ± 0.4	<0.001
Serum albumin, g/dl	3.7 ± 0.2	4.2 ± 0.5	<0.001
Serum total bilirubin	2.0 ± 0.3	0.8 ± 0.1	<0.001
AST, IU/l	243.4 ± 56.8	36.9 ± 5.2	<0.001
ALT, IU/l	206.9 ± 30.5	39.5 ± 5.3	<0.001
ALP, IU/l	277.3 ± 73.7	255.8 ± 64.8	0.24

DIH = drug-induced hepatotoxicity; AST = aspartate transaminase; IU = international unit; ALT = alanine transaminase; ALP = alkaline phosphatase.

Table 1 shows that pre-treatment BMI, MUAC and serum albumin were significantly different between the cases and controls. Table 2 gives a comparison of maximum abnormality in LFT among cases and controls during treatment with anti-tuberculosis drugs.

Plasma drug levels of RMP, INH and PZA were estimated at 0, 1, 2 and 4 h on days 1, 7 and 14 (total samples = 7920). Comparison of C_{max} and $AUC_{0–4}$ of both free and total drug levels between cases and controls is shown in Tables 3 and 4. Both the C_{max} and $AUC_{0–4}$ of plasma RMP levels (free and total) at days 1, 7 and 14 were significantly higher among cases than in controls. Day 7 mean total RMP C_{max} and $AUC_{0–4}$ were 26.73 (SD 5.72) and 47.58 (SD 33.10) among cases vs. 7.87 (SD 10.95) and 14.01 (SD 10.69) among controls. The $AUC_{0–4}$ of free drug concentration of INH on day 1 achieved borderline statistical significance ($P < 0.05$). The C_{max} and $AUC_{0–4}$ of the remaining drugs on days 1, 7 and 14 show no statistically significant difference among cases and controls.

The ability of these drug levels to predict the development of DIH later during the course of treatment was analysed using ROC curve analysis, as shown in Table 5, Appendix Table A.2 and Appendix Table A.3. C_{max} and $AUC_{0–4}$ of free and total plasma RMP levels on days 1, 7 and 14 predicted subsequent development of DIH during the course of treatment. Plasma drug levels on day 7 were the best predictor among these, as shown in Figures 1–4. The C_{max} of day 7 total RMP, with a cut-off value of 12.50 mg/l, predicted 93.3% of the patients who eventually developed DIH, with an adjusted hazard ratio of 39.1. Plasma drug levels of INH and PZA did not correlate with the development of DIH.

DISCUSSION

Hepatotoxicity is the most common side effect of anti-tuberculosis drugs, leading to the interruption

Table 3 Plasma free drug concentration of hepatotoxic anti-tuberculosis drugs

	C_{max}, mg/l			$AUC_{0–4}$, mg/l.h		
Free drug	DIH (n = 15) mean ± SD	No DIH (n = 95) mean ± SD	P value	DIH (n = 15) mean ± SD	No DIH (n = 95) mean ± SD	P value
Day 1						
RMP	0.35 ± 0.37	0.18 ± 0.56	<0.001	0.54 ± 0.61	0.29 ± 0.99	<0.001
INH	1.83 ± 1.48	1.50 ± 1.75	0.09	3.77 ± 3.30	3.11 ± 4.12	0.05
PZA	48.08 ± 20.15	57.67 ± 30.91	0.31	107.01 ± 60.72	139.45 ± 91.04	0.29
Day 7						
RMP	1.06 ± 1.36	0.09 ± 0.27	<0.001	1.65 ± 2.16	0.14 ± 0.50	<0.001
INH	1.91 ± 1.17	2.36 ± 2.41	0.90	3.54 ± 1.99	4.86 ± 6.14	0.91
PZA	52.29 ± 25.35	65.99 ± 33.67	0.12	147.67 ± 89.14	164.10 ± 8.22	0.37
Day 14						
RMP	0.94 ± 1.23	0.14 ± 0.53	<0.001	1.52 ± 2.04	0.18 ± 0.67	<0.001
INH	2.86 ± 2.05	1.94 ± 1.56	0.10	5.87 ± 4.92	3.76 ± 3.14	0.11
PZA	55.91 ± 26.38	64.13 ± 36.34	0.46	151.36 ± 86.17	156.80 ± 93.84	0.78

$C_{max,}$ = maximum concentration among 0, 1, 2 and 4 h; $AUC_{0–4}$ = area under the drug concentration curve from 0 to 4 h; DIH = drug-induced hepatotoxicity; RMP = rifampicin; INH = isoniazid; PZA = pyrazinamide.

Table 4 Plasma total drug concentration of hepatotoxic anti-tuberculosis drugs

	C_{max}, mg/l			$AUC_{0–4}$, mg/l.h		
Total drug	DIH (n = 15) mean ± SD	No DIH (n = 95) mean ± SD	P value	DIH (n = 15) mean ± SD	No DIH (n = 95) mean ± SD	P value
Day 1						
RMP	12.18 ± 2.33	5.90 ± 5.17	<0.001	21.53 ± 7.80	11.37 ± 10.57	<0.001
INH	2.83 ± 1.89	2.41 ± 2.05	0.17	5.47 ± 2.97	5.02 ± 4.01	0.32
PZA	51.60 ± 21.23	50.19 ± 22.86	0.72	122.20 ± 57.31	122.64 ± 61.67	0.89
Day 7						
RMP	26.73 ± 5.72	7.87 ± 10.95	<0.001	47.58 ± 33.10	14.01 ± 10.69	<0.001
INH	3.30 ± 1.72	3.03 ± 2.32	0.32	7.03 ± 4.06	6.80 ± 5.96	0.49
PZA	65.34 ± 22.34	61.12 ± 21.50	0.57	149.99 ± 65.69	153.43 ± 50.47	0.70
Day 14						
RMP	18.06 ± 8.82	5.99 ± 5.02	<0.001	36.64 ± 15.93	12.61 ± 10.19	<0.001
INH	3.40 ± 1.85	2.75 ± 2.23	0.12	7.56 ± 4.65	5.69 ± 4.09	0.11
PZA	63.10 ± 19.29	61.98 ± 27.77	0.51	171.16 ± 64.63	146.42 ± 60.12	0.30

C_{max} = maximum concentration among 0, 1, 2 and 4 h; $AUC_{0–4}$ = area under the concentration curve from 0 to 4 h; DIH = drug-induced hepatotoxicity; RMP = rifampicin; INH = isoniazid; PZA = pyrazinamide.

Table 5 Receiver operating characteristic curve analysis: plasma drug concentration of rifampicin

Day, parameter	Cut-off value	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)	Unadjusted HR % (95%CI)	Adjusted HR % (95%CI)
Free drug							
Day 1							
C_{max}	0.09	73.3 (44.9–92.2)	75.8 (65.9–84.0)	32.4 (17.4–50.5)	94.7 (87.1–98.6)	7.1 (2.3–22.5)	5.4 (1.3–21.6)
$AUC_{0–4}$	0.14	73.3 (44.9–92.2)	75.8 (65.9–84.0)	32.4 (17.4–50.5)	94.7 (87.1–98.6)	7.1 (2.3–22.5)	5.4 (1.3–21.5)
Day 7							
C_{max}	0.16	86.7 (59.5–98.3)	85.3 (76.5–91.7)	48.2 (28.7–68.1)	97.6 (91.6–99.7)	26.0 (5.9–115.7)	15.4 (2.4–98.9)
$AUC_{0–4}$	0.19	86.7 (59.5–98.3)	86.3 (77.7–92.5)	50.0 (29.9–70.1)	97.6 (91.7–99.7)	27.7 (6.2–123.4)	13.8 (2.1–89.7)
Day 14							
C_{max}	0.11	85.7 (57.2–98.2)	82.1 (72.9–89.2)	41.4 (23.5–61.1)	97.5 (91.3–99.7)	21.2 (4.7–95.1)	20.0 (3.1–129.4)
$AUC_{0–4}$	0.21	85.7 (57.2–98.2)	85.3 (76.5–91.7)	46.2 (26.6–66.6)	97.6 (91.6–99.7)	25.5 (5.7–114.4)	19.7 (3.2–122.0)
Total drug							
Day 1							
C_{max}	10.20	86.7 (59.5–98.3)	83.2 (74.1–90.1)	44.8 (26.5–64.3)	97.5 (91.4–99.7)	22.3 (5.0–99.2)	9.2 (1.5–57.4)
$AUC_{0–4}$	16.78	80.0 (51.9–95.7)	77.9 (68.2–85.8)	36.4 (20.4–54.9)	96.1 (89.0–99.2)	11.0 (3.1–39.1)	3.9 (0.9–16.7)
Day 7							
C_{max}	12.50	93.3 (68.1–99.8)	92.6 (85.4–97.0)	66.7 (43.0–85.4)	98.9 (93.9–99.9)	93.4 (12.2–714.6)	39.1 (4.5–342.7)
$AUC_{0–4}$	23.05	80.0 (51.9–95.7)	82.1 (72.9–89.2)	41.4 (23.5–61.1)	96.3 (89.6–99.1)	13.4 (3.8–47.4)	6.4 (1.5–27.9)
Day 14							
C_{max}	10.90	92.9 (66.1–99.8)	90.5 (82.8–95.6)	59.1 (36.4–79.3)	98.9 (93.8–99.9)	34.6 (7.7–155.3)	15.7 (2.9–82.8)
$AUC_{0–4}$	22.46	85.7 (57.2–98.2)	84.2 (75.3–90.9)	44.4 (25.5–64.7)	97.6 (91.5–99.7)	23.1 (5.1–103.4)	13.3 (1.9–89.8)

C_{max} = maximum concentration among 0, 1, 2 and 4 h; $AUC_{0–4}$ = area under the concentration curve from 0 to 4 h; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value; HR = hazard ratio.

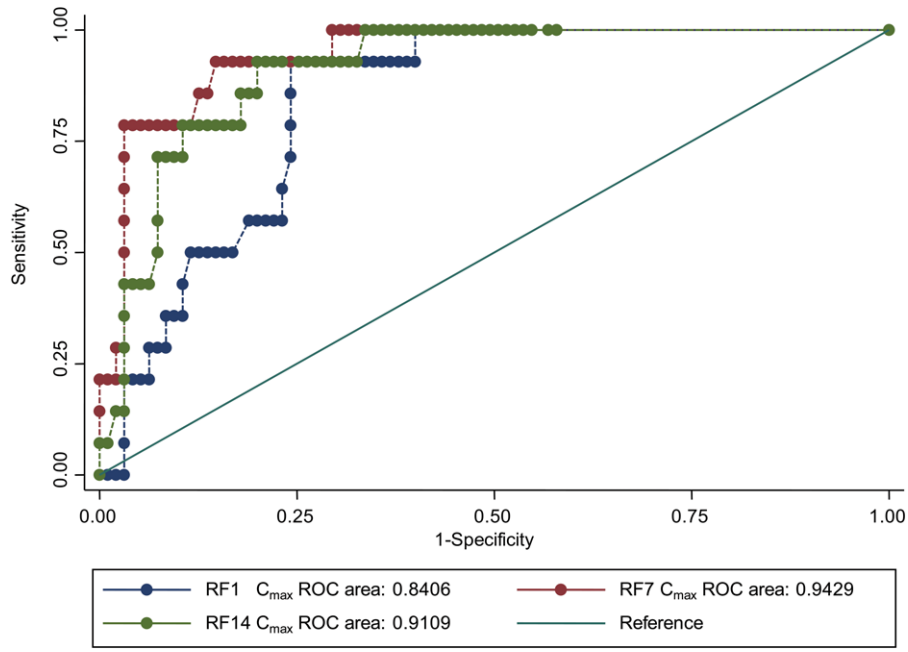

Figure 1 ROC curve analysis: C_{max} for free RMP. ROC = receiver operating characteristic; C_{max} = maximum concentration at 0, 1, 2 and 4 h; RF1 = plasma free RMP level at Day 1; RF7 = plasma free RMP level at Day 7; RF14 = plasma free RMP level at Day 14; RMP = rifampicin.

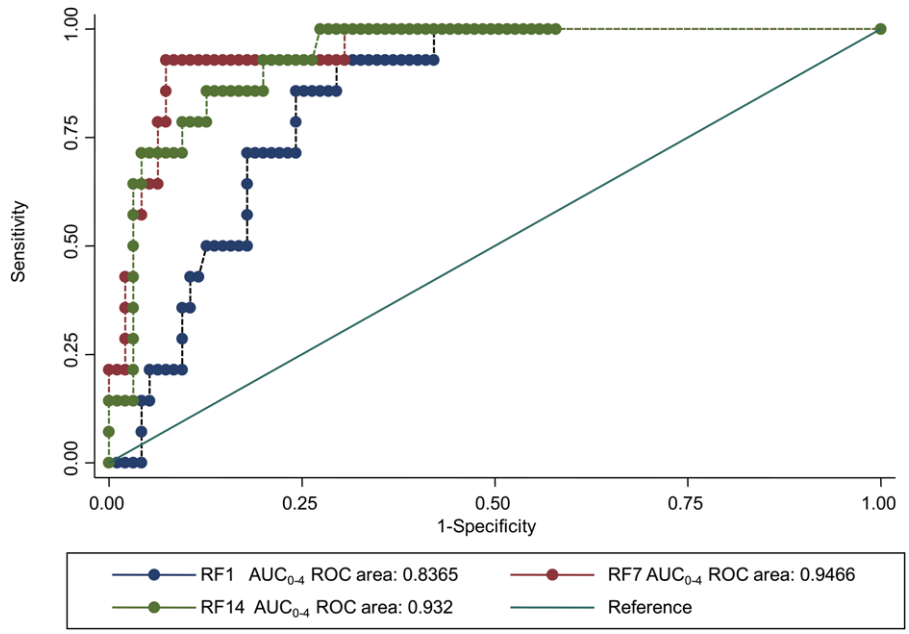

Figure 2 ROC curve analysis: AUC_{0-4} for free rifampicin. ROC = receiver operating characteristic; AUC_{0-4}, area under the concentration curve from 0 to 4 h; RF1 = plasma free RMP level at Day 1; RF7 = plasma free RMP level at Day 7; RF14 = plasma free RMP level at Day 14; RMP = rifampicin.

of treatment. Various mechanisms have been postulated for the development of DIH;[7,17,18] hypoalbuminaemia is a significant clinical predictor of DIH.[4,7,15] As RMP at therapeutic levels is significantly (about 80%) bound to plasma proteins, primarily albumin,[19,20] free drug exerting pharmacological action is increased in the case of hypoalbuminaemia. However, in our study we observed that both free and total drug levels of RMP (C_{max} and AUC_{0-4}) on days 1, 7 and 14 were higher in patients who developed hepatotoxicity. RMP concentrations significantly increased from day 1 to day 7 and 14 in patients who

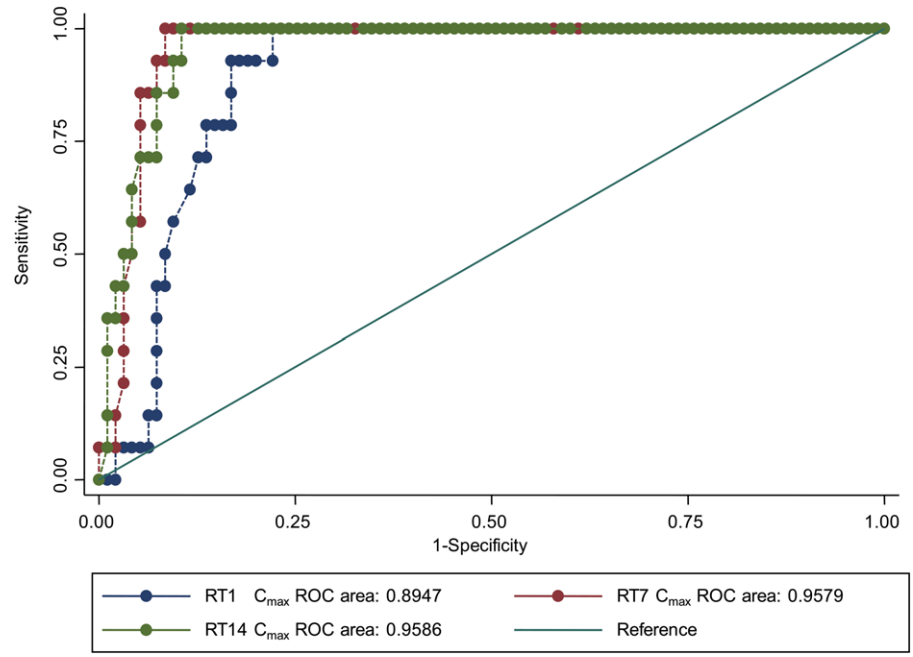

Figure 3 ROC curve analysis: C_{max} for total rifampicin;. ROC = receiver operating characteristic; C_{max} = maximum concentration at 0, 1, 2 and 4 h; RF1 = plasma free RMP level at Day 1; RF7 = plasma free RMP level at Day 7; RF14 = plasma free RMP level at Day 14; RMP = rifampicin.

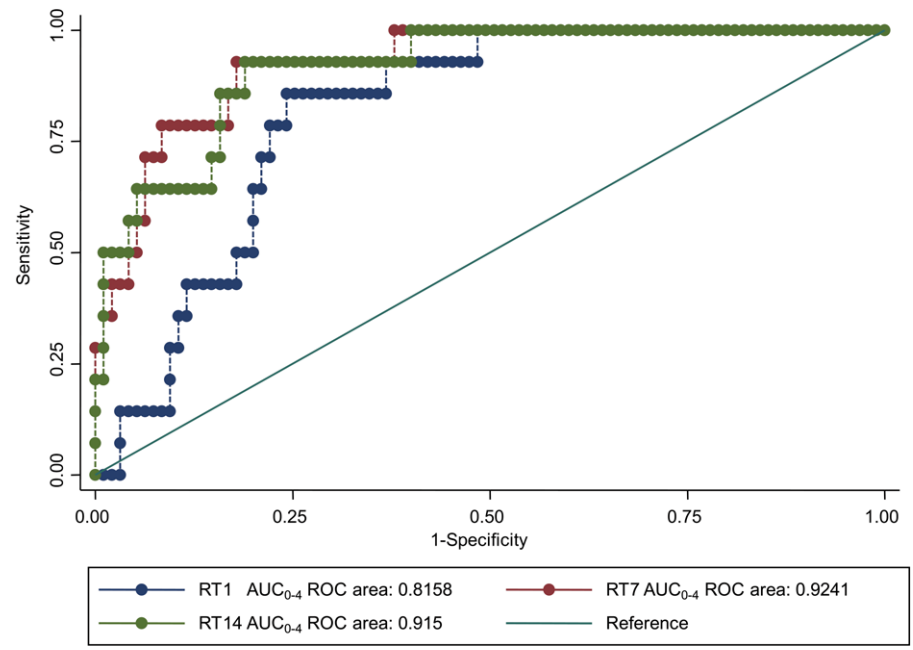

Figure 4 ROC curve analysis: AUC $_{0-4}$ for total rifampicin. ROC = receiver operating characteristic; AUC_{0-4}, area under the concentration curve from 0 to 4 h; RF1 = plasma free RMP level at Day 1; RF7 = plasma free RMP level at Day 7; RF14 = plasma free RMP level at Day 14; RMP = rifampicin.

developed DIH compared to controls. This suggests that the auto-induction of metabolism that generally takes place in the case of RMP is not occurring to a significant degree among cases, leading to the accumulation of the drug that is responsible for development of DIH. Some studies that have used higher doses of RMP have reported fewer adverse reactions or less severe hepatotoxicity (but a high incidence of mild hepatotoxicity) without requiring discontinuation of the offending drug.[21,22] This in contrast to our

study, in which the increased incidence of DIH noted in Indian patients may be due to various factors such as ethnic or genetic differences, or malnutrition. Our results also indicate that we should consider the potential risks of a higher incidence of DIH if a higher dose of RMP is used.

With regard to INH, the present study showed no statistically significant difference between cases and controls at days 1, 7 and 14, except for AUC_{0-4} at day 1, which was of borderline significance. Previous studies on INH hepatotoxicity are in line with our results.[2,23] Hepatotoxic metabolites of INH-like monoacetyl hydrazine may thus play a significant role in INH-related hepatotoxicity.[24]

Hepatotoxicity occurs at a greater frequency when INH and RMP are co-administered than when either drug is given alone.[6,7] On one hand, RMP induces the metabolism of INH to form hepatotoxic hydrazine metabolites,[25] and on the other, increased plasma RMP levels may occur due to the displacement of the drug from plasma protein binding sites by INH. These pharmacokinetic interactions between the two drugs may explain their added toxicity.

In the present study, PZA levels at days 1, 7 and 14 did not show any statistically significant difference between the two groups. Although dose-related hepatotoxicity has been reported in earlier studies using high doses of PZA,[26,27] hepatotoxicity related to the currently prescribed PZA dose of 20–30 mg/kg/day appears to be either idiosyncratic or due to the generation of hepatotoxic free radicals.

Drug-induced liver injury is a spectrum that ranges from hepatic adaptation to severe hepatotoxicity. Hepatic adaptation is a physiological phenomenon whereby the activation of various constitutional genes helps in averting drug- or toxin-related injury.[28] It manifests as asymptomatic transient elevations of transaminases, and with persisting insult, may lead to hepatocellular injury.[7,29] All of the patients who developed DIH in our study had clinical symptoms with elevated serum bilirubin levels, necessitating interruption of treatment and modification of anti-tuberculosis treatment, representing true hepatotoxicity. Although 14 of 15 patients tolerated the re-introduction of anti-tuberculosis drugs, it is difficult to say whether this was due to hepatic adaptation or hepatotoxicity, as this is a disease spectrum and patients could have developed acute hepatic failure if these drugs were continued. As plasma drug levels subsequent to challenge with drugs were not estimated, this remains conjectural and could be an area for future research.

In our present study, both free and total RMP levels at days 1, 7 and 14 prospectively predicted the development of DIH later during the course of anti-tuberculosis treatment, with day 7 values being the most predictive. Measurement of total RMP levels can therefore be used in clinical practice to predict subsequent development of DIH. However, as measurement of free RMP levels is cumbersome and costly, we suggest estimating drug levels in patients at risk of DIH development as indicated in Table 1; patients with C_{max} of total RMP level at day 7 with a cut-off value of 12.50 mg/l (positive predictive value 66.7% and negative predictive value 98.9%) should be closely monitored for abnormal liver function.

Our study has several merits. This is the first of its kind to measure drug levels of hepatotoxic drugs and follow patients for subsequent development of hepatotoxicity. It provides the first concrete evidence that hepatotoxicity is mediated by increased plasma RMP levels. The laboratory team involved in measuring plasma drug levels was blinded to the DIH diagnosis. We carefully excluded acute viral hepatitis and other factors responsible for coincident hepatotoxicity. The limitations of the present study were as follows: as patients were receiving concurrent INH and PZA, it is difficult to predict whether DIH was caused by increased plasma RMP levels alone or whether they were due to synergistic action of RMP with other anti-tuberculosis drugs. Acetylator status and plasma concentration of toxic metabolite of INH were not estimated to correlate with hepatotoxicity.

In conclusion, the present study, which shows an association between higher plasma RMP levels and DIH, possibly due to the failure of auto-induction of RMP metabolism, helps in furthering our understanding of the development of DIH. Plasma INH and PZA drug levels contributed little to DIH. Plasma levels of total RMP, being a better predictor, can also be used as a predictor for subsequent development of DIH later during the course of treatment. These preliminary findings require confirmation using a large multicentre study.

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APPENDIX A
EXCLUSION CRITERIA

Patients were excluded from the study if one of the following criteria were present: human immunodeficiency virus (HIV) infection; history of alcohol consumption >48 g/day for at least 1 year; history of chronic diarrhoea or intestinal TB; concurrent administration of other hepatotoxic drugs (e.g., methotrexate, phenytoin, valproate, fluconazole, paracetamol) and known inhibitors (grapefruit juice, calcium channel blockers) or inducers (anticonvulsants) of cytochrome P450 or use of herbal medications; abnormal baseline liver function (aspartate aminotransferase [AST] or alanine aminotransferase [ALT] >50 IU) or kidney function tests. Patients with serological evidence of acute viral hepatitis (A, B, C or E) and carriers of hepatitis B or C virus were also excluded.

APPENDIX B
CRITERIA USED TO ESTABLISH A DIAGNOSIS

Briefly, the diagnosis of pulmonary TB was based on the presence of acid-fast bacilli on sputum smear or bronchoalveolar lavage fluid, or *Mycobacterium tuberculosis* on sputum culture. In patients with smear-positive TB, culture was not performed unless multidrug-resistant TB was strongly suspected. Sputum cultures were performed in all patients with smear-negative pulmonary TB.

In patients with negative smears and cultures, the diagnosis of TB was based on symptoms, chest radiographic infiltrates in the upper lobes, and clinical and radiographic response to anti-tuberculosis drugs. Focal disseminated TB was diagnosed on the basis of histopathological and/or microbiological evidence of TB from two non-contiguous sites.[1,2] A group of patients with disseminated TB exhibiting classic miliary mottling on chest radiographs were included as miliary TB.[3]

Lymph node TB was diagnosed by the demonstration of *M. tuberculosis* in the smear and/or culture of lymph node aspirates or biopsy specimens or histopathology compatible with the diagnosis of TB.

The diagnosis of tuberculous pleural effusion was made as follows:[4] definitive when *M. tuberculosis* was demonstrated in pleural fluid smear (Ziehl-Neelsen method) and/or culture (Löwenstein Jensen using BACTEC™ 460, BD, Sparks, MD, USA) and/or pleural biopsy, or *M. tuberculosis* polymerase chain reaction (PCR) was positive in pleural fluid. Tuberculous pleural effusion was considered probable when any one of the following criteria 1 or 2 along with 3 were positive: 1) exudative, lymphocyte predominant effusion with increased pleural fluid protein (>3.5 g/dl) and elevated pleural fluid adenosine deaminase (ADA) activity (>35 IU/l); 2) demonstration of caseating granulomas in pleural biopsy specimens; and 3) good clinical and radiological response to anti-tuberculosis treatment during follow up.

APPENDIX C
SAMPLE PREPARATION AND QUANTIFICATION OF DRUGS

Blood samples were collected in vials containing both ethylenediaminetetraäcetic acid and ascorbic acid and stored at 4°C. Plasma was harvested from the blood samples within 2–3 h of collection. Plasma was separated by centrifuging the blood samples at 1800 rpm for 5 min. The plasma samples were immediately transferred and stored at −80°C until further analysis. For sample preparation and quantification of all anti-tuberculosis drugs, the method reported by Sang Hoon Song et al. was adopted with minor modifications using liquid chromatography–tandem mass spectrometry (LC-MS/MS).[5] The method used by Sang Hoon Song et al. was revalidated in the present experimental set up and the validated parameters were found to be within the acceptable range. Thermo Finnigan Ultra High Performance Liquid Chromatographic System (Thermo Electron Corp, Waltham, MA, USA) with photodiode array detector controlled by ChromQuest™ (Ver 4.5) Software (Thermo Scientific) coupled with electrospray ionization–mass spectrometry (ESI-MS/MS; 4000QTrap, ABI Sciex, Framingham, MA, USA) was used for the analysis. The compound and source-dependent parameters were optimised to obtain maximum ion intensity for the analysis using the inbuilt algorithm in the software. The protocol was validated per guidelines and used for the analysis. All plasma samples were stored at −80°C until analysis.

Plasma samples were subjected to direct deproteinisation and ultra-filtration for the determination of total and free drug levels, respectively. Briefly, samples amounting to 100 μl were added to 200 μl of extraction solvent (70% methyl alcohol with 0.1% formic acid in water containing 100 ng/ml of internal standard) and vortexed to precipitate proteins (1 min), followed by centrifugation at 7000 rpm for 15 min; the supernatant was then analysed. Free drug levels were quantified from the ultra-filtrates prepared using Vivaspin centrifilters (Vivaspin, Littleton, MA, USA) using a molecular weight cut-off value of 3 kD.

APPENDIX D
DIAGNOSTIC CRITERIA FOR DRUG-INDUCED HEPATOTOXICITY

DIH was diagnosed if any one of criteria 1, 2 or 3 was present along with criteria 4 and 5 in a clinically presumptive patient. Although the second and third criteria do not fit American Thoracic Society (ATS)

criteria, clinical symptoms with elevated serum bilirubin levels would help detect true hepatotoxicity.

1 An increase of 5× ULN (50 IU/l) of serum AST and/or ALT on one occasion, or by >3× ULN on three consecutive occasions (one week apart), as well as clinical symptoms
2 An increase in the level of total serum bilirubin to >2× ULN (1 mg/dl), with significant serum transaminase elevation (>3× ULN)
3 Any increase in serum ALT and/or AST above pretreatment values, with presence of all symptoms (anorexia, nausea, vomiting, abdominal pain and jaundice; serum bilirubin >2 mg/dl)
4 Absence of serological evidence of infection with hepatitis A, B, C or E
5 Improvement in liver function (serum bilirubin <1 mg/dl, AST and ALT <100) after withdrawal of anti-tuberculosis drugs.

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Table A.1 Method used to establish the diagnosis of tuberculosis

Type of TB, method	*n*
Pulmonary TB	
Sputum smear-positive AFB	36
BAL smear-positive AFB	2
BAL *M. tuberculosis* PCR-positive	2
M. tuberculosis (BACTEC 460) culture-positive	4
Pleural effusion	
Definitive: *M. tuberculosis* PCR-positive	4
Probable: lymphocytic, exudative with elevated adenosine aminase	12
Disseminated TB	
Sputum smear-positive AFB with necrotic mesenteric lymph node	4
Lymph node TB	
Smear-positive AFB	28
Caseating necrotising granulomas	10
Abdominal TB	
Smear-positive for AFB (FNAC from mesenteric lymph nodes)	8

TB = tuberculosis; AFB = acid-fast bacilli; BAL = bronchoalveolar lavage; PCR = polymerase chain reaction; FNAC = fine-needle aspiration cytology.

Table A.2 ROC curve analysis: plasma drug concentration of isoniazid

Day, parameter	Cut-off value	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)	Unadjusted HR (95%CI)	Adjusted HR (95%CI)
Free drug							
Day 1							
C_{max}, mg/l	1.20	60.0 (32.3–83.7)	58.9 (48.4–68.9)	18.8 (9.0–32.6)	90.3 (80.1–96.4)	2.0 (0.7–5.6)	0.6 (0.2–1.8)
AUC_{0-4}, mg/l.h	2.26	60.0 (32.3–83.7)	61.1 (50.5–70.9)	19.6 (9.3–33.9)	90.6 (80.7–96.5)	2.1 (0.7–6.0)	0.9 (0.3–2.8)
Day 7							
C_{max}, mg/l	1.67	53.3 (26.6–78.7)	54.7 (43.2–64.0)	15.4 (6.9–28.1)	87.9 (76.7–95.0)	1.3 (0.5–3.7)	1.0 (0.3–3.1)
AUC_{0-4}, mg/l.h	3.10	53.3 (26.6–78.7)	54.7 (44.2–65.0)	15.7 (7.0–28.6)	88.1 (77.1–95.1)	1.3 (0.5–3.7)	0.6 (0.2–1.7)
Day 14							
C_{max}, mg/l	1.72	57.1 (28.9–82.3)	57.9 (47.3–68.0)	16.7 (7.5–30.2)	90.2 (79.8–96.3)	1.7 (0.6–5.0)	2.0 (0.6–6.3)
AUC_{0-4}, mg/l.h	3.43	57.1 (28.9–82.3)	57.9 (47.3–68.0)	16.7 (7.5–30.2)	57.9 (79.8–96.3)	1.7 (0.6–5.0)	1.9 (0.6–5.9)
Total drug							
Day 1							
C_{max}, mg/l	1.89	53.3 (26.6–78.7)	54.7 (43.2–64.0)	15.4 (6.9–28.1)	87.9 (76.7–95.0)	1.4 (0.5–3.7)	1.0 (0.3–3.5)
AUC_{0-4}, mg/l.h	4.42	53.3 (26.6–78.7)	53.7 (43.2–64.0)	15.4 (6.9–28.1)	88.0 (76.7–95.0)	1.3 (0.5–3.6)	1.1 (0.3–4.2)
Day 7							
C_{max}, mg/l	2.53	46.7 (21.3–73.4)	48.4 (37.0–57.9)	12.3 (5.1–23.7)	84.9 (72.4–93.3)	0.8 (0.3–2.2)	0.9 (0.3–3.0)
AUC_{0-4}, mg/l.h	5.34	46.7 (21.3–73.4)	48.4 (38.0–58.9)	12.5 (5.2–24.0)	85.2 (72.9–93.4)	0.8 (0.3–2.2)	0.8 (0.3–2.5)
Day 14							
C_{max}, mg/l	2.69	64.3 (35.1–87.2)	64.2 (53.7–73.8)	20.9 (10.0–36.0)	92.4 (83.2–97.5)	3.0 (1.0–8.9)	2.2 (0.6–7.5)
AUC_{0-4}, mg/l.h	5.83	64.3 (35.1–87.2)	64.2 (53.7–73.8)	20.9 (10.0–36.0)	92.4 (83.2–97.5)	3.0 (1.0–8.9)	2.1 (0.6–7.2)

ROC = receiver operating characteristic; CI= confidence interval; PPV = positive predictive value; NPV = negative predictive value; HR = hazard ratio; C_{max} = maximum concentration among 0, 1, 2 and 4 h; AUC_{0-4} = area under the concentration curve from 0 to 4 h.

Table A.3 ROC curve analysis: plasma drug concentration of pyrazinamide

Day, parameter	Cut-off value	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)	Unadjusted HR (95%CI)	Adjusted HR (95%CI)
Free drug							
Day 1							
C_{max}, mg/l	48.40	53.3 (26.6–78.7)	53.7 (43.2–64.0)	15.4 (6.9–28.1)	87.9 (76.7–95.0)	0.8 (0.3–2.1)	1.1 (0.3–4.1)
$AUC_{0–4}$, mg/l.h	105.50	53.3 (26.6–78.7)	53.7 (43.2–64.0)	15.4 (6.9–28.1)	87.9 (76.7–95.0)	0.8 (0.3–2.2)	1.4 (0.4–4.9)
Day 7							
C_{max}, mg/l	56.10	57.9 (47.3–68.0)	53.3 (26.6–78.7)	16.7 (7.5–30.2)	88.7 (78.1–95.3)	0.7 (0.2–1.8)	1.1 (0.3–4.4)
$AUC_{0–4}$, mg/l.h	155.35	53.3 (26.6–78.7)	53.7 (43.2–64.0)	15.4 (6.9–28.1)	87.9 (76.7–95.0)	1.3 (0.5–3.6)	2.0 (0.5–7.6)
Day 14							
C_{max}, mg/l	54.40	57.1 (28.9–82.3)	57.9 (47.3–64.0)	16.7 (7.5–30.2)	90.2 (79.8–96.3)	0.6 (0.2–1.6)	0.8 (0.2–3.2)
$AUC_{0–4}$, mg/l.h	124.77	42.9 (17.7–71.1)	45.3 (35.0–55.8)	10.3 (3.9–21.2)	84.3 (71.4–93.0)	0.7 (0.2–1.9)	0.4 (0.1–1.8)
Total drug							
Day 1							
C_{max}, mg/l	48.30	53.3 (26.6–78.7)	52.6 (42.1–63.0)	15.1 (6.8–27.6)	87.7 (76.3–94.9)	1.2 (0.4–3.3)	0.5 (0.2–1.9)
$AUC_{0–4}$, mg/l.h	115.40	46.7 (21.3–73.4)	48.4 (38.0–58.9)	12.5 (5.2–24.1)	85.2 (72.9–93.4)	0.8 (0.3–2.3)	0.4 (0.1–1.7)
Day 7							
C_{max}, mg/l	56.90	46.7 (21.3–73.4)	46.3 (36.0–56.9)	12.1 (5.0–23.3)	84.6 (71.9–93.1)	0.7 (0.3–2.0)	0.4 (0.1–1.4)
$AUC_{0–4}$, mg/l.h	152.89	46.7 (21.3–73.4)	48.4 (37.0–57.9)	12.3 (5.1–23.7)	84.9 (72.4–93.3)	0.8 (0.3–2.2)	0.2 (0.1–0.9)
Day 14							
C_{max}, mg/l	62.00	57.1 (28.7–82.3)	57.9 (47.3–68.0)	16.7 (7.5–30.2)	90.2 (79.8–96.3)	1.8 (0.6–5.1)	0.5 (0.1–1.8)
$AUC_{0–4}$, mg/l.h	158.25	57.1 (28.9–82.3)	54.7 (44.2–65.0)	15.7 (7.0–28.6)	89.7 (78.8–96.1)	1.6 (0.5–4.5)	0.5 (0.2–1.8)

ROC = receiver operating characteristic; CI= confidence interval; PPV = positive predictive value; NPV = negative predictive value; HR = hazard ratio; C_{max} = maximum concentration among 0, 1, 2 and 4 h; $AUC_{0–4}$ = area under the concentration curve from 0 to 4 h.

RÉSUMÉ

OBJECTIFS : Comparer les concentrations plasmatiques libre et totale de la rifampicine (RMP), de l'isoniazide et du pyrazinamide chez des sujets porteurs ou non d'hépatotoxicité induite par des médicaments antituberculeux (DIH).

MÉTHODES : Au total 110 patients tuberculeux ont reçu un traitement anti-tuberculeux quotidien et ont été suivis à la recherche d'une DIH. Les taux plasmatiques des médicaments ont été mesurés lors de l'administration, puis 1, 2 et 4 heures plus tard les jours 1, 7 et 14 du traitement. Les taux des 15 patients qui ont développé une DIH (cas) ont été comparés aux autres patients (témoins).

RÉSULTATS : L'analyse univariée a mis en évidence les facteurs prédictifs suivants de DIH : le sexe féminin, un indice de masse corporelle < 17 kg/m^2 et un taux d'albumine sérique initial < 4 g/dl. Les taux de RMP plasmatique libre et totale (C_{max} et $AUC_{0–4}$) étaient significativement plus élevés chez les cas que chez les témoins, et prédisaient le développement d'une DIH. Le 7e jour, la concentration totale C_{max} et l'$AUC_{0–4}$ de la RMP étaient plus élevés chez les cas (moyenne 26,73 ; SD 5,72 et moyenne 47,58 et SD 33,10) que chez les témoins (moyenne 7,87 ; SD 10,95 et 14,01 ; SD 10,69, respectivement).

CONCLUSIONS : Les taux plasmatiques de RMP se sont avérés plus élevés chez les cas et prédisaient à eux seuls le développement d'une DIH. La C_{max} au 7e jour de la RMP totale (valeur seuil 12,50 mg/l) prédisait le développement d'une DIH chez 93,3% des patients qui ont eu une DIH.

RESUMEN

OBJETIVOS: Comparar las concentraciones plasmáticas totales y libres de rifampicina (RMP), isoniazida y pirazinamida entre los pacientes que presentaban hepatotoxicidad inducida por los medicamentos antituberculosos (DIH) y los pacientes sin esta afección.

MÉTODOS: Se practicó el seguimiento de 110 pacientes tuberculosos que recibían tratamiento diario con medicamentos antituberculosos a fin de detectar la aparición de hepatotoxicidad. Se determinaron las concentraciones plasmáticas a las 0, 1, 2 y 4 horas de haber recibido el medicamento en los días 1, 7 y 14 de tratamiento. Se compararon las concentraciones plasmáticas de los 15 pacientes que presentaron DIH (casos) con los 95 pacientes sin DIH (testigos).

RESULTADOS: El análisis monofactorial puso en evidencia que el sexo femenino, un índice de masa corporal < 17 kg/m^2 y una albúmina sérica < 4 g/dl eran factores de riesgo de padecer DIH. La diferencia de la concentración plasmática libre y total de RMP (C_{max} y $AUC_{0–4}$) en los días 1, 7 y 14 entre los casos y los testigos fue significativa y pronosticó la aparición de DIH. En el séptimo día, se observó en los casos una concentración total de RMP C_{max} (media 26,73 ± desviación estándar 5,72) y un $AUC_{0–4}$ (47,58 ± 33,10) más altas que en los testigos (C_{max} 7,87 ± 10,95; $AUC_{0–4}$ 14,01 ± 10,69).

CONCLUSIÓN: Las concentraciones plasmáticas de RMP fueron más altas el grupo de casos que en el grupo de testigos y además pronosticaron de manera independiente la aparición posterior de DIH. La concentración total de RMP C_{max} en el séptimo día (valor discriminatorio 12,50 mg/l) pronosticó la aparición ulterior de DIH en 93,3% de los pacientes.