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The Effect of Pyrazinamide and Rifampicin on Isoniazid Metabolism in Rats

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ABSTRACT: Hepatotoxicity is the main concern during tuberculosis chemotherapy with the first-line drugs isoniazid (INH), rifampicin (RMP) and pyrazinamide (PYR). Since these hepatotoxic events have been associated with INH metabolites, the study aimed to measure the area under curve (*AUC*) parameter for INH and its metabolites acetylisoniazid (AcINH), hydrazine (Hz) and acetylhydrazine (AcHz), when groups of rats were pre-treated for 21 days with INH alone or in combination with RMP and/or PYR, in the following amounts per kg body weight: INH 100 mg; INH 100 mg + RMP 100 mg; INH 100 mg + PYR 350 mg; INH 100 mg + PYR 350 mg + RMP 100 mg. It was found that co-administration of RMP, PYR and RMP + PYR caused a significant decrease in the *AUC* for INH. Co-administration of PYR was the only treatment that caused a significant increase in the *AUC* for Hz and a decrease in the *AUC* for its acetylated product AcHz. The *AUC* for AcINH was not significantly altered in any experimental group. In conclusion, the increased metabolism of INH in all the drug combinations and the significantly higher production of Hz in the group INH + PYR might be linked with exacerbated hepatotoxic effects of these drug associations. Copyright © 2007 John Wiley & Sons, Ltd.

Key words: isoniazid; rifampicin; pyrazinamide; acetylisoniazid; acetylhydrazine

Introduction

Hepatotoxicity is the main concern during tuberculosis (TB) chemotherapy with the first-line drugs isoniazid (INH), rifampicin (RMP) and pyrazinamide (PYR) [1–3]. In this regard, the products of biotransformation of INH have been said to be responsible for these adverse effects. Such derivatives include acetylisoniazid (AcINH) and acetylhydrazine (AcHz), the products of acetylation of INH and hydrazine (Hz) respectively, catalysed by *N*-acetyltransferase (NAT-2) [4–6]. In fact, the involvement of acetylated metabolites of INH in hepatotoxicity has given rise to a huge controversy about the role of the acetylator phenotype status and the development of liver damage [7,8]. Another toxic metabolite that deserves attention is hydrazine (Hz), the product of direct hydrolysis of INH, catalysed by isoniazid hydrolase [9,10]. The enzyme cytochrome P450 isoform 2E1 (CYP2E1) is also important in the biotransformation pathways of INH. This oxidase is responsible for posterior oxidation of these metabolites, leading to the generation of reactive species that are supposed to damage intracellular proteins [11–13].

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It is well known that successful treatment of TB is highly dependent on co-administration of INH, RMP and PYR, and it is evident that drug interactions could exacerbate the hepatotoxicity. This is the case for RMP and INH, which are both inducers of CYP2E1, and reports have argued that the combination of INH and RMP can be even more deleterious [14–18]. For these reasons, the aim was to study the effect of the drug associations INH + RMP, INH + PYR and INH + RMP + PYR on the kinetics of the disposal of INH and its metabolites AcINH, Hz and AcHz. The area under curve (*AUC*) parameter for the serum profiles of INH, AcINH, Hz and AcHz were monitored, when rats were pre-treated with INH alone or in combination with RMP and/or PYR. Aiming to simulate the real therapeutic regimen, the rats were exposed to the drugs for 21 days before the pharmacokinetic study.

Materials and Methods

Chemicals

Isoniazid, pyrazinamide, rifampicin and *trans*-cinnamaldehyde were purchased from Sigma-Aldrich (St Louis, MO, USA), hydrazine and acetylhydrazine from Acros Organics (New Jersey, USA). Trichloroacetic acid from Labsynth (São Paulo, SP Brazil). Acetylisoniazid was synthesized by acetylation of the precursor by standard methods. The reagents for buffers and solvents were of HPLC grade.

Animals

Male Wistar rats (weighing around 250 g) were housed at constant temperature ($23 \pm 1^{\circ}C$), and relative humidity ($55 \pm 5\%$) in a 12:12 h light/dark photoperiod, with food and water *ad libitum*. Daily doses of the anti-tuberculosis drugs were administered by gavage to the animals for one period of 21 days as follows: INH 100 mg/kg (group I); INH 100 mg/kg + RMP 100 mg/kg (group II); INH 100 mg/kg + PYR 350 mg/kg (group III); INH 100 mg/kg + PYR 350 mg/kg +*RMP* 100 mg/kg (group IV). These doses were based on recent studies in which hepatotoxicity was evaluated in rats [19–21].

After 21 days of exposure to the anti-tuberculosis drugs, the animals were killed by decapitation before (time zero) and 0.25, 0.5, 0.75, 1, 1.5, 3, 6, 12, 24 h after administration. Five animals were used for each point. The blood samples were centrifuged and analysed as described next.

The study protocol was approved by The Research Ethics Committee of the School of Pharmaceutical Science, UNESP, Araraquara (Process 55/2004).

Analytical protocol

The concentrations of INH and Hz in the rat serum were measured by liquid chromatography after derivatization with *trans*-cinnamaldehyde [22]. Briefly, 300 µl serum was mixed with 300 µl 10% trichloroacetic acid, vortexed and centrifuged at 2500 rpm for 10 min. To the deproteinized supernatant were added 30 µl H_2O and 60 µl of a 1% solution of *trans*-cinnamaldehyde in methanol. After vortexing and incubation for 10 min at room temperature, the samples (50 µl) were injected into the HPLC system (Alliance separation model and 2487 UV-Vis detector, Waters). The mixture was separated on a reverse phase C_{18} column (5 µm particle, 3.9×300 mm, Symmetry®, Waters®) by running a linear gradient of isopropanol 8–14%, acetonitrile 32–56% and 0.05 mol/l phosphate buffer 60–30% for 15 min at a flow rate of 1 ml/min and detected at 340 nm. For determination of the acetylated metabolites, the supernatant samples were first hydrolysed by addition of 30 µl 6 mol/l HCl and incubated at 80°C for 1 h. The samples were then derivatized as before and quantified as INH or Hz.

Calibration curve, stability, sensitivity, precision and accuracy

Samples of serum spiked with stock methanol solutions of INH, Hz and their acetylated metabolites were prepared and submitted to the analytical protocol. For INH the calibration curve was linear ($r = 0.9999$, $n = 8$) in the range 0.78–50.0 µg/ml, and the limit of quantitation was 70 ng/ml. Precision, expressed as the inter- ($n = 5$) and intraday ($n = 10$) coefficients of variation, was $\leqslant 2.5\%$ on the same day and $\leqslant 7.2\%$ between days for each quality control

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sample of 1, 10 and 40 μg/ml. Accuracy, expressed as the inter- and intraday % bias, was −7.6 to 1.2 on the same day and −7.3 to 1.1 between days for each quality control sample.

For Hz the calibration curve was linear ($r = 0.9983$, $n = 6$) in the range 50–400 ng/ml, and the limit of quantitation was 40 ng/ml. Precision, expressed as the inter- ($n = 5$) and intraday ($n = 10$) coefficients of variation, was $\leqslant 3.2\%$ on the same day and $\leqslant 6.0\%$ between days for each quality control sample of 75, 150 and 300 ng/ml, respectively. Accuracy, expressed as the inter- and intraday % bias, was −11.6 to −0.2 on the same day and −11.8 to 3.1 between days for each quality control sample.

For AcHz the calibration curve was linear ($r = 0.9999$, $n = 6$) in the range 70–400 ng/ml, and the limit of quantitation was 60 ng/ml. Precision, expressed as the inter- ($n = 5$) and intraday ($n = 10$) coefficients of variation, was $\leqslant 11.1\%$ on the same day and $\leqslant 14.8\%$ between days for each quality control sample of 100, 200 and 300 ng/ml. Accuracy, expressed as the inter- and intraday % bias, was −13.9 to 3.4 on the same day and −14.6 to −6.2 between days for each quality control sample.

For AcINH the calibration curve was linear ($r = 0.9999$, $n = 8$) in the range 0.78–50.0 μg/ml, and the limit of quantitation was 100 ng/ml. Precision, expressed as the intraday coefficient of variation ($n = 10$), was $\leqslant 7.3\%$ on the same day for each quality control sample of 5, 15 and 30 μg/ml. Accuracy, expressed as the intraday % bias, was 0.3 to 14.8 on the same day, for each quality control sample.

Statistical analysis

Data were expressed as mean and SEM, and analysed by one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparison between the groups (Sigma-Stat software).

Results

Figure 1 shows the time-dependent plots of the serum concentration of INH, AcINH, Hz and AcHz for the animals treated with isoniazid and drug combinations. Table 1 displays the *AUC*s for the experimental groups. Animals that received INH + RMP exhibited a decrease in the plasma concentrations of INH and Hz, compared with the control (INH alone). In the group that received INH + PYR, the plasma concentrations of INH and AcHz fell and that of Hz rose. Animals that received INH + RMP + PYR showed a decrease in the plasma concentrations of all compounds except AcHz, for which an increase was observed.

Discussion

Although it is still the primary drug for TB treatment, the use of INH is not uncommonly associated with cases of hepatotoxicity. The mechanism by which such anti-tuberculosis drugs exert their toxic effects has been the subject of research for more than 25 years, but it remains controversial. On the other hand, there is no doubt that the metabolic products of INH have an important role in this scenario. This is the first study of the effect of the drug associations INH + RMP, INH + PYR and INH + RMP + PYR on the kinetics of the disposal of INH and its metabolites AcINH, Hz and AcHz. To simulate classical anti-tuberculosis treatment, the animals were pre-treated with the drug combinations for 21 days. The kinetic parameter *AUC* was chosen, as it expresses the total exposure of the animals to isoniazid and its metabolites over the period of dose administration.

Here, the co-administration of RMP (group II) caused a strong decrease in the *AUC* of INH. However, during this diminished bioavailability of INH there was no concomitant rise in *AUC* for its metabolites. Indeed, the *AUC*s for AcINH and AcHz were not significantly altered, and that for Hz actually fell, in comparison with the control (INH group). Although the oxidative products of biotransformation of Hz were not measured, these results suggest an increase in the downstream metabolism of Hz. Corroborating these findings, plasma hydrazine in rats co-treated with RMP was decreased by 18% relative to the INH group [19]. Moreover, these results are consistent with the well known characteristic of RMP as an inducer of isoniazid hydrolase and,

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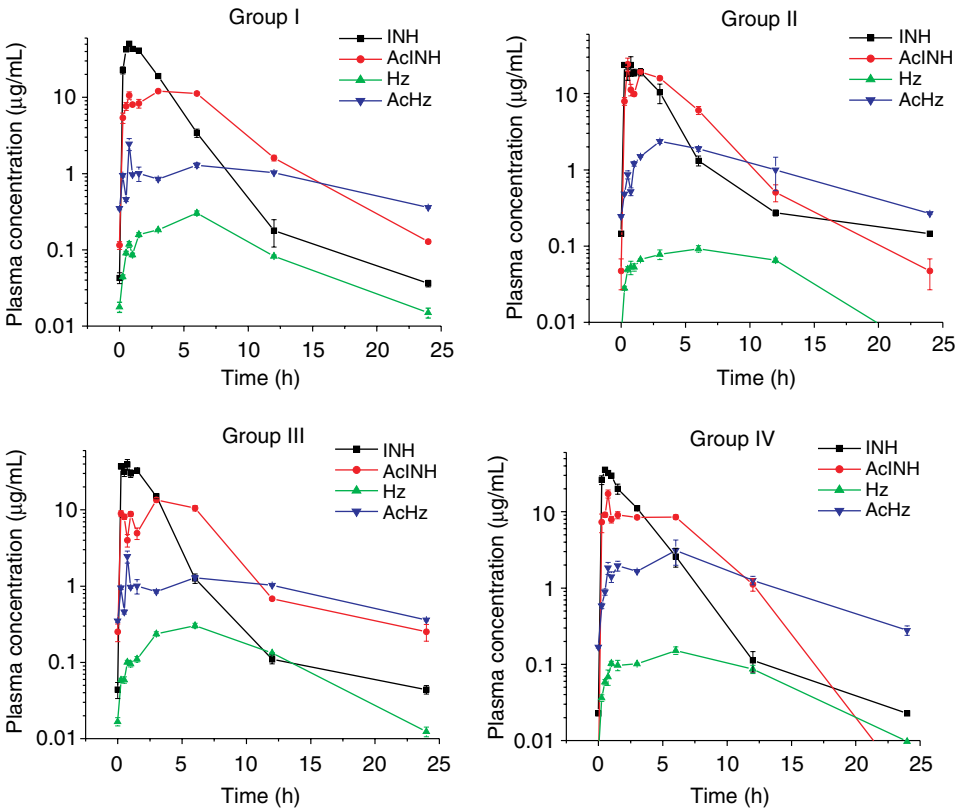

Figure 1. Time-dependent plasma concentrations of isoniazid and its metabolites. Results are expressed as mean and SEM ($n = 5$)

Table 1. Area under curve (*AUC*) for the animals treated with isoniazid and its metabolites

AUC_{0-24} ($\mu g\,ml^{-1}\,h$)	Group I (INH)	Group II (INH + RMP)	Group III (INH + PYR)	Group IV (INH + RMP + PYR)
INH	146 ± 3	75 ± 5^{a}	$112 \pm 6^{a,b}$	93 ± 5^{a}
AcINH	110 ± 2	102 ± 1	99 ± 5	88 ± 5^{a}
Hz	2.9 ± 0.1	1.3 ± 0.1^{a}	$4.6 \pm 0.1^{a,b}$	$1.9 \pm 0.2^{a,b,c}$
AcHz	20 ± 1	27 ± 4	$6.8 \pm 0.3^{a,b}$	$34 \pm 7^{a,c}$

The results are expressed as mean and SEM ($n = 5$).
[a] $p < 0.05$ relative to the control (group I).
[b] $p < 0.05$ relative to group II.
[c] $p < 0.05$ relative to group III.

microsomal enzymes, the latter being responsible for oxidation of the formed Hz [3,23]. In short, the increased metabolism of INH and Hz in rats treated with INH + RMP might be directly linked with the exacerbated hepatotoxicity of this drug association [24].

The effect of PYR (group III) on INH metabolism differed from that observed for RMP, since the production of AcHz was decreased significantly. The most likely explanation would be an increase in the rate of metabolism of AcHz to Hz, which actually was augmented. Taking into account that Hz is a potentially hepatotoxic substance [14], this finding reinforces the potential risk of co-administration of PYR with INH. This is consistent with a report by Yee *et al*. that

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the incidence of all major adverse events was 1.48 per 100 person-months of exposure to PYR, compared with 0.49 for INH and 0.43 for RMP [25]. Moreover, in a Singaporean study, all the patients with fatal drug-induced hepatotoxicity had followed PYR-containing regimens [26].

Finally, the effect of the triad INH + RMP + PYR (group IV) on INH metabolism was studied. The main finding here was that RMP had a dominant effect, since no significant differences were observed between groups II and IV. Relative to the control, the *AUC* of AcINH decreased. Indeed, the *AUC*s of AcINH for groups II and III were also diminished, although not significantly. It is suggested that the trend was accentuated when the drugs were administered together in group IV.

In summary, the bioavailability of INH was significantly diminished when rats were exposed to RMP and/or PYR. Additionally, co-administration with PYR caused the accumulation of Hz and a decrease in the serum level of AcHz. These findings might explain the differences in the risk of hepatotoxicity when the combined drugs are used.

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