Indian Journal of Pharmacology

ISSN 0253-7613

Official Publication of the Indian Pharmacological Society
December 2007 Vol 39 Issue 6

CONTENTS

Gastrointestinal permeability studies using combinations of rifampicin and nucleoside analogue reverse transcriptase inhibitors in rats

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Received: 19.11.2007
Revised: 28.12.2007
Accepted: 08.01.2008

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ABSTRACT

Objectives: To investigate the gastrointestinal tract (GIT) permeability of five nucleoside analogue reverse transcriptase inhibitors (NRTIs), viz., zidovudine, stavudine, abacavir sulphate, lamivudine and didanosine, individually and in the presence of rifampicin in rats by ligated-loop technique.

Materials and Methods: The permeability studies were carried out on Sprague-Dawley rats in the weight range of 240-260 g. The drug contents were estimated by a validated gradient HPLC method. Degradation and solubility studies were also carried out on the drugs, alone and in combination, to correlate with the results of in situ experiments.

Results: The results showed that rifampicin was better absorbed from stomach and duodenum; zidovudine was moderately absorbed throughout GIT; stavudine and lamivudine were absorbed better through the intestine; abacavir was well absorbed through duodenum; and didanosine completely disappeared through stomach and was absorbed moderately from proximal parts of the intestine. In drug combinations, NRTIs did not influence permeability/absorption of rifampicin and vice versa. The disappearance of didanosine through stomach could be ascribed to decomposition of the drug at pH 2.

Conclusion: The study reaffirms that rifampicin and NRTIs do not influence gastrointestinal permeability of each other.

KEY WORDS: Rifampicin, NRTIs, gastrointestinal permeability, solubility, degradation

Presently, infection with *Mycobacterium tuberculosis* is considered as the most common human infection. Every year, tuberculosis (TB) infects 8 million people worldwide. Every second human being around the world is getting infected with TB. Each year, approximately 2 million new cases are added to the evergrowing list of TB-infected individuals. Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV), which also continues to present a significant global public health crisis. At the end of the year 2001, an estimated 40 million people worldwide (adults, 37.2 million and children, 2.7 million) were living with AIDS. During 2001, approximately 5 million new HIV infections have occurred worldwide, i.e., approximately 14,000 infections each day. In the last year, HIV-associated illnesses resulted in approximately 3 million deaths worldwide. After invading the human body, the HIV gradually erodes the ability of the immune system to resist various pathogens, thus making the patient increasingly vulnerable to a number of opportunistic infections, the most dreaded one of which is TB.

During the last 10-15 years, TB cases have increased by 300-400% in countries with high HIV prevalence; this is mainly because HIV increases the risk of disease reactivation in people with latent *M. tuberculosis* and HIV-infected persons are more susceptible to new TB infections. HIV-positive patients often exhibit a host of symptoms while under going anti-TB treatment, such as fever, chest infection, recurrent diarrhea, *Candida*, bacteremia, cryptococcosis and Kaposi's sarcoma. Adverse reactions to anti-TB drugs are more frequent, leading to interruptions of treatment and occasional fatalities. An increase has been observed in the recurrence rate of TB in HIV-positive patients, particularly occurring after applying the 'standard treatment' and after the discontinuation of treatment due to drug reactions. The maximum number of cases of multi-drug resistant-TB (MDR-TB) has been reported from India. HIV itself does not cause MDR-TB, but augments the spread of this dangerous condition by increasing the susceptibility to infection and accelerating the progression from the infection to disease stage.

HIV and TB when combined, can induce physiological and immunological alterations that have pronounced effects on the absorption, metabolism and protein binding of drugs employed in their therapy. The anti-TB drugs have been categorized into two types, namely, the first- and second-line drugs. First-line drugs are further classified into essential first-line (rifampicin

and isoniazid) and the supplemental first-line (pyrazinamide, ethambutol and streptomycin). Second-line drugs include capreomycin, anamycin, ethionamide, para-aminosalicylic acid, cyclosporine, thiacetazone, ciprofloxacin, levofloxacin, ofloxacin and sparfloxacin. Antiretroviral agents fall under three categories, namely, nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). These categories include the following drugs: NRTIs—zidovudine, didanosine, stavudine, lamivudine, abacavir, tenofovir and zalcitabine; NNRTIs—delaviridine, nevirapine and efavirenz; and PIs—ritonavir, saquinavir, indinavir, amprenavir, nelfinavir and lopinavir. Antiretroviral drug regimens consist of concomitant use of three or four antiretroviral drugs; they may even include more drugs depending upon the condition of the patient i.e., viral RNA count and CD4+ cells count. *M. tuberculosis* infection worsens the condition of the patient taking rifampicin, isoniazid, pyrazinamide and/or ethambutol along with other antiretroviral drugs.

Management of HIV-infected patients having concomitant TB is complicated by drug interactions between rifamycins and two classes of antiretroviral medications, *viz.*, PIs and NNRTIs. Rifampicin is known to induce the cytochrome P450 activity, which lowers the plasma concentration of PIs and NNRTIs (substrates for cytochrome P450) to subtherapeutic level.[1] Therefore, low plasma levels of antiretroviral drugs are associated with incomplete viral suppression and the emergence of drug resistance. Other multifactorial interactions such as modulation of P-glycoprotein by rifampicin, e.g., induction of the efflux pump, while ritonavir and indinavir inhibit the efflux transporters. Therefore, concomitant administration of rifampicin with PIs and NNRTIs is not recommended.

Accordingly, NRTIs are the drugs used in HIV patients coinfected with TB. However, no comprehensive and conclusive *in vivo* studies are yet available regarding the absence of interactions between rifampicin and NRTIs. Hence, the purpose of this study was to confirm the same through *in situ* gastrointestinal permeability studies. We carried out additional studies regarding the decomposition and solubility of rifampicin in the presence of NRTIs to correlate with the results of permeability studies.

Materials and Methods

Materials

Rifampicin was a gift sample from Panacea Biotec Ltd. (Lalru, India). Zidovudine and stavudine were gift samples from Ranbaxy Laboratories Ltd. (Gurgaon, India) and Cipla Ltd. (Mumbai, India), respectively. Abacavir sulphate, lamivudine and didanosine were supplied by Aurobindo Pharma Ltd. (Hyderabad, India). Buffer materials and all other chemicals were of analytical-reagent grade. High performance liquid chromatography (HPLC)-grade acetonitrile and methanol were procured from J.T. Baker (Mexico City, Mexico) and Mallinckrodt Baker Inc. (Paris, KY, USA), respectively. We used Zorbax C-18 HPLC column of size 250 × 4.6 mm and particle size 5 μm (Agilent Technologies, Wilmington, USA). Ultra-pure water was obtained from an ELGA water purification unit (Elga Ltd., Bucks, England). Decomposition reactions were carried out using a precision water bath (Julabo, Germany). Digital shaking water bath (Julabo SW 21, Germany) was used for solubility studies. The pH values were recorded on a research pH meter (MA 235; Mettler Toledo, Switzerland). Other equipment used were a sonicator (Branson, Germany), a vapour pressure osmometer (Vapro, USA), an analytical balance (AG 135; Mettler Toledo, Switzerland), a homogenizer (PT-3100 POLYTRON; Switzerland), centrifuge (Biofuge 15; Germany) and autopipettes (Tripette, Merck, Germany).

Development and validation of analytical methods for the simultaneous determination of rifampicin along with various NRTIs

Several HPLC methods have been reported in the literature for the analysis of rifampicin[2-7] and NRTIs.[8-12] However, to date, no HPLC methods are available in the literature for the simultaneous analysis of NRTIs and rifampicin. One of the objectives of the present study was to determine the gastrointestinal permeability of NRTIs alone and in combination with rifampicin; development of simple HPLC methods was considered necessary to simultaneously estimate the investigated NRTIs along with rifampicin in the presence of artifacts obtained from permeability studies. For this purpose, various mobile phase compositions comprising acetonitrile and water were investigated using a C-18 column. A gradient elution was attempted keeping in view the polar nature of NRTIs and the non-polar nature of rifampicin.

The HPLC methods developed were validated for linearity, precision, accuracy and specificity. For establishing linearity, different concentrations of rifampicin, zidovudine, stavudine, lamivudine, abacavir and didanosine were prepared separately in triplicate from the primary stock solutions (1 mg/ml) of each compound. Ten-point standard plots within the concentration range of 1 to 100 μg/ml were constructed by plotting concentration *versus* mean area responses for individual drugs. Intraday precision of the method was assessed by injecting three different concentrations of each drug, three times on the same day. The experiment was repeated for three consecutive days at three different concentrations to determine the interday precision. The %R.S.D. values were calculated in each case. The percentage recovery of different amounts of each drug was determined by spiking the drugs separately into the blank matrix obtained from permeability studies. The blank matrix used in this study was a mixture of the matrices obtained from the stomach, duodenum, jejunum and ileum when the blank buffer solution was injected into different loops of rat GIT and kept them undisturbed for 60 min. The complete experimental protocol for the permeability study is described in the subsequent section. The recovery of the drugs in each sample was calculated from the established linearity equations. Drug specificity was determined by spiking each NRTI together with rifampicin into the blank matrix obtained from different segments of the GIT. The resolution of different drugs was assessed on the HPLC column.

Permeability studies

For permeability studies, Sprague-Dawley rats weighing 240-260 g were used. All animal experiments were performed under approved protocol (IAEC/04/031) of the Institutional Animal Ethics Committee. Gastrointestinal permeability studies were carried out using ligated loop technique. The

animals were fasted for 12 to 16 h before the experiments. They were anesthetized by intraperitoneal administration of urethane (1.5 g/kg) and kept on a wax tray. The temperature was maintained at 37°C using a table lamp. After making an abdominal incision, the GIT was assessed and divided into loops comprising the stomach, duodenum, jejunum and ileum. For preparing the loops, the stomach was exposed without injuring any blood vessels and ligated immediately adjacent to the cardiac sphincter. A second ligature was placed adjacent to the pyloric sphincter. A needle with silicone tubing was carefully introduced through the duodenum to project into the stomach *via* the pyloric sphincter, before finally securing the ligature. The three intestinal loops measured 10 cm each. The duodenal loop was made from approximately 1 cm from the pylorus. The jejunal loop was made immediately adjacent to the duodenal loop. The ileal loop was made approximately 5 cm above the ileo-cecal junction. Care was taken to avoid injury to any blood vessels during dissection. The intestinal loops were washed with 10 ml saline, and a needle with a silicone tubing was inserted in each loop.

Using a syringe, 1 ml of the drug suspension/solution was injected into each loop slowly through the needles. After an hour, the loops were excised and rinsed in ice-cold saline. We rejected the stomach loops in which food material was observed after dissection. The contents of all other loops were emptied into a separate 25 ml volumetric flask. The mucosal side of each loop was rinsed with the buffer and added to the respective flask and the volume was made up to 25 ml. The resulting solution was filtered and analyzed by HPLC. During analyses, all the samples were stored at 4°C in an HPLC auto sampler.

Following are the concentrations of various drugs used in this study: rifampicin, 2.40 mg/ml; zidovudine, 1.20 mg/ml; stavudine, 0.16 mg/ml; abacavir, 1.20 mg/ml; lamivudine, 0.60 mg/ml and didanosine, 0.80 mg/ml. These were calculated based on the human single dose strength of each drug, gastric volume in humans in the fasted state (250 ml) and average weight of the animals (~250 g). The extent of absorption was considered as the amount of drug disappeared from the loop. The latter was obtained by deducting the sum of the amount of the drug present in the loop and the amount accumulated in the mucosa from the initial amount injected. Separate sets of animals ($n = 5$) were used for each drug. The studies were also extended to determine the influence of each NRTI on the permeability of rifampicin individually and in combination. The differences in the results were statistically evaluated using one-way analysis of variance (ANOVA).

Decomposition studies

Decomposition studies were carried out for individual drugs as well as the combinations containing each NRTI and rifampicin. The pH values of the buffers employed were 2, 5.5 and 7. The individual drug solutions/suspensions or the mixtures of drugs were vortexed and a sample withdrawn immediately. The remaining mixtures were maintained in a water bath at 37°C for 60 min. All samples were analyzed by HPLC. The percentage degradation of the drugs was calculated from the difference between the peak areas of samples drawn at 0 and 60 min. All experiments were performed in triplicate.

Solubility studies

The solubility of rifampicin at pH employed in the gastrointestinal permeability studies (pH 2.0, 5.5 and 7.0) was determined, when present alone and in combination with NRTIs. For this purpose, solutions of zidovudine (1.2 mg/ml), stavudine (0.16 mg/ml), lamivudine (0.6 mg/ml), abacavir (1.2 mg/ml) and didanosine (0.8 mg/ml) were prepared separately in each buffer. An excess amount of rifampicin was then added to each drug solution and incubated at 37°C in a shaking water bath. As a control, rifampicin alone was dissolved in different pH buffers and incubated at the same conditions to determine its solubility in the absence of NRTIs. Samples were withdrawn after 3 h and analyzed by HPLC after diluting appropriately. All the experiments were conducted in triplicate.

The solubility of NRTIs in the presence of rifampicin was not investigated because the human single dose of all five NRTIs used in the present study are reported to be completely soluble in 250 ml of buffer at pH 1-7.5.[13]

Results

Figure 1 shows the representative chromatograms of the samples obtained by spiking the combination of rifampicin and each NRTI to the blank matrices recovered from the stomach, duodenum, jejunum and ileum. The gastrointestinal permeability behavior of rifampicin and NRTIs in different segments of rat GIT is shown in Figure 2. Figure 3 shows the permeability behavior of rifampicin in the presence of various NRTIs. The permeability of NRTIS in the presence of rifampicin is shown in Figure 4.

Table 1 provides the data for the decomposition of NRTIs when present alone and in combination with rifampicin, at the pH employed for gastrointestinal permeability studies (pH 2, 5.5 and 7). Table 2 lists % decomposition values of rifampicin in the absence and the presence of NRTIs. The solubility data of rifampicin in buffers with pH values 2, 5.5 and 7 are presented in Table 2. The table also lists the solubility of the drug in the presence of various NRTIs.

Discussion

Development and validation of the HPLC method

In the initial trials using an isocratic method employing acetonitrile/water and a C-18 column, rifampicin and NRTIs were eluted either together or at an unacceptable time gap. Accordingly, a gradient method was decided to be used to obtain the optimum separation of rifampicin with NRTIs from the samples obtained in the permeability studies. It was possible to achieve an acceptable resolution of all compounds using a mobile phase composed of acetonitrile and water, which was run in a gradient mode where the percentage of acetonitrile was varied by 7%, 7%, 43%, 43%, 7% and 7% at 0, 2, 3, 18, 18.01 and 25 min, respectively. The flow rate was maintained at 1 ml/min, detection was carried out at 254 nm and injected volume was 20 µl. The experiments were carried out under appropriate column temperature conditions.

The drug responses were strictly linear in the investigated concentration ranges ($R^2 > 0.99$) and the %R.S.D. values for intra- and interday precisions were within the acceptable limits of 1% and 2%, respectively. The drug recoveries were in the range 99%-101.5%, thus confirming the accuracy of the

Figure 1: Overlay chromatograms showing separation of rifampicin and NRTI drugs, *viz.*, zidovudine, stavudine, lamivudine, abacavir and didanosine from blank matrices recovered from the stomach (a), duodenum (b), jejunum (c) and ileum (d) of rat

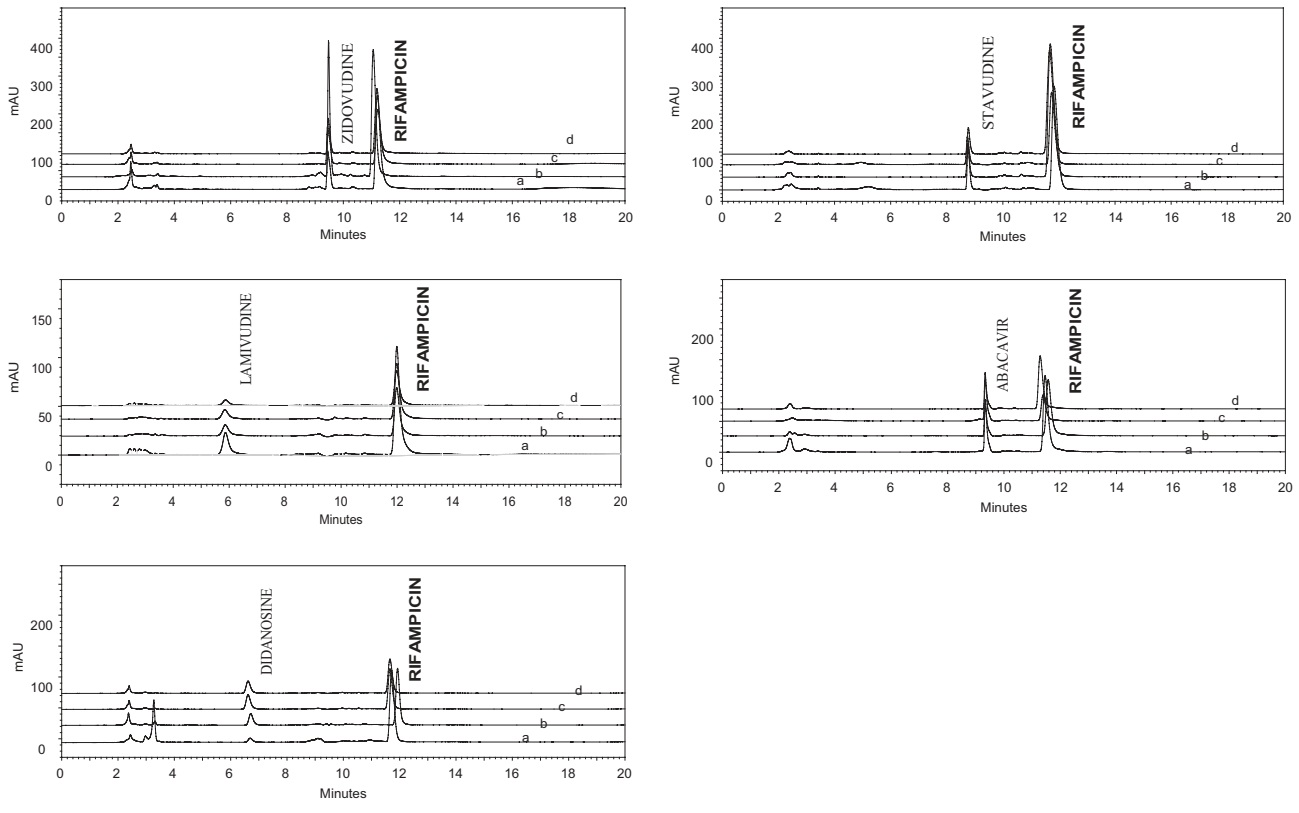
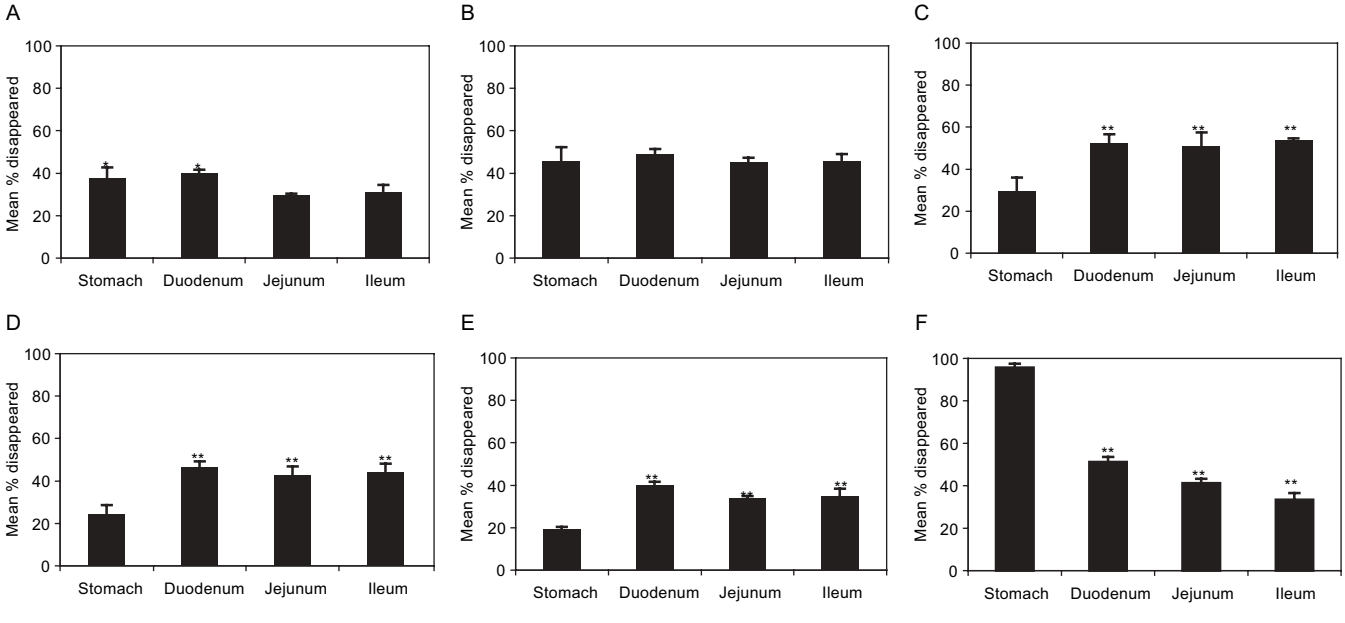


Figure 2: Permeability behavior of rifampicin (A), zidovudine (B), stavudine (C), lamivudine (D), abacavir (E) and didanosine (F) alone in different segments of GIT in rats in 60 min. *P < 0.05, significantly different when compared to jejunum and ileum; **P < 0.05, significantly different when compared to stomach. The statistical analysis was carried out by employing one-way analysis of variance (ANOVA)



method.

Figure 1 shows no interfering peaks near the retention time of the drugs. The retention time of rifampicin was ~12 min and that of zidovudine, stavudine, lamivudine, abacavir and didanosine was 9.5, 9.0, 5.5, 9.2 and 7.0 min, respectively. A small peak of the artifact appeared at ~2.0-3.0 min in all

Figure 3: Permeability behavior of rifampicin in the presence of zidovudine (A), stavudine (B), lamivudine (C), abacavir (D) and didanosine (E) in different segments of rat GIT in 60 min. Key: ■, drug alone; □ , drugs in combination

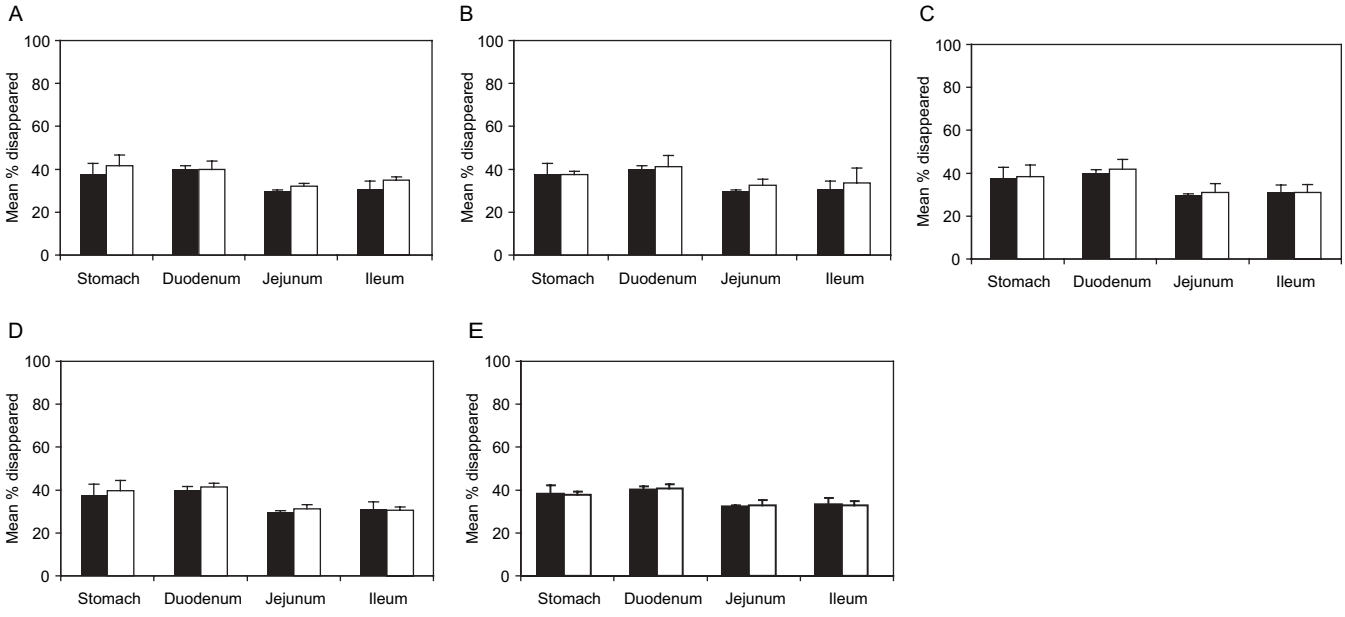
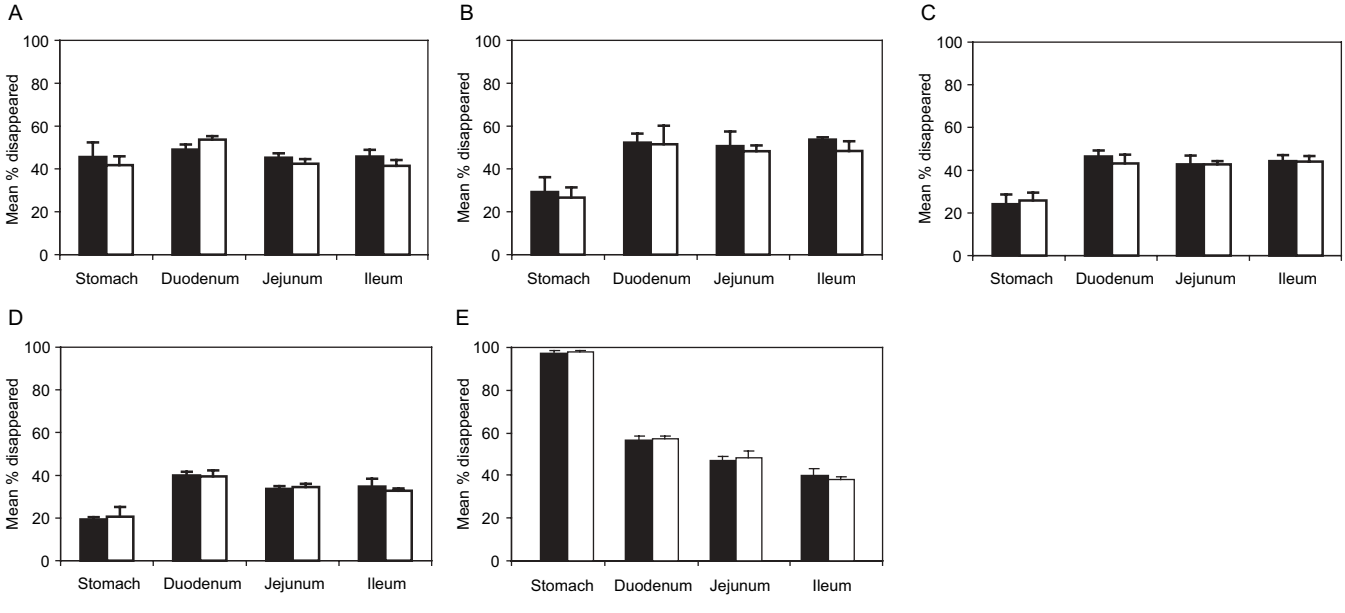


Figure 4: Permeability behavior of zidovudine (A), stavudine (B), lamivudine (C), abacavir (D) and didanosine (E) in the presence of rifampicin in different segments of rat GIT in 60 min. Key: ■, drug alone; □ , drugs in combination



the cases, the level of which was slightly higher in the fluid obtained from the stomach as compared to that from other segments.

Permeability studies

It is evident from Figure 2 that rifampicin was better absorbed through the stomach and duodenum than through the distal regions of the intestine. On the other hand, zidovudine was absorbed moderately throughout the GIT, stavudine and lamivudine were absorbed better from the intestine as compared to the stomach. Didanosine completely disappeared from the stomach. The drug also disappeared significantly through the proximal regions of the intestine.

The permeability behavior of rifampicin in the presence of various NRTIs did not alter in the combination [Figure 3]. Similarly, the permeability behavior of NRTIs in the presence of rifampicin did not exhibit any change, as indicated in Figure 4. Thus, rifampicin or NRTIs did not influence the permeability of each other.

Table 1

Extent of decomposition of NRTIs alone and in combination with rifampicin in 60 min at pH values corresponding to those in different regions of gastrointestinal tract

Drugs	*Mean % decomposed ± S.D. (n = 3)*					
	Alone			*Combination with rifampicin*		
	pH 2.0	*pH 5.5*	*pH 7.0*	*pH 2.0*	*pH 5.5*	*pH 7.0*
Zidovudine	0.28 ± 0.35	0.12 ± 0.09	0.10 ± 0.28	0.29 ± 0.25	0.42 ± 0.45	0.29 ± 0.21
Stavudine	1.07 ± 0.22	0.48 ± 0.16	1.24 ± 0.42	1.14 ± 0.82	1.67 ± 0.27	0.87 ± 0.34
Lamivudine	1.42 ± 0.64	0.84 ± 0.59	0.28 ± 0.77	0.92 ± 0.37	0.88 ± 0.07	0.07 ± 0.10
Abacavir	0.18 ± 0.44	0.12 ± 0.02	0.27 ± 0.25	0.06 ± 0.02	0.73 ± 0.29	0.80 ± 0.12
Didanosine	99.58 ± 0.15	2.49 ± 0.41	2.01 ± 0.17	99.10 ± 0.19	4.28 ± 0.90	3.12 ± 1.62

Table 2

Decomposition and solubility of rifampicin alone and in the presence of NRTIs in 60 min at pH values corresponding to those in different regions of gastrointestinal tract

pH	*Alone*	*Combination with NRTIs*				
		Zidovudine	*Stavudine*	*Lamivudine*	*Abacavir*	*Didanosine*
		Mean % decomposed ± S.D. (n = 3)				
2.0	3.92 ± 0.21	3.77 ± 0.31	3.62 ± 0.14	3.50 ± 0.23	3.58 ± 0.25	3.31 ± 0.26
5.5	1.09 ± 0.08	1.28 ± 0.43	1.19 ± 0.70	1.17 ± 0.66	1.34 ± 0.79	1.54 ± 0.69
7.0	0.61 ± 0.13	0.51 ± 0.19	0.56 ± 0.12	0.72 ± 0.25	0.03 ± 0.14	0.97 ± 0.09
		Solubility (mg/ml) ± S.D. (n = 3)				
2.0	20.44 ± 1.27	19.86 ± 1.42	20.77 ± 1.80	19.51 ± 0.52	20.95 ± 0.37	19.52 ± 0.81
5.5	0.65 ± 0.02	0.65 ± 0.03	0.66 ± 0.01	0.65 ± 0.02	0.62 ± 0.01	0.67 ± 0.04
7.0	0.84 ± 0.07	0.80 ± 0.06	0.83 ± 0.07	0.83 ± 0.10	0.83 ± 0.06	0.86 ± 0.07

Decomposition studies

Various NRTIs, except didanosine, demonstrated decomposition to a very small extent (<2%) when present alone or in combination with rifampicin at pH values 2, 5.5 and 7 [Table 1]. Didanosine decomposed almost completely at acidic pH, while its decomposition was minimal (~2-2.5%) at pH values 5.5 and 7. This clearly explains its complete disappearance from the stomach during permeability studies [Figure 2F]. No significant difference was evident in the decomposition behavior of NRTIs in the presence of rifampicin.

Rifampicin alone exhibited decomposition of ~4% at pH 2, whereas it was negligible at pH 5.5 and 7 [Table 2]. Rifampicin showed similar decomposition pattern even in the presence of NRTIs.

Solubility studies

As shown in Table 2, rifampicin demonstrated very high solubility at pH 2, while the solubility decreased with an increase in pH, similar to the reported behavior.[14] It is also evident from the table that the presence of NRTIs did not alter the solubility of rifampicin at the investigated pH, which explains the absence of the effect of NRTIs on the permeability of rifampicin.

In conclusion, while describing the absorption sites of NRTIs through GIT, we reaffirm that rifampicin and NRTIs do not influence the gastrointestinal permeability of each other.

Acknowledgments

We thank the Ministry of Chemicals and Fertilizers, Government of India, for providing funds for this study under the Pharmaceutical Research and Development Project.

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