

Simultaneous HPLC Assay of Paracetamol and Sulfapyridine as Markers for Estimating Gastrointestinal Transit of Amphotericin B-containing Nanoparticles in Rat Plasma

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Abstract

A simple and sensitive two-step reversed-phase HPLC method was developed and validated for determining amphotericin B, paracetamol and sulfapyridine in rat plasma using piroxicam as internal standard. This was subsequently employed in a pharmacokinetic study in rats following simultaneous oral administration of solid lipid nanoparticle formulations of amphotericin B, paracetamol and sulfasalazine. The paracetamol and sulfapyridine served as markers for estimating gastric emptying and cecal arrival times. A gradient elution was employed in the analysis and detection at 405 nm for amphotericin B and 254 nm for paracetamol and sulfapyridine. The internal standard was detectable at both wavelengths. The gastrointestinal study was conducted on male Sprague-Dawley rats. Calibration curves were linear for the three drugs ($r^2 \geq 0.9998$) with the detection and quantification limits comparable with those in literature. Drug recovery from rat plasma was more than 92%. The accuracy of the method was < 8% for the three drugs whilst the precision was 10%. Paracetamol was rapidly absorbed from the small intestine reaching its C_{max} in 1 hr while amphotericin B was absorbed slowly reaching peak levels in 8 hr. Sulfapyridine was absorbed only after a lag time of 2 hr due to delayed arrival and hydrolysis of sulfasalazine at the colon by azo-reductase bacteria. The HPLC method is rapid and applicable for the simultaneous estimation amphotericin B, paracetamol and sulfapyridine in rat plasma, which may serve for an indirect estimation of gastric emptying and orocecal transit properties of amphotericin B within dosage forms after oral administration.

Keywords: Amphotericin B; Paracetamol; Sulfapyridine; Sulfasalazine; Gastrointestinal transit

Introduction

Amphotericin B (AmB) is a broad-spectrum antifungal drug chiefly used for managing life-threatening systemic fungal infections. A micellar suspension of the sodium deoxycholate salt of amphotericin B (Fungizone[®]) is widely used currently as the conventional option. However, three other lipid-based formulations are available but used to a lesser extent: AmB lipid complex (AmB complexed with dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, Abelcet[®]); AmB colloidal dispersion (AmB complexed with cholesteryl sulfate, Amphocil[®]) and liposomal AmB (AmB complexed with hydrogenated soy phosphatidylcholine, distearoyl, phosphatidylglycerol and cholesterol, AmBisome[®]) [1,2].

The mode of administration of AmB in these formulations is limited to intravenous injection due to its poor aqueous solubility, which makes systemic absorption from the gastrointestinal (GI) tract negligible. Thus, research has intensified in formulating AmB into a suitable oral preparation that addresses the above constraint since intravenous administration of is associated with several adverse effects such as fever, chills, rigors, malaise, headache, generalized aches, nausea, vomiting and hypoxia [3]. These adverse reactions are partly related to the induction of pro-inflammatory cytokines produced by AmB and also the release of TNF-alpha from macrophages [4,5].

Solid lipid nanoparticles (SLN) can be formulated to incorporate hydrophobic drugs such as AmB [6-10]. Due to their hydrophobicity and relatively high surface area to volume ratio, SLN can be effectively taken up by the Peyer's patches within the GI tract which improves drug bioavailability [11]. Furthermore, SLN exhibit mucoadhesion to the gut wall which therefore creates a desirable platform for the establishment of a concentration gradient with blood and further enhances drug absorption [12,13].

Data on the transit behavior of dosage forms through the GI tract is essential in determining possible absorption windows of the drug payload. This information can in turn be used to optimize desirable properties from the dosage form with a view to improving drug bioavailability.

The GI transit properties of dosage forms can be studied using gamma scintigraphy [14] and nuclear magnetic resonance [15]. An indirect means of studying GI transit properties of dosage forms using marker drugs is less costly and has been used successfully by several researchers [16-18]. The indirect approach is based on the premise that certain drugs have preferential absorption at specific locations within the GI tract. For example, following oral administration, the absorption of paracetamol (PAR) which is preferential in the small intestine, is related to the rate of gastric emptying of a dosage form containing it [19]. On the other hand, when sulfasalazine (SSZ) is administered orally, it is hydrolyzed by the large bowel flora to produce sulfapyridine (SP) and 5-aminosalicylic acid. The rate of appearance of absorbed SP in the blood can also be used to estimate the orocecal transit time [20,21] of a SSZ-loaded dosage form. Therefore, these two drugs can be used as markers to estimate the gastric emptying and orocecal transit times [16-18] of AmB-containing SLN.

A crucial aspect of utilizing the indirect GI transit estimation of dosage forms based on marker drugs is that the analytical method must be validated and be sufficiently sensitive to detect minute concentrations of the marker drugs. A further desirable requirement is the simplicity of the method, given that several drugs would have to be analyzed. In this paper, we propose a simple and sensitive HPLC method for the simultaneous assay of AmB, PAR and SP in rat plasma. This method was later used in a GI transit study to determine the GI transit and pharmacokinetic data of AmB following oral administration of AmB SLN as well as the marker drugs, PAR SLN and SSZ SLN [with SP to be released from SSZ *in-vivo*] using rats as the animal model.

Experimental

Chemicals and reagents

Acetonitrile was purchased from Duksan Pure Chemicals Co. Ltd. (Ansan, Korea). Paracetamol, dimethyl sulfoxide (DMSO) and sulfapyridine were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC., St. Louis, USA) and amphotericin B from Nacalai Tesque (Kyoto, Japan). Methanol and ethanol were purchased from Fisher Scientific (Waltham, USA). Water was Milli-Q 18.2 MΩ.cm at 25 °C (Millipore Corp., Bedford, USA). Acetic acid and sodium acetate trihydrate were purchased from R & M Chemicals (Essex, UK). All reagents and solvents used were of analytical and HPLC grades respectively.

HPLC instrumentation and conditions

A Perkin Elmer HPLC system (Series 200 auto-sampler, Series 200 UV/ VIS detector, Waltham, USA) configured to TotalChrom® Navigator software was used to analyze drug samples in the plasma. Separation of peaks was achieved using an Eclipse Plus® C18 (250 x 4.6 mm, 5 µm) column (ZORBAX, Agilent, USA). The mobile phase was filtered through a 0.45 µm membrane filter before use and comprised of acetate buffer (pH 4), prepared by mixing 847 mL of 0.1M acetic acid and 153 mL 0.1M sodium acetate (trihydrate): [A] and acetonitrile: [B] and run in gradient mode as follows: 100% A at 0 - 0.5 min; 60% A at 0.5 - 7 min; 20% A at 7 - 9 min; 60% A at 9-12 min and 100% A at 12-15 min, at a flow rate of 1 mL/min. The HPLC was run in a two-step injection mode to detect PAR and SP at 254 nm and then AmB at 405 nm. Piroxicam as internal standard (IS) was dissolved in a 1:1 methanol-ethanol solution to prepare a 5 µg/mL stock solution. The IS was detectable at both wavelengths. The methanol-ethanol mixture also served as deproteinizing solvent for the plasma samples.

Plasma standards

Stock plasma solutions containing AmB, PAR and SP at 100 µg/mL, 200 µg/mL and 1000 µg/mL respectively were prepared by spiking appropriate amounts of the respective drug solutions into the appropriate volumes of plasma. The stock solutions were further diluted with plasma to prepare the calibration standards for each drug, at three concentration levels representing low (LC), mid-range (MC) and high (HC) concentrations. The LC, MC and HC for AmB were 0.05, 0.25 and 10 µg/mL; for PAR were 0.5, 2.5 and 100 µg/mL; and for SP were 0.1, 0.5 and 20 µg/mL.

Plasma sample preparation

Samples for drug analyses were prepared by transferring 100 µL of plasma and 150 µL of the IS solution into microcentrifuge tubes. The tubes were vortex-mixed for 5 min and the extracts centrifuged at 14,000 rpm for 10 min. The clear supernatants were aspirated and filtered through a 0.20 µm syringe filter followed by direct injection onto the HPLC system.

Linearity

Calibration curves for AmB, PAR and SP in drug spiked-plasma were obtained over the following concentration ranges; 0.05 - 10 µg/mL, 0.5 - 100 µg/mL and 0.1 - 20 µg/mL respectively. The calibration curves were constructed using the ratio of the peak signal of each drug concentration to that of the internal standard versus corresponding drug concentration. Each analysis represents a replica of six runs.

Precision and accuracy

The within-day and between-day precision of the analysis at each concentration for each drug was determined as the percentage coefficient of variation (%CV) using the following relationship:

$$\%CV = \frac{\text{Standard deviation (SD)}}{\text{Mean concentration}} \times 100\% \dots \dots \dots (1)$$

The mean represents a replica of six determinations.

The within-day and between-day accuracy of the analyses was determined as the relative error at each concentration for each drug, using the following relationship:

$$\text{Relative error (E}_r\text{)} = \frac{\bar{x} - x_t}{x_t} \times 100\% \dots \dots \dots (2)$$

Where: \bar{x} =measured value(average); x_t =true value

Recovery

The recoveries of AmB, PAR and SP from spiked plasma following the extraction procedure were determined by comparing the peak signal of each drug from the prepared plasma samples with that of solutions of the pure drug samples in a solvent mix of DMSO and methanol. Six determinations were done for each drug, covering the three concentration levels explained in section 4.3. The percentage recovery was determined using the following relationship:

$$\% \text{ Recovery} = \frac{\text{Concentration obtained from spiked plasma}}{\text{Concentration of pure drug solutions}} \times 100\% \dots \dots \dots (3)$$

Specificity and sensitivity

The specificity of the analysis was performed by observing the chromatogram for blank plasma samples to detect any possible peak interference.

The sensitivity was determined in terms of limit of detection (LOD) and limit of quantization (LOQ), which were determined as the lowest concentration of each analyte at which the signal-to-noise ratio (S/N) were 3:1 and 10:1 respectively [22].

Pharmacokinetic and GI transit studies in rats

Pharmacokinetic studies on AmB, PAR and SSZ and GI transit studies on the SLNs was performed using three male Sprague-Dawley rats (300-350 g), fasted overnight. The protocol used for this study was approved by the Animal Ethics Committee of the University of Science, Malaysia. The animals were obtained from the Animal Holding Unit of University of Science, Malaysia.

Drug concentrations for AmB, PAR and SP in plasma were obtained after oral administration of drug-loaded SLNs to the rats. The three SLNs (AmB, PAR and SSZ) were similarly formulated and characterized physicochemically as regards to similarity in particle size, surface charge, morphology and *in-vitro* release properties in our previous work [8] prior to the *in-vivo* study. SP absorption into plasma was preceded by cleaving of SSZ in the colon by colonic bacteria to produce SP and 5-aminosalicylic acid. Each dose contained an equivalent of 10 mg/kg of the three individual SLNs dispersed in 1 mL of distilled water and then administered by oral gavages to the rats.

Blood samples (300 µL) were collected from the tail of the rats at 0, 1, 2, 4, 8, 12 and 24 hr post-administration into heparinized microcentrifuge tubes. The plasma was immediately harvested from the blood samples by centrifugation and analyzed for drug content using the above HPLC method.

Statistical analysis

The data have been presented as mean \pm standard deviation (SD) where indicated. GraphPad Prism 5 software was used in the statistical analyses and also to obtain the pharmacokinetic data presented.

Results and Discussion

Validation of the method

A number of HPLC methods specific to the detection of only AmB and suitable internal standards in biological fluids have been reported [23-27]. In the present study, we propose a method for the simultaneous analysis of AmB as well as PAR, SP and the IS, suitable for the indirect GI transit monitoring of AmB. Piroxicam was employed as the IS in the present study because it was found to absorb significantly at the two detection wavelengths used (254 and 405 nm). The retention times for PAR, SP, AmB and the IS were 8.5, 9.9, 11.7 and 12.8 min respectively. No interfering peaks were observed at the retention times for all four drugs in blank rat plasma as shown in Figures 1(a) and (b), indicating that the method is specific for the drugs. Furthermore, the peaks obtained for the respective drugs were well-resolved and separate from each other. The baselines are indicative of minimal noise interference (Figures 2 and 3).

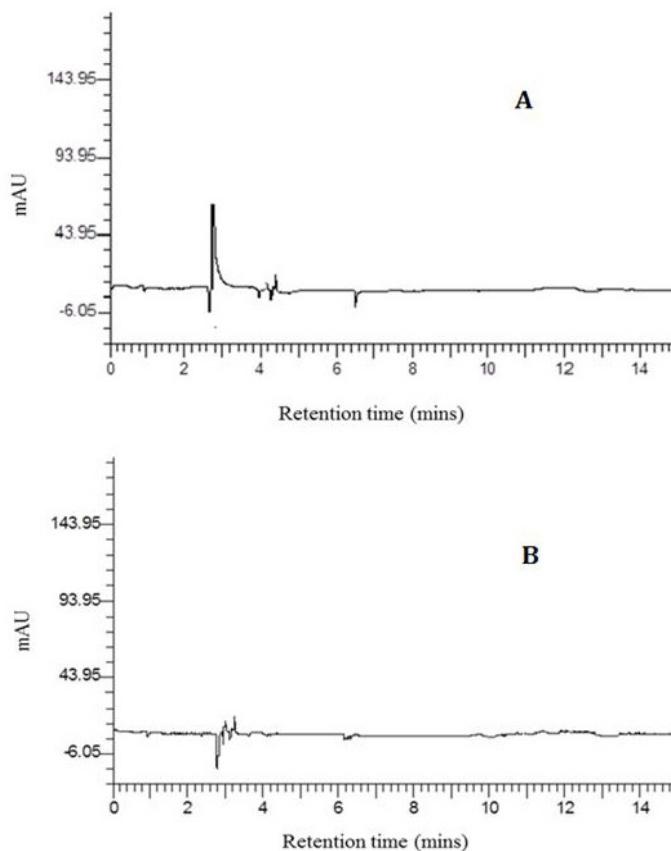


Figure 1: Chromatogram of blank plasma (a) at 254 nm and (b) at 405 nm

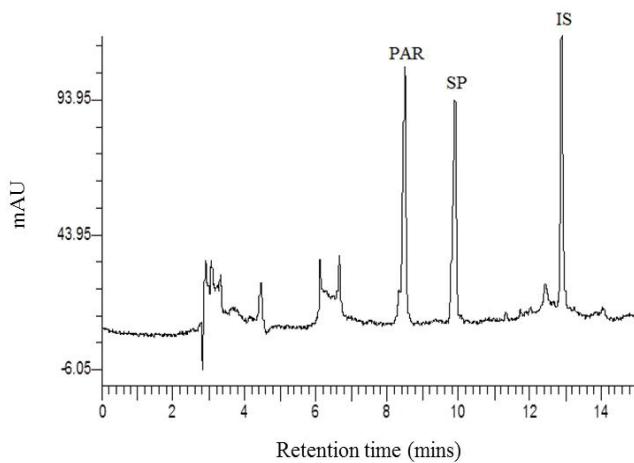


Figure 2: Chromatogram of plasma spiked with PAR, SP and IS at 254 nm

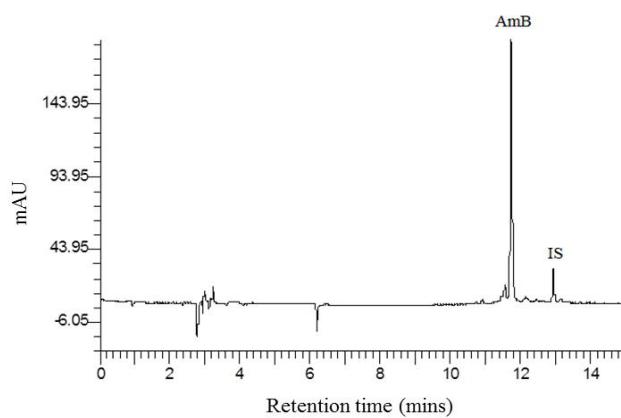


Figure 3: Chromatogram of plasma spiked with AmB and IS at 405 nm

The calibration curves for AmB, PAR and SP in plasma, were found to be linear over the concentration ranges of 0.05 - 10 µg/mL; 0.5 - 100 µg/mL and 0.1 - 20 µg/mL respectively. The coefficients of determination (R^2) were found to be 0.9999 for AmB; 0.9990 for PAR; and 0.9999 for SP.

The recovery of each analyte from plasma at the concentrations studied is shown in Table 1, pointing to a good drug recovery of the method with a minimum value of 91.70% (<9% matrix affect).

Level	% Recovery (mean ± SD; n = 6)		
	AmB	PAR	SP
LC	97.8 ± 3.15	98.7 ± 1.53	97.5 ± 5.42
MC	95.6 ± 6.12	100.1 ± 5.33	99.2 ± 4.76
HC	92.1 ± 0.58	99.4 ± 1.34	91.7 ± 1.82

Table 1: Recovery values for AmB, PAR and SP in rat plasma

The percent accuracies, which estimates the closeness of the observed concentrations to the expected values are shown in Table 2, with the maximum error obtained being 7.50%. The precision of the method, expressed as % coefficient of variation was assessed in terms of intra-day and inter-day variability's and the values are expressed in Table 3.

Level	% Accuracy (% Relative error)(n = 6)					
	AmB		PAR		SP	
	Intra-day	Inter- day	Intra-day	Inter- day	Intra-day	Inter- day
LC	2.40	0.27	0.90	5.06	1.55	1.29
MC	3.20	3.90	1.44	1.81	1.60	7.50
HC	0.20	1.20	0.27	2.90	0.08	1.67

Table 2: Intra-day and inter-day accuracy of the HPLC method

Level	% Precision (% CV)(n = 6)					
	AmB		PAR		SP	
	Intra-day	Inter- day	Intra-day	Inter- day	Intra-day	Inter- day
LC	4.39	9.41	2.53	6.08	7.31	1.13
MC	7.17	2.36	4.01	2.87	9.89	4.79
HC	5.78	0.93	4.28	5.13	3.77	3.16

Table 3: Intra-day and inter-day precision of the HPLC method

The %CVs were found to be less than 10% in all cases, indicating an acceptable degree of variation at the respective concentrations.

The limits of detection (LOD) and quantification (LOQ) were respectively, 5.7 ng/mL and 19 ng/mL for AmB; 13.0 ng/mL and 44.8 ng/mL for PAR; and 15.1 ng/mL and 51.0 ng/mL for SP. The LOD and LOQ values for AmB and PAR were found to be comparable with or lower than those from published data [23-28] in which similar mobile phase systems were used for AmB. In the present study, all four drugs were analyzed in a two-step procedure, thus minimizing analysis time. Furthermore, this HPLC method has the advantage of a simple sample preparation procedure as compared to solid-phase extraction [25] and also has a relatively short run time of 15 min, considering that four different compounds (including the IS) can be simultaneously analyzed.

Pharmacokinetic and GI transit study

In order to test the viability of the method, a pilot pharmacokinetic and GI study was conducted using three male Sprague-Dawley rats. The three SLNs exhibited identical physicochemical characteristics [8]. *In-vitro* release in phosphate buffered saline (pH 7.4) over an 8 hr period showed a potential of prolonged drug release for AmB (11.8%) and SSZ (31.4%) in contrast to a faster release for PAR (65.9%) from their respective SLNs [8]. Figure 4 and Table 4 show the plasma drug concentration-time profiles and pharmacokinetic data respectively, for AmB, PAR and SP following simultaneous oral administration of AmB, PAR and SSZ SLNs to the rats. A T_{max} of about 1 hr was observed from PAR, indicating that it was rapidly released from the PAR SLN due to its preferred solubility in the surrounding aqueous medium as compared with AmB and SSZ which are more hydrophobic. The rapidly released and absorbed PAR in the small intestine therefore estimates the gastric emptying time of the SLNs. AmB was slowly absorbed from the GI tract, reaching a T_{max} in 8 hr. This is indicative of a slow uptake process most likely via the Peyer's patches, which are located mainly in the ileum, prior to drug emptying into systemic circulation [11]. There was a lag time of about 2 hr prior to appearance of SP in the plasma, which estimates the cecal arrival time of the SLNs. The delay in the absorption of SP is due to a combination of delayed microbial degradation of SSZ in the colon by microbial flora to produce SP and 5- amino salicylic acid followed by its absorption.

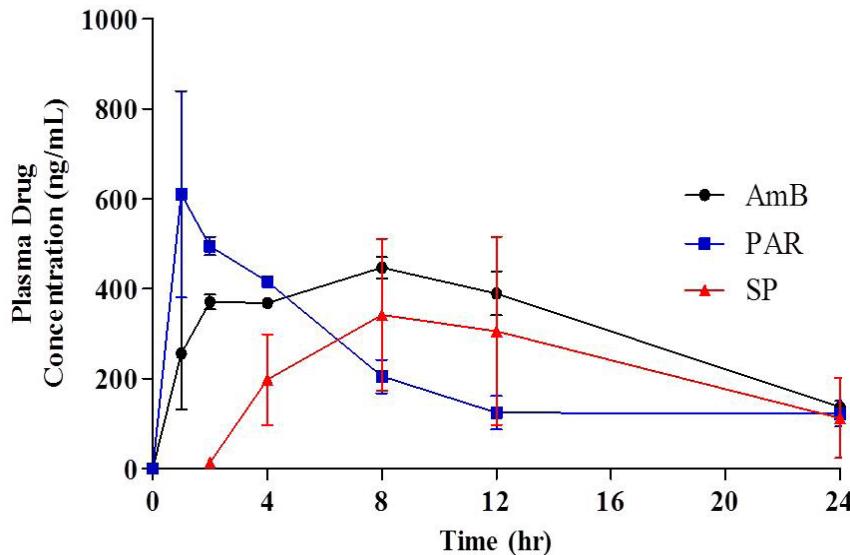


Figure 4: Mean plasma concentration-time profiles of AmB, PAR and SP after a single simultaneous oral gavage of AmB, PAR and SSZ SLNs (10 mg/kg each) simultaneously (mean±SD; n=3)

Drug	Dose (mg/kg)	T _{max} (hr)	C _{max} (ng/mL)	AUC ₀₋₂₄ (ng·hr/mL)
AmB	10	8	446.7 ± 23.6	7638.4 ± 440.3
PAR	10	1	609.8 ± 229.5	5146.2 ± 356.2
SP (from SSZ)	10	8	341.7 ± 169.1	5095.3 ± 3186.2

Table 4: Pharmacokinetic data of AmB, PAR and SP following simultaneous oral administration of AmB, PAR and SSZ SLNs (n = 3)

The AUC₀₋₂₄ was significantly high for AmB compared to PAR and SP, indicating a favorable albeit slow, uptake process. The high magnitude of C_{max} for PAR is indicative of a rapid rate of absorption from the small intestine which is likely to be more as a result of released PAR than due to uptake of the SLNs by Peyer's patches followed by emptying. The pharmacokinetic data obtained is comparable with those reported for lipid based AmB formulations [10,29,30]. We may conclude that the developed HPLC method is suitable for analyzing AmB, PAR and SP in rat plasma and may therefore be employed in pharmacokinetic studies on these drugs and in the indirect estimation of GI transit properties of dosage forms.

Conclusion

The above analytical method for the simultaneous detection of AmB, PAR and SP is rapid, accurate and sensitive for determining the concentrations of these drugs in plasma, using piroxicam as the IS. The peaks for all the analytes are well resolved and the run time is considerably short, given that four drugs were simultaneously analyzed. The sample volume is small, which is crucial during pharmacokinetic studies on rodents. Crucially, the method can potentially be applied to studying the pharmacokinetics and the GI transit properties of AmB as well as other dosage forms by the indirect approach.

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