RESEARCH PAPER

Development and Validation of an RP-HPLC Method for Simultaneous Estimation of Metformin and Amphotericin B in Liposomal Formulation

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ABSTRACT

This study aimed to develop and validate a simple, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of metformin and amphotericin B in a liposomal formulation. Chromatographic separation was achieved using a C18 column with a mobile phase comprising acetonitrile and water (70:30 v/v) at a flow rate of 1.0 mL/min. Detection was performed at 240 nm. The retention times for metformin and amphotericin B were found to be 4.60 and 3.60 minutes, respectively. The method was validated following ICH guidelines for linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ). Calibration curves were linear in the range of 10–60 µg/mL with correlation coefficients exceeding 0.999. The method demonstrated satisfactory accuracy (98–102%) and precision (%RSD < 2). The developed method was successfully applied to analyse both drugs in liposomal formulation. It can be utilised for routine quality control analysis.

KEY WORDS: Metformin, Amphotericin B, Liposomal Formulation, RP-HPLC

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INTRODUCTION:

Lung cancer is one of the primary causes of cancer-associated mortality and a huge burden on health worldwide. Survival rates have poor outcomes, especially with non-small cell lung cancer (NSCLC), which comprises 85% of the disease, despite improvement in the field of diagnostics and treatment. Side effects, resistance, and relapse are some of the challenges facing the standard therapies, including chemotherapy, radiation, and immunotherapy. Mechanisms such as drug repurposing and formulating drugs to make them more effective and less toxic are being investigated. It is important to note that a liposomal metformin/amphotericin B (AmB) combination has been promising. Approximately, the number of new cases and deaths in lung cancer in 2022 is 2.48 million and 1.8 million cases respectively.[1]Approximately 70 per cent of the patients of NSCLC are diagnosed at an advanced stage with only 7 per cent five-year survival rate of the metastatic disease.[2] New therapies have made poor prognosis and short median overall survival (10 12 months) remain common among the majority of NSCLC patients, particularly those without actionable mutations [3,4].

Re-purposing or repositioning of drugs means using drugs that have been developed to different medical uses. This method has a lower cost of development, shorter time to market and well-developed safety profiles, which are especially attractive in cancer treatment. The example of metformin, an oral biguanide that is typically used to treat diabetes type 2, is a good example of a drug used to be repurposed because of its possible anticancer effect. It is not only exhibiting anti-proliferative properties in different types of cancers, such as lung cancer, but also activates AMP-activated protein kinase (AMPK) and suppresses the mTOR pathway, as well as changes cellular metabolism [5]. According to clinical studies, metformin can be used to increase chemotherapy and decrease cancer cell resistance [6]. Therefore, the interest of combination repurposing approaches that address several ways at once has emerged, as it is commonly noted that the complexity of oncogenic signaling can undermine the efficacy of single-target therapy.[7,8]

The ample scientific focus on the potential repurposing of metformin hydrochloride, a crucial antidiabetic medication and a vital drug in the World Health Organization Model List, has elicited significant scientific interest in repurposing the drug. According to epidemiological investigations, the application of metformin to diabetic patients lowers the chances of developing cancer and enhances their survival chances on diagnosis of cancer [9]. The anticancer actions of Metformin are well documented and they involve AMPK activation, inhibition of mTOR, inhibition of lipogenesis and cholesterol synthesis, arresting of cell-cycle, and immunomodulation [10, 11]. Synergistic antiproliferative actions between metformin and conventional chemotherapeutic drugs (cisplatin, paclitaxel, pemetrexed) in NSCLC models have demonstrated synergistic effects in the preclinical model of lung cancer [12]. There is clinical evidence that combination of metformin with carboplatin, paclitaxel, and bevacizumab has better success rates of one-year progression-free survival than combination of standard therapy alone (47% vs. 15) [13]. The positive safety profile, low cost, and long history of clinical usage of metformin qualify it as one of the most contemporary active repurposing targets in the field of oncology.

The amphotericin B (AmB) is a polyene macrolide antifungal that was initially discovered in Streptomyces nodosus in 1955. It is mainly applied in management of severe systemic fungal diseases, including aspergillosis, cryptococcosis, candidiasis, and mucormycosis [14]. Its use in oncology has also been pointed out in research. As an illustration, it can increase the permeability of tumor cell membranes, augment the uptake of chemotherapeutic substances [15], and cause cytotoxicity in different tumor cell lines, such as lung cancer [16]. This is partly because its anticancer effects are mediated by the inhibition of membrane ergosterol and cholesterol dynamics. It also has immunostimulatory effects because it activates the macrophage, and sensitizes tumor cells to platinum-based drugs [17]. Even though traditional amphotericin B deoxycholate is also effective, it is not used extensively in clinical practice. Its use is limited by dose-limiting nephrotoxicity, infusion-related adverse reactions, and severe electrolyte imbalance, such as hypokalemia and hypomagnesemia, among others [18]. To overcome these complications, liposomal formulation (LAmB; AmBisome5) is developed, which is a vesicle made of amphotericin B and is encircled by small unilamellar vesicles. Such a formulation is significantly superior in terms of therapeutic index. Liposomal encapsulation enhances increased drug concentrations in target organs, decreased renal and systemic toxicity and plasma half-life [19]. It is important to note that LAmB shows positive diffusion into lung tissue as well as accumulates with time in the pulmonary spaces following intravenous injection. They are particularly pertinent when it comes to the treatment of malignancies of the lungs [20].

The combination of complementary mechanisms of action of metformin and amphotericin B in a liposomal delivery system is a promising and rational approach to treating lung cancer. Another anti-diabetic drug, Metformin, stimulates the AMPK in cancer cells and suppresses the mTOR signaling pathway, which causes anti-proliferative and pro-apoptotic effects. Antifungal therapy Amphotericin B interferes with cellular membranes, making the cells more permeable in their membranes and making chemotherapeutics more lethal to cancerous cells. Further, amphotericin B entrapped in liposomes enhances the pharmacokinetics, increases tumor-targeting and decreases systemic toxicity. The metabolic reprogramming of Metformin makes tumor cells more susceptible to the disruption of the membrane of amphotericin B, which may trigger an increase in apoptotic and immune-mediated responses. This mechanistic complementation forms the basis of the possible additive or synergistic effect and endorses the reduction of doses thereby reducing the adverse effects which is a major consideration in combination therapies. It is interesting to note that there have been no systematic studies on the co-liposomal formulation of this dual agent liposomal to investigate pharmaceutical development, characterization or validation when used as an oncological agent, which makes this dual-agent liposomal formulation novel and translationally promising.

Validated, accurate analytic method should be used to ensure reliable development and quality control of combination drug products by being able to measure both of the active ingredients. HPLC is the conventional method of pharmaceutical quantification, which is appreciated due to precision, selectivity, and reproducibility. There are RP-HPLC solutions to conduct individual analysis of metformin and amphotericin B using C_{18} columns with both validated and guideline-compliant protocols. Nevertheless, an approved RP-HPLC procedure to analyze both metformin and liposomal amphotericin B in one formulation does not exist as yet. There is an increased use of liposomal drug delivery in cancer treatment. It enhances solubility, bioavailability and duration of

drug. Liposomes have the ability to entrap hydrophilic and hydrophobic medications, and release them slowly and specifically. Liposomal amphotericin B is meant to reduce kidney toxicity in lung cancer. The EPR effect may enhance the stability and tumor targeting by liposomal metformin. Proper drug measurement is used to promote reliable testing and drug repurposing. HPLC is the choice of method of measuring drug quality and quantity, even in liposomal preparations, because it is sensitive and repeatable. To perform testing of metformin and amphotericin B together in a liposomal mixture through HPLC, the mobile phase, column and detection settings are to be carefully adjusted. The earlier HPLC technique has worked successfully with individual drugs. Detection at the same time is not easy as the methodology should be able to differentiate the two drugs clearly. The validation of the working of the HPLC method would imply the accuracy, specificity, linearity, detection limits, and robustness. These checks are in line with ICH Q2(R1). This makes the method reliable in the detection of these drugs in liposomal preparations.

In this study, a strong and precise RP-HPLC methodology of quantitative determination of metformin and amphotericin B in liposomal formulation is developed and validated as per the ICH Q2(R1) standards.

Materials and Methods

1. Materials

Amphotericin B and metformin hydrochloride were procured from Sigma-Aldrich (Mumbai, India). Cholesterol, sodium deoxycholate, and hydrogenated soy phosphatidylcholine (HSPC) were obtained from TCI Chemicals Pvt. Ltd. (Mumbai, India). HPLC grade methanol from Merck® (Mumbai, India), HPLC grade acetonitrile and dimethylsulfoxide (DMSO) from Tedia® (Mumbai, India); Formic acid from Sigma-Aldrich® (Mumbai, India), and ultra-purified water obtained using a Milli-Q Plus system (Merck-Millipore®, Mumbai, India).

2. Preparation and Characterization of Metformin and Amphotericin Liposomes

First prepared the Metformin- and amphotericin B-based ionic liquids. Preparing metformin and amphotericin B ionic liquids in liposome formulations offers significant advantages in solubility, bioavailability, stability, targeted delivery, and therapeutic efficacy, making it a promising approach for drug delivery systems. Briefly, the previously described method MET-IL and AMB-IL were prepared by acid–base ion-pair formation using sodium docusate as the counterion. Metformin or amphotericin B (0.1 mmol) was dissolved in methanol; for AMB-IL, a few drops of hydrochloric acid were added before stirring. Sodium docusate was then added gradually, and the mixtures were stirred for 6 h at room temperature to form ionic liquids. Solvents were removed under reduced pressure, followed by purification using dichloromethane and acetonitrile. The final clear, viscous ionic liquids were collected and stored in a desiccator for further characterization and formulation studies. [12-13]

3. HPLC instrumentation and chromatographic conditions

The chromatographic system used to perform analytical method development and validation of this assay method was comprised of (SHIMADZU, HPLC) with LC-20 AD pump, with software LC software and UV-Visible detector SPD-20A. Chromatographic analysis was performed on a C_{18} (THERMO BDS) 4.6 x 250 mm. Standard stock solution (100 µg/ml) of metformin and amphotericin B was prepared in optimized mobile phase. Accurately weighed 10 mg of each drug is transferred to a 10 ml volumetric flask and dissolved in the selected mobile phase. It was then sonicated for 20 min. The solution was diluted up to volume with the same mobile phase. In order to obtain the test solution, stock solutions were further diluted to make 1 µg/ml. (250 mm 4.6 mm ID, 5 µm particle size) column. The mobile phase consisted of water at pH 2.51 with 0.1 % (v/v) formic acid (v/v): acetonitrile 0.1 % (v/v) formic acid (v/v) (70:30). The flow rate of the mobile phase was adjusted to 1.0 ml/min, and the injection volume was 20 µl. Detection was performed at 245 nm. An isocratic analytical method was used.

4. Preparation of Standard Stock Solution

Standard stock solution of AMP was prepared by dissolving 10 mg of drug in 10 ml methanol to get a concentration of 1 mg/ml. Standard stock solution of METH was prepared by dissolving 10 mg of drug in 10 ml methanol to get concentration of 1 mg/ml. From these solutions 1 ml was further diluted to 10 ml with mobile phase to get working standard stock solution of concentration 100 µg/ml of each.

5. Selection of Detection Wavelength

From the standard stock solution (1000 µg/ml) further dilutions were made using methanol and scanned over the range of 200-400 nm and the spectra was obtained. It was observed that both the drug showed linear, stable and considerable absorbance at 245 nm. Representative overlain UV spectrum of AMP and METH is shown in Fig 5.1.1.

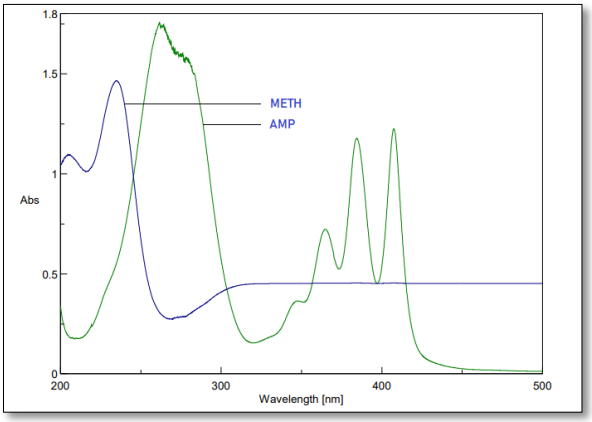

Fig. 1: Overlain spectra of AMP (50 µg/ml) and METH (50 µg/ml)

6. Selection of Mobile Phase:

The solutions of Amphotericin B (AMP) and Metformin (METH) working standards were injected into the HPLC system and run in different solvent systems. Different mobile phases containing methanol, water, acetonitrile and formic acid in different proportions were tried and finally Acetonitrile (0.1 % Formic acid): water (0.1 % Formic acid) (80 v/v) was selected as an appropriate mobile phase which gave good resolution and acceptable peak parameters for both.

7. Checking the Resolution of Two Drugs

The column was saturated with the mobile phase (indicated by constant back pressure at desired flow rate). A mixed standard solution of AMP and METH was injected to get the chromatogram. Wavelength 245 nm was selected at which both the drugs have considerable absorbance. Chromatograms of AMP and METH of different concentrations are shown in (Fig. 2 –7), which confirmed the baseline separation of the mixture of the two drugs.

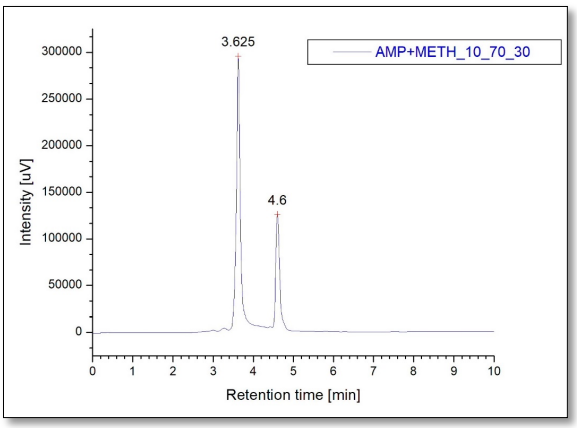

Fig 2: Chromatogram of standard mixture contaning AMP 10 µg/ml (3.62 min) and METH 10 µg/ml (4.60 min).

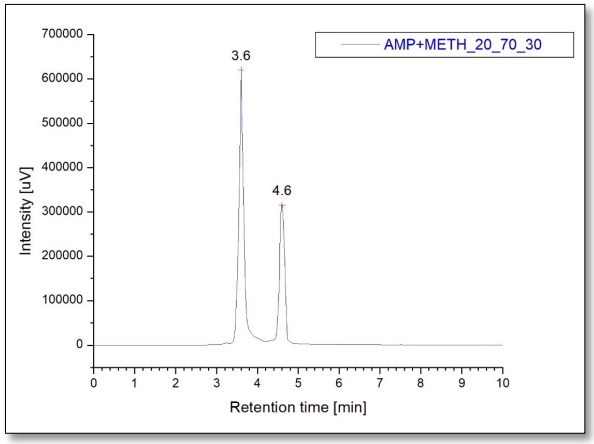

Fig 3*:* Chromatogram of standard mixture contaning AMP 20 µg/ml (3.60 min) and METH 20 µg/ml (4.60 min).

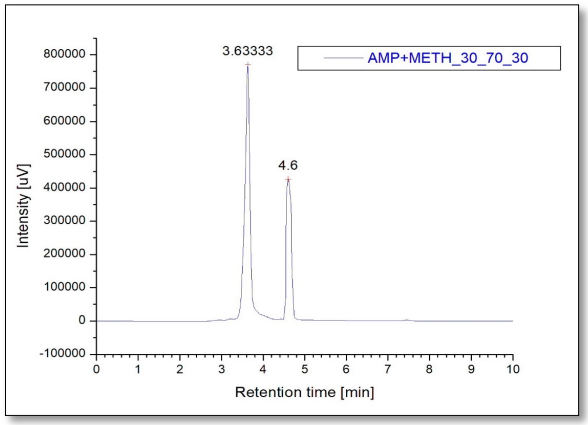

Fig 4*:* Chromatogram of standard mixture contaning AMP 30 µg/ml (3.63 min) and METH 30 µg/ml (4.60 min).

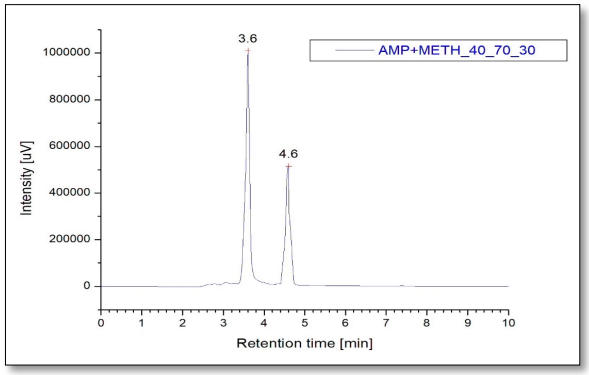

Fig 5: Chromatogram of standard mixture contaning AMP 40 µg/ml (3.63 min) and METH 40 µg/ml (4.60 min).

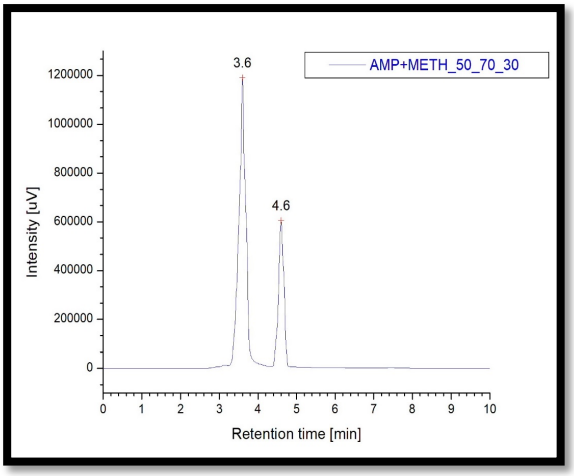

Fig 6: Chromatogram of standard mixture contaning AMP 50 µg/ml (3.63 min) and METH 50 µg/ml (4.60 min).

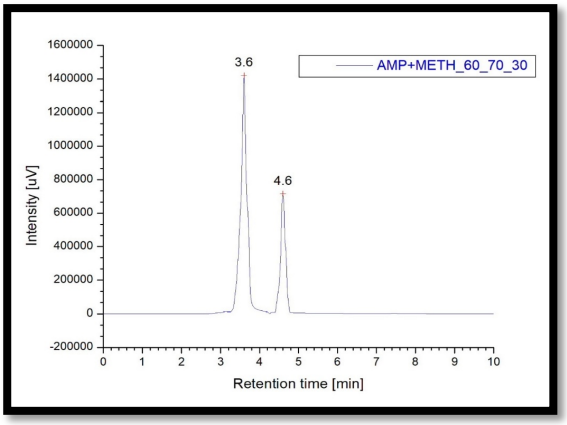

Fig 7: Chromatogram of standard mixture contaning AMP 60 µg/ml (3.60 min) and METH 60 µg/ml (4.60 min).

Table 2: Details of chromatogram of AMP and METH

Sr. No.	Drug	RT (min)
1	AMP	3.60±02
2	METH	4.60±02

8. Preparation of Sample Solution-

A 100 mg of liposome was weighed and powdered. A powder of liposome was transferred to a 10 ml volumetric flask containing 5 ml of methanol. The mixture was ultra sonicated for 10 min and the resulting sample stock solution was filtered with Whatman filter paper 41 and the volume was made up with the methanol to get concentration of 1000 µg/ml. Further dilution was done to get concentration 40µg/ml of AMP and 40 µg/ml of METH.

8.1. Summary of Chromatographic Parameters Selected:

Table 3: Summary of chromatographic parameters

Sr. No.	Parameter	Conditions used for Analysis
1	Column	C_{18} (THERMO BDS) 4.6 x 250 mm
2.	Mobile phase	Acetonitrile (0.1 % Formic acid) : water (0.1 % Formic acid) (70:30 v/v)
3.	Flow rate	1.00 mL
4.	Detection Wavelength	UV -245 nm
5.	Sample injector	20 µl
6.	Column temperature	Ambient
7.	Particle size packing	5µm

8.2Calibration Curve:

Calibration curves were constructed by plotting peak area versus concentration. The regression equations were found to be:

- Metformin: y = 50883x + 502596 (R^2 = 0.999)
- Amphotericin B: y = 200228x + 847026 (R^2 = 0.999)
-

9. Validation of Analytical Method:

9.1 Linearity-

From the standard stock solution (1000 µg/ml) of AMP and METH, solutions were prepared containing 100 µg/ml of each. Six replicates per concentration were injected and chromatograms were recorded. The peak area of AMP and METH were recorded and respective calibration curves were plotted of peak area against concentration of each drug. Linear response was observed in the concentration range of 10-60 µg/ml for AMP and METH. The results obtained are shown in Table 4 and 5 respectively. Excellent correlation exists between peak area and concentration of drugs within the Concentration range indicated above. Calibration curve for and are shown in Figs. 8 & 9 respectively.

Table 4: Observation table for calibration curve of AMP (n = 3)

Replicates	Concentrations of AMP (µg/ml)					
	10	20	30	40	50	60
	Peak Area					
1	2821559	4845570	6844364	8942388	10834982	12856575
2	2822560	4851358	6835789	8935834	10801765	12830392
3	2847932	4834385	6852685	8951593	10870384	12807621

Mean	2830684	4843771	6844279	8944109	10835701	12831529
Std. Dev.	14945.88	8628.324	8448.318	6789.543	34315.3	24496.81
%RSD	0.527995	0.178132	0.123436	0.055911	0.316687	0.190911

Table 5: Observation table for calibration curve of METH (n = 3)

Replicates	**Concentrations of METH (µg/ml)**					
	10	**20**	**30**	**40**	**50**	**60**
	Peak Area					
1	1003315	1541287	2154398	2514543	3060676	3534330
2	1000250	1510378	2015864	2524010	3026765	3587503
3	1007307	1502957	2010850	2511512	3040354	3556798
Mean	1003624	1582077	2060371	2516688	3042598	3559544
Std. Dev.	3538.633	20329.07	81468.64	6519.344	17066.54	26692.62
%RSD	0.352586	1.339018	3.954077	0.259045	0.560920	0.749889

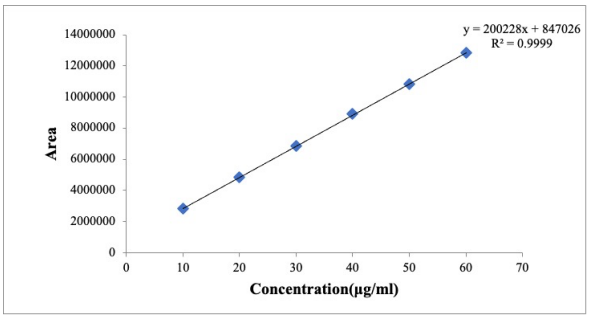

Fig 8: Calibration curve of AMP

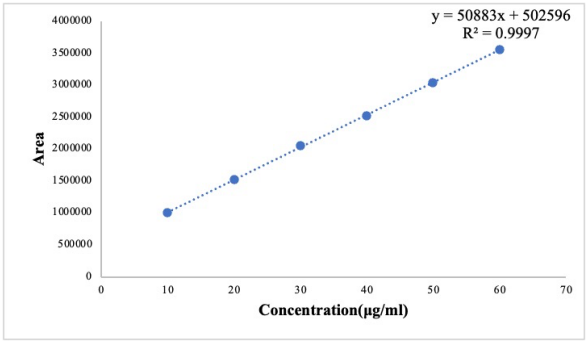

Fig. 9: Calibration curve of METH

9.2 Precision-

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 3 replicates of 3 different concentrations were analyzed in a day and percentage RSD was calculated. For the inter day variation studies, 3 different concentrations were analyzed on 3 consecutive days and percentage RSD was calculated. The results obtained for intraday and inter day variations are shown in Table 6 and 7 for PIO and table 8 and 9 for METH respectively.

Table 6: Intra-day precision study of AMP

Replicates	**Conc. (µg/ml)**
	40
1	19499819
2	19751987
3	19601724
4	19559671
5	19652765
6	19706917
Mean	19628813.83
SD	93807.94
%RSD	0.4779
Mean	19628813.83

Table 7: Inter-day precision of AMP

Replicates	**Conc. (µg/ml)**
	40
1	19394998
2	19738719
3	19622717

4	19597159
5	19676527
6	19702547
Mean	19622111.17
SD	122663.57
%RSD	0.6251
Mean	19622111.17

Table 8: Intra-day precision study of METH

Replicates	Conc. (µg/ml)
	40
1	2514543
2	2511387
3	2550995
4	2524010
5	2547542
6	2531432
Mean	2529984.83
SD	16563.84
%RSD	0.6547
Mean	2529984.83

Table 9: Inter-day precision of METH

Replicates	Conc. (µg/ml)
	40
1	2554476
2	2550415
3	2541963
4	2537542
5	2528953
6	2516892
Mean	2538373.5
SD	13914.49
%RSD	0.5482
Mean	2538373.5

9.3 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ are calculated from the formula: -

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\ \sigma}{S}$$

Where,

σ = S.D of the response at lowest concentration or standard deviation of Y intercept;

S = Average of slope of the calibration curve

Table 10: LOD and LOQ of AMP

Method	Avg. slope	S. D.	LOQ (µg/ml)	LOD (µg/ml)
Using S. D of	200228.22	111740.98	5.58	1.84

Table 11: LOD and LOQ of METH

Method	Avg. slope	S.D.	LOQ (µg/ml)	LOD (µg/ml)
Using S.D. of	50883.11	44559.69	8.76	2.89

9.4 Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 995, indicating the no interference of any other peak of degradation product, impurity or matrix.

9.5 Assay

Liposomal formulation analysis was carried out as mentioned under section preparation of sample solution. Procedure was repeated for six times. Sample solution was injected and area was recorded. Concentration and % recovery was determined from linear equation. The results obtained are shown in Table 12.

Table 12: Assay of Liposomal formulation

Sr. No.	AMP			METH		
	Peak area	Amount Recovered	% Assay	Peak area	Amount Recovered	% Assay
1	8332271	37.38	93.46	2373245	36.67	91.91

2	8316982	37.31	93.27	2393256	37.16	92.89
3	8387657	37.66	94.15	2398768	37.27	93.16
Mean			93.63			92.65
SD			0.46			0.66
%RSD			0.50			0.71

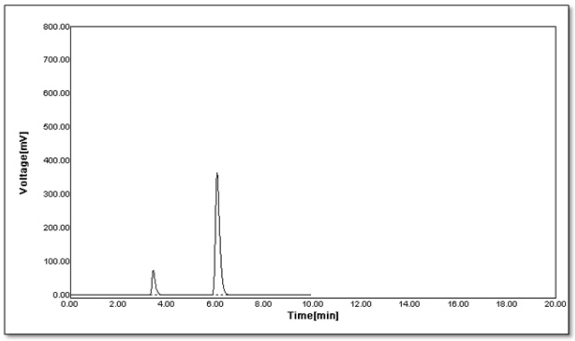

Fig.9: Optimized Chromatogram of formulation

9.6 Accuracy

To check accuracy of the method, recovery studies were carried by spiking the standard drug to the Liposome formulation sample solution, at three different levels around 80, 100 and 120 %. Basic concentration of sample solution chosen was 3.73µg/ml for AMP and 16µg/ml for METH. % recovery was determined from linearity equation. The results obtained are shown in Table 13 and 14.

Table 13: Accuracy of AMP

Level	Conc. of Sample solution	Conc. of Standard solution	Area	Amount recovered (µg/ml)	% recovery (Mean ±%R
80 %	50	40	196798	81.62	81.17
			19611987	80.99	
			19601724	80.90	
100 %	50	50	21696245	100.23	100.06
			21675012	100.03	
			21662204	99.92	
120 %	50	60	23849945	120.11	120.07
			23789081	119.55	
			23898284	120.55	

Table 14: Accuracy of METH

Level	Conc. of Sample solution	Conc. of Standard solution	Area	Amount recovered (µg/ml)	% recovery (Mean ±%RSD)
80 %	50	40	5500489	80.78	80.55
			5489487	80.42	
			5490576	80.46	
100 %	50	50	6117145	101.05	99.62
			6103289	100.59	
			6000925	97.23	
120 %	50	60	6702392	120.29	119.77
			6678306	119.49	
			6679739	119.54	

9.7 Robustness-

Robustness of the method was checked by carrying out the analysis under conditions during which mobile phase composition (± 1% Composition), detection wavelength (± 1 nm), flow rate (± 0.1 ml/min) were altered and the effect on the area were noted. Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest remained unaffected by small changes of the operational parameters indicating that the method is robust.

Table 15: Robustness study

% RSD Found For Robustness Study (peak area)									
	Column temp. (30)			Detection Wavelength (± 1 nm)			FLOW RATE (± 0.1 ml/min)		
DRUG	28	30	32	244	245	246	0.8	1.0	1.2
AMP	0.62	0.39	0.77	0.68	0.32	0.74	0.77	0.52	0.59
METH	0.66	0.43	0.92	0.70	0.41	0.74	0.99	0.54	0.97

9.8 Summary of Validation Parameters:

Table 16: Summary of validation parameters by HPLC

Sr. No.	Validation Paramet	Results	
		AMP	METH
1.	Linearity	10-60	10-60
2.	Assay (Mean ± % RSD)	93.63±0.50	92.65±0.71

3.	**Precision**	(% RSD)		(% RSD)	
	Intraday precision	**Conc (µg/ml)**	**% RSD**	**Conc (µg/ml)**	**% RSD**
		40	0.4780	40	0.6547
		40		40	
		40		40	
	Interday precision	**Conc (µg/ml)**	**% RSD**	**Conc (µg/ml)**	**% RSD**
		40	0.6551	40	0.5482
		40		40	
		40		40	
4.	**Accuracy**	**% Recovery (Mean ± % RSD)**		**% Recovery (Mean ± % RSD)**	
	80 %	81.17±0.48		80.55±0.25	
	100 %	100.06±0.16		99.62±2.09	
	120 %	120.07±0.42		119.77±0.37	
5.	**LOD**	1.84		2.89	
6.	**LOQ**	5.58		8.76	
7.	**Specificity**	Specific		Specific	
8.	**Robustness**	Robust		Robust	

9.9 System Suitability

System suitability parameters were evaluated before analysis. The results are summarised Table no.17 below:

Table 17: System suitability parameters

Parameter	Metformin	Amphotericin B
Retention time	4.60	3.60
Theoretical plates	>2000	>2000
Tailing factor	<2	<2
Resolution	>2	—

RESULTS & DISCUSSION:

The developed RP-HPLC method provided good separation of metformin and amphotericin B with well-resolved peaks and acceptable retention times. The validation results confirmed that the method is linear, precise, accurate, and robust as per ICH guidelines. The system suitability parameters were within acceptable limits, indicating proper functioning of the chromatographic system. The method was successfully applied for the estimation of both drugs in liposomal formulation without interference from excipients.

CONCLUSION:

A simple, precise, and accurate RP-HPLC method was developed and validated for the simultaneous estimation of metformin and amphotericin B in liposomal formulation. The method complies with ICH validation guidelines and can be effectively used for routine quality control analysis.

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