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Characterization, thermal stability studies, and analytical method development of Paromomycin for formulation development

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Paromomycin (PM) is an aminoglycoside antibiotic, first isolated in the 1950s, and approved in 2006 for treatment of visceral leishmaniasis. Although isolated six decades back, sufficient information essential for development of pharmaceutical formulation is not available for PM.

The purpose of this paper was to determine thermal stability and development of new analytical method for formulation development of PM. PM was characterized by thermoanalytical (DSC, TGA, and HSM) and by spectroscopic (FTIR) techniques and these techniques were used to establish thermal stability of PM after heating PM at 100, 110, 120, and 130 °C for 24 h. Biological activity of these heated samples was also determined by microbiological assay. Subsequently, a simple, rapid and sensitive RP-HPLC method for quantitative determination of PM was developed using pre-column derivatization with 9-fluorenylmethyl chloroformate. The developed method was applied to estimate PM quantitatively in two parenteral dosage forms.

PM was successfully characterized by various stated techniques. These techniques indicated stability of PM for heating up to 120 °C for 24 h, but when heated at 130 °C, PM is liable to degradation. This degradation is also observed in microbiological assay where PM lost ∼30% of its biological activity when heated at 130 °C for 24 h. New analytical method was developed for PM in the concentration range of 25–200 ng/ml with intra-day and inter-day variability of <2%RSD. Characterization techniques were established and stability of PM was determined successfully. Developed analytical method was found sensitive, accurate, and precise for quantification of PM.

Keywords: paromomycin; characterization; analytical method; thermal stability.

Introduction

Visceral leishmaniasis (VL) is a communicable disease that is endemic and causes high morbidity and mortality worldwide. Various antileishmanial agents like antimony compounds, amphotericin B, etc., have been used in chemotherapy of VL. The major challenges in management of VL are the poor oral bioavailability, dose-limiting toxicity, and cost of these antileishmanial agents and their available dosage forms.[1, 2]

Paromomycin (PM) is a broad spectrum aminoglycoside antibiotic with antiparasitic activity. Its antileishmanial activity was established in 1980 and thereafter phase III clinical trials were conducted during 2003/2004 in India. In 2005, the US Food and Drug Administration and the European Commission granted orphan drug status to PM, and in 2007 it was included in 15th edition of *WHO Model List of Essential Medicines*. In 2006, PM was approved by the Indian government for the treatment of VL. Presently, phase IV clinical trials are in process in India.[3, 4] Though PM has been approved as an intramuscular injection for treatment of VL, being an aminoglycoside it causes local toxicity at site of injection along with ototoxicity and nephrotoxicity. PM also causes temporary tubular damage leading to calcium wasting in urine and hypocalcaemia resulting in tetany.[5] Thus, there is an urgent need for improved formulation of PM which can provide a better efficacy and reduced toxicity.

In the present approach, PM-loaded albumin microspheres (of size ≤5 μm) were formulated by authors as a parenteral delivery system for targeting drug to macrophages.[6] These microspheres were prepared using spray drying method. In this process, albumin microspheres need to be stabilized using heat treatment, and thus the prepared microspheres were processed at high temperature (100–160 °C) for 6–24 h according to thermal stability of the drug.[7] Drug stability is highly dependent on the temperature and time of heating, therefore for successful development of such new delivery systems, well established preformulation studies including characterization techniques, thermal behaviour and feasible analytical method [8–10] are required. In the past six decades, attempts have been made to formulate PM in one or other dosage forms,[11, 12] but sufficient information is not available with respect to characterization and thermal stability of PM. According to literature search, merely one patent [13] reporting the FTIR of PM and one report discussing % elemental composition (carbon and nitrogen content) of PM are available.[14]

In view of analytical method, PM neither contains UV chromophore nor fluorophore (Figure 1). To overcome this problem, numerous methods have been tried by various authors, but all were associated with one or other complexity. Some of the most widely used methods and their limitations are given in Table 1. Microbial method of *United States Pharmacopoeia* (USP 23) was conventionally used; this method is simple, inexpensive, can be used to determine antimicrobial activity of drug, but this method

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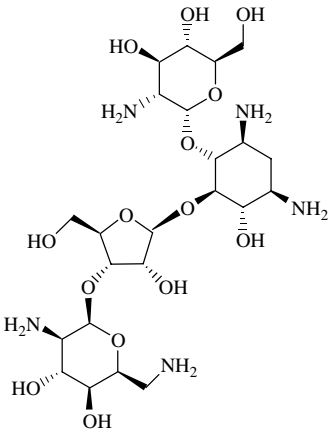

Figure 1. Chemical structure of Paromomycin.

is time-consuming, non-specific, and it is less sensitive.[15] GC method [16, 17] can be used for determination of PM, but it is a less sensitive due to the fact that aminoglycosides are non-volatile and require derivatization at elevated temperature.[15] Most of the HPLC methods for PM described in literature are based on derivatization of PM. They are either inconvenient due to post-column derivatization[14] or tedious pre-column derivatization.[12, 18] There are few other methods available where a complex instrument set-up is required, for example, pulsed electrochemical detection method,[19] fast fourier continuous cyclic voltammetry method,[20] and LC-MS method.[21–23] In addition, they also lack ensitivity.

It was realized that for development of new formulation of PM, preformulation studies are essential and thus it is important to know the stability of PM during the processing steps with a workable and sensitive analytical method for analysis. Thus, in the present work, PM was characterized by thermoanalytical and spectroscopic methods and these characterization techniques along with microbiological assay were used to establish the thermal stability and biological activity of PM. With this, a simple and workable analytical method based on pre-column derivatization with 9-fluorenylmethyl chloroformate was developed.

Experimental

Reagents and materials

Paromomycin sulfate (labelled potency 758 µg/mg) was obtained from Gland Pharma Limited (Hyderabad, India) as a gift sample. FMOC-Cl was purchased from Sigma (Banglore, India). Bovine serum albumin (Fraction V) and sodium metabisulphite were procured from sd fine-chem. Ltd (Chandigarh, India). Acetonitrile was purchased from J.T. Baker (Mumbai, India). All other chemicals and reagents used in the experiments were procured from Indian vendors. Ultrapure water was obtained using the Elgastat water purification system (Cheshire, UK).

Characterization and thermal stability studies of paromomycin

PM was characterized using DSC, TGA, FTIR, and HSM. These techniques were also used to determine the thermal stability of PM heated at different temperatures (100 °C, 110 °C, 120 °C, and 130 °C) for 24 h. Biological activity of these heated samples was also determined by microbiological assay.

Differential scanning calorimetry (DSC)

Differential scanning calorimeter (DSC 821e, Mettler Toledo, Zurich, Switzerland) was used for thermal characterization of PM. In this method, individual samples (3–5 mg) were weighed directly in DSC aluminum pan and scanned at heating rate of 10 °C/min in a temperature range of 0–300 °C.[24] All experiments were carried out in an inert atmosphere and thermograms obtained were observed for the effect of heating on PM samples.

Table 1. Analytical methods available and currently in use for determination of PM in bulk/formulations

Formulation/analyte	Derivatization agent/ method	Detection	Comments	Published Year	Ref.
Bulk-PM	Post-col., OPA–ME	Fluorimetric	Chromatogram run time 40 min	1997	[14]
PM Liposomes	Microbial method	–	Time consuming, non specific, poor sensitivity	2004, 2009	[11, 33]
Oral PM Formulation	Pre-col., DNFB	UV	Tedious derivatization, extraction steps involved, run time 30 min	2008	[34]
PM Liposomes	Pre-col., DNFB	UV	Derivatization at 80 °C for 45 min, followed by extraction step	2009	[12]
PM-IM Injection PM-MS	Pre-col., FMOC-Cl	Fluorimetric	Derivatization at RT for 10 min, no extraction step, run time 13 min	*2010*	*Present Manuscript*

Abbreviations: PM, Paromomycin; PM-IM Inj, Paromomycin intramuscular injection; PM-MS, Paromomycin loaded albumin microspheres; Post-col., post-column; Pre-col., pre-column; DNFB, 1-fluoro-2, 4-dinitrobenzene; OPA–ME, *o*-phthaldialdehyde–2-mercaptoethanol; FMOC-Cl, 9-fluorenylmethyl chloroformate; RT, room temperature.

Thermogravimetric analysis (TGA)

TGA was performed on Mettler Toledo (TGA/SDTA 851^e) with STAREe software to study the thermal decomposition of PM with perspective to weight loss. In this method, individual samples (3–5 mg) were weighed directly in crucibles and scanned at the heating rate of 10 °C/min from 25 to 300 °C under nitrogen purging [25] and thermograms obtained were observed for the effect of heating on PM samples.

Hot stage microscopy (HSM)

HSM was carried out using Leica DMLP polarized microscope and Leica LMV hot stage (Leica Microsystems, Wetzlar, Germany). Photographs were taken with a JVC colour video camera (Victor, Yokohama, Japan) and analyzed using LINKSYS 32 software. Samples were mounted in silicone oil and heated from 25 to 350 °C. Samples were studied using a programmable heating cycle (10 °C/min upto 50 °C, followed by heating rate of 5 °C/min upto 350 °C).

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the samples were obtained on IR spectrophotometer (Perkin–Elmer, California, USA) in the range of 4000–450 cm^{-1} using conventional potassium bromide discs method. The FTIR spectrum obtained was used for chemical characterization of PM samples.

Microbiological assay

Microbiological activity of PM in heated and unheated samples was determined by agar-diffusion inhibition assay.[11] Cylinder-plate method as described in the *United States Pharmacopoeia* (USP 23) was used to observe potency, i.e. inhibition in the growth of *Staphylococcus epidermides* (ATCC 12228). Briefly, after 18 h incubation of the plates at 37 °C, the zone of inhibition was measured for each sample. Standard curve was prepared with PM concentrations 2, 4, 8, 12, and 16 µg/ml in 0.1 M phosphate buffer (pH 8.0) and was used to determine the thermal stability of PM in unheated and heated samples (at different temperatures 120 °C and 130 °C for 24 h).

RP-HPLC analysis of PM

Preparation of borate buffer, PM, FMOC-Cl, and glycine solutions

Boric acid was used to prepare borate buffers (pH 8.0, 0.4M) and pH was adjusted with 50% *w/v* potassium hydroxide solution. PM (5 mg) was dissolved in 100 ml of water and further diluted to form a stock solution of 1 µg/ml. FMOC-Cl (10.3 mg) was dissolved in 10 ml of acetonitrile (MeCN) to make 4 mM FMOC-Cl solution. Glycine (75 mg) was dissolved in borate buffer to obtain 0.1M glycine solution.

Chromatographic conditions

Chromatographic separation and subsequent quantification of PM in different samples was carried out using HPLC system (Shimadzu, Kyoto, Japan) fitted with a solvent pump (LC-10AT*VP*), online degasser (DGU-14A) and temperature controller (CTO-10AS*VP* column oven). CLASS-VP software was used for data analysis. After FMOC-Cl derivatization of PM, 10 µl samples were injected using auto injector (SIL-10AD*VP*) and PM-FMOC derivative was separated at 30 °C using reversed phase HPLC Inertsil C_{18} column. The mobile phase used was acetonitrile-water (87 : 13, *v/v*) in an isocratic mode and flow rate was 1 ml/min. The effluent was monitored using fluorescence detector (RF-10AXL) set at excitation wavelength of 260 nm and emission wavelength of 315 nm.

Optimization of derivatization conditions

Derivatization of PM with FMOC-Cl was influenced by four parameters *viz.* (1) concentration of FMOC-Cl; (2) concentration of acetonitrile in reaction medium; (3) reaction medium pH (i.e. borate buffer pH); and (4) time for completion of the reaction. These parameters were studied to find the optimum condition for derivatization. FMOC-Cl concentration in the reaction mixture was varied from 0.25 mM to 4.0 mM, acetonitrile concentration was varied from 30 to 70%, pH was kept at 7.0, 7.5, 8.0, and 8.5; and reaction time was varied from 2 to 20 min. For derivatization reaction, the required quantity of PM stock solution was diluted with boric acid buffer and this solution was mixed with FMOC-C1 solution. The reaction mixture was then vortexed repeatedly and reacted for desired time in dark at RT. The reaction was stopped by adding 50 µl glycine (0.1M). 10 µl of the reaction mixture was injected in HPLC and the relative fluorescence of the samples was studied to optimize the derivatization conditions.

Preparation of calibration curves

A six-point calibration curve was constructed over the concentration range of 25–200 ng/ml using optimized derivatization conditions. Concentration range of 25–200 ng/ml was selected on the basis of linearity of calibration curve. Different volumes of PM stock solution were mixed upto 450 µl with boric acid buffer (0.4M), then samples were vortexed repeatedly and derivatized with 500 µl FMOC-C1 (4 mM in acetonitrile) at RT for 10 min in dark. The reaction was stopped by adding 50 µl glycine (0.1M) and was further kept for 2 min. 10 µl of the reaction mixture was injected and the relative fluorescence of the test samples was measured. The interpolation of areas obtained for each analyte in the calibration curve was used for the quantification of PM in samples.

Method validation

RP-HPLC method was validated on three different days to determine linearity, range, accuracy, precision, system suitability, specificity, and sensitivity as per ICH guideline for analytical method development. Calibration curves were used to determine linearity of the method which was characterized by regression coefficient, slope and intercept in the concentration range of 25–200 ng/ml. Precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) for quality control (QC) samples; repeatability was evaluated by assaying 3 QC samples of same concentration (n=3) during same day whereas, intermediate precision was calculated by comparing the assays on 3 different days. Percentage recoveries obtained from QC samples were used to express accuracy of the method. Various parameters were tested for the system suitability included capacity factor, asymmetry, tailing, retention time, and percentage RSD of 100% assay concentration i.e. 150 ng/ml (n=5). System suitability for the proposed method was evaluated by using CLASS-VP software and by manual calculation. Specificity of the method was determined by injecting two parenteral formulations (PM-IM injection and PM loaded albumin MS) and sensitivity of method was determined by calculating LOD and LOQ separately using formula based on the slope of calibration curve and SD of response, as per ICH guidelines of analytical method development. LOD is the lowest concentration of the analyte detected by the method, whereas LOQ is the minimum quantifiable concentration.[26, 27]

Quantification of PM in injectable dosage forms

The developed analytical method was used to determine PM concentration in PM-IM injections and PM-MS formulation which was developed in the lab.[6] IM injections were diluted (n=3) in borate buffer and aliquots were used for derivatization reaction to determine the PM content. To quantify the amount of PM in MS, 2 mg MS were dispersed in 10 ml phosphate buffer (pH 7.4) in triplicate and 0.2% trypsin was used to degrade the microsphere in order to release PM at 37 °C. Thereafter, 0.5 ml of this sample was mixed with 1 ml MeCN to precipitate albumin and centrifuged at 12000g for 10 min. 0.75 ml of supernatant solution was diluted upto 5 ml with borate buffer (pH 8), filtered and used for determination of PM in formulation.

Statistical analysis

Heated and unheated sample groups were compared before and after heating for significant difference in antimicrobial activity using one way analysis of variance followed by Dunnett's and Duncan's tests. Differences were considered significant for P value less than 0.05.

Result and discussion

Thermal stability studies of paromomycin

PM loaded albumin microsphere were formulated using spray drying method. Briefly, in this method, drug with other excipient get exposed to high temperature in spray drying chamber during particulate formulation. Prepared particulate formulation was then stabilized by heating at temperature >100 °C. The heating temperature was selected in such a way that the drug is stable during heating. Thus, it was important to know the effect of heating on the drug or formulation to dictate the quality of the formulation. Hence, in such cases, it is of prime importance to know the thermal stability of the drug to have better control of the processing part of the formulation development. Keeping this in mind, thermal stability of PM was evaluated at different temperatures.

Differential scanning calorimetry, thermogravimetric analysis, and hot stage microscopy

PM is very hygroscopic and contains approximately 4% adsorbed water (determined by IR balance). DSC and TGA analysis of PM (Figures 2A and 3A) show two endothermic events: diffused endotherm which starts from 0 °C in DSC and 25 °C in TGA upto 100 °C; and endotherm in a temperature range of 210–230 °C in both DSC and TGA. The first diffused endotherm may be attributed to a gradual loss of adsorbed water. Whereas, the second endotherm (shown by dotted rectangle in Figures 2 and 3) was attributed due to the pyrolysis of PM which results in a drastic reduction of sample weight as observed from TGA. Loss of water and pyrolysis during heating were further confirmed by HSM.

In HSM experiments, PM was heated from 25 to 350 °C in a procedural manner as given in the Method section. During heating of PM, no event was observed till 100 °C (Figures 4A and 4B) and loss of water was observed above 100 °C (water evolves in the form of bubble) as shown by arrow in Figure 4C. After this, no change was observed up to 210 °C; however at >210 °C, a colour change was observed and the sample became black in colour with evolution of gas bubbles (Figure 4D). This suggests the charring of PM. Thus, HSM results indicate that endotherm observed at >210 °C in DSC and TGA thermograms were due to the pyrolysis of PM i.e. degradation of the drug above this temperature.

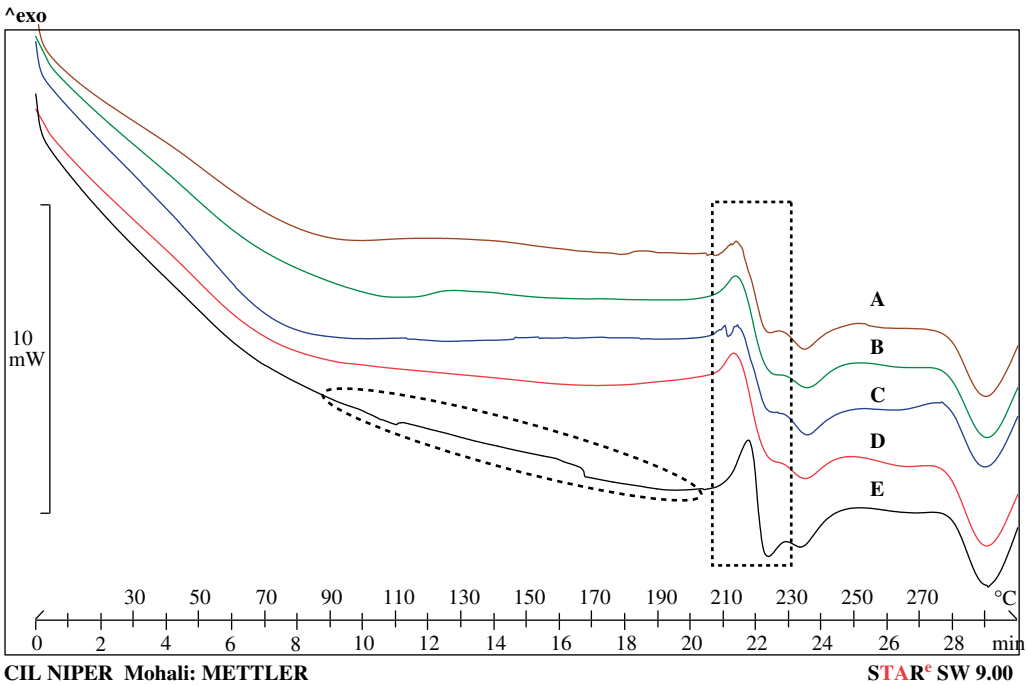

Figure 2. DSC thermograms of (A) PM unheated, (B) PM heated at 100 °C for 24 h, (C) PM heated at 110 °C for 24 h, (D) PM heated at 120 °C for 24 h and (E) PM heated at 130 °C for 24 h. PM showed two endothermic events: first endotherm up to 100 °C due to gradual moisture loss and second endotherm between 210 and 230 °C due to pyrolysis. In 130 °C, 24 h heated sample heat flow signal drop and deviation from normal DSC pattern was observed in the temperature range 80–210 °C.

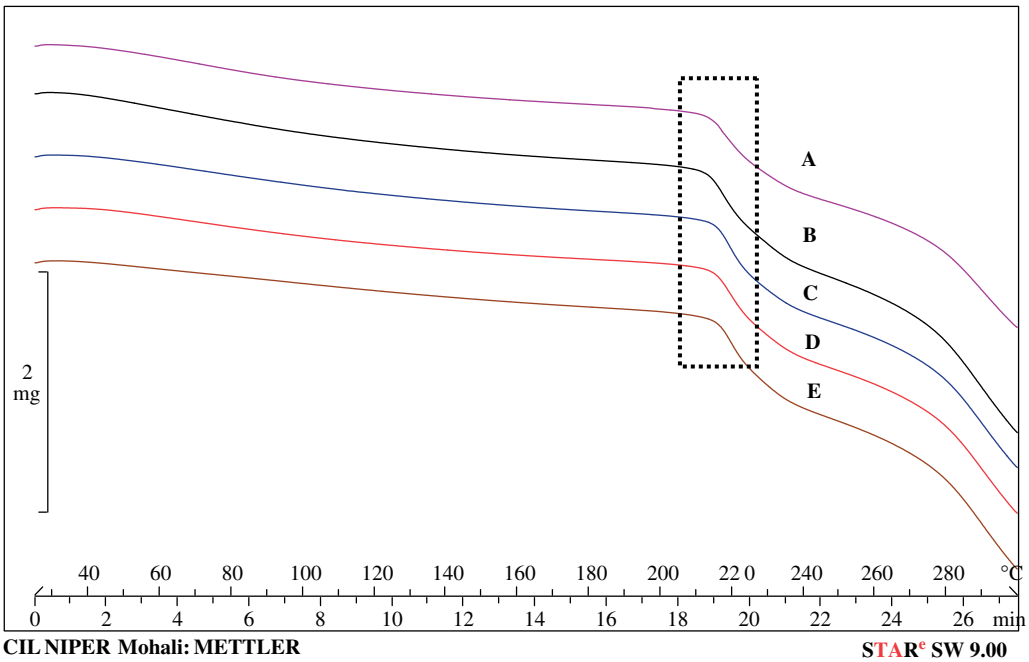

Figure 3. TGA thermograms of (A) PM unheated, (B) PM heated at 100 °C for 24 h, (C) PM heated at 110 °C for 24 h, (D) PM heated at 120 °C for 24 h and (E) PM heated at 130 °C for 24 h. PM showed two endothermic events: first endotherm from 25 to 100 °C due to gradual moisture loss and second endotherm between 210 and 230 °C due to pyrolysis. Same endothermic events were observed in all samples.

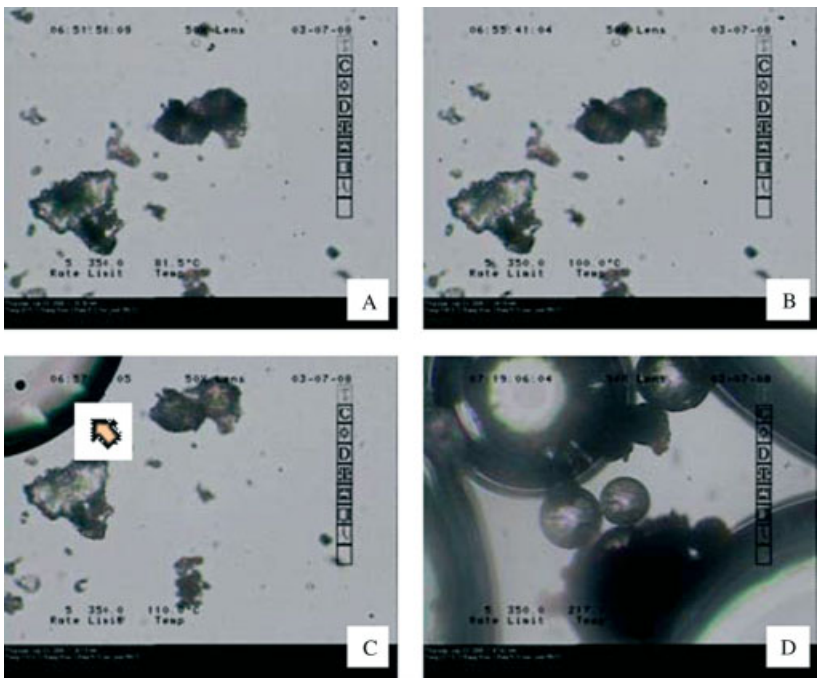

Figure 4. Hot stage microscopy photographs for PM mounted in silicone oil. Events observed: No change was observed when PM sample was heated upto 100 °C (A and B). Further increase in temperature led to loss of adsorbed moisture in form of bubble shown by arrow (C), when PM was further heated; above 210 °C pyrolysis of PM occurs with evolution of vigorous bubbles without melting (D).

After thermal analysis of native PM, it was desirable to see the thermal behaviour of the samples which were kept at different temperature (100 °C, 110 °C, 120 °C, and 130 °C) for 24 h. Figure 2 shows thermal stability of PM in samples heated at varied temperature (100 °C, 110 °C, 120 °C, and 130 °C) for 24 h. As observed from DSC, both the first and second endotherm patterns obtained were same for unheated and heated samples at 100 °C, 110 °C, 120 °C, 130 °C for 24 h as given in Figures 2A, 2B, 2C, 2D and 2E, respectively. But, samples heated at 130 °C for 24 h showed a heat flow signal drop in the temperature range of 80–210 °C and deviation from normal DSC pattern of PM was observed (shown by dotted circle in Figure 2E).

Figure 3 shows TGA results in different samples heated at varied temperature. TGA technique depends on the weight loss and

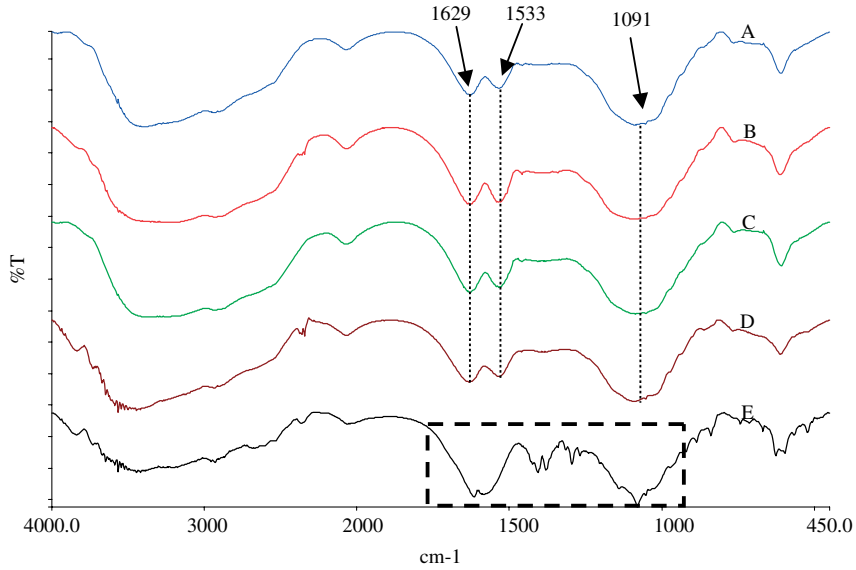

Figure 5. FTIR spectrum of (A) PM unheated, (B) PM heated at 100 °C for 24 h, (C) PM heated at 110 °C for 24 h, (D) PM heated at 120 °C for 24 h and (E) PM heated at 130 °C for 24 h. In 130 °C, 24 h heated sample intensity of normal peaks altered and various new unknown peaks have emerged (shown in dotted rectangle).

thus it does not recognize any change in thermal behaviour of the sample. The first endotherm which attributed to the loss of adsorbed water is the same in all the samples which was not expected in heated samples (since heated samples should not contain adsorbed water). It is also pertinent to note that these dehydrated samples are very hygroscopic and adsorb water instantly. This adsorption may take place in the processing of the sample preparation and thus TGA results confirms the same thermal behaviour of PM in unheated and heated samples.

Fourier transform infrared spectroscopy (FTIR)

The characterization of samples heated at different temperatures was also done with FTIR and results are shown in Figure 5. The characteristic peaks of PM are observed at 1629 cm^{-1} (due to N-H bending coupled with the C-N stretch), 1533 cm^{-1} (CH_2 bending), and at 1091 cm^{-1} (C-O-C stretch). The bands at 2928 and 3377 cm^{-1} typical for hydroxyl and primary amino-groups are seen in the spectrum of PM. It is observed that FTIR spectra remain the same for the samples which were heated up to 120 °C for 24 h (Figures 5A–5D); however a change in FTIR pattern is observed for the sample heated at 130 °C for 24 h. In this case, intensity of peaks altered, especially intensity of the ether peak present at 1091 cm^{-1} reduces (Figure 5E) and other unknown peaks appeared. It indicates a change in the functional groups for the samples heated at 130 °C for 24 h.

Microbiological assay

For any drug during the formulation processing step, there may be a loss of biological activity. Therefore, it is important to know the biological activity of the drug before and after dosage form development.[28] For this purpose, microbiological assay was performed and potency of the PM was estimated by comparing the inhibition of the growth of a sensitive micro-organism produced by known concentrations of the antibiotic being examined with a test substance. A linear relationship between area of the inhibition zone and logarithm of the PM concentration was observed for

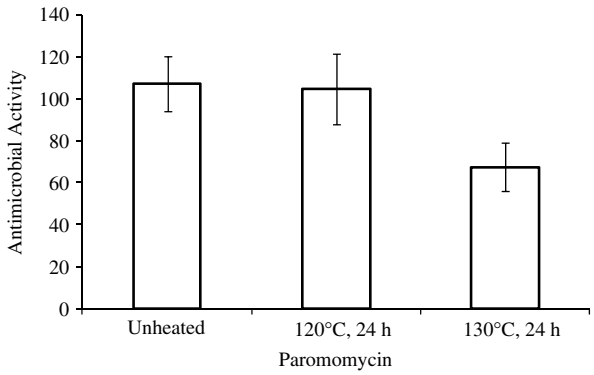

Figure 6. Antimicrobial activity (%) of different unheated and heated samples of PM.

untreated PM. The standard curve obtained was linear with equation $y = 16.463x+104.62$; $R^2 = 0.993$ for PM concentrations between 2 and 16 µg/ml. Thermal stability of PM was correlated with the biological activities by analyzing the unheated samples and heated samples (at different temperatures 120 °C and 130 °C for 24 h) as shown in Figure 6. The result obtained showed no reduction in antimicrobial activity for PM heated at 120 °C for 24 h as compared to the unheated sample, whereas nearly 30% reduction in antimicrobial activity was observed for the samples of PM heated at 130 °C for 24 h. Microbiological assay also revealed no significant difference in antimicrobial activity when mean values were compared before and after heating at 120 °C for 24 h using one way analysis of variance, whereas when mean values were compared before and after heating at 130 °C for 24 h, differences in the mean values among the groups was statistically significant ($P = 0.023$) in antimicrobial activity.

RP-HPLC analysis of PM

Taking into account various reports available for analysis of PM, initially pre-column derivatization with OPA-ME

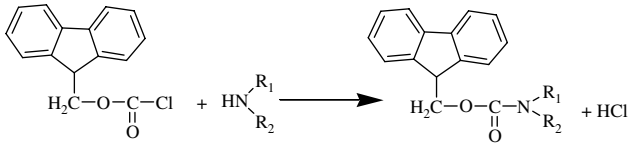

Figure 7. Reaction scheme for the derivatization of amine with FMOC-Cl, both primary and secondary amines can be derivatized with FMOC-Cl. In PM, five primary amine groups are present which undergo derivatization.

(o-phthaldialdehyde–2-mercaptoethanol) as a derivatizing agent was tried, but this method was found to be unsuitable due to instability of OPA-ME derivative in C_{18} HPLC columns. To improve the stability of OPA-ME derivative, ME was replaced with NAC (N-Acetyl-L-Cysteine) as the bulky thiol group to obtain OPA-NAC derivative. In spite of this change, fast elution of the components and unresolved reagent peaks still existed in chromatogram (data not shown in present manuscript). Thereafter, methods using universal detectors like ELSD and LC-MS were tried, but these methods were less sensitive and required ion pairing agent to retain the drug in reversed phase column. Thus, an RP-HPLC method using FMOC-Cl as the derivatizing reagent was developed for PM and this method was found quite simple, sensitive, and most suitable for determination of PM.

Chromatographic separation

PM showed strong fluorescence after derivatization with FMOC-Cl (Figure 7) and appeared as a well-resolved peak at 10.2 min. Representative chromatograms of blank and PM samples after FMOC derivatization are shown in Figure 8. The run time of 13 min was sufficient for proper conditioning of the column and is also appropriate for routine sample analysis. It was observed (Figure 8) that this method showed adequate separation of PM (Peak 2) from FMOC-Cl (Peak 1) to allow quantification of PM. Peak resolution obtained was 1.9 for PM peak from other FMOC-Cl peaks; obtained resolution was found to be desirable for any new method development. It is reported that PM is very stable and even excreted as such from the body,[29] thus no degradation products were considered in its method development.

Optimization of derivatization conditions

PM was reacted with FMOC-Cl and for this reaction, borate buffer pH, reaction time, and concentrations of acetonitrile and FMOC-Cl in reaction medium were independently varied to obtain optimized parameters. The effect of these parameters on relative fluorescence obtained by derivatives of PM is shown in Figure 9. It was found that optimal reaction occurred within the pH range 7.5–8.5 and was completed in 5 min and derivative formed were stable up to 20 min, therefore 10 min was taken as the optimum time for completion of the reaction. It was also observed that the yield of prepared derivative was maximized when the reaction was carried out in 40% to 70% acetonitrile concentration. Below 40% (*v*/*v*) acetonitrile concentration, FMOC-Cl was not completely soluble while acetonitrile above 70% (*v*/*v*) leads to precipitation of water soluble components of reaction mixture. For the fourth parameter (FMOC-Cl concentration), it was observed that a sufficiently high concentration of FMOC-Cl ($\geq$1 mM) was required for the reaction to proceed efficiently and therefore 2 mM was chosen as optimized parameter. All these optimization experiments were carried out at highest point of the calibration curve (i.e. 200 ng/ml) so that optimized reactant concentration would be sufficient for lower concentrations of PM to precede the reaction to the completion. It was also observed that optimal reaction conditions of PM with FMOC-Cl were in a similar range to other aminoglycoside drug (gentamicin) reported previously [30, 31] using FMOC-Cl derivatization reaction. Optimized parameters obtained are shown in Table 2, and were used for further experiments.

Method validation

Range and linearity. Six-point calibration curves for PM were constructed at the concentration range of 25–200 ng/ml and regression parameters *viz.* slope, intercept and correlation coefficient of these plots were calculated as shown in Table 3. Linear regression analysis showed correlation coefficient ($R^2 = 0.999$) which was suitable for further analysis of PM using developed analytical method.

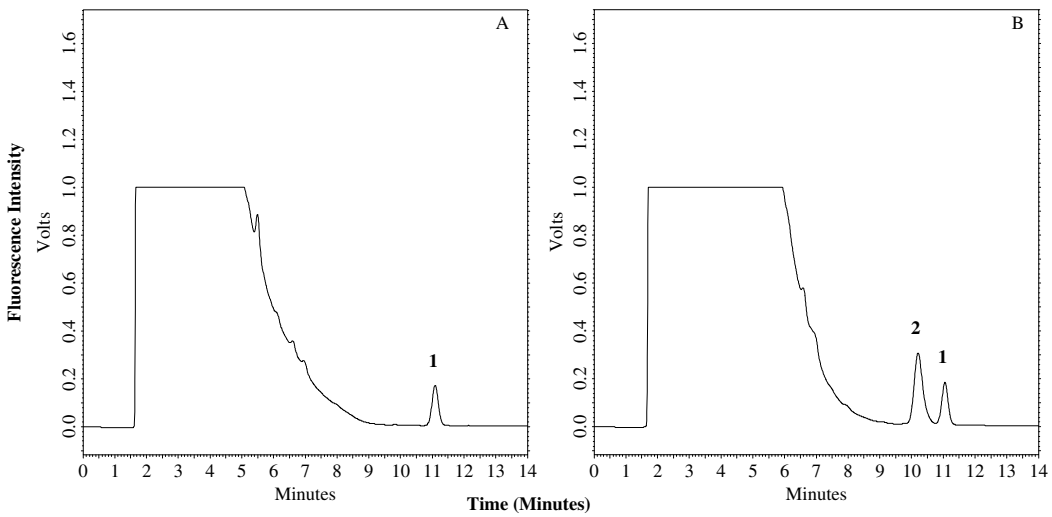

Figure 8. Representative HPLC chromatograms of FMOC-Cl derivative (A) Blank and (B) PM sample. Peak 1 corresponds to FMOC-Cl component, whereas Peak 2 is for PM-FMOC complex. Both chromatograms were obtained with HPLC conditions specified in the present paper.

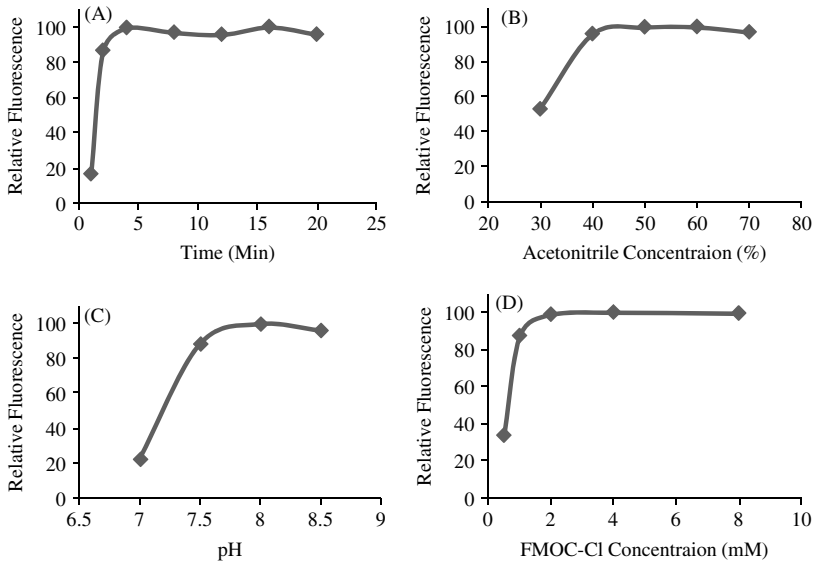

Figure 9. Optimization of derivatization conditions (A) reaction time, (B) acetonitrile concentration, (C) pH and (D) FMOC-Cl concentration for derivatization of PM with FMOC-Cl.

Table 2. Optimized parameters for the derivatization of PM with FMOC-Cl

S. No.	Parameter	Optimized values
1.	Time	10 min
2.	PH	8.0
3.	* MeCN	50%
4.	* FMOC-Cl	2 mM

* Total of final reaction mixture.

Table 3. Validation parameters for the determination of PM

Parameter	Values
Analytical wavelength	Ex. 260 nm, Em. 315 nm
Linearity range	25–200 ng/ml
Linearity (%RSD)	0.999 (0.01)
Slope (%RSD)	50661 (2.07)
Intercept (%RSD)	51191.66 (45.42)
LOD	1.51 ng/ml
LOQ	4.59 ng/ml

Accuracy and precision. Accuracy and precision of the developed method were assessed by analyzing QC samples at three different concentrations in triplicates ($n = 3$) within the calibration range. The experimental values obtained are presented in Table 4. The percent recovery (accuracy) of this method was found to be $100 \pm 2\%$ indicating fair agreement between true and obtained values. The intra and inter-day precision values showed %RSD values to be $<2\%$ indicating repeatability and intermediate precision of the developed method.

System suitability. While developing a new analytical method, it is necessary to know the system suitability which depends on the mobile phase selection and is determined by peak

Table 4. Inter-day and intra-day accuracy and precision for calibration curve of PM by HPLC

* Concentration (ng/ml)	Accuracy (%)	Precision (%RSD)	Precision (%RSD)
	Inter-day		Intra-day
40	100.40	1.68	0.32
80	99.63	1.68	0.50
160	99.78	1.00	0.57

* Triplicate of three QC samples within the concentration range of standard curve were determined, the concentrations were different from those in calibration curve.

Table 5. System suitability parameters for chromatographic analysis of PM

S. No.	Parameter	Observed values	Recommended values [32]
1.	Capacity factor	5.39	>2
2.	Asymmetry	1.33	<1.5
3.	Tailing	1.16	≤2
3.	Retention time	10.2 min	–
4.	%RSD*	0.66	≤1

* %RSD of 100% assay concentration (n=5).

parameters such as capacity factor, asymmetry, tailing, run time, and %RSD of 100% assay concentration. If parameters obtained under system suitability are within the acceptable limit then the method is acceptable. For this, in the present method, mobile phase consisting two solvents, acetonitrile and water, was tested at different ratios. Finally, acetonitrile-water (87 : 13, *v/v*) was selected as a suitable mobile phase and system suitability parameters obtained at this mobile phase ratio are summarized in Table 5. It is observed that all the parameters are in acceptable limits.[32]

Table 6. Analysis of PM concentration in dosage forms using proposed HPLC method

PM spiked (ng/ml)	Amount detected (ng/ml)	Relative Recovery (%)	%RSD
Sample PM Injection			
150	147.37	98.25	0.27
Sample PM loaded albumin MS			
150	149.35	99.57	0.41

Specificity and sensitivity. For any drug sample analysis, it is necessary that drug peak should be specific without interference of other peaks and method should be sensitive enough to determine the lower concentration of the drug in test samples. Specificity of the method was determined by injecting two parenteral dosage forms (PM-IM injection and PM-MS). No interfering peaks of FMOC-Cl and other formulation ingredients were observed at retention time of PM which indicates the specificity of the method. Sensitivity of the method was determined by calculating LOD and LOQ. ICH guideline suggests three methods for determining LOD and LOQ: the first method is based on visual evaluation; the second is based on signal to noise ratio; and the third is based on SD of response and slope of standard curve. In this work, the third method was used for calculating LOD and LOQ. LOD and LOQ were calculated by $3.3\sigma/S$ and $10\sigma/S$ respectively, where σ is SD of response and S is the slope of standard curve. LOD and LOQ were found to be 1.51 and 4.59 ng/ml, respectively.

Quantification of paromomycin in injectable dosage form

The developed method was applied to estimate PM quantitatively in two parenteral dosage forms. For this purpose, 150 ng/ml concentration of PM was selected as 100% assay content. It is observed that mean assay recovery of PM in both formulations is $100 \pm 2\%$ with %RSD $<1\%$ (Table 6), indicates that the developed method is suitable for determining PM content in these dosage forms and can be extended to other routine analysis procedures for PM.

It is important to mention here that changes observed in heated samples during thermal analysis and microbial activity assay were not observed using this developed RP-HPLC method. The prime reason for this discrepancy is that proposed RP-HPLC was developed using derivatization of amino group with fluorescent FMOC-Cl; if no change occurs in the amino group of PM during heating then degradation cannot be detected using the present method. However, thermal properties and biological activity are dependent on the structure and configuration of the molecule. During the heat change from 120 °C to 130 °C, the amino functional group may not be affected; however, there may be changes in the structural orientation of the molecules and other functional groups as ether peak (as observed from FTIR). Therefore these changes were detected by thermal characterization and microbiological assay but not by developed HPLC method.

Conclusion

Under the preformulation study of PM, the drug was characterized successfully by thermoanalytical and spectroscopic techniques. These characterization techniques and microbiological assay were applied for determining the thermal stability of PM. Results obtained indicated that PM is stable when heated up to 120 °C for 24 h, whereas above this temperature as observed by FTIR, DSC, and microbiological assay, it is liable to degradation. A new RP-HPLC method for quantitative determination of PM was also developed. The proposed method was found to be simple, accurate, sensitive, and reproducible for quantitative analysis of PM. This method is rapid, cost-effective, and feasible due to easy availability of instruments required in the analysis of PM and hence can be applied on routine basis during all steps of the preparation of dosage form of PM as a quality control method for an optimized formulation of the drug. These preformulation studies were further used by authors in formulating a new formulation of PM-MS for targeting PM to macrophages.

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