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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF BLEOMYCIN SULPHATE

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ABSTRACT

Bleomycin is an anti-neoplastic drug that has recently been used for the treatment of vascular anomalies. An expedient method was developed for the determination of plasma bleomycin levels using ion-paired reversed phase high performance liquid chromatography (HPLC). The concentration was found to be proportional to the area and the response of the detector was determined to be linear over the range of 1-6 µg/ml for both Bleomycin A1 and Bleomycin B2. Recovery was approximately 100%. This method provides a simple and rapid way of determining the levels of bleomycin A2 and B2 in human and rat plasma

INTRODUCTION

Bleomycins are a mixture of glycopeptide antibiotics isolated from the fermentation broth of *Streptomycesverticillus.* These antibiotics are effective against a variety of human neoplasms, particularly squamous-cell carcinoma, lymphoma and testicular carcinoma. More recently, intralesional bleomycin (IB) has been used successfully to treat vascular anomalies. Due to its efficacy and apparent lack of side-effects, the drug has prompted much clinical research into making it the drug of choice for vascular anomalies. Although pulmonary toxicity, the major side-effect of this drug which manifests as pulmonary fibrosis, has been well established in cancer patients, this toxicity in patients after direct injection of bleomycin into the lesions has not been studied. The monitoring of bleomycin levels in body fluids following such intralesional therapy is imperative to determine spill-over levels and for the establishment of safety of use [1 – 3].

Various analytical methods have been developed to assay bleomycin fractions in biological fluids. Broughton and Strong in 1976 used a radioimmunoassay method to assay this compound in phosphate buffered saline (PBS) and in serum. This radioimmunoassay method was inadequate as it did not distinguish between the various components of the bleomycin mixture. Bleomycins are a mixture of active fractions (A1-A6; B1-B5) and the pharmacology of the different composite fractions are clinically important. Indeed, clinically administered bleomycin (Bleomycin Sulphate USP) consists 55-70%w/w of bleomycin A1 (25-32%w/w of bleomycin B2) and the remaining percentage divided among the other sub-fractions [4 – 6].

In 1980 Shiu and Goehl published a high performance liquid chromatography (HPLC) method for the specific determination of one of the major component of the bleomycin mixture, namely bleomycin A1, in plasma [7]. Ten years later another group developed a more sensitive HPLC method using a fluorescence detector in a linear gradient, ion-paired reversed phase procedure to assay bleomycin A1 in human plasma and rat hepatocytes [8].

These HPLC methods for the determination of BLM in plasma were validated for the A2 fraction only. Furthermore, these long assay methods would not be optimally applicable for monitoring a large number of patients' plasma samples. In the present study, a simple, rapid and sensitive method for the separation and quantitation of both major fractions, bleomycins A1 and B2 in human plasma is reported [9, 10].

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MATERIALS AND METHODS

Analytical procedure

The analytical method for the determination of Bleomycin Sulphate in human and rat plasma using internal standard was developed and validated. Sample preparation process was performed solid phase extraction technique. The processed samples were chromotographed on DISCOVERY C18100A (250 x 4.6 mm) column, using Methonal: Buffer-1(80:20, v/v) as mobile phase [11].

Experimental animals

Experiments were carried out on Wistar rats of either sex weighing 150-180gm. They were procured from authorized dealer. The animals were housed in polypropylene cages. Paddy husk was provided as bedding material, which was changed every day. The cages were maintained clean and hygienic. They were fed with standard pellet diet and water. They were kept in a well-aerated room and a 12-hour light and dark cycle was maintained. The room temperature was maintained at 22- 24^{0}C. The experiments were conducted in noise free environment between 9:00 AM to 2:00 PM. All procedures were approved and carried out as per the guidelines of Committee for the purpose of control and supervision of experimental animals (CPCSEA). Prior approval from Institutional Animal Ethics Committee (IAEC) was obtained for conduction of experiments. {CPCSEA Approval No.: RIP/IAEC/2014-15/05}.

Experimental Design

Procedure:

Healthy Wistar rat weighing about150- 180gms (either sex), kept under room temperature with standard diet and water *add libitum*. Rat dose was calculated according to BSA or body weight. The rats were divided into 2 groups of 6 animals each.

Grouping

- Group I Test group–Market Formulation (Suspension form bleomycin (15 unit i.p)
- Group II Standard group–Pure drug (Bleocip-15 unit- i.p)

Analysis of Bleomycin in Plasma Samples:

Blood Sample was collected in tube containing EDTA/Heparinised micro centrifuge, so as to prevent blood coagulation. The blood was centrifuged at 5000 RPM for 20 min, the plasma will be separated and stored at 2-8°C until further analysis of sample by HPLC. Different Pharmacokinetic parameter such as Cmax, Tmax, AUC, AUC0-24, AUC0-∞, t1/2 will be determined by using statistical analysis.

TABLE 1: EXPERIMENTAL CONDITIONS

Analyte	Bleomycin sulphate
Biological matrix	Plasma
Retention time	37.618-46.512
Mobile Phase	Pentane sulphonic acid sodium buffer (pH-4.3): methanol in gradient elution
Absorption maxima	254 nm
Flow rate	1.2 ml/min.
Injection volume	20 µl
Column temperature	25 °C
Column	Discovery C18 100A (250x4.6mm)
Parameters	Results
Analytical range	1-6 µg/ml
Bleomycin A1	
LOD	0.92µg/ml
LOQ	2.81µg/ml
Bleomycin B2	
LOD	5.6µg/ml
LOQ	17.06 µg/ml5

RESULT

HPLC Chromatogram of Bleomycin A1 and Bleomycin B2:

HPLC analysis of mixture of Bleomycin A1 and Bleomycin B2 chromatogram was optimized in which Retention time of both drug was given in Fig. 1

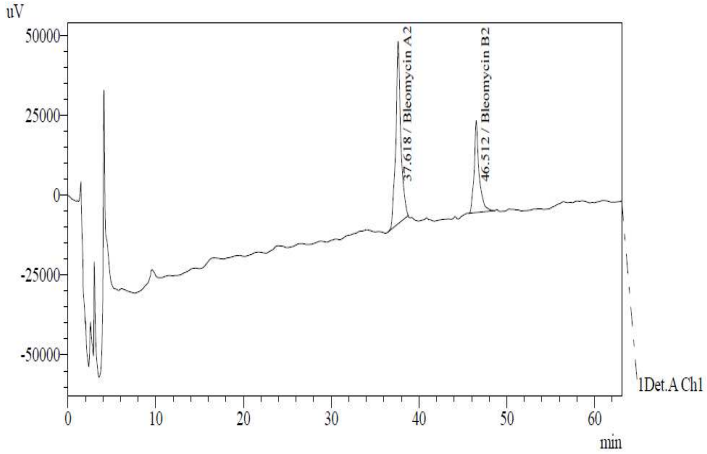

Fig: 1 Chromatogram of Bleomycin sulphate pure drug (6 µg/ml)

Method development: The bio-analytical method developed is simple and shows good accuracy, specificity and reproducible.

Formulation Table: 2 Pharmacokinetic Parameters of Bleomycinsulphate

Pure drug solution			**Formulation**	
Pharmacokinetic parameters	**Bleomycin A1**	**Bleomycin B2**	**Bleomycin A1**	**Bleomycin B2**
T_{max}(hr)	0.2	0.31	1.31	1.39
C_{max}(µg/ml)	33.15803182	10.13416537	19.24375	87.77291
$t_{1/2}$ (hr)	3.12	3.56	6.81	7.23
AUC (hr/µg/ml)	2.04303346	0.736983229	19.24375	87.77291
Relative bioavailability	-	-	9.419205	119.0975677

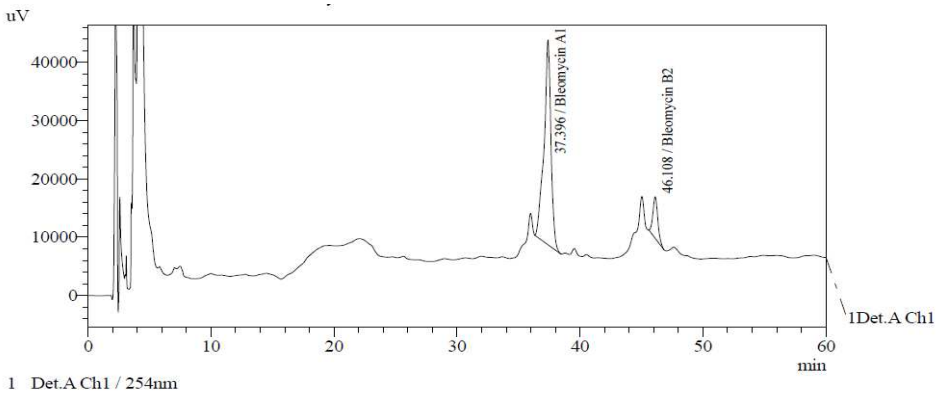

Fig: 2 Chromatogram of Bleomycin sulphate Formulation drug

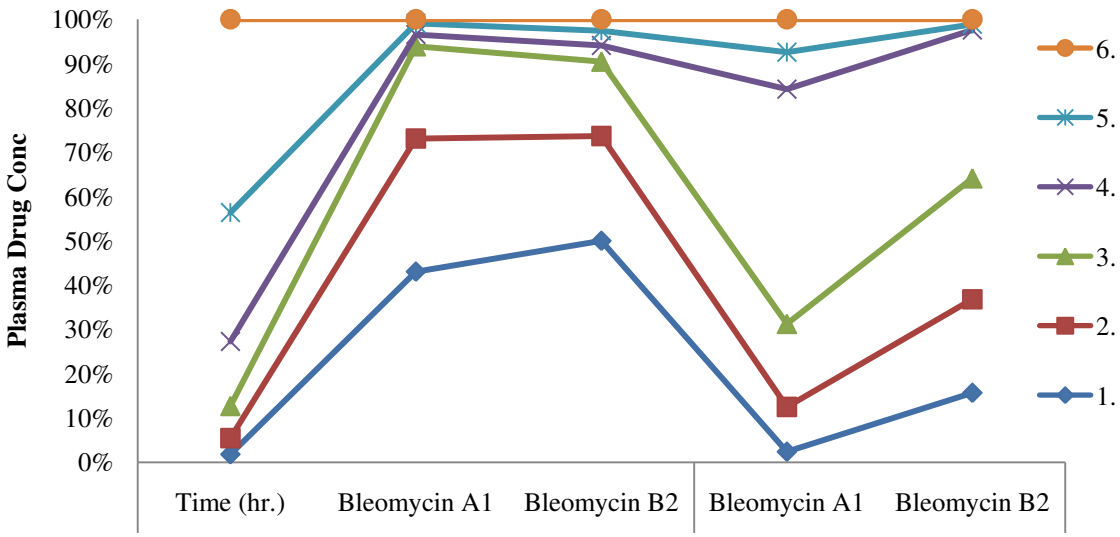

Fig: 3 Plasma drug concentration curves of Bleomycin solution and Bleomycin

Table: 3 Chromatographic conditions:

Mobile Phase	Pentane sulphonic acid sodium buffer (pH-4.3): methanol in gradient elution
Absorption maxima	254 nm
Flow rate	1.2 ml/min.
Injection volume	20 µl
Column temperature	25 °C

Linearity: The correlation coefficient (r) obtained was calculated and it was found to be greater than 0.99 for Bleomycin A1 and Bleomycin B2 given, which is well within the acceptance criteria. The concentration was found to be proportional to the area and the response of the detector was determined to be linear over the range of 1-6 µg/ml for both BleomycinA1 and Bleomycin B2.

Accuracy: The results indicate that the recoveries are well within the acceptance range of 80 – 120%, therefore, method is accurate and it can be used for the simultaneous estimation of Bleomycin A1 and Bleomycin B2.

Precision: It is a measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD). The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits. Similarly % RSD for Inter day Precision and Intraday Precision was given below that was found to be within the specified limits. The percentage RSD values for Area and Retention Time in precision study were calculated.

LOD and LOQ: The Limit of detection and limit of quantification of the method were calculated basing on standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the limit of detection and limit of quantification. The data were represented in Table13. The results obtained were within the limit.

Ruggedness: The Ruggedness of the method was found out by testing the effect of small deliberate changes in the analyst. The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions. The system suitability parameters were within the limits.

Pharmacokinetic and statistical analysis: Standard pharmacokinetic parameters obtained from each of the individual rat plasma concentration-time profiles of Bleomycin sulphate were calculated by non-compartmental methods using the computer program WinNonlin. The values of C_{max} and T_{max} were read directly from the arithmetic plot of time versus serum concentration of Bleomycin sulphate. The relative oral bioavailability of the different formulations has been evaluated by the equation:

$$\text{Relative bioavailability} = \frac{\text{AUC of belomycin pure drug solution}}{\text{AUC of belomycin formulation solution}}$$

DISCUSSION

A HPLC method for the estimation of Bleomycin sulphate in human and rat plasma was developed and validated. Sample preparation process was accomplished by solid phase extraction. The reconstituted samples were subjected to auto injector on a DISCOVER C18, 20 µl at 25 °C using a mobile phase consisting of methonal: Buffer-1(80:20,v/v). Transfer 0.96 g of sodium l-pentanesulphonate to a 1000-ml volumetric flask, add 1.86 g of disodium edetate and 5.0 ml of glacial acetic acid in 900 volumes of water and then Adjust the volume with water up to 1000 ml Mix well and adjust the pH to 4.3 filter through membrane filter with pore size 0.45 µm and degas before use. The method was subjected to complete validation and all the validation parameters showed results which were within the acceptance limits. A linear response was obtained between concentration and peak area ratio of the drug in human and rat plasma.The concentration was found to be proportional to the area and the response of the detector was determined to be linear over the range of 1-6 µg/ml for both Bleomycin A1 and Bleomycin B2 for Bleomycin sulphate. The accuracy and precision of the method was evaluated by peak area ratio response of the drug and internal standard.

CONCLUSION

The analytical method described is valid for the determination of bleomycin in human and rat plasma. The analytical method for the determination of Bleomycin Sulphate in human plasma and rat plasma using as internal standard was developed and validated. The concentration was found to be proportional to the area and the response of the detector was determined to be linear over the range of 1-6 µg/ml for both Bleomycin A1 and Bleomycin B2.Sample preparation process was performed on solid phase extraction technique. The processed samples were chromotographed on DISCOVERY C18,using Methonal: Buffer-1(80:20,v/v) as mobile phase.

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