A validated HPLC method for the simultaneous determination of bleomycin A2 and B2 in human plasma

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ABSTRACT

Bleomycins are a mixture of glycopeptide antibiotics isolated from the fermentation broth of Streptomyces verticillus [1,2]. These antibiotics are effective against a variety of human neoplasms, particularly squamous-cell carcinoma, lymphoma and testicular carcinoma [3]. More recently, intralional 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 [4-7]. 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 [8]. The monitoring of bleomycin levels in body fluids following such intralional therapy is imperative to determine spill-over levels and for the establishment of safety of use.

Various analytical methods have been developed to assay bleomycin fractions in biological fluids [9-12]. 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 [12]. Bleomycins are a mixture of active fractions (A1-A6; B1-B5) and the pharmacology of the different composite fractions are clinically important [13]. Indeed, clinically administered bleomycin (Bleomycin Sulphate USP) consists 55-70%w/w of bleomycin A2 (Fig.1A), 25-32%w/w of bleomycin B2 (Fig.1B), and the remaining percentage divided among the other sub-fractions [10].

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 A2, in plasma [11]. 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 A2 in human plasma and rat hepatocytes [3]. These HPLC methods for the determination of BLM in plasma were validated for the A2 fraction only. Furthermore, these long

1. INTRODUCTION

Bleomycins are a mixture of glycopeptide antibiotics isolated from the fermentation broth of Streptomyces verticillus [1,2]. These antibiotics are effective against a variety of human neoplasms, particularly squamous-cell carcinoma, lymphoma and testicular carcinoma [3]. More recently, intralional 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 [4-7]. 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 [8]. The monitoring of bleomycin levels in body fluids following such intralional therapy is imperative to determine spill-over levels and for the establishment of safety of use.
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 A\(_2\) and B\(_2\) in human plasma is reported.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Bleomycin A\(_2\) (BWS-18) was donated by the National Institute of Health, Japan, and Bleomycin B\(_2\) (BMT 049 B2) was donated by Nippon Kayaku, Co, Ltd. (Japan). Methanol and acetonitrile were of HPLC grade; acetic acid was of reagent grade (Radchem, Johannesburg, South Africa). Sodium heptanesulfonate was purchased from African Biotech Consultants (Johannesburg, SA). Water was purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Instrumentation

The HPLC system (Waters, Milford, USA) consisted of a model 600 gradient quaternary pump, 715 autosampler and 486 variable UV detector. The chromatographic system was controlled through a Galaxie chromatography workstation version 1.5 (Varian Inc., USA). The chromatographic separations were performed on a Phenomenex (Phenomenex Corp., Sunnyvale, CA, USA) C18 analytical column (250 × 4.6 mm i.d., 5 µm). The analytical column was protected by an inline guard column (4 × 2 mm i.d., 5 µm).

2.3. Chromatographic conditions

The mobile phase consisted of de-ionised water: acetonitrile: acetic acid (70: 25: 5) and contained sodium heptanesulfonate (0.0085M) as an ion-pairing agent. Isocratic conditions were used for all the work with a flow rate of 1mL/min and a detector wavelength setting of 295nm. The resultant column backpressure was approximately 2000psi. The temperature of the column was 40°C.

2.4. Preparation of standards

Bleomycin stock solutions were prepared by dissolving 0.8mg/mL bleomycin A\(_2\) and 1mg/mL bleomycin B\(_2\) in 0.1M sodium-phosphate buffer, pH 6.8. The stock solutions were kept at -20°C until use. Frozen stock solutions were thawed and diluted with milli-Q water to yield concentrations of 1, 2, 4, 6, 8, 200, 250, 300µg/mL bleomycin A\(_2\); 2, 4, 6, 8, 10, 300, 350, 400µg/mL bleomycin B\(_2\) required for the preparation of plasma standards. The stock solution was further diluted in drug-free plasma to yield concentrations of 0.1, 0.2, 0.4, 0.6, 0.8µg/mL bleomycin A\(_2\); 0.2, 0.4, 0.6, 0.8, 1µg/mL bleomycin B\(_2\).

2.5. Sample preparation

Samples were deproteinised with methanol (1 part plasma in two parts methanol), vortexed, and then centrifuged for 10min at 1864 X g. The supernatant was filtered twice through a 0.2µm acetate filter to remove interfering substances. 20µL of the filtered sample was injected onto the column. Ethical approval for the use of patient samples was granted by the University of Pretoria ethics committee.

2.6. Method validation

Aliquots of plasma were spiked with known amounts of the bleomycin stock solution to give a range of concentrations (Table 1). Samples were assayed in triplicate and coefficients of variation and relative errors for the assay were calculated (Table 1). The recovery of bleomycin was determined by comparing the peak heights resulting from spiked plasma standards, and the peak heights obtained from...
direct injection of the same amount of drug in aqueous solution (Table 1).

Table 1
Method validation data for bleomycin A2 and B2 in human plasma

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration (µg/mL)</th>
<th>%Recovery</th>
<th>CV %</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual</td>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BLM A2</td>
<td>30.0</td>
<td>31.1 ± 0.16</td>
<td>103.7</td>
<td>0.52</td>
</tr>
<tr>
<td>BLM B2</td>
<td>40.0</td>
<td>39.0 ± 0.65</td>
<td>97.5</td>
<td>1.67</td>
</tr>
<tr>
<td>BLM A2</td>
<td>25.0</td>
<td>26.8 ± 0.51</td>
<td>107.2</td>
<td>1.90</td>
</tr>
<tr>
<td>BLM B2</td>
<td>35.0</td>
<td>34.5 ± 0.30</td>
<td>98.7</td>
<td>2.08</td>
</tr>
<tr>
<td>BLM A2</td>
<td>20.0</td>
<td>25.1 ± 0.51</td>
<td>125.5</td>
<td>2.03</td>
</tr>
<tr>
<td>BLM B2</td>
<td>30.0</td>
<td>31.8 ± 0.21</td>
<td>106.0</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Table 2
Mean levels of bleomycin in patient plasma samples collected over a 24 hour period

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose</th>
<th>Bleomycin A2 levels</th>
<th>Bleomycin B2 levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5 mg/kg IV</td>
<td>292.02µg/mL</td>
<td>132.94µg/mL</td>
</tr>
<tr>
<td>2</td>
<td>0.4 mg/kg IV</td>
<td>10.58µg/mL</td>
<td>2.82µg/mL</td>
</tr>
<tr>
<td>3</td>
<td>0.6 mg/kg IB</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.6 mg/kg IB</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

IV – intravenous; IB – intralesional bleomycin; (n = 12)

The linearity of this assay was assessed by comparison of calibration curves from analyses of spiked samples of bleomycin A2 at 0.1 to 0.8µg/mL and of bleomycin B2 at 0.2 to 1.0µg/mL on three different days. The limit of quantitation was defined as the lowest drug concentration in each sample which could be quantitatively determined from the resultant calibration curves.

3. RESULTS AND DISCUSSION

3.1. Chromatograms of plasma samples

Peaks of bleomycin A2 and B2 were separated successfully (Fig.2A). Using these assay conditions, BLM A2 and BLM B2 had retention times of 5.22 and 5.86 minutes, respectively. The selectivity of the method was determined by analyzing spiked human plasma samples (Fig.2B and Fig.2C). Calibration curves were constructed by plotting the detector response (peak heights) against the corresponding bleomycin concentrations using a polynomial second order fit to account for slight non-linearity at the highest concentrations. Bleomycin concentrations correlated with detector response (regression coefficients of 0.9997 (A2) and 0.9996 (B2)).

3.2. Method validation

The accuracy and precision for the assay are reported in Table 1. The coefficient of variation ranged from 0.52 to 2.03 for BLM A2, and from 0.66 to 2.08 for BLM B2. The mean relative error was 1.50 for BLM A2 and 2.17 for BLM B2. To study the precision of the method, known amounts of the bleomycin stock were added to control plasma. The samples were assayed in triplicate (n = 3).

Previously reported work on the HPLC separation of bleomycin A2 required long elution times of up to 45 minutes [3,10,11]. With this method, elution times have been reduced considerably, with a total HPLC run-time of 8 minutes for both elution of the sample and equilibration of the system. In addition, the resolution between BLM A2 and BLM B2 was good (R = 1.8). The relative recovery of bleomycin A2 and B2 was approximately 100%.

The limit of quantitation was 0.1 and 0.2µg/mL for bleomycin A2 and B2 respectively, and a good correlation between peak height and concentration was obtained for BLM A2 and BLM B2 (r² = 0.9997 and 0.9996) over the concentration ranges 0.1 to 0.8µg/mL and 0.2 to 1µg/mL, respectively.
3.3. Application to real plasma samples

The developed and validated HPLC method was employed to measure the levels of bleomycin in patients undergoing intralesional bleomycin (IB) treatment and those receiving bleomycin intravenously (IV) (Fig. 2D). High levels of bleomycin A_2 and B_2 were measured following IV treatment, while the bleomycin analogues were barely detectable in the plasma of the IB treated patients (Table 2).

4. CONCLUSIONS

Discrete separation of two bleomycin analogues, A_2 and B_2, from components of human plasma was achieved using isocratic reversed phase chromatographic conditions. This study also provides the first information about bleomycin plasma concentration-time profiles in patients treated with bleomycin intralesionally. The present method for the determination of both BLM A_2 and B_2 in human plasma is simple, rapid, sensitive and accurate, and may thus be useful for routine monitoring of drug levels in plasma samples of patients undergoing IB therapy.

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