Method Validation in Determination of Mitoxantrone by HPLC
Mitoksantron’un HPLC ile Miktar Tayininde Method Validasyonu

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Abstract
The HPLC method used for determination of mitoxantrone was validated by linearity, sensitivity, precision, accuracy and specificity. Retention time for mitoxantrone was 4.3 min and that for haloperidol (internal standard) 7.9 min. Calibration curves were constructed for mitoxantrone over the concentration range 0.25-5 μg mL⁻¹ (haloperidol, 2 μg mL⁻¹). For six replicate samples at 0.5, 2.4, 5 μg mL⁻¹, intra-day precision values were within 1.63 %. Inter-day precision values at the same concentrations were within 1.90 %. The limit of detection of mitoxantrone was 0.1 μg mL⁻¹. The method was proved to be applicable to the determination of mitoxantrone.

Key words: Mitoxantrone, HPLC assay, validation

Introduction
Mitoxantrone (MTZ), 1,4-dihydroxy-5,8-bis[2-[(2-hydroxyethyl) amino] ethyl]-amino-9,10-anthracenedione dichloride, is an analogue of the anthracycline synthesized by Murdock et al. in the 1970’s. It is a potent inhibitor of DNA and RNA synthesis. MTZ shows better antitumour activity than the parent compounds (daunorubicin, doxorubicin) against a wide range of murine tumours without presenting induced cardiotoxic side effects in treated patients (Payet et al., 1988).

There are several high performance liquid chromatographic (HPLC) methods for the determination of MTZ and its metabolites in biological fluids and tissues (Ehninger et al., 1985; Choi et al., 1987; Czejka et al., 1988; Payet et al., 1988; Yoa-Pu Hu et al., 1990; De Vries et al., 1991; Catalin et al., 1994; Priston et al., 1994) and MTZ in pharmaceutical formulations (Ficarra et al., 1992). HPLC, together with various sample clean-up procedures, may better provide the required sensitivity and specificity to elucidate the pharmacokinetic characteristics of MTZ (Ehninger et al., 1990). Few of the published methods have utilised an internal standard method, cresyl violet (Ostrow et al., 1980), bisantrene (Choi et al., 1987), methylene blue (Lin et al., 1989), ametantrone (Van Belle et al., 1985; Steward et al., 1986) and haloperidol (Yoa-Pu Hu et al., 1990) have been used.

The stability of MTZ in human plasma has been discussed (Yoa-Pu Hu et al., 1990). Besides the problem of stability, a significant amount of MTZ adsorbed on the surface of glassware during sample preparation is another obstacle to be solved.

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The aim of this study was to describe an HPLC method used for the quantitation of MTZ which was an antitumour drug of the antitumour antibiotic classification, and to determine the related validation parameters.

Experimental

Materials and Methods

MTZ was purchased from Sigma (St. Lois, USA). Haloperidol was supplied by Heumann (UK). The sodium salt of 1-pentanesulphonic acid, potassium phosphate disodium salt and HPLC grade methanol were purchased from Merck (Darmstadt, Germany).

Chromatography: Determination of MTZ was performed by HPLC according to the modified method of Yoa-Pu Hu et al (1990). HPLC system consisted of a pump (Waters 510, Millipore), an autosampler (Waters 717 Autosampler, Millipore), a UV detector (set at 242 nm) (Waters 490-E, Millipore), a column (μBondapak C18, 10μm particle size, 25 cm x 4 mm I.D.) and an integrator (IBM PC/ 2,80, 386, NEC). The column was preceded by a guard column. MTZ and haloperidol were eluted isocratically with methanol –10mM KH₂PO₄ buffer (pH 3.0) (55:45, v/v), containing 0.09 % 1-pentanesulphonic acid sodium salt as the mobile phase at a flow rate of 1.5 mL.min⁻¹. The mean peak area ratio used throughout this study was calculated by dividing the MTZ peak area by the haloperidol peak area.

In this study, all stock solutions and dilutions of MTZ were prepared in mobile phase. To avoid loss of MTZ due to adsorption on the surface of glassware, polypropylene flasks were used throughout the study.

Method Validation

Accuracy and precision: For intra-day and inter-day accuracy and precision the samples (n=6) which contained MTZ (concentration of 0.5, 2, 4, 5 μg.mL⁻¹) and haloperidol (internal standard, 2 μg.mL⁻¹) were injected into the HPLC system at a single day and three consecutive days.

For evaluation of accuracy, the relative error percentage was calculated from the formula [(mean of detected concentration – added concentration) / added concentration] x 100, while precision was determined as the coefficient of variation (C.V.) (Tomiyama et al., 2000).

Limit of detection and limit of quantification: The limit of detection (LOD) was defined as the lowest concentration of MTZ (Signal/Noise(S/N)=3). The quantification limit was set at the lowest standard concentration on the calibration curve.

Linearity of calibration curve: For each standard curve set (n=6), a separate weighing of powders (MTZ and haloperidol) were made and then followed by serial dilutions to the appropriate concentrations (MTZ concentration range 0.25- 5 μg.mL⁻¹ and haloperidol 2 μg.mL⁻¹).

Stability study: In order to evaluate the MTZ stability during the experiments, solutions containing low (0.5 μg.mL⁻¹) and high (4 μg.mL⁻¹) concentration of MTZ were prepared and then, injected into HPLC column immediately after its preparation and 6 h.

Results and Discussion

Retention time for MTZ was 4.3 min. and that for haloperidol was 7.9 min and smooth peaks were obtained by the HPLC method described (Figure 1).

The LOD (S/N=3) of MTZ was 0.1μg.mL⁻¹. The linear response of the method was evaluated in the range of the concentrations (0.25- 5 μg.mL⁻¹) used. A good determination coefficient (r²> 0.999) was obtained with the calibration curve, where the mean peak area ratio (MTZ/haloperidol) was plotted versus concentration of MTZ (μg.mL⁻¹). The parameters are shown in Table 1.
Intra-day and inter-day accuracy and precision were determined to evaluate reliability of the analytical method. The results are listed in Table 2. Intra-day accuracy for MTZ at concentrations of 0.5, 2, 4, 5 μg.mL⁻¹ was between −1.80 and 0.36, with CV being 1.63 % or less. Inter-day accuracy ranged from −1.93 to −1.14 and inter-day precision was less than 1.94 %. Validation experiments showed very good precision and accuracy of the method with coefficients of variation and relative errors of less than 2 %. Stability studies revealed no appreciable degradation of MTZ (Table 3). Finally, results demonstrated that the method was accurate, precise and sufficiently specific and sensitive for the quantitation of MTZ.

Figure Legends

![HPLC chromatogram of mitoxantrone. (A: mitoxantrone, 1μg.mL⁻¹; B: haloperidol, 2 μg.mL⁻¹)](image)

**Figure 1.** HPLC chromatogram of mitoxantrone. (A: mitoxantrone, 1μg.mL⁻¹; B: haloperidol, 2 μg.mL⁻¹)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range of concentrations</td>
<td>0.25 μg.mL⁻¹ - 5 μg.mL⁻¹</td>
</tr>
<tr>
<td>Slope⁹</td>
<td>1.4933 ± 0.0596</td>
</tr>
<tr>
<td>Intercept⁸</td>
<td>-0.0715 ± 0.1262</td>
</tr>
<tr>
<td>(Correlation coefficient)² (r²)</td>
<td>0.9997</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>0.1 μg.mL⁻¹</td>
</tr>
<tr>
<td>Limit of quantitation</td>
<td>0.25 μg.mL⁻¹</td>
</tr>
</tbody>
</table>

⁹ ± confidence interval of the slope (P = 0.05)

⁸ ± confidence interval of the intercept (P = 0.05)
Table 2. Intra-day and inter-day assay of MTZ.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration added (µg.mL⁻¹)</th>
<th>Concentration determined (µg.mL⁻¹)</th>
<th>Accuracy a</th>
<th>Precision b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>0.4981</td>
<td>-0.3605</td>
<td>1.5600</td>
</tr>
<tr>
<td>Intra-day</td>
<td>2</td>
<td>1,9733</td>
<td>-1.3340</td>
<td>1.6362</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.9276</td>
<td>-1.8083</td>
<td>1.5694</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.9272</td>
<td>-1.4543</td>
<td>1.4764</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.4903</td>
<td>-1.9344</td>
<td>1.9470</td>
</tr>
<tr>
<td>Inter-day d</td>
<td>2</td>
<td>1.9752</td>
<td>-1.2357</td>
<td>1.9729</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.9395</td>
<td>-1.5123</td>
<td>1.5423</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.9428</td>
<td>-1.1437</td>
<td>1.6065</td>
</tr>
</tbody>
</table>

a Relative error (%)  
b C.V. (%)  
c n = 6 for each concentration for intra-day assay  
d n=3 for each concentration for 3 days (inter-day assay)

Table 3. Stability results of MTZ during the experiments

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Concentration added (µg.mL⁻¹)</th>
<th>Concentration determined (µg.mL⁻¹) (X±SD)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.4761 ± 0.0076</td>
<td>1.6004</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>0.4845 ± 0.0055</td>
<td>1.1488</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>3.8547 ± 0.0645</td>
<td>1.6746</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>3.8037 ± 0.0212</td>
<td>0.5593</td>
</tr>
</tbody>
</table>

X : mean  
SD: Standard deviation

Acknowledgements
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Özet
MTZ’ün HPLC ile miktar tayininde kullanılan yöntem doğrulanık, duyarlılık, kesinlik, doğruluk ve özgünlük çalışmalar ile valide edildi. Alkonma zamanları MTZ için 4.3 dakika ve haloperidol (internal standart) için 7.9 dakika olarak tespit edildi. Kalibrasyon doğruları MTZ konsantrasyonunun 0.25-5 µg.mL⁻¹ (haloperidol, 2 µg.mL⁻¹) olduğu aralıktı elde edildi. 0.5, 2, 4, 5 µg.mL⁻¹ konsantrasyonu 6 örnek için gün içi kesinlik % 1.63 olarak tespit edildi. Aynı konsantrasyonlarda günler arası kesinlik % 1.90 olarak bulundu. MTZ için sapma sınırı 0.1 µg/mL olarak tayin edildi. Kullanılan metotun MTZ’ün miktar tayini için uygun olduğunu gösterildi.

References


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