High Performance Liquid Chromatographic Estimation of Drotaverine Hydrochloride and Mefenamic Acid in Human Plasma

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Abstract

The present work describes a reverse phase HPLC method for the quantitation of drotaverine hydrochloride and mefenamic acid in human plasma. Organic solvent system used for liquid extraction composed of dichloromethane and isopropyl alcohol in the ratio 80:20 (v/v). The compounds were separated on a Thermo BDS Hypersil C8 (25.0 cm × 4.6 mm, 5 µm particle size) column in isocratic mode with a mixture of acetonitrile and ammonium acetate buffer (20 mM, pH 3.5 ± 0.05 adjusted with 85% phosphoric acid) in a ratio of 55:45 (v/v), as the mobile phase, at a flow rate of 1.0 mL min⁻¹. The effluent was monitored by UV detection at 230 nm. The method was validated for accuracy, precision, specificity, linearity, and sensitivity. The total chromatographic analysis time was approximately 10 min with diclofenac sodium (internal standard), drotaverine hydrochloride and mefenamic acid, eluting with retention times of 6.89, 8.21 and 9.67 min, respectively. Calibration plots were linear within the range of 32-960 ng mL⁻¹ and 100-3000 ng mL⁻¹ for drotaverine hydrochloride and mefenamic acid, respectively. The limit of detection values were 11 and 33 ng mL⁻¹ and the limit of quantitation values were 32 and 100 ng mL⁻¹ for drotaverine hydrochloride and mefenamic acid, respectively. Results from analysis of quality control samples at concentrations of 90, 450 and 750 ng mL⁻¹ for drotaverine hydrochloride and 300, 1600 and 2400 ng mL⁻¹ for mefenamic acid, were indicative of good repeatability and precision. Recovery from plasma was 98.68% for drotaverine hydrochloride and 94.51% for mefenamic acid.

Keywords: Drotaverine hydrochloride; Mefenamic acid; Plasma; HPLC; Method validation.

Introduction

Drotaverine hydrochloride, 1-[(3,4-diethoxy phenyl) methylene]-6,7-diethoxy-1,2,3,4-tetrahydro isoquinolone (Figure 1) is an analogue of papaverine (1). It acts as an antispasmodic agent by inhibiting phosphodiesterase IV enzyme, specific for smooth muscle spasm and pain, used to reduce excessive labor pain (2). Drotaverine hydrochloride is official in Polish Pharmacopoeia (3). A few UV spectrophotometric (4-9) and HPLC (10-16) methods have been reported for estimation of drotaverine hydrochloride. HPLC methods (10-13), using perchlorate ions, have shortcomings of reduced column efficiency over a period of time and the long time required for equilibrating the system (up to 30-45 min).
Other reported methods (14-16) provide (Limit of detection) of around 15-30 ng mL⁻¹.

Mefenamic acid, 2-[(2,3-dimethyl phenyl)amino] benzoic acid (Figure 1), is an orally active analgesic and anti-inflammatory drug, used to relieve pain (17-18). Mefenamic acid is official in IP (19), BP (20) and USP (21). Several UV spectrophotometric (22-24), HPLC (25-29) and HPTLC (30) methods for the estimation of mefenamic acid have been reported. A combination of drotaverine hydrochloride and mefenamic acid is used to treat excessive labor pain (31).

Literature survey revealed a need for a method capable of simultaneous estimation of drotaverine hydrochloride and mefenamic acid. The proposed method can estimate both these drugs over an entire therapeutic plasma concentration range. The described method avoids the use of perchlorate in mobile phase and is suitable for simultaneous pharmacokinetic study of both drugs.

**Experimental**

*Standard and reagents*

Drotaverine hydrochloride, mefenamic acid, and diclofenac sodium (internal standard) were obtained from Glen-Mark Pharmaceuticals (Nasik). All solvents were of HPLC grade (E-Merck, Mumbai). HPLC grade water was obtained by double distillation in glass and purification through a RO water purification system (Canpex, Mumbai). Water was filtered through 0.22 µm filters (Millipore). Blood samples were collected from healthy volunteers and separated plasma was stored at -20 °C.

*Chromatographic system and conditions*

The Jasco HPLC system comprised of an Intelligent UV/Vis detector (UV - 1575), a 3-line degasser (DG-1580-53), an Intelligent HPLC pump (PU-1580) and Borwin Chromatograph software. Chromatograms were run at ambient temperature on a steel, C₈ Thermo BDS Hypersil (250 mm × 4.6 mm, 5 µm particle size) column. The mobile phase was a mixture of acetonitrile and ammonium acetate buffer (20 mM, pH 3.5 ± 0.05 adjusted with glacial acetic acid) in a ratio of 55:45 (v/v). The flow rate was 1.0 mL min⁻¹ at a pressure of 102 kg cm⁻². The column elute was monitored at 230 nm. Organic solvent system used for extraction was a mixture of dichloromethane and isopropyl alcohol in a ratio of 80:20 (v/v).

*Choice of internal standard*

To select a suitable internal standard (IS) for analysis, seven drug substances (viz. ketoprofen, ibuprofen, chlorzoxazone, acetaminophen, caffeine and diclofenac sodium) were examined. Among these, diclofenac sodium met all the typical requirements of a compound to be used as an IS. It was stable during analysis, readily available, well resolved from drotaverine hydrochloride, its peak shape was good (tailing factor 1.25), and its elution time was shorter than that of the last eluting analyte peak, mefenamic acid (saving run time per sample).

*Standard solutions*

The stock solutions of IS, drotaverine
hydrochloride, and mefenamic acid were made up in methanol to a concentration of 50, 1036.15 and 921.14 µg mL⁻¹. HQC, MQC, LQC and linearity range dilutions were obtained by serially diluting stock solution with mobile phase. The amount of internal standard, diclofenac sodium in each sample tube was 2500 ng. Drotaverine hydrochloride and mefenamic acid were determined by the ratio of the peak areas of each drug to the peak areas of internal standard, plotted against drug concentration. Working standard was always made up on the day of analysis.

**Extraction protocol**

One mL blank plasma was added to glass 15 mL conical centrifuge tubes (J-sil Borosil, Mumbai). 25 µL of each drug solution was added separately. The mixture was vigorously vortex-mixed for 1 min. 50 µL of internal standard solution was then added and the sample, and vortex-mixed for 30 sec. 1 mL buffer solution was then added and again vortex-mixed for 60 sec, followed by the addition of 5 mL extraction solvent. The mixture was vigorously vortex-mixed for 2 min and then centrifuged for 10 min at 3000 rpm. The organic layer was then separated (4 mL) and evaporated to dryness with a nitrogen sample evaporator (Takahe Instruments, Mumbai) at 30°C. The residue was reconstituted with mobile phase (200 µL) and 20 µL aliquots were injected into the HPLC column.

**Calibration**

To determine the linearity of the method, various spiked standard concentrations ranging from 32 to 960 ng mL⁻¹ for drotaverine hydrochloride and 100 to 3000 ng mL⁻¹ for mefenamic acid were prepared by adding 25 µL of each working standard drug solutions to 1 mL blank plasma, followed by addition of 50 µL internal standard.

**Validation procedures**

The method was validated in accordance with published guidelines (32). Within-run precision and standard deviation, as a measure of accuracy, were examined by supplementing blank plasma with appropriate amounts of each of the two compounds to yield quality control (QC) samples containing 90, 450 and 750 ng mL⁻¹ of drotaverine hydrochloride and 300, 1600 and 2400 ng mL⁻¹ of mefenamic acid. The QC samples were divided into equal portions, and each analyzed (n=10) as a separate sample, using the procedure described above. To avoid the risk of possible drug degradation during storage, QC samples were prepared each day from the stock solutions prepared on the first day of the study.

Inter-day reproducibility was determined on three separate occasions by replicate analysis of each of the QC samples. Results were regarded as satisfactory, if they were within ±15% of the actual value, except for the limit of quantification, for which 20% was regarded as satisfactory.

Specificity was assessed in the presence of ranitidine hydrochloride, ibuprofen and aceclofenac. To determine long-term freezer stability of drotaverine hydrochloride and mefenamic acid in plasma, QC solutions were analyzed in triplicate after storage of the solutions at -80°C for 30 days. The drug was regarded as stable if more than 90% was intact at the end of the study period.

**Data analysis**

Student’s t-test was used to examine differences between sets of results. Differences were regarded as significant if P < 0.05.

**Results and Discussion**

Extraction of both drugs from plasma was achieved by organic solvent mixture of DCM and IPA. This results in easy, rapid, and convenient separation of the analytes. The chromatograms obtained under the assay conditions used were clean, despite injection of the sample on to the column without pre-purification. This HPLC method enabled rapid simultaneous measurement of both drugs in plasma samples. Total analysis time, including sample pretreatment and rapid elution, was less than 20 min.

**Selectivity**

We demonstrated the absence of interfering endogenous compounds in blank plasma. A representative chromatogram has been shown.
in Figure 2 a, which confirms the presence of blank plasma peaks at 3.62, 4.26, 5.15 and 8.9 min. Addition of the two compounds to plasma samples showed that they were well separated, with no interfering peaks being observed in blank plasma samples. Retention times were 6.89, 8.21 and 9.67 min for IS, drotaverine hydrochloride and mefenamic acid, as shown in Figure 2 b. During therapeutic treatment, drotaverine hydrochloride is often co-administered with ranitidine hydrochloride. When samples were spiked with the latter drug, no interference was observed in the chromatogram, as shown in Figure 2 c.

**Linearity**

Calibration standards spiked in plasma for each drug were prepared at concentrations of 32, 64, 160, 320, 480, 640, 800 and 960 ng mL\(^{-1}\) for drotaverine hydrochloride and 100, 200, 500, 1000, 1500, 2000, 2500 and 3000 ng mL\(^{-1}\) for mefenamic acid. All calibration standards contained 2500 ng mL\(^{-1}\) IS. Separate calibration plots for the drugs were constructed by plotting peak area ratios, against respective concentrations. Typical calibration plots for plasma extracts had good correlation coefficients (0.9994 for drotaverine hydrochloride and 0.9989 for mefenamic acid; n=8 calibration points).

**Limits of quantification and detection**

The limit of quantification, defined as the lowest concentration that could be measured with accuracy and precision, i.e. within ±20% of the actual value (32), were 32 and 100 ng mL\(^{-1}\) for drotaverine hydrochloride and mefenamic acid, respectively. The lower limits of detection of drotaverine hydrochloride and mefenamic acid (three times the baseline noise) were 11 and 33 ng mL\(^{-1}\), respectively. As a simultaneous estimation in plasma, the values for limit of quantification and limit of

Table 1. Intra-day precision and accuracy of the HPLC method.

<table>
<thead>
<tr>
<th>Actual value (ng mL(^{-1}))</th>
<th>Drotaverine hydrochloride (ng mL(^{-1}))</th>
<th>Mefenamic acid (ng mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90</td>
<td>450</td>
</tr>
<tr>
<td>Mean concentration found</td>
<td>86.3</td>
<td>449.9</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>4.38</td>
<td>4.45</td>
</tr>
<tr>
<td>Coefficient of variation (CV%)</td>
<td>5.08</td>
<td>0.99</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>-4.11</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

\(^{a}\text{CV} = \frac{(\text{SD}/\text{Mean}) \times 100}{100}

\(^{b}\left[\frac{\text{(Amount found)-(Amount added)}}{\text{(Amount added)}}\right] \times 100
detection were smaller than any other reported simultaneous estimation method for drotaverine hydrochloride and mefenamic acid.

**Intra-day accuracy and precision**

Method performance was evaluated as intra-day accuracy and precision, determined by replicate analysis of QC samples. The results obtained have been listed in Table 1. These results show good repeatability of the method, used including both sample processing and chromatographic measurement. The coefficient of variation (%) is a ratio of standard deviation to mean in percent. Small deviations from perfect accuracy were observed (i.e. -10% at most) whereas coefficient of variation (%) was at most 5.08.

**Inter-day accuracy and precision**

As is apparent from Table 2, inter-day coefficients of variation determined from experiments performed on three different days (n = 6) were < 5%, whereas coefficient of variation (%) was at most 3.57. These statistical data are indicative of good precision.

**Recovery**

Recovery was determined by dividing the peak area obtained from analysis of each of the two compounds added to plasma by that observed for the same amount of each compound injected directly into the chromatograph. Recovery of drotaverine hydrochloride and mefenamic acid from plasma was 98.68 and 94.51%, respectively. These values were constant in the concentration range studied.

**Stability**

Experiments conducted in our laboratory showed that QC solutions of drotaverine hydrochloride in plasma were stable for at least 30 days at -80°C. The amount of the initial concentration remaining after this time interval was 97.57±1.85% and 95.23±1.15%

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**Table 2. Inter-day precision and accuracy of the HPLC method.**

<table>
<thead>
<tr>
<th>Actual value (ng mL$^{-1}$)</th>
<th>Drotaverine hydrochloride (ng mL$^{-1}$)</th>
<th>Mefenamic acid (ng mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90</td>
<td>450</td>
</tr>
<tr>
<td>Mean concentration found</td>
<td>88.4</td>
<td>434.7</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>3.16</td>
<td>5.15</td>
</tr>
<tr>
<td>Coefficient of variation (CV%)$^a$</td>
<td>3.57</td>
<td>1.19</td>
</tr>
<tr>
<td>Accuracy (%)$^b$</td>
<td>-1.78</td>
<td>-3.4</td>
</tr>
</tbody>
</table>

$^a$CV = (SD/ Mean) ×100  
$^b$[(Amount found)-(Amount added)] / (Amount added)] ×100

<table>
<thead>
<tr>
<th>Actual value (ng mL$^{-1}$)</th>
<th>Drotaverine hydrochloride (ng mL$^{-1}$)</th>
<th>Mefenamic acid (ng mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90</td>
<td>450</td>
</tr>
<tr>
<td>Mean initial concentration</td>
<td>86.5</td>
<td>448.7</td>
</tr>
<tr>
<td>Coefficient of variation (CV%)$^a$</td>
<td>4.52</td>
<td>1.21</td>
</tr>
<tr>
<td>Number of replicates</td>
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<td>6</td>
</tr>
<tr>
<td>Mean final concentration$^b$</td>
<td>82.9</td>
<td>437.5</td>
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<tr>
<td>Recovery (%)$^c$</td>
<td>95.8</td>
<td>97.5</td>
</tr>
<tr>
<td>Coefficient of variation (CV %)$^d$</td>
<td>3.85</td>
<td>1.34</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$CV = (SD/ Mean) ×100  
$^b$Data obtained after 30 days  
$^c$[(Final concentration)/(Initial concentration)] ×100
for drotaverine hydrochloride and mefenamic acid, respectively. These results are shown in Table 3.

Acknowledgements

Authors wish to thank Dr. S. J. Surana (R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur), and Dr. R. A. Fursule (H. R. Patel Women’s College of Pharmacy, Shirpur) for their kind assistance.

References


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