A rapid, sensitive, and direct quantification of tamosulosin in human plasma through LC-ESI-MS/MS for the purposes of a bioequivalence study

M. L. Kundlik¹, B. H. Zaware² S. R. Kuchekar ¹*

¹Padmashri Vikhe Patil College, Pravaranagar, Loni Kurd, Pin-413713, Dis-Ahmednagar, Maharashtra State, India
²New Arts, Commerce and Science College of Ahmednagar-414 001, Maharashtra State, India.

Received August 26, 2010, accepted September 9, 2010

A high-throughout bioanalytical method based on a solid phase extraction (SPE) and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been developed and validated for the quantification of tamosulosin in heparinized human plasma. Plasma samples, without a drying and reconstitution step, were extracted by a simple SPE. The analytes and tamosulosin D4 isotope (internal standard, IS) were chromatographed on a Betabasic-8 column. The total chromatographic run time was 1.8 min per sample. The response was a linear function of concentration in the range of 0.075–50.0 ng/ml, with correlation coefficient $\geq 0.9992$. The assay has excellent characteristics and was successfully applied to bioequivalence study samples for estimation of tamosulosin in healthy human subjects.

Key words: human plasma; LC-MS/MS; tamosulosin; positive ion electrospray.

INTRODUCTION

The symptoms, associated with benign prostatic hyperplasia (BPH), are related to the bladder outlet obstruction, which is comprised of two underlying components: static and dynamic. The static component is related to the increase in the prostate size, caused partly by a proliferation of smooth muscle cells in the prostatic stroma. However, the severity of BPH symptoms and the degree of the urethral obstruction do not correlate well with the size of the prostate [1, 2]. The dynamic component is a function of increase in the smooth muscle tone in the prostate and bladder neck, leading to constriction of the bladder outlet. Smooth muscle tone is mediated by the sympathetic nervous stimulation of the alpha 1 adrenoceptors, which are abundant in the prostate, prostate capsule, prostatic urethra, and bladder neck. Blockage of these adrenoceptors can lead to smooth muscles in the bladder neck and relaxation in the prostate which results in improvement of the urine flow rate and reduction in the BPH symptoms [1, 2].

Several chromatographic methods, including LC-fluorescence detector [3] and LC-MS/MS [4-9], have been developed to measure tamosulosin in the biological fluids. LC-fluorescence detector [4] and LC-MS/MS [4-6] methods are inadequate because of the low sensitivity (LLOQ>0.2 ng/ml), high injection volume (>10 µl), long chromatographic run time (>2.5 min) and large volume of plasma (>0.5 ml), required for the analysis. Hence, these methods allow for limited numbers of sample analysis in a day, which is not enough for day to day analysis and commercial utilization in the pharmacokinetics studies. The reported LC-MS and LC-MS/MS methods [7-9] are sensitive enough but all these technique require laborious extraction procedure such as the liquid-liquid extraction (LLE), involving time-consuming and error-prone solvent evaporation and reconstitution steps, high injection volume, and large volume of plasma for the analysis. Therefore, it was necessary to develop a simple, rapid and sensitive analytical method with minimum plasma requirement for extraction and short run time method to quantify the tamosulosin in the human plasma.

We report a new validated LC-MS/MS method for quantitation of tamosulosin in the human plasma that includes a simple SPE technique without drying and following reconstitution steps. Proposed method reduces sample preparation and analysis time relative to other commonly employed techniques with an improved limit of quantitation (LOQ) 0.075 mg/ml. Present method has run time of 1.8 min per
Table 1a. Experimental condition for liquid chromatography

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Chromatographic parameters</th>
<th>Optimized parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mobile Phase Delivery</td>
<td>Isocratic mode</td>
</tr>
<tr>
<td>2</td>
<td>Mobile Phase</td>
<td>80:10:10 (v/v/v) methanol: acetonitrile: 3mM ammonium acetate, pH adjusted to 3.0 with formic acid.</td>
</tr>
<tr>
<td>3</td>
<td>Analytical column</td>
<td>Betabasic-8, 50 mm X 4.6 mm, 3.0 µm</td>
</tr>
<tr>
<td>4</td>
<td>Mobile phase flow rate</td>
<td>0.45 ml/min</td>
</tr>
<tr>
<td>5</td>
<td>Autosampler tray temperature</td>
<td>20°C</td>
</tr>
<tr>
<td>6</td>
<td>Column temperature</td>
<td>45°C</td>
</tr>
<tr>
<td>7</td>
<td>Injection volume</td>
<td>5 µl</td>
</tr>
<tr>
<td>8</td>
<td>Autosampler rinsing volume</td>
<td>500 µl before and after injection</td>
</tr>
<tr>
<td>9</td>
<td>Analytical run time</td>
<td>1.8 min</td>
</tr>
</tbody>
</table>

Table 1b. Experimental condition for mass spectrometric detection.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>ESI source optimized parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spray voltage : 3500V</td>
</tr>
<tr>
<td>2</td>
<td>Sheath gas : 40 (arbitrary units)</td>
</tr>
<tr>
<td>3</td>
<td>Auxiliary gas : 20 (arbitrary units)</td>
</tr>
<tr>
<td>4</td>
<td>Capillary temperature : 350°C</td>
</tr>
<tr>
<td>5</td>
<td>Collision gas pressure (Argon) : 1.5 m Torr</td>
</tr>
<tr>
<td>6</td>
<td>Scan type : Selected Reaction Monitoring (SRM)</td>
</tr>
<tr>
<td>7</td>
<td>Polarity for analytes : Positive ion polarity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scan parameter for analyze</th>
<th>Parent (m/z)</th>
<th>Product (m/z)</th>
<th>Scan Time (sec.)</th>
<th>CE</th>
<th>Q1 PW</th>
<th>Q3 PW</th>
<th>Tube lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamosulosin</td>
<td>409.18</td>
<td>228.18</td>
<td>0.2</td>
<td>32</td>
<td>0.7</td>
<td>0.7</td>
<td>89</td>
</tr>
<tr>
<td>IS</td>
<td>413.28</td>
<td>228.21</td>
<td>0.2</td>
<td>34</td>
<td>0.7</td>
<td>0.7</td>
<td>96</td>
</tr>
</tbody>
</table>

sample; 300 µl of plasma needed for the analysis, and the injection volume required is 5.0 µl, which helps to increase the electrospray ionization (ESI) source life and reduces the column backpressure during the analysis of the clinical sample. Tamosulosin D4 isotope was used as an internal standard.

EXPERIMENTAL

Chemicals, Reagents, Standards

Pharmaceutical grade of tamosulosin was kindly supplied by CIPLA (Mumbai, Maharashtra, India) and IS was procured from MEDICAL ISOTOPES INC (Pelham, New Hampshire, USA). Both analytes were certified to contain more than 98.00%, and were used without further purification. Organic solvents used were of gradient grade and were obtained from Ranbaxy (Delhi, India). Water was obtained from the Milli-Q Gradient water purification system (Millipore, Bedford, Massachusetts, USA). Formic acid was of suprapur grade and was obtained from Merck (Darmstadt, Germany). Ammonium acetate used for mobile phase preparation was of molecular biology-tested grade from Sigma-Aldrich (Steinheim, Germany). O-phosphoric acid was of suprapur grade and was obtained from Merck (Darmstadt, Germany). Oasis SPE cartridges were obtained from Waters (Massachusetts, Ireland). Control human plasma was obtained from Green Cross Laboratory and was stored below -70°C prior to use.

Stock solutions of tamosulosin and IS were prepared in methanol at free base concentration of 1 mg/ml, respectively. Secondary and working standard solutions were prepared from stock solutions by dilution in water. These diluted working standard solutions were used to prepare the
calibration curve and quality control samples in human plasma.

**Sample Preparation**

A 0.3 ml aliquot of human plasma was mixed with 25 µl of IS working solution (30 ng/ml of IS). Then 500 µl of 2% (v/v) O-phosphoric acid in water was added by stirring. The sample mixture was loaded into an Oasis HLB (1 cm³/30mg), equipped with an extraction cartridge that was pre-conditioned with 1.0 ml of methanol, and consequently with 1.0 ml water. The extraction cartridge was washed with 2 ml water, followed by washing with 1.0 ml 20% methanol. Tamosulosin and IS were eluted with 0.5 ml of methanol, and 5.0 µl of the eluate was injected into LC-MS/MS system.

**LC-MS/MS**

High-performance liquid chromatography was performed with a Prominence pump, autosampler, autoinjector, and column oven Shimadzu (Kyoto, Japan) made. Mass spectrometry was performed with a TSQ Quantum triple-quadrupole mass spectrometer by Thermo Finnigan (Thermo Electron, San Jose, CA, USA). All LC and MS/MS conditions were controlled by LCquan software, version 2.5.6.

Compounds were separated on Betabasic-8 reversed-phase column (Thermo Electron Corporation, 50 mm × 4.6 mm, 3.0-µm particles. The column temperature was 45°C and the autosampler tray temperature was 10°C. The mobile phase was (80:10:10, v/v/v) acetonitrile: methanol: 3 mM ammonium acetate, pH adjusted to 3.0 with formic acid, at a flow rate of 0.45 ml/min. The chromatographic condition and mass spectrometric parameter are presented in Table No.1a and 1b.

The LCquan software provided a standard method for calculations for quantitative analysis. Peak-area ratios of the calibrators were used in linear regression analysis with a weighting factor of 1/x². The response curve was used to calculate the concentration of the calibrators, QC, and stability samples.

![Representative chromatograms for tamosulosin](image)

**Fig. 1.** Representative chromatograms for tamosulosin: (a) extracted blank plasma; (b) extracted tamosulosin LLOQ (0.075 ng/ml).

**RESULTS AND DISCUSSION**

**Method Development**

The objective of this method was to develop and validate [10] a rapid, sensitive and simple assay method for the extraction and quantification of tamosulosin. During method development different detection, chromatographic, and sample-extraction condition were evaluated to achieve maximum response and good peak shape. Initially, tuning of the MS conditions in both, positive and negative modes, was performed for tamosulosin and IS, and the response was found to
be much higher in positive-ionization mode. Use of Betabasic-8 (50 mm x 4.6 mm i.d., 3.0 µ) column enabled usage of 0.45 ml/min flow rate, which resulted in run time as low as 1.8 min with better peak symmetry and signal of analytes. The optimum column oven temperature was 45°C, which resulted in symmetrical peaks.

In order to achieve cleanliness in the extract, the solid-phase extraction was optimized for extraction of analytes from plasma. Tamsulosin and IS showed good retention when eluted with methanol. In order to eliminate time-consuming and error-prone solvent evaporation and the reconstitution steps for concentration of samples after elution with methanol, the elution volume of methanol was reduced to 0.5 ml to concentrate the sample in the eluate. The optimized extraction condition was enabled to reduce processing and analysis time. The sample volume of 5.0 µl, avoided column backpressure and ESI source contamination during sample analysis in the clinical studies.

**Method Validation**

**Specificity and selectivity**

The specificity of the method was investigated by comparing chromatograms obtained from six different sources of plasma. The limit of detection (LOD) was 6 pg/ml. The selected reaction monitoring (SRM) transitions, 409.18→228.18 and 413.28→228.21, were chosen for quantification of tamsulosin and IS, respectively. The area observed at the tamsulosin retention time was much less than 20 % than that of the LLOQ (ng/ml) area (Fig. 1a and Fig.1b).

**Table 2. Intra and Inter accuracy and precision for tamsulosin**

<table>
<thead>
<tr>
<th>Quality control samples</th>
<th>Conc. added (ng/ml)</th>
<th>Conc. found (ng/ml) (a)</th>
<th>SD</th>
<th>Precision % CV</th>
<th>Accuracy (%)</th>
<th>Conc. found (ng/ml) (b)</th>
<th>SD</th>
<th>Precision % CV</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ</td>
<td>0.075</td>
<td>0.069</td>
<td>0.006</td>
<td>8.44</td>
<td>92.00</td>
<td>0.073</td>
<td>0.007</td>
<td>9.57</td>
<td>97.33</td>
</tr>
<tr>
<td>LQC</td>
<td>0.225</td>
<td>0.243</td>
<td>0.013</td>
<td>5.44</td>
<td>108.00</td>
<td>0.235</td>
<td>0.030</td>
<td>12.64</td>
<td>104.44</td>
</tr>
<tr>
<td>MQC</td>
<td>15.0</td>
<td>15.789</td>
<td>2.058</td>
<td>6.96</td>
<td>105.26</td>
<td>15.354</td>
<td>1.571</td>
<td>10.23</td>
<td>102.36</td>
</tr>
<tr>
<td>HQC</td>
<td>37.0</td>
<td>36.250</td>
<td>3.108</td>
<td>3.94</td>
<td>97.97</td>
<td>34.726</td>
<td>3.219</td>
<td>9.27</td>
<td>93.85</td>
</tr>
</tbody>
</table>

(a) = mean of six replicates; (b) = mean of thirty replicates; Conc. = Concentration

**Linearity**

Five linearity curves, containing nine non-zero concentrations, were analyzed. The calibration curves appeared linear and were well described by least square lines. A weighting factor of 1/concentration i.e. 1/x² was chosen to achieve homogeneity of the variance for tamsulosin. The correlation coefficient was ≥0.9992 (n=5) for tamsulosin.

**Sensitivity**

The LLOQ is defined as the lowest concentration of the calibration standard yielding accuracy ±20% RE and precision of ≤20% RSD. The intra-run precision of LLOQ plasma samples, containing tamsulosin, was 8.84%. The mean intra-run accuracy of LLOQ plasma, containing tamsulosin, was 92.44%.

**Recovery**

Recovery of tamsulosin was calculated by comparing the peak area of the analyte from extracted plasma standard with that obtained from unextracted standard at the same concentration for the QC samples, containing 0.225, 15 and 37 ng/ml. The percentage mean recovery for tamsulosin was observed to be 74.12 %. The mean recovery of IS was 76.50% at concentration of 30 ng/ml.

**Accuracy and precision**

The intra-assay precision and accuracy were calculated in six replicate analyses for tamsulosin at four concentration levels viz. LLOQ (0.075 ng/ml), low quality control, (0.225 ng/ml), medium quality control, (15 ng/ml) and high quality control (37 ng/ml), each on the same analytical run. Inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The results are given in Table 2.
Matrix effects

Matrix effects were investigated for six different samples of plasma which comprise four lots of normal control heparinized plasma, one lot of lipemic plasma, and one lot of haemolyzed plasma. Three samples, each at low and high quality control levels, were prepared from different lots of plasma (i.e. a total of 36 QC samples) and checked for accuracy to see whether the matrix affected the back-calculated value of the nominal concentrations for these different plasma samples. The results obtained were well within the acceptable limit of ±15%.

Stability

Exhaustive experiments were performed to assess the stability of tamosulosin in stock solution and in plasma samples under different conditions, simulating the conditions which occurred during study analysis. The stock solutions of tamosulosin and IS were stable at a room temperature for 20 h and at 2–8°C for 28 days. Tamosulosin, in control human plasma, were stable for 14 h at a room temperature. Both analytes in the extracted plasma samples were stable for 54 h in an autosampler at 10°C. The tamosulosin was found to be stable at least for four freeze–thaw cycles. Tamosulosin spiked plasma samples, stored at −70°C to test long-term stability, were stable for at least 58 days.

The design of the study comprised an open randomized, two period, two sequence, replicate, crossover, comparative evaluation of the relative bioavailability of tamosulosin test formulation with reference in 12 healthy adult human subjects under fasting condition. Plasma samples were collected in 0.00, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 8.00, 10.00, 12.00, 14.00, 16.00, 18.00, 20.00, 24.00 and 48.00 h after the administration of a single oral dose of a 0.4 mg tamosulosin capsule to 12 male volunteers in each phase.

APPLICATIONS

The proposed validated method was successfully applied, for the assay of tamosulosin, to 12 healthy adult male human volunteer samples who received 0.4 mg of tamosulosin capsule under fasting condition. Current method is applicable for a large number of pharmacokinetics sample analysis because of its rapid sample preparation technology and short chromatographic run time. Tamosulosin presence in the volunteer’s blood circulation was noticed in about 5.0 h after dose administration (Fig. 2).

CONCLUSION

The objective of this work was to develop a simple, specific, rugged and a high throughput method for estimation of tamosulosin in the
human plasma. The advantages of the SPE technique used in the present work are:
(i) minimized sample extraction time;
(ii) only 300 µL of human plasma were used, hence, reduced amount of blood withdrawn from the volunteers during the study;
(iii) significantly less labor consuming (compared to the labor commonly associated with the LLE technique) in terms of the flash freezing of the aqueous part.

REFERENCES

БЪРЗО, ЧУВСТВИТЕЛНО И ПРЯКО КОЛИЧЕСТВЕНО ОПРЕДЕЛЯНЕ НА ТАМУЛОЗИН В ЧОВЕШКА ПЛАЗМА ЧРЕЗ LC-ESI-MS/MS ПРИ ИЗСЛЕДВАНЕТО НА БИОЕКВИВАЛЕНТНОСТ

М.Л. Кундлик1, Б.Х. Зауаре2, С.Р. Кучекар1,*

1 Колеж Падмашири Вике Патил, Праваранаагар, Лони Кърд, Пин-413713, Дис-Ахмеднаагар, Махараксастра, Индия
2 Колеж за нови изкуства, търговия и наука в Ахмеднаагар -414 001,

Постъпила на 26 август, 2010, приета на 9 септември, 2010

(Резюме)

Разработен е експресен биоаналитичен метод, основан на твърдо-течна екстракция (TTE) и бърза течна хроматография, съчетана с мас-спектрометрия (LC-MS/MS). Методът е изпитан и утвърден за количественото определяне на тамулозин в хепаринизирана човешка плазма. Пробите от плазма, без сушене и повторно разтваряне, се екстрахират чрез TTE. Пробите и вътрешният стандарт от тамулозин (изотоп D4) се подлагат на течна хроматография на колона Betabasic-8. Цялото време за хроматографското определяне е 1.8 мин. за проба. Отговорът е линейна функция на концентрацията при концентрации в интервала 0.075–50.0 ng/ml, с корелационен коефициент ≥0.9992. Методът има отлични характеристики и е използван успешно за изследвания на биоеквивалентност на проби от плазма от здрави хора за наличие на тамулозин.