An isocratic liquid chromatography-electrospray ionization tandem mass spectrometric determination of varenicline in human plasma and dosage form

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A simple, sensitive and accurate liquid chromatography tandem mass spectrometric (LC/MS/MS) method has been developed and validated for determination of varenicline (VRC) in human plasma and pharmaceutical tablets as a tool for therapeutic drug monitoring. The VRC and internal standard (paracetamol, IS) were extracted by liquid-liquid extraction technique. The separation was achieved on C18 column (150mm x 4.6 mm, 5 μm, maintained at 25°C) by isocratic mode at a flow rate of 0.7 ml/min using a mobile phase consisted of a mixture of 5 mM ammonium formate, pH 7.5 (A) and (acetonitrile: methanol, 50:50, v/v) (B) in a ratio of A:B (15:85, v/v) for 10 min. The analytes were monitored by electrospray ionization in positive ion multiple reaction monitoring (MRM) mode. Optimization of MRM mode and chromatographic conditions were applied to eliminate the interference peaks and increase of sensitivity. The method was linear (\( r^2 = 0.9998 \)) at concentration range of 20.0 to 500.0 ng/ml with lower limit of detection of 6.0 ng/ml. The method was statistically validated for linearity, accuracy, precision and selectivity following Food and Drug Administration (FDA) guidelines. The mean extraction recovery of VRC from human plasma was 87.06 ± 2.47%. The reproducibility of the method was reliable with the intra- and inter-day precision was < 5% and average accuracy of 103.54%. The validated method was successfully applied to quantify VRC in human plasma as well as bulk and dosage form in quality control laboratory.

**Key words:** Liquid chromatography tandem mass spectrometric (LC/MS/MS), varenicline, human plasma, dosage form

INTRODUCTION

Varenicline (VRC) \((7,8,9,10\text{-tetrahydro-} 6,10\text{-methano-} 6H\text{-pyrazino}[2,3-h][3] \text{benzazepine})\) (Figure 1) was recently introduced as a novel efficacious smoking cessation aid that acts as an \(\alpha 4\beta 2\) nicotinic acetylcholine receptor (nAChRs) partial agonist, centrally acting as a highly selective partial agonist for the nicotinic acetylcholine receptor (Rollema et al., 2007). Varenicline has mixed agonistic-antagonistic properties, thus it has the therapeutic benefit of relieving the symptoms of nicotine withdrawal and cigarette craving during abstinence while blocking the reinforcing effect of nicotine in those who lapse (Dani and De-Biasi, 2001; Picciotto et al., 1988; Tapper et al., 2004). Varenicline tartrate (Champix® and Chantix®; Pfizer) has been approved by the Food and Drug Administration (FDA) as an aid to smoking cessation (Zieler-Brown et al., 2007). Maximum plasma concentrations of varenicline tartrate occur typically within 3 to 4 h after oral administration. Mean ± standard deviation (SD) \(C_{\text{max}}\) was 9.22 ± 2.05 ng/ml at the recommended dose. The approved regime of VRC is 1 mg.
for 12 weeks, starting with a 1-week titration period (Champix, 2001).

The quality of pharmaceutical product of VRC, in terms of purity and stability of the active substance and/or finished product is vital for the effective and safest delivery of its therapeutic values to the smokers. A detailed understanding of correlations of drug levels with drug action is an important aspect of the routine use of drug. The accurate quantification of agents in biological matrices such as blood, serum, urine and tissue samples is the cornerstone of therapeutic drug monitoring. Therefore, detailed specific, reproducible and accurate method for the quantification of VRC is necessary. Additionally, examining the matrix effects represents an important issue in liquid chromatography tandem mass spectrometric (LC-MS/MS), particularly when dealing with biological matrices such as biological fluids. These phenomena can be reduced by an efficient sample preparation (Souverain et al., 2004) and an adequate chromatographic separation with the elution of the analytes outside the matrix effect time window generally observed at the beginning of the chromatogram (Marchi et al., 2007).

However in quantitative analysis, these conditions might be insufficient to reduce interferences, and other approaches should be combined to compensate residual matrix effects; the use of multiple reaction monitoring (MRM) mode can be one of these approaches (Chambers et al., 2007; Addona et al., 2009). Two LC-MS/MS methods have been published for the quantification of varenicline in human plasma, the first one was done by Obach et al., (2006) which has then been applied for the study of varenicline pharmacokinetics (Faessel et al., 2006; Faessel et al., 2007). The second method was developed by Dobrinas et al. (2011) for determination of nicotine, cotinine, trans-3’-hydroxycotinine and varenicline, which was performed as a procedure for a clinical study on smoking cessation to confirm abstinence from smoking and detection of overdose.

In dosage form, three methods were reported using reversed phase high powered liquid chromatography (RP-HPLC) (Katakam et al., 2012; Kadi et al., 2011) and ultra performance liquid chromatography (UPLC) (Satheesh et al., 2010) with detection limit in microgram level. The calibration ranges were in the range of 2.5 to 7.5 µg/ml (Katakam et al., 2012), 2 to 14 µg/ml (Kadi et al., 2011), and 0.005 to 0.30% (Satheesh et al., 2010).

The present study describes, for the first time, the development and validation of an isocratic LC-MS/MS with highly efficient, more specific and highly sensitive method for quantitative determination of VRC in both human plasma and dosage form.

**METHODOLOGY**

**Chemicals and reagents**

Varenicline tartarate reference standard (purity, 99.5%) was purchased from Weihua Pharma Co. Ltd. (Zhejiang, China), paracetamol reference standard (purity, 99.5%) was purchased from Sigma-Aldrich (Buchs, Switzerland), Champix® 0.5 mg and 1 mg tablets (Pfizer Inc. New York, USA) were procured from a local pharmacy. High power liquid chromatography (HPLC)-grade solvents and reagent-grade ammonium formate were purchased from Merck (Darmstadt, Germany). De-ionized water was purified using cartridge system (Milford, USA) [Ultra pure water of 18 µΩ was obtained from Milli-Q plus purification system, Millipore, Waters (Milford, USA)]. Human blood was obtained from King Khalid University hospital (Riyadh, KSA) and was kept frozen until use after gentle thawing.

**Instrumentation and chromatographic conditions**

Chromatographic separation was performed on an Agilent 1200 series system consisting of G1311A binary pump, G1322A degasser, G1367B HIP-ALS autosampler, G1316 thermostat station column compartment and an Agilent 6410 triple quadrupole LC/MS (Agilent Technologies, Palo Alto, CA, USA). Binary chromatography was carried out on Agilent eclipse plus C18 analytical column (150 mm x 4.6 mm, 5 µm) (Agilent Technologies, Palo Alto, CA, USA). Column temperature was kept constant at 25 ± 2°C. The most suitable chromatographic conditions were achieved at a flow rate of 0.7 ml/min with a mobile phase consisted of A; 5 mM ammonium formate buffer, and an apparent pH was adjusted to 7.5 using formic acid: B (acetonitrile and methanol; 50:50%) in a ratio of A: B, 15:85, v/v. Sample injection volume was 10 µl. Detection was performed on a triple quadrupole MS detector (6410 QQQ), operated with an ESI interface in the positive ionization mode. Nitrogen was used as desolvation gas at a flow rate of 12 L/min and as collision gas at a pressure of 30 psi. Source temperature was set at 350°C, capillary voltage at 4 kV, and dwell time for each ion was 200 ms. Quantification was achieved using multiple reaction monitoring (MRM) of the transitions 212→183; 212→169 for VRC and 152→110; 152→93 for paracetamol as an internal standard (IS). These transitions were previously reported in other publications for the detection of VRC (Obach et al., 2006; Tan et al., 2010). Fragmentor voltage was set to 130 and 75 V with collision energy of 21 and 13 V for VRC and paracetamol, respectively. Mass Hunter software (Agilent Technologies, Palo Alto, CA, USA) was used to control the instruments and data acquisition.

**Preparation of standard solutions**

Varenicline standard stock solution was prepared in de-ionized distilled water to give a final concentration of 1 mg/ml. The working standard solution was prepared by diluting 1 ml of stock solution into 10 ml measuring flask in de-ionized water to give a 100 µg/ml concentration. The internal standard (IS) paracetamol stock solution was prepared in methanol to produce a concentration of 1.0 mg/ml.
One ml of stock solution (IS) was prepared into 10 ml measuring flask in methanol to produce a working solution of a 100 µg/ml concentration, and then an appropriate amount was diluted in methanol to give working stock solution of 480 ng/ml. All working solutions were stored at -20°C until required for analysis.

**Sample preparation and construction of the calibration curve**

The calibration standard samples were prepared by spiking blank human plasma with varenicline to yield final concentrations of 50 Low Quality control (LQC), 200 medium quality control (MQC), and 400 high quality control (HQC) ng/ml. To an aliquot of plasma (300 µl), 1 N sodium hydroxide solution (500 µl) was added. The alkalized samples were subjected to liquid extraction, where (15 µl) paracetamol internal standard (480 ng/ml) was added with diethyl ether (3 ml). The organic phase was separated and evaporated to dryness under nitrogen.

The residue was reconstituted with the mobile phase. Similarly, blank and blank with IS were also prepared. Ten (10 µl) of each calibration sample was injected into the LC-MS system. The drug free plasma was processed with similar procedure using de-ionized water instead of VRC. Blank plasma was then tested to ascertain the absence of any endogenous interference at the retention time of VRC and internal standard. An eight-point calibration curve (20, 50, 100, 150, 200, 300, 400 and 500 ng/ml) was constructed by plotting the peak area ratio of VRC to paracetamol (IS) versus VRC concentration (x). Analysis of calibration samples at each concentration was performed in triplicates. Slope, intercept, and \( r^2 \) values were calculated as regression parameters by linear regression. The linear regression equation was used to calculate the concentrations of VRC in spiked plasma based on their peak area ratios.

**Preparation of tablet solutions**

Twenty tablets were weighed and the average weight was calculated. Tablets were crushed to a fine powder, and a quantity of the powdered tablets, equivalent to 10 mg of VRC, was transferred to 50 ml volumetric flasks. A 25 ml of methanol was added, the contents of the flask were shaken for 10 min by a mechanical shaker, and the volume was diluted to 50 ml with methanol. This solution (0.2 mg/ml) was diluted to give a concentration 10 µg/ml. This solution was filtered through a 0.45 µm membrane filter and the filtrate was subjected to the analysis by the LC-MS/MS method.

**Method validation**

The method validation was based on the recommendations of International Conference on Harmonisation (ICH) (ICH Guidance for Industry, 2000) and on the guidelines for analytical procedures and methods validation by the Food and Drug Administration (FDA) (FDA, 2000).

**Selectivity**

The selectivity of an analytical method may be defined as the ability to obviously determine the analyte in the presence of additional components such as impurities, degradation products and matrix. Method selectivity was tested by analyzing 10 blank plasma batches from different sources for interfering peaks. Additionally, selectivity was checked by analyzing 10 placebo tablet samples comparing them with the prepared tablet solutions. Possible carryover effects were reduced by increasing run time after elution of the analytes.

**Linearity and sensitivity**

Using the aforementioned optimum chromatographic conditions, three independent calibration curves were constructed correlating the calculated peak area ratio of VRC to the internal standard (paracetamol) versus the nominal concentrations of VRC. Calibration plots for VRC in plasma were prepared daily at eight concentration points; each concentration was injected in triplicates. Regression analysis for the results was carried out using the least-square method. The method is extensively validated as per the United States Food and Drug Administration (FDA, 2000) guidelines and ICH (ICH Guidance for Industry, 2000).

**Precision and accuracy**

Precision was measured in accordance with ICH recommendation (ICH Guidance for Industry, 2000). Intra-day accuracy and precision were determined in six replicates by analyzing QC samples at low, medium and high concentrations (50, 200 and 400 ng/ml) across the linear range. Inter-day accuracy and precision were evaluated on three consecutive days. Precision was expressed as the relative standard deviation of the determined concentrations. Percent accuracy was reported as:

\[
\text{Error \%} = \frac{[\text{mean measured concentration} - \text{nominal concentration}]}{\text{nominal concentration}} \times 100.
\]

Precision less than 5.3 % and accuracy within 97.8 to 104.6% were accepted.

**Limit of detection and lower limit of quantification**

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the signal-to-noise ratio (ICH Guidance for Industry, 2000). The intercept was then equal to SD (the estimated SD at a concentration of zero). LOD and LOQ were then defined as 3SD, 10SD, respectively.

**Robustness and ruggedness**

In order to measure the extent of the method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged, and in parallel the chromatographic profile was observed and recorded. The chromatographic parameters were interchanged within the range of 1 to 10% of the optimum recommended conditions. The studied parameters were: the composition of the mobile phase, pH, flow rate, and column temperature. Ruggedness of the method was determined by using two different analyzing and different instruments.

**Recovery**

The percentage recovery of VRC in human plasma and pharmaceutical tablets was assessed as the ratio of the mean peak area of the VRC spiked before extraction to the mean peak area of the same concentration spiked post-extraction in the same matrix multiplied by 100.

**Stability studies**

Stability experiments were performed with low, medium and high QC samples to evaluate the varenicline stability under different conditions. Experiments were performed in triplicate to determine stability of bench top (6 h) and auto sampler (24 h) sample at room
RESULTS AND DISCUSSION

Method development

Mass spectrometric conditions were optimized so as to achieve the maximum stable response of the parent ions and the major product ions of the analytes. Multiple reaction monitoring (MRM) afforded by MS/MS had a greater advantage in reducing interference and enhancing sensitivity over selected ion monitoring (SIM). ESI operated in positive ion mode for the LC-MS/MS analysis to provide optimum sensitivity and selectivity. The mass spectrum of VRC showed protonated molecular ions ([M+H]+) at m/z 212. Two major fragments were observed at m/z 169 and 183, which were selected for the subsequent monitoring in the third quadrupole. The mass spectrum of the IS, paracetamol, showed a protonated molecular ions ([M+H]+). Two major fragments were observed at m/z 110 and 93 (Figure 2).

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution, sensitivity and symmetric peak shapes for varenicline and IS. Different percentages of acetonitrile and methanol solution containing ammonium formate buffer at different pH using formic acid, were tested. The presence of formic acid in the mobile phase can aid in ionization the analytes, enhancing ion response, and modifying the peak shape. Finally, mixture of formate, pH 7.5 (A) and (acetonitrile: methanol, 50:50, v/v) (B) in a ratio of A:B (15:85, v/v) was adopted as mobile phase because of its better separation, high sensitivity and more stable MS signal. Varenicline and IS were detected at retention times of 7.7 and 5.4 min, respectively, using the optimized LC-MS/MS condition and not interfered by endogenous compounds.

The matrix effects were also evaluated by comparing the peak areas ratio of varenicline from the spiked after extraction of the samples (the blank plasma samples were obtained from six different sources) to those obtained for the standards in the mobile phase at equivalent concentrations. The ratio from low to high dose levels was 88.17 to 87.05%. These results indicate that the matrix effect should not have a significant impact on assay performance. Choosing the appropriate IS is important for active high accuracy and to deal with sample matrix effect where LC-MS/MS is used for the assay. Paracetamol was selected as the IS because of its chromatographic behavior similar to that of varenicline. Varenicline was found to be stable at bench top for 6 h, and then for 24 h in an auto sample at room temperature.

Under the optimal LC conditions, VRC eluted at 7.7 min, and the IS at 5.4 min, with a total chromatographic run time within 10 min. Carryover was not obvious in either blank matrices or zero-level standard (blank with IS). A representative total ion chromatogram of VRC and IS in multiple reaction monitoring (MRM) mode is shown in Figure 3.

Our proposed study, in comparison with the reported methods is highly efficient, more specific and highly sensitive for quantitative determination of VRC in both human plasma and dosage form compared with published methods (Obach et al., 2006; Dobrinas et al., 2011). Both methods (Obach et al., 2006; Dobrinas et al., 2011) were used as gradient elution. In the first method (Obach et al., 2006), the column was washed and re-equilibrated after each injection and the method was not fully validated. In the second method (Dobrinas et al., 2011), UPLC was used with gradient mode followed by recondition with 95% of solution B (acetonitrile with 0.1% formic acid) for 8.0 min, as requested for HPLC columns.

The present study describes, for the first time, the development and validation of an isocratic LC-MS/MS with highly efficient, more specific and highly sensitive method for quantitative determination of VRC in both human plasma and dosage form also, the method may be useful for therapeutic drug monitoring of VRC in plasma as well as pharmacokinetic studies of VRC.

Validation of the method

Linearity, sensitivity and selectivity

The method is extensively validated according the FDA guidelines and ICH and is rugged and adequately sensitive for routine subject sample of analysis. The linear regression analysis for the results was carried out using the least-square method. The relative standard deviation values of each concentration point (triplicates) did not exceed 5.13%. The results revealed a good linear calibration fit in the range of 20 to 500 ng/ml, with a correlation coefficient (r) ≥ 0.998. A typical calibration curve has the regression equation of \( y = 1.5209x - 0.0006 \) \( (r^2 = 0.9998) \). The high \( r^2 \) value was indicative for the good linearity, and the low values of standard deviations of the intercept and the slope were indicative for the significant validity of the calibration points used for constructing the calibration curve. The method is selective as no interference was observed in drug-free plasma and placebo tablets samples at the retention time of VRC. Additionally, no carry-over effect was observed in our system. Varenicline and IS were well separated under the HPLC conditions applied and retention times were 7.7 and 5.4 min, respectively. No interferences were observed in drug free human plasma or excipients commonly co-formulated with drug (Figure 2). Otherwise, there are no peaks detected at the retention time of varenicline and internal standard paracetamol.

Limit of detection and lower limit of quantification

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the signal-to-noise ratio
Figure 2. MS scan of (a) varenicline (b) parectamol (IS) and MRM spectra of (c) varenicline (d) paracetamol (IS).
Figure 3. Total ion chromatogram of MRM scan of VRC and IS in spiked plasma.

(ICH Guidance for Industry, 2000). The intercept was then equal to SD (the estimated SD at a concentration of zero). LOD was then defined as 3SD, and LOQ was defined as 10 SD. The LOD and LOQ values were 6.0 and 20.0 ng/ml, respectively.

**Precision and accuracy**

The results of intra-day and inter-day accuracy and precision are presented in Table 1. The intra- and inter-day precisions were less than 2.32 and 4.1%, respectively. Similarly, the average intra-day and inter-day accuracy was 103.7 and 103.38%, respectively. Moreover, accuracy and precision were determined by the recovery study of known amounts (20 to 500 ng/ml) of VRC standard added to a placebo matrix for tablets. The samples were analyzed (6 replicates were injected) by one analyst, and the added amounts were calculated from a calibration curve. The accuracy values ranged from 97.28 to 101.65% and precision values ranged from 0.54 to 2.39 (Table 2). These results indicated the acceptable accuracy and precision of the method (ICH Guidance for Industry, 2000).

**Robustness and Ruggedness**

In order to measure the extent of robustness, the most critical parameters were interchanged while keeping the other parameters unchanged, and the chromatographic profile was observed and recorded, in parallel. The chromatographic parameters were interchanged within the range of 1 to 10% of the optimum recommended conditions. The studied parameters were: the composition of the mobile phase, pH, flow rate, and column temperature. The results indicated that (relative standard deviation (RSD) was 0.3 to 0.4%) the small change in the conditions did not significantly affect the determination of VRC. Ruggedness of the method was determined by using mobile phase components from two different manufactures, two different analyst, and two different instruments. There was no significant change observed in the retention time of VRC; RSD was 0.26 to 0.39%, indicating the ruggedness of the method.
Table 1. Intra-day (n = 6) and inter-day (n = 6) precision and accuracy of varenicline.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/ml)</th>
<th>Intra-day</th>
<th></th>
<th></th>
<th>Inter-day</th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
<td>Mean±SD</td>
<td>Accuracy (%)</td>
<td>RSD (%)</td>
<td></td>
</tr>
<tr>
<td>LQC (50)</td>
<td>52.3±1.2</td>
<td>104.60</td>
<td>2.29</td>
<td>51.4±2.1</td>
<td>102.8</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>MQC (200)</td>
<td>205.7±2.9</td>
<td>102.85</td>
<td>1.45</td>
<td>206.8±2.6</td>
<td>103.4</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>HQC (400)</td>
<td>414.6±9.6</td>
<td>103.65</td>
<td>2.32</td>
<td>415.8±7.7</td>
<td>103.95</td>
<td>1.9</td>
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</tbody>
</table>

Table 2. Accuracy and precision data of varenicline.

<table>
<thead>
<tr>
<th>Nominal concentration (ng/ml)</th>
<th>Mean±SD</th>
<th>Recovery (%)</th>
<th>Precision (RSD %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>19.67±1.01</td>
<td>98.35</td>
<td>5.13</td>
</tr>
<tr>
<td>50</td>
<td>48.64±0.94</td>
<td>97.28</td>
<td>1.93</td>
</tr>
<tr>
<td>100</td>
<td>99.02±1.54</td>
<td>99.02</td>
<td>1.56</td>
</tr>
<tr>
<td>150</td>
<td>152.35±1.88</td>
<td>101.56</td>
<td>1.23</td>
</tr>
<tr>
<td>200</td>
<td>200.84±1.11</td>
<td>100.42</td>
<td>0.55</td>
</tr>
<tr>
<td>300</td>
<td>301.97±2.41</td>
<td>100.65</td>
<td>0.80</td>
</tr>
<tr>
<td>400</td>
<td>401.78±3.02</td>
<td>100.44</td>
<td>0.75</td>
</tr>
<tr>
<td>500</td>
<td>497.01±2.67</td>
<td>99.40</td>
<td>0.54</td>
</tr>
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</table>

Table 3. Determination of VRC in tablets (Chantix® 0.5 and 1.0 mg).

<table>
<thead>
<tr>
<th>CHAMTIX® 0.5 mg</th>
<th>determined tablet concentration (mg)</th>
<th>recovery (%)</th>
<th></th>
<th>CHAMTIX® 1.0 mg</th>
<th>determined tablet concentration (mg)</th>
<th>recovery (%)</th>
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<tbody>
<tr>
<td></td>
<td>0.491</td>
<td>98.2</td>
<td>0.986</td>
<td>101.94±3.18</td>
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<td></td>
<td>0.495</td>
<td>99.0</td>
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<td></td>
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<tr>
<td></td>
<td>0.492</td>
<td>98.4</td>
<td>1.021</td>
<td></td>
<td>102.1</td>
<td></td>
</tr>
<tr>
<td>Mean recovery</td>
<td>98.6±0.7</td>
<td></td>
<td>Mean recovery</td>
<td>101.94±3.18</td>
<td></td>
<td></td>
</tr>
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</table>

Application to pharmaceutical formulations and in plasma

The accuracy data of back-calculated concentration of calibration samples for varenicline was evaluated by recovery studies using the standard addition method. The obtained recovery values were 97.28 to 101.65% and the RSD was 0.5 to 2.39% (Table 2). The method was proven to be highly accurate. Results obtained for the analysis of VRC in each formulation by the proposed HPLC is given in Table 3. The recovery of varenicline was in the range of ~98.6 ± 0.7 to 101.98 ± 3.18%.

Recovery of VRC was also determined by analysis of plasma spiked with standard VRC under the optimum conditions. As shown in Table 4, average percentage recovery 87.057% for linearity was in the range of 20.0 to 500.0 ng/ml of VRC.

Conclusion

The optimized LC/MS/MS method was validated for measuring varenicline in human plasma and pharmaceutical formulations. Good linearity was observed from 20 to 500 ng/ml. The assay has a simple satisfactory extraction procedure for sample preparation and a relatively rapid run time of 10 min. The validated method described here, utilizing an isocratic HPLC separation and positive ionization tandem MS detection is rapid, robust, highly selective, and sufficiently sensitive. The method may be useful for therapeutic drug monitoring of VRC in plasma as well as pharmacokinetic studies of VRC.
Table 4. Varenicline recovery from spiked plasma

<table>
<thead>
<tr>
<th>Nominal concentration (ng/ml)</th>
<th>Determined concentration (ng/ml)</th>
<th>Recovery (%)</th>
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<tr>
<td>20</td>
<td>17.63</td>
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<td>50</td>
<td>44.85</td>
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<td>85.57</td>
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<td>150</td>
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<td>500</td>
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<tr>
<td></td>
<td>Mean recovery</td>
<td>87.05</td>
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ACKNOWLEDGMENTS

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REFERENCES


