Rapid LC-MS/MS method for determination of scopolamine in human plasma

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Abstract

Sensitive, simple, and fast LC-MS/MS method for the determination of Scopolamine in human plasma was developed and validated. Liquid-Liquid extraction technique was used for sample preparation. Cyano bonded phase column (150 × 4.6 mm, 5 µm) was used for the separation with an isocratic elution of ammonium format buffer:methanol (60:40) mobile phase at a flow rate of 1 ml.min⁻¹ over 3.8 min run time. Scopolamine and [¹³C,²H₃]-Scopolamine, as internal standard, were detected and quantified in positive ion mode via MRM at m/z 304/138 and m/z 308/142, respectively. The developed method was validated according to FDA and EMA guidelines. The standard calibration curve was linear over the concentration range of 3.03–315.76 pg.ml⁻¹ (r² = 0.999). The intra-day and inter-day precision was in the range 1.28–10.46% and accuracy 96.89–110.53%. The recovery of analyte and IS was 78.63% and 76.21%, respectively. Scopolamine in plasma was stable at benchtop (short term) for 18 h, in autosampler tray for 43 h, in instrumentation room for 43 h (post-preparative), after 4 freeze-thaw cycles (−70 °C), and 3 days in the freezer (−70 °C). The validated method was successfully applied to a bioequivalence study of scopolamine transdermal patch of 1 mg for 3 days for 16 healthy Jordanian volunteers.

Keywords

LC–MS/MS, scopolamine, bioanalytical, pharmacokinetics, bioequivalence

Introduction

Scopolamine, also known as hyoscine, is a natural alkaloid drug obtained from several plants such as Datura stramonium, Hyoscyamus niger, Scopolia carniolica and many other plants from the family Solanaceae, plants from this family produce natural toxic belladonna alkaloids (i.e. Scopolamine) as a protective mechanism (Pergolizzi et al. 2012). Scopolamine is an alkaloid drug with a structure shown in Fig. 1, which works as a nonselective muscarinic antagonist by inhibiting acetylcholine binding to a muscarinic receptors, this generate peripheral antimuscarinic characteristics, central sedative, antiemetic, and amnestic actions (Zhang et al. 2017). The parasympatholytic scopolamine, which is structurally related to atropine, is used in situations that need reduced parasympathetic activity, particularly for its effects on the eye, gastrointestinal system, heart, salivary, and bronchial secretory glands, and in rare cases for a CNS impact (Renner et al. 2005). As a result, scopolamine is best used for premedication and antiemetic effects before anesthesia (White et al. 2007). Scopolamine recommended as the most effective single
drug used to prevent motion sickness and vomiting (Cróinin et al. 1982). Sleepiness, impaired vision, dilated pupils, and dry mouth are all common adverse effects of Scopolamine (Honkavaara et al. 1995; Pergolizzi et al. 2012). However, the agent’s effectiveness when taken orally or parenterally has been limited due to it is short duration of action and it is high incidence of adverse effects, thus a Scopolamine transdermal formulation has been developed (Atkins 2003; Whelan and Apfel 2013; Bailey et al. n.d.).

**Experimental**

**Chemicals and reagents**

Scopolamine hydrobromide and internal standard (IS) \([\text{[}^{13}\text{C}\text{,}^2\text{H}_3\text{]}\text{-Scopolamine oxalate salt}) were obtained from ALSACHIM (Illkirch-Graffenstaden, France). LC-MS grade Methanol, n-Hexane, and Ethyl acetate were obtained from Honeywell (Charlotte, NC, United States). Sodium hydroxide and Formic acid were obtained from ISOLAB (Eschau, Germany). Ammonium format was obtained from Fischer (Schaffhausen, Switzerland). HPLC Water was obtained from Avantor (Radnor, PA, United States). Human K3EDTA plasma obtained from Pharmaceutical Research Unite (PRU) clinical site (Amman, Jordan).

**Instrumentation and chromatographic conditions**

Chromatographic separation was achieved using a Shimadzu Nexera XR system (Kyoto, Japan) using cyano column \((150 \times 4.6\text{ mm}, 5\text{ µm})\) obtained from ACE (Reading, UK). The auto-sampler temperature was 6 °C. The analyte and IS were separated with an isocratic elution of ammonium format buffer:methanol \((60:40)\) mobile phase, using flow rate of 1 ml.min⁻¹. The mass spectrometric data were collected on a Shimadzu LC-MS 8060 (Shimadzu, Japan) with a triple quadrupole mass analyzer. Multiple reactions monitoring (MRM) mode and positive mode of ESI interface were intended for Scopolamine \((m/z\ 304/138)\) and \([^{13}\text{C}\text{,}^2\text{H}_3\text{]}\text{-Scopolamine} (m/z\ 308/142)\). The separation of analyte spray droplets was accomplished by adjusting the nitrogen gas at a flow rate of 3 L.min⁻¹. The analysis data were obtained by Lab solution software, version 5.9.1 from Shimadzu (Kyoto, Japan).

**Preparation of standards and quality control samples**

Standard solutions of scopolamine were prepared from stock solution \((160\ µg.mL^{-1})\) and IS \((80\ µg.mL^{-1})\) in methanol which stored at –20 °C. All standard solutions of Scopolamine and IS were prepared by diluting the stock solution using methanol. Quality control (QC) samples and calibration standard solutions were prepared by spiking blank plasma with Scopolamine at concentrations of 3.03, 6.06, 16.17, 40.42, 78.94, 189.46, 284.18, and 315.76 pg.mL⁻¹. QC samples concentrations were 3.03 pg.mL⁻¹ for lower limit of quantification (LLOQ), 9.09 pg.mL⁻¹ for low quality control-1 (QL1), 60.63 pg.mL⁻¹ for low quality control-2 (QL2), 126.30 pg.mL⁻¹ for middle quality control, (QCM), and 236.82 pg.mL⁻¹ for high quality control (QCH).

**Sample preparations**

For extracting Scopolamine and IS from human plasma, Liquid-Liquid extraction was performed using ethyl acetate and n-hexane \((70:30)\) as extraction solvent. 500 µL spiked human plasma sample and 50 µL \([^{13}\text{C}\text{,}^2\text{H}_3\text{]}\text{-Scopolamine oxalate salt}) were obtained from ALSACHIM (Illkirch-Graffenstaden, France). LC-MS grade Methanol, n-Hexane, and Ethyl acetate were obtained from Honeywell (Charlotte, NC, United States). Sodium hydroxide and Formic acid were obtained from ISOLAB (Eschau, Germany). Ammonium format was obtained from Fischer (Schaffhausen, Switzerland). HPLC Water was obtained from Avantor (Radnor, PA, United States). Human K3EDTA plasma obtained from Pharmaceutical Research Unite (PRU) clinical site (Amman, Jordan).

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\[^{2}\text{H}_1\]\text{-Scopolamine was transferred to an Eppendorf micro}
\begin{align*}
tube (2 \text{ ml}) \text{ then vortex-mixed for 30 s then 100 \mu l}
of \text{NaOH solution (0.2 M) was added and vortex for 30 s.}
\end{align*}
\text{After that, 3.0 ml of extraction solvent was added to the}
sample and vortexed for 5 min and centrifuged (5 min @
3000 rpm, 2–8 °C). The organic solvent was decanted and
evaporated under vacuum for 15 min, then it was recon-
stituted with 300 \mu L mobile phase. Finally, 180 \mu L of ali-
quots were injected into the LC-MS/MS unit.

**Method validation**

The developed method was validated according to the
in the FDA and EMA guidelines. Selectivity, sensitivi-
ty, linearity, matrix effect, precision, accuracy, integrity,
stability, and dilution recovery were all evaluated. The
selectivity was evaluated by injection of eight different
lots of hemolyzed and hyperlipidemic blank plasma.
Caffeine, Paracetamol, Diclofenac, ascorbic acid, Nico-
tine, Aspirin, and Ibuprofen were all examined as poten-
tial concomitant medication interference. The stan-
dard calibration curves were evaluated by plotting eight
different levels. Sensitivity was tested by analyzing six
triplicates of LLOQ against a calibration curve. The matrix
effect was evaluated at two different levels (QCL-1 and
QCH) using eight different lots of blank plasma in-
cluding hemolyzed, and hyperlipidemic. For intra-
and inter-day precision and accuracy six determinations at
LLOQ, QCL-1, QCL-2, QCM, and QCH were extracted
and assessed against the calibration curve. The peak area
of non-extracted standard was compared to extracted
standard to establish Scopolamine and IS recoveries. The
bioanalytical method’s recovery was calculated for Sco-
polamine at three concentration levels (QCL-1, QCH,
and QCM), and for IS at the QCH concentration. Dil-
ution integrity at a concentration of two times the con-
centration of QCH. Six replicates of each concentration
were tested. Stability tests were evaluated to the stock
solutions and plasma samples to assess Scopolamine
stability under various conditions. The stability of stock
solution was evaluated in two conditions: at room tem-
perature and −20 °C, by comparing the area of Scopol-
amine in stability sample to the area in freshly prepared
solution. Six duplicates at QCL-1 and QCH levels were
used to examine bench top stability (18 h), freeze-thaw
(four cycles), autosampler stability (43 h), and long-term
stability (3 days).

**Pharmacokinetic study**

The pharmacokinetic parameters of Scopolamine were
measured in sixteen healthy Jordanian volunteers in a by
measuring the rate and extent of scopolamine after using
scopolamine transdermal patch. This study was approved
by the Institutional Review Board/Independent Ethics
Committee (IRB/IEC) (Bošnjak 2001). Informed consent
containing the purpose, procedures, risks that could be
happening and all information needed about this study
was taken from all subjects as directed according to the
Declaration of Helsinki for biomedical research. Each vol-
unteer received one transdermal patch (2.5 cm^2) contain-
ing approximately 1 mg scopolamine and it was applied
for 72 hours. 8 mL of blood samples were collected from
a forearm vein in labelled K$_3$EDTA blood tubes at (pre-
dose) and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 12,
14, 16, 20, 24, 30, 36, 48, 60, 72, 84, 96, 108, and 120 hours
post dosing. Samples were centrifuged (3500 rpm for
10 min) and supernatant was transferred to pre-labeled
polypropylene tubes then stored in a freezer at −70 °C.
WinNonlin version 8.3 software form Scientific Consulting
Inc. (Asheville, NC, USA) was used to assess all phar-
macokinetic parameters.

**Results and discussion**

**Method development**

Both positive and negative ionization modes were inves-
tigated for Scopolamine. The positive mode response was
more suitable than negative mode. Chromatographic pa-
rameters were optimized to achieve high resolution and
improved scopolamine signal intensity yet maintaining a
short run time. Scopolamine detection was enhanced after
addition of formic acid to the mobile phase. Different ra-
tios of mobile phase were evaluated, and the optimum ra-
tio was 60:40 of ammonium format buffer and methanol.
Many columns stationary phases and brands were tested
and ACE Cyano (150 × 4.6 mm, 5 µm) was the optimum
one and was used for chromatographic separation. The
retention time of Scopolamine and IS was about 2.3 min.
Liquid-Liquid extraction approach was used for sample
preparation as it facilitates high selectivity separation and
cleaner matrix than direct protein precipitation.

**Method validation**

**Selectivity**

Selectivity reflects the degree of interference of Sco-
polamine and IS with endogenous plasma components. It
was demonstrated by examining chromatograms from
processed blank plasma samples and endogenous com-
ponents in drug-free plasma. no interference with ana-
lyte peak was observed as illustrated in Fig. 2. Likewise,
commonly used medications (caffeine, paracetamol, di-
clofenac, ascorbic acid, nicotine, aspirin, and ibuprofen)
also showed no interference (data not showed).

**Sensitivity**

The sensitivity of the developed method was determined
using LLOQ sample by evaluating signal to noise ratio in
order to ensure more than five times response as com-
pared to blank. The value of 3.03 pg.ml$^{-1}$ was chosen as
the LLOQ for Scopolamine. At LLOQ concentrations,
Scopolamine precision and accuracy were determined to
be 10.1% and 102.3%, respectively, which revealed a good
sensitivity of the method.
Matrix effect

Matrix effect quantitative evaluation of Scopolamine and IS, the peak area response for aqueous samples (representing 100% recovery at QCL-1 and QCH levels), were compared to the extracted post-spiked blank with aqueous samples QCL-1 and QCH, respectively. The precision for Scopolamine was found to be 1.94%, and 0.10% at QCL-1 and QCH concentration, respectively, indicating no significant matrix effect was detected in the method.

Linearity

Over the concentration range of 3.03–315.76 pg.ml⁻¹ the method was found linear. A regression equation with a weighting factor (1/x²) of Scopolamine to the IS concentration provided the best match for the concentration–detector response relationship for scopolamine in human plasma. The calibration curves created during validation have a mean correlation coefficient of 0.999.

Precision and accuracy

The accuracy of the developed method was evaluated in terms of % Recovery, and it was found to be ranged from 96.89 to 107.57 % for intra-day accuracy and from 97.74 to 110.53 % for inter-day accuracy. The inter- and intra-day precision were evaluated using 18 replicates and six replicates, respectively. The CV% for both precisions was in the range of 1.28 to 10.46%. The results summarized in Table 1.
Extraction efficiency

Liquid-Liquid extraction technique with Ethyl acetate and n-Hexane (70:30) was robust, effective, and simple. The percentage recovery was assessed by comparing the peak area ratio of scopolamine in the treated samples with those in the untreated samples, as showed by the following equation:

\[
\text{Recovery (\%)} = \frac{\text{Average area ratio of drug for untreated samples}}{\text{Average area ratio of drug for treated samples}} \times 100\%
\]

The recoveries of scopolamine and IS were satisfactory and reproducible. The mean overall recoveries of scopolamine and IS were 78.63% and 76.21%, respectively. The CV was less than 3.25% and 4.92% for scopolamine and IS, respectively.

Dilution integrity

The dilution integrity was assessed for samples whose concentration was as double as ULOQ concentration, which were named as QC dilution. The QC dilution samples were further diluted with interference-free plasma dilutions for determining the dilution integrity of samples. The method found to be accurate and precise up to 473.64 pg.ml⁻¹, with a dilution factor of 2.

Stability

The stability test shows that scopolamine was stable in human plasma at 25 °C for 18 h. The result of the extracted plasma samples indicates that samples were stable in the auto-sampler (25 °C) for 43 h, and they were stable after subjected to four freeze and thaw cycles. QC samples were stable when long term stability was investigated at −70 °C after 3 days. No significant loss of scopolamine can be seen during sample storage, repeated thawing and freezing conditions as shown in Table 2.

Application to a pharmacokinetic study

The suggested and validated LC-MS/MS method has been successfully implemented for measuring the pharmacokinetic parameters of scopolamine in 16 healthy male volunteers. Scopolamine concentration/time profiles from all volunteers after receiving Scopolamine transdermal patch (1 mg/3 days) are presented in Fig. 3.

The results of the pharmacokinetic parameters illustrated that the average maximum plasma concentration (Cmax) of scopolamine for the twenty subjects was 95.787 ± 36.877 pg.mL⁻¹ and reached at the average time of 36.03 ± 21.86 h. The other parameters were the area under the curve (AUC₀₋₅₀₇ and AUC₀₋∞) for scopolamine and those were found to be 1945.255 ± 783.994 pg.h.ml⁻¹ and

![Figure 3](image-url). Mean plasma concentration following administration of a single transdermal patch of Scopolamine (1 mg/3 days).
5425.862 ± 1259.914 pg.h.ml⁻¹ for AUC₀-t and AUC₀-∞, respectively as illustrated in Table 3.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Scopolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₀ (ng.ml⁻¹)</td>
<td>95.787 ± 36.877</td>
</tr>
<tr>
<td>T₁/₂ (h)</td>
<td>30.03 ± 21.86</td>
</tr>
<tr>
<td>T₁/₄ (h)</td>
<td>6.553 ± 2.690</td>
</tr>
<tr>
<td>AUC₀ (ng.h.ml⁻¹)</td>
<td>1945.255 ± 788.994</td>
</tr>
<tr>
<td>AUC₀⁻∞ (ng.h.ml⁻¹)</td>
<td>5425.862 ± 1259.914</td>
</tr>
<tr>
<td>Kel (h⁻¹)</td>
<td>0.0498 ± 0.0137</td>
</tr>
</tbody>
</table>

**Table 3.** Pharmacokinetic parameters of Scopolamine transdermal patch (1 mg/3 days) (Mean ± SD).

### Conclusion

A rapid, simple, and sensitive LC-MS/MS method for scopalamine quantification in human plasma was developed and fully validated according to FDA and EMA guideline. The used liquid-liquid extraction technique gave consistent and reproducible recoveries for Scopolamine. The method was accurate and precise for the determination of scopalamine in human plasma throughout a concentration range of 3.03–315.76 pg.ml⁻¹ and should be useful for regular monitoring of drug concentrations in pharmacokinetic investigations. The method was successfully applied to determine scopalamine transdermal patches in healthy subjects and pharmacokinetic parameters were calculated.

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### References


