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Determination of N-Butylscopolamine in Human Plasma by Solid-Phase Extraction and UHPLC-ESI-MS/MS: Development, Validation and Application to a Bioequivalence Study

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Abstract

A sensitive ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) method for measurements of N-butylscopolamine in plasma was developed and validated. A SPE extraction was proposed for the clean up of plasma and N-butylscopolamine-d9 was added as internal standard. The analyses were carried out using a phenyl column and mobile phase of acetonitrile: 2 mM ammonium formate + 0.02% formic acid (20:80 v/v). The mass spectrometer equipped with an electrospray ion source in positive mode, was set up in selective reaction monitoring, to detect precursor → product ion with m/z 360 to 194 and 360 to 203 transitions, for analyte and IS, respectively. The linearity was checked between 5 to 500 pg/mL for N-butylscopolamine, with R^2=0.9951 and LOD= 5 pg/mL. Satisfactory selectivity, linearity, precision, accuracy and showed no matrix effect or column carryover were obtained for the UPLC-ESI-MS/MS method. The proposed method was successfully applied to a bioequivalence study of healthy human volunteers.

Keywords: N-Butylscopolamine; UHPLC-ESI-MS/MS; SPE; Development; Validation; Bioequivalence

Introduction

N-butylscopolamine according to Figure 1A, is the free base of scopolamine butylbromide, it belongs to the group of quaternary ammonium compounds and is a substance derived from scopolamine. This compound has low solubility in water (0.00166 mg/mL), pKa (strongest acid -15.2 and strongest basic -2.3) and logP -0.6. N-butylscopolamine is also known as scopolamine butylbromide, butylhyoscine, and hyoscine butylbromide [1,2].

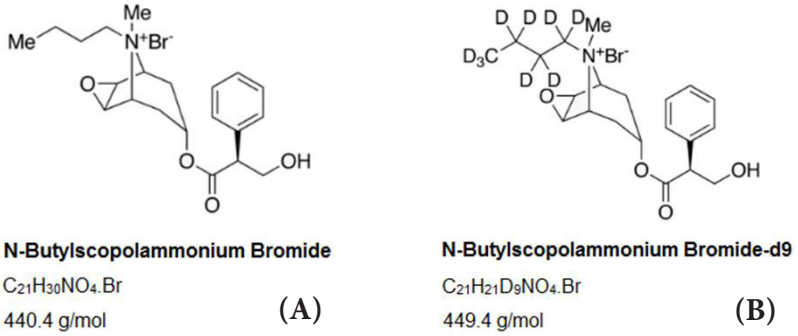


Figure 1: Chemical Structure of: **A)** Analyte and **B)** Internal Standard

The alkaloid scopolamine is a kind of tropane alkaloid separated from various solanaceous species, such as the roots of Chinese traditional medicine *Anisodus tanguticus (Maxim.) Pascher. Scopolamine* has widespread physiological activities such as spasmolytic, anesthetic, acesodyne, and ophthalmic effects. In recent years, further more pharmacological activities of scopolamine have been investigated and widely noticed. Compared with the comprehensive investigations into its therapeutic purpose, the study of its metabolism *in vivo* or *in vitro* is limited, although the metabolic study of scopolamine plays an important role in the development of new drugs and their clinical applications [2]. A number of analogs have been synthesized, the most common being homatropine,

ipratropium, oxitropium, flutropium and N-butylscopolamine, Figure 2 shows the molecular structure, molecular formula and molar mass of these drug. These derivatives have similar structures and are generally used for anticholinergic purposes [3].

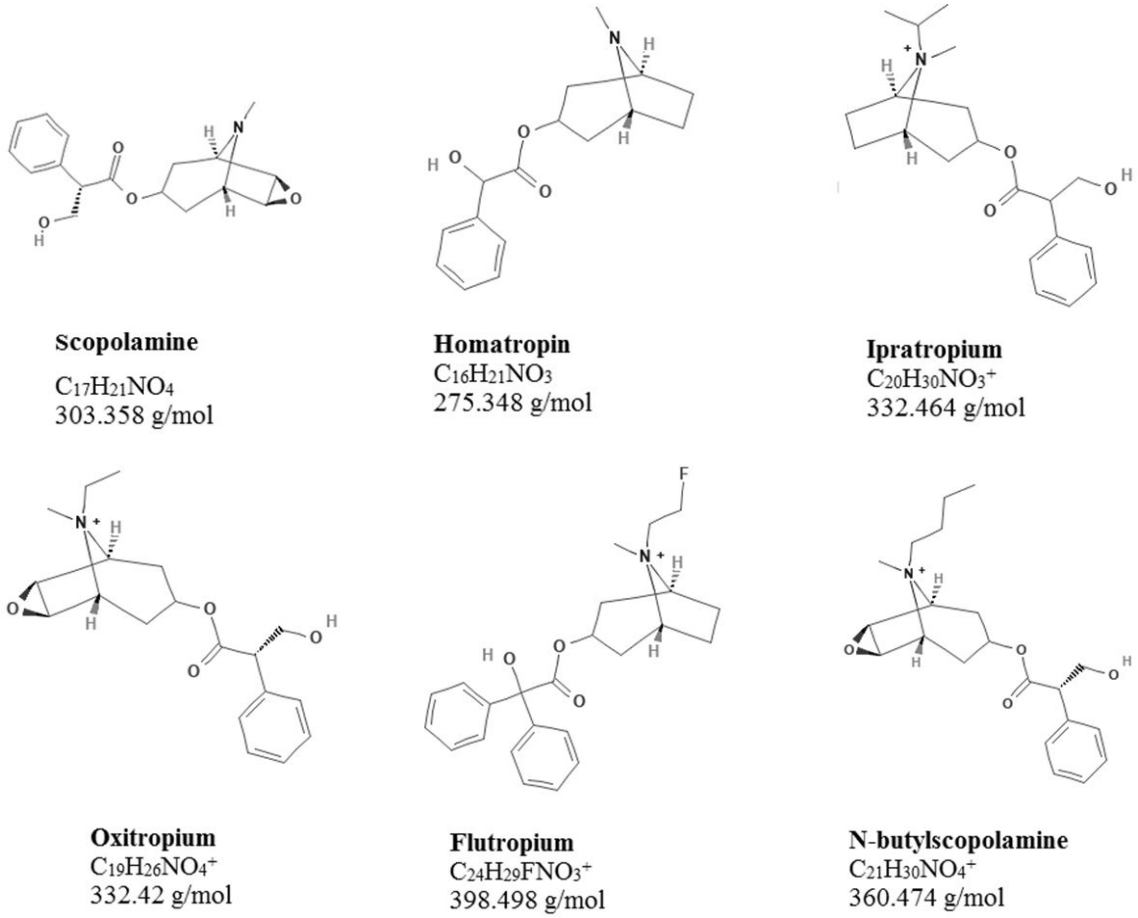


Figure 2: Molecular Structure, Molecular Formula and Molar Mass of the Most Common Synthesized Drugs Analogous to Scopolamine

The pharmacokinetic parameters of scopolamine or N-butylscopolamine following oral administration are generally highly variable, the compound has limited bioavailability because of its high first-pass metabolism [4,5]. The maximum drug concentration (T_{max}) occurs approximately = 4.26 ± 1.48 h after oral administration, and the maximum plasma concentration levels (C_{max}) for N-butylescopolamine are in the order of pg/mL or ultra-trace level (ppt), which range from 0.29 ± 0.23 ng/mL, with an upper RSD of 70% for a dose of 10 mg [6,7].

Chromatographic methods have been reported for the determination of scopolamine in plant and pharmaceutical samples, especially liquid chromatography (LC) [8,9]. Capillary electrophoresis methods and micellar electrokinetic chromatography were developed for the separation and determination of tropane alkaloids and scopolamine derivatives in plants [3,10,11].

A pharmacokinetic study has been based by chromatographic techniques coupled to mass spectrometry. The method was reported by Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS), the scopolamine and the internal standard (IS) mexiletine were extracted from serum by using a single step liquid/liquid extraction; however, after that, a derivatization step was necessary [12]. Most methods are reported by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [6,7,12-14]. In the method reported by Oertel *et al*., [13] the scopolamine and atropine (IS) were extracted and cleaned up by using solid-phase extraction (SPE). This type of extraction has a high variability and is more expensive than liquid/liquid human plasma extraction or protein precipitation.

SPE is a very popular technique currently in sample preparation. Over the years, the use and application of SPE had stable growth, using simple and relatively inexpensive procedures, proportional selectivity and good recovery to several classes of analytes reduced the use of organic solvents and also had its process automated. It has several purposes of use in purification, trace, enrichment, desalting and sample fractionation. The purpose of solid phase extraction method development is to tackle several problems that occur during sample preparation in order to get high selectivity and precise compound detection [15].

Ultra-performance liquid chromatography (UHPLC), a novel advance in rapid, sensitive, and high-resolution liquid chromatography, offers the possibility of significantly increased efficiency of the chromatographic separation through the utilization of columns packed with smaller diameter particles (1.8 μm) that can withstand higher pressures compared to the conventional packing materials [16].

In this paper was to validate a simple, linear selective and sensitive, precise, accurate and reproductible UHPLC-ESI-MS/MS method for determination of N-butylscopolamine in human plasma using deuterated IS, according to Figure 1B. The method uses a SPE extraction, which is an improvement over the most recent published procedures, mainly using the technique of precipitation extraction of proteins by Manfio *et al.*, Favreto *et al.*, and liquid-liquid extraction by Suenaga *et al.,* [6,7,17-22]. The method was also shown to be applicable to the determination of N-butylscopolamine in bioequivalence study.

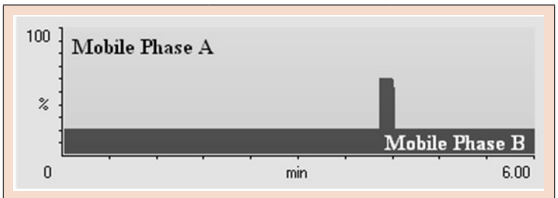
Experiment

Chemical and Reagents

(a) Acetonitrile (C_2H_3N) - MS grade (Merck, Darmstadt, Germany), batch: I764029.
(b) Ammonium formate (NH_4HCO_2) - for HPLC ≥ 99.0% (Fluka, Geneva, Switzerland), batch: BCBN4321V.
(c) Anticoagulant - Vacutainer® tubes with sodium heparin (Becton & Dicskson, New Jersey, NJ, USA).
(d) Formic acid (CH_2O_2) - ACS for Analysis (Sharlau, Barcelona, Spain), batch: 13891502.
(e) Isopropanol (C_3H_8O) - HPLC grade (Sharlau, Barcelona, Spain), batch: 14943109.
(f) Methanol (CH_4O) - MS grade (Sharlau, Barcelona, Spain), batch: 14484518.
(g) N-butylscopolamine bromide - Reference standard 99.8% (British Pharmacopoeia), batch: 3114, view Figure 1A.
(h) N-butylscopolamine-d9 bromide - Reference internal standard 96.0% (Toronto Research Chemicals), batch: 24-GHZ-90-1, view Figure 1B.
(i) Water (H_2O) - Obtained from Milli-Q Elix system (Millipore, Bedford, MA, USA), batch: daily.

Apparatus and Analytical Conditions

The UPLC system utilized was a Waters Acquity H-Class FTN system (Milford, MA, USA). The experiments were carried out on a reversed phase CSH Waters (Milford, MA, USA) Phenyl column (100 X 2.1 mm i.d.; 1.7 mm) and a Phenyl Kit Security Guard Cartridges was used to protect the analytical column. The UPLC system was operated at 40 °C. Mobile phase consisted of two solution, mobile phase A: 2 mM ammonium formate + 0.02% formic acid and mobile phase B: acetonitrile, with mobile phase proportion gradient (Table 1) and 0.4 mL/min as flow rate. The mobile phases were sonicated for 5 minutes under vacuum. The autosampler was set at 20 °C and the injection volume was 1 μL.



Time	Flow (mL/min)	(%) Mobile Phase A	(%) Mobile Phase B
0.00	0.400	80	20
4.00	0.400	80	20
4.01	0.400	40	60
4.20	0.400	40	60
4.21	0.400	80	20
6.00	0.400	80	20

Mobile Phase A: Buffer formate, 2 mM ammonium formate + 0.02% formic acid
Mobile Phase B: Acetonitrile MS grade
Table 1: Mobile Phase Proportion Gradient

Analyte and IS were detected on a mass spectrometer (Xevo TQ-S Atmospheric Pressure Ionization, Waters, Milford, MA, USA) equipped with electrospray ion source, operating in the positive ion mode. MassLynx software version 4.1 was used to control all parameters of LC and MS. Quantitation was performed using selective reaction monitoring (SRM) mode to study precursor → product ion transitions for N-butylscopolamine (m/z 360 → 194) and for N-butylscopolamine-d9 (m/z 369 → 263). Source-dependent parameters optimized were gas 1 (nebulizer gas): 150 L/h; gas 2 (heater gas): 750 L/h flow; ion spray voltage: 1.5 kV; temperature: 450 °C. Compound-dependent parameters set were declustering potential (DP), 50 eV (N-butylscopolamine) and 50

eV (N-butylscopolamine-d9); collision energy (CE), 22 eV (N-butylscopolamine) and 22 eV (N-butylscopolamine-d9). Argon was used as collision-induced dissociation (CID) gas was set at 4.0 mbar and the electron multiplier was set at 515 eV and gain 1.0. The MS was maintained at unit resolution and dwell time was set at 0.3 s.

Preparation of Stock Solutions, Calibration Standards and Quality Control Samples

The stock solutions of N-butylscopolamine and IS were prepared by dissolving accurately weighed amounts in methanol to give a final concentration of 0.02 µg/mL respectively. The standard stock solution of N-butylscopolamine was diluted with methanol to obtain a series of working solutions from 0.25 – 100.00 ng/mL concentration range. All the solutions were stored at -20 °C and were left in room temperature before use. N-butylscopolamine-d9 was diluted with ultrapure water to obtain a IS working solutions with the concentrations of 1000 pg/mL (this solution was prepared daily).

Blank human blood was collected into a heparin vacutainer from healthy and drug-free volunteers. After centrifugation, blank plasma was collected and stored at -20 °C until further used. The calibration standards and Quality Control (QC) samples were prepared by spiking blank plasma with working solution. Calibration standards were made at 5, 40, 80, 150, 200, 300, 400 and 500 pg/mL for N-butylscopolamine. The Quality Control samples were prepared in pooled plasma, with the concentrations of 15 (low), 200 (medium) and 400 pg/mL (high) for N-butylscopolamine. They were divided in aliquots that were stored at -20 °C until further analysis.

Plasma Extraction Procedure

N-butylscopolamine and the IS were extracted from human plasma by solid liquid extraction (SPE) technique. All frozen human plasma samples were thawed at room temperature.

In order to perform sample extraction, 0.6 mL of the sample (human plasma) was added into 2 mL graduated microtube, and 100 µL of 1000 pg/mL N-butylscopolamine-d9 standard solution (diluent: water), 500 µL ultrapure water were added to all microtubes and homogenized for 3 min in a microtube shaker. After the sample was extracted with cartridges (Oasis® HLB µElution Plate, 30 µg, Waters Inc., Ireland), using Positive Pressure-96 Processor under nitrogen gas flow. (Waters Inc., Ireland).

Before starting SPE extraction, the cartridge were activated with 500 µL isopropanol, 500 µL methanol, 500 µL methanol 50% and then equilibrated by 500 µL ultrapure water. Thereafter, the sample was loaded (2 x 550 µL) to pass through the cartridge and then washed with (2 x 500 µL) ultrapure water and (2 x 500 µL) methanol 5%.

Finally, the analyte was eluted with 400 µL methanol 50% into a collection plate 2 mL (Waters Inc., Ireland). The eluate was transferred to a microtube and evaporated under nitrogen gas at 45 °C. The residues were dissolved in 100 µL ultrapure water and homogenized for 2 min in a microtube shaker. The supernatant was transferred into the sample insert vials, and an aliquot of 1 µL was injected into the UHPLC-ESI-MS-MS system.

Bio Analytical Method Validation

The method was validated by the determination of the following parameters: matrix effects, selectivity, carryover, linearity, range, recovery, accuracy, precision, lower limit of quantitation (LLOQ) and stability studies [6,17].

Note: Robustness is not a regulatory requirement, any change in the extractive or instrumental process requires a critical evaluation of the risks involved, requiring at least partial validation. If the risk is unknown, a full validation may be required.

Selectivity and Cross-Talk

The selectivity of the method was evaluated by analyzing four regular, one hyperlipemic, and one hemolyzed blank samples of human plasma obtained from six individuals, and the results were compared with those obtained from a sample extracted containing the desired LLOQ concentration (5 pg/mL) without addition of IS, and an IS sample without analyte. The cross-talk was evaluated with an extracted white sample added with IS, and an upper limit of quantitation (ULOQ, 500 pg/mL) sample without addition of IS.

Additionally, the potential interferences from the common drugs were determined, especially for drugs that could be administered concurrently during execution of the clinical phase. A blank sample was contaminated with a pool of the following drugs: acetaminophen, ibuprofen, caffeine, scopolamine butylbromide, ondansetron, metoclopramide and dipyrone.

Matrix Effect

Matuszewski *et al*., [18] reported that matrix components that coelute with analytes may adversely affect the reproducibility of analyte ionization in a mass spectrometer's ESI source. The matrix effect was checked at quality control lower (QCL) and quality control high (QCH) levels, using 8 different blank plasma lots (including two hemolytic and two lipemic lots). The matrix factor for analyte and IS was calculated in each lot by comparing the peak responses of post-extraction samples (blank extracts spiked after extraction) against those of equivalent aqueous samples, prepared in the ultrapure water. The normalized matrix factor (NMF), equation 1, in each lot was evaluated by comparing the matrix factor of analyte and IS, assuming RSD 15% between samples for a controlled matrix effect.

$$NMF = \frac{(\text{response of the analyte in matrix / response of the analyte in solution})}{(\text{response of the IS in matrix / response of the IS in solution})} \quad \text{(Equation 1)}$$

Carryover

The carryover (memory effect) is the increase of the analyte signal or IS caused by contamination of the equipment or its components by previously analyzed samples To evaluate the parameter was used 3 (three) injections of the same white sample, one before and two after the injection of a processed sample of the ULOQ. The results were compared with the chromatographic response obtained from a processed sample of LIQ. The acceptance criterion adopted was: the interfering peak responses in the analyte retention time should be less than 20% (twenty percent) of the analyte response in the processed LLOQ samples. Peak interfering responses at IS retention time should be less than 5% (five percent) of the IS response.

Calibration Curve

Matrix Match calibration curve were used (plasma sample without analyte or blank sample, a zero sample (a plasma sample processed with IS) and eight concentrations including the LLOQ, ranging from 5 to 500 pg/mL for n-butylscopolamine. A blank sample (matrix sample processed without IS) and a zero sample (matrix sample processed with IS) were used to exclude contamination.

The peak area ratio of the drug to the IS against the respective standard concentrations was used for plotting the graph and the linearity evaluated by a weighted ($1/x^2$) least squares regression analysis. This linear regression was used due to curve fitting, as well as, increasing the accuracy of the low concentration (QC) levels. Linear regression analysis of acyclovir was performed by plotting the peak area ratio (y) against to the inverse of the analyte quadratic concentration ($1/x^2$) in ng/mL; $1/x^2$ weight regression was applied after assessment of heterocedasticity through the evaluation of the residuals (deviations) with and without weighting according to the methodology described by Almeida *et al.*, [19].

The acceptance criteria for each calculated standard concentration was not more than 15% deviation from the nominal value, except for the LLOQ which was set at 20%.

Recovery

Extraction efficiency (recovery), we compared QC samples before and after extraction in 6 replicate samples, using 3 different acyclovir concentrations (12, 375, 750 ng/mL) and 1000 pg/mL for the IS. Recovery was determined by comparing peak areas obtained from the plasma sample, and the standard solution spiked with the blank plasma residue. Results were compared using the mean peak areas of the analytes (analyte and IS) separately, as Equation 1.

Note: Only the absolute recovery of the method, only the extract after the final elution of the sample by the SPE, was evaluated.
Recovery % = (extracted mean / unextracted mean) x 100 (Equation 1)

Accuracy and Precision

The intra-day and inter-day precision and accuracy of the method were assessed by sextuplicate (06 replicates) analysis of five QC samples (low, medium, dilution and high) and the LOQ sample. The calibration standards and quality controls were analysed in three different batches to determine intrabatch and interbatch precision and accuracy. The evaluation of precision was based on the criteria [17] that the deviation of each concentration level should be within ± 15%, except for the LLOQ, that should be within ± 20%. Similarly for accuracy, the mean value should not deviate by ± 15% of the nominal concentration except the LLOQ where it should not deviate by ± 20% of the nominal concentration.

Stability in Plasma

The stability of n-butylscopolamine in human plasma was studied by using two QC standards at low and high concentrations analyzed in six replicates. Stability samples were calculated with a freshly prepared calibration curve. The stability was demonstrated when it was not observed deviation of more than 15% of the average concentrations obtained with respect to the nominal value (except for time zero stability, which for this purpose, three replicates of each solution subjected to stress was diluited in the water and injected into the UPLC/MS/MS system was allowed deviation of 10%).

Time zero stability, was determined before proceeding with the stability study to verify the integrity of the samples. Long-term stability was determined by assaying QC plasma samples after storage at -20 °C for 89 days. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 5 h, which exceeded the routine preparation time of samples. Freeze-thaw stability was investigated after four freeze (-20 °C) thaw (room temperature) cycles. Post preparative stability was assessed by analyzing the extracted QC plasma samples kept in the autosampler at 10 °C for 45 h.

Stock Solution Stability and of Work

The stability of the n-butylscopolamine and IS stock solutions (higher concentration) and work solutions (lower concentration) was evaluated by comparison of results from a solution kept for about 11 days at -20 °C and from a solution that was extemporaneously

prepared. The stability of n-butylescopolamine and IS stock solutions kept 5 h on a rack at room temperature in the presence of ambient light was also tested. For this purpose, three replicates of each solution subjected to stress was diluted in the water and injected into the UPLC/MS/MS system. Results were compared to those from freshly prepared solutions at corresponding concentrations. The samples qualified if the test deviation was within ±10%. Note: the solutions were diluted and analyzed within the detector linearity range (stock solution 1:39999 v/v) (work solution 1:49 v/v).

Bioequivalence Study

Seventy-two healthy volunteers (n: 72), 36 males and 36 females, ranging in age from 18 to 46 years (mean ± S.D., 30.7 ± 7.4 years), in weight from 47.0 to 92.0 kg (mean ± S.D., 69.8 ± 10.6 kg), and in height from 1.55 to 1.85 m (mean ± S.D., 1.67 ± 0.08 m), and within 15% of their ideal body weight, were enrolled. The clinical protocol was approved by the local Ethics Committee and the volunteers gave written informed consent to participate in the study. Volunteers were healthy and had no history of heart, kidneys, neurological or metabolic diseases, no history of drug hypersensitivity, were not undergoing any pharmacological treatment and female volunteers were not pregnant. The study was an open, randomized, two-period, two-group crossover trial with a 1-week washout interval. During the first period, volunteers from group A received 1 tablet with 10 mg dose of Buscopan® (reference product), while volunteers from group B received 1 tablets with 10 mg dose of Scopolamine butylbromide (test product). During the second period, the procedure was repeated on the groups in reverse. The tablets were administered to the volunteers in the morning, after an overnight fast, with 200 mL of water. Volunteers received standard lunch and afternoon snacks, respectively, 5 and 8 h after drug administration. Volunteers did not ingest any alcoholic drink, coffee or other xanthine-containing drinks during the trial. Furthermore, they did not take any other drug, 1 week before the study and during its execution. Blood samples were collected at 0:00 (pre-dose); 0:15; 0:30; 0:45; 1:00; 1:20; 1:40; 2:00; 2:20; 2:40; 3:00; 3:30; 4:00; 4:30; 5:00; 6:00; 8:00; 10:00; 12:00; 14:00; 24:00; 36:00 hours after dosing. The samples were centrifuged and the plasma was stored at -20°C until N-butylscopolamine extraction and quantification.

Statistical Analysis and Pharmacokinetic Parameters

Individual plasma concentration-time curves were constructed the C_{max} and T_{max} values were directly obtained from these curves, and the area under the plasma concentration-time curve from time baseline to the last measurable concentration (AUC_{0-t}) was calculated by a non-compartmental method using the linear trapezoidal rule [21]. From the terminal log-decay phase, the elimination rate constant (k_e) was estimated using linear regression, and the $t_{½}$ was estimated using the following Equation 2:

$t_{½} = \ln 2/k_e$, where ln was defined as the natural logarithm (Equation 2).

The extrapolation of AUC from baseline to infinity ($AUC_{0-\infty}$) was calculated as follows Equation 3, where C_t was the last measurable plasma concentration.

$AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$ (Equation 3).

To assess the bioequivalence between the test and reference formulations, C_{max}, AUC_{0-16h} and $AUC_{0-\infty}$ were considered as the primary variables. Using log-transformed data for these parameters, ANOVA for a 2 x 2 crossover design, was carried out at the 5% significance level ($\alpha = 0.05$).

The 90% confidence intervals (CIs) of the geometric mean ratios (test/reference) of the C_{max}, AUC_{0-16h} and $AUC_{0-\infty}$ were calculated using log-transformed data. The test and the reference formulations were considered bioequivalent if the 90% CIs of these parameters fell within a predetermined range of 80% to 125%. All pharmacokinetic and statistical analyses were performed using WinNonlin Version 5.0.1 (Pharsight, Mountain View, California) and Excel.

Results and Discussion

Method Development

Selection of the I.S.: The best IS, in a LC-MS/MS assay is a deuterated form of the analyte. Although the method is due to the level pg/mL (equivalent ppt), biological matrix and by extraction in solid phase, which contribute greatly to the increase in variability, the deuterated form of the IS was adopted for this project. Another important factor to always use deuterated IS in bio analytical methods is the reduction in the possibility of interference of the IS against possible concomitant drugs or drugs co-administered during the clinical stage to treat adverse events.

Extraction Condition

Methanol and acetonitrile were tested as precipitating agents. In liquid-liquid extraction different kinds of organic solvents and mixtures of solvents, such as ethyl acetate, ethyl ether, dichloromethane, chloroform, methyl tert-butyl ether and hexane were used, resulting in different polarities for extractor phases, but without success. Protein precipitation and liquid-liquid extraction tests are not satisfactory at the required concentration levels (LLOQ 5 pg/mL). It was observed that a presence of matrix components in both tests compete for ionization with the analyte by suppressing the chromatographic response.

The Sunega *et al*., method [22], which uses the liquid-liquid extraction technique using dichloromethane, was also tested, however by using a generic/similar filter to filter the resuspended sample we did not succeed in reproduction.

So the method required a more advanced extraction technique and that a sample cleaning step is possible to eliminate as much as possible matrix components, which are requirements for solid phase extraction. For a choice of extraction cartridge filling taken into account that one molecule has the main functional group amine quaternary (always ionized), and the other end of the apolar molecule (hydrophobic), being ideal for ion exchange or retention in reverse phase (lipophilic).

The tests were performed by ionic solid phase extraction using Oasis MCX is a Mixed-mode, strong Cation-exchange, reversed-phase, water-wettable polymer. Oasis MCX is selective for bases (pKa > 10) and stable in organic solvents. However, after several attempts to improve the recovery of the method, many matrix constituents elute with final extract, compromising the chromatographic analysis.

Oasis HLB is an all purpose, strongly hydrophilic, reversed-phase, water-wettable polymer with a unique Hydrophilic-Lipophilic Balance. Oasis HLB maintains high retention and capacity even if it runs dry after conditioning. This sorbent is ideal for acidic, basic and neutral analytes as it is stable from pH 0-14. In this way, it was the filling model chosen. Due to the large number of samples, we chose to work with extraction in plates and not in cartridges, because the number of samples extracted simultaneously is 96 against 20, increase productivity.

Due to the need for sample concentration and material availability we used the μElution plates, ideal for analyte concentration. However, during the development of the solid phase extraction method was observed several times clogging or decrease in elution after sample loading. This fact was related to the addition of IS with diluent (methanol), which generates a small proportion precipitation, even with pre-treatment of the sample. In this way, IS (work) was diluted in water, even diluent used in the treatment of the sample before its loading. The plate of micro-elution is very useful for concentration of the sample; however, care must be taken during the step of preparation of the sample or washing of the sample after the loading. We recommend that for methods that require stage of concentration, dependent on a large sample volume during loading, has a lot of attention in the pretreatment of the sample stage, or use plates with higher amount of sorbent.

As for the eluents of activating, washing and recovering the analyte were optimized based on the maximum possible elimination of contaminants from the matrix interfering with the method, or that were present in the cartridge filling. The use of iso-propanol was necessary in the activation step to eliminate interference from the filling, while the washing and recovery stages optimized different mixtures by combining methanol and water in order to maintain maximum recovery versus elimination of matrix constituents and interferents.

Optimization of Chromatographic Conditions

The choice of the phenyl column was defined mainly because the functional group was also present in the molecule, favoring the interaction during the elution of the analyte and IS. It was also decided to choose the filling of the column with specific technology for basic compounds, which are capable of extending the shape of the chromatographic peak via unwanted interactions with free silanols present in reverse phase columns. This type of undesired interaction reduces chromatographic separation efficiency as well as sensitivity. As mass spectrometry is not possible to add chemical additives, such as triethylamine for peak shape correction, the choice of a suitable column was essential to achieve the required sensitivity.

The options led to the choice the 1.7 μm Charged Surface Hybrid (CSH ™) particle is Waters third generation hybrid particle technology. Based on Waters Ethylene Bridged Hybrid (BEH) particle technology, CSH particles incorporate a low level surface charge that improves sample loadability and peak asymmetry in low-ionic-strength mobile phases, while maintaining the mechanical and chemical stability inherent in the BEH particle technology. Among the main advantages of choosing this technology for the filling of the column are: superior peak shape for basic compounds, increased loading capacity, rapid column equilibration after changing mobile-phase pH, improved batch-to-batch reproducibility and better stability at low and high pH.

To avoid background contamination, and taking into account that the molecule remains ionized in any pH range (between 1 - 14), the minimum possible chemical additive was used in the mobile phase to promote the desired separation as a function of sensitivity. The main chromatographic challenge was to find a relation in the proportion of mobile phase and mobile flow that would promote the separation of the analytes against the matrix constituents to avoid the strong ionic suppression of the chromatographic response of the analyzed analytes. After elution of the analytes, it was necessary to include a fast gradient of mobile phase ratio, and the main objective was to eliminate matrix constituents that were strongly retained in the column, and interfered in the analysis of later samples.

The coupling of UHPLC with MS/MS detection showed high selectivity because only the ions derived from the analytes of interest were monitored and the comparison of the chromatograms of the blank and spiked human plasma indicated that no interferences was detected from endogenous substances in Figure 3A, or interference of the IS in the mass transition of the analyte in Figure 3B. Typical chromatograms obtained from the proposed UHPLC-MS/MS method, with the resolution of the symmetrical peak corresponding to N-butylscopolamine and IS are shown in Figure 3B. The mean retention times of N-butylscopolamine and IS, 3.54 and 3.43 min (Figure 3C), respectively allow a rapid determination of the drugs, which is an important advantage for the routine analysis at trace level.

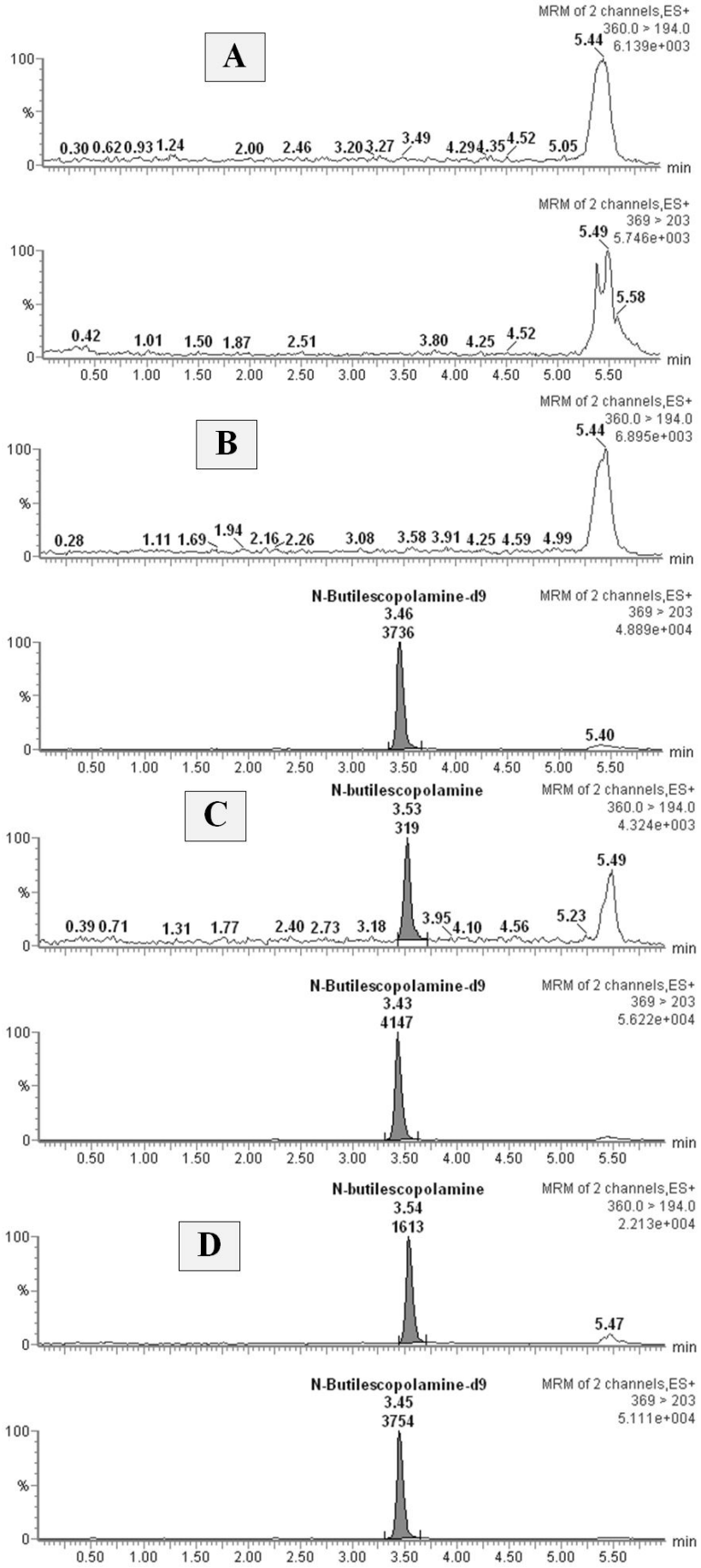


Figure 3: MRM Chromatograms of: **A)** Blank Normal Pooled Human Plasma; **B)** Blank Matrix Chromatogram of Internal Standard; **C)** LLOQ Sample (5 pg/mL) and **D)** A Representative Volunteer Sample Time 2:00 Hours.

Mass spectrometric analysis - MS/MS optimization

In order to detect N-butylscopolamine and N-butylscopolamine-d9 (IS) using MRM, full scan mass spectrum and product ions mass spectrum of each compound was investigated. Under the mass spectrometric conditions, described above, the protonated molecular ions, [M + H] +, were the most abundant ions for both N-butylscopolamine and N-butylscopolamine-d9 (IS) observed at m/z 360 and 369, respectively, in their full-scan mass spectra. It was not verified the presence of adduct ion formation. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rates of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecule of analyte. The mass spectra obtained using SCAN mode showed high abundance fragment ions at m/z 194 and 203 for N-butylscopolamine and N-butylscopolamine-d9, respectively. The product-ion spectra of these compounds are shown in Figure 4 A-D, and the proposed fragmentation of the ion monitored product is presented in Figure 5 A-B. The gas pressure and collision energy were optimized for maximum response of the fragmentation of m/z 194 for analyte. The ion transitions of m/z 360 → 194 for N-butylscopolamine and m/z 309 → 203 for N-butylscopolamine-d9 were chosen for multiple reaction monitoring. For qualitative effect, we evaluated the acquisition of two transitions for each compound resulted in high confidence regarding the identity of the analyte and IS, where ion transitions of m/z 360 → 121 for analyte and m/z 369 → 121 for IS.

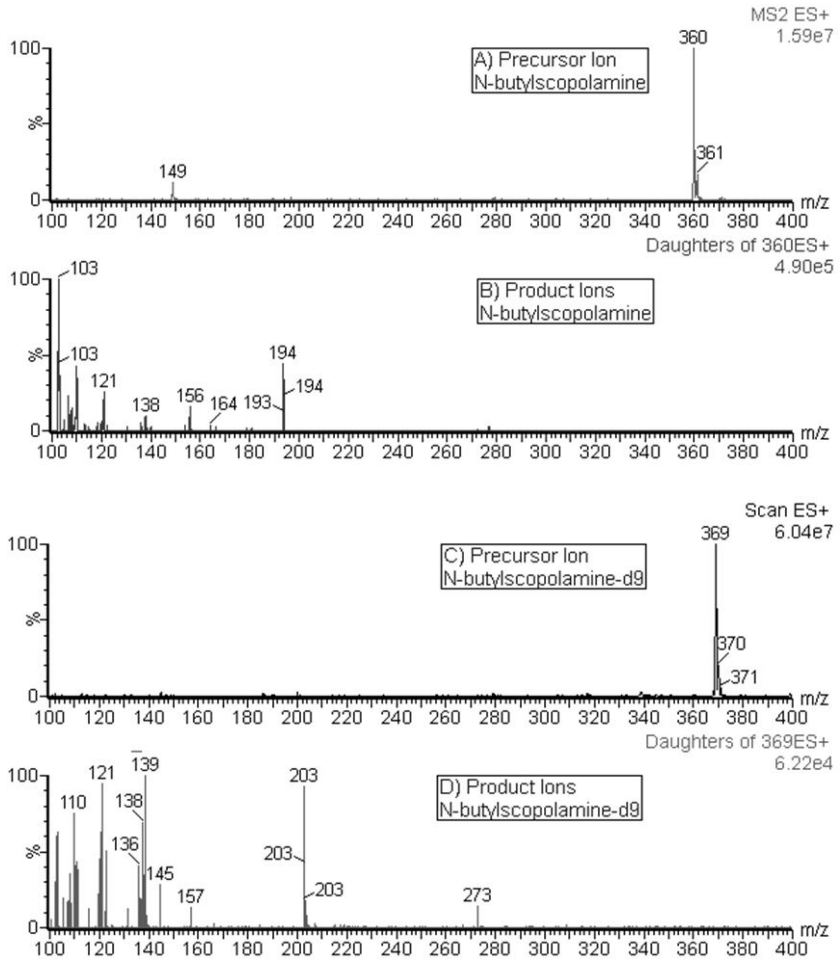


Figure 4: Full Scan Mass Spectra of **(A)** N-butylscopolamine and **(C)** N-butylscopolamine-d9 I.S; Product Ions Spectra of **(B)** N-butylscopolamine and **(D)** N-butylscopolamine-d9 I.S

Bio analytical Validation

Selectivity: No significant interference with the drug or IS was found, Figure 3A. Figures 3B and C show MRM chromatogram channels of non-zero 5 pg/mL N-butylscopolamine sample extracted (with an S/N ≥ 39 for the LOQ) and IS obtained from a regular analytical run. No cross-talk effect was also observed on the monitored mass transitions. The blank plasma sample containing all possible interferents did not detect any peak in the RT of the analyte or IS.

Recovery: The comparison between the unextracted samples, spiked plasma residues, and the extracted sample was done in order to eliminate matrix effects, giving a true recovery (Table 2). Recovery of the IS was also tested concomitantly using the same methodology, with similar average recovery between analyte and IS were greater than 93%. A recovery was considered adequate to obtain required sensitivity.

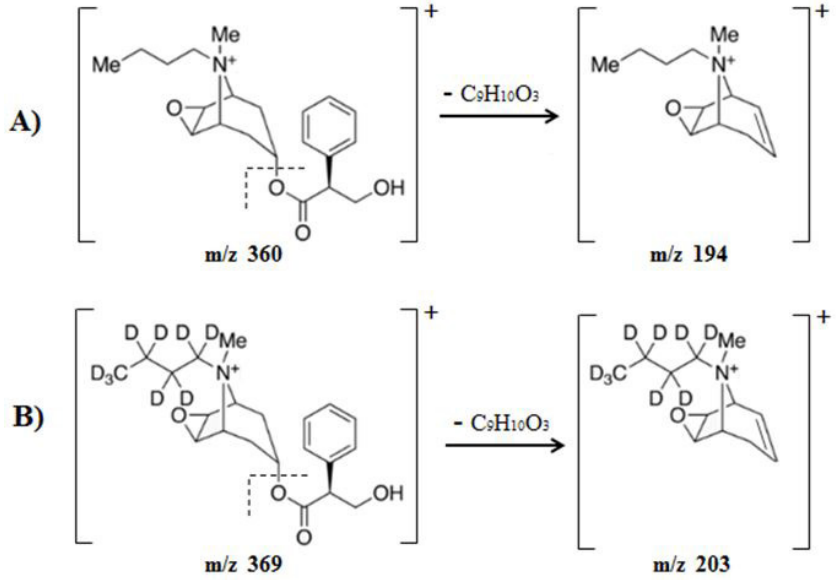


Figure 5: Proposed Fragmentation Pathways of **(A)** N-Butylscopolamine and **(B)** N-Butylscopolamine-d9 I.S.

QC	Recovery (%) (Mean ± RDS %)	
	N-Butylscopolamine[a]	IS[a]
Low	94.1 ± 5.8	93.4 ± 4.5
Medium	93.5 ± 3.6	90.9 ± 3.9
High	97.2 ± 3.2	95.8 ± 2.3
Mean	**94.9 ± 2.0**	**93.4 ± 2.5**

[a]Mean of six replicates
QC: 15 (low), 200 (medium), 400 pg/mL (high).
Table 2: Recovery of N-Butylscopolamine and N-Butylscopolamine-d9 (Is) After the Extraction Procedure

Carryover: The sample carryover comparator showed less than 5.0% for the analyte and less than 0.2% for the IS.

Matrix effect: The normalized matrix factor found was 1.055 ± 0.147 with RSD = 13.9% of the mean peak areas of N-butylscopolamine and IS, indicating control difference in ionization efficiency using different plasma samples. Besides, the results were higher than 93%, suggesting that the ion suppression by endogenous components was not interfering in the repeatability of the method.

Analytical curve (Linearity): The linearity of the curves was verified over the LOQs and found to comply the least squares regression equation y = (0.0069 ± 0.0004)x + (0.0145 ± 0.0103), with a linear correlation coefficient, (r2) = 0.9951 ± 0.0009 (mean n = 3). As the error variance is not constant throughout the quantification range of the analytical method, we used the weighting that presented the smallest value for sum of the relative errors of the nominal values of the standards off calibration versus its values obtained by the curve equation. The method used polynomial type is linear, point of origin is excluded, evaluating fit weighting linearity: without (none), 1/x and 1/x2 axis transformation, obtaining the following results none 779, 1/x 414 and 1/x2 368. The results show that the weighting of 1/x2 is the most adequate for the application of the bioanalytical method.

Precision and accuracy: The results of validation demonstrated good precision and accuracy (Table 3), in the linearized range presented, its RSD values were between 2.1% and 13.9% for precision and among 101.9-108.3% for accuracy in relation to the nominal concentration of the analyte, in accordance with regulatory guidelines.

Quality control (QC)	Intra-day		Inter-day	
	RSD (%)	Accuracy (%)	RSD (%)	Bias[a] (%)
LLQ	13.9	108.3	6.9	108.0
Low	8.1	105.6	3.2	101.9
Medium	3.5	103.7	4.6	104.3
Dilution	2.5	102.4	2.1	103.3
High	3.4	106.6	3.8	103.3

[a]Bias = [(measured concentration - nominal concentration) / nominal concentration] x 100.
QC: 5 (LLQ) 15 (low), 200 (medium), 400 (high) and 600 pg/mL (Dilution).
Table 3: Intra-Day and Inter-Day Precision and Accuracy for the Determination of N-Butylscopolamine in Human Plasma

Stability in plasma: As shown in Table 4, the plasma samples were stable for at least 89 days at -20 °C (long term) and also after four freeze-thaw cycles demonstrating that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

N-butylscopolamine is stable in plasma for up to 6h at room temperature (short term). The results demonstrated that extracted samples could be analysed after keeping in the autosampler for at least 45h at control temperature 6 °C with an acceptable precision and accuracy.

Stability	QC	RSD (%)	Bias (%)[a]
Zero time	**Low**	7.9	-4.5
	High	3.4	6.2
Autosampler 45 hours	**Low**	4.4	4.9
	High	4.5	-0.2
Four freeze-thaw cycles	**Low**	6.8	-6.3
	High	9.0	-6.1
Long term[b] 89 days	**Low**	9.6	-9.8
	High	7.4	-6.3
Short term[c] 6 hours	**Low**	9.3	-3.8
	High	5.7	-4.8

[a]Bias = (measured concentration - nominal concentration / nominal concentration) x 100
[b]Long term = stability for at least 89 days at -20 °C
[c]Short term = stability in neat plasma for up to 6h at room temperature
QC: 15 (low) and 400 pg/mL (high).
Table 4: Summary Of Stability of N-Butylscopolamine in Human Plasma

Stability in solution: The stability of the stock and methanol working solutions obtained were 11 days stored in a freezer at -20 °C and 5 hours in a bench at room temperature. It was observed deviations below 5% of the stressed samples when compared to freshly prepared samples (Figure 5).

Application of the bioanalytical method and statistical analysis and pharmacokinetic parameters

The validated method was successfully used to quantify n-butylscopolamine in human plasma samples in a bioequivalence study. Average concentration versus time curves after administration of Buscopan® (reference product) and scopolamine butylbromide (test product) to 72 healthy volunteers are shown in Figure 6. Table 5 shows the average values of calculated pharmacokinetic parameters and descriptive statistics of the results obtained.

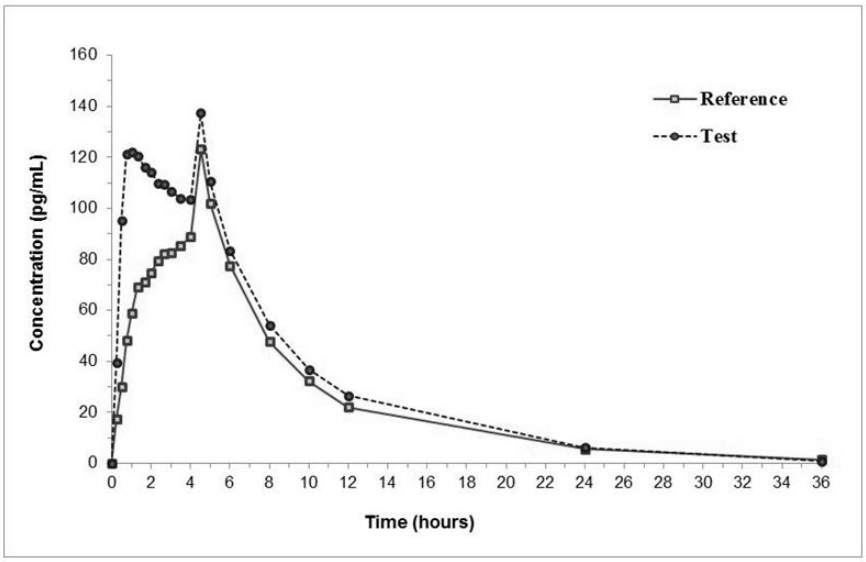


Figure 6: Average Values of Plasmatic Concentrations (pg/ml) After Administration of Reference Product (Buscopan, 10 mg) and Test Product (N-Butylscopolamine Bromide, 10 mg) to 72 Healthy Volunteers

FORMULATION	PARAMETER	$T_{máx}$ (h)	$C_{máx}$ (pg/mL)	K_{el} (1/h)	$T_{1/2}$ (h)	ASC_{0-t} (h x pg/mL)	ASC_{0-inf} (h x pg/mL)
TEST	**Average**	2.90	187.74	0.21	4.28	1103.66	1226.87
	Stand. Dev.	1.80	184.40	0.12	2.06	1111.56	1149.52
	Minimum	0.25	25.03	0.07	0.94	76.16	170.52
	Maximum	5.00	931.45	0.74	9.68	4106.43	4219.49
	RSD (%)	62.25	98.22	56.35	48.01	100.72	93.70

FORMULATION	PARAMETER	$T_{máx}$ (h)	$C_{máx}$ (pg/mL)	K_{el} (1/h)	$T_{1/2}$ (h)	ASC_{0-t} (h x pg/mL)	ASC_{0-inf} (h x pg/mL)
REFERENCE	Average	3.71	144.56	0.21	5.14	875.73	1073.65
	Stand. Dev.	1.51	140.23	0.13	3.66	961.67	1021.71
	Minimum	0.25	15.35	0.04	0.89	51.68	120.58
	Maximum	5.00	774.43	0.78	19.17	4631.16	4730.58
	RSD (%)	40.74	97.00	65.08	71.23	109.81	95.16

Table 5: Primary And Secondary Pharmacokinetic Parameters of N-Butylscopolamine, Calculated on the Basis of Nominal Time, For Formulation Test and Reference

All of the 90% CIs of the geometric mean ratios of the pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) were found to be within the predetermined range of bioequivalence are between 80% and 125. The reference and test formulation were considered not bioequivalent, according to the results of the statistical analysis because it is higher than the limits established for all analyzed pharmacokinetic parameters.

Conclusion

A sensitive and fast UHPLC-ESI-MS/MS method for the determination of N-butylscopolamine in human plasma was developed and validated. This method involves a solid-phase extraction procedure, using N-butylscolamine-d9 as IS. The results of the validation studies show that the optimized method possesses selectivity, sensitivity, linearity, precision and accuracy over the entire range of significant therapeutic plasma concentrations. Moreover, the proposed method was successfully applied to a pharmacokinetic study of healthy human volunteers and the results showed that the two scopolamine butylbromide formulations studied are not bioequivalent.

Acknowledgments

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References

1. Drugbank (2017) Butylscopolamine, Accession number: DB09300 (DB11535), Canada.

2. Chen H, Chen Y, Du P, Han F (2008) Liquid Chromatography–Electrospray Ionization Ion Trap Mass Spectrometry for Analysis of in Vivo and in Vitro Metabolites of Scopolamine in Rats. J Chromatogr Sci 46: 74-80.

3. Cherkaoui S, Mateus L, Christen P, Veuthey JL (1998) Validated capillary electrophoresis method for the determination of atropine and scopolamine derivatives in pharmaceutical formulations. J Pharm Biomed Anal 17: 1167-76.

4. Renner UD, Oertel R, Kirch W (2005) Pharmacokinetics and Pharmacodynamics in Clinical Use of Scopolamine. Ther Drug Monit 27: 655-65.

5. Ebert U, Grossmann M, Oertel R, Gramatté T, Kirch W (2001) Pharmacokinetic-Pharmacodynamic Modeling of the Electroencephalogram Effects of Scopolamine in Healthy Volunteers. J Clin Pharmacol 41: 51-60.

6. Favreto WAJ, Pinto AMP, Manfio JL, Fiametti KG, Percio M.F, et al. (2012) Development and validation of a UPLC-ESI-MS/MS method for the determination of N-butylscopolamine in human plasma: Application to a bioequivalence study. Drug Test Anal 4: 215-21.

7. Manfio JL, Santos MB, Favreto WA, Hoffmann FI, Mertin AC (2009) Validation of a Liquid Chromatographic/TandemMass Spectrometric Method for the Determination of Scopolamine Butylbromide in Human Plasma: Application of the Method to a Bioequivalence Study. J AOAC Int 92: 1366-72.

8. Mandal S, Naqvi AA, Thakur RS (1991) Simultaneous determination of atropine and scopolamine in plants by mixed-column high performance liquid chromatography. Phytochem Anal 2: 208-10.

9. Kursinszki L, Hank H, László I, Szoke E (2005) Simultaneous analysis of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine in Solanaceous hairy roots by reversed-phase high-performance liquid chromatography. J Chromatogr A 14: 32-9.

10. Ye N, Zhu R, Gu X, Zou H (2001) Determination of scopolamine, atropine and anisodamine in Flos daturae by capillary electrophoresis. Biomed Chromatogr 15: 509-12.

11. Mateus L, Cherkaoui S, Christen P, Oksman-Caldentey K (2000) Simultaneous determination of scopolamine, hyoscyamine and littorine in plants and different hairy root clones of Hyoscyamus muticus by micellar electrokinetic chromatography. Phytochem 54: 517-23.

12. Oertel R, Richter K, Ebert U, Kirch W (1996) Determination of scopolamine in human serum by gas chromatography-ion trap tandem mass spectrometry. J Chromatogr B 682: 259-64.

13. Oertel R, Richter K, Ebert U, Kirch W (2001) Determination of scopolamine in human serum and microdialysis samples by liquid chromatography–tandem mass spectrometry. J Chromatogr B 750: 121-8.

14. Xu A, Havel J, Linderholm K, Hulse J (1995) Development and validation of an LC/MS/MS method for the determination of L-hyoscyamine in human plasma. J Pharm Biomed Anal 14: 33-42.

15. Abd-Taliba N, Mohd-Setapara SH, Khamisb AK (2014) The Benefits and Limitations of Methods Development in Solid Phase Extraction: Mini Review. Jurnal Teknologi 69: 69-72.

16. Brouwer VD, Storozhenko S, Stove CP, Daele JV, Straeten DVD (2010) Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the sensitive determination of folates in rice. J Chromatogr B 878: 509-13.

17. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) (2001) Bioanalytical Method Validation, Guidance for Industry, USA.

18. Matuszewski BK, Constanzer ML, Chavez-Eng CM (1998) Matrix Effect in Quantitative LC/MS/MS Analyses of Biological Fluids: A Method for Determination of Finasteride in Human Plasma at Picogram Per Milliliter Concentrations. Anal Chem 70: 882-9.

19. Almeida AM, Castel-Branco MM, Falcão AC (2002) Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. J Chromatogr B 774: 215-22.

20. Abbott RW, Townshend A, Gill R (1987) Determination of morphine in body fluids by high-performance liquid chromatography with chemiluminescence detection. Anal 112: 397-406.

21. Chow SC, Liu JP (2000) Design and analysis of bioavailability and bioequivalence studies (2nd Edn) Marcel Dekker Inc., New York, USA.

22. Suenaga EM, Val LC, Tominaga M, Filho JHS, Soares G (2016) A fast and sensitive UHPLC–MS/MS method for the determination of N-butylscopolamine in human plasma: application in a bioequivalence study. Biomed Chromatogr 31: 10.1002/bmc.3823