Development of a new ultra-high performance liquid chromatography––tandem mass spectrometry method for determination of ambroxol hydrochloride in serum with pharmacokinetic application

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Abstract
Ambroxol hydrochloride is an expectorant agent, successfully applied in mucolytic therapy for acute and chronic bronchopulmonary diseases. The drug regulates not only mucus secretion but also showed antioxidant, anti-inflammatory and local anesthetic properties. To supplement the pharmacokinetic and toxicological studies of ambroxol, a rapid ultra-high performance liquid chromatography–tandem mass spectrometry method for the quantitation of ambroxol in rabbit serum was developed. A validation of the method was performed as per the ICH guidelines for the validation of bioanalytical methods. The chromatographic separation was achieved in a submicron Kinetex RP-C$_{18}$ column (2.1 mm×50 mm, 1.3 μm) using the no buffer mobile phase. The ESI mass spectrometry in the MRM mode was used with a typical transitions *m/z* 378.9→263.8 for ambroxol and *m/z* 455.2→165.0 for IS. Linearity was determined with an average coefficient of determination >0.999 over the dynamic range from 0.5–200 ng/mL with *LOD* and *LOQ* of 0.25 and 0.5 ng/mL, respectively. The results of the intra- and inter-day precision and accuracy determined in different days were all found to be within the acceptable limits ±15%. The present method was successfully applied to pharmacokinetic study in the rabbits after a single oral dose administration.

Keywords: ambroxol hydrochloride, UHPLC–MS/MS, serum, pharmacokinetics.

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During the last three decades, the ambroxol hydrochloride has been widely used as a mucoregulatory agent in different pharmaceutical formulations. Ambroxol is the drug that is used as a mucolytic agent alone and in combination with antibiotics. The pharmacology of ambroxol is apparently complicated considering that it is known several mechanisms of its action. In addition to the well-known expulsion of the mucus and clearance facilitation, the ambroxol also facilitates productive cough and had shown antioxidant, anti-inflammatory, analgesic, local anesthetic, antiviral and antibacterial effects [1–5]. Ambroxol is applied in mucolytic therapy of acute and chronic diseases associated with increased production and disruption of the formation and transportation of mucus (mucociliary clearance). The increase in the fluid secretion and mucociliary clearance facilitate the expectoration [6]. Local anesthetic effect of ambroxol observed in the rabbit eye model is interpreted as the ability to block sodium channels [7]. *In vitro* studies have shown that ambroxol blocks the cloned neuronal sodium channel, and this binding was reversible and concentration-dependent [8]. It has been shown that the ambroxol *in vitro* also significantly reduces the release of the cytokines from the blood, as well as the mononuclear and polymorphonuclear cells associated with the tissue [9]. These pharmacological properties are consistent with the accompanying observations in the clinical trials for efficacy of the ambroxol treatment of upper respiratory tract symptoms, which leads to the relief of pain and discomfort associated with pain in the region of the ear-nose-trachea [10]. Ambroxol is often combined with antibiotics (amoxicillin, doxycycline and erythromycin), even in a single tablet or syrup, because it increases the concentration of antibiotics in bronchopulmonary secretions and sputum [11].

Pharmacokinetics of the ambroxol in humans is well documented. After oral administration, the ambroxol is rapidly and completely absorbed, and the maximum plasma concentration (c_{max}) is reached within two hours after administration of immediate-release tablets. Distribution of the ambroxol is rapid, with the largest concentration achieved in the lungs. It is highly bound to binds to the plasma proteins (70–80%) [12]. Approximately 30% of a given oral dose is eliminated by the first-pass metabolism. The ambroxol is metabolized mostly in the liver by glucuronidation and decomposition of the dibromantranilic acid in the pre-

sence of CYP3A4. The ambroxol is eliminated with a half-life of 10 h [13].

Several chromatographic and non-chromatographic methods have been developed for the determination of the ambroxol in different pharmaceutical formulations (syrup, tablets, lozenges) and biological samples. Using electrochemical properties of the ambroxol, non-chromatographic methods such as amperometric with carbon film resistor electrodes [14] and cyclic voltammetry with boron-doped diamond electrode method [15] were reported, both used in the pharmaceutical research and development. The capillary gas-liquid chromatography [16], capillary zone electrophoresis and fluorescent detection [17,18] with reached limit of detection of 2 and 8 ng/ml, were successfully applied for the quantification of ambroxol in human plasma. The most employed chromatographic method for this purpose is a high-performance LC with UV/Vis spectrophotometric [19,20] and photodiode array detection [21]. The separation LC chromatographic methods are usually used for the ambroxol determination in the pharmaceuticals as well as in human, rat or dog plasma [22–26]. Using a high mobile phase flow rate (>1 mL//min, except EC 0.2 mL/min), long chromatographic run time (>15 min) and large sample injection volume (>50 mL) to reach a low quantification limits (>10 ng/ml), make this methods unsuitable for extensive and sensitive validation procedures. To analyze ambroxol in the biological specimens several highly sensitive and precise LC–MS and LC–MS/MS methods have also been reported. The achieved limits of detection were in the range of 0.2–1.0 ng/mL with a sample volume of 0.5–1 mL [27–30]. The presented MS methods have already enabled pharmacokinetic studies of ambroxol in the human volunteers [31,32].

The purpose of this study was to develop an ultra--high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the simple determination of the ambroxol in rabbit serum. Further method application should contribute to a new pharmaceutical and pharmacokinetic studies of the ambroxol in animals and humans.

The experimental procedure included evaluation of the optimal parameters for sample preparation, chromatographic conditions and MS/MS detection. Through the validation process, several significant advances were achieved: 1) a chromatographic separation with a shorter run time in 1.3 μm submicron column, 2) a high sensitivity with a very small amount of sample and injection volume and 3) a high purity of the sample extracts obtained with a solid-phase extraction procedure which has provided the MS/MS measurements without the interferences. The developed UHPLC––MS/MS method was successfully implemented in a pharmacokinetic study of the ambroxol in rabbits.

MATERIAL AND METHODS

Chemicals and reagents

Ambroxol hydrochloride, standard and verapamil hydrochloride, internal standard (IS) were provided by Fluka (Dorset, UK). Methanol (MeOH), acetonitrile (ACN), formic acid (gradient grade for LCMS) were purchased from Promochem (Wesel, Germany), while ammonium hydroxide (NH_4OH), hydrochloric acid (HCl) and potassium hydrogen phosphate (analytical grade) were purchased from Sigma–Aldrich (Dorset, UK). Ultra-pure Optima™ LC/MS water was obtained from Fisher Chemical (Waltham, MA, USA). Strata™-X-C polymeric strong cation exchange solid phase extraction cartridges (30 mg, 1 mL) were obtained from Phenomenex (Torrance, CA, USA).

Instrumentation and chromatographic conditions

The data were collected with a liquid chromatography–tandem quadrupole Shimadzu LCMS-8030 mass spectrometer equipped with the atmospheric pressure chemical ionization (APCI), the electrospray ionization (ESI) and the dual ion source (DUIS) mode. The MS detection was carried out in a positive (+) electrospray ionization mode with the multiple reaction monitoring (MRM) of the transitions for each compound. An automated wizard was used to perform the MRM optimization and determination of the optimum ion optics voltages and collision energies, as well as the highest intensity of the product ions. The desolvation line (DL) and heater block (HB) temperatures were 270 and 400 °C, respectively. Argon was used as collision gas (230 kPa) and nitrogen as the nebulizer and dry gas at flow rates of 3 and 15 L/min. The selected MRM transitions were: m/z 378.9→263.8 for the ambroxol and m/z 455.2→165.0 for the internal standard (verapamil). The compounds were identified by matching the retention times and mass spectral data with those of the calibration standards while the method of internal standard was used for the quantification of ambroxol in all samples.

The Shimadzu UHPLC system with binary pumps (LC-30AD, Nexera), column oven (CTO-30A, Nexera), degasser (DGU-20A, Prominence), communication interface (CBM-20A, Prominence) and autosampler (SIL-30AC, Nexera) were used. Optimal chromatographic efficiency was achieved at reversed-phase column Kinetex™ C_{18}-column (2.1 mm×50 mm, 1.3 μm, Phenomenex, USA) with an isocratic mobile phase methanol––0.01% formic acid aqueous solution (90:10, *V/V*) at a flow rate of 0.3 ml/min. Before the use, the mobile phase was filtered through 0.22 μm membrane filter paper. The operating temperature of the column was set to 40 °C and the cooler of autosampler at 15 °C. The

measured signal was monitored and processed using Lab Solution software.

Preparation of stock and standard solutions

Stock solutions of the ambroxol and internal standard were prepared at a concentration of 1 mg/mL in methanol and were serially diluted to get working standard solutions for the preparation of calibration curves. The stock solutions were stored at –20 °C and used up to three weeks for preparation while working dilutions were freshly made when needed.

For the preparation of calibrators (CCs) and quality control samples (QC$_S$), the standard solutions of ambroxol and internal standard (20 ng/mL) were spiked to 250 µl of blank serum in 10 ml glass tube, within calibration limit. Calibrators for this method were prepared at the following concentrations: 0.5, 2, 5, 10, 20, 50, 100, 150 and 200 ng/mL and the quality control samples at levels of lower limit of quantitation (*LLOQ*): 0.5 ng/mL, low (*LQC*): 20 ng/mL, medium (*MQC*): 100 ng/mL and high (*HQC*): 200 ng/mL. All samples were stored at 4 °C and analyzed during the same day.

Sample preparation

All calibrators (CCs), quality controls (QC$_S$), and serum samples were prepared in the same manner using polymeric strong cation exchange solid phase extraction cartridges (Strata™-X-C). Summarily, after stirring with the vortex mixer, the 250 µl diluted, pH adjusted (1:1 phosphate buffer, pH 6.7/25 °C) and added IS (20 ng/mL) samples were loaded onto cartridges. The cartridges were previously conditioned and equilibrate with 1 ml of methanol and 1 ml of 0.1 M HCl. Washing step was also performed using 1 ml of 0.1 M HCl. The SPE tubes were then dried under full vacuum for 5 min and eluted with 2×500 µl 90:10 MeOH/ACN containing 5% of concentrated NH$_4$OH solution applying a light vacuum. The residue was evaporated to dryness under a stream of nitrogen at 40 °C and dissolved in 250 µl of the mobile phase. After filtration (0.22 µm syringe filter), 2 µl was injected into the UHPLC–MS/MS system.

Method validation

The validation of the UHPLC–MS/MS method for the determination of ambroxol in rabbit serum was performed in accordance with ICH Q2A/Q2B guidelines [33] and included the following parameters: sensitivity, linearity, accuracy and precision, specificity, stability tests, carry-over and matrix effects.

Extraction recovery

The absolute recovery of ambroxol from serum by SPE was determined for three different standard concentrations by spiking the analyte into the blank serum. The extraction recovery was calculated by comparing the peak areas extracted from spiked samples with those of the same quantities added to the mobile phase. The recovery of three QCs (20, 100 and 200 ng/mL) concentrations in serum was repeated six times.

Animal experiment

Male *Oryctolagus cuniculus* rabbits (3.0–3.5 kg) were bred and housed in the Laboratory Animal Services Centre. All experiments were conducted to comply with the Good Laboratory Practice (GLP, 40 CFR Part 160: U.S. EPA) regulations [34] and approved by the Animal Research Ethics Committee. The animals were housed with free access to food and water in a temperature controlled room (23±2 °C) with a 12 h light-dark cycle. In the animal study, rabbits were fasted overnight with free access to the water before administration of the ambroxol. After administration of a single tablet containing 30 mg of ambroxol directly to each rabbit's stomach, 1 ml of the blood samples were collected from the auricular marginal vein at 0.25, 0.5, 1, 2, 4, 6 and 24 h time-points. Serums were separated by the centrifugation 7000×*g* for 10 min at room temperature and then stored at –70 °C prior to analysis. Aliquots of 0.25 ml serum samples were processed with defined SPE method and analyzed for the ambroxol content.

Pharmacokinetic and statistical analysis

Statistical analyzes of the validation parameters were performed by using Microsoft Office Excell 2013. Each value was expressed as the mean ±*SD* and the *RSD* (%). The pharmacokinetic parameters for ambroxol were calculated using a non-compartmental model of the Thermo Scientific™ Kinetica software for PK/PD data analysis (version 5.0, Thermo Electron Corporation, USA).

RESULTS AND DISCUSSION

Method development

Chromatographic parameters

All the LC chromatographic conditions were optimized to achieve the symmetric peak shapes, good resolution and short run time. Three types of the columns C-18 (1.3, 1.7 and 2.6 µm) were tested and finally a reversed-phase Kinetex RP-C-18 column (2.1 mm×50 mm; 1.3 µm, Phenomenex, USA) with security guard C-8 column (Kinetex, Phenomenex, USA) was applied. The stationary UHPLC phase with 1.3 µm particle size, which use has not been recorded in the literature data so far, enabled the separation of the analytes with high sensitivity in a very short chromatographic run time. Using data from published works, the various concentrations of formic buffer solutions in the methanol and acetonitrile were tested as a mobile phase

[27,28,31,32]. Finally, the modified bicomponent mixture of 90% methanol and 10% formic acid solution in water (0.01%), was found as appropriate for the chromatographic separation. The retention times of the ambroxol and IS (verapamil) were 0.79 and 0.91 min, respectively, with the total chromatographic run time of 1.5 min.

Spectrometric parameters

The electrospray MS detection was performed in the full-scan and the multiple reaction ion monitoring mode (MRM). First, the full tuning of tandem quadrupole control parameters was performed to obtain the accurate ion mass calibration and the measurement of mass spectrums with high sensitivity and resolution. Then, the *m/z* values of the precursor (Q1 quadrupole) ions, product (Q3 quadrupole) ions and the voltages of collision energies were input into the MRM event table and auto-optimized for the each compound. The MS detecting conditions were operated according to the MS signal response of the target compound. The full-scan mode results showed that the positive mode of ionization was much more sensitive than the negative mode with a typical protonated MS ion mass spectra [M+H]$^+$; *m/z* 378.9 for ambroxol and IS *m/z* 455.2 at 100 mg/L. Furthermore, the MRM mode was used for the quantification of analytes with the appropriate transitions and run time segments; *m/z* 378.9→263.8 (0.4–1.2 min) and *m/z* 455.2→165.0 (0.5–1.5 min) for ambroxol and IS, respectively, as present Figure 1. All applied transitions were according to the literature data [30]. The total run time was 1.5 min with a dwell/ /pause time of 200/1 and 100/2 ms for each compound. The MRM ion chromatograms of a blank, zero, ambroxol standard at 20 ng/mL and rabbit serum sample were presented in Figure 2. Other optimized MS conditions were as follow: interface voltage: 4.5 kV; conversion dynode voltage: 6 kV; detector: −1.92 kV

and exit lens: −4.0 V. The collision energy was optimized and applied to −20 and −25 V; Q1 precursor bias/Q3 product bias: −28/−18 and −11/−10 V for AMB and IS, respectively.

Sample preparation

Sample preparation with the high recovery and purity without matrix interferences is the key to successful MS/MS determination. The most common problems faced in the extraction of an analyte from biological matrices are solubility and natural organic substances in the samples that reduce extraction efficiency and hinder detection [35]. The MS/MS analysis of low purity biological samples can cause ion suppression, elevated background, and other negative matrix effects. Results may significantly influence on the lack of selectivity. Moreover, the use of columns with submicron particles (<2 μm) which leads to very high back pressures require very pure solvents and sample extracts. The residual matrix components also limit the lifespan of these columns much more than they limit the lifespan of conventional columns.

According the literature data, extraction techniques published for this purpose are protein precipitation (PPE) and liquid–liquid extraction (LLE) with the extraction recovery 70–90% [27–32]. In order to achieve better extracts purity and recovery with a smaller sample volume, in this study was developed a four-step solid phase extraction (SPE) procedure. Considering the solubility and pK_a values of ambroxol and verapamil (IS), the strong cation exchange cartridges (Strata^{TM} – X-C) showed the satisfactory results and therefore used in further proceedings of the sample preparations. The relative extraction efficiency (*RE*) was determined by comparing peak areas obtained from extracted serum samples with those found by extracting blank matrices through the extraction procedure and spiking with a known amount of the ambroxol. The examined extract
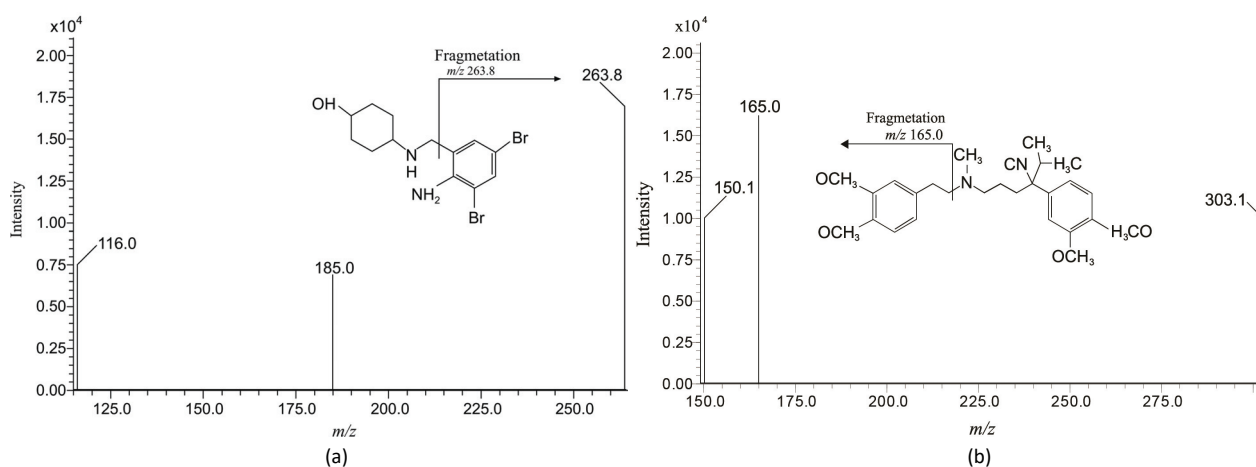

Figure 1. Typical product MS ion mass spectra of the protonated [M+H]$^+$ for: a) ambroxol, m/z 378.9→263.8, and b) internal standard (verapamil), m/z 455.2→165.0, in MRM mode with structural fragmentation.
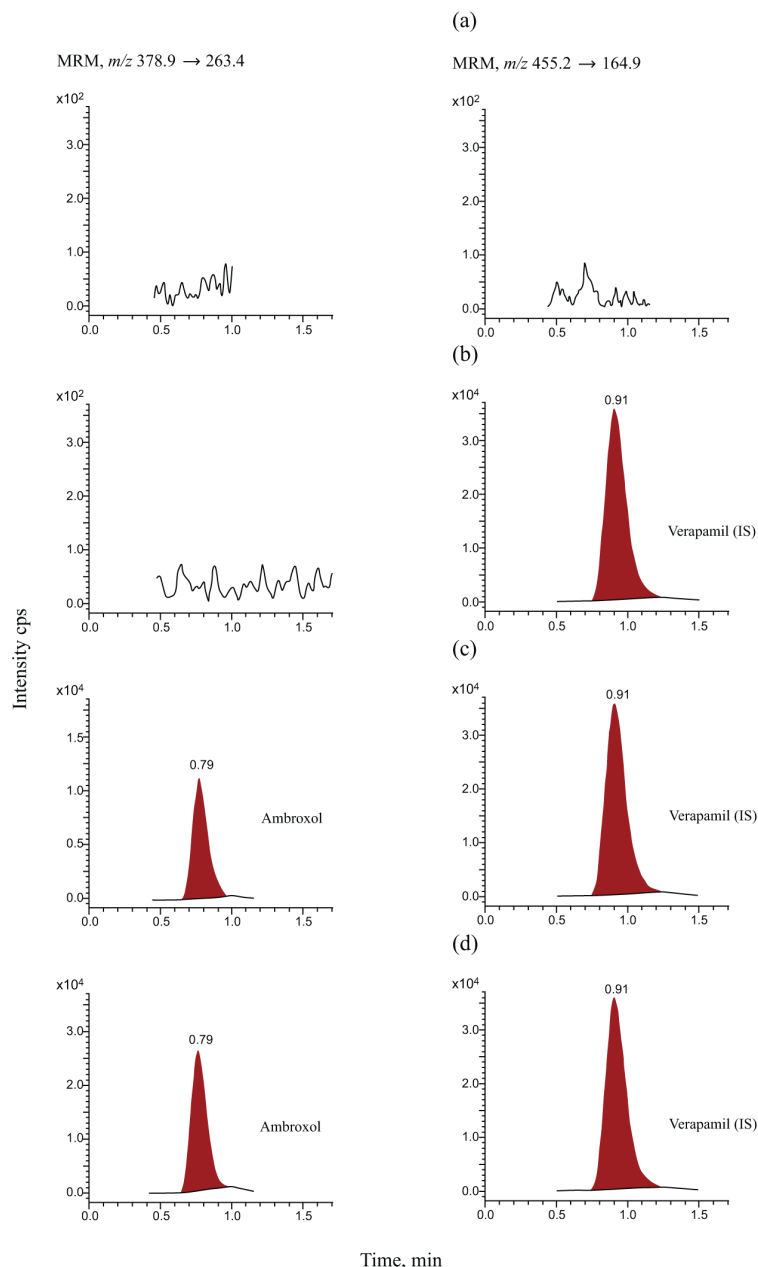
Figure 2. MRM ion chromatogram of: a) sample without internal standard (blank), b) sample with internal standard (zero), c) ambroxol at 20 ng/mL (m/z 378.9→263.8) and d) rabbit serum at 4 h after administration of 30 mg single oral dose of ambroxol.

efficiency was evaluated over three concentration levels, 20, 100 and 200 ng/mL, with the mean relative recovery of 85.96±9.05%.

Method validation

Linearity-calibration curve

Linearity was assessed by three calibration curves over nine-point calibration with a concentration range from 0.5–200 ng/mL, without zero points. The calibration samples were injected six times (n = 6) on three different days. The high correlation coefficients (r^2 > > 0.99) for all calibration curves indicated good correlations. The corresponding average regression line was:

y = (0.0125±0.0003)x − (0.0013±0.0008), where y is the peak ratio of ambroxol/IS and x the concentration of the ambroxol. A least square regression model was fitted to each standard curve with the mean value of the correlation coefficient (r^2) of 0.9995 and standard deviation of 0.00045. Under certain assumptions required for valid linear regression, reliable results were obtained, and suitable prediction function was defined. First, the linear relationship between the concentrations and the peak areas is evident from the corresponding scatter plots. No significant "outliers" were noticed. Further, homoscedasticity of the error terms was established using Hartley's test, and the

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independence of the residuals was proved by using the Durbin-Watson autocorrelation test. Also, the normality of the error terms of the regression line was not violated as Shapiro–Wilk test showed. The "lack of the fit" test was also performed and showed the high coefficients of the correlation ($r > 0.999$) between the two variables. The value of $p < 0.001$ indicated that the regression model statistically significantly predicts the outcome variable, *i.e.*, is a good fit for the data.

Sensitivity

The lowest standard on the calibration curves, 0.5 ng/mL was accepted as the *LLOQ* with accuracy criteria within ±20% of the signal to noise ratio $S/N \geq 1:10$. The accepted quantitation limit showed the satisfactory levels of the published LC–MS/MS methods for determination of ambroxol in human plasma [30]. The accuracy of the other CC samples at all concentration levels were within the acceptable RSD range of 1.63–10.72%. The *LOD*s were set at three times the noise level of the baseline in the chromatogram ($S/N \geq 1:3$) and the concentration of 0.25 ng/mL was confirmed as a limit of detection for the method.

Accuracy and precision (repeatability)

To evaluate accuracy and precision of the method, the six replicates at four concentration levels (*LLOQ*: 0.5 ng/mL; low: 20 ng/mL, medium: 100 ng/mL and high: 200 ng/mL) were obtained with one batch for intra-day and over the 3 days for inter-day. Acceptance criteria were $RSD \leq 20\%$ for *LLOQ* and low concentrations and $\leq 15\%$ for medium and high concentrations. The mean intra-day *RSD* (%) was 3.38% (range 0.69––5.74%) and the corresponding value for inter-day was 5.43% (range 3.18–7.77%). The mean intra-day accuracy was 107.6% (range 100.3–118.6%) and corresponding value for inter-day was 106.7% (range 99.9––110.1%). The satisfactory measured results indicated that the established method is high reproducible and repeatable, as listed in Table 1.

Matrix effect and selectivity

For completely bioanalytical method validation, the influence of matrix effect was evaluated according the Matuszewski *at al.* (2003). The matrix factors for the ambroxol and IS, was calculated as the ratio between the areas in the presence (A) and absence (B) of the matrix. The calculated matrix factors ($A/B > 0.9$) indicates that no statistically significant ion suppression occurred. Furthermore, the assay selectivity was assessed by extracts from the free lots of serums by different untreated rabbits. The endogenous and exogenous peaks at the retention times of analytes were not observed in any of the evaluated blank serum lots as showed Figure 2a. In addition, to prove absence of a cross interferences between MS/MS channels used for the ambroxol and IS monitoring, it was separately injected each of the compound at a high concentration (200 ng/ml) and monitored in opposite channel. No cross-talk effect was observed.

Carryover effect

The carryover effects were studied by analysis of the three replicates of the blank sample, which was injected immediately after the sample at concentration 1.5 higher than HQC (300 ng/mL). The response of the blank at a retention time of ambroxol has not been exceeded 20% of the average responses of the analyte at *LLOQ* concentration level.

Stability tests

The stock solution stability test was evaluated at one concentration (20 ng/mL, $n = 3$). The average area of stored standard solution (5 h/room temperature) compared with the freshly prepared standard solution showed no significant static difference (not higher than 5%). Aqueous work standards solutions of the ambroxol were stable for at least 20 days stored at the fridge temperature (4 °C). The short-term, long-term and on the instrument stability test of QC samples were evaluated at two concentration levels, low (20 ng/mL) and high (200 ng/mL). Six replicates of each level were achieved at the initial time point and after a period of

Table 1. Intra- and inter-day precision and accuracy (n = 6); SD, standard deviation; RSD, relative standard deviation

Solution stability	Nominal concentration, ng/mL	Precision		Accuracy, %
		Mean±*SD*	*RSD* / %	
Intra-day	0.5	0.59±0.03	5.74	118.59
	20	20.92±1.16	5.52	104.58
	100	106.87±1.69	1.59	106.87
	200	200.63±4.71	0.69	100.32
Inter-day (3 days)	0.5	0.55±0.04	7.77	110.10
	20	21.35±1.07	5.01	106.78
	100	109.92±3.50	3.18	109.92
	200	199.72±11.53	5.77	99.86

storage (1 h) at a room temperature for short-term, at –20 °C/10 days for long-term, and after 24 h sample standing on an autosampler stability test. The same concentrations and replicates were applied for analysis freeze/thaw stability after three cycles. Concentrations of sample stability were back-calculated against freshly prepared calibration curve. The evaluated relative standard deviations were obtained in acceptable ranges between ±15% at a high levels and ±20% at a low level, as showed in Table 2. All of the applied stability tests did not show significant degradation level, which indicates the constant stability of ambroxol in serum under different conditions at least ten days.

ention times.

The ruggedness of the method was studied by comparison of calibrators and QC samples at low and high concentrations with two analysts on different days. The calculated relative standard deviation values less than 5% indicate the ruggedness of the proposed method with no statistically significant differences in measurement.

Pharmacokinetic study

Analytical applicability of the developed UHPLC––MS/MS method was tested in the pharmacokinetic study in rabbits. After single oral dose administration of

Table 2. Stability of quality controls in serum (n = 6), mean ± standard deviation, %

Conditions	c / ng mL^{-1}	
	20	200
Short-term stability (1 h)	111.79±2.58	104.88±1.41
Long-term stability (–20 °C/10 days)	113.42±4.35	112.72±1.10
Autosampler stability (24 h)	96.94±3.49	96.22±1.18
Three freeze/thaw cycles	99.66±8.07	93.71±17.08

Robustness and ruggedness

According to the validation guideline, the robustness of an analytical procedure represent its capacity to remain unaffected by small variations of critical chromatographic parameters [33]. In present work, effects of minor variations of a flow rate, organic percentage share of the mobile phase and column oven temperature were examined around the parameters set in the validated method to demonstrated reliability during normal usage. Chromatographic parameters were changed through the values; 90±0.5% of methanol, 0.3±0.05 mL/min and 40±5 °C for mobile phase organic strength, flow rate, and column oven temperature, respectively. The measurements were performed in three replicate injections (*n* = 3) with one parameter changing at a time. The obtained results, as represented in Table 3, indicated no significant difference between the results and confirmed the robustness of the developed method, with less variability in the ret-

30 mg ambroxol tablets per rabbit, the serum concentrations were determined over a period of 24 h after administration. The maximum serum concentration (c_{max}), time to reach c_{max} (T_{max}) and the area under the plasma concentration–time curve (AUC) were calculated for each animal and as a mean values. The AUC from time zero to the last quantifiable time point, 24 h (AUC$_{0-24}$) and from time zero to infinity (AUC$_{0-\infty}$) were calculated by the log-linear trapezoidal rule. The elimination half-life ($t_{1/2}$) as 0.693/λz, where λz is the elimination rate constant, was derived from the terminal linear portion of the log plasma concentration–time profile. All above pharmacokinetic parameters, as well as volume of distribution based on the terminal phase (V_d) and total body clearance (Cl), were directly obtained from the experimental data processed by the Kinetica Software, version 5.0. The main pharmacokinetic parameters of ambroxol in rabbit serums are presented in Table 4 and the average concentration–time

Table 3. Robustness evaluation of UHPLC–MS/MS method at concentration of ambroxol standard 20 and 200 ng/mL (n = 3)

Parameter	Value	Mean accuracy[a], %	Precision (*RSD* / %)	Mean accuracy[a], %	Precision (*RSD* / %)
		20 ng /mL		200 ng/ mL	
Column oven emperature	–5 °C	101.45	1.24	99.14	1.31
(40±5 °C)	5 °C	100.25	1.75	96.46	0.41
Mobile phase organic strength (90±5% methanol)	–5% methanol	111.25	0.12	107.91	0.55
	5% methanol	97.83	1.07	94.89	0.44
Flow rate (0.30±0.05 ml/min)	–0.05 ml/min	111.58	1.96	106.72	0.71
	0.05 ml/min	97.29	1.27	89.43	0.89

[a]Mean accuracy at evaluated parameter was calculated by comparison with mean value at validated condition

profile is showed in Figure 3. The average c_{max} and T_{max} of ambroxol in rabbits were 185.52±3.25 ng/mL and 1.61±0.35 h.

Table 4. Mean pharmacokinetic parameters of ambroxol for rabbits following administration of a single oral dose of 30 mg (n = 3)

Parameter	Mean value ± *SD*
c_{max} / ng mL^{-1}	185.52±3.25
AUC0-24, h ng/mL	536.39±67.16
AUC0-inf, h ng/mL	538.74±66.85
T_{max} / h	1.61±0.35
MRT / h	3.15±0.21
$T_{1/2}$ / h	3.53±0.18
K_{el} / h^{-1}	0.19±0.01
Cl, mg h / ng mL^{-1}	0.06±0.01
Vd, mg / ng mL^{-1}	0.29±0.05
Vss, mg / ng mL^{-1}	0.18±0.03

without employing buffer solutions as a component of the mobile phase. The lower limit of detection was achieved at 0.5 ng/mL with only 2 µl of injection volume and 250 µl of sample volume. Repeatability of all concentration levels was high precise with *RSD* less than 5.5% and accuracy greater than 100%. The reported UHPLC–MS/MS method was proved to be reliable and highly sensitive for the determinations of ambroxol in short analysis run time for an only minute and a half. Consequently, the method is enabled a hundred of injections per day which makes the validation process faster and more efficient. The method was also successfully applied to the analysis of ambroxol levels in rabbit serum and determination of pharmacokinetic parameters after dosing a single 30 mg of ambroxol tablet to rabbits.
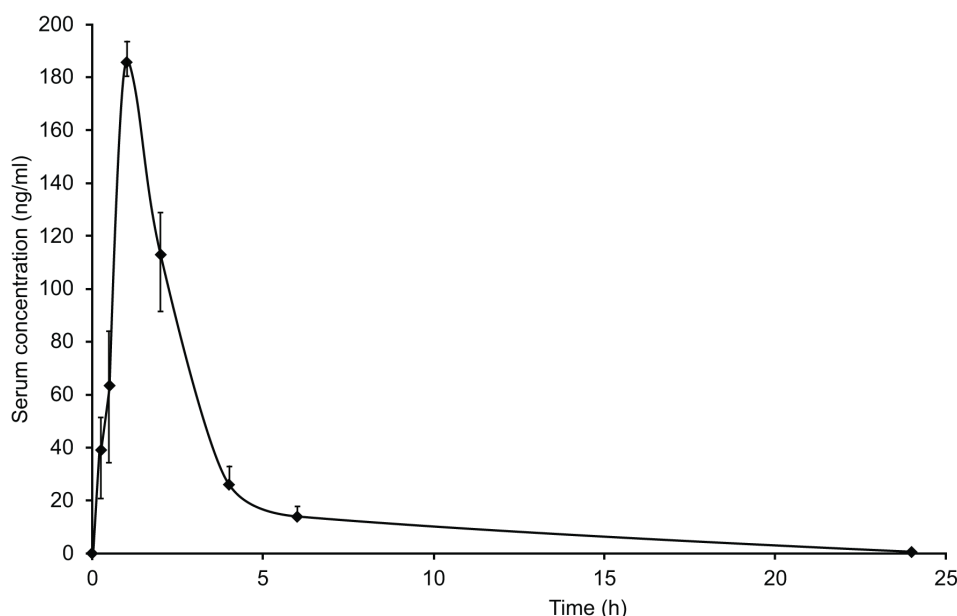

Figure 3. Mean serum concentration-time curve of ambroxol after single oral administration of 30 mg tablet formulation to rabbits at seven time-points (0.25, 0.5, 1, 2, 4, 6 and 24 h).

CONCLUSION

In this study, the determination of ambroxol by UHPLC–MS/MS method in serum after SPE extraction have been established and validated. The validation of the method was performed according the ICH Q2A/ /Q2B guidelines for the validation of bioanalytical methods. Linearity, precision, extraction recovery, carryover, and stability test of the spiked serums stored under different conditions satisfy the acceptance criteria. The presented method was found to be appropriate for all validation parameters. The method was developed in novel 1.3 µm submicron C-18 column

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IZVOD

RAZVOJ NOVE ANALITIČKE METODE ULTRA-BRZE TEČNE HROMATOGRAFIJE SA TANDEM MASENIM DETEKTOROM ZA ODREĐEVANJE AMBROKSOL-HIDROHLORIDA U SERUMU I FARMAKOKINETIČKA PRIMENA

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Ambroksol-hidrohlorid je ekspektorans koji se uspešno primenjuje u muko-litičkoj terapiji više od trideset godina. Lek reguliše sekreciju mukoze ali pokazuje i antioksidativno, antiinflamatorno, analgetičko, lokalno anestetičko, antivirusno i antibakterijsko dejstvo. Primenjuje se u mukolitičkoj terapiji kod akutni i hro-ničnih bronhopulmonalnih bolesti povezanih sa povećanim stvaranjem i poreme-ćajem formiranja i transporta mukusa. Cilj ovog naučnog istraživanja je razvoj nove analitičke metode za određevanje ambroksola u serumu primenom ultra--brze tečne hromatografije sa tandem masenim detektorom (UHPLC–MS/MS) i njena farmakokinetička primena. Validacija analitičke metode sprovedena je u skladu sa priručnikom Internacionalne Komisije za harmonizaciju i validaciju bio-analitčkih metoda ICH (1996). Hromatografsko razdvajanje ambroksola i verapa-mila (IS) izvršeno je na submikrometarskoj koloni Phenomenex Kinetex[TM] RP - C$_{18}$ (2.1 mm×50 mm, 1.3 µm), sa mobilnom fazom metanol – 0.01% mravlja kiselina u vodi (90:10, *V/V*) pri brzini protoka od 0.3 ml/min. Za MS detekciju primenjena je elektrosprej pozitivna jonizacija (ESI) masenog spektrometra u multi-reakcionom monitoring modu (MRM) sa tipičnim jonskim tranzicijama *m/z* 378,9→263,8 za ambroksol i *m/z* 455,2→165,0 za IS pri kolizionim energijama od −20 i −25 V. Električni naponi na interfejsu i detektoru bili su 4,5 i −1,92 kV. Linearnost metode je utvrdjena sa srednjim koeficijentom korelacije (*r* > 0,999) u koncentracionom opsegu 0.5–200 ng/mL sa limitom detekcije (*LOD*) od 0.25 ng/mL i donjim limitom kvantifikacije (*LLOQ*) 0,5 ng/mL. Rezultati intra- i inter-dnevne preciznosti i tač-nosti izmerene u tri različita dana pokazali su prihvatljive vrednosti od ±15%. Raz-vijena metoda je uspešno primenjena za određevanje farmakokinetičkih parame-tara u serumu kunića nakon primene jednokratne oralne doze ambroksol-hid-rohlorida od 30 mg.

Ključne reči: Ambroksol-hidrohlorid • UHPLC–MS/MS • Serum kunića • Farma-kokinetika