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Development and validation of an HPLC method for quantification of solifenacin in spiked human breast milk

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Abstract: An efficient and reliable reversed phase high performance liquid chromatography (HPLC) method was established and validated for the quantitative analysis of solifenacin succinate in fortified human breast milk samples. Chromatographic separation was carried out on a C18 column (150 × 4.6 mm, 5 μm particle size) and a isocratic mobile phase composed of acetonitrile and phosphate buffer (pH 3.5) in a 65:35 (v/v) ratio. The flow rate was at 1.1 mL/min, and detection was performed at 225 nm using a ultraviolet (UV) detector. The method exhibited excellent linearity in the range of 1.0 to 40.0 ng/mL (r^2 = 0.9999). The validation process was performed in accordance with European Medicines Agency (EMA) bioanalytical guidelines, and included selectivity, accuracy, precision, sensitivity, recovery, robustness, and stability assessments. The liquid-liquid extraction (LLE) procedure used for sample pretreatment provided satisfactory recovery (mean: 99.32 %) and minimized matrix interferences from breast milk. The method showed high reproducibility, did not require an internal standar, and offered a rapid analysis time of approximately 3.4 minutes. This study offers a simple, cost-effective, and sensitive HPLC-UV method for monitoring solifenacin levels in breast milk matrices, providing a valuable tool for evaluating drug safety during lactation.

Key words: solifenacin, HPLC-UV, validation, human breast milk, method development

1. Introduction

Overactive bladder (OAB) syndrome is a common clinical condition that negatively affects quality of life and is characterized by increased urinary frequency, sudden urgency, and, in some cases, urge incontinence. In the pathophysiology of OAB, involuntary contraction of the detrusor muscle and the resulting overactivation of muscarinic receptors play a central role.[1,2] For this reason, antimuscarinic agents are considered first-line pharmacological treatments and are widely used in clinical practice.[3,4] However, classical anticholinergic

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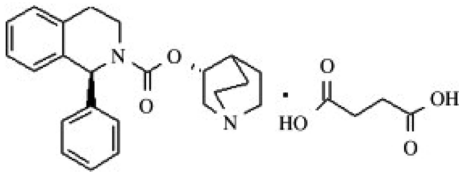

Fig. 1. Chemical structure of solifenacin succinate.

agents often lack receptor selectivity, which frequently leads to undesirable side effects such as dry mouth, blurred vision, and constipation.[4]

Solifenacin succinate ((+)-(1*S*,3'*R*)-quinuclidin-3'-yl 1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carboxylate monosuccinate) (*Fig.* 1) is a selective muscarinic receptor antagonist with higher affinity for the bladder than for the salivary glands. Due to this property, it is expected to provide effective treatment for OAB with a lower incidence of anticholinergic side effects compared to older agents.[5,6] Following oral administration, solifenacin reaches peak plasma concentrations within approximately 4–6 hours and is primarily metabolized in the liver via the cytochrome P450 3A4 (CYP3A4) enzyme. The terminal elimination half-life is approximately 50 hours, supporting once-daily dosing.[7]

Several chromatographic methods have been reported for the determination of solifenacin in biological samples, mainly in plasma, often using liquid chromatography-tandem mass spectrometry (LC-MS/MS) due to its high sensitivity and selectivity.[8-10] However, for non-pharmacokinetic applications, simpler and more accessible techniques such as HPLC-UV remain valuable.[11]

In this study, a LLE approach was used to prepare human breast milk samples, offering a practical and effective solution for removing matrix interferences such as proteins and lipids. Unlike more complex or resource-intensive methods, LLE was found to be suitable for isolating solifenacin with sufficient recovery and minimal matrix effects.

Since breast milk is the primary source of nutrition for infants, monitoring the transfer of drugs into this matrix is critical for evaluating potential exposure and associated risks in nursing babies. Solifenacin is a highly lipophilic drug with a long elimination half-life (approximately 50 hours), which increases the likelihood of excretion into breast milk. Given the overactive bladder syndrome frequently affects women of childbearing and breastfeeding age, and that no data are currently availeble regarding the presence of solifenacin in breast milk, establishing a reliable analytical method for its quantification is of particular clinical and toxicological importance.

Importantly, this work does not involve pharmacokinetic analysis. Instead, known concentrations of solifenacin standard were spiked into breast milk to support method development and validation. The aim was to establish a reliable HPLC-UV method for the selective and accurate determination of solifenacin in human milk, as a basis for future investigations into drug transfer during lactation.

2. Experimental

2.1. Chemicals, solutions, and reagents

Solifenacin succinate was obtained from Shanghai Yingxuan Pharmaceutical Science & Technology Co Ltd. (China). Acetonitrile, sodium dihydrogen phosphate, disodium hydrogen phosphate, hydrochloric acid (all HPLC grade), and n-hexane (analytical grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using a Human ultrapure water system (Japan). A stock solution of solifenacin was prepared at a concentration of 0.1 μg/mL in water and further diluted to obtain standard solutions ranging from 1.0 to 40.0 ng/mL. The phosphate buffer (pH 4.0) was prepared by dissolving 2.0209 g of disodium hydrogen phosphate and 0.3394 g of sodium dihydrogen phosphate in 50 mL of water. The pH was adjusted to 4.0 using 0.1 M hydrochloric acid, and the final volume was brought to 100 mL with ultrapure water.

2.2. Instrumentation and chromatographic conditions

Spectrophotometric and chromatographic analyses were performed to optimize the detection and quantification of solifenacin. UV absorbance measurements were carried out using a Shimadzu UV-160A spectrophotometer (Japan) equipped with 1 cm quartz

cuvettes, and the maximum absorbance for solifenacin was observed at 225 nm.

High performance liquid chromatography was conducted on a Shimadzu LC-20 system (Japan), consisting of an LC-20AT pump, SIL-20A HT autosampler, SPD-20A UV detector, and a CTO-10AC column oven. Various mobile phase compositions, column types and dimensions, flow rates, and column temperatures were evaluated to achieve optimal chromatographic separation. The final method was based on isocratic elution using a GL Sciences C18 (ODS) column (150 × 4.6 mm, 5 μm) at ambient temperature. The mobile phase consisted of acetonitrile and phosphate buffer (pH 4.0) in a 65:35 (v/v) ratio, delivered at a flow rate of 1.1 mL/min. Detection was carried out at 225 nm.

2.3. Sample preparation and extraction procedure

Breast milk samples were obtained from a healthy 27-year-old volunteer after securing informed consent, in accordance with ethical committee approval. The samples were collected using polyethylene storage bags and preserved at -20 °C until analysis. Various extraction procedures were evaluated for isolating the target drug, including both LLE using different solvents and solid-phase extraction (SPE) employing cartridges of different types and sizes.

Initially, the LLE technique was tested with different extraction solvents (acetonitrile, methanol, chloroform, dimethyl sulfoxide, and dimethylformamide), solvent mixtures (1:1 and 1:2, v/v), and varying volumes of extraction solvents in order to optimize the efficient extraction of solifenacin from breast milk. Following the LLE phase, SPE techniques were also experimented with, using various cartridge types and sizes (C18-N, C8, NH2, and C18 Resprep cartridges; 6 mL, 1.000 mg) together with different elution solvents. However, compared to LLE, the SPE approaches did not provide any significant improvement in recovery or matrix elimination. Therefore, the LLE procedure with hexane was selected as the final sample pretreatment method for subsequent validation and application.

To isolate solifenacin from human breast milk, a 2 mL aliquot of the sample was treated with 250 μL of 0.1 M sodium hydroxide to adjust the pH. Following alkalinization, 5 mL of hexane was added to the mixture. The contents were vortexed at a moderate speed for 5 minutes and then centrifuged at 4500 × g for 3 minutes. After centrifugation, the aqueous phase was discarded, and the organic (hexane) layer was carefully collected. This organic phase was evaporated under a gentle nitrogen stream at ambient temperature. The resulting residue was reconstituted in 300 μL of acetonitrile and vortexed briefly for 30 seconds. Finally, a 20 μL portion of the prepared solution was injected into the HPLC-UV system for analysis.

2.4. Evaluation of measurement uncertainty

Key uncertainty components affecting the analytical results were identified and quantified. These included uncertainties arising from the reference material's purity ($u_{standard}$), sample mass measurement ($u_{weighing}$), the recovery process ($u_{recovery}$), and calibration curve fitting (u_{curve}). Repeatability was also taken into consideration where applicable. The overall combined standard uncertainty ($u_{combined}$) was calculated using the root sum of squares approach, as shown below:

$$u_{Combined} = \sqrt{(u_{standard})^2 + (u_{weighing})^2 + (u_{recovery})^2 + (u_{curve})^2}$$

The expanded uncertainty ($u_{expanded}$) was determined by multiplying the combined uncertainty by a coverage factor k = 2, corresponding to a 95 % confidence level. All uncertainty estimations were carried out in alignment with the recommendations outlined in the EURACHEM Guide[12] and supported by relevant scientific literature.[13]

3. Results and Discussion

3.1. Chromatographic process

An isocratic elution method was employed to achieve optimal separation of solifenacin. *Fig.* 2 illustrates representative chromatograms of: a) blank breast milk sample, and b) breast milk sample spiked with 10 ng/mL solifenacin.

The retention time for solifenacin was observed at 3.4 minutes. *Table* 1 summarizes the system suitability

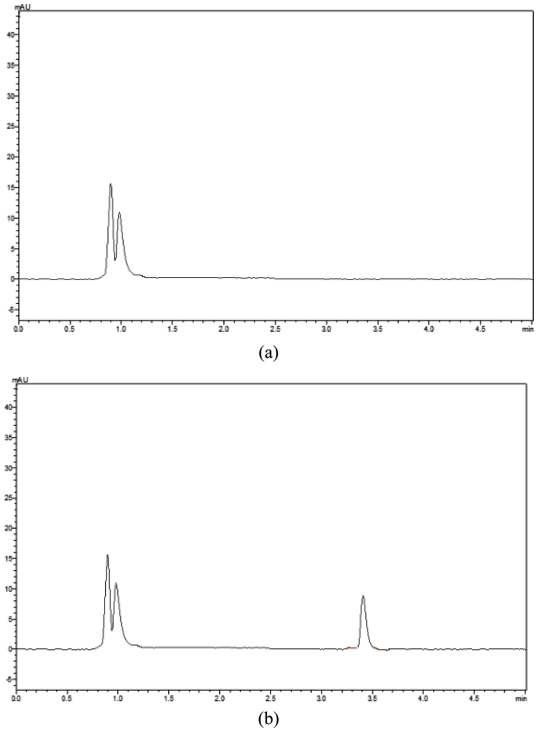

Fig. 2. (a) blank breast milk sample, (b) breast milk samples spiked with 10 ng/mL standard solifenacin.

parameters, confirming the performance and reliability of the chromatographic method for detecting solifenacin in human breast milk.

3.1.1. Optimization of chromatographic and extraction conditions

During method development, several chromatographic parameters (mobile phase composition, flow rate, and column temperature) and extraction solvents were evaluated. Gradient elution and alternative columns testes resulted in longer retention times (>6 min) and asymmetric peaks (tailing factor > 1.5). The final isocratic system using acetonitrile-phosphate buffer (65:35, v/v) at 1.1 mL/min yielded sharp and symmetric peaks (tailing factor 1.09) with a short retention time (3.4 min). For sample pretreatment, various solvents including acetonitrile, methanol, chloroform, dimethyl sulfoxide, and dimethylformamide were compared. Among them, hexane provided the highest recovery (99.3&) and minimal matrix interference, while other solvents produced lower recovery values (<85%) or incomplete protein/lipid removal. Accordingly, hexane-based liquid-liquid extraction was selected as the final method. A summary of the optimization results is provided in *Table* 1.

3.2. Validation of the method

The procedure was validated in accordance with the guidelines set forth by the EMA.[14] Based on the method validation results, the high reproducibility of the method rendered the use of an internal standard unnecessary.

3.2.1. Linearity and sensitivity

The calibration curve for solifenacin in breast milk was established by plotting the peak area of the analyte against solifenacin concentrations using a linear least-squares regression method. Based on six replicate measurements at five different concentration levels, the resulting calibration equation was: $y = 2428.6x + 101.64$, with an excellent correlation coefficient or $r = 0.9999$. The method demonstrated a linear response within the concentration range of 1.0 to 40.0 ng/mL in breast milk samples.

The limits of detection (LOD) and quantitation (LOQ) were calculated using the equation LOD or LOQ = k × SDa/b, where k is 3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope of the calibration curve. A summary of the analytical performance parameters of the proposed method is presented in *Table* 2, including the linear concentration range, regression equation, correlation coefficient, and sensitivity values (LOD and LOQ). The obtained results demonstrate an

Table 1. Chromatographic system suitability parameters

Capacity factor*	Resolution*	HETP (μm)*	Tailing factor*	Asymmetry factor*
6.22	1.65	0.28	1.09	1.02

*Mean values of the parameters of all the points in the calibration study are mentioned

Table 2. Analytical parameters of the method

Parameters	Method
Concentration range[a] (ng mL^{-1})	1.0–40.0
Regression equation[b]	y = 2428.6 × +101.64
Intercept ± SD	2428.6 ± 121
Slope ± SD	101.64 ± 68
Correlation coefficient (r^2)	0.9999
LOD (ng mL^{-1})	0.3
LOQ (ng mL^{-1})	1.0

[a]Average of six determinations
[b]$y = xC$ +b where C is the concentration in ng mL^{-1} and y is the peak area

excellent linear response ($r^2 = 0.9999$) over the tested range (1.0 – 40.0 ng/mL) and indicate that the method offers high sensitivity, with LOD and LOQ values of 0.3 and 1.0 ng/mL, respectively. These findings confirm the suitability of the method for accurate quantification of solifenacin in human breast milk.

3.2.2. Accuracy, precision, and recovery

Quality control (QC) samples were analyzed at three concentration levels to evaluate the accuracy and precision of the method. QC samples were prepared in both aqueous solution and breast milk at low (1.0 ng/mL), medium (10.0 ng/mL), and high (40 ng/mL) concentrations (n = 3 for each level). Accuracy was expressed as recovery values and relative mean error (RME), while precision was evaluated using the relative standard deviation (RSD).

To asses absolute recovery, solifenacin-spiked breast milk samples were subjected to extraction procedures, and the resulting peak areas were compared to those obtained from equivalent concentrations of unextracted aqueous solifenacin solutions. The mean absolute recovery of solifenacin from breast milk was found to be 99.80 %. The mean relative recovery, calculated by comparing the known added amounts in spiked samples to the concentrations measured via the calibration curve, was determined to be 99.32 %.

Intraday accuracy and precision were assessed by analyzing three replicated of each concentration level on the same day, while interday evaluations were performed over three consecutive days. All intraday and interday RSD values were below 2.47 %, indicating high precision. These results, summarized in *Table* 3, demonstrate that the method provides reliable accuracy and precision for the quantification of solifenacin in human breast milk.

3.2.3. Robustness

As described in the validation section, the robustness of the method was assessed by analyzing QC samples at three concentration levels (n = 3). To evaluate the robustness, deliberate variations were introduced to key chromatographic parameters, including flow rate, column oven temperature, and the composition of the mobile phase consisting of acetonitrile and phosphate buffer.

Specifically, the column temperature was adjusted from the optimized 30 °C to 25 °C and 35 °C; the mobile phase ratio (acetonitrile:phosphate buffer) was modified from 65:35 (v/v) to 70:30 and 60:40; and the flow rate was varied from 1.1 mL/min to 1.2 mL/min and 1.0 mL/min. These changes were made to determine the method's resilience under slightly altered analytical conditions. The results

Table 3. Accuracy and precision of the method

Existant concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) (Mean ± SD[1])	Recovery (%)	RSD of recovery	RSD of intra-day variation	RSD of inter-day variation
10	1.0	10.88 ± 0.02	98.90	1.11	2.05	2.47
	10	19.85 ± 0.01	99.25	0.79	1.84	2.35
	40	49.90 ± 0.01	99.80	0.66	1.78	2.26
Mean relative recovery = 99.32						

For each concentration n = 3

Table 4. Robustness of the method

Condition	Value	Recovery %	RSD %
Flow rate mL min^{-1}	1.0	98.4	2.20
	1.2	97.6	2.61
Mobile phase composition (acetonitrile and phosphate buffer)	60:40	98.1	2.30
	70:30	97.9	2.55
Column temperature	25	99.7	0.77
	35	99.8	0.74

n = 3 for all QC sample levels

showed that neither the peak area nor the resolution of solifenacin in human breast milk samples was significantly affected by these variations. The low RSD values observed across the measurements confirm the robustness of the method, as summarized in *Table* 4.

3.2.4. Stability

The stability of working standard solifenacin solutions at QC concentrations was evaluated under different storage conditions using three replicated per condition. Test conditions included storage at room temperature in the dark for 24 hours, placement in the autosampler for 24 hours, and refrigeration at 4 °C for one month. The corresponding recovery rates under these conditions were 98.2 %, 98.7 %, and 99.1 %, respectively, all of which exceeded the values reported in previous studies.[15] Among all tested scenarios, the highest observed RSD was 1.85 %, indicating minimal variability. These findings confirm that SLN remains stable across all examined storage conditions.

In addition to standard solution stability, matrix stability of solifenacin in breast milk was evaluated at QC levels (1.0, 10.0, and 40.0 ng/mL). Spiked samples stored at -20 °C for one month, subjected to three freeze-thaw cycles, and kept at room temperature for 6 hours all demonstrated recoveries within 95-105 % of the nominal concentration, with RSD values below 3.0 %, indicating good stability in the matrix. Processed-sample stability was assessed by re-injecting extracted QC samples that had been stored in the autosampler at 4 °C for 24 hours. The recoveries ranged from 98.4 % to 101.2 %, with RSD values less than 2.5 %. These results confirm that solifenacin is stable under typical sample storage and analytical conditions.

3.3. Assessment of uncertainty

The uncertainty associated with the analytical method was evaluated and expressed as a percentage (%) at a 95 % confidence level for all analytes and calculated parameters. The observed values, summarized in *Table* 5, indicate that the uncertainty levels were within acceptable limits. The contribution of sample weighing to the overall uncertainty was found to be negligible and was therefore reported for completeness, although its impact was minimal.

4. Conclusions

In conclusion, this study presents the first validated HPLC-UV method specifically developed for the quantification of solifenacin in human breast milk. Currently, no published data exist on solifenacin transfer into breast milk, despite its widespread use in the treatment of overactive bladder. The present work did not include pharmacokinetic investigations; rather, the primary aim was to establish a reliable, rapid, and cost-effective analytical tool. The proposed method, based on a straightforward liquid-liquid extraction procedure followed by isocratic reversed-phase HPLC, offers excellent sensitivity, robustness, and recovery, with a short retention time of 3.4 minutes. Although pharmacokinetic was beyond the scope of this study, the validated method provides a solid foundation for

Table 5. Uncertainity assessment for the developed method

Uncertainty (U) in %					
$U_{Standard}$	$U_{Calibration}$	$U_{Recovery}$	$U_{Repeatability}$	$U_{Combined}$	$U_{Expanded}$
0.451	1.223	0.144	0.865	1.340	2.982

future research, including pharmacokinetic or drug safety studies during lactation, where understanding the excretion profile of solifenacin will be of clinical importance.

Conflict of Interest Statament

The authors declare no conflicts of interest/competing interests.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Bezmialem Vakıf University was approved by the Clinical Trials Ethics Committee (No: 2022/33 – E.63030).

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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