# Determination of Guaifenesin in Spiked Human Breast Milk: HPLC-UV Method Development, Validation, and Uncertainty Evaluation

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*A simple, sensitive, and reliable isocratic reversed-phase high-performance liquid chromatography (HPLC-UV) method was developed, validated, and applied for the quantitative determination of guaifenesin in spiked human breast milk. Chromatographic separation was achieved on a C18 column (150 x 4.6 mm, 5 µm) using a mobile phase composed of methanol and water (50:50, v/v), where the aqueous phase was acidified with orthophosphoric acid (pH=3.2). The flow rate was 0.8 mL $min^{-1}$, and detection was performed at 230 nm. The method exhibited excellent linearity over the concentration range of 5.0-30.0 ng $mL^{-1}$ with a correlation coefficient of $r^2$=0.9999. Liquid-liquid extraction (LLE) was used for sample preparation and resulted in a mean relative recovery of 98.82% with an absolute recovery of 99.52%, while effectively minimizing matrix interferences associated with breast milk. Method validation was performed in accordance with European Medicines Agency (EMA) bioanalytical guidelines, including assessments of selectivity, accuracy, precision, sensitivity, robustness, and stability. The method demonstrated strong reproducibility, did not require an internal standard, and provided a short analysis time suitable for routine application. This study presents the first simple, cost-effective, and sensitive HPLC-UV method for the determination of guaifenesin in human breast milk, offering a valuable analytical tool for evaluating drug safety during lactation.*

**Keywords:** guaifenesin, HPLC-UV, human breast milk, method development

## Introduction

Guaifenesin, chemically known as 3-(2-methoxy-phenoxy)-1,2-propanediol and shown in Figure 1, is a well-established expectorant commonly included in numerous over-the-counter cough and cold formulations. It works by increasing the volume and decreasing the viscosity of bronchial secretions, thereby facilitating mucus clearance from the airways [1, 2]. Due to its wide availability and frequent therapeutic use, guaifenesin is likely to be consumed by breastfeeding women. Nevertheless, information regarding its transfer into human breast milk and the potential exposure of breastfed infants remains scarce [3].

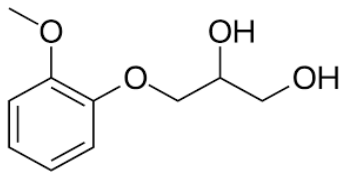


**Fig. 1.** Chemical structure of guaifenesin.

Previous analytical studies involving guaifenesin have focused primarily on pharmaceutical dosage forms or various biological samples such as plasma and urine [4-6]. It has been determined alone or in combination with other active ingredients using spectrophotometric approaches [7, 8], RP-HPLC [9, 10], HPTLC [11], LC-MS/MS [12], GC-MS/MS [13], and chemometric techniques [14]. In particular, several studies have highlighted the relevance of separating guaifenesin from structurally related impurities or degradation products such as guaiacol [15]. Despite these efforts, no validated analytical method has been reported for the complex matrix with substantial protein and lipid content.

Given the complexity of breast milk and the need to minimize matrix interferences, a selective, sensitive, and reliable analytical technique is required. Such a method would support drug safety evaluations during lactation, contribute to pharmacokinetic studies, and provide data critical for assessing infant exposure. In light of the gaps identified in the literature, the present study aims to develop, validate, and perform uncertainty evaluation of a novel HPLC-UV method for the quantitative determination of guaifenesin in spiked human breast milk. To the best of our knowledge, this is the first analytical approach specifically tailored for guaifenesin analysis in lactational matrices.

## Experimental part

*Chemicals, solutions and reagents*

Guaifenesin was supplied by Shanghai Yingxuan Pharmaceutical Science & Technology (China). The

certified purity of the guaifenesin reference standard was 99.8%, as stated in the certificate of analysis provided by the manufacturer, and this value was incorporated into the uncertainty calculations. HPLC-grade methanol and orthophosphoric acid were obtained from Merck (Darmstadt, Germany), while analytical-grade reagents and ultrapure water used throughout the study. Ultrapure water was produced using a Human ultrapure water system (Japan). A primary guaifenesin stock solution was prepared in water at a concentration of 0.1 μg $mL^{-1}$, and serial dilutions were carried out to make calibration solutions in the range of 5-30 ng $mL^{-1}$.

*Instrumentation and chromatographic conditions*

Spectrophotometric and chromatographic evaluations were carried out to establish suitable conditions for detecting and quantifying guaifenesin. UV measurements were obtained with a Shimadzu UV-160A spectrophotometer (Japan) using 1 cm quartz cells, and guaifenesin displayed its strongest absorbance at 230 nm. Chromatographic analysis was performed using a Shimadzu LC-20 HPLC system (Japan), equipped with an LC-20AT pump, an SIL-20A HT autosampler, an SPD-20A UV detector (bandwidth: 8 nm, sampling rate: 10 Hz), and a CTO-10AC column oven. Different mobile-phase compositions, methanol-to-water ratios, and flow rates were systematically examined to identify conditions that provided clear and reproducible separation.

The finalized procedure employed an isocratic elution program using a C18 column (150 x 4.6 mm, 5 μm; [Thermo Hypersil Gold]) maintained at 30 °C. The mobile phase consisted of methanol and water (50:50, v/v), with the aqueous portion adjusted to pH 3.2 using orthophosphoric acid. A flow rate of 0.8 mL $min^{-1}$ was selected as optimal, and UV detection was carried out at 230 nm. The injection volume was 20 μL.

*Sample preparation and extraction procedure*

Breast milk samples were obtained from a healthy volunteer with informed consent and kept at -20 ºC in polypropylene containers until analysis. Different extraction options were examined, but hexane-based LLE provided the most consistent recovery for guaifenesin and was therefore selected as the sample-cleaning approach.

For extraction, 2 mL of breast milk was combined with 250 μL of 0.1 M sodium hydroxide to alkalize the matrix. Then 5 mL of hexane was added, and the mixture was vortexed for 5 minutes followed by centrifugation at 4500 x g for 3 minutes. The upper organic phase was collected and evaporated under a mild nitrogen stream at approximately 200 mL $min^{-1}$ for 10 minutes at room temperature. The residue was reconstituted in 300 μL of the mobile phase and briefly vortexed. A 20 μL portion of this solution was injected into the HPLC-UV system for the determination of guaifenesin.

*Evaluation of measurement uncertainty*

The primary sources contributing to uncertainty in the analytical results were identified and quantified individually. Factors considered included the certified purity of the reference standard ($u_{standard}$), uncertainties associated with weighing the sample ($u_{weighing}$), the extraction efficiency ($u_{recovery}$), and the calibration model used to generate the analytical curve ($u_{curve}$). When relevant, the influence of repeatability was also incorporated.

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

All components were combined to obtain the overall standard uncertainty, calculated using the square-root-of-sum-of-squares method. The expanded uncertainty was then derived by applying a coverage factor of k = 2, representing approximately 95% confidence. The approach for estimating uncertainty followed the principles and guidance provided in the EURACHEM Guide, supported by additional literature where appropriate.

## Results and discussion

### Chromatographic procedure

A constant-composition (isocratic) mobile phase was used to ensure stable and reproducible separation. Figure 2 presents typical chromatograms obtained for (a) a blank breast milk sample and (b) a sample fortified with 30.0 ng $mL^{-1}$ guaifenesin. As shown in the blank breast milk chromatogram (Figure 2a), no interfering peaks were observed at the retention time of guaifenesin, demonstrating the selectivity of the proposed method.

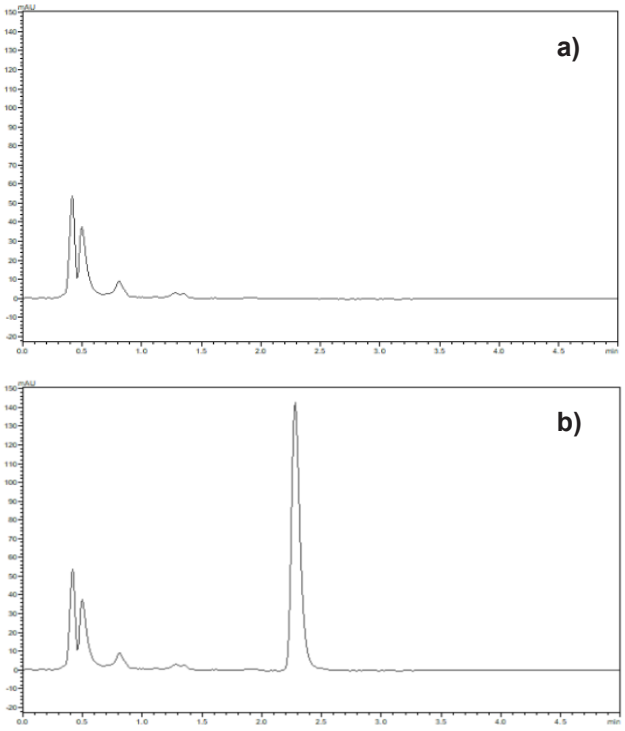


**Fig. 2.** Chromatograms of a: blank breast milk, b: breast milk samples spiked with 30.0 ng $mL^{-1}$ standard guaifenesin.

Under the finalized conditions, guaifenesin eluted at approximately 2.3 minutes. System suitability results are listed in Table 1, demonstrating that the chromatographic setup met the required performance criteria and was suitable for routine quantification of guaifenesin in human breast milk. The capacity factor (k') for guaifenesin was calculated as approximately 5.6, which falls within the recommended range for reversed-phase HPLC. The height equivalent to a theoretical plate (HETP) was found to be approximately 15 µm, consistent with expected values for a 5 µm particle size C18 column. These results confirm that the chromatographic system was operating under optimal and theoretically acceptable conditions.

***Optimization of chromatographic and extraction conditions***

Several chromatographic variables such as mobile-phase ratio, flow rate, and column temperature were examined during method refinement. During method optimization, different reversed-phase columns with varying stationary phases and dimensions, including C8 and C18 columns with different lengths and particle sizes from different manufacturers, were evaluated. However, these columns did not provide significant improvement in retention time or peak symmetry compared to the selected column; therefore, the final chromatographic conditions were maintained as described. The final isocratic setup, employing the selected methanol-water mobile phase at the optimized flow rate, generated well-defined and symmetric guaifenesin peaks with a short elution time.

Different extraction solvents were also compared to enhance sample cleanliness and recovery. Among the tested options, hexane consistently yielded the best extraction efficiency and minimized matrix effects. As a result, hexane-based LLE was chosen as the definitive sample-preparation procedure. A concise overview of the optimization outcomes is presented in Table 1.

**Table 1.** Chromatographic system suitability parameters.

| Parameter | Value |
|---|---|
| Capacity factor* | 5.62 |
| Resolution* | 2.13 |
| HETP (µm)* | 14.98 |
| Tailing factor* | 0.96 |
| Asymmetry factor* | 1.22 |

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

**Method validation**

The method was validated following the EMA recommendations [16]. The outcomes of the validation demonstrated that the procedure was highly reproducible, making the inclusion of an internal standard redundant.

***Linearity and sensitivity***

Calibration for guaifenesin in breast milk was obtained by relating peak area to analyte concentration using linear regression. Six replicates at five concentration levels produced a calibration line with excellent linearity ($r^2 = 0.9999$) across 5.0 – 30.0 ng mL$^{-1}$. Detection (LOD) and quantification (LOQ) limits were determined from the standard deviation of the intercept and the slope of the curve (k=3 for LOD; k=10 for LOQ). LOD and LOQ were calculated based on the standard deviation of the y-intercept and the slope of the calibration curve. The method showed analytical sensitivity, with LOD and LOQ values of 1.7 and 5.0 ng/mL, respectively, confirming that this approach is suitable for reliable measurement of guaifenesin in human breast milk. The corresponding validation parameters are summarized in Table 2. Calibration curves were constructed using weighted linear regression (1/x) to account for heteroscedasticity across the concentration range. Excellent linearity was obtained with a correlation coefficient of $r^2 = 0.9999$

**Table 2.** Analytical parameters of the method.

| Parameters | Value |
|---|---|
| Concentration range [a] (ng mL$^{-1}$) | 5.0-30.0 |
| Regression equation[b] | y = 727.53x+267.08 |
| Slope ± SD | 727.53±108.12 |
| Intercept ± SD | 267.08±278.25 |
| Correlation coefficient ($r^2$) | 0.9999 |
| LOD (ng mL$^{-1}$) | 1.7 |
| LOQ (ng mL$^{-1}$) | 5.0 |

[a] Average of six determinatons.

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

**Accuracy, precision, and recovery**

Accuracy and precision were assessed using quality control samples of guaifenesin at low (5.0 ng mL$^{-1}$), medium (15.0 ng mL$^{-1}$), and high (30.0 ng mL$^{-1}$) concentrations, analysed in triplicate. Accuracy was evaluated through recovery and relative mean error, while precision was determined from relative standard deviation values. Absolute recovery was calculated by comparing extracted guaifenesin-spiked breast milk samples with unextracted aqueous standards, yielding a mean recovery of 99.52%. Mean relative recovery, based on measured concentrations versus nominal values, averaged 98.82%. Intraday precision was established from repeated measurements on a single day, and interday precision over three days; all RSD values remained below 1.88%, confirming the method's robustness. These accuracy, precision, and recovery results are presented in Table 3.

**Table 3.** Accuracy and precision of the method.

| Existent concentration (ng mL$^{-1}$) | Added concentration (ng mL$^{-1}$) | Found concentration (ng mL$^{-1}$) (Mean±SD[1]) | Recovery (%) | RSD of recovery | RSD of intraday variation | RSD of interday variation |
|---|---|---|---|---|---|---|
| 10.0 | 5.0 | 14.68 ± 0.01 | 97.86 | 2.61 | 1.22 | 1.88 |
| | 15.0 | 24.77 ± 0.02 | 99.08 | 2.34 | 1.05 | 1.71 |
| | 30.0 | 39.81 ± 0.04 | 99.52 | 2.17 | 0.94 | 1.50 |
| Mean relative recovery = 98.82 | | | | | | |

[1] For each concentration n=3.

***Robustness***

Robustness was examined by testing QC samples of guaifenesin at three concentrations (n=3) while intentionally altering key chromatographic conditions. Small changes were applied to the flow rate, column temperature, and mobile-phase composition to assess how well the method performs under slight deviations from the optimized settings. The column temperature was varied from the original 30 ºC to 25 ºC and 35 ºC, the methanol-water mobile phase ratio was adjusted from 50:50 to 40:60 and 60:40 (v/v), and the flow rate as modified from 0.8 mL min$^{-1}$ to 0.7 and 0.9 mL min$^{-1}$. These adjustments produced no meaningful changes in peak area or chromatographic resolution for guaifenesin in breast milk. Consistently low RSD values further confirmed the method's robustness. A summary of these outcomes is provided in Table 4.

**Table 4.** Robustness of the method.

| Condition | Value | Recovery % | RSD % |
|---|---|---|---|
| Flow rate mL min$^{-1}$ | 0.7 | 98.1 | 1.92 |
| | 0.9 | 98.5 | 1.88 |
| Mobile phase composition (methanol/ aqueous phase) | 40:60 | 97.0 | 2.21 |
| | 60:40 | 97.4 | 2.13 |
| Column temperature | 25 | 99.6 | 0.95 |
| | 35 | 99.7 | 0.93 |

n=3 for all QC sample levels.

***Stability***

The stability of guaifenesin working standards at QC concentrations was examined under several storage conditions, each tested in triplicate. Solutions kept at room temperature for 24 hours, held in the auto sampler for 24 hours, or stored at 4 ºC for one month showed recoveries of 97.5%, 98.2%, and 99.2%, respectively, with the highest RSD remaining below 1.9%. These results indicate that the standard solutions maintain their integrity under all evaluated conditions.

Matrix stability was also assessed in breast milk at 5.0, 15.0, and 30.0 ng mL$^{-1}$. Spiked samples frozen at – 20 ºC for one month, subjected to three freeze-thaw cycles, or left at room temperature for 6 hours all produced recoveries within 95-105% and RSD values under 2.4%, confirming adequate stability in the matrix. Processed-sample stability was verified by re-injecting extracted QC samples stored in the autosampler at 4 ºC for 24 hours, which yielded recoveries between 96.1% and 99.7% with RSD below 3.3%. Overall, guaifenesin remained stable under the storage and handling conditions commonly used in analysis.

***Assessment of uncertainty***

The measurement uncertainty for guaifenesin was calculated at the 95% confidence level and reported as a percentage for all evaluated parameters. As shown in Table 5, the obtained uncertainty values remained well within acceptable analytical limits. The contribution of sample-weighing steps was minimal and included only for completeness, as its effect on total uncertainty was insignificant.

**Table 5.** Uncertainty assessment for the developed method.

| Uncertainty (U) | Value, % |
|---|---|
| $U_{standard}$ | 0.314 |
| $U_{calibration}$ | 0.701 |
| $U_{recovery}$ | 0.286 |
| $U_{repeatability}$ | 0.230 |
| $U_{combined}$ | 0.851 |
| $U_{expanded}$* | 1.70 |

*k=2.

## Conclusion

In conclusion, this study presents the first validated and uncertainty evaluated HPLC-UV method specifically developed for the quantitative determination of guaifenesin in human breast milk. The optimized isocratic procedure provided a short time of 2.3 minutes, excellent linearity ($r^2$ = 0.9999, 5-30 ng mL$^{-1}$), and analytical sensitivity with an LOD of 1.7 ng mL$^{-1}$ and LOQ of 5.0 ng mL$^{-1}$. The hexane based LLE yielded a high absolute recovery of 99.52%, while intraday and interday precision remained below 1.88%, demonstrating robust analytical performance.

All validation steps were fully compliant with EMA bioanalytical guidelines, ensuring reliability,

traceability, and regulatory suitability of the method. Additionally, the comprehensive measurement uncertainty evaluation revealed and expanded uncertainty of 1.70% (k = 2), confirming that the method operates within acceptable analytical limits for routine quantification.

Overall, this simple, sensitive, and cost-effective methodology provides a valuable analytical tool for assessing guaifenesin exposure during lactation and forms a strong foundation for future pharmacokinetic or drug safety studies in breastfeeding populations.

**Authors' Contributions**

All the authors contributed equally. The author(s) read and approved the final manuscript.

**Conflicts of Interest/Competing Interests**

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 approved by the Clinical Trials Ethic Committee (No: 2022/33).

**Informed consent**

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

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