Validation of Analysis of S-Phenyl Mercapturic Acid in Urine by High-Performance Liquid Chromatography – Photodiode Array

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Abstract

An easy, eclectic, precise, and high-performance liquid chromatography (HPLC) analysis was developed and validated for the estimation of S-phenyl mercapturic acid in urine. The chromatographic separation was achieved using a separation column of the ODS-3 Inertsil type with dimensions of (5μ, 150mm x 4.6mm). The column temperature was set at 45° C. A mobile phase composed of acetonitrile: methanol: deionized water acidified with perchloric acid 60% (v/v/v ratio of 4:1:5, pH=3) was used at a flow rate of 1.5 ml/min and a wavelength of 225 nm for UV detection. System Suitability tests (SSTs) are typically performed to assess the suitability and effectiveness of the entire chromatography system. Retention times of S-phenyl mercapturic acid and Benzoic acid were found to be 17.3 and 15.6 minutes, respectively. The detection limit was 0.126 μg/ml, the quantitative estimation unit was 0.38 μg/ml, and the recovery was 97.183%. S-phenyl mercapturic acid showed a linear signal in the domain of 0.5-20 μg/mL.

The developed method has been validated through linearity, precision, accuracy, specificity, LOD, and LOQ. Therefore, the proposed analytical method proved its applicability and efficiency in estimating the benzene metabolite in urine.

Keywords: Benzoic acid, HPLC, S-phenyl mercapturic acid, System Suitability Test, Validation.

Introduction

Numerous toxic substances are emitted into the environment by various industries, including toxic heavy metals like lead1, gases such as NO2, and volatile organic compounds (BTEX), of which benzene is one of the most significant3. Benzene is a colorless liquid that is a highly volatile liquid chemical and slightly soluble in water, with a sweet odor4. According to the International Agency for Research on Cancer (IARC, 1989), benzene is a group 1 carcinogen due to its mutagenic and carcinogenic properties5. Benzene is present in oil refineries and burning processes6, and its emission into the environment results in significant health risks. Indeed, long-term exposure to low concentrations of benzene may increase the frequency of cancer and leukemia7.

As a result, it is important to monitor environmental benzene concentrations in the air8,9 and biological exposure to benzene in the human body, particularly in occupational workplaces10,11. Human uptake of benzene from the occupational environment primarily occurs through inhalation, oral, and dermal contact12. Benzene undergoes liver metabolism, resulting in the formation of a series of hydroxyl compounds and metabolites that conjugate with glutathione. Other products containing opened rings are also formed, which are eliminated through urine13.

Phenol, trans, trans-muconic acid (tt-MA), and S-phenyl mercapturic acid (SPMA as shown in Fig. 1 are among the well-known benzene metabolites excreted through urine as shown in Fig. 214.
However, phenol is excreted at a much higher rate, but it is not a reliable biomarker for benzene exposure below 1 ppm, as it is also present in the urine of people without benzene exposure at lower rates\textsuperscript{15}. On the other side, 3.9\% and 0.11 \% of the inhaled dose are excreted as tt-MA and SPMA respectively\textsuperscript{16}. Therefore, it is essential to develop a sensitive method to measure human exposure to a wide range of benzene concentrations in the body, as even low doses of benzene can be toxic\textsuperscript{17,18}. 

tt-MA is non-specific biomarker of benzene exposure as this chemical is also present in the diet, cosmetics, and pharmaceuticals and can be formed endogenously\textsuperscript{19}. SPMA can be used as a biomarker for low concentrations of benzene in urine, as it is not affected by contamination or loss of material due to volatility. Moreover, some studies have shown SPMA to be the most specific biomarker of benzene exposure\textsuperscript{19}.

In order to excretion of SPMA in small amounts, highly sensitive methods like GC-MS and LC-MS should be used to determine SPMA levels\textsuperscript{20,21}. However, these methods are not suitable for routine analysis in clinical diagnostics due to their high cost. Alternatively, HPLC-UV analysis of SPMA in urine is a simple and accurate method with good precision, as demonstrated in several previous studies in literature\textsuperscript{22,23}.

This article presents a validated HPLC method, in combination with PDA (photodiode array) detection, which can be used for high throughput analyses in clinical routines. The method was distinguished from the methods in previous studies by the ease of use and simplicity of the devices, greater accuracy, and better separation of the mentioned material by using three components of the mobile phase. It surpassed previous studies that used PDA technology which provides multiple wavelengths and chooses the best of them, in addition to using liquid-liquid extraction with a higher extraction recovery that is close to solid-phase extraction, which has a higher cost and requires a special separation column and solvents. The results demonstrated the rapidity and validity of the proposed method.

**Figure 1. Structure of S-phenyl mercapturic acid**

**Figure 2. benzene metabolism in human body\textsuperscript{14}**
Materials and Methods

**Instrumentation and Chemicals**

A Shimadzu Prominence SPD-20A HPLC PDA, Japan and an auto sampler (Shimadzu, Japan, model SIL-10AD) and a model SPD-20AV UV-VIS detector were used for chromatographic measurements. Working standard S-phenyl mercapturic acid (98% w/w) was purchased from Biosynthesis (UK), while Benzoic acid (99% w/w) was gifted by Medico Labs (a Syrian pharmaceutical company in Homs countryside). The main source of Benzoic acid was Sigma Aldrich (Germany).

Sulfuric acid 9 N (which was prepared from H₂SO₄ 98%), KOH 7.8N and Ethyl acetate were obtained from EMSURE (India). Perchloric acid 70% was obtained from Emparta (India).

Pro HPLC grade methanol, Acetonitrile were obtained from LiChrosolv (Germany).

Tools used: micro filter 45µm - extraction funnel - glassware (becher + arlen) - graduated micropipette. Devices used: pH meter from EZDO (Taiwan) – Centrifuge from Hettich (USA) - Ultrasonic device from Jeken (China) - Vacuum drying oven (Vacucell) from BMT (USA).

**Preparation of standard stock and working solutions**

* Preparing the mobile phase:

Mix 400 mL of acetonitrile, then add 100 mL of methanol and complete the volume to 1 liter by deionized water acidified with perchloric acid (8.5-9mL) to reach a total pH = 3.

*Preparation of Standard Solutions:

Standard solution of the substance to be analyzed "SPMA" (External Standard):

A quantity of the substance of 20 mg was dissolved in 100 mL of methanol (where the solubility is 30 mg/mL) and a stock solution was obtained at a concentration of 200µg/mL.

Through this solution, a series of standard solutions with different concentrations are prepared.

Standard solution of Benzoic acid (Internal Standard "IS"):

A solution of Benzoic acid was prepared as an internal standard at a constant concentration of 5 µg/mL for inclusion in each assay and in the analytical method validation calculations.

It was prepared by dissolving 1.5 mg of Benzoic acid in 50 mL methanol and then completing the volume up to 100 mL in a 100 mL calibration balloon.

The IS is added to previous concentration of 3 mL urine sample, and it became 5 µg/mL that is extracted after sample preparation.

**Analytical conditions of HPLC method**

The HPLC system (Shimadzu Prominence HPLC, Japan) consisted of a pump that was set to inject 20 µl per injection at once. The detector (SPD-20A PDA) consisted of UV/VIS in the range of 190 to 700 nm which can be used for all UV analyses. (Stainless steel 150 mm in length and 4.6 mm in inner diameter) was packed with Inertsil ODS-3 (5 µm; GL Science, Tokyo, Japan) (stationary phase) was utilized for LC separations. The mobile phase contained {400mL acetonitrile: 100 methanol: 500 deionized water acidified with perchloric acid up to1 liter, pH = 3}. Degassing and then filtering procedures were performed on the mobile phase before using it. The pump was set at a flow rate of 1.5mL/min. All measurements were optimized at 45°C temperature with a detection wavelength of 225.²⁴ Wavelength is selected because it showed a low background level, high absorption by SPMA and clear separation from other peaks into consideration.

**Validation of the HPLC method**

A system suitability test should be performed before starting validation of the HPLC method, where the standard solutions of both material and internal standard are injected sundry times and the relative standard deviation is calculated, which should be less than %2 at all.

**System Suitability Test (SST)**

SST was particularly fulfilled to decide the column efficiency, resolution (Rs), and repeatability of a specific chromatographic system to affirm its ability for a described evaluation. SST is an important characteristic of all HPLC analytical systems. When the system suitability for the evaluation method uses high-performance liquid chromatography, the
subsequent parameters have to be inappropriate outlines. 

**Retention time (Rt):** It is the interval time between the injection of the sample into the tool and the advent of the maximal peak at the detector.

**Capacity factor:** It expresses the overlap of the injected sample material with the column filling and the mobile phase and has to be more than 2.

**Resolution:** It demonstrates the dissociation power of the whole chromatographic system to the specific compounds of the commixture. It is expressed as the proportion of the range between the peak to the peak width value. If R is identical to or greater than one, then components are absolutely separated. If R is much less than one, then components are overlapped.

**Theoretical plates:** The quantity of theoretical plates is a degree of the overall performance of the column and has to be more than 2000.

The HPLC procedure was established with appreciation to the subsequent Parameters according to the ICH guidelines.

**Linearity:** The analytical method’s ability to provide measurement results that are directly proportional to the concentration of the analyzed material within the given range, either immediately or after performing precise mathematical transformations, was evaluated. The linearity of the standard series preparation within the range of 0.5-20 µg/ml was determined by generating linear calibration curves. These curves were obtained by plotting the peak area of the metabolite against its concentration.

The capacity of this analytical method is to offer measurement outcomes immediately proportional to the concentration of the analyzed material within the sample in the given range, either immediately or after performing precise mathematical transformations. The linearity of a standard series preparation within the range of 0.5-20 µg/mL has been determined. A linear standardization curve had been generated with the aid of using plotting the peak area in opposition to the concentration of the metabolite.

**Accuracy:** The HPLC procedure accuracy was carried out inside the procedure range as follows: three weights of SPMA were prepared to give concentration (5, 10 and 15) µg/mL. The percentage for every sample and the relative standard (RSD) were calculated. The relative standard value should not be greater than 2%.

**Precision:** Studies of repeatability and intermediate precision were carried out to analyze the precision of the method. Repeatability studies had been done by evaluation of three different concentrations of 5, 10 and 15 µg/mL of the sample material using HPLC. Procedure repeatability was performed from RSD% values gained by duplicating the measure several times around the same time for intra-day accuracy. The intermediate (inter-day) precision of the procedure was tested by a seemingly similar framework on various days under similar trial conditions.

**Specificity:** It was prescribed by injecting the internal standard solution.

**Limit of Detection (LOD):** It means the lowest quantity of the material that may be detected.

**Limit of Quantification (LOQ):** It is expressed by the lowest quantity of the material that may be quantitatively determined with appropriate precision and accuracy.

LOD and LOQ had been calculated by the subsequent equations:

\[
LOD = \frac{3.3 SD}{m} 1
\]

\[
LOQ = \frac{10 SD}{m} 2
\]

In which SD is the standard deviation of the intercept of the regression line, and m is the slope of the regression line.

**Analysis of Urine Samples (Assay):**

Sample preparation:

SPMA extraction from urine sample: liquid-liquid extraction (LLE) method was used for a 15 mL urine sample taken in a special plastic container for urine collection and incubated in a sample container equipped with ice to keep the samples at a low temperature (to stop bacterial metabolism that may affect the number of metabolites in the sample) until samples are extracted and analyzed.

The method of work:

3 mL of urine was taken from the sample and sulfuric acid H2SO4 was added at a concentration of 9 N until the medium became pH=1 (This stage is achieved in order to convert the substance from the salt form to its free form "Pre SPMA to SPMA")21.
For the next step, a waiting period of 10 minutes should be achieved, then the work continues by adding potassium hydroxide KOH at a concentration of 7.8N until it becomes pH=2 in order to adjust the remaining amount of sulfuric acid\(^{22,23}\).

Extraction was performed using 3 mL of ethyl acetate, repeating the process three times to increase the recovery. The extraction funnel was shaken cyclically for one minute during each extraction. Previous studies have demonstrated that ethyl acetate is the most effective solvent for extracting SPMA. The resulting organic phases were collected and then centrifuged at 5000 rpm for 30 seconds. The organic phase containing ethyl acetate (in the upper layer) is taken and dried by a vacuum evaporator \(\text{N}_2\) at room temperature, as in the study of Purwanto et al. \(^{22}\), because it is not available in Syria.

After obtaining the dry residue, it is dissolved in 0.5 mL distilled water and 2.5 mL mobile phase, and the solution is filtered with an HPLC micro filter with a diameter of 0.45 µm.

**Results and Discussion**

**Validation of HPLC method**

**System Suitability Test (SST)**

The system suitability test was conducted by the injection of six injections of extracted spiked urine samples. The dissociation was carried out by setting the pump at a flow rate of 1.5 mL/min. The retention times for Benzoic acid as internal standard (IS) and SPMA as external standard (ES) were found to be 15.6 and 17.3 min, respectively, see Fig. 3

Acceptable retention time (Rt), theoretical plates, tailing factor and good resolution for SPMA were obtained as shown in Table 1.

![Chromatogram of standards](image)

**Figure 3. Chromatogram of standards that contains 20 µg/mL of SPMA (Rt 17.3) and 10 µg/mL of Benzoic acid (Rt 15.6)**

**Table 1. SST for SPMA.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Theoretical plates</th>
<th>Resolution</th>
<th>Tailing Factor</th>
<th>Retention Time (min)</th>
<th>SPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>19151</td>
<td>3.09</td>
<td>1.08</td>
<td>17.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Linearity:** The plotting standard concentrations against peak areas for the compound showed a linear relationship. The curve equation was \(y=mx+b\) with linear regression method to estimate SPMA concentration. S-phenyl mercapturic acid showed a linear signal in the domain of 0.5-20 µg/mL for the compound. The corresponding a linear regression equation was \(y=1.4168x+0.0697\) and \((R^2)\) of 0.9995 for SPMA, see Fig. 4 An excellent correlation existed among the peak areas and concentrations of SPMA was presented in Table 2.
Table 2. Linear regression data for calibration curve.

<table>
<thead>
<tr>
<th>SPMA µg/mL</th>
<th>SPMA Area</th>
<th>IS (Benzoic acid) µg/mL</th>
<th>IS (Benzoic acid) Area</th>
<th>SPMA/IS Area</th>
<th>SPMA/IS concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>11.56</td>
<td>5</td>
<td>67.4</td>
<td>0.171513353</td>
<td>0.1</td>
</tr>
<tr>
<td>1</td>
<td>21.21</td>
<td>5</td>
<td>63.5</td>
<td>0.343015748</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>41.17</td>
<td>5</td>
<td>66.2</td>
<td>0.621903323</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>98.09</td>
<td>5</td>
<td>64.1</td>
<td>1.530265211</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>196.23</td>
<td>5</td>
<td>65.9</td>
<td>2.977693475</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>376.25</td>
<td>5</td>
<td>66.1</td>
<td>5.692133132</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 4. Calibration curve of SPMA

Accuracy: The accuracy test affirmed appropriate retrievals % with small (RSD%) against concentrations. The outcomes signify that the procedure is highly precise for the estimation of the mentioned material, as shown from the data in Table 3.

Table 3. Accuracy studies.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>AUC&lt;sub&gt;SPMA&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;Benzoic acid&lt;/sub&gt;</th>
<th>Determined Conc.</th>
<th>Mean Conc.</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.389</td>
<td>4.615</td>
<td>4.808</td>
<td></td>
<td>96.16</td>
</tr>
<tr>
<td></td>
<td>1.442</td>
<td>4.805</td>
<td></td>
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<td></td>
<td>1.435</td>
<td>4.78</td>
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<td></td>
<td>1.486</td>
<td>4.96</td>
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<td></td>
<td>1.463</td>
<td>4.88</td>
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<td>10</td>
<td>2.67</td>
<td>9.155</td>
<td>9.751</td>
<td></td>
<td>97.51</td>
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<tr>
<td></td>
<td>2.91</td>
<td>10.005</td>
<td></td>
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<td></td>
<td>2.89</td>
<td>9.935</td>
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<td></td>
<td>2.85</td>
<td>9.795</td>
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<td></td>
<td>2.87</td>
<td>9.865</td>
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<tr>
<td>15</td>
<td>4.23</td>
<td>14.685</td>
<td>14.683</td>
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<td>97.88</td>
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<td></td>
<td>4.16</td>
<td>14.435</td>
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<td></td>
<td>4.21</td>
<td>14.61</td>
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<td></td>
<td>4.19</td>
<td>14.54</td>
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<td></td>
<td>4.36</td>
<td>15.145</td>
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Average of Recovery=97.183 %
SD=0.905
RSD=0.932
**Precision:** The results of the repeatability and intermediate precision experiments are presented in Table 4. The proposed method was precise according to the RSD values which were less than 2%, as advised by the International Council for Harmonization guidelines.

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
<th>Intermediate precision Between-day (n=5)</th>
<th>Repeatability Within-day (n=5)</th>
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<tr>
<td></td>
<td>RSD</td>
<td>SD</td>
</tr>
<tr>
<td>5</td>
<td>1.10</td>
<td>1.08</td>
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<tr>
<td>10</td>
<td>1.25</td>
<td>1.23</td>
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<td>15</td>
<td>1.16</td>
<td>1.14</td>
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</table>

**Specificity:** The specificity was realized by completing the demarcation of SPMA peak in the existence of urine as shown in Fig. 3. No overlap was noticed due to any unknown materials of urine samples at the retention time of SPMA. The peak obtained was sharp and had clear baseline demarcation, and no peak was obtained at the retention time of SPMA in a blank urine sample as shown in Fig. 5.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** (LOD) was 0.126 µg/mL and (LOQ) was 0.380 µg/mL for SPMA.

![Figure 5. Chromatogram of the Blank Urine.](image-url)
Conclusion

Previously, several methods have been described for the determination of SPMA but most of these methods were with the high cost and some instruments do not exist in Homs City. The proposed HPLC method is a simple, precise, and reproducible technique for quantitatively analyzing SPMA in urine samples. The HPLC method was validated in accordance with ICH guidelines, and it was found to be distinct, with no overlap with other sample compounds. The developed procedure offers several benefits, like: being fast and cost-effective since it uses cheap IS, using easy-to-implement mobile phase, providing sample preparation steps by LLE, improved sensitivity (LOD=0.126-LOQ=0.380), a relatively moderate run time (approximately 22 minutes), a very good recovery (97.183%), and excellent linearity (R²=0.9995). These advantages make the method specific, reliable, and easily reproducible in any quality control setting, as long as all parameters are appropriately followed for their intended use. In addition, this method showed a new way to analyze SPMA with different equipment and proved superior in chromatogram separation with clear peaks.

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Authors’ Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Damascus.

Authors’ Contribution Statement

M.N.A and S.B contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript.

References


التحقق من مصدوقية طريقة تحليل S-phenyl mercapturic acid بالبول باستخدام الاستشراب السائل عالي الأداء مع كاشف الصمام الثنائي الضوئي

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قسم الأدوية والسموم، كلية الصيدلة، جامعة دمشق، دمشق، سوريا.

الخلاصة
تم تطوير وتوثيق مصدوقية طريقة تحليلية باستخدام جهاز الاستشراب السائل عالي الأداء HPLC تتمتع بالبساطة والانتقائية والدقة HPLC وذلك من أجل تعيين حمض س-فينيل ميركابتوريك. خُطّقت الفصل الاستشاري بواسطة استخدام عمود فصل من نوع (ODS-3) في لمبة (Inertsil) ببعض من (5μ, 150 mm x 4.6 mm). وكانت درجة حرارة العمود 45 درجة مئوية. واستخدم طور متحرك مؤلف من أسيتونتريل: ميثانول: ماء منزوع الشوارد محمض بحمض فوق الكلور (4/1/5 حجم/حجم/حجم pH=3) بمعدل جريان 1.5 مل/دقيقة واستخدام طول موجة مكشاف 225 نانومتر. أجري اختبارات ملاءمة النظام لتقييم مدى ملاءمة وفعالية النظام الاستشرابي بأكمله. خشب زمن احتباس مادة حمض س-فينيل ميركابتوريك وحمض البنزوئيك فقد وجدتا 17.3 و 15.6 دقيقة، على التوالي، وأجري التحقق من مصدوقية الطريقة المطورة من حيث الخطية والدقة والمضبوطة والنوعية وحد الكشف بعد التقدير الكمي. حيث كان حذ الكشف 0.126 مكغ/مل وحد التقدير الكمي: 0.38 مكغ/مل لمادة س-فينيل ميركابتوريك، ومردو: 97.183% ظهرت المادة استجابة خطية ضمن المجال (0.5-20 مكغ/مل). تهدف هذه الدراسة إلى تطوير طريقة تحليلية باستخدام جهاز الاستشراب عالي الأداء والتحقق من مصدوقيتها، ويمكن استخدام الطريقة المقترحة لتقدير مستقلب البنزن هذا في البول كونها بسيطة ودقيقة وانتقائية.

الكلمات المفتاحية: حمض البنزوئيك، الاستشراب السائل عالي الأداء، HPLC، حمض س-فينيل ميركابتوريك، اختبار ملاءمة النظام.