We are IntechOpen, the world’s leading publisher of Open Access books
Built by scientists, for scientists

6,600
Open access books available

177,000
International authors and editors

195M
Downloads

154
Countries delivered to

TOP 1%
Our authors are among the most cited scientists

12.2%
Contributors from top 500 universities

WEB OF SCIENCE™
Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com
Chapter
Rapid Qualitative and Quantitative HPLC/MS Analysis of an Antioxidant Couple Consisted of Glutathione and Ascorbic Acid in a Pharmaceutical Product

Stanislav V. Yefimov

Abstract
Vitamin C and glutathione are mostly found together in pharmaceutical products. These two components protect each other from oxidation by forming an antioxidant couple and mutually reinforcing each other’s actions. This paper describes a method for determining the activity of the antioxidant couple in pharmaceutical products using HPLC/MS. An Agilent 6125 HPLC equipped with MSD and DAD detectors was used. The first detector is for the detection of glutathione, and the second is for the detection of ascorbic acid. The MS spectrum of glutathione (Glut) was dominated by the signal m/z = 308.2, which corresponds to the Glut-H⁺ cation. The MS spectrum of ascorbic acid (AA) was dominated by signals m/z = 177 and 375, which corresponds to the cations AK-H⁺ and 2AK-Na⁺. The use of HPLC with two detectors (MS and DAD) made it possible to simultaneously determine both components of the antioxidant couple in pharmaceutical products without derivatization and any preliminary sample preparation. The method meets the FDA criteria for accuracy, selectivity, robustness, and reproducibility, and has a low detection limit of both components of the antioxidant couple.

Keywords: antioxidant couple, glutathione, ascorbic acid; potency, HPLC/MS; Validation

1. Introduction
In living cells, glutathione protects cells from oxidative damage and maintains redox balance [1]. Vitamin C (ascorbic acid) is one of the powerful reducing agents, it works as a neutralizer of oxidizing free radicals in a living cell [2]. Glutathione and
vitamin C often coexist in pharmaceutical products. Both components enhance each other's actions and protect each other from oxidation. This combination is called the antioxidant couple [3].

Determination of the activity of an antioxidant couple in pharmaceutical products is carried out using modern instruments and specially developed methods. In this work, we used HPLC/MS from Agilent [4].

When developing methods of analysis, it is customary to follow the recommendations of the International Conference on Harmonization (ICH), which contains the necessary criteria for validating methods [5]. It is also important for developers to achieve simplicity (fewer stages) and speed of analysis. Both factors affect its cost.

The complexity of the simultaneous analysis of glutathione and ascorbic acid is that Glutathione (Figure 1) is relatively poorly visible to UV-VIS detectors. To make it highly visible, chemists are forced to derivatize glutathione by adding a radical containing a benzoic or furan ring through the sulfhydryl group.

Ellman's reagent (5,5'-dithiobis (2-nitrobenzoic acid) [6, 7], O-phthalic aldehyde [8], 4-vinyl pyridine, 4-fluoro-7-sulfobenzofurazan ammonium salt, 4- (aminosulfonyl)-7-fluorobenzofurazan and N- (9-acridinyl) maleimide [9] are used as derivatizing agent. However, the glutathione molecule contains two carboxyl groups and can be ionized at (ESI) and detected by MSD [10, 11], or by HPLC/MS/MS [12] without derivatization.

The ascorbic acid molecule (Figure 1) contains a furan ring and is well detected by the UV-Vis detector. There are some literature data about methods for the determination of ascorbic acid and ascorbates by HPLC in fruits, blood serum, and pharmaceuticals [13–17].

Usually, the UV detector is used [13, 18–20], some authors prefer an electrochemical detector [16]. The tandem of MS and UV-VIS detectors is a useful and effective tool [14, 17, 21, 22].

As a mobile phase for HPLC/MS, methanol and acetonitrile volatile solutions are most popular [14, 17, 21, 22]. The not volatile sodium dihydrogen orthophosphate solutions may be used as a mobile phase for HPLC-UV [13, 16–19].

To simultaneously analyze several components in a pharmaceutical product, chemists select the appropriate detector and suitable chromatography conditions. In the case when the components differ in nature and their concentration is very different, a good result has given the use of two detectors, each of which is focused on the detection of its group of components. In the present study, we used two detectors, one for glutathione and the other for ascorbic acid. This approach allowed us to obtain a satisfactory result of the analysis with a tenfold difference in the concentration of the components and to exclude derivatization.

Figure 1.
Structures of the molecules forming an antioxidant couple.
The antioxidant couple analysis method presented in this article was developed for the analysis of nasal spray and injection solution. The specificity, precision, reproducibility, reliability, and selectivity of the method have been validated. The recommendations of the European Medicines Agency [5], as well as the FDA guidelines for the validation of analytical procedures [23–25], were used.

2. Materials and methods

Chemicals. L-ascorbic acid analytical standard from Sigma-Aldrich; formic acid 98-100% analytical grade from Merck; glutathione from European Pharmacopoeia Reference Standard, water HPLC grade purchased from Agilent. All the solvents used were of HPLC grade. Branded pharmaceutical formulation, in form of nasal spray and injection solution, was obtained from commercial sources and used as received, without any further purification.

Samples. All the samples were from a freshly prepared product. The products were tested: Glutathione injection solution, containing 20% of glutathione and 2% of ascorbic acid and, glutathione nasal spray, containing 20% of Glutathione and 3.5% ascorbic acid.

The instruments. The Agilent instrument (Agilent Single Quadrupole LC/MS instrument, 2019) includes the following components: quaternary pump 600 bar maximal pressure; single quadrupole (SQ) mass selective detector (MSD) with electrospray ionization (ESI), 150 V fragmentor, gas flow: 7 L/min, gas temperature 300°C, capillary 4000 V and nebulizer 15 psi; OpenLAB CDS Version 2.2. software. Reversed-phase (RP) column Poroshell 120 EC-C18, 50 × 4.6 mm, particles size 2.7 μm, with guard precolumn. Isocratic elution was performed with mobile phase: 0.1% formic acid water solution and flow rate of 1.0 mL/min.

A qualitative analysis was made using MDS spectrum. Glutathione shows the predominant signal of m/z+=308 which corresponds to the cation Glut-H+. Ascorbic acid (AA) shows m/z+=177, 199, and 375 which corresponds to the AA-H+ cation, AA-Na+ cation, and to the 2AA-Na+ cation respectively.

A quantitative analysis was made based on the standard calibration curve.

Preparation of standard stock solution. An accurately weighed of ascorbic acid and glutathione was dissolved in water. The solution was filtered through a 0.45 μm cellulose acetate membrane filter. The stock solution was stepwise diluted to make a set of standard solutions.

System suitability was tested and validated according to the Centre for Drug Evaluation and Research [24] and Agilent recommendations [25].

Calibration curve. Five standard concentration solutions were tested in the range of glutathione concentrations from 0.1 g/l to 1.0 g/l and AA concentrations from 0.04 g/l to 2.1 g/l. Each point of the calibration curve was the mean of five measurements. Slope (a), intercept (b), and correlation coefficient (r) were calculated by least squares using OpenLAB CDS software. The range of concentrations for which the correlation coefficient was equal to or greater than 0.999 was taken as the working range [24]. For MSD, the type of calibration curve (linear or parabolic) was chosen so that the correlation coefficient was maximum.

Accuracy was expressed as mean absolute recovery and percent relative standard deviation (RSD), for AA and glutathione samples in five copies for each concentration.

The precision of the method was determined by comparing the measurement results of five samples under the same experimental conditions. Intra- and inter-day tests were carried out.
Limits of detection (LOD) and quantification (LOQ). LOD is the minimum measurable amount of a substance, LOQ is the minimum measurable amount with acceptable linearity, accuracy, and precision. For a linear calibration curve, LOD is calculated using the formula (1) [5] where \( \sigma \) is the standard deviation of the response, and \( a \) is the slope of the line.

\[
LOD = 3.3 \times \frac{\sigma}{a} \quad (1)
\]

If the calibration curve is a parabola (\( y = ax^2 + bx + c \)), then the LOD is the root of the quadratic equation, which is calculated by the formula (2).

\[
LOD = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \quad (2)
\]

The standard deviation of the response \( \sigma \) is determined based on the calibration curve as the residual standard deviation of the regression line [5]. The LOQ may be estimated as 3 times LOD.

**Repeatability** was evaluated in the same tests as for the accuracy and recovery. The measure of repeatability is RSD.

To assess the **specificity** of the method, samples of the pharmaceutical compound and a standard solution of approximately the same concentration of the tested components were compared in terms of tail coefficient, retention time, and the number of theoretical plates. The difference was expressed in RSD.

The robustness of the method was evaluated by the effect of small changes in flow rate, column temperature, and mobile phase composition on the measurement result. The system suitability parameters (T and N) were determined, and the results were compared with acceptable limits.

**Statistical analysis.** Each experimental point was the average of five measurements. The results \( p < 0.05 \) were considered statistically significant. Data variation was expressed as standard deviation (SD) and relative standard deviation. A regression analysis based on the Least Squares Method was used to construct a calibration curve. The correlation coefficient \( r \) and the coefficient of determination \( r^2 \) were calculated.

### 3. Results

**System suitability.** The solution of a standard sample with a volume of 1 μl was injected five times. The relative standard deviation of the peak area, retention time, the number of theoretical plates, and tail factor were determined and compared with the acceptable limits (Table 1) according to the recommendations [26, 27].

The MS spectra and chromatograms are shown in Figures 2 and 3. As we can see in Figure 2B, the ascorbic acid DAD signal is clearly visible, while the glutathione peak is barely visible. The situation is the reverse of Figure 2A, the glutathione peak is high, and the ascorbic acid peak is very small. As a result of electrospray ionization, glutathione is converted into a single cation \( m/z=308 \) (Figure 3A), and ascorbic acid into several cations, including those with \( m/z=177 \) and 375 (Figure 3B).

**Linearity, range, and limit of detection.** The working range for glutathione was from 0.2 to 1.0 μg, and for AA from 0.5 to 1.0 μg. The limit of detection was calculated based on the standard deviation of the response (\( \sigma \)) and slope (\( a \)) according to Eq. (1) for ascorbic acid, and Eq. (2) for glutathione. The results of the LOD calculation are
**Table 1. System suitability.**

<table>
<thead>
<tr>
<th>Test parameters</th>
<th>Mean</th>
<th>S.D.</th>
<th>% RSD</th>
<th>Acceptable limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA Peak area (mAU min)</td>
<td>1,023.90</td>
<td>18.95</td>
<td>1.9</td>
<td>RSD ≤ 2</td>
</tr>
<tr>
<td>Glut. Peak area (counts min)</td>
<td>2,543,221.44</td>
<td>22,859.33</td>
<td>0.9</td>
<td>RSD ≤ 2</td>
</tr>
<tr>
<td>AA Retention time (min)</td>
<td>0.78</td>
<td>0.00</td>
<td>0.0</td>
<td>RSD ≤ 2</td>
</tr>
<tr>
<td>Glut. Retention time (min)</td>
<td>1.02</td>
<td>0.00</td>
<td>0.2</td>
<td>RSD ≤ 2</td>
</tr>
<tr>
<td>AA Theoretical plates (N)</td>
<td>*30,128.50</td>
<td>4,273.05</td>
<td>1.6</td>
<td>&gt; 2,000</td>
</tr>
<tr>
<td>Glut. Theoretical plates (N)</td>
<td>*47,000.00</td>
<td>4,242.64</td>
<td>9.0</td>
<td>&gt; 2,000</td>
</tr>
<tr>
<td>AA Tailing factor (T)</td>
<td>*1.29</td>
<td>0.01</td>
<td>0.6</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Glut. Tailing factor (T)</td>
<td>*1.60</td>
<td>0.01</td>
<td>0.4</td>
<td>≤ 2</td>
</tr>
</tbody>
</table>

Ascorbic acid (AA) is detected by DAD and glutathione (Glut.) is detected by MSD.
Ascorbic acid standard solution 2.7 μg/μL, injection volume 0.1 μL. Values are presented as mean ± S.D., n = 5. *p < 0.05
Glutathione standard solution 0.5 μg/μL, injection volume 1.0 μL. Values are presented as mean ± S.D., n = 5. *p < 0.05.

**Figure 2.**
(a) Chromatogram detected by MSD, high peak corresponds to glutathione. (b) Chromatogram detected by DAD (272 and 380 nm), high peak corresponds to ascorbic acid. Concentration of glutathione is 0.62 μg/μL, concentration of ascorbic acid is 0.122 μg/μL; mobile phase is aqua solution of 0.1% formic acid.

**Figure 3.**
Extracted spectrum (m/z+ value) of glutathione (A) and ascorbic acid (B). Concentration of glutathione is 0.62 μg/μL, concentration of ascorbic acid is 0.122 μg/μL; mobile phase is aqua solution of 0.1% formic acid.
shown in Table 2. Linear regression in the case of MSD does not have a good correlation coefficient, so parabolic regression was used for MSD, it gives a satisfactory correlation coefficient ($r \geq 0.999$).

**Accuracy/recovery and precision.** The accuracy of the method was validated for recovery at 3 different concentrations in five replicate tests. Recovery was determined based on the calibration curve. The results are shown in Tables 3 and 4. Interday analysis shows the stability of the AA and Glut samples during the day.

**Selectivity analysis (Table 5).** Two peaks of both components were compared, one for the standard solution and the other for the drug. It has been shown that the presence of other ingredients in the composition of the drug does not affect the recovery of glutathione and ascorbic acid. In particular, the relative standard deviation of recovery does not exceed 0.72%. Thus, the method is specific to the antioxidant couple.
To test for robustness, the flow rate, column temperature, and formic acid concentration in the mobile phase were varied. The system suitability parameters (T and N) were determined; they were within acceptable values for all changes in the analysis conditions (Table 6). Thus, the method is robust.

4. Discussion

The use of two detectors (MSD and DAD) for the analysis of the antioxidant couple is efficient and extends the range of HPLC analysis. In the tandem of detectors,
**5. Conclusion**

The HPLC/MS method has been developed for the determination of the antioxidant couple consisting of glutathione and ascorbic acid in pharmaceutical products. The use of a tandem of DAD and MSD detectors is substantiated. The method has been validated for accuracy, stability, and precision. The method has a low detection limit. The presence of foreign components in samples including sodium hydroxide, disodium EDTA, and benzyl alcohol does not impair the accuracy of the analysis. The method provides a fast, sensitive, accurate, and reproducible means of determining the antioxidant couple in pharmaceuticals. Preliminary special preparation of samples is not required.

**Conflict of interest**

The authors claim that there is no conflict of interest.

**Abbreviations**

- ACN: acetonitrile
- AA: Ascorbic acid
- DAD: Diode Array Detector
Rapid Qualitative and Quantitative HPLC/MS Analysis of an Antioxidant...
DOI: http://dx.doi.org/10.5772/intechopen.102975

ESI electrospray ionization
FDA Food and drug administration
Glut. Glutathione
ICH International Conference on Harmonization
LOD limit of detection
m/z mass per charge unit
MSD mass selective detector
MW Molecular weight
RP reversed-phase
SQ single quadrupole
UV-VIS Ultraviolet-Visible

Author details

Stanislav V. Yefimov
Pharmetric Laboratory, St. Petersburg, FL, USA

*Address all correspondence to: stanislav@pharmetriclab.com

IntechOpen

© 2022 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
References


