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Original Article

Rapid qualitative and quantitative HPLC/MS analysis of an antioxidant couple consisted of glutathione and ascorbic acid in a pharmaceutical product

[Análisis cualitativo y cuantitativo rápido por HPLC/MS del par antioxidante glutatión y ácido ascórbico en un producto farmacéutico]

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Abstract

Context: In pharmacy products, glutathione and vitamin C are often present together. Both components not only enhance each other's action but also protect each other from oxidation, forming an antioxidant couple. The work is devoted to the development of an HPLC method for determining the activity of pharmaceutical preparations containing the antioxidant couple as an active substance.

Aims: Develop and validate an HPLC/MS method for the determination of an antioxidant couple of glutathione and ascorbic acid in pharmaceutical products. The method should be fast and simple, that is, analyze both components in one run without derivatization and any preliminary sample preparation.

Methods: The Agilent 6125 C SQ LS/DAD/MS instrument was used to detect the two active components simultaneously. MSD was used to detect glutathione; DAD was used to detect ascorbic acid.

Results: The run time was 2.0 min. Glutathione was eluted with a retention time of 1.48 min. Its limit of detection was 0.03 µg. Ascorbic acid was eluted with a retention time of 1.02 min. Its limit of detection was 0.01 µg. Recovery of glutathione varied from 92 to 105%, and the recovery of ascorbic acid varied from 99 to 100%. In positive electrospray ionization mode, the spectra of glutathione (Glut.) showed the predominant signals at m/z+ of 308.2, which corresponds to cation (Glut.-H+). Ascorbic acid (AA) is converted into several cations, including AA-H+ and 2AA-Na+, m/z+= 177 and 375.

Conclusions: The developed HPLC/MS method for the determination of antioxidant couple consisted of glutathione and ascorbic acid in nasal spray and injection solution was validated for accuracy/recovery, precision, and selectivity. The use of a tandem of a mass spectrometric detector and a diode-array detector is substantiated. The method is fast, simple, sensitive and reproducible, and is suitable for the determination of the antioxidant couple in pharmaceutical products without derivatization or any preliminary sample preparation.

Keywords: antioxidant couple; ascorbic acid; glutathione; LC/MS; potency; RP-HPLC; validation.

Resumen

Contexto: En los productos farmacéuticos, el glutatión y la vitamina C suelen estar presentes juntos. Ambos componentes no solo mejoran la acción del otro, sino que también se protegen entre sí de la oxidación formando una pareja antioxidante. El trabajo está dedicado al desarrollo de un método HPLC para determinar la potencia de los productos farmacéuticos que contienen el par de antioxidantes como componente activo.

Objetivos: Desarrollar y validar un método HPLC/MS para la determinación del par antioxidante compuesto por glutatión y ácido ascórbico en productos farmacéuticos. El método es rápido y simple, pues analiza ambos componentes en una sola ejecución sin derivatización y sin preparación preliminar de la muestra.

Métodos: Se utilizó el instrumento Agilent 6125 C SQ LS/DAD/MS para detectar los dos componentes activos simultáneamente. Se utilizó un detector de espectrometría de masas para detectar el glutatión. Se utilizó un detector de arreglo de diodos para detectar el ácido ascórbico.

Resultados: El tiempo de ejecución fue de 2,0 min El glutatión se eluyó con un tiempo de retención de 1,48 min, su límite de detección fue de 0,03 µg. El ácido ascórbico se eluyó con un tiempo de retención de 1,02 min, su límite de detección fue de 0,01 µg. La recuperación de glutatión varió de 92 a 105% y la recuperación de ácido ascórbico varió de 99 a 100%. En el modo de ionización por electropulverización positiva, los espectros de glutatión (Glut) mostraron las señales predominantes en m/z+ de 308,2 que corresponde al catión (Glut.-H+). El ácido ascórbico (AA) se convierte en varios cationes, incluidos AA-H+ y 2AA-Na+, m/z+ = 177 y 375.

Conclusiones: El método HPLC/MS desarrollado para la determinación del par de antioxidantes glutatión y ácido ascórbico en aerosol nasal y solución de inyección fue validado por exactitud/recuperación, precisión y selectividad, así como bajo límite de detección y cuantificación. Se comprobó la validez del uso de detectores de arreglo de diodos y espectrometría de masas. El método es rápido, simple, sensible y reproducible, y es adecuado para la determinación del par antioxidante en productos farmacéuticos sin derivatización ni preparación previa de muestras.

Palabras Clave: ácido ascórbico; glutatión; LC/MS; par antioxidante; potencia; RP-HPLC; validación.

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INTRODUCTION

Glutathione protects cells from oxidative damage and maintains redox balance (Forman et al., 2009). Vitamin C (ascorbic acid) is one of the potent reducing agents and scavengers of free radicals in biological systems, working as a scavenger of oxidizing free radicals (Pehlivan, 2017). In pharmacy products, glutathione is often present along with ascorbic acid. Both components enhance each other's action and protect each other from oxidation, forming a so-called antioxidant couple (Meister, 1994).

Glutathione (Fig. 1) is relatively poor visible for UV-VIS or Diode Array Detectors (DAD) at wavelength 200 nm and more. To make it good visible for UV-VIS or fluorescent detectors, chemists derivatize glutathione attaching through sulfhydryl group of glutathione a radical containing a benzoic ring, or furan ring.

Among the derivatizing agent are popular: Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (Florholmen-Kjær et al., 2014; Appala et al., 2020), O-phthalaldehyde (Hou et al., 2018), 4-vinylpyridine, 4-fluoro-7-sulfobenzofurazan ammonium salt, 4-(aminosulfonyl)-7-fluorobenzofurazan and N-(9-acridinyl) maleimide (The Protein Man's Blog, 2020). From another site, glutathione contains two carboxyl groups in his molecule. This fact suggests that the molecule may be easily charged and good detectable by MSD (Rellán-Alvarez et al., 2006; Mika et al., 2012) and using HPLC/MS/MS technique (Herzog et al., 2019).

In contrast to glutathione, ascorbic acid (Fig. 1), whose molecule contains a furan ring, is clearly visible for a UV-VIS detector. Literature provides some information about the quality and quantity methods for the determination of ascorbic acid and ascorbates by HPLC in fruits (Frenich et al., 2005; Sawant et al., 2010; Boonpangrak et al., 2016) in blood serum and in pharmaceutical samples (Gazdik et al., 2008; Mitić et al., 2011). Most often the UV detectors are used (Watada, 1982; Lloyd et al., 1987; Racz et al., 1990; Sawant et al., 2010), but some authors prefer an electrochemical detector (Gazdik et al., 2008). Mass spectrometry detection is used in tandem with UV-VIS or Diode Array Detector (Frenich et al., 2005; Mitić et al., 2011; Szultka et al., 2014; Yefimov, 2019). The mobile phase composition is varied: Sodium dihydrogen orthophosphate solutions are often used for HPLC-UV (Watada, 1982; Lloyd et al., 1987; Racz et al., 1990; Gazdik et al., 2008; Sawant et al., 2010; Mitić et al., 2011), but for HPLC/MS, methanol acetonitrile-based solutions are most popular (Frenich et al., 2005; Mitić et al., 2011; Szultka et al., 2014; Yefimov, 2019). The direct way to analyze an antioxidant couple in a pharmaceutical product is to choose the appropriate detector and appropriate chromatographic conditions to reach good detection of both components. But if the analytes differ by nature and their amount in the formulation also differs significantly, 10 times as in our case, using the two detectors is more effective. In the present study, we have developed and validated HPLC/MS method for the qualification and quantification of an antioxidant couple composed of glutathione together with ascorbic acid in pharmaceutical products. We used two detectors, one for the first component and the other for the second component, which provided good analysis with a 10-fold difference in the concentration of the components.

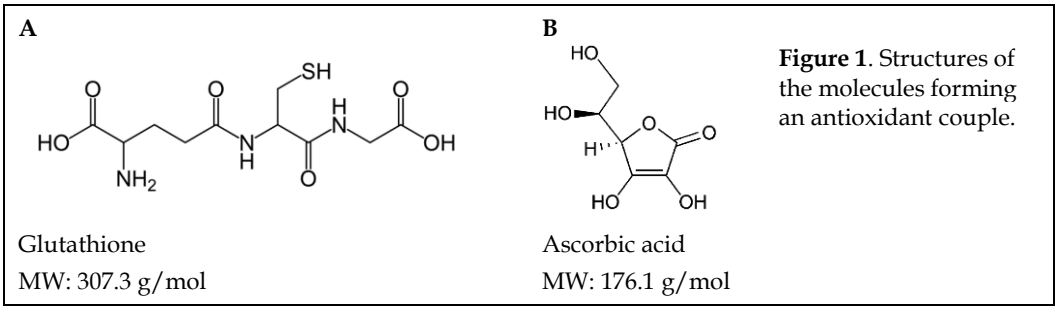


Figure 1. Structures of the molecules forming an antioxidant couple.

The purpose of the present work is to develop a fast method for qualitative and quantitative estimation of the antioxidant couple in pharmaceutical products, validate the method for accuracy, precision, repeatability, selectivity, specificity, and robustness according to (European Medicines Agency. ICH, 2006; FDA Guidance for Analytical Procedures and Methods Validation, 2015).

MATERIAL AND METHODS

Chemicals

Water HPLC grade purchased from Agilent, glutathione from European Pharmacopoeia Reference Standard, L-ascorbic acid analytical standard from Sigma-Aldrich, formic acid 98-100% analytical grade from Merck. HPLC grade solvents were used. Nasal sprays and injection solutions were obtained from commercial sources and used as received.

Samples

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

Chemical analysis

Instrument

Agilent LC/MS instrument consists of the following components: Single quadrupole (SQ) mass selective detector (MSD) with electrospray ionization (ESI) and 150 V fragmentor, plus Diode Array Detector (DAD). Gas temperature is 300°C, capillary voltage 4000 V and nebulizer 15 psi. Reversed-phase (RP) Column is Poroshell 120 EC-C18, 50 ×v4.6 mm, particles size 2.7 µm, with guard precolumn; quaternary pump with flow: 1.0 mL/min, high-pressure limit: 600 bar. Isocratic elution was performed with mobile phase: 0.1% formic acid water solution.

Quantitative analysis

A qualitative analysis of the antioxidant couple was made by the value of mass/charge (m/z+). The glutathione (Glut.) showed the predominant signal of 308 m/z+ corresponding to the cation Glut-H+. Ascorbic acid (AA) showed 177 m/z+, which corresponded to the AA-H+ cation, 199 m/z+ AA-Na+ cation, and 375 m/z+, which corresponded to the 2AA-Na+ cation.

The compound was quantified using a compound-specific calibration curve.

Preparation of standard stock solution

An accurately weighed 50 mg of glutathione and 150 mg of ascorbic acid was dissolved in 50 mL of water. A 0.45µm cellulose acetate membrane filter was used to filtrate the solution. A set of standard solutions of the antioxidant couple with various concentrations were prepared from the stock by dilution with water.

The system suitability was validated according to the Center for Drug Evaluation and Research (CDER, 1994) and the System Suitability Assessment Guidelines (Evaluating System Suitability CE, GC, LC and A/D ChemStation, 2019). Parameters were peak area, retention time, number of theoretical plates (N), and tailing factor (T).

Calibration curve and coefficient of correlation

The sets of dilutions of standard stock solution in a range of glutathione concentrations from 0.1 g/L to 1.0 g/L and of AA concentrations from 0.04 g/L to 2.1 g/L were tested. The correlation coefficient (r) of peak areas with the corresponding concentrations was calculated by the least square method, and a calibration curve was plotted. Calculation of the calibration curve was performed automatically by OpenLAB CDS. Each point was the average of five measurements. The range of concentrations for which the correlation coefficient was equal to or greater than 0.999 was taken as the working range (CDER, 1994). For the MSD, the type of the calibration curve (linear or parabolic) was chosen so that to maximize the correlation coefficient.

Accuracy

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 value of the ratio of the measured amount of the analyte to the true amount of the analyte, expressed as a percentage, was used as the recovery value.

The precision/accuracy of the method was determined by the RSD value from the analysis of five samples of the same concentration under the same experimental conditions. The intraday and interday analysis was compared by RSD and recovery.

Limits of detection (LOD) and quantitation (LOQ)

LOD characterizes the sensitivity of a method. It is the minimum amount of a substance that can be measured by a given method, whereas the LOQ is the lowest concentration with acceptable linearity, accuracy, and precision. If the equation of the calibration curve is an equation of the first degree (straight line), then LOD is calculated by formula [1] (European Medicines Agency. ICH, 2006). Here (σ) is the standard deviation of the response, and (a) is the slope of the line.

$$LOD = 3.3^* \sigma/a \quad [1]$$

Similarly, if the equation of the calibration curve is an equation of the second degree, parabola ($y = ax^2 + bx + c$), then the LOD is the root of the quadratic equation, which is calculated by formula [2].

$$LOD = (-b+SQRT(b^*b-4^*a^*(c-3.3^*\sigma)))/(2^*a) \quad [2]$$

Where the (σ) is the residual standard deviation of the regression line (European Medicines Agency. ICH, 2006). LOQ is 3 times LOD.

Repeatability, specificity, and robustness

A measure of repeatability was the RSD of the mean of five independent tests of samples of the same concentration.

To estimate the specificity of the method, pairs of the antioxidant couple in a pharmaceutical compound and of standard solution about the same concentration of tested components compared by retention time, tailing factor, and number of theoretical plates. The difference in these pairs was expressed as RSD.

To demonstrate the robustness of the method, flow rate, column temperature, and mobile phase composition were varied. The tailing factor (T) and the number of theoretical plates (N) were calculated from the robustness test results. The results were compared with the acceptable limits.

Statistical analysis

Statistical analysis included calculating mean, standard deviation (SD), relative standard deviation (RSD), and correlation coefficient (r). Results $p<0.05$ were considered statistically significant. The least-squares regression analysis was used. In most cases, the calculation was performed automatically by the OpenLAB CDS program.

RESULTS AND DISCUSSION

System suitability

Test parameters were assessed by injecting 1 µL of analyte standard solution 5 times. The acceptable limit was chosen according to recommendations (Dr. Deepak, 2013; Bose, 2014). The parameters such as relative standard deviation of peak area, retention time, number of theoretical plates, and tailing factor were determined and calculated automatically by OpenLAB CDS software and stated in Table 1.

The chromatograms and extracted MS spectra are presented in Figs. 2 and 3.

As can be seen, using the DAD, the AA signal is clearly visible (Fig. 2B), and the peak of glutathione is hardly visible. The situation is reverse if MSD is used: the peak of glutathione is dominant, and the AA peak is very small (Fig. 2A). MS specters of both components are presented in Fig. 3. As a result of electrospray ionization, glutathione is converted into the only cation m/z+ = 308 (Fig. 3 A), and AA is converted into several cations, including m/z+ = 177 and 375 (Fig. 3B).

Table 1. System suitability.

Test parameters	Mean	SD	% RSD	Acceptable limit
AA Peak area (mAU·min)	1023.90	18.95	1.9	RSD ≤2
Glut. peak area (counts·min)	2543221.44	22859.33	0.9	RSD ≤2
AA retention time (min)	0.78	0.00	0.0	RSD ≤2
Glut. retention time (min)	1.02	0.00	0.2	RSD ≤2
AA theoretical plates (N)	30128.50*	4273.05	1.6	>2000
Glut. theoretical plates (N)	47000.00*	4242.64	9.0	>2000
AA tailing factor (T)	1.29*	0.01	0.6	≤2
Glut. tailing factor (T)	1.60*	0.01	0.4	≤2

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

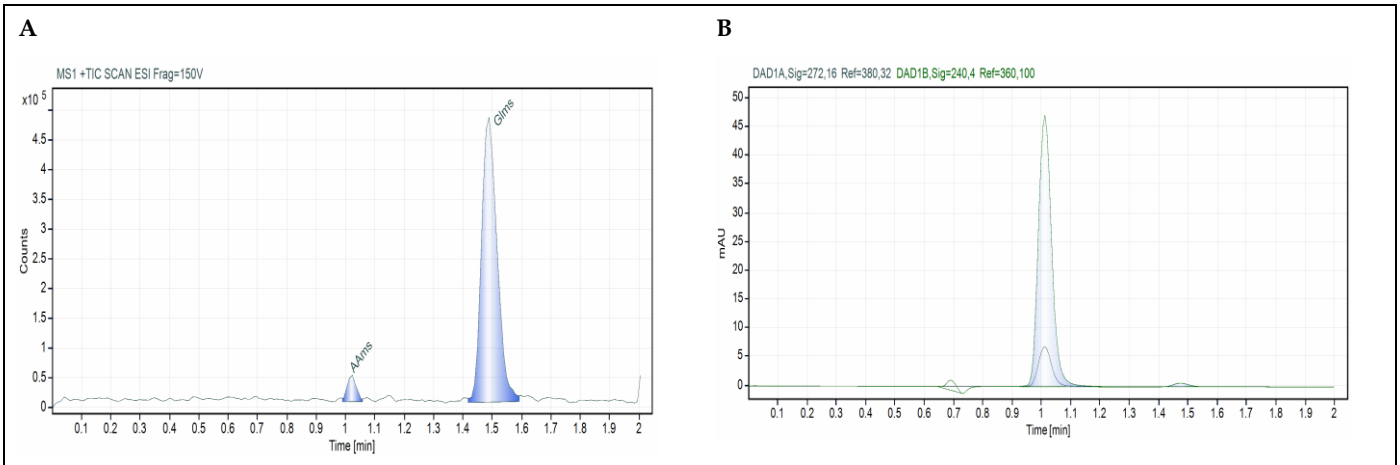


Figure 2. **(A)** Chromatogram detected by MSD, high peak corresponds to glutathione. **(B)** Chromatogram detected by DAD (272 and 380 nm), high peak corresponds to ascorbic acid.

The concentration of glutathione was 0.62 µg/µL, the concentration of ascorbic acid was 0.122 µg/µL; mobile phase was water solution of 0.1% formic acid.

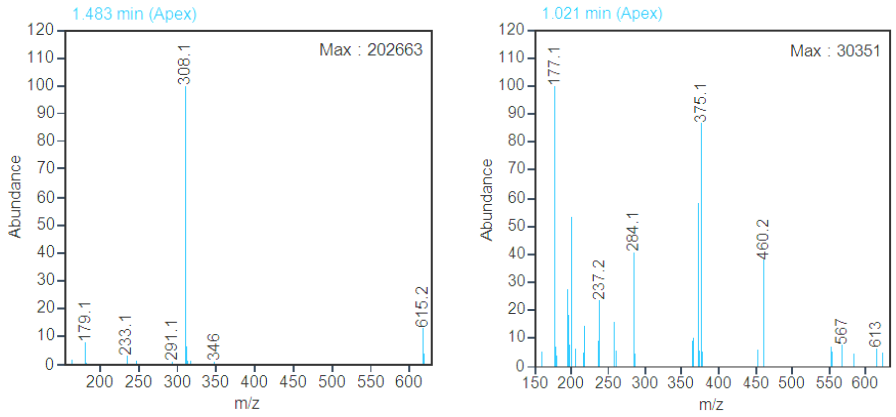


Figure 3. Extracted spectrum (m/z+ value) of glutathione (**A**) and ascorbic acid (**B**). The concentration of glutathione was 0.62 µg/µL, the concentration of ascorbic acid was 0.122 µg/µL; mobile phase was water solution of 0.1% formic acid.

Linearity, range, and limit of detection

The working range was determined for glutathione from 0.2 to 1.0 µg and for AA from 0.5 to 1.0 µg. The limit of detection was calculated by equation [1] for ascorbic acid and by [2] for glutathione. The results are shown in Table 2. Parabolic regression has always been used for MSD and gives a satisfactory correlation coefficient (r≥0.999). The theoretical rationale for this decision is outside the scope of this work. It will be presented in a subsequent publication.

Accuracy/recovery and precision

To establish the accuracy of the method, samples that contained three different concentrations of the component of interest were measured five times, the mean and RSD were calculated. The recovery was determined based on the calibration curve (Table 2). Tables 3 and 4 present data were confirming the accuracy/reproducibility and precision of the method. Inter-day analysis (next day test) does not show any significant degradation of AA and Glut.

Selectivity assay

The results of the analysis of the standard solution and the test solution with the same concentration of the tested components were compared. The presence of other ingredients does not affect the recovery of glutathione and ascorbic acid. In particular, the relative standard deviation of the recovery does not exceed 0.72%. Thus, the method is specific for the antioxidant couple.

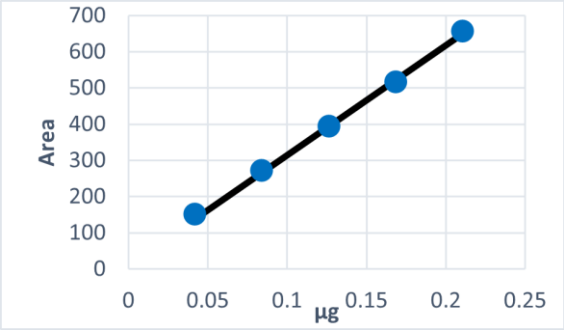
Within the framework of establishing the robustness of the method, the components of the antioxidant couple were determined by changing the flow rate or temperature of the column or the composition of the mobile phase. The parameters N and T were calculated. These parameters were in the permissible range. Thus, this method is robust (Table 6).

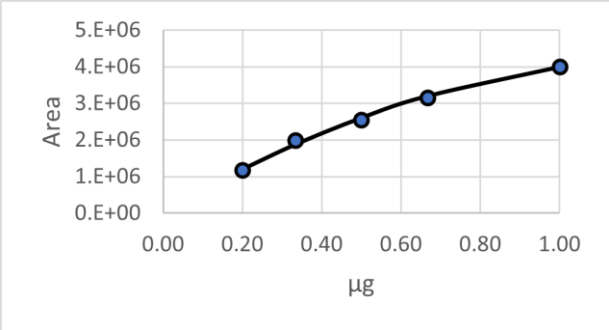
In our previous work (Yefimov, 2019), an improved method for the determination of ascorbic acid using HPLC/MS with two detectors, DAD and MSD, was validated. In this paper, we develop this approach and extend it to the analysis of two components simultaneously. The advantage of the method is that one of the components (glutathione) is poorly recorded by DAD but good by MSD. In addition, the advantage of using MSD as a second detector is that it provides information on the qualitative composition of the mixture. However, the use of MSD for quantitative analysis has several limitations. First, it is not always easy to select the analysis parameters to have a satisfactory correlation coefficient (r>0.999). The situation is improved using parabolic regression. Second, in complex multicomponent mixtures, when several

different molecules are ionized, there is a mutual repulsion of the particles. This repulsion can be the source of incorrect analysis results if the calibration curves plotted for one component are used.

Table 2. Linearity, Range and LOD.

AA (µg)	Mean Y (n = 5)	Y calc.	ΔY	Glut. (µg)	Mean Y (n = 5)	Y calc.	ΔY
0.0422	150	145	4.8	0.20	1174797	1193679	-18882
0.0844	270	271	-1.0	0.33	1984510	1869527	114983
0.1266	392	397	-4.8	0.50	2543221	2596191	-52970
0.1688	516	523	-6.6	0.67	3147655	3190815	-43160
0.211	656	648	7.6	1.00	4005954	3985687	20268





y = ax + b		y = ax² + bx + c	
a	2981.04	a	-2370063
b	19.4	b	6334085
c	-	c	21664.53
r	0.9991	r	0.99905
Mean ΔY (n = 5)	-1.2	Mean ΔY (n = 5)	4047.61
S.D. ΔY (n = 5)	6.31	S.D. ΔY (n = 5)	68145.19
LOD (µg)	**0.01**	LOD (µg)	**0.032**

"x" - the content of AA or Glut. in the sample; "Y"- the peak area; "Y calc." – the calculated peak area; "ΔY" – the residues; "a"- the slope of the regression line; "b"- the intercept; "r" – the correlation coefficient; "SD. ΔY" – the residual standard deviation of the regression line (σ). Ascorbic acid (AA) was detected by DAD, and glutathione (Glut.) was detected by MSD. Calibration curves are in the table. Standard errors of the calibration points are represented by the size of the circles ($p<0.05$).

Table 3. Accuracy, recovery, repeatability.

AA (µg)	Mean recovery (µg)	± SD	RSD (%)	Recovery (%)
1.335	1.316	0.077	6.0	99*
1.780	1.776	0.054	0.0	100*
2.670	2.677	0.092	0.0	100*
1.780#	1.70	0.010	4.0	96*

Recovery data presents an average value of five independent determinations (n = 5). #The bottom row corresponds to the inter-day analysis. Samples were kept overnight in closed vials at 19°C. *p<0.05. Ascorbic acid (AA) was detected by DAD.

Table 4. Accuracy, recovery, repeatability.

Glut. (µg)	Mean recovery (µg)	SD	RSD (%)	Recovery (%)
0.534	0.54	0.03	5.0	101*
0.84	0.88	0.02	3.0	105*
1.335	1.23	0.00	0.0	92*
0.84#	0.84	0.03	4.0	100*

Recovery data presents an average value of five independent determinations (n = 5). #The bottom row corresponds to the inter-day analysis. Samples were kept overnight in closed vials at 19°C. *p<0.05. Glutathione (Glut.) is detected by MSD.

Table 5. Specificity.

Active component, detector	Active component (µg/µL)	Mean peak area (standard) (n = 3)	Mean peak area (drug) (n = 3)	RSD (%)
Glut.	0.200	1755849	1759024	0.13
AA	0.054	218	220	0.72

Two peaks are compared: one for a standard solution and the other for a dilute solution for injection containing the following components: glutathione 0.2 g/L, ascorbic acid 0.054 g/L, disodium EDTA 0.001 g/L, NaOH 0.02 g/L, benzyl alcohol 0.015 g/L. Ascorbic acid (AA) was detected by DAD, and glutathione (Glut.) was detected by MSD.

Table 6. Robustness.

Parameter	Ascorbic acid (0.1 µg)				Glutathione (0.5 µg)			
	T	%RSD	N	%RSD	T	%RSD	N	%RSD
Flow rate 1.0 mL/min	1.24	1.5	30709	1.8	1.59	1.6	44504	1.9
Flow rate 1.05 mL/min	1.30	1.5	28320	1.7	1.60	1.7	50693	1.9
Temperature 22°C	1.24	1.5	30709	1.8	1.59	1.6	44504	1.9
Temperature 24°C	1.42	1.7	29552	1.5	1.81	1.8	43332	1.7
Mobile phase								
Formic acid 0.10%	1.24	1.5	30709	1.8	1.59	1.6	44504	1.9
Formic acid 0.13%	1.21	1.4	30889	1.6	1.56	1.5	44651	1.8

T: Tailing factor (mean); N: Theoretical plates (mean); n = 5. Ascorbic acid was detected by DAD, and glutathione was detected by MSD.

CONCLUSIONS

The HPLC/MS method for the determination of the antioxidant couple composed of glutathione and ascorbic acid in pharmaceutical products containing various other ingredients including excipients sodium hydroxide, disodium EDTA, benzyl alcohol was developed. The use of two detectors (MSD and DAD) has been substantiated. The method has been validated for accuracy, robustness, and precision. It has a low value of limits of detection. The method is suitable for the determination of an antioxidant couple in pharmaceutical formulations without prior sample preparation.

CONFLICT OF INTEREST

The author declares no conflicts of interests.

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