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Express qualitative and quantitative HPLS/MS analysis of the ascorbic acid in pharmaceutical product

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method has been developed and validated for qualitative and quantitative estimation of ascorbic acid in pharmaceutical products. A C18 column was used with elution of 0.1% (v/v) Formic acid solution in HPLC-grade water as mobile phase at a flow rate of 0.6 mL/ min. UV lamp and Diode-array detection (DAD) was performed at 272 nm. The method was validated for accuracy, precision, linearity, specificity and sensitivity in accordance with International Conference on Harmonization guidelines. Total run time was 1.0 min. Ascorbic acid was eluted with a retention time of 0.22 min. Validation revealed that the method is specific, accurate, precise, reliable and reproducible. Calibration plots were linear over the working ranges 0.01–1.00 µg per injection. The limit of detection was 0.01 µg per injection. Recovery was 100% and the relative standard deviation (R.S.D.) was <1.0%. In positive ESI mode, the spectra showed the predominant signals at m/z of 177.1 for pure ascorbic acid and m/z of 199.1 for ascorbic acid when relative high concentration of Na is presented in the sample. The high percentage recovery, low coefficient of variation and short time of analysis confirm the suitability of the method for express analysis of Ascorbic acid in pharmaceutical products.

Keywords: RP-HPLC; LC-MS; Validation; Ascorbic acid, Ascorbates.

1. INTRODUCTION

Quality control and potency determination is an important and necessary part of pharmaceutical products manufacturing. Using a modern highly effective analytical instrument such as Agilent HPLC/MS make this task easily done [1]. International Conference on Harmonization (ICH) guidelines give necessary analytical criteria for the validation of analytical methods [2]. There is an additional and very important consideration for analytical laboratory criteria - time and cost of an analysis. Literature provides some information about quality and quantity methods for determination of Ascorbic acid and ascorbates by HPLC in fruits [3,4,9,10] blood serum [7] and in pharmaceutical samples [7,11]. Most often the UV detectors are used [3-6] but some authors prefer an electrochemical detector [7]. Mass spectrometry detection is used in tandem with UV-VIZ or Diode Array Detector (DAD) [8-10]. The mobile phase composition is varied: Sodium dihydrogen orthophosphate aq. Solutions are often used for HPLC-UV [3-7,11], but for HPLC/MS, methanol and acetonitrile-based solutions are most popular [8,9,10]. The aim of the presented work was to improve the analytical procedure and to have fast and simple method which gives us the chance to process a large bunch of samples daily.

In the present study we have developed and validated a fast, sensitive and selective HPLC/MS method for the qualification and quantification of ascorbic acid in pharmaceutical products with using a new opportunity that the Agilent HPLC/MS instrument gave us.

2. MATERIALS AND METHODS

Water for HPLC purchased from Agilent; Ascorbic acid (AA) C6H8O6 99.7% was purchased from Fagron Inc. USA; HCl aq. 36.5 - 38.0% solution from VWR LLC USA; Formic acid 98-

100% analytical grade from Merck; Sodium bicarbonate, EDTA disodium and Benzyl alcohol from Medica Inc. USA.

2.1. Optimized chromatographic condition

Instrument: Agilent 6125 C SQ LS/MS [1]

Software: OpenLAB CDS Version 2.2

Detectors: Diode array detector, Single Quadrupole Mass Selective Detector (MSD) with Electrospray ionization (ESI).

Column: Agilent ZORBAX SB-C18, 2.1 x 50 mm, particle size:1.8 µm.

Quat. Pump Method:

Flow: 0.600 mL/min

High Pressure Limit: 600.00 bar

Maximum Flow Gradient: 100.000 mL/min^2

DAD Method

Peakwidth: > 0.1 min (2 s response time) (2.5 Hz)

UV Lamp Required: YES

Analog Zero Offset: 5 %

Analog Attenuation: 1000 mAU

Signal A (nm)/ Bandwidth (nm): 272/16

Ref Wavel. (nm)/ Ref Bandw. (nm): 380/32

Spectrum Range WL (nm): from 190 to 400

SQ Mass Spectrometer:

Ion Source: ESI

Fragmentor (V): 150

Polarity: positive

Gas Temperature (°C): 300

Gas Flow (l/min): 7

Nebulizer (psi): 15

Capillary (V): 4000

Qualitative analysis:

Recovery of the analyte was made by MS and DAD peak retention time. Dominated MS signal of +177.1 m/z or +199.1 m/z show presence of Ascorbic acid which is ionized in the form of +C6H8O6H (m/z= 177.1) or in form + C6H8O6Na (m/z= 199.1) if a lot of Na is presented in the sample.

Quantitative analysis:

All the quantitative data was made based on the area of DAD detected peaks of the analyte.

Method validation:

The method was validated for linearity, accuracy, precision, repeatability, selectivity and specificity according to International Conference on Harmonization guidelines [2].

Standards preparation:

116, 49 and 37 mg of Ascorbic acid was weighed precisely and dissolved in 50 mL of 1.0 mM HCl water solution to obtain a stock concentration of 2.32, 0.98 and 0.74 g/L. Standards were freshly prepared.

Linearity assay:

To obtain the working solution, aliquots of standard Ascorbic acid stock solutions were serially diluted by 1.0mM HCl solution to a concentration of from 0.1 to 1.0 g/L. The linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to the concentration. Least square regression analysis was done for the data obtained. The linearity was studied over a concentration range of 0.01 – 1.0 g/L. Replicates of three injections were performed for each sample. Linearity data were computed automatically in OpenLAB CDS Version 2.2 Agilent software.

Accuracy/recovery and precision assay:

The accuracy of the method is the closeness of the measured value to the true value for the sample. Accuracy was assessed as percent relative error and mean percent recovery. The accuracy of the method was checked by determining recovery values. Accuracy/ recovery was calculated for three runs of each solution. The precision was determined by measuring three sample probes under the same experimental conditions. To calculate precision, intra- and inter-day tests were performed and the results were expressed as relative standard deviation (RSD, %).

Limits of detection (LOD) and quantitation (LOQ) assay:

The limits of detection and quantitation were determined by serial dilutions of ascorbic acid solutions in order to obtain signal/noise ratios of 3:1 for LOD and 10:1 for LOQ. Appropriate amounts of standard Ascorbic acid solution were diluted to the required concentrations of 0.01, 0.5, 1.0 g/L. Working standard solutions were prepared in triplicate. To determine the LOD for each detector (DAD and MSD), three separate series of ascorbic acid solutions were prepared from the stock solutions and analyzed. The data was plotted in coordinates µg of AA versus peak area. Least square regression analysis was done for the data obtained (OpenLAB CDS software). The abscissa of intersection point of the regression line and horizontal line corresponding to the 3 times noise average value was taken as LOD. Replicates of three injections were

performed for each sample. Linear regression data were computed automatically (OpenLAB CDS).

Selectivity assay:

The specificity of the HPLC method for ascorbic acid quantitation in pharmaceutical/veterinary preparations was investigated in order to obtain an indication of possible interference from excipients in topical preparations. For specificity and selectivity of method, ascorbic acid solutions (0.5 g/L) were prepared as said above with and without common ingredients (Sodium bicarbonate, EDTA Disodium, Benzyl alcohol) and tested.

3. RESULTS AND DISCUSSION

The basic parameters all the tests were as follows: Mobile phase: 0.1% Formic acid aqua solution. Injection volume: 1.00 µL; Column temperature: 40°C; Flow speed: 0.6 ml/min; Detection time: 1min. Retention time of Ascorbic acid detected by DAD was 0.221±0.002 min., MS detector is on the line after DAD, so MS peak appears after (0.03 min.) of DAD. The chromatograms and extracted MS spectra of Ascorbic acid are present in figures 1, 2 and 3.

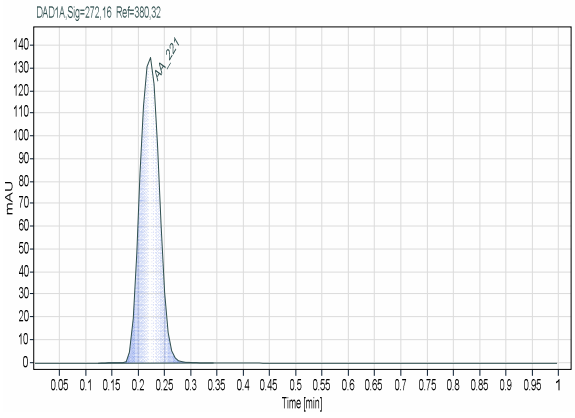

Figure - 1: DAD chromatogram 0.5 µg Ascorbic acid. Retention time: 0.221 min.

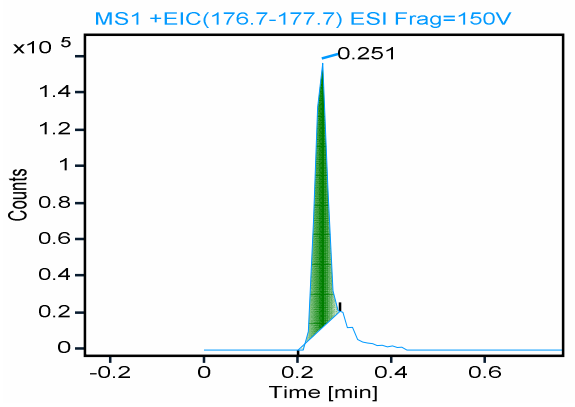

Figure - 2: MS extracted chromatogram 0.5 µg Ascorbic acid, m/z =+(176.7 – 177.7).

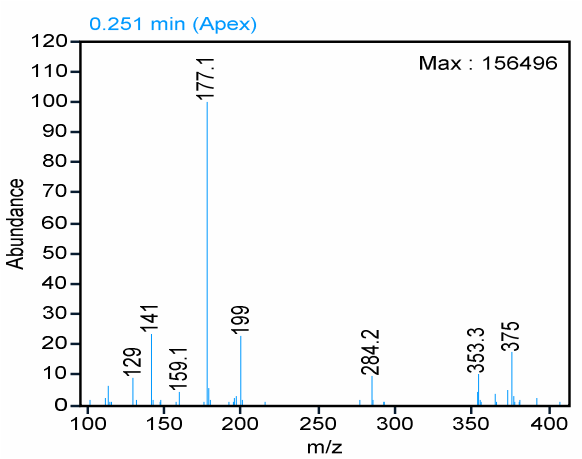

Figure - 3: MS extracted spectra for MS peak 0.251 min (figure II). 0.5 µg Ascorbic acid.

3.1. Linearity

The linearity was checked on samples of standard ascorbic acid at different concentrations (0.1 – 1.0 g/L). For DAD, see Figure IV and for MSD Figure V. Plots are the output of the OpenLAB CDS.

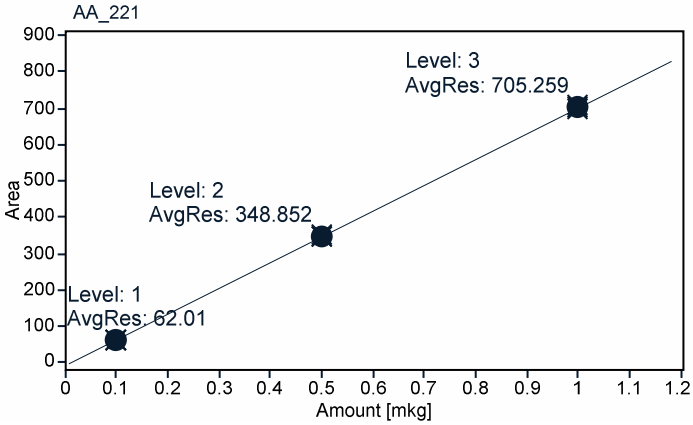

Figure – 4: Calibration curve on basis of DAD (peaks area. Signal: DAD1A. R: 0.99993; R^2: 0.99987; Formula: y = ax + b; a: 708.8; b: 4.4778).

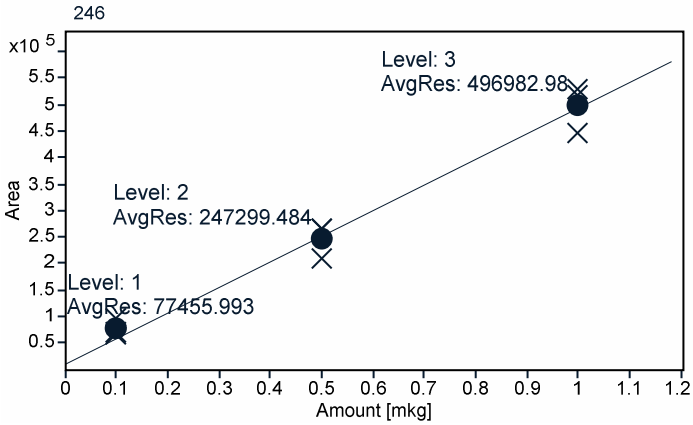

Figure – 5: Calibration curve on basis of MSD peaks area. Signal: MS1 +EIC(176.7-177.7 m/z)

R: 0.99835; **R^2:** 0.99669; Formula**:** y = ax + b; **a:** 483359.6; **b:** 12090.8

3.2. Accuracy/recovery and precision

The accuracy of the method was confirmed by conducting a recovery study for different concentrations (1.0, 0.98, 0.74 and 0.5 g/L) by replicate analysis (N = 3), in accordance with ICH guidelines. DAD peak had retention time 0.221 ±0.002 min. and a MS signal of m/z=+177.1

The results of accuracy/recovery and precision experiments are recorded in Table 1. The data indicates an adequate percentage of accuracy/recovery for the HPLC method for the quantitation of ascorbic acid in the pharmaceutical preparations. Inter-day analysis (overnight test) does not show any degradation of Ascorbic acid. Samples were kept in closed vials at 19 °C. In our previous tests the degradation after more than 24 hours was detected (data is not shown). It is reported about significant Ascorbic acid degradation after 24 hours in media of 0.2M sodium dihydrogen orthophosphate [6].

Recovery is an average value of three tests. Standard deviation (STDV) and RSD are presented. * Bottom line is the Inter-day analysis.

Table - 1: Accuracy of the HPLC method for ascorbic acid determination

Ascorbic acid (µg)	% Recovery (mean)	% Stdv	% RSD
1.00	100.3	0.58	0.58
0.98	100.0	1.02	1.02
0.74	100.0	0.00	0.00
0.50	100.0	0.00	0.00
*1.00	101.0	0.00	0.00

3.3. Limits of detection (LOD) and quantitation (LOQ)

LOD is defined as the lowest active substance concentration that can be determined by a method. In figure 6 (a) and (b) results of LOD estimation are presented in graphical form. The amount of noise was estimated during process of peaks integration and was equal to parameter b of calibration curve (See Figure 4 and 5). An estimation of the limits, which was achieved by the determination of the signal/noise ratios of 3:1 were 0.01 µg of Ascorbic acid per injection for DAD and 0.05 µg for MSD. The LOD of the method should be estimated as 0.5 ppm because the 20 times diluted samples and injection volume increased up to 20 µL give us the same 0.01 µg per injection. The noise level is assumed to be constant. LOQ =3.3*LOD.

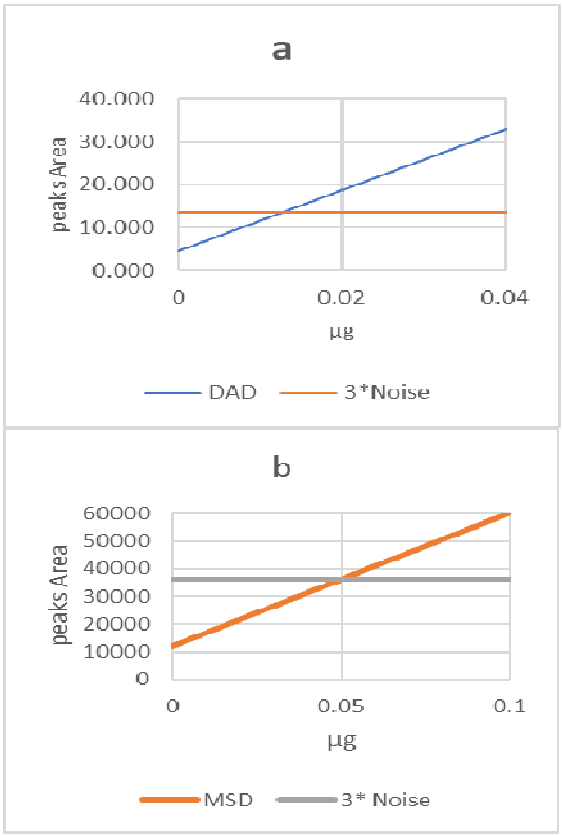

Figure - 6: Graphical determination of LOD. The point of intersection of calibration curve with 3 times noise line (horizontal). DAD (a) and MSD (b).

3.4. Selectivity assay

The specificity of the HPLC method for vitamin C quantitation in the pharmaceutical formulations is an indication of possible interference from excipients in the preparations. The presence of other ingredients in the formulations did not cause any interference with the ascorbic acid peak. Under the test conditions, ascorbic acid was observed to be well resolved from the other components of the formulations and potential degradation products of vitamin C. Thus, the method is specific for Vitamin C. For specificity and selectivity of method, ascorbic acid solutions (0.5 g/L) were prepared in the 1.0 mM HCl solution with and without common ingredients (Table 2). For HPLC/MS analysis concentrations all the compounds were 1000 times less. Test results are as follow: Percent of recovery of AA in presence of all the other compounds was 101.4 % and standard deviation (STDV) was 1.3%. Percent of recovery of AA in absence of all the other compounds was 100.0 % and (STDV) was 0.0%. Chromatograms and MS spectrum are presented in figures 7, 8 and 9 (compare with figures 1, 2 and 3).

Table – 2: Average composition of Sodium ascorbate injection solution

Name	MW	g/L	M
Ascorbic Acid	176.12	500	2.84
NaHCO3	84	240	2.86
Na2EDTA	338.2	1.0	0.003
Benzyl alcohol	108.1	9.0	0.08

It is important to point out that the samples containing significant amount of Na shows predominantly +199.1 m/z which corresponds to +C6H8O6Na ion. Figure IX.

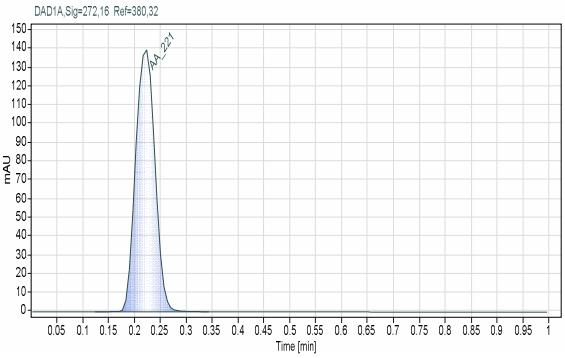

Figure – 7: DAD chromatogram of Ascorbic acid 0.5 µg with presence of other components of the formulations. Retention time: 0.221 min.

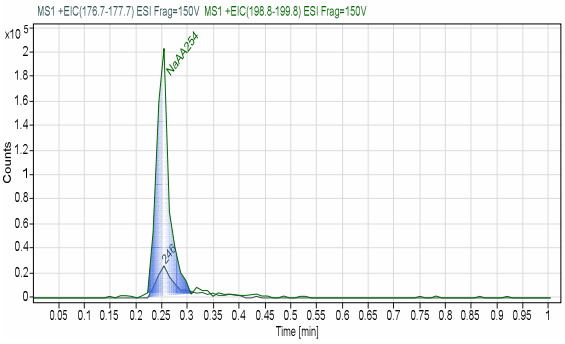

Figure – 8: MS chromatogram of Ascorbic acid 0.5 µg with presence of other components of the formulations.

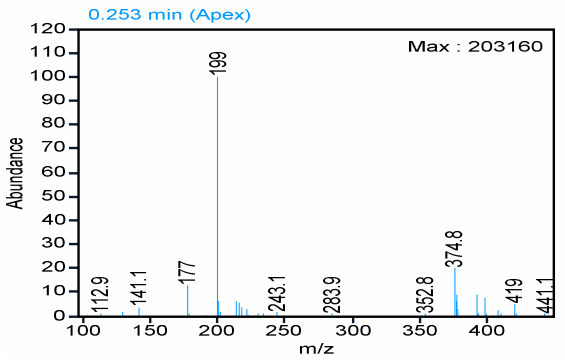

Figure - 9: MS extracted spectrum of Ascorbic acid 0.5 µg with presence of other components of the formulations.

4. CONCLUSION

The developed HPLC/MS method for the determination of ascorbic acid in pharmaceutical preparations containing various other ingredients including excipients (NaHCO3, Na2EDTA, Benzyl alcohol) has been validated for linearity, accuracy/recovery and precision, as well as low values of limits of detection and quantitation. The method provides a rapid (1 minute), simple, cheap, sensitive, accurate, and reproducible means of determining Vitamin-C in pharmaceutical formulations without prior sample preparation.

Acknowledgements

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