Development and Validation of RP-HPLC Method for Simultaneous Determination of Ascorbic Acid and Salicylamide in their Binary Mixtures: Application to Combined Tablets

M. Sharaf El-Din, M Eid and A M Zeid*

Department of Analytical Chemistry, Faculty of Pharmacy, University of Mansoura, 35516, Mansoura, Egypt

Abstract

A new simple, rapid and sensitive reversed-phase liquid chromatographic method was developed and validated for the simultaneous determination of ascorbic acid (ASC) and salicylamide (SAL) in their combined dosage form. The analysis was carried out on C18 Shim-pack C8 column (250 x 4.6 mm, 5 µm particle size) using a mobile phase consisting of methanol: 0.03 M phosphate buffer mixture (55: 45, v/v) of pH 4.0. The mobile phase was pumped at a flow rate of 1 mL/min with ultraviolet detection at 255 nm. The selectivity, linearity of calibration, accuracy, intra and inter day precision and recovery were examined as parts of the method validation. The concentration–response relationship was linear over a concentration range of 0.50-10.00 and 5.00-50.00 µg/mL for ASC and SAL, respectively with limits of detection of 0.048 and 0.676 µg/mL. The proposed method was applied for the simultaneous determination of the two studied drugs in their combined tablets with average recoveries of 100.04 ± 0.75% and 100.11 ± 1.04% for ASC and SAL, respectively. The results were favorably compared to those obtained by the comparison methods.

Keywords: Ascorbic acid; Salicylamide; HPLC; Ultra violet detection; Tablets

Introduction

Ascorbic acid (ASC, vitamin C), 2, 3-Didehydro-L-threo-hexono-1, 4-lactone (Figure 1a), is a water-soluble anti-oxidant vitamin that is essential for the synthesis of collagen and intercellular material. ASC is used in the treatment and prevention of vitamin C deficiency (scurvy) that is characterized by capillary fragility, bleeding and slow healing of wounds [1]. Both the United States Pharmacopeia(USP) [2] and the British Pharmacopeia (BP) [3] recommended iodimetric titration methods for determination of ASC in raw material using standard iodine as a titrant. The (USP) [2] recommended a titration method for its determination in tablets by adding known volume of metaphosphoric-acetic acids TS, and titrate with standard dichlorophenol-indophenol blue as indicator. Several analytical methods are described for the determination of SAL, whether in dosage forms or in biological fluids. Such methods include; spectrophotometry [16], spectrofluorimetry [17,18], HPTLC [19], HPLC [20,21], and capillary electrophoresis [22]. ASC and SAL are co-formulated in tablets to treat common cold associated with fever and muscular pain. ASC strengthens connective tissue, increasing resistance to viral invasion. ASC also strengthens the body’s immune system, neutralizes free radicals, and kills viruses. These four important functions of ASC work together to safely and effectively reduce the frequency, severity, and duration of a cold [23,24]. SAL as analgesic and anti-pyretic agent can be used in combination with ASC to prevent fever and pain associated with common cold.

To the best of our knowledge, no analytical methods have been reported for the simultaneous determination of both compounds. Therefore, the proposed method was developed and validated for the simultaneous determination of such binary mixture.

The aim of the present study is to develop and validate a new sensitive and accurate HPLC method for simultaneous determination of ASC and SAL in tablets. The proposed method has the advantage of

*Corresponding author: A. M. Zeid, Department of Analytical Chemistry, Faculty of Pharmacy, University of Mansoura, 35516, Mansoura, Egypt; Tel: +20502247406; E-mail: abdallah_zeid@hotmail.com

Received July 22, 2012; Accepted September 05, 2012; Published September 09, 2012

Citation: El-Din MS, Eid M, Zeid AM (2012) Development and Validation of RP-HPLC Method for Simultaneous Determination of Ascorbic Acid and Salicylamide in their Binary Mixtures: Application to Combined Tablets. J Chromat Separation Techniq 3:137. doi: 10.4172/2157-7064.1000137

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being very rapid where the total analytical run time does not exceed 6 min.

**Experimental**

**Apparatus**

Separation was performed using a Merck Hitachi L-7100 chromatograph equipped with a Rhodyne injector valve with a 20 µL loop and an L-7400 UV detector (Darmstadt, Germany). Chromatograms were recorded on a Merck Hitachi D-7500 integrator.

Mobile phases were degassed using Merck L-7612 solvent degasser.

A Consort pH-Meter (Belgium) was used for pH adjustments.

**Materials**

- Ascorbic acid pure sample, batch # 1006813004, was provided by Hebei Welcome Pharmaceutical Co., Ltd., China.
- Salicylamide pure sample, batch # 1107012 was provided by Shenzhen Gosun Pharma Co., Ltd, Shenzhen, China.
- Cidal C® tablets, batch # 151209, labeled to contain 50 mg ascorbic acid and 500 mg salicylamide per tablet. Product of Chemical Industries Development “CID” Co., Cairo, Egypt, was purchased from local pharmacy.
- Methanol (HPLC grade) was purchased from Sigma-Aldrich (Germany).
- Orthophosphoric acid (85% w/v) and triethylamine (TEA) were obtained from Riedel-deHäen (Sleeze, Germany).
- Sodium dihydrogen phosphate was obtained from Adwic Co. (Cairo, Egypt).

**Chromatographic conditions**

- **Column:** CLC Shim-pack C8 column (250 x 4.6 mm, 5 µm particle size), Shimadzu Corporation, Japan.
- **Mobile phase:** A mixture of methanol and 0.03 M sodium dihydrogen orthophosphate (55:45, v/v), the pH was adjusted to pH 4.0 with 0.03 M orthophosphoric acid. The mobile phase was filtered through a 0.45 µm membrane filter (Millipore, Ireland).
- **Flow rate:** 1mL/min.
- **UV detection:** 255 nm.

**Preparation of standard solutions**

Standard solutions containing 100 µg/mL of ASC, SAL and salicylic acid (SALA) were prepared separately in methanol. Working solutions were prepared by appropriate dilution of the standard solutions with the mobile phase. The standard solution of ASC shows stability problems upon exposure to light or air due to photo-degradation or oxidation so it is used freshly prepared in dark brown flasks or in flasks wrapped with aluminum foil. On the other hand, SAL was found to be stable for 1 week when kept in the refrigerator.

**Procedures**

**Construction of the calibration graphs:** Working solutions containing (5.00-50.00) µg/mL of SAL were prepared by serial dilution of standard SAL solution together with an aliquot of SALA standard solution as internal standard (final concentration of 10.0 µg/mL) with the mobile phase. For SAL, working solutions containing (5.00-50.00) µg/mL of SAL were prepared by serial dilution of standard SAL solution together with an aliquot of SALA standard solution as internal standard (final concentration of 10.0 µg/mL) with the mobile phase. In all cases, 20 µL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The average peak area ratio of each drug and the internal standard were plotted versus the final concentration of the drug in µg/mL to get the calibration graph. Alternatively, the corresponding regression equation was derived.

**Analysis of laboratory-prepared mixtures of ASC and SAL**

Aliquots of the standard solutions containing different concentrations of ASC and SAL in the ratio of (1:10) respectively were transferred into a series of 10 mL volumetric dark brown flasks containing 10.0 µg/mL of SALA internal standard. The volumes were completed with the mobile phase and the solutions were mixed well. Twenty µL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The % recoveries of each drug were determined either from the previously constructed calibration graphs or from the corresponding regression equations.

**Analysis of Cidal C® tablets:** Ten Cidal C® tablets were accurately weighed, finely pulverized and thoroughly mixed, then a quantity of the powder equivalent to 50 mg ASC and 500 mg of SAL (in their pharmaceutical ratio of 1:10) was transferred into a small conical flask and extracted with 3 x 30 mL of methanol. The extract was filtered into a 100 mL volumetric flask and wrapped with aluminum foil. The conical flask was washed with few milliliters of methanol. The washings were passed into the same volumetric flask and completed to the volume with the same solvent. Aliquots covering the working concentration ranges were transferred into 10 mL dark brown volumetric flasks containing 10.0 µg/mL of the internal standard, completed to volume with the mobile phase and mixed well. Twenty µL aliquots were injected (triplicate) and eluted with the mobile phase under the previously described chromatographic conditions. The nominal content of ASC and SAL in the tablets was determined either from the previously plotted calibration graphs or from the corresponding regression equations.

**Results and Discussion**

An HPLC method coupled with UV detection was developed and fully validated for the simultaneous determination of ASC and SAL.
The proposed method provided sensitive and rapid assay for the quality control of ASC and SAL in tablets, where it allowed separation of the two drugs with satisfactory resolution factor and good sensitivity within a reasonable analysis time. Figure 2 illustrates a typical chromatogram indicating good resolution of ASC (t_R = 3.10 min), SAL (t_R = 5.13 min) and SALA (t_R = 5.86 min) under the described chromatographic conditions.

**Chromatographic performance**

Well-resolved symmetrical peaks were obtained upon measuring the response of the eluent under the optimized chromatographic conditions after thorough experimental trials that can be summarized as follows:

**Choice of appropriate wavelength:** The absorption spectra of both ASC and SAL were investigated in order to determine the optimum wavelength for the assay. ASC was found to have a λ_max at 243 nm, meanwhile, SAL was found to exhibit maximum absorption maxima at 205, 235 and 300 nm upon their measurements in the mobile phase at pH 4.0. Figure 3 illustrates the absorption spectra of ASC and SAL in the mobile phase. UV scan analysis showed best quantitative analysis at 255 nm, since it provided a good compromise in terms of sensitivity for both ASC and SAL. The choice depended on the ratio of both drugs in their pharmaceutical formulations, where at 255 nm the sensitivity of ASC is about 10 times more than SAL.

**Mobile phase composition:** Several modifications in the mobile phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the pH of the mobile phase, the type and ratio of the organic modifier, the strength of phosphate buffer and the flow rate. The results achieved are summarized in table 1.

**pH of the mobile phase:** The effect of changing the pH of the mobile phase on the selectivity and retention times of the test solutes was investigated using mobile phases of pH values ranging from 2.5 to 5.0. The change in the pH value of the mobile phase did not significantly affect the retention of SAL, while, the retention time of ASC increased with the increase in the pH of the mobile phase. Additionally, increasing the pH value of the mobile phase caused broadening of the peak of ASC. Therefore, pH 4.0 was selected as the optimum pH value yielding well separated symmetrical peaks of the two drugs with high sensitivity within a reasonable analytical run time.

**Ratio of organic Modifier:** The effect of changing the ratio of organic modifier on the selectivity and retention times of the two drugs was investigated using mobile phases containing 40-60% (v/v) of methanol. As the ratio of methanol in the mobile phase decreases, the retention of SAL increases without a significant effect on the retention of ASC. A mobile phase containing methanol: 0.03 M phosphate buffer in the ratio of (55: 45, v/v) was chosen as the best mobile phase since it allowed the separation of ASC and SAL within a short analytical run time with reasonable resolution factor and excellent sensitivity.

- **Ionic strength of phosphate buffer:** The effect of changing the ionic strength of phosphate buffer on the chromatographic performance was investigated using mobile phases containing [A] SAL (5.0 µg/mL) in the mobile phase at pH 4.0 [B] ASC (5.0 µg/mL) in the mobile phase at pH 4.0

| Parameter          | No. of theoretical plates (N) | Resolution (R_s) | Relative retention (α) |
|--------------------|-------------------------------|------------------|------------------------|
| ASC                | 973                           | 5069             | 6.80                   | 1.71                   |
| SAL                | 1362                          | 6128             | 10.34                  | 2.12                   |
| 3.5                | 1011                          | 2796             | 7.62                   | 2.08                   |
| 4.0                | 1708                          | 7066             | 6.00                   | 1.69                   |
| 4.5                | 967                           | 4679             | 5.80                   | 1.65                   |
| 5.0                | 576                           | 1220             | 4.07                   | 1.75                   |
| Ratio of A: B*     | 40: 60                        | 1643             | 4822                   | 14.10                  | 2.86                   |
| 50: 50             | 1457                          | 3355             | 9.18                   | 2.17                   |
| 55: 45             | 1665                          | 5069             | 5.88                   | 1.71                   |
| 60: 40             | 858                           | 2776             | 4.48                   | 1.57                   |
| Conc. of H3PO4 (M) | 0.01                          | 961              | 1744                   | 4.71                   | 1.68                   |
| 0.02               | 1030                          | 4032             | 5.34                   | 1.71                   |
| 0.03               | 1223                          | 4391             | 5.88                   | 1.62                   |
| 0.04               | 1365                          | 2663             | 6.00                   | 1.69                   |
| Flow rate (mL/min) | 0.8                           | 1886             | 5094                   | 7.80                   | 1.72                   |
| 1.0                | 1708                          | 7066             | 6.00                   | 1.69                   |
| 1.2                | 1346                          | 5890             | 5.44                   | 1.56                   |

*A: methanol. B: 0.03 M phosphate buffer.

Table 1: Optimization of the chromatographic conditions for separation of ASC and SAL by the proposed method.
concentrations of 0.01-0.04 M of phosphate buffer. It was found that, the change in the ionic strength of phosphate buffer did not have a significant effect on the chromatographic performance. Finally, 0.03 M phosphate buffer was used throughout this study.

- **Effect of flow rate:** The effect of flow rate of the mobile phase on the separation of the studied drugs was investigated and a flow rate of 1 mL/min was found to be the optimal one for the good separation within a reasonable time (Table 1).

**Method validation:** To assess the validity of the proposed method, it was tested for linearity, ranges, limits of quantification, limits of detection, accuracy, precision, specificity, selectivity, solution stability, mobile phase stability and system suitability.

- **Linearity and ranges:** Under the above described chromatographic conditions, a linear relationship was established by plotting the peak area ratio of each drug to the internal standard versus the corresponding drug concentration (µg/mL). The concentration ranges were found to be 0.50-10.00 and 5.00-50.00 µg/mL for ASC and SAL, respectively. The high values of the correlation coefficients with small values of intercept indicate the good linearity of the calibration graphs. Statistical analysis of the data [25] gave small values of the standard deviation of the residuals ($S_y$), of slope ($S_a$) and of intercept ($S_b$), and the % relative error (Table 2). Thus, indicating low scattering of the points around the calibration graphs.

- **Limits of quantification (LOQ) and limits of detection (LOD):** The limits of quantification (LOQ) and limits of detection (LOD) were determined according to ICH Q2 (R1) recommendations [26]. The results are summarized in table 2. LOQ and LOD were calculated according to the following equations: 

$$LOQ = 10S_y/b$$

$$LOD = 3.3S_y/b$$

Where, $S_y$ is the standard deviation of the intercept of regression line and $b$ is the slope of the regression line.

- **Accuracy:** To test the validity of the proposed method it was applied to the determination of pure samples of both ASC and SAL over the concentration range of (0.05-10.00) and (5.00-50.00) µg/mL, respectively. The results obtained were in good agreement with those obtained using the two comparison HPLC methods [14,21] for ASC and SAL, respectively. Statistical evaluation of the results using the Student t-test and the variance ratio F-test revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Table 3).

- **Precision:** The intra-day precision was evaluated through replicate analysis of three concentrations of each drug in raw material on three successive times. The inter-day precision was also evaluated through replicate analysis of three concentrations of each drug over a period of 3 successive days. The results of intra-day and inter-day precision are summarized in table 4. The small values of % Error and % RSD indicate high accuracy and precision of the proposed method, respectively.

- **Specificity:** The specificity of the proposed HPLC method was proved by its ability to determine the studied drugs in their combined tablets confirming that, there was no interference by common additives or preservatives such as; microcrystalline cellulose, lactose, pregelatinised starch, crospovidone and magnesium stearate. Figure 4 shows a typical chromatogram for determination of the two drugs in tablets. No interference from common additives or preservatives was observed. Small well-resolved peak appeared at tR of 4.2 min, this peak may be due to one of the added preservatives.

### Table 2: Performance data of the proposed method.

| parameters | Concentration taken (µg/mL) | Concentration found (µg/mL) | % Found | Comparison methods [14,21] |
|------------|----------------------------|-----------------------------|---------|---------------------------|
| 1-ASC      |                            |                             |         |                           |
| 0.50       | 0.4969                     | 99.38                       | 99.08   |                           |
| 1.00       | 0.9883                     | 98.83                       | 99.78   |                           |
| 2.00       | 2.0191                     | 100.96                      | 100.22  |                           |
| 3.00       | 3.0165                     | 100.55                      | 98.97   |                           |
| 4.00       | 4.0147                     | 100.37                      |         |                           |
| 5.00       | 4.9675                     | 99.35                       |         |                           |
| 8.00       | 7.9703                     | 99.63                       |         |                           |
| 10.00      | 10.0267                    | 100.27                      |         |                           |
| X          |                            |                             | 99.92   | 99.52                     |
| SD         | ± 0.73                    | ± 0.57                      |         |                           |
| t-test     | 0.960 (2.23)*              | F value 1.502 (8.89)*       |         |                           |
| F value    | 5.00                       | 5.0353                      | 100.71  | 100.15                    |
| 10.00      | 10.1193                    | 101.19                      | 98.38   |                           |
| 20.00      | 20.1581                    | 100.79                      | 100.65  |                           |
| 25.00      | 24.9836                    | 99.93                       | 101.11  |                           |
| 30.00      | 29.5075                    | 98.36                       |         |                           |
| 40.00      | 39.9341                    | 99.84                       |         |                           |
| 50.00      | 50.2745                    | 100.55                      |         |                           |
| X          |                            | 100.20                      | 100.32  |                           |
| SD         | ± 0.94                    | ± 0.75                      |         |                           |
| t-test     | 0.230 (2.26)*              | F value 1.610 (8.94)*       |         |                           |

*Figures between parenthesis are the tabulated t and F values, respectively at p=0.05 (25)

### Table 3: Application of the proposed method to the determination of the studied drugs in the raw material.
- **Selectivity**: The proposed HPLC method is selective for each of the studied drugs in presence of the other. The proposed method was also selective for each drug in presence of co-administered drugs like, aspirin (ASP) and methocarbamol (MET) as shown in figure 5.

**Solution stability and mobile phase stability**

The stability of the stock solutions was determined by quantitation of ASC and SAL and comparison to freshly prepared standard solutions. No significant change was observed in standard solution response, relative to freshly prepared standard in case of SAL but ASC showed stability problems upon exposure to light and or air due to photo-oxidation. ASC is readily oxidized to dehydroascorbic acid, so, its solutions were prepared daily in dark brown flasks or flasks wrapped with aluminum foil and used freshly to overcome stability problems. Similarly, the stability of the mobile phase was checked. The results obtained in both cases proved that SAL solution and mobile phase used during the assay were stable up to 7 and 3 days, respectively but ASC solution was stable for only one day under the recommended conditions mentioned above.

**System suitability**: System suitability test (SST) parameters were performed during the development and optimization of the method (Table 1). In addition, SST parameters were checked to ensure that, the system is working correctly during the analysis. The test was performed by injecting the standard mixture in triplicate and the parameters were calculated as reported by the USP (2) and ICH guidelines. SST parameters including selectivity factor ($\alpha$), resolution factor ($R_s$) and column efficiency (number of theoretical plates, $N$) were shown in table 5.

| Concentration added (µg/ml) | % Recovery | % RSD | % Error |
|----------------------------|------------|-------|---------|
| ASC                        |            |       |         |
| Intra-day                  |            |       |         |
| 2.00                       | 99.43 ± 0.70 | 0.71  | 0.41    |
| 4.00                       | 100.19 ± 0.95 | 0.95  | 0.55    |
| 8.00                       | 99.56 ± 0.88 | 0.88  | 0.51    |
| Inter-day                  |            |       |         |
| 2.00                       | 100.51 ± 0.95 | 0.94  | 0.54    |
| 4.00                       | 99.40 ± 0.82 | 0.83  | 0.48    |
| 8.00                       | 100.55 ± 0.0 | 0.89  | 0.52    |
| SAL                        |            |       |         |
| Intra-day                  |            |       |         |
| 10.00                      | 99.66 ± 0.54 | 0.54  | 0.31    |
| 20.00                      | 99.24 ± 0.39 | 0.39  | 0.23    |
| 40.00                      | 100.69 ± 0.94 | 0.93  | 0.54    |
| Inter-day                  |            |       |         |
| 10.00                      | 100.77 ± 0.40 | 0.40  | 0.23    |
| 20.00                      | 99.71 ± 0.68 | 0.68  | 0.39    |
| 40.00                      | 100.13 ± 0.35 | 0.53  | 0.20    |

Table 4: Validation of the proposed method for determination of ASC and SAL raw materials using the proposed method.

| Parameter | ASC | SAL |
|-----------|-----|-----|
| No of theoretical plates, $N$ | 1706 | 7066 |
| Selectivity factor, $\alpha$ | 1.69 |
| Resolution, $R_s$ | 6.00 |

Table 5: System suitability parameters.

**Robustness**: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. To test the robustness of the proposed HPLC method, one chromatographic variable was varied while keeping all the others constant. The studied variables included; pH of the mobile phase (4.0 ± 0.2) and concentration of phosphate buffer (0.03 ± 0.005 M). These minor changes did not affect the separation and resolution of ASC and SAL indicating the reliability of the proposed method during normal usage. It was observed that, the most critical factor for the separation process is the ratio of methanol: 0.03 M phosphate buffer in the mobile phase, where small variations in such ratio (55:45, v/v) caused significant changes in the resolution and separation of the test solutes. This should be taken in consideration during preparation of the mobile phase.
### Table 6: Application of the proposed method for determination of the studied drugs in their laboratory prepared mixtures.

| Preparation                  | Concentration taken (µg/mL) | Concentration found (µg/mL) | % Found | Comparison methods [14, 21] |
|------------------------------|----------------------------|-----------------------------|---------|---------------------------|
| ASC and SAL mixture          | ASC SAL ASC SAL ASC SAL | ASC SAL ASC SAL ASC SAL | ASC SAL | ASC SAL                  |
| 2.00                         | 20.00                      | 1.9898                      | 20.0920 | 99.49 100.46             |
| 3.00                         | 30.00                      | 3.0207                      | 29.8170 | 100.69 99.39             |
| 4.00                         | 40.00                      | 3.9896                      | 40.0920 | 99.74 100.23             |
| X                            |                            |                             |         |                           |
| ± SD                         | ± 0.63                     | ± 0.92                      | ± 1.03  | ± 0.61                   |
| % RSD                        | 0.63                       | 0.93                        | 1.03    | 0.61                     |
| % Error                      | 0.37                       | 0.46                        |         | 0.33                     |

Each result is the average of three separate determinations.

### Table 7: Application of the proposed method for determination of the studied drugs in their tablets.

| Application   | Dosage form analysis                                                                 |
|---------------|--------------------------------------------------------------------------------------|
| Dosage form analysis | The proposed method was successfully applied to the simultaneous determination of ASC and SAL in laboratory prepared mixtures and in their combined tablets (Cidal C® tablets). The average percent recoveries of different concentrations were based on the average of three replicate determinations (Table 6 and 7). Figure 4 shows representative chromatogram for simultaneous determination of the two studied drugs in tablets. |

### Conclusion

An accurate, sensitive, and precise HPLC method with ultra violet detection was developed and fully validated for quality control analysis of ASC and SAL in their combined tablets. The proposed method is very rapid, where the total analytical run time for both drugs and the internal standard is less than 6 min. The method can be also applied for the determination of each drug in different matrices without interference and in presence of different co-administered drugs like, aspirin (ASP) and methocarbamol (MET).

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