Creation with Validation of Reverse Phase- High Performance Liquid chromatography Assay (Content) Method for Sorbic Acid in Oral Solutions

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ABSTRACT
Sorbic acid is one of the antimicrobial agent used as an antibacterial and antifungal, which is used as preservative in food, pharmaceutical formulations (oral solutions and external products) and in cosmetic products to prevent mold, yeast, fungi and bacterial growth. Creation (Development or Foundation or Establishment) of RP-HPLC method for the determination of sorbic acid in oral solutions. The standard and sample were extracted with the solvent mixture methanol and glacial acetic acid (95:5). The chromatographic condition: Column: L1,(25 cm × 4.6 mm),5μm,Detector Wavelength : 240 nm, Flow Rate: 1 ml/min, Injection Volume was 20 μl. The validation of the created RP-HPLC method was done according to USP, WHO and ICH guidelines with respect to Specificity, Linearity, Accuracy, Precision, Range, Limit of detection (LOD) and limit of quantitation (LOQ), System Suitability Determination and Robustness. The validation acceptance criteria were met in all cases.

Keywords: Sorbic acid, antimicrobial agent, RP-HPLC

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INTRODUCTION
The pharmaceutical formulations consist of an aqueous part like solution, powders for oral suspensions and external products require protection against microbial colonization (growth), that led to changes in stability of products or infect the users thus the antimicrobial agents can be added to the mixture of products to prevent the growth of several microorganisms during manufacture or use. (1-4)

The sorbic acid is an antimicrobial agent has antibacterial and antifungal activities used as a preservative in food, pharmaceutical formulations that consist of oral solutions and external products, in addition to cosmetic products to prevent the growth of microorganisms. Sorbic acid is a natural preservative, isolated at 1859 during rowanberry oil processing by Hofmann. This made parasorbic acid, that is hydrolysis to sorbic acid. (5-9) Studies improved that sorbic acid is safe for using in different applications included foods, drugs, and cosmetics, it is metabolized in body through pathway oxidation as the 5-carbon saturated fatty acid caproic acid. It has low toxicity in mammalian cells because of high LD50 (7.4 - 10 g/kg), also studies improved that this acid didn’t has any carcinogenic activity and each individuals can be uptake more than 25 mg/kg which detected by Joint FAO / WHO Expert Committee on Food Additives, for Oral and topical pharmaceutical formulations about 0.05–0.2% was used at pH below 6.5. (8-10) The chemical formula of sorbic acid is C6H8O2 (Figure 1) known as (E,E)-Hexa-2,4-dienoic acid, with white or creamy–white crystalline powder, tasteless with low characteristic odor, has 134°C melting point and it Soluble in water (1/1000), of ethanol (1/10), chloroform (1/15), ether (1/30), methanol (1/8) and propylene glycol (1/19). (6,9,11)
Different methods have been used to detection sorbic acid in products such as capillary zone electrophoresis, colorimetric like spectrophotometric, chromatography technologies like gas chromatography, micellarelectrokinetic chromatography, and finally high performance liquid chromatography (12-19).

1- Present study aims to create and optimization validate the RP-HPLC-UV-Detector-Assay Method For Sorbic Acid In Oral Solutions.

2- methodology and results

2-1- Determination Of RP-HPLC Assay Method For Sorbic Acid; (19-21)

Several types of extraction solvent were investigated. The recovery averages were calculated and compared. methanol and glacial acetic acid mixture (95:5 V/V) was improved that its suitable solvent for sorbic acid extraction from oral solutions with the average recovery (97-103) % , for obtaining optimum separation of sorbic acid and matrix interferences in oral solutions the chromatographic conditions were optimized.

A- procedure

(1) Solvent Mixture: methanol and glacial acetic acid 95:5 (V/V).
(2) Standard Preparation: about 0.1 mg/ml of Sorbic Acid RS in Solvent mixture.
(3) Assay Preparation: about 10 mg of Sorbic Acid was added To a 100-ml volumetric flask, then 75 ml of Solvent mixture was Mixed, then volume was complete to 100 ml by solvent mixture.
(4) Mobile Phase: consist of methanol, glacial acetic acid and water 69:28:3 (V\V\V).

B- chromatograph conditions:
- Column: L1, (25 cm × 4.6 mm), 5μm
- Detector Wavelength: 240 nm
- Flow Rate: 1 ml/min
- Injection Volume: 20 μl

C- determination of contents :
   Acceptance Criteria: (85-115)% of the presented amount

2-2- Validation of Created RP-HPLC Method For Sorbic Acid

Assay:
For created RP-HPLC method validation, the USP, WHO and ICH guidelines were dependence . (20-28)

The following parameters were assessed for defining the performance of the chromatographic system:
1- Specificity
2- Linearity
3- Accuracy (Recovery)
4- Precision
5. Range
6. Limit of Detection and Quantitation (LOD and LOQ)
7. System Suitability Determination
8. Robustness

2-2-1. Specificity
Its ability to estimate unequivocally the mixture in the presence of other compounds which may be present. It measures only the target component without interference with other types.

Specificity estimated by analyzing blank (solvent mixture used in standard and sample preparation), control (sample matrix), standard solution and sample solution to estimated interference happened. Table (1), Figures (2-5)

Experimental Procedure
- Inject the blank two times.
- Prepared two control.
- each control were injected.
- ensure that no peaks may be interference with blank or control.
- Labeled any peaks appeared by relative retention time indexed to the active component.
- The standard of Sorbic Acid were twice injected.
- Prepare two separated samples containing Sorbic Acid at 100%
- Twice inject each sample to confirm specificity.

Acceptance Criteria
There was no interference occurred in peak purity index for Sorbic Acid (table 1)

2-2-2. Linearity
Its mean that capability to summarized results directly, or using mathematical conversation, proportional to the concentration of mixture in samples within a given range.

Linearity Experimental Determination: (Table 2)
- six solutions were prepared at 25%, 50%, 75%, 100%, 125% and 150% of the actual concentration of analyte in the solution.
- samples were injected (lowest - upper concentration)
- The concentration versus the response was plotting.
- Linear regression analysis was implemented, the origin was excluded.

Acceptance Criteria
- determination Coefficient ($R^2$) must be not less than 0.999 (Figure 6)
- residuals plot didn’t has curvature.

2-2-3. Accuracy (Recovery)
Its mean agreement between the values found with the accepted value as a conventional true value or an accepted reference value. Samples were prepared as a 50%, 100% and 150% of the concentration. Then the recoveries calculated as following:

\[
\% \text{ Recovery} = \frac{\text{Amount Recovered}}{\text{Amount Prepared}} \times 10
\]

Experimental Procedure of Accuracy (Recovery) for Assay: (Table 3)
- Samples were prepared as 50%, 100% and 150% of the amount of the sorbic acid
- Prepare three samples for each concentration
- Prepare standard solution for each sample
- Each sample was injected three times next its analyze based on the analytical method.
- Samples were injected (lowest to upper concentration).
- The recovery was calculated for each sample
- Calculate the % RSD and Confidence Interval for all results (Table 4)

Acceptance Criteria
- Each individual sample % recovery must be within (98% -102%) (100% ± 2.0%)
- Average recovery for all samples must be within (98%-102%) (100% ± 2.0%)
- % RSD of all samples should be NMT 1%
Analysis implemented at Confidence Interval 95 %.

2-2-4 Precision
It is the agreement degree among test results for repeated the same procedures to multiple homogeneous samplings. Precision can get from replicated homogeneous samples at the prescribed conditions. It considered as :

A - Repeatability
Its mean that accurate express in the same conditions of operating during short time interval. It is implemented by analyzing multiple replicates for an assay composite sample. The recovery value calculated and reported for each value.

Determination of experiment : (Table 5)
- six samples prepared
- standard solution prepared
- samples analyze using analytical method
- Results assay were calculated (% recovery) for each samples.
- the statics were calculated (% RSD).

Acceptance Criteria
The assay % RSD values should be NMT 2.0%

B - Intermediate precision (Ruggedness)
Its variations of expresses in laboratory, like different days, analysts, and equipment. A second analyst repeats the repeatability analysis at different day and conditions. The recovery values reported. A statistical comparison is implemented to the first analyst’s results.

Experimental Determination:
- the repeatability analysis on different days with different analyst were performed
- The repeatability analysis by different operating conditions and different instruments were performed.
- six samples were prepared
- Standard solution was prepared.
- samples were analyzed according to the analytical method
- results (% recovery) for each sample were calculated.

Acceptance Criteria
- the % RSD made by a single analyst should be NMT 2.0%.
- the % RSD of the combined assay were calculated for each analysts, over both days should be NMT 3.0%.

2-2-5 Range
Its mean that the interval between upper and lower levels of the analyte including these levels. it is expressed normally in the same units of test results.

2-2-6 Determination limit of detection (LOD) and limit of quantitation (LOQ)
Limit of detection is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero.Limit of quantitation is the lowest concentration of analyte that can be determined with an acceptable repeatability and trueness.

2-2-7 System Suitability Test Determination:
Its implemented through optimization and development of the method during the validation procedure, the parameters consist of factor of capacity (k'), selectivity (α), resolution (Rs), efficiency of column (number of theoretical plates, N), relative retention time (RRT) and tailing factor (T) which were done using 10 replicates. The % RSD values calculating for 10 replicates should be not more than 2%. (Table 7)

2-2-8 Robustness
It is capacity to still unaffected by small, deliberate divers in method parameters and give an indication of its robustness during normal usage. it's tested through phase development by a lightly
variation of injection volume (± 5 μl), wavelength (±2 nm), flow rate (± 0.1 ml/min) and little variations of the mobile phase parts ratio.

3- RESULTS and DISCUSSION:
For specificity parameter results, there are no interferences occurred with the blank, and other excipients and the peak purity index for Sorbic Acid is satisfactory (Table 1, Figures 2-5).

Table (1) Specificity Confirmation Parameters

<table>
<thead>
<tr>
<th>Injection</th>
<th>Retention Time</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>No Peak</td>
<td>No interference</td>
</tr>
<tr>
<td>Control</td>
<td>No Peak</td>
<td>No interference</td>
</tr>
<tr>
<td>Standard Of Sorbic Acid</td>
<td>Pure Peak</td>
<td>Observation (16.36 min)</td>
</tr>
<tr>
<td>Sample</td>
<td>Pure Peak</td>
<td>Observation (16.5 min)</td>
</tr>
</tbody>
</table>

Figure (2) HPLC chromatogram of Blank

Figure (3) HPLC chromatogram of Control

Figure (4) HPLC chromatogram of Sorbic Acid Standard
According to the results shown in Figure (6) and Table (2), the method corresponds with linearity acceptable criteria.

<table>
<thead>
<tr>
<th>Level</th>
<th>Response</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>7177</td>
<td>Slope = 284.72x</td>
</tr>
<tr>
<td>50%</td>
<td>14223</td>
<td>Intercept = +17.333</td>
</tr>
<tr>
<td>75%</td>
<td>21331</td>
<td>$R^2 = 1$</td>
</tr>
<tr>
<td>100%</td>
<td>28532</td>
<td></td>
</tr>
<tr>
<td>125%</td>
<td>35556</td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>42763</td>
<td></td>
</tr>
</tbody>
</table>

The results of % Recovery of nine samples show that a good recovery of Sorbic Acid from the Solutions matrix. True value is embraced by the 95% Confidence Interval, thus the obtained results of recovery are closed to the true value and the method is more accurate for Sorbic Acid assay in oral solutions. Tables (3,4).
Table (3) Experimental Procedure Results

<table>
<thead>
<tr>
<th>Spiking Level</th>
<th>Standard Concentration Response</th>
<th>Recovered Concentration Response</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>14235</td>
<td>14245</td>
<td>100.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14223</td>
<td>99.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14211</td>
<td>99.83</td>
</tr>
<tr>
<td>100%</td>
<td>28553</td>
<td>28528</td>
<td>99.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28515</td>
<td>99.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28581</td>
<td>100.1</td>
</tr>
<tr>
<td>150%</td>
<td>42755</td>
<td>42763</td>
<td>100.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42732</td>
<td>99.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42725</td>
<td>99.93</td>
</tr>
</tbody>
</table>

Table (4) Accuracy parameters and results

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Recovery (%)</td>
<td>99.96</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.09029</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.09023%</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>9</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>0.06</td>
</tr>
<tr>
<td>95% Confidence Interval (lower)</td>
<td>99.9%</td>
</tr>
<tr>
<td>95% Confidence Interval (upper)</td>
<td>100.02%</td>
</tr>
</tbody>
</table>

Repeatability and Intermediate Precision considered precise method for sorbic acid assay in oral solutions according to present results. Tables (5,6)

Table (5) Repeatability tests replicates and their statics

<table>
<thead>
<tr>
<th>Test No.</th>
<th>(%) recovery for each sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100.12</td>
</tr>
<tr>
<td>2</td>
<td>100.02</td>
</tr>
<tr>
<td>3</td>
<td>99.76</td>
</tr>
<tr>
<td>4</td>
<td>99.81</td>
</tr>
<tr>
<td>5</td>
<td>100.07</td>
</tr>
<tr>
<td>6</td>
<td>100.18</td>
</tr>
<tr>
<td>Mean</td>
<td>99.99%</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.17</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.17%</td>
</tr>
</tbody>
</table>

Table (6) Ruggedness Confirmation Parameters

<table>
<thead>
<tr>
<th>Test No.</th>
<th>1st Analyst</th>
<th>2nd Analyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of the labeled amount</td>
<td>% of the labeled amount</td>
</tr>
<tr>
<td>1</td>
<td>100.02</td>
<td>100.12</td>
</tr>
<tr>
<td>2</td>
<td>98.93</td>
<td>100.02</td>
</tr>
<tr>
<td>3</td>
<td>99.91</td>
<td>99.76</td>
</tr>
<tr>
<td>4</td>
<td>100.11</td>
<td>99.81</td>
</tr>
<tr>
<td>5</td>
<td>100.06</td>
<td>100.07</td>
</tr>
<tr>
<td>6</td>
<td>99.15</td>
<td>100.18</td>
</tr>
<tr>
<td>Mean</td>
<td>99.7%</td>
<td>99.99%</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.517</td>
<td></td>
</tr>
<tr>
<td>% RSD</td>
<td>0.519%</td>
<td></td>
</tr>
<tr>
<td>Mean for 12 tests</td>
<td>99.85%</td>
<td></td>
</tr>
<tr>
<td>Standard Deviation for 12 tests</td>
<td>0.399</td>
<td></td>
</tr>
<tr>
<td>% RSD for 12 tests</td>
<td>0.3995%</td>
<td></td>
</tr>
</tbody>
</table>

According to the results of precision, accuracy, and linearity, the range in this method is more suitable for sorbic acid assay in oral solutions.
Limit of Detection (LOD) was 0.27 μg/ml and Quantitation (LOQ) was 0.87μg/ml. These results demonstrate that this method can be applied to determine very small concentrations of sorbic acid in oral solutions.

For System Suitability Parameters evaluation, the % RSD values calculating for 10 replicates were less than 2%. All parameters were satisfactory and met the acceptable criteria (Table 7).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity factor ($k'$)</td>
<td>3.79</td>
<td>1.0 - 10.0</td>
</tr>
<tr>
<td>Selectivity factor ($α$)</td>
<td>1.47</td>
<td>1.0 - 2.0</td>
</tr>
<tr>
<td>Resolution (Rs)</td>
<td>3.32</td>
<td>NLT 2</td>
</tr>
<tr>
<td>Theoretical plate (N)</td>
<td>3538</td>
<td>NLT 2500</td>
</tr>
<tr>
<td>Relative Retention Time (RRT)</td>
<td>0.76%</td>
<td>% RSD: NMT 2%</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1</td>
<td>0.9 – 1.0</td>
</tr>
</tbody>
</table>

The results of your study should be discussed with other studies (??????????)

4- CONCLUSION:

The present study concluded that RP-HPLC-UV detector is sensitive, simple and specific method for the sorbic acid assay in Oral solutions. The mixture of methanol and glacial acetic acid (95:5, V/V) most suitable solvent for extracting sorbic acid from oral solutions. According to the results of Specificity, range Linearity, Accuracy (Recovery), Precision, limit of quantitation (LOQ)in addition to Limit of detection (LOD), System Suitability Determination and Robustness, the method deal with good laboratory practice requirements and validation criteria in USP, WHO and ICH guidelines.

The System Suitability parameters kept on unaffected over the deliberate changes in the chromatographic conditions illustrating that the method is robust.

References