Validation of Spectrophotometric Methods for Quantitative Determination of 7-ADCA in Pharmaceutical Formulations

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ABSTRACT
Two simple, sensitive and accurate methods are described for the determination of 7-Amino deacetoxy cephalosporanic acid (7-ADCA) in bulk drug and in formulations. Method M1&M2 are based on the oxidizing reaction between 7-ADCA and Ninhydrin-Ascorbic acid (NIN-AA) (M1); β-Naphthol-H2O2-Cupric nitrate-2, 4-DNP-Ammonium hydroxide (β-NPT,H2O2-DNP)(M2) solutions. The chromogen being extractable with chloroform could be measured quantitatively at 400 nm (M1) and 420 nm (M2). All variables were studied to optimize the reaction conditions. Regression analysis of Beer’s Law plot showed good correlation in the concentration range 0.5-3.0 for M1 and 0.8-4.8 for M2 µg/ml. The calculated molar absorptivity values are 6.701×104 and 7.015×104 L/mol/cm for M1 and M2, respectively. The methods were successfully applied to the determination of 7-ADCA in formulations and the results tallied well with the label claim. The results were statistically compared with those of a literature method by applying the Student’s t-test and F-test. No interference was observed from the concomitant substances normally added to preparations. The accuracy and validity of the methods were further ascertained by performing recovery experiments via standard-addition method.
Key words: 7-Amino deacetoxy cephalosporanic acid, oxidation products, spectrophotometric methods, statistical analysis, recovery studies

INTRODUCTION
7-ADCA (7-Amino deacetoxy cephalosporanic acid) is an important intermediate for preparing cephalosporin antibiotics, is prepared by a novel bioprocess in which a transformed Penicillium chrysogenum strain is cultured in the presence of an adipate feedstock to produce adipoyl-6-APA
(6-amino penicillanic acid); and the in situ expression of an expandase gene, e.g., from Streptomyces clavuligerus, with which the P. chrysogenum has been transformed, converts the adipoly-6-APA by ring expansion to adipoyl-7-ADCA. The final product 7-ADCA, is then prepared by cleavage of the adipoyl side chain using an adipoyl acylase. The entire synthesis, accordingly, is carried out using bioprocesses, and is efficient and economical.

A very few physico-chemical methods appeared in the literature for the assay of 7-ADCA in biological fluids and pharmaceutical formulations. The methods so far reported include HPLC [1-8], CE [9], GC-MS [10-11], and UV-Visible spectrophotometric methods [12]. Existing analytical methods reveal that relatively little attention was paid in developing visible spectrophotometric methods by exploiting thoroughly the analytically useful functional groups in 7-ADCA. Hence there is a need to develop sensitive and flexible visible spectrophotometric methods, which prompted the author to choose 7-ADCA for the investigation. Based on the different chemical reactions two methods have been developed. These methods were based on the reactivity of 7-ADCA with reagents such as NIN-AA (M$_1$); β-NPT, H$_2$O$_2$; 2,4-DNP (M$_2$). All these methods have been extended to pharmaceutical formulations as well. The author has developed two simple and sensitive UV methods (CH$_3$OH as solvent) and adopted it as a reference method to compare the results obtained by proposed methods. The analytical utility of the proposed chromogenic reagents

**EXPERIMENTAL SECTION**

**Instruments used:** An Elico, UV – Visible digital spectrophotometer with 1cm matched quartz cells were used for the spectral and absorbance measurements. An Elico LI-120 digital pH meter was used for pH measurements.

**Preparation of standard drug solutions:** A 1 mg/ml solution was prepared by dissolving 100 mg of pure 7-ADCA in 100ml of distilled water and this stock solution was diluted step wise with distilled water to get the working standard solutions of concentration of 100 µg/ml (M$_1$), 200 µg/ml (M$_2$)

Preparation of reagents: All the chemicals and reagents used are of analytical grade and solutions were prepared in triply distilled water

**Method M$_1$:** Ninhydrin solution (BDH 1%, 5.605×10$^{-5}$M): Prepared by dissolving 1 gm of ninhydrin in 100 ml of acetone.

Ascorbic acid solution (BDH; 0.1%, 5.678×10$^{-3}$M): Prepared by dissolving 100mg of ninhydrin in 100 ml of distilled water.

Buffer solution (pH 5.0): Prepared by diluting a mixture of 200 ml of 0.5 M citric acid and 200 ml of 1.0 M NaOH solutions to 500 ml with distilled water and the pH was adjusted to 5.0.

**Method M$_2$:** β-Napthol H$_2$O$_2$; A 2% solution of β-napthol in ethanol. Dilute 0.1ml of 30 % aqueous solution of Hydrogen peroxide to 200ml with ethanol.

Cupric nitrate: 0.1% solution of cupric nitrate trihydrate in ethanol.

Conc.HCl: 1:9 mixtures of Conc. HCl and ethanol.

2,4-DNP: A saturated solution of 2,4-DNP in ethanol.

Ammonium hydroxide: 1: 4 mixtures of aqueous ammonium hydroxide and ethanol.
Recommended Procedures

**Method M1:** Aliquots of standard 7-ADCA solution (0.5 - 2.5 ml; 400 µg) was transferred into a series of calibrated tubes containing 4.0 ml of buffer (pH 5.0), 1.0 ml ninhydrin solution and 0.5 ml of ascorbic acid solution. The volume in each tube was adjusted to 8.0 ml with distilled water and was kept in boiling water bath. After 15 min tubes were removed and chilled in ice water. The solution in each tube was made up to 10.0 ml with distilled water. The absorbances were measured at 560 nm after 10 min against a reagent blank prepared similarly. The amount of 7-ADCA was calculated from its calibration graph.

**Method M2:** To 0.6 ml of β-Napthol, add 0.1 ml of aqueous solution of H₂O₂, 0.5 ml of aliquots of standard 7-ADCA (0.5-3.0ml, 100 µg/ml) solution in ethanol and 0.1 ml of cupric nitrate trihydrate. Heat at 60°C for 10 minutes and cool for 2 min in a water bath. Add 0.1 ml of Conc. HCl, 1ml of 2,4- DNP & let stand for 2 min. Add 0.2 ml of 1:4 mixture of aqueous NH₄OH and ethanol and 4 ml of dimethyl formamide. The absorbances were measured at 630 nm.

**Reference Method** [13]: An accurately weighed portion of the powdered tablets equivalent to 100 mg of drug was dissolved in 30 ml of isopropyl alcohol, shaken well and filtered and the filtrate was diluted to 100 ml with isopropyl alcohol to get 1mg/ml solution of drug in formulations. Five ml of this solution was further diluted to 200 ml to get 25 µg/ml solution. The absorbance of the solution was determined at λ_{max} 229 nm. The quantity of the drug was computed from the Beer’s law plot of the standard drug in isopropyl alcohol.

For pharmaceutical formulations: An accurately weighed portion of tablet content equivalent to about 100 mg of 7-ADCA was transferred into a 100 ml volumetric flask. Added about 80 ml of warm isopropyl alcohol and shaken well for about 20 min. The contents were diluted with isopropyl alcohol up to the mark and mixed thoroughly. The solution was filtered. The filtrate was evaporated to dryness. The residue was used for the preparation of formulation solutions for different methods as given under standard solutions preparations. These solutions were analyzed as under procedures described for bulk solutions.

**RESULTS AND DISCUSSIONS**

**Spectral Characteristics:** In order to ascertain the optimum wavelength of maximum absorption (λ_{max}) of the colored species formed in the above methods, specified amounts of 7-ADCA were taken and colors were developed separately by following the above procedures. The amounts of 7-ADCA present in total volume of colored solutions were 40 µg/ml (M₁) and 16 µg/ml (M₂). The absorption spectra were scanned on a spectrophotometer in the wavelength region of 340 to 900 nm against similar reagent blank or distilled water. The reagent blank absorption spectrum of each method was also recorded against distilled water. The absorption curves of the colored species in each method show characteristics absorption maxim where as the blank in each method has low or no absorption in this region.

**Optimum conditions fixation in procedures:** The optimum conditions for the color development of methods M₁, M₂ were established by varying the parameters one at a time, keeping the others fixed and observing the effect produced on the absorbance of the colored species. The following experiments were conducted for this purpose and the conditions so obtained were incorporated in recommended procedures.

**Optical Characteristics:** In order to test whether the colored species formed in the above methods, adhere to Beer’s law the absorbance’s at appropriate wave lengths of a set of solutions
containing varying amounts of 7-ADCA and specified amounts of reagents (as given in the recommended procedures for each method) were recorded against the corresponding reagent blanks. The Beer’s law plots of these systems are recorded against the corresponding reagent blanks. The Beer’s law plots of these systems are recorded graphically. Beer’s law limits, molar absorptivity, Sandell’s sensitivity and optimum photometric range for 7-ADCA in each method developed. With mentioned reagents were calculated. Least square regression analysis was carried out for getting the slope, intercept and correlation coefficient values (Table 1).

**Precision:** The precision of each proposal methods was ascertained from the absorbance values obtained by actual determination of six replicates of a fixed amount of 7-ADCA in total solution. The percent relative standard deviation and percent range of error (at 0.05 and 0.01 confidence limits) were calculated for the proposed methods (Table 1).

**Accuracy:** To determine the accuracy of each proposed method, different amounts of bulk samples of 7-ADCA within the Beer’s law limits were taken any analyzed by the proposed method. The results (percent error) are recorded in (Table 1).

**Interference studies:** The effect of wide range of excipients and other active ingredients usually present in the formulations for the assay of 7-ADCA in methods (M1, M2) under optimum conditions were investigated. The commonly used excipients and other active ingredients usually present in formulations did not interfere even if they were present in amount than they usually exist.

**Analysis of formulations:** Commercial formulations (tablets) containing 7-ADCA were successfully analyzed by the proposed methods. The values obtained by the proposed and reference methods for formulations were compared statistically with F and t tests and found not to different significantly. Percent recoveries were determined by adding standard drug to preanalyzed formulations. The results of the recovery experiments by the proposed methods are also listed in Table 2.

**Chemistry of the colored species:** The chemistry of the colored species formed in each one of the proposed methods for the assay of 7-ADCA has been presented scheme 1 and 2

**Method M1:** In the present investigation, the drug 7-ADCA that possesses NH₂ in β-lactam portion, when heated with Ninhydrin in presence of ascorbic acid afforded a blue violet color product. The reaction pathway can be represented in scheme 1.
**Method M$_2$:** The oxidation of $\beta$-naphthol with $\text{H}_2\text{O}_2$ in a solution of 7-ADCA, which contains $-\text{NH}_2$ group and copper nitrate in methanol, gives amino substituted 1,2–naphthoquinone. It further reacts with 2, 4-dinitro phenyl hydrazine under acid conditions to give colored phenyl hydrazine derivative (scheme 2).

![Scheme 2](image)

**Table 1. Optical and regression characteristics, precision and accuracy of the proposed methods for 7-ADCA**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$M_1$</th>
<th>$M_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>400</td>
<td>420</td>
</tr>
<tr>
<td>Beer’s law limits ($\mu$g/ml)</td>
<td>0.5-3.0</td>
<td>0.8-4.8</td>
</tr>
<tr>
<td>Detection limit ($\mu$g/ml)</td>
<td>0.2088</td>
<td>0.2007</td>
</tr>
<tr>
<td>Molar absorptivity (L.mol/cm)</td>
<td>$6.701 \times 10^4$</td>
<td>$7.015 \times 10^4$</td>
</tr>
<tr>
<td>Sandell’s sensitivity ($\mu$g/cm$^2$/0.001 absorbance unit)</td>
<td>$3.708 \times 10^{-2}$</td>
<td>$3.546 \times 10^{-2}$</td>
</tr>
<tr>
<td>Optimum photometric range ($\mu$g/ml)</td>
<td>1.26-3.0</td>
<td>1.6-4.4</td>
</tr>
<tr>
<td>Regression equation ($Y=a+bc$)</td>
<td>0.1735</td>
<td>0.1561</td>
</tr>
<tr>
<td>slope (b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation on slope ($S_b$)</td>
<td>$5.772 \times 10^{-3}$</td>
<td>$3.811 \times 10^{-3}$</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>$6 \times 10^{-3}$</td>
<td>$4.999 \times 10^{-3}$</td>
</tr>
<tr>
<td>Standard deviation on intercept ($S_a$)</td>
<td>$9.572 \times 10^{-3}$</td>
<td>$10.11 \times 10^{-3}$</td>
</tr>
<tr>
<td>Standard error on estimation ($S_e$)</td>
<td>$9.127 \times 10^{-3}$</td>
<td>$9.642 \times 10^{-3}$</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9979</td>
<td>0.9992</td>
</tr>
<tr>
<td>Relative standard deviation (%)*</td>
<td>0.9059</td>
<td>0.5311</td>
</tr>
<tr>
<td>% Range of error (confidence limits)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 level</td>
<td>1.041</td>
<td>0.6116</td>
</tr>
<tr>
<td>0.01 level</td>
<td>1.633</td>
<td>0.9576</td>
</tr>
</tbody>
</table>

*Average of three determinations
Table 2. Assay of 7-ADCA in Pharmaceutical Formulations

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Amount taken (mg)</th>
<th>Amount found by proposed methods</th>
<th>Reference method</th>
<th>Percentage recovery by proposed methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₁</td>
<td>M₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tablet I</td>
<td>20</td>
<td></td>
<td>19.74±0.64 F = 1.373 t = 0.55</td>
<td>19.96±0.75</td>
</tr>
<tr>
<td>Tablet II</td>
<td>20</td>
<td></td>
<td>19.65±0.63 F = 1.9511 t = 0.73</td>
<td>19.97±0.88</td>
</tr>
<tr>
<td>Tablet III</td>
<td>20</td>
<td></td>
<td>19.72±0.52 F = 1.421 t = 0.60</td>
<td>19.92±0.62</td>
</tr>
<tr>
<td>Tablet IV</td>
<td>20</td>
<td></td>
<td>19.52±0.56 F = 1.841 t = 1.18</td>
<td>19.97±0.76</td>
</tr>
</tbody>
</table>

aTablets from four different pharmaceutical companies. bAverage ± standard deviation of six determinations, the t- and F-test values refer to comparison of the proposed method with the reference method. Theoretical values at 95% confidence limit, F = 5.05, t = 2.57; cRecovery of 10 mg added to the pre-analyzed pharmaceutical formulations (average of three determinations).

CONCLUSIONS

The proposed methods exploit the various functional groups in 7-ADCA molecule. The decreasing order of sensitivity ($\varepsilon_{\text{max}}$) and the $\lambda_{\text{max}}$ among the proposed methods are $M_2 > M_1$ respectively. The concomitants, which do not contain the functional groups chosen in the present investigation, do not interfere in the color development by proposed methods. Thus the proposed methods are simple, sensitive and selective with reasonable precision and accuracy and constitute better alternatives to the reported ones in the assay of 7-ADCA in bulk form and pharmaceutical formulations.

REFERENCE

[6] L Antonio; Doadrio; Antonio Mayorga; Regina Orenge, Journal of the Brazilian Chemical Society, 2002, 13 (1), 95-100