Research Article

# NEW SPECTROPHOTOMETRIC METHODS FOR QUANTITATIVE DETERMINATION OF 7-ADCA IN PHARMACEUTICAL FORMULATIONS

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**ABSTRACT**

Three simple, sensitive and accurate methods are described for the determination of 7-Amino deacetoxy cephalosporanic acid (7-ADCA) in bulk drug and in formulations. Methods ($M_a$ to $M_c$) are based on ion association complex between 7-ADCA and Safranin O ($M_a$) Methylene blue (MB) ($M_b$) and Methylene Violet (MV) ($M_c$) solutions. The chromogen being extractable with chloroform could be measured quantitatively at 530 ($M_a$) and 655 nm ($M_{b\&c}$). All variables were studied to optimize the reaction conditions. Regression analysis of Beer's Law plot showed good correlation in the concentration range 1.0 to 5-6.0 for $M_a$, 1.25 -7.5 for $M_b$ and 2-12µg/mL for $M_c$. The calculated molar absorptivity values are 2.998 x $10^4$, 2.971 x $10^4$, and 1.423 x $10^4$ L/mol/cm for $M_a$ and $M_c$, 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.

**Keywords:** 7-Amino deacetoxy cephalosporanic acid, ion association complex, 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 Safranin O ($M_a$) MB ($M_b$) and MV ($M_c$). All these methods have been extended to pharmaceutical formulations as well. The author has developed three simple and sensitive UV methods ($CH_3OH$ 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

## MATERIALS AND METHODS

**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 1.0 µg/mL ($M_a$), 0.5 µg/mL ($M_{b\&c}$).

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

Safranin O solution (Fluka; 0.2%, w/v 5.714 x $10^{-3}$M): Prepared by dissolving 200 mg of safranin O in 100 ml of

distilled water and subsequently washed with chloroform to remove chloroform impurities.

MB solution (Fluka; 0.2%, w/v $6.25x10^{-3}$M): Prepared by dissolving 200 mg of Methylene Blue in 100 ml of distilled water and subsequently washed with chloroform to remove chloroform soluble impurities.

MV solution: Prepared by dissolving 200 mg of Methylene Violet in 100 ml of distilled water and subsequently washed with chloroform to remove chloroform impurities.

Buffer solution pH 9.8 ($NH_4OH$ – $NH_4$ Cl): 7g of $NH_4Cl$ and 6.8ml of liquid Ammonia solutions were mixed and diluted to 100 ml with distilled water and pH was adjusted to 9.8.

**Recommended Procedures**

**Method $M_a$, $M_b$& $M_c$:** Aliquots of standard drug solution 1.0-5.0 ml for method $M_a$, $M_b$ & $M_c$ (0.5-3.0 mL, 25 μg/mL) and 1.0 mL of pH 9.8 buffer solutions were placed separately in a series of 125 mL separating funnels. A volume of 1.0 mL of Safranin O ($M_a$), 0.5 mL of MB ($M_b$) and 0.5 mL of MV ($M_c$) was added respectively. The total volume of aqueous phase in each funnel was adjusted to 10.0 mL with distilled water. Then 10 mL of chloroform was added in each separating funnel and the contents were shaken for 2 min and allowed to separate. The organic layer was collected through cotton plug and the absorbance was measured immediate at 530 nm ($M_a$) and at 655 nm ($M_b$ & $M_c$) against reagent blank. Both the colored species were stable for 2 hours. The amount of drug in a sample was obtained from the Beer's Lambert plot.

**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 $\lambda_{max}$ 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 fro bulk solutions.

**RESULTS AND DISCUSSION**

**Spectral Characteristics:** In order to ascertain the optimum wavelength of maximum absorption ($\lambda_{max}$) 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 1.25 μg/mL ($M_a$,$M_b$&$M_c$). 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 in these methods were fixed based on the study of the effects of various parameters such as type of acid for buffer, conc. of acid, conc. of dye Safranin O ($M_a$), MB ($M_b$), and MV ($M_c$) choice of organic solvent, ratio of organic phase to aqueous phase, shaking time, temp, intensity and stability of the colored species in organic phase. The author performed controlled in pediments by measuring absorbance at λmax 530 nm ($M_{2a}$) and 655 nm ($M_b$, $M_c$) of a series of solutions varying one and fixing the other parameter and the results are recorded in Table 1.

**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 (Table 2) 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.

**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 2).

**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 (% error) are recorded in Table 2.

**Table 1:** Optimum conditions established for the proposed methods

| Parameter | Optimum range | Conditions in procedure | Remarks |
|---|---|---|---|
| $\lambda_{max}$ (nm)<br>$M_a$<br>$M_b$<br>$M_c$ | <br>525-535<br>650-660<br>650-660 | <br>530<br>655<br>655 | -- |
| Effect of buffer on color development | 9.0-10.0 | pH-9.8 | Variations of the pH<6 and >11 resulted in low absorbance values |
| Volume of buffer required for maximum intensity of color (ml) | 0.5-1.5 | 1.0 | Optimum volume of 1.0ml of buffer was sufficient for maximum color development |
| Effect of vol of dye SFNO ($M_a$)<br>MB ($M_b$)<br>MV($M_c$) | <br>1.0-5.0<br>0.1-1.0<br>0.1-1.0 | <br>1.5<br>0.5<br>0.5 | 1.5ml of SFNO ($M_a$), 0.5 ml of MB ($M_b$) and 0.5 ml of MB ($M_c$) dye was necessary for covering the broad range of beer's law limits |
| Choice of organic solvent for extraction of colored complex | Chloroform | Chloroform | The other water immiscible solvents tested for the extraction of the colored complex into roganic phase include chlorobenzene, dichloro methane, $CCl_4$, $C_6H_6$ butanol $CHCl_3$ was preferred for its selective extraction of the colored drug-dye complex from the aqueous phase. |
| Effect of the ratio of organic to aqueous phase on extraction | 1:1 | 1:1 | The extraction of the colored species in to Chloroform layer was in complete when the ratio of chloroform to aqueous phase was more than the specified ratio in each case. |
| Effect of shaking time (min) | 1-5 | 2 | Constant absorbance values were obtained for the shaking period of 1-5 min. |
| Effect of temperature on the colored species ($C^0$) | Lab-Temp (28±5) | Lab-Temp (28±5) | At low temperature (<$20^0$C) and at high temperature (>$35^0$C) the extraction of the colored species was found to be improper and the stability of the colored species was found to be very less. |
| Stability of the colored species | Immediate to 60 min | 10 min | The colored species after separation from organic phase was stable for 60 min, after wards the absorbance gradually decreases. |

**Table 2:** Optical and regression characteristics, precision and accuracy of the proposed methods for 7-ADCA

| Parameter | $M_1$ | $M_2$ | $M_2$ |
|---|---|---|---|
| $\lambda_{max}$ (nm) | 530 | 655 | 655 |
| Beer's law limits (µg/mL) | 1.0-6.0 | 1.25-7.5 | 2-12 |
| Detection limit (µg/mL) | 0.07374 | 0.06312 | 0.7475 |
| Molar absorptivity (L.moL/cm) | $2.998 \times 10^4$ | $2.971 \times 10^4$ | $1.423\ 10^4$ |
| Sandell's sensitivity (µg/$cm^2$/0.001 absorbance unit) | $6.486 \times 10^{-2}$ | $6.238 \times 10^{-2}$ | 0.1027 |
| Optimum photometric range (µg/mL) | 2.5-4.5 | 3.6-7.5 | 5.0-12 |
| Regression equation (Y=a+bc)<br>slope (b) | <br>0.0655 | <br>0.0645 | <br>0.0302 |
| Standard deviation on slope ($S_b$) | $8.705 \times 10^{-4}$ | $3.2485 \times 10^{-4}$ | $1.1335 \times 10^{-3}$ |
| Intercept (a) | $6.75 \times 10^{-3}$ | $4.999 \times 10^{-4}$ | $4.999 \times 10^{-12}$ |
| Standard deviation on intercept ($S_a$) | $1.443 \times 10^{-3}$ | $1.347 \times 10^{-3}$ | $7.519 \times 10^{-3}$ |
| Standard error on estimation ($S_e$) | $1.376 \times 10^{-3}$ | $1.2841 \times 10^{-3}$ | $7.169 \times 10^{-3}$ |
| Correlation coefficient (r) | 0.9999 | 0.9996 | 0.9999 |
| Relative standard deviation (%)* | 0.2428 | 1.350 | 1.557 |
| % Range of error (confidence limits) | | | |
| 0.05 level | 0.2791 | 0.15 | 1.79 |
| 0.01 level | 0.4378 | 2.43 | 2.80 |
| % error in Bulk samples ** | 0.10 | 0.164 | -0.260 |

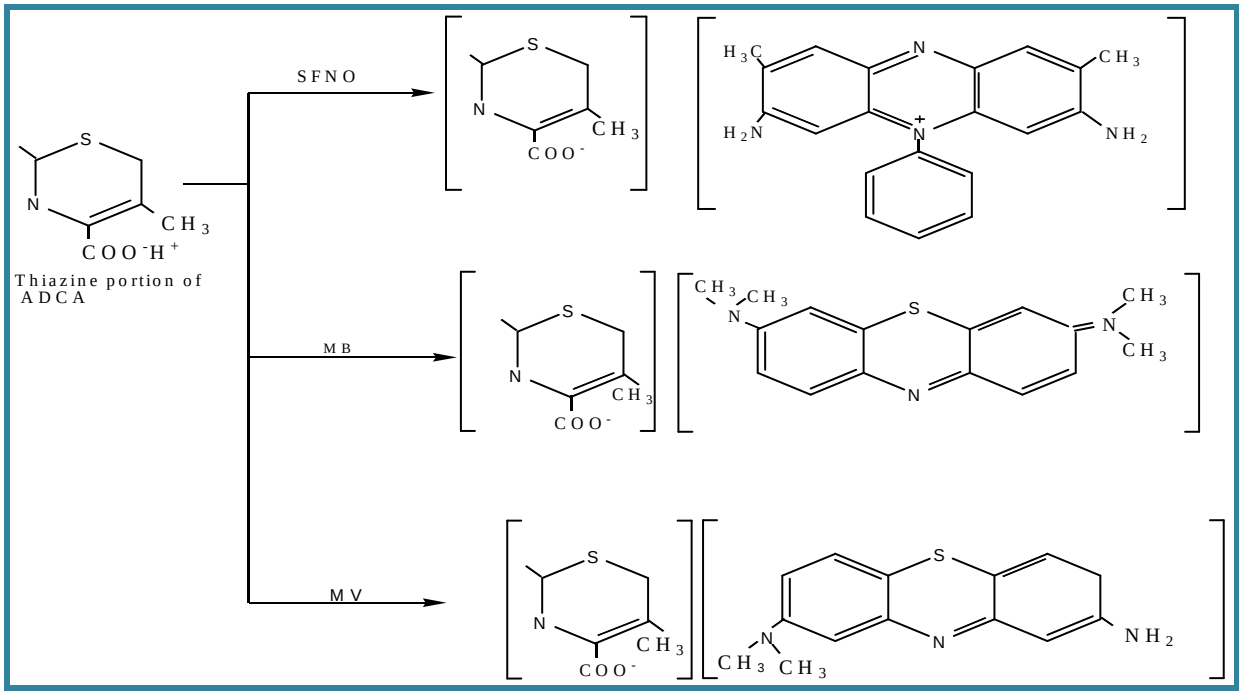
*average of three determinations ** Average of six determinations

**Table 3:** Assay of 7-ADCA in Pharmaceutical Formulations

| Formulations | Amount taken (mg) | Amount found by proposed Methods | | | | Percentage recovery by proposed methods | | |
|---|---|---|---|---|---|---|---|---|
| | | $M_a$ | $M_b$ | $M_c$ | Reference method | $M_a$ | $M_b$ | $M_c$ |
| Tablet I | 20 | 19.91±0.57<br>F=2.019<br>t=0.5 | 19.83±0.63<br>F=1.653<br>t=0.69 | 19.88±0.69<br>F=1.378<br>t=0.55 | 20.12±0.81 | 99.61±0.56 | 99.80±0.83 | 99.87±0.45 |
| Tablet II | 20 | 19.56±0.49<br>F=2.342<br>t=1.061 | 19.32±0.58<br>F=1.672<br>t=1.614 | 19.45±0.66<br>F=1.291<br>t=1.21 | 19.94±0.75 | 99.71±0.62 | 99.82±0.96 | 99.55±0.99 |
| Tablet III | 20 | 19.67±0.38<br>F=1.945<br>t=1.1 | 19.72±0.42<br>F=1.592<br>t=0.95 | 19.77±0.44<br>F=1.450<br>t=0.75 | 19.98±0.53 | 99.72±0.19 | 99.36±0.35 | 99.74±0.46 |
| Tablet IV | 20 | 19.49±0.52<br>F=1.5147<br>t=1.28 | 19.63±0.46<br>F=1.9357<br>t=0.91 | 19.58±0.49<br>F=1.705<br>t=1.05 | 19.92±0.64 | 99.85±0.16 | 99.76±0.52 | 99.66±0.46 |

[a]Tablets from four different pharmaceutical companies. [b]Average ± 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; [c]Recovery of 10 mg added to the pre-analyzed pharmaceutical formulations (average of three determinations)

**Scheme 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 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 3.

**Chemistry of the colored species**: As 7-ADCA possesses carboxyl group (acidic), in dihydrothiazine is responsible for color formation in ion association complex with basic dyes (Safranin O, Methylene blue and Methylene violet),

which is extractable into chloroform from aqueous phase. The carboxylate anion (negative charge) of 7-ADCA is expected to attract the oppositely charged part of the dye (positive charge, safranine O, methylene blue and methylene violet) and behave as single unit being held together by electrostatic attraction. It is supported by slope ratio method, which was obtained as 1:1 in each method ($M_a$, $M_b$, and Mc). Based on analogy the structure of ion association complexes are shown in scheme 1.

## CONCLUSION

It is concluded that the newly developed spectrophotometric methods for the determination of 7-ADCA are reliable economical. The results are in good agreement with reference method. The literature indicated that this color reaction have not been reported previously. 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.

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