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 NQS (M_a), vanillin (M_b) and Ninhydrin (M_c) solutions. The chromogen being extractable with chloroform could be measured quantitatively at 480 (M_a) and 560 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 4-24 for M_a, 0.4-2.4 for M_b and 0.5-3.0 µg/mL for M_c. The calculated molar absorptivity values are 5.945 x 10^3, 1.722 x 10^5, and 6.701 x 10^4 L/mol/cm for M_a to 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.
Key words: 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 NQS (M_a); Vanillin (M_b); Ninhydrin (M_c). All these methods have been extended to pharmaceutical formulations as well. The author has developed two 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

EXPERIMENTAL

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

Method M_a: *NQS solution* (Loba, 0.5%, $1.92x10^{-2}$M): 500 mg of 1,2- naphthaquinone-4-sulfonic acid sodium salt (NQS)in 100 ml of distilled water

NaOH solution (E.Merck, 20%, 5M): Prepared by dissolving 20gms of sodium hydroxide in 100ml of distilled water

Method M_b: *Vanillin solution* (BDH, 0.4%, 2.63 x 10^{-3}M): Prepared by dissolving 400 mg of Vanillin in 100 ml of CH_3OH

H_2SO_4 (Merck,Conc.): Used as it is.

Method M_c: *Ninhydrin solution* (BDH 1%, $5.605x10^{-5}$m): Prepared by dissolving 1 gm of ninhydrin in 100 ml of acetone

Ascorbic acid solution (BDH; 0.1%, $5.678x10^{-3}$M): Prepared by dissolving 100mg of ninhydrin in 100 ml of distilled water

Buffer solution (pH 5.0): Prepared by diluting a mixture of 200ml 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

Recommended Procedures

Method M_a: Aliquot of standard 7-ADCA solution (0.5ml - 3ml; 50µg/ml) were transferred into a series of calibrated tubes containing 0.2 ml of0.02N NaOH and 0.2 ml of 0.5% NQS reagent solution was added in each tube and the contents were heated at 50^0c for 5 min and cooled for 2 min in ice water. This operation was performed in the dark. The contents were transferred to 25 ml separating funnel containing 6.5 ml of dichloromethane, rinsed the tube with 1 ml of water and poured the rinsing into the funnel and shaken immediately for 5 sec. The whole bulk of the organic layer from the bulk was collected after 2 min and 3 ml of DNPH was added. It was heated for 10 min at 50^0c by using air condenser and chilled in ice water. Then 0.5 ml of con H_2SO_4 was added slowly, mixed and the absorbance were measured after 5 min at 480 nm against a reagent blank prepared similarly. The amount of 7-ADCA was calculated from its calibration graph.

Method M_b: To each of 25ml calibrated tubes, aliquots (0.5-2.5 ml, 20µg/ml) of standard 7-ADCA solution, 2.0 ml of vanillin and 3 ml of con sulphuric acid were added successively and the total volume in each flask was brought to 20 ml by the addition of methanol and placed in heating water bath (maintained at 50^0c) for 15 min. Then the flasks were colored and made up to the mark with methanol and the absorbance's were measured at 560 nm against a reagent blank prepared in a similar way. The con of drug in a sample was computed from Beer-Lambert plot.

Method M_c: Aliquot of standard 7-ADCA solution (0.5ml 2.5ml; 400µg) was transferred into a series of calibrated tubes containing 4.0 ml of buffer (pH 5.0), 1.0 ml ninhydrin solution and 0.5ml of ascorbic acid solution. The volume in each tube was adjusted to 8.0ml 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 560nm after 10 min against a reagent blank prepared similarly. The amount of ADCA was calculated from its calibration graph.

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 fro 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 12 µg/ml (M_a), 1 µg/ml (M_b), 40 µg/ml (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 for the color development of methods (M_a, M_b, & M_c) 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.

Method M_a: In developing this method a systematic study of the effects of various parameters were under taken by varying one parameter at a time and controlling all other fixed. The effects of various parameters such as time, volume and strength of NQS & NaOH, solvent for final dilution on the stability and intensity of colored species were studied and the optical condition are incorporated in Table 1a

Methods M_b: Among different aldehydes (Vanillin) tried for developing the color in alcoholic medium (MeOH), Vanillin was found to be superior for it's sensitivity. This method involves the condensation of the ADCA with Vanillin in the presence of acid. The effect of various parameters, such as concentration and volume of Vanillin, nature and strength of acid, order of addition of reagents, solvent for final dilution were studied and the optimum conditions developed and actual conditions chosen for the procedure are recorded in Table 1b.

Method M_8: The method involves the reaction between drug and ninhydrin reagent to produce blue color. The conditions were fixed basing on the study of the effects of various parameters such as volume of ninhydrin, nature and conc. of reducing agent, pH and volume of the buffer, heating time and temp, order of addition of the reagents, solvent for final dilution and stability of the colored products after final dilution. The optimum conditions were established by measuring the absorbances at 560 nm and the results are presented in Table 1c.

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).

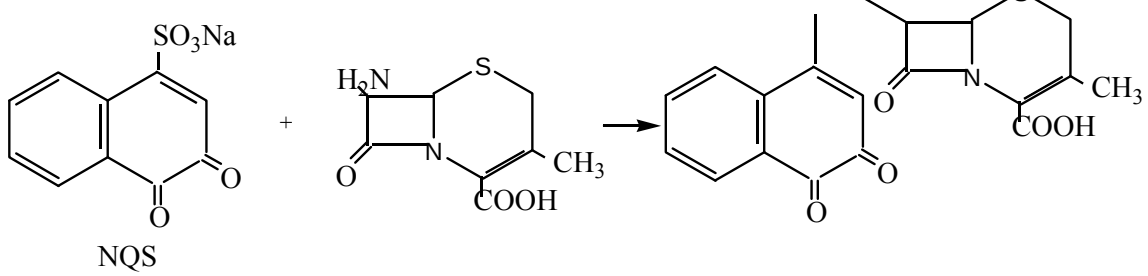
Accuracy: To determine the accuracy of each proposed method, different amounts of bulk samples of 7-ADCA within the Beer's law limits were taken any analyzed by the proposed method. The results (% error) are recorded in Table 2.

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.

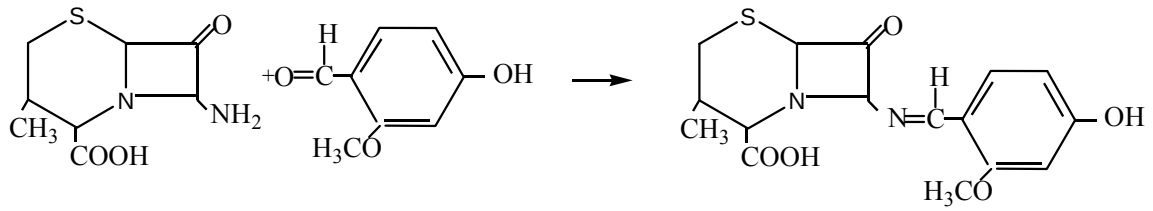
Chemistry of the colored species

Method M_a: Formation of colored complex, when ADCA is treated with NQS appears to be due to the presence of amino substituent linked to β-lactam portion yielding the colored product as shown in scheme 1.



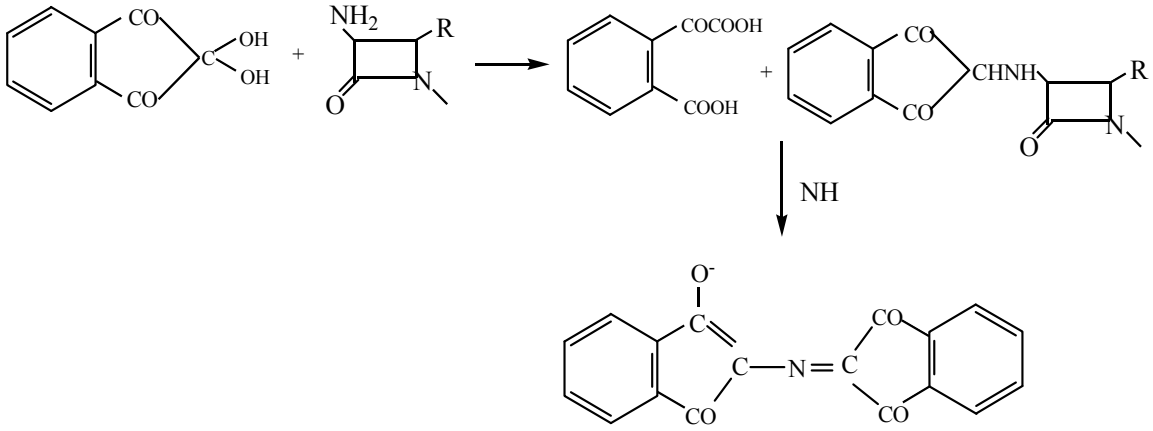
Scheme 1

Method M_b: In the present work, aromatic primary amine group in 7-ADCA condenses with Vanillin. The colored species postulated as follows. The azomethine formation in the reaction between vanillin and 7-ADCA may be represented as follows.



Scheme 2

Method M_c: The drug 7-ADCA that possesses NH_2 in β-lactam portin, when heated with ninhydrin in presence of ascorbic acid afforded a blue violet color product. The reaction pathway can be represented in scheme 3.



Scheme 3

CONCLUSIONS

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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Table 1a: Optimum conditions established for the proposed method M_a

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	480-490	480	
Effect of NQS on color development ($1.92x10^{-2}M$)	6.25-0.75ml	0.5ml	The use of < 0.25 ml NQS resulted in a decrease in absorbance,> 0.75ml resulted in cloudiness.
Effect of NaOH,5M on the absorbance of the final colored species	1.4-2.8ml	2ml	<1.4ml and>2.8ml was found to disturb Beer's law obeyance in a broad range.
Solvent for final dilution	H_2O	H_2O	Other water viscible solvent did not enhance the color of final colored solution.
Stability period after final dilution	8-45 min	10 min	8 minutes was necessary for the attainment of max color. It remains stable upto 45 min.

Table 1b: Optimum conditions established in method M_b

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	415-42	420	
Volume of Vanillin ($2.63 \times 10^{-3}M$)	1.5-3.0ml	2.0ml	Two ml of Vanillin ($2.63x10^{-3}M$) was necessary for covering broad range of Beer's law limits
Effect of vol. Of con H_2SO_4 on color development	2-4 ml	3ml	<1.5 ml of con H_2SO_4 results in low absorbance values and >4 ml results in instability of the colored product.
Effect of the order of addition reagents on color development.	ADCA, Vanillin, Con. H_2SO_4	ADCA, Vanillin, Con. H_2SO_4	If the order of addition is changed, low absorbance values resulted.
Effect of temperature and time	40-50^0c 10-20 min	50^0c 15 min.	Above 50^0c methanol evaporates.
Stability period after final dilution	Immediate - 30 min	5 min	--

Table 1c: Optimum conditions established in method M_c

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	555-565	560	--
Volume of Ninhydrin ($5.605x10^{-2}$M) in acetone required	0.8-1.2 ml	1.0 ml	One ml of Ninhydrin was found to be necessary for color product formation and to cover broad range of Beer's law limits. No added advantage was observed even when excess ninhydrin was used.
Volume and pH of the buffer	3.5-4.5 ml pH 4.8-5.2	4.0 ml pH 5.0	Four ml of pH 5.0 buffers was found to be optimum for maximum color development.
Nature of the reducing agent	AA	AA	Among the two reducing agents tried (AA, $Sncl_2$), AA was found to me more efficient with respect to stability attainment
Volume of AA solution ($5.678x10^{-3}$M)	0.4 – 0.6 ml	0.5 ml	Half ml of AA was found to be adequate for maximum color development
Order of addition	Buffer, Ninhydrin, AA	Buffer, Ninhydrin, AA	Order of addition has no significant effect
Time and temperature for maximum color development	10-15 min boiling water bath	10-15 min boiling water bath	Heating in water bath for 10 min was required to obtain better results (sensitivity & reproducibility
Solvent for final dilution	Water	Water	No advantage was observed with usage of other water miscible solvents (acetone and MeOH) instead of water.
Stability of the colored species	5-60 min	10 min	The absorbance of the colored product decreased slowly with time after 1 hour.

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

Parameter	M_a	M_b	M_c
λ_{max} (nm)	480	560	560
Beer's law limits (μg/ml)	4-24	0.4-2.4	0.5-3.0
Detection limit (μg/m)	0.9330	0.0347	0.2088
Molar absorptivity (L.mol/cm)	5.945×10^3	1.722×10^5	6.701×10^4
Sandell's sensitivity (μg/cm^2/0.001 absorbance unit)	0.1838	2.231×10^{-2}	3.708×10^{-2}
Optimum photometric range (μg/ml)	5-17.78	1.1-2.4	1.26-3.0
Regression equation (Y=a+bc)			
slope (b)	0.0137	0.3768125	0.1735
Standard deviation on slope (S_b)	2.980×10^{-4}	3.123×10^{-3}	5.772×10^{-3}
Intercept (a)	9.999×10^{-4}	0.01875	6×10^{-3}
Standard deviation on intercept (S_a)	3.953×10^{-4}	4.143×10^{-3}	9.572×10^{-3}
Standard error on estimation (S_e)	3.769×10^{-3}	3.950×10^{-3}	9.127×10^{-3}
Correlation coefficient (r)	0.9993	0.9998	0.9979
Relative standard deviation (%)*	1.807	0.6271	0.9059
% Range of error (confidence limits)			
0.05 level	2.07	0.7211	1.041
0.01 level	3.25	1.130	1.633
% error in Bulk samples **	0.102	0.166	0.10

*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	19.79±0.70 F=1.338 t=0.75	19.62±0.47 F=2.546 t=0.962	19.74±0.64 F=1.373 t=0.55	19.96±0.75	99.83±0.99	99.62±0.76	99.90±0.95
Tablet II	20	19.63±0.62 F=3.808 t=0.7	19.54±0.59 F=2.224 t=1.01	19.65±0.63 F=1.9511 t=0.73	19.97±0.88	99.31±0.93	99.66±0.55	99.46±0.82
Tablet III	20	19.56±0.39 F=1.846 t=1.5	19.81±0.41 F=2.286 t=1.161	19.72±0.52 F=1.421 t=0.60	19.92±0.62	99.90±0.32	99.63±0.98	99.94±0.73
Tablet IV	20	19.65±0.43 F=2.215 t=0.88	19.73±0.48 F=3.008 t=0.67	19.52±0.56 F=1.841 t=1.18	19.97±0.76	99.54±0.60	99.68±0.98	99.86±0.65

[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)