VALIDATION OF 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 Redox / complex formation reaction between 7-ADCA and ammonium Molybdate and sulphuric acid (AM/H_2SO_4) (M_a), Ferric chloride/ Ortho Phenonthraline (Fe^{+3}/o-Phen)(M_b) and $FeCl_3$/potassium Ferricyanide ($Fe^{+3}/K_3Fe(CN)_6$) (M_c) solutions. The chromogen being extractable with chloroform could be measured quantitatively at 625 (M_a), 510 (M_b) and 740 nm (M_c). All variables were studied to optimize the reaction conditions. Regression analysis of Beer's Law plot showed good correlation in the concentration ranges 10-60 for M_a, 1.25-7.5 for M_b and 2.5-15 µg/ml for M_c. The calculated molar absorptivity values are 2.835×10^3, 2.277×10^4, and 1.715×10^4 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-ADCA, Redox / complex formation reactions, 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 (Yang FL et al, 1999, Velasco J et al 2000, Yamazaki T et al,1976, Deshmukh P et al, 1998, Schroën CGPH et al 2002, Antonio L et al 2002, Kovačić-Bošnjak N et al 1987, Dengchao Li et al, 2008), CE (Nierstrasz AV et al 1997), GC-MS (Jette Thykaer et al 2002, Aki at al 1976), and spectrophotometric methods (Medikondu Kishore et al 2010, Dutta N et al 2005). 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 7-7-ADCA with reagents such as AM/H_2SO_4 (M_a); (Fe (III)/o-Phenanthroline) (M_b); $Fe^{+3}/K_3Fe(CN)_6$ (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: AM Solution (Loba; 2%, 1.618 x 10^{-2}M): Prepared by dissolving 2g of Ammonium Molybdate in 100 ml of distilled water

H_2SO_4 solution (Conc H_2SO_4 to Qualigens, 1M): Prepared by diluting 105 ml Conc H_2So_4 to 100 ml of distilled water initially followed by diluting to 1000ml with distilled water.

Method M_b: Fe (III) solution (Wilson labs; (0.9%, $1.1x10^{-2}$ M): Prepared by dissolving 900mg of anhydrous Ferric chloride in 100 ml of distilled water.

PHEN solution (Merck, 0.2%, $1.10x10^{-2}$M): Prepared by dissolving 200mg of o-phenanthroline in 100 ml of distilled water with warming.

o-Phosphoric acid solution (Qualigens, $2.0x10^{-2}$M): Prepared by mixing 1.27ml of o-phosphoric acid with 100ml of distilled water. Ten millilitres of this stock solution was diluted to 100ml with distilled water.

Method M_c: Potassium ferricyanide solution (BDH; 0.1%, $3.02x10^{-3}$ M): Prepared by dissolving 100mg of $K_3Fe(CN)_6$ in 100 ml of distilled water.

Fe(III) solution (Wilson labs; (0.054%, $3.32x10^{-3}$ M): Prepared by dissolving 54mg of anhydrous Ferric chloride in 100 ml of distilled water

Recommended Procedures

Method M_a: Aliquots of standard drug solution (0.5-2.5 ml, 100 µg/ml) were delivered in to a series of 10 ml calibrated tube. To each tube 1.0ml of AM (1.618 x 10^{-2} M) reagent and 0.5 ml of 1 M H_2SO_4 were added to each tube and the contents were heated for 20 min in boiling water bath. After cooling the volume was made up to 10 ml with distilled water. The resulting absorbance of the green color was measured at 625 nm against a reagent blank. The amount of drug was computed from to appropriate calibration graph.

Method M_b: Aliquots (0.5-3.0ml, 10µg/ml) of standard 7-ADCA solution were transferred into a series of 10ml calibrated tubes and then solutions of 0.5ml ($1.1x10^{-2}$M) of Fe (III), 2 ml of ($1.10x10^{-2}$M) o-Phenonthraline were added successively. The total volume in each tube was brought to 5.0ml with distilled water. The tubes were kept on a boiling water bath for 30 min. The tubes were removed and cooled to room temperature.
Two milliliters of ($2.0x10^{-2}$M) o-phosphoric acid was added and volume in each tube was made up to the mark (10ml) with distilled water. The absorbance of the colored complex solution was measured after 5 min. At 510 nm against a reagent blank prepared similarly. The content of the drug was computed from the appropriate calibration graph.
Method M_c: Into a series of 10 ml calibrated tubes, aliquots of standard 7-ADCA solution (0.5- 2.5 ml, 200 µg.ml^{-1}) were transferred and 1 ml of 3.32 x 10^{-3} M $FeCl_3$ solution was added .The tubes were stopper immediately and shaken well for 5 min. Then 0.5 ml of $3.02x10^{-3}$ M Potassium ferricyanide solution was added into each tube and was closed with lids immediately. After 5 min. 1 ml of 1 N HCl was added and the final volume was added upto 10 ml with distilled water. The absorbance of the solution in each tube was measured immediately at 740 nm against a similar reagent blank. The amount of the drug 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 10 µg/ml (M_a), 1 µg/ml (M_b), 20 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: The method involves the reaction of the drug 7-ADCA with AM in acid medium. The effect of various parameters, such as concentrated H_2SO_4 and AM, nature and strength of acid, order of addition of reagents, solvent for final dilution were studied and the optimum conditions developed and recorded in Table1a.

Table1a: Optimum conditions established in M_a

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	620-630	625	
Effect of vol of ($1.61x10^{-2}$M) AM	0.7-1.3ml	1.0 ml	One ml of AM ($1.61x10^{-2}$M) was necessary for covering broad range of beer's law limits
Effect of volume of Conc. H_2SO_4 on color development	3.0-5.0	4.0ml	<3.0 ml of conc. H_2SO_4 results in low absorbance values and >5.0 ml has no additional value.
Effect of the order of addition of reagent on color development	Drug, AM Conc. H_2SO_4	Drug, AM conc. H_2SO_4	The change in the order of addition has no effect.
Effect of temperature and time	Boiling water bath 20-30min	Boiling water bath 20min	It was found that boiling water bath was necessary for uniform temperature and maximum color development. Heating on a boiling water bath for 20 min is necessary for maximum colour development
Solvent for final dilution	Ethanol	Ethanol	The absorbance of the test solution decreased when water was used instead of ethanol for dilution.
Stability period after final dilution	5min-24 hours	5min	

Method M_b (Fe (III)/o-Phenanthroline)**:** In order to establish optimum conditions necessary for rapid and quantitative formation of the colored complex with maximum stability and sensitivity, the author has performed control experiments by varying one and fixing the other parameters, such as effect of pH of the buffer solution, volume of buffer solution, volume of Fe (III) and o-phenanthroline solution, temperature, heating time, order of addition of reagents and nature of solvents for final dilution. The optimum conditions are incorporated in Table1b.

Method M_c: The optimum conditions in this method were fixed basing on the study of the effects of various parameters such as volumes of $3.32x10^{-3}$ M ferric chloride solution, 3.02 x 10^{-3} M potassium ferricyanide solution and 1N HCl, time and temperature necessary for complete color development, the stability and intensity of the colored species after final dilution were established by measuring absorbance's at 740 nm and results were incorporated in Table1c.

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).

Table 1b: Optimum conditions established in M_b

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	500-520	510	
Effect of volume of ($1.1x10^{-2}$M) Fe(III) solution on color development	0.25-0.5 ml	0.5 ml	Variation of volume below and above of this range gave erratic results
Effect of volume of ($1.1x10^{-2}$M) PHEN on colour development	1.5 –3.0 ml	2.0 ml	Variation of volume below and above of this range gave erratic results
Effect of temperature on colored species	90-99^{0}C	Boiling Water Bath	It was found that boiling water bath was necessary for uniform temperature and maximum color development. Below this temperature the intensity of the colored complex was less.
Effect of heating time	25-40 min	30 min	Below 25 min the colored complex was not completely formed.
Effect of volume of ($2.0x10^{-2}$M) o-phosphoric acid	1.0-3.0 ml	2.0 ml	To complex excess of Fe(III) ions, a minimum of 1.0 ml of o-phosphoric acid was required.
Effect of order of addition of reagents on color development	Drug, Fe(III) and PHEN before heating & phosphoric acid after heating	Drug, Fe(III) and PHEN before heating & phosphoric acid after heating	Interchanging the order of drug, Fe (III) and PHEN has no effect on absorbance of the colored species.
Nature of solvent for final dilution	Distilled Water	Distilled Water	Other water miscible solvents, like Acetonitrile, methanol, ethanol, acetone and 1,4-dioxan did not enhance the intensity of the final colored product.
Stability of the colored species after final dilution.	5-60 min	5 min	The absorbance of the colored product decreased slowly beyond 60 min.

Table 1c: Optimum conditions established in M_c

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	730-750	740	-
Volume of ferric chloride ($3.32x10^{-3}$M) required for oxidation.	0.8-1.5	1.0ml	Optimum conditions furnished in column 2 were preferred for broad coverage of Beer's Law limits and stability of colored species formed.
Vol. of Potassium ferricyanide ($3.02x10^{-3}$M) for formation of ferrous ferricyanide.	0.4-0.7 ml	0.5 ml	-
HCl (1N) necessary for maintenance of acidity prior to dilution.	0.8-1.5ml	1.0 ml	-
Temperature and time necessary for complete development of color.	5-15 min. Room temp.	10 min. Room temp.	-
Stability period	Immediate-1 hr.	Immediate-1 hr.	-

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 2.Optical and regression characteristics, precision and accuracy of the proposed methods

Parameter	M_a	M_b	M_c
λ_{max} (nm)	625	510	740
Beer's law limits (µg/ml)	10 - 60	1.25 - 7.5	2.5-15.0
Detection limit (µg/m)	7.754	0.01352	7.0517
Molar absorptivity (L.mol/cm)	2.835×10^3	2.277×10^4	1.715×10^4
Sandell's sensitivity (µg/cm^2/0.001 absorbance unit)	0.2994	7.507×10^{-2}	9.104×10^{-2}
Optimum photometric range (µg/ml)	20 - 50	2.5-7.5	6 – 14
Regression equation (Y=a+bc) slope (b)	0.01088	0.4878	0.03984
Standard deviation on slope (S_b)	4.586×10^{-3}	5.293×10^{-4}	1.016×10^{-2}
Intercept (a)	4.999×10^{-3}	1×10^{-3}	3.999×10^{-3}
Standard deviation on intercept (S_a)	1.5211×10^{-2}	2.194×10^{-3}	8.424×10^{-2}
Standard error on estimation (S_e)	1.450×10^{-2}	2.092×10^{-3}	8.038×10^{-2}
Correlation coefficient (r)	0.9997	0.9998	0.9997
Relative standard deviation (%)*	1.6015	1.256	0.6644
% Range of error (confidence limits)			
0.05 level	1.841	1.444	0.763
0.01 level	2.887	2.264	1.198
% error in Bulk samples **	-0.29	0.348	-0.298

*average of three determinations ** Average of six determinations

Table 3 Assay of 7-ADCA in Pharmaceutical Formulations

Formulations*	Amount taken	Amount found by proposed Methods**				Percentage recovery by proposed methods***		
	(mg)	M_a	M_b	M_c	Reference method	M_a	M_b	M_c
Tablet I	60	59.63±0.60	59.72±0.65	59.56±0.53	60.05±0.82	99.62±0.76	99.90±0.95	99.83±0.99
		F=1.86	F=1.59	F=2.393				
		t=1.024	t=0.777	t=1.257				
Tablet II	60	59.62±0.62	59.43±0.58	59.58±0.60	60.04±0.7	99.66±0.55	99.46±0.82	99.31±0.93
		F=1.502	F=1.717	F=1.604				
		t=1.104	t=1.628	t=1.222				
Tablet III	60	59.63±0.67	59.33±0.70	59.42±0.58	60.06±0.8	99.63±0.98	99.94±0.73	99.90±0.32
		F=1.725	F=1.5804	F=2.302				
		t=0.983	t=0.964	t=1.54				
Tablet IV	60	59.47±0.58	59.71±0.59	59.32±0.60	60.08±0.9	99.54±0.60	99.68±0.98	99.86±0.65
		F=2.167	F=2.326	F=2.025				
		t=1.427	t=0.8601	t=1.755				

*Tablets from four different pharmaceutical companies. **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; ***Recovery of 10 mg added to the pre-analyzed pharmaceutical formulations (average of three determinations)

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: The tetrahedral anion Mo O_4^{-2} in aqueous medium, which is strongly oxidized form, on acidification with conc. H_2SO_4 exist as isopolyanionic species as a result of polymerization and condensation reaction having an arrangement Mo_6 octahedral as exemplified by $Mo_7O_{24}^{-6}$ and $Mo_6O_{26}^{-4}$. The oxidisable portions in ADCA probably effects the reduction of 1,2 or 3 oxygen atoms from exemplified molybdate, thereby producing one, two or more of the possible reduced species which have intense blue color (Molybdenum blue).

Method M_b& M_c: The reduced form of Fe (III) (i.e. Fe (II) has a tendency to give a colored complex on treatment with O-Phenonthraline (M_b) or $[Fe(CN)_6]^{3-}$(M_c).

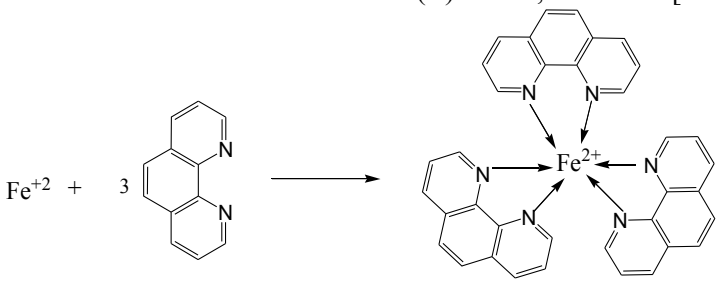
Step I

7-ADCA + Fe(III) → oxidation products + Fe (II) + unreacted Fe (III)

As Fe (III) interferes although only to slight extent in the determination of Fe (II), the reactivity of the interfering entity has to be made insignificant by complexing it with o-phosphoric acid (M_b).

Fe (III) + o-phosphoric acid → Complex (un-reactive)

The second step concerns with the estimation of Fe (II) with 1,10 PTL or $[Fe(CN)_6]^{3-}$(M_c).



Ferroin Complex

$$3Fe^{+2} + 2[Fe(CN)_6]^{3-} \rightarrow Fe_3[Fe(CN)_6]_2 \quad (M_{21})$$

Scheme1

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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