CHAPTER - IV

ANALYTICAL UTILITY OF VARIOUS CHROMOGENIC REAGENTS FOR THE SPECTROPHOTOMETRIC DETERMINATION OF AZELAIC ACID IN BULK AND DOSAGE FORMS

Azelaic acid (ay-ze-LAY-ik AS-id) is used to treat mild to moderate acne. It works in part by stopping the growth of skin bacteria that can help cause acne. Azelaic acid also helps to lessen acne by keeping skin pores (tiny openings on the skin's surface) clear.

Its official status has been presented in Table 1.01 (p. 2). The structural features, category; certain characteristics, therapeutic importance and commercially available formulations of AZA are compiled in Tables 4.01 (p. 222); 4.02 (p.223) and 4.03 (p. 224) respectively.

A very few physico – chemical methods appeared in the literature for the assay of AZA in biological fluids and pharmaceutical formulations (less). The methods so far reported include UV and visible[290-292] spectrophotometric methods, chromatography, HPLC, in biofluids, pharmacological and clinical aspects, applied radiation[293] methods. The analytically useful functional groups in AZA have not been fully exploited for designing suitable, visible spectrophotometric methods and so still offer a scope to develop more visible spectrophotometric methods with better sensitivity, selectivity, precision and accuracy. The author has made some attempts in this direction and succeeded in developing three methods i.e., SFNO (M_{2a}); MB (M_{2b}); MV (M_{2c}). All these methods have been extended to pharmaceutical formulations as well.

A reported UV spectrophotometric method has been adopted for the determination of AZA in pharmaceutical formulations as a reference method (Table 4.04,p.225) to compare the results obtained by the proposed methods.

Experimental:

1. 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.

Table 4.01

Structural features of selected drugs

Drug Name / Abbreviation	Category	Chemical Name	Structure, Molecular formula and Molecular Weight	Analytically important moieties / functional groups
Azelaic acid	Antiacne agent,topical, Hypopigmentation agent	Nonanedioic acid	HOOC- $(CH_2)_7$ - COOH	Dicarboxylic acid

Table 4.02

Physico chemical characteristic and therapeutic importance of Azelaic acid

Category	Characteristic	Therapeutic importance
Nonanedioic acid	Molecular formulas – $C_9 H_{16} O_4$ Molecular weight - 188.22 Appearance - White to cream solid Solubility – Slightly soluble in water, freely soluble in CH_3OH, ethyl alcohol, isopropyl alcohol, 0.1N NaOH. Storage : Refrigerator UV_{max} : 216 nm	Azelaic acid gel is used to clear the bumps, lesions and swelling caused by rosacea (a skin disease that causes redness, flushing and pimples on the face). Azelaic acid cream is used to treat acne. It is in a class of medications called dicarboxylic acids. It works to treat acne by killing the bacteria that infect pores and by decreasing production of keratin, a natural substance that can lead to the development of acne. The way Azelaic acid works to treat rosacea is not known.

Table4.03

Commercially available formulations

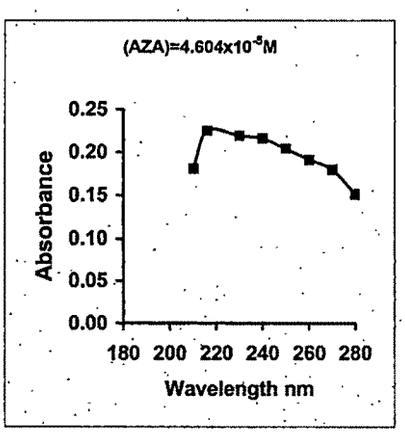
Generic Name	Pharmaceutical concern	Formulation	Strength of formulation	Other ingredients	
				Active	Inactive
Azelaic acid	Metronidazole	gel	40gm	--	--
	20% cream	cream	30gm	--	--

2. Preparation of standard drug solutions:

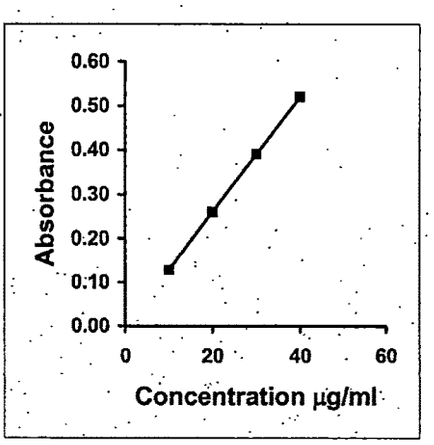
A 1 mg/ml solution was prepared by dissolving 100 mg of pure AZA in 100 ml of 0.1N HCl and this stock solution was diluted step wise with distilled water to get the working standard solutions of concentration of 25 µg/ml (M_{2a}); 20 µg/ml (M_{2b}, M_{2c}).

Table 4.04
Procedure for the assay of AZA in formulations

Pharmaceutical formations	Reference Method
Tablets	An accurately weighed amount of tablet powder equivalent to 100mg of drug was transferred into a 100ml volumetric flask. Added about 75 ml of ethyl alcohol and shaken well for about 15 min. The contents were diluted with ethanol up to the mark and mixed thoroughly. The solution was filtered. Then 2 ml of filtrate was pipetted out into a 100 ml volumetric flask and made up the solution up to the mark with ethanol for obtaining a concentration of 20 µg/ml. Into a series of 5 ml-graduated tubes, aliquots of drug solution ranging from 0.5-3.0 µg/ml were taken and diluted to mark with ethanol. Read the absorbance at 216 nm against a solvent blank. The drug was read from its calibration graph



(Fig. 4.01) Absorption spectrum of AZA in Ethanol (UV reference method)



(Fig. 4.02) Beer's law plot of AZA in Ethanol (UV reference method)

3. Preparation of reagents:

All the chemicals and reagents used were of analytical grade and solutions were prepared in triply distilled water, chloroform or isopropyl alcohol .

Method M_{2a},M_{2b},M_{2c}

SFNO solution (Fluka; 0.2%, w/v 5.714×10^{-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 6.25×10^{-3}M)	:	Prepared by dissolving 200 mg of MB in 100 ml of distilled water and subsequently washed with chloroform to remove chloroform soluble impurities.
MVsolution (Fluka; 0.2%, w/v 6.25×10^{-5}M)	:	Prepared by dissolving 200 mg of MV in 100 ml of distilled water and subsequently washed with chloroform to remove chloroform impurities.
Buffer solution P^H 9.8 $NH_4OH - NH_4Cl$	:	7gms of NH_4Cl and 6.8 ml of liquid Ammonia solutions were mixed and diluted to 100 ml with distilled water and pH was adjusted to 9.8.

Recommended procedures:

After systematic and detailed study of the various parameters involved, as described under results and discussions in this chapter, the following procedures [Methods M_{2a} (SFNO), M_{2b} (MB), M_{2c} (MV)] were recommended for the assay of AZA in bulk samples and pharmaceutical formulations.

Method M_{2a}, $M_{2b}$and M_{2c}

Aliquots of standard drug solution 1.0-5.0 ml for method M_{2a}, M_{2b} or M_{2c} (0.5-3.0ml 25µg/ml, 20µg/ml and 20µg/ml) and 1.0ml of P^H 9.8buffer solution were placed separately in a series of 125ml separating funnels. A volume of 1.0ml of Safranin o (for method M_{2a}) or 0.5ml of MB (for method M_{2b}) or MV (for method

M_{2c}) 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 immediately at 530 nm (for method M_{2a}) and at 655 nm (for method M_{2b}, M_{2c}) against reagent blank. All the colored species were stable for 2 hours. The amount of drug in a sample solution was obtained from its Beer's Lambert plot [Fig 4.06 (M_{2a}), 4.07 (M_{2b}), 4.08(M_{2c}) p. 232].

For pharmaceutical formulations:

An accurately weighed portion of tablet content equivalent to about 100 mg of AZA was transferred into a 100 ml volumetric flask. Added about 80 ml of warm ethyl alcohol and shaken well for about 20 min. The contents were diluted with ethyl alcohol up to the mark and mixed thoroughly. The solution was filtered and the filtrate was evaporated to dryness. The residue was used for the preparation of sample solutions in the same way as under standard solutions preparations. These solutions were analyzed as under procedures described for bulk solutions.

Results and Discussions:

i. 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 AZA were taken and colors were developed separately by following the above procedures. The amounts of AZA present in total volume of colored solutions were 1.25 µg/ml (for method M_{2a}, M_{2b} M_{2c}). 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 results were graphically represented in fig. 4.03 to 4.05, p.228-229.The absorption curves of the colored species in each method show characteristic absorption maxima.

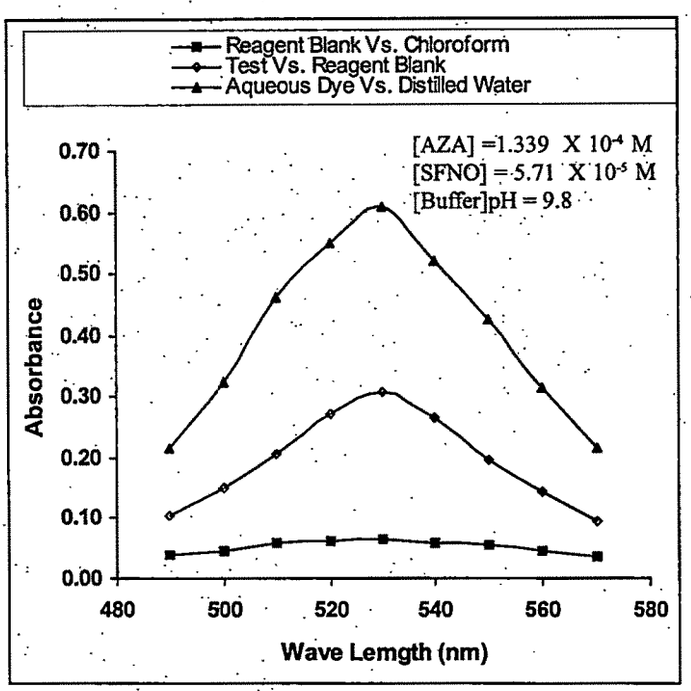


Fig. 4.03 Absorption spectrum of AZA - SFNO (M_{2a})

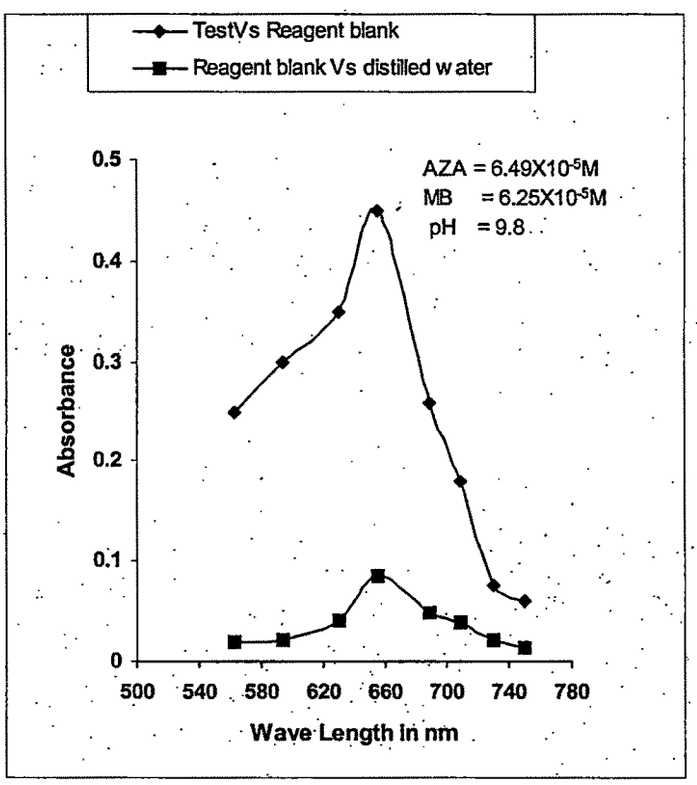


Fig. 4.04 Absorption spectrum of AZA -MB (M_{2b})

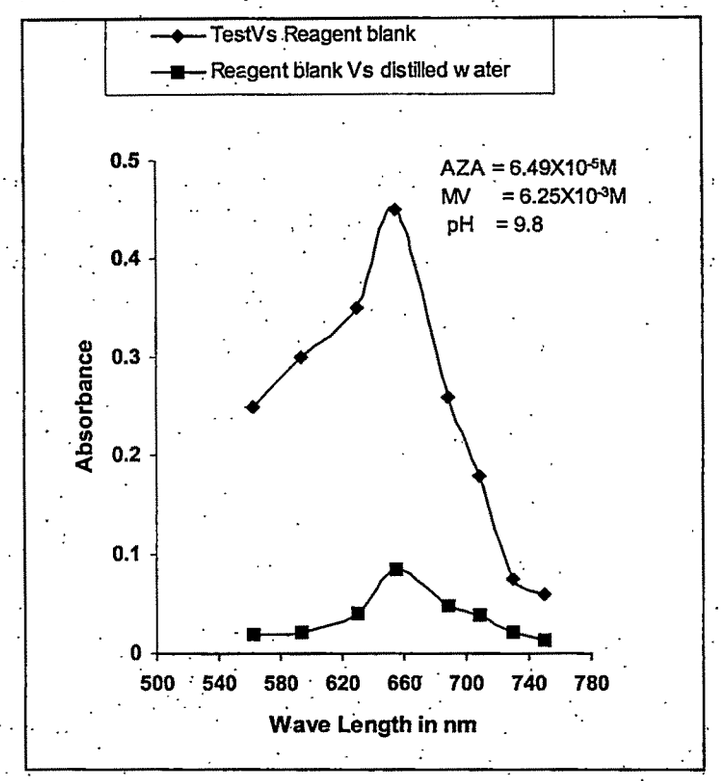


Fig. 4.05Absorption spectrum of AZA-MV (M_{2b})

ii. Optimum conditions fixation in procedures:

The optimum conditions for the color development of methods (M_{2a}, M_{2b}, M_{2c}) 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_{2a}, M_{2b} and M_{2c}

The optimum conditions in these methods were fixed based on the study of the effects of various parameters such as type of buffer, conc. of dye SFNO (M_{2a}), MB (M_{2b}), or MV (M_{2C}) 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 experiments by measuring absorbance at λmax 530 nm (M_{2a}) or 655 nm (M_{2b}, M_{2C}) of a series of solutions varying one and fixing the other parameter and the results are recorded in Table. 4.05, p. 231.

Optical Characteristics:

In order to test whether the colored species formed in above methods adhere to Beer's law, the absorbance's at appropriate wavelength of a set of solutions containing varying amounts of AZA and specified of amounts of reagents were recorded against the corresponding reagent blanks. The Beer's law plots of these recorded graphically. Beer's law limits, molar absorptivity, Sandell's sensitivity and optimum photometric range for AZA in each method were calculated. Least square regression analysis was carried out for getting the slope, intercept and the correlation coefficient values (Table 4.06a, p. 236).

Precision:

The precision of the proposed methods was ascertained from the absorbance values obtained by actual determination of six replicates of a fixed amount of AZA 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 4.06a, p. 236).

Table4.05

Optimum conditions established for methods M_{2a}, M_{2b} and M_{2c} (for AZA)

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm) M_{2a} M_{2b} M_{2c}	525-535 650-660 650-660	530 655 655	–
Effect of buffer on color development	9.0-10.0	p^H-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_{2a}) MB (M_{2b}) MV(M_{2c})	1.0-5.0 0.1-1.0 0.1-1.0	1.5 0.5 0.5	1.5ml of SFNO (for M_{2a}), 0.5 ml of MB (for M_{2b}) and 0.5 ml of MB (for M_{2c}) 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 immicible 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 (^{0}C)	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, in organic phase was stable for 60 min. After wards the absorbance gradually decreases.

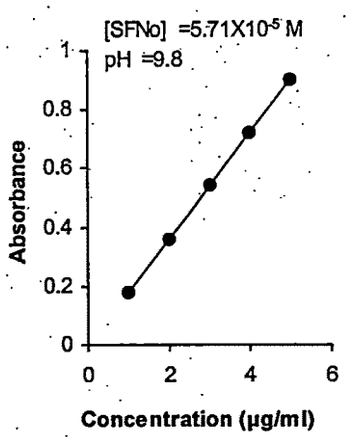


Fig.4.06 : Beer's Law plot of AZA with SFNO system (M_{2a})

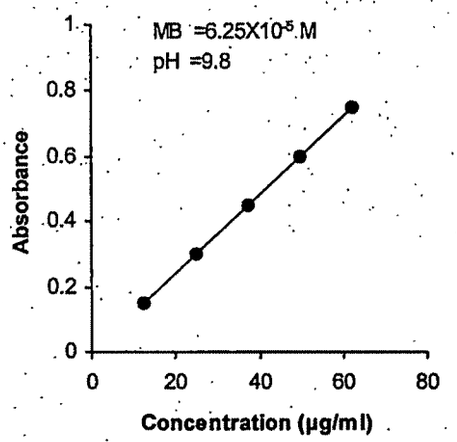


Fig.4.07 : Beer's Law plot of AZA with MB system (M_{2b})

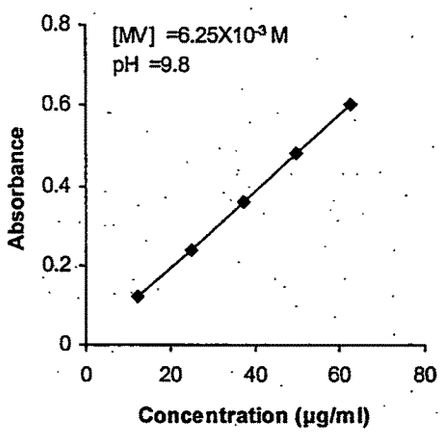


Fig.4.08 : Beer's Law plot of AZA with MV system (M_{2b})

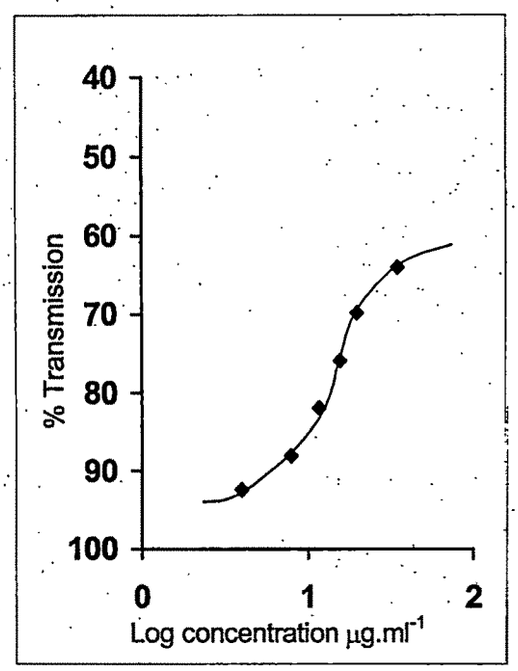


Fig 4.09 Ringbom plot of AZA with Saffranine O (M_{2a})

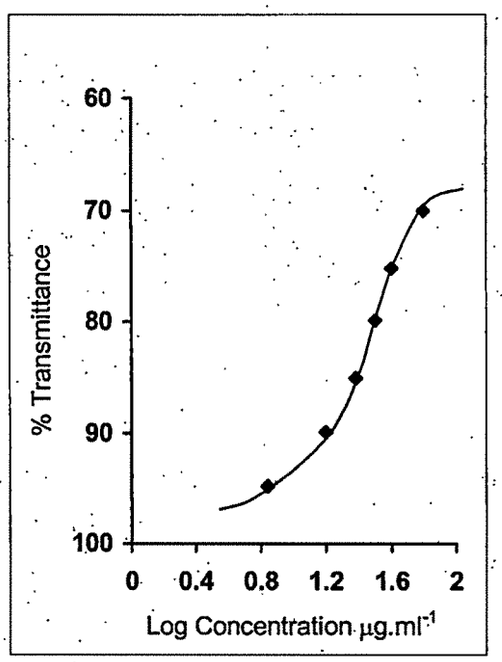


Fig 4.10 Ringbom plot of AZA with MB (M_{2b})

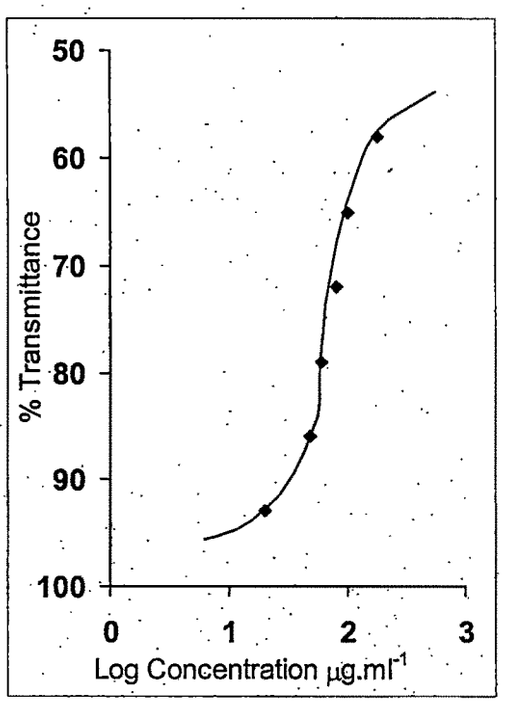


Fig 4.11 Ringbom plot of AZA with MV (M_{2c})

Accuracy:

To determine the accuracy of each proposed method, different amounts of bulk samples of AZA within the Beer's law limits were taken and analyzed by each proposed method. The results (percent error) are recorded in (Table 4.06a, p. 236).

Interference studies:

The effect of wide range of concomitants and other additives usually present in the formulations for the assay of AZA (in methods M_{2a}, M_{2b}, M_{2c}) under optimum conditions were investigated. The commonly used concomitants and additives in the preparation of formulation even when added in excess fold (as mentioned in paranthesis) did not interfere with the assay of AZA by proposed methods. However, for avoiding the interference of concomitants such as starch, lactose (if present), in few methods the formulations were selectively extracted with appropriate organic solvent initially, since the interfering concomitants are insoluble in it.

Analysis of formulations:

Commercial formulations (Creams) containing AZA 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 differ significantly. The results are summarized in Table 4.10a (p. 237). Percent recoveries were determined by adding standard drug to preanalysed formulations. The results of the recovery experiments by the proposed methods are also listed in Table 4.10a (p.237).

Chemistry of the colored species:

The chemistry of the colored species formed in each one of the proposed methods for the assay of AZA has been presented in chapter IV.

Conclusions:

The proposed methods exploit the various functional groups in AZA molecule. The decreasing order of sensitivity (ϵ_{max}) and the λ_{max} among the proposed methods are (M_{2b}>M_{2a}>M_{2c}) and (M_{2b}=M_{2c}>M_{2a}) 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 or selective with reasonable precision and accuracy and constitute better alternatives to the reported ones in the assay of AZA in bulk form and pharmaceutical formulations.

Table 4.06a

Optical and regression characteristics, precision and accuracy of the proposed methods for AZA

Parameter	M_{2a}	M_{2b}	M_{2c}
λ_{max} (nm)	530	655	655
Beer's law limits (μg/ml)	1-5	15-70	10-70
Detection limit (μg/ml)	2.9445	4.2746	02118
Molar absorptivity (1 $mol^{-1}.cm^{-1}$)	3.901×10^4	5.259×10^4	8.768×10^3
Sandell's sensitivity (μg.cm^{-2}/0.001 absorbance unit)	0.5472	6.171×10^{-2}	5.357×10^{-2}
Optimum photometric range (μg/ml)	32-125	7-14	1.8-7.1
Regression equation (Y=a+bc) slope (b)	8.238×10^{-3}	8.116×10^{-3}	1.4306×10^{-2}
Standard deviation on slope (S_b)	0.1950×10^{-3}	0.2789×10^{-3}	0.0243×10^{-3}
Intercept (a)	2.25×10^{-3}	1.449×10^{-2}	5.000×10^{-3}
Standard deviation on intercept (S_a)	8.085×10^{-3}	1.156×10^{-4}	8.25×10^{-3}
Standard error on estimation (S_e)	7.7094×10^{-3}	1.102×10^{-4}	2.752×10^{-3}
Correlation coefficient (r)	0.9993	0.9986	0.9999
Relative standard deviation (%)*	0.7034	0.8706	0.5390
% Range of error (confidence limits)			
0.05 level	0.8088	1.007	0.6197
0.01 level	1.2683	1.580	0.9719
% error in bulk samples **	-0.360	0.157	0.120

* Average of six determinations considered

** Average of three determinations

Table 4.10a

Assay of AZA in Pharmaceutical Formulations

Formulations*	Amount taken (g)	Amount found by proposed Methods**			Reference method	Percentage recovery by proposed methods***		
		M_{2a}	M_{2b}	M_{2c}		M_{2a}	M_{2b}	M_{2c}
Tablet I	40	40.25±0.69 F=1.935 t=0.612	38.85±0.76 F=1.595 t=1.910	39.56±0.29 F=1.404 t=0.630	39.91±0.96	99.94±0.22	99.90±0.31	99.96±0.11
Tablet II	40	39.58±0.43 F=4.577 t=0.451	38.95±0.57 F=2.605 t=1.824	40.45±0.69 F=1.777 t=1.184	39.82±0.92	98.83±0.25	99.94±0.17	99.92±0.33
Cream I	30	28.84±0.71 F=1.905 t=1.924	30.15±0.63 F=1.994 t=0.388	28.57±0.62 F=2.498 t=0.635	29.93±0.98	98.95±0.22	98.97±0.34	98.98±0.17
Cream II	30	28.82±0.57 F=2.437 t=0.380	30.84±0.63 F=1.995 t=1.558	31.15±0.68 F=1.713 t=2.148	30.02±0.91	99.96±0.28	99.93±0.16	99.92±0.22

* Tablets from four different pharmaceutical companies.

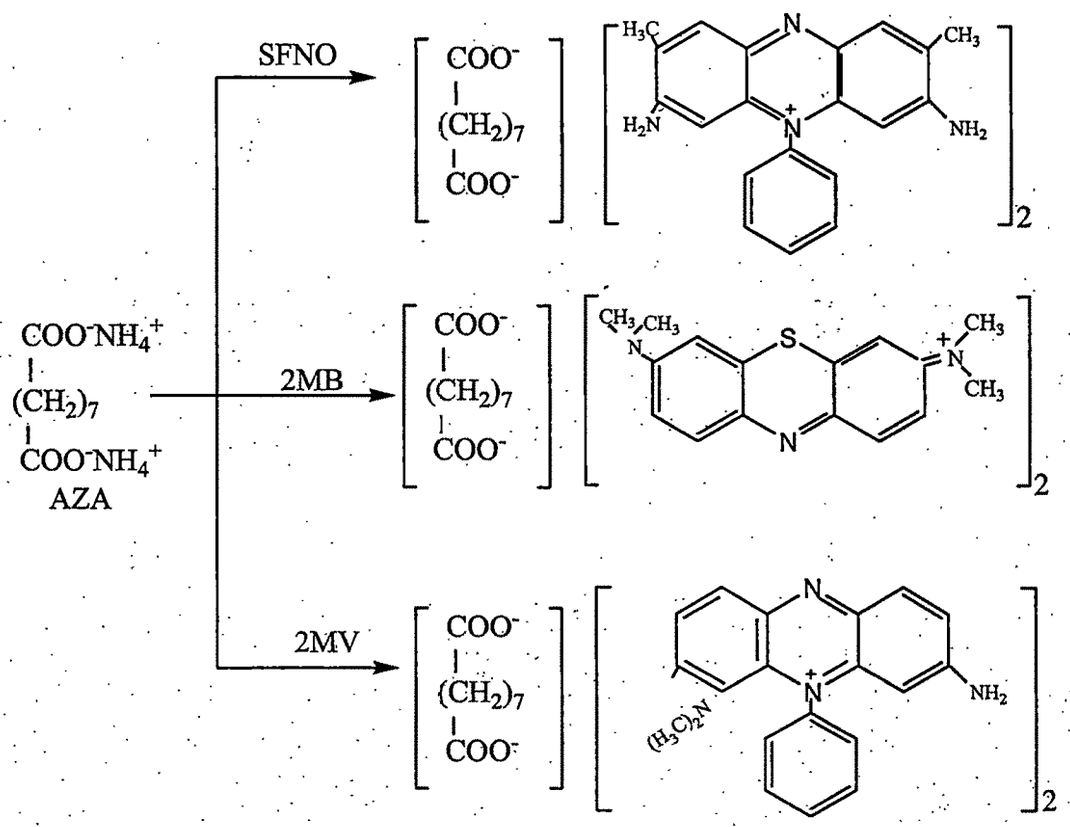
** Average ± standard deviation of six determinations, the t-and F-test values refer to comparison of the proposed method with the reference method. Theoretical values at 95% confidence limit, F = 5.05, t = 2.262

*** Recovery of 10mg added to the preanalysed pharmaceutical formulations (average of three determinations).

Azelaic acid (nonanedioic acid) is an aliphatic dicarboxylic acid and so responds to characteristic color reactions involving carboxylic acid group(s).

Method M_{2a}, M_{2b}, M_{2c}

AZA forms an ion association complex with basic dye (Saffranin O, Methylene blue or Methylene violet), which is extractable into chloroform from aqueous phase. The carboxylate anion (negative charge) of AZA is expected to attract the oppositely charged part of the dye (positive charge, safranine O, methylene blue or Methylene violet) and behave as single unit being held together by electrostatic attraction. It is supported by slope ratio method, which was obtained as 2:1 in each method (M_{2a}, M_{2b}, M_{2c}). Based on analogy the structure of each ion association complex is shown in scheme 4.01.



Scheme 4.01