RAPID SPECTROPHOTOMETRIC EVALUATION OF DIHYDROARTEMISININ IN BULK AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A simple and sensitive spectrophotometric method for the determination of Dihydroartemisinin (DHA) in bulk and tablet formulation is described. The method is based on the reduction of Fe^{3+} to Fe^{2+} by DHA and the subsequent interaction of the Fe^{2+} with potassium fericyanide to produce a blue coloured complex (Prussian blue) which is measured spectrophotometrically at λmax 710nm. A plot of concentration against absorbance gave a calibration curve which obeyed Beer's law in the range of 2.0-50µg/ml. The regression equation was A = mc + b and the correlation coefficient obtained via the least square method was 0.9998. The limit of detection and quantification determined as per the current ICH guidelines were 0.39µg/ml and 1.11µg/ml respectively. The precision and accuracy were determined as the relative standard deviation and relative error and found to be ≤1.75 and ≤3.75 respectively. The molar absorptivity was found to be $2.9 \times 10^3 Lmol^{-1}cm$ and Sandell sensitivity was found to be $0.095 \mu g cm^{-2}$.The method was statistically compared with the official international pharmacopoeia method, the t and F values were below the tabulated value showing great congruence with the official method. Finally, the validity, accuracy and the utility of the method was further ascertained by recovery studies via the standard addition method showing excellent recoveries of > 100%. The pharmaceutical excipients /additives did not affect the analysis of DHA via this method.

INTRODUCTION

Malaria remains the foremost cause of mortality and morbidity from infectious disease in the tropical regions of Africa, Asia and Latin America. It is a source of great concern as it has extended to over 40% of the world's population (Robert *et al*, 2001). It affects all manner of vulnerable people especially women and children under the age of five. Currently WHO (2012) report reveals that African region accounts for 85% of malaria cases and 90% of malaria death worldwide. As international travels become more and more frequent, malaria is no longer confined to the tropics as imported malaria is becoming an increasing problem. Furthermore, if global warming remains unchecked, malaria will re-establish itself in Europe and North America (Drew *et al*, 2006).

The emergence and spread of multidrug resistant malaria parasite raises the concern that untreatable malaria may soon be encountered. To prevent the prevalence of this resistant mutant, proper use of artemisinin and its derivative have been advocated by WHO.

Presently, reduced susceptibility of parasite to artemisinin based component of ACTs (Artemisinin combination therapy) has developed in Thai - Cambodia boarder and other known foci in South East Asia (O'Neil, 2010).The reduced susceptibility is due largely to the manufacture and distribution of counterfeit, fake and adulterated artesunate in South East Asia

(Green *et al,* 2000).This has also become a major problem in Africa as demonstrated by a study concluded in Kenya and DR Congo (Atemkeng *et al,* 2007).

Once absorbed, the Artemisinin derivatives are converted primarily to dihydroartemisinin DHA was a potent antimalarial. Since DHA is the central metabolite of these artemisinin derivatives that is most active against the multidrug resistant parasite, it has become the best candidate for adulteration and outright faking. Hence the need to develop simple affordable method for the assay of DHA.

Officially, dihydroartemsinin is assayed by HPLC and UV-vis spectrophotometry (International Pharmacopoeia, 2003). Many analytical methods have been developed for the determination of artemisinin derivatives some of these methods are those reported by Gabriel and Plaizer-Vercamen (2004); Van Quckelbergheit *et al,* (2008), Naik *et al,* (2005), Na Bangchang *et al* 1998, Teja-Isavadharm 2010. Some of these methods are very expensive, others are quite simple and accurate but have some obvious faults. A typical example of this is the colorimetric field method for the assay of artesunate in tablets, by Green *et al* (2000). The method though simple and affordable, a situation where 1% of the tablet is used for the assay by scrapping the surface of the tablet was a good idea but this cannot be full proof as sophisticated drug adulterers and fakers can coat the outer surface of the tablet with a thin layer of active ingredient (in which case could be 1% of active ingredient). The second method by Green *et al* 2001 for the authentication of artemether, artesunate and dihydroartemisinin antimalarial tablets using simple colorimeter using the dye Fast Red TR Salt. The resultant yellow colour is not specific as other drugs such as tetracyclines, doxicyclines and amodiaquine also produce yellow colour. The iodometric titration method proposed by Attih *et al* (2012) is quite simple but can best be a complementary method to this spectrophotometric method.

METHODOLOGY

In the proposed method dihydroartemisinin reduces Iron III to Iron II and then made to react with Potassium Ferricyanide in acid medium leading to the formation of the Prussian blue colour which is then determined by uv spectrophotometry at λ_{max} 710nm.The method was then used to assay DHA in bulk and in tablet formulation brands procured locally from pharmacies in Uyo, Nigeria.

All spectral and absorbance measurements were carried out using labomed double and dual split beam, UVD 2950 spectrophotometer, Labomed Inc, USA. All reagents used in this work were analytical grade chemicals and bidistilled water was used for preparing and diluting the reagents. Ferric chloride hexahydrate (Merck, Germany) 0.2% w/v was prepared using distilled water. Potassium ferricyanide $K_3Fe(CN)_6$ (Merck, Downstadt Germany) 0.2% w/v was prepared using distilled water. Sulphuric acid (10M) was prepared by adding 555.0ml of the concentrated acid (sp.Gr 118) to 445ml of bidistilled water.

Test Drugs

Pure dihydroartemisinin was obtained as a kind gift from the Directorate of Pharmacy, University of Uyo Teaching Hospital and used as received. Standard DHA solution (100µg/ml) was prepared by dissolving 100mg in enough absolute ethanol to obtain 1mg/ml which diluted appropriately to obtain 100µg/ml. Tablets were procured from local pharmacies in different locations within Uyo metropolis, Southern Nigeria.

Recommended Procedure

Different aliquot 0.1-5.0 ml of standard solution (100µg/ml) were accurately transferred into a series of 10ml calibrated standard volumetric flask, using a micro burette. The volume in the flask was adjusted to 4ml, then 2ml of 0.2% w/v Feric chloride hexahydrate was added followed by 2ml of 0.2 potassium ferricyanide and the content of the flask were shaken to mix well. Finally 1ml of 10M sulphuric acid was added to each flask and the contents made up to the mark using bidistilled water allowed to stand in water bath maintained at 40^{o}C for 5 mins.

The absorbance of the resulting blue solution was measured at 710nm against a reagent blank prepared in the same way but in containing no DHA. A standard calibration graph was prepared by plotting the absorbance values against drug concentration. The unknown concentration of the DHA read from the generated standard curve or deduced (evaluated) from the regression equation derived from the Beer's Law data.

Procedure for Tablets

Twenty tablets of each of the commercial brand selected randomly were weighed individually and the average weight was determined. Then the twenty tablets were pulverised into a fine powder. An amount of the powder equivalent to 100mg of dihydroartemisinin was accurately measured and transferred into 100ml capacity analytical volumetric flask containing 50ml of absolute ethanol. The mixture in the flask was sonicated for 10 minutes. Then 10ml of absolute ethanol was added and shaken vigorously and finally made up to the mark with the absolute ethanol. The resulting mixture was filtered using Whatman filter paper No 42. The extracted drug solution contain 1mg/ml was diluted stepwise to obtain a working concentration of 100µg/ml from where the desired aliquot was taken and analysed as described in the general procedure above.

Analysis of Blank

A blank preparation made of pharmaceutical excipients of the composition, starch (10mg), glucose (10mg), talc (20mg), sodium citrate (10mg), sodium alginate (10mg) and lactose (10mg) were weighed out and mixed properly and a solution made.

Procedure for the Evaluation of DHA in Synthetic Mixture

Ten (10) milligrams of DHA was accurately weighed and transferred into a ceramic mortar containing the blank with the composition as described above and triturated using ceramic plastic to finer powder. The resulting mixture transferred into a 100ml standard volumetric flask and a solution of this mixture prepared as described under "Procedure of Tablets".

The solution was shaken to mix well and filtered using Whatman filter paper No 42. The first 10ml portion of the filtrate was discarded. The resulting solution with the concentration of 100µg/ml of DHA was assayed for five times via the same "procedure for tablets' described above.

RESULTS AND DISCUSSION

Absorption Spectra of the Coloured Complexes

This determination is based on the reduction of Fe^{3+} ion by dihydroartemisinin to Fe^{2+} ion and the subsequent interaction of the Iron II salt with potassium ferricyanide to form the blue complex which absorbed at 710nm. Fig 1, the reactions of potassium ferrycyanide (hexacyanoferate (III)) and potassium ferrocyanide (hexacyanoferate (II)) with Iron salt form the basis for the qualitative determination of Iron and the ultimate differentiation of Fe^{2+} from Fe^{3+}. Iron II ions form the (Tunbulls) blue $[KFe(CN)_6Fe]$ complex with potassium fericyanide while Iron III ions forms Prussian blue $KFe(CN)_6Fe$ with potassium ferocyanide.

Turbulls blue $[KFe(CN)_6Fe]$ and Prussian blue $[KFe(CN)_6Fe]$ are the same compound (Knew, Rogers and Sampson) but are only slightly different in their structure. In the Turnbulls's blue the Iron II is bonded to cyanide molecule through the Carbon atom while in the Prussian blue complex the Iron (III) is branded with the cyanide molecule through the nitrogen atom (33). The Prussian blue and the Turnbull's blue have distinct marked absorbance hence their use in 2005., Abdel-Gaber *et al* ,2003). The absorbance of Prussian blue complex formed is directly proportional to the concentration of dihydroartemisinin and hence its determination via spectrophotometry. The intensity of the developed colour is dependent on the reaction conditions. The experimental conditions and variables were carefully studied and optimized.

Effect of Ferric Chloride and Ferriccyanide

All other experimental parameters were kept constant while the volume and concentration of Fe^{3+} ions was varied. It was observed that absorbance of the complex increased as the volume of 0.2% Fe^{3+} increased reaching a maximum of 2ml of 0.2% Fe^{3+} solution (Fig. 2). Similarly the volume and concentration of Ferricyanide was studied. The absorbance increased with increase concentration of ferricyanide reaching a maximum at 0.2%. For this work 2ml of 0.2% ferricyanide was found to be adequate and further addition lead to the decrease in absorbance (Fig. 2). Finally 2ml of 0.2% Ferric chloride and 2ml 0.2% Ferricyanide in a 10ml aliquot of the final concentration of the DHA gave the optimum colour of the complex required. Further increase in the concentration of both FeCl and Ferricyanide had no serious change in the absorbance of the coloured complex.

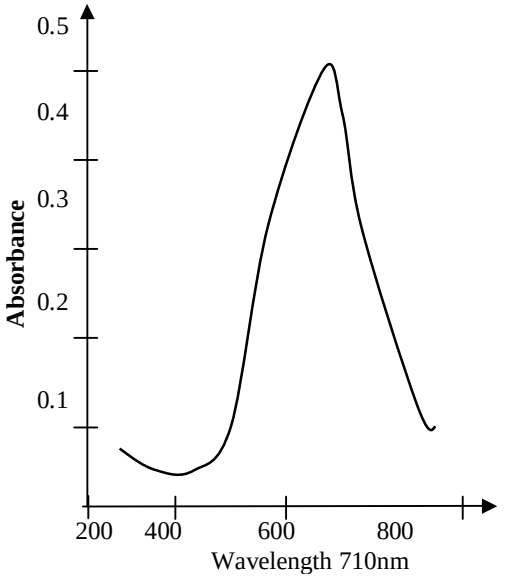


Fig 1: Spectrum of the blue complex formed from the reaction of 5µg/ml of DHA, 2ml of 0.2%Ferric chloride and 2ml of 0.2% of Potassium Ferricyanide

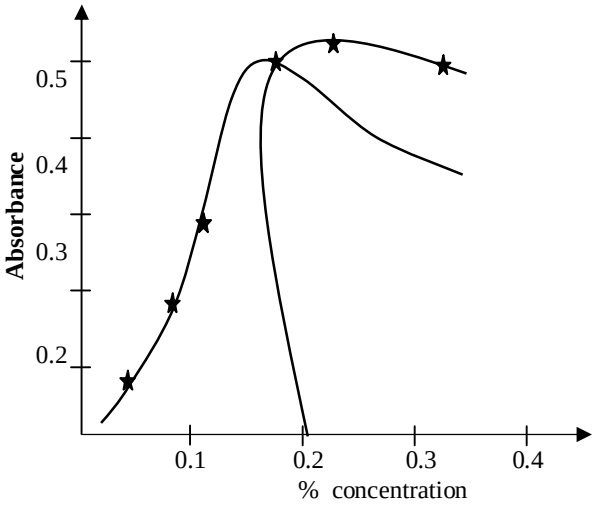


Fig 2: Effect of Ferric chloride and Ferricyanide x--x--x Effect of Ferric chloride concentration o--o--o Effect of Ferricyanide concentration on Absorbance of the blue complex measured at 710nm, of 5µg/ml of DHA at 45^{o}C.

Effect of Acid Type and Concentration

Sulphuric acid, hydrochloric and phosphoric acid were used, of these sulphuric acid gave the most stable colour complex. A volume of 1.0ml of 10M sulphuric acid in a total volume of 10ml of solution containing a fined concentration of DHA was found adequate. The complex formed some flock at about 25-30 minutes after formation of the blue coloured complex. To control the flocculation acid was added when the colour was fully developed and then the dilution was made.

Effect of Temperature (Fig. 4)

The reaction was slow at room temperature, but was enhanced at higher temperature. The reaction was therefore studied between 30^{o}C and 80^{o}C using the water bath. It was found that 40^{o}C was best for these reactions, because, though at this temperature the reaction was relatively slower than higher temperature, the stability of the coloured complex was better at 40^{o}C and the absorbance was higher while at higher temperature the absorbance decreased due to formation of precipitate.

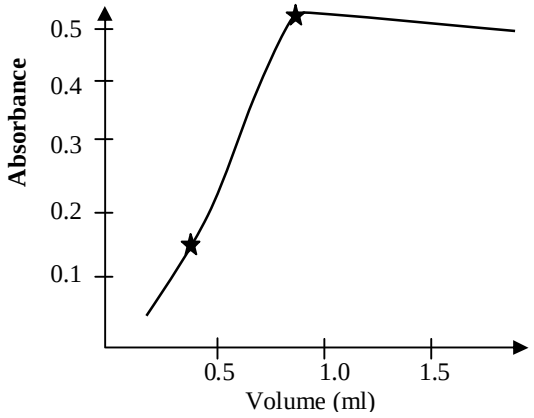


Fig. 3: Effect of Volume of 10M H_2SO_4 used on the absorbance of 5µg/ml of DHA at 710nm

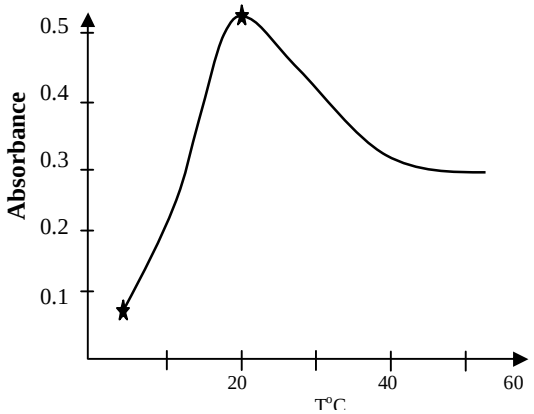


Fig. 4: Effect of Temperature on the absorbance of the blue complex at 710nm, when 5µg/ml of DHA reacted with 2ml of 0.2% $FeCl_3$ and 2ml of 0.2% Ferric chloride.

Effect of Heating Time on Stability of Coloured Complex (Fig. 5)

The effect of heating time on formation and stability of the coloured complex was studied between 5-60 minutes (Fig 5). Initially an olive green colour was observed, but by 10min the Prussian blue colour started developing and peaked at 15 minutes. This colour was stable for one hour after which the absorbance decreased due to the formation of precipitate.

Effect of Order of Addition of Reagent

Keeping other experimental variables constant the order of addition of the reagent were studied as shown in Table 1

S/N	Order of Addition	Absorbance at 710nm
1	DHA + $FeCl_3$ + $K_3Fe(CN)_6$ + H_2SO_4	0.558
2	$FeCl_3$ + $K_3Fe(CN)_6$ + DHA + H_2SO_4	0.440
3	$K_3Fe(CN)_6$ + $FeCl_3$ + DHA + H_2SO_4	0.235
4	DHA + $K_3Fe(CN)_6$ + $FeCl_3$ + H_2SO_4	0.543
5	$FeCl_3$ + DHA + $K_3Fe(CN)_6$ + H_2SO_4	0.556
6	$K_3Fe(CN)_6$ + DHA + $FeCl_3$ + H_2SO_4	0.480

It was observed that the order DHA + $FeCl_3$ + $K_3Fe(CN)_6$ + H_2SO_4 gave the best absorbance.

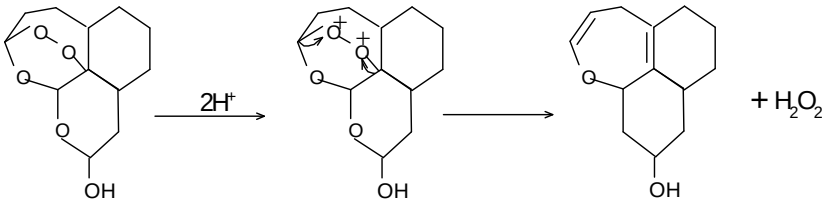
Mechanism of Reaction

Two mechanism of reaction leading to the production of blue coloured complex is possible. In acid medium there is the protonation of the two oxygen centres of the endoperoxide bond in the dihydroartemisinin molecule, leading to the release of hydrogen peroxide.

(2) The hydrogen peroxide interacts with the Fe^{3+} ion reducing it to Fe^{2+}.

(3) On addition of Potassium Ferricyanide, the Ferrous ion is first oxidized to Ferric ion while the Ferricyanide ion is reduced to Ferrocyanide ion. Then the Ferric ions react with the Ferricyanide ions to form the complex Turnbull's blue $KFe^{3+}[Fe^{2+}(CN)_6]$.

DIAGRAM OF CHEMICAL STRUCTURE



$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + H_2O$$

$$Fe^{2+} + [Fe(CN)_6]^{3+} \longrightarrow [Fe(CN)_6]^{4} + Fe^{3+}$$

$$[Fe(CN)_6]^{4} + Fe^{3+} \longrightarrow Fe(III)\ Fe(II)\ (CN)_2$$

Potassium ferric ferricyanide (Turnbull's blue)

The second mechanism is a two step mechanism the first step is the oxidation of Fe(II).

$$Fe^{2+} + [Fe(CN)_6]^{3} \longrightarrow Fe^{3+} + [Fe(CN)_6]^{4}$$

The second step is the formation of the Prussian blue

$$4Fe^{3+} + 3[Fe(CN)_6]^{4} + \text{acidic hydrolytic product of DHA} \longrightarrow Fe(II)\ Fe(III)\ (CN)_6$$

Prussian blue

Both Prussian blue and Turnbull's blue are hydrated salts of $Fe^{3}_{4}[Fe^{2}(CN)_6]_3 \cdot \varkappa H_2O$ ($\varkappa = 14$) and related to them is $KFe[Fe(CN)_6]$ soluble Prussian blue (8).

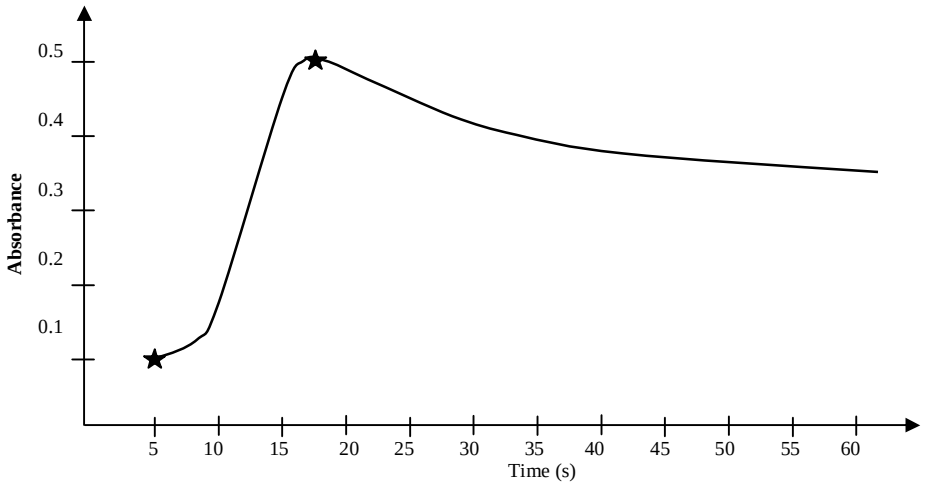


Fig 5: Effect of heating time on the absorbance of the blue complex, formed from 5µg/ml of DHA, 2ml of 0.2% $FeCl_3$ and 2ml of 0.2% Ferricyanide

Linear and Sensitivity

Under optimum experimental conditions, standard calibration curve was generated by plotting the absorbance versus concentration of DHA. Straight line graphs were obtained confirming the linear relationship between the absorbance vs the DHA concentration. Beer's Law was obeyed within the range of 2.0-50µg/ml of DHA. The equation for the calibration curve obeyed the normal straight line equation of A = mx + b where A = absorbance, m = slope, c =

concentration and b = intercept obtained by least square method. The correlation coefficient, intercept and slope of the standard calibration curves are recorded in table 1. The sensitivity parameters include molar absorptivity, Sandell sensitivity values. The Limit of Detection (LOD) and Limit of Qualification (LOQ) were evaluated based on the ICH guidelines; the formular

$$LOD = 3.3\sigma/S \quad \text{and} \quad LOQ = 10\sigma/S$$

where σ is the standard deviation of five blank determination and S the slope of the calibration curve. All the sensitivity parameters are also recorded in Table 1.

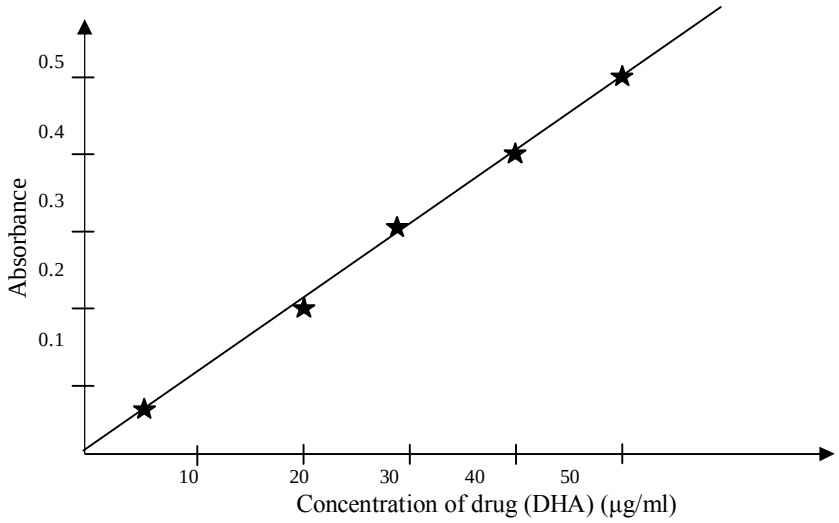


Fig 6: Calibration graph of absorbance vs concentration of the drug (DHA)

Table 1: Analytical parameters and optical characteristics of proposed method

Parameters	
λmax (nm)	710
Beer's Law limit	2.0-50
Molar absorptivity ($Lmol^{-1}cm^{-1}$)	$2.98x10^3$
Sandell sensitivity (μgcm^{-2})	0.095
Limit of detection (µg/ml)	0.39
Limit of qualification (µg/ml)	1.11
Regression equation	y = 0.018x
Slope	0.018
Intercept	-
Correlation coefficient	0.9998

Accuracy and Precision

The proposed method was evaluated for accuracy and precision by carrying out replicate analysis (n = 5) of calibration standards at three concentration levels. The analysis was carried out five times within same day (intra-day) and consecutive days for 5 days. The percentage Relative Standard Deviation (RSD%) as precision and relative error (Er%) as accuracy. The relative percentage error was calculated using the equation

$$Er\ \% = \left[\frac{found - taken}{taken}\right] x \quad 100$$

The results of the intra and inter day precision is recorded in Table 2.

Table 2: Evaluation of Intra-day and Inter-day Precision and Accuracy

S/N	Amount of DHA Taken (μg/ml)	Intra-day Accuracy and Precision			Inter-day Accuracy and Precision		
		Amount of DHA Found	RE%	RSD %	Amount of DHA Found	RE%	RSD %
1	40	41.5	3.75	1.75	41.31	3.28	1.41
2	80	82.5	3.50	1.56	82.6	3.25	1.63
3	120	123.6	3.17	1.50	123.6	3.17	1.30

Table 2: Results of Analysis of Tablets by the Proposed Spectrophotometric Method

S/N	Tablets Analyzed	Label Claim (mg)	Reference Method	Result of the Proposed* Method
1	Alaxin	60	110.0±1.09	111.0±1.10
				F = 1.02, t = 1.02
2	Cotecxin	60	110.0±1.25	111.3±1.30
				F = 1.08, t = 1.61
3	Santecxin	60	110.0±1.30	111.1±1.34
				F = 1.10, t = 1.32
4	Codisin	60	110.0±0.98	111.2±1.11
				F = 1.26, t = 1.80

*Mean value of five determinations.

The value of t (tabulated at 95% confidence level and at four degree of freedom) = the value of F (tabulated at 95% confidence level at four degree of freedom) = 6.37

Table 3: Results of the Recovery Study by the Standard Addition Method

Tablets Studied	Amount of Drug (DHA) (μg/ml)	Amount of DHA Drug Added (μg/ml)	Total Amount Found (μg/ml)	Result of the* Proposed Method
Alaxin	40.20	20.00	61.00	104.0±2.00
	40.20	40.00	81.10	102.3±1.00
	40.20	60.00	101.00	103.0±1.09
Cotecxin	41.00	20.00	61.55	102.0±1.75
	41.00	40.00	81.40	101.0±0.50
	41.00	60.00	102.30	103.0±1.25
Santecxin	45.10	20.00	65.70	103.0±1.45
	45.10	40.00	86.10	102.5±1.25
	45.10	60.00	107.20	103.5±1.75
Codisin	50.10	20.00	70.65	102.7±1.63
	50.10	40.00	91.30	103.0±1.50
	50.10	60.00	112.40	103.8±1.92

*The value of 3 determinations.

Specificity/Selectivity

The developed method was evaluated for selectivity/specificity by preparing and analyzing placebo blank and synthetic mixture. A specific aliquot of the placebo blank and synthetic mixture as prepared earlier were analyzed according to the recommended procedure also mentioned earlier. In both cases there was no reasonable interference from pharmaceutical experiments present. Yet there were excellent recoveries when the synthetic mixture containing the pure drug was analyzed ranging between 96.5-110 with standard derivation of 1.19.

Application to Table Formulation

To evaluate the application of the developed method in the determination of quality of DHA in tablets, the results obtained from this method was statistically compared with the reference uv-spectrophotometric method in the International Pharmacopoeia via student's t-test for accuracy and F-test for precision. (Method 2 - uv-vis determination of DHA-Atenimol in the IP). The t and F tests were at 95% confidence level and at 4 degree of freedom. The results obtained were

below the tabulated values showing a good congruence with the reference method as per accuracy and precision.

Recovery Studies

The recovery study of the proposed method was performed via standard addition method. Pure DHA was used to spike up a preanalyzed tablet powder at three concentration levels and analyzed by the proposed method and percentage recovery of the added pure drug ranged 101-104 with standard deviation (Table 3). The recovery study was done to ascertain the accuracy, validity and obvious utility of the propose method for analysis of DHA.

CONCLUSION

A simple spectrophotometric method was developed for the determination of DHA in bulk and pharmaceutical preparation. This method has advantage of high precision and accuracy. The method also has the advantage of not being as expensive as the HPLC method described in the International Pharmacopoeia. It is environment friendly devoid of the use of toxic solvents for extraction. The analyst faces no exposure to hazardous chemicals and reagents, and therefore recommended as a direct method or as a complementary method for the assay of DHA in third world countries still battling with counterfeit and fake DHA.

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