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Development of a Validated Ultraviolet Spectrophotometric Technique for The Assay of Dihydroartemisinin Content of Dihydroartemisinin-Piperaquine Tablet Combination

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ABSTRACT

A new ultraviolet spectrophotometric method was developed and subsequently validated for the estimation of dihydroartemisinin in dihydroartemisinin-piperaquine phosphate tablet combination. The proposed method is a modification of the official difference ultraviolet spectrophotometric procedure for dihydroartemisinin with determination at 292nm after reaction with 0.05M sodium hydroxide. The method was developed and validated in the presence of piperaquine and two commonly used tablet excipients; starch and talc. The assay was linear at the three concentrations 0.04, 0.08 and 0.12mg/ml selected for the study (r = 0.9984), with good intraday (recovery 93.77–101.33%) and interday accuracy (recovery 94.14–101.0%), precision and no interference from the excipients used. Application of the method to three commercially available tablet dosage forms gave good accuracy (recovery 93.25–103.0%) and precision (RSD < 2%). Similarly, inter-analyst application of the procedure on the same brands of tablets by two analysts on different days gave good reproducibility ($p > 0.05$). This simple, validated and low cost analytical technique could be used in the assay of dihydroartemisinin content of dihydroartemisinin – piperaquine phosphate tablet formulations.

Key words: Dihydroartemisinin, Ultraviolet spectrophotometric method, Dihydroartemisinin-piperaquine phosphate tablet, Analytical technique

INTRODUCTION

Malaria is one of the world's debilitating infectious disease resulting in about 228million cases, mortality of 405,000 annually, with about 93% prevalence in African region as at 2018 (WHO, 2019a). The chemotherapeutic management has been seriously compromised by the development of resistance to most antimalarial drugs by the parasite resulting in search for new drug compounds, of which artemisinin based therapies are the current drug of choice. Artemisinin derivatives which are fast acting have short biological half-lives, which thus preclude long lasting activity, hence, the adoption of these drugs with longer acting antimalarial drugs known as artemisinin-based combination therapy (ACT) (WHO, 2014). Artemisinin-based therapies comprise of the combination of artemisinin or any of its semi synthetic derivatives such as artesunate, dihydroartemisinin, and arthemether with other longer acting antimalarials (WHO, 2015). Artemisinin based combination therapies while giving rapid therapeutic responses also protects against the development of resistance (White 1999, Liwang and Xin-zhuan 2009). Fixed dose combination of dihydroartemisinin (DHA) and piperaquine phosphate (PPQ) is one of the ACTs recommended by the WHO for the treatment of uncomplicated malaria (WHO, 2014). Dihydroartemisinin (DHA), 3R,5As,6r,8aS,9R,10S,12R,12aR)-3,6,9-trimethyldecahydro-3,12-epoxy [1,2] dioxepino [4,3-i] isochromen-10-ol (Figure 1) is the active metabolite of most artemisinin derivatives which is also available as a semisynthetic compound obtained by reduction of artemisinin (RSC, 2006, Staines and Krishna, 2012). Piperaquine phosphate (PPQ) (1,3-bis[1-(7-chloro-4′quinolyl)-4′-piperazinyl]-propane) which is a 4-aminoquinoline is an orally active bisquinoline that is structurally related to chloroquine (Olaniyi, 2005). It has a large volume of distribution at steady state, good bioavailability, long elimination half-life and clearance rate that is markedly higher in children than adults. Hence, the ACT combination of DHA and PPQ has been quite useful in the management of malaria since its introduction. Effective management of any disease is subject to the assurance of the quality of available drugs. Quality assurance of drugs depends on maintaining established quality standards based on standardized laboratory tests and procedures prior to, during and after manufacture to ensure the identity and purity of a particular pharmaceutical product (WHO, 2019b). The pharmaceutical sector is faced with the challenge of good quality products, this vary from country to country with the status of the regulatory bodies as well as resources available for production in such countries playing a critical role (Newton, et al, 2011).

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An important challenge in ensuring quality of drug in most developing countries including Nigeria is the non-availability of infrastructures and sophisticated equipment specified in most official monographs. Thus, the high prevalence of fake and substandard drugs in such countries compared to developed countries. Antimalarial drugs have become the target of a very sophisticated and highly profitable counterfeit drug trade which includes packaging, holograms and barcodes (Atemnkeng, et al., 2007, Bate, et al., 2008). The increasing prevalence of fake and substandard artemisinin products is a challenge that needs urgent attention in order to maximize the public health benefits of ACTs. Many developing countries lack infrastructures and sophisticated resources for specified analytical methods, hence the need for rapid, inexpensive but reliable analytical techniques to screen such drugs. The official method as well as reported analytical procedures for dihydroartemisinin as a monotherapy formulation in dosage forms and biological fluids involves the use of UV-VIS spectrophotometry, colorimetry involving derivatization, LC-MS and LC-MS-MS (IP, 2016, Attih, et al. 2015, Naik, et al., 2005, Modi, et al, 2012, Adegoke and Osoye, 2011, Babalola et al., 2014). All these reported procedures are sophisticated analytical techniques which are beyond the reach of most poor-resourced economies where malaria is endemic. However, reports on validated analytical procedures for DHA in combination dosage forms are almost non-existent, although there are reports on its field identification (Roth, et al., 2019, Vickers, et al., 2018, Wilson, et al., 2017) their ability to effectively detect fake or substandard drug samples is a source of concern to regulatory authorities. This study thus report a simple, validated and low cost analytical procedure for DHA in DHA-PPQ combination in tablet dosage form.

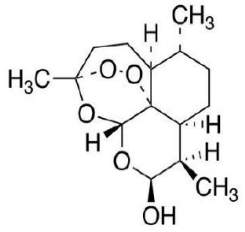

Figure 1: Dihydroartemisinin structure

MATERIALS AND METHODS

Chemicals and Reagents

Ethanol, methanol, chloroform, diethyl ether, glacial acetic acid, petroleum spirit, potassium iodide, hydrochloric acid, silver nitrates (all chemicals and reagents were of analytical grade), pharmaceutical grade starch and talc powder. Pure dihydroartemisinin was a gift from Bond Chemicals Industry, Aawe, Oyo State, Nigeria. Four brands (Codison-Plus®, P-Alaxin®, Viskart-P® and Amnoquine®) of dihydroartemisinin-piperaquine phosphate tablets were obtained from Pharmacies in Ibadan.

Identification of pure dihydroartemisinin powder (1^o Standard)

The pure DHA was identified by chemical test, thin layer chromatography (TLC) and infrared spectrometry (Infrared Spectrometer, Buck Scientific, Buck M530, USA) (IP, 2016) and melting point determination (Jansen, 2010).

Purification and identification of pure piperaquine phosphate (PPQ) powder (2^o Standard)

Pure piperaquine phosphate powder (2^o standard) was extracted from one of the brands of DHA-PPQ tablet (Codison-Plus®). Equivalent of 0.025g piperaquine phosphate (PPQ) was extracted with 30ml diethyl ether in an extraction tube, washed with 2 x 50ml diethylether, the diethyl ether was discarded. Obtained powder was dried in vacuum oven at 40^oC. The procedure was repeated to obtain 2g PPQ to which was added 50ml diethylether, allowed to stand for 10minutes followed by filtering using vacuum pump and drying in oven at 40^oC for 2hours. The obtained PPQ (2^o standard) was identified using chemical identification test, thin layer chromatography (TLC), melting point, ultraviolet spectrophotometry (UV/Visible spectrophotometer, (Lambda 25 Perkin Elmer Inc., Singapore) (Kissi, 2010, Sunderland, et al., 2001, Hung, 2002, Richard, et al., 2011) and infrared spectroscopy (KBr method).

Proposed assay method development for DHA in DHA-PPQ combination

This was based on a slight modification of the International Pharmacopoeia (2016) method for the determination of DHA in tablet dosage forms.

Dihydroartemisinin (DHA) calibration curve (IP 2016): Dihydroartemisinin solution in ethanol (0.1%w/v) was prepared in 25ml volumetric flask. Aliquots were transferred into 25ml volumetric flasks and made up to volume with 0.05M NaOH to obtain 0.04-0.12mg/ml respectively. Corresponding blank solutions were prepared using ethanol and 0.05M NaOH. The solutions were incubated in a thermostated water bath at 50^oC for 30minutes and absorbance readings were taken at 292nm using UV-Visible spectrophotometer against reagent blank solutions. Triplicate determination was made and used to generate a calibration curve.

Development of proposed assay method for DHA in DHA-PPQ combination

Presence of talc and starch: Dihydroartemisinin pure powder (0.025g) was mixed with starch (0.05g) and talc (0.01g) in a mortar and kept covered for 30 minutes; the mixture was transferred into an extraction tube and extracted with diethyl ether (1x30ml, 2x20ml). The combined filtrate was filtered and evaporated to dryness at 25^{o}C under nitrogen gas. The dried extract was dissolved in 10ml absolute ethanol, transferred to 25ml volumetric flask and made up to volume with ethanol. Aliquots of the ethanol solution was transferred into different 25mls volumetric flask and made up to volume with 0.05M NaOH to produce 0.04, 0.08 and 0.12mg/ml. Corresponding blank solutions were prepared using ethanol and 0.05M NaOH. The solutions were placed in a thermostated water bath at 50^{o}C for 30minutes and absorbance determined at 292nm using UV-Visible spectrophotometer. Dihydroartemisinin contents were calculated from the calibration curve and triplicate determination was done.

Presence of PPQ, talc and starch: Dihydroartemisinin pure powder (0.025g) was mixed with piperaquine phosphate (0.05g), starch (0.05g) and talc (0.01g) in a mortar and kept covered for 30 minutes. The mixture was transferred into an extraction tube and extracted with diethyl ether (1x30ml, 2x20ml). The combined filtrate was filtered and evaporated to dryness at 25^{o}C under nitrogen gas. The obtained dried extract was analysed using the procedure above. Dihydroartemisinin contents were calculated from the calibration curve and triplicate determination was done.

Validation of the proposed method

The proposed method was subjected to validation using the current ICH guidelines through parameters such as linearity, accuracy, precision, recovery studies, specificity and reproducibility (ICH, 2005). The obtained mean DHA content (n=3) at the three different concentrations were plotted against the expected concentrations to assess the linearity. The accuracy was determined using recovery studies at the selected three concentrations 0.04, 0.08 and 0.12mg/ml to represent low, medium and high concentrations of the calibration curve. Precision assessment was carried out by replicate determination (n=4) on the same day (intra-day) and on three consecutive days (inter-day) using the described procedure.

Assay of DHA in commercial DHA-PPQ tablets: Three commercially available brands of DHA-PPQ tablets containing DHA (40mg) and PPQ (320mg) per tablet obtained from pharmacies were used for the study. Uniformity of weight was determined, while dihydroartemisinin and piperaquine phosphate content in each tablet were identified using TLC (IP, 2016, Kissi, 2010, Richard, et al., 2011). Equivalent of DHA (0.025g) in powdered tablets was extracted with diethyl ether (1x30ml, 2x20ml). The combined filtrate was filtered and evaporated to dryness at 25^{o}C under nitrogen gas. The dried extract was dissolved in 10ml absolute ethanol, transferred to 25ml volumetric flask and made up to volume with ethanol. Two millilitres of the ethanol solution was transferred into 25mls volumetric flask and made up to volume with 0.05M NaOH. Corresponding blank solutions were prepared using ethanol and 0.05M NaOH. The solutions were placed in a thermostated water bath at 50^{o}C for 30minutes and absorbance determined at 292nm using UV-Visible spectrophotometer against the blank. Replicate determinations were made with the dihydroartemisinin contents calculated from the calibration curve. Intra-day (n=5) and inter-day (three days) (n=9) assays were carried out on the tablet brands. Also, the assay was repeated by another analyst on the same samples on separate days.

RESULTS AND DISCUSSION

The pure DHA conforms to the reported specifications of 164^{o}C (Jansen, 2010), chemical tests and infrared spectrometry (IP, 2016). Similarly, the purity of the water soluble, pale yellow crystalline piperaquine phosphate powder (2^{o} Standard) was confirmed using melting point (248-249^{o}C), chemical test, ultra violet absorption peaks at 225, 239 and 346nm in 0.01M HCl (Sunderland, et al., 2001, Hung, 2002) and IR. The proposed procedure for the assay of DHA content in the combined formulation of DHA-PPQ tablets was a slight modification of the official method of analysis of DHA in tablet formulations (IP, 2016). The method which is a difference ultraviolet spectrophotometric technique was based on the reaction of DHA with 0.05M NaOH to generate chromophoric group which gave good ultraviolet light absorption at 292nm. A linear calibration curve; $y = 5x-0.016$ ($r^2 = 0.9948$) was obtained for the pure DHA at 0.04 - 0.12mg/ml using the IP method. The proposed method was validated by determining the accuracy, linearity, precision, recovery and specificity based on ICH guidelines (ICH, 2005). Dihydroartemisinin content determination in the presence of talc and starch which are two basic components of tablet dosage forms as well as PPQ was linear across the three concentrations; 0.04, 0.08 and 0.12mg/ml representing low, medium and high concentrations

respectively. The obtained linear equation for DHA in the presence of starch and talc was $y = 1.01x - 0.0036$ ($r^2 = 0.9901$), while $y = 0.9525x + 0.002$ ($r^2 = 0.9969$) was obtained in the presence of PPQ, starch and talc. The non-significant ($p < 0.05$) difference in the obtained amount of DHA in the presence of excipients (talc and starch) and PPQ across the three concentrations was an indication of specificity and selectivity of the method in the quantitative determination of DHA in the presence of PPQ (Table 1). The obtained correlation coefficient (r = 0.9984) obtained in the presence of PPQ (DHA-Talc-Starch-PPQ) also shows linearity across the three concentrations. The accuracy determination for the proposed method using recovery studies of samples containing known amount of pure DHA is presented in Table 1. Interday and intraday

Table 1: Assessment of interference effects of PPQ and two common excipients on the proposed method for the assay of DHA

Expected Concentration (mg/ml)	DHA-T-S			DHA-T-S-PPQ		
	Obtained Concentration (mg/ ml)	% Recovery	RSD	Obtained Concentration (mg/ ml)	% Recovery	RSD
0.04	0.0391± 0.0005	97.67± 1.258	1.29	0.0389± 0.0003	97.30± 0.8411	0.86
0.08	0.0725± 0.0005	90.58± 0.6291	0.63	0.0805± 0.0008	100.58± 1.0408	1.03
0.12	0.1199± 0.0008	99.94± 0.6736	0.67	0.1151± 0.0064	92.39± 2.78	2.94

Note: DHA-T-S = DHA-Talc-Starch, DHA-T-S-PPQ = DHA-Talc-Starch-PPQ
[a] *Mean ±SD,* [b] *% Recovery stated as a function of amount of sample used*

variation studies in the presence of PPQ, starch and talc showed very good precision at the three different concentrations over a period of three day determination (Table 2). Three commercially available brands of DHA-PPQ tablets; P-Alaxin®, Vinscart® and Amnoquine® containing 40 and 320mg of DHA and PPQ respectively were selected for the application of the proposed method to determine the DHA content. The TLC results showed the presence of DHA and PPQ in the tablet brands which conformed to the uniformity of weights specifications and were within their shelf lives and registered by NAFDAC. In view of the validation determination results obtained with the proposed method using the three concentrations, 0.08mg/ml was selected for the assay of three brands of commercial tablets formulation. The accuracy and precision over a three-day period at 0.08mg/ml gave mean recovery between 100.6 and 101.3% for intra-day determination, while inter-day assessment gave 101.0% (Table 2). Both inter-day and intra-day precision assessments gave RSD less than the 2% specification for precision. All the validation parameters determined were within the specification according to the current ICH guidelines (ICH, 2005). Inter-day and intra-day assay of the commercial tablets showed good accuracy and precision across the three brands (Table 3). Application of the proposed method in the analysis of the same brands of the commercial tablets by two different analysts on separate days gave very good accuracy and precision (Table 4). Statistical comparison of assay outcome of the two analysts on the same brands at 95% confidence level at 5 degree of freedom gave *t* and *F* values below the tabulated values indicating accuracy and precision of the proposed method (Table 4). This shows that the method could be used for routine analysis of DHA content in DHA-PPQ tablet formulations.

Table 2: Intraday and interday accuracy and precision assessment of proposed UV spectrophotometry method in the assay of DHA in the presence of PPQ, talc and starch

Expected Concentration (mg/ml)	Inter-day (n=3)						Intraday (n=9)	
	Day 1		Day 2		Day 3		% Recovery	RSD (%)
	% Recovery	RSD (%)	% Recovery	RSD (%)	% Recovery	RSD (%)		
0.04	97.30± 0.841	0.86	99.92± 1.665	1.67	97.88± 1.84	1.88	98.37± 1.770	1.80
0.08	100.58± 1.041	1.03	101.08± 1.283	1.27	101.33± 1.283	1.27	101.00± 1.097	1.09
0.12	94.39± 2.77	2.94	93.77± 1.779	1.90	94.28± 2.80	2.97	94.14± 2.180	2.32

[a] *Mean ±SD*
[b] *% Recovery stated as a function of amount of sample used*

Table 3: Intraday and Interday assay of three brands of DHA-PPQ tablet dosage forms using the proposed method

Brand	Inter-day (n=3)						Intraday (n=9)	
	Day 1		Day 2		Day 3		% Recovery ± SD [a]	RSD (%)
	% Recovery ± SD [a]	RSD (%)	% Recovery ± SD [a]	RSD (%)	% Recovery ± SD [a]	RSD (%)		
P-Alaxin®	95.08± 0.382	0.40	94.17± 1.233	1.31	93.25± 0.50	0.53	94.17± 1.053	1.12
Viscart P®	99.17± 0.722	0.73	102.58± 0.144	0.14	103.00± 0.250	0.24	101.58± 1.862	1.83
Amnoquine®	93.58± 0.382	0.41	93.75± 0.250	0.27	94.5± 0.500	0.53	93.94± 0.542	0.58

[a] *Mean ±SD;* [b] *% Recovery stated as a function of amount of sample used*

Table 4 Inter-analysts application of the proposed assay method on three brands of DHA-PPQ tablets

Brand	Analyst 1[a]		Analyst 2[a]		Statistics (p-value)[c]	
	% Recovery± SD [b]	RSD (%)	% Recovery± SD [b]	RSD (%)	t-value	F-value
P-Alaxin®	94.96± 0.697	0.73	94.29± 1.08	1.14	1.28	2.40
Viscart P®	100.88± 1.928	1.91	100.75± 2.151	2.13	0.11	1.25
Amnoquine®	93.83± 0.438	0.47	94.04± 0332	0.35	0.94	1.74

[a] *Mean ±SD (n = 6) % Content of DHA in tablets stated by IP, 2008 ranges from 90 – 110%*
[b] *% Recovery stated as a function of amount of sample used*
[c] *Statistical analyses done between the results of the two analysts; tabulated 't' and 'F' values at 95% confidence level and 5 degree of freedom are 2.571 and 5.05 respectively*

Most of the earlier reports on the assay of DHA in combination with PPQ dosage forms require the use HPLC with UV detection (Reddy, et al., 2013, Vemula et al., 2013). A previous HPLC report on the assay of pure DHA in combination with PPQ did not reflect the presence of excipients and dose combination ratio of the two drugs in formulation (Deokate, et al., 2014), while two other reported methods were neither validated nor referenced (Olajide, et al., 2017, Yunarto, et al., 2016). This proposed UV method for the determination of DHA content in combined formulation of dihydroartemisinin-piperaquine phosphate is hereby reported to be simple, fast, selective, reproducible with very good accuracy and precision. The obtained DHA content in the analyzed commercial tablet samples were statistically comparable ($p>0.05$) ranging from 94.29± 1.08 to 100.88± 1.93%w/w, which was within the official specification of 90 – 110%w/w for DHA tablets (IP, 2016). Thus, this method could be very useful especially in most resource-deficient economies where malaria has become an endemic disease, and fake or sub-standard antimalarials is prevalent because of inadequate quality control facilities.

CONCLUSION

A new simple ultraviolet spectrophotometric method successfully developed and validated is hereby presented to monitor DHA content in in-process bulk

samples as well as tablet dosage forms of dihydroartemisinin-piperaquine phosphate combinations. This method would easily find application in the control of DHA-PPQ tablets in poor-resourced economies.

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