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Development and Validation of High-Performance Liquid Chromatography-Tandem Mass Spectrometric Method for Simultaneous Determination of Artemether and its metabolite, Dihydroartemisinin in Human Plasma: A Pharmacokinetic Study Application

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ABSTRACT

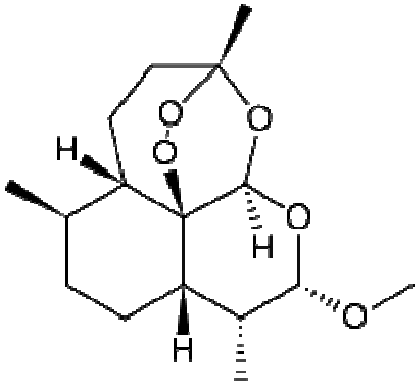
An analytical procedure was developed/validated for the quantification of Artemether and its metabolite, Dihydroartemisinin (DHA), in human plasma. The analyte and its metabolite were extracted from human plasma by solid phase extraction technique, followed by simple isocratic chromatographic condition with mobile phase Acetonitrile: Ammonium formate buffer 2 mM (80:20 v/v) and mass spectrometric detection that enables detection at nanogram levels. Artemisinin was used as the internal standard. A Polaris A, C18, 50 X 4.6 mm, 5µm column provided chromatographic separation of the analyte which was followed by detection with mass spectrometry. The mass transition ion pair was followed as m/z 316.1 / 267.1 for Artemether, m/z 302.1 / 267.1 for Dihydroartemisinin and m/z 300.2 / 209.2 for internal standard using ESI Positive ionization mode. The retention time of analyte (Artemether and Dihydroartemisinin) and internal standard was 2.7, 1.6 and 1.8 minutes respectively. The proposed method has been validated with linear range of 2.386-200.787 ng/mL for Artemether and 2.391- 201.252 ng/mL for Dihydroartemisinin. Estimation of Artemether and its metabolite dihydroartemisinin in biological matrices has usually been difficult, with sensitivity being unease. The absence of any matrix effects was observed. Both intra-day and inter-day accuracy and precision showed good reproducibility. The method developed produce recovery of for Artemether was 92.57%, and for Dihydroartemisinin was 93.89%. The LC-MS/MS method described is sensitive, selective and linear for the wide range of concentrations for Ethambutol in human plasma. The validated method well suited for application in bioequivalence study of 20 mg Artemether tablet.

Key words: Artemether and its metabolite Dihydroartemisinin, Human plasma, Solid-phase extraction; LCMS/MS.

INTRODUCTION

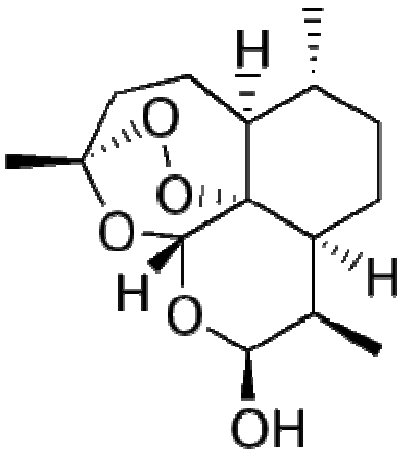
The chemical name of Artemether is (3R,5aS,6R,8aS,9R,10S,12R,12aR)-decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepine. Artemether is a white, crystalline powder that is freely soluble in acetone, soluble in methanol and ethanol, and practically insoluble in water. It has the empirical formula $C_{16}H_{26}O_5$ with a molecular weight of 298.4, and the following structural formula Refer Figure 1 (1):

Figure 1: Chemical Structure of Artemether



Dihydroartemisinin has the empirical formula $C_{15}H_{24}O_5$ with a molecular weight of 284.35, and the following structural formula Figure 2:

Figure 2: Chemical Structure of Dihydroartemisinin



Quite a lot of LCMS methods have been published for the quantitative analysis of Artemether and Dihydroartemisinin in human plasma (2-12) and by HPLC method (13-17). The aim of this work was to develop a quick, simple and relatively sensitive LCMS method. Although recently a similar application has been reported compared to the earlier methods, in addition we have proved stability experiments in blood, wet extract, wet extract bench top and extended batch verification.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and methanol of HPLC grade were supplied by JT Becker. Water HPLC grade was obtained from a Milli-Q water purification system. Ammonium Acetate was procured from CDH. A reference standard of Artemether, Dihydroartemisinin & Artemisinin internal standard were procured from SeQuent Research Limited, Mangalore, India. Fresh frozen human plasma (K3-EDTA as anticoagulant) was used during validation was supplied by Sai Laxmi Labs Hyderabad.

Instrumentation and Chromatographic Conditions

Ultra flow liquid chromatography Coupled with Mass Spectrometry was used for the method development and validation. Mass Spectrometry Model API 4000, UFLC model UFLC XR equipped with a model LC-20ADXR a binary pump, SIL-20ACXR auto sampler was used to keep temperature at 5°C, CTO-20AC column oven used to keep temperature at 35° C and CBM-20Alite system controller. Detection was made at m/z 316.1 / 267.1 for Artemether, m/z 302.1 / 267.1 for Dihydroartemisinin and m/z 300.2 / 209.2 for internal standard using ESI Positive ionization mode. Analyst 1.5.1 software was used for the quantification. The stationary phase was Polaris A, C18, 50 X 4.6 mm, 5µm.

Method Development and Optimization

The procedure was developed to validate a method for the determination for Artemether and Dihydroartemisinin in human plasma using K_3EDTA as an anti coagulant. The standard stock solution was diluted with methanol to50 ng/mL before injecting into the Polaris A, C18 column with different ratios of Acetonitrile and Ammonium acetate buffer. The optimum mobile phase was found to be Acetonitrile: Ammonium formate buffer 2 mM (80:20 v/v). The separation was carried out at ambient temperature with a flow rate of 0.5 mL. The injection volume was 10 µL and run time was 3.5 minutes. The RT of analyte (Artemether and Dihydroartemisinin) and internal standard was 2.7, 1.6 and 1.8 minutes.

Preparation of extracted samples

Aliquoted 300 µL of plasma from the pre-labelled polypropylene tubes into RIA vials and added 25 µL of Artemisinin internal standard (approx.500 ng/mL) and vortexed 20 seconds. The cartridges {Strata X (30 mg/1cc)} were conditioned with 1.0 mL methanol, equilibrated with the 1.0 ml of Milli-Q water and samples were loaded, cartridges were washed with 1.0 mL of 20% Acetonitrile and were eluted with 1 mL of dilution solution.

Standards and working solutions

Individual stock standard solution of Artemether and Dihydroartemisinin and IS containing 0.5 mg/mL was prepared by dissolving in methanol. Working standard solutions of varying concentrations of Artemether and Dihydroartemisinin were prepared on the day of analysis by diluting the stocks with dilution solution.

Calibration standards were established between 2.386-200.787 ng/mL for Artemether and 2.391-201.252 ng/mL for Dihydroartemisinin of using eight non-zero concentrations levels. Quality control (QC) standards of four different levels (QCLLQ) (2.394 ng/ mL), (QCL) (7.010 ng/ mL), (QCM) (85.484 ng/mL) and (QCH) (157.052 ng/ mL) for Artemether and QCLLQ) (2.399 ng/ mL), (QCL) (7.026 ng/ mL), (QCM) (85.681 ng/mL) and (QCH) (157.415 ng/ mL) for Dihydroartemisinin were also prepared. These samples were stored below -50 °C until used. Twelve sets of QCL and QCH were stored to below -20 °C freezer for generation of below -

20 °C stability. All these stock solutions, calibration standards and QC samples were stored at 4±2°C. These solutions were found to be stable and used for the complete method validation.

System Suitability

The system suitability was performed before starting each day's activity according to in-house and it was within acceptance criteria less than or equal to 2 % for area ratio and less than or equal to 4 % for RT.

Method Validation

The method was validated over a concentration range of 2.386-200.787 ng/mL for Artemether and 2.391-201.252 ng/mL for Dihydroartemisinin. This validation provides the results of specificity and selectivity, carryover, matrix effect, calibration standards and quality control samples data, precision and accuracy data, the results of various stabilities, dilution integrity, reinjection reproducibility, ruggedness, extended batch verification, effect of haemolysed and lipemic plasma and blood stability.

Aqueous solution linearity

Aqueous solution linearity of calibration standards i.e. spiking solution checking was assessed by subjecting the spiked concentrations and the respective peak areas using $1/x^2$ (x-concentration) linear least-squares regression analysis. The calibration curves had a correlation coefficient (r) of 0.9990 for Artemether and 0.9993 for Dihydroartemisinin. In aqueous solution linearity test all calibration standards accuracy were within 85-115%, except LLOQ where it was 80-120%.

Specificity / Selectivity

Specificity and selectivity were performed from nine different lots of plasma and they were extracted and analyzed for the assessment of potential interferences with endogenous substances.

Matrix Effect

Blank samples (plasma) from six independent sources of matrix were processed in duplicate and then spiked with analyte at QCL and QCH level and internal standard at the concentration used in the method being validated just before injection into the LC-MS/MS. An aqueous solution of analyte was prepared at QCL and QCH with internal standard in diluent (Reference solution). Peak area ratios of the plasma samples were compared with that of reference solution to ensure that the matrix factor was consistent for different sources of matrix.

Signal to Noise Ratio

Signal to Noise ratio was obtained at the lower limit of quantification (LLOQ) from the chromatogram by comparing the area obtained at LLOQ for each lot used in the specificity / selectivity experiment with area obtained in respective blank samples. The signal-to-noise ratio obtained for the samples was greater than 5 for all the plasma lots tested.

Carry Over

Processed and injected Blank, 2LLOQ and 2ULOQ samples and re-injected blank samples to check carry over.

Linearity

A regression equation with a weighting factor of $1/x^2$ of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Artemether and Dihydroartemisinin in human plasma. The representative calibration curves for regression analysis are illustrated in Figure 5 & 6. Correlation coefficient (r^2) was greater than 0.9975 and

0.9960 for Artemether and Dihydroartemisinin in the concentration range of 2.386-200.787 ng/mL for Artemether and 2.391-201.252 ng/mL for Dihydroartemisinin.

Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of QCLLOQ, QCL, QCM and QCH samples respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage. For results refer Table No 1 & 2.

Table No. 1: Precision and Accuracy for Artemether

Within Batch Precision	PA1	2.21 % - 6.08 % (QCM-QCLLQ)
	PA2	1.84 % - 6.32 % (QCM-QCLLQ)
	PA3	1.53 % - 3.68 % (QCM-QCLLQ)
Within Batch Accuracy	PA1	101.80 % - 106.16 % (QCLLQ -QCM)
	PA2	100.02 % - 104.99 % (QCH-QCM)
	PA3	98.28 % - 108.80 % (QCLLQ -QCM)
Intraday Batch Precision		2.02 % - 5.91 % (QCM -QCLLQ)
Intraday Batch Accuracy		101.29 % - 105.57 % (QCL-QCM)
Between Batch Precision		2.34 % - 5.54 % (QCM- QCLLQ)
Between Batch Accuracy		100.58 % - 106.65 % (QCL- QCLLQ)

Table No. 2: Precision and Accuracy for Dihydroartemisinin

Within Batch Precision	PA1	2.69 % - 8.72 % (QCH-QCLLQ)
	PA2	2.87 % - 7.30 % (QCL-QCLLQ)
	PA3	2.27 % - 5.45 % (QCH-QCL)
Within Batch Accuracy	PA1	101.72 % - 105.87 % (QCL -QCM)
	PA2	101.74 % - 107. 17 % (QCM-QCLLQ)
	PA3	97.99 % - 105.72 % (QCLLQ -QCH)
Intraday Batch Precision		2.90 % - 7.67 % (QCM -QCLLQ)
Intraday Batch Accuracy		101.82 % - 106.52 % (QCLLQ-QCM)
Between Batch Precision		2.84 % - 6.89 % (QCM-QCLLQ)
Between Batch Accuracy		100.54 % - 105.59% (QCLLQ - QCM)

Recovery

Prepared 6 sets of recovery comparison samples by spiking 5 µL of dilution of quality control samples (QCL, QCM, QCH) of Artemether and Dihydroartemisinin, 20 µL of internal standard dilution (approx. 500 ng/mL) and 975 µL of diluent, representing 100 % extraction and injected. The recovery comparison samples of Artemether and Dihydroartemisinin were compared against extracted samples of QCL, QCM and QCH samples of PA2 batch.

Dilution Integrity

Twelve dilution integrity samples were prepared by spiking approximately 1.7 times (341.339 and 342.128 ng/mL for Artemether and Dihydroartemisinin respectively) of the highest standard concentration. Six dilution integrity samples were processed by diluting them twice and another six samples by diluting them four times using pooled plasma. These samples were analyzed along with a PA2 batch .The sample concentrations were calculated using appropriate dilution factor. Results demonstrated acceptable dilution integrity for two times and four times dilution.

Re-injection Reproducibility
One precision and accuracy batch (PA1) was retained in the auto sampler at 5°C for 49 hours to test the re-injection reproducibility of the method.

Stabilities
Freeze-Thaw Stability Three Cycles
The stability in human plasma was determined for three freeze-thaw cycles. Six replicates of QCL and QCH were analyzed after undergoing three freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards and % change calculated against fresh quality control samples.

Bench Top Stability
Bench top stability was determined for 22 hours, using six sets each of QCL and QCH. The quality control samples were quantified against the freshly spiked calibration curve standards and % change calculated against fresh quality control samples.

Long Term Stability at below -20° C and -50°C
To assess the stability of the analytes in the biological matrix under the same conditions of storage as that of the study samples the following test was performed. Six samples of each quality control samples at low and high concentrations were stored below -20°C and -50°C in the freezer for 68 days. These samples were quantified against the freshly spiked calibration curve standards and % change calculated against fresh quality control samples.

Wet Extract Stability
To assess the wet extract stability, six sets of QCL and QCH samples were extracted and retained in the auto sampler to prove wet extract / auto sampler stability. These samples were injected after a period of 37 hours and were quantified against freshly spiked calibration curve standards and % change calculated against fresh quality control samples.

Wet Extract Bench Top Stability
To assess the wet extract stability on bench top at room temperature, six sets of QCL and QCH were processed, reconstituted and kept on bench top at room temperature. These samples were injected after a period of 4.5 hours and were quantified against freshly spiked calibration curve standards and % change calculated against fresh quality control samples. The results demonstrate that the processed samples were stable for 4.5 hours on bench top at room temperature.

Stock Dilution Stability
The stability of stock dilutions of analytes and the internal standard was evaluated at room temperature. Aqueous stock dilutions of the analytes and the internal standard were prepared. One portion of the stock dilution was placed in the refrigerator between 2-8°C, while the other portion was placed at room temperature for 27 hours.

Stock Solution Stability
Stock solution stability was carried out for 29 days by injecting six replicates of stock dilution of stability standards (analyte and internal standard which prepared and stored in the refrigerator between 2 - 8° C) and freshly prepared stock dilutions of Comparison standard (analyte and internal standard). The response of stability sample was corrected by multiplying with correction factor.

Hemolysed and Lipemic Effect

To assess the effect of hemolysed and lipemic biological matrix on analyte the following test was performed. Six samples of each quality control samples at low and high concentrations were freshly prepared in hemolysed and lipemic matrix. These samples were quantified against the freshly spiked calibration curve standards. The stability of the analytes was evaluated by comparing each of the back calculated concentrations of stability QCs with the nominal concentration of QCs.

Extended Batch Verification

To check the batch size of the run during the study sample analysis following experiments was preformed. Processed one set of CC and six sets of QCL, QCM and QCH along with 102 blank samples. These QC samples were interspersed with the blank samples and processed.

Blood Stability

To assess the stability of the analyte in the blood at room temperature the following test was performed. Three sets of each quality control samples at low and high concentrations were spiked in to whole blood and kept on bench top at room temperature for 3.5 hrs.

Chromatography

Representative chromatograms of blank plasma, QCM & calibration curve of Artemether and Dihydroartemisinin are given in Figure.3 to 6.

Figure 3 - A Representative Chromatogram of Artemether and Dihydroartemisinin for Blank

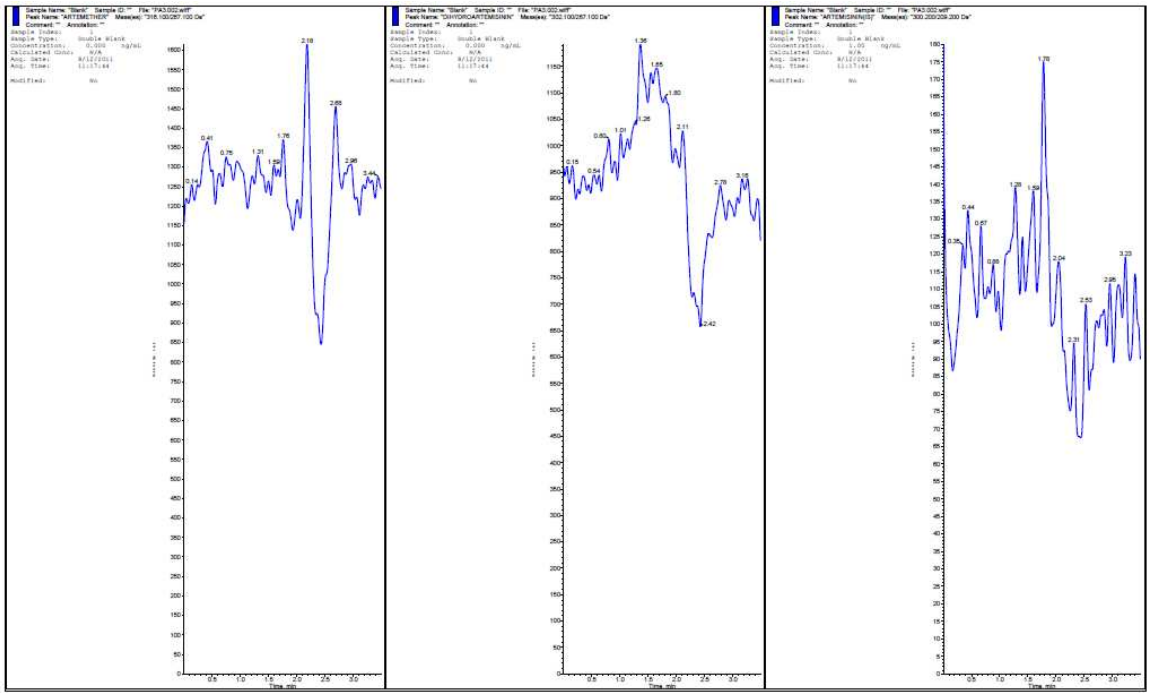


Figure 4 - A Representative Chromatogram of Artemether and Dihydroartemisinin for QCL

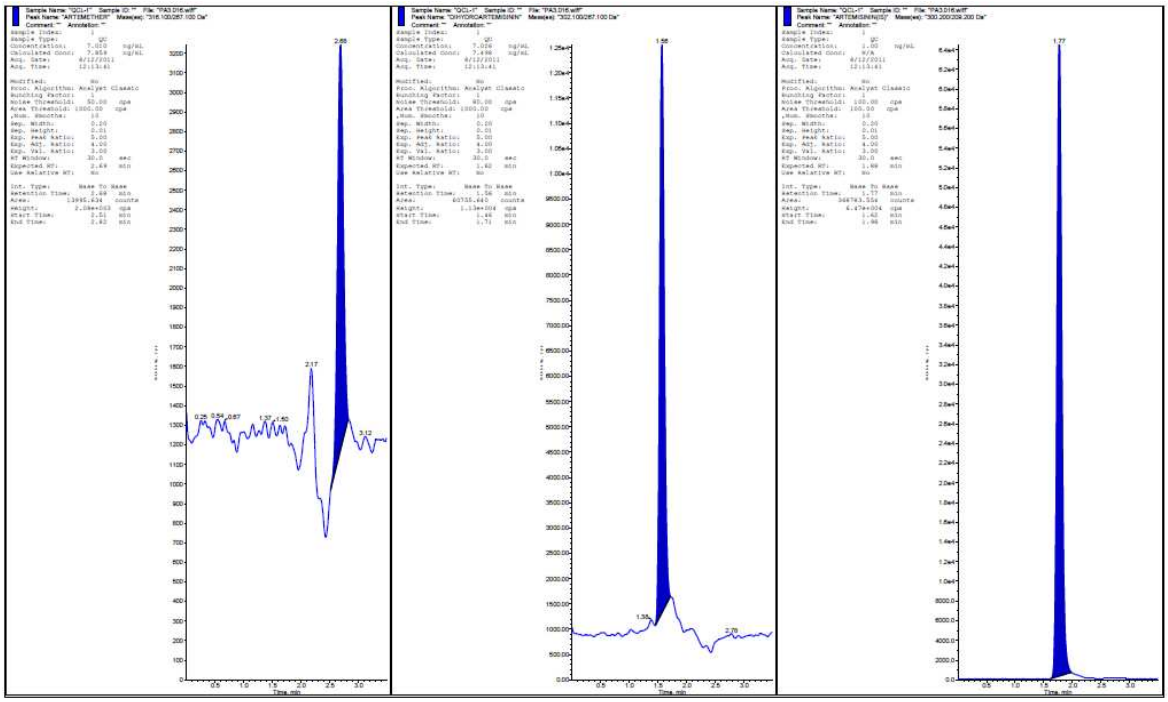


Figure 5 - A Representative Calibration Curve for Artemether

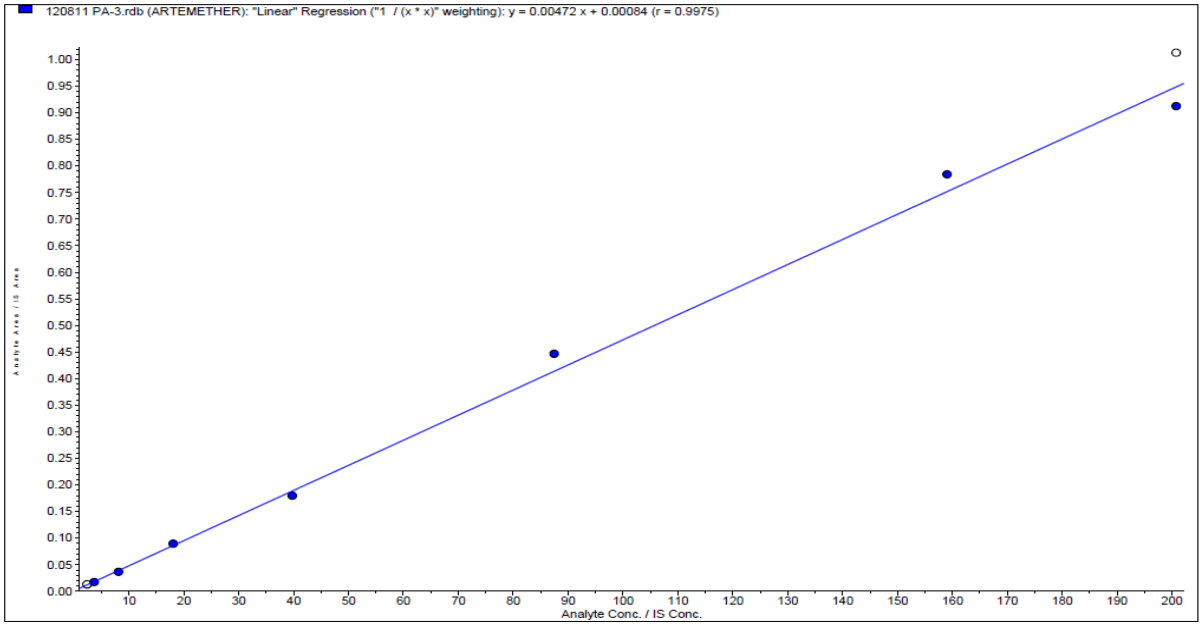
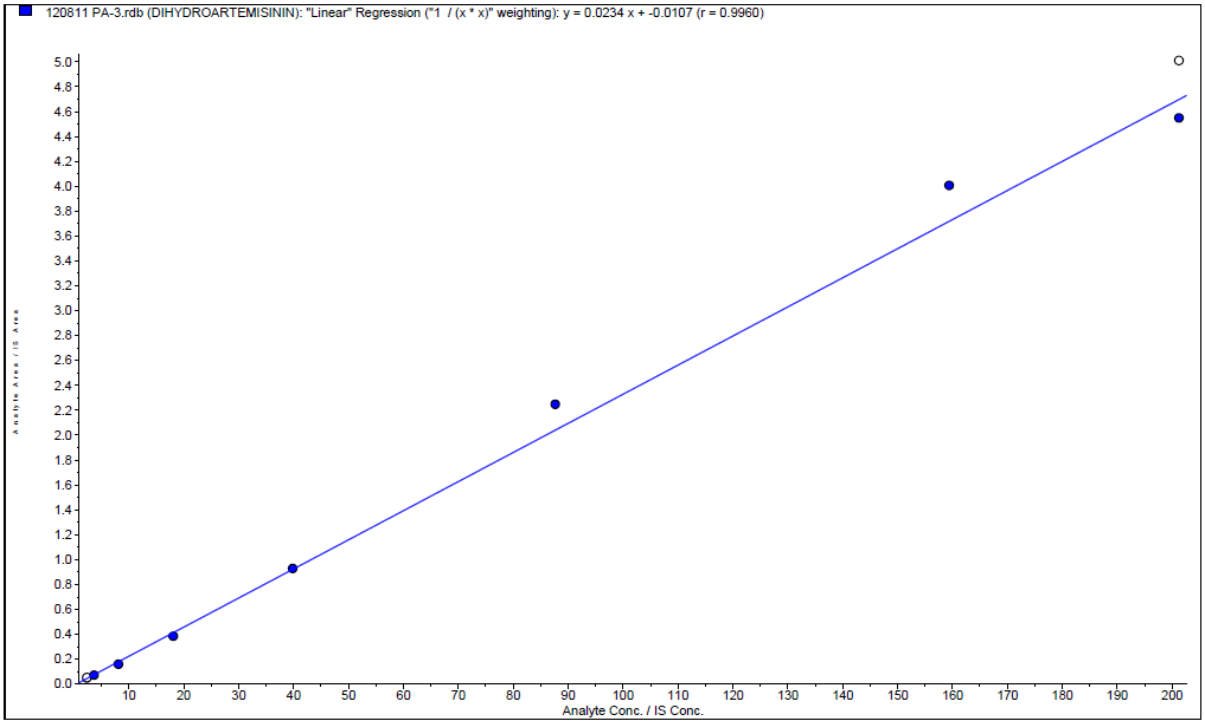


Figure 6 - A Representative Calibration Curve for Dihydroartemisinin



Data Processing

The chromatograms were acquired and were processed by peak area ratio method using the Analyst Version 1.5.1 Software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked standard with the reciprocal of the ratio of the (drug concentration)^2 to internal standard concentration as a weighing factor ($1/x^2$):

$y = mx + c$

Where,
y = peak area ratio of Artemether and Dihydroartemisinin to internal standard
m = slope of calibration curve
x = concentration of Artemether and Dihydroartemisinin
c = y-axis intercept of the calibration curve

RESULTS

No interference was observed at the RT of Artemether and Dihydroartemisinin and Internal standard. The IS-normalized matrix factor was found to be 0.9710, 0.9674 for QCL and 0.9850, 0.9826 for QCH (close to unity) for six different matrix lots for Artemether and Dihydroartemisinin and the % CV was 0.92, 0.89 for QCL and 0.95, 0.91 for QCH. The % carry over was found to be 0.00, 0.00 and 0.00 for Artemether, Dihydroartemisinin and Artemisinin. The mean and precision of overall recovery for Artemether was 92.57%, 3.19% and for Dihydroartemisinin was 93.89%, 2.60 %. The mean recovery of internal standard was 96.52%.

The within batch precision and accuracy, for a 2 dilution factor of Artemether was 3.68%, 109.89% and for Dihydroartemisinin was 5.01% and 99.67%. The within batch precision and accuracy, for a 4 dilution factor of Artemether was 1.41%, 89.39 % and for Dihydroartemisinin

was 3.49 % and 106.75%. For Re-injection Reproducibility mean accuracy for Artemether ranged from 91.57% (QCL) to 101.14% (QCH) and precision ranged from 1.25% (QCL) to 3.42% (QCM). Dihydroartemisinin mean accuracy ranged from 101.74% (QCLLQ) to 111.38% (QCL) and the precision ranged from 3.96% (QCH) to 9.40% (QCLLQ). The results demonstrate that the reinjection of the sample was reproducible for 49 hours.

For freeze-thaw cycles % nominal for Artemether ranged from 93.71 % to 108.45 % and for Dihydroartemisinin ranged from 89.01 % to 103.67 % and precision for Artemether ranged from 0.34 % to 4.20 % and for Artemether ranged from 1.03 % to 7.38 %. The % change ranged from 0.54 to 1.01 for Artemether and 0.17 to 0.87 for Dihydroartemisinin.

For Artemether % nominal ranged from 91.12% to 110.11% and 89.19% to 106.17% for Dihydroartemisinin. The precision ranged from 3.01% to 5.74% for Artemether and 0.89% to 2.40% for Dihydroartemisinin. The % change ranged from 1.08 to 3.25 for Artemether and 2.01 to 4.39 for Dihydroartemisinin. The results display samples was stable on bench top for 22 hours.

The % nominal ranged from 89.21% to 91.02% for Artemether and 93.09% to 109.15% for Dihydroartemisinin. The precision ranged from 0.96% to 3.21% for Artemether and 2.03% to 3.96 % for Dihydroartemisinin. The % change ranged from 0.15 to 2.96 for Artemether and 1.39 to 1.96 for Dihydroartemisinin respectively for samples stored at -20°C. The % nominal ranged from 87.36% to 106.39% for Artemether and 96.29% to 109.36% for Dihydroartemisinin. The precision ranged from 0.99% to 3.71 % for Artemether and 2.01% to 3.02 % for Artemether. The % change ranged from 1.00 to 2.37 for Artemether 0.21 to 1.02 for Dihydroartemisinin respectively for samples stored at -50°C.

The % nominal at around ranged from 91.01% to 100.20% for Artemether and 98.39% to 103.26 for Dihydroartemisinin. The precision ranged from 0.98% to 3.00% for Artemether and 1.52% to 7.32% for Dihydroartemisinin. The % change ranged from 0.81 to 1.75 for Artemether and 1.99 to 3.63 for Dihydroartemisinin. The result put on show samples was stable on wet extract for 37 hours.

The % nominal at around ranged from 91.32% to 107.11% for Artemether and 89.11% to 98.00 for Dihydroartemisinin. The precision ranged from 0.65% to 3.99% for Artemether and 3.22% to 3.69% for Dihydroartemisinin. The % change ranged from 4.38 to 5.68 for Artemether and 1.06 to 3.60 for Dihydroartemisinin. The result put on show samples was stable on wet extract bench top for 4.5 hours.

For stock dilution stability percent change for Artemether and Dihydroartemisinin was 1.94% and 2.01% for Artemisinin is 0.92%, respectively. For Stock solution stability percent change for Artemether and Dihydroartemisinin was 0.92%, 1.33% and for Artemisinin is 1.04%.

For hemolysed and lipemic effect percent nominal ranged from 97.99% to 103.79% for Artemether and 89.33% to 109.25% for Dihydroartemisinin. The precision ranged from 3.66% (QCH) to 5.00% (QCL) for Artemether and 0.96% (QCH) to 3.11% (QCL) for Dihydroartemisinin. The percent change ranged 0.39 to 5.61 for Artemether and 1.62 to 6.31 for Dihydroartemisinin.

The % nominal for calibration curve standards ranged from 91.29% to 101.99% for Artemether and 95.06% to 101.98% for Dihydroartemisinin. The accuracy of QCL, QCM and QCH for Artemether was found to be 92.11, 109.21 and 107.11% respectively and 92.01, 96.33 and

102.32 respectively for Dihydroartemisinin. The precision of QCL, QCM and QCH was found to be 0.89, 2.81 and 4.02 for Artemether and 3.05, 0.89, and 3.07 for Dihydroartemisinin.

For ruggedness he mean accuracy ranged from 99.02% (QCLLQ) to 103.22% (QCL) for Artemether and 89.25% (QCLLQ) to 109.23% (QCL) for Dihydroartemisinin. The precision ranged from 2.01% (QCH) to 5.72% (QCLLQ) for Artemether and 1.93% (QCH) to 4.02% (QCLLQ) for Dihydroartemisinin.

CONCLUSION

The elaborate method proved to be fast, precise, accurate and sensitive and has been successfully used in analyzing plasma samples from healthy human volunteers. The proposed have short analysis time with good resolution, sensitivity and great reproducibility.

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