New liquid chromatographic method for simultaneous quantification of Atovaquone and Proguanil with its active metabolite Cycloguanil in human plasma

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ABSTRACT

Objective: The aim of the present study is to develop HPLC method for simultaneousquantification of atovaquone and proguanil with its active metabolite cycloguanilin human plasma. **Methodology:** A specific and accurate highperformance liquid chromatographic method has been developed using Phenyl (150 × 4.6 mm, 5 μm) column maintained at 18 °C. The separation was achieved using a mobile phase composed of phosphate buffer pH 7.2 and methanol (45:55%). The mobile phase was maintained at flow rate of 0.8 mL/min. The analytes were monitored at 254 nm usingultra-violet detector. The plasma samples extractions was carried out using tert-Butyl Methyl Ether: Dichloromethane (80:20% v/v) mixture. Tramadol was used as an internal standard (ISTD). **Result:** The developed method was validated as per US FDA guidelines and found to be highly specific, precise and accurate. Moreover, atovaquone, proguanil and cycloguanilwere stable in plasma at various stability conditions. **Conclusion:** The developed method is simple, economic, and can be used for quantification of said drugs human plasma samples.

Key words: Atovaquone, Cycloguanil, Proguanil, Human plasma, Bioanalysis, HPLC.

INTRODUCTION

Malaria is parasitic disease that involves high fevers, shaking chills, flu-like symptoms, and anemia caused by a parasite plasmodium, which infects red blood cells. The estimated annual mortality attributed to malaria ranges from 700000 to 2.7 million globally.[1] Malaria is treated with anti-malarial drugs and by supportive measures. There are several classes of anti-malarial drugs available, chloroquine being one of them as a standard treatment option. Among all four types of plasmodium, in particular, plasmodium falciparum rapidly develops resistance against anti-malarials. This fact unavoidably entails poly-pharmacy for the treatment and prophylaxis of malaria to slow the emergence of resistance. The above cited fact is the primary reason for emergence of many fixed dose combinations in the treatment of malaria, Atovaquone (ATV) and Proguanil (PGN) being one of them.[2-6]

ATVisahydroxyl-1,4-naphthoquinone[CAS: 95233-18-4], an analog of ubiquone with anti-pneumocystic activity. It acts by selectively affecting mitochondrial electron transport and pyridine biosynthesis in plasmodium species. PGN (chloroguanide) [CAS: 500-92-5] is a biguanide, a synthetic derivative of pyrimidine. PGN is converted into an active metabolite called Cycloguanil pamoate (CYG). It exerts its anti-microbial action by inhibiting parasitic dihydrofolate reductase enzyme and thereby blocks the biosynthesis of purines and pyrimidines, which are essential for DNA synthesis and cell multiplication of plasmodium. There are certain scientific facts, which make ATV (250 mg) plus PGN, a popular fixed dose combination Firstly, the combination has

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100% protective efficacy. Secondly, there is no recorded adverse drug event even in chemo-suppressed patients with this combination. Thirdly, simple administration with single daily dose increases the patient compliance.[3,5,7] The Therapeutic Drug Monitoring (TDM) of ATV plus PGN requires a fast, reliable and validated analytical method for estimation in human plasma. The complexity of dosage forms including the presence of multiple drug entities pose challenge during the development of assay procedure. The combination of ATV plus PGN is efficient in controlling the malarial infection. The PGN undergoes the CYP P450 metabolism and forms the active metabolite CYG. It is necessary to estimate the active metabolites with parent compunds.

Literature survey revealed that there are plenty methods have been published for estimation of either ATV or PGN in biological matrix. All methods except one estimate either ATV or PGN and its metabolite CYG. Only one method reported by Berggrist et al. for simultaneous estimation of ATV, PGN and CYG in single run.[8] This method used mixed mode column for separation of three analytes. The common problem with mixed mode column is that, optimization of chromatographic parameters with different buffer ratios usually turn out to be cumbersome, as it requires to optimize the concentration of sodium and potassium ions into mobile phase to achieve good retention and moreover mixed mode column is not usually available and comes under specialty requirement in analytical lab. These facts disqualify aforementioned method on the grounds of simplicity and cost effectiveness. Many methods also utilized the technique of dry spot analysis for extraction of analytes, which require much less matrix volume for processing and further analysis.[9-11] Several LC-MS/MS methods also have been reported for quantification of ATV or PGN and CYG, which are more specific and sensitive.[12,13] However these methods are not affordable for most laboratories because of their specialty requirements and high equipment costs, moreover no LC-MS/MS method has been reported for simultaneous estimation of ATV, PGN and CYG in one single run so far. Under the scope of this view, the aim of our research work was to develop and validate alternative, simple and cost-effective method for simultaneous estimation of ATV, PGN and CYG in single run.

MATERIALS AND METHODS

Chemicals and Reagents

Micronized Atovaquone (99.80%) (Figure 1a), Proguanil hydrochloride (99.49%) (Figure 1b) and Cycloguanil hydrochloride (98.00%) (Figure 1c) were purchased from USV limited (Mumbai, Maharashtra, India), Varda biotech private limited (Hyderabad, Andhra Pradesh India) and Torrent research center (Ahmedabad, Gujarat, India) respectively. Tramadol hydrochloride (100.40%) (Figure 1d) was selected as an internal stan-

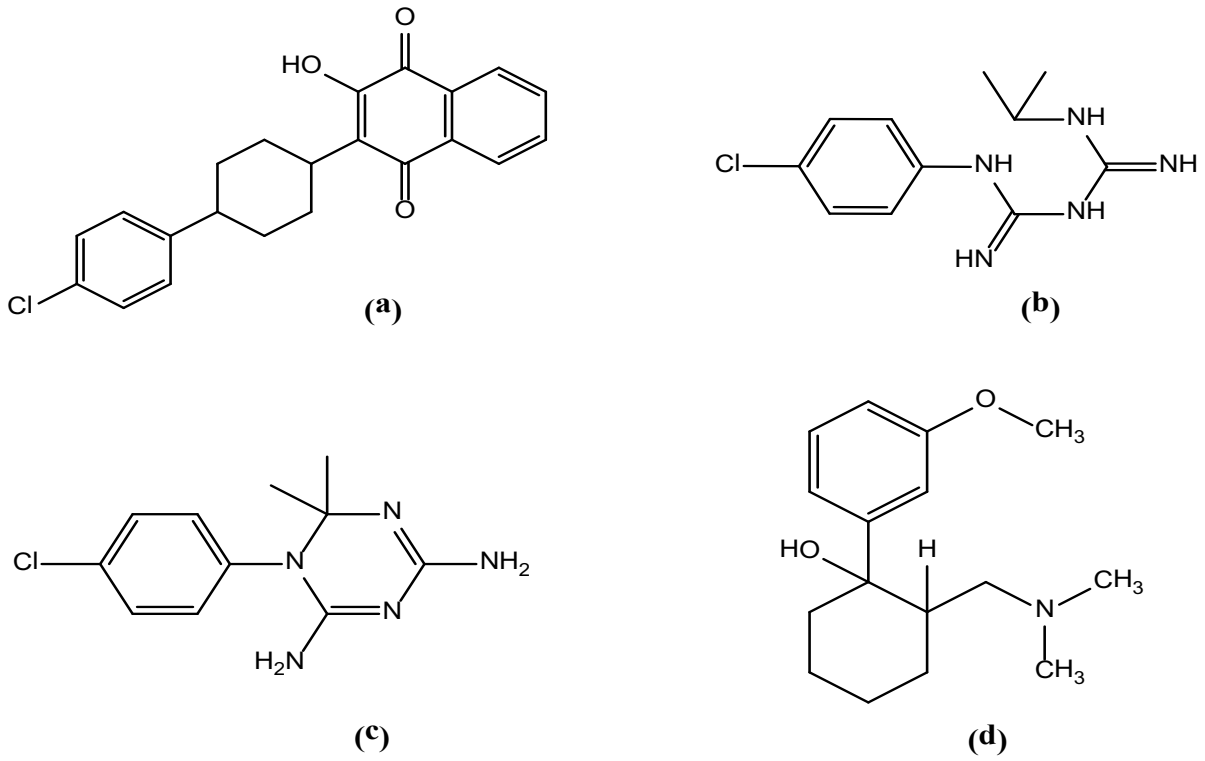

Figure 1: Structures of Atovaquone (a), Proguanil (b), Cycloguanil (c) and Tramadol(d)

dard, which was purchased from Jubilant Organosys (New Delhi, India). Analytical grade reagents such as potassium dihydrogen phosphate, potassium hydroxide and sodium bicarbonate were obtained from SD fine chemicals (Mumbai, Maharashtra, India). HPLC grade reagents such as methanol, methyl tertiary butyl ether (TBME) and dichloromethane (DCM) were purchased from LabScan. Millipore water (0.22 µm membrane filtered) was produced in the laboratory by Millipore system (Model: Direct-Q®3 water purification system) Millipore Corporation, (Billerica, MA, U.S.A.).

Instrumentation

The analysis was carried out on a Shimadzu LC-10 series chromatographic system (Shimadzu Corporation, Kyoto, Japan) consisting of a SCL-10A controller unit, DGU-2A degasser unit, a LC-20AD quaternary gradient pump, SIL10AD autosampler with SPD-20A UV detector. System control, data acquisition and processing were performed with a PC-Pentium IV Processor personal computer operated with Microsoft Windows XP and Shimadzu LC solution 1.24 SP1 software. Standard substances were weighed on Sartorius analytical balance. A glass vacuum-filtration apparatus (Alltech Associates) was employed for the filtration of buffer solution using 0.45 µm filter obtained from Pall Life Sciences (Bangalore, Karnataka, India). Degassing of the mobile phase was performed by sonication in Oscar Micro clean-103 Ultrasonic bath. Thermo 995-Forma-86C freezer (Thermo electron corporation, USA) was used to store the plasma samples. A model Genie-2 Spinix vortex mixer, a Heraeus refrigerated centrifuge (Thermo electron corporation, USA) and TurboVap LV Evaporator (Caliper Life Sciences, Hopkinton, MA, USA) were employed for sample pre-treatment.

Chromatographic conditions

The chromatographic separation was achieved using a Thermo phenyl (150×4.6 mm ID×5 µm) column with mobile phase comprising of 15.0 mM potassium dihydrogen phosphate buffer, pH 7.2 (45%) and methanol (55%). The flow rate was 0.8 mL/min and column temperature was set to 18ºC. Ultra-violet measurements were done at 254 nm wavelength with total run time of 12.5 min.

Preparation of standard solutions

ATV, CYG and PGN were dissolved in methanol to prepare a primary stock solution at a concentration of 1000 µg/mL. Corrections to the theoretical concentration were performed according to the degree of standard substance impurities. The mixed intermediate stock of these drugs was prepared by approximately diluting the stock solutions of ATV, CYG and PGN to get a concentration of 600.0 µg/mL, 45.0 µg/mL, and 22.4 µg/mL respectively.

The mixed intermediate stock was used to prepare spiking stock solution for the construction of nine point calibration curve for ATV(4687.5, 9375.0, 15000.0, 30000.0, 60000.0, 120000.0, 180000.0, 240000.0, 300000.0 µg/ml); (351.5, 703.1, 1125.0, 2250.0, 4500.0, 9000.0, 13500.0, 18000.0, 22500.0 µg/ml) PGN and Cycloguanil (175.0, 350.0, 560.0, 1120.0, 2240.0, 4480.0, 6720.0, 8960.0, 11200.0 µg/ml). QC samples at three different levels for ATV (14.06 µg/ml, 150.00 µg/ml and 270.00 µg/ml), for PGN (1.05 µg/ml, 11.25 µg/ml, 20.25 µg/ml) and for CYG (0.525 µg/ml, 5.60 µg/ml and 10.08 µg/ml) were prepared by taking appropriate volumes of mixed intermediate stock.

Working stock solutions of ATV, CYG and PGN were prepared using a mixture of methanol–water, 50:50 % v/v (diluent). The ISTD working solutions were prepared in a similar manner, providing finally a plasma concentration of 14.3 µg/mL. The intermediate stock solutions were prepared weekly, while working stock solutions used for the calibration curves were prepared daily. Stock solutions, intermediate stock solutions and spiking stock solutions were kept at 4°C and remained stable for at least 7 days.

Calibration standards in plasma for ATV (93.75, 187.50, 300.00, 600.00, 1200.00, 2400.00, 3600.00, 4800.00, 6000.00 ng/mL), CYG (3.50, 7.00, 11.20, 22.40, 44.80, 89.60, 134.40, 179.20, 224.00 ng/mL), PGN (7.03, 14.06, 22.50, 45.00, 90.00, 180.00, 270.00, 360.00, 450.00 ng/mL) were prepared by spiking 686.00 µL blank human plasma with 14.00 µL of mixed working stock solutions of ATV, CYG and PGN as percentage of spiking was 2% (v/v).

Quality Control (QC) samples at four different levels were independently prepared at LOQ-QC with same concentration as the lowest non-zero standard, LQC lower quality control sample, MQC middle quality control sample and HQC higher quality control sample of ATV, CYG and PGN in the same manner. The quality control samples were prepared from a stock solution that was different from the one used to generate standard curve samples. These quality control samples were used to investigate intra- and inter-run variations.

Sample preparation

700.0 µL of blank plasma or standard spiked plasma was taken in 15.00 mL glass centrifuge tube followed by addition of 10.00 µL of tramadol (ISTD) of 1.0 mg/mL and vortexed for 15 sec. Further, 700.00 µL of 5.0 M Sodium hydroxide solution was added and vortexed for 30sec. Subsequently, total 1400.00 µL sample was extracted with 5.500 mL of extracting solvent

TBME:Dichloromethane (80:20% v/v) mixture. The mixture was vortexed again for 15 min and centrifuged at 3500 rpm for 10 min. The supernatant organic phase was collected and evaporated to dryness in a water bath at 40°C under a gentle stream of nitrogen. The dried residue was reconstituted with 100.0 µL of diluent and vortexed for 1 min and a volume of 25.0 µL was injected into the HPLC system.

Method validation[14]

Selectivity

Selectivity was performed to ensure that the bioanalytical method was able to measure and differentiate the analytes in the presence of components that may be expected to be present (metabolites, impurities, degradants or matrix components). It was evaluated by injecting extracted blank human plasma of six different sources and comparing the response of extracted LLOQ with ISTD. There should be no interfering peak present either at drug or at ISTD retention time. If the peak was present then, its response should be ≤ 20% of mean extracted LLOQ area for analyte(s) and 5% for mean extracted ISTD area.

Linearity (calibration curve)

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. Linearity of the method was assessed by constructing five calibration curves, each with one blank plasma (matrix sample processed without ISTD), zero sample (matrix sample processed with ISTD) and nine non-zero samples covering the range 93.75-6000.00 ng/mL for ATV, 3.50-224.00 ng/mL for CYG and 7.00 – 450.00 ng/mL for PGN. Calibration curves were constructed by linear least-squares regression analysis, plotting of peak-area ratios (drug /ISTD) versus the drug concentrations. Weighing factor $1/x^2$ was used for the determination of curve fitting. The calibration model was accepted, if (a) coefficient of correlation was greater than or equal to 0.9800; (b) residuals were within±20% at the lower limit of quantification and within±15% at all other calibration levels; (c) no two consecutive calibration curve standards fail to meet the above acceptance criteria; and (d) at least 2/3 of the standards meet this criterion.

Detection & Quantification limit (Sensitivity)

The detection limit (LOD) is defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The limit of quantification (LLOQ) was determined as the lowest concentration on the standard calibration curve that provided a peak area with a signal-to-noise ratio higher than 5 with a precision 20% and accuracy of 80–120% of its nominal value.

Assay precision & Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. As per the US-FDA guideline, accuracy and precision were done at four concentration levels viz LOQ-QC, LQC, MQC and HQC. Six determinations were made at each quality control level on three different days. The accuracy (%bias) was calculated from the mean value of observed concentration (Cobs) and the nominal concentration (Cnom) as follows

$$Accuracy\ (\%\ Bias) = \frac{Concentration\ found}{Nominal\ Concentration} \times 100$$

The percent coefficient of variation, % CV was calculated from the observed concentrations as follows:

$$\%\ CV = \frac{Standard\ diviation}{Mean} \times 100$$

The accuracy determined at each concentration level must be within ±15% of the respective nominal value except at LOQ-QC where it must be within±20% of the nominal value. The precision around the mean value must not exceed 15% of the %CV except for LOQ-QC where it must not exceed 20% of the %CV.

Recovery

Recovery pertains to the extraction efficiency of a bioanalytical method within the limits of variability. Recovery of analytes was evaluated by comparing the mean peak areas of the three extracted LQC, MQC, and HQC samples to mean peak areas of three reference solutions (Unextracted). Recovery of ISTD was evaluated by comparing the mean peak areas of extracted samples to mean peak areas of reference solutions (unextracted) of the same concentration.

$$Recovery = \frac{Peak\ area\ response\ of\ extracted\ samples\ at LQC, MQC, HQC}{Peak\ area\ response\ of\ unextracted\ amples\ at LQC, MQC, HQC}$$

Stability studies

Stock solution stability

The drug stability is a function of the storage conditions and the chemical properties of the drug. Stock solution stability was performed at room temperature for 6.0 h and at 2 to 8ºC for 7 days. Stock solution stability was assessed by comparing freshly prepared samples of analyte(s) and ISTD with that of stability samples at MQC level by performing five injections of each. Mean percentage change was calculated for both analyte(s)

and ISTD. Stock solution of analyte(s) and ISTD is deemed stable if mean percentage change of ISTD and analyte(s) was within ±10%.

Stability in matrix

Stability studies on analyte(s) which include bench top, freeze and thaw, and long term have been evaluated during validation.

Bench top stability

Six replicates of LQC and HQC in biological matrix were withdrawn and thawed unassisted at room temperature and kept unprocessed for 7 h (stability samples). After 7 h fresh calibration curve was prepared with one set of low and high QC samples (comparison samples). Both comparison samples and stability samples were processed and analyzed in single run.

Freeze thaw stability

Freeze thaw stability in plasma was assessed by analyzing six replicates of LQC and HQC samples after three freeze and thaw cycles. Samples were stored at the intended storage temperature for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 h under the same conditions. The freeze–thaw cycle was repeated two more times, samples were then analyzed on the third cycle. Calibration standards and quality control samples (comparison samples) and stability samples were processed and analyzed in single run.

Long term stability

Six replicates of LQC and HQC in biological matrix were withdrawn from deep freezer (-70°C) after 90 days and thawed at room temperature (stability samples). Fresh calibration was prepared with six replicates of low and high QC samples (comparison samples). Both comparison samples and stability samples were processed and analyzed in single run.

Analyte(s) were deemed stable in matrix if mean percentage change of analyte(s) was within ± 15%.

$$\text{Mean \% change} = \frac{\text{Calculated concentration of stability samples}}{\text{Calculate concentration of comparison samples}} \text{ -1 X 100}$$

RESULTS AND DISCUSSION

Selection of chromatographic parameters

Due to the difference between log P values of ATV (5.8) and CYG (1.0), which is in particular very high, the selection of either reversed phase C_{18} column or normal phase column is crucial. Selection of reversed phase thermo Betasil C_{18} column was tried with 50:50 %v/v aqueous methanol as a mobile phase which was resulted in very long retention time for ATV and less retention for CYG. The trial with Genesis C_8 with intermediate polar stationary phase was taken with the intent to negotiate the considerable log P difference between ATV and CYG. The peak shape of analytes was improved and retention was increased with ATV eluting almost at retention time 16 min. Further in order to decrease the total chromatographic run time thermo Betasil phenyl column was tried. Owing to the intermediate polarity and □-□ electron interaction between analyte and phenyl stationary phase, all three analytes were retained on thermo Betasil phenyl column with run time reduced to 12.5 min. The potassium dihydrogen phosphate buffer was used due to its buffer capacity over wide range of pH. Upon increasing the pH of the buffer from 4.5 through 6 to 7.2, the retention time of PNG and CYG were increased whilst retention for ATV was decreased. Buffer methanol in the ratio of 40: 60v/v

was tried as mobile phase, which was resulted in poor resolution between CYG and PGN, further the organic phase was reduced to 55% in order to increase the resolution between CYG and PGN. The retention time of ATV was found to be dependent on the proportion of organic content in the mobile phase. Upon decreasing of organic content in mobile phase, retention time of ATV was increased. In nutshell, the optimized mobile phase composition for proper resolution between analytes was 45:55% (v/v) of potassium dihydrogen phosphate pH 7.2 methanol.

Selection of extraction technique

Due to the high to intermediate polarity of analytes, solid phase extraction was thought to be a good sample clean up technique. By using OASIS HLB i.e. hydrophilic lipophilic balance cartridges, which has the capability to retain analytes ranging from low polarity to high polarity was tried. The recovery of PGN was found good, but interference was observed at the retention time of ATV. Further by keeping in mind the high cost associated with solid phase extraction, the optimization efforts were abandoned. The conventional Liquid-Liquid Extraction (LLE) technique was given a priority for sample clean-up step. But the problem faced with LLE was, again the wide variation in polarity values for analytes in question, which entailed the approach of using cocktail of two different extractions solvent to manipulate the final polarity of resulting extraction solvent. TBME was used to decrease the polarity, while DCM was used for otherwise purpose. Different ratios of TBME and DCM were tried to get good, consistent recovery, and further to avoid any interference on retention time of analytes. The combination of TBME : DCM (80:20% v/v) was finalized as an extraction solvent (Table 1).

Table 1: Effect of extraction solvent and buffer on recovery of atovaquone, cycloguanil and proguanil in human plasma.

Extraction Solvent	Buffer	Recovery		
		Atovaquone	Cycloguanil	Proguanil
Combination of TBME:DCM (50:50%)	KH_2PO_4 (0.5M)	52%	15%	65%
	NaOH (0.5M)	55%	40%	70%
	KOH (0.5M)	30%	20%	68%
	Na_2CO_3 (0.5 M)	50%	25%	65%
	$NaHCO_3$ (0.5M)	55%	20%	65%
	NaOH(1M)	50%	42%	80%
	NaOH(5M)55%	65%	83%	94%
Combination of TBME:DCM				
60.40	NaOH(5M	65%	75%	88%
70:30	NaOH(5M	72%	78%	90%
80:20	NaOH(5M)	83%	78%	90%

The Pka values for ATV, CYG and PGN are 8.23, 10.12 and 9.24 respectively. Since all three Pka values are lying towards the basic side, the basic buffers were chosen to optimize the recovery of analytes. Potassium dihydrogen orthophosphate (KH_2PO_4), sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) with 7.00 µL of 0.5 M concentration were tried initially by keeping TBME:DCM ratio 50:50% v/v. Amongst all buffers, NaOH was further chosen due to satisfactory recovery of analyte i.e 55% ATV, 40% CYG and 70% PGN. In order to increase the recovery, buffer strength was increased from 0.5 to 1.0 M initially and to 5.0 M eventually. Due to an interesting finding of increased in recovery of all three analytes after increasing the buffer strength, 5.0 M buffer strength with highest recovery at TBME:DCM 50:50% v/v was taken up for further development. To increase the recovery further, changes in the proportion of TBME and DCM were tried. Increase in the proportion of TBME was followed by increase in the recovery of ATV and PGN, though the recovery of CYG was fallen. The recovery of CYG was found 83% and 78% at 50% and 80% of TBME proportion respectively. It is evident that fall in the recovery of CYG is not significant in light of the significant increase in the recovery of other two analytes at 80:20% v/v proportion and hence the TBME:DCM (80:20% v/v) proportion was finalized for extraction.

Method validation

Selectivity

The developed method was found selective as no significant interference observed at respective retention

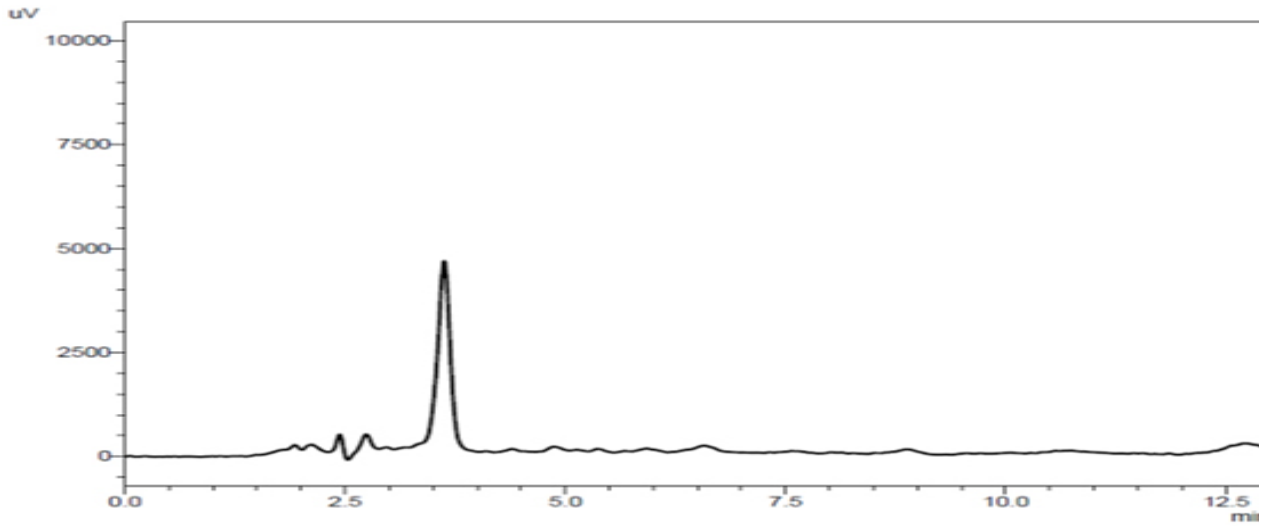

Figure 2: Representative chromatogram of blank human plasma

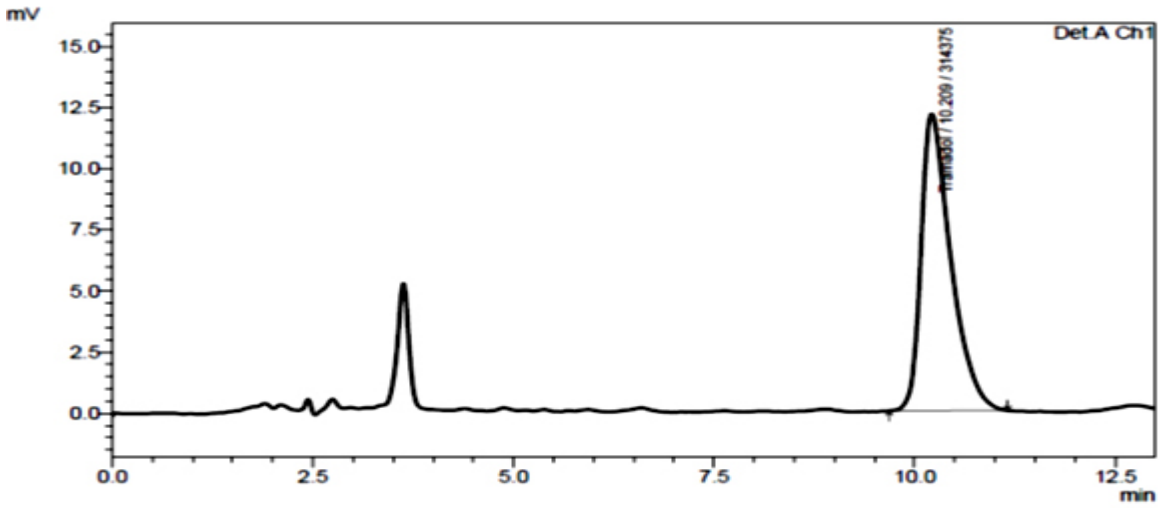

Figure 3: Representative chromatogram of blank human plasma spiked with internal standard

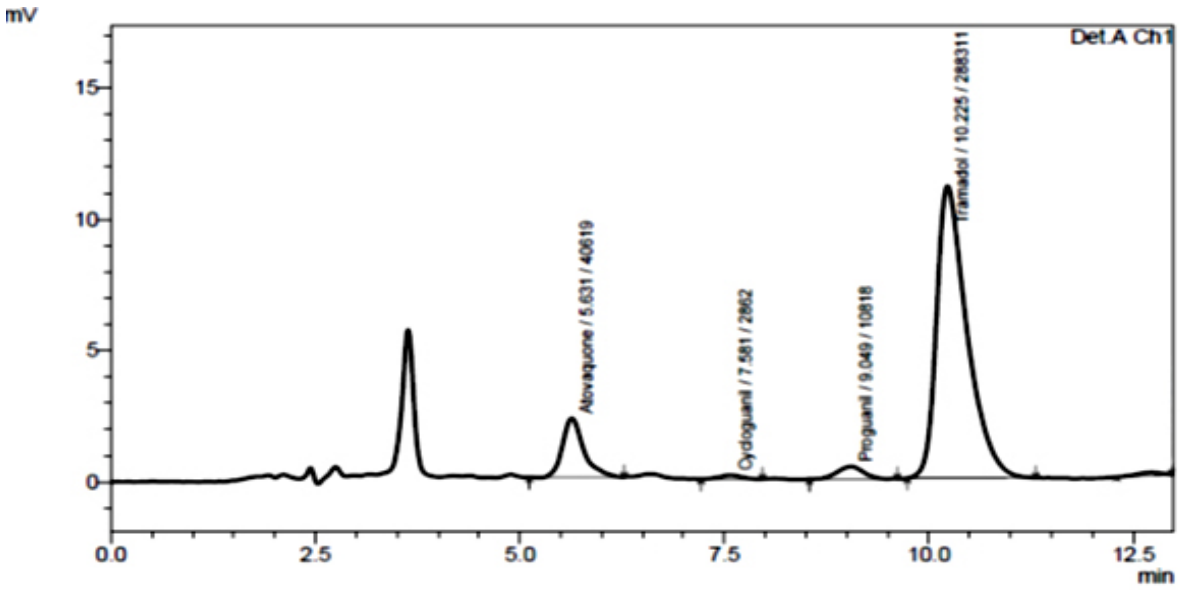

Figure 4: Representative chromatogram of blank human plasma spiked at LLOQC level of atovaquone, Proguanil, Cycloguanil and internal standard

time of ATV, CYG and PGN from the plasma. The representative chromatograms of extracted blank human plasma and plasma spiked with ISTD are shown in figure 2 and figure 3 respectively.

Linearity

Calibration curves were found linear for ATV over 93.75-6000 ng/mL, CYG over 3.5-224 ng/mL and PGN over 7.03-450 ng/mL. The r^2 between 0.9854 – 0.9916 for ATV, was 0.9864-0.9943 for CYG and 0.9841-0.9964. for PGN All the calibration levels nominal concentration and %CV was within the limit.

Detection and quantification limit

In alignment with the criteria for clinical and preclinical bioequivalence studies, which demand LLOQ of bioassay to be at least 10 % of the Cmax or five $t_{1/2}$ of the drug under consideration, whichever is smallest, the LLOQs of present method were found out to be 93.75, 3.50 and 7.03 ng/mL for the ATV, CYG and PGN respectively (Figure 4). The LOD considering the signal to noise ratio of 3:1, was estimated to be 5.0, 2.0 and 5.0. ng/mL for the ATV, CYG and PGN respectively.

Assay precision and accuracy

To determine intra-day accuracy and precision, six replicate analyses were carried out at each of the four quality control levels. The inter-day accuracy and precision were assessed by the analysis of six samples (two samples per day at each level of four QC samples), back calculated from freshly spiked calibration curves for the

Table 2a: Intra-day Accuracy and Precision data for atovaquone, cycloguanil and proguanil in human plasma (n=6)

QC Sample	Concentration (ng/ml)	Mean measured concentration (ng/ml) ± SD	Accuracy (%)	Precision (% CV)
ATV				
LLOQC	93.75	89.94 ± 4.22	95.93	4.69
LQC	281.25	260.36 ± 14.74	92.57	5.66
MQC	3000	2960.17 ± 123.04	98.67	4.16
HQC	5400	5299.61 ± 110.24	98.14	2.08
CYG				
LLOQC	3.50	3.41+0.35	97.33	10.40
LQC	10.50	10.57±0.74	100.69	7.03
MQC	112.00	109.42 ± 9.48	97.69	8.66
HQC	201.60	189.51 ± 13.70	94.00	7.23
PGN				
LLOQC	7.03	6.85 ± 0.39	97.46	5.75
LQC	21.09	21.08 ± 0.88	99.94	4.18
MQC	225.00	213.61 ± 11.75	94.94	5.50
HQC	405.00	396.00 ± 15.47	97.78	3.91

Table 2b: Inter-day Accuracy and Precision data for atovaquone, cycloguanil and proguanil in human plasma (n=6)

QC Sample	Concentration (ng/ml)	Mean measured concentration (ng/ml) ± SD	Accuracy (%)	Precision (% CV)
ATV				
LLOQC	93.75	94.71 ± 6.86	101.03	7.24
LQC	281.25	277.72 ± 20.90	98.75	7.52
MQC	3000	2914.17 ± 193.81	97.14	6.65
HQC	5400	5386.43 ± 359.16	99.75	4.57
CYG				
LLOQC	3.50	3.55 ± 0.28	101.30	7.83
LQC	10.50	10.12 ± 0.67	96.33	6.59
MQC	112.00	111.81 ± 7.02	99.83	6.28
HQC	201.60	198.62 ± 15.66	98.52	7.89
PGN				
LLOQC	7.03	6.93 ± 0.40	98.62	5.83
LQC	21.09	21.29 ± 1.03	100.92	4.84
MQC	225.00	217.18 ± 12.87	96.52	5.93
HQC	405.00	396.62 ± 21.22	97.93	5.35

analyte on three different days. The intra-day and inter-day accuracy and precision values of the assay method are shown in (Table 2a and 2b) respectively. The precision of the method was calculated as coefficient of variation (%CV) of the concentrations determined in all replicates. The intra-day %CV values were below 3.63%, 10.40% and 5.75% for ATV, CYG and PGN respectively. All inter-day %CV were below 7.89%. The accuracies were determined by comparing the mean calculated concentration with the spiked target concentration of the quality control samples. The inter-day accuracies for all three analytes were found to be within 96.33% to 101.30% of the target values. Whilst intra-day accuracies for all three analytes were found to be within 92.57% to 100.69%.

Recovery

Mean recoveries across the QC levels were found 88.15, 87.20 and 97.52% for ATV, CYG and PGN respec-

Table 3. Mean percentage recovery for atovaquone, cycloguanil and proguanil in human plasma (n=6)				
Sample/Level	**ATV**	**CYG**	**PGN**	**ISTD[a]**
LQC	91.98 ± 1.42	90.75 ± 2.05	96.74 ± 0.49	85.4 ± 2.07
MQC	85.44 ± 1.73	87.62 ± 2.71	99.12 ± 0.41	
HQC	87.08 ± 2.30	83.21 ± 2.33	96.70 ± 0.91	
Mean Recovery	88.17 ± 3.40	87.19 ± 3.79	97.52 ± 1.39	
a (n=18)				

tively. The mean recovery of ATV, CYG and PGN were found to be 88.17 ± 3.40, 87.19 ± 3.79 and 97.52 ± 1.39% respectively in human plasma whereas the ISTD recovery was 85.4 ± 2.07%. Recoveries for all three analytes along with ISTD were found consistent (Table 3).

Stability studies

The stock solutions of ATV, CYG, PGN and ISTD were found stable at room temperature (25°C) for 6 h with mean percentage change for each analyte less than 10%. There was no significant change observed in concentrations of ATV, CYG and PGN in matrix, when tested for bench top stability for 10 h, freeze thaw stability over 3 cycles and long term stability for 90 days. The mean percentage change for each analyte at LQC and HQC level for said stability conditions was found to be less than ±10% shown in (Table 4a, 4b and 4c) for ATV, CYG and PGN in human plasma respectively.

CONCLUSION

The objective of present study was to develop a HPLC method for the simultaneous estimation of Atovaquone, Proguanil and its active metabolite Cycloguanil in human plasma. In the present study, method was optimized in terms of chromatographic parameter and extraction of drug from the biological matrix. The developed method was validated as per US FDA guideline to prove its efficiency. The proposed method was found to be highly accurate and precise with good and consistent recovery of all analytes. The method was validated by evaluating linearity, accuracy, precision, limit of quantification and stability and found to be acceptable in terms of its predetermined acceptance criteria according to the US FDA guideline.

The method has shown acceptable precision, accuracy and adequate sensitivity and thus can be of use in the clinical studies.

Table 4a: Stability study results for atovaquone in human plasma						
Stability	**Spiked conc.**	**Calculated conc (comparison sample)**		**Calculated conc (Stability sample)**		**Mean % Change**
		Mean ± SD	**%CV**	**Mean ± SD**	**%CV**	
Bench top	281.25	301.58 ± 5.73	1.90	297.31 ± 11.58	3.90	-1.42
	5400.00	5522.57 ± 87.42	1.58	5289.41 ± 307.85	5.28	-4.22
Freeze and Thaw	281.25	302.61 ± 4.56	1.51	288.07 ± 7.47	2.59	-4.80
	5400.00	5518.07 ± 88.63	1.61	5201.39 ± 247.15	4.75	-5.74
Long term	281.25	281.97 ± 7.63	2.71	266.76 ± 9.79	3.67	-5.39
	5400.00	5577.06 ± 42.32	0.76	5457.66 ± 52.71	0.97	-2.14

Table 4b: Stability study results for cycloguanil in human plasma						
Stability	**Spiked conc.**	**Calculated conc (comparison sample)**		**Calculated conc (Stability sample)**		**Mean % Change**
		Mean ± SD	**%CV**	**Mean ± SD**	**%CV**	
Bench top	10.50	10.95 ± 0.70	6.41	10.71 ± 0.44	4.16	-2.17
	201.60	207.76 ± 4.71	2.27	199.97 ± 8.68	4.34	-3.75
Freeze and Thaw	10.50	10.96 ± 0.70	6.36	10.24 ± 0.80	7.82	-6.50
	201.60	206.31 ± 3.56	1.72	196.59 ± 9.42	4.79	-4.71
Long term	10.50	10.61 ± 0.57	5.41	10.11 ± 0.41	4.10	-4.71
	201.60	204.49 ± 5.29	2.59	202.69 ± 3.18	1.57	-0.88

Table 4c: Stability study results for Proguanil in human plasma						
Stability	**Spiked conc**	**Calculated conc (comparison sample)**		**Calculated conc (Stability sample)**		**Mean % Change**
		Mean ± SD	**%CV**	**Mean ± SD**	**%CV**	
Bench top	21.09	21.15 ± 0.78	3.70	20.72 ± 1.22	5.89	-2.05
	405.00	416.41 ± 16.70	4.01	404.09 ± 20.15	4.99	-2.96
Freeze and Thaw	21.09	21.12 ± 0.79	3.73	20.29 ± 0.63	3.10	-3.94
	405.00	416.41 ± 16.70	4.01	392.11 ± 17.33	4.42	-5.83
Long term	21.09	21.77 ± 1.06	4.89	19.91 ±0.80	4.03	-8.55
	405.00	416.29 ± 11.47	2.75	398.91 ± 5.35	1.34	-4.17

The developed method has advantage for simultaneous estimation of Atovaquone, Proguanil and its active metabolite Cycloguanil in human plasma which can be used by common laboratories for various purposes like bioavailability and bioequivalence studies, drug-drug interaction studies etc.

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