A SENSITIVE LIQUID CHROMATOGRAPHIC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF LUMEFANTRINE AND ARTEMETHER IN HUMAN PLASMA

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ABSTRACT

This paper presents the development of a new RP-HPLC method for the separation of lumefantrine and artemether in human plasma. Sample preparation involved the extraction using protein precipitation method with acetonitrile. Chromatography was performed with mobile phase containing a mixture of methanol and 0.025M ammonium acetate (38:52, v/v) with a flow rate of 1 ml min⁻¹. Column effluent was monitored at 216 nm using a UV detector. The calibration graphs were linear in the range of 2.4-16.8 µg ml⁻¹ for lumefantrine and 0.4-2.8 µg ml⁻¹ for artemether with limits of quantification of 3.0 µg ml⁻¹ and 0.6 µg ml⁻¹ for lumefantrine and artemether respectively. Intra and inter-assay precision provided relative standard deviation lower than 10% for the analytes. Extraction recoveries of lumefantrine and artemether in plasma were 94.32-97.15% and 94.75-98.55% respectively. The proposed HPLC with UV detection is simple, rapid, precise and accurate. Therefore it is appropriate for the routine quantification of therapeutic levels of lumefantrine and artemether in human plasma.

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INTRODUCTION

Malaria, one of the most serious, complex and refractory health problem being faced by humans, is a leading cause of morbidity and mortality in the present scenario. It affects the largest number of people (between 300 to 500 million annually) in the world, with more than 2 million deaths worldwide. Artemisinin – based combination therapy (ACT) has been promoted as a means to reduce malaria transmission due to their ability to kill both asexual blood stages of malaria parasites which sustain infections over long periods and the immature sexual stages responsible for infecting mosquitoes and onward transmission. Artemisinins are primarily active against mature ring stage of P. falciparum, when the parasites are metabolically active. They are also active against gametocytes, which are transmitted from humans to mosquitoes. The long duration of treatment with artemisinins can be reduced to three days when given in combination with slowly eliminated antimalarials [1-2].

Artemether -lumefantrine is the only fixed-dose artemisinin containing formulation after WHO recognized guidelines. Artemether and lumefantrine have different modes of action and act at different points in parasitic life cycle. Lumefantrine prevents detoxification of haem, so that toxic haem and free radicals induce parasite death. Artemether on the other hand, interferes with parasite transport protein, disrupts parasite mitochondrial function, inhibits angiogenesis and modulates host immune function. This combination is currently available as fixed dose formulation containing 20 mg artemether and 120 mg lumefantrine [3].

Lumefantrine (LUM), chemically 2, 7-Dichloro-9-[(4-chlorophenyl)methylene]-a-(dibutylamino) methyl]-9H-fluorene-4-methanol, inhibits the formation of β-hematin by forming a complex with hemin and inhibits nucleic acid and protein synthesis (Fig. 1a). Artemether (ART), chemically (3R, 5aS, 6R, 8aS, 9R, 10S, 12R, 12aR)-Decahydro-10-methoxy-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyran-4-(3-j)-1, 2 benzoxodioxepin, is a potent blood schizonticide agent against P. falciparum (Fig. 1b). Detailed survey of analytical literature of LUM revealed different LC methods in plasma [4-10]. Similarly, a survey of analytical literature for ART for plasma and dosage forms revealed methods like GC-MS [11], LC with UV detection [12-14], LC with ECD electro chemical detection [15-17] and HPTLC [18-20]. Two LC-MS/MS and one each of LC and HPTLC methods have been reported for simultaneous determination of LUM and ART in formulation and plasma respectively [21-24]. However there is no method regarding the simultaneous quantification of LUM and ART by HPLC-UV in plasma.

Figure 1a: Chemical structure of lumefantrine. Figure 1b: Chemical structure of artemether.

Chromatographic separation and simultaneous quantification of LUM and ART is challenging, because of poor molar absorptivity of ART in comparison with LUM. In addition, the lower dose proportion of ART with LUM, does not allow unified dilution. All the challenges were considered in the present study and a validated LC-method was developed for simultaneous determination of LUM and ART in human plasma. The mobile phase was optimized for simple isocratic elution system with economic combination.

MATERIALS AND METHODS

Instrumentation

HPLC analysis was performed on a LC-20AT (Shimadzu, Japan) liquid chromatograph equipped with an SPDM-20A UV-detector (Shimadzu, Japan). The separation was performed on a Hypersil BDS C18 column (250 × 4.6 mm i.d, 5µ m particle). A UV-Visible spectrophotometer model-1700 (Shimadzu, Japan) was used to determine UV-spectra of both compounds.

Chromatographic conditions

An isocratic mobile phase containing methanol and 0.025M ammonium acetate (38:52, v/v pH adjusted to 3.8 using 0.2% glacial acetic acid) was used at a flow rate of 1.0 ml min\(^{-1}\). The injection volume was 20 µl and the effluents were monitored a wavelength of 216 nm with a run time of 2.12 ± 0.03 and 4.10 ± 0.02 min for ART and LUM respectively (Fig. 2).
Calibration standards and quality control samples

Stock solution of LUM and ART were prepared by dissolving 10 mg each compound in 100 ml methanol (100 µg ml⁻¹). The working solutions were prepared by appropriate dilution in methanol just before use. Volumes of 20 µl of the prepared working solutions were added to 960 µl of drug free human plasma to obtain drug concentration levels of 2.4-16.8 µg ml⁻¹ LUM and 0.4-2.8 µg ml⁻¹ of ART respectively.

The quality control samples used in validation study were prepared in the same way as the calibration standard, by spiking drug-free human plasma with appropriate volume of working solutions to obtain three different concentrations, near the low, middle and high values of calibration curve (2.4, 9.6, 16.8 µg ml⁻¹ LUM and 0.4, 1.6, 2.8 µg ml⁻¹ of ART). The samples were stored in a freezer at -20 °C until analysis. A calibration curve was constructed from a blank sample and seven non-zero samples covering the range including LOQ. Calibration curves were generated using the peak area by least squares linear-regression.

Sample preparation

The stored plasma samples were thawed at room temperature before processing. In a 15 ml screw capped polypropylene centrifuge tubes, vortexed for 30 sec and 1.5 ml of acetonitrile was added. The mixture was further vortexed for 1 min, and then centrifuged for 5 min at 10,000 × g, supernatant was transferred into clean tubes and evaporated to dryness at 37 °C under a steady stream of nitrogen. The residue was reconstituted with 500 µl mobile phase and 20 µl was injected into the column (Fig. 3).

Method validation

Each bioanalytical technique has its own characteristics, which will vary from analyte to analyte. So specific validation criteria has to be developed for successful method development. The proposed method was validated for selectivity, precision, accuracy, linearity, limit of detection, limit of quantification and recovery according to international conference on harmonization and food and drug administration (FDA) guidelines [25-26].

Specificity

Randomly selected six blank human plasma samples, which were collected under controlled conditions, were extracted through protein precipitation procedure and chromatographed individually to determine whether endogenous constituents’ co eluted with the analyte of interest. The specificity was studied from peak resolution factor.
Calibration curve

The calibration curves were constructed from blank plasma and seven concentrations of the drugs including LLOQ. The peak area of the drug against the respective standard concentrations was used for plotting the graph and the linearity was evaluated by least squares regression analysis.

Recovery

The analytical recovery was determined by comparing chromatographic peak areas of standard samples prepared from post-extracted blank plasma and standard spiked samples at three different concentrations of LUM and ART.

Accuracy and precision

To study the inter-day precision and accuracy, the quality control samples were evaluated for three consecutive days, while intra-day precision and accuracy were evaluated through analysis of validation control samples at three different concentrations (2.4, 9.6, 16.8 µg ml⁻¹ of LUM and 0.4, 1.6, 2.8 µg ml⁻¹ of ART) in six replicates in the same day. Inter- and intra-day precision were expressed as relative standard deviation (RSD). The accuracy was expressed as relative error (RE) for the determination studied drugs in human plasma sample. The precision was based on the deviation of each concentration level and should be within ± 15%. The accuracy was based on the mean value which should not deviate by ± 15% of the nominal concentration, except LLOQ, where it should not deviate by ± 20% of the nominal concentration.

Lower Limit of quantification and detection

The lower limit of quantification (LLOQ) was taken as the lowest limit of concentration that can be accurately (80-120% for biological samples) and precisely determined (RSD <20 % for biological samples). The limit of detection (LOD) was the concentration with a signal-to-noise ratio of 3.

Stability

The freeze-thaw stability was determined at low, medium and high quality control samples, over three freeze-thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 hours and refrozen for 24 hours. After completion of each cycle, the samples were analyzed and the results were compared with that of zero cycle. Short-term stability study was conducted with three aliquots each of low, medium and high QC samples and kept at room temperature for 24 hours. After 24 hours, samples were analyzed and results are compared with that of zero cycles. For long-term stability also three aliquots each of low, medium and high QC samples were frozen at -4 °C for 30 days.

RESULTS AND DISCUSSION

For instance, at the best of our knowledge, no method has been reported for the simultaneous analysis of LUM and ART in human plasma. So a successful attempt has been made to develop a simple and precise HPLC-UV method for the determination of LUM and ART in human plasma. Poor molar absorptivity of ART presented a significant challenge in simultaneous determination with LUM. In preliminary studies, the wavelength was optimized at 216 nm for maximum sensitivity for ART determination, as detection at other wavelengths showed poor quantification limits. To obtain best chromatographic conditions different columns and mobile phases with different pH in combination with different organic modifiers were tested. The influence of both organic modifier concentrations and pH was carefully studied. Organic modifier concentration improves peak shape and decreases retention time. Variation of pH played an imported role in the separation process. At pH 3.8 good resolution was obtained with better peak shape for both compounds. So the same was chosen as the optimum pH for resolution of drugs from endogenous biological matrixes in a reasonable run time of below 10 min. Based on above findings, the best chromatographic separation was achieved on a Hypersil BDS C₁₈ (250 × 4.6 mm × 5µ) column with a mobile phase composition of methanol-0.025M ammonium acetate, pH 3.8 adjusted with 0.2% acetic acid (38:52, v/v) at a flow rate of 1 ml min⁻¹ and UV detection at 216 nm. The selected chromatographic conditions provided optimum resolution of LUM and ART.

Table 1: Data for recovery studies.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal concentration (µg ml⁻¹)</th>
<th>Recovery (%) ± SD</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUM</td>
<td>2.4</td>
<td>94.32 ± 3.21</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>97.15 ± 5.10</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>16.8</td>
<td>96.46 ± 3.06</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>98.55 ± 5.03</td>
<td>2.05</td>
</tr>
<tr>
<td>ART</td>
<td>1.6</td>
<td>94.75 ± 2.07</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>97.61 ± 2.92</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Before the preparation of pooled calibration and QC samples, six lots of blank plasma were screened for interferences. Each sample of blank plasma was eluted three times individually and they found be free from co eluting peaks at the retention time range of LUM and ART.
The linearity of the method was studied by seven-point standard curves. The regression parameters were calculated by least squares linear-regression (Table 1). Good linearity was obtained over the concentration range of 2.4-16.8 µg ml\(^{-1}\) for LUM and 0.4-2.8 µg ml\(^{-1}\) for ART respectively. The linear regression equation of the calibration curves produced are: \(y = 852.5x + 13159\) for LUM and \(y = 2537x + 1908\) for ART. LLOQ, LOD and other parameters for the assay were listed in Table 1.

The absolute recovery of the studied drugs were determined by comparing the peak area of QC sample spiked in human plasma and defined in three runs with those of post extracted plasma blanks fortified with the known amount of analytes. Table 2 indicates high ability of the proposed method to recover LUM and ART from human plasma.

The inter- and intra-day precisions were measured as the relative standard deviation (RSD) expressed as percentage over the concentration range of LUM and ART during the course of validation. Table 3 summarizes precision studies, which indicates an acceptable precision for all concentrations assayed for both intra- and inter-day sample. The % RSD of intra- and inter-day precisions were found to be < 4 and < 5 for LUM and ART respectively. The low values of % RSD reflect the precision of the method. The accuracy of the method was determined by calculating relative error (RE) and summarized in Table 3, indicates good accuracy of the proposed method.

### Table 2: Regression characteristics of LUM and ART by the proposed HPLC method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LUM</th>
<th>ART</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg ml(^{-1}))</td>
<td>2.4-16.8</td>
<td>0.4-2.8</td>
</tr>
<tr>
<td>Detection limit (µg ml(^{-1}))</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Quantification limit (µg ml(^{-1}))</td>
<td>3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Standard deviation of slope</td>
<td>0.002</td>
<td>0.008</td>
</tr>
<tr>
<td>Relative standard deviation of slope</td>
<td>1.85</td>
<td>1.60</td>
</tr>
<tr>
<td>Standard deviation of intercept</td>
<td>0.032</td>
<td>0.027</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9991</td>
<td>0.9980</td>
</tr>
<tr>
<td>Standard error of regression</td>
<td>0.051</td>
<td>0.042</td>
</tr>
</tbody>
</table>

The stability of the drug spiked human plasma samples were studied at three levels of freeze-thaw cycles. The results indicate the stability of the studied drugs in freeze-thaw cycles. Also stability of the drugs in human plasma was assessed by analyzing six replicate samples at the low, medium and high concentration levels at ambient temperature over 24 hour and \(-4^\circ\)C for 30 days for a short term and long term stability analysis. The results in Table 4 indicate the high stability of drugs under short and long term stability studies.

### Table 3: Data for precision studies (n = 6).

<table>
<thead>
<tr>
<th>Precision</th>
<th>Analyte</th>
<th>Nominal concentration (µg ml(^{-1}))</th>
<th>Recovery (µg ml(^{-1}) ± SD)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td>LUM</td>
<td>2.4</td>
<td>2.31 ± 0.08</td>
<td>3.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.6</td>
<td>9.12 ± 0.21</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.8</td>
<td>15.80 ± 0.54</td>
<td>3.42</td>
</tr>
<tr>
<td></td>
<td>ART</td>
<td>0.4</td>
<td>0.38 ± 0.01</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6</td>
<td>1.57 ± 0.06</td>
<td>3.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8</td>
<td>2.65 ± 0.07</td>
<td>2.64</td>
</tr>
<tr>
<td>Inter-day</td>
<td>LUM</td>
<td>2.4</td>
<td>2.30 ± 0.06</td>
<td>2.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.6</td>
<td>9.15 ± 0.22</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.8</td>
<td>15.76 ± 0.36</td>
<td>2.28</td>
</tr>
<tr>
<td></td>
<td>ART</td>
<td>0.4</td>
<td>0.39 ± 0.014</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6</td>
<td>1.61 ± 0.08</td>
<td>4.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8</td>
<td>2.60 ± 0.09</td>
<td>3.46</td>
</tr>
</tbody>
</table>
**Table 4: Stability studies of lumefantrine and artemether (n=3).**

<table>
<thead>
<tr>
<th>Stability study</th>
<th>Lumefantrine concentration (µg ml⁻¹)</th>
<th>Artemether concentration (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>%RSD</td>
</tr>
<tr>
<td>Freeze and thaw</td>
<td>2.4</td>
<td>2.32</td>
</tr>
<tr>
<td>Short-term</td>
<td>2.4</td>
<td>2.54</td>
</tr>
<tr>
<td>Long-term</td>
<td>2.30</td>
<td>2.07</td>
</tr>
</tbody>
</table>

**CONCLUSION**

The HPLC method developed is rapid, sensitive and specific. It can be employed for the estimation of LUM and ARTM from spiked samples of plasma with ease of sensitivity and repeatability in the analysis. Hence the method could be used for estimation of these drugs in human plasma, and further extended for bioavailability and pharmacokinetic studies.

**REFERENCES**