SIMULTANEOUS HPLC AND HPTLC ASSAY METHOD FOR ANTIMALARIAL AGENTS CHLOROQUINE, PRIMAQUINE AND BULAQUINE
Introduction

The relapse of malaria is a major danger in the regions, which are prone to malaria, or regions where it is endemic. A combination kit [1,2] for anti-relapse treatment of *P. vivax* malaria, consisting of chloroquine tablets and bulaquine capsules has been recently developed, and marketed in India under the trade name Aablaquine.

There are various works reported on antimalarials like detection and determination of antimalarial drugs by Bergqvist and Churchill [3] and whereas others have developed sensitive and accurate methods of estimation by HPLC [4]. Dwight et.al. [5] and Betschart et.al. [6] have developed field-adapted method for HPTLC detection and estimation of chloroquine and its metabolite. Rimoy et.al. checked bioavailability of oral sugar coated and plain formulation of chloroquine phosphate in the market [7]; Paci et.al. have done simultaneous determination of chloroquine and proguanil [8]; Some other HPLC methods are reported for *in vitro* or *in vivo* analysis of chloroquine by Ramana Rao et.al.[9], Yngve Bergqvistfrederick et.al.[10], Elfatih et.al.[11], Pirkko Volin et.al.[12], Julie Ducharme et.al.[13].

Many have developed methods of estimation of primaquine [14,15]. Workers like Dua et.al. have developed a HPLC method for separation and identification of oxidation products of primaquine [16] as well as determination of primaquine and carboxyprimaquine in plasma and blood cells [17]; Kristensen et.al. have checked the influence of molecular oxygen and oxygen radicals on the photosensitivity of primaquine [18]; Li et.al. have determined primaquine and hepatic targeting neoglycoalbumin-primaquine in blood and liver of mouse [19]; Borissova et.al. have
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prepared microspheres of primaquine and checked its degradation in rat liver [20,21]; Dean et.al. have used electrochemical detection for primaquine [22]; Ittarat et.al. have tried to link suppression of enzymatic activity with drug dosage [23,24]; Endoh et.al. have determined pamaquine, primaquine and carboxyprimaquine in calf plasma by EC detection [25]. Others have developed HPLC methods for primaquine using UV detection [26], Electrochemical assay in plasma and urine [27], excretion distribution and metabolism in rats [28], and its determination using reversed phase chromatography in biological fluids [29]. Whereas YC Ni et.al. have developed HPTLC and HPLC methods for analysis of major metabolites of primaquine[30]. A combine method for primaquine and chloroquine has also been developed by Das Gupta et.al.[31].

Dwivedi et.al. have developed a method for simultaneous estimation of Primaquine and Bulaquine by TLC densitometry and UV spectrophotometry[32]. Whereas method of estimation of Bulaquine in serum [33] and Bulaquine along with its primary metabolite Primaquine has been given by Paliwal et.al.[34], but no method for the simultaneous estimation for all the three drugs has yet been reported. Therefore, present study was undertaken to develop a simple, sensitive and reproducible HPLC as well as HPTLC assay method for chloroquine, primaquine and bulaquine.

This will be particularly useful as several combination kits are now coming up in the market for anti-relapse of malaria. We recognised the need for a combined method of estimation for the three drugs for combined multiple drug therapy. In the following section we discuss the procedure followed for developing the method for estimation of three drugs by HPLC and HPTLC.
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Experimental

Reagents and standards

Standard samples of bulaquine and primaquine phosphate were supplied by Chemical Technology Division, and chloroquine diphosphate was obtained from Parasitology Division of this Institute. HPLC grade acetonitrile, and methanol were obtained from Merck. All other solvents and reagents used were of analytical grade. Triply distilled water was obtained from an all glass apparatus. The TLC plates used were pre-washed with methanol.

Apparatus and Chromatographic conditions

The HPLC system was equipped with 250 binary gradient pump (Perkin-Elmer), a Rheodyne model 7125 injector with a 20 μl loop and LC-235 diode array detector. HPLC separation was achieved on a RP select-B C₈ endcapped Lichrospher (Merck) analytical column (250mmX 4mm internal diameter, 5μm particle size). Column effluent was monitored at 265 nm. Data was acquired and processed using an IndTech HPLC interface and software. The mobile phase was 0.01M sodium acetate buffer pH 5.6 and acetonitrile (45:55). The flow rate was maintained at 1ml/minute. Both the solutions were filtered and degassed before use. Chromatography was performed at 27 ± 3°C.

The HPTLC workstation comprised of Automatic TLC Sampler-III (CAMAG) equipped with 50μl hamilton syringe, TLC Scanner-3 (CAMAG) equipped with Mercury, Tungsten and Deuterium lamp for scanning of TLC plate and twin trough glass chamber with lid for the development of plate. The separation of three drugs
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was achieved on thin layer plates of precoated Si-gel 60 F$_{254}$ (20x20 cm, 0.2 mm from Merck). The Solvent system used for developing the plate was hexane: diethyl ether: methanol: diethylamine (37.5: 37.5: 25: 0.5). The plates were scanned at wavelength of 254 nm. Data was acquired and processed using a CATS4 - HPTLC software. TLC plate was developed at 27±3°C.

Preparation of stock and working standard solutions

Stock standard solutions of primaquine and chloroquine were prepared by weighing 25 mg of each of the drug, mixing in both 50 mg of potassium carbonate, then dissolving in 1 ml of water followed by finally making the volume to 25 ml with methanol. The stock standard solution of bulaquine was prepared by dissolving 25 mg of the drug in methanol containing 0.5% dimethyl octyl amine. Working standard solutions were prepared in methanol by serial dilution in the range of 1-20 µg/ml. Stock sample solutions of primaquine, bulaquine and chloroquine were prepared by weighing the samples equivalent to 10 mg of each of the drug and following the same procedure as mentioned above and finally making the volume to 10 ml with methanol.

Accuracy and precision

The accuracy of the method was calculated on the basis of the difference in the mean calculated and added concentrations and the precision was obtained by calculating the intra and inter-day relative standard deviations (R.S.D.s)[35,36].

Known amounts of primaquine/ bulaquine/ chloroquine were spotted on the silica plate to give mixture of the samples at one spot and the quantity of the respective drug was determined by interpolation on the corresponding calibration graphs. The accuracy of the method was calculated on the basis of the difference in
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the mean calculated and added concentrations and the precision was obtained by calculating the inter-plate relative standard deviations (R.S.D.s)[35-38].

Analysis of drug samples

The samples of Chloroquine, Primaquine and Bulaquine formulations were obtained from the retail pharmacist. The samples were weighed and crushed evenly to give powder. The powder equivalent to 5 mg of the drug was dissolved in 9 ml methanol containing 10 mg of potassium carbonate in 1 ml of water and 0.5 ml of dimethyloctylamine and filtered to remove any insoluble matter. The drug solution in methanol was then injected into the HPLC and as well spotted on TLC plate and analysed for the drug content.
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Results and Discussion

Chromatography

Initial experiments with columns like C18 endcapped (250 mm, 4mm, 5μm, E. Merck) or CN (250 mm, 4mm, 5μm, E. Merck) using several solvent systems, including buffers were tried, but proper resolution was not achieved. A RP select-B C8 lichrospher (Merck) column was used to separate the three drugs from each other and all other ingredients. The HPLC method described herein provides a good separation of primaquine, bulaquine and chloroquine [Figure 4]. Proper separation of primaquine, bulaquine and chloroquine was obtained by this method. Along with the peak of bulaquine initial hump is observed which is due to the tautomer of bulaquine. Under the chromatographic conditions, other constituent did not interfere since they eluted either before or after the peak of interest. The photodiode array detector gave a peak purity index, which indicated a pure peak without any interference from other substances.

In HPTLC a Silica gel 60 F254 (Merck) TLC plate was used to separate primaquine, bulaquine and chloroquine from all other ingredients. Initial experiments with different solvent systems like (MeOH: CHCl3), (CHCl3: MeOH: NH3), (ethyl acetate: methanol) etc. were tried. No satisfactory resolution was obtained. The best resolution was obtained by developing the plate in solvent system of hexane: diethyl ether: methanol: diethylamine (37.5: 37.5: 25: 0.5).

The HPTLC method described herein provides a good separation of primaquine, bulaquine and chloroquine [Figure 5]. Proper separation of primaquine, bulaquine and chloroquine was obtained by this method. One additional peak between
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chloroquine and primaquine was observed, which was due to the dimethyloctylamine added in the solution of bulaquine. The peak shape of bulaquine is not symmetrical due to the tautomer of bulaquine under the chromatographic conditions, other constituent did not interfere since their Rf has no interference with the spots of interest.

Selectivity and specificity

In HPLC assay the retention time, of chloroquine, primaquine and bulaquine were 4.22, 5.72 and 17.26 minutes respectively. In HPTLC the retention factor of bulaquine, primaquine and chloroquine were 0.88, 0.28 and 0.39 respectively. No interference was detected with the spots of bulaquine, primaquine and chloroquine.

Linearity and reproducibility

External standardization by peak area was used for the quantitative determination of bulaquine, primaquine and chloroquine in both the methods.

In HPLC assay, based on the noise to signal ratio of 3, the detection limit of bulaquine, chloroquine, and primaquine were 0.4, 1.6, and 0.5 µg/ml respectively. However the lower limits of quantitation were set at 2, 4.1, and 1.0 µg/ml respectively. The calibration curves were linear in the range of 1.9 µg/ml to 15 µg/ml for bulaquine (r=0.9988) [Figure-6], 4 µg/ml to 33 µg/ml for chloroquine (r=0.9995) [Figure-7], and 1 µg/ml to 21 µg/ml for primaquine (r=0.9998) [Figure-8].
A typical standard curve could be described by the equation:

\[
\text{Unknown conc. (\(\mu\text{g/ml}\))} = A + (B \times \text{peak area})
\]

where \(A\) & \(B\) are linear regression constants taking \(x = \text{peak area}\), \(y = \text{concentration}\) for the different drugs with values (by HPLC):

For Bulaquine: \(A = 0.01466, B = 1.8192\times10^{-7}\)

For Chloroquine: \(A = 1.139; B = 4.333\times10^{-7}\)

For Primaquine: \(A = 0.04157; B = 2.2764\times10^{-7}\)
Figure 5: HPTLC Separation of Bulaquine (4), Chloroquine (3) and Primaquine (1).
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While in HPTLC, based on the noise to signal ratio of 3, the detection limit of bulaquine, chloroquine, and primaquine were 0.52, 0.59 and 0.53 µg respectively while the lower limits of quantitation were found to be 0.52, 1.21, 1.07 µg respectively. The calibration curves were linear in the range of 0.52 µg to 21 µg for Bulaquine (r=0.99) [Figure-9], 1 µg to 23 µg for chloroquine (r=0.99) [Figure-10], and 1 µg to 21 µg for primaquine (r=0.99) [Figure-11].

A typical standard curve could be described by the equation

**Unknown conc. (µg/ml) = A + (B * peak area)**

Where A & B are linear regression constants for the different drugs (by HPTLC):

- **For Bulaquine**, \(A = -11.40595\); \(B = 0.00075\)
- **For Chloroquine**, \(A = -2.0335\); \(B = 0.00152\)
- **For Primaquine**, \(A = -3.09601\); \(B = 0.00049\)

The methods provided adequate sensitivity for the determination of bulaquine, primaquine and chloroquine in bulk drug substance, in dosage forms and to check primaquine, as an impurity in the bulaquine samples.

The reproducibility and accuracy of the methods were determined by intra and inter assay variation (Table-1-6). Table-1, 2 and 3 give the % Deviation from actual (DFA) and % Coefficient of variation (CV) of Primaquine, Chloroquine and Bulaquine respectively by HPLC. The lower limit of quantitation for each of the drug was set on the basis of linearity of the graph. At the lower limit of detection, the drugs did not show linearity and the %DFA and %CV was very high. The values of reproducibility and accuracy of the method were within the acceptable limits [35-38].
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Figure 6: Calibration graph of Bulaquine.

![Calibration graph of Bulaquine](image)

Figure 7: Calibration graph of Chloroquine

![Calibration graph of Chloroquine](image)
**Simultaneous HPLC and HPTLC assay method**

Figure 8: Calibration graph of Primaquine

![Calibration of Primaquine by HPLC](image)

Y = A + B * X
A = 0.04157
B = 2.2764E-7
R^2 = 0.99938

Figure 9: Calibration graph of Bulaquine by HPTLC

![Calibration of Bulaquine by HPTLC](image)

Y = A + B * X
A = 0.00030596
B = 0.00030722
R^2 = 0.99968
N = 6
Simultaneous HPLC and HPTLC assay method

Figure 10: Calibration graph of Chloroquine by HPTLC

![Calibration graph of Chloroquine by HPTLC]

Figure 11: Calibration graph of Primaquine by HPTLC

![Calibration graph of Primaquine by HPTLC]
The result obtained from the analysis of drug samples are given in Table- 7. The results were consistent with the label claim.

Table 1- Inter and Intra Assay Variations of Primaquine by HPLC

<table>
<thead>
<tr>
<th>Conc. Taken (μg/ml)</th>
<th>INTRA ASSAY VARIATIONS</th>
<th>INTER ASSAY VARIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con.Found* (μg/ml) Mean±SD</td>
<td>%C.V.</td>
</tr>
<tr>
<td>1.04</td>
<td>1.06±0.05</td>
<td>4.717</td>
</tr>
<tr>
<td>2.6</td>
<td>2.68±0.14</td>
<td>5.223</td>
</tr>
<tr>
<td>5.2</td>
<td>5.302±0.23</td>
<td>4.338</td>
</tr>
<tr>
<td>10.4</td>
<td>9.95±0.21</td>
<td>2.110</td>
</tr>
<tr>
<td>15.6</td>
<td>15.62±0.44</td>
<td>2.82</td>
</tr>
<tr>
<td>20.8</td>
<td>20.39±0.87</td>
<td>4.266</td>
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</table>

*n = 3

Table 2- Intra and inter Assay Variations of Chloroquine by HPLC

<table>
<thead>
<tr>
<th>Conc. Taken (μg/ml)</th>
<th>INTRA ASSAY VARIATIONS</th>
<th>INTER ASSAY VARIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con.Found* (μg/ml) Mean±SD</td>
<td>%C.V.</td>
</tr>
<tr>
<td>1.6</td>
<td>1.684±0.112</td>
<td>6.65</td>
</tr>
<tr>
<td>4.14</td>
<td>4.261±0.114</td>
<td>2.675</td>
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<tr>
<td>8.28</td>
<td>8.425±0.23</td>
<td>2.73</td>
</tr>
<tr>
<td>16.56</td>
<td>15.975±0.21</td>
<td>1.314</td>
</tr>
<tr>
<td>24.84</td>
<td>24.62±0.44</td>
<td>1.787</td>
</tr>
<tr>
<td>33.12</td>
<td>33.39±1.22</td>
<td>3.654</td>
</tr>
</tbody>
</table>

*n = 3
### Table 3- Intra and Inter Assay Variations of Bulaquine by HPLC

<table>
<thead>
<tr>
<th>Conc. Taken (μg/ml)</th>
<th>INTRA ASSAY VARIATIONS</th>
<th>INTER ASSAY VARIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con.Found* (μg/ml) Mean±SD</td>
<td>% C.V.</td>
</tr>
<tr>
<td>0.772</td>
<td>0.81±0.025</td>
<td>3.086</td>
</tr>
<tr>
<td>1.93</td>
<td>1.862±0.085</td>
<td>4.564</td>
</tr>
<tr>
<td>3.86</td>
<td>3.97±0.167</td>
<td>4.206</td>
</tr>
<tr>
<td>7.72</td>
<td>8.024±0.216</td>
<td>2.691</td>
</tr>
<tr>
<td>11.58</td>
<td>12.125±0.521</td>
<td>4.295</td>
</tr>
<tr>
<td>15.44</td>
<td>16.024±0.49</td>
<td>3.058</td>
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*n = 3

### Table 4- Inter and Intra Assay Variations of Bulaquine by HPTLC

<table>
<thead>
<tr>
<th>Conc. Taken (μg/ml)</th>
<th>INTRA ASSAY VARIATIONS</th>
<th>INTER ASSAY VARIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con.Found* (μg/ml) Mean±SD</td>
<td>% C.V.</td>
</tr>
<tr>
<td>0.525</td>
<td>0.544±0.024</td>
<td>4.411</td>
</tr>
<tr>
<td>1.05</td>
<td>1.10±0.012</td>
<td>1.091</td>
</tr>
<tr>
<td>2.1</td>
<td>2.202±0.023</td>
<td>1.044</td>
</tr>
<tr>
<td>10.1</td>
<td>10.28±0.25</td>
<td>2.432</td>
</tr>
<tr>
<td>15.75</td>
<td>15.92±0.26</td>
<td>1.633</td>
</tr>
<tr>
<td>21</td>
<td>21.22±0.38</td>
<td>1.791</td>
</tr>
</tbody>
</table>

*n = 3
Simultaneous HPLC and HPTLC assay method

Table 5- Inter and Intra Assay Variations of Chloroquine by HPTLC

<table>
<thead>
<tr>
<th>CONC. TAKEN (µg/ml)</th>
<th>INTRA ASSAY VARIATIONS</th>
<th>INTER ASSAY VARIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con. Found* (µg/ml)</td>
<td>% CV</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td>0.59</td>
<td>0.58±0.013</td>
<td>2.241</td>
</tr>
<tr>
<td>1.18</td>
<td>1.21±0.011</td>
<td>0.91</td>
</tr>
<tr>
<td>2.36</td>
<td>2.278±0.07</td>
<td>3.072</td>
</tr>
<tr>
<td>11.8</td>
<td>11.58±0.45</td>
<td>3.886</td>
</tr>
<tr>
<td>17.7</td>
<td>17.532±0.25</td>
<td>1.426</td>
</tr>
<tr>
<td>23.6</td>
<td>23.416±0.11</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*n = 3

Table 6- Inter and Intra Assay Variations of Primaquine by HPTLC

<table>
<thead>
<tr>
<th>Conc. Taken (µg/ml)</th>
<th>INTRA ASSAY VARIATIONS</th>
<th>INTER ASSAY VARIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con. Found* (µg/ml)</td>
<td>% CV</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td></td>
</tr>
<tr>
<td>0.535</td>
<td>0.553±0.014</td>
<td>2.532</td>
</tr>
<tr>
<td>1.07</td>
<td>1.021±0.014</td>
<td>1.371</td>
</tr>
<tr>
<td>2.14</td>
<td>2.121±0.031</td>
<td>1.461</td>
</tr>
<tr>
<td>10.7</td>
<td>10.53±0.24</td>
<td>2.279</td>
</tr>
<tr>
<td>16.05</td>
<td>16.116±0.24</td>
<td>1.489</td>
</tr>
<tr>
<td>21.4</td>
<td>20.95±0.54</td>
<td>2.577</td>
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</tbody>
</table>

*n = 3

[144]
Table-7 Analysis of the samples of Bulaquine, Chloroquine and Primaquine

<table>
<thead>
<tr>
<th>Sample* name</th>
<th>Amount mentioned in mg</th>
<th>Amount found by HPLC in mg</th>
<th>Amount found by HPTLC in mg</th>
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</thead>
<tbody>
<tr>
<td>Primaquine</td>
<td>7.5</td>
<td>7.51± 1.12</td>
<td>7.355± 2.21</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>250</td>
<td>245± 2.64</td>
<td>239.3± 4.51</td>
</tr>
<tr>
<td>Bulaquine</td>
<td>25</td>
<td>25.2± 2.31</td>
<td>25.31± 3.24</td>
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</table>

*n=3
Simultaneous HPLC and HPLC assay method

Conclusion and Application of Method in Pharmaceutical Analysis

Both the methods developed are simple, easy to use and precise and are in use for routine analysis and quality control of bulaquine, primaquine and chloroquine formulations and bulk samples. Several samples of bulk preparation of bulaquine, required for pharmacological and toxicological activities were also analyzed by the present methods.

The separation achieved for all three drugs for simultaneous estimation has significance in the fact that this method can be of use for analysis of combination kit coming in the market.
REFERENCES

1. Indian patent No. 186617.


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