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Short communication

HPLC and HPTLC assays for the antimalarial agents Chloroquine, Primaquine and Bulaquine

A.K. Dwivedi *, D. Saxena, S. Singh

Division of Pharmaceutics, Central Drug Research Institute, Chhattar Manzil Palace, M.G. Marg, Lucknow 226001, India

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Abstract

A combination Kit for antirelapse treatment of *P. vivax* malaria, consisting of Chloroquine phosphate tablets and Bulaquine capsules has been recently developed, and marketed under the trade name Aablaquine. Bulaquine is prepared from Primaquine. Several methods of analysis are reported for each drug separately as well two drugs in combination but no method for simultaneous estimation of these three drugs is known. Therefore, the present study was undertaken to develop a sensitive and reproducible high performance liquid chromatographic as well as high performance thin layer chromatographic assay method for the simultaneous estimation of Chloroquine, Primaquine and Bulaquine.

Keywords: Antirelapse; Antimalarial; Bulaquine; Primaquine; Chloroquine; Simultaneous estimation; HPLC; HPTLC

1. Introduction

A combination Kit [1] for antirelapse treatment of *P. vivax* malaria, consisting of Chloroquine phosphate tablets and Bulaquine capsules has been recently developed, and marketed in India under the trade name Aablaquine. Bulaquine, is derived from Primaquine. Moreover Bulaquine is susceptible to acidic pH [2] and is converted to Primaquine.

Bergqvist and Churchill [3] have reported the detection and determination of several antimalarial drugs by different techniques and Taylor et al. have developed sensitive and accurate methods of estimation of antimalarials by high performance liquid chromatography (HPLC) [4]. Several methods are reported for the in vitro or in vivo analysis of Chloroquine [5–9]. Many methods for estimation of Primaquine are also reported. [10,11], including a HPLC method for the separation and identification of the oxidation products of Primaquine [12] as well as determination of Primaquine and Carboxyprimaquine in plasma and blood cells [13]. Dean et al. have used electrochemical detection for Primaquine [14] and Endoh et al. have determined Pamaquine, Primaquine and Carbox-

* Corresponding author. Tel.: +91-522-221-2411–18x4301, 4302; fax: +91-522-222-3938/3405.
E-mail addresses: a_k_dwivedi1957@rediffmail.com, a_k_dwivedi@yahoo.com, deepu272000@yahoo.com (A.K. Dwivedi).

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yprimaquine in calf plasma by EC detection[15]. Others have developed HPLC methods for Primaquine using various detection techniques [16–19]. high performance thin layer chromatographic (HPTLC) and HPLC methods for the analysis of major metabolites of Primaquine have been described [20]. A combined method for Primaquine and Chloroquine has been developed [21] as a method for the simultaneous estimation of Primaquine and Bulaquine by TLC densitometry and UV spectrophotometery [22]. The estimation of Bulaquine in serum [23] and Bulaquine along with its primary metabolite Primaquine has been reported [24].

Although the simultaneous estimation of the Primaquine and Bulaquine combination and Primaquine and Chloroquine are reported in the literature no method is reported for the simultaneous estimation of all three drugs. Since the development of Aablaquine, the need has arisen for an analytical method for the simultaneous estimation of Bulaquine, Chloroquine and Primaquine (an impurity/degradation product of Bulaquine). Therefore, the present study was undertaken to develop a simple, sensitive and reproducible HPLC as well as HPTLC assay method for the simultaneous estimation of Chloroquine, Primaquine and Bulaquine.

2. Experimental

2.1. Reagents and standards

Standard samples of Bulaquine and Primaquine diphosphate were supplied by the Chemical Technology Division of our Institute, and Chloroquine phosphate was obtained from Dr S.K. Puri of this Institute. HPLC grade acetonitrile, and methanol were obtained from M/S Merck (India) Limited (Mumbai, India). All other solvents and reagents used were of analytical grade. Triple distilled water was obtained from an all quartz apparatus. The TLC plates obtained from M/S Merck (India) Limited (Mumbai, India), were pre-washed with methanol. All glassware were washed with detergent, rinsed thoroughly with triple distilled water and dried prior to use.

2.2. Apparatus and chromatographic conditions

The HPLC system was equipped with 250 binary gradient pump (Perkin–Elmer), a Rheodyne (Cotati, CA) model 7125 injector with a 20 μl loop and 235 diode array detector (Perkin–Elmer). HPLC separation was achieved on a RP select-B C_8 lichrospher (Merck) analytical column (250 mm × 4 mm i.d., 5-μm 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 0.01-M sodium acetate buffer pH 5.6 and acetonitrile (45:55 v/v). Both the solutions were filtered and degassed before use. Chromatography was performed at 27 ± 3 °C at a flow rate of 1.0 ml/min.

The HPTLC workstation comprised of Automatic TLC Sampler-III (from CAMAG) equipped with 50 μl Hamilton syringe, TLC Scanner-3 (from CAMAG) equipped with mercury, tungsten and deuterium lamp for scanning of TLC plate. The separation was achieved on thin layer plates of precoated Si-gel 60 F_{254} (20 × 20 cm, 0.2 mm from Merck). The plates were scanned at wavelength of 254 nm. Data was acquired and processed using a CATS4-HPTLC software. The Solvent system used was hexane:diethyl ether:methanol:diethylamine in the ratio of (37.5:37.5:25:0.5 v/v). TLC was performed at 27 ± 3 °C in a Camag TLC twin trough glass chamber.

2.3. Preparation of stock and working standard solutions and sample solutions

Stock standard solutions of Primaquine diphosphate and Chloroquine phosphate were prepared individually by weighing 25 mg of drug, adding 50 mg of potassium carbonate, in 1 ml of water, followed by making the volumes to 25 ml with methanol. This was done to liberate the free base of the drugs. The stock standard solution of Bulaquine was prepared by dissolving 25 mg of the drug in methanol containing 0.5% dimethyl octyl amine in order to prevent the drug solution from degradation [22]. Working standard solutions (calibration concentrations), containing all three drugs, were prepared in methanol by serial dilution containing Bulaquine in the range of 0.5–

35 μg/ml, Chloroquine (free base) in the range of 1.5–50 μg/ml and Primaquine (free base) in the range of 0.5–35 μg/ml.

The samples of tablets of Primaquine diphosphate and samples of Aablaquine containing capsules of Bulaquine and tablets of Chloroquine phosphate were obtained from the retail pharmacist. Ten tablets of Primaquine diphosphate or Chloroquine phosphate were weighed and crushed evenly to give powder. The powder equivalent to 5 mg of each of the drug was taken and to this 25 mg of potassium carbonate in 1 ml of water was added and the final volume was made 10 ml with methanol. Capsule material equivalent to 5 mg of Bulaquine was taken and extracted with three times 3 ml of methanolic solution of 1% dimethyloctylamine and the final volume was made to 10 ml with the same solvent. All the sample solutions were filtered before analysis to remove any insoluble matter. Then 0.5 ml of these were further diluted to 25 ml with methanol. The drug solution in methanol was then injected into the HPLC and as well spotted on to a TLC plate and analysed for the drug content.

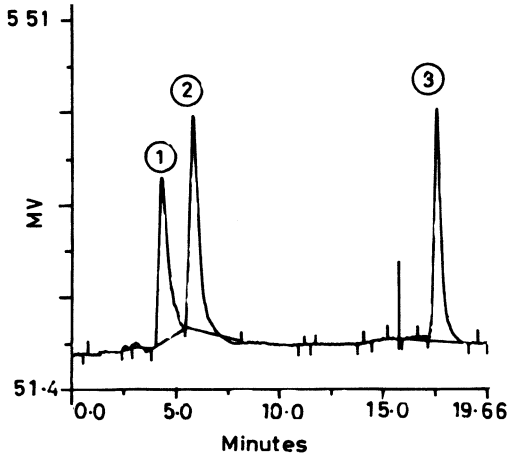

Fig. 1. Separation of Chloroquine (1) Rt. = 4.22 min, Primaquine (2) Rt. = 5.72 min and Bulaquine (3) Rt. = 17.26 min by HPLC and the amounts shown are 20.7, 10.4 and 11.58 μg/ml, respectively.

2.4. Accuracy and precision

The accuracy of the HPLC and HPTLC methods were calculated on the basis of the difference in the mean calculated and concentrations taken (% deviation from actual concentration, DFA) and the precision was obtained by calculating the intra- and inter-day relative standard deviations, the assay was done on the basis of six replicates of each calibration concentration [25]. For the recovery studies known amounts of Bulaquine or Primaquine or Chloroquine were added to the known amount of mixed contents of tablets or capsule material and the solutions were made as given in Section 2.3. The quantity of the respective drug was determined by interpolation on the corresponding calibration graphs.

3. Results and discussion

3.1. Chromatography

A RP select-B C_8 lichrospher (Merck) column was used to separate the three drugs from each other and all other ingredients. Initial experiments with columns like C_{18} end capped (250, 4 mm, 5 μm, Merck) or CN (250, 4 mm, 5 μm, Merck) using several solvent systems, including buffers were tried, but adequate resolution was not achieved.

The HPLC method described herein provides a good separation of Chloroquine (1), Primaquine (2) and Bulaquine (3) (Fig. 1). Along with the peak of Bulaquine an initial hump is observed which is due to the tautomer of Bulaquine. Under these chromatographic conditions, other constituents 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. As given in the manual of photodiode array detector, a peak purity index less than 1.5 indicates a pure peak. In this case the peak purity index was found to be less than 1.3.

In HPTLC, a Silica gel 60 F_{254} (Merck) TLC plate was used to separate Primaquine, Bulaquine

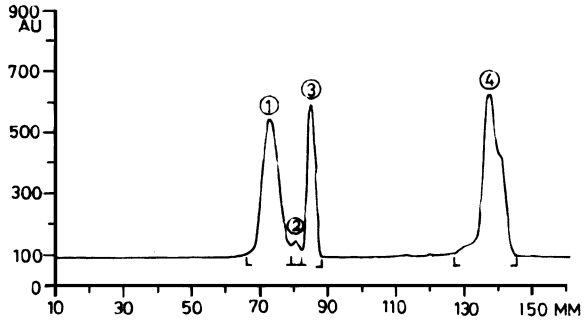

Fig. 2. Separation of Primaquine (1) at 72 mm, Chloroquine (3) at 85 mm and Bulaquine (4) at 136 mm by HPTLC and the amounts shown are 16, 17 and 15.7 μg, respectively. The origin is at 10 mm and solvent front is at 155 mm.

and Chloroquine from all other ingredients. Initial experiments with different solvent systems e.g. MeOH:$CHCl_3$, ethyl acetate:methanol and $CHCl_3$:MeOH:NH_3 previously used [22] for the separation of Primaquine and Bulaquine were also tried but could not achieve satisfactory resolution especially between Chloroquine and Primaquine. 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 good separation of Primaquine (1) Chloroquine (3) and Bulaquine (4) (Fig. 2). One additional peak (peak no. 2) between 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 these chromatographic conditions; other constituents did not interfere.

3.2. Selectivity and specificity

In HPLC assay the retention time of Chloroquine, Primaquine and Bulaquine were 4.22, 5.72 and 17.26 min, 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.

3.3. Linearity and reproducibility

External standardization by peak area was used for the quantitative determination of Bulaquine, Primaquine and Chloroquine in both the methods.

In the 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 1.9–21 μg/ml for Bulaquine ($r = 0.9976$), 4–33 μg/ml for Chloroquine ($r = 0.99947$), and 1–21 μg/ml for Primaquine ($r = 0.99938$).

For HPTLC, based on the noise to signal ratio of 3, the detection limit of Bulaquine, Chloroquine, and Primaquine were 0.25, 0.59 and 0.53 μg, respectively while the lower limits of quantitation were found to be 0.52, 1.21 and 1.07 μg, respectively. The calibration curves were linear in the range of 0.52–21 μg for Bulaquine ($r = 0.9986$), 1–23 μg for Chloroquine ($r = 0.9993$), and 1–21 μg for Primaquine ($r = 0.9984$).

A typical standard curve could be described by the equation

$$\text{Unknown conc.} = A + (B \times \text{peak area})$$

Where A and B are linear regression constants taking x, peak area, y, concentration for the different drugs with values:

For Bulaquine	$A = 0.01466$, $B = 1.8192\text{E-}7$ by HPLC $A = -11.40595$; $B = 0.00075$ by HPTLC
For Chloroquine	$A = 1.139$; $B = 4.333\text{E-}7$ by HPLC $A = -2.0335$; $B = 0.00152$ by HPTLC
For Primaquine	$A = 0.04157$; $B = 2.2764\text{E-}7$ by HPLC $A = -3.09601$; $B = 0.00049$ by HPTLC

Table 1
Inter and intra assay variations of Primaquine by HPLC

Conc. taken (μg/ml)	Intra assay variations			Inter assay variation		
	Conc. Found[a] (μg/ml) mean ± S.D.	% CV	% DFA	Conc. Found[a] (μg/ml) mean ± S.D.	% CV	% DFA
1.04	1.05 ± 0.041	3.905	0.96	1.031 ± 0.032	3.104	−0.86
2.6	2.64 ± 0.061	2.311	1.54	2.53 ± 0.082	3.241	−2.69
5.2	5.32 ± 0.14	2.632	2.31	5.4 ± 0.152	2.815	3.85
10.4	10.25 ± 0.22	2.146	−1.44	10.53 ± 0.231	2.194	1.25
15.6	15.52 ± 0.42	2.706	−0.513	15.62 ± 0.321	2.055	0.13
20.8	20.61 ± 0.56	2.717	−0.91	21.25 ± 0.54	2.54	2.16

[a] $n = 6$.

Table 2
Intra and inter assay variations of Chloroquine by HPLC

Conc. taken (μg/ml)	Intra assay variations			Inter assay variation		
	Conc. Found[a] (μg/ml) mean ± S.D.	% CV	% DFA	Conc. Found[a] (μg/ml) mean ± S.D.	% CV	% DFA
4.14	4.21 ± 0.114	2.708	1.69	4.25 ± 0.09	2.117	2.66
8.28	8.45 ± 0.23	2.722	2.05	8.35 ± 0.02	0.24	0.84
16.56	16.75 ± 0.21	1.254	1.15	17.04 ± 0.54	3.17	2.89
20.70	21.021 ± 0.42	1.99	1.55	20.872 ± 0.521	2.496	0.831
24.84	25.2 ± 0.44	1.746	1.45	25.12 ± 0.87	3.463	1.13
33.12	34.01 ± 1.02	2.99	2.69	32.87 ± 0.52	1.582	−0.76

[a] $n = 6$.

Table 3
Intra and inter assay variations of Bulaquine by HPLC

Conc. taken (μg/ml)	Intra assay variations			Inter assay variation		
	Conc. Found[a] (μg/ml) mean ± S.D.	% CV	% DFA	Conc. Found[a] (μg/ml) mean ± S.D.	% CV	% DFA
1.93	1.88 ± 0.065	3.46	−2.59	1.87 ± 0.015	0.802	−3.11
3.86	3.92 ± 0.141	3.59	1.55	3.79 ± 0.038	1.002	−1.81
7.72	8.011 ± 0.212	2.646	3.77	7.62 ± 0.24	3.15	−1.29
11.58	12.02 ± 0.43	3.577	3.79	11.43 ± 0.24	2.09	−1.29
15.44	15.76 ± 0.43	3.728	2.07	15.01 ± 0.34	2.265	−2.78
21.5	22.01 ± 0.52	2.362	2.37	21.83 ± 0.56	2.565	1.53

[a] $n = 6$.

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.

Table 4
Inter and intra assay variations of Bulaquine by HPTLC

Conc. taken (μg)	Intra assay variations			Inter assay variation		
	Conc. Found[a] (μg) mean ± S.D.	% CV	% DFA	Conc. Found[a] (μg) mean ± S.D.	% CV	% DFA
0.525	0.51 ± 0.015	2.941	−2.86	0.53 ± 0.017	3.207	0.95
1.05	1.08 ± 0.013	1.203	2.86	1.01 ± 0.026	2.574	−3.81
2.1	2.06 ± 0.022	1.068	−1.91	2.08 ± 0.031	1.49	−0.95
10.1	10.32 ± 0.23	2.23	2.18	10.32 ± 0.26	2.519	2.18
15.75	16.02 ± 0.31	1.935	1.71	15.35 ± 0.35	2.28	−2.54
21	21.67 ± 0.42	1.938	3.19	20.97 ± 0.46	2.193	−0.14

[a] $n = 6$.

Table 5
Inter and intra assay variations of Chloroquine by HPTLC

Conc. taken (μg)	Intra assay variations			Inter assay variation		
	Conc. Found[a] (μg) mean ± S.D.	% CV	% DFA	Conc. Found[a] (μg) mean ± S.D.	% CV	% DFA
0.59	0.58 ± 0.014	2.413	−1.69	0.576 ± 0.015	2.604	−2.37
1.18	1.21 ± 0.013	1.074	2.54	1.15 ± 0.025	2.174	−2.54
2.36	2.27 ± 0.052	2.291	−3.81	2.4 ± 0.015	0.625	1.69
11.8	11.48 ± 0.36	3.135	−2.71	12.15 ± 0.26	2.139	2.97
17.7	18.2 ± 0.21	1.153	2.82	18.14 ± 0.40	2.205	2.48
23.6	23.16 ± 0.21	0.907	−1.86	24.02 ± 0.78	3.24	1.78

[a] $n = 6$.

Table 6
Inter and intra assay variations of Primaquine by HPTLC

Conc. taken (μg)	Intra assay variations			Inter assay variation		
	Conc. Found[a] (μg) mean ± S.D.	% CV	% DFA	Conc. Found[a] (μg) mean ± S.D.	% CV	% DFA
0.535	0.523 ± 0.016	3.059	−2.24	0.542 ± 0.015	2.767	1.31
1.07	1.1 ± 0.017	1.545	2.81	1.08 ± 0.017	1.574	0.93
2.14	2.17 ± 0.036	1.658	1.40	2.2 ± 0.062	2.818	2.81
10.7	11.03 ± 0.21	1.903	3.08	10.3 ± 0.26	2.524	−3.74
16.05	16.16 ± 0.21	1.299	0.68	16.31 ± 0.16	0.98	1.62
21.4	20.95 ± 0.56	2.673	−2.10	21.75 ± 0.08	0.367	1.64

[a] $n = 6$.

The reproducibility and accuracy of the methods were determined by intra and inter assay variation (Tables 1–6). Reproducibility and accuracy of the method were within the acceptable limits [26–28]. The recovery studies indicated a recovery of more than 95% in the case of all three drugs.

Table 7
Analysis of the samples of Bulaquine, Chloroquine and Primaquine

Sample[a] name	Amount mentioned in mg	amount found by HPLC in mg	Amount found by HPTLC in mg
Primaquine	7.5	7.51 ± 1.12	7.355 ± 2.21
Chloroquine	250	245 ± 2.64	239.3 ± 4.51
Bulaquine	25	25.2 ± 2.31	25.31 ± 3.24

[a] $n = 3$.

3.4. Analysis of drug samples

In the analysis of drug samples, there was no interference from the excipients of the formulation of each drug substance. The results obtained from the analysis of drug samples are given in Table 7. The results were consistent with the label claim.

4. Conclusion

Both the methods developed are simple, easy to use, 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 their simultaneous estimation has significance in the fact that this method can be of use for the analysis of the combination kit coming to the market.

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