APPENDIX
LIST OF PUBLICATIONS


4. Fingerprinting of sapindosides in Sapindus mukorosii saponin (reetha saponin) and quantitative determination of sapindoside-B; D.Saxena, R.Pal, A.K.Dwivedi and S.Singh; Accepted in J SIR.
LIST OF PAPERS PRESENTED/ACCEPTED
IN VARIOUS CONFERENCES

Paper accepted at AAPS Annual meeting and exposition:


List of Papers Presented at Indian Pharmaceutical Congress:

Year 2002

- 'CDRI compound no.97/78 a new antimalarial agent - Development of HPLC assay method and physicochemical studies'; Deepali Saxena, A.K.Dwivedi, Chandan Singh and Satyawan Singh


- 'Simultaneous HPLC and HPTLC assay method for antimalarial agents chloroquine, primaquine and bulaquine'; A.K.Dwivedi, D. Saxena and S.Singh
'Inhalation delivery of microparticles reduces serum burden of toxic bactericidal drugs'; Pavan Muttil, Rolee Sharma, D. Saxena and Amit Misra

Year 2001

'High performance thin layer chromatographic method for quantitative determination and fingerprinting of Sapindoside-B in *Sapindus mukorosii* saponin (reetha saponin); D. Saxena, A.K. Dwivedi, R. Pal, and S. Singh

Year 2000


Characterisation of Sapindosides in *Sapindus mukorosii* saponin (*reetha saponin*) and Quantitative Determination of Sapindoside B'

D Saxena, R Pal, A K Dwivedi* and S Singh

Division of Pharmaceutics, Central Drug Research Institute (CDRI), Lucknow, India

High performance thin layer chromatography (HPTLC) method as well as high performance liquid chromatographic (HPLC) method combined with ES-MS are developed and validated for fingerprinting (profiling) *Sapindus saponin* and quantitative determination of Sapindoside B in bulk drug samples of *Sapindus saponin* and its formulation Consap cream. The separation of saponins using TLC is achieved on precoated silica gel plates using chloroform: methanol: water as mobile phase. Detection of the spots is done at the 630 nm. The identification of saponins is done by LC-MS, using Electro Spray Ionisation [ESI] technique. The HPLC method involves chromatography of the saponins on reverse phase (C-18) column, using acetonitrile-water gradient as mobile phase. The detection is done by UV-visible detector at a wavelength of 215 nm. Calibration graphs are found to be linear over the range 11μg-220 μg in HPTLC and 30 μg/mL-200 μg/mL in HPLC method. The methods developed are being used for the analysis of the bulk drug samples and consap cream samples.

**Keywords:** *Sapindus saponin*, Sapindoside-B, Spermicidal, HPLC, HPTLC, LC-MS.

# CDRI Communication No. 6316; Accepted in AAPS Annual Meeting and Exposition, Toronto, November 2002.

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Introduction

The *Reetha* plant *Sapindus mukorosii* is found across the Indian subcontinent. It has been used in the Indian household since long, as a cleansing agent. Its fruit was found to contain various saponins, which are mainly responsible for its cleansing properties. *Sapindus saponin*\(^1,2\), (reetha saponin) is the n-butanol soluble fraction, obtained from the ethanolic extract of the fruit pericarp of the plant *Sapindus mukorosii*. The saponin fraction consists of several saponins and most of them are derived from hederagenin\(^3\). The spermicidal action of the saponins was found to be associated with the β-amyrin C-28 carboxylic acid type of sapogenins linked to a particular sequence of sugar moieties\(^4\). The mixture of saponins\(^5,6\) isolated from *Sapindus mukorosii* were taken for further drug development at CDRI, Lucknow. For this, it was incorporated in a cream referred to as *Consap cream*, and its phase III multi-centric clinical trials are complete in seven State Medical Colleges and three Family Welfare Centres. The cream has been found to be safe and efficacious, and permission for marketing from the Drug Controller General of India is received. The process has been handed over to industry for manufacturing.

There are several methods used for saponin determination in plant materials. It is observed that the biological and spectrophotometric methods which are being used for saponin determination provide, to a large extent, valuable results on saponin concentrations in plant material but these determinations do not provide accurate data and have to be recognized as approximate. GLC, TIC on normal and reversed-phases is being used for routine determination of saponins in plant material. HPLC is the most-widely used method for saponin determination. However the lack of chromophores limits the choice of detection method. The pre-column derivatisation\(^7\) with benzoyl chloride, coumarin or 4-bromophenacyl bromide has been used in some cases, allowing UV detection. New hyphenated techniques, combining HPLC with MS and NMR are developing rapidly and allow on-line identification of separated saponins\(^8\) among other methods being developed\(^9,10\). A colourimetric assay method for the estimation of the total saponin content of the spermicidal *reetha saponin*\(^11\) was also reported. The present study describes two different methods for analysis of sapindosides within the saponin mixture. The identity of six sapindosides, including sapindoside B was established in the sapindus saponin mixture by LC-MS technique. We have developed and used HPLC and HPTLC methods for the fingerprinting and estimation of sapindoside-B, one of the major active constituents, in bulk drug samples of *sapindus saponin* and its formulation consap cream.

2 Experimental Procedure

2.1 Reagents and standards

Standard *sapindus saponin*, was provided by Chemical Technology Division, of this Institute. HPLC grade acetonitrile, methanol and chloroform were obtained from M/s E Merck (India) Limited. Triple distilled water, from an all glass apparatus, was used as solvent. Other reagents like, naphthorescorcinol, ethanol and \(\text{H}_2\text{SO}_4\) (96 percent) used were of analytical grade.

2.2 Isolation and Purification of Sapindoside B

Pure sapindoside B was obtained by column chromatography of the *sapindus saponin* and the identity of sapindoside B was established by ES-MS. To isolate pure sapindoside B, from the saponin mixture, enriched fraction of sapindoside B was prepared by column chromatography using silica column and mixture of chloroform,
methanol and water as eluent. This enriched fraction was again loaded on a silica column. The fractions were eluted with chloroform: methanol: water (89.5:10:0.5 - 84.5:15:0.5) and those corresponding to sapindoside B were pooled, concentrated to dryness under reduced pressure. Final purification of sapindoside B was done by prep-TLC using solvent system of chloroform: methanol: water (50:14:1). The MS of this purified sapindoside B (Figure I) using ES MS showed a major peak at 905 (M+Na).

2.3 Apparatus and Chromatographic Conditions

The HPTLC system was equipped with auto-TLC sampler-3 with air pressure injector (CAMAG), syringe injector with 50 μL loop, twin chamber TLC developer and Camag Scanner ATS-3 with tungsten, mercury and deuterium lamps. HPTLC separation was achieved on a silica gel pre-coated plate (20x20cm, Si gel 60 F 254) from Merck, for detection of the spots CAMAG TLC scanner was used. Data was acquired and processed, using a Camag HPTLC software CATS-4.

The HPLC workstation used was from Shimadzu, Japan; equipped with SCL-10A VP system controller, LC-10AT VP twin pump, SPD-10A VP UV-VIS detector, and Rheodyne injector with 20 μL injection loop. The separation was achieved on a 5μ Lichrosphere RP C-18 (25 cm x 0.4 cm) Lichrocart reverse phase analytical column (Merck). The data acquisition and processing was done by using Shimadzu HPLC software class-VP (V5.03).

The LC-MS experiment was performed on a Micromass Quattro II triple quadrupole mass spectrometer. The ESI capillary was set at 3.5 kV and the cone voltage was 40V. The spectra was collected in 6s scans and the final prints were the averaged spectra of 6-8 scans.

2.3.1 Preparation of Stock and Working Standard Solution

Stock standard solution containing 1 mg/mL of standard solution was prepared by dissolving 10 mg of sapindoside B in 10 mL of methanol. The working standards were prepared by serial dilution from the stock solution of sapindoside B accordingly in the range of 30 to 200 μg/mL in methanol.

2.3.2 Preparation of Sample Solution

The samples of the sapindus saponin (25 mg) each from different batches were dissolved in 10 mL methanol and analysed.

2.3.2.1 Extraction of Saponin from Consap Cream

It was done as described by Dwivedi et.al\textsuperscript{11}.

2.3.3 HPTLC Method

Various spots of standard sapindoside B in the range of 5.0-220 μg and three spots of 100μL each of the sample were loaded on the silica plate. The plates were developed in the solvent system - Chloroform: Methanol: Water :: 50:14:1. TLC was performed at 27 ± 3° C. The plates were removed, dried and the spots were visualized by post chromatography derivatization with naphthorescorcinol reagent\textsuperscript{12} {naphthorescorcinol 20 mg in 10 mL ethanol + 0.4 mL H₂SO₄ (96percent)} and heating the plate at 100° C for 10 min. The blue coloured spots were scanned at 630 nm.
2.3.4 HPLC method

The separation of different saponins was achieved on C-18 (25x 0.4 cm, 5\(\mu\)m) reverse phase column (Merck) and also on C-18 (30x 0.39, 6\(\mu\)m) reverse phase column (Waters). The solvent system was Acetonitrile:Water gradient starting with 90 water to 20 water in 30 min. The eluents were detected at 215 nm, and chromatography was performed at 27±3°C.

LC-MS of Sapindosides in Sapindus saponin

Using the above described method of HPLC, and \(\mu\)-bondapack C-18 column (Waters), the LC-MS was performed for enriched fractions of saponins obtained from column chromatography. The identity of saponins was established by Selected Ion Monitoring (SIM) of the Total Ion Current (TIC) at the known mass numbers (Figure 2).

2.4 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 inter- and intra-day relative standard deviations (percentage R. S. D.)

2.5 Recovery

Known amount of sapindoside B was added to the mixed contents of the samples/cream and sapindoside B in the sample/cream was determined by interpolation on the corresponding calibration graphs.

3 Result and Discussion

3.1 Chromatography

Sapindus saponin is a mixture of more than ten components. They are structurally related\(^4\) and only number and type of sugar moieties attached constitute the different saponins. Therefore proper resolution of the individual components for quantitation as well as estimation, among the saponins becomes difficult task.

Initial experiments, using silica gel TLC plates and several solvent systems with different ratio of ethyl acetate, methanol, chloroform including water were tried. No proper separation of the individual components was obtained. Chloroform: Methanol: Water (50:14:1) gave the best separation of the spot corresponding to sapindoside B from ten other prominent spots (Figure 3).

The identity of purified fraction of sapindoside B from column chromatography was confirmed by LC-MS. Further the acid hydrolysis of this purified fraction revealed the presence of three sugars xylose, rhamnose, and arabinose. For HPLC, a C-18 reverse phase analytical column and the acetonitrile-water gradient system gave proper separation of the Sapindoside B from the other sapindosides (Figure 4).

Fingerprinting of Sapindus saponins

For LC-MS, HPLC method, using C-18 column from Waters and MS were used. Authors were able to recognise six saponins (Figure 1): sapindoside A (M+Na=773) at 12.56 min, sapindoside B (M+Na=905) at 11.6 min, sapindoside C (M+Na=1067) at 5.79min, sapindoside D (M+Na=1375) at 10.91min, mukorozisaponin E1 (M+1=924) and mukurozisaponin Y1 (M+1=1207) at 4.13min (Table 1).
3.2 Selectivity and Specificity

The retention factor on HPTLC of sapindoside B was 0.48, and other sapindoside was found to be, 0.19, 0.26, 0.41, 0.59, 0.67, 0.75, 0.83, 0.90, 0.94, respectively (Figure 3). Based on the noise to signal ratio of 3, the detection limit in HPTLC was 5 µg, however the limit of quantitation was set at 10 µg.

The retention time on HPLC of I was found to be about 14 min. Under the chromatographic conditions mentioned above, peaks corresponding to other constituents did not interfere with the peak of I. The detection limit in this case was 10 µg and the limit of quantitation was set at 30 µg.

3.3 Linearity and Reproducibility

In HPTLC and HPLC both, external standardization by peak area was used for the quantitative determination of I. In HPTLC the calibration curves were linear in the range of 11 to 220 µg with r=0.99949.

Unknown conc. = A + (B * peak area), where A= -12.62364 and B = 0.0008, while in HPLC the calibration curve was linear in the range of 30 to 210 µg with r= 0.9998.

Unknown conc. = A + (B * peak area), where A= -4.73921 and B = 0.00049.

The reproducibility and accuracy of both the methods were determined by intra- and inter-day assay variation. Reproducibility and accuracy of the method were within the acceptable limit13-15.

3.4 Recovery

The recovery from the consap cream was checked by extraction of saponins from the cream and estimation by the described method. It was found that the amount of saponins recovered from the cream was more than 90 percent.

3.5 Application of Method in Pharmaceutical Analysis

Estimation of sapindoside B in the bulk samples for formulation development was also done (Table 2). Estimation of Sapindus saponin and Sapindoside B in consap cream for vaginal irritation and efficacy studies after storage was also checked, using this method (Table 3).

4 Conclusions

The HPTLC method so developed is simple and can be easily used. Further, due to post-chromatographic derivatisation, using naphthorescorcinol reagent significant improvement in the sensitivity and limit of quantitation of Sapindoside B was achieved. By this method proper resolution between Sapindus saponins was also obtained.

HPLC method also provides good resolution between the Sapindus saponins. The method developed is sensitive and can be used for the analysis of bulk samples of Sapindus saponins and Consap cream. Further, using LC-MS, fingerprinting of sapindus saponin can be done.

Acknowledgement

The authors thank Director CDRI, Lucknow, for providing fellowship to one of them (D. S.).
References:


Figure 1: ES-MS spectra of Sapindoside B, m/z=905 by ES-MS
Figure 2- Selected ion monitoring of sapindosides by LC-MS. (a) Y1; (b) C; (c) E1; (d) B; (e) A; (f) D.

(a) mukurozisaponin-Y1

(b) sapindoside-C

(c) mukurozisaponin-E1

(d) sapindoside-B

(e) sapindoside-A

(f) sapindoside-D
Figure 3 HPTLC separation of Sapindoside-B and other saponins

(a) Spots (1-6) and (13-18) of Sapindoside-B concentration range 5µg - 220µg
(b) Spots (7-8) of sample 1 (c) Spots (9-10) of sample 2 (d) Spots (11-12) of sample 3.

Figure 4 - The HPLC separation of Sapindoside B and other constituents

Detector A - 1 (215nm)
Table 1 - Finger-printing of Sapindosides in *Sapindus* saponin by LC-MS

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Sapindosides</th>
<th>Mass</th>
<th>M + Sodium ion</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sapindoside-A</td>
<td>750</td>
<td>773</td>
<td>12.56</td>
</tr>
<tr>
<td>2</td>
<td>Sapindoside-B</td>
<td>882</td>
<td>905</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>Sapindoside-C</td>
<td>1044</td>
<td>1067</td>
<td>5.79</td>
</tr>
<tr>
<td>4</td>
<td>Sapindoside-D</td>
<td>1352</td>
<td>1375</td>
<td>10.91</td>
</tr>
<tr>
<td>5</td>
<td>Mukurozisaponin-E1</td>
<td>924</td>
<td>947</td>
<td>13.52</td>
</tr>
<tr>
<td>6</td>
<td>Mukurozisaponin-Y1</td>
<td>1206</td>
<td>1207(M+1)</td>
<td>4.13</td>
</tr>
</tbody>
</table>

Table 2 - Estimation of Sapindoside-B in *Sapindus saponin* bulk drug samples.

<table>
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<tr>
<th>Batch No.</th>
<th>Year of manufacture</th>
<th>Percent Sapindoside B found</th>
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<tbody>
<tr>
<td>5-1997</td>
<td>1997</td>
<td>5.4</td>
</tr>
<tr>
<td>4-1998</td>
<td>1998</td>
<td>7.2</td>
</tr>
<tr>
<td>2-2002</td>
<td>2002</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 3 - Estimation of Sapindoside-B in consap cream formulation after storage

<table>
<thead>
<tr>
<th>Year of preparation</th>
<th>Label claim (per cent w/w)</th>
<th>Saponin extractive found (per cent w/w)</th>
<th>Sapindoside-B per cent estimated in extracted saponin</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>2.5</td>
<td>0.97</td>
<td>2.22</td>
</tr>
<tr>
<td>95</td>
<td>2.5</td>
<td>1.38</td>
<td>6.33</td>
</tr>
<tr>
<td>97</td>
<td>2.5</td>
<td>2.39</td>
<td>8.16</td>
</tr>
</tbody>
</table>
Assay method for quality control and stability studies of a new CVS disorder agent (compound 93/478)*

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Abstract

1-[4-(4-Fluorophenyl)-piperazine-1-yl]-3-(2-oxopyrrolidin-1-yl)-propane hydrochloride, (I), (CDRI code No. 93/478) is a new potent anti-ischemic and anti-hypertensive agent, being developed at the Central Drug Research Institute (CDRI), Lucknow, India. A sensitive high performance liquid chromatographic assay method has been developed and validated for in process quality control and for stability studies. HPLC separation was achieved on a C18 Purospher (Merck) column using a gradient of 0.02% tetra-methyl ammonium hydroxide (pH 7.5) and acetonitrile as mobile phase. The eluents were monitored by diode array detector at 240 and 290 nm. The lower limit of detection of I was 0.62 µg/ml, while the lower limit of quantitation was set to be 1.5 µg/ml. The calibration curves were linear in the range 1.5–62 µg/ml. Reproducibility of the method was determined by inter and intra assay variation, which were <10%.

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1. Introduction

Among the 1-[4-piperazine-1-yl]-3-[2-oxopyrrolidin-1-yl] propanes designed and synthesized for treatment of cardiovascular system (CVS) disorders, including hypertension, ischemia and dyslipidemia, mediated through α-adrenergic receptors, particularly α1a, the compound 1-[4-(4-fluorophenyl)-piperazine-1-yl]-3-(2-oxopyrrolidin-1-yl)-propane hydrochloride (CDRI Compound No. 93/478) (I, Fig. 1) has shown a good profile for development as a drug. This compound has now been patented [1–3] and taken up for further development at our organization. The present study was undertaken to develop a sensitive and reproducible high performance liquid chromatography assay method, for this compound, for in process quality control and stability studies.
Fig. 1. Structure of CDRI compound No. 93/478 (I).

2. Experimental

Standard compound 93/478 (I), starting materials and intermediates were supplied by the Medicinal Chemistry Division of this Institute. HPLC grade acetonitrile, methanol, tetra-methyl ammonium hydroxide (analytical grade) was obtained from E. Merck (India) Ltd. (Mumbai, India). Triply distilled water, from an all glass apparatus, was used in analysis. Other reagents used were of analytical grade.

The HPLC system consisted of a binary gradient pump (model 250, Perkin-Elmer), a Rheodyne model 7125 injector with a 20 μl loop and diode array detector (model 235, Perkin-Elmer). HPLC separation was achieved on a C18 Puruspher (Merck) column (250 x 4 mm I.D., 5 μm particle size). Column effluent was monitored at 240 and 290 nm. Data was acquired and processed using an IndTech HPLC interface and software. The mobile phase was a gradient of 0.02% tetra-methyl ammonium hydroxide (adjusted to pH 7.6 with orthophosphoric acid) and acetonitrile. The gradient was 10–90% acetonitrile in 20 min followed by 90% acetonitrile for 10 min. Both the solutions were filtered and degassed before use. Chromatography was performed at 27 ± 3 °C at a flow rate of 1.5 ml/min.

Stock standard solutions containing 200 μg/ml of I as hydrochloride were freshly prepared in water. Working standard solutions were prepared in water in the range of 0.62–62 μg/ml by serial dilution. Quantitation was based on a six-point calibration curve.

Samples (5 mg) from various batches of the compound were dissolved in 25 ml of water. The solution was filtered and 1 ml further diluted to 10 ml with water to obtain a sample solution of ≈20 μg/ml.

Acid-base degradation studies were carried out to obtain the pH for maximum stability. This information is required for the preparation of the final formulations. Effect of pH on this compound was checked by the following method. Some 1 ml each of stock solution of I was taken in 10 ml volumetric flasks and the volumes were made up with buffers of pH 3–9. Samples were withdrawn at different time intervals and 20 μl were injected on to the HPLC column to analyze as described above. The reaction rate constants were calculated by LINREG program [4].

3. Results and discussion

Acid-base degradation studies were carried out to obtain the pH for maximum stability. This information is required for the preparation of the final formulations. Effect of pH on this compound was checked by the following method. Some 1 ml each of stock solution of I was taken in 10 ml volumetric flasks and the volumes were made up with buffers of pH 3–9. Samples were withdrawn at different time intervals and 20 μl were injected on to the HPLC column to analyze as described above. The reaction rate constants were calculated by LINREG program [4].

Initial experiments with columns, such as C18 endcapped (250 mm, 4 mm, 5 μm, E. Merck) or CN (250 mm, 4 mm, 5 μm, E. Merck), using
several solvent systems including buffers were tried. The C18 endcapped (250 mm, 4 mm, 5 μm, E. Merck), column with 0.05 M phosphate buffer (pH 3):acetonitrile gave a single peak in the chromatogram (Fig. 2). The UV spectrum of this peak was, however, found to differ at peak onset, maximum and decline (Fig. 3). The peak purity index also indicated that peak was impure. HPLC of individual starting materials (1-(3-bromopropyl)-4-(4-fluorophenyl)-piperazine, 1-(3-chloropropyl)-2-pyrrolidone and 2-pyrrolidone) under the same conditions did not show any overlap with the elution of I (data not shown). When the NMR spectrum of the sample was analysed by a protocol of deletion of peaks corresponding to I and stating materials, it was found that the peaks remaining in the processed spectrum corresponded to 1-(3-hydroxy-propyl)-4-(4-fluoro-phenyl)-piperazine, a side product formed during preparation of I.

The use of a C18 Purospher column with a gradient of acetonitrile in water containing 0.02% TMAH (pH 7.6) resulted in good separation of I from contaminants arising from starting material as well as possible degradation products (Fig. 4). Base line separation of I and the process impurity 1-(3-hydroxy-propyl)-4-(4-fluoro-phenyl)-piperazine was obtained (Fig. 5). Under the chromatographic conditions, other constituents did not interfere, since they eluted either before or after the peak of interest.

The retention times of I, 1-(3-hydroxy-propyl)-4-(4-fluoro-phenyl)-piperazine, 1-(3-bromo-propyl)-4-(4-fluoro-phenyl)-piperazine, 1-(3-chloropropyl)-2-pyrrolidone and 2-pyrrolidone were ≈ 13, 12, 18.5, 9 and 2.5 min, respectively. It would be recalled that 1-(3-bromopropyl)-4-(4-fluorophenyl)-piperazine, 1-(3-chloropropyl)-2-pyrolidone and 2-pyrrolidone are the starting materials, while 1-(3-hydroxypropyl)-4-(4-fluorophenyl)-piperazine is the process impurity in the preparation of the final compound. None of the above interfered with the peak corresponding to I [6–8].

External standardization by peak area was used for the quantitative determination of I. Based on a signal to noise ratio of 3, the detection limit was 0.62 μg/ml. The limits of quantitation were derived from extremes of concentration in which the interpolating polynomial was of the first order, i.e. linear. Thus, a straight line with a regression coefficient of 0.999 could be fit to data in the range

Fig. 2. Peak of the compound I in 0.05 M phosphate buffer (pH 3): acetonitrile.
1.5–62 μg/ml (Fig. 6). Below 1.5 or above 62 μg/ml, significant deviation from linearity was observed. A typical standard curve ($r = 0.99966$) could be described by the equation

$$\text{Unknown conc. (μg/ml)} = [(4.8012 \times 10^{-7}) \times \text{peak area}] - 0.09215.$$ 

The method showed adequate sensitivity for the determination of I in bulk preparations and checking pH stability of the compound.

The reproducibility and accuracy of the method were determined by intra and inter assay variation (Table 1). This data also illustrates that our choice of the lower limit of quantitation (1.56 μg/ml) is correct. At a concentration level of 0.62 μg/ml, the standard curve back-calculated a value showing > 29% deviation from the actual (DFA), indicating that such low concentrations cannot be accurately determined by the standard curve.
Calibration curve for compound 93/478

\[ Y = A + B \times X \]

\[ A = 21672.97155 \]

\[ B = 2081376.2417 \]

\[ R = 0.99966, N = 7 \]

Fig. 6. Calibration graph of compound 93/478.

The % C.V. and % DFA were calculated on the basis of replicate injections of the calibration standards on the same day (intra day) and on different days (inter assay) and to check the variation in the analysis. All these were found to be within acceptable limits [5–8]. As is commonly observed, the % C.V. was always higher at lower concentrations.

Several samples of bulk preparation, required for pharmacological and toxicological studies were analyzed by the reported method. The method developed is simple and is being routinely used for quality control of I in bulk preparations, preformulation studies and for ongoing stability studies under stress conditions.

Table 2
First order degradation parameters of the compound No. 93/478 at different pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Rate constant*</th>
<th>Half life (h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.061</td>
<td>10.43</td>
</tr>
<tr>
<td>4</td>
<td>0.030</td>
<td>21.41</td>
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<tr>
<td>5</td>
<td>0.038</td>
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<tr>
<td>6</td>
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<td>7</td>
<td>0.086</td>
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<tr>
<td>8</td>
<td>0.042</td>
<td>15.24</td>
</tr>
<tr>
<td>9</td>
<td>0.083</td>
<td>07.73</td>
</tr>
</tbody>
</table>

* No. of experiments; rate constant, first order degradation rate constant of the compound 93/478; half life, first order degradation half life of the compound 93/478. pH of 1% solution of the compound in triple distilled water is \( \approx 4.3 \).

Table 1
Inter and intra assay variations

<table>
<thead>
<tr>
<th>Conc. taken (µg/ml)</th>
<th>Intra assay variations</th>
<th>Inter assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. found* (µg/ml) mean ± S.D.</td>
<td>% C.V.</td>
<td>% D.F.A.</td>
</tr>
<tr>
<td>0.62</td>
<td>0.80 ± 0.011</td>
<td>1.37</td>
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<tr>
<td>1.56</td>
<td>1.62 ± 0.016</td>
<td>0.988</td>
</tr>
<tr>
<td>3.12</td>
<td>3.16 ± 0.11</td>
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</tr>
<tr>
<td>7.8</td>
<td>8.10 ± 0.31</td>
<td>3.83</td>
</tr>
<tr>
<td>15.6</td>
<td>15.37 ± 0.61</td>
<td>3.97</td>
</tr>
<tr>
<td>31.2</td>
<td>32.23 ± 0.79</td>
<td>2.45</td>
</tr>
<tr>
<td>62.4</td>
<td>63.36 ± 1.24</td>
<td>1.96</td>
</tr>
</tbody>
</table>

* n = 3.
kept in mind during designing of other drug delivery systems for this compound.

Acknowledgements

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References


Short communication

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

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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.

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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 Carboxyprimaquine...
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 spectrophotometry [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 diphasphate 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 C8 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 F254 (20 × 20 cm, 0.2 mm from Merck). The plates were scanned at wavelength of 254 nm. Data was acquired and processed using a CAT54-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 diphasphate 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–
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.

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 C₈ lichrospher (Merck) column was used to separate the three drugs from each other and all other ingredients. Initial experiments with columns like C₁₈ 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 F₂₅₄ (Merck) TLC plate was used to separate Primaquine, Bulaquine

![Fig. 1. Separation of Chloroquine (1) \( R_t = 4.22 \text{ min} \), Primaquine (2) \( R_t = 5.72 \text{ min} \) and Bulaquine (3) \( R_t = 17.26 \text{ min} \) by HPLC and the amounts shown are 20.7, 10.4 and 11.58 μg/ml, respectively.](image-url)
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:CHCl₃, ethyl acetate:methanol and CHCl₃:MeOH:NH₃ 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

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

\[ A = 0.01466; \quad B = 1.8192E-7 \]

HPLC

\[ A = -11.40595; \quad B = 0.00075 \]

HPTLC

For Chloroquine

\[ A = 1.139; \quad B = 4.333E-7 \]

HPLC

\[ A = -2.0335; \quad B = 0.00152 \]

HPTLC

For Primaquine

\[ A = 0.04157; \quad B = 2.2764E-7 \]

HPLC

\[ A = -3.09601; \quad B = 0.00049 \]

HPTLC
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

<table>
<thead>
<tr>
<th>Conc. taken (µg)</th>
<th>Intra assay variations</th>
<th>Inter assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. Found* (µg) mean ± S.D.</td>
<td>% CV</td>
</tr>
<tr>
<td>0.525</td>
<td>0.51 ± 0.015</td>
<td>2.94 ± 2.86</td>
</tr>
<tr>
<td>1.05</td>
<td>1.08 ± 0.013</td>
<td>1.203 ± 2.86</td>
</tr>
<tr>
<td>2.1</td>
<td>2.06 ± 0.022</td>
<td>1.068 ± 1.91</td>
</tr>
<tr>
<td>10.1</td>
<td>10.32 ± 0.23</td>
<td>2.23 ± 2.18</td>
</tr>
<tr>
<td>15.75</td>
<td>16.02 ± 0.31</td>
<td>1.935 ± 1.71</td>
</tr>
<tr>
<td>21</td>
<td>21.67 ± 0.42</td>
<td>1.938 ± 3.19</td>
</tr>
</tbody>
</table>

* n = 6.

Table 5
Inter and intra assay variations of Chloroquine by HPTLC

<table>
<thead>
<tr>
<th>Conc. taken (µg)</th>
<th>Intra assay variations</th>
<th>Inter assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. Found* (µg) mean ± S.D.</td>
<td>% CV</td>
</tr>
<tr>
<td>0.59</td>
<td>0.58 ± 0.014</td>
<td>2.41 ± 1.69</td>
</tr>
<tr>
<td>1.18</td>
<td>1.21 ± 0.013</td>
<td>1.074 ± 2.54</td>
</tr>
<tr>
<td>2.14</td>
<td>2.17 ± 0.02</td>
<td>2.291 ± 3.81</td>
</tr>
<tr>
<td>11.8</td>
<td>11.48 ± 0.36</td>
<td>3.135 ± 2.71</td>
</tr>
<tr>
<td>17.7</td>
<td>18.2 ± 0.21</td>
<td>1.153 ± 2.82</td>
</tr>
<tr>
<td>23.6</td>
<td>23.16 ± 0.21</td>
<td>0.907 ± 1.86</td>
</tr>
</tbody>
</table>

* n = 6.

Table 6
Inter and intra assay variations of Primaquine by HPTLC

<table>
<thead>
<tr>
<th>Conc. taken (µg)</th>
<th>Intra assay variations</th>
<th>Inter assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. Found* (µg) mean ± S.D.</td>
<td>% CV</td>
</tr>
<tr>
<td>0.535</td>
<td>0.523 ± 0.016</td>
<td>3.059 ± 2.24</td>
</tr>
<tr>
<td>1.07</td>
<td>1.1 ± 0.017</td>
<td>1.545 ± 2.81</td>
</tr>
<tr>
<td>2.14</td>
<td>2.17 ± 0.026</td>
<td>1.658 ± 1.40</td>
</tr>
<tr>
<td>10.7</td>
<td>11.03 ± 0.21</td>
<td>1.903 ± 3.08</td>
</tr>
<tr>
<td>16.05</td>
<td>16.16 ± 0.21</td>
<td>1.299 ± 0.68</td>
</tr>
<tr>
<td>21.4</td>
<td>20.95 ± 0.56</td>
<td>2.673 ± 2.10</td>
</tr>
</tbody>
</table>

* 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.
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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The authors thank Director CDRI, Lucknow, for providing fellowship to one of them (D.S.). The authors are also thankful to Ms Asmita and Ms Priyanka for their technical assistance. The authors like to thank the referees for their encouraging comments for further improvement of the present work.

References


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>
</tr>
</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>
</tr>
</tbody>
</table>

* n = 3.