DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF PARACETAMOL, DICLOFENAC POTASSIUM AND CHLORZOXAZONE IN BULK DRUG AND TABLET DOSAGE FORM

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ABSTRACT
Objective: Research study was undertaken to develop and validate simple, rapid, precise, accurate, robust High Performance Thin Layer Chromatographic (HPTLC) method for simultaneous determination of paracetamol (PARA), diclofenac potassium (DCL) and chlorzoxazone (CHL) in bulk drug and tablet dosage form.

Methods: The chromatographic separation was performed on precoated silica gel G 60 F_{254} plates with toluene: ethyl acetate [55:45, v/v] as mobile phase. The detection was carried out at 271 nm.

Results: Retention factors of PARA, DCL and CHL were found to be 0.21 ± 0.01, 0.54 ± 0.01, and 0.74 ± 0.01, respectively. Linearity of PARA, DCL and CHL was found to be in the concentration range of 1000-3500 ng band^{-1}, 100-350 ng band^{-1} and 500-1750 ng band^{-1}, respectively. The % assay (Mean ± S.D.) was found to be 100.63 % ± 1.2, 103.46 ± 1.58 and 101.85 % ± 1.92 for PARA, DCL and CHL, respectively. Method was validated for linearity, accuracy, precision, specificity, robustness in accordance with International Conference on Harmonisation (ICH) guidelines.

Conclusion: The proposed HPTLC method has been successfully applied for the analysis of drugs in tablet dosage formulation and applicable to routine analysis PARA, DCL and CHL in bulk drug and tablet dosage form.

Keywords: Paracetamol, Diclofenac potassium, Chlorzoxazone, Validation, High Performance Thin Layer Chromatography.

INTRODUCTION
Paracetamol, (p-hydroxy acetanilide) is a compound with analgesic and antipyretic activity [1, 2]. Diclofenac potassium is potassium-[2, 6-dichlorophenyl] amino] phenylacetate. It is potassium salt of an aryl acetic acid derivative and official in USP [3-6]. It inhibits prostaglandin synthesis by interfering with the action of prostaglandin synthetase (cyclooxygenase) [7, 8]. Chlorzoxazone, (5-chloro-2(3H)-benzoxazolone), is a skeletal muscle relaxant used to decrease muscle tone and tension and thus relieve spasm and pain associated with musculoskeletal disorders [9, 10]. Literature survey revealed various analytical methods for analysis of paracetamol [11-16], diclofenac potassium [17] and chlorzoxazone [18-20], either individually or in combination with other drugs. The objective of the present research study was to develop a simple, reliable, rapid, sensitive, and accurate procedure for simultaneous densitometric analysis of paracetamol, diclofenac potassium and chlorzoxazone in combined tablet dosage form.

MATERIALS AND METHODS

Chemicals and reagents
Reference standards of paracetamol, diclofenac potassium and chlorzoxazone were gifted by Emcure Pharmaceuticals Ltd. (Pune), R.P. Drugs Ltd., (Pune, India) and Aristo Pharma Ltd. (Mumbai), respectively. Brand of tablet DICLOPOT-MR [500 mg paracetamol], 50 mg diclofenac potassium and 250 mg chlorzoxazone was purchased from local market. All chemicals and reagents used were of analytical grade and purchased form Merck Chemicals Corporation Ltd. Mumbai, India.

Chromatographic system and conditions
HPTLC analysis were carried out on precoated silica gel aluminium plate 60F_{254} (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) in the form of bands of 6 mm width with a Hamilton syringe (100 µL) using a Camag Linomat V (Switzerland) sample applicator. Preflashing of HPTLC plate was done with methanol followed by activation in an hot air oven at 105°C for 20 min, then cooling to room temperature. The slit dimension was kept at 5mm × 0.45 mm and scanning speed of 10 mm/s was employed. Plate was then developed, at constant temperature, with 20 mL mobile phase consisting of toluene: ethyl acetate [55:45, v/v]. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The chamber saturation time for mobile phase was 20 min at room temperature (25 ± 2°C) at relative humidity of 60 ± 5%. The length of chromatographic run was 8 cm. After development the plate was removed from the chamber and air-dried followed by densitometric scanning at 271 nm using a Camag TLC Scanner-3 with winCATS software version 1.4.4 in the reflectance mode.

Preparation of standard stock solutions
Preparation of Linearity Solutions
Standard stock solutions were prepared by dissolving 250 mg of paracetamol, 2.5 mg diclofenac potassium and 125 mg chlorzoxazone in 25 ml methanol, separately. The resulting solutions were used for further analysis.

Preparation of standard Solutions for assay
Standard stock solutions for assay were prepared by dissolving 500 mg of PARA, 50 mg DCL and 250 mg CHL in 100 ml methanol, separately. The stock solutions were further diluted with methanol to obtain a concentration of 500 µg/ml for PARA, 50 µg/ml for DCL and 250 µg/ml for DCL.

Selection of detection wavelength
After chromatographic development bands were scanned over the range of 200-400 nm and the spectra were overlain. It was observed that paracetamol, diclofenac potassium and chlorzoxazone showed considerable absorbance at 271 nm and hence it was selected as the wavelength for detection (Fig1).

Method validation
The proposed HPTLC method was optimized and validated as per ICH Q2 (R1) guidelines [21].

Linearity
For preparation of calibration plots appropriate dilutions of the stock solution were prepared and applied to HPTLC plate to give a...
concentration range of 1000-3500 ng spot⁻¹ for PARA, 100-350 ng spot⁻¹ for DCL, 500-1750 ng spot⁻¹ for CHL. The plate was developed as per the above mentioned method and scanned densitometrically. Each standard in six replicates was estimated and peak areas were recorded. Peak area versus concentration was subjected to least square linear regression analysis and the intercept, slope and correlation coefficient for the calibration were determined.

**Fig. 1:** Overlaid UV spectrum of paracetamol, diclofenac potassium and chlorzoxazone

**Precision**

One set of three different concentrations of mixed standard solutions of paracetamol, diclofenac potassium and chlorzoxazone were prepared. All the solutions were analyzed thrice, in order to record any intraday variations in the results. For inter day variations study, three different concentrations of mixed standard solutions in linearity range were analyzed on three consecutive days. The peak areas were recorded and relative standard deviation (RSD) was calculated.

**Accuracy**

Recovery studies were carried out by standard addition method in which the known amount of standard solution was added to preanalyzed sample solution at three different levels 80 %, 100 % and 120 %. These samples were then analyzed as per the procedure mentioned above and percentage recoveries estimated.

**Limit of detection (LOD) and limit of quantitation (LOQ)**

Limit of detection (LOD) and limit of quantitation (LOQ) were determined as 3.3 σ/S and 10 σ/S, respectively. Where S is the slope of the calibration plot and σ is the standard deviation of the response (y-intercept).

**Robustness studies**

The effect of small, deliberate variation of the analytical conditions on the peak areas of the drugs was examined. Factors varied were mobile phase composition (± 0.1 %) volume of mobile phase (± 0.5 %), time from application to development (+ 20 min) and from development to scanning (+ 20 min). One factor at a time was changed to study the effect. The robustness of the method was tested at 500 µg/ml for paracetamol, 50 µg/ml for diclofenac potassium and 250 µg/ml for chlorzoxazone, respectively. The RSD (%) of peak area was calculated for each change of condition and was found to be within the range stipulated by ICH guidelines.

**Specificity**

Specificity of the method was assessed by comparing the densitograms obtained from standard drugs with those obtained from sample solutions. The developed method was found to be specific as no interference from excipients was found.

**Assay of the marketed formulations**

Twenty tablets were accurately weighed, their average weight was calculated and then finely powdered. A portion equivalent to about one tablet was accurately weighed and transferred to a 100 ml volumetric flask containing 50 ml methanol. It was ultrasonicated for 30 min and then diluted to 100 ml with methanol. The sample solution was then filtered using 0.45 µ filter (Millipore, Milford, MA). The stock solution was further diluted to obtain final concentration of 500 µg/ml for paracetamol, 50µg/ml for diclofenac potassium and 250 µg/ml for chlorzoxazone, respectively. Procedure was repeated six times for the analysis of marketed formulation.

**Fig. 2:** Representative densitogram of mixed standard solution of PAR, CHL and DCL.
RESULTS AND DISCUSSION

Different mobile phases containing various ratios of methanol, ethanol, ethyl acetate, acetone and toluene were examined. After analysis of the results the mobile phase containing toluene:ethyl acetate [55:45, v/v] was selected for analyses. The wavelength used for detection and quantitation was 271 nm. The retention factors for paracetamol, diclofenac potassium and chlorzoxazone were found to be 0.21 ± 0.01, 0.54 ± 0.01, and 0.74 ± 0.01, respectively. Representative densitogram obtained from a mixed standard solution of paracetamol, diclofenac potassium and chlorzoxazone is shown in Fig 2. The results were found to be linear over a range of 1000-3500 ng band\(^{-1}\) for PAR, 100-350 ng band\(^{-1}\) for CHL and 500-1750 ng band\(^{-1}\) for DCL. The correlation coefficients (r) for the plots were 0.9987, 0.9985 and 0.9985 for PAR, CHL and DCL, respectively. Results of the accuracy study showed satisfactory recoveries of 99.89 - 100.09 %, 99.03 - 100.28 %, 99.74 - 100.18 % for PAR, CHL and DCL, respectively indicating the reliability of the proposed HPTLC method for the quantification of these drugs in the marketed formulation (Table 1). Robustness of the proposed HPTLC method checked after deliberate variation of the analytical parameters indicate that areas of peaks of interest and retention factor remained unaffected by small changes of the operational parameters (% RSD < 2) which demonstrated that the developed HPTLC method is robust. The % assay (mean ± S.D.) of marketed formulations was found to be 100.63 ± 1.2, 103.46 % ± 1.58 and 101.85 % ± 1.92 for paracetamol, diclofenac potassium and chlorzoxazone, respectively. The summary of validation parameters of proposed method are given in Table 3.

Table 1: Recovery studies of PAR, CHL and DCL.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount taken (ng/band)</th>
<th>Amount added (ng/band)</th>
<th>Total amount</th>
<th>Total Amount found (ng/band)</th>
<th>% Recovery</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>1500</td>
<td>1200</td>
<td>2700</td>
<td>2697.03</td>
<td>99.89</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>1500</td>
<td>3000</td>
<td>3000.3</td>
<td>100.01</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>1800</td>
<td>3300</td>
<td>3302.97</td>
<td>100.09</td>
<td>1.16</td>
</tr>
<tr>
<td>Diclofenac Potassium</td>
<td>150</td>
<td>120</td>
<td>270</td>
<td>270.756</td>
<td>100.28</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>150</td>
<td>300</td>
<td>297.57</td>
<td>99.19</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>180</td>
<td>330</td>
<td>326.799</td>
<td>99.03</td>
<td>1.23</td>
</tr>
<tr>
<td>Chlorzoxone</td>
<td>750</td>
<td>600</td>
<td>1350</td>
<td>1346.49</td>
<td>99.74</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>750</td>
<td>1500</td>
<td>1502.7</td>
<td>100.18</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>900</td>
<td>1650</td>
<td>1648.515</td>
<td>99.91</td>
<td>1.63</td>
</tr>
</tbody>
</table>

*Average of three determinations.

Table 3: Summary of validation parameters of proposed method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Paracetamol</th>
<th>Diclofenac Potassium</th>
<th>Chlorzoxazone</th>
</tr>
</thead>
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<tr>
<td>Linearity range</td>
<td>1000-3500</td>
<td>100-350</td>
<td>500-1750</td>
</tr>
<tr>
<td>Correlation coefficient (r(^2))</td>
<td>0.9987</td>
<td>0.9985</td>
<td>0.9985</td>
</tr>
<tr>
<td>LOD (ng/band)</td>
<td>122.92</td>
<td>13.50</td>
<td>65.81</td>
</tr>
<tr>
<td>LOQ (ng/band)</td>
<td>372.5</td>
<td>40.92</td>
<td>199.44</td>
</tr>
<tr>
<td>Accuracy (%) Recovery</td>
<td>99.99 ± 0.95</td>
<td>99.94 ± 1.31</td>
<td>99.5 ± 0.916</td>
</tr>
<tr>
<td>Robustness</td>
<td>Specific</td>
<td>Specific</td>
<td>Specific</td>
</tr>
<tr>
<td>Intraday (n = 6)</td>
<td>1.133</td>
<td>1.343</td>
<td>1.15</td>
</tr>
<tr>
<td>Interday (n = 6)</td>
<td>1.153</td>
<td>1.436</td>
<td>1.136</td>
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<tr>
<td>Specificity</td>
<td>Robust</td>
<td>Robust</td>
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</table>

CONCLUSION

The validated HPTLC method employed here proved to be simple, fast, robust, precise, accurate and thus can be used for routine analysis of paracetamol, diclofenac potassium, and chlorzoxazone in combined tablet dosage form.

ACKNOWLEDGEMENTS

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REFERENCES