3. MATERIALS AND METHODS

Pharmaceutical grade tolperisone hydrochloride, paracetamol, Rosuvastatin calcium, fenofibrate were taken as gift sample from Emcure Pharmaceuticals Limited, Pune, India and chlorpheniramine maleate, ambroxol hydrochloride, guaifenesin, phenylephrine hydrochloride were received from Centaur Pharmaceuticals Limited, Pune, and Maharashtra, India. They were used as it is without further purification and certified to contain 98.85 % of tolperisone hydrochloride, 99.09 % paracetamol, 98.87 % rosuvastatin calcium, 99.6 % fenofibrate, 99.28 % chlorpheniramine maleate, 99.8 % ambroxol hydrochloride, 98.5 % guaifenesin and 99.4% phenylephrine hydrochloride. In analysis marketed formulations used were Myotop P Tablet (tolperisone 150 mg, paracetamol 500 mg) Emcure Zuventus Healthcare Limited., Pune, India, Arvast F Tablet, (rosuvastatin calcium 10 mg, fenofibrate 67 mg) Intas Pharmaceuticals Limited, Ahmedabad, India, Solvin Cold tablet, (paracetamol 500 mg, guaifenesin 100 mg, ambroxol hydrochloride 30 mg, phenylephrine hydrochloride 10 mg, and chlorpheniramine maleate 2 mg) Ipca Laboratories Limited, Mumbai, India, were purchased from the market.

Analytical grade and HPLC grade chemicals and reagents were used for HPTLC work and for LC/MS/MS analyses respectively and were purchased from Merck Specialities Private Limited, India. Double distilled water filtered through 0.45 µ filter paper was used in analysis.

Chromatographic (HPTLC) system consisted of a Camag twin-trough chamber (20 × 10 cm), a Camag Linomat V sample applicator (Camag, Muttenz, Switzerland), Camag winCATS software (Version 1.4.4), with a 100 µL Camag syringe (Hamilton, Bonaduz, Switzerland). The sample application was done on aluminium HPTLC plates precoated with silica gel 60 F254 with dimensions 20 × 10 cm, thickness 250µm. HPTLC analysis was performed by using Camag TLC scanner III. The slit dimension was set at 5 × 0.45 mm with scanning speed 10 mm s⁻¹. The samples were spotted using a Camag 100 µL syringe in the form of bands with 6 mm width on HPTLC plate.
Tandem mass (LC/MS/MS) system comprised of a HPLC system -1260 infinity with (20 µL) auto injector. Agilent mass hunter workstation data acquisition software (version 1.18.03), Agilent mass spectrometric 6460 triple quadruple mass spectrometer, Poroshell 120 EC – C18 (4.6 x 50 mm 2.7 µ) column of Thermo Technologies Corporation, Japan was used.

Following are the details of mass spectrometry acquisitions

<table>
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<th>Sr. No.</th>
<th>Parameters</th>
<th>Details</th>
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Simultaneous determination of tolperisone hydrochloride and paracetamol in combined tablet dosage form by validated HPTLC method.

**Chromatographic Parameters**

The following optimized chromatographic parameters were used for analysis.

**Stationary phase**
Merck aluminum plates precoated with silica gel 60 F$_{254}$

**Mobile phase**
Toluene: ethyl acetate: methanol (1: 7: 3, v/v/v)

**Plate size**
20 X 10 cm

**Band size**
6 mm (Distance between two bands: 10 mm)

**Development chamber**
Twin-trough glass chamber, 20 X 10 cm with stainless steel lid

**Saturation time**
15 min

**Migration distance**
80 mm

**Room temperature**
25 ± 2 °C

**Scanning mode**
Absorbance/reflectance

**Slit dimensions**
5 × 0.45 mm

**Detection wavelength**
256 nm

\[ R_f = 0.39 ± 0.02 \text{ TOLP and } 0.79 ± 0.02 \text{ PCM} \]

**Method Validation:**

**Standard stock solutions preparation**
TOLP and PCM standard stock solutions were prepared separately by dissolving 5 mg each of standard drug in 10 mL methanol (solvent) to obtain a 500 μg/mL concentration. This solution was diluted, used for further analysis.

**Detection wavelength Selection**
Once chromatographic development over, bands were scanned over the range of 200-700 nm and the spectra were overlain. At 256 nm both drugs showed significant absorbance hence was selected for HPTLC analysis.
Sample solutions preparation
Twenty tablets were accurately weighed for analysis of marketed formulation. The average weight of tablets was calculated. Tablets were finely powdered. Powder equal to 50 mg of TOLP was weighed, transferred to volumetric flask (50 mL) containing 30 mL solvent methanol. Then solution was sonicated for 15 min, diluted up to the mark with methanol. The resulting solution was filtered using Whatman (No.41) filter paper.

Validation of analytical method
The developed HPTLC method was validated as per the ICH guideline for following validation parameters.
1. Linearity and Range
Stock solutions were applied on the HPTLC plate in the range of 50-800 ng/band and 100-800 ng/band for TOLP and PCM respectively, to evaluate linearity. The plate was developed using the optimized chromatographic conditions. Peak area against concentration was plotted, followed by least square linear regression analysis and the correlation coefficient, slope, intercept for the calibration were estimated. Residual analysis was also carried out to confirm linearity.

2. Detection limit (LOD) and quantification limit (LOQ)
LOD, LOQ was calculated using formula 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the linearity.

3. Precision studies
Precision was established by intra and inter-day precision studies. TOLP (100 ng/band) and PCM (333.33 ng/band) sample were prepared and analyzed six times on the same day in order to trace any variations in the results, to verify intraday analysis. For interday study, the above mentioned drug samples were analyzed on three successive days. Precision was measured in terms of RSD (%).

4. Robustness studies
To study robustness small, intentional changes in the analytical parameters (optimized) of the projected method were carried out and its effect on the peak areas of the drugs was observed. Parameters changed were solvent system amount (±5 %), solvent system composition (ethyl acetate ±0.1 mL), time (+10 min) from band application to chromatographic development and time (+10 min) from chromatography to scanning, only one parameter was changed at a time.
Concentration of 100 ng/band for TOLP and 333.33 ng/band PCM in six replicates were used to study the robustness of the method. The standard deviation of peak areas along with % RSD was determined.

5. Specificity

Specificity was confirmed by assessing test sample as well as standard drug. On a HPTLC plate TOLP and PCM standard solutions and the sample solutions were applied. The plate was developed in mobile phase and scanned. By comparing the UV spectrum of TOLP and PCM at three peak areas at start, apex and end positions of the band, the peak purity of drugs was tested.

6. Accuracy studies

Accuracy was performed by the % recoveries method where the known quantity of mixtures of TOLP and PCM added to solutions of marketed formulation. The samples were added with 80, 100 and 120 % of 100 ng/band of TOLP and 333.33 ng/band of PCM standard solutions. Recovery was calculated from the equation.

7. Solution stability

Solution stability of TOLP and PCM standard solutions (100 ng/band) was studied after 0, 6, 12, 24, 48 h of storage at room temperature. The stability of the solutions was determined by comparing peak areas against freshly prepared standard solutions at each time hour.

Analysis of marketed formulation

The proposed HPTLC method is applied to the marketed formulation to determine the content of TOLP and PCM in tablet formulation (Myotop-P tablets), Label claim: 150 mg of TOLP and 500 mg of PCM. Powder equivalent to 50 mg of TOLP was weighed, transferred to (50 mL) volumetric flask containing solvent (methanol), sonicated for 15 min and diluted up to the mark with methanol. From this stock solution, 1 mL was diluted to 10 mL (100 ug/mL) and used for further analysis. The % assay was determined.
HPTLC method for simultaneous determination of rosuvastatin calcium and fenofibrate in bulk and pharmaceutical dosage form.

**Stationary phase**  
silica gel 60 f$_{254}$ Merck aluminum plates

**Mobile phase**  
Ethyl acetate: acetic acid (20: 0.2, v/v)

**Plate size**  
20 X 10 cm

**Band size**  
6 mm (Distance between two bands: 10 mm)

**Development chamber**  
Twin-trough glass chamber, 20 X 10 cm with stainless steel lid

**Saturation time**  
15 min

**Migration distance**  
80 mm

**Room temperature**  
25 ± 2°C

**Scanning mode**  
Absorbance/reflectance

**Slit dimensions**  
$5 \times 0.45$ mm

**Detection wavelength**  
246 nm

\[ R_f = 0.31 \pm 0.02 \text{ ROS and } 0.76 \pm 0.02 \text{ FEN} \]

**Standard stock solutions preparation**

10 mg rosuvastatin and fenofibrate were precisely weighed, dissolved and diluted with methanol up to 100 mL separately, to get 100 μg/mL concentrations.

**Detection wavelength selection**

Different concentrations of rosuvastatin (ROS) and fenofibrate (FEN) were spotted on the plate and plate was developed under optimized chromatographic conditions. Drug bands (over the range of 200-700 nm) were scanned. Their UV-spectra were overlain. Both drugs showed significant absorbance at 246 nm hence was selected for further analysis.
Sample solutions preparation
Marketed twenty tablets were weighed exactly; average weight was calculated and finely powdered. Tablet powder equivalent to 10 mg of ROS and FEN was weighed, transferred to a volumetric flask (100 mL) and 50 mL of methanol solvent was added. The solution was sonicated for 30 min. Volume was made up of methanol and resulting solution was filtered using Whatman no.1 filter paper.

Method Validation
Validation of the developed densitometric method was performed in accordance with the (ICH) Q2 (R 1) guideline.

1. Linearity and Range
To evaluate linearity, stock solutions of ROS and FEN were applied on the HPTLC plate in the range of 50-800 ng/band. The HPTLC plate was developed using the optimized mobile phase. Peak area against concentration was plotted, and it is subjected to least square linear regression analysis to obtain correlation coefficient, intercept, slope for the calibration. Residual analysis was carried out to confirm linearity.

2. LOD and LOQ
Standard deviation-slope method was applied for estimation of detection and quantitation limit. Formula 3.3 $\sigma$/S and 10 $\sigma$/S, used to determine LOD and LOQ respectively. Where, S is the slope of the linearity plot and $\sigma$ is the standard deviation of the response.

3. Precision
Repeatability and intermediate precision studies were carried out to verify precision of the method. 100 ng/band concentrations, six times on the same day for both ROS and FEN were used to perform repeatability studies. The intermediate precision of the method was checked by repeating studies on three consecutive days. % RSD of peak areas was calculated.

4. Robustness
Little but intentional variations in the optimized method parameters were made and its effects on the results were observed. Factors like mobile phase composition, amount of mobile phase, time from band application to chromatographic development and time from chromatography to scanning were varied. Single parameter was changed at a time.
100 ng/band concentrations of both drugs analyzed six times to determine robustness of the HPTLC method. The % RSD and standard deviation of peak areas were calculated.

5. **Specificity studies**

Sample and standard solutions of ROS and FEN were applied on to a HPTLC plate to prove specificity of the method. The plate was developed, scanned as mentioned above. The band for ROS and FEN in the samples was confirmed by comparing the Rf and spectrum of the band of a standard drug. By comparing the spectrum at different regions (i.e. peak start (S), peak apex (M) and peak end (E) of the band) the peak purity of drugs was determined.

6. **Accuracy**

The standard addition method was used to determine the accuracy of ROS and FEN. Known amounts of ROS and FEN standard solutions were spiked at 80, 100 and 120 % to the selected concentration of sample solution (100 ng/band for ROS and FEN). Dilutions were done accordingly.

**Study of Solution stability**

To confirm the stability, standard solutions of ROS and FEN of 100 ng/band were tested after specific time period (0, 6, 12, 24 and 48 h of storage) at room temperature. The stability of the solutions was estimated by comparing peak areas of ROS and FEN against freshly prepared standard solutions at each time hour.

**Marketed tablet form analysis**

The HPTLC analysis was carried out on tablet dosage form i.e., Arvast F Tablet (10 mg ROS and 67 mg FEN) by Intas Pharmaceuticals Limited, (Ahmadabad), India. To perform assay, twenty tablets were weighed accurately and the average weight was calculated. The tablets were finely powdered. Tablet powder equivalent to 10 mg of ROS was accurately weighed, transferred to a 100 mL calibrated volumetric flask. Approximately 50 mL of methanol was added, and the solution was sonicated for 30 min. After volume adjustment the solution was filtered and the assay was performed.
Tandem mass (LC-MS/MS) method development and validation for the concurrent analysis of paracetamol, guaifenesin, phenylephrine hydrochloride, chlorpheniramine maleate, and ambroxol hydrochloride in bulk and combined tablet formulation.

LC/MS/MS analysis of this combination of drugs was performed by using following optimized chromatographic parameters.

**LC Conditions:**

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Gradient mobile phase of water: methanol</th>
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</thead>
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<td>Time (min.)</td>
<td>(%)/Water (%)</td>
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</tr>
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<td>4.00</td>
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<td>5.01</td>
<td>60 (40)</td>
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<tr>
<td>9.00</td>
<td>60 (40)</td>
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</table>

**Column**

C18 (4.6 × 50 mm 2.7 µ) – Poroshell 120 EC

**Column temperature**

20 °C

**Flow rate**

0.3 (mL/min)

**Injection volume**

5 µL

**Run time**

9 min

**MS Conditions:**

**Gas temperature**

250 °C

**Gas flow**

11 (Lit/min)

**Nebulizer**

45 psi

**Sheath gas heater**

300 °C
**Materials and Methods**

**Sheath gas flow** 9 (Lit/min)

**Nozzle voltage** 500 ev

**Software system** Agilent mass hunter workstation data acquisition

**Mass spectrometer** 6460 Triple quadruple

**Standard solution preparation**
10 mg of accurately weighed PCM, GUA, PE, CPM, and AMB were dissolved in the methanol: water solvent mixture in 80:20, v/v ratio to obtain 1000 µg/mL concentration of standard solution. These stock solutions were properly diluted and used for further analysis.

**Sample solution preparation**
The marketed formulation Solvin Cold tablets were used in the research study. Tablets (Twenty) were weighed precisely. The average weight was calculated and then tablets were powdered finely. Powder equivalent to 1 mg of CPM was accurately weighed, transferred to a volumetric flask (100 mL). 70 mL of diluent was added. The solution was sonicated for 30 min and the volume was adjusted to the mark with the solvent. The solution was filtered, properly diluted and used in the analysis.

**LC/MS/MS Method Development**
Methanol: water (80:20 v/v) mixture is used to dissolve all drugs as it was found to be a common solvent. For the selection of mobile phase 10 ng/mL concentrations of all standards were used and injected into the column. Different solvents including water and methanol in different ratios were used in isocratic mode but results were non-reproducible. Hence the gradient system was tried with the methanol, water mobile phase. The composition of the mobile phase was optimized through several trials to get a linear response for concentration, symmetrical peak shape, and selective MRM transition.
Materials and Methods

Method Validation (LC/MS/MS):
The tandem mass spectrometric method was validated with respect to ICH guideline.

1. Linearity
Successive dilutions in a concentration range of 10-200 ng/mL were prepared from 1000 µg/mL standard stock solutions of 5 drugs. Solutions were injected, developed through optimized chromatographic conditions. Five concentrations were determined in hexaplicate. To get calibration curves concentrations vs. peak response were plotted.

2. Detection limit and quantification limit
Detection limit and quantification limit were calculated by formulae using the standard deviation of y-intercept and slopes of calibration curves.

3. Precision studies
The precision of the proposed method was established by intraday and inter day precision. Intraday studies (100 ng/mL) were performed six times on the same day for all drugs. To study inter day precision of the method a concentration of 100 ng/mL of each drug was analyzed for 3 consecutive days. The (%) relative standard deviation was calculated to assess precision.

4. Robustness
Small but deliberate changes in the chromatographic conditions were done and the results were examined to estimate robustness of the method. The result of alteration in mobile phase ratio and flow rate on peak areas was studied. For all drugs 100 ng/mL solutions was injected in triplicate into sample injector. The % relative standard deviation of peak areas was calculated.

5. Specificity
The specificity of the LC/MS/MS method was studied in Multiple Reaction Monitoring mode. The standard, blank, and sample solutions of PCM, GUA, PE, CPM, and AMB were injected in the LC/MS/MS system and the retention time values for the respective drugs were studied.

6. Recovery studies
Recovery was determined through the % recoveries where the known amount of mixture of PCM, GUA, PE, CPM, and AMB, added to the solution of marketed formulation. Sample stock solution was prepared from tablet formulation. 80, 100 and
120% of the standard drug solutions were added to the sample solution diluted suitably and studies were performed.

**Stability of solution**

The stability of all standard solutions of 10 ng/mL was tested after 0, 6, 12, 24 and 48 h of storage at room temperature. The stability of the solutions was determined by comparing peak response against freshly prepared standard solutions at each time hour.

**Marketed formulation analysis**

The drug content of Solvin cold tablet was determined in hexaplicate. Twenty tablets were weighed accurately; their average weight was calculated and then powdered finely. Tablet powder equivalent to 1 mg of CPM was precisely weighed and transferred to a volumetric flask (100 mL). 70 mL of methanol was added, and the solution was sonicated for 30 min. Volume was adjusted to the mark with the solvent. The solution was filtered and further diluted to get a concentration of 10, 50, 150 ng/mL for CPM, PE, AMB and 25, 125 ng/mL for GUA, PCM used in the study. The percent content of each drug was determined.