Analytical Method Development and Validation for Simultaneous Estimation of Tolperisone Hydrochloride and Diclofenac Sodium in Bulk and Pharmaceutical Formulation

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ABSTRACT
Quantitative analysis of any drug is an important tool in an industry. It is important to determine that the raw material, intermediate products as well as final products meet its specifications and are of required quality. The number of drugs and drug formulations introduced into the market has been increasing at an alarming rate. These drugs or formulations may be either new entities or partial structural modification of the existing ones or novel dosage forms.
Spectrophotometry and HPLC methods are considered to be most suitable for estimation of the drugs present in pharmaceutical dosage forms.
· Literature review reveals that several spectroscopic and Chromatographic method have been reported for estimation of TOL and DIC alone as well as with other drugs.
· Simultaneous equation, dual wavelength difference UV spectrophotometry and First derivative spectroscopic method is available for this combination.
· The aim of work is to develop and validate cost effective First derivative method in water and RP-HPLC method for simultaneous estimation of TOL and DIC in bulk and Tablet dosage form.
· Development of UV spectrophotometric method.

Keywords: Tolperisone, Diclofenac, Analytical Method, Validation

INTRODUCTION
Muscle spasms, which can affect any part of the body, are an involuntary contraction in the muscle tissue. Depending on the muscle’s size and location, it might be sharp and painful or nearly imperceptible. A series of spasms or permanent spasms are called a spasmism. A spasm may lead to muscle strains or tears of tendons and ligaments, if the force of the spasm exceeds the tensile strength of the underlying connective tissues, such as with a particularly forceful spasm, or in the case of weakened connective tissues. An effective treatment might come from physical therapy, dietary changes, medical intervention, or some combination of the three.
Most muscle spasms fall into one of two categories[1]. There may not be enough of certain chemicals necessary for a muscle to function properly, called electrolytes, which can cause nerve signals to not travel correctly. Alternately, the nerve that triggers the muscle might be at fault, whether due to a problem with the nerve itself or with the brain. The common denominator is that the muscle is contracting inappropriately and without the person’s control[2].

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In medicine a spasm is a sudden, involuntary contraction of a muscle, a group of muscles, or a hollow organ such as a heart, or a similarly sudden contraction of an orifice. It most commonly refers to a muscle cramp which is often accompanied by a sudden burst of pain, but is usually harmless and ceases after a few minutes. There is a variety of other causes of involuntary muscle contractions, which may be more serious, depending on the cause.

**Causes of Muscle Spasm**[^3]

There are a number of reasons for muscle spasms. These include:
- Muscular fatigue, overuse or excessive stretching of muscles and prolonged periods of no movement – eventually, muscle cells run out of energy and fluid, become hyper excitable and develop a forceful contraction/spasm involving part of a muscle, the whole muscle, or even adjacent muscles.
- Dehydration and depletion of electrolytes also lead to muscle spasm and cramping.
- Abnormal supply of water, glucose, sodium, potassium, calcium, and magnesium upsets protein regulation required for normal contraction causing a muscle spasm.
- Systemic illnesses like diabetes, low red blood cell count, kidney disease and other hormonal concerns are potential causes of muscle spasms.

**Classification of Drug Used for Muscle Spasms**[^4]:

Table 1: Classification of drugs used for Muscle Spasms

<table>
<thead>
<tr>
<th>Peripherally acting Muscle relaxants</th>
<th>Non-depolarizing agent</th>
<th>Curare alkaloids</th>
<th>Tubocurarine, Dimethyltubocurarine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Depolarizing agent</td>
<td>Choline derivatives</td>
<td>Succinylcholine,</td>
</tr>
<tr>
<td></td>
<td>Ach release inhibitors</td>
<td></td>
<td>Botalinum toxin</td>
</tr>
<tr>
<td>Centrally acting Muscle relaxants</td>
<td>Carbamic esters</td>
<td>Meprobamate, Methocarbamol, Tybamate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzodiazepines</td>
<td>Diazepam, Lorazepam, Nitrazepam</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anticholinergics</td>
<td>Orphenadrine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piperidine derivatives</td>
<td>Tolperisone, Eperisone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Quinine, Baclofen, Thiocolchicoside</td>
<td></td>
</tr>
<tr>
<td>Directly acting Muscle relaxants</td>
<td></td>
<td></td>
<td>Dantrolene</td>
</tr>
<tr>
<td>NSAIIDS</td>
<td></td>
<td></td>
<td>Diclofenac, Ibuprofen, Lorxicam</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHOD**

Table 2: Materials

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Ingredient</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tolperisone (TOL)</td>
<td>Orbit pharmaceuticals Ltd, Ahmedabad</td>
</tr>
<tr>
<td>2</td>
<td>Diclofenac Sodium (DIC)</td>
<td>Orbit pharmaceuticals Ltd, Ahmedabad</td>
</tr>
<tr>
<td>3</td>
<td>Ortho Phosphoric acid</td>
<td>AR grade</td>
</tr>
<tr>
<td>4</td>
<td>Tri ethylamine</td>
<td>AR grade</td>
</tr>
<tr>
<td>5</td>
<td>Acetonitrile</td>
<td>HPLC grade, Merck</td>
</tr>
<tr>
<td>6</td>
<td>Methanol</td>
<td>HPLC grade, Merck</td>
</tr>
<tr>
<td>7</td>
<td>Water</td>
<td>HPLC grade</td>
</tr>
</tbody>
</table>
EXPERIMENTAL METHOD
DEVELOPMENT AND VALIDATION OF FIRST ORDER DERIVATIVE SPECTROSCOPIC METHOD FOR ANALYSIS OF TOLPERISONE HYDROCHLORIDE AND DICLOFENAC SODIUM IN TABLET

Apparatus and Instruments
- Double beam UV-visible Spectrophotometer: Shimadzu, 1800.
  · System controller: UV Probe 2.31
  · Mode: Spectrum
  · Scan speed: Medium
  · Wavelength range: 400-200 nm
- Weighing balance: Shimadzu AUX 220
- Ultra Sonicator
- Borosil-Volumetric flasks of 10, 25, 50 and 100 ml (Borosil)
- Pipettes of 1, 2, 5 and 10 ml (Borosil)

Method Development:
Determination of the zero crossing points (Selection of wavelength)
From the overlaid first order derivative spectra of both the drug, DIC and TOL showed zero crossing at 248 and 226 nm respectively. At 248 nm DIC showed zero absorbance and TOL showed reasonable absorbance, while at 226 nm TOL showed zero absorbance and DIC showed reasonable absorbance. So these two wavelengths were selected for further measurement.

Method Validation [6-8]
As per ICH guidelines Q2 (R1), the method validation parameters studied were so, linearity, accuracy, precision, limit of detection and limit of quantitation.

Linearity:
D1 Absorbance of standard solutions of DIC (2, 4, 6, 8, 10 µg/ml) were measured at ZCP of TOL (226 nm) and D1 Absorbance of standard solutions of TOL (5, 10, 15, 20, 25 µg/ml) were measured at ZCP of DIC (248 nm). D1 Absorbance for both the drugs were plotted against their respective concentrations to get linear regression line.

Precision
The repeatability was checked by repeatedly (n = 6) measuring D1 absorbances of DIC (6 µg/ml) and TOL (15 µg/ml).
The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of DIC (2, 6 and 10 µg/ml) and TOL (5, 10 and 15 µg/ml) respectively. The results were reported in terms of relative standard deviation.

Accuracy (Recovery study)
The accuracy of the method was determined by calculating recovery of DIC and TOL by the standard addition method. Known amounts of standard solutions of DIC (0, 2, 4 and 6) and TOL (0, 5, 10 and 15) were added to prequantified sample solution of DIC (4 µg/ml) and TOL (10 µg/ml). The solutions were measured at 226 nm for DIC and 248 nm for TOL and % recovery of the each sample was calculated.

Limit of Detection and Limit of Quantification
Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of intercept (σ) and slope (S) of the calibration curve.
LOD = 3.3 x σ/S
LOQ = 10 x σ/S
Where, σ = the standard deviation of the response and S = slope of the calibration curve.

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF TOL AND DIC
Apparatus and instrumentation
- HPLC: Shimadzu 20-AT
Column: BDS hypersil C18, (250mm × 4.6mm, 5µ)
Manual Injector: Rheodyne Injector (Fixed Capacity Loop of 20 µl)
Syringe: Hamilton syringe
Pump: Binary pump, (Shimadzu, LC 20 AT)
Detector: UV detector (PET), (SPD 20 AT)
Weighing balance: Shimadzu AUX 220
Digital pH meter: Chemiline
Sonicator: Ultra sonicator
Pipettes of 1, 2, 5 and 10 ml (Borosil)
Volumetric flasks of 10, 25, 50 and 100 ml (Borosil)
Measuring cylinder of 100 ml. (Borosil)

Linearity
Standard diluted stock solutions (0.2, 0.4, 0.6, 0.8, and 1.0 ml equivalent to 2.0, 4.0, 6.0, 8.0 and 10.0 µg/ml of DIC and 0.6, 1.2, 1.8, 2.4 and 3.0 ml equivalent to 6.0, 1.2, 1.8, 2.4 and 3.0 µg/ml of TOL) were transferred in a series of 10 mL volumetric flasks and diluted to the mark with methanol. An aliquot (20 µl) of each solution was injected under the operating chromatographic conditions as described earlier [9]. Chromatograms were recorded. Methanol (20 µl) blank was also injected under the same conditions and chromatogram of methanol was recorded for the correction of the response of methanol in the chromatograms containing responses of DIC and TOL. Calibration curves were constructed by plotting peak areas versus concentrations, and the regression equations were calculated. Each response was average of three determinations [10].

Precision
Repeatability was checked by repeatedly (n = 6) injecting the solution containing DIC (6 µg/ml) and TOL (18 µg/ml) and recording the chromatograms [11].

Intra-day and inter-day precisions of the developed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentration of DIC (3.0, 6.0 and 9.0 µg/ml) and TOL (9.0, 18.0 and 27.0 µg/ml).

Accuracy
Accuracy of the method was determined by calculating percentage recovery of DIC and TOL by the standard addition method. Known amount of standard solutions of DIC (0, 4.8, 6 and 7.2 µg/ml) and TOL (0, 14.4, 18 and 21.6 µg/ml) were added to a pre-analyzed sample solution of DIC (6 µg/ml) and TOL (18 µg/ml). Each solution (20 µl) was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves [13].

Limit of Detection and Limit of Quantification
Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of intercept (σ) and slope (S) of the calibration curve [14].
LOD = 3.3 x σ/S
LOQ =10 x σ/S

RESULTS AND DISCUSSION
Method Development
The working standard solution of DIC and TOL were prepared separately in distilled water. They were scanned in the wavelength range of 200-400 nm. From the overlaid first order derivative spectra of both the drugs, it was observed that DIC and TOL show a zero crossing point at 248 nm and 226 nm respectively. These two wavelengths were employed for the determination of DIC and TOL. Overlain derivative spectra of both the drugs are shown in Figure 1.
VALIDATION OF THE DERIVATIVE SPECTROSCOPY METHOD

Linearity
The Beer’s law was obeyed. Linear correlation was obtained between $D_1$ absorbance and concentration of DIC (2-10 µg/ml) and TOL (5-25 µg/ml). The linearity of the calibration curve was validated by the value of correlation coefficient of the regression ($r$). The optical and regression characteristics are listed in Table 3.
Table 3: Optical and regression characteristics (n=3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DIC</th>
<th>TOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/ml)</td>
<td>2-10</td>
<td>5-25</td>
</tr>
<tr>
<td>Linearity equation</td>
<td>( y = -0.0263x + 0.0108 )</td>
<td>( y = 0.0237x + 0.0077 )</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.1886</td>
<td>0.3111</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.5659</td>
<td>0.9429</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9973</td>
<td>0.9954</td>
</tr>
</tbody>
</table>

**Precision**

The % RSD for repeatability of DIC and TOL were found to be 1.8618 and 0.8999 respectively. The value of % RSD for intra-day precision was found to be in the range of 0.93 – 1.06% and inter-day precision was found to be in the range of 1.19 - 1.31%, which indicated that the method was precise.

Table 4: Repeatability Data (n=6)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Concentration (µg/ml)</th>
<th>D1 Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DIC</td>
<td>TOL</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>

Mean: -0.1428  0.369
SD: 0.0021  0.0033
%RSD: 1.8618  0.8999
Table 5: Intraday precision data for DIC and TOL

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>D$_1$ Abs Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
<th>Conc (µg/ml)</th>
<th>D$_1$ Abs Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-0.045 ± 0.00057</td>
<td>1.273</td>
<td>5</td>
<td>0.120 ± 0.001</td>
<td>0.8333</td>
</tr>
<tr>
<td>6</td>
<td>-0.139 ± 0.0015</td>
<td>1.096</td>
<td>15</td>
<td>0.366 ± 0.0035</td>
<td>0.95778</td>
</tr>
<tr>
<td>10</td>
<td>-0.255 ± 0.0030</td>
<td>1.194</td>
<td>25</td>
<td>0.586 ± 0.0045</td>
<td>0.7686</td>
</tr>
</tbody>
</table>

Table 6: Interday precision data for DIC and TOL

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>D$_1$ Abs Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-0.041 ± 0.00057</td>
<td>1.385</td>
</tr>
<tr>
<td>6</td>
<td>-0.142 ± 0.0015</td>
<td>1.073</td>
</tr>
<tr>
<td>10</td>
<td>-0.257 ± 0.0035</td>
<td>1.364</td>
</tr>
</tbody>
</table>

Accuracy

The recovery experiments were performed by the standard addition method. The mean recoveries were found to be 99.087 – 100.35 % and 99.93 – 100.46% for DIC and TOL, respectively. The recoveries results indicate that the proposed method is accurate. Results of recovery studies are shown in Table 5 and 6

Table 7: Recovery data of DIC (n = 3)

<table>
<thead>
<tr>
<th>Level</th>
<th>Sample Conc. (µg/ml)</th>
<th>Amt of Drug added (µg/ml)</th>
<th>Total Conc. (µg/ml)</th>
<th>Amt of Drug recovered (µg/ml)</th>
<th>Recovery %</th>
<th>Mean ± SD (%)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>5.922</td>
<td>98.716</td>
<td>100.35 ± 1.457</td>
<td>1.452</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>6.091</td>
<td>101.517</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>6.049</td>
<td>100.817</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8.047</td>
<td>100.599</td>
<td>99.401 ± 1.068</td>
<td>1.074</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>7.883</td>
<td>98.545</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>7.924</td>
<td>99.059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>10.013</td>
<td>100.13</td>
<td>99.087 ± 0.984</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>9.895</td>
<td>98.957</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8: Recovery data of TOL (n = 3)

<table>
<thead>
<tr>
<th>Sample Conc. (µg/ml)</th>
<th>Amt of Drug added (µg/ml)</th>
<th>Total Conc. (µg/ml)</th>
<th>Amt of Drug recovered (µg/ml)</th>
<th>Recovery %</th>
<th>Level</th>
<th>Mean ± SD (%)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>15</td>
<td>14.851</td>
<td>99.00</td>
<td>50%</td>
<td>99.93 ± 0.803</td>
<td>0.806</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>15</td>
<td>15.054</td>
<td>100.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>15</td>
<td>15.065</td>
<td>100.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>20</td>
<td>20.173</td>
<td>100.86</td>
<td>100%</td>
<td>100.88 ± 1.637</td>
<td>1.623</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>20</td>
<td>19.853</td>
<td>99.265</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LOD and LOQ
LOD and LOQ values for DIC found to be 0.1886 and 0.5659 µg/ml at 226 nm, and TOL were found to be 0.3111 and 0.9429 µg/ml at 248 nm. Low value of LOD & LOQ indicates that the method is sensitive. Results are shown in Table 6

Analysis of Tablet Dosage Form
The proposed UV spectrophotometric method was successfully applied for determination of DIC and TOL in tablet dosage form. The percentage of DIC and TOL were found to be satisfactory, which was comparable with the corresponding label claim.

Table 9: Analysis of DIC and TOL in Tablet dosage form (n=3)

<table>
<thead>
<tr>
<th>TOLERITAS-D®</th>
<th>Label claim (mg)</th>
<th>Amount found (mg)</th>
<th>% Label claim (mg) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC TOL</td>
<td>DIC TOL</td>
<td>DIC TOL</td>
<td>DIC TOL</td>
</tr>
<tr>
<td>1</td>
<td>50 150</td>
<td>4.93 14.52</td>
<td>98.6 98.00</td>
</tr>
<tr>
<td>2</td>
<td>50 150</td>
<td>5.01 14.90</td>
<td>100.2 99.34</td>
</tr>
<tr>
<td>3</td>
<td>50 150</td>
<td>5.08 14.99</td>
<td>101.6 99.93</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td></td>
<td>100.13 99.09</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>1.501 0.988</td>
</tr>
<tr>
<td>% RSD</td>
<td></td>
<td></td>
<td>1.49 0.99</td>
</tr>
</tbody>
</table>

RESULT AND DISCUSSION
Table 10: Trials for the selection of different mobile phase

<table>
<thead>
<tr>
<th></th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DIC Water : Methanol (50 : 50)</td>
</tr>
<tr>
<td>2</td>
<td>TOL Water : Methanol (50 : 50)</td>
</tr>
<tr>
<td>3</td>
<td>DIC - TOL Water : Methanol (30 : 70)</td>
</tr>
<tr>
<td>4</td>
<td>DIC - TOL Water : ACN (30 : 70)</td>
</tr>
<tr>
<td>5</td>
<td>DIC - TOL Water : ACN (15 : 85)</td>
</tr>
<tr>
<td>6</td>
<td>TOL Buffer (pH 4.5) : ACN (30 : 70)</td>
</tr>
<tr>
<td>7</td>
<td>DIC - TOL Buffer (pH 4.5) : ACN (30 : 70)</td>
</tr>
<tr>
<td>8</td>
<td>DIC - TOL Buffer (pH 4.5) : ACN (40 : 60)</td>
</tr>
<tr>
<td>9</td>
<td>DIC - TOL Buffer (pH 4.5) : ACN (50 : 50)</td>
</tr>
<tr>
<td>10</td>
<td>DIC - TOL Buffer (pH 5.0) : ACN (60 : 40)</td>
</tr>
<tr>
<td>11</td>
<td>DIC - TOL Buffer (pH 4.0) : ACN (60 : 40)</td>
</tr>
<tr>
<td>12</td>
<td>DIC - TOL Buffer (pH 4.0) : ACN (60 : 40)</td>
</tr>
<tr>
<td>13</td>
<td>DIC - TOL Buffer (pH 3.5) : ACN (60 : 40)</td>
</tr>
<tr>
<td>14</td>
<td>DIC - TOL Buffer (pH 3.5) : ACN (60 : 40)</td>
</tr>
<tr>
<td>15</td>
<td>DIC - TOL Buffer (pH 3.5) : ACN (50 : 50)</td>
</tr>
</tbody>
</table>
Fig 6: Chromatogram of DIC Mobile Phase – Water: Methanol (50: 50 v/v)

Fig 7: Chromatogram of TOL Mobile Phase – Water: Methanol (50: 50 v/v)

Fig 8: Chromatogram of DIC – TOL Mobile Phase – Water: Methanol (30: 70 v/v)

Fig 9: Chromatogram of DIC – TOL Mobile Phase – Water: ACN (30: 70 v/v)
Fig 10: Chromatogram of DIC – TOL Mobile Phase – Water: ACN (15: 85 v/v)

Fig 11: Chromatogram of TOL Mobile Phase – 20 mM Phosphate Buffer (pH 4.5): ACN (30: 70 v/v)

Fig 12: Chromatogram of DIC – TOL Mobile Phase – 20 mM Phosphate Buffer (pH 4.5): ACN (30: 70 v/v)

Fig 13: Chromatogram of DIC – TOL Mobile Phase – 20 mM Phosphate Buffer (pH 4.5): ACN (40: 60 v/v)
Fig 14: Chromatogram of DIC – TOL Mobile Phase – 20 mM Phosphate Buffer (pH 4.5): ACN (50: 50 v/v)

Fig 15: Chromatogram of DIC – TOL Mobile Phase – 20 mM Phosphate Buffer (pH 4.5): ACN (60: 40 v/v)

Fig 16: Chromatogram of DIC – TOL Mobile Phase – 20 mM Phosphate Buffer (pH 5.0): ACN (60: 40 v/v)

Fig 17: Chromatogram of DIC – TOL Mobile Phase – 20 mM Phosphate Buffer (pH 4.0): ACN (60: 40 v/v)
**METHOD DEVELOPMENT AND OPTIMIZATION**

Table 11: Optimized chromatographic conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chromatographic Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase</td>
<td>BDS hypersil C$_{18}$, (250mm × 4.6mm × 5µm)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>20 mM Phosphate Buffer (pH 3.5 ± 0.02 with OPA) : ACN (50:50 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Wave length</td>
<td>268 nm</td>
</tr>
<tr>
<td>Run time</td>
<td>20 min</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µl</td>
</tr>
<tr>
<td>Pump</td>
<td>LC-20AT</td>
</tr>
<tr>
<td>Detector</td>
<td>UV detector, SPD-20AT</td>
</tr>
<tr>
<td>Temperature</td>
<td>26 ± 2°C</td>
</tr>
</tbody>
</table>
METHOD DEVELOPMENT:

Validation of the HPLC method

Linearity:
Linear correlation was obtained between peak area and concentration of DIC and TOL in the range of 2-10 µg/ml and 6-30 µg/ml respectively, the linearity of the calibration curves were validated by the value of correlation coefficient of the regression (r), the regression analysis of the calibration curves is listed in Table 13.

Table 12: Linearity data for DIC

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Conc. (µg /ml)</th>
<th>Area Mean ± S.D. (n=6)</th>
<th>% R.SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2</td>
<td>815.013 ± 6.832</td>
<td>0.8421</td>
</tr>
<tr>
<td>2.</td>
<td>4</td>
<td>1281.084 ± 8.952</td>
<td>0.6954</td>
</tr>
<tr>
<td>3.</td>
<td>6</td>
<td>1629.355 ± 11.398</td>
<td>0.6983</td>
</tr>
<tr>
<td>4.</td>
<td>8</td>
<td>2007.033 ± 13.347</td>
<td>0.6609</td>
</tr>
<tr>
<td>5.</td>
<td>10</td>
<td>2450.012 ± 17.556</td>
<td>0.7218</td>
</tr>
</tbody>
</table>
Table 13: Linearity data for TOL

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Conc. (µg /ml)</th>
<th>Area Mean ± S.D. (n=6)</th>
<th>% R.SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>6</td>
<td>1144.916 ± 6.132</td>
<td>0.5329</td>
</tr>
<tr>
<td>2.</td>
<td>12</td>
<td>1711.452 ± 10.208</td>
<td>0.5938</td>
</tr>
<tr>
<td>3.</td>
<td>18</td>
<td>2289.258 ± 16.663</td>
<td>0.7236</td>
</tr>
<tr>
<td>4.</td>
<td>24</td>
<td>2805.833 ± 18.129</td>
<td>0.6427</td>
</tr>
<tr>
<td>5.</td>
<td>30</td>
<td>3445.196 ± 25.019</td>
<td>0.7273</td>
</tr>
</tbody>
</table>

Table 14: Optical and regression characteristics (n=3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DIC</th>
<th>TOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/mL)</td>
<td>2-10</td>
<td>6-30</td>
</tr>
<tr>
<td>Linearity equation</td>
<td>$202.95x + 406.25$</td>
<td>$94.916x + 570.85$</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>0.141</td>
<td>0.347</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>0.429</td>
<td>1.053</td>
</tr>
<tr>
<td>Correlation coefficient(r)</td>
<td>0.9995</td>
<td>0.9992</td>
</tr>
</tbody>
</table>

System Suitability Test:
Following parameters were calculated for system suitability of RP-HPLC method.

Table 15: Data of System suitability Parameters

<table>
<thead>
<tr>
<th>System suitability parameters</th>
<th>DIC</th>
<th>TOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tailing Factor</td>
<td>1.455</td>
<td>1.424</td>
</tr>
<tr>
<td>Theoretical Plates</td>
<td>7290</td>
<td>7126</td>
</tr>
<tr>
<td>Retention Time (minutes)</td>
<td>3.50</td>
<td>5.26</td>
</tr>
<tr>
<td>Resolution</td>
<td></td>
<td>8.480</td>
</tr>
</tbody>
</table>
Precision:
The % RSD for repeatability of DIC and TOL were found to be 1.86 and 0.89 respectively. The results are shown in Table 6.
The value of % RSD for intra-day precision was found to be in the range of 0.850-1.003% and 0.851-1.010% while inter-day precision was found to be in the range of 1.049-1.151% and 1.050-1.153% for DIC and TOL respectively, which indicated that the method was precise. The results are shown in Table 17 and 18.

Table 16: Repeatability data for DIC and TOL

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Concentration (µg/ml)</th>
<th></th>
<th>DIC</th>
<th>TOL</th>
<th></th>
<th>DIC</th>
<th>TOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>15</td>
<td>-0.142</td>
<td>0.369</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>15</td>
<td>-0.145</td>
<td>0.372</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>15</td>
<td>-0.141</td>
<td>0.365</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>15</td>
<td>-0.140</td>
<td>0.371</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>15</td>
<td>-0.145</td>
<td>0.374</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>15</td>
<td>-0.144</td>
<td>0.367</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean: -0.1428 0.369
SD: 0.0021 0.0033
%RSD: 1.8618 0.8999

Table 17: Intraday precision data for DIC and TOL

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>Area Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
<th>Conc (µg/ml)</th>
<th>Area Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>812.839 ± 8.156</td>
<td>1.003</td>
<td>9</td>
<td>1142.421 ± 11.539</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>1627.357 ± 16.312</td>
<td>1.002</td>
<td>18</td>
<td>2286.221 ± 22.905</td>
<td>1.06</td>
</tr>
<tr>
<td>10</td>
<td>2445.919 ± 20.809</td>
<td>0.850</td>
<td>27</td>
<td>3439.557 ± 29.293</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Table 18: Inter-day precision data for DIC and TOL

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>Area Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
<th>Conc (µg/ml)</th>
<th>Area Mean ± S.D. (n=3)</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>815.549 ± 9.00</td>
<td>1.103</td>
<td>9</td>
<td>794.730 ± 12.843</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>1633.328 ± 17.140</td>
<td>1.049</td>
<td>18</td>
<td>2296.086 ± 24.127</td>
<td>1.06</td>
</tr>
<tr>
<td>9</td>
<td>2457.381 ± 28.292</td>
<td>1.151</td>
<td>27</td>
<td>3455.172 ± 39.871</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Accuracy (Recovery):
The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 100.01-100.12% and 99.61-100.31% for DIC and TOL respectively, which indicated accuracy of the method. The results are shown in Table 19 and 20.
Table 19: Accuracy Data for DIC

<table>
<thead>
<tr>
<th>Level</th>
<th>Sample Conc. (µg/ml)</th>
<th>Amt of Drug added (µg/ml)</th>
<th>Total Conc. (µg/ml)</th>
<th>Amt of Drug recovered (µg/ml)</th>
<th>Recovery %</th>
<th>Mean ± SD (%),(n=3)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>6 4.8 10.8</td>
<td>10.789</td>
<td>99.712</td>
<td>100.06 ± 0.34</td>
<td>0.348</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>6 4.8 10.8</td>
<td>10.819</td>
<td>99.409</td>
<td>100.409</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>6 4.8 10.8</td>
<td>10.803</td>
<td>99.712</td>
<td>100.06</td>
<td>0.348</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>6 6 12</td>
<td>11.935</td>
<td>98.925</td>
<td>100.12 ± 1.32</td>
<td>1.323</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>6 6 12</td>
<td>12.092</td>
<td>98.925</td>
<td>101.547</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>6 6 12</td>
<td>11.995</td>
<td>99.916</td>
<td>100.066</td>
<td>0.348</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120%</td>
<td>6 7.2 13.2</td>
<td>13.121</td>
<td>98.907</td>
<td>100.01 ± 1.02</td>
<td>1.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120%</td>
<td>6 7.2 13.2</td>
<td>13.215</td>
<td>98.907</td>
<td>100.217</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120%</td>
<td>6 7.2 13.2</td>
<td>13.266</td>
<td>98.907</td>
<td>100.930</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 20: Accuracy Data for TOL

<table>
<thead>
<tr>
<th>Level</th>
<th>Sample Conc. (µg/ml)</th>
<th>Amt of Drug added (µg/ml)</th>
<th>Total Conc. (µg/ml)</th>
<th>Amt of Drug recovered (µg/ml)</th>
<th>Recovery %</th>
<th>Mean ± SD (%),(n=3)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>18 14.4 32.4</td>
<td>32.394</td>
<td>99.962</td>
<td>99.613 ± 1.21</td>
<td>1.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>18 14.4 32.4</td>
<td>32.488</td>
<td>99.962</td>
<td>100.615</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>18 14.4 32.4</td>
<td>32.510</td>
<td>99.962</td>
<td>98.264</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>18 18 36</td>
<td>35.833</td>
<td>99.073</td>
<td>100.31 ± 1.37</td>
<td>1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>18 18 36</td>
<td>36.321</td>
<td>99.073</td>
<td>101.784</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>18 18 36</td>
<td>36.013</td>
<td>99.073</td>
<td>100.074</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120%</td>
<td>18 21.6 39.6</td>
<td>39.324</td>
<td>98.724</td>
<td>100.05 ± 1.21</td>
<td>1.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120%</td>
<td>18 21.6 39.6</td>
<td>39.677</td>
<td>98.724</td>
<td>100.357</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120%</td>
<td>18 21.6 39.6</td>
<td>39.836</td>
<td>98.724</td>
<td>101.094</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 21: Data for robustness (change in pH of mobile phase)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parameter</th>
<th>Change 1</th>
<th>Change 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3.7 (n=3)</td>
<td>pH 3.3 (n=3)</td>
<td></td>
</tr>
<tr>
<td>DIC</td>
<td>Area</td>
<td>1621.92</td>
<td>1630.597</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>11.519</td>
<td>16.287</td>
</tr>
<tr>
<td></td>
<td>% RSD</td>
<td>0.710</td>
<td>0.998</td>
</tr>
<tr>
<td>TOL</td>
<td>Area</td>
<td>2279.995</td>
<td>2292.093</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>15.963</td>
<td>22.863</td>
</tr>
<tr>
<td></td>
<td>% RSD</td>
<td>0.700</td>
<td>0.997</td>
</tr>
</tbody>
</table>
Limit of Detection and Limit of Quantification:
The Limit of detection (LOD) was found to be 0.141 and 0.347 µg/mL while the Limit of quantification (LOQ) was 0.429 and 1.053 µg/mL for DIC and TOL respectively. The results are shown in Table 14.

Assay of the Tablet dosage form:
The proposed RP-HPLC method was successfully applied for determination of DIC and TOL from combined tablet dosage form [15]. The percentage of DIC and TOL were found to be satisfactory; which was comparable with the corresponding label claim. The results are shown in Table 15.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Marketed Preparation</th>
<th>Label claim</th>
<th>Amount of drug estimated</th>
<th>%Label Claim</th>
<th>S.D</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC</td>
<td>TOLPERITAS-D®</td>
<td>50 mg</td>
<td>49.879</td>
<td>99.759</td>
<td>0.5466</td>
<td>0.544</td>
</tr>
<tr>
<td>TOL</td>
<td></td>
<td>150 mg</td>
<td>150.737</td>
<td>100.491</td>
<td>0.4799</td>
<td>0.481</td>
</tr>
</tbody>
</table>

**CONCLUSION**

A HPLC method has been developed and validated for the determination of DIC and TOL in tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. The proposed method was found to be simple, accurate, precise and robust. Hence, it can be used successfully for the routine analysis of DIC and TOL in pharmaceutical dosage forms.

- Statistical Comparison of The Developed Methods

**Comparison of Developed Methods by Statistical t - TEST**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DIC</th>
<th>TOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>HPLC</td>
<td>UV</td>
</tr>
<tr>
<td>Drug ± SD, % (n=3)</td>
<td>99.67 ± 1.63</td>
<td>100.49 ± 0.54</td>
</tr>
<tr>
<td>Tabulated t- Value</td>
<td>2.131</td>
<td>2.131</td>
</tr>
<tr>
<td>Calculated t- Value</td>
<td>0.317</td>
<td>0.714</td>
</tr>
</tbody>
</table>

The assay results for DIC and TOL in tablet dosage form, obtained using UV and HPLC methods were compared statistically by applying the two tail paired t-test. The calculated t- value for DIC (0.317) and TOL (0.714) is less than the tabulated t- value (2.131) at the 95% confidence interval.

$t_c$ calculated < $t_t$ tabulated

$P$ - Value for the DIC and TOL were found to be 0.38 and 0.25 respectively. $P$ - Value should be more than 0.05.

Therefore no significant difference was found in the content of DIC and TOL determined by the proposed UV and HPLC methods.

A UV spectrophotometric method has been developed and validated for the determination of DIC and TOL in tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. Distilled water was used as a solvent. Hence, proposed method is a cost effective. The proposed method was found to be simple, accurate, precise and robust. Hence, it can be
used successfully for the routine analysis of DIC and TOL in pharmaceutical dosage forms. A HPLC method has been developed and validated for the determination of DIC and TOL in tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. The proposed method was found to be simple, accurate, precise and robust. Hence, it can be used successfully for the routine analysis of DIC and TOL in pharmaceutical dosage forms.

**REFERENCES**

4. drugs.com/international/tolperisone.html

* Both the methods were validated as per ICH Q2R1 guideline.
* Spectrophotometric and RP-HPLC methods were compared by statistical t - Test.