METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF TOLPERISONE HCl AND ETODOLAC IN BULK AND ITS PHARMACEUTICAL DOSAGE FORMULATIONS

Introduction

Muscle relaxants are not really a class of drugs, but rather a group of different drugs that each has an overall sedative effect on the body. These drugs do not act directly on the muscles; rather they act centrally (in the brain) and are more of a total body relaxant. Typically, muscle relaxants are prescribed early in a course of back pain, on a short-term basis, to relieve low back pain associated with muscle spasms. A muscle relaxant is a drug which affects skeletal muscle function and decreases the muscle tone. It may be used to alleviate symptoms such as muscle spasms, pain, and hyperreflexia. The term "muscle relaxant" is used to refer to two major therapeutic groups: neuromuscular blockers and spasmotics.

Neuromuscular blockers act by interfering with transmission at the neuromuscular end plate and have no central nervous system (CNS) activity. They are often used during surgical procedures and in intensive care and emergency medicine to cause temporary paralysis. Spasmotics, also known as "centrally acting" muscle relaxants, are used to alleviate musculoskeletal pain and spasms and to reduce spasticity in a variety of neurological conditions. While both neuromuscular blockers and spasmotics are often grouped together as muscle relaxants\textsuperscript{1,2} the term is commonly used to refer to spasmotics only\textsuperscript{3,4}.

Muscle relaxation and paralysis can theoretically occur by interrupting function at several sites, including the central nervous system, myelinated somatic nerves, unmyelinated motor
nerve terminals, nicotinic acetylcholine receptors, the motor end plate, and the muscle membrane or contractile apparatus. Most neuromuscular blockers function by blocking transmission at the end plate of the neuromuscular junction. Normally, a nerve impulse arrives at the motor nerve terminal, initiating an influx of calcium ions, which causes the exocytosis of synaptic vesicles containing acetylcholine. Acetylcholine then diffuses across the synaptic cleft. It may be hydrolysed by acetylcholine esterase (AchE) or bind to the nicotinic receptors located on the motor end plate.

The binding of two acetylcholine molecules results in a conformational change in the receptor that opens the sodium-potassium channel of the nicotinic receptor. This allows $\text{Na}^{+}$ and $\text{Ca}^{2+}$ ions to enter the cell and $\text{K}^{+}$ ions to leave the cell, causing a depolarization of the end plate, resulting in muscle contraction. Following depolarization, the acetylcholine molecules are then removed from the end plate region and enzymatically hydrolysed by acetylcholinesterase.

Non depolarizing agents, such as Tubocurarine, block the agonist, acetylcholine, from binding to nicotinic receptors and activating them, thereby preventing depolarization. Alternatively, depolarizing agents, such as Succinylcholine, are nicotinic receptor agonists which mimic Ach, block muscle contraction by depolarizing to such an extent that it desensitizes the receptor and it can no longer initiate an action potential and cause muscle contraction. The generation of the neuronal signals in motor neurons that cause muscle contractions are dependent on the balance of synaptic excitation and inhibition the motor neuron receives. Spasmolytic agents generally work by either enhancing the level of inhibition, or reducing the level of excitation. Inhibition is enhanced by mimicking or enhancing the actions of endogenous inhibitory substances, such as GABA.
Because of the enhancement of inhibition in the CNS, most spasmolytic agents have the side effects of sedation, drowsiness and may cause dependence with long-term use. Several of these agents also have abuse potential, and their prescription is strictly controlled. Spasmolytics such as carisoprodol, cyclobenzaprine, metaxalone, and methocarbamol are commonly prescribed for low back pain or neck pain, fibromyalgia, tension headaches and myofascial pain syndrome. However, they are not recommended as first-line agents; in acute low back pain, they are not more effective than Paracetamol or nonsteroidal anti-inflammatory drugs (NSAIDs) and in fibromyalgia they are not more effective than antidepressants. Nevertheless, some (low-quality) evidence suggests muscle relaxants can add benefit to treatment with NSAID’s.

As the above class name suggests, Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce inflammation but are not related to steroids which also reduce inflammation. NSAIDs work by reducing the production of prostaglandins. Prostaglandins are chemicals that promote inflammation, pain, and fever. They also protect the lining of the stomach and intestines from the damaging effects of acid, promote blood clotting by activating blood platelets, and promote normal function of the kidneys.

The enzymes that produce prostaglandins are called cyclooxygenase (COX). There are two types of COX enzymes, COX-1 and COX-2. Both enzymes produce prostaglandins that promote inflammation, pain, and fever; however, only COX-1 produces prostaglandins that activate platelets and protect the stomach and intestinal lining. NSAID’s block COX enzymes and reduce production of prostaglandins. Therefore, inflammation, pain, and fever are reduced. Since the prostaglandins that protect the stomach and promote blood clotting also are reduced,
NSAIDs that block both COX-1 and COX-2 can cause ulcers in the stomach and intestines, and increase the risk of bleeding.

Prostaglandins are produced within the body's cells by the enzyme cyclooxygenase (COX). There are two COX enzymes, COX-1 and COX-2. Both enzymes produce prostaglandins that promote inflammation, pain, and fever. However, only COX-1 produces prostaglandins that support platelets and protect the stomach. Nonsteroidal anti-inflammatory drugs (NSAIDs) block the COX enzymes and reduce prostaglandins throughout the body. As a consequence, ongoing inflammation, pain, and fever are reduced. Since the prostaglandins that protect the stomach and support platelets and blood clotting also are reduced, NSAIDs can cause ulcers in the stomach and promote bleeding. The development of new muscle relaxants and NASAID’s drugs are increasing very much widely and rapidly and is an ongoing field of research. The author identified a new, widely used promising drug namely Tolperisone HCl & Etodolac as the most commendable drug from the above categories enlisted, for analytical investigations.

Tolperisone (TOL), a piperidine derivative, is a centrally-acting muscle relaxant. Tolperisone is indicated for use in the treatment of pathologically increased tone of the cross-striated muscle caused by neurological diseases (damage of the pyramidal tract, multiple sclerosis, myelopathy, encephalomyelitis) and of spastic paralysis and other encephalopathies manifested with muscular dystonia¹⁴,¹⁵.

Etodolac (ETD) is a nonsteroidal anti-inflammatory drug (NSAID). NSAIDs are used for the management of mild to moderate pain, fever, and inflammation. They work by reducing
the levels of prostaglandins, which are chemicals that are responsible for pain and the fever and tenderness that occur with inflammation. Etodolac blocks the enzyme that makes prostaglandins (cyclooxygenase), resulting in lower concentrations of prostaglandins. As a consequence, inflammation, pain and fever are reduced. Post-marketing studies demonstrated that Etodolac inhibition of cyclooxygenase is somewhat COX-2 selective\(^\text{16}\) similar to celecoxib and other "COX-2 inhibitors." Unlike rofecoxib, both Etodolac and celecoxib can fully inhibit COX-1 and are designated as having "preferential selectivity" toward COX-2. The (inactive against COX) r-enantiomer of Etodolac inhibits β-catenin levels in hepatoma cells\(^\text{17}\).

From the above context, the author has selected a combination drug consisting of TPS and EDL, with a novel approach for muscle relaxant & anti-inflammatory activity, for analytical investigations.

**Pharmacology & Mechanism Actions of Tolperisone (TOL) and Etodolac (ETD)**

**Tolperisone (TOL):** Tolperisone is a centrally-acting muscle relaxant that acts at the reticular formation in the brain stem\(^\text{14}\) by blocking voltage-gated sodium and calcium channels\(^\text{18, 19}\). Voltage-gated Na\(^+\) channels (VGSC, Na\(_v\)) are critically important for electrogensis and nerve impulse conduction. Certain Na\(^+\) channel iso-forms are predominantly expressed in peripheral sensory neurons associated with pain sensation, and the expression and functional properties of Na\(_v\) in peripheral sensory neurons can be dynamically regulated following axonal injury or peripheral inflammation\(^\text{20}\). Tolperisone is a centrally acting muscle relaxant (muscle relaxant acting on spasticity by interaction with upper motor neurons), which is also used for the treatment of chronic pain\(^\text{21, 22}\).
It is mainly used for treating muscle spasticities of neurological origin and painful muscle spasms due to rheumatologic conditions.

Besides being an effective antispastic agent\textsuperscript{23, 24}, Tolperisone also has analgesic activity in rodents\textsuperscript{25} and in humans\textsuperscript{26}. It possesses relatively few side effects in humans\textsuperscript{24}. Tolperisone is an ion channel blocker that acts, at micro molar concentrations, as an acute blocker of seven different iso-forms of Na\textsubscript{v} expressed in Xenopus oocytes. The drug concentrations needed to exert 50\% block differ between the different iso-forms are 116, 802, 96, 131, 326, 394 and 49 µM for Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.4, Na\textsubscript{v}1.5, Na\textsubscript{v}1.6, Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 channel, respectively\textsuperscript{27, 28}. Na\textsubscript{v}1.8, which is the most prominently affected channel, is known to be up regulated in an animal model of neuropathic pain\textsuperscript{29}. The inhibitory effect is reversible and develops rapidly, with an IC\textsubscript{50} value of 198 µM in dorsal root ganglion (DRG) cells\textsuperscript{30}.

**Etodolac (ETD):** NSAID’s are used for the management of mild to moderate pain, fever, and inflammation. They work by reducing the levels of prostaglandins, which are chemicals that are responsible for pain and the fever and tenderness that occur with inflammation. Etodolac blocks the enzyme that makes prostaglandins (cyclooxygenase), resulting in lower concentrations of prostaglandins. As a consequence, inflammation, pain and fever are reduced.

**Effects, contraindications and adverse effects of TOL & ETD**

Tolperisone should not be used in patients with myasthenia gravis. Only limited data are available regarding the safety in children, youths, during pregnancy and breastfeeding. It is not known whether TOL is excreted into mother's milk\textsuperscript{14}. Adverse effects occur in less than 1\% of patients and include muscle weakness, headache, arterial hypotension, nausea, vomiting, dyspepsia, and dry mouth. All effects are reversible. Allergic reactions occur in less
than 0.1% of patient and include skin rash, hives, Quincke's edema, and in some cases anaphylactic shock.

Etodolac should be avoided by patients with a history of asthma attacks, hives, or other allergic reactions to aspirin or other NSAIDs. Upset stomach, nausea, diarrhoea, drowsiness, or dizziness may occur. Other side effects include: easy bruising/bleeding, difficult/painful swallowing, hearing changes (such as ringing in the ears), mental/mood changes, swelling of the ankles/feet/hands, sudden/unexplained weight gain, change in the amount of urine, unexplained stiff neck, vision changes, unusual tiredness. It also should be avoided by patients with peptic ulcer disease or poor kidney function, since this medication can worsen both conditions. Etodolac is used with caution in patients taking blood thinning medications (anticoagulants), such as warfarin (Coumadin), because it increases the risk of bleeding.

Patients taking both lithium and Etodolac may develop toxic blood lithium levels. Additionally, Etodolac has been found to interact with certain anti-depressant medications, such as sertraline or fluoxetine, which can increase risks of stroke, heart attack, and other cardiovascular conditions. NSAIDs should be discontinued prior to elective surgery because of a mild interference with clotting that is characteristic of this group of medicines. Etodolac is best discontinued at least four days in advance of surgery.

**Current therapeutic and clinical uses of TOL & ETD**

Tolperisone is indicated for use in the treatment of pathologically increased tone of the cross-striated muscle caused by neurological diseases (damage of the pyramidal tract, multiple sclerosis, myelopathy, encephalomyelitis) and of spastic paralysis and other encephalopathies manifested with muscular dystonia. Other possible uses are: Spondylosis, Spondylarthrosis,
Cervical and lumbar syndromes, Arthrosis of the large joints obliterating atherosclerosis of the extremity vessels, Diabetic angiopathy, Thromboangiitis obliterans, Raynaud's syndrome. Etodolac is used to relieve pain from various conditions. It also reduces pain, swelling, and joint stiffness from arthritis. This medication is known as a nonsteroidal anti-inflammatory drug (NSAID). It works by blocking your body's production of certain natural substances that cause inflammation. Its medication may also be used to treat gout attacks.

**Dosage Regimen:** CTRI/2009/091/001021, Cadila Healthcare Ltd, Ahmedabad, Gujarat, India (Under Clinical Trials: Phase IV)

**Table 3.1: Structural features of Tolperisone (TOL):**

<table>
<thead>
<tr>
<th>Official Name</th>
<th>Chemical Name(s)</th>
<th>Structure</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolperisone HCl</td>
<td>(R, S) 2-methyl-1-(4-methyl phenyl)-3-(1-piperidyl) propan-1-one</td>
<td><img src="image.png" alt="Structure" /></td>
<td>Methyl derivative, piperidyl ring, carbonyl derivative, chlorine moiety</td>
</tr>
</tbody>
</table>
Table 3.2: Structural features of Etodolac (ETD)

<table>
<thead>
<tr>
<th>Official Name</th>
<th>Chemical Name(s)</th>
<th>Structure</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etodolac</td>
<td>(RS)2—(1,8-Diethyl-4,9-dihydro-3H-pyrano[3,4-b]indo-1-yl)acetic acid</td>
<td><img src="image" alt="Etodolac Structure" /></td>
<td>Indole ring derivative, pyranosyl derivative, carbonyl group, acetate moiety</td>
</tr>
</tbody>
</table>

**Drug/Characteristic Profile of Tolperisone HCl (TOL)**

**Name**: Tolperisone HCl

**Description**: TOL is a piperidine derivative, is a centrally-acting muscle relaxant

**Brand names**: Tolifast(150mg), Tolflex(150mg), Synaptol(150mg), Myotop(SR (450mg), Myotop(150mg), Tolfree((150mg), TolpidolFC(150 mg), Tolpidol OD, Tolfree (100 mg), Synaptol (100 mg)

**Categories**: Muscle relaxant, Spondylosis

**Molecular Weight**: 281.82gms/mol

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Chemical Formula : C_{16}H_{24}ClNO

IUPAC Name : 2-methyl-1-(4-methylphenyl)-3-piperidin-1-ylpropan-1-one; Hydrochloride

CAS Number : 728-88-1

Physicochemical descriptive properties:

State : white colour solid

Solubility : Soluble in Chloroform, Methanol and Water

Melting Range : 181-183°C

pKa : 3

Odour & taste : Odourless & saline bitter

Storage : Store between 15°C-25°C and protect from moisture

Pharmacological Actions & Clinical Pharmacology

Indication : TOL is indicated for use in the treatment of pathologically increased tone of the cross-striated muscle caused by neurological diseases (damage of the pyramidal tract, multiple encephalomyelitis) and of spastic paralysis and other encephalopathies manifested with muscular dystonia.

Mechanism of Action: TOL is a centrally-acting muscle relaxant that acts at reticular formation in the brain stem by blocking voltage-gated sodium & calcium channels
**Pharmacokinetics**

**Absorption**: TOL is absorbed nearly completely from the gut and reaches its peak blood plasma concentration after 1.5 hours.

**Toxicity**: The symptomatic adverse reactions produced by TOL are more or less tolerable and if they become severe, they can be treated symptomatically, these include muscle weakness, somnolence etc..

**Metabolism**: The most prominent effect of TOL is its inhibitory action on pathways of spinal reflexes. It suppresses the mono and polysynaptic reflex transmission by both pre-synaptic and post-synaptic mechanisms.

**Elimination**: The substance is excreted via the kidneys in two phases; the first with a half-life of two hours, and the second with a half-life of 12 hours.

**Half-life**: 2-12 hrs.

**Year of Introduction**: 2006 A.D

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**Drug/characteristic Profile for Etodolac (ETD)**

**Name**: Etodolac

**Description**: ETD is a non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic and antipyretic properties. Its therapeutic effects are due to its ability to inhibit prostaglandin synthesis. It is indicated for relief of signs and symptoms of rheumatoid arthritis and osteoarthritis.
Brand names: Eccoxolac, Edopain ER,

Categories: Anti-Inflammatory Agents, Non-Steroidal, Cyclooxygenase-2 Inhibitors

Molecular Weight: 287.3535 gms/mol

Chemical Formula: $C_{17}H_{21}NO_{3}$

IUPAC Name: $2\{-1,8$-diethyl-1H,3H,4H,9H-pyrano[3,4-b]indol-1-1\}aceticacid$

CAS Number: 41340-25-4

**Physicochemical Properties**

State: Etodolac is a white crystalline compound

Solubility: Insoluble in water but soluble in alcohols, chloroform, dimethyl sulfoxide, and aqueous polyethylene glycol.

Melting Range: 146.5 °C

pKa: 4.65

Odour/taste: Odourless & tasteless

Storage: Store between 15°C-25°C and protect from moisture

**Pharmacological Actions & Clinical Pharmacology**

Indication: For acute and long-term management of signs and symptoms of osteoarthritis and rheumatoid arthritis, as well as for the management of pain.
**Mechanism of Action:** Similar to other NSAIDs, the anti-inflammatory effects of Etodolac result from inhibition of the enzyme cyclooxygenase (COX). This decreases the synthesis of peripheral prostaglandins involved in mediating inflammation. ETD binds to the upper portion of the COX enzyme active site and prevents its substrate, arachidonic acid, from entering the active site. ETD was previously thought to be a non-selective COX inhibitor, but it is now known to be 5 – 50 times more selective for COX-2 than COX-1. Antipyresis may occur by central action on the hypothalamus, resulting in peripheral dilation, increased cutaneous blood flow, and subsequent heat loss.

**Pharmacokinetics**

**Absorption**

Based on mass balance studies, the systemic bioavailability of Etodolac from either the tablet or capsule formulation is at least 80%.

**Toxicity**

Selective COX-2 inhibitors have been associated with increased risk of serious cardiovascular events (e.g. myocardial infarction, stroke) in some patients. Current data is insufficient to assess the cardiovascular risk of Etodolac. Etodolac may increase blood pressure and/or cause fluid retention and edema. Risk of GI toxicity including bleeding, ulceration and perforation. Risk of direct renal injury, including renal papillary necrosis. Anaphylactoid and serious skin reactions (e.g. exfoliative dermatitis, Stevens-Johnson syndrome, and toxic epidermal necrolysis) have been reported. Common adverse events include abdominal pain,
constipation, diarrhoea, dyspepsia, flatulence, GI bleeding, GI perforation, nausea, peptic ulcer, vomiting, renal function abnormalities, anaemia, dizziness, edema, liver function test abnormalities, headache, prolonged bleeding time, pruritus, rash, tinnitus. Symptoms of overdose include lethargy, drowsiness, nausea, vomiting, and epigastric pain.

**Metabolism**

ETD is extensively metabolized in the liver. Renal elimination of ETD and its metabolites is the primary route of excretion (72%). Metabolites found in urine (with percents of the administered dose) are: unchanged ETD (1%), ETD glucuronide (13%), hydroxylated metabolites (6-, 7-, and 8-OH; 5%), hydroxylated metabolite glucuronides (20%), and unidentified metabolites (33%). Faecal excretion accounts for 16% of its elimination.

**Elimination**

It is not known whether etodolac is excreted in human milk; however, based on its physical-chemical properties, excretion into breast milk is expected. Etodolac is extensively metabolized in the liver. The hydroxylated-etodolac metabolites undergo further glucuronidation followed by renal excretion and partial elimination in the feces (16% of dose). Approximately 1% of ETD dose is excreted unchanged in the urine with 72% of the dose excreted into urine as parent drug plus metabolite.

**Half-life**

Terminal $t_{1/2}$, 7.3 ± 4.0 hours. Distribution $t_{1/2}$, 0.71 ± 0.50 hours

**Year of Introduction:** 2001 A.D
OBJECTIVE OF INVESTIGATION

The objective of current investigation is to provide combined estimation of the selected drugs in dosage forms as well as in its pure forms by using a simple, reliable, precise, accurate, robust and reproducible analytical method with stability indication. The stability indication nature of the analytical method provides confidence to use the developed method in a regulatory environment of pharmaceutical industry without any further modification. This makes that the developed analytical method will be use full for the estimation of these drugs in either combined dosage forms or in its pure form.

LITERATURE SURVEY

Both, Tolperisone HCl (TOL) and Etodolac (ETD) are official in Japan Pharmacopeia (JP), USP & BP respectively. The official methods of analysis for TOL & ETD are Colorimetry and Liquid Chromatography. The JP prescribes Colorimetry & HPLC method for the analysis of TOL and ETD. A survey of literature reveals that, other reported methods for TOL include Spectrophotometry, HPLC and Tandem Mass Spectroscopy in varied Matrices like formulations and biological fluids. The details of the HPLC methods are given below.

The analytical methods reported for ETD are based upon Spectrophotometry, HPLC and other related analytical techniques like Tandem Mass Spectroscopy. The details of HPLC methods are presented below. As highlighted earlier, the use of the above two drugs in combination has become, very wide spread. It is however, surprising to note that, not even a single method is available till now, for the simultaneous estimation of these two drugs in combination products. The present project seeks to bridge this gap by developing such methods. The details of these successful efforts, by the author are incorporated in this chapter.
Past Studies on Tolperisone (TOL) and Etodolac (ETD)

1. A rapid, specific and sensitive UV Spectrophotometric method was developed for the determination of Tolperisone hydrochloride in bulk and tablet dosage form. The absorbance was measured at 260 nm using purified water as a solvent and the calibration curve was found to be linear in the concentration range of 3 – 18 μg/ml. The molar absorptive and Sandell’s sensitivity were obtained 1.33 X 104 liter/mol/cm and 0.019 μg/cm2/0.001 absorbance units respectively. The detection limit and quantization limit were found to be 0.032 and 0.096 μg/ml respectively. The % recovery was detected 99.09 – 101.26 %. The method was found to be precise as the value of % RSD was remained below 2 %. There was not found any interference of common excipients used in the tablet formulation. Hence the proposed UV Spectrophotometric method seems to be suitable for the routine quality control analysis in analytical lab.(Koladiya Bhavesh B and Vaghela Vipul M., 2012) (32)

2. Tolperisone HCl is a central muscle relaxant, which was incorporated in a matrix system formulated with poly(ethylene oxide)–PEO, in order to achieve adequate gastric residence time. This tablet presents considerable analytical difficulties in the quantitative determination of the drug, because the PEO matrix causes significant increase of viscosity in the samples. Our purpose was to develop a reproducible sample preparation method, which is adapted from parameters of the in vitro dissolution test and validate an LC-UV analytical method, which allows good recovery of the drug (99.97%). The developed analytical method was suitable for quantitative analysis of tolperisone HCl in matrix tablets.(Bernadett Stiedl, Dorottya Kovács-Kiss, Krisztina Ludányi, Attila Bódis, Imre Klebovich, István Antal, 2010) (33)
This present study reports the simultaneous quantification of Tolperisone hydrochloride and Diclofenac sodium in the bulk drug and tablet dosage form employing simultaneous equation and absorbance ratio method. This method allows determination of Tolperisone hydrochloride and Diclofenac sodium at their $\lambda_{\text{max}}$ 254 nm and 282 nm respectively and at the iso-absorptive wavelength of 238 nm in methanol. Tolperisone at $\lambda_{\text{max}}$ of 254 nm obeyed Beer’s Law in the concentration range 4-12 $\mu$g/mL and Diclofenac sodium at $\lambda_{\text{max}}$ of 282 nm obeyed Beer’s Law in the concentration range 8-16 $\mu$g/mL. The accuracy and reliability of the method was assessed by linearity, precision (intra-day % RSD and inter-day % RSD of Tolperisone hydrochloride and Diclofenac sodium) and specificity in accordance with ICH guidelines. (Amita S Ashokan1, Mary Mathew1, Shajahan Puthusseri, 2013) (34)

4. Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reducing pain in certain conditions. Tolperisone hydrochloride is a piperidine derivative, is a centrally-acting muscle relaxant. Two simple, accurate and economic methods; Q analysis and first order derivative method have been described for the simultaneous Spectrophotometric estimation of Diclofenac sodium and Tolperisone hydrochloride in tablet dosage form. Absorption maxima of Diclofenac sodium and Tolperisone hydrochloride in distilled water were found to be 275.0 nm and 260.0 nm respectively. Beer’s law was obeyed in the concentration range 5-50 $\mu$g/mL for Diclofenac and 5-60 $\mu$g/mL for Tolperisone hydrochloride. In Q analysis method, absorbance’s were measured at the selected wavelengths, 237.0 nm (isoabsorptive point) and 260.0 nm ($\lambda_{\text{max}}$ of Tolperisone). In first order derivative method, zero crossing point of Diclofenac sodium and Tolperisone hydrochloride were selected at 275.0 nm and 260.0 nm respectively. The analysis of binary pharmaceutical formulation was carried by both methods. Results of two methods were validated statistically by
recovery studies and were found to be satisfactory. (Mahaparale S. P., Shinde S. S., Nirmal P. N., 2013) (35)

5. Two simple, sensitive, rapid, accurate and economical methods were developed for the estimation of Paracetamol (PCM) and Tolperisone Hydrochloride (TOL) in their combined dosage forms. The first method is based on the simultaneous equation and second method is based on the First order derivative method. For the method I, the estimation of PCM and TOL were carried out in 243.2 nm ($\lambda_{\text{max}}$ of PCM) and 261 nm ($\lambda_{\text{max}}$ of TOL) respectively. For method II, estimation of PCM was carried out at 227 nm (ZCP of TOL) and of TOL at 243.2 nm (ZCP of PCM). The developed methods were validated in terms of linearity, accuracy, precision, sensitivity as per ICH Q2A guidelines. The developed methods could be routinely applied for quality control and analysis of PCM and TOL in their marketed formulation. (Umang Shah, Krupa Thula, Manan Raval, Pankti Desai, 2012) (36)

6. A simple high performance liquid chromatographic (HPLC) method was developed for the determination of Tolperisone in human plasma. Tolperisone and internal standard (chlorphenesin) were isolated from 1 mL of plasma using 8 mL of dichlormethane. The organic phase was collected and evaporated under nitrogen gas. The residue was then reconstituted with 300 mL aliquot of mobile phase and a 100 mL aliquot was injected onto the C18 reverse-phased column. The mobile phase, 45% methanol containing 1% glacial acetic acid and 0.05% 1-hexanesulfonic acid was run at a flow rate of 1 mL/min. The column effluent was monitored using UV detector at 260 nm. The retention times for Tolperisone and the internal standard were approximately 7.1 and 8.4 min, respectively. The standard curve was linear with minimal intra-day and inter-day variability. The quantification limit of Tolperisone in human plasma was 10 ng/mL. The proposed method has been applied to the determination of pharmacokinetic profile
of Tolperisone in Koreans. The $T_{max}$ of Tolperisone in Koreans (0.94 +/- 0.42 h) was not significantly differ from that reported in Europeans (0.5-1 h), but the mean half-life in Koreans (1.14 +/- 0.27 h) was shorter than that in Europeans (2.56 +/- 0.2 h). The proposed HPLC method is simple, accurate, reproducible and suitable for pharmacokinetic study of Tolperisone. (Bae JW, Park YS, Sohn UD, Myung CS, Ryu BK, Jang CG, Lee SY., 2006) (37)

7. A simple, rapid reverse phase high performance liquid chromatographic method has been developed and validated for estimation of Tolperisone in pharmaceutical dosage form. The estimation was carried out on Inertsil ODS C$_{18}$, 5µm column having 250 x 4.6mm internal diameter column with a mixture of methanol: acetonitrile in the ratio of 90:10:(v/v) as mobile phase. UV detection was performed at 232 nm. The method was validated for linearity, accuracy, precision and specificity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 2.48 min. for Tolperisone and total run time was 5 min. at a flow rate of 1.0ml/min. The calibration curve was linear over the concentration range of 40-100 ppm for Tolperisone. The LOD and LOQ values were found to be 0.5 and 3 ppm respectively. The high percentage of recovery confirms the suitability of the method for the estimation of Tolperisone in pharmaceutical dosage form. (Murali. M, Satyanarayana. P. V. V, 2011) (38)

8. A simple, precise and accurate reverse phase liquid chromatographic method has been developed for the simultaneous estimation of Tolperisone Hydrochloride and Paracetamol in tablet formulations. The chromatographic separation was achieved on Symmetry C$_{18}$ (250 mmx4.6 mm) analytical column. A mixture of acetonitrile: water (40:60 v/v) (pH 3.0) was used as the mobile phase at the flow rate of 0.7 ml/min and detector wavelength at 258 nm (Isobestic Point) respectively. The retention time of Tolperisone HCl and Paracetamol were found to be
2.25 and 3.29 minutes respectively. The validation of the proposed method was carried out for linearity, accuracy, recovery, precision, limit of detection and limit of quantification and robustness. The linear dynamic ranges were 2-10 μg/mL for Tolperisone HCl and Paracetamol. The percentage recovery of Tolperisone Hydrochloride and Paracetamol were obtained from the range of 99.85-100.26% w/w and 99.88-100.69% w/w respectively. Limit of detection and quantification for Tolperisone HCl were 0.003 μg/mL and 0.03 μg/mL and Paracetamol 0.002 μg/mL and 0.02 μg/mL respectively. The developed method can be used for routine quality control analysis of titled drugs in combination of tablet formulation. (I.Carolin Nimila, P.Balan, N.Chiranjeevi, Vinnakota.V.V.M.Kumar, 2012). (39)

9. A simple, accurate, precise, rapid and economical Spectrophotometric method for simultaneous estimation of Etodolac and Paracetamol in combined tablet dosage form has been developed utilizing simultaneous equation method. In this method, absorbance is measured at two wavelengths, one being $\lambda_{\text{max}}$ of Etodolac at 223.5 nm ($\lambda_{\text{1}}$) and the other being $\lambda_{\text{max}}$ of Paracetamol 242.5 nm ($\lambda_{\text{2}}$). Both the drugs obey Beer's Lambert law in the concentration ranges employed for this method. Result of the method was validated statistically as well as by recovery studies. Proposed method for simultaneous estimation of Etodolac and Paracetamol in combined sample solutions was found to be simple, accurate and reproducible. Once the equations were determined, analysis required only the measurement of the absorbance of the sample solution at the two wavelengths selected, followed by a few simple calculations. It is a novel method that can be employed for routine analysis in quality control.( Balan, P.; Nimila, I. Carolin; Prasanna, M. Lakshmi; Rani, M. Vanaja; Rajasekar, S.,2011) (40)

10. Two simple, rapid and reproducible simultaneous equation and Q-analysis UV-Spectrophotometric methods have been developed for simultaneous estimation of Etodolac
(ETD) and Thiocolchicoside (THC) in combined tablet dosage form. The methods involved solving simultaneous equations and Q-value analysis based on measurement of absorbance at wavelengths, $223(\lambda_{\text{max}}$ of ETO), $259.4 (\lambda_{\text{max}}$ of THC) and 236 nm (iso-absorptive point). Linearity was found in the concentration range of 1–6 $\mu$g/mL and 4–24 $\mu$g/mL for ETD and THC, respectively, with correlation coefficients 0.9998 and 0.9992. The amounts of drugs estimated by the proposed methods are in excellent agreement with the label claimed. Furthermore, the methods were applied for the determination of ETD and THC in spiked human urine. The degradation behavior of ETD and THC was investigated under acid hydrolysis, alkali hydrolysis, photo- and oxidative degradation. The subsequently generated samples were used for degradation studies using the developed method. THC was found to degrade extensively under alkali hydrolysis and unaffected by other stress conditions, while ETD was found to be stable in all stress conditions. The methods were validated according to ICH guidelines. The method, suitable for routine quality control, has been successfully applied to the determination of both drugs in commercial brands of tablets. (Ramchandra Pandey, Pravin O. Patil1Sanjay B. Bari Dinesh M. Dhuma, 2014) (41)

11. The objective of study was to study and develop a simple accurate, precise and cost effective UV-Vis Spectrophotometric method for the estimation of Etodolac in bulk and pharmaceutical dosage form. The solvent used was methanol and the absorption maximum of the drug was found to be 226 nm. A linear response was observed in the range of 2-20 $\mu$g/mL with a regression coefficient of 0.999. The method was then validated for different parameters as per the ICH (International Conference for Harmonization) guidelines. This method can be used for the determination of Etodolac in quality control of formulation without interference of the excipients. Etodolac was subjected to stress degradation under different conditions recommended
by ICH. The degradation studies was carried out by using the developed method.(Aniruddha J Palande, Shailaja B Jadhav, Amit S Tapkir, Pravin D Chaudhari, Bhimashankar H Survase and Pundlik M. Rachamale, 2013) (42)

12. A reversed-phase liquid chromatographic (RP-HPLC) method was developed for the simultaneous determination of Tolperisone hydrochloride (TOL) and Etodolac (ETD) in a combined fixed dose oral formulation. The analysis was carried out using a phenomenax C\textsubscript{18}, pre-packed column. A mobile phase containing a phosphate buffer (pH 5.5): Methanol: Acetonitrile: Tri-ethylamine (40: 40: 20: 1.5), with the pH adjusted to Orthophosphoric acid, was pumped at a flow rate of 1.0 ml min\textsuperscript{-1} with a UV-detector and PDA detection at 257 nm. Retention time was 3.91 minutes and 6.89 minutes for TOL and ETD, respectively. The method was validated for linearity, accuracy, precision, sensitivity, and specificity. The method showed good linearity in the range of 3 – 21 μg/mL for TOL μg/mL and 8 – 56 μg/mL for ETD. The detection limit of the proposed method was 0.16 μg/mL and 0.58 μg/mL for TOL and ETD, respectively. The quantification limit of the proposed method was 0.51 μg/mL and 1.7 μg/mL for TOL and ETD, respectively. The % recovery was within the range of 99.42 – 101.15 for TOL and 98.63 – 100.94 for ETD. The percentage RSD for precision of the method was found to be less than 2%. The method was validated as per the International Conference on Harmonization (ICH) guidelines. The developed method could be applied for routine analysis of TOL and ETD in tablet dosage form.(Mit J. Patel, R. Badmanaban, and C. N. Patel, 2011) (43)
PROPOSED WORK

As mentioned earlier, inspite of the wide therapeutic use of the TOL & ETD combinations, no analytical methods for the simultaneous quantification by stability studies, of these two drugs are available till date. The author has made efforts to fulfil this need and succeeded in developing a simple, precise, accurate and sensitive RP-HPLC method for this purpose. The protocols and guidelines prescribed by the ICH\textsuperscript{44} have been followed in the development and validation\textsuperscript{45} of these procedures. The details are presented in the following sections as mentioned below.

The present project has been focused on the development of simple and precise analytical method for the world’s first dosage formulation combination - CTRI/2009/091/001021, Cadila Healthcare Ltd, Ahmadabad, Gujarat, India (Under Clinical Trials: Phase IV)\textsuperscript{®} containing two drugs (Tolperisone HCl & Etodolac) meant for the treatment of muscle relaxant & Anti-inflammatory therapeutic activity.

This part of the thesis, reports sensitive and precise RP-HPLC method for the determination of drug in bulk samples and also in pharmaceutical formulations. The reported methods are applicable for the estimation of either for TOL or ETD individually or in combination with other drugs from pharmaceutical dosage forms or biological fluids. But till date there were not even a single method reported on degradation studies to prove that the method is stability indicating method. The author was very much inclined & interested in achieving the methodology for the above said drugs and also for routine analytical purpose in both industrial and academic levels. The present work describes the development of a validated
stability indicating analytical RP-HPLC method, which can quantify these components simultaneously from a combined dosage form.

EXPERIMENTAL

Materials and Instrumentation

Drugs

Tolperisone and Etodolac gift samples were procured from Chandra Labs, Hyderabad.

Marketed formulations

CTRI/2009/091/001021, Cadila Healthcare Ltd, Ahmadabad, Gujarat, India (Under Clinical Trials: Phase IV)®

Each tablet contains:

Tolperisone HCl ........................................... 150 mg

Etodolac ..................................................... 400 mg

Chemicals & Reagents Used

▸ HPLC grade Acetonitrile (Merck specialities Private Limited, Mumbai)

▸ Sodium Hydroxide (Merck specialities Private Limited, Mumbai)

▸ Laboratory grade Potassium Dihydrogen phosphate (Merck chemicals limited)

▸ HPLC grade Double distilled water (Merck specialities Private Limited, Mumbai)

▸ Laboratory grade Ortho phosphoric acid, (SD Fine Chemicals, Mumbai)
► All dilutions were performed in standard class-A, volumetric glassware. (Borosil)

**Instrumentation**

Agilent 1120 compact LC chromatographic system, with DAD detector and a fixed injector equipped with 20 µL loop was used for the chromatographic separation. The chromatogram was recorded at and peaks quantified by means of Ezchrome software. Chromatographic separation was carried out on a C\textsubscript{18} column [Sunsil, 250 mm x 4.6 mm 5µ particle size]. Sartorius electronic balance was used for weighing the samples. Ultra-sonic bath sonicator was used for degassing and mixing of the mobile phase.

**Chromatographic conditions**

Chromatographic separation of Tolperisone and Etodolac was carried on a C\textsubscript{18} column. The mobile phase was composed of acetonitrile and phosphate buffer (pH 2.6) in the ratio of 30:70 v/v. It was filtered through a 0.45 µ membrane filter and degassed for 15 minutes. The flow rate of the mobile phase was maintained at 1 ml/min. Detection was carried out at 267 nm at ambient temperature.

**Analytical Methodology**

**Preparation of Primary Standard Stock Solutions**

Standard stock solutions were prepared by dissolving 50 mg of Tolperisone and 40 mg Etodolac working standard in two separate 100 ml and 50 ml volumetric flasks using 15 ml of mobile phase and made up to the mark with mobile phase to obtain a final concentration of 500 µg/mL and 400 µg/mL of each TOL and ETD. From the above stock solutions, 5 and 10 ml
aliquots each were pipette in to a 100 mL volumetric flask and dissolved in 25 mL of the mobile phase and made up to the mark with the solvent to obtain a final concentration of 30 µg/mL and 80 µg/mL for Tolperisone and Etodolac respectively.

**Preparation of Sample solutions**

Twenty tablets were weighed and finely powdered. Accurately weighed and transferred equivalent to 150 mg of TOL and 400 mg of ETD into a 200 ml volumetric flask, added 150 ml of diluent, and sonicated for 30 minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mixed. Filter the solution through 0.45 µm membrane Filter. Transferred 4.0 ml of the above solution into a 100 ml volumetric flask and diluted to volume with diluent to obtain a concentration of 30 µg/mL and 80µg/mL of TOL and ETD respectively.

**Table: 3.3: Chromatographic Conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Sunsil, 250 mm x4.6 mm i.d; 5µ particle size</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Acetonitrile and phosphate buffer (pH 2.6) in the ratio of 30:70 v/v</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Run time</td>
<td>25 minutes</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>Detection &amp; Wavelength</td>
<td>DAD detector, 267 nm</td>
</tr>
<tr>
<td>Retention times</td>
<td>2.957 &amp; 4.193 minutes for TOL &amp; ETD</td>
</tr>
</tbody>
</table>
**Recommended Procedure**

After systematic and detailed study of the various parameters involved as described under the results and discussion in this chapter, the following procedure was adopted for the determination of TOL and ETD in bulk samples and pharmaceutical formulations.

**Procedure**

Initially the mobile phase was pumped for about 30 minutes to saturate the column thereby to set the baseline corrected. Then 20 µL of the standard and sample solutions were injected separately. A quantitative determination of the active ingredients was made by comparison of the peak area of the sample injection with the corresponding peak area of the standard injection. The amount of TOL and ETD present in the sample were calculated through the standard calibration curve.

**RESULTS AND DISCUSSIONS**

The appropriate wavelength in the UV-region (267 nm), was selected for the measurement of the active ingredients in the proposed method. The method was validated by linear fit curve and all the other parameters were calculated similar to the Spectrophotometric method and were discussed in the following pages. The typical chromatogram indicating the separation of TOL & ETD with Sunsil RP-C\textsubscript{18} column (250 mm x 4.6 mm i.d; 5µ particle size) and mobile phase consisting of Acetonitrile and phosphate buffer (pH 2.6) in the ratio of 30:70 v/v in gradient mode was shown in Figures: 3.1 and 3.2 respectively. Chromatogram of Blank Standard samples tested by the same procedure showed no interfering peaks as shown in below Figure: 3.1.
Figure: 3.1: A typical HPLC Chromatogram showing the no interference of diluent for Tolperisone and Etodolac

Figure: 3.2: A typical HPLC Chromatogram showing the peak of Tolperisone and Etodolac
Parameters Fixation

In developing this method, systematic study of the effects of various parameters were undertaken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

Mobile Phase Characteristics

In order to get sharp peaks and baseline separation of the components, the author has carried out a number of experiments by varying different components like composition of organic phase in mobile phase, pH of the aqueous phase, total pH of the selected mobile phase, modifiers and flow rate by changing one at a time and keeping all other parameters constant respectively. The optimum conditions evolved from the above studies were incorporated in the recommended procedure.

Detection Characteristics

The schematic experimentation has been carried out to test whether TOL & ETD have been linearly eluted from the column successively and systematically. In this method, different amounts of the active ingredients were taken and all the solutions were analyzed by respective procedures separately. Quantitative determinations were made by comparing the peak area from a sample injection to the corresponding peak area from the standard injection in the method. The linear fit was illustrated graphically in Figures: 3.5-3.6. Least linear correlation and regression data analysis for the method was carried out for the slope; intercept, standard error of estimate; correlation coefficient & % RSD were illustrated in Tables: 3.4 and 3.5 respectively. The results are represented in Table: 3.6.
Table: 3.4 Linear Correlations and Regression Data Sheet / Summary for Tolperisone

\[ \sum X = 150, \sum X^2 = 4860, \quad \sum Y = 1731.986, \quad \sum Y^2 = 642339.3826 \]

Therefore, \( \sum XY = 55863.264 \)

<table>
<thead>
<tr>
<th>( r )</th>
<th>( r^2 )</th>
<th>Slope</th>
<th>Y Intercept</th>
<th>Standard error of estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.999</td>
<td>0.999</td>
<td>10.844</td>
<td>21.0902</td>
<td>4.2593</td>
</tr>
<tr>
<td>( t )</td>
<td>df</td>
<td>( P )</td>
<td>One-tailed</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>48.305</td>
<td>3</td>
<td></td>
<td>Two-tailed</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

0.95 and 0.99: Confidence Intervals of rho

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<th>( \rho )</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
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<tr>
<td>0.95</td>
<td>0.986</td>
<td>1</td>
</tr>
<tr>
<td>0.99</td>
<td>0.963</td>
<td>1</td>
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</table>

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<th>Y</th>
<th>Residuals</th>
</tr>
</thead>
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<tr>
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<td>18</td>
<td>212.082</td>
<td>-4.192</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>284.081</td>
<td>2.745</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>349.659</td>
<td>3.262</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>413.469</td>
<td>2.01</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>472.695</td>
<td>-3.825</td>
</tr>
</tbody>
</table>
Table: 3.5 Linear Correlations and Regression Data Sheet / Summary for Etodolac

\[ \sum X = 400, \quad \sum X^2 = 34560, \quad \sum Y = 17282.164 \quad \sum Y^2 = 64013165.9622 \]

Therefore \[ \sum XY = 1487209.792 \]

<table>
<thead>
<tr>
<th>R</th>
<th>( r^2 )</th>
<th>Slope</th>
<th>Y Intercept</th>
<th>Standard error of estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.999</td>
<td>0.999</td>
<td>40.874</td>
<td>186.5368</td>
<td>23.3775</td>
</tr>
<tr>
<td>T</td>
<td>df</td>
<td>P</td>
<td>One-tailed</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>88.464</td>
<td>3</td>
<td></td>
<td>Two-tailed</td>
<td>&lt;.0001</td>
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0.95 and 0.99 : Confidence Intervals of rho

<table>
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<tr>
<th>( \rho )</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
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<tr>
<td>0.95</td>
<td>0.985</td>
<td>1</td>
</tr>
<tr>
<td>0.99</td>
<td>0.963</td>
<td>1</td>
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</table>

Values entered:

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<th>Y</th>
<th>Residuals</th>
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<tbody>
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<td>1</td>
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<td>2124.641</td>
<td>-23.833</td>
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<td>2</td>
<td>64</td>
<td>2834.196</td>
<td>31.742</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>3459.154</td>
<td>2.721</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>4105.076</td>
<td>-5.336</td>
</tr>
<tr>
<td>5</td>
<td>112</td>
<td>4759.097</td>
<td>-5.294</td>
</tr>
</tbody>
</table>
Table: 3.6: Least Linear Regression Data Analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tolperisone HCl</th>
<th>Etodolac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. Range (µg/mL)</td>
<td>18 - 42</td>
<td>48 - 112</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>10.844</td>
<td>40.874</td>
</tr>
<tr>
<td>Intercept (b)</td>
<td>21.0902</td>
<td>186.5368</td>
</tr>
<tr>
<td>Standard error of estimate</td>
<td>4.2593</td>
<td>23.3775</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.66</td>
<td>0.41</td>
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</table>

Performance Calculations

To ascertain the system suitability for the method, a number of parameters such as retention time, theoretical plates, and HETP, tailing factor, % Peak area, and LOD, LOQ, resolution and peak asymmetry have been calculated with the observed readings and the results are recorded in Table: 3.7.

Table: 3.7: Validation Summary / System Suitability

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tolperisone HCl</th>
<th>Etodolac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical Plates (N)</td>
<td>5183</td>
<td>4143</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.39</td>
<td>1.44</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>2.957</td>
<td>4.193</td>
</tr>
<tr>
<td>Resolution</td>
<td>-----</td>
<td>5.82</td>
</tr>
<tr>
<td>% Peak Area</td>
<td>9.08</td>
<td>90.92</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>1.30</td>
<td>1.88</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>3.93</td>
<td>5.70</td>
</tr>
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</table>
Method validation

The following parameters were used to validate the method for the proposed assay procedure of TOL & ETD in pharmaceutical dosage forms. The developed HPLC method for the simultaneous determination of Thiocolchicoside and Aceclofenac was validated as per the ICH guidelines\(^{44,45}\).

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision was considered at different levels, i.e. method, system, Inter day and intraday. Precision of the developed method was assessed by measuring the response on the same day (intraday precision) and next two consecutive days (inter day precision). The precision of the method was assessed by six replicate injections of 100% test concentration. Intra and inter-day precision of the method was assessed by determination of standard deviation and % RSD for the analyte response. The result was given in Table 3.8. Similarly the representative chromatograms for method precision were shown in Figure: 3.3.
Figure: 3.3: Representative Chromatograms for Method Precision (Six Replicates)
Table: 3.8: Method Precision (Inter and Intraday) studies for Tolperisone and Etodolac by proposed method

<table>
<thead>
<tr>
<th>Summary showing Method Precision by Proposed Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolperisone HCl</td>
</tr>
<tr>
<td>Method Precision (Inter &amp;Intra Day)</td>
</tr>
<tr>
<td>99.1</td>
</tr>
<tr>
<td>99.2</td>
</tr>
<tr>
<td>99.5</td>
</tr>
<tr>
<td>99.4</td>
</tr>
<tr>
<td>98.5</td>
</tr>
<tr>
<td>99.4</td>
</tr>
<tr>
<td>Overall Avg.</td>
</tr>
<tr>
<td>Overage Std Dev.</td>
</tr>
<tr>
<td>Over all %RSD</td>
</tr>
</tbody>
</table>
Linearity and range

The standard curve was obtained in the concentration range of 18 - 42 μg/mL for TOL and 48 - 112 μg/mL for ETD. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient \( r^2 \) of standard curve were calculated and given in Figure-3.5 (Tolperisone HCl) and Figure-3.6 (Etodolac) to demonstrate the linearity of the proposed method. The result of regression analysis was given in the Table 3.6. Similarly the representative chromatograms for linearity were shown in Figure: 3.4. From the data obtained which given in Table-3.9 (Tolperisone HCl & Etodolac) the method was found to be linear within the proposed range.

Figure: 3.4 Representative Chromatograms for Linearity
Table: 3.9: Linearity studies for Tolperisone and Etodolac by proposed method

<table>
<thead>
<tr>
<th>% Level</th>
<th>Conc. µg/mL</th>
<th>Area</th>
<th>Conc. µg/mL</th>
<th>Area</th>
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<td>48.00</td>
<td>2124.641</td>
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<td>80</td>
<td>24.00</td>
<td>284.081</td>
<td>64.00</td>
<td>2834.196</td>
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<td>100</td>
<td>30.00</td>
<td>349.659</td>
<td>80.00</td>
<td>3456.154</td>
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<tr>
<td>120</td>
<td>36.00</td>
<td>413.469</td>
<td>96.00</td>
<td>4105.076</td>
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<td>140</td>
<td>42.00</td>
<td>472.695</td>
<td>112.00</td>
<td>4759.097</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Slope</th>
<th>Intercept</th>
<th>% Y-Intercept</th>
<th>Residual Sum of Squares</th>
<th>CC(r)</th>
<th>RSQ(r2)</th>
<th>LOD</th>
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<tbody>
<tr>
<td>10.8</td>
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<td>194.5</td>
<td>4.3</td>
<td>0.9994</td>
<td>0.9987</td>
<td>1.30</td>
<td>3.93</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>
System suitability

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), Resolution, Tailing
factor. The HPLC system was equilibrated using the initial mobile phase composition, followed by 5 injections of the standard solution of 100% concentration containing 30 µg/mL of TOL and 80 µg/mL of ETD. These 5 consecutive injections were used to evaluate the system suitability on each day of method validation. The result was given in the below table 3.10.

Table 3.10: System suitability parameters for Tolperisone and Etodolac by proposed method

<table>
<thead>
<tr>
<th>Name of the Compound</th>
<th>Retention Time</th>
<th>Theoretical plate</th>
<th>Tailing factor</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
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<td>Tolperisone</td>
<td>2.957</td>
<td>1.39</td>
<td>5183</td>
<td>-</td>
</tr>
<tr>
<td>Etodolac</td>
<td>4.193</td>
<td>1.44</td>
<td>4143</td>
<td>5.82</td>
</tr>
</tbody>
</table>

Specificity & Interference studies

The effect of wide range of excipients & additives were studied and determined under optimum conditions. A study to establish the interference of blank was conducted. Diluent was injected into the chromatograph in the defined above chromatographic conditions and the blank chromatograms were recorded. Chromatogram of Blank solution (Figure: 3.1) showed no peaks at the retention time of TOL and ETD peaks. This indicates that the diluent solution used in sample preparation do not interfere in estimation of TOL and ETD in tablets. Similarly typical representative chromatogram of standard is also shown (Figure: 3.2).
Analysis of Formulation

To find out the suitability of the method for the assay of pharmaceutical formulation containing TOL and ETD were analyzed by the proposed method. It was found that the proposed method do not differ significantly in the precision and accuracy from the reference method. The results are recorded in Table: 3.11.

Accuracy

To determine the accuracy of the proposed method, different amounts of bulk samples of TOL & ETD within linearity limits were taken and analyzed by the proposed method. The results are presented in Table: 3.11 & 3.12 and Figure 3.15. The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%), which is determined by the standard addition method. In the current study recovery at three spike levels 50%, 100% and 150% were carried out. The % recovery at each spike level was calculated and was given in Tables: 3.11 & 3.12. Similarly the representative chromatograms for recovery studies at different levels were shown in Figures: 3.7 – 3.9.
Figure 3.15: Representative Assay chromatograms
Recovery Studies

Recovery studies were conducted by analyzing the formulations in the first instance for the active ingredients in the concentration of 50%, 100% & 150% of the working standard solution for both the two drugs (TOL & ETD) by the proposed method. Each concentration was injected three times and the peak areas were recorded. The known amount of the pure drug 10% of the working standard solution contains was added to each three previously analyzed formulations and the total amount of the drug was again determined by the proposed method (each concentration was injected three times) by keeping the active ingredient concentration within the linearity limits. The chromatograms are shown in Figures: 3.7 – 3.9 and the results are recorded in Table: 3.11, 3.12 & 3.14.

Figure: 3.7: Reference chromatogram for Recovery-50% level
Figure: 3.8: Reference chromatogram for Recovery-100% level

Figure: 3.9: Reference chromatogram for Recovery-150% level
<table>
<thead>
<tr>
<th>% Level</th>
<th>Recovery Range</th>
<th>% RSD at each level</th>
<th>Over all % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>97.8-98.9</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>98.2-99.8</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>150</td>
<td>97.8-98.9</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

Table: 3.12: Recovery studies for Etodolac by proposed method

<table>
<thead>
<tr>
<th>% Level</th>
<th>Recovery Range</th>
<th>% RSD at each level</th>
<th>Over all % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>98.1-98.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>98.6-99.6</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>150</td>
<td>99.2-100.5</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

**LOD and LOQ**

The detection limit of the method was investigated by injecting standard solutions CTRI/2009/091/001021® into the HPLC column. By using the signal-to-noise (S/N) method, the peak-to-peak noise around the analyte retention time is measured. Subsequently, the concentration of the analyte which would yield a signal equal to certain value of noise to signal...
A signal-to-noise ratio (S/N) of 3 was generally accepted for estimating LOD and signal-to-noise (S/N) ratio of 10 was used for estimating LOQ.

The LOQ can be determined by signal-to-noise ratio of 10:1, or even approximated by multiplying the LOD by 3. This method is commonly applied to analytical methods that exhibit the baseline noise. The LOD was found out to be 1.30 & 1.88 µg/mL for TOL & ETD respectively. The LOQ was found to be 3.93 & 5.70 µg/mL for TOL & ETD.

**Robustness**

The robustness of the method was determined by assessing the ability of the developed method to remain unaffected by the small changes in the parameters such as percent organic content, pH of the mobile phase, buffer concentration, temperature, injection volume and flow rate. A deviation of ± 2 nm in the detection wavelength, ± 0.2 ml/min in the flow rate, ± 5% change in the organic phase were tried individually. The result was given in the Table 3.13. Similarly the representative chromatograms for robustness parameters like flow variation and wavelength variation were shown in Figure. 3.10.

**Figure: 3.10 - Representative Chromatograms for robustness parameters**
Table: 3.13: Robustness studies for Tolperisone and Etodolac by proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tolperisone</td>
</tr>
<tr>
<td>Wavelength ( ±2)</td>
<td></td>
</tr>
<tr>
<td>261 nm</td>
<td>0.22</td>
</tr>
<tr>
<td>265 nm</td>
<td>0.34</td>
</tr>
<tr>
<td>Flow Rate (ml /min) (±0.2)</td>
<td></td>
</tr>
<tr>
<td>0.8 ml/min</td>
<td>0.68</td>
</tr>
<tr>
<td>1.2 ml.min</td>
<td>0.54</td>
</tr>
</tbody>
</table>

FORCED DEGRADATION STUDIES

Control Sample:

Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 150 mg of Tolperisone and 400 mg of Etodolac into a 200 ml volumetric flask, add 150 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 4.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 3.2)

Acid Degradation Sample:

Weigh and finely powder not fewer than 20 tablets. Accurately weigh and transfer equivalent to 150 mg of Tolperisone and 400 mg of Etodolac into a 200 ml volumetric flask, add 150 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5ml of 1N HCl, refluxed for 30 min at 60°C, then cooled to room
temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 4.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 3.11)

**Base Degradation Sample:**

Weigh and finely powder not fewer than 20 tablets. Accurately weigh and transfer equivalent to 150 mg of Tolperisone and 400 mg of Etodolac into a 200 ml volumetric flask, add 150 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5ml of 1N NaOH, refluxed for 30 min at 60°C, then cooled to room temperature, neutralize with 1N HCl and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 4.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 3.12)

**Peroxide Degradation Sample**

Weigh and finely powder not fewer than 20 tablets. Accurately weigh and transfer equivalent to 150 mg of Tolperisone and 400 mg of Etodolac into a 200 ml volumetric flask, add 150 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 ml of Hydrogen Peroxide, refluxed for 30 min at 60°C, then cooled to room temperature, and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 4.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 3.13)

**Thermal Degradation Sample**

Powder collected from 20 tablets is exposed to heat at 105°C for about 5 days. Accurately weigh and transfer equivalent to 150 mg of Tolperisone and 400 mg of Etodolac into
a 200 ml volumetric flask, add 150 ml of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 4.0 ml of the above solution into a 100 ml volumetric flask and dilute to volume with diluent. (Figure: 3.14). Similarly Humidity, UV-Light exposure, Sunlight exposure and Water hydrolysis stress samples are prepared and checked for their purity by proposed method.

**Figure: 3.11: A typical HPLC Chromatogram showing the Control Sample profile of Tolperisone and Etodolac in Acidic hydrolysis by proposed method**
Figure: 3.12: A typical HPLC Chromatogram showing the Control Sample profile of Tolperisone and Etodolac in Base hydrolysis by proposed method

Figure: 3.13: A typical HPLC Chromatogram showing the Control Sample profile of Tolperisone and Etodolac in Peroxide hydrolysis by proposed method
SUMMARY: RESULTS AND DISCUSSION

Column chemistry, solvent selectivity, solvent strength (volume fraction of organic solvent(s) in the mobile phase), detection wavelength and flow rate were varied to determine the chromatographic conditions for giving the best separation. Several mobile phase compositions were tried to resolve the peaks of Tolperisone and Etodolac. The optimum results were attained with acetonitrile: phosphate buffer (pH 2.6) in the ratio 30:70 (v/v) because it could resolve the peaks of Tolperisone with retention time at 2.957 min and Etodolac retention time at 4.193 min. The two peaks were symmetric and sufficiently resolved. System suitability was carried out by
injecting 5 replicate injections of 100 % concentration of Tolperisone and Etodolac. The resolution was found to be greater than 2 and the other parameters are presented in Table 3.10.

Specificity of the chromatographic method was tested by injecting mobile phase as blank and sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Tolperisone and Etodolac at 2.957 min and 4.193 min respectively without any interference. Thus the developed method was specific for analyzing the commercial formulations for Tolperisone and Etodolac. An optimized chromatogram with the retention times of Tolperisone and Etodolac was shown in the Figure. 3.2.

The peak areas corresponding to the concentration range of Tolperisone 18-42 µg/mL and Etodolac 48-112 µg/mL prepared in triplicate were plotted against the respective concentrations. The calibration curves were linear in the range studied for Tolperisone and Etodolac, respectively, with mean correlation coefficients (n=3) of 0.999 and higher, the representative calibration curves were shown in Figure 3.5 & 3.6. The regression analysis was given in Table 3.6.

Accuracy of the proposed method was assessed by standard addition method at 50 %, 100 % and 150 % levels of recovery to the pre analyzed sample in triplicate. The recovery of the added standard to the sample was calculated and it was found to be 97.8-99.8 % w/w for Tolperisone and 98.1-100.5 % w/w for Etodolac respectively and the % RSD was less than 2 for both the drugs which indicates good accuracy of the method. The result of recovery was given in Table 3.11 & 3.12.
LOD and LOQ were calculated from the average slope and standard deviation of Y intercepts of the calibration curve. Limit of Detection for Tolperisone and Etodolac were 1.30 µg/mL and 1.88 µg/mL respectively where as Limit of Quantitation of Tolperisone and Etodolac were 3.93 µg/mL and 5.70 µg/mL respectively indicating high sensitivity of the method. LOD and LOQ value was given in tables 3.9 & 3.11. The method is precise with a % RSD of less than 2 for both Tolperisone and Etodolac respectively. The results of intraday and inter day precision was given in table 3.8. Robustness was carried out by change in the flow rate (±0.2 ml/min), mobile phase variation (±5%) and variation in wavelength (± 2 nm). Solution of 100 % concentration is prepared and injected in triplicate for each varied operational condition and % R.S.D was found to be less than 2. The result was given in table 3.6. The proposed method was applied for the assay of commercial formulation containing Tolperisone and Etodolac. Each sample was analyzed in triplicate. The mean recovery values were 98.73 and 97.25 % for Tolperisone and Etodolac. The results of estimation were given in table 3.11 & 3.12.
Table 3.14: Assay studies for Tolperisone and Etodolac by proposed method on Marketed sample

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount Claimed in mg per Tablet</th>
<th>Amount obtained (mg) by proposed method</th>
<th>% recovery by the proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolperisone HCl</td>
<td>150</td>
<td>148.12</td>
<td>98.73</td>
</tr>
<tr>
<td>Etodolac</td>
<td>400</td>
<td>389.28</td>
<td>97.25</td>
</tr>
</tbody>
</table>
CONCLUSION

There are no reports on the simultaneous HPLC determination of TOL & ETD in combination pharmaceutical formulations from the literature prior to the commencement of these investigations. The author has developed a sensitive, accurate and precise RP-HPLC procedure for the simultaneous estimation of TOL & ETD in bulk drug and also in pharmaceutical formulations.

The proposed RP-HPLC method for simultaneous assay TOL and ETD in combined dosage forms was validated, and found to be applicable for routine quantitative analysis. In the HPLC method, the standard and sample preparations required less time and no tedious extraction were involved thereof. The low values of standard deviations are indicative of the high precision of the method developed. The results of linearity, precision, accuracy and specificity, were proved to be within the limits.

The absence of additional peaks in the chromatogram indicated non-interference of the common excipients used in the tablets. It is thus, demonstrated that the developed RP-HPLC method is simple, linear, accurate, sensitive and reproducible. Thus, the developed method can easily be used for the routine quality control of bulk and pharmaceutical formulations of TOL & ETD with a short analysis time.

It can be seen from the results presented that the proposed procedure has good precision and accuracy. The above proposed method obviates the need for any preliminary treatment and is simple, sensitive and reliable. Thus, the present procedures constitute the first ever reported RP-HPLC method with good precision, accuracy and sensitivity for the simultaneous estimation of TOL & ETD in pure stage and also in combination products.
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