A NOVEL RP–HPLC METHODOLOGY FOR METHOD DEVELOPMENT AND VALIDATION OF ACECLOFENAC AND TIZANIDINE PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A simple and selective LC technique is chosen for the determination of Aceclofenac and Tizanidine in pill indefinite quantity forms. Chromatographic process separation was achieved on a c18 column victimization mobile part consisting of a combination of fifty volumes of Triethylamine buffer, fifty volumes of acetonitrile with detection of 230nm. Dimensionality was discovered within the vary 5-15 µg/ml for aceclofenac ($r^2 =0.999$) and 1-3 µg /ml for tizanidine ($r^2 =0.998$) for the number of medicine calculable by the planned strategies was in smart agreement with the label claim. The planned strategies have a sound procedure. At three completely different levels the accuracy of the strategies was assessed by recovery studies. The recovery experiments indicated the absence of interference from unremarkably encountered pharmaceutical additives showing %RSD below a pair of this technique was found to be precise as indicated by the repeatability analysis. All applied mathematics information proves all ways have valid procedure and might be used for routine analysis of pharmaceutical dose kind.

INTRODUCTION

Pharmaceutical analysis just means examination of pharmaceuticals. Webster' word reference portrays a pharmaceutical is a medication. An additionally fitting term for a pharmaceutical is dynamic pharmaceutical fixing to remember it from a planned thing or prescription thing is set up by figuring a solution substance with idle concentration (excipients) to set up a drug thing that is sensible for association to patients [1].

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Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Reverse phase chromatography uses hydrophobic bonded packing, usually with an octadecyl or octyl functional group and a polar aqueous mobile phase. Polar substances prefer the mobile phase and eluted first. Because the hydrophobic character of the solutes will increase, and followed retention will also increase. Generally, the lower the polarity of the mobile section, the upper is its eluent strength. The elution order of the classes of compounds in table is reversed (thus the name reverse-phase chromatography) [2].

Drug Profile

Aceclofenac is a non-steroidal calming drug (NSAID) with stamped mitigating and pain relieving properties. It is accounted for to have a higher mitigating activity or if nothing else tantamount impacts than ordinary NSAIDs in twofold visually impaired investigations. Aceclofenac intensely hinders the cyclo-oxygenase protein (COX) that is engaged with the combination of prostaglandins, which are fiery middle people that reason torment, swelling, irritation, and fever [3]. It is orally regulated for the alleviation of agony and aggravation in osteoarthritis, rheumatoid joint pain and ankylosing spondylitis. Aceclofenac has a place with BCS Class II as it has poor watery dissolvability. It shows high porousness to infiltrate into synovial joints where in patients with osteoarthritis and related conditions, the loss of articular ligament in the territory causes joint torment, delicacy, solidiness, crepitus, and nearby aggravation [4]. Aceclofenac is likewise answered to be compelling in other excruciating conditions, for example, dental and gynecological conditions. In 1991, aceclofenac was produced as a simple of a usually recommended NSAID, Diclofenac, by means of substance adjustment in push to enhance the gastrointestinal mediocrity of the medication. It is an all the more generally recommended medicate in Europe [5]. Through COX-2 hindrance, aceclofenac downregulates the generation of different provocative go betweens including prostaglandin E2 (PGE2), IL-1β, and TNF from the arachidonic corrosive (AA) pathway. Restraint of IL-6 is believed to be interceded by diclofenac changed over from aceclofenac [6]. Stifled activity of incendiary cytokines diminishes the creation of receptive oxygen species. Aceclofenac is appeared to diminished generation of N2O in person articular chondrocytes. Also, aceclofenac meddles with neutrophil bond to endothelium by diminishing the statement of L-selectin (CD62L), which is a cell grip atom communicated on lymphocytes. Aceclofenac is proposed to fortify the amalgamation of glycosaminoglycan in human osteoarthritic ligament which might be interceded through its inhibitory activity on IL-1 creation and movement. The chrondroprotective impacts are created by 4'-hydroxyaceclofenac which stifles IL-1 interceded generation of promatrix metalloproteinase-1 and metalloproteinase-3 and meddles with the arrival of proteoglycan from chondrocytes [7]. Tizanidine is a short-acting medication for the administration of spasticity. Tizanidine is an agonist at a2-adrenergic receptor, and it is very close to clonidine and apparently lessens spasticity by expanding presynaptic hindrance of engine neurons. In creature models, tizanidine has no immediate impact on skeletal muscle filaments or the neuromuscular intersection, and no significant impact on monosynaptic spinal reflexes. The impacts of tizanidine are most prominent on polysynaptic pathways. The general impact of these activities is thought to decrease assistance of spinal engine neurons [8]. Tizanidine reduces spasticity by causing presynaptic inhibition of motor neurons via agonist acts at Alpha-2 adrenergic receptor sites. This drug is centrally acting and leads to a reduction in the release of excitatory amino acids like glutamate and aspartate, which cause neuronal firing that leads to muscle spasm. The above reduction and excitatory neurotransmitter release results in presynaptic inhibition of motor neurons. The strongest effect of tizanidine has been shown to occur on spinal polysynaptic pathways. The anti-nociceptive and anticonvulsant activities of tizanidine may also be attributed to agonist action on Alpha-2 receptors. Tizanidine also binds with weaker affinity to the Alpha-1 receptors, explaining its slight and temporary effect on the cardiovascular system [9, 10]. In young nursing rats, abnormal results were obtained in tests indicative of central nervous system function. Various developmental changes that may have been attributable to the drug were observed. It is unknown whether tizanidine is excreted in human milk. It is a lipid-soluble drug, however, and likely to be excreted into breast milk [11].

Quality investigation plays a very important role in quality specification establishment of chemical drugs. The numbers of drugs launch into the retail every year very often there's a time lag from the time of launch of a drug into the market to the date of its insertion in pharmacopoeias. Hence, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, so to develop newer analytical strategies for such medication [12].
To develop new RP HPLC method, of pharmaceutical dosage form. The plan of work includes solubility determination of aceclofenac and tizanidine various solvents and buffers. Establish the maximum absorbance of each of the medication in UV-Visible region in numerous solvents/buffers and choosing the dissolving agents for HPLC methodology development. Optimize the mobile phase and flow rates for proper resolution and retention times. Validate the developed method as per ICH guidelines.

**MATERIAL AND METHOD**

**Instruments Used**
The instruments which were used for this work were, UV Visible apparatus which was manufactured by Nicolet evolution 100, UV-Visible Software that was developed by Vision Pro, HPLC software that was developed by Spin chrome (LC solutions), HPLC and Electronic balance manufactured by Shimadzu(LC 20 AT VP), Ultra sonicator which was manufactured Citizen, Digital Ultrasonic Cleaner, pH meter manufactured by Global digital, Syringe was used for injection which was manufactured by Hamilton, HPLC Column was obtained from Inertsil ODS 3V(250x4.6mm) 5μm

**Reagents Used**
Water, Methanol, was used according to HPLC Grade; Ammonium acetate was used as per AR Grade.

**Drugs Used**
Aceclofenac and Tizanidine drugs are obtained as gift Samples obtained from Chandra labs; Hyd. ACENT (100/2mg) was obtained from local pharmacy.

**Preparation of Mobile Phase**
A concoction of fifty volumes of triethylamine buffer maintaining a pH of 3.0 and same volume of acetonitrile were created. The mobile section was sonicated for 10min to get rid of gases and filtered through 0.45µ membrane for degassing from the mobile section.

**Determination of Wavelength using UV Visible Spectroscopy [13]**

**Preparation of stock solution of acenocafen**
Accurately weighed 10 mg of acenocafen was pass on to a 100 ml flask and aceclofenac was dissolved in methanol, make up to the 100ml with same solvent to prepare 100 μg /ml. From this solution pipette out 1ml and prepare up to 10ml with methanol to prepare 10 μg /ml of drug solution.

**Preparation of stock solution of tizanidine**
Weighed 10 mg of drug was shifted in to a 100ml volumetric flask and tizanidine was dissolved in methanol, make up to the 100ml with same solvent to prepare 100μg /ml. From this suspension pipette out 1ml and prepare up to 10ml with methanol to prepare 10 μg /ml of drug solution.

**Method Development**

**Trial – 1**
The Mobile phase that is used are Phosphate buffer: Acetonitrile: Methanol maintaining a pH of 5.0 in an ratio, 40:30:30 weigh accurately 5 mg of acenocafen and 5 mg of tizanidine in 100 ml of meter flask and add 10ml of mobile phase and dissolve, and make up the volume with mobile phase. From above stock solution, to prepare, 10µg/ml of acenocafen and 20 µg/ml of tizanidine, it is prepared by diluting 2.4ml of stock solution in 10ml of mobile phase. This liquid is better used for chromatogram recording [14].

**Trial- 2**
The mobile phase that is used are Triethylamine: Acetonitrile maintaining a pH of 3.0 in an ratio, 50:50. Weigh accurately 5 mg of acenocafen and 5 mg of tizanidine in 100 ml of volumetric flask and add 10ml of mobile phase and dissolve and make up the capacity with mobile phase. From above stock solution, to prepare, 10µg/ml of acenocafen and 20 µg/ml of tizanidine, it is prepared by diluting 2.4ml in 10ml of mobile phase. This liquid is better used for chromatogram recording [15].

**Assay [16]**

**Preparation of mixed standard solution**
Weigh accurately 5 mg of acenocafen and 5 mg of tizanidine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10µg/ml of acenocafen and 20µg/ml of tizanidine is prepared by diluting 2.4ml in 10ml of mobile phase. This liquid is better used for chromatogram recording.

**Tablet sample**
10 tablets (each tablet contains aceclofenac -100 mg and tizanidine – 100 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Stock solutions of tizanidine and acenocafen tablets (μg/ml) were made by solubilizing acenocafen and tizanidine drugs equivalent to 5 mg in sufficient mobile phase. After that filter the solution using 0.45-micron wheel filter and sonicate for five minutes and mix it up to 10 ml with mobile phase. Further dilutions are prepared
in 5 replicates of 20 μg/ml of tizanidine and 10 μg/ml of aceclofenac was made by adding 2.4 ml of stock solution upto 10 ml of mobile phase. Validation [17].

**Linearity and range**

Standard stock solutions of aceclofenac and tizanidine (μg/ml) were prepared by dissolving 10 mg of aceclofenac and tizanidine in 10 ml of mobile phase and dilute and make up the volume to 100 ml with mobile phase.

**Accuracy**

Accuracy of the method is determined by recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were dole out three times and also the percentage recovery and percentage mean recovery were calculated. To check the accuracy of the maneuver, recovery studies were disbursed by addition of ordinary drug resolution to pre- analyzed sample solution at 3 totally different levels 50%, 100%, 150%.

**Precision**

Method precision. Prepared sample preparations of tizanidine and aceclofenac as per test method and injected 6 times in to the column. The percentage recovery of tizanidine and aceclofenac should not be more than 2.0%.

**Robustness**

To demonstrate the strength of the method, prepared solution as per test method and injected at completely different variable conditions like flow and wavelength. System suitableness parameters were compared there upon of technique preciseness.

**Ruggedness**

The strength of the tactic was studied by the determining the analyst to analyst variation by performing the Assay by 2 totally different analysts. The percentage relative standard deviation should be not more than 2.0%.

**RESULTS**

**Determination of Wavelength of Aspirin and Clopidogrel using UV Visible Spectroscopy**

(X – Axis – Wavelength and Y – axis – Absorbance)

The wavelength (λmax) at where maximum absorbance of the drug of 10 μg/ml concentration using methanol as solvent was scanned using UV-Visible spectrophotometer in the range of 200–400 nm comparing methanol as blank. and the isobestic value was observed to be 230 nm for mixture of the medications.

**Method Development of Aceclofenac and Tizanidine**

**Trial – 01** (X – Axis – Time and Y – axis – Voltage)

Trial – 01 - The Efficiency was not satisfactory for aceclofenac and the baseline was not proper. Hence it was not taken for optimization.
Trial – 02 (X – Axis – Time and Y – axis – Voltage)

Trial – 02 – In this trial, all the system suitability requirements were met. The peak asymmetry factor was below 2 for both tizanidine and aceclofenac. The efficiency was more than 2000 tizanidine and aceclofenac. Resolution between two peaks is >1.5. Hence, the method is taken for optimization.

**Assay Results**

|          | ACECLOFENAC |  | TIZANIDINE |  |
|----------|-------------|  |------------|  |
| Injection-1 | 2334.362 |  | 2344.463 |  |
| Injection-2 | 2323.199 |  | 2351.614 |  |
| Injection-3 | 2337.863 |  | 2337.863 |  |
| Injection-4 | 2331.502 |  | 2344.463 |  |
| Injection-5 | 2328.483 |  | 2341.801 |  |
| Average Area | 2331.082 |  | 2342.095 |  |
| Standard deviation | 6.489006 |  | 2.004044 |  |
| %RSD | 0.276506 |  | 0.952806 |  |
| Assay(%purity) | 100.27 |  | 102.61 |  |
The amount of Aceclofenac and Tizanidine present in the taken dosage form was found to be 100.27% and 102.61% respectively.
Linearity of Aceclofenac and Tizanidine

| S. No. | Aceclofenac | Tizanidine |
|--------|-------------|------------|
|        | Conc.(µg/ml) | Area       | Conc.(µg/ml) | Area       |
| 1      | 5           | 1044.606   | 1            | 90.783     |
| 2      | 7.5         | 1549.853   | 1.5          | 129.306    |
| 3      | 10          | 2038.421   | 2            | 174.849    |
| 4      | 12.5        | 2563.186   | 2.5          | 222.682    |
| 5      | 15          | 3106.591   | 3            | 263.873    |

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of aceclofenac and tizanidine is 0.99 and 0.98. The relationship between the concentration of aceclofenac and tizanidine and area of aceclofenac and tizanidine is linear in the range examined since all points lie in a straight line and hence the correlation coefficient is well within limits.

Accuracy results for Aceclofenac and Tizanidine

| Recovery level | Accuracy ACECLOFENAC | Accuracy TIZANIDINE |
|----------------|-----------------------|---------------------|
|                | Amount taken (mcg/ml) | Area | % Recovery | Average % Recovery | Amount taken (mcg/ml) | Area | % Recovery | Average % Recovery |
| 50%            | 5                     | 2326.313 | 96.27 | 100.28          | 1 | 224.953 | 103.20 |
|                | 5                     | 2211.866 |       |                 | 1 | 280.286 |          |
|                | 5                     | 2194.643 |       |                 | 1 | 280.051 |          |
| 100%           | 10                    | 2608.241 | 101.86 | 100.28          | 2 | 236.388 | 97.60  |
|                | 10                    | 2609.160 |       |                 | 2 | 234.158 | 102.28 |
|                | 10                    | 2605.517 |       |                 | 2 | 242.445 |          |
| 150%           | 15                    | 3112.744 | 102.72 |                 | 3 | 3112.744 | 102.72 |
|                | 15                    | 3109.681 |       |                 | 3 | 267.053 |          |
|                | 15                    | 3106.682 |       |                 | 3 | 269.916 |          |
The percentage mean recovery should lie between 98 to 103 % and the percentage mean recovery of aceclofenac and tizanidine is 100.28 % and 102.25 % respectively.
Results for Method Precision of Aceclofenac and Tizanidine

| S. No. | ACECLOFENAC | TIZANIDINE |
|--------|-------------|------------|
|        | Rt  | Area     | Rt  | Area     |
| 1      | 2.510| 2192.147 | 4.397| 267.545  |
| 2      | 2.523| 2322.573 | 4.410| 211.442  |
| 3      | 2.523| 2321.138 | 4.413| 202.102  |
| 4      | 2.523| 2333.196 | 4.413| 200.853  |
| 5      | 2.507| 2350.119 | 4.397| 202.888  |
| 6      | 2.497| 2341.355 | 4.390| 198.551  |
| Avg    | 2.513833| 2310.088 | 4.403333| 213.8968 |
| stdev  | 0.010926| 58.82541 | 0.009893| 21.512   |
| %RSD   | 0.433746| 1.021     | 0.224216| 0.0213   |

The test results for Aceclofenac and tizanidine are showing that the %RSD within the acceptable limits.
Result of Robustness study

| Parameter | ACECLOFENAC | TIZANIDINE |
|-----------|-------------|------------|
| Flow Rate |             |            |
| 0.8 ml/min| 3.130       | 5.443      |
| 1.2 ml/min| 2.090       | 3.663      |
| Wavelength|             |            |
| 233nm     | 2.513       | 4.380      |
| 237nm     | 2.517       | 4.380      |

From the observation it was found that the system suitability parameters were within the acceptable limits at all variable conditions.

Results for Ruggedness

| Parameter | ACECLOFENAC | TIZANIDINE |
|-----------|-------------|------------|
| Analyst 01| 96.28%      | 99.30%     |
| Analyst 02| 99.30%      | 95.97%     |

From the observation between the two analysts, it has been found out that, the percentage relative standard deviation should not greater than 2.0%, hence the method is considered as rugged.
The HPLC Technique is a very powerful separation tool in the determination of aceclofenac and tizanidine in pharmaceutical dosage forms. The analysis has been performed with various mobile phases which include, phosphate buffer: Acetonitrile: Methanol, and Triethylamine: Acetonitrile, which have provided optimization.

Post optimization, validation parameters including, linearity and range, accuracy, precision, robustness, ruggedness, have been carried out according to ICH guidelines and indicated the procedure to be valid, specific, accurate and more easily reproducible within the limits, agreeable with the label claim. The low values of %RSD for method precision suggested that the method is precise. Linearity evaluated for the analyte peak showed a good linear response over a wide range of concentration.

CONCLUSION
From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Aceclofenac and Tizanidine was found to be simple, precise, accurate and the higher resolution and shorter retention time makes this method more acceptable and cost effective. As the advanced research is being continued followed up by developing drug combinations and multiple dosage forms, the method which has been performed, it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories studies in near future.

FINANCIAL ASSISTANCE
Nil

CONFLICT OF INTEREST
The authors declare no conflict of interest.
AUTHOR CONTRIBUTION
Nenavath Adilakshmi and Nellore Dharani Sai Sreekanth conceived and planned the research experiment. Nenavath Adilakshmi did detailed literature review for the plan of the experiment. Nellore Dharani Sai Sreekanth had carried out the method development procedure trials until it reached optimization. Based on the trials, Nenavath Adilakshmi and Nellore Dharani Sai Sreekanth performed the validation parameters respectively, further contributing to the interpretation of final results. Nellore Dharani Sai Sreekanth took the lead in writing the manuscript with discussions with Nenavath Adilakshmi.

REFERENCES
[1] Hamilton RJ. Tarascon Pocket Pharmacopoeia. Jones & Bartlett Learning, London, (2014).
[2] Sanjay KD, Kumar HD. Importance of RP-HPLC in analytical method development: A review. Int. J. Pharm. Sci. Res., 3, 4626–33 (2012).
[3] Legrand E. Aceclofenac in the management of inflammatory pain. Expert Opin Pharmacother 5, 1347-57. (2004).
[4] Moore RA, Derry S, McQuay HJ. Single dose oral aceclofenac for postoperative pain in adults. Cochrane Database Syst Rev, 8, CD007588. (2009)
[5] Raza K, Kumar M, Kumar P, Malik R, Sharma G, Kaur M, Katare OP. Topical delivery of aceclofenac: challenges and promises of novel drug delivery systems. Biomed Res Int 2014, 406731. (2014).
[6] Pareek A, Chandurkar N. Comparison of gastrointestinal safety and tolerability of aceclofenac with diclofenac: a multicenter, randomized, double-blind study in patients with knee osteoarthritis. Curr Med Res Opin 29, 849-59. (2013).
[7] Pareek A, Chandurkar N, Gupta A, Sirsikar A, Dalal B, Jesalpura B, Mehrotra A, Mukherjee A. Efficacy and safety of aceclofenac-cr and aceclofenac in the treatment of knee osteoarthritis: a 6-week, comparative, randomized, multicentric, double-blind study. J Pain 12, 546-53. (2011).
[8] National Center for Biotechnology Information (2020). PubChem Compound Summary for CID 5487, Tizanidine. Retrieved October 18, 2020 from https://pubchem.ncbi.nlm.nih.gov/compound/Tizanidine.
[9] Tizanidine, Drug Bank, https://www.drugbank.ca/drugs/DB00697
[10] Ghanavatian S, Derian A. Tizanidine. [Updated 2020 Aug 11]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK519505/
[11] Tizanidine Canadian monograph, https://s3-us-west-2.amazonaws.com/drugbank/cite_this/attachments/files/000/004/471/original/Canadian_monograph_Tizanidine.pdf?1556041234
[12] Taylor RB, Shakoor O, Behrens RH, Everard M, Low AS, Wangboonskul J, Reid RG, Kolawole JA. Pharmacopoeial quality of drugs supplied by Nigerian pharmacies. Lancet 357, 1933-6. (2001).
[13] Siva Kumar R, Kumar Nallasivan P, Vijai Anand PR, Akelesh T, Venkatnarayanan R. Spectrophotometric methods for simultaneous estimation of aceclofenac and tizanidine. Int. J. PharmTech Res., 2, 945–9 (2010).
[14] Vaidya V V, Singh GR, Choukekar MP, Kekare MB. Simultaneous RP HPLC determination of aceclofenac, paracetamol and tizanidine in pharmaceutical preparations. E-Journal Chem., 7, 260–4 (2010).
[15] Patil PP, Ware AL, Hon PA. Development and validation of RP-HPLC method for simultaneous determination of diclofenac sodium and tizanidine hydrochloride in bulk and tablet formulation. J. Anal. Pharm. Res., 7, (2018).
[16] Sinha S, Rajput MS. Validated simultaneous multicomponent spectrophotometric determination of paracetamol, aceclofenac and tizanidine in tablets. Int. J. ChemTech Res., 3, 963–6 (2011).
[17] Siva Kumar R, Nathan Senthil P, Nallasivan KP, Sam Solomon WD, Venkatnarayanan R. A validated reversed phase HPLC-method for the determination of aceclofenac and tizanidine in tablets. Asian J. Pharm. Res. Heal. Care, 2, 84–94 (2010).