Development & validation of reversed phase HPLC method for quantification of water insoluble API

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Abstract: In the present work a specific, accurate, precise, and reproducible UV-HPLC method was developed and validated for the analysis of Aceclofenac. This method involved elution of Aceclofenac in a mobile phase which is composed of buffer pH 6.8 (i.e. using 0.01N KH_2PO_4) and HPLC grade Acetonitrile (60:40). Separation of the analyte was achieved using HPLC isocratic pump attached to the UV-VIS detectorC_{18}, guard column and C_{18} column. The injection volume was 20µL, detected at 274 nm; flow rate: 1mL/min. Standard calibration curve was measured and found linear from 0.1 to 40µg/ml. The validation parameters were measured according to FDA guidelines and successful results were obtained. The presented analytical method could be employed for pharmacokinetic studies.

Keywords: Aceclofenac, method development, validation.

INTRODUCTION

Aceclofenac [[2-(2', 6'-dichlorophenyl) amino] phenylacetoxyacetic acid], is orally administered drug; which belongs to non-steroidal anti-inflammatory (NSAIDs) group (Sheikh *et al*., 2011). It is analog to Dicolfenac and produces anti- inflammatory, anti-pyretic and analgesic activities. It is a prostaglandin synthetase (COX-2) inhibitor, and reduces the production of prostaglandins (E_2) and leukotrienes (Ballesteros *et al*., 1990; Gonzalez *et al*., 1994; Grau *et al*., 1991; www.micromedix.com, 16/9/2016). It's wider use for the treatment of inflammatory conditions (i.e. for >75 million patients) is due to lesser GI discomforts (Legrand, 2004).

Previously many researchers have described method development and validation of Aceclofenac alone (Bhinge *et al*., 2008; Hinz *et al*., 2003; Naz *et al*., 2011; Zawilla *et al*., 2002) in combination with other drugs (Lee *et al*., 2000) and in pharmaceutical dosage forms (Battu & Reddy, 2009; Gandhi *et al*., 2012; Gowramma *et al*., 2010; Joshi & Sharma, 2008; Sekar *et al*., 2010; Shah *et al*., 2008). In present study specific, accurate, precise, and reproducible HPLC method has been described which may be used for pharmacokinetic and bioequivalence study of Aceclofenac formulations.

MATERIALS AND METHODS

Materials and reagents

For this study Aceclofenac was gifted by Sami Pharm (Pvt.) Ltd. Acetonitrile (i.e. HPLC grade), KH_2PO_4 (i.e. potassium-di-hydrogen phosphate) were purchased from Merck. Distilled water was freshly obtained from distillation plant (Hamilton Lab, Kent, England).

Equipment

The separation of analyte was achieved on HPLC system connected with isocratic pump: LC-10A, Shimadzu Corp; Detector: SPD-10A, Shimadzu Corp; C_{18} guard column, C_{18} column: Teknokroma. Mediterranea SEA® 18 5µm 25x 0.46cm; Communication Bus Module: CBM 102, Shimadzu Corp and Rheodyne injector with 20µL loop. Software: Class-GC 10, Shimadzu Corp was used for detection and processing of data. Further equipments included: pH meter (Mettler Toledo), Filtration Assembly (Sartorius, Gottingen, Germany), Membrane Disc Filters: 0.45µm pore size (Millipore Corporation), Vacuum pump (England) and Ultrasonic Bath (Clifton, Nickel Electro Ltd, Somerset, England).

Chromatographic conditions

The analysis of Aceclofeanc was carried out at λ=274 nm; using injection volume 20µL and flow rate: 1mL/min. A combination of phosphate buffer and acetonitrile was used as mobile phase in a ratio of 60:40. Buffer was prepared using 0.01N potassium di-hydrogen phosphate solution and pH was adjusted to 6.8 using 1M NaOH solution.

Preparation of sample in mobile phase

100mg of Aceclofenac was weighed and transferred in a volumetric flask of 100mL with 5ml methanol. This mixture was shaken for five minutes and finally diluted to 100ml (q.s.) with mobile phase. This stock solution was subsequently diluted in mobile phase using serial dilution method to make 0.05, 0.1, 0.5, 1, 5, 10, 15, 20 and 40µg/mL, for constructing calibration curve.

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Preparation of sample in plasma

Spike stock solution of Aceclofenac was prepared in drug free human plasma at a concentration of 1mg/mL. This plasma sample was further diluted in blank plasma to produce: 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, and 40μg/mL. For separation of protein, acetonitrile and plasma samples in 1:1 ratio were vortex for 1 minute and centrifuged at high speed (i.e. 10,000 rpm) for ten minutes. Next filtration of supernatant layer was carried out by 0.45μm Swinney filter prior injection.

Method development

A new method was designed for analysis of Aceclofenac using mobile phase at pH6.8.Separation of analyte was carried out using C_{18} column (Teknokroma18 5μm 25x 0.46cm), connected with C_{18} guard column. Samples prepared in mobile phase were analysed at λ=274; which produced sharp peak with less retention time of Aceclofenac (i.e. around 8 minutes).The appropriate wave length of analysis was previously selected by using the standard solution of Aceclofenac and scanned on UV Spectrophotometer (UV-1800 Shimadzu Corporation Kyoto, Japan)at wave length: 273, 274, 282 and 286. At λ=274 light absorption of samples was much better therefore rest of the analysis was carried out at this wave length.

Validation

Method validation was the next stage after method development. FDA guidelines were followed for method validation (FDA, 2001).

Selectivity

Selectivity was investigated by analyzing blank plasma from six different volunteers and were compared with a nominal concentration of 1μg/mL in mobile phase and LLOQ in plasma.

Linearity

Three calibration curves of aceclofenac at different concentrations (i.e. 0.1-40μg/mL in mobile phase & plasma) were plotted. Linearity between the peak area and concentration of analyte were examined.

Accuracy and precision

For intraday, interday accuracy & precision determination; four different concentrations of aceclofenac (five replicates of each concentration) were analyzed. Mean, standard deviation, precision and percentage accuracy was calculated according to FDA, 2001 guide lines.

Limit of quantification (LOQ) and limit of detection (LOD)

Three samples of Aceclofenac at six different concentrations were prepared to determine these parameters. Sample results analyzed by applying regression and r^2 value. Mean, standard deviation and percentage coefficient of variation were estimated.

Absolute analytical recovery

Absolute analytical recovery was estimated in mobile phase and human plasma by examining the peak area of three different concentrations of Aceclofenac in plasma and mobile phase and their percentage recoveries were measured.

Stability of stock solution

Stability was determined by injecting 4 replicate injections of 25μg/mL Aceclofenac solution in mobile phase. Whereas, stability of Aceclofenac stock solution was determined by keeping the samples at -20°C for 1, 2 and 4 week intervals. Four replicate injections of the prepared solutions were used to evaluate stability of the sample.

Freeze and thaw stability

Freeze- thaw stability was estimated by 3 freeze/ thaw cycles. Serial dilutions method was employed to prepare a maximum of fifteen spike samples of low drug concentration i.e. 1μg/mL and high drug concentration i.e. 35μg/mL. These prepared samples were stored for 24 hours at -20°C. Only 5 samples out of 15 were thawed and analysed while remaining samples were again frozen for 24-hour. This procedure was replicated for 2 more cycles. Samples were then tested and a comparison was made with five fresh samples of similar concentration. Finally mean, standard deviation, and % coefficient of variance were evaluated.

RESULTS

Results of conducted study are mentioned in tables and figures. Table 1 represents the system suitability, sensitivity, and linearity (i.e. from 0.1-40μg/mL). Absolute analytical recovery is given in table 2. Table 3 indicates intraday/interday accuracy & precision of developed method in plasma. In table 4 freeze & thaw stability of Aceclofenac at lower and higher concentration in plasma is given. Mean calibration curves in plasma is given in fig. 1 and fig. 2 shows the chromatographs of blank plasma and plasma spiked with Aceclofenac low and high concentrations i.e. 1μg/mL and 10μg/mL.

Table 1: Method validation and system suitability studies

Parameters	Results
Linearity Range	0.1-40[ug/ml]
Regression equation	Y=mx+C
Correlation coefficient [R^2]	0.9995
Tailing factor	1.12
Resolution factor	2.92
Theoretical plates	10445
LOD	0.05ug/ml
LLOQ	0.1ug/ml

Table 2: Absolute analytical recovery in mobile phase and plasma

Theoretical concentration	Average measured conc. (ug/ml)		Recovery (%)	
(ug/ml)	Mobile phase	Plasma	Mobile phase	Plasma
1	1.031	0.961	103.100	96.100
20	20.24	19.797	101.200	98.985
35	35.412	34.873	101.177	99.637

Table 3: Intraday and interday accuracy and precision of developed method in plasma

Intraday				Interday			
Theoretical con. (ug/ml)	Average measured con. (ug/ml) in plasma	Precision	Accuracy (%)	Theoretical con. (ug/ml)	Average measured con. (ug/ml) in plasma	Precisio n	Accuracy (%)
20	19.98±0.207	1.03	99.94	20	19.98±0.043	0.21	99.93
10	09.99±0.161	1.60	99.90	15	15.00±0.054	0.35	100.04
5	05.08±0.053	1.03	101.72	10	09.96±0.173	1.73	99.68
1	0.98±0.095	9.73	98.00	1	0.98±0.022	2.27	98.80

Table 4: Freeze and Thaw stability of Aceclofenac in plasma

Low conc.1 ug/ml				High conc.35 ug/ml			
Sample	Average measured con. (ug/ml)	% CV	Recovery (%)	Sample	Average measured con. (ug/ml)	% CV	Recovery (%)
Fresh Sample	1.000±0.016	1.581	100.00	Fresh Sample	35.144±0.146	0.415	100.41
FT Cycle I	0.990±0.013	1.320	98.80	FT Cycle I	35.062±0.129	0.367	100.17
FT Cycle II	0.982±0.011	1.116	98.20	FT Cycle II	35.056±0.182	0.520	100.16
FT Cycle III	0.970±0.011	1.171	97.40	FT Cycle III	34.922±0.056	0.161	99.77

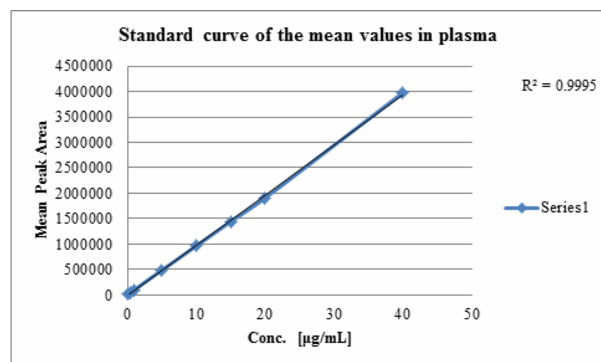

Fig. 1: Standard curve in plasma

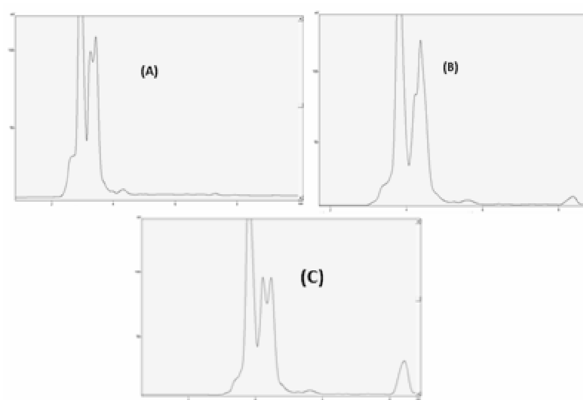

Fig. 2: Chromatograms of (a) blank plasma (b) plasma sample spiked with 1μg/ml Aceclofenac (c) b) plasma sample spiked with 10μg/ml Aceclofenac

DISCUSSION

Analysis of Aceclofenac in human plasma has been reported by many researchers in different countries. In this study a previously reported method was adopted to analyse the Aceclofenac concentration in human plasma by taking potassium-di-hydrogen phosphate buffer/ acetonitrile mixture in a ratio of 55:45 as mobile phase at pH 2.8 (Naz *et al.*, 2011). At wave length 286nm, the retention time of the drug was too long (i.e. 20-21min) on C_{18} column (Teknokroma 18 5μm 25x 0.46cm) with broad peaks. Therefore the pH of mobile phase was raised and tested at pH 3.8 and pH 5 using same composition of mobile phase but high retention time and poor peak shape was observed.

Thus in the present study a new method was designed and validated for the analysis of Aceclofenac in mobile phase and human plasma. For this purpose mobile phase containing phosphate buffer (0.01N potassium di-

hydrogen phosphate) and acetonitrile in a ratio60:40 at pH 6.8 was used. Appropriate separation of analyte was carried out at λ=274nm with least retention time i.e. 8 minutes. FDA bioanalytical method guidelines were followed for validation of new method. This method showed excellent results of validation parameters such as: linearity (from 0.1µg/ml to 40µg/ml with r^2=0.9995), selectivity (no interrupting peak observed at retention time of API), sensitivity, stability, absolute analytical recovery (in mobile phase and plasma respectively), accuracy and precision. These findings were in agreement with the existing reported result.

CONCLUSION

This study described a simple and specific analytical method for analysis of Aceclofenac in mobile phase and human plasma. Results of validation parameters revealed the selectivity, accuracy, precision and reproducibility of the developed method. Thus, this method could be used for pharmacokinetic and bioequivalence study of Aceclofenac in human volunteers.

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