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VALIDATED UPLC/Q-TOF-MS METHOD FOR SIMULTANEOUS DETERMINATION OF ACECLOFENAC AND PARACETAMOL IN HUMAN PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ABSTRACT

An ultra-performance liquid chromatographic/quadrupole time-of-flight mass spectrometric (UPLC/Q-TOF-MS) method has been developed and validated for simultaneous determination of aceclofenac and paracetamol in human plasma. Both the drugs were analyzed by Acquity UPLC BEH C18 (100.0 x 2.1 mm, 1.7 µm) column using isocratic mobile phase consisting of acetonitrile-2mM ammonium acetate (50:50, v/v) at a flow rate of 0.20 mL/min. The Q-TOF mass spectrometer was operated in positive ionization mode and quantitation was done using the MS/MS transitions m/z 354.07 to 215.07 for aceclofenac, and 152.07 to 110.06 for paracetamol. The calibration curves were linear over the concentration range of 1–1000 ng/mL for both the drugs. The developed method was validated according to ICH guidelines. The method was applied for pharmacokinetic study of FDC tablets containing aceclofenac and paracetamol in human plasma.

KEYWORDS: UPLC/Q-TOF-MS, Aceclofenac, Paracetamol, Pharmacokinetic Study.

INTRODUCTION

Aceclofenac (ACF), is chemically [[[2-[(2,6-Dichlorophenyl)amino] phenyl]acetyl]oxy] acetic acid, belongs to a group of non-steroidal anti-inflammatory drugs (NSAIDS). It has anti-inflammatory and analgesic properties and is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis, ankylosing spondylitis.[1] Paracetamol (PCM) is also known as acetaminophen, and chemically N-(4-hydroxy phenyl) acetamide. It is a potent

analgesic and antipyretic drug used worldwide for management of pain and fever.[2] Fixed dose combination (FDC) tablets containing aceclofenac, and paracetamol have been approved for acute painful condition in adults for relief from various diseases related with pain, inflammation and muscle spasm. Determination of aceclofenac alongwith its metabolite or degradation product were reported by several analytical methods such as UV spectrophotometry,[3-5] HPLC, [6-8] and LC-MS.[9] Several analytical methods have been reported for determination of paracetamol alongwith its metabolite or degradation product by UV spectrophotometry,[10-12] HPLC,[13] and LC-MS.[14,15] Simultaneous determination of aceclofenac and paracetamol in tablets has been reported by UV,[16] HPLC,[17-19] and densitometry.[20] In our previous investigation, an UPLC/Q-TOF-MS method was developed and validated for simultaneous determination of aceclofenac, paracetamol, and their degradation products in tablets. In this method, simultaneous identification and quantitative determination was done for aceclofenac, paracetamol and their main degradation products, diclofenac and para-aminophenol, respectively.[21]

The UHPLC/Q-TOF-MS technique is relatively new technique and has been used worldwide in drug discovery and development. It has been applied in pharmaceutical development particularly in the identification and quantitative analysis of drug products. The metabolite profiling has been investigated in various biological samples by applying UHPLC/Q-TOF coupled with MetaboLynx™ software. The Q-TOF mass spectrometry gives the accurate mass, reliable chemical fragmentation of synthetic compounds.[22-25] In the presented work, a rapid and specific UPLC/Q-TOF-MS method was developed and validated for the simultaneous determination of aceclofenac and paracetamol in human plasma. The method was applied for pharmacokinetic study of combination tablets containing aceclofenac and paracetamol.

EXPERIMENTAL

Chemicals and Reagents

Aceclofenac ($C_{16}H_{13}Cl_2NO_4$ and Molecular weight 354.20), and Paracetamol ($C_8H_9NO_2$ and Molecular weight 151.16), were kindly supplied as gift sample by Moraceae Pharmaceuticals Ltd. (Uttarakhand, India). Diclofenac ($C_{14}H_{11}C_{l2}NO_2$, Molecular weight 295.01, and purity 99.98%) was supplied by Arti Drugs Ltd. (Mumbai, India). Para-aminophenol (C_6H_7NO, Molecular weight 109.13, and purity 99.98%) was supplied by Hema Pharmaceuticals Ltd. (Gujarat, India). Tablets, Aceclo-Plus (Aristo Pharmaceuticals Ltd., Mumbai, India) was

obtained commercially within their shelf lives with labeled amounts of 100mg of aceclofenac, and 500mg of paracetamol. LC-MS grade acetonitrile (purity 99.98%; Lot No:9170S), methanol (purity 99.99%; Lot No:SZBA010S), ammonium acetate (purity 99.95%; Lot No:1411594), and formic acid (purity 99.98%; Lot No:1439632) were purchased from Fluka analytical, Sigma-Aldrich Corporation, St. Louis, MO, USA). Milli-Q water was used throughout the analysis, which was prepared from Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). All other reagents used were of LC-MS grade.

Q-TOF-MS and UPLC Conditions

Mass spectrometry was performed on a Waters Synapt Q-TOF Premier (Micromass MS Technologies, Manchester, UK) mass spectrometer. The capillary voltage, sampling cone voltage, extraction cone voltage, source temperature, desolvation temperature, cone gas flow, desolvation gas flow, trap gas flow, and source gas flow were set to 3.0 kV, 40 V, 4 V, 80^{o}C, 350^{o}C, 50 L/hr, 800 L/hr, 1.50mL/min, and 0.50mL/min, respectively for all the drugs. Argon was employed as the collision gas at a pressure of 2.5 x 10^{-4} mbar. Trap collision energy and transfer collision energy were set to 12 and 6 V, respectively for all the drugs. The Q-TOF mass spectrometer was operated in positive ionization mode and quantification was done using the MS/MS transitions at m/z 354.07 to 215.07 for aceclofenac, and 152.07 to 110.06 for paracetamol. UPLC was performed with Waters Acquity UPLC system (Waters Corporation, MA, USA) equipped with a binary solvent manager, an auto-sampler, column manager and a tunable MS detector.

Preparation of Standard Solutions

Each of aceclofenac and paracetamol were weighed accurately and transfer to 50 mL volumetric flasks separately. The powders were then dissolved with approximately 25 mL of methanol and ultrasonicated for 5 min. The final volume was made up with methanol. The solutions were further diluted with methanol: water (50:50, v/v) to give a series of standard solutions containing required concentrations for each compound.

Preparation of sample solutions

500 µL of plasma sample was transferred to 10 mL glass tube. To this 5 mL of extraction solvent (diethyl ether: dichloromethane 70:30, v/v) was added. The sample was mixed by vortexer for 5 min. The organic layer was transferred to another glass tube. The solid residue was evaporated to dryness using evaporator at 40 ºC under a stream of nitrogen. The dried

extract was reconstituted in 200 µL of diluent (methanol: water, 50:50, v/v). This solution was filtered through 0.45 µm nylon membrane filter to remove all the particulate materials. 20 µL aliquot was injected in to UPLC system.

Validation of the Method

The developed method was validated according to ICH validation guidelines.[26] The validation parameters addressed were linearity and range, limit of detection and quantitation, precision, accuracy, and specificity.

Linearity, Range, LOD and LOQ

Different standard concentrations each of aceclofenac, and paracetamol in the range of 1-1000 ng/mL (1, 10, 50, 100, 200, 500, and 1000 ng/mL) was spiked to 100 µL of blank human plasma separately in methanol: water (50:50, v/v). Similarly the low, medium and high concentration QC samples containing 100, 200 and 500 ng/mL for each drug were prepared independently using the same procedure. The solutions were filtered through 0.20 µm nylon syringe filter and injected in to the UPLC/QTOF-MS system for analysis. Average peak area at each concentration level was subjected to linear regression analysis with the least squares method.

Accuracy and Precision

Intraday and interday accuracy and precision was evaluated by analyzing low, medium and high concentration QC samples containing 100, 200 and 500 ng/mL of each drug concentration (n=6) on three consecutive days. The mean of percentage recoveries and the RSD (%) was calculated.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of sample matrix. The specificity of the method was examined by analyzing blank plasma extract. The chromatogram of drug free plasma was compared with the chromatograms obtained from plasma spiked with all the drugs.

Stability of samples

Sample stability was tested by analyzing QC samples containing 100 ng/mL of each drug after short-term (6 h) storage at 25 ºC, 12 h storage in an autosampler at 25 ºC, after three freeze-thaw (-20 ºC) cycles, and after long-term (15 days) storage at -20 ºC. The results were

compared with those QC samples freshly prepared and RSD (%) was calculated.

Pharmacokinetic Study

The method was applied for pharmacokinetic study to determine the plasma concentrations of all the three drugs from a clinical trial in which 3 healthy male volunteers received a FDC tablet (Aceclo-Plus) containing 100 mg aceclofenac, and 500 mg paracetamol. Blood samples were collected before and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h post-dosing. Plasma was separated by centrifugation of the heparinized samples at 2000 × g for 10 min and was stored at -20ºC until analysis.

RESULTS AND DISCUSSION

Optimization of Q-TOF-MS Conditions

All the compounds have strong responses in the positive ionization mode and they form protonated molecules in the full scan mass spectra. Therefore, the positive ions, $[M+H]^+$ at m/z 354.07 for aceclofenac, and m/z 152.07 for paracetamol, were selected as the precursor ions. Moreover, under the selected MS/MS conditions the precursor ions were fragmented to major product ions at m/z 215.07 for aceclofenac, and m/z 110.06 for paracetamol, as shown in Figures 1, and Figures 2, respectively. Quantitation was done on the basis of major product ions. The product ion spectra of aceclofenac suggested that the fragmentation of molecules occurs from carboxylic group and loss of carbon dioxide results in the formation of one common production, which was identified as $C_6H_3Cl_2NHC_7H^{+5}$ at m/z 250.05, is further fragmented in to another product ion, $C_6H_4ClNC_7H^{+5}$ with higher intensity at m/z 215.07. Spectra of paracetamol was due to the fragmentation of molecule from acetamide group and loss of neutral molecule, namely ketene (CH_2=C=O) results in the formation of major product ion at m/z 110.06. On the basis of product ion spectrum, the fragmentation patterns of drugs were established. The proposed fragmentation mechanisms of aceclofenac, and paracetamol are presented in Figures 3 and Figures 4, respectively.

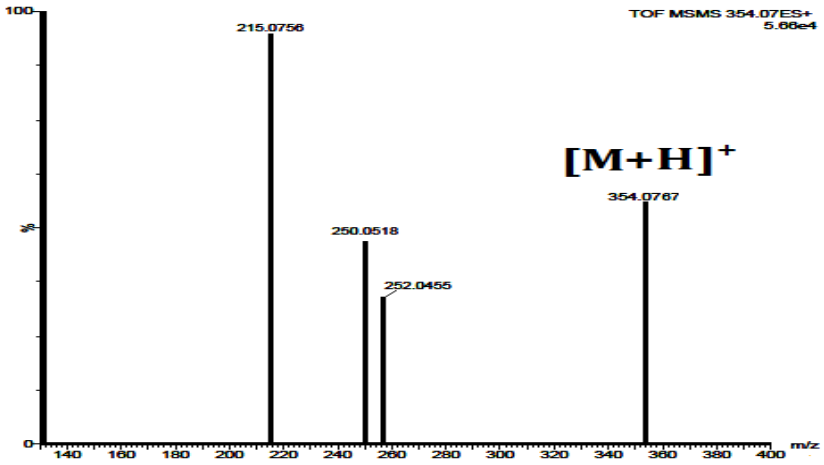

Figure 1: Q-TOF-MS/MS Spectra of Aceclofenac.

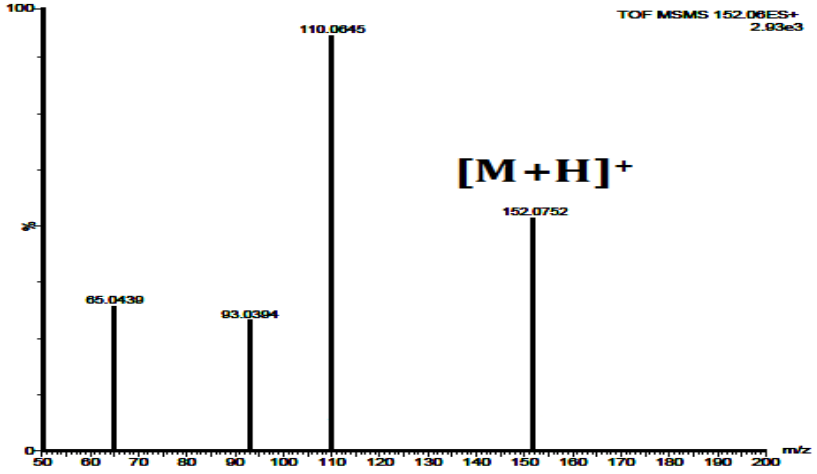

Figure 2: Q-TOF-MS/MS Spectra of Paracetamol.

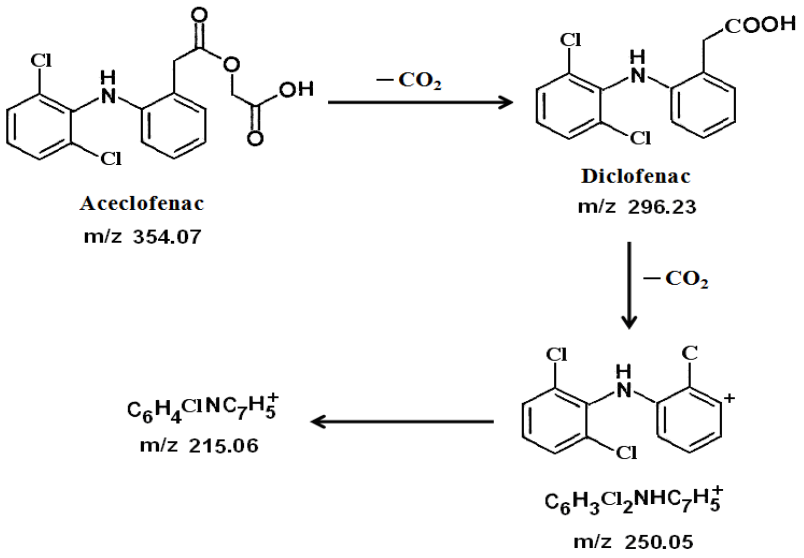

Figure 3: Fragmentation mechanism of Aceclofenac by Q-TOF-MS/MS technique.

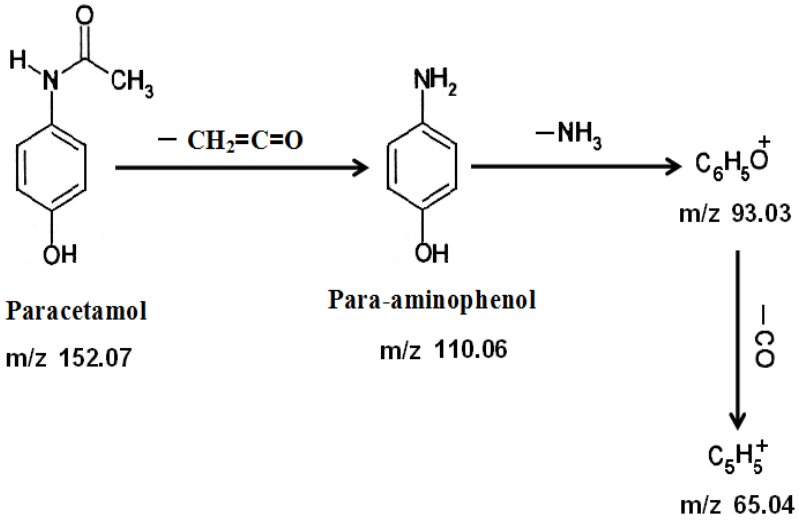

Figure 4: Fragmentation mechanism of Paracetamol by Q-TOF-MS/MS technique.

Optimization of UPLC Conditions

The isocratic mobile phase containing acetonitrile-2mM ammonium acetate (50:50, v/v) at a flow rate of 0.20 mL/min provide peaks with short retention times. The retention time was found to be 1.50 min for aceclofenac, and 0.50 for paracetamol with the total chromatographic run time of 2.00 min for each compound. UPLC-TOF-MS/MS chromatogram obtained from mixed standards (1ng/mL each) of all the drugs is shown in Figure 5.

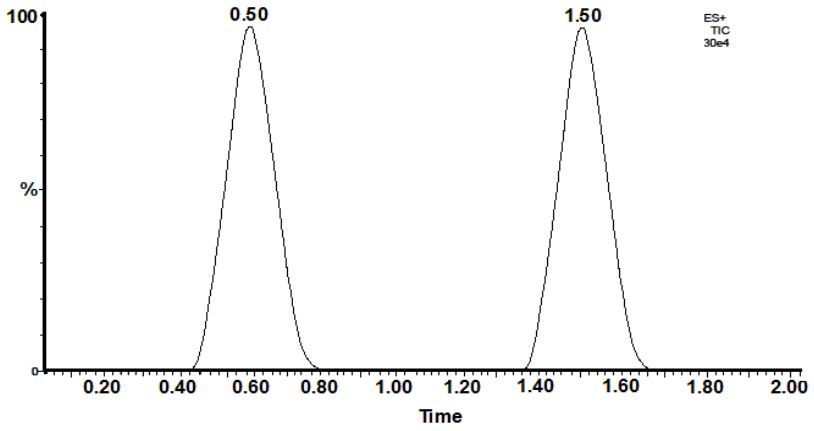

Figure 5: UPLC-TOF-MS/MS chromatogram obtained from mixed standards (1ng/mL each) of Aceclofenac (R_t 1.50 min), and Paracetamol (R_t 0.50 min).

Validation of the method

The results of linearity, LOD and LOQ are presented in Table 1. The obtained results indicated that higher sensitivity of the method. The RSD less than 2% were obtained for all

the compounds by evaluation of intraday, interday, and different analysts precision suggested an acceptable precision of the method.

Table 1: Results obtained from Linearity, LOD, and LOQ.

Parameters	Aceclofenac	Paracetamol
Linear range (ng/mL)	1-1000	1-1000
Correlation coefficient[a]	0.9995	0.9997
LOD (ng/mL)	0.01	0.01
LOQ (ng/mL)	1	1

Stability of samples

The stability of drugs in human plasma under various storage conditions and time period are presented in Table 2. The results indicated that no significant change in the concentration of drugs over the period of 12 h at room temperature which was covered the entire chromatographic procedure. There were no significant differences in the concentration of drugs when the samples were subjected to three freeze-thaw (-20 ºC) cycles and after long-term (15 days) storage at -20ºC ($p>0.05$, ANOVA).

Table 2: Results obtained from Stability Studies.

Storage conditions	Analyte	Conc. Added (ng/mL)	Conc. Found (ng/mL)	RSD (%)
Storage for 6 h at 25ºC	ACF	100	100.01	1.45
	PCM	500	499.95	1.72
Three freeze-thaw (-20 ºC) cycles	ACF	100	100.11	1.25
	PCM	500	499.92	1.52
Storage for 15 days at -20 ºC	ACF	100	99.98	1.15
	PCM	500	499.99	1.78

Pharmacokinetic Study

The method was applied to pharmacokinetic study of aceclofenac and paracetamol in human plasma. Diclofenac and para-aminophenol as the main metabolite of aceclofenac and paracetamol, respectively, were also identified structurally in human plasma by Q-TOF-MS mass spectrometer. The quantitation was done using the MS/MS transitions m/z 354.07 to 215.07 for aceclofenac, 296.23 to 214.06 for diclofenac, 152.07 to 110.06 for paracetamol, and 110.07 to 65.04 for para-aminophenol.[21] The results of pharmacokinetic parameters obtained from mean plasma concentration time curve after administration of single FDC tablet (Aceclo-MR) are presented in Table 3. The results obtained from pharmacokinetic parameters were not significantly different from the reported methods of each drug administered separately.[8,9,14,15-17]

Table 3: Results obtained from Pharmacokinetic Studies.

Pharmacokinetic Parameter	Aceclofenac	Paracetamol
Tmax (hr)	1.25-3	1.00 ± 2.25
Cmax (ng/mL)	4525 ± 225	5550 ± 1550
AUC (ngh/mL)	11250 ± 1.12	13250 ± 250
$T_{1/2}$ (hr)	4-4.5	4-5

CONCLUSION

This is the first method described for identification and quantification of three drugs namely aceclofenac, and paracetamol in human plasma by application of UPLC/Q-TOF-MS technique. The developed method has shown acceptable precision, accuracy and sensitivity of all the drugs in human plasma samples obtained by pharmacokinetic studies. It gives fast, better chromatographic separation and shorter chromatographic run time. Q-TOF mass spectrometry gives accurate mass measurement and reliable chemical fragmentation, which are ultimately helpful in structure elucidations of the drugs in plasma samples. The use of isocratic chromatographic separation without any internal standard makes it an advantageous analytical method over other methods. The proposed method can be applied for pharmacokinetic study of aceclofenac and paracetamol alongwith their main metabolites diclofenac and para-aminophenol in human plasma.

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