High-Throughput Ultra-Performance LC–MS-MS Method for Analysis of Diclofenac Sodium in Rabbit Plasma

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A new UPLC–MS-MS method was developed and validated for quantification of diclofenac sodium in rabbit plasma. Acetonitrile-based protein precipitation method was used to extract the drug from plasma samples. Chromatographic separation was carried out on Acquity UPLC® BEH phenyl C18 1.7 μm, 2.1 × 50 mm column. Drug elution was facilitated by using mobile phase containing acetonitrile (0.1% glacial acetic) and water (pH 3.5), in a ratio of 75 : 25, flowing at 0.2 mL/min. Molecular ions were generated by using the positive electrospray ionization mode (ESI+) and analyzed on a triple-quadrupole mass spectrometer. The ionic transitions of diclofenac (m/z 296 > 214 and 249.9) and flufenamic acid (internal standard) (m/z 282.1 > 166.9 and 244) were measured in multiple reaction modes. Developed method is simple, quick, precise and accurate over a linearity range of 80–4,000 ng/mL. The lower limit of quantification (LLOQ) for diclofenac was 80 ng/mL. The percentage recoveries of diclofenac at three quality control samples were 54 ± 6.1, 67.1 ± 5.4 and 62.3 ± 1.4%. Precision and accuracy of the assay at LLOQ, middle limit of quantitation and higher limit of quantitation were 100 ± 7.0, 100 ± 1.0 and 100 ± 2.0% and 81.8 ± 4.6, 106.8 ± 3.1 and 103.3 ± 4.0%, respectively.

Introduction

Diclofenac sodium is a non-steroidal anti-inflammatory drug and chemically designated as 2-[(2,6-dichlorophenyl)amino]benzenacetic acid, monosodium salt. It is indicated for the relief of mild-to-moderate pain, osteoarthritis and rheumatoid arthritis. It is indicated for the relief of mild-to-moderate pain, osteoarthritis and rheumatoid arthritis. The oral absorption of diclofenac is rapid and complete. Diclofenac binds extensively to plasma albumin. The molecular weight of diclofenac is 296.18. Chemical structures of diclofenac sodium and flufenamic acid are presented in Figure 1.

Numerous analytical methods have been identified for quantification of diclofenac (2–4). Yilmaz et al., Nasir et al. and Dahivelkar et al. developed HPLC methods for the analysis of diclofenac in human plasma (5–7). Emara et al. (8) reported a sensitive HPLC method with long retention time. El-Sayed et al. (9) reported another HPLC method for diclofenac analysis in serum. The observations reveal that many of published methods are less sensitive and long retention time and involve multiple processing steps of sample preparation.

Lee et al. developed an HPLC method combined with column-switching technique (10, 11). Sioufi et al. (12) reported a fully automated system based on liquid–solid extraction combined with column liquid chromatography. An automated robotics system was also developed that aliquots biological sample, adds the internal standard, extracts the drug from acidified biological matrix into an organic phase and concentrate extracts for analysis through directly interfaced HPLC system (13).

A very sensitive (1 ng/mL) HPLC method based on electrochemical detection was reported by Zecca et al. (14) for the analysis of diclofenac in biological fluid. Chmielewska et al. (15) also reported another HPLC method with electrochemical detection for the quantitative determination of diclofenac potassium in plasma. Another electrochemical detection based HPLC method was developed by Plavsic et al. (16), which involves double extraction of drug by using organic solvent. Brombacher et al. (17) reported a gas chromatographic method for determination of drug in human plasma.

Cui et al. (18) developed a liquid–liquid extraction (LLE)-based UPLC–MS-MS method in rat plasma. Another LC–MS-MS method for the diclofenac analysis in mouse plasma was also reported (19).

To the best of our knowledge, the method for the analysis of diclofenac sodium in rabbit plasma is not available. The present article provides a high-throughput, simple, accurate and precise UPLC–MS-MS method for determination of diclofenac in rabbit plasma samples. The method was validated for accuracy, precision, specificity, recovery, linearity and stability parameters. The advantages of present method include quick and simple extraction procedure through precipitation by using just one solvent, short run-time, less noise, highly sensitive and selectivity.

Generally, the LC–MS-MS-based methods use deuterated internal standards, most of the time these deuterated compounds are not easily available, if available then their cost is high. The present method was developed by using non-deuterated compound as internal standard, and it was successfully used for present investigations.

Materials and methods

Chemicals and reagents

Diclofenac sodium was obtained as gift sample Tabuk Pharmaceutical Manufacturing Co., Saudi Arabia and manufactured by Amoli Organics, India. Flufenamic acid (internal standard) was obtained from Winlab, Edgware, UK. The HPLC grade methanol and acetonitrile were purchased from Fisher Chemical; Loughborough, UK. Glacial acetic acid was supplied by Fluka Chemika, Switzerland.

Instrumentation

LC–MS-MS analysis was done by using a Waters Acquity H-Class UPLC-tandem quadrupole spectrometer (TQD). The H-Class
Three different concentration of lower limit of quantitation (LLOQ), middle concentration and highest concentration.

Preparation of standard plot

Rabbit plasma was prepared in house from its blood. Blood was collected through middle ear vein. The study protocol was approved and ethical permission was granted by the Director of "Experimental Animal Care Centre, College of Pharmacy, King Saud University, Riyadh" in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publication No 80-23; 1996). The approved protocol reference no. is CPR-3567. The 100 μL of rabbit plasma was transferred into centrifuge tube. Plasma samples were spiked with known amount of diclofenac (10 μL), and further added 10 μL of internal standard. Plasma samples comprising diclofenac and internal standard were vortexed for 30 s, and then precipitated with 380 μL of acetonitrile. These precipitated plasma samples were vortexed for another 30 s for complete precipitation and uniform distribution of drug in the solvent. These precipitated samples were centrifuged for 8 min at 12,000 rpm. The upper clear layer was transferred into fresh eppendorf tubes and again centrifuged for another 6 min to avoid any debris. The 180 μL of each sample was transferred into separate glass inserts of 200 μL capacity. The 10 μL of sample was injected for the analysis.

Validation procedure

Plasma samples were spikes according to the procedure discussed above. The US Food and Drug Administration (FDA) guidelines were adopted for validation of UPLC–MS-MS analytical method (20). The fundamental parameters for validation such as accuracy, precision, specificity and stability were investigated. Apart from fundamental parameters, the LLOQ, linearity and extraction recoveries were also determined. The linearity of the method was investigated by using linear regression analysis.

Accuracy

Accuracy of the method was determined by replicate (n = 6) analysis of quality control samples (LLOQ = lower, MQ = middle and HQ = higher limits of quantitation) containing known amounts of the analyte. Accuracy was calculated and expressed as percentage by comparing the mean values with the respective true concentration values. For acceptability of assay, the mean value should be within ±15% of the actual concentration, with the exception at LLOQ, where the limit is ±20%. The deviation of mean from the actual value is considered as the measure of accuracy.

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\text{Accuracy} = \frac{\text{mean measured concentration}}{\text{actual concentration}} \times 100\%.
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Precision

Precision was determined by measuring six replicates of quality control samples. Precision was characterized by percent coefficient of variation (%CV). The limits of acceptable variability at LLOQ were 20%, while for other concentrations were set at 15%.
Selectivity
The selectivity of the method was determined by analysing the drug-free plasma from six different batches and confirming the absence of any endogenous (metabolites, decomposition products and concomitant medication administered) or exogenous interference at retention times of analyte and internal standard. The interfering peak area needed to be <20% of the peak area for analyte at the LLOQ.

Recovery
The extraction efficiency (recovery) of method for analyte was determined at three quality control concentration levels by replicate analysis ($n = 6$) of actual samples and plasma spiked samples with known concentrations of analyte and internal standard. The peak area ratio of analyte in plasma was compared with peak area ratio of actual analyte (in mobile phase) at the same final concentrations. The recovery was expressed as percentage value, and the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible.

Calibration curve
Calibration curve was prepared by plotting the peak area ratios of analyte vs. internal standard against the known concentrations of the spiked analyte in plasma. The 20% deviation at LLOQ and 15% deviation at other concentrations were accepted. Regression parameters such as slope, intercept and correlation coefficient ($r^2$) were calculated. The minimally acceptable correlation coefficient ($r^2$) for the calibration curves was 0.98. The calibration curve in the range of analysis was replicated six times.

Lower limit of quantitation
The LLOQ was determined as the lowest amount of analyte that gave a peak response at least 10 times the response compared with blank response, and the peak was identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120%.

Stability
The benchtop stability of diclofenac was determined by storing extracted quality control samples at room temperature for 3 days to mimic sample manager storage conditions (20 °C). Stability of quality control samples in plasma was also assessed after three freeze-and-thaw cycles (−80 °C). The peak area ratio of diclofenac at the quality control concentration levels at the initial condition was used as reference to determine the relative stability.

Results
UPLC method development
An isocratic UPLC method was developed for the elution of diclofenac sodium and flufenamic acid. A 5-cm long column (Acquity UPLC® BEH phenyl C18, 1.7 μm, 2.1 × 50 mm column) and mobile phase acetonitrile (0.1% glacial acetic acid) : water (pH 3.2, by glacial acetic acid) (75: 25, v/v) running at 0.2 mL/min rate was used to achieve fast elution and to shorten the analysis time. Both diclofenac and internal standard were eluted at ~1.0 min, and the run-time was set for 2.0 min. The column temperature was kept at 40 ± 5 °C.

Mass spectrometry
Tuning parameters were optimized for detection of parent and daughter ions of analyte (diclofenac sodium) and internal standard (flufenamic acid). During positive electrospray ionization the protonated diclofenac ions ($m/z$ 296) and flufenamic acid ions ($m/z$ 282.1) were produced. Protonated diclofenac ion produces two daughter ions of $m/z$ 214 and 249.9. Daughter ion of $m/z$ 214 was selected for quantification and $m/z$ 249.9 was considered for qualitative purpose. Protonated flufenamic acid produces two daughter ions of $m/z$ 169.9 and $m/z$ 244.

Validation
Multiple-reaction monitoring chromatograms of blank plasma, spiked analyte and internal standard are shown in Figures 2 and 3, respectively.

Accuracy
Accuracy data of three quality control samples are presented in Table I. The data are acceptable as all the mean values of assays are within ±15% of the actual concentration. The mean value of assay at LLOQ is also within the permissible limit (±20%).

Precision
The interday precision for quality control samples at LLOQ, middle limit of quantitation (MLQ) and HLQ was 100 ± 7.05, 100 ± 1.09 and 100 ± 2.06%, respectively. The precision was within acceptable limits of 15% (for LLOQ 20%).

Recovery
Average recovery of diclofenac at three levels of quality control ($n = 6$) samples was 54.07 ± 6.1% at low level, 67.1 ± 5.1% at medium level and 62.3 ± 1.4% at high level. The coefficient of variation at low level, medium level and high level was 11.29, 7.6 and 2.27%, respectively.

Selectivity
No interfering peaks were observed in any MS/MS traces at the retention time of diclofenac and internal standard in the chromatograms of blank rabbit plasma obtained from six different animals. There was no coeluting peak of any endogenous substance in the blank plasma samples having peak area >20% of analyte area at LLOQ. Representative chromatograms of blank rabbit plasma, spiked plasma with diclofenac and internal standard are presented in Figures 2 and 3. Average retention times for diclofenac and flufenamic acid were 1.04 and 1.14 min, respectively. The level of blank plasma response compared with LLOQ was <10%.

Stability
Percentage recovery of bench-top stability of diclofenac at three quality control samples was 85.9 ± 9.9, 99.6 ± 3.5 and 97.6 ± 3.8%, respectively. The percentage recovery of diclofenac after three freeze–thaw cycles in the rabbit plasma at three quality control samples was 88.9 ± 8.3, 101.2 ± 4.8 and 101.3 ± 8.3%, respectively. Diclofenac sodium was stable up to 3 days on autosampler storage temperature (20 °C). No statistically significant degradation of diclofenac sodium was observed in rabbit plasma so the stability of diclofenac was acceptable under stated conditions. The stability results are presented in Table I.

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Calibration curve, LLOQ and linearity

The peak area ratio of diclofenac and internal standard versus actual concentration of diclofenac showed a linear relationship in the range of 80–4,000 ng/mL. Therefore, the calibration curve ranges from 80 to 4,000 ng/mL, wherein the accuracy and precision of three quality control samples were found to be in accordance with the FDA guidelines (Table I). The calibration curve was found to be linear over the range of plasma concentration.

Figure 2. Representing chromatograms of blank plasma.
80–4,000 ng/mL, with a correlation coefficient ($r^2$) 0.999. At all points of calibration curve, the back calculation of concentration from the equation of regression analysis ($Y = 0.0005X + 0.0082$) was always within $\pm 15\%$ of actual value. The LLOQ was established at 80 ng/mL.

**Discussion**

A sensitive, simple and high-throughput UPLC–MS-MS method for the quantification of diclofenac sodium in plasma was developed and validated according to the FDA guidelines. The drugs in biological samples (blood, plasma or serum) are being analyzed.
after extraction by using LLE or solid-phase extraction (SPE) or protein precipitation or online extraction procedures. The LLE and SPE were the most commonly used procedures. For present investigation, the protein precipitation was used to extract the diclofenac sodium from plasma samples. Protein precipitation process is simple, time saving and economical as it involves fewer steps and consumes less solvent when compared with LLE, SPE and online extraction procedures. In the present method, the retention time for diclofenac and flufenamic acid was ~1 min, and the total run-time of a sample was 2 min.

Despite very simple sample preparation process (protein precipitation and centrifugation), a fairly low quantitation limits was achieved. The LLOQ was 80 ng/mL, which was quite fare as the reported $C_{\text{max}}$ of voltaren, arthrotec was ~1,200 ng/mL at 50 mg dose and ~2,000 ng/mL at 75 mg dose. Generally, the deuterated compounds are recommended to be used as internal standard. In contrary, the present investigation successfully uses a non-deuterated compound (flufenamic acid) as internal standard. The molecular mass and structural nucleus of diclofenac ($m/z$ 296) and flufenamic acid ($m/z$ 282) were close and similar (Figure 1). Both diclofenac and flufenamic acid comprise aniline group attached with phenyl group. Before validation the method was optimized for mass spectrometric parameters such as cone voltage, capillary voltage, CE, daughter ions stability and intensity. Daughter ions of diclofenac and flufenamic acid were obtained through automatic optimization technology (IntelliStart®). The machine optimized parameters were further fine-tuned by manual optimization. The manually optimized values are listed under the Materials and Method section.


| Table I |
| Showing Validation Results |
| Parameter | LLOQ (%) | MLQ (%) | HLQ (%) |
| Accuracy | 81.8 ± 4.6 | 106.6 ± 3.1 | 103.3 ± 4.0 |
| Precision | 100 ± 7.0 | 100 ± 1.0 | 100 ± 2.0 |
| Interfering peak area | 6.2 ± 80.7 | – | – |
| Recovery | 54.0 ± 6.1 | 67.1 ± 5.1 | 62.3 ± 1.4 |
| Benchtop stability | 85.9 ± 9.9 | 99.6 ± 3.5 | 97.7 ± 3.8 |
| Freeze–thaw stability | 88.9 ± 8.3 | 101.2 ± 4.8 | 101.3 ± 8.3 |

Coefficient of variation: $r^2 = 0.999$
Regression equation: $Y = 0.0005X + 0.0082$

Conclusion

A simple, high-throughput and sensitive LC–MS-MS method was developed and validated for the analysis of diclofenac in rabbit plasma. The developed method can quantify the diclofenac at very low level. The present validated UPLC–MS-MS method was found to be reliable and reproducible for the intended applications.

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