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Bioanalytical Method Development and Validation of Valdecoxib in Human Plasma Using LC-MS/MS

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Abstract: A selective, sensitive, and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the determination of Valdecoxib (VXB) in human plasma. VXB and its internal standard, Valdecoxib-D3 (VXB-D3), were extracted from 300 µL of plasma using solid-phase extraction (SPE) with Strata-X cartridges. Chromatographic separation was achieved on an ACE C8-300 column (50 × 4.0 mm, 3.0 µm) with a mobile phase consisting of methanol and 1.0 mmol ammonium acetate solution in an 80:20 (v/v) ratio. Detection was carried out in negative ionization mode, with precursor-to-product ion transitions of m/z 380.0 → 315.9 for VXB and 387.0 → 323.0 for VXB-D3.Method validation demonstrated system suitability with area ratio CV% < 3 and retention time CV% < 2, within the acceptance criteria. The method was linear over the concentration range of 20.017 ng/mL to 4503.846 ng/mL, exhibiting excellent precision and accuracy. Stability studies showed that VXB was stable under various conditions, including five freeze-thaw cycles, 45 hours of bench-top storage, and up to 6 days for long-term storage in both matrix and stock solution. The drug was also stable in whole blood for up to 60 minutes.This method is robust and rugged, showing reliable performance despite changes in column and analyst. It is suitable for quantifying Valdecoxib in K2EDTA human plasma, making it a reliable tool for pharmacokinetic studies and clinical monitoring.

Keywords: Valdecoxib, Validation, Stability, Precision,Accuracy

I. Introduction

Valdecoxib (VXB) is a selective cyclooxygenase-2 (COX-2) inhibitor and was the first of its class introduced into clinical practice. It is approved worldwide for the relief of signs and symptoms associated with osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis. In addition, it is used for the management of primary dysmenorrhoea and for the treatment of acute pain in adults [1].Following oral administration of a 200 mg dose of valdecoxib to healthy volunteers, the maximum plasma concentration (C_max; 450–800 ng/mL) is reached within 1.5–3 h. The low aqueous solubility of the drug appears to prolong the absorption phase, leading to greater variability in terminal half-life estimates, particularly under fasted conditions.

Several analytical methods have been reported for the determination of VXB in biological fluids, including liquid chromatography with spectrophotometric detection (LC) [2], liquid chromatography–mass spectrometry (LC-MS) [3], ultra-performance liquid chromatography–inductively coupled plasma mass spectrometry (UPLC-ICP-MS), and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [4].The aim of the present study was to simplify the sample preparation process using solid-phase extraction (SPE) while simultaneously reducing chromatographic run time through the development of a more selective LC-MS/MS method. Furthermore, to improve method precision and accuracy, an isotopically labeled valdecoxib internal standard was employed, rather than the conventional internal standards used in previous studies [5]. These improvements enabled the development of a rapid, selective, and sensitive LC-MS/MS method for the determination of VXB in human plasma. The validated method was successfully applied to a pharmacokinetic study under both fed and fasting conditions.

II. Materials and Methods

Chemicals and materials Reference standards of VXB (99.47 %) and Valdecoxib-D3 (IS, 98.36 %) were purchased from Maravai Life Sciences (Mumbai, India). HPLC grade methanol, analytical grade formic acid and ammonium acetate were obtained from SD Fine Chemicals Ltd. (Mumbai, India). Deionized water for LC-MS/MS was prepared using Milli Q water purification system from Millipore (Bangalore, India). Samples were centrifuged on Eppendorf 5810 centrifuge machine (Hamburg, Germany).

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LC-MS/MS instrumentation and conditions:

Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) Conditions

The liquid chromatography system (Shimadzu, Kyoto, Japan) comprised an LC-10ADvp pump, an SIL-HTc autosampler, and a DGU-14A on-line degasser. Chromatographic separation was achieved using an ACE C8-300 column (50 × 4.0 mm, 3.0 µm; Chromatopak Analytical Instrumentation Pvt. Ltd., Mumbai, India). The mobile phase consisted of methanol and 1.0 mmol ammonium acetate (80:20, v/v) and was delivered at a flow rate of 0.5 mL/min. The autosampler temperature was maintained at 4 °C, with an injection volume of 2.0 µL. The total run time was 2.5 min. Detection was performed using a triple quadrupole mass spectrometer (API-4000, MDS SCIEX, Toronto, Canada) equipped with a Turbo Ion Spray® source, operated in negative ionization mode. Quantitation was carried out in multiple reaction monitoring (MRM) mode by monitoring the transitions m/z 380.0 → 315.9 for VXB and m/z 387.0 → 323.0 for the internal standard (IS). Data acquisition and processing were performed using Analyst software version 1.6.2.Source-dependent parameters were as follows: nebulizer gas (Gas 1), 55 psi; heater gas (Gas 2), 60 psi; ion spray voltage (ISV), −4500 V; turbo heater temperature, 500 °C; collision activation dissociation (CAD), 8 psi; and curtain gas (CUR), 25 psi. Compound-dependent parameters, including declustering potential (DP), collision energy (CE), entrance potential (EP), and cell exit potential (CXP), were optimized at −120, −39, −10, and −10 V for VXB, and −120, −35, −10, and −20 V for the IS, respectively. Quadrupoles Q1 and Q3 were maintained at unit resolution, and the dwell time was set to 500 ms.

Preparation of Standard Stock and Plasma Samples

Separate standard stock solutions of VXB and VXB-D3 (500 µg/mL) were prepared in methanol. The VXB stock solution was further diluted with methanol to obtain an intermediate concentration of 80.0 µg/mL. Working solutions for calibration standards (CSs) and quality control (QC) samples were prepared by appropriate dilution of the stock and intermediate solutions using methanol:water (90:10, v/v).

The internal standard working solution (1500 ng/mL) was prepared by diluting the IS stock solution with deionized water. Calibration standards were prepared by spiking blank plasma at concentrations of 10.0, 20.0, 50.0, 100, 200, 400, 800, 1600, 3200, and 4000 ng/mL.

Quality control samples were prepared at 10.0 ng/mL (LLOQ QC), 30.0 ng/mL (LQC), 300 ng/mL (MQC-2), 1200 ng/mL (MQC-1), 3000 ng/mL (HQC), and 4000 ng/mL (ULOQ QC). Standard stock and working solutions were stored at 2–8 °C, while plasma CSs and QC samples were stored at −20 °C until analysis. [6]

Sample Preparation

Prior to analysis, plasma samples were thawed at room temperature and vortex-mixed thoroughly. Aliquots of 300 µL plasma were transferred into RIA vials, followed by the addition of 15 µL methanol:deionized water (90:10, v/v) and 100 µL of IS working solution (1500 ng/mL). Samples were vortexed, and 100 µL of 2% formic acid was added and mixed again. [7]

Solid-phase extraction (SPE) cartridges were preconditioned with 1.0 mL methanol followed by 1.0 mL deionized water and centrifuged for 1 min at 1811 × g. Plasma samples were loaded onto the cartridges and centrifuged for 2 min at 1811 × g. The cartridges were washed with 1.0 mL deionized water and centrifuged for 1 min.

Elution was performed using 2 × 250 µL of methanol–1.0 mmol ammonium acetate (80:20, v/v), with centrifugation for 1 min at 1811 × g after each elution step. An aliquot of 2.0 µL of the final extract was injected into the LC–MS/MS system using partial-loop mode[8].

Methodology for validation

Method validation for VXB in human plasma was performed in accordance with the USFDA bioanalytical method validation guidelines [8], and the procedures were consistent with previously reported methods [9]. The method was validated for selectivity, sensitivity, interference, carryover, linearity, precision and accuracy, reinjection reproducibility, recovery, matrix effect, ion suppression/enhancement, stability, dilution integrity, ruggedness, robustness, and extended batch verification.

Selectivity was evaluated using eight different lots of normal human plasma, four lots of lipemic plasma, and four lots of haemolysed plasma containing K_2EDTA as the anticoagulant. Sensitivity was assessed using six replicates of lower limit of quantification (LLOQ) samples.

Linearity was established using calibration curve standards, and quality control (QC) samples at LLOQ QC, LQC, MQC, and HQC levels were

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analyzed across three precision and accuracy batches. Precision was expressed as percent coefficient of variation (%CV), while accuracy was calculated as the ratio of the mean measured concentration to the nominal concentration. Goodness-of-fit analysis was conducted using back-calculated concentrations of calibration standards with weighting factors of 1/x and $1/x^2$; the $1/x^2$ weighting provided the best regression fit. Linearity was determined using a regression equation based on the analyte-to-internal standard (DRN D4) concentration ratio.

Matrix effect was assessed using processed blank plasma samples from six normal plasma lots (two aliquots per lot), three lipemic plasma lots, and three haemolysed plasma lots [11]. After extraction and drying, the samples were reconstituted with aqueous low-quality control (LQC) and high-quality control (HQC) solutions. For comparison, six replicates each of aqueous LQC and HQC were injected. Matrix factor was calculated as the ratio of peak response in the presence of matrix ions to that in the absence of matrix ions, and percent matrix effect was determined using the equation: % Matrix effect = (1 − mean matrix factor) × 100.

Stability studies included stock solution and stock dilution stability at refrigerated and room temperatures, autosampler stability, reinjection reproducibility, freeze–thaw stability, long-term stability at −65 ± 10 °C and −22 ± 5 °C, bench-top stability, reagent stability, dry ice stability, dry extract stability, extended bench-top stability, wet extract stability under refrigerated conditions, and stability in lipemic and haemolysed plasma. Blood stability and the effect of potentially interfering drugs were also evaluated, all meeting USFDA acceptance criteria [10].

Stock solution and stock dilution stability for DRN and the internal standard (DRN D4) were assessed for 8 days under refrigerated conditions and for 21 hours at room temperature. Photodegradation studies were conducted for 22 hours in the dark. For aqueous stability studies, two sets of aqueous mixtures were prepared—one from stability stock solutions and the other from freshly prepared comparison stock solutions. Six replicates from each set were injected, and responses from stability samples were corrected using an appropriate correction factor.

Correction factor= (conc. of fresh standard sol.)/(conc. of stability standard sol.)

Corrected response=stability stock response X correction factor

% Change=[(mean response of comparison samples - mean corrected response of stability samples)]/(mean response of comparison samples) X 100

Aqueous recovery comparison samples (LQC, MQC and HQC) were prepared by adding 8µL each of aqueous dilution of DRN from respective quality control samples, 50µL of internal standard dilution (2500.000 ng/mL) and 242µL of mobile phase (representing 100% extraction). The aqueous samples (LQC, MQC and HQC) of DRN were compared against 6 sets of extracted LQC, MQC and HQC samples . Recovery of internal standard was compared at LQC, MQC and HQC level [12].

% Recovery = (mean peak area response of extracted sample) / (corrected mean peak area response of unextracted sample) X 100

The effect of potentially interfering drugs (PIDs)—ibuprofen, caffeine, acetaminophen, and acetylsalicylic acid—on DRN analysis was evaluated by spiking each PID at approximately its reported Cmax concentration into LLOQ samples in triplicate. Bench-top stability was assessed for 13 hours using six sets each of LQC and HQC samples, while extended bench-top stability was evaluated in spiked samples to assess DRN stability at each extraction step. Freeze–thaw stability was determined over five freeze–thaw cycles by analyzing six sets of LQC and HQC samples after completion of the cycles.

Long-term stability was evaluated in plasma stored at −65 ± 10 °C and −22 ± 5 °C for 22 days using six sets of LQC and HQC samples. Dry extract stability was assessed by processing six sets of LQC and HQC samples and storing them at −22 ± 5 °C without reconstitution, whereas wet extract stability was evaluated by storing reconstituted extracts at 2–8 °C. Both wet and dry extract stability samples were analyzed after 51 hours of storage.All stability QC samples were analyzed against freshly prepared calibration curve standards along with six sets of freshly spiked LQC and HQC samples prepared from fresh stock solutions. Percentage change was calculated by comparing stability QC samples with comparison QC samples.Method robustness was evaluated by varying chromatographic conditions, including column temperature (38 °C and 42 °C), flow rate (1.17 and 1.23 mL/min), and mobile phase composition (acetonitrile:milli-Q/HPLC-grade water:formic acid at 92:8:0.1 and 88:12:0.1, v/v/v). Ruggedness was assessed by analyzing precision and accuracy batches processed by a different analyst using a different column and independent sets of solutions [13].

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III. RESULTS AND DISCUSSION

The objective of the present study was to develop and validate a simple, rugged, selective, and sensitive LC–MS/MS method for the quantification of Valdecoxib (VXB) in human plasma for routine sample analysis. The sensitivity of the method was designed to be sufficient to monitor at least five half-lives of Valdecoxib with acceptable accuracy and precision in subject samples.

During method development, electrospray ionization (ESI) of Valdecoxib and the internal standard Valdecoxib-D7 was evaluated in negative ionization mode, as both compounds exhibit basic characteristics due to the presence of secondary and tertiary amine groups. A tuning solution of 10.0 ng/mL was used. Valdecoxib and the IS produced predominant singly charged deprotonated precursor ions $[M–H]^-$ at m/z 380.0 and 387.0, respectively, in Q1 full-scan spectra, consistent with previously reported data.

Collision-induced dissociation (CID) was achieved using nitrogen as the collision gas, with a curtain gas pressure of 25.0 psi to facilitate precursor ion fragmentation. The most abundant and reproducible product ions observed in Q3 were m/z 315.9 for Valdecoxib and m/z 323.0 for the IS. To achieve a stable Taylor cone and optimal signal response, the nebulizer gas pressure was set to 55 psi. Fine optimization of the nebulizer and CAD gas flows resulted in a consistent and stable analyte response. Ion spray voltage and source temperature showed no significant influence on signal intensity and were therefore maintained at –4500 V and 500 °C, respectively. A dwell time of 500 ms was selected for both Valdecoxib and the IS. No cross-talk was observed between the monitored MRM transitions.

Method specificity was assessed by evaluating potential interference at the retention times of Valdecoxib and the internal standard. Blank plasma samples were compared against samples spiked at the LLOQ concentration (20.025 ng/mL). No significant endogenous interference was observed. Potential interference of the IS at the Valdecoxib retention time was evaluated by injecting six replicates of blank plasma containing IS only, and no interfering peaks were detected. Similarly, interference of Valdecoxib at the IS retention time was assessed using six replicates of MQC samples (2252.817 ng/mL), with no interference observed.

The lower limit of quantification (LLOQ-QC) was established at 20.132 ng/mL, demonstrating an accuracy of 93.06% and a precision (CV) of 4.61%, which met acceptance criteria.

Carryover was evaluated by alternately injecting blank plasma samples (seven injections) and ULOQ samples (4505.635 ng/mL, six injections). The response in blank samples was compared with the mean response at the LLOQ level. No significant carryover was observed for either Valdecoxib or the IS.The method showed linearity over the concentration range of 20.017 ng/mL to 4503.846 ng/mL. Calibration curves were constructed using a $1/x^2$ weighted linear regression model, yielding correlation coefficients (r) greater than 0.99 across all analytical runs, confirming excellent linearity.Freeze–thaw stability was evaluated after five cycles at −20 °C ± 4 °C, with samples thawed at room temperature (<27 °C). Concentrations were compared with freshly prepared QC samples and showed acceptable stability.Autosampler stability was assessed by storing processed QC samples at 15 °C for 73.00 hours. The percentage stability was 110.48% for LQC and 100.44% for HQC. Wet extract stability was evaluated after 68.00 hours at ambient temperature, yielding stability values of 104.86% (LQC) and 100.18% (HQC).Bench-top stability of thawed plasma QC samples was determined after 45.00 hours at ambient conditions, resulting in stability values of 102.85% (LQC) and 101.57% (HQC). Long-term matrix stability was established after five days of storage at −20 °C ± 4 °C, with stability values of 100.80% and 100.64% for LQC and HQC, respectively.

Stock solution stability of Valdecoxib and Valdecoxib-D3 was evaluated over six days at 2–8 °C. Valdecoxib showed stability of 102.55% and 100.01% at aqueous LQC and HQC levels, respectively, while the IS exhibited 100.12% stability, confirming adequate stock solution stability.

IV. Conclusion

System suitability testing during method validation demonstrated acceptable performance, with CV values below 3% for area ratios and below 2% for retention times. Based on the validation results, the developed LC–MS/MS method is precise, accurate, selective, and robust for the quantification of Valdecoxib in K_2EDTA human plasma over a

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concentration range of 20.017 ng/mL to 4503.846 ng/mL. Valdecoxib was found to be stable under all tested conditions, including freeze–thaw, bench-top, autosampler, wet extract, long-term matrix, and stock solution stability. The method also demonstrated ruggedness with respect to changes in analyst and column, making it suitable for routine bioanalytical and pharmacokinetic studies.

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Table 1. System suitability parameters of Valdecoxib

Parameters	Result
Limit of Quantification	20.015 ng/mL
Average recovery of drug (%)	99.81%
Average recovery of IS (%)	104.83%
Standard curve concentrations (pg/mL)	20.016, 40.033, 100.084, 300.255, 1000.854, 2502.136, 3502.992, 4503.855 ng/mL
QC concentration (pg/mL)	LQC = 59.853 ng/mL GMQC = 372.498 ng/mL MQC = 2264.892 ng/mL HQC = 3422.501ng/Ml

International Journal of Pharmaceutical research and Applications
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QC intra batch precision range (%)	LLOQ = 1.64% to 5.42 % LQC = 2.82 % to 5.35% MQC = 2.28% to 2.77% HQC = 2.09% to 3.06%
QC intra batch accuracy range (%)	LLOQ = 88.08% to 95.48% LQC = 91.71% to 104.73% MQC = 96.33% to 104.19% HQC = 96.72% to 106.88%
QC Inter Batch precision range (%)	LLOQ = 4.62% LQC = 6.73% MQC = 4.07% HQC = 4.94%
QC Inter Batch accuracy range (%)	LLOQ = 93.05% LQC = 96.08% MQC = 98.66% HQC = 99.54%

Table 2. Stability parameters of Valdecoxib

Parameters	Result
Bench top stability (hrs)	45.00 hours at room temperature % stability of LQC = 102.85% and HQC = 105.57%
Wet extract stability (hrs)	68.00 hours at ambient temperature % stability of LQC = 104.86% and HQC 100.18%
Dry extract stability (hrs)	73.00 hours at ambient temperature % stability of LQC = 110.48% and HQC = 100.44%
In injector stability (hrs)	77.00 hours at 15^0 C peltier temperature % stability of LQC = and HQC 101.07%
Stock solution stability (short term) (hrs)	After 22.00 hrs at room temperature % stability of LQC = 99.89% and HQC = 100.04% ISTD = 99.53%
Working solution stability (hrs)	After 25.00 hrs at room temperature in 60% Acetronitrile in water, % stability of LQC = 104.80% and HQC = 93.16% ISTD = 99.88%
Freeze thaw stability (cycles)	After five cycles % stability of LQC = 102.79% and HQC = 99.90%
Stock solution stability (Long term) (Days)	6 days at 2 to 8°C % stability of LQC = 102.55% and HQC 100.01% ISTD 100.12%

International Journal of Pharmaceutical research and Applications
Volume 10, Issue 6, Nov-Dec 2025, pp:1441-1447 www.ijprajournal.com ISSN: 2456-4494
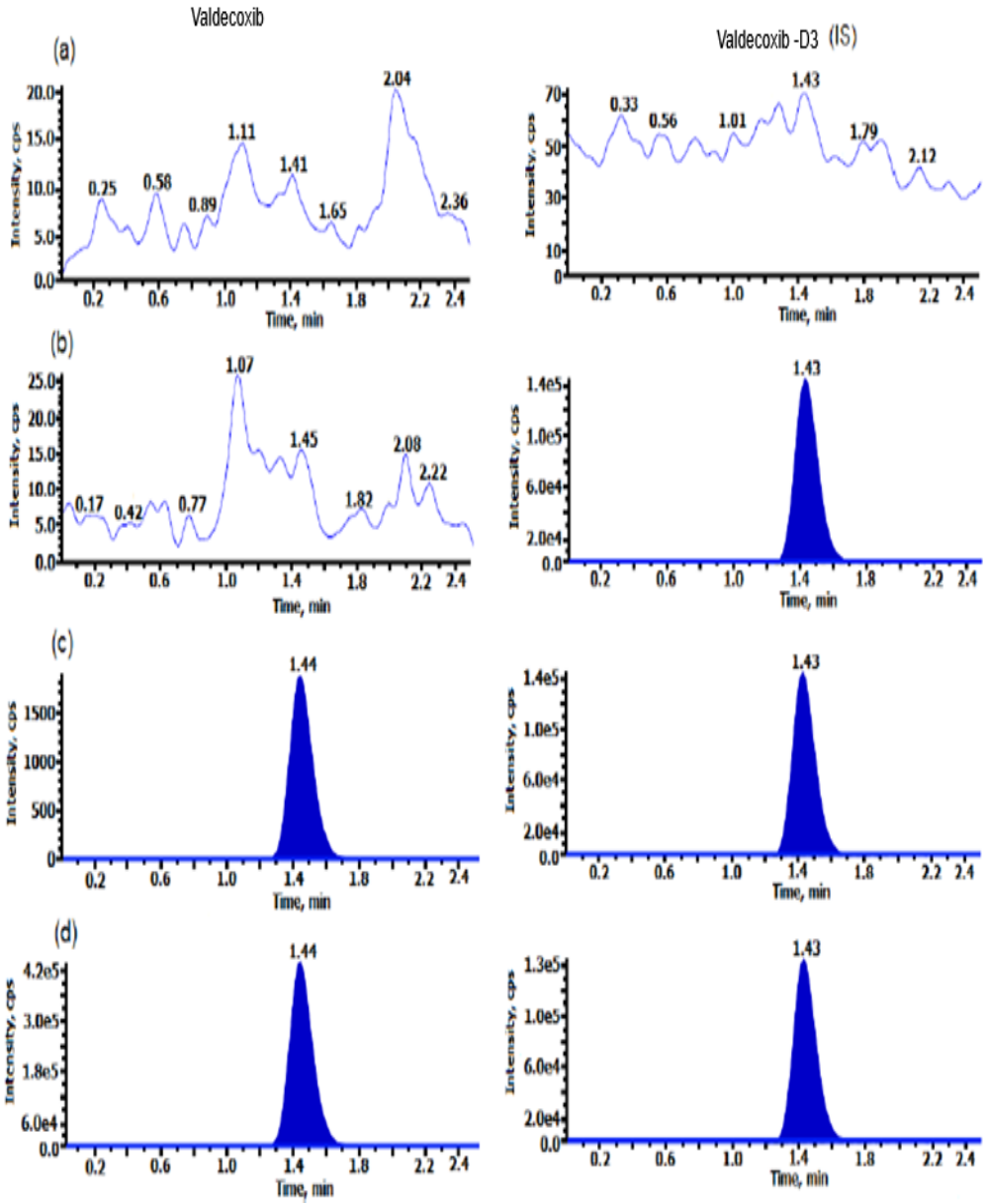
Figure. 1: MRM ion-chromatograms of Valdecoxib(m/z 380.0 → 315.9) and ValdecoxibD3 (IS, m/z 387.0 → 323.0) in (a) double blank plasma (without analyte and IS), (b) blank plasma with IS, (c) Valdecoxib at LLOQ and IS (d) real subject sample