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DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL HPLC-DAD METHOD FOR KETOPROFEN QUANTIFICATION IN RAT PLASMA AND BRAIN TISSUE: APPLICATION TO PHARMACOKINETIC STUDIES AND TISSUE DISTRIBUTION ANALYSIS

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Abstract

Ketoprofen (KTP) is used as an anti-inflammatory agent and has been studied as a potential treatment for neurodegenerative diseases. This study developed and validated a sensitive high-performance liquid chromatography with diode-array detection method for quantifying KTP in rat plasma and brain tissue. Sample preparation was performed via solvent extraction assisted by protein precipitation, using acetonitrile acidified with 10% HCl in plasma or brain homogenate (1:10, v/v). Chromatographic separation was carried out on a Waters C18 reversed-phase column (150 × 4.6 mm, 5 µm) with an isocratic mobile phase composed of water, 0.3% triethylamine, acetonitrile, and methanol (50:40:10, v/v/v). The aqueous phase was adjusted to pH 3.5 with orthophosphoric acid, and the flow rate was set at 1 mL/min. Detection wavelengths were set at 255 nm for KTP and 355 nm for the internal standard, with a total run time of 5 min. The method demonstrated lower limits of quantification of 0.5 µg/mL for plasma and 0.25 µg/mL for brain tissue, with linearity ranges of 0.5–20 µg/mL and 0.25–8 µg/mL, respectively, and correlation coefficients above 0.999. Intra- and interday accuracy and precision ranges were within ±15% for both matrices, in accordance with the official guidelines of Brazil's National Health Surveillance Agency and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. The method also demonstrated adequate selectivity, an absence of endogenous interferences, and consistent recovery in plasma and brain tissue. Stability studies confirmed the analyte content under different storage and processing conditions. Hence, the validated method proved sensitive, precise, accurate, and selective, supporting its application in preclinical pharmacokinetic and tissue-distribution research on KTP.

Keywords: Chromatography. NSAIDs. Solvent extraction.

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1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drug classes worldwide. They are employed in both veterinary medicine, particularly post-surgically, and human medicine (Tyumina et al. 2023). The significant rise in NSAID consumption among humans is a crucial topic, given the increased incidence of neurodegenerative and neuroinflammatory diseases. These diseases, often linked to oxidative stress, include Alzheimer's, Parkinson's, and Huntington's diseases, as well as amyotrophic lateral sclerosis, and they are associated with changes in the body's inflammatory responses (Teleanu et al. 2022). Consequently, NSAIDs have gained increasing prominence in scientific studies (Jayawickreme et al. 2024), alongside their standard use for treating muscle pain, osteoarthritis, and other inflammatory conditions. However, the tissue distribution of these drugs is not always efficient due to challenges such as crossing biological barriers (e.g., the blood-brain barrier) or a generally low systemic distribution, which can compromise treatment effectiveness.

Ketoprofen (KTP) is noteworthy for its analgesic, anti-inflammatory, and antipyretic properties, and it is widely used to manage inflammatory and degenerative changes in rheumatic diseases (Rajamohan et al. 2024). Nonetheless, it may cause several adverse effects at the gastric, renal, and cardiac levels (Kuczynska and Nieradko-Iwanicka 2022). It is understood that the S-enantiomer primarily inhibits prostaglandin synthesis, while the R-enantiomer primarily mediates analgesia (Czub et al. 2022). Additionally, a conversion of R to S occurs upon administration (Lorier et al. 2016), making enantiomer isolation optional depending on the study objectives. The half-life varies by species, being approximately 6 h in horses (Knych et al. 2024), 1–4 h in humans, and roughly 5 h in rats (Choi et al. 2006), making it a drug with rapid absorption and elimination.

To ensure effective and safe treatments, a bioanalytical method capable of quantifying drug concentrations in plasma and various tissues must be developed. The validation of this method is crucial to demonstrate its suitability for its intended use, thereby ensuring it can provide precise, accurate, and reproducible results in different biological matrices (Woziński et al. 2024). Such reliable data are vital for pharmacokinetic studies, including bioavailability, bioequivalence, pharmacodynamic, and toxicological studies, in which analytes must be quantified in biological matrices such as urine or plasma. Notably, several regulatory agencies have established guidelines for conducting these studies, including the United States Food and Drug Administration, the European Medicines Agency, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), and Brazil's National Health Surveillance Agency (ANVISA) (Vazvaei-Smith et al. 2024).

High-performance liquid chromatography (HPLC) is commonly employed to quantify drugs in biological matrices. For KTP quantification, the most frequently cited studies have utilized quantification via derivatization (Péhourcq et al. 1995, Qiu et al. 2007). However, methods developed for other matrices, such as dog plasma and rainbow trout (David et al. 2014), did not reproduce well when tested in rat plasma (Tang et al. 2026), or they employed powerful and potentially toxic solvents, such as dimethyl sulfoxide, in the sample extraction process (Xi et al. 2005).

Given these considerations and the relevance of KTP quantification in rat plasma samples for characterizing formulations in preclinical studies, this study aimed to optimize and validate an HPLC with diode-array detection (HPLC-DAD) method. Ensuring adequate sensitivity and precision for the characteristic concentrations of KTP in plasma and brain matrices is pivotal to comply with specific validation regulations.

2. Material and Methods

Chemicals and Reagents

Ketoprofen (99% purity) was purchased from Sigma-Aldrich (São Paulo, Brazil), as was piroxicam (PI), which served as the internal standard (IS) for developing the bioanalytical method. Ultrapure water was obtained using a Milli-Q Plus system (Millipore, USA). Acetonitrile and HPLC-grade methanol were procured from Dinâmica Química Contemporânea (São Paulo, Brazil). All other solvents, reagents, and chemicals used were of analytical grade.

Chromatographic Conditions

The quantification of KTP in plasma and brain samples was carried out using an HPLC system (LC-10, Shimadzu, Japan) equipped with an SPD-M20A DAD, an LC-20AT pump, a CBM-20A system controller, a DGU-20A3 degasser, and an SIL-20 automatic injector. Data acquisition and processing were conducted using LC Solution software (v. 1.22 SP1). A Waters C18 reversed-phase column (150 mm × 4.6 mm, 5 µm particle size) connected to a precolumn was used for chromatographic separation. The mobile phase consisted of water containing 0.3% triethylamine adjusted to pH 3.5 using orthophosphoric acid, acetonitrile, and methanol in a 50:40:10 (v/v/v) ratio, delivered at 1.0 mL/min over a 5-min run. The injection volume was 20 µL, with the UV detection wavelengths set at 255 nm for KTP (Vozniuk et al. 2024) and 355 nm for PI (Amin 2002), at 25 °C.

Stock Solution Preparation

Stock solutions for KTP and PI were prepared by dissolving accurately weighed samples in HPLC-grade methanol to achieve a final concentration of 1 mg/mL. To obtain the working concentrations, the stock solutions were serially diluted in methanol. These solutions were prepared at concentrations ten times higher than the final target concentrations for plasma, extracted brain samples, and quality control (QC) points. All stock and working solutions were aliquoted and stored at -20 °C in the dark. Methanol was selected based on literature recommendations and preliminary assessments performed during method development.

Sample Preparation

Solvent extraction assisted by protein precipitation was used to prepare the samples. This procedure consisted of adding 90 µL of thawed rat plasma to 10 µL of KTP at various calibration curve points (0.5, 1, 4, 8, 16, and 20 µg/mL). For each calibration curve point or sample, 10 µL of 10% HCl and 10 µL of the IS (PI, 25 µg/mL) were added. The mixture was initially homogenized by vortexing for 20 s. Subsequently, 1000 µL of acetonitrile (1:10, v/v) was added to each sample, which was then shaken on an orbital shaker (SH2000, FinePCR, Korea) at 2000 rpm for 5 min to ensure consistent, uniform mixing under controlled, reproducible conditions. The samples were centrifuged at 14,000 rpm for 10 min. During centrifugation, precipitated proteins and particulate matrix components formed a pellet, while the KTP remained dissolved in the acetonitrile-aqueous supernatant. An 800-µL aliquot of the supernatant was collected, transferred to an appropriate tube, and evaporated in a vacuum centrifuge at 60 °C for 50 min. The dried samples were resuspended in 100 µL of the mobile phase. For tissue samples, a small, thawed piece of brain tissue (0.1 g) was homogenized in 1 mL of acetonitrile for 30 s. After centrifugation at 14,000 rpm for 5 min, 90 µL of the supernatant was collected and processed using the same preparation procedure as for the plasma samples.

Selectivity and Specificity

Method selectivity and specificity were evaluated following the ANVISA and ICH M10 (2023) guidelines, which recommend analyzing blank plasma chromatograms without the addition of any drug (i.e., no KTP or IS). Blank plasma samples and blank brain tissue homogenates were analyzed to confirm the absence of endogenous interferences. Samples spiked with KTP and IS were also analyzed to confirm the absence of interference from co-eluting peaks in the chromatograms. The purity of the peaks was assessed in both plasma and brain tissue samples.

Linearity

The linearity of the method was assessed at six different concentrations by adding 10 µL of KTP and PI working solutions of known concentrations to 90 µL of drug-free rat plasma. These solutions were subjected to the protocol described in Section 2.4, resulting in a concentration curve of 0.5–20 µg/mL in rat

plasma. The IS concentration was set at 25 µg/mL. Three different curves were prepared and evaluated in triplicate over three days, and the calibration curve was plotted by relating the analyte concentration to the IS concentration ratio. Microsoft Excel software was then used to calculate the slope, intercept, and regression coefficient (Harish et al. 2022). Lastly, brain linearity (0.25–8 µg/mL) was prepared and analyzed in the same manner as the rat plasma.

Accuracy and Precision

The accuracy and precision of the method were evaluated using QC samples, which consisted of blank plasma and brain tissue matrices fortified with KTP at three concentration levels within the calibration range (0.5, 10, and 20 µg/mL for plasma, and 0.25, 4, and 8 µg/mL for brain tissue). For each concentration level, five independently prepared QC samples were subjected to the complete sample preparation procedure described in Section 2.4 and subsequently analyzed by HPLC-DAD. Intraday accuracy and precision were evaluated using five independently prepared QC samples analyzed on the same day, whereas interday assessments were performed using independently prepared QC samples analyzed over three consecutive days. Method performance was assessed using the mean concentration, standard deviation, accuracy, and relative standard deviation (RSD%), which did not exceed ±15% (Lambarki et al. 2025).

Recovery and Matrix Effect Study

A recovery study was conducted to evaluate the extraction efficiency of KTP from plasma and brain tissue and to assess the matrix effect. KTP was added to blank plasma or brain tissue matrices at concentrations of 0.5 and 20 µg/mL for plasma, and 0.25 and 8 µg/mL for brain tissue. The fortified samples were subjected to the complete sample preparation procedure (see Section 2.4) and subsequently analyzed under the chromatographic conditions (see Section 2.2). Simultaneously, samples from different plasma matrices (hemolyzed and lipemic) were prepared at the same concentrations. For the brain tissue, lipemic samples and those from aged animals were chosen. Recovery was calculated based on the ratio of KTP peak areas in samples with and without matrix alteration, with variations not exceeding ±15% (Hollander et al. 2024).

Ketoprofen Stability in Plasma

The bioanalytical method for KTP in rat plasma was validated according to current regulatory guidelines and subsequently applied in a pharmacokinetic study. As part of the validation process, KTP stability in rat plasma and brain tissue was assessed under various operational conditions to ensure reliable quantification throughout the analytical workflow. Stability tests included: (1) short-term stability, where spiked samples were maintained at room temperature (25 °C) for 6 h before analysis, simulating routine sample handling; (2) autosampler stability, which evaluated processed samples kept in the autosampler for 12 h at 25 °C, mimicking possible analytical delays; (3) freeze-thaw stability, where samples were subjected to three consecutive freeze (-20 °C for 12 h) and thaw (1 h at room temperature) cycles, simulating typical storage and reanalysis conditions; and (4) long-term stability, assessed after storing spiked samples at -20 °C for 60 days to verify the analyte's stability during extended storage. Under all tested conditions, KTP concentrations in both matrices were compared to nominal values, with accuracy and RSD% remaining within acceptable limits (Gniazdowska et al. 2022).

Method Applicability

The protocol for this animal study was approved by the Ethics Committee on Animal Use of the Federal University of Pampa. Plasma was obtained from male Wistar rats weighing 180 ± 40 g (approximately 60 days old), sourced from the Animal Facility of the Federal University of Pampa. The animals were maintained under guidelines from the National Council for the Control of Animal Experimentation, with the study protocol approved by the Ethics Committee of the Federal University of Pampa (CEUA no. 029/2024).

To demonstrate the robustness of the developed method, plasma samples from three rats were analyzed after intravenous administration of KTP at a dose of 5 mg/kg. The drug was previously solubilized in an aqueous solution containing 2% polysorbate 80 at a final concentration of 1 mg/mL and administered via the lateral caudal vein. Blood samples were collected in heparinized tubes at various intervals post-administration (0.083, 0.16, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, and 24 h).

The administration protocol for assessing tissue distribution in the brain matched that used for plasma, except 18 animals were used (3 per experimental time point). Brain samples were collected at times of 0.5, 1, 2, 4, 8, and 12 h, and the animals were euthanized at each specific time for brain removal. Afterward, the brains were carefully wrapped in aluminum foil, labeled, and stored at -20 °C until processing.

3. Results

Method Development and Validation

We began by working with the racemic mixture of KTP using a conventional C18 reversed-phase column. In this column type, a single peak is expected since the R- and S-enantiomers share identical polarity and thus co-elute without separation, which was our aim. To achieve optimal chromatographic separation and peak resolution within the shortest run time, we conducted numerous tests, drawing on guidance from the literature and pharmacopeias (Peng et al. 2022). Various ratios of water, triethylamine, acetonitrile, and methanol, in addition to pH adjustments, were tested to optimize the separation of plasma interferences from both KTP and IS. We also assessed several extraction techniques, including protein precipitation, solvent extraction, and plasma acidification, until the optimal chromatographic conditions were achieved, as described in Sections 2.2 and 2.4.

Separation of KTP and IS in the samples was performed via isocratic elution. The chromatographic overlap shown in Figure 1 indicates sharp peaks for KTP and IS at their respective wavelengths. Accordingly, absorbance was monitored at 255 nm for KTP and 355 nm for IS. Figure 2 presents these parameters in brain tissue samples.

KTP was efficiently extracted from plasma, resulting in a symmetrical peak (Figure 1) when a mobile phase composed of water with 0.3% triethylamine (adjusted to pH 3.5 with orthophosphoric acid), acetonitrile, and methanol in a 50:40:10 (v/v) ratio was used.

Extraction Optimization During Sample Preparation

Solvent extraction assisted by protein precipitation was selected as the sample preparation strategy, as it provided clean extracts, minimized chromatographic interferences, and yielded satisfactory KTP recovery. Acetonitrile was employed as the extraction solvent due to its well-established capacity to precipitate proteins from biological matrices while maintaining KTP in solution. After centrifugation, the precipitated proteins and tissue debris formed a pellet, whereas KTP remained dissolved in the acetonitrile-aqueous supernatant. The supernatant was collected, evaporated, and reconstituted prior to chromatographic analysis. Acetonitrile is widely used in bioanalytical sample preparation, and organic solvents such as acetonitrile and methanol are commonly employed for protein precipitation in biological fluids (Shendy et al. 2025).

Selectivity and Specificity

The specificity of the developed method was assessed by comparing chromatograms of blank plasma/organ homogenates with those containing KTP and IS. The peak purity of KTP was quantified using LC solution software and exceeded 0.999. No interfering peaks were observed at the retention times of KTP (3.90 min) or IS (3.49 min) at either wavelength (Figures 1a–c and 2), confirming the method's specificity.

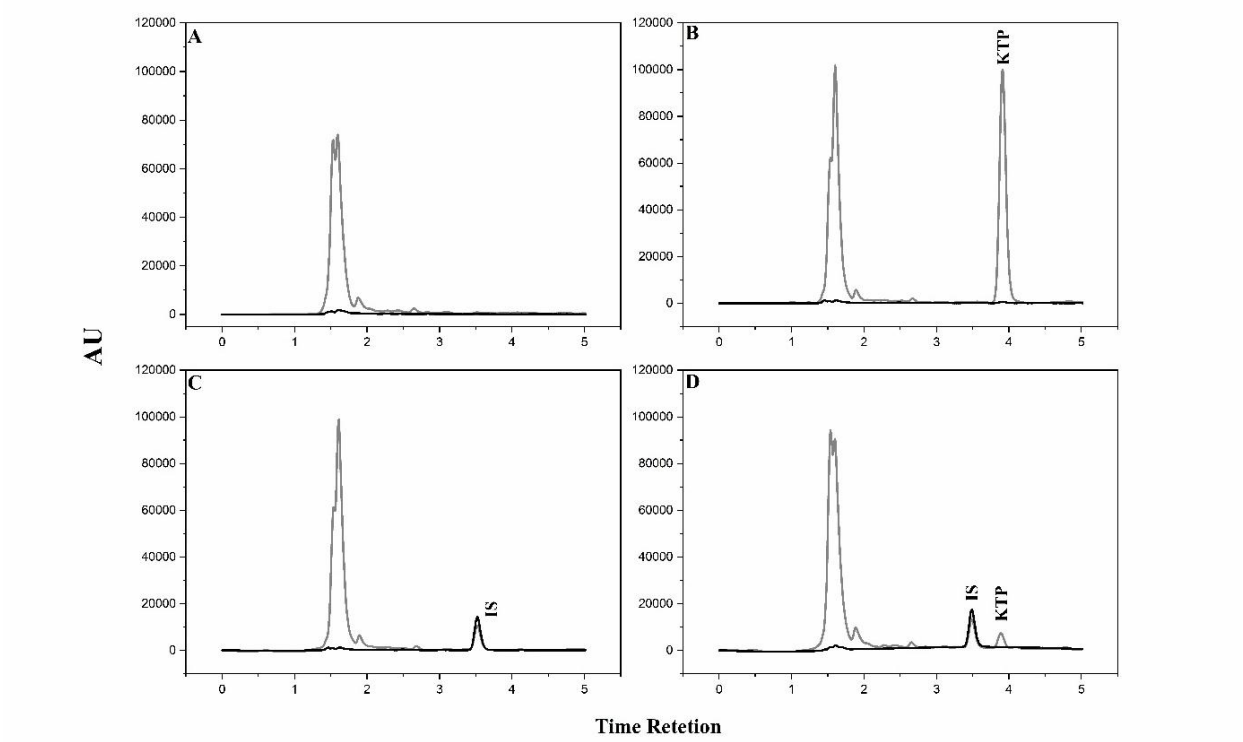

Figure 1. Representative HPLC chromatograms of plasma samples: (A) blank plasma sample without ketoprofen (KTP) or internal standard (IS); (B) blank plasma sample fortified with KTP (10 µg/mL); (C) blank plasma sample fortified with piroxicam (IS); and (D) rat plasma sample collected 24 h after intravenous administration of KTP (5 mg/kg), showing both KTP and IS peaks. Detection wavelengths were 255 nm for KTP and 355 nm for IS.

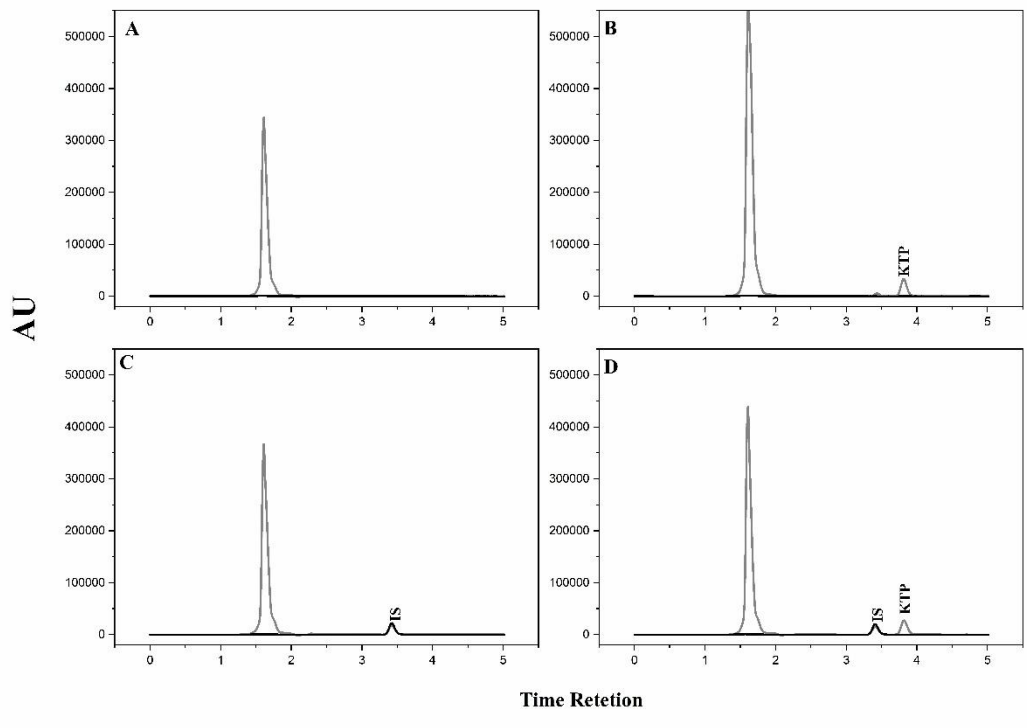

Figure 2. Representative HPLC chromatograms of brain tissue homogenate samples: (A) blank brain tissue homogenate without ketoprofen (KTP) or internal standard (IS); (B) blank brain tissue homogenate fortified with KTP (4 µg/mL); (C) blank brain tissue homogenate fortified with piroxicam (IS); and (D) rat brain tissue homogenate collected 12 h after intravenous administration of KTP (5 mg/kg), showing both KTP and IS peaks. Detection wavelengths were 255 nm for KTP and 355 nm for IS.

Linearity and Lower Limit of Quantification

The linearity range in any bioanalytical method reflects the direct relationship between test results and drug content within a specified range of biological samples, such as plasma or tissues (Sinha et al. 2024). To assess this, a six-point calibration curve for KTP was constructed in both matrices, with the chosen concentrations reflecting likely plasma levels of KTP encountered during pharmacokinetic studies. Plasma and brain tissue samples were fortified with known KTP concentrations, and the corresponding peak area ratios were determined. The calibration curve was plotted as KTP concentration against the peak area ratio of KTP to the internal standard (IS), covering concentration ranges of 0.5–20 µg/mL in plasma and 0.25–8 µg/mL in brain tissue. Linear regression equations for plasma and brain tissue were derived from their respective plots and are summarized in Table 1. A representative standard curve for KTP in rat plasma is presented in Figure 3. The calibration curves for KTP in plasma and brain tissue demonstrated linearity within the expected parameters, with R^2 values of 0.9995–0.9997. The linearity equation for plasma was y = 0.7985x + 0.0436; for brain tissue, it was y = 0.4724x + 0.0717, with R^2 values of 0.9992–0.9996. Limits of detection (LOD) for each matrix were also calculated, indicating the minimum measurable concentration for which the analyte's presence can be reliably inferred with statistical confidence (Cerioni et al. 2024). The percentage variation at the lowest reproducible concentration, with precision of 20% and accuracy within 80–120%, was also determined.

Table 1 Linear regression data for the calibration curve of ketoprofen in rat plasma.

Parameter	Plasma	Brain tissue
Range (µg/mL)	0.5–20	0.25–8
Correlation coefficient (R^2)	0.9996 ± 0.0001	0.9994 ± 0.0002
Slope	0.7985 ± 0.0072	0.0703 ± 0.0063
Intercept	0.0436 ± 0.0040	0.4702 ± 0.0017
LLOQ (µg/mL)	0.05	0.08
LOD (µg/mL)	0.01	0.04

LLOQ: lower limit of quantification; LOD: limit of detection

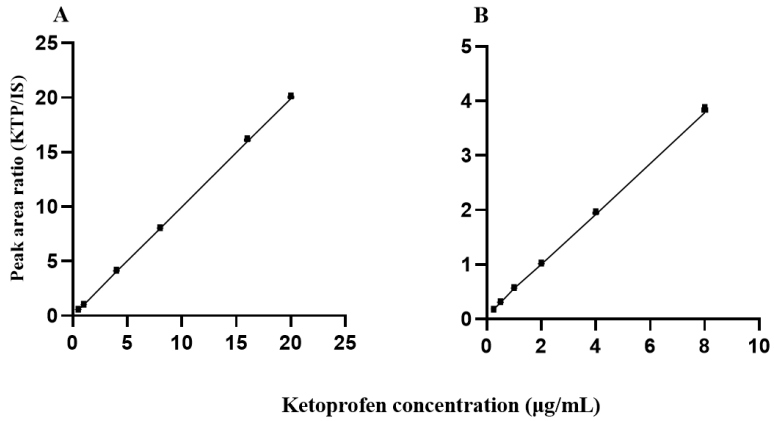

Figure 3. Representative calibration curves for ketoprofen (KTP) in rat plasma (0.5–20 µg/mL) (A) and rat brain tissue (0.25–8 µg/mL) (B). Results are expressed as mean ± standard deviation obtained over three separate days.

Accuracy and Precision

Inter- and intra-day accuracy and precision were assessed at different concentrations. RSD values ranged from 0.25% to 3.80%, and accuracy remained within the acceptable range (Table 2), indicating the method's reliability across different days and biological matrices.

Recovery and Matrix Effect on Plasma and Brain

Recovery tests performed with lipemic, hemolyzed, and elderly samples showed no significant matrix interference, with recovery values ranging from 94.99% to 106% (Table 3). Matrix effects from endogenous

substances can complicate drug analysis in biological samples, regardless of the detection method. According to ANVISA guidelines, at least 6 different matrix sources must be evaluated, including 1 hyperlipidemic source if using whole blood and 1 hyperlipidemic and 1 hemolyzed source if using plasma (both conditions apply to our experiment). Additionally, interference must be less than 20% of the analyte response at the lower limit of quantification (LLOQ) and 5% of the IS response (Vazvaei-Smith et al. 2024). Both aged and lipemic samples met these criteria (Gniazdowska et al. 2023). These samples represent various populations studied, particularly those relevant to neurodegenerative or inflammatory diseases in numerous species (Xi et al. 2005).

In our study, the recovery of KTP in plasma and brain tissue was assessed using the standard addition method at two concentrations (0.5 and 20 µg/mL) spanning the entire linear range. Table 3 lists the results at lower and higher concentrations of quality control samples, indicating that the recovery rate ranged from 94.99 to 106%, regardless of the matrix evaluated. This demonstrates that the matrices did not affect the final response, the recovery rate of KTP in plasma and brain tissue.

Table 2. Plasma and brain KTP precision and accuracy data results.

	Concentration (µg/mL)	Inter-day				Intra-day			
		Day	Mean (µg/mL) and SD	Accuracy (%)	RSD%	Day	Mean (µg/mL) and SD	Accuracy (%)	RSD%
Plasma	0.5	1	0.50 ± 0.01	100.54	1.52	1	0.52 ± 0.01	100.56	1.54
		2	0.51 ± 0.01	102.19	1.68	2	0.51 ± 0.01	101.10	1.50
		3	0.51 ± 0.02	101.14	1.26	3	0.50 ± 0.02	101.06	1.23
	10	1	9.98 ± 0.46	99.89	0.79	1	9.93 ± 0.45	99.83	0.74
		2	9.81 ± 0.07	99.81	0.76	2	10.01 ± 0.46	101.10	0.81
		3	9.95 ± 0.06	99.96	0.25	3	10.02 ± 0.53	100.10	0.89
	20	1	19.94 ± 0.18	99.68	0.89	1	19.92 ± 0.13	99.64	0.80
		2	20.08 ± 0.02	100.38	0.12	2	20.04 ± 0.02	100.30	0.18
		3	20.02 ± 0.09	100.10	0.44	3	20.02 ± 0.09	100.30	0.60
Brain	0.25	1	0.22 ± 0.01	89.16	1.94	1	0.21 ± 0.01	90.01	1.90
		2	0.26 ± 0.01	103.51	3.80	2	0.26 ± 0.01	103.51	3.79
		3	0.23 ± 0.02	89.32	0.32	3	0.22 ± 0.02	90.30	0.34
	4	1	3.97 ± 0.04	99.30	1.06	1	3.99 ± 0.02	99.89	1.04
		2	4.02 ± 0.03	99.69	1.03	2	4.02 ± 0.03	99.69	1.02
		3	3.98 ± 0.64	99.40	1.44	3	3.98 ± 0.64	99.40	1.40
	8	1	8.06 ± 0.08	100.71	0.94	1	8.06 ± 0.08	100.71	0.90
		2	8.74 ± 0.83	102.46	0.87	2	8.74 ± 0.83	102.46	0.45
		3	8.01 ± 0.02	101.40	0.26	3	8.03 ± 0.05	100.08	0.20

Five replicates of quality control samples in the concentration range of the calibration curve. The results were expressed as means ± standard deviations (SD), and percentage relative standard deviation (RSD%)

Table 3. Extraction of ketoprofen from rat plasma and brain tissue.

Plasma				
	Lipemic sample		Hemolyzed sample	
Concentration	Concentration measured	Recovery (%)	Concentration measured	Recovery (%)
0.5	0.53 ± 0.04	106.88 ± 0.05	0.47 ± 0.02	94.99 ± 0.02
20	20.19 ± 0.52	100.95 ± 0.52	19.66 ± 0.02	98.34 ± 0.02
Brain tissue				
	Lipemic sample		Sample elderly	
Concentration	Concentration measured	Recovery (%)	Concentration measured	Recovery (%)
0.25	0.22 ± 0.02	102.03 ± 0.01	0.24 ± 0.02	98.79 ± 0.02
8	8.04 ± 0.50	100.95 ± 0.52	8.86 ± 0.04	98.34 ± 0.02

Concentrations are expressed in µg/mL

Stability

The study established that KTP exhibited good stability under various conditions, including short-term stability (6 h), autosampler stability (12 h), three freeze-thaw cycles, and extended storage for 60 days at -20 °C (Table 4). These conditions were selected to simulate real-world scenarios during sample

preparation, sequential analysis, and the interval between collection and HPLC processing. Results indicated that KTP remained stable in both plasma and brain under all tested conditions, with an RSD% below ± 3% of the actual value.

The accuracy values in plasma and brain consistently ranged from 97.30% to 103.63% and 97.30% to 103.62%, respectively, highlighting the reliability of the analytical method. Notably, the compound's stability persisted after multiple freeze-thaw cycles and extended storage, supporting its suitability for pharmacokinetic and bioanalytical studies. This finding is particularly significant, as it confirms the method's practical applicability and ensures the precision required for analysis of biological matrices such as plasma and brain tissue.

Table 4. Stability of ketoprofen in rat plasma and brain under operational conditions.

Stability study	Plasma		Brain tissue	
	Concentration (µg/mL)	Accuracy (Mean ± RSD%)	Concentration (µg/mL)	Accuracy (Mean ± RSD%)
Short-term stability (6 h)	0.5	101.38 ± 2.86	0.25	101.38 ± 2.86
	20	100.61 ± 0.28	8	100.61 ± 0.28
Auto-sampler stability (12 h)	0.5	100.06 ± 2.29	0.25	100.09 ± 2.29
	20	103.63 ± 2.74	8	103.62 ± 2.74
Freeze-thaw stability (3 cycles)	0.5	97.30 ± 1.29	0.25	97.30 ± 1.28
	20	99.70 ± 1.81	8	99.70 ± 1.81
Long-term stability (60 days, -20 °C)	0.5	101.77 ± 1.08	0.25	100.06 ± 1.47
	20	99.06 ± 1.94	8	101.63 ± 1.41

Application of the HPLC Method for Ketoprofen Quantification in Rats

The method was applied to quantify KTP in rat plasma and brain. The mean plasma half-life was 7.11 ± 0.33 h, and clearance was 0.014 ± 0.001 mL/h/kg. In the brain, the half-life was 1.66 h, with a penetration factor of 0.02. The LLOQ was low enough to characterize drug elimination in both matrices (Figure 4). The area under the curve ($AUC_{0-\infty}$), calculated using the trapezoidal rule, was 17.3 ± 7.73 µg·h/mL. These results indicate that the LLOQ of the method was sufficient to accurately define the elimination phase of KTP. Sampling for approximately three half-lives yielded an extrapolated AUC of 6.88 ± 1.70%. In the brain, the estimated half-life was 1.66 h, with a Ke of 0.42 h, an AUC_{0-t} of 1.57 µg·h/mL, and a penetration factor of 0.02. These findings demonstrate that the LLOQ was sufficient to characterize the elimination phase of the drug in the brain. Sampling over a period of roughly three half-lives resulted in an extrapolated $AUC_{0-\infty}$ of 3.71%.

Table 5. Comparison of representative analytical methods previously reported for ketoprofen determination in biological matrices and the method developed in the present study.

Study	Matrix	Detector	Sample preparation	Run time (min)	LLOQ	Main characteristics
(Péhourcq et al. 1995)	Human plasma	HPLC-UV	LLE + derivatization	~14 min	0.1 µg/mL	Enantioselective, complex sample preparation
(Xi et al. 2005)	Rat plasma/GI tissues	HPLC-UV	LLE	NR	NR	GI distribution study
(Allegrini et al. 2011)	Human plasma	HPLC-DAD	SPE	5 min	0.01 µg/mL	Plasma only, SPE required
(Granero and Amidon 2008)	Dog plasma	HPLC-UV	Protein precipitation	17 min	0.70 µg/mL	Pharmacokinetic study; enterohepatic recycling
Present study	Rat plasma and brain tissue	HPLC-DAD	Protein precipitation/solvent extraction	5 min	Plasma 0.05 µg/mL Brain 0.08 µg/mL	Simultaneous applicability to plasma and brain tissue

LLE, liquid-liquid extraction; SPE, solid-phase extraction; LLOQ, lower limit of quantification; NR, not reported.

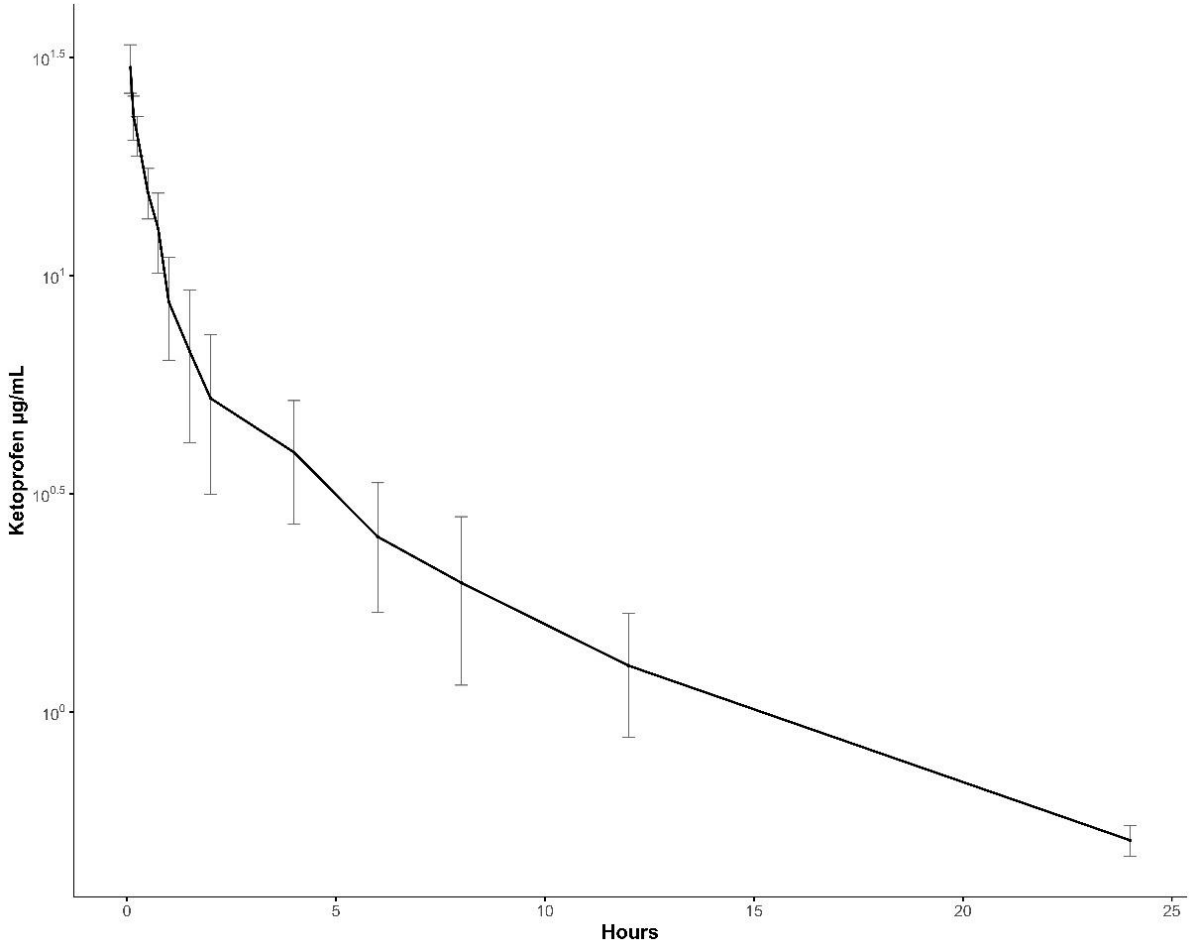

Figure 4. Mean plasma concentration versus time profile of ketoprofen (KTP) after an intravenous dose of 5 mg/kg in male Wistar rats. Points show mean ± standard deviation for three animals.

4. Discussion

The efficiency of the HPLC method depends directly on the proper separation and resolution of chromatographic peaks. Factors influencing this efficiency include the type of stationary phase, detector selection, detection wavelength, and the chemical composition of the mobile phase. The choice of IS was based primarily on its short retention time, compatibility with plasma and tissue matrices, and a detection wavelength distinct from that of KTP, thereby enhancing the method's selectivity (Satyavert et al. 2020). To better contextualize the present method within the available literature, a comparison with representative analytical methods previously reported for KTP quantification is presented in Table 5.

As shown in Table 5, previously published methods for KTP quantification were predominantly developed for plasma samples and often included derivatization procedures, solid-phase extraction, or were limited to a single biological matrix. In contrast, the present method was validated for both rat plasma and brain tissue using a straightforward sample preparation procedure, a short chromatographic run time, and HPLC-DAD instrumentation. Although some previously reported methods achieved lower LLOQ values, the sensitivity obtained in this study was sufficient for its intended pharmacokinetic and tissue distribution applications while maintaining methodological simplicity and accessibility. Moreover, the method was successfully applied to pharmacokinetic and tissue distribution studies, confirming its suitability beyond the validation stage. Notably, limited information is available on validated HPLC-DAD methods that can be simultaneously applied to rat plasma and brain tissue for pharmacokinetic and tissue distribution studies. Considering the importance of evaluating KTP distribution beyond systemic circulation, particularly in preclinical investigations involving central nervous system exposure, the development of a simple, reliable, and accessible HPLC-DAD method remains highly relevant.

Selectivity, or specificity, is understood as the accuracy of a bioanalytical method to accurately differentiate the analyte from all other sample components, such as impurities or endogenous compounds. This should be evaluated and compared using matrix-enriched QCs in addition to the response of blank

plasma (Attwa et al., 2025). Typically, the chromatogram of the blank sample is compared with that of the analyte-spiked sample. In our study, the specificity of the developed method was evaluated by comparing chromatograms from blank plasma and organ homogenates with those spiked with KTP and IS in blank plasma and organ homogenates.

The method demonstrated linearity over a wide concentration range, consistent with the expected pharmacokinetic profiles of KTP in preclinical studies. High R^2 values and low variation at lower concentrations demonstrate the method's sensitivity and appropriateness for determining KTP at trace levels. The correlation coefficients obtained ($R^2 > 0.999$) for both matrices indicated excellent proportionality between analyte concentration and detector response, supporting the reliability of the method for quantitative applications in pharmacokinetic and tissue distribution studies. The validated calibration ranges adequately covered the concentrations observed during in vivo experiments, eliminating the need for additional sample dilution or concentration steps.

Accuracy reflects how closely the experimentally obtained value matches the true value. Freshly prepared samples were analyzed in five replicates on the same day for intra-day assessment, and this procedure was repeated on three consecutive days for inter-day assessment, as described in the literature (Liu et al. 2023) and shown in Table 3. Accuracy and precision were determined as percent recovery and RSD, respectively. Inter-day accuracy and precision ranged from 99.29% to 100.54%, with RSDs of 0.47% to 1.52%. Similarly, intra-day accuracy and precision ranged from 99.83% to 101.29%, with RSDs from 0.60% to 1.54% for plasma and brain tissue samples (Table 2). All values were well within the acceptance thresholds established by current bioanalytical validation guidelines, demonstrating excellent method reproducibility and robustness. The consistently low variability across days further supports the method's suitability for routine analysis of large sample sets generated during pharmacokinetic studies (El Orche et al. 2024).

The demonstrated stability of KTP under all tested conditions further supports the method's practical utility, supporting sample storage and batch analysis over extended periods. This is especially relevant for studies involving repeated sampling or long-term pharmacokinetic evaluations. The stability results indicated that potential delays between sample collection, preparation, and chromatographic analysis would not compromise measurement reliability, which is particularly important in preclinical studies with multiple sampling time points and tissue collection steps (Gomez-Rioja et al. 2023).

Effective characterization of KTP pharmacokinetics in rat plasma and brain tissue suggested that the developed bioanalytical method can be applied to both matrices with equal efficiency. The results indicated that the LLOQ of the method was sufficient to accurately characterize the elimination phase of KTP. These findings demonstrate that the LLOQ was low enough to accurately characterize the drug's elimination phase in the brain. Successful application of the method to real biological samples confirmed its selectivity, sensitivity, and practical applicability beyond validation. The ability to quantify KTP in rat plasma and brain tissue using a simple HPLC-DAD approach, without derivatization or solid-phase extraction, offers an attractive alternative to more complex and costly analytical techniques (Mahdavijalal et al. 2024). These results confirm that the bioanalytical method is appropriate for measuring drug concentrations in plasma and tissue during preclinical trials.

5. Conclusions

An HPLC-DAD bioanalytical method was developed and validated for the quantification of KTP in rat plasma and brain tissue utilizing PI as the IS. The solvent extraction procedure efficiently recovered the drug without interference from endogenous interference. The method showed linearity ($R^2 > 0.999$), precision, and accuracy within regulatory limits (±15%), with adequate sensitivity for detailed pharmacokinetic evaluation. The lower limits of quantification were 0.05 µg/mL for plasma and 0.08 µg/mL for brain tissue, allowing reliable quantification of ketoprofen. Pharmacokinetic analysis revealed a plasma half-life of 7.11 h and a brain penetration factor of 0.02. Stability tests confirmed analyte integrity under various conditions. The method proved suitable for tissue and plasma distribution studies. These findings will support the quantification of KTP in future pharmacokinetic investigations of different formulations, facilitating the development of improved therapeutic strategies.

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Ethical approval: Animals were used according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publication no. 8023, revised 1978). All efforts were made to minimize animal suffering and reduce the number of animals used. The study was approved by the Committee on Care and Use of Experimental Animal Resources of the Federal University of Pampa, Uruguaiana, Brazil (protocol no. 029/2024).

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