Original Research
Pharmacology

Effective method for the detection of piroxicam in human plasma using HPLC

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Abstract: Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used by the general population to alleviate inflammation and pain after oral surgeries. Piroxicam is among the most commonly used NSAIDs and excels in controlling pain, swelling, trismus and other common symptoms of inflammation. This study aimed to evaluate different concentrations of piroxicam and its major metabolite, 5'-hydroxypiroxicam, in human plasma samples over time using high performance liquid chromatography (HPLC) after liquid-liquid extraction. Briefly, 10 volunteers participated in this study after approval by the Ethics Committee of Bauru School of Dentistry, *Universidade de São Paulo* – USP, Brazil. Volunteers received a single dose oral of piroxicam (20 mg) and had blood collected at various times following an established protocol. The methodology of liquid-liquid extraction was effective for determining concentrations of piroxicam in plasma using HPLC in 10 out of 10 volunteers while 5'-hydroxypiroxicam was only detected in 2 out of 10 volunteers.

Keywords: Chromatography, High Pressure Liquid; Liquid-Liquid Extraction, Piroxicam.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) include a large number of drugs which, although chemically diverse, are regularly consumed by the general population to alleviate inflammation thereby often alleviating pain, fever and edema.[1,2,3] Piroxicam, in particular, is a commonly used NSAID which inhibits prostaglandin production, often employed to manage pain, swelling and trismus after oral surgeries.[4,5,6] Cytochrome P4502C9 (CYP2C9) is the main enzyme involved with metabolizing piroxicam to 5'-hydroxypiroxicam. Moreover, urinary excretion of unmetabolized piroxicam is rare.[7] High-performance liquid chromatography (HPLC) is frequently used to analyze the concentration of piroxicam in samples of plasma.

HPLC, briefly, is a physicochemical separation method which partitions a sample into two chemical phases – a stationary phase and a mobile phase which percolates through the stationary phase. In particular, the stationary phase may be a solid or a liquid, while the mobile phase can be a liquid, gas or supercritical gas. Not only does HPLC separate and identify substances, but it can quantify compounds by interpolating the concentration of an

unknown sample with a standard curve of purified compounds at known concentrations.[8]

Liquid-liquid extraction is a widely used technique which partitions compounds based on their relative solubilities, which is useful in the preparation of pharmaceutical samples. Some important features of liquid-liquid extractions include reasonable selectivity of compounds and rapid solvent extractions. This type of extraction involves adding an immiscible solvent to the samples followed by selective partitioning analysis of analytes versus contaminants between two phases.[9,10]

The primary aim of this study was to evaluate the efficacy of the liquid-liquid extraction method for piroxicam and its major metabolite in human plasma for use with HPLC analysis.

Methodology

Materials

Piroxicam, 5'-hydroxypiroxicam and naproxen (internal standard – IS) were purchased from Sigma-Aldrich (São Paulo, Brazil). Methanol, tricloacetic acid, acetonitrile and other chemicals used in the tests were purchased from Merck (Hobenbrunn, Germany), chromatographic grade. Additionally, water from a Milli-Q Plus purification system (Millipore, Belford, USA) was used exclusively throughout the experiments.

Stock solutions of piroxicam (100 mg/mL methanol), 5'-hydroxypiroxicam (10 mg/mL methanol) and naproxen (100 mg/mL methanol) were prepared. More specifically, the necessary dilutions were made from rates of stock solutions to construct a standard curve. When not in use, all standards were stored in the dark at -20°C in polypropylene tubes. All stages of the research were conducted under a sodium lamp to prevent photodecomposition of piroxicam, 5'-hydroxypiroxicam and naproxen.

HPLC analyses

The HPLC system (Shimadzu, model LC-20AD, Shimadzu Corp., Kyoto, Japan) was fitted with a UV-light absorbance detector (Detector SPD-10A, Shimadzu Corp.), and piroxicam, 5'-hydroxypiroxicam, and naproxen were detected using a UV absorbance of 330 nm. Briefly, separations were performed using reversed-phase chromatographic using a HPLC column LiChroCART® 60 RP-select B (LiChrospher®, Merck, Darmstadt, Germany 205 × 4.6 mm, particle size of 5 µm) with protective column LiChroCART® 4-4, 60RP-select B (LiChrospher®, Merck, Darmstadt, Germany, particle size of 5 µm). The mobile phase consisted of 0.1 M phosphate buffer and acetonitrile (70:30),[11] and the pH was adjusted with 0.1 M orthophosphoric acid (pH 3.2) while the column was maintained at 24 °C. Lastly, a flow rate of 1.0 mL/min, with an injection volume of 70 µL was used.

Quantification of piroxicam and 5'-hydroxypiroxicam

To interpolate the concentrations of piroxicam and its metabolite, a standard curve was constructed according to methods described previously.[1,9,11,12] Briefly, a twelve point scale was created using values from 0.05 to 20.0 mg/mL (piroxicam) and 0.05 to 10.0 mg/mL (5'-hydroxypiroxicam).[9] These predetermined concentrations of piroxicam and 5'-hydroxypiroxicam were aliquoted in methanol, dried, and then resuspended in 100 µL of the mobile phase (0.1 M phosphate buffer pH 3.2 and acetonitrile, 7:3 ratio) for analysis in HPLC injection flow 1 mL/min, 24°C with UV light (330 nm). All samples were analyzed during the same period and automatically injected using an injector (automatic injector SIL model, 20 AC HT, Shimadzu Corp., Kyoto, Japan).

A second standard curve in plasma was prepared using ten different concentrations of the stock solutions in the mobile phase. Briefly, 400 µL of each plasma sample were mixed with 25 µL of an internal standard (naproxen 0.1 mg/mL) and 50 µL of trichloroacetic acid 10 mM for acidification and sedimentation. Next, each sample was vortexed for 40 s and then centrifuged at 3,000 rpm for 15 min at 25°C. Then supernatants of the samples were aliquoted (100 µL) and 70 µL was injected into the HPLC machine using the same conditions used to create the first standard curve. The areas from each of the signals' peaks were interpolated from each of the standard curves for piroxicam and 5'-hydroxypiroxicam (Figure 1).

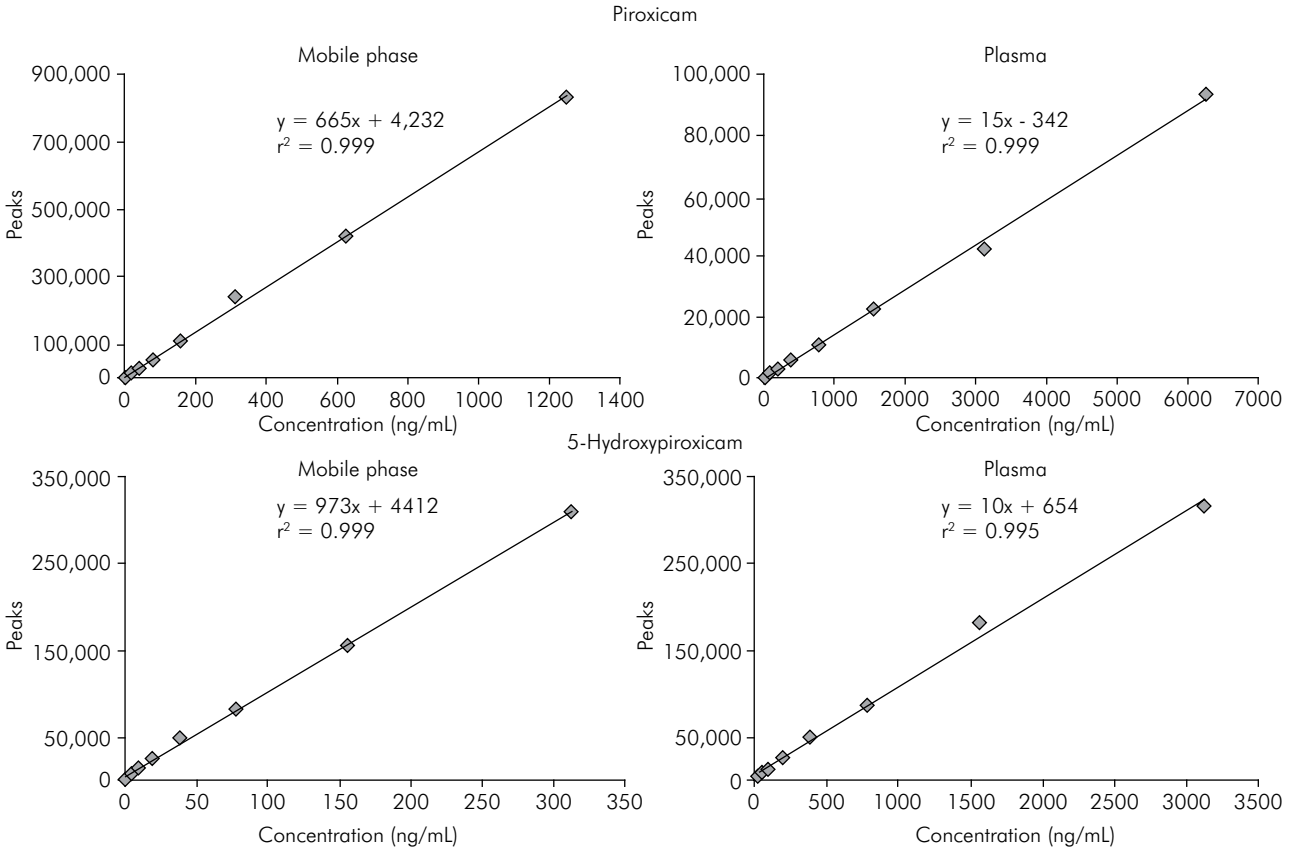

Figure 1. Standard curves of piroxicam and 5-hydroxypiroxicam in mobile phase (left) and in human plasma (right).

Patients and administration

Each of the 10 volunteers signed a consent form approved by the Ethics Committee in Human Research of the Bauru School of Dentistry, *Universidade de São Paulo* – USP (CAAE: 227527714.8.0000.5417, this research was an important part of a clinical trial registered at www.clinicaltrials.gov, NCT02450487). Eligibility criteria included the absence of inflammation or infection and no systemic diseases that could interfere with the recorded data. Criteria for exclusion consisted of any history of allergy, history of gastrointestinal bleeding or ulcers, kidney disease, asthma, sensitivity or allergy to aspirin or other nonsteroidal anti-inflammatory agents, pregnancy or lactating women. Additionally, it was confirmed with a questionnaire that patients did not use antidepressants, diuretics or aspirin in the days prior to collection, since these medicines can influence the results.

The plasma concentrations of piroxicam, and its main metabolite, 5'-hydroxypiroxicam were determined by validated procedures for high-performance liquid chromatography (HPLC).[1,9,11,12] Furthermore, naproxen was used as an internal standard.[9,12] Briefly, volunteers consumed a single dose of piroxicam (20 mg). The blood collection protocol consisted of blood samples taken before and after ingestion of this single 20 mg dose of piroxicam – immediately, 1, 2, 3, 4, 5, 6, 8, 11, 24, 48 and 72 h after intake.[1,11] The first 9 collections were collected using a catheter (BD Cateter Insyte 22 g × 1.00" – 0.9 × 25 mm – 35 mL/min) in a dorsal vein from each patient's hand. Next, the collected blood was transferred to a tube containing anticoagulant (BD Vacutainer – Buff In Citrate – 9NC). Following these collections, conventional blood samples were taken at 24, 48 and 72 hours. All samples were centrifuged for 30 min and stored at -20°C until analyzed. Drug concentrations of all samples for each patient were simultaneously analyzed. The plasma samples, more

specifically, were acidified by the addition of 10 mM trichloroacetic acid.

The analysis of plasma concentration after drug administrations allowed the collection of the following pharmacokinetic parameters: maximum plasma concentration of piroxicam (Cmax) and time of maximum concentration (Tmax); area under the concentration-time curve (AUC) from time 0 to 72 h (reported in Table and Figure 2, using software PK Solutions 2.0 – Noncompartmental Pharmacokinetics Data Analysis, Ashland, USA).

Statistical analysis

The standard curves for piroxicam and 5′-hydroxypiroxicam were analyzed statistical using WinNonlin® 4.0 software (Pharsight Corp., Mountain View, USA). Standard curves of piroxicam and 5′-hydroxypiroxicam were evaluated through linear regression equations and the correlation coefficients (r2) were obtained from peak area ratios (analyte/internal standart) plotted as a function of the respective plasma concentrations. Data are expressed as a mean ± one standard deviation (SD).

Results

The choice of the mobile phase proportion of 70% 0.1 M phosphate buffer (pH 3.2) to 30% acetonitrile showed results consistent and efficient for separating piroxicam and 5′-hydroxypiroxicam (data not shown). The average retention time for piroxicam was ~11 min, 5′-hydroxypiroxicam ~12 min, and the internal standard (naproxen) ~23 minutes.

The heights of each signal peak obtained for each of the standard curve points provided the basis for the equation for piroxicam and 5′-hydroxypiroxicam (Figure 1). After this experiment, the values obtained from the standard mobile phase curve for piroxicam and 5′-hydroxypiroxicam were used to make the standard curves in plasma (Figure 1). The two highest points of the curve (5,000 and 2,500 ng/mL) were saturated and, for this reason, were not considered for analysis in the next steps.

The standard curves were nearly linear in the concentration range evaluated, 10 ng/mL to 2,500 ng/mL (Figure 1). The combination of the HPLC machine and the extraction method used was unable to detected concentrations of piroxicam and 5′-hydroxypiroxicam outside of this range. Within the detectable range, pharmacokinetic analysis of piroxicam using HPLC was an effective method for separating mixtures (Figure 2, Table). For all volunteers, this methodology was effective for detecting piroxicam in plasma samples during all periods after volunteers ingested a single 20 mg tablet. However, 5′-hydroxypiroxicam was only effectively identified in the plasma samples from 2 patients – 5′-hydroxypiroxicam was found in the 72 h sample from patient 6 and in the 48 and 72 h samples from patient 7 (Figure 3).

Table. Pharmacokinetic parameters of piroxicam concentrations in the human plasma of each volunteer.

Volunteer	Tmax (h)	Cmax (ng/mL)	$AUC_{0–72}$ (h*ng/mL)
1	2.0	273	6,827
2	4.0	207	6,756
3	3.0	272	7,267
4	4.0	518	11,316
5	4.0	673	20,809
6	2.0	460	13,400
7	3.0	552	11,377
8	2.0	435	12,769
9	4.0	403	11,082
10	3.0	371	7,245
Average	3.1 ± 0.7	417 ± 111	10,885 ± 3,089

Maximum retention time (Tmax) and maximum concentration (Cmax) observed; respectively; area under the concentration versus time curve (AUC0–72) from the first to the last observed concentration (mean ± SD).

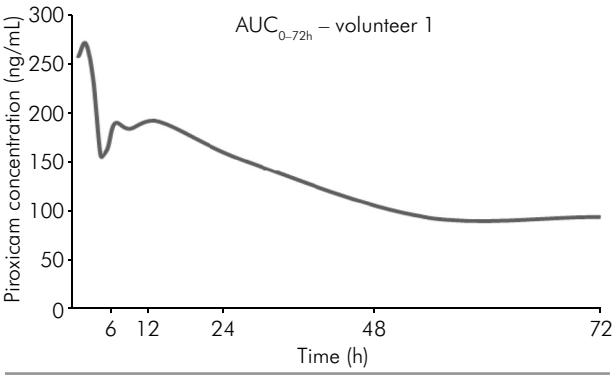

Figure 2. Area under the concentration versus time curve ($AUC_{0–72h}$) of piroxicam (0 to 72 h) from a representative volunteer (volunteer 1).

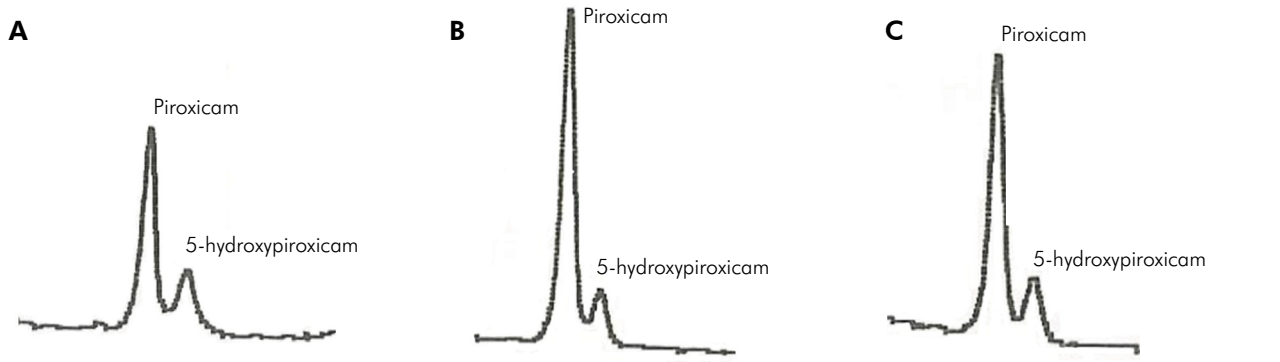

Figure 3. Representative chromatograms of the spectrophotometric signal versus time for piroxicam (retention time ~11 min) and 5-hydroxypiroxicam (retention time ~12 min). (A) chromatogram from the analyte of volunteer 6 at 72 h and (B) 48 h; (C) chromatogram from the analyte of volunteer 7 at 72 hours.

Discussion

Understanding the specific pharmacokinetics of a drug assists in its general understanding and use. In particular, drugs in the blood stream can be in a bound form or free form, which affects its function in the body. More specifically, drugs often bind to albumin, a large protein found in the blood. Only the unbound drug, free form of the drug, can efficiently act on tissues in the body via diapedesis. Commonly, ~99% of a drug may bind to albumin leaving only ~1% able to directly act on tissues outside of the blood vessels.

NSAIDs are widely used throughout the world to treat symptoms present in inflammatory conditions such as fever, pain and edema. This study evaluated piroxicam, a NSAID widely used for tooth extractions due to its efficient performance. That is, a single 20 mg dose of piroxicam can be taken orally only once a day to effectively manage pain, inflammation and swelling resulting from third molar extraction.[4,5,6] Moreover, piroxicam is metabolized by CYP2C9, a cytochrome P450 enzyme, creating 5′-hydroxypiroxicam by hydrolysis. This study's objective was to evaluate different concentrations of piroxicam and its major metabolite, 5′-hydroxypiroxicam, in human plasma samples over time using HPLC after liquid-liquid extraction.

The extraction methods used in this pilot study allowed efficient quantification of piroxicam in plasma samples with HPLC, but was insufficient for efficiently quantifying 5′-hydroxypiroxicam under the same conditions. Moreover, the sensitivity of HPLC for detecting and separating piroxicam and 5′-hydroxypiroxicam depends on the drug concentrations in the blood samples. In particular, the HPLC machine used was unable to detect 5′-hydroxypiroxicam concentrations in all plasma samples, and it is possible that the amount of 5′-hydroxypiroxicam was outside of the limits for the HPLC machine used (approximately 50 to 10,000 ng/mL). However, this HPLC machine's detection limits for piroxicam correspond to limits previously reported. In essence, proper 5′-hydroxypiroxicam identification may require higher volumes of plasma samples, other extractions techniques or devices with greater efficiency and sensitivity for identifying smaller metabolite concentrations. Mass spectrometry (LC-MS / MS) could potentially meet these needs and, thus, effectively quantify 5′-hydroxypiroxicam in plasma samples.[13]

Conclusion

In this study, the main objective was to test an extraction methodology for piroxicam and 5′-hydroxypiroxicam in human plasma samples, widely used in pharmacokinetic analyses. The method of liquid-liquid extraction of the plasma samples for HPLC analysis of piroxicam was effective for the lower limit of detection and quantification of this drug. In relation to piroxicam's major metabolite, 5′-hydroxypiroxicam, this approach was ineffective. A different analysis method such as mass spectrometry may be needed to accurately quantify 5′-hydroxypiroxicam.

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