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Pharmacogenetic and Pharmacokinetic Assays from Saliva Samples Can Guarantee Personalized Drug Prescription

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Saliva is widely used for clinical and laboratory analysis. This study proposed to use DNA extracted from saliva for genotyping and pharmacokinetics of piroxicam. A fast and efficient genotyping method was used to determine relevant allelic variants of CYP2C9 (**2 and *3*), since genetic factors can influence in non-steroidal anti-inflammatory drugs (NSAIDs) metabolization. DNA Extract All Reagents Kit® was used for DNA extraction and genotyping was performed using TaqMan® GTXpress™ Master Mix, SNP genotyping assays and a Viia7 Real-Time PCR system. Volunteers performed sequential collections of saliva samples before and after taking a single dose of piroxicam (0.25 to 72 h) which were used for pharmacokinetics assays. Piroxicam concentrations were analyzed using LC–MS/MS. Sixty-six percent of volunteers were ancestral homozygous (*CYP2C9*1/*1*), and 34% showed one or both polymorphisms. Of these 34%, 22 individuals showed *CYP2C9*2* polymorphism, 8 *CYP2C9*3*, and 4 *CYP2C9*2/*3*. Piroxicam pharmacokinetics were performed in 5 subjects. Areas under the curve (AUC0-t(h*ng/mL)) for *CYP2C9*1/*1, *1/*2 and *1/*3* were, respectively, 194.33±70.93, 166 and 303. Maximum concentrations (Cmax(ng/mL)) for these genotypes were respectively 6.46±2.56, 4.3 and 10.2. Saliva sampling was a very effective matrix for both pharmacogenetic and pharmacokinetic tests, ensuring the speed of the procedure and the well-being and agreement of the participants. Once having the knowledge about the slow and fast metabolizers, it is possible to make an adequate prescription in order to avoid the adverse effects of the medication and to guarantee greater analgesic comfort to the patients respectively.

Key Words: genotyping, DNA, pharmacokinetic, NSAID, saliva.

Introduction

Individualization of drug prescriptions has gained interest in the pharmacological research field due to the need to minimize side effects and has become a reality in several countries. According to a 2001 study conducted in the United States, non-steroidal anti-inflammatory drugs (NSAIDs) accounted for 70 million prescriptions, and 30 billion nonprescription NSAIDs were sold annually (1,2). In addition, according to WHO data and statistics from several countries, some NSAIDs are among the most widely used medicines in the world (3). One category of NSAIDs, enolic acids, is comprised of non-selective COX inhibitors such as piroxicam, which is mostly used to treat pain and inflammation (4,5).

Use of NSAIDs is associated with serious adverse drug reactions in the gastrointestinal, renal, and cardiovascular systems including stomach pain, gastrointestinal bleeding, ulcers, hyperkalemia, acute renal failure, hypernatremia, impaired glomerular filtration rate, and edema (1–4). Although several factors such as age, sex, weight, medical history, lifestyle, and drug interactions may influence individual responses to drugs, genetic factors can also influence drug pharmacokinetics (PK) and pharmacodynamics (PD), thus altering drug toxicity and the efficacy profile (2,4,6).

Due to widespread use of NSAIDs and the associated adverse effects, it is critical to understand individual genetic variations and promote individualized dosing (2,4). However, personalization of drug therapy would likely not be universally adopted due to the costs of pharmacogenetic testing (4). Methodologies that minimize cost, time, and difficulty of pharmacogenetic testing prior to prescription of medication have been widely studied.

The effects of genetic polymorphisms are described for most PK and PD parameters of drugs (2,4). *CYP2C9*, a cytochrome P450 enzyme, metabolizes approximately 15% of commercially available drugs, including most NSAIDs such as piroxicam, and is highly polymorphic, showing 56 allelic variants and several characterized subunits (2,4,7,8). In this study, we evaluated *CYP2C9*2* (430C → T, rs1799853) and *CYP2C9*3* (1075A → C, rs1057910), with DNA extracted from saliva, which are present at varying frequencies in different ethnic groups (2).

In this study, we genotyped volunteers for the

clinically relevant *CYP2C9* polymorphisms, *CYP2C9*2* and *CYP2C9*3*, using a rapid and efficient method, and performed pharmacokinetic analysis of piroxicam in five individuals who agreed to participate in the experiment. Saliva was chosen as a source of DNA and as the sample for pharmacokinetics analysis for a number of reasons including ease of collection, low cost, non-invasive and painless nature and low risk of infection (5,9–12). Therefore, the hypothesis of the present study is that saliva can be reliably used for pharmacogenetic and pharmacokinetic studies. In addition, the present work idea is to establish this relationship between the polymorphism in *CYP2C9* and the agility in NSAIDs metabolization, with saliva as starting material. A personalized prescription could be used so that fast metabolizers have the desired efficacy in controlling pain by adjusting the medication dose if necessary and slow metabolizers presenting less adverse effects with the usual doses that sometimes need to be reduced for this population.

Material and Methods

All laboratory procedures were performed at the Laboratory of Pharmacology and Genetics of the Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo (FOB / USP), Brazil.

Subjects

One hundred volunteers aged 18-60 were selected by the Laboratory of Pharmacology and Clinical Physiology (LAFFIC- FOB / USP). From this pool, the first five participants underwent pharmacokinetic analysis for piroxicam. All study participants signed the "Free and Informed Consent Term," approved by the Human Research Ethics Committee (CAAE numbers: 20657913.7.0000.5417 and 88326918.3.0000.5417, ClinicalTrials.gov NCT02450487, ReBEC RBR-6qgc6m), and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Criteria used to select participants for this study were good general health, absence of infection or inflammation, and lack of systemic diseases. These parameters were determined through an oral questionnaire administered prior to reading and signing the "Free and Informed Consent Form". Exclusion criteria were pregnancy or lactation; existence of systemic diseases; inflammation or ongoing infection; history of gastrointestinal bleeding or ulceration; presence of cardiovascular, renal, or hepatic diseases; use of antidepressants, diuretics, or anticoagulants; and history of allergy to NSAIDs.

Saliva Samples Collection for Genotyping

After receiving verbal instructions, study participants received a 50 mL falcon tube, to which they added 4 mL of saliva. To obtain the largest possible number of cells in saliva, participants were instructed to rub their tongues against the buccal mucosa for a few seconds prior to saliva collection. Samples were initially stored on ice. Saliva was equally distributed to sterile 2 mL microcentrifuge tubes and immediately stored in a freezer at -20 °C until DNA extraction. Saliva samples were thawed in a refrigerator at 4 °C, homogenized using a shaker (Quimis Aparelhos Científicos Ltda., Diadema, SP, Brazil) and centrifuged at 10,000 rpm at 4 °C for 5 min (High-speed Micro Centrifuge CF16RN, Himac, Hitachi Koki Co., Ltd., Japan). The supernatant was removed and the pellet was retained.

DNA Extraction

DNA Extract All Reagents Kit (catalog number 4402616, Applied Biosystems®, Foster City, California, United States) was used for DNA extraction according to the manufacturer's instructions. The extraction procedure summarized in Figure 1 was carried out as follows: I - The pellet was eluted in 200 µL of phosphate buffered saline (PBS 1X) and vortexed using a shaker. II - Three µL of each sample were pipetted into 96-well plates. III - Twenty microliters of the cell lysis solution were added to each well, resulting in cell and nuclear lysis, and the plates were

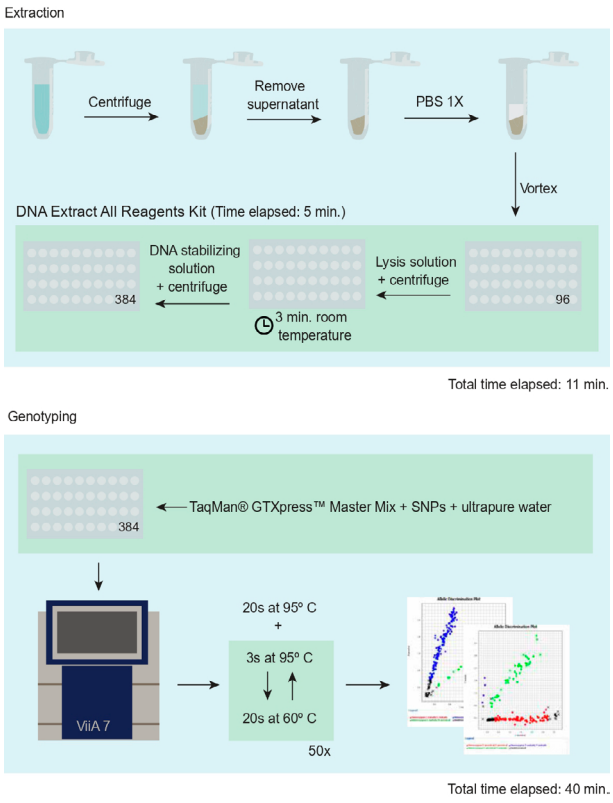

Figure 1. Flowchart representing the time spent in the steps of DNA extraction with the DNA Extract All and the sequencing using Taqman® GTXpress™ Master Mix (Applied Biosystem®) in Viia 7

centrifuged at 2,000 rpm at 18 °C for 1 min. IV - After centrifugation, plates were kept at 23 °C for 3 min and 20 µL of DNA stabilization solution was added to each well. Plates were centrifuged at 2,000 rpm at 18 °C for 1 min.

CYP2C9 Genotyping

To detect the different *CYP2C9* genotypes the Taqman® GTXpress™ Master Mix was used and DNA were analyzed using a Viia 7 Real-Time PCR (Applied Biosystems®) system. Assays were validated by Applied Biosystems®. The experiment resulted in three conditions: I - Only DNA fragments labeled with the ancestral probe were amplified, which indicated that the individual was an ancestral homozygous for the gene, showing that the gene had not mutated. II – Only DNA fragments labeled with the polymorphism probe were amplified, which indicated a mutated homozygous gene. III - DNA fragments labeled with both probes were amplified, which indicated a heterozygous genotype due to the presence of the polymorphism on only one allele.

The procedure described above was used to prepare two 384 well plates (catalog number 4343370, Applied Biosystems®) using the following kit: TaqMan® GTXpress™ Master Mix (catalog number 4401892, Applied Biosystems®) containing Taq polymerase, magnesium chloride, and nitrogenous bases, which are necessary for amplification. A premanufactured and validated assay kit containing primers and probes required for each target polymorphism was also used. In this study, primers and probes corresponded to *CYP2C9*2* rs1799853 (430C → T) and *CYP2C9*3* rs1057910 (1075A → C) (catalog numbers C_25625805_10 and C_27104892_10, respectively).

After preparing each sample in duplicate, two sets of 384-well plates containing SNPs of one of the polymorphisms were analyzed using a Viia 7 Real-Time PCR system. Cycling conditions were as follows: the initial temperature of 95 °C was held for 20 s to allow for activation of Taq polymerase, following 50 cycles at 95 °C for 3 s each. Samples were then held at 60 °C for 20 s. The process is illustrated in Figure 1.

Pharmacokinetics of Piroxicam

All the participants were invited to participate in a pharmacokinetic study using saliva samples following ingestion of piroxicam, but only five participants (26,6 ± 7,09 y) agreed. These participants had not taken any NSAIDs in the month prior to the study. Saliva was collected from participants immediately before the administration of a single 20 mg dose of piroxicam and during the next 72 hours following a preset scheme (0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 11, 24, 48, and 72 h). Piroxicam concentrations measured in saliva at each collection time were plotted.

The methodology used in this study was validated and published by our research group and is summarized below, in accordance of U.S. Guidance for Industry: Bioanalytical Method Validation (5). Piroxicam was analyzed using LC–MS/MS following separation using a LiChroCART 125-4 RP-select B Sorbent C18 (LiChrospher®, Merck, Darmstadt, Germany; 205 × 4.6 mm, particle size 5 µm) maintained at 25 °C. The mobile phase consisted of methanol with 2% phosphoric acid (pH 2.7) (70:30, v/v) and flow rate was set at 1 mL/min. Total analytical run time was 4 min. The eluate was analyzed using a Quattro Micro LC triple quadrupole mass spectrometer (MS/MS) (Micromass UK Ltda., Manchester, UK). Results were analyzed using WinNonlin 4.0 software (Pharsight Corp., Mountain View, CA, USA) and non-compartmental modeling with first order elimination (5).

Results

DNA from all saliva samples were effectively extracted and genotyped. Results obtained from 100 participants (57 women and 43 men, 18 to 60 years of age) are described in detail in Table 1. None of the participants were mutated homozygous for *CYP2C9*3*, and no individuals with both mutations (*CYP2C9*2* and *CYP2C9*3*) were homozygous. Sixty-six percent of participants did not show the *CYP2C9* gene polymorphisms investigated in this study *(*2 and *3)*. These participants were ancestral homozygotes (*CYP2C9**1/*1). Thirty-four participants presented one or both of the polymorphisms evaluated in this study. Of these 34 participants, 22 showed the polymorphism *CYP2C9*2* (20 participants were heterozygous (*CYP2C9**1/*2) and 2 were homozygous (*CYP2C9*2*/*2)), 8 showed the *CYP2C9*3* polymorphism, of whom all were heterozygous (*CYP2C9**1/*3), and 4 were heterozygous for both polymorphisms (*CYP2C9*2*/*3). Subjects who underwent pharmacokinetic analysis were comprised of 3 women and 2 men, aged 22 to 39. Of these 5 participants, 3 were ancestral homozygotes (*CYP2C9**1/*1) and two were heterozygous (one *CYP2C9**1/*2 and one *CYP2C9**1/*3).

Pharmacokinetic parameters are expressed as a function of *CYP2C9* polymorphisms in Figure 2 and Table 2, obtained

Table 1. Polymorphisms of CYP2C9 in study participants (n=100)

CYP2C9	%
CYP2C9 *1/*1	66
CYP2C9 *1/*2	20
CYP2C9 *2/*2	2
CYP2C9 *1/*3	8
CYP2C9 *3/*3	0
CYP2C9*2/*3	4

from piroxicam concentration in saliva samples determined by LC / MS and analyzed using by the WinNonlin 4.0 software (Pharsight Corp.). Although there was no intention to statistically analyze the 5 volunteers' pharmacokinetic results who joined the research, it is possible to observe the differences in the area under the piroxicam concentration curve, in clearance and in saliva piroxicam half-life. These data confirm the hypothesis of the present study since saliva can be reliably used for pharmacogenetic and pharmacokinetic studies.

Table 2. Pharmacokinetic data of piroxicam relative to the CYP2C9 alleles (*1, *2 and *3) of the participants (*CYP2C9 *1 / *1* (n=3), *1 / *2* (n=1), and *1 / *3* (n=1)), provided by the WinNonlin 4.0 software from LC/MS analysis concentrations of saliva samples

PIROXICAM	CYP2C9		
	*1 / *1 (n=3) Mean±SD	*1 / *2 (n=1)	*1 / *3 (n=1)
T_{max} (h)	3.33±1.52	11	2
C_{max} (ng/mL)	6.46±2.56	4.3	10.2
AUC_{0-t} (h*ng/mL)	194.33± 70.93	166	303
AUC_{INF} (hr*ng/mL)	327±186.83	261	497
Vd/F (L)	4507.66±1259.47	5132	2112
Clt/F (L/h)	69.66±30.36	74	31
Kel (1/h)	0.0153±0.0047	0.0146	0.0149
$t_{1/2}$ (h)	48.66±17.21	47	46

Tmax: maximum time observed; Cmax: maximum concentration observed; AUC0-t: area under the curve from zero to the last quantifiable time; AUC_{INF}: area under the curve from zero to the last quantifiable concentration; Vd/F: estimated distribution volume in the total AUC; Clt/F: total clearance; Kel: constant of the elimination rate estimated from the regression line representing the terminal phase of the concentration-time profile; $t_{1/2:}$ terminal half-life of the drug for saliva samples.

Discussion

Use of saliva as a matrix for both genotyping and pharmacodynamic experiments was critical to this study. Pharmacogenetic, pharmacokinetic and pharmacodynamic studies using saliva samples instead of other biological matrices are increasingly found in the literature due to various intrinsic characteristics of this compound and its collection (13).

Part of the components found in blood may also be present in saliva. This makes its use possible for pharmacogenetic testing, since there are several cell types immersed in saliva, including desquamated epithelial cells and leukocytes, that can be used as a source of DNA (10,12,14,15). In addition, various drug concentrations can also be measured in saliva, depending on molecular weight, liposolubility, pH, among other characteristics (15).

Use of saliva as a matrix is not always possible, since there should be a correlation between drug concentrations present in saliva and those present in blood, as well as a relationship of this concentration to the proposed

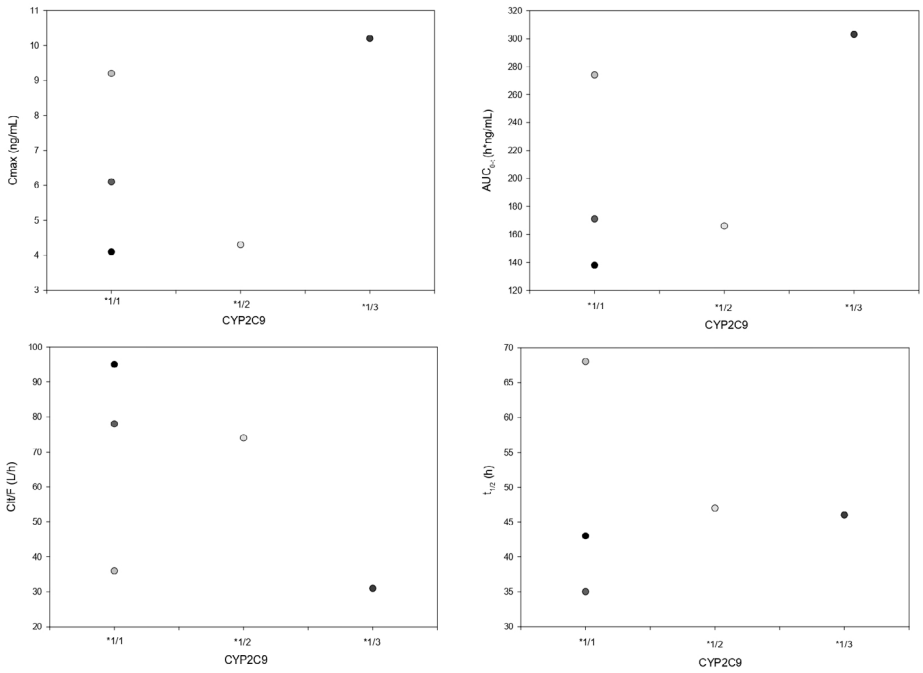

Figure 2. Pharmacokinetic parameters of piroxicam (Cmax: maximum concentration observed, AUC0-t: area under the curve from zero to the last quantifiable time, Clt/F: total clearance; t1/2: terminal half-life of the drug) for saliva samples relative to the CYP2C9 alleles (*1, *2 and *3) of the volunteers. CYP2C9 *1 / *1 (n=3), *1 / *2 (n=1), and *1 / *3 (n=1).

analyzes (14). However, when it is possible to use saliva for pharmacokinetic and pharmacodynamic experiments, this compound can prove to be an asset. In addition to being a cheaper method, with low technical costs for collection and storage, saliva collection is rapid, painless, with lower risks of infection and an ease to patient compliance (5,14,16). These characteristics become the use of saliva, especially in relation to blood samples, advantageous not only to ensure no damage or to facilitate the acceptance of the protocol, but also to therapeutic drug monitoring in patients, especially in susceptible patients such as children, cancer patients and patients with severe infections, ensuring the safest prescription, the best dosage and the lowest risk of adverse reactions (13–15,17–19).

Despite the benefits of saliva in relation to blood collection for participant acceptance, in our study we found difficulties in adherence in the second phase, pharmacokinetics analyses, due to the frequency of saliva collection, which discouraged many participants. Our research group is working on developing protocols that promote greater adherence.

The correlation between plasma and saliva was considered satisfactory for the analysis of NSAID pharmacokinetic parameters in a previous study conducted by our research group and in international literature (5). The pharmacokinetic experiment was performed using the saliva samples of 5 participants. Despite the reduced number of participants, it is possible to observe the differences in the area under the curve, in clearance and in half-life. Pharmacokinetic studies with a greater number of heterozygous and homozygous individuals for the studied variants (*CYP2C9*2* and *CYP2C9*3*) should be performed to confirm this trend.

Therefore, saliva sampling ensured effectiveness for the procedures, since it was rapid and noninvasive compared to methods such as blood sampling (5). The DNA Extract All Reagents Kit allowed for effective genotyping because it required fewer steps than other methods, allowing for completion of the first phase of the experiment in 2h at a lower cost (12). Pharmacokinetic methods validated by Calvo et al (5) through comparison with blood samples were also efficient in our study.

The present study hypothesis was confirmed once saliva sampling was a very effective matrix for both pharmacogenetic and pharmacokinetic tests, ensuring the speed of the procedure and the well-being and agreement of the participants. With the development of methodologies that reduce cost, facilitate and speed genotyping, pharmacogenetic and pharmacokinetic, these methodologies may become more available to health professionals, allowing for individualized prescriptions for patients. Once establishing the relationship of the mutation in the *CYP2C9* gene and the rate at which NSAIDs are metabolized, only with a simple molecular test, using saliva as the starting material, it will be possible to prescribe individually, thus ensuring analgesic efficacy for fast metabolizers and less adverse effects for slow metabolizers.

Resumo

Saliva é amplamente utilizada para análises clínicas e laboratoriais. Este estudo propôs o uso de DNA extraído da saliva para genotipagem e farmacocinética do piroxicam. Um método de genotipagem rápido e eficiente foi usado para determinar as variantes alélicas clinicamente relevantes de *CYP2C9* (* 2 e * 3), uma vez que fatores genéticos podem influenciar nas respostas metabólicas individuais a medicamentos como anti-inflamatórios não esteroides (AINEs). DNA Extract All Reagents Kit® foi usado para extração de DNA e a genotipagem foi realizada usando TaqMan® GTXpress ™ Master Mix, ensaios de genotipagem SNP e um sistema Viia7 Real-Time PCR. Os voluntários realizaram coletas sequenciais de amostras de saliva antes e após a ingestão de uma única dose de piroxicam (0,25 a 72 h) que foram utilizadas para ensaios farmacocinéticos. As concentrações de piroxicam foram analisadas usando LC – MS / MS. Sessenta e seis por cento dos voluntários eram homozigotos ancestrais (*CYP2C9* * 1 / * 1) e 34% apresentaram um ou ambos os polimorfismos. Destes 34%, 22 indivíduos apresentaram polimorfismo *CYP2C9* * 2, 8 *CYP2C9* * 3 e 4 *CYP2C9* * 2 / * 3. A farmacocinética do piroxicam foi realizada em 5 indivíduos. As áreas sob a curva (AUC0-t (h * ng / mL)) para *CYP2C9* * 1 / * 1, * 1 / * 2 e * 1 / * 3 foram, respectivamente, 194,33±70,93, 166 e 303. Concentrações máximas (Cmax (ng / mL)) para esses genótipos foram, respectivamente, 6,46±2,56, 4,3 e 10,2. A amostra de saliva foi uma matriz muito eficaz tanto para os testes farmacogenéticos quanto para os farmacocinéticos, garantindo a agilidade do procedimento e o bem-estar e concordância dos participantes. Com o conhecimento dos metabolizadores lentos e rápidos, é possível fazer uma prescrição adequada para evitar os efeitos adversos da medicação e garantir maior conforto analgésico aos pacientes respectivamente.

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