SALIVA AS A NONINVASIVE BIOLOGICAL SAMPLE TO COMPARE BIOAVAILABILITY OF PHENYTOIN FORMULATIONS BY LC-MS/MS

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ABSTRACT: The use of noninvasive biological samples such as saliva, it is of great interest for therapeutic drug monitoring (TDM), including the anticonvulsants Phenytoin (PHT). A simple analytical methodology by liquid chromatography-tandem mass spectrometry was developed and validated to carry out a relative comparative bioavailability (RBA) study of two PHT formulations in human plasma and saliva and the subsequent correlation between both matrices. A single-dose, randomized-sequence, open-label, two-way crossover study, was conducted in 24 healthy Latin American male volunteers. The bioequivalence of 200 mg PHT tablet was evaluated using plasma and saliva to determine C max, T max, AUC 0→∞, and AUC 0→∞. Figures of merit of the proposed methodology were as follows: linear ranges of 40-5055 ng/mL and 5-1340 ng/mL for plasma and saliva, respectively, with a correlation coefficient of (r)>0.999. The LOD and LOQ were 15 ng/mL -40ng/mL for plasma and 1.5 ng/mL - 5.0 ng/mL for saliva. Accuracy, precision (as %CV) and recovery were accepted according the bioequivalence criteria. Stability showed %bias<15%. The PHT saliva/PHT plasma ratios for all parameters were: 5.4, 2.2, 1.4 for C max, and 1.7, 1.2, 1.1 for AUC. Plasma and saliva results were correlated (R=0.9889 and R=0.9947 for Reference and Test, respectively).

INTRODUCTION: Epilepsy is one of the most serious disorders of the central nervous system. It affects over 50 million people worldwide and it may cause persistent deformity and a decrease in quality of life.

The drug 5,5-diphenyl-imidazoline-2,4-dione, commonly known as phenytoin (PHT), is one of the drugs most widely used as anticonvulsant to treat many of the epileptic seizure types and it is usually given in daily oral doses of 200 to 600 mg. PHT is highly bound to serum albumin protein and is excreted almost completely in bile as inactive metabolites, which is then reabsorbed from the intestinal tract and excreted in the urine.

In the last 30 years, the use of saliva for therapeutic drug monitoring (TDM), including anticonvulsants such as PHT, has gained considerable interest.

Keywords: Phenytoin; Saliva; Bioequivalence; LC-MS/MS; TDM; Pharmacokinetics.
This oral fluid can be easily obtained without patient discomfort, and in most used anticonvulsant drugs it has shown relationships between saliva and blood concentrations. Saliva contains only unbound drug which is able to interact with the intracellular receptor sites and to exert effect.

Thus, a correlation between PHT data in saliva and plasma would be very useful for comparative bioavailability (RBA) studies. Moreover, TDM of anticonvulsant received early attention due to its pharmacokinetics and pharmacodynamics properties and several recent developments have provided renewed impetus for the use of this biological fluid in the TDM.

Different analytical methods to determine drug concentrations in biological samples include reverse-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection or tandem mass spectrometry (MS/MS). Therefore, the aims of the present study were: i) to develop and validate a simple method by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for PHT determination in human plasma and saliva, ii) to determine correlations between plasma and saliva PHT concentrations in the same health volunteers and its potential application in RBA studies and TDM.

**MATERIALS AND METHODS:**

**Chemicals and Reagents:**

PHT and the internal standard (IS) clonazepam (CZP) were supplied by a pharmaceutical company and stored in a vacuum desiccator. Acetonitrile (ACN) HPLC grade and ammonium acetate for analysis (ACS, Reag. Ph. Eur.) were from Carlo Erba Reagents and Merck, respectively. Ultrapure water HPLC grade was from Tedia (Fairfield, USA). Blank human saliva was obtained from healthy, drug-free volunteers. The test and reference formulations contained 100mg of PHT were supplied by the pharmaceutical company.

**LC-MS/MS conditions:**

The detection was performed by an Applied Biosystems Sciex (API 3200) triple quadrupole MS/MS with an electrospray (ESI) interface operating in negative and positive mode (switch system), and using the multiple reaction monitoring (MRM) mode (Table 1). Desolvatation temperature (°C), nebulizer gas (psi), desolvatation gas (psi) and entrance potential (V), were set at 500, 50, 40 and -10 (PHT) 10.5 (CZP), respectively. Data were acquired and processed using ANALYST (version 1.5.1; ABSciex, Toronto, Canada).

The high performance liquid chromatography (HPLC) system was a Shimadzu LC-20AD consisting of a binary pump equipped with autosampler SIL-HTc. Chromatographic separation was performed with a Hypersil Gold C\textsubscript{18} column (50 mm x 2.1 mm i.d, 1.9 µm particle size for plasma and 100 mm x 2.1 mm i.d, 3.0 particle size for saliva) maintained at 40 °C.

The two mobile phase components were as follows. Mobile Phase (A):ACN and Mobile Phase (B): 3 mM ammonium acetate. A linear gradient was programmed as 50% mobile phase A, it was held isocratically for 0.40 min increasing to 68% mobile phase A at 0.90 min, then it was maintained isocratically to 1.70 min and returning at 1.71 min to 50% mobile phase A to the end of the run. The injection volume, run time and flow rate for plasma analysis were: 20 µL, 4 min and 0.25 mL/min; while for saliva analysis were 50 µL, 3 min and 0.21 mL/min. The autosampler was kept at room temperature.

**TABLE 1: LC-MS/MS CONDITIONS**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ions (m/z)*</th>
<th>Dwell Time (msec)</th>
<th>Collision Energy (V)</th>
<th>Cone Voltage (mV)</th>
<th>Ion Source</th>
<th>Ionization Method</th>
<th>Scan Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHT</td>
<td>251.1</td>
<td>102.0 (Q)</td>
<td>300.0</td>
<td>-30.00</td>
<td>-4500</td>
<td>ESI</td>
<td>Negative</td>
<td>MRM</td>
</tr>
<tr>
<td>CZP (IS)</td>
<td>316.0</td>
<td>269.9</td>
<td>300.0</td>
<td>-24.00</td>
<td>5500</td>
<td>ESI</td>
<td>Positive</td>
<td>MRM</td>
</tr>
</tbody>
</table>

*m/z: mass-charge ratio; Q: quantification transition; C: confirmation transition
Preparations of standards and quality control samples:

Stock solutions of PHT and IS were prepared in ACN-water (50:50, v/v) at concentrations of 1 mg/mL. An aliquot of PHT stock solution was diluted with ACN to obtain the working solutions (WS) and it were prepared fresh daily. Calibration curves of PHT were prepared by spiking blank human plasma and saliva in a concentration range of 40–5050 ng/mL and 5–1340 ng/mL, respectively. Blank human plasma and saliva were obtained from healthy, drug-free volunteers. Each concentration was analyzed in triplicate. Plasma quality control (QC) samples at 101.6, 1016.1 and 4549.7 ng/mL were prepared using WS to blank human plasma. Saliva QC samples at 13.4, 446.7 and 893.3 ng/mL were prepared using WS to blank human saliva. Five QC samples for each level (low QC (LQC), medium QC (MQC) and high QC (HQC)) were analyzed on each validation day (n = 3).

The spiked plasma and saliva samples (calibration points and QC) were extracted in each analytical batch along with the unknown samples.

Sample Preparation:

Samples and QC were thawed at room temperature on the day of analysis. Plasma: Aliquots of 300 μL of sample or QC were mixed with 100 μL 2.61 μg/mL WS-IS (final concentration of 870 ng/mL in human plasma) into a 2 mL centrifuge tube. A 500μL ACN aliquot was added in order to induce protein precipitation. Samples were homogenized by vortex agitation and further centrifuged at 3000 x g for 5 min. A 500 μL aliquot of the supernatant was transferred to an auto sampler vial, diluted with 500 μL of HPLC water, and injected into a LC-MS/MS system for analysis as described above.

Analytical method validation:

The method was validated by determining the following parameters: specificity, linearity, working range, recovery, accuracy, precision, limit of quantification (LOQ) and stability studies, according to the regulations for validation of bioanalytical methods. Volunteers:

Twenty four healthy Latin American male volunteers participated in this study, which was conducted at the Biopharmaceutical Research Center Dominguez Lab. The volunteers were between 18 – 40 years old (25.3 ± 6.9 years), their body weights were between 65 – 90 kg (78.1 ± 7.4 kg) and their heights were between 161 – 186 cm (176.0 ± 0.1 cm). All volunteers were selected after being screened by physical examination and clinical laboratory tests, including renal and liver functions and routine blood. Volunteers with a history of drug allergies, renal or hepatic impairment, or drug or alcohol abuse were excluded. The consumption of alcohol or beverages and food containing xanthines was not permitted for the volunteers 48 h prior to the study and after drug administration, until the last blood sample was collected in the respective study phase. Volunteers were instructed to abstain from taking any medication for at least 2 weeks prior to and during the study period.

The informed consent was approved by the Ethic Committee and was obtained from the subjects after explaining the nature and purpose of the study which was performed according to the principles of the Declaration of Helsinki and was previously approved by the Public Health Institute of the country for which the study was conducted.

Study Design:

Drug administration:

A relative bioavailability (RBA) study of two PHT Sodium 200 mg tablets (2 x 100 mg tablets) was performed under fasting conditions in a randomized, open-label, two-period crossover design with a 20-day washout interval between doses. The volunteers were confined to the center.
20 h before drug administration and for 24 h after administration. The volunteers were continuously monitored and under medical supervision throughout the confinement period of the study.

**Sample collection:**
During the first day of each period of the study, fasted volunteers received a single oral 200 mg dose of PHT (test or reference product, according to the group assigned) at 08:00 in the morning, with 240 mL of water. Meals were standardized and programmed to be taken at 12:00–16:00–20:00 clock h during the first day of the study and at 08:00 clock h the next day. Blood and saliva samples were obtained at the following intervals: 0 (pre-dose), 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16, 24, 46, 72 and 96 h post-dosing. Blood samples were collected in to heparin zed polypropylene 8 mL tubes (NAHEP PLH 13X75 4.0 PLBL GN, BD Vacutainer®, Broken Bow NE 68822, 20-daywashout US). Plasma was separated by centrifugation at 3000 x g for 10 minutes at room temperature (20°C).

It was then transferred into 2 mL polypropylene tubes and frozen at -20°C until assayed. Unstimulated saliva samples were collected in glass jars and immediately transferred into labeled centrifuge tubes and frozen at -20°C until assayed. After a 20-day washout period, the study was repeated under the same conditions to complete the cross-over design. No adverse reactions were reported by any volunteer during the study.

**Pharmacokinetics and statistical analysis:**
The following pharmacokinetic (PK) parameters were calculated using a validated PK software, Win Nonlin version 6.02 (Pharsight Corp. Mountain View, CA): area under the curve from time zero to the last measurable PHT concentration in plasma or saliva (AUC∞), using the linear trapezoidal rule; (AUC0–∞), calculated as the sum of AUC0–t plus Clast/ke (where Clast is the last measurable plasma or saliva concentration, and ke is the terminal rate constant of elimination); maximum measured concentration of PHT in plasma and saliva (Cmax); time to maximum plasma or saliva concentration (Tmax); and terminal elimination half-life (T1/2). After logarithmic transformation, AUC0–∞, AUC0–t and Cmax values were subjected to analysis of variance (ANOVA). The bioequivalence between the two formulations was evaluated based on the 90% CI transformed back for the geometric mean ratios of AUC0–∞, AUC0–t and Cmax, which were within acceptance range of 80-125% according to the local and international guidelines 21, 22, 23, 24.

**RESULTS AND DISCUSSION:**
**Analytical performance and method validation:**
The proposed analytical methods were evaluated in terms of linearity, recovery, accuracy and precision, specificity and study stability. The unweighted calibration curves were linear in the ranges of 40-5055 ng/mL and 5-1340 ng/mL for plasma and saliva, respectively; with a correlation coefficient values (r) > 0.999 for both type of samples. The retention times in plasma for PHT and IS were 1.72 min and 1.90 min, respectively, whereas in saliva the retention times were 2.27 min and 2.54 min, respectively. Specificity was assessed in six different batches of plasma and saliva samples by analyzing blanks and spiked samples at LOQ levels. No significant chromatographic signals of endogenous PHT were observed neither in plasma nor saliva batches at the target analytes retention times (PHT and IS). The limits of detection (LOD) for the analytical methodology were 15 ng/mL and 1.5 ng/mL for plasma and saliva, respectively. The LOQ were 40 ng/mL and 5 ng/mL for plasma and saliva, respectively and was defined as the lowest concentration in the calibration curve that can be measured with acceptable accuracy and precision17, 18, 19.

The criteria to evaluate accuracy and precision was as follows: mean concentration value should be within 15% of the spiked value, excepting LOQ, where it should not deviate by more than 20%17, 18, 19. Table 2 shows accuracy and precision intra-day and inter-days values. They were obtained by analyzing three batches (LQC, MQC and HQC).

The recovery test was carried out by comparison of analytical response of samples spiked before starting sample preparation, against analytical response of samples spiked just before instrumental analysis. The recovery values achieved at different spiked PHT levels were within the range of 70-80% and 95-105% for plasma and saliva, respectively.
Table 3 shows the stability of PHT in plasma and saliva. It was carried out at three QC levels (LQC, MQC and HQC) under the following conditions: auto sampler stability (samples in the injector at room temperature); freeze-thaw stability (after 3 cycles of freezing and thawing the enriched samples); and long term stability (aliquots of each sample). The tested product mainly, prob (test and reference products) in plasma and saliva. Pharmacokinetics parameters for both formulations were significantly correlated (Fig 3A-B). On the other hand, an in vivo dissolution test was performed in oral cavity to demonstrate that concentration discrepancies of saliva (at earlier stages of the study) were coming from residues of active ingredients in the mouth and not from the bloodstream. This test was conducted like the bioequivalence study, with the exception that the volunteers (n = 3) took the medication and kept the pill in the mouth for 1-3 s, and then they spat it. The results showed extremely high concentration levels for 4 h with the test product comparing with the reference product (data not shown). Cawello et al. have recently reported the same problem with syrups formulations. Moreover, according to the prospectus the test...
product contains an excipient sodium starch glycolate, which is used as a disintegrant in prompt-release drug \cite{27,28}, and probably this difference could explain the high values reached in saliva samples at the beginning of the study. In this sense, the test product could be disintegrated in the mouth faster than the reference and it could also be adhered to the mouth, thus preventing its entry to the blood stream. After several mouthwashes, the discordant values disappeared. Although the amount of drug that did not enter the body had no therapeutic consequences, this fact had a great analytical impact.

Therefore, the use of saliva as noninvasive alternative for bioequivalence studies should be carried out with some previous considerations for prompt release drugs. Our study also highlights the importance of the protocol design (mainly in the first sampling points) to ensure a valid result in the study of comparative bioavailability. Results from the RBA studies in plasma and saliva, showed that both products are not interchangeable (Table 5). Dominguez Lab adopted a normal-release medication for the study as reference product, while the test product was a prompt release drug, according to the legislation of the Public Health Institute.

It could be an explanation for the nonequivalence between the two products in plasma and in saliva. A similar outcome was observed for PHT in plasma during a bioavailability study with a prompt-release and an extended-release preparation\cite{29}. Based on these results, the prompt-release drug (test product) was adopted as an innovator product by the Public Health Institute of the country. In contrast, Ruiz et al.\cite{30} reported bioequivalence between PHT products in saliva. The differences in findings could be due to their different and less sensitive detection technology used for the comparison between prompt-release products (test and reference).

In addition, the PHT concentrations in saliva showed a good correlation with the PHT concentrations in plasma (approximately 10% of plasmatic values, consistent with the literature) and the PHT fraction in saliva corresponded with the unbound-protein fraction\cite{3,9}. This point emphasizes the usefulness of the analytical methodology herein presented as a tool for MTD in narrow therapeutic range drugs.

FIG.1: COMPARATIVE CURVES OF THE REFERENCE AND TEST PRODUCTS, PLOTTED WITH THE MEAN VALUES (NOT LOG TRANSFORMED) vs. TIME AFTER A SINGLE 200 mg ORAL DOSE ADMINISTRATION TO 24 HEALTHY VOLUNTEERS. A) TIME PROFILE OF PHT IN PLASMA. B) TIME PROFILE OF PHT IN SALIVA.
FIG. 2: COMPARATIVE CURVES OF THE REFERENCE (R) AND TEST (T) PRODUCTS, PLOTTED WITH THE MEAN VALUES (NOT LOG TRANSFORMED) vs. TIME (EXCLUDING THE TIMES BEFORE 4 H) AFTER A SINGLE 200 mg ORAL DOSE ADMINISTRATION OF PHT TO 24 HEALTHY VOLUNTEERS IN SALIVA.

FIG. 3: PHT CORRELATION CURVES BETWEEN SALIVA AND PLASMA MATRIX (ng/mL). A) REFERENCE AND B) TEST. NOTE: DATA WERE OBTAINED FROM A COMPARATIVE BIOAVAILABILITY STUDY.

TABLE 4: PHARMACOKINETIC PARAMETERS FOR PHT OF REFERENCE AND TEST PRODUCTS IN PLASMA AND SALIVA (EXCLUDING THE TIMES BEFORE 4 H), AFTER SINGLE ORAL ADMINISTRATION OF A 200 mg TABLET (MEAN ± S.D.; N = 24 VOLUNTEERS).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference</th>
<th>Test</th>
<th>Reference</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-t}$ (ng h/mL)</td>
<td>79429 ± 23506</td>
<td>79430 ± 22999</td>
<td>7899 ± 2793</td>
<td>8612 ± 2513</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng h/mL)</td>
<td>76391 ± 24110</td>
<td>81182 ± 24963</td>
<td>8242 ± 3049</td>
<td>8822 ± 2682</td>
</tr>
<tr>
<td>C$_{\text{max}}$ (ng/mL)</td>
<td>1997 ± 523</td>
<td>2600 ± 638</td>
<td>263 ± 100</td>
<td>377 ± 205</td>
</tr>
<tr>
<td>T$_{\text{max}}$ (h)</td>
<td>12.09 ± 7.74</td>
<td>5.91 ± 4.94</td>
<td>11.38 ± 6.75</td>
<td>7.37 ± 3.34</td>
</tr>
<tr>
<td>k$_{e}$ (1/h)</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>T$_{1/2}$ (h)</td>
<td>14.03 ± 4.60</td>
<td>13.84 ± 5.12</td>
<td>15.00 ± 5.53</td>
<td>14.40 ± 4.69</td>
</tr>
</tbody>
</table>
TABLE 5: GEOMETRIC MEAN OF THE INDIVIDUAL C_{max}, AUC_{0-4}, AND AUC_{0-∞} RATIOS (TEST/REFERENCE FORMULATION), THE RESPECTIVE 90% CONFIDENCE INTERVALS (CI) AND A COMPARATIVE SALIVA/PLASMA PHT RATIO (n = 24 VOLUNTEERS).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ratio % Ref</th>
<th>90% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA PHT</td>
<td>C_{max}</td>
<td>130.9</td>
</tr>
<tr>
<td>SALIVA PHT (all times)</td>
<td></td>
<td>710.2</td>
</tr>
<tr>
<td>SALIVA PHT (excluding times before 2 h)</td>
<td></td>
<td>284.2</td>
</tr>
<tr>
<td>SALIVA PHT (excluding times before 4 h)</td>
<td></td>
<td>178.9</td>
</tr>
<tr>
<td>PLASMA PHT</td>
<td>AUC_{0-4}</td>
<td>106.3</td>
</tr>
<tr>
<td>SALIVA PHT (all times)</td>
<td></td>
<td>179.0</td>
</tr>
<tr>
<td>SALIVA PHT (excluding times before 2 h)</td>
<td></td>
<td>129.5</td>
</tr>
<tr>
<td>SALIVA PHT (excluding times before 4 h)</td>
<td></td>
<td>113.7</td>
</tr>
<tr>
<td>PLASMA PHT</td>
<td>AUC_{0-∞}</td>
<td>106.2</td>
</tr>
<tr>
<td>SALIVA PHT (all times)</td>
<td></td>
<td>175.5</td>
</tr>
<tr>
<td>SALIVA PHT (excluding times before 2 h)</td>
<td></td>
<td>127.1</td>
</tr>
<tr>
<td>SALIVA PHT (excluding times before 4 h)</td>
<td></td>
<td>111.8</td>
</tr>
</tbody>
</table>

CONCLUSION: An efficient, simple and rapid analytical methodology based on LC-MS/MS analysis to determine PHT level in plasma and saliva was developed and validated according to the international guideline. Correlation between plasma and saliva PHT concentrations was demonstrated. Therefore, saliva not only is a suitable matrix for RBA studies and TDM, but also a more convenient tool since it provides analogous information with major analytical, ethical, and biosafety advantages. However, an additional pre-study of product dissolution in oral cavity is suggested before carrying out complete RBA studies for prompt-release drugs.

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REFERENCES:


