USEFULNESS OF SALIVARY DRUG MONITORING FOR DETECTING EFFLUX TRANSPORTER OVEREXPRESSION

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Running title:
SALIVA FOR DETECTING EFFLUX TRANSPORTER OVEREXPRESSION

ABSTRACT

Venous (V) / artery (A) concentration ratio of a drug could be a reliable index of its clearance (CL) if measurements of plasma concentration were performed during a period of time where the absorption process was not longer operative, then during a pure elimination phase. A novel subrogate using two protocolized saliva samples sequentially collected (first, S1, and second, S2) was designed in order to replace V and A free plasma drug concentrations, respectively. Two drugs, phenytoin (PHT) and carbamazepine (CBZ), which are well-known for their inducer properties and their dose-dependent CL variations, were studied.

A multicentre two-phase collaborative study was done. The first phase was performed with healthy volunteers (single dose) and the second phase was carried out with epileptic patients under CBZ or PHT monotherapy (multiple doses). S1/S2 saliva drug concentration ratio was sensitive enough for detecting systemic CL changes from single to multiple dose administrations, and because of daily dose modifications. Both CBZ and PHT would modify their bioavailability and clearance by inducing efflux transporter throughout chronic treatments. Sex-related pharmacokinetic impacts of this inductive effect were also observed.

Keywords: Saliva drug concentration – Efflux transporter expression – Sex of individuals – Bioavailability – Clearance – Carbamazepine – Phenytoin

INTRODUCTION

Pharmacological management of epilepsy

Antiepileptic drugs (AEDs) such as carbamazepine, phenytoin, valproic acid, ethosuximide, phenobarbital and benzodiazepines were the mainstays of seizure treatment until the 1990s, when newer AEDs emerged and were approved mainly as add-on therapy. Many structures and processes are involved in the development of seizures: ion channels, receptors, inhibitory and excitatory neurotransmitters. AEDs were designed to act at these targets in order to stop or prevent seizure activity.

Most AEDs have more than one mechanism of action. Blockade of voltage-dependent sodium channel is a primary action of phenytoin (PHT), carbamazepine (CBZ), oxcarbamazepine (OXC), lamotrigine (LTG), topiramate (TPM), zonisamide (ZNS), and felbamate (FBM).

As gamma-aminobutyric acid (GABA) is the main brain inhibitory neurotransmitter, many AEDs can act by enhancing GABAergic inhibition, exerting this effect by inhibiting GABA transaminase avoiding GABA degradation (vigabatrin, VGT); by blocking the reuptake of GABA in the synapsis (tiagabine, TGB); by acting on the $GABA_A$ receptor-chloride channel complex (benzodiazepines, BZP; phenobarbital, PHB).

Glutamate is the main excitatory neurotransmitter in the central nervous system and blocking its receptors: N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate is the mechanism shared by some AEDs such as FBM (acting on NMDA receptor) and TPM (acting on AMPA receptor).

Blockade of T-type calcium channels in thalamic neurons is the main mechanism of ethosuximide (ESM) and such action contributes to the efficacy of valproic acid (VPA) in absence seizures as well. Gabapentin (GBP) and pregabalin (PGB) modulate excitatory neurotransmitters release by blocking N-type and P/Q type calcium channels. Retigabine (RTG) may affect potassium currents. Recent data suggest that levetiracetam (LEV) acts modulating SV2A, a synaptic vesicle protein involved in exocytosis.

Carbonic anhydrase inhibitor: acetazolamide has been used in refractory seizures with catamenial pattern. TPM and ZNS are weak inhibitors of this enzyme.

Despite the many advances made in neurobiology and molecular dysfunction of epilepsy and the introduction of new AEDs, there are still patients that do not completely respond to current therapeutic agents. Not only uncontrolled seizures are the cause of refractory epilepsy but also the induction properties of some drugs that can impact overexpressing efflux transporters and consequently diminishing their concentration at the action site. So a pharmacokinetic insight into this topic must be given to understand how drugs themselves are involved in the installment or maintenance of refractory epilepsy.

Plasma drug concentration

Plasma drug concentration is not homogeneous within the intravascular space, while all arteries have the same drug level value, each vein coming from different organs may have different drug concentrations, among them and in relation with its respective artery. This circulatory issue has been well referenced in the literature both in animals [1] and in humans [2], and gives evidence for understanding the discrepancy between plasma venous drug concentrations, which are commonly measured, and drug effects [2,3].

During the input of a drug, arteries have higher plasma drug concentrations than all the veins of the large circulation, except for the vein through which the substance enters the body. So, while drug is entering the body arteries are transporting an amount of substance that exceeds the one previously eliminated. This is repeated after each circulatory cycle until the steady state is reached. At this point, the amount of drug that enters the body is the same as the one eliminated, and the concentrations in veins and arteries become equal.

Once the administration is interrupted, drug decay proceeds from all branches of the circulatory apparatus. After drug input ceases, the veins exhibit higher concentrations than the arteries, except for those veins coming from eliminating organs. This inversion during the elimination phase is because the

blood entering the arteries of the large circulation suffered a dilution caused by the lesser content of solute that veins coming from the eliminatory organs had.

It is important to bear in mind that not only the absorption or elimination of a drug rules the A-V difference in drug concentration but also changes in the distribution of cardiac output [4]. For instance, a great migration of substances outside the vessels takes place during physical activity of individuals, while the situation reverts once subjects stop doing muscular activity.

Clearance

In order to evaluate the impact that depuration of substances from the body could have on the A-V difference, the following reasoning needs to be done. Over the mono-exponential descending part in both artery and vein profiles, a constant value of V/A drug concentration ratio could be assumed. This V/A ratio gives information about the extent of drug clearance. The higher drug clearance is, the higher V/A ratio becomes.

This V/A concentration ratio would be a reliable index of drug clearance, if measurements of plasma concentration were performed once both the absorption and the fast disposition pharmacokinetic phases are finished. It should be kept in mind that the same clock time needs to be maintained when different individuals or different daily dose are compared. The cases to be presented in this article precisely deal with drugs that modify the pharmacokinetic system during the time course of the treatment and when daily doses are changed.

Bioavailability

Absorption of the intact drug from the administration site is normally assumed when the substance appears in the arterial blood stream. The result of this process is commonly called systemic bioavailability or bioavailability in plasma [5]. According to the previous section, the most precise determination of bioavailability should be accomplished when arterial plasma drug concentration is monitored. At this moment, all quantified molecules in the arteries would be available to pursue their transference to each extravascular point.

When drug entrance persists for a long period of time, even longer than the interval of administration in multiple dose regimens, V/A concentration ratios could have a value inverse of those observed at the pure elimination phase.

Saliva drug concentration

Saliva is the fluid produced by the salivary glands. As other organs, salivary glands receive substances from the arterial zone of capillaries and thereafter solutes are transferred through the basal and apical membranes of acinar cells into the upper zone of salivary ducts. The fluid recently formed in acini has a similar composition in free substances to the plasma in the artery [6]. This is true for substances which have no restriction in their passage through lipophilic membranes. During its transit by the luminal space of ducts the fluid interchanges solutes with the interior of ductal cells through the apical membrane, and thereafter with the interstitial space through the basal membrane [7,8]. So, drug molecules located in the arterial space of the circulatory system pass through the acini into the salivary conducts, returning to the venous space from the ductal cells.

When saliva is secreted into the oral cavity, drug concentration in this fluid differs sensitively from the value it had at the upper zone of ducts. If during salivation the sample were consecutively collected in two fractions: 1) first portion (S1), coming from the lower part of ducts; and 2) second portion (S2), coming from the upper part of ducts; both vein and artery concentrations could be, in some way, subrogated by S1 and S2 concentrations, respectively. In a more exhaustive sampling protocol, several fractions of saliva secreted by continuous stimulation, chewing parafilm® or placing small crystals of citric acid over the tongue, could be obtained. The first small portion of saliva would contain a drug concentration that resembles the venous one. Discarding intermediate collected samples a final fraction of saliva could be obtained, with a concentration practically the same as that flowing free within the arterial vessels.

As it was referenced in the literature [9,10] the variability in saliva drug concentration could be diminished by using stimulated saliva sampling procedure. This was possible since the higher collected

volumes of saliva approaches its composition with the final fraction of secreted fluid, and then more closely related with the corresponding arterial free plasma drug concentration. Otherwise, without stimulation, variable volumes of saliva would determine a higher variance in drug concentration values.

Summing up, it could be expected that the S1/S2 saliva drug concentration ratio would play a reliable role of subrogating the V/A plasma free drug concentration ratio.

It is currently known that efflux pumps belonging to the ABC (ATP binding cassette) family of transporters, such as P-glycoprotein (Pgp) and multidrug resistance protein 2 (MRP2), are located at the apical membrane of both acinar and ductal cells [11]. Those proteins are important determinants of interindividual differences in intestinal absorption and hence plasma levels of carbamazepine and phenytoin. Furthermore the role of ABCC2 polymorphisms on MRP2 expression is less well established, but recent data indicate that hepatic MRP2 expression is influenced by two SNPs in positions 1188 (V1188E) and 1515 (C1515Y) of the MRP2 protein. Specifically, the glutaminic acid and the tyrosine in positions 1188 and 1515, respectively, were associated with significantly higher hepatic MRP2 protein expression levels than the corresponding wildtype proteins [12].

Some drugs, like the ones to be studied in the present work, are substrate of these transporters, but their active transportation to the luminal space of salivary ducts are to be of minimal effect in the S1/S2 concentration ratio, because their participation in both the numerator and denominator of the quotient compensates. Conversely, saliva/plasma concentration ratio (either S1 or S2) could be effectively affected by changes in the activity or expression of efflux transportation. For this reason, this ratio will be used for assessing some systemic modification in efflux carriers.

Scope of the current investigation

The objective of the present investigation was to detect whether the clearance and/or the bioavailability of two well-known efflux transporter substrate antiepileptic drugs [13, 14]: carbamazepine (CBZ mainly for MRP2) and phenytoin (PHT mainly for MRP2), could change throughout the course of the therapy, from the first dose to multiple dose administration, whether the induction of efflux transporter could be involved in this change, and whether the sex of individuals could influence these pharmacokinetic modifications.

MATERIALS AND METHODS

Subjects

The present multicentre two-phase collaborative study took place in Argentine (National University of La Plata) and Uruguay (University of the Republic).

The first phase was performed with healthy volunteers in order to determine pharmacokinetic parameters after single dose of drugs. No patients were enrolled in these single dose trials because of ethical considerations. Subjects were considered to be healthy based on their medical history, physical examination, urine and blood analysis, routine biochemistry tests, hepatitis B and C, and HIV status.

The second phase was carried out with epileptic patients under monotherapy of each drug studied. This second clinical phase belonged to a larger multinational (Uruguay-Argentine-Mexico) research program, which deals with the therapeutic management of multidrug refractory epileptic patients.

Phase I: Single dose study

Trial design and setting

The trials were designed and monitored in accordance with Good Clinical Practices and the Declaration of Helsinki.

The study protocols and informed consents were designed according to the ethical guidelines for human studies and were approved by the Institutional Ethics Review Committees of the Faculty of Chemistry of Uruguay, for the CBZ study, and of the Italian Hospital of La Plata, Argentine, for the PHT study.

Written informed consent was obtained from all subjects before entry the study. The CBZ study was performed in the Bioavailability and Bioequivalence Centre for Medicine Evaluation, situated in "Dr. Juan J. Crottogini" Hospital. The PHT study was carried out in the Faculty of Exact Sciences, National University of La Plata.

Volunteers were given oral doses of CBZ, 400 mg (2 tablets x 200 mg, immediate release product), and PHT, 100 mg (1 capsule, immediate release product) at 8 a.m. Volunteers fasted for 8 hours before dose administration. Volunteers were also required to refrain from strenuous physical exercise, smoking, caffeine-containing drinks and food, alcohol, grapefruit-containing products and other medications.

Salivary sampling

Mixed salivary samples (2 mL approximately) were taken before and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16 and 24 hours after CBZ administration, and before and at 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10, 12, 24, 36 and 48 hours after PHT administration.

Saliva samples were stimulated by chewing paraffin wax (parafilm®) for 1 minute before spitting into tubes. Then the samples were centrifuged at 3000 rpm for 5 minutes and stored at -20°C until assay.

Determination of saliva drug concentrations

CBZ and its active metabolite carbamazepine-10,11-epoxide (EPOX) and PHT in saliva were analyzed by the use of HPLC methods previously validated and published [15,16] with some modifications. A brief description of the techniques and the equipments used can be summarized as follows:

CBZ and EPOX were assayed using Prominal as internal standard (IS). Drugs and IS were extracted from the matrix by means of ethylacetate. Then the organic phase was separated and evaporated to dryness under nitrogen current. The residue was dissolved with methanol and 20 µL injected into a C18 reversed phase column (5 µ particle size, 150x4.6 mm, Spherex-Phenomenex). The mobile phase consisted of water / methanol / acetonitrile (58:38:4). A Shimadzu chromatograph with LC-6A pump, SPD-6A UV detector set at 240 nm, SLC-6A system controller, and C-R6A Chromatopac processor, was used. Lower limit of quantifications (LLOQ) of 0.02 and 0.05 mg/L were determined for CBZ and EPOX, respectively. Linearity of the method was determined up to 4 and 2 mg/L for CBZ and EPOX respectively. Intra and inter-day precision at the upper, medium and lower sections of the calibration curves were below 5 % for CBZ and 7 % for EPOX. The accuracy of the method was above 90 % for both substances.

PHT was assayed using Propylparaben as IS. Drug and IS were extracted from the matrix by means of chloroform. Then the organic phase was separated and evaporated to dryness under nitrogen current. The residue was dissolved with methanol and 20 µL injected into a C18 reversed phase column (5 µ particle size, 125x4 mm, LiChrocart Merck). The mobile phase consisted of water / methanol / acetonitrile / isopropanol (57:20:20:3) adjusted to pH 3 with phosphoric acid. A Gilson SAS chromatograph with 322-H2 binary infusion pump, 156 UV-visible detector, and UniPoint LC 3.3 data processing, was used. A LLOQ of 0.01 was determined. Linearity of the method was assessed up to 4 mg/L. Intra and inter-day precision at the upper, medium and lower sections of the calibration curves were under 10%. Accuracy of the method was above 90 %.

Pharmacokinetics and statistical evaluation

Maximum observed CBZ and PHT saliva concentration (Cmax) with the corresponding sampling time (Tmax) were computed directly from the data. The elimination rate constant (λ_z) was determined by least squares linear regression analysis (log C versus t) of the last data point (C_{last}). The half-life ($t_{1/2}$) was calculated by the equation $t_{1/2}=0.693/\lambda_z$. The area under the saliva concentration-time curve from time 0 to the time of the last quantifiable concentration (AUC_T) was calculated by the linear-log trapezoidal rule. The area from zero to infinity (AUC_∞) was calculated by adding C_{last}/λ_z to AUC_T. Apparent clearance was calculated as dose/AUC_∞. Exposure parameters were corrected by bodyweight in order to compare pharmacokinetics between both sexes.

Phase II: Multiple dose study

Trial design and setting

Informed consents were obtained from the patients that were selected to be included in this prospective therapeutic salivary drug monitoring. Selection was made considering their understanding to follow the experimental protocol strictly and drug dosing compliance. Responsive and non responsive patients were enrolled since saliva antiepileptic level were under investigation in order to determine if there were any relationships between their salivary levels and the clinical response to the treatments. Specially, S1/S2 drug concentration ratio was intended to give information about the induction of efflux transporter expression due to the antiepileptic dose and/or the extent of uncontrolled seizures in the case of refractory patients [17, 18]. This study was carried out in the Therapeutic Drug Monitoring Service of the University Hospital, Uruguay.

Salivary sampling

Only trough saliva samples were withdrawn from patients. Two salivary fractions sequentially collected, S1 (1 mL) and S2 (1 mL), were obtained prior to morning dose (predose). Saliva was stimulated with citric acid, and collected in recipients with sodium bicarbonate when CBZ and EPOX were intended to be quantified. Non buffered collection was needed for PHT determination.

Determination of saliva concentrations

The same HPLC techniques mentioned above were used in patients.

Pharmacokinetics and statistical evaluation

Daily doses were divided by the body weight of patients in order to compare S2 concentrations between sexes. Mean salivary S1/S2 drug concentration ratios were compared between sexes, clinical response, and analyzed in accordance to S2 concentrations. Statistical analysis was performed with SPSS 13.0 for Windows NT software package (Chicago, IL, USA).

RESULTS

Subject characteristics

Healthy adult volunteers 21 to 26 years old for the CBZ study and 22 to 37 years old for the PHT study entered and completed the trial. Six men and six women participated in the CBZ study. The mean body weights (± standard deviation) were 81 (±14) kg and 57 (±5) kg respectively. Ten men and fourteen women participated in the PHT study. The mean weights (± standard deviation) were 77 (±6) kg and 59 (±5) kg respectively.

Adult epileptic patients on CBZ or PHT monotherapy were enrolled. Fifteen women and eleven men, 18 to 40 years old and 42 to 117 kg, took part in the CBZ study. Eleven women and eleven men, 18 to 77 years old and 40 to 105 kg, participated in the PHT study.

Single and multiple dose pharmacokinetics

Tables 1 and 2 show the pharmacokinetic parameters obtained in both sexes after single dose administration of CBZ and PHT in healthy volunteers, respectively. CBZ and PHT salivary concentrations after multiple dose administration in patients can be observed in tables 3 and 4, respectively. Figures 1 and 2 illustrate the relationships between saliva drug S1/S2 concentration ratios and S2 levels, for CBZ and PHT.

DISCUSSION

Single dose

No significant differences between sexes in Tmax were observed after single doses of both drugs. Nevertheless, Cmax and AUC were significantly higher in women than men. In the case of CBZ, these

exposure differences disappeared when these parameters were multiplied by the weights of individuals (in tables 1 and 2 the corrected AUC_{∞} are shown).

So, body size was responsible for the differences found. In other words, the apparent clearance per unit of body weight was similar in both sexes, which means that bioavailability (F) and systemic clearance (CL) would have actually the same value in women and men, or both F and CL would change similarly when sexes are compared. It is known that women have higher efflux transporter expression [19] at the enterocyte and hepatocyte. So, probably they could have lower F than men because of a higher first pass metabolism.

This could be envisaged in the case of CBZ regarding the higher EPOX/CBZ concentration ratio found in women (table 1), and explained by a higher content of CYP3A4 isozyme, responsible for the CBZ epoxidation, in the gut compared to liver [20]. If there was a lower oral bioavailability in women their systemic clearance should be lower too. Since no differences in half-lives were found between sexes (table 1), the lower CL in women should be explained by their lower distribution volume, which is compatible with their lower muscular mass.

A similar behavior could be anticipated for PHT. However, it should be mentioned that either CYP2C9 or CYP2C19 isozymes, which are responsible for PHT metabolism, are ten-fold lower expressed in the gut than in the liver [21]. Then, a slightly lower bioavailable dose may be envisaged in women after oral administration, since the high drug concentration inside the enterocyte could easily saturate the enzymes. Table 2 shows a significant difference ($p<0.05$) in the corrected AUC_{∞}, and hence the female CL/F would be slightly higher than the male one. If only F was responsible for this difference, no sex-related difference in systemic clearance would be expected. Otherwise, also a higher CL for women should be considered.

On the other hand, a non significant difference was observed in half-lives. In order to explain the similar or even higher distribution volume [22] that female must have in relation with male, in agreement with their similar or higher CL respectively, the important role that gastrointestinal lumen has on PHT distribution needs to be considered. A higher MRP2 expression in women extruding PHT to the intestinal lumen could enlarge the space for including the drug, and hence, compensate or even override her lower muscle capacity.

Multiple doses

According to figure 1, S1/S2 saliva drug concentration ratios for CBZ are positively correlated with S2 saliva levels. This result confirms that CBZ CL increases with daily doses, or with systemic drug concentrations. Interestingly, significant differences in drug exposure between genders were observed after multiple dose administration (see table 3). Even though S2 CBZ concentration did not differ between male and female epileptic patients, the doses received by women were significantly higher (approximately 2-fold higher). Based on the higher EPOX/CBZ concentration ratio obtained in women, 2-fold higher than that observed after single dose administration, and on the similar CL inferred through the figure 1, an extensive presystemic metabolism of CBZ should be the main cause for both the lower drug oral bioavailability and the higher EPOX formation in female in relation to male subjects under CBZ monotherapy regimens [23]. In conclusion, women would have an important presystemic loss of CBZ when multiple dose administrations are given. It would seem that the induction of both efflux transporter and enzyme in the gut during chronic drug administration impacts more on the bioavailability than on the clearance. This could be due to a higher concentration of CBZ in the enterocyte in relation to the hepatocyte, after oral doses, as well as the MRP2 drug induction in the hepatobiliary membrane, increasing in this way the intestinal fraction of both presystemic and systemic clearance of the drug.

As it can be observed in figure 2, a negative correlation between S1/S2 PHT concentration ratio and its corresponding S2 level was obtained, contrasting with the previously mentioned CBZ findings. This was expected since S1/S2 quotient reflects drug clearance, and PHT reduces its clearance with increasing daily doses. Since PHT daily doses were similar in both genders, the higher S2 drug level in women is in accordance with their lower S1/S2 ratio (table 4). In conclusion it could be said that, conversely to CBZ, the clearance plays the main role in sex-related pharmacokinetic differences after PHT multiple dosing.

The usefulness of S1/S2 drug concentration ratio to determine systemic clearances in patients is really important, since up to now it was impossible to separate the contribution of F and CL from the apparent

clearance experimentally determined after oral dose administration. To note is the fact that two drugs, CBZ and PHT, which share the same inductive mechanism for enzymes and transporter expression, would have different pharmacokinetic response to increasing doses: 1) less than proportional in the case of CBZ; and 2) more than proportional in the case of PHT. A plausible explanation was recently done [13] throughout the induction of MRP2 efflux transporter at the hepatobiliary membrane, which drives molecules from the liver to a high or to a low metabolizing intestinal space in the case of CBZ or PHT respectively, either by considering presystemic (bioavailability) or systemic (clearance) loss of drug.

Significance of saliva levels in the context of routine plasma drug monitoring

In the last year, plasma concentration (P) of 62 epileptic adult patients (13 men and 49 women), between 18 and 40 years old, under CBZ monotherapy, were monitored in our Therapeutic Drug Monitoring Service. Significantly higher ($p<0.01$) mean CBZ plasma concentration (9.7 mg/L) were observed in men in comparison with women (6.7 mg/L) after receiving the same daily dose (16 mg/kg).

In a previous work the relationship between CBZ plasma concentration and daily oral dosing was established [23]. Accordingly, plasma drug concentrations for male and female patients were inferred for 11 and 19 mg/kg/day, respectively. Dividing the experimental S2 saliva drug concentrations obtained in the present work by the respective calculated plasma levels, the following S2/P ratios were obtained: 0.33 and 0.23 for male and female patients, respectively. The higher S2/P CBZ concentration ratio is in accordance with a higher plasma level observed in men, which is in agreement with a concentration-dependent MRP2 inductive effect of this drug at the salivary glands [24].

During the same period, plasma concentration of 160 epileptic adult patients (72 men and 88 women), between 18 and 40 years old, under PHT monotherapy, were monitored. Even though different ($p<0.01$) daily doses were received by the patients (male: 4.5 mg/kg/day and female: 5.3 mg/kg/day), mean plasma concentrations (13.5 mg/L) of PHT were identical in both sexes. Since PHT dosing received by women (4.9 mg/kg/day) and men (4.6 mg/kg/day) in the present salivary multiple dose trial were very near to that observed in patients under plasma routine drug monitoring, the following S2/P concentration ratios could be informed without the need of extrapolations: 0.067 and 0.14 for male and female patients respectively. This higher S2/P PHT concentration ratio observed in women could be probably related to an amplification of the natural higher salivary MRP2 female expression caused by the presence of PHT.

The latter statement is based on the near-to-one female/male weight-corrected AUC_{∞} saliva drug concentration ratio observed after PHT single dose administration, and on the two-fold increase of this sex-related ratio when steady state saliva drug concentrations after similar multiple dosing per kg of body weight are considered. Conversely, in the case of CBZ, a decrease from 1 to 0.67 in the female/male saliva drug level ratio could be informed when moving from single to multiple doses. This systemic CBZ concentration-dependent induction of MRP2 detected in male may have overridden its natural lower transporter expression.

As a conclusion, S1/S2 and S2/P drug concentration ratio would become interesting indexes for detecting the systemic induction of efflux transporter.

Refractory epilepsy and efflux transporter overexpression

Strong evidence in the literature [25-27] supports the hypothesis of an overexpression of efflux transporter, not only in the brain but also in the rest of the body, caused by uncontrolled seizures. It would be interesting to analyze the feasibility of S1/S2 concentration ratio to differentiate responsive from non-responsive epileptic patients. Individuals of the present trials were divided into two groups according to the frequency of seizures: 1) one or less seizure every six months (responsive patients with controlled seizures); 2) more than one seizure episode per week (non responsive patients with uncontrolled seizures).

In the CBZ trial, patients under combined CBZ and valproic acid (VPA) therapy were included in order to equilibrate daily doses of CBZ between responsive (n= 20, 17 mg/kg/day) and non-responsive (n=28, 17 mg/kg/day) groups. This was possible as VPA does not induce efflux transporter and does not interfere with free CBZ clearance. A significant difference ($p<0.05$) in S1/S2 between responsive (1.03) and non-responsive (1.09) was found. This result shows a higher systemic clearance in non-responsive patients, which agrees with a higher elimination rate, promoted by efflux transporter overexpression in the splanchnic organs, specifically at the hepatobiliary membrane.

In the PHT trial, daily doses were similar in both groups (4.8 mg/kg/day). Only a decreasing tendency in the S1/S2 ratio for non-responsive patients (1.07 to 1.01) was observed (p=0.1), probably because of the lower number of subjects having a bad control of seizures (n=8). This trend precludes a lower clearance in non-responsive patients, probably due to the same transporter-related cause aforementioned, but in the case of PHT with a dissimilar pharmacokinetic consequence.

In conclusion, our results with S1/S2 saliva drug concentration ratios give new evidence supporting the generalized overexpression of efflux transporter promoted by uncontrolled seizures.

Clinical implications

More research in this field should be done in order to confirm our findings, but some issues arise in giving us a new opportunity of dealing more efficiently with the pharmacological treatment of refractory epilepsy. It seems mandatory to start as soon as possible not only with an effective antiepileptic drug in order to avoid seizures exacerbation, but also with a new dosage scheme of the antiepileptic drug for regulating the development of drug resistance provoked by the induction capacity of the drug itself. Because of the time-dependency and concentration-dependency of this induction, maybe intermittent administration of loading doses of antiepileptic drugs could enhance the treatment success. A non inducer antiepileptic drug should be included in the therapy as a baseline control of seizures, allowing a less frequent administration of the inducer antiepileptic drug.

Relative importance of efflux transporters in clearance and bioavailability of drugs among individuals

In both the drug absorption and elimination processes, enzymes and transporters are playing relevant roles. Enzymes determine the rate and the extension of drug pre-systemic and systemic elimination, but either influx or efflux transporter regulate the actual activity of the enzymes. In other words, enzyme activity genetically determined could be diminished or enhanced by membrane transporters.

The CYP2C9 and CYP2C19 genes, responsible for PHT metabolism, are members of the CYP2C gene cluster located on chromosome 10q24.1-q24.3, which include CYP2C8 , −2C9, −2C18 and −2C19 At the present time, 34 and 28 sequence variants have been reported for CYP2C9 and CYP2C19 , respectively . The gene encoding CYP2C9 is highly polymorphic; it is expressed with more than 20 variant alleles. It has been reported that the major allelic variants, CYP2C9*1 (wildtype), *2 and *3 might cause interindividual and interethnic variability in the disposition of its substrates. The frequencies of these genetic variations in CYP2C9 have been described for Caucasian and Asian populations showing interethnic variability [28]. In a Spanish population, the frequencies of CYP2C9*1, *2 ,and *3 alleles were 0.74 (95 % CI: 0.68 – 0.80), 0.16 (95 % CI:0.11 – 0.21) and 0.10 (95 % CI: 0.06 – 0.15), respectively; however, a lower frequency of CYP2C9*2 was found among Mexican-Americans [29]. The authors hypothesized that the frequency of CYP2C9*2 might be related to Amerindian ancestry.

Since CYP3A4, the main responsible for CBZ metabolism, has not polymorphic variation, the efflux transporter expression becomes relevant for understanding both clearance and bioavailability variations among individuals. Simon et al [30] noted that intestinal expression levels of MDR1 and MRP2 were possible determinants of CBZ plasma levels and dose requirement in epileptic patients. Furthermore, dose requirements of CBZ and PHT and intestinal MDR1 content may be influenced by frequent ABCB1 polymorphisms.

Taken together these findings and our results, regarding drug-related and seizure-related overexpression of efflux transporters, additional explanations for the large interindividual variability observed in the pharmacokinetic profiles of CBZ and PHT and for the inter/intra-individual variability of drug bioavailability and clearance could be assessed.

CONCLUSION

Women had an important presystemic loss of CBZ when multiple dose administrations were given. It would seem that the induction of both efflux transporter and enzyme in the gut during chronic drug administration impacted more on the bioavailability than on the clearance.

Conversely to CBZ, the clearance played the main role in the sex-related pharmacokinetic differences observed after PHT multiple dosing.

The usefulness of S1/S2 drug concentration ratio to determine systemic clearance in patients has been one of the most important finding developed in this work, not only from a pharmacokinetic point of view but also as new index to phenotype epileptic patients as refractory to the pharmacological treatment.

Working with both S1/S2 and S2/P drug concentration ratios could be a good approach for detecting efflux transporter induction at the membranes of the local entero-hepatic zone and at the membranes of other organs systemically distributed.

Salivary drug concentration measurement is a good clinical practice for monitoring epileptic patients. This cost-saving and easy-to-obtain fluid gives systemic information of drug fate beyond the local processes taking place at the salivary glands.

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Table **1**
Saliva pharmacokinetic parameter means [or medians] of Carbamazepine after a single oral dose of 400 mg**.**

	Male (n=6)	**Female (n=6)**	**Significance**
T_{max} (h)	6 [3-8]	5 [2-6]	NS
C_{max} (mg/L)	1.37 (0.19)	2.06 (0.16)	p<0.001
$AUC_{0-\infty}$ (mg.h/L)	60.0 (11.6)	78.6 (18.8)	p<0.05
AUCxW (mg.h.kg/L)	4897 (1401)	4409 (941)	NS
$t_{1/2}$ (h)	36.6 (8.9)	35.4 (14.0)	NS
AUC_{EPOX}/AUC_{CBZ}	0.0626 (0.0123)	0.0913 (0.0179)	p<0.05

T_{max}: time-to-peak drug concentration
C_{max}: peak concentration of drug
$AUC_{0-\infty}$ (or AUC): area under drug concentration-time curve from zero to infinite
$t_{1/2}$: elimination half-life
W: body weight
[ ]: range
( ): standard deviation
NS: non significant

Table **2**
Saliva pharmacokinetic parameter means [or medians] of Phenytoin after a single oral dose of 100 mg.

	Male (n=10)	**Female (n=14)**	**Significance**
T_{max} (h)	4 [2-8]	4 [2-7]	NS
C_{max} (mg/L)	0.188 (0.020)	0.220 (0.020)	p<0.05
$AUC_{0-\infty}$ (mg.h/L)	3.63 (0.52)	4.42 (1.04)	p<0.05
AUCxW (mg.h.kg/L)	278 (25)	261 (25)	p<0.05
$t_{1/2}$ (h)	12.6 (1.3)	13.0 (3.3)	NS

T_{max}: time-to-peak drug concentration
C_{max}: peak concentration of drug
$AUC_{0-\infty}$ (or AUC): area under drug concentration-time curve from zero to infinite
$t_{1/2}$: elimination half-life
W: body weight
[ ]: range
( ): standard deviation
NS: non significant

Table **3**
Carbamazepine and its epoxide metabolite salivary mean exposure after multiple dose administration.

	Male (n=11)	**Female (n=15)**	**Significance**
Daily Dose (mg/kg)	11 (4.0)	19 (7.7)	p<0.01
[S2] $_{CBZ}$ (mg/L)	2.00 (0.90)	1.82 (0.57)	NS
S1/S2 ratio $_{CBZ}$	1.03 (0.08)	1.08 (0.14)	NS
[S2] $_{EPOX}$ (mg/L)	0.55 (0.30)	0.94 (0.39)	p<0.05
mean[S2]$_{EPOX}$/mean[S2]$_{CBZ}$	0.275	0.87	____

[S2]: saliva drug concentration in the collected second sample (see on the text)
S1/S2: first-to-second-sample ratio of saliva drug concentrations (see on the text)
( ): standard deviation
NS: non significant

Table **4**
Phenytoin salivary mean exposure after multiple dose administration.

	Male (n=11)	**Female (n=11)**	**Significance**
Daily Dose (mg/kg)	4.58 (1.20)	4.87 (0.83)	NS
[S2] $_{PHT}$ (mg/L)	0.90 (0.39)	1.91 (1.04)	p<0.01
S1/S2 ratio $_{PHT}$	1.09 (0.08)	1.01 (0.08)	p<0.05

[S2]: saliva drug concentration in the collected second sample (see on the text)
S1/S2: first-to-second-sample ratio of saliva drug concentrations (see on the text)
( ): standard deviation
NS: non significant

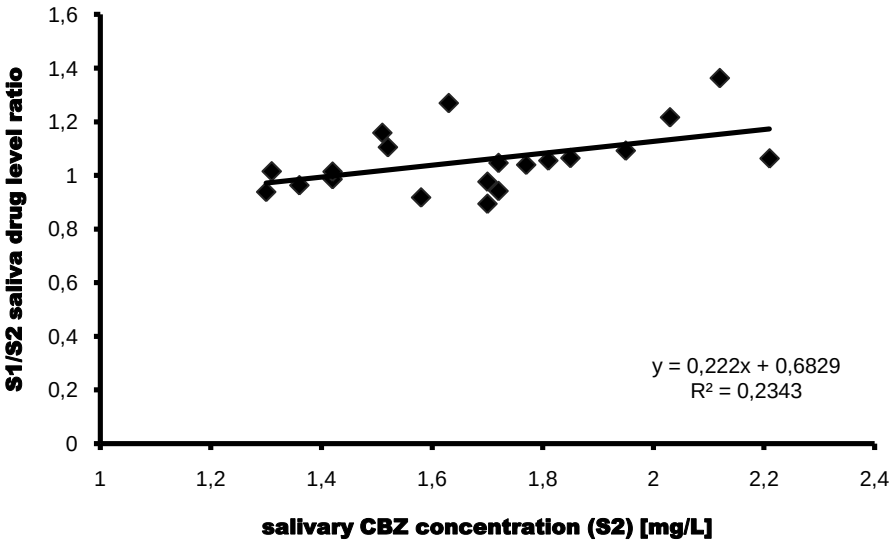

Figure **1**:
S1/S2 saliva carbamazepine concentration ratio in relation with its salivary levels or daily dose.

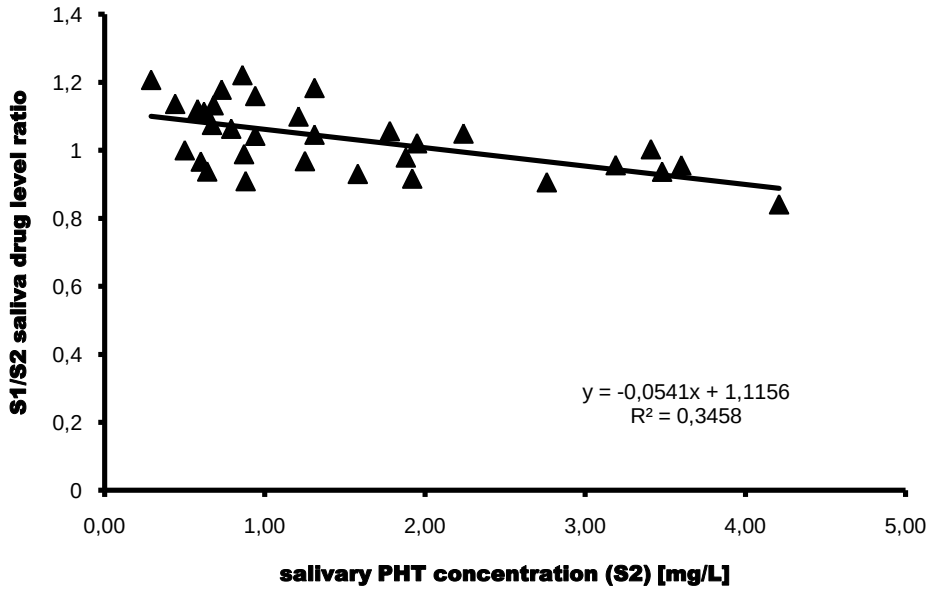

Figure **2**:
S1/S2 saliva phenytoin concentration ratio in relation with its salivary levels or daily dose.