Saliva versus Plasma Bioequivalence of Valsartan/Hydrochlorothiazide in Humans: Validation of Classes II and IV Drugs of the Salivary Excretion Classification System

Introduction

The protein binding of drug and membrane permeability were previously investigated for several drugs as major factors for salivary excretion where a Salivary Excretion Classification System was proposed (SECS) [1]. High intestinal permeability corresponds to fraction absorption (Fa) > 0.9, while high protein binding corresponds to low fraction unbound (fu) of < 0.1. Based on SECS, class I drugs of high intestinal permeability and low protein binding, such as paracetamol, are subject to salivary excretion. Class II drugs of low permeability and low protein binding, such as metformin, are subject to salivary excretion since low permeability is counterbalanced by low protein binding. Class III drugs of high intestinal permeability and high protein binding, such as ronovastatin, are subject to salivary excretion since high protein binding is counterbalanced by high permeability. Class IV drugs of low intestinal permeability and high protein binding, such as montelukast, are not subject to salivary excretion [1].

Salivary excretion of some drugs has been reported previously as a good indicator for drug bioavailability, therapeutic drug monitoring, drug abuse and pharmacokinetics. Saliva sampling method is a simple, non-invasive, and cheap with less stress or pain and no risk of infection compared with plasma sampling method [2–11].

Valsartan is a non-peptide angiotensin II type 1 (AT1) receptor blocker [12]. It is rapidly absorbed after oral administration and eliminated mainly as unchanged drug via biliary excretion [13, 14]. In case of renal dysfunction, there is no effect on the pharmacokinetics of valsartan. Also the pharmacokinetics is not affected by age [15]. Valsartan is used for the treatment of hypertension either alone or in combination therapy and its effect in reducing blood pressure persists throughout the 24-h after dosing. It is also effective for heart failure and post myocardial infarction patients [16]. Hydrochlorothi-
azide, it’s a thiazide diuretic and its well absorbed after oral admin-
istration with a bioavailability ranging from 60–80% [17]. Hydro-
chlorothiazide is widely used for the treatment of hypertension ei-
ther alone or in combination with other antihypertensive drugs.
Hydrochlorothiazide also used for the treatment of edema associat-
ed with heart failure, liver cirrhosis and nephrotic syndrome [18].
The combination of valsartan and hydrochlorothiazide provides fur-
ther blood pressure lowering than the individual components [16].

Objectives
The aim of this study is to investigate the robustness of using non-
invasive saliva sampling method instead of plasma sampling meth-
method for this combination (valsartan and hydrochlorothiazide) in bi-
eoequivalence and in pharmacokinetic studies for drugs that are ex-
creted in saliva according to SECS.

Methods

Study Design
Saliva pharmacokinetics were compared with plasma pharmacoki-
netics in 12 healthy male subjects under a fasted state after sign-
ing the informed consent and passing the laboratory test to par-
ticipate in a two-way, cross-over design study with wash-out peri-
od of 7 days. Medical history, vital signs, physical examination
showed no evidence of clinically significant deviation from normal
medical condition as evaluated by the clinical investigator. This
study was conducted at Red Crescent Hospital as per the Interna-
tional Conference on Harmonization (ICH), Good Clinical Practice
(GCP) and Helsinki declaration guidelines, after Institutional Re-
view Board (IRB) of Jordan Center for Pharmaceutical Research
(JCPR) and Jordan Food and Drug Administration (JFDA) approvals.

A single oral dose of valsartan/hydrochlorothiazide 160/12.5 mg
either test drug Co-Diovan® tablets, batch no. T9169 or refer-
dence drug Co-Diovan® tablets, batch no. 160159 or refer-

Assay Methodology
Plasma and saliva samples that kept frozen were assayed by a vali-
dated liquid chromatography-mass spectrometry (LC-MS) assay
method. The chromatographic conditions were, column type:
ACE 5 C8 (50 × 2.1 mm), 5 µm, the, mobile phase was, A: (0.04 %
Ammonia (10 %) & 0.04 % Formic acid) and B: 85.0 % methanol
and saliva samples were kept frozen at −20 °C until analysis.

Add 300 µL of blank and spiked plasma samples into the
appropriately labeled tubes.

Add 6 mL of extraction solvent (ethyl acetate) and vortex for
5.0 min.

Centrifuge the samples for 6 min at 4400 rpm.

Freeze the samples for about 30 min, and then decant the
organic layer in a clean evaporating glass tube.

Evaporate the extraction solvent by compressed air in water
bath at 40 °C, then reconstitute with 250 µL of reconstitution
solution (water: methanol) (35:65 %; v/v) and vortex for 1 min.
( this step should be conducted in the fume hood)

Transfer the samples into a flat bottom insert’s vials, and
inject to instrument.

Data analysis
Pharmacokinetic parameters
Individual pharmacokinetic parameters for drug concentration of
both analytes (valsartan and hydrochlorothiazide) in plasma and
saliva were calculated by non-compartmental analysis (NCA), using
WinNonlinV5.2. Pharmacokinetic parameters were area under
the concentration curves to last collection time (AUC0→t), area under
the concentration curves to infinity (AUC0→∞), maximum measured
concentration(Cmax), time to maximum concentration(Tmax), elimi-
nation rate constant (Kel) and half-life (t1/2). Statistical t-tests were
done for pharmacokinetic parameters (AUC0→24, AUC0→∞, Cmax,Kel
andt1/2), while Wilcoxon test was done for Tmax.

Bioequivalence analysis
Analysis of variance (ANOVA) was done according to EMA guideline
on bioequivalence. It includes sequence and subject (sequence) as
random effects, treatment and period as fixed effects without in-
teraction terms. Level of significance used was 0.05 for all effects.
Also, 90 % confidence intervals and intra-subject variability esti-
mates for primary pharmacokinetic parameters (AUC0→t, AUC0→∞,
andCmax) for testversusreference after logarithmic transformation
were calculated by WinNonlinprogram V5.2.

Dimensional and correlation analysis
Saliva versus plasma concentrations up to median Tmax were cor-
related by linear regression using Microsoft Excel program. Dimen-
sional analysis was done on an individual basis for each volunteer.

Dimensional analysis offers the advantage of more clear com-
parisons since ratios are unit less. The following dimensionless ra-
tios were calculated:

\[
AUC^* = \frac{\text{saliva AUC}_{0→t}}{\text{plasma AUC}_{0→t}} \\
T_{max}^* = \frac{\text{saliva } T_{max}}{\text{plasma } T_{max}} \\
C_{max}^* = \frac{\text{saliva } C_{max}}{\text{plasma } C_{max}} \\
C^* = \frac{\text{saliva concentration/plasma concentration}}{C_i/C_p}
\]

However, C* is calculated by using C_i/C_p at each sampling time
for 12 subjects.

Optimized effective intestinal permeability
Effective intestinal permeability (P_{el}) values were estimated by PK-
Sim/Mobi program V5.6. This was done by searching for the best
parameter values that produce plasma concentration that matches the actual plasma concentration at the same time.

Fraction absorption (Fa) was calculated according to equations below:

\[ Fa = 1 - e^{-2An} \]

\[ An = P_{av} t_{res} / R \]

Where An is the absorption number, R and t_{res} are radius, set at 1.75 cm, and mean residence time, set at 3 h, in the human small intestine respectively.

Results and Discussion

Valsartan falls into SECS class IV with low permeability (Fa = 0.46) and high protein binding (Fu = 0.05). As a result valsartan didn’t appear in saliva. Mean plasma valsartan concentrations of test and reference formulations are shown in ▶ Fig. 1. The pharmacokinetic parameters of test and reference formulations were calculated and showed no significant differences since \( P > 0.05 \), as shown in ▶ Table 1.

Bioequivalence metrics and intra-subject variability values for primary pharmacokinetic parameters \( AUC_{0\rightarrow24} \), \( AUC_{0\rightarrow\infty} \) and \( C_{\text{max}} \) in plasma were calculated. The 90\% confidence intervals didn’t fall within the acceptance range of 80–125\% because the small sample size used and this is expected due to the high intra-subject variability observed in this study as shown in ▶ Table 2.

> Table 2 Bioequivalence metrics: point estimate (90\% lower limit–90\% upper limit), intra-subject variability \% for valsartan after log transformation.

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Parameter} & \text{Plasma} & \text{Test} & \text{Reference} \\
\hline
AUC_{0\rightarrow24} (\mu g/ml h) & 105.5 (81.2–136.9), 36.4 & 19.01 & 17.41 \\
AUC_{0\rightarrow\infty} (\mu g/ml h) & 106.1 (82.4–136.6), 35.2 & 20.32 & 18.63 \\
C_{\text{max}} (\mu g/ml) & 108.3 (75.8–154.8), 51.2 & 2.73 & 2.55 \\
\hline
\end{array}
\]

> Table 3 ANOVA P values of \( (AUC_{0\rightarrow24}, AUC_{0\rightarrow\infty}, C_{\text{max}}) \) for valsartan in plasma *

\| \text{Source} | \text{Plasma} | \text{Test} | \text{Reference} | \text{P value} \|
\hline
\text{Sequence} & 0.864, 0.674, 0.489 & & & \\
\text{Subject (Sequence)} & 0.573, 0.541, 0.781 & & & \\
\text{Treatment} & 0.719, 0.682, 0.694 & & & \\
\text{Period} & 0.256, 0.227, 0.258 & & & \\
\hline
* ANOVA analysis of variance; \( AUC_{0\rightarrow24} \) area under concentration curves to last collection time; \( AUC_{0\rightarrow\infty} \) area under concentration curves to infinity; \( C_{\text{max}} \) maximum measured concentration. Level of significance is 0.05.
Table 4: Plasma pharmacokinetic parameters of hydrochlorothiazide test and reference formulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0→24&lt;/sub&gt;(ng/ml h)</td>
<td>392.19</td>
<td>380.05</td>
<td>0.544</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt;(ng/ml h)</td>
<td>432.41</td>
<td>421.38</td>
<td>0.640</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;(ng/ml)</td>
<td>62.27</td>
<td>52.30</td>
<td>0.252</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;(h)</td>
<td>2.30</td>
<td>2.35</td>
<td>0.678 *</td>
</tr>
<tr>
<td>K&lt;sub&gt;el&lt;/sub&gt;(h&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.09</td>
<td>0.09</td>
<td>0.988</td>
</tr>
<tr>
<td>t&lt;sub&gt;0.5&lt;/sub&gt;(h)</td>
<td>7.61</td>
<td>7.69</td>
<td>0.897</td>
</tr>
</tbody>
</table>

*: Wilcoxon test was done for T<sub>max</sub>

Table 5: Saliva pharmacokinetic parameters of hydrochlorothiazide test and reference formulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0→24&lt;/sub&gt;(ng/ml h)</td>
<td>87.66</td>
<td>91.38</td>
<td>0.372</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt;(ng/ml h)</td>
<td>116.57</td>
<td>111.95</td>
<td>0.646</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;(ng/ml)</td>
<td>15.65</td>
<td>18.12</td>
<td>0.548</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;(h)</td>
<td>5.42</td>
<td>4.25</td>
<td>0.262 *</td>
</tr>
<tr>
<td>t&lt;sub&gt;0.5&lt;/sub&gt;(h)</td>
<td>5.96</td>
<td>6.43</td>
<td>0.704</td>
</tr>
<tr>
<td>K&lt;sub&gt;el&lt;/sub&gt;(h&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.15</td>
<td>0.19</td>
<td>0.681</td>
</tr>
</tbody>
</table>

*: Wilcoxon test was done for T<sub>max</sub>

Table 6: Bioequivalence metrics: point estimate (90% lower limit-90% upper limit), intra-subject variability for hydrochlorothiazide in plasma and saliva after log transformation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0→t&lt;/sub&gt;</td>
<td>103.1 (94.7–112.3), 11.6</td>
<td>93.9 (82.8–106.5), 17.2</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt;</td>
<td>102.4 (94.6–110.9), 10.8</td>
<td>103.8 (90.4–119.3), 18.9</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>114.8 (94.4–139.7), 26.9</td>
<td>91.9 (71.188–118.801), 35.7</td>
</tr>
</tbody>
</table>

Table 7: ANOVA P values of (AUC<sub>0→t</sub>, AUC<sub>0→∞</sub>, C<sub>max</sub>) for hydrochlorothiazide in plasma and saliva *.

<table>
<thead>
<tr>
<th>Source</th>
<th>Plasma</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject (Sequence)</td>
<td>0.689, 0.699, 0.302</td>
<td>0.464, 0.472, 0.342</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.531, 0.598, 0.231</td>
<td>0.389, 0.635, 0.566</td>
</tr>
<tr>
<td>Period</td>
<td>0.207, 0.070, 0.163</td>
<td>0.615, 0.205, 0.754</td>
</tr>
</tbody>
</table>

*: ANOVA analysis of variance; AUC<sub>0→24</sub> area under concentration curves to last collection time; AUC<sub>0→∞</sub> area under concentration curves to infinity; C<sub>max</sub> maximum measured concentration. Level of significance is 0.05

Table 8: Saliva to plasma ratios of test and reference formulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sup&gt;−&lt;/sup&gt;</td>
<td>0.23</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>0.27</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>2.67</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.26</td>
<td>0.23</td>
<td></td>
</tr>
</tbody>
</table>

AUC<sup>−</sup> = saliva AUC<sub>0→24</sub>/plasma AUC<sub>0→24</sub> ; C<sub>max</sub> * = saliva C<sub>max</sub>/plasma C<sub>max</sub> ; T<sub>max</sub> * = saliva T<sub>max</sub>/plasma T<sub>max</sub> ; C = saliva concentration/plasma concentration = C<sub>s</sub>/C<sub>p</sub>; Area under concentration curves to last collection time, C<sub>max</sub> maximum measured concentration, T<sub>max</sub> time to maximum concentration

From a regulatory point of view, bioequivalence studies using saliva matrix is not against international guidelines. For example, the US FDA guidance for industry stated, "The statutory definitions of BA and BE, expressed in terms of rate and extent of absorption of the active ingredient or moiety to the site of action, emphasize the use of pharmacokinetic measures in an accessible biological matrix such as blood, plasma, and/or serum to indicate release of the drug substance from the drug product into the systemic circulation" and "Biological matrix: A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues." [http://www.fda.gov/cder/guidance/index.htm]. Also, saliva matrix is mentioned clearly in the Japanese guidance [http://www.nihs.go.jp/drug/BEguide-E.html]. Dimensional analysis for the ratios of saliva to plasma is shown in Table 8. It showed low saliva/plasma ratios in the AUC and C<sub>max</sub> with a longer T<sub>max</sub>. This could be due to the low permeability of hydrochlorothiazide that led to low saliva to plasma ratios. Fig. 4 shows valsartan observed versus PK-Sim/Mobi predicted plasma concentration with good fitting line between observed and predicted. Optimized effective intestinal permeability estimated was equal to 5.00598 × 10<sup>−5</sup> cm/s. valsartan has low permeability despite the high partition coefficient (log P) that was correlated with permeability classification according to BCS (Biopharmaceutics Classification System) that classified drugs according to permeability and solubility [19]. It was found that valsartan is exposed to intestinal efflux transporter p-glycoprotein that limits its transport.

Fig. 3 Correlations of plasma and saliva hydrochlorothiazide mean concentrations.

Fig. 4 Correlations of plasma and saliva hydrochlorothiazide pharmacokinetic parameters.

Table 7 ANOVA P values of (AUC<sub>0→t</sub>, AUC<sub>0→∞</sub>, C<sub>max</sub>) for hydrochlorothiazide in plasma and saliva *.

Table 8 Saliva to plasma ratios of test and reference formulations.

from the intestinal lumen and lead to low intestinal permeability [20, 21]. Fig. 5 shows hydrochlorothiazide observed versus PK-Sim/Mobi predicted plasma concentration with good fitting line between observed and predicted and the estimated optimized effective intestinal permeability was equal to $7.60281 \times 10^{-9}$ cm/s.

**Conclusion**

The data collected suggest that salivary hydrochlorothiazide can be used as alternative to plasma sample in pharmacokinetic studies and in bioequivalence when adequate sample size is used.

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**Conflict of interest**

Authors declare no conflict of interest. This work was done in partial fulfillment of master of science in Pharmaceutics at Petra University.

**References**

