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Original Article

# COMPARATIVE BIOAVAILABILITY (BIOEQUIVALENCE) STUDY FOR FIXED DOSE COMBINATION TABLET CONTAINING AMLODIPINE, VALSARTAN, AND HYDROCHLOROTHIAZIDE USING A NEWLY DEVELOPED HPLC-MS/MS METHOD

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**ABSTRACT**

**Objective:** The aim of the study was to evaluate the bioequivalence between a newly developed generic tablet containing fixed-dose combination of amlodipine besylate, valsartan and hydrochlorothiazide (10/160/25 mg), and the reference brand product Exforge HCT® tablet; using a newly developed HPLC-MS/MS method for simultaneous determination of these drugs in human plasma.

**Methods:** The brand (reference) and the test (generic) products were administered to thirty-nine healthy subjects. A fasting, laboratory blind, single-dose, two-treatment, two-period, two-sequence, randomized crossover design was conducted with 14 d washout period between dosing. Serial blood samples were withdrawn from each subject immediately before dosing (zero time), and then at 0.33, 0.66, 1.0, 1.33, 1.66, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 11, 12, 14, 16, 24, 48 and eventually at 72 h post dosing. Plasma samples were analyzed for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide by a newly developed HPLC coupled with MS/MS detector. The linearity of the method was established for plasma concentration ranges of 0.2-12 ng/ml, 50-8000 ng/ml, and 2-250 ng/ml for amlodipine, valsartan, and hydrochlorothiazide, respectively.

**Results:** Plasma concentration-time data of each individual were analyzed by non-compartmental method to measure the pharmacokinetics parameters; $C_{max}$, $T_{max}$, $AUC_{0-t}$, $AUC_{0-\infty}$, $\lambda_z$, $T_{1/2}$. For amlodipine truncated $AUC_{72hr}$ was calculated. The 90% confidence interval for the pharmacokinetic parameters used for bioequivalence evaluation ($C_{max}$ and AUC) for amlodipine, valsartan, and hydrochlorothiazide were well within FDA acceptable ranges of 80-125%.

**Conclusion:** It is concluded that the newly devolved generic product is bioequivalent with the brand product Exforge HCT® tablet. Thus, both products are clinically interchangeable.

**Keywords:** Amlodipine, Valsartan, Hydrochlorothiazide, Pharmacokinetics, Bioequivalence, HPLC-MS/MS



## INTRODUCTION

Exforge HCT® is a brand film-coated tablet formulated for oral administration in five strengths which are available in the market as fixed dose combinations of 5/160/12.5 mg, 10/160/12.5 mg, 5/160/25 mg, 10/160/25 mg, and 10/320/25 mg of amlodipine besylate, valsartan, and hydrochlorothiazide. The maximum recommended dose of Exforge HCT® is 10/320/25 mg. Exforge HCT® tablets are produced by Novartis, Switzerland, and approved by FDA on April 30, 2009 [1].

Exforge HCT® is indicated for the treatment of hypertension. The three components; amlodipine, valsartan, and hydrochlorothiazide lower blood pressure through complementary mechanisms, each working on a separate site and blocking different effector pathways. Many patients require more than one drug to achieve blood pressure goals, such as patients with diabetes or hyperlipidemia, and such patients would be expected to benefit from more aggressive treatment to lower blood pressure. Some antihypertensive drugs have smaller blood pressure effects when given as monotherapy, therefore, selection of combination therapy is recommended [1].

The active ingredients of Exforge HCT® target three separate mechanisms involved in blood pressure regulation. Specifically, amlodipine blocks the contractile effects of calcium on cardiac and vascular smooth muscle cells. Valsartan blocks the vaso-constriction and sodium retaining effects of angiotensin II on cardiac, vascular smooth muscle, adrenal and renal cells. Hydrochlorothiazide directly promotes the excretion of sodium and chloride in the kidney leading to reductions in intravascular volume [1].

Several studies indicated that dual therapy with amlodipine and valsartan achieved significantly greater reduction in blood pressure than monotherpay by amlodipine or valsartan [1-3]. Additionally, many clinical studies demonstrated that triple therapy with amlodipine, valsartan, and hydrochlorothiazide in a single tablet contributes additional advantages to fixed dose combinations of two drugs by achieving higher and more rapid reduction in blood pressure in safe and well-tolerated manner [4-10]. Besides, the effectiveness is maintained over the entire 24 h dosing period [6]. The most frequent adverse effects of Exforge HCT® that occurred in controlled clinical trial in at least 2% of patients treated with a maximal dose (10/320/25 mg of amlodipine/v alsartan/ hydrochlorothiazide) are; dizziness, edema, headache, dyspepsia, muscle spasms, fatigue, back pain, nasopharyngitis [1]. It cannot be determined whether these events were causally related to Exforge HCT® [1]. The triple fixed dose combination is well tolerated and most adverse events are transient and of mild to moderate severity [2-10]. Furthermore, the use of double [11] or triple [12] fixed dose combinations that reduce pill burden could help the patients to continue treatment, decreased total healthcare costs and medication compliance and adherence, and consequently result in improved clinical outcomes in hypertensive patients [11, 12].

No clinically relevant pharmacokinetic drug interactions were reported between amlodipine, valsartan, and hydrochlorothiazide in triple combination [13]; and the corresponding double combination valsartan, and hydrochlorothiazide [14]. Besides, clinically significant interactions were not observed between the individual components; amlodipine [15, 16], valsartan [17-20], or hydro-chlorothiazide [21, 22] and other drugs.

Following oral administration of Exforge HCT® tablet to normal healthy adults; peak plasma concentrations of amlodipine, valsartan, and hydrochlorothiazide are reached in about 6 h, 3 h, and 2 h, respectively. The rate and extent of absorption of individual dosage forms of amlodipine, valsartan or hydrochlorothiazide are similar to the triple fixed combination tablets (Exforge HCT®). The bioavailability of amlodipine, valsartan, and hydrochlorothiazide was not altered when Exforge HCT® was administered with food, therefore, it may be administered with or without food [1].

The absolute bioavailability of amlodipine in monotherapy has been estimated to be between 64%-90%, and the apparent volume of distribution is about 1500 L. After oral administration of 5, and 10 mg amlodipine besylate tablets, the maximum plasma was found to be about 3, and 6 ng/ml, respectively. The time to reach peak concentration ranged 6-12 h. The drug is approximately 93% bound to plasma proteins, and it is extensively (about 90%) converted to inactive metabolites via hepatic metabolism. The elimination of amlodipine from the plasma is biphasic with a terminal elimination half-life of about 30 to 50 h [23-38]. Amlodipine tablet was found to be bioequivalent to amlodipine solution [24], amlodipine capsule [30], amlodipine orodispersible tablet [30], or amlodipine suspension [38].

The absolute bioavailability of valsartan in monotherapy is about 25% (range 10% to 35%), and the steady-state volume of distribution of after intravenous administration is 17 L indicating that valsartan does not distribute into tissues extensively. The drug is highly bound to serum proteins (95%), mainly serum albumin. After oral administration of 160 mg valsartan tablet, the maximum concentration of the drug in plasma range from 2000-6000 ng/ml, which achieved within 2 to 4 h. The drug shows biexponential decay kinetics following intravenous administration with an average terminal elimination half-life of about 6 h, and 6-9 h after oral intake. Valsartan is mainly excreted unchanged with only about 20% of the dose recovered as metabolites [27-29, 39-42].

The absolute bioavailability of hydrochlorothiazide as monotherapy is about 70%, and the concentration of the drug is reached after 2 to 5 h of drug intake. There is no clinically significant effect of food on the bioavailability of hydrochlorothiazide. The drug binds to albumin (40% to 70%) and distributes into erythrocytes. Hydrochlorothiazide concentrations decline biexponentially, with a mean distribution half-life of about 2 h and an elimination half-life of about 10 h, about 70% of an orally administered dose of hydrochlorothiazide is eliminated in the urine as unchanged drug [42-44].

Several analytical methods are available for individual determination of amlodipine, valsartan, hydrochlorothiazide, or the corresponding double and triple fixed dose combinations in bulk powder and pharmaceutical dosage forms [45-55]. On the other hand, many bioanalytical methods are found for monitoring plasma concentrations of amlodipine, valsartan, or hydrochlorothiazide administered as monotherapy, or as the corresponding double fixed-dose combination [56-59]. However, recently a bioanalytical method is presented for simultaneous determination of plasma concentrations of amlodipine, valsartan and hydrochlorothiazide in triple fixed dose combination for pharmacokinetic, bioavailability, and bioequivalence studies [13].

As per international guidance, documentation of bioequivalence are considered as pivotal and crucial part for registration of generic drug products since bioequivalence studies are conducted to show that the rate and extent of bioavailability of the generic product are identical to the brand product. Consequently, the effect(s) and side effect(s) of the generic product are essentially equivalent to the brand product, and hence both products are considered interchangeable in clinical practice [60-64].

The present investigation was aimed to conduct a comparative bioavailability (bioequivalence) study between, a newly developed generic tablet containing fixed-dose combination of amlodipine besylate, valsartan, and hydrochlorothiazide (10/160/25 mg), and the reference brand product (Exforge HCT® tablet, produced by Novartis, Switzerland), applying FDA guidance on bioavailability and bioequivalence [60, 61]; by utilizing a newly developed HPLC-MS/MS method for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide in plasma applying FDA bioanalytical method validation guidance [65].

## MATERIALS AND METHODS

### Study products

Reference product: Exforge HCT® tablet, produced by Novartis, Switzerland, containing fixed-dose combination of amlodipine besylate, valsartan, and hydrochlorothiazide (10/160/25 mg).

Test product: a newly developed generic tablet containing fixed combination of amlodipine besylate, valsartan, and hydro-chlorothiazide (10/160/25 mg).

### Ethical considerations

The study was conducted according to the provisions of the declaration of Helsinki [66], and ICH guidelines for good clinical practice [67]. The subjects provided written and signed informed consent before the commencement of the study. As per the above-mentioned guidelines, the study protocol was written, and then approved by the principal investigator, clinical investigator, Institutional Review Board (IRB), the sponsor, and eventually the local health authority before conducting the study. For ethical considerations, the subjects were free to leave the study for any reason and at any time. Moreover, according to the clinical investigator's decision, subject's withdrawal at any time during the study was also considered to protect the health of the subjects.

### Study design

As per study protocol, the study was designed as; fasting, laboratory blind, single-dose, two-treatment, two-period, two-sequence, randomized crossover design with 14 d washout interval between period I and period II dosing. Forty subjects enrolled in the study. An equal number of subjects (20 subjects) were randomly assigned to each dosing sequence of the test and the reference formulations [60-64].

### Inclusion criteria

Forty male adult subjects were selected from the pool of the Jordan Center for Pharmaceutical Research, Amman, Jordan. The subjects were judged healthy and considered eligible for participation in the study based on the following inclusion criteria: age between 18-50 y; normal body mass index 18.5-30{BMI=weight (kg)/height$^2$ (meter); nonsmokers or light smokers (less than 10 cigarette per day); no drug or alcohol abuse; no history of contraindication and/or allergy to the investigational drugs; physical and clinical examinations including normal vital signs, renal, hepatic, cardiovascular/ECG, pulmonary, gastrointestinal, neurological and psychiatric; normal clinical laboratory tests including biochemistry, hematology, routine urine analysis, negative HIV, hepatitis B and C; no clinically significant illness within 4 w before the start of the study; no concomitant intake or administration of any prescribed systemic or topical medications within 2 w prior to the study conduct; the subjects have not been participated in another trial (clinical, pharmacokinetic, bioavailability or bioequivalence) within the last 2 mo prior the study; no treatment with drugs known to alter the major metabolic systems within the last 30 d of starting the study; no major surgery; no donation of blood or plasma within the last two months of the study; the supine blood pressure (after resting for 5 min) is not higher than 140/90 or lower than 110/70 mmHg; the supine pulse (after resting for 5 min) is not outside the range of 60-90 beats/min; not vegetarian; and there is no evidence of uncooperative attitude of the subject.

### Study conduct

The subjects attended the clinical site at about 6:00 p. m. the day before drug products dosing and remained confined (hospitalized) in the clinical site until 24 h post-dosing. Alcohol abuse test and drug abuse tests were performed during admission to exclude any subject with positive result. Before administration of study medication, the principal or the clinical investigator with the aid of nurses checked

the label against the randomization schedule to ensure that the subject number on the label corresponds to the number allocated to each recipient. The drug product (the test or the reference) was administered with 240 ml of water after an overnight fasting of 10 h. Hand check and mouth check were achieved in order to insure that the drug was taken by the subject as directed. No water was allowed 1 hour before and 1 hour after dosing. Standard diets (breakfast, lunch and dinner) were served after 4, 9, and 14 h, respectively post dosing. The diets were identical in both periods of the study. Xanthine-containing drinks were not allowed 7 d before dosing till the follow-up examination. Grapefruit juice or beverages containing grapefruit were not allowed within the past week before the study and until the completion of the study (end of period II). The subjects were remained seated upright and not allowed to sleep or lie during the first six hours of drug administration. At the end of period II, clinical examinations including clinical laboratory tests, ECG and vital signs were performed for each subject before subjects discharge.

**Blood sampling**

Serial blood samples were collected from each subject via an Indwelling cannula placed into the forearm anticubital vein for the first 24 h post dosing to avoid multiple skin puncture, whereas, the rest blood samples were collected using direct vein puncture by disposable syringe. The cannula was kept patent by flushing with 1 ml of heparinized saline (2 IU per ml) after each blood sample withdrawal. Besides, before each blood sampling withdrawal, about 0.2 ml of blood was discarded from the cannula to get rid of residual blood from previous sampling. Blood was sampled immediately before drug product administration (zero/blank sample), and then at 0.33, 0.66, 1.0, 1.33, 1.66, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 11, 12, 14, 16, 24, 48 and eventually at 72 h post dosing. A total of 28 blood samples were obtained from each subject in each period. Blood samples were immediately placed in a heparinized tube, shacked gently, and then centrifuged for 5 min at 4000 rpm to separate the plasma. The plasma samples were stored at-20º C until analysis for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide. All tubes used for blood and plasma samples were labeled by confidential coding system according to in-house standard operating procedures of the research unit. The principal investigator and the quality assurance responsible only have the excess of the labeling system.

**Clinical observations**

Vital signs (blood pressure and pulse) were recorded few minutes before drug product administration, and then at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4, 6, 8, 10, 12, 14, 16, 24 and eventually at 72 h post administration. The adverse events (if any), were registered by the clinical staff at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 24, 48 and 72 h post drug product administration. All clinical observations were followed for each subject and for each test and reference drug product. The adverse events and serious adverse events if any were recorded based on vital signs, clinical observations, and direct questioning. For follow-up, clinical laboratory tests, ECG and vital signs were done before and then after period II.

**Drugs bioanalysis**

A new HPLC-MS/MS method was developed and validated in Jordan Center for Pharmaceutical Research, Amman, Jordan for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide in human plasma. The method was validated according to FDA and EMEA bioanalytical method validation guidance [62, 65]. Amlodipine was detected in plasma in the range of 0.2-12 ng/ml with a lower limit of quantification (LLOQ) of 0.2 ng/ml. Valsartan detection range was 50-8000 ng/ml with LLOQ of 50 ng/ml. Hydrochlorothiazide detection limit was 2-250 ng/ml with LLOQ of 2 ng/ml.

Amlodipine with its internal standard amlodipine-d4, valsartan with its internal standard valsartan-d3, and hydrochlorothiazide with its internal standard hydrochlorothiazide–d2 c13 were extracted from human plasma samples by liquid-liquid extraction technique. Samples were analyzed by reversed-phase liquid chromatography coupled with MS/MS detector. As per FDA and EMEA guidance [62, 65], the validation include; selectivity, lower limit of quantification (LLOQ), calibration range, accuracy, precision, matrix effects, stability of the analytes (amlodipine, valsartan and hydrochlorothiazide) in the biological matrix (plasma), and stability of the analytes and of the internal standards in the stock and working solutions and in extracts under the entire period of storage and processing conditions.

The materials used in this study include the following: The HPLC system was Agilent, model: 1200 series, USA. The detector was applied biosystems, MDS SCIEX, model: API 4000, USA. The computer system was windows 7 professional. The data management software was Analyst 1.6. The micropipettes were Eppendorf 20-200 µl, and 100-1000 µl, USA. The dispenser was Eppendorf 2-10 ml, USA. The centrifuge was Eppendorf, model: 5702 R, USA. The analytical balance was Sartorius, model: ME235S, Germany. The freezer was Frigidaire-20 ºC, USA. The refrigerator was Daewoo 2-8 °C, Korea.

The HPLC was optimized according to the following chromatographic conditions; flow rate (0.55 ml/min), column temperature (30 º C), autosampler temperature (10º C), injection volume (10 microliters), total run time (3.5 min), and column Ace 5 C8 (50*2.1) mm. The retention time of hydrochlorothiazide with its internal standard was 0.34 min, amlodipine with its internal standard was 1. 4 min and valsartan with its internal standard was 2.49 min. The HPLC system used was Agilents 1200 series coupled with API 4000, Applied Biosystems, MDS SCIEX detector; and computer system windows 7 professional. Data management was achieved by software Analyst 1.6.

As recommended by FDA and EMEA bioanalytical method validation guidance [62, 65], plasma samples were analyzed for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide after completing the clinical phase of the study (period II). Plasma samples obtained from each subject and for both periods (after test and reference products administration) were analyzed together, with the calibration curve including blank matrix and quality control (QC) samples, as one batch and in a single run. In each run, six quality control samples (dispersed evenly in a low-high and high-low sequence throughout the batch) were analyzed. No calculation was done for measuring concentration by extrapolation below the lower limit of quantitation (LLOQ) and above the upper limit of quantitation (ULOQ) of the standard calibration curve.

**Pharmacokinetic (PK) analysis**

Plasma concentration-time data of each individual was analyzed by Kinetica software (v5.1) to calculate the PK parameters applying non-compartmental method [68]. The actual sampling times of blood were used for PK analysis. The PK parameter $C_{max}$ (maximum concentration of drug in plasma) was obtained directly from concentration versus time profile of each individual. The $T_{max}$ (time to attain $C_{max}$) was obtained from concentration versus time curves. The $AUC_{0-t}$ (area under plasma concentration-time curve from time zero to $t_{last}$) was calculated by trapezoidal rule. The $AUC_{t-\infty}$ (extrapolated area under the plasma concentration-time curve from $t_{last}$ to infinity was measured as $C_{last}/\lambda_z$. The $AUC_{0-\infty}$ (total area under the plasma concentration-time curve from time zero to infinity) was calculated from the sum of $AUC_{0-t}$+$AUC_{t-\infty}$. The $\lambda_z$ (first order terminal elimination rate constant) was estimated by linear regression of not less than 3 points of the last points at the terminal phase of the log-concentration versus time plot of each subject. The $T_{0.5}$ (first-order terminal elimination half-life) was calculated as $0.693/\lambda_z$. The $C_{last}$ is the last measurable concentration of the drug which meets or exceeds the lower limit of quantification (LLOQ). The $t_{last}$ is the time at which $C_{last}$ occur. Since amlodipine is a drug of long terminal elimination half-life which is about 30-50 h, thus truncated $AUC_{0-72}$ was used, as per international guidance on bioavailability and bioequivalence concerning long elimination half-lives drugs [60-64].

**Statistical analysis**

Statistical data analysis was achieved using kinetica software (v5.1). Descriptive statistics including arithmetic mean, geometric mean, standard deviation (±SD) and coefficient of variation (CV) were

done. For the purpose of bioequivalence testing, analysis of variance (ANOVA) and 90% confidence interval were applied. ANOVA was carried out to account for the effects of treatment, period, sequence and subjects nested in sequence on the PK parameters; $C_{max}$, $T_{max}$, $AUC_{0-t}$, $AUC_{0-\infty}$, $\lambda_z$ and $T_{1/2}$. Besides, ANOVA was also executed for the Ln-transformed values of the parameters; $C_{max}$, $AUC_{0-t}$, $AUC_{0-\infty}$ and ($AUC_{0-72\ h}$ for amlodipine). The difference between PK parameters of the test (T) and the reference (R) drug products were declared statistically insignificant at 5% significance level ($\propto = 0.05$) when $P \geq 0.05$. Evaluation of bioequivalence was based on 90% confidence interval (CI) for geometric mean ratios T/R of the primary PK parameters used for bioequivalence evaluation (namely $C_{max}$ and AUC) of amlodipine, valsartan, and hydrochlorothiazide [65-67, 74]. Both products were concluded bioequivalent if 90% CI range between 80-125%, as recommended by international guidance [60-64, 69]. The mean±SD for plasma concentrations-time data and the log-mean values were plotted in regular and semilog graph types, respectively using excel software.

## RERSULTS AND DISCUSSION

### Subjects demography

As per study protocol, forty subjects were selected to participate in the study; however, one subject was dropped out before drug administration in period I due to clinical investigator decision. The demographic characteristics of the thirty-nine subjects participated in the study were as follow; mean age 31 y (range 18-46), mean weight 76 kg (range 54-95), mean height 1.73 meters (range 1.60-1.90), and mean BMI 25.1 (range 18.7-29.7).

### Safety assessment

The test and reference drug products were generally safe and well tolerated by all subjects. No incidence of adverse events or serious adverse events was registered during the study. All subjects who participated in the investigation (thirty-nine subjects) had successfully completed the study without any drop out or withdrawal. Clinical examinations and clinical laboratory test before subject discharge showed no clinically significant changes in the base line.

### Bioanalytical method validation

The newly developed HPLC-MS/MS method used in the present investigation was rapid, sensitive, precise, accurate and selective/specific for simultaneous determination of amlodipine, valsartan, and hydrochlorothiazide in human plasma. Therefore, the current method can be reliably and successfully applied to analyze a large number of plasma samples obtained from patients receiving fixed-dose triple combination of these drugs for pharmacokinetic (PK), bioavailability (BA) and bioequivalence (BE) studies.

The method was proved to be specific/selective since no interferences from endogenous plasma samples were found. Concerning amlodipine assay in plasma samples, the validation results are summarized as follow: the detection limits were 0.2-12 ng/ml with a lower limit of quantification (LLOQ) of 0.2 ng/ml. The linearity and calibration curve range were established for concentrations range of 0.2-12 ng/ml with high coefficient of determination ($R^2$) of 0.9987. The within run accuracy ranged 93.78-101.89 % and for LLOQ it was 106.50%. The between run accuracy was 91.46-99.92% and it was107.47% for LLOQ. The within-run precision coefficient of variation (% CV) ranged 2.67-5.56, and for LLOQ it was 8.87 The between-run precision (%CV) was between 3.98-5.78 and 7.63 for LLOQ. Besides, no considerable effect of the matrix on amlodipine and its internal standard was detected.

The validation of valsartan assay in plasma samples are summarized as follow: the detection limits were 50-8000 ng/ml with LLOQ of 50 ng/ml. The linearity and calibration curve range were established for the concentrations range of 50-8000 ng/ml with high ($R^2$ =0.9996). The within-run accuracy was between 95.73-102.07 % and for LLOQ it was 97.74%. The between-run accuracy was between 97.07-99.92 %, and it was 103.04% for LLOQ. The within-run precision (% CV) was between 1.11-4.01, and for LLOQ it was 3.43. The between-run precision (%CV) ranged 2.43-3.60, and it was 5.38 for LLOQ. Moreover, no remarkable effect of the matrix on valsartan and its internal standard was found.

The main validation characteristics of hydrochlorothiazide assay in plasma samples are: the detection limits were 2.0-250.0 ng/ml with LLOQ of 2 ng/ml. The linearity and calibration range was 2.0-250.0 ng/ml and $R^2$ was high (0.9991). The within-run accuracy was between104.28-108.14 %, and for LLOQ it was 100.85%. The between-run accuracy ranged100.31-103.99 %, and it was 106.04 % for LLOQ. The within-run precision (CV %) was between 1.15-8.0 and for LLOQ it was 5.97. The between-run precision (CV %) was between 4.70-5.94, and 11.40 for LLOQ. Furthermore, No significant effect of the matrix on hydrochlorothiazide and its internal standard was observed.

The results of stability studies of the analytes (amlodipine, valsartan, and hydrochlorothiazide) in the biological matrix (plasma), and the stability of the analytes and their internal standards in the stock and working solutions and in extracts under the entire period of storage and processing conditions were within the acceptable ranges. The long-term stability of the analytes at-20º C covered the period from the first blood sample withdrawal from the subjects and until the date of last sample assay of the analytes. The long-term stability for recovered amlodipine was 99.42-101.00%, for valsartan 86.79-99.87%, and for hydrochlorothiazide 93.26-96.75% which is very acceptable.

### Plasma concentrations of the drugs

The mean±SD for plasma concentrations-time data and the corresponding log-mean values for amlodipine, valsartan and hydrochlorothiazide are depicted in regular and semilog graph types as shown in fig. 1-6. The mean plasma concentration-time profiles of the test product were almost similar and superimposable with the reference product for the three components (amlodipine, valsartan, and hydrochlorothiazide) suggesting equivalent absorption and disposition (distribution and elimination) characters (fig. 1-6).

The drugs amlodipine, valsartan, and hydrochlorothiazide were not detected in any plasma sample obtained before drug products administration (zero/blank sample) of any subject which insures the absence of carryover effects, and indicating that a washout period of two weeks is sufficient for almost complete removal of the drugs from the body. Thus, reliable estimation of concentrations of these drugs in plasma and their pharmacokinetics could be guaranteed.

The following blood sampling strategy (0, 0.33, 0.66, 1.0, 1.33, 1.66, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 11, 12, 14, 16, 24, 48 and eventually at 72 h post dosing) performed in this investigation was adequate enough to show the entire pharmacokinetic behaviors of the drugs (amlodipine, valsartan, and hydrochlorothiazide) including absorption, distribution and elimination. The sampling schedule was early enough to insure reliable estimation of the absorption phase/rate, and the onset of appearance of the drugs in plasma. The sampling was very frequent in order to detect and show any fluctuation (if any) in the concentration of the investigated drugs in plasma. Besides, the sampling was for an adequate time (72 h post dosing) in order to insure reliable estimation of the terminal elimination half-lives of valsartan, and hydrochlorothiazide. Concerning amlodipine, since it is long half-life drug (30-50 h), thus, blood sampling for 72 h post dosing is acceptable as recommended by international guidance for bioequivalence [60-64].

After administration of the test product, amlodipine was detected in plasma samples after 40 min post dosing in 25 subjects out of the total 39 subjects participated in the study. The same observations were noticed after administration of the reference product in which the drug was detected in 21 subjects after 40 min post dosing. The drug was detected in all subjects and for both products after 1 hour after administration. This indicates the close similarity in the rate of absorption of amlodipine from both products. Besides, this finding demonstrates rapid appearance of the drug in the systemic circulation after oral administration. Interestingly, similar absorption behaviors were also noticed for valsartan and hydrochlorothiazide and for both products.

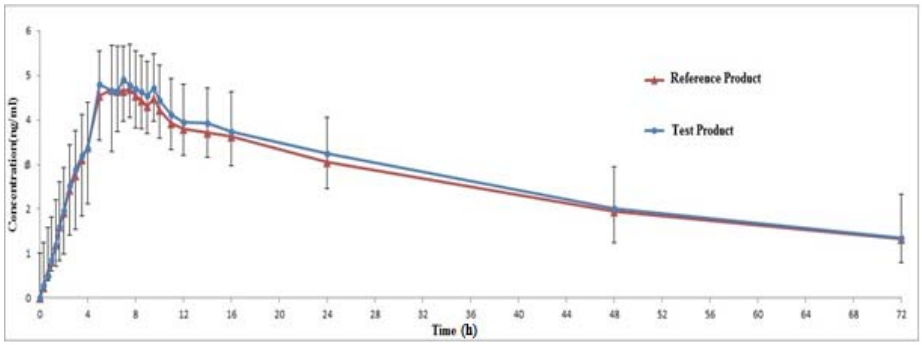


**Fig. 1: Amlodipine plasma concentrations-time profile after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects. Data points represent** mean±**SD (N=39)**

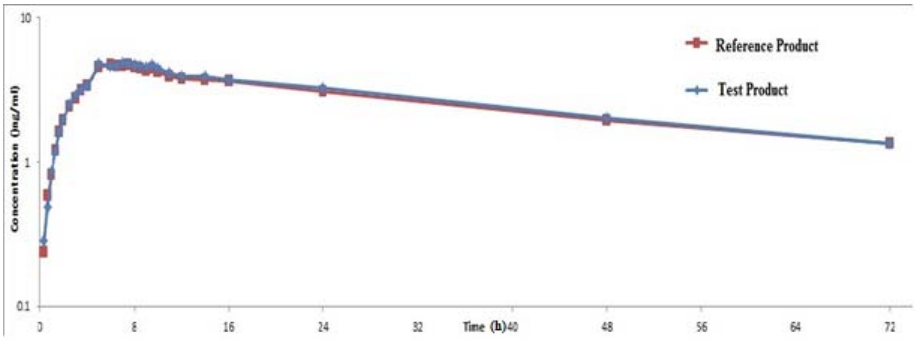


**Fig. 2: Amlodipine mean log plasma concentrations-time profile after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects**

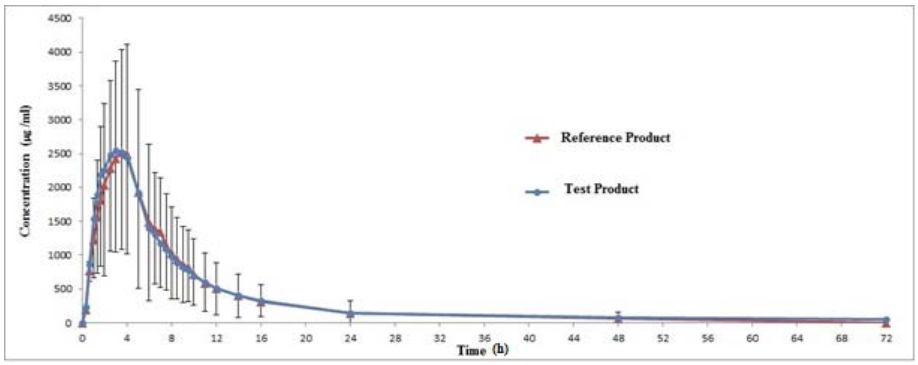


**Fig. 3: Valsartan plasma concentrations-time profile after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects. Data points represent** mean±**SD (N=39)**

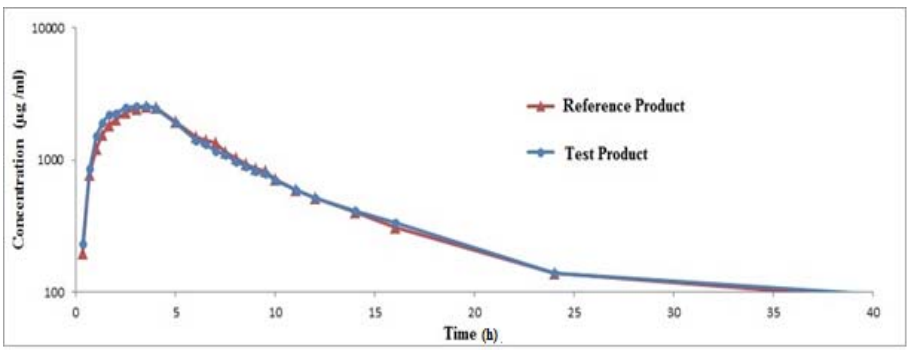


**Fig. 4: Valsartan mean log plasma concentrations-time profile after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects**

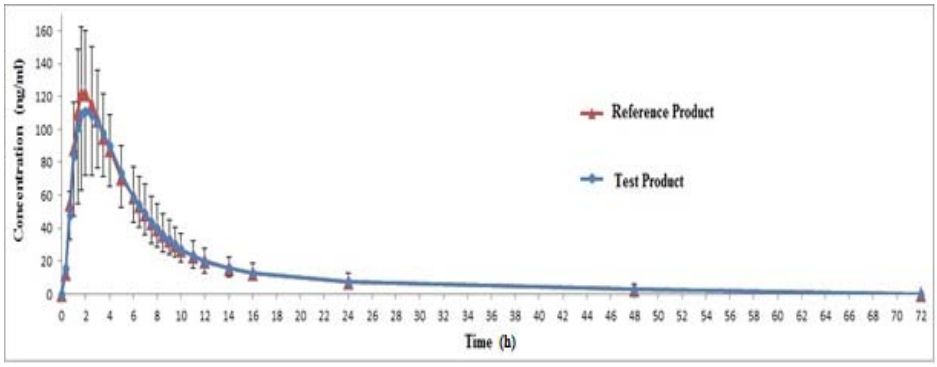


**Fig. 5: Hydrochlorothiazide plasma concentrations-time profile after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects. Data points represent** mean±**SD (N=39)**

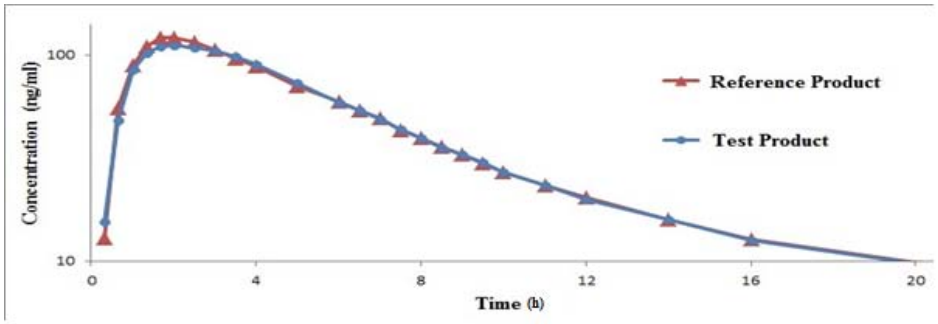


**Fig. 6: Hydrochlorothiazide mean log plasma concentrations-time profile after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects**

**Pharmacokinetic (PK) characteristics**

The average PK parameters obtained from PK analysis of the individual plasma concentration versus time data of the reference and the test products for amlodipine, valsartan and hydrochlorothiazide are summarized in tables 1-3. The results indicate that both products demonstrate almost similar PK behaviors. The primary PK parameters which reflect the rate of drug absorption (namely $C_{max}$ and $T_{max}$) indicates that both products exhibit almost similar absorption behaviors. Besides, the primary PK parameters which reflect the extent of drug absorption and the systemic exposure (namely $C_{max}$ and AUC) were approximately identical. Moreover, the terminal phase/decay of the concentration-time profiles of both products are parallel indicating similar elimination behaviors of both products. Thus, both test and reference formulations are equivalent in term of absorption from GIT (rate and extent) and disposition regarding all components amlodipine, valsartan, and hydrochlorothiazide.

Moreover, the interindividual variations in the PK parameters of amlodipine (table 1), valsartan (table 2) and hydrochlorothiazide (table 3) of the test product are almost similar to the corresponding parameters of the reference product. Valsartan (table 2) demonstrated high intersubject variability (%CV about 50%) in all PK parameters. Hydrochlorothiazide elucidated %CV range of 20-50% (table 2). Whereas, amlodipine showed relatively low %CV of about 20% (table 1).

For both valsartan (table 2) and hydrochlorothiazide (table 3), the % $AUC_{extrapolated}$

($AUC_{t-\infty}/AUC_{0-\infty}$ x 100) in all subjects and for both test and reference products had very low contribution to the total AUC since % $AUC_{extrapolated}$ was less than 10%. Thus, blood sampling time for 72 h post dosing, and a lower limit of quantitation of 50 ng/ml for valsartan and 2 ng/ml for hydrochlorothiazide used in this study are adequate enough for PK, BA and BE studies of these drugs.

Many PK, BA, and BE studies were conducted for amlodipine administered alone [24, 26, 30-38], valsartan alone [39-41] or hydrochlorothiazide alone [43, 44]. Besides, PK, BA, and BE investigations were also achieved for combination of two or three drugs, such as amlodipine with olmesartan [25], amlodipine with valsartan [27-29], or valsartan with hydrochlorothiazide [14, 42]. However, very limited PK, BA or BE information is presented in literature after a single dose administration of fixed dose combination of amlodipine, valsartan, and hydrochlorothiazide tablet [1]. Comparing the results obtained from the current investigation (table 1) with that presented in literature after a single oral doses of amlodipine show some differences in $C_{max}$ and AUC, whereas good similarity is observed for $T_{max}$ and $T_{1/2}$ The mean $C_{max}$ found in the current study is about 5.0 ng/ml (table 1). The average $C_{max}$ obtained in other investigations are; about 6 ng/ml [24, 27, 29, 31, 32], 7 ng/ml [25, 30], 4 ng/ml [26, 36], 2 ng/ml [33], 5 ng/ml [34], and about 8 ng/ml [35]. The average $AUC_{0-72\ h}$ calculated in this study is approximately 180 ng. hr/ml (table 1). Previous investigations showed mean values of about 216 ng. hr/ml [24], 245 ng. hr/ml [25], and 190 ng. hr/ml [27]. The higher differences in average $C_{max}$ values found between studies relative to the differences in AUC is expected from PK point of view since $C_{max}$ is a single data point in comparison to AUC which represent the whole concentration-time profile, in addition to individual variations. The average $T_{max}$ and $T_{1/2}$ found in the current study are about 7 and 40 h, respectively (table 1); which are almost similar to that obtained from many previous investigations [24-35, 37].

The mean $C_{max}$ of valsartan found in this investigation is about 3000 ng/ml and the mean total AUC is about 20000 ng. hr/ml (table 1). The approximate $C_{max}$ and total AUC values presented in other studies were as follow: 4000 ng/ml and 43000 ng. hr/ml [14], 5000 ng/ml and 33000 ng. hr/ml [39], 3500 ng/ml and 23000 ng. hr/ml [27], 5000 ng/ml and 35000 ng. hr/ml [28], 3800 ng/ml and 28000 ng. hr/ml [29], 2000 ng/ml and 24000 [40]. It is obvious from these results that valsartan exhibit some individual variation in these PK parameters. On the other hand, The average $T_{max}$ and $T_{1/2}$ found in this study are about 3 and 10 h, respectively (table 1); which are almost similar to that obtained from many other investigations [14, 27-29, 39, 40].

The average $C_{max}$ of hydrochlorothiazide found in the current study

is about 130 ng/ml and the average total AUC about 1000 ng/ml. The reported average $C_{max}$ were about 150 ng/ml [14] and 170 ng/ml [44], the reported total AUC were approximately 1800 ng. hr/ml [14] and 1200 ng. hr/ml [44]. This difference in these parameters is attributed mainly to individual variability. The average $T_{max}$ and $T_{1/2}$ obtained in this study are about 2 and 9 h, respectively which are almost identical to that presented in a previous study [14].

**Table 1: Pharmacokinetic parameters of Amlodipine after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg tablet) and a test product to thirty-nine healthy male adult subjects**

| Parameter | | Test product | Reference product |
|---|---|---|---|
| $C_{max}$ | Geometric Mean | 5.408 | 5.214 |
| (ng/ml) | Arithmetic Mean | 5.491 | 5.302 |
| | ±SD (1) | 0.9940 | 0.9529 |
| | CV % (2) | 18.10 | 17.97 |
| | N (3) | 39 | 39 |
| $AUC_{0-72}$ | Geometric Mean | 186.145 | 179.468 |
| (ng. hr/ml) | Arithmetic Mean | 190.083 | 182.867 |
| | ±SD | 40.2090 | 34.5963 |
| | CV % | 21.15 | 18.92 |
| | N | 39 | 39 |
| $T_{max}$ | Median | 7.00 | 7.00 |
| (hrs.) | Range | 5.0-10.0 | 4.0-16.0 |
| | Arithmetic Mean | 6.83 | 6.96 |
| | ±SD | 1.695 | 2.199 |
| | CV % | 24.80 | 31.58 |
| | N | 39 | 39 |
| Lz | Geometric Mean | 0.01828 | 0.01755 |
| (1/hr.) | Arithmetic Mean | 0.01864 | 0.01814 |
| | ±SD | 0.003728 | 0.004402 |
| | CV % | 20.00 | 24.26 |
| | N | 39 | 39 |
| $T_{½}$ | Arithmetic Mean | 38.69 | 41.16 |
| (hrs.) | ±SD | 8.046 | 13.898 |
| | CV % | 20.80 | 33.77 |
| | N | 39 | 39 |

(1)SD=StandardDeviation.(2)CV%=CoefficientofVariation.(3)N=Numberofsubjects

**Table 2: Pharmacokinetic parameters of Valsartan after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects**

| Parameter | | Test product | Reference product |
|---|---|---|---|
| $C_{max}$ | Geometric Mean | 2545.442 | 2637.339 |
| (ng/ml) | Arithmetic Mean | 2981.250 | 3032.849 |
| | ±SD (1) | 1593.5828 | 1622.4859 |
| | CV % (2) | 53.45 | 53.50 |
| | N (3) | 39 | 39 |
| $AUC_{0-t}$ | Geometric Mean | 18019.463 | 17450.671 |
| (ng. hr/ml) | Arithmetic Mean | 20809.792 | 20098.508 |
| | ±SD | 11259.1828 | 10785.8111 |
| | CV % | 54.11 | 53.66 |
| | N | 39 | 39 |
| $AUC_{0-\infty}$ | Geometric Mean | 19046.778 | 19337.613 |
| (ng. hr/ml) | Arithmetic Mean | 21916.131 | 21825.207 |
| | ±SD | 11661.7626 | 10866.0725 |
| | CV % | 53.21 | 49.79 |
| | N | 38 | 37 |
| $T_{max}$ | Median | 3.00 | 3.00 |
| (hrs.) | Range | 1.33-7.0 | 1.0-5.0 |
| | Arithmetic Mean | 2.98 | 2.90 |
| | ±SD | 1.220 | 1.120 |
| | CV % | 40.90 | 38.62 |
| | N | 39 | 39 |
| Lz | Geometric Mean | 0.10554 | 0.11103 |
| (1/hr.) | Arithmetic Mean | 0.11504 | 0.11483 |
| | ±SD | 0.042384 | 0.027181 |
| | CV % | 36.84 | 23.67 |
| | N | 38 | 37 |
| $T_{½}$ | Arithmetic Mean | 7.50 | 6.52 |
| (hrs.) | ±SD | 5.164 | 2.261 |
| | CV % | 68.85 | 34.68 |
| | N | 38 | 37 |

**Table 3: Pharmacokinetic parameters of Hydrochlorothiazide after a single dose administration of the reference product (Exforge HCT® containing fixed-dose combination of Amlodipine 10 mg, Valsartan 160 mg, and HCT 25 mg) and a test product to thirty-nine healthy male adult subjects**

| Parameter | | Test product | Reference product |
|---|---|---|---|
| $C_{max}$ | Geometric Mean | 122.304 | 129.257 |
| (ng/ml) | Arithmetic Mean | 127.235 | 133.435 |
| | ±SD | 36.1699 | 34.6043 |
| | CV % | 28.43 | 25.93 |
| | N | 39 | 39 |
| $AUC_{0-t}$ | Geometric Mean | 849.526 | 857.493 |
| (ng. hr/ml) | Arithmetic Mean | 874.028 | 888.595 |
| | ±SD | 221.4740 | 245.0147 |
| | CV % | 25.34 | 27.57 |
| | N | 39 | 39 |
| $AUC_{0-\infty}$ | Geometric Mean | 926.286 | 938.198 |
| (ng. hr/ml) | Arithmetic Mean | 950.370 | 968.633 |
| | ±SD | 224.1029 | 248.9472 |
| | CV % | 23.58 | 25.70 |
| | N | 38 | 39 |
| $T_{max}$ | Median | 2.00 | 1.66 |
| (hrs.) | Range | 0.66-6.5 | 1.0-5.0 |
| | Arithmetic Mean | 2.24 | 1.92 |
| | ±SD | 1.142 | 0.756 |
| | CV % | 51.06 | 39.46 |
| | N | 39 | 39 |
| Lz | Geometric Mean | 0.08100 | 0.07561 |
| (1/hr.) | Arithmetic Mean | 0.08231 | 0.07694 |
| | ±SD | 0.014778 | 0.014284 |
| | CV % | 17.95 | 18.56 |
| | N | 38 | 39 |
| $T_{½}$ | Arithmetic Mean | 8.70 | 9.34 |
| (hrs.) | ±SD | 1.614 | 1.875 |
| | CV % | 18.55 | 20.08 |
| | N | 38 | 39 |

**Table 4: Statistical analysis of pharmacokinetic parameters**

| Parameter | Ratio of geometric mean (%) | | | 90% confidence interval (%) | | | $F^{(1)}$(%) | |
|---|---|---|---|---|---|---|---|---|
| | $C_{max}$ | $AUC_{0-t}$ | $AUC_{0-\infty}$ | $C_{max}$ | $AUC_{0-t}$ | $AUC_{0-\infty}$ | $AUC_{0-t}$ | $AUC_{0-\infty}$ |
| Amlodipine | 103.73 | 103.72 | - | 99.30–108.37 | 100.95–106.56 | - | 103.9 | - |
| Valsartan | 96.52 | 103.26 | 100.44 | 82.06–113.51 | 89.83–118.69 | 87.26–115.61 | 103.5 | 100.4 |
| Hydrochlorothiazide | 94.62 | 99.00 | 98.74 | 87.97–101.77 | 94.01–104.27 | 93.94–103.79 | 98.4 | 98.1 |

(1)F: Ratio of arithmetic means (relative bioavailability)

## CONCLUSION

The current investigation demonstrated that the pharmacokinetic characteristics of a newly developed generic tablet containing fixed-dose combination of amlodipine/valsartan/hydrochlorothiazide (10/160/25 mg) are clearly similar to the brand product Exforge HCT® tablet manufactured by Novartis, Switzerland. The rate and extent of bioavailability of both products are identical. The range of 90% CI of $C_{max}$ and AUC for amlodipine, valsartan, and hydro-chlorothiazide were well within BE acceptance range of 80-125. Thus, both products is bioequivalent and clinically interchangeable.

Besides, the present study present outline information of a newly developed HPLC-MS/MS bioanalytical method for simultaneous determination of amlodipine, valsartan, and hydrochlorothiazide in human plasma after administration of fixed-dose combination tablet containing amlodipine/ valsartan/hydrochlorothiazide (10/160/25 mg). The method is validated according to FDA and EMEA bioanalytical method validation guidance. The method is proved to be rapid, sensitive, precise, accurate and specific/ selective. Therefore, this method can be successfully applied for measuring large number of plasma samples containing these drugs together to be used for pharmacokinetic, bioavailability, and bioequivalence studies.

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## CONFLICT OF INTERESTS

Declared none

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