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Pharmacokinetics of 600 mg loading dose of clopidogrel in patients undergoing percutaneous coronary intervention

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Clopidogrel is an oral antiplatelet drug. Loading dose of 600 mg clopidogrel was shown to improve clinical outcome in patients following percutaneous coronary intervention (PCI). Wide inter-individual variation has been detected in clopidogrel response that can be related to variation in Clopidogrel serum concentration. The aim of this study was to assess the pharmacokinetics parameters of 600 mg loading dose clopidogrel among Jordanian patients undergoing PCI. Additionally, the development of a simple and a validated High-performance liquid chromatography (HPLC) method for the quantification of clopidogrel carboxylate was described. 80 patients who received a loading of 600 mg Clopidogrel were included in our study, several blood samples were collected at different time points. Validated reverse phase HPLC method was used to determine Clopidogrel carboxylic acid metabolite. Non-compartmental analysis was used to determine peak plasma concentration (C_{max}), time to peak plasma concentration (T_{max}), elimination half-life ($t_{1/2e}$), and area under the curve (AUC). The pharmacokinetic-parameters were characterized by considerable inter-individual differences [C_{max}=24.49±11.64 µg/ml, T_{max}=2.02±1.52 h, $AUC_{0\rightarrow\infty}$= 123.17± 54.6 mg/ml.h, and t1 / 2e=4.29±2.92 h]. 15% of the patients who had less than one third of the $_{Cmax}$ 8.09±2.34 µg/ml had delayed T_{max} of 4.17±1.76 h, which was not explained by standard in vitro dissolution test. Pharmacokinetic parameters of 600 mg Clopidogrel showed marked inter-individual differences. The low plasma concentrations in some of the patients and the high inter-individual variability may contribute to reported cases of resistance to Clopidogrel therapy. Further studies are needed to explain low C_{max} and delayed T_{max} values in some patients.

Key words: Clopidogrel carboxylic acid, loading dose, percutaneous coronary intervention, reverse phase High-performance liquid chromatography (HPLC), pharmacokinetics.

INTRODUCTION

Clopidogrel, a thienopyridine antiplatelet agent, is marketed worldwide as Plavix®/Iscover® and is used in the prevention of myocardial infarction, stroke and death in patients with acute coronary syndromes. In patients

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undergoing percutaneous coronary intervention (PCI), pretreatment with a loading dose of clopidogrel (300 to 600 mg) was shown to improve clinical outcome (Steinhubl et al., 2002; Lotrionte et al., 2007).

Clopidogrel is an inactive prodrug that is converted by hepatic bio-transformation via cytochrome P450 (CYP) pathway. Two sequential CYP-dependent oxidative steps are required to convert clopidogrel to its active metabolite (a thiol compound) that react with and irreversibly inhibit the platelet P_2Y_{12} ADP receptor, which is involved in platelet activation and stabilization of the platelet aggregate (Savi et al., 2000; Sugidachi et al., 2000). The active metabolite is extremely labile and unstable and it remained undetected in plasma for many years (Pereillo et al., 2002) until recently (Takahashi et al., 2008).

Though, the quiet nature of the active metabolite and its determination is still not settled. The published dependable pharmacokinetic parameters of clopidogrel are related to its inactive carboxylic acid metabolite that represents approximately 85% of the total amount. Since neither the parent drug nor the active metabolite is easily detected in plasma, the quantification of carboxylic acid metabolite would be an indirect approach for studying the pharmacokinetics of clopidogrel (Caplain et al., 1999; Singh et al., 2005). High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, have been used widely to determine the inactive metabolite of clopidogrel in the biological fluids (Bahrami et al., 2008). Recently, several liquid chromatography-tandem mass spectrometry (LC-MS-MS) methods for the study of pharmacokinetics of clopidogrel parent compound and the inactive carboxylic metabolite have also been published (Ksycinska et al., 2006). Whatever the method to detect clopidogrel parent compound or its metabolites, a high inter-individual variation has been noticed in both clopidogrel response (Muller et al., 2003; Angiolillo et al., 2005) and clopidogrel concentrations in plasma. This poor response to clopidogrel is associated with an increased risk of recurrent ischemic events (Gurbel et al., 2005). It has been suggested that the high variability in response to clopidogrel is caused by variation in pharmacokinetics parameters that could be caused by different level of activity of the activation enzymes (Heestermans et al., 2006; Brandt et al., 2007; Mega et al., 2009). The aim of this study was to investigate the pharmacokinetics of clopidogrel 600 mg loading dose in Jordanian patients undergoing PCI by measuring the inactive carboxylic acid metabolite. To the best of our knowledge, this is the first study to evaluate pharmacokinetics of 600 mg clopidogrel among relatively large number of patients undergoing PCI.

MATERIALS AND METHODS

Clopidogrel carboxylic acid was obtained from Toronto Research Chemicals in Canada. Hydrochlorothiazide (internal standard, IS) was obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and methanol were HPLC-grade and purchased from ACRÕS (Darmstadt, Germany). All other chemicals and solvents (phosphoric acid 85%, perchloric acid, sodium hydroxide) were gradient grade and used without any further purification (Merk, Darmstadt, Germany). Plavix® 75 mg tablets containing clopidogrel were obtained from Sanofi-Aventis France (batch no. 2273).

Study population

Patients who were admitted to Jordan University Hospital for catheterization and underwent PCI were eligible for the entry into this study if they were given 600 mg of clopidogrel (8 tablets of plavix®). Patients were excluded if they were known to have hepatitis B infection or carrier of respective antigen; donated blood within last 2 months; have allergic diathesis or any significant allergic disease; have gastro intestinal (GI) diseases or hepatic disease; were pregnant; have been on statin that has been altered within 14 days; have creatinine Cl < 25 ml/min; or diagnosed with heart failure with New York Heart Association (NYHA) class 4. Participants were classified into overweight (BMI ≥ 25 kg/m^2) or normal weight (BMI < 25 kg/m^2). This stratification agrees with the definition of overweight patients by the World Health Organization (Willett et al., 1999). The study was approved by local Research Ethics Committees of the Jordan University Hospital and informed consent was obtained from all participants after having been informed verbally by the medical supervisor about the need to withdraw extra blood samples for pharmacokinetic analysis. The decision to give or not to give 600 mg clopidogrel was solely the responsibility of the treating cardio-surgeon performing the PCI.

Intervention and sample collection

Whole blood samples from patients were drawn into heparinized test tubes immediately before (0) and at 20, 40, 60, 120, 240 min and 8 h after the intake of clopidogrel. Blood was centrifuged at 4000 g for 5 min and plasma samples were separated and immediately stored at -80°C until analysis. Perchloric acid ($HCLO_4$) (50 µl of 1%) was added to plasma (100 µl). Internal standard was then added (50 µl of 15.0 µg/ml hydrochlorothiazide), the mixture was vortex-mixed, then methyl-tert-butyl ether (3.0 ml) was added, vortexed for 6.0 min and then centrifuged for 10.0 min at 4,400 rounds per min (rpm). The organic layer was quantitatively transferred to another test tube and a volume of 200 µl of 50 mM sodium hydroxide (NaOH) was added and vortex-mixed for 1.0 min. After 7 min of centrifugation at 4,400 rpm, the lower layer was carefully transferred to 350 µl flat bottom insert and 25 µl of this sample was injected on LiChosphere RP-select B column (5 µm, 150 × 4.6 mm). Clopidogrel metabolite and the internal standard were separated from endogenous substances.

Chromatographic conditions

The plasma level of clopidogrel carboxylic acid were determined by reverse-phase high-performance liquid chromatographic method, where the separation achieved using isocratic mobile phase consisted of 80% of 5 mM potassium dihyrogen phosphate (KH_2PO_4) and 20% acetonitrile at pH = 3 prepared daily and degassed by passing through a 0.45 µm filter. A Dionex HPLC auto-sampler system was used and composed of the following: a constant solvent delivery system (P580); a 100 µl fixed volume injector [Rheodyne 7125; UV Detector (UVD340S)]; autosampler (ASI-100) and operated by Microsoft Windows 2000. Flow rate was 1.0 ml/min, and pressure was 70 bar. All separations were performed at room

temperature. Detection was monitored at 220 nm.

Validation of analytical methods

Specificity/selectivity

Specificity was verified by the absence of any co-eluted peak of endogenous plasma components at the retention times of the clopidogrel carboxylic acid and the internal standard. Moreover, selectivity was affirmed by the absence of interference from commonly used drugs (aspirin, acetaminophen, ascorbic acid, caffeine, nicotine and ibuprofen).

Linearity

Standard calibration curves of 8 points (0.5, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0 µg/ml) in addition to the blank sample and standard zero sample were prepared. In each day of the validation course, a calibration curve was prepared and its goodness of fit was calculated by weighted least square linear regression equation (Strutz, 2011). The sample preparation and HPLC analysis were performed as described. Calibration curves were constructed by plotting the measured peak area ratios of metabolite to the internal standard (IS) versus concentrations of standard samples, and statistical analysis was performed. The lowest standard concentration in the calibration curve is considered the lower limit of quantification (LLOQ), given that its detector's response is at least three times that of the blank.

Accuracy and precision

Accuracy and precision evaluation was held over the first three days of the validation time course employing the regression of the calibration curve that was carried out at the same day. To establish the intra-day and inter-day accuracy and precision of the method, three replicates of standard plasma solutions at three different concentrations (1.5, 20.0 and 40.0 µg/ml) were assayed per day, for two consecutive days. Four replicates of standard plasma solutions were assayed on the third day.

Recovery

The absolute recoveries were calculated for clopidogrel carboxylic acid and hydrochlorothiazide by comparing the relevant peak areas of the extracted and unextracted samples. The absolute analytical recovery of clopiogrel carboxylic acid was calculated at three different concentrations (1.5, 20.0, 40.0 µg/ml), while for the internal standard, the nominal concentration (4.5 µg/ml) was used.

Pharmacokinetic calculations

The pharmacokinetic parameters of clopidogrel carboxylic acid metabolite were estimated by standard non-compartmental methods using Kinetica™ 2000 Version 4.2 (Innaphase, Philadelphia, PA, USA) computer program. The C_{max} and the T_{max} of clopidogrel carboxylic acid were taken directly from the measured data. The $AUC_{0\rightarrow t}$ was calculated from measured data points from the time of administration to the time of last quantifiable concentration (C_{last}) by linear trapezoidal rule. The following equations were utilized to calculate the remaining pharmacokinetic parameters (Gibaldi and Perrier, 1982).

$$AUC_{0\rightarrow\infty} = AUC_{0\rightarrow t} + \frac{C_{last}}{k_e}$$

$$t_{1/2e} = \frac{0.693}{-b}$$

$$\mathrm{AUMC}_{0\rightarrow\infty} = \sum_{i=0}^{n-1}\left(\frac{t_{i+1}-t_i}{2}(C_i t_i + C_{i+1}t_{i+1})\right) + \frac{C_{last}t_{last}}{k_e} + \frac{C_{last}}{k_e^2}$$

$$\frac{Vd}{F} = \frac{D}{k_e * \mathrm{AUC}_{0\rightarrow\infty}}$$

$$MRT = \frac{\mathrm{AUMC}_{0\rightarrow\infty}}{\mathrm{AUC}_{0\rightarrow\infty}}$$

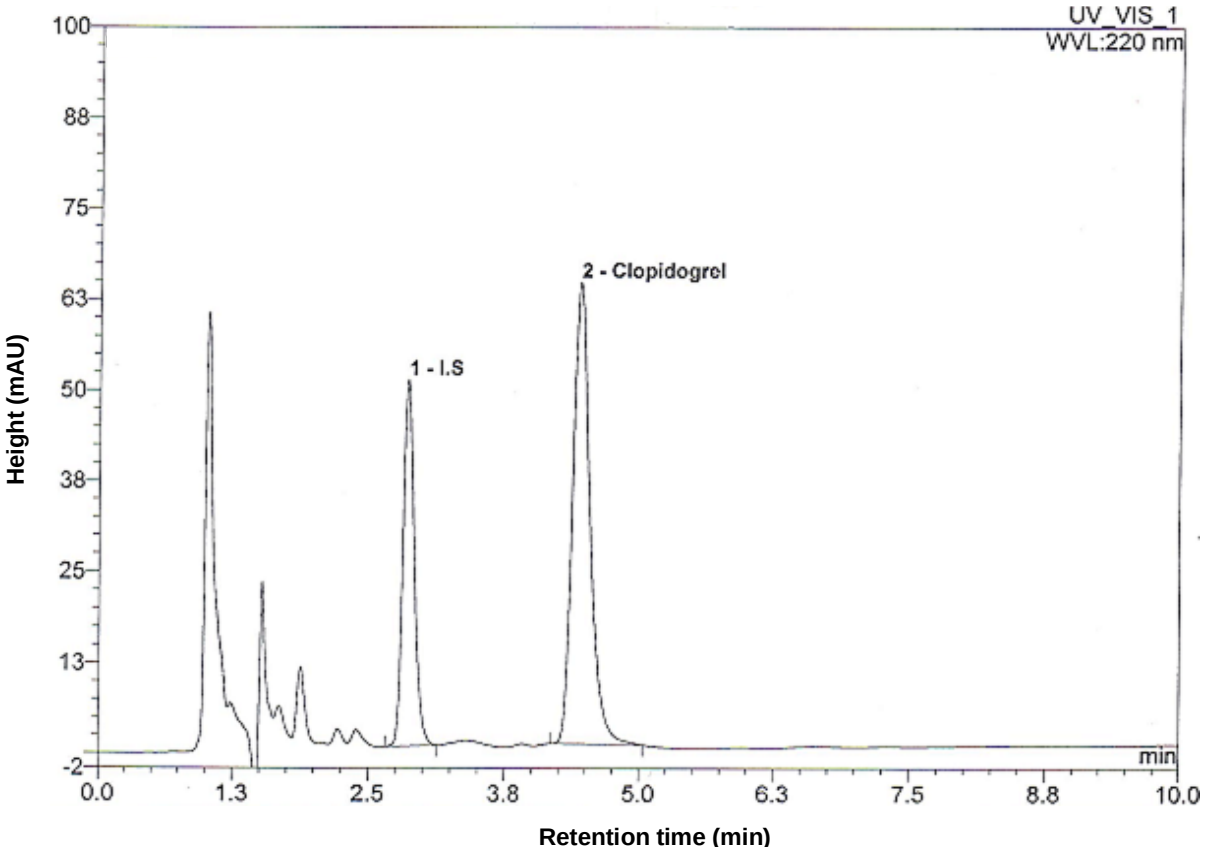
Where ($AUC_{0\rightarrow\infty}$) is the area under the plasma concentration-time curve extrapolated to infinity; k_e is the elimination rate constant; ($t_{1/2e}$) is the elimination half life; b is the slope of the linear regression of the Ln-transformed plasma concentration versus time in the terminal period of the plasma curve; ($AUMC_{0\rightarrow\infty}$) is the area under the first momentum curve; Vd is the apparent volume of distribution; F is bioavailability; and MRT is mean residence time.

Dissolution studies

The dissolution medium consisted of 900 ml of buffer solution, pH 2.0, prepared by dissolving 14.91 g of potassium chloride in 119.0 ml of 0.2 M hydrochloric acid and further diluted with water. The reference solution was prepared by dissolving 75.0 mg of clopidogrel working standard in 100.0 ml of methanol; 5.0 ml of this solution were diluted to 50.0 ml with the dissolution medium. The dissolution medium was thermostatically controlled at 37°C. The rotation speed of the paddles was 50 rpm. A volume of 10 ml was taken at 5, 10, 15, 20, 25, 30, 35, 40 and 60 min and analyzed in the spectrophotometer. Initially, the values obtained at 30 min were used to evaluate the differences between the samples. Based on the results of the reference and the acceptance criteria of the United States Pharmacopeia (USP) (USP, 2007), the Q-value was proposed to be 75%. This implied that the percentage of active ingredient dissolved after 30 min, for each of the six tablets examined might not be less than 80% (Q + 5%) of the theoretical clopidogrel content. If the tablets did not pass the test, another 6 units were examined. The result was satisfactory if the average of the 12 units was not less than 75% and no unit was less than 60%. If the tablets did not pass the test using these 12 units, another 12 units were tested. The batch was accepted if the average of the 24 units was not less than 75%; not more than 2 units were less than 60% and no unit was less than 50%.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS© software version 11.0; SPSS, Inc, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Coefficient of variation (CV) was calculated by (SD/mean × 100). Kruskal-Wallis test was utilized to detect differences in T_{max} and $t_{1/2e}$ while analysis of the variance (ANOVA) was used to detect the differences in C_{max}, $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$ among 3 categories.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount
1	2.85	I.S	50.415	6.038	33.51	n.a.
2	4.44	Clopidogrel	63.481	11.983	66.49	n.a.
Total:			113.896	18.021	100.00	0.000

Figure 1. HPLC chromatogram of carboxylic acid metabolite of clopidogrel and internal standard (IS). Plasma sample spiked with clopidogrel carboxylate metabolite and IS; Peaks: 1, IS; 2, clopidogrel carboxylate acid metabolite.

RESULTS

Chromatographic condition

Under the chromatographic conditions described, the drug and IS were well resolved in plasma samples and eluted at 4.44 and 2.85 min, respectively. No interfering peaks of endogenous plasma components were found at the retention time of metabolite or internal standard in blank plasma (Figure 1)

Method development and validation

Linearity

A linear relationship was obtained between the peak area ratio of metabolite and that of the internal standard versus the corresponding concentration over the range 0.5 to 50.0 µg/ml (Table 1).

Accuracy and precision

Precision and accuracy of intra-day and inter-day are presented in Table 2. Variation ranged from 3.05 to 5.97%, and accuracy ranged from 100.33 to 110.04%.

Extraction recovery

The recovery of metabolite and internal standard was determined. The mean recoveries of carboxylic acid metabolite of clopidogrel at concentrations of 1.5, 20.0

Table 1. Statistical data of calibration curves of carboxylic acid metabolite of clopidogrel in spiked human plasma.

Parameter	Clopidogrel metabolite
Range of linearity	0.5-50 µg/ml
Regression equation	Peak area ratio = 3.89×10^{-2} X + 1.48×10^{-2}
SD of slope	4.2×10^{-4}
RSD of slope	1.08%
SD of intercept	$6.2*10^{-3}$
Correlation coefficient	0.9997

SD: standard deviation, RSD: relative standard deviation

Table 2. Precision and accuracy of method for determination of carboxylic acid metabolite of clopidogrel in spiked plasma.

Concentration added (µg/ml)	Concentration found (mean ± SD) (µg/ml)	Precision as CV (%)	Accuracy (%)
Within-day (n = 3)			
1.5	1.62±0.06	3.95	108±4.27
20.0	20.71±1.02	4.95	103.56±5.12
40.0	40.81±2.35	5.75	102.03±5.86
Between-day (n = 10)			
1.5	1.65±0.06	3.65	110.04±4.00
20.0	20.07±1.20	5.97	100.33±5.99
40.0	41.39±1.26	3.05	103.46±3.16

and 40.0 µg/ml were 81.95, 79.2, and 81.9%, respectively with mean recovery of 80.3 ± 1.91. The mean recovery of internal standard was 77.48%.

Patients' characteristics

From a total of 100 patients who received 600 mg clopidogrel loading dose, only eighty patients were selected and accepted to take part in the study. Table 3 summarizes characteristics of recruited subjects and Table 4 summarizes their laboratory findings.

Pharmacokinetic parameters

The primary pharmacokinetic parameters of clopidogrel carboxylic acid metabolites are reported in Table 5. Figure 2a represents the average plasma concentration of clopidogrel carboxylate as it changes over time after oral administration of 600 mg of clopidogrel to patients undergoing PCI. The pharmacokinetic parameters of participants were characterized by considerably inter-individual differences [C_{max} = 24.49 ± 11.64 µg/ml (CV = 47.5%), T_{max} = 2.02 ± 1.52 h (CV = 75.2%), $AUC_{0\rightarrow\infty}$ = 123.17 ± 54.6 µg/ml × h (CV = 44.3%), and $t_{1/2e}$ = 4.29 ± 2.92 h (CV = 68%)]. The data were further subdivided into three groups based on the C_{max} value: "Lower 15%"; "Upper 15%" and the remaining data were termed "Trimmed". The three groups differed in their C_{max} ($p < 0.0001$, ANOVA), $AUC_{0\rightarrow t}$ ($p < 0.0001$, ANOVA), and $AUC_{0\rightarrow\infty}$ ($p < 0.0001$, ANOVA) (Table 5). The three groups did not differ with their $t_{1/2e}$ ($p = 0.44$, Kruskal-Wallis); AUMC ($p = 0.195$, Kruskal-Wallis); MRT ($p = 0.736$, Kruskal-Wallis); or Vd/F ($p = 0.61$, Kruskal-Wallis), though there was significant difference in T_{max} ($p<0.0001$, Kruskal-Wallis). The data indicate that the differences in C_{max} are not caused by changes in elimination rate among the three groups; rather it may be due to differences in absorption phase (extent and/or rate) (Figure 2b).

To evaluate the robustness of our findings, two other C_{max}-value dependent sub-classifications were assessed for pharmacokinetics parameters. The first one: "Lower 12.5%"; "Upper 12.5%" and the remaining data were termed "Trimmed". The second one: "Lower 17.5%"; "Upper 17.5%" and the remaining data were termed "Trimmed". In both cases, the three groups differed in their C_{max} ($p < 0.0001$, Kruskal-Wallis), $AUC_{0\rightarrow t}$ ($p < 0.0001$, Kruskal-Wallis), and $AUC_{0\rightarrow\infty}$ ($p < 0.0001$, Kruskal-Wallis). The three groups did not differ with their

Table 3. Demographic and clinical characteristics of recruited patients in current study as compared to published characteristics of Jordanian patients undergoing PCI.

Parameter	Number/mean (percent ± SD)	
	Current study (N=80)	**Typical patients (Yousef et al., 2008) (N=159)**
Age (years)	60.5 (±8.1)	57.6 (±10.2)
≥ 60	41 (52)	76 (47.8)
Gender		
Female	33 (41)	38 (24)
Body mass index (kg/m^2)	28.6 (±4.7)	28.3 (±4.3)
< 25	23 (28.8)	33 (21)
Smoking		
Current smokers	22 (27.5)	32 (20)
Quit < 12 months	3 (4)	19 (12)
Quit > 12 months	12 (15)	49 (31)
Does not smoke	43 (53.5)	59 (37)
Concomitant diseases	8 are medically free	
Hypertension	65 (81)	110 (69)
Diabetes mellitus	44 (55)	100 (63)
Dyslipidemia	32 (40)	118 (74)
Heart failure	10 (10)	25 (15.7)
Arrhythmia (include atrial fibrillation)	7 (8.5)	24 (15)
Peripheral vascular disease	4 (5)	24 (15)
Cerebrovascular accident-history	6 (7.5)	19 (11.9)
Myocardial infarction-history	12 (15)	94 (59)
Prior to admission medications history		
Aspirin	65 (81)	148 (93)
Clopidogrel	10 (12.5)	27 (17)
Beta adrenergic receptors blockers	48 (60)	78 (49)
ACEIs/ARBs	33/18 (41/22.5)	64/3 (40/2)
Diuretics	22 (27.5)	50 (31.4)
Calcium channel blockers	24 (30)	40 (25)
HMGCo-reductase inhibitors	50 (62.5)	110 (69.2)
Fibric acid derivatives	5 (6)	10 (6)
Proton pump inhibitors	23 (28.75)	16 (10)
H2 receptor blockers	16 (20)	40 (16)
Anti-diabetic agents (oral)	35 (43.75)	60 (38)
Anti-diabetic agents (injection)	25 (31)	37 (23)
Cardiac glycosides (Digoxin)	6 (7.5)	16 (10)

$t_{1/2e}$ ($p = 0.21$, Kruskal-Wallis) but there was significant difference in T_{max} ($p < 0.001$, Kruskal-Wallis). The data indicate that the differences in C_{max} are not caused by changes in elimination rate among the three groups; rather it may be due to differences in absorption phase.

Authors investigated the hypothesis that lower C_{max} and delayed T_{max} may be due to dissolution problems. To test this hypothesis, dissolution test was carried out according to published compendia of United State, with two modifications. Rather than testing one 75 mg tablet at a time, four 75 mg tablets and eight 75 mg tablets were tested together in the dissolution vessel. Additionally, the 4 tablets and 8 tablets were evaluated for dissolution once in 900 ml total dissolution medium and once in 330

Table 4. Laboratory findings of patients included in the study.

Parameter	Mean ± SD
Kidney function	
Na conc. (mEq/L)	138.8±2.9
K conc. (mEq/L)	4.3±0.42
Scr (mg/dl)	0.74±0.22
BUN (mg/dl)	16.55±6.0
Liver function	
Total protein (g/L)	6.7±0.6
Albumin (g/L)	3.9±0.4
ALT (IU/L)	22.7±12.9
AST (IU/L)	24.7±9.9
ALP (IU/L)	81.7±11.8
GGT (IU/L)	40.26±66.2
Complete blood count	
RBC (10^6/mm^3)	4.6±0.6
WBC (10^3/mm^3)	8.8±2.7
Platelets (10^3/mm^3)	232.7±61.3
Hgb (g/dl)	12.7±1.7
Other tests (mg/dl)	
Total cholesterol	165.9±44.3
LDL-cholesterol	108.8±57.6
HDL-cholesterol	35.7±8.23
Triglycerides	169±87.6

Scr: Serum creatinine; BUN: blood urea nitrogen; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phophatase; GGT: gamma-glutamyltransferase ; RBC: red blood cell; WBC: white blood cell; Hgb: hemoglobin.

ml. We were not able to utilize less than 330 ml, as the level of the dissolution medium will be lower than the paddle level. The dissolution tests were repeated in triplicates for each variation from the compendia procedure. None of the modifications on the dissolution test resulted in significant changes in dissolution behavior. In all cases, more than 90% of the nominated clopidogrel contents were dissolved in the first 15 min.

DISCUSSION

Clopidogrel is an important antiplatelet agent that has application in the primary and secondary prevention of cardiovascular complications, especially among patients unable to take aspirin. In patients who unedrgo percutaneous coronary intervention, dual antiplatelet consisting of aspirin and clopidogrel is the regimen of choice to prevent thrombotic complications (De Backer et al., 2003; Gibbons et al., 2003; Patrono et al., 2004)

This is the first report of the pharmacokinetics of 600 mg clopidogrel among Jordanians. While native Jordanians are mostly descended from people of villagers and Bedouin descent originating in the Arabian Peninsula (Lowi, 1995), ethnically, the Jordanians represent a mixed stock. Most of the population is Arab (approximately 98%), with 1% of the population is Armenian, and another 1% is Circassian. There are also small Kurd, Druze, and Chechen minorities (The Royal Hashemite Court, 2012)

Although, there was a previous study in young healthy Jordanian, this is the first to be conducted among patients undergoing PCI. The participants characteristics were comparable with published data of Jordanian patients undergoing PCI (Yousef et al., 2008). With regard to laboratory findings of recruited subjects, patients had normal or close to normal kidney function (serum creatinine, blood urea nitrogen and sodium); liver function (total proteins, albumin, aminotransferases, and alkaline phosphatase); lipid profile and complete blood count, indicating minimal if any effect on current findings (Table 4).

Very few HPLC methods were reported for the determination of carboxylic acid metabolite of clopidogrel (Singh et al., 2005; Souri et al., 2006; Bahrami et al., 2008). HPLC is the most convenient and common analytical method for therapeutic drug monitoring, and it is readily available in majority of laboratories. We reported simple isocratic reversed phase HPLC method with UV detection for the determination of inactive metabolite of clopidogrel. The statistical evaluation of the proposed HPLC method revealed its good linearity and reproducibility and led us to the conclusion that it could be used for the rapid and reliable determination of clopidogrel carboxylic metabolite in plasma in pharmacokinetic studies.

The high pharmacokinetic variability (at the level of C_{max}, AUC, T_{max} and $T_{1/2e}$) observed by us and others (Caplain et al., 1999; Taubert et al., 2004) may explain the reported inter-individual variability of ADP-induced inhibition of platelet aggregation to clopidogrel (Matetzky et al., 2004; Snoep et al., 2007). The observation is of importance as it is estimated that laboratory clopidogrel non-responsiveness can be found in 1 of 5 patients undergoing PCI (Muller et al., 2003; Snoep et al., 2007). Patients *ex vivo* labeled non-responsive are likely to be also clinically “non-responsive” with increased cardiovascular risk (Muller et al., 2003; Matetzky et al., 2004). Although there are a number of postulated causes of clopidogrel resistance phenomenon, the major underlying etiology is still unresolved (Fontana et al., 2003; Lau et al., 2004; Gurbel et al., 2005; Heestermans et al., 2006; Angiolillo et al., 2007; Snoep et al., 2007; Kim et al., 2008; Ford, 2009). It has been suggested that the high variability of clopidogrel and its metabolite may be due to differences in activation process (Angiolillo et al., 2007; Brandt et al., 2007; Kim et al., 2008; Ford,

Table 5. Pharmacokinetic parameters of clopidogrel carboxylic acid in patients after taking 600 mg clopidogrel.

Parameter	Mean ± SD			
	All (N=80)	Lower 15%[a] (N=12)	Trimmed[c] (N=56)	Upper 15%[b] (N=12)
C_{max} (µg/ml)	24.49±11.64	8.09±2.34	23.88±6.1	43.76±9.05
T_{max} (h)	2.02 ± 1.52	4.17±1.76	1.71±1.2	1.35±0.55
$t_{1/2e}$ (h)	4.29 ±2.92	3.99±0.95	4.05±2.37	4.45±3.38
AUC_{0-t} (µg/ml×h)	90.40±32.64	42.27±12.96	91.29±21.77	127.11±34.66
$AUC_{0-\infty}$ (µg ml/h)	123.17±54.60	58.09±19.36	125.54±51.22	163.89±45.07
$AUMC_{0-\infty}$ (µg/ml×h^2)	899±1186	1452±2807	754±651	1082±772
MRT (h)	6.0±2.7	5.3±1.6	5.9±2.5	6.9±4.0
Vd/F (L)	32.7±24	37±30.	32.7±24.1	29.3±19.3

[a,b] Based on C_{max} value, data were subdivided into the lower 15% and upper 15%. [c]Data excluding the lower 15% and the upper 15%.

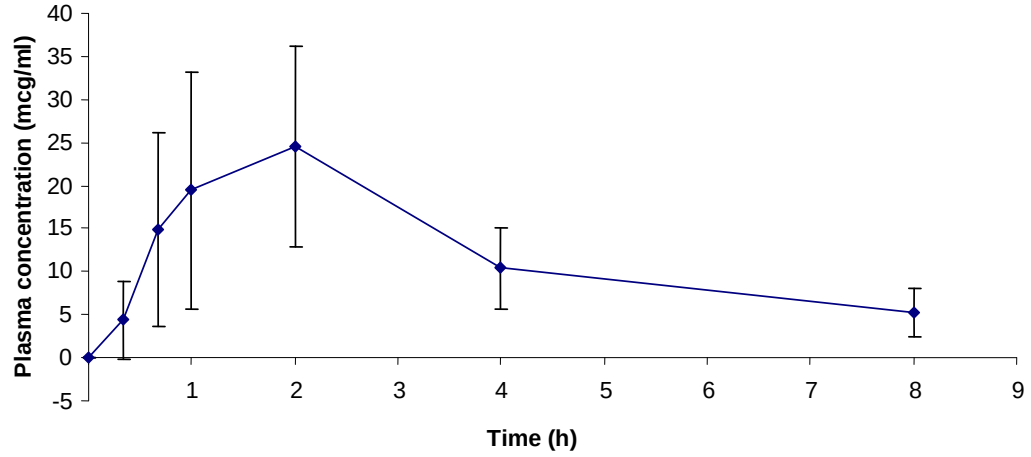
2009).

The interindividual differences in the activity of the cytochrome P450 isoenzymes 3A4, 3A5 (Lau et al., 2003; Lau et al., 2004) and 2C19 (Brandt et al., 2007; Kim et al., 2008; Mega et al., 2009) that metabolize the inactive clopidogrel prodrug to its active thiol form were suggested to underlie the response variability. Additionally, polymorphisms of P_2Y_1 and P_2Y_{12} genes, differences in receptor expression or posttranscriptional regulation or the downstream signaling cascades are proposed to contribute to individual variations in ADP-induced response to P_2Y_{12} inhibition with clopidogrel (Fontana et al., 2003; Hechler et al., 2003; Ford, 2009).

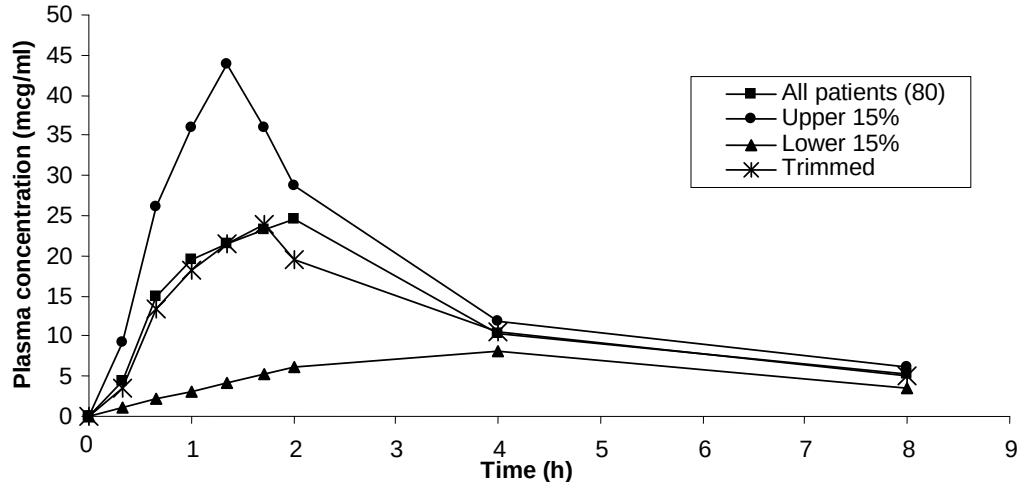
In addition to the high variability in carboxylic acid metabolite concentrations, our results concerning pharmacokinetics parameters differed from what has been published previously (Taubert et al., 2004; von Beckerath et al., 2005). Taubert and co-authors investigated the pharmacokinetics of clopidogrel after administration of a high loading dose (600 mg) to ten fasting young healthy volunteers, and revealed higher C_{max} and $AUC_{0\rightarrow\infty}$ (C_{max}: 43 µg/ml ± 16.9; T_{max}: 1.6 ± 0.9 h; $AUC_{0\rightarrow\infty}$: 198.6 ± 52.4; $t_{1/2e}$: 1.9 ± 0.9 h) as compared to our study (Taubert et al., 2004). Nicolas von Beckerath and colleagues compared three different loading doses (300, 600, 900 mg) of already crushed tablets that were given in solution form to sixty patients with suspected or documented coronary artery disease, and their reported C_{max} (44 µg/ml) was higher than ours (von Beckerath et al., 2005). The previous two studies did not aim to assess the crushed tablets versus non crushed tablets in the same study side by side. In a recent study (Zafar et al., 2009), plasma levels of clopidogrel carboxylic acid metabolite were followed in healthy subjects who were given 300 mg clopidogrel in crushed form via nasogastric tube with 30 mL water. Two weeks later the same subjects swallowed 300 mg clopidogrel. Plasma concentrations peaked earlier after crushed delivery than after oral intake and the median peak was 80% higher (Zafar et al., 2009). It should be noted that current study recruited patients rather than healthy volunteers who were given non-crushed and non dissolved whole eight (75 mg) tablets. Published pharmacokinetic parameters of clopidogrel administered at doses other than 600 mg were considered. Upon extrapolation to 600 mg dosing, there were similarities between C_{max} and current study C_{max} (Ksycinska et al., 2006; Souri et al., 2006; Bahrami et al., 2008).

Several studies suggested that the high variability of clopidogrel and its metabolite may be due to differences in absorption rate (Taubert et al., 2004; Taubert et al., 2006; Zafar et al., 2009). In a subset of our patients (n = 12) who had the lowest C_{max} values, they had $t_{1/2e}$ similar to the rest of the subjects but had significantly delayed T_{max}. The data indicate that the differences in C_{max} are not caused by changes in elimination process; rather it may be due to differences in absorption phase. Authors investigated the hypothesis that lower C_{max} and delayed T_{max} may be due to dissolution problems. The hypothesis was proposed for a number of reasons: Patients undergoing PCI are recommended by physicians not to drink or eat, starting from the night before the operation. This means that water content in the stomach by the time of PCI is very little, which may affect dissolution. Studies conducted on crushed tablets reported noticeably higher levels of clopidogrel carboxylic acid C_{max} (Taubert et al., 2004; von Beckerath et al., 2005; Zafar et al., 2009). To test this hypothesis, dissolution test was carried out with slight modification from published USP (USP, 2007). None of the modifications on the dissolution test resulted in significant changes in dissolution behavior. Further research has to include different ways of administering clopidogrel; as whole tablets, as crushed tablets and as crushed and dissolved tablets. Also, *in situ* disintegration and dissolution have to be evaluated.

Yet another potential explanation is the variable intestinal



(A)



(B)

Figure 2. Average plasma drug concentration versus time profile determined after oral administration of clopidogrel (dose 600 mg). (A) Plasma concentration profile for total 80 patients; (B) plasma concentration profile for 3 subgroups (upper, lower and trimmed) compared to the 80 patients. Two additional time points were assumed (time to reach C_{max} for both trimmed (1.75 h) and upper 15% (1.35 h).

absorption mediated through P-glycoprotein (P-gp) along the intestinal mucosa. Inhibition of P-gp activity by different modulators increased the absorptive clopidogrel flux across Caco-2 monolayers. C_{max} and AUC values were lower in subjects homozygous for the *MDR1 3435T* variant compared with subjects with the *3435C/T* and *3435C/C* genotypes (Taubert et al., 2006). Other than genetic polymorphism, many factors have been found to alter P-gp expression including but not limited to food intake (Deferme et al., 2002; Zhang et al., 2009), diseases (Liu et al., 2008; Dopp et al., 2009), and drugs (Fiegenbaum et al., 2005) .

The findings of current study should be read with caution, as the parent compound and the active metabolite were not measured. However, previous studies showed a good relationship between serum concentrations of inactive metabolite, active metabolite and parent compound (Taubert et al., 2004). Some other published bioequivalence studies also used data for carboxylic acid metabolite in their results. Additionally, the findings of this study are limited by the fact that the population being studied suffered from multiple medical problems and were on long lists of medications, all of which may influence the pharmacokinetic behavior of clopidogrel. Still, this population does reflect some certain groups of patients in community practice who are often

prescribed clopidogrel. Moreover, the sampling time in the current study was short as it was limited to 8 h post treatment. More time is needed (24 to 48 h) to have a better estimation of terminal half-life. Unfortunately, this is not feasible in real life situation where real patients are being recruited, and most of them get discharged after much less than 24 h post stenting. It should be noted that several other studies terminated sampling by 8 h or even less (Deferme et al., 2002; Zhang et al., 2009).

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Abbreviations: ADP, Adenosine diphosphate; **P_2Y_{12},** platelet receptor; **C_{max},** maximum plasma concentration; **$AUC_{0\rightarrow\infty}$,** area under the "plasma concentration vs. time" curve; **BMI,** body mass index; **Hb,** hemoglobin; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **T_{max},** time to peak plasma concentration; **C_{last},** last quantifiable concentration; **$t_{1/2e}$,** elimination half life; **AUMC,** area under the first momentum curve; **MRT,** mean residence time; **Vd,** apparent volume of distribution; **Δ,** accepted margin of error; **SD,** standard deviation; **CV,** coefficient of variation.

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