Research Article

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Exploring the effect of khat (*Catha edulis*) chewing on the pharmacokinetics of the antiplatelet drug clopidogrel in rats using the newly developed LC-MS/MS technique

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Abstract: Clopidogrel (CLOP) is widely used worldwide for cardiovascular complications. CLOP is highly metabolized in the liver to its active metabolite by cytochrome P450 enzymes. Studies have shown that khat, an addictive substance, is a powerful inhibitor of cytochrome P450 enzymes and can influence the metabolism of drugs that are concomitantly used. Therefore, this study was designed to evaluate the effects of khat on the pharmacokinetics of CLOP in rats. In this study, rats were administered either CLOP alone or CLOP combined with khat and their plasma were obtained at different time intervals and analyzed using the newly developed and validated liquid chromatography with tandem mass spectrometry (LC-MS/MS) method using foretinib (FTB) as the internal standard. The corresponding peak area of the analyte versus FTB was used for calculating the peak ratio. The validated LC-MS/MS method resulted in the separation of the well-defined quantifiable peaks of CLOP, FTB, and CLOP metabolite within 7 min. Results showed a significant influence of khat on the peak ratio of CLOP metabolite, which was found to be significantly decreased ($P < 0.05$) in comparison to CLOP alone, suggesting significant decrease in the conversion of CLOP to its active metabolite due to the inhibition of CYP450 enzymes by khat. Therefore, there might be a need for dose adjustment for regular khat chewers using CLOP.

Keywords: clopidogrel, Khat, pharmacokinetics, metabolism, LC-MS/MS

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1 Introduction

Khat (*Catha edulis* [Vahl] Endl) is a plant that is grown in some parts of the South Western Arab Peninsula and East Africa [1]. Generally, the leaves of the khat plant are chewed on a daily basis by more than 20 million people in the South Western Arabian Peninsula and Eastern part of Africa [2,3]. A large number of people chew khat leaves owing to their amphetamine-like properties with a variety of pleasurable and stimulating effects [4]. The stimulating and euphoric effects of khat are due to the presence of alkaloids, cathine and cathinone [5,6]. These alkaloids are categorized as sympathomimetic amines, the category similar to amphetamine [7,8].

Previously, a number of studies have been carried out to establish the effects of khat on the cytochrome P450 (CYP450) enzyme family [9–11]. In one of these studies, the effects of the khat extract on the activity of three *in vitro* human recombinant cytochrome P450 (CYP)2C9, CYP2D6, and CYP3A4 enzymes were checked and compared with that of cathinone, a constituent of khat [12]. Interestingly, the khat extract was observed to inhibit all three subenzymes with IC_{50} of 42, 62, and 18 μg/mL, whereas cathinone showed negligible inhibition of the tested CYPs [12].

Clopidogrel (CLOP), an antiplatelet thienopyridine-derivative drug, has been implicated as an effective therapeutic strategy against atherosclerosis, is used for both management and prevention of ischemic stroke [13,14], and is widely prescribed to patients with cardiovascular and cerebrovascular diseases in clinical practice. Figure 1 shows the

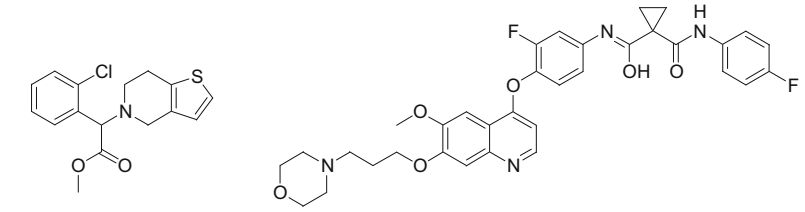

Figure 1: Chemical structures and molecular weights of CLOP and FTB (IS).

structures of CLOP and foretinib (FTB) along with their molecular weights. CLOP irreversibly inhibits P2Y12 receptor, a subtype of adenosine diphosphate G protein-coupled receptor (P2Y receptor) family [14]. Functioning as a prodrug, CLOP is converted into its active form through two steps by the CYP450 family of enzymes in the liver. CLOP is first converted into 2-oxo-CLOP by CYP2C19, CYP1A2, and CYP2B6 before it is further biotransformed into its active metabolite by CYP3A4, CYP2B6, CYP2C19, and CYP2C9 subenzymes [15].

As CYP450 regulates the metabolism of a large number of drugs, herbs, and dietary chemicals including CLOP, coadministration of CLOP with inhibitors or inducers of CYP450 might alter the pharmacokinetic profile of CLOP and its conversion to active metabolite, which will eventually influence the efficacy of CLOP [16,17]. For instance, previously it has been reported that the pharmacokinetic profile of CLOP was effectively decreased by coadministration of fluvoxamine, a potent CYP1A2 and CYP2C19 inhibitor [18]. Similarly, the concomitant use of CYP3A4 inhibitors and atorvastatin with CLOP also exhibited a marked reduction in the clinical efficacy of CLOP via inhibition of the CYP3A4-mediated oxidation of CLOP [19]. Moreover, CYP2C19-mediated metabolism of CLOP was also revealed to be decreased by omeprazole, one of the proton pump inhibitors acting as a CYP2C9 inhibitor [20]. Therefore, uncovering the potential alteration in the pharmacokinetics of CLOP by other coadministered drugs is particularly of clinical interest.

Owing to the growing consumption of herbal medicines, the assessment of herb–drug interaction has become an urgent clinical issue, particularly for patients on combination therapy. The interactions of khat with other coadministered chemical drugs including CLOP have never been previously investigated and are particularly important for the patients who regularly chew khat and are on medications. Since khat chewing itself is one of the reasons for cardiovascular diseases, many khat chewers remain on cardiovascular drug therapy. A significant number of khat users are known to have developed some kind of cardiovascular complications due to either khat or some other reason. Cardiovascular drugs are widely prescribed to these patients for the prevention of stroke. Therefore, it is very important to study the effect of khat on these drugs and to establish their interaction. Since khat is a known inhibitor of CYP450 enzymes, it was anticipated that the pharmacokinetic properties of CLOP could be affected by the concomitant use of khat. This study is very important for attracting the attention of physicians and clinical practitioners when prescribing the optimal dose of CLOP for khat-chewing patients. Therefore, we developed a sensitive, rapid, and robust liquid chromatography with tandem mass spectrometry (LC-MS/MS) technique to quantify CLOP in plasma samples using FTB (GSK1363089), which is a CYP450-3A subfamily substrate as International Standard (IS) to investigate the potential effect of khat on pharmacokinetics of CLOP and its metabolite.

2 Experimental

2.1 Chemicals and reagents

CLOP and FTB (IS) reference standards were purchased from Med Chem Express (USA). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN), analytical reagent-grade ammonium formate ($HCOONH_4$), formic acid (HCOOH), and microsomes from human liver (pooled; M0567) were purchased from Sigma Aldrich (St. Louis, MO, USA). The HPLC-grade water was used throughout the study and was produced in our lab using Milli-Q® plus filtration system (Missouri city, TX, USA).

2.2 Plant material

Khat (*C. edulis* [Vahl] Endl) leaves were obtained from the Substance Abuse and Toxicology Research Centre of Jazan University. Freshly cultivated khat leaves that were grown at the western border of Jazan province were

selected for the study. In order to have more cathinone content, smaller leaves present at the apex of the branch were used. The doses of the khat leaves administered to rats were calculated according to the general human intake of khat on a daily basis.

2.3 LC-MS/MS methodology

2.3.1 Operating parameters

The best chromatographic separation with high resolution was obtained for CLOP and FTB (IS) by optimizing all LC-MS/MS parameters (Table 1). For the analysis of CLOP in plasma samples, FTB was chosen as the IS in the light of the fact that similar extraction technique could effectively be utilized for the two drugs. A triple quadrupole (QqQ) mass spectrometer working in the positive electrospray–ionization interface (ESI) source mode was used for the detection of peaks. In the ESI source, the low-purity nitrogen (12 L/min) was used as the drying gas, whereas high-purity nitrogen (55 psi) was utilized as the collision gas. Moreover, the ESI temperature "*T*" and the capillary voltage "*V*" were maintained at 350°C and 4,000 V, respectively. MassHunter software (Agilent Technologies, Santa Clara, CA, USA) was used to control the instruments and for data acquisition.

The mass reactions (parent to fragment ions) from 322 → 212 and 322 → 184 for CLOP, 633 → 128 for FTB, and 308 → 113 for the CLOP metabolite (Figure 2) were observed to quantify the CLOP using the analyzer mode of multiple reaction monitoring (MRM). The fragmentor voltage (FV) was set at 135 and 130 V with collision energy (CE) of 15 and 20 for CLOP and FV of 140 V with a

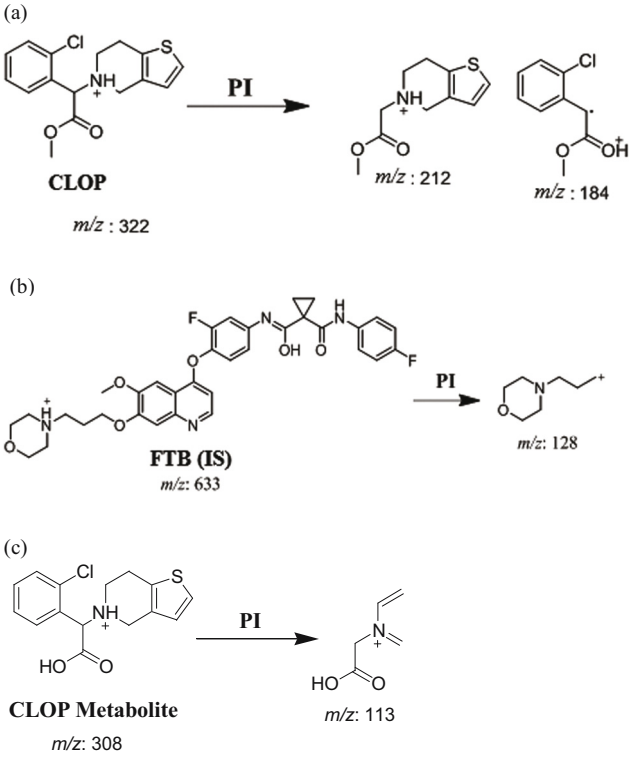

Figure 2: MRM mass spectra of (a) CLOP, (b) FTB, and (c) CLOP metabolite.

Table 1: LC-MS/MS analytical parameters

LC			MS	
RRLC	**Agilent 1200**		**MS**	**Agilent 6410 QqQ**
Isocratic mobile phase	75% ACN		ESI	Positive ESI
	10 mM NH_4COOH	25% pH: 4.2		Drying gas: N_2 of low purity at 12 L/min
	Flow rate: 0.2 mL/min			Pressure (60 psi)
	Injection volume: 5 µL			
Agilent eclipse plus C_{18} column	Length: 100 mm			Source temp.: 350°C
	Internal diameter: 2.1 mm			Capillary voltage: 4,000 V
	Particle size: 1.8 µm		Collision cell	N_2 gas (highly pure)
	Temp.: 20 ± 2°C		Mode	MRM
Mass spectra segment	0.0–4.0 min	CLOP MRM	CLOP	*m/z* 322 → *m/z* 212, FV:[a] 135 V, CE:[b] 15 eV
				m/z 322 → *m/z* 184, FV: 130 V, CE: 20 eV
	0.0–4.0 min	CLOP-metabolite MRM	CLOP-metabolite	*m/z* 308 → *m/z* 113, FV: 130 V, CE: 20 eV
	4.0–7.0 min	FTB MRM	FTB (IS)	*m/z* 633 → *m/z* 128, FV: 145 V, CE: 20 eV

[a] Fragmentor voltage. [b] Collison energy.

CE of 20 for FTB. The MRM mass analyzer mode was used for CLOP quantification to omit any possible interference caused by the plasma constituents as well as to increase the sensitivity of the method.

2.3.2 CLOP calibration stock solutions

The stock solution of CLOP (1 mg/mL) was prepared in water and was further diluted using the mobile phase to get 100 µg/mL (working solution 1) and 10 µg/mL (working solution 2) solutions. Similarly, the IS stock solution of 100 µg/mL concentration was prepared in DMSO and was further diluted using the mobile phase to obtain the IS working solution of 1 µg/mL.

2.3.3 Preparation of CLOP calibration standards

A calibration plot of 12 points (5, 10, 15, 30, 50, 80, 100, 150, 200, 300, 400, and 500 ng/mL) was constructed by spiking CLOP working solution 2 into rat plasma. For quality control study, four calibration levels were selected, namely, lower limit of quantification (LLOQ) of concentration 5 ng/mL, low limit (LQC) of 15 ng/mL, medium limit (MQC) of 150 ng/mL, and high limit (HQC) of 400 ng/mL quality control samples. A fixed volume of IS working solution (100 µL) was added to all samples followed immediately by the addition of ACN to precipitate the protein. The resulting solution was vortexed and then followed by centrifugation at 1,000 rpm for 4 min. The supernatant was siphoned off and injected into the LC-MS system. All the LC-MS analyses were performed in triplicate.

2.3.4 Method validation

Various parameters used for the validation of the newly proposed LC-MS/MS method for CLOP assay were per the guidelines provided by Food and Drug Administration [21]. All the parameters including linearity, sensitivity, assay recovery, specificity, reproducibility, limit of detection (LOD), and limit of quantification (LOQ) were calculated. The least-squares statistical method was used to compute the calibration curve equation ($y = ax + b$), and a linear fit was confirmed by r^2, which showed linearity in the range of 5–500 ng/mL.

2.3.5 Dose selection and corresponding calculations

The homogenate of khat leaves was prepared by mincing fresh khat leaves in 100% distilled water. The estimated amount of khat leaves consumed daily by an adult human was used to calculate the corresponding dose to be administered orally to rats. The daily use of khat in Yemen, Saudi Arabia, and some African countries has been reported earlier [2,22,23]. This was also established by additional research on the average content of fresh leaves of khat from cathinone, cathine, and norephedrine. A psychoactive dose of fresh khat leaves should contain about 0.8 mg cathinone per kg body weight [24]. The chewing dose of fresh khat leaves is estimated to be ~5 g/kg per chewing session [23]. Therefore, the current study uses the daily use rate of fresh khat rather than the extracted khat. This will provide a more practical conclusion on the pharmacokinetic effects of the whole khat leaves.

2.3.6 Pharmacokinetic study

Male Wistar rats of 200–300 g were used in this study. The study was conducted following the ethical guidelines of the Jazan University ethical committee. Rats were placed individually in cages, allowed to recover, and kept on fasting for 12 h prior to the pharmacokinetic study. Eighteen rats were randomized into three equal groups. The first group served as the control and received the control vehicle (0.5% sodium carboxymethyl cellulose) only. The second group (CK) rats were orally administered with khat (12.4 g/kg dose) first and CLOP (7.75 mg/kg dose) after 15 min. The third group (CA) received oral dose of CLOP alone (7.75 mg/kg dose). Blood samples (250 µL) were collected into heparinized tubes before the start of the study (0 h) and at the following times: 0.5, 1.0, 2.0, 3.0 , and 4.0 h. All the blood samples obtained were immediately centrifuged at 1,000 rpm at 4°C, and the plasma separated was siphoned off and stored at −80°C till further analyses.

3 Results and discussion

3.1 LC-MS/MS methodology

The mass spectral transitions obtained in MRM mode of CLOP, FTB (IS), and CLOP metabolite are presented in Figure 3. The total run time taken for complete elution of CLOP and FTB was 7 min and the peaks showed good resolution. Also, no carryover peaks were observed in the blank rat plasma matrix sample. Figure 4 represents

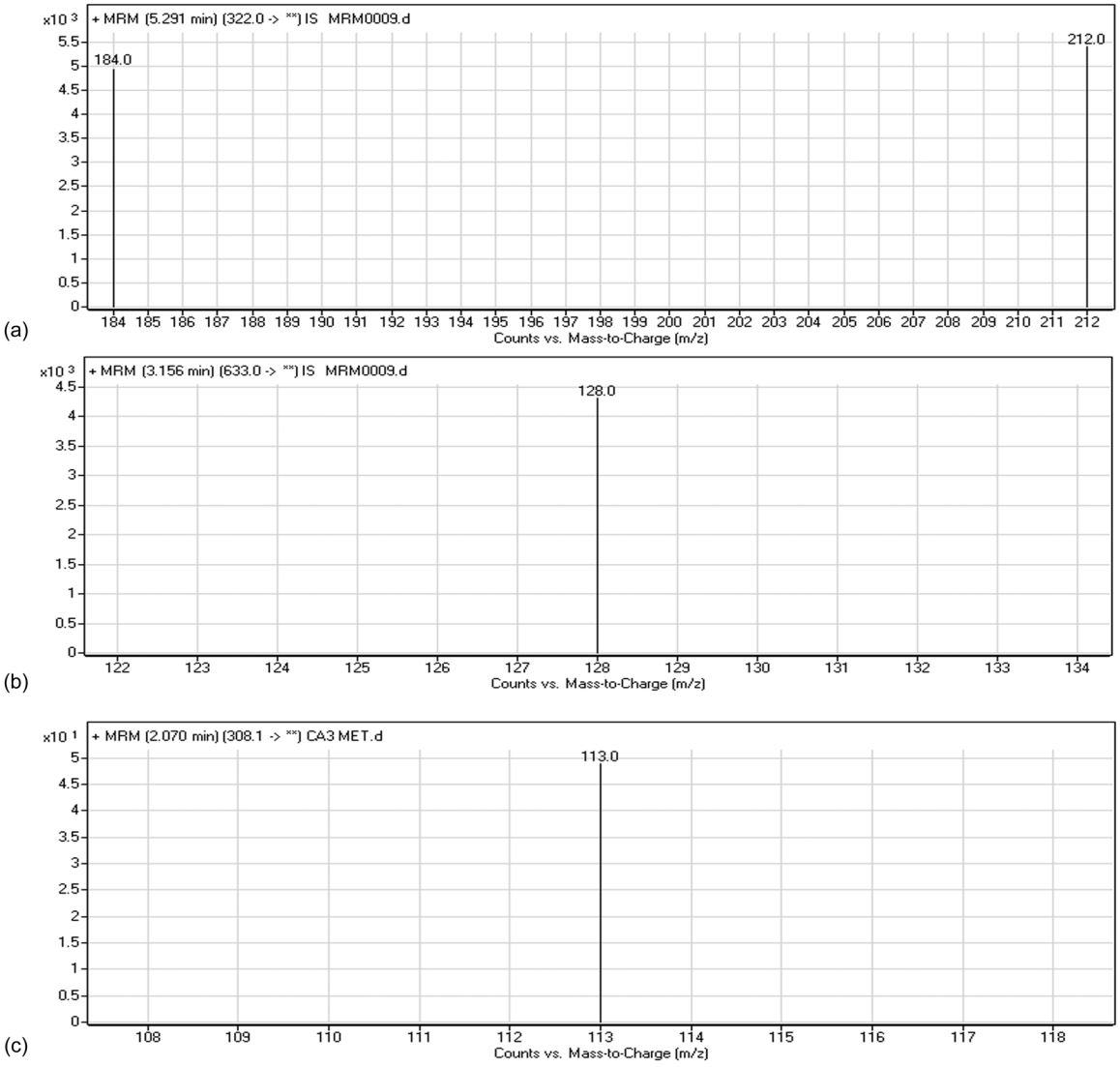

Figure 3: MRM mass spectral transitions of (a) CLOP, (b) FTB (IS), and (c) CLOP metabolite.

the MRM mode chromatogram of CLOP standard (500 ng/mL) and FTB (50 ng/mL). CLOP metabolite has been eluted before CLOP prodrug as shown in Figure 5.

3.2 Validation of the established LC-MS/MS method

The HPLC-MS/MS was developed to determine the remaining amount of CLOP prodrug, which could not metabolize due to the influence of khat. As evident in Figure 6, optimum separation of CLOP and FTB peaks was obtained in the absence of any peak in the blank CLOP matrix at their elution times, which confirmed the specificity of the newly established method. Moreover, no carryover effect of CLOP and FTB was observed in the MS chromatograms.

The linearity range and r^2 for the proposed method were found to be 5–500 ng/mL and ≥0.9994, respectively, in the rat plasma matrix, and the linear regression equation for the CLOP calibration plot was $y = 0.5942x - 1.264$. The LOD and LOQ values were calculated from the slope of the curve and were found to be 1.12 and 3.39 ng/mL, respectively. The lower limit quality control (LLQC) chromatogram displayed a peak with good shape and high signal to noise (S/N) ratio, indicating good sensitivity of the established LC-MS/MS method.

The precision (percentage of relative standard deviation [%RSD]) and the accuracy of the developed method were tested on different quality control samples. The concentrations of CLOP obtained from rat plasma

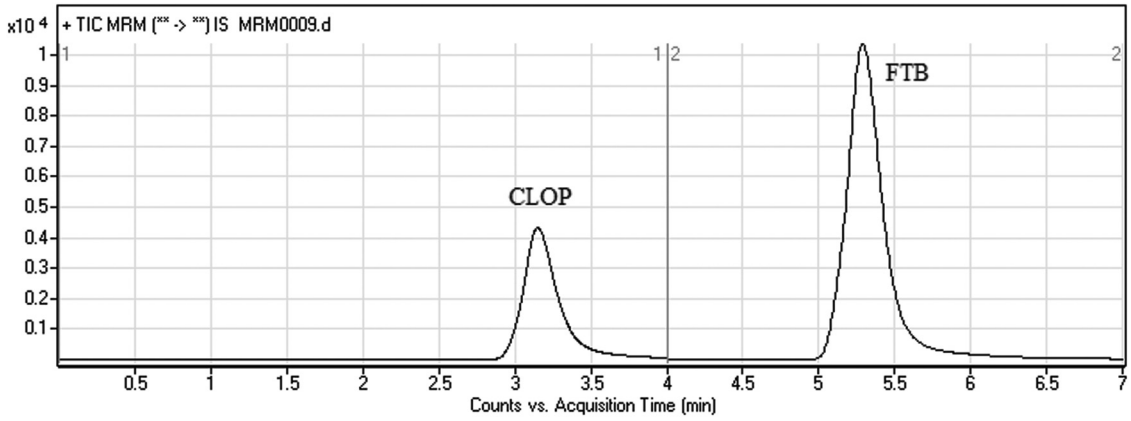

Figure 4: MRM chromatograms of CLOP standard (500 ng/mL) and FTB (50 ng/mL).

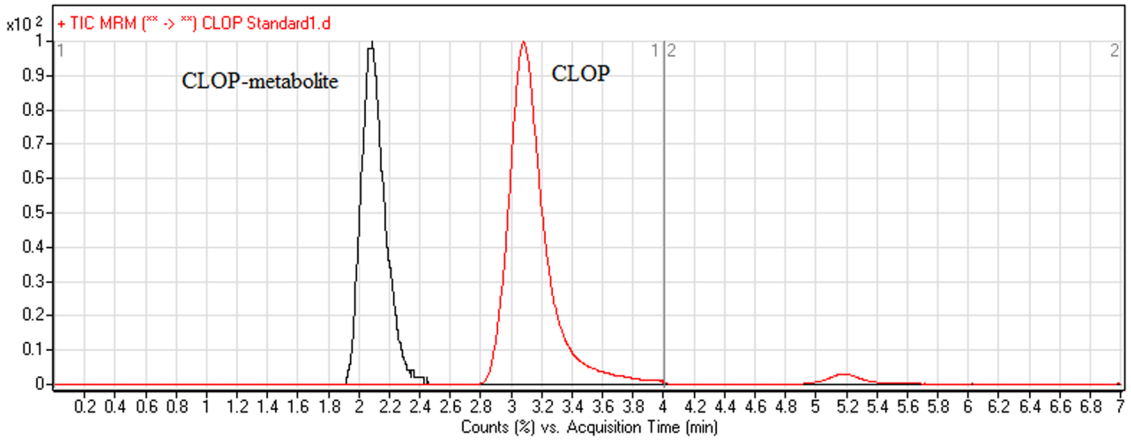

Figure 5: MRM chromatograms of CLOP prodrug (red line, RT: 3.1 min) and its metabolite (black line, RT: 2.1 min).

matrix were back calculated and the results are summarized in Table 2. Different LLQC, LQC, MQC, and HQC samples were used for the study and the %RSD and %accuracy were calculated.

The three quality control samples, namely, LQC, MQC, and HQC, were also analyzed interday as well as intraday to see the interference and deviation, if any. The %RSD values and %accuracy were calculated for LQC, MQC, and HQC samples. The results for interday and intraday samples are shown in Table 3. All the values obtained were acceptable per the International Conference on Harmonisation (ICH) guidelines Q2R1.

3.3 Pharmacokinetic study

The developed LC-MS/MS method was applied to study the effect of khat on the pharmacokinetics of CLOP in plasma following their oral administration in rats. Plasma samples obtained from the rats at different time points were brought to room temperature and spiked with IS FTB before injecting into the LC-MS/MS system. Control, CLOP alone, and CLOP + khat groups of samples were analyzed at different times) 0, 0.5, 1, 2, 3, and 4 h) and the analyte to IS peak area ratio was calculated. Values obtained are shown in Table 4 and represented in Figure 7. As evident from Table 4, the peak corresponding to the CLOP disappeared in both CLOP alone and CLOP with khat samples. This is due to the fact that CLOP gets highly metabolized by CYP450 enzymes present in the blood and is converted to its different metabolites. Another peak corresponding to CLOP metabolite was observed from which the peak ratio was calculated and used for comparison. The CLOP metabolite powder was utilized for the MS/MS optimization.

As shown in Table 4 and Figure 7, the decreased peak ratios of CLOP metabolite and khat samples indicate the significant influence of khat on CLOP

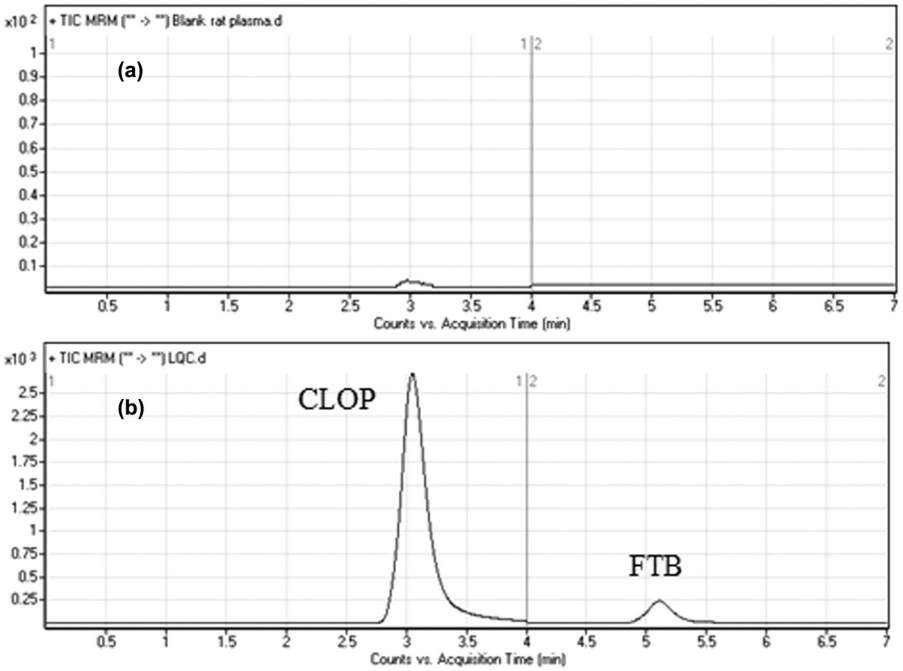

Figure 6: MRM chromatograms of (a) blank rat plasma and (b) CLOP (15 ng/mL; LQC). The blank rat plasma did not reveal any matrix interference.

Table 2: CLOP back-calculated calibration standard concentrations from rat plasma matrix

Samples	CLOP nominal concentrations (ng/mL)	Mean[a]	SD	RSD (%)	Accuracy (%)
LLQC	5	5.25	0.06	1.18	5.03
	10	9.82	0.41	4.21	–1.85
LQC	15	14.79	0.19	1.29	–1.42
	30	32.25	0.36	1.13	7.49
	50	51.56	1.54	2.99	3.12
	80	77.02	1.34	1.74	–3.72
	100	97.17	1.85	1.91	–2.83
MQC	150	147.35	2.06	1.40	–1.77
	200	197.52	2.42	1.22	–1.24
	300	295.67	3.19	1.08	–1.44
HQC	400	386.53	4.52	1.17	–3.37
	500	498.21	5.78	1.16	–0.36

[a] Mean of six replicates; RSD: relative standard deviation

metabolism. In CLOP only samples, the metabolite appeared at 0.5 h interval which increased at 1 and 2 h of the administration and decreased thereafter. The highest peak area was observed at 2 h. On the other hand, CLOP with khat samples showed the appearance of a metabolite peak at 1 h, instead of 0.5 h as observed in the case of CLOP alone. The metabolite peak area at 1 h for CLOP with khat was also observed to be far less than the CLOP alone. It increased at 2 and 3 h under the influence of khat but was still lesser than CLOP alone. The highest peak area was obtained at 3 h which decreased thereafter at 4 h. It was clear from the results that the highest peak area of CLOP with khat which was obtained at 3 h was almost equal to the peak area of CLOP only at 0.5 h. It shows that the concentration of CLOP metabolite decreased under the influence of khat and less CLOP metabolite was available in the blood when compared to CLOP only. This is because of the fact that khat has potentially inhibited the CYP450 enzymes which are responsible for the metabolism of CLOP to its

Table 3: Intraday and interday (accuracy and precision) of the established method

Rat plasma matrix	LQC (15 ng/mL)		MQC (150 ng/mL)		HQC (400 ng/mL)	
	Intraday assay	Interday assay	Intraday assay	Interday assay	Intraday assay	Interday assay
Mean	14.79*	14.90**	147.35	148.43	386.53	384.74
SD	0.19	0.19	2.06	3.11	4.52	3.54
Precision (%RSD)	1.29	1.27	1.40	2.09	1.17	1.05
% accuracy	−1.42	−0.65	−1.77	−1.05	−3.37	−1.34

*Mean of 12 replicates on the same day. **Mean of six replicates for 3 days.
LQC: low-quality control; MQC: medium-quality control; HQC: high-quality control.

Table 4: Concentration of CLOP and its metabolite at different time intervals

Sample	CLOP	Metabolite
CA* 0 h	0.0	0.0
CA 0.5 h	0.0	128.67
CA 1 h	0.0	141.33
CA 2 h	0.0	360.67
CA 3 h	0.0	194.67
CA 4 h	0.0	125.33
**CK 0 h	0.0	0.0
CK 0.5 h	0.0	0.00
CK 1 h	0.0	28.00
CK 2 h	0.0	117.33
CK 3 h	0.0	126.43
CK 4 h	0.0	48.00
Control 0 h	0.0	0.0
Control 0.5 h	0.0	0.0
Control 1 h	0.0	0.0
Control 2 h	0.0	0.0
Control 3 h	0.0	0.0
Control 4 h	0.0	0.0

*CA = CLOP alone. **CK = CLOP + khat.

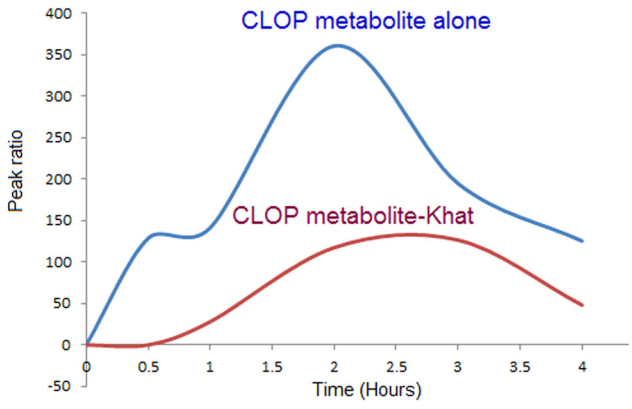

Figure 7: Influence of khat on the level of CLOP active metabolite over time in rat plasm.

active metabolite. However, the CLOP prodrug peak in the presence of khat did not appear, and this probably was due to the fact that khat could not influence the first step of CLOP prodrug metabolism and could not inhibit the enzymes that convert the CLOP prodrug to its metabolites.

Khat intake is a major problem associated with a number of people worldwide particularly in the Gulf region. Generally, the leaves of khat are chewed on a daily basis by more than 20 million people on the Arabian Peninsula and Eastern part of Africa [25,26]. A large number of people chew khat leaves due to their amphetamine-like properties involving a variety of pleasurable and stimulating effects [27]. It is expected that around 500 g of fresh khat leaves are chewed by the user per day depending upon the variety of khat, nature of the user, and the availability in market. This amount usually increases on certain occasions such as cultural/social festivals and ceremonies [28]. More than 60 phytochemicals have been isolated and identified from khat [25]. Our previous research using GC-MS has also identified a number of constituents of khat plants including cathine and cathinone alkaloids [26,27]. The stimulating and euphoric effects of khat are due to the presence of these alkaloids in the plants. These alkaloids are categorized as sympathomimetic amines, the category similar to amphetamine. They result in the feeling of well-being and mental alertness as well as excitement in users, but they have a number of untoward effects as well, which is why their use is banned in many countries. As aforementioned, the previous research shows neither cathine nor cathinone appears to be responsible for the inhibition of cytochrome subenzymes [9,10,12,28]. Therefore, investigating the molecular mechanism of cytochrome subenzymes' inhibition by khat is still very much needed.

Khat has been used by millions of people around the world, but the effect of khat use on the metabolism of

various drugs has not been addressed. Khat has a clear influence on the metabolism and pharmacokinetics of various drugs administered concomitantly including CLOP, as evident from this study. The activity and toxicity of any drug depend upon the concentration that reaches the site of action, and generally, the drug concentration in plasma is related to its pharmacological response. Like other drugs, dosing of CLOP is based on the assumption that a particular dose will produce a predefined blood concentration and, thereby, create the anticipated therapeutic effect. Therefore, this study emphasizes that physicians need to be cautious when prescribing CLOP to khat using patients who should be monitored to determine the optimal dose of CLOP.

4 Conclusions

A sensitive, newly developed, and validated LC-MS/MS method was successfully used to evaluate the effects of khat, a known inhibitor of CYP group of enzymes, on the pharmacokinetics of CLOP, on rats. The use of khat is prevalent in Middle-Eastern countries; and many people, who are suffering from cardiovascular disorders, use CLOP as an antiplatelet drug. Therefore, in khat users, the dose of CLOP should be monitored to achieve the optimal dose. Meanwhile, the physicians and pharmacists should educate the patient about the consequences of the use of khat on the efficiency of drugs prescribed for the chronic diseases.

Author contributions

HAA was involved with LC-MS/MS and manuscript writing; AAK contributed to LC-MS/MS and study design; MWA was involved in LC-MS/MS and study design; WA performed the pharmacokinetic study; MMET contributed to pharmacokinetic study and manuscript writing; and AK contributed to study design and manuscript writing.

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Conflict of interest: The authors declare no conflict of interest.

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