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Original Article

Determination of Serum Crocetin After Oral Administration of Krocina Tablets Using HPLC and LC–MS Methods

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ABSTRACT

Background: Saffron, derived from *Crocus sativus*, has long been used in traditional medicine because of its antioxidant, anti-inflammatory, and neuroprotective properties, which are primarily attributed to crocin, its major bioactive pigment. Crocin is extensively hydrolyzed to crocetin in the gastrointestinal tract, where it exhibits improved systemic absorption and acts as the principal circulating metabolite. However, pharmacokinetic data regarding commercially available crocin formulations remain limited.

Objectives: Crocin is the main active pigment in saffron and is responsible for its many therapeutic effects. A pharmaceutical product called Krocina is available on the Iran market, and this study aims to investigate the pharmacokinetics and absorption of Krocina 15-mg tablets.

Methods: A stock solution of crocetin at a concentration of 5 µg/mL in methanol was prepared. Serum and urine standards were then prepared from this stock in the concentration range of 0.025 to 2.5 µg/mL. Each standard was analyzed with high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) with multiple reaction monitoring (MRM). A mobile phase composed of methanol, water, and acetic acid (85%, 14.5%, 0.5%), at a detection wavelength at 423 nm with a flow rate of 0.8 mL/min was employed. LC-MS measurements were performed in positive electrospray ionization mode (ESI^+) using a Supelco analytical column (150×4.6 mm, 3 µm Discovery HS C18, USA).

Results: The intraday coefficient of variation (CV) measured by HPLC <1.28%, and the interday CV<3.44%. For the LC-MS/MRM method, intraday CV<1.05% and interday CV was below 2.87%. The average peak serum concentration of crocetin after Krocina tablet administration occurred at 240 minutes, measured as 177.11 ng/mL by HPLC and 184.33 ng/mL by LC-MS/MRM.

Conclusion: Both HPLC and LC-MS methods demonstrated adequate capability for blood analysis and determination of pharmacokinetic parameters, and the obtained data were considered reliable. The clinical effects of Krocina tablets should be related to absorbed crocetin.

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Introduction

Crocetin is the aglycone of crocin, a natural product of saffron. It can be interchangeably found as hydrolyzed crocin in biological systems as a bioactive metabolite [1]. Crocetin may play its role via different mechanisms like promoting oxygen transfers and velocities of diffusion, blocking inflammatory mediators, safeguarding the cells against harm by reactive oxygen species (ROS), and causing apoptosis of cancer cells. Crocetin ($C_{20}H_{24}O_4$; molecular mass 328.4 g/mol) possesses conjugated acid structure in a multiple unsaturated bonds, 4 side groups of methyl, and 7 conjugated double bonds, including cis and trans [2].

Owing to its long series of conjugated carbon–carbon double bonds, crocetin is highly responsive to shifts in temperature, exposures to light, and variations in pH, irradiation with light and heating promote oxidation and isomerization in the compound. Furthermore, crocetin is frequently stabilized through esterification with gentiobiose, glucose, and other standard sugar moieties [3]. Conventionally, the trans isomer is more stable than the cis isomer. The compound exhibits limited solubility in water as well as in most organic solvents, save for pyridine and dimethyl sulfoxide (DMSO) [4]. Among the analytical techniques employed for crocetin investigation are high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) [5]. Significantly, crocetin is endowed with substantial pharmaceutical value and displays cardioprotective, hepatoprotective, neuroprotective, antidepressant, antiviral, anticancer, antidiabetic, and memory-enhancing effects [6].

HPLC gains greater efficiency by employing very fine particles under 10 μ in diameter, a tight particle-size distribution, and surface-modified column packings. Such particles of minute size impose pronounced resistance to liquid flow. Hence, advanced pumps are employed to convey the liquid at a steady, reproducible flow rate, thereby elevating system pressure. Moreover, a custom designed sample injection port is necessary. HPLC employs sample handling and detector technologies comparable to those employed in gas chromatography (GC). Detectors of GC may likewise be adapted for use in HPLC. Nevertheless, in GC only the mobile phase functions as a simple carrier, exerting no influence on the sample molecules. However, in HPLC, both the stationary and mobile phases may interact with the sample. Moreover, in HPLC, the mobile phase may engage with the sample through complex formation or hydrogen bonding, thereby improving separation capacity [7].

Owing to the intricate nature of biological samples, liquid-chromatography/mass spectrometry (LC/MS) has become the method of choice for metabolomics in recent years. Because of its high sensitivity, molecular weight detection, low cost, and compatibility with separation techniques, such as liquid chromatography (LC), GC, or capillary electrophoresis (CE), MS remains the instrument of choice. Within the array of mass spectrometers, including ion trap, time-of-flight, orbitrap, and quadrupole models, the triple quadrupole instrument stands out for targeted metabolomics owing to its superior sensitivity, high degree of specificity, and superior quantification performance.

A number of targeted metabolomics methodological studies have been documented. LC/MS with multiple reaction monitoring (MRM) was applied to examine Isoflavone pharmacokinetics (PK) in men [8]. Employing analogous MRM approaches [9] enhanced the screening capacity in forensic toxicology research. An MRM assay was devised for screening 238 drugs in blood [10]. This method was adopted as a survey scan for self-assays of 301 drugs [11]. In a recent study, the Rabinowitz research team described an LC/MS/MRM approach capable of detecting 174 metabolites through both positive and negative ESI MS (electrospray ionization MS) analyses [12]. Nevertheless, the current approaches are limited to scrutinizing comparable classes of metabolites or compounds via a single chromatographic run and a single ionization mode.

In another study, a UHPLC apparatus furnished with a diode array detector was employed to enhance the resolution and detection of crocin and other polar constituents in saffron extract [13]. The separation was carried out at 440 nm with a mobile phase buffered at 1% formic acid. Optimization was performed through an artificial neural network in conjunction with a Luna Omega C18 column that was packed with a novel stationary phase endowed with modified polar surfaces. Consequently, the technique ensured sharper resolution of neighboring peaks in the chromatogram and facilitated the identification of a larger number of crocetin esters than those detected with conventional HPLC methods.

Crocetin was supplied at 7.5, 15, and 22.5 mg to 5 male and 5 female volunteers, then tracked its plasma concentrations at 1, 2, 4, 6, 8, 10, and 24 hours after dosing, quantified by HPLC [14]. Crocetin displayed rapid absorption, with the mean time to peak concentration (Tmax) recorded as being between 4.0 and 4.8 hours. The mean peak concentration (Cmax) varied from 100.9 to 279.7 ng/mL, whereas area under the curve (AUC0–

24h) spanned 556.5 to 1720.8 ng·h/mL, with both parameters rising in direct proportion to the given dose. The mean plasma clearance of crocetin was approximated at 6.1–7.5 hours. In an animal study conducted by Asai et al., crocetin was swiftly absorbed, then conveyed through the portal vein to enter systemic circulation. Following its peak, the plasma concentration of crocetin fell gradually and could still be detected 24 hours after administration [15].

Weak affinity of crocetin toward serum albumin allows it to disseminate more broadly across body tissues [16], a characteristic evidenced by the drug's comparatively large volume of distribution (Vd/F). According to another study, HPLC method was devised and validated to determine crocetin [17]. Blood withdrawals were performed at 0, 20, 40, 60, 90, 120, 150, 180, and 240 minutes following the intake of a 15 mg crocetin capsule by 5 healthy volunteers. Crocetin attained a Cmax of 0.35 µg/mL after 60 minutes and was cleared from the bloodstream by 240 minutes. The present study contrasted two extraction methods: Direct precipitation and solid-phase extraction (SPE). Using direct precipitation yielded higher extraction recoveries (>70% versus 59%), superior sensitivity (lower limit of quantification [LLOQ]=0.05 µg/mL as opposed to 0.5 µg/mL), and superior linearity (0.999 versus 0.990), thereby permitting analysis of samples at very low concentrations. Variations in LLOQ were ascribed to greater drug loss during SPE as opposed to precipitation.

SPE-HPLC method was validated and developed to detect crocetin in human plasma after consuming saffron tea (200 mg saffron steeped in 80 °C water 5 minutes) [10]. Three female and 1 male volunteers were sampled after 0, 2, and 24 hours. The procedure produced sharp peaks at 10.7 minutes (trans-crocetin) and 18.6 minutes (cis-crocetin), and the calibration curve was linear with good linearity of total crocetin in plasma over 0.020-20 µM (R^2=0.999).

To assess crocin and crocetin PKs in plasma, it was monitored in 64 rats—each housed in separate groups of 8 (4 males and 4 females)—after intravenous (IV) and intragastric (IG) dosing by employing UPLC-MS/MS [11]. By IV administration to an additional cohort of 8 rats (4 males, 4 females), a group received an IV bolus of a crocin solution in PEG-400 normal saline (3:7 v/v) at 0.98 mg/kg. For PK analysis, oral doses of 30, 60, and 120 µmol/kg were contrasted with an IV injection of 1.0 µmol/kg. Bioavailability (F) for oral crocin doses stood at 0.6±0.25%, 0.44±0.29%, and 0.28±0.11%, respectively; when doses were enriched with crocetin, it rose correspondingly to 3.4±0.84%, 3.64±1.13%, and 2.26±1.77%. At the three tested doses, crocetin had absolute oral bioavailability of 11.8±6.1%, 8.8±5.7%, and 5±2.5%, respectively, points that exceeded those of crocin (3.4±0.84%, 3.64±1.13%, 2.26±1.77%).

In a study on mice a lyophilized saffron aqueous extract (SFE) reconstituted in water and administered intragastrically, with crocetin concentrations being quantified by HPLC-PDA [18]. Serum crocetin derived from in vivo crocin hydrolysis, together with its tissue concentrations (both conjugated and total), was quantified in C57/Bl6J mice after IV and oral SFE administration (60 mg/kg, reconstituted with water for injection). PK evaluation was performed through both compartmental and non-compartmental methodologies. Administration of SFE orally yielded a one-compartment PK model with first-order absorption that adequately characterized the kinetics of crocetin and crocin. Relative oral bioavailability of total crocetin was determined to be 1.17. Non-compartmental tissue PK analysis revealed wide distribution of crocetin in both liver and kidneys.

For the current investigation, Krocina tablets (Samisaz, Mashhad, Iran; 15 mg crocin roughly corresponding to 5 mg crocetin) were administered orally, and human subjects' blood samples were subsequently examined to assess the absorbed crocetin generated from the intestinal microbial metabolism of crocin. The Krocina tablets are employed as an antioxidant and a potent antidepressant [19]. The compound has been shown to exert effects in multiple other conditions, including withdrawal syndrome [20], osteoarthritis [21, 22], and fertilization [23].

Materials and Methods

Voluntarily, 5 healthy men were enrolled in the present study meeting the following criteria. Complete blood count analyses were carried out and evaluated. The volunteers filled out a questionnaire to provide information on their health, medication history, allergic background to food and pharmaceuticals, and tobacco habits. The participants chosen for the study were physiologically healthy adults aged between 24 and 41 years. Their body weight varied from 73 to 92 kg. Before blood assessment, the participants had not fasted and were supplied with the same snack during the ensuing rest period.

The serum crocetin concentration was determined through reversed-phase high performance Shimadzu liquid chromatography (HPLC) quipped with a C18 column (150×4.6 mm, 320 apore size) and a UV detector [24]. For the stock solution, 2.5 g of crocetin were

weighed and dissolved in methanol, then diluted to 250 mL in a volumetric flask, resulting in a crocetin solution containing 10000 ng/mL. The stock solution was subsequently diluted in sequential steps to obtain other standard solutions with concentrations of 10, 100, 500, 1000, 2000, 5000, and 10000 ng/mL.

Accordingly, by diluting 1 mL of the stock solution with 1 mL of serum, we obtained serum standards containing 5, 50, 250, 500, 1000, 2500, and 5000 ng/mL concentrations. Preparation of the samples was carried out via the direct precipitation method. Under this protocol, 0.5 mL of serum was combined with 0.5 mL of the methanolic standard solution to precipitate plasma proteins. The tube containing the mixture was sealed with Teflon tape and vortexed for 1 minute, afterward being subjected to 15 minutes of sonication in a bath sonicator. Subsequently, the samples were centrifuged three times for 10 minutes each at 13000 rpm. The supernatant was carefully removed and kept at 4 ˚C for 5 hours. The next day, should they reveal no visible precipitate, they were injected directly into the HPLC system. If precipitate was evident, the pellet was centrifuged three more times for 10 minutes each and the resulting supernatant was subsequently injected.

For every prepared concentration, the HPLC system received a minimum of three injections, each at 25 µL. The spectrophotometric instrument calculates the area under the crocetin peak for the serum standards.

A Shimadzu UFLC LC-AD20 chromatography system manufactured by Shimadzu, Japan was employed within the LC-MS apparatus [25]. Inside the apparatus, there were 1 DGU-20A3R degasser, 2 LC-20AD pumps, 1 SIL-20AC HT auto sampler, and 1 CTO-20AC column oven. Analysis was carried out using a C18 column supplied by Supelco analytical (150×4.6 mm, 3 µm, PA, USA) with a size of 150 mm by 4.6 mm, coupled with a 3-µm particle size. The column oven temperature was established at 35 ˚C. The mobile phase comprised pure methanol with a concentration of 0.1% formic acid. Five microliters were chosen as the injection volume, while the flow rate was fixed at 0.25 mL per minute. Target compounds were analyzed in the MS/MS mode by a 3200 QTRAP mass spectrometer (AB Sciex, MA, USA) operating in positive electrospray ionization (ESI^+) mode. Nitrogen was used as the gas and its source was kept at 10 psi pressure. The pressure in both ion source gases 1 and 2 were set to 40 psi. Both the ion spray voltage and the source temperature remained set at 3000 V and 450 ˚C, respectively. All the calculations were carried out with AB Sciex Analyst software version 1.6.3. The instrument operated in MRM mode [26], with an assignment of one precursor and one product ion for the monitoring of crocetin. One hundred milliseconds were the dwell time employed for all analyses. A fragmentation event was triggered by applying a collision energy (CE) of 35 eV. A survey of the m/z spectrum from 327 (the protonated species) to 283 (the fragment ion) was employed to monitor crocetin.

In order to verify the method, precision—measured as intraday and interday repeatability—along with accuracy, LLOQ, limit of detection (LOD), and limit of quantification (LOQ) were computed according to the respective Equations 1 and 2.

1. LOD=3.3×(STEYX/Slope)

2. LOQ=10×(STEYX/Slope)

Five preparations of every standard concentration were made on 5 different days for evaluating interday repeatability. For assessment of intraday repeatability, 5 samples per concentration were prepared and analyzed on the same day with the HPLC. The coefficient of variation (CV%) of the areas under the curve for every standard concentration was computed. Both intraday and interday repeatability corresponds to precision, which is the closeness of data achieved from repeated measurements of the same analyte. At this stage, a CV acceptable for each concentration must remain less than 15%.

For determining accuracy, the true concentrations of the samples were compared with the values calculated from the calibration curve. Thereafter, bias percentage and accuracy were determined using the Equation 3:

3. Bias%=(actual concentration−measured concentration)/actual concentration)×100 Accuracy=100+Bias%

The acceptable range for Bias is ±15% [27].

The highest concentration having intraday and interday CVs less than 20% was regarded as the LLOQ.

For computation of the recovery percentage, a defined volume of serum is introduced into the titrated methanolic solution, vortexed, centrifuged, and subsequently injected into the device. Subsequently, the recovery percentage is calculated as per the Equation 4.

4. $$\frac{AUC_{\text{Derived from the serum product}}}{AUC_{\text{Derived from the metabolite product}}} = \text{Recovery \%}$$

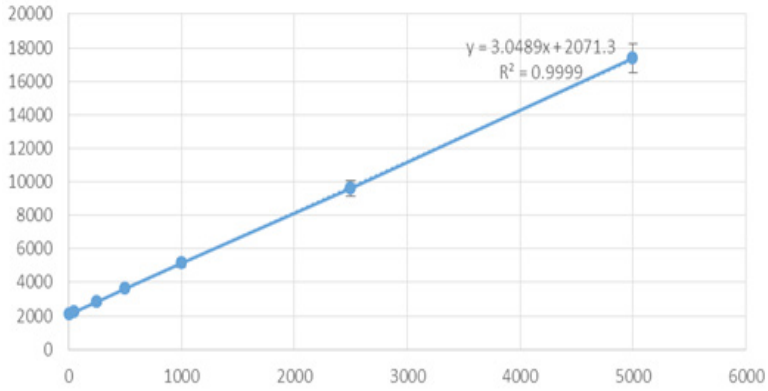

Figure 1. Calibration curve of prepared concentrations in 1:1 methanol-serum using the LC device

Results

To determine the calibration curve before injection into the LC device, 7 concentrations (5, 50, 250, 500, 1000, 2500 and 5000 ng/mL) were prepared in 1:1 mixture of methanol and serum. The standard samples were then injected in the device. Figure 1 shows the calibration curve of crocetin and the corresponding equation.

Figure 1 shows that the linear equation derived from the injection of this concentration range is (Equation 5):

5. y=3.0489x+2071.3 and R^2=0.9999.

Before running the analyte on the HPLC system, calibration standards containing 5, 50, 250, 500, 1000, 2500 and 5000 ng/mL were prepared in 1:1 methanol:serum, together with a corresponding 1:1 methanol:serum blank sample. Thereafter, the working standards were injected into the instrument. Figure 2 presents the calibration curve of crocetin together with the associated equation.

Based on Figure 2, the linear equation obtained from the injection of this concentration range was as Equation 6:

6. y=260.63x+332.49 and R^2=0.9993.

Results concerning interday and intraday variations for LC and HPLC methods were presented in Table 1, 2, 3 and 4, while LOD and LOQ data for both techniques were summarized in Table 5.

As calculated from the methodology's designated formula, the recovery percentages were 75.1±3.4% for the HPLC method and 87±3.8% for the LC-MS method, respectively.

PK data acquired through HPLC (Figure 3) and LC-MS/MS (Figure 4) were analyzed on PK-Solver software using the one-compartment model, thereby yielding the PK parameters. The t-test statistical comparison of the PK results derived from HPLC and LC-MS was executed in GraphPad Prism software, version 8. The statistical analyses revealed no significant variance between the two data sets. All results are shown in Table

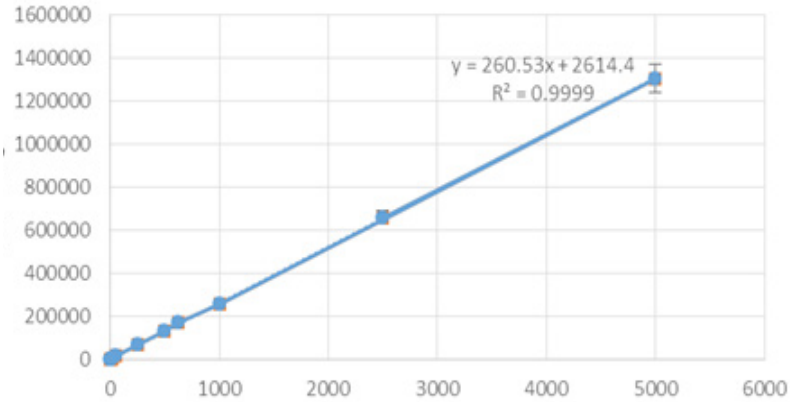

Figure 2. Calibration curve of prepared concentrations in 1:1 methanol:serum using the HPLC system

Table 1. Intraday and interday variations in serum crocetin measurement analyzed by LC-MS (n=5)

Concentration (ng/mL)	CV (%)	
	Intraday Variation	Interday Variation
50	0.78	1.38
250	0.45	2.87
500	1.05	1.68
100	0.32	2.21
2500	0.65	1.64
5000	0.89	2.41

LC-MS: Liquid chromatography-mass spectrometry.

Table 2. Intraday and interday variations of serum crocetin measurement analyzed by HPLC (n=5)

Concentration (ng/mL)	CV%	
	Intraday Variation	Interday Variation
50	0.92	1.62
250	0.54	3.44
500	1.28	2.03
1000	0.38	2.67
2500	0.77	1.97
5000	1.03	2.90

HPLC: High-performance liquid chromatography.

6. The t-test findings revealed no statistical significance between the two analytical approaches, thereby confirming that both procedures were valid.

Discussion

For the present investigation, direct precipitation was utilized to precipitate serum proteins. To serve as both a protein denaturant and a precipitant, methanol was added to the serum solution in equal proportions, a ratio of 1:1. On the other hand, an identical proportion of acetonitrile in another study was employed [17]. Likewise, a 3:1 methanol solution was employed for precipitating plasma proteins. Linear calibration profiles generated by HPLC and LC-MS employing methanol-serum (1:1) mixtures verified a linear correlation between concentration and peak area for both techniques [28]. Recovery values of 75% (HPLC) and 87% (LC-MS) were obtained,

Table 3. Accuracy results of concentrations measured by LC-MS

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Bias (%)	Accuracy
50	56	-12	88
250	256	-2.4	97.6
500	495	1	101
1000	998	0.2	100.2
2500	2512	-0.48	99.52
5000	4980	0.4	99.6

LC-MS: Liquid chromatography-mass spectrometry.

Table 4. Results related to accuracy of concentrations by HPLC device

Actual concentration (ng/mL)	Calculated concentration (ng/mL)	Bias (%)	Accuracy
50	57.18	-14.34	85.64
250	257.55	-3.02	96.98
500	507.72	-1.54	98.46
1000	995.92	0.41	100.41
2500	2525.51	-1.02	98.98
5000	4997.53	2.47	102.47

HPLC: High-performance liquid chromatography.

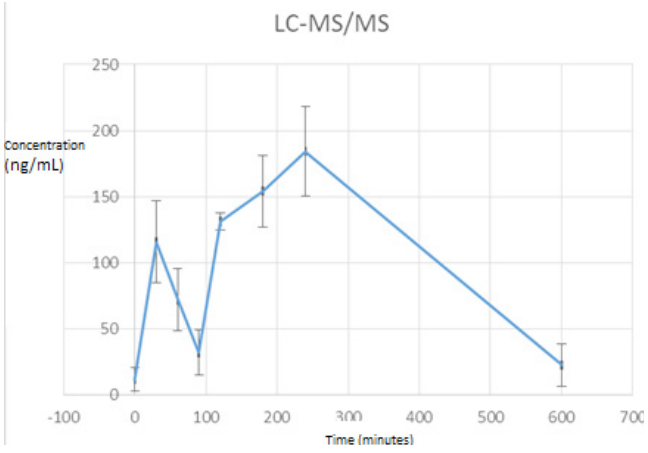

Figure 3. Crocetin concentration versus time analyzed by LC-MS/MS

LC-MS/MS: Liquid chromatography-tandem mass spectrometry.

therefore confirming that both analytical techniques are optimal for this analysis. Data validation constitutes the most pivotal element in a PK analysis. Accuracy, precision, the LOQ, and the LOD for both HPLC and LC-MS methods were all within acceptable thresholds. Coefficients of variation (CV) below 15% thus underline the reliability of these techniques for measuring unknown concentrations across various time points.

PKs of crocetin is generally evaluated with higher doses in animal studies. For instance, The quails were administered an average daily dose of 50 mg/kg crocetin, where-

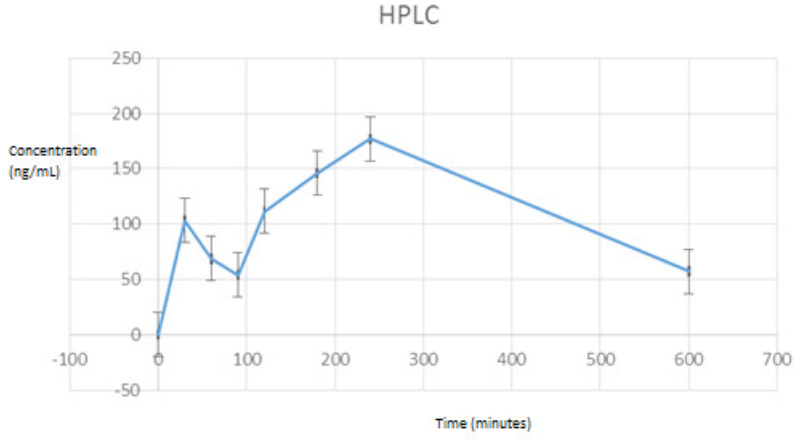

Figure 4. Crocetin concentration versus time curve analyzed by HPLC

HPLC: High-performance liquid chromatography.

Table 5. Method sensitivity with calculation of LOQ and LOD

Method	Mean±SD	
	LOD (ng/mL)	LOQ (ng/mL)
HPLC	15.06±2.65	45.18±3.7
LC-MS-MS	6.24±1.68	18.74±2.71

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Abbreviations: HPLC, high-performance liquid chromatography; LC-MS-MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantification; LOD, limit of detection.

as 15 mg/kg crocetin was used for every rabbit [29, 30]. Nevertheless, because the doses approved for human use are far lower (0.5 mg/kg rather than 50 mg/kg), the resulting plasma concentrations in humans are anticipated to be considerably lower than those recorded in the animal models. Therefore, the analytical technique might prove incapable of detecting low concentration levels in animal datasets. Therefore, this underscores the need for PK investigations carried out in humans themselves.

An oral study conducted using crocin-1 (1 mg/kg) in mice demonstrated swift absorption of crocin-1 in the form of crocetin, keeping its plasma levels above 50 ng/mL for roughly ten hours. A rise in blood concentration emerged at 20 minutes, declined quickly, and afterward peaked again 6 hours later. Certain phytochemicals, such as isoflavonoids, display a characteristic biphasic absorption pattern that may arise from hepatic recycling [8] or from a first peak arising from absorption in the small intestine, followed by a second peak stemming from absorption further down in the colon [31].

An investigation conducted on 5 healthy male participants who were given 7.5 mg oral crocetin observed a Cmax of 100.9±50.2 ng/mL. Consequently, our investigation of 15 mg crocin, equivalent to 5 mg of crocetin, recorded a Cmax of 136±5 ng/mL. The plasma concentration time course documented a single peak, in contrast with the two observed in our study. This divergence might be attributable to gut bacterial-mediated absorption of crocin in the gastrointestinal tract, which produces the initial peak. Further PK parameters for 7.5 mg orally crocetin—Tmax, T1/2, clearance (CL/F), and Vd/F—were recorded as 4.8±1.0 h, 6.1±7.2 h, 13.8±5.4 L/h, and 82.8±59.0 L, respectively [14].

Table 6. Results obtained from LC-MS/MS and HPLC

Parameter	Value of LC-MS	Value of HPLC
t1/2ka (min)	132.4319271	153.418969
$t_{1/2}$k10 (min)	142.5353408	164.1011262
V_d/F (mg)/(ng/mL)	0.013456447*	0.013256177
CL/F (mg)/(ng/mL)/min	6.54385E-05**	5.59928E-05
T_{max} (min)	198.1683797	228.8694719
C_{max} (ng/mL)	141.7471874	143.4529633
AUC 0-t (ng/mL×min)	61473.9489	65759.67855
AUC 0-inf (ng/mL×min)	76407.62645	89297.19436
AUMC (ng/mL×min²)	30310440.37	40905675.53
MRT(=AUMC/AUC) (min)	396.6939137	458.0846668

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LC-MS/MS: Liquid chromatography-tandem mass spectrometry; HPLC: High-performance liquid chromatography.
*0.013456447×1000=13.45 L, **6.54385E-05×60000=39.26 L/h

In a study, 6 healthy men were given a saffron solution and found intraday and interday repeat abilities of 15% alongside an LLOQ of less than 20 ng/mL [11]. Among the study's chief strengths was the successful identification of human-sample isolated crocetin and crocin separate isomers [11].

Conclusion

This investigation effectively carried out a PK assessment, confirming that HPLC and LC-MS constitute sound techniques for blood analysis and are dependable means of determining PK parameters. The observed clinical effects attributed to Krocina tablets can be attributed to the crocetin that is absorbed.

Ethical Considerations

Compliance with ethical guidelines

This work was approved by Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran (Code: IR.MUMS.REC.1401.016). This study was registered by the Iranian Registry of Clinical Trials (IRCT), Tehran, Iran (Code: IRCT20130507013263N2).

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Authors' contributions

Conceptualization and supervision: Mojgan Nejabat and Farzin Hadizadeh; Methodology: Hossein Kamali and Majid Ghayour Mobarhan; Investigation and data collection: Faezeh Ghorbanzadeh; Data analysis: Hossein Kamali; Funding acquisition and resources: Farzin Hadizadeh; Writing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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