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Comparative pharmacokinetic profiles of selected irreversible tyrosine kinase inhibitors, neratinib and pelitinib, with apigenin in rat plasma by UPLC–MS/MS

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ABSTRACT

Neratinib (NER) and pelitinib (PEL) are irreversible tyrosine kinase inhibitors (TKIs) that have been recently employed in cancer treatment. Apigenin (API), among other flavonoids, is known to have antioxidant, anti-proliferative, and carcinogenic effect. API can potentiate the antitumor effect of chemotherapeutic agents and/or alleviate the side effects of many anticancer agents. Since TKIs are mostly metabolized by CYP3A4 enzymes and that API could alter the enzymatic activity, potential drug interactions could be expected following their co-aministration. In the present study, a bioanalytical UPLC–MS/MS method has been developed and validated for the quantification of NER and PEL in rat plasma, using domperidone (DOM) as an internal standard. Sample preparation was carried out using solid phase extraction (SPE) with C18 cartridges with good extraction recovery of not less than 92.42% (NER) and 89.73% (PEL). Chromatographic analysis was performed on a Waters BEH C18 column with a mobile phase composed of acetonitrile and water, (70:30, v/v), each with 0.1% formic acid. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions from protonated precursor ions $[M+H]^+$, at *m/z* 557.30 (NER), *m/z* 468.21 (PEL), and at *m/z* 426.27 (DOM), to selected product ions at *m/z* 112.05 (NER), *m/z* 395.22 (PEL), and at *m/z* 175.18 (DOM). The method was fully validated as per the FDA guidelines over the concentration range of 0.5–200 ng/mL with very low lower limit of quantification (LLOQ) of 0.5 ng/mL for both NER and PEL. The intra- and inter-day assay precision and accuracy were evaluated for both drugs and the calculated values of percentage relative standards deviations (%RSD) and relative errors ($\%E_r$) were within the acceptable limits (<15%) for concentrations other than LLOQ and 20% for LLOQ. The applicability of the method was extended to study the possibility of drug interactions following the oral co-administration of NER/PEL with API. Thus, this study could be readily applied in therapeutic drug monitoring (TDM) of cancerous patients receiving such drug combinations.

1. Introduction

Recently, the anticancer strategy has been shifting from cytotoxic chemotherapeutic agents to targeted therapies. Molecular-targeted drugs are designed to target overexpressed or mutated molecules within the tumor cells. Because of their high selectivity, these drugs were thought to exhibit more efficiency and less toxicity, compared with the conventional chemotherapeutic agents [1]. Among the most promising drugs in targeted therapy are tyrosine kinase inhibitors (TKIs). It is well known that dysfunction in the kinase activity results in the normal regulation of the cellular phosphorylation signaling with successive pathological effects ranging from inflammation to cancer development. Thus tyrosine kinases have been recognized as prime targets for controlling the cancer growth. The majority of TKIs show their anticancer effect by targeting the ATP binding site of the protein kinases. Based on the binding mechanism, TKIs could be broadly classified into reversible and irreversible inhibitors [2]. ATP-competitive drugs bind to the kinase domain of the targeted receptor in a reversible manner via weak interactions (e.g. hydrogen bonding, dipole–dipole interaction). However, the more potent and selective irreversible TKIs inhibit their target protein by the formation of covalent bonds. Covalent irreversible TKIs are generally more superior compared with the conventional reversible competitors. Among their advantages are; improved selectivity, prolonged pharmacological effect,

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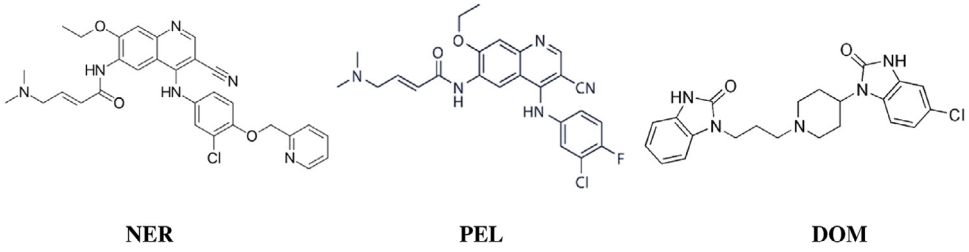

Fig. 1. Chemical structure of the studied compounds; neratinib (NER), pelitinib (PEL), and domperidone (DOM).

and more potential to overcome resistance [2]. The progress of the introduction of irreversible TKIs to clinical trials is steadily increasing. Neratinib (NER) and pelitinib (PEL), Fig. 1, are examples of this class of irreversible TKIs that have clinical implications. NER has shown antitumor activity in patients with breast cancer, phase II study, [3] and non- small cell lung cancer (NSCLC), phase II study [4]. PEL has also been evaluated for its efficacy in NSCLC and colorectal cancer, phase II study [2].

All TKIs are orally administered and thus they are generally characterized by convenient and flexible administration, in addition to better quality of life. Moreover, being taken on a regular daily basis, this contributes to the consistent exposure of the tumor cells to the active drug [5]. On the other hand, their oral administration generates a complex step in the pharmacokinetics (PK) of these drugs [6,7]. Intra and inter-subject variability with differences in drug exposure could markedly affect the treatment benefits or increase the severity of drug toxicity [7]. This variability could be attributed to the pharmacogenetics characteristics of the patient (e.g. polymorphism in ABC efflux transporters and drug metabolizing enzymes cytochrome CYP P450 enzymes) and the genetic heterogeneity of drug targets [6]. Phenotyping drug disposition has gained interest in oncology practice [8]. Another important factor contributing to PK variability is patient characteristics including smoking, habits, patient compliance to therapy, and environmental factors (e.g. drug–drug interactions) [6].

Drug-drug interactions are a matter of potential concern in oncology practice. They can affect the bioavailability of the anticancer agents with an alteration in the antitumor effect, gained resistance, or increased drug toxicities to normal cells (side effects) [1]. This is extremely important since most cancerous patients are on multiple non-cancer medications including self-prescribed over-the-counter drugs, supportive care medications used to alleviate the side effects of anticancer drugs, in addition to complementary and alternative medicine (CAM) including plant or natural product medications [1].

The concomitant use of natural products with many of the anticancer medications could eventually contribute to the problem of potential drug interactions. Although dangerous, this problem is often underestimated by the patients. Being natural, they are usually thought of as being "safe" and hence are usually taken without consulting the physician as "self-care products". Thus natural products present a potential problem with CAM users with the increasing risk of food-drug interactions [1]. Ever-increasing use of natural products among different populations increases the potential of food-drug interactions which may affect drug bioavailability via their effect on the absorption, metabolism, distribution, and excretion of the co-administered drugs.

Flavonoids are the most abundant polyphenols in the human diet. They are constituents of many vegetables and fruits and are also ingredients of many teas, juices, and herbal products. Based on the chemical structure, flavonoids can be classified into several classes, among which are flavones. Apigenin (API) is among the most common examples of flavones that could be found in a diverse variety of vegetables and fruits. Many studies describe the role of flavonoids in the prevention of degenerative diseases, particularly cardiovascular diseases [9]. Moreover, the dietary supplementation with flavonoids could be extremely beneficial in cancer treatment since they can potentiate the antitumor effect of chemotherapeutic agents and/or alleviate the side effects of many anticancer agents [9]. Also, polyphenols could inhibit the growth of cancer cells due to their ability to modulate the activity of multiple targets involved in the carcinogenesis [10]. API, 4′, 5, 7-trihydroxy flavone, is found in many fruits and vegetables, including onions, parsley, green apple, citrus fruits, guava, chamomile, tea, and in some seasonings. API exhibits several biological activities including antioxidant, anti-inflammatory, antibacterial, antihypertensive, antidiabetic, and anticancer effect [10–13]. It also has proven to have anti-proliferative action against some cancer cell lines including colon, thyroid, breast, multiple myeloma, pancreatic [11], glioma [12], and melanoma [13].

It has been recently shown that flavonoids, including API, contribute to several food-drug interactions. Such interactions are due to alteration in the activity of metabolizing enzymes and/or transporter proteins [14,15]. Over the past few years, many clinically important interactions between API and co-administered drugs have been reported. The effect of API on the PK of co-administered drugs resulted from its inhibitory effect either on CYP-mediated metabolism (e.g. raloxifene [16], imitanib and its metabolite [17], venlafaxine [18]) or on P-gp-mediated efflux (e.g. paclitaxel [19]). It has also been reported that the bioavailability of etoposide could be attributed to the combined inhibitory effect of API on both CYP3A4 enzymes and P-gp efflux pump [20].

Given the widespread availability of API, it was essential to investigate the effect of its concomitant use on the bioavailability of selected irreversible TKIs; NER and PEL. Since API is an inhibitor of both CYP 450 enzymes and P-gp transporters, it could potentially affect the systematic exposure of TKIs which are CYP3A4 and P-gp substrates [1]. Review of the literature revealed that only few LC–MS/MS methods have been reported for the analysis of NER [21–23] or PEL [24] in biological samples. To our knowledge, the effect of food on the PK of NER/PEL has not been investigated yet.

This work aims at the development and validation of a rapid and highly selective UPLC–MS/MS method for the determination of NER/PEL in rat plasma samples. The method was successfully applied to the pharmacokinetic study of NER/PEL when administered alone, and in comparison with their concomitant administration with API.

2. Experimental

2.1. Materials and reagents

REFERENCES standards of NER and PEL (purity: >99%) were obtained from Pfizer Inc. (NY, USA). The internal standard (IS), domperidone (DOM), (purity: >98%), was supplied by Amoun Pharmaceutical Company (El-Obour City, Cairo, Egypt). A reference standard of API was purchased from Atlantic Research Chemicals (Stratton, UK).

All solvents used in the study, including acetonitrile and methanol (Panreac, Barcelona, Spain) were HPLC grade. Formic acid (Sigma-Aldrich, Chemie GmbH, Munich, Germany) was involved in the study. Ultrapure water was obtained from Ultrapure water Milli-Q Advantage water purification system (Millipore, Molsheim, France).

2.2. Instrumentation and UPLC–MS/MS conditions

Chromatographic analysis was performed on UPLC–MS/MS Waters Model Xevo TQ-S separation system (Waters, Singapore, Singapore) equipped with binary solvent manager and sample manager (Aquity TM Ultra-performance LC). A triple-quadrupole mass spectrometric detector (STEP WAVE TM, Ultra-performance LC) with electrospray ionization (Zspray TM ESI-APCI-ESCI, Ultra-performance LC) and multiple reaction monitoring (MRM)-mode was applied. Masslynx™ Version 4.1 software (Micromass, Manchester, UK) was used to process and manipulate the data.

UPLC separation was achieved on Acquity UPLC BEH™ C 18 analytical column (100 × 1.0 mm, i.d., 1.7 μm particle size) (Waters, Dublin, Ireland). All samples were analyzed using a mobile phase consisting of acetonitrile: water (70: 30, v/v), each with 0.1% formic acid. The flow rate was set at 0.2 mL/min and injection volumes of 5 μL of solutions were injected with the full loop mode. The column and auto-sampler temperatures were maintained at 45° and 10 °C, respectively.

MS/MS detection was performed using electrospray ionization (ESI) source operated at the positive ion mode with optimized MS parameters including a source temperature of 150 °C, the dwell time of 0.025 s, and desolvating gas, cone gas, and collision gas flow rates adjusted at 800 L/h, 150 L/h, and 0.15 mL/min, respectively. Also, the MS analyzer was set at the resolutions; LM of 2.8 and HM of 14.86, for both ion energy 1 and 2. Quantitation was performed using MRM of the transitions from protonated precursor ions $[M+H]^+$, at *m*/*z* 557.30 (NER), *m*/*z* 468.21 (PEL), and at *m*/*z* 426.27 (DOM), to selected product ions at *m*/*z* 112.05 (NER), *m*/*z* 395.22 (PEL), and at *m*/*z* 175.18 (DOM).

For sample preparation, two types of solid-phase extraction (SPE) cartridges were tried including Bond Elut C 18 cartridges (100 mg, 1 mL), (Agilent, Santa Ckara, CA, USA), and Spe-ed cartridges C 8 (200 mg, 3 mL), (Applied Separations, Allentown, Pennsylvania, USA). SPE was performed using a J.T. Bakers vacuum system. Nitrogen evaporator N-EVAP 112 with heating system OA-SYS (Organomation Assocciates, Inc, Berlin, Massachusetts, USA) was used for sample preparation. Disposable syringe filters (CHROMAFIL ® Xtra PA-20/25) polyamide filters, pore size: 0.2 μm, filter-θ: 25 mm (MACHEREY NAGEL, GmbH & Co. KG, Duren, Germany) were used for sample filtration.

2.3. Stock and standard solutions

Stock solutions of 10 μg/mL of NER, PEL, and DOM (IS) were prepared in methanol. Further dilutions were made to get standard solutions of different concentrations of NER and PEL. Also, a standard solution of concentration 5 ng/mL of DOM was prepared.

2.4. Calibration standards and quality control samples

Separate volumes of 50 μL blank plasma samples were spiked with eight different volumes of standard solutions of NER and PEL to cover the concentration range of 0.5–200 ng/mL of both NER and PEL. To each spiked plasma sample, a volume of 100 μL of 5 ng/mL DOM (IS) was added. All samples were then made up to final volumes of 1 mL with methanol. These spiked samples were used to construct matrix-based calibration graphs for the quantification of both NER and PEL in rat plasma. Blank samples were simply prepared by adding 950 μL methanol to 50 μL plasma samples. For the validation purpose, quality control (QC) samples were prepared by spiking 50 μL volumes of drug-free rat plasma samples at four different concentrations within the linearity range, namely very low (LLOQ, 0.5 ng/mL), low (5 ng/mL), medium (50 ng/mL), and high (150 ng/mL) concentrations, along with DOM (IS).

2.5. Sample preparation

Blank and spiked plasma samples were vortex-mixed for 5 min at 6000 rpm. The supernatant of each sample was separated to a glass tube and the residue was further washed with 0.5 mL volumes of methanol. The combined methanolic solutions obtained from the supernatants and the washings were further purified by passing onto C 18 Bond Elut cartridges used for SPE, previously preconditioned with 1.0 mL methanol followed by 1.0 mL ultrapure water. The retained drugs were then eluted with 0.5 mL methanol which was further evaporated to dryness under nitrogen using the nitrogen evaporator. The obtained residue was then reconstituted in 0.5 mL acetonitrile and volumes of 5 μL were injected into the UPLC–MS/MS system for analysis.

2.6. Assay validation

Method validation was carried out based on the FDA guidelines for bioanalytical methods [25]. Accordingly, different validation parameters were evaluated. They include specificity, linearity, lower limit of detection and of quantification, extraction recovery, matrix effects, accuracy and precision, dilution integrity, and stability studies.

2.6.1. Specificity

The specificity of the method was assessed by comparing the chromatograms of plasma extracts collected from six different rats with those spiked with very low drug concentrations equivalent to the LLOQ of NER and PEL, along with DOM (IS). The analytical responses obtained at the retention times of both drugs at their LLOQ and of IS were then compared to that obtained from drug-free samples.

2.6.2. Linearity

Linearity was assessed by analyzing rat plasma samples (50 μL) spiked with eight different concentrations of the studied drugs (NER and PEL) in the range 0.5–200 ng/mL plasma, along with DOM (IS), 100 μL of 5 ng/mL DOM (IS). Matrix-based calibration graphs were then constructed by relating the peak area ratios of each drug in spiked plasma samples to that of DOM (IS) using the method of least squares. Alternatively, the regression equation was separately derived for each of NER and PEL.

2.6.3. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

LLOD of both NER and PEL were calculated based on the analyte concentrations that produced analytical responses of at least three times that of the blank signals. However, for LLOQ, the ratio of five times that of the blank signals was used instead and taking into consideration that the signals produced should be identifiable with acceptable accuracy and precision (at least ±20%).

2.6.4. Recovery and matrix effect

The extraction recovery of NER and PEL from plasma samples was determined at the four QC levels; very low (0.5 ng/mL), low (5 ng/mL), medium (50 ng/mL), and high (150 ng/mL). This was achieved by comparing the peak area obtained from spiked plasma samples with those obtained from standard drug solutions of the same nominal concentrations (n = 6).

To evaluate the matrix effect, the same procedure as per evaluation of extraction recovery was repeated but with the exception that recovery calculations were based on using processed standard samples (without plasma) as a reference. Moreover, the extraction recovery and matrix effect of DOM (IS) at the specified concentration used in actual analysis was calculated.

2.6.5. Precision and accuracy

Precision and accuracy were evaluated by analyzing QC samples previously prepared at four different concentration levels. These samples were then treated and analyzed six times on the same day or on three consecutive days for evaluating the intra-day and inter-day precision and accuracy, respectively. The peak area ratios of each drug to that of DOM (IS) were used to calculate the actual drug concentration found in each sample which was then compared with the nominal values. Accordingly, accuracy in terms of percentage relative error (E_r%) and precision in terms of percentage relative standard deviation (%RSD) were assessed.

2.6.6. Dilution integrity

Dilution of highly concentrated plasma samples was evaluated for its effect on the recoveries of both drugs. This was assessed by using plasma samples spiked with high concentrations of both NER and PEL (300 ng/mL). Prior to analysis, these samples were diluted with blank rat plasma (1:2 and 1:5). Diluted samples were then processed exactly as described above. In each case, the found concentrations were related to the nominal values and the recovery% (±RSD) was calculated.

2.6.7. Stability studies

The stability of NER and PEL in rat plasma was assessed by analyzing QC samples at two concentration levels, low (5 ng/mL) and high (150 ng/mL), (n = 6). Plasma samples were subjected to different conditions for assessing autosampler stability, short-term (bench-top), long-term stability, and stability to freeze-thaw cycles. In each case, recoveries were calculated by relating the found concentration of both drugs to that of the nominal concentrations. To evaluate post-preparation stability, the extracted samples left in the autosampler at 10 °C for 56 h prior to the injection were analyzed. However, short-term and long-term stability were assessed using samples left at room temperature (25 °C) for 6 h or at −30 °C for 30 days, respectively. Finally, the stability of both drugs following three freeze-thaw cycles (freeze at around −30 °C then thaw at room temperature) was evaluated.

2.7. Application to pharmacokinetic studies

All experiments were performed as per the WHO regulations in Saudi Arabia with reference to ethical guidelines for experimental studies with animals. Wistar healthy male rats weighing 250 ± 30 g were provided by the animal house, Women Student-Medical studies & Sciences Sections, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The rats were placed in cages kept at standard laboratory conditions; a well-ventilated room, a regular 12 h day–night cycle, an average temperature of 24–27 °C, and a relative humidity of 40–60%. All the rats had free access to water while diet was prohibited for 12 h before conducting the experiment. The rats were acclimatized for 7 days to laboratory conditions before initiating the study. The animals were divided into four groups of five rats each. Suspensions of 30 mg/mL (NER), 10 mg/mL (PEL), and 100 mg/mL (API) were prepared by triturating an appropriate weight of each drug with aqueous methyl cellulose (0.5%, w/v). Separate volumes (0.25 mL) of the drugs suspensions were given to the treated animals orally using a gavage needle as follows; NER, 30 mg/kg (Group I), PEL, 10 mg/kg (Group II). Rats of groups III and IV were first separately given an oral dose of API (100 mg/kg). After a period of 30 min, the rats were subjected to an oral dose of NER, 30 mg/kg (Group III), or PEL, 10 mg/kg (Group IV). Blood samples (0.3 mL) were collected from the retro-orbital sinus of each rat into heparinized tubes following drug administration at different time intervals; 0 (prior to dosing), 0.5, 1, 2, 3, 4, 6, 10, and 24 h. All samples were immediately centrifuged for 30 min using 4500 rpm at 4 °C. The resulting plasma samples were kept frozen at −20 °C until analysis. Volumes of 50 μL of each plasma sample were separately spiked with a constant volume of 100 μL of DOM, IS (5 ng/mL), then completed to a final volume of 1 mL with methanol. All samples were then treated exactly as described under sample preparation. The found concentrations were calculated, using the corresponding matrix-based calibration graph, by relating the peak area ratio of each drug to that of the IS.

3. Results and discussion

3.1. Optimization of UPLC–MS/MS conditions

Analysis method was performed by optimizing UPLC and MS/MS conditions for maximum specificity and sensitivity. At first, standard solutions of the analytes; NER, PEL, and DOM (IS) (10 ng/mL), were individually introduced into the mass spectrometer via syringe infusion. All the analytes were more efficiently ionized in the positive mode than in the negative mode. Thus positive ESI was employed as the ionization mode in this study. MRM was used to monitor both the precursor and product ions. Different MS/MS parameters such as ESI source temperature, cone and capillary voltage, flow rate of desolvation gas, and desolvation temperature were optimized in order to obtain the highest intensity of the protonated precursor ions. It was observed that a source temperature of 150 °C and desolvation gas flow rate of 80 L/h resulted in the highest response of the protonated precursor ions. Also, an increase in the relative abundance of the precursor ions was associated with the increase in the cone voltage or capillary voltage till optimum values after which a remarkable decrease was noticed. On the other hand, the collision energy was optimized for maximum intensity of the product ions. The intensity of a particular fragment ion increased with increasing the collision gas energy till optimum values above which a decrease in its intensity was noticed. The optimized MS/MS conditions of the studied drugs were summarized in Table 1. Full scan product ion spectra of protonated precursor ions $[M+H]^+$ for NER, PEL, and DOM were shown in Fig. 2. Precursor ions were selected at *m*/*z* 557.30 (NER), *m*/*z* 468.21 (PEL), and at *m*/*z* 426.27 (DOM) while the product ions were at *m*/*z* 112.05 (NER), *m*/*z* 395.22 (PEL), and at *m*/*z* 175.18 (DOM).

The second phase of the optimization procedure involves studying the effect of mobile phase composition on the analytes' response. Different mobile phases composed of mixtures of different ratios of acetonitrile (30–90%), water, and formic acid (0.005-0.15%) were evaluated for the chromatographic elution of standard mixtures of NER, PEL, and DOM (IS). Initially, mobile phases of different ratios of acetonitrile (30–90%) and water, each with 0.1% formic acid, were tried. Experimental trials revealed that acetonitrile percentage of 60% or less resulted in distortion of NER peaks. Sharp and symmetric peaks of both drugs, along with DOM (IS) were obtained with acetonitrile percentage of 70%. However, higher acetonitrile percentage resulted in peak distortion including peak tailing (NER) and peak broadening (PEL). Also, increased response of PEL peaks was noticed at acetonitrile percentage of 60% or higher. In addition, an increase in the acetonitrile content in the mobile phase was associated with a slight increase in the retention time of the three compounds. Thus, 70% acetonitrile in the mobile phase produced best results regarding the runtime, peak response, and peak shape of NER, PEL and DOM peaks. The second phase of

Table 1
LC–MS/MS optimized parameters for the determination of the studied drugs.

Target compound	Precursor ion[M+H]$^+$	Daughter ion	Cone voltage(V)	Capillary voltage(KV)	Collision energy(eV)	Desolvation Temperature(°C)
NER	557.30	112.05	90	4	30	200
PEL	468.21	395.22	3	3	30	200
DOM (IS)	426.27	175.18	30	3.5	30	200

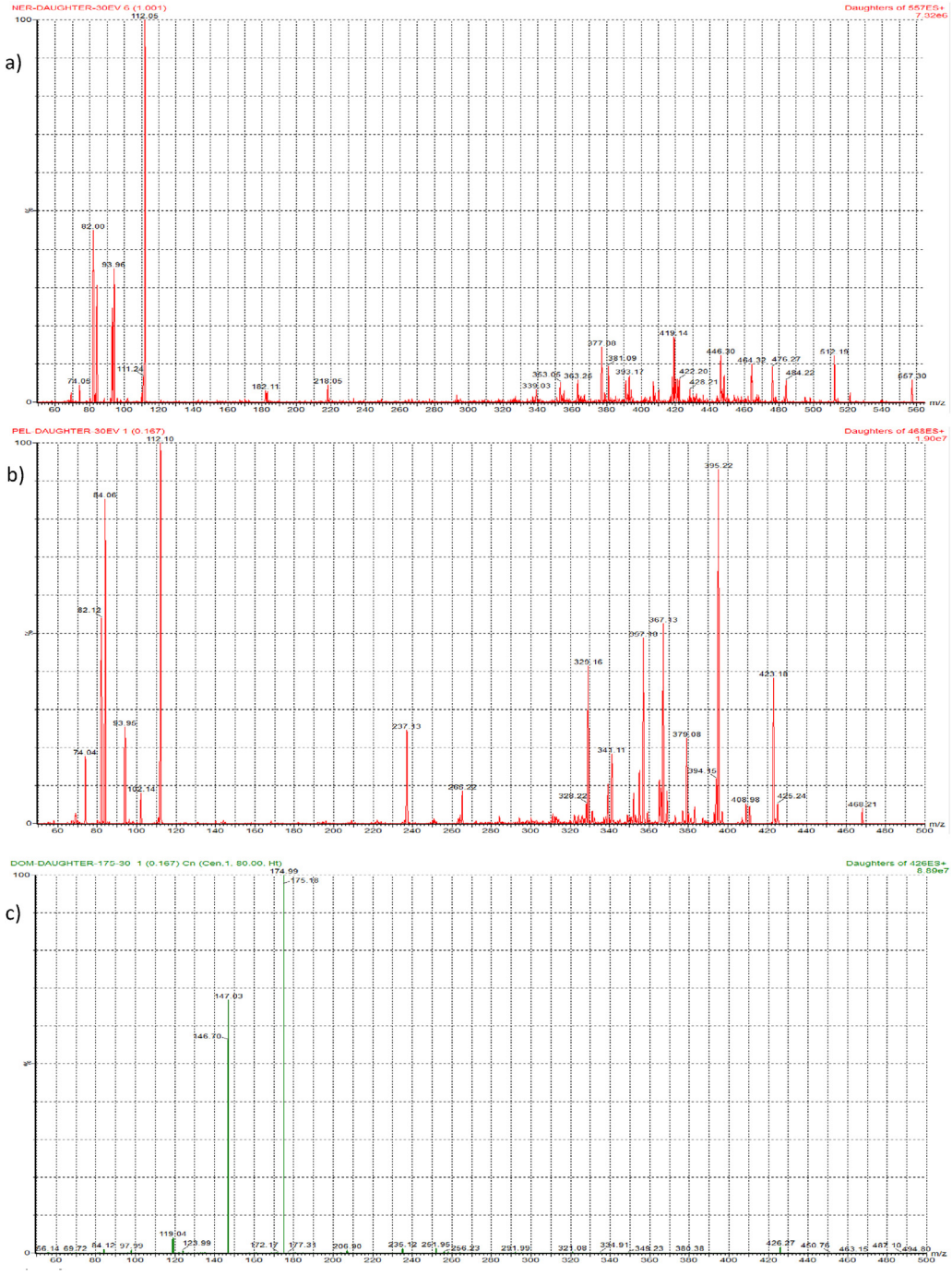

Fig. 2. Product ion spectra of NER, a), PEL, b), and DOM, c).

mobile phase optimization involved the effect of formic acid content (0.005–0.15%) in the mobile phase. Formic acid was found to be essential to get sharp peaks of both NER and PEL. It was also noticed that a decrease in the retention time of both NER and PEL was associated with an increase in the formic acid content in the mobile phase till 0.1% above which some tailing was recorded for NER and PEL peaks. Best results were achieved with 0.1% formic acid content in the mobile phase. Final analysis was performed with a mobile phase consisting of acetonitrile: water, each with 0.1% formic acid, (70: 30, v/v) for the whole runtime of 1.5 min. DOM had a suitable retention and chromatographic behavior, compared with NER/PEL. Thus it was used as the IS in this study. Under these chromato-

graphic conditions, sharp and symmetric peaks of all drugs were obtained (NER eluted at 0.59 ± 0.03 min, PEL at 0.57 ± 0.02 min, and DOM (IS) at 0.51 ± 0.020 min).

3.2. Sample preparation

Sample preparation is very important parameter in the development of bioanalytical techniques. To overcome the interfering effect of many endogenous components that could largely hinder the selective analysis, different sample preparation techniques are applied. They include protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). Among which, SPE has been widely applied in LC–MS/MS analysis of many biological matrices. Its superior potential to minimize the ion suppression could be referred to its ability to eliminate most endogenous serum components [26–28]. Thus, SPE was applied for sample preparation in this study.

Plasma samples were treated with methanol then the obtained clear supernatants were further purified using SPE. Initially, two SPE cartridges namely, Bond Elut C 18 (100 mg, 1 mL) and octyl C 8 (200 mg, 3 mL) were tested for their extraction efficiency using plasma samples spiked with both NER and PEL at the concentration level of 50 ng/mL. It was found that better peak shape was obtained using C 18 compared with C 8 cartridges since the latter resulted in a significant peak splitting of both NER and PEL peaks. SPE recovery of C18 cartridges was further evaluated by replicate analysis (n = 6) of plasma samples spiked with NER and PEL at the QC levels 0.5, 5, 50, and 150 ng/mL. C 18 cartridges provided excellent recovery for both drugs (89.73–94.84%).

3.3. Method validation

3.3.1. Specificity

The chromatograms of drug-free plasma samples and plasma samples spiked with both NER and PEL at their LLOQ level are shown in Fig. 3. High degree of method specificity was confirmed by the absence of any interference at the retention times of both analytes and IS. Moreover, the required criteria for the peak response for both drugs at their LLOQ and for the IS were met; at least five times and twenty times compared with the blank, respectively.

3.3.2. Linearity

The linear regression of the peak area ratios calculated for each of NER and PEL to that of DOM (IS) versus spiked concentrations were fitted over the range (0.5–200 ng/mL plasma) for both drugs. Regression and statistical parameters were presented including; correlation coefficients (r), intercept (a), slope (b), standard deviations of residuals ($S_{y/x}$), of the intercept (S_a), of the slope (S_b), in addition to the variance ratio (F values), Table 2. Low degree of scatter of the experimental data points around the regression line was indicated by the high F values and low values of $S_{y/x}$ [29]. For both drugs, high degree of linearity of the proposed method was indicated from the high values of the correlation coefficients ($r \geq 0.9983$) along with small intercepts indicated.

3.3.3. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

For both NER and PEL, LLOQ and LLOD were found to be 0.5 and 0.3 ng/mL, respectively (Table 2). The MRM chromatograms of drug-free plasma and plasma samples spiked with NER and PEL at their LLOQ are shown in Fig. 3. The low value of LLOQ obtained for both drugs ensures the ability of the method to be applied in the trace analysis of the two drugs in further clinical studies. Compared with the previous literature, the proposed method provided the lowest LOQ values obtained so far in the analysis of NER [21–23] and PEL [24].

Table 2
Regression and statistical parameters for the determination of NER and PEL in rat plasma by the proposed UPLC–MS/MS method.

Parameter	NER	PEL
Linearity range (ng/mL)	0.5–200	0.5–200
LLOQ[a] (ng/mL)	0.5	0.5
LLOD[b] (ng/mL)	0.3	0.3
Intercept (a)	−0.0222	0.0078
Slope (b)	0.1002	0.0289
CorrelationCoefficient (r)	0.9997	0.9983
S_a[c]	0.0341	0.0075
S_b[d]	0.0008	0.0006
$S_{y/x}$[e]	0.0748	0.0157
F[f]	14434.24	2288.29
Significance F	2.8785×10^{-8}	1.1425×10^{-6}

[a] LLOQ: lower limit of quantification.
[b] LLOD: lower limit of detection.
[c] S_a: standard deviation of intercept.
[d] S_b: standard deviation of slope.
[e] $S_{y/x}$: standard deviation of residuals.
[f] F: variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals).

3.3.4. Recovery and matrix effect

Recovery values of not less than 92.42% (NER) and 89.73% (PEL) were obtained following the analysis of plasma samples spiked at four concentration levels (QC samples) as described under the experimental section. In addition, the mean recovery obtained for DOM (IS) at the specified concentration applied in the analysis was 89.555. Recovery results summarized in Table 3 revealed that the proposed method for sample treatment provided high degree of extraction efficiency of both NER and PEL from plasma samples.

The matrix effect for NER and PEL at concentrations (0.5, 5, 50, 150 ng/mL) was found to be not more than 5.79% and 3.44%, respectively (Table 3). Also, the matrix effect for DOM (IS) at the specified concentration level was found to be 1.88%. Thus, the matrix effect of plasma samples was negligible using the applied method. This is extremely important for the determination of very low concentrations and trace analysis of both NER and PEL in plasma samples.

3.3.5. Precision and accuracy

The data for intra-day and inter-day accuracy and precision for both NER and PEL were summarized in Table 4. For both drugs, the calculated relative errors were in the range (−0.33–(−9.92) %) and (−0.91–(−8.62) %) for intra-day and inter-day errors, respectively, while, the calculated RSD values were in the range (1.52–6.33%) and (2.70–10.02%) for intra-day and inter-day deviations, respectively. Since both relative error and deviation values did not exceed ±15.0%, for concentrations other than LLOQ and ±20% for LLOQ, this indicated acceptable degree of accuracy and precision of the proposed method.

3.3.6. Dilution integrity

The calculated recovery% (±RSD) obtained for both NER and PEL following the dilution of plasma samples are shown in Table 4. Since these results did not exceed the (±15%) acceptance limits, this indicates the integrity of both drugs up to five times dilution of plasma samples.

3.3.7. Stability studies

The results obtained from stability studies are summarized in Table 5. Since the calculated recoveries did not exceed ± 15%, this indicated the stability of NER and PEL under the studied storage and handling conditions. Moreover, the results also revealed the stabil-

Table 3
Evaluation of the extraction efficiency of C-18 cartridges and matrix effect in the UPLC–MS/MS analysis of standard mixtures of NER and PEL, with DOM (IS).

	Concentration spiked (ng/ml)	Mean recovery (%) ± RSD[a]	E_r(%)[b]
Extraction recovery			
NER	0.5	93.01 ± 5.85	−6.99
	5	92.42 ± 3.44	−7.58
	50	93.25 ± 6.75	−6.75
	150	94.84 ± 0.86	−5.16
PEL	0.5	90.99 ± 8.87	−9.01
	5	89.73 ± 7.01	−10.27
	50	92.89 ± 4.88	−7.11
	150	94.56 ± 9.44	−5.44
Matrix effect			
NER	0.5	94.21 ± 3.63	−5.79
	5	98.81 ± 1.73	−1.19
	50	97.32 ± 1.56	−2.68
	150	100.23 ± 1.39	0.23
PEL	0.5	98.50 ± 0.64	−1.50
	5	98.44 ± 3.21	−1.56
	50	99.69 ± 2.86	−0.31
	150	96.56 ± 1.77	−3.44

[a] Mean recovery (%) ± RSD of six determinations.
[b] Percentage relative error.

Table 4
Evaluation of the intra-day and inter-day accuracy and precision as well as the dilution integrity for the determination of NER and PEL in rat plasma by the proposed UPLC–MS/MS method.

Accuracy and Precision					
		Intra-day (n = 6)		Inter-day (n = 18)	
	Concentration added (ng/mL)	Mean recovery (%) ±RSD [a]	E_r(%)[b]	Mean recovery (%) ±RSD [a]	E_r(%)[b]
NER	0.5	98.07 ± 2.85	−1.93	98.57 ± 4.69	−1.43
	5	98.26 ± 4.99	−1.74	95.47 ± 4.89	−4.53
	50	92.18 ± 6.33	−7.82	97.67 ± 4.87	−2.33
	150	100.72 ± 1.56	0.72	97.98 ± 2.76	−2.02
PEL	0.5	97.44 ± 4.84	−2.56	101.34 ± 2.70	1.34
	5	90.08 ± 2.19	−9.92	99.09 ± 2.70	−0.91
	50	91.08 ± 5.60	−8.92	94.18 ± 6.10	−5.82
	150	99.67 ± 1.52	−0.33	91.38 ± 10.02	−8.62
Dilution integrity (n = 6)					
		1:2		1:5	
	Concentration added (ng/mL)	Mean recovery (%) ±RSD [a]	E_r(%)[b]	Mean recovery (%) ±RSD [a]	E_r(%)[b]
NER	300	98.38 ± 2.67	−1.62	99.61 ± 2.69	−0.39
PEL	300	99.49 ± 1.62	−0.51	97.88 ± 4.28	−2.12

[a] Mean recovery (%) ± RSD of multiple determinations.
[b] Percentage relative error.

Table 5
Evaluation of the stability of NER and PEL in rat plasma.

Stability	Concentration added (ng/mL)	Mean recovery (%) ±RSD[a]	
		NER	PEL
Auto-sampler stability (10 °C, 56 h)	5	98.88 ± 1.00	97.85 ± 2.67
	150	100.43 ± 1.15	98.78 ± 1.58
Short-term stability (25 °C, 6 h)	5	98.83 ± 3.92	99.04 ± 2.24
	150	97.75 ± 1.16	99.47 ± 1.16
Long-term stability (−30 °C, 30 days)	5	95.42 ± 3.13	97.68 ± 7.26
	150	96.30 ± 0.93	97.17 ± 0.80
Freeze-thaw stability(−30 °C, 3 cycles)	5	98.95 ± 0.63	100.06 ± 3.15
	150	97.22 ± 1.75	101.25 ± 2.24
Refrigerator (4 °C, 3 months)	5	95.04 ± 1.81	97.73 ± 2.88
	150	100.83 ± 1.93	98.26 ± 3.35

[a] Mean recovery (%) ± RSD of six determinations.

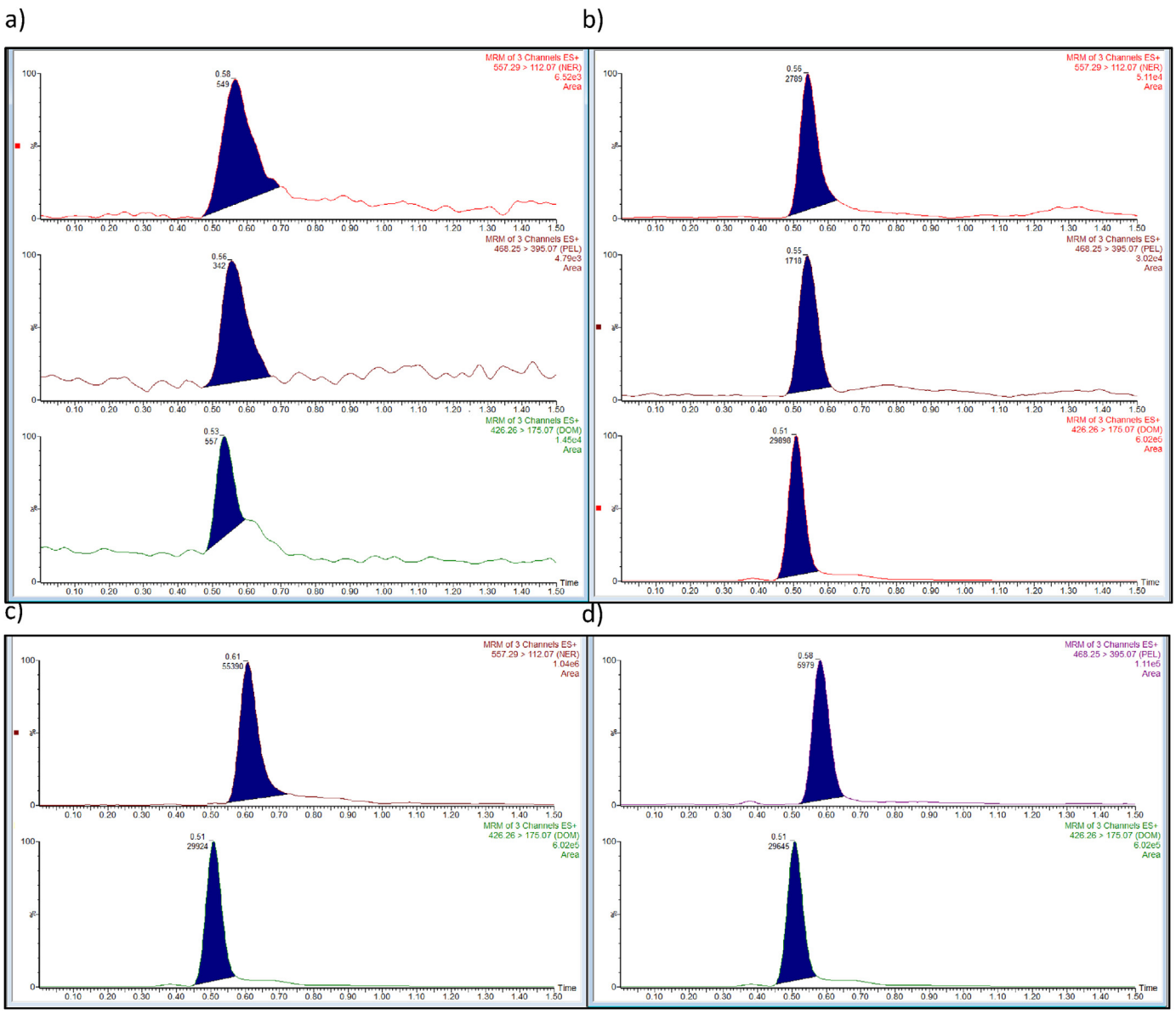

Fig. 3. Multiple reaction monitoring (MRM) of a blank plasma, a), and a plasma sample spiked with a standard mixture of NER and PEL at their LLOQ levels with DOM (IS), b), plasma sample of treated rats collected 3 h after the oral administration of a combination of NER (30 mg/kg) and API (100 mg/kg), c), plasma sample of treated rats collected 4 h after the oral administration of a combination of PEL (10 mg/kg) and API (100 mg/kg), d).

ity of both drugs solutions stored at the refrigerator temperature (4 °C) for 3 months and at room temperature for 6 h.

3.4. Application to pharmacokinetic studies

Food-drug interactions could be widely classified into physicochemical, PK, and pharmacodynamics interactions. Among which, PK interactions is the most common. Despite the widespread use of API, as antioxidant and anticancer flavonoid, among cancerous patients receiving NER/PEL therapy, no studies have been found in the literature studying their PK interactions. Thus, the applicability of the validated UPLC–MS/MS method developed in this work was extended to study the possibility of pharmacokinetic interaction that could be encountered following the concomitant administration of NER/PEL with API. NER is clinically given in the oral dose of 240 mg daily. While for PEL, a daily oral dose of 75 mg is established for cancer treatment. Thus the design of this study involved the treatment of two groups of Wistar rats with NER (30 mg/kg), group I, or PEL (10 mg/kg), group II. In addition, for the purpose of studying the possibility of PK interactions with API, further two groups of Wistar rats were administered combinations of API (100 mg/kg) with either NER (30 mg/kg), group III, or PEL (10 mg/kg), group IV. As per the experimental section, blood samples were withdrawn from each group separately at different time intervals and analyzed for NER and PEL content. Fig. 3 shows representative MRM chromatograms of rat plasma samples withdrawn 4 h after the concomitant administration of NER/PEL with API. Fig. 4 shows the mean drug plasma concentration time profiles reported for each of NER/PEL combination with API, compared with their single administration. Different PK parameters were calculated for both drugs as shown in Table 6. They include the area under the curve (AUC), maximum plasma concentration (C_{max}), time to reach the maximum plasma concentration (t_{max}), and half-life ($t_{1/2}$). Although there was no significant change in the t_{max} of NER, a significant increase in the C_{max} (56%) and AUC (58%) was reported following its co-administration with API suggesting the role of API on NER

Table 6
Main pharmacokinetic parameters after oral administration of NER and PEL to rats ($n = 5$).

Drug	Pharmacokinetic parameter			
	C_{max} (ng/mL) ±SD	t_{max} (h)±SD	$t_{½}$ (h) ±SD	AUC (ng.h/mL) ±SD
Group I NER (30 mg/kg)	483.34 ± 63.41	3.33 ± 0.58	7.00 ± 1.50	4295 ± 99.100
Group II PEL (10 mg/kg)	184.78 ± 27.05	4.00 ± 1.25	5.80 ± 0.22	1290 ± 112.21
Group III NER(30 mg/kg) +API (100 mg/kg)	755.64 ± 65.91	3.67 ± 0.58	5.20 ± 0.66	6787 ± 158.55
Group IV PEL (10 mg/kg) +API (100 mg/kg)	214.94 ± 20.53	5.30 ± 1.15	9.20 ± 2.33	1970 ± 212.02

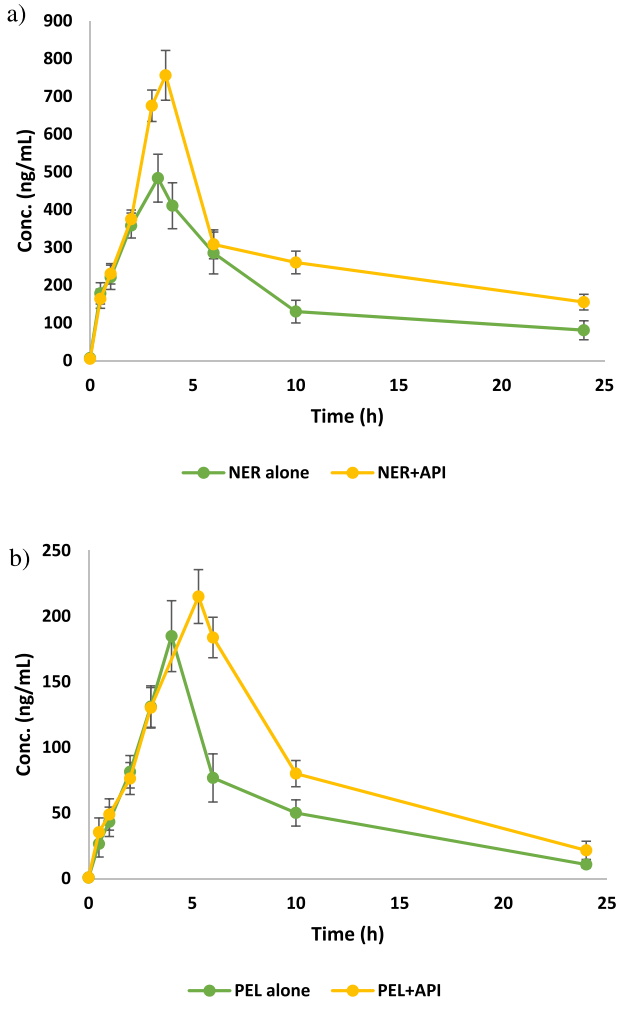

Fig. 4. Plasma concentration– time profile of the studied drugs in rats after an oral administration of a combination of NER (30 mg/kg) and PEL (10 mg/kg) along with API (100 mg/kg), compared with their single oral administration at the same doses, NER, a) and PEL, b). Suitable dilutions of prepared plasma samples were made before analysis.

clearance. Also, an increase in the C_{max} (16%) and AUC (53%), along with an increase in the t_{max} and $t_{1/2}$ was noticed with PEL combination with API, compared with its single administration. From these results, the effect of API on the PK of both NER and PEL was considered significant. It is clearly indicated that API slowed down the metabolism of both drugs. This could be attributed to the fact that both NER and PEL are mainly metabolized by CYP3A4 enzymes and like other TKIs they are substrates of P-gp efflux transporters [30]. Thus API-induced increased bioavailability of NER/PEL could be attributed to the inhibitory effect of API on both CYP3A4 enzymes as well as P-gp efflux proteins. Because this type of PK interactions could lead to increased systematic drug exposure, excessive side effects could be encountered with this combination. This is extremely important since NER is characterized by diarrhea as the dose limiting toxicity. Also PEL suffers from gastrointestinal and cutaneous side effects that could lead to drug withdrawal in many clinical studies [2]. Although this study was conducted in rats and not on humans and that differences in their PK pattern could exist, the possibility of occurrence of PK interactions between NER/PEL and API could exist when shifting to clinical studies. Thus, TDM of both drugs is significantly important in cancerous patients receiving NER/PEL with API.

4. Conclusion

In the present study, a UPLC–MS/MS method has been developed and validated for the quantification of the two irreversible TKIs, NER and PEL, in plasma samples. The proposed method has many advantages over the previously reported methods for the determination of either NER [21–23] or PEL [24] including higher sensitivity, smaller sample volume, and shorter analysis time. This superior sensitivity permits the determination of very low drug concentrations, an important issue in terminal phase elimination during PK studies. Also, smaller sample volume is preferable where large volumes of plasma samples are not available in some clinical cases, e.g. children and elderly people. In addition, short analysis time is necessary for high throughput bioanalysis. Another great advantage of the proposed method over previous literature is the use of SPE for sample clean-up and preparation with its great potential in eliminating interference and producing high degree of purity of prepared samples. The applicability of the developed SPE-UPLC–MS/MS method was extended to studying the possibility of PK interaction between NER/PEL and the widely used flavonoid, API. The results showed that API could affect the CYP-mediated metabolism of NER/PEL with increased drug plasma levels and enhanced drug-induced toxicity. Thus TDM of these drugs, when co-administered with API, is very important for the sake of public health.

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