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UPLC–ESI–MS/MS study of the effect of green tea extract on the oral bioavailability of erlotinib and lapatinib in rats: Potential risk of pharmacokinetic interaction

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ABSTRACT

Green tea (GT) is one of the most consumed beverages worldwide. Tyrosine kinase inhibitors (TKIs) belong to the oral targeted therapy that gained much interest in oncology practice, among which are erlotinib (ERL) and lapatinib (LAP). Since green tea polyphenols (GTP) are known to be inhibitors of receptor tyrosine kinases, GTE could likely potentiate the anticancer effect of TKIs, but with a possibility of pharmacokinetic (PK) interaction with co-administered TKIs. In this study, the effect of GTE on the PK of ERL/LAP in rats was studied. UPLC–ESI–MS/MS method has been developed and validated for the quantification of ERL and LAP in rat plasma, using gefitinib (GEF) as the internal standard. Plasma samples were treated extensively by protein precipitation (PPT) followed by solid phase extraction (SPE) using octadecyl C 18/14% cartridges. Chromatographic analysis was carried out on Acquity UPLC BEH^{TM} C18 column with a mobile phase consisting of water: acetonitrile (20: 80, v/v), each with 0.15% formic acid. Quantification was performed in the positive electrospray ionization (ESI+) mode with multiple reaction monitoring (MRM) of the transitions m/z 394.29 → 278.19 (ERL), m/z 581.07 → 365.13 (LAP), and m/z 447.08 → 128.21 (GEF). The method was fully validated as per the FDA guidelines showing linearity over the range of 0.4–1000 (ERL) and 0.6–1000 (LAP) ng/mL with very low lower limit of quantification (LLOQ) of 0.4 and 0.6 ng/mL for ERL and LAP, respectively. The applicability of the method was extended to perform a comparative study of the PK of ERL/LAP following short-term and long-term administration of GTE, compared with their single oral administration. The results revealed that a significant reduction in the oral bioavailability was recorded with both ERL and LAP following the ingestion of GTE particularly for short-term administration. A reduction in C_{max} (AUC) by 67.60% (69.50%) and 70.20% (73.96%), was recorded with short-term administration of GTE, compared with only 16.03% (21.09%) and 13.53% (22.12%) reduction for ERL and LAP, respectively, with long-term administration. Thus patients taking TKIs should preferably avoid drinking GT or ingesting GTE capsules during the period of treatment with TKIs.

1. Introduction

Food-drug interactions have gained much attention in the recent years. The influence of dietary substances on drug exposure constitutes a major challenge during drug development, particularly for oral drugs. Green tea (GT) is among the most consumed beverages worldwide. It is derived from the non-fermented leaves of the *Camellia sinensis* plant. GT has shown promising health beneficial effects and it has been investigated as one of the most important nutraceuticals used as new treatment approaches for oral cancer [1]. GT polyphenols (GTP), particularly catechin (−)-epigallocatechin-3-gallate (EGCG), which constitutes about 50–80% of the total catechins in GT, are reported to have antioxidant [2], and anti-proliferative effect in different types of human malignancies [1–3]. Several interventional studies have explained the anti-carcinogenic effect of tea catechins, with EGCG being the most active, by several mechanisms. Among which are down regulation of the cell cycle, inhibition of receptor tyrosine kinases [3–5], anti-metastatic effect [6], and modulation of the immune system. Multi-targeted anticancer effect of GTP has shown to exhibit promising results against different types of cancer cells including,

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hepatoma cells [7], human placental choriocarcinoma cells [8], and estrogen-receptor positive breast cancer [9]. In addition, GTP are reported to have potent cardio-protective properties besides their protective effect against drug-induced hepatoma [6,10]. Moreover, many studies have shown the synergistic anticancer effect of GT with other chemo-preventive drugs e.g. tamoxifen and 5-FU [11]. Also, GTP could enhance the anti-metastatic effect of TKIs via synergistic inhibition of the epidermal growth factor receptor (EGFR), e.g. gefitinib [12]. Thus combinations of GTE with TKIs could gain prospective action in clinical trials against the progression of oral cancer [12].

Broad evidence of the beneficial effects of GTP in different health aspects starting from enhancing weight loss to their anticancer effect has led to their increased popularity among different age groups whether as beverages or as food supplements. The exposure to high doses of these bioactive compounds daily could be widely encountered. Therefore, many studies have been concerned with investigating the possible effect of GTP on the therapeutic efficacy and toxicity of other co-administered drugs. This is extremely beneficial particularly that GTP could significantly affect the drug-metabolizing enzymes and/or drug transporters on the intestinal and hepatic levels [13–16]. Moreover, it was strongly proposed that GTP (particularly EGCG) could inhibit the activity of p-gp transporter showing reversal of multidrug resistance [14]. Previous studies have reported the effect of GT on the PK of co-administered drugs. Based on different mechanisms, GT could enhance the bioavailability of some drugs (e.g. tamoxifen [17], simvastatin [18], 5-fluorouracil [19]), while decrease the bioavailability of others (e.g. sunitinib [20], bortezomib [21], quetiapine [22], nadolol [23]). Thus ingestion of high doses GTP could significantly affect the PK of concomitantly administered drugs which is considered a matter of public concern [24].

Tyrosine kinase inhibitors (TKIs) are among the oral targeted therapy that gained much interest in oncology practice, among which are erlotinib (ERL) and lapatinib (LAP), Fig. 1. ERL is an orally active selective inhibitor of the ErbB-1 receptor. ERL has been approved for the treatment of $EGFRm^{+}$ NSCLC [25]. Further clinical trials have demonstrated the antitumor activity of ERL in different cancer cases including, head and neck carcinoma, glioma, squamous cell skin carcinoma, and bladder cancer [25–27]. LAP is an orally active dual −kinase inhibitor with specific activity for EGFR, ErbB-1 and ErbB-2 (HER2) [28,29]. LAP has shown significant anticancer effect either as monotherapy or in combination with other chemotherapeutic agents in different cases of malignancies, e.g. HER2-positive metastatic breast cancer [28,29], pancreatic cancer [30], and ovarian carcinoma [31]. Moreover, ERL and LAP are shown to be potent reversal agents of multidrug resistance (MDR) [32]. This suggests the likeliness of their use in combination with other chemotherapeutic agents to overcome the problem of MDR commonly encountered in oncology practice [32]. However, being oral drugs, the PK characteristics of TKIs show large intra and inter-individual variation [33,34]. Different factors could affect their bioavailability including, genetic heterogeneity of drug targets, patient adherence to treatment, in addition to the patient habits. Moreover, drug–drug interactions (DDI) and food-drug interactions (FDI) are widely encountered with this group of oral anticancer drugs. This could be related to the fact that TKIs are mostly substrates of CYP450 metabolizing enzymes as well as drug transporters [33,34].

The effect of co-administered drug/food on the bioavailability of TKIs has attracted much attention. Since tea catechins are known to be inhibitors of receptor tyrosine kinases [3–5], GTE could likely potentiate the anticancer effect of TKIs, but with a possibility of PK interaction with co-administered TKIs (e.g. ERL, LAP). ERL PK interaction studies with corticosteroids, antiemetics [35], and tamoxifen [36] was conducted with our research group using UPLC–MS/MS. Moreover, other PK interactions were reported for ERL with other drugs (e.g. warfarin [37] and aprepitant [38]). Also, PK interaction studies using LC–MS/MS have been previously performed to investigate the possible interaction of LAP with other co-administered drugs, namely tamoxifen [29], carboplatin [31], sorafenib [39], docetaxel [40], and different food types [41]. Different LC–MS/MS methods have been also reported for the determination of either ERL [35,36,42–44] or LAP [42,44–46] in plasma samples.

In spite of the importance of studying the effect of GTE on the PK of co-administered ERL/LAP, there is no study available in the literature so far dealing with this respect. Therefore the present study aims at studying the effect of GTE on the PK of ERL/LAP in rats using a newly developed UPLC–MS/MS method. The applicability of the method was extended to perform a comparative study of the PK of ERL/LAP following short-term and long-term administration of GTE, compared with their single oral administration.

2. Experimental

2.1. Chemicals and reagents

The reference standards of ERL (purity >99%) and gefitinib (GEF), being used as the internal standard (IS), (purity >99%), were purchased from Pfizer Inc. (NY, USA). LAP reference standard (purity >99%) was supplied by Haoyuan Chemexpress Co., Ltd., Shanghai, P.R. China. HPLC grade solvents namely methanol and acetonitrile (Panreac, E.U.) were involved in the study. Formic acid (Sigma Aldrich, Chemie GmbH, Steinheim, Germany) was also used in the analysis. Capsules of GTE 400 mg, Veg capsules EGCg (NOW FOODS, Bloomingdale, IL, USA) were involved in the study. The standardized extract was labelled to contain a minimum of 80% total catechins and 50% EGCG, 200 mg, in addition to up to 4 mg of naturally occurring caffeine.

Ultrapure water used throughout the study was prepared using a Milli-Q Advantage water purification system (Millipore, Molsheim, France) supplied with 0.22 μm filter.

2.2. Instrumentation and analytical conditions

Analysis was performed on Waters Model Xevo TQ-S UPLC–MS/MS separation system (Singapore) equipped with binary solvent manager (Acquity ™ Ultra-performance LC) and sample manager (Acquity ™ Ultra-performance LC). Mass spectrometric detection was carried out using triple-quadrupole mass spectrometric detector (STEP WAVE ™, Ultra-performance LC) with multiple reaction monitoring (MRM)-mode and supplied with different ionization modes (Zspray ™ ESI-APCI-ESCI, Ultra-performance LC). Data acquisition was performed with Masslynx™ Version 4.1 (Micromass) software.

J.T. Bakers vacuum system was used in the solid-phase extraction (SPE) procedure using octadecyl C 18/14% (200 mg, 3 mL) Spe-ed cartridges (Applied Separations, Allentown, Pennsylvania, USA). Nitrogen evaporator N-EVAP 112 with heating system OA-SYS (Organomation Assocciates, Inc, MA, USA) was used in sample preparation. Sample filtration was performed using 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).

Chromatographic analysis was carried out on Acquity UPLC BEH™ C 18 column (100 × 1.0 mm, i.d., 1.7 μm particle size) (Waters, Ireland). Isocratic elution was carried out using a mobile phase consisting of water: acetonitrile (20: 80, v/v), each with 0.15% formic acid, at a flow rate of 0.2 mL/min. The injection volume was 5 μL with the full loop mode. The auto-sampler and column temperature were maintained at 10° and 45 °C, respectively.

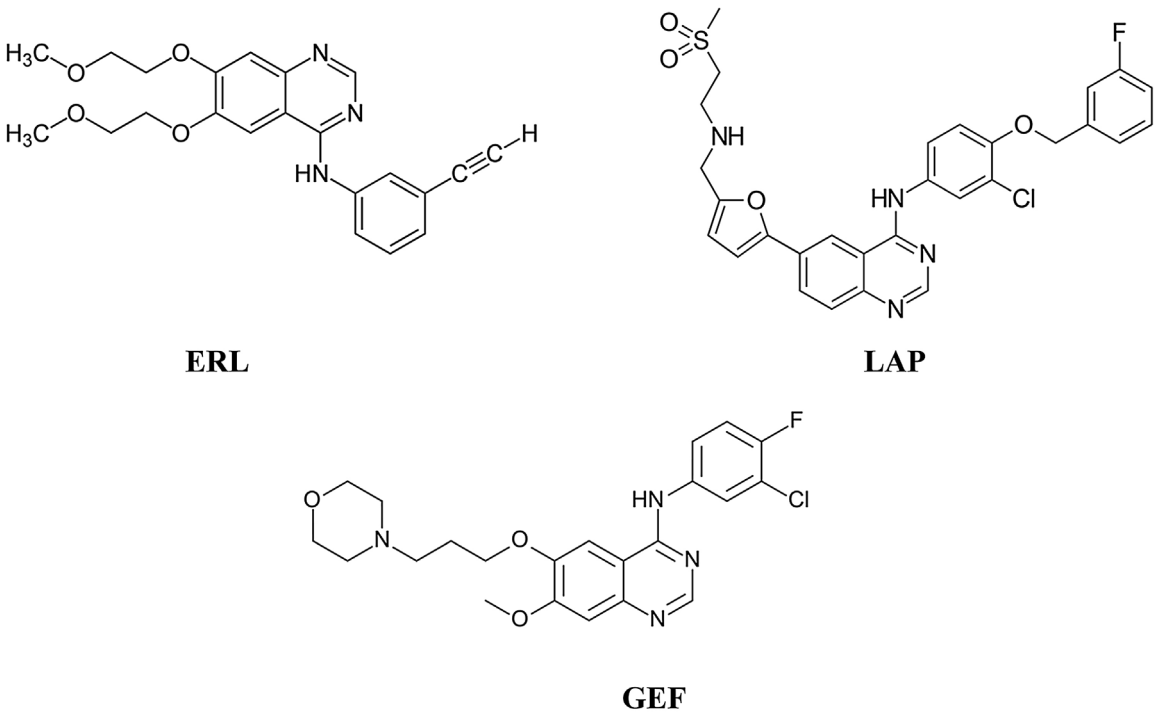

Fig. 1. Chemical structure of the studied compounds; erlotinib (ERL), lapatinib (LAP), and gefitinib (GEF).

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)
ERL	394.29	278.19	25	3.5	30	200
LAP	581.07	365.13	4	3.5	35	500
GEF (IS)	447.08	128.21	3	3.5	24	300

The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode using MRM of the parent $[M+H]^+$ to selected product ions. Quantification was performed using the transitions m/z 394.29 → 278.19 (ERL), m/z 581.07 → 365.13 (LAP), and m/z 447.08 → 128.21 (GEF). Different MS parameters were separately optimized for each compound (Table 1). They include the cone voltage (V), capillary voltage (KV), collision energy (eV), and desolvation temperature (°C). Other MS parameters were as follows, desolvating gas flow 800 L/h, cone gas flow 150 L/h, collision gas flow 0.15 mL/min, source temperature 150°C, and dwell time 0.025 s. Besides, the MS analyzer was set at the resolution of 2.8 (LM) and 14.86 (HM).

2.3. Preparation of stock solutions, calibration standards, and quality control (QC) samples

Stock solutions of 1 mg/mL of ERL, LAP, and GEF (IS) were prepared in methanol. Further dilutions were carried out in methanol to get standard solutions of suitable concentrations. On the other hand, GEF standard solution of concentration 5 ng/mL was prepared. Eight calibration solutions were prepared by spiking separate volumes of 50 μL plasma samples with predetermined volumes of ERL and LAP standard solutions to get final concentrations of 0.4–100 and 0.6–1000 ng/mL plasma for ERL and LAP, respectively, along with 100 μL of 5 ng/mL GEF (IS). All samples were then made up to final volumes of 1 mL with methanol. Blank samples were simply prepared by adding 950 μL methanol to 50 μL volumes of plasma samples. QC samples were separately prepared at four different concentration levels, very low, low, medium, and high concentrations corresponding to LLOQ of 0.4 ng/mL (ERL), 0.6 (LAP), 10 ng/mL, 200 ng/mL, and 800 ng/mL for both drugs.

2.4. Sample preparation

Spiked and blank plasma samples, prepared in the above section, were vortex-mixed at 6000 rpm for 5 min, the supernatant of each sample was separated and the residues were separately washed with volumes of 0.5 mL methanol. For sample clean-up, the combined methanolic solutions (the supernatant and the washing) were passed onto C 18/14% (200 mg, 3 mL) Spe-ed cartridges for SPE. These cartridges were previously preconditioned with 3.0 mL methanol followed by 3.0 mL ultrapure water. Separate volumes of 0.5 mL methanol were used for the elution purpose. The eluate was then evaporated to dryness under nitrogen and the residue was then reconstituted in 0.5 mL acetonitrile. Finally, volumes of 5 μL of reconstituted samples were injected into the UPLC–MS/MS system under the optimized analytical conditions. The peak area ratios of both ERL/LAP to that of GEF (IS) were used to construct the matrix-based calibration graph of each compound and the corresponding regression equations were derived.

2.5. Assay validation

Method validation was performed with reference to the FDA guidelines for bioanalytical methods [47]. Different validation aspects were assessed. The studied parameters included specificity, linearity, lower limit of detection and of quantification, accuracy

and precision, extraction recovery, matrix effects, dilution integrity, and stability studies.

2.5.1. Specificity

The specificity of the method was evaluated by analyzing reconstituted plasma extracts obtained from six different batches. To check for the endogenous interference, the obtained chromatograms were compared with those of plasma samples spiked with both ERL and LAP at their LLOQ levels, along with GEF (IS).

2.5.2. Linearity, lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

Linearity of the method was assessed by analyzing rat plasma samples (50 μL) spiked with eight different concentrations of the studied drugs in the range 0.4–1000 (ERL) and 0.6–1000 (LAP) ng/mL plasma, each with GEF (IS). Using the method of least squares, the obtained peak area ratios of each drug to the IS was related to the spiked concentration to derive the matrix-based calibration graph and the regression equation for each of ERL and LAP.

LLOD and LLOQ of both ERL and LAP were selected based on the concentrations that produced analytical responses of at least three or five times that of the blank signals, for LLOD and LLOQ, respectively. Moreover, the concentrations selected as LLOQ produced identifiable signals with acceptable accuracy and precision (at least ±20%).

2.5.3. Accuracy and precision

Intra-day and inter-day accuracy and precision were evaluated by carrying out replicate analysis (n = 6) of QC samples previously prepared at the four QC concentration levels, very low, low, medium, and high concentrations corresponding to LLOQ of 0.4 ng/mL (ERL), 0.6 (LAP), 10 ng/mL, 200 ng/mL, and 800 ng/mL for both drugs. Analysis was performed either on the same day or on three consecutive days for the intra-day and inter-day levels, respectively. The actual drug concentration found in each sample was calculated using a calibration curve obtained on the day of analysis, and then compared with the nominal spiked concentrations. Accuracy was assessed in terms of percentage relative error (E_r%) while precision was assessed in terms of percentage relative standard deviation (%RSD).

2.5.4. Extraction recovery and matrix effect

The extraction recovery of ERL and LAP from plasma samples was assessed at three QC levels; low (10 ng/mL), medium (200 ng/mL), and high (800 ng/mL). The peak area obtained from plasma samples spiked pre-extraction were compared with those obtained from plasma samples spiked post extraction with the same nominal concentrations (n = 6). The recovery of the IS at the same concentration level used in actual analysis was also evaluated.

The matrix effect of the plasma on the response of the analytes was determined by calculating the ratio of the mean peak area of each of ERL and LAP spiked post-extraction to those of standard solutions prepared directly in acetonitrile at the three QC concentration levels previously used in the evaluation of the extraction recovery. Moreover, matrix effect of GEF (IS) at the specified concentration used in actual analysis was calculated.

2.5.5. Dilution integrity

Dilution of highly concentrated plasma samples was evaluated for its effect on the recoveries of both drugs. Plasma samples spiked with high concentrations of both ERL and LAP (1600 ng/mL) were used to assess the effect of dilution of plasma samples originally containing high concentrations, beyond the linearity range of the proposed method, on the obtained recovery values. These concentrated samples were initially diluted with blank rat plasma (1:2 and 1:5 fold dilution) then analyzed exactly as described under "sample preparation". For each drug, the recovery% (±RSD) was finally calculated.

2.5.6. Stability studies

The stability of ERL and LAP in rat plasma was evaluated by analyzing QC samples prepared at two concentration levels, low (10 ng/mL) and high (800 ng/mL), (n = 6). Plasma samples were exposed to different conditions: at room temperature (25 °C) for 6 h (short-term, bench-top stability), at −30 °C for 30 days (long-term stability), and after three freeze-thaw cycles by freezing the samples at around −30 °C then thawing at room temperature (freeze-thaw stability). Moreover, post-preparation stability was evaluated by leaving the processed samples in the auto-sampler at 10 °C for 56 h prior to the injection (auto-sampler stability). Finally, the stability of stock solutions of both drugs when kept in the refrigerator at 4 °C for 3 months was also evaluated. In each case, recoveries were calculated by relating the found concentration of both drugs to that of the nominal concentrations.

2.6. Application to pharmacokinetic studies

Wistar healthy male rats (250 ± 30 g) were supplied by the animal house, Women Student-Medical studies & Sciences Sections, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. All the animals were kept in cages under standard laboratory conditions; a well-ventilated place, a regular 12 h day–night cycle, a controlled room temperature (25 ± 2 °C), and a relative humidity of 50 ± 10%. The rats had free access to tap water while diet was prohibited for 12 h before the drug administration. They were acclimatized for 7 days to laboratory conditions before starting the experiments. All experiments were carried out according to the WHO regulations in Saudi Arabia with reference to ethical guidelines for experimental studies with animals. Six groups, each of five rats, were used in the study. Initially, an appropriate weight of each of ERL and LAP was triturated with aqueous methyl cellulose (0.5%, w/v) to prepare suspensions of 20 mg/mL (ERL) or 25 mg/mL (LAP). However, for GTE (200 mg/mL), water was used as a solvent. The treated animals received an oral dose of 0.25 mL volumes of the drugs suspensions using a gavage needle as follows; ERL, 20 mg/kg (Group I), LAP and 25 mg/kg (Group II). Rats of groups III and IV were first separately given an oral dose of GTE (200 mg/kg). Then immediately, the rats were administered an oral dose of ERL, 20 mg/kg (Group III), or LAP, 25 mg/kg (Group IV). On the other hand, rats of groups V and VI were given a daily oral dose of GTE (200 mg/kg) for one week. Following this week, diet was prohibited for 12 h, and then the rats were subjected to an oral dose of ERL, 20 mg/kg (Group V), or LAP, 25 mg/kg (Group VI). For the six groups and following drug administration, blood samples (0.3 mL) were collected from the retro-orbital sinus of each rat into heparinized tubes at different time intervals; 0 (prior to dosing), 0.5, 1, 2, 3, 5, 24, and 48 h (post dosing). All samples were immediately centrifuged for 30 min using 4500 rpm at 4 °C and plasma samples were kept frozen at −20 °C until analysis. For each plasma sample, 50 μL volumes were separately spiked with a 100 μL volume of GEF, IS (5 ng/mL), then completed with methanol to a final volume of 1 mL. Spiked samples were processed exactly as mentioned under sample preparation. The peak area ratio of each drug to that of the IS was then used to calculate the found drug concentration in each analyzed sample.

3. Results and discussion

3.1. Optimization of UPLC–MS/MS conditions

Optimization of both mass spectrometric and chromatographic conditions was carried out. For selecting the most optimum MS/MS

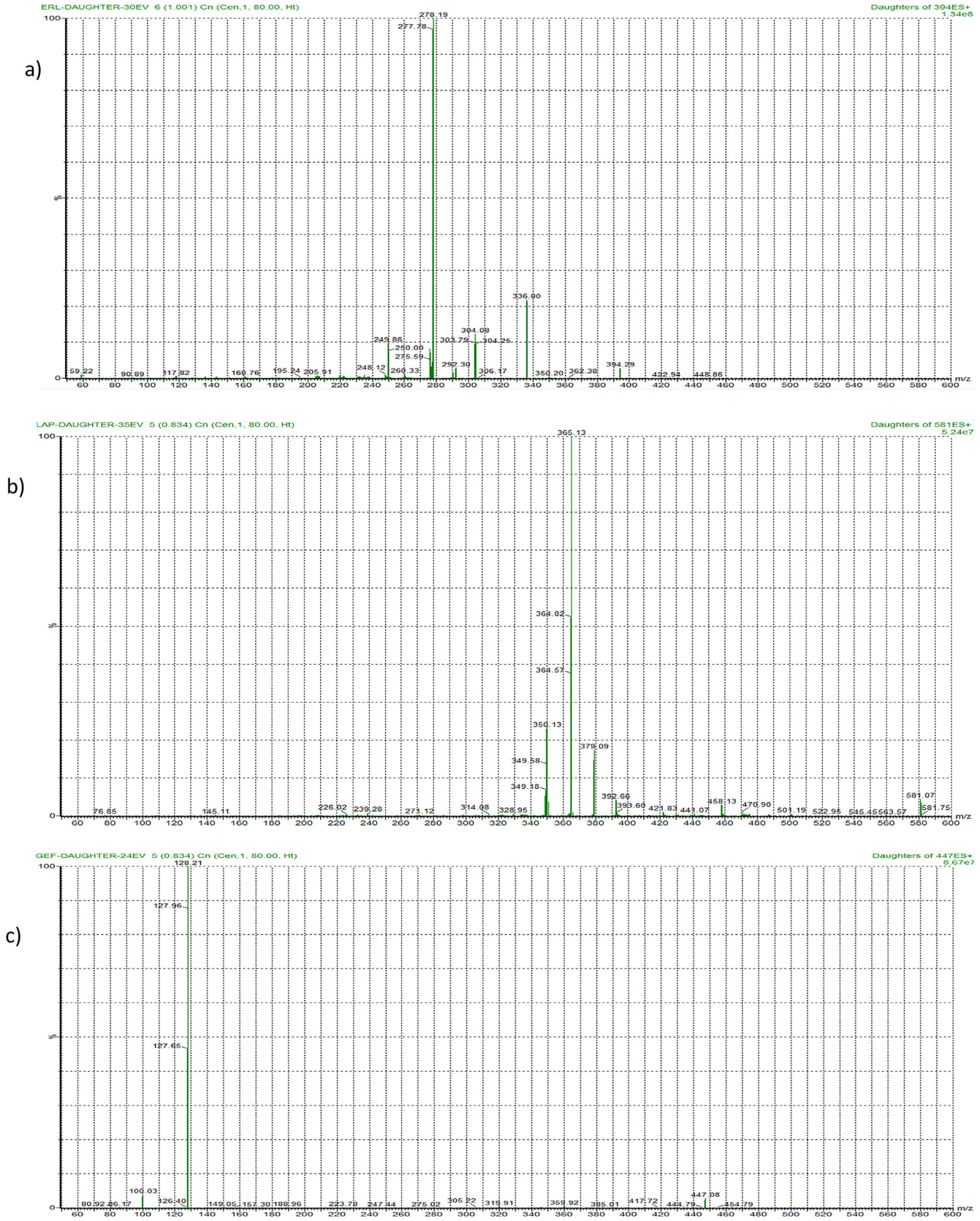

Fig. 2. Product ion spectra of ERL, a), LAP, b), and GEF, c).

conditions, standard solutions of ERL, LAP, and GEF (IS) (1 ng/mL) were individually introduced via syringe infusion into the mass spectrometer. Positive ESI mode was employed since it produced more efficient ionization of the three compounds relative to the negative ionization mode. Because of the high sensitivity and selectivity provided by MRM, it was applied to monitor both the

precursor and product ions. Full scan product ion spectra of protonated precursor ions $[M+H]^+$ for ERL, LAP, and GEF were shown in Fig. 2. Precursor ions were selected at *m/z* 394.29 (ERL), *m/z* 581.07 (LAP), and at *m/z* 447.08 (GEF) while the product ions were at *m/z* 278.19 (ERL), *m/z* 365.13 (LAP), and at *m/z* 128.21 (GEF). To obtain the highest intensity of the protonated precursor ions, different MS/MS parameters were optimized. The highest response of the protonated precursor ions was obtained using a desolvation gas flow rate of 80 L/h and a source temperature of 150°C. In addition, cone voltage and capillary voltage were tested for their effect on the intensity of the precursor ions where 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 followed by a significant decrease. On the other hand, the intensity of the product ions was controlled by the magnitude of the collision energy since an increase in the collision energy till optimum values resulted in increasing the intensity of a particular fragment ion. However, further increase in the collision energy above optimum resulted in a consequent decrease in the intensity of the selected product ions. The optimized MS/MS conditions of the studied drugs were mentioned in Table 1.

The second phase of the study involved optimization of chromatographic conditions. To achieve the optimum peak shape with highest possible response, and within reasonable runtime, mobile phases of different composition were tested. In this respect, mobile phases of mixtures of different ratios of acetonitrile (30–90%), water, and formic acid (0.05–0.2%) were investigated for their effect on the chromatographic behavior of standard mixtures of ERL, LAP, and GEF (IS). Mobile phases of different ratios of acetonitrile (30–90%) and water, each with 0.1% formic acid, were initially tried. It was observed that distortion of ERL, LAP, and GEF peaks was recorded with acetonitrile percentage of 60% or less and that the most symmetric and sharpest peaks of the studied drugs were obtained with acetonitrile percentage of 80%. Moreover, a significant increase in the retention time of the three drugs was associated with an increase in the acetonitrile percentage in the mobile phase (above 80%). Also, the response of LAP was noticed to increase dramatically with the increase in acetonitrile percentage (60–80%). As a result, mobile phases with 80% acetonitrile content were selected for further optimization since it provided the best results regarding the peak shape, response, and runtime for the three compounds, ERL, LAP, and GEF. As the next step of mobile phase optimization, formic acid content (0.05–0.2%) in the mobile phase was studied. Practical experimentation revealed that formic acid was essential to get sharp peaks of both ERL and LAP. In addition, formic acid content in the mobile phase had a significant effect on the retention of both ERL and LAP where a decrease in their retention time was associated with an increase in the formic acid percentage in the mobile phase till 0.15%, with no further increase above this percentage. Thus, 0.15% formic acid content in the mobile phase was selected as optimum. In conclusion, a mobile phase consisting of acetonitrile: water, (80: 20, v/v), each with 0.15% formic acid, was selected for final analysis. In this study, GEF was selected as an IS since it provided a suitable chromatographic and retention behavior, compared with ERL/LAP. Using the optimized UPLC–MS/MS conditions, the whole runtime was 2 min where all compounds were eluted with sharp and symmetric peaks within reasonable retention time (ERL eluted at 0.56 ± 0.01 min, LAP at 0.82 ± 0.03 min, and GEF (IS) at 0.86 ± 0.01 min).

3.2. Sample preparation

Selectivity and sensitivity of bioanalytical techniques depend mainly on the efficiency of sample preparation. Prior to actual analysis, the endogenous interfering components in plasma samples should be removed to the largest possible extent that could be achieved practically. Protein precipitation (PPT) followed by solid phase extraction (SPE) has been used by our research group as a combined sample clean-up procedure and it was found extremely beneficial in this respect [38,39]. In this work, protein precipitation was carried out using methanol then the clear supernatants were passed on the SPE cartridges for further purification. Two types of SPE cartridges namely, octadecyl C 18/14% (200 mg, 3 mL) and octyl C 8 (200 mg, 3 mL) were tested using plasma samples spiked with both ERL and LAP at the concentration of 200 ng/mL and the extraction efficiency in each case was evaluated. As found in our previous research with ERL [35], better peak shape was obtained using C 18 cartridges for both ERL and LAP peaks.

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

Parameter	ERL	LAP
Linearity range (ng/mL)	0.4–1000	0.6–1000
LLOQ[a] (ng/mL)	0.4	0.6
LLOD[b] (ng/mL)	0.2	0.4
Intercept (a)	0.0749	0.1090
Slope (b)	1.1580	0.3595
Correlation Coefficient (r)	0.9994	0.9991
S_a[c]	0.0403	0.0159
S_b[d]	0.0158	0.0062
$S_{y/x}$[e]	0.0630	0.0249
F[f]	5380.57	3337.09
Significance F	5.5839×10^{-6}	1.1428×10^{-5}

[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. Method validation

3.3.1. Specificity

The typical chromatograms of blank plasma samples and blank plasma samples spiked with both ERL and LAP at their LLOQ level are shown in Fig. 3. The peak response for both ERL and LAP at their LLOQ provided at least five times compared with the blank signal. However, the IS concentration was selected so that it yielded a signal with at least twenty times that of the blank. The absence of any interference at the retention times of both analytes and IS ensures high degree of method specificity.

3.3.2. Linearity, lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

Using the method of least squares, the peak area ratios calculated for each of ERL and LAP to that of GEF (IS) were linearly related to the spiked concentrations over the concentration range of 0.4–1000 and 0.6–1000 ng/mL plasma for ERL and LAP, respectively. The average linear regression equation for both ERL and LAP was derived, intercept (a), slope (b), and the correlation coefficients (r) was separately calculated. The high values of the correlation coefficients obtained from the regression of both drugs ($r \geq 0.9991$) along with the small intercepts indicated high degree of method linearity. Moreover, other statistical parameters including, standard deviations of residuals ($S_{y/x}$), of the intercept (S_a), of the slope (S_b), and the variance ratio (F values) were also assessed as mentioned in Table 2. The obtained low values of $S_{y/x}$ as well as the high F values indicated the low degree of scatter of the experimental data points around the regression line [48].

Using the proposed method, LLOD and LLOQ were set at 0.2 and 0.4 ng/mL (ERL), 0.4 and 0.6 ng/mL (LAP) (Table 2). The LLOQ were low enough to ensure the applicability of the method for

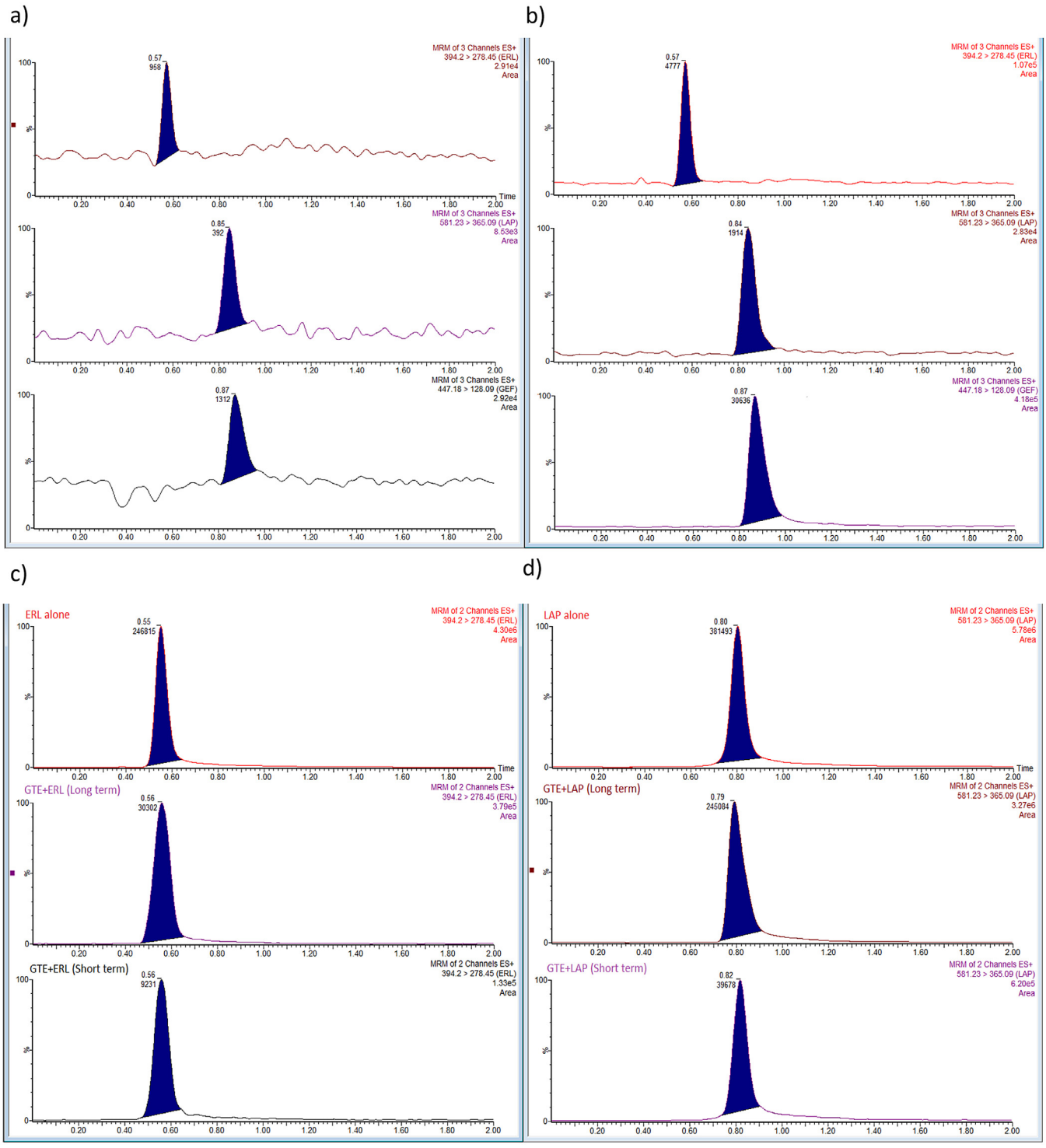

Fig. 3. Multiple reaction monitoring (MRM) chromatograms of a blank plasma, a), and a plasma sample spiked with a standard mixture of ERL and LAP at their LLOQ levels with GEF (IS), b), plasma sample of treated rats collected 1 h after the oral administration of ERL (20 mg/kg) or combined with GTE (200 mg/mL), c) and plasma sample of treated rats collected 1 h after the oral administration of LAP (25 mg/kg) or combined with GTE (200 mg/mL), d).

the quantitative detection of the two drugs in pharmacokinetic studies. Fig. 3 shows the MRM chromatograms of plasma samples spiked with ERL and LAP at their LLOQ, compared with drug-free plasma.

3.3.3. *Accuracy and precision*

As shown in Table 3, the intra-day and inter-day accuracy, calculated in terms of percentage relative errors were found to be in the range (−8 to (−1) %) and (−9 to (−0.5) %), respectively. However, intra-day and inter-day precision, calculated in terms of %RSD were in the range (1–8%) and (1–9%), respectively. The method provided high degree of accuracy and precision since all obtained relative errors and deviation values were within the acceptance criteria of ≤15.0% (for concentrations other than LLOQ) or ≤20.0% (for LLOQ).

Table 3
Evaluation of the intra-day and inter-day accuracy and precision for the determination of ERL and LAP in rat plasma by the proposed UPLC–MS/MS method.

		Intra-day (n = 6)		Inter-day (n = 18)	
	Concentration added (ng/mL)	Mean recovery (%) ±RSD	E_r(%)[a]	Mean recovery (%) ±RSD	E_r(%)[a]
ERL	0.4	96 ± 7	−4	91 ± 6	−9
	10	92 ± 7	−8	95 ± 1	−5
	200	95 ± 1	−5	91 ± 9	−9
	800	99 ± 3	−1	100 ± 5	−0.5
LAP	0.6	95 ± 8	−5	98 ± 6	−2
	10	97 ± 5	−3	93 ± 6	−7
	200	98 ± 1	−2	97 ± 3	−3
	800	98 ± 3	−2	99 ± 2	−1

[a] Percentage relative error.

Table 4
Extraction efficiency of C-18 cartridges in the UPLC–MS/MS analysis of standard mixtures of ERL and LAP, with GEF (IS).

	Concentration added (ng/mL)	Mean recovery (%) ±RSD[a]	E_r(%)[b]
ERL	0.4	87 ± 9	−13
	10	98 ± 4	−2
	200	91 ± 7	−9
	800	94 ± 7	−6
LAP	0.6	87 ± 7	−13
	10	90 ± 6	−10
	200	98 ± 2	−2
	800	95 ± 2	−5

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

Table 5
Evaluation of the matrix effect for the determination of ERL and LAP in rat plasma by the proposed UPLC–MS/MS method.

	Concentration added (ng/mL)	Mean recovery (%) ±RSD[a]	E_r(%)[b]
ERL	0.4	97 ± 6	−3
	10	102 ± 2	2
	200	100 ± 1	0
	800	98 ± 2	−2
LAP	0.6	99 ± 4	−1
	10	99 ± 5	−1
	200	99 ± 3	−1
	800	98 ± 1	−2

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

3.3.4. Recovery and matrix effect

Extraction recovery for both ERL and LAP was evaluated using plasma samples spiked at four concentration levels (QC samples), (0.4, 10, 200, 800 ng/mL) for ERL, (0.6, 10, 200, 800 ng/mL) for LAP, as mentioned in Table 4. Recoveries of not less than 87% for both ERL and LAP were obtained. Also, the mean recovery of 89% was recorded for GEF (IS) at the concentration level used in actual analysis indicating a satisfactory extraction procedure of all studied compounds from plasma samples.

The matrix effect was evaluated using samples of the same four QC levels as those used for assessing the extraction recovery. Matrix effects of not more than 3% and 2% were obtained for both ERL and LAP, respectively (Table 5). For GEF (IS), matrix effect of 2% was obtained indicating negligible matrix effect using the applied method. This permitted trace analysis of both ERL and LAP in plasma samples.

3.3.5. Dilution integrity

Dilution integrity was evaluated to investigate the effect of dilution of plasma samples containing high concentrations of ERL and LAP beyond the linearity range of the proposed method on the recovery of the studied drugs following dilution of the concentrated samples. For both drugs, the recovery% (±RSD) following the dilution procedure was calculated and presented in Table 6. The integrity of both ERL and LAP up to five times dilution of concentrated plasma samples was revealed by the accepted values of the obtained results, error values ≤15%.

Table 6
Evaluation of the dilution integrity of ERL and LAP in rat plasma.

Concentration spiked (ng/mL)	Dilution fold	Mean recovery (%) ±RSD[a]	E_r(%)[b]
ERL	1:2	98 ± 4	−2
	1:5	98 ± 3	−2
LAP	1:2	99 ± 4	−1
	1:5	99 ± 2	−1

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

3.3.6. Stability studies

Room temperature (short-term) stability, long-term stability, freeze-thaw stability, auto-sampler stability, and stock solution stability were assessed by calculating the recovery% (±RSD) using two concentration levels, low (10 ng/mL) and high (800 ng/mL), (n = 6). The obtained results were all within the acceptance criteria for both errors and RSD (≤15%) indicating the stability of both drugs under the studied conditions (Table 7). Also, solutions of both ERL and LAP were found stable when kept at room temperature for 6 h or refrigerated (4 °C) for 3 months. Results of stability studies matched those found in previous literature for the determination of either ERL [35,36,42,44] or LAP [42,44,46].

3.4. Application to pharmacokinetic studies

The effect of GT on the bioavailability of co-administered drugs is diverse showing large variation, depending on the amount ingested, the duration of GT ingestion, and the most important is the nature of the co-administered drug itself. Since TKIs are oral drugs with many PK interaction problems and since that GTP have recently proved to have TK inhibition, GT has been postulated to have synergistic action when combined with TKIs [12]. However, the possibility of their PK interaction may constitute a major problem and studies have started to suggest that GT could unexpectedly antagonize the in-vivo anticancer effect of some drugs [20,21]. The proposed UPLC–MS/MS method developed in this work was applied to investigate the possibility of PK interaction between GTE and the selected TKIs (ERL, LAP). For this purpose, the study was designed to include six animal groups. The first two groups were administered only the TKI; ERL (20 mg/kg), group I, or LAP (25 mg/kg), group II. Since the duration of ingestion of GTE could affect the extent of PK interaction, the study comprised both short term and long-term designs. In this respect, further four groups of Wistar rats were administered combinations of GTE (200 mg/kg) with either ERL (20 mg/kg) or LAP (25 mg/kg) either immediately,

Table 7
Evaluation of the stability of ERL and LAP in rat plasma.

Stability	Concentration added (ng/mL)	Mean recovery (%) ±RSD[a]	
		ERL	LAP
Auto-sampler stability (10 °C, 56 h)	10	100 ± 2	97 ± 2
	800	98 ± 4	100 ± 2
Short-term stability (25 °C, 6 h)	10	100 ± 5	98 ± 5
	800	98 ± 2	98 ± 5
Long-term stability (−30 °C, 30 days)	10	100 ± 2	96 ± 4
	800	97 ± 3	97 ± 4
Freeze-thaw stability (−30 °C, 3 cycles)	10	101 ± 3	97 ± 7
	800	101 ± 4	99 ± 1
Refrigerator (4 °C, 3 months)	10	99 ± 5	97 ± 4
	800	98 ± 6	99 ± 1

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

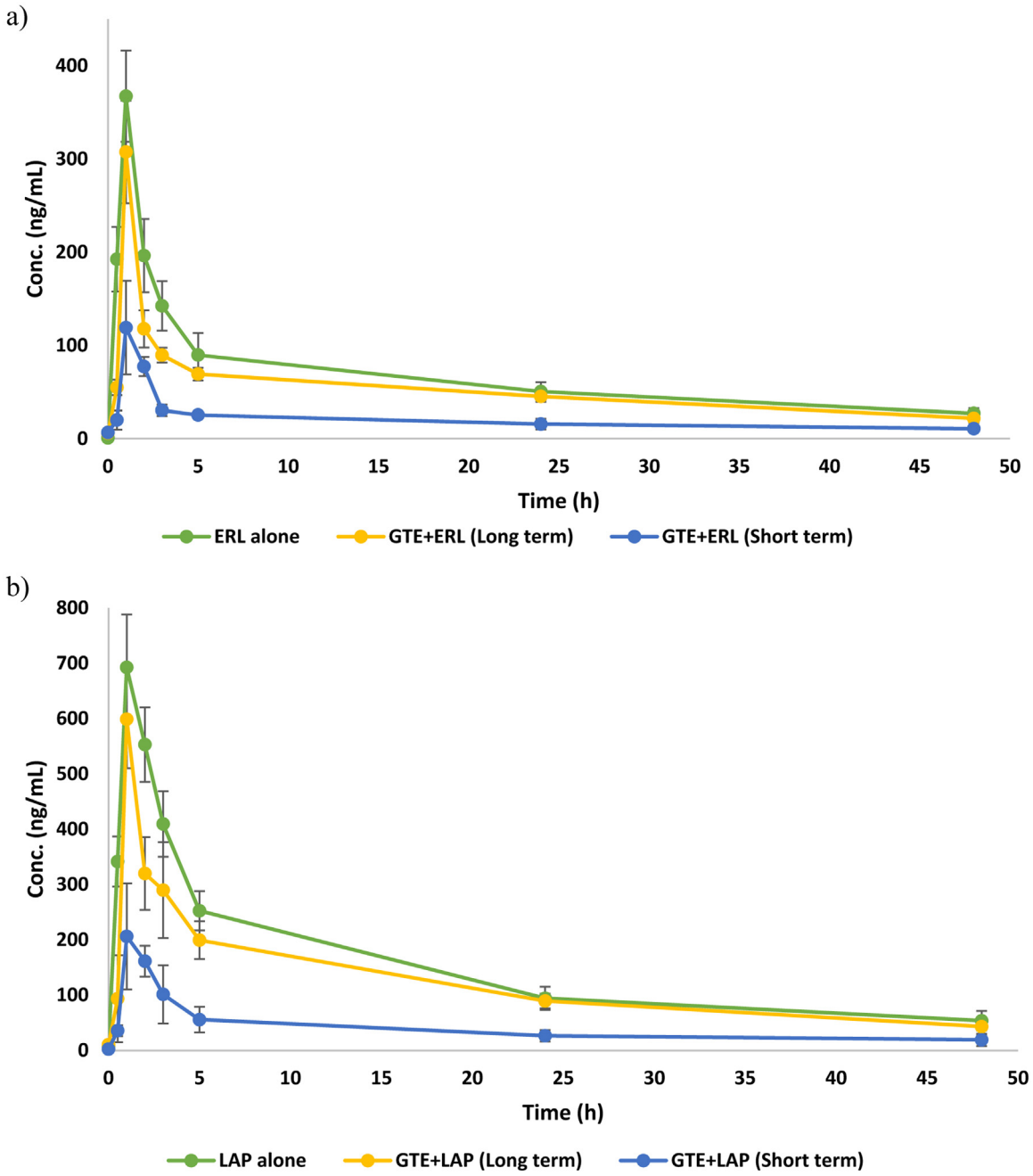

Fig. 4. Plasma concentration versus time (h) of the studied drugs in rats after an oral administration of a combination of ERL (20 mg/kg) and LAP (25 mg/kg) along with GTE (200 mg/kg), compared with their single oral administration at the same doses, ERL, a) and LAP, b). (1: 2) fold dilutions of prepared plasma samples were made before analysis.

Table 8
Main pharmacokinetic parameters after oral administration of ERL and LAP 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 ERL (20 mg/kg)	367 ± 49	1.0 ± 0.6	2.1 ± 0.4	3129 ± 569
Group II LAP (25 mg/kg)	692 ± 96	1.0 ± 0.2	3.3 ± 1	7182 ± 1458
Group III GTE + ERL (20 mg/kg) Short term	119 ± 50	1.0 ± 0.7	2.3 ± 0.5	953 ± 152
Group IV GTE + LAP (25 mg/kg) Short term	206 ± 96	1.0 ± 0.7	3.1 ± 1	1870 ± 201
Group V GTE + ERL (20 mg/kg) Long term	307 ± 55	1.0 ± 0.3	2.0 ± 0.2	2469 ± 195
Group VI GTE + LAP (25 mg/kg) Long term	599 ± 89	1.0 ± 0.5	3.0 ± 0.6	5593 ± 789

groups III, IV or after one week administration of GTE (200 mg/kg), groups V, VI, for short-term and long-term administrations, respectively. The selected doses of the administered ERL/LAP used in this study were in accordance with those used in previous mice studies [35,36,46]. Also, the dose of GTE was calculated in terms of its EGCG content (100 mg/mL) in accordance with previous studies [20] which corresponds to drinking approximately six cups of GT per day by an adult male. In each study group, blood samples were withdrawn from each rat at the specified time interval and analyzed for its content of ERL or LAP. Representative MRM chromatograms of rat plasma samples withdrawn 1 h following the concomitant administration of ERL/LAP with GTE are shown in Fig. 3. Fig. 4 shows the mean plasma concentration versus time profiles following the administration of ERL/LAP with GTE after short term and long term administration, compared with their single oral administration at the same doses. The associated PK parameters including, maximum plasma concentration (C_{max}), the area under the curve (AUC), time to reach the maximum plasma concentration (t_{max}), and half-life ($t_{1/2}$), were presented in Table 8. The results indicated that the administration of GTE resulted in a significant decrease in the bioavailability of both ERL and LAP. A reduction in C_{max} (AUC) by 67.60% (69.50%) and 70.20% (73.96%), for ERL and LAP, respectively, was recorded with short-term administration of GTE. However, for long term administration, the effect was much less compared with short-term administration with only 16.03% (21.09%) and 13.53% (22.12%) reduction in the corresponding C_{max} (AUC) of ERL and LAP, respectively. On the contrary, no significant change in the t_{max} and $t_{1/2}$ was recorded for either drug suggesting that GTE had no effect on the elimination of the studied drugs and that its effect is attributed mainly to a process occurring in the gut rather than a systematic effect on the systematic clearance [22]. Interestingly, analogous PK interaction studies reported the antagonistic action of GTP on the anti-proliferative effect of bortezomib on myeloma cells [21]. This was explained by a direct chemical reaction between EGCG and bortezomib. Also, another study reported the antagonistic effect of GT on sunitinib, a TKI, through reduction of its absorption [20]. In an attempt to explain the nature of interaction between GTE and the selected TKIs, in-vitro interaction was tested by mixing aqueous solutions of ERL/LAP (5 mg/mL) with GTE (200 mg/mL), in a ratio of 1: 1, v/v. No physical change was observed, even after acidification of the mixture to pH 1.0 with hydrochloric acid to mimic the pH of the stomach. In contrast to bortezomib, there has not been any reported incidence of chemical reaction between GT polyphenols and ERL/LAP or even the previously studied sunitinib [20]. This suggests that, as per sunitinib, the possibility of physiochemical interaction between GTP and ERL/LAP is less likely to occur and that GT-induced reduction of ERL/LAP absorption could be an explanation of the reduced bioavailability of the studied TKIs. In this context, further studies should be conducted to emphasize the nature of this PK interaction. Again, matching with what was previously found with sunitinib [20], compared with long-term administration, short-term administration of GT resulted in much decrease in the bioavailability of co-administered TKIs. Based on these results, we suggest that patients taking TKIs should preferably avoid drinking GT or ingesting GTE capsules during the period of treatment with TKIs. Since actually some variation could exist in the PK parameters of many drugs studied in animal models compared with humans, further experimentation should be conducted with human volunteers. Thus, TDM is a matter of concern in cancerous patients receiving ERL/LAP while drinking GT or ingesting capsules of GTE during the period of treatment.

4. Conclusion

To our knowledge, this work was the first to investigate the effect of GTE on the PK behavior of the selected TKIs, ERL/LAP. This study showed that GTE, particularly short-term administration, resulted in a significant decrease in the bioavailability of both drugs. This finding has significant clinical implications for tea drinking habits during the period of treatment with TKIs, ERL/LAP. The developed UPLC–MS/MS method has been validated for the quantification of the ERL and LAP in plasma samples. Compared with previous literature, the proposed method has many advantages for the determination of either ERL [42,43,46] or LAP [42,44–46] regarding higher detectability, shorter analysis time, in addition to improved sample preparation (SPE compared with PPT or LLE). In addition, the use of SPE for sample preparation contributed to the enhanced sensitivity and selectivity of the proposed method.

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