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Simultaneous determination of erlotinib and tamoxifen in rat plasma using UPLC–MS/MS: Application to pharmacokinetic interaction studies

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ABSTRACT

Tamoxifen (TAM) is a non-steroidal estrogen receptor antagonist that enhances erlotinib (ERL)-induced cytotoxicity in the treatment of NSCLC. ERL and TAM are metabolized by CYP3A4 enzymes. In addition, both drugs have the potential of altering the enzymatic activity through either inhibition (ERL) or induction (TAM). Thus it was expected that pharmacokinetics (PK) drug–drug interactions (DDIs) could be encountered following their co-administration. In this respect, a bioanalytical UPLC–MS/MS method has been developed and validated for the simultaneous determination of ERL and TAM in rat plasma samples, using ondansetron (OND) as an internal standard (IS). Plasma samples were prepared using mixed mode cationic solid phase extraction (SPE) STRATA^{TM} –X-C 33 μm cartridges with good extraction recovery of both drugs from rat plasma (E_r% from −13.92 to −3.32). The drugs were separated on a Waters BEH^{TM} C18 column with an isocratic elution using a mobile phase composed of a mixture of acetonitrile and water, each with 0.15% formic acid, in the ratio of 80: 20, v/v. Quantitation was carried out using the positive ionization mode with multiple reaction monitoring (MRM) at *m/z* 394.20 > 278.04 (ERL), *m/z* 372.25 > 72.01 (TAM), and *m/z* 294.18 > 170.16 (OND). The method was fully validated as per the FDA guidelines over the concentration range of 0.2–50 ng/mL with very low lower limit of quantification (LLOQ) of 0.2 ng/mL for both ERL and TAM. The intra- and inter-day assay precision (in terms of relative standard deviation, RSD) and accuracy (in terms of percentage relative error, % E_r) were evaluated for both drugs and the calculated values evaluated at four different concentration levels were within the acceptable limits (<15%) for concentrations other than LLOQ and 20% for LLOQ. The method was successfully applied to the study of possible PK-DDI following the oral administration of ERL and TAM in a combination, compared to their single administration.

1. Introduction

Lung cancer is currently the leading cause of cancer death in men and women in most parts of the world. Almost all stages of lung cancer have not more than 15% survival rate [1]. Non-small cell lung cancer (NSCLC) is known to be the most frequent type of lung cancer, accounting for nearly 85% of all cases [2]. Generally, dysregulation of protein kinases is marked in different types of cancer. The most famous of which is the human epidermal growth factor receptor (EGFR) which is overexpressed in NSCLC with enhanced cell proliferation, antiapoptosis, and metastasis [2,3]. This usually results in poor disease prognosis. Erlotinib (ERL), Fig. 1, is an orally active tyrosine kinase inhibitor (TKI), used mainly in the treatment of EGFR active lung cancer. ERL is a reversible TKI which forms a complex with the ATP-binding site of the EGFR with a subsequent targeted inhibition. ERL is approved by the FDA for the treatment of locally advanced or metastatic NSCLC [4].

Tamoxifen (TAM), Fig. 1, is a non-steroidal estrogen receptor (ER) antagonist that has been widely used in the treatment of breast cancer [2,5]. Moreover, it has been recently suggested that TAM could be effective in the management of NSCLC. A series of prospective studies have shown the relation between NSCLC and estrogen. Estrogen could significantly result in triggering cell proliferation or inhibiting apoptosis, contributing to the development and genesis of NSCLC in the same way as its contribution to breast cancer [1,5].

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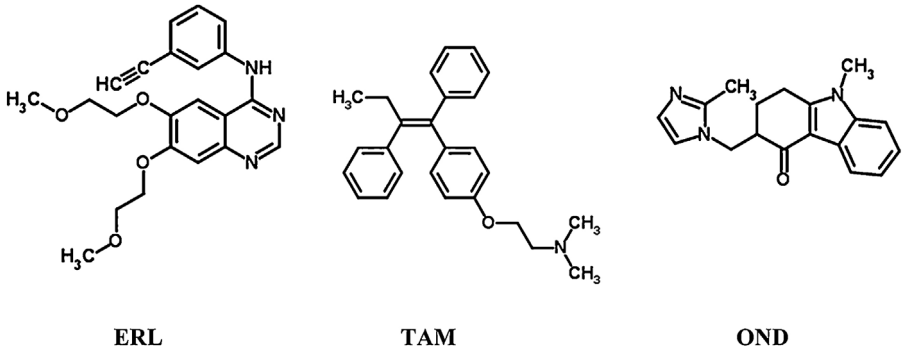

Fig. 1. Chemical structure of the studied compounds; erlotinib (ERL), tamoxifen (TAM), and ondansetron (OND).

A combination of an ER antagonist with a TKI could be beneficial in the management of NSCLC in different ways. A previous study reported that TAM along with gefitinib (TKI) showed synergistic anti-proliferative effects with the suggestion that a functional cross-signaling between ER and EGRR pathways is pronounced in NSCLC [1]. Recently, it has been reported that TAM can enhance the cytotoxic effect of ERL through down-regulation of thymidine phosphorylase expression. This postulates that a combination of TAM with ERL can be beneficial as NSCLC therapy, particularly in women with lung adenocarcinoma or cases with sensitive EGFR mutations [2].

Drug-drug interactions (DDIs) are a matter of great concern in oncology practice. Pre-calculated plasma levels of a particular anticancer agent should be attained in order to provide the required cytotoxic action with minimum side effects. Most cancerous patients receive more than one chemotherapeutic agents, in addition to non-cancerous medications including over-the-counter drugs, drugs used for the treatment of underlying diseases, besides complementary and alternative medicine (CAM) that are mostly used to alleviate the side effects of the anticancer medications. These anticancer and non-cancerous medications could significantly contribute to increased risk of DDIs.

Cytochrome P450 enzymes play an important role in the biotransformation of different exogenous and endogenous chemicals [5]. Specific cytochrome P450 enzymes are involved in the oxidative metabolism of many anticancer drugs. Among which are ERL and TAM which are mainly metabolized by CYP3A4 mostly in the liver and intestine [6,7]. Since both drugs are substrates of the CYP 3A4 enzymes, any co-administered agents which have been identified as an inhibitor or an inducer of the CYP 450 enzymes could lead to DDIs.

As other targeted therapies, TKIs are usually prescribed for prolonged periods of time with an incidence of inter-individual pharmacokinetic (PK) variability. This variability may arise from genetic factors (activity of CYP3A enzymes), environmental factors (DDIs), in addition to some habitual characteristics of the patients (smoking) [8]. Many DDIs have been reported with ERL. Ketoconazole and aprepitant, potent CYP3A4 inhibitors, increase plasma concentrations of co-administered ERL by about 2-fold [9,10]. Also ciprofloxacin, an inhibitor of CYP 3A4, resulted in a significant increase of ERL AUC by about 39%, with no significant change in C_{max}. Also, different reports suggested that CYP3A4 or CYP2C8 substrates such as phenytoin, simvastatin, and warfarin should be given with caution when co-administered with ERL [11–13].

Previous studies showed that ERL is a potent inhibitor of CYP1A1 and a moderate inhibitor of CYP 3A4 [7]. Also, TAM and its main metabolite 4-hydroxy tamoxifen are reported to induce CYP3A4 activity in cell lines [14]. It was observed that TAM increased clearance of co-administered letrozole and anastrozole [14].

Since both ERL and TAM are substrates of CYP3A4/5 and that both drugs can alter the enzymatic activity, DDIs could be expected. Thus drug plasma levels should be measured when the two drugs are co-administered in the management of NSCLC. This is extremely important in therapeutic drug monitoring (TDM) to ensure the desired anticancer effect with minimum toxicity. Thus development of analytical methods capable of simultaneous analysis of ERL and TAM in plasma samples is extremely beneficial in the field of TDM.

Different liquid chromatographic techniques have been reported in the literature dealing with the determination of ERL in biological samples. They include HPLC-UV [15,16], HPLC-DAD [17], HPLC–MS/MS [18–21], and UPLC–MS/MS [22,23]. Also, TAM has been analyzed in biological matrices using numerous liquid chromatographic techniques, namely HPLC-fluorimetry [24–26], HPLC-DAD [27], HPLC-UV [28], HPLC–MS/MS [26,29–32], and UPLC–MS/MS [33–36]. Since UPLC–MS/MS is nowadays the technique of choice in bioanalysis, regarding selectivity and sensitivity, it was successfully applied in studying PK interaction studies of ERL [23] and TAM [31,36].

Review of the literature revealed that, to our knowledge, no method has been found so far dealing with the simultaneous determination of ERL and TAM. Thus, the purpose of the present study was to develop and validate UPLC–MS/MS method for the simultaneous determination of ERL and TAM in rat plasma. The validated method was successfully applied to PK interaction studies as a result of possible co-administration of the two drugs in the clinical management of NSCLC.

2. Experimental

2.1. Materials and reagents

ERL reference standard was purchased from (Pfizer Inc., NY, USA). Tamoxifen (TAM) and ondansetron (OND) used as the internal standard (IS), were supplied by Sigma Aldrich Co. (St. Louis, MO, USA).

Solvents of HPLC grade namely methanol and acetonitrile (Panreac, E.U.) were used in the study. Formic acid (Sigma Aldrich, Chemie GmbH, Steinheim, Germany) was involved in the analysis.

Ultrapure water was used throughout the study. It was obtained from Milli-Q Advantage water purification system (Millipore, Molsheim, France) with 0.22 μm filter.

2.2. Instrumentation

Waters Model Xevo TQ-S UPLC–MS/MS separation system (Singapore) was applied in the study. The instrument was supplied with binary solvent manager (Aquity ^{TM} Ultra-performance

LC) and sample manager (Aquity ™ Ultra-performance LC). Triple-quadrupole mass spectrometric detector (STEP WAVE ™, Ultra-performance LC) with different ionization modes (Zspray ™ ESI-APCI-ESCI, Ultra-performance LC) and multiple reaction monitoring (MRM)-mode was used. Masslynx™ Version 4.1 (Micromass) software was used for data processing.

Strata ™–X-C 33 μm polymeric strong cation (200 mg/3 mL) solid-phase extraction (SPE) tubes supplied by phenomenex (Torrance, USA) were used for sample preparation.

Nitrogen evaporator N-EVAP 112 with a heating system OA-SYS (Organomation Assocciates, Inc, MA, USA) was involved in sample preparation.

Disposable syringe filters (CHROMAFIL ® Xtra PA-20/25 polyamide filters), pore size: 0.2 μm, filter-⊖: 25 mm (MACHEREY NAGEL, GmbH & Co. KG, Duren, Germany) were used for sample filteration.

2.3. Chromatographic conditions

Chromatographic analysis was carried out using a mobile phase consisting of acetonitrile: water (80: 20, v/v), and so that each phase contained 0.15% formic acid. Acquity UPLC BEH™ C 18 column (100 × 1.0 mm, i.d., 1.7 μm particle size) (Waters, Ireland) was applied for the analysis. The auto-sampler temperature was maintained at 10 °C and the column temperature was adjusted at 45 °C. Volumes of 5 μL were injected into the system with the full loop mode. The flow rate of 0.2 mL/min was applied for the whole runtime of 2.0 min.

2.4. Mass spectrometric conditions

Positive electrospray ionization (ESI+) mode was used in the study. Quantification of the compounds was performed using multiple reaction monitoring (MRM) of the parent $[M+H]^+$ to selected daughter ions. MS parameters including the cone voltage (V), capillary voltage (KV), collision energy (eV), as well as the desolvation temperature (°C) were separately optimized for each compound (Table 1). A source temperature of 150 °C was applied. Desolvating gas, cone gas, and collision gas flow was set at 800 L/h, 150 L/h, and 0.15 mL/min, respectively. The dwell time of all compounds were kept at 0.025 s. The MS analyzer was set for both ion energy 1 and 2 at the following resolutions; LM of 2.8 and HM of 14.86.

2.5. Preparation of stock and standard solutions

Stock solutions of 1 mg/mL of ERL, TAM, and OND (IS) were prepared in methanol. Further dilutions were carried out in methanol to get standard solutions of different concentrations of ERL and TAM. On the other hand, OND standard solution of concentration 5 ng/mL was prepared.

2.6. Construction of calibration graphs and spiking of plasma samples

Matrix-based calibration graphs were constructed from drug-free rat plasma samples spiked with pre-determined amounts of ERL and TAM, along with OND (IS). Volumes of 50 μL plasma samples were separately spiked with different volumes of standard solutions of ERL and TAM to get final concentrations of 0.2–50 ng/mL of ERL and TAM, along with 50 μL of 5 ng/mL OND (IS). Then a volume of 150 μL 2% formic acid was added to each sample. All samples were then made up to final volumes of 1 mL with methanol. Blank samples were simply prepared by adding 0.8 mL methanol and 150 μL 2% formic acid to 50 μL plasma samples. Samples were vortex-mixed at 1000 g for 5 min. The methanolic centrifugate was then purified by pouring onto STRATA™ –X-C 33 μm cartridges used for SPE, previously preconditioned with 1.0 mL methanol followed by 1.0 mL ultrapure water. Loaded SPE cartridges were then washed with 1.0 mL 2% formic acid. The analytes were finally eluted with 1.0 mL 5% solution of ammonium hydroxide in methanol. The eluate was evaporated to dryness under nitrogen then reconstituted in 0.5 mL acetonitrile before being injected into the UPLC–MS/MS system under the optimized UPLC–MS/MS conditions. The peak area ratios of each compound to that of OND (IS) were used to construct the calibration graph of each compound and the corresponding regression equations were derived.

2.7. Assay validation

Validation was performed as per the FDA guidelines for bioanalytical methods [37]. Different parameters were validated including extraction recovery, linearity, lower limit of detection and of quantification, accuracy and precision, specificity, recovery, matrix effects, dilution integrity, and stability studies.

2.7.1. Extraction recovery

Recovery of ERL and TAM from plasma samples was evaluated by spiking drug-free rat plasma samples with the two studied analytes at three different concentration levels; low (0.5 ng/mL), medium (1.0 ng/mL), and high (5.0 ng/mL), along with OND (IS). Spiked plasma samples were processed and the drugs were extracted as under construction of the calibration graphs. Processed samples were than analyzed under the above mentioned UPLC–MS/MS conditions and the peak area ratios of each drug to the IS were compared with those of standard solutions of the drugs having the same concentrations. Mean percentage recoveries (n = 6) obtained for both drugs were then calculated. Also, the extraction recovery of OND (IS) at the specified concentration of the assay was also calculated.

2.7.2. Linearity

Matrix-based calibration graphs were constructed by spiking drug-free rat plasma samples (50 μL) with eight different concentrations of the studied drugs (ERL and TAM) in the range 0.2–50 ng/mL plasma for both drugs, along with OND (IS), 50 μL of 5 ng/mL OND (IS). Calibration graphs were constructed by relating the peak area ratios calculated for each drug to that of OND and spiked concentrations in the specified range for both drugs. The calibration graphs were best fitted using least-squares linear regression model with the regression equation $y = a + bx$, where y is the peak area ratio, a is the intercept, b is the slope, and x is the concentration of a particular analyte.

2.7.3. Lower limit of detection (LLOD) and of quantification (LLOQ)

The analyte concentrations that gave analytical responses of at least three times or five times the blank signals were used to estimate the LLOD and LLOQ, respectively. In addition, concentrations selected as LLOQ should be identifiable with acceptable precision and accuracy (within ±20%).

2.7.4. Selectivity

Method selectivity was assessed by analyzing plasma extracts from six different batches of rat plasma obtained from different sources. This was assessed by comparing the chromatograms of drug-free blank plasma samples with those spiked with concentrations equivalent to the LLOQ of ERL and TAM, along with OND (IS). The analytical responses obtained at the retention times of both

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	394.20	278.04	30	3.5	30	200
TAM	372.25	72.01	40	4.0	25	150
OND (IS)	294.18	170.16	35	4.0	25	200

drugs at LLOQ and of IS were then compared to that obtained from blank samples.

2.7.5. Precision and accuracy

The concentrations of drugs in the quality control (QC) samples were selected to cover the whole range of the calibration graph as follows; very low (LLOQ), low, medium, and high concentrations. Drug-free rat plasma samples were spiked with ERL and TAM at four different concentration levels (0.2, 1, 5, 40 ng/mL) then analyzed in replicates (n = 6) under the optimized UPLC–MS/MS conditions. In each case, the found concentrations were compared with the nominal concentrations. The results obtained were used for the assessment of the accuracy, in terms of percentage relative error (E_r%), and precision, in terms of percentage relative standard deviation (%RSD). Intra-day and inter-day accuracy and precision were validated by repeating the assay six times on the same day or on three consecutive days, respectively.

2.7.6. Matrix effect

Since the matrix of biological samples could affect the ionization of the analyzed drugs either by suppression or enhancement of the ionization, it is very important to validate the bioanalytical method in this respect. This was assessed by carrying out the same procedure as per recovery evaluation but processed samples (without plasma) were used as a reference for comparison. The% matrix factors at four different concentration levels (0.2, 1, 5, 40 ng/mL) of both drugs and of IS at the specified concentration level, were then calculated.

2.7.7. Stability studies

Processed plasma samples were tested for the stability of ERL and TAM under different conditions including; autosampler stability, short-term (bench-top), and long-term stability, in addition to freeze-thaw stability. This was assessed using plasma samples spiked at two different concentration levels, low (0.5 ng/mL) and high (1 ng/mL), each analyzed in six replicates. Autosampler stability was assessed by leaving the processed samples in the autosampler at 10 °C for 56 h prior to the injection. However, samples left at room temperature (25 °C) for 6 h or at −30 °C for 30 days were used to evaluate short-term and long-term stability, respectively. Finally, the stability of the drugs following three freeze-thaw cycles were tested (freeze at around −30 °C then thaw at room temperature). In each case, the calculated concentrations were compared with the nominal concentrations and the corresponding recoveries were calculated.

2.7.8. Dilution integrity

To ascertain whether the dilution of highly concentrated samples could affect the accuracy of the assay, a blank plasma sample, plasma samples spiked with high concentrations of ERL and TAM (100 ng/mL), diluted with blank rat plasma (1:2 and 1:5) before analysis were used. The calculated concentrations were then compared with the expected concentrations and the calculated recovery% (±RSD) was reported.

2.8. Application to pharmacokinetic studies

All experiments were performed with reference to ethical guidelines for experimental studies with animals as per the WHO regulations in Saudi Arabia [38]. The study was conducted using healthy male Wistar rats of weight 250 ± 30 g. The animals were kept in cages placed in a well-ventilated room and maintained at standard laboratory conditions; an average temperature of 24–27 °C, a regular 12 h day–night cycle, and a relative humidity of 40–60%. The rats were left in their cages for one week before conducting the study. All the animals had free access to water while diet was prohibited for 12 h before the beginning of the experiment. The animals were divided into four cages of five rats each. One group (Group I) was used as the control while the other groups (II to IV) were the treated groups. Suspensions of 20 mg/mL (ERL) and 8 mg/mL (TAM) were prepared by triturating an appropriate weight of each drug with aqueous methyl cellulose (0.5%, w/v). All treated animals received pre-calculated oral doses (0.25 mL) of these suspensions using a gavage needle as follows; ERL, 20 mg/kg (Group II), TAM, 8 mg/kg (Group III), and a combination of ERL, 20 mg/kg, and TAM, 8 mg/kg (Group IV). Blood samples (0.3 mL) were collected from the retro-orbital sinus of each rat into K3-EDTA 1.5 mL polythene tubes at different time intervals; 0, 0.25, 0.5, 1, 2, 4, 5, 24, and 48 h following drug administration. Blood samples were immediately centrifuged for 30 min (2300 g, 4 °C). The resulting plasma samples were stored at −20 °C till the day of analysis. Volumes of 50 μL of each plasma sample were separately spiked with a constant volume of 50 μL of OND, IS (5 ng/mL). All samples were then treated exactly as described under the construction of calibration graphs.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Initial trials involved the use of mobile phases consisting of mixtures of organic modifiers (methanol or acetonitrile) with water, each with 0.1% TFA. However, systems with acetonitrile, water were adopted since they provided better peak shape and response, compared with methanol: water systems. Different mobile phases composed of mixtures of different ratios of acetonitrile (30–90%), water, and formic acid (0.01–0.2%) were used for the elution of standard mixtures of ERL, TAM, and OND (IS). These mobile phases were evaluated regarding peak shape, peak response, as well as the retention time. Initially, mobile phases of different ratios of acetonitrile and water, each with 0.1% formic acid, were tried. It was observed that acetonitrile percentage of less than 50% resulted in distortion of ERL peaks. Also, increased response of TAM peaks was noticed at acetonitrile percentage of 70% or higher. However, with regards to the analysis runtime, 80% acetonitrile resulted in the least runtime, compared with 70% and 90% acetonitrile content in the mobile phase. Thus, 80% acetonitrile in the mobile phase produced best results regarding the runtime and peak shape of ERL and TAM peaks. Then, the percentage of formic acid in the mobile phase was evaluated in the range (0.01–0.2%). It was noticed that formic acid was essential to get sharp peaks with a decrease in the retention time of both drugs with increasing formic acid content in the mobile phase till 0.15% above which the retention time of all

drugs was increased. Best results were achieved with 0.15% formic acid. Final analysis was performed with a mobile phase consisting of acetonitrile: water, each with 0.15% formic acid, (80: 20, v/v) for the whole runtime of 2.0 min. Under these chromatographic conditions, sharp and symmetric peaks of all drugs were obtained (ERL eluted at 0.65 ± 0.030 min, TAM at 1.03 ± 0.020 min, and OND (IS) at 0.65 ± 0.030 min).

3.2. Optimization of mass spectrometric conditions

In order to select the optimum MS/MS conditions, standard solutions of ERL, TAM, and OND (IS) (5 ng/mL) were directly infused along with the mobile phase into the mass spectrometer with ESI as the ionization mode. Compared with the negative ionization, the positive ionization mode provided better response for all studied compounds. Thus it was applied for final analysis. In the full scan spectra of protonated precursor ions $[M+H]^+$, fragments were selected at *m/z* 394.20 (ERL), *m/z* 372.25 (TAM), and at *m/z* 294.18 (OND). The product ions used for quantitation were at *m/z* 278.04 (ERL), *m/z* 72.01 (TAM), and at *m/z* 170.16 (OND) (Fig. 2). Different MS/MS parameters were optimized in order to get the highest response of the protonated molecules of the studied compounds. They included cone voltage, capillary voltage, desolvation temperature, and gas flow rate besides the ESI source temperature. Increasing the capillary voltage or cone voltage resulted in an increase in the relative abundance of the parent ions till certain optimum values (Table 1) after which a remarkable decrease was noticed. It was also found that desolvation gas flow rate of 80 L/h and a source temperature of 150 °C resulted in the highest response of the protonated parent ions of the cited drugs. However, the response of the daughter fragment ions was significantly affected by the collision energy. By analogy, the intensity of a certain 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 recorded in Table 1.

3.3. Sample preparation

SPE was selected for sample preparation because of its potential in eliminating matrix components with subsequent minimization of ion suppression. Different types of SPE cartridges were tested for their extraction recovery, namely octadecyl C 18, octyl C 8, ethyl C 2, and cyanopropyl CN (200 mg, 3 mL) (Spe-ed SPE cartridges, Applied Separations, Allentown, PA). Moreover, Strata™ −X-C 33 μm strong cation cartridges (200 mg, 3 mL) (Phenomenex, Torrance, USA) were used. Different extraction procedures were tried with each type of SPE cartridges and the extraction efficiency was assessed by calculating the average recovery of a spiked mixture of 1.0 ng/mL (ERL, TAM) compared with a standard mixture of the same concentration. Experimental trials showed that extraction recoveries obtained for ERL with octadecyl C 18, octyl C 8, ethyl C 2, and cyanopropyl CN cartridges did not exceed 90.56, 69.22, 55.22, and 17.21%, respectively. Also, for TAM, the extraction recoveries were 39.80, 42.50, 49.11, and 52.05% for octadecyl C 18, octyl C 8, ethyl C 2, and cyanopropyl CN cartridges, respectively.

Although some previous work applied C 18-for 3-hydroxy tamoxifen- [39], C2 [28,32], or CN [25] cartridges for TAM extraction, good recovery could not be attained in the preliminary trials. It is very important to mention that basic compounds (e.g. TAM) are mostly retained with an ionic interaction as a type of secondary interactions parallel to the reversed phase interactions. It was previously postulated that the use of neutral eluents containing a displacing cation could be helpful to eliminate this subtype of ionic interactions. Other studies suggested that strongly lipophilic compounds e.g. TAM (log P = 6.63, pka = 8.85) cannot be easily eluted using high concentration of ammonium acetate in methanol/water mixture even with the more polar C2 cartridge [40]. This leads to the attempt of using extraction cartridges acting on two interaction modes, reversed-phase and ionic interactions. Since both ERL (pka 5.42) and TAM are neutral and weakly basic compounds, respectively, cation mixed mode SPE cartridges seemed promising in this respect. It is noteworthy to mention that this type of cartridges was previously applied in the analysis of TAM [26,36] and TKIs including ERL [22].

Table 2
The extraction efficiency of STRATA™ −X-C−33 μm cartridges in the UPLC–MS/MS analysis of standard mixtures of ERL and TAM, with OND (IS).

	Concentration spiked (ng/mL)	Mean recovery (%) ± RSD[a]	E_r(%)[b]
ERL	0.5	96.68 ± 1.72	−3.32
	1	89.38 ± 5.76	−10.62
	5	86.60 ± 4.70	−13.40
TAM	0.5	92.58 ± 6.05	−7.42
	1	86.08 ± 6.46	−13.92
	5	92.31 ± 3.02	−7.69

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

Different trials were tested for their extraction efficiency of ERL and TAM from spiked samples and the final analysis was carried out based on the following procedure; cation SPE (Strata™ −X-C 33 μm) cartridges were preconditioned with 1 mL methanol then 1 mL water. Volumes of 50 μL plasma samples spiked with ERL and TAM, along with 50 μL of OND, IS (5 ng/mL in methanol) were acidified with 150 μL 2% formic acid followed by the addition of up to 1 mL methanol. The solutions were then loaded on the SPE cartridge. Washing was performed with 1 mL 2% formic acid followed by the elution of the analytes using 1 mL 5% ammonia in methanol. The eluent was evaporated to dryness before being reconstituted into 0.5 mL acetonitrile then injected into the UPLC system. In this case, a good average% recovery with good peak shape was obtained for both ERL (89%) and TAM (86%). Table 2 shows the extraction efficiency of STRATA™ −X-C 33 μm cartridges using this optimized procedure which was applied for actual analysis.

Based on the mechanism of the analytes' retention on this mixed-mode cation cartridges, basic analytes should be positively charged for better retention through cation exchange mechanism with sulfonic acid ligands embedded in the cartridges which constitute the major interaction mechanism in addition to JI—JI bondings, and hydrophobic interactions. Thus initial treatment of the samples with formic acid was essential as a preliminary step. Then for the purpose of elution, these ionic interactions should be overcome by using ammonia for alkalinization. Thus the retained drugs were eluted with a mixture of ammonia and methanol.

3.4. Method validation

3.4.1. Extraction recovery

Table 2 summarizes the percentage recovery of ERL and TAM, obtained from plasma samples spiked at three concentration levels. Recovery values of not less than 86.60 (ERL) and 86.08 (TAM) were obtained. Also, the mean recovery obtained for OND (IS) at the specified concentration applied in this assay was 87.33. These results indicated good extraction efficiency of the proposed sample treatment procedure.

3.4.2. Linearity

Calibration graphs constructed by relating the peak area ratios calculated for each drug to that of OND and spiked concentrations were found to be linear in the range (0.2–50 ng/mL plasma) for both ERL and TAM. High degree of linearity of the method was assessed

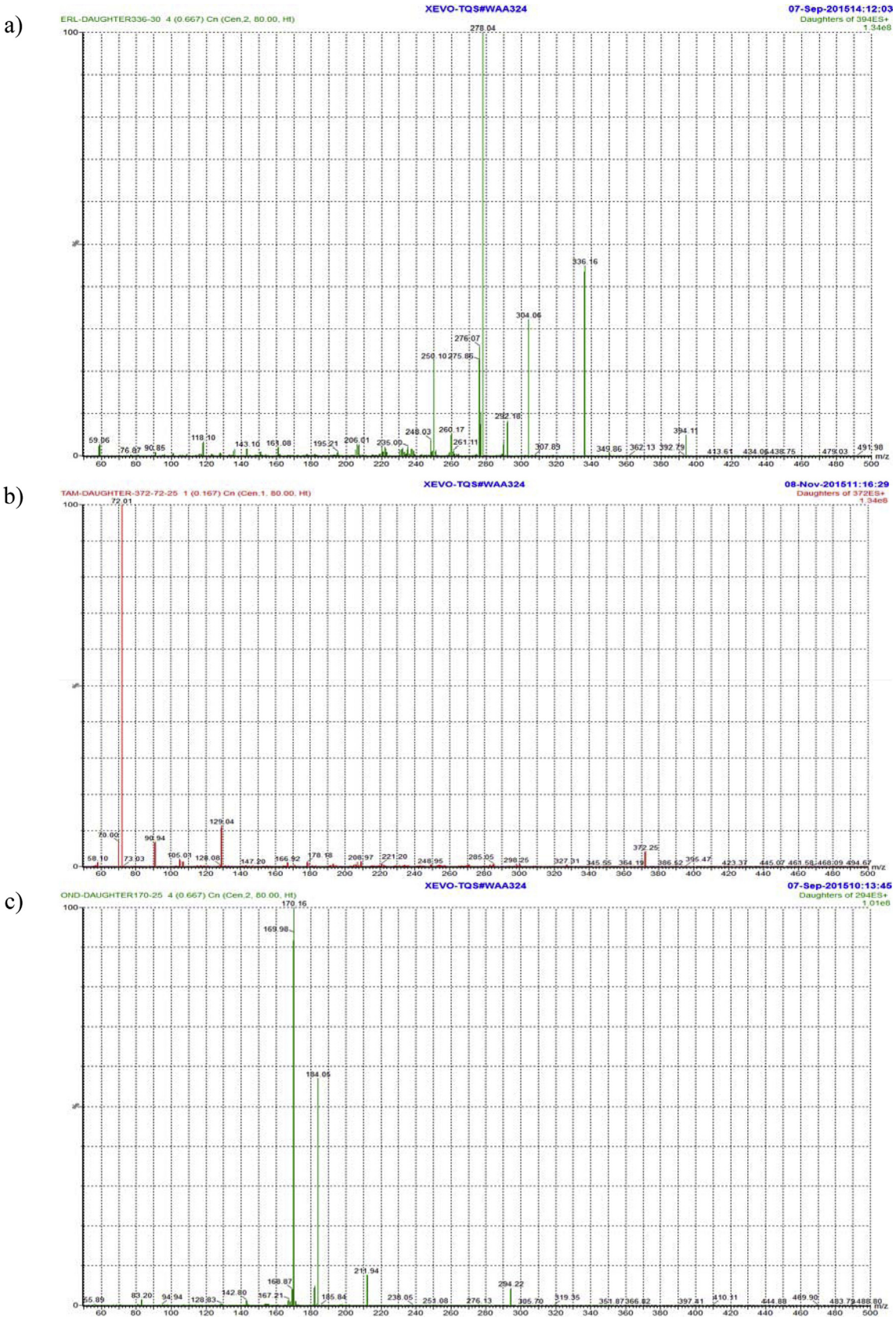

Fig. 2. Product ion spectra of ERL, a), TAM, b), and OND, c).

Table 3
Regression and statistical parameters for the determination of ERL and TAM rat plasma by the proposed UPLC–MS/MS method.

Parameter	ERL	TAM
Linearity range (ng/mL)	0.2–50	0.2–50
LLOQ[a] (ng/mL)	0. 2	0. 2
LLOD[b] (ng/mL)	0.03	0.03
Intercept (a)	0.1240	0.1134
Slope (b)	3.2477	9.3268
CorrelationCoefficient (r)	0.9989	0.9971
S_a[c]	0.0058	0.0297
S_b[d]	0.0227	0.1159
$S_{y/x}$[e]	0.0088	0.0450
F[f]	20443.90	6477.78
Significance F	4.8911×10^{-5}	0.00015

[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).

by the high values of the correlation coefficients ($r > 0.9971$) for both analytes along with small intercepts. Other statistical parameters were presented including standard deviations of residuals ($S_{y/x}$), of the intercept (S_a), and of the slope (S_b). Moreover, variance ratio (F values) were also calculated. High F values along with low values of $S_{y/x}$ indicated low degree of scatter of the experimental data points around the regression line [41]. All calculated parameters were shown in Table 3.

3.4.3. Lower limit of detection (LLOD) and of quantification (LLOQ)

LLOQ values were set at 0.2 ng/mL for ERL and TAM while LLOD were set at 0.03 ng/mL for both drugs (Table 3). MRM chromatograms of blank plasma and plasma samples spiked with ERL and TAM at their LLOQ were shown in Fig. 3. Using this method, the achieved LLOQ values of both drugs were lower than those of previously reported LC–MS/MS methods for ERL [18–22] and TAM [26,29–36]. These low values of LLOQ allowed the successful application of this method in the trace analysis of the two drugs in clinical studies.

3.4.4. Selectivity

Comparing the chromatograms of drug-free plasma samples with those spiked with concentrations equivalent to the LLOQ of ERL and TAM revealed that the analytical response obtained at the retention times of both drugs at LLOQ was at least five times of that obtained from blank samples while that of IS was not less than twenty times compared with the blank (Fig. 3). Thus no minimal interference was detected at the retention times of analytes and IS indicating high degree of selectivity of the proposed method.

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

	Concentration added (ng/mL)	Mean recovery (%) ± RSD[a]	E_r(%)[b]
ERL	0. 2	98.75 ± 1.91	−1.25
	1	101.19 ± 1.29	1.19
	5	97.41 ± 4.04	−2.59
	40	99.39 ± 2.18	−0.61
TAM	0. 2	99.94 ± 3.45	−0.06
	1	98.83 ± 3.03	−1.17
	5	96.13 ± 4.91	−3.87
	40	97.47 ± 1.25	−2.53

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

3.4.5. Precision and accuracy

The data for intra-day and inter-day precision and accuracy were presented in Table 4. For both ERL and TAM, the calculated relative errors were in the range (1.18–(−6.43)%) and (5.04–(−10.09)%) for intra-day and inter-day errors, respectively, while, the calculated RSD values were in the range (0.86–10.52%) and (1.89–9.52%) for intra-day and inter-day deviations, respectively. Since both 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.

3.4.6. Matrix effect

The% matrix factors at the four selected concentration levels (0.2, 1, 5, 40 ng/mL) of not more than 2.59 (ERL) and 3.87 (TAM) were reported (Table 5). Also, the% matrix factor found for OND (IS) at the specified concentration applied in the assay was 3.22. This emphasized that negligible matrix effect was encountered for plasma samples analyzed with the optimized method which is extremely important to carry out trace analysis of the cited drugs.

3.4.7. Stability studies

The results shown in Table 6 indicated that all the calculated recoveries did not exceed the allowed limits (±15%.). Thus both ERL and TAM were considered stable under these storage and handling conditions. In addition, stock and standard solutions of both drugs were stable when kept at room temperature for 6 h or stored at the refrigerator (4 °C) for 3 months.

3.4.8. Dilution integrity

The calculated recovery% (±RSD) results of diluted plasma samples given in Table 7 were within the acceptance limits (±15%) indicating the integrity of both drugs up to five times dilution of plasma samples.

Table 4
Evaluation of the intra-day and inter-day accuracy and precision for the determination of ERL and TAM in rat plasma by the proposed UPLC–MS/MS method.

	Concentration added (ng/mL)	Intra-day		Inter-day	
		Mean recovery (%) ± RSD (n = 6)	E_r(%)[a]	Mean recovery (%) ±RSD (*n = 18*)	E_r(%)[a]
ERL	0. 2	99.85 ± 4.16	−0.15	97.94 ± 4.25	−2.06
	1	98.28 ± 6.08	−1.72	98.50 ± 3.60	−1.50
	5	97.31 ± 0.86	−2.69	99.71 ± 1.89	−0.29
	40	99.75 ± 4.53	−0.25	97.11 ± 5.56	−2.89
TAM	0. 2	99.38 ± 5.35	−0.62	105.04 ± 3.23	5.04
	1	97.31 ± 10.52	−2.69	96.66 ± 3.08	−3.34
	5	101.18 ± 7.09	1.18	89.91 ± 5.12	−10.09
	40	93.57 ± 2.26	−6.43	95.92 ± 9.52	−4.08

[a] Percentage relative error.

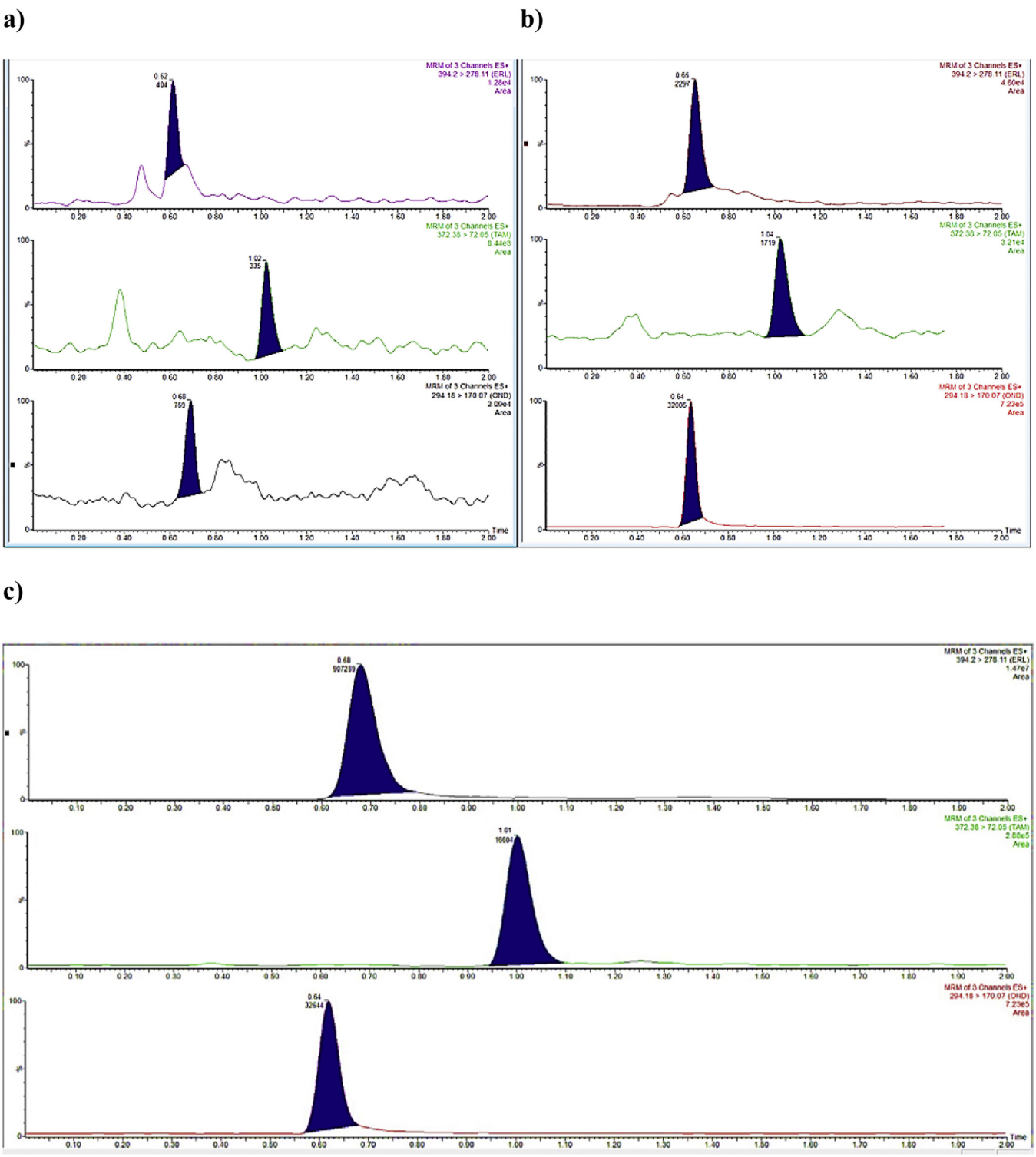

Fig. 3. Multiple reaction monitoring (MRM) of a blank plasma, a), and a plasma sample spiked with a standard mixture of ERL and TAM at their LLOQ levels with OND (IS), b), plasma sample of treated rats collected 1 h after oral administration of a combination of ERL (20 mg/kg) and TAM (8 mg/kg), c).

3.5. Comparison between the proposed method over previously reported bioanalytical methods

This study describes, for the first time, the simultaneous determination of ERL and TAM in plasma samples. The developed method was applied to study possible PK interactions between the two drugs that may arise following their co-administration as a suggested treatment protocol in the management of NSCLC. This method utilized the state-of – the-art SPE-UPLC–MS/MS technology for plasma analysis. Our proposed method has many advantages compared with the previously reported LC–MS/MS methods described for the determination of either ERL or TAM in biological samples. First of all, this method had a marked sensitivity with the ability to analyze very low concentrations of the drugs. With the exception of ERL which was previously determined by our research team in rat plasma samples, in combinations with

Table 6
Evaluation of the stability of ERL and TAM in rat plasma.

Stability	Concentration added (ng/mL)	Mean recovery (%) ± RSD[a]	
		ERL	TAM
Autosampler stability (10 °C, 56 h)	0.5	96.01 ± 2.90	87.03 ± 3.45
	1	90.25 ± 6.58	99.05 ± 3.10
Short-term stability (25 °C, 6 h)	0. 5	93.81 ± 7.64	99.43 ± 2.10
	1	96.40 ± 4.14	93.65 ± 5.10
Long-term stability (−30 °C, 30 days)	0. 5	91.16 ± 8.69	93.89 ± 4.16
	1	87.17 ± 7.72	90.25 ± 6.32
Freeze-thaw stability (−30 °C, 3 cycles)	0. 5	98.82 ± 2.25	84.95 ± 5.74
	1	96.69 ± 1.71	94.52 ± 3.41
Refrigerator (4 °C, 3 months)	0. 5	97.75 ± 3.81	100.31 ± 4.39
	1	102.62 ± 4.28	98.14 ± 6.12

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

gefitinib, dexamethasone, prednisolone, and ondansetron [23], the current method provided the lowest LLOQ (0.2 ng/mL) described so far for the analysis of TAM. Compared with previously published methods for the determination of ERL [18–22] or TAM [26,29–36], this method was of a remarkable sensitivity. This is extremely important for trace analysis of the studied drugs and for accurate determination of terminal phase elimination during PK studies. Moreover, some of these previous reports utilized less advanced sample preparation techniques like protein precipitation [18–20,29,30,33,35] or liquid-liquid extraction [31,34], had longer analysis time [20,22,29–35], or even utilized larger sample volume for analysis [20,22,31,33–36]. Our developed method depends on the analysis of only very small sample volume (50 μL) which is extremely beneficial in cases where only limited amounts of samples are available. Moreover, the whole analysis time did not exceed 2 min which guarantees the suitability of the method in high throughput bioanalysis.

3.6. Application to pharmacokinetic studies

There has been considerable scientific interest in the role of TAM in a combination with ERL for the management of NSCLC because they may have synergistic anti-proliferative and cytotoxic effects [2]. However, no studies have been previously published regarding their PK interactions. In this respect, the validated UPLC–MS/MS method was applied to study the possibility of DDI when ERL and TAM following their co-administration. ERL is clinically given in the oral dose of 150 mg daily. While for TAM, a daily oral dose of 20 mg, up to 60 mg, is established for breast cancer treatment with no accurate data available for TAM dosing in NSCLC. Thus Wistar rats being treated with combinations of ERL (20 mg/kg) and TAM (8 mg/kg), group IV, were involved in the study. Moreover, for studying possible PK interactions, separate rats were treated orally with ERL (20 mg/kg), group II, and TAM (8 mg/kg), group III,

Table 7
Evaluation of the dilution integrity of ERL and TAM in rat plasma.

Concentration spiked (ng/mL)	Dilution fold	Mean recovery (%) ± RSD[a]	E_r(%)[b]
ERL	1:2	98.73 ± 6.68	−1.27
	1:5	97.41 ± 4.04	−2.59
TAM	1:2	94.46 ± 8.14	−5.54
	1:5	96.31 ± 5.20	−3.69

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

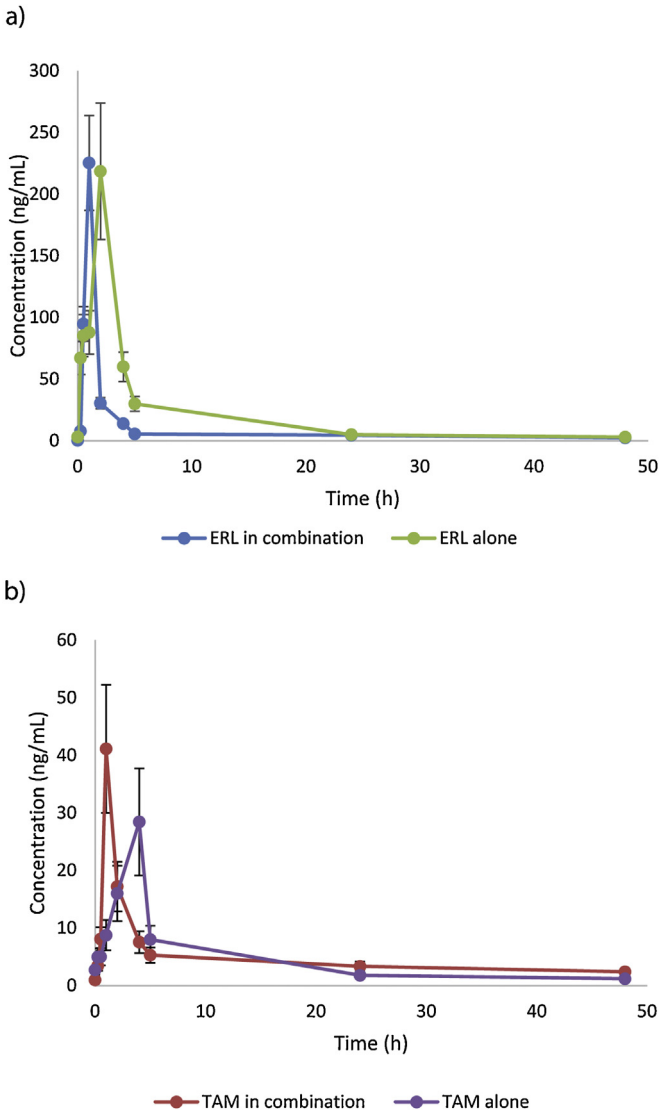

Fig. 4. Plasma concentration– time profile of the studied drugs in rats after an oral administration of a combination of ERL (20 mg/kg) and TAM (8 mg/kg), compared with their single oral administration at the same doses, ERL, a) and TAM, b). Suitable dilutions of prepared plasma samples were made before analysis.

respectively. Representative MRM chromatograms of rat plasma samples 1 h following the oral co-administration of ERL/TAM were presented in Fig. 3. The mean plasma concentration time profiles of ERL/TAM, either alone or in a combination, were presented in Fig. 4. Since both ERL and TAM are substrates of CYP3A4 enzymes and that TAM and its metabolite 4-hydroxy derivative are CYP3A inducers while ERL is a moderate CYP inhibitor, it was expected to report DDI when the two drugs are given together in a combination. Moreover, different PK parameters including maximum plasma concentration (C_{max}), time to reach the maximum concentration (t_{max}), half-life ($t_{1/2}$), and area under the curve (AUC) for ERL/TAM were calculated as presented in Table 8. Although there was no significant change in the C_{max} of ERL, a significant decrease in the AUC (54%) was reported upon its co-administration with TAM suggesting the role of TAM in CYP3A4 induction with subsequent increase in ERL clearance. On the other hand, an increase in the C_{max} of TAM of about 46% with no significant increase in the AUC (10%) was noticed with TAM combination with ERL, compared with its single administration. This could be explained by the fact that ERL is a moderate CYP3A inhibitor with subsequent

Table 8
Main pharmacokinetic parameters after oral administration of ERL and TAM 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 II				
ERL (20 mg/kg)	218.46 ± 55.30	2.0 ± 0.33	7.5 ± 1.52	976.2 ± 89.22
Group III				
TAM (8 mg/kg)	28.42 ± 9.31	4.0 ± 1.10	3.5 ± 0.23	201.4 ± 52.65
Group IV				
ERL (20 mg/kg)	225.27 ± 38.53	1.0 ± 0.29	1.8 ± 0.22	452.0 ± 82.22
TAM (8 mg/kg)	41.12 ± 11.12	1.0 ± 0.51	1.7 ± 0.35	226.0 ± 18.85

suppression of TAM metabolism and subsequent increase in its plasma levels. Since the present study was conducted on rats, and not humans, and since their pharmacokinetics could show some differences, this study suggested the possibility of occurrence of DDIs between ERL and TAM when shifted to clinical studies. As a result, TDM of both drugs is significantly important in NSCLC patients receiving ERL/TAM regimen. This is extremely essential to get the required cytotoxic effect while minimizing dose-dependent side effects. Moreover, the large inter-individual variation in the PK of both drugs potentiates the necessity for their continuous plasma monitoring in patients receiving these drugs during the treatment protocols.

4. Conclusion

In this work, a simple and sensitive UPLC–MS/MS method has been developed and validated for the simultaneous determination of ERL and TAM in rat plasma. Mixed-mode cationic SPE cartridges have been applied for sample preparation. The method was fully validated as per the FDA guidelines for validating bioanalytical methods. The proposed method offered very low LLOQ so far for the determination of both ERL and TAM. The method was successfully applied to study the PK parameters of ERL and TAM when given in a combination, compared with their single administration. Considerable DDIs were noticed with their co-administration suggesting the importance of carrying out TDM with the administration of such drug combination in the treatment of NSCLC.

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