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Original article

New bioanalytical microemulsion Electrokinetic chromatography method for the simultaneous determination of Trifluridine with its metabolites and Tipiracil in rat plasma: Application to pharmacokinetic studies

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ABSTRACT

A Microemulsion Electrokinetic Chromatography method coupled with diode array detector (MEEKC-DAD) was developed for the first time and found to be efficient, sensitive, and selective for the simultaneous analysis of Trifluridine (FTD), and its metabolites 5-(trifluoromethyl) uracil (FTY) and 5-carboxy-2′-deoxyuridine (5CDU), and Tipiracil (TIP) in rat plasma. Sample pre-treatment involved a simple protein precipitation from plasma using acetonitrile. The separation was achieved using a fused silica capillary (65 cm total length, 55 cm effective length and 50 μm i.d.) and a microemulsion solution consisted of 1.66% sodium dodecyl sulfate (SDS), 0.91% heptane, 6.61% 1-butanol, and 90.72% borate buffer (20 mM, pH 9.5). Electrophoretic separation was carried out at 20 °C and 20 kV. The samples were injected for 40 s at 20 mbar and detected simultaneously at 205 nm. The electrophoretic parameters indicated that the developed MEEKC-DAD method permitted complete resolution of the analytes within 13 min. The developed method was fully validated according to the FDA guidelines for bioanalytical method validation. The method was linear in the range 200–4000 ng/ml for FTD, FTY, 5CDU, and 100–1000 ng/ml for TIP. The intra/inter-day accuracy and precisions were ≤4% for all drugs. Extraction recovery and stability were also assessed and were within acceptable range. After being validated, the method was applied for the determination of the studied drugs in plasma samples collected from rats injected intraperitoneally with a combination of FTD and TIP. The results obtained were used to study the pharmacokinetics of FTD with its metabolite and TIP in rat plasma.

1. Introduction

Colorectal cancer (CRC) is the third common cancer and the second cause of death by cancer globally, with 1,849,518 new cases and 880,792 deaths in 2018 (Globocan, 2018). Treatment with first-line and second-line therapies has led to declines in death rate in patients with metastatic colorectal cancer (mCRC). Although the median overall survival (OS) has reached around 30 months, effective treatments for patients with mCRC refractory or intolerant to these therapies are still limited, and the development of new drugs is of great importance (Heinemann et al., 2014; Loupakis et al., 2014; Yamada et al., 2013; Yamazaki et al., 2014).

In 1960s, a thymidine-based nucleic acid analogue was developed (Heidelberger and Anderson, 1964). The anti-tumor activity of trifluridine (FTD) is due to inhibition of cellular proliferation and cell death by incorporation of its triphosphorylated thymidine-based nucleoside form into the DNA. It has poor bioavailability due to its degradation by thymidine phosphorylase to the inactive metabolites 5-(trifluoromethyl) uracil (FTY) and 5-carboxy-2′-deoxyuridine (5CDU). Tipiracil (TIP) is a potent and specific inhibitor of thymidine phosphorylase (a major metabolizer of FTD) which allows FTD to reaches high plasma levels for a longer time when administered concurrently (Fukushima et al., 2000; Tanaka et al., 2014).

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Based on the ability of TIP to inhibit thymidine phosphorylase and increases the bioavailability of FTD, TAS-102 was developed (Fukushima et al., 2000). It is an orally administered combination of Trifluridine/Tipiracil (FTD/TIP) in a molar ratio of 1:0.5. The combination has been approved for patients with refractory mCRC in Japan based on phase 1 and 2 studies (Doi et al., 2012; Yoshino et al., 2012; Tanaka et al., 2014). In phase 3 RECOURSE trial, which compared FTD/TIP with placebo in patients with mCRC refractory to standard treatment, FTD/TIP significantly prolonged OS (median, 7.1 months vs. 5.3 months; $P < 0.001$), prolonged progression-free survival (PFS) (median, 2.0 months vs. 1.7 months; $P < 0.001$), and increased disease control rate (DCR) (44% vs. 16%; $P < 0.001$) compared with placebo (Mayer et al., 2015). The results of this RECOURSE trial led to the approval of Trifluridine/Tipiracil combination for treatment of patients with refractory mCRC by the US Food and Drug Administration (US FDA, 2015).

The pharmacokinetics of TAS-102 has also been studied. The study concluded that the area under the curve (AUC) and the maximum plasma concentration (Cmax) of FTD were about 37- and 22-fold higher, respectively, when administered with TIP (Cleary et al., 2017). The results of this pharmacokinetics study are supported by the previous trials (Doi et al., 2012; Yoshino et al., 2012; Tanaka et al., 2014; Mayer et al., 2015).

The monitoring of plasma levels of anticancer drugs is of potential importance. Drug monitoring allows adaptation of dose to improve efficacy and reduce side effects. This can be achieved by the development of advanced and sensitive analytical methods. Among the advances in analytical techniques, capillary electrophoresis (CE) has developed as a powerful separation technique based on different separation approaches. It has distinct advantages in terms of high selectivity and efficiency, fast analysis, smaller quantities of samples, minimal solvent consumption, and overall lower expenses. More importantly, the separation is achievable over a wide pH range. These advantages allow the determination of drugs in presence of impurities or metabolites in biological samples (Altria and Rogan, 2006).

Microemulsion electrokinetic chromatography (MEEKC) was emerged as a mode of CE that separates neutral analytes using background electrolyte (BGE) consisted of microemulsion (ME). It has been introduced in CE separation by Watarai (Watarai, 1991). In addition, MEEKC is suitable for separation of cationic and anionic analytes. Microemulsion consists of two immiscible liquids dispersed in each other; either oil-in-water [o/w] or water-in-oil [w/o]. Oil-in-water MEs are most commonly used in MEEKC. The addition of surfactant and cosurfactant lowers the surface tension between the two liquids. The oil droplets act as a pseudostationary phase, and the separation is based on partitioning of analytes between the aqueous phase and the oil droplets (Schmitt-Kopplin, 2016). The possibility of applying the same operating conditions for separating different analytes is a significant advantage of MEEKC. Broad classes of analytes can be separated by MEEKC ranging from pharmaceuticals to vitamins, polycyclic hydrocarbons, natural products, sugars, amino acids, proteins, fatty acids, nucleosides, and chiral compounds (Schmitt-Kopplin, 2016). When equipped with a diode array detector (DAD), electrophoretic techniques become a potent analytical technique. DAD allows a range of wavelengths to be sensed simultaneously, and all analytes that absorb within this range can be detected in one run (Altria and Rogan, 2006; Manjula Devi and Ravi, 2012).

Based on extensive literature review, no MEEKC method has been published for the determination of FTD and TIP, and a limited number of methods have been described applying liquid chromatography. Few reported methods employed HPLC technique for the determination of FTD and TIP in pharmaceutical dosage forms (Mainaaz et al., 2017; Prathap et al., 2017; Sahu and Akula, 2017; Jogi et al., 2017; Goday et al., 2017; Hazra et al., 2018), two of which are stability-indicating (Jogi et al., 2017; Goday et al., 2017). In addition, an HPLC method for the simultaneous estimation of FTD and TIP in spiked human plasma has also been reported (Phani et al., 2017). Review of the literature- to our knowledge- revealed the absence of any analytical method for plasma determination of FTD/TIP. Therefore, we develop and validate for the first time, a sensitive, selective, and simple MEEKC method for the determination of FTD, and its metabolites FTY and 5CDU, and TIP simultaneously in rat plasma. The developed method has been applied to study the pharmacokinetics of the drugs in plasma after intraperitoneal administration of the combination to rats.

2. Materials and methods

2.1. Materials

Authentic standards of FTD, TIP, FTY, 5CDU, and Uridine (UD) as an Internal Standard (IS) were purchased from (Toronto research chemicals, Toronto, Canada). Analytical grade ammonium acetate, potassium diphosphate, dipotassium phosphate, triethylamine, trifluoracetic acid, sodium tetraborate, boric acid, Sodium dodecyl sulfate (SDS), heptane, 1-butanol, sodium hydroxide, hydrochloric acid and glacial acetic acid were purchased from (Sigma Aldrich, Chemic GmbH, Steinem, Germany). HPLC grade methanol and acetonitrile were purchased from (Panreac, Barcelona, Spain). Deionized water and Millipore membrane filter (0.2 mm, Nihon, Millipore) were used throughout the experiments. Adult male rats were provided by the Experimental Animal Care Centre at the College of Pharmacy, King Saud University.

2.2. Methods

2.2.1. Instrumentation and electrophoretic conditions

An Agilent CE instrument (Agilent Technologies Deutschland, Germany) was used for the analysis. The instrument equipped with data processing Agilent Chem Station® software and IBM computer. Analytes were detected at 205 nm by DAD. A fused silica capillary (Agilent Technologies, Fullerton, CA) with the dimensions: 65 cm total length, 55 cm effective length, and 50 μm internal diameter, was used throughout the analysis. The capillary and the sample were maintained at 20 °C. The background electrolyte (BGE) is a microemulsion consisted of 1.66% Sodium dodecyl sulfate (SDS) as surfactant, 0.91% heptane as oil phase, 6.61% 1-butanol as cosurfactant, and 90.72% borate buffer (20 mM, pH 9.5) as aqueous phase. Samples were introduced into the capillary at 20 mbar for 40 s. A 20 kV voltage was applied to the capillary. The capillary was washed with 0.1 M NaOH (5 min), water (5 min), and BGE (5 min) before the first run. To ensure reproducibility of the assay, the capillary was washed between injections with 0.1 M NaOH (2 min), water (2 min), and BGE (2 min).

2.2.2. Preparation of stock and standard solutions

Stock solutions of FTD, TIP, FTY, 5CDU, and UD (IS) in a concentration equal 100 μg/ml were prepared in water. Working standard solutions of drugs and IS were prepared by appropriate dilution of the stock solutions with the same solvent. All solutions were found to be stable for 1 month if kept in refrigerator at 2–8 °C.

2.2.3. Preparation of microemulsion (ME) solution

For preparation of microemulsion (ME) solution, aqueous borate buffer was prepared and its pH was adjusted (20 mM, pH 9.5). SDS was dissolved completely in the borate buffer applying magnetic stirring. Then 1-butanol was added, followed by the addition of heptane. The solution was stirred for 5 min, and sonicated

until it became clear, then left at room temperature for 1 h. Before use it has to be filtered using 0.2 μm nylon syringe filters. Finally, the prepared ME consists of 1.66% SDS, 6.61% 1-butanol, 0.91% heptane, and 90.72% borate buffer (20 mM, pH 9.5).

2.2.4. Preparation of plasma quality control samples

Four quality control samples (QCs), including LLOQ, low L (three times the LLOQ), mid M (mid-range), and high H (high-range) were prepared by spiking the rat plasma with appropriate volumes of individual FTD, TIP, FTY, 5CDU, and UD (IS) working standard solutions.

2.2.5. Plasma samples extraction procedure

Aliquots of the individual drugs working standards were added to 50 μl of rat plasma in a 1.5 ml Eppendorf tubes. Then 50 μl of the IS working solution (1000 ng/ml) was added to each tube followed by addition of 500 μl of Acetonitrile for deproteinization. The samples were completed to 1 ml with water and vortexed then centrifuged for 30 min at 6000 rpm. Then the supernatant layer was injected (in triplicate) into the CE system.

2.2.6. Validation of the analytical method

2.2.6.1. Linearity and LLOQ. Matrix-based calibration curves for the studied drugs were constructed. Four calibration curves were in the concentration range 200–4000 ng/ml and 100–1000 ng/ml for FTD, FTY, 5CDU, and for TIP, respectively. The calibration standards were prepared by spiking the rat plasma with suitable volumes of drugs′ working solutions and 50 μl of the IS working solution. Then the drugs were extracted from the plasma and analyzed. Calibration curves were created using ratios of analyte peak area to IS peak area versus analyte concentrations. From the Least-square linear regression data, the regression equation was derived for each analyte. The lower limit of quantification (LLOQ) is defined as the lowest amount of an analyte that can be quantitatively determined with acceptable precision and accuracy. It should be determined and included in the quantitation range.

2.2.6.2. Selectivity and specificity. A blank rat plasma samples and zero calibration standards (blank samples with IS) were prepared from six different batches of rat plasma and used to assess the selectivity and specificity of the method. The electropherograms of these samples were compared with electropherograms obtained by analyzing rat plasma samples spiked with the analytes and the IS. To confirm selectivity, blank and zero calibration standards should be free of interference at the retention times of the analytes and the IS. Spiked samples should be ±20% LLOQ. The IS response in the blank should not exceed 5% of the average IS response of the calibrators and QCs. Specificity is the ability of the method to assess the analyte in the presence of other components (impurities, degradation products, metabolites, etc.).

2.2.6.3. Sensitivity. The lowest nonzero standard on the calibration curve defines the sensitivity (LLOQ). The analyte response at the LLOQ should be ≥five times the analyte response of the zero calibration standards with accuracy and precision ±20%.

2.2.6.4. Accuracy and precision. Accuracy and precision should be established with four QC levels (LLOQ, L, M, H) and five replicates per QC level over three days. The QCs concentrations covered the full range of the calibration curves. The derived regression equations were applied to calculate concentrations of drugs in QCs. Accuracy and precision were described as % relative error [% Error = (measured − nominal)/nominal × 100], and % relative standard deviation [%RSD = (SD/mean) × 100], respectively.

2.2.6.5. Recovery. Recovery refers to the extraction efficiency of an analytical process, and reported as a percentage. It should not be 100 percent, but should be consistent and reproducible. The recovery of each drug from rat plasma was calculated by comparing the analytical results obtained from extracted samples at L, M, and H QC concentrations versus extracts of blanks spiked with the analytes post extraction (at L, M, and H QC levels).

2.2.6.6. Stability. Stability is a measure of the intactness of an analyte in a particular matrix under specific storage and use conditions. The auto-sampler, bench-top, extract, freeze-thaw, and long-term stability, were examined using L and H QC concentrations of FTD, TIP, FTY, and 5CDU. The peak areas of the studied drugs at different conditions were compared to freshly prepared drugs to determine their stability. The auto-sampler stability was evaluated by keeping the reconstituted plasma samples in the auto-injector at the temperature of 10 °C for 56 h prior to injection. Freeze–thaw stability was assessed using three cycles of freezing plasma samples at −30 °C then thawing at room temperature (25 °C). To evaluate short-term (bench top) and long-term stability, samples were kept at room temperature (25 °C) for 6 h and were left frozen at −30 °C for 30 days before analysis, respectively. The stability was evaluated as % recovery with accuracy ±15% at each QC level.

2.3. Pharmacokinetic study in rats

Ten to twelve-week-old adult male rats weighing 200–250 gm were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were acclimated in our laboratory for 2 days under standard humidity, temperature (25 ± 2 °C), and light (12 h light/12 h dark). Rats were fed with a standard pellet diet and had free access to water. After 2 days of acclimation, the rats were divided into 2 groups each consisting of 4 rats. Rats of one group were injected intraperitoneally with combination of FTD and TIP standard solutions while the rats of the control group were injected with saline solution. The dose is 35 mg/m^2 at ratio of 1:0.5 (FTD: TIP) which was converted to 5.6 mg/kg according to the Meeh′s formula (Gouma et al., 2012). Orbital blood samples were withdrawn from control and treated groups at 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hr after injection, and were transferred into heparin tube. Plasma were separated from blood by centrifugation at 3000 rpm at 4 °C for 10 min, and then stored at −2°C. A quantity of 50 μl of plasma sample was transferred to 1.5 ml Eppendorf tube, and was spiked and analyzed as described above. All animal procedures obeyed the standards set forth in the guidelines for care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2003). The study protocol was approved by the Animal Ethics Committee of Pharmacology Department, College of Pharmacy, King Saud University (Ref. No. KSU-SE-18-18).

3. Results and discussion

3.1. Optimization of electrophoretic conditions

The effect of electrophoretic parameters (buffer pH and ionic strength, surfactant concentration, co-surfactant concentration, organic modifier, capillary, applied voltage, temperature, injection time and pressure, detection wavelength) on resolution of FTD, TIP, FTY, and 5CDU were examined.

3.1.1. Buffer pH

Using buffer in electrophoretic analysis is of great importance as it affects the electrophoretic performance of analytes. The effect of ionization of analytes, pH range, and ionic strength of buffer should be studied (Marina et al., 2005; Li, 1992). Two buffer systems that suitable for microemulsions were chosen; borate buffer and phosphate buffer. Borate buffer showed better results than phosphate buffer and was used in the analysis (Fig. 1S). Change in buffer pH affects the ionization of analytes and internal wall of the capillary, which leads to differences in electrophoretic mobility and electroosmotic flow (Li, 1992). Therefore, borate buffers (20 mM) at different pH (6.5–10.5) were tested. Buffer with pH values $\leq$8.5 lead to incomplete separation of the studied drugs and the IS. Increasing the pH to 9.5 resulted in a complete resolution of the four drugs with the IS (Fig. 2S).

3.1.2. Buffer ionic strength

The resolution of analytes was affected by the buffer's ionic strength. Therefore, borate buffers of different strength (10–100 mM) at pH 9.5 were examined. Overlapped peaks of FTD and its metabolites resulted when using buffers with ionic strength <20 mM. However, higher values of ionic strength increased the resolution with sharp and symmetric peaks. Buffers strength $\geq$30 mM lead to long analysis time (>24 min). The optimum resolution of the studied drugs was reached using 20 mM borate buffer in less than 13 min (Fig. 3S).

3.1.3. Surfactant

Surfactants significantly affect the electrophoretic resolution through changing electroosmotic flow and micelle's charge and size. Increasing surfactant concentrations can either decrease or increase analyte's migration time or resolution depending on specific interaction between them (Altria et al., 2000; Harang et al., 2004). SDS was used commonly as anionic surfactant in MEEKC. The effects of different concentrations (1.66%, 2.66%, and 3.66%) of SDS on the resolution of the drugs were tested. High concentration of SDS extended migration times, therefore, 1.66% was chosen as an optimum concentration (Fig. 4S).

3.1.4. Cosurfactant

The separation selectivity of analytes is affected by the presence of a cosurfactant. In MEEKC, different alcohols have been employed as a cosurfactant (Klampfl, 2003; Hansen et al., 2001; Pompiono et al., 2003). Increasing cosurfactant concentration alters migration times. The viscous alcohol increases the viscosity of the ME which reduces the EOF velocity. Also, electrophoretic mobility reduced as a result of increase in the micelle's size (Altria et al., 2000; Gorski et al., 1996). Three alcohols; 1-butanol, 2-pentanol, and cyclohexanol were chosen as a cosurfactants. They resulted in different migration orders of the studied drugs. 1-butanol gave the best results and was used as the optimum cosurfactant in concentration equals 6.61% (Fig. 5S).

3.1.5. Organic modifier

Generally, hexane, heptane and octane have been used as organic modifier in MEEKC. Octane gave high peak resolution, high efficiency, and high precision (Baker, 1995). Heptane, ethyl acetate, and octane were evaluated as organic modifier in our study. The migration order of FTD, TIP, FTY, 5CDU, and IS was significantly affected by their presence. The best result was obtained when using 0.91% heptane (Fig. 6S).

3.1.6. Capillary

The length and diameter of the capillary have a great influence on electrophoretic resolution of analytes. The selection of smaller internal diameter of capillaries is effective for dissipating heat generated during the analysis. This allows application of high voltage, thereby increasing resolution and shortening analysis time. Increase in the capillary length improves resolution, but prolongs migration time while, a separation performed in a short capillary is more likely to experience a reduction in analysis time and a reduction in efficiency. Thus, we must do a compromise between the optimum time and efficiency of the analysis (Landers, 2008; Xuan et al., 2004). Furthermore, the chemical nature of the capillary's internal surface has a vital effect on the resolution of analytes (Marina et al., 2005). A capillaries of 50, 75, 100 μm i.d., and different effective lengths (35, 55, 75 cm) were chosen to study their effects on resolution of drugs. The use of 100 μm i.d. capillary resulted in overlapped and broad peaks, while the 50 μm i.d. gave sharp peaks. Capillaries of different length and fixed internal diameter (50 μm) were tested. The analysis times were 24 and 12 min, for 75 and 55 cm capillaries, respectively. Thus, the capillary with 55 cm effective length and 50 μm i.d. was used for the analysis of FTD, TIP, FTY and 5CDU.

3.1.7. Applied voltage

The applied voltage largely influences the resolution of analytes in electrophoretic methods. The resolution is proportional to the square root of voltage; hence a fourfold increase in voltage will lead to a twofold increase in resolution (Marina et al., 2005). However, benefits subsequent to high voltage can be lost after exceeding the optimum voltage utilized for analysis due to the generation of Joule heat which leads to peak broadening and low efficiency. Efficient heat dissipation is essential to retain the efficiency. Narrow-diameter and longer capillaries, with effective cooling systems will minimize the generation of joule heat and are favorable when applying high voltage. A voltage of 10–30 kV was tested. Higher voltage led to higher efficiencies and shorter migration times however, applying voltages >20 kV led to production of Joule heat and broadening of peaks. Decreasing the voltage to 10 kV resulted in overlapped FTD and FTY peaks and elongated analysis time (25 min). Efficient and complete resolution was obtained with 20 kV voltage within 13 min.

3.1.8. Temperature

The repeatability of the results of electrophoretic separation depends on maintaining a fixed temperature. Higher BGE temperature led to low viscosity and high electroosmotic flow. Consequently, the migration time and the separation efficiency were reduced. Therefore, effective controlling of temperature inside the capillary is of great importance in electrophoretic separation (Knox and McCormack, 1994). Temperatures from 10 to 40 °C were studied. At temperature higher than 30 °C, the migration time decreased with overlapped peaks. Temperatures lower than 20 °C resulted in increased viscosity of the BGE and subsequent late and broad peaks. The temperature of 20 °C was selected as optimum for the analysis.

3.1.9. Injection time and applied pressure

Sample injection time (10–90 s) at pressure (10–50 mbar) were tried. Injection time starting from 10 to 40 s increased the response of all drugs. Increased time (>40 s) resulted in broad peaks. The best results were obtained with injection time of 40 s at 20 mbar pressure.

3.1.10. Detection wavelength

Solutions containing FTD, TIP, FTY and 5CDU were measured at different wavelengths (200–300 nm). Based on the absorption spectra, all drugs showed strong UV absorption at 205 nm. There-

fore, it was selected for detection of the studied drugs simultaneously.

Under the above optimized conditions, a complete resolution of the studied drugs was achieved. The migration times for FTD, and its metabolite (FTY, 5CDU), TIP, and IS were 6.34 ± 0.21, 7.68 ± 0.44, 8.48 ± 0.53, 10.40 ± 0.30, and 12.44 ± 0.30, respectively (Fig. 1).

3.2. Validation of the analytical method

The method was validated according to the FDA guidelines on Bioanalytical Method Validation (Bioanalytical Method Validation, 2018). This includes linearity and LLOQ, selectivity, sensitivity, accuracy and precision, recovery, and stability

3.2.1. Linearity and LLOQ

Calibration curves were found to be linear over the concentration ranges of 200–4000 ng/ml for FTD, FTY, 5CDU, and 100–1000 ng/ml for TIP. Linearity and regression parameters are presented in Table 1. The high determination coefficient values (r^2) indicated excellent linearity. The lowest amount that can be quantitatively determined (LLOQ) was 200 ng/ml for FTD, FTY, 5CDU, and 100 ng/ml for TIP with acceptable accuracy and precision.

3.2.2. Selectivity and specificity

The selectivity and specificity of the method was tested by analyzing blank rat plasma samples and zero calibration standards. The electropherograms of these samples were compared with elec-

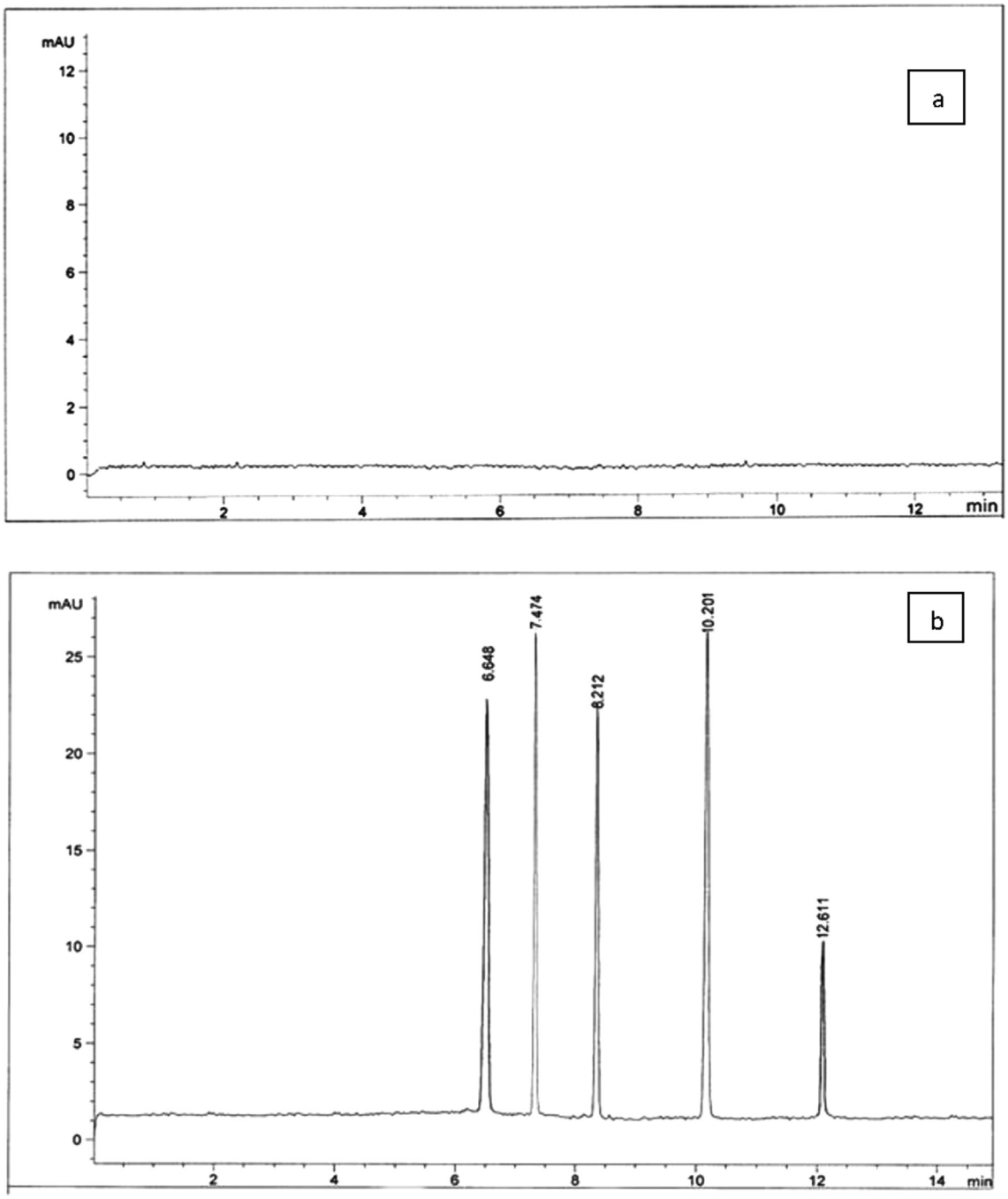

Fig. 1. MEEKC electropherogram of (a) blank rat plasma and (b) rat plasma spiked with standard mixture of FTD (2000 ng/ml), FTY (2000 ng/ml), 5CDU (2000 ng/ml), TIP (1000 ng/ml), and IS (1000 ng/ml).

Table 1
Regression parameters for the determination of FTD, FTY, 5CDU, and TIP, by the developed MEEKC method.

	FTD	FTY	5CDU	TIP
Range (ng/ml)[a]	200–4000	200–4000	200–4000	100–1000
Intercept	−0.0057	0.0178	0.0959	0.0025
Slope	0.0011	0.0024	0.0033	0.0050
Determination coefficient (r^2)	0.9993	0.9989	0.9993	0.9987
LLOQ (ng/ml)[b]	200	200	200	100

[a] Mean of three measurements.
[b] LLOQ lower limit of quantification ≥five times the response of the zero calibrator.

tropherograms of spiked rat plasma samples. No interferences were detected at the migration times of the drugs and IS. In addition, the purity of the peaks was assessed by using DAD to ensure that no related compounds (impurities, degradation products, metabolites, etc.) were co-eluted with the drugs (Fig. 1).

3.2.3. Sensitivity

The lowest non-zero calibration standard (LLOQ) that can be quantitatively determined and defined the sensitivity of the method were 200 ng/ml for FTD, FTY, 5CDU, and 100 ng/ml for TIP with accuracy and precision less than 4% (Fig. 2).

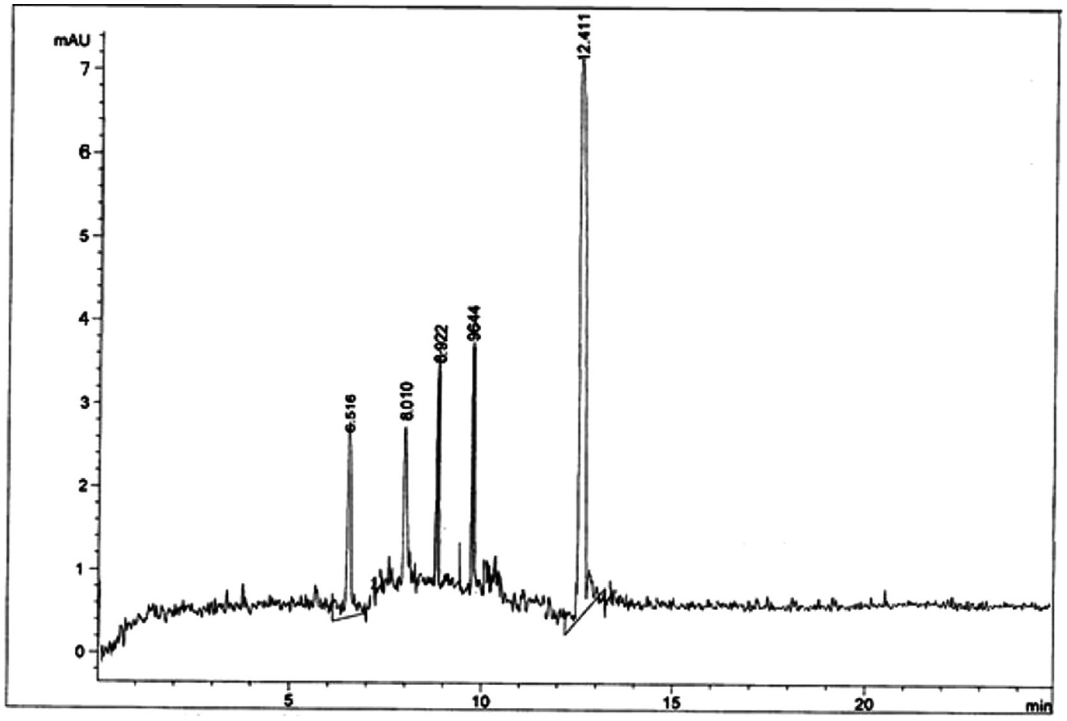

Fig. 2. MEEKC electropherogram of rat plasma sample spiked with standard mixture of FTD, FTY, 5CDU, and TIP at LLOQ level with IS (1000 ng/ml).

Table 2
Intra- and inter-day accuracy and precision for FTD, FTY, 5CDU, and TIP in rat plasma.

		Nominal conc. (ng/ml)	% Recovery	% RSD	% Error
FTD	Intra-day[a]	200	96.17	1.59	−3.83
		600	98.89	0.76	−1.11
		2000	99.32	0.86	−0.68
		4000	99.47	0.78	−0.53
	Inter-day[b]	200	96.00	1.88	−4.00
		600	98.83	0.85	−1.16
		2000	99.18	0.74	−0.82
		4000	99.44	0.32	−0.56
FTY	Intra-day[a]	200	99.50	0.50	−0.50
		600	99.67	0.29	−0.33
		2000	99.72	0.13	−0.28
		4000	99.93	0.12	−0.07
	Inter-day[b]	200	99.33	0.77	−0.67
		600	99.61	0.35	−0.39
		2000	99.70	0.15	−0.30
		4000	99.92	0.10	−0.08

Table 2 (*continued*)

		Nominal conc. (ng/ml)	% Recovery	% RSD	% Error
5CDU	Intra-day[a]	200	99.34	0.77	−0.67
		600	99.67	0.29	−0.33
		2000	99.77	0.21	−0.23
		4000	99.97	0.06	−0.03
	Inter-day[b]	200	99.50	0.50	−0.50
		600	99.61	0.35	−0.39
		2000	99.78	0.19	−0.22
		4000	99.95	0.05	−0.05
TIP	Intra-day[a]	100	99.83	0.29	−0.17
		300	99.83	0.29	−0.17
		500	99.85	0.15	−0.15
		1000	99.99	0.52	−0.01
	Inter-day[b]	100	99.67	0.29	−0.33
		300	99.72	0.46	−0.28
		500	99.83	0.35	−0.17
		1000	99.97	0.42	−0.03

[a] Mean of five measurements over 1 day.
[b] Mean of five measurements over 3 days.

3.2.4. Accuracy and precision

Accuracy and precision are assessed as percentage error (%E) and relative standard deviation (%RSD), respectively. Four quality control samples (200, 600, 2000, 4000 ng/ml for FTD, FTY, and 5CDU) and (100, 300, 500, 1000 ng/ml for TIP) were assayed in five replicates over one day (intra-day) and in five replicates over three days (inter-day) for each drug. The %E and %RSD for intra-and inter-day accuracy and precision were found to be ≤4% for all drugs, indicating that the method is accurate and precise (Table 2).

Table 3
Recovery of FTD, FTY, 5CDU, and TIP from rat plasma.

	Nominal conc. (ng/ml)	Mean recovery (%) ± SD[a]	%RSD
FTD	600	88.66 ± 4.44	5.01
	2000	92.25 ± 2.11	2.29
	4000	91.53 ± 2.04	2.23
FTY	600	86.50 ± 6.21	7.18
	2000	90.35 ± 4.35	4.81
	4000	92.58 ± 3.00	3.24
5CDU	600	89.17 ± 3.44	3.86
	2000	91.30 ± 2.47	2.71
	4000	92.78 ± 2.15	2.32
TIP	300	90.00 ± 3.89	4.32
	500	92.83 ± 3.78	4.07
	1000	92.25 ± 3.54	3.84

[a] Mean of three measurements.

3.2.5. Recovery

Table 3 presents the % recoveries of FTD, TIP, FTY, and 5CDU after extraction from rat′s plasma. Recovery mean values of 90.81 for FTD, 89.81 for FTY, 91.08 for 5CDU and 91.69 for TIP indicated a high degree of extraction recovery of the applied method for all analytes.

3.2.6. Stability

The stabilities of FTD, TIP, FTY and 5CDU with IS were evaluated in rat plasma at different conditions; auto-sampler stability (10 °C, 56 hr), bench-top stability (25 °C, 6 hr), extract stability (10 °C, 48 hr), freeze–thaw stability (−30 °C, 3 cycles), and long-term stability (−30 °C, 30 days). The resultant peak areas were compared to peak areas of the drugs that freshly prepared. Table 4 showed that all drugs were stable under the studied conditions with accepted mean % recovery.

3.3. Application to pharmacokinetic studies

The developed MEEKC method was applied to study the pharmacokinetic behavior of FTD and TIP in rats after I.P. administration. This was accomplished by analyzing rat plasma samples taken at different time intervals following drugs administration. Fig. 3 showed that there were no peaks observed in plasma samples collected from control rats similar to those obtained when

Table 4
Stability of FTD, FTY, 5CDU, and TIP in rat plasma under different storage and use conditions.

	Mean recovery (%) ± RSD [a]					
	Nominal conc. (ng/ml)	Auto-sampler stability (10 °C, 56 hr)[b]	Bench-top stability (25 °C, 6 hr)[c]	Extract stability (10 °C, 48 hr)[d]	Freeze–thaw stability (−30 °C, 3 cycles)[e]	Long-term stability (−30 °C, 30 days)[f]
FTD	600	98.20 ± 0.31	99.21 ± 0.22	95.66 ± 2.65	94.77 ± 0.43	90.82 ± 9.34
	4000	98.98 ± 0.11	100.00 ± 0.14	97.11 ± 0.54	94.99 ± 2.98	90.50 ± 7.55
FTY	600	98.89 ± 0.54	100.00 ± 6.43	97.00 ± 1.54	94.00 ± 6.83	90.65 ± 7.32
	4000	99.04 ± 0.25	100.00 ± 4.21	96.76 ± 4.75	93.97 ± 5.08	92.55 ± 4.99
5CDU	600	99.43 ± 0.34	99.52 ± 1.11	97.86 ± 2.11	93.12 ± 7.61	92.11 ± 7.34
	4000	99.00 ± 1.21	99.87 ± 1.41	97.69 ± 1.56	92.69 ± 1.43	91.04 ± 5.21
TIP	300	98.76 ± 0.21	100.00 ± 2.15	96.87 ± 0.43	94.39 ± 1.22	88.43 ± 3.54
	1000	99.21 ± 0.34	99.96 ± 0.33	96.00 ± 6.05	95.32 ± 2.13	91.06 ± 8.54

[a] Mean of three measurements.
[b] Assess stability of extracts in the autosampler only if the autosampler storage conditions are different.
[c] Assess stability of samples under the laboratory handling conditions that are expected for the study samples.
[d] Assess the stability of processed samples, including the residence time in the autosampler.
[e] Assess the stability of the sample after a minimum of three freeze-thaw cycles.
[f] Assesses the long-term stability of the sample over a period of time equal to or exceeding the time between the date of first sample collection and the date of last sample analysis.

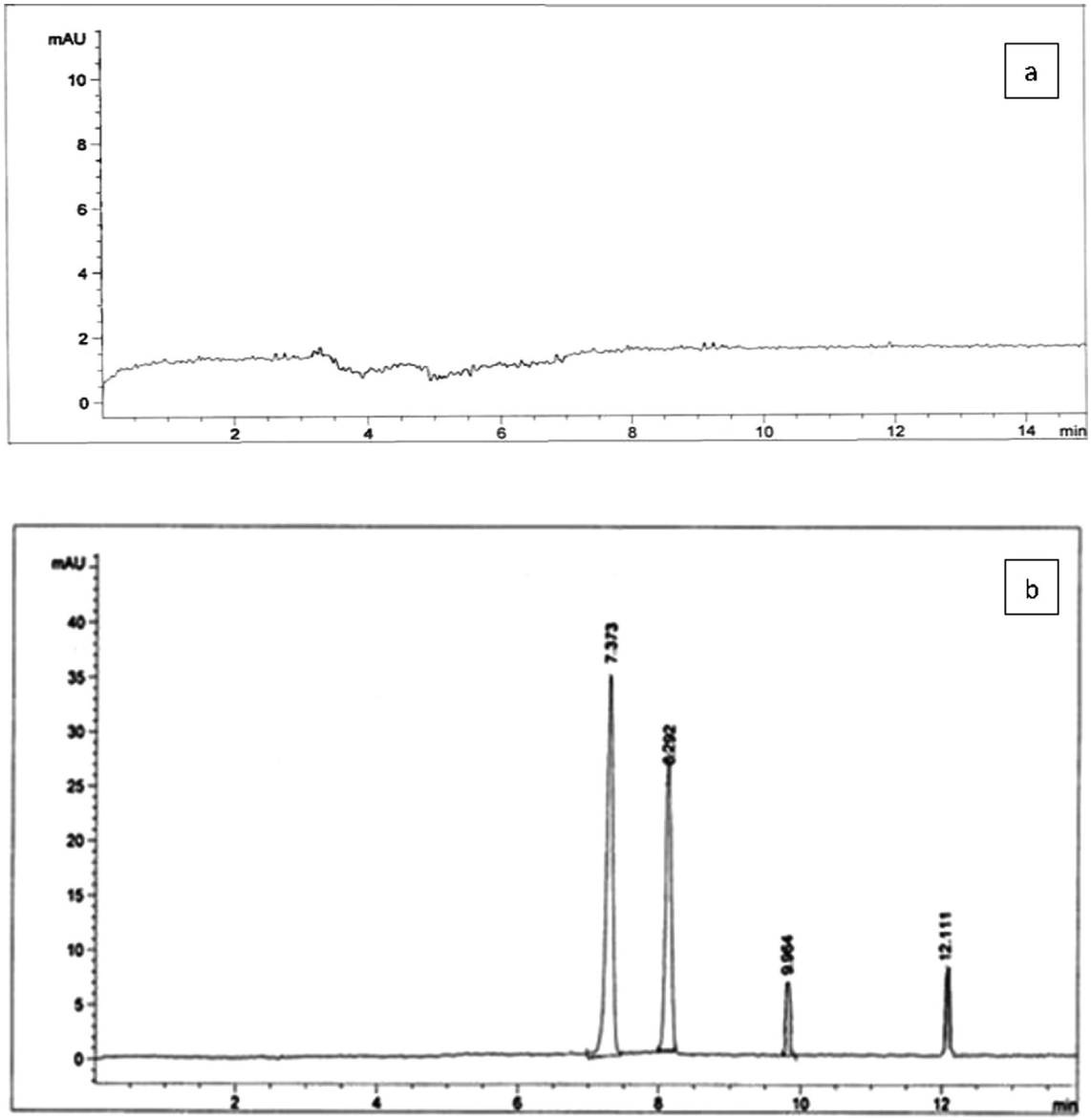

Fig. 3. MEEKC electropherogram of (a) blank rat plasma and (b) rat plasma samples taken 1 h after intraperitoneal administration of combination of FTD and TIP standard solutions to rats (5.6 mg/kg).

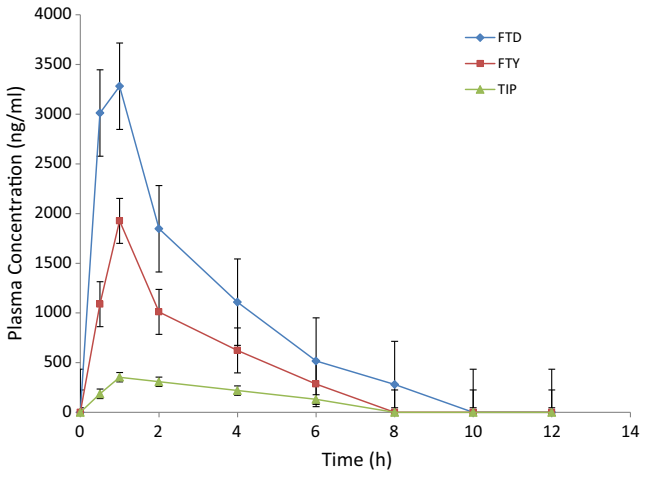

Fig. 4. Concentration–time profiles of FTD and its metabolite FTY and TIP after intraperitoneal administration of combination of FTD and TIP standard solutions to rats (5.6 mg/kg). Each point represents mean of 4 rats ± SD.

Table 5
Pharmacokinetic parameters of FTD and its metabolite FTY, and TIP in rat plasma after intraperitoneal injection of combination of FTD and TIP standard solutions (5.6 mg/kg).

Pharmacokinetics parameters	FTD[g]	FTY[g]	TIP[g]
$AUC_{0\text{-}24}$ (ng h/ml)[a]	7579.3 ± 1092.9	3887.2 ± 811.8	932.7 ± 118.3
$AUC_{0\text{-}\infty}$ (ng h/ml)[b]	7579.3 ± 1092.9	3887.2 ± 811.8	932.7 ± 118.3
C_{max} (ng/ml)[c]	3228.0 ± 1071.7	1936.0 ± 65.2	347.3 ± 57.6
T_{max} (h)[d]	0.8 ± 0.3	1 ± 0	1.7 ± 0.6
$t_{1/2}$ (h)[e]	1.25 ± 0.44	1.04 ± 0.01	1.73 ± 0.09
Ke (1/h)[f]	0.55 ± 0.21	0.40 ± 0.01	0.66 ± 0.03

[a] Area under the curve up to the last sampling time.
[b] Area under the curve extrapolated to infinity.
[c] Maximum plasma concentration.
[d] Time taken to reach the maximum plasma concentration.
[e] Half-life.
[f] Elimination rate constant.
[g] Mean of 4 rats ± SD.

analyzing plasma samples collected from treated rats. The mean plasma concentration–time curves for FTD and its metabolite FTY, and TIP are constructed (Fig. 4). The 5CDU metabolite of FTD (minor metabolite 15%) was not detected. The pharmacokinetic parameters were calculated from the curve. The mean value of Tmax and Cmax for FTD with its metabolite FTY, and TIP were 0.8 ± 0.3 h and 3228.0 ± 1071.7 ng/ml, 1 ± 0 h and 1936.0 ± 65.2 ng/ml, and 1.7 ± 0.6 h and 347.3 ± 57.6 ng/ml, respectively (Table 5).

4. Conclusion

A MEEKC-DAD method for simultaneous quantification of FTD, and its metabolites FTY and 5CDU, and TIP in rat plasma has been successfully developed and validated. The results showed that this developed method is simple, rapid, sensitive, and selective. It also demonstrated good accuracy and precision and was fully validated based on the FDA guidelines. After being validated, the method was applied for the determination of the studied drugs in plasma samples collected from rats injected intraperitoneally with a combination of FTD and TIP. The results obtained were used to study the pharmacokinetics of the drugs in rats.

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Declaration of competing interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2019.09.004.

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