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ORIGINAL RESEARCH PAPER

Development and validation of a rapid UHPLC-MS/MS method for the determination of fenofibric acid in human plasma: Application to a pharmacokinetic study of fenofibrate tablet in Chinese subjects

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ABSTRACT

An ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) method was developed to determine the fenofibric acid (FA) in human plasma and applied to a pharmacokinetic study of fenofibrate tablet (Lipanthyl® supra, 160 mg) on Chinese subjects which had not been reported. Bezafibrate was used as an internal standard (IS), and the plasma samples were precipitated by methanol. Multiple reaction monitoring (MRM) mode was used to quantitatively analyzed FA *m/z* 317.2 → 230.7 and the IS *m/z* 360.0 → 274.0 in the electrospray ionization (ESI) negative interface. The calibration curves were linear over the range of 50–30,000 ng/mL ($r^2 \geq 0.996$). The intra-day and inter-day precision (coefficient of variation, CV%) was less than 2.7 and 2.5%, respectively. The accuracy (relative error, RE%) ranged from −4.5 to 6.9%. The average recovery was higher than 86.2%, and the matrix effect was between 95.32 and 110.55%. The simple, rapid, and selectivity method was successfully applied to the pharmacokinetic study of fenofibrate tablets on Chinese subjects.

KEYWORDS

UHPLC-MS/MS, fenofibrate, fenofibric acid, pharmacokinetic study

INTRODUCTION

Fenofibrate is a lipid-regulating agent that is widely used in the treatment of primary hypercholesterolemia or mixed dyslipidemia. It can reduce the levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglycerides (TG), and apolipoprotein (apo) B, and increase the level of high-density lipoprotein cholesterol (HDL-C) and apo A. Recent bench studies have demonstrated an array of cardiovascular and renal pleiotropic beneficial activities of fenofibrate, besides its foremost lipid-lowering action [1, 2]. There are many available formulations of fenofibrate on the market, such as micronized capsules and tablets, nanocrystal tablets, and fenofibric acid (FA) capsules (delayed-release) [3–5]. The pharmacokinetic studies of these preparations have been conducted in many countries, however, the pharmacokinetic studies of fenofibrate tablets (Lipanthyl® supra, 160 mg) in Chinese subjects have not been reported.

Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) is an excellent method for the selective and sensitive determination of small molecular weight compounds in biological samples [6, 7]. Fenofibrate is a prodrug that is hydrolyzed

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immediately after absorption by tissue and plasma esterases to its active major metabolite, FA. No unchanged fenofibrate is found in human plasma [8]. Therefore, FA was the detected drug in many pharmacokinetic studies of fenofibrate after oral administration [9–11]. The lower quantification limit of the FA in plasma could be quantified to 5 ng/mL by LC-MS/MS with a lipid–lipid extraction method [12–14]. In this study, plasma samples were processed by protein precipitation to shorten the sample processing time. Although the lower limit of quantification (50 ng/mL) of this method was not the lowest, it was sufficient for pharmacokinetic analysis.

EXPERIMENTAL

Chemicals and reagents

Fenofibric acid (Purity: 99.18%, Lot: LF80N35) was purchased from Bailingwei Technology Co., Ltd. (Peking, China). Bezafibrate (purity: 99.9%, Lot: 100732–201602; internal standard, IS) was obtained from China Food and Drug Institute (Peking, China). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA, USA), formic acid was from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). High-pure water was prepared by an ultrapure water system from Heal Force Biological and Medical Scientific Co., Ltd. (Hongkong, China). Blank human plasma was from healthy donors, following the local ethics guidelines.

Analytical instrumentation

The UHPLC-MS/MS system was performed using a Nexera X2 UHPLC system (Shimadzu, Japan) coupled with a QTRAP® 4500 triple-quadrupole mass spectrometer (AB MDS Sciex, USA). The mass spectrometer was equipped with a turbo electrospray ionization (ESI) source for mass analysis and detection. Analyst 1.6.2 software (AB MDS Sciex) was used for data acquisition.

Liquid chromatographic conditions

Chromatographic separation was achieved on An Agilent Zorbax SB-C18 column (2.1 × 100 mm I.D.,3.5 μm)connected with a Phenomenex C18 pre-column (4 × 3.0 mm I.D.,5 μm). The column temperature was maintained at 35 °C. The mobile phase consisted of acetonitrile and 0.1% formic acid (58:42, v/v) at a flow rate of 0.4 mL/min and the injection volume was 5.0 μL.

Mass spectrometric conditions

The mass spectrometer was operated using ESI source in negative ion mode. Quantitation was performed with multiple reaction monitoring (MRM) mode. The mass transition of FA and bezafibrate were *m/z* 317.2→230.7 and *m/z* 360.0→274.0, respectively. The ionspray voltage was set at 4,500 V. Nitrogen was used as nebulizer gas (GAS1), auxiliary air (GAS2), and curtain gas (CUR), and the pressure values were set at 50, 50, and 25 psi, respectively. The source temperature was 450 °C. The pattern of the Collision Gas Collisionally Activated Dissociation (CAD) was medium. The declustering potential (DP) of FA and IS was optimized at −55 and −70 V. The collision energy (CE) was set at −20 V for FA and −24 V for IS.

Preparation of standard work solutions

The stock solutions of FA (1 mg/mL) and IS (1 mg/mL) were prepared with methanol. Subsequently, the stock solutions were diluted in methanol–water at a ratio of 50:50 (v/v) to get a series of standard work solutions (500–300,000 ng/mL) and IS work solution (10.0 μg/mL). All the stock and work solutions were stored at −20 °C.

Preparation of standard curve

Standard plasma samples were prepared by spiking 10 μL of the serial standard working solutions into 100 μL of human blank plasma to prepare samples with concentrations of 50, 100, 500, 1,000, 5,000, 10,000, 30,000 ng/mL. Similarly, Quality control (QC) samples were prepared at the concentration of 100, 1,000, 22,500 ng/mL as low, medium, and high level, respectively.

Sample preparation

One hundred microliter plasma sample was mixed with 10 μL of IS work solution in a 2.0 mL tube. Then, 300 μL methanol was added as protein precipitation reagent. After vortex for 2 min, the sample was centrifuged at 10,000 rpm for 10 min. Twenty microliter of supernatant was diluted by 380 μL mobile phase before testing.

Method validation

The method was validated for selectivity, linearity (calibration curve), precision and accuracy, recovery and matrix effect, stability according to the guidelines of the US Food and Drug Administration (FDA) and National Medical Products Administration (NMPA) for the biological sample analysis [15, 16].

Selectivity was evaluated by analyzing the chromatograms of the blank plasma samples from six different sources with those of lower limit of quantification (LLOQ) samples and plasma samples after administration.

Linearity was constructed using a $1/x^2$ weighted linear regression of the peak area ratios (FA/IS) vs the plasma concentration ratios (FA/IS). The LLOQ was defined as the lowest concentration on the calibration curve.

The intra-day and inter-day precision and accuracy were assessed by determining the 6 replicates of QC samples at a low, medium, and high level in 3 different validation days. The precision was expressed as a variable coefficient (CV%) and the accuracy as the relative error (RE%).

The recovery of FA was calculated by comparing the peak areas of extracted QC samples at a low, medium, and high level with those of corresponding unextracted QC samples (spiked QC work solutions to the supernatant of the blank plasma after protein precipitation).

Matrix effect (normal, hemolytic, and high-fat plasma) was determined by comparing the peak areas of blank

plasma extracts spiked with the analytes at low, medium, and high level with the pure standard solutions at equivalent concentrations. Hemolytic plasma was prepared by adding 20 µL of whole blood frozen in the refrigerator at −70 °C for 30 min to 980 µL of blank plasma. High-fat plasma was obtained by taking blood from a subject 2 h after eating a high-fat meal (~1,000 kcal, ~50% from fat).

The stability of FA in plasma was assessed by analyzing the QC samples at low, medium, high level under different storage conditions: short-term stability at ambient temperature for 6.0 h, processed sample stability in autosampler (15 °C) for 24 h, three freeze–thaw stability (−70–25 °C), long-term stability stored at −70 °C for 74 days.

Pharmacokinetic study

Twenty four Chinese subjects (including male and female) were participated in a pharmacokinetic study of fenofibrate tablets (Lipanthyl® supra, 160 mg) in Chinese subjects under fasting and fed conditions. The protocol was approved by the medical ethics committee of Bethune International Peace Hospital (Shijia Zhuang, China) and was performed in the phase I clinical center of Bethune International Peace Hospital in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines. All the volunteers were provided written informed consent before the screening.

A total of 15 blood samples were collected under both fasting and fed conditions including a baseline sample predose and those taken at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0, 24.0, 36.0, 48.0, 72.0, 96.0 h post-dose. The blood samples were centrifuged at 4,000 rpm at 2–8 °C for 15 min. The supernatant was transferred and stored at a −70 °C until analysis.

RESULTS AND DISCUSSION

Method optimization

According to the previous research, FA was analyzed by ESI source in the positive or negative mode. In this study, the signal intensity of FA in the negative mode was higher and the noise response was lower compared with the positive mode. The precursor ion of FA and IS in negative ESI was *m/z* 317.2 and *m/z* 360.0 in the full-scale spectrum. Optimization of the CE value from −5 to −35 V, the most abundant product ions of FA and IS were *m/z* 230.7 and *m/z* 274.0, respectively. Fig. 1 showed the product ion spectra of $[M-H]^-$ ions from FA and IS. The values of DP and CE were optimized in MRM mode. The best DP values of FA and IS were −55 and −70 V. CE values were optimized at −20 V (FA) and −24 V (IS).

In the optimization of LC conditions, 0.1% formic acid was added to the aqueous phase to improve sensitivity and reproducibility. Acetonitrile was used as an organic phase for its good elution ability. The ratios of the acetonitrile–water (0.1% formic acid) were investigated at 80/20, 75/20, 65/35, 60/40, 58/42, 55/45 (v/v). The optimal ratio

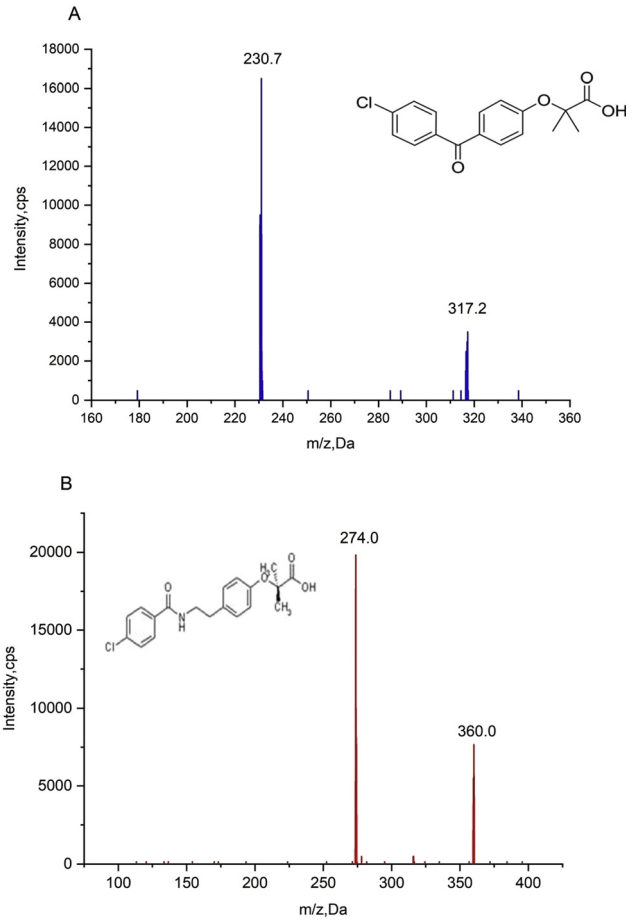

Fig. 1. Product ion spectra of $[M-H]^-$ ions from fenofibric acid (A) and IS (B)

(58/42, v/v) was preferable to realize suitable retention time and good peak shape.

METHOD VALIDATION

The typical MRM chromatograms of blank human plasma, blank plasma sample spiked with FA at the LLOQ (50 ng/mL) and IS (10.0 µg/mL), and a plasma sample obtained from a subject at 4.0 h after administration of fenofibrate tablet were shown in Fig. 2. No significant interference from endogenous substances was observed at the retention times of the FA and IS, demonstrating the good selectivity of the method.

The calibration curves showed good linear over the range of 50–30,000 ng/mL of FA in plasma by 7 levels of calibration standards. A typical equation of calibration curve was $y = 14.5x - 0.00352$ ($r = 0.9994$). RE% of all the standards were within ±15%. The LLOQ was 50 ng/mL in three validated batches in which the precision was less than 2.7%, and the accuracy was −4.7–8.4%.

As shown in Table 1, the intra-day and inter-day precision (CV%) was less than 2.7 and 2.5%, respectively. The accuracy (RE%) ranged from −4.5 to 6.9%. The mean recovery of FA was between 86.2 and 88.2%, which conformed to the requirement of analysis. The matrix effect of the FA

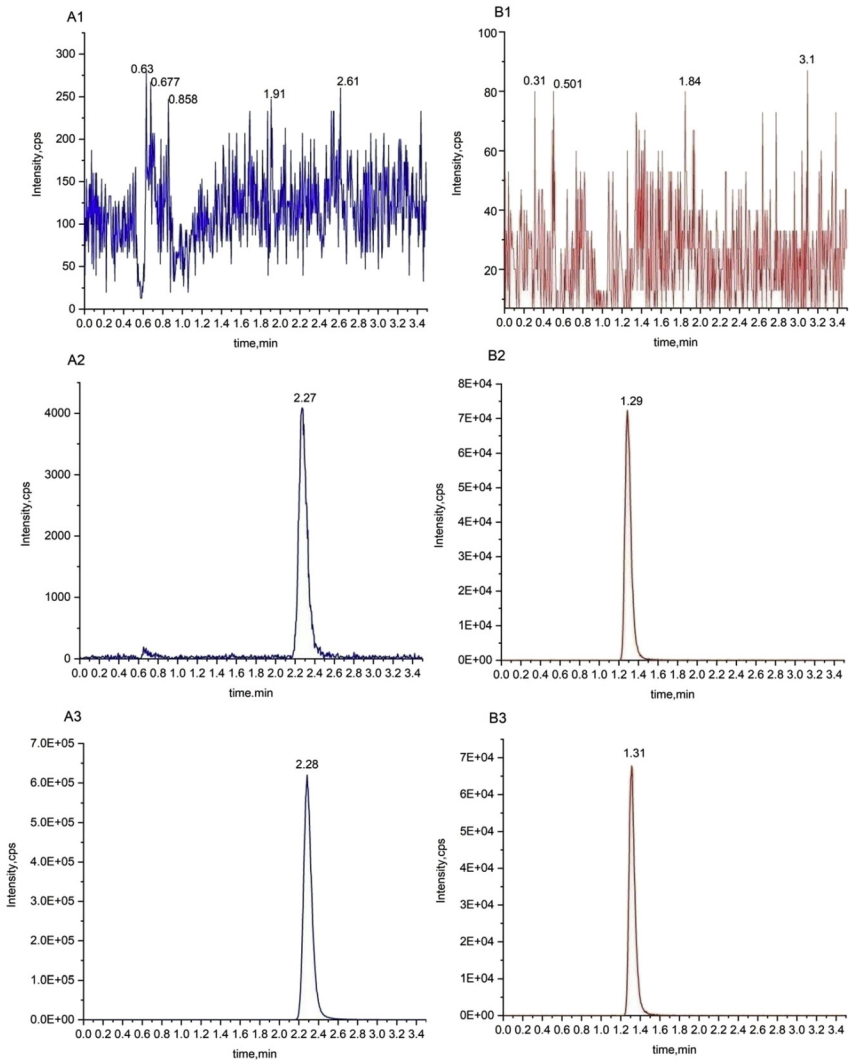

Fig. 2. Typical MRM chromatograms of fenofibric acid (A) and IS (B) in human plasma: (1) blank human plasma; (2) blank plasma sample spiked with fenofibric acid at the LLOQ (50 ng/mL) and IS (10.0 μg/mL); (3)plasma sample obtained from a subject at 4.0 h after administration of a fenofibrate tablet (Lipanthyl® supra, 160 mg)

Table 1. Intra- and inter-day precision and accuracy, recovery and matrix effect of fenofibric acid in human plasma

Concentration (ng/mL)	Accuracy (RE%)		Precision (CV%)		Recovery	Matrix effect		
	Intra-day	Inter-day	Intra-day	Inter-day		Normal plasma	Hemolytic plasma	High-fat plasma
100	6.9	6.9	1.8	2.1	86.2	104.65	110.55	96.81
1,000	0.5	1.4	1.8	1.8	86.4	98.03	102.00	95.32
22,500	−4.1	−4.5	2.7	2.5	88.2	98.69	103.96	96.85

ranged from 95.32 to 110.55% in normal, hemolytic, and high-fat plasma which indicated the matrix effect under different physiological states was negligible.

The stability of FA in human plasma was investigated under different conditions in which the samples may be processed or stored: at room temperature for 6.0 h, auto-sampler (15 °C) for 24 h, −70 °C for 74 days and three freeze–thaw cycles. The results summarized in Table 2 showed that the variation of FA under different conditions was between −6.2 and 10.2% and the CV was less than 3.3%.

Application to a pharmacokinetic study

The validated method was successfully applied to a pharmacokinetic study on Chinese subjects to assay the FA in plasma after oral administration of a fenofibrate tablet (Lipanthyl® supra, 160 mg) under fasting and fed condition. The mean mean plasma concentration-time curve of FA was shown in Fig. 3. The pharmacokinetic (PK) parameters were calculated by Phoenix WinNonlin version 8.0 (Certara USA. Inc, United States) with a non-compartmental model. The

Table 2. Stability of fenofibric acid in human plasma

Storage conditions	Concentration (ng/mL)	Accuracy (RE%)	Precision (CV%)
Bench-top stability for 6.0 h	100	6.8	2.6
	1,000	−1.6	5.1
	22,500	4.2	2.2
15 °C in autosampler for 24 h	100	2.7	3.3
	1,000	−3.2	1.3
	22,500	−4.8	3.0
Three freeze–thaw cycles	100	10.2	0.8
	1,000	5.0	3.3
	22,500	1.5	1.9
−70 °C for 74 days	100	−1.9	2.3
	1,000	−6.2	1.8
	22,500	−4.2	1.4

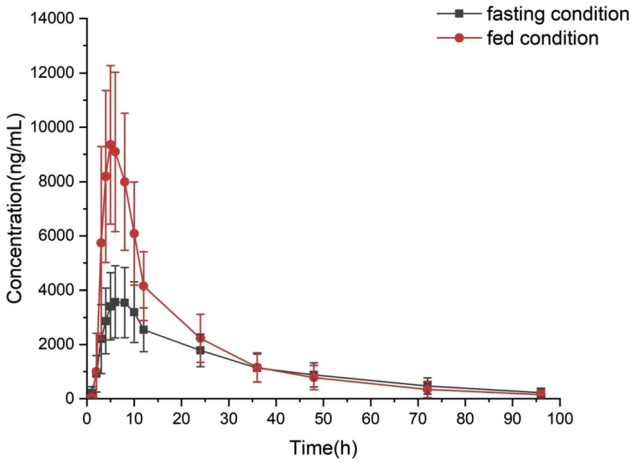

Fig. 3. Mean plasma concentration-time profiles of fenofibric acid in human plasma after oral administration of a fenofibrate tablet (Lipanthyl® supra, 160 mg) on Chinese subjects under fasting and fed conditions

results were listed in Table 3. The C_{max} and area under the curve (*AUC)* of FA under fed condition were significantly higher than those of the fasting group, suggesting a positive food effect on the absorption of fenofibrate. Guivarc'h et al. [17] conducted six single-dose clinical Studies, 7 male subjects were recruited into a bioavailability of fenofibrate tablet (Lipidil Supra®, 160 mg, Canada). The C_{max} and $AUC_{0-\infty}$ under fasting and fed condition were 2667.4 vs 8657.2 ng/mL, 113681.1 vs 158099.2 h ng/mL. Their results were slightly different from ours, due to different pharmaceutical preparations, different races, genders, and numbers of subjects, but the conclusion was similar, that was, the absorption of fenofibrate under high-fat meal conditions was higher than that under fasting condition.

There are also some studies on the pharmacokinetics or bioequivalence of fenofibrate tablets with different preparation methods and dosages in human subjects [10, 14, 18, 19]. The results form Sauron et al. [18] showed that food had no effect on the C_{max} and AUC of a 145-mg fenofibrate nanoparticle tablet formulation. Park et al. [19] compared the pharmacokinetics and bioequivalence of a new 135-mg choline fenofibrate tablet and its original capsule. They found that the t_{max} of both formulations was significantly prolonged under fed condition compared to fasting condition, and all other PK parameters were comparable between the fed and the fasting studies. Considering the potential disadvantages of a high-fat diet for patients with hypertriglyceridemia, fenofibrate preparations that can be taken without food are more favorable and have been marketed in some countries, but not in China [3]. The analytical method we have established can be used in future pharmacokinetic studies of these preparations in Chinese subjects.

CONCLUSION

A simple, rapid, and selective UHPLC-MS/MS method was developed and validated to determine the concentration of FA in human plasma. The protein precipitation extraction procedure can shorten the processing time of blood samples, thereby improving the efficiency of processing a large number of biological samples. The method was successfully applied to a pharmacokinetic study of fenofibrate tablets in Chinese subjects which have not been reported. The results

Table 3. Pharmacokinetic parameters of fenofibric acid in human plasma after oral administration

PK parameters	Fasting condition (Mean ± SD)	Fed condition (Mean ± SD)
C_{max} (ng/mL)	3786.383 ± 1281.224	10369.279 ± 2736.320
AUC_{0-96h} (h·ng/mL)	112752.302 ± 42100.595	160362.351 ± 54444.816
$AUC_{0-\infty}$(h·ng/mL)	121998.067 ± 48076.863	167377.562 ± 60036.723
T_{max} (h)	5.564 ± 2.106	3.669 ± 1.166
$T_{1/2}$ (h)	24.072 ± 6.483	19.380 ± 5.601
λ_z (h^{-1})	0.031 ± 0.011	0.038 ± 0.010

may be useful in further development of fenofibrate formulations for the Chinese market.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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REFERENCES

1. Jadwiga, N. *Clin. Ther.* **2002**, *24*, 2022–50.
2. Balakumar, P.; Sambathkumar, R.; Mahadevan, N.; Muhsinah, A. B.; Alsayari, A.; Venkateswaramurthy, N.; Dhanaraj, S. A. *Pharmacol. Res.* **2019**, *144*, 132–41.
3. Ling, H.; Luoma, J. T.; Hilleman, D. *Cardiol. Res.* **2013**, *4*, 47–55.
4. Xu, H.; Shi, Y.; Vela, S.; Marroum, P.; Gao, P. *J. Pharm. Sci.* **2018**, *107*, 476–87.
5. Verbeeck, R. K.; De Niet, S.; Lebrun, S.; Tremege, M.; Rennie, T. W.; Coffiner, M.; Streel, B.; Cahay, B. *J. Pharm. Pharm. Sci.* **2015**, *18*, 61–7.
6. Wei, X.; Li, P.; Liu, M.; Du, Y.; Wang, M.; Zhang, J.; Wang, J.; Liu, H.; Liu, X. *Biomed. Chromatogr.* **2017**, *31*, .
7. Salvagno, G. L.; Danese, E.; Lippi, G. *Clin. Biochem.* **2017**, *50*, 582–6.
8. Sharp, M.; Ormrod, D.; Jarvis, B. *Am. J. Cardiovasc. Drugs* **2002**, *2*, 125–32.
9. Li, G.; Zhao, M.; Qiu, F.; Sun,Y.; Zhao, L. *Drug Des. Devel. Ther.* **2018**, *13*, 129–39.
10. Chachad, S. S.; Gole, M.; Malhotra, G.; Naidu, R. *Clin. Ther.* **2014**, *36*, 967–73.
11. Vlase, L.; Popa, A.; Muntean, D.; Leucuta, S. E. *Arzneimittelforschung* **2010**, *60*, 560–3.
12. Li, G.; Yang, F.; Liu, M.; Su, X.; Zhao, M.; Zhao, L. *Biomed. Chromatogr.* **2016**, *30*, 1075–82.
13. Wang, G.; Guo, J.; Meng, F.; Song, X.; Zhong, B.; Zhao, Y. *Biomed. Chromatogr.* **2012**, *26*, 497–501.
14. Godfrey, A. R.; Digiacinto, J.; Davis, M. W. *Clin. Ther.* **2011**, *33*, 766–75.
15. Chen, L.; Zhang, Q.; Lin, Y.; Lu, X.; Zhong, Z.; Ma, J.; Wen, C.; Ding, C. *Acta Chromatogr.* **2020**, *32*, 44–8.
16. Han, A.; Lin, G.; Cai, J.; Wu, Q.; Geng, P.; Ma, J.; Wang, X.; Lin, C. *Acta Chromatogr.* **2019**, *31*, 99–104.
17. Guivarc'h, P. H.; Vachon, M. G.; Fordyce, D. *Clin. Ther.* **2004**, *26*, 1456–69.
18. Sauron, R.; Wilkins, M.; Jessent, V.; Dubois, A.; Maillot, C.; Weil, A. *Int. J. Clin. Pharmacol. Ther.* **2006**, *44*, 64–70.
19. Park, J. M.; Chae, S. I.; Noh, Y. S.; Lee, S. J.; Shim, W. S.; Yoon, J. M.; Hwang, S. J.; Lee, K. T.; Chung, E. K. *Int. J. Clin. Pharmacol. Ther.* **2019**, *57*, 217–28.