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A Simple and Efficient Method to Determine Montelukast in Rat Plasma Using Liquid-Liquid Extraction and Tandem Mass Spectrometry

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Abstract : While montelukast (ML), a cysteinyl-leukotriene type 1 receptor ($CysLT_1$) antagonist is widely used to treat symptoms of rhinitis or asthma, its formulations are mainly limited to solid preparation due to its instability. Recently, there have been attempts to develop various ML dosage forms, and this situation increases the demand of sensitive and creditable methods to determine ML in various samples such as plasma. Thus, here, a simple and efficient method to determine ML in rat plasma using liquid-liquid extraction (LLE) and multiple reaction monitoring was presented. The mixture of DCM:EtOAc (25:75, v/v), the optimized extract solvent for LLE was found to be effective to extract ML without hydrophilic salts and proteins from the sample with limited volume. Also, the use of zafirlukast, instead of expensive ML-d_6, as the internal standard makes the present method economical. The developed method was successfully validated in terms of selectivity, matrix effects (-14.8--6.9%), linearity (r^2≥0.998 within 0.5-500 ng/mL), sensitivity (the limit of detection and the lower limit of quantitation, ≤0.5 ng/mL), accuracy (88.4-100.6%), precision (3.0-13.3%), and recovery (80.8-86.3%) by following the FDA guidelines. Finally, the applicability of the validated method to pharmacokinetics (PK) studies was confirmed by the successful determination of PK parameters through it following oral administration of Singulair® granule in rats. Therefore, the present method can contribute to the development of new ML formulations through its performance to determine ML in rat plasma efficiently and sensitively.

Keywords : montelukast, multiple reaction monitoring, liquid-liquid extraction, rat plasma, pharmacokinetics

Introduction

Montelukast (ML, Figure 1), a cysteinyl-leukotriene type 1 receptor ($CysLT_1$) antagonist is widely used to treat symptoms of rhinitis or asthma.[1,2] However, since ML is sensitive to light and humidity, it is used mainly as a solid preparation.[3] Recently, there have been attempts to develop various ML dosage forms, ranging from syrup to transdermal dosage forms,[4] and as a result, it is essential to develop sensitive methods to determine ML in various samples including biological samples like plasma. According to documents reported to date, protein preparation and a liquid chromatography and tandem mass spectrometry (LC-MS/MS) system employing multiple reaction monitoring (MRM) have been a good choice to analyze trace amounts of ML in plasma with high sensitivity.[5-8] The use of protein precipitation as sample preparation method has the advantage of saving time and improving the convenience of experimenters. However, its problem is that too many components of the sample are introduced to the mass spectrometer without being removed in the pretreatment process due to its lack of selectivity.[9,10] Among those substances, compounds ionized with analytes at the same time during electrospray ionization can cause suppression effect (ionization interference and sensitivity alteration, generally, reduction).[11] Also, nonvolatile salts still dissolved in the sample solution even after protein precipitation can be deposited at the orifice of the curtain plate of the mass spectrometer.[12-14] In this case, the reproducibility and the reliability of results are reduced, and cleaning and tuning the mass spectrometer is time-consuming. While Muppavarapua *et al*.'s method based on liquid-liquid extraction (LLE) seems to be effective to handle these issues, it still has a couple of limitations.[15] First, since it requires the relatively large volume (200 μL) of human plasma, it is difficult to apply it to rat plasma. Also, the use

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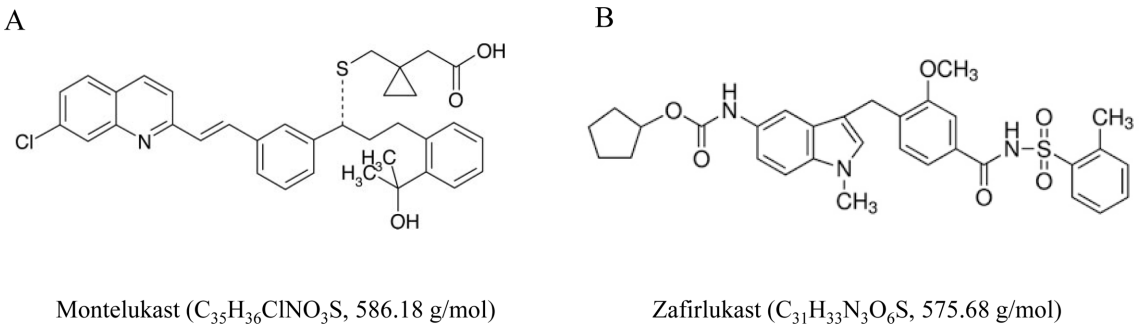

Montelukast ($C_{35}H_{36}ClNO_3S$, 586.18 g/mol) Zafirlukast ($C_{31}H_{33}N_3O_6S$, 575.68 g/mol)

Figure 1. Chemical structures of montelukast (A) and zafirlukast (B)

of ML-d_6 in which six hydrogens of ML are substituted with six deuteriums is a factor of increasing the experimental cost.

Therefore, here, a simple and efficient method to determine ML in rat plasma using LLE and MRM was developed. Its optimized extract solvent for LLE was found to be effective to extract ML without hydrophilic salts and proteins from the sample with limited volume. Also, employing zafirlukast (Figure 1), another $CysLT_1$ antagonist much cheaper than ML-d_6 as the internal standard (IS) makes the present method economical. The developed method was successfully validated according to FDA guidelines and its applicability to pharmacokinetics (PK) studies was also confirmed.

Experimental

Chemicals and reagents

ML sodium (≥ 99.0%), zafirlukast (≥ 99.0%), and ammonium formate (LC-MS grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile, ethyl acetate (EtOAc), dichloromethane (DMC), methyl *tert*-butyl ether (MTBE), methanol, and water were obtained from J. T. Baker (Phillipsburg, NJ, USA).

Preparation of calibration and quality control samples

To prepare stock solutions, ML and zafirlukast (IS) were dissolved at 1 mg/mL in methanol and acetonitrile, respectively. The ML stock solution was diluted with methanol to 500 ng/mL (the ML working solution), and the extraction solvent was prepared by the dilution of the IS stock solution with a mixture of DCM:EtOAc (25:75, v/v) to 60 ng/mL. All stock solutions and working solutions were stored at -27°C, until use. Samples for quality control (QC) and calibration were prepared by spiking the ML working solution to blank rat plasma for a specific concentration of ML. A total of four QC samples (0.5, 1.5, 200, and 400 ng/mL for lower limit of quantification (LLOQ), low QC (LQC), middle QC (MQC) and high QC (HQC), respectively) and six calibration samples (0.5, 10, 50, 100, 250, and 500 ng/mL) were prepared.

Liquid-liquid extraction

An aliquot (20 μL) of a rat plasma sample, a calibration sample, or a quality control sample was mixed with 500 μL of the extraction solvent including IS using a vortex mixer for a minute. Then, the mixture was centrifuged at 12,000 × g and 4°C for 10 minutes and the top layer was transferred to a micro-centrifuge tube. The extract solution taken was dried at room temperature under nitrogen stream, and the residue was reconstituted in 100 μL of methanol. After the centrifugation of the final solution at 12,000 × g and 4°C for 10 minutes, a part of its supernatant was analyzed by LC-MS/MS.

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

For LC-MS/MS, a Shimadzu Nexera UPLC system (Tokyo, Japan) and a Shimadzu LCMS 8050 triple quadrupole mass spectrometer were interfaced through electrospray ionization (ESI) in positive ion mode. For LC separation, a Phenomenex Luna C18 column (2.0 × 150 mm, 5 μm, Torrance, CA, USA) and the gradient mobile phase program between 5 mmol/L of an aqueous ammonium formate solution and methanol were used (Supporting Information). A run of a sample analysis was carried out for seven minutes at the MP flow rate of 0.25 mL/min. During the analysis, the column and the autosampler were kept at 40°C and 4°C, respectively. In the case of ESI, source parameter values were as follows: nebulizing gas flow at 3 L/min, heating gas flow at 10 L/min, drying gas flow at 10 L/min, interface temperature at 300°C, DL temperature at 250°C, and heating block temperature at 400°C. For highly selective and highly sensitive determination of ML using the mass spectrometer, MRM was selected among various MS/MS scans. Three MRM transitions per target compound were monitored: one with the highest sensitivity was the screening transition for quantitation and the others were the confirmatory transition for the target identity confirmation. In the case of ML, 586.3 *m/z* (precursor ion) / 422.3 *m/z* (product ion)/ -25 V (collision energy), 586.3 *m/z* / 278.1 *m/z* / -35 V, and 586.3 *m/z* / 440.2 *m/z* / -23 V were the screening transition,

the confirmatory transition 1, and the confirmatory transition 2, respectively. Also, the screening transition of 576.3 *m/z* / 337.2 *m/z* / -22 V, the confirmatory transition 1 of 576.3 *m/z* / 319.2 *m/z* / -33 V, and the confirmatory transition 2 of 576.3 *m/z* / 464.2 *m/z* / -14 V were applied for IS. All mass spectrometry data were acquired and analyzed using Lab Solutions (version 5.93, Shimadzu). For quantitation, three pre-requirements (all three transition peaks should have the same retention time; the signal to noise ratio (S/N) of the screening transition peak should be higher than 10; all confirmatory transition peaks should have the S/N values higher than 3) were tested. When all they were satisfied, a screening transition peak area ratio of ML to IS was calculated and used for quantitation.

Results and Discussion

Method development

Liquid chromatography and multiple reaction monitoring

For sensitive analysis, $[M+H]^+$ ions (586.3 *m/z* and 576.3 *m/z* for ML and IS, respectively) were chosen as precursor ions. Product ions for MRM were selected from product ion scan (PIS) results of individual precursor ions. The strongest fragment ions (422.3 *m/z* and 337.2 *m/z* for ML and IS, respectively) were chosen for screening transitions. As confirmatory transitions, the second and third strongest fragment ions (278.1 and 440.2 *m/z* for ML and 319.2 and 464.2 *m/z* for IS) were selected. In the case of separation, a C18 column and a gradient mobile phase program between 5 mM of an aqueous ammonium formate solution and methanol was used for 1) longer retention on the column and less suppression effect of IS and 2) less selectivity factor (but baseline separation) between ML and IS even with good peak shapes as well as high sensitivity (4.8 minutes for ML and 2.4 minutes for IS, Figure 2). Both peaks could not be located closer due to the suppression effect on IS. However, the resulting relatively large retention time difference between both peaks was found not to affect the quantitation accuracy of the present method from the method validation followed. Therefore, the cost advantage of the present method was proved.

Sample preparation

For the development of a simple and efficient sample preparation method which can be applied to rat plasma, LLE was considered as the only candidate, and the decision of its extraction solvent was the key part of this study. As the first step, common non-polar extraction solvents for LLE, EtOAc and MTBE were tested at the ML concentration of 200 ng/mL and their ML and IS recovery

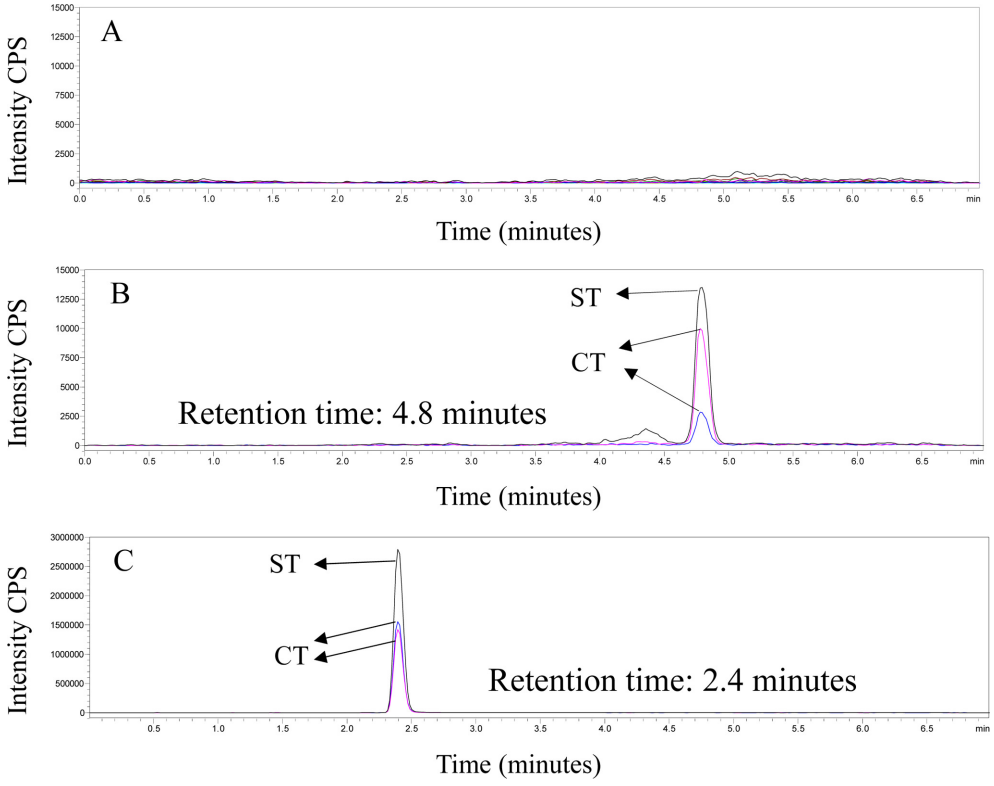

Figure 2. Multiple reaction monitoring chromatograms of montelukast (ML) and zafirlukast (IS) in rat plasma. Blank plasma (A), plasma including 0.5 ng/mL of ML (B), and plasma including 1.5 μg/mL of IS (C). "ST" and "CT" stand for the screening transition peak and the confirmatory transition peaks, respectively.

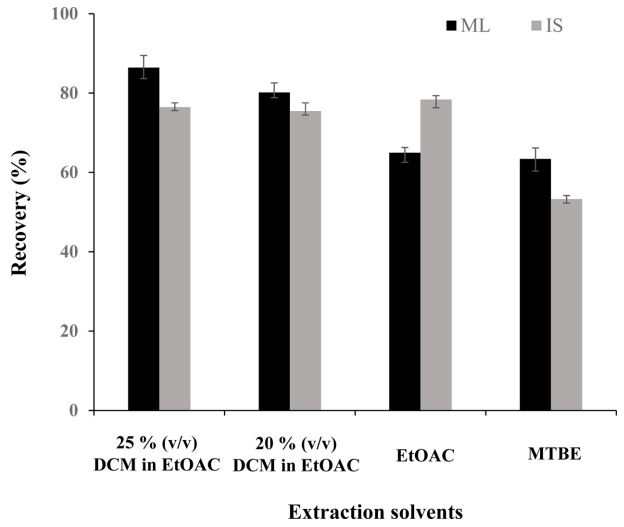

Figure 3. Effect of different extraction solvents on the recovery of montelukast (ML, 200 ng/mL) and zafirlukast (IS, 1.5 μg/mL) in rat plasma (n=6). DCM, dichloromethane; EtOAc, ethyl acetate; MTBE, methyl *tert*-butyl ether.

results were compared.[16,17] As shown in Figure 3, there was not any significant difference in ML recovery values from both solvents (65.0% from EtOAc and 63.4% from MTBE), but EtOAc showed the advantage over MTBE in the aspect of IS recovery (78.5% from EtOAc and 53.3% from MTBE). Thus, while EtOAc was selected as the 'interim' extract solvent, it still had the margin at ML recovery to be improved. In order to find a better LLE solvent which can extract ML more efficiently, DCM, another common non-polar solvent for LLE was mixed with EtOAc, and the effects of their mixing ratios to ML and IS recovery were evaluated at the ML concentration of 200 ng/mL. A total three different mixing ratios (DCM:EtOAc of 30:70, 25:75, and 20:80, v/v) were tried to be compared, 25:75 was found to be the best mixing ratio showing the best recovery (86.43% and 76.48% for ML and IS, respectively, Figure 3). Actually, 30:70 was excluded from the study due to the 'sinking' property of its resulting solvent. Since DCM is relatively dense (the density of 1.33 g/mL at 4°C), the larger portion of DCM in the mixed solvent makes the resulting solvent denser.[18] At the DCM:EtOAc of 30:70, the mixed solvent became heavier than plasma, and as a result, it was located at the bottom layer after centrifugation. Since it is difficult to take/transfer the bottom layer for downstream experiments, this condition was excluded from the study. The actual LLE extraction solvent of the present method includes zafirlukast, another $CysLT_1$ antagonist as the IS. Since similar methods have employed expensive ML-d_6 as the IS, the present method is relatively economical.[15,19,20] The volume of rat plasma used in this method was decided to 20 μL, the minimal volume which showed precise and linear results from comparison tests of various plasma volumes (data not shown). The plasma volume used in the present method is less than the smallest volume used in previous ML studies (50 μL) and suitable for the application to rat.[7,21] As the instability of a ML solution was reported, all centrifugation steps were carried out at 4°C and nitrogen purging steps were applied under dark condition.[3] Finally, the possibility of the deposit of involatile salts, which may be transferred from plasma to the extraction solvent during LLE, on the curtain plate of the mass spectrometer during analyses was tested. Various plasma sample LLE extracts were continuously analyzed over 150 runs and there was not any significant sign of salt deposit (data not shown).

Method validation

According to FDA guidelines, the developed method was validated in terms of selectivity, matrix effects, linearity, sensitivity, accuracy, precision, and recovery (Supporting Information).[22] Briefly, selectivity was confirmed by observing no peak at the retention times of ML (4.8 minutes) and IS (2.4 minutes) from the blank plasma analysis (Figure 2). While only the limited matrix effect between -14.83 and -6.91% was observed at four different concentrations (Table 1), matrix-matched calibration was used in the present method for better accuracy. Since the coefficient of determination (r^2) values were at least 0.998 from all calibration curves covering ML plasma levels from 0.5 to 500 ng/mL, the linearity of the method was confirmed. Also, at all concentration levels, the signal to noise ratio values of the ML screening transition peak and the ML confirmatory transition peak are higher than 10 and 3, respectively. Thus, the limit of detection and the limit of quantitation of the method is lower than 0.5 ng/mL, the lowest concentration at which the linearity was confirmed. In the case of accuracy and precision, they were evaluated from four QC samples and all related values are as below: 88.37-100.64% of intra-day accuracy, 92.22-100.02% of inter-day accuracy, ≤ 13.25% of intra-day precision, and ≤ 11.29% of inter-day precision (Table 2). Finally, recovery values at four different concentrations were obtained and they were between 80.78% and 86.34%

Table 1. Matrix effect and recovery of montelukast (ML) in rat plasma (n = 6)

ML concentration (ng/mL)	Matrix effect (%) (mean ± standard deviation)	Recovery (%) (mean ± standard deviation)
0.5	-14.83 ± 5.75	84.57 ± 9.06
1.5	-11.29 ± 5.59	80.78 ± 3.96
200	-7.97 ± 4.13	86.34 ± 2.58
400	-6.91 ± 3.15	84.24 ± 3.11

Table 2. Intra- and inter-day accuracy and precision from LC-MRM of montelukast (ML) in rat plasma (n = 6)

Types	Nominal concentration of ML (ng/mL)	Calculated concentration of ML (ng/mL) (mean ± standard deviation)	Accuracy (%)	Precision (%)
Intra-day	0.5	0.50 ± 0.07	100.64	13.25
	1.5	1.40 ± 0.07	93.43	5.32
	200	176.67 ± 5.27	88.37	2.99
	400	373.05 ± 13.77	93.26	3.69
Inter-day	0.5	0.49 ± 0.05	97.01	11.29
	1.5	1.45 ± 0.13	96.65	8.78
	200	192.44 ± 15.14	92.22	7.87
	400	400.08 ± 29.61	100.02	7.1

Table 3. Pharmacokinetic parameters of montelukast following oral administration of Singulair® granule in rats at a dose of 2 mg/kg. Data represent mean ± standard deviation (n = 5).

Parameters	Singulair® granule
$AUC_{0–24\ h}$ (ng·h/mL)	420.9 ± 106.5
$AUC_{0–\infty}$ (ng·h/mL)	472.8 ± 144.5
C_{max} (ng/mL)	110.4 ± 82.7
T_{max} (h)	1.5 ± 1.4
$t_{1/2}$ (h)	6.1 ± 2.3

(Table 1). Since all validation results satisfied the FDA guidelines, the present method is proven to be a novel method to determine ML in rat plasma.[22]

Application to pharmacokinetic studies in rats

To confirm the applicability of the present method to actual PK studies of ML in rat plasma, the plasma concentration profile of ML following oral administration of Singulair® granule in rats at a dose of 2 mg/kg was obtained through the present method (Supporting Information). Also, based on the PK data acquired, PK parameters of ML such as $AUC_{0\text{-}24h}$, $AUC_{0\text{-}\infty}$, C_{max} , T_{max}, and $t_{1/2}$ were successfully determined (Table 3). Thus, this result shows that the present method is good enough to be used for actual PK studies of ML in rat plasma.

Conclusions

A simple and efficient method to determine ML in rat plasma using LLE and MRM was developed. The mixture of DCM:EtOAc (25:75, v/v), the optimized extract solvent for LLE was found to be effective to extract ML without hydrophilic salts and proteins from the sample with limited volume. Also, the use of zafirlukast, instead of expensive ML-d_6, as the IS makes the present method economical. The developed method was successfully validated in terms of selectivity, matrix effects, linearity, sensitivity, accuracy, precision, and recovery by following the FDA guidelines. Finally, the applicability of the validated method to PK studies was confirmed by the successful determination of PK parameters through it following oral administration of Singulair® granule in rats. Therefore, the present method can contribute to the development of new ML formulations through its performance to determine ML in rat plasma efficiently and sensitively.

Supporting Information

Supporting information is available at https://drive.google.com/file/d/1P4a45v60sG8Q2Ec-DXkU_pmlJ_vXQrLD/view?usp=sharing.

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References

1. Diamant, Z.; Mantzouranis, E.; Bjermer, L. *Expert Rev. Clin. Immunol*. **2009**, 5, 639, DOI: 10.1586/eci.09.62
2. Lambiase, A.; Bonini, S.; Rasi, G. *JAMA Ophthalmol.* **2003**, 121, 615, DOI: 10.1001/archopht.121.5.615.
3. Al Omari, M. M.; Zoubi, R. M.; Hasan, E. I.; Khader, T. Z.; Badwan, A. A. *J. Pharm. Biomed. Anal*. **2007**, 45, 465, DOI: 10.1016/j.jpba.2007.07.014.
4. Lim, S. H. *Design of montelukast nanocrystals for enhanced transdermal delivery*, master's thesis, Dankook University, Cheonan, South Korea. **2018**.
5. Ezzeldin, E.; Abo-Talib, N. F.; Tammam. M. H.; Shahat, A. A. *Chem. Cent. J.* **2014**, 8, 17, DOI: 10.1186/1752-153X-8-17.
6. Huang, Y.; Ding, L.; Liu, Y. Y.; Liu, H.; Wen, A.; Yang, L. *J. Chinese Pharm. Sci.* **2009**, 18, 261.
7. Kim, D. W.; Kim, Y. H.; Yousaf, A. M.; Kim, D. S.; Kwon, Y. K.; Park, J. H.; Kim, Y. I.; Park, J. H.; Jin, S. G.;

Kim, K. S.; Cho, K. H.; Li, D. X.; Kim, J. O.; Yong, C. S.; Woo, J. S.; Choi, H. G. *Arch. Pharmacal Res.* **2016**, 39, 539, DOI: 10.1007/s12272-015-0664-x.
8. Challa, B. R.; Awen, B. Z.; Chandu, B. R.; Khagga, M.; Kotthapalli, C. B. *Sci. Pharm.* **2010**, 78, 411, DOI: 10.3797/scipharm.1002-07.
9. Moein, M. M.; Beqqali, A. E.; Abdel-Rehim, M. *J. Chromatogr. B* **2017**, 1043, 3, DOI: 10.1016/j.jchromb.2016.09.028.
10. Ashri, N. Y.; Abdel-Rehim, M. *Matrix Biol*. **2011**, 3, 2003, DOI: 10.4155/bio.11.201.
11. Park, J. S.; Cho, H. R.; Kang, M. J.; Choi, Y. S. *Arch. Pharmacal Res*. **2016**, 39, 73, DOI: 10.1007/s12272-015-0681-9.
12. Jessome, L. L.; Volmer, D. A. *LC GC North Am.* **2006**, 24, 498.
13. Annesley, T. M. *Clin. Chem.* **2003**, 49, 1041, DOI: 10.1373/49.7.1041.
14. Crescenzi, C.; Corcia, A. D.; Marchese, S.; Samperi, R. *Anal. Chem.* **1995**, 67, 1968, DOI: 10.1021/ac00109a010.
15. Muppavarapu, R.; Guttikara, S.; Rajappan, M.; Kamarajan, K.; Mullangi, R. *Biomed. Chromatogr.* **2014**, 28, 1048, DOI: 10.1002/bmc.3114.
16. Lee, H. C.; Kim, D. Y.; Choi, M.-J.; Jin, S. G.; Choi, Y. S. *Mass Spectrorm. Lett*. **2019**, 10, 66, DOI: 10.5478/MSL.2019.10.2.66.
17. Kim, C. H.; Kang, T. H,; Kim, B. D.; Lee, T. H.; Yoon, H. Y.; Goo, Y. T.; Choi, Y. S; Kang, M. J.; Choi, Y. W. *Int. J. Pharm.* **2020**, 583: 119393, DOI: 10.1016/j.ijpharm.2020.119393.
18. Curran, D. P.; Werner, S. *Org. Lett.* **2004**, 6, 1021, DOI: 10.1021/ol0499248.
19. Karonen, T.; Neuvonen, P. J.; Backman, J. T. *Br. J. Clin. Pharmacol.* **2012**, 73, 257, DOI: 10.1111/j.1365-2125.2011.04086.x.
20. Zaid, A. N.; Mousa, A.; Ghazal, N.; Bustami, R. *Clin. Transl. Allergy* **2015**, 5, 37, DOI: 10.1186/s13601-015-0081-8.
21. Papp, R.; Luk, P.; Mullet, W. M.; Kwong, E. *J. Chromatogr. B* **2007**, 858, 282, DOI: 10.1016/j.jchromb.2007.08.003.
22. Food and Drug Administration. *Boanalytical method validation: guidance for industry FDA-2013-D-1020*. **2019**.

