

Development and validation of a simultaneous analytical method for non-steroidal therapeutic compounds in cosmetics using LC-MS/MS

In Suk Woo, Jun Hyung Kim, You Kyung Kim, Hyung Il Kim, Dong Woo Shin, Jin Ho Kim, and Sun Young Baek*

Center for Advanced Analysis, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong Health Technology Administration Complex, Cheongju-si, Chungcheongbuk-do, Republic of Korea

*Correspondence

Center for Advanced Analysis, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Osong Health Technology Administration Complex, 187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, 28159, Republic of Korea.

Email address: stepany9828@korea.kr

ABSTRACT

Atopic dermatitis is a typical chronic inflammatory skin disease that affects all age groups and requires basic skin care for treatment. Anti-inflammatory and antiallergy steroids are the most frequently used treatments but they are limited due to their side effects caused by a weakening of the immune system. Many consumers focus on performance as a criterion for selecting cosmetics. However, steroids have been illegally used to improve the performance of cosmetics, and consumers have been adversely affected by the corresponding side effects.

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In this paper, we propose a simple and rapid method using **liquid chromatography–tandem mass spectrometry** to simultaneously analyze ten non-permitted atopic therapeutic compounds in cosmetic products: chlorpheniramine maleate, ketotifen fumarate, doxepin hydrochloride, azelastine hydrochloride, bufexamac, clotrimazole, tranilast, fusidic acid, tacrolimus, and pimecrolimus. Additionally, the major characteristic fragment ions for tacrolimus, pimecrolimus, and clotrimazole were identified by **time-of-flight mass spectrometry**. The specificity, linearity, limit of detection, limit of quantification, recovery, precision, accuracy, and stability of the proposed method were validated. The limit of detection and quantification were in the ranges of 5.05–203.30 pg/mL and 15.15–609.90 pg/mL, respectively. The proposed analysis method could help improve the safety management of cosmetics.

Keywords

Illegal cosmetics, atopic dermatitis, therapeutics, topical calcineurin inhibitors, liquid chromatography tandem mass spectrometry

Abbreviations:

AD, Atopic dermatitis; TCI, topical calcineurin inhibitor; UHPLC, ultra-high performance liquid chromatography; LC-Q-TOF-MS, liquid chromatography quadrupole time of flight mass spectrometry; MRM, multiple reaction monitoring

1. Introduction

Atopic dermatitis (AD) refers to a chronic refractory inflammatory skin disease caused by a combination of genetic, environmental, and immunological factors of an individual; AD afflicts children (up to 20%) and adults (2–8%) in most countries around the world [1–3]. Several guidelines for the treatment of AD have been published by working groups in different countries. Such guidelines state that age, gender, location, and the severity of the lesion should be considered when determining a patient's treatment [2,5,6]. These guidelines recommend the use of moisturizers, topical steroids, and topical calcineurin inhibitors (TCIs) as the main treatments for AD [2–6].

Topical calcineurin inhibitors (TCIs) are typically used to treat skin diseases, such as mild-to-moderate AD [4,7]. They are non-steroidal immune modulators to reduce the inflammation caused by AD and can significantly reduce itchiness [3,8]. Tacrolimus and pimecrolimus are TCIs that are used for atopic treatment and are approved for use on patients over the age of two in the European Union [3,4]. Concerns about TCI have been raised following the announcement of the US Food and Drug Administration (FDA) regarding the lack of long-term safety of TCI and the corresponding black box warning [4,9–12]. Moreover, the non-steroidal components of antihistamines and antibiotics, which have been used as therapeutic agents for atopy, may also cause side effects.

AD treatment requires a suitable moisturizer with the ability to recover the function of the damaged skin barrier in tandem with topical anti-inflammatory drugs. Several moisturizing products have been sold with emphasis on their therapeutic effects on AD in the cosmetics market. However, prohibited ingredients (such as steroids and ketoconazole) may

be included to enhance their therapeutic effects. These illegal cosmetics are either confiscated or distribution is seized when they are found to be distributed commercially. The number of such cases has been increasing steadily every year. As such, consumers must be protected from cosmetics mixed with pharmaceutical ingredients that can cause side effects when used in the long term.

In the Republic of Korea, commercial cosmetics are currently managed in accordance with the Regulation on the Safety Standards, etc. of Cosmetics (Notice No. 2017-114), which was announced by the Ministry of Food and Drug Safety. Recently, pharmaceutical ingredients for atopy treatment (pimecrolimus and tacrolimus) have been added to the list of ingredients that are prohibited in cosmetics (Notice No. 2017-41). To prevent the illegal use of the main ingredients intended for therapeutic purposes, the management of commercial cosmetics is strengthened through these regulations. Accordingly, a verifiable analytical method is necessary to detect such prohibited components in commercial products.

Many studies have been conducted for the quantitative analysis of corticosteroids that have been illegally included in cosmetic products. In these studies, the method was validated and established using ultra high performance liquid chromatography (UHPLC) [13] and liquid chromatography with tandem mass spectrometry (LC-MS/MS) [14,15]. Nam et al. [16] and Giaccone et al. [14] completed applicability experiments using established methods for actual manufactured products. However, it is uncommon for analytical methods to determine if a cosmetic product contains non-steroidal ingredients that can only be used as medicines. Some researchers [15,17–19] have conducted the analysis of tacrolimus and pimecrolimus, which are immunosuppressants used in topical treatment. They have determined a means to elucidate the penetration behavior of these compounds on human skin samples treated with commercial ointments using LC-MS and/or LC-MS/MS. Recently, a method for the

simultaneous analysis of the immunosuppressant components (including tacrolimus) from a drug using reversed-phase HPLC was developed by Sharma et al. [20].

Methods for the simultaneous analysis of steroid components in products such as creams, ointments, and gels have been developed, and the risks associated with such components have been continuously emphasized. Therefore, it is essential to develop analytical methods for other pharmaceutical ingredients besides steroids. In this study, we aimed to develop a multi-component simultaneous analysis method to detect prohibited substances (such as pharmaceutical ingredients used in atopy treatment) in cosmetics to strengthen the safety management of commercial cosmetics.

2. Materials and methods

2.1 Chemicals and reagents

The following standards were purchased: chlorpheniramine maleate, bufexamac, and fusidic acid from TCI (Tokyo, Japan); ketotifen fumarate from Santa Cruz Biotechnology (Dallas, TX, USA); doxepin HCl from US Pharmacopoeia (UPS, Rockville, MD, USA); and azelastine HCl, clotrimazole, tranilast, tacrolimus, and pimecrolimus from Sigma-Aldrich (St. Louis, MO, USA). The purity of each chemical was greater than 95%. Formic acid of LC-MS grade was procured from Wako Pure Chemical (Tokyo, Japan). Acetonitrile and methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). To prepare the stock solutions, the standards were dissolved in methanol to concentrations of approximately 1

mg/mL and stored in a refrigerator (2–8 °C) until use. In the case of tacrolimus, the stock solution was prepared by dissolving tacrolimus in acetonitrile. Working mixtures were prepared by diluting the stock solutions in methanol to concentrations that were appropriate for the calibration curves and method validation.

2.2 Sample preparation

All cosmetic samples were domestically traded products. Each sample (0.100 g for toner and face cleanser products, 0.010 g for lotion) was precisely weighed and placed in a 50.0 mL clean and dry volumetric flask with a stopper. On each flask, 100% methanol was filled to the mark, and the mixture was shaken for 1 min. After sonicating the mixture for 30 min in a water bath, the extracted solution was centrifuged at 4500 rpm for 5 min. Then, the supernatant was filtered using a 0.2 µm PTFE filter (Millipore, Milford, USA) prior to LC-MS/MS analysis.

2.3 LC-Q-TOF-MS and LC- MS/MS conditions

Liquid chromatography quadrupole time of flight mass spectrometry (LC-Q-TOF-MS, Waters Synapt G2 HDMS, Milford, MA, USA) analysis was used to confirm fragmentation of the analyte, and the results were used as basic data for optimizing the multiple reaction monitoring (MRM) conditions of LC-MS/MS. Electrospray ionization (ESI) was operated in the positive and negative ion modes. The capillary voltage was 2.5 kV and the temperature

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and gas (N₂) flow for desolvation were set to 500 °C and 800 L/h, respectively. The nebulizer and collision gas flow were maintained at 7.0 bar and 0.15 mL/min, respectively. Cone gas (N₂) flow was not applied.

In the LC-MS/MS, Waters ACQUITY ultra-pure liquid chromatograph (UPLC) and a Xevo TQ-S (Milford, MA, USA) system were used to establish simultaneous analysis of 10 non-steroidal therapeutic compounds. UPLC was carried out using an ACQUITY UPLC BEH C₁₈ (100 × 2.1 mm id., 1.7 µm, Waters, MA, USA) column with the temperature maintained at 35 °C. The injection volume and flow rate were set to 2.0 µL and 0.4 mL/min, respectively. The mobile phase consisted of 0.1% formic acid in water (A) and methanol (B). The gradient program was set as follows: initial, 10% B; 0–2.0 min, 40% B; 2.0–6.0 min, 70% B; 6.0–6.5 min 90% B; 6.5–7.5 min, 100% B; 7.5–7.6 min, 10% B; and 7.6–10.0 min, 10% B for equilibration. Most of the MS parameters used the same values as in LC-Q-TOF-MS. All other detection settings, such as the collision energy, cone voltage, parent ion, and quantification ion for each compound, are shown in Table 1. Data acquisition and qualitative analysis for the LC-MS/MS experiments were performed using the MassLynx Software (Ver. 4.1, Waters).

2.4 Method validation

To establish an analytical method for the detection of illegally included pharmaceutical ingredients in cosmetics, the samples were pretreated according to cosmetic type (toner, lotion, and face cleanser). Method validation was performed on all cosmetic sample types. The proposed method was validated for specificity, linearity, limit of detection (LOD), limit

of quantification (LOQ), recovery, accuracy, precision, and stability as per FDA and International Council of Harmonization (ICH) guidelines [21,22].

The linearity was evaluated with concentrations of six points evenly distributed over the expected concentration range of the analytes. **In this study, the influence of the matrix was minimized using a matrix-matched calibration technique.** The calibration curves of the samples were obtained by adding accurate amounts of mixed standards into cosmetic blank samples (toner, lotion, cleanser) of different concentrations (**0.02–121.98 ng/mL for all blank samples**). The lowest concentration for calibration was considered as the LOQ. **To evaluate recovery, accuracy, precision, and stability, all compounds were added at low (near the LOQ), medium (approximately 48-times the LOQ) and high (approximately 190-times the LOQ) concentrations to three blank samples that had been analyzed previously.**

3. Results and discussion

3.1 MS fragmentation patterns

For the LC-Q-TOF-MS analysis, ESI was selected as the ionization method and 1 $\mu\text{g/mL}$ of the working solution was directly injected to confirm the ion mode and precursor ions for each component. Here, only the MS^2 spectra for tacrolimus and pimecrolimus are shown because other components can be easily found in many studies, and no differences were found in our study. Except for three components (clotrimazole, tacrolimus, pimecrolimus), all other components have a precursor ion of $[\text{M}+\text{H}]^+$ or $[\text{M}-\text{H}]^-$. As shown

in Fig. 1, the precursor ions of tacrolimus and pimecrolimus were observed in the form of $[M+Na]^+$ and were found at m/z 826.4732 (calculated m/z 826.4730) and 832.4393 (calculated m/z 832.4391), respectively.

In the case of clotrimazole, the parent molecular ion was detected at m/z 345.1158 ($[M+H]^+$), but the sensitivity was considered to be too low under the proposed conditions. In the present study [23], the analysis was carried out under acidic conditions; hence, clotrimazole was detected with high sensitivity at m/z 277.0764, which corresponds to the intermediate ($[M-N_2C_2H_3]^+$) of clotrimazole (Scheme 1).

Initial MS scans resulted in the detection of the precursor ions $[M+H]^+$, $[M-H]^-$, and $[M+Na]^+$ of the analyzed compounds. The 2 to 3 product ions derived from the precursor ion were selected in the MS/MS spectra. The representative product ion exhibits high intensity among those derived from precursor ions. The results obtained from the LC-Q-TOF-MS analysis were used to establish the MRM conditions for LC-MS/MS, as shown in Table 1, which allowed the detection of 10 therapeutic compounds for atopy. To interpret the LC-MS/MS analysis results, qualitative and quantitative ions were selected by comparing the intensity of the daughter ions of each standard component. The ion with the highest sensitivity was used as the quantitative ion.

3.2 Specificity

To optimize the LC conditions, formic acid was added to the mobile phase. Compared to ammonium acetate, formic acid exhibited a higher separation and sensitivity for each component. Validation experiments were performed under this optimized condition.

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Specificity was estimated by comparing the chromatograms of blank samples with those of samples of standard solutions using optimized assays. For each type (toner, lotion, cleanser) of cosmetic sample, all the target components were well separated according to retention time without interference from the matrix components of the sample, which was confirmed using MRM chromatogram (Fig. 2). There were no significant differences between cosmetic types.

3.3 LOD, LOQ, and Calibration curves

The LOD is the minimum concentration at which the presence of an analyte in the sample can be confirmed. The LOQ is the lowest concentration that can yield quantitative measurement results with reasonable reliability (accuracy and precision) for the analyte. Based on the signal-to-noise ratio (S/N), the LOD corresponds to a concentration at which $S/N = 3:1$, and the LOQ corresponds to a concentration three times the LOD. The LOD and LOQ were evaluated for toner-, lotion-, and face cleanser-type samples. The LOD and LOQ were calculated using an analyzer software (Waters MassLynx), and the value of S/N was calculated using the baseline noise. Under the optimized analytical conditions, the overall LODs and LOQs ranged between 5.05–203.30 and 15.15–609.90 pg/mL, respectively, for toner-, lotion-, and face cleanser- products. For some components (chlorpheniramine, azelastine, bufexamac, and pimecrolimus), the LODs and LOQs were different for each cosmetic type. Doxepin and clotrimazole exhibited lower LODs and LOQs for the cleanser-type products (Table 2). These results indicate that the detectable concentration of each target component can vary depending on the type of cosmetic.

Linearity refers to the ability to obtain linear measurements in proportion to the amount (or concentration) of the analyte. The calibration curves obtained by plotting the peak areas versus the six individual concentrations (all above the LOQ). The concentration ranges of each standard solution used in the linearity experiment are listed in Table 2, wherein the concentrations ranged from the LOQ to about 200 times the LOQ. The regression correlation coefficients for the 10 atopic therapeutics were in the ranges of 0.9993–0.9998, 0.9990–1.0000, and 0.9990–0.9999 for toner, lotion, and face cleanser cosmetics, respectively. The detailed data are summarized in Table 2.

3.4 Recovery

The recovery was evaluated by adding standard solutions of each analyte of known low, medium, and high concentrations (within the linearity range) to blank samples. The three concentrations correspond to the concentration range used to obtain the calibration curve and include the LOQ. The recovery was the percentage ratio of the measured concentration to the theoretical concentration of the analyte. Blank samples of the three cosmetic types were examined, and the analysis was repeated three times. Table 3 summarizes the recoveries of the 10 therapeutic compounds for atopy. The recoveries were in the ranges of 80.26%–118.98%, 80.11%–117.50%, and 80.07%–109.69% for toner-, lotion-, and cleanser-type samples, respectively. The RSDs were $\leq 14.22\%$ for all samples.

3.5 Accuracy and precision

Accuracy indicates the closeness of the measured value to the true value. The accuracy of the proposed method was evaluated based on two factors: intra- and inter-day accuracy. To evaluate the accuracy and precision, the analysis was conducted in one day (intra-day) and over three days (inter-day). The accuracy was calculated as the percentage ratio of the mean of the quantified concentrations from the calibration curve and the theoretical concentration of each analyte.

The precision of the method corresponds to the proximity between each repeated measurement under the established conditions and is usually expressed as the standard deviation (SD) or relative standard deviation (RSD) of the repeated measurements ($n = 3$). Precision is described by the RSD, which is the percentage ratio of the standard deviation and the mean. As shown in Table. 4, the accuracy values for the toner-type cosmetics were in the ranges of 81.45%–119.50% (intra-day) and 83.92%–118.71% (inter-day), and the precision values were $\leq 13.79\%$ (intra-day) and $\leq 8.70\%$ (inter-day). For lotion-type cosmetics, the accuracy values were in the ranges of 80.17%–117.68% (intra-day) and 84.44%–118.61% (inter-day), and the corresponding precision values were less than 12.92% and 10.00%, respectively. For the cleanser-type cosmetics, the accuracy values were in the ranges of 82.56%–119.70% (intra-day) and 81.78%–119.83% (inter-day), and the corresponding precision values were $\leq 14.31\%$ (intra-day) and $\leq 12.00\%$ (inter-day), respectively.

3.6 Stability

Stability is an indicator of the reliability of an analyte, pertaining to changes that may occur during pretreatment and analysis. Stability was evaluated using solutions spiked with three concentrations of each analyte for cosmetic matrices. The stability was confirmed within 0 to 48 h after storage at room temperature (25 °C) for 6 h. The RSDs of the peak areas of each component were 12.37% (toner), 14.98% (lotion), and 14.92% (cleanser), and the test solutions were determined to be stable for at least 48 h. The corresponding results are listed in Table 5.

4. Conclusions

In this study, we developed a method for the rapid, sensitive and accurate analysis of 10 therapeutic compounds for atopy in basic cosmetics (toner, lotion) and face cleansers. The developed LC-MS/MS method was validated using the matrices of commercially available cosmetics. Validation parameters (LOD, LOQ, linearity, recovery, precision, accuracy, and stability) were found to be within the appropriate ranges provided by FDA and ICH guidelines, thereby confirming the suitability of the proposed method for multicomponent simultaneous analysis. In further applications of this method to these or similar cosmetic preparations, we recommend the researchers to be sure that their instruments work with good system-suitability parameters, if external standard method will be used. In the distribution of cosmetics in the market, the proposed analytical method could be used to screen illegally included pharmaceutical ingredients. Furthermore, we believe that the proposed method may contribute to the prevention of accidents related to the use of cosmetics.

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Conflict of interest statement

There are no conflicts to declare.

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Figure 1. MS² spectra of (A) tacrolimus ([M+Na]⁺; m/z 826.4732) and (B) pimecrolimus ([M+Na]⁺; m/z 832.4393) using LC-Q-TOF-MS in positive ion mode.

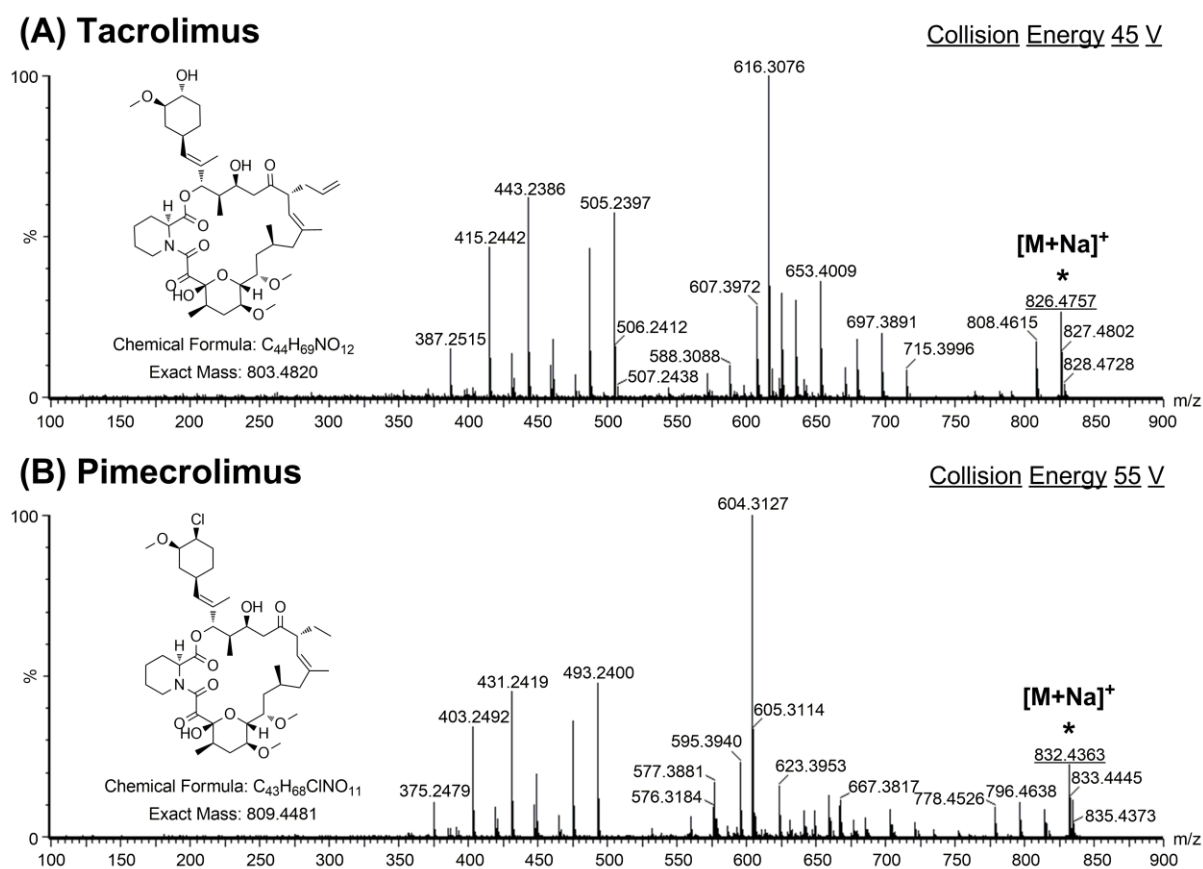


Figure 2. MRM chromatogram of 10 therapeutic compounds spiked at LOQ levels in the lotion matrix. The line below the peak is the signal of the lotion blank matrix. Peak 1: Ketotifen (MRM 310.24 > 96.07, 3.07 min), Peak 2: Chlorpheniramine (MRM 275.28 > 230.15, 3.10 min), Peak 3: Doxepin (MRM 280.29 > 107.05, 3.89 min), Peak 4: Azelastine (MRM 382.30 > 112.12, 4.49 min), Peak 5: Bufexamac (MRM 224.22 > 107.05, 4.90 min), Peak 6: Clotrimazole (MRM 277.19 > 165.09, 5.03 min), Peak 7: Tranilast (MRM 326.19 > 266.09, 6.20 min), Peak 8: Tacrolimus (MRM 826.64 > 616.38, 7.32 min), Peak 9: Fusidic acid (MRM 515.42 > 455.35, 7.43 min), Peak 10: Pimecrolimus (MRM 832.61 > 604.38, 7.45 min).

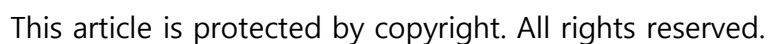
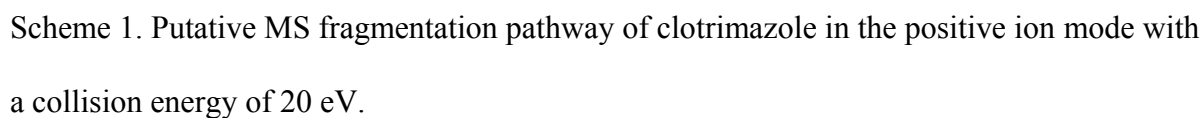


Table 1. Optimized parameters for the MRM transitions of 10 therapeutic compounds for atopy in LC-MS/MS.

Compound	Chemical formula	Ion mode	RT (min)	Precursor ion (m/z)	Cone voltage (V)	Product ion (m/z)	Collision energy (V)
Chlorphenir-amine	C ₁₆ H ₁₉ ClN ₂	+	3.10	275.27	15	167.11	35
						201.07	35
						230.13 [*]	15
Ketotifen	C ₁₉ H ₁₉ NOS	+	3.07	310.24	25	96.07 [*]	20
						213.11	25
Doxepin	C ₁₉ H ₂₁ NO	+	3.86	280.29	25	107.05 [*]	20
						202.12	30
						220.15	25
Azelastine	C ₂₂ H ₂₄ ClN ₃ O	+	4.51	382.30	30	112.10 [*]	25
						159.06	40
						271.15	30
Bufexamac	C ₁₂ H ₁₇ NO ₃	+	4.89	244.25	15	107.07 [*]	20
						163.14	10
						150.08	15
Clotrimazole	C ₂₂ H ₁₇ ClN ₂	+	5.01	277.21	25	165.09 [*]	20
						199.08	25
						241.16	25
Tranilast	C ₁₈ H ₁₇ NO ₅	–	6.19	326.21	25	144.04	20
						266.12 [*]	20
						282.16	15
Fusidic acid	C ₃₁ H ₄₈ O ₆	–	7.43	515.37	35	221.20	25
						393.36	20
						455.36 [*]	20

Tacrolimus	$C_{44}H_{69}NO_{12}$	+	7.30	826.57	40	505.31	35
						616.36 [*]	35
						697.45	35
Pimecrolimus	$C_{43}H_{68}ClNO_{11}$	+	7.46	832.57	40	403.30	50
						431.31	45
						604.35 [*]	35

RT: Retention Time; * Quantitative ion

Table 2. Linearity of the six concentrations, limits of detection (LODs), and limits of quantification (LOQs) of 10 therapeutic compounds for atopy analyzed using LC-MS/MS.

Compound	Blank sample	Linear range (ng/mL)	LOD (pg/mL)	LOQ (pg/mL)
Chlorpheniramine	Toner	0.28–53.87	89.78	269.34
	Lotion	0.16–29.93	49.88	149.64
	Cleanser	0.06–11.97	19.95	59.85
Ketotifen	Toner	0.02–3.03	5.05	15.15
	Lotion	0.02–3.03	5.05	15.15
	Cleanser	0.02–3.00	5.05	15.15
Doxepin	Toner	0.10–18.04	30.06	90.18
	Lotion	0.10–18.04	30.06	90.18
	Cleanser	0.06–12.02	20.04	60.12
Azelastine	Toner	0.16–30.00	50.00	150.00
	Lotion	0.32–60.00	100.00	300.00
	Cleanser	0.25–48.00	80.00	240.00
Bufexamac	Toner	0.16–30.05	50.08	150.24
	Lotion	0.16–30.05	45.07	135.21

	Cleanser	0.10–18.03	30.05	90.15
Clotrimazole	Toner	0.06–12.07	20.11	60.33
	Lotion	0.06–12.07	20.11	60.33
	Cleanser	0.03–6.04	10.06	30.18
Tranilast	Toner	0.32–60.00	100.00	300.00
	Lotion	0.32–60.00	100.00	300.00
	Cleanser	0.32–60.00	100.00	300.00
Fusidic acid	Toner	0.64–121.98	203.30	609.90
	Lotion	0.64–121.98	203.30	609.90
	Cleanser	0.64–121.98	203.30	609.90
Tacrolimus	Toner	0.16–30.00	50.00	150.00
	Lotion	0.16–30.00	50.00	150.00
	Cleanser	0.16–30.00	50.00	150.00
Pimecrolimus	Toner	0.16–30.38	50.63	151.89
	Lotion	0.11–20.25	30.38	91.14
	Cleanser	0.14–27.34	45.56	136.68

Linear range (6 points): ×1 (low), 5, 14, 48 (medium), 95, 190 (high)

$R^2 \geq 0.999$ (for all compounds)

Table 3. Recovery efficiency of 10 therapeutic compounds for atopy analyzed using LC-MS/MS.

Compound	Spiking	Toner		Spiking	Lotion		Spiking	Cleanser	
	Conc. (ng/mL)	Mean, %	RSD, %	Conc. (ng/mL)	Mean, %	RSD, %	Conc. (ng/mL)	Mean, %	RSD, %
Chlorphenir- amine	0.28	110.14	7.46	0.16	98.78	12.19	0.06	95.07	2.52
	13.47	95.73	1.25	7.48	80.11	14.22	2.99	87.48	2.45
	53.87	88.55	2.53	29.93	84.68	8.40	11.97	99.99	2.81

Ketotifen	0.02	103.23	12.91	0.02	117.50	5.60	0.02	89.14	11.70
	0.76	96.04	4.20	0.76	95.85	3.57	0.75	84.65	1.91
	3.03	91.68	1.98	3.03	91.62	0.92	3.00	89.66	1.35
Doxepin	0.10	98.42	9.30	0.10	83.68	10.59	0.06	90.62	7.95
	4.51	90.62	1.16	4.51	85.07	3.71	3.01	100.41	0.98
	18.04	95.20	0.53	18.04	89.45	2.75	12.02	109.69	0.71
Azelastine	0.16	89.69	4.21	0.32	85.26	1.61	0.25	101.51	1.51
	7.50	82.98	1.68	15.00	90.92	2.68	12.00	101.27	1.53
	30.00	89.00	0.24	60.00	86.35	0.96	48.00	109.61	0.57
Bufexamac	0.16	107.99	11.31	0.16	114.78	1.77	0.10	83.11	1.52
	7.51	95.70	4.96	7.51	99.29	1.32	4.51	94.24	4.06
	30.05	105.65	2.70	30.05	96.05	1.81	18.03	99.00	3.23
Clotrimazole	0.06	105.48	5.29	0.06	89.15	1.78	0.03	107.88	13.71
	3.02	102.28	1.46	3.02	95.52	1.02	1.51	93.98	1.38
	12.07	99.20	0.68	12.07	93.56	1.14	6.04	100.76	0.38
Tranilast	0.32	90.69	4.08	0.32	87.07	5.25	0.32	105.59	9.06
	15.00	94.68	1.65	15.00	91.39	1.36	15.00	101.64	2.49
	60.00	100.69	1.60	60.00	96.64	0.43	60.00	100.34	0.79
Fusidic acid	0.64	80.26	12.69	0.64	99.70	4.80	0.64	95.07	10.88
	30.50	84.34	4.18	30.50	92.79	2.73	30.50	90.69	3.35
	121.98	97.54	1.50	121.98	98.19	0.79	121.98	96.70	0.52
Tacrolimus	0.16	118.98	14.01	0.16	99.23	12.39	0.16	85.92	13.70
	7.50	101.27	1.24	7.50	92.23	1.20	7.50	80.07	0.92
	30.00	94.45	0.72	30.00	99.80	0.90	30.00	80.77	2.96
Pimecrolimus	0.16	85.68	8.75	0.11	94.31	1.63	0.14	96.58	1.33
	7.59	88.33	0.77	5.06	95.73	1.58	6.83	80.75	9.51
	30.38	89.09	0.71	20.25	99.90	0.68	27.34	83.88	0.18

Conc.: Concentration

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Table 4. Intra- and inter-day assay precision and accuracy for 10 therapeutic compounds for atopy in three cosmetic samples ($n = 3$) analyzed using LC-MS/MS.

Compound	Toner					Lotion					Cleanser				
	Conc.	Intra-day		Inter-day		Conc.	Intra-day		Inter-day		Conc.	Intra-day		Inter-day	
	(ng/mL)	Precisi	Accura	Precisi	Accura	(ng/mL)	Precisi	Accura	Precisi	Accura	(ng/mL)	Precisi	Accura	Precisi	Accura
		on	cy	on	cy		on	cy	on	cy		on	cy	on	cy
		(RSD, %)	(%)	(RSD, %)	(%)		(RSD, %)	(%)	(RSD, %)	(%)		(RSD, %)	(%)	(RSD, %)	(%)
Chlorpheniramine	0.28	9.00	107.98	4.82	83.92	0.16	7.73	97.26	4.34	101.99	0.06	11.15	119.70	7.63	83.73
	13.47	0.96	100.87	3.09	95.08	7.48	4.73	97.74	1.11	97.63	2.99	1.54	96.77	0.47	104.98
	53.87	1.44	95.81	2.00	97.29	29.93	4.71	100.86	2.01	102.78	11.97	1.41	100.07	0.50	107.19
Ketotifen	0.02	4.61	118.74	5.60	117.46	0.02	12.72	80.17	1.53	117.21	0.02	14.31	116.90	9.61	109.93
	0.76	3.31	88.09	4.78	91.83	0.76	6.45	86.49	5.23	87.03	0.75	6.18	87.08	3.34	89.62
	3.03	0.67	81.45	4.03	83.96	3.03	1.73	82.33	3.99	85.14	3.00	2.37	90.65	0.87	89.80
Doxepin	0.10	3.80	81.83	3.03	87.34	0.10	7.64	92.13	3.31	89.99	0.06	3.84	99.69	3.43	102.76
	4.51	0.41	88.95	2.66	91.27	4.51	0.47	87.53	2.47	88.73	3.01	0.91	107.38	2.46	109.96
	18.04	0.95	87.63	1.30	88.47	18.04	0.53	84.96	2.28	85.44	12.02	0.56	111.90	0.50	112.44
Azelastine	0.16	3.92	107.10	1.23	118.71	0.32	4.34	82.32	2.86	84.61	0.25	1.91	102.44	2.21	110.07
	7.50	1.58	90.58	4.25	94.33	15.00	3.82	117.68	5.38	118.94	12.00	1.48	101.16	1.41	109.51
	30.00	0.70	84.60	3.90	87.84	60.00	0.67	100.73	4.91	104.28	48.00	0.61	95.01	0.96	95.44
Bufexamac	0.16	12.38	82.58	3.48	81.11	0.16	4.14	117.40	3.33	118.61	0.10	3.57	105.22	12.00	83.52
	7.51	2.22	91.11	3.35	92.87	7.51	1.50	89.03	0.98	89.98	4.51	1.50	112.97	3.28	116.90
	30.05	0.83	90.16	1.89	90.67	30.05	1.32	87.71	1.22	88.95	18.03	1.82	82.56	0.83	81.78
Clotrimazole	0.06	6.32	113.82	8.70	105.35	0.06	4.26	90.15	7.73	97.36	0.03	11.26	107.61	6.03	110.02
	3.02	0.93	97.99	1.85	99.42	3.02	0.62	98.34	1.04	98.51	1.51	0.44	110.95	0.47	111.52
	12.07	1.44	98.31	0.89	98.30	12.07	0.96	95.59	0.53	97.28	6.04	2.34	108.67	0.65	108.72
Tranilast	0.32	6.37	94.36	4.75	94.97	0.32	6.61	80.63	10.0	85.91	0.32	7.21	115.43	6.26	119.83
	15.00	1.31	104.64	3.74	100.31	15.00	6.47	108.86	8.07	86.64	15.00	1.99	107.51	0.22	107.51
	60.00	1.12	104.23	4.36	99.26	60.00	1.73	103.89	6.05	84.87	60.00	0.98	112.83	0.93	111.80
Fusidic acid	0.64	13.79	90.63	4.64	85.62	0.64	3.65	92.67	5.73	108.58	0.64	6.40	107.40	10.22	101.79
	30.50	4.86	100.84	2.66	97.84	30.50	2.12	97.95	3.10	97.67	30.50	3.24	106.23	0.92	105.24

	121.9 8	3.48	98.23	4.74	94.11	121.9 8	1.35	96.85	1.56	95.59	121.9 8	3.47	107.99	2.52	106.93
Tacrolimus	0.16	4.73	92.74	5.15	97.42	0.16	2.58	105.33	2.21	94.00	0.16	12.67	112.79	1.81	113.78
	7.50	3.22	93.58	4.33	95.42	7.50	2.32	88.50	1.93	89.67	7.50	1.95	95.01	3.16	98.56
	30.00	1.01	97.06	5.10	96.29	30.00	2.14	87.43	2.31	89.14	30.00	0.41	103.23	3.90	105.35
Pimecrolimus	0.16	4.67	110.43	3.64	114.15	0.11	12.92	90.49	9.88	84.44	0.14	10.9	104.21	4.53	108.02
	7.59	3.37	111.29	2.70	109.88	5.06	1.80	112.53	5.55	106.64	6.83	4.61	95.83	1.42	97.26
	30.38	0.85	119.50	4.11	115.69	20.25	2.00	106.42	1.97	108.49	27.34	0.56	105.94	1.80	107.10

Table 5. Stability of 10 therapeutic compounds for atopy over 48 h as determined by LC-MS/MS.

Compound	Conc. (ng/mL)	Toner (RSD, %)			Conc. (ng/mL)	Lotion (RSD, %)			Conc. (ng/mL)	Cleanser (RSD, %)		
		0 h ¹⁾	24 h	48 h		0 h ¹⁾	24 h	48 h		0 h ¹⁾	24 h	48 h
Chlorpheniramine	0.28	3.61	0.53	0.93	0.16	7.46	3.38	9.39	0.06	3.09	7.12	12.10
	13.47	0.11	3.32	5.58	7.48	3.24	7.63	8.53	2.99	6.28	0.13	2.09
	53.87	0.37	2.22	2.47	29.93	4.24	2.40	3.80	11.97	5.53	9.94	11.00
Ketotifen	0.02	9.84	5.01	11.52	0.02	12.07	3.86	7.52	0.02	6.31	9.64	13.28
	0.76	3.76	0.37	2.16	0.76	3.34	9.51	14.08	0.75	0.45	12.20	14.06
	3.03	3.67	4.76	3.61	3.03	5.42	1.79	7.79	3.00	6.83	12.74	13.66
Doxepin	0.10	11.78	6.00	8.11	0.10	8.21	10.43	13.09	0.06	2.06	5.61	9.17
	4.51	5.89	4.72	2.48	4.51	1.25	11.97	14.98	3.01	2.17	7.61	11.32
	18.04	6.88	5.47	4.66	18.04	0.59	9.10	12.77	12.02	4.28	11.81	14.92

Azelastine	0.16	1.96	2.00	6.57	0.32	2.25	4.33	11.9	0.25	5.08	5.63	8.47
								0				
	7.50	8.39	3.52	0.10	15.00	1.53	11.8	13.8	12.00	0.15	8.05	7.83
Bufexamac	30.00	7.52	4.60	3.15	60.00	2.01	6.13	8.91	48.00	3.02	12.2	13.6
											2	8
	0.16	0.00	1.80	8.74	0.16	11.0	2.94	9.64	0.10	1.12	7.89	1.65
Clotrimazole	7.51	3.71	4.81	3.60	7.51	4.97	6.61	12.6	4.51	2.97	9.56	13.8
								2				1
	30.05	0.73	3.27	2.28	30.05	1.89	1.85	10.1	18.03	1.09	5.85	9.84
Tranilast	0.06	6.34	8.83	7.45	0.06	0.70	5.75	12.0	0.03	4.31	5.46	14.8
								6				0
	3.02	9.10	8.66	8.73	3.02	3.46	11.1	14.8	1.51	2.49	8.10	14.0
Fusidic acid							5	4				7
	12.07	9.07	8.10	8.87	12.07	1.13	7.46	12.5	6.04	2.52	9.95	13.6
								5				7
Tacrolimus	0.32	9.43	11.5	10.1	0.32	9.43	11.5	10.1	0.32	14.4	4.56	8.84
			7	9			7	9		0		
	15.00	2.27	7.80	5.37	15.00	2.27	7.80	5.37	15.00	5.13	9.95	14.5
Azelastine												1
	60.00	2.57	6.71	7.68	60.00	2.57	6.71	7.68	60.00	2.47	7.49	14.5
												7
Bufexamac	0.64	4.33	12.2	10.9	0.64	4.33	12.2	10.9	0.64	4.33	5.71	4.33
			4	8			4	8				
	30.50	8.58	5.35	5.21	30.50	8.58	5.35	5.21	30.50	2.83	3.02	10.5
Clotrimazole												0
	121.9	6.50	0.93	0.78	121.9	6.50	0.93	0.78	121.9	0.26	3.98	10.6
	8				8				8			9
Tranilast	0.16	12.3	1.02	11.5	0.16	8.58	11.3	9.18	0.16	6.56	11.4	4.95
		7		1			2				8	
	7.50	6.24	5.86	7.50	7.50	3.58	0.08	1.05	7.50	0.26	5.71	3.43
Fusidic acid	30.00	5.52	5.29	5.89	30.00	1.41	0.68	1.17	30.00	1.43	5.04	3.38

Pimecrolimus	0.16	1.05	11.0 8	9.87	0.11	11.8 1	6.35	5.86	0.14	1.05	11.0 8	9.87
	7.59	3.53	2.21	1.60	5.06	11.0 2	6.44	1.19	6.83	0.46	7.74	6.71
	30.38	4.95	4.46	3.69	20.25	5.03	1.10	2.08	27.34	1.43	3.59	4.84

0 h¹⁾: after storage at room temperature for 6 h