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Determination of Fluticasone Propionate in Nasal Sprays by a Validated Stability-Indicating MEKC Method

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Abstract

A micellar electrokinetic chromatography method (MEKC) is developed and validated for the analysis of fluticasone propionate (FP) in nasal sprays. The MEKC method is performed on a fused-silica capillary (50 µm i.d.; effective length, 40 cm). The background electrolyte consists of 25 mM borate and 25 mM anionic detergent SDS solution at pH 9. The capillary temperature is maintained at 35°C, and the applied voltage is 20 kV. The injection is performed using the hydrodynamic mode at 50 mbar for 6 s with detection at 238 nm. The method is linear in the range of 2–80 µg/mL (r^2 = 0.9956). The specificity and stability-indicating capability are proven through forced degradation studies inclusive by mass spectrometry, which also shows that there is no interference of the excipients. The limit of detection and limit of quantitation are 0.56 and 2 µg/mL, respectively. Moreover, method validation demonstrates acceptable results for accuracy, precision, and robustness. The proposed method was successfully applied for the quantitative analysis of FP nasal sprays, and the results were compared to a validated reversed-phase liquid chromatographic method, showing non-significant difference (P > 0.05).

Introduction

The corticosteroids are natural and synthetic compounds structurally related to hydrocortisone, which bind to a single class of endogenous corticosteroid receptors involved in anti-inflammatory activity (1).

Fluticasone propionate (FP) (Figure 1) is a trifluorinated glucocorticoid based on the androstane nucleus with potent anti-inflammatory activity. Clinically, it is used intranasally as effective therapy for seasonal and allergic perennial rhinitis, and by inhaled route for the management of asthma (2,3). Rhinitis is one of the major chronic upper respiratory tract disorders, recognized to substantially affect quality of life and to impair both physical and cognitive functioning in adults (4,5).

A radioimmunoassay with solid-phase extraction (SPE) method was used to determine the concentrations of FP in plasma in the early phases of the clinical trials (6). The liquid chromatography–tandem mass spectrometry (LC–MS–MS) method with atmospheric pressure chemical ionization (APCI) were developed and validated for the quantitation of FP in human plasma using a combination of protein precipitation with SPE and also coupled to an automated SPE system over the concentration range of 25–500 pg/mL (7,8). A sensitive and selective LC–APCI-MS–MS method was also validated to quantify FP at levels lower than 10 pg/mL (9). Besides, a LC–MS–MS method with electrospray ionization (ESI) using automated SPE was also developed to monitor systemic concentrations of inhaled FP at therapeutic doses (10). An LC–ESI-MS–MS method was developed for the simultaneous detection and quantification of the 14 most frequently used synthetic corticosteroids, including FP, in human serum, plasma, urine, and tablets (11). A reversed-phase liquid chromatographic method (RP-LC) method was developed and validated for the simultaneous determination of salmeterol xinafoate and FP in combined pressurized metered dose inhaler using a RP ODS-2 base-deactivated column, showing the application for the quantification of basic, acidic, and neutral compounds (12). A RP-LC method for the analysis of nasal spray formulations was also studied and performed on a RP ODS-2 column including FP only in a chromatogram (13). Recently, a RP-LC method was developed and validated using a CLC-ODS column with UV detection at 240 nm and applied for the determination of FP in pharmaceutical formulations, showing high sensitivity and fast analysis time (14).

Capillary electrophoresis (CE) has emerged as a powerful analytical technique for pharmaceutical analysis. It allows the determination of the active pharmaceutical ingredients, impurities, and chiral analysis and has some advantages related to the existing methodologies (15,16). Presently, there is no published CE method for the determination of the drug. The aim of the present study was to develop and validate a stability-indicating

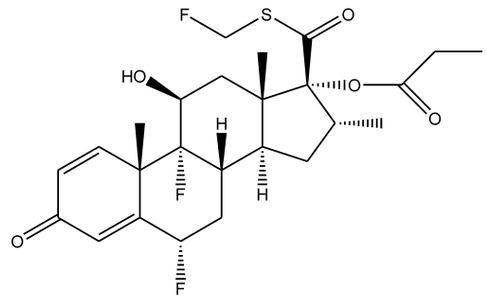

Figure 1. Chemical structure of fluticasone propionate.

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micellar electrokinetic chromatography (MEKC) method for the determination of FP in nasal sprays, establishing comparison with the validated RP-LC method, and contributing to the research of new alternatives with advantages for the quality control of pharmaceutical formulations.

Experimental

Chemicals and reagents

The FP reference substance was supplied by Sigma Aldrich (St. Louis, MO), and prednisolone acetate (PA) reference substance (IS) was purchased from United States Pharmacopoeia (Rockville, MD). A total of four batches of Flixonase (GlaxoSmithKline, Rio de Janeiro, Brazil) nasal sprays, containing 50 µg of fluticasone propionate per dose, were obtained from commercial sources within their shelf-life period and identified by Arabic numbers from 1 to 4. Ultrapure SDS and analytical-grade boric acid were acquired from Bio-Rad Labs (Hercules, CA) and Merck (Darmstadt, Germany), respectively. All chemicals used were of pharmaceutical- or special analytical-grade. For all of the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Bedford, MA). All solutions were degassed by ultrasonication (Tecnal, São Paulo, Brazil) and filtered through a 0.22-µm Millex filter (Millipore).

Apparatus

CE experiments were performed on an Agilent ^{3D}CE apparatus (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a photodiode array (PDA) detector, a temperature controlling system (4–60°C), and power supply able to deliver up to 30 kV. CE ChemStation software was used for instrument control, data acquisition and analysis. The pH of the solutions was measured by a pH meter (Thermo Orion, Beverly, MA).

The RP-LC method was carried out on a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A$_{VP}$ system controller, a LC-10 AD$_{VP}$ pump, a DGU-14A degasser, a SIL-10AD$_{VP}$ autosampler, and a SPD-M10A$_{VP}$ PDA detector. The peak areas were integrated automatically by computer using a Shimadzu Class VP V 6.14 software program.

The mass spectrometry (MS) experiments were performed on a triple quadrupole mass spectrometer Quattro LC (Micromass, Manchester, UK) equipped with an atmospheric pressure chemical ionization (APCI) source in positive mode, set up in scan mode, using a Masslynx (version 3.5) software program. A syringe pump (KD Scientific, Holliston, MA) was used to infuse the samples.

Preparation of reference substance solutions

The reference substance solutions were prepared by accurately weighing 10 mg of FP and PA reference substances, transferring the solution to individual 10-mL volumetric flasks, and diluting to volume with acetonitrile, obtaining concentrations of 1 mg/mL. The stock solutions were stored at 2–8°C, protected from light, and diluted daily to an appropriate concentration with background electrolyte (BGE) solution.

Preparation of sample solutions

The sample solutions of FP nasal spray were prepared by weighing accurately 3.2 g of the formulation equivalent to 1600 µg of FP and diluting to the final volume of 10 mL in a volumetric flask with acetonitrile. Then the mixture was vortex mixed for 2 min, sonicated for 5 min, and centrifuged at 5000 × *g* for 15 min. The sample solution prepared with the final concentration of 160 µg/mL of the FP was stored at 2–8°C, protected from light, and diluted daily to an appropriate concentration with BGE. The sample was injected, and the amount of the drug was calculated against the reference substance.

Preparation of BGE solution

The optimized BGE solution was prepared by separately weighing boric acid and SDS quantities, respectively equivalent to 25 mM and dissolving in 40 mL of ultrapure water. The pH was adjusted to 9 by adding 0.1 M sodium hydroxide and the volume completed to 50 mL with ultrapure water. The solution was daily prepared and filtered.

Electrophoretic procedure

All experiments were carried out on a fused-silica capillary with 50 µm i.d. and 48.5 cm of total length (effective length 40 cm), thermostatized at 35°C, and with detection set at 238 nm using a PDA detector. At the beginning of each working day, the capillary was conditioned by rinsing with 0.1 M sodium hydroxide for 20 min, followed by water for 20 min, and then with running electrolyte solution for 20 min. To achieve high migration time reproducibility between injections, the capillary was conditioned with 0.1 M sodium hydroxide (2 min), water (1 min), and a running BGE solution (5 min). Samples were injected using the hydrodynamic injection for 6 s at 50 mbar, and a constant voltage of 20 kV (current about 16 µA) was applied during the analysis. Because electrolysis can change the electroosmotic flow (EOF) and affect the migration time, efficiency, and selectivity, the running electrolyte solution was replaced by a fresh solution after each three injections.

RP-LC procedure

The validated RP-LC method applied for the analysis of FP in nasal sprays is described elsewhere (14). The elution was carried out on a RP Shimadzu (Kyoto, Japan) Shim-pack CLC-ODS column (150 mm × 4.6 mm i.d., particle size 4 µm). A security guard holder was used to protect the analytical column. The Shimadzu LC system was operated isocratically at controlled temperature of 35°C using a mobile phase of acetonitrile–methanol–phosphate buffer (0.01 M, pH 4) (35:35:30, v/v/v) run at a flow rate of 1.0 mL/min and using PDA detection at 240 nm. The injection volume was 20 µL of the solutions, containing 50 µg/mL for both the reference substance and the samples.

Mass spectrometry procedure

The mass spectrometer conditions were optimized with the direct injection of the FP reference solution (1000 ng/mL) into the system. The best response was obtained with a corona potential of 3.5 kV, a cone voltage of 25 V, a RF lens voltage of 0.6 V, a source temperature of 130°C, and APCI probe temperature of 450°C, respectively. Before the analysis, the samples were diluted

to 1:10 in methanol–water (80:20, v/v) and introduced into the MS by direct infusion at 10 µL/min. The mass spectrometry data were acquired in the *m/z* range between 50 and 600.

Validation of the MEKC method

The method was validated using samples of pharmaceutical formulation of nasal spray by determinations of the following parameters: specificity, linearity, range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness, stability, and system suitability test, according to the International Conference on Harmonization (ICH) guidelines (17,18).

The stability-indicating capability of the method was determined by subjecting a reference substance solution (200 µg/mL) to accelerated degradation by acidic, basic, neutral, oxidative, and photolytic conditions to evaluate the interference in the quantitation of FP. A sample solution prepared in 0.1 M hydrochloric acid was used for acidic hydrolysis and a sample solution in 0.1 M sodium hydroxide for the basic hydrolysis evaluation. Both solutions were maintained at ambient temperature for 6 h and neutralized with acid or base as necessary. For the study under neutral condition, the reference solution was diluted in water and heated at 80°C for 4 h. Oxidative degradation was induced by storing the solutions in 5% hydrogen peroxide at ambient temperature for 24 h and protected from light. Photodegradation was induced by exposing the sample to 200 Wh/m^2 of near-UV light for 24 h. The solutions were diluted with the electrolyte solution to final concentrations of 40 µg/mL. The interference of the excipients of the pharmaceutical formulation was determined by the injection of a sample containing only placebo (in-house mixture of all the spray excipients) and a sample containing placebo added with FP at a concentration of 40 µg/mL. Then, the specificity of the method was established by determining the peak purity of FP in the degraded samples using a PDA detector. Additionally, the FP reference standard and the degraded samples were respectively analyzed by MS.

Linearity was determined by constructing three calibration curves each one with seven calibration points of FP, including the LOQ, in the range of 2–80 µg/mL prepared in BGE solution. Three replicate injections of each reference substance solution spiked with PA 40 µg/mL as IS were made to verify the repeatability of the detector response. The peak-area ratio of FP reference substance to the IS, against the respective reference concentrations, was used for plotting the graph, and the linearity was evaluated by the least square regression analysis.

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by nine evaluations of the same concentration sample of FP on the same day and under the same experimental conditions, calculating the relative standard deviation (RSD) of the migration time and peak area ratio for the concentration of 40 µg/mL. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis on the same day and in the same laboratory (between-analysts).

The accuracy was evaluated by applying the proposed method to the analysis of an in-house mixture of the tablet excipients with known amounts of each drug to obtain solutions at concentrations of 32, 40, and 48 µg/mL, equivalent to 80, 100, and 120% of the analytical concentrations, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

The LOQ and the LOD were calculated from the slope and the standard deviation of the intercept of the mean of three calibration curves, determined by a linear regression model as defined by ICH (17). The LOQ was also evaluated in an experimental assay.

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis (19,20). The robustness was determined by analyzing the same samples of the FP reference solution containing 40 µg/mL in triplicate by the one-variable-at-a-time approach, changing electrolyte composition (± 1 mM), capillary temperature (± 2ºC), voltage applied (± 1 kV), injected time (± 1 s), and detection wavelength (± 2 nm).

To assess the stability of FP, the stability studies were performed maintaining the FP reference solution in acetonitrile for two months at 2–8°C, protected from light. The stability of FP in BGE was assessed after the storage of the samples for 48 h at 2–8°C and placed in the autosampler for 24 h at room temperature.

A system suitability test was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed using five replicates injections of a reference substance solution containing 40 µg/mL of FP. The parameters measured were peak area, retention time, theoretical plates, and tailing factor (peak symmetry).

Analysis of FP in nasal sprays

For the quantitation of FP in the nasal sprays, the respective stock solutions were diluted to appropriate concentration with BGE, filtered, injected in triplicate. The percentage recoveries of the drug were calculated against the reference substance.

Results and Discussion

Optimization of the electrophoretic conditions

To compensate for injection errors and improve the reproducibility of the MEKC method, several drugs including betamethasone acetate, hydrocortisone acetate, clobetasol propionate, and deflazacort were tested as IS for validation procedures of FP. Prednisolone acetate, also a corticosteroid, was selected as IS due to its suitable peak resolution and migration time.

To obtain the optimum capillary electrophoretic conditions, some electrolyte solutions containing sodium phosphate, sodium citrate, sodium acetate, sodium tetraborate, or boric acid, respectively, were investigated by adding the surfactant SDS for the solubilization of the analyte by the micelles in the pH range of 4–10.5. Best results were obtained with the borate solution. The pH of the BGE solution containing 25 mM boric acid and SDS, which affects the ionization of the analyte, and the magnitude of the EOF was evaluated as shown in Figure 2. The pH 9 was selected because higher pHs resulted in decrease of the migration time and peak width but not with peak asymmetry. The effect of BGE concentration was studied in the range from

15 to 40 mM at a constant 25 mM SDS concentration at pH 9. As the migration time increased with the increase of the borate solution concentration, the 25 mM borate solution was selected. Then, the influence of the SDS was investigated (Figure 3), which showed that the migration time of FP increased with the increase of the concentrations due to the interaction of the analyte with the micelles, achieving better efficiency combined with short analysis time with 25 mM SDS. To investigate the effect of organic modifiers, 5–20% (v/v) concentrations of methanol and acetonitrile were added to the 25 mM borate and 25 mM SDS solution (pH 9), showing no improvements. The effect of the voltage was investigated from 10 to 30 kV, which showed shorter analysis time, better separation efficiencies, and higher currents with up to 20 kV potential, giving also the best peak parameters and acceptable current (about 16 µA) without Joule heating. The capillary temperature was investigated within 20–45°C, and the temperature of 35°C was chosen. The sample solutions were hydrodynamically injected at 50 mbar, changing the injection time between 3 to 8 s. This showed increased peak width and shape deformed after 6 s, so 6 s was selected.

As demonstrated, the MEKC method parameters were optimized to obtain the best peak area, resolution, symmetry, and short migration time with 25 mM borate and 25 mM SDS solution at pH 9 with detection at 238 nm. The electropherogram of FP reference substance and IS with migration times of 5.6 and 5.1 min, respectively, is shown in Figure 4.

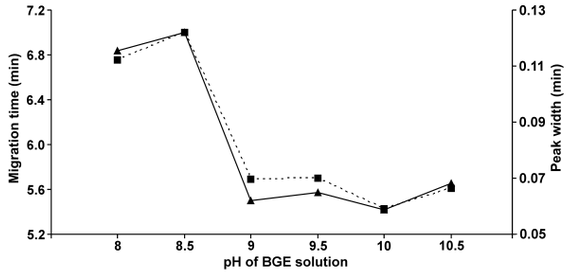

Figure 2. Effect of BGE solution pH on migration time (■) and peak width (▲) of FP. Operating condition: 25 mM borate and 25 mM SDS solution (pH 9), hydrodynamic injection (6 s at 50 mbar), 20 kV, 35ºC, and 238 nm. FP: 40 µg/mL.

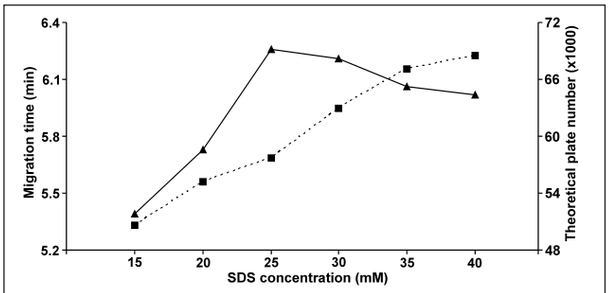

Figure 3. Effect of SDS concentration on migration time (■) and theoretical plate number (▲) of FP.

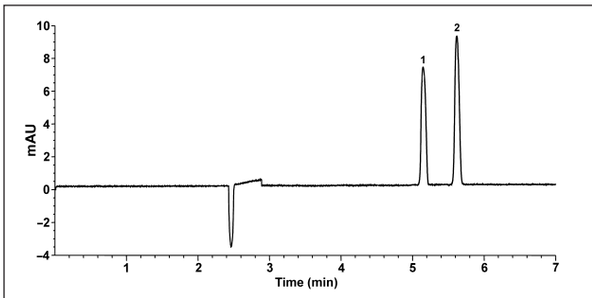

Figure 4. Representative electropherogram of a reference substance solution containing 1, prednisolone acetate (IS) and 2, fluticasone propionate (FP) at a concentration of 40 µg/mL.

Method validation

A stability-indicating method is defined as an analytical method that accurately quantifies the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities (21). The stressed acidic condition (Figure 5A) showed significant decrease of the peak area without any additional peak as well the basic condition (data not shown). The neutral heated degradation hydrolysis also showed significant decrease at 4 h without any additional peak (Figure 5B). Under the photolytic condition (Figure 5C), nearly 51% of the FP was degraded, but no additional peak was identified. For the oxidative condition (Figure 5D), 76% of the FP was degraded at 24 h without any additional peak. The basic and photolytic conditions showed, respectively, and an additional peak was detected by RP-LC method (14), indicating that the degradation products did not migrate in the MEKC method. Moreover, if or when present they will not interfere in the analysis as the specificity of the method towards the drug was also established through determinations of peak purity of the analyte obtained by

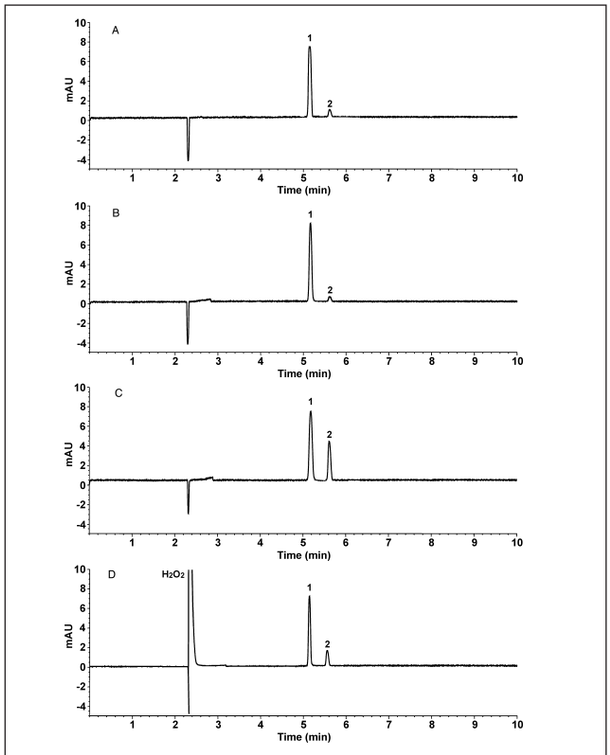

Figure 5. MECK electropherograms of FP. After: acidic hydrolysis (A), neutral heated hydrolysis (B), photodegradation condition (C), oxidative condition (D). Peak 1, prednisolone acetate (IS); peak 2, fluticasone propionate.

overlay of the spectra captured at the apex, upslope, and downslope using a PDA detector. The FP reference substance and degradation samples were also analyzed by APCI-MS in the positive mode, but the degraded products were not ionizated/detected or their molecular weights were out of the lower mass range used in the study. Besides, no interference from formulation excipients, mainly benzalkonium chloride and phenylethyl alcohol, was demonstrated, indicating that the peak was free from any coeluting peak, peak purity index values in the range of 0.999–1, confirming that the proposed method is specific for the analysis of FP.

The calibration curves constructed for FP were found to be linear in the 2–80 µg/mL range. The value of the calculated determination coefficient (r^2 = 0.9956, y = 0.0341 ± 0.0017x – 0.0706 ± 0.0064, where x is concentration and y is the peak area ratio of FP to IS) indicated the linearity of the calibration curve for the method.

The precision, evaluated as the repeatability of the method, was studied by obtaining RSD values of 0.22 and 0.84% for the migration time and the peak-area ratio, respectively. The intermediate precision was assessed by analyzing two samples of the nasal sprays on three different days (inter-days) giving mean values of 99.86 and 99.87% with RSD and 0.73 and 1.17%, respectively. Between-analysts precision was determined by calculating the mean values and the RSD for the analysis of two samples of the nasal spray by three analysts; the values were found to be 100.06 and 99.82% with RSD, 1.35 and 0.96%, respectively. The results are shown in Table I.

The accuracy was assessed from three replicate determinations of the three different solutions, and the absolute means obtained for FP are shown in Table II with a mean value of 100.37% and bias lower than 1.47%, demonstrating that the method is accurate within the desired range (22).

Table II. Accuracy of MEKC for FP in Samples of Nasal Spray

Nominal conc. (µg/mL)	Mean conc. found* (µg/mL)	RSD† (%)	Accuracy (%)	Bias‡ (%)
32	32.21	1.77	100.67	0.67
40	40.59	1.05	101.47	1.47
48	47.50	0.43	98.96	–1.04

* Mean of three replicates. † RSD = relative standard deviation.
‡ Bias = [(measured conc. – nominal conc.) / nominal conc.] × 100.

Table I. Inter-day and Between-analysts Precision Data of MEKC for Fluticasone Propionate in Samples of Nasal Spray

	Inter-day			Between-analysts		
Sample	Day	Recovery* (%)	RSD† (%)	Analysts	Recovery* (%)	RSD† (%)
1	1	99.01	0.73	A	98.62	1.35
	2	100.25		B	100.27	
	3	100.31		C	101.30	
2	1	100.27	1.17	A	99.08	0.96
	2	98.55		B	99.49	
	3	100.79		C	100.90	

* Mean of three replicates. † RSD = relative standard deviation.

For calculating the LOD and LOQ, the calibration equations were generated by using the mean values of the three independent calibration curves. The obtained values were 0.56 and 1.86 µg/mL, respectively. The LOQ, evaluated experimentally, with a precision lower than 5% and an accuracy within ± 5% (23,24), was found to be 2.0 µg/mL, suitable for the quality-control analysis, but the low sample injection volume and the short optical path-length can be related to the low sensitivity of the MEKC method.

The results and the experimental range of the selected variables evaluated in the robustness assessment are given in Table III with the optimized values. There were non-significant changes of the results and in the electropherogram pattern. Testing was also done with different capillary batches, indicating that the proposed method is robust under the conditions tested.

Table III. MEKC Conditions and Range Investigated for Robustness Testing

Variable	Range investigated	FP peak area ratio FP* (%)	FP peak area ratio RSD† (%)	FP migration time (min)	Optimized condition
Electrolyte solution pH	8.9	99.66	0.39	5.52	9
	9	100.14	0.55	5.62	
	9.1	99.96	0.63	5.67	
Electrolyte solution conc. (mM)	24	100.48	0.26	5.60	25
	25	100.12	0.47	5.62	
	26	99.41	1.07	5.64	
SDS conc. (mM)	24	100.64	1.87	5.57	25
	25	99.46	0.45	5.61	
	26	101.23	0.74	5.64	
Temp. (°C)	33	98.74	1.80	5.65	35
	35	100.38	0.62	5.62	
	37	100.82	1.21	5.59	
Voltage (kV)	19	98.18	0.83	5.66	20
	20	100.25	0.53	5.62	
	21	100.43	1.35	5.58	
Time injection (s)	5	98.75	1.68	5.61	6
	6	100.49	0.71	5.62	
	7	101.49	1.72	5.64	
Wavelength (nm)	236	100.75	0.94	5.62	238
	238	101.02	0.65	5.62	
	240	100.40	1.82	5.62	

*Mean of three replicates.
† RSD = relative standard deviation.

Table IV. Comparison Between MEKC and RP-LC Methods Applied for the Analysis of FP in Nasal Sprays

Theoretical amount		Experimental amount MEKC*			Experimental amount RP-LC*		
Sample	µg/dose	µg	%	RSD† (%)	µg	%	RSD† (%)
1	50	51.72	103.45	0.98	50.76	101.51	0.29
2	50	50.60	101.02	0.77	49.46	98.93	0.43
3	50	51.15	102.31	1.02	50.20	100.41	0.54
4	50	49.06	98.12	0.83	48.04	96.08	0.31

* Mean of three replicates. † RSD = relative standard deviation.

The stability studies showed the stability of the working solution with loss of content lower than 0.5% for two months. The stability of FP in BGE was assessed after the storage of the samples for 48 h at 2–8°C and placed into the autosampler for 24 h at room temperature. It showed non-significant change (< 2%) relative to freshly prepared samples as suggested (25).

The system suitability test carried out for the migration time, peak area and peak symmetry showed RSD values of 1.04, 1.49, and 1.92%, respectively. The number of theoretical plates was about 63,954 with RSD of 1.45%. The parameters tested were within the acceptable range (RSD < 2.0%), indicating that the method is suitable for the analysis intended.

Method application

The validated MEKC method was applied for the determination of FP in nasal sprays, and the results compared to those obtained using a validated RP-LC method gave a mean value 1.97% higher as shown in Table IV. The experimental values of the two methods were compared statistically by ANOVA, showing non-significant difference ($P > 0.05$). The LOQ found by RP-LC method is forty times more sensitive than the MEKC method, but the proposed method can be useful for the determination of FP without prior separation of the excipients of the formulation with the added advantage of small sample volumes, without consumption of organic solvents and short analysis time. The application for the analysis of spiked human plasma samples after extraction procedure (7) was also considered, but due to the very low levels of FP achieved, the more sensitive existing methods by mass spectrometry are useful.

Conclusion

The results of validation studies show that the MEKC method with a LOQ of 2 µg/mL is accurate with a mean value of 100.37% and bias lower than 1.47%. It is also economic, specific, and stability-indicating. It possesses significant linearity ($r^2 = 0.9956$) and precision of the peak area ratio with a RSD of 0.84% without any interference from the excipients or degradation products as demonstrated also by MS studies. Therefore, the proposed method was successfully applied for the quantitative analysis of FP in pharmaceutical formulations of nasal sprays, representing an alternative for the quality control and also contributing to assure the therapeutic efficacy of the drug.

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