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Quantitation of active ingredients and excipients in nasal sprays by high-performance liquid chromatography, capillary electrophoresis and UV spectroscopy

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Abstract

A study on the use of different analytical methodologies to determine active ingredients and excipients found in commercial nasal sprays is presented. Two of the developed methodologies consisted of separation techniques, i.e. high-performance liquid chromatography and capillary electrophoresis, and the third one involved a UV-spectroscopic multicomponent procedure. The samples studied are characterized by a high viscosity and the existence of a large number of particles in suspension; therefore, special emphasis is paid on the sample preparation required by each methodology. Advantages and drawbacks of each analytical technique are also discussed in terms of speed of analysis, sensitivity and reproducibility. From this work it is observed that although the UV method needs the most laborious sample preparation, the total time required per analysis is the shortest one. The best reproducibility in terms of analysis time and quantitation of the analyzed compounds is obtained using HPLC. CE allows the determination of more components in the same sample.

Keywords: Nasal sprays; Pharmaceutical analysis; Beclomethasone dipropionate; Fluticasone dipropionate; Benzalkonium chloride

1. Introduction

Nowadays, identification and quantification of the different substances that form a pharmaceutical formulation is carried out using a large variety of analytical methods. These methods are described in the appropriate pharmacopoeia and they can indistinctly consist of classical procedures (for example, gravimetric analysis, colorimetric reactions, etc), or analyses involving more sophisticated instrumentation (for instance, spectrometric determinations, chromatographic methods, etc.).

A common problem when analysing pharmaceutical preparations is the different nature of the various substances found in these samples (e.g. salts, carbohydrates, detergents, alcohols, etc). Thus, active ingredients and excipients usually have very different chemical and spectroscopic properties which in many cases makes their simultaneous determination very difficult (or even impossible) using a single analytical procedure. Moreover, the numerous tests (e.g. identity, assay, evaluation) required to study the purity, composition and efficacy of each pharmaceutical formulation have brought about the development of a large number of methodologies available to address each problem. Thanks to this develop-

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ment, the analyst can select the adequate methodology depending on, for example, his experience, available instrumentation at the laboratory, etc. Therefore, comparative studies between different analytical methodologies employed to examine pharmaceutical formulations are useful since they provide suitable information to analysts about advantages and drawbacks of each technique.

In this work, three analytical methodologies (see below) are compared for the determination of different substances found in two commercial nasal sprays, namely Beconase and Flixonase. The substances that are the focus of this study are: (1) the active ingredients beclomethasone dipropionate (BDP) or fluticasone dipropionate (FDP) depending on the nasal spray studied, i.e. Beconase or Flixonase. respectively; (2) a family of surfactants, i.e., benzalkonium chloride (BKC), that act as emulsifiers and preservatives; and (3) phenylethyl alcohol (PEA) that mainly works as a solvent. A brief description of the three techniques employed to determine such substances is given next.

High-performance liquid chromatography (HPLC) has shown to be a valuable analytical tool for the separation and analysis of a large number of pharmaceuticals [1,2]. Among its numerous applications, this technique has been employed for the routine analysis of BDP and related products in pharmaceutical compounds [3]. In this case, the only sample treatment is dilution either with methanol or with the mobile phase to be used. For nasal sprays it would be important to study if the selected conditions used to quantitate the active ingredient also allow the quantitative determination of the other two compounds, BKC and PEA, obtaining in this way additional information about the sample.

The use of capillary electrophoresis (CE) in its different formats has found numerous applications in the separation of small compounds, drugs and metabolites [4–7]. The simplicity of this relatively new technique, together with both its minimal operating costs and separation power have made CE the technique of choice for many applications in the pharmaceutical industry. In this work, a similar CE procedure to that mentioned elsewhere [8] is employed for the separation and quantitation of several compounds found in a commercial nasal spray. From an electrophoretic point of view, the compounds above mentioned range from positively charged, i.e. BKC, to non-charged, e.g. BDP, this one being a very insoluble compound even in aqueous solutions containing large quantities of sodium dodecyl sulfate (SDS). In a previous paper [8], we have shown the intrinsic difficulties of this technique when applied to the separation of neutral and very insoluble compounds appearing, in the same sample, together with other compounds of high positive electric charge. A good approach to solve this problem seems to be the combined use of high contents of organic solvents and surfactants. It has been shown that by using both types of substances in the separation buffer, the so called separation window in micellar electrokinetic chromatography (MECC) can be enlarged [9,10], and they can increase the solubility of hydrophobic substances while modifying the selectivity of their separation [11–17]. More interestingly, in this work we address the use of this type of 'highly-organic' CE buffers to quantitate compounds from real samples, which to our knowledge has received scarce attention.

Another interesting analytical trend at the present time is the combined use of multicomponent analysis with UV spectroscopy [18–25] applied to determine simultaneously different compounds found in a complex matrix. The main feature of these methods is that they do not require any previous separation for determining the different analytes and they seem to be specially recommended for those cases in which the sample composition can only vary into a relatively narrow range. Besides, much faster analyses can be obtained compared to those given by other analytical procedures involving separation methods. This type of analysis using UV-spectroscopy is usually carried out by multivariate calibration models, such as principal component regression that utilize the full spectrum [19,20]. Nevertheless, it has been recently demonstrated that better results can be obtained by a proper selection of the spectral range to be included into the calculations [21,22]. A correct selection of the spectral range can be done through different procedures based on mathematical methods and algorithms such as principal component regression and classical least-squares [23], simplex methods [24] and others [25]. However, the observation of anomalous results and other error sources [25] makes difficult the application of these procedures in many cases.

The goal of this work is, therefore, to compare

three different analytical methodologies, i.e. HPLC, CE and UV spectroscopy, developed to analyze the composition of commercial nasal sprays. The analytical complexity of this matrix (i.e. high viscosity together with a large number of particles in suspension) makes necessary to establish a first comparison in terms of sample preparation requirements of the three techniques. Moreover, a further comparison is established in terms of speed of analysis, sensitivity, quantification capability and reproducibility obtained with each technique.

2. Experimental

2.1. Instrumentation

2.1.1. HPLC

The HPLC system consists of a CM 4100 quaternary pump, an AS-3000 autosampler, a SCM 1000 membrane degasser and a SM 5000 UV spectromonitor, all of them from TSP (San José, CA, USA). The chromatographic conditions were slightly different to those described in the pharmacopoeia for the analysis of BDP [3]. They were as follows: a 150× 4.6 mm Spherisorb 5 ODS-2 column from Phenomenex (Torrance CA, USA), mobile phase acetonitrile–water (55:45, v/v) at a flow-rate of 1 ml/min. The injected volume was 20 μl and the detection took place at 250 nm. The chromatographic column and the mobile phase were maintained at 25°C.

2.1.2. CE experiments

For the CE experiments a P/ACE System 5000 (Beckman Instruments, Fullerton, CA, USA) electrophoresis apparatus controlled by a Pentium/100 MHz personal computer was used. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 37 cm (30 cm effective length)×75 μm I.D.×360 μm O.D. were used. The temperature of the capillaries was kept at 45°C. The injection was carried at anode using N_2 pressure (0.5 p.s.i.) for 1.6 s (1 p.s.i.=6894.76 Pa). The detection took place at 214 and 254 nm. All the data was collected and analyzed using the System Gold software from Beckman running on the Pentium/100 MHz computer. Solutions containing 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) and surfactant were prepared by calculating the required amounts of CHES and surfactant in Milli-Q water and adding 1 *M* sodium hydroxide until pH 10. All the amounts were weighed in order to obtain, after diluting with the organic solvent, the final concentrations indicated in each case. The CE electrolyte consisted of 50% of buffer 100 m*M* CHES at pH 10, 30% acetonitrile, 20% methanol and 30 m*M* SDS. The buffer was stored at 4°C and equilibrated at room temperature before use.

In order to increase the reproducibility between separations, capillaries were rinsed after each injection with acetonitrile for 1.5 min, water for 0.5 min and separation buffer for 1 min. Capillaries were stored overnight with acetonitrile inside.

2.1.3. UV-spectroscopic method

An HP8452A diode array UV–Vis spectrophotometer and a Chemstation with the G1116AA UV–visible advanced software all from Hewlett-Packard (Palo Alto, CA, USA) were used in all the UV experiments. Multicomponent analysis was performed using first-order derivative mode with a wavelength range of 228–270 nm.

2.2. Samples and chemicals

The nasal sprays Beconase and Flixonase as well as the standards of active ingredients and excipients were a gift from Glaxo Wellcome (Aranda de Duero, Burgos, Spain). Standards were dissolved in acetonitrile (Scharlau, Barcelona, Spain) at the concentrations indicated in each case and stored at −4°C. SDS and methanol were from E. Merck (Darmstad, Germany). CHES was purchased from Sigma (St. Louis, MO, USA).

2.3. Sample preparation

2.3.1. HPLC analysis

1 g of Beconase nasal spray was dissolved in 10 ml of acetonitrile. The mixture was shaken using a vortex mixer for 1 min, then centrifuged for 5 min at 3200 *g*. After sample preparation they were shortly analyzed by directly injecting the supernatant into the HPLC instrument. Similar procedure was followed for the nasal spray Flixonase.

2.3.2. CE analysis

1 g of nasal spray Beconase was weighted and

acetonitrile added to a final volume of 5 ml. The mixture was shaken using a vortex mixer for 1 min, sonicated for 1 min and centrifuged at 8000 rpm for 10 min. After samples preparation they were shortly analyzed by directly injecting the supernatant into the CE capillary. Standards were dissolved in acetonitrile.

2.3.3. UV-multicomponent analysis

2.3.3.1. Blank preparation

All the substances that compose the excipient were weighed and mixed according to the confidential information provided by the manufacturer. The mixture was dissolved in 200 ml of methanol and the suspension was sonicated for 7 min. Next, acetonitrile was added to the mixture to achieve a final volume of 500 ml and the solution was filtered out using a 0.5 μm filter. The filtrate obtained was used for obtaining the spectrum of the blank and for dissolving the standards.

2.3.3.2. Sample preparation

1 ml of sample was dissolved with methanol up to a final volume of 10 ml. The solution was sonicated for 30 s and then acetonitrile added up to 25 ml of final volume. An aliquot of 5 ml was centrifuged at 2000 *g* and the supernatant was used for obtaining the spectrum (compared with the blank).

3. Results

3.1. HPLC results

Fig. 1 shows two chromatograms, one obtained from a standard mixture (Fig. 1A) and the second one obtained from the nasal spray Beconase (Fig. 1B). Peak number 4 at the chromatogram given in Fig. 1A corresponds to fluticasone propionate (FP), the active ingredient found in the spray Flixonase. Peaks 2 and 3 at that figure correspond to PEA and BDP, respectively. Therefore, this HPLC method would be useful to analyze the active ingredient found in both nasal sprays Beconase and Flixonase. As shown in Fig. 1B, the sample treatment applied together with the HPLC separation conditions selected also allows to determine another compound found in both sprays, PEA, eluting at 2.3 min as can be seen in Fig. 1. However, it has to be mentioned that, under these separation conditions, the surfactant BKC could not be detected at the wavelength chosen. It was observed that for detecting that compound, which eluted at 3.8 min, it was necessary to inject a BKC concentration 10 times higher than the normal one found in the nasal spray and to change the wavelength to shorter values, i.e. 205–210 nm, which prevented its determination in the real sample. Fig. 1B shows, therefore, only the separation of BDP and PEA found in the nasal spray Beconase. As can be seen, there is a good match of the elution times of these substances obtained from standards and real samples. When this method was used to analyze the second nasal spray, i.e. Flixonase, similar results to those aforementioned were obtained, that is, PEA and fluticasone dipropionate were detected (data not shown). In order to establish the comparison with the other two techniques we arbitrarily selected the nasal spray Beconase as the target sample to be analyzed.

In Table 1 the results obtained in terms of analysis time reproducibility, quantitative calibration for the three compounds and sensitivity is given. These results, together with those given in Table 2 regarding validation of the HPLC method, are discussed below and compared to those obtained using the other two techniques.

3.2. CE results

In Fig. 2, the electrophoregram obtained from a standard mixture (Fig. 2A) containing BKC, PEA and BDP is compared to that from a commercial nasal spray (Fig. 2B). The use of a complex buffer constituted by SDS, methanol, acetonitrile and CHES acid at pH 10 allowed the solubilization and simultaneous determination of the three compounds by CE. This could be done, besides, due to the detection wavelength was changed during each analysis, i.e. 214 nm for BKC and PEA and 254 nm for BDP. The wavelength change was possible due to this capability being automatically carried out by the CE system. Moreover, as can be seen in Fig. 1, the surfactant BKC was separated in different peaks (mainly peaks 1 and *) which agrees with that observed previously [8,26,27], that is, BKC is a

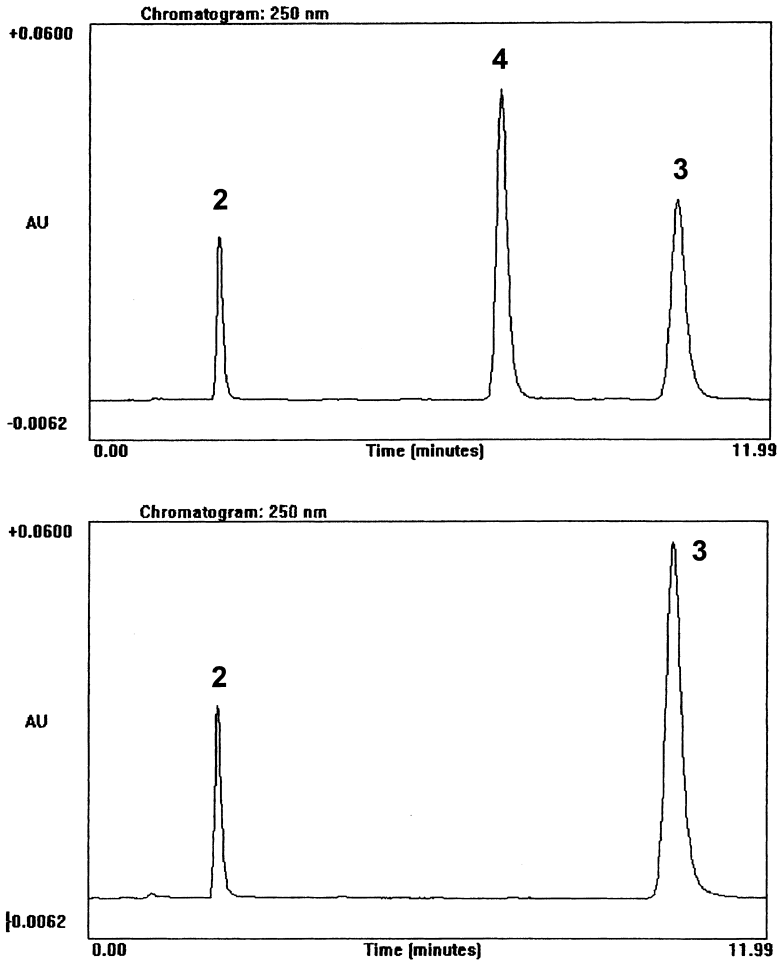

Fig. 1. Chromatograms of: (A) a standard mixture of PEA (2), FP (4) and BDP (3); and (B) a real sample of Beconase spray.

Table 1
HPLC, CE and UV results for separation time reproducibility, quantitative calibration and sensitivity

Parameters	HPLC		CE			UV–Vis	
	PEA	BDP	BKC	PEA	BDP	PEA	BDP
Separation time (min)	2.26	10.35	4.22	7.47	8.26	–	–
R.S.D. (%) (n=6)	0.19	0.07	0.75	0.57	0.61	–	–
Range (mg/l)	185–315	20–70	21–1050	55–1100	40–1000	185–315	20–70
Intercept (b)	−3719	3287	4.98	−3.79	−3.26	0.061	0.004
s_b [a]	±2470	±3611	±3.81	±17.37	±8.07	±0.007	±0.015
Slope (a)	955	19711	470	1354	719	0.0015	0.028
s_a [a]	±9.66	±76	±7.78	±30.9	±15.8	±2e−5	±4e−4
Correlation coefficient	0.9998	0.9999	0.9995	0.9992	0.9993	0.9997	0.9996
LOD[b] (mg/l)	0.50	0.04	3.0	10.0	8.0	1.60	0.20

[a] Standard deviation values of intercept (s_b) and slope (s_a).
[b] Limit of detection.

Table 2
Repeatability, reproducibility and accuracy values obtained for the quantitative determination carried out using HPLC, CE and UV spectroscopy

Parameters	HPLC		CE			UV–Vis	
	PEA	BDP	BKC	PEA	BDP	PEA	BDP
Repeatability R.S.D. (%) (n=8)	1.52	1.21	5.65	1.95	0.90	1.94	1.60
Reproducibility R.S.D. (%) (n=18)	2.65	2.45	6.51	2.50	3.78	3.20	2.92
Accuracy (error, %) (n=6)	1.96	2.07	6.27	4.84	2.90	3.06	4.15

long-chain cationic surfactant constituted of different homologues. At ca. 6.5–7 min a perturbation of the base line can be observed, which corresponds to the time at which the electroosmotic flow appears in both Fig. 2A, B.

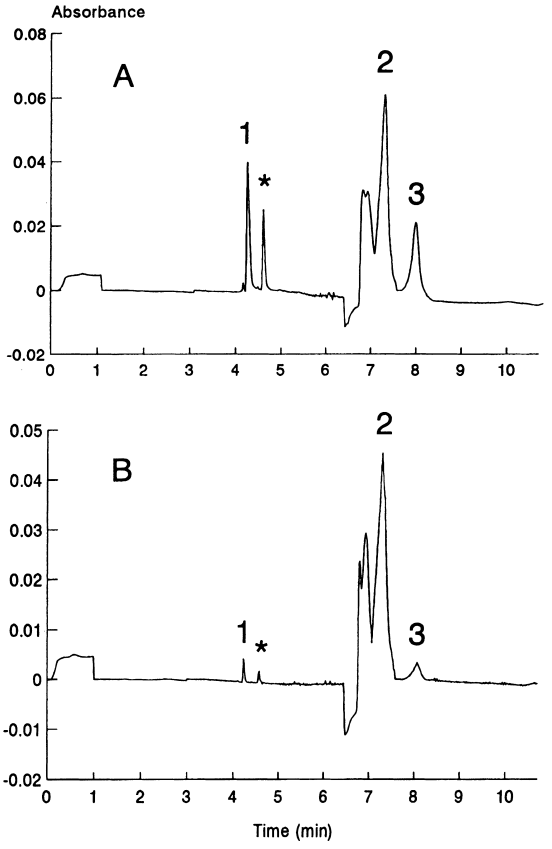

Fig. 2. Separation of BKC (1 and *), PEA (2) and BDP (3) in a standard mixture (A) and from a sample of Beconase spray (B). Injection of BKC (0.53 mg/ml), PEA (0.55 mg/ml) and BDP (0.50 mg/ml). Run voltage 12 kV. Detection UV 214 nm till migration time t_m=7.6 min, 254 nm from t_m=7.6 min to the end of analysis.

The results of the quantitative calibration obtained employing this procedure and using different concentrations of BKC, PEA and BDP are shown in Table 1. In the same table other figures of merit such as separation time reproducibility and sensitivity are summarized. These parameters together with those ones shown in Table 2 regarding the validation of the CE method will be discussed below.

3.3. UV results

Fig. 3 shows the superimposed UV spectra of BKC, BDP and PEA dissolved in acetonitrile. As can be seen there was an important overlap between the three spectra that initially made difficult the direct evaluation of each species. This overlap induced us to use multicomponent analysis. In order to select the optimal conditions, i.e. wavelengths to be used and sample treatment, several tests were carried out employing standard mixtures of known concentrations. Subsequently, the selected conditions were applied to real samples of different batches, which had been previously analyzed by HPLC. The validity

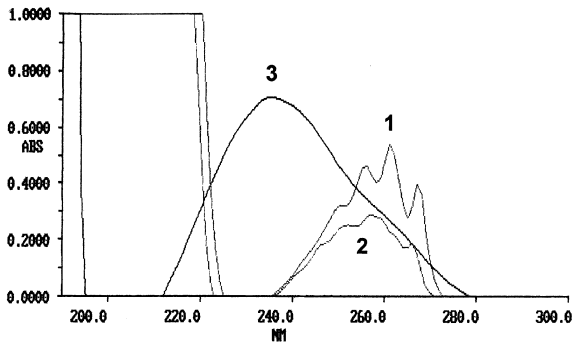

Fig. 3. UV–Vis spectra of standards of BKC (1 mg/l), BDP (0.1 mg/l) and PEA (0.2 mg/l) dissolved in acetonitrile.

of the selected parameters was only admitted when both type of quantitative results, i.e. HPLC vs. UV, showed a difference of 2.5% as maximum. The choice of wavelengths and sample treatment was done as follows.

For the selection of wavelengths (λ), and as a first approach, individual λ values were assigned to each compound and other λ values of reference were used to eliminate the background interference. Neither, the use of the maximum of absorbance for each compound as analytical λ nor those λ corresponding to the maximum difference of absorbance between compounds gave acceptable results even when they were applied to standards of known concentration. Therefore, this approach was discarded. As a second method, the use of a broad range of λ values to carry out the measurements was studied. To do this, two optimization tools of the software were employed. These tools allow both to minimize the matrix influence and to optimize the analytical λ range, so, through statistics calculations the different calibration parameters are obtained showing the expected results depending on the different λ range selected. The best results were obtained with this method and they consisted of using a λ range from 230 to 310 nm.

For the sample treatment, it was observed that the physical characteristics of the nasal spray, i.e. high viscosity and particles in suspension, made practically impossible the direct application of multicomponent analysis, even when different orders of derivation together with the aforementioned software tools for minimizing the matrix effect were employed. For this reason, various sample treatments were tested trying to reach a maximum agreement between expected and obtained values for the real samples. The best results were obtained dissolving the standards in a solution simulating the matrix (as described in Section 2 which was also employed to evaluate the background signal. Under these conditions the range of λ values that gave the best results was finally 228–270 nm. In order to get results representative of the sample the minimum volume of spray that should be used was determined to be equal to 1 ml.

The low absorptivity of BKC prevented its determination in the sample. In order to detect this compound much higher quantities should be added while the λ range employed should be modified. Due to these difficulties, we abandoned the simultaneous determination of BKC with the other compounds; therefore, we focused on the determination of BDP and PEA.

The results of the quantitative calibration, as well as the results of the sensitivity study obtained using this UV-spectroscopic method are given in Table 1. In Table 2 results on the validation of this UV method to quantitavely determine PEA and BDP are given. All these results are next discussed.

4. Discussion

In Table 1 the reproducibility of the separation time for the same day (logically applicable only to HPLC and CE) given as relative standard deviation (R.S.D.) is shown. It can be seen that by using HPLC the R.S.D. values obtained are better than those obtained using CE. The calculated reproducibility of the analysis time for three different days ($n=18$) gave the same behavior; low R.S.D. values for HPLC, i.e., up to 0.3% like those ones obtained for one day, and for CE values up to 2.5%. This can be due to the difficulty to obtain a reproducible inner capillary wall between experiments when bare fused-silica is employed. This point has been considered in this work and we observed that under our conditions, the CE intra-day reproducibility was improved using the washing procedure described in Section 2. Likewise, higher day-to-day reproducibility was obtained storing the capillary overnight with acetonitrile, and making the next day two equilibration runs, ca. 30 min of equilibration time. This negative effect has been broadly studied [28–31], and it seems to be related to hysteresis phenomena [29,30] as well as the history of each capillary [31].

Also in Table 1 the quantitative results of the calibration carried out with these three techniques are given. As can be seen, the three techniques seem to be suitable to carry out the quantitative determination of the active ingredient BDP plus the alcohol PEA in the usual concentrations that they are found in the analyzed sample, that is, considering the original quantities in the nasal spray Beconase plus the dilution due to the sample preparation. Besides, CE allows the quantitative determination of BKC whose

estimation was carried out considering together the area of the peaks 1 and * of BKC (see Fig. 2).

The best sensitivity, calculated as three times the signal-to-noise ratio and given as limit of detection (LOD) in Table 1, was obtained by using HPLC. It can also be observed that such value is ca. 2 orders of magnitude better for BDP than that obtained with CE with which the poorest sensitivity values were obtained. Considering that the obtained LOD values are in any case much smaller than the concentration values expected to be observed from real samples, it can be concluded that the three techniques are suitable to carry out this type of determination.

The validation of the quantitative methods was done calculating the repeatability ($n=8$), reproducibility ($n=18$) and accuracy ($n=6$) of each methodology. The results obtained are summarized in Table 2. The two first parameters were calculated injecting a commercial nasal spray Beconase treated as indicated in Section 2. The accuracy was determined preparing a synthetic matrix, viz., placebo, of similar qualitative and quantitative composition to that theoretically found in the spray. From this preparation six aliquots were taken and they were treated as indicated in Section 2, depending upon the analytical technique which later was going to be employed.

From the values given in Table 2 it can be deduced that HPLC and UV spectroscopy render similar repeatability values for PEA and BDP, while HPLC gives better results in terms of reproducibility and accuracy of the method. From Table 2, it is also evident that CE provides in general worse values than HPLC; however, these CE values are in some cases similar to those obtained with UV spectroscopy. The influence of the matrix, as mentioned above, can be responsible of the poor reproducibility and exactitude values obtained with UV. The partial resolution obtained by CE between peak 2 (PEA) and the closest system peak, as shown in Fig. 2, seems not to affect the reliability of the integration for peak 2. This can be deduced from the better repeatability, reproducibility and accuracy values for PEA compared with those obtained for BKC as can be deduced from Table 2. Moreover, reproducibility for PEA was better than that obtained for BDP, i.e., 2.50% vs. 3.78%, respectively, as can be seen in Table 2. In spite of these results, some negative effect of such a system peak on the precise determination of PEA can not be ruled out. For CE it has been broadly discussed the problems related to the use of this technique for quantitative aims [32–34], arguing that many parameters have a negative influence on the reproducibility of the injection. Thus, injection by pressure or electromigration is dramatically influenced among other factors by the temperature of the sample solution. Also, the velocity of pass of the samples through the detector, which is influenced by the state of the capillary wall, affects the peak area obtained upon integration [35]. All these negative effects bring about poor reproducibility, repeatability and accuracy values. The use of an internal standard would improve the accuracy and injection repeatability of this procedure, while it would also reduce other undesired effects originated by, for example, the different surface tension and viscosity of standards and samples [36,37].

5. Conclusions

The determination of the active compound, i.e. BDP, found in samples of nasal spray, a matrix characterized by the existence of particles in suspension and high viscosity, can be done by HPLC, CE and multicomponent UV spectroscopy. From the comparative examination of the three techniques it is concluded that (under the experimental conditions chosen) CE gives a more complete information about the three compounds object of this study, i.e. BKC, PEA and BDP, while HPLC and UV allow only the evaluation of BDP and PEA requiring another sample preparation with much higher quantity in order to determine BKC. However, in terms of analysis time reproducibility, both in the same day and day-to-day, the comparison between HPLC and CE shows that HPLC provides better values compared to those from CE.

When the comparison is established in terms of quantification capabilities of the three methodologies it seems clear that HPLC renders the best results, followed by UV and giving CE the worst results. A similar trend is observed when the sensitivity of these three methods is compared.

Although, the complexity of the sample preparation is higher when UV is used, the total time per

analysis is slightly shorter by using this technique than that required when HPLC or CE are employed.

In summary, these three techniques can work in a complementary fashion and it seems from our results that HPLC is more suitable to carry out quantitative determinations, CE provides more information about the sample and UV spectroscopy gives the fastest analysis.

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