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A new validated method for the simultaneous determination of benzocaine, propylparaben and benzyl alcohol in a bioadhesive gel by HPLC

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Abstract

A new HPLC-RP method has been developed and validated for the simultaneous determination of benzocaine, two preservatives (propylparaben (nipasol) and benzyl alcohol) and degradation products of benzocaine in a semisolid pharmaceutical dosage form (benzocaine gel). The method uses a Nucleosil 120 C18 column and gradient elution. The mobile phase consisted of a mixture of methanol and glacial acetic acid (10%, v/v) at different proportion according to a time-schedule programme, pumped at a flow rate of 2.0 ml min^{-1}. The DAD detector was set at 258 nm. The validation study was carried out fulfilling the ICH guidelines in order to prove that the new analytical method, meets the reliability characteristics, and these characteristics showed the capacity of analytical method to keep, throughout the time, the fundamental criteria for validation: selectivity, linearity, precision, accuracy and sensitivity. The method was applied during the quality control of benzocaine gel in order to quantify the drug (benzocaine), preservatives and degraded products and proved to be suitable for rapid and reliable quality control method.

Keywords: Benzocaine; Propylparaben; Benzyl alcohol; Liquid chromatography; Pharmaceutical dosage forms; Gel; Validation; Quality control

1. Introduction

Benzocaine, *p*-aminobenzoic (ethyl 4-aminobenzoate) acid ester is a local anasthesic used for superficial anasthesia, for the local and temporal relief of pain related, among other disorders, to buccal affections [1]. For such reasons, it is a drug extensively used in odontology [2,3].

A considerable number of publications have appeared describing different strategies to quantify benzocaine in tissues and biological fluids of several fish species [4,5]; also there are papers for the determination of the active in different pharmaceutical dosage forms such as tablets [6–8], solutions and suspensions [9–11], semisolids preparations [12–14], suppositories [15], sunscreens and after-sun products [16–17].

A new pharmaceutical dosage form, consisting of a bioadhesive gel of the active has been developed. The gel increases the residence time of the active in the buccal mucosa, thus achieving a prolonged action [18]. This dosage form apart from the drug, also contains two preservatives to keep its microbiological quality and meet the challenge test [19–21], following the requirements needed for the development of a dosage form in the pharmaceutical industry [22,23].

The focus of this study has been to develop and validate a rapid HPLC-RP method for the quality control of benzocaine, its degradation products and propylparaben (4-hidroxybenzoic acid propyl ester, nipasol®) (benzoic acid, 4-hydroxy-, propyl ester) and benzyl alcohol as preservatives in the pharmaceutical preparation.

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This work describes the validation parameters stated either by USP 27 [24] or by the ICH guidelines [25,26] to achieve an analytical method with acceptable characteristics of suitability, reliability and feasibility.

The proposed method is applicable for routine analysis as well during the production: quantification of benzocaine, preservatives and degradation products of benzocaine gel and complies with the validation requirements in the pharmaceutical industry.

2. Experimental

2.1. Equipment

A high-performance liquid chromatographic system consisted of a Hewlett-Packard 1100 (Agilent Technologies) featuring a column oven (79856A), a quaternary pump (G1311A), an automatic injector (G1313A) and a DAD detector (G1315A), which is set at 258 nm. Data adquisition is performed using a chromatography software package (Chemstation version A.07).

2.2. Materials and reagents

Methanol was of HPLC grade and was purchased from Panreac-Quimica S.A., Barcelona, Spain. Glacial acetic acid for analysis and ethanol 96° were also purchased from Panreac-Quimica. The water used was redestilled from a purelab Plus system by Vivendi. The reagents used for the stress-testing study of the active and preservatives (HCl 0.1 N, NaOH 0.1 N, H_2O_2 (33%, v/v), potassium permanganate) were of analytical grade and supplied by Roig Farma S.A. and Panreac-Quimica S.A.

Benzocaine (batch 021198) was purchased from Roig Farma S.A. and benzocaine reference standard was purchased from European Pharmacopoeia (batch 1b). Ethyl ester of *p*-nitrobenzoic acid (EPNB) and *p*-aminobenzoic acid (PABA) were provided by Archimica Limited. Nipasol® (propylparaben) and benzyl alcohol were purchased from Roig Farma S.A. Benzocaine gel was provided by laboratories KIN S.A. This pharmaceutical preparation contains 5% of benzocaine, 1% of benzyl alcohol and 0.3% of propylparaben. The rest of excipients of the gel are: medium-chain triglycerides, colloidal silicon dioxide and pineapple flavour. A placebo for the validation study is prepared with these excipients.

2.3. Chromatographic conditions

Chromatographic separation of the active, degradation products and preservatives was performed using a Nucleosil 120 C18 column 250 mm × 4.6 mm i.d., 10 μm particle size, made of stainless steel. The mobile phase consisted of methanol and solution of glacial acetic acid (10%, v/v) that were carried as a gradient programme (Table 1). Both methanol and glacial acetic acid solution were degassing by filtering through a 0.45 μm GH-membrane filter. The flow rate was 2.0 ml min^{-1}. The DAD detector was operated at 258 nm. The injection volume was 10 μl. During the analysis the column was equilibrated at 40 °C. Each determination required 30 min.

Table 1
Gradient profile programme to carry out the chromatographic method

Time (min)	Buffer solution (%)	Methanol HPLC (%)
0	90	10
9	90	10
13	60	40
25	60	40
28	90	10
30	90	10

2.4. Stock and working standard solutions

Working standard solution of benzocaine was prepared at a concentration of 2000 μg ml^{-1} dissolving the appropriated amount of the compound in ethanol. This standard solution was used to quantify the active on the final product. Based on this solution and by means of an adequate dilution, a 20 μg ml^{-1} was prepared to quantify unknown degradation products. These solutions could be stored at 25 °C (room temperature) for 24 h.

Stock standard solution of benzyl alcohol was prepared at a concentration of 4000 μg ml^{-1} dissolving the appropriated amount of the compound in ethanol. This solution could be stored at 25 °C (room temperature) for 72 h. A working standard solution of benzyl alcohol was prepared by the appropriate dilution of the above mentioned stock standard solution in ethanol to reach a concentration of 400 μg ml^{-1}, that is used to quantify the preservative on the final product.

Working standard solution of propylparaben was prepared at a concentration of 120 μg ml^{-1} dissolving the suitable amount of the compound in ethanol. This standard solution is used to quantify the preservative on the final product. This solution could be stored at 25 °C (room temperature) for 72 h.

Finally, stock standard solutions of degradation products (EPNB and PABA) were prepared at a concentration of 200 μg ml^{-1} dissolving the appropriated amount of the compounds in ethanol. These solutions should be stored at 4 °C and were found to be stable for several weeks. Working standard solutions of these degradation products were prepared by the appropriate dilution of the above-mentioned stock standard solutions in ethanol to reach a concentration of 20 μg ml^{-1}.

Out of the solutions obtained, a proportion was taken and filtered through a PVDF membrane filter (0.45 μm). The resulting filtered solutions were placed in HPLC vials. Each of the solutions prepared were injected by duplicate into the chromatograph, recording later the results obtained.

2.5. Assay of the pharmaceutical preparation

Four grams of benzocaine gel were weighed and placed in a 100 ml Erlenmeyer flask with 50 ml of ethanol. The solution was magnetically stirred for 90 min. After that, the solution was transferred to a 100 ml volumetric flask and 30 ml of additional ethanol were added and the sample was ultrasonicated for 10 min. Once the volumetric flask reached the environmental temperature (25 °C), it was diluted up to 100 ml with additional ethanol. This solution could be stored at 25 °C (room temperature) for 48 h.

Out of the solution obtained, a proportion was taken and filtered through a PVDF membrane filter (0.45 μm). The resulting filtered solution was placed in an HPLC vial. Each of the solutions prepared were injected by duplicate into the chromatograph, recording later the results obtained.

2.6. Validation study

2.6.1. Specificity

In order to determinate the specificity of the method, identification of the active was studied, comparing raw material with a standard reference. Another study was carried out to check the absence of interference by the excipients, which take part in the pharmaceutical preparation (placebo solution), as well as the study which was carried out to determine the absence of interference of the impurities or degradation products from benzocaine.

In order to assure the specificity and provide an indication of the stability properties of the proposed method, forced degradation studies were performed under various stress conditions. Thus, appropriate amounts of gel and placebo were stressed with HCl 0.1 N and NaOH 0.1 N (keeping the solutions to both, environmental temperature and 105 °C). Bioadhesive gel was also subjected to the effect of temperature (105 °C), UV light, IR light, high relative humidity (79% HR) for 24 h and exposed to daylight for a period of 15 days. Moreover, samples of gel and placebo were exposed to an oxidizing treatment with H_2O_2 and $KMnO_4$ for 24 h. When the degradation treatments were completed, samples were analyzed according to sample preparation assay.

2.6.2. Linearity

To carry out this study, seven levels of concentration within the range 70–130% of the working-concentration of the active and preservatives (benzocaine: 2000 μg ml^{-1}, benzyl alcohol: 400 μg ml^{-1}, propylparaben: 120 μg ml^{-1}) were prepared. Analysis was performed in triplicate, individually weighing the amount of active and preservatives and the corresponding amount of placebo. The experimental results were represented graphically, obtaining a calibration curve and carrying out the corresponding statistic study (ANOVA).

2.6.3. Precision

For the precision study, four different tests were carried out. The first one consisted of checking the instrumental system precision injecting 10 times the same solution of three levels of concentration (70%, 100% and 130% of the working concentration) one day and repeating the same procedure on the second day. The second test consisted of testing the standard solution precision where three solutions were prepared at 70%, three solutions at 130% and seven solutions at 100% of the working concentration, studying the relative standard deviation obtained for the response factor (relationship between the experimental area and the studied concentration). The third test consisted of checking the precision of the method, operating as described in the standard solution previously mentioned: seven individual samples were prepared and the relative standard deviation was studied for the response factor obtained. Lastly, the intermediate precision, where the variability of the analyst and days was studied.

2.6.4. Accuracy (recovery method)

The recovery method was studied at the concentration levels of 70% (three samples), 100% (seven samples) and 130% (three samples) where a known amount of the active and preservatives were added to a determined amount of placebo and it was calculated the quantity of benzocaine, benzyl alcohol and propylparaben recovered in relation to the added amount.

2.6.5. Robustness

In order to evaluate the robustness of the proposed method, the influence of small deliberate variations of the method parameters in the determination of benzocaine, benzyl alcohol and propylparaben in gel (percentage of active and preservatives) was examined throughout. The factors selected to examine were the wavelength (nm), temperature (°C), flow rate (ml min^{-1}), mobile phase (percentage methanol) and volume of injection (μl). Each factor was charged at three levels (−1, 0 and 1). One factor at a time was changed to estimate the effect. In each assay seven samples were studied together with a working standard solution.

3. Results and discussion

3.1. System suitability

The chromatographic separation, as explained above, was carried out with a C18 column (Nucleosil 120, 250 mm × 4.6 mm i.d., 10 μm particle size). The gradient used to elute all compounds of the pharmaceutical preparation allows to quantify the compounds in the same chromatographic method and only within 30 min. In Fig. 1 a representative chromatogram with all possible compounds (active, preservatives and degradation products) is shown, where it was possible to show the well-separated peaks of the different compounds.

It was concluded that the developed method is the optimum according to the studied parameters and complies with the accepted values for USP or ICH guidelines. Therefore,

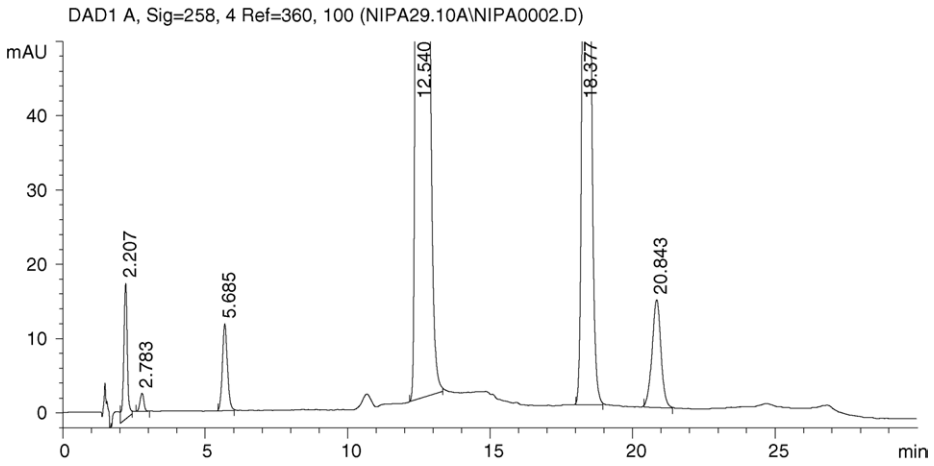

Fig. 1. Representative chromatogram obtained from a system suitability study, where preservatives and degradation products appear together with benzocaine. Benzocaine, PABA, EPNB, benzyl alcohol and propylparaben elute at 12.54, 2.207, 20.843, 5.685 and 18.377, respectively.

this method can be applied to routine with no problems, as its suitability being proved.

3.2. Validation study

3.2.1. Specificity

It was checked that benzocaine raw material as well as benzocaine reference standard elute at the same retention time indicating a positive identification of the drug. In Fig. 1 it is observed that all the compounds of the injected sample elute at different retention times and do not interfere between them. The study of the purity of the peaks shows that the three spectrums obtained at different times are within the established threshold for these peaks. The placebo chromatogram shows that there were no interference between the excipients of the pharmaceutical preparation and the active, preservatives and degradation products.

The degradation studies carried out as stated in Section 2.6.1 is the last test to demonstrate the specificity of the method.

During the stress-testing study in acid media, at 25 °C (Fig. 2a) as well as 105 °C (Fig. 2b) appeared a degraded product, which eluted at 2.1 min, and it corresponded to PABA. An unknown degraded product of benzocaine was also formed which eluted at 11 min (25 °C). The peak which has a retention time of 3.196 in acid media at 105 °C corresponded to a degraded product of propylparaben. In basic media at 25 °C (Fig. 2c) a degraded product, which eluted at 11.287 min was observed, apart from observing the presence of degraded product PABA, which was mainly formed when de basic solution was submited at 105 °C (Fig. 2d) [27,28]. A peak appeared at 3.243 min in both stress conditions, which corresponded to a degraded product of propylparaben.

In the high temperature treatment (105 °C) apart from the degradation products already seen, appeared another one, which was eluted at 16.326 min. In the literature the degradation of benzocaine is described by the temperature [29]. When the gel was submited to an oxidizing treatment the presence of several unknown degraded products was observed, some of them already seen and other new ones which eluted at 15.429 and 16.246 min (treatment with $KMnO_4$ (Fig. 2e), apart from the degraded product EPNB which eluted at 21.598 min (treatment with $KMnO_4$ and H_2O_2 (Fig. 2f)).

When treating the sample with IR light, UV light, high relative humidity (79% HR) and daylight, three unknown peaks appeared, which eluted at 11.312, 15.428 and 16.324 min.

Moreover, a purity test of the three compounds is performed, indicating that the three spectrums obtained at different times are within the threshold fixes for this study, 990.

To conclude, it can be stated that none of the peaks that could be generated by the stress treatment interfere with the peaks corresponding to the active and the preservatives, therefore showing that it was a selective method and suitable for routine work.

3.2.2. Linearity

Linear relationship was obtained between the peak area of benzocaine, benzyl alcohol and propylparaben and the corresponding concentrations, as shown by the equations presented in Table 2. The correlation coefficient, determination coefficient, standard error ($S_{x,y}$) of regression line are also given, along with the standard deviations of the slope and intercept.

3.2.3. Precision

Inter-day data of instrumental system precision was studied at three level concentration (70%, 100% and 130%) and the R.S.D. obtained for the response factor are presented in Table 3, for the active as for both the preservatives. In all these cases, the R.S.D. obtained was far below 1%, the limit percentage set for the precision study of the instrumental system (AEFI: Asociación Española de Farmacéuticos de la Industria) [30], thus showing that the equipment used for the study

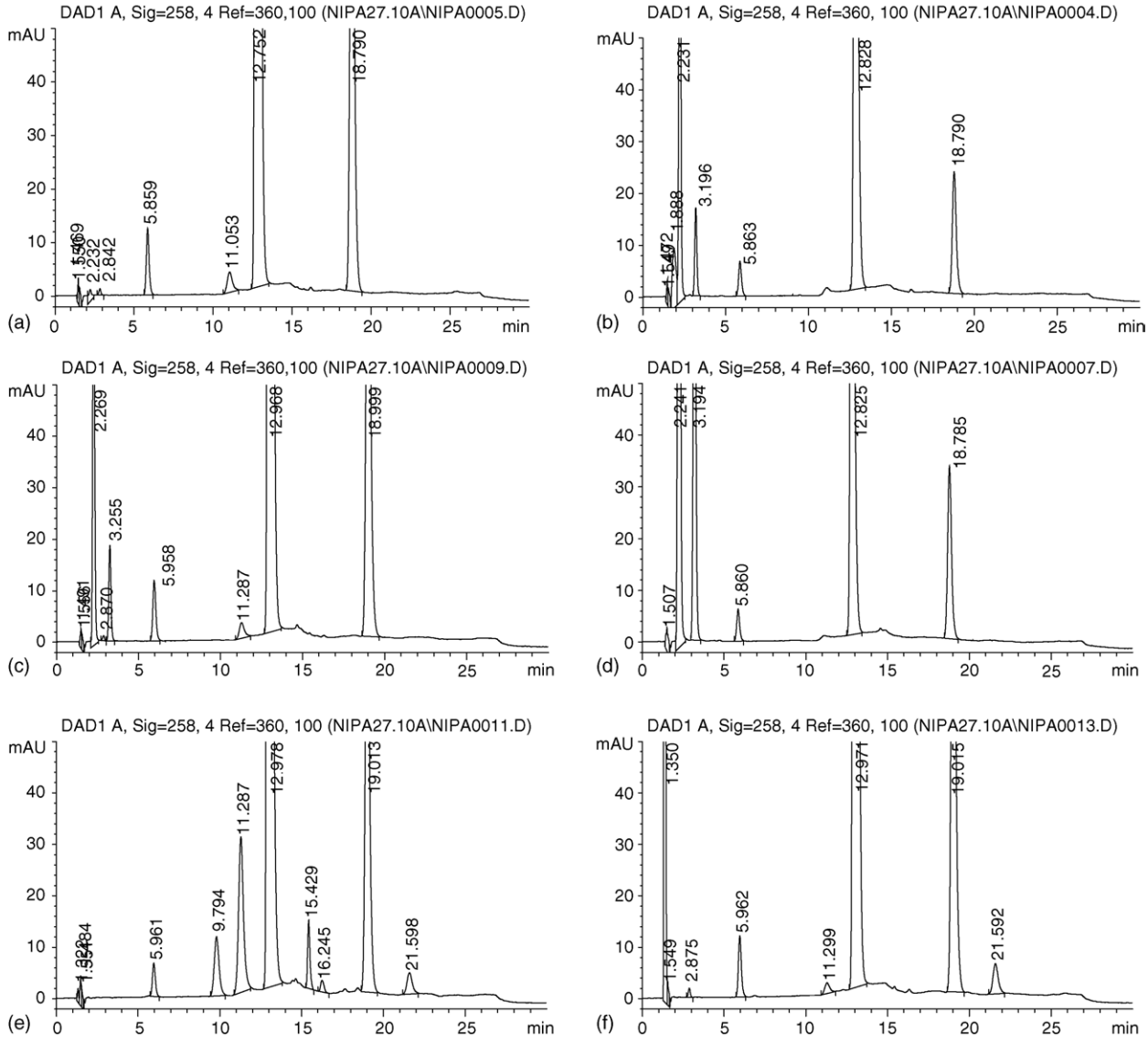

Fig. 2. Representative chromatograms obtained from the stress testing study.

Table 2
Calibration curves for the determination of benzocaine and two preservatives by high-performance liquid chromatography

Compound	Equations	r	r^2	$S_{(x,y)}$	S.D. intercept	S.D. slope
Benzocaine	$Y = 7.8339X - 42.8246$	0.9997	0.9994	91.8106	177.4736	0.0869
Benzyl alcohol	$Y = 0.4566X - 0.7480$	0.9994	0.9987	0.9985	2.8573	0.0072
Propylparaben	$Y = 27.1497X - 135.2745$	0.9993	0.9986	29.1321	56.4546	0.4579

Table 3
Results obtained for the precision of instrumental system

Compound	70% ($n = 20$): R.S.D. R.F.[a]	100% ($n = 20$): R.S.D. R.F.[a]	130% ($n = 20$): R.S.D. R.F.[1]
Benzocaine	0.34	0.24	0.18
Benzyl alcohol	0.71	0.50	0.85
Propylparaben	0.58	0.60	0.86

[a] Response factor.

Table 4
Results obtained for the precision study of the working standard solution

Compound	Benzocaine	Benzyl alcohol	Propylparaben
Mean R.F.[a]	7.6337	0.4569	26.1200
S.D.	0.1452	0.0060	0.3900
R.S.D.	1.90	1.31	1.50

[a] Response Factor.

works correctly for the developed analytical method, being highly repetitive.

The results obtained for the working standard precision study from 13 samples studied ($n = 3$ for 70%, $n = 7$ for 100% and $n = 3$ for 130%) for the active and both preservatives are indicated in Table 4. For the study of the precision of the method ($n = 7$) the value of R.S.D. obtained for benzocaine was 0.807%, for benzyl alcohol was 0.885% and for propylparaben was 1.495%. Both studies with values far below the value established (2.7%) at the beginning of the study (AOAC: Association of Official Analytical Chemists) [31].

For the intermediate precision, a study carried out by the same analyst working on different days ($n = 7$ number of samples per day). The results were given both individually and as a whole observing that the inter-day R.S.D. corresponded to 1.928% for benzocaine, 2.460% for benzyl alcohol and 2.297% for propylparaben. The same study was carried out for different analysts ($n = 7$ number of samples per analyst) obtaining a R.S.D. of 2.092% for benzocaine, 2.011% for benzyl alcohol and 2.429% for propylparaben. Both results together with the individual results are below the established limit according to the AOAC (2.7%) [31], thus showing that the proposed analytical method has a good intermediate precision.

Table 5
Accuracy study of the determination of benzocaine and preservatives by this chromatographic system

Compound	Recovery (%)		
	Mean ± S.D.	R.S.D. (%)[a]	E_r[b]
Benzocaine	99.64 ± 1.63	1.64	−0.43
Benzyl alcohol	101.57 ± 1.05	1.03	1.53
Propylparaben	97.82 ± 1.97	2.02	−2.18

[a] Relative standard deviation percentage.
[b] Relative error percentage.

3.2.4. Accuracy (recovery method)

The results obtained for the accuracy study (recovery method) from 13 samples studied ($n = 3$ for 70%, $n = 7$ for 100% and $n = 3$ for 130%) are presented in Table 5 for the three studied compounds. The standard deviation, relative standard deviation and relative percentage error are also given. The recovery obtained, individually and the mean for every compound studied are between the 97 and 103% for benzocaine and benzyl alcohol and 95–105% for propylparaben established according to the AOAC [31].

Therefore, it can be concluded that the recovery study of the active and preservatives in the matrix of the developed method for the assessment of the active and preservatives in final product was correct, and therefore, the proposed analytical method was sufficiently accurate.

Table 6
Robustness evaluation of the high-performance liquid chromatographic method developed

Chromatographic change		Recovery (%) (mean ± S.D.)		
Factor	Level	Benzocaine	Benzyl alcohol	Propylparaben
A: wavelength				
255 nm	−1	99.66 ± 1.30	99.00 ± 0.93	96.56 ± 0.92
258 nm	0	98.87 ± 1.01	101.38 ± 1.28	96.90 ± 1.18
261 nm	1	99.68 ± 1.29	99.01 ± 0.80	96.52 ± 0.93
B: temperature				
37 °C	−1	100.03 ± 0.93	99.19 ± 1.32	97.21 ± 0.80
40 °C	0	98.87 ± 1.01	101.38 ± 1.28	96.90 ± 1.18
43 °C	1	99.77 ± 1.30	99.51 ± 0.86	96.88 ± 0.62
C: flow rate				
1.9 ml min^{-1}	−1	98.84 ± 1.04	100.32 ± 1.51	95.97 ± 0.84
2.0 ml min^{-1}	0	98.87 ± 1.01	101.38 ± 1.28	96.90 ± 1.18
2.1 ml min^{-1}	1	100.17 ± 0.93	100.43 ± 1.13	97.00 ± 0.56
D: mobile phase				
85:15	−1	100.09 ± 1.31	98.00 ± 0.53	98.31 ± 0.89
90:10	0	98.87 ± 1.01	101.38 ± 1.28	96.90 ± 1.18
95:5	1	98.43 ± 0.88	98.43 ± 0.88	97.35 ± 0.58
E: injection volume				
5 μl	−1	100.14 ± 1.14	100.14 ± 1.14	95.67 ± 0.38
10 μl	0	98.87 ± 1.01	101.38 ± 1.28	96.90 ± 1.18
15 μl	1	99.45 ± 1.20	99.47 ± 1.20	96.98 ± 0.32

Table 7
Determination of benzocaine and preservatives in pilot batches of the gel by the new HPLC method developed

Pilot batch number	Dosage form (gel) (mean ± S.D. (n = 9))		
	Benzocaine 5%	Benzyl alcohol 1%	Propylparaben 0.3%
1	99.36 ± 0.59	100.72 ± 0.79	98.67 ± 0.57
2	101.56 ± 1.49	100.26 ± 1.99	99.21 ± 1.71
3	100.64 ± 1.02	98.15 ± 0.99	101.59 ± 0.91

3.2.5. Robustness

Results obtained in this study are presented in Table 6. In this table the mean obtained (n = 7) for every level and factor studied is indicated, showing that the selected factors remained unaffected by small variations of these parameters because the recovery obtained, individually and the mean were between 97% and 103% for benzocaine and benzyl alcohol and 95–105% for propylparaben established according to the AOAC [31]. Therefore, it can be concluded that the method is consistent in front of the wavelength, the temperature, the flow rate, the mobile phase and the injection volume.

3.2.6. Detection limit and quantitation limit

This study was carried out to determinate the limit of detection (LOD) and limit of quantitation (LOQ) in order to apply this method for the quantification of degraded products (known and unknown products) in the pharmaceutical preparation. It employed an Eurachem method (AEFI) being the R.S.D. for the precision of LOQ 3.7% and accuracy (95–105%). The LOD for PABA was 5.03 $\mu g\,ml^{-1}$, for EPNB was 5.99 $\mu g\,ml^{-1}$ and for unknown degraded products was 6.06 $\mu g\,ml^{-1}$. The LOQ was established at 5.03 $\mu g\,ml^{-1}$ for PABA, 7.99 $\mu g\,ml^{-1}$ for EPNB and 8.06 $\mu g\,ml^{-1}$ for unknown degraded products.

3.3. Label claim recoveries from benzocaine gel

The proposed method was evaluated in the assay of the available pilot batches of benzocaine gel containing 5% of benzocaine, 1% of benzyl alcohol and 0.3% of propylparaben. Nine replicate determinations were carried out on an accurately weighed amount of the gel as is reflected in Section 2.5. Three different pilot batches of benzocaine gel containing the three compounds mentioned above, were analyzed using the proposed procedure and the results are summarised in Table 7.

4. Conclusion

A new simple and quick, analytical method has been developed to be applied in routine to determinate benzocaine, benzyl alcohol, propylparaben and degraded products in a bioadhesive gel. The proposed high-performance liquid chromatographic method has been evaluated over the linearity, precision, accuracy and specificity and proved to be convenient and effective for the quality control of benzocaine in the pharmaceutical dosage form studied (bioadhesive gel).

It has been proved that it was selective, linear, precise and accurate over the concentration range tested (70–130% of the working concentration), with a correlation coefficient higher than 0.9990. It has also proved its robustness regarding the wavelength, flow rate, mobile phase, injection volume and temperature.

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