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New approaches with two cyano columns to the separation of acetaminophen, phenylephrine, chlorpheniramine and related compounds

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Abstract

The development of new pharmaceutical forms with classical active compounds generates new analytical problems. That is the case of sugar-free sachets of cough-cold products containing acetaminophen, phenylephrine hydrochloride and chlorpheniramine maleate. Two cyanopropyl stationary phases have been employed to tackle the problem. The Discovery cyanopropyl (SUPELCO) column permitted the separation of the three actives, maleate and excipients (mainly saccharine and orange flavour) with a constant proportion of aqueous/ organic solvent (95:5, v/v) and a pH gradient from 7.5 to 2. The run lasted 14 min. This technique avoids many problems related to baseline shifts with classical organic solvent gradients and opens great possibilities to modify selectivity not generally used in reversed phase HPLC. On the other hand, the Agilent Zorbax SB-CN column with a different retention profile permitted us to separate not only the three actives and the excipients but also the three known related compounds: 4-aminophenol, 4-chloracetanilide and 4-nitrophenol in an isocratic method with a run time under 30 min. This method was validated following ICH guidelines and validation parameters showed that it could be employed as stability-indicating method for this pharmaceutical form.

Keywords: Common cold drugs; Impurities; pH gradient; HPLC

1. Introduction

Safety concerns require tests to ensure the absence of degradation products at a determined level during stability studies of the pharmaceutical formulations. Analytical methods have to assure that they are selective to related compounds when they are developed as stability-indicating methods. Pharmaceutical formulations for the relief common cold symptoms usually contain a high proportion of acetaminophen and small amounts of phenylephrine hydrochloride and chlorpheniramine maleate. Acetaminophen presents known impurities commercially available such as 4-aminophenol, 4-chloracetanilide and 4-nitrophenol. Moreover, formulations such as sachets contain flavouring agents among the excipients and more recently sucrose, a non UV absorbing compound, has been replaced by saccharine in formulations developed for diabetic or dieting people. All these compounds with very different properties and proportions have to be separated in a HPLC method developed as stability-indicating method. This, although classical, is a problem, not yet solved, and can be considered a useful example to check the performance of stationary phases since the mixture contains not only acidic, neutral and basic compounds and with very different polarities, but also different functional groups as could be observed in Table 1.

Three methods in the literature report the simultaneous quantification of acetaminophen, chlorpheniramine and phenylephrine, but they do not include related compounds. The method of Senyuva and Ozden [4] permits the rapid de-

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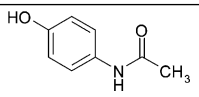
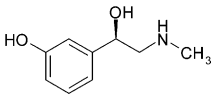
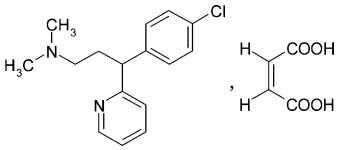
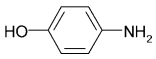
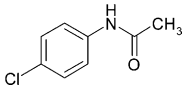
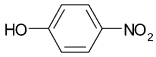
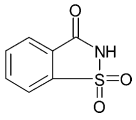
Table 1
Chemical structures of the assayed compounds and their values for the acid–base constants

	Name	Molecular form	Structural form	Molecular weight	pK_a	Reference	pK_a (ACD laboratory)
Actives	Acetaminophen	$C_8H_9NO_2$		151.17	9.5		15.32 ± 0.70 (NH), 9.86 ± 0.13 (OH), -0.14 ± 0.50 (NH_2^+)
	Phenylephrine	$C_9H_{13}NO_2$		167.21	8.9 and 10.1	[1]	14.32 ± 0.20 (OH), 9.76 ± 0.10 (OH phenol), 9.22 ± 0.20 (NH_2^+)
	Chlorpheniramine, maleate	$C_{16}H_{19}ClN_2$, $C_4H_4O_4$		274.80, 116.07	9.1	[2,3]	9.33 ± 0.28 (NH^+), 3.77 ± 0.19(NH^+ pyridine)
Impurities	4-Aminophenol	C_6H_7NO		109.13	10.46		10.17 ± 0.13 (OH), 5.28 ± 0.10(NH_3^+)
	4-Chloracetanilide	C_8H_8CINO		169.61			14.25 ± 0.70 (NH), -1.97 ± 0.50 (NH_2^+)
	4-Nitrophenol	$C_6H_5NO_3$		139.11			7.23 ± 0.13 (OH)
Others	Saccharine	$C_7H_5NO_3S$		183.19			

termination of the three actives in combined pharmaceutical dosage forms using a Bondapak CN column, nevertheless acetaminophen is not separated from the solvent front, with the corresponding quantification problems, and there is no space for the impurities. The method of Kanumula et al. [5] that uses wavelength programming and pseudoephedrine hydrochloride as internal standard and the method developed by Krieger [6] for the separation of acetaminophen in analgesic preparations containing chlorpheniramine maleate, phenylephrine hydrochloride, and other active components by HPLC were not adequate for the present problem.

Our group has long been working with these active compounds in different formulations. Previously, we developed and validated a HPLC method for the simultaneous determination of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations such as capsules and sachets, including the separation of impurities and excipients with a gradient elution in a SymmetryShield RP8 column [7]. The run lasted 20 min. The method has been employed for stability assays, but it presented the problems related to gradient elution. The change in solvent composition causes baseline shifts because of the change in optical properties of the eluent, which is troublesome at low wavelengths. Moreover, it also generates the irregular elution of unknown retained compounds, usually coming from water, when the organic solvent proportion increases, which complicates the impurities analysis.

Afterwards, we developed and validated an isocratic HPLC method for the simultaneous determination of the three actives in capsules as pharmaceutical formulations, including the separation of impurities and excipients [8]. The run time was less than 12 min and it was developed by using new polar reversed phase stationary phases in HPLC, such as polyethyleneglycol, that provide a unique selectivity. The method was a great success to facilitate stability studies for capsules, which contained non UV absorbing excipients. But, when the method was applied to new formulations in the form of sachets containing saccharine, the excipients overlapped with the active compounds. The mobile phase was phosphate buffer 20 mM at pH 7.0/acetonitrile 90:10 (v/v), and the resolution could not be increased either by increasing the polarity nor by any other change such as pH or concentration of the buffer.

Therefore, the main goal of this study was the development and validation, following ICH guidelines, of an isocratic HPLC method for the determination of acetaminophen, phenylephrine hydrochloride and chlorpheniramine maleate, as well as 4-aminophenol, 4-chloracetanilide and 4-nitrophenol in the presence of usual excipients in soluble formulations such as orange flavour and saccharine. Furthermore, it was of great interest to use the formulation as a

real test mixture to compare the performance of two cyano columns with different chemistries and to develop a less common separation in reversed phase HPLC based on pH gradient instead of organic solvent gradient. Indeed, while this manuscript was in preparation a paper from Kaliszan et al. has been published reporting pH gradient HPLC as a new original mode of reversed-phase high-performance liquid chromatography [9] and supplies rational basis and a mathematical model. Therefore, the present work, although almost simultaneous in time, can be the first application to a real sample of pH gradient approach in HPLC.

2. Experimental

2.1. Chemicals

Standards of actives (acetaminophen, phenylephrine and chlorpheniramine maleate) all of them >99.9% purity and impurities (4-aminophenol, 4-chloracetanilide and 4-nitrophenol) as well as excipients of the formulations were kindly provided by CINFA, S.A. (Pamplona, Spain). NaOH (>99%) was from Panreac (Barcelona, Spain), H_3PO_4 (85%), and CH_3CN (HPLC grade) were from Merck (Darmstadt, Germany), and water was purified with a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.2. HPLC analysis

2.2.1. Method 1

LaChrom Elite HPLC equipment from VWR (Barcelona, Spain) consisted of quaternary pump, an automatic injector, a single wavelength detector and a column oven. The column was a Discovery cyanopropyl with 5 μm particle size, 4.6 mm × 250 mm, kept at 35 °C. Different mobile phases were tested with 10 mM phosphoric acid adjusted to different pH values with KOH and acetonitrile at a flow rate of 1 ml/min. Finally the best conditions were a linear gradient changing from 95% A to 95% B in 10 min, being A phosphate buffer pH 7.5 and B phosphate buffer pH 2.0. The initial conditions were then recovered during 3 min. The mobile phase contained also a constant proportion of 5% acetonitrile during the run entering through C. Detection was performed at 215 nm.

2.2.2. Method 2

The HPLC was an Agilent Technologies 1100 liquid chromatograph (Las Rozas, Madrid, España) with a quaternary pump, an automatic injector, a diode-array detector and a column oven. The column was an Agilent Zorbax SB-CN with 5 μm particle size, 4.6 mm × 250 mm, kept at 35 °C. The mobile phase was phosphate buffer 10 mM at pH 3.0/acetonitrile, 96:4 (v/v). The phosphate buffer was prepared from H_3PO_4 by adding KOH to reach the pH 3.0. The flow rate was 1.5 ml/min and the injection volume was 5 μl. UV detection was performed at 215 nm, for chlorphenamine and impurities, 270 nm for phenylephrine and 310 nm for acetaminophen. The last wavelength was employed to avoid the saturation in the signal that occurs at lower wavelengths due to the high proportion of acetaminophen in the sample.

2.3. Standards and sample preparation

In all cases the solvent solution (SS) for standards and samples was water/acetonitrile 80:20 (v/v). A stock solution of phenylephrine was prepared with 250 mg of phenylephrine hydrochloride exactly weighed and dissolved with SS in a 25 ml volumetric flask. For chlorpheniramine maleate stock solution, 100 mg were made up 25 ml with SS. For the reference stock standard, 650 mg of acetaminophen were weighed in a 50 ml volumetric flask and 1 ml of phenylephrine and chlorpheniramine solutions were added. The mixture was made up by the corresponding volume with SS and treated with magnetic stirring for 10 min.

For sugar-free sachets, 4 g of sample (the approximate weight of one sachet) coming from 20 sachets homogenate were dissolved in a 50 ml volumetric flask with SS. After 10 min of magnetic stirring an aliquot was filtered with a 0.45 μm syringe filtration disk to the vials for injection in the HPLC system. In all cases three replicates were processed. When different formulations were tested the amount of sample was calculated to keep constant the concentration of the actives.

2.3.1. Validation

The selectivity was tested by running solutions containing the excipients of the formulation in the same quantities and conditions that the samples to show that there is no peak in the retention times corresponding to the analytes. Moreover, solutions of the standards with the identified impurities, 4-aminophenol 4-nitrophenol and chloracetanilide, at the 1% level added were also run to show both the resolution and selectivity of the method. In all cases the spectra of the peaks were compared with those of the pure standards.

The linearity was tested by preparing standard solutions at five concentration levels, from 75 to 120% of the target analyte concentration. In this case, acetaminophen concentrations were from 9.75 to 15.60 mg/ml, phenylephrine hydrochloride from 0.15 to 0.24 mg/ml and chlorpheniramine maleate from 0.060 to 0.096 mg/ml. They were prepared in 50 ml volumetric flasks by weighting the exact amount of acetaminophen (from 487.5 to 780 mg) and by adding the corresponding volumes of phenylephrine and chlorpheniramine, stock standards (0.750–1.20 ml each) and completed the total volume with SS. Each point was analysed three times.

The accuracy of the method was tested by applying it to synthetic mixtures of the drug components to which known quantities of the drug substances to be analysed have been added. It was tested by triplicate in three levels (80, 100 and 110%) and in parallel with the linearity assay for the three main components. The percent recovery and R.S.D. were then calculated.

Instrumental precision was tested by running consecutively 10 injections of the same standard. *Intra-assay precision* data were obtained by repeatedly analyzing, in one laboratory on 1 day, 10 aliquots of a homogeneous sample, each of which were independently prepared according to the method procedure. *Intermediate precision* data were obtained by repeating the intra-assay experiment on a different day with newly prepared mobile phase and samples.

The limit of detection (LOD) was evaluated from the three signal-to-noise ratio procedure and *the limit of quantification* (LOQ) with 10 signal-to-noise ratio. These are the common estimations, nevertheless to obtain the experimental limit of quantification, the validation was performed with the actives from 0.05 to 0.5% of the theoretical content of a sample, i.e. acetaminophen concentrations from 6.5 to 65 μg/ml, phenylephrine hydrochloride from 0.1 to 1 μg/ml, and chlorpheniramine maleate from 0.04 to 0.4 μg/ml.

3. Results and discussion

Our previous experience with these compounds showed us that in order to develop an isocratic method to separate all of them in one run it was necessary to employ a stationary phase with high retention of polar compounds and lower retention of hydrophobic compounds. Once the possibilities with the polyethyleneglycol column were explored and the separation was found not to be adequate, mainly due to the presence of the sweetener, the cyano columns were studied.

The first approach was the use of a Discovery cyanopropyl 5 μm, 4.6 mm × 250 mm (SUPELCO). The bonded phase is $-O-Si(CH_3)_2CH_2CH_2CH_2CN$, it is endcapped and has a pore size of 180 Å. Some of the characteristics according to the manufacturer are the low hydrophobicity for rapid elution of hydrophobic molecules, the retention and separation of strongly basic analytes, the compatibility with highly aqueous mobile phases and its excellent stability and reproducibility.

The three actives (acetaminophen, phenylephrine hydrochloride and chlorpheniramine maleate) and the excipients of sachets including saccharine were assayed. Isocratic conditions were tested with 95:5 (v/v) 10 mM phosphate buffer (pH 2.5, 4.6 and 7)/acetonitrile. This high proportion of aqueous buffer was considered necessary in preliminary runs to provide enough retention of more polar compounds. In this mixture of compounds (as an example of many chromatographic situations) pH influences selectivity decisively. The chemical structures of the assayed compounds and most of their values for the acid–base constants are shown in Table 1. While maleate and saccharine (the acidic ones) were more retained at acidic pHs, acetaminophen (neutral) was not affected and phenylephrine and chlorpheniramine (basic) were less retained. The key problem in the separation was that at acidic pH values phenylephrine coeluted with saccharine and/or with acetaminophen, while at higher pH values the retention time for chlorpheniramine was too high.

In such conditions it was possible to develop a separation with a constant proportion of aqueous phase and a pH gradient, starting with pH 7.5 to obtain a better resolution of phenylephrine with the compounds eluting first and changing to pH 2 to decrease the retention time of the most hydrophobic (and basic) compounds, mainly chlorpheniramine. Fig. 1 shows the chromatogram corresponding to the three actives, the excipients and the pH gradient profile. This approach of a constant organic solvent proportion, with a change in the pH of the aqueous phase is not usual in reverse phase separations, but it results in a flat baseline, avoiding some of the problems of common solvent gradients. The problem arose with impurities because 4-aminophenol could not be separated from one of the excipients peaks. Therefore, this method could be employed for quality control, but not for stability tests. In order to establish the reliability of the method, some pre-validation assays were performed. The method was linear with correlation coefficients >0.999 for paracetamol and >0.99 for phenylephrine and chlorpheniramine. Accuracy ranged from 102.0 to 108.2% and intra-assay precision from 0.45 to 1.53%. Therefore, preliminary results showed that the method could be fully validated. However, this approach was not pursued further in order to develop a method including the three impurities.

It has been previously described that the properties of the CN columns are governed by the type of cyano phase (trifunctional or monofunctional) on the silica gel support and endcapping [10,11]. Therefore, a very different column was tested. Agilent Zorbax SB-CN is described by the manufacturer as a densely covered, sterically protected, diisopropyl cyanopropyl stationary phase, not endcapped, with pore size of 80 Å. The behaviour proved to be different and, in this case, highly satisfactory for the objective of our work. This column permitted us to work with isocratic conditions to separate all the compounds in less than 30 min. Final chromatographic conditions employed an Agilent Zorbax SB-CN with 5 μm particle size, 4.6 × 250 mm, kept at 35 °C. The mobile phase was phosphate buffer 10 mM at pH 3.0/acetonitrile 96:4 (v/v) at a flow rate of 1.5 ml/min. UV detection was performed at 215, 270 and 310 nm for quantification. The chromatograms corresponding to the separation of the active compounds (acetaminophen, phenylephrine and chlorpheniramine) at 100% level, the impurities plus acetaminophen at 0.2% level (4-aminophenol, 4-chloracetanilide and 4-nitrophenol) and the excipients (including saccharine and orange flavour) are illustrated in Fig. 2 at 215 nm. The different performance of the Discovery cyanopropyl column for the three active compounds and saccharine with the same chromatographic conditions can be observed in Fig. 3. Phenylephrine, maleate and saccharine, the more polar compounds, were more retained, while chlorpheniramine showed lower retention, as could be expected with a more polar stationary phase. In such conditions all the analytes, finally coeluted.

Once the separation was developed, the method was validated. As can be observed in Fig. 2, there is no peak in

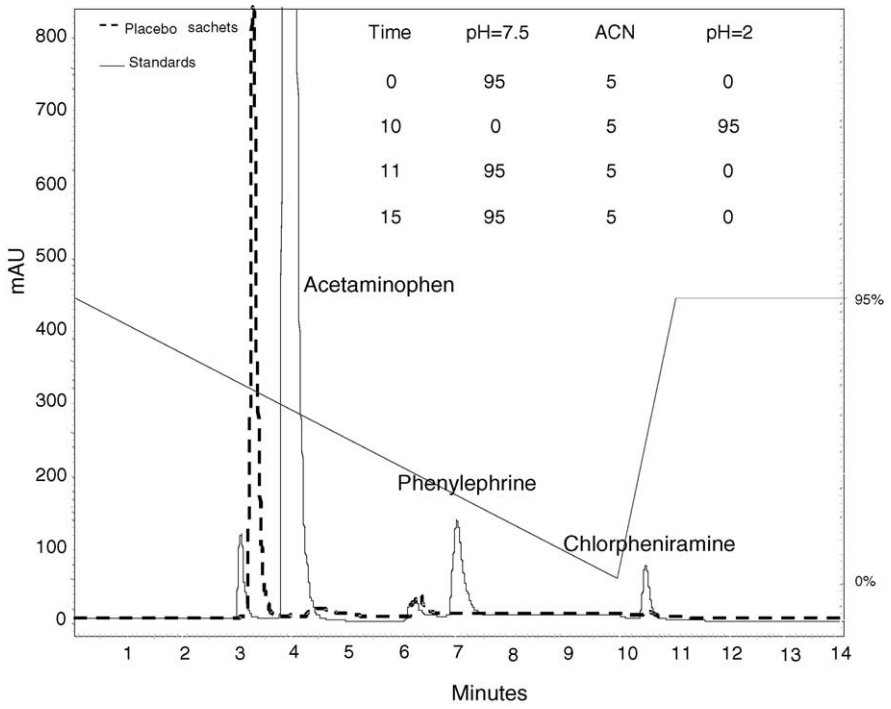


Fig. 1. Chromatogram of the active compounds (acetaminophen, phenylephrine and chlorpheniramine) and excipients sachets at 215 nm with phosphate buffer 10 mM/acetonitrile 95:5 (v/v) in a pH gradient from 7.5 to 2.0 in 10 min on a Discovery CN column (5 μm particle size, 4.6 mm × 250 mm) kept at 35 °C. Detection at 215 nm.

the placebo of the speciality corresponding to the retention times of the analytes. The hold up time was 0.8 min and retention times were: 4-aminophenol 1.8 min, phenylephrine 2.1 min, acetaminophen 5.5 min, chlorpheniramine 14.5 min, 4-nitrophenol 15.1 min and chloracetanilide 26.8 min. On the other hand, the known impurities of acetaminophen, 4-aminophenol and 4-chloroacetanilide, which were run together with the standards, showed both the resolution and selectivity of the method. The minor value obtained for resolution in the method corresponded to the 4-aminophenol-

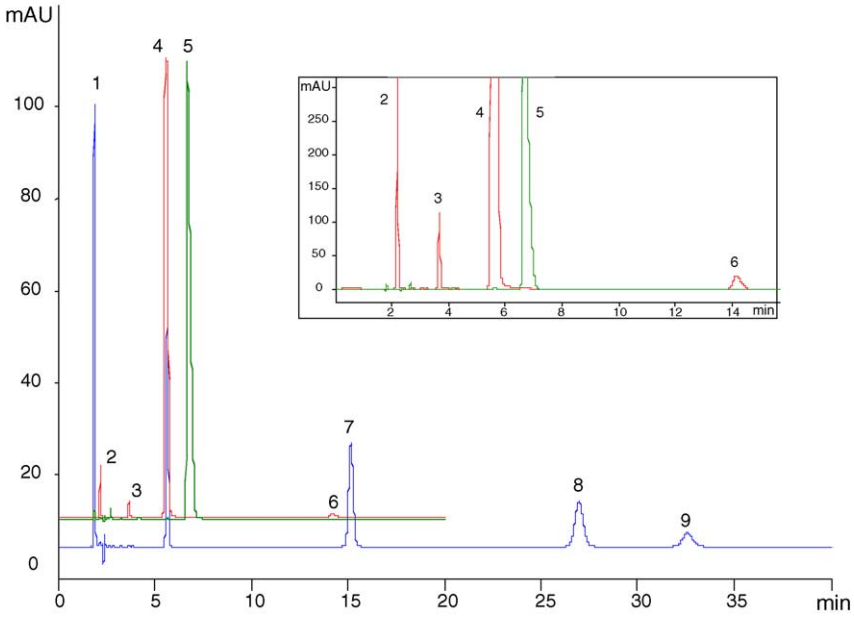


Fig. 2. Chromatograms of the active compounds, the impurities and the excipients on an Agilent Zorbax SB-CN column corresponding to: (1) 4-aminophenol; (2) phenylephrine; (3) maleate; (4) acetaminophen; (5) placebo sachets (saccharin); (6) chlorpheniramine; (7) 4-nitrophenol; (8) 4-chloracetanilide; (9) acetanilide (impurity of chloracetanilide standard). The mobile phase: phosphate buffer 10 mM at pH 3.0/acetonitrile, 96:4 (v/v). Detection at 215 nm.

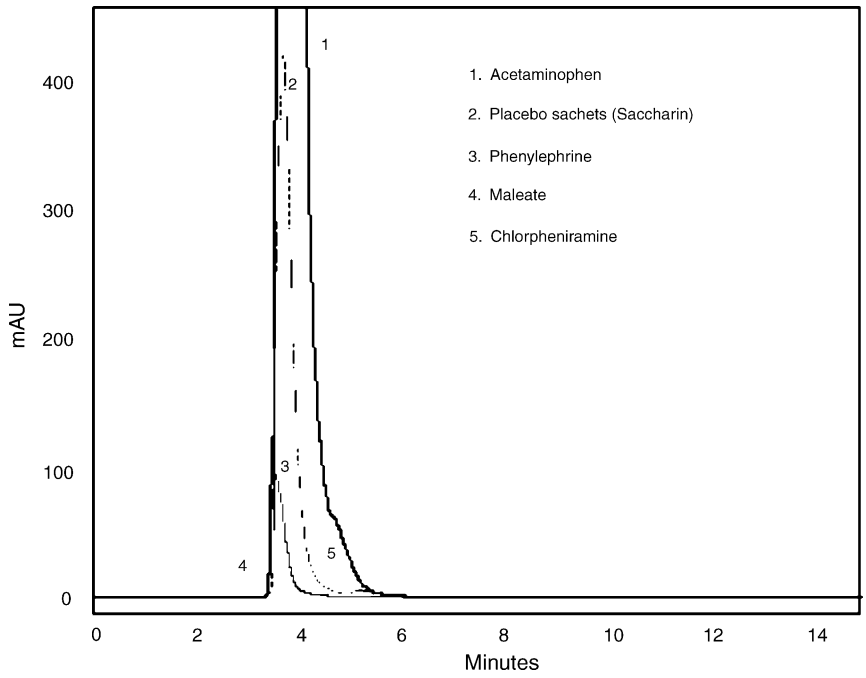


Fig. 3. Chromatograms of the active compounds and the excipients on a Discovery cyanopropyl (SUPELCO) column corresponding to: (1) acetaminophen; (2) placebo sachets (saccharin); (3) phenylephrine; (4) maleate; (5) chlorpheniramine; the mobile phase: phosphate buffer 10 mM at pH 3.0/acetonitrile, 96:4 (v/v). For other conditions see the text.

phenylephrine pair, and it was 2.95. This means a baseline separation of both peaks.

Validation parameters for *linearity* at the quantification level are shown in Table 2. It includes the experimental values plus the specifications to pass the test, established following generally accepted criteria [12]. These parameters showed a good linearity, with correlation coefficients equal to 0.999 for the three analytes, although only 0.99 would be enough for phenylephrine and chlorpheniramine due to their low concentration level in samples. A small bias was found in all the regression lines, because the intercepts with their limits of confidence did not include the zero value. It could be mostly justified by the good fit of the points to the regression lines, which makes the limits of confidence for the intercept very narrow. Although statistically significant (Student *t*-test, $p > 0.05$), the bias did not pose practical consequences, considering the results for the recoveries. Linearity results for impurities are included in the same Table 2 and similar comments can be given for them.

For *precision*, values in the *intra*-assay instrumental precision ranged from 0.09 to 0.43% for the three actives when considering a single day, which means a very small variation due to instrumental causes and from 0.30 to 0.50% when the areas of two different days were tested together.

For the *intra*-assay precision of the method R.S.D.s for the three actives ranged from 1.19 to 2.09% in separated days and from 1.62 to 2.70% for intermediate precision.

Recoveries were $102.7 \pm 0.4\%$ for phenylephrine, $100.1 \pm 0.3\%$ for acetaminophen and $102.3 \pm 0.8\%$ for

Table 2
Validation parameters for linearity in the actives and impurities

Variable	Specification	Results		
		Phenylephrine	Acetaminophen	Chlorpheniramine
Range (mg/ml)		0.15–0.24	9.75–15.60	0.060–0.096
Correlation coeficient (*r*)	>0.999	0.999	0.999	0.999
Response factor R.S.D. (%)	<5%	1.35%	1.03%	1.15%
Slope		1546.61	67.77	5899.38
Intercept		−11.0	52.21	−21.34
		4-Aminophenol	4-Nitrophenol	4-Chloracetanilide
Range (μg/ml)		6.5–65.5	6.5–65.5	6.5–65.5
Correlation coeficient (*r*)	>0.99	0.9999	0.9998	0.9999
Response factor R.S.D. (%)	<5%	1.92%	2.07%	5.01%
Slope		38404.81	157394.36	121937.27
Intercept		23.31	−65.97	58.15

chlorpheniramine, which can be considered acceptable for the levels of each analyte in samples.

The detection limits were 4.6, 1.6 and 1.8 ng/ml for phenylephrine, acetaminophen and chlorpheniramine, respectively, which corresponded to 0.002, 0.00001, and 0.002% of the theoretical content of each active in the formulation.

The quantification limit were 15.3, 5.3 and 6 ng/ml for phenylephrine, acetaminophen and chlorpheniramine, respectively, which corresponded to 0.008, 0.00004, and 0.008% of the theoretical content of each active in the formulation. Although the experimental quantification limit were 0.05% for each compounds, obtained by validating the method in these levels of concentration. Obviously the active compounds will never be measured at their LOQ, but this parameter permits one to evaluate the performance of the method to detect their degradation products during a stability assay. Impurities were validated to the lower limit where they have to be reported.

The method has been applied through 6 months in our laboratory without any change in column performance. Moreover, an Agilent Zorbax SB-CN column with 3.5 μm particle size, 4.6 mm × 150 mm was also checked. At a flow rate 0.6 ml/min retention times with this column were the same as with the longer column with higher particle size. Therefore, although lot-to-lot column reproducibility was not thoroughly investigated, this experiments suggests that the method may be reproduced.

Once validated the method was applied to different commercial market brands containing these actives and four out of six products tested could be measured without interferences. In two formulations excipients, usually colorants and flavouring used in sachets, interfered with some analyte, mainly phenylephrine.

4. Conclusions

As frequently shown with conventional C18 stationary phases, cyano bonded phases from different manufacturers and with different chemistries, can also provide with different selectivities. That was the case of Discovery CN from SUPELCO and Zorbax SB-CN from Agilent related to the substances included in the present study, and that must be considered during method development. The compounds presented in the pharmaceutical formulation considered in this work provided a good test mixture containing compounds with different functional groups (alcoholic, carboxylic, primary, secondary and tertiary amine, amide, nitro and chloro).

On the other hand, pH gradient with a constant proportion of organic solvent has shown to be a powerful tool to modify the retention of compounds with different acid–base properties in the same mixture unexplored in reverse phase separations. That overcomes many problems related to the usual organic solvent gradient mainly due to baseline shifts.

Finally, the Zorbax SB-CN column from Agilent has shown higher retention of more polar compounds while lower retention of more basic and hydrophobic compounds to permit us the development of an isocratic HPLC method to separate acetaminophen, phenylephrine and chlorphenyramine present in the pharmaceutical as actives, maleate as contraion, saccharine and orange flavour as part of the excipients and three impurities *p*-aminophenol, *p*-nitrophenol and *p*-chloracetanilide. Moreover, validation parameters of the method permitted us to consider it reliable as stability-indicating method.

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