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Development and validation of a high-performance liquid chromatography method for the determination of cold relief ingredients in chewing gum

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Abstract

An isocratic high-performance liquid chromatography (HPLC) method for the simultaneous determination of the active compounds paracetamol, pseudoephedrine and chlorpheniramine in chewing gum samples has been developed and validated. The method required a simple liquid–liquid extraction using *n*-hexane and a mixture of water–acetonitrile prior to HPLC analysis. The chromatographic separation was achieved with an aqueous solution containing hexylamine (pH 3)–acetonitrile as the mobile phase, a Spherisorb C_{18} column and UV detection at 220 nm. As an application, the proposed method has been used to evaluate the release into saliva of these compounds from samples under a controlled human chewing process.

Keywords: Chewing gum; Pharmaceutical analysis; Paracetamol; Pseudoephedrine; Chlorpheniramine

1. Introduction

The common cold has been considered as the most frequent disease acquired by people in all age groups. The frequency depends episodically on the kind of population: 4–6 times a year for children, 3–5 times for youths and close to once a year for adults, and the symptomatology takes a mean period of 7–10 days. It is associated with a great number of different viruses, although 50% of the cases are estimated to be caused by rhinovirus. Approximately a hundred different antigens are well known. For this reason, its prevention is inefficient in most cases. The therapy is based on the delivery of active ingredients with action against the most relevant symptoms. Nowadays, only treatments that reduce or eliminate symptomatology, with a lack of specificity, are used [1].

Paracetamol, pseudoephedrine (PE) and chlorpheniramine (CLP) (molecular structures and UV spectra are shown in Fig. 1) are used in numerous pharmaceutical preparations that help to relieve or fight against the symptomatology of the common cold. Paracetamol has analgesic and antipyretic effects. PE is a sympathomimetic, acting as a nasal decongestive, and CLP has anti-allergic effects, which reduce or relieve cold symptomatology, such as congestion, rhinorrhea or nasal irritation. There-

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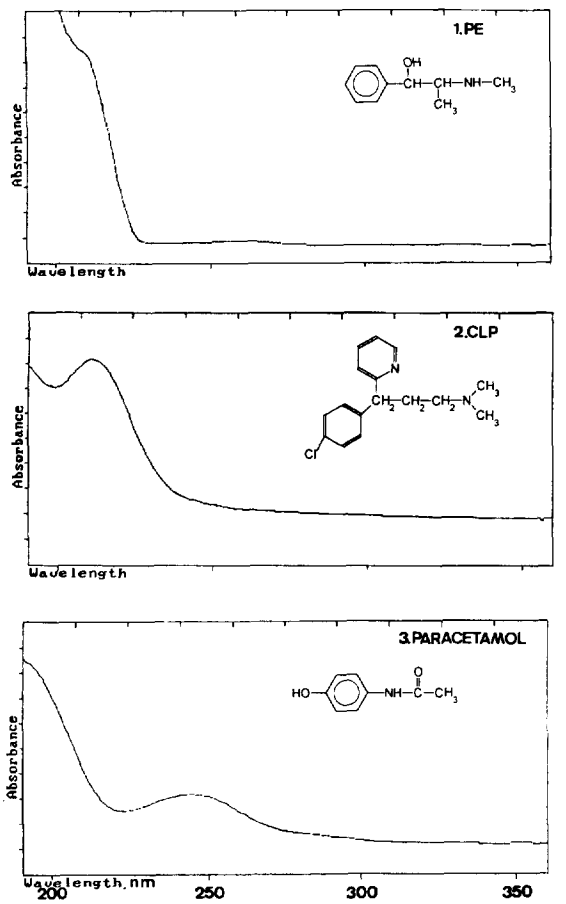

Fig. 1. Structures and UV spectra for paracetamol, pseudoephedrine (PE) and chlorpheniramine (CLP).

fore, PE and CLP are the ideal complements of paracetamol for an overall cold relief action [2].

Several analytical methods to determine these compounds in different pharmaceutical preparations and biological samples, with only one or mixtures of the compounds, are described in the literature. CLP has been determined by complexometric-titration spectrophotometry in capsules [3] and spectrophotometry [4]. PE has been determined by gas chromatography–mass spectrometry (GC–MS) in urine [5], by potentiometry with perchloric acid in raw materials [4], by HPLC in urine [6–8], plasma [9] and in drugs used for the treatment of colds [10]. Paracetamol has been determined by spectrophotometry in raw materials and HPLC in pharmaceuticals [4], serum [11] and by spectrofluorimetry in pharmaceutical preparations and biological fluids [12]. Mixtures of CLP and PE have been simultaneously determined by HPLC in plasma [13] and by using a diode array spectrophotometer under concrete conditions for several pharmaceutical tablet preparations [14]. This last method was further expanded to include a third component (dextrometorphan) and the resulting mixture was then simultaneously determined by second-derivative photodiode array spectroscopy and the results compared favorably with those obtained by a validated HPLC method [14]. However, no method is currently available to quantitate paracetamol, PE and CLP in chewing gum formulations. The simultaneous determination of the active ingredients in multicomponent pharmaceutical products normally requires the use of a separation technique, such as GC or HPLC, followed by quantitation.

In this paper, a simple, rapid and selective reversed-phase (RP)-HPLC method for the simultaneous determination of CLP, paracetamol and PE in a new chewable pharmaceutical formulation has been developed, validated and used to evaluate the release into saliva of these compounds from samples under a controlled human chewing process. However, PE (pK_a=9.8) [15] has a nitrogen group on a β-carbon and CLP (pK_a=9.1) [15] also shows basic character [6,13]. Typically, such compounds exhibit severe peak tailing, broad bands and small plate numbers. In addition, retention often varies from column to column, and dramatic changes in the separation are observed as the column ages. These changes vary with the structure of the basic compounds to be separated and are commonly ascribed to "silanol effects". These effects are mainly due to the interaction of the basic drug with free silanols and/or heavy metals [16]. Many methods have been developed to reduce (or eliminate) untoward manifestations due to silanophilic retention of several basic solutes in RP-HPLC by adding surfactants [17] and amines [18–20] to the eluent. For these reasons, the HPLC method development includes a study of the retention of these compounds using different mobile phases containing different organic modifiers and hexylamine. This study also includes a sample pretreatment method to deal with the unusual pharmaceutical formulation.

2. Experimental

2.1. Chemicals and reagents

Paracetamol, PE sulphate and CLP maleate were from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were from Promochem (Wesel, Germany). A water–methanol (90:10, v/v) mixture was used to prepare standard solutions of paracetamol, PE and CLP. Phosphoric acid was of analytical-reagent grade from Merck (Darmstadt, Germany) and hexylamine was from Aldrich (Alcobendas, Madrid, Spain). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore 0.45-μm nylon filters (Bedford, MA, USA) were also used. Other chemicals used were of analytical-reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components, all of which were from LDC Analytical (Riviera Beach, FL, USA): A Constametric 4100 solvent delivery system; a spectromonitor 5000 photodiode-array detector, covering the range 190–360 nm and interfaced to a computer for data acquisition; recorder Model CI 4100 data module. A Rheodyne 20-μl loop injector (Cotati, CA, USA), a Jones-Chromatography block heated series 7960 (Seagate Technology, Scotts Valley, CA, USA) for thermostating columns at 40°C and a Spherisorb ODS, bonded-silica (250×4.6 mm I.D.), 5 μm column from Phenomenex (Torrance, CA, USA) were used. For pH measurements, a Crison (Barcelona, Spain) digital pH meter with an Ingold 10-402-253 glass combined electrode was also used.

2.3. Mobile phase

The mobile phase consisted of an aqueous solution of hexylamine (4.5 m*M*)–acetonitrile (88:12, v/v). Phosphoric acid was added to the aqueous solution containing hexylamine to adjust the pH to 3. Acetonitrile and water were previously filtered under vacuum through 0.45-μm nylon filters and were degassed using helium.

2.4. Sample preparation

Chewing gum samples were from Farmalider (Carriches, Spain). These samples, containing theoretical amounts of paracetamol (325 mg), PE (30 mg) and CLP (2.0 mg) per sampling unit (SU) and non-active ingredients such as sugar, glucose, glycerin and a calcium carbonate type gum-base [consisting of a mixture of elastomeric polymers, resins, refined wax, esters, glyceride esters from edible fatty acids, calcium carbonate and the antioxidant, 2,3-di-*tert.*-butyl-*p*-cresol (BHT)] were cut into small pieces of roughly 3×3×2 mm and weighed. They were then placed in a separatory funnel containing 40 ml of *n*-hexane. The mixture was vigorously shaken for 5 min, after which 50 ml of a mixture containing aqueous phosphoric acid (pH 3)–acetonitrile (60:40, v/v) were added and the mixture was shaken for 15 min. The mixture was left for 30 min to allow the phases to separate. Under such conditions, the solutes were extracted into the hydro-organic phase, which was separated from hexane. Finally, 230 μl were diluted with water–methanol (90:10, v/v) to 5 ml and 20 μl were introduced into the HPLC system. Base-gum (placebo sample) was processed in the same way as samples containing active ingredients, to get a blank solution.

2.5. Release of active ingredients from chewing gum

Samples were chewed for 15–300 s by healthy individuals from the researchers team (four male and six female, 20–35 years old). After this, samples were washed with deionised water. The amounts of the active ingredients remaining in the gum were analyzed by HPLC after sample preparation.

2.6. Chromatographic analysis

Once the column had been conditioned with the mobile phase at 40°C (for reproducible measurements), a standard solution containing one of the compounds or a mixture of them (5 μg/ml) was injected (20 μl). The flow-rate was 1.0 ml/min and UV-diode array detection (DAD) in the range 190–360 nm was used. Identification of compounds and

peak purity were performed by comparison between the UV spectra of the chromatographic peaks with those of compounds previously registered by injection of each one individually. Analyses were monitored at 220 nm.

3. Results and discussion

3.1. Optimization of mobile phases

In a previous study on the HPLC separation of basic drugs, hexylamine (7.5 m*M*) was added as a modifier to a mobile phase containing acetonitrile–phosphoric acid (pH was adjusted to 3.2) and it was found to be an excellent additive to mask silanols when a Spherisorb C_{18} column was used [21]. Under such conditions, a relative effectiveness of 100% was assigned to hexylamine when compared with other amines of different structure and concentration, because it afforded the lowest retention times. On this basis, mobile phases containing phosphoric acid–4.5 m*M* hexylamine (pH 3) and methanol, tetrahydrofuran and acetonitrile have been tested as organic modifiers in order to prevent silanophilic retention of PE [6] and CLP [13]. Acetonitrile was finally chosen to optimize the separation of the mixture of PE, paracetamol and CLP. Among different contents (10–30%), 12% of acetonitrile was finally selected. In Fig. 2A the chromatogram obtained with this mobile phase is shown for a standard mixture containing PE (20 μg/ml), CLP (1.7 μg/ml) and paracetamol (284 μg/ml).

3.2. Separation performances

The separation obtained in the chromatogram (Fig. 2A) and estimates of the mean and R.S.D. values ($n=6$) using peak areas obtained from the same standard containing PE (26 μg/ml), CLP (1.7 μg/ml) and paracetamol (284 μg/ml) are shown in Table 1. The R.S.D. ($n=6$) of the retention factors

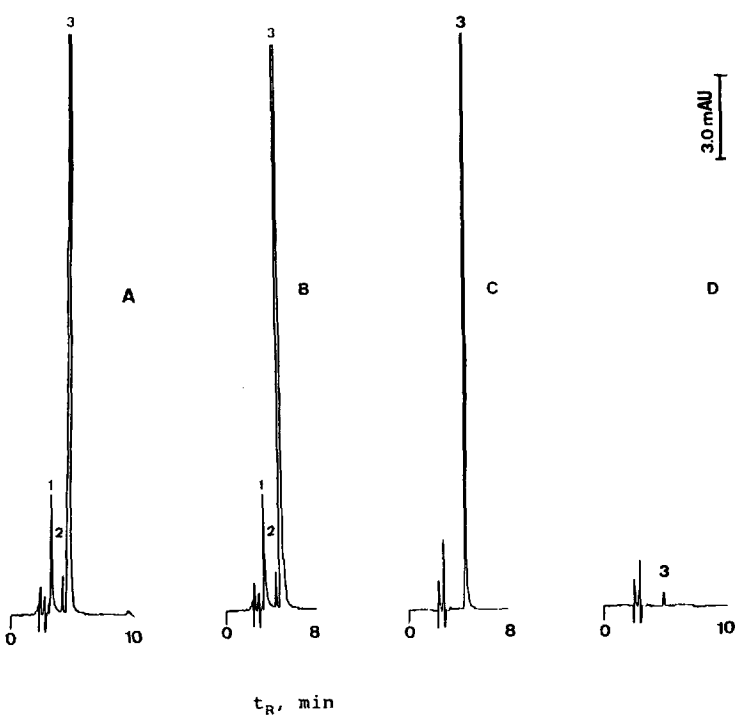

Fig. 2. Chromatograms obtained from (A) a standard mixture containing PE (20 μg/ml), CLP (1.7 μg/ml) and paracetamol (284 μg/ml) and samples containing these active components after sample preparation before (B) and after chewing for 50 s (C) and 3 min (D).

Table 1
Performances of the HPLC separation involving the active ingredients PE, CLP and paracetamol

	PE		CLP		Paracetamol
k	0.41		0.94		1.11
R.S.D. (%) (n=6)[a]	1.1		6.4		0.40
N	2266		38 573		7273
ASF	1.8		1.0		1.1
α		2.29		1.18	
R_s		6.70		2.25	

Conditions: Spherisorb C_{18} column. Mobile phase: aqueous solution containing phosphoric acid–4.5 m*M* hexylamine (pH 3) and acetonitrile (88:12, v/v). k is the retention factor, N is the number of theoretical plates of the column, ASF is the asymmetry factor of the peaks, α is the separation factor and R_s is the resolution between the peaks.
[a] R.S.D. of peak areas.

for the compounds was lower than 1% for each one. As can be observed, the data obtained from these compounds are adequate to develop an analytical method.

3.3. Calibration graphs and detection limits

Standards containing mixtures of PE, CLP and paracetamol were prepared at eight different concentrations, in the range shown in Table 2. These solutions were analyzed with the optimized mobile phase, using a flow-rate of 1.0 ml/min, a Spherisorb column and UV–DAD at 220 nm. The calibration equation, $y=A+Bx$ (μg/ml), was obtained for each compound by plotting peak area (y) versus the concentration (x). In Table 2, the parameters A (intercept), B (slope) and r (regression coefficient) are given.

The detection limits (LODs) obtained for a signal-to-noise (S/N) ratio of 3 are also shown in Table 2.

4. Analysis of chewing gum samples and method validation

4.1. Liquid–liquid extraction of active ingredients

Procedures to extract the active ingredients contained in chewing gum samples required for sample analysis are not described in the literature. However, the first supplement to the United States Pharmacopeia has included a liquid chromatographic (LC) method for the analysis of nicotine in chewing gum [22] and, recently, a LC method for the determination of nicotine in pharmaceutical formulations, nicotine chewing gum and in a nicotine transdermal system has been reported [23].

For the kind of matrix under study and taking the polar nature of the active ingredients into account, appropriate sample pretreatment was required. For this purpose, hexane was selected for the initial extraction step, which was completed using aqueous phosphoric acid (pH 3)–acetonitrile (60:40, v/v). Under such conditions, solutes are extracted into the hydro-organic phase, which was separated from hexane (see Section 2).

In order to evaluate the recovery of active ingredients, samples were processed according to the described procedure. Recoveries (n=6) obtained by means of the calibration curves were as follows: PE (98.7±2.8%), CLP (99.6±4.4%) and paracetamol (101±3.4%).

Table 2
Linear regression equations ($y=A+Bx$) for PE, CLP and paracetamol

	Range (μg/ml)	B (μg/ml)$^{-1}$	A	r	LOD (ng)
Paracetamol	10–500	71 981	16 178	0.9970	0.03
PE	20–100	28 078	−8473	0.9940	0.07
CLP	1–30	96 353	−10 624	0.9997	0.02

y is the peak area and x=μg/ml of active ingredients.
r=correlation coefficient.
LOD=detection limit.

4.2. Linearity

A calibration similar to the one performed above was carried out in the concentration range of interest for the determination of the active ingredients contained in the chewing gum samples. The linearity test was performed using seven different amounts of PE, CLP and paracetamol in the range of 70–130% around the theoretical values. The correlation coefficients, r, found were 0.980, 0.990 and 0.992 for PE, CLP and paracetamol, respectively, indicating good linearity.

4.3. Precision (repeatability and reproducibility)

The precision was examined by analyzing six different chewing gum samples ($n=6$) by only one operator, using the calibration curves.

The repeatability (within-run precision) was evaluated by only one analyst within a day, whereas reproducibility (between-run precision) was evaluated for three different days. The results obtained are shown in Table 3.

4.4. Accuracy

Placebo samples were spiked with different amounts of the active ingredients at 90, 100 and 110% (in triplicate for each one, $n=9$) over the theoretical values. After this, the mixtures obtained were processed according to the extraction procedure (see Section 2) and the active ingredients were determined. The mean values of the percentage recoveries obtained [Recovery (%)±SD] were 102±11 for PE, 97±9.2 for CLP and 96.5±7.1 for paracetamol. As expected, these results are consistent with those obtained above (see Section 4.1).

Table 3
Within-run and between-run precision

	Sample content (mg/SU)		
	PE	CLP	Paracetamol
Within-run precision			
Mean	30.1	2.1	324
R.S.D. (%) ($n=6$)	3.5	4.6	2.2
Between-run precision			
Mean	29.6	2.0	325
R.S.D. (%) ($n=18$)	6.6	7.6	1.6

SU is the sampling unit.

Table 4
Robustness test for chewing gum samples containing PE, CLP and paracetamol, carried out by three analysts

Operator	Statistics	Active ingredient (mg/SU)		
		PE	CLP	Paracetamol
1	Mean ($n=6$)	30.1	2.1	324
	R.S.D. (%)	3.5	4.6	2.2
2	Mean ($n=6$)	32.1	2.0	315
	R.S.D. (%)	8.2	2.1	2.2
3	Mean ($n=6$)	27.3	1.8	328
	R.S.D. (%)	14.6	7.9	4.1
Mean		29.8	2.0	322
R.S.D. (%)		10.1	5.4	3.9

Chromatographic conditions for analyst No. 2 (mobile phase, 4.6 m*M* hexylamine (pH 3.1) and acetonitrile (13%); flow-rate, 1.1 ml/min; column temperature, 38°C; detection, at 219 nm) and operator No. 3 (mobile phase, 4.4 m*M* hexylamine (pH 2.9) and acetonitrile (11%); flow-rate, 0.9 ml/min; column temperature, 42°C; detection, at 221 nm).

4.5. Selectivity

Selectivity was assessed by a qualitative comparison of the chromatograms obtained from samples and from a placebo. It was found that possible interferences due to substances present in chewing gum were not observed.

4.6. Robustness

In order to test the robustness of the method, six samples were analyzed by two operators (Nos. 2 and 3) under different chromatographic conditions than those used in the present method (operator No. 1). The working conditions for the operators and the results obtained shown are in Table 4.

5. Release test of active ingredients into saliva

A study of the release into saliva of active components contained in chewing gum was carried out by a controlled chewing process (see Section 2.5). The chewing process was carried out by ten volunteers for a given chewing time (e.g. 60 s) at the

Table 5
Mean values (%) of active ingredients contained in chewing gum after chewing for various times

	Chewing time (s)							
	15	30	60	90	120	180	240	360
PE	41.1	1.3	0.5	0.4	0.3	0	0	0
CLP	34.6	0.9	1	0.4	0.2	0	0	0
Paracetamol	85.5	68.6	45.6	31.9	22	11.1	3	0.05

same time of the day over eight days, to avoid variability between samples. The results obtained from this study are summarized in Table 5. As can be seen, 3 min is enough time for all of the active ingredients to be released, therefore, chewing for at least 5 min is recommended. Fig. 2 shows the chromatograms obtained from chewing gum samples before (B) and after chewing [50 s (C) and 3 min (D)]. 3 min was enough time for the active components to be completely released from chewing gum.

6. Conclusions

A simple, sensitive, accurate and reproducible HPLC method was developed for the analysis of PE, CLP and paracetamol in chewing gum samples. Moreover, the robustness test indicates that different working conditions are possible due to the fact that small variations in the main variables of the method do not significantly affect the results and the applicability of the proposed method under several working conditions are also shown. The method required a simple liquid–liquid [(*n*-hexane–water)–acetonitrile] extraction procedure prior to the LC analysis. In addition, it was found that the chewing of samples afforded a rapid release of active ingredients into saliva and, therefore, the formulation seemed appropriate for pharmaceutical purposes.

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