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Migration behaviour and separation of acetaminophen and *p*-aminophenol in capillary zone electrophoresis: Analysis of drugs based on acetaminophen

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Abstract

The migration behaviour of acetaminophen and *p*-aminophenol was investigated by capillary electrophoresis. The influence of different parameters (pH, nature and concentration of the running buffer and applied voltage) on the migration time, peak symmetry, efficiency and resolution was systematically investigated. The two analytes can be well separated within 4 min in a 57 cm fused-silica capillary at a separation voltage of 18 kV in a 50 mM borate buffer adjusted to pH 9.5. Correlation coefficients for calibration curves in the range 0.2–200 $\mu g\,ml^{-1}$ for acetaminophen and 0.3–3 $\mu g\,ml^{-1}$ for *p*-aminophenol were higher than 0.999. The sensitivity of detection is 4.2 $ng\,ml^{-1}$ for acetaminophen and 11.2 $ng\,ml^{-1}$ for *p*-aminophenol. The method was applied to the analysis of various commercially available acetaminophen dosage forms with recoveries of 98.4–100.7%.

Keywords: Acetaminophen; *p*-Aminophenol; Capillary zone electrophoresis; Pharmaceuticals

1. Introduction

Acetaminophen (*N*-acetyl-*p*-aminophenol) (AC) is a popular analgesic, antipyretic and non-steroidal anti-inflammatory drug [1]. It is the preferred alternative to aspirin, particularly for patients who cannot tolerate aspirin [2]. Under strong conditions (heat, pH, etc.) AC is slowly hydrolysed to a mixture of *p*-aminophenol (PAP) and acetic acid [3–5]. This reaction can also be carried out by enzymatic cleavage. PAP has high toxicity, including teratogenic effects and nephrotoxicity. It may be present in the pharmaceutical preparations of AC as a synthetic intermediate or as a degradation product.

The International Conference on Harmonization (ICH) has published guidelines on impurities in new drugs products and new drug substances. These guidelines state that all impurities and/or degradants should be identified when present at a level varying from 0.1% to 1% of the main drug, depending on the maximum daily dose [6,7]. Therefore, there is a sustained interest in the development of simple and reliable methods for the determination of the degradation products of the active ingredients in pharmaceutical preparations.

Several methods have been reported for the determination of AC and PAP in pure form, pharmaceutical preparations and biological fluids, including titrimetry [8], fluorimetry [9,10], colorimetry based on oxidation [11,12], diazo coupling [13,14] and nitration [15,16], electrochemical procedures [17] and flow injection [18–21]. Many of these methods suffer from interferences from other active ingredients or additives, and chromatographic separation is usually required in order to obtain good results [22–23].

To our knowledge, measurement of the mixture of AC and PAP has only been carried out by spectrophotometry [4],

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liquid chromatography [23–25] and capillary electrophoresis [26,27]. The spectrophotometric method uses the reaction of PAP with sodium sulfide in the presence of an oxidant to produce a dye. AC can be determined by this spectrophotometric method only after it is hydrolysed to PAP. Liquid chromatography (HPLC) uses SDS or porous graphitized carbon columns with UV and amperometric detection. Both HPLC methods are effective for the simultaneous determination of the two compounds, although the alkyl bonded silica-based stationary phases suffer from a number of drawbacks, including poor stability at extremes of pH and a variety of unwanted interactions [24].

Capillary electrophoresis (CE) is gaining widespread acceptance as a standard analytical technique in pharmaceutical analysis owing to the advantages of minimal sample volume, short analysis time, high separation efficiency, high selectivity and low costs. The separation of AC and PAP by capillary zone electrophoresis (CZE) with amperometric detection has been reported by Yang et al. [26] and Chen et al. [5]. The use of phosphate buffers adjusted to pH 7.2 or 6.5 has been proposed in case where AC is neutral and when its determination in real samples may be a problem due to the presence of electroactive neutral substances. However, according to its pK_a value of 9.56 [27] AC is an ionisable compound, which renders it amenable to CZE separation using middle basic running electrolytes. Thus, despite the major progress in CZE separation of AC and PAP there is still room for optimization of CZE separation. Accordingly, the aim of the present investigation was to optimize the CE conditions for the determination of AC and PAP using photometric detection. The effects of pH, type of buffer and its concentration and applied voltage on mobility, efficiency, resolution, sensitivity and speed were carefully evaluated. The assay was validated by determining its accuracy, precision, linearity, specificity and robustness. This approach may eventually be useful for the quality control of commercial pharmaceuticals because PAP can be measured easily even when present at less than 1% of the concentration of AC.

2. Experimental

2.1. Reagents

AC and PAP were obtained from Sigma (St. Louis, MO, USA). All aqueous solutions were made up in ultrapure water from a Milli-Q plus system (Millipore Ibérica, Madrid, Spain) and filtered through a 0.45 μm filter (Millisolve Kit, Millipore), and then degassed by sonication and evacuation. The running buffer used as the separation medium was prepared by dissolving the adequate amount of sodium tetraborate in ultrapure water and then adjusted to pH 9.5 with sodium hydroxide. Phosphate, CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid) and CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) buffers were also prepared.

2.2. Apparatus

Separations were performed with a P/ACE 5500 automated CE system equipped with a diode array detector, fluid-cooled column cartridge and automatic injector. Fused-silica capillaries (Beckman) of 57 cm (length to detector 50 cm) × 75 μm i.d. and 375 μm o.d. were used. New capillaries were first rinsed with 0.1 M sodium hydroxide for 5 min at high pressure (20 psi), followed by rinsing with the running buffer. It was then equilibrated with the buffer for 10 min by applying a separation voltage of 18 kV. Between experiments, the capillary was rinsed with the running buffer for 1 min. Absorbance was monitored at 250 nm and data were collected and processed using the System Gold data station.

An HPLC Beckman Coulter instrument composed of a System Gold 125 NM Solvent Module, a Rheodyne 7725i manual injection valve and a System Gold 168 diode array detector was also used for the analysis of AC in pharmaceuticals formulations.

2.3. Determination of AC in pharmaceutical preparations

Five tablets were finely powdered and an amount equivalent to 20 mg of AC was accurately weighed and dissolved in a 1 l volumetric flask with ultrapure water. After 10 min of sonication, an aliquot was filtered with a 0.45 μm syringe filtration disk to the vial for injection in the CE system.

3. Results and discussion

3.1. Effect of buffer pH

Manipulation of buffer pH is a key strategy for optimizing the separation of ionizable analytes in CZE because buffer pH determines the extent of the ionization of each analyte and the magnitude of the electroosmotic flow (EOF) that is directed towards the negative end of fused-silica capillary.

AC has only one functional group relevant to acid–base equilibrium in aqueous solution, the phenolic group (pK_a = 9.56 [27]); the acetamino group can only be hydrolyzed under prolonged heating with either acid or basic catalysis [3,28]. PAP has two functional groups, each in acid–basic equilibrium, namely an amino group (pK_a = 5.5 [29]) and a phenolic group. The presence of the amino group, which is electron-donating by resonance, increases the electron density on the benzene ring, intensifying the negative charge. Thus, this phenolic group is a very weak acid (pK_a = 10.46 [29]).

The pH dependence of the migration time of AC, PAP and the neutral marker mesityl oxide is shown in Fig. 1. The EOF velocity indicated by the neutral marker increases with increased pH, which can be ascribed to the increasing dissociation of silanol groups on the capillary wall.

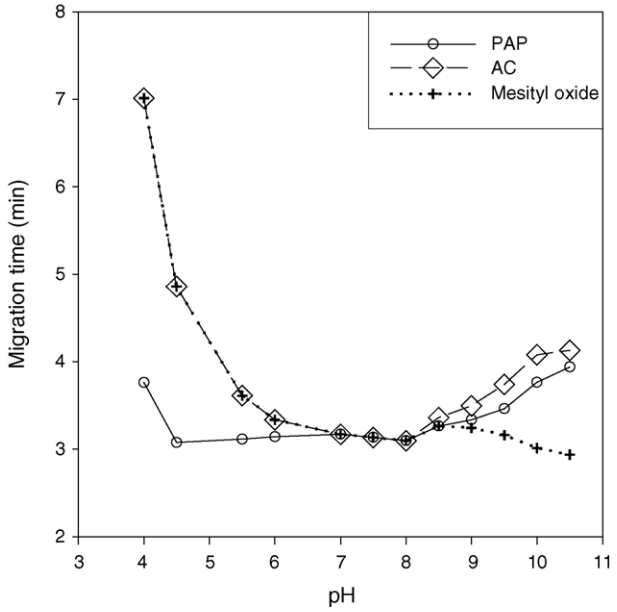

Fig. 1. Dependence of migration time of AC (◊), PAP (◯) and neutral markers (+) on the buffer pH. The electrolyte was 20 mM phosphate solutions of various pH. The effective capillary length was 50 cm, the total length 57 cm and the applied voltage 18 kV. Hydrodynamic injection was performed for 5 s at 3.45 kPa. Detection wavelength, 250 nm.

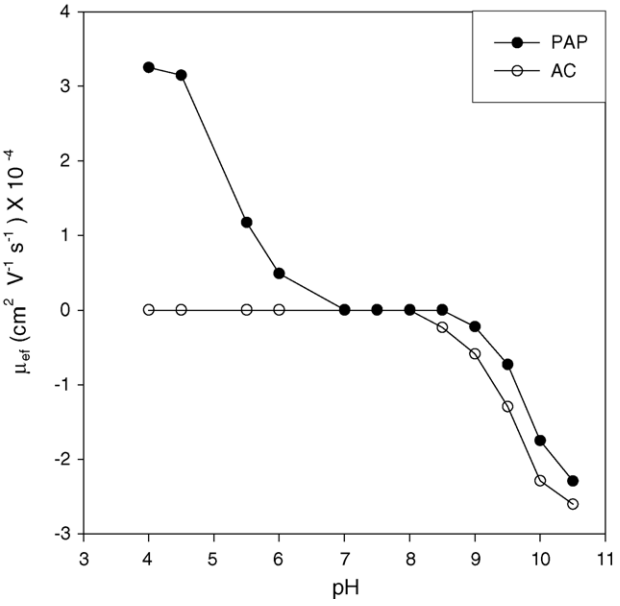

Fig. 2. Variation of the electrophoretic mobility of AC (◯) and PAP (●) as a function of buffer pH in a phosphate buffer (20 mM).

When the pH of the buffer was below the isoelectric point of PAP, it was positively charged and migrated towards the negative electrode in the same direction as EOF due to the protonated amino group, and consequently eluted before the neutral solutes. In contrast, at pH above the isoelectric point, the negatively charged PAP eluted after the neutral marker owing to the dissociation of the phenolic group.

AC remained uncharged up to pH 8.0 and therefore migrated with the EOF. Separation of AC from EOF began at pH 8.5 when its phenolic group started to be sufficiently dissociated.

The electrophoretic mobility of AC and PAP obtained at various pH values in the range 3.5–10.5 is shown in Fig. 2. As expected, the effective electrophoretic mobility (μ_{eff}) of PAP possessing both acid and basic character is given by [30]:

$$\mu_{\text{eff}} = \alpha_{\text{HPAP}^+}\mu_{\text{HPAP}^+} + \alpha_{\text{HPAP}^-}\mu_{\text{HPAP}^-} \quad (1)$$

where μ_{HPAP^+} and α_{HPAP^+} and μ_{PAP^-} and α_{PAP^-} are, respectively, the electrophoretic mobility and mole fraction of the cationic and anionic forms of PAP. It should be noted that μ_{HPAP^+} is a positive value and μ_{PAP^-} is a negative value.

From Eq. (1) one can derive the following equation:

$$\mu_{\text{eff}} = \frac{[\text{H}^+]^2\mu_{\text{HPAP}^+} + K_{\text{a}_1}K_{\text{a}_2}\mu_{\text{HPAP}^-}}{[\text{H}^+]^2 + K_{\text{a}_1}[\text{H}^+] + K_{\text{a}_1}K_{\text{a}_2}} \quad (2)$$

where K_{a_1} and K_{a_2} are the dissociation constant of the protonated amino and phenolic groups, respectively.

Similarly, for AC, a monoprotic weak acid, the effective electrophoretic mobility can be expressed as

$$\mu_{\text{eff}} = \mu_{\text{AC}^-}\alpha_{\text{AC}^-} = \frac{K_{\text{a}}\mu_{\text{AC}^-}}{[\text{H}^+] + K_{\text{a}}} \quad (3)$$

where μ_{AC^-} is the electrophoretic mobility of the fully dissociated anion of AC and K_{a} the dissociation constant of its phenolic group.

The trends in the variation of the effective electrophoretic mobility of AC and PAP as a function of buffer pH (Fig. 2) are in accordance with the prediction of the migration behaviour based on Eqs. (2) and (3). Taking into account that the peak of PAP is overlapped with the system peak at pH values lower than 6.5, the separation of AC and PAP was carried out within the pH range 8.5–10.5.

3.2. Effect of buffer nature and ionic strength

Four different aqueous buffers were tested: borate, phosphate, CHES and CAPSO, all adjusted to the optimum pH range (8.5–10.5). The resolution between PAP and AC was achieved using any of one of these buffers as electrolyte electrophoretic. However, only borate buffer adjusted to pH 9.5 yielded the necessary separation between PAP and the electroosmotic break-through time of the buffer; therefore, this buffer was selected for further studies.

The ionic strength of the run buffer was tested at a constant pH of 9.5. The concentration of the borate buffer affected the EOF and the current produced in the capillary. When it was varied from 10 to 90 mM, the separation between the system peak and PAP and between PAP and AC increased with increasing buffer concentration up to 50 mM, above which it

decreased. Accordingly, a 50 mM borate buffer concentration was considered as suitable.

3.3. Effect of buffer additives

Because the separation between the system peak corresponding to the 50 mM borate buffer adjusted to pH 9.5 and the PAP peak was not sufficiently great, different modifiers were tested in order to improve it. The addition of organic solvents (acetonitrile, ethanol or methanol) did not improve this separation. However, the presence of urea led to an enhancement both in the separation between the system and PAP peak and in the resolution of PAP and AC. Thus, the influence of the concentration of urea in the electrophoretic electrolyte was studied from 2 to 20 mM (Fig. 3). As a result, a concentration of 5 mM urea was selected because it provided the best results.

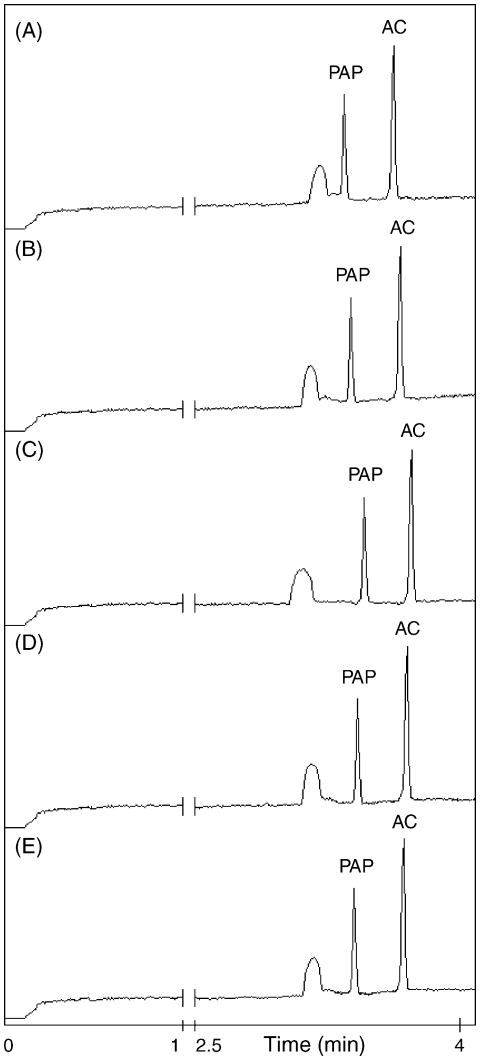

Fig. 3. The effect of urea concentration on the separation of adjacent peaks. Electrolyte, 50 mM borate buffer of pH 9.5. Urea concentration (mM): (A) 0; (B) 2; (C) 5; (D) 10; (E) 20. Other conditions were same as for Fig. 1.

3.4. Influence of applied voltage

The effect of varying the voltage from 10 to 25 kV was investigated under the conditions selected above. As expected, on increasing the applied voltage there was an increase in EOF, leading to shorter run times and higher efficiencies. However, higher voltages also exhibited higher currents and increased Joule heating. To limit this heating inside the capillary, the maximum applied voltage was chosen from an Ohm plot. The best separation was achieved with a voltage of 18 kV (current 36 μA).

3.5. Injection time

The length of the sample injection time was examined in an effort to achieve a low detection limit. Sample solutions were hydrodynamically injected at a pressure of 3.45 kPa while the injection time was varied from 2 to 10 s. Peak area of AC increased linearly on increasing the injection time, but the relationship of PAP was only linear up to 6 s. The injection time selected was 5 s, which corresponded to a plug length of ca. 1.2% of the capillary length.

3.6. Method validation

Fig. 4 represents an electropherogram obtained under the conditions selected for the separation of AC and PAP. Under these conditions, the two compounds are separated in less than 4 min and appear on the electropherogram as highly efficient and symmetrical peaks.

The suitability of the optimized method for the analysis of AC purity was evaluated with validation studies, including stability of the solutions, specificity, linearity, precision, limits of detection and quantification, accuracy and robustness.

3.6.1. Stability of the solutions

This test is often considered as a part of ruggedness of the procedure, however, it should be carried out at the beginning of the validation procedure because it conditions the validity of the data of the other tests.

The response factors of standard solutions of AC and PAP were found to be unchanged for at least up to 15 days if they were stored in brown bottles and kept in a refrigerator at 4 °C.

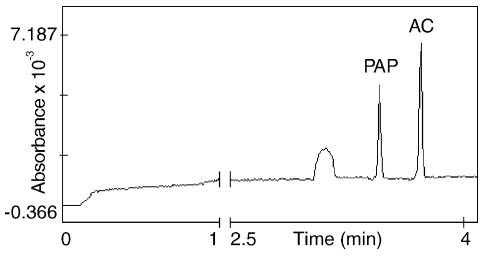

Fig. 4. Separation of AC (4 μg ml^{-1}) and PAP (5 μg ml^{-1}). Electrolyte, 50 mM borate buffer of pH 9.5 and 5 mM urea. Other electrophoretic conditions were same as for Fig. 1.

Less than 1% concentration difference was found between the freshly prepared solutions and those aged 15 days. The solutions can therefore be used within this period without the results being affected.

3.6.2. Specificity

As the procedure described is for application to pharmaceutical preparations, the response of AC and its degradation product, PAP, in the test mixtures containing both compounds and aminoacids, vitamins and excipients were studied. No significant electrophoretic interference was found. Due to the reproducibility of the migration time, this CZE method allowed the discrimination of AC and PAP in these samples. The peak purity technique with diode array detection was used to confirm the consistency of the spectra of the obtained peaks. The selectivity was further assessed by analysis of standard solutions containing freshly prepared AC and AC that had been degradated after hydrolysis with 5 M sulfuric acid for 40 min at 60 °C. The analysis of the hydrolysed sample indicates the presence of a second peak in addition to AC peak. According to the migration time and absorption spectrum, this peak corresponded to PAP. So the proposed CZE method is capable of resolving AC from the degradation product that may be formed during hydrolysis.

3.6.3. Linearity

The concentrations examined were between 0.2 and 200 $\mu g\,ml^{-1}$ for AC and between 0.3 and 3 $\mu g\,ml^{-1}$ for PAP. External calibration was used because no improvement was observed when an internal standard was used. The calibration curves were defined by the following equations: $y = (-9.21 \times 10^{-4} \pm 2.82 \times 10^{-4}) + (2.36 \times 10^{-2} \pm 3.22 \times 10^{-5})\ x$, $r^2 = 0.9999$ for AC and $y = (8.86 \times 10^{-4} \pm 1.25 \times 10^{-4}) + (9.14 \times 10^{-3} \pm 3.51 \times 10^{-4})\ x$, $r^2 = 0.9992$ for PAP, where y is the peak area and x the drug concentration expressed in $\mu g\,ml^{-1}$.

3.6.4. Precision

The repeatability was tested with 11 injections of 2 sample solutions containing the analytes at 2 concentration levels. The relative standard deviations (R.S.D.) for the peak area were 0.18% and 0.44% at 10 and 50 $\mu g\,ml^{-1}$ level, respectively for AC and 1.90% and 1.57% at 0.5 and 1 $\mu g\,ml^{-1}$, respectively for PAP.

The intra-day precision was studied by analysing six different samples containing 0.5 $\mu g\,ml^{-1}$ of PAP and 60 $\mu g\,ml^{-1}$ of AC. The R.S.D. for peak area were 1.97% for PAP and 0.51% for AC.

The inter-day precision of the method was studied by analysing three samples (containing 80 $\mu g\,ml^{-1}$ of AC and 0.5 $\mu g\,ml^{-1}$ of PAP), injected six times every day on six consecutive days. The R.S.D. were 0.73% for AC and 2.31% for PAP.

The migration times were reproducible. The intra and inter-day precision, expressed as R.S.D., were 0.69% and 1.50% for AC and 0.61% and 0.96% for PAP.

Table 1
Robustness of the analytical method upon variation of CE separation conditions

Parameter	Resolution
Voltage	
16	8.7
18	11.3
20	10.5
Buffer pH	
8.5	10.9
9.5	11.3
10.5	9.8
Buffer concentration (mM)	
45	11.0
50	11.3
55	10.7

3.6.5. Limit of detection (LOD) and limit of quantification (LOQ)

The LODs, defined as the concentration where the signal-to-noise (S/N) ratio is 3:1, were found to be 4.2 and 11.2 $ng\,ml^{-1}$ for AC and PAP, respectively. The LOQs, defined as the lowest concentration that can be measured with acceptable precision and accuracy (S/N = 10) were 14.3 $ng\,ml^{-1}$ (R.S.D., 1.17%) and 37.3 $ng\,ml^{-1}$ (R.S.D., 3%) for AC and PAP, respectively.

Ideally, the concentration of the degradation products in the commercial products should be significantly lower than that of the original compounds. Accordingly, the challenge is to determine PAP in the presence of the major component, AC. Unfortunately, PAP could not be quantified at concentrations lower than 0.5% corresponding to AC because the peak was largely overlapped by AC.

3.6.6. Accuracy

The accuracy of the proposed method was tested with several synthetic mixtures containing both compounds in different proportions. PAP/AC mixtures in the ratios from 10:1 to 1:200 were analysed by the proposed CZE procedure. The results obtained were excellent because the recoveries ranged between 97% and 103.8%.

3.6.7. Robustness

The robustness of the method was evaluated by deliberate variation of the method parameters, such as pH, borate buffer concentration and voltage. The change in the CZE results of the same sample was monitored by varying these parameters, and it was found that there was little change in the efficiency and resolution of the two compounds when variations in the electrophoretic parameters were ±10% of the optimum value (Table 1).

3.7. Analysis of AC formulations

The developed method was used for the analysis of commercially available AC containing formulations from differ-

Table 2
Determination of AC in pharmaceutical formulations using the proposed method and a HPLC reference method [25]

Pharmaceutical formulation[a] (supplier)	Stated concentration (mg unit^{-1})	Found concentration[b] (mg unit^{-1})	
		CZE method	HPLC method
Dolostop (Bayer)	650	648.8 ± 7.3	651.6 ± 4.2
Gelocatil (Gelos)	650	655.1 ± 2.9	653.5 ± 1.5
Propalgina Plus (Roche)	500	491.9 ± 1.8	494.4 ± 2.8

[a] Composition of samples: Dolostop: 650 mg acetaminophen, starch, cellulose, polyvinylpyrrolidone, magnesium stearate; Gelocatil: 650 mg acetaminophen, silicon dioxide, cellulose, magnesium stearate, starch; Propalgina Plus: 500 mg acetaminophen, chlorphenamine maleate, phenylephrine hydrochloride, dextrometorphan bromhydrate, ascorbic acid, mannitol, saccharose.
[b] Average of four determinations ± S.D.

Table 3
Recovery data for AC spiked to pharmaceuticals

Pharmaceutical formulation	Added (mg unit^{-1})	Found[a] (mg unit^{-1})	Recovery (%)
Dolostop	50	50.1	100.2
	100	96.3	96.3
	200	191.9	96.0
			$\bar{x}$ = 97.5%
			R.S.D. = 1.38
Gelocatil	50	48.3	96.6
	100	100.0	100
	200	195.5	97.9
			$\bar{x}$ = 98.16
			R.S.D. = 1.00
Propalgina Plus	50	49.7	99.5
	100	98.5	98.5
	200	198.5	99.2
			$\bar{x}$ = 99.06
			R.S.D. = 0.29

[a] Average of four determinations.

ent manufactures. Additives and excipients did not interfere. The quantification of AC in pharmaceuticals was carried out from the calibration graph obtained using the standard solutions. The data of Table 2 clearly indicate that the AC content, as measured by the proposed CZE method, was in excellent agreement with those obtained by the HPLC method [25]. Student's *t*-test was used to compare the average contents of the pharmaceutical formulations. The calculated value of *t* (1.79) is less than the critical value of *t* (2.20) at $P = 0.05$ and 11 degrees of freedom.

The recovery studies were carried out with the three pharmaceuticals fortified with AC over the range 8–40% according to the label. Each sample was analysed four times. As can be seen in Table 3 the recoveries obtained were close to 100%.

The present method was also tested to determine PAP in pharmaceutical formulations. The concentration of this degradation product in all samples was found to be below the limit of detection. This means that the PAP concentration is less than 0.5% the amount of AC.

Confirmation analyses were made in order to make sure that the method is reliable for the determination of PAP, when its concentration is higher than 0.5% that of AC. This was carried out by spiking a sample of one of the pharmaceuticals ("Gelocatil") with different amounts of PAP. The results obtained showed that PAP could be determined at percentages 0.5% or higher respect to AC.

4. Conclusions

The electrophoretic behaviour of AC and PAP showed that the migration times were dependent on pH and the nature of the running buffer. A 50 mM borate buffer adjusted to pH 9.5 was found to be optimal for separating and determining AC and PAP by CZE using UV detection. Such conditions represent a great improvement over previous methods in terms of simplicity, efficiency and sensitivity. The present method is well suited to satisfying the demands for qualitative and quantitative analysis of AC in formulations and for controlling its degradation product within the limits specified.

When this method was compared to the other CE methods, its LOD was seen to be slightly better than that reported for other methods [26,5]. However, the present assay is much more reliable because it works in alkaline medium where AC has negative charge and does not co-migrate with EOF. In addition, it is simpler than the other methods because it

uses absorbance detection instead a homemade amperometric detection.

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