Validated capillary electrophoretic method for the quantitative analysis of histamine acid phosphate and/or benzalkonium chloride

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Abstract

A novel capillary electrophoresis method has been developed and validated for the quantitative determination of histamine acid phosphate (HAP) and/or benzalkonium chloride (BKC). The solutes were separated using a pH 2.5 phosphate electrolyte with detection at 200 nm. Acceptable precision was obtained using internal standardisation. The method was also acceptable for determining levels of histidine which is an impurity in HAP. Profiling of BKC homologues was demonstrated for batch identity purposes. This method is used routinely and it is intended to register this method in the British Pharmacopoeia to supplement current test methods of TLC and HPLC.

Keywords: Histamine acid phosphate; Benzalkonium chloride; Histidine
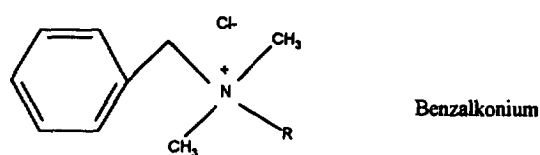
1. Introduction

Capillary electrophoresis (CE) is continuing to expand its application within the area of pharmaceutical analysis. Much of this advance is due to its simplicity and low cost of operation. Often [1–3] simple buffer solutions can be used to separate simultaneously a range of different substances. For example, a simple low-pH phosphate buffer has been validated to quantify a range of basic drugs and excipients [4].

CE has been used [5,6] to quantify levels of anionic detergents such as sodium dodecylbenzenesulphonate (SDBS) and is capable of resolving SDBS homologues. Benzalkonium chloride (BKC) is a widely used antiseptic detergent within the pharmaceutical industry and is composed of alkylbenzyldimethylammonium chlorides with chain lengths in the range C_8–C_{18} (Fig. 1). BKC is
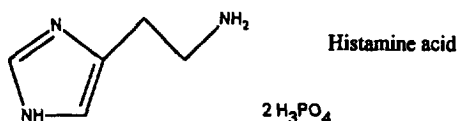
extensively used as a preservative in liquid pharmaceutical formulations. The distribution of the various BKC homologues is characteristic of the input batch of BKC and can be used for batch identity confirmation. It is possible to determine BKC by a variety of analytical techniques including titrimetry [7] and HPLC [8]. Given the potential benefits of adopting CE methods discussed earlier, it was decided to investigate the possibility of assaying and profiling the homologues of BKC by CE.

Histamine acid phosphate (HAP) is a widely used drug whose structure is shown in Fig. 1. The compound is highly basic and such solutes have problems of peak tailing when analysed by HPLC [9]. HAP has only a limited chromophore and this also represents a problem in terms of detection in HPLC where pre-separation derivatisation [10] or electrochemical detection [11] is necessary to determine histamine and similar amines. The testing requirements specified in the British Pharmacopoeial (BP) monograph [12] include separate tests for

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Benzalkonium

R = C_8H_{17} to $C_{18}H_{37}$

Histamine acid

$2 H_3PO_4$

Histidine

Imidazole

Fig. 1. Chemical structures of solutes.

in CE compared to HPLC and internal standards [16–18] are widely used to improve CE precision. The use of CE to determine levels of impurities in various drug substances has been reported [3,14,15,19]. The principal impurity in HAP drug substance is histidine which is determined by two-dimensional TLC [12] with an LOQ of 1% specified for histidine content. The use of low-UV detection wavelength such as 190–210 nm in CE can enable sensitive detection of compounds such as HAP with limited chromophores. The use of low pH electrolytes for the analysis of basic drugs has been shown to be beneficial in several reports [15,19,20] as good peak shapes are often obtained which can allow useful selectivity to be obtained with simple electrolyte compositions. Therefore, it was also decided to assess the use of a low-pH electrolyte in combination with low-UV wavelength detection for the identity confirmation, assay of HAP and determination of histidine levels by CE.

Liquid formulations containing various levels of both HAP and BKC have been produced. Clearly it would be of great benefit if a single method could be used to quantify levels of both constituents. In some circumstances this type of dual analysis is possible by HPLC. For example the levels of both BKC and tramadol were determined [8] by HPLC in ophthalmic solutions. It was decided to investigate the performance of CE in the dual analysis of BKC and HAP in liquid formulations. During this investigation it was noted that high concentrations of HAP (up to 16 mg/ml) caused a decrease in the resolution of BKC homologues but still allowed acceptable quantitation of BKC levels. This resolution decrease is due to the increased ionic strength of the sample solution [21] causing localised perturbation of the electric-field strengths in the sample zone resulting in band broadening and loss of resolution.

All the testing requirements described above for analysis of BKC raw material, HAP drug characterisation and dual analysis of HAP and BKC in formulations could be accomplished using a simple pH 2.5 phosphate buffer. We have therefore validated this testing of HAP and BKC raw materials and for combination products. The validation has shown acceptable performance for linearity, selectivity, precision, recovery, sensitivity and robustness.
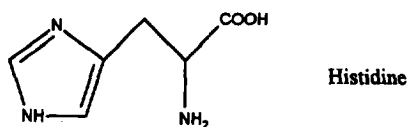
identity confirmation, assay and determination of related impurities. It was anticipated that all of these individual tests could be replaced by a single CE method. The use of CE for identity confirmation by use of concordance of the migration time of the sample with the migration time of a standard is established [13]. CE has been used in many instances to assay the drug content in samples [3,14,15]. However, the injection precision is generally lower

2. Experimental

A 37-cm-long (30 cm to detector), 75-μm capillary filled with 25 mM NaH$_2$PO$_4$ solution (pH adjusted to pH 2.5 with concentrated H$_3$PO$_4$) was used for separation. The capillary was rinsed between injections; initially for 1 min with 0.1 M NaOH, followed by 1 min with the electrolyte. Sample was introduced by pressure application for 5 s. The separation voltage was +15 kV with detection at 200 nm and an operating temperature of 30°C. Sample solutions were prepared in an internal standard solution (ISS) consisting of imidazole (0.1 mg/ml) dissolved in Milli Q water. For raw material testing 0.5 mg/ml HAP and 0.1 mg/ml BKC concentrations were used. Formulations containing both 0.125–16 mg/ml HAP and 0.01% (w/v) BKC were analysed using standards containing 0.1 mg/ml HAP and 0.01% BKC. Sample solutions were diluted accordingly (with water and ISS) to give equivalent HAP and internal standard concentrations to that of the standards. Samples of all test compounds were obtained from within GlaxoWellcome R&D. Inorganic reagents were obtained from Sigma (Poole, UK). Analysis was performed on Beckman P/ACE 5000 CE instruments (Fullerton, CA, USA). Uncoated fused-silica capillaries were purchased from Composite Metal Services (Hallow, UK).

3. Results and discussion

Fig. 2 shows a typical separation of a test mixture containing 0.1 mg/ml of each of the test solutes dissolved in water. Histidine migrates later than HAP due to its increased size and the presence of an ionised carboxylic acid group.

The basis of the method has previously been extensively validated [4] for the testing of a range of basic drugs such as salbutamol, lamuvidine and sumatriptan. Therefore, the general robustness (evaluated using an experimental design protocol), repeatability (between days, analysts and laboratories) and shelf life of electrolytes have been well-established. The validation and analytical performance of the method was established for the three testing requirements of HAP raw material testing,
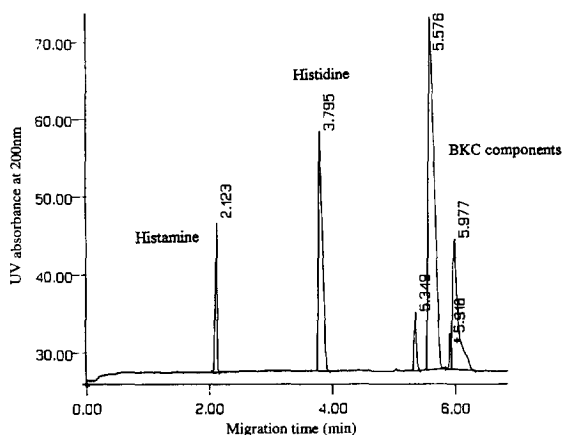

Fig. 2. Separation of a test mixture containing the test solutes. Separation conditions: 37-cm-long (30 cm to detector), 75 μm capillary, 25 mM NaH$_2$PO$_4$ solution (pH adjusted to pH 2.5 with conc. H$_3$PO$_4$), pressure injection for 5 s, +15 kV with detection at 200 nm and an operating temperature of 30°C.

BKC raw material testing and analysis of formulations containing both BKC and HAP.

3.1. HAP raw material testing

Fig. 3 shows an expanded section of an electropherogram of a HAP batch spiked with 1% w/w
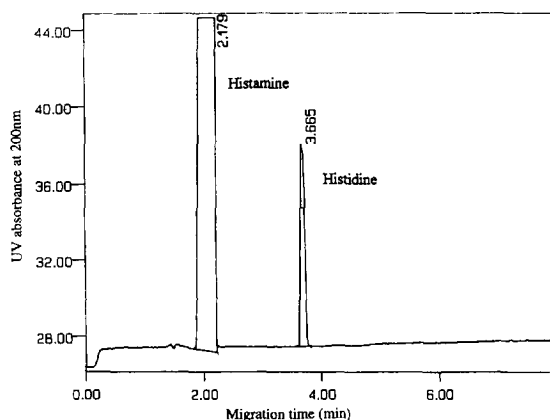

Fig. 3. Separation of histamine acid phosphate (HAP) spiked with 1% histidine. Separation conditions: as Fig. 2 except aqueous sample containing 1 mg/ml HAP and 10 mg/l histidine.
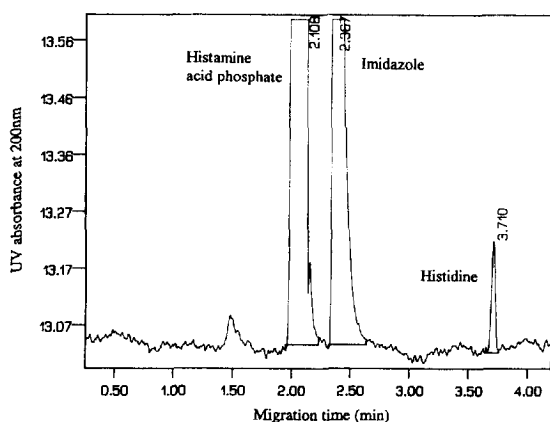
Fig. 4. Separation of histamine acid phosphate (HAP) spiked with 0.1% histidine in internal standard solution. Separation conditions: as Fig. 2 except 0.1 mg/ml imdazole solution containing 1 mg/ml HAP and 1 mg/l histidine.

of histidine. This solution was injected 10 times and an acceptable R.S.D. of 5.7% was obtained for percent area/area measurements of histidine content. Histidine has an appreciably higher UV absorbance at 200 nm compared to HAP. The current BP test specifications for histidine content, performed by TLC, requires a sensitivity of 1% w/w for histidine [12]. Fig. 3 clearly shows that this is readily achievable by CE. Fig. 4 shows that 0.1% (w/w) histidine content can be readily detected in a sample containing both HAP and the internal standard imidazole.

Identity confirmation for HAP was achieved by concordance of the migration times [or relative

migration time (RMT), compared to the migration time of the internal standard, imidazole] of the main peak for the sample with that obtained in the analysis of the HAP standard. Assay of HAP content was performed using peak area ratios of the sample compared to HAP standards. Table 1 shows that acceptable precision data was obtained to allow both identity confirmation and assay. Repeatability of sample preparation was shown by preparation of ten individual HAP samples, each sample solution being injected in duplicate. Table 1 shows an acceptable precision for the response factors for the 20 injections. Response factor precision for calibrations within analytical sequences was found to be in the order of 1–2% R.S.D. Linearity data covering the range 0.05–0.15 mg/ml HAP in internal standard solution (ISS) was acceptable (Table 1). Accuracy was shown (Table 1) by assaying a HAP batch against HAP material previously tested to BP specifications [12] using a titrimetric assay. The simple sample preparation, acceptable analytical performance and rapid analysis time make this CE method a very suitable test method for HAP raw material characterisation.

3.2. BKC raw material testing

Fig. 5 shows separation of two batches of BKC. The method allows resolution of a range of BKC homologues, the longer-chain homologues migrating later than the short-chain homologues. The ratios and distribution of the BKC homologues are characteristic of the batch identity. Improved resolution of the

Table 1
HAP raw material testing results

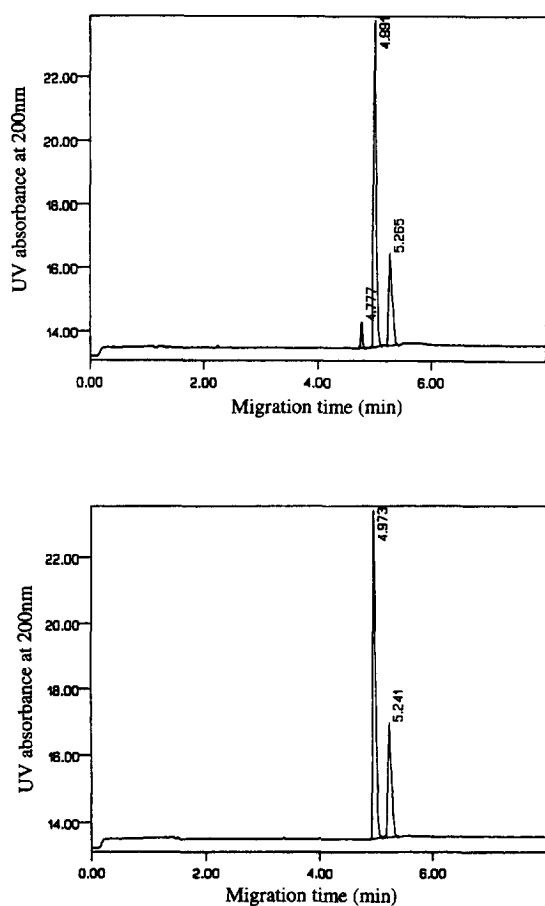| Parameter		Result
Precision	Histamine time	0.38% R.S.D.
	Histamine area	1.55% R.S.D.
	Histidine time	1.6% R.S.D.
Linearity (50–150% target) five samples injected in duplicate	Correlation	0.99994
	Slope	1.81
	Intercept	−0.04%
Accuracy	Sample 1 (CE)	100.5% (w/w)
	Sample 2 (CE)	99.8% (w/w)
	Titration	99.6% (w/w)
Repeatability of ten sample preparation (n=2)	Response factor	0.91% R.S.D.

Fig. 5. Profiling of two BKC batches. Separation conditions: as Fig. 2 except aqueous sample containing 0.1 mg/ml BKC.

homologues would be achieved by sample dilution. However, it is preferable to have higher sample concentrations as these give [18] improved peak area precision values.

For assay purposes the sum of all the peak areas is used to calculate response factors and total BKC content. However, if results are calculated as percent (w/w) of the input BKC batch using standards prepared with that particular batch, then calculations may be performed using a single homologue peak.

Excellent linearity in the range of 0.1 to 0.3 mg/ml BKC in ISS was obtained (50–150%, correlation 0.99915, intercept 0.15%). Precision was assessed by performing 10 injections of a BKC solution. Response factor precision for calibrations within analytical sequences was in the order of

1–2% R.S.D. This performance is similar to that obtained for HPLC [8].

3.3. Analysis of combination BKC and HAP formulations

The method is capable of resolving all the required components in a combination formulation (Fig. 1). Table 2 shows performance data from analysis of prepared formulations containing BKC at a single concentration (0.01%, w/v) and HAP over a variety of concentrations (0.125–16 mg/ml). The test results (Table 2) show the method is capable of generating accurate and precise results for both BKC and HAP contents. Placebo formulations are often prepared and it was therefore necessary to determine the sensitivity of the method for both HAP and BKC. The limits of detection (LOD) and limits of quantitation (LOQ) are given in Table 2 for both BKC and HAP. The sensitivity for BKC is better than for HAP as BKC has an improved UV absorbance at 200 nm. The LOD was calculated as the concentration giving a peak at three times the signal-to-noise ratio. The LOQ was determined by analysing a sample solution ten times (an acceptable R.S.D. of <20% was obtained).

As discussed previously in the introduction, the presence of high concentrations of HAP in the sample solutions caused a deterioration in the separation efficiencies and resolution obtained for the BKC homologues. Therefore in the analysis of BKC content in concentrated HAP solutions the sum of the areas of all the BKC homologues was used for calculation purposes.

3.4. Solution stability

The CE buffer used has a three-month shelf life stored in a plastic container at room temperature [4]. Stored samples were re-analysed after eight days and gave acceptable and comparable data to freshly prepared samples. Assay figures were within 1% agreement for stored and freshly prepared samples and no degradation peaks were observed in the analysis of the stored sample solutions. Thus, a sample solution shelf-life of eight days was assigned. A longer shelf-life was not required in this study and

Table 2
HAP–BKC formulation testing results

Parameter	Factor	Result
Precision	Normalised area ($n=10$)	1.60%
Accuracy (two samples at each concentration injected in duplicate)	BKC 0.010% (w/v)	0.011% (w/v)
	HAP 0.12 mg/ml	0.12 mg/ml
	0.25 mg/ml	0.25 mg/ml
	0.50 mg/ml	0.49 mg/ml
	1.00 mg/ml	0.98 mg/ml
	2.00 mg/ml	1.99 mg/ml
	4.00 mg/ml	4.03 mg/ml
	8.00 mg/ml	8.07 mg/ml
	16.00 mg/ml	15.42 mg/ml
Sensitivity (LOQ determined from ten injections)	HAP LOD	0.5 μg/ml
	HAP LOQ	1.5 μg/ml
	BKC LOD	0.05 μg/ml
	BKC LOQ	0.15 μg/ml
Response factor precision	BKC	2.2% R.S.D. ($n=12$)
	HAP	0.5% R.S.D. ($n=8$)

therefore a more extensive shelf-life determination was not performed.

4. Advantages of the method

The CE method is capable of performing a variety of testing using a relatively simple electrolyte and a standard, inexpensive capillary. Use of the CE method in preference to methods specified in the BP monograph [12], or HPLC alternatives [10,11], can have significant benefits in terms of analysis time, costs, improved quality and solvent purchase/disposal. Table 3 shows an analysis time and performance

Table 3
Comparison of CE method with testing specified in BP monograph for HAP

Parameter	CE	BP testing
Identity confirmation	4 min	2 h (colour test)
HAP assay	4 min	2 h (titration)
Histidine assay	4 min	3 h (TLC)
Absence of HAP	Yes	No
Histidine sensitivity	0.1% or less	~1%
Reagents preparation time	10 min	2 h
Retrievable raw data	Yes	No
Automated testing	Yes	No
Analysis time (one sample)	1 h	1 day
Analysis time (five samples)	2 h	1.5

breakdown for the testing of HAP raw material by CE and the test methods specified in the BP monograph.

It should be noted that a single sample preparation is needed for all analyses by CE. Three separate sample and standard preparations are specified in the BP monograph. Use of the CE method results in a higher quality analysis as it removes the somewhat subjective colour test and histidine quantitation in the TLC test. The TLC analysis time is particularly long as it involves a double development procedure.

The CE method is applicable to both assay of histidine and BKC which can result in significant savings, compared to HPLC, in terms of analysis time and the cost of consumables and reductions in solvent disposal and purchase.

5. Conclusions

A novel CE method has been developed and validated for the quantitative determination of both BKC and HAP. The single method allows identity testing of HAP and BKC raw materials, identity confirmation in BKC–HAP combination formulations, BKC batch profiling, assay of both HAP and BKC, histidine assay in HAP and confirmation of absence for both HAP and BKC. The method has

significant advantages over current BP-specified testing and HPLC alternatives.

Satisfactory validation data was achieved for sensitivity, linearity, precision, recovery, robustness and selectivity. This method is now in routine use within our laboratories and is to be submitted to the British Pharmacopoeia for future inclusion.

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