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Quality control of histamine and methacholine in diagnostic solutions with capillary electrophoresis

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Abstract

Solutions of histamine and methacholine bromide in different matrices for diagnostic purposes were analyzed for stability and quality control using capillary electrophoresis. Histamine (2-[4-imidazolyl]ethylamine) [CAS No. 51-45-6] was determined using a 0.1 M Tris-borate buffer of pH 8.3 with $5 \cdot 10^{-5}$ M cetyltrimethylammonium bromide (CTAB) and 0.005% poly(vinyl alcohol) (PVA) and detected at 214 nm using clenbuterol [4-amino- α -(*tert.*-butylaminomethyl)-3,5-dichlorobenzyl alcohol] [37148-27-9] as an internal standard. Metacholine bromide (acetyl- β -methacholine bromide) [333-31-3] was determined with a 0.01 M creatinine-chloride buffer of pH 4.85 and detected with indirect UV at 230 nm using potassium as an internal standard. Histamine solutions were stable for a prolonged period of time, whereas under enforced degradation conditions methacholine was hydrolyzed, yielding acetic acid and (tentatively) β -methylcholine as reaction products.

Keywords: Capillary electrophoresis; Quality control; Histamine; Metacholine

1. Introduction

Allergic reactions are developed by one in every six individuals in industrialized countries [1]. For the treatment of different kinds of allergies, a number of solutions containing allergenic extracts are used. In an earlier study [2], capillary isotachopheresis was used as a qualitative technique to monitor the (glyco)protein profile of allergenic extracts, e.g. from pollen or house dust mites. Samples were dialyzed prior to analysis in order to remove low-molecular-mass components.

Prior to treatment with the above-mentioned allergenic extracts, specifically for diagnostic pur-

poses, the skin response to intracutaneous injections of solutions of histamine and methacholine is often determined in order to obtain valuable qualitative (and sometimes semi-quantitative) information with regard to allergic response in general and that in which especially histamine plays a crucial role [3]. Mostly these solutions also contain buffering substances. With respect to the consistency of the information obtained from their use, short- and long-term stabilities of these solutions are of prime importance. In the present study, capillary zone electrophoresis was used as a routine quantitative method for the quality control of diagnostic solutions of histamine and metacholine and, in addition, to monitor the thermal degradation of these solutions in dependence of their initial matrix composition.

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2. Experimental

2.1. Equipment

All measurements were carried out on a Beckman P/ACE 2200 capillary electrophoresis instrument (Beckman Instruments, Fullerton, CA, USA). A fused-silica capillary of 75 μm I.D., 330 μm O.D. was purchased from Scientific Glass Engineering (Milton, Keynes, UK). Operating conditions were: voltage, 20 kV; sample introduction was performed with pressure injection for 10 s at $33 \cdot 10^2$ Pa. Prior to each injection, the capillary was rinsed at $1.3 \cdot 10^5$ Pa for 1 min with 0.1 M HCl, for 1 min with 0.01 M KOH, and for 2 min with buffer. Buffers were prepared from analytical grade reagents (Merck, Darmstadt, Germany), dissolved in Milli-Q purified water (Millipore, Bedford, MA, USA). The data were collected on a personal computer and analyzed by the program CAESAR V3.0 (CE Solutions, Long Branch, NJ, USA).

2.2. Chemicals

All chemicals needed for the electrolyte systems were purchased from Merck (Darmstadt, Germany), with the exception of poly(vinyl alcohol) (PVA) which was from Hoechst (Frank-

furt, Germany). Histamine and methacholine standards were provided by HAL (Haarlem Allergenen Laboratorium, Haarlem, Netherlands).

2.3. Sample solutions

Samples were provided as aqueous solutions in 3 or 5 ml ampules (HAL). These solutions contained a number of other components (e.g. for stabilization purposes) such as phenol, monobasic sodium phosphate, dibasic sodium phosphate, ϵ -aminocaproic acid (ϵ -ACA), glycerol, and human serum albumin. The samples and standard solutions were diluted with distilled water and internal standard solution on mass basis.

2.4. Histamine analysis

The capillary was 57 cm long (50 cm to the detector), the detection wavelength was 214 nm. A higher wavelength of detection resulted in an insufficient signal-to-noise ratio. The electrophoresis buffer was 0.1 M Tris–borate pH 8.3 with $5 \cdot 10^{-5}$ M cetyltrimethylammonium bromide (CTAB) and 0.005% poly(vinyl alcohol) (PVA; Hoechst, Frankfurt, Germany) (Table 1). The

Table 1
Operational systems for the analysis of diagnostic solutions of histamine (system A) and methacholine (systems B and C)

Parameter	System A	System B	System C
Capillary length, overall (mm)	570	870	870
Length to detector (mm)	500	800	800
I.D. (μm)	75	75	75
O.D. (μm)	330	330	330
Buffer co-ion	0.1 M Tris	0.01 M creatinine	0.01 M histidine
Buffer counter-ion	borate	HCl	phthalic acid
Buffer pH	8.3	4.85	5.3
Additives	$5 \cdot 10^{-5}$ M CTAB, 0.005% PVA	none	$5 \cdot 10^{-5}$ M CTAB
Polarity	positive	positive	negative
Voltage (kV)	20	20	20
Detection wavelength (nm)	214	230, indirect	230, indirect

Tris = Tris(hydroxymethyl)aminomethane [77-86-1]; CTAB = cetyltrimethylammonium bromide [57-09-0]; PVA = poly(vinyl alcohol) [CAS No. 9002-89-5].

concentration of histamine in the sample solutions ranged between 1 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$.

2.5. Methacholine bromide analysis

The capillary was 87 cm long (80 cm to the detector). As the component of interest does not show UV absorption, detection was carried out at 230 nm in the indirect mode, using creatinine as the UV-absorbing buffer co-ion. For this reason, a background electrolyte of 0.01 M creatinine-HCl, pH 4.85, was used. As an internal standard potassium was used which also shows a negative peak in the indirect mode of detection (see Table 1). The concentration of methacholine bromide in the sample solutions ranged between 0.1 and 20 mg/ml.

3. Results and discussion

3.1. Histamine analysis

For histamine, a 1 g/l histaminediphosphate-monohydrate stock solution in water was made. This was diluted on a mass basis with water and a 0.5 g/l clenbuterol solution. Final concentrations were between 0.1 and 0.8 g/l for histamine and 0.06 g/l for clenbuterol. Calibration graphs thus obtained had correlation coefficients of 0.9995 or better. Samples were diluted in a comparable way to give concentrations within the corresponding calibration range.

Histamine could be detected by its native absorption. In order to improve the inter-variation, a cationic internal standard was required with UV absorbance at 214 nm and an effective mobility lower than $25 \cdot 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$. Clenbuterol was found to be a suitable candidate. Although histamine ($\text{p}K_{\text{a}}\text{s}$ 5.94 and 9.75) is positively charged over a broad pH range, analysis at pH 8.3 is preferred because of two reasons. Although at neutral pH the difference in mobility between histamine and sodium, which is also present in the samples, is considerable, the sodium zone is a broad triangle with a sloping front because of the low effective mobility of the buffer co-ion [4]. The UV absorbance at 214 nm

of the sodium zone is due to a local increase of the counter-ion concentration (borate). The steep rear boundary of the sodium consequently migrates at a considerably lower mobility, and it should be baseline resolved from the histamine peak. This is achieved by analyzing at a pH somewhat closer to the $\text{p}K_{\text{a}}$ of the histamine. Initial experiments without additives to the background electrolyte showed insufficient resolution between sodium and histamine (Fig. 1a). This was caused by considerable electroosmosis, directed towards the detector, a problem often encountered when analyzing cations at high pH. This can be seen from the well-known resolution equation [5,6]:

$$R_s = \frac{1}{4\sqrt{2}} \Delta\mu \sqrt{\frac{VL_d}{DL_t(\mu_{\text{av}} + \mu_{\text{eo}})}} \quad (1)$$

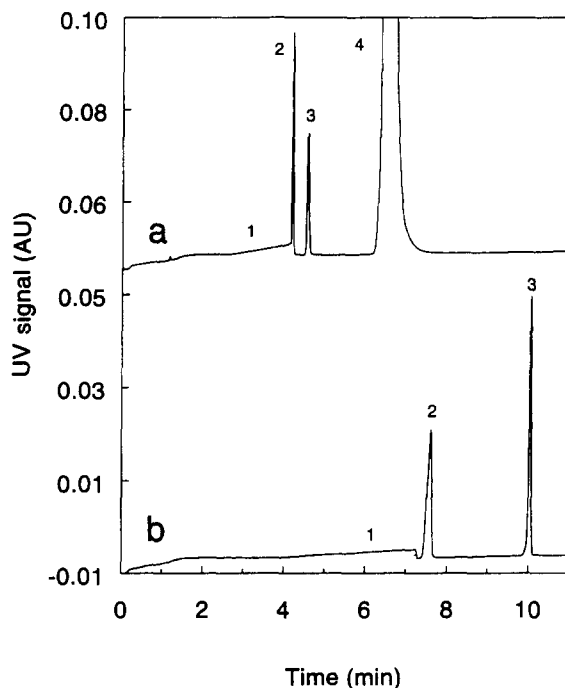


Fig. 1. Typical analysis of a diagnostic solution of histamine, using system A (Table 1) without additives (a) and with PVA and CTAB as additives (b). Peaks: 1 = sodium, 2 = histamine, 3 = clenbuterol (I.S.), and 4 = phenol.

in which R_s is the resolution, $\Delta\mu$ the difference in effective mobility, V the voltage, L_d the length to the detector, D the average diffusion coefficient, μ_{av} the average effective mobility, L_t the total capillary length, and μ_{eo} the electroosmotic mobility. Although the difference in effective mobility $\Delta\mu$ is significant, the electroosmotic mobility μ_{eo} , with the same sign as μ_{av} , is considerable, resulting in a low resolution (Fig. 1a).

Addition of $5 \cdot 10^{-5}$ M CTAB and 0.005% PVA effectively suppresses electroosmosis [7], even at the relatively high pH of the background electrolyte used. The result is a better resolution with the same mobility difference $\Delta\mu$ (Fig. 1b).

The efficiency of the histamine peak is lower (apparent plate numbers 88 000 and 21 000, respectively) because part of the isotachopheretic (ITP) stacking is lost, but the resolution between histamine and sodium is much better, in accordance with Eq. 1.

Figs. 1a and 1b also show that, in spite of the fact that clenbuterol reaches the detector much later with suppressed electroosmosis, its peak height is larger. This can be explained by a reduction of the solute–wall interactions caused by the CTAB–PVA combination. The efficiency of the clenbuterol peak is much higher (apparent plate numbers 40 000 and 130 000, respectively).

To correct for the drift in migration time, the peak areas of both the separand and the internal standard can be divided by the migration times. Calibration graphs thus obtained are often slightly better than those obtained without such correction. Such a correction is especially useful in cases where run-to-run fluctuations of the electroosmotic flow are encountered. In general, the correction is based on a constant linear velocity of each sample component during analysis.

However, in the case of histamine analysis in a system with suppressed electroosmosis, the migration time of especially histamine, but also of the internal standard, depends on the sodium content in the sample. This is caused by the fact that in the initial stage of separation an isotachopheretic configuration is present due to the mobility sequence sodium > histamine > clenbuterol > Tris. This is clearly visualized in Fig. 2,

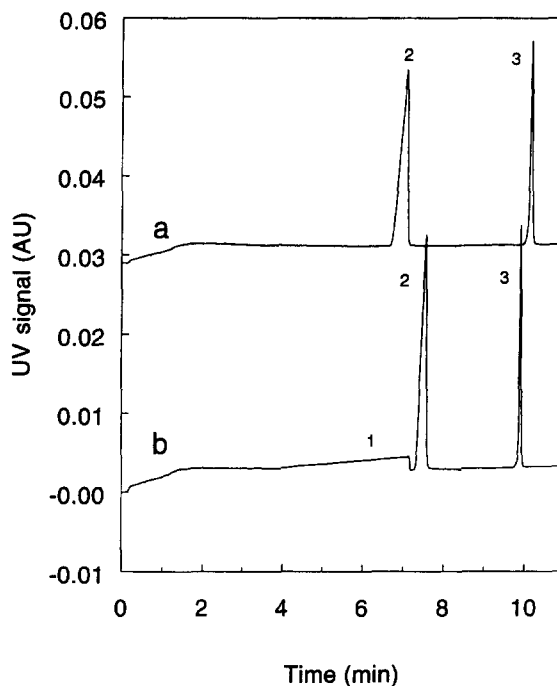


Fig. 2. Comparison of the analysis of histamine in the absence (a) and in the presence (b) of sodium, using system A (Table 1) with PVA and CTAB as additives. Sample component numbers as in Fig. 1.

where Fig. 2a shows the analysis without sodium. Comparison with the analysis of a sample with sodium (Fig. 2b) shows that both the histamine and the clenbuterol peak have significantly higher efficiencies caused by initial ITP stacking, as expected [4]. Apparent plate numbers increase from 8600 to 14 000 for histamine and from 135 000 to 236 000 for clenbuterol. The migration times, however, show a shift that cannot be attributed to electroosmosis. From Fig. 2b it is obvious that a considerable part of the capillary contains sodium. The histamine peak comes later because histamine was forced to migrate at a lower field strength due to the presence of sodium. Clenbuterol, however, moves faster, due to a higher field strength in the part of the capillary not containing sodium. In this case it was found that correction of the (relative) peak areas with migration times does not lead to improved recovery. Thus, in the case of ITP stacking, migration time correction for relative

peak area determination is not advised, unless standards and samples have the same matrix and consequently the same stacking behavior.

In an inter-variation assay for histamine, the following errors were included: weighing, pipetting, deviation of calibration curve, matrix effects, analytical separation, detection, and integration. The results are summarized in Table 2. Recoveries between 90 and 98% were achieved with a 2% relative standard deviation (R.S.D.) at the 10 mg/ml concentration level.

3.2. Methacholine analysis

For methacholine bromide, a 0.5 g/l stock solution in water was made. This was diluted on a mass basis with water and a 1 g/l KCl solution. Final concentrations for the calibration solutions were between 0.064 and 0.45 g/l for methacholine bromide and 0.1 g/l for KCl.

Calibration graphs thus obtained were linear within the concentration range mentioned. The coefficients of correlation were at least 0.9995. Samples were diluted in a comparable way to give concentrations within the corresponding calibration range.

Methacholine migrates as a cation at acidic pH but it does not show any UV absorption. Therefore it was decided to perform detection in the indirect UV mode. A buffering cation with UV absorption was found in creatinine. A 0.01 M creatinine solution was buffered with HCl at the pK_a value of creatinine: 4.85. As seen in a typical analysis (Fig. 3a), separation from bulk sodium content is satisfactory. As an internal standard, potassium was used for the following reasons: it has no UV absorption, was absent in all samples,

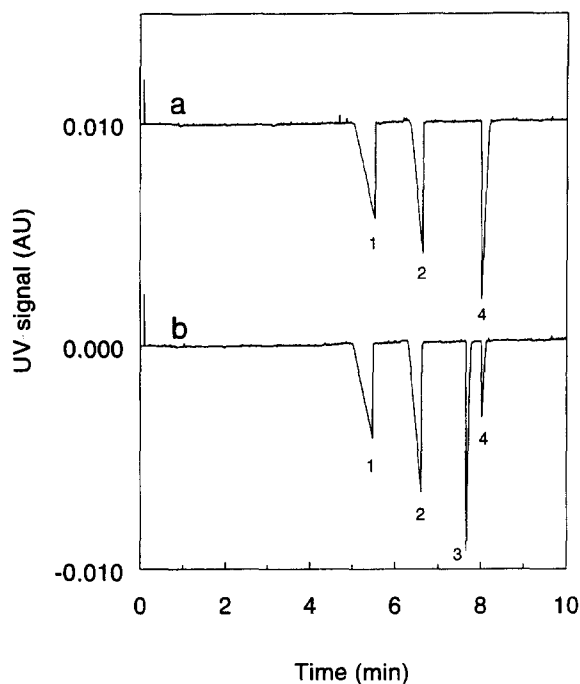


Fig. 3. Typical analysis of a diagnostic solution of methacholine, using system B (Table 1). A fresh solution (a) was compared with a degraded solution (b). Peaks: 1 = potassium (I.S.), 2 = sodium, 3 = degradation product (probably β -methylcholine), and 4 = methacholine.

and has a mobility of $76.2 \cdot 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ and so migrates sufficiently ahead of sodium.

The results of an inter-variation assay for methacholine, including all aforementioned experimental errors, are summarized in Table 3. Recoveries between 98.6 and 100% were achieved with a 2% R.S.D. at the 10 mg/ml concentration level.

A number of samples with different matrices were stored for several months at different

Table 2
Inter-variation assay of the analysis of histamine

Concentration (mg/ml)	Matrix	Recovery (%)	R.S.D. of recovery
10	phenol–water	98	2% ($n = 9$)
10	phenol–Na-phosphate– ϵ -ACA–water	98	
0.1	phenol–Na-phosphate– ϵ -ACA–water	90	
0.003	phenol–Na-phosphate– ϵ -ACA–water	91	

Table 3
Inter-variation assay of the analysis of methacholine

Concentration (mg/ml)	Matrix	Recovery (%)	R.S.D. of recovery
10	phenol–Na-phosphate– ϵ -ACA–water	100	2% ($n = 8$)
1	phenol–Na-phosphate– ϵ -ACA–water	98.6	
0.1	phenol–Na-phosphate– ϵ -ACA–water	100	

temperatures. An example of enforced degradation (24 months at room temperature) of a methacholine solution is shown in Fig. 3b. An additional peak is observed, whereas the peak area of methacholine, relative to that of the internal standard, is decreased. The effective mobility of the additional peak, calculated with sodium as a mobility reference, was $32.5 \cdot 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$. It was suspected that hydrolysis of methacholine results in the formation of acetic acid (not visible in the cationic analysis) and β -methylcholine. The latter component was not

available, but the effective mobility of choline under the operational conditions used appeared to be $36 \cdot 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, so that the peak of $32.5 \cdot 10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ might very well be β -methylcholine, the additional methyl group accounting for the mobility difference.

Hydrolysis of methacholine to acetic acid and β -methylcholine was suspected. In order to verify this degradation reaction, additional anionic analysis of the above-mentioned samples was carried out. Acetate has to be analyzed with indirect UV as well, using electrolyte system C (Table 1). The results are shown in Fig. 4. In Fig. 4a, an anionic analysis is shown of a fresh methacholine solution, corresponding to the cationic analysis in Fig. 3a. The chloride (from the internal standard) and bromide peaks (from the buffer and the sample) coincide, which was verified with separate injections of pure KCl and KBr, respectively. In addition there is a phosphate peak (sample) and system peaks, originating from analyzing the chloride/bromide combination in a twin co-ion buffer: phthalate/bromide [8]. No acetic acid is detected in the fresh sample. Fig 4b shows an anionic analysis of the sample corresponding to Fig. 3b. The migration time of the additional peak corresponds to the migration time of acetate. Consequently, the proposed hydrolysis reaction into β -methylcholine and acetic acid is very likely.

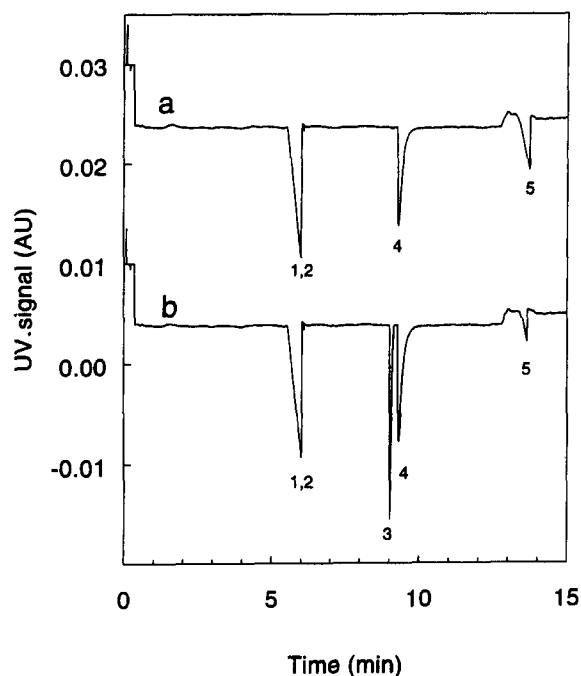


Fig. 4. Forced degradation of a methacholine solution, using anionic system C (Table 1); a fresh solution (a) versus a degraded solution (b). Peaks: 1 = chloride (from I.S.), 2 = bromide, 3 = acetate (degradation product), 4 = phosphate, 5 = system peaks.

4. Conclusions

Capillary zone electrophoresis has proved to be a fast and flexible tool for monitoring quality control of diagnostic solutions of histamine and methacholine. Long-term stability of solutions is easily monitored: forced degradation of methacholine showed hydrolysis into acetic acid and

(tentatively) β -methylcholine, whereas histamine solutions were stable under these conditions.

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