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Capillary electrophoretic determination of acetic acid and trifluoroacetic acid in synthetic peptide samples

Kanthi Hettiarachchi*, Shane Ridge

Analytical Chemistry Department, Biopharmaceutical Development Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA

Abstract

Synthetic peptide samples may contain counter-ions such as acetate or trifluoroacetate as a result of their method of preparation. Furthermore, because acetic acid (HOAc) and trifluoroacetic acid (TFA) are frequently used reagents in peptide synthesis, these acids may be found in synthetic peptide samples as impurities. This paper describes a method validation to determine HOAc and TFA in synthetic peptide samples by capillary electrophoresis (CE) using an internal standard (I.S.) with indirect UV detection. Typical analytical parameters such as precision, linearity, accuracy, specificity, limit of detection and ruggedness were evaluated during the validation. In addition, the contents of HOAc and TFA in two synthetic opioid peptide samples, TIPP[ψ] and Orphanin FQ, were determined using the validated method. A unique feature of the method is that it offers determination of both acids in a single assay using a common I.S. The method is very efficient because of relatively short electrophoretic migration times (typically 2 to 8 min) for the acids investigated. This paper also discusses the factors that affect precision in a CE assay.

Keywords: Acetic acid; Trifluoroacetic acid; Peptides; Opioid peptides; TIPP[ψ]; Orphanin FQ; Glutamic acid

1. Introduction

Peptides have a wide range of applications, and synthetic peptides are currently in great demand. Hormonal peptides such as oxytocin and calcitonin have been known for a long time and their roles in performing specific biochemical functions are well understood. More recently discovered peptides include enkephalins such as Met-enkephalin and Leu-enkephalin. These are neuroactive peptides that belong to a new class called opioid peptides. Peptides such as calcitonin and secretin are medicinally important as therapeutic agents and are manufactured on a large scale. The development of new peptides as potential therapeutic agents is also growing rapidly. Peptides are also increasingly used as model compounds in protein research. The need for synthetic peptides and also technological advances in peptide synthesis has grown dramatically over the past few decades with the rapid growth of newly discovered peptides and proteins.

Trifluoroacetic acid (TFA) is a reagent that has various applications in peptide chemistry, including synthesis, purification and analysis. Peptides are chemically synthesized either by conventional methods or by solid-phase synthesis. Irrespective of the method used, synthesis of peptides always involves many steps, such as protection, anchoring, coupling, deprotection, cleaving and purification. In peptide synthesis, TFA is commonly used as a deprotection reagent in the acidolysis of Boc (*tert*.-butyloxycarbonyl) groups. For this purpose, rather dilute solu-

*Corresponding author.

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tions such as 25% TFA in dichloromethane or chloroform at room temperature are used. Under these conditions, most of the Boc groups are removed in about 30 min. Treatment with higher TFA concentrations such as 50% is preferred to reduce reaction times. Either TFA or a milder acid such as acetic acid (HOAc) is commonly used to extract the peptide after the final cleavage step.

Preparative high-performance liquid chromatography (HPLC) using reversed-phase methods is frequently performed to purify synthetic peptides. In these HPLC systems TFA is often used in the mobile phases and under these circumstances peptide salts such as the trifluoroacetates are formed, upon lyophilization. For basic peptides, sometimes, synthesis is designed so that the final product contains one or two counter-ions such as acetates and trifluoroacetates.

TFA is a chemical that is corrosive and toxic. Because of its toxicity, it can profoundly alter the biological actions during preclinical and clinical studies of synthetic peptides that contain TFA. Thus it is imperative that any residual TFA be removed from the peptide prior to the final formulation. With regard to peptide acetates, the presence of acetic acid may not cause any adverse toxic effects. However, its presence may lower the biological potency of the peptide. Therefore, development of reliable and efficient analytical methods for determining acids such as HOAc and TFA in synthetic peptide samples is very important.

A sample of a synthetic peptide in addition to its major component, the peptide, and its counter-ion, often contain contaminants such as moisture, organic solvents, and peptide impurities. The potency of a synthetic peptide is lowered by the presence of a large amount of inactive species including the counter ions and impurities. The formulations prepared for biological evaluations or therapeutic purposes are based on the peptide content, and hence it is critical that chemical composition be evaluated preceding the formulation process.

To obtain an overall assessment of sample composition during peptide analysis, determination of the peptide content and its counter-ion is necessary. Furthermore, the amount of water and any other constituents that may be present as impurities should also be determined. An extra benefit of such an analysis is the built-in control that all these must add up to a 100%. Therefore, complete analysis of a peptide necessarily includes determination of acids such as HOAc and TFA if the peptide is prepared as an acetate or a trifluoroacetate, or if they are present as impurities.

Until recently, ion chromatography (IC) using anion-exchange columns and conductivity detectors was predominantly used for the separation of anions such as acetate and trifluoroacetate [1–7]. With this technique, in the majority of cases, HOAc co-elutes with early-eluting anions such as fluorides and chlorides unless special columns or modified methods such as gradient elution [2], or coupled separation systems [3] are employed. Application of IC was shown to be a straightforward method to determine TFA in peptide samples [4], however, co-elution of HOAc with chloride was evident.

In recent years, capillary electrophoresis (CE) has become an efficient and reliable technique to determine organic anions. Jones and Jandik [8,9] have worked extensively on ion analysis using both IC and CE and compared the two techniques. According to their findings, CE has many advantages over IC for ion analysis in terms of selectivity, peak capacity, ease of adaptability and rapid analysis times.

In CE, because the online detector window of the capillary is very small, indirect detection becomes an excellent choice for detecting analytes such as formic acid and HOAc that have insufficient UV absorption to permit direct detection. The feasibility of indirect detection of small organic acids such as formic acid and HOAc by CE was first demonstrated by Hjertén et al. [10] in 1987. Since then, analysis of HOAc has been successfully achieved by making use of the excellent separation capabilities of CE coupled with indirect detection [11–20]. In most of these cases, UV-absorbing background electrolytes such as chromate or phthalate and electroosmotic flow modifiers such as cetyltrimethylammonium bromide (CTAB) or tetradecyltrimethylammonium bromide have been utilized to achieve good separation and detection of anions in various sample matrices.

This paper describes the validation of a CE method for the determination of HOAc and TFA in synthetic peptide samples using L-glutamic acid (L-Glu) as the internal standard (I.S.). The CE method is time efficient for two reasons, (a) it allows simulta-

neous determination of both acids from a single assay using a common internal standard instead of requiring two different assays, and (b) the electrophoretic run times are much shorter, requiring less than 10 min. The capability of further reduction of electrophoretic run times to about 4 min, is also demonstrated in this paper, thereby making the assay extremely efficient.

2. Experimental

2.1. Instrumentation

Two CE systems, a BioFocus 3000 with a programmable fast-scanning UV–Vis detector and BioFocus 3000 software for system control and data collection and analysis (Bio-Rad, Hercules, CA, USA) and an HP ^{3D}CE with a built-in diode-array detector and a ChemStation for system control, data collection and analysis (Hewlett-Packard, Wilmington, DE, USA), were used in this work. Fused-silica (uncoated) capillaries were purchased from Bio-Rad and J&W Scientific (Folsom, CA, USA).

2.2. Reagents

Sodium trifluoroacetate (NaTFA) and sodium acetate (NaOAc) were used as the reference standards. The I.S. used was L-Glu. Phthalic acid was used as the UV-absorbing electrolyte and CTAB as the electroosmotic flow modifier. All chemicals (reagent grade) were purchased from Aldrich (Milwaukee, WI, USA).

2.3. Peptide samples

The synthetic opioid peptide samples investigated in this study were TIPP[ψ], supplied by Research Triangle Institute (Research Triangle Park, NC, USA) and Orphanin FQ, supplied by Multiple Peptide Systems (San Diego, CA, USA).

3. Procedure

For method validation and to determine HOAc and TFA in peptide samples, reference standard solutions were prepared using NaOAc and NaTFA (equivalents of HOAc and TFA) and I.S. solution using L-Glu. Test solutions were prepared by mixing equal volumes of reference standard solutions and I.S. solution. Sample solutions were prepared by dissolving the peptides in water, 0.5 mg/ml, and then by mixing each solution with an equal volume of I.S. All CE experiments were performed at a voltage of 18 kV under negative polarity, i.e., with the electrode at the inlet end of the capillary maintained at a negative voltage. Indirect detection was monitored at 200 nm UV. Other experimental conditions are given in Tables 1–3.

The buffer solution used was 5 m*M* sodium phthalate, pH 5.85 (*o*-phthalic acid $pK_{a1}=2.89$ and $pK_{a2}=5.51$) containing 5 m*M* CTAB.

All reference standard solutions, sample and buffer solutions were prepared in distilled, deionized water.

4. Results and discussion

4.1. Precision

To validate the CE method presented in this paper, the precision was first determined by electrophoresing five aliquots of a single test solution, and then by electrophoresing five test solutions prepared separately.

The detection response factor, defined as the ratio of analyte peak area to I.S. peak area per unit mass was calculated for each electropherogram and these results are given in Table 1. From these two sets of precision data, the relative standard deviation (R.S.D.) values obtained were 1.5% and 1.7% for HOAc and 0.9% and 1.6% for TFA ($n=5$ for both sets).

Peak area reproducibility is a major factor that determines precision in a separation-based assay.

For a continuous set of electrophoretic runs, the migration time of an analyte either gradually increases [21] or decreases (Table 2, part A) depending on the conditions used, until it reaches a steady value. For the experiment shown in Table 2, part A, the decrease in migration time is very prominent for the first four runs. Between the first and the second runs, the changes in migration times are 0.09, 0.11

Table 1
Precision data (75 cm×50 μm J&W Scientific uncoated capillary; load: 10 p.s.i. s; BioFocus 3000; 1 p.s.i.=6894.76 Pa)

Run No.	$W_{HOAc\ mg/200\ ml}$	$W_{TFA\ mg/200\ ml}$	Peak area			Detection factor response	
			HOAc	I.S.$_{1\ mg/20\ ml}$	TFA	HOAc	TFA
(A) Obtained using the same solution							
1	7.399	14.947	382 444	379 734	636 958	0.1361	0.1122
2	7.399	14.947	394 610	386 886	635 081	0.1378	0.1098
3	7.399	14.947	389 652	395 337	659 240	0.1332	0.1116
4	7.399	14.947	390 646	391 895	657 701	0.1347	0.1123
5	7.399	14.947	410 478	402 810	670 566	0.1377	0.1114
					Mean:	0.1359	0.1115
					S.D.:	0.0020	0.0010
					% R.S.D.:	1.5	0.9
(B) Obtained using different solutions							
1	7.399	14.947	382 444	379 734	636 958	0.1361	0.1122
2	7.795	15.165	405 366	389 527	670 003	0.1335	0.1134
3	8.531	14.022	446 026	381 184	594 503	0.1372	0.1112
4	7.250	14.584	383 063	382 034	629 878	0.1383	0.1131
5	7.777	14.386	414 257	381 331	636 013	0.1397	0.1159
					Mean:	0.1370	0.1132
					S.D.:	0.0023	0.0018
					% R.S.D.:	1.7	1.6

and 0.13 min for HOAc, L-Glu and TFA, respectively, in contrast to the constant migration times observed for the ninth and tenth runs. The R.S.D. value of migration times calculated for the last six runs (runs 5–10) is 0.5%, which is much better than the 1.6% calculated for all ten runs. From this observation it could be easily predicted that the R.S.D. values for individual peak areas would behave in a similar way [21]. In fact, comparison of peak area R.S.D. values for the last six runs with those for all ten runs showed improved trends (1.2, 1.3 and 1.3% for HOAc, L-Glu and TFA, respectively, in the last six runs compared to 1.6, 1.3 and 1.5%, respectively, for all ten runs). R.S.D. values for the peak area ratios also showed similar trends (0.6, 0.4 and 0.7% for the last six runs compared to 0.9, 0.7 and 0.6% all ten runs). An important conclusion that can be drawn from these results is that in quantitative analysis using CE, it is best to condition the capillary by electrophoresing a test solution containing similar analytes a few times until the migration time becomes stable before starting the assay.

Results shown in Table 2, parts B and C further confirm this conclusion. These results are from two different CE systems, a Bio-Rad BioFocus 3000 and a Hewlett-Packard HP ^{3D}CE. Two aliquots from the same test solution and two pieces of a J&W Scientific uncoated capillary from the same batch were used to study the electrophoretic behavior of HOAc, TFA and L-Glu. The R.S.D. results obtained from the Bio-Rad instrument (Table 2, part B) are very similar to those obtained from the Hewlett-Packard instrument (Table 2, part C), indicating that the CE method is very reproducible. At least ten trial runs were made with both systems thereby allowing the capillary to condition sufficiently before obtaining the data, and accordingly, the migration times of Table 2 parts B and C show much better precision than those shown in Table 2, part A. The R.S.D. values for peak area ratios showed a slight improvement than those for peak areas alone. However, the R.S.D. values for the latter alone were also acceptable. One reason for this is that the good precision in migration times would have caused a better precision of peak areas. As demonstrated earlier [21], shorter migration times, or in other words, higher mobilities could have caused the analytes to have reproducible peak areas without contributing to peak area distortion. The results presented in Table 2, parts B and C demonstrate that the predominant factor that contri-

Table 2
CE precision of migration time, peak area and peak area ratio for HOAc, L-Glu and TFA

	Migration time (min)			Peak area			Peak area ratio		
Run No.	HOAc	L-Glu	TFA	HOAc	L-Glu	TFA	HOAc/L-Glu	TFA/L-Glu	TFA/HOAc
(A) 75 cm×50 μm Bio-Rad uncoated capillary; load: 10 p.s.i. s; BioFocus 3000									
1	6.42	7.71	8.21	253 434	183 321	366 502	1.3825	1.9992	1.4461
2	6.33	7.60	8.08	251 047	184 122	364 012	1.3635	1.9770	1.4500
3	6.27	7.53	8.00	246 005	180 900	356 401	1.3599	1.9702	1.4488
4	6.22	7.48	7.94	244 937	182 307	354 974	1.3435	1.9471	1.4492
5	6.19	7.44	7.90	243 672	178 947	354 234	1.3617	1.9795	1.4537
6	6.17	7.41	7.87	249 168	182 076	359 754	1.3685	1.9758	1.4438
7	6.16	7.40	7.85	239 377	176 337	347 984	1.3575	1.9734	1.4537
8	6.14	7.38	7.83	245 678	182 381	357 819	1.3471	1.9619	1.4565
9	6.13	7.37	7.82	246 093	182 512	361 717	1.3484	1.9819	1.4698
10	6.13	7.37	7.82	245 622	181 938	361 180	1.3500	1.9852	1.4705
Mean (1–10)	6.22	7.47	7.93	246 503	181 484	358 458	1.3583	1.9751	1.4542
S.D.	0.10	0.11	0.13	3929	2278	5371	0.0117	0.0139	0.0092
% R.S.D.	1.6	1.5	1.6	1.6	1.3	1.5	0.9	0.7	0.6
Mean (5–10)	6.15	7.40	7.85	244 935	180 699	357 115	1.3555	1.9763	1.4580
S.D.	0.03	0.04	0.04	3024	2316	4802	0.0078	0.0075	0.0096
% R.S.D.	0.5	0.5	0.5	1.2	1.3	1.3	0.6	0.4	0.7
(B) 75 cm×50 μm J&W Scientific uncoated capillary; load: 18 p.s.i. s; BioFocus 3000									
1	6.49	7.38	8.14	646 431	195 388	115 012	0.3308	0.5886	1.7792
2	6.48	7.37	8.14	648 376	194 283	113 049	0.3337	0.5819	1.7436
3	6.48	7.38	8.15	637 377	193 042	111 306	0.3302	0.5766	1.7463
4	6.48	7.38	8.15	641 645	192 858	111 420	0.3327	0.5777	1.7365
5	6.48	7.39	8.16	637 310	192 433	110 601	0.3312	0.5748	1.7354
6	6.48	7.39	8.16	636 038	193 030	111 124	0.3295	0.5757	1.7471
7	6.48	7.39	8.16	638 421	194 387	111 236	0.3284	0.5722	1.7424
8	6.48	7.39	8.17	633 510	190 921	110 569	0.3318	0.5791	1.7453
9	6.48	7.4	8.17	632 011	191 594	109 412	0.3299	0.5711	1.7312
10	6.48	7.4	8.18	636 246	189 864	108 661	0.3351	0.5723	1.7078
Mean (1–10)	6.48	7.39	8.16	638 737	1927 803	1 112 395	0.3313	0.5770	1.7415
S.D.	0.003	0.009	0.013	5277	16 755	17 766	0.0020	0.0053	0.0176
% R.S.D.	0.0	0.1	0.2	0.8	0.9	1.6	0.6	0.9	1.0
Mean (5–10)	6.48	7.393	8.166	635 589	192 038	1 102 676	0.3310	0.5742	1.7349
S.D.	0.000	0.005	0.008	2397	16019	10 187	0.0024	0.0030	0.0146
% R.S.D.	0.0	0.1	0.1	0.4	0.8	0.9	0.7	0.5	0.8
(C) 50.5 cm×50 μm J&W Scientific uncoated capillary; load: 500 mbar s; HP ^{3D}CE									
1	2.25	2.56	2.79	111.771	354.643	194.978	0.3152	0.5498	1.7444
2	2.25	2.56	2.78	113.963	357.184	194.473	0.3191	0.5445	1.7065
3	2.24	2.55	2.78	113.288	352.687	192.559	0.3212	0.5460	1.6997
4	2.24	2.55	2.78	113.339	350.959	192.865	0.3229	0.5495	1.7017
5	2.24	2.55	2.77	112.740	352.125	193.147	0.3202	0.5485	1.7132
6	2.24	2.54	2.77	112.450	356.937	190.743	0.3150	0.5344	1.6962
7	2.23	2.54	2.77	113.070	353.775	191.477	0.3196	0.5412	1.6934
8	2.23	2.54	2.76	113.607	358.665	194.183	0.3167	0.5414	1.7093
9	2.23	2.54	2.76	112.820	358.154	193.836	0.3150	0.5412	1.7181
10	2.23	2.54	2.76	111.386	350.471	191.694	0.3178	0.5470	1.7210
Mean (1–10)	2.24	2.55	2.77	112.843	354.560	192.996	0.3183	0.5443	1.7104
S.D.	0.009	0.007	0.009	0.8008	3.0194	1.3958	0.0028	0.0048	0.0150
% R.S.D.	0.4	0.3	0.3	0.7	0.9	0.7	0.9	0.9	0.9
Mean (5–10)	2.23	2.54	2.77	112.679	355.021	192.513	0.3174	0.5423	1.7085
S.D.	0.005	0.004	0.005	0.7432	3.3884	1.4013	0.0022	0.0050	0.0114
% R.S.D.	0.2	0.2	0.2	0.7	1.0	0.7	0.7	0.9	0.7

Table 3
Linearity data (75 cm×50 μm J&W Scientific uncoated capillary; load: 10 p.s.i. s; BioFocus 3000)

Run No.	Mass of NaOAc (mg/100 ml)	Mass of HOAc (mg/100 ml)	Sample peak area	I.S. peak area	Peak area ratio	Back calculated mass[a]	Deviation[b] (%)
(A) Acetic acid							
1	2.006	1.453	65 599	264 354	0.2481	1.482	1.989
2	3.881	2.810	115 035	251 013	0.4583	2.787	−0.826
3	6.106	4.422	182 599	259 274	0.7043	4.314	−2.442
4	7.882	5.708	237 885	257 252	0.9247	5.682	−0.452
5	9.776	7.079	299 393	256 184	1.1687	7.197	1.665
6	12.157	8.803	365 627	251 203	1.4555	8.977	1.981
7	14.423	10.444	416 116	249 892	1.6652	10.279	−1.578
Equation:	$y=0.1611x+0.0094$; $R^2=0.9986$; error[c]=1.6%						
(B) Trifluoroacetic acid	Mass of NaTFA	Mass of TFA					
1	10.064	8.267	245 341	264 354	0.9281	8.205	−0.757
2	19.008	15.615	470 578	251 013	1.8747	15.967	2.260
3	29.710	24.406	753 395	259 274	2.9058	24.423	0.070
4	39.075	32.099	963 206	257 252	3.7442	31.298	−2.494
5	50.630	41.591	1287 426	256 184	5.0254	41.805	0.515
6	61.002	50.111	1535 878	251 203	6.1141	50.733	1.241
7	70.274	57.728	1730 582	249 892	6.9253	57.385	−0.594
Equation:	$y=0.1219x-0.0724$; $R^2=0.9993$; error[c]=1.10%						

Note: Data for Table 2, parts A and B were generated from the same experiment.
[a] Back calculated mass=result for x when the peak area ratio y is substituted in above equation.
[b] Deviation=[Mass−back calculated mass]/mass×100.
[c] Error=Mean absolute deviation.

butes to peak area reproducibility is the migration time and not the injection volume. The reason for using an I.S. in a CE assay is to counteract the peak area distortion supposed to be caused by inaccurate sample injection. However, as seen from data presented in Table 2, parts B and C good peak area precision for the analytes can be achieved provided migration times are kept very short. This may indicate that the pressure injections offered by commercial CE instruments are sufficiently accurate.

Typical electropherograms obtained from the Bio-Rad and the Hewlett-Packard CE systems are shown in Fig. 1A Fig. 1B, respectively. Because a lower electric field strength (E) of 24 V/cm was used with a capillary of 75 cm length in the Bio-Rad instrument, migration times were longer (Fig. 1A) than those observed using a higher E of 36 V/cm with a capillary of 50 cm length used in the Hewlett-Packard instrument (Fig. 1B). As shown in Fig. 1B, HOAc, L-Glu and TFA are completely baseline resolved with very short migration times of 2.25, 2.56 and 2.78 min respectively, making it a very time efficient assay.

4.2. Linearity and accuracy

Electropherograms were obtained from seven separate test solutions that covered a range from 50% to 150% of the acid content typically found in a small peptide salt. The ratios of test solution acid peak area to I.S. peak area were calculated for both acids (HOAc and TFA) in each electropherogram. The mass of each acid was plotted against the corresponding peak area ratio to construct working curves for HOAc and TFA. Linear regression analysis of the data for each curve yielded R^2 values of 0.9986 and 0.9993 for HOAc and TFA, respectively. The equations obtained for the two curves were $y=0.1611x+0.0094$ and $y=0.1219x-0.0724$, with corresponding errors of 1.6% and 1.1%, for HOAc and TFA, respectively. The linearity data for both HOAc and TFA were obtained from a single experi-

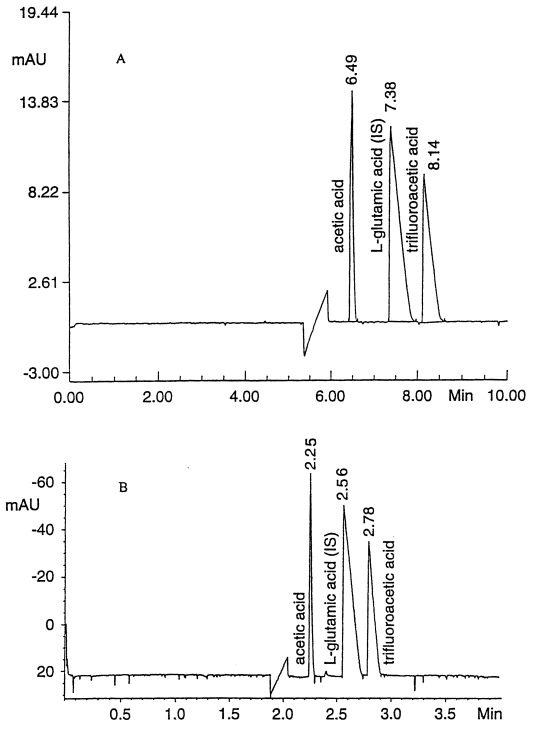

Fig. 1. Typical CE profile of acetic acid, L-glutamic acid and trifluoroacetic acid (5.0 mg sodium acetate, 10.0 mg L-glutamic acid and 12.0 mg sodium trifluoroacetate dissolved in 60 ml water) (A) obtained under an electric field strength of 24 V/cm and at a capillary temperature of 23°C using a Bio-Rad instrument and (B) obtained under an electric field strength of 36 V/cm and at a capillary temperature of 23°C using a Hewlett-Packard instrument. For other conditions of (A) and (B), please see Table 2, parts B and C, respectively.

ment and are shown in Table 3. These results indicate good linearity and accuracy (errors <2.0%).

4.3. Ruggedness and robustness

Because the above precision experiments (Section 4.1) were carried out by two analysts on different days, using different capillaries (Bio-Rad and J&W Scientific), separately prepared test solutions, buffer solutions and two different CE systems (Bio-Rad BioFocus 3000 and Hewlett-Packard HP ^{3D}CE), they also confirm the ruggedness of the method.

The fact that the R.S.D. of the peak area ratio remained unaffected by changes in method parameters such as capillary length and the amount of sample loaded (Table 2, parts B and C) is a clear indication of the robustness of the method.

4.4. Specificity and sensitivity

Succinate is another counter ion commonly found in basic drugs [20]. Specificity of the CE method was examined by using succinic acid together with HOAc, TFA and the I.S., L-Glu. An electropherogram of a solution prepared from a mixture of these acids and the I.S. is shown in Fig. 2. The good resolution obtained between each constituent indicates the specificity of the method.

Based on 3-times the signal-to-noise ratio, the lower detection limits for HOAc and TFA are 240 and 40 ppm, respectively, under the conditions used for these experiments.

4.5. Determination of counter-ions in synthetic peptide samples

CE offers determination of organic acids found as counter-ions in basic drugs and indeed it is a simple and an inexpensive alternative to IC. This is very well demonstrated for the analysis of succinate in sumatriptan and maleate in chlorpheniramine [20], and acetate in bradykinin [22].

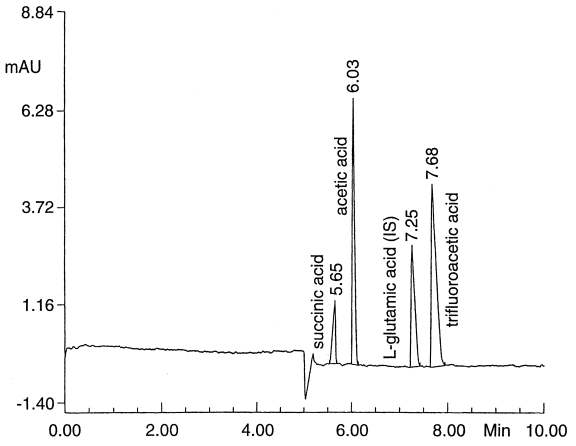

Fig. 2. Typical CE profile of acetic acid, trifluoroacetic acid, succinic acid and L-glutamic acid, showing specificity (2 mg sodium acetate, 8 mg sodium trifluoroacetate, 3 mg sodium succinate and 3 mg L-glutamic acid dissolved in 50 ml water).

4.5.1. Determination of HOAc and TFA in TIPP[ψ]

One opioid peptide sample subjected to analysis by the validated method described above was TIPP[ψ]. Because of the method of preparation, this peptide was a mixture of 50% acetate and 50% trifluoroacetate salts. Analysis of acids in the peptide was performed by using two working curves constructed for HOAc and TFA by electrophoresing reference standard solutions as described earlier. Three accurately prepared peptide sample solutions each mixed with I.S. were also electrophoresed to obtain peak area ratio for the acids present in the peptide. From the two linear equations obtained for the reference solutions and the corresponding responses obtained for the peptide samples, the amounts of HOAc and TFA calculated were 2.1±0.2% and 16.4±0.5%, respectively. Considering the experimental errors involved in each analysis, these values have a reasonable correlation with the found peptide content of 75.1% and the solvated water content of 4.9% in the sample. A typical CE profile of HOAc and TFA present in the peptide and the I.S. is shown in Fig. 3.

4.5.2. Determination of TFA in Orphanin FQ

This peptide sample was a TFA salt. Experiments performed similar to those for TIPP[ψ] excluding NaOAc in the reference standard solution, yielded a value of 22.8±1.3% for TFA. This value was again in reasonable agreement with the found peptide content of 71.7% and water content of 3.8%. A typical CE profile of TFA present in the sample and the I.S. is shown in Fig. 4.

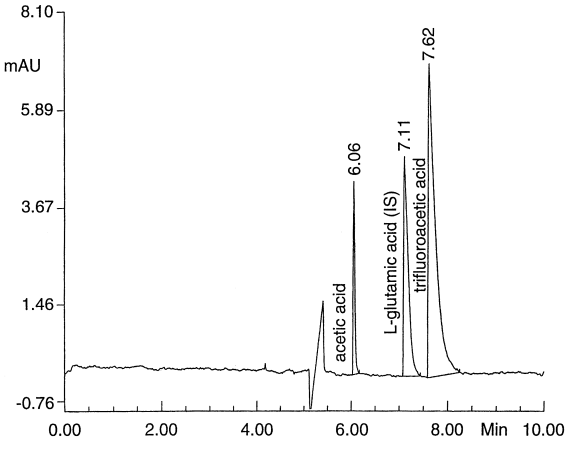

Fig. 3. CE profile of TIPP[ψ] sample showing acetic acid, trifluoroacetic acid and internal standard, L-glutamic acid (TIPP[ψ] 0.5 mg/ml water, L-glutamic acid 0.5 mg/10 ml water). For other conditions, please see Table 2, part B.

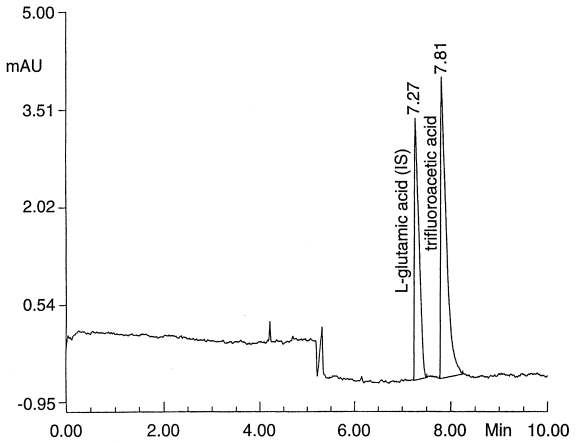

Fig. 4. CE profile of Orphanin FQ sample showing acetic acid and internal standard, L-glutamic acid (Orphanin FQ 0.5 mg/ml water, L-glutamic acid 0.5 mg/10 ml water). For other conditions, please see Table 2, part B.

5. Conclusions

The method presented here for the determination of HOAc and TFA by CE in synthetic peptide samples is precise (R.S.D. 1.6 and 1.3% for HOAc and TFA, respectively), linear, accurate (error <2.0%), specific, rugged and robust. A unique feature of this method is that both these acids can be determined in a single assay, eliminating the need to perform separate analyses. Furthermore, the electrophoretic run time can also be reduced to 4 min (Fig. 1B) still allowing baseline resolution between the acids and the I.S.

In a CE assay, to obtain a good peak area precision, it is important to have steady migration times for the analytes which require capillary conditioning before commencing the assay. This can be achieved by electrophoresing a test solution several times repeatedly under the same conditions. Shorter migration time (higher mobility) of the analyte is a predominant factor that contributes to better precision. This can be easily accomplished by reducing

the capillary length, by increasing the voltage or by manipulating the buffer conditions as appropriate while maintaining resolution between the sample constituents.

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