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Quantitative determination of a dipeptide in personal wash liquid by capillary electrophoresis

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ABSTRACT

The dipeptide alanylglutamine is quantified in a commercial personal cleaning fluid using micellar electrokinetic capillary chromatography. Quantitation is achieved using an internal standard approach with either normalised peak height or area measurements. Correlation coefficients for the calibration graphs were typically 0.999 with data generated over a two-month period and using different capillaries. The data was in good agreement with that of an HPLC approach which utilised pre-column derivatisation with dabsyl chloride.

INTRODUCTION

Since Virtanen [1] described the advantages of using small diameter tubes in 1974, capillary electrophoresis (CE) has enjoyed an exponential growth rate in terms of published papers. In 1991 a CE data base [2] estimated that there were in excess of over 700 publications in the open literature. However, an analysis of these papers [3] reveals that a sizeable proportion are either reviews or deal with instrumental modification, techniques or theory. Papers that detail analytical applications are therefore in the minority, and those that describe quantitative assays are relatively few [4–6]. Although remarkable qualitative separations have been demonstrated [7], in order to exploit CE to the full, it will have to be proven as a robust technique capable of providing quantitative data of a comparable quality to that of HPLC and GLC.

In this paper, CE has been investigated as a means of quantifying the level of a dipeptide (alanylglutamine) in a personal cleaning fluid undergoing stability trials. This particular problem was chosen not just as a suitable test-bed to examine quantitative aspects of the technique, but also because it represents a relatively complex sample matrix. Hence it provides a good test of the techniques quantitative ability in a "real sample" situation. For comparative purposes, data produced by CE are compared with that generated via an HPLC approach that utilises pre-column derivatisation.

EXPERIMENTAL

Equipment

Electrophoresis was carried out on a model 270A capillary electrophoresis system from Applied Biosystems using a 122 cm × 50 μm I.D. fused-silica capillary. Data collection, as for HPLC, was undertaken with Multichrom software from VG Instruments running on a μVAX computer.

HPLC was carried out using a Spectra Physics Model SP8800, a LiChrospher 100 reversed-phase column from Merck (12.5 cm × 0.5 cm I.D.) and a Model SA6500 UV–Vis detector from Severn Analytical. Sample injection was carried out using a Model 710 WISP autosampler from Millipore.

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Materials

Tricine [N-tris(Hydroxymethyl)methylglycine], sodium dodecyl sulphate, triglycine and norvaline (2-aminopentanoic acid) were all purchased from Sigma and the dipeptide alanylglutamine from Nova Biochem. Acetonitrile was HPLC grade and the water was Milli Q (Millipore) with a resistivity >18 MΩ. Dabsyl chloride (4-dimethylaminoazobenzene-4′-sulphonylchloride) was double recrystallised from Pierce. All other reagents were of Analar grade.

Capillary electrophoresis

Conditions. The running buffer was 20 m*M* tricine pH 7.5 with varying levels of sodium dodecyl sulphate (SDS). An applied voltage of 25 kV equivalent to a field strength of 205 V cm^{-1} was used throughout. The column was maintained at a temperature of 55°C and detection was at 200 nm (rise time 0.5 s). Hydrodynamic injections of 3 s were carried out and a wash cycle consisting of 2 min of 0.1 *M* NaOH followed by 4 min running buffer was done after each injection.

Sampling. Approximately 0.6 g personal cleaning fluid was accurately weighed into a 25 cm^3 flask and 1 cm^3 of a 2.1 mg cm^{-3} solution of triglycine internal standard added. The flask was made up to volume with running buffer and thoroughly mixed. An aliquot of this solution was passed through a 0.45-μm filter prior to electrophoresis.

Calibration. Dipeptide levels in cleaning fluid were quantified using a series of dipeptide calibration standards dissolved in running buffer and containing triglycine internal standard. Initial work incorporated the cleaning fluid base into these standards but it was subsequently removed for a comparative experiment. Using independent stock solution of dipeptide and triglycine, both at a concentration of 2 mg cm^{-3}, calibration standards containing 80 μg cm^{-3} triglycine and from 24 to 240 μg cm^{-3} dipeptide were formulated. Cleaning fluid base was present at a level of 24 mg cm^{-3} (*i.e.* the same as for the samples) and the solutions were made up to volume with running buffer.

Quantitation. All quantitative work was undertaken with the use of internal standard normalisation using both peak height and area measurements. Calibration plots of dipeptide normalised peak height or area *versus* concentration were obtained over a two-month period.

HPLC

Sampling. Cleaning fluid (0.5 g) was accurately weighted into a volumetric flask (in duplicate), norvaline internal standard solution added, and the volume made up to 100 cm^3 with water. After mixing, an aliquot of this solution was filtered through a Whatman 541 and the filtrate used for derivatisation.

Calibration. As with the electrophoresis work, dipeptide levels in cleaning fluid were quantified using a series of dipeptide calibration standards containing cleaning fluid base and norvaline internal standard. The dipeptide concentration range was from 5 to 40 μg cm^{-3} with an internal standard concentration of 10 μg cm^{-3}.

Derivatisation. Both samples and standards were derivatised in an identical manner. Aliquots of 200 μl of sample or standard were dispensed into a low volume autosampler vial and then taken to dryness. The residue was dissolved in 20 μl of 50 m*M* sodium bicarbonate buffer pH 8.1 and then derivatised with 40 μl of dabsyl chloride solution in acetonitrile (4 mmoles cm^{-3}). The tubes were then stoppered, vortexed and incubated at 70°C for 12 min. After cooling, 440 μl of 50 m*M* sodium phosphate pH 7.0–ethanol (1/1, v/v) was added to each tube, and after vortexing, an aliquot of this solution taken for chromatography. The dipeptide derivative was separated on a reversed-phase column using a 40-min gradient from 20 m*M* sodium acetate–dimethylformamide (96:4) pH 6.4 to acetonitrile, with detection in the visible at 436 nm.

Quantitation. Dipeptide peak areas for both calibration standards and samples were determined and then normalised by dividing by the area of the internal standard. A calibration graph of normalised dipeptide peak area *versus* concentration was constructed and used to calculate dipeptide concentration in samples.

RESULTS AND DISCUSSION

Electrophoretic conditions

Cleaning fluids are complex formulations comprising a combination of surfactant types in

combination with other functional components such as conditioning agents, preservatives and pearlising agents. Using tricine buffer at pH 7.5, the dipeptide was found to migrate close to a number of other cleaning fluid constituents (Fig. 1a). By the addition of SDS above the critical micelle concentration, the separation mode can be changed from free solution electrophoresis to micellar electrokinetic capillary chromatography (MECC). In this mode, the negatively charged micelles migrate towards the anode, carrying with them any species that can partition into the micelle. Since at pH 7.5 however, the electro-osmotic flow is very rapid, the micelles will still eventually be swept to the cathode. Although both the dipeptide and cleaning fluid surfactants can partition into the micelle, the latter does so far more strongly.

The net effect is to retard the migration of the surfactant more than that of dipeptide (Fig. 1b). At 50 m*M* SDS most of the residual material migrating underneath the dipeptide can be removed and a clean separation achieved with the internal standard (Fig. 1c).

Migration time

Table I shows a typical set of data obtained from a calibration run, with dipeptide levels in

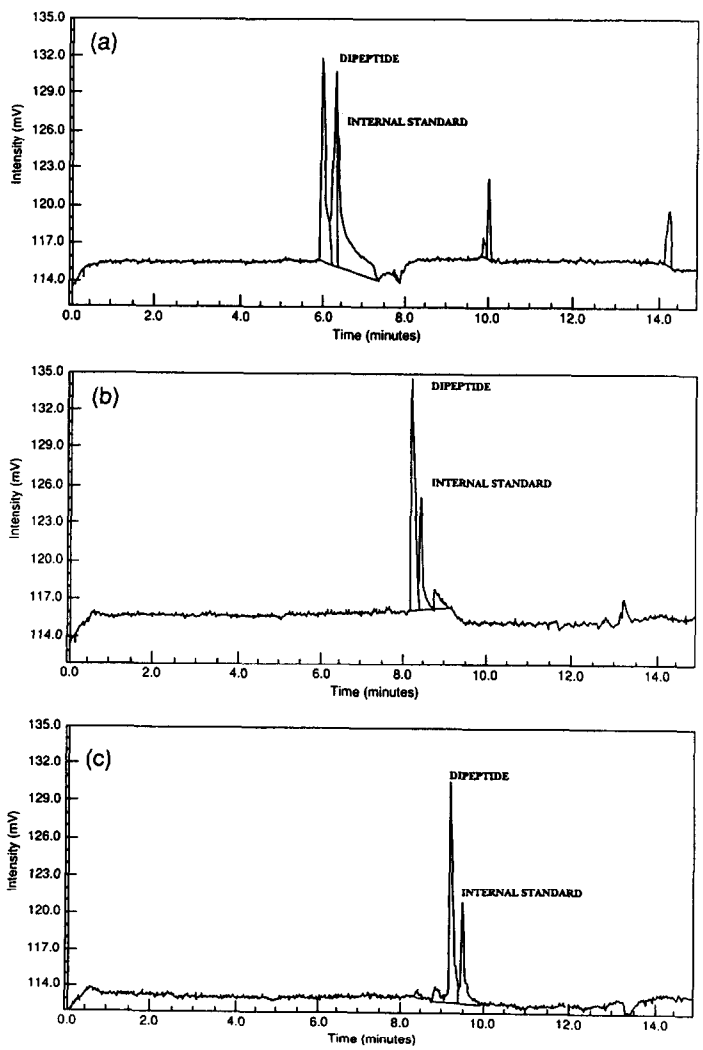

Fig. 1. Separation of dipeptide from cleaning fluid components. (a) Tricine buffer only; (b) tricine buffer with 20 m*M* SDS; (c) tricine buffer with 50 m*M* SDS.

TABLE I

MIGRATION TIME AND PEAK HEIGHT/AREA DATA FROM A CE CALIBRATION RUN

Internal standard (I.S.)			Dipeptide				Dipeptide/I.S.	
Migration time (min)	Peak height ($\mu V \cdot 10^{-2}$)	Peak area ($\mu Vs \cdot 10^{-2}$)	Migration time (min)	Peak height ($\mu V \cdot 10^{-2}$)	Peak area ($\mu Vs \cdot 10^{-2}$)	Level in cleaning fluid (%, w/w)	Normalised height	Normalised area
14.74	67.4	440.5	14.0	14.3	75.4	0.10	0.21	0.17
14.53	67.0	434.8	13.8	14.0	79.9	0.10	0.21	0.18
14.19	66.7	435.7	13.5	35.1	206.0	0.24	0.53	0.47
13.95	68.2	445.9	13.3	33.7	190.3	0.24	0.49	0.43
13.69	64.8	403.7	13.0	63.8	363.5	0.52	0.99	0.90
13.50	64.6	414.4	12.9	65.1	366.3	0.52	1.01	0.88
13.23	63.2	394.8	12.6	96.2	536.3	0.72	1.52	1.36
13.08	63.9	405.7	12.5	65.4	526.3	0.72	1.49	1.30
12.68	60.7	367.2	12.1	123.8	677.7	1.01	2.04	1.85

cleaning fluid ranging from 0.1 to 1.01%. It is important to note that with on-column detection in CE, any change in migration time will affect the speed at which the band passes the detector window. Hence a slower band will exhibit an apparently larger peak area [8]. Consequently it is important to ensure that the migration times within a run are reproducible (good repeatability) or to correct (normalise) the areas using *e.g.* the migration time of the band, or a suitable internal standard. From all the data generated during this study, this problem can be seen most clearly from the data in Table I. The migration times for both the triglycine and dipeptide progressively decrease; resulting in a steady increase to peak areas. All quantitative work was therefore conducted on peak height or area measurements that had been normalised with respect to that of the internal standard.

Calibration curves

Linearity of response, *i.e.* peak height/area of dipeptide *versus* concentration, was determined over three separate days during a two-month period for standards containing cleaning fluid base. The data in Table II shows the results for the correlation coefficients obtained following a least squares linear regression analysis. For both normalised height or area measurements good linearity is obtained indicating either approach is suitable for quantitation. The data from run 4 was obtained for a calibration standard in the absence of cleaning fluid base, *i.e.* in run buffer only, and demonstrates an improvement in linearity. The matrix in which the sample is injected can pose problems in CE [9]. If it is different between sample and standard then it can cause a change to the band migration time. In this particular assay, however, no such problems are encountered indicating that omission of the cleaning fluid base is not only a simpler approach but that the quality of the data is improved. The calibration graphs obtained from run 4 are shown in Figs. 2 and 3.

As a final point it should be noted that two different capillaries of identical dimensions were used to generate the above data, demonstrating

TABLE II

LINEAR REGRESSION ANALYSIS OF CE CALIBRATION DATA

Run No.	Correlation coefficient	
	Peak height	Peak area
1	0.9981	0.9981
2	0.9964	0.9949
3	0.9992	0.9984
4	0.9998	0.9998

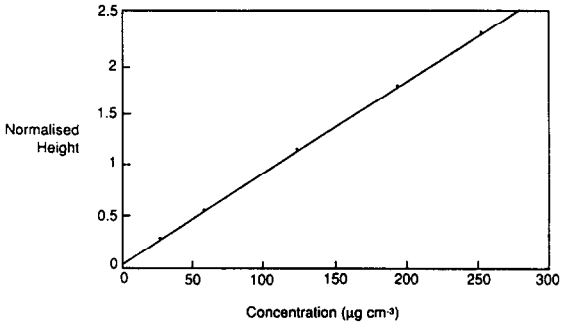

Fig. 2. Dipeptide concentration *versus* normalised peak height.

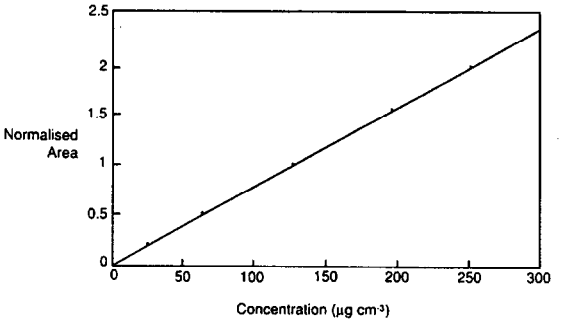

Fig. 3. Dipeptide concentration *versus* normalised peak area.

that variation in capillary characteristics does not pose problems of reproducibility.

Levels of dipeptide in cleaning fluid

The calibration graphs obtained in the preceding section were used to calculate alanyl-glutamine levels in cleaning fluid undergoing stability trials. The dipeptide was incorporated at a nominal 0.4% (w/w) and the sample divided in two. One of these was frozen down (−20°C) and the other placed in a fixed temperature cabinet (37°C). Samples were taken from these two storage trials and CE data generated using both normalised peak height and area measurements. The data obtained are presented in Table III and shows that essentially there is no difference between the two approaches.

The 17-week data was generated on two consecutive days, with the first −20°C and 37°C data set obtained with run 3 calibration (Table II) and the second set with run 4. The difference between these, was that run 3 was generated with cleaning fluid base in the calibration standard, whilst for run 4 it was omitted. Clearly both approaches give the same result and since an improved correlation coefficient is obtained for standards with no added base, this is the preferred procedure.

The data in Table III can be condensed by averaging the peak height and area result, as well as the two sets of 17-week data. Table IV gives the averaged CE data compared to that obtained via HPLC and shows good agreement between the two techniques. As far as the dipeptide is concerned, storage at 37°C leads to a rapid loss from 0.39% to 0.28% after four weeks and then to 0.24% after ten weeks. At this point the decomposition levels off.

CONCLUSIONS

From this study it is apparent that CE can be a viable technique for quantitative analysis, and more importantly is capable of quantifying ana-

TABLE III

CALCULATION OF DIPEPTIDE LEVELS IN CLEANING FLUID BY CE

Sample	Level of dipeptide in cleaning fluid (% w/w)			
	Normalised peak height (Mean)		Normalised peak area (Mean)	
Time 0	0.39, 0.36, 0.40	(0.38)	0.41, 0.40, 0.35	(0.39)
37°C/10 wks	0.25, 0.24, 0.25	(0.25)	0.22, 0.23, 0.24	(0.23)
−20°C/17 wks	0.34, 0.36	(0.35)	0.33, 0.35	(0.34)
37°C/17 wks	0.22, 0.23	(0.23)	0.21, 0.23	(0.22)
−20°C/17 wks	0.36, 0.34	(0.35)	0.36, 0.36	(0.36)
37°C/17 wks	0.25, 0.24	(0.25)	0.25, 0.25	(0.25)

TABLE IV

COMPARISON OF CE AND HPLC DATA

Sample	Level of dipeptide in cleaning fluid (% w/w)	
	CE	HPLC
Time 0	0.39	0.39
37°C/4 wks	ND[a]	0.28
37°C/10 wks	0.24	ND
−20°C/17 wks	0.35	0.33
37°C/17 wks	0.25	0.24

[a] ND = Not determined.

lytes in relatively complex matrices with the minimum of sample pre-treatment.

For this application data from the calibration graphs yielded correlation coefficients comparable to those which are typically achieved via the established techniques of HPLC and GLC. Peak height or area measurements were found to be equally suitable. One major requirement in CE however is the use of internal standards. Migration times are found to vary with CE, most likely due to changes in the zeta potential at the capillary wall. Consequently some form of normalisation must be adopted to allow for this variation, and this is probably best approached via the use of internal standards.

The data generated via the separate approaches of CE and HPLC was in good agreement, although admittedly on a limited data set. For this particular assay, however, the former technique is the preferred method since it is rapid and simple and obviates the need for pre-column derivatisation.

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