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Journal of Chromatography A, 768 (1997) 89–95

JOURNAL OF CHROMATOGRAPHY A

Gradient capillary electrochromatography of drug mixtures with UV and electrospray ionisation mass spectrometric detection

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Abstract

A simple method for forming reproducible mobile phase gradients with capillary electrochromatography (CEC) has been demonstrated. It allows short columns and low ionic strength buffers to be used and it lends itself to mass spectrometric detection. Mixtures of benzodiazepines, corticosteroids and thiazide diuretic drugs were separated using UV absorbance and electrospray ionisation mass spectrometric (ESI-MS) detection. Separations were performed on fused-silica capillary columns (33–50 cm×50–75 μm I.D.) packed with CEC Hypersil ODS (3 μm) and Apex ODS (3 μm) particles. A stainless steel tee connector was used to interface the CEC column with samples and mobile phase delivered from a HPLC autosampler and pump. A voltage (30 kV) was applied to the tee connector causing electrokinetic transfer of samples and mobile phase into the CEC column. Pre-conditioning of the CEC column with mobile phase of low organic content allowed dilute samples to be stacked at the head of the column prior to elution.

Keywords: Electrochromatography; Gradient elution; Benzodiazepines; Corticosteroids; Thiazide diuretics

1. Introduction

The advent of capillary electrochromatography (CEC) and recent introduction of commercial instruments has brought a new and powerful separation technique to the armoury of the analytical laboratory. The high separation efficiency of CEC has recently been well documented [1–3]. With HPLC, the separation of analytes of widely differing polarity, such as drugs and metabolites, can necessitate long run times when using isocratic mobile phases and gradient elution is often used. To date the same restrictions apply to CEC since gradient elution is not easily achieved using CE instruments in which the samples and mobile phase are held in vials into which the CEC column and electrodes are placed. These systems are also of limited use if post-column detection techniques such as mass spectrometry (MS) are employed. Owing to the design of the instrumentation, removing one end of the CEC column from a CE autosampler and placing the other end in a MS interface requires a column length of typically 1 m [4,5]. This restricts the field which can be applied across the capillary with a standard 30 kV power supply, lengthening run times and reducing efficiency.

Low ionic strength buffers are commonly used in CEC to reduce the problem of bubble formation induced by Joule heating. A further disadvantage of using vials to hold the mobile phase is that electrolytic depletion occurs with low ionic strength buffers causing reproducibility problems. The problem has been partly overcome by pressurising the mobile phase vials and using higher concentrations of zwitterionic buffers [3]. However we have found

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PII S0021-9673(96)00942-9

that these buffers tend to give a high background signal which can reduce MS detection sensitivity.

HPLC pumps have been utilised to deliver mobile phase gradients and pressurise the CEC column inlet [6–8]. Behnke and Bayer [6] designed an injection device for CEC which utilised a gradient HPLC pump for mobile phase delivery. The system was applied to peptide mixtures using short CEC columns and electrospray ionisation mass spectrometric (ESI-MS) detection [7]. The exact design of the HPLC–CEC interface was not revealed but, since the flow-rate through the injection device was below 5 nl min^{-1}, we would expect that a coupling of very low dead volume was required to prevent band broadening due to pre-column mixing.

Eimer et al. [8] used an isocratic HPLC pump to generate hydrodynamic flows in CEC columns of 200 μm I.D.. A Latek tee piece functioned as the HPLC–CEC interface and flow splitter. HPLC flow-rates of 0.5 to 1.0 ml min^{-1} were split via a restriction capillary to generate hydrodynamic flows of 0.5 to 3.0 μl min^{-1} in the CEC column. The tee was grounded and the capillary outlet placed in a vial containing electrolyte and a platinum electrode held at −6 to −7 kV. The applied potential was found to improve peak efficiencies and affect the selectivity with charged analytes.

We have constructed a gradient HPLC–CEC interface consisting of a stainless steel tee connector and restriction capillary (Fig. 1). Samples are introduced into the mobile phase stream, which flows coaxially past the CEC column inlet, using the HPLC autosampler via a fused-silica capillary. Voltages of up to 30 kV were applied to the tee to effect an electrokinetic flow in the CEC column, the outlet of which is effectively grounded. The split ratio and HPLC flow-rate are adjusted such that the CEC column is pressurised, to prevent bubble formation with the high applied fields, without generating a significant hydrodynamic flow therein. Pressurisation of both ends of the CEC column was possible using this approach. The construction of the device is simplified by the fact that the dead volume is swept by a high flow of mobile phase relative to that in the CEC column.

The system is applied to model drug mixtures using UV and ESI-MS detection.

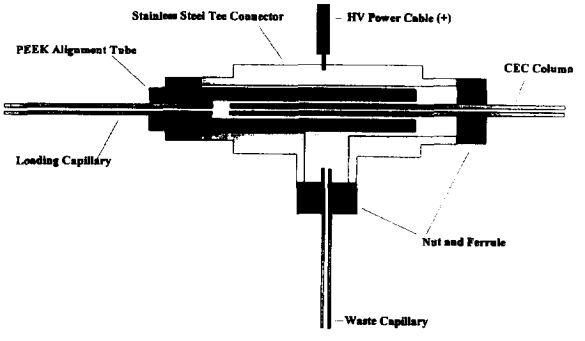

Fig. 1. Cross-section of the CEC sampling interface. Sample and mobile phase are delivered to the tee, which is held at high voltage, through the loading capillary using an autosampler and gradient HPLC pump. The liquid is sampled electrokinetically by the CEC column, the majority passing to waste through the waste capillary which has dimensions such that a flow restriction and consequently a back pressure is produced within the tee housing.

2. Experimental

2.1. Reagents and materials

Diazepam and nitrazepam were obtained from Roche (Welwyn, UK), hydroflumethiazide from Glaxo-Wellcome (Stevenage, UK), methylclothiazide from Abbot (Queensborough, UK), metolozane from Pennwalt (Dublin, Republic of Ireland), epitizide and bendrofluazide from Berk (Eastbourne, UK) adrenosterone, betamethasone, cortisone, dexamethasone, fluocortolone, hydrocortisone, methylprednisolone, triamcinolone and triamcinolone acetonide from Sigma (Poole, UK). Standards were prepared in ammonium acetate (2 m*M*), acetonitrile (5%, v/v) was added to corticosteroid mixtures to assist solubility.

Acetonitrile, acetone, isopropanol and methanol (HPLC grade) were obtained from Rathburn (Walkerburn, UK). Acetic acid (AnalaR grade) was obtained from BDH (Poole, UK). Ammonium acetate (98%) was obtained from Sigma (Poole, UK).

2.2. CEC

CEC separations were performed in fused-silica capillary columns (375 μm O.D.×50 μm I.D.) packed with CEC Hypersil ODS particles (3 μm) which were gifts from Hypersil, Runcorn, UK and Glaxo-Wellcome. Fused-silica capillary columns

(375 μm O.D.×75 μm I.D. and 50 μm I.D. packed in-house with Apex ODS, 3 μm (Jones Chromatography, Hengoed, UK) were also used. Mobile phases were acetonitrile–water mixtures containing ammonium acetate, 5 m*M*. The ammonium acetate concentration was therefore constant throughout gradient elution experiments. Mobile phase flow-rates were typically 10–20 μl min^{-1} during sample loading increasing to 100 μl min^{-1} during the CEC run.

CEC columns were sleeved in PEEK tubing (1.5 cm×400 μm I.D.) and connected to a Swagelok stainless steel tee union, 1/16 in. I.D. (part No. 100–3, Great Yarmouth Valve and Fitting, Great Yarmouth, UK, Fig. 1; 1 in.=2.54 cm). The loading and waste capillaries were constructed of fused-silica (2 m×375 μm O.D.×75 μm I.D., Composite Metal Services, Hallow, UK) and were connected to the tee using nuts and Vespel ferrules. The loading capillary was butted with the CEC column in the PEEK alignment tube and connected to the column outlet port of the injection valve of a HP1050 HPLC (Hewlett-Packard, Stockport, UK) fitted with autosampler and quaternary gradient pump. The loading and waste capillaries were of narrow I.D. and sufficient length to reduce the risk of grounding.

The tee was connected to a Crystal 300 CE power supply (30 kV, Thermo Unicam, Cambridge, UK) and placed in a sealed plastic box fitted with a safety cut-out switch. The tee was continuously purged with mobile phase from the HPLC system and a constant voltage of 30 kV applied to the tee throughout the analysis to effect transfer of sample and mobile phase into the CEC column. Samples were injected using the HPLC autosampler. The power supply, HPLC and detectors were linked through a common earth.

2.3. ESI-MS detection

The waste capillary tip was placed in a glass collection bottle. The CEC column was interfaced to a Platform mass spectrometer using a triaxial inlet probe (VG Biotech, Altringham, UK). The MS was operated in selected ion recording (SIR) mode. Sheath liquid was introduced at 5–10 μl min^{-1} from a pressurised reservoir. Nitrogen was used as the nebulising gas, flow-rate was adjusted to attain beam stability. A voltage of 4 kV was applied to the probe tip with 10 V cone voltage. The benzodiazepines were detected in positive ion mode using acetic acid (1% in methanol) as the sheath liquid. The thiazide diuretics were detected in negative ion mode using isopropanol–water (80:20) as the sheath liquid.

2.4. UV detection

Pressurisation of the outlet end of the CEC column was necessary to prevent bubble formation and consequent field breakdown with UV detection. This was achieved using a second interface held at ground potential and connected to the CEC column outlet. A Spectra 100 variable wavelength UV detector (Thermo Unicam) was used.

3. Results and discussion

The coupling system allowed mobile phase gradients, generated by a conventional HPLC pump, to be used. Samples were loaded electrokinetically onto the CEC column from a mobile phase containing a low concentration of organic modifier, promoting retention at the head of the column. After loading, the mobile phase gradient was applied automatically by the HPLC system causing the analytes to be eluted. The focusing effect of the mobile phase gradient helped overcome band broadening due to pre-column mixing and on-column electrophoretic dispersion. Hydrodynamic flow in the CEC column induced by the head pressure in the sampling interface (typically 100–200 kPa) was found to be negligible. Average linear flow velocity in the CEC column was typically 1–2 mm s^{-1}. The actual flow velocity was dependent on the mobile phase composition and increased slightly with increasing acetonitrile concentration.

The formation of the gradients was studied using UV detection (254 nm) to monitor the baseline with one of the mobile phase reservoirs spiked with acetone (5%, Fig. 2). The applied acetone gradients were reproducible and the baseline changes matched the applied solvent programs. A time delay of approximately 17 min was observed between a change in the mobile phase at the pump and its detection using a flow-rate of 100 μl min^{-1} through the interface. It is envisaged that this delay could be reduced if a low dead volume gradient pumping

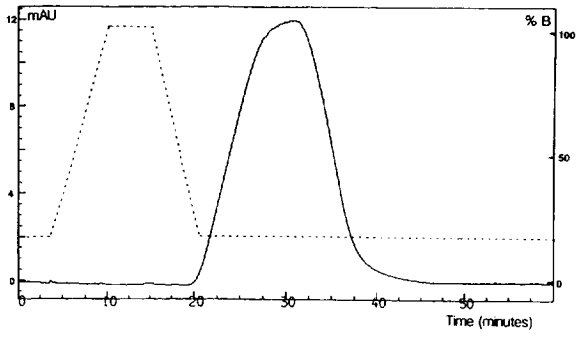

Fig. 2. CEC–UV baseline (254 nm) obtained with a mobile phase gradient program. Solvent A=ammonium acetate, 5 m*M* in acetonitrile–water (1:1). Solvent B=solvent A spiked with acetone, 5%. Voltage=30 kV, column=Hypersil ODS, 3 μm, 42 cm total length, 30 cm packed length, 30.1 cm to detection window. Dashed line=applied gradient, solid line=detected gradient.

system were used. Mobile phase composition will also affect the measured gradient profile as the electroosmotic flow is dependent on a number of factors such as pH, buffer ion mobility and organic modifier concentration.

Chromatographic plate theory is unsuitable for application to gradient systems. Peak width at half height is a more appropriate measure of peak sharpness. A mixture of two benzodiazepines (100 μg ml^{-1}) were analysed by CEC–ESI-MS in positive ion mode using both isocratic and gradient elution (Fig. 3). A simple step gradient, from 50% to 80% acetonitrile at 2 min after injection provided a three-fold reduction in peak width when compared to isocratic elution with 50% acetonitrile. Shallower linear gradients provided comparable results in terms of peak width but analysis run times were lengthened compared to the simple step gradient.

The injection volume linearity was determined for diazepam standard using ESI-MS detection with step gradient elution. Diazepam peak area was subjected to linear regression analysis ($y=0.36x+0.38$, $r=0.997$). Diazepam peak width (at half height) was unaffected by injection volume over the 1–5 μl range (8.4 s). A widening of the peak was observed with 10 μl (9.6 s) and 20 μl (11.4 s) injections. Column overloading, indicated by peak shouldering, was evident with the 20 μl injection volume. Retention time reproducibility was 2.8% R.S.D. ($n=4$) for diazepam over the applied injection volume range.

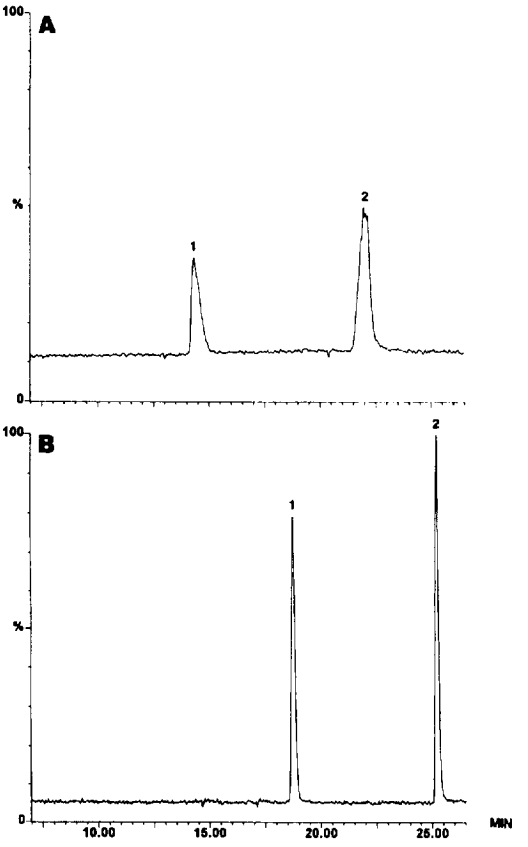

Fig. 3. CEC–ESI-MS chromatogram recorded in selected ion recording mode (TIC) of a mixture of two benzodiazepines (100 μm ml^{-1}) using A=isocratic elution with ammonium acetate, 10 m*M* in acetonitrile–water (1:1) and B=step gradient elution. CEC conditions: voltage=30 kV (effective field=565 V cm^{-1}), HPLC injection volume=10 μl, flow-rate=10 μl min^{-1} for 3 min then increased to 100 μl min^{-1}. Gradient program=initial: ammonium acetate, 5 m*M* in acetonitrile–water (1:1) held for 3 min then ramped to 80% acetonitrile in 0.1 min, maintained for 35 min. Column=Hypersil ODS, 3 μm, 46 cm fully packed. MS conditions as described in Section 2.2. 1=nitrazepam, 2=diazepam.

The CEC–MS system was also applied in negative ion mode with a simple step gradient elution. A mixture of thiazide diuretics were separated (Fig. 4). Measured peak widths at half height were typically below 0.3 min. The HPLC flow-rate during sample loading affected the peak areas. Metolazone peak area (100 μg ml^{-1}) increased by a factor of 1.5

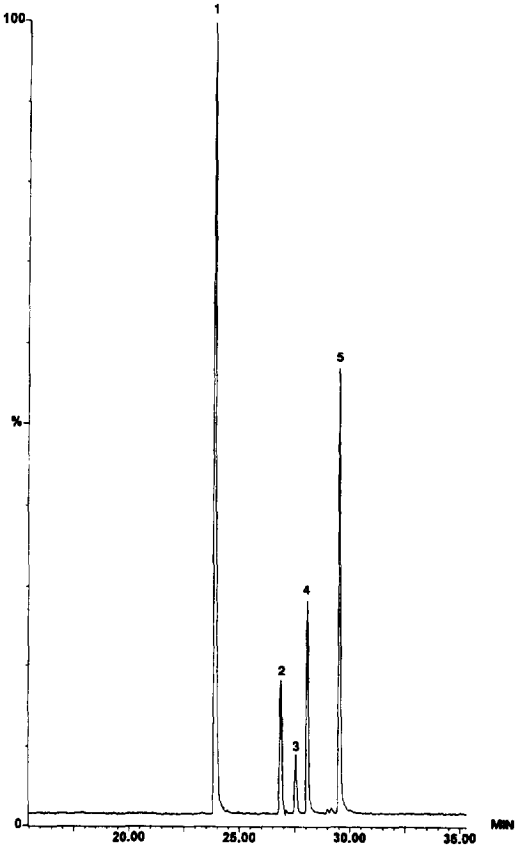

Fig. 4. CEC–ESI-MS chromatogram recorded in selected ion recording mode (TIC) of a mixture of thiazide diuretics (100 μg ml^{-1}) using a step gradient elution program. Voltage=30 kV (effective field=739 V cm^{-1}), HPLC injection volume=5 μl, flow-rate=10 μl min^{-1} for 3 min then increased to 100 μl min^{-1}. Gradient program=initial: ammonium acetate 5 m*M* in acetonitrile–water, (1:1) held for 3 min then ramped to 80% acetonitrile in 0.1 min, maintained for 35 min. Column=Hypersil ODS, 3 μm, 46 cm fully packed. 1=hydroflumethiazide, 2=methylclothiazide, 3=metolazone, 4=epitizide, 5=bendrofluazide.

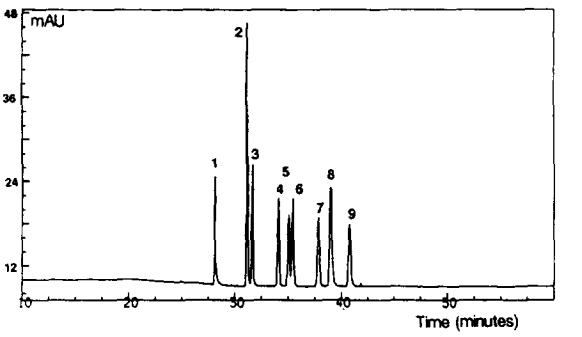

Fig. 5. CEC–UV chromatogram (240 nm) of a mixture of 10 corticosteroids (100 μg ml^{-1}) using a linear gradient elution program. Voltage=30 kV, HPLC injection volume=10 μl, flow-rate=10 μl min^{-1} for 3 min then increased to 100 μl min^{-1}. Gradient program=initial: ammonium acetate, 5 m*M*, in acetonitrile–water (17:83), held for 3 min then ramped to 38% acetronitrile at 15 min and maintained to end of run. Column=Hypersil ODS, 3 μm, 42 cm total length, 30 cm packed length, 30.1 cm to window. 1=triamcinolone, 2=hydrocortisone and prednisolone co-eluting, 3=cortisone, 4=methylprednisolone, 5=betamethasone, 6=dexamethasone, 7=adrenosterone, 8=fluocortolone, 9=triamcinolone acetonide.

when the HPLC flow-rate during loading was reduced from 20 to 10 μl min^{-1}. We found that there was no need for capillary pressurisation with ESI-MS detection.

A mixture of ten corticosteroids was analysed using the CEC–UV system, experimenting with different gradient profiles in order to separate the mixture within a reasonable run time. It contained species of very similar polarity, such as betamethasone and dexamethasone, and also of widely differing polarity, such as triamcinolone and triamcinolone acetonide. A separation of nine of the ten was achieved (Fig. 5). The injection reproducibility of the CEC–UV system with mobile phase gradient elution was demonstrated by repetitive injections ($n=6$) of a mixed standard solution on two different days (Table 1). The injection linearity of the gradient CEC–UV system in terms of analyte concentration and injection volume were evaluated (Table 2).

The ability to focus dilute samples at the head of the CEC column and then elute them with a mobile phase of increasing organic modifier concentration was demonstrated by injecting a large volume (250 μl) of a corticosteroid mixed standard (100 ng ml^{-1}, Fig. 6). The CEC column was conditioned with a mobile phase containing acetonitrile (17%) which was maintained for 3 min before applying a linear mobile phase gradient to 45% acetonitrile at 8 min after injection. The analytes were clearly detected despite the baseline drift, presumably caused by a difference in UV transmission of the aqueous mobile phase. Comparison to Fig. 5 demonstrates an approximately 25-fold increase in sensitivity which is in direct relation to the increased loading volume

Table 1
Precision (100 μg ml^{-1}, n=6) data for separation of five corticosteroids by CEC with mobile phase gradient elution using UV detection (240 nm)

Drug	Day 1		Day 2	
	Retention Time % R.S.D.	Peak Area % R.S.D.	Retention Time % R.S.D.	Peak Area % R.S.D.
Hydrocortisone	0.51	2.21	0.79	1.78
Dexamethasone	0.49	2.24	0.78	1.06
Adrenosterone	0.47	2.49	0.93	1.53

CEC performed in=Apex ODS, 3 μm, column, 24 cm total length, 16 cm packed length, 16.1 cm to detector window, voltage=25 kV, HPLC injection volume=50 μl, flow-rate=100 μl min^{-1}. Gradient program=initial: ammonium acetate, 5 m*M*, in acetonitrile–water (1:99) ramped to 40% acetonitrile at 8 min and maintained to end of run.

(time). Increasing the sensitivity further leads to excessive loading times.

With the sampling interface employed most of the sample is flushed to waste during injection. However, excess sample could be reclaimed from the waste effluent if required. Alternatively, smaller injections could be made and the pump stopped as the sample enters the tee, restarting the pump after the desired injection time. The injection of larger sample volumes than are actually sampled may be seen as a disadvantage compared to conventional CEC systems where sub-μl samples, typically a few nl, are introduced electrokinetically into the column. However, the total volume required in the sample vial is typically greater than 30 μl in these systems to allow a sufficient depth of liquid to accommodate both CEC column and electrode. The use of a HPLC system to deliver samples to the CEC column has some potential benefits. Established HPLC techniques such as heart cutting and on line sample extraction may now be interfaced with CEC.

Table 2
Injection volume and concentration linear regression data for separation of three corticosteroids by gradient CEC with UV detection (240 nm)

x=HPLC injection volume (10–250 μl, n=4)
y=Peak area (mAU s)
Drug concentration=1 μg ml^{-1}

Drug	Slope	Intercept	r
Methylprednisolone	5.96	−7.09	0.999
Dexamethasone	5.13	−5.70	0.999
Fluocortolone	4.44	−7.30	0.999

x=Drug concentration (1–100 μg ml^{-1}, n=5)
y=Peak area (mAU s)
HPLC injection volume=10 μl

Drug	Slope	Intercept	r
Methylprednisolone	0.57	1.58	0.999
Dexamethasone	0.49	2.20	0.998
Fluocortolone	0.48	2.47	0.998

CEC performed in=Apex ODS, 3 μm, column, 32.5 cm total length, 24 cm packed length, 24.1 cm to detector window, 75 μm I.D., voltage=30 kV, HPLC injection volume and flow-rate varied. Gradient program=initial: ammonium acetate, 5 m*M*, in acetonitrile–water (17:83) held for 3 min then ramped to 45% acetonitrile in 5 min and maintained to end of run.

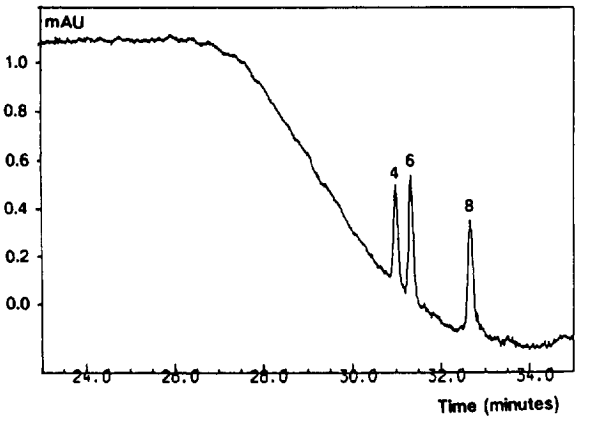

Fig. 6. CEC–UV chromatogram (240 nm) of a mixture of 3 corticosteroids (100 ng ml^{-1}) using a linear gradient elution program as described for Fig. 5. HPLC injection volume=250 μl, Column=Apex ODS, 3 μm, 32.5 cm total length, 20.5 cm packed length, 20.6 cm to detector window. 4=methylprednisolone, 6=dexamethasone, 8=fluocortolone.

4. Conclusion

A simple method for forming reproducible mobile phase gradients with CEC has been demonstrated, it allows short columns and low ionic strength buffers to be used. Mobile phase gradients can be employed to stack dilute samples at the head of the CEC column, focus the eluting bands and reduce run times. The interface is of simpler construction than published devices for gradient elution in CEC [6–8]. The sampling region is swept by a high flow of mobile phase, relative to the electroosmotic flow in the CEC column, and therefore there is no need to use an ultra-low dead volume coupling and buffer depletion does not occur. Since the interface does not have to be fitted inside a CE instrument, short capillaries may be used with end column detection techniques such as mass spectrometry. There is development potential for automated on-line sample preparation and HPLC–CEC (heart cutting) using well established and commercially available systems for HPLC.

Acknowledgments

The authors would like to thank Dr. Steven Westwood (Horseracing Forensic Laboratory, UK) and Professor David Perrett (St Bartholomews Hospital Medical College, UK) for their advice, Ms. Orla Weir and Ms. Helen Lomax (Hypersil, UK), Mr. Robert Boughtflower and Ms. Clare Paterson (Glaxo-Wellcome, UK) for donation of CEC columns.

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