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Simplification of capillary electrochromatography procedures

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Abstract

A simplified packing and operating procedure for capillary electrochromatography (CEC) has been developed which draws from some of the more accessible features of published practice. The key elements of this methodology are use of an end-fitting and frit from a 1-mm microbore column in the initial phase of the packing, use of a conventional constant-flow HPLC pump for capillary packing, conditioning of the packed capillary using a conventional constant-flow HPLC pump with flow splitting, bedding down of the packed capillary under an electric field while fitted into the CE instrument and operation without pressurisation and without an end frit. Working in such a manner, CEC was carried out on a commercial test mixture and mixtures of steroids. © 1998 Elsevier Science B.V.

Keywords: Electrochromatography; Capillary columns; Packing procedures; Steroids

1. Introduction

In simple terms, capillary electrochromatography (CEC) may be considered to be capillary LC in which an electric field drive is used instead of a pressure drive. Alternatively, it may be perceived as capillary electrophoresis conducted in capillaries which are not open but are packed with porous microparticles. It has attracted much interest [1] because, inter alia, it offers much higher efficiencies than equivalent pressure-driven LC systems and allows the separation of neutral compounds using more MS-friendly conditions than those required in micellar capillary electrophoresis (CE) techniques.

Despite these obvious advantages, the uptake of the technique in analytical laboratories has not been what might have been expected. A principal reason for this is that much of the pioneering work in the field has been carried out using packing procedures and operating conditions that are not readily available in most laboratories. For example, capillaries have been packed by methods which require a slurry chamber fitted with an ultrasound probe [2], customised equipment set up in a Faraday cage and attached to hi-fi speakers [3] or the use of supercritical fluids [4]. More recently, it has been possible to purchase commercially-packed capillaries so that a more pertinent factor in limiting wider use of CEC may now be the long held view [5] that it is necessary to use modified instrumentation for the actual operation of the CEC. More specifically, it is common to modify commercial CE instrumentation so that both the inlet and outlet vials may be pressurised up to 500 p.s.i. (1 p.s.i.=6894.76 Pa).

The general aim of this study was therefore to

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address these practical shortcomings in such a way as to make CEC much more readily accessible.

2. Experimental

2.1. Apparatus

Separations were performed using a P/ACE 2050 capillary electrophoresis system with data being collected using the related Gold software version 8.01. (Beckman Instruments, High Wycombe, UK). The capillaries of 50-μm diameter (Composite Metals, Hallow, UK) were packed using a LDC Constametric III HPLC pump (ThermoSeparations Products, Stone, UK). The packing process was observed using a UniLux-11 microscope (Kyowa, Tokyo, Japan). Frits were fused and windows burnt using a frit-making device which was a gift from Glaxo Wellcome (Ware, UK). This device is now also commercially available from Innovatech (Stevenage, UK). In later work the filament casing of this device was adapted so that it could accept alternative filaments. Initially filaments purchased from Capital HPLC (Broxburn, UK) were used. Later, filaments were prepared in-house from Nuralloy 368 wire gauge which has a thickness of 0.0076 in. (1 in.= 2.54 cm). A length of 16 mm of this wire was wound inside the filament casing in a loop around the aperture for the capillary. Packed capillaries were conditioned using a Philips PU4010 pump (Unicam, Cambridge, UK) followed by flow splitting via a T-piece with the flow balanced between one arm connected to a conventional LC column and the other to which the packed capillary was connected.

2.2. Materials

The packing material used throughout was ODS Hypersil (3 μm) (Hypersil, Runcorn, UK). All solvents were HPLC grade obtained from Merck (Leeds, UK) and were filtered before use through a 0.45-μm nylon syringe filter (Alltech, Canforth, UK). Disodium hydrogenphosphate was obtained from Sigma (Poole, UK). Thiourea, benzamide, benzophenone and biphenyl were obtained from Lancaster Synthesis (Morecambe, UK). Polyaromatic hydrocarbons were a gift from The City University. Steroids were either purchased from Sigma or were a gift from Glaxo Wellcome. All water used throughout was doubly purified using an Elga Option 3 and then the Elgastat UHQPS systems (Elga, High Wycombe, UK).

2.3. Methods

2.3.1. Initial preparation of packed capillaries

Capillaries were cut to approximately 40 cm in length and one end was connected to an end fitting, containing a standard frit, for a 1-mm I.D. microbore LC column by the use of a short length of polyether ether ketone (PEEK) as a sleeve. The other end of the capillary was placed in a slurry chamber (15-cm×1-mm I.D. empty column) so that at least 6 cm of the capillary was protruding into the chamber. A stock of a slurry of 90 mg ODS Hypersil (3 μm) in 3 ml of propan-2-ol was prepared and a portion (about 100 μl) of this was added to the slurry chamber until it was full. The other end of the capillary was connected to a HPLC pump and the packing solvent, propan-2-ol, was pumped through the chamber and capillary assembly at a flow-rate of 60 $\mu l\,min^{-1}$. Whilst the packing procedure was taking place the capillary and the slurry chamber were kept horizontal with the capillary being held as straight as possible.

The packing process was observed through a microscope and allowed to continue until the required column length plus approximately a 1/3 excess had been packed. Water was then pumped through the system for 2–3 h. Before switching off the pump and removing from the packing assembly, the mechanical frit was removed and very rapidly replaced with a stationary phase frit made by heating the end section of stationary phase in the frit burner. In this way the frit was formed before any of the stationary phase bed could be lost by being pumped out of the capillary. The optimum heating conditions to use were determined by prior trial frit-making exercises. For the Glaxo burner fitted with filaments from Capital, this was 10 s at each heat setting rising from 0 to 8. 7 s at each heating setting rising from 0 to 7 was used for the laboratory-prepared Nuralloy filaments.

After removal from the packing assembly, a window was made in the capillary at a position appropriate for a Beckman instrument (20 cm from

inlet frit) to be used for subsequent CEC. This was done by using the Glaxo burner at setting 5 for about 5 s. Conditioning of the packed capillary prior to CEC was carried out with the capillary placed in its Beckman cartridge. By use of a conventional constant-flow LC pump and flow splitting so that a setting of 0.2 ml min^{-1} gave rise to a flow of about 5 μl min^{-1} through the packed capillary (back pressure of about 100 p.s.i. registered at the pump), water was pumped through the capillary for 24 h followed by acetonitrile for 24 h. Before use the CEC mobile phase was pumped through for 2–3 h.

2.3.2. Capillary electrochromatography

Once set up in the Beckman CE instrument, voltages of 5 kV for 60 min, followed by 10 and 15 kV for 30 min, were applied to the capillary. The initial mobile phase for all attempts at separations was acetonitrile–2 m*M* aqueous phosphate, pH 7.8 (80:20, v/v). This was always filtered and sonicated prior to use. Injection was electrokinetic, 5 kV for 5 s, on samples which generally contained 0.1 mg ml^{-1} of each component present dissolved in mobile phase. The samples were then run by applying a voltage of 10 or 15 kV across the capillary which typically gave a current of 0.8–1.2 μA. Detection was post-bed UV at either 214 nm or 254 nm. The separation voltage and mobile phase composition were altered as necessary to optimise the separation. When the packed capillaries were not in use they were stored with their ends immersed in acetonitrile–water.

3. Results and discussion

3.1. Appraisal of options

This study was initiated to evaluate whether CEC could be readily implemented in a typical separation science laboratory. The situation in the laboratory in which the study was taking place was similar to that in many other such separation science laboratories. There no longer was a dedicated HPLC column packer. However there were commercial CE systems, albeit unmodified, and a heating device for preparing windows on capillaries.

Initial attempts at implementing CEC showed that as well as the (anticipated) problems associated with not having a specialised capillary packing system and with using an unmodified CE system, much practice was required to develop the skills needed for frit making whether by heating a silica paste or heating the packed bed. Therefore as the study continued, it was attractive to consider the work of Lord [6] in which no outlet frit at all was used. Although originally designed specifically to deal with CEC–MS related problems, this approach should also be applicable to CEC with UV detection, and could also give additional benefits since according to Rebschler [7], air bubble formation is particularly a problem at the interfaces on either side of the frit. Therefore attempting to develop readily accessible methodology for packing capillaries utilising nonspecialized equipment, minimisation of reliance on frits could be important. However, there were a number of other practical issues that also had to be considered.

3.2. Initial preparation of packed capillaries

In published packing procedures e.g. [8], it is common to first prepare a temporary frit at one end of the capillary by heating silica paste. This is later replaced by a frit made by heating silica in the packed bed using the frit burner since this type of frit can be made more reproducibly. Even after much practice at making the type of frit made from silica paste there is scope for considerable variability in the quality of frit prepared. Here variability was eliminated and it was found much more convenient using a stainless steel frit of the type used in end fittings for 1-mm I.D. microbore LC columns [9]. This was held in place by seating it in a microbore end fitting and connecting the capillary by the use of a short length of PEEK as a sleeve.

The ultimate intention was to work with no outlet frit when the packed capillary was used in the CEC instrument since the electroendoosmotic flow could be relied upon not only to hold the packed bed in place by the attraction of the negatively-charged silica particles to the anode at the inlet end of the capillary but also to draw it in to produce a more dense bed. It was therefore felt that it would be sufficient in the initial pressure packing stage, to obtain a reasonably dense bed rather than to make

great efforts to obtain the best possible packing with the equipment available. Accordingly a constant-flow reciprocating piston pump was used in the absence of a constant-pressure packing pump. Although this type of pump does not generate a very high initial packing velocity there was evidence [10] that it could be used for packing capillaries. Nonetheless some measures were necessary to ensure that the speed of packing was not too slow. It was found that the optimum configuration was for the capillary and the slurry chamber to be kept horizontal with the capillary being held as straight as possible. Also the open end of the capillary was placed in the slurry chamber so that at least 6 cm of the capillary was protruding into the chamber.

3.3. Frit making

It was necessary to make one frit i.e. at the inlet end for CEC of the capillary prior to conditioning and as soon as the microbore frit was removed. This was done according to the method of Smith [11] by fusing the packed bed at the very end of the capillary while water was the liquid in the capillary. At first it was found there were problems in preparing consistent frits using the frit-burning device even when using a predetermined optimum heating rate. It was not easy to tell if the filament was functioning as it should since they had a tendency to burn out slowly, altering the heating profile required to produce the frits. This was not the case when the filament casing was slightly adjusted so that it could accept a different type of filament provided by Capital HPLC which is much less prone to burn out. This solution to the problem was discontinued for safety reasons. While the Capital filaments are ideal for Capital's own frit-burning device, the Glaxo Wellcome device is of quite a different electrical design wherein it is actually beneficial for the filaments to frequently burn out in order that the power supply may be protected. It was therefore decided that, rather than purchasing a new Capital burning device, regular consumption of filaments would be accepted but the cost of this would be defrayed by preparing filaments in-house from Nuralloy 368 wire. With experience it was possible to recognise when this type of filament was beginning to burn out and thereby replace it rather than proceed with a modified heating protocol on a filament close to the end of its lifetime.

3.4. Conditioning of packed capillaries

The conditioning of the capillary column prior to use in CEC is vitally important. At worst if the capillary is dry it will not conduct the current through the capillary. However, even if there are slightly dried-out patches in the capillary this can lead to the formation of bubbles or capillary blockage through accelerated drying out at local 'hot spots'. It was found that partial drying took place even during the process of inserting the packed capillary into its cartridge. Therefore conditioning, with more water then acetonitrile and then the CEC mobile phase to be used initially, on a conventional LC pump with flow splitting was carried out with the packed capillary already inserted into its cartridge. The conditioning profile used was fairly standard and no attempt was made to reduce the time involved for each solvent. Clearly the times involved would be impractical for commercial production but they were perfectly acceptable in the research laboratory situation with capillary packing being an ongoing process with capillary production exceeding capillary consumption or with capillaries simply being left conditioning until required. Any 'consumption', whether for 'laboratory' or commercially prepared packed capillaries, was by breakages. Conditioning is also an issue in this respect in that the capillary seemed less susceptible to breakages when the packing material was completely 'wetted'.

As indicated earlier, the critical phase of the 'conditioning' was the bedding down which took place while the instrument was subjected to electric field on fitting into the CE instrument. This was continued for 2 h prior to any samples being loaded and its effectiveness could clearly be observed. Perhaps the most vivid evidence that the EOF should be strong enough to bed down the packing material and hold it in place without a frit during operation was that on one occasion when the polarity was accidentally reversed the capillary unpacked in under a minute! Also the fact that the efficiency continued to improve after the initial 2-h period as shown in Table 1 suggests that with any capillary packed by any other means or using an outlet frit, it would have

Table 1
Change in efficiency from time of application of electric field

Peaks	N (run 1)	Plates per metre (run 1)	N (run 2)	Plates per metre (run 2)
0	4300	22000	5200	26000
1	8500	42000	12000	58000
2	3700	18000	4600	23000
3	600	2800	900	4700
4	8700	44000	18000	90000
5	34000	170000	42000	210000
6	32000	160000	38000	190000
7	8900	45000	11000	54000
8	180000	890000	210000	1100000

$N=5.54(t_r/w^{1/2h})^2$; t_r=retention time; $w^{1/2h}$=width at half height.
Plates per metre=N/L; L=length of capillary in metres.

to be exceptionally well packed for void formation at the outlet frit not to occur.

3.5. Capillary electrochromatography

To date, capillaries packed using the methodology described here have been used for CEC of the range of neutral compounds such as steroids and polyaromatic hydrocarbons and test mixtures that have typically been used to illustrate the powers of the technique. For example the separation of eight steroids shown in Fig. 1 is a considerable improvement over what can be obtained by LC, or indeed even by micellar electrokinetic chromatography. Two main points of interest were noted from the electrochromatograms. Despite the relatively low sample concentrations, 0.1 mg ml^{-1} of each analyte, anomalously low efficiency similar to what might be observed with overloading effects was obtained for peaks 3 and 7. This phenomenon was repeatable and therefore not likely to arise from simultaneous passage through the window with an air bubble. The other point was that, apart from peaks 3 and 7, the efficiencies obtained for the other peaks generally increased with retention time and were very high, so high in fact that at least for betamethasone valerate (peak 8) it needs to be considered whether or not some focusing effect was taking place. However it also provided the salutary lesson that it is not always possible to overpower all separation problems by the use of efficiency alone. Using the mobile phases usually used with acetonitrile and aqueous phosphate buffer in different proportions it was not possible to completely baseline resolve hydrocortisone (peak 1) and prednisolone (peak 2) while still eluting all eight steroids from the column. Nonetheless the degree of separation is very close to a target that has already been observed to be difficult by other CEC users [12]. It seems that even in CEC there is still a need to study solvent-selectivity issues. As well as selectivity and polarity, there is a need to consider how the solvent affects electroosmotic flow [13] and therefore run times. For that reason, i.e. to obtain shorter run times, acetonitrile was always used instead of methanol, for example in a separation of a commercial test mixture of neutral analytes (Fig. 2) to give a separation comparable to that obtained on commercial columns.

4. Conclusions

The key elements of the overall procedure, which when combined make for a very simple and easily accessible methodology for the practice of CEC, may be identified as the use of an end-fitting and frit from a 1-mm microbore column in the initial phase of the packing, use of a conventional constant-flow HPLC pump, conditioning of the packed capillary using a conventional constant-flow HPLC pump with flow splitting, inserting the packed capillary into its cartridge before conditioning on the LC pump, bedding down of the packed capillary under an electric field while fitted into the CE instrument and operation without pressurisation and without an end frit. Use of this methodology should allow many

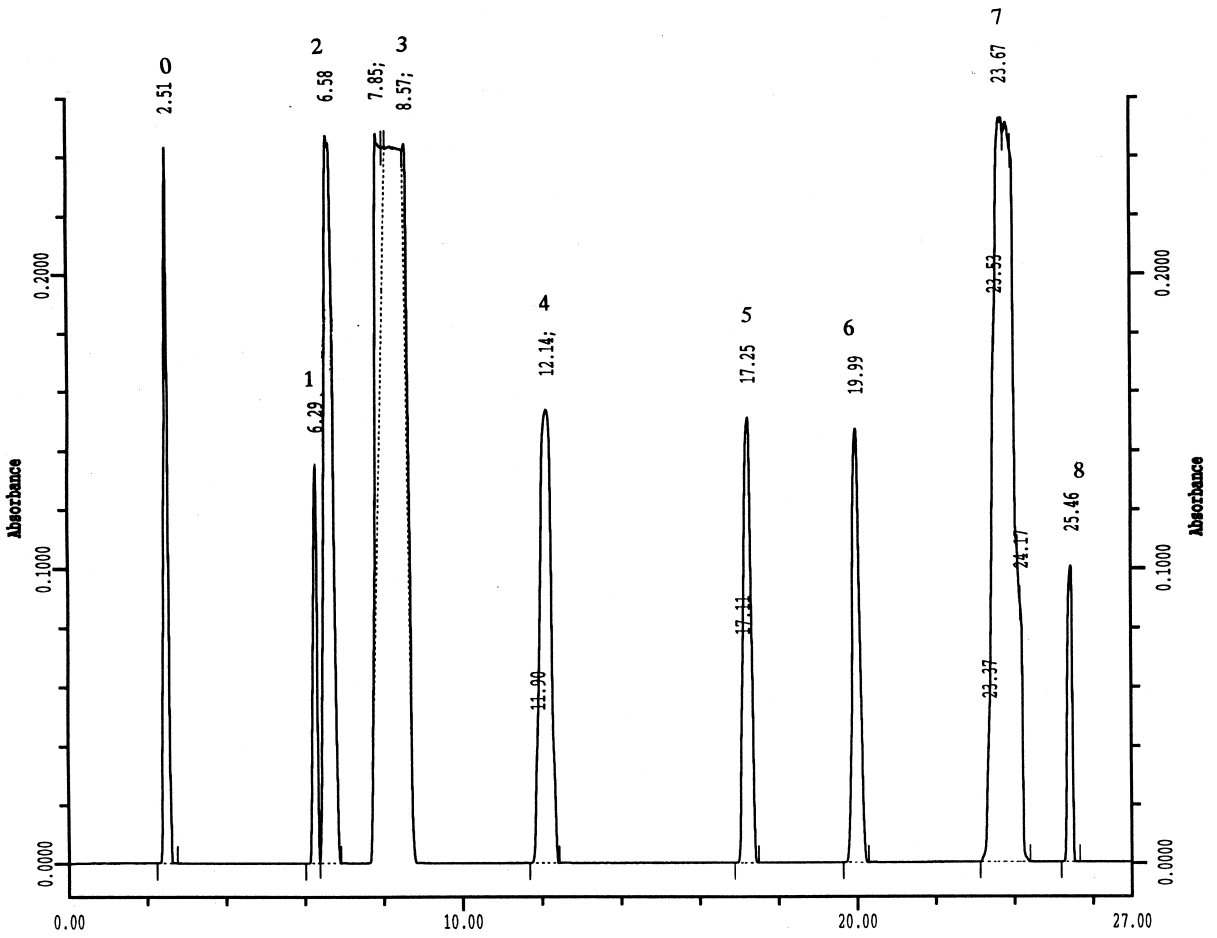

Fig. 1. Separation of steroids. Capillary: 27-cm (20 cm packed bed)×50-μm I.D. capillary packed with ODS Hypersil (3 μm); mobile phase: acetonitrile–2 m*M* aqueous phosphate, pH 7.8 (80:20, v/v); detection: UV, 214 nm; voltage: 10 kV (no pressurisation); injection: electrokinetically, 5 kV for 5 s. Sample: 0.1 mg ml^{-1} each of eight steroids and neutral marker dissolved in mobile phase. Thiourea (0), hydrocortisone (1), prednisolone (2), betamethasone (3), betamethasone dipropionate (4), clobetasol butyrate (5), fluticasone propionate (6), clobetasone butyrate (7) and betamethasone-17-valerate (8).

practitioners of separation science to enter the field of CEC much earlier than otherwise would be possible. Even if they initially prefer to attempt to use a commercial packed capillary and at some stage break it at the outlet frit end, they can continue to use a shorter length without having to make a new outlet frit. The only possible limitation of the methodology is that when operating at the higher end of the voltage range there is greater risk of capillary blockages than when operating with a pressurised CEC system. In practice however this proved to be hardly a limitation at all since high efficiencies and short run times were easily obtained when working with voltages of 15 kV and below.

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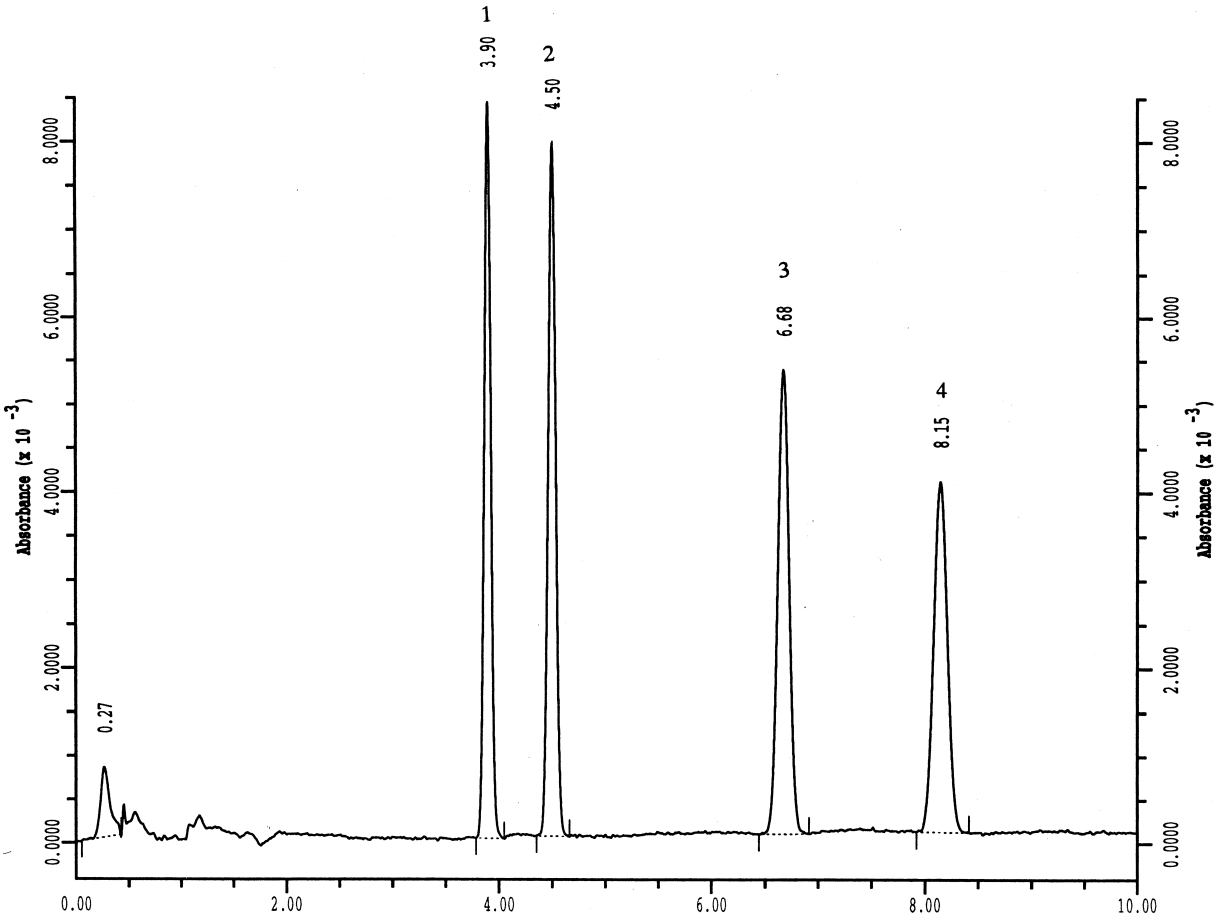

Fig. 2. Separation of Commercial Test Mixture of Neutral Analytes. Capillary: 27-cm (20 cm packed bed)×50-μm I.D. capillary packed with ODS Hypersil (3 μm); mobile phase: acetonitrile–2 m*M* aqueous phosphate, pH 7.8 (80:20, v/v); detection: UV, 214 nm; voltage: 15 kV (no pressurisation); injection: electrokinetically, 5 kV for 5 s. Sample: 0.1 mg ml^{-1} each of thiourea, benzamide, benzophenone and biphenyl dissolved in mobile phase.

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