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At-line solid-phase extraction for capillary electrophoresis: application to negatively charged solutes

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Abstract

The analysis of complex biological samples with capillary electrophoresis (CE) requires proper sample pretreatment. In this paper the applicability of solid-phase extraction (SPE) coupled at-line with CE is studied, by using a laboratory-made interface. A fresh (disposable) SPE cartridge is used for each sample to prevent carry-over effects. The sample handling procedure is performed parallel with the analysis of the previous sample, to improve sample throughput. Using this set-up, negatively charged test compounds (some non-steroid anti-inflammatory drugs) can be determined in serum and urine. The method is linear over at least two decades and detection limits are around 40 μg/l. A single capillary, flushed only once a week with a sodium hydroxide solution, was used without problems for the analysis of ca. 900 samples during 1 year. The robustness of the system was very good: no blocking of loop, interface or capillary was found during this period. Furthermore, the system was successfully used for overnight runs.

Keywords: Solid-phase extraction; Sample pretreatment; Flurbiprofen; Ketoprofen; Naproxen; Non-steroidal anti-inflammatory drugs

1. Introduction

Because of its high efficiency, capillary electrophoresis (CE) is very suitable for the analysis of complex biological samples. There are, however, several distinct problems to be solved. Large amounts of salts, as present in urine (50–500 m*M* of sodium chloride) and serum (150 m*M* of sodium chloride), will disturb the analysis because of the high electrical conductivity of the sample which may result in peak splitting or even in a complete loss of resolution [1]. Furthermore, proteins, as present in serum (ca. 75 g/l), can bind irreversibly to the silanols of the capillary wall, which can cause dramatic changes of the migration times. Proteins can also bind with drug molecules and therefore reduce their recovery. Finally, particulate matter can cause clogging of the CE capillary. In addition, it should be noted that the analyte concentrations usually dealt with are rather low compared with the detectability that can be obtained in CE. Obviously, the development of appropriate sample treatment procedures for biological matrices is urgently required. Most sample treatment procedures available today can be performed either manually (off-line) or

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in an automated fashion (at-line or on-line). If large numbers of samples have to be analysed, the latter alternative is to be preferred. Sample dilution [2] (to lower the salt concentration), filtration [3] and centrifugation [4] (to remove particulate matter), liquid–liquid extraction (LLE) [5,6] (to remove salts and other interferences) and the addition of acetonitrile [6] (to precipitate proteins) are generally carried out off-line. Isotachophoresis is one of the electrophoretic techniques considered for sample concentration [7,8]. Unfortunately, it can only be used for samples with a constant matrix.

Solid-phase extraction (SPE) is a more promising option. Two different modes for the coupling of SPE and CE have been described in the literature, the off-line use of disposable cartridges [4,9,10] and the use of an in-line (in-capillary) microcolumn [11,12] or particle-loaded membranes [13,14]. Using off-line disposable SPE cartridges does not create any technical problems. However, the inherent disadvantages that it requires much time and is a rather laborious technique are well-known. In-line SPE–CE systems can be fully automated, but also have some disadvantages. Desorption of the analytes as a narrow plug is difficult, the sample may contaminate the capillary or the SPE device, and finally the choice of CE buffer is limited. And furthermore, only relatively clean samples (no proteins or particulate matter and low concentration of interferences) can be analysed. Therefore, urine and serum are pretreated with off-line sample preparation methods, such as SPE, LLE or precipitation.

The purpose of the present paper is to develop a fully automated sample treatment procedure coupled at-line with CE, which is able to analyse urine and serum directly without additional sample pretreatment, to overcome the above problems. In an at-line system the SPE unit is connected with the CE unit by means of a laboratory-made interface, which was also used for the coupling of an LC system with a CE unit [1]. Non-steroid anti-inflammatory drugs (NSAIDs), were used as test compounds and urine and serum as biological matrices. NSAIDs are weak acids with pK_a values of about 4.5 [15]. The NSAIDs were selected because, so far, mainly manual pretreatment procedures were used for their CE determination in biological fluids.

2. Experimental

2.1. Materials

Ibuprofen, ketoprofen, naproxen and flurbiprofen were purchased from Sigma (St. Louis, MO, USA), acetic acid (>99.8%) and sodium acetate came from Riedel-de Haën (Seelze, Germany) and formamide (99.8%), methanol (99.8%) and acetonitrile (>99%) were from J.T. Baker (Deventer, The Netherlands). Water was demineralized and distilled before use.

2.2. Samples

Urine was collected from five healthy volunteers on 3 subsequent days. The samples were pooled and divided into 100-ml portions and frozen at −18°C. Bovine serum of untreated objects was purchased from Sigma; it was divided into 10-ml portions and frozen at −18°C. Urine and serum samples were stored at −18°C for, at maximum, 6 months.

2.3. Off-line CE

Optimization of the CE buffer conditions, which led to using a 20 m*M* acetate CE buffer (pH 4–5), was performed using a HPCE system (Hewlett-Packard, Waldbronn, Germany) with a capillary (Hewlett Packard) of 48.5 cm (effective length, 40 cm), an I.D. of 50 μm and an O.D. of 375 μm. The capillary was conditioned by flushing (at 900 mbar) with, successively, 1 *M* sodium hydroxide aqueous (5 min), water (15 min) and CE buffer (30 min). Before each analysis, the CE buffer in the electrophoresis vials was replaced and the capillary was flushed with CE buffer for 2 min. Sample injection was performed for 20 s at a pressure of 10 mbar. Electrophoresis was performed by applying a voltage of −30 kV for 10 min. The CE runs were performed at 20°C, using absorbance UV detection at 200 nm (band width, 10 nm).

2.4. At-line SPE–CE

At-line SPE–CE experiments were performed using an automated SPE (ASPEC XL) system (Gilson, Villiers-le-Bel, France) which automates all

solvent handling procedures. The desorption liquid from the SPE cartridge was injected into a 100 μl loop via the injection port, which was connected to the six-port valve of the ASPEC system. The six-port valve was connected with the interface as shown in Fig. 1; it has been discussed in detail before [1]. The interface constructed from 7 mm of green polyetherether ketone (PEEK) tubing (0.75 mm I.D., 1/16″ O.D.) was positioned on top of 30 cm of red PEEK tubing (0.13 mm I.D., 1/16″ O.D.). Both pieces of PEEK were placed inside a piece of PTFE tubing with a length of 37 mm (1/16″ I.D., 1/8″ O.D.). The red PEEK tubing was inserted into the PTFE tubing over a length of 18 mm, while the other end was connected to the valve. The CE capillary was inserted into, and the electrode placed on top of, the green PEEK tubing (see insert of Fig. 1).

The CE part of the SPE–CE experiments was performed using a PrinCE CE system (Prince Technologies, Emmen, The Netherlands). After completing the SPE procedure by the ASPEC, an 'SPE ready' signal is sent to the PrinCE system, while the ASPEC waits in its turn for the 'CE ready' signal. In the next step, the six-port valve is switched and the content of the loop is flushed to the interface by the CE buffer. When the effluent plug containing the analytes, passes the tip of the capillary (between 0.2 and 0.5 min after switching of the valve), injection is performed by applying an underpressure of −40 mbar at the other end of the capillary. It should be realized that only a minor part of the desorbed SPE effluent is introduced into the CE capillary; by far the largest fraction is flushed to waste. The CE capillary 96 cm (effective length 40 cm)×50 μm I.D., 375 μm O.D., (LC-Service, Emmen, The Netherlands), was equipped with a thermostating device, set at 20°C, [16] to avoid Joule heating effects. The capillary was conditioned at a pressure

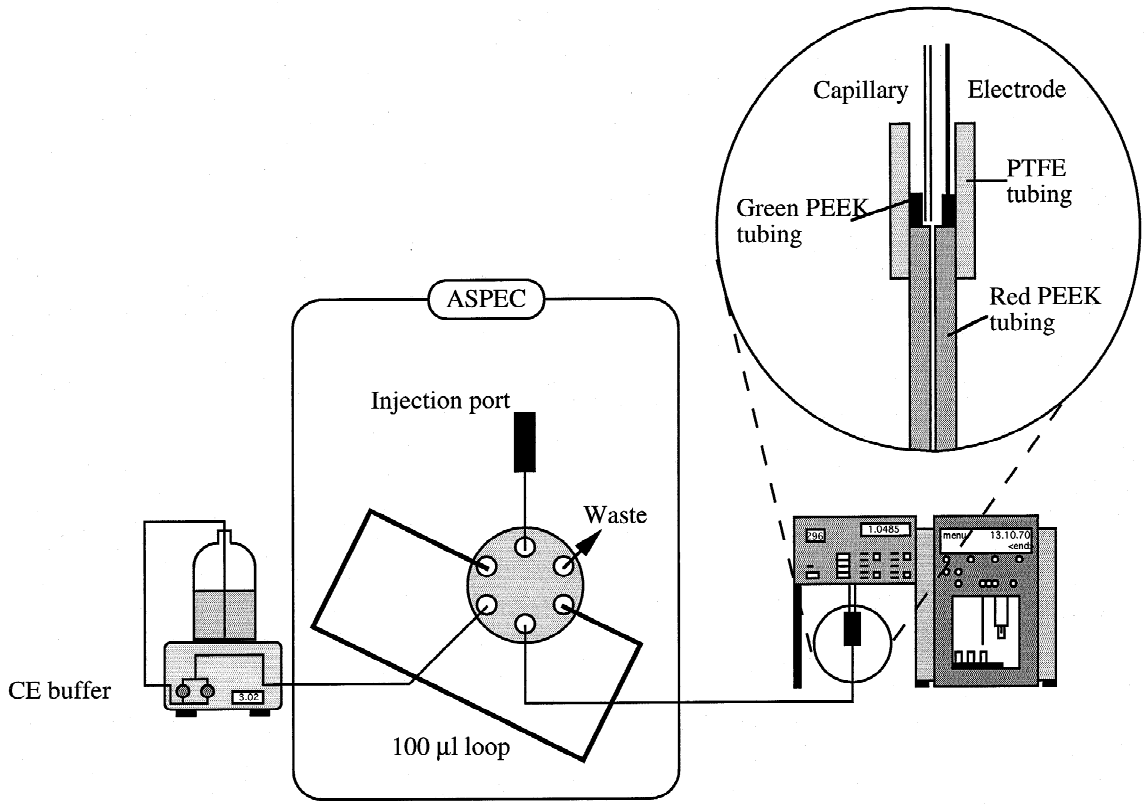

Fig. 1. At-line SPE–CE configuration. For more details, see text.

of 2000 mbar by means of the same procedure as described for the HPCE system. Before injection, the capillary was flushed with the optimized CE buffer. The analysis was performed using a voltage of −30 kV (the electrode in the interface was grounded) and detection was performed using a Model 759A UV–Vis detector (Applied Biosystems, Foster City, CA, USA), set at 200 nm.

2.5. SPE procedure

Disposable LC-18 cartridges (Supelco, Bellefonte, PA, USA), packed with 100 mg of C_{18}-bonded silica, were conditioned with 2 ml of methanol and, subsequently, 2 ml of 10 m*M* phosphate buffer (pH 2). Next, the cartridges were loaded with urine (8 ml) or serum (1 ml). In the latter case 1% (v/v) of formic acid was added to release the drugs from the proteins. Then for the urine samples the cartridges were washed with 3 ml of wash buffer (10 m*M* phosphate buffer (pH 4.5)–acetonitrile 80:20, v/v); with serum, the cartridges were washed first with 1 ml of 10 m*M* phosphate buffer (pH 2), followed by 3 ml of wash buffer and, again, 1 ml of 10 m*M* phosphate buffer (pH 2). Finally, the analytes were desorbed with 400 μl of desorption solvent (10 m*M* phosphate buffer (pH 7.8)–acetonitrile, 25:75, v/v).

3. Results and discussion

In the present study, after optimization of the CE separation of the test compounds, the SPE procedure was optimized and, next, the conditions for the at-line SPE–CE coupling were determined. Finally, the total SPE–CE set-up was used for the analysis of biological samples.

3.1. Optimization of CE analysis

However, the CE analysis of the test compounds was optimized again [17–21], because a buffer system without micelles and chiral additives had to be developed to avoid interfacing disturbances. Analyte separation in CE is based on differences in effective mobilities, which mainly depend on the size/charge ratios. All test compounds (pK_a around 4.5) have about the same size but, at pH values of

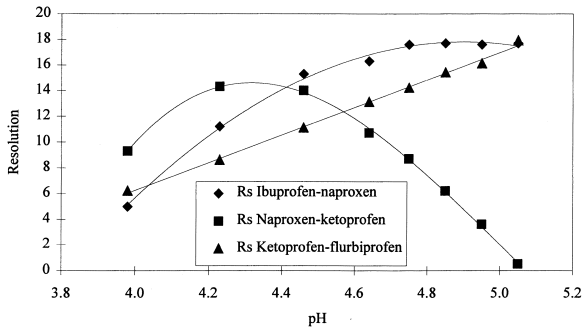

Fig. 2. Dependence of NSAID resolution on pH of CE buffer.

4–5, their charges are slightly different. The resolution data of Fig. 2 indeed show that optimum conditions are obtained at a pH of 4.5–4.7. A pH value of 4.6 was chosen for all further experiments. The resolution is less at lower pH values because of decreasing charge of all analytes which results in smaller differences of the charge/size ratios.

3.2. Interfacing SPE with CE

The SPE module, connected to the CE by means of the PEEK-tubing interface (Fig. 1) allows a continuous flow of CE buffer through the interface. At the end of the SPE procedure the analytes are desorbed and the solvent is flushed through the interface via a loop. The dimensions of the loop, the loop filling and injection of the sample into the CE capillary had to be optimized.

3.2.1. Loop dimensions

Since the loop is filled by flushing it with solvent and the loop content is subsequently flushed to the interface, mixing effects may occur which will depend on the loop diameter and volume applied. Loops with a volume of 100 μl (constructed of PEEK tubing) and I.D.s of 0.25, 0.50 and 0.75 mm were used. Since the syringe pump of the ASPEC can only handle pressures less than 4 bar, diameters smaller than 0.25 mm cannot be used because of pressure build-up or unacceptable washing and loading times. The loops were flushed with a 1 vol. % formamide solution and flushed by switching the six-port valve in the solvent stream (flow, 0.2 ml/min). A 50 cm×75 μm I.D. fused-silica capillary was used instead of the interface. The tested loop

diameters resulted in peak widths of 1.2, 1.4 and 1.7 min, respectively. Therefore, the PEEK tubing with the smallest diameter (0.25 mm) was applied in all further experiments.

To monitor the influence of the loop volume, the loops were flushed with 1 ml of solvent (1% (v/v) formamide). After switching the six-port valve the contents of the loop were flushed to the interface and the elution profile recorded. The dilution effect can be visualised by plotting the maximum detector response (i.e. peak height) versus the loop volume. Fig. 3A shows that the peak height increases with larger loop volumes, indicating reduced mixing effects. Plateau conditions are reached for a loop volume of 70–80 μl. A loop volume of 100 μl in combination with a diameter of 0.25 mm I.D. was used in all further experiments.

3.2.2. Loading of the loop

In the above experiments the loops were flushed with 1 ml of solvent. Because of the limited sample extract volume available after desorption of the analytes from an SPE cartridge, it is necessary to reduce this volume significantly. In order to study this aspect, a 100-μl loop was flushed with different volumes of 1% (v/v) of formamide and the peak area was recorded. The area, which indicates the total amount of sample loaded on the loop, was plotted versus the volume loaded. Fig. 3B shows that the peak area becomes constant after at least 180–200 μl are loaded. To be on the safe side, in all further experiments the loop was flushed with 190 μl of desorption solvent. The flow-rate of the CE buffer did not affect the plug profile in the range from 0.1 to 0.4 ml/min (data not shown). The time period during which the underpressure has to be applied should not be too short, because this will adversely affect the precision of the injection. A flow-rate of 0.2 ml/min was chosen as a compromise.

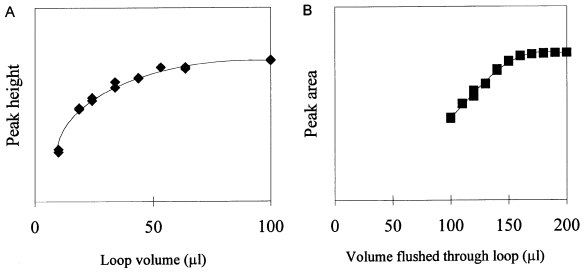

Fig. 3. Influence of (A) loop volume on height of eluted peak and (B) volume flushed into 100-μl loop on area of peak.

3.2.3. Injection parameters

Injection of an aliquot of the SPE desorption plug into the CE capillary is started by applying an underpressure at the detection side of the CE capillary. The parameters of interest are the timing of the injection and the injection pressure. Two approaches can be used. In the first method the injection starts before, and ends after all of the eluted compounds have passed the interface (between 0 and 1.2 min after switching the valve). The other method is to inject an aliquot out of the middle of the elution plug, i.e. when the maximum analyte concentrations in the plug passing the tip of the capillary are maximal, i.e. between 0.2 and 0.5 min after switching the valve. The injection pressure was varied between −5 and −60 mbar for the former, and between −10 and −110 mbar for the latter approach. The criteria used to select the optimum time and pressure were: (i) the efficiency, expressed as the number of theoretical plates and (ii) the detectability, expressed as the peak height (since the noise was about the same during all experiments).

The loop was filled with a solution containing 30 μg/ml of each of the test compounds in acetonitrile–phosphate buffer pH 7.8 (75:25, v/v). In Fig. 4 the results for ibuprofen are shown; the other compounds showed similar behaviour. If 100 000 plates are arbitrarily taken as the minimum value for providing

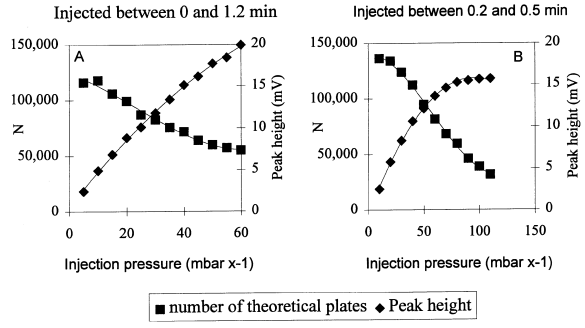

Fig. 4. Dependence of the number of theoretical plates and peak height, on injection procedure used, (A) 0–1.2 min and (B) 0.2–0.5 min. Test compound is ibuprofen. For further details, see text.

sufficient resolution, the second injection procedure (Fig. 4B) is seen to be more favourable: the peak height is 12 mV which is about two times higher than when using the first approach (Fig. 4A). For this reason the 0.2–0.5 min injection procedure, applying a pressure of −40 mbar, was selected.

3.3. Optimizing the SPE procedure

The breakthrough of the analytes on the SPE cartridge was studied using a test solution of the NSAIDs in water (10 μg/ml each). Even after loading of 8 ml of the standard solutions on the cartridge, no breakthrough was observed. Larger volumes were not tested because of the limited availability of biological samples. Because of this it can be concluded that this type of cartridge is able to retain the analytes sufficiently, and therefore, this cartridge is used in further experiments. After loading of urine (8 ml) or serum (1 ml) no breakthrough of the analytes was observed.

In order to obtain low detection limits, the analytes should be desorbed in as small a volume as possible. To achieve this, a high modifier concentration (elution strength) and $pH \geq pK_a + 2$ (fully charged analytes) were used. Two organic solvents were used as a modifier, methanol and acetonitrile. The concentration of organic modifier was limited to 75% (v/v) to avoid precipitation of (in)organic salts. The buffer concentration (40 m*M* phosphate, pH 7.8) was high enough to ensure sufficient buffer capacity; higher concentrations should not be used since they will make the stacking procedure less efficient.

After loading of the sample, the cartridges were desorbed with different volumes (i.e. 0.2–1.0 ml) of either methanol–40 m*M* phosphate buffer, pH 7.8 (75:25, v/v) or acetonitrile–40 m*M* phosphate buffer, pH 7.8 (75:25, v/v). Fig. 5 shows that with methanol the required desorption volume is about 800 μl, while with acetonitrile ca. 400 μl will suffice. For this reason desorption with 400 μl acetonitrile–40 m*M* phosphate buffer, pH 7.8 (75:25, v/v) was selected.

3.4. At-line SPE–CE of urine and serum samples

Spiked urine samples were analysed using both direct CE and the present at-line SPE–CE procedure.

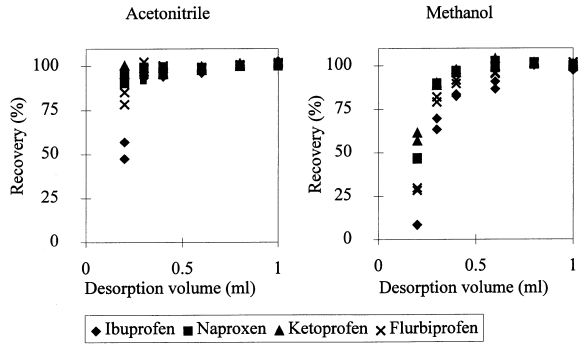

Fig. 5. Influence of desorption volume on NSAID recovery during SPE, when using (left) acetonitrile or (right) methanol in modifier–40 m*M* phosphate pH 7.8 (75:25, v/v) mixture.

Fig. 6A shows that direct CE, i.e. without sample pretreatment, is not possible. Because of the high salt concentration in the sample, the plug containing a part of the analytes migrates together with the EOF at about 8 min [1]. Incorporating the SPE pretreatment step using 1 ml of 10 m*M* phosphate buffer (pH 2.5) to wash the cartridge and the desorption procedure described above, resulted in a significant decrease of the interferences in the electropherogram. Still, the number of interfering peaks was quite large (Fig. 6B); therefore, a more effective washing procedure had to be developed. This aspect was studied varying the pH (2.5–6.5), percentage of modifier (0–20% acetonitrile, v/v) and volume (1–3 ml) of the washing buffer, as is shown in Table 1. The results for pH 2.5 solutions are in most cases rather poor. The better results found at higher pH values can be explained by the increased charge of the interferences, which are therefore more easily removed under these conditions. However, a too large increase of the pH results in desorption of the analytes (compare pH 6.5 and pH 4.5 with 10% acetonitrile, v/v). The optimum was found by using 3 ml of acetonitrile–10 m*M* phosphate buffer, pH 4.5 (20:80, v/v). The fully satisfactory result then obtained is shown in Fig. 6C for the analysis of a spiked and a blank urine sample.

Serum protein binding of analytes can be a serious problem during the analysis of serum samples. The test compounds are known to be bound for over 99% to serum proteins and indeed no analyte peaks showed up in SPE–CE electropherograms for serum

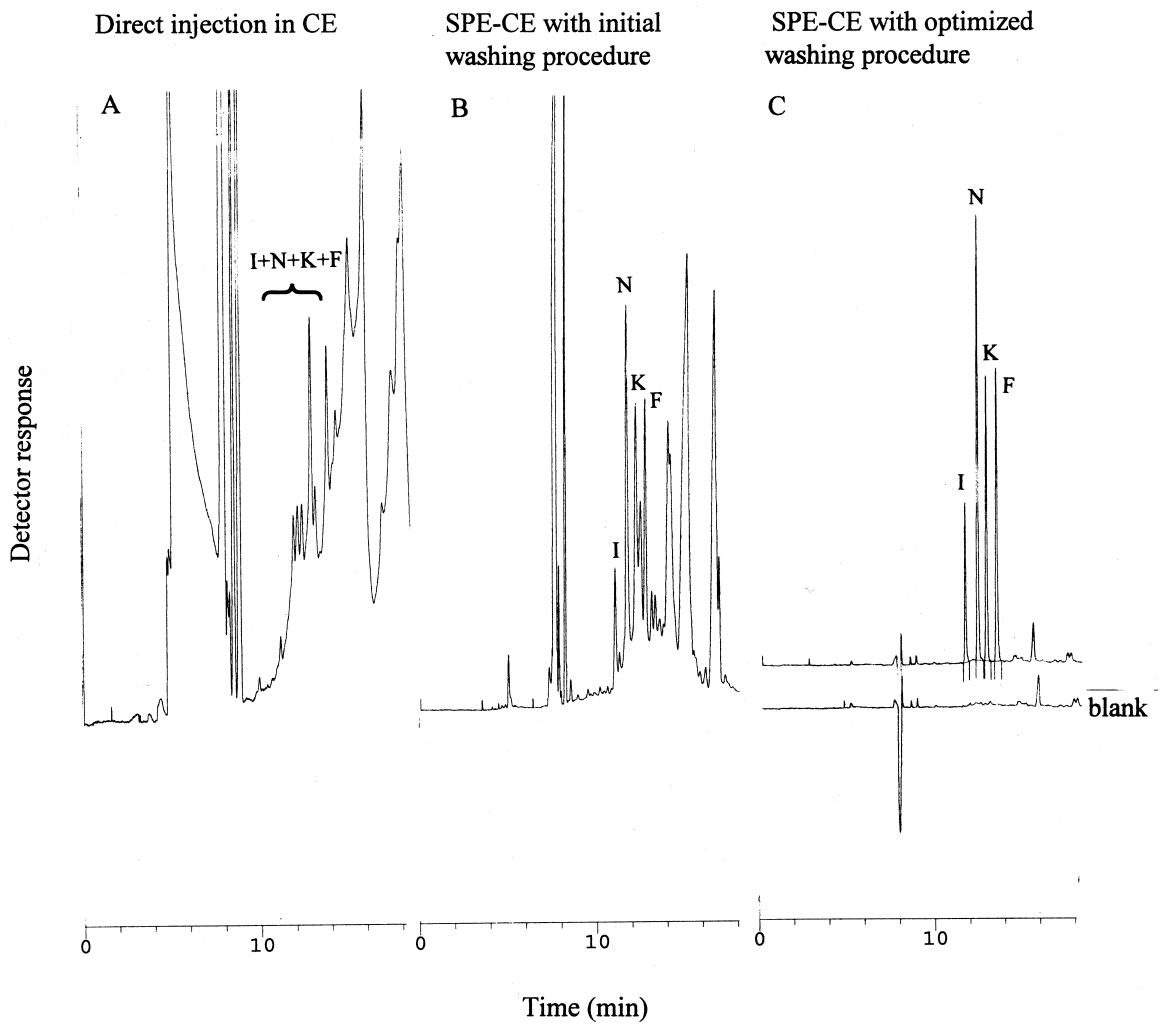

Fig. 6. Electropherograms of spiked urine (8 ml sample; 10 μg/ml of each NSAID) with (A) direct CE analysis, (B) SPE–CE using 1 ml phosphate buffer (pH 2) in washing procedure and (C) SPE–CE using 3 ml of acetonitrile–phosphate buffer pH 4.5 (20:80, v/v) in washing procedure; blank, blank urine.

Table 1
Influence of volume, pH and percentage of acetonitrile of the washing buffer on at-line SPE–CE of urine samples[a]

Acetonitrile (%)	pH								
	2.5			4.5			6.5		
	1 ml	2 ml	3 ml	1 ml	2 ml	3 ml	1 ml	2 ml	3 ml
0	4(0)	4(0)	4(0)	4(0)	4(0)	4(0)	4(0)	4(0)	4(1)
10	4(0)	4(0)	4(0)	4(1)	4(2)	4(2)	4(1)	2(1)	1(1)
20	–	–	–	4(2)	4(3)	4(4)	–	–	–

[a] Results are given in the format *a*(*b*), with *a*, number of analyte peaks observed and *b*, number of analyte peaks baseline-separated from the interferences.

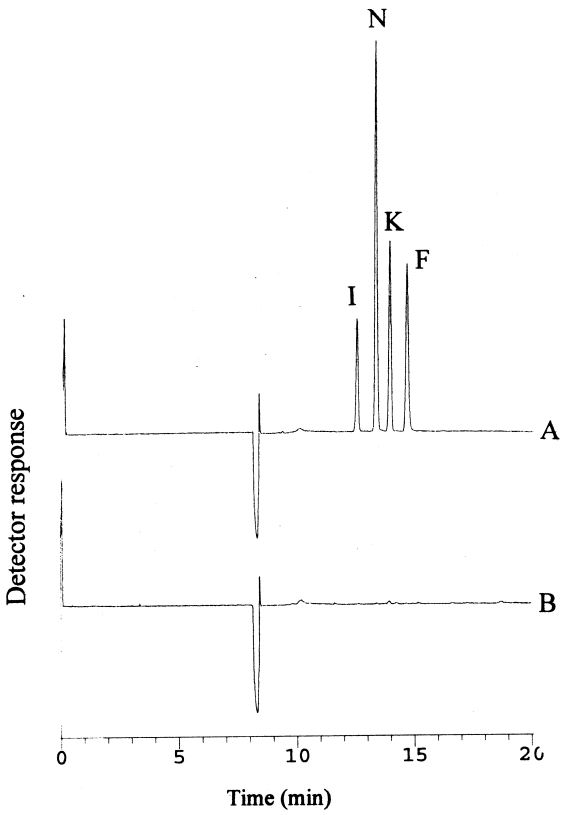

Fig. 7. SPE–CE analysis of (A) 1 ml serum spiked with 10 μg/ml of each NSAID and (B) blank. In both cases 1% (v/v) of formic acid was added to the sample prior to sample preparation.

spiked with 10 μg/ml of each of the NSAIDs [22,23]. To disrupt the analyte–protein bonds, 1% (v/v) of formic acid was added to the sample before loading it on the SPE cartridge [22,23]. An almost quantitative release (>99%) of the test compounds was obtained as is shown in Fig. 7, trace A. The optimized washing procedure described above efficiently removed all low molecular-mass interferences (Fig. 7, trace B). Two additional washing steps with 1 ml of water before and after removal of these interferences by the washing buffer, were necessary to remove the bulk of endogenous high-molecular-mass interferences.

3.5. Quantitative data

Calibration plots were constructed both for serum and urine using analyte concentrations from the detection limit (signal-to-noise ratio=3) up to 10 μg/ml. The data of Table 2 show that the plots are linear (r^2>0.99) for all test compounds in both matrices. The detection limits are 0.04–0.08 μg/ml in urine, and 0.2–0.4 μg/ml in serum. The differences in the detection limits for the various test compounds can be explained by their differences in molar absorptivity at the detection wavelength, 200 nm. The somewhat higher detection limits obtained for serum can be attributed to the smaller sample volumes loaded on the SPE cartridges, 1 ml for serum and 8 ml for urine. Nevertheless, the analyte detectability is sufficient for therapeutic drug monitoring.

The within-day and day-to-day precision were determined using solutions of the test compounds in water and the biological matrices containing high (10 μg/ml) and low (five times the detection limit) concentrations of the NSAIDs. The results are summarized in Table 3. As is to be expected, in most cases a better precision is obtained for aqueous samples. However, the differences with the real

Table 2
Calibration plots of the test compounds in urine (1 ml loaded) and serum (8 ml loaded) recorded from the detection limit up to 10 μg/ml

	Compound	Intercept (std.)	Slope (std.)	Correlation coefficient (r^2)	Detection limit (μg/ml)
Serum (n=7)	Ibuprofen	−0.01 (0.03)	0.30 (0.01)	0.9934	0.2
	Ketoprofen	−0.01 (0.06)	1.12 (0.01)	0.9983	0.4
	Naproxen	−0.01 (0.03)	0.52 (0.01)	0.9983	0.4
	Flurbiprofen	−0.02 (0.04)	0.49 (0.04)	0.9969	0.2
Urine (n=8)	Ibuprofen	−0.2 (0.1)	2.01 (0.02)	0.9984	0.08
	Ketoprofen	0.5 (0.1)	6.61 (0.03)	0.9997	0.04
	Naproxen	1.4 (0.2)	4.01 (0.05)	0.9978	0.04
	Flurbiprofen	−0.2 (0.1)	4.11 (0.02)	0.9997	0.04

Table 3
Day-to-day and within-day reproducibility for SPE-CE of NSAIDs in urine, serum and water (n=5)

Volume loaded on cartridge:		8 ml			1 ml		
Matrix:		Urine	Water		Serum	Water	
Concentration:		5×LOD[a]	5×LOD[a]	10 μg/ml	5×LOD[a]	5×LOD[a]	10 μg/ml
Day-to-day reproducibility (%)	Ibuprofen	11.0	7.6	5.8	5.5	2.2	3.5
	Ketoprofen	6.6	6.8	4.6	4.7	3.7	3.7
	Naproxen	8.6	4.6	4.8	4.2	1.2	3.5
	Flurbiprofen	8.3	3.3	2.8	7.6	3.5	3.8
Within-day reproducibility (%)	Ibuprofen	8.2	3.0	3.1	6.7	3.7	1.0
	Ketoprofen	7.9	3.3	2.8	2.8	2.8	1.1
	Naproxen	7.7	3.9	3.3	2.9	3.4	2.5
	Flurbiprofen	6.0	3.0	1.6	2.6	3.2	1.2

[a] Concentrations five times LOD for urine and serum, respectively (see Table 2).

samples — which are of course primarily due the presence of residual endogenous interferences — are not too large, and sometimes even surprisingly small. In any case, the R.S.D. values of the precision are lower than 15%, which is the often used acceptance level for quantitative data in biomedical analysis [24].

4. Conclusions

The present study shows that SPE can be coupled at-line to CE using a home-made interface. The construction of the interface and the SPE-to-CE transfer procedure is performed in such a way that the only parameter to be adapted if analytes other than the present test compounds have to be determined, will be the injection pressure.

Our set-up was used to determine NSAIDs in serum and urine. The optimized sample pretreatment procedure causes the release of analytes from protein binding, their preconcentration, and the removal of essentially all interfering compounds. The SPE–CE method is linear over two decades and detection limits down to 0.04 μg/ml can be obtained. The detection limits are sufficiently low to perform therapeutic drug monitoring studies.

A single CE capillary was used for the analysis of at least 150 serum, 300 urine and 400 standard samples over a period of 1 year. The capillary was flushed once a week with a sodium hydroxide solution. To avoid carry-over effects and clogging of the interface and CE system, the SPE cartridges were used only once when biological samples were analysed. The robustness of the total SPE–CE system was enough to perform unattended analyses.

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