On-line dialysis–SPE–CE of acidic drugs in biological samples

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A fully automated method is presented for the determination of acidic drugs in urine and serum using on-line dialysis–solid-phase extraction (SPE)–capillary electrophoresis (CE) with UV detection. With non-steroidal anti-inflammatory drugs (NSAIDs) as test compounds, detection limits in the biological samples were 0.05–1.0 μg ml^{-1}. Calibration plots were linear over two orders of magnitude and the within-day and between-day repeatability were better than 10%. The CE capillary and SPE column were used for over 500 analyses; the dialysis membrane was replaced after 250 analyses. A general protocol for dialysis–SPE–CE which can be used for amphoteric and acidic drugs was devised. The present results show that this protocol has general validity and can be recommended for future work on other classes of drugs.

Introduction

Capillary electrophoresis (CE) has a high separation power and can be used for the separation of charged analytes present in biological samples. Unfortunately, such matrices contain a number of compounds which can interfere with the analysis, such as salts, proteins and particulate matter. These compounds must be removed prior to injection by applying a suitable sample preparation procedure. Off-line methods such as liquid–liquid extraction (LLE), precipitation and off-line solid-phase extraction (SPE) are most frequently used. These are, however, time consuming and degradation of the analytes can occur because of (nearly) irreversible adsorption effects and if solvent evaporation steps cannot be avoided. However, in bioanalysis, large numbers of samples often have to be processed, which means that automated procedures are preferred.

Papers describing CE procedures for the determination of NSAIDs are mainly directed at the separation of chiral isomers,[1,2] aqueous standards[3] and pharmaceutical formulations.[4] The determination of NSAIDs using CE in serum has been reported using off-line sample preparation methods such as LLE[5,6] and protein precipitation.[7] Detection limits varied from 1 to 10 μg ml,[6,7] which are not sufficient for therapeutic drug monitoring for some of the compounds (see Table 1).

The principle of using an automated dialysis system connected to an HPLC system has been presented.[8] In a recent study, on-line dialysis–SPE–CE was applied for the first time to the determination of sulfonamides in urine and serum.[9] Dialysis is applied to remove proteins and particulate matter. The analytes are diluted during this procedure; therefore, in a second step, they are trapped on an SPE column while salts are simultaneously removed. During the subsequent washing step, compounds that will interfere during the CE run can be removed. Finally, the analytes are desorbed and injected into the CE capillary via a laboratory-made interface. In this paper, it will be shown that this set-up and the optimization strategy in general can be used also to determine other compound classes such as strongly acidic drugs, with non-steroidal anti-inflammatory drugs (NSAIDs) as test compounds. The result is a fully automated method for the direct determination of NSAIDs in biological samples, presented here for the first time.

Table 1 Therapeutic levels of the NSAIDs in serum and their absorbance maxima

Compound	Concentration/ μg ml^{-1}	Absorbance maximum/nm
Ibuprofen	25–50	264
Naproxen	25–70	273
Fenoprofen	20–50	273
Ketoprofen	0.5–6	261
Flurbiprofen	2–12	247

Experimental

Chemicals and samples

Ibuprofen, naproxen, fenoprofen, ketoprofen and flurbiprofen were obtained from Sigma (St. Louis, MO, USA), acetonitrile, boric acid, methanol and phosphoric acid from J. T. Baker (Deventer, The Netherlands), decanoic acid, sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate dodecahydrate from Merck (Darmstadt, Germany) and acetic acid and sodium acetate from Riedel-de Haën (Seelze, Germany). Water was de-mineralized and distilled before use. In all cases, chemicals of the best available quality were used.

Urine was collected from five healthy volunteers on three consequent days. The samples were pooled and divided into 100 ml portions and frozen at −18 °C. Bovine serum from untreated animals was purchased from Sigma; it was divided into 10 ml portions and frozen at −18 °C. The biological samples were stored for a maximum period of 3 months.

Methods

The set-up of the dialysis–SPE–CE system and the instruments used have been described in detail,[9] and here just a schematic diagram of the set-up is presented in Fig. 1. The experimental parameters are discussed in the next section and are summarized in Table 2.

Results and discussion

The main aim of this project was to devise a generally valid optimization strategy for dialysis–SPE–CE. An overview of all

relevant parameters is given in Table 2; data relating to an earlier first attempt are included for convenience. Because of practical aspects, several parameters were kept the same. These included the sample injection into the dialysis block (which can certainly be improved if there were insufficient analyte detectability), the dimensions of the CE capillary and the CE capillary rinsing procedure. The method development steps are briefly introduced below and the results found for the NSAIDs are presented and, if relevant, compared with those of the sulfonamides.

(i) In order to optimize the CE analysis, the first choice of buffer composition is a relatively high pH to ensure complete ionization of the analytes. If co-migration is observed, optimization is carried out using pH values closer to the pK_a values of the analytes involved. With the NSAIDs, complete resolution required pH adjustment to 4.6, a value very close to the pK_a of all test analytes; furthermore, 10% v/v of methanol had to be added to reduce the electroosmotic flow and to improve the separation. NSAIDs have absorption maxima in the 247–273 nm range (*cf.*, Table 1), but the absorption wavelengths of the maxima differ considerably. Therefore, to detect all analytes, 'end absorption', *i.e.*, detection at 200 nm, had to be used, a wavelength choice which, of course, adversely affects the selectivity of the detection.

(ii) The composition of the dialysis acceptor solution and the type of SPE cartridge largely determine the selection range of most of the other parameters. For example, the acceptor solution should not contain compounds which can interfere in the CE analysis (such as cationic ion-pairing reagents). To facilitate dialysis and increase analyte retention on hydrophobic SPE sorbents, it should also have a pH at which the analytes are neutral. As regards analyte trapping, in many instances the use of a small SPE cartridge (20 × 2.1 mm id) packed with a 5 μm sorbent is a good choice. To be on the safe side, the SPE cartridge should have a breakthrough volume for the analytes that is sufficient to retain them from a volume twice the volume of the acceptor phase generated during the dialysis step (typically 5–20 ml). For obvious reasons, an acceptor solution with low pH was selected for the NSAIDs. No breakthrough then occurred for any analyte after loading up to 50 ml of a pH 2 phosphate buffer when using C_{18}-bonded silica as SPE sorbent. This is in marked contrast with the sulfonamides, which required a hydrophobic styrene–divinylbenzene copolymer, PLRP-S, to achieve sufficient retention.

(iii) Interfacing of the SPE and CE systems involves three steps, desorption of the analytes from the SPE column, their transport to the interface and their injection into the CE capillary. A critical aspect in SPE–CE interfacing is the selection of an organic desorption buffer which, preferably, should have a low ionic strength to ensure a good stacking effect in the CE system. In order to desorb the analytes in a relatively small volume, at least 70% v/v of acetonitrile should be added to the desorption buffer. In addition, the pH should be increased to 7 to ensure that all analytes are fully deprotonated during the desorption step. Because of the relatively small volume of the SPE cartridge, the time between starting the elution from the SPE cartridge and the analytes passing the tip of the CE capillary, Δt, and the total time in which the analytes pass the tip of the CE capillary, t_{inj}, are critical. Since a decrease in t_{inj} requires a significantly higher pressure for a short period during

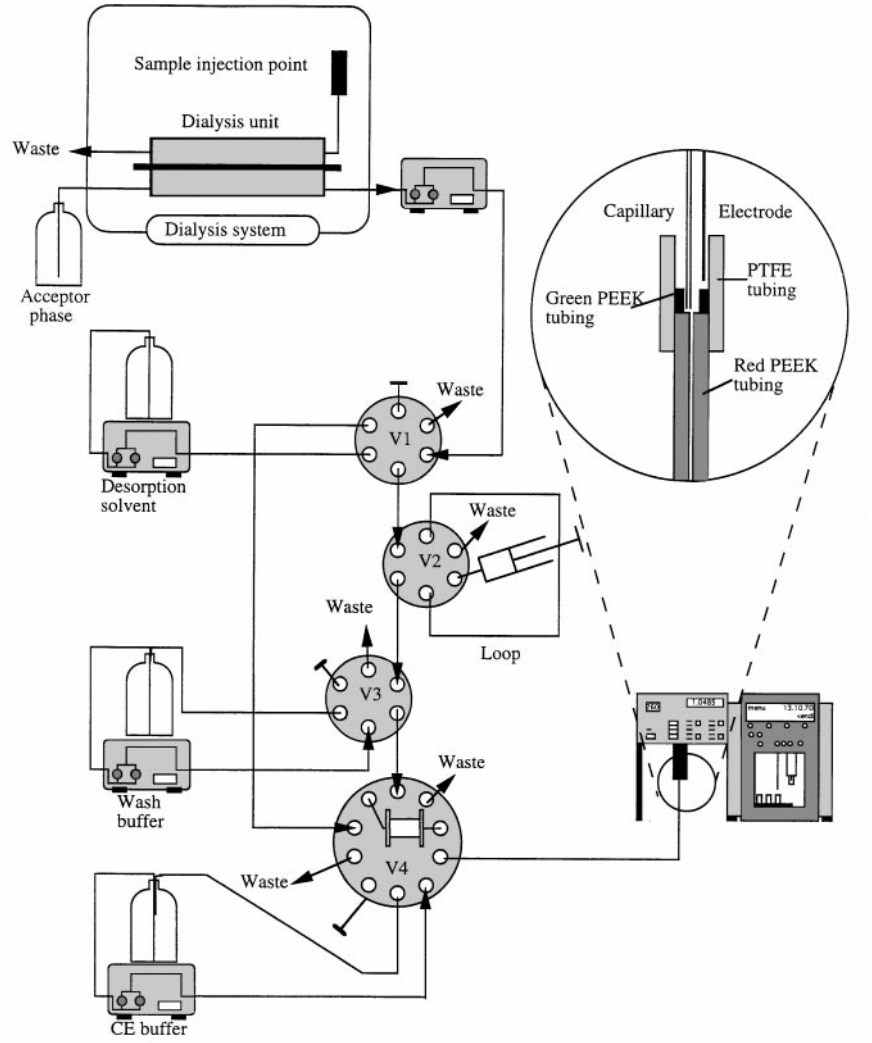

Fig. 1 Set-up of the on-line dialysis–SPE–CE–UV system.

injection, the repeatability of the procedure will be adversely affected. As a result, the maximum desorption flow rate is 0.2 ml min^{-1}.

(iv) To improve sensitivity, more sample can be injected. Because the time during which injection is performed has already been set (t_{inj}; see above), the only way to inject more analyte is to increase the pressure during injection. Optimization can be performed by injecting increasing amounts of sample and plotting the peak height *versus* the injection pressure. With our set-up, analyte detectability can be improved by injecting at pressures of up to 80 mbar without a dramatic decrease in efficiency due to band broadening. Increasing the pressure eightfold resulted in an eightfold increase in the peak heights, with a concomitant decrease in plate number of only 20%.

(v) Optimization of the dialysis step usually involves the selection of a suitable (often pulsed-type) dialysis mode and the sample volume for analysis. Regarding the mode, pulsed dialysis is often preferred since it improves the concentration detection limits. To facilitate the comparison of the protocols for NSAIDs and sulfonamides, the above dialysis parameters were kept the same as in previous work.[9] However, because of the good retention of the NSAIDs on the SPE cartridge, a higher acceptor solution flow rate was used to decrease the dialysis time, as the risk of early analyte breakthrough was negligible (see above).

(vi) The dialysis–SPE–CE–UV system is used for the analysis of urine. If too many interferences show up in the electropherogram, the SPE cartridge has to be subjected to a wash step prior to desorption of the analytes. The volume of the wash solution, its pH (often around the pK_a values of the analytes) and the fraction of organic modifier (more rapid removal of interferences *versus* increasing loss of analytes) are the main variables. Fig. 2(A) and (B) show that a number of interfering peaks occur near those of the analyte if no wash step is included. The beneficial effect of a 1.5 min clean-up procedure in the presence of 10% v/v of organic modifier and with a phosphate buffer of the expected pH value becomes obvious from Fig. 2(C) and (D).

(vii) The analysis of serum generally requires a modified strategy because of the more complex sample configuration and its higher viscosity (which tend to affect analyte detectability) and drug–protein bonding. If total drug concentrations in serum have to be determined, a significant amount of an organic solvent and/or a properly selected displacer have to be added to the sample to disrupt the drug–protein bonding. For the acidic drugs concerned, the addition of acetonitrile and decanoic acid (final concentrations 20% v/v and 8 mM, respectively) was found to give the best results. If such a step has to be included in the final protocol, it is recommended that the duration of the dialysis procedure is re-optimized. For the NSAIDs, the optimum was found to be 19 min. The increase in the dialysis time can probably be attributed to an unfavourable drug–protein equilibrium which is shifted during the dialysis procedure. With serum, a wash step was also necessary, even though the situation is less critical than with urine [Figs. 3(A) and (B)].

Table 2 Experimental conditions used for NSAIDs (this study) and sulfonamides[9]

Determination of	NSAIDs	Sulfonamides
Sample treatment—		
Urine	None	
Serum, free concentration	None	
Serum, total concentration	Addition of 20% ACN + 8 mM decanoic acid	
Dialysis—		
Dialysis block	Donor phase 100 µl, acceptor phase 170 µl	
Membrane	Cellulose acetate, MMCO[a] 15 kDa	
Sample injection	0.4 ml prior to starting dialysis and 0.6 ml at 0.18 ml min^{-1} after starting dialysis	
Acceptor solution	10 mM phosphate buffer (pH 2)	20 mM phosphate buffer (pH 3)
Acceptor solution flow rate/ml min^{-1}	1.0	0.5
Dialysis time for urine/min	10	9
Dialysis time for serum/min	19	9
SPE—		
Column	20 × 2.1 mm id, 5 µm C_{18}	50 × 2 mm id, 5 µm PLRP-S
Desorption buffer	27 mM phosphate buffer (pH 7)–ACN (30 + 70 v/v)	water–THF (25 + 75 v/v)
Desorption buffer flow rate/ml min^{-1}	0.2	0.4
Wash buffer	12.5 mM phosphate buffer (pH 4.5)–ACN (80 + 20 v/v)	10 mM phosphate buffer (pH 6)–ACN (90 + 10 v/v)
Wash buffer flow rate/ml min^{-1}	1.0	
Wash time/min	1.5	2.0
SPE–CE interfacing—		
Interface	Laboratory-made interface	
CE buffer flow rate/ml min^{-1}	0.2	
Δt[b]	18	20
t_{inj}[c]	18	15
CE—		
Capillary	Bare silica, 50 µm id, 375 µm od, total length 106 cm, effective length 40 cm	
Capillary rinsing	2000 mbar for 2 min	
Injection pressure	−80 mbar during t_{inj}	
CE buffer	50 mM acetate buffer (pH 4.6)–MeOH (90 + 10 v/v)	20 mM phosphate buffer (pH 7.0)
UV detection/nm	200	260
Analysis time/min	15	10

[a] Molecular mass cut-off. [b] Time between start of elution and analytes passing the tip of the CE capillary. [c] Total length of time in which the analytes pass the tip of the CE capillary.

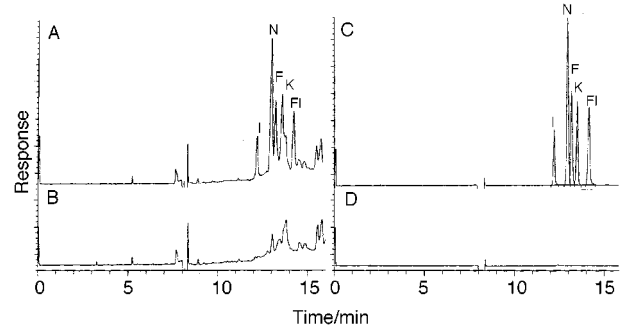

Fig. 2 On-line dialysis–SPE–CE of (B and D) blank and (A and C) spiked (10 µg ml^{-1} level of each NSAID) urine. (A and B) no washing of the SPE cartridge after loading or (C and D) washing with 1.5 ml of 12.5 mM phosphate buffer (pH 4.5)–acetonitrile (80 + 20 v/v).

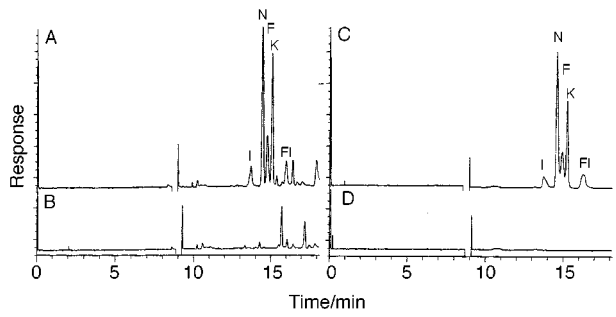

Fig. 3 On-line dialysis–SPE–CE of (B and D) blank and (A and C) spiked (10 µg ml^{-1} level of each NSAID) serum. (A and B) no washing of the SPE cartridge after loading or (C and D) washing with 1.5 ml of 12.5 mM phosphate buffer (pH 4.5)–acetonitrile (80 + 20, v/v).

Table 3 Calibration data for the determination of NSAIDs using dialysis–SPE–CE. Concentration range: from detection limit to 10 μg ml^{-1} for urine and from detection limit to 100 μg ml^{-1} for serum

Analyte	Detection limit/ μg ml^{-1}	Within-day precision (%)[a]	Between-day precision (%)[a]	Intercept[b]	Slope[b]	Correlation coefficient (r^2)
Urine (n = 7–8)—						
Ibuprofen	0.10	5.9	6.5	−0.03 (0.01)	0.46 (0.002)	0.9997
Naproxen	0.05	1.5	4.0	0.06 (0.05)	1.47 (0.01)	0.9992
Fenoprofen	0.05	2.5	3.6	0.01 (0.02)	0.84 (0.004)	0.9997
Ketoprofen	0.10	2.2	5.1	0.00 (0.05)	0.69 (0.01)	0.9973
	0.10	8.0	4.7	−0.01 (0.02)	0.73 (0.004)	0.9997
Serum (n = 7–10)—						
Ibuprofen	1.00	3.5	6.3	−0.08 (0.04)	0.10 (0.001)	0.9997
Naproxen	0.10	3.0	5.6	−0.04 (0.11)	0.52 (0.003)	0.9997
Fenoprofen	0.50	2.8	8.5	−0.13 (0.10)	0.20 (0.002)	0.9991
Ketoprofen	0.20	4.6	7.7	0.03 (0.11)	0.29 (0.003)	0.9992
Flurbiprofen	1.00	6.1	8.8	−0.10 (0.08)	0.12 (0.002)	0.9988

[a] Determined using a concentration of five times the LOD. [b] Standard deviation in parentheses.

When the same washing procedure as for urine was applied, satisfactory results were obtained [Fig. 3(C) and (D)].

Finally, the optimized procedure for the determination of the NSAIDs was validated with serum and urine. The detection limits were 0.05–0.1 μg ml^{-1} for urine and 0.1–1.0 μg ml^{-1} for serum (Table 3). As indicated above, the higher detection limits in serum can be attributed to small losses caused by drug–protein binding and to a higher background. Calibration curves were constructed from the detection limits up to 10 μg ml^{-1} (urine) or 100 μg ml^{-1} (serum). They were linear (Table 3) and covered the therapeutic levels in serum (0.5–70 μg ml^{-1}) listed in Table 1. The within-day and between-day precisions (RSDs) were $<8\%$ for urine and $<9\%$ for serum (Table 3). This is significantly better than the value of 15% which is an often used acceptance level for quantification results for biological samples.[10]

Obviously, the present protocol can be used to optimize dialysis–SPE–CE procedures for the low-μg ml^{-1} determination of different classes of drugs. Since the set-up is fully automated, analyses can be, and indeed were, run unattended. The CE capillary and SPE cartridge were used without any problems for over 500 analyses. The dialysis membrane was replaced every 250 analyses.

Conclusions

This study has shown that the optimization procedure used earlier for sulfonamides can be applied also for other classes of drugs; there are, of course, differences in the numerical values for several parameters, but no major changes occur. However, as we shall show in a subsequent paper, complicating effects do occur if unusual CE conditions are encountered. This was observed when cationic drugs were studied and a non-standard CE buffer had to be used.[11]

On-line dialysis–SPE–CE of NSAIDs with UV detection was performed in a fully automated way, being controlled by the SPE unit and dialysis module. The procedure is robust, and 250–500 analyses can be performed without any need for maintenance or exchange of parts. The analytical characteristics are satisfactory for both urine and serum and allow the use of dialysis–SPE–CE–UV for metabolic studies and clinical analyses.[10]

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