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Separation of fifteen non-steroidal anti-inflammatory drugs using micellar electrokinetic capillary chromatography

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Abstract

Micellar electrokinetic capillary chromatography with sodium dodecylsulfate as anionic surfactant was used to separate simultaneously fifteen non-steroidal anti-inflammatory drugs. UV detection was performed at 254 nm. The electroosmotic flow was carefully adjusted for optimal separation. Resolution of the drugs was obtained using a buffer containing 40 mmol l^{-1} NaH_2PO_4, 0.104 mol l^{-1} sodium dodecylsulfate and 3% (v/v) methanol, adjusted to pH 8 with sodium hydroxide. Lack of interference was checked with a number of drugs and metabolites. Between-day coefficients of variation ranged between 2 to 10%.

1. Introduction

Assays of non-steroidal anti-inflammatory drugs (NSAIDs) in body fluids are needed for pharmacokinetic and toxicologic studies. Identification and quantification of NSAIDs in blood may be particularly useful in patients with upper gastrointestinal haemorrhage [1], which is the major adverse effect caused by NSAIDs. Recently, the separation of a number of NSAIDs by micellar electrokinetic capillary chromatography (MECC) was described [2]. However, the sensitivity and reproducibility of the method were not reported. In the present study we have searched for the optimal buffer by varying the NaH_2PO_4 and sodium dodecylsulfate (SDS) concentrations, temperature, pH and methanol content to separate fifteen NSAIDs and the performance of the method was assessed.

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2. Experimental

2.1. Instrumentation

MECC was carried out with a P/ACE System 2000 (Beckman Instruments, Palo Alto, CA, USA), equipped with an on-column UV detector, set at 254 nm, and a temperature regulation system. Separation was performed with a fused-silica capillary (58.7 cm × 75 μm I.D., 50 cm from capillary tip to optical window) suspended between two buffers reservoirs. An adjustable high-voltage power supply was used to apply 0–30 kV across platinum electrodes placed into the buffer reservoirs.

The capillary was washed every morning with 0.1 *M* NaOH for 10 min. The system was programmed for the following successive operations: a 2-min wash-out with assay buffer, a 2-s hydrodynamic injection of the sample, and a return to the assay buffer before application of

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the high voltage. Under the conditions used, the direction of the electrophoretic migration of anions was opposite to the electroosmotic flow [3].

2.2. Reagents

All chemical used were of analytical grade. The assay buffer contained $NaH_2PO_4 \cdot H_2O$ (Merck, Darmstadt, Germany), SDS (Sigma, St. Louis, MO, USA) and methanol (Carlo Erba, Milano, Italy). Methanol was used as a neutral marker of electroosmotic flow which was expressed as μ_{eo} [3]. NaOH was used to adjust the pH of the buffer between 7 and 11. The buffer solution was filtered through a 0.45-μm membrane filter before use. Sudan III was used to calculate the capacity factor k' [3,4] and the resolution [4].

The NSAIDs were gracious gifts from the following laboratories: 1, diclofenac (Ciba-Geigy, Huninge, Switzerland); 2, diflunisal (Merck, Sharp and Dohme Research Lab., Rahway, NJ, USA); 3, etodolac (Wyeth France, Paris, France); 4, fenbufen (Lederle, Oulins, France); 5, fenoprofen (Eli Lilly, Basingstoke, UK); 6, flurbiprofen (Boots Pharma, Courbevoie, France); 7, ibuprofen (Boots Pharma); 8, indomethacin (purchased from Sigma); 9, ketoprofen (Rhône-Poulenc Santé Propharm, Paris, France); 10, naproxen (Cassenne, Osny, France); 11, niflumic acid (UPSA, Agen, France); 12, piroxicam (purchased from Sigma); 13, sulindac (Merck, Sharp and Dohme Research Lab.); 14, tenoxicam (Roche, Neuilly-sur-Seine, France); and 15, tiaprofenic acid (Roussel, Paris, France). Benzoyl-4-phenyl-2-butyric acid (BPBA), used as internal standard, was a gift from Rhône-Poulenc Research Center (Alfortville, France).

2.3. Solutions

Standard solutions of all NSAIDs except ibuprofen, piroxicam and tenoxicam were prepared in methanol at a concentration of 1 mg ml^{-1}. A working solution was prepared by mixing 100 μl of the 15 standard solutions, which resulted in a concentration of 67 μg ml^{-1}. For ibuprofen the initial concentration was 10 mg ml^{-1}, because of its low absorption at 254 nm. Piroxicam and tenoxicam (1 mg ml^{-1}) were dissolved in benzene and in 0.01 *M* NaOH solution respectively. All solutions were stored in the dark at + 4°C. A working solution prepared by mixing equal volumes of each standard solution was used for the assays.

2.4. Sample treatment

The method used for sample treatment was derived from that described by Lapicque *et al.* [5]. Aliquots of working solution were placed in a tube, the methanol was evaporated under a stream of nitrogen and the residue was incubated with 100–1000 μl of blank plasma. The spiked samples were transferred to another tube and 2 μg of internal standard (BPBA) and 0.4 ml of 1 *M* HCl were added. The drugs and BPBA were extracted by mechanical agitation for 10 min after addition of 10 ml of diethyl ether. The organic phase was separated by centrifugation (3500 *g* for 10 min), transferred to another tube and evaporated under a stream of nitrogen. The dry residue was dissolved in a mixture of a NaH_2PO_4 solution (concentration equal to that of the working buffer) and methanol (1:1).

The influence of pH (range 7–11), NaH_2PO_4 concentration (25–50 mmol l^{-1}), methanol volume (0–10%), SDS concentration (0.069–0.174 mol l^{-1}) and temperature (19–24°C) were investigated by varying them one by one, the others maintained constant.

3. Results and discussion

3.1. Influence of pH

NSAIDs are lipophilic acid compounds. The use of an appropriate pH allows the separation of NSAIDs depending on the differences between their partition coefficients. Fig. 1 shows that the retention times of all compounds increased with increasing pH. This is probably caused by a decrease in the micellar solubilisa-

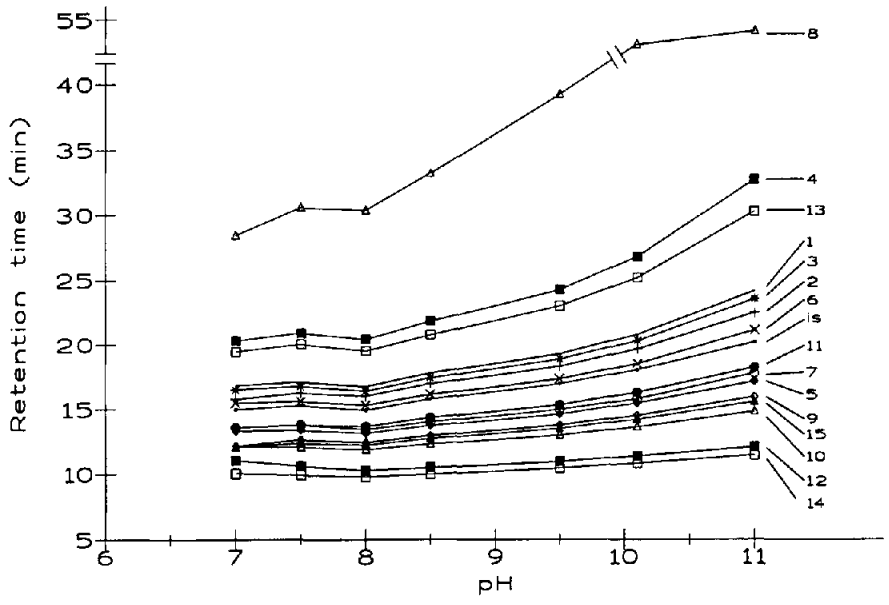

Fig. 1. Retention times of NSAIDs as a function of pH. Conditions: 40 mmol l^{-1} NaH_2PO_4; 0.104 mol l^{-1} SDS; 3% methanol (v/v); 20 kV; 22°C; 254 nm. Code numbers are as in the Reagents section.

tion of the NSAIDs with the increase of their ionisation: the negatively charged solute is subject to electrostatic repulsion by the negatively charged SDS micelles [3].

3.2. Influence of NaH_2PO_4 concentration

Resolution of the 15 NSAIDs *versus* NaH_2PO_4 concentration is shown in Fig. 2a. The resolution of the 15 peaks was much better at 50 mmol l^{-1} NaH_2PO_4 than at 25 mmol l^{-1}. The electroosmotic flow (μ_{eo}) decreased as the buffer concentration increased (Fig. 2b). Since the presence of surfactant has only little effect on the electroosmotic flow because of the electrostatic repulsion [3], the observed improvement in resolution with increasing NaH_2PO_4 concentration may be due to the reduction of the electroosmotic flow. On the other hand, the decrease in electroosmotic flow with increasing buffer concentration is linked to a decrease in counter-ion layer thickness [6]. In addition, there is an increase in the coverage of negative sites on the silica surface which reduces the charge per unit area at the interface between the capillary wall and the buffer [6].

3.3. Influence of methanol

In the absence of methanol, diclofenac and etodolac (1 and 3) on the one hand and fenoprofen and ibuprofen (5 and 7) on the other hand were not completely separated (Fig. 3a). With 3% methanol, all peaks were baseline resolved. The resolution was further improved using 4% of methanol (Fig. 3b), but the retention times were increased. A decrease in electroosmotic flow may explain the improvement in resolution (Fig. 3c). This was reported by Bushey and Jorgensen [7] and attributed to changes in the zeta potential, as well as changes in viscosity and dielectric constant of the buffer.

3.4. Influence of SDS concentration

The influence of SDS concentration on the retention times is shown in Fig. 4a. Changing the SDS concentration from 0.069 to 0.174 mol l^{-1} increased the retention times of the NSAIDs and improved resolution. Unlike the effect of NaH_2PO_4 or methanol, there were no changes in the order in which the compounds eluted. Indomethacin had the longest retention time, prob-

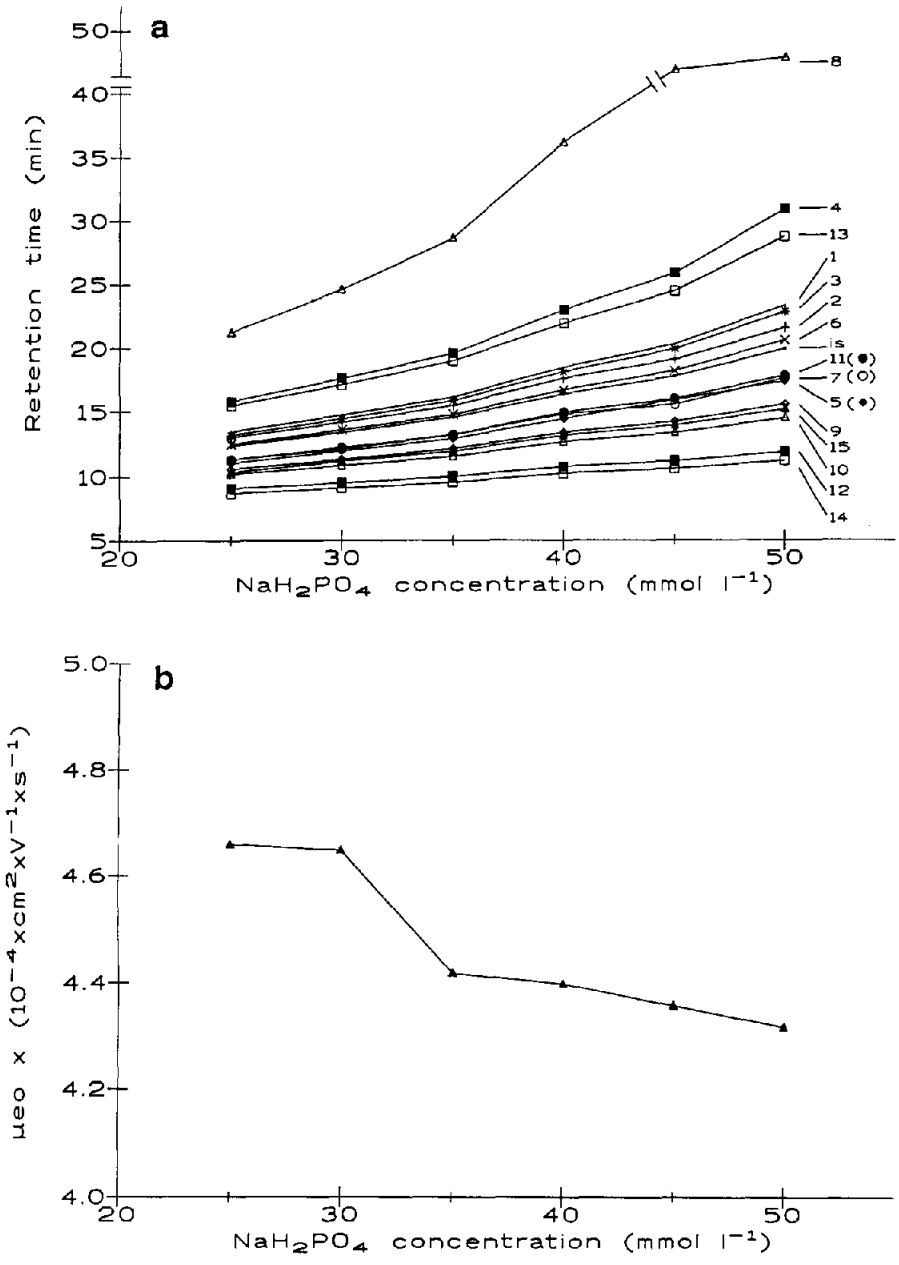

Fig. 2. Effect of NaH_2PO_4 concentration on (a) retention times and (b) electroosmotic mobility (μ_{eo}). Conditions: pH 8; other experimental conditions are the same as in Fig. 1.

ably because of its higher hydrophobicity which led to higher micellar solubility. Since all NSAIDs are hydrophobic compounds, their capacity factor increases with SDS concentration (Fig. 4b). According to Otsuka *et al.* [8], this increase in capacity factor should decrease the velocity of non-ionised solutes. Therefore, the improved resolution of the NSAIDs with increasing SDS concentration may be due to their hydrophobicity.

3.5. Influence of temperature

When the temperature was increased, the retention times of all compounds decreased at the expense of the resolution of the NSAIDs (Fig. 5). One factor explaining the decrease in retention times may be the reduction in buffer viscosity.

3.6. Optimal conditions

In consequence of the assays mentioned

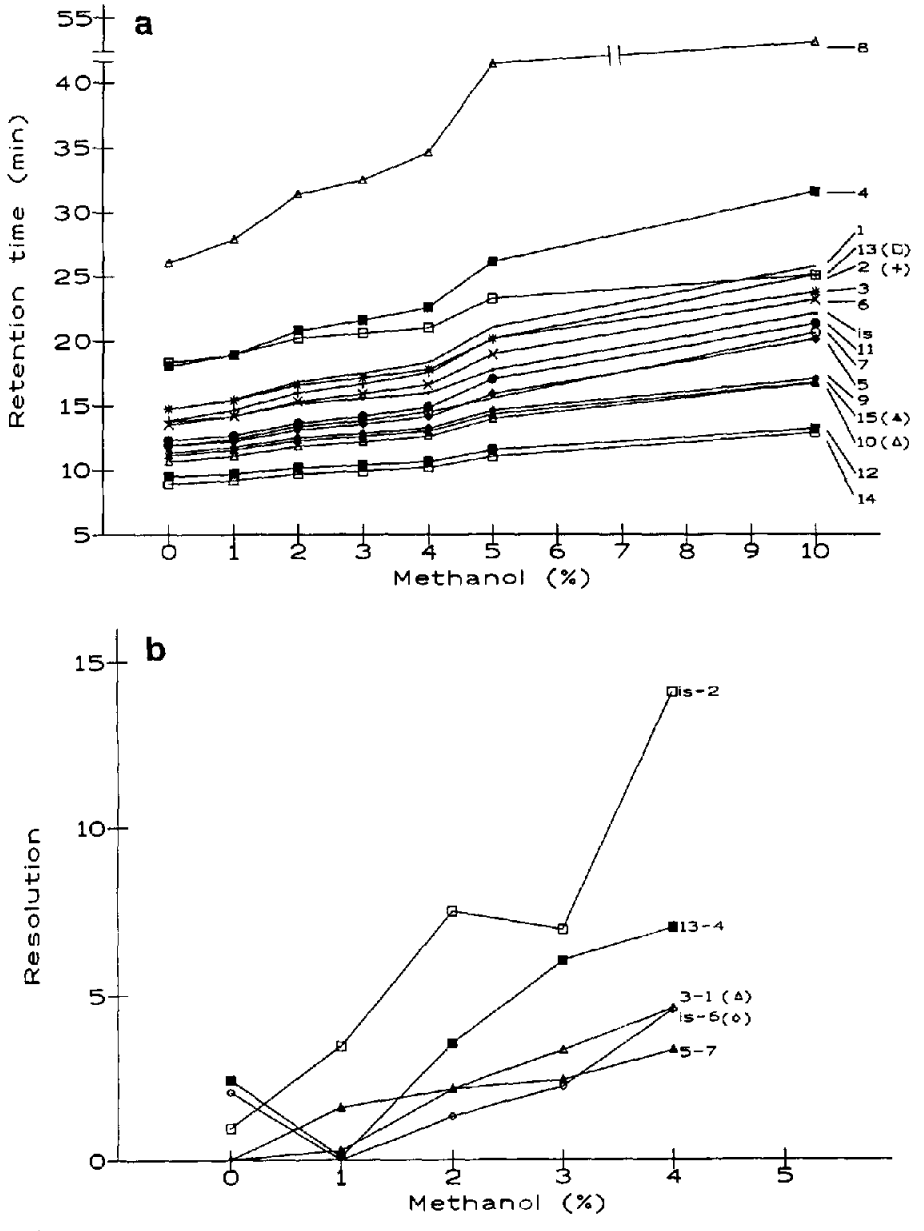

Fig. 3. Effect of methanol concentration on (a) retention times, (b) resolution, and (c) electroosmotic mobility (μ_{eo}). Conditions: pH 8; other experimental conditions are the same as in Fig. 1. (Fig. 3 continued on next page).

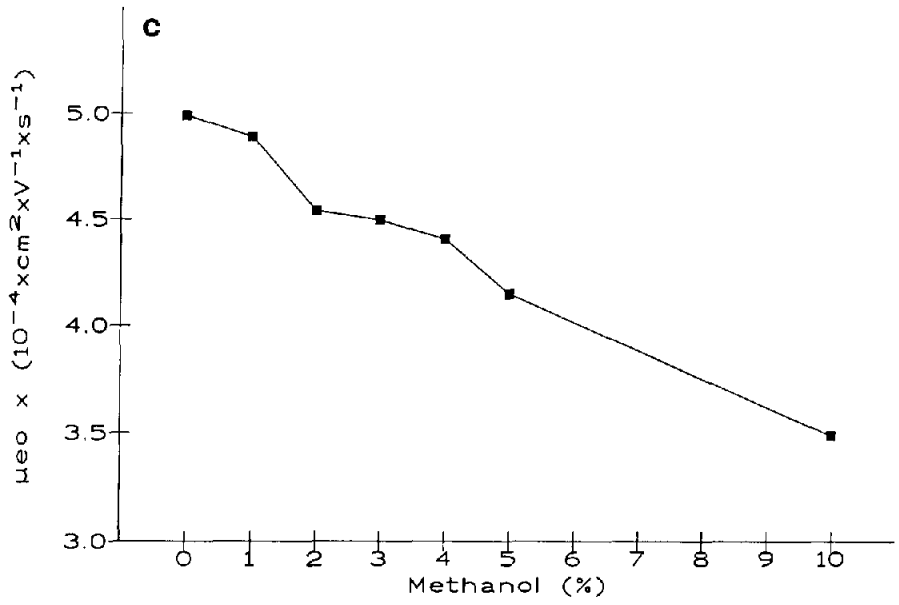

Fig. 3. (*Continued*).

above, we chose to use the following conditions for electrophoresis: buffer, 40 mmol l^{-1} NaH_2PO_4; SDS, 0.104 mol l^{-1}; methanol, 3%; pH 8. Separation of the NSAIDs is shown in Fig. 6. Fig. 7 shows the chromatogram obtained from a plasma sample of a subject who was given naproxen. The concentration of drug was 17.65 $\mu g\ ml^{-1}$. These optimal conditions were used during the validation of the method.

3.7. Detection limits

The detection limits (signal-to-noise ratio of 5) were 0.13 $\mu g\ ml^{-1}$ for flurbiprofen, ketoprofen, piroxicam, tenoxicam and tiaprofenic acid, 0.33 $\mu g\ ml^{-1}$ for fenbufen, 0.33 $\mu g\ ml^{-1}$ for diflunisal and etodolac, 0.67 $\mu g\ ml^{-1}$ for diclofenac, indomethacin, naproxen, niflumic acid and sulindac, 1 $\mu g\ ml^{-1}$ for fenoprofen and 10 $\mu g\ ml^{-1}$ for ibuprofen. Differences in the absorption spectra of the NSAIDs may explain the differences in the detection limits. With HPLC, the detection limits of NSAIDs were *ca.* 0.01–0.05 $\mu g\ ml^{-1}$ [5]. A lower performance of capillary electrophoresis compared with HPLC has also been reported for tricyclic antidepressants [6]. These higher detection limits may result from differences in the optical path and in loading capacity between the two methods.

3.8. Interferences

Interference with the following drugs were tested: amitryptiline, caffeine, clomipramine, desipramine, diazepam, imipramine, maprotiline, nortriptiline, paracetamol, phenobarbital, phenytoin, sulfametoxazole, theophylline and trimipramine. In addition, 5′-hydroxytenoxicam and deoxysulindac, the main metabolites of tenoxicam and sulindac, were also tested. None of the drugs or metabolites interfered with NSAIDs separation or quantification.

3.9. Linearity

For the chosen concentration range, which includes the concentrations observed under therapeutic conditions [5], regression analysis of the peak-area ratios between the drugs and the internal standard showed that the correlation coefficient for 14 out of the 15 drugs was above 0.99 (Table 1).

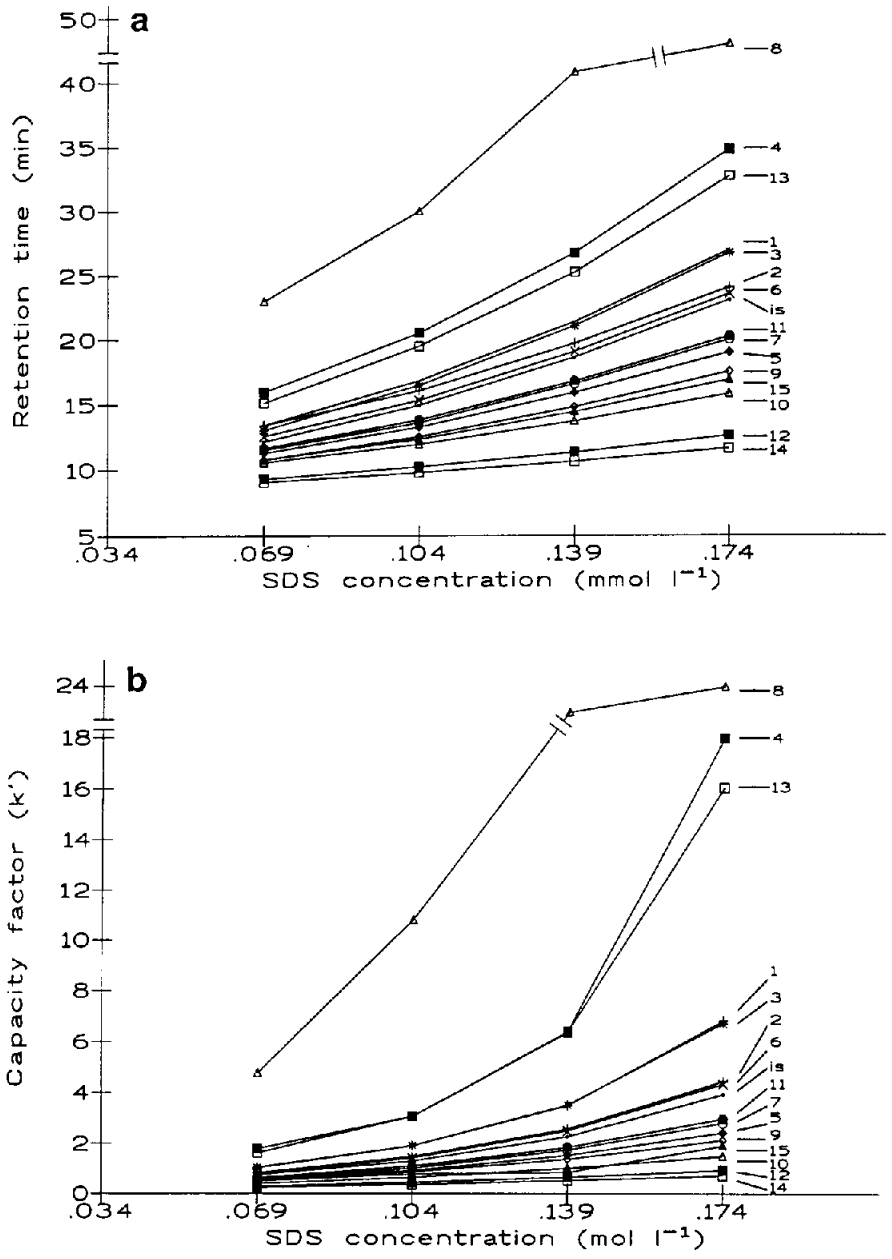

Fig. 4. Effect of SDS concentration on (a) retention times and (b) capacity factor (k'). Conditions: pH 8; other experimental conditions are the same as in Fig. 1.

3.10. Accuracy and reproducibility

The accuracy of the assay is displayed in Table 2. The reproducibility was determined at a concentration of 6.7 μg ml^{-1} for all drugs, except for ibuprofen which was tested at 66.7 μg ml^{-1} (Table 2). The between-day coefficient of variation ranged from 2 to 10%. The retention times were stable since the coefficients of variation were found to be less than 3.4% for within-

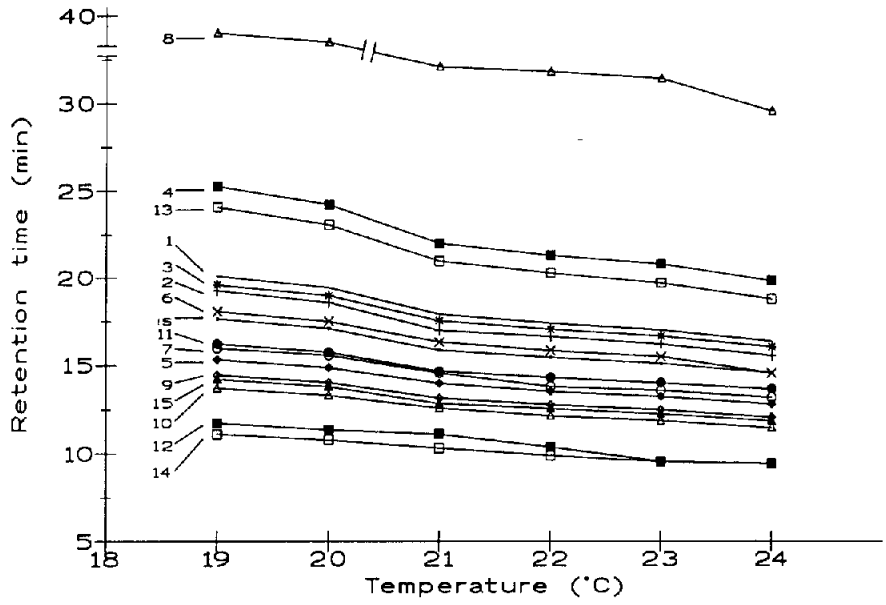

Fig. 5. Effect of temperature. Conditions: pH 8; other experimental conditions are the same as in Fig. 1.

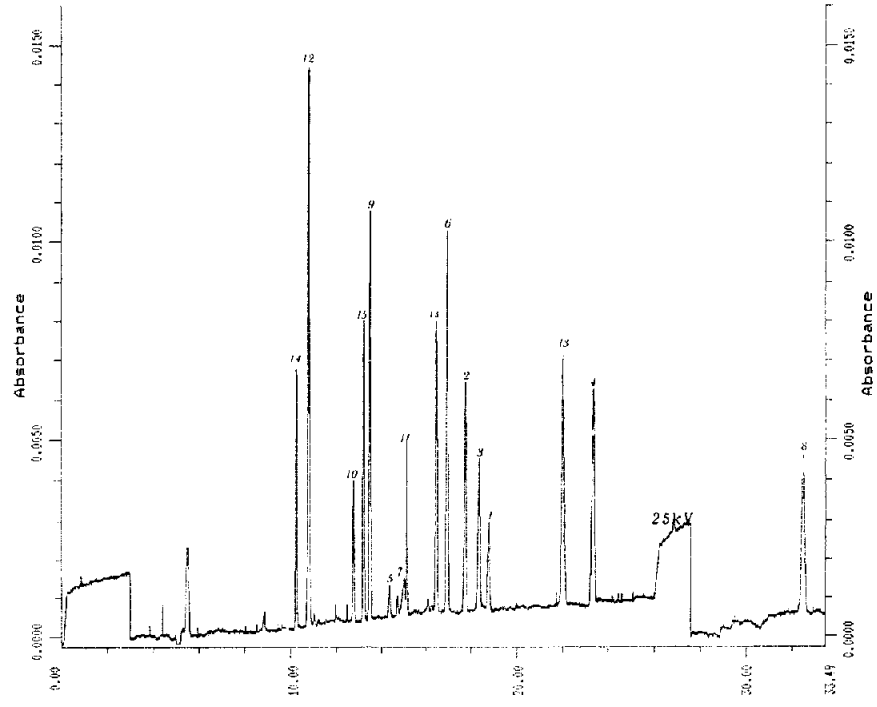

Fig. 6. Separation of 15 NSAIDs. Conditions: 40 mmol l^{-1} NaH_2PO_4, pH 8, 0.104 mol l^{-1} SDS; 3% methanol (v/v); 20 kV, 22°C. Code numbers are: 1 = diclofenac; 2 = diflunisal; 3 = etodolac; 4 = fenbufen; 5 = fenoprofen; 6 = flurbiprofen; 7 = ibuprofen; 8 = indomethacin; 9 = ketoprofen; 10 = naproxen; 11 = niflumic acid, 12 = piroxicam, 13 = sulindac; 14 = tenoxicam; 15 = tiaprofenic acid.

Table 1
Calibration linear regression for NSAIDs[a]

NSAID	*a*	*b*	*r*
Concentration: 0.133–6.700 μg ml^{-1} ($n = 6$)			
Diclofenac	0.0558	0.0378	0.9816
Diflunisal	0.1780	−0.0025	0.9970
Etodolac	0.0588	0.0026	0.9987
Fenbufen	0.2517	0.0349	0.9997
Fenoprofen	0.0189	0.0360	0.9986
Flurbiprofen	0.2708	−0.0083	0.9974
Ibuprofen[b]	0.1508	−0.0180	0.9989
Indomethacin	0.3170	−0.0502	0.9990
Ketoprofen	0.1678	0.0387	0.9992
Naproxen	0.9197	0.0153	0.9998
Niflumic acid	0.2226	0.0258	0.9997
Piroxicam	0.1153	0.0639	0.9995
Sulindac	0.3014	0.0403	0.9991
Tenoxicam	0.0962	0.1709	0.9993
Tiaprofenic acid	0.1611	−0.0253	0.9997
Concentration: 6.70–33.35 μg ml^{-1} ($n = 8$)			
Diclofenac	0.0708	−0.2660	0.9804
Diflunisal	0.1577	−0.5860	0.9938
Etodolac	0.0548	−0.1069	0.9984
Fenbufen	0.1313	−0.1497	0.9991
Fenoprofen	0.0124	−0.0271	0.9969
Flurbiprofen	0.2045	−0.2545	0.9971
Ibuprofen[c]	0.0394	0.0011	0.9981
Indomethacin	0.2558	−0.6032	0.9976
Ketoprofen	0.1501	−0.1357	0.9992
Naproxen	0.0499	−0.0760	0.9985
Niflumic acid	0.0449	−0.0284	0.9983
Piroxicam	0.0771	−0.0390	0.9995
Sulindac	0.1565	−0.1985	0.9988
Tenoxicam	0.0628	−0.0772	0.9996
Tiaprofenic acid	0.0669	−0.0838	0.9989

[a] $y = ax + b$, where y is the peak-area ratio between drug and internal standard, x is the drug concentration, and r is the correlation coefficient.
[b] 1.33–66.70 μg ml^{-1} for ibuprofen.
[c] 66.7–333.5 μg ml^{-1} for ibuprofen.

day and 4.2% for between-day assays (Table 3). The run time was 35 min, which is considerably shorter than that reported for HPLC [5].

4. Conclusion

We have shown that it is possible to separate a mixture of 15 NSAIDs and demonstrated the high resolution power of MECC. Because of the ease of operation and the good reproducibility, the method may be useful in toxicological and presumably in pharmacokinetic studies. In the latter case, the higher observed detection limits may be a drawback compared to results reported with HPLC. Better detection limits might be obtained by improving the sensitivity of the detectors for narrow-bore capillary tubes.

Table 2
Accuracy and between-day reproducibility of peak-area ratio between drug and internal standard

NSAID	Accuracy[a]			Reproducibility[b]		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
Diclofenac	0.469	0.021	4.47	1.098	0.098	8.60
Diflunisal	0.962	0.022	2.29	2.228	0.053	2.40
Etodolac	0.451	0.011	2.41	1.077	0.022	2.00
Fenbufen	1.098	0.034	3.12	2.742	0.081	2.95
Fenoprofen	0.271	0.005	1.85	0.635	0.015	2.40
Flurbiprofen	1.605	0.047	2.91	3.767	0.139	3.70
Ibuprofen	0.516	0.034	6.64	1.113	0.112	10.10
Indomethacin	1.904	0.065	3.44	4.256	0.190	4.50
Ketoprofen	1.415	0.042	2.97	3.268	0.120	3.70
Naproxen	0.443	0.017	3.94	1.056	0.048	4.55
Niflumic acid	0.448	0.020	4.57	1.026	0.073	7.14
Piroxicam	0.694	0.022	3.19	1.667	0.057	3.40
Sulindac	1.454	0.052	3.59	3.263	0.108	3.30
Tenoxicam	0.595	0.024	4.09	1.403	0.061	4.33
Tiaprofenic acid	0.719	0.020	2.85	1.617	0.051	3.15

[a] C.V. = coefficient of variation of five measurements; S.D. = standard deviation. Concentration: 3 μg ml^{-1}, except for ibuprofen: 30 μg ml^{-1}.
[b] C.V. = coefficient of variation for nine measurements. Concentration: 6.7 μg ml^{-1}, except for ibuprofen: 67 μg ml^{-1}.

Table 3
Reproducibility of retention times

NSAID	Within-day			Between-day		
	Mean (min)	S.D.[a] (min)	C.V.[b] (%)	Mean (min)	S.D. (min)	C.V. (%)
Tenoxicam	10.2	0.2	1.8	10.1	0.2	2.0
Piroxicam	10.7	0.2	2.0	10.7	0.2	2.2
Naproxen	12.7	0.3	2.1	12.6	0.3	2.3
Tiaprofenic ac.	13.1	0.3	2.2	13.0	0.3	2.4
Ketoprofen	13.4	0.3	2.2	13.3	0.3	2.4
Fenoprofen	14.2	0.3	2.3	14.0	0.4	2.5
Ibuprofen	14.5	0.4	2.5	14.5	0.4	2.9
Niflumic ac.	14.9	0.4	2.4	15.1	0.5	3.1
IS[c]	16.3	0.4	2.6	16.2	0.4	2.6
Flurbiprofen	16.7	0.4	2.5	16.6	0.4	2.7
Diflunisal	17.6	0.5	2.7	17.4	0.5	2.7
Etodolac	18.1	0.5	2.7	17.9	0.5	3.0
Diclofenac	18.5	0.5	2.7	18.3	0.6	3.2
Sulindac	21.6	0.8	3.5	21.6	0.8	3.7
Fenbufen	22.9	0.7	3.1	22.7	1.0	4.2
Indomethacin	30.5	0.3	1.1	31.3	0.8	2.7

[a] S.D. = standard deviation.
[b] C.V. = coefficient of variation for 5 and 6 measurements on the same day and on subsequent days respectively.
[c] IS = internal standard.

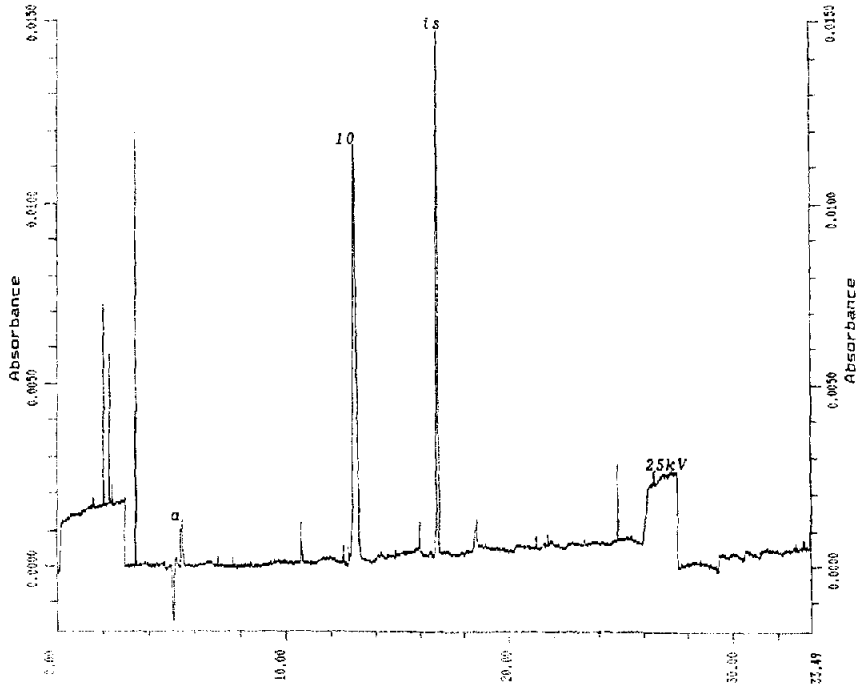

Fig. 7. Electropherogram from a subject given a 550-mg dose of naproxen (10). The sample was drawn 30 min after dosing. a: methanol; IS: internal standard. Conditions as for Fig. 6. Naproxen concentration was 17.65 $\mu g\ ml^{-1}$.

5. Acknowledgement

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