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Separation of platinum(II) anti-tumour drugs by micellar electrokinetic capillary chromatography

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Abstract

Micellar electrokinetic capillary chromatography was used for the separation of platinum anti-tumour drugs in aqueous solutions. With the use of sodium dodecyl sulfate (SDS) the neutral drugs cisplatin, carboplatin and lobaplatin and charged hydrolysis products could be separated very easily in only one run. The pH of buffer solution, SDS concentration, applied voltage and injection conditions were optimized. The limit of detection and linearity for the determination of each complex were determined. The developed method was used for stability measurements of the complexes in solution.

Keywords: Micellar electrokinetic chromatography; Cisplatin; Carboplatin; Lobaplatin; Platinum anti-tumour drugs

1. Introduction

In the last two decades, many platinum complexes have been evaluated in an attempt to improve the anti-tumour activity and to reduce the severe side-effects of cisplatin such as neurotoxicity and nephrotoxicity [1]. Besides cisplatin [*cis*-diamminedichloroplatinum(II); CDDP] the second-generation drug carboplatin (*cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II); CBDCA] is used in cancer chemotherapy [2]. As a third-generation drug lobaplatin (1, 2-diaminomethylcyclobutaneplatinum(II)lactate; D19466) has been tested in clinical trials in the hope of obtaining less toxicity and increased activity [3].

For pharmaceutical and biomedical analysis, a large number of analytical techniques have been used in the study of platinum anti-tumour agents in aqueous solutions and biological media. For the analysis of platinum-based anti-cancer drugs it is essential to distinguish between the various forms in which platinum may be present in the sample.

The intact platinum drugs and the related complexes, e.g., degradation and biotransformation products, have been separated chromatographically in the reversed-phase and normal-phase modes, with ion exchangers or solvent-generated ion exchangers and by gel permeation [4].

New platinum drugs are synthesized in order to increase the water solubility and drug stability. The stability of cisplatin is well documented, but stability data for the second- and third-generation anti-tumour drugs are rare [2]. In aqueous

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solutions mainly positively charged species exist in addition to the neutral complexes. For the separation and determination of these species, HPLC with isocratic elution is most often used, but to obtain better separations and shorter analysis times gradient techniques are helpful. Here gradients of organic modifier concentration [5,6], ionic strength [7] or both are used [8]. However, often the problem still remains of separating neutral in addition to cationic and anionic platinum species.

Capillary electrophoresis (CE) could be a promising method for solving this problem because of its well known advantages. In capillary zone electrophoresis (CZE), charged species are separated according to their electrophoretic mobility in an electric field. In micellar electrokinetic capillary chromatography (MECC), another separation effect occurs, the distribution of the analyte between the pseudo-stationary micellar phase and the aqueous buffer phase. In MECC neutral analytes could also be separated in addition to charged species. Because of this advantage, many MECC techniques have been investigated for a number of applications within the area of pharmaceutical analysis [9,10].

Initial results using the high performance of CE for the analysis of different platinum drugs and hydrolysis and reaction products are presented. Fig. 1 depicts the structures of the studied platinum(II) complexes and the main hydrolysis species for cisplatin.

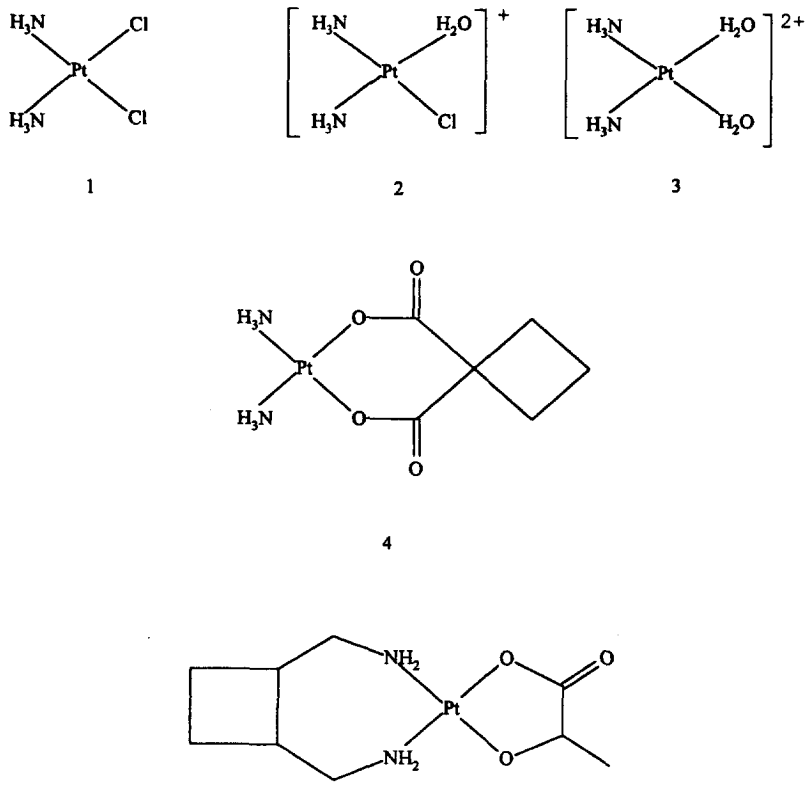

Fig. 1. Structural formulae of the anti-tumour drugs *cis*-diamminedichloroplatinum(II) (**1**), the hydrolysis products *cis*-diammineaquachloroplatinum (**2**) and *cis*-diamminediaquaplatinum (**3**), diammine (1,1-cyclobutane-1,1-dicarboxylato)platinum(II) (carboplatin) (**4**) and 1,2-diamminomethylcyclobutaneplatinum(II)lactate (lobaplatin) (**5**).

2. Experimental

2.1. Materials

Sodium dodecyl sulfate (SDS), hexydecyltrimethylammonium bromide (HTAB), sodium dihydrogenphosphate and ammonium acetate were of analytical-reagent grade (all obtained from Merck, Darmstadt, Germany). Adenosine was purchased from Fluka (Buchs, Switzerland) and cisplatin and carboplatin from Heraeus (Karlsruhe, Germany). Lobaplatin was donated by Asta Medica (Frankfurt, Germany).

All solutions and reaction mixtures were prepared in deionized, doubly distilled water.

2.2. Apparatus and chromatographic conditions

The CE experiments were carried out using laboratory-made equipment [11], consisting of a high-voltage power supply (HCN 35-35000 fug; FuG Electronik, Rosenheim, Germany), a UV–Vis detector (V4 absorbance detector; ISCO/Colora, Lincoln, NE, USA) and an integrator (SP 4270; Spectra-Physics, Darmstadt, Germany). Electrodes used were 60 mm × 0.8 mm I.D. platinum–iridium (90:10) and kept together with the buffer reservoirs in a Plexiglas box to avoid contact with electrical current. MECC was performed in 0.75 m × 75 μm I.D. (365 μm O.D.) fused-silica capillaries (CS Chromatographie Service, Langerwehe, Germany) with an effective length of 0.50 m to the detector. The sample was introduced by hydrostatic pressure for the desired time.

Buffer solutions were SDS in 25 m*M* $Na_2B_4O_7$–50 m*M* NaH_2PO_4 or 50 m*M* NaH_2PO_4 adjusted to pH 4.5, 7 or 9. Before each run, the capillary was purged with buffer for 2 min. After a few runs the reproducibility of retention was better than 1% relative standard deviation from run to run.

3. Results and discussion

To separate neutral platinum drugs it is necessary to use the micellar technique. With a cationic surfactant (HTAB) no separation was possible, but with the anionic surfactant (SDS) neutral and charged complexes could be separated. Fig. 2 shows the electropherogram of a platinum complex standard.

The effect of SDS concentration on migration behaviour was studied and the capacity factors of the analytes were calculated using the equation

$$k' = \frac{t_i - t_0}{t_0[1 - (t_i/t_{mc})]} \quad (1)$$

where t_i = migration time of a solute i, t_0 = elution time of a neutral substance with no interaction with the micelle [electroosmotic flow (EOF) marker, e.g., methanol] and t_{mc} = migration time of the micelle, measured with Sudan III.

Fig. 3 shows that the capacity factors of cisplatin and carboplatin are very low, also at high SDS concentrations. This behaviour corresponds to the polar uncharged structure of the complex-

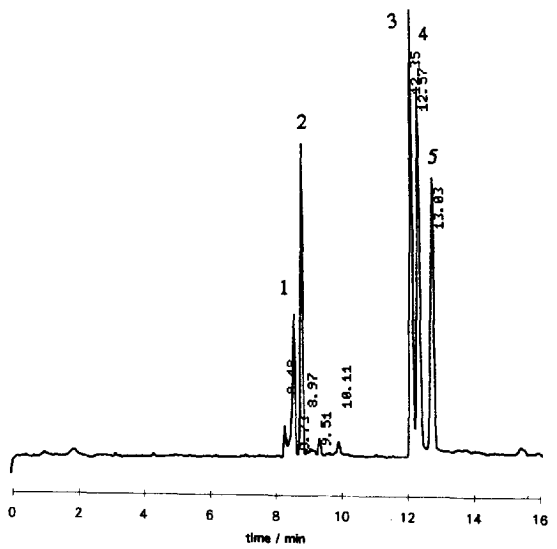

Fig. 2. Separation of (1) cisplatin, (2) carboplatin, the (3) *SSS* and (4) *RRS* diastereomers of lobaplatin and a lobaplatin precursor complex (5). Buffer, 50 m*M* SDS in 25 m*M* $Na_2B_4O_7$–50 m*M* NaH_2PO_4 (pH 7.0); capillary, 750 × 0.75 mm I.D.; effective length, 500 mm; applied voltage, 15 kV, 50 μA; detection wavelength, 210 nm.

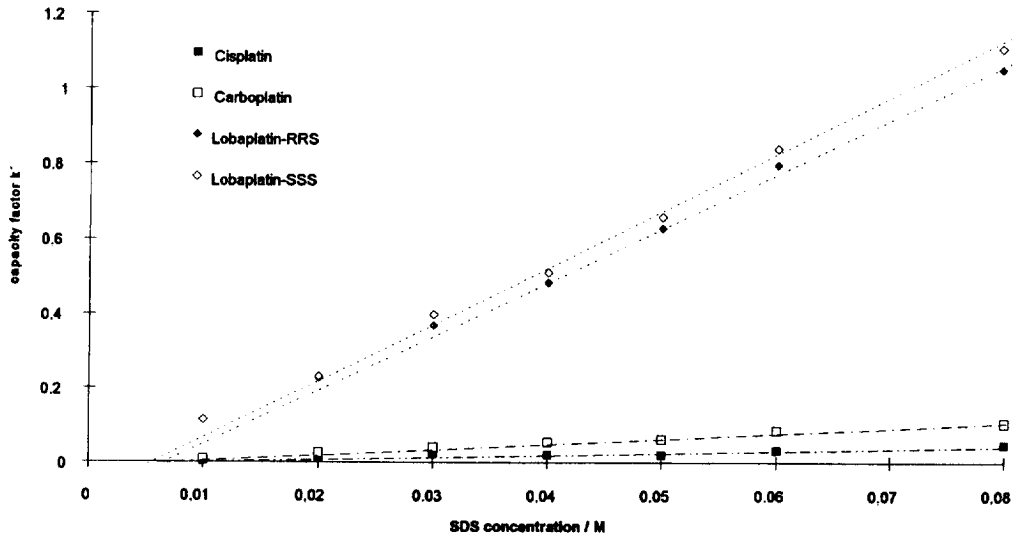

Fig. 3. Dependence of capacity factors (k') of cisplatin, carboplatin and lobaplatin on SDS concentration. Experimental conditions as in Fig. 2 except SDS concentrations.

es. There is only a slight attraction to the SDS micelles, both to the lipophilic inside and to the positively charged hydrophilic micelle surface. In contrast, the capacity factor of the more lipophilic lobaplatin increases with increasing micelle concentration.

The critical micelle concentration (CMC) of SDS under these conditions was calculated from the intercepts of the graphs in Fig. 3 on the abscissa as 5 mM. This value is comparable to the reported value of 6 mM in 20 mM sodium phosphate buffer (pH 8) [12]. With the molar volume ($v = 0.2525\ \mathrm{l\ mol^{-1}}$) of SDS the partition coefficient P, which is the distribution coefficient of the analyte between the pseudo-stationary micellar phase and the aqueous buffer phase, can be calculated from the slopes as given in the equation [12]

$$k' = P \cdot (0.2525\ \mathrm{l\ mol^{-1}}) \cdot ([\mathrm{SDS}] - \mathrm{CMC}) \qquad (2)$$

Mostly the platinum complexes contain lipophilic amine ligands and more or less hydrophilic leaving groups, and therefore it is difficult to assess the polarity of the whole drug. Measuring the partition coefficient could be an easy way to describe the polarity. For the given MECC system the partition coefficients at 25°C were calculated as $P = 3$ for cisplatin, 6 for carboplatin and 59 for lobaplatin, which means that cisplatin and carboplatin with low P values are more polar, whereas lobaplatin with a high P value is more lipophilic.

For further optimization of the MECC system, the influence of pH, buffer solution, injection technique and volume, SDS concentration and applied voltage on the resolution of the complexes was investigated.

3.1. pH of buffer solution

In a more acidic buffer solution the EOF decreases, which results in very long analysis times for all analytes. At pH 4.5 cisplatin has a migration time of 17.7 min, carboplatin 19.4 min and lobaplatin >50 min. With buffers of pH 9.5 the neutral complexes could be separated, but there was no separation of the hydrolysis products of cisplatin.

With phosphate–borate buffer (pH 7) the best resolution for all complexes was obtained in a short analysis time.

3.2. Injection technique and injection volume

Because the intact drugs are uncharged, there should be no discrimination when electrokinetic injection is used. However, different charged species have to be determined in stability measurements of the complexes and discrimination of these species could occur. Table 1 shows the results of the electrokinetic and hydrodynamic injection techniques when analysing a hydrolysed cisplatin solution.

With the injection parameters used for hydrodynamic and electrokinetic injection, similar absolute sample amounts were introduced into the capillary, as can be seen from the overall peak areas in Table 1. The injection volume for hydrodynamic injection was calculated as 8 nl. The volume was determined by continuous injection of Sudan III in a buffer–methanol solution when hydrodynamic pressure (height difference 10 cm) was applied.

The reproducibility of percentage peak areas from run to run for hydrodynamic injection is better than 2.5%. Because of the manual injection procedure, the reproducibility for the total injected sample amount is not as good as for commercial automated systems (2.3–3.6% for hydrodynamic and 3.2–10.3% for electrokinetic injection [12]). This disadvantage could be negligible when using an internal standard.

The difference in percentage peak areas between electrokinetic and hydrodynamic injection is a consequence of the different electrophoretic mobilities of the charged species and of the different attraction to the SDS micelles.

Because of the species discrimination with electrokinetic injection, we chose hydrodynamic injection for the further measurements. Here the optimum injection time was found to be 10 s at a 10-cm height difference. With larger injection volumes cisplatin, carboplatin and the lobaplatin diastereomers overlapped more and with smaller injection volumes the limit of detection was too high.

3.3. SDS concentration and applied potential

As can be seen in Fig. 3, the capacity factors of the intact drugs cisplatin and carboplatin are very low and there is only a slight climb of the graph with increasing SDS concentration. The reason for this behaviour is the less lipophilic structure of the complexes. Although the resolution of the complexes is not significantly affected by SDS concentration, the minimum concentration for baseline separation of the complexes is 30 m*M* SDS.

In contrast to the intact drugs, the k' values of the cisplatin hydrolysis products are determined by the SDS concentration and the capacity factors increase more with increasing surfactant concentration. Although the main hydrolysis products are positively charged, their electrophoretic mobility is directed towards the anode in an SDS–buffer solution. We assume that the positively charged complex is attracted electrostatically to the negatively charged surface of the micelle, resulting in an electrophoretic mobility (ΔEPF; see Fig. 4a) towards the anode. A similar behaviour was found for metal ions, such as

Table 1
Comparison of the total and percentage peak areas for hydrodynamic and electrokinetic injection techniques

Analyte	Peak area (%)[a]	
	Hydrodynamic injection (10 s, 10 cm)	Electrokinetic injection (10 s, 4 kV, 11 μA)
Cisplatin	59.0 (0.8)	59.3 (2.4)
Hydrolysis product 1	8.4 (0.2)	11.5 (3.5)
Hydrolysis product 2	32.6 (2.4)	29.2 (4.3)
Overall peak area ($\times 10^3$ units)	5136 (11)	4947 (17)

[a] Results are mean values ($n = 3$) with R.S.D. (%) in parentheses.

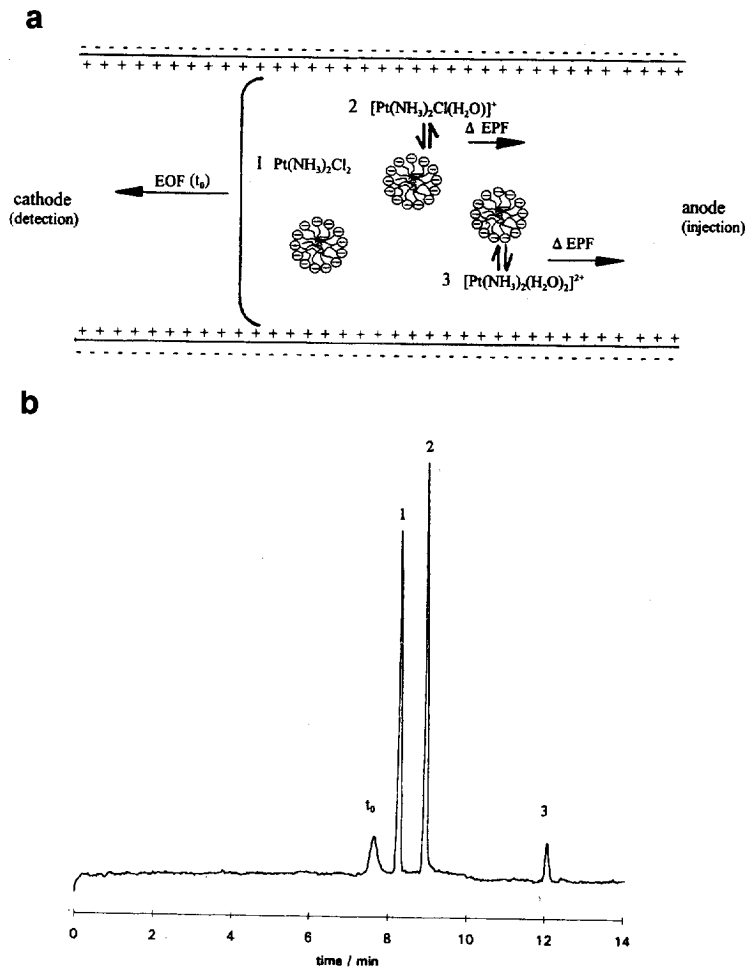

Fig. 4. (a) Schematic representation of the principle of MECC for cisplatin analysis. The directions of electroosmotic flow (EOF) and electrophoretic flow (EPF) are shown. (b) Electropherogram of a cisplatin solution in water after 2 h at 37°C. Peaks: 1 = cisplatin; 2 = [*cis*-diammineaquachloroplatinum]$^{+}$; 3 = [*cis*-diamminediaquaplatinum]$^{2+}$. Experimental conditions as in Fig. 2 except 100 m*M* SDS.

Cu(II), Zn(II) and Mg(II), which were used as metal additives in the separation of nucleosides in MECC [13]. Because of the dominant EOF, the net velocity of all the analytes is towards the cathode. The different lengths of the arrows for EPF and EOF in Fig. 4a demonstrate this behaviour schematically. Fig. 4b shows an electropherogram of cisplatin in water after 2 h at 37°C. Besides cisplatin (peak 1), two hydrolysis products are detectable, which correspond to the mono- (2) and diaquated (3) complexes.

The influence of SDS concentration and the applied potential on the resolution of the lobaplatin diastereomers is shown in Fig. 5. Here the peak separation function (f/g) (where f is the depth of the valley below the straight line connecting the two adjacent peak maxima and g is the height of the straight line above the baseline at the valley [14]) is presented as a value for overlap of the diastereomers. At a value of unity the two analytes are baseline separated.

Fig. 5 shows that the peak separation function

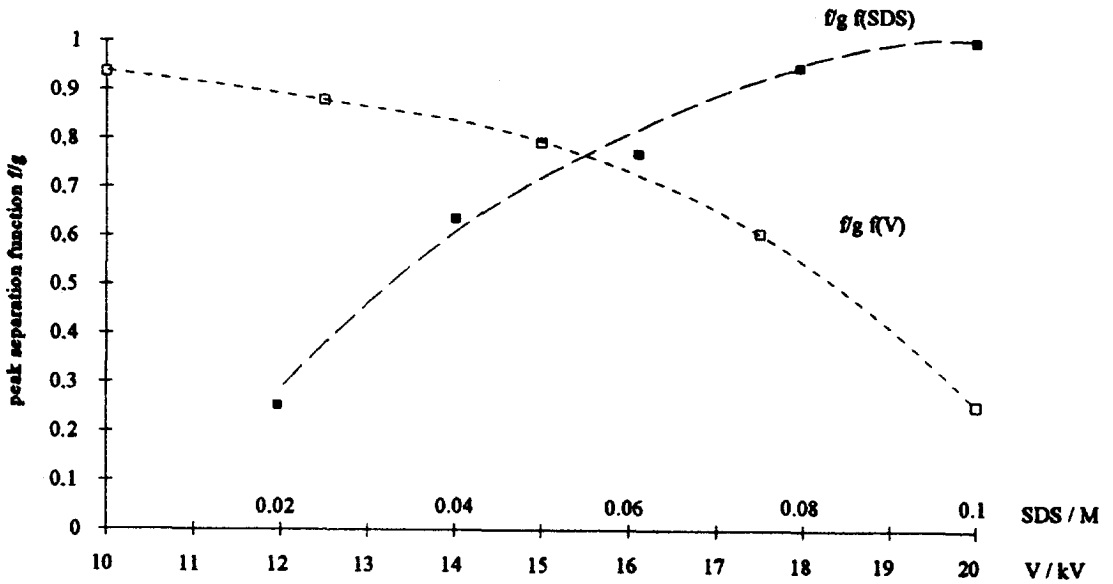

Fig. 5. Peak separation function (f/g) for the separation of the lobaplatin diastereomers as a function dependence of SDS concentration and potential.

increases continuously with increasing SDS concentration and with decreasing voltage. At high voltages and current, the Joule heating increases, resulting in greater band broadening and less separation. The optimum parameters are an applied voltage of 15 kV and an SDS concentration of 80 m*M*. Here the migration times of the analytes are very short and the lobaplatin diastereomers are almost baseline separated ($f/g = 0.95$).

For stability measurements and investigations on hydrolysis of the platinum complexes, optimization of all steps leads to the following method: fused-silica capillary, 0.70 m × 75 μm I.D. (0.50 m effective length); detection wavelength, 210 nm; hydrodynamic injection, 10 s, 10 cm; 80 m*M* SDS in 50 m*M* phosphate–25 m*M* tetraborate buffer (pH 7.0); and applied potential, 15 kV.

3.4. Limit of detection and linearity

For method validation, the linearity and limit of detection (LOD) of the intact drugs were determined. The peak areas were correlated to the concentration of complex standards in the range 10–1000 μg ml^{-1}. The correlation coefficient is 0.999 for cisplatin in the range 20–250 μg ml^{-1} and better than 0.998 in the range 20–500 μg ml^{-1} for carboplatin and lobaplatin. At higher concentrations there is a shift to lower peak areas and the correlation coefficient is worse. We calculated the LOD (twice the baseline noise) as 20 μg ml^{-1} for cisplatin and 15 μg ml^{-1} for carboplatin and lobaplatin. Because of the low detection pathlength in CE, the limit of detection is 1–2 orders of magnitude higher than in HPLC. However, compared with HPLC, the absolute detectable amount of platinum complex is very low (120–160 ng) because of the small injection volume of 8 nl.

3.5. Stability measurements of platinum complexes

The developed MECC method was used for kinetic measurements of complex stability in

aqueous solutions at 37°C. Besides the stability in water, the stability in solutions containing 100 m*M* chloride (the concentration in blood) respectively 4 m*M* chloride (the cytoplasm concentration) was investigated.

Fig. 6 shows that cisplatin decline depends highly on the chloride concentration. Addition of chloride to aqueous solutions of cisplatin stabilize the drug, as there was 42% hydrolysis in 4 m*M* NaCl and only 2% hydrolysis in 100 m*M* NaCl compared with 86% hydrolysis in water after 8 h at 37°C.

Although water is in large excess, the hydrolysis of cisplatin does not follow simple reaction kinetics. Because the forward reaction rate constant k_1 is about $100k_{-1}$ [15] pseudo-first-order reaction kinetics could be assumed. The reaction constant (k_1) at 37°C was calculated from the slope of the logarithmic concentration (% of initial value) versus time curve. For hydrolysis in water k_1 was calculated as $8.0 \cdot 10^{-5}$ s^{-1} from the linear part of the graph (linear up to 4 h of hydrolysis, $r = 0.997$, $n = 4$). This value is identical with the published value of $8 \cdot 10^{-5}$ s^{-1} [16]. With longer hydrolysis times the reaction rate constant decreases.

Fig. 7 shows the electropherograms of (a) lobaplatin in 4 m*M* NaCl and (b) carboplatin in 100 m*M* NaCl after 3 weeks at 37°C. Both complexes are much more stable than cisplatin in aqueous solutions. In contrast to cisplatin, increasing chloride concentrations accelerate the hydrolysis of the complexes.

4. Conclusions

MECC has been used for the separation of the platinum anti-tumour drugs cisplatin, carboplatin and lobaplatin in aqueous solutions. The effects of SDS concentration, pH, applied voltage and injection conditions on the resolution of the complexes were elucidated and the conditions were optimized. With the optimized parameters [80 m*M* SDS in 50 m*M* phosphate–25 m*M* borate buffer (pH 7); applied voltage 15 kV; 52 μA) the intact drugs and hydrolysed species could be separated with high resolution in a short analysis time. When using SDS as micellar phase, the positively charged hydrolysis products of cisplatin migrate electrophoretically towards the

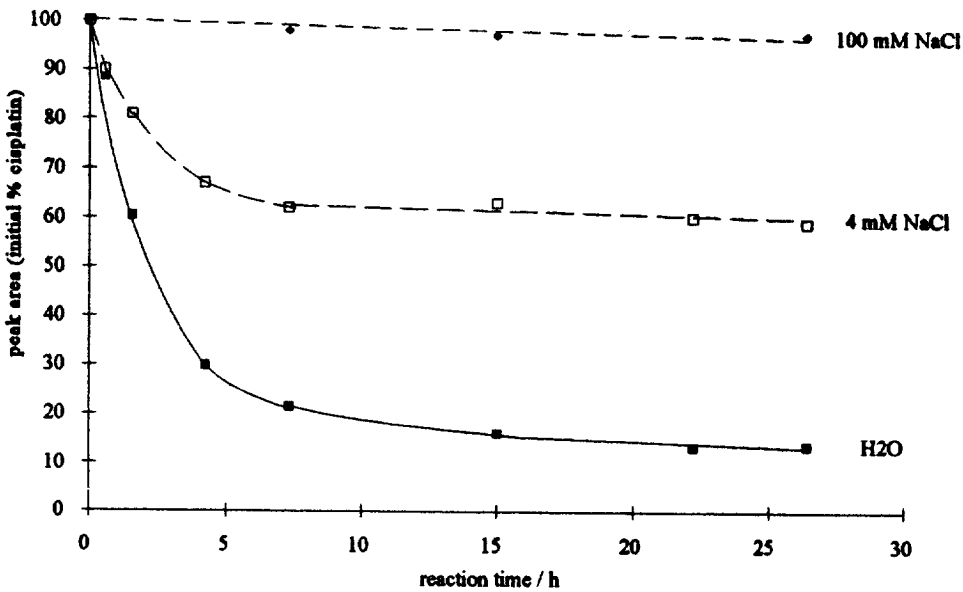

Fig. 6. Kinetic curve for cisplatin hydrolysis at 37°C in aqueous solutions containing different amounts of sodium chloride.

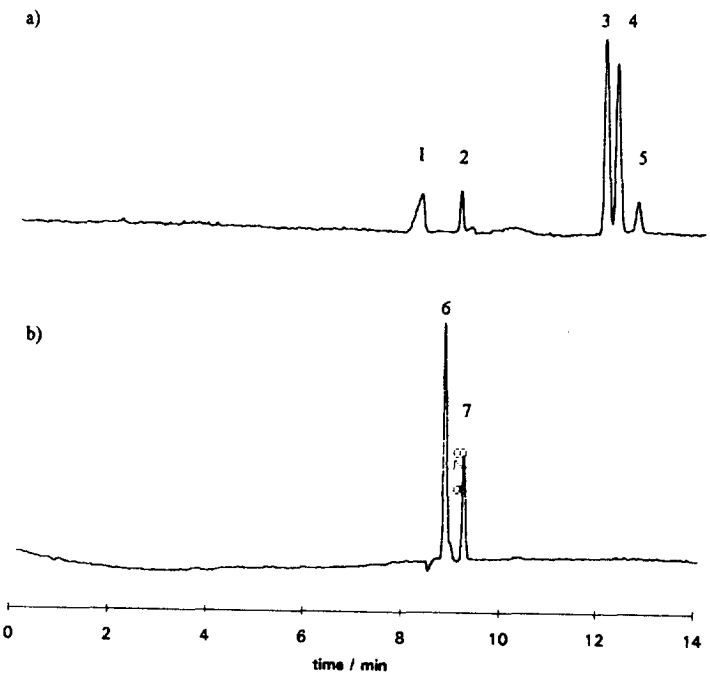

Fig. 7. Electropherograms of (a) lobaplatin solution in 4 m*M* NaCl with the hydrolysis products **1**, **2** and **5** and the lobaplatin diastereomers **3** and **4** and (b) carboplatin solution in 100 m*M* NaCl with carboplatin (**6**) and one hydrolysis product (**7**). Experimental conditions as in Fig. 2 except 80 m*M* SDS.

anode, because they are attracted to the surface of the negatively charged micelles. The net velocity of the analytes is still towards the cathode because of the dominant EOF.

The stability of the platinum drugs in aqueous solutions were determined. In chloride-free solutions cisplatin is less stable ($k_1 = 8.0 \cdot 10^{-5}$ s^{-1}) than carboplatin and lobaplatin (both stable for several days at 37°C). Higher chloride concentrations prevent the hydrolysis of cisplatin, whereas the hydrolysis of carboplatin and lobaplatin increases.

The developed method offers new possibilities in the analysis of platinum complexes, including stability measurements, kinetic measurements of complex hydrolysis and investigations of complex reactivity. The main advantage of the MECC method is in the easy separation of neutral species besides charged platinum species in one run, whereas HPLC requires more runs with different techniques. Although the detection limit is still higher than in HPLC, shorter analysis times and easier handling are significant advantages for MECC.

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