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Application of micellar electrokinetic chromatography for the separation of retinoids

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Abstract

The applicability of micellar electrokinetic chromatography (MEKC) using sodium dodecylsulphate (SDS) as pseudo-stationary phase for the separation of five retinoids (retinol, retinal, retinyl acetate, retinyl palmitate, retinoic acid), was investigated. The effects of the acetonitrile content, the SDS concentration, the pH and the addition of Brij 35 to the background electrolyte on the migration behaviour of the retinoids were determined. It was found that the effective mobilities of retinol, retinal and retinyl acetate could be easily regulated through the ACN content and the SDS concentration of the BGE. The electrophoretic behaviour of the very hydrophobic retinyl palmitate was abnormal. Under various conditions this compound showed up as a late, very sharp peak. A strong indication was found that the retinyl palmitate forms a stable, charged complex with SDS during sample preparation. The mobility of the retinyl palmitate peak could be regulated, independently from the other peaks, through the Brij concentration of the BGE. Using a running buffer consisting of Tris–buffer (pH 8), 20 mmol l^{-1} SDS, 1 mmol l^{-1} Brij 35 and 35% (v/v) acetonitrile, a complete separation of the five retinoids could be realised in less than 20 min.

Keywords: MEKC; Retinoids; Vitamin A

1. Introduction

Vitamins are essential compounds for the life of man that are not synthesised in the organism or formed in insufficient quantities. For this reason vitamins have to be consumed regularly via the food or by intake of pharmaceutical vitamin preparations [1]. Vitamin A (retinol) is only required in exceptionally small amounts, but plays an important role in the organism [2]. An inadequate supply of this vitamin leads to typical pathological deficiency symptoms, such as night blindness, atrophies and growth retardation. Retinoids are compounds that are structurally related to retinol. Retinal and retinoic acid are metabolites of retinol formed in the body, while esters of retinol, retinyl acetate and retinyl palmi-

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tate, are used in pharmaceutical formulations or as food additives. Also, approximately 90% of vitamin A in the body are stored in the liver as retinyl palmitate.

Since the discovery of the importance of vitamins for the human health, numerous analytical techniques have been developed to determine vitamins in preparations, foodstuffs and body fluids. Of the various techniques applied, HPLC has become to be the method of choice for the analysis of both water-soluble and fat-soluble vitamins [3,4]. For retinoids, HPLC with UV detection is most often applied, due to their favourable absorbance characteristics [5]. Still, the separation of the fat-soluble retinoids by HPLC is problematic because of their analogue structure.

Capillary electrophoresis (CE) is a relatively new analysis technique, with as main advantages its high separation efficiency and high speed. The applicability of CE for the separation of water-soluble vitamins, based on their migration as ions, has been demonstrated by various researchers [6–9]. The CE methods developed have been shown to have good sensitivity, linearity and precision, and their application in pharmaceutical industry is increasing [10].

An attractive variant of CE is micellar electrokinetic chromatography (MEKC) [11,12]. The separation in this technique is based on the differential partition of the solutes between a migrating, pseudo-stationary micellar phase and the (aqueous) solution. MEKC is applicable for the separation of neutral substances. For ionic compounds an MEKC-system is also frequently used, since the combination of ionic migration and differential partition allows an easy control of the separation selectivity. MEKC has been applied frequently for the separation of water-soluble vitamins [9,13–16], and occasionally also for fat-soluble vitamins [17].

Pedersen-Bjergaard et al. separated retinyl palmitate from other vitamins in a MEKC system with tetradecylammonium bromide as the (positively charged) micellar phase [18,19]. Profumo et al. used sodium cholate as micelle former for the MEKC separation of three retinoids [20]. Recently, Hsieh and Kuo realised the separation of five retinoids with MEKC [21]. In this work the authors used a mixture of sodium deoxycholate (SDC) and Brij 35 as micellar phase. The method was applied for the analysis of retinyl acetate and retinyl palmitate in pharmaceutical products. A disadvantage of the use of the cholate surfactant is that a relatively high concentration appeared to be needed; this could lead to high electrophoretic currents in more demanding applications. Sodium dodecylsulphate (SDS), a more usual surfactant in MEKC, could not be used since all retinoids were then completely absorbed into the pseudo-stationary phase, and migrated with the velocity of the micelles.

In this paper we report the results of an investigation to apply MEKC with SDS as (main) surfactant for the separation of the five most important retinoids. Attention was given to the effect of the surfactant and ACN concentration on the migration of the retinoid solutes, and of the addition of the neutral surfactant Brij 35.

2. Experimental

2.1. Apparatus

The CE set-up consisted of a Prince injector (Prince Technologies, Emmen, The Netherlands) in combination with an on-column UV detector (Spectroflow 757, Kratos Analytical, Ramsey, NJ, USA). The detection wavelength was 325 nm. The fused silica capillaries used, obtained from Polymicro Technologies (Phoenix, AZ, USA), had an I.D. of 75 μm and an O.D. of 360 μm. The total length of the capillary was 0.80 m while the effective length was 0.46 m. The separations were performed with an applied voltage of 20 kV at ambient temperature. In all experiments hydrodynamic injection was applied (50 mbar, 6 s).

To identify the peaks in the electropherograms, in some experiments use was made of a HP^{3D}CE system (Hewlett Packard, Waldbronn, Germany) equipped with a diode array detector.

2.2. Chemicals and solutions

Retinol, retinal, retinyl acetate, retinyl palmitate and retinoic acid and sodium dodecyl sul-

phate (SDS) were obtained from Sigma (St Louis, MO, USA). Brij 35, acetonitrile (ACN), ethanol, methanol, tris-(hydroxymethyl)-methylammonium chloride (Tris–HCl) and tris-(hydroxymethyl) aminomethane (Tris) were obtained from Merck (Darmstadt, Germany) and Sudan III from Aldrich Chemie (Steinheim, Germany). Other chemicals, obtained from various standard suppliers, were of analytical quality and used as received. All solutions were prepared with subboiled water and filtered over a 0.45-μm membrane prior to use.

The running buffer contained 10 mmol l^{-1} Tris–HCl and 18 or 28 mmol l^{-1} Tris for buffer solutions with a pH of 8 or 8.5, respectively, and various amounts of ACN, SDS and Brij 35. For the measurements at pH 7 a sodium phosphate buffer was used and for pH 9 a sodium borate buffer. The concentrations of these buffers were chosen such that the running buffers all had the same ionic strength (10 mmol/l without the SDS). The pH of the buffers was measured in the aqueous phase before mixing with organic solvent. Sudan III was used as marker to determine the electrophoretic mobility of the micelles.

Stock solutions of the retinoids (2.5 mg ml^{-1}) were prepared in ethanol and stored at -20°C in the dark. The analytical solutions of the retinoids were daily prepared by diluting aliquots of the standard solutions in a phosphate buffer (pH 7.0) containing 10 mmol l^{-1} SDS. The final concentrations of the retinoids in the sample solution were 3 μg ml^{-1} (retinol), 8 μg ml^{-1} (retinal), 2.5 μg ml^{-1} (retinoic acid), 2.5 μg ml^{-1} (retinyl acetate) and 10 μg ml^{-1} (retinyl palmitate). The solutions were stored at 0°C in the dark during the day.

2.3. Procedures

New capillaries were successively flushed with 1 mol l^{-1} NaOH (10 min), 0.1 mol l^{-1} NaOH (10 min), water (5 min) and running buffer (20 min). When applying a different running buffer the capillary was first flushed with 0.1 mol l^{-1} NaOH (15 min), water (5 min) and than with the new running buffer (10 min). Prior to use the running buffer was filtered through a 0.45-μm filter and degassed by ultrasonification for 20 min.

3. Results and discussion

The use of SDS in MEKC has certain advantages. SDS has a relatively low critical micellar concentration (cmc), so that low concentrations can be applied and the electrophoretic current can be limited. It can be used at low and high pH so that the retention window can be adapted through the choice of the BGE pH. In a high-pH solution, the pseudo-stationary SDS phase migrates against the electro-osmotic flow, which generally gives the best resolution. Compared to for instance cholates, SDS micelles have a high solubilising power. For strongly hydrophobic solutes, such as the retinoids, this may be problematic, since it may mean that for such compounds the selectivity of MEKC is lost. In experiments with a background electrolyte (BGE) containing only Tris buffer (pH 8) and 10 mmol l^{-1} SDS, it was indeed observed that all four neutral retinoids migrated with the mobility of the micelles, as determined with Sudan III, against the EOF. Only retinoic acid was separated from the other compounds, since it is ionised at higher pH and will not be absorbed in the micellar pseudo-phase.

It has been shown that the affinity of SDS micelles for very hydrophobic compounds can be conveniently regulated by the addition of acetonitrile (ACN) or another organic modifier to the buffer used in MEKC [22–24]. The presence of ACN decreases the volume fraction of the micellar phase and changes the distribution characteristics of the SDS micelles [25]. To decrease the affinity of the micellar phase for the neutral retinoids, ACN was added to the BGE in various volume fractions. The EOF mobility gradually decreased with increasing ACN content of the BGE, from 6.6×10^{-8} m^2 s^{-1} V^{-1} in a purely aqueous buffer to 3.0×10^{-8} m^2 s^{-1} V^{-1} in a buffer with 60% ACN. The mobility of retinoic acid also decreased with the ACN content. Moreover, as is shown in Fig. 1, the ACN volume fraction had a strong influence on the effective mobilities (corrected for the electro-osmotic mobility) of the neutral retinoids. With 10% ACN the mobility was hardly affected, but when the ACN concentration was increased in the range 10–30% the effective mobilities of retinol, retinal

and retinyl acetate decreased steeply. With higher ACN volume fractions (40–60%) a further, gradual decrease of the mobilities was found. Similar behaviour has been observed for various other hydrophobic compounds in a SDS-MEKC system [25]. It has been concluded that with increasing ACN content, up to approximately 40%, the SDS micelles are gradually broken down to smaller aggregates with a lower solubilising power and possibly a lower electrophoretic mobility. At high ACN content, the micelles may be completely disintegrated and the remaining effective migration of hydrophobic, neutral compounds may then be attributed to solvophobic interaction with individual surfactant ions [26].

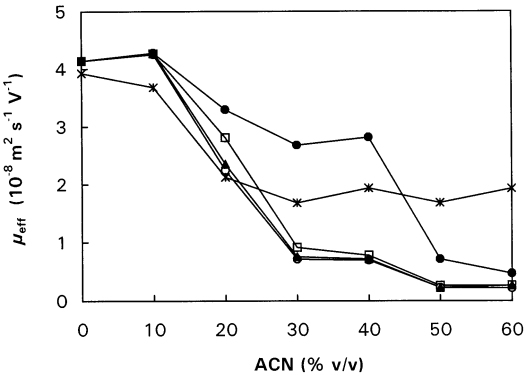

Fig. 1. Influence of the ACN content of the BGE on the effective mobilities of the retinoids. BGE: Tris buffer (pH 8), 10 mmol l^{-1} SDS and 0–60% (v/v) ACN. For other experimental conditions see text. (○) retinol; (▲) retinal; (*) retinoic acid; (□) retinyl acetate; (●) retinyl palmitate.

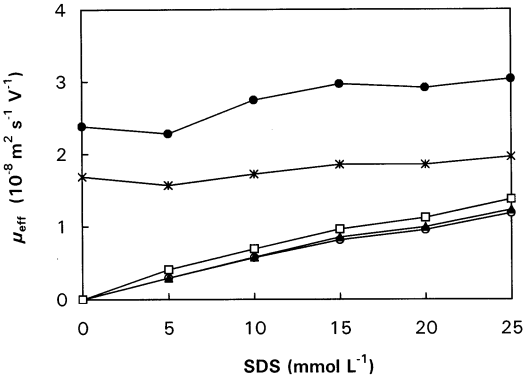

Fig. 2. Influence of the SDS concentration of the BGE on the effective mobilities of the retinoids. BGE: Tris buffer (pH 8), 35% (v/v) ACN and 0–25 mmol l^{-1} SDS. For other experimental conditions see text. (○) retinol; (▲) retinal; (*) retinoic acid; (□) retinyl acetate; (●) retinyl palmitate.

The electrophoretic behaviour of retinyl palmitate was found to be abnormal. Up to 40% ACN could be added to the BGE without drastical change of the effective mobility of the retinyl palmitate ester. Moreover, with 0–40% ACN in the BGE, the retinyl palmitate peak was found to be unusually sharp, with an apparent plate number in the order of 2 million. The spike-like peak was identified as the retinyl palmitate peak by its UV spectrum measured with a diode-array detector. Since the retinyl palmitate peak is even narrower than can be expected on basis of the injected sample volume, some kind of stacking effect must have taken place. For a significant decrease of the effective mobility of retinyl palmitate at least 50% of ACN was required. With 50 or 60% ACN retinyl palmitate migrated as a broad, asymmetrical peak. However, with these high ACN contents the other neutral retinoids were not separated.

The effect of the pH of the running buffer was investigated with 35% (v/v) ACN and 10 mmol l^{-1} SDS in the BGE. While the migration of the three more hydrophilic retinoids and retinoic acid was hardly affected in the pH range 7–9, the behaviour of retinyl palmitate was again different. At pH 7 retinyl palmitate did not elute from the capillary. In the pH range 8–8.5 the retinyl palmitate migrated with an effective mobility of 2.4×10^{-8} m^2 s^{-1} V^{-1} as a sharp peak. At pH = 9 the effective migration mobility was increased (to 3.0×10^{-8} m^2 s^{-1} V^{-1}) and the retinyl palmitate showed up as a very broad disturbed peak.

The effect of the SDS concentration on the EOF and the migration of the retinoids was investigated with a BGE containing Tris buffer (pH 8) and 35% (v/v) ACN. The EOF mobility decreased to some extent with the SDS concentration, from 5.0×10^{-8} m^2 s^{-1} V^{-1} without SDS to 3.8×10^{-8} m^2 s^{-1} V^{-1} at a concentration of 25 mmol l^{-1}. The results for the retinoids are shown in Fig. 2. The (ionic) mobility of retinoic acid is hardly influenced by the presence of SDS. The effective mobilities of retinol, retinal and retinyl acetate follow the expected pattern: an increase

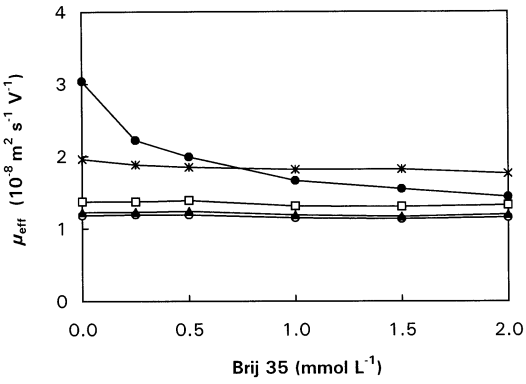

Fig. 3. Influence of the Brij 35 concentration of the BGE on the effective mobilities of the retinoids. BGE: Tris buffer (pH 8), 20 mmol l^{-1} SDS, 35% (v/v) ACN and 0–2 mmol l^{-1} Brij. For other experimental conditions see text. (○) retinol; (▲) retinal; (*) retinoic acid; (□) retinyl acetate; (●) retinyl palmitate.

with the SDS concentration with a trend to approach an asymptotic value at high SDS concentrations. The effective mobilities of these retinoids were close to that of Sudan III, which was used as the micellar marker. Again, an abnormal behaviour was observed for retinyl palmitate. Its effective mobility appeared to be independent of the SDS concentration. Surprisingly, the presence of SDS in the BGE was not even required. A possible explanation for this behaviour can be sought in the formation of a stable complex of the retinyl palmitate with SDS present in the sample solution. In order to disperse the retinoids in an aqueous solution, SDS had to be added to the buffer in a concentration above the critical micellar concentration. The neutral retinoids dissolve well in the micellar phase. With the injection of this solution into a running buffer without SDS, containing ACN, the micelles will disintegrate and the neutral, more hydrophilic retinoids will migrate with the electro-osmotic mobility. However, the extremely hydrophobic retinyl palmitate could interact strongly with the hydrocarbon chain of SDS to form a stable, negatively charged complex, independent of the presence of SDS in the background electrolyte. The observed stacking effect, leading to extremely sharp peaks for the retinyl palmitate, may also be related in some way to this process of micelle disintegration and complex formation.

From the previous experiments it is clear that the selectivity of the separation system can be regulated by the SDS concentration and the ACN content of the BGE. The critical separation is that of retinol and retinal. The best resolution for this pair was found with 20 mmol l^{-1} SDS and 35% (v/v) of ACN in the BGE. However, under these conditions the migration time of retinyl palmitate is rather long (approximately 40 min), because it's effective mobility almost opposes the electro-osmotic mobility in the direction of the detector.

In order to shorten the migration time of retinyl palmitate while preserving the selectivity for the more hydrophilic retinoids, the effect of addition of a neutral surfactant (Brij 35) was investigated. Brij 35 forms neutral micelles in water, but can be also incorporated in SDS micelles. Hsieh and Kuo studied the effect of Brij for the MEKC-separation of retinoids with sodium deoxycholate (SDC) as micellar phase [21]. These authors found that the addition of Brij 35 decreased the retention times of all retinoids considerably. The effect of the addition of Brij 35 (0–2 mmol l^{-1}) in the present SDS micellar system on the migration of the retinoids is shown in Fig. 3. As can be seen here, the addition of Brij 35 has an insignificant effect on the migration of the hydrophilic retinoids and retinoic acid, but strongly decreased the migration velocity of retinyl palmitate. A possible explanation of this effect is the replacement of SDS molecules in the SDS-retinyl palmitate complex by neutral Brij 35 molecules. With Brij in the BGE the retinyl palmitate peak is fairly broad, which indicates that the dynamics of this process is slow. With 1–2 mmol l^{-1} Brij 35 the retinyl palmitate peak can be made to elute before the retinoic acid peak. Since the peak width with 2 mmol l^{-1} Brij 35 appeared to be somewhat larger than with 1 mmol l^{-1} Brij 35, the latter concentration was finally chosen. Fig. 4 shows the optimised separation of the five retinoids. The analysis time under the conditions chosen was 18 min.

4. Conclusions

In contrast to what has been reported before, this investigation shows that it is possible to use SDS as pseudo-stationary phase to separate the five most important retinoids (retinol, retinal, retinyl acetate, retinyl palmitate and retinoic acid) by MEKC. The use of SDS has certain advantages over systems proposed before; it can be used in a low concentration (giving low electrophoretic currents) and it gives an advantageous retention window. The selectivity of the separation can be easily controlled through the concentration of SDS and the ACN content of the running buffer. Brij 35 can be used to adjust the migration of retinyl palmitate without influencing the migration of the other retinoids.

There is a strong indication that the migration behaviour of retinyl palmitate is not governed by distribution into the micellar phase but by the formation of SDS-complexes in the sample solution. This implies that in routine use of the separation technique proposed, the sample preparation method should be carefully controlled, and that one has to be alert for possible interferences by, e.g. excipients in pharmaceutical formulations.

The sensitivity of UV detection as applied here is sufficient when the method proposed is applied for commercial formulations. However, for samples such as foodstuffs or blood plasma fluorescence detection will be required. Work in this direction is now being carried out.

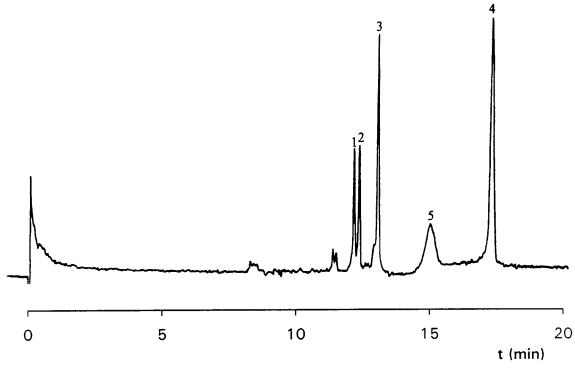

Fig. 4. Optimised separation of the retinoids. BGE: Tris buffer (pH 8), 20 mmol l^{-1} SDS, 35% (v/v) ACN and 1 mmol l^{-1} Brij. For other experimental conditions see text. (1) retinol; (2) retinal; (3) retinyl acetate; (4) retinoic acid; (5) retinyl palmitate.

References

[1] T.K. Basu, J.W.T. Dickerson, Vitamins in Human Health and Disease, CAB International, Wallingford, 1996.
[2] M.B. Sporn, A.B. Roberts, D.S. Goodman, The Retinoids: Biology, Chemistry and Medicine, second ed., Raven Press, New York, 1994.
[3] A.P. De Leenheer, W.E. Lambert, H.J. Nelis (Eds.), Modern Chromatographic Analysis of Vitamins, Marcel Dekker, New York, 1992.
[4] A. Rizzolo, S. Polesello, J. Chromatogr. 624 (1992) 103–152.
[5] R. Wyss, J. Chromatogr. B 671 (1995) 381–425.
[6] R. Huopalahti, J. Sunell, J. Chromatogr. 636 (1993) 133–135.
[7] J. Schiewe, Y. Mrestani, R. Neubert, J. Chromatogr. A 717 (1995) 255–259.
[8] J. Schiewe, S. Göbel, M. Schwarz, R. Neubert, J. Pharm. Biomed. Anal. 14 (1996) 435–439.
[9] L. Fotsing, M. Fillet, I. Bechet, P. Hubert, J. Crommen, J. Pharm. Biomed. Anal. 15 (1997) 1113–1123.
[10] K.D. Altria, M.A. Kelly, B.J. Clark, Trends Anal. Chem. 17 (1998) 204–214.
[11] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111–113.
[12] S. Terabe, K. Otsuka, T. Ando, Anal. Chem. 57 (1985) 834–841.
[13] S. Fujiwara, S. Iwase, S. Honda, J. Chromatogr. 447 (1988) 133–140.
[14] H. Nishi, N. Tsumagari, T. Kakimoto, S. Terabe, J. Chromatogr. 465 (1989) 331–343.
[15] C.P. Ong, C.L. Ng, H.K. Lee, S.F.Y. Li, J. Chromatogr. 547 (1991) 419–428.
[16] D.B. Gomis, L.L. Gonzalez, D.G. Alvarez, Anal. Chim. Acta 396 (1999) 55–60.
[17] B.J. Spencer, W.C. Purdy, J. Chromatogr. A 782 (1997) 227–235.
[18] S. Pedersen-Bjergaard, K.E. Rasmussen, T. Tilander, J. Chromatogr. A 807 (1998) 285–295.
[19] O. Naess, T. Tilander, S. Pedersen-Bjergaard, K.E. Rasmussen, Electrophoresis 19 (1998) 2912–2917.
[20] A. Profumo, V. Profumo, G. Vidali, Electrophoresis 17 (1996) 1617–1621.
[21] Y.-Z. Hsieh, K.-L. Kuo, J. Chromatogr. A 761 (1997) 307–313.
[22] J. Gorse, A.T. Balchunas, D.F. Swaile, M.J. Sepaniak, J. High Resolut. Chromatogr. Chromatogr. Commun. 11 (1988) 554–559.
[23] J. Vindevogel, P. Sandra, Anal. Chem. 63 (1991) 1530–1536.

[24] E.S. Ahuja, J.P. Foley, J. Chromatogr. A 680 (1994) 73–83.
[25] R.M. Seifar, J.C. Kraak, W.Th. Kok, Anal. Chem. 69 (1997) 2772–2778.
[26] Y. Walbroehl, J.W. Jorgenson, Anal. Chem. 58 (1986) 479–481.