Journal of Chromatography, 447 (1988) 133–140
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 519

ANALYSIS OF WATER-SOLUBLE VITAMINS BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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(First received February 5th, 1988; revised manuscript received April 5th, 1988)

SUMMARY

Seven water-soluble vitamins were determined simultaneously by micellar electrokinetic capillary chromatography with UV detection. All these compounds were separated from each other within *ca.* 22 min by using a carrier containing sodium dodecyl sulphate as the surfactant. On-column detection at 254 nm with ethyl *p*-aminobenzoate as the internal standard allowed sensitive, accurate and reproducible determination of these compounds. Five principal constituents of a vitamin injection were determined with relative standard deviations of less than 2.1%.

INTRODUCTION

Capillary zone electrophoresis (CZE) is a remarkable method of separation, since it allows efficient separation of ionic substances, with large numbers of theoretical plates ranging from 10^5 to 10^6 plates/m of column length, *e.g.*, refs. 1–6. The addition of a surfactant in the carrier electrolyte solution has brought forth another mode of separation, based on differential partition of solutes between the aqueous and micellar phases in a rapid electroosmotic flow. This mode, initiated by Terabe *et al.*[7,8] and designated micellar electrokinetic capillary chromatography (MECC) by Burton *et al.*[9], is also efficient and has the advantage that it can be applied also to non-ionic substances[10,11]. Recently, several papers have demonstrated the usefulness of MECC for separation of various groups of compounds, including phenylthiohydantoin derivatives of amino acids[12], pyridoxine as well as its metabolites[9], purines[13] and nucleic acid-related compounds[14]. We also succeeded in separating antipyretic analgesics and in quantifying small amounts of these drugs[15]. Our success in this micro determination may be valuable, since it suggested the use of MECC in biomedical analysis. Therefore, we have extended the application of MECC to various drugs and biological substances. The present paper describes the results obtained for the analysis of water-soluble vitamins.

0021-9673/88/$03.50

EXPERIMENTAL

Reagents

Thiamine hydrochloride (VB_1), nicotinamide (VB_3), nicotinic acid (VB'_3), pyridoxine hydrochloride (VB_6), cyanocobalamin (VB_{12}) and L-ascorbic acid (VC) were obtained from Wako Pure Chemicals (Dosho-machi, Higashi-ku, Osaka, Japan). The sodium salt of riboflavine phosphate (VB_2PNa) was obtained from Tokyo Kasei Kogyo (Nihonbashi-honcho, Chuo-ku, Tokyo, Japan). Sodium dodecyl sulphate (SDS) and ethyl *p*-aminobenzoate (EAB) used as the surfactant and the internal standard, respectively, were also from Wako Pure Chemicals. All other chemicals were of the highest grade commercially available and were used without further purification. All these vitamins were used as aqueous solutions. EAB was used as a solution in methanol–water (1:1, v/v). Phosphate solutions were made by mixing $2.0 \cdot 10^{-2}$ *M* disodium hydrogenphosphate with $2.0 \cdot 10^{-2}$ *M* potassium dihydrogenphosphate in appropriate proportions (for pH 7, 8 and 9) or by adding 1 *M* sodium hydroxide to the former solution (for pH 10). SDS-containing carriers were prepared by dissolving 2.5–10 mmol of SDS in 100 ml of phosphate solutions obtained as above, and were used after filtration through a membrane filter having a pore size of 0.5 μm.

Apparatus

MECC was performed in a fused-silica capillary tube (80 cm × 100 μm I.D.; Scientific Glass Engineering, Australia). An high-voltage d.c. power supplier in an IP-2A isotachophoresis apparatus (Shimadzu, Nishinokyo, Nakakyo-ku, Kyoto, Japan), capable of delivering up to 30 kV, was used to generate the electric field. On-column UV detection was carried out with a SPD-2A double-beam variable-wavelength detector (Shimadzu). The polymer coating on the capillary tube was partly removed by burning at a position 50 cm from the anodic end, and the transparent portion was set on an 100 μm × 1 mm handmade slit attached to the detector block. A Chromatopac C-R1B (Shimadzu) was used for the measurement of retention time and peak area.

Procedure for MECC

The capillary tube was filled with a carrier solution by suction. Both ends of the tube were separately dipped in the anodic and cathodic solutions having the same composition as the carrier solution, and the surfaces of these electrode solutions were adjusted to the same level. For the introduction of a sample solution into the tube, the anodic end was quickly moved into a sample solution and the level of the sample solution was raised to about 5 cm higher than that of the cathodic solution. After 5 s the end of the tube was returned to the anodic solution, and an high voltage was applied in the constant-current mode. The current applied was 100 μA in the experiments using SDS-containing carriers, whereas it was reduced to 75 μA in those using SDS-free carriers, to obtain approximately the same voltage throughout the work.

Procedure for determination of the ingredients of a vitamin injection

For the determination of VB_1, VB_2PNa, VB_3 and VB_6, 5 ml of a commercial

sample of vitamin injection were accurately taken, to which were added 5.00 ml of a $1.00 \cdot 10^{-2}$ *M* solution of EAB (internal standard), and the volume was adjusted to 50.0 ml with distilled water. For the determination of VC, the commercial sample was diluted 1:25 in distilled water, and a 5-ml aliquot was processed in the manner described above. Standard solutions were prepared by dissolving authentic VB_1, VB_2PNa, VB_3, VB_6 and VC in distilled water. EAB was added to give approximately the same concentration as that in the sample solution.

The sample and the standard solutions were analyzed by MECC according to the procedure described above. The sample-to-standard peak area ratio of each vitamin was measured, from which its content was calculated.

RESULTS AND DISCUSSION

Optimization of separation

Fig. 1 shows the pH dependence of the retention time of seven water-soluble vitamins in 0.02 *M* phosphate solutions. Since SDS was absent from the carriers under these conditions, separation was performed simply by electrophoresis. The almost neutral vitamins (VB_3 and VB_{12}) were driven from the anode (inlet) to the cathode only by the electroosmotic flow generated in the electric field by the interaction of the carrier solution with the material of the capillary inner wall. However, VB_1, which gave a positively charged species in the carrier solution in the pH range examined, was eluted more rapidly than the neutral vitamins due to a positive electrophoretic effect. In contrast, VB_2PNa, VB'_3 and VC, giving negatively charged species, were eluted more slowly than the neutral vitamins, because they were pulled back electrophoretically to the anode (negative electrophoretic effect). The retention time of VB_6 was the same as that of VB_3 or VB_{12} at pH 7.0, but was greater than that of VB_3 and VB_{12} at higher pH values, due to dissociation of the phenolic hydroxyl group. Although the retention time increased almost linearly with increasing pH for all these vitamins, the extent varied depending on the ionic state and weight. As a result, VB_1, VB_2PNa, VB'_3, VB_6 and VC, which gave electrically charged species,

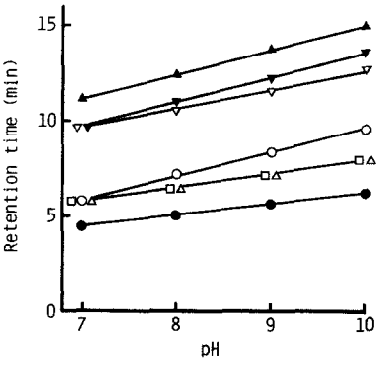

Fig. 1. Effect of the pH on the retention time in CZE of VB_1 (●), VB_2PNa (▼), VB_3 (□), VB'_3 (▲), VB_6 (○), VB_{12} (△), VC (▽). Capillary tube: fused silica (80 cm × 100 μm I.D.). Carrier: 0.02 *M* phosphate solution. Current applied: 75 μA. Detection wavelength; 254 nm. Sample concentrations: $0.25 \cdot 10^{-3}$ (VB_2PNa and VB_{12}), $0.50 \cdot 10^{-3}$ (VB_1 and VC), $1.00 \cdot 10^{-3}$ *M* (VB_3, VB'_3 and VB_6).

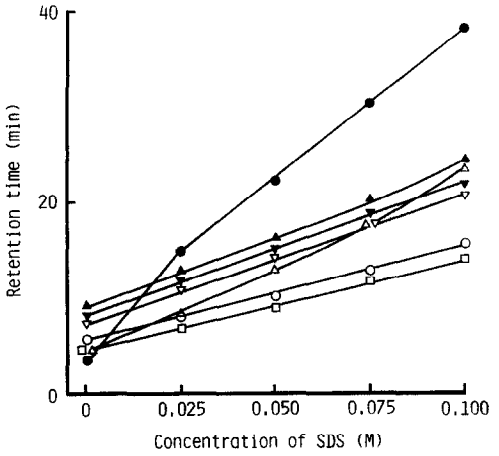

Fig. 2. Effect of the SDS concentration on the retention time in MECC. Carrier: 0.02 *M* phosphate solution, pH 9.0, containing 0.025–0.10 *M* SDS. Current applied: 100 μA. Other experimental conditions as in Fig. 1.

were all separated from each other at pH 9.0 and 10.0. However, the two neutral vitamins, VB_3 and VB_{12}, were not resolved electrophoretically at any pH value examined.

MECC gave elution profiles quite different from those in CZE. In this mode these vitamins were distributed between the aqueous and the micellar phases moving in a capillary tube, in the order of increasing hydrophobicity, resulting in good separation. Fig. 2 shows the effect of the SDS concentration on the retention time of each vitamin, obtained by adding various amounts of SDS to the phosphate solution, pH 9.0. The retention time of each vitamin increased with increasing SDS concentration. VB_3 and VB_{12}, a combination not resolvable in CZE, were readily separated from each other over the whole range of SDS concentration examined. VB_{12} gave longer retention times and a greater increase than did VB_3, because the former was more easily incorporated into SDS, presumably due to higher hydrophobicity. VB_1 was greatly retarded by the addition of SDS, being eluted last, probably due to the interaction between the positive charge of VB_1 and the negative charge of SDS. At a SDS concentration of 0.05 *M* all these compounds were well separated from each other. Good separation was also obtained at 0.1 *M* but the retention times of all these compounds were longer than those at 0.05 *M*.

Fig. 3 shows the effect of the pH of the phosphate solution on retention time, obtained when the SDS concentration was 0.05 *M*. The retention time of each compound increased almost linearly with increasing pH in this constant-current mode. At pH 7.0, the pairs VB_3–VB_6 and VC–VB_2PNa were not separated. The separations of VC from VB_2PNa at pH 8.0 and of VB_{12} from VC at pH 10.0 incomplete. The phosphate solution of pH 9.0 gave the best separation.

Based on these results, the 0.02 *M* phosphate solution, pH 9.0, containing 0.05 *M* SDS, was used for the analysis of these vitamins. EAB was chosen as the internal standard, because it was well separated from these vitamins under the separation conditions adopted. Fig. 4 shows an electropherogram of these water-soluble vit-

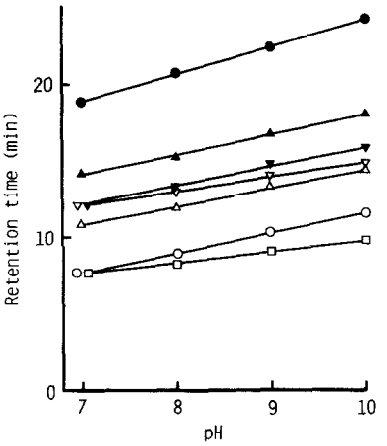

Fig. 3. Effect of the pH on the retention time in MECC. Carrier: 0.02 *M* phosphate solution containing 0.05 *M* SDS. Other experimental conditions as in Fig. 2.

amins together with the internal standard. The seven vitamins were completely resolved and eluted within *ca.* 22 min. The relative standard deviation of the relative retention time was within 0.6% for all these vitamins, indicating highly reproducible separation. The numbers of theoretical plates calculated as 5.54 $t_R^2\ w^{-2}$, where t_R is the retention time of a compound and w is the peak width at half the peak height, for VB_1, VB_2PNa, VB_3, VB_3', VB_6, VB_{12} and VC were 40 000, 40 000, 90 000, 30 000, 60 000, 120 000 and 70 000, respectively.

Quantification

The absorption maxima of these vitamins obtained under the aforementioned conditions ranged rather widely from 210 to 270 nm. Although individual vitamins could be monitored at the wavelength of their absorption maxima because a multi-

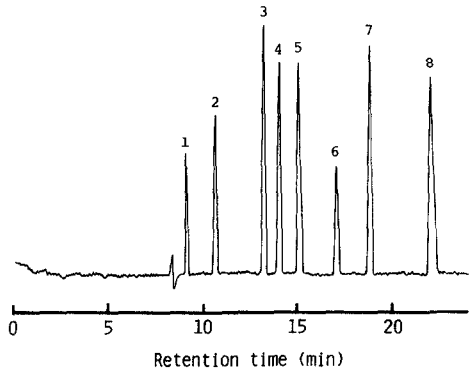

Fig. 4. Electropherogram of water-soluble vitamins. Carrier: 0.02 *M* phosphate solution, pH 9.0, containing 0.05 *M* SDS. The analytical conditions were as in Fig. 2. Peaks: 1 = VB_3; 2 = VB_6; 3 = VB_{12}; 4 = VC; 5 = VB_2PNa; 6 = VB_3'; 7 = EAB (internal standard); 8 = VB_1. The concentration of EAB was $1.00 \cdot 10^{-3}$ *M*.

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF WATER-SOLUBLE VITAMINS BY THE INTERNAL STANDARD METHOD

Sample concentration: $0.25 \cdot 10^{-3}$ (VB_2PNa and VB_{12}), $0.50 \cdot 10^{-3}$ (VB_1 and VC), $1.00 \cdot 10^{-3}$ M (VB_3, VB'_3 and VB_6). Internal standard: $1.00 \cdot 10^{-3}$ M EAB.

	*Sample-to-standard peak area ratio**						
	VB_1	VB_2PNa	VC	VB_3	VB'_3	VB_6	VB_{12}
$\bar{x}$**	1.18	1.02	0.81	0.36	0.55	0.64	0.82
s***	0.014	0.013	0.012	0.005	0.010	0.013	0.011
$s/\bar{x}$§	1.2	1.3	1.5	1.4	1.8	2.0	1.3

* Ten determinations for each sample.
** Mean.
*** Standard deviation.
§ Relative standard deviation.

wavelength UV detector was employed in the present work, multiple switching of wavelengths was laborious and accompanied by baseline fluctuation. Therefore, we selected 254 nm, where commercial mercury lamps emit most abundantly, as the common wavelength for the present system. Use of this single wavelength is profitable, since UV detectors employing mercury lamps are economical. At this wavelength, VB_1, VB_2PNa, VB_3, VB'_3, VB_6, VB_{12} and VC gave 62, 65, 29, 32, 25, 18 and 72%, respectively, of their maximum absorptions.

The reproducibility of the determination of these vitamins was examined by use of the peak-area ratio mode. Table I gives the relative standard deviation for ten

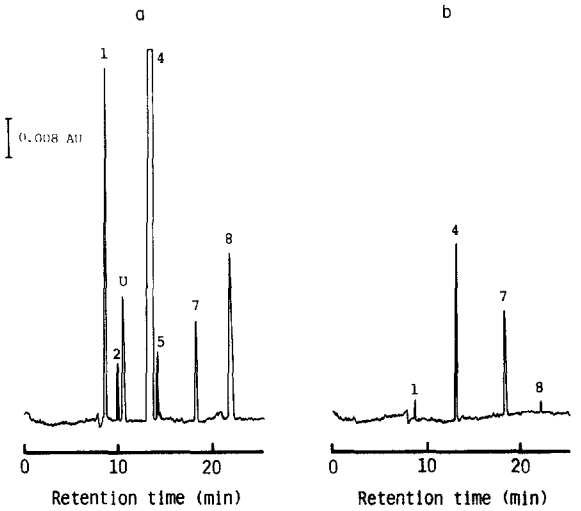

Fig. 5. Electropherograms of a commercial vitamin injection: (a) intact sample; (b) sample diluted 25-fold. The analytical conditions were as in Fig. 3. U = Unknown. Peaks as in Fig. 4.

TABLE II

ACCURACY AND REPRODUCIBILITY OF THE DETERMINATION OF THE INGREDIENTS OF A VITAMIN INJECTION

Ingredient	*Amount indicated on the label (w, mg/ml)*	*Amount found*			*Found/indicated ($\bar{x}$/w, %)*
		$\bar{x}$ (mg/ml)	*s (mg/ml)*	*$s/\bar{x}$ (%)*	
VB_1	5	5.07	0.062	1.2	101
VB_3	10	10.4	0.114	1.1	104
VB_6	1	1.00	0.013	1.3	100
VB_2PNa	0.5	0.52	0.011	2.1	104
VC	25	24.7	0.207	0.8	98.8

repeated determinations. In this experiment the concentration of the internal standard was fixed at $1.00 \cdot 10^{-3}$ *M*, and those of the vitamins were varied $0.25 \cdot 10^{-3}$, or $0.50 \cdot 10^{-3}$ or $1.00 \cdot 10^{-3}$ *M*, dependent on their molar absorptivities. The values of the relative standard deviation were in the range of 1.2–2.0%. In contrast to the peak-area ratio mode, the absolute peak-area mode gave higher values of the relative standard deviations, ranging from 5.5 to 9.7%. The calibration plots showed excellent linearity in the molar ratio ranges of 0.125–0.5 for VB_2PNa and VB_{12}, 0.25–1.0 for VB_1 and VC and 0.5–2.0 for VB_3, VB_3' and VB_6. The detection limits were at the 0.5, 1 and 4 pmol levels for the first, second and last groups of vitamins, respectively.

Analysis of the ingredients of a vitamin injection

We applied this method to the analysis of water-soluble vitamins in a commercial injection. Since the concentration of VC was extraordinarily high, it was determined after 25-fold dilution of the sample. Fig. 5a and b show the electropherograms of the intact and diluted samples, respectively. An unknown peak presumably attributable to an additive was observed at *ca.* 11 min, but it did not interfere with the analysis.

The ingredients of this preparation were quantified by the peak-area ratio mode, based on the results mentioned above. The data on the accuracy and reproducibility of the determination are given in Table II. The $\bar{x}$ value for each ingredient was very close (98.8–104%) to the indicated value, and the relative standard deviation, $s/\bar{x}$, in its estimation was less than 2.1%, demonstrating that this method is sufficiently accurate and reproducible.

A few reports have been published on high-performance liquid chromatography of water-soluble vitamins, *e.g.*, refs. 16–20. However, these methods were disadvantageous, because they either required gradient elution[17,18], gave imperfect separation[19] or suffered from peak broadening[20] as well as tailing[16–18]. The MECC method proposed allowed complete separation of seven water-soluble vitamins with extremely high numbers of theoretical plates. The analysis time was only *ca.* 22 min. Furthermore, quantification was highly accurate and reproducible.

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