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Analytica Chimica Acta 570 (2006) 8–14
ANALYTICA CHIMICA ACTA
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Determination of avermectins in commercial formulations using microemulsion electrokinetic chromatography

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Received 22 February 2006; received in revised form 30 March 2006; accepted 30 March 2006
Available online 18 April 2006

Abstract

Microemulsion electrokinetic chromatography (MEEKC) was developed for quantitative analysis of avermectins, such as abamectin, doramectin and ivermectin, in commercial formulations, using the microemulsion buffer containing a 50 mM phosphate buffer at pH 2.5, 1.1% (v/v) *n*-octane as oil droplets, 180 mM sodium dodecylsulphate as surfactant, 890 mM 1-butanol as co-surfactant and 30% (v/v) ethanol as organic co-solvent. High accuracy and precision of the method were obtained. The contents of avermectins in commercial formulations determined by MEEKC were found to be insignificantly different with those determined by high performance liquid chromatography (HPLC). Therefore, MEEKC can be used an alternative method to HPLC for quantitative determination of avermectins.

Keywords: Avermectins; Abamectin; Doramectin; Ivermectin; Microemulsion electrokinetic chromatography; Capillary electrophoresis

1. Introduction

Avermectins are antibiotic macrocyclic lactone compounds produced by fermentation of soil bacterium *Streptomyces avermitilis* [1,2]. They are widely used in agriculture and farm animals for treatment of a broad spectrum of parasitic diseases. The structures of typical avermectins, such as abamectin, ivermectin and doramectin, are shown in Fig. 1. Abamectin or avermectin B_1, used as insecticide and acaricide, consists of avermectin B_{1a} as major component (more than 80%) and avermectin B_{1b} as minor component (less than 20%). Ivermectin or 22,23-dihydroavermectin B_1 is commonly used as antiparasitic or anthelmintic agent for many farm animals such as cattle, horse, sheep, swine and dog. Doramectin or 25-cyclohexylavermectin B_1 is usually used for cattle and some farm animals [3]. Much of previous work on analysis of avermectins involved avermectin residue using high performance liquid chromatography (HPLC) with direct UV detection [4,5], mass spectrometry (MS) [6–13], and fluorescence detection of derivative avermectins [14–16]. Advantages of MS and fluorescence over UV for detection in HPLC are better selectivity and sensitivity. In addition, MS can provide structural information on the resolved peaks. Therefore, HPLC with MS or fluorescence detection is suitable for trace analysis of avermectins.

A conventional technique used for quantitative analysis of each avermectin in commercial formulations is HPLC with UV detection. Since the commercial formulations of avermectins also consist of organic solvent, emulsifier and oil, the direct method for HPLC analysis of samples requires long analysis time to remove highly hydrophobic compounds that strongly retain in an HPLC column. Capillary electrophoresis (CE) has been shown to be a powerful and alternative method to HPLC for direct analysis of samples without removing matrix, and with fast flushing matrix after each run [17–20]. Additional advantages of CE include high efficiency, short analysis time and many applications for both hydrophobic and hydrophilic compounds.

Since avermectins are neutral and hydrophobic, CE analysis of avermectins is challenging. Recently, microemulsion electrokinetic chromatography (MEEKC) has been reported to be an alternative way for analysis of varieties of hydrophobic compounds such as fat-soluble vitamins [21], steroid [22], UV filter in sunscreen lotion [23], polymer additives [24] and curcuminoids [20]. Up to date, CE has not been previously reported for analysis of avermectins. Therefore, the aims of this work are to

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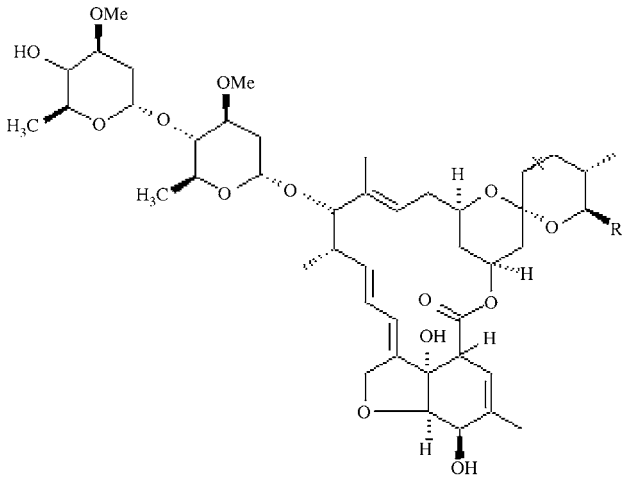
doi:10.1016/j.aca.2006.03.110



Avermectims	R	X
Abamectin B_{1a}	sec-butyl	-CH=CH-
Abamectin B_{1b}	isopropyl	-CH=CH-
Ivemectin	> 80% sec-butyl < 20% isopropyl	$-CH_2=CH_2-$
Doramectin	cyclohexyl	-CH=CH-

Fig. 1. Chemical structures of avermectins.

develop MEEKC as an alternative method for analysis of avermectins and to compare MEEKC and HPLC for determination of avermectins in commercial formulations. MEEKC separation was carried out using the microemulsion buffer containing a phosphate buffer at pH 2.5, *n*-octane as oil droplets, sodium dodecylsulphate (SDS) as surfactant, 1-butanol as co-surfactant and appropriate types and amounts of organic co-solvent.

2. Experimental

2.1. Chemicals

Standards of abamectin B_{1a}, ivermectin and doramectin were purchased from Dr. Ehrenstorfer (Augsburg, Germany), SDS and ethyl 4-hydroxybenzoate from Sigma–Aldrich (Steinheim, Germany), and 1-butanol and *n*-octane from Fluka (Buchs, Switzerland). All organic solvents at least HPLC grade and phosphoric acid were obtained from Merck (Darmstadt, Germany). Commercial formulations of avermectins were purchased from a market in Thailand, and the actual brand names cannot be disclosed.

2.2. Preparation of microemulsion buffer

The microemulsion buffer was prepared by pipetting the appropriate amount of stock aqueous solutions of 500 mM SDS and 500 mM phosphate buffer at pH 2.5 into a 10 ml volumetric flask, followed by pipetting 110 μl of *n*-octane, and 814 μl of 1-butanol [20]. The final solution was made up to 10 ml with Milli-Q water. In case of preparation of the microemulsion buffer containing organic co-solvent such as methanol, ethanol, 2-propanol or acetonitrile, the appropriate volume of organic co-solvent was added before addition of water. All microemulsion buffers were sonicated for 30 min to obtain clear and highly stable microemulsion. The microemulsion buffers were filtered through 0.45 μm membrane filters prior to MEEKC analysis.

2.3. Preparation of standard solutions and diluted sample solutions

Stock solutions of 5000 ppm abamectin, ivermectin and doramectin were separately prepared by weighing an appropriate amount of each standard and then dissolving this in ethanol. For CE analysis, the working standard solutions containing abamectin, ivermectin and doramectin in a range of 25–400 ppm were prepared by pipetting the appropriate amounts of the stock standard solutions, 100 μl of 5000 ppm dodecylbenzene (DB) in ethanol and 100 μl of 5000 ppm ethyl-4-hydroxybenzoate in ethanol, and then diluting the mixture with the microemulsion to have final volume of 5.0 ml. Therefore, each final working standard solution contained 100 ppm DB as a microemulsion marker and 100 ppm ethyl-4-hydroxybenzoate as internal standard (ISTD). The microemulsion used for diluting working solution contained 180 mM SDS, 890 mM 1-butanol and 1.1% (v/v) *n*-octane. For HPLC analysis, the working standard solutions containing abamectin, ivermectin and doramectin in a range of 10–100 ppm were prepared by pipetting the appropriate amounts of the stock standard solutions and then diluting the mixture with methanol to have final volume of 5.0 ml.

Stock sample solutions for MEEKC analysis were prepared by weighing the appropriate amounts of samples and then dissolving these in ethanol. The appropriate amounts of stock sample solutions were then diluted, typically 10-fold, with the microemulsion without organic co-solvent in a 5.0 ml volumetric flask, to give the diluted sample solutions containing 100 ppm DB and 100 ppm ISTD. For HPLC analysis, the diluted sample solutions were obtained by diluting stock sample solutions with methanol. All solutions were filtered through 0.45 μm membrane filters prior to MEEKC or HPLC analysis.

2.4. Instrumental analysis

2.4.1. CE conditions

All CE experiments were performed on a Beckman Coulter MDQ-CE system equipped with a photodiode array detector scanning from 190 to 400 nm and monitoring at 214 and 245 nm. An uncoated fused silica capillary used was 40.2 cm in length (30 cm to detector) × 50 μm I.D., thermostated at 25 °C. Voltage was set at −15 kV. A sample solution was introduced by 0.5 psi pressure injection for 3 s. The microemulsion buffer contained a 50 mM phosphate buffer at pH 2.5, 1.1% (v/v) *n*-octane, 180 mM SDS, 890 mM 1-butanol and 30% (v/v) ethanol, unless otherwise stated. Prior to MEEKC analysis each day, the capillary was rinsed with ethanol, 0.1 M NaOH and the microemulsion buffer for 15 min each. Between consecutive runs, the capillary was rinsed with ethanol and the microemulsion buffer for 2 min each. After analysis each day, the capillary was rinsed with ethanol for

5 min, water for 5 min, 0.1 M NaOH for 10 min and then water for 10 min.

2.4.2. HPLC conditions

All HPLC experiments were carried out on a Waters™ HPLC system equipped with a Waters™ 600 controller pump, a Waters™ 996 photodiode array detector scanning from 190 to 400 nm and monitoring at 245 nm, and a Waters™ 717 plus autosampler. The injection volume of samples was 10 μl. The analytical column used was 250 mm × 4.6 mm I.D., 5 μm Hypersil BDS C_{18} from Thermo Finnigan. Mobile phase and elution conditions used in this work were carried out according to the HPLC analysis of abamectin formulation reported by the Department of Agriculture, the Ministry of Agricultural and Cooperatives, Thailand. Solvents A, B and C are acetonitrile, methanol and water, respectively. The gradient elution conditions with flow rate 1 ml/min and room temperature were initially A:B:C with 60:0:40, programming to 60:30:10 over 40 min, programming to 90:0:10 over 10 min, and programming to 60:0:40 over 1 min and holding for 9 min. The total analysis time used is 60 min each run. Before HPLC analysis each day, HPLC column equilibration was accomplished by using the initial mobile phase condition for at least an hour, and the HPLC column was flushed with methanol for an hour after HPLC analysis each day.

3. Results and discussion

3.1. MEEKC optimization

Since avermectins are hydrophobic compounds, MEEKC analysis was performed using an acidic buffer at pH 2.5 in order to suppress electroosmosis and to obtain short analysis time [20,21,25,26]. In initial work, MEEKC analysis of a mixture of abamectin B_{1a} (A), ivermectin (I) and doramectin (D) standards was carried out using a microemulsion buffer without organic co-solvent (Fig. 2a). Dodecylbenzene (DB), a highly hydrophobic compound, spiked into a sample solution is used as a marker for migration time of negatively charged oil droplet [20,27]. Ethyl 4-hydroxybenzoate was spiked as an internal standard for quantitative analysis of avermectins. Without organic co-solvent (Fig. 2a), no resolution of avermectins was obtained, and co-retention of these compounds and DB was observed. This indicates that these compounds strongly and completely partitioning into the oil droplet phase.

Organic solvents at 0–35% (v/v), such as acetonitrile, methanol, ethanol and 2-propanol, were separately added in the microemulsion buffer, in order to obtain the difference in partitioning of these analytes into the aqueous phase [20,21,27]. Figs. 2b–e show an example of electropherograms of avermectins using 30% (v/v) organic solvent in the MEEKC buffer. All three peaks of avermectins were observed to have UV spectra with the maximum absorbance at wavelength of 245 nm. DB slightly absorbs UV light at wavelength 245 nm, and therefore the DB peak is not observed in Figs. 2b–e. Fig. 3 compares the effects of types and concentrations of organic solvents on the migration time of abamectin B_{1a} and resolution of doramectin and abamectin. Addition of organic co-solvent resulted in an

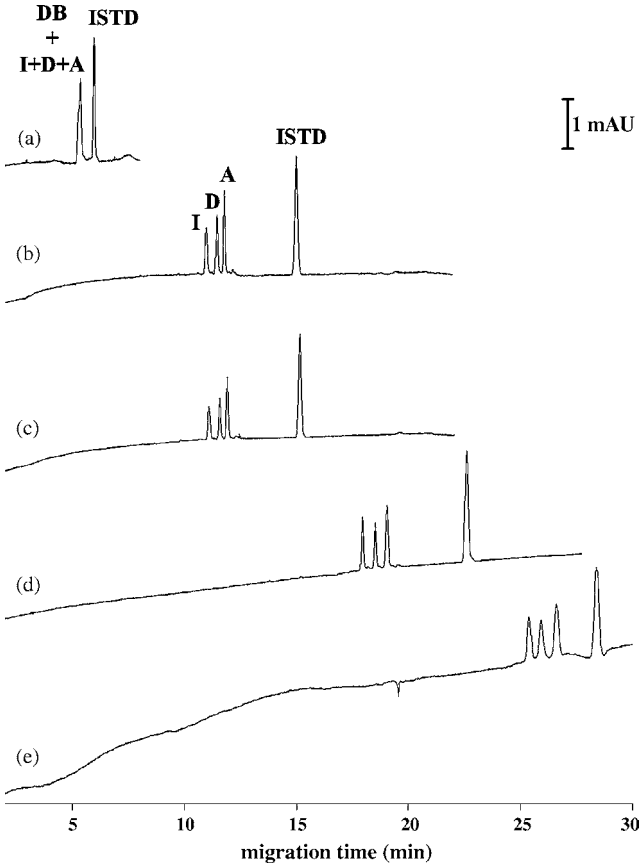


Fig. 2. MEEKC separation of avermectins using (a) no organic co-solvent and 30% (v/v) organic co-solvent as (b) acetonitrile, (c) methanol, (d) ethanol, and (e) 2-propanol in a pH 2.5 50 mM phosphate buffer containing 1.1% (v/v) *n*-octane, 180 mM SDS and 890 mM 1-butanol. CE conditions: uncoated fused silica capillary 50 μm I.D. × 40.2 cm (30 cm to detector), temperature 25 °C, voltage −15 kV, 0.5 psi pressure injection for 3 s and UV detection at 214 nm (a) and 245 nm (b–e).

increase in migration time due to a decrease in retention factor of analytes and an increase in the viscosity of the microemulsion buffer [20,27]. It should be noted that, in MEEKC with suppressed electroosmosis as in this work, the smaller the retention factor, the longer the migration time as discussed in our previous work [20]. Migration time order was found to be I < D < A in the microemulsion buffer containing organic co-solvents. In a range of 20–35% (v/v) co-solvent in the microemulsion buffer, 2-propanol gave the slight difference in resolution of D:A (Fig. 3b) and also resolution of I:D which is not shown in this work. Addition of 20–30% (v/v) methanol, ethanol or acetonitrile in the microemulsion buffer resulted in an increase in resolution of analytes, while the slight difference in resolution was obtained at 30 and 35% (v/v) organic co-solvent. Ethanol at 30% (v/v) provided highest resolution of analytes and was chosen as organic co-solvent in the microemulsion buffer in this work, with baseline resolution values of 4.9 and 4.5 for I:D and D:A, respectively (Fig. 3b). In addition, peaks of I, D and A were separated from peaks of other compounds in test samples.

Fig. 4 shows the effect of SDS concentration in the microemulsion buffer on migration time and resolution of avermectins. An increase in SDS concentration resulted in

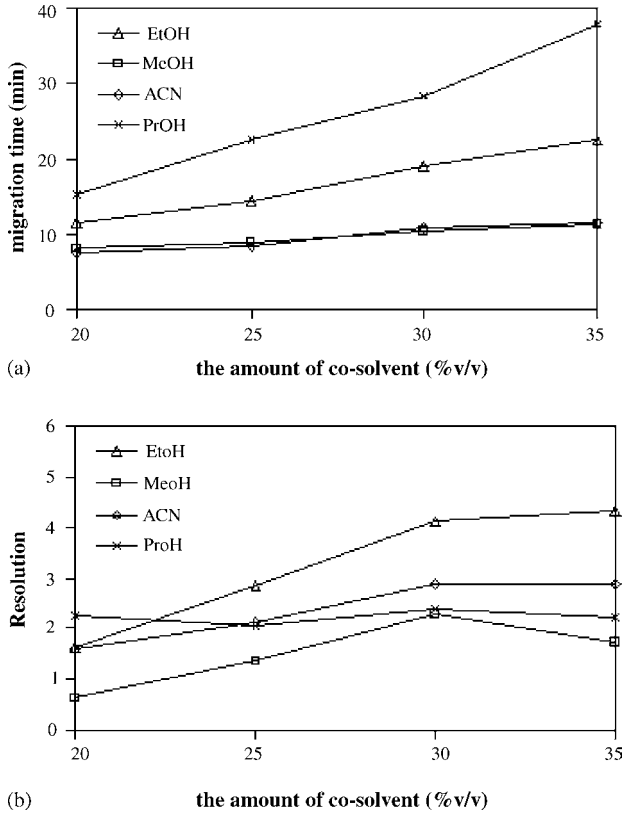


Fig. 3. Effect of types and concentrations of organic co-solvent on (a) migration time of A and (b) resolution of D:A. Acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), and 2-propanol (PrOH). Other CE conditions as shown in Fig. 2d.

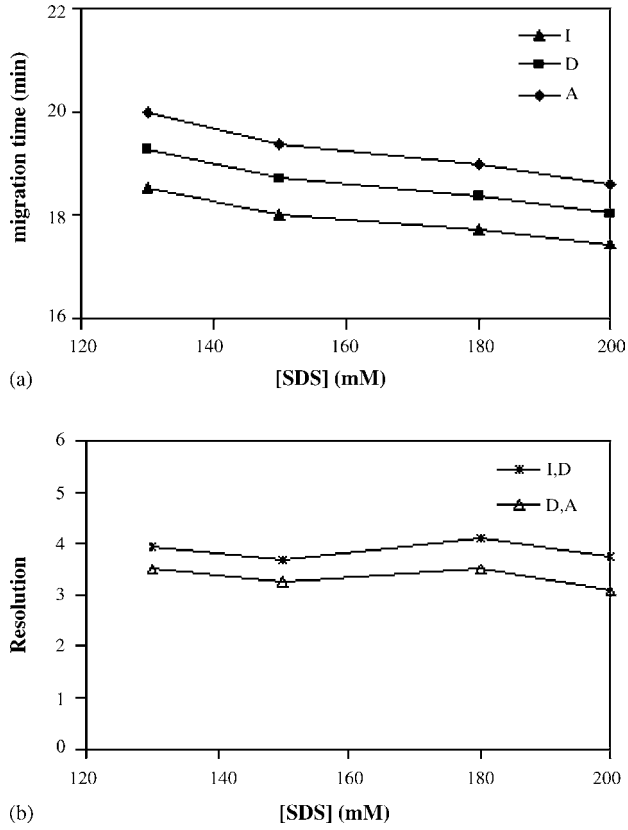


Fig. 4. Effect of SDS concentration on (a) migration time and (b) resolution of avermectins. Other CE conditions as shown in Fig. 2d.

faster analysis time due to the greater retention factor caused by an increase in phase ratio [20]. With increasing 130–200 mM SDS, a slight change in the resolution of analytes was found due to a slight change in selectivity and an increase in retention factor and efficiency (Fig. 4b), calculated using equations as reported in our previous work [20]. Therefore, the following MEEKC conditions were chosen for quantitative determination of avermectins: 50 mM phosphate buffer at pH 2.5, 1.1% (v/v) *n*-octane as oil droplets, 180 mM SDS as surfactant, 890 mM 1-butanol as co-surfactant and 30% (v/v) ethanol as organic co-solvent; applied voltage of −15 kV; and separation temperature 25 °C. Faster analysis time can be obtained using higher temperature and/or voltage, but poorer resolution of analytes was found due to Joule heating, which was also observed in our previous work on MEEKC analysis of curcuminoids [20].

3.2. Validation of method

Limit of detection (LOD) and limit of quantitation (LOQ) for analytes are defined as the analyte concentration giving signal-to-noise of 3 and 10, respectively. Results of LOD and LOQ for avermectins in MEEKC are shown in Table 1. For HPLC analysis, LODs of I, D and A were obtained to be 0.28, 0.30 and 0.26 ppm, respectively, approximately 20 times better than LODs in MEEKC. It is well known that LOD in CE is typically higher than that in HPLC, due the larger amount of analytes injected and the longer cell path length in HPLC. LOD in CE may be improved by using a bubble cell capillary to increase cell path length and/or using off-line and/or on-column sample preconcentration techniques. However, it is not necessary for this work because the large amount of avermectins in formulation is determined.

For quantitative analysis, ethyl 4-hydroxybenzoate was used as an internal standard due to its UV absorption in the region of

Table 1
Calibration plot, LOD and LOQ for analytes in MEEKC

Avermectins	Concentration range (ppm)	Linear equation			LOD[a] (ppm)	LOQ[a] (ppm)
		Slope	Intercept	r^2		
I	25–400	0.00330	0.00133	0.9985	5.3	16.8
D		0.00311	−0.00172	0.9993	6.0	18.2
A		0.00468	−0.02884	0.9993	4.5	14.6

[a] The average values were obtained from triplicate runs.

Table 2
MEEKC analysis of the amounts of standard avermectins spiked in the microemulsion and the diluted solution of samples

Matrix	Avermectins	Spiked (ppm)	Mean recovery (%)	R.S.D. (%)
Microemulsion[a]	I	50	99.7	1.1
		200	99.0	1.3
	D	50	101.3	1.7
		200	98.7	1.1
	A	50	99.3	1.0
		200	99.6	1.3
A diluted solution of sample[b]	I	50	99.2	1.4
		200	98.7	1.5
	D	50	103.4	1.3
		200	100.4	1.8
	A	50	99.2	1.0
		200	102.6	1.4

$n = 5$ runs.
[a] Microemulsion contained 180 mM SDS, 1.1% (v/v) *n*-octane, 890 mM 1-butanol, 30% (v/v) ethanol and water.
[b] An appropriate amount of sample was diluted with microemulsion.

245 nm and its migration time separating from avermectins and other compounds in samples. Calibration plots were established from the ratio of corrected peak area of the analyte to that of internal standard, $A_{corr,ratio}$, against the analyte concentration for six levels in a range of 25–400 mM, where corrected peak area is defined as the peak area divided by migration time [28]. High linear relationship of $A_{corr,ratio}$ and the concentration of each avermectin was obtained with $r^2 \approx 0.999$, as shown in Table 1.

The effect of sample matrix on accuracy of the method was investigated by spiking a mixture of avermectin standards with known amounts in the microemulsion and separately spiking each avermectin with known amounts in the diluted solution of samples containing an interested avermectin. Each MEEKC experiment was carried out for five runs. Results in Table 2 show high accuracy of the method with the recoveries for spiked standard ranging from 98.7 to 103.4% with R.S.D. < 2.0%. In addition, the sample matrix was found to give no effect on the accuracy and precision due to the similar range of the recovery and R.S.D. for standard spiked in the microemulsion and samples.

The intraday and interday precision in migration time and $A_{corr,ratio}$ was determined using 100 ppm avermectin standards. For the intraday precision, the values of the mean and R.S.D. were obtained from five separate runs each day, while 5 days for the interday precision. Results in Table 3 indicate high precision in migration time and $A_{corr,ratio}$ for the intraday, with R.S.D. < 2.0%. The values of R.S.D. for interday precision were found to be <3.0 and <2.0 for migration time and $A_{corr,ratio}$, respectively, also indicating high interday precision.

3.3. Application to real samples

The amounts of avermectins in commercial samples were determined by MEEKC and HPLC. Fig. 5 shows an example of MEEKC electropherograms. Similar patterns of electropherograms monitoring at 245 nm were found for abamectin formulations of samples A1, A2 and A3 (Figs. 5b–d). Peaks of analytes in samples were identified by comparing their UV spectra in a range of 190–400 nm with the UV spectra of standards and using a spiking technique. From UV detection at 214 nm as shown in Fig. 5a, several peaks of highly hydrophobic compounds in abamectin formulations A1, A2 and A3 were observed to overlap and to be near the DB peak. This is a reason why direct HPLC analysis of avermectin formulations requires long time to

Table 3
Intraday and interday precision in migration time and $A_{corr,ratio}$ of 100 ppm avermectins

Precision	R.S.D. (%) and mean of t_m (min)			R.S.D. (%) and mean of $A_{corr,ratio}$		
	I	D	A	I	D	A
Intraday[a]						
Day 1	0.7 (18.07)	0.7 (18.77)	0.8 (19.42)	2.1 (0.341)	1.8 (0.319)	0.5 (0.437)
Day 2	1.2 (17.54)	1.3 (18.20)	1.3 (18.82)	1.8 (0.339)	0.8 (0.313)	0.7 (0.434)
Day 3	0.4 (17.59)	0.4 (18.26)	0.4 (18.88)	1.7 (0.337)	2.0 (0.313)	0.9 (0.435)
Day 4	0.9 (18.44)	1.0 (19.17)	1.0 (19.85)	0.6 (0.346)	0.4 (0.313)	1.1 (0.432)
Day 5	0.7 (18.52)	0.7 (19.26)	0.7 (19.95)	1.0 (0.345)	1.2 (0.316)	1.4 (0.437)
Interday[b]						
Overall	2.5 (18.03)	2.6 (18.73)	2.7 (19.38)	1.8 (0.342)	1.5 (0.315)	1.0 (0.435)

The values of mean are in parentheses.
[a] $n = 5$ runs for each day.
[b] $n = 5$ days.

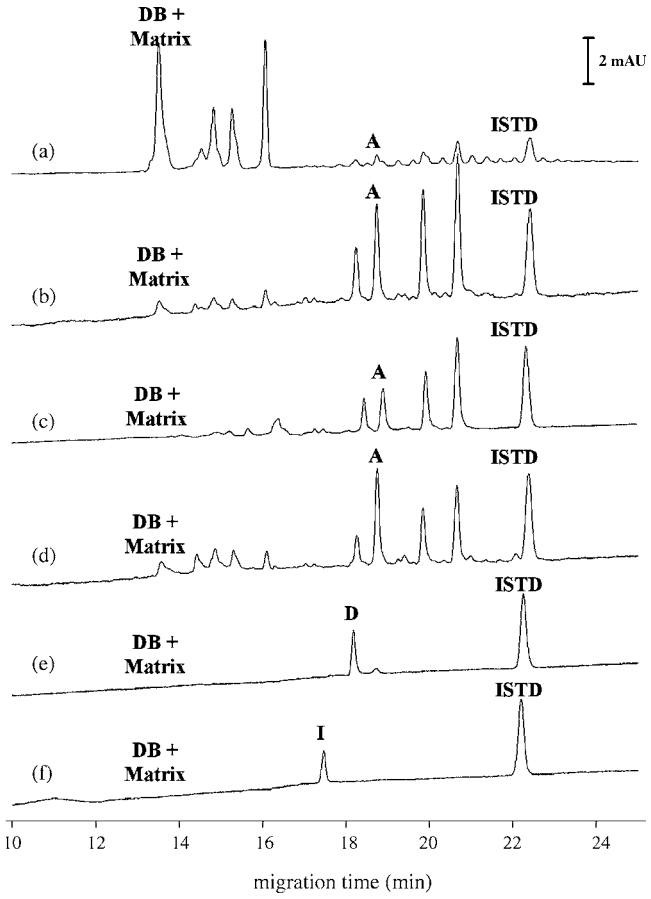


Fig. 5. An example of electropherograms of avermectin formulations of samples A1 (a and b), A2 (c), A3 (d), D1 (e) and I1 (f). UV detection at 214 nm (a) and 245 nm (b–f). Other CE conditions as shown in Fig. 2d.

remove these compounds from an HPLC column. By separately spiking ivermectin and doramectin standard in samples A1, A2 and A3, results from MEEKC and HPLC analyses showed that these samples contained neither ivermectin nor doramectin. Table 4 lists a comparison of the contents of avermectins in commercial formulations determined by MEEKC and HPLC. Using paired *t*-test analysis at 95% confidence interval of the mean, MEEKC and HPLC gives non-significant difference in the determined amounts of avermectins in each sample. Good agreement was obtained for determined and labeled amounts of avermectins in each sample, except for sample I1 where the determined amount of ivermectin was found to be approximately a half of the labeled amount. The experiment for sample I1 was repeated, and same results were obtained. This difference of determined and labeled amounts for sample I1 is not known.

Table 4
The amounts of avermectins in samples determined by MEEKC and HPLC

Sample	Avermectins	Content (%, w/v)		
		Labeled	Determined	
			MEEKC	HPLC
A1	A	1.8	1.70 ± 0.01	1.71 ± 0.01
A2	A	1.8	1.58 ± 0.01	1.56 ± 0.01
A3	A	1.8	1.76 ± 0.01	1.77 ± 0.02
D1	D	1.0	0.99 ± 0.01	0.98 ± 0.01
I1	I	1.5	0.77 ± 0.01	0.76 ± 0.01

4. Conclusion

This work is first reported that MEEKC can be used as an excellent method for quantitative determination of avermectins in commercial formulations. High accuracy and precision of the method was obtained with the recovery of 98.1–103.4% and R.S.D. < 2% for avermectin standards spiked in the microemulsion buffer and the diluted samples. High precision in migration time and the ratio of corrected peak area of the analyte to that of internal standard was found for intraday and interday. In addition, no difference in the determined amounts of avermectins in samples was obtained using MEEKC and HPLC analysis. Thus, MEEKC can be used an alternative method for quantitative determination of avermectins in commercial formulations. Advantages of MEEKC over HPLC include the less amount of organic solvent consumption and faster analysis time, 30 min for MEEKC and 60 min for HPLC each run. The MEEKC method will be used for analysis of avermectins in fermentation broth in our future work.

Acknowledgements

This work was financially supported by the Graduate School, Chulalongkorn University.

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