ANEXO II

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Journal of Chromatography B, 752, 2001, 99-105

ELSEVIER

Journal of Chromatography B, 752 (2001) 99–105

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Resolution of isomers of sorbitolparaben esters by chromatographic and electrophoretic techniques

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Received 13 June 2000; received in revised form 21 September 2000; accepted 21 September 2000

Abstract

Pharmaceutical preparations usually contain preservatives and sweeteners. When parabens are used as preservatives and a polyol as a sweetener, a transesterification reaction may happen, yielding the transester polyol-paraben. The products formed in the transesterification reaction of methylparaben and sorbitol were analyzed by micellar electrokinetic chromatography and by HPLC. Up to six positional isomers of sorbitolparaben (SPB) can be produced. However, only three peaks were found by HPLC. The higher efficiency and resolution power of MEKC allowed one to resolve five peaks. Results were compared with those obtained by capillary zone electrophoresis in borate buffer, where the separation of isomers occurred in a different way, because of a complexation between SPB and borate.

Keywords: Sorbitol; Methylparaben

1. Introduction

The joint use of parabens as preservatives and polyols as sweeteners or stabilizers is very extensive in the pharmaceutical, cosmetic and food industries. However, it has been proved that this mixture is not stable, because a slow transesterification reaction takes place [1–3], yielding different degradation products that should be controlled to guarantee the preservative capability. Sorbitol is one of these frequently used polyols, which reacts with methylparaben (MPB) to form the ester sorbitolparaben (SPB). MPB and SPB can also hydrolyze to form *p*-hydroxybenzoic acid (PHBA) and both reactions reduce the preservative capability of the formulation, since PHBA has lower preservative properties. Because of the six sorbitol hydroxyl groups, and the different conformation of the secondary carbons, six different positional isomers of SPB can be formed. Runesson and Gustavii [1] and Hensel et al. [3] studied this reaction by high-performance liquid chromatography (HPLC), but failed in an attempt to resolve the mixture of isomers, since only three peaks, partially overlapped, were obtained.

In a previous paper, we described the application of capillary zone electrophoresis (CZE) to the analytical control of a pharmaceutical aqueous formulation, which contained MPB as preservative and an excess of sorbitol as sweetener [4]. We demonstrated the efficiency of CZE in the analytical control of the formulation stability, determining the degradation

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00526-0

products of MPB. However, the mixture of SPB isomers was not completely resolved.

There are several works on the use of capillary electrophoresis (CE) for the separation of geometrical and positional isomers. The effects of experimental parameters on the separation of positional isomers of aminobenzoic acid [5], the separation of phenylenediamine isomers [6] and the separation of *cis* and *trans* double-bond isomers [7] are some of these works that have been developed by CZE. More recently, micellar electrokinetic chromatography (MEKC) has been applied to separate positional and geometrical isomers of several analytes: dichlorophenols [8], nitrophenols [9] and retinoic acid [10].

The aim of this work is to compare the capacity of resolution of HPLC with electrophoretic techniques, like CZE and MEKC, in the separation of neutral positional isomers. A mixture of SPB isomers was used in this work as test substances to demonstrate the potential of MEKC in these kind of analysis. MEKC combines electrophoretic and chromatographic characteristics [11] so a better resolution than obtained by CZE or HPLC is expected.

2. Experimental

2.1. Apparatus

CE measurements were made on a Hewlett-Packard Model ^{3D}CE instrument (Waldbronn, Germany) equipped with a diode-array detector, automatic injector and sampler, and a system for thermostating the capillary within ±0.1°C over the range 10–60°C. Hydrodynamic injection at the anode end (accomplished by applying a pressure of 50 mbar for 5 s to the injection vial) was used throughout. A Sugelabor (Madrid, Spain) fused-silica capillary of 54.5 cm (effective length 56 cm)×50 μm I.D. with a normal lightpath was used in all experiments. The experimental set-up was governed, and data acquired and processed, using a HP ^{3D}CE Chemstation.

The HPLC system consisted of: a Shimadzu Model LC-10AD pumping system (Kyoto, Japan), a Rheodyne six-way injection valve with 20-μl injection loop (Cotati, CA, USA), C_{18} Tracer Spherisorb column, 5 μm, 15×0.4 cm from Teknokroma (St. Cugat del Vallés, Spain) and a UV–Vis diode-array detector (Hewlett-Packard Model 1040A HPLC). Data were acquired and processed with a Data Station (Hewlett-Packard Model 9153C).

The preparative HPLC system used was a Waters HPLC 600 (Milford, MA, USA) with a Waters 486 UV–Vis diode-array detector. Data were acquired in a Waters Data Module 746 and the column used was a Bondapak C_{18} cartridge, 15–20 μm, 100×25 mm, integrated in a Waters LC25 preparative module.

pH measurements were made by means of a Crison micropH 2001 pH meter (Alella, Spain).

2.2. Reagents

The reagents used included: methylparaben (methyl 4-hydroxybenzoate) from Fluka (Buchs, Switzerland), D-(−)-sorbitol from Panreac (Montcada i Reixac, Spain), disodium tetraborate decahydrate and disodium hydrogenphosphate dihydrate from Merck (Darmstadt, Germany), sodium dodecyl sulfate (SDS) from Panreac, HPLC-grade methanol from Promochem (Wesel, Germany), and NaOH from Carlo Erba (Milan, Italy). Milli-Q water from a Millipore water purification system (Molsheim, France) was used throughout.

Sorbitolparabens were not commercially available so we had to prepare them by reaction of methylparaben with an excess of sorbitol. Two solutions containing 35% of sorbitol, and 2.2 mg/ml of MPB were prepared, adjusting the pH to 8, in order to accelerate the transesterification reaction. These solutions were kept for 3 months, one at room temperature and the other at 60°C inside an oven. After this time, they were analyzed by HPLC, CZE and MEKC.

2.3. Procedures

2.3.1. MEKC

Background electrolyte (BGE) was prepared by dissolving disodium hydrogenphosphate dihydrate in Milli-Q water, and adjusting pH to 7. When the BGE was to contain surfactant, appropriate amounts of SDS, always over the critical micellar concentration (CMC), were dissolved in the electrolyte. All solutions were passed through a filter of 0.2 μm pore-size and degassed prior to insertion into the capillary.

Prior to each experiment batch, the capillary was

successively flushed with 1 *M* NaOH, 0.1 *M* NaOH, Milli-Q water and BGE for 5 min each, and then equilibrated at an applied voltage of 25 kV for 10 min. Between batch runs, the capillary was flushed with 0.1 *M* NaOH, Milli-Q water and BGE for 3, 5 and 5 min, respectively. Then the capillary was equilibrated at a voltage of 25 kV for 10 min. Detection was carried out at 255 nm.

2.3.2. HPLC

A mobile phase of MeOH–water (30:70) at a constant flow of 1 ml/min and a detection wavelength of 254 nm was used. Injection volume was 20 μl.

2.3.3. CZE

The same conditions as described in a previous work [4] were used. These conditions consisted of: 20 m*M* borate buffer at pH 10 as BGE, 25°C, 22 kV. The detection was carried out at 300 nm, the wavelength at which parabens presented an absorption maximum in basic medium.

3. Results

Since SPBs were not commercial products, they had to be prepared by reaction of MPB and sorbitol. The structures of sorbitol and SPB are shown in Fig. 1. From the two starting solutions, the one heated at 60°C could not be used, because the hydrolysis of MPB and SPB yielding PHBA was also accelerated and the concentration of SPBs obtained by this procedure was lower than that obtained keeping the reaction at room temperature. The injected solution contained SPB esters, unreacted MPB and PHBA formed in the hydrolysis of esters.

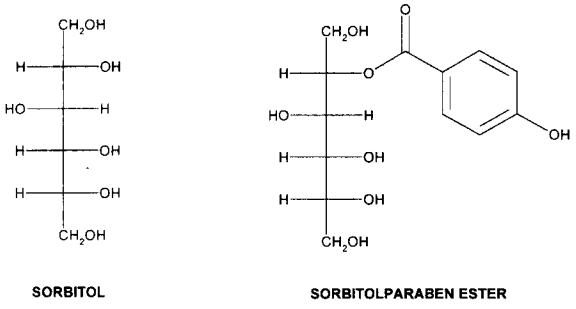

Fig. 1. Structures of sorbitol and one of the six possible isomers of sorbitolparaben esters.

Since the same solution was analyzed by the three techniques and the MPB peak was always well resolved and could be precisely measured, the ratio SPBs/MPB has been used as an indicator of the total amount of SPBs eluted. If all the isomers were measured, the total amount of SPB should be the same in the three cases and so should the area ratio.

3.1. Analysis by HPLC

Fig. 2 shows a typical chromatogram obtained with the working conditions. The first peak eluted was identified as PHBA. The next three peaks corresponded to the different isomers of SPB, formed in the transesterification, and the last peak was the remaining MPB. The area ratio of the three SPB peaks was the same of those obtained by Runesson and Gustavii [1]: 5%, 20% and 75%. Runesson and Gustavii [1] analyzed the major peak by nuclear magnetic resonance (NMR) and assigned it to a mixture of the two primary monoesters so the minor peaks corresponded to the secondary transesters. These proportions can be explained by the fact that primary hydroxyl group has a lower steric impediment than the secondary group and should react to a greater extent. Also, the ratio SPBs/MPB was calculated to be 7.0%.

3.2. Analysis by CZE

The isomers of SPB elute later than MPB in the form of two peaks, with approximately the same area (Fig. 3). The order of elution can be explained on basis of a complexation reaction between the polyol chain and tetraborate ion from the buffer solution [12,13], which increases the negative charge on SPB.

The ratio SPBs/MPB in the electropherogram was calculated to be about 7.1%, similar to that obtained in the HPLC analysis. The ratio of the two peaks of SPB obtained by CZE did not correspond to any possible combination of the ratio of the peaks obtained by HPLC. It seems reasonable to think that SPB esters were eluted with quite different separation mechanisms and this led to the differences in the number of peaks and the ratio in which they were

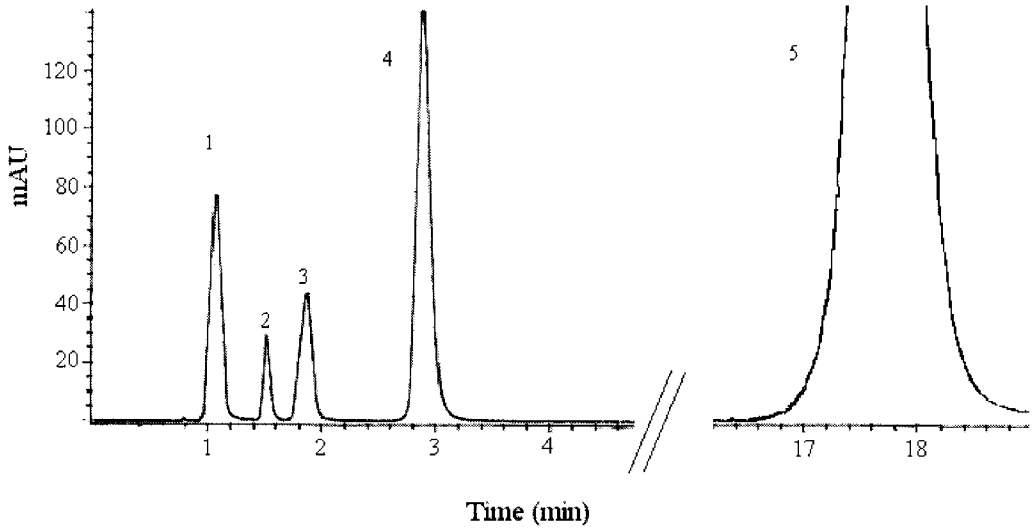

Fig. 2. Chromatogram obtained after injecting the solution containing sorbitolparabens. Peaks: 1=*p*-hydroxybenzoic acid; 2, 3, 4= sorbitolparaben isomers; 5=methylparaben. Conditions: MeOH–water (30:70), 1 ml/min. Detection wavelength: 254 nm.

obtained. To get a better understanding of the separation process involved and to find a relation between the different proportions in which the SPB peaks were obtained by HPLC and by CZE, a preparative chromatography separation, using the same conditions of the analytical chromatography, was carried out and the major SPB peak collected.

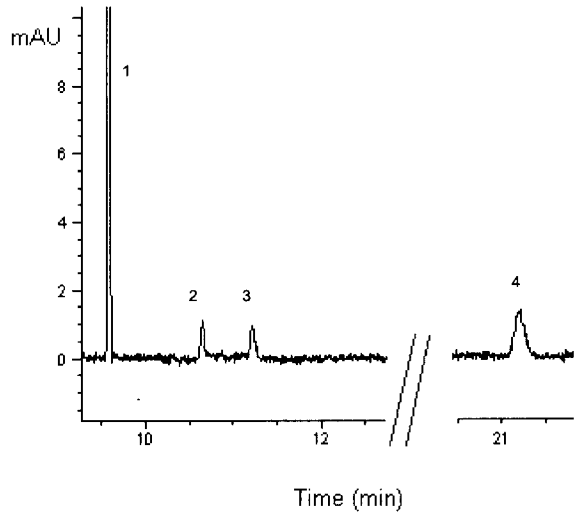

Fig. 3. Electropherogram obtained for the solution containing SPBs. Peaks: 1=methylparaben; 2, 3=sorbitolparaben esters; 4= *p*-hydroxybenzoic acid. Conditions: 20 m*M* borate buffer, pH 10, 22 kV (43 μA), 25°C, detection wavelength: 300 nm.

This fraction was injected again into the HPLC system and only one, well defined peak was obtained. After that, it was injected into the CZE system, where one obtained two peaks of SPB at the same migration times of those obtained in the CZE separation of the solution containing all the SPB isomers. It seems reasonable to think that the two primary monoesters were separated and appeared in a proportion of 64% and 36%, probably due to different steric hindrances between the benzoic ring and the polyol chain in the two isomers. These data suggest that the SPB peak at 11.3 min, in Fig. 3, having injected all the SPB esters, corresponded to the sum of secondary isomers (25% of the total SPBs) plus the minor primary isomer (also 25% of the total SPBs), while the peak at 10.6 min was the major primary isomer. However an explanation for the separation of the two primary esters and the overlapping of the minor primary ester with secondary esters could not be found with the data available.

3.3. Analysis by MEKC

Since parabens have a pK_a between 8 and 8.5, it is clear that at pH 7 most of them must be in the neutral form and able to interact with the surfactant micelles. Without adding the surfactant, no separation of parabens was obtained: MPB and SPBs

eluted as a single and well defined peak. Adding increasing amounts of SDS, always over the CMC (8 m*M* for SDS in water) [14], SPBs and MPB eluted separately.

The effect of increasing the SDS concentration from 50 to 100 m*M* on the separation of isomers of SPBs is shown in Fig. 4, where it can be seen that upon increasing the SDS concentration, a better resolution for the SPB isomers was obtained. This fact can be easily explained as the increase of the micellar structures that could interact with the different isomers. At 50 m*M* SDS, the separation achieved by MEKC for SPBs was the same that obtained by HPLC. Three peaks were resolved with the same ratio of HPLC: 5, 20 and 75%, so the first and the second peaks were assigned to the secondary esters and the last peak to the primary esters. When the SDS concentration was increased, the first peak was well resolved and the resolution of the secondary isomers in the second peak was enhanced. At 100 m*M* SDS, which was the maximum SDS concentration that allowed us to work under reproducible conditions, the four possible secondary isomers (minor peaks) appeared separated, but not the two primary isomers, which eluted together in the major peak. However, a shoulder seemed to formed in this peak, but it was not possible to resolve it completely. In this case, the ratio of SPBs/MPB was 7.0%, which was the same as that obtained in HPLC and CZE. This fact demonstrates that the total amount of isomers were obtained by the three separation techniques, but each resolved different peaks. A summary of the results obtained by each technique can be seen in Table 1, where one can see the order of elution of all the compounds, the number of SPB peaks obtained, the ratio of the SPB peaks and the efficiency obtained by each technique. MEKC obtained the best resolution: five peaks of SPB isomers were resolved with the same order of elution and ratio as obtained by HPLC: the four secondary isomers that suppose 25% of the total amount of SPBs eluted first followed by the primary isomers, overlapped in one peak that suppose 75% of the total amount of SPBs. The ratio of the five peaks separated by MEKC from the mixture of isomers of sorbitolparaben have been calculated and reported in Table 2.

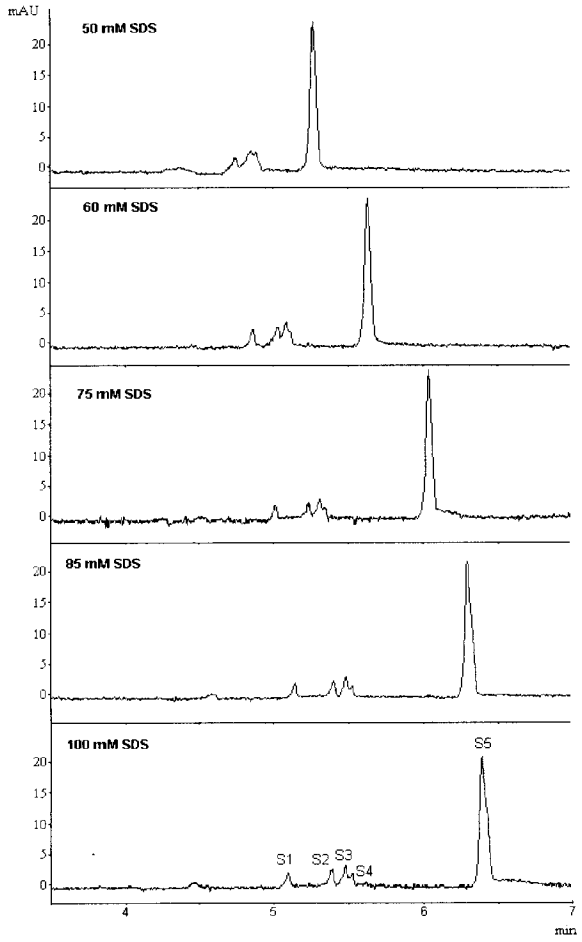

Fig. 4. Electropherograms of solution containing SPBs, increasing the SDS concentration. Conditions: 25 m*M* phosphate buffer, pH 7, 25 kV, 25°C, detection wavelength: 255 nm. Current obtained: 44 μA (50 m*M* SDS), 53 μA (60 m*M* SDS), 59 μA (75 m*M* SDS), 64 μA (85 m*M* SDS), 71 μA (100 m*M* SDS).

The efficiency of the three separation systems can be compared through the number of theoretical plates $[N=16(t_R/w_b)^2]$, calculated in the three cases using the retention time (t_R) and band width (w_b) of the MPB peak. The values obtained were $N_{HPLC}=1200$; $N_{MEKC}=51\,000$ (with 100 m*M* SDS) and $N_{CZE}=120\,000$.

4. Conclusions

Three different separation techniques were tested to separate a mixture of positional isomers. None of them were able to completely resolve all the possible isomers. With CZE, the separation took place in a

Table 1
Summary of the results obtained by the three techniques tested[a]

Technique	Conditions	Order of elution	SPB peaks	SPBs ratio (%)	Efficiency (*N*)
HPLC	Water–MeOH (70:30), 1 ml/min, 254 nm	PHBA–SPBs–MPB	3	5:20:75	1200
CZE	20 m*M* Borate, pH 10, 22 kV, 25°C, 300 nm	MPB–SPBs–PHBA	2	50:50	120 000
MEKC	25 m*M* Phosphate, pH 7+ 100 m*M* SDS, 25 kV, 25°C, 255 nm	SPBs–PHBA–MPB	5	6:7:8:3:76	51 000

[a] Peak area and corrected peak area have been used to calculate the SPBs ratio by HPLC and CZE–MEKC, respectively.

very different way than with HPLC and MEKC, even having a higher efficiency, the mixture of isomers eluted only forming two symmetric peaks. The separation mechanism of MEKC seemed to be similar to that of HPLC, since the same order of elution of the different isomers of SPB esters was obtained by both techniques. However, MEKC yielded a higher resolution for the separation of SPB isomers than that obtained by HPLC: five peaks of SPB were separated while in HPLC only three could be separated. With MEKC, secondary isomers were all resolved, and the primary isomers appeared together in the major peak. When the peak corresponding to the primary isomers was injected into the CZE system with borate buffer, two different peaks, possibly corresponding to the two primary SPB esters, were separated. Combining chromatographic and electrophoretic techniques, we demonstrated the existence of the six isomers of SPB. MEKC using a BGE containing SDS as surfactant, was demonstrated to be an effective choice to separate mixtures of positional isomers, having a higher efficiency than the other chromatographic techniques in these separations.

Table 2
Ratio of the five peaks resolved by MEKC with 100 m*M* SDS[a]

Peak	Ratio (%)
S1 (secondary isomer)	5.9
S2 (secondary isomer)	7
S3 (secondary isomer)	8.1
S4 (secondary isomer)	3.2
S5 (primary isomers)	75.8

[a] Corrected peak areas have been used to calculate the ratios.

Acknowledgements

The authors are grateful to Spain's DGICyT (Project PB96-1180) for funding this work. M.A R. also acknowledges additional funding from Spain's Ministry or Education and Culture in the form of an FPI grant.

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