Separation of Parabens on a Zirconia-Based Stationary Phase in Superheated Water Chromatography

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A superheated water chromatography (SWC) method for the separation of alkyl esters of 4-hydroxybenzoic acid (parabens) using a zirconia-based stationary phase was developed and applied to real sample analysis. First, the SWC system was optimized in terms of the proper length of the preheating coil for establishing thermal equilibration of the mobile phase entering the column at the oven temperature. Next, the effect of the column temperature on the retention was investigated at 100–180°C. The elution time for all parabens decreased with increasing column temperature, and linear relationships between $\ln k$ and $1/T$ were obtained. At higher column temperatures, the elution time was further shortened because of the increased mobile-phase flow rate. Nevertheless, the loss of column efficiency at the higher flow rates was not significant. The application of the present method to the analysis of commercial lotions was then demonstrated. The quantification results obtained from SWC showed good agreement with those from a conventional HPLC method.

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Introduction

The alkyl esters of 4-hydroxybenzoic acid (parabens) are commonly used as preservatives to protect foods, cosmetics, and pharmaceuticals from microbial and fungal attacks.¹ These compounds have been successfully used for the preservation of cosmetics for over half a century. They are well suited for this role because they have no perceptible odor or taste, have neutral pH, do not produce discoloration, and do not cause hardening. In addition, the use of parabens as preservatives in cosmetics is also widespread because of their broad spectrum of activity, inertness, biodegradability, and low cost. At present, they are accepted for use in cosmetics by various regulatory agencies worldwide, including those in Japan.²

For individuals with normal skin, parabens are generally not irritating or sensitizing. However, the application of products containing parabens to damaged or broken skin has resulted in sensitization.¹ Moreover, parabens were found to have endocrine activity,³⁻⁵ and have been detected in human tissues.⁶ Thus, the analysis of parabens in cosmetics is crucial not only for controlling the quality of the products, but also for evaluating the risk they pose. For these tasks, HPLC is generally applied under reversed-phase (RP) conditions.^{7,8}

In RP-HPLC, mixtures of relatively polar organic solvents (*i.e.*, methanol or acetonitrile) and water are generally used as the mobile phase, and the retention time of the analytes can be easily controlled by changing the composition of the mobile phase. However, most organic solvents are not environmentally

friendly and, therefore, minimizing the amount of organic solvent required or developing an organic-solvent-free separation technique is of great interest. Water is a nonhazardous, inexpensive, and environmentally friendly solvent. Although the solubility of organic compounds in water is poor under ambient conditions, it increases with increasing temperature under a pressure modulated to maintain the liquid state of water.^{9,10} Thus, at elevated temperatures (typical temperature range: 100–250°C), water is considered to be capable of replacing organic solvents as the mobile phase for HPLC.^{11,12} This chromatographic method is known as superheated water chromatography (SWC), subcritical water chromatography, or pressurized hot water chromatography. Many researchers have demonstrated the SWC of various organic compounds using modified HPLC systems.¹³⁻¹⁵ This method has several advantages over conventional RP-HPLC. One typical advantage is its ability to extend detection techniques, because it requires water alone as the mobile phase. In particular, universal detection of organic compounds was carried out with flame ionization detection^{16,17} or UV detection at a low wavelength of 190 nm.¹⁸ Moreover, increasing the column temperature can potentially improve the separation efficiency.¹⁹ The viscosity of the water mobile phase and the back pressure of the chromatographic system decrease at high temperatures, so a high flow rate can be achieved. Thus, SWC is potentially advantageous for high-speed separation.²⁰ However, few published studies to date have demonstrated high-speed separation using this method.²⁰⁻²³

One of the major constraints on the development of SWC has been the poor stability of many stationary phases. The octadecylsilyl-silica gel (ODS) stationary phase, which is widely used in conventional RP-HPLC under ambient conditions, was

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used in early works on SWC. However, the retention of the solutes decreased with elution time. We have suggested that this decrease is due to the cleavage of octadecylsilyl groups from the silica gel, because the carbon content of the ODS decreased from 21.7 to 18.7% during 169 h of SWC operation at 100°C.²⁴ Although the poly(styrene-divinylbenzene) stationary phase was very stable in superheated water,^{18,24} it had higher retention capacities and provided poorer peak resolution in comparison with ODS. To overcome the thermal instability of ODS, a zirconia-based stationary phase intended for use in RP-HPLC has been investigated. Up to now, several types of zirconia-based columns whose packings were encapsulated or coated with different organic materials have been used in RP-HPLC, particularly for relatively high column temperatures. Moreover, they have also been used with success in SWC, where they provided sufficient peak resolution.¹³

Very early attempts to separate parabens using SWC were made with ODS¹² and poly(styrene-divinylbenzene)^{11,25,26} stationary phases. A zirconia-based stationary phase was also used for similar separations using water with 5% tetrahydrofuran as the mobile phase.²⁷ In this study, we developed an SWC method for the separation of parabens using a zirconia-based stationary phase that was made by covalently bonding octadecyl ligands to the carbon-clad zirconia surface. The SWC system was optimized in terms of the length of the preheating coil used for thermal exchange of the mobile phase. Having investigated the effect of the operation parameters (column temperature and mobile phase flow rate) on the separation, we successfully applied the developed method to the quantification of parabens in commercial lotion samples.

Experimental

Reagents and materials

Methyl 4-hydroxybenzoate (methylparaben, MP), ethyl 4-hydroxybenzoate (ethylparaben, EP), isopropyl 4-hydroxybenzoate (isopropylparaben, IPP), propyl 4-hydroxybenzoate (propylparaben, PP), isobutyl 4-hydroxybenzoate (isobutylparaben, IBP), and butyl 4-hydroxybenzoate (butylparaben, BP) were the target analytes in this study. Reagent-grade MP, EP, PP, and BP obtained from Kanto Chemical (Tokyo, Japan) and reagent-grade IPP and IBP obtained from Wako Pure Chemical (Osaka, Japan) were used as standards. HPLC-grade acetonitrile purchased from Kanto Chemical was used as the eluent for HPLC. For other purposes, pesticide-grade acetonitrile purchased from Kanto Chemical was used. Ultrapure water was prepared in our laboratory using a Millipore (San Jose, CA) Milli-Q Integral system at an output of 18.2 MΩ cm. Other reagents were of reagent grade, and were used as received. As real samples, eight kinds of lotions (samples A – H) were purchased from the market.

Preparation of sample solutions

Standard paraben solutions were prepared by dissolving the standards and/or uracil into a mixture of water and acetonitrile (7:3, v/v). These solutions were used to optimize the SWC system and to investigate the effect of the operation parameters on SWC separation.

For the analysis of commercial lotions, sample solutions of the eight kinds of lotion (each concentration was 0.5 g/mL) were prepared by dissolving each one into acetonitrile. These solutions were injected into the SWC and HPLC systems without any cleanup process, because they did not appear to be cloudy.⁷ The paraben standards were also dissolved into

acetonitrile to prepare calibration solutions.

Superheated water chromatography

The SWC system employed in this study consisted of a Shimadzu (Kyoto, Japan) LC-10ADvp pump, a Rheodyne (Cotati, CA) Model 7725i sample injector with a 5-μL sample loop, a column oven from a Hitachi (Tokyo, Japan) G-3000 gas chromatograph, a Shimadzu SPD-10Avp UV-VIS detector equipped with a high-pressure cell, and a JASCO (Tokyo, Japan) 880-81 back-pressure regulator that was designed for supercritical fluid chromatography. A ZirChrom Separations (Anoka, MN) Diamondbond-C18 (150 × 2.1 mm i.d.; particle size, 3 μm; pore diameter, 30 nm) was used as a separation column. According to the manufacturer, this column is stable up to 200°C.²⁸ A stainless-steel pipe (0.17 mm i.d.) placed between the sample injector and the separation column was used as a preheating coil. The length of the coil from the sample injector to the entrance of the column oven was constant at 0.3 m, and the length inside the column oven was varied in the range of 0.3 – 1.8 m. An EZChrom Elute data processor (Ver. 3.1.0, GL Sciences, Tokyo, Japan) was used to process the chromatographic data.

The water mobile phase was degassed in advance with helium gas. The mobile-phase flow rate was set so that the pressure of the pump head was below 25 MPa. The back pressure was kept at 1.0 MPa. The injection volume of the sample solution was 5 μL for analysis of lotions and 2 μL for the other runs. The UV detection wavelength was set to 254 nm.

High-performance liquid chromatography

To investigate the effect of the column temperature on the retention factors of the parabens in RP-HPLC, the same SWC system was employed, except without the back-pressure regulator. A mixture of water and acetonitrile (70:30, v/v) was used as the mobile phase at a flow rate of 0.2 mL/min. The injection volume of the sample solution was 2 μL. The column temperature was varied in the range of 50 – 80°C. The UV detection wavelength was set to 254 nm.

For the analysis of commercial lotions, a Shimadzu LC-20 series HPLC system equipped with an LC-20AB binary pump, DGU-20A5 degasser, SIL-20ACHT autosampler, CTO-20A column oven, SPD-20A UV-VIS detector, CBM-20AB communication module, and LC-solution (Ver. 1.22 SP1) workstation was employed. An L-column ODS (150 × 2.1 mm i.d.; particle size, 5 μm; pore diameter, 12 nm) obtained from Chemicals Evaluations and Research Institute, Japan (Tokyo, Japan) was used as a separation column. The injection volume was 5 μL. The UV detection wavelength was set to 254 nm. The other conditions were set following the literature,⁸ as follows: the mobile phase was composed of 50 mmol/L potassium dihydrogenphosphate (adjusted to a pH of 3.5 with diluted phosphoric acid) and acetonitrile (60:40, v/v), the mobile-phase flow rate was 1 mL/min, and the column temperature was 35°C.

Results and Discussion

Because the reactivity of water generally increases with increasing temperature, the SWC system, including the separation column, must be stable against attacks by superheated water. In this study, although the column temperature was elevated to 180°C, the retention time for the parabens was constant when the system was operated under the same chromatographic conditions (column temperature and mobile

Fig. 1 Effect of the length of the preheating coil on the observed adjusted retention time. Analyte, PP; column temperature, 180°C; mobile phase flow rate, 2.0 mL/min.

phase flow rate). That is, the present system was stable against superheated water during the course of the experiment.

Optimization of length of the preheating coil

A large difference between the temperatures of the mobile phase and the column can cause peak broadening due to incomplete thermal equilibration of the cold water at the entrance of the column.²⁹ To avoid this, either an extended preheating coil in the hot air oven or a heater on the inlet line is generally used in SWC systems. Although the former is not superior to a rapid heat transfer system,²³ it is easier to operate. Thus, in this study, a preheating coil was placed in the column oven.

Seven different preheating coils were equipped in the SWC system, with lengths inside the column oven ranging from 0.3 to 1.8 m. Then, the effect of the coil length on the adjusted retention time of PP was investigated using these coils, as shown in Fig. 1. The experimental conditions were set to the maximum column temperature (180°C) and the maximum mobile-phase flow rate (2.0 mL/min) during this study. The adjusted retention time was obtained as a difference between the elution time of PP and that of uracil. When preheating coils whose lengths inside the oven were 1 m or less were used, the observed adjusted retention time increased with decreasing length. Thus, it seems that the mobile phase entering the column was not thermally equilibrated at the oven temperature. The observed adjusted retention time was constant when the length of the preheating coil inside the oven was 1.5 m or more. On the other hand, the use of an excessively long preheating coil would cause band broadening of the analytes. Therefore, a length inside the oven of 1.5 m was considered to be optimal. Subsequent experiments were conducted using this preheating coil.

Effect of the column temperature on SWC separation

The effect of the column temperature on the retention of parabens was also investigated. Figure 2 shows chromatograms of the standard paraben solution obtained at column temperatures of 120, 140, 160, and 180°C. The elution of the target compounds required more than 280 min of run time when the column temperature was set to 120°C; however, it significantly decreased as the column temperature was elevated. Moreover, the elution order of the parabens was unchanged for all column temperatures. Thus, reasonable elution conditions were achieved by setting a higher column temperature (160 or 180°C).

Next, the mechanism of the retention of parabens in SWC was

Fig. 2 Chromatograms obtained from SWC separation of the standard paraben solution at column temperatures of: (A) 120, (B) 140, (C) 160, (D) 180°C. Mobile phase flow rate, 0.5 mL/min. Peak identification and concentrations: 1, MP (0.50 g/L); 2, EP (0.50 g/L); 3, IPP (0.50 g/L); 4, PP (0.50 g/L); 5, IBP (1.0 g/L); 6, BP (1.0 g/L).

Fig. 3 Effect of the column temperature on the retention factor of parabens in: (●) SWC and (◆) HPLC. Mobile phase flow rate of SWC, 0.5 mL/min. The peak identification is the same as in Fig. 2.

investigated using a thermodynamic theory that describes retention in conventional RP-HPLC. The temperature dependence of the retention of solutes can be given by

$$\ln k = -\Delta H^\circ/RT + \Delta S^\circ/T + \ln \phi, \quad (1)$$

where k is the retention factor of the solute, ΔH° the enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS° the entropy of transfer of the solute from the mobile phase to the stationary phase, R the gas constant, T the absolute temperature, and ϕ the phase ratio of the separation column. The k value is defined as the ratio of the time an analyte is retained in the stationary phase to the time it is retained in the mobile phase. In this study, the former time was calculated as a difference between the elution time of the paraben and that of uracil. Then, the latter time was calculated as a difference between the elution times of uracil obtained with and without the separation column, because the extra column dead volume, including the volume inside the preheating coil, might be disregarded. A van't Hoff plot of $\ln k$ vs. $1/T$ is presented in Fig. 3. Linear relationships with correlation coefficients greater than 0.998 were obtained for all parabens. This suggests that

Fig. 4 Chromatograms obtained from SWC separation of the standard paraben solution at mobile-phase flow rates of: (A) 0.3, (B) 0.75, (C) 1.5 mL/min. Column temperature, 160°C. The peak identifications and concentrations are the same as in Fig. 2.

ΔH° , ΔS° , and ϕ remained constant over the temperature range examined. The ΔH° values for each analyte were calculated from the slopes of the plots as follows: -40.2 kJ/mol for MP, -43.8 kJ/mol for EP, -45.1 kJ/mol for IPP, -49.1 kJ/mol for PP, -53.1 kJ/mol for IBP, and -54.7 kJ/mol for BP.

To compare the effect of the temperature in SWC to that in RP-HPLC, separations of parabens by RP-HPLC using the zirconia-based stationary phase were performed. Water/acetonitrile (70:30, v/v) was used as the mobile phase. The relationship between $\ln k$ and $1/T$ for RP-HPLC is also shown in Fig. 3, and linear relationships with correlation coefficients greater than 0.999 were found for all analytes. The ΔH° values for the parabens were also calculated, as follows: -17.6 kJ/mol for MP, -18.4 kJ/mol for EP, -17.8 kJ/mol for IPP, -20.3 kJ/mol for PP, -21.3 kJ/mol for IBP, and -22.8 kJ/mol for BP. The absolute values of the standard enthalpies for the individual parabens were much larger in SWC. This suggests that the retention of parabens was more sensitive to changes in temperature in SWC separation. Because SWC uses water alone as the mobile phase, a gradient elution program, which is widely used in HPLC, cannot be applied. Instead, column temperature programming, in which the column temperature is increased during the chromatographic run, seems to be useful for shortening the elution time of the late-eluting parabens such as IBP and BP.

Effect of the flow rate of the mobile phase on SWC separation

Since the viscosity of water decreases with increasing temperature, separation with a high flow rate of the mobile phase is potentially available in SWC at higher column temperatures. In this study, the effect of the flow rate on the separation was investigated at a column temperature of 160°C. As could be easily anticipated, the pressure drop across the column increased almost in proportion to increases in the mobile-phase flow rate. For example, the pressures at the column inlet at mobile-phase flow rates of 0.3, 0.75, and 1.5 mL/min were 5.5, 11.3, and 21.2 MPa, respectively. These results indicate that under these chromatographic conditions, SWC could supply a mobile-phase flow rate of 7.5-times as much as the conventional flow rate in RP-HPLC (0.2 mL/min).

Figure 4 shows chromatograms of the standard paraben solution obtained at the above-mentioned mobile-phase flow rates. The elution time of the parabens was mostly in inverse proportion to the mobile-phase flow rate. Under these conditions, the change in the flow rate hardly affected the symmetry of the peaks. For example, the symmetry factor (5%

Fig. 5 Chromatogram obtained from SWC separation of the standard paraben solution. Column temperature, 150 to 180°C (held for 1.5 min) at 3°C/min. Mobile phase flow rate, 1.7 to 2.0 mL/min (held for 1.5 min) at 0.3 mL/min². The peak identifications and concentrations are the same as in Fig. 2.

peak height) of the EP peak was 1.8 for all flow rates. Moreover, the theoretical plate numbers for PP obtained at mobile-phase flow rates of 0.3, 0.75, and 1.5 mL/min were 2699, 2166, and 2074, respectively, so the loss of column efficiency at the higher flow rates was not significant. These results indicate that SWC has advantages over conventional RP-HPLC for high-speed separation. Although the peak shapes for the parabens observed using the present method seemed to be poorer than those in conventional RP-HPLC separations,⁸ they were suitable for the quantification of parabens in commercial lotion samples, as described later.

Column temperature and mobile-phase flow rate program

As already shown, elevating the column temperature and increasing the mobile-phase flow rate can shorten the elution time and narrow the peak widths of the solutes. Therefore, SWC separation was demonstrated under programmed temperature and flow rate conditions. Figure 5 shows a chromatogram of the standard paraben solution. The column temperature and mobile-phase flow rate were initially set to 150°C and 1.7 mL/min, and then they were increased to 180°C and 2.0 mL/min. The results clearly show that the program allowed the parabens that elute early in the run (e.g., MP and EP) to separate adequately while shortening the time it took for late-eluting parabens (e.g., IBP and BP) to pass through the column. As a result, all target parabens were eluted within 11 min. Under these chromatographic conditions, the pressure at the column inlet was almost constant in the range of 24.0 – 24.5 MPa. The baseline rose slightly as the column temperature was elevated, which might have prevented the detection of small peaks. However, the baseline could be adjusted by a correction with a blank chromatogram obtained under the same chromatographic conditions.

Fig. 6 Chromatograms obtained from SWC separation of the commercial lotion samples. Column temperature, 160°C; mobile phase flow rate, 1.5 mL/min. Peak identification: 1, MP; 2, EP; 3, PP.

Fig. 7 Chromatograms obtained from HPLC separation of the commercial lotion samples. The peak identification is the same as in Fig. 6.

Application to analysis of commercial lotions

To test its feasibility for real sample analysis, the developed SWC method was applied to the quantification of parabens in

commercial lotions. The separations were conducted under isothermal conditions at 160°C with a mobile-phase flow rate of 1.5 mL/min. Under these conditions, the detection limits ($S/N =$

Table 1 Comparison of SWC and HPLC quantifications of parabens in commercial lotion samples

Analyte	Method	Concentration, % (mass fraction) ^a							
		Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	Sample H
Methylparaben	SWC	0.213 (1.1)	0.048 (1.2)	0.231 (1.2)	0.121 (0.9)	0.177 (0.2)	0.150 (1.1)	0.163 (0.7)	0.148 (0.2)
	HPLC	0.212 (0.07)	0.048 (0.1)	0.224 (0.08)	0.119 (0.06)	0.178 (0.04)	0.147 (0.03)	0.162 (0.03)	0.146 (0.04)
Ethylparaben	SWC	—	0.037 (1.0)	—	—	0.005 (0.6)	—	0.013 (1.5)	—
	HPLC	—	0.037 (0.1)	—	—	0.005 (0.1)	—	0.012 (0.2)	—
Propylparaben	SWC	0.022 (1.2)	—	0.023 (0.8)	—	—	—	—	—
	HPLC	0.021 (0.11)	—	0.022 (0.07)	—	—	—	—	—

a. Values in parentheses are the relative standard deviation (%) of the chromatographic measurement ($n = 3$).

3) were obtained as follows: 1.2 mg/kg for MP, 1.8 mg/kg for EP, 2.5 mg/kg for IPP, 3.7 mg/kg for PP, 7.8 mg/kg for IBP, and 10 mg/kg for BP. The relatively poor values for IBP and BP were probably due to their broad peak shapes, which could be improved by applying a higher column temperature and/or a higher flow rate. Moreover, calibration curves were obtained for concentrations (mass fractions in the lotion samples) up to 0.32% for MP and up to 0.025% for the other parabens. Linear relationships with correlation coefficients greater than 0.999 were obtained for all analytes.

Figure 6 shows SWC chromatograms of the commercial lotion samples. Although the sample solutions were injected into the system without any extraction or cleanup process, no overlapping peaks were observed. All tested lotions were found to contain parabens, although the types detected were limited to MP, EP, and PP; MP was the major component. These samples were also analyzed using HPLC, as shown in Fig. 7. Table 1 compares the quantification results of the two methods, which agreed well. The repeatability with respect to the peak area was a little poorer for SWC than for RP-HPLC. This is probably because the SWC setup was a laboratory-made system with a manual injector. Because the SWC system is operated without any organic solvent, the present method is safe for the operators and provides low operating costs.

The amount of parabens was less than 0.26% for all samples. These concentrations conformed to the regulation value in Japan (1%). Moreover, most of the parabens contained in the lotions were displayed on attached labels. An exception was that the existence of EP in sample G was not displayed on its label.

Conclusions

Advances in thermally stable stationary phases are expanding the applicability of SWC. In this study, the SWC of parabens was carried out using a zirconia-based stationary phase that provided suitable peak resolution. The $\ln k$ values of all parabens decreased linearly with decreases in $1/T$; thus, separations at higher column temperatures (160 or 180°C) provided good analysis times. In addition, under these conditions, the analytical time could be further shortened by increasing the flow rate of the mobile phase. The present method was also successfully applied to the quantification of parabens in commercial lotion samples, and the analytical results obtained were comparable with those found using conventional HPLC. These results indicate that the SWC method is not only environmentally friendly, but is also a potential alternative to conventional HPLC methods.

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