Introduction

Pravastatin (Fig. 1) is a cholesterol-lowering compound that competitively inhibits the microsomal enzyme 3-hydroxy-3-methylglutaryl-co-enzyme A (HMG-CoA) reductase.1,2 Its acid dissociation constant (K_a) has been determined by capillary zone electrophoresis (CZE) in pressure-assisted mode using the effective electrophoretic mobility with changing pH of the separation buffer.3 Pravastatin is degradable under acidic pH conditions, and its lactonized and/or C-6-epimerized forms are supposed to be generated.1,2 The quantification of pravastatin has been conducted in the acidic degradation conditions,2 and micellar electrokinetic chromatography (MEKC) has been performed to resolve the degraded species.5

As for the determination of K_a values, CZE analysis is convenient using the effective electrophoretic mobility of an analyte with changing pH of the separation buffer.4 The CZE includes the electrophoretic separation, and therefore, any impurities and/or degraded species are resolved from the equilibrium species of a particular analyte.5 Acid dissociation constants of degradable substances have been determined with alkaline-degradable phenolphthalein,6 labile drug of pyridinyl-methyl-sulfinyl-benzimidazoles,7 and photo-degradable haloperidol.8

In this study, pravastatin was adopted as an example of an acid-degradable analyte to determine its K_a value under the degradation conditions, in order to examine the availability of the CZE analysis. Pravastatin was subjected to an acidic solution (pH = 2.0) for 5 h for its degradation. Although the amount of the pravastatin obviously decreased along with the standing time in the acidic solution, its K_a value was successfully determined by the CZE analysis with the residual pravastatin. The pK_a value determined under the degraded conditions was 4.46 ± 0.03 (I = 0.01 – 0.02; error: standard error), which agreed well with the one obtained with freshly prepared solution, as well as the reported values.

Experimental

Reagents and chemicals

Pravastatin was purchased from Tokyo Chemical Industries.
Apparatus

An Agilent Technologies 3DCE (Agilent Technologies, Waldbronn, Germany) was used as a CZE system. Photometric detection was made by an attached diode-array detector. A capillary from GL Sciences was cut at a length of 64.5 cm, and it was attached to the system after preparing the detection window by burning the polyimide coating. The dimensions of the capillary were 64.5 cm in total length, 56 cm in its effective length from the injection point to the detection point, and 50 μm i.d. The capillary was held in a thermostat compartment controlled at 25°C. An Agilent Technologies ChemStation software (Ver. B.04.02) was used to control the 3DCE system and to record and analyze the electropherograms.

A JASCO (Tokyo, Japan) spectrophotometer V-630 equipped with 1 cm quartz cells was used to measure the absorption spectra of the analyte and to determine the detection wavelength in the CZE. A TOA DKK (Tokyo, Japan) HM-25 pH meter with a combined glass electrode was calibrated daily with standard pH solutions; it was used for the pH measurements of the separation buffers.

Procedures

After the capillary was flushed with 0.1 mol dm⁻³ NaOH and then with the purified water, it was filled with a separation buffer and equilibrated. A sample solution containing pravastatin at 1 × 10⁻⁵ mol dm⁻³ and 2% (v/v) ethanol was injected into the capillary from its anodic end by applying pressure for 5 s. Both ends of the capillary were then dipped again in the buffer vials, and the electrophoresis was started by applying +25 kV DC voltage. Pravastatin was photometrically detected at 238 nm. Effective electrophoretic mobility of pravastatin, μₑₑ′, was calculated by the usual manner with the migration times of the electroosmotic flow (ethanol) and pravastatin, as well as with the CZE conditions.⁵ For monoprotic pravastatin, its effective electrophoretic mobility is given with its acid dissociation constant (Kₐ) and pH condition of the separation buffer as in Eq. (1):⁵

\[
\muₑₑ' = \frac{Kₐ \times \muₑ}{[H⁺] + Kₐ},
\]

where \(\muₑₑ'\) is the electrophoretic mobility of mono-anionic species of pravastatin. In the analysis of the \(Kₐ\) value by a software of R program,⁶ Eq. (2) was used instead.

\[
\muₑₑ' = 10^{pKₐ} \times \muₑ \times \frac{10^{-pH}}{10^{-pKₐ}}.
\]

A series of pairs of pH and \(\muₑₑ'\) were put into Eq. (2), and the values of \(pKₐ\) and \(\muₑₑ'\) were optimized by the program.

Pravastatin was degraded in acidic solution by dissolving it at pH 2.0 in purified water to terminate the degradation reaction. An aliquot of 1-NS was added to the diluted solution as an internal standard for the quantification. The diluted solution was subjected to the quantification by CZE, where a 10 mM phosphate buffer (pH 7.9) was used as a separation buffer.

A separation buffer containing 10 mM borate buffer (pH 8.33) and 50 mM SDS was used in the MEKC separation of the diluted solution of degraded pravastatin. The MEKC conditions with the apparatus were: 5 s sample injection, 25°C capillary temperature, +25 kV applied voltage, 238 nm detection wavelength, 25°C capillary temperature.

Results and Discussion

Determination of acid dissociation constants of pravastatin by CZE

Prior to the \(Kₐ\) determination of pravastatin under the degradation conditions, the acid dissociation constant of pravastatin was determined by CZE with its freshly prepared solution. The pH of the separation buffer was changed in the pH range between 3.0 and 8.0 using appropriate separation buffers. Ionic strength (I) of the separation buffer was controlled in the range between 0.01 and 0.02. The CZE can detect pravastatin at 1 × 10⁻⁵ mol dm⁻³, and therefore, measurements under such low concentration is predominant over potentiometric titrations. Changes in the effective electrophoretic mobility of pravastatin are shown in Fig. 2. Neutral pravastatin at acidic pH condition dissociates a proton with increasing pH to be mono-anionic. An acid dissociation constant was determined by analyzing the mobility data by fitting them to Eq. (2). An acid dissociation constant of \(pKₐ = 4.47 \pm 0.02\) (I = 0.01 – 0.02; error: standard error) was obtained by the analysis. The curve sampled periodically, and it was diluted by 10-fold with the purified water to terminate the degradation reaction. An aliquot of 1-NS was added to the diluted solution as an internal standard for the quantification. The diluted solution was subjected to the quantification by CZE, where a 10 mM phosphate buffer (pH 7.9) was used as a separation buffer.

A separation buffer containing 10 mM borate buffer (pH 8.33) and 50 mM SDS was used in the MEKC separation of the diluted solution of degraded pravastatin. The MEKC conditions with the apparatus were: 5 s sample injection, 25°C capillary temperature, +25 kV applied voltage, 238 nm detection wavelength, 25°C capillary temperature.
in Fig. 2 is the simulated results of the analysis.

**Acid degradation of pravastatin**

Pravastatin is known to be degradable under acidic pH conditions.\(^1,2\) Pravastatin was stored in 0.01 mol dm\(^{-3}\) HCl solution (pH 2.0) at ambient temperature (25 ± 2°C). The concentration of pravastatin was set at 1 × 10\(^{-3}\) mol dm\(^{-3}\) to be able to detect it even after its degradation and 10-fold dilution. Degraded species would also be detected by CZE or MEKC separations with the pravastatin concentration. The solution was periodically sampled and diluted. The degradation profile was monitored by determining the residual pravastatin with its peak area by the CZE analysis, where \(5 \times 10^{-3}\) mol dm\(^{-3}\) 1-NS was used as an internal standard of the quantification. Changes in the concentration of pravastatin under acidic pH are shown in Fig. S1 (Supporting Information). The amount of pravastatin decreased to about 60% from its initial concentration after 5 h reaction. Electropherograms obtained with the degraded pravastatin are shown in Fig. 3 at several pH conditions. No additional signal except pravastatin and EOF was observed in the CZE electropherograms. It would be because the degraded product is of lactone form and it is neutral.\(^3\)

To ascertain the degradation species of pravastatin, the degradation solution was measured by MEKC using SDS as an anionic micelle matrix. The results are shown in Fig. 4. By the comparison between Figs. 4(a) and 4(b), it is noticed that the signal for pravastatin decreased by the acid degradation and that two new signals indicated as I and II are detected. Nigovic\& Vegar have already examined the MEKC separation of the degraded pravastatin,\(^2\) and a similar MEKC chromatogram is also obtained in this study. They assigned the emerged signals to be C-6 epimer of pravastatin and pravastatin lactone. In this study, signals I and II would be C-6 epimer of pravastatin and pravastatin lactone, respectively. Since total concentration of the equilibrium species of pravastatin decreases by the degradation and the degraded species are photometrically detected by the MEKC, the spectrophotometric titration would be quite difficult on determining the \(pK_a\) value of pravastatin. The UV spectra of the degraded species at uncertain concentrations would interfere with those of the equilibrium species of pravastatin.

**Determination of acid dissociation constants of pravastatin under degraded conditions**

The acid dissociation constant of pravastatin was also determined with the degradation solution. Electropherograms shown in Fig. 3 were used for the analysis. Signals for residual pravastatin are detected even under the degradation, and thus equilibrium analysis is still feasible under the degraded conditions. It has been reported in our previous study that \(pK_a\) values of alkaline-degradable phenolphthalein were determined under alkaline pH conditions and that the degraded species of phenolphthalein is resolved from the equilibrium species under electrophoresis.\(^6\) On the other hand, the degradation of pravastatin does not occur under the pH range of the CZE measurements, and sharp signals have been detected as in Fig. 3. Changes in the effective electrophoretic mobility of residual pravastatin are also shown in Fig. 2 with solid circles. The mobility data were analyzed with Eq. (2), and an acid dissociation constant of \(pK_a = 4.46 ± 0.03\) (\(I = 0.01 – 0.02\); error: standard error) was obtained. The curve in Fig. 2 is also the simulated result of the analysis. The \(pK_a\) value determined under the degraded condition is almost identical to the one determined with the freshly prepared solution. The \(pK_a\) value determined under the degraded condition is also comparable.
with the reported data (p$_{Ka}$ = 4.36, 4.2, 4.6). The difference in the p$_{Ka}$ values by the CZE analyses between 4.36 (Ref. 3) and 4.46 (this study) would come from the ionic strength and the analysis technique used. Actually, in Ref. 3, air pressure is applied to the inlet (anode) vial to shorten the measurement time. When a p$_{Ka}$ value determined in this study is extrapolated to $I = 0$ as in Ref. 3, the value increases by ~0.10, i.e., p$_{Ka}$ ≈ 4.56. The p$_{Ka}$ value determined under the degraded condition (4.46 ± 0.03, $I = 0.01 – 0.02$) is close to the one determined under the ordinary CZE measurement (4.47 ± 0.02, $I = 0.01 – 0.02$, no degradation). Thus, the value would be reliable.

Conclusions

The acid dissociation constant of pravastatin was determined by CZE under its degraded conditions in an acidic solution. While the electropherogram of pravastatin was similar to the one obtained with its freshly prepared solution, the signal height for pravastatin decreased by the degradation and the degraded species were detected by an MEKC format. The degraded species are neutral, and they migrated at the EOF time. Thus, the CZE separation can be utilized for the p$_{Ka}$ determination of pravastatin with this degradation format by electrophoretically separating the degraded species. The acid dissociation constant of pravastatin determined with the degraded solution agreed well with the one determined with the freshly prepared solution, as well as the reported values.

Acknowledgements

This study was supported in part by the Grant-in-Aid for Scientific Research Program (KAKENHI) of the Japan Society for the Promotion of Science (JSPS), Grant Number 26410154.

Supporting Information

Changes in the residual concentration of pravastatin under the degradation in acidic solution (Fig. S1). This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

1. D. Mulvana, M. Jemal, and S. C. Pulver, J. Pharm. Biomed. Anal., 2000, 23, 851.
2. B. Nigović and I. Vegar, Croat. Chem. Acta, 2008, 81, 615.
3. Y. Ishihama, M. Nakamura, T. Miwa, T. Kajima, and N. Asakawa, J. Pharm. Sci., 2002, 91, 933.
4. M. G. Khaledi, S. C. Smith, and J. K. Strasters, Anal. Chem., 1991, 63, 1820.
5. T. Takayanagi, Bunseki Kagaku, 2015, 64, 105.
6. T. Takayanagi and S. Motomizu, Chem. Lett., 2001, 14.
7. E. Örnskov, A. Linusson, and S. Folestad, J. Pharm. Biomed. Anal., 2003, 33, 379.
8. N. Shimakami, T. Yabutani, and T. Takayanagi, Bunseki Kagaku, 2014, 63, 643.
9. The R Project for Statistical Computing, http://www.r-project.org/.
10. A. T. M. Serajuddin, S. A. Ranadive, and E. M. Mahoney, J. Pharm. Sci., 1991, 80, 830.
11. Y. Adachi, Y. Okuyama, H. Miya, H. Matsusita, M. Kitano, T. Kamisako, and T. Yamamoto, J. Gastroen. Hepatol., 1996, 11, 580.