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Comparison of CE and HPLC Methods for Determining Lovastatin and Its Oxidation Products after Exposure to an Oxidative Atmosphere

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Lovastatin is a cholesterol-lowering agent, which competitively inhibits the enzyme HMG-CoA reductase. Several HPLC methods for its analysis have been developed but there is no report of its determination using capillary electrophoresis (CE). In this paper, we report the development of a simple CE method for lovastatin determination, which is selective with respect to its degradation products and useful for routine analyses. Since the molecule of lovastatin in its lactone form is uncharged and is only slightly soluble in water, base hydrolysis was used to open the lactone ring and transform the compound into a water-soluble acid form, which is negatively charged. Different solvents, different amounts of NaOH added, different hydrolysis times and different temperatures for sample preparation were tested. The CE and HPLC methods are compared in terms of susceptibility, precision, linearity and accuracy. HPLC method was found to be more susceptible and more precise.

Key words
• lovastatin
• lovastatin acid
• analytical method
• capillary electrophoresis, CE
• high performance liquid chromatography, HPLC

INTRODUCTION

Lovastatin, simvastatin, pravastatin and mevastatin are structurally similar cholesterol-lowering agents called statins. They competitively inhibit the enzyme HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase, a rate-limiting enzyme, which catalyzes the conversion of hydroxymethylglutarate to mevalonate, an early and rate limiting step in the biosynthesis of cholesterol.[1,2] Liver is the main site of cholesterol synthesis in the body.[1] The drug is administered to patients as the prodrug lactone, which is converted to its hydroxy acid form *in vivo*.[1]

Several HPLC methods for the analysis of lovastatin have been developed[3–6] but there is no report on the use of capillary electrophoresis (CE).

The expiry time of many pharmaceutical products is limited by the susceptibility of the drug substance to oxidative degradation. The susceptibility of statins to oxidative degradation is well established.[7–10] Apart from the loss of drug potency, other stability problems, such as changes in the dissolution rate, discolouration, generation of toxic degradation compounds of the final pharmaceutical formulation, are also very common. To assure the efficacy and safety of pharmaceutical products with drugs susceptible to oxidative degradation, a precise method is needed. Volative degradation products and degradation products without chromophores are possible and therefore also monitoring of the main compound is recommendable.

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In this paper, we report the development of a CE method for lovastatin, which is selective towards its degradation products. The CE and HPLC methods[11] are compared in terms of susceptibility, precision, linearity and accuracy.

EXPERIMENTAL

Materials

Lovastatin was obtained from Krka, d.d., Novo Mesto (Slovenia), and was at least 99 % pure. All solvents and reagents were of analytical grade.

Oxidation Procedure

The sample was oxidized in a Perkin Elmer differential scanning calorimeter (DSC), type DSC-7, equipped with water cooling.

Experimental conditions: 50-µl aluminium crucibles with four holes, dry oxygen atmosphere with 40 ml/min flow rate, isothermal heating at 135 °C for 15 minutes. Sample masses were 2.0–2.4 mg. Oxidized samples were analyzed by HPLC and CE.[12]

Determination of Lovastatin by CE

The sample from an aluminium crucible was transferred quantitatively into a 5 ml Erlenmayer flask, dissolved in 200 µl of methanol (Merck, HPLC grade), using an ultrasound bath if necessary (sample solution 1). 50 µl of solution 1 was transferred into a 2-ml vial and 50 µl of 0.1 M NaOH (Riedel-deHaen, *p.a.*) was added. In order to complete the hydrolysis of lovastatin lactone into hydoxy acid, the vial was covered and left at room temperature for at least 30 minutes. After that, 250 µl of water was added and the CE analysis was performed.

For calibration purposes, a standard solution of lovastatin was prepared at a concentration of about 10 mg ml^{-1} in methanol (standard solution 1) and treated in the same way as described for the oxidized sample solutions.

The CE analyses were performed on a Hewlett-Packard, HP 3D CE apparatus (Walbronn, Germany) using the ChemStation software. The fused silica capillary was 64.5 cm long (56 cm to the detector) with an internal diameter of 50 µm and with a bubble cell (150 µm light path length). Separation buffer was prepared by mixing 65 ml of 0.2 M borate buffer solution, pH = 9.3 (solution A) (Fluka Chemie AG, *p.a.*) and 35 ml of 0.05 M borate buffer solution, pH = 9.3, with 0.1 M sodium dodecyl sulphate, SDS (solution B) (Hewlett-Packard, CE grade). The optimal separation parameters were: buffer, $\psi_{A,B}$ = 65 : 35; injection, 40 mbar, 10 s; voltage, 30 kV; temperature, 30 °C; detection, 238 nm (band width 16 nm). The current was about 87 µA and the migration time of lovastatin acid about 14 minutes. The migration times of oxidized products ranged from 5 to 14 minutes and from 15 to 16 minutes. The capillary was rinsed for 3 minutes with running buffer before each run.

Determination of Lovastatin by HPLC

The sample from an aluminium crucible was transferred quantitatively and dissolved in 2.0 ml of acetonitrile (J. T. Baker, HPLC grade). Standard solutions used for calibration purposes were prepared in the same way as described for sample solutions.

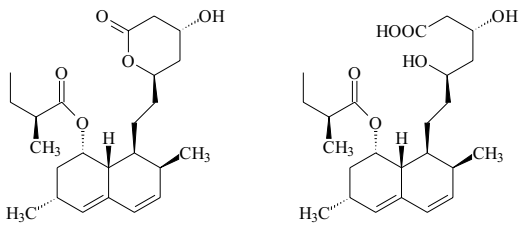
Quantitative HPLC analyses according to The European Pharmacopoeia[11] were performed on a Hewlett-Packard 1100 VWD HPLC system using a Eurosphere C18 (endcapped octadecylsilane) 250 mm, 4.6 mm i.d., column with 5 µm particles equilibrated at 25 °C. Acetonitrile (J. T. Baker, HPLC grade) was used as mobile phase A, and mobile phase B was aqueous 0.1 % H_3PO_4(aq) (Merck). The flow rate was 1.5 ml min^{-1}, injection volume 5 µl and peak detection was at 238 nm. Analysis started with 60 % A and 40 % B for 5 minutes and then increased to 65 % B in 2 minutes. The gradient was then ramped up to 90 % B in 5 minutes and held there for the next 9 minutes. After 22 minutes (stop time), the mobile phase B was decreased to 40 % and the chromatographic system was equilibrated to the starting condition for about 5 minutes.

Lovastatin eluted in approximately 10 minutes. The retention times of oxidized products ranged from 2 to 16 minutes.

RESULTS AND DISCUSSION

The development of an alternative analytical method using CE to determine lovastatin and its oxidation products after exposure to an oxidative atmosphere was based on its chemical characteristics. The molecule of lovastatin (Scheme 1) in lactone form, as it is administered to patients as a prodrug, does not have any charge and is only slightly soluble in water (approx. 0.0044 mg ml^{-1}).[13] Base hydrolysis was used to open the lactone ring. The compound was transformed to lovastatin acid and its water solubility increased dramatically. Lovastatin acid in its ionic form is negatively charged, which enables it to be separated from other compounds in the sample.

Addition of the surfactant sodium dodecyl sulphate (SDS) enables the neutral compounds in the sample solution to partition between SDS micelles and buffer solution based on their polar/nonpolar characteristics. It



lovastatin (lactone form) lovastatin acid

Scheme 1. Structures of lactone and hydroxy acid forms of lovastatin.

enables the separation of the uncharged compounds which result from the oxidation processes.

The shortest analysis time and the appropriate resolution between lovastatin acid, impurities and oxidation products were sought by selecting the appropriate electrophoresis buffer and the optimal electrophoresis conditions. At the same time, we attempted to narrow the peak width of lovastatin acid, which would also contribute to better separation.

Selection of Optimal Electrophoretic Conditions

An electrophoresis buffer based on borate ions was chosen in order to increase the selectivity by creating complexes with hydroxyl groups of the sample molecules, which were then drawn to the cathode.[14]

Buffer solutions with different ionic strengths were tested and later modified by addition of different concentrations of SDS. The criteria for the optimal electrophoresis buffer were the migration time of lovastatin acid, and the resolution between lovastatin acid and its oxidation products, and between the oxidation products themselves.

Increasing the concentration of borate ions and decreasing the concentration of SDS in the buffer decreased the migration time, but unfortunately, decreased the resolution as well (Figures 1 and 2).

The migration time of lovastatin acid decreased when higher concentrations of borate buffer solution were used, but a significant amount of heat was generated, even at low voltages.

The optimal compromise chosen between these parameters was an electrophoresis buffer of the following composition: 147 mmol dm^{-3} borate buffer solution and 35 mmol dm^{-3} SDS.

Further, we have proved that the migration time could be decreased dramatically, without any significant loss of resolution. For this purpose, the analyses were modified by reversing the electrodes and injecting the sample on the capillary side where the path to the detector cell was shorter by aprox. 6.6 fold. Different concentrations of borate buffer pH = 9.3 and different concentrations of SDS were tested in this inverted configuration. The migration time of lovastatin acid and the resolution between its peak and the nearest neighbouring peaks were followed. The migration time of lovastatin acid was shortened, about 5.8 times on average (Figure 3), but the resolution between individual peaks and lovastatin acid was not suitable for quantitative evaluation.

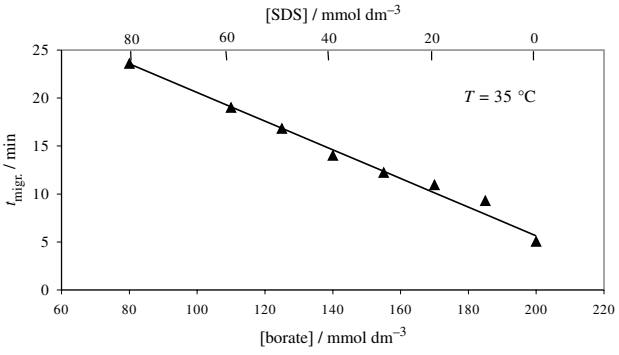


Figure 1. Dependence of lovastatin acid migration time ($t_{migr.}$) on the concentration of borate buffer and SDS.

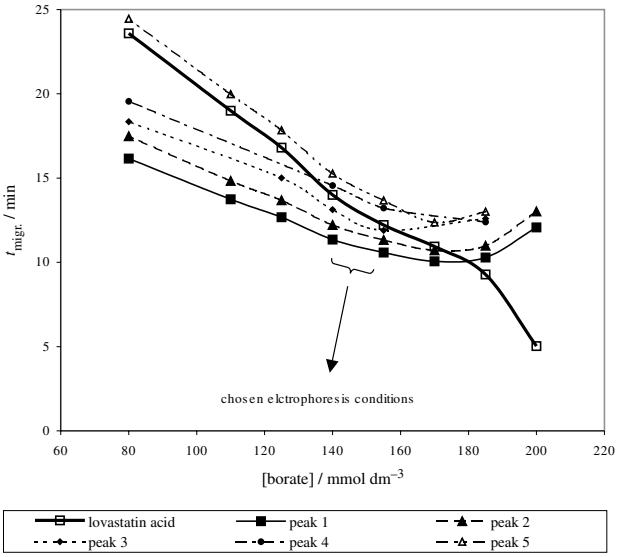


Figure 2. Dependence of migration time ($t_{migr.}$) of the five peaks nearest to lovastatin acid on the composition of the electrophoresis buffer.

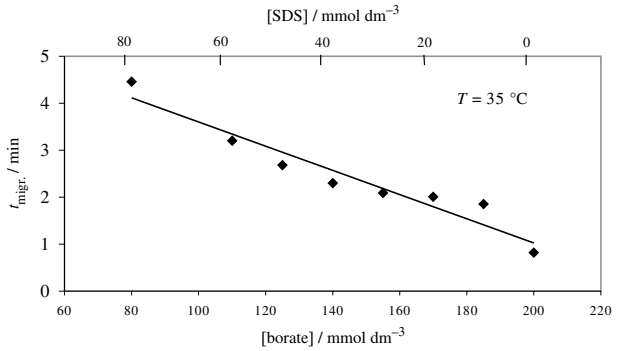


Figure 3. Dependence of lovastatin acid migration time ($t_{migr.}$) on the concentration of borate and SDS in inverted configuration.

On increasing the temperature, the migration time of lovastatin acid decreased to about 20 minutes at 30 °C and about 14 minutes at 35 °C. The temperature of 30 °C was chosen as a compromise between suitable resolution and migration time.

By shortening the injection time and/or decreasing the pressure, using a hydrodynamic type of injection, the injection volume of the sample was reduced, resulting in lower sensitivity of the method. In the case of the chosen hydrodynamic type of injection, with a pressure of 40 mbar and injection time of 10 s, the width of the lovastatin acid peak at the baseline was about 0.7 min.

Optimization of Sample Preparation

Influence of Solvent in Preparing the Lovastatin Solution. – We tried to prepare the lovastatin solution at concentrations of about 10 mg ml^{-1}. Methanol and acetonitrile were tested as solvents. Both solutions were handled in the same manner (15 minutes in an ultrasound bath). Lovastatin dissolved completely in methanol, but only partially in acetonitrile. No such difference in solubility is reported in the literature.[7]

Influence of NaOH on Lovastatin Hydrolysis. – We expected that higher concentrations of lovastatin would require more NaOH for its hydrolysis. This implies also higher ionic strength and undesired spreading of the lovastatin acid peak. For hydrolysis of lovastatin to lovastatin acid excess NaOH was added to the lovastatin solution. At least 60 % (mole fractions) excess is needed to complete the hydrolysis at room temperature within 30 minutes. With a decreased amount of NaOH (excess less than 25 %), we observed that at a lovastatin concentration higher than 5 mg ml^{-1} the hydrolysis was not complete. It is seen as a plateau on the curve (Figure 4) where the data are presented as detector response to the concentration of lovastatin. Incomplete hydrolysis is also seen as the precipitate of non-hydrolyzed lovastatin after dilution with water.

Time Dependence of Lovastatin Hydrolysis. – The time dependent course of lovastatin hydrolysis was followed as the detector response. The results given in Table I show that lovastatin hydrolysis (conc. 10 mg ml^{-1}) was complete within 30 minutes under the above described experimental conditions.

Influence of Temperature on Lovastatin Hydrolysis. – Lovastatin (conc. 10 mg ml^{-1}) was hydrolyzed at room temperature and at 37 °C. Temperature was found to have no significant effect on the rate of hydrolysis (Table II), so lovastatin hydrolysis was carried out at room temperature.

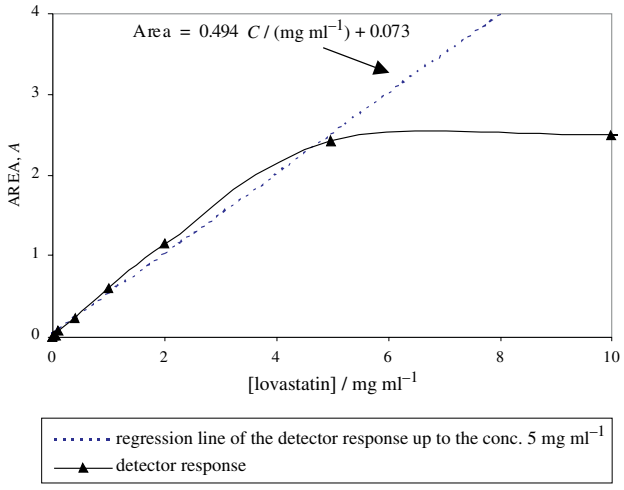


Figure 4. Dependence of detector response on the concentration of the lovastatin starting preparation (solution 1) after the hydrolysis procedure. Lovastatin at concentrations > 5 mg ml^{-1} is not hydrolyzed because of an insufficient amount of NaOH. Detector response measured as absorbance, A (A = log I_0 / I; I_0, incident light intensity; I, transmitted light intensity).

TABLE I. Time dependence of lovastatin hydrolysis followed as detector response

t / min	$A_{\text{lovastatin acid}}$[a]	(A / A_{30})[b] / %
30	11.92131	100
60	12.47367	105
120	11.16885	94
180	13.14566	106
300	12.15077	102
480	11.56391	97
1440 (24h)	12.04013	101
average	12.06633	101
RSD	4.9	3.9

[a] Detector response measured as absorbance A.
[b] A_{30}, A value after 30 min.

TABLE II. Temperature and time dependence of lovastatin hydrolysis process

	Room temperature		T = 37 °C	
t / min	$A_{\text{lovastatin acid}}$	(A/c) / ml mg^{-1}	$A_{\text{lovastatin acid}}$	(A/c) / ml mg^{-1}
30	11.92131	0.6000	11.6482	0.5755
60	12.47367	0.6278	13.3338	0.6588
120	11.16885	0.5621	12.0051	0.5931
average	12.1721	0.5966	12.3290	0.6091
RSD	6.0	5.5	7.2	7.2

Comparison of CE and HPLC Methods

Selectivity Study. – The specificity and selectivity of the chromatographic and electrophoretic methods was examined (Figure 5). For quantitative evaluation of non-oxidized lovastatin in solutions exposed to oxygen, it is important to ensure separation of the lovastatin peak from those of its oxidation products. In the »peak purity test«, using a diode array detector, the UV spectra of different parts of the peak do not differ significantly, indicating homogeneity of the lovastatin acid (CE) and lovastatin (HPLC) peaks.

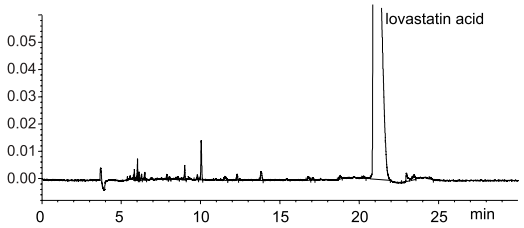


Figure 5. Analysis of lovastatin solution exposed to an oxygen atmosphere. Detector response (y_{axis}) measured as absorbance (dimensionless unit).

TABLE III. Comparison of CE and HPLC methods in terms of regression parameters

Parameter of regression analysis	HPLC	CE
Lovastatin conc. range / mg ml^{-1}	0.0002–1.2	0.02–25.0
Correlation coefficient, r^2	0.9992	0.9993
Slope, a / ml mg^{-1}	20.88740 ± 0.23141	0.605039 ± 0.011851
Intercept on y axis, b	0.12568 ± 0.13181	0.046736 ± 0.121017
Intercept on y axis, b / %[(a)]	0.60	0.75
Intercept on y axis, b / %[(b)]	0.60	6.7

[(a)] b is the intercept on y axis expressed in percents relative to the detector response at lovastatin concentrations of about 1.0 mg m^{-1} (HPLC) and 10.0 mg ml^{-1} (CE). These concentrations were chosen as working concentrations for calibration purposes.

[(b)] b is the intercept on y axis expressed in percents relative to the detector response at the lovastatin concentration of about 1.0 mg ml^{-1} (HPLC and CE).

Quantitative Performance. – The quantitative aspects of both methods were examined and the data are shown in Table III.

Limits of Detection and Quantitation. – Limits of detection (LOD) and limits of quantitation (LOQ) were estimated from the signal to noise ratios (*S*/*N*). In the limit of detection (*S*/*N* = 3) and limit of quantitation (*S*/*N* = 10) tests, a series of diluted solutions of lovastatin was prepared. The concentration of 0.025 μg ml^{-1} and 0.10 μg ml^{-1} for the HPLC method and 10.0 μg ml^{-1} and 40.0 μg ml^{-1} for the CE method were found to correspond to LOD and LOQ, respectively.

Linearity. – The CE method is linear in the range from LOD (10 μg ml^{-1}) to about 25 mg ml^{-1} of lovastatin while the HPLC method is linear in the range from LOD (0.025 mg ml^{-1}) to about 1.2 mg ml^{-1} of lovastatin.

The correlation coefficients (peak area *vs*. sample concentration) are shown in Table III. In both cases they exceed 0.999, indicating good correlation. Detector responses at zero concentration, expressed in percents relative to the detector response at working concentration, are acceptable in both cases (0.6 % for HPLC and 0.75 % for CE), but the working concentration using CE is 10 times higher than for HPLC.

Precision. – The results of 6 successive injections of lovastatin solution (HPLC, conc. = 1.0 mg ml^{-1}) and lovastatin acid solution show that the injection precision of the HPLC method is, as expected, better (RSD = 0.1 %) than that of CE (RSD = 1.1 %). We suppose that the reason for the lower precision of CE is the type of injection, which is hydrodynamic.

The repeatability of the method was determined by the analysis of at least six sample solutions, prepared from the same lovastatin sample. Due to the limited amount of oxidized lovastatin available (in aluminium crucibles about 5 mg substance can be oxidized at a time, which is not enough for 6 sample solutions), the repeatability of the method was performed on non-oxidized lovastatin. The lovastatin assay was performed six times using HPLC (RSD = 0.4 %) and eight times using the CE method (RSD = 3.5 %). The CE determination of lovastatin was based on the conversion of lovastatin into lovastatin acid, which takes place irreversibly in alkaline media in a relatively short time. In order to check the repeatability of the conversion of lovastatin into lovastatin acid, a lovastatin solution was divided into six aliquots. Each solution was hydrolyzed and analyzed using CE. The RSD of six determinations of lovastatin acid was 3.2 %. These results confirmed that the repeatability of lovastatin conversion into lovastatin acid in alkaline media was adequate.

These results show that the HPLC method for determining lovastatin is more precise and repeatable than the CE method.

A comparison of HPLC and CE results of three lovastatin samples after exposure to an oxygen atmosphere (135 °C, 15 minutes) is shown in Table IV.

The difference between the CE and HPLC results is relatively high, but considering the RSD value of the analytical method repeatability (about 4 %) and the repeatability of the oxidation processes,[14] the difference is within the expected limits.

The amount of lovastatin after exposing the sample to an oxygen atmosphere decreased by about 40 %, but the total content of impurities did not reach the level of degraded lovastatin (Figure 5). We suppose that the volatile oxidation products and the products without chromophores (doubly bound in naphthalenediene ring) result from the oxidation processes.

TABLE IV. Comparison of the results of HPLC and CE analytical methods

Ser. no.	Non-oxidized lovastation / %		
	HPLC	CE	$\Delta_{HPLC-CE}$
1	70	66	4
2	78	70	8
3	60	56	4

CONCLUSION

The CE method developed in the present work is suitable for the routine analysis of lovastatin and its oxidation products. It involves a simple transformation of the lactone form of lovastatin into a hydroxy acid using base hydrolysis. Comparison of the CE and HPLC methods has shown that the latter is more precise, reproducible and sensitive than the former, although the many advantages of CE, such as small injection volume, simplicity and wide applicability, should be taken into account.

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SAŽETAK

Usporedba metoda CE i HPLC pri određivanju lovastatina i njegovih oksidativnih produkata nakon izlaganja oksidirajućoj atmosferi

Sandra Javernik Rajh, Samo Kreft, Borut Štrukelj i Franc Vrečer

Lovastatin je spoj koji smanjuje nastajanje kolesterola, kompetitivno inhibirajući enzim HMG-Co-reduktazu. Poznato je nekoliko HPLC-metoda za njegovu analizu, ali nema podataka o njegovom određivanju kapilarnom elektroforezom (CE). U ovom radu izvještavamo o razvoju jednostavne CE-metode za određivanje lovastatina, koja je selektivna prema njegovim degradacijskim produktima i uporabljiva u rutinskim analizama. Budući da su lovastatin i njegov laktonski oblik nenabijeni i slabo topljivi u vodi, upotrebljena je lužnata hidroliza za otvaranje laktonskoga prstena i pretvaranje spoja u negativno nabijeni kiselinski oblik topljiv u vodi. Ispitana su različita otapala, dodatak različitih količina NaOH, različita vremena hidrolize i različite temperature pripreme uzoraka. Metode CE i HPLC su uspoređene u smislu osjetljivosti, preciznosti, linearnosti i točnosti. Ustanovljeno je da je HPLC osjetljivija i preciznija metoda.