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Short communication

Fast analysis of pravastatin in production media

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Abstract

High throughput methods (high performance liquid chromatography and capillary electrophoresis) were developed to determine pravastatin in production media. The analyses were performed on particle column, monolithic column and silica capillary filled with borate buffer pH 9.3 containing 20 mM SDS. All three methods successfully separate pravastatin from interfering compounds (matrix, mevastatin and 6-*epi* pravastatin) and runtimes are shorter than 1 min. Solvent consumptions for methods using small particle column, monolith column and MECK were 132, 510 and 1.5 mL h^{-1}. The most sensitive was the method using particle column (LOD was about 10^{-5} mg mL^{-1}), followed by the system using monolith column (LOD was 2×10^{-4} mg mL^{-1}) and the MECK method (LOD was about 0.02 mg mL^{-1}).

Keywords: Pravastain; Fast analysis; Pharmaceutical analysis; Monolith column; Small particle column; Fast MECK method

1. Introduction

Cholesterol lowering statin drugs (atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, simvastatin) are most frequently prescribed substances for reducing mortality related to coronary heart disease (CHD). The elevated plasma cholesterol level and low-density lipoprotein cholesterol levels have been recognized as a major risk factor for atherosclerotic disease, specifically for CHD. Pravastatin is as other statin drugs a cholesterol-lowering agent that competitively inhibits the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzim A reductase [1–4]. Some of the statins are completely synthetic compounds (atorvastatin, fluvastatin, cerivastatin); however, pravastain is like lovastatin a natural product. It is produced in two-step fermentation. In the first step, mevastatin is produced by *P. citrinum*, and in the second step, bioconverted to pravastatin by *S. carbophylus* [4]. The structures of mevastatin (a), pravastatin (b) and 6-*epi* pravastatin (c) are shown in Fig. 1.

Instead of conventional HPLC columns, short columns with small particles or monolithic columns are used in modern chromatographic methods [5,6]. By reducing the particle diameter of the packing material, the resolution is increased and analysis time shorter. The only limit is relatively high backpressure for standard instruments, which is now more or less solved with the UPLC system.

In the last few years, monolithic stationary phases have also attracted much attention. The monolithic columns can be described as a single large "particle" without interparticular voids. The monolith C18 silica column consists of single silica rod. Because of high porosity of such material, analysis can be performed at much higher flow rates (up to 9 mL min^{-1}, for 4.6 mm I.D. columns), which shortens analysis time [7–9]. Recently, monoliths have been successfully used for fast separation of β-blocking drugs [10], metabolites in biological fluids [11–13], diastereoisomers [14], biological macromolecules [15] etc.

CE is a complementary or an alternative technique for analysis of drugs. It is used for impurity profiles, chiral separation, protein analysis, determination of metal ions and inorganic anions [16]. Several articles are dealing with analysis of drugs by CE [16,17]. Most of CE separations are performed in

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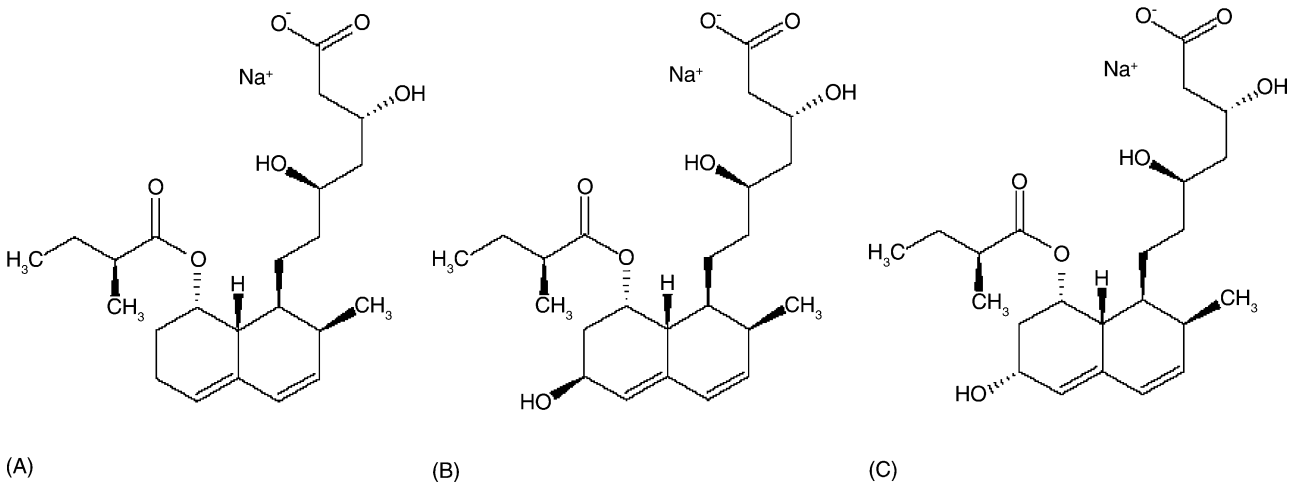

Fig. 1. Structures of mevastatin (A), pravastatin (B), 6-*epi* pravastatin (C).

uncoated silica capillaries in the presence of electroosmotic flow (EOF). Micelar electrokinetic chromatography (MECK) is one of widely used CE modes. The most important requirements for fast CE analysis are usage of short and narrow capillaries, high intense electric field, low ionic strength of buffers, strong EOF (alkaline buffer) and parallel hydrodynamic flow [18].

Chromatography and capillary electrophoresis are as analytical separations techniques widely used in pharmaceutical research and development. For determination of investigated compounds conventional HPLC method could be used [19]. To optimize the production of pravastatin in bioreactors fast analytical methods are needed. Good separation of pravastatin from interfering compounds (matrix, mevastatin and main impurity 6-*epi* pravastatin) is important. In our work, we present high throughput HPLC systems with conventional particle and monolithic column and a high throughput electrophoretic system based on micellar electrokinetic chromatography (MECK). All three methods are shorter than 1 min and allow baseline separation of all tree compounds of interest.

2. Experimental

2.1. Materials

Pravastatin fermentation broth was produced in Lek Pharmaceutical company d.d. (Ljubljana, Slovenia). Pravastatin, 6-*epi* pravastatin sodium salts and mevastatin tert-butyl amine salt were from Lek Pharmaceutical company d.d. (Ljubljana, Slovenia). Acetonitrile, sodium hydroxide and sodium dihydrogen phosphate monohydrate were obtained from Merck (Darmstadt, Germany). 20 mM borate buffer pH 9.3 and 50 mM borate buffer pH 9.3 with 100 mM SDS were from Hewlett Packard (Waldbronn, Germany). Deionized water of at least 18 MΩ was purified by an Elga UHQ (High Wycombe, UK) apparatus.

2.2. Sample preparation

Standard solutions of pravastatin sodium, mevastatin tert-butyl amine and 6-*epi* pravastatin sodium in concentration of 0.1 mg mL^{-1} were prepared in deionized water.

Standard solutions of pravastatin were prepared in fermentation broth without mevastatin in concentration range from 0.1 to 10^{-5} mg mL^{-1}. For the determination of LOD, solutions were diluted to obtain signal to noise ratio in-between 3 and 5.

The sample of pravastatin fermentation broth was filtrated using Millex 0.45 μm PVDF filter (Millipore, USA) and analyzed without any pretreatment. The same fermentation broth with concentration of pravastatin 0.4 mg mL^{-1} was used for determination of R.S.D. for all three methods.

2.3. HPLC method I

Analyses were performed on Agilent Technologies 1100 System (Waldbronn, Germany). Zorbax SB-C18 1.8 μm (50 × 4.6) mm (Agilent Technologies, Newport, Delaware, USA) column was used for separation. The two mobile phases were 2.5 mM phosphate buffer pH 7.0 (mobile phase A) and acetonitrile (mobile phase B). The mobile phase gradient started at 20% of B, increased to 60% B within 0.4 min and to 85% within 0.1 min and than held for 0.4 min at that level. The flow rate of mobile phase was 2.2 mL min^{-1} and the injection volume was 20 μL. The UV detector was set to 238 nm.

2.4. HPLC method II

Analytes were separated using Agilent Technologies 1100 System (Waldbronn, Germany), with a Chromolith SpeedROD RP-18 e (50 × 4.6) mm column (Merck, Darmstadt, Germany) at the flow rate of 8.5 mL min^{-1}. Gradient elution was carried out with 17% acetonitrile (mobile phase A) and 85% acetonitrile (mobile phase B) in 2.5 mM phosphate buffer pH 7.0. The mobile phase gradient was started at

0% of B, and after 0.3 min increased to 65% B within 0.05 min and held at that level for 0.2 min. The injection volume was 20 μl. The UV detector was set to 238 nm.

2.5. Capillary electrophoresis

CE experiments were performed on HP^{3D}CE instrument (Waldbroon, Germany) in an uncoated fused-silica capillary (50 μm I.D., effective length to detector 23 cm, total length 31.5 cm). The run voltage was set at 30 kV (∼130 μA) and the column temperature controlled at 20 °C. The sample was loaded by pressure injection 50 mbar 4 s. Borate buffer (26 mM, pH 9.3) with 20 mM SDS was used for MECK. Capillary was flushed every ten runs with running buffer for 3 min. UV detection was at 237 nm.

3. Result and discussion

3.1. HPLC method I

Smaller particles of stationary phase must be used in shorter column to obtain similar efficiency as at the longer column. The main drawbacks of short columns with small particles (lower than 2 μm) are very high backpressure; gradient equilibration times and stabilization times are longer than with monolith column. Flow rate of 2.2 mL min^{-1} was used because most of the commercially available instruments are designed for maximal backpressure of 400 bar. Although, the work on higher backpressure limits is possible, we have to consider that pump will wear out much faster than when working at usual 150–200 bar. The chromatogram of investigated compounds analyzed using 1.8 μm particle column is shown in Fig. 2. All three compounds are eluted within 1 min. The method using small particle column was the most sensitive (LOD was about 10^{-5} mg mL^{-1}) and had good reproducibility (R.S.D. of peak areas was 0.2% for $n = 26$).

3.2. HPLC method II

Monolith columns contain a single piece of porous silica gel, which gives them greater porosity and permeability than conventional particle columns. This enables high flow rate due to low backpressure. Run times, gradient equilibration times and stabilization times are proportionally shorter. Chromatographic properties (selectivity, plate count) of the monolithic columns are similar to conventional 5 μm particle columns. The main drawback of monolith column is high solvent consumption. The separation of investigated compounds is presented in Fig. 3. The elution profile is similar with HPLC method I; first, eluting compound is 6-*epi* pravastatin ($t_R = 0.27$ min) followed by pravastatin ($t_R = 0.34$ min) and mevastatin ($t_R = 0.56$ min). The method described has very high solvent consumption (510 mL h^{-1}), but is very reproducible (R.S.D. of peak areas is about 0.1% for $n = 33$) and sensitive (LOD was 2×10^{-4} mg mL^{-1}).

3.3. Capillary electrophoresis

A short capillary, strong electric field (30 kV) and alkaline buffer (pH 9.3) generate strong electroosmotic flow, allowing the separation of all three compounds of interest within 0.9 min. Relatively low concentration of micelles was used to shorten the separation time. Without SDS the separation time decreases, but also the baseline separation between 6-*epi* pravastatin and pravastatin is lost. On

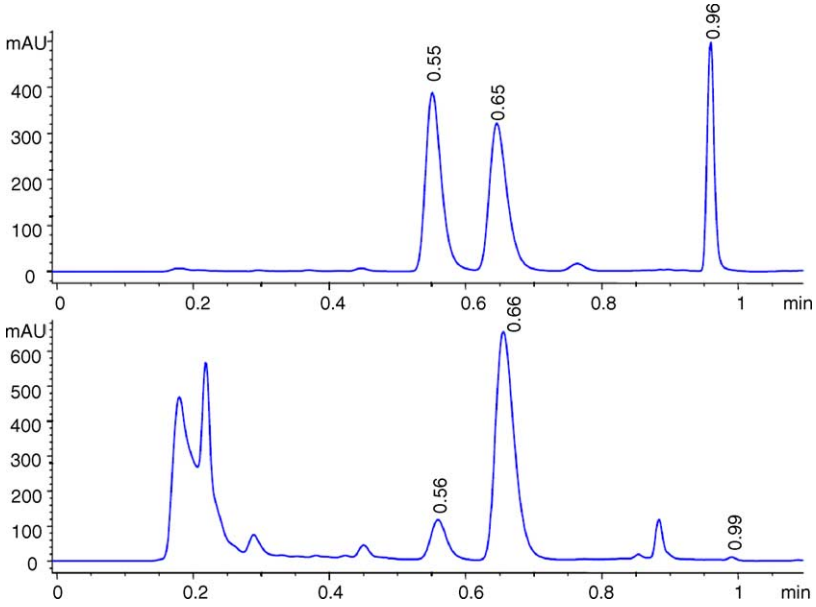

Fig. 2. Chromatogram (particle column) of standard solution (upper trace); 6-*epi* pravastatin ($t_R = 0.55$ min), pravastatin ($t_R = 0.65$ min) and mevastatin ($t_R = 0.96$ min) and chromatogram of fermentation broth.

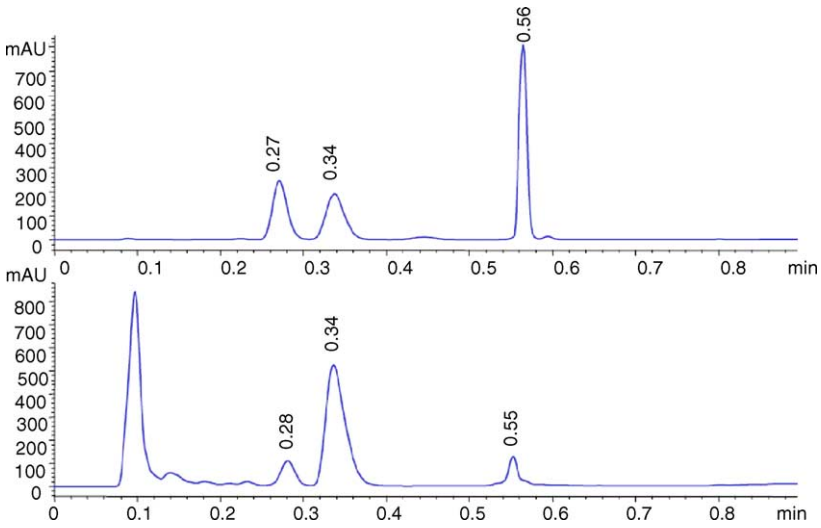

Fig. 3. Chromatogram (monolith column) of standard solution (upper trace); 6-*epi* pravastatin (t_R = 0.27 min), pravastatin (t_R = 0.34 min) and mevastatin (t_R = 0.56 min) and chromatogram of fermentation broth.

Fig. 4 electropherogram of 6-*epi* pravastatin (t_R = 0.59 min), pravastatin (t_R = 0.62 min), mevastatin (t_R = 0.88 min) and fermentation broth is presented. The content of mevastatin in fermentation broth is under LOD, which was 0.02 mg mL^{-1}. CE method had very low solvent consumption (1.5 mL of buffer per hour), but was weak on limit of detection (0.02 mg mL^{-1}) and reproducibility. Relative standard deviation (R.S.D.) of peak areas was about 4.1% (n = 27). Limit of detection can be improved with sample pre-concentration or increased sample load. Also so-called "bubble" cell capillaries (Agilent Technologies, Waldbronn, Germany) with extended light path can be used to improve sensitivity 3- to

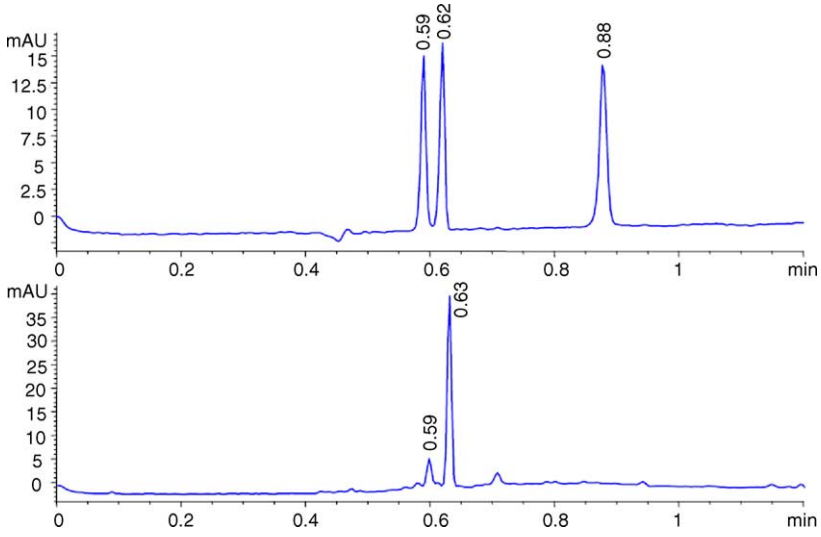

Fig. 4. Electropherogram of standard solution (upper trace); 6-*epi* pravastatin (t_R = 0.59 min), pravastatin (t_R = 0.62 min) and mevastatin (t_R = 0.88 min) and electropherogram of fermentation broth.

Table 1
Summerized results, number analysis per hour, relative standard deviation (R.S.D.) of peak areas of pravastatin, solvent consumption (per hour) and limit of detection (LOD)

	Samples per hour	R.S.D. (%) [pravastatin]	Solvent consumption (mL) per hour	LOD (mg mL^{-1})
CHROMATOGRAPHY I	26	0.2	132	10^{-5}
CHROMATOGRAPHY II	33	0.1	510	2×10^{-4}
CAPILLARY ELECTROPHORESIS	27	4.1	1.5	0.02

5-fold over standard capillary. The attained LOD was sufficient for the purpose of developed screening method.

Parameters of interest for all three high-speed separation methods: number of analyses per hour, relative standard deviation (R.S.D.), solvent consumption and limit of detection are summarized in Table 1.

4. Conclusion

The need for massive screening of different processes in bioreactors demands the development of fast, reliable and inexpensive analytical procedures. The use of small porous spherical particles (<2 μm) column or monolithic column enables fast HPLC analysis. CE methods with short capillaries and alkaline buffers generating strong EOF are also often used for fast analytical procedures.

Two HPLC methods using small particles and monolithic column, and MECK method were developed for the determination of pravastatin in fermentation broth. Methods were optimized for high throughput analysis. All methods have similar throughput but differ in consumption of chemicals, sensitivity and reproducibility. Reproducibility is very important factor for determination of pravastatin in fermentation broth because of small variations between samples. The HPLC methods were more reproducible and sensitive with R.S.D. 0.1–0.2% in comparison with MECK method with R.S.D. 4%. The limit of detection was 10^{-5} mg mL^{-1} for the particle column HPLC method, 2×10^{-4} mg mL^{-1} for monolithic column HPLC method compared to 0.02 mg mL^{-1} achieved at MECK method, respectively. The main drawback of monolith column HPLC method was in high solvent consumption.

We use particle column HPLC method because of its good reproducibility and relatively low solvent consumption.

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References

[1] P. O'Connor, J. Feely, J. Shepard, Br. Med. J. 300 (1990) 667.
[2] S. Vickers, C.A. Duncan, J.W. Chen, A. Rosegay, D.E. Duggan, Drug Metab. Dispos. 18 (1990) 138.
[3] T. Fujioka, F. Nara, Y. Tsujika, J. Fukushige, M. Fukami, Mi. Kuroda, Biopshys. Acta 1254 (1995) 7.
[4] M. Arai, N. Serizawa, A. Terahara, Y. Tsujita, M. Tanaka, H. Masuda, S. Ishikawa, Sankyo Kenkyusho Nempo 40 (1988) 1.
[5] K. Heinig, J. Henion, J. Chromatogr. B 732 (1999) 445.
[6] A.D. Jerkovich, J.S. Mellors, J.W. Jorgenson, LCGC Eur. 16 (2003) 20.
[7] N. Tanaka, H. Kobayashi, N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Hosoya, T. Ikegami, J. Chromatogr. A 965 (2002) 35.
[8] F. Svec, LC GC Eur. 16 (2003) 24.
[9] D. Lubda, K. Cabrera, W. Kraas, C. Schaefer, D. Cunningan, LCGC Eur. 14 (2001) 730.
[10] K. Cabrera, D. Lubda, H.M. Eggenweiler, H. Minakuchi, K. Nakanishi, J. High. Resol. Chromatogr. 23 (1) (2000) 93.
[11] G. Dear, R. Plumb, D. Mallett, Rapid Commun. Mass Spectrom. 15 (2001) 152.
[12] H. Zeng, Y. Deng, J.T. Wu, J. Chromatogr. B 788 (2) (2003) 331.
[13] M.M. Hefnawy, H.Y. Aboul-Enein, Anal. Chim. Acta 504 (2) (2004) 291.
[14] M. Schulte, J. Dingenen, J. Chromatogr. A. 923 (2001) 17.
[15] D. Josic, A. Buchacher, A. Jungbauer, J. Chromatogr. B 752 (2001) 191.
[16] K.D. Altria, J. Chromatogr. A 856 (1999) 443.
[17] M.A. Jenkins, M.D. Guerin, J. Chromatogr. B 682 (1996) 23.
[18] P. Bartak, P. Bednar, D. Friedecky, A. Haviger, J. Ševčik, J. Chromatogr. B 758 (2001) 323.
[19] European Pharmacopoeia 4.5, 3760.