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High-performance liquid chromatographic analysis of mevinolin as mevinolinic acid in fermentation broths☆

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Abstract

High-performance liquid chromatographic analysis of mevinolin in fermentation broth was initially performed after addition of acid and extraction with methanol using a mobile phase at pH 3.0. Under such conditions mevinolin was present in three different forms: as a lactone, as the corresponding β-hydroxy acid (mevinolinic acid) and as its methyl ester. To achieve accurate and reproducible results the method was modified such that only one form was present: mevinolinic acid. The fermentation broth samples were adjusted to pH 7.7 before the extraction with methanol, and the pH of the mobile phase was adjusted to 7.7 as well. For the separation a 250 × 4 mm I.D. column, termostated at 40°C, packed with Spherisorb ODS 2 of 5 μm particle size was used. Under these conditions mevinolin was detected at 237 nm as a single peak of a β-hydroxy acid, which has the lowest retention time of all three forms.

1. Introduction

Mevinolin (monacolin K, lovastatin) is an important fungal secondary metabolite inhibiting the enzyme which catalyses a rate-limiting step in the biosynthesis of cholesterol; it is an effective drug for the treatment of atherosclerosis and ischemic heart disease. Commercially this substance is produced biotechnologically by the fungi *Aspergillus terreus* or *Monascus rubber*.

The basic chemical structure of mevinolin contains a naphthalene ring system, a β-hydroxylactone and methyl butyric acid [1] as shown in Fig. 1a. The physiologically active form of the drug is the β-hydroxy acid which is formed by hydrolysis of the lactone ring (Fig. 1b).

Different analytical methods were developed for measuring mevinolin in blood [2,3], in bile [2], in tablets [4] and in fermentation broths [5,6]. Except for a method employing gas chromatography in combination with mass spectrometry [3], HPLC was used for mevinolin

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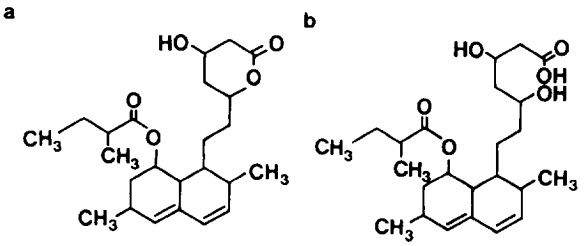

Fig. 1. Structural formulae of (a) mevinolin and (b) mevinolinic acid.

analyses. The separation was performed on reversed-phase columns with a mobile phase composed of a buffer solution and an organic modifier, and the drug was detected spectrophotometrically. To detect mevinolin in fermentation broths, Gullo et al. [5] derivatized the compounds to 4-nitrobenzoate derivatives. Mevinolin and the related compound hydroxymevinolin together with their β-hydroxy acids could be detected. The essential advantage of derivatization was the possibility to measure dihydromevinolin which has low UV absorption. Recently, a simple and fast method was described for analysing mevinolin directly without prior derivatization [6]. Kysilka and Křen [6] omitted the extraction of the broth, which is usually performed with methanol [5,7]. The substance was simultaneously present in two forms, as a lactone and as an acid and detected as two separate peaks.

In our laboratory biosynthesis of mevinolin by fungi was studied. For the detection during fermentation, the HPLC method by Monaghan et al. [8] had to be modified. This paper presents a new method for analysis of mevinolin in fermentation broths.

2. Experimental

2.1. Reagents and chemicals

As pure mevinolin was not available, a tablet of Mevacor (MSD, West Point, PA, USA) was used to prepare the standard solution. The tablet was dissolved in methanol, analytical-reagent grade (Serva, Heidelberg, Germany) and deionized water (1:1). The mobile phase consisted of HPLC-grade acetonitrile (Serva) and 0.02 *M* potassium phosphate buffer prepared from KH_2PO_4 and K_2HPO_4, both analytical-reagent grade (Kemika, Zagreb, Croatia). The initial pH value of the buffer was 3.0 and the proportion of acetonitrile–buffer was 35:65 (v/v). These values were changed during the experiments as indicated under Results and discussion. The mobile phase was filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA).

2.2. Equipment

All analyses were performed on a Knauer (Berlin, Germany) isocratic system equipped with a data acquisition station of the same producer. The 250 × 4.0 mm I.D. column, thermostated at 40°C, packed with Spherisorb ODS 2 of 5 μm particle size, was from BIA (Ljubljana, Slovenia). During all experiments the flow-rate of the mobile phase was 0.7 ml/min. The detection was at 237 nm (UV detector, Knauer).

2.3. Sample preparation

After fermentation, the broth was harvested and the pH was adjusted to the appropriate value as indicated under Results and discussion. An equal volume of methanol was added and the suspension was shaken for 1 h on a rotary shaker at 200 rpm and 30°C. The suspension was then filtered, first through a filter paper ("black ribbon") and the filtrate obtained then through a micro filter (Millipore) of 0.22 μm pore diameter. The samples were injected into the HPLC column with a 20-μl sample loop injector.

To obtain the lactone form standard, a tablet of Mevacor was crushed in a mortar and suspended in a 1:1 mixture of deionized water (adjusted to pH 3.0 with 1 *M* HCl) and methanol. The suspension was sonicated for 20 min and filtered through a 0.22-μm filter. Subsequently, the filtrate was diluted to a concentration of 5 μg/ml and used in the analyses.

To obtain the acid form, the lactone was converted to acid through sodium salt according

to Brown et al. [9]. The crushed tablet was suspended in 0.1 *M* NaOH, sonicated and heated at 50°C for 1 h. Subsequently, the suspension was adjusted to pH 7.7 with 1 *M* HCl, filtered through a 0.22-μm filter (Millipore) and diluted to the concentration of 5 μg/ml.

3. Results and discussion

Initially we intended to determine mevinolin in the lactone form. According to the literature [8], mevinolinic acid can be converted to the lactone form at acidic pH. Therefore the broth samples were acidified to pH 3.0. Since the lactone was not soluble in water, methanol was added to the broth for extraction. After carrying out some analyses, it was found that mevinolin was not efficiently converted to the lactone and that a larger part was still present as mevinolinic acid. In addition to these two forms, a third unknown peak was observed in the samples as well as in the standard solution after some days of storing in the refrigerator (Fig. 2a). It could be the methyl ester of mevinolinic acid. To confirm this assumption, Mevacor tablets were dissolved in water–methanol (1:1, v/v), in water–ethanol (1:1, v/v) and in water–*n*-propanol (1:1, v/v). The solutions were stored at room temperature for three days and were subsequently analysed. It was shown that the retention time (t_R) of the unknown peak increased with the molecular mass of the alcohol used from 6 min 50 s to 8 min 7 s and 9 min 49 s, respectively. The results are shown in Fig 2. From these results it was concluded, that after extraction with methanol, mevinolin was present in the broth samples in three forms: as a lactone, as the corresponding β-hydroxy acid and as its methyl ester. The same forms were found in the aged solution of the standard, indicating that under acidic conditions the lactone slowly transformed to acid, which further reacted with methanol to form an ester (Fig. 2a).

A modified method for the analysis of mevinolin was developed. The following facts were taken into account:

(1) In the samples of our fermentation broth

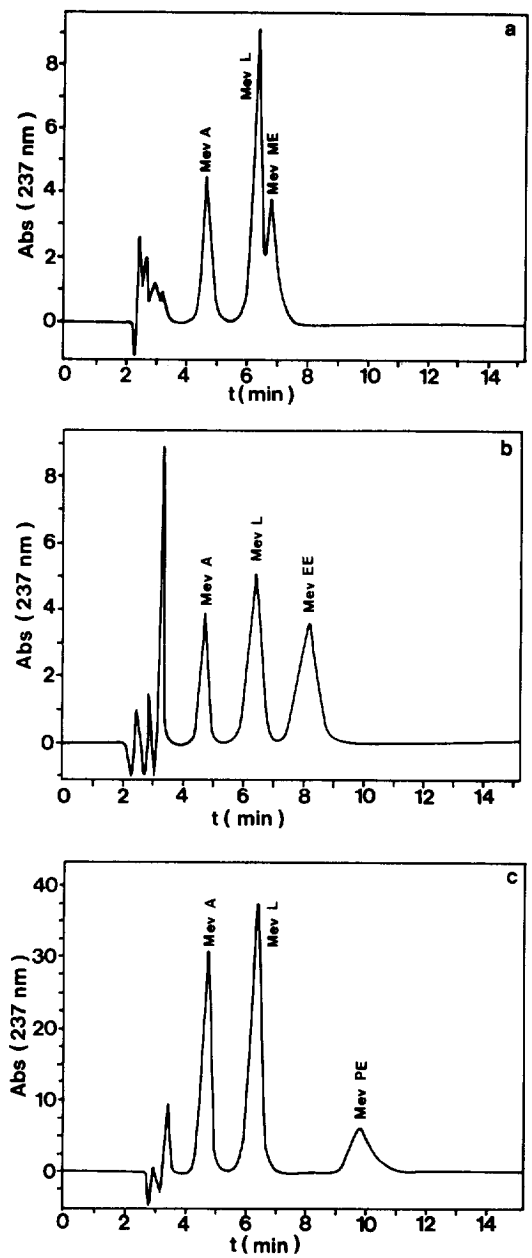

Fig. 2. HPLC analysis of a standard prepared in a lactone form, solubilized in (a) water–methanol (1:1), (b) water–ethanol (1:1), (c) water–*n*-propanol (1:1), after storing the solutions at room temperature for three days. Peaks: Mev A = mevinolinic acid; Mev L = mevinolin as lactone; Mev ME, Mev EE, Mev PE = methyl, ethyl and propyl ester of mevinolinic acid, respectively. Column: Spherisorb ODS 2, mobile phase: potassium phosphate buffer (pH 3.0)–acetonitrile (30:70, v/v), flow-rate: 0.7 ml/min, UV detection at 237 nm.

and according to the data found in the literature [8], the substance was "present in the fermentation broth largely as the hydroxycarboxylate (open lactone) form".

(2) From our experiments it followed that the acid was more stable in acidic water–methanol mixture than the lactone. The lactone form was stable only in pure methanol. Since water can not be avoided in the samples of fermentation broth, it is convenient to convert mevinolin to mevinolinic acid.

(3) The acid form had a lower t_R than the lactone.

A method was developed in which the measured substance was present in the acid form as shown in Fig. 3. Since mevinolinic acid is stable at pH 7.7, the samples were prepared at this pH. The pH of the mobile phase had to be adjusted to the same value as well. For this purpose acetonitrile was mixed with 0.02 *M* potassium phosphate buffer at pH 7.7 in a 65:35 proportion. The broth samples were further treated as described above. The corresponding separation is shown in Fig. 4.

In order to check the necessity of extraction with methanol, some experiments were done by omitting the extraction step. However, the yield

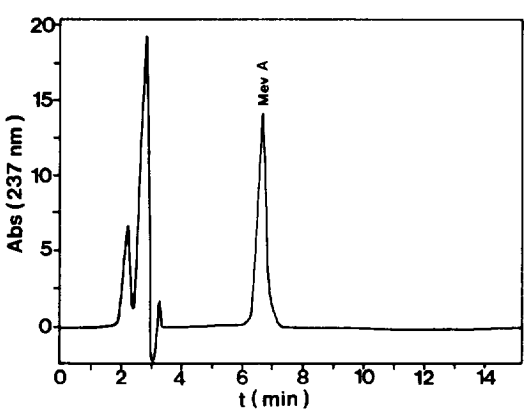

Fig. 3. Chromatogram of a standard in the acid form. Column: Spherisorb ODS 2, mobile phase: potassium phosphate buffer (pH 7.7)–acetonitrile (65:35, v/v), flow-rate: 0.7 ml/min, UV detection at 237 nm. Peak: Mev A = mevinolinic acid.

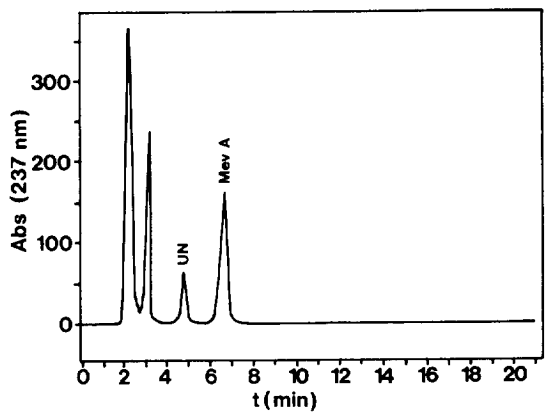

Fig. 4. HPLC chromatogram of a broth sample. Column: Spherisorb ODS 2, mobile phase: potassium phosphate buffer (pH 7.7)–acetonitrile (65:35, v/v), flow-rate: 0.7 ml/min, UV detection at 237 nm. Peaks: UN = unknown; Mev A = mevinolinic acid.

was much lower, attaining less than 60% of that obtained in the extracted samples.

From these results it can be assumed that a part of the substance was present as a lactone and solubilized only after addition of methanol, or that the substance was bound to the mycelium and released from it after methanol had changed the cell membrane composition and its permeability.

The effect of storage time on the standard solution was studied as well. The HPLC analysis was prolonged from 15 to 60 min in order to eliminate any doubt about the existence of the lactone form. The standard in the acid form was stable since no additional peak was detected after storing the solution for 10 days (data not shown).

Comparing this method with the one described by Kysilka and Křen [6], it can be seen that in our experiments the extraction of the broth was necessary. Without extraction of the fungal biomass with methanol, a great part of mevinolin was not detected. An additional advantage is the neutral pH, where all substance is in the form of one single compound. This proved to be the most stable, and had the lowest retention time during HPLC analysis under these conditions. In

the fermentation broth extracts mevinolin is found predominantly in its acid form and only in a small amount as the closed lactone [7]. Therefore the best way to determine the amount of mevinolin in fermentation studies is to measure it in the form of mevinolinic acid.

4. Conclusions

The HPLC analysis of mevinolin in fermentation broths extracted with methanol under acidic conditions results in three peaks corresponding to acid, lactone and methyl ester of mevinolin. By adjusting the pH of the samples and the mobile phase to 7.7, the substance can be detected as a single peak of mevinolinic acid, which is stable and has the lowest retention time of all three forms. It is not convenient to omit the extraction of the broth with methanol, since the yield is reduced by nearly half of the value detected after extraction.

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