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Note

High-performance liquid chromatographic analysis of derivatized hypocholesteremic agents from fermentation broths

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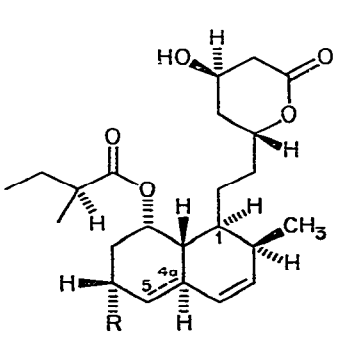
Substances which reduce cholesterol synthesis in humans are of great interest because of the possible connection between high blood cholesterol and atherosclerosis[1,2]. The fermentation metabolites of *Aspergillus terreus*, mevinolin (I)[3] and dihydromevinolin (II)[4], inhibit cholesterol biosynthesis at the level of the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). The analysis of these hypocholesteremic agents in fermentation broths by high-performance liquid chromatography (HPLC) is of great interest.

The determination of low concentrations of mevinolin in broth extracts by HPLC is conveniently carried out using ultraviolet (UV) detection at 237 nm. However, dihydromevinolin lacks the diene chromophore and has no appreciable absorption at this wavelength. An additional problem is that mevinolin and dihydromevinolin exist in the lactone and free acid forms which makes the chromatographic separation of all four substances from impurities difficult.

The formation of benzoate esters for UV detection in HPLC has been reported for hydroxylated compounds such as hydroxy steroids[5–8] and carbohydrates[9]. In this paper we report a method for the HPLC analysis of mevinolin and dihydromevinolin by the efficient formation of the 4-nitrobenzoate derivative. The method we employ yields the reaction products in the lactone form which reduces chromatographic difficulties. The derivatives can be readily separated from the reagents and broth impurities by reversed-phase HPLC with detection limits in the ng range. The method as presented in this paper demonstrates a simple derivatization procedure for fermentation broth extracts with minimal sample preparation before HPLC analysis.



I	Mevinolin	R = CH_3, $\Delta^{5,4a}$
II	Dihydromevinolin	R = CH_3
III	Compactin	R = H, $\Delta^{5,4a}$
IV	Dihydrocompactin	R = H

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The utility of forming 4-nitrobenzoate derivatives is not limited to the quantitative analysis of known hydroxylated compounds. This method can also be employed for observing hydroxylated compounds which cannot be readily detected because of the lack of a significant UV chromophore. When the procedure was applied to the fermentation broth of *Penicillium citrinum* which produces compactin (III)[10,11], the previously undetected compound dihydrocompactin (IV) was discovered[12].

EXPERIMENTAL

Reagents

4-Nitrobenzoylchloride (practical grade, Eastman, Rochester, NY, U.S.A.) was recrystallized from hexanes (b.p. 65.8°–68.8°C; Fisher, Pittsburgh, PA, U.S.A.). 4-Dimethylaminopyridine was obtained from Aldrich (Milwaukee, WI, U.S.A.). All solvents for extraction and derivatization were ACS certified (Fisher). The HPLC solvents were all HPLC Grade (Fisher).

Instrumentation

For HPLC analyses the following system was employed: a Micromeritics Model 725 autoinjector with a 6-μl injection loop, a Spectra-Physics SP 4100 Computing Integrator, a Laboratory Data Control Spectromonitor III detector set at 260 nm, a DuPont Zorbax C_8 column thermostatted at 60°C and a Spectra-Physics 740B pump set a 2.0 ml/min for the solvent system: acetonitrile–methanol–water (69:2:29).

Extraction and derivatization

Fermentation broth (2 ml), adjusted to pH 4.0 with HCl, was extracted with an equal volume of ethyl acetate. A 1-ml volume of the ethyl acetate was separated and evaporated to dryness under a stream of nitrogen. The sample was redissolved in 1 ml of methylene chloride and reacted with 50 μl of a 500 mg/ml solution of 4-nitrobenzoylchloride and 100 μl of a 200 mg/ml solution of 4-dimethylaminopyridine in the same solvent. After 5 min at room temperature, 100 μl of methanol were added followed by the addition of 1 ml of 0.75 *M* ammonium phosphate, pH 3.0. After mixing, the sample was then centrifuged at 1200 *g* for 30 sec and the lower methylene chloride phase was placed in an autoinjector vial. A 6-μl volume of sample was injected into the HPLC system. Injection of larger volumes caused peak splitting in the chromatography, apparently due to the incompatibility of the methylene chloride with the HPLC solvent system.

Preparation of standards

Standards were prepared by dissolving a known quantity of mevinolin and dihydromevinolin in methylene chloride. A 1-ml volume of the standard solution was nitrobenzoylated as described above for the fermentation extracts.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatographic separation of the 4-nitrobenzoate derivative of mevinolin and dihydromevinolin for a standard and a fermentation broth

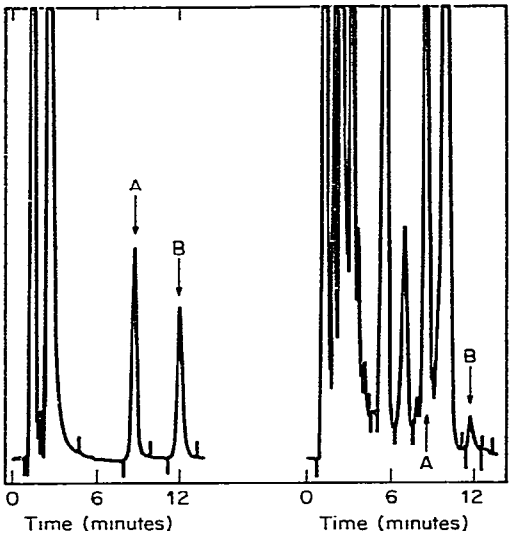

Fig. 1. Typical chromatograms of a standard (left) and a fermentation broth extract (right). A = Mevinolin; B = dihydromevinolin. The concentrations of the injected solutions are: A = 298 μg/ml, B = 268 μg/ml (left); A = 823 μg/ml, B = 62 μg/ml (right).

extract. The capacity factors (k') are 9.0 and 12.4 for mevinolin and dihydromevinolin nitrobenzoates, respectively. As shown in the figure most of the impurities, including excess reagents, side products, etc., elute very close to time = 0. Analysis time for each sample is less than 20 min and resolution of the mevinolin and dihydromevinolin nitrobenzoates is satisfactory for quantitative analyses by peak height measurements.

Derivatization of the acid forms of mevinolin and dihydromevinolin yielded the lactonized 4-nitrobenzoate forms of these compounds. The mechanism of this lactonization reaction is most likely to occur via the mixed anhydride. The concomitant lactonization is advantageous, when assaying fermentation broths which can contain both the acid and lactone forms of mevinolin and dihydromevinolin, because the quantitation and separation of two components from a complex mixture is more precise than four components. In addition, the analysis can be carried out isocratically in a reasonable time whereas the separation of four components could require a gradient analysis and increased time.

Linearity and precision

The calibration curves for the derivatives of mevinolin and dihydromevinolin are linear within the concentration range of 0–4 mg/ml and have zero intercepts.

The precision of the benzoylation procedure was determined by repeatedly assaying a single fermentation broth extract. The relative standard deviation for mevinolin is 2.2% and for dihydromevinolin is 2.7%.

Stability of the derivatized products

For this family of compounds, the hydroxyl group is located β to the lactone carbonyl and ideally poised for elimination when derivatized. For mevinolin, the

elimination of the 4-nitrobenzoate group is quite rapid in the reaction solution at room temperature (94% in 5.5 h). Extensive studies were performed to determine the best method for stabilizing the derivatization products. Interestingly, dihydromevinolin was more stable with only a 10% loss in 5.5 h. The additional $\Delta^{5,4a}$ double bond in mevinolin which flattens the decalin system, possibly interacts with the transition state to facilitate the β elimination. Various other acylating agents were explored; *i.e.*, 4-fluoro- and 4-bromobenzoylchloride and benzoyl chloride. The products were more stable (a 17% loss of the bromobenzoate group in 7 h for mevinolin) but the reaction times were slower. For the 4-fluoro- and 4-bromobenzoylchloride, the reaction times at room temperature were approximately 20 min and for benzoyl chloride much greater than 20 min. The most satisfactory method to prevent β elimination involved the addition of methanol after the desired reaction was complete, followed by extraction with pH 3.0 ammonium phosphate buffer. The 4-nitrobenzoate derivatives were stable for days at room temperature in the methylene chloride solution. Buffer extraction also removed other materials which resulted in improved chromatograms.

Discovery of dihydrocompactin

When the procedure was applied to the fermentation broth of *P. citrinum*, which produces compactin (III), a new peak was observed. A comparison of the relative retention times of mevinolin and dihydromevinolin as compared with compactin and this unknown peak suggested that the unknown peak was dihydrocompactin (IV). Isolation and structure determination of the material corresponding to this derivatized compound indeed proved this identification to be correct[12].

CONCLUSION

The formation of 4-nitrobenzoate derivatives of the HMG-CoA reductase inhibitors in the mevinolin family has been demonstrated as a practical method for sensitive HPLC analysis. The compounds of interest can be derivatized in a fermentation broth extract in a short time, at room temperature. Separation of the complex mixture can be achieved isocratically on a microparticulate C_8 column. Compounds previously with only low UV absorption can now be quantitatively observed at 260 nm with detection limits in the ng range.

There are numerous practical applications of this method. As demonstrated by the discovery of dihydrocompactin, compounds which were previously undetected can now be observed. The method can also be applied to analyses for fermentation development studies and drug metabolism studies. The 4-nitrobenzoylchloride derivatization technique should be applicable to many organic extractable hydroxylated compounds which occur in fermentation broths.

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