Scientific Paper

Identification of an Impurity in Pravastatin by Application of Collision-Activated Decomposition Mass Spectra

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Abstract

Pravastatin is produced by a two step fermentation. Mevastatin and *6-epi* pravastatin are the main impurities in the fermentation broth as well as in final product. An unknown impurity with *m/z* 437 for $(M-H)^-$ was detected in analysis of pravastatin sodium sample by reversed phase HPLC-MS. CAD spectrum of the impurity was obtained using HPLC-MS/MS equipped with negative APCI. The CAD mass spectra of impurity was similar with CAD mass spectra of statins. The structure of an impurity was finally determined on the basis of proposed fragmentation mechanisms for statins.

Keywords: HMG-CoA reductase inhibitors, statins, tandem mass spectrometry, impurity identification

1. Introduction

Pravastatin, simvastatin, lovastatin and mevastatin are 3-hydroxy-3-methylglutaryl coencime A (HMG-CoA) reductase inhibitors used in the treatment of hypercholesterolemia. HMG-CoA reductase is a key enzyme in the biosynthesis of cholesterol. Statins are most often prescribed substances for reducing mortality related to coronary heart diseases.[1-5] Treatment with statins is very long, so high purity of drug substance and the knowledge of the impurity profile are important criterions in a manufacturing process. All drugs contain impurities. Organic impurities can originate from raw materials and reagents, intermediates, synthetic or bio-synthetic by-products, or degradation products. It is important to know that some impurities can have safety and efficacy effects. In general, drug impurities in excess of 0.1% should be identified and quantified by selective methods.[6,7] Development of selective methods for the determination of impurities is one of the most important field of analytics in pharmaceutical industry. Chromatographic techniques (thin-layer-chromatography - TLC, high-performance liquid chromatography - HPLC, gas chromatography - GC, capillary electrophoresis – CE , and capillary electro chromatography - CEC) can be used for determination of known impurities. Unknown peaks on the chromatograms which represent unknown impurities should be identified and quantified. For the identification and structure elucidation of unknown impurities, spectroscopic techniques (mass spectrometry - MS, UV-spectrometry, NMR spectrometry, FTIR spectrometry) and hyphenated techniques (GC-MS, HPLC-MS, CE-MS, HPLC-NMR) are used.[8-11]

Mass spectrometry is a sensitive and selective spectroscopic technique which provides data for structural elucidation of organic molecules, especially when they are present in low concentration. With soft ionisation techniques, such as Atmospheric Pressure Chemical Ionisation (APCI) or Electrospray (ESI), molecular mass and fragment information is obtained. MS has been widely used in identifying of impurities and degradation products of drugs in pharmaceutical research. Several articles are dealing with quantitative determination of statins and biotransformation products in biological samples.[12-15] Mass spectrometric studies of lovastatin and simvastatin were performed initially with positive electrospray ionisation by Wang et al..[16] Fragmentation schemes for statins in both positive and negative mode were also proposed by Grahek et al. [17] . Negative ionisation and MS/MS data were used for the determination of simvastatin hydroxyl acid and related species by Xue-Zhi Quin.[18]

The experience of the collision-activated decomposition (CAD) of known HMG-CoA reductase inhibitors helped to identify an impurity in pravastatin. By reversed phase high-performance liquid chromatography mass spectrometry analysis of pravastatin, the unknown impurity was initially detected. The CAD spectra were subsequently acquired by HPLC-MS/MS. The

fragmentation pattern of the impurity was interpreted and the structure of the impurity was determined.

2. Experimental

2.1. Materials

Pravastatin, simvastatin, lovastatin and mevastatin were obtained by Lek Pharmaceutical company d.d. (Ljubljana, Slovenia). All four drugs were obtained as a lactone and acid form. Methanol, acetonitrile, ammonium acetate were obtained from Merck (Darmstadt, 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, simvastatin, lovastatin and mevastatin were prepared in concentration of 0.1 mg·mL^{-1} in 10 mM ammonium acetate, 70% acetonitrile.

2.3. Mass spectrometry

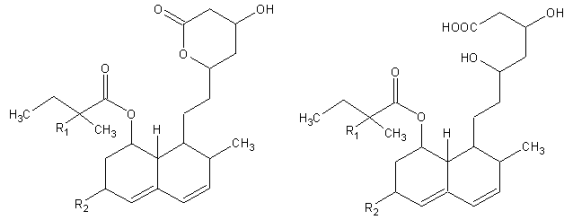
MS/MS measurements were performed with Finnigan TSQ 7000 instrument (San Jose, CA, USA) interfaced with an APCI ion source. APCI source parameters were: vaporiser temperature set to 520 °C, capillary temperature 220 °C, sheat gas (nitrogen) 90 psi, auxiliary gas (nitrogen) 5 mL·min^{-1}, corona 4 μA. Sample was introduced with loop injection 5 μL. Mobile phase (10 mM ammonium acetate, 30% methanol) flow rate was 1 mL·min^{-1}. Argon (2.5 mTorr) was used as collision gas. Collision energy was set in-between 20-27 eV.

2.4. HPLC-MS and HPLC-MS/MS

Analyses were performed using HPLC system consisted of SpectraSystem SCM 1000 Vacuum Degasser, SpectraSystem P4000 Quarternary Pump, SpectraSystem AS3000 Auto sampler with column oven and SpectraSystem UV6000 LP PDA Detector (Thermo Separation Products, San Jose, CA, USA). Separation was achieved on Betasil C18 (250 × 4.6) mm column (Keystone Scientific Inc., PA, USA) at the flow rate of 1 mL·min^{-1}. Column temperature was 25 °C. Gradient elution was carried out with 30% methanol with 10 mM ammonium acetate (mobile phase A) and 100% methanol with 10 mM ammonium acetate (mobile phase B). The mobile phase gradient was started at 25% of B, increased to 65% B within 8 min and to 100% B within 2 min. The injection volume was 20 μL.

3. Results and Discussion

Pravastatin is a natural product and is produced in a two-step fermentation. First mevastatin is produced by *P. citrinum* and then bioconverted to pravastatin by *S. carbophylus*. Raw material is then purified with different techniques (e.g. industrial preparative HPLC). Main impurities that usually occurred in raw material (fermentation broth) were mevastatin and *6-epi* pravastatin.[19] The structures of statins (pravastatin, simvastatin, lovastatin and mevastatin) are shown in Figure 1.



Pravastatin,	R_1 = H, R_2 = OH
Simvastatin,	R_1 = CH_3, R_2 = CH_3
Lovastatin,	R_1 = H, R_2 = CH_3
Mevastatin,	R_1 = H, R_2 = H

Figure 1. Structural formulas of pravastatin, simvastatin, lovastatin and mevastatin (lactone and acid form)

Sample of pravastatin sodium was analysed using reversed phase HPLC-MS (negative APCI ionization). At retention time of 10 min, an unknown impurity with molecular mass 438 Da was detected. HPLC-MS chromatograms (TIC and extracted ion *m/z* 437 chromatogram) are shown in Figure 2.

The main fragmentation pathways for pravastatin, simvastatin, lovastatin and mevastatin in lactones and acid form in both positive and negative ionisation modes was determined by studying low energy collision activated decomposition (CAD) of protonated $(M+H)^+$ and deprotonated $(M-H)^-$ molecules [16-18]. Ester side chain elimination from acid or lactones moiety is observed at all studied compounds. In negative ionization technique more relevant information are acquired for studying structure of statins in salt or acid form. Carboxylic group is deprotonated under negative APCI conditions. Less fragments are observed in negative product ion spectra. By all investigated molecules de-esterification is the first fragmentation step, forming *m/z* 321, 101 ions from pravastatin, *m/z* 319, 115 from simvastatin, *m/z* 319, 101 from lovastatin and *m/z* 305, 101 from mevastatin deprotonated molecule. In opposite to positive ionization, also ester part ($C_5H_8O_2R_1$) of the molecule can form an anion. So, with negative APCI also a structure modification on the ester side chain can be observed. CAD spectra of $(M-H)^-$ of pravastatin and $(M+H)^+$ are shown in Figure 3.

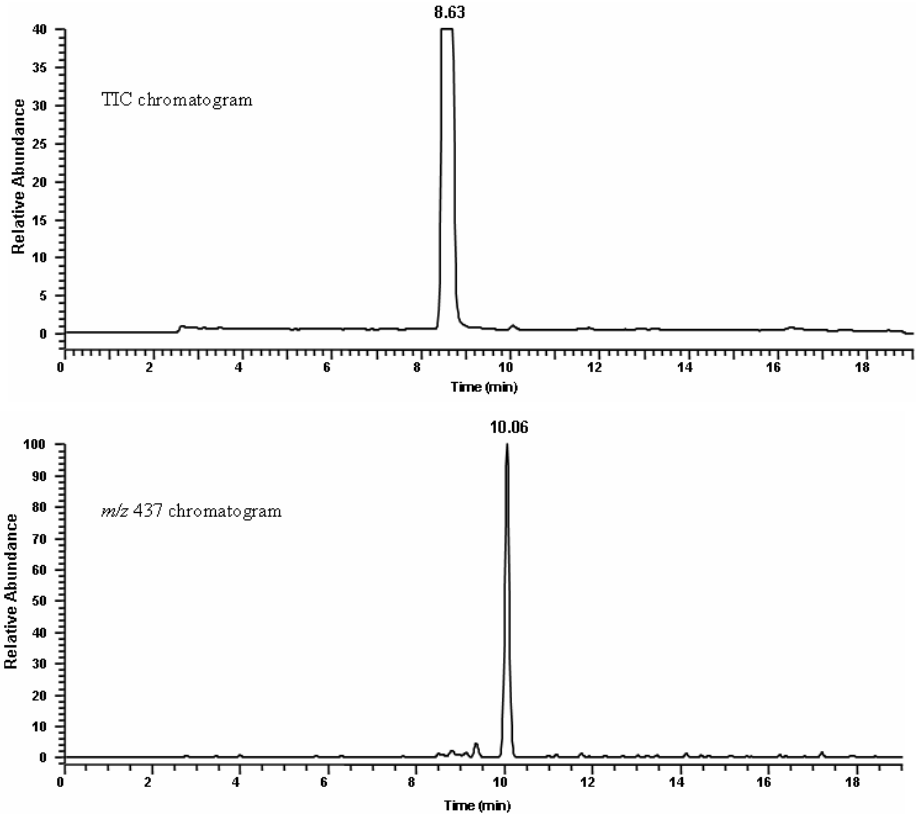


Figure 2. HPLC-MS chromatograms (TIC and extracted ion chromatogram *m*/*z* 437) of pravastatin sample

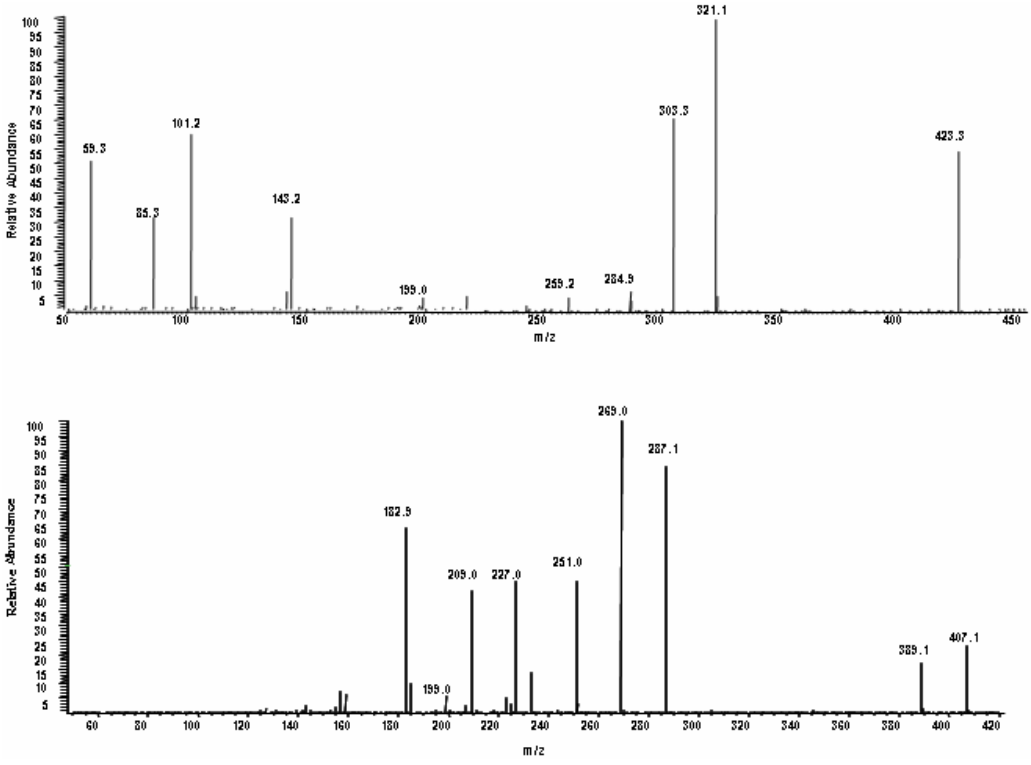


Figure 3. CAD spectrum of pravastatin acid form $(M-H)^-$ (upper trace) and CAD spectrum of pravastatin lactone $(M+H)^+$ (lower trace)

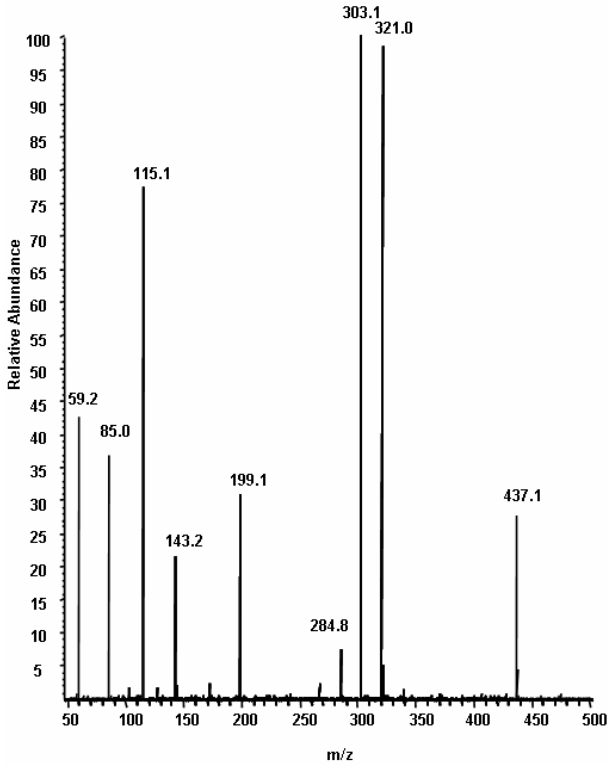


Figure 4. CAD spectrum (negative ionisation mode) of unknown impurity eluting at t_R of 10 min

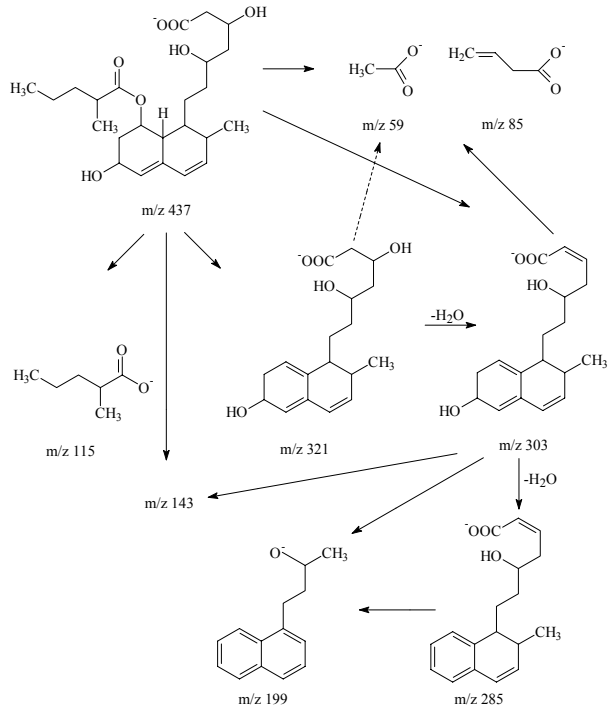


Figure 5. Proposed fragmentation scheme of unknown impurity

Using HPLC-MS/MS in product ion mode, we obtained CAD spectrum (Figure 4) of an impurity of pravastatin eluting at retention time 10 min (Figure 2). The main fragments *m/z* 321 and 303 indicate that the impurity is somehow a structural analogue to pravastatin.

By comparison of fragmentation sheme of $(M+H)^+$ and $(M-H)^-$ [17] of all statins, also fragmentation of impurity could be predicted. It can be pointed out that after de-esterification product ions typical for naphthalene part of molecule are obtained. Fragment ion *m/z* 115 can be described as 2-methyl-1-oxobutoxy group with an additional functional group (+ 14 Da). In the further study the impurity was isolated using preparative HPLC to obtain more pure compound (approximately 10 mg). By NMR measurements we confirmed that the impurity has 2-methyl-1-oxopentoxy group on naphthalene position 8. Proposed fragmentation scheme and structure of the impurity are presented in Figure 5.

4. Conclusion

By reversed phase HPLC-MS method a new impurity with $(M-H)^-$ *m/z* 437 in a sample of pravastatin sodium was detected. For identification of impurity more selective detection with MS/MS was applied. The structure of impurity was obtained by comparison of fragmentation pathway of impurity with known fragmentation pathways $(M+H)^+$ and $(M-H)^-$ ions of other statins. An impurity is an analogue to pravastatin with an additional methyl group on ester side chain.

5. Acknowledgements

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6. References

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Povzetek

Pravastatin pridobivamo z dvostopenjsko biosintezo. Vsebuje dve glavni nečistoči: mevastatin in *6-epi* pravastatin. S HPLC-MS analizo, ki se največkrat uporablja za določanje nečistoč v učinkovinah, smo detektirali novo nečistočo v vzorcu natrijevega pravastatina. Pri MS analizi smo uporabili negativno kemijsko ionizacijo pri atmosferskem tlaku (APCI). Molekulski ion $(M-H)^-$ nečistoče je bil m/z 437 . To ni zadoščalo za identifikacijo nečistoče. S trkovno razgradnjo iona $(M-H)^-$ nečistoče in nadaljnjo masnospektrometrično analizo smo ugotavljali strukturo neznane spojine. MS/MS spekter neznane spojine je bil podoben MS/MS spektrom drugih statinov. Na osnovi predpostavljene fragmentacijske sheme statinov smo določili strukturo neznane nečistoče, ki je imela na naftalenskem položaju 8 vezano 2-metil-1-okso-pentoksi-skupino.