Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 998 (2003) 133–141
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of cholesterol-lowering statin drugs in aqueous samples using liquid chromatography–electrospray ionization tandem mass spectrometry

Xiu-Sheng Miao, Chris D. Metcalfe*
Water Quality Center, Trent University, 3720 Nassau Mills Road, Peterborough, ON K9J 7B8, Canada

Received 3 March 2003; received in revised form 1 April 2003; accepted 9 April 2003

Abstract

Cholesterol-lowering statin drugs are among the most frequently prescribed agents for reducing morbidity and mortality related to coronary heart disease. Four major statin drugs, atorvastatin, lovastatin, pravastatin and simvastatin, were determined using liquid chromatography–electrospray ionization tandem mass spectrometry with methylammonium acetate as an additive in the mobile phase. Protonated atorvastatin, and methylammonium-adducted lovastatin, pravastatin and simvastatin were selected as precursor ions, and product ions were detected by selected reaction monitoring in positive-ion mode. The instrumental detection limits of atorvastatin, lovastatin, pravastatin and simvastatin are 0.7, 0.7, 8.2 and 0.9 pg, respectively. A solid-phase extraction method was developed to enrich the analytes from aqueous samples. All of the statins were detected in an untreated sewage sample at 4–117 ng/l and in a treated sewage sample at 1–59 ng/l; but only atorvastatin was detected in a surface water sample at 1 ng/l.

Keywords: Environmental analysis; Cholesterol reducers; Statins

1. Introduction

Cardiovascular disease, in particular coronary heart disease (CHD), is the principal cause of morbidity and mortality [1]. Elevated plasma total cholesterol and low-density lipoprotein cholesterol levels have been shown repeatedly to be predictive of premature CHD [2]. The ‘‘statin’’ class of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors represent the most efficient drugs for the treatment of hypercholesterolemia, and they can significantly reduce the morbidity and mortality associated with CHD [3]. The statin class of cholesterol-lowering drugs, including atorvastatin (Lipitor®), cerivastatin (Baycol®), fluvastatin (Lescol®), lovastatin (Mevacor®), pravastatin (Pravachol®) and simvastatin (Zocor®), have been introduced since 1987. Lovastatin is a natural product; simvastatin and pravastatin are semi-synthetic products; and atorvastatin, fluvastatin and cerivastatin are completely synthetic compounds.

Statins are now among the most frequently prescribed drugs [4]. For example, worldwide sales of simvastatin were $6.67 billion in 2001 [5]. Choles-

*Corresponding author. Tel.: +1-705-748-1272; fax: +1-705-748-1587.
E-mail address: cmetcalfe@trentu.ca (C.D. Metcalfe).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00645-9

terol-lowering drugs of the ''fibrate'' class, including bezafibrate, clofibrate, etofibrate, fenofibrate and gemfibrozil have been frequently detected in the aquatic environment [6–8]. Therefore, the more highly prescribed statins may also be of environmental concern. In this study, an analytical method was developed using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS), coupled with solid-phase extraction (SPE) for determination of the statins in aqueous samples. Cerivastatin and fluvastatin were not included in this study because they are not major cholesterol-lowering drugs used therapeutically in North America.

2. Experimental

2.1. Materials

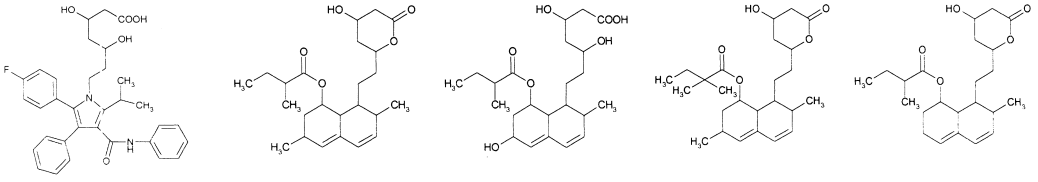
Lovastatin and mevastatin were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada). Atorvastatin, pravastatin and simvastatin were purchased as their formulations. Fig. 1 shows the chemical structures of the statins. Each analyte was dissolved in an appropriate volume of acetonitrile/aqueous methylammonium acetate (3:2, 2 m*M* methylammonium acetate, pH 4.5). The instability of the lactone compounds, such as lovastatin and simvastatin, can be caused by hydrolysis in aqueous media; while acidic compounds, such as atorvastatin and pravastatin, can form esters by reaction with alcohols (e.g. methanol). Thus, alcohols were avoided in the preparation of stock and working solutions of statins, and all standard solutions were stored in a −10 °C freezer to minimize the interconversion of statins.

Methylamine (40%) and acetic acid (99%) were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile and methanol were purchased from Caledon Laboratories (Georgetown, ON, Canada), and HPLC grade water was purchased from EM Science Industries (Gibbstown, NJ, USA).

The SPE cartridges, Oasis® HLB (Hydrophilic–lipophilic Balance), Bond Elute C8 and DSC-18 were purchased from Waters, Varian and Supelco, respectively. The 1.5-μm glass microfiber filters were purchased from Whatman (Clifton, NJ, USA).

2.2. Mass spectrometry

Mass spectrometry was performed using a Quattro LC™ tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source. The capillary voltage was 4.0 kV, and the voltages of extractor and RF lens were 3.0 and 0.8 V, respectively. The entrance and exit energies of the collision cell were set at 15.0 V. The cone voltage was operated at an optimal value for each analyte in positive-ion mode. Nitrogen was used as the drying and nebulizing gas at flow rates of 500 and 70 l/h,



Atorvastatin	Lovastatin	Pravastatin	Simvastatin	Mevastatin[b]
$C_{33}H_{35}N_2O_5F$	$C_{24}H_{35}O_5$	$C_{23}H_{36}O_7$	$C_{25}H_{37}O_5$	$C_{23}H_{34}O_5$
558.25[a]	404.26	424.25	418.27	390.24

[a] Lowest isotopomer

[b] Internal standard

Fig. 1. Chemical structures of statins.

respectively. The source and desolvation temperatures were optimized under LC–MS–MS operation, and were kept at 100 and 350 °C, respectively.

During method development, individual standard solutions were infused through a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow-rate of 10 μl/min into the mass analyzer. Following the selection of precursor ions by the first quadrupole mass analyzer, collision-induced dissociation (CID) was carried out using 2.0×10^{-3} mbar UHP argon (Praxair Products, Peterborough, ON, Canada) in the hexapole collision cell. Product ion mass spectra were obtained at a series of collision energies so as to characterize each compound's fragmentation behavior. When LC was used for analyte separation prior to tandem spectrometry, the mass spectrometer was operated in selected reaction monitoring (SRM) mode with 1.3 of low mass resolution (LM Res) and high mass resolution (HM Res) on both of the first and second analyzers. A dwell time of 200 ms per ion pair was used, and the inter-channel delay was 10 ms.

2.3. Liquid chromatography

Analytes were separated using a Waters 2695 liquid chromatograph (Waters, Milford, MA, USA) with a Genesis C_{18} column (2.1×50 mm, 3 μm) (Jones Chromatography, Hengoed, UK). The two mobile phase solvents, A and B, were acetonitrile and water, respectively, containing 2 m*M* methylamine with 0.1% acetate acid. The mobile phase gradient was started at 60% of A, which was increased linearly to 100% within 3 min and held for 2 min. The flow-rate of the mobile phase was 0.2 ml/min and the injection volume was 20 μl.

2.4. Sample collection and preparation

Samples of influent and effluent were collected from the sewage treatment plant (STP) in Peterborough, ON, Canada and surface water was collected from the Otonabee River on November 6, 2002. To remove suspended material, aqueous samples were vacuum filtered through 1.5-μm glass microfiber filters, which had been pre-washed with hexane/dichloromethane (1:1) in a Soxhlet apparatus for 2 h. After filtration, the pH of samples was adjusted to 4.5 with 3.0 *M* H_2SO_4.

SPE cartridges with different packing materials, HLB, Bond Elute C8 and DSC-18 were tested for the best recovery of the analytes. The cartridges were installed on a vacuum manifold and sequentially preconditioned with 6 ml acetone, 6 ml methanol and 6 ml HPLC grade water (pH 4.5). Thereafter, the aqueous samples (500 ml STP influent, 1000 ml STP effluent and 2000 ml surface water) were allowed to pass slowly through the cartridges at a rate of approximately 10 ml/min. After passage of the samples, each sample bottle was rinsed with 10 ml of pH 4.5 HPLC grade water, and the rinse was allowed to flow through the cartridge. The cartridges were eluted using three successive 3-ml aliquots of methanol. Each aliquot of methanol was eluted through the column for a minimum of 10 min. The eluates were collected in a 10-ml collection tube and concentrated to almost dryness with a vacuum centrifuge. Then the samples were reconstituted to 1.0 ml with acetonitrile: aqueous methylammonium acetate (3:2, 2 m*M* methylammonium acetate, pH 4.5).

2.5. Method validation

Spiked recovery experiments were performed to determine the precision and accuracy of the method. Matrix effects were investigated using HPLC grade water, surface water, and STP effluent and influent, and instrumental detection limits (IDLs) and method detection limits (MDLs) of the analytes were calculated. Surface water, and STP effluent and influent were analyzed to evaluate the method.

3. Results and discussion

3.1. LC–ESI-MS–MS

Lovastatin and simvastatin were administrated as their lactone forms; while atorvastatin and pravastatin were administered as calcium and sodium salts, respectively, of the active hydroxy acids. Therefore, atorvastatin and pravastatin were monitored as free acidic forms, and negative-ion mode was expected to be more appropriate for these two analytes. However, as we determined previously for atorvastatin,

positive-ion mode with $[M+H]^+$ as a precursor ion was more sensitive than negative-ion mode for LC–MS–MS [9]. Due to the high signal intensity of $[M-H]^-$, pravastatin has normally been analyzed in negative-ion mode [10]. Positive-ion mode is appropriate for the lactone compounds, so the positive–negative switch function available on most LC–MS–MS instruments can be used to simultaneously monitor statin drugs as acidic and lactone forms. However, acids (e.g. formic acid or acetic acid) are usually added to the mobile phase to improve sensitivity in positive-ion mode [11]. Therefore, positive-ion mode would be preferable for both the acidic and lactone compounds within the class of statins.

Various adduct ions, such as $[M+Na]^+$ and $[M+NH_4]^+$, have been used as precursor ions for LC–ESI-MS–MS analysis in positive-ion mode to improve method sensitivity [12,13]. Zhao et al. investigated the effects of ammonium and alkylammonium acetate as mobile phase additives on the ionization of simvastatin and simvastatin hydroxy acid, and observed that methylammonium acetate could much improve the ion signal intensity of simvastatin [14]. So, in our study, methylammonium acetate was used as a mobile phase additive in positive-ion mode, and $[M+CH_3NH_3]^+$ was selected as the precursor ion for lovastatin, pravastatin and simvastatin. $[M+H]^+$ was chosen for detection of atorvastatin due to the low signal intensity of the methylammonium adducts for this compound.

Capillary, cone, extractor and RF voltages were optimized during the full scan MS experiment based on the signal intensities of precursor ions. At the optimized conditions, methylammonium adduct ions, $[M+CH_3NH_3]^+$ of lovastatin, pravastatin and simvastatin were observed as major ions in the full scan mass spectra, while $[M+H]^+$ and $[M+Na]^+$ were suppressed. Fig. 2 shows the full scan mass spectrum of lovastatin, where the methylammonium adduct ions of lovastatin, $[M+CH_3NH_3]^+$, were observed as the base peak.

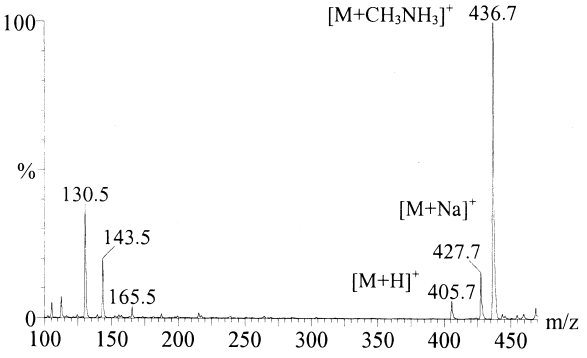


Fig. 2. Full scan mass spectrum of lovastatin in ESI positive-ion mode.

Fig. 3 illustrates the product ion mass spectra of $[M+CH_3NH_3]^+$ from lovastatin, pravastatin and simvastatin. They were similar to the product ion mass spectra of $[M+H]^+$ (data not shown). Major product ions at m/z 199, 267, 269, 285 were observed for the compounds, and their generating pathways were proposed by Wang et al. [15]. As for lovastatin, according to the signal intensities and structure-specificities of the product ions, ion of m/z 285, generated by losses of CH_3NH_2, the ester side-chain ($C_5H_{10}O_2$, 102 Da) and H_2O from the precursor ion, $[M+CH_3NH_3]^+$, was selected to set an SRM transition channel for monitoring lovastatin. Similar optimization processes were conducted for pravastatin and simvastatin. The sensitivity for analysis of pravastatin was compared in positive-ion mode with $[M+CH_3NH_3]^+$ as a precursor ion and m/z 456>269 as an SRM channel, with negative-ion mode with $[M-H]^-$ as a precursor ion and m/z 423>101 as an SRM channel. The former monitoring mode was approximately eight times more sensitive than the latter. Table 1 lists the optimized LC–ESI-MS–MS parameters together with the IDLs of the statins in SRM mode.

Based on achieving a balance between sensitivity, separation efficiency and analysis time, a flow-rate of 0.2 ml/min was selected for the separation of statins. Fig. 4 (left panel) illustrates the time-scheduled SRM chromatograms of a standard mixture of statins, in which the separation of the analytes together with the internal standard was achieved within 5 min.

3.2. Extraction efficiency and matrix effects

A variety of SPE cartridges, including HLB, Bond Elute C8 and DSC-18, were investigated to find the most efficient extraction method for statins. Mean recoveries of the analytes from surface water with different cartridges are summarized in Table 2. Bond

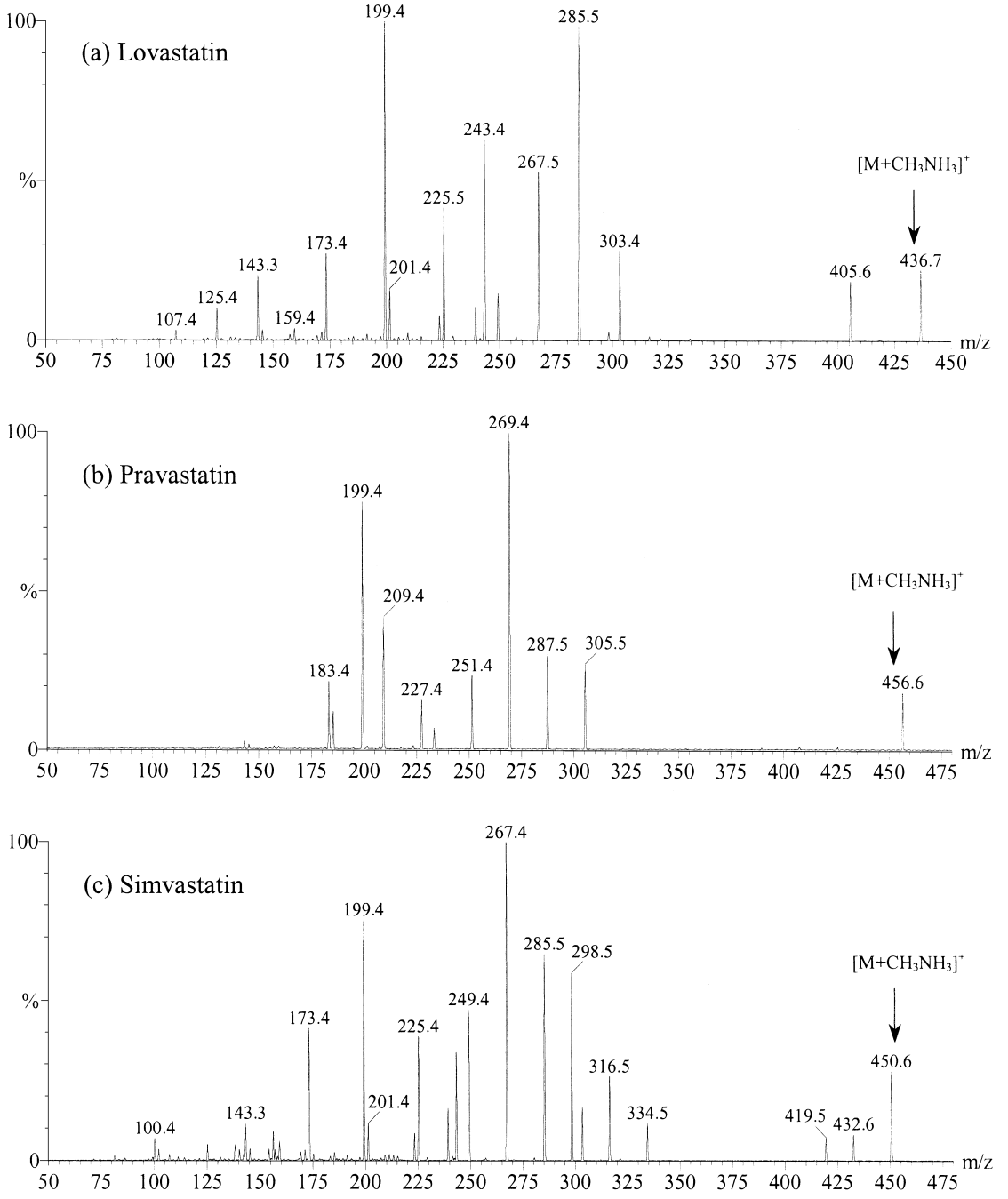


Fig. 3. Product ion mass spectra of $[M+CH_3NH]$ of lovastatin, pravastatin and simvastatin. The selected precursor ions are denoted by vertical arrows. (a) lovastatin, cone 25 V and collision energy 15 eV; (b) pravastatin, cone 18 V and collision energy 11 eV; (c) simvastatin, cone 25 V and collision energy 16 eV.

Table 1
Optimized LC–ESI-MS–MS parameters used for statin analysis in positive-ion mode

Analyte	Precursor ion	SRM channel m/z	Cone (V)	Collision energy (eV)	IDL[a] (pg)
Atorvastatin	$[M+H]^+$	559>440	35	24	0.7
Lovastatin	$[M+CH_3NH_3]^+$	436>285	25	15	0.7
Pravastatin	$[M+CH_3NH_3]^+$	456>269	18	12	8.2
Simvastatin	$[M+CH_3NH_3]^+$	450>199	25	18	0.9
Mevastatin[b]	$[M+CH_3NH_3]^+$	422>185	22	16	–

[a] Instrumental detection limit.
[b] Internal standard.

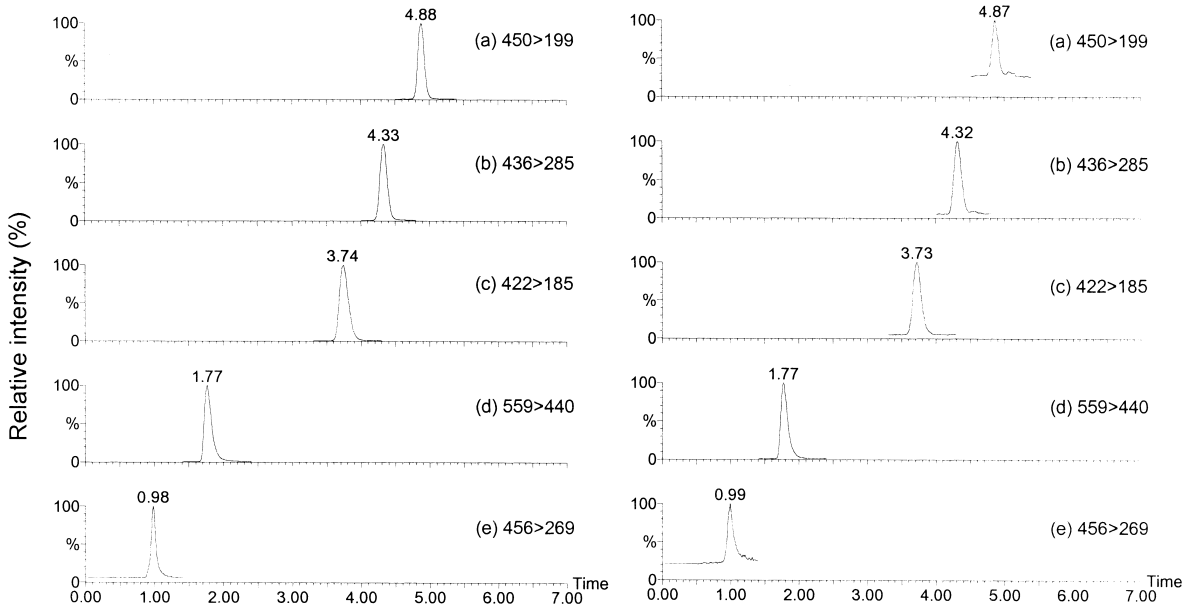


Fig. 4. Time-scheduled SRM chromatograms of a standard solution of statins (50 μg/ml) (left panel) and an effluent (right panel) of the STP in Peterborough, ON.

Elute C8 and DSC-18 had better recoveries for lovastatin, pravastatin and simvastatin, but they could recover only about 40% of atorvastatin. In addition, less ion suppression for the analytes was observed for extracts prepared from the HLB cartridge than the extracts from Bond Elute C8 and DSC-18 cartridges; and therefore, HLB cartridges were used to develop the extraction method. Table 3 lists the recoveries of analytes, which range from 71 to 86% in surface water, 64–87% in STP effluent, and 61–91% in STP influent.

Table 2
Mean recoveries of statins from surface water with different SPE cartridges (%)

Analyte	HLB	Bond Elute C8	DSC-18
Atorvastatin	86	40	42
Lovastatin	79	84	90
Pravastatin	71	73	90
Simvastatin	73	78	82

The degree of signal suppression was determined in different matrices, HPLC grade water, surface water, and STP effluent and influent. Fig. 5 demonstrates the ion signal suppression of analytes in different matrices. The matrix effects are reflected by the ion signal suppression ratio, where a factor of 1.0 is indicative of no signal suppression or enhancement. Little signal suppression was observed for

Table 3
Mean recoveries and MDLs of statins from different matrices[a]

Analyte	Surface water		STP effluent		STP influent	
	Recovery (%)	MDL (ng/l)	Recovery (%)	MDL (ng/l)	Recovery (%)	MDL (ng/l)
Atorvastatin	86 (5)	0.1	64 (6)	0.5	86 (3)	1.2
Lovastatin	79 (5)	0.1	81 (7)	0.9	91 (7)	1.2
Pravastatin	71 (6)	1.0	87 (4)	9.8	61 (7)	15.4
Simvastatin	73 (8)	0.1	69 (6)	0.2	84 (7)	1.0

[a] The average of triplicates of spiked concentrations of 100 and 500 ng/l.

HPLC grade water, but progressively more severe suppression was observed in analytes from surface water, to STP effluent, and then influent. However, the same matrix may cause different degrees of ion signal suppression to different analytes. For example, very severe signal suppression was observed for pravastatin in surface water (only ~27% of ions were monitored), but suppression was less for atorvastatin, lovastatin or simvastatin.

3.3. Method detection limits and quantification

Atorvastatin, lovastatin and simvastatin showed low MDLs in the aqueous samples compared to pravastatin, which exists in an acidic form (Table 3). The tendency for formation of $[M+CH_3NH_3]^+$ among lactone forms leads to higher sensitivity. Higher MDLs were observed for analytes in STP influent and effluent because of the signal suppression within these matrices. Due to the varying signal suppression in different matrices, quantitative calibration curves were constructed for the analytes in each type of aqueous sample, and the linearity factors (r^2) were within 0.999.

Stability of analytes can significantly affect the accuracy of an analytical method. Statins can undergo lactonization and/or hydrolysis under conditions of high or low pH, and such interconversions make quantitation less accurate. It was reported that the

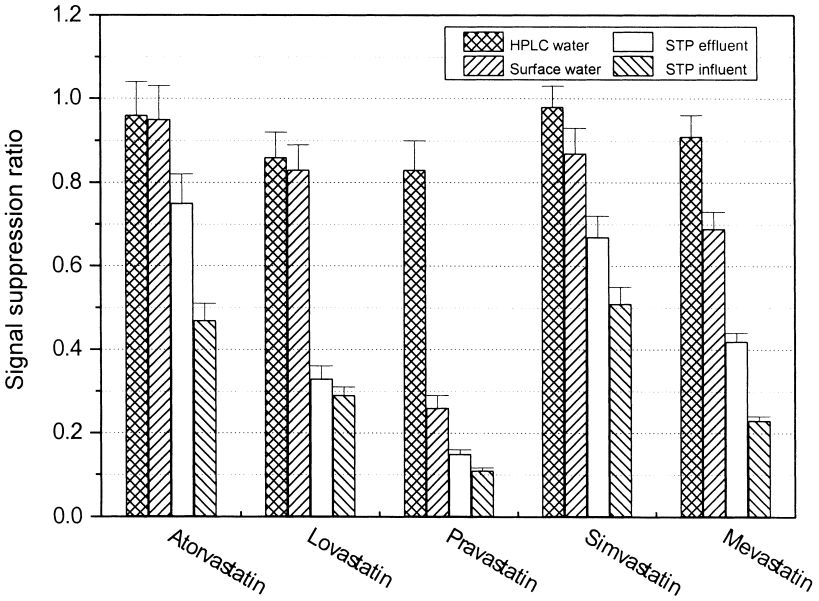


Fig. 5. Ion signal suppression of stains in different matrices.

Table 4
Mean concentrations and standard deviations (in brackets) of statins in STP influent and effluent, and in surface water (ng/l, $n=3$)

Analyte	STP influent[a]	STP effluent[a]	Surface water[b]
Atorvastatin	76 (3)	37 (2)	1 (0)
Lovastatin	49 (2)	14 (1)	ND[c]
Pravastatin	117 (6)	59 (2)	ND
Simvastatin	4 (0)	1 (0)	ND

[a] Samples were collected from the STP in Peterborough, ON, Canada on November 6, 2002.
[b] Samples were collected from the Otonabee River at a sampling site about 100 m below the outlet of the STP.
[c] Not detected.

concentration of atorvastatin in a stock solution varied by 10% after refrigeration for 190 days [16]. Simvastatin and its acid form showed 1% interconversion at 4 °C after 4 weeks, but the interconversion was only 0.05% at −20 °C [17]. Therefore, after the extraction of aqueous samples, the extracts were concentrated and reconstituted with acetonitrile and 4 m*M* aqueous methylammonium acetate to replace methanol as soon as possible in order to eliminate the ester formation of acidic statins by reaction with methanol. In addition, LC–MS–MS analysis was conducted as soon as possible to reduce the interconversion of statins between acid and lactone forms. Our investigation showed that the variations in responses of the four analytes in the reconstitution solvent varied by 10% within 24 h at ambient temperature (approx. 18 °C), indicating stability of the analytes during the typical period between sample storage and analysis.

3.4. Application

To show the practicability of the method, STP influent and effluent, and surface water samples were analyzed. Fig. 4 (right panel) shows the time-scheduled SRM chromatograms of the statins in the STP effluent (treated sewage) sample, where the analytes were clearly present. All of the four statins were detected in the samples of STP influent and effluent at 4–117 ng/l and 1–59 ng/l, respectively (Table 4). However, only atorvastatin was detected in the surface water at 1 ng/l. The statins were detected at similar concentrations to fibrate drugs in effluents from the same STP, as reported in our previous study [18], where bezafibrate and gemfibrozil were detected at 68 and 33 ng/l, respectively. The concentrations of atorvastatin detected in sewage and the Otonabee River were consistent with concentrations of this drug reported previously [9].

4. Conclusion

An LC–ESI-MS–MS method coupled with SPE was developed and validated for the quantitative determination of cholesterol-lowering statin drugs in aqueous environmental samples. By using methylammonium acetate as a mobile phase additive, methylammonium adducted ions of lovastatin, pravastatin and simvastatin were generated as precursor ions, while protonated atorvastatin was used as a precursor ion. The MDLs of the method were at ng/l levels, which are suitable for investigations of the distribution of statin drugs in the aquatic environment.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada through the Strategic Research Grant Program. We thank John Kennedy at the Environmental Protection Division of the city of Peterborough for his cooperation in collection of sewage samples.

References

[1] World Health Organization, World Health Report, Report of the Director-General, Geneva, WHO, 1998.
[2] P.J. Barter, Atherosclerosis (Suppl.) 1 (2000) 21.
[3] P. O'Connor, J. Feely, J. Shepherd, Br. Med. J. 300 (1990) 667.

[4] D.W. Kaufman, J.P. Kelly, L. Rosenberg, T.E. Anderson, A.A. Mitchell, J. Am. Med. Assoc. 287 (2002) 337.
[5] A.M. Rouhi, Chem. Eng. News 23 (2002) 37.
[6] T.A. Ternes, R. Hirsch, J. Mueller, K. Haberber, Fresenius J. Anal. Chem. 362 (1998) 329.
[7] T.A. Ternes, Water Res. 32 (1998) 3245.
[8] C.D. Metcalfe, X.-S. Miao, B.G. Koenig, J. Struger, Environ. Chem. Toxicol. (2003) in press.
[9] X.-S. Miao, C.D. Metcalfe, J. Mass Spectrom. 38 (2003) 27.
[10] M. Jemal, Y. Qing, D.B. Whigan, Rapid Commun. Mass Spectrom. 12 (1998) 1389.
[11] M. Jemal, Z. Ouyang, D.S. Teitz, Rapid Commun. Mass Spectrom. 12 (1998) 429.
[12] Y. Wu, J. Zhao, J. Hennion, W.A. Korfmacher, A.P. Lapiguera, C.C. Lin, J. Mass Spectrom. 32 (1997) 379.
[13] D. Mulvana, M. Jemal, S.C. Pulver, J. Pharm. Biomed. Anal. 23 (2000) 851.
[14] J.J. Zhao, A.Y. Yang, J.D. Rogers, J. Mass. Spectrom. 37 (2002) 421.
[15] H. Wang, Y. Wu, Z. Zhao, J. Mass Spectrom. 36 (2001) 58.
[16] W.W. Bullen, R.A. Miller, R.N. Hayes, J. Am. Soc. Mass Spectrom. 10 (1999) 55.
[17] J.J. Zhao, I.H. Xie, A.Y. Yang, B.A. Roadcap, J.D. Rogers, J. Mass Spectrom. 35 (2000) 1133.
[18] X.-S. Miao, B.G. Koenig, C.D. Metcalfe, J. Chromatogr. A. 952 (2002) 139.