Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 987–992
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Liquid chromatography–negative ion electrospray tandem mass spectrometry method for the quantification of ezetimibe in human plasma

Shuijun Li [a], Gangyi Liu [a], Jingying Jia [a], Xiaochuan Li [b], Chen Yu [a,*]

[a] *Central Laboratory, Shanghai Xuhui Central Hospital, Shanghai 200031, China*
[b] *Department of Pharmacology, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China*

Received 29 April 2005; received in revised form 13 July 2005; accepted 29 July 2005
Available online 19 October 2005

Abstract

A simple, reliable and sensitive liquid chromatography–tandem mass spectrometry method (LC–MS/MS) was developed and validated for quantification of free and total ezetimibe in human plasma. The analyte and internal standard (^{13}C6-ezetimibe) were extracted by liquid–liquid extraction with methyl *tert*-butyl ether. The reversed-phase chromatographic separation was performed on a Capcell C18 column, and the plasma extract was eluted with a gradient consisting of acetonitrile and 5 mM ammonium acetate. The analyte was detected using negative ionization by multiple reaction monitoring mode. The mass transition pairs of m/z 408.5 → 270.8 and m/z 414.5 → 276.8 were used to detect ezetimibe and internal standard, respectively. The assay exhibited linear ranges from 0.02 to 20 ng/ml for free ezetimibe and 0.25 to 250 ng/ml for total ezetimibe in human plasma. Acceptable precision and accuracy were obtained for concentrations of the calibration standard and quality control. The validated method was successfully used to analyze human plasma samples for application in a pharmacokinetic study.

Keywords: Ezetimibe; LC–MS/MS; Negative electrospray ionization

1. Introduction

Ezetimibe is a novel and selective cholesterol absorption inhibitor that effectively blocks intestinal absorption of dietary and biliary cholesterol [1]. The chemical structure (Fig. 1) of ezetimibe is 1-(4-fluorophenyl)-3(*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxypropyl]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone with a molecular weight of 409.4. Clinical trials showed that administering ezetimibe at a dose of 10 mg once daily resulted in a significant reduction in low-density lipoprotein cholesterol (LDL-C) by approximately 18% [2–6]. When combined with statins, ezetimibe produces more significant reductions in LDL cholesterol [7–9]. Moreover, ezetimibe has a favorable safety and tolerability profile without any clinically important drug interactions. Therefore, it is an option for monotherapy in patients with mild hypercholesterolemia or in those requiring adjunctive drug therapies for reduction of LDL-C levels.

After oral administration ezetimibe is rapidly absorbed and extensively conjugated to ezetimibe-glucuronide, which is a pharmacologically active phenolic glucuronide in vivo [10–11]. Ezetimibe and its glucuronide are the major drug-derived compounds detected in plasma, constituting approximately 10–20 and 80–90% of the total drug in plasma, respectively [12]. The conjugated glucuronide undergoes enterohepatic circulation resulting in repeated delivers of drug back to the site of action.

For the pharmacokinetic study of ezetimibe in man, a simple and validated method for biological fluids such as plasma is required. To our knowledge no methods for ezetimibe determination in bio-fluid samples, with detailed and comprehensive validation have been published. In this article, we provide a LC–MS/MS method with negative ESI for quantification of free and total ezetimibe (after hydrolysis). This method was applied to a pharmacokinetic study in healthy volunteers.

2. Experimental

2.1. Chemicals

Ezetimibe (99.7% purity) and ^{13}C6-ezetimibe (internal standard, 99.9% purity) were obtained from Schering Company

* Corresponding author. Tel.: +86 21 5403 0254; fax: +86 21 5403 0254.
E-mail address: chen-yu@online.sh.cn (C. Yu).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.07.053

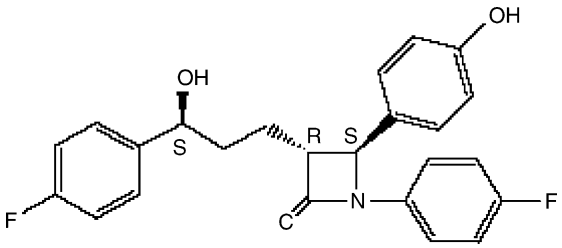

Fig. 1. Chemical structure of ezetimibe.

(USA), HPLC-grade methanol, acetonitrile and β-glucuronidase (crude solution) were from Sigma–Aldrich (Germany), methyl *tert*-butyl ether and ammonium acetate were from TEDIA Company (USA). All other reagents were of analytical grade.

2.2. LC–MS/MS equipment and conditions

The liquid chromatography system (Shimadzu, Japan) was equipped with two LC10ADvp pumps, a vacuum degasser, a SILHTC autosampler and a controller module. The chromatographic separation was achieved on a Capcell Pak C_{18} MG column (5 μm, 50 mm × 2 mm i.d., Shiseido, Japan) and a Phenomenex C18 guard column at room temperature. The mobile phase consisted of acetonitrile (A) and 5 mM ammonium acetate (B), which was pumped at a flow rate of 0.25 ml/min with binary gradient elution, the time program was as follows: 0–0.1 min, 70–0% B, 0.1–1.5 min, 0% B, 1.5–1.6 min, 0–70% B, 1.6–5 min, 70%B. The samples were kept at 4 °C in an autosampler and a volume of 5 μl was injected.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A Turbo Spray interface in negative ionization mode was used. The main working parameters are summarized in Table 1. The data were acquired and processed using the Analyst 1.3.1 software package.

Table 1
Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature (°C)	450
Dwell time per transition (ms)	200
Nebulizer gas (NEB)	7
Curtain gas (CUR)	8
Collision gas (CAD)	12
Ion spray voltage (IS, V)	−2000
Declustering potential (DP, V)	−34
Focusing potential (FP, V)	−170
Entrance potential (EP, V)	−10
Collision energy (CE, V)	−25
Collision cell exit potential (CXP, V)	−12
Polarity of analysis	Negative
Ion transition for ezetimibe (*m*/*z*)	408.5/270.8
Ion transition for IS (*m*/*z*)	414.5/276.8

2.3. Sample preparation

All frozen human plasma samples were thawed at ambient temperature. For the analysis of free ezetimibe, 200 μl of plasma sample was transferred to a 2-ml polypropylene test tube. After the addition of 10 μl of internal standard (10 ng/ml) solution, the tube was briefly vortexed then extracted with 1 ml of methyl *tert*-butyl ether. For the analysis of total ezetimibe 100 μl of plasma sample and 10 μl of internal standard (250 ng/ml) solution were transferred to a 5 ml polypropylene test tube, then 250 μl of sodium acetate buffer (0.5 M, pH 5.0) and 50 μl of β-glucuronidase (100,000 IU/ml) were added into the tube. After vortexing for 30 s, the tube was incubated at 50 °C for 60 min and 250 μl of sodium borate solution (0.1 M) was added into it. The mixture was extracted with 2 ml of methyl *tert*-butyl ether for 10 min by vortexing and then centrifuged at 4000 rpm for 10 min. After centrifugation samples for free and total ezetimibe analysis were prepared under same conditions. The supernatant was transferred to a clean polypropylene tube and dried with a stream of nitrogen gas at 40 °C. The residue was reconstituted with 100 μl of methanol and 5 μl volume was injected into LC–MS/MS system.

2.4. Bioanalytical method validation

2.4.1. Matrix effect

To assess the matrix effect, two concentration solutions of standard (0.5, 25 ng/ml) with IS (1, 25 ng/ml) were dried and reconstituted in 100 μl of methanol (neat standard). Two groups of six blank plasma samples of different sources were processed as in Section 2.3 and the residues were reconstituted with 100 μl of methanol containing 0.5, 25 ng/ml of standard and 1, 25 ng/ml of IS for free and total ezetimibe, respectively. By calculation the ratio of the mean areas of standard and IS spiked into plasma after extraction to the peak areas of the neat standard and IS, the matrix effect (%) was evaluated.

2.4.2. Calibration and quality control samples

Stock standard solutions of ezetimibe and ^{13}C6-ezetimibe (internal standard) were prepared in 100 μg/ml with methanol and stored at −20 °C. Working solutions for calibration and quality control were prepared from the stock solution by adequate dilution using double-distillated water. The IS working solutions were prepared by diluting its stock solution with water to 10 and 250 ng/ml for free and total ezetimibe analysis, respectively. Working solutions were added to blank plasma to obtain concentrations from 0.02 to 20 ng/ml for free ezetimibe and 0.25 to 250 ng/ml for total ezetimibe. Quality control (QC) samples were prepared as the same procedure, at concentrations of 0.02, 0.025, 8.5 and 17 ng/ml for free ezetimibe and 0.25, 0.3, 120 and 200 ng/ml for total ezetimibe.

2.4.3. Calibration curve

Calibration samples were prepared with human plasma consisting of two known concentration series of 0.02–20 and 0.25–250 ng/ml for free and total ezetimibe, respectively. In addition, two blank plasma samples were analyzed in order to

detect possible interferences from the matrix. Calibration curves were constructed by calculating peak area ratios (analyte/IS) as a function of plasma ezetimibe concentration. These data were fitted with a weighted ($1/x$) linear regression equation.

2.4.4. Precision and accuracy

The within-batch precision and accuracy was determined by analyzing six sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing six sets of quality control samples on three different batches. The quality control samples were randomized daily, processed and analyzed together with calibration samples.

2.4.5. Recovery

The recovery of free or total ezetimibe and internal standard was evaluated by comparing the mean peak areas of six extracted quality control samples to those of six unprocessed standard solutions containing the corresponding concentrations.

2.4.6. Stability

Stability of free ezetimibe was investigated by placing processed samples into the autosampler (4 °C) for 0, 12, 24 h and by subjecting samples to three freeze/thaw (−20 °C to room temperature) cycles. To further assess the stability of total ezetimibe samples with low and high analyte concentrations were also analyzed by placing them at room temperature for 16 h.

The variability in the glucuronide hydrolysis step was assessed by analyzing real plasma samples containing low and high ezetimibe-glucuronide concentrations six times. Samples were processed same as total ezetimibe analysis (Section 2.3). The precision data are expressed as coefficients of variation (CVs, %).

3. Results and discussion

3.1. Selection of MS and LC conditions

LC–MS/MS with negative ESI was selected to detect free and total ezetimibe in human plasma. In the present study, to obtain an appropriate ionization mode in analysis, ezetimibe and IS were scanned with negative and positive ion mode by injection of standard solutions. Compared to the positive ion mode, negative mode gave higher peak intensity in Q1 and product ion scan when the same concentration (1 μg/ml) of standard was infused into the mass spectrometer. In addition, a prominent fragment with m/z 270.8 was observed in the product ion scan with negative ESI (Fig. 2). Two ion transition pairs for ezetimibe and the IS were presented at m/z 408.5 → 270.8 and 414.5 → 276.8, respectively. Excellent specificity and sensitivity were observed under the present MS condition by combination with a liquid chromatographic separation. The gradient mobile phase consisted of acetonitrile and 5 mM ammonium acetate solution at a flow rate of 0.25 ml/min. The retention time of both analyte and IS was approximately 2.6 min and total run time for each sample was 5 min. Fig. 3 shows representative LC–MS/MS chromatograms for free and total ezetimibe in plasma samples.

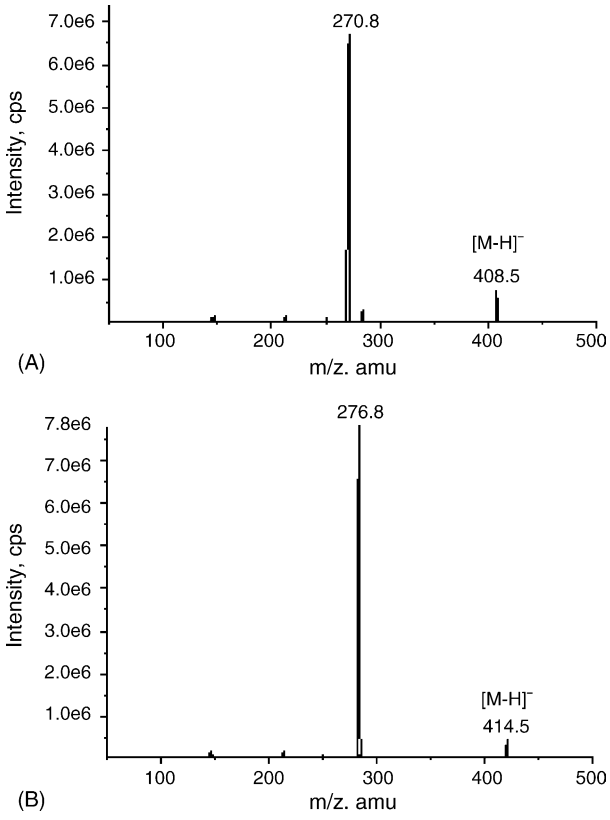

Fig. 2. Product ion mass spectra of deprotonated ions obtained from (A) ezetimibe and (B) the internal standard.

3.2. Validation of the method

3.2.1. Matrix effect

Matrix effects and the possibility for ionization suppression for ezetimibe and internal standard in different plasma samples were examined. The coefficients of variation (CVs, %) of the mean peak areas of free and total ezetimibe and IS in six different plasma were small (<6%), indicating little difference in ionization efficiency of the analyte and IS from different plasma sources. In addition, by comparing peak areas of standard and internal standard for samples spiked after extraction from plasma with those obtained by injecting neat standard and IS directly, the matrix effect was assessed. The result for both free and total ezetimibe and IS were above 92.5%, suggesting ion suppression by endogenous components was low.

3.2.2. Calibration curves

Calibration curves were linear in the concentration ranges 0.02–20 ng/ml for free ezetimibe and 0.25–250 ng/ml for total. The nine-point calibration curves gave acceptable results for both free and total ezetimibe. A correlation coefficient (r) of above 0.999 was obtained during method validation. Results of calibration curves of ezetimibe in human plasma are presented in Table 2. For both free and total ezetimibe the CVs were less than 7.56%, accuracy ranged from 92.0 to 102.8%. The lower limit of quantification was 0.02 ng/ml for free ezetimibe and 0.25 ng/ml for total ezetimibe with a signal to noise ratio >10.

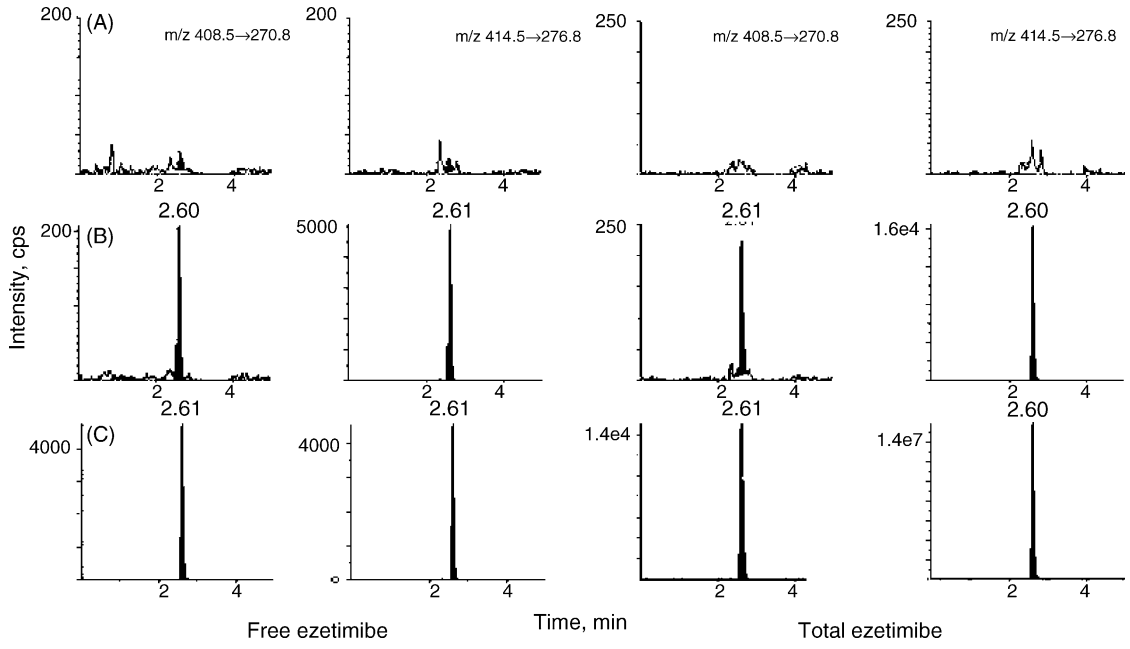

Fig. 3. Representative LC–MS/MS chromatograms for free and total ezetimibe in human plasma. (A) Blank plasma sample; (B) LLOQ plasma sample with 0.02 ng/ml and 0.25 ng/ml of free and total ezetimibe, respectively; and (C) human plasma sample collected 3 h after oral intake 10 mg of ezetimibe. m/z 408.5 → 270.8 for ezetimibe and m/z 414.5 → 276.8 for IS.

Table 2
Precision and accuracy of calibration samples for free and total ezetimibe in human plasma

Free ezetimibe			Total ezetimibe		
Nominal concentration (ng/ml)	CV (%)	Accuracy (%)	Nominal concentration (ng/ml)	CV (%)	Accuracy (%)
0.02	6.99	98.7	0.25	3.32	100.4
0.05	3.36	92.0	0.5	2.14	102.4
0.1	7.56	101.9	1.5	2.05	98.1
0.25	1.10	103.5	7.5	1.1	99.9
0.5	0.44	101.8	25	0.72	98.1
1.0	1.01	102.7	50	0.70	99.9
5.0	1.55	98.5	100	0.74	102.8
10	0.47	101.0	150	0.71	98.2
20	0.38	99.6	250	0.30	100.3

3.2.3. Precision and accuracy

The accuracy and precision of the assay are summarized in Table 3. The within-batch precision (CV) of the assay was no more than 11.1% for free and total ezetimibe at four concentration levels of the quality control samples, the CVs for between-batch precision were no more than 7.9%, the within-batch accuracy and between-batch accuracy of the assay ranged from 91.6 to 106.4 and 97.9 to 105.0%, respectively.

Table 3
Precision and accuracy of quality control samples for free and total ezetimibe in human plasma

Analyte	Concentration (ng/ml)	Within-batch ($n = 6$)		Between-batch ($n = 18$)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Free	0.02	11.1	101.8	4.3	103.7
	0.025	8.8	91.6	7.9	100.5
	8.5	2.1	106.4	2.4	105.0
	17	2.3	104.1	1.1	103.1
Total	0.25	5.1	94.9	5.5	98.1
	0.3	5.1	95.1	2.5	97.9
	120	1.5	102.8	0.6	102.4
	200	0.9	103.8	0.5	103.2

Table 4
Stability results of quality control samples for free and total ezetimibe in human plasma

Analyte	Concentration (ng/ml)	Autosampler ($n = 6$)		Freeze-thaw ($n = 6$)		Room temperature ($n = 6$)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Free	0.02	13.2	98.2	9.2	106.1		
	0.025	6.1	102.8	10.8	104.5		
	8.5	1.4	107.6	6.4	103.9		
	17	1.1	102.8	5.8	102.8		
Total	0.25	4.5	98.2	2.6	106.3		
	0.3	3.2	92.9	6.8	96.1	6.2	100.7
	120	2.2	102.1	2.2	105.2		
	200	1.6	103.2	0.5	105.0	1.3	104.8

3.2.4. Recovery

Free ezetimibe in human plasma was directly extracted with methyl *tert*-butyl ether by liquid–liquid extraction, whereas total ezetimibe must be hydrolyzed by β-glucuronidase before extraction because of ezetimibe-glucuronide conjugation. The extraction recovery of four concentration levels of QC samples was 78.6% on average for free ezetimibe and 76.7% for internal standard. Values of corresponding QC samples for total ezetimibe and IS were 48.3 and 42.9%, respectively. Recovery of total ezetimibe was low, but it was consistent and reproducible with a CV of 6.57% at all concentration levels of QC samples.

3.2.5. Stability

Stability of free and total ezetimibe and its internal standard was assessed at four concentrations of QC samples after processing in autosampler (4 °C). The results indicated that the analyte and IS were stable in the autosampler for at least 24 h (Table 4), which enables the assay of a large number of plasma samples in a working day. The freeze-thaw stability was determined by measuring the assay precision and accuracy for the QC samples. Both free and total ezebimibe in human plasma were considered to be stable after three freeze-thaw cycles. Since the analysis of total ezetimibe requires a procedure of hydrolysis, a possibility of long-term exposure to room temperature before plasma processing will occur. Stability of QC samples with low and high concentrations at room temperature for 16 h was evaluated. Little effect on the quantification was observed.

The glucuronide hydrolysis efficiency was investigated by analysis of replicates ($n = 6$) of plasma samples containing known concentrations (low and high) of total ezetimibe. The CVs of samples in the hydrolysis step were 0.90 and 3.45% for the low and high concentration, respectively. The results indicated that hydrolysis with β-glucuronidase was repeatable and consistent.

3.3. Application study

The validated method was successfully used to determine the free and total ezetimibe in human plasma samples from a pharmacokinetic study after a single oral dosing of ezetimibe (10 mg) to 20 healthy male volunteers. Fig. 4 show mean plasma concentration profiles of free and total ezetimibe in the 20 subjects. C_{max} of free and total ezetimibe was 3.61 ± 1.88 and 39.8 ± 17.4 ng/ml, respectively. T_{max} of both forms was reached at 1 h after oral administration. The plasma concentration-time profiles exhibited significant multiple peaks, suggesting enterohepatic recycling.

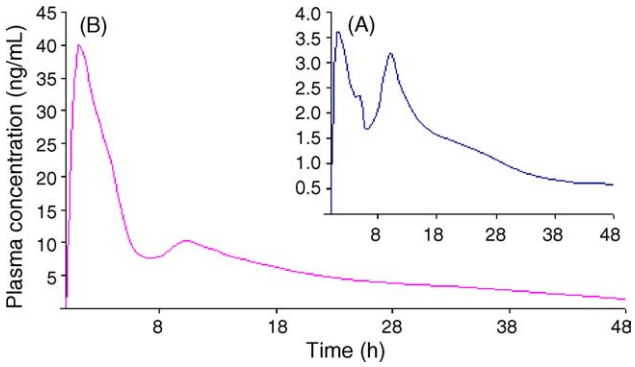

Fig. 4. Profiles of mean plasma concentration of ezetimibe versus time after oral administration of single dose of 10 mg of ezetimibe. (A) Free ezetimibe and (B) total ezetimibe.

4. Conclusions

A simple, reliable and sensitive LC–MS/MS method, with negative ESI, was developed and validated for quantification of free and total ezetimibe in human plasma. Liquid–liquid extraction with methyl *tert*-butyl ether was performed on 100–200 μl plasma. Acceptable precision, adequate reliability and sensitivity were obtained in the proposed method with the range from 0.02 to 20 ng/ml and 0.25 to 250 ng/ml for free and total analyte, respectively. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic studies.

References

[1] S.B. Rosenblum, T. Huynh, A. Afonso, H.R. Davis Jr., N. Yumibe, J.W. Clader, D.A. Burnett, J. Med. Chem. 41 (1998) 973–980.
[2] R.H. Knopp, C.A. Dujovne, A. Le Beaut, L.J. Lipka, R. Suresh, E.P. Veltri, Int. J. Clin. Pract. 57 (2003) 363–368.
[3] J. Jurado, R. Seip, P.D. Thompson, Am. J. Cardiol. 93 (2004) 641–643.
[4] L. Masana, P. Mata, C. Gagne, W. Sirah, M. Cho, A.O. Johnson-Levonas, A. Meehan, J.K. Troxell, B. Gumbiner, Clin. Ther. 27 (2005) 174–184.

[5] H.E. Bays, P.B. Moore, M.A. Drehobl, S. Rosenblatt, P.D. Toth, et al., Clin. Ther. 23 (2001) 1209–1230.
[6] C.A. Dujovne, M.P. Ettinger, J.F. McNeer, L.J. Lipka, A.P. LeBeaut, R. Suresh, B. Yang, E.P. Veltri, Am. J. Cardiol. 90 (2002) 1092–1097.
[7] H.E. Bays, L. Ose, N. Fraser, et al., Clin. Ther. 26 (2004) 1758–1773.
[8] B. Kerzner, J. Corbelli, S. Sharp, et al., Am. J. Cardiol. 91 (2003) 418–424.
[9] C.M. Ballantyne, M.A. Blazing, T.R. King, Am. J. Cardiol. 93 (2004) 1487–1494.
[10] M. Van Heek, C. Farley, D.S. Compton, et al., Br. J. Pharmacol. 129 (2000) 1748–1754.
[11] M. Van Heek, C. Farley, D. Compton, et al., Atherosclerosis 18 (2000) 151–155.
[12] C. Simard, J. Turgeon, Can. J. Clin. Pharmacol. 10 (2003) 13A–20A.