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# Liquid chromatography-tandem mass spectrometric assay for pravastatin and two isomeric metabolites in mouse plasma and tissue homogenates

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ABSTRACT

A bioanalytical assay for pravastatin and two isomeric metabolites, 3′α-isopravastatin and 6′-epipravastatin, was developed and validated. Mouse plasma and tissue homogenates from liver, kidney, brain and heart were pre-treated using protein precipitation with acetonitrile containing deuterated internal standards of the analytes. The extract was diluted with water and injected into the chromatographic system. This system consisted of a polar embedded octadecyl silica column using isocratic elution with formic acid in a water–acetonitrile mixture. The eluate was transferred to an electrospray interface using negative ionization and the analytes were detected and quantified with the selected reaction monitoring mode of a triple quadrupole mass spectrometer. The assay was successfully validated in a 3.4–7100 ng/ml concentration range for pravastatin, 1.3–2200 ng/ml for 3′α-isopravastatin and 0.5–215 ng/ml for 6′-epipravastatin using only plasma for calibration. For plasma samples, subjected to full validation, within and between day precisions were 1–7% (9–18% at the LLQ level) and accuracies were between 91% and 103%. For tissue homogenates, subjected to partial validation, within and between day precisions were 2–12% (6–19% at the LLQ level) and accuracies were between 87% and 113% (81 and 113% at the LLQ level). Drug and metabolites were shown to be chemically stable under most relevant analytical conditions. Finally, the assay was successfully applied for a pilot study in mice. After intravenous administration of the drug, all isomeric compounds were found in plasma; however, in liver and kidney homogenate only the parent drug showed levels exceeding the LLQ.

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## 1. Introduction

Pravastatin (Fig. 1) is a well known 3-hydroxy-3methylglutaryl-coenzyme-A reductase inhibitor (statin). Widely used in the treatment of hypercholesterolemia, pravastatin exhibits unique pharmacokinetic properties among the members of its class [1]. The pharmacokinetics of pravastatin are characterized by low absorption and bioavailability, a fast, absorption rate limited elimination and a relatively low protein binding. Further, Cytochrome P450 mediated biotransformation is less important for pravastatin compared to other statins [2]. The main metabolite of pravastatin is 3′α-isopravastatin, which is formed in the stomach, the gut wall and the liver [3,4]. Pravastatin absorption, distribution and elimination are determined by active transport mechanisms, especially by organic anion transporting polypeptide (OATP) 1B1 mediated transport [5]. Genetic variation of the transporter proteins involved can lead to altered pharmacokinetics [6] and increased toxicity [7,8]. Altered active transport mechanisms can be studied using knock-out mice [9–11]. The Oatp1b2(−/−) mouse was the first developed Oatp knock-out mouse and was found to be a valuable tool in the investigation of pravastatin disposition [9,10,12]. For the evaluation of new Oatp knock-out mice [13] and humanized mice expressing for example OATP1B1 [14] using pravastatin a sensitive bioanalytical assay will be required for this drug.

Validated bioanalytical assays for pravastatin have been reported earlier for human plasma and serum [15,16], using for example LC–UV or GC–MS [15] methods. However, since 1998 LC/MS/MS assays [16–25] have become available for pravastatin. In addition to pravastatin, the 3′α-isomeric metabolite [19–24] (Fig. 1) and pravastatin-lactone [22,23] (Fig. 1) could also be quantified in some assays. Positive electrospray ionization was used for both assays capable of quantifying the lactone metabolite,

*Abbreviations:* i.v., intravenous; LLQ, lower limit of quantification; OATP/Oatp, Organic anion transporting polypeptide.

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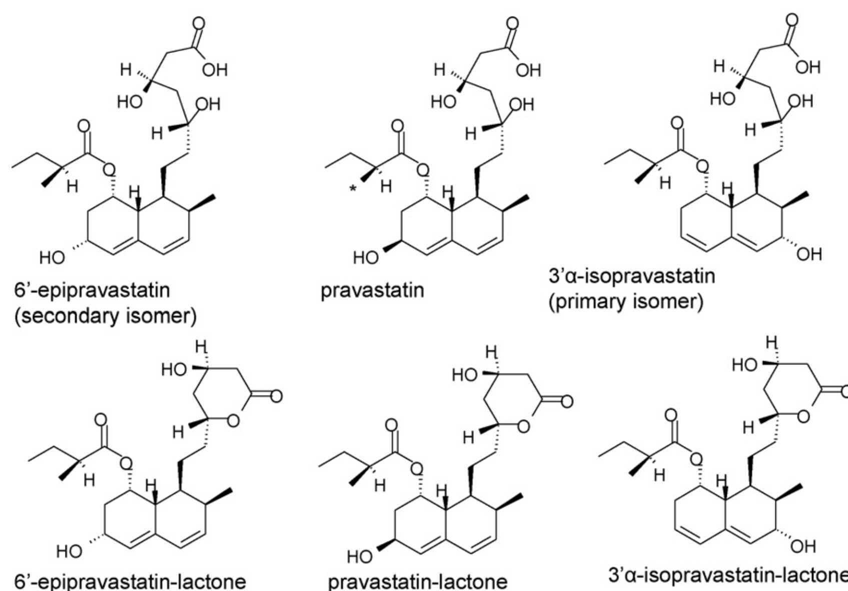


**Fig. 1.** Pravastatin and its expected reaction products after isomerization and/or lactonization under acidic conditions. * Position of $CD_3$ group in labeled pravastatin.

negative ionization with APCI [20,21,24] or ESI [17–19,25] was used in other LC/MS/MS assays. All these LC/MS/MS methods [17–25] use reversed-phase chromatography and solid-phase extraction on a reversed-phase sorbent. Bioanalytical LC/MS/MS assays for samples of other species have only been reported, concisely, for mice [9–11] and rat [5] using a simple protein precipitation procedure without the ability to quantify metabolites. We therefore developed and validated a sensitive bioanalytical assay for pravastatin and the isomeric metabolites 3′α-isopravastatin, 6′-epipravastatin, using protein precipitation of mouse plasma and four tissue homogenates (liver, kidney, brain, heart).

## 2. Experimental

### 2.1. Chemicals

Pravastatin-sodium (≥98%), sodium acetate, sodium chloride and bovine serum albumin were all obtained from Sigma–Aldrich (St. Louis, MO, USA); pravastatin-$d_3$ sodium salt (Fig. 1; chemical purity 95%; isotopic purity 98%) was obtained from Medical Isotopes (Pelham, NH, USA). LC–MS grade water, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were obtained from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home-purified by reversed osmosis on a multi-laboratory scale. Formic acid was of analytical grade and originated from Merck (Darmstadt, Germany). Hydrochloric acid was supplied by Fisher Scientific ('s-Hertogenbosch, The Netherlands) and pooled mouse EDTA plasma by Innovative Research (Southfield, MI, USA).

Pravastatin isomers (Fig. 1) were obtained by partial isomerization of pravastatin for both, target compounds and internal standards. To 200 μl of a pravastatin(-$d_3$) stock solution (250,000–1,000,000 ng/ml) in water, 200 μl of 10 mM hydrochloric acid was added. After vortex-mixing shortly, the mixture was incubated for *ca.* 15 min at 37 °C. The reaction was terminated by adding 100 μl of 59 mM sodium acetate and vortex-mixing shortly. Remaining pravastatin and isomers formed were quantified using LC–UV based on their equal UV responses and the conversion of pravastatin to only isomers and lactones. For each target compound the UV response at 238 nm was divided by the total UV response of the peaks in the chromatogram and multiplied with the initial pravastatin concentration.

The procedure was used for two pravastatin and one pravastatin-$d_3$ stock solutions, these three partially isomerized stock solutions were used throughout the complete analytical study. The isomerized internal standard stock solution (250,000 ng/ml) was diluted to 100 ng/ml in acetonitrile to obtain the precipitating agent, containing the internal standards for the sample pre-treatment.

Blank mouse tissue homogenates were prepared by adding different volumes of ice-cold 4% (w/v) bovine serum albumin solution in water to the (weighted) organ, followed by homogenizing with a Polytron PT1200 blender (Kinematica, Littay, Switzerland) for 1 min at position 23. The volumes of albumine solution used were 5 ml for a liver, 3 ml for a kidney, and 1 ml for both brain and heart.

### 2.2. Equipment

The LC/MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-μ pumps, a SPD10-Avp spectrophotometric UV–vis detector (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA, USA). For data recording and system controlling a Dell Optiplex GX270 personal computer, equipped with the Finnigan Xcalibur software (version 1.4, Thermo Electron), was used.

### 2.3. LC/MS/MS conditions

Partial-loop injections (30 μl) were made on an Atlantis dC18 column (150 mm × 2.1 mm, $d_p$ = 3 μm, average pore diameter = 10 nm, Waters, Milford, MA, USA) with a Polaris 3 C18-A pre-column (10 mm × 2 mm, $d_p$ = 3 μm, Varian, Middelburg, The Netherlands). The column temperature was maintained at 30 °C and the autosampler rack was maintained at 4 °C. The eluent comprised a mixture of acetonitrile (29%, v/v) and 0.1% (v/v) formic acid in water that was pumped at 0.5 ml/min. The whole eluate was transferred to the electrospray probe, starting at 4 min after injection by switching the MS inlet valve, until the end of the analytical run at 10 min. The electrospray was tuned in the negative ionization mode by introducing 0.5 ml/min of a mixture of methanol (50%, v/v) and 0.1% formic acid in water (50%, v/v) and 5 μl/min of 10 μg/ml pravastatin. The highest response was obtained with

a −4000 V spray voltage, a 255 °C capillary temperature and the nitrogen sheath, ion sweep and auxiliary gasses set at 43, 40 and 12 arbitrary units, respectively; the source CID voltage was set off. The SRM mode was used with argon as the collision gas at 2.0 mTorr. The tube lens off set was −111 V for all compounds. Pravastatin and isomers were monitored at *m*/*z* 423.3 > 321.3, 303.2, internal standards were monitored at *m*/*z* 426.3 > 321.3, 303.2, all using 16 V collision energies, 0.2 s dwell times and mass resolutions at 0.7 full width at half height (unit resolution) for both separating quadrupoles.

### 2.4. LC–UV conditions

Partial-loop injections (20 μl) were made on a Vydac 218 MS C18 column (50 mm × 2.1 mm, $d_p$ = 5 μm, Grace Davison, Hesperia, USA) with an Ascentis C18 Supelguard pre-column (20 mm × 2 mm, $d_p$ = 5 μm, Supelco, Bellefonte, PA, USA). The column temperature was maintained at 30 °C and the autosampler rack was maintained at 4 °C. The eluent comprised a mixture of acetonitrile (24% ,v/v) and 0.1% (v/v) formic acid in water that was pumped at 0.5 ml/min. The eluate was monitored at 238 nm using the UV absorbance detector.

### 2.5. Sample pre-treatment

To a volume of 20 μl mouse plasma or tissue homogenate, pipetted into a 1.5 ml polypropylene tube, 30 μl of partially isomerized 100 ng/ml pravastatin-$d_3$ in acetonitrile was added, containing 65.2 ng/ml $d_3$-pravastatin, 23.9 ng/ml $d_3$-3′α-isopravastatin and 2.1 ng/ml $d_3$-6′-epipravastatin. The tube was closed and shaken vigorously for *ca.* 5 s using vortex-mixing. After centrifugation of the sample at $10 \times 10^3 \times g$ at 20 °C for 1 min, *ca.* 40 μl of the supernatant was pipetted into a 250 μl glass insert placed in an auto-injector vial. Before closing the vial, 80 μl of water was added and finally, 30 μl of the mixture was injected onto the Atlantis column.

### 2.6. Validation

A laboratory scheme based on international guidelines [26–28] was used for the validation procedures using a full validation for mouse plasma and a partial validation for the four homogenates from mouse liver, kidney, brain and heart tissue. In accordance with the FDA guidelines [26] a partial validation is sufficient if a full validation of the same compounds with the same method for another matrix of the same species was already performed. We used a less extensive partial validation protocol for the brain and heart homogenates compared to the liver and kidney homogenates because they were expected to be less relevant for future studies in the mouse.

#### 2.6.1. Calibration

Stock solutions of pravastatin, one at 1,000,000 ng/ml for calibration samples, another at 500,000 ng/ml for quality control (QC) samples and pravastatin-$d_3$ at 250,000 ng/ml as internal standard were prepared in water. The stock solutions were stored at −30 °C.

The 1,000,000 ng/ml stock solution of pravastatin was partially isomerized once to 71.0% remaining pravastatin, 23.8% 3′α-isopravastatin and 2.15% 6′-epipravastatin using the procedure reported (these percentages do not equal 100% due to lactone formation). The partially isomerized solution was diluted to a 10 μg/ml calibration sample in pooled mouse EDTA plasma, stored at −30 °C. Additional calibration samples were prepared daily at 5000, 1000, 500, 100, 50, 10 and 5 ng/ml of the initial pravastatin concentration. The 5, 10 and 10,000 ng/ml calibration samples were processed in duplicate for each daily calibration, whereas the levels in between were processed only once. Least-squares linear regressions with $1/x^2$ (reversed square of the concentration) as the weighting factor were employed to define the calibration curves using the ratios of the peak area of pravastatin and pravastatin-$d_3$, 3′α-isopravastatin and its labeled internal standard and finally 6′-epipravastatin and pravastatin-$d_3$. For pravastatin both product ions were used for quantification, for both isomers only the *m*/*z* 321.3 product was used.

#### 2.6.2. Precision and accuracy

The 500,000 ng/ml stock solution of pravastatin was partially isomerized once to 68.3% pravastatin, 26.1% of the primary isomer and 2.30% of the secondary isomer using the procedure reported. The partially isomerized solution was diluted to obtain validation (quality control (QC)) samples in pooled mouse EDTA plasma, liver homogenate and kidney homogenates at 8000 (QC-high), 400 (QC-med), 20 (QC-low) and 5 ng/ml (QC-LLQ) of the initial pravastatin concentration. QC-med and -low were additionally prepared in mouse brain and heart homogenates. The QC samples were stored in polypropylene tubes at −30 °C. Precisions and accuracies were determined by sextuple analysis of each plasma QC or quintuple analysis of each homogenate QC in three analytical runs on three separate days; however, half of the tissue homogenate QCs were assayed on only 1 day. In addition, within day precision and accuracy were assessed for 5000 ng/ml (not isomerized) pravastatin in plasma ($n$ = 6) and all four tissue homogenates ($n$ = 5 each) and finally for QC-med and -low samples in plasma, prepared by diluting the isomerized plasma QC-high sample with plasma containing 5000 ng/ml (not isomerized) pravastatin. Relative standard deviations of the concentrations were calculated for both the within day precision (repeatability) and, if assessed, the between day precision (reproducibility).

#### 2.6.3. Selectivity

Six individual mouse EDTA plasma samples and five samples of each tissue homogenate, without pravastatin, its isomers or internal standard present, were processed to investigate the presence of potential interferences in the pravastatin SRM transitions as a demonstration of the selectivity of the assay.

#### 2.6.4. Recovery

Extraction efficiencies were determined in quadruplicate for plasma by comparing processed samples at three validation levels (QC-high, -med and -low) with extracts of drug-free human EDTA plasma spiked with the analytes at these levels after extraction. Ionization efficiency (ion suppression or enhancement) was assessed by comparing the same spiked blank extracts with reference material solutions in water – acetonitrile (4/1, v/v), again at the same three validation levels. The extraction and ionization efficiencies from tissue homogenates were assessed using identical procedures but only at the QC-med level.

Ionization efficiency was also studied by monitoring SRM responses after injection of all 26 blank extracts (6 from plasma, 5 of each tissue) from the selectivity study with post-column mixing of the eluate with 2 μl/min of a 2000 ng/ml pravastatin solution in water prior to ionization.

#### 2.6.5. Stability

The stability of pravastatin and its isomers was investigated in QC-high and -low plasma samples. Quadruplicate analysis of these samples was performed after storage at ambient temperature (26 h), three additional freeze–thaw cycles (thawing at ambient temperature during ca. 1 h and freezing again at −30 °C for at least 1 day), storage at −30 °C and storage at −80 °C, both for 2 months, respectively. The stability at ambient temperature (for 22 h) was also investigated in QC-med samples for all tissue homogenates and (for 24 h) for 5000 ng/ml (not isomerized) pravastatin in all five matrices. Furthermore, plasma QC extracts were re-injected after additional storage of the extracts at 4 °C for 8 days. Finally, all stock

solutions, before and after isomerization, were investigated after additional storage for 3 months at −30 °C and for 3 days at ambient temperature.

### 2.7. *In vivo mouse samples*

Mice were housed and handled according to guidelines of the Netherlands Cancer Institute and complying with the Dutch legislation. Animals used in this study were male wild-type mice of FVB genetic background, between 9 and 15 weeks of age. Animals were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water (to pH 2.4–2.5 using hydrochloric acid to suppress bacterial growth) *ad libitum*.

Male FVB mice ($n$ = 5 per time point) were treated with a dose of 5 mg pravastatin/kg bodyweight by intravenous (i.v.) administration in the tail vein. Pravastatin solution was obtained by dissolving the drug in 0.9% sodium chloride (w/v) in water. Blood samples were collected in EDTA tubes via cardiac puncture 5 or 30 min after administration of the drug. Samples were kept on melting ice. After centrifugation at 2100 × $g$ for 6 min at 4 °C, plasma was pipetted and stored at −30 °C. In addition, livers and kidneys were collected and weighted after cardiac puncture and after homogenization in 5 or 3 ml, respectively of the bovine serum albumin solution; they were also stored at −30 °C.

## 3. Results and discussion

### 3.1. *Method development*

Because pravastatin metabolites are very expensive and the main metabolite in human is the isomeric 3′α-pravastatin which is known to be formed under acidic conditions [1,4,29] we started to study pravastatin reactions in the presence of hydrochloric acid. Using LC in combination with both, UV detection and scanning positive electrospray MS detection, we observed that under mild acidic conditions (reported in Section 2.1) pravastatin is solely converted into isomeric compounds and their lactones. The assumed structures are shown in Fig. 1. This quantitative conclusion was made because the total UV response of pravastatin and the reaction products formed remained constant (Fig. 2) during conversion

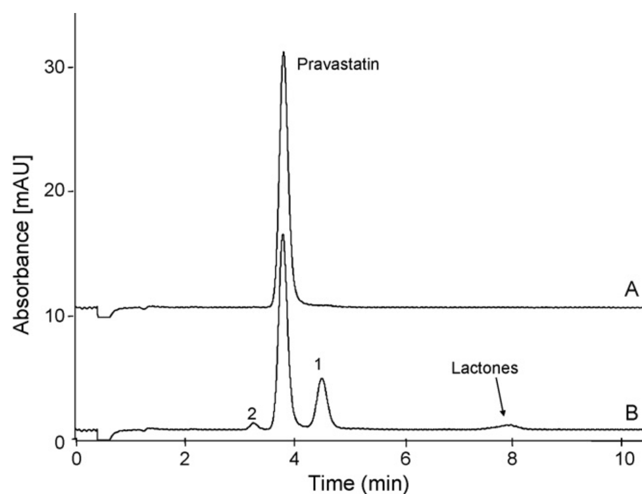


**Fig. 2.** UV chromatograms (238 nm) showing the isomerization and lactonization of a stock solution of pravastatin under acidic conditions, injected after dilution to 10,000 ng/ml pravastatin. Reaction conditions have been reported in Section 2.1. (A) Before reaction with 10 mAU off set; (B) after reaction; (1) primary isomer (3′α-isopravastatin); (2) secondary isomer (6′-epipravastatin).

and because UV spectra of these compounds have been reported to be identical [29]. Reaction conditions (hydrochloric acid concentration, reaction temperature and reaction time) were optimized to obtain pravastatin and its primary isomer in a ratio of *ca.* 3:1 within a relatively short time. Lactones formed did not cause any response in the pravastatin SRM traces. Additional reaction (degradation) products seemed to be formed under stronger acidic conditions and after longer reaction times because the total UV responses at 238 nm were reduced after treatment under these conditions. Two isomeric compounds, product spectra are shown in Fig. 3, were formed in sufficient amounts to be used for development of a bioanalytical assay and could both be separated from the parent compound using the Vydac column (Resolution ≥1.9, Fig. 2B). The primary isomer is assumed to be 3′α-isopravastatin and the secondary isomer 6′-epipravastatin based on Kitazawa et al. [4] and Everett et al. [29]. Unfortunately, the first *in vivo* mouse samples showed, after oral administration of the drug, an additional compound, probably also a pravastatin isomer based on its prod-

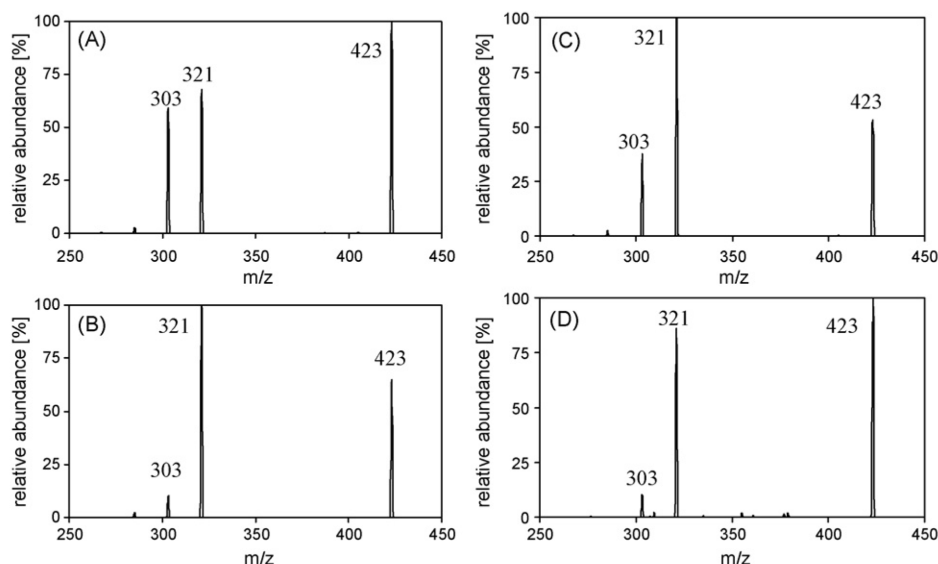


**Fig. 3.** Product spectra ($m/z$ 423.3@16 V) of pravastatin (A), its primary isomer (3′α-isopravastatin; B), its secondary isomer (6′-epipravastatin; C) and the additional isomer in *in vivo* mouse samples (D).

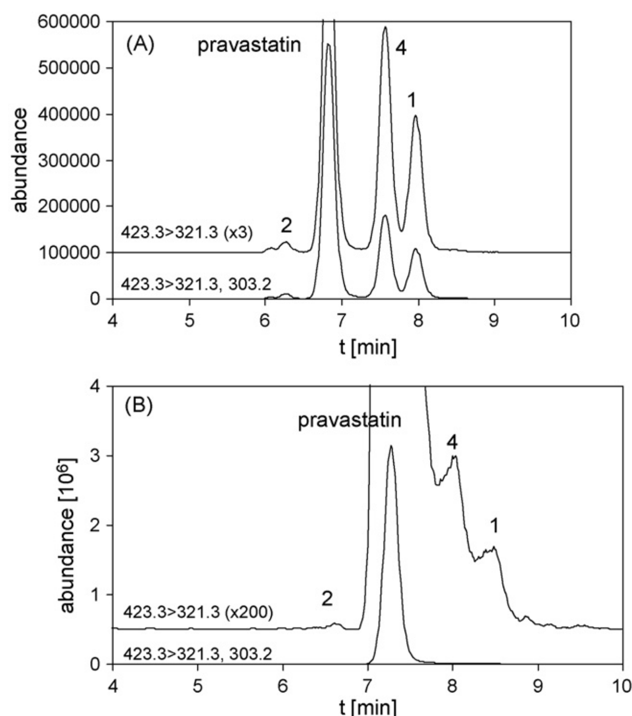


**Fig. 4.** SRM chromatograms of (A) an *in vivo* mouse kidney sample taken 2 h after administration of 5 mg/kg pravastatin orally to a female wild-type mouse and (B) an *in vivo* mouse liver sample taken 5 min after administration of 5 mg/kg pravastatin i.v. by a male wild-type mouse, this sample contained 2119 ng/ml pravastatin, 4.63 ng/ml 3′α-isopravastatin and no 6′-epipravastatin exceeding the LLQ (1 ng/ml). The upper graphs in both figures have an artificial off-set and are multiplied by a factor 3 in A and a factor 200 in B. Isomeric compounds: (1) primary isomer (3′α-isopravastatin); (2) secondary isomer (6′-epipravastatin); (4) additional isomer in mouse samples.

uct spectrum (Fig. 3D), in several matrices. This new isomer could not be separated from 3′α-isopravastatin using the Vydac column. The Atlantis C18 column with a higher separation power due to its length and particle diameter was then successfully used to acquire separation between pravastatin and the three isomers from mouse samples (Fig. 4A). The kidney sample was only chosen as an example to show the additional metabolic compound, further results of oral studies will be reported separately. The acetonitrile content of the eluent and column temperature were optimized for this column, keeping the run time within 10 min. Alternative chromatographic conditions like using methanol instead of acetonitrile or using a third column, a Polaris 3 C18-A column (50 mm × 2 mm, $d_p$ = 3 μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) were unsuccessful in obtaining sufficient resolution between pravastatin and the three isomers.

Finally, the isomerized labeled pravastatin also showed an additional isomer (Fig. 5B), this isomer was also produced by unlabeled pravastatin, but only after longer reaction times (data not shown). This additional pravastatin-$d_3$ isomer showed a negative product spectrum identical to 3′α-isopravastatin-$d_3$ and both these spectra were analogous to the product spectrum of 3′α-isopravastatin (shown in Fig. 3B) with the expected mass shift for the parent ion due to presence of deuterium.

Atmospheric ionization of pravastatin and its isomers was optimal using electrospray ionization in the negative mode using acetonitrile as organic modifier, forming the deprotonated molecule. APCI, positive ionization and methanol were also investigated. Acetonitrile was additionally preferred because of its lower viscosity and the possibility to separate all five isomers observed (Figs. 4 and 5). Product spectra of pravastatin and three isomers are shown in Fig. 3. Typically, pravastatin showed almost equal responses for two product ions ($m/z$ 321 and 303), both used for quantification, while the isomers each show the same prominent product ion ($m/z$ 321) with a minor $m/z$ 303 ion under these conditions (Fig. 3). The product ion $m/z$ 321 can be explained by loss of 2-hydroxy-propanoic acid, which is followed by water loss for $m/z$ 303. Because 6′-epipravastatin and the additional isomer in mouse samples both show a low abundant signal at $m/z$ 303, it is likely that both possess the 3′-hydroxy group that will probably be more difficult to remove by collision induced water loss from the $m/z$ 321 ion than a 6′-hydroxy group because of the presence of the neighboring 2′-methyl group. For optimal sensitivity, two transitions with the two different product ions were used for pravastatin and only the transition to $m/z$ 321.3 for the isomers. No other transitions approaching the abundance of these two prominent product ions were available for the development of a sensitive assay for pravastatin and its metabolites.

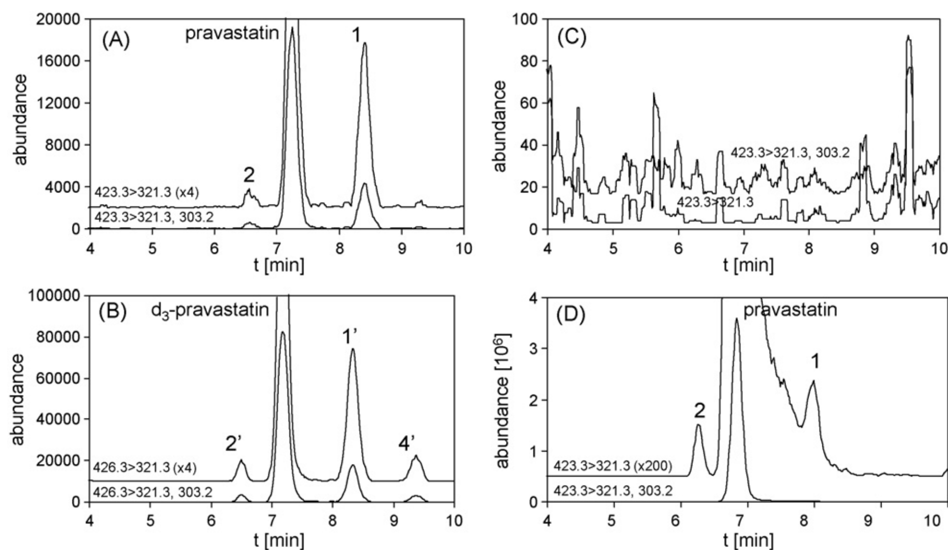


**Fig. 5.** SRM chromatograms of QC-low spiked plasma (A and B), blank plasma (C) and an *in vivo* plasma sample taken 5 min after administration of 5 mg/kg pravastatin i.v. by a male wild-type mouse (D), this sample contained 4375 ng/ml pravastatin, 13.9 ng/ml 3′α-isopravastatin and 5.9 ng/ml 6′-epipravastatin. The upper graphs in each figure have an artificial off-set. The upper traces in A and B are multiplied by a factor 4, in D a factor 200. Isomeric compounds: (1) primary isomer (3′α-isopravastatin); (2) secondary isomer (6′-epipravastatin); (3′) additional isomer in internal standard mixture. Accent indicates $d_3$-labeled compounds.

### 3.2. Validation

SRM chromatograms of pravastatin, its isomeric metabolites and the labeled internal standards are depicted in Fig. 5, showing chromatograms of a blank and a QC-low spiked plasma sample and an *in vivo* mouse plasma sample.

#### 3.2.1. Calibration

After the partial isomerization of pravastatin the calibration ranges used were 3.6–7099 ng/ml for pravastatin, 1.2–2383 ng/ml for 3′α-isopravastatin and 0.22–215 ng/ml for 6′-epipravastatin by excluding the lowest calibration sample only for 6′-epipravastatin. For pravastatin and its primary isomer the corresponding deuterated internal standards could be used for a linear calibration. Because of the low amount of labeled secondary isomer pravastatin-$d_3$ was used as internal standard for 6′-epipravastatin, also resulting in a linear calibration. For all calibration samples in six calibration curves the concentrations were back-calculated from the ratios of the peak area (of analyte and internal standard) using the calibration curves of the run in which they were included; no deviations of the averages of each level higher than 7.4% were observed (data not shown), indicating the suitability of the regression model. The average regression parameters of the linear regression functions ($n = 6$; concentration in ng/ml) were $y = 0.0018(\pm 0.0025) + 0.0167(\pm 0.0007)x$ with a regression coefficient of $0.994 \pm 0.003$ for pravastatin, $y = -0.018(\pm 0.004) + 0.0499(\pm 0.0023)x$ with a regression coefficient of $0.990 \pm 0.007$ for 3′α-isopravastatin and $y = -0.0021(\pm 0.0003) + 0.0143(\pm 0.0007)x$ with a regression coefficient of $0.991 \pm 0.008$ for 6′-epipravastatin. Both isomers show a significant ($P < 0.005$) intersection at the $y$-axis that could not be explained but all the functions show reproducible calibration parameters.

**Table 1**
Assay performance data of pravastatin and both isomers in mouse EDTA plasma resulting from 18 validation (QC) samples in 3 analytical runs.

| Nominal concentration [ng/ml] | Within day precision [%] | Between day precision [%] | Accuracy [%] |
|---|---|---|---|
| *Pravastatin* | | | |
| 5466 | 1.3 | 1.4 | 94.4 |
| 273.3 | 3.2 | 3.2 | 101.1 |
| 13.66 | 3.8 | 4.0 | 103.2 |
| 3.42 | 9.5 | 10.8 | 94.0 |
| *Primary isomer* | | | |
| 2086 | 2.8 | 3.2 | 94.2 |
| 104.3 | 4.1 | 4.4 | 100.0 |
| 5.21 | 6.3 | 6.8 | 102.9 |
| 1.30 | 17.3 | 17.7 | 96.8 |
| *Secondary isomer* | | | |
| 184.0 | 2.3 | 3.5 | 103.0 |
| 9.20 | 3.8 | 3.9 | 100.7 |
| 0.460 | 14.3 | 14.6 | 91.5 |

#### 3.2.2. Precision and accuracy

Assay performance data from the plasma validation samples at 4 concentrations are reported in Table 1. Between day variations and deviations of the accuracy lower than 7% were observed for all compounds for the QC-high, -med and -low levels. Precisions and deviations of the accuracy had to meet the required ±15% (±20% for the lower limit of quantification (LLQ)) [26–28]. Precisions for pravastatin and is isomeric metabolites were in the same order as in previously reported assays for human plasma and serum [17–25].

Tissue homogenate validation data also met the same criteria with only one exception (precision of 0.46 ng/ml of 6′-epipravastatin in liver homogenate). These assay performance data are reported in Tables 2 and 3. These criteria were also met for the (not isomerized) 5000 ng/ml pravastatin samples in all

**Table 2**
Assay performance data of pravastatin and both isomers in mouse liver and kidney homogenates resulting from 15 validation (QC) samples in 3 analytical runs or from 5 samples in one run.

| Nominal concentration [ng/ml] | Within day precision [%] | | Between day precision [%] | | Accuracy [%] | |
|---|---|---|---|---|---|---|
| | Liver | Kidney | Liver | Kidney | Liver | Kidney |
| *Pravastatin* | | | | | | |
| 5466 | 2.2 | 2.0 | | | 88.1 | 95.2 |
| 273.3 | 2.7 | 4.2 | 4.4 | 4.7 | 88.8 | 93.9 |
| 13.66 | 8.3 | 1.8 | | | 91.4 | 106.5 |
| 3.42 | 6.4 | 5.5 | 7.5 | 9.1 | 81.0 | 93.0 |
| *Primary isomer* | | | | | | |
| 2086 | 4.9 | 5.9 | | | 91.6 | 96.5 |
| 104.3 | 4.2 | 4.9 | 4.2 | 5.3 | 90.0 | 94.4 |
| 5.21 | 12.0 | 8.8 | | | 96.7 | 101.3 |
| 1.30 | 14.1 | 14.1 | 15.5 | 19.2 | 85.1 | 93.5 |
| *Secondary isomer* | | | | | | |
| 184.0 | 3.2 | 5.2 | | | 91.1 | 97.8 |
| 9.20 | 6.8 | 5.1 | 7.6 | 6.0 | 91.0 | 90.5 |
| 0.460 | **21.2** | 16.1 | | | 80.1 | 87.9 |

The bold value exceeds 20%, required for the LLQ.

**Table 3**
Assay performance data of pravastatin and both isomers in mouse brain and heart homogenates resulting from 15 validation (QC) samples in 3 analytical runs or from 5 samples in one run.

| Nominal concentration [ng/ml] | Within day precision [%] | | Between day precision [%] | | Accuracy [%] | |
|---|---|---|---|---|---|---|
| | Brain | Heart | Brain | Heart | Brain | Heart |
| *Pravastatin* | | | | | | |
| 273.3 | 2.0 | 3.3 | 2.2 | 3.9 | 101.9 | 94.7 |
| 13.66 | 4.8 | 2.9 | | | 105.8 | 99.3 |
| *Primary isomer* | | | | | | |
| 104.3 | 4.0 | 5.0 | 4.6 | 5.3 | 101.7 | 96.1 |
| 5.21 | 7.9 | 8.1 | | | 112.8 | 104.0 |
| *Secondary isomer* | | | | | | |
| 9.20 | 5.5 | 3.5 | 7.0 | 5.0 | 99.5 | 96.6 |
| 0.460 | 11.4 | 17.3 | | | 88.4 | 88.2 |

**Table 4**
Assay performance data of pravastatin and both isomers in all mouse matrices at a 5000 ng/ml pravastatin level ($n = 6$ for plasma, $n = 5$ for each tissue homogenate).

| Matrix | Within day precision [%] | | | Concentration found [ng/ml] | | Accuracy [%] |
|---|---|---|---|---|---|---|
| | Pravastatin | Primary isomer | Secondary isomer | Primary isomer | Secondary isomer | Pravastatin |
| Plasma | 2.6 | 45 | 2.9 | 3.4 | 5.46 | 97.3 |
| Liver | 3.1 | 28 | 6.3 | 4.7 | 5.35 | 96.3 |
| Kidney | 4.3 | 30 | 4.1 | 4.2 | 5.80 | 100.2 |
| Brain | 2.2 | 22 | 9.8 | 3.7 | 5.70 | 100.0 |
| Heart | 1.0 | 16 | 5.8 | 5.0 | 5.77 | 99.9 |

matrices; however, these samples appeared also to contain a small amount of both validated isomers (Table 4). The poor precision for the primary isomer is due to the tailing of the large pravastatin peak, accompanied by an increased noise level on its peak tail. The metabolite concentrations in Table 4 (average ($n = 26$): 4.16 ng/ml primary isomer and 5.16 ng/ml secondary isomer) were successfully used to correct the levels found in the QC-med and -low samples diluted with 5000 ng/ml pravastatin in plasma (Table 5), investigated to assure the possibility to measure low isomeric metabolite levels in the presence of a high pravastatin concentration.

### 3.2.3. Selectivity

The analysis of six batches of blank plasma samples and five of each tissue homogenate showed no interfering peaks in all SRM traces. Blank responses could not be distinguished from the detector noise for all compounds and were below 1% of the pravastatin LLQ, 2% of the LLQ of the primary isomer, 10% of the QC-low of the secondary isomer, 0.01% of the normal pravastatin-$d_3$ and 0.1% of the normal 3′α-isopravastatin-$d_3$ responses.

### 3.2.4. Limits of quantification

Based on the results of calibration, precision and accuracy, the highest levels of the calibration ranges could be assigned as the upper limits of quantification (ULQ): 7100, 2400 and 215 ng/ml for pravastatin and its primary and secondary isomers, respectively in all matrices. The results of the precision, accuracy and selectivity experiments proved that the validated LLQ levels for pravastatin (3.4 ng/ml) and 3′α-isopravastatin (1.3 ng/ml) could indeed be assigned as the LLQ in all matrices. For 6′-epipravastatin, the QC-low level proved to be the real LLQ level in plasma (0.46 ng/ml), this level was however not totally met in tissue homogenates (from the liver). Therefore, 1 ng/ml was attributed as the LLQ of the secondary isomer in tissue homogenates. These sensitivities do not totally meet the sensitivities (0.1–0.625 ng/ml) [18–25] of the LC/MS/MS assays for human serum and plasma samples using SPE and at least a 10-fold of the current sample volume but are better compared to the LC/MS/MS methods of Chen et al. [9,10] also using protein precipitation for mouse plasma (10 ng/ml) and mouse tissue samples (40 ng/ml).

Further, one additional limitation was observed: quantification of the primary isomer is only possible if the concentration is at least 0.2% of the concentration of pravastatin. Finally, the LLQ values in tissue homogenate (3.4, 1.3 and 1.0 ng/ml, respectively for the three compounds) can all be multiplied by 6, 7, 3 and 4.33 for liver, kidney, brain and heart respectively to obtain the LLQ values in the original tissues (in ng/g). These multiplication factors were derived from the average weights of 1, 0.5, 0.5 and 0.3 g of the four organs respectively that were diluted by homogenization with the bovine serum albumin solution to obtain samples that could easily be processed as a liquid.

### 3.2.5. Recovery

Extraction recoveries of the protein precipitation procedure are reported in Supplementary Table 1 and ionization efficiencies (ion suppression or enhancement) in Supplementary Table 2. In general, appropriate data were obtained; however, two remarks can be made. All extraction recoveries are relatively high (109–136%). This can, at least partially, be explained by the sample volume reduction during pre-treatment. Mixing water with acetonitrile and precipitation of the plasma proteins both cause such a reduction of the total sample solvent volume. Further, the data of the secondary isomer seem to show some ion enhancement (+34%) and also a high variation of the extraction recovery (RSD = 34%) at the QC-low level. However, because for 6′-epipravastatin the QC-low level is also the plasma LLQ concentration, a relatively high variation of the absolute SRM response can be expected and will therefore be accepted.

Further, post-column MS/MS infusion experiments with different batches of all matrices ($n = 26$) did not show any ionization suppression (or enhancement) effects later than 4 min after injection. This observation explains the accurate validation results of the tissue homogenate samples using plasma calibration samples and assures accurate measurements in individual samples of all matrices.

### 3.2.6. Stability

Recoveries of pravastatin and its isomers in plasma after different storage procedures are shown in Supplementary Table 3, in tissue homogenates at the QC-med level after 22 h at ambient temperature in Supplementary Table 4, and in all matrices containing 5000 ng/ml pravastatin after 24 h at ambient temperature in Table 6.

Excellent recoveries were observed for the QC samples under all storage condition tested (Supplementary Tables 3 and 4). However, levels of the primary isomer are increased after storage of 5000 ng/ml pravastatin for 24 h at ambient temperature in both

**Table 5**
Assay performance data of both isomers of pravastatin in mouse plasma QC samples diluted with 5000 ng/ml pravastatin resulting from 6 validation (QC) samples in one run.

| Expected concentration [ng/ml] | Within day precision [%] | Accuracy [%] |
|---|---|---|
| *Primary isomer* | | |
| 108.2 | 5.6 | 96.8 |
| 9.36 | 11.6 | 91.4 |
| *Secondary isomer* | | |
| 14.53 | 6.0 | 104.2 |
| 6.06 | 10.6 | 103.2 |

**Table 6**
Stability data (recovery [%]; ±SD; $n = 4$) of pravastatin (5000 ng/ml) and its two isomeric metabolites (primary isomer ca. 4.2 ng/ml; secondary isomer ca. 5.6 ng/ml) in all matrices after 24 h at ambient temperature, reporting the percentage of the initial concentration.

| Matrix | Pravastatin | Primary isomer | Secondary isomer |
|---|---|---|---|
| Plasma | 101.7 ± 2.6 | 83 ± 27 | 104.4 ± 9.6 |
| Liver | 97.7 ± 3.5 | 173 ± 36 | 97.7 ± 4.3 |
| Kidney | 95.4 ± 1.5 | 134 ± 27 | 92.4 ± 4.6 |
| Brain | 98.7 ± 2.7 | 224 ± 31 | 103.5 ± 5.6 |
| Heart | 94.7 ± 2.4 | 107 ± 7 | 96.9 ± 4.4 |

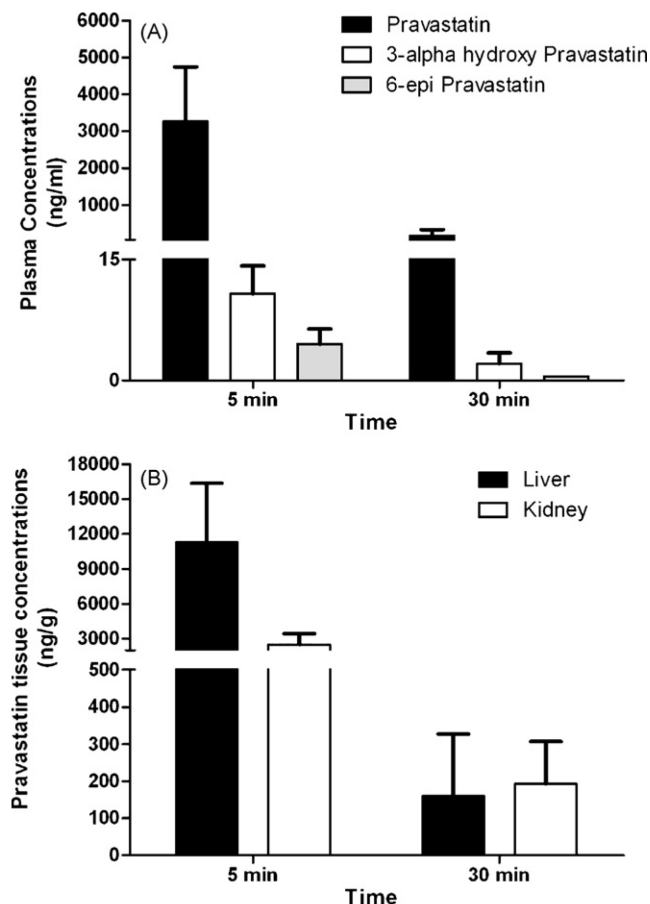


**Fig. 6.** *In vivo* mouse plasma pravastatin (solid bars), primary isomer (3′α-isopravastatin; white bars) and secondary isomer (6′-epipravastatin; grey bars) concentrations (A) and tissue concentrations of pravastatin in liver (solid bars) and kidney (white bars) (B) at 5 and 30 min after intravenous administration of 5 mg/kg pravastatin to male wild-type mice ($n = 5$). The *in vivo* liver and kidney concentrations of the two isomers were below the quantification limit for all mice with one exception at 5 min in liver (Fig. 4B).

liver and heart homogenate (Table 6). In these matrices ca. 0.1% of the pravastatin seems to be converted to the primary metabolite under these conditions. Despite the high variation of the data of the primary metabolite in Table 6, due to co-elution with the peak tail of pravastatin, an advice to limit exposure to ambient conditions of liver and brain homogenate samples that may contain <1% of the primary metabolite relative to the pravastatin concentration seems appropriate.

Re-injection of calibration and validation samples after additional storage at 4 °C for 8 days resulted again in successful performances, these results allowed storage of the diluted extracts for a prolonged period of time before final analysis.

The original stock solutions both showed recoveries higher than 95% for pravastatin after both conditions tested. The partially degraded stock solutions showed recoveries higher than 93% for pravastatin and both isomers and an average increase of 20% for the amount of lactones during additional storage for 3 months at −30 °C. The degraded stock solutions showed an average recovery of 91% for pravastatin and a slight increase of the amounts of isomers (not higher than 5%) and lactones after additional storage for 3 days at ambient temperature. Therefore, pravastatin and its isomers showed to be sufficiently stable in all stock solutions; however, the partially degraded stock solutions should not be used for more than 3 months when stored at −30 °C.

### *3.3. In vivo mouse samples*

The distribution of pravastatin was investigated in a short pilot experiment, starting with only two tissues, in which 5 mg/kg of pravastatin were administered i.v. to male wild-type mice, to demonstrate the applicability of the presented assay. Five and 30 min, respectively, after administration, plasma (Fig. 5D), liver and kidney concentrations (Fig. 4B) of pravastatin and its isomers were measured (Fig. 6).

Upon i.v. administration, pravastatin is cleared rapidly from the plasma, most probably due to rapid uptake and excretion via the liver mediated by active transport mechanisms [1]. Isomeric metabolite levels are low in plasma after i.v. administration and even absent in liver and kidney with one exception (Fig. 4B). These metabolite levels have been shown to be higher after oral administration of the drug in ongoing studies due to the first-pass-effect and the passage of the acidic stomach. Other tissues than liver and kidney are also being investigated in these ongoing studies.

## 4. Conclusions

The first validated assay for pravastatin in mouse plasma and tissue homogenate samples has now been reported. The LC/MS/MS assay uses a simple sample pre-treatment method and a low sample volume and meets commonly accepted criteria for precision, accuracy, recovery and stability [26–28]. Isomeric metabolites could also be quantified in this assay after their simple synthesis under acidic conditions. The sensitivity of the method is better compared to earlier assays using the same pre-treatment method [9,10] by at least a factor 3. The compounds are stable under most conditions, only at ambient temperature a minor amount of 3′α-isopravastatin was formed from the parent drug in liver and brain homogenate. The presented assay is being a valuable tool for ongoing pre-clinical studies investigating the impact of active transport mechanisms on the pharmacokinetics and toxicity of pravastatin and its isomeric metabolites in new mouse models.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.08.015.

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