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High-performance liquid chromatographic method with UV photodiode-array, fluorescence and mass spectrometric detection for simultaneous determination of galantamine and its phase I metabolites in biological samples

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Abstract

Galantamine, an alkaloid isolated from the bulbs and flowers of Caucasian snowdrop (*Galanthus woronowii*, Amaryllidaceae) and related species, is employed in human medicine for the treatment of various neuromuscular and neurodegenerative diseases. After the administration, the products of oxidative biotransformation (*O*-desmethyl-galantamine, *N*-desmethyl-galantamine, galantamine-*N*-oxide) and chiral conversion (epigalantamine) are formed in various concentrations from parent compound. For the identification and determination of galantamine and its *phase I* metabolites in blood plasma and tissues, a new bioanalytical method based on a reversed-phase high-performance liquid chromatography with UV photodiode-array, fluorescence and mass spectrometric detection was developed, validated and applied to pharmacokinetic and biotransformation studies. Sample preparation included a homogenization of the rat tissues (liver, brain, hypophysis) in a phosphate buffer 0.05 mol/L pH 7.4. Plasma samples and tissue homogenates were purified using a mixed-mode solid-phase extraction (Waters Oasis MCX cartridges). Galantamine, its above-mentioned metabolites and the internal standard codeine were separated on a Discovery HS F5 column (Supelco, 150 mm × 4.6 mm I.D., 5 μm) at flow rate of 1 mL/min using a linear gradient elution. UV photodiode-array and mass spectrometric detection were employed for the identification of individual galantamine metabolites in various biomatrices, the fluorescence detection (λ_{excit} = 280 nm/λ_{emiss} = 310 nm) was chosen for the quantification of galantamine and its metabolites. The developed method was applicable in liver tissue in the range from 0.50 to 63.47 nmol/g of galantamine, from 0.32 to 41.42 nmol/g of *O*-desmethyl-galantamine, from 0.54 to 69.40 nmol/g of *N*-desmethyl-galantamine and from 0.70 to 89.03 nmol/g of epigalantamine. Limit of detection was found to be 0.04 nmol/g for galantamine, 0.19 nmol/g for *O*-desmethyl-galantamine, and 0.07 nmol/g for *N*-desmethyl-galantamine and epigalantamine.

Keywords: High-performance liquid chromatography; Fluorescence detection; Mass spectrometry; Galantamine; Metabolites

1. Introduction

The alkaloid galantamine is a competitive and reversible cholinesterase inhibitor. It is used in neuromuscular diseases such as myasthenia gravis [1], in antagonism of skeletal neuromuscular blockade [2] and drug-induced respiratory depression [3]. Galantamine was approved for the treatment

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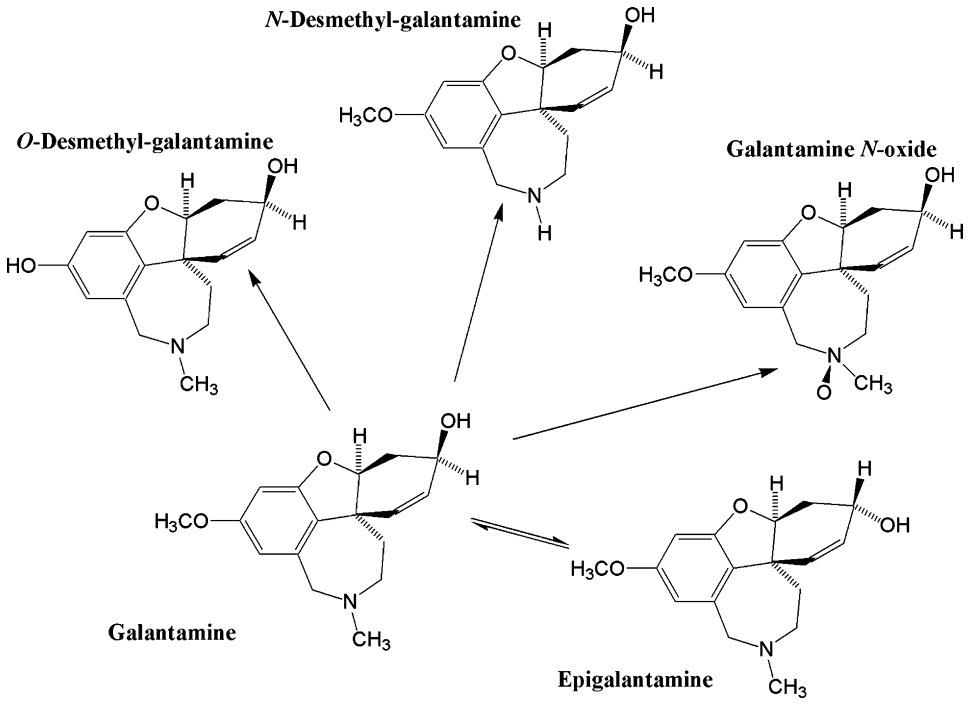

Fig. 1. Metabolic pathways of galantamine.

of mild-to-moderate Alzheimer's disease [4]. The cognitive deficits in patients with Alzheimer's disease are thought to be related to a decline in hippocampal and neocortical cholinergic neurotransmission, reduced activity of choline acetyltransferase and decreased number of nicotinic acetylcholine receptors in the brain of patients [5–8]. The galantamine mode of action covers both acetylcholinesterase inhibition and positive modulation of nicotinic acetylcholine receptors [9,10]. Galantamine is metabolized (75%) to pharmacologically inactive or less-active metabolites [11]. Principal metabolic pathways (see Fig. 1) are *O*-desmethylation, *N*-desmethylation, *N*-oxidation, epimerization, glucuronidation and sulfate conjugation [12]. Cytochrome P450 (CYP) 2D6 and 3A4 are the major CYP isoenzymes involved in the hepatic metabolism of this drug. CYP2D6 is responsible for the formation of *O*-desmethyl-galantamine, and CYP3A4 is involved in the production of galantamine *N*-oxide [13,14].

Various analytical methods were described for the determination of galantamine. A capillary zone electrophoresis [15], high-performance liquid chromatography with ultraviolet detection [16,17] or fluorescence detection [12,18], high-performance liquid chromatography tandem mass spectrometry HPLC/MS/MS [19] and HPLC-UV photodiode-array radiometric detection [12,18] have already been presented. All these bioanalytical methods required at least 1–2 mL of plasma, with the exception of HPLC/MS/MS. For the sample preparation, single-step liquid–liquid extraction of galantamine into toluene was applied [12,18], or a procedure employing the precipitation with trichloracetic acid followed by alkalization of the sample and by the liquid–liquid extraction into chloroform was described [17]. A mixed-mode solid-phase extraction procedure preceding a bioanalytical HPLC method for the determination of galantamine and its metabolites has not been tested yet. Also an HPLC determination of galantamine and its metabolites in tissues has not been described.

The aim of this study was to develop and validate an HPLC method for the determination of galantamine in rat plasma and galantamine and its metabolites in rat liver tissue. The method was employed in pharmacokinetic and biodistribution studies of galantamine and its metabolites. This bioanalytical method was used to investigate the possible targeting of galantamine to central nervous system by pretreatment with L-carnitine or other drugs [20,21]. The identity of galantamine and metabolites in the extract from samples was confirmed using HPLC–PDA–MS experiments.

2. Experimental

2.1. Chemicals and reagents

Galantamine hydrobromide [(4*aS*,6*R*,8*aS*)-4*a*,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3*a*,3,2-*ef*] [2] benzazepin-6-ol hydrobromide, $C_{17}H_{22}BrNO_3$, MW = 368.26 g/mol, CAS 1953-04-4] was purchased from ICN Biomedicals Inc. (Irvine, CA, USA). Epigalantamine ($C_{17}H_{21}NO_3$, MW = 287.36 g/mol) and *N*-desmethyl-galantamine ($C_{16}H_{19}NO_3{\cdot}HCl{\cdot}2H_2O$, MW = 345.82 g/mol) were obtained from Janssen Pharmaceutica (Beerse, Belgium). *O*-Desmethyl-galantamine ($C_{16}H_{19}NO_3$, MW = 273.33 g/mol, CAS 60755-80-8) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Galantamine *N*-oxide ($C_{17}H_{21}NO_4$, MW = 303.35 g/mol) was prepared using synthetic procedures described previously [22] and the structure was confirmed by mass spectrometry. Internal standard codeine

phosphate (pharmacopoeial purity) was supplied by Tamda (Olomouc, Czech Republic).

Albumin from human serum (approximately 99% agarose gel electrophoresis, lyophilized powder) and acetonitrile (Chromasolv for HPLC, gradient grade) were obtained from Sigma Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q Plus system (Millipore, Bedford, USA). Sodium dihydrogenphosphate (p.a.), disodium hydrogenphosphate (p.a.), ammonium acetate (GR for analysis), ammonia solution (25% GR for analysis), hydrochloric acid (32% GR for analysis) and methanol (for liquid chromatography, LiChrosolv) were supplied by Merck (Darmstadt, Germany).

2.2. Preparation of the stock solutions and the calibration samples

2.2.1. Calibration samples in rat plasma

Galantamine hydrobromide (0.724 mg/mL) and internal standard codeine phosphate (1 mg/mL) were dissolved in methanol and stored at −20 °C. The stock solution of galantamine hydrobromide (0.1 mL) was immediately before the analysis diluted by 2% of human serum albumin in a phosphate buffer 0.05 mol/L at pH 7.4 (HSA solution, 9.9 mL). The phenomenon of galantamine adsorption to the glass was eliminated using a 2% human serum albumin solution in a phosphate buffer (0.05 mol/L) at pH 7.4. Bovine albumin was not suitable because of a little interference at the retention time of galantamine.

Calibration standards were prepared daily by spiking 0.2 mL aliquots of diluted stock solution of galantamine (using another half dilution) with a blank heparinized rat plasma (0.1 mL) resulting in a galantamine concentration ranging from 0.08 to 19.66 μmol/L. An internal standard solution was prepared daily at a concentration of 224 μmol/L in 2% HSA solution.

2.2.2. Calibration samples in rat tissues

In case of analysis of the rat liver tissues, galantamine hydrobromide (2 mg/mL), *O*-desmethyl-galantamine (1 mg/mL), *N*-desmethyl-galantamine (1.68 mg/mL) and epigalantamine (2.26 mg/mL) were dissolved in methanol, and 0.2 mL each of the stock solutions of individual compounds were diluted to final volume 10 mL by 2% human serum albumin in a phosphate buffer 0.05 mol/L pH 7.4 (HSA solution). Samples for the calibration were prepared using drug-free liver tissue. Homogenates were obtained using homogenization of liver tissue (1 g) in 10 mL of a phosphate buffer 0.05 mol/L (pH 7.4). The volume 5 mL of liver drug-free homogenates were spiked with 0.3 mL aliquots of diluted stock solution of galantamine and its metabolites (HSA solution) resulting in a galantamine concentration in the range 0.50–63.47 nmol/g, *O*-desmethyl-galantamine in the range 0.32–41.42 nmol/g, *N*-desmethyl-galantamine in the range 0.54–69.40 nmol/g and epigalantamine in the range 0.70–89.03 nmol/g. The concentration samples in brain and hypophysis homogenates were prepared in a similar way.

The stock solution of codeine phosphate in methanol (2 mg/mL) for the tissue analysis was diluted to obtain a concentration of 345 μmol/L in a 2% HSA solution.

The quality control samples (QC) were independently prepared using drug-free plasma and liver tissue homogenates and stored at −72 °C until analysis.

2.3. Sample preparation

Waters Oasis MCX cartridges (3 cc/60 mg) based on a mixed-mode cation–exchange reversed-phase polymeric sorbent were chosen for the solid-phase extraction (SPE) of galantamine and its metabolites.

HSA solution 2% (0.2 mL) and 0.05 mL of I.S. solution (224 μmol/L) were added to 0.1 mL aliquots of rat plasma. After this step, the sample was acidified with 0.35 mL of hydrochloric acid (0.6 mol/L). The cartridges were rinsed with 1 mL of methanol and 1 mL of water. The acidified samples (0.6 mL) were passed through the SPE cartridges without vacuum. The SPE cartridges were then washed with 1 mL of hydrochloric acid (0.1 mol/L) and with 1 mL of methanol. The captured analytes were eluted with 1 mL of 5% ammonium hydroxide in methanol. The elutes were evaporated in the centrifugal evaporation system (RC 10.10, Jouan) at 60 °C. The residues were reconstituted in 240 μL of the 15% solution of ammonium acetate (5 mmol/L) in methanol.

Sample preparation of the rat tissues included their homogenization (Potter-Elvehjem homogenizer, 6 °C) in a phosphate buffer 0.05 mol/L pH 7.4 (10 mL of mentioned buffer per 1 g of liver or brain tissues, 3 mL of buffer per 10 mg of hypophysis). The solution of internal standard (345 μmol/L, 0.2 mL) was added to 5 mL aliquots of rat liver or brain samples. The homogenization was followed by a solid-phase extraction of diluted homogenate using Oasis MCX cartridges (6 cc/150 mg, Waters). The sample was acidified with 5 mL of hydrochloric acid (0.6 mol/L). The cartridges were treated using 2 mL of methanol and 2 mL of water. Then the acidified homogenate (10 mL) was loaded. The washing phase was made using 4 mL of hydrochloric acid (0.1 mol/L) and 4 mL of methanol. Next procedure was identical to the plasma sample procedure.

Sample preparation of the rat hypophysis was similar with the exception of a smaller sample volume (2 mL of rat hypophysis homogenate was used). The sample was acidified after the addition of 0.1 mL of I.S. solution (345 μmol/L) using 2 mL of hydrochloric acid (0.6 mol/L) and 4 mL of this sample was loaded on an Oasis MCX (3 cc/60 mg) cartridge and treated according to the above-described rat plasma procedure.

2.4. HPLC with fluorescence detection

Analysis was performed on a 2695 Waters Separations Module (Waters Corp., Milford, MA, USA) equipped with 996 photodiode array detector (PDA), Waters 2475 fluorescence detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). Empower Software (Waters Corp., Milford, MA, USA) was employed for the data acquisition and processing.

The separation of galantamine and its metabolites was performed on the analytical column Discovery HS F5

(Supelco, Bellefonte, PA, USA) 5 μm particle size (4.6 mm I.D. × 150 mm). A Waters Symmetry C18 5 μm particle size Guard Column (3.9 mm I.D. × 20 mm) was used as the analytical precolumn.

The flow rate of mobile phase was 1 mL/min. A linear gradient of mobile phase was applied during the analysis. The elution started from 15% of acetonitrile and 85% of aqueous ammonium acetate (5 mmol/L) pH 6.8 (v/v) to 25% of acetonitrile and 75% of aqueous ammonium acetate (5 mmol/L) over 4 min. This linear gradient of mobile phase was followed by isocratic flow until the end of analysis in 30 min. The equilibration to the starting elution conditions lasted 5 min. Temperature of the column was set at 30 °C. The injection volume was 4 μL.

The chromatographic analysis of galantamine and its metabolites was performed using a fluorescence detector at excitation and emission wavelengths of 280 and 310 nm.

2.5. HPLC/MS analysis

High-performance liquid chromatography–mass spectrometry (HPLC/MS) analysis was performed on an Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) and a Waters liquid chromatograph consisting of a model 616 pump, a model 717+ autosampler, a thermostated column compartment and a model 996 photodiode-array detector (all from Waters, Milford, MA, USA). HPLC conditions are mentioned in the previous paragraph. Electrospray ionization (ESI) mass spectra were recorded in the mass range *m/z* 50–1200 using positive-ion mode and the following setting of tuning parameters: target mass *m/z* = 300, compound stability = 100%, pressure of the nebulizing gas = 482.6 kPa, the drying gas flow rate = 12 L/min and temperature of ion source = 365 °C. The selected precursor ions were further analyzed by MS/MS experiments using the isolation width *m/z* = 4 and the collision amplitude 1.0 V. UV spectra of all chromatographic peaks were recorded in the range 200–600 nm using a diode-array UV detector with the highest resolution at 1.2 nm. The standards and samples were dissolved in the mobile phase and 100 μL of the solution was injected into the HPLC/MS system.

2.5.1. ESI mass spectra

Galantamine *N*-oxide (MW = 303, retention time 5.6 min). Positive-ion ESI–MS: *m/z* 607 $[2M+H]^+$, 304 $[M+H]^+$ (100%), 231 $[M+H\text{-}CH_2CHNH(CH_3)O]^+$, 213 $[M+H\text{-}CH_2CHNH(CH_3)O\text{-}H_2O]^+$.

Positive-ion ESI–MS/MS of *m/z* 304: *m/z* 286 $[M+H\text{-}H_2O]^+$, 245 $[M+H\text{-}CH_2N(CH_3)O]^+$, 231 $[M+H\text{-}CH_2CHNH(CH_3)O]^+$ (100%), 213 $[M+H\text{-}CH_2CHNH(CH_3)O\text{-}H_2O]^+$.

Positive-ion ESI–MS/MS of *m/z* 231: *m/z* 213 $[M+H\text{-}CH_2CHNH(CH_3)O\text{-}H_2O]^+$ (100%).

2.5.2. O-Desmethyl-galantamine (MW = 273, retention time 13.4 min)

Positive-ion ESI–MS: *m/z* 274 $[M+H]^+$ (100%), 256 $[M+H\text{-}H_2O]^+$, 225 $[M+H\text{-}H_2O\text{-}CH_3NH_2]^+$, 217 $[M+H\text{-}CH_2CHNHCH_3]^+$, 199 $[M+H\text{-}CH_2CHNHCH_3\text{-}H_2O]^+$.

Positive-ion ESI–MS/MS of *m/z* 274: *m/z* 256 $[M+H\text{-}H_2O]^+$, 225 $[M+H\text{-}H_2O\text{-}CH_3NH_2]^+$ (100%), 217 $[M+H\text{-}CH_2CHNHCH_3]^+$, 199 $[M+H\text{-}CH_2CHNHCH_3\text{-}H_2O]^+$.

2.5.3. N-Desmethyl-galantamine (MW = 273, retention time 17.6 min)

Positive-ion ESI–MS: *m/z* 547 $[2M+H]^+$, 312 $[M+K]^+$, 296 $[M+Na]^+$, 274 $[M+H]^+$ (100%), 231 $[M+H\text{-}CH_2CHNH_2]^+$, 213 $[M+H\text{-}CH_2CHNH_2\text{-}H_2O]^+$.

Positive-ion ESI–MS/MS of *m/z* 274: *m/z* 231 $[M+H\text{-}CH_2CHNH_2]^+$, 213 $[M+H\text{-}CH_2CHNH_2\text{-}H_2O]^+$ (100%).

Positive-ion ESI–MS/MS of *m/z* 231: *m/z* 213 $[M+H\text{-}CH_2CHNH_2\text{-}H_2O]^+$ (100%).

2.5.4. Galantamine (MW = 287, retention time 21.7 min)

Positive-ion ESI–MS: *m/z* 326 $[M+K]^+$, 310 $[M+Na]^+$, 288 $[M+H]^+$ (100%), 270 $[M+H\text{-}H_2O]^+$, 231 $[M+H\text{-}CH_2CHNHCH_3]^+$, 213 $[M+H\text{-}CH_2CHNHCH_3\text{-}H_2O]^+$.

Positive-ion ESI–MS/MS of *m/z* 288: *m/z* 270 $[M+H\text{-}H_2O]^+$, 231 $[M+H\text{-}CH_2CHNHCH_3]^+$, 225 $[M+H\text{-}H_2O\text{-}CH_3CH_2NH_2]^+$ (100%), 213 $[M+H\text{-}CH_2CHNHCH_3\text{-}H_2O]^+$.

Positive-ion ESI–MS/MS of *m/z* 231: *m/z* 213 $[M+H\text{-}CH_2CHNHCH_3\text{-}H_2O]^+$ (100%).

2.5.5. Codeine (internal standard, MW = 299, retention time 28.4 min)

Positive-ion ESI–MS: *m/z* 322 $[M+Na]^+$, 300 $[M+H]^+$ (100%).

Positive-ion ESI–MS/MS of *m/z* 300: *m/z* 282 $[M+H\text{-}H_2O]^+$, 243 $[M+H\text{-}CH_2CHNHCH_3]^+$, 225 $[M+H\text{-}CH_2CHNHCH_3\text{-}H_2O]^+$, 215 $[M+H\text{-}CH_2CHNHCH_3\text{-}CH_2CH_2]^+$ (100%).

2.6. Validation of the bioanalytical method

2.6.1. Limit of detection, linearity of the method, precision and accuracy

Validation experiments were performed according to the FDA guideline [23]. The limit of detection (LOD) was determined using equation LOD = 3.3 σ/S, where σ is the standard deviation of baseline noise, evaluated by measurement of blank plasma samples and rat liver homogenates from six drug-free rats, and S was the slope of calibration curve.

The calibration experiments were based on six calibration curves and nine various concentration samples covering the expected concentration range of galantamine in plasma (0.08–19.66 μmol/L) or using eight concentration levels during analysis of rat liver tissue samples (see Section 2.2.2). The linearity of the calibration curve was tested and evaluated using equation $y = kx + q$, where y is the peak-area ratio of galantamine (metabolite) to codeine and x the corresponding concentration ratio of galantamine (metabolite) to codeine. The regression equation of the calibration curve was calculated by a least-squares procedure. During development, a calibration curve was accepted a limit of 15% deviation of calculated value of calibration standard from spiked concentration.

The precision was calculated as relative standard deviation (R.S.D. = 100 S.D./mean). The precision should not exceed the

limit of 15% except of the lower limit of quantification. The accuracy was defined as $R(\%) = 100$ (arithmetic mean of concentrations determined/concentration spiked). The precision and the accuracy were determined using the quality control samples at 11 (intra-batch, 0.10–104.40 μmol/L) or 9 (inter-batch, 0.08–19.66 μmol/L) concentration levels of galantamine in rat plasma. Parameters in case of analysis of rat liver homogenates were established using eight concentration levels. The accuracy was measured by replicate analysis of quality control samples, and the mean value at each concentration level should be within 15% of the spiked value except of the lower limit of quantification.

2.6.2. The range of applicability and recovery

The range of applicability of the analytical method was enclosed by the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). LLOQ was established as the lowest standard concentration on the calibration curve characterized of a precision of 20% and an accuracy of 80–120%. ULOQ was set as the highest standard concentration featured a precision within the limit of 15% and an accuracy of 85–115%.

Recovery of a solid-phase extraction procedure for galantamine in plasma was tested at nine concentration levels of quality control samples (0.08–19.66 μmol/L). Each sample at each concentration level was analyzed in triplicate. The mean extraction recovery of galantamine and internal standard codeine (224 μmol/L) was determined by comparing the peak area before and after extraction, using the dilution of compounds in a phosphate buffer 0.05 mol/L pH 7.4. The same procedure was used to establish this parameter during analysis of galantamine and metabolites in the rat liver tissue. Galantamine extraction recovery was also evaluated from the slope of an external standard calibration curve before and after the extraction.

2.6.3. Selectivity and stability

Selectivity was documented by comparison of chromatograms from the analysis of drug-free samples and samples spiked by analytes under the study.

The stability of galantamine in plasma samples was studied at three various concentrations of quality control samples −0.56, 2.24 and 8.94 μmol/L. The QC samples were analyzed in triplicate immediately and after 1 month storage at −72 °C.

A similar procedure was performed to evaluate the stability for galantamine and metabolites in the rat liver tissue. The four QC samples were analyzed at eight amount levels after storage at −72 °C for 6 weeks. To prove stability, the arithmetic mean of galantamine and metabolites concentration at each level had to be within a limit of 15% deviation of the spiked concentration.

2.6.4. In vivo studies

The experiments were performed under the supervision of the Ethical Committee of Academy of Sciences of the Czech Republic. The Wistar male rats weighing up to 0.3 kg were used in the study. The rats ($n = 6$) were housed under a 12-h light cycle at constant temperature and humidity with a standard laboratory pellet diet. The animals were anaesthetized with diethyl ether. Galantamine hydrobromide was administered in a single intramuscular dose of 10 mg/kg. The sampling was done with the use of PE-cannula introduced *via* vena jugularis before and subsequently after drug administration in the selected time range (5–360 min). The blood (300 μL) was withdrawn into heparinized tubes and centrifuged at 2000 × *g*. Plasma and tissues samples were immediately frozen and stored at −72 °C. The tissue (brain, liver and hypophysis) was obtained from two rats at each time point.

Plasma concentrations of galantamine were averaged per time point and area-under-the curve (AUC) value until the last sampling point was calculated using the linear logarithmic trapezoidal rule.

3. Results and discussion

3.1. Sample preparation development

Various experiments were carried out for the optimization of the sample preparation procedure. Both liquid–liquid extraction into toluene according to the procedure described previously [12] and the solid-phase extraction were tested. Solid-phase extraction is usually characterized by advantages such as a simplicity and low consumption of solvents. SPE also provides clean samples for the separation on chromatographic column. Taking into account the environmental and economic aspects, the solid-phase extraction was chosen. Oasis MCX extraction cartridges based on a new polymeric SPE sorbent copolymer [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] were employed for the SPE. This sorbent is characterized by more reproducible recoveries for a wide range of analytes and by high selectivity for basic compounds such as galantamine (its metabolites) and codeine. The matter of the retention of above-mentioned compounds consists in a strong cation-exchange sulfonic acid groups on the surface of [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] copolymer. Reproducibility and recoveries are not affected by drying time because the sorbent is able to maintain surface conditioning. Due to high permeability of these SPE cartridges, it was not necessary to apply the vacuum. The excellent flow was achieved for viscous homogenized tissue samples using 60 μm large particle size sorbent. SPE procedure using Waters Oasis MCX cartridges provided an acceptable extraction recovery after the optimization and clean samples for separation on an analytical column.

The conversion of *N*-oxide metabolite to galantamine did not occur during a mixed-mode solid-phase extraction method. This observation was verified using the addition of galantamine *N*-oxide to drug-free samples. This problem has been mentioned during usage of Bondelut Certify cartridges from Varian [19].

3.2. HPLC with ultraviolet and fluorescence detection

Optimal chromatographic conditions were investigated in order to reach the acceptable resolution of individual concentration zones of all analytes under study (see compounds in Fig. 1 and codeine as an internal standard) and to avoid their interferences with matrix components.

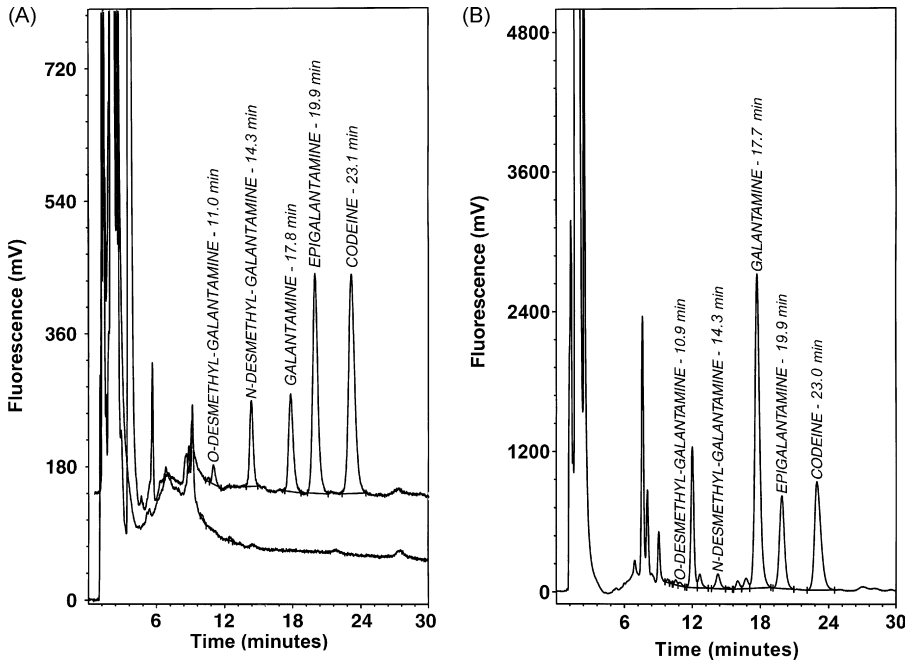

Fig. 2. (A) Chromatograms of the extracts from rat liver tissue: a blank sample (lower chromatogram in (A) and the drug-free rat liver tissue homogenate spiked with the standards (upper chromatogram in (A)). The displayed peaks corresponds to 3.97 nmol/g of galantamine, 5.56 nmol/g of epigalantamine, 2.59 nmol/g of *O*-desmethyl-galantamine, 4.34 nmol/g of *N*-desmethyl-galantamine and 172.67 nmol/g of codeine. (B) Chromatogram of the extract from the rat liver tissue 30 min after a single intramuscular dose of 10 mg/kg of galantamine hydrobromide. The concentration of *O*-desmethyl-galantamine was 0.86 nmol/g, *N*-desmethyl-galantamine was 1.71 nmol/g, galantamine was 26.12 nmol/g, epigalantamine was 4.47 nmol/g and codeine 172.67 nmol/g. (A and B) The chromatograms were acquired using a fluorescence detector at $\lambda_{excit} = 280$ nm and $\lambda_{emiss} = 310$ nm. See Section 2.4 for the chromatographic conditions.

Various chromatographic columns were tested during the prevalidation experiments. The best peak shape, acceptable resolution and reasonable retention time were achieved with the analytical column Discovery HS F5 (Supelco, Bellefonte, PA, USA) 5 μm particle size (4.6 mm I.D. × 150 mm) using a gradient elution described in Section 2.4. Discovery HS F5 column contains pentafluorophenylpropyl bonded silica-based phase with a very good selectivity, as it is demonstrated in Figs. 2 and 3. Chromatogram of the extract from drug-free liver homogenate in a comparison with the chromatogram of the extract from the same biomatrix spiked with the analytes under study is presented in Fig. 2A. The actual presence of galantamine and its individual metabolites in liver tissue and blood plasma 30 min after the intramuscular administration of galantamine to a rat is apparent from the chromatograms in Figs. 2B and 3.

Galantamine and its metabolites had the same chromophore and very similar ultraviolet spectra with the maximum within the range of wavelengths from 211 to 215 nm.

3.3. HPLC/MS analysis

First, electrospray ionization (ESI) mass spectra of all available standards (galantamine *N*-oxide, *O*-desmethyl-galantamine, *N*-desmethyl-galantamine, galantamine, epigalantamine and codeine as an internal standard) were measured using HPLC/MS and HPLC/MS/MS. The base peak of all first-order positive-ion ESI mass spectra was the protonated molecule $[M+H]^+$, which enabled an easy determination of molecular weight of all analyzed compounds (examples in

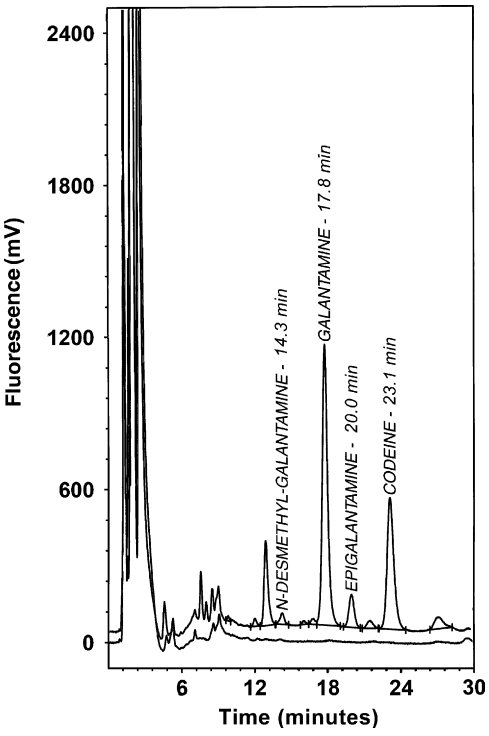

Fig. 3. The chromatograms of the extracts from the rat drug-free plasma (lower chromatogram) and from the rat plasma 30 min after a single intramuscular dose of 10 mg/kg of galantamine hydrobromide (upper chromatogram). The concentrations were 7 μmol/L for galantamine and 0.44 μmol/L for both *N*-desmethyl-galantamine and epigalantamine.

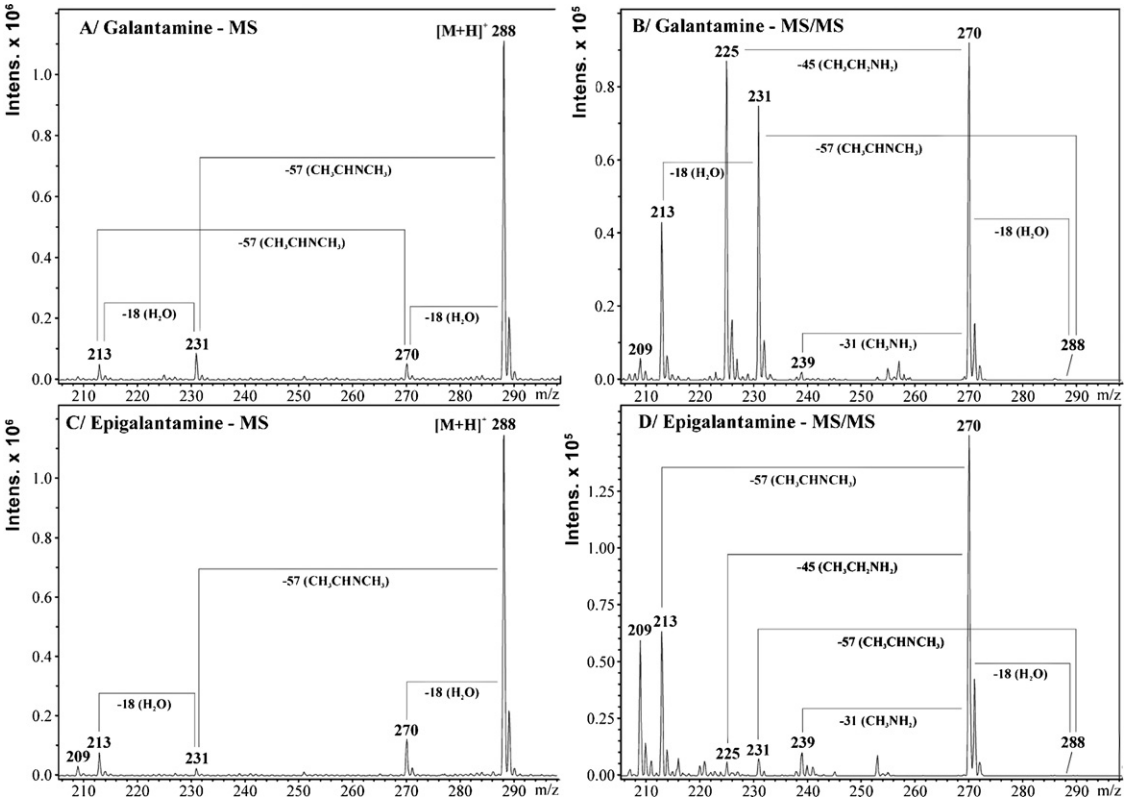

Fig. 4. ESI mass spectra of (A) first-order spectrum of galantamine, (B) MS/MS spectrum of precursor ion *m/z* 288 for galantamine, (C) first-order spectrum of epigalantamine and (D) MS/MS spectrum of precursor ion *m/z* 288 for epigalantamine.

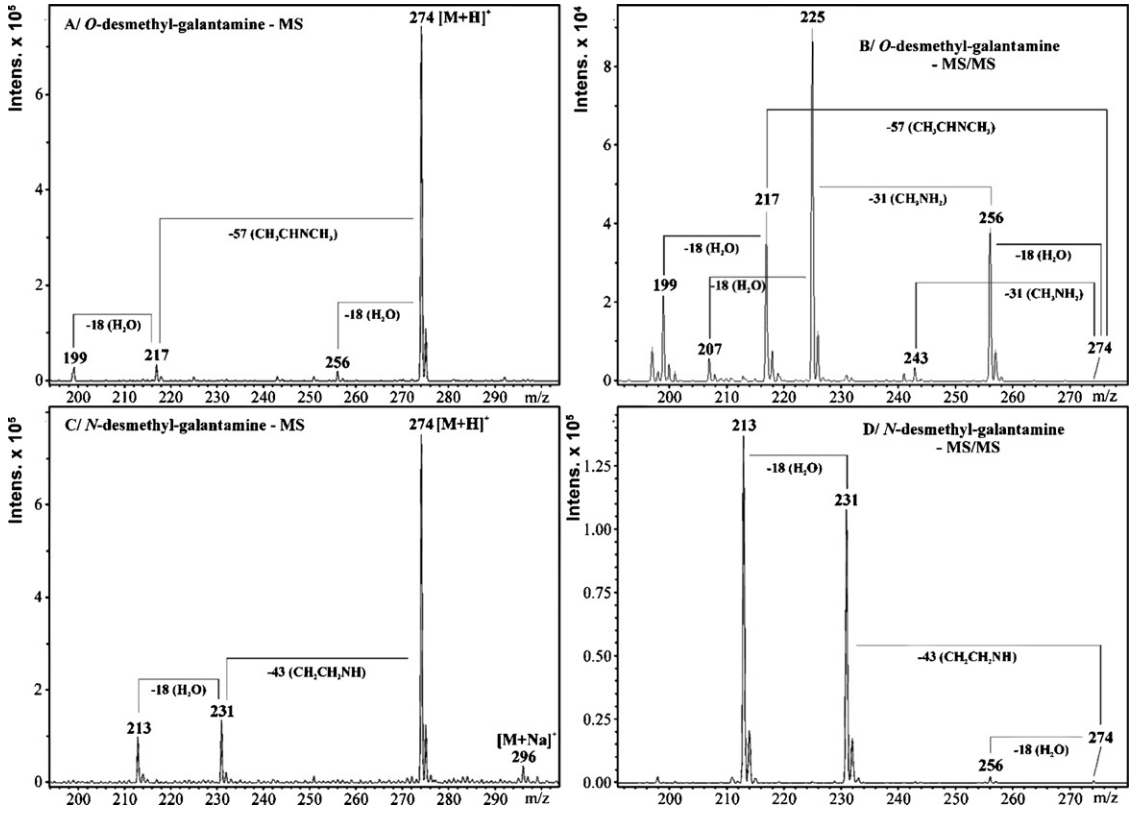

Fig. 5. ESI mass spectra of (A) first-order spectrum of *O*-desmethyl-galantamine, (B) MS/MS spectrum of precursor ion *m/z* 274 for *O*-desmethyl-galantamine, (C) first-order spectrum of *N*-desmethyl-galantamine and (D) MS/MS spectrum of precursor ion *m/z* 274 for *N*-desmethyl-galantamine.

Table 1
Intra-batch accuracy and precision from independently prepared plasma quality control samples

Spiked concentration of galantamine (μmol/L)	Mean concentration of galantamine (± S.D., μmol/L)	Intra-batch accuracy (%)	Intra-batch precision R.S.D. (%)	*n*
0.10	0.10 ± 0.003	100.0	3.0	7
0.20	0.20 ± 0.01	100.0	5.0	8
0.41	0.40 ± 0.02	97.6	5.0	8
0.82	0.83 ± 0.03	101.2	3.6	8
1.63	1.60 ± 0.07	98.2	4.4	8
3.26	3.36 ± 0.13	103.1	3.9	8
6.53	6.37 ± 0.10	97.5	1.6	8
13.05	13.07 ± 0.49	100.2	3.7	8
26.10	26.36 ± 0.97	101.0	3.7	8
52.20	51.97 ± 1.51	99.6	2.9	8
104.40	104.46 ± 2.55	100.1	2.4	7

Figs. 4A, C, 5A and C). Moreover, the presence of low abundant molecular adducts with alkali metal ions, such as $[M+Na]^+$ and $[M+K]^+$, and sometimes dimeric ions $[2M+H]^+$ confirmed determined molecular weights. The characteristic fragment ions were observed in tandem with a mass spectra of $[M+H]^+$ ions (Figs. 4B, D, 5B and D) and some fragment ions observed already in the first-order ESI mass spectra. The differences in the first-order mass spectra of galantamine and epigalantamine are negligible (Fig. 4A and C), but MS/MS spectra measured under identical conditions show some significant differences useful for distinguishing these isomers, in addition to their different retention times. The main difference is found for the fragment ions $[M+H\text{-}CH_3CHNCH_3]^+$ at *m/z* 231 and $[M+H\text{-}H_2O\text{-}CH_3CH_2NH_2]^+$ at *m/z* 225, which have relative abundances higher than 70% for galantamine (Fig. 4B) but lower than 10% for epigalantamine (Fig. 4D). Fig. 5 demonstrates the differentiation of desmethylation at different positions. Apparently, *O*-desmethylation is indicated by increased relative abundance of fragment ion corresponding to the neutral loss of water $[M+H\text{-}H_2O]^+$ at *m/z* 256, which is well pronounced mainly on the comparison of MS/MS spectra in Fig. 5B and D. On the other hand, the cleavage in the heterocyclic 7-membered ring is important for the identification of *N*-substitution, where $\Delta m/z$ 43 neutral loss (CH_2CHNH_2) corresponds to *N*-desmethyl, $\Delta m/z$ 57 ($CH_2CHNHCH_3$) to *N*-methyl and $\Delta m/z$ 73 ($CH_2CHNH(CH_3)O$) to *N*-oxide. Consequent neutral loss of water was observed as well. Based on the comparison of retention times and ESI mass spectra, *O*-desmethyl-galantamine, *N*-desmethyl-galantamine, galantamine and epigalantamine were positively identified in studied extracts from liver tissue.

The galantamine *N*-oxide was identified in the standard samples from synthetic procedure but was not found in the rat liver tissue extracts. Concentrations of metabolites in the extracts from plasma, brain and hypophysis tissues were below the limit of detection of method. Only galantamine and internal standard were identified in these samples.

3.4. The validation characteristics

Analytical method development was followed by the validation to ensure a suitable performance of the assay. The validation parameters were determined by HPLC method with fluorescence detection for the measurement of galantamine in the rat plasma samples. The lower limit of detection was determined to be 0.03 μmol/L. The range of the applicability of method was enclosed within the lower limit of quantification 0.08 μmol/L and the upper limit of quantification 19.66 μmol/L.

The inter- and intra-batch accuracies and precisions reached acceptable values (see Tables 1 and 2).

The linearity of calibration curve was established in the concentration range from 0.08 to 19.66 μmol/L. Table 2 presents the concentrations of galantamine in calibration samples. The regression equation was found to be $y = (17 \pm 0.68)x - (0.57 \pm 0.22)$, with the correlation coefficient of $r = 0.999$ and the standard error of 1.23 ($n = 6$).

Table 2
Inter-batch accuracy and precision from independently prepared plasma quality control samples

Spiked concentration of galantamine (μmol/L)	Mean concentration of galantamine (± S.D., μmol/L)	Inter-batch accuracy (%)	Inter-batch precision R.S.D. (%)	Extraction recovery (%)	*n*
0.08	0.08 ± 0.01	100.0	12.5	74	6
0.15	0.15 ± 0.01	100.0	6.7	81	6
0.31	0.30 ± 0.01	96.8	3.3	85	6
0.61	0.61 ± 0.01	100.0	1.6	79	6
1.23	1.22 ± 0.02	99.2	1.6	71	6
2.46	2.45 ± 0.04	99.6	1.6	72	6
4.92	4.88 ± 0.05	99.2	1.0	71	6
9.83	9.73 ± 0.22	99.0	2.3	73	6
19.66	19.69 ± 0.11	100.2	0.6	74	6

Table 3
Validation characteristics of HPLC method with a fluorescence detection for the determination of galantamine (GAL), epigalantamine (EPIGAL), *O*-desmethyl-galantamine (ODMG) and *N*-desmethyl-galantamine (NDMG) in the rat liver tissue

Parameter	ODMG	NDMG	GAL	EPIGAL
Lower limit of detection (nmol/g)	0.19	0.07	0.04	0.07
Lower limit of quantification (nmol/g)	0.32	0.54	0.50	0.70
Upper limit of quantification (nmol/g)	41.42	69.40	63.47	89.03
Precision (R.S.D., %)				
Intra-batch (8 levels, $n=8$)	2.9–7.7	2.5–8.0	2.9–8.0	3.2–8.4
Inter-batch (8 levels, $n=6$)	1.7–5.4	1.3–9.1	1.5–4.4	2.6–6.4
Accuracy (mean recovery, %)				
Intra-batch (8 levels, $n=8$)	98.7–102.0	93.4–103.4	95.2–104.5	96.2–106.5
Inter-batch (8 levels, $n=6$)	99.0–103.1	95.3–102.9	97.1–102.6	97.5–105.7
Recovery of extraction procedure (%)	57.8	49.7	81.0	81.3
Linearity of calibration curve (nmol/g) (six calibration curves)	0.32–41.42	0.54–69.40	0.50–63.47	0.70–89.03
Regression equation				
Slope (± S.D.)	3.45 ± 0.23	9.64 ± 1.01	15.25 ± 0.81	24.70 ± 1.18
Intercept (± S.D.)	0.05 ± 0.09	−0.21 ± 0.87	0.24 ± 1.14	1.11 ± 2.12
Standard error	0.14	1.08	1.35	3.81
Correlation coefficient	0.999	0.999	0.999	0.999

The extraction recoveries of galantamine, which were determined by comparing the peak area before and after the extraction, are shown in Table 2. The average extraction recovery of codeine was 73%. The extraction recovery of galantamine evaluated from the slope of external calibration curve before and after extraction was 78%.

During the evaluation of selectivity, the retention time region of galantamine, metabolites and the internal standard was free from any interferences in six blank samples of plasma and tissue from brain, liver and hypophysis. The chromatograms of the extracts from HPLC analysis with fluorescence detection illustrate these observations for drug-free plasma and liver tissue and show the retention time region of *O*-desmethyl-galantamine, *N*-desmethyl-galantamine, galantamine, epigalantamine and codeine (Figs. 2 and 3).

Validation characteristics of HPLC method with a fluorescence detection for the determination of galantamine (GAL), epigalantamine (EPIGAL), *O*-desmethyl-galantamine (ODMG) and *N*-desmethyl-galantamine (NDMG) in the rat liver tissue are displayed in Table 3.

The stability of galantamine in QC samples prepared from drug-free plasma and liver tissue homogenates was tested and evaluated. The mean recovery of galantamine in QC plasma samples was in the range from 98.7 to 100.2% (instant analysis) and from 95 to 99.6% (after 1-month storage at −72 °C).

After 6 weeks storage at −72 °C, galantamine and its metabolites in QC rat liver tissues samples did not exceed the limit of R.S.D. 15%, and accuracy was within ±15% deviation of the spiked concentration. These results were in agreement with a detailed stability investigation [19]. According to the mentioned study, galantamine was stable in human plasma even after four freeze–thaw cycles, after 72 h at room temperature and at least 308 days at −18 °C.

3.5. *In vivo studies*

The HPLC method based on fluorescence detection was used for pharmacokinetic studies of galantamine. Plasma concentration–time profile of galantamine after a single intramuscular dose of 10 mg/kg of galantamine hydrobromide administered to the rats ($n=6$) is shown in Fig. 6. The plasma AUC reached the value of 5.44 μg h/mL as compared to the value of 1.466 μg h/mL from previously presented study [24]. The different experimental conditions were used in the above-mentioned study (for example animals were treated subcutaneously with a dose of 5 mg/kg of galantamine). Therefore, the comparison of plasma AUC was difficult.

The validated analytical method was applied to a biodistribution study of galantamine and its metabolites in tissue samples from liver, brain and hypophysis. The tissue concentration–time profiles for galantamine, epigalantamine, *N*-desmethyl-galantamine and *O*-desmethyl-galantamine in the liver are presented in Fig. 7. The peak concentrations for analytes

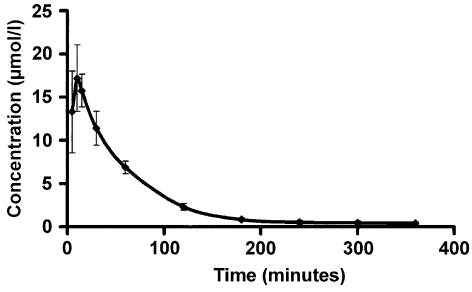

Fig. 6. Galantamine plasma concentration–time profile (mean ± S.D.) after a single intramuscular dose of 10 mg/kg of galantamine hydrobromide administered to rats ($n=6$).

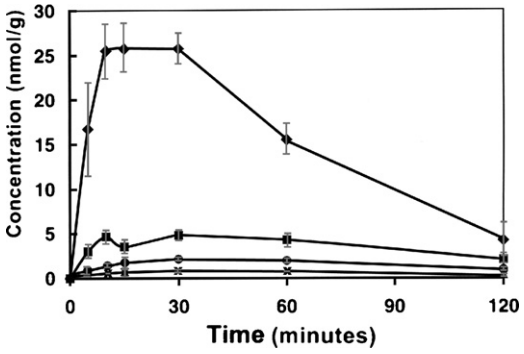

Fig. 7. The time course of the concentration (arithmetic mean ± S.D.) of galantamine (♦), epigalantamine (■), *N*-desmethyl-galantamine (●) and *O*-desmethyl-galantamine (×) in the rat liver tissue following a single intramuscular dose of 10 mg/kg of galantamine hydrobromide administered to the rats (two rats at each time period).

under the study were reached 30 min after the administration of dose.

4. Concluding remarks

A new bioanalytical HPLC method with fluorescence detection involving the mixed-mode solid-phase extraction procedure for the determination of galantamine and its phase I metabolites was developed and validated. The analytical method was employed in *in vivo* studies and was applicable for quantification of galantamine not only in plasma but also in tissue samples such as liver, brain and hypophysis.

Galantamine, epigalantamine, *O*-desmethyl-galantamine and *N*-desmethyl- galantamine were identified by HPLC–PDA–MS experiments in the rat liver tissue extracts. Galantamine *N*-oxide was not found in these samples. This metabolite concentration could be below the limit of detection. Validation experiments certified possible suitability of bioanalytical method in the determination of galantamine, *O*-desmethyl-galantamine, *N*-desmethyl-galantamine and epigalantamine in the rat liver tissue. The new method was found to be a beneficial contribution to the further progress of the pharmacokinetic and biodistribution studies of galantamine.

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