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Article

Quantification of Galantamine in Human Plasma by Validated Liquid Chromatography–Tandem Mass Spectrometry using Glimepride as an Internal Standard: Application to Bioavailability Studies in 32 Healthy Korean Subjects

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A simple, rapid and selective liquid chromatography method coupled with tandem mass spectrometry is developed and validated for the quantification of galantamine in human plasma using a commercially available compound, glimepride, as an internal standard (IS). Following simple one-step liquid–liquid extraction by ethyl acetate, the analytes are separated using an isocratic mobile phase consisting of acetonitrile and 0.01M ammonium acetate (95/5, v/v) on a reverse-phase C18 column and analyzed by tandem mass spectrometry in the multiple reaction monitoring mode using the transitions of respective $(M + H)^+$ ions, m/z 288.22 → 213.20 and m/z 491.17 → 352.30 for the quantification of galantamine and IS, respectively. The standard calibration curves show good linearity within the range of 4 to 240 ng/mL ($r^2 = 0.9996$, $1/x^2$ weighting). The lower limit of quantification is 4 ng/mL. The retention times of galantamine and IS are 1.1 and 0.71 min, which showsthe high throughput potential of the proposed method. In addition, no significant metabolic compounds are found to interfere with the analysis. Acceptable precision and accuracy are obtained for the concentrations over the standard curve range. The validated method is successfully applied for pharmacokinetic and bioequivalence studies of 24 mg of a galantamine hydrobromide capsule in 32 healthy Korean subjects.

Introduction

Galantamine ([4aS,6R,8aS]-5,6,9,10,11,12-hexahydro-3-methoxy-11-methyl-4aH-[1]benzofuro[3a,3,2-ef]-[2] benzazepin-6-ol (Figure 1) belongs to the cholinergic drugs with acetylcholinesterase (AChE) inhibition and is approved for the treatment of mild to moderate Alzheimer's disease (AD) and various memory impairments, particularly those of vascular origin (1–3). Galantamine works by a competitive and reversible cholinesterase inhibition and an allosteric modulation for nicotinic AChE (4). It reduces the action of AChE and therefore tends to increase the concentration of acetylcholine in the brain. It is hypothesized that this effect might relieve some of the symptoms of AD. Although galantamine was first used as a reverser of neuromuscular blockade (5, 6), it has recently been studied and shown to be effective for use in the treatment of AD (7–9), vascular dementia and AD with cerebrovascular disease (10). In double-blind placebo-controlled clinical trials, galantamine has shown benefits in the domains of cognition, global function, ability to perform activities of daily living and behavioral symptoms (7–9, 11). The oral bioavailability of galantamine is approximately 90–100% and co-administration of food has little effect on galantamine absorption, delaying the rate but not affecting the extent (12). Approximately 75% of a dose of galantamine is metabolized to a pharmacologically inactive or less-active metabolite (13) by major metabolic pathways with O-desmethylation, *N*-desmethylation, *N*-oxidation, epimerization, glucuronidation and sulfate conjugation (14), and approximately 20% of the dose is excreted as unchanged in the urine in 24 h, representing a renal clearance of approximately 65 mL/min, 20–25% of the total plasma clearance (300 mL/min), representing low protein binding and a relatively large volume of distribution (3). Cytochrome P450 (CYP) 2D6 and 3A4 are the major isozymes involved in the hepatic metabolism. CYP2D6 is responsible for the O-desmethylgalantamine, and CYP3A4 is involved in the production of galantamine *N*-oxide (3, 15). Galantamine demonstrates biexponential elimination, with a mean plasma terminal elimination half-life of 5.26 h (16) to 5.68 h (17) in healthy subjects (18). Total plasma clearance in patients with AD appears to be approximately 30% less than that in healthy volunteers (0.25 and 0.34 L/h/kg, respectively) (19). Various analytical methods such as a capillary zone electrophoresis (20), high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (21, 22) or fluorescence detection (14, 23), HPLC-UV with photodiode-array radiometric detection (14, 23) and HPLC with tandem mass spectrometry (HPLC–MS-MS) (24–26) have been developed to support clinical and pharmacokinetic studies of galantamine in biological fluids. The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, selectivity, high sensitivity, small sample volume requirement and short run time for high throughput process. Two bioanalytical methods using HPLC with UV detection required 2 mL of plasma with either phenacetin or codeine as the internal standard (IS). Sample preparation consisted of protein precipitation followed by liquid–liquid extraction, either one-step or using back-extraction and re-extraction (22, 27). In other methods, an HPLC method with fluorescence detection was used to quantify the galantamine in plasma

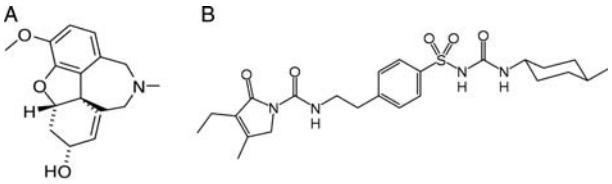

Figure 1. Chemical structures of galantamine {MW = 287.354 g/mol, $C_{17}H_{21}NO_3$, (4a*S*,6*R*,8a*S*)-5,6,9,10,11,12-hexahydro-3-methoxy-11-methyl-4a*H*-[1]benzofuro[3a,3,2-*ef*] [2] benzazepin-6-ol} (A); glimepride {IS, MW = 490.617 g/mol, $C_{24}H_{34}N_4O_5S$, 3-ethyl-4-methyl-*N*-(4-[*N*-((1*r*,4*r*)-4-methylcyclohexylcarbamoyl)sulfamoyl]phenethyl)-2-oxo-2,5-dihydro-1*H*-pyrrole-1-carboxamide} (B).

throughout the preclinical and the earlier clinical trials (14, 15). Codeine was used as IS and samples were extracted by one-step liquid–liquid extraction of galantamine into toluene (14, 23), or by a precipitation processes with trichloracetic acid followed by alkalization of the sample and by liquid–liquid extraction into chloroform (22). Although the method proved very robust, the plasma volume requirement was still quite high, requiring at least 1 ~ 2 mL of plasma, and the throughput was limited due to the relatively long analytical run times above 7 min. The ever-shortening timelines in drug discovery and development have brought about the need for high-throughput approaches to methods used to determine drugs, metabolites and endogenous biomolecules in biological matrices such as blood-derived samples, urine, and *in vitro* biological samples. Because of the large amount of samples generated in recent clinical trials, such as pharmacokinetic or therapeutic drug monitoring studies, it was inevitable to develop and validate a new bioanalytical method using LC–MS-MS that would provide higher sample throughput than previous reported methods. Quantification of drugs in biological samples by LC–MS-MS is becoming more common, owing to the improved sensitivity and selectivity of this method (27). Recently, two bioanalytical methods using LC–MS-MS have been described in the literature for galantamine (24, 26). One reported LC–MS-MS method for the quantification of galantamine in human plasma used a stable isotope IS with a lower limit of quantification of 1 ng/mL (24). Even if the stable isotope-labeled compound as the IS makes LC–MS-MS a reliable and robust analytical method, the isotope-labeled compound is not commercially available, and the cost of custom synthesis may bottleneck its application. Another LC–MS-MS method for quantification of galantamine in human plasma used a commercially available compound, loratadine, as the IS and one-step sample preparation by liquid–liquid extraction using toluene and high sensitivity with a lower limit of quantification (LLOQ) of 0.5 ng/mL (26). The purpose of this work was to explore the simple and high-throughput applicable methods of a triple-quadruple MS system operated in MS-MS mode with an electrospray ionization (ESI) interface for development and validation of a robust reversed-phase LC–MS-MS method in the multiple reaction monitoring (MRM) mode for the quantification of galantamine in human plasma using commercially available compound, glimepride, as the IS. The proposed LC–MS-MS method allowed a very fast and sensitive determination of galantamine in pharmacokinetic and bioavailability studies after oral dose of the 24-mg galantamine hydrobromide capsules in 32 healthy Korean male subjects.

Experimental

Chemicals and reagents

Galantamine ($C_{17}H_{21}NO_3$, MW = 287.354 g/mol) and glimepride (IS, $C_{24}H_{34}N_4O_5S$, MW = 490.617 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO) (Figure 1). Purity was found to be more than 99% in all compounds. Acetonitrile, ammonium acetate and methanol were all HPLC-grade and purchased from Sigma, while other reagents and solvents used were analytical grade. All aqueous solutions, including the buffer for the HPLC mobile phase, were prepared with water that was purified by a Milli-Q water purification system (Millipore; Milford, MA). Reference drug (Reminyl, extended release galantamine hydrobromide capsule, 24 mg/capsule) was obtained from Janssen Korea Pharmaceutical Co. (Seoul, South Korea) and test drug (Tamilin, extended release galantamine hydrobromide capsule, 24 mg/capsule) was provided by Hyundae Pharmaceutial Co. (Seoul, South Korea).

Stock solutions and standards

Primary stock solutions of galantamine and IS were prepared with methanol solution to final concentrations of 1 and 10 μg/mL, respectively, and stored at −20°C. A set of six non-zero calibration standards, ranging 4 to 300 ng/mL, were prepared in blank human plasma with an appropriate amount of galantamine. The quality control (QC) samples were prepared in blank human plasma at concentrations of 4, 12 (low), 120 (middle) and 240 ng/mL (high). Blank human plasma was tested before spiking to ensure that no endogenous interference was found proximal to retention times of galantamine and IS.

Preparation for plasma samples

After dilution of stock solution of galantamine to its concentration of 4.0 ~ 300 ng/mL with blank plasma, a 50-μL aliquot of human plasma was pipetted into a screw-cap glass tube. Briefly, a 50-μL aliquot of IS working solution (IS, 10 μg/mL) and 2 mL of ethyl acetate were added to the 50-μL aliquot of human plasma and the galantamine and IS were extracted by agitating in a vortex mixer for 10 min. After centrifugation at 4,000 rpm for 5 min, the organic phase was transferred to another set of clean glass tubes and then evaporated to dryness under nitrogen gas at 40°C. The dried residue was dissolved with 500 μL of 50% methanol. After centrifugation at 4,000 rpm for 5 min, 3 μL of the reconstituted solution were directly injected into the LC–MS-MS system.

LC–MS-MS conditions and quantifications

The LC system used was an Agilent (Palo Alto, CA) chromatograph quipped with an isocratic pump (1100 series) and interfaced with an autosampler (Reliance; Spark, Holland). The analytical column was a YMC Hydrosphere C18 (50 × 2 mm i.d., 3 μm; YMC Co., Japan). The mobile phase consisted of acetonitrile–0.01M ammonium acetate (95:5, v/v) and the flow rate was 0.2 mL/min. MS analysis was performed using an API 2000 mass spectrometer system (Applied Biosystems; Foster City, CA) equipped with an ESI interface and operated in the positive ionization mode. The ion source

parameters were set as the following: curtain gas, 35 psi; GS1, 50 psi and GS2, 50 psi; ion spray voltage, 4,500 V; ion source temperature, 250°C; collision-activated dissociation (CAD), 8.0. This system was set up in MRM mode, monitoring the transitions m/z 288.22 → 213.20 and m/z 491.17 → 351.30 for quantification of galantamine and IS, respectively. Data acquisition and analysis were performed using the analyst software Peak Simple Chromatography Data system version 1.4.1. (Applied Biosystems).

Method assay validation

Assay validation was performed according to the Food and Drug Administration (FDA) guidance on bioanalytical method validation (29). Linearity was determined using a linear least-squares regression with $1/x^2$ weighting, which was performed on the peak area ratios of galantamine and IS versus galantamine concentrations of the six human plasma standards. The sensitivity of the method was expressed as LLOQ that could be quantitatively determined with acceptable accuracy

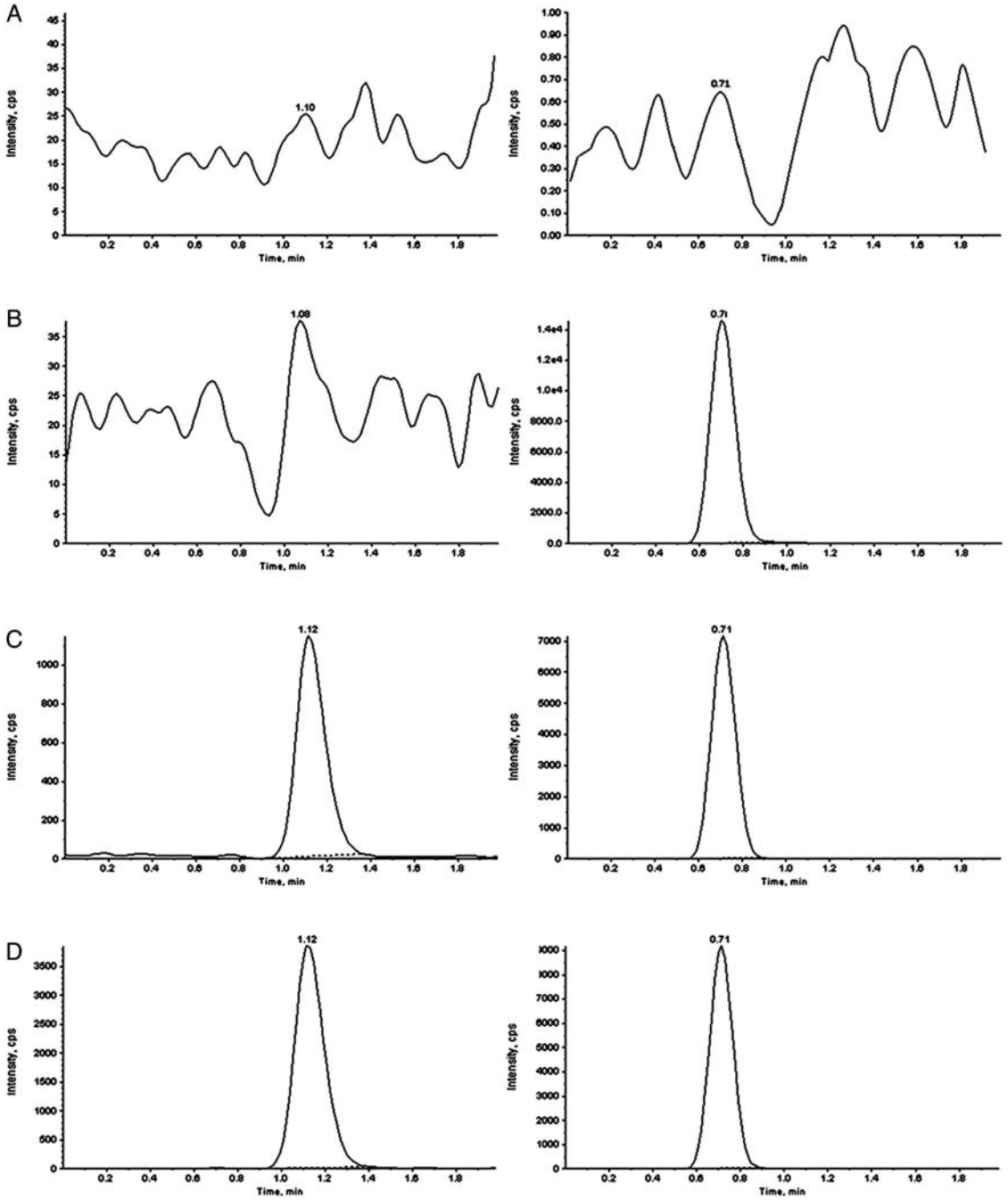

Figure 2. Chromatograms of: double blank plasma (A); blank plasma with IS (10 μg/mL) (B); blank plasma with galantamine (LLOQ, 4 ng/mL) and IS (10 μg/mL) (C); human plasma taken 2 h after a single oral dose of 24 mg galantamine hydrobromide capsule spiked with IS (10 μg/mL) (D).

and precision. The accuracy and precision were assessed by analyzing four concentrations of QC samples from five different validation batches and calculated using one-way analysis of variance (ANOVA). The specificity was performed and six randomly selected blank human samples, which were collected under controlled conditions, were carried through a similar extraction procedure and analyzed to determine the extent to which endogenous plasma components could interfere with the analyte or IS at the retention time. To evaluate the inter-day precision and accuracy, validation control samples with drug concentration of 4, 12, 120 and 240 ng/mL galantamine and IS (10 μg/mL) solutions were analyzed together with one independent calibration curve. The accuracy and precision of the inter-day assay were evaluated at the same concentration and calculated for five different days. Inter-day and intra-day precision were expressed as relative standard deviation (RSD). The accuracy was expressed as the percent ratio between the experimental and nominal concentrations for each sample. The lowest limit of quantification was defined as the lowest plasma concentration of each galantamine analyzed with an error of 20% or lower that corresponds to a signal five times greater than the analytical background noise in our experiment (28, 29).

Pharmacokinetic and bioequivalence study in healthy volunteers

A randomized, single-dose, two-period, two-sequence and crossover design was used for the assessment of the pharmacokinetics and bioequivalence. One capsule of 24-mg galantamine hydrobromide was randomly given to 32 healthy male volunteers. Participants had not taken other medications (including over-the-counter drugs) for two weeks before or during the study period. The study was carried out according to the World Medical Association Declaration of Helsinki (1997) for biomedical research involving human subjects (30) and the Guideline for Good Clinical Practice (2008) (32). Based on this description, they provided written informed consent before participating in the study. In addition, the Institutional Review Board of Hanyang University Medical Center approved the protocol before the start of the study. Thirty-two volunteers aged between 19 and 29 years (24.1 ± 2.5 years), with heights

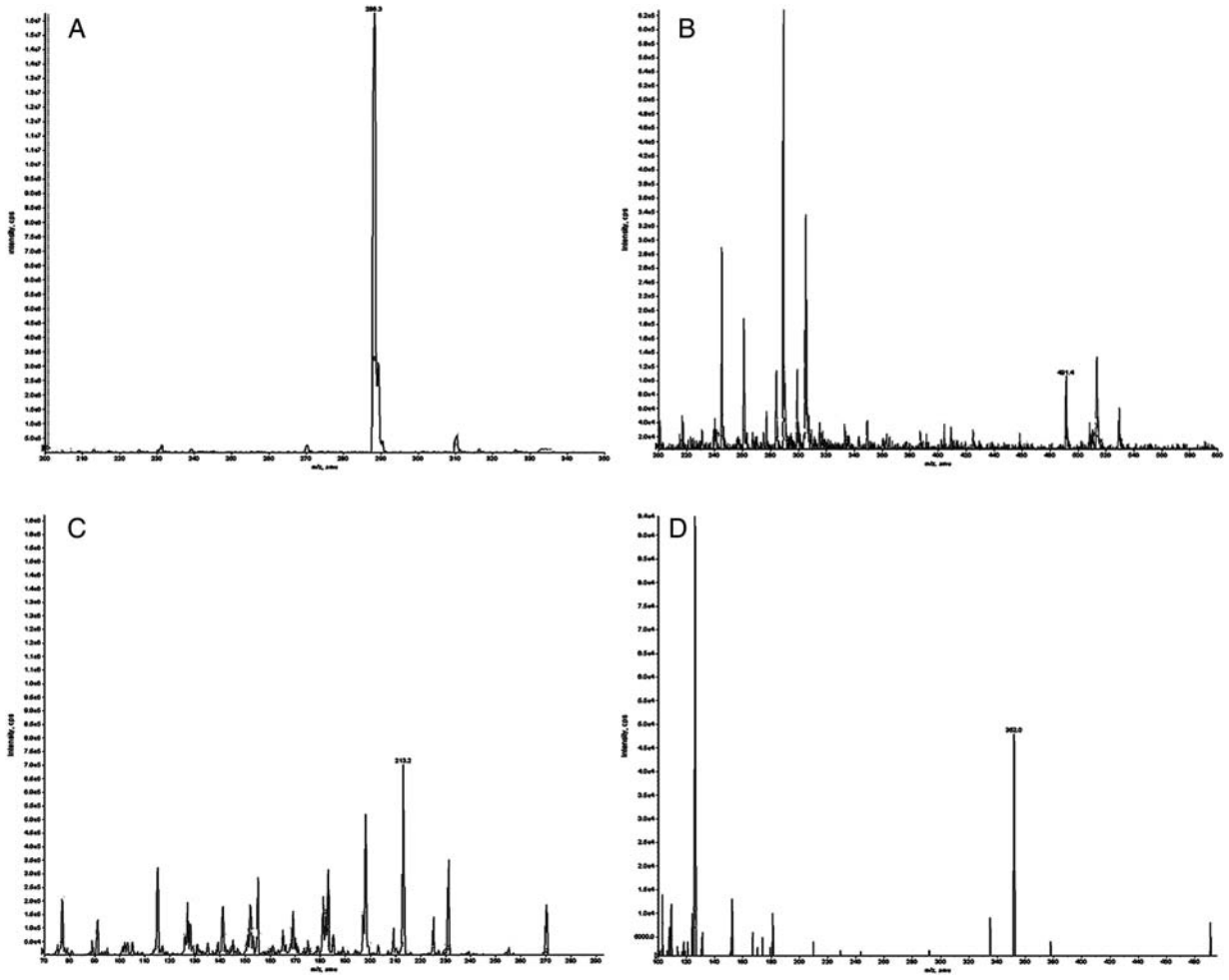

Figure 3. Full-scan mass spectra of precursor ions of galantamine (A) and glimepride (B); product ions of galantamine (C) and glimepride (D)

between 165.4 and 192.0 cm (176.2 ± 5.9 cm) and body weights between 59.0 and 92.0 kg (72.7 ± 8.4 kg), who were non-alcoholics and free from disease, were assessed as having a healthy status by clinical evaluations. including a physical examination and routine clinical laboratory analysis. During each period, the participants were hospitalized at the Hanyang University Medical Center at 17:00 p.m. and had an evening meal before 20:00 p.m. After an overnight fast, they received a test or reference drug (single 24-mg galantamine hydrobromide/capsule) at 7:00 a.m. along with 240 mL of water. A standard lunch and evening meal were provided at four and 10 h after dosing. Liquid consumption was allowed *ad libitum* after lunch, except for liquids that contained xanthine and acidic beverages, including tea, coffee and cola. Before and at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 36, 48 and 60 h after dosing, vital signs were recorded. Blood samples (9 mL) were withdrawn via an indwelling catheter into heparin-containing tubes from a suitable antecubital vein. The blood samples were centrifuged at 3,000 rpm for 10 min at room temperature and plasma was stored at −70°C until analysis. The total plasma galantamine levels were determined and the maximal concentration (C_{max}) and T_{max} were determined by visual inspection from each subject's plasma concentration of galantamine versus time plots. Other pharmacokinetic parameters were calculated by non-compartmental PK data analysis using PKCAL computer software as follows. The area under the plasma concentration versus time curve ($AUC_\infty = \int_t C_t\ d_t + C_{last}/\lambda$, where λ = terminal phase slope) was calculated for the total galantamine level (C_t) using the linear trapezoidal rule extrapolated to infinity according to a pharmacokinetic analysis program multiline fitting (33), where C_{last} was the last measurable concentration and the terminal phase slope (λ) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration versus time profile (33). The terminal half-life ($T_{1/2}$) was calculated by $0.692/\lambda$ (34). The first moment versus time curve ($AUMC_\infty$) was calculated by integration of time (t) of first moment ($C_t \times t$) ($AUMC = \int C_t \times t \times dt + Cp \times t/\lambda_z + Cp/\lambda_z^2$). The mean residence time (MRT) of the galantamine in the body was calculated by AUMC/AUC and λ_z indicates the elimination rate constant (*Ke*) (33, 35–36).

Table I
Method Validation for the Analysis of Galantamine in Human Plasma and Recovery of Galantamine and IS (Glimepride) after the Extraction Procedure (n = 5)

Nominal concentration (ng/mL)	Precision (%RSD)		Accuracy (%)	
	Inter-day	Intra-day	Inter-day	Intra-day
4 (LLOQ)	12.79	8.97	104.77	105.31
12 (low)	3.74	9.26	103.29	97.23
120 (middle)	5.47	4.61	101.99	99.65
240 (high)	5.25	4.22	106.00	106.59

Results and Discussion

Separation

The molecular structures of galantamine hydrobromide and IS are shown in Figure 1. The simple preparation procedures, including the liquid–liquid extraction of galantamine with ethyl acetate and evaporation of extracted sample and reconstitution with 50% methanol, were used before reverse-phase HPLC separation. The chromatograms of blank plasma (Figure 2A), blank plasma spiked with 10 μg/mL of IS (Figure 2B), blank plasma spiked with 4 ng/mL of calibration standard of galantamine and 10 μg/mL of IS (Figure 2C), and a subject's plasma taken after 2 h of a single oral dose of 24 mg galantamine hydrobromide spiked with 10 μg/mL of IS (Figure 2B) are shown in Figure 2. The retention times of galantamine and IS were 1.11 and 0.71 min, respectively. Blank human plasma had no significant endogenous peaks at the retention time of galantamine or IS (Figure 2A). To avoid interference from exogenous/endogenous compounds co-eluting with the target compound, MS-MS detection was performed. Ionization of analytes was performed using the ESI technique with positive polarity and MRM mode. From full-scan mass spectra via the Q1 mass filter (Figures 3A and 3B), the protonated molecular ions, $[M + H]^+$, at m/z 288.22 for galantamine and m/z 492.17 for IS, were chosen for the precursor ion. The MS-MS fragmentation was achieved by introducing the $[M + H]^+$ ions into the second quadrupole (Q2) cell with the best collision energy set of 29.0 eV for galantamine and 23.0 eV for IS. After collision-induced

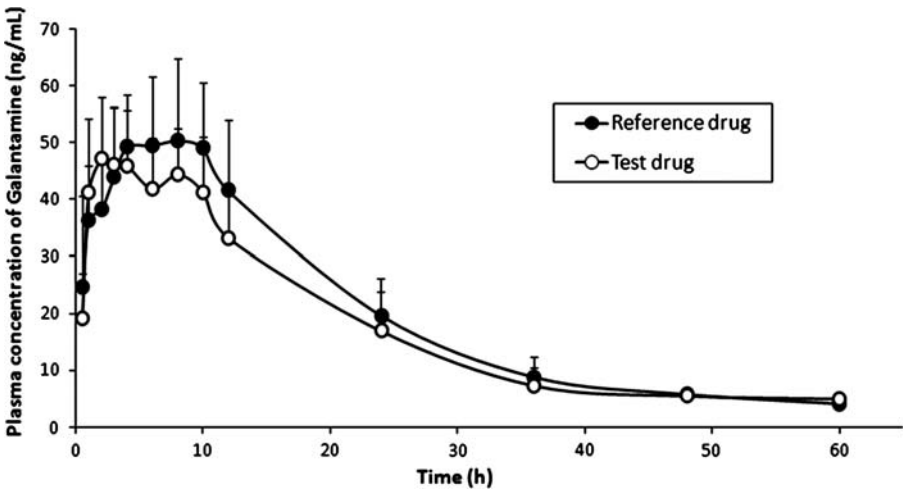

Figure 4. Mean (± SD) plasma concentrations versus time plots after a single oral dose of 24-mg galantamine hydrobromide capsule to the 32 healthy male subjects.

Table II
Pharmacokinetic Parameters (mean ± SD, $n = 32$) of Two Formulations of 24-mg Galantamine Capsules Based on Blood Galantamine Concentrations in the 32 Healthy Subjects

Parameters	Reference	Test
AUC_{60h} (μg-h/mL)	1079.83 ± 322.60	931.99 ± 270.13
AUC_{∞} (μg-h/mL)	1181.33 ± 320.06	1023.37 ± 274.15
Extrapolation ($AUC_{60h-\infty}/AUC_{\infty}$, %)	9.34 ± 4.98	9.37 ± 4.46
AUMC60h (μg-h^2/mL)	32170.6 ± 1007.8	48817.64 ± 2351.9
AUMC∞ (μg-h^2/mL)	33564.6 ± 1021.0	53604.12 ± 2413.5
MRT (h)	28.42 ± 6.37	52.38 ± 13.78
C_{max} (μg/mL)	55.35 ± 12.51	53.86 ± 10.62
$T_{1/2\alpha}$ (h)	2.96 ± 1.78	3.38 ± 2.76
kα (h^{-1})	0.234 ± 0.056	0.205 ± 0.037
T_{max} (h)	7.22 ± 2.29	3.93 ± 2.73
$T_{1/2\beta}$ (h)	9.83 ± 1.91	9.72 ± 2.18
Λ_z (*ke*, h^{-1})	0.167 ± 0.047	0.176 ± 0.039

dissociation, the MS-MS transition m/z 288.22 → 213.20 for galantamine and m/z 491.17 → 352.30 for IS was selected. The most abundant ions in the product ion mass spectrum at m/z 213.20 for galantamine and m/z 352.30 for IS were monitored for quantification (Figures 3C and 3D).

Method validation and linearity of calibration

The standard calibration curves showed good linearity within the range of 4 ~ 300 ng/mL using least-squares regression analysis ($y = 0.0138x + 0.00556$, $r^2 \geq 0.996$, $1/x^2$ weighting). Intra-day and inter-day precisions and accuracies were determined by analyzing QC samples against a calibration curve on the same day ($n = 5$) and on different days ($n = 5$). As shown in Table I, this method offered good precision and accuracy. The intra-day and inter-day RSD values were below 12.79 and 9.26%, respectively. Intra-day and inter-day accuracies were 101.99 ~ 106.00 and 97.23 ~ 106.56%, respectively. Under the described analytical conditions, the LLOQ, defined as the lowest concentration of galantamine at which both the precision and accuracy were less than or equal to 20% (28), was 4 ng/mL. Because the oral administration of 24 mg galantamine hydrobromide resulted in a mean plasma concentration of 24.49 ± 16.11 ng/mL at 0.5 h and 5.68 ± 1.25 ng/mL at 48 h for the reference drug, and 19.05 ± 8.00 ng/mL at 0.5 h and 5.43 ± 1.38 ng/mL at 48 h for the test drug, the LLOQ of this method appeared to have enough sensitivity.

Clinical application in healthy volunteers

The proposed method was applied to the determination of galantamine hydrobromide in plasma samples for the purpose of establishing the pharmacokinetic and bioequivalence study of 24-mg galantamine hydrobromide capsule formulations in 32 healthy Korean volunteers. The pharmacokinetic parameters for the reference and test drug obtained are described as follows. The profiles of the plasma galantamine concentration versus time are shown in Figure 4. Plasma concentrations of galantamine were in the standard curve range and remained above the LLOQ (4 ng/mL) for the entire sampling period, except for three subjects at 0.5 h after dosing. Because the bioavailability of orally administered galantamine is good, mean maximal galantamine plasma concentrations (C_{max}) were 97 ng/mL within 10 h for the 24 mg/day dose (7, 8). These concentrations were higher than those observed in healthy young volunteers in this study. The plasma profiles of the mean galantamine concentration versus time after oral administration of a single dose of both formulations in 32 subjects exhibited some different patterns. The mean estimated pharmacokinetic parameters derived from the plasma concentration profiles of galantamine are shown in Table II. The bioequivalent parameters are overlapped between test and reference. In previous reports, absorption of galantamine after oral administration was rapid: maximum concentrations are reached in approximately 1 h and elimination half-life is approximately 7 to 8 h in fasting conditions (37, 38). Our experimental results showed some differences from the results of those studies. The mean ratio of the $AUC_{t\rightarrow\infty}$ divided by AUC_{∞} was above 9.34 and 9.37% for reference and test, respectively. The 90% confidence intervals (CI) of the test/reference percentage ratios were 97.8 (92.73 ~ 103.21%) for C_{max} and 86.8% (78.79 ~ 97.64%) for AUC_{60h}.

Conclusion

The proposed method of combining a simplified plasma extraction procedure and a sensitive LC–ESI-MS-MS method provided a rapid and sensitive detection technique for galantamine in human plasma. We achieved shorter retention times (1.12 min for galantamine, 0.71 min for IS) and smaller volumes (50 μL) of human plasma than previous reports (26) with sufficient LLOQ (4 ng/mL) for application in pharmacokinetic, bioavailability or bioequivalence studies. The precision and accuracy for calibration and QC samples were well within the acceptable limits. This method was sensitive enough to monitor galantamine plasma concentrations up to 60 h after dosing and provided us with a successful application in a pharmacokinetics and bioequivalence study of the two types of 24-mg galantamine formulations in 32 healthy Korean volunteers.

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