Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 789 (2003) 337–346
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a liquid chromatographic–tandem mass spectrometric method for the determination of galantamine in human heparinised plasma

T. Verhaeghe*, L. Diels, R. de Vries, M. De Meulder, J. de Jong

Department of Bioanalysis, Johnson & Johnson Pharmaceutical Research and Development, a Division of Janssen Pharmaceutica N.V., Turnhoutseweg 30, 2340 Beerse, Belgium

Received 11 October 2002; received in revised form 23 January 2003; accepted 23 January 2003

Abstract

Galantamine is an acetylcholinesterase inhibitor, recently approved for the treatment of mild-to-moderate Alzheimer's disease. To allow a higher throughput of samples, a new bioanalytical method for the determination of galantamine in human plasma was developed and validated. A stable isotope labelled internal standard was used. Sample preparation consisted of a simple one-step liquid–liquid extraction with toluene. The extracts were analysed with positive ion TurboIonspray tandem mass spectrometry (LC–MS–MS). The method was validated in the 1–500-ng/ml range. The accuracy, precision, selectivity, lower limit of quantification, upper limit of quantification, linearity and extraction recovery were evaluated, as well as the stability of the compound in plasma, blood, methanol and 2% BSA solutions under different conditions. The method proved very rugged during the analysis of large numbers of samples from clinical trials.

Keywords: Galantamine

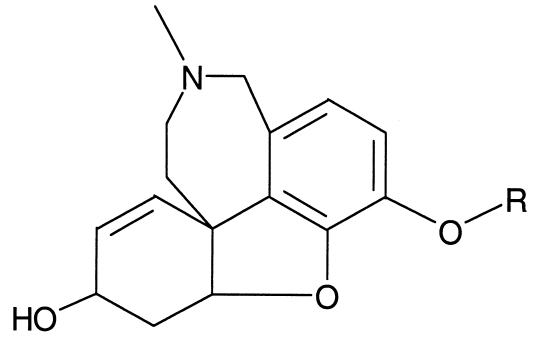
1. Introduction

Galantamine (Reminyl) (Fig. 1) is a tertiary alkaloid that can be isolated from a number of plants (e.g., daffodils bulbs). More recently, a fully synthetic version was approved. Galantamine is a cholinergic drug, approved for the treatment of mild-to-moderate Alzheimer's disease (AD) in Europe and the USA. The compound exhibits a dual mode of action by inhibiting acetylcholinesterase and positively modulating nicotinic acetylcholine (ACh) receptors, which augments responses to the neurotransmitter ACh [1–3].

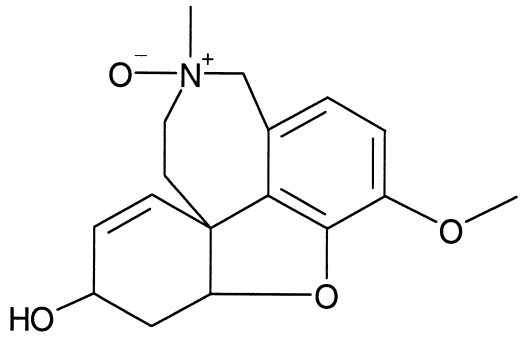
Two bioanalytical methods using HPLC with UV detection have been described in the literature for galantamine [4,5]. Both methods required 2 ml of plasma, with either phenacetin or codeine as the internal standard. Sample preparation consisted of protein precipitation followed by liquid–liquid extraction, either single-step or using back-extraction and re-extraction. Within our lab, throughout the preclinical program and the earlier clinical trials, an HPLC method with fluorescence detection was used to quantify the drug in plasma [6,7]. Codeine was used as the internal standard and samples were

*Corresponding author.
E-mail address: tverhaeg@prdbe.jnj.com (T. Verhaeghe).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00129-6



Galantamine: R = CH_3
I.S.: R = $^{13}C^2H_3$



Galantamine N-oxide

Fig. 1. Structures of galantamine *N*-oxide and the isotopically labelled internal standard.

extracted with toluene. Although the method proved very robust, the required volume of plasma was still quite high (1-ml aliquots) and the throughput was limited due to the relatively long chromatographic run times (>7 min). Because of the large number of samples generated in the recent clinical trials, it was decided to develop and validate a new bioanalytical method using LC–MS–MS that would enable a higher sample throughput. In order to make the method as reliable and robust as possible, a stable isotope labelled internal standard (Fig. 1) was obtained from the Synthesis group of the Pre-Clinical Pharmacokinetics department of Johnson & Johnson Pharmaceutical Research and Development. The synthetic route is described in detail elsewhere [8]. The new bioanalytical method was validated according to the FDA guidelines [9].

2. Experimental

2.1. Chemicals and reagents

Galantamine and [$^{13}C^2H_3$]galantamine, used as the internal standard (I.S.) (Fig. 1), were obtained from Johnson & Johnson Pharmaceutical Research and Development (Beerse, Belgium). Toluene (Pestanal grade) was from Riedel-de Haën (Seelze, Germany), acetonitrile (for HPLC far UV, >99.9%) was from Acros Organics (Geel, Belgium) and methanol (UV spectrophotometric grade) was from J.T. Baker (Deventer, The Netherlands). BSA (albumin, bovine, lyophilized powder, fraction V, >96%) was from Sigma (St. Louis, MO, USA). Disodiumhydrogenphosphate (p.a.), potassiumdihydrogenphosphate (p.a.), sodiumhydroxide 0.1 *M* solution (p.a.), potassiumchloride (p.a.) and ammoniumacetate (Fractopur) were all obtained from Merck (Darmstadt, Germany). Control plasma was from Bio Media (Boussens, France). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standard and quality control samples

Stock solutions of galantamine and the I.S. were prepared in methanol (0.1 mg/ml) and stored at <−18 °C for a maximum period of 6 months.

Serial dilution in 2% BSA (prepared by dissolving BSA in a pH 7.5, 0.05 *M* phosphate buffer) was used to prepare calibration standard spiking solutions from the stock solution (range 2–1000 ng/ml). An I.S. spiking solution was prepared at a concentration of 50 ng/ml in 2% BSA. All spiking solutions were stored at 4 °C for a maximum period of 14 days.

Calibration standards were prepared daily by spiking 0.2-ml aliquots of blank, non-pooled human heparinized plasma with 100-μl aliquots of the calibration standard spiking solutions in 2% BSA, resulting in nine different concentrations (1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 100, 250 and 500 ng/ml).

Quality control (QC) samples were prepared in bulk from independently prepared methanolic solutions spiked to blank human plasma and stored at <-18 °C until analysis. The final concentration of methanol in these samples was less than 2%. QC samples were prepared at four different levels: at the lower level of quantification (LLOQ, 1.00 ng/ml), at the low level (<3 times LLOQ; 2.82 ng/ml), at the medium level (20.7 ng/ml) and at the high level (80–85% of the upper limit of quantification; 391 ng/ml).

2.3. Sample preparation

Aliquots of 0.2 ml of human heparinized plasma were spiked with 5 ng of internal standard and 100 μl of blank 2% BSA solution was added to match with the calibration samples. After adding 1 ml of NaOH 0.1 *M* and 0.5 ml of a saturated KCl solution (to prevent formation of emulsions), the samples were extracted (manually) with 5 ml of toluene by over-the-top mixing at 15 rpm for 10 min. After centrifugation at 2000 *g* for 10 min, the upper organic layer was removed and evaporated under nitrogen at 65 °C. The dry residue was dissolved in 200 μl of the HPLC solvent mixture.

2.4. LC–MS–MS

HPLC was performed on a Hewlett-Packard (Waldbronn, Germany) 1100 series instrument consisting of a vacuum degasser, quaternary pump and autosampler. The injection volume was 20 μl. The analytical column was a Waters (Milford, MA, USA) C_{18} Symmetry Shield 3.5 μm (4.6 mm I.D.×50 mm). The isocratic mobile phase was composed of 0.01 *M* ammoniumacetate–acetonitrile (85:15, v/v) and pumped at 1.5 ml/min. Separation was performed at ambient temperature. The chromatographic run time was 2 min, with a retention time of 1.12 and 1.10 min for galantamine and the internal standard, respectively. The column eluent was split at 100 μl/min to the mass spectrometer. A PE Sciex (Foster City, CA, USA) API 3000 triple quadrupole mass spectrometer, equipped with a TurboIonspray interface, was operated in the positive-ion mode. The ionspray needle was maintained at 4500 V. The turbo gas (nitrogen) temperature was 350 °C at a flow of 6.5 l/min. The nebulizing gas (air) was set at 12, the curtain gas (nitrogen) was set at 12 and the collision gas (nitrogen) was set at 3 (all arbitrary units). The orifice (OR) and ring (RNG) potentials were 31 and 230 V, respectively. The mass spectrometer was operated in SRM mode with a collision energy (Q0-RO2) of 32 V. Galantamine and the internal standard were monitored at transitions m/z 288.1→213.0 and 292.1→217.0, respectively, with a dwell time of 300 ms each. Data acquisition and processing was through Sample Control (version 1.4) and MacQuan (version 1.6) running on a Power Macintosh G4 workstation. A linear regression on log–log transformed axes was used to generate calibration curves from standards and to calculate the concentrations of quality control samples. These calculations were done by a purpose-built software program (JRF regression, version 2.0.5.).

3. Results and discussion

3.1. Mass spectrometry

The positive ion Ionspray Q1 mass spectrum and product ion mass spectrum of galantamine are shown in Fig. 2. The protonated molecular ion at m/z 288.1 was used as the precursor ion to obtain a product ion spectrum. The most intense precursor to fragment transition m/z 288.1→213.0 was selected for monitoring the compound. This fragment is probably formed by cleavage of the azepine ring, followed by loss of a water molecule. The proposed fragmentation pattern is displayed in Fig. 3.

3.2. Method development

The method to be developed had to be very robust, enabling the analysis of large numbers of samples from clinical trials with short run times that would not hamper the selectivity of the method.

The metabolism of galantamine played a predominant role in the strategy employed during the development phase. The original attempts to develop a mixed-mode solid-phase extraction method (using Bondelut Certify cartridges from Varian) failed because of the presence of an *N*-oxide metabolite in clinical samples (Fig. 1). Using SPE, this metabolite

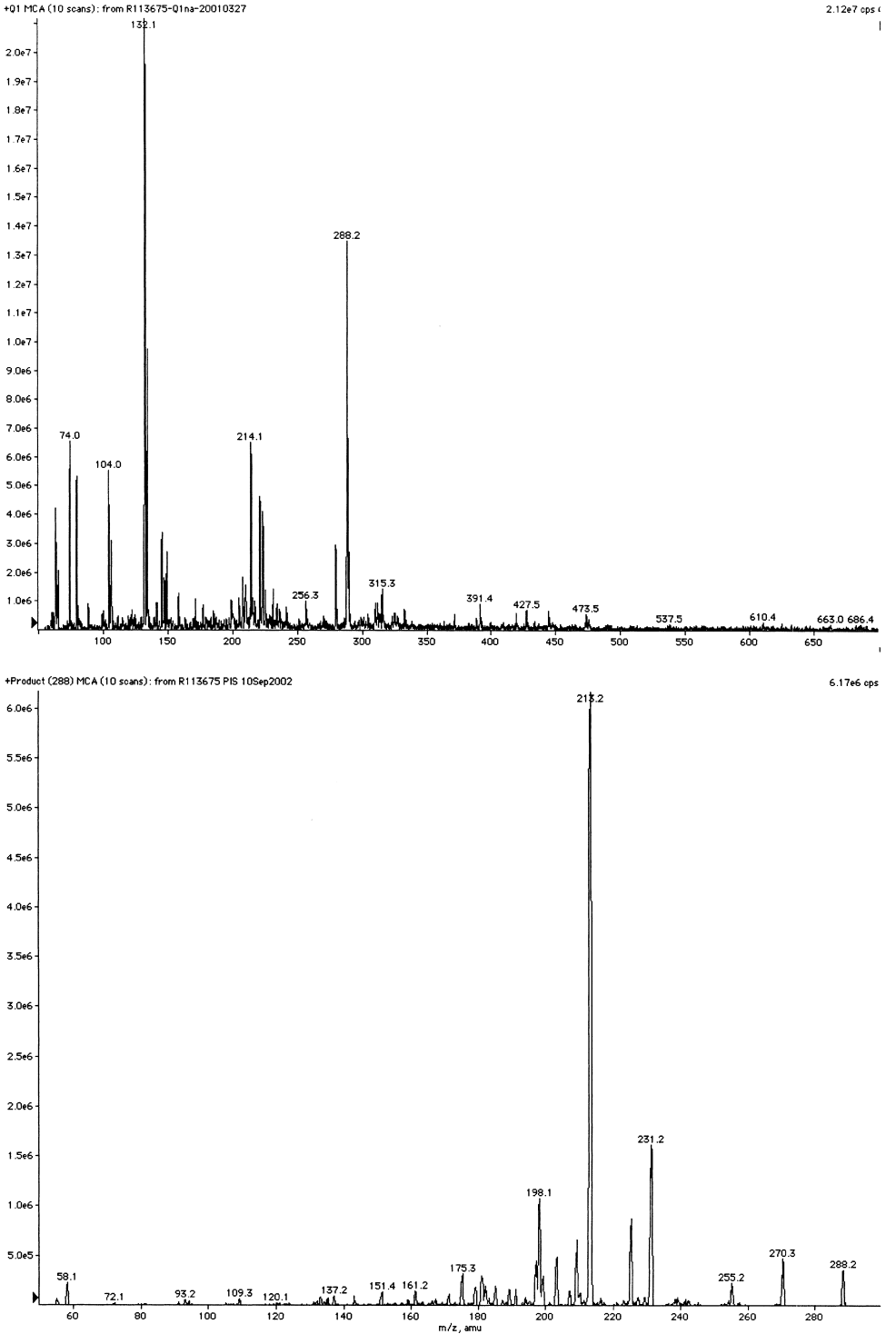


Fig. 2. Positive ion TurboIonspray Q1 mass spectrum (upper panel) and product ion mass spectrum (lower panel) for galantamine.

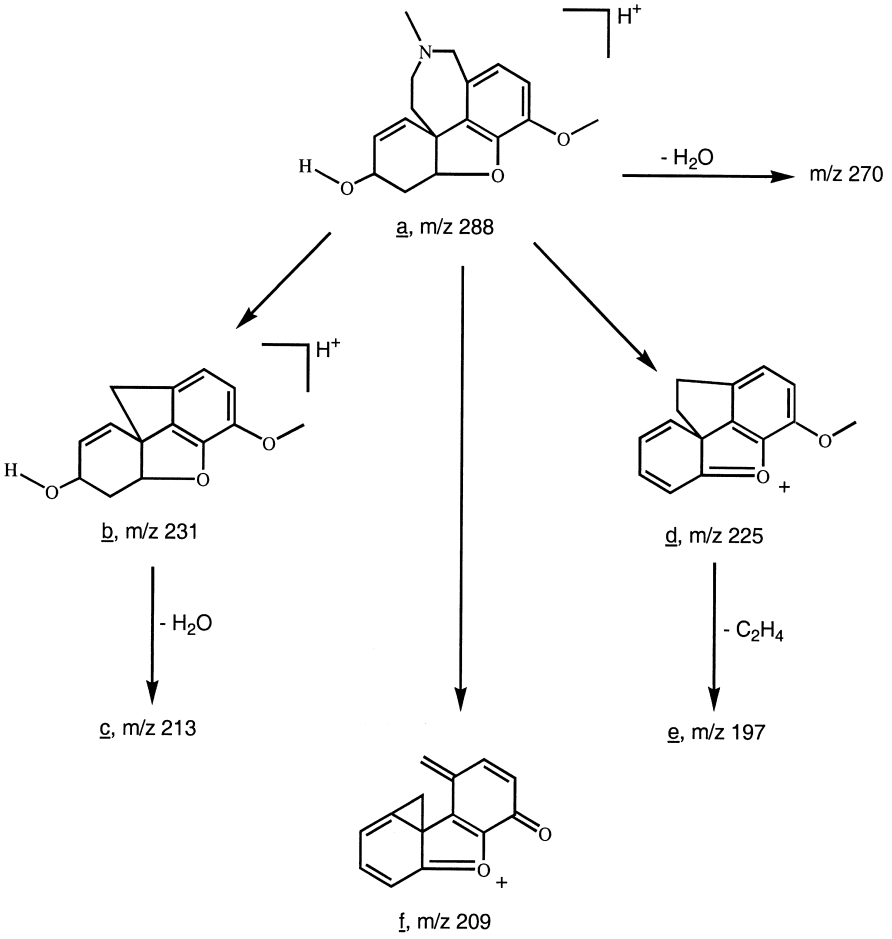


Fig. 3. Proposed fragmentation pattern for the protonated molecular ion of galantamine (from Ref. [6]).

is co-extracted and is partially (up to 15%) converted back to galantamine during the evaporation step. When using liquid–liquid extraction, however, this problem did not occur. Several conditions were tested by varying both pH and extraction solvent type. Best results were obtained by extraction with toluene at a pH of 13. Under these conditions the extraction recovery of the *N*-oxide metabolite was less than 0.1% while the recovery of galantamine was high (>90%). Epigalantamine, a stereoisomer of galantamine, also played an important role in the method development. Because both compounds have exactly the same mass transition, an adequate chromatographic separation was needed. Eight different column types were tested for optimal separation and peak shape. Best results were obtained with a Waters Symmetry Shield column. Fig. 4 shows a chromatogram of a dosed subject, illustrating the separation between galantamine and epigalantamine.

During the first validation batch, the QC samples at the LLOQ were overestimated by more than 20%. It was found that galantamine was adsorbing to the glass container wall in the methanolic solutions that were originally used for spiking the calibration lines. This problem was of course most important at the low end of the calibration line. This phenomenon did not occur in the QC samples, since these were prepared by spiking a large pool of plasma with more concentrated methanolic solutions. The adsorption problem was circumvented by using a 2% BSA solution in phosphate buffer for spiking the calibration standards. The albumin proteins form a

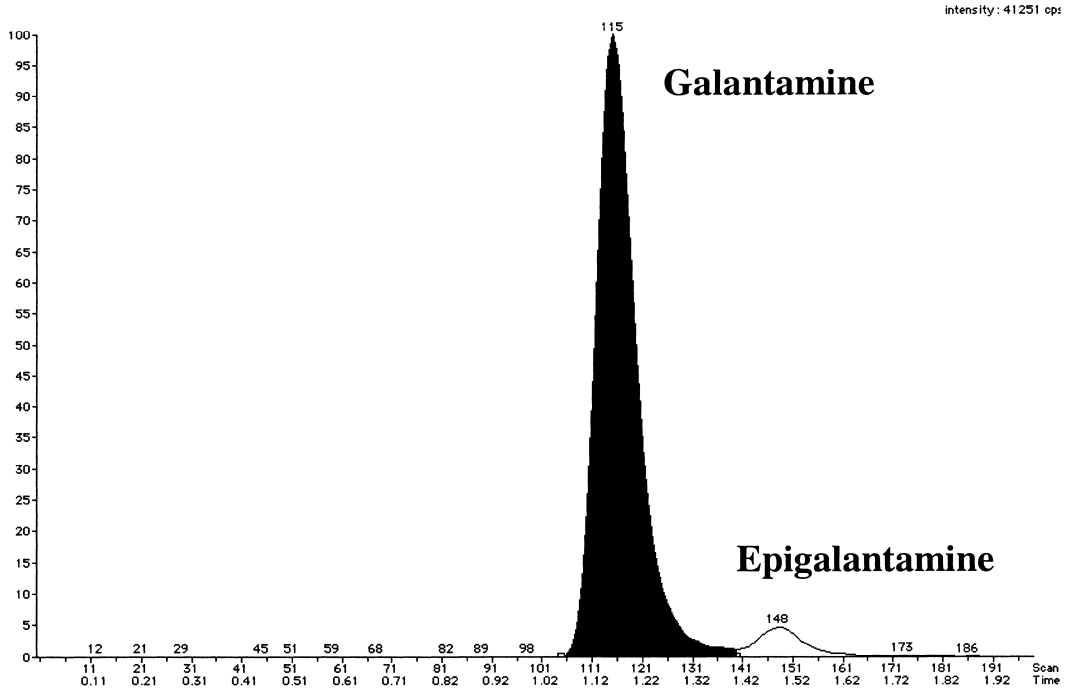


Fig. 4. Ion chromatogram from a dosed subject containing 78.8 ng/ml of galantamine, illustrating the separation between galantamine and epigalantamine.

coating on the glass container walls, thus preventing the adsorption of galantamine. As an alternative, preparing the methanolic spiking solutions in polystyrene plastic tubes was also examined, but this could not prevent adsorption. No further attempts were made to select other plastics as container material.

3.3. Validation experiments

3.3.1. Accuracy and precision

The accuracy and precision (both inter- and intra-batch) were determined from the results of the quality control samples. The QCs were analysed at four levels, in duplicate in five independently processed batches, each containing a calibration curve. Each calibration curve was prepared in a unique source of plasma that differed from the one used for preparing the QCs.

The results are displayed in Table 1. The overall precision ranged from 1.6 to 3.7% and the overall accuracy ranged from 100.6 to 105.5%. These excellent results also demonstrate the absence of matrix effects or the compensation for it by the internal standard.

3.3.2. Selectivity

Selectivity was evaluated by analysing blanks, zeros (blanks spiked with I.S.) and spiked samples at LLOQ level from six different sources of human plasma. For each source, the interference was evaluated by comparing the signal in the blanks with the signal of the corresponding LLOQ spiked samples.

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

Spiked conc. (ng/ml)	*n*	Inter-batch accuracy (%)	Inter-batch precision (% C.V.)
1.00	10	105.5	3.7
2.82	10	101.4	3.2
20.7	10	100.6	1.8
391	10	101.5	1.6

Chromatograms of a blank and a zero are shown in Fig. 5. For all six blanks the galantamine retention time region was free from endogenous interfering peaks, as could be expected given the high selectivity of MS–MS detection. For the zeros, a signal can be observed for galantamine, arising from the internal standard which contained about 0.3% of unlabelled material. Since the I.S. is spiked at a level of 25 ng/ml, this results in a contribution to the signal of galantamine of about 7% at the LLOQ level, which is acceptable.

3.3.3. LLOQ

The lower limit of quantification for galantamine was set at 1.00 ng/ml.

The LLOQ was determined from the LLOQ QCs, spiked samples at LLOQ level in six different sources of plasma and from 6-fold spiked samples at LLOQ level in one source of blank matrix. The results for the QCs at this level are shown in Table 1. Accuracy and precision were all within the generally accepted limits of 20% [9]. In Table 2 the results of the spiked samples in different plasma sources are displayed. The back-calculated values for the six different plasma sources were all within 7% of the spiked value, again demonstrating the absence of inter source variability in matrix effects. The within-source accuracy and precision (spiked in plasma source A) at this level were 106.0 and 4.0%, respectively.

3.3.4. Linearity

Calibration curves were constructed by applying a least-squares fit to the log-transformed peak area ratios versus the log-transformed concentrations. The calibration lines were linear over the range 1.00–500 ng/ml as was demonstrated by the slopes which were very close to unity (range 0.985–1.001) [10]. The linearity was evaluated by means of the back-calculated values of the calibration samples. The pooled results for the five calibration curves are shown in Table 3. All individual back-calculated values were well within 15% of the spiked value, the highest deviation being 6.7%.

Although FDA guidelines [9] state that the ''simplest'' regression model should be used to fit the regression curve, the question remains which model is the simplest. When a calibration range, spanning multiple orders of magnitude is needed, a simple linear regression model is not appropriate to achieve the acceptance criteria for the individual calibration points, especially at the lower end of the calibration range. This is due to the fact that for many analytical procedures, the condition of uniform variance along the calibration curve is not fulfilled (heteroscedasticity). This problem can be overcome in two ways: either by applying a weighting factor to each point that is inversely proportional to the variance at that point or by performing a transformation of the variables in such a way that homoscedasticity is obtained [11]. For historical reasons, we have always opted for the latter approach by doing a logarithmic transformation on the axes. A number of years ago, a comparison was done in our laboratory on historical data from GC–NPD, GC–MS, HPLC-UV, HPLC-fluorescence and LC–MS–MS methods, using different regression algorithms with different weighting factors. The conclusion was that the log–log model is at least equal to a linear regression model of the form $y = ax + b$ with $1/x^2$ as the weighting factor. In this test, the mean percent deviation of the back-calculated values of the calibrators was used to evaluate the fits.

Fig. 5 shows an example of a chromatogram of a calibration sample at the lower limit of quantification. The peak shape was quite good, illustrating the progress made in column manufacturing. In former methods, additives like dibutylamine [5] or diethylamine [6,7] were added to the mobile phase in order to get an acceptable peak shape whereas in the current method, such an additive was not needed.

3.3.5. Extraction recovery

The extraction recoveries of galantamine and the I.S. were determined by comparing the peak area for plasma samples that were spiked before and after extraction. The extraction recovery for galantamine was constant over the entire calibration range with average recoveries of 97.6, 93.8 and 92.1% at 2.50, 25.0 and 250 ng/ml, respectively. The extraction recovery of the I.S. was 93.3% at 25.0 ng/ml.

3.3.6. Stability

The stability of galantamine in the methanolic stock solution and 2% BSA spiking solutions was evaluated at a concentration of 20 μg/ml by compar-

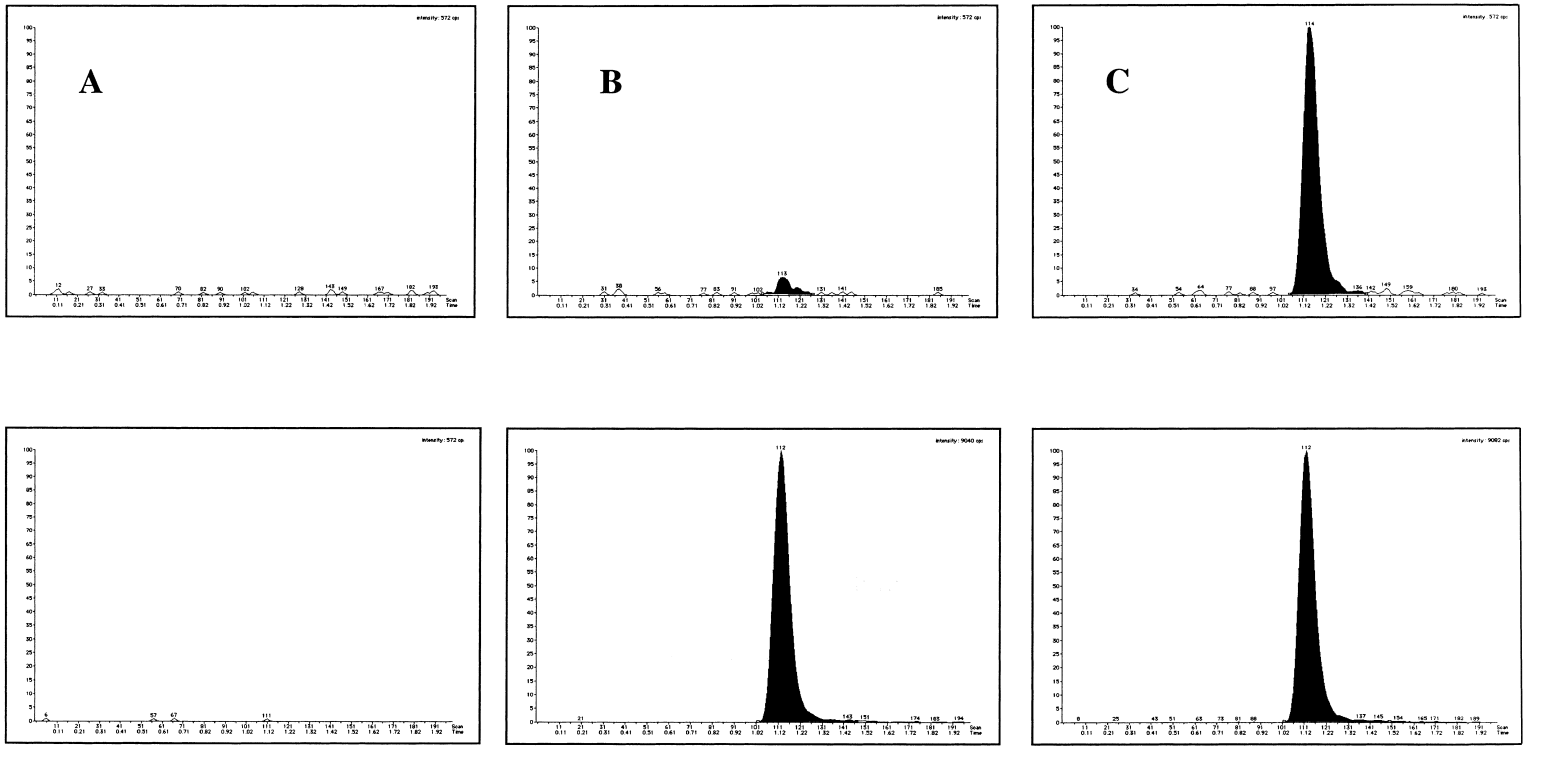


Fig. 5. Representative ion chromatograms displaying (A) blank plasma, (B) blank plasma spiked with 25 ng/ml of I.S., (C) blank plasma spiked with 1.0 ng.ml of galantamine and 25 ng/ml of I.S. The upper panel shows the galantamine trace, the lower panel is the I.S trace.

Table 2
Evaluation of the lower limit of quantification: six different sources of plasma spiked with galantamine at 1.00 ng/ml

Spiked (ng/ml)	Plasma source	Found (ng/ml)	% Accuracy
1.00	A	1.03	103.0
	B	0.992	99.2
	C	1.04	104.0
	D	1.07	107.0
	E	1.07	107.0
	F	1.01	101.0
Mean		1.04	
% C.V.		3.0	
% Accuracy		103.5	

ing the peak areas of the aged versus freshly prepared solution using a generic HPLC method with UV detection. Stability was considered proven if the peak area of the aged solution was within 10.0% of the peak area for the fresh solution. The compound was stable in methanol after storage for 6 months at −20 °C, 1 month at 4 °C and 3 days at room temperature, both in the dark and exposed to light. Galantamine was also stable in a 2% BSA solution after 14 days at 4 °C and 3 days at room temperature, both in the dark and exposed to light.

The stability of the extracted samples was determined by reinjecting six QC samples and six study samples and calculating the results by using the parameters of the original calibration curve. These results were compared to the results obtained from the first injection. If on average, the measured concentrations did not differ by more than 15. 0% between the two injections, stability was proven. This experiment proved that the extracted samples are stable for at least 4 days.

Galantamine was stable in human plasma after four freeze–thaw cycles and after 72 h at room temperature and at least 308 days at −18 °C. This was examined at two concentrations (5.01 and 391 ng/ml). The concentrations found (determination in triplicate) were compared to the nominal concentrations. To prove stability, the average concentration of the three replicates had to be within 15.0% of the nominal concentration.

Finally, the compound proved stable in blood (tested at 5.04 and 237 ng/ml blood in triplicate) after storage at room temperature for up to 4 h, at 4 °C for up to 72 h and at 37 °C for 2 h. The stability was evaluated by comparison with the plasma concentrations of reference blood that was spun off immediately after spiking. The average concentration of the three replicates had to be within 10.0% of the average concentration of the replicates of the reference sample in order to prove stability.

3.4. Method application

The method was successfully used to analyse over 3000 clinical samples. Fig. 6 shows plasma concentration–time profiles after multiple dosing of galantamine at three different dose levels for a healthy volunteer. Although the method involves a manual extraction step, the throughput was acceptable. During a recent bioequivalence trial, 750 samples were analysed by two lab technicians within 4 working days.

Table 3
Accuracy and precision for the five calibration curves

Spiked (ng/ml)	Found (ng/ml)	Accuracy (%)	Precision (% C.V.)	*n*
1.00	1.03	103.0	3.1	5
2.50	2.48	99.3	1.2	4
5.00	4.97	99.4	2.9	5
10.0	9.77	97.7	1.0	5
25.0	24.8	99.0	1.7	5
50.0	50.2	100.4	2.1	5
100	98.9	98.9	1.9	5
250	252	100.6	1.2	5
500	508	101.6	1.9	5

4. Conclusions

A new LC–MS–MS method was developed and validated for the determination of galantamine in human plasma. The use of an isotopically labelled internal standard made the method very robust and enabled short chromatographic run times. Combined with the simple sample clean-up this allowed for a much higher throughput than the original HPLC-fluorescence method. An additional advantage was the reduction of the amount of plasma required from 1.0 to 0.2 ml.

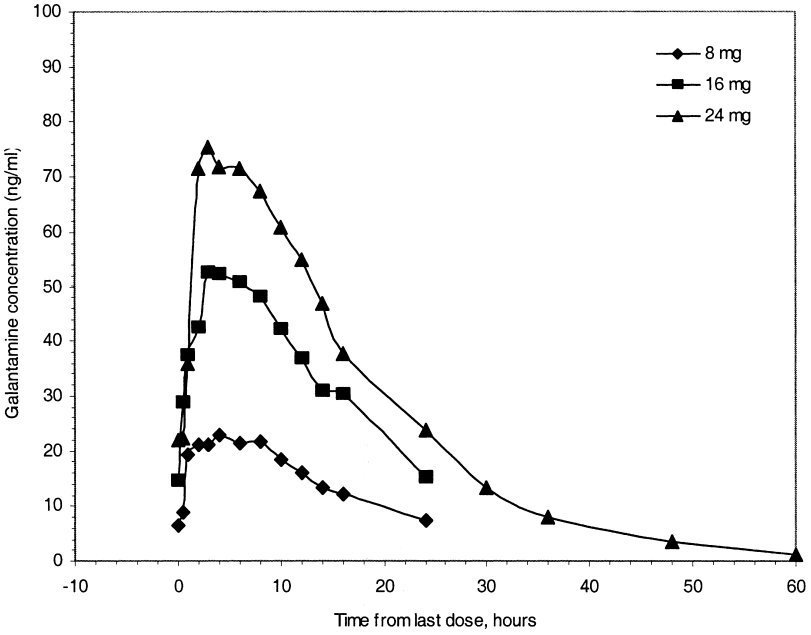


Fig. 6. Individual plasma concentration–time profile of galantamine after multiple doses of 8, 16, and 24 mg once daily of a galantamine controlled release capsule.

Acknowledgements

The authors would like to express their gratitude to Dr. Cor Janssen and collaborators (Synthesis group of the Pre-Clinical Pharmacokinetics department of Johnson & Johnson Pharmaceutical Research and Development) for the synthesis of the stable isotope labelled internal standard.

References

[1] V.D. Tonkopii, V.B. Prozorovskii, I.M. Suslova, Bull. Exp. Biol. Med. 82 (1976) 1180.
[2] A. Maelicke, E.X. Albuquerque, Eur. J. Pharmacol. 393 (2000) 165.
[3] G.K. Wilcock, S. Lilienfield, E. Gaens, Br. Med. J. 321 (2000) 1.
[4] H.A. Claessens, M. Van Thiel, P. Westra, A.M. Soeterboek, J. Chromatogr. 275 (1983) 345.
[5] J. Tencheva, I. Yamboliev, Z. Zhivkova, J. Chromatogr. 421 (1987) 396.
[6] G.S.J. Mannens, C.A.W. Snel, J. Hendrickx, T. Verhaeghe, L. Le Jeune, W. Bode, L. van Beijsterveld, K. Lavrijsen, J. Leempoels, N. Van Osselaer, A. Van Peer, W. Meuldermans, Drug Metab. Dispos. 30 (2002) 553.
[7] Q. Zhao, G.R. Lyer, T. Verhaeghe, L. Truyen, J. Clin. Pharmacol. 42 (2002) 428.
[8] C.G.M. Janssen, J.B.A. Thijssen, W.L.M. Verluyten, J. Label. Comp. Radiopharm. 45 (2002) 841.
[9] Guidance for Industry-Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug administration, Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM), May 2001
[10] C.A. Dorschel, J.L. Ekmanis, J.E. Oberholtzer, F.V. Warren, B.A. Bidlingmeyer, Anal. Chem. 61 (1989) 951A.
[11] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Data handling in science and technology, in: Chemometrics: A Textbook, Vol. 2, Elsevier, Amsterdam, 1988, p. 84, Ch. 5.