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Quantitative determination of rivastigmine and its major metabolite in human plasma by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry

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Abstract

A sensitive, specific, accurate and reproducible LC–MS–MS method was developed and validated for the simultaneous determination of rivastigmine and its major metabolite NAP 226-90 in human plasma according to International Regulatory Requirements. After addition of their respective labelled internal standards, the compounds were extracted from plasma using methyl-*tert*.-butyl ether at basic pH with a simultaneous derivatization of NAP 226-90 with propionic anhydride, and backextracted into an acidic solution. After re-extraction the compounds were analyzed on a 3-μm Purospher Star RP-18 column interfaced with a MDS Sciex API 3000 triple quadrupole mass spectrometer. Positive atmospheric chemical ionization was employed as the ionization source. The analytes and their internal standards were detected by use of multiple reaction monitoring mode. Intra- and inter-day accuracy and precision were found suitable over the range of concentrations between 0.200 and 30.0 ng/ml. The LC–MS–MS method was crossvalidated with a previously developed in-house GC–MS method by the analysis of plasma samples obtained from patients after administration of Exelon® capsules and showed excellent correlation between the methods.

Keywords: Rivastigmine; NAP

1. Introduction

Rivastigmine (ENA713, Exelon®), (*S*)-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methyl-phenylcarbamatehydrogentartrate, is an acethylcholinesterase inhibitor of the carbamate type approved for the treatment of Alzheimer's disease. Prior to elimination, rivastigmine is rapidly and extensively metabolized, principally via esterase-mediated hydrolysis of the carbamate moiety, to the decarbamylated metabolite, NAP 226-90.

A previous gas chromatographic–mass spectrometric (GC–MS) method which was developed in-house and validated according to Guidelines for analytical method establishment [1] was used to determine rivastigmine and its major metabolite in toxicokinetic, preclinical and clinical studies with a

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limit of quantitation of 0.200 ng/ml for both compounds [2–5].

Our goal was to modify the previous method

- to reduce the volume of plasma (1 ml in GC–MS) without modifying the LLOQ
- to reduce the time of analysis (injection every 20 min in GC–MS).

The described LC–MS–MS method was validated according to Guidance for Bioanalytical Method Validation [6,7] including determination of matrix effect, selectivity, accuracy, precision, recovery, calibration curve, stability of analyte in spiked samples, stability over freeze–thaw cycles and reinjection reproducibility. A crossvalidation with the GC–MS method is also reported.

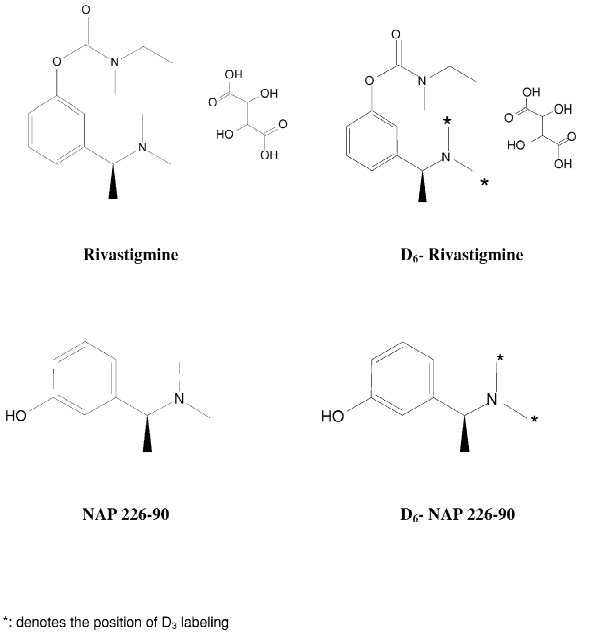

Fig. 1. Chemical structures of rivastigmine, NAP 226-90 and their respective labelled internal standards.

2. Experimental

2.1. Chemicals and reagents

The chemical structures of rivastigmine, its major metabolite NAP 226-90 and their corresponding deuterium-labelled internal standards are shown in Fig. 1; they were supplied by Novartis (Basle, Switzerland).

All the chemicals were of analytical grade: methyl-*tert*.-butyl ether (MTBE, ref. 20247), physostigmine hemisulfate (ref. E8625) and propionic anhydride (ref. 81942) from Sigma–Fluka (Saint-Quentin, Fallavier, France), 0.1 *M* hydrochloric acid (Titrisol 109973), sodium hydroxide (ref. 106469) and sodium carbonate (ref. 106392) were from Merck (Darmstadt, Germany).

Drug-free human plasma was obtained from Les Etablissements Français du Sang (Rungis, France) where blood was collected from volunteers into tubes containing heparin. After centrifugation, the plasma was stored at −18 °C.

The enzymatic cholinesterase activity of the biological material, which leads to an in vitro hydrolysis of rivastigmine to NAP 226-90, was competitively inhibited for analytical purposes by addition of 10 μl of 0.01 *M* physostigmine hemisulfate (eserine) solution for every 1 ml of plasma collected and used for the preparation of calibration and quality control samples.

2.2. Standard solutions

The stock solution of rivastigmine was prepared by dissolving 1.60 mg of substance in 10 ml of methanol–10^{-4} *M* hydrochloric acid (1:9, v/v). The stock solution of NAP 226-90 was prepared by dissolving 1 mg of substance in 10 ml methanol–10^{-4} *M* hydrochloric acid (1:9, v/v). Appropriate serial dilutions of the stock solutions with water were then made in order to prepare the spiking solutions to be used for calibration samples, at concentrations ranging from 0.200 to 30.0 ng/ml of rivastigmine base and NAP 226-90.

The I.S. stock solutions were prepared by dissolving 1.60 mg of d_6-rivastigmine and 1 mg of d_6-NAP 226-90 in 10 ml of methanol–10^{-4} *M* hydrochloric acid (1:9, v/v). Further dilution of the stock solutions with water resulted in the internal standards spiking solution (10 ng/50 μl).

All the solutions were stored at about +5 °C for 1 month.

2.3. Equipment

2.3.1. Chromatography

The HPLC system consisted of two Perkin-Elmer (Les Ulis, France) Micropums 200 with a autosampler Perkin-Elmer series 200. Chromatographic separations were performed on a 3-μm Purospher Star RP-18 (Merck) column (55×2 mm I.D.) operated at ambient temperature. The mobile phase of methanol–0.02 *M* ammonium acetate (55:45, v/v) was delivered at a flow-rate of 0.2 ml/min.

2.3.2. Mass spectrometry

The MS–MS system was a MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Les Ulis, France) equipped with an APCI interface used to generate positive ion $[M+H]^+$. The vaporizer temperature was set at 350 °C. Operating conditions were optimized by flow injection of a mixture of all analytes and were determined as follows: nebulizing gas pressure, 100 p.s.i. (1 p.s.i.= 6894.76 Pa); auxiliary gas pressure, 50 p.s.i.; curtain gas pressure, 55 p.s.i.; nebulizing current, 4 μA; orifice voltage, 31 V; ring voltage 220 V; collision gas (nitrogen) flow, 4. Unit resolution (at half peak height) was used for both Q1 and Q3. Multiple reaction monitoring (MRM) in the positive ionization mode was performed using a dwell time of 500 ms per transition to detect ion pairs at *m/z* 251/206 (rivastigmine), *m/z* 257/206 (d_6-rivastigmine), *m/z* 222/177 (NAP 226-90 propionate) and *m/z* 228/177 (d_6-NAP 226-90 propionate). The analytical data were processed by ANALYST software (version 1.1).

2.4. Calibration standards and quality control samples

For calibration standards, aliquots of working solutions were added to 0.5 ml of drug-free human plasma to produce reference samples in the range of concentrations 0.200–30.0 ng/ml for rivastigmine and NAP 226-90.

For quality control samples, aliquots of working solutions were added to 0.5 ml of drug-free human plasma to produce reference samples in the range of concentrations 0.200–25.0 ng/ml for rivastigmine and NAP 226-90.

For calibration standards and quality control samples, a constant amount of I.S. (10 ng/50 μl) was added to each sample.

2.5. Extraction from plasma

After addition of their respective labelled I.S.s, the compounds were extracted from plasma using MTBE at basic pH with a simultaneous derivatization of NAP 226-90 with propionic anhydride, backextracted in an acidic solution before re-extraction.

The procedure is detailed in Table 1.

3. Results and discussion

3.1. MS–MS development with derivatization of NAP 226-90

The ion efficiency was tested in APCI and TIS mode for rivastigmine and NAP 226-90.

Preliminary works have shown an important matrix effect and high baseline noise with TIS mode. These investigations led us to believe that NAP 226-90 could not be detected with sufficient sensitivity. Best conditions resulting in minimal baseline noise and maximal response were obtained in derivatizing the phenolic metabolite using propionic anhydride to give propionate derivative in yields suitable for LC–MS–MS.

3.2. Mass spectra

Precursor ions for rivastigmine, NAP 226-90 propionate and their respective internal standards were determined from spectra (at Q1) obtained during the infusion of neat solutions into mass spectrometer using APCI source, operating in the positive ionization mode with the collision gas off. Under these conditions, the analytes yielded predominantly protonated molecules at *m/z* 251, 257, 222 and 228 for rivastigmine, d_6-rivastigmine, NAP 226-90 propionate and d_6-NAP 226-90 propionate, respectively (Figs. 2 and 3).

Table 1
Description of the sample preparation

Extraction and derivatization	0.5 ml of plasma (treated with eserine 10 μl 0.01 *M* eserine/ml plasma) +50 μl of the working solution of the internal standards (10 ng) +0.5 ml of sodium hydroxide–sodium carbonate (0.7:0.5 *M*) +5 ml methyl-*tert*.-butyl ether (MTBE) +100 μl propionic anhydride Shaken mechanically for 15 min Centrifugation at 4000 rpm for 5 min The organic phase was transferred into another tube
Washing	Organic phase + 1 ml 0.1 *M* HCl Shaken mechanically for 5 min The aqueous phase was frozen by dipping the tube into dry ice The organic phase was discarded
Re-extraction	Aqueous phase + 0.1 ml of sodium hydroxide–sodium carbonate (0.7:0.5 *M*) + 5 ml MTBE Shaken mechanically for 15 min The aqueous phase was frozen by dipping the tube into dry ice The organic phase was evaporated to dryness under nitrogen at 40 °C
Dilution	The residue was reconstituted in 150 μl mobile phase
Injection	A 5-μl volume was injected onto the analytical column

By selecting these parents and scanning for daughter ions in Q3 after collision with nitrogen in Q2, both rivastigmine and d_6-rivastigmine gave the same daughter ion of m/z 206, both NAP 226-90 propionate and d_6-NAP 226-90 propionate gave the same daughter ions of m/z 177, suitable for quantification (Figs. 2 and 3).

3.3. Specificity

The specificity of the analytical method was investigated by preparing and analyzing blank samples prepared from six different batches of human plasma.

The specificity was assessed by comparing the apparent signal for Rivastigmine and NAP 226-90 propionate and for d_6-rivastigmine and d_6-NAP 226-90 propionate in blank samples to the mean signal obtained for samples ($n=3$) spiked with a concentration of Rivastigmine and NAP 226-90 at LLOQ (0.200 ng/ml) and d_6-rivastigmine and d_6-NAP 226-90 at the working concentration. The following criteria for specificity was used to assess the method suitability: interference in the blank at the retention of analyte should not exceed 20% of the response at the LLOQ and interference at the retention of I.S. should not exceed 5% of the response at the working concentration. The results are summarized in Table 2.

Representative selected ion current profiles from extracts of drug-free human plasma and of the same plasma spiked with Rivastigmine, NAP 226-90 and I.S. are shown in Figs. 4 and 5, respectively. Similar profiles were observed for six different batches of human plasma and the criteria mentioned were respected.

No crosstalk of rivastigmine and d_6-Rivastigmine (NAP 226-90 and d_6-NAP 226-90) and no interferences with endogenous compounds were detected in either case.

3.4. Matrix effect

The ion suppression caused by the plasma matrix was evaluated during the development of the analytical method. Use of the APCI mode considerably

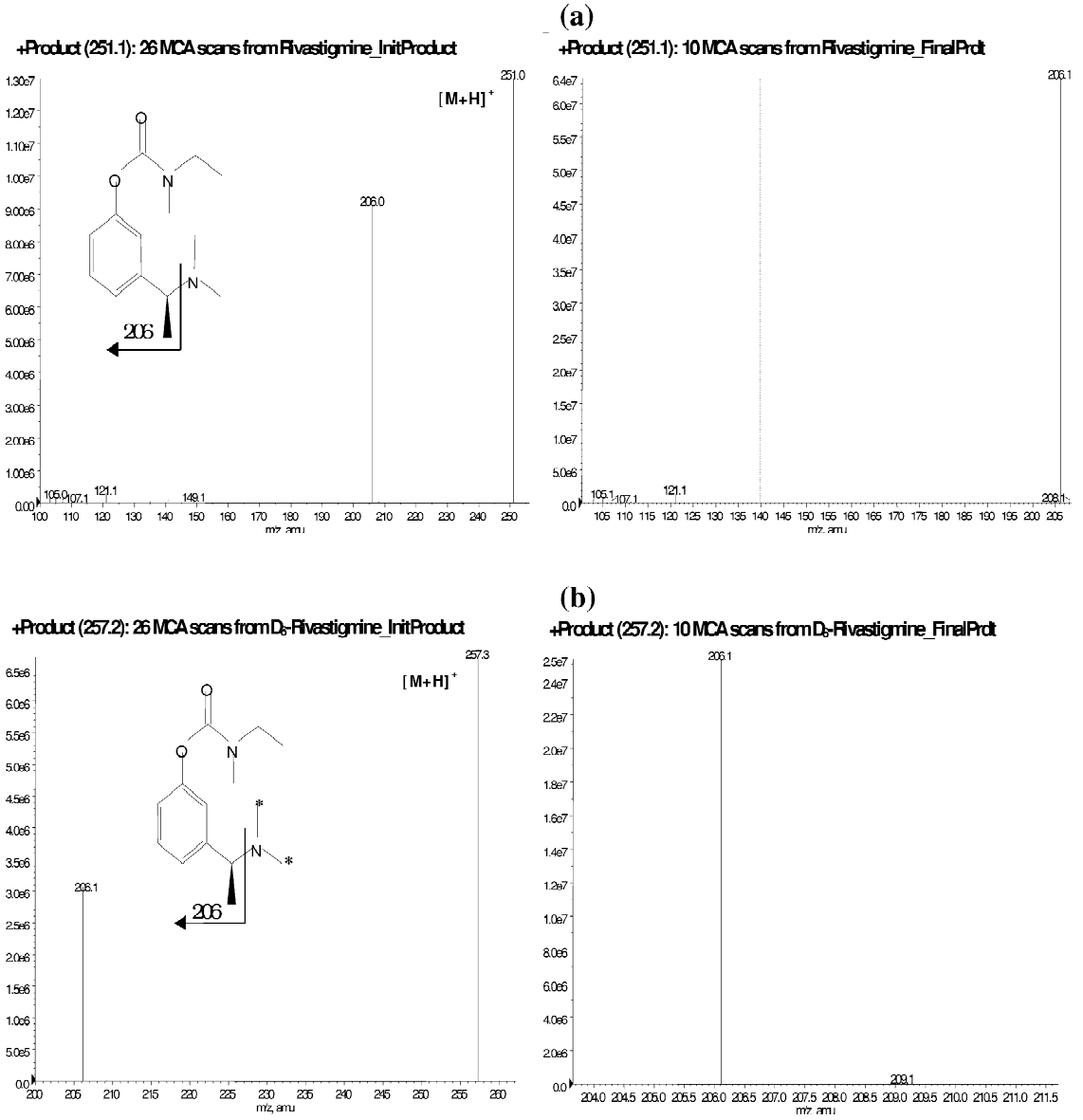

Fig. 2. MS and MS–MS mass spectra of (a) rivastigmine and (b) d_6-rivastigmine using APCI ionisation mode.

reduced the matrix effect and by eliminating from plasma extracts a number of endogenous components with additional washing and re-extraction steps during the sample preparation, the matrix effect was suppressed.

3.5. Extraction and ionisation recovery

The extraction yields from plasma were estimated at three different concentrations levels: 0.400, 5.00 and 25.0 ng/ml for rivastigmine and NAP 226-90.

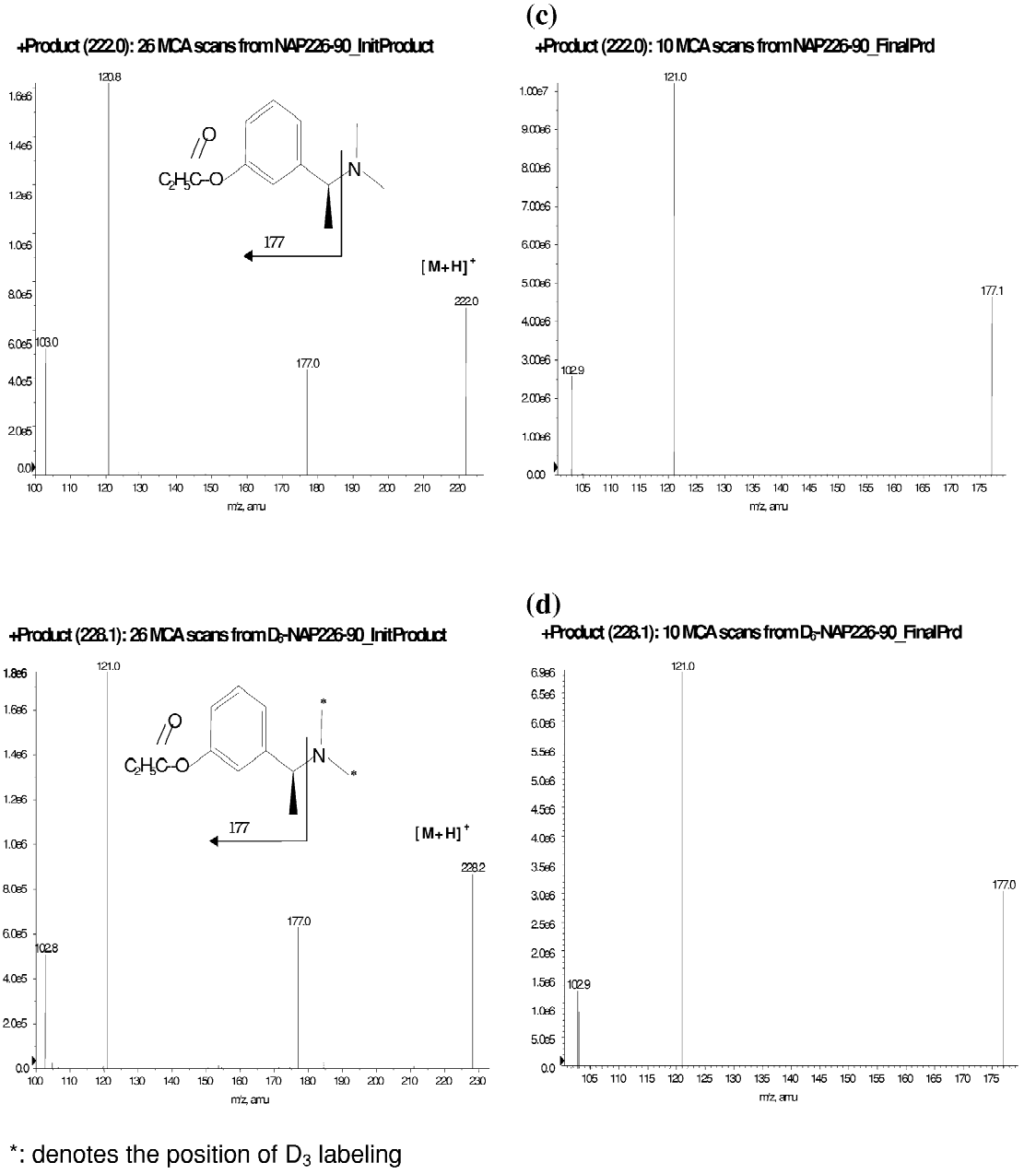

Fig. 3. MS and MS–MS mass spectra of (c) NAP 226-90 propionate and (d) d_6-NAP 226-90 propionate using APCI ionisation mode.

The extraction and ionisation yields were given as the ratio of mean peak area obtained from extracted human plasma sample and the mean of expected peak area from injections of derivatized reference solutions. The results are summarized in Table 3. The mean extraction efficiencies were 115 and 88% for rivastigmine and NAP 226-90, respectively. I.S. efficiencies estimated at the working concentration level (10 ng) were 116 and 86% for d_6-rivastigmine and d_6-NAP 226-90, respectively.

Table 2
Specificity of the analytical method

Batch (blank human plasma)	Response at LLOQ ($n=3$)				Response at working concentration ($n=3$)			
	3204 Rivastigmine		2531 NAP 226-90		232 943 d_6-Rivastigmine		240 118 d_6-NAP 226-90	
	Apparent response	Interference (%)	Apparent response	Interference (%)	Apparent response	Interference (%)	Apparent response	Interference (%)
67011041729	20	0.62	0	0.00	6	0.00	19	0.01
67011029164	36	1.12	27	1.07	8	0.00	37	0.02
67011064087	13	0.41	32	1.26	24	0.01	1	0.00
67011041331	16	0.50	0	0.00	12	0.01	8	0.00
67011041972	6	0.19	22	0.87	0	0.00	0	0.00
67010666348	45	1.40	29	1.15	23	0.01	23	0.00

3.6. Calibration curves

Daily calibration standards were prepared at six different concentrations in duplicate in the range 0.200 to 30.0 ng/ml for Rivastigmine and NAP 226-90. Calibration curves ($y = ax + b$) were represented by the plots of the peak area ratios (y) of Rivastigmine and NAP 226-90 to I.S. versus the concentrations (x) of the calibration standards and were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model. They correspond to the regression equation $y = 0.0708x + 0.00231$ with a correlation coefficient of 0.9998 for rivastigmine and $y = 0.0514x - 0.00099$ with a correlation coefficient of 0.9997 for NAP 226-90. Concentrations in quality control samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve.

Inter-day (inter-run) repeatability was determined on 3 different days. A good agreement between nominal and backcalculated concentration for calibration samples has been observed. Inter-day variability is presented in Table 4, the precision ranged from 1.4 to 8.5% and mean accuracies were within 2% of the nominal value for the two compounds.

3.7. Accuracy and precision

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest (0.200 ng/ml), near the lowest (0.400 ng/ml), near the middle (5.00 ng/ml) and the highest (25.0 ng/ml) concentration values of the calibration range. Accuracy was determined by calculating the mean recovery for the found concentrations as a percent of the nominal concentrations in standard samples. Precision was assessed from the relative standard deviation (RSD) expressed as a percentage of the mean recoveries. The following validation criteria for accuracy and precision were used to assess the method suitability: mean recoveries should be within 85–115% except at the LLOQ where it should be within 80–120%; the RSD value should not exceed 15%, except at the LLOQ where it should not exceed 20% [5,6].

A series of five quality control samples were prepared at four different concentrations in the range 0.200–25.0 ng/ml for rivastigmine and NAP 226-90, by spiking drug-free plasma with the corresponding working solutions.

As shown in Table 5, the results were satisfactory and met the acceptance criteria.

3.8. Lower limit of quantitation

The analyte response at LLOQ should be at least five times the response of interfering peaks at the retention time of the analytes of blank human plasma.

The LLOQ is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. As indicated in the previous section, the mean recovery should be within 80–120% of the expected value with a RSD not exceeding 20%. The lowest concentration value of 0.200 ng/ml, whose accuracy and precision (Table 5) were within the proposed criteria,

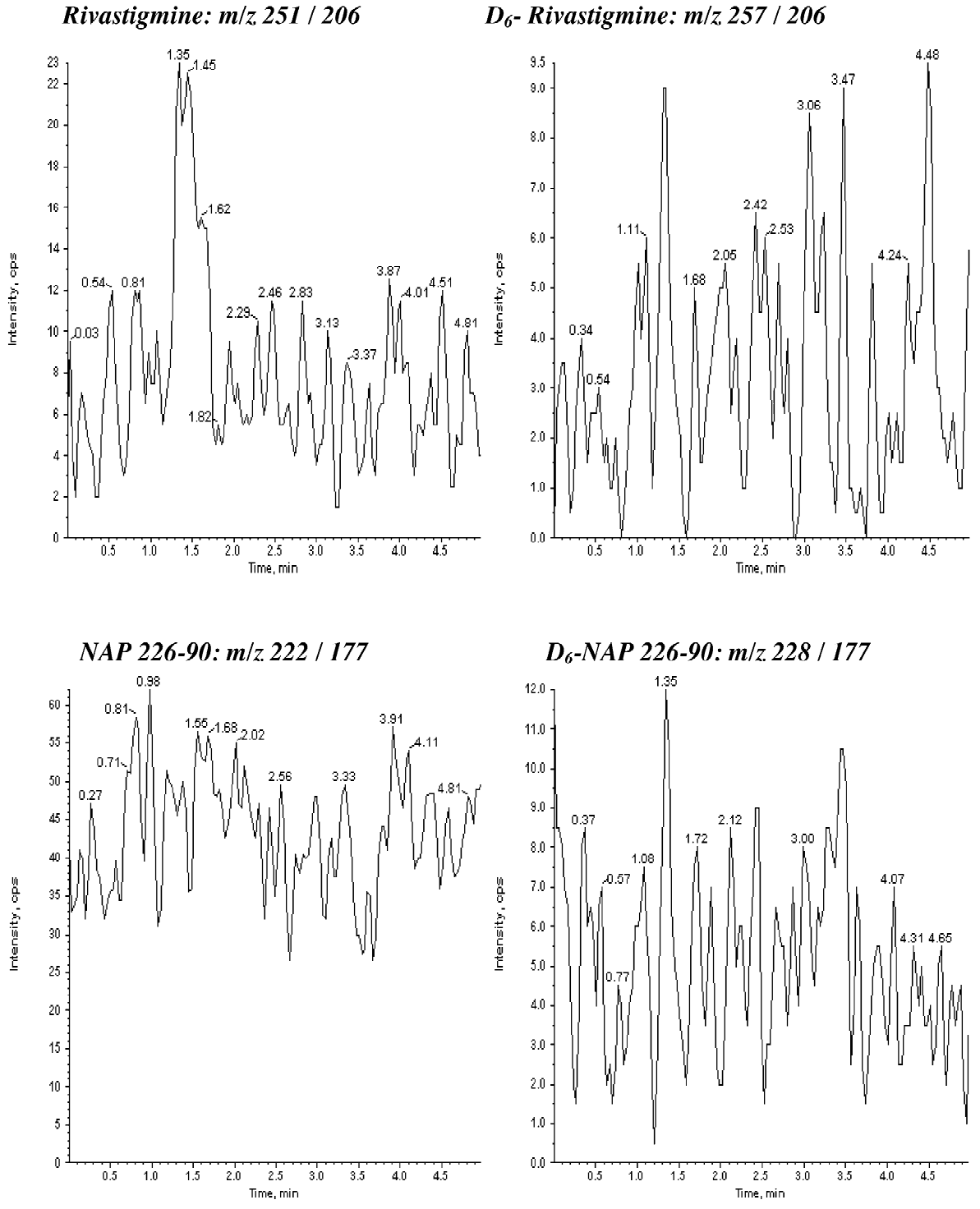

Fig. 4. Examples of selected ion current profiles of an extract of 0.5 ml drug free human plasma.

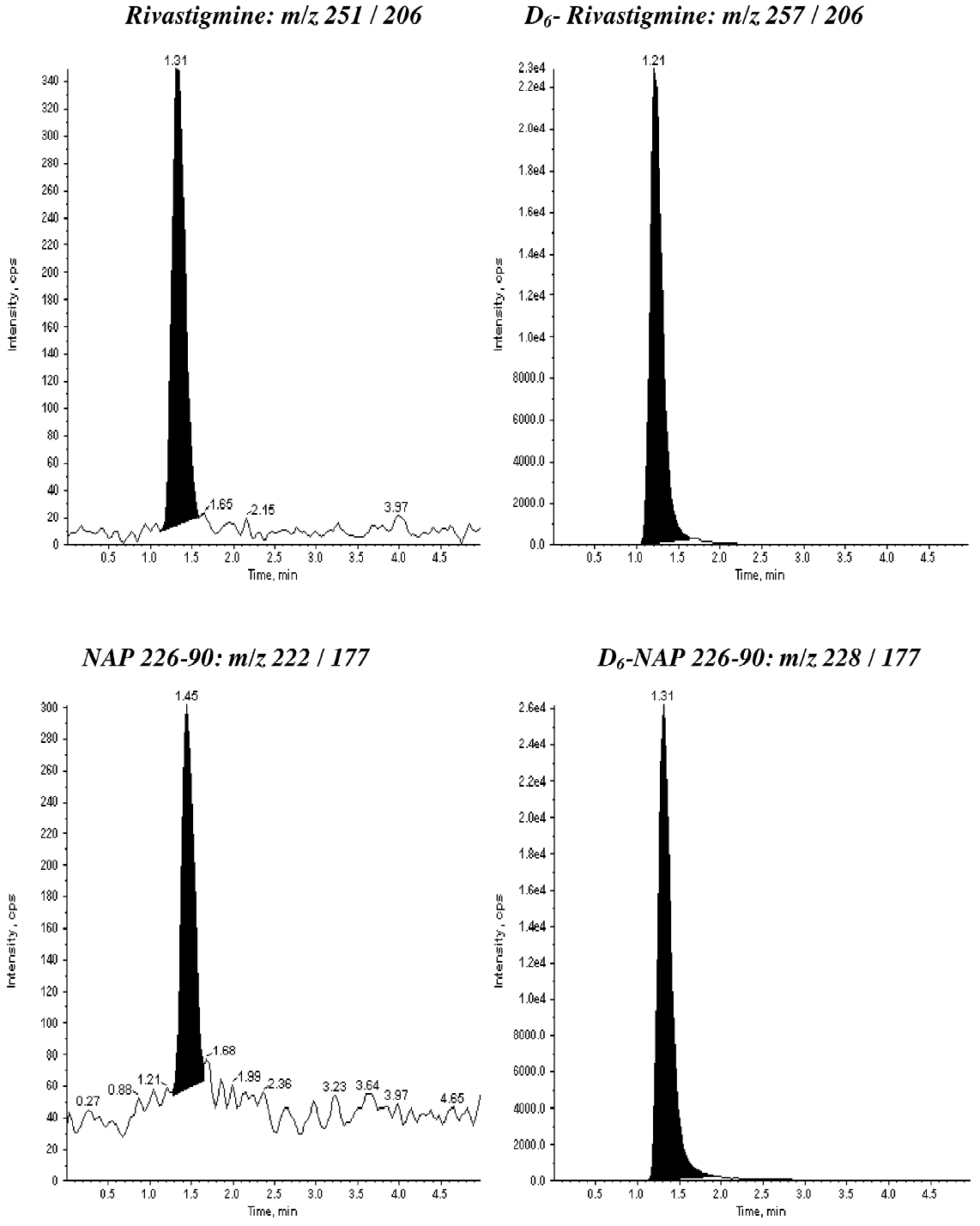

Fig. 5. Examples of selected ion current profiles: an extract of 0.5 ml plasma spiked with 0.100 ng (LLOQ) of rivastigmine and NAP 226-90 and 10 ng of labelled internal standards.

Table 3
Extraction and ionisation recovery

	Compound						Internal standard	
	Rivastigmine			NAP 226-90			d_6-Rivastigmine	d_6-NAP 226-90
Concentration (ng/ml)	0.400	5.00	25.0	0.400	5.00	25.0	10.0	10.0
Mean peak area[a]	6068	73 567	375 019	5337	59 042	319 602	217 881	255 710
Expected peak area[a]	5353	62 109	331 520	5769	70 528	364 275	188 406	296 967
Recovery (%)	113	118	113	93	84	88	116	86
Mean recovery (%)	115			88				

[a] $n=4$ for compounds and 12 for I.S.

Table 4
Backcalculated concentrations of calibration curves

Nominal concentration (ng/ml)	0.200	0.500	2.00	10.0	20.0	30.0
Rivastigmine						
Mean accuracy[a] (%), $n=6$	99.4	102	99.9	99.6	100	99.5
Precision[b] (%)	7.5	2.8	2.1	1.6	1.7	1.8
NAP 226-90						
Mean accuracy[a] (%), $n=6$	100	99.4	101	100	99.5	100
Precision[b] (%)	8.5	4.3	3.0	3.7	2.2	1.4

[a] Accuracy, found concentration expressed as percentage of the nominal concentration.
[b] RSD, relative standard deviation (%).

is quoted as the LLOQ for both analytes.

3.9. Stability

Stock solution were found stable at least 1 month at +5 °C.

The stability of rivastigmine and NAP 226-90 was investigated by analyzing quality control human plasma samples and reconstituted extracts, which were stored under varying conditions, in triplicate at low (0.400 ng/ml) and high concentration (25.0 ng/ml), together with freshly prepared C standards and QC samples. The stability data are summarized in Table 6 with an acceptance criteria of mean accuracy in the range 85–115%.

At room temperature, rivastigmine and NAP 226-

Table 5
Intra- and inter-day accuracy and precision of the method

Measurements	Given (ng/ml)	Rivastigmine		NAP 226-90	
		Mean accuracy[a] $n=6$, (%)	Precision RSD[b] (%)	Mean accuracy[a] $n=6$, (%)	Precision RSD[b] (%)
Intra-day	0.200	88.2	8.2	82.5	6.3
	0.400	91.3	2.8	87.8	5.2
	5.00	93.0	1.9	98.3	2.2
	25.0	94.6	1.2	97.7	1.3
Inter-day	0.200	90.6	8.7	96.2	11.8
	0.400	91.7	4.9	94.1	9.3
	5.00	93.7	3.7	99.0	5.3
	25.0	95.1	2.8	100	3.1

[a] Accuracy, found concentration expressed as percentage of the nominal concentration.
[b] RSD, relative standard deviation (%).

Table 6
Stability of rivastigmine and NAP 226-90 in the sample preparation and during storage

Storage period	Nominal concentration (ng/ml)	Mean accuracy (%)	
		Rivastigmine $n=3$	NAP 226-90
Stability in human plasma stored at room temperature			
4 h	0.400	98.1	98.5
	25.0	96.5	103
24 h	0.400	85.4	97.0
	25.0	90.0	97.4
Stability in extract on autosampler stored at room temperature			
1 h	0.400	88.0	88.0
	25.0	94.4	99.4
30 h	0.400	97.5	97.4
	25.0	100	107
Stability in spiked human plasma stored at or below −18 °C			
2.5 months	0.400	87.3	98.8
	5.00	95.8	92.0
	25.0	97.5	96.0
19 months	0.400	86.8	88.4
	8.00	94.8	101.0
	16.0	98.8	104.0

Effect of freeze–thaw cycles

Number of freeze–thaw cycles	Nominal concentration (ng/ml)	Mean accuracy (%)	
		Rivastigmine ($n=3$)	NAP 226-90
1	0.400	97.5	110
	25.0	93.0	101
2	0.400	85.4	86.0
	25.0	94.9	100
3	0.400	95.3	99.0
	25.0	95.8	97.9

90 were found to be stable for at least 24 h. Extracts (rivastigmine and NAP 226-90 propionate) were found stable on the autosampler at room temperature for at least 30 h. Rivastigmine and NAP 226-90 were found to be stable in human plasma after three freeze–thaw cycles. At or below −18 °C, in frozen human plasma rivastigmine and NAP 226-90 were found to be stable for at least 19 months in spiked and real samples.

3.10. Crossvalidation of the LC–MS–MS and GC–MS methods

The assays were crossvalidated by the analysis of 38 plasma samples obtained from one healthy subject at steady state after administration of 6 mg Exelon® IR capsule twice daily in clinical study. Six QCS were measured simultaneously on the same day with GC–MS and LC–MS–MS.

An example of the very similar pharmacokinetic profiles obtained in one patient with GC–MS and LC–MS–MS is depicted in Fig. 6 for rivastigmine and NAP 226-90.

Unweighted linear regression analysis of the data yielded an equation of $y = 1.050x + 0.1227$ for Rivastigmine and $y = 1.016x + 0.0148$ for NAP 226-90 where y and x were concentrations (ng/ml) obtained by LC–MS–MS and GC–MS analysis, respectively. The correlation coefficient (r) of 0.9983 for rivastigmine and 0.9936 for NAP 226-90 indi-

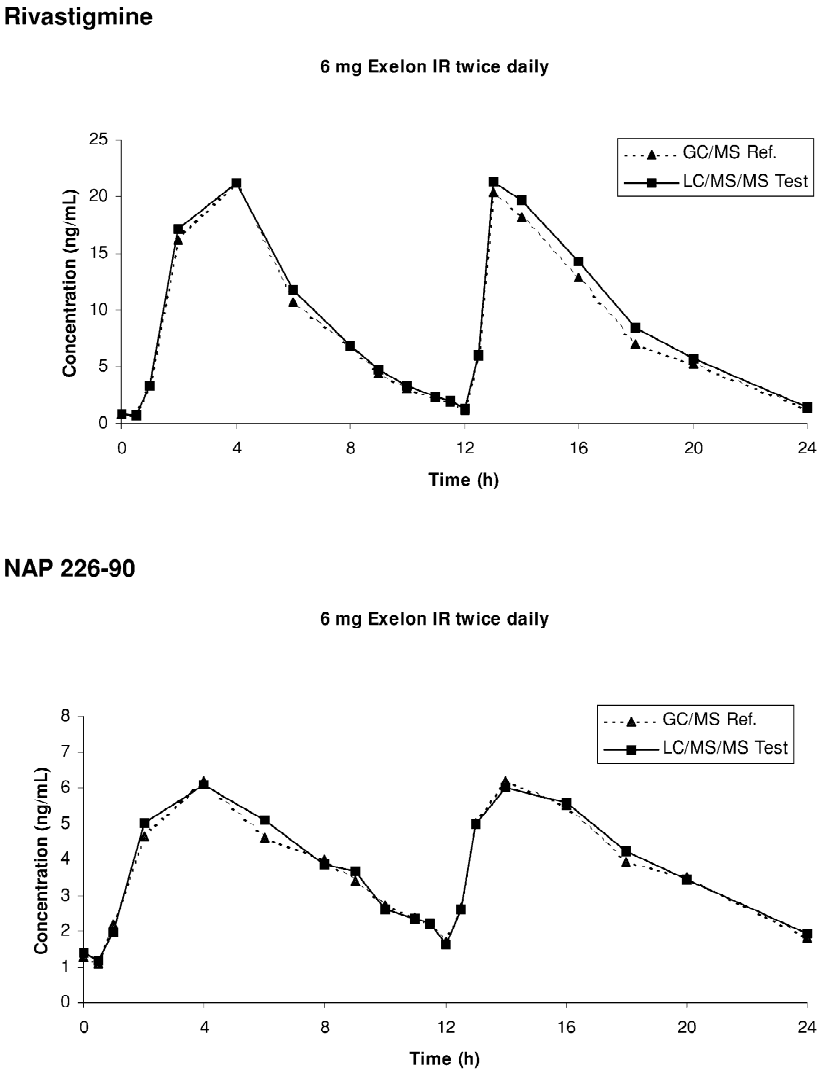

Fig. 6. Comparison of plasma concentration profiles for the determination of rivastigmine and NAP 226-90 by LC–MS–MS and GC–MS in one patient.

cated excellent correlation between the two analytical methods in the concentration range 0.6–21.0 ng/ml for ENA713 and 1–6.2 ng/ml for NAP 226-90, the LOQ being 0.2 ng/ml for both methods. The mean ratio [LC–MS–MS(test)/GC–MS(reference)] was 1.07 (RSD: 3.7%) for rivastigmine and 1.02 (RSD: 4.9%) for NAP 226-90.

4. Conclusions

A proposed LC–MS–MS technique was developed and validated for quantifying rivastigmine and its major metabolite NAP 226-90 in human plasma over the range 0.200–30.0 ng/ml according to internationally accepted criteria. Compared to the GC–MS method, the volume of plasma was halved and the time of analysis was reduced by four with injections every 5 min instead of every 20 min. Crossvalidation data performed by the analysis of actual plasma samples showed good correlation between GC–MS and LC–MS–MS method. Both methods yielded very similar pharmacokinetic profiles for rivastigmine and NAP 226-90 in man

following oral administration of Exelon. Thus, use of GC–MS and LC–MS–MS methods are interchangeable in the pharmacokinetic evaluation of rivastigmine and NAP 226-90. However, the LC–MS–MS method is now the preferred choice due to its faster chromatographic procedure and much higher sample throughput than the GC–MS method.

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