High performance liquid chromatography coupled with mass spectrometry for simultaneous determination of rivastigmine and its metabolite in rat plasma

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ABSTRACT

Several studies on the pharmacokinetic parameters of antidementia drugs have reported that plasma concentration is linked to the drugs’ efficacy and adverse effects. At present, there is no quantitation method that is highly sensitive and can be applied to simultaneous monitoring of the pharmacokinetics of rivastigmine and its metabolites (NAP 226-90) in rat plasma. No methods fulfilling the assay validation requirements of the US Food and Drug Administration and the European Medicines Agency was also established. Therefore, this study developed a quantitative method for measuring rivastigmine and NAP 226-90 concentrations using high-performance liquid chromatography and tandem mass spectrometry, examining plasma samples after rivastigmine administration. Rat plasma samples were prepared via the protein precipitation method. The methods for measuring rivastigmine and NAP 226-90 concentrations showed good fit over wide ranges of 1–100 ng mL⁻¹ and 0.5–50 ng mL⁻¹, with lower limits of quantification at 1 ng mL⁻¹ and 0.5 ng mL⁻¹, respectively. The plasma concentrations of rivastigmine and NAP 226-90 in six healthy rats were successfully determined, demonstrating the feasibility of applying the developed method. Thus, this research has successfully developed a sensitive, selective method, to simultaneously quantify rivastigmine and NAP 226-90 concentrations in rat plasma and be applicable to a pharmacokinetic study.

KEYWORDS

rivastigmine, NAP 226-90, tandem mass spectrometry, high performance liquid chromatography

INTRODUCTION

Rivastigmine is a cholinesterase inhibitor of the carbamate type approved for the treatment of Alzheimer’s disease (AD). Rivastigmine is rapidly and extensively metabolized, principally via esterase-mediated hydrolysis of the carbamate moiety, to NAP 226-90, which exerts pharmacological activities and is the major metabolite of rivastigmine detectable in both animals and humans [1, 2]. Rivastigmine is often prescribed to stabilize AD progression; it shows an additive effect and has proven to be the most effective therapy for AD patients with moderate to severe dementia [3, 4].

A quantitative method has been established to measure rat plasma concentrations of rivastigmine and NAP 226-90 [5]. This method has employed high-liquid chromatography (HPLC) combined with mass spectrometry using a solid-phase extraction (SPE), that requires
laborious extraction procedures involving time-consuming and error-prone solvent evaporation and reconstitution steps. This method is not highly sensitive for the determination of NAP 226-90 in plasma samples and cannot be applied for simultaneously monitoring of rivastigmine and NAP 226-90 pharmacokinetics as the range is very low: between 10–100 pmol mL$^{-1}$ (2.6–26 ng mL$^{-1}$). Additionally, no method for simultaneously measuring rivastigmine and NAP 226-90, while meeting the US Food and Drug Administration (FDA) [6] and the European Medicines Agency (EMA) [7] recommended criteria, is currently available. Therefore, it was necessary to develop a simple, and sensitive analytical method for the simultaneously quantification of rivastigmine and NAP 226-90 in rat plasma. Protein precipitation extraction via SPE and liquid-liquid extraction (LLE), including methanol and acetonitrile typically provide cleaner extracts with fewer matrix effects, minimum ion suppression, and a low propensity for building backpressure in the chromatographic column. The presented method is well applicable when studying plasma concentrations of NAP 226-90 following rivastigmine administration in rats.

This study, therefore, developed a high sensitive and selective quantitative method to simultaneously measure rivastigmine and NAP 226-90 in the plasma samples of healthy male rats via HPLC-MS/MS and used for pharmacokinetics. Our method will provide other alternatives for already existing LC-MS method with an advantage of higher resolution.

**EXPERIMENTAL**

**Chemicals and instrumentation**

Standard rivastigmine tartrate [3-[(1S)-1-(Dimethylamino)ethyl] phenyl N-ethyl-N-methylcarbamate tartrate, purity ≥ 98%; NAP 226-90 [3-[(1S)-1-(Dimethylamino)ethyl] phenol, purity ≥ 98%], and the isotopically labeled internal standard (IS) (rivastigmine-d$_4$, purity ≥ 98%) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Acetonitrile, methanol, and formic acid (99%) were purchased from Acros Organics (New Jersey, USA). Acetonitrile, methanol, ultrapure water, and formic acid were of LC-MS grade, and all other reagents were of analytical grade. Samples were analyzed using an Alliance 2695 System and a triple-stage quadrupole mass spectrometer (Qtrto microAPI Tandem Quadrupole System). The mass spectrometer was tuned automatically to rivastigmine, NAP 226-90, and the IS using the MassLynx V4.0 software package (Waters).

**Stock and working solutions**

Stock solutions of both rivastigmine and NAP 226-90, at concentrations of 0.5 mg mL$^{-1}$, were prepared by weighing them in separate measuring flasks and dissolving them in methanol. The quality control (QC) stock solution was prepared separately from the solution intended for the calibration curve. Stock solutions were stored at −20°C. The IS stock solution, at 0.5 mg mL$^{-1}$, was also prepared in methanol and stored at −20°C. It was then diluted with acetonitrile to give a single IS working solution of 100 ng mL$^{-1}$. Calibration solutions at concentrations of 1–100 ng mL$^{-1}$ and 0.5–50 ng mL$^{-1}$ for measuring rivastigmine and NAP 226-90, respectively, were prepared by diluting the calibration stock solution in 0.01 N hydrochloride (HCl). For both rivastigmine and NAP 226-90, QC solutions representing their lower limits of quantification (LLOQs), as well as low, medium, and high QC solutions, were prepared by diluting the QC stock solution with 0.01 N HCl to reach concentrations of 1, 3, 15, and 75 ng mL$^{-1}$ for rivastigmine and 0.5, 1.5, 7.5, and 37.5 ng mL$^{-1}$ for NAP 226-90.

**Sample preparation**

The plasma samples from healthy male rats were prepared via protein precipitation with acetonitrile. To measure rivastigmine and NAP 226-90 concentrations, the QC stock solution and blank plasma for calibration (90 μL total) were placed in a 1.5 mL polypropylene tube. Then, this mixture was added to 10 μL each of eight calibrating solutions – the LLOQ, low, medium, and high QC solutions for both rivastigmine and NAP 226-90, prepared as described in previous section followed by 300 μL of the IS (100 ng mL$^{-1}$) prepared in acetonitrile. Afterwards, the samples were vortex-mixed and centrifuged for 10 min at 14,000×g in a 0.5 mL polypropylene tube. The supernatants were transferred into 1.5 mL polypropylene tubes and evaporated to dryness (dinitrogen [N$_2$] flow, 40°C). The solid residues were reconstituted in 100 μL of the mobile phase at initial conditions (0.1% formic acid–acetonitrile 80:20, v/v), vortex-mixed, and again centrifuged for 10 min at 14,000×g. Finally, the supernatants (90 μL) were transferred into glass vials prior to being injected into the HPLC-MS/MS system.

**Liquid chromatography**

The analytes were chromatographically separated via an InertSustain C18 column (3.0 μm, 1.5 × 50 mm). The mobile phase used for this chromatographic separation consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile. The total analysis time was 5 min per injection, and the flow rate was 0.2 mL min$^{-1}$. The gradient program of mobile phase in the LC is shown in Table 1. The column and sample temperatures were kept at 20°C throughout all experiments. The injection volume for each sample was 10 μL.

**Mass spectrometry**

Samples were ionized using the following parameters: electrospray voltage: 3.5 keV; cone voltage: 25 eV; source

Table 1. The gradient program of mobile phase in the Liquid Chromatography

| Minutes | 0 | 1.7 | 2.0 | 3.0 | 4.0 | 5.0 |
|---------|---|-----|-----|-----|-----|-----|
| Solvent A (%) | 80 | 65 | 65 | 20 | 20 | 80 |
| Solvent B (%) | 20 | 35 | 35 | 80 | 80 | 20 |

Solvent A: 0.1% aqueous formic acid.
Solvent B: Acetonitrile.
temperature: 120 °C; cone gas flow (N₂): 50 L h⁻¹; desolvation gas flow (N₂): 600 L h⁻¹; and desolvation temperature: 350 °C. Selective reaction monitoring (SRM) was carried out using N₂ as a collision gas, and the MS/MS transitions monitored in the positive ion mode for rivastigmine, NAP 226-90, and the IS were m/z 251.0 → m/z 206.0 at 15 eV; m/z 166.0 → m/z 121.0 at 15 eV; and m/z 255.0 → m/z 206.0 at 15 eV, respectively. A dwell time of 200 msec was given for each transition.

Validation of the method

The validation procedure was carried out to determine whether the proposed method met the acceptance criteria for bioanalytical methods from the FDA [6] and the EMA [7].

Accuracy and precision

The LLOQs for rivastigmine and NAP 226-90 were the lowest analyte concentrations on the calibration curve that could be determined with precision < 20%, and accuracy < 40%. The response of the blank plasma sample should be at least five times lower than that of a spiked sample at the LLOQ. Three validation batches, each containing eight calibration and 24 QC samples at different concentrations (LLOQ, low, medium, and high QCs in sextuplicate preparation), were analyzed. Precision and accuracy were calculated and expressed as coefficient of variation (CV%), relative error (RE%), and total analytical error (%) for each analytical batch (within-run) and across the three validation batches (between-run).

Recovery rate

Blank plasma was spiked at three different QC concentrations and extracted (A). Additionally, three QCs were prepared in the extracted blank plasma (B). Theses samples were prepared in triplicate and subjected to HPLC-MS/MS. The peak areas of samples A and B were obtained, and the extraction recovery rate (%) was calculated as A/B × 100.

Matrix effect

The corresponding peak areas of the three QCs prepared in extracted blank plasma (B) and three other QCs diluted in matrix-free LC eluent (C) were obtained in triplicate. Then, the matrix effect (%) on ionization efficacy was calculated as B/C × 100.

Selectivity

Selectivity was evaluated by comparing chromatograms of the plasma samples obtained from six healthy male rats, without adding rivastigmine, NAP 226-90, or the IS. The baseline signals at the expected analyte retention times were evaluated as interfering peaks.

Linearity

The calibration samples and analyte-specific SRM quantifier transitions were used to construct calibration curves for the rivastigmine and NAP 226-90 measurements. Peak areas of the respective analytes were divided by those of the IS. Weighted linear regression (1/x) was performed for each analytical batch. Method linearity was validated by performing simple linear regression between the introduced concentrations and the back-calculated concentrations via the selected calibration curve.

Application of the HPLC-MS/MS method

The developed HPLC-MS/MS method was successfully applied to determine the plasma concentrations of rivastigmine and NAP 226-90, following intravenous rivastigmine administration, in healthy male Sprague Dawley rats (n = 6) (CLEA Japan, Inc., Japan). The animals were housed in temperature- and humidity-controlled rooms with a 12 h/12 h light/dark cycle. They were kept on a standard chow diet and were given free access to water. All animal experiments were carried out according to the guidelines of the Institutional Animal Ethical Committee (approval no: 2021-A-18) at Fukuyama University. Rivastigmine (1 mg kg⁻¹) was intraperitoneally administered as a suspension in saline to three of the sampled rats. This rivastigmine dose was equivalent to the clinical dose, calculated according to FDA guidelines for human-to-animal dose extrapolation. Blood samples were drawn from the jugular vein and collected in heparinized polypropylene tubes at 30 min, 60 min, 90 min, 180 min, 6 h, and 12 h post-dose under light pentobarbital anesthesia. After each blood sample was taken, the plasma was separated by centrifuging the blood at 2,000 × g for 20 min. The extracted plasma was kept frozen at −80 °C until analysis.

Plasma concentration-time profile and pharmacokinetics parameters of rivastigmine and NAP 226-90 were calculated by moment analysis. The pharmacokinetic parameters includes the area under the plasma concentration-time curve from zero to infinity (AUC₀-∞); the mean residence time (MRT); the terminal elimination half-life (t½); the distribution volume on steady state (Vdss) and total clearance (CL).

RESULTS AND DISCUSSIONS

Mass spectrometric and chromatographic characteristics

During MS tuning, [M+H]⁺ signals for rivastigmine and NAP 226-90 were more intensely detected in the positive ion mode. Figure 1 shows the precursor and product ion mass spectra of the [M+H]⁺ ion for rivastigmine and NAP 226-90. Sensitive detection via SRM was used to choose identical fragment ions for rivastigmine, NAP 226-90, and the IS. The identical fragment ions of rivastigmine and the IS were m/z 206.0, and NAP 226-90 was m/z 121.0, respectively.

The chromatographic conditions were optimized to rapidly and efficiently separate rivastigmine, NAP 226-90, and the IS from the plasma components. Figure 2 shows the chromatographic characteristics of rivastigmine, NAP 226-90, and the IS for blank plasma and for the LLOQs. Under optimized chromatographic conditions, the retention times of rivastigmine, NAP 226-90, and the IS were all about 1.3 min.
Method validation

No interfering peaks derived from the plasma matrix were observed in the plasma samples collected from the six rats, which indicated that the method had acceptable selectivity (Fig. 2). For the calibration curves, the regression coefficient ($r^2$) of the rivastigmine concentrations from 1 ng mL$^{-1}$ to 100 ng mL$^{-1}$ was $\geq 0.9982$, and that for the NAP 226-90 concentrations from 0.5 ng mL$^{-1}$ to 50 ng mL$^{-1}$ was $\geq 0.9983$, confirming the linearity of the method’s eight-point calibration curves. The LLOQs determined for rivastigmine and NAP 226-90 fulfilled the FDA and EMA criteria for both within-run and between-run accuracy (88.8% and 106.6%, respectively, for rivastigmine; 98.1% and 98.1%, respectively, for NAP 226-90) and precision ($\leq 14.60\%$ and $7.53\%$, respectively, for rivastigmine; $\leq 11.31\%$ and $4.53\%$, respectively for NAP 226-90) (Tables 2 and 3). All analyte signals of the LLOQs were at least five times higher than the signals detected in plasma samples without rivastigmine and NAP 226-90. Tables 2 and 3 present within-run and between-run accuracy and precision data for rivastigmine and NAP 226-90 measurement. In the rivastigmine measurements, the within-run the accuracy for the three QCs was 101.1–107.8%; and the precision was 4.34–11.27%. The between-run the accuracy was 103.2–114.9%; and the precision was 1.66–7.56%. For the NAP 226-90 measurements, the within-batch the accuracy for the three QCs was 99.1–105.8%; and the precision was 99.1–105.8%. The between-run the accuracy was 96.7–102.1%; and the precision was 3.44–7.78%.

The rivastigmine extraction recovery rates from the rat plasma in the low, medium, and high QCs were 107.4% ± 12.03%, 101.2% ± 9.82%, and 103.6% ± 3.47% (mean ± standard deviation), respectively. The NAP 226-90 extraction recovery rates from the rat plasma in the low, medium, and high QCs were 109.8% ± 8.05%, 102.1% ± 10.23%, and 91.9% ± 11.62%, respectively. The matrix effects of rat plasma for rivastigmine in the low, medium, and high QCs were 87.3% ± 12.04%, 95.4% ± 8.88%, and 103.8% ± 3.47%, respectively. The matrix effects of rat plasma for NAP 226-90 in the low, medium, and high QCs were 106.8% ± 8.05%, 102.3% ± 10.21%, and 99.1% ± 11.62%, respectively. There were no significant differences in matrix effect among the three QCs for rivastigmine and NAP 226-90 measurements (Table 4).

Protein precipitation was chosen as the method for extracting rivastigmine and NAP 226-90 from the rat plasma. However, previous reports have employed protein precipitation extraction via SPE. SPE and LLE typically provide cleaner extracts with fewer matrix effects, minimum ion suppression, and a low propensity for
building backpressure in the chromatographic column. Nevertheless, in our preliminary investigation using SPE or LLE as the extraction procedure, extracts with satisfactory analyte recoveries were not obtained. Therefore, in this study, the procedure was simplified, and protein precipitation with acetonitrile was used, as it costs less and allows for faster sample preparation. In addition, the amount of required plasma was reduced from 100 μL [5] to 90 μL. The extraction recoveries ranged between 91.9% and 109.8%. The repeatability was also good for rivastigmine and NAP 226-90, when an analyte CV < 12.03% was considered.

**Plasma concentrations of rats receiving rivastigmine administration**

Figure 3 shows the profiles of rivastigmine and NAP 226-90 concentrations, as measured by the HPLC-MS/MS method...
developed in this study. All measured drug concentrations fell within the range (rivastigmine; 2.1–80.2 ng mL\(^{-1}\), NAP 226-90; 1.0–11.2 ng mL\(^{-1}\)). The corresponding values of the pharmacokinetic parameters by non-compartment model are shown in Table 5.

Regarding rivastigmine measurement in rat plasma, a previous report using LC-MS [5] has found a range from 10–100 pmol mL\(^{-1}\) (2.6–26 ng mL\(^{-1}\)). In another report, the range is 0.1–100 ng mL\(^{-1}\) [8]. The concentration range of the calibration curve in the current research stretched from 1–100 ng mL\(^{-1}\), and in vivo pharmacokinetic study has suggested that plasma concentrations of rivastigmine are dose proportional. Karasova et al. [8] summarizes the results of previously published investigations, reporting the values of pharmacokinetic parameters and the plasma and brain concentrations of rivastigmine after single intramuscular injections. According to that report, the \(C_{max}\) of rivastigmine, after a single injection of 137 mg kg\(^{-1}\) in healthy rats was 4.96 ± 0.67 ng mL\(^{-1}\). Recently, many reports have demonstrated the efficacy and safety of maintenance doses (9 or 18 mg/body) of rivastigmine for dementia [9, 10]. In the current study, rivastigmine doses were calculated according to recommended human doses and were given to each rat via injections of 1 mg kg\(^{-1}\) or 2 mg kg\(^{-1}\). Taking these findings into consideration, the calibration curve range for rivastigmine determination might be set with an upper limit of 100 ng mL\(^{-1}\). The current method of rivastigmine measurement, therefore, has the advantage of a wider calibration range compared to the methods applied in previous research.

A study using LC-MS to measure NAP 226-90 in rat plasma has found a range from 10–100 pmol mL\(^{-1}\) (2.6–26 ng mL\(^{-1}\)) [5]. However, pharmacokinetics data on NAP 226-90, especially in terms of rat plasma targeting, have not been previously reported. Preclinical and clinical data about NAP 226-90 are limited to the results of

Table 2. Validation results for measuring rivastigmine concentrations in rat plasma measured by the method developed in the present study

| Parameters          | Rivastigmine (ng mL\(^{-1}\)) | NAP 226-90 (ng mL\(^{-1}\)) |
|---------------------|-------------------------------|-------------------------------|
|                     | LLOQ (1.0) LQC (3.0) MQC (15) | LLOQ (0.5) LQC (1.5) MQC (7.5) | HQC (75) | HQC (37.5) |
| Within-run (n = 6)  |                               |                               |         |
| Mean calculated conc., ng mL\(^{-1}\) (SD) | 0.887 (0.156) 3.23 (0.364) 15.17 (0.937) | 0.456 (0.261) 1.45 (0.283) 7.30 (1.38) |
| Accuracy (%)        | 88.8                          | 98.1                          |
| Precision (CV%)     | 14.60                         | 11.31                         |
| Between-run (n = 6) |                               |                               |         |
| Mean calculated conc., ng mL\(^{-1}\) (SD) | 1.06 (0.080) 3.44 (0.057) 15.77 (1.19) | 0.491 (0.221) 1.45 (0.222) 7.37 (1.38) |
| Accuracy (%)        | 106.6                         | 98.1                          |
| Precision (CV%)     | 7.53                          | 4.53                          |

LLOQ, lower limit of quantitation; LQC, low quality control; MQC, medium quality control; HQC, high quality control; SD, standard deviation; CV, coefficient of variation. [SD/mean × 100].
acetylcholinesterase/butyrylcholinesterase inhibition studies, which do not report the pharmacokinetic profile of NAP 226-90. The LLOQ of our method was 0.5 ng mL$^{-1}$, which was smaller than that of a previous report [5]. In our pharmacokinetics study, $C_{\text{max}}$ and $C_{\text{min}}$ of NAP 226-90 was 8.8–11.1 ng mL$^{-1}$ and 1.0–3.2 ng mL$^{-1}$, especially when a rat is given rivastigmine at higher doses of more than 1 mg kg$^{-1}$. The range of the calibration curve for the current method of NAP 226-90 measurement is 0.5–50 ng mL$^{-1}$, which fills the recommended range, including the LLOQ and the upper limit.

CONCLUSION

The authors have successfully developed a simple method for simultaneously quantifying plasma rivastigmine and NAP 226-90 concentrations. This method has the following features: calibration curves covering wide concentration ranges and capable of measuring pharmacokinetics in rats; short measurement and analysis times; and good accuracy, precision, selectivity, recovery rates, and matrix effects. This method will be very useful in studies concerned with the pharmacokinetic aspects that follow rivastigmine administration.

Conflict of interest: The authors declare no conflicts of interest.

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