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ORIGINAL RESEARCH

A simple, rapid and sensitive method for simultaneous determination of rivastigmine and its major metabolite NAP 226-90 in rat brain and plasma by reversed-phase liquid chromatography coupled to electrospray ionization mass spectrometry

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ABSTRACT: A simple and sensitive reversed-phase liquid chromatography coupled with electrospray–mass spectrometry was developed and validated for the simultaneous determination of rivastigmine, a cholinesterase inhibitor, and its major metabolite NAP 226-90 in rat plasma and brain homogenates. Rivastigmine and NAP 226-90 were extracted from plasma and brain by ethyl acetate and, after drying under nitrogen, re-dissolved in acetonitrile and separated isocratic by HPLC on a C_{18} column and quantified by single ion monitoring mass spectrometer. The mean (±SD) extraction efficiency for rivastigmine in plasma and brain was 93 ± 2 and 95 ± 2% (n = 5) of NAP 226-90 in a drug range of 10–100 pmol/mL or pmol/g. The method proved to be linear within the tested range (regression coefficient, r = 0.9999, n = 5). Intra- and inter-day precision coefficients of variation and accuracy bias were acceptable (within 15%, n = 5) over the entire range for both compounds using plasma or brain samples. The limits of quantification were 0.5 pmol/mL plasma and 2.5 pmol/g brain for rivastigmine and 1 pmol/mL plasma and 5 pmol/g brain for NAP 226-90, respectively. The analytical technique was used to determine the concentrations of rivastigmine and its metabolite NAP 226-90 in rat plasma and brain after oral drug administration. The concentrations of the parent drug and its major metabolite were compared to a pharmacodynamic parameter, the *ex vivo* inhibition of acetylcholinesterase. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: acetylcholinesterase; carbamate inhibitor; Exelon; Alzheimer's disease

INTRODUCTION

Rivastigmine is a cholinesterase (ChE) inhibitor of the carbamate type and is therapeutically used for the symptomatic treatment of Alzheimer's disease (Corey-Bloom *et al.*, 1998). Rivastigmine inhibits acetylcholinesterase (AChE) as well as butyrylcholinesterase (BChE) to a similar degree by carbamylation of a serine in the catalytic triad (Bar On *et al.*, 2002). Carbamate inhibitors like rivastigmine mimic the substrate by forming a carbamylated rather than an acylated complex with the enzyme that is hydrolyzed considerably more slowly than the acylated form. Sequestration of the ChE in its carbamylated form thus precludes further enzyme-catalyzed hydrolysis of acetylcholine (ACh) for an extended period of time (Enz *et al.*, 1993, Enz and Floersheim, 1997). This enzymatic interaction with rivastigmine generates a cleavage product, NAP 226-90, which is the major metabolite of rivastigmine detectable both in animals and man (Cutler *et al.*, 1998; Gottwald and Rozanski, 1999). The concentration of this metabolite obviously depends on the extent of ChE inhibition and therefore its quantification, together with the determination of the parent drug concentration, is very useful. This is of particular importance for measuring the degree of ChE inhibition in target organs such as brain in animals *ex vivo*. We here report on the development of an analytical method to simultaneously determine the parent compound (rivastigmine) and its major metabolite (NAP 226-90) in rat brain and plasma by using an LC-MS methodology. A similar method based on LC-MS-MS determination of the two compounds in human plasma has been recently published (Pommier and Frigola, 2003). The present method uses a cyclic analog of rivastigmine as internal standard instead of deuterated rivastigmine and NAP 226-90, and does not need a derivatization step for the determination of the metabolite NAP 226-090.

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Abbreviations used: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChE, cholinesterase; SIR, selective ion recording.

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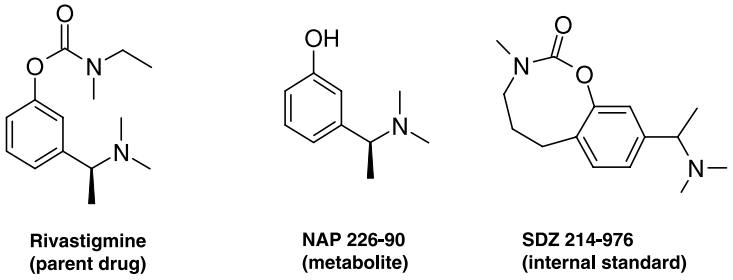

Figure 1. Chemical structures of rivastigmine, (*S*)-*N*-Ethyl-*N*-methyl-3-[1-(dimethylamino)ethyl]-phenyl carbamate, its metabolite NAP 226-90, (*S*)-3-(1-dimethylamino-ethyl)-phenol and the internal standard used SDZ 214-976, 9-(1-dimethylamino-ethyl)-3-methyl-3,4,5,6-tetrahydro-benzo[g][1,3]oxazocin-2-one.

EXPERIMENTAL

Drugs and reagents. Rivastigmine, (*S*)-*N*-Ethyl-*N*-methyl-3-[1-(dimethylamino)ethyl]-phenyl carbamate hydrogen (2*R*,3*R*) tartrate, NAP 226-90, (*S*)-3-(1-dimethylamino-ethyl)-phenol and SDZ 214-976, 9-(1-dimethylamino-ethyl)-3-methyl-3,4,5,6-tetrahydro-benzo[g][1,3]oxazocin-2-one (Amstutz *et al.*, 1990) (internal standard) were from Novartis Pharma AG, Basel Switzerland. The structures of the three chemical entities are show in Fig. 1. All reagents used were of analytical grade and purchased from Sigma (Merck, Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Chromatography and mass-spectrometry. The HPLC system consisted of a vacuum degasser, an auto sampler (ambient temperature, Gilson 233XL), a pump (LKB 2150), and a thermostated column with a Nucleosil CC-125/2 C_{18} reversed-phase column (Macherey & Nagel). The separation was run under isocratic conditions: eluent acetonitrile 80% in H_2O (v/v) containing 0.1% formic acid at a constant temperature of 40°C in the column compartment. The flow rate was held constant at 0.35 mL/min. Sample injection volume was 10 µL. Under these conditions, the retention time for rivastigmine was 3.3 min and for NAP 226-90 2.6 min. A complete analysis cycle took 8 min. Column efflux was directly introduced into the ion source of a Micromass Platform II LC detector (single quadrupole, Micromass Ltd, Manchester, UK). The MS conditions were as follows: ionization ESI-positive polarity, capillary voltage set to 1.4 kV, fragmentor voltage (cone) 40 V, source temperature 150°C. Quantitative analysis was performed by selected ion recording (SIR) over the respective protonated molecular ions [M-H$^+$] of rivastigmine (m/z 251.4 ± 0.5), NAP 226-90 (m/z 166.3 ± 0.5) and SDZ 214-976 (internal standard; m/z 263.4 ± 0.5). The respective peaks were automatically integrated using the software facilities of the analytical system MassLynx (Micromass Ltd, Manchester, UK). It was the peak area which was chosen as the chromatographic signal for quantification.

Preparation of standards. Standard solutions of rivastigmine and NAP 226-90 in drug-free rat plasma and rat brain homogenates were prepared by suitable dilutions from aqueous stock solutions of rivastigmine (10 mM), NAP 226-90 (20 mM) and SDZ 214-976 (20 mM).

Sample preparation. Frozen plasma and brain samples were thawed out. To 100 µL plasma 20 µL NH_4OH (25%) and 10 µL internal standard (0.3 pmol) were added and extracted three times with 500 µL ethyl acetate. The combined extracts were then dried under a stream of nitrogen and redissolved in 100 µL acetonitrile. Brains were weighed and then homogenized in water (1:5 w/v) using an Ultra-Turax Mod T8 for 30 s. Per homogenate, two 100 µL aliquots + 20 µL NH_4OH (25%) + 10 µL internal standard (0.3 pmol) were extracted three times with 500 µL ethyl acetate and then further processed as the plasma samples.

Analytical calibration (standard curves). Standard curve: calibration or standard curves were prepared by spiking rat plasma and brain homogenate samples originating from drug-free animals. Aliquots of 100 µL of rat plasma were supplemented with 10 µL acetonitrile containing the internal standard SDZ 214-976 at a concentration of 30 pmol/mL; to these samples external standard solution with six different concentrations was then added. The samples containing the mixture of plasma, internal and external standard were further processed as described for the plasma and brain samples from drug-treated rats.

A second set of standards in acetonitrile was directly analyzed to estimate the yield of the extraction. The calibration set was prepared on the day of analysis and the standards were run prior and at the end of each analytical batch. A linear calibration was calculated for each analytical batch using the ratio between calibrant and internal standard (y-axis) and the calibrant in spiked blood or brain samples (x-axis). Linear regression through the origin was performed using Origin Version 7.03 (OriginLab Corporation, Northampton, USA) software.

Linearity. Linearity of the assay was determined using duplicates of six concentrations per compound (applied range was 10–100 pmol/mL or pmol/g). The linear correlation coefficients of the curves had to be 0.995, as minimum.

Limit of detection and limit of quantification. The limit of detection (LOD) was defined as the lowest concentration of the extracted standard sample with a signal to noise ratio of ~3. The limit of quantification (LOQ) was defined as the lowest concentration of the extracted standard sample with

a precision (coefficient of variation, CV) better than 30% (triplicate determinations).

Analytical method validation. The validation of the HPLC-MS method was based on proportionality (linearity assay), recovery, precision and accuracy using spiked rat plasma and brain samples.

Linearity. The same concentration range as the calibration curve was used and these were 10, 20, 40, 60, 80 and 100 pmol/mL or pmol/g brain tissue for rivastigmine and NAP 226-90, respectively. Analyses were run as duplicate (individual extractions).

Recovery. The absolute recovery (extraction efficiency) of rivastigmine and NAP 226-90 from rat plasma and brain homogenates was evaluated using six different concentrations covering the linear range of the standard curve and was established by comparing the peak area responses obtained from spiked plasma and brain extracts with those of unextracted standards prepared in the eluent, which represents 100%. For reliable quantification the recovery was defined to be between 85 and 115%.

Precision (repeatability) and accuracy (bias). Analyses of a low (10 pmol/mL or pmol/g), a medium (50 pmol/mL or pmol/g) and a high concentration (100 pmol/mL or pmol/g) were performed. Five standard solutions of each concentration were spiked to plasma and brain samples from drug-free animals and extracted and analyzed for intra-day assay. The analyses were also repeated over 5 days to determine inter-day variability.

***Ex vivo* determination of drug concentrations and of the inhibition of acetylcholinesterase activity in rat brain samples.** Male OFA rats (200 g) were used. Rivastigmine was dissolved in water. Groups of $n = 6$ rats were killed 30 min after oral administration of 1, 2, 3, 6, 10, 15 or 30 μmol/kg rivastigmine. Rats of the control group ($n = 7$) received water only. Rats were killed by decapitation, the removed brain immediately dissected according the method described by Glowinski and Iversen (1961) and the brain regions immediately frozen on dry ice. All samples were stored at –70°C until analysis. The frozen tissue was homogenized and AChE activity was measured according to the method described by Ellman *et al.* (1961), using slight adaptations to optimally determine the enzyme activity in rat brain homogenates.

RESULTS

Chromatography and mass spectroscopy

Figure 2 depicts the mass spectra of the parent compound rivastigmine (a), its major metabolite NAP 226-90 (b) and of the internal standard SDZ 214-976 (c), as obtained under the conditions described in the Experimental section. The respective molecular [M-H$^+$] ions used for recording the compound specific chromatographic peak area were m/z 251.4 ± 0.5 for rivastigmine, m/z 166.3 ± 0.5 for NAP 226-90 and m/z 263.4 ± 0.5 for the internal standard SDZ 214-976, respectively. In Fig. 3, representative ion chromatograms of an extracted spiked plasma and brain sample are shown which illustrate the clean baseline and separation profile of the two compounds. The graph also contains the profile of the added internal standard SDZ 214-976. Note that, by the applied reversed-phase HPLC separation, both compounds were resolved and quantified acceptably; the retention times were 3.5 min for rivastigmine, 2.8 min for NAP 226-90 and 3.3 min for the internal standard SDZ 214-976.

Assay validation

Linearity. The calibration curves before and after extraction and resulting from the ratio of calibrant/internal standard were linear over the tested dose range (10–100 pmol/mL or pmol/g) for both rivastigmine and NAP 226-90. The mean correlation coefficients, obtained from unweighted least-square linear regression (trough origin), were 0.9998 for rivastigmine and 0.9999 for NAP 226-90, respectively. Linearity of the calibration curve, using the ratio between calibrant and internal standard, is reflected by a mean correlation coefficient of $r = 0.9999$ for both rivastigmine and NAP 226-90. These values thus point to a very good linear relationship between peak areas and concentrations with both compounds.

Recovery. Within the range of the calibration standards, the analytical recovery using rat plasma samples was 92 ± 3% for rivastigmine and 95 ± 2% for NAP 226-90, respectively. The corresponding values with brain samples were 93 ± 2% for rivastigmine and 95 ± 2% for NAP 226-90.

Precision and accuracy. The evaluation for intra-day and inter-day precision as well as for accuracy when using three different concentrations are summarized in Table 1. Precision is reported as the coefficient of variation (CV) and accuracy by bias. Maximal CV values were 5% (with plasma) and 6.5% (with brain) for rivastigmine, and 12% (with plasma) and 7% (with brain) for NVP 226-90 for intra-day precision. The intra-day bias was ≤8% for all concentrations of either of the two compounds (Table 1). For inter-day precision maximal CV values were 3% (with plasma) and 4% (with brain) for rivastigmine and 5% (with plasma) and 7% (with brain) for NAP 226-90. The inter-day bias was ≤7% for all concentrations and both compounds (Table 1).

Limit of quantification. A minimum signal-to-noise ratio of 3:1 was obtained with 10 and 20 fmol rivastigmine and NAP 226-90 respectively injected, giving rise to a limit of quantification of 0.5 pmol/mL (0.13 ng/mL) in plasma and 2.5 pmol/g (0.6 ng/g) in brain for

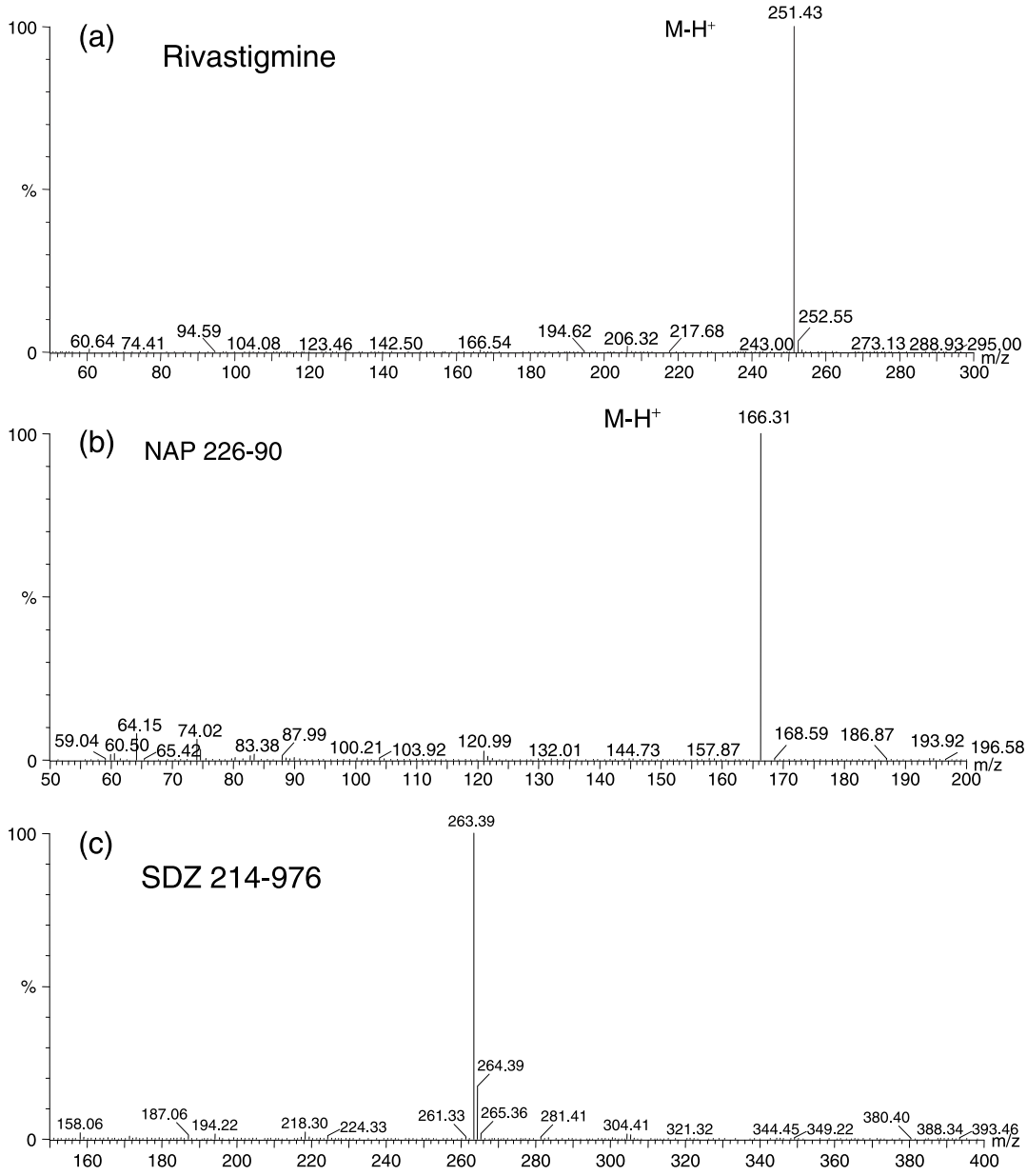

Figure 2. (a) Mass spectrum of rivastigmine (*m*/*z* 251.4). (b) Mass spectrum of NAP 226-90, the major metabolite of rivastigmine (*m*/*z* 166.3). (c) Mass spectrum of the internal standard SDZ 214-976 (*m*/*z* 263.4).

rivastigmine and of 1 pmol/mL (0.17 ng/mL) in plasma and 5 pmol/g (0.83 ng/g) in brain for NAP 226-90.

Ex vivo determination of AChE inhibition in rat brain samples and its relation to drug concentration in plasma and brain

The applied method allowed to determine plasma and brain concentrations of rivastigmine and NAP 226-90 following a single oral dose administered to rats. Based on the pseudo-irreversible AChE inhibition by rivastigmine it was possible to determine the remaining enzyme activity of AChE in rat brain *ex vivo*. The detailed results of this study will be summarized within a separate, more biologically focused manuscript. Therefore, we here just present some preliminary data which clearly demonstrate that the actual brain concentration of parent drug, rivastigmine and its metabolite NAP 226-90 are clearly related to AChE inhibition in rat cortex *ex vivo* (Figs 4 and 5). With increasing concentrations of rivastigmine and NAP 226-90 the activity of the enzyme AChE dose-dependently declined. Thereby, the nonlinear increase of the metabolite NAP 226-90 correlates well with the enzyme inhibition. Obviously, the inhibition of AChE by rivastigmine generates NAP 226-90 via decarbamylation and thus the

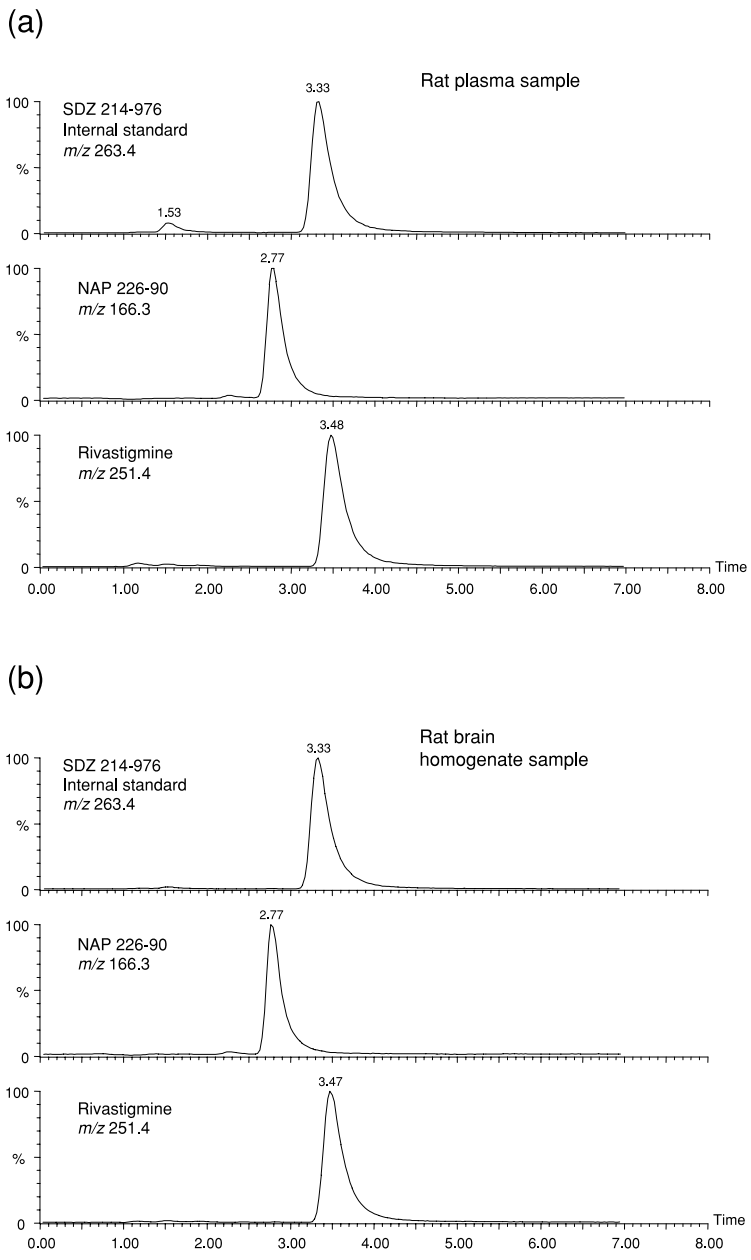

Figure 3. Representative chromatogram of a rat plasma sample (a) and of a rat brain sample (b) spiked with rivastigmine, NAP 226-90 and the internal standard SDZ 214-976 (10 pmol/mL or pmol/g each). Channel SIR 1: *m*/*z* 251.4; channel SIR 2: *m*/*z* 166.3; and channel SIR 3: *m*/*z* 263.4. The compounds' retention times are indicated on the top of each peak.

concentration of NAP 226-90 more reliably reflects the extent of inhibition. Thirty minutes after oral drug-administration the brain concentration was about 15–18% of the corresponding amount of NAP 226-90.

DISCUSSION

Rivastigmine, the cholinesterase inhibitor therapeutically used as Exelon since 1999 for the symptomatic treatment of Alzheimer's disease, is mainly metabolized to NAP 226-90 via the target enzymes, the cholinesterases. Each NAP 226-90 molecule thereby represents an inhibited enzyme molecule. Therefore, it is of obvious importance and relevance to simultaneously measure the concentration of both the parent drug rivastigmine and the major metabolite NAP 226-90. The analytical methodology summarized here consists of a simple organic extraction procedure for both compounds with a subsequent reversed-phase HPLC

Table 1. Precision and accuracy of rivastigmine and NAP 226-90 determination in rat plasma and brain

Compound/ tissue matrix	Spiked concentration (pmol/mL or pmol/g)	Intra-day (n = 5)			Inter-day (n = 5)		
		Data (pmol/mL or pmol/g)	CV %	Bias (%)	Data (pmol/mL or pmol/g)	CV %	Bias (%)
Rivastigmine							
Plasma	10	10.0 ± 0.3	3.0	0	10.2 ± 0.2	2.0	2.0
	50	51.7 ± 1.6	3.1	3.4	50.5 ± 0.6	1.2	1.0
	100	99.6 ± 4.9	4.9	−0.4	99.1 ± 3.0	3.0	−0.9
Brain	10	10.7 ± 0.7	6.5	7.0	9.6 ± 0.2	2.1	−4.0
	50	52.9 ± 1.8	3.4	5.8	49.9 ± 1.6	3.2	−0.2
	100	100.5 ± 3.5	3.5	0.5	98.6 ± 3.6	3.7	−1.4
NAP 226-90							
Plasma	10	9.5 ± 0.4	4.2	−0.5	10.1 ± 0.4	4.0	1.0
	50	51.0 ± 6.1	12.0	2.0	51.9 ± 2.1	4.0	3.8
	100	98.8 ± 3.8	3.8	−1.2	99.0 ± 4.9	4.9	−1.0
Brain	10	9.7 ± 0.7	7.21	−3.0	9.2 ± 0.5	5.4	−8.0
	50	51.5 ± 3.8	7.4	3.0	48.6 ± 3.3	6.8	−2.8
	100	100.8 ± 6.2	6.2	0.8	98.0 ± 2.5	2.6	−2.0

Data are means ±SD, n = 5.
CV (%) = (SD/mean) × 100; bias (%) = [(measured concentration–spiked concentration)/spiked concentration] × 100.

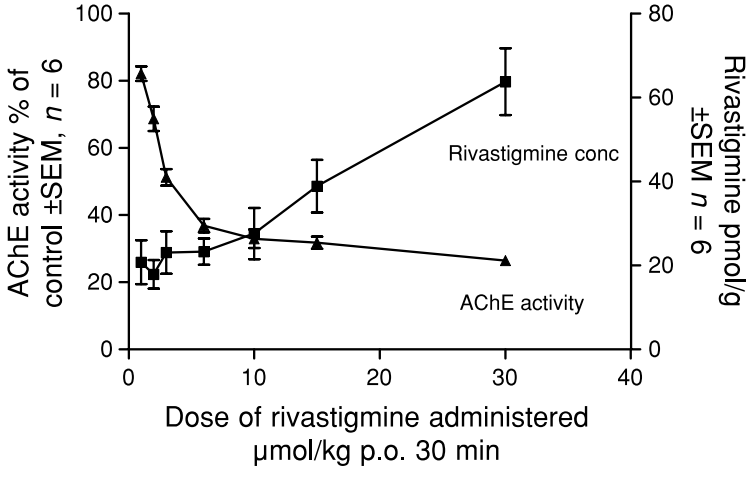

Figure 4. Effect of various doses of rivastigmine on its concentration and the activity of AChE. The concentration of the parent compound and the enzyme activity were determined in brain cortex of rats, which had received various doses of rivastigmine 30 min prior to being killed. Each point represents mean ±SEM of n = 6 rats.

analysis with mass spectrometric detection. The presented method is, according to the validation parameters, selective, precise and accurate and well applicable when studying plasma and tissue concentrations of rivastigmine and its major metabolite NAP 226-90 following a systemic administration of rivastigmine to laboratory animals such as rats. The described methodology has already been successfully applied when evaluating the relation between AChE activity as the pharmacodynamic parameter with drug and metabolite concentration in the rat brain. Certainly, the method will also be very useful in studies focusing on pharmacokinetic aspects following administration of rivastigmine. A similar method for the determination of rivastigmine and NAP 226-90 has been published very recently (Pommier and Frigola, 2003). This method is based on LC-MS-MS detection and uses deuterated internal rivastigmine and NAP 226-90 as internal standards. NAP 226-90 is derivatized before re-extraction. With both methods comparable results in terms of analytical and validation parameters were obtained.

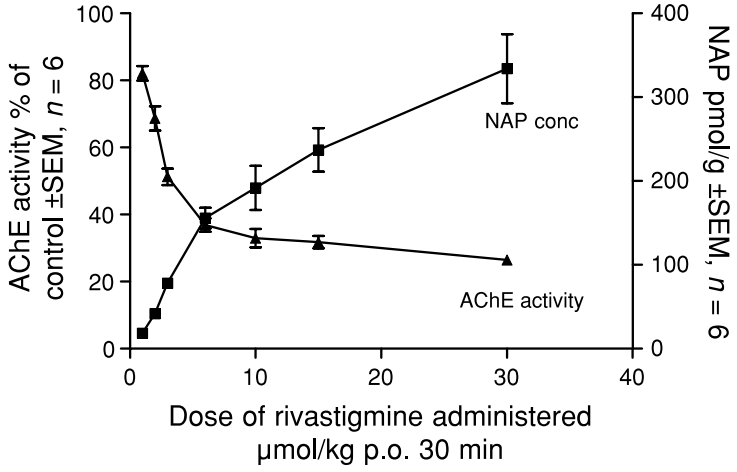

Figure 5. Effect of various doses of rivastigmine on the concentration of its metabolite NAP 226-90 and the activity of AChE. The metabolite concentration and the enzyme activity were determined in the brain cortex of rats, which had received various doses of rivastigmine 30 min prior to being killed. Each point represents mean ±SEM of $n = 6$ rats.

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