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ORIGINAL RESEARCH

Simultaneous liquid chromatographic assay of amantadine and its four related compounds in phosphate-buffered saline using 4-fluoro-7-nitro-2,1,3-benzoxadiazole as a fluorescent derivatization reagent

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ABSTRACT: Simultaneous HPLC assay of 1-adamantanamine hydrochloride (amantadine) and its four related compounds [2-adamantanamine hydrochloride (2-ADA), 1-adamantanmethylamine (ADAMA), 1-(1-adamantyl)ethylamine hydrochloride (rimantadine) and 3,5-dimethyl-1-adamantanamine hydrochloride (memantine)] in phosphate-buffered saline (pH 7.4) after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was developed. Phosphate-buffered saline samples were mixed with borate buffer and NBD-F solution in acetonitrile at 60°C for 5 min and injected into HPLC. Five derivatives were well separated from each other. The lower limits of detection of amantadine, 2-ADA, ADAMA, rimantadine and memantine were 0.008, 0.001, 0.0008, 0.0015 and 0.01 μg/mL, respectively. The coefficients of variation for intra- and inter-day assay were less than 6.4 and 8.2%, respectively. The method presented was applied to a binding study of these compounds to human α_1-acid glycoprotein. While affinity constants and capacities for ADAMA, rimantadine and memantine were calculated by means of Scatchard plots, those for the others were not determined. ADAMA, rimantadine and memantine were bound with different affinities and capacities. These results indicate that NBD-F is a good candidate as a fluorescent reagent to simultaneously determine amantadine and its four related compounds by HPLC after pre-column derivatization. Our method can be applied to binding studies for protein. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: amantadine; amantadine-related compound; 4-fluoro-7-nitro-2,1,3-benzoxadiazole; pre-column derivatization; α_1-acid glycoprotein

INTRODUCTION

1-Adamantanamine hydrochloride (amantadine, AMA) has been clinically used as an anti-parkinsonism agent as well as an antiviral drug (Bryson, 1982; Paci *et al.*, 2001). 2-Adamantanamine hydrochloride (2-ADA) has been found to be a possible drug candidate for a new class of insulin secretagogues in *in vitro* experiments (Garrino and Henquin, 1987), although it is not recognized as an agent for Parkinson's disease. 1-(1-Adamantyl)ethylamine hydrochloride (rimantadine, RIM) exhibits equal efficacy and fewer adverse reactions than AMA, and it has been reported to be effective against influenza (Wingfield *et al.*, 1969; Dolin *et al.*, 1982). 3,5-Dimethyl-1-adamantanamine hydrochloride (memantine, MEM) has potent abilities to treat Parkinson's disease (Evidente *et al.*, 1999; Merello *et al.*, 1999). RIM and MEM have been used for the treatment of Alzheimer's disease and infections by the influenza virus, respectively (Englund *et al.*, 1998; Miguel-Hidalgo *et al.*, 2002; Colgan *et al.*, 2003; Reisberg *et al.*, 2003). 1-Adamantanemethylamine (ADAMA) is an AMA-related compound, while its pharmacological effects remain unknown.

A gas chromatographic procedure employing flame ionization, electron capture or mass selective detection and high-performance liquid chromatographic (HPLC) assay coupled with mass spectrometry has been developed for the determination of the described compounds in biological fluids (Bleidner *et al.*, 1965; Biandrate *et al.*, 1972; Sioufi and Pommier, 1980; Stumph *et al.*, 1980; Belanger and Grech-Belanger, 1982; Koeberle *et al.*, 2003). [^{3}H]-AMA and [^{14}C]-RIM have been utilized for their transport and pharmacokinetic studies (Spector, 1988; Goralski *et al.*, 1999; Hoffman *et al.*, 1988). In the various experiments, complicated equipment and special facilities for using radioactive

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Abbreviations used: AAG, α_1-acid glycoprotein; 2-ADA, 2-adamantanamine hydrochloride; ADAMA, 1-adamantanmethylamine; AMA, 1-adamantanamine hydrochloride (amantadine); IS, internal standard; MEM, 3,5-dimethyl-1-adamantanamine hydrochloride (memantine); NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; RIM, 1-(1-adamantyl)ethylamine hydrochloride (rimantadine).

compounds are necessary. Therefore, a more sensitive and simple method is required.

We have shown the simple and quantitative analysis of AMA by HPLC after pre-column derivatization with *o*-phthalaldehyde and 1-thio-β-D-glucose using 2-ADA as an internal standard (IS) (Higashi and Fujii, 2004). Also, we have developed an HPLC assay that simultaneously measures AMA and RIM after the derivatization with *o*-phthalaldehyde and 2-mercaptoethanol using 2-ADA as IS (Higashi *et al.*, 2005a). However, our preliminary tests showed that RIM and MEM derivatives with *o*-phthalaldehyde and these thiol agents co-eluted. On the other hand, when using dansyl chloride as the reagent, AMA and 2-ADA derivatives exhibited the same retention times (Higashi and Fujii, 2005). Thus, there is no information in the literature on a labeling agent for the simultaneous determination of AMA, 2-ADA, ADAMA, RIM and MEM by HPLC after pre-column derivatization.

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was synthesized as a fluorescent reagent by Imai *et al.* (Watanabe and Imai, 1981, 1983; Imai, 2003). It selectively reacts toward the primary or secondary amino group, and the method was adapted for sensitively detecting various endogenous and exogenous compounds (Watanabe and Imai, 1981, 1983; Honda *et al.*, 2000; Aoyama *et al.*, 2004; Fukushima *et al.*, 2004; Higashi *et al.*, 2005b). However, the derivatization of the five compounds described has not been performed using NBD-F.

We report in this paper the simultaneous and quantitative analysis of AMA, 2-ADA, ADAMA, RIM and MEM in phosphate-buffered saline by HPLC after pre-column derivatization with NBD-F. The reaction scheme is presented in Fig. 1. Afterwards, we compared the binding behaviors of five compounds to α_1-acid glycoprotein (AAG), which is recognized as a potential protein to bind various basic drugs and as a determinant factor responsible for their disposition kinetics.

EXPERIMENTAL

Reagents. AMA, 2-ADA, ADAMA and RIM were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). MEM, NBD-F and AAG from human, acetonitrile and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). The Ultrafree-MC Centrifugal Filter Unit was supplied from Millipore Corporation (Bedford, MA, USA).

Equipment. The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati, CA, USA) with a 20 µL loop and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. The HPLC column (Kanto Chemical, Tokyo, Japan) was 150 × 4.6 mm i.d. with 5 µm particles of C_{18} packing material.

Derivatization. Phosphate-buffered saline containing NaCl (140 mM), K_2HPO_4 (2.5 mM) and NaH_2PO_4 (7.5 mM) was adjusted to pH 7.4 by the addition of NaOH (0.1 M). Borate buffer (0.1 M) containing ethylenediaminetetraacetic acid disodium salt (1 mM) was adjusted to pH 8.0 by the

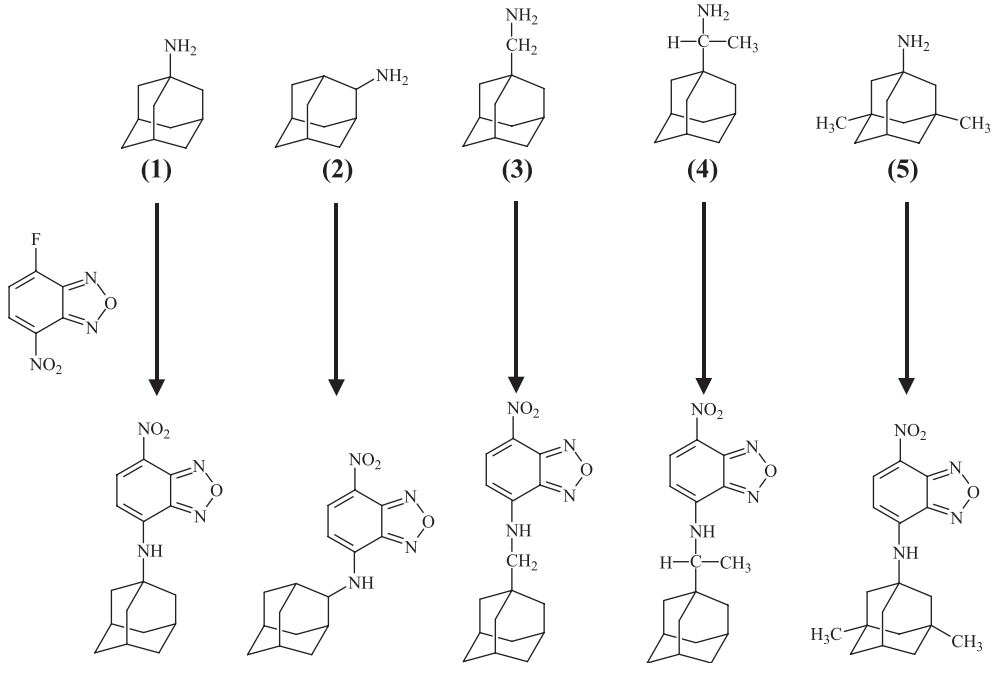

Figure 1. Fluorescent AMA, 2-ADA, ADAMA, RIM and MEM derivatives by NBD-F. (**1**) AMA; (**2**) 2-ADA; (**3**) ADAMA; (**4**) RIM; and (**5**) MEM.

addition of NaOH (0.1 M). Borate buffer (300 μL) was added to phosphate-buffered saline samples (100 μL). NBD-F solution in acetonitrile (20 mM, 100 μL) was added and vortexed. The mixture was allowed to react for 5 min at 60°C. Then, it was placed on ice to stop the derivatization reaction before ice-cold HCl (0.05 M, 400 μL) was immediately added. The derivative sample (20 μL) was injected into the HPLC system. NBD-F solution was prepared weekly in acetonitrile (100 mM), stored at –20°C as a stock solution, and diluted with acetonitrile before use.

Chromatographic conditions. Quantification of the peaks of interest was performed using a Chromatopac Model CR-8A integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared according to our previous method with a minor modification (Higashi *et al.*, 2005b). Briefly, it contained acetonitrile (400 mL), ethanol (200 mL) and trifluoroacetic acid (0.1% v/v) in water (400 mL), and was degassed. The samples were eluted from the column at 25°C at a flow rate of 1.0 mL/min.

Calibration curves. Each solution of AMA, 2-ADA, RIM and MEM in water (1 mg/mL) and an ethanol solution of ADAMA (1 mg/mL) was mixed and diluted with phosphate-buffered saline. The range of concentrations of these compounds varied from 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1 to 2.5 μg/mL. All samples were analyzed using the procedures described above.

Binding study to AAG. AAG solution (80 mg/dL) was prepared in phosphate-buffered saline, and warmed to 37°C prior to incubation with the tested compounds. The protein solution (0.4 mL) was transferred into an incubation container and mixed with each drug solution (0.1 mL) in phosphate-buffered saline. The mixture was vortex-mixed and stood for 10 min. Then, the sample was transferred to an Ultrafree-MC tube and the centrifuged at 3000 ***g*** for 15 min. Derivatization of the filtrate (100 μL) was carried out, and the derivatized sample (20 μL) was injected into the HPLC system.

Binding kinetics. The kinetic parameters (affinity constants and binding capacities) of the five compounds to AAG were calculated by means of Scatchard plots (Scatchard, 1949).

RESULTS AND DISCUSSION

Time courses

For the time course study, as shown in Fig. 2, the reaction time was set at 1, 2, 3, 5, 7 and 10 min. AMA, 2-ADA, ADAMA, RIM and MEM solutions (each 1 μg/mL) in phosphate-buffered saline were derivatized as described in the Derivatization section. The derivatization of AMA and MEM, reached the plateau level at 5 min. The derivatization of 2-ADA, ADAMA and RIM reached a plateau more rapidly. However, the peak areas at 1–2 min exhibited a greater standard deviation, and the areas of 2-ADA, ADAMA and RIM derivatives at 10 min tended to reduce, compared with those at 5 min. Therefore, the derivatization time of 5 min was chosen for a complete reaction in this study.

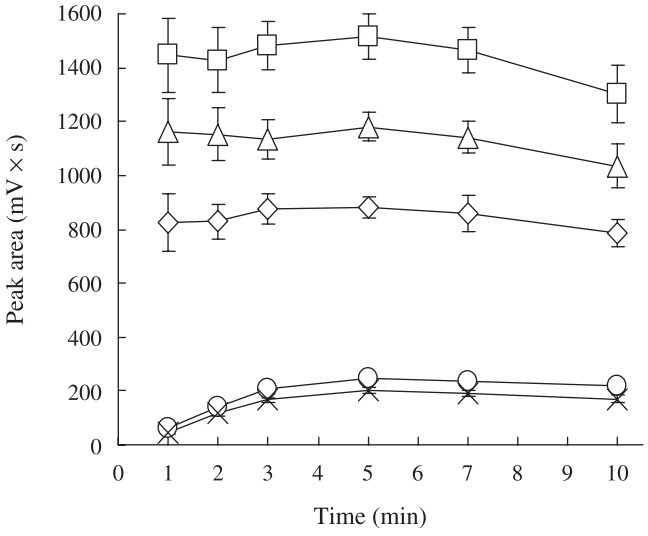

Figure 2. Reaction time course of AMA, 2-ADA, ADAMA, RIM and MEM derivatives. (○) AMA; (△) 2-ADA; (□), ADAMA; (◇) RIM; (×) MEM. Data are expressed as the mean ± SD of three experiments.

The peak area for AMA and MEM was about 4–8 times smaller than that of 2-ADA, ADAMA or RIM. We consider that monitoring the peaks at same excitation and emission wavelengths might be a factor, although further studies are needed on this point.

Chromatogram

Figure 3 shows the chromatograms obtained from (A) drug-free phosphate-buffered saline and (B) phosphate-buffered saline spiked with AMA, 2-ADA, ADAMA, RIM and MEM. The retention times of AMA, 2-ADA, ADAMA, RIM and MEM derivatives were 15.5, 16.6, 20.9, 27.4 and 34.3 min, respectively. The derivatized analytes were well separated from each other and from a large system peak, which was apparently produced by NBD-F hydrolysis.

Standard curves

Standard curves of AMA, 2-ADA, ADAMA, RIM and MEM were constructed by plotting the integrated peak area vs each concentration. Linearity was displayed for AMA ($y = 217.83x - 0.0503$), 2-ADA ($y = 1206.9x - 0.0674$), ADAMA ($y = 1521.8x - 0.0156$), RIM ($y = 849.98x - 0.0201$) and MEM ($y = 194.94x - 0.0151$) concentrations ranging from 0.025 to 2.5 μg/mL. Square regression coefficients (r^2) of AMA, 2-ADA, ADAMA, RIM and MEM were 0.9974, 0.9997, 0.9996, 0.9995 and 0.9997, respectively. The lower limits of detection for AMA, 2-ADA, ADAMA, RIM and MEM were

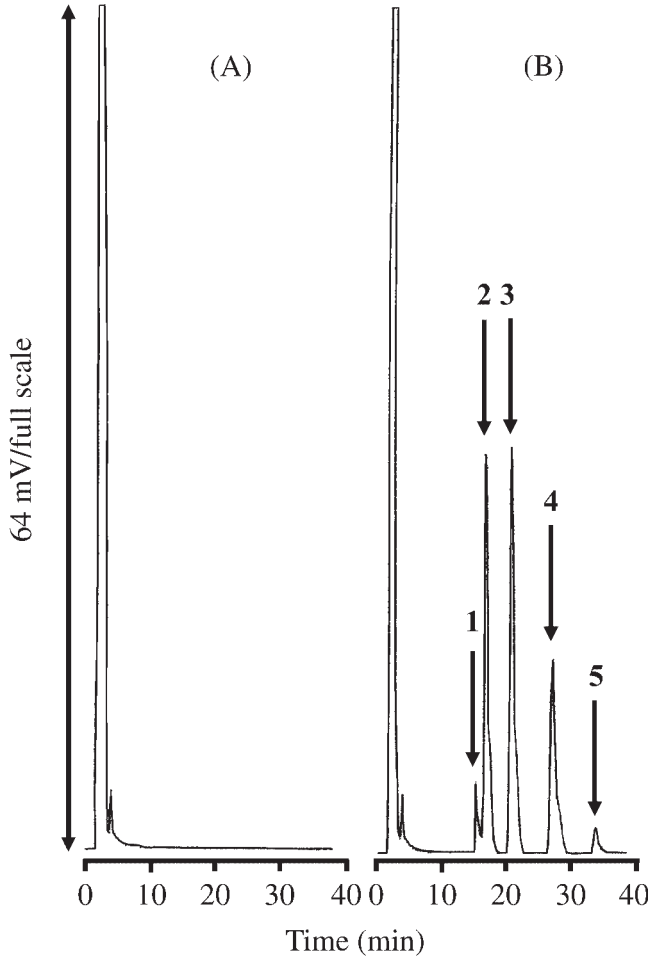

Figure 3. Chromatograms of blank phosphate-buffered saline and derivatives of AMA, 2-ADA, ADAMA, RIM and MEM. (A) Chromatogram obtained from phosphate-buffered saline sample (no AMA, 2-ADA, ADAMA, RIM and MEM peaks). (B) Standard chromatogram containing peaks from the AMA, 2-ADA, ADAMA, RIM and MEM derivatives (each 1 μg/mL). Peaks **1**, **2**, **3**, **4** and **5** represent AMA, 2-ADA, ADAMA, RIM and MEM derivatives, respectively. The attenuation for two chromatograms is 64 mV/full scale.

established at 0.008, 0.001, 0.0008, 0.0015 and 0.01 μg/mL, respectively (signal-to-noise ratio of 3:1).

Our data presented were 1.3 and 4.3 times more sensitive in 2-ADA and RIM, respectively, compared with the detection limits of previous results (Higashi and Fujii, 2005). On the other hand, they were 3.0 and 9.3 times lower in terms of sensitivity in AMA and MEM, respectively (Koeberle *et al.*, 2003; Higashi and Fujii, 2005). To our knowledge, there has been no information in the literature on the sensitivity of ADAMA.

Precision and accuracy

The precision and accuracy for intra- and inter-day assays of AMA, 2-ADA, ADAMA, RIM and MEM derivatives are shown in Table 1. In the intra-day assay, the range of standard deviation to the average of AMA, 2-ADA, ADAMA, RIM and MEM was within 3.8–6.4%. The recoveries were within 96.4–105.6%. In the inter-day assay, the range of standard deviation to the average of AMA, 2-ADA, ADAMA, RIM and MEM was within 5.4–8.2%. The recoveries were within 96.8–105.2%.

Table 1. Intra- and inter-day assay precision and accuracy for the analysis of AMA and its four related compounds

Concentration (μg/mL)	Measured (μg/mL) (mean ± SD, n = 5)	CV (%)	Recovery (%)
Intra-day assay			
AMA			
0.025	0.0264 ± 0.0017	6.4	105.6
0.25	0.247 ± 0.013	5.3	98.8
2.5	2.41 ± 0.15	6.2	96.4
2-ADA			
0.025	0.0241 ± 0.0013	5.4	96.4
0.25	0.259 ± 0.013	5.0	103.6
2.5	2.62 ± 0.10	3.8	104.8
ADAMA			
0.025	0.0252 ± 0.0013	5.2	100.8
0.25	0.260 ± 0.011	4.2	104.0
2.5	2.51 ± 0.12	4.8	100.4
RIM			
0.025	0.0254 ± 0.0014	5.5	101.6
0.25	0.257 ± 0.011	4.3	102.8
2.5	2.52 ± 0.10	4.0	100.8
MEM			
0.025	0.0244 ± 0.0015	6.1	97.6
0.25	0.253 ± 0.011	4.4	101.2
2.5	2.43 ± 0.12	4.9	97.2
Inter-day assay			
AMA			
0.025	0.0246 ± 0.0020	8.1	98.4
0.25	0.257 ± 0.019	7.4	102.8
2.5	2.49 ± 0.17	6.8	99.6
2-ADA			
0.025	0.0242 ± 0.0018	7.4	96.8
0.25	0.255 ± 0.018	7.1	102.0
2.5	2.63 ± 0.18	6.8	105.2
ADAMA			
0.025	0.0253 ± 0.0019	7.5	101.2
0.25	0.262 ± 0.017	6.5	104.8
2.5	2.60 ± 0.14	5.4	104.0
RIM			
0.025	0.0244 ± 0.0018	7.4	97.6
0.25	0.249 ± 0.014	5.6	99.6
2.5	2.58 ± 0.16	6.2	103.2
MEM			
0.025	0.0245 ± 0.0020	8.2	98.0
0.25	0.243 ± 0.017	7.0	97.2
2.5	2.63 ± 0.17	6.5	105.2

Binding study to AAG

The data for binding of AMA, 2-ADA, ADAMA, RIM and MEM to AAG in phosphate-buffered saline at various times were examined. Unbound concentration

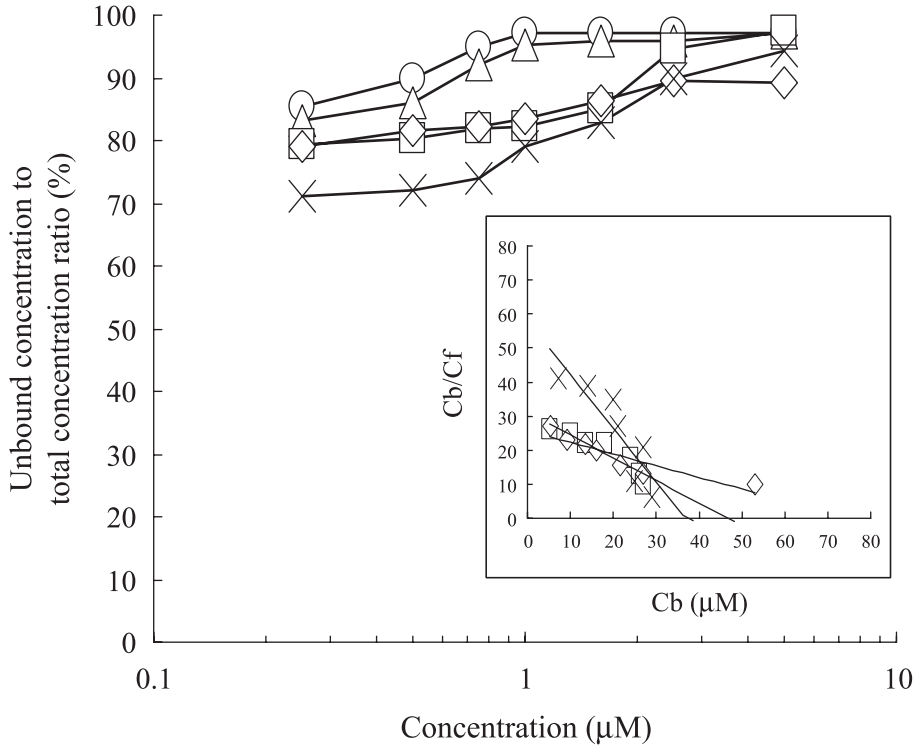

Figure 4. Concentration-dependent binding of AMA, 2-ADA, ADAMA, RIM and MEM to AAG in phosphate-buffered saline. (○), AMA; (△), 2-ADA; (□), ADAMA; (◇), RIM; (×), MEM. AAG concentration: 64 mg/dL (16 μM). Scatchard plots for the binding of ADAMA, RIM and MEM to AAG are illustrated as an insert. Data are expressed as the mean of two experiments. (Cb) bound concentration; (Cf) free concentration.

Table 2. Affinity constants (K_d) and capacities (C) of tested compounds to AAG

	K_d (μM)	C (mol/mol)
AMA	ND	ND
2-ADA	ND	ND
ADAMA	0.64	2.3
RIM	2.9	4.7
MEM	1.5	2.9

ND, not determined. AAG concentration: 64 mg/dL (16 μM).

to total concentration ratios of AMA, 2-ADA, ADAMA, RIM and MEM were plotted. The AAG–five drug binding process appeared to occur virtually instantly and the binding remained constant after the initial 10 min incubation time (data not shown). On the basis of these results, a 10 min incubation time was adopted as the standard to determine the concentration dependency of binding of the five drugs.

The concentration-dependent data for binding of AMA, 2-ADA, ADAMA, RIM and MEM to AAG are shown in Fig. 4. The association constants (K_d) and capacities (C) based on the data were calculated by means of Scatchard plots (insert), and the parameters are listed in Table 2. Those parameters for AMA and 2-ADA were not determined because they exhibited weak binding behavior to AAG. The drugs (ADAMA, RIM and MEM) investigated here were bound with different affinities and capacities. ADAMA exhibited the highest affinity and the lowest capacity of the three, while RIM showed the opposite. The parameters of MEM were in the middle.

CONCLUSION

Our preliminary tests showed that RIM and MEM derivatives with *o*-phthalaldehyde and thiol agents co-eluted (Higashi and Fujii, 2004). Also, our previous results using LC assay after pre-column derivatization with dansyl chloride showed that AMA and 2-ADA derivatives exhibited the same retention times (Higashi *et al.*, 2005a). Thus, there has not been a labeling agent for the simultaneous determination of AMA, 2-ADA, ADAMA, RIM and MEM by HPLC after pre-column derivatization. The results presented demonstrate that NBD-F is a good candidate as a fluorescent reagent for simultaneous HPLC assay of AMA and its four related compounds after pre-column derivatization. The method presented is simple, sensitive and reproducible,

and can be applied to binding studies of these drugs to AAG.

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