Simultaneous Determination of the Binding of Amantadine and Its Analogues to Synthetic Melanin by Liquid Chromatography After Precolumn Derivatization with Dansyl Chloride

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Abstract

For the determination of amantadine (1-ADA), 2-adamantanamine (2-ADA), memantine (MEM), and rimantadine (RIM) in melanin binding studies, the simultaneous determination of 1-ADA or 2-ADA, MEM, and RIM is investigated by high-performance liquid chromatographic assay with dansyl chloride as a fluorescent derivative reagent. Dansyl derivatives with fluorescent intensity are detected at an excitation wavelength of 370 nm and an emission wavelength of 506 nm. Retention times of 1-ADA, 2-ADA, MEM, and RIM derivatives are 12.2, 12.2, 15.2, and 16.6 min, respectively. The peak of 1-ADA derivative coelutes with the 2-ADA derivative. The limits of detection for 1-ADA, 2-ADA, MEM, and RIM are 0.014, 0.007, 0.012, and 0.020µM, respectively (signal-to-noise ratio of 3:1). In the intra- and interday assay, the range of standard deviation to the average of 1-ADA, 2-ADA, MEM, and RIM is 4.6–12.7%. Their recovery is also good. The ranking order for synthetic melanin binding among these compounds is RIM > MEM > 2-ADA ≒ 1-ADA. The method is simple, sensitive, and reproducible for simultaneously measuring 1-ADA or 2-ADA, MEM, and RIM. Also, it is useful to investigate their binding kinetics to melanin.

Introduction

Amantadine (1-adamantanamine hydrochloride, 1-ADA) is clinically used as an antiparkinsonism drug as well as an antiviral drug (1–3). Also, memantine (1-amino-3,5-dimethyladamantane hydrochloride, MEM) and rimantadine [1-(1-adamantyl)ethylamine hydrochloride, RIM], which are derivatives of 1-ADA, have potent abilities to treat Parkinson's disease (4,5). Currently, MEM and RIM are used for the treatment of Alzheimer's disease and infections of influenza virus, respectively (6–9). Then, 2-adamantanamine hydrochloride (2-ADA) has been found to be a possible candidate for a new class of insulin secretagogues in in vitro experiments (10), although it is not recognized as an agent to treat Parkinson's disease. These drugs are primary amines possessing no prominent absorption properties in the UV spectrum and no fluorescence properties.

For the pharmacokinetic and transport studies, the determination of the levels was performed by the gas–liquid chromatographic procedure employing flame ionization and electron capture detection in biological fluids (11–15). According to Siouffi and Pommier, the former procedure lacked the sensitivity for a reliable determination of plasma levels of 1-ADA (14). Hesselink et al. have investigated the brain penetration of 1-ADA by microdialysis study using gas chromatographic analysis coupled with mass selective detection (16). [^{3}H]-1-ADA and [^{3}H]-RIM are frequently utilized for studies on the transport mechanism through the blood-brain barrier and on the reabsorptive mechanism in the kidney (17,18). In the various described experiments, complicated equipment, special facilities, and expensive radioactive compounds are required, and the disposal of isotopes presents a problem in these experiments. Therefore, a more convenient, sensitive, and simple method is needed. The use of a derivatization reagent, which is reactive toward a primary amino group, seems to be one of the most suitable procedures for the sensitive determination of these compounds. Dansyl chloride (Dns-Cl) has been used as a popular derivative reagent for a primary or secondary amino group (19–21). Also, its derivative is generally stable over the long term. This approach leads to a possible, attractive method not only to detect more drugs possessing a primary or secondary amino group but also to promote therapeutic drug monitoring and transport studies of these compounds.

The accumulation of a drug in melanin-rich tissues such as eyes, skin, etc. may have serious side effects (22–25). Despite several investigations into the nature of drug–melanin binding, the

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exact mechanism of the interaction remains unknown (26–29). Drug–melanin binding is a phenomenon that has been observed with structurally and pharmacologically unrelated drugs following administration by ocular and other routes. Presumably, the factors for drug–melanin bindings were the drug's positive charge at physiological pH, lipophilicity, van der Waals forces, and ability to form charge–transfer complexes. Thus, the binding of MEM to melanin has been illustrated by a liquid chromatography (LC)–mass spectrometric (MS) method (30). Interestingly, the differences in the extent of MEM–melanin binding depend on the melanin type and the nature of the incubation medium. Very recently, our high-performance liquid chromatography (HPLC) method precolumn derivatization using *o*-phthalealdehyde and 1-thio-β-D-glucose as fluorescent derivative reagents was found to be appropriate for the determination of 1-ADA levels in human plasma (31). In addition, the binding of four antidepressants, amoxapine, desipramine, maprotiline, and nortriptyline, to synthetic melanin by HPLC assay after derivatization with Dns-Cl is examined (32). Nortriptyline was the strongest of the four compounds for binding to melanin, suggesting that the drug's lipophilicity and cationic charge might be mainly involved in the melanin binding mechanisms. However, there have been no reports on the binding studies of 1-ADA, 2-ADA, and RIM to melanin.

In the present study, Dns-Cl was used to form a fluorescent derivative with 1-ADA, 2-ADA, MEM, and RIM according to the reaction shown in Figure 1. This derivatization technique is utilized along with reversed-phase HPLC to simultaneously measure 1-ADA or 2-ADA, MEM, and RIM. Moreover, the binding behavior of melanin to the four drugs was examined.

Experimental

Equipment

The HPLC system consisted of a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati, CA) with a 25-µL loop and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan), operating at an excitation wavelength of 370 nm and an emission wavelength of 506 nm. The HPLC column (KANTO Chemical Co., Tokyo, Japan) was 150- × 4.6-mm i.d. with 5-µm particles of C_{18} packing material. Derivatization was performed using a Personal-10 incubator (TAITEC Co., Koshigaya, Japan).

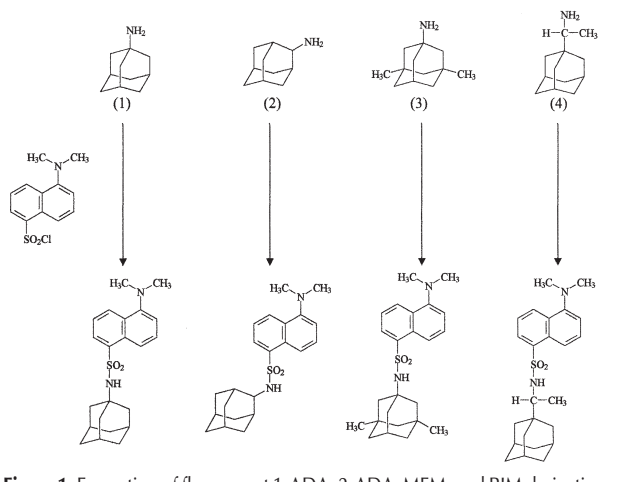

Figure 1. Formation of fluorescent 1-ADA, 2-ADA, MEM, and RIM derivatives by Dns-Cl.

Reagents

Synthetic melanin was purchase from Sigma-Aldrich Co. (St. Louis, MO). Samples of 1-ADA, 2-ADA, RIM, amoxapine, and maprotiline hydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). MEM, desipramine hydrochloride, nortriptyline hydrochloride, methanol for HPLC, and other general reagents were supplied from Wako Pure Chemical Industries (Osaka, Japan). Dns-Cl was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Derivatization

Phosphate-buffered saline (PBS) contained NaCl (140mM), K_2HPO_4 (2.5mM), and NaH_2PO_4 (7.5mM). The pH of the buffer was adjusted to 7.4 using NaOH. Dns-Cl solution (2 mg/mL in acetonitrile, 100 µL) was added to sample solutions in PBS (100 mL) and vortexed. The mixture (final concentration of 0.040 to 5µM) was allowed to react for 30 min at 50°C, and the derivatized sample (25 µL) was injected on the column.

Chromatographic conditions

Quantitation of the peaks was performed with a Chromatopac, model CR-3A integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared by addition of methanol (450 mL) to a solution of 50 mL containing acetic acid (0.2 v/v%) in water adjusted to a pH 7.0 by NaOH. The derivatives were eluted from the column at 25°C at a flow rate of 0.3 mL/min until 18 min. The flow rate was then automatically adjusted to 1.5 mL/min until 29 min.

Melanin binding study

The binding study was based on the previous method (30,32). Suspensions (2 mg/mL) of synthetic melanin were prepared in PBS, sonicated for 15 min, and warmed up to 37°C prior to incubation with 1-ADA or 2-ADA, MEM, and RIM. While being stirred, a volume (0.2 mL) of the melanin suspension was transferred into an incubation container and mixed with each sample solution (0.2 mL) of PBS. The samples were vortexed for 1 min and placed horizontally in the temperature-controlled shaker, set to 37°C and 100 rpm. After incubation for 10 min, the samples were centrifuged at 10,000 *g* for 15 min. The supernatant (100 µL) was then utilized for the previously described derivatization.

Results and Discussion

Chromatogram of 1-ADA, MEM, and RIM derivatives

A blank chromatogram and a chromatogram of the standard mixture of 1-ADA, MEM, and RIM are shown in Figure 2. Retention times (t_R) of 1-ADA, MEM, and RIM derivatives were 12.2, 15.2, and 16.6 min, respectively. The t_R of 2-ADA derivative was also 12.2 min. Although 1-ADA or 2-ADA, MEM, and RIM

derivatives can be completely resolved from each other, the 2-ADA derivative coeluted with the 1-ADA derivative. The chromatographic run time was 29 min.

Standard curves of 1-ADA, 2-ADA, MEM, and RIM

The standard curves were constructed by plotting the integrated peak area versus 1-ADA, 2-ADA, MEM, and RIM concentrations as show in Figure 3. All linearity for 1-ADA ($y = 467.06x + 18.462$), 2-ADA ($y = 775.21x + 28.325$), MEM ($y = 563.41x + 14.203$), and RIM ($y = 869.11x + 48.505$) concentrations were displayed, ranging from 0.040 to 5µM. Square correlation coeffcient (r^2) of 1-ADA, 2-ADA, MEM, and RIM were 0.9956, 0.9982, 0.9990, and 0.9960, respectively. Limits of detections (LODs) for this procedure were determined for each compound. The LODs for 1-ADA, 2-ADA, MEM, and RIM were established at 0.014, 0.007, 0.012, and 0.020µM, respectively (signal-to-noise ratio of 3:1). Compared with those of previous results, the proposed method was 9.5, 7.6, and 120 times more sensitive in 1-ADA (15), 2-ADA (31), and RIM (33), respectively. However, our method was 24 times less sensitive to the LC–MS procedure according to Koeberle et al. in regards to MEM (30).

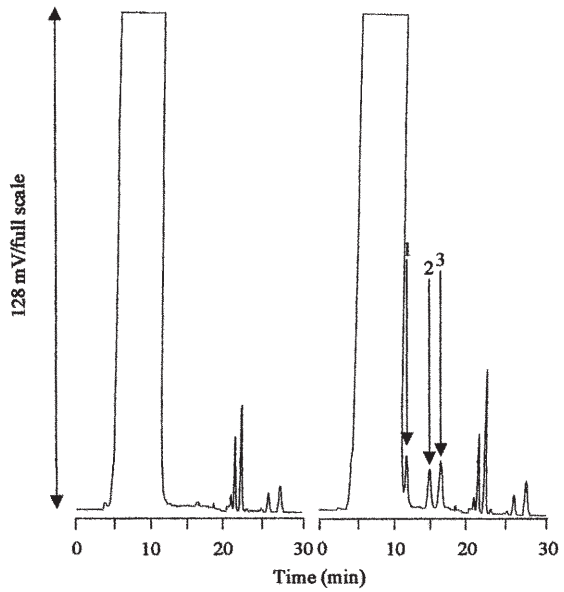

Figure 2. Chromatograms of blank PBS and 1-ADA, MEM, and RIM derivatives. (A) Chromatogram obtained from PBS (no peak of 1-ADA, MEM, and RIM derivatives). (B) Standard chromatogram containing peaks from the 1-ADA, MEM, and RIM derivatives (0.2µM). The attenuation for all chromatograms is 128 mV/full scale. Peaks: 1-ADA (1), MEM (2), and RIM (3).

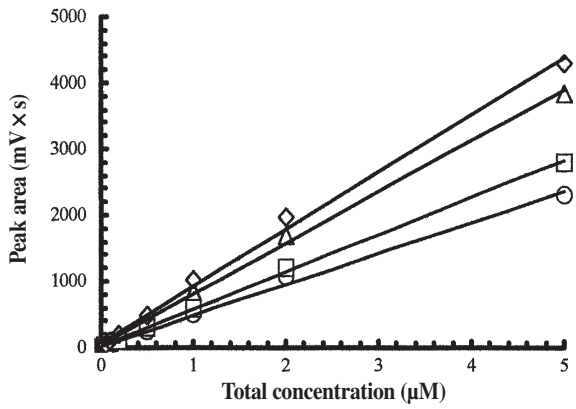

Figure 3. Standard curves of 1-ADA, 2-ADA, MEM, and RIM. Compounds represented as follows: 1-ADA (○), 2-ADA (△), MEM (□), and RIM (◇).

Precision and accuracy of 1-ADA, 2-ADA, MEM, and RIM

Precision and accuracy for intra- and interday assays of Dns-Cl derivatives are shown in Tables I and II. In the intraday assay, the range of the standard deviation of the average of 1-ADA, 2-ADA, MEM, and RIM was 4.6–8.8%. The recovery of 1-ADA, 2-ADA, MEM, and RIM was 95.8–104.6%. In the interday assay, the range of the standard deviation of the average was 8.2–12.7%. The recovery of 1-ADA, 2-ADA, MEM, and RIM was 95.4–104.8%, respectively. It was ascertained that the precision and accuracy of the measurement of 1-ADA, 2-ADA, MEM, and RIM by HPLC are satisfactory.

Interferences for the assay of 1-ADA, 2-ADA, MEM, and RIM

The interference of compounds (2µM) containing a primary or secondary amino group on the derivatization of 1-ADA, 2-ADA, MEM, and RIM (each 0.2µM) is examined. Retention times of Dns derivatives of the compounds are listed in Table III. The peaks of the derivatives of L-dopa (clinically coadministered with 1-ADA) and dopamine (an active metabolite of L-dopa) coeluted when the blank peaks overlapped. Therefore, L-dopa and dopamine did not affect the recovery of 1-ADA, 2-ADA, MEM, and RIM. The retention times of amoxapine, desipramine, maprotiline, and nortriptyline derivatives with Dns-Cl were 18.2, 18.2, 18.2, and 19.7 min, respectively. Because amoxapine, desipramine, maprotiline,

Table I. Intraday Assay Reproducibility for Determination of 1-ADA, 2-ADA, MEM, and RIM

Concentration (µM)	Measured (µM) (mean ± SD* *n* = 10)	%CV†	Recovery (%)
1-ADA			
0.05	0.497 ± 0.0040	8.0	99.4
0.5	0.517 ± 0.039	7.5	103.4
5	5.11 ± 0.29	5.7	102.2
2-ADA			
0.05	0.0509 ± 0.0038	7.5	101.8
0.5	0.503 ± 0.036	7.2	100.6
5	4.79 ± 0.33	6.9	95.8
MEM			
0.05	0.0479 ± 0.0042	8.8	95.8
0.5	0.495 ± 0.031	6.3	99.0
5	5.17 ± 0.34	6.6	103.4
RIM			
0.05	0.0486 ± 0.0038	7.8	97.2
0.5	0.499 ± 0.031	6.2	99.8
5	5.23 ± 0.24	4.6	104.6

* Standard deviation.
† Coefficients of variation.

and nortriptyline did not interfere with the derivatization of 1-ADA, 2-ADA, MEM, and RIM, the simultaneous and quantitative determination of 1-ADA or 2-ADA, MEM, RIM, (amoxapine, desipramine, and maprotiline), and nortriptyline is possible by HPLC after the derivatization with Dns-Cl. Moreover, there was no interference from amino acids such as L-leucine, L-phenylalanine, and L-valine because of coelution with the blank peaks.

Binding to melanin

The data for binding of 1-ADA, 2-ADA, MEM, and RIM to synthetic melanin in PBS for 10 min are shown in Figure 4. The ratios of unbound concentration to total concentration ($f_{1\text{-}ADA}$, $f_{2\text{-}ADA}$, f_{MEM}, and f_{RIM}) of 1-ADA, 2-ADA, MEM, and RIM were plotted. The values of $f_{1\text{-}ADA}$, $f_{2\text{-}ADA}$, f_{MEM}, and f_{RIM} varied from 0.90–0.98, from 0.89–0.95, from 0.71–0.91, and from 0.62–0.87, respectively. The ranking order for melanin binding among the tested compounds was RIM > MEM > 2-ADA ≒ 1-ADA. Recently, Koeberle et al. reported that approximately 40% of MEM bound to synthetic melanin in PBS containing NaCl (154mM), KH_2PO_4 (1.5mM), and Na_2HPO_4 (8.5mM), using the final MEM concentration at 0.75µM by LC–MS (30). In our PBS, which is more appropriate to physiological conditions, the value of f_{MEM} was approximately 0.8 in the presence of MEM (0.75µM), suggesting that 20% of MEM bound to the melanin. Although determinants for the difference (20%) remain unknown, electrolytes in PBS were considered to be involved to some degree. Further studies will be required to resolve the difference.

In this study, the potency of 1-ADA, 2-ADA, MEM, and RIM for binding to melanin is shown for the first time. It is well known that melanin is present in hair, eyes, skin, etc. in the body (22–25). These results indicate that RIM and MEM may be remarkably cumulative in the tissues after administration and that there is slower elimination from the body. Therefore, repeated dosages may cause adverse effects in these tissues because of melanin binding.

Table II. Interday Assay Reproducibility for Dertemination of 1-ADA, 2-ADA, MEM, and RIM

Concentration (µM)	Measured (µM) (mean ± SD* n = 10)	%CV†	Recovery (%)
1-ADA			
0.05	0.0505 ± 0.0064	12.7	101.0
0.5	0.502 ± 0.046	9.2	100.4
5	4.81 ± 0.48	10.0	96.2
2-ADA			
0.05	0.0521 ± 0.0063	12.1	104.2
0.5	0.499 ± 0.052	10.4	99.8
5	4.77 ± 0.43	8.2	95.4
MEM			
0.05	0.0516 ± 0.0054	10.5	103.2
0.5	0.524 ± 0.061	11.6	104.8
5	4.81 ± 0.47	9.8	96.2
RIM			
0.05	0.0491 ± 0.0058	11.8	98.2
0.5	0.520 ± 0.053	10.2	104.0
5	5.10 ± 0.49	9.6	102.0

* Standard deviation.
† Coefficients of variation.

Table III. Retention Times of Dns Derivatives of Endogenous and Exogenous Compounds

Compounds	t_R of Dns derivative (min)
1-ADA	12.2
2-ADA	12.2
MEM	15.2
RIM	16.6
Dopamine	ND*
L-Dopa	ND
Amoxapine	18.2
Desipramine	18.2
Maprotiline	18.2
Nortriptyline	19.7
L-Leucine	ND
L-Phenylalanine	ND
L-Valine	ND

* ND, not determined.

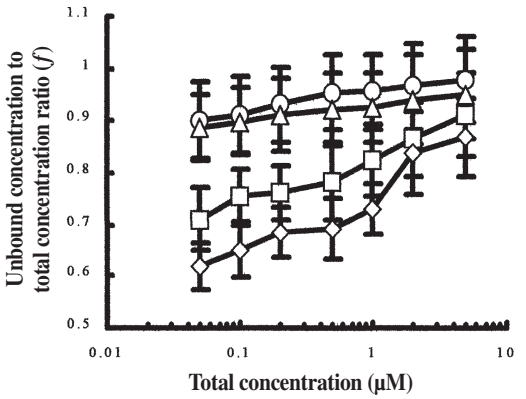

Figure 4. Binding studies of 1-ADA, 2-ADA, MEM, and RIM to synthetic melanin. Compounds represented as follows: 1-ADA (○), 2-ADA (△), MEM (□), and RIM (◇). Each point represents mean ± SD of four experiments.

Conclusion

The procedure using HPLC after precolumn derivatization with Dns-Cl is simple, sensitive, and reproducible for simultaneously measuring 1-ADA or 2-ADA, MEM, and RIM. The ranking order for binding to synthetic melanin was RIM > MEM > 2-ADA ≒ 1-ADA. Our method can be adopted for analyses in binding studies of drugs to melanin.

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