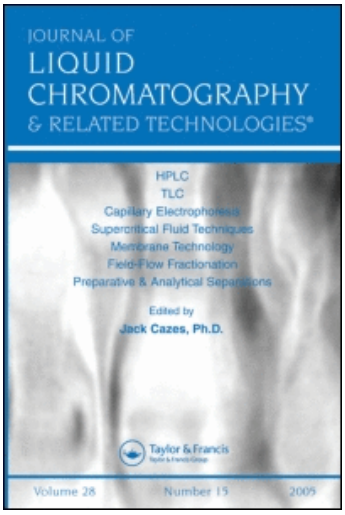

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Sensitive Determination of the Binding of Antidepressants to Synthetic Melanin by Liquid Chromatography After Pre-column Derivatization with Dansyl Chloride

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Sensitive Determination of the Binding of Antidepressants to Synthetic Melanin by Liquid Chromatography After Pre-column Derivatization with Dansyl Chloride

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ABSTRACT

Desipramine (DES), nortriptyline (NOR), amoxapine (AMO), and maprotiline (MAP) are clinically used as antidepressants that contain a secondary amino group and are commercially available. We investigated high performance liquid chromatographic (HPLC) analysis of DES, NOR, AMO, and MAP by derivatization with dansyl chloride (Dns-Cl) in phosphate-buffered saline (PBS). These samples were immediately mixed with Dns-Cl at 50°C for 30 min and injected into HPLC (excitation and emission wavelength: 370 and 506 nm, respectively). Retention times of DES, NOR, AMO, and MAP derivatives were 14.2, 15.5, 14.2, and 14.2 min, respectively. Linearity was displayed for DES, NOR, AMO,

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and MAP concentrations ranging from 10 to 2000 nM ($r^2 = 0.9977$, 0.9992, 0.9992, and 0.9994, respectively. The lower limits of detection of DES, NOR, AMO, and MAP were 5, 5, 7, and 5 nM, respectively. The coefficients of variation for their intra- and inter-day assays were less than 3.6–4.4% and 3.9–4.9%, respectively. The recovery of DES, NOR, AMO, and MAP was good. DES, AMO, and MAP were not found to interfere with the peak of the NOR derivative. The binding of antidepressants to synthetic melanin was measured by determining the unbound concentration ratio to total concentration of DES, NOR, AMO, and MAP. NOR showed the strongest binding to melanin of the four antidepressants. Our results indicated that the HPLC assay of DES, AMO, MAP, and NOR by derivatization with Dns-Cl is simple, sensitive, and reproducible in PBS. In addition our assay system is applied for the binding studies of antidepressants to melanin.

Key Words: Antidepressant; Derivatization; Dansyl chloride; Binding study; Melanin.

INTRODUCTION

Antidepressants have been the mainstay of treatment for major depression for over 30 years. Rapid detection of antidepressants is important in both clinical and forensic toxicology, as there are frequent cases of patients who have taken overdoses of these drugs. Several methods have been published on the simultaneous chromatographic detection of antidepressants and related drugs. Some methods are based on TLC,[1,2] but most are based on high performance liquid chromatography (HPLC).[2–7] As ultraviolet (UV) detection is used in most existing HPLC methods, sensitivity is limited and somewhat critical.[8,9] Multicomponent analysis[6] and dual UV detection[7] have also been used for identification.

The accumulation of a drug in melanin-rich tissues may have serious physiological consequences, as it could lead to potentially toxic effects.[10–14] Despite several investigations into the nature of drug–melanin binding, the exact mechanism of the interaction remains unknown.[15–18] Drug–melanin binding is a phenomenon that has been observed with structurally and pharmacologically unrelated drugs, following administration by ocular and other routes. Of the drugs with known melanin affinity, many are positively charged at physiological pH, and it is generally accepted that ionic interactions are a major contributor. Other factors involved in the reversible binding are the drug's lipophilicity, van der Waals forces, and the ability to form charge-transfer complexes. In addition, there has been a report that tricyclic antidepressant agents might be responsible for blue-gray cutaneous pigmentation, as a possible

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cause of binding of desipramine (DES) to melanin.[19] Therefore, binding studies of antidepressants to melanin are required.

Up to now, dansyl chloride (Dns-Cl), as an analytical reagent, has been used for the determination of some drugs such as methamphetamine,[20] polyamines,[21,22] and some others,[23–25] which possess secondary or primary amines in their structures. However, HPLC determination of antidepressants such as DES, nortriptyline (NOR), amoxapine (AMO), and maprotiline (MAP), possessing a secondary amino group, has not been carried out by derivatization using Dns-Cl. This derivative procedure seems very useful for sensitive determination to estimate unbound concentration of antidepressants to melanin, because the concentrations are very low.

In the present study, Dns-Cl was used to form a fluorescent derivative with DES, NOR, AMO, and MAP, as shown in Fig. 1. We have utilized this derivatization technique and reversed-phase HPLC to measure DES, AMO, MAP, and NOR in phosphate-buffered saline (PBS). Afterwards, binding studies of DES, NOR, AMO, and MAP to synthetic melanin were carried out in the buffer.

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

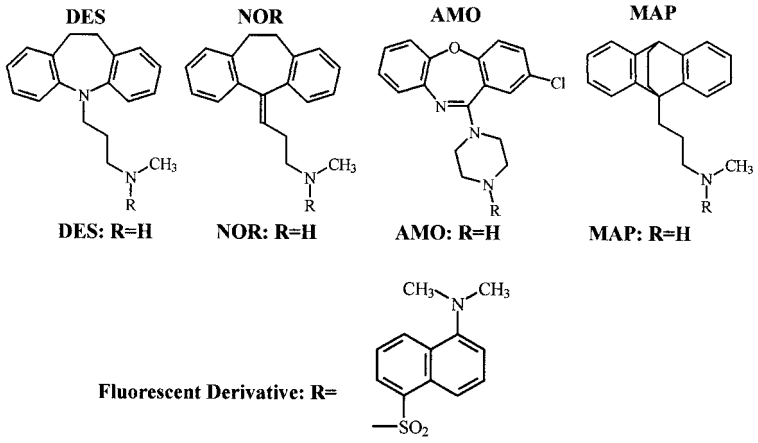

Figure 1. Fluorescent DES, NOR, AMO, and MAP derivatives by Dns-Cl.

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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, Tokyo, Japan) had 150 × 4.6 mm i.d. and 5 μm particles of C_{18} packing material.

Reagents

DES, NOR, methanol for HPLC, and general reagents were supplied by Wako Pure Chemical Industries (Osaka, Japan). AMO, MAP, and synthetic melanin were obtained from Sigma (St Louis, MO). Dns-Cl was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Derivatization

PBS contained NaCl (144 mM), K_2HPO_4 (2 mM), and NaH_2PO_4 (8 mM). The pH of the buffer was adjusted to 7.4 using NaOH. Stock solutions (each 1 mM) of DES, NOR, AMO, and MAP were prepared in acetonitrile (50%) and stored at 4°C for at least 1 week. Then, the solution was diluted by the PBS for standard curves (2000, 1000, 400, 200, 100, 40, 20, 10, and 0 nM). A 100-μL aliquot of PBS containing the antidepressant and 100 μL of Dns-Cl solution (2 mg/mL in acetonitrile) was mixed and vortexed. The mixture was allowed to react for 30 min at 50°C, and the derivatized sample (25 μL) was injected into the column.

Chromatographic Conditions

Quantification 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, at pH 7.0 by NaOH. The derivatives were eluted from the column at a flow rate of 0.4 mL/min for 17.5 min. Then, the flow rate was automatically transformed into 1.6 mL/min for 27 min.

Binding Study to Melanin

Suspensions (2 mg/mL in PBS) of synthetic melanin were prepared in PBS, sonicated for 15 min, and warmed up to 37°C prior to incubation with the antidepressant. While being stirred, a 0.2 mL volume of the melanin

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suspension was transferred into an incubation container and mixed with each solution (0.2 mL) of PBS. The mixtures 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 derivatization described above.

RESULTS AND DISCUSSION

Chromatogram

A blank chromatogram and a chromatogram of the standard mixture of DES and NOR are shown in Fig. 2. Retention times (RTs) of DES and NOR derivatives were 14.2 and 15.5 min, respectively. In addition, RTs of AMO and MAP derivatives were both 14.2 min. The DES derivative overlapped with the AMO and MAP derivatives. It was found that (DES, AMO, or MAP) and NOR derivatives were completely resolved from each

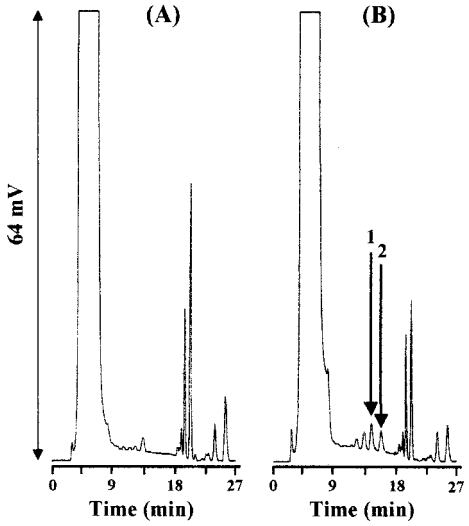

Figure 2. Chromatograms of blank PBS and derivatives of DES and NOR. (A) Chromatogram obtained from PBS sample (no DES and NOR peaks). (B) Standard chromatogram containing peaks from the DES and NOR derivatives (each 100 nM). Peaks 1 and 2 represent DES and NOR derivatives, respectively. The attenuation for both chromatograms is 64 mV/full scale.

other. Then, the chromatographic run time was 27 min by automatically controlling the flow rate.

Linearity and Lower Limit of Detection

Standard curves of DES, NOR, AMO, and MAP are shown in Fig. 3 and were constructed by plotting integrated peak area vs. DES, NOR, AMO, and MAP concentrations. Linearity was displayed for DES ($y = 1.0868x - 1.8411$), NOR ($y = 0.9268x - 0.7699$), AMO ($y = 0.6840x - 1.4569$), and MAP ($y = 0.9907x - 1.7854$) concentrations ranging from 10 to 2000 nM. Square regression coefficients (r^2) of DES, NOR, AMO, and MAP were 0.9977, 0.9992, 0.9992, and 0.9994, respectively. Lower limits of detections for this procedure were determined for each antidepressant. The limits for DES, NOR, AMO, and MAP were established at 5, 5, 7, and 5 nM (signal-to-noise ratio of 3 : 1). HPLC procedures with UV detection of antidepressants have been frequently performed. The limits of quantifications of DES was within 10–20 ng/mL (33–67 nM).[8,9] In addition, lower limits of detections by the gas-chromatography-mass spectrometric assay showed 25 ng/mL (83 nM).[19] Previously, Oztunc et al. have reported that lower limits of detections of antidepressant (DES, NOR, MAP, paroxetine, and floxetine) derivatives, using 7,7,8,8-tetracyanoquinodimethane as a derivative agent, by HPLC were less than 36 ng/mL (120 nM), and the run time was 40 min.[2] It was considered that our method is superior to previous procedures in terms of sensitivity and rapidity.

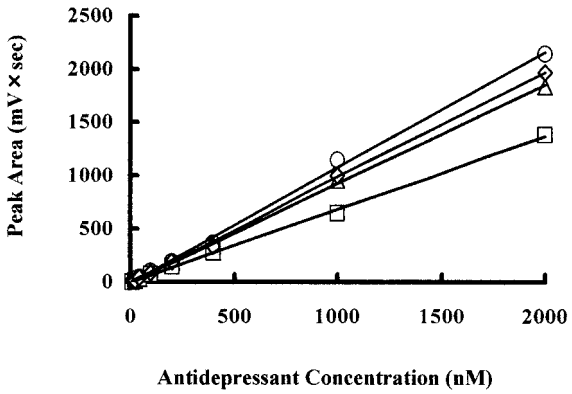

Figure 3. Standard curves of DES, NOR, AMO, and MAP derivatives. (○) DES, (△) NOR, (□) AMO, (◇) MAP.

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Storage Stability

The storage stability of DES, NOR, AMO, and MAP in PBS was examined by the analysis of PBS, containing known amounts of analytes stored at −18°C for 7 days. The stabilities of DES, NOR, AMO, and MAP derivatives were 96.2%, 95.4%, 96.7%, and 97.2%, respectively.

Precision and Accuracy

Precision and accuracy for intra-day and inter-day assays of Dns-Cl derivatives are shown in Tables 1 and 2. In the intra-day assay, the range of standard deviation to the average of DES, NOR, AMO, and MAP was within 3.3–3.6%, 2.6–4.4%, 3.0–4.1%, and 1.9–3.6%, respectively. These recoveries of DES, NOR, AMO, and MAP were within 96.5–101.9%. In the inter-day assay, the range of standard deviation to the average of DES, NOR, AMO, and MAP was within 3.9–4.9%, 4.2–4.6%, 3.0–3.9%, and 3.1–4.1%, respectively. These recoveries of DES, NOR, AMO, and MAP were within 96.5–104.1%, respectively. It was ascertained that the precision and accuracy of the measurement of DES, NOR, AMO, and MAP by HPLC are satisfactory.

Table 1. Intra-day assay reproducibility for determinations of DES, NOR, AMO, and MAP.

Concentration (nM)	Measured (nM) (mean ± SD, $n = 4$)	CV (%)	Recovery (%)
DES			
20	19.7 ± 0.7	3.6	98.5
200	195 ± 6	3.1	97.5
2000	2026 ± 67	3.3	101.3
NOR			
20	20.3 ± 0.9	4.4	101.5
200	201 ± 7	3.5	100.5
2000	1964 ± 51	2.6	98.2
AMO			
20	19.3 ± 0.8	4.1	96.5
200	203 ± 6	3.0	101.5
2000	2038 ± 63	3.1	101.9
MAP			
20	19.4 ± 0.7	3.6	97.0
200	196 ± 6	3.1	98.0
2000	2018 ± 38	1.9	100.9

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Table 2. Inter-day assay reproducibility for determinations of DES, NOR, AMO, and MAP.

Concentration (nM)	Measured (nM) (mean ± SD, $n = 4$)	CV (%)	Recovery (%)
DES			
20	20.5 ± 1.0	4.9	102.5
200	197 ± 8	4.1	98.5
2000	1948 ± 76	3.9	97.4
NOR			
20	19.6 ± 0.9	4.6	98.0
200	195 ± 9	4.6	97.5
2000	2082 ± 87	4.2	104.1
AMO			
20	20.4 ± 0.7	3.4	102.0
200	198 ± 6	3.0	99.0
2000	2066 ± 81	3.9	103.3
MAP			
20	19.3 ± 0.8	4.1	96.5
200	203 ± 7	3.4	101.5
2000	2056 ± 64	3.1	102.8

Interference

Furthermore, we examined the interference of antidepressants (each 2000 nM) on the derivatization (each 200 nM) with Dns-Cl. The peaks of Dns-Cl derivatives of AMO and MAP overlapped with the peak of the DES derivative described above. Therefore, the recovery of DES derivative was about 1100% and 800% in the presence of AMO and MAP, respectively. The values of recovery of AMO and MAP were increased in the presence of DES. In addition, interferences of NOR for DES, AMO, and MAP derivatives, and of DES, AMO, and MAP for NOR derivative were not observed. These results indicate that the NOR levels can be precisely measured when DES, AMO, and MAP co-exist in PBS. Our results strongly indicate that the determination of (DES, AMO, or MAP) and NOR will be possible. However, further studies are needed for the simultaneous determination of these four antidepressants.

Binding Studies to Synthetic Melanin

The data for binding of DES, NOR, AMO, and MAP to synthetic melanin in PBS for various times are shown in Fig. 4. Unbound concentration to total

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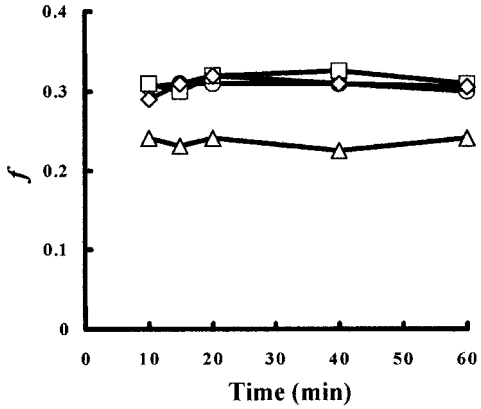

Figure 4. Binding of DES, NOR, AMO, and MAP to synthetic melanin in PBS. All samples contained antidepressants (1000 nM) and melanin (1 mg/mL). (○) f_{DES}, (△) f_{NOR}, (□) f_{AMO}, (◇) f_{MAP}. Data are expressed as the means of two experiments.

concentration ratios (f_{DES}, f_{NOR}, f_{AMO}, and f_{MAP}) of DES, NOR, AMO, and MAP were plotted. The melanin-antidepressants binding process appeared to occur virtually instantly, and the binding remained constant after the initial 10-min incubation time. On the basis of these results, a 10-min incubation time was adopted as the standard to determine the concentration dependency of binding of antidepressants.

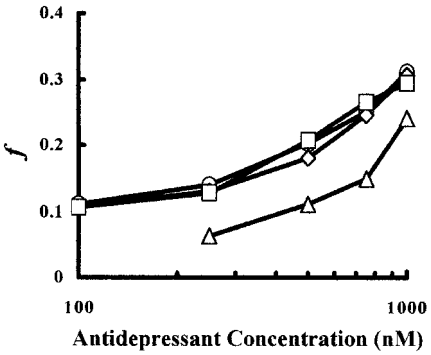

Figure 5. Concentration dependent binding of DES, NOR, AMO, and MAP to synthetic melanin in PBS. (○) f_{DES}, (△) f_{NOR}, (□) f_{AMO}, (◇) f_{MAP}. Data are expressed as the means of two experiments.

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The concentration dependent data for binding of DES, NOR, AMO, and MAP to the melanin are shown in Fig. 5. When each antidepressant was incubated at the final concentration of 1000–100 nM, the values of f_{DES}, f_{NOR}, f_{AMO}, and f_{MAP} varied from 0.310 to 0.110, from 0.242 to 0.062 (not determined at 100 nM), from 0.305 to 0.106, and from 0.293 to 0.105, respectively. The potent order for the melanin binding among the tested compounds was NOR $>$ MAP $\fallingdotseq$ AMO $\fallingdotseq$ DES, suggesting that NOR may be a more responsible drug for blue-gray cutaneous pigmentation than the others. Then, judging from RTs of DES, NOR, AMO, and MAP derivatives, as shown in Fig. 2, it seems that NOR possesses the highest lipophilicity of the four drugs. It was considered that the lipophilicity would be, in part, involved in the melanin binding. It is well known, that melanin is present in hairs, eyes, skin, and so on in the body.[10–14] Our results indicate that tested antidepressants may be remarkably cumulative in melanin-rich tissues after oral or systemic administration, percutaneous and ocular route; side effects will occur. Especially, this is likely to be more frequent for NOR.

CONCLUSION

In conclusion, we established the sensitive HPLC assay of DES, AMO, MAP, and NOR by derivatization with Dns-Cl in PBS. Our procedure is simple, sensitive, and reproducible in the buffer. Our assay system is applied for the binding studies of antidepressants to melanin. NOR is the most potent drug to bind to melanin among the tested antidepressants.

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