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PII S0024-3205(99)00629-3

Life Sciences, Vol. 66, No. 7, pp. 563-574, 2000

0024-3205/00/$–see front matter

HAIR ANALYSIS FOR DRUGS OF ABUSE XXI. EFFECT OF PARA-SUBSTITUENTS ON BENZENE RING OF METHAMPHETAMINE ON DRUG INCORPORATION INTO RAT HAIR

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(Received in final form September 20, 1999)

Summary

In order to study the effect of para-substituents on the benzene ring of methamphetamine on drug incorporation into hair from blood, the plasma AUCs and hair concentrations of 7 methamphetamines [methamphetamine(MA), p-hydroxymethamphetamine(OHMA), p-bromomethamphetamine (BMA), p-aminomethamphetamine (AMA), p-nitromethamphetamine (NMA), p-methoxymethamphetamine (MOMA) and 3,4-methylenedioxymethamphetamine (MDMA)] plus propylhexedrine(PHX) in DA rats was determined after intraperitoneal injection at 5 mg/kg, with single dose for the plasma AUC and 10 doses for the hair concentration. Drug incorporation rates into hair (ICRs) were calculated by dividing each hair concentration by each plasma AUC. Comparing the highest value (NMA) to the lowest one (OHMA), the ICR of NMA was 31.7 times larger than that of OHMA. Using the ICR of MA which has no substitute on the benzene ring as a base point, nitro, bromo, methylenedioxy, methoxy and amino groups raised the drug incorporation into rat hair in this order. On the other hand, hydroxy substitution showed a negative effect on the ICR. In comparison between the ICRs of MA and PHX, it was found that the benzene ring shows higher affinity to melanin and less lipophilicity than the cyclohexyl ring. Our results showed that there is a relatively strong effect of the functional groups on drug incorporation into hair. The combination of melanin affinity and lipophilicity are clearly correlated with their ICR.

Key Words: **hair analysis, methamphetamine analogs, rat plasma, drug incorporation, hair**

In forensic and clinical toxicology, the importance of hair analysis for drugs has been increasing. It is necessary to study drug incorporation mechanisms to correctly evaluate the results obtained from hair analysis. It is empirically known that there are large differences among drugs in their incorporation into hair from blood which depend on their physicochemical properties (1). Until now, it has been shown that hree main factors affect drug incorporation and retention in hair,

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namely melanin affinity (2-13), lipophilicity (1, 4, 14-16) and basicity (4, 8). Since the melaninmolecule is acidic due to the many carboxylic acid groups in its structure, basicity may be also linked with melanin affinity. Therefore, to be simplistic, it would be possible to interpret drug incorporation into hair on the basis of just two factors, melanin affinity and lipophilicity. In addition to these factors, a few reports (4, 17,18) have described membrane permeability as an important factor of drug incorporation into hair. Membrane permeability is basically determined by lipophilicity and protein binding. Thus, membrane permeability is also linked with one of two main factors, lipophilicity.

In our previous papers, we demonstrated that melanin affinity and lipophilicity clearly relate to drug incorporation into hair (4) and the structural factors such as alkyl chain length, aromatic rings, hydroxy groups, keto groups, triple bonds or halogens are relevant in this phenomenon (16). In particular, the addition of aromatic rings to the original structure strongly raised their drug incorporation rates into hair (ICR). For example, the ICRs of benzphetamine, the N-benzyl derivative of methamphetamine(MA), and furfenorex, the N-furfuryl derivative of MA were 5 - 11 times higher than that of MA (16). In addition, the introduction of methylenedioxy and methoxy groups on benzene ring also greatly raised their ICR (16). At that time, it was considered that substituents on the benzene ring had a definite effect on the drug incorporation into hair.

In order to study in detail the effect of functional groups on the benzene ring, the 6 para-substituted MA (nitro-, hydroxy-, bromo-, amino-, methoxy- and methylenedioxy-methamphetamines) were compared with MA in terms of ICR, melanin affinity and lipophilicity. Moreover, in order to evaluate the effect of the aromatic ring, propylhexedrine (PHX) was compared with MA in these three aspects.

Materials and Methods

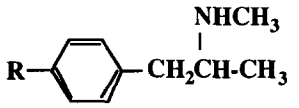
Chemicals The following 8 compounds were used in this study (structures in Fig. 1); methamphetamine (MA), p-hydroxymethamphetamine (OHMA), p-bromomethamphetamine (BMA), p-aminomethamphetamine (AMA), p-nitromethamphetamine (NMA), p-methoxymethamphetamine(MOMA), 3,4-methylenedioxymethamphetamine(MDMA), and propylhexedrine(PHX). All were synthesized in house except for MA which was purchased from Dainippon pharmaceutical Co. Ltd (Osaka, Japan). OHMA, MOMA and MDMA were prepared by condensation of the corresponding aldehydes and nitroethane followed by reduction and methylation as previously reported (4). BMA and NMA were prepared from p-bromo and p-nitrophenyl acetone by reductive amination with methylamine and sodium cyano-borohydride. AMA was prepared from NMA by reduction with lithium aluminium hydride. PHX was prepared from cyclohexylmethylchloride and Shiff's base of acetaldehyde and methylamine by modified Grignard reaction (19). PHX-d4 as an internal standard (IS) was also prepared by the same method as above except acetaldehyde-d4 was used (19). MA-d4 synthesized in our previous report (19) was also used as an IS.

The structures of these compounds were confirmed by melting points, gas chromatography-mass spectrometry(GC-MS) and nuclear magnetic resonance. The purities of these drugs were over 99.5% when checked by high-performance liquid chromatography.

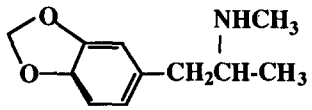
Animals Three male Dark-Agouti(DA) rats (Japan SLC, Shizuoka, Japan) weighing 95 - 105 g (age 5 weeks) were used throughout the experiment. All animal experiments were conducted according to the guidelines of the Committee on Animal Experimentation of the National Institute of Health Sciences (Tokyo, Japan). Before drug administration, the back hair of the rats was shaved

with an animal electric shaver. The rats (3 per drug) were intraperitoneally administered with the drug at 5 mg/kg once a day for 10 successive days. Hair samples. The newly growing back hair was collected by the electric shaver 28 days after the first administration.

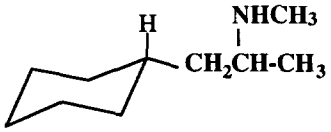
Plasma samples. Blood(400 – 500 μl) was collected at 5, 15, 30, 60, 120 and 360 min after injection from orbital vein plexus on the first day of administration. The blood was collected into plastic tubes containing heparin and sodium fluoride cooled in ice. The plasma samples were obtained by centrifugation at 10,000 rpm for 3 min and stored at -20℃ until analysis.



R=H: Methamphetamine(MA)
R=OH: p-Hydroxymethamphetamine (OHMA)
R=Br: p-Bromomethamphetamine (BMA)
R=NH_2: p-Aminomethamphetamine (AMA)
R=NO_2: p-Nitromethamphetamine (NMA)
R=OCH_3: p-Methoxymethamphetamine(MOMA),



Methylenedioxymethamphetamine(MDMA)



Propylhexedrine(PHX)

Fig. 1
The structures of methamphetamines used in this study

Extraction procedures Plasma; To 100 μl of plasma, 100 μl of 2.5% NH4OH and 100 μl of the internal standard(IS) aqueous solution containing 100 ng of MA-d4 were added except in the analysis of propylhexedrine which used propylhexedrine-d4 as an IS instead of MA-d4. The solution was extracted with 1 ml of dichloromethane using a vortex mixer for 1 min. After the organic layer was transferred into a new tube and 100 μl of methanol-5M HCl (20:1) added, the solvent was evaporated under nitrogen at below 40℃.

Hair; Hair samples were washed three times with 0.1% sodium dodecyl sulfate and rinsed three times with water, dried, and weighed(5 mg). 100 ng of MA-d4 or PHX-d4 were added and the samples incubated in 1.5 ml of methanol-5M HCl(20:1) with ultra-sonication for 1 h and stored overnight at room temperature as previously reported (4, 16). After filtration, the filtrate was evaporated as above.

Derivatization and GC/MS Analysis The extracts from plasma and hair were dissolved in 200 μl of trifluoroacetic anhydride/ethyl acetate(1:1) and heated at 60℃ for 20 min. After evaporation of solvent, the residue was redissolved in 50 μl of ethyl acetate and 1 μl injected onto GC/MS. GC/MS analysis was performed using a Hewlett Packard 5890 SERIES-II gas chromatograph equipped with a 7673A autosampler and MSD 5971. The gas chromatography (GC) was carried out using a 20 m x 0.25 mm I.D., 0.25 μm cross linked methylsilicone fused silica TC-1 column

(GL Sciences Inc., Tokyo, Japan). The injection port temperature was 200℃ (splitless mode) and helium was the carrier gas (5.5 psi head pressure). The injector temperature was 250℃ and splitless injection was employed with a split valve on time of 1.0 minutes. The oven temperature was held at 60℃ for 0.5min following injection and programmed to 280℃ at a rate of 20℃/min. Mass chromatograms were recorded in the selected ion monitoring mode using the following ions ; TFA-MA m/z: 154, 118; TFA-OHMA m/z: 230, 154; TFA-BMA m/z: 198, 196, 154; TFA-AMA m/z: 202, 229, 154; TFA-NMA m/z: 154, 110; TFA-MOMA m/z: 154, 148, 121; TFA-MDMA m/z: 339, 204, 162; TFA-PHX m/z: 236, 182, 154. For quantitative analysis, the drug concentrations in the biological specimens were calculated using their peak-area ratios of the ions monitored; for MA (m/z 154), BMA (m/z 154), AMA (m/z 154) and NMA (m/z 154) with MA-d4 (m/z 158), for OHMA (m/z 154) and MOMA (154) with OHMA-d4 (m/z 158), for MDMA (m/z 204) with MDMA-d4 (m/z 208) and for PHX (m/z 154) with PHX-d4 (m/z 158).

Pharmacokinetic Calculations. The areas under the plasma concentration vs. time curve(AUCs)$[PL]_{\infty}$ were calculated by the trapezoidal rule over the time of measurement with the remainder of the curve estimated as $b \cdot C_{last}$ where b is the terminal rate constant and C_{last} is the concentration at the last time point observed.

Definition of Drug Incorporation Rate (ICR) We have designed the rat model experiment for determining the drug incorporation into hair by ratios of hair concentrations to plasma AUCs (4, 16). After shaving animal hair just before the first administration and administering the drugs daily for 10 days at 5 mg/kg, the newly grown hair was collected on Day 28. The ratio of drug concentration in the newly grown hair to plasma AUC is regarded as a drug incorporation rate (ICR) into hair.

Measurement of Lipophilicity (log Po/w) The n-octanol-water partition parameter (log Po/w) was determined as a representation of lipophilicity. A test solution (1 mL) containing 10 nmole of each drug in 0.05M phosphate buffer (pH 7.4) was extracted with n-octanol(1 mL) by vortex mixing for 3 min at room temperature. To the aqueous phase (0.5 mL) from the lower layer, IS (500 ng) was added and the solution basified with 25% NH4OH and extracted with dichloromethane (1 mL). The drug concentration in dichloromethane was analyzed as mentioned in the analytical procedure. The drug concentration in the buffer was regarded as $[drug]_{water}$ and the subtraction of $[drug]_{water}$ from the total amount (10 nmole) as $[drug]_{octanol}$. The log Po/w was equal to log $[drug]_{octanol}/[drug]_{water}$.

Measurement of melanin affinity Twenty mg of synthetic melanin (Sigma Chemical Co., St.Louis, MO,USA) was dissolved in 0.5 ml of dimethylsulfoxide according to the method of Kaliszan et al.(20) and the solution diluted with 50 ml of sodium phosphate buffer (0.1 M, pH 7.00). Series of 5 buffer solutions were prepared for each of the test drugs with concentrations ranging from $6.25 \cdot 10^{-9}$ M to $1.0 \cdot 10^{-7}$ M. One ml of test solution was added to 1 ml of melanin solution and the mixture incubated at 36℃ for 2 h in the dark allowing 0.4 mg of melanin to interact with 3.125, 6.25, 12.5, 25 and 50 nM of drug. After 2 h, 1 ml of the mixture was transferred to a Centrifree membrane ultrafilter cone (Amicon Division, Beverley, MA, USA) and centrifuged at 1800 g for 15 min. The drug concentration in the filtrate was determined by GC/MS

as described above.

Results

Effect of substituents on AUC and incorporation rates

To obtain AUCs of 8 drugs, the blood was collected at 5, 15, 30, 60, 120 and 360 min after the administration. The time courses of rat plasma levels of the 8 drugs are shown in Figures 2 and 3. The times of maximum concentration were 5 min for MA, OHMA and AMA, 15 min for MOMA, MDMA and PHX, and 30 min for BMA and NMA.

The AUCs of each of the eight drugs fell into 3 groups as shown in Table 1. MA and MDMA were in the large AUC group (around 120 μg•min/mL); BMA, AMA, NMA, MOMA and PHX were in the middle; and OHMA was in the smallest. This could be due to differences in metabolism, distribution and excretion. The order of Cmax was NMA > MDMA > MOMA > PHX > MA > NMA > OHMA > BMA.

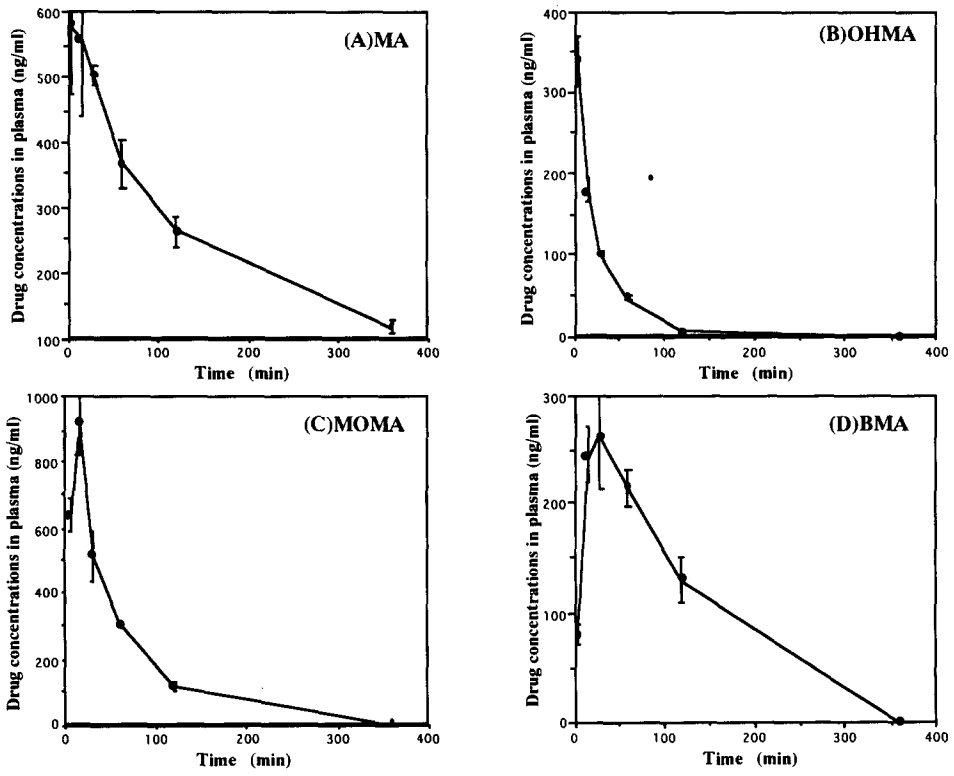


FIG. 2

The time courses(A) of rat plasma levels after the administration of (A) MA, (B) OHMA, (C) MOMA and (D) BMA. (5 mg/kg, n=3)

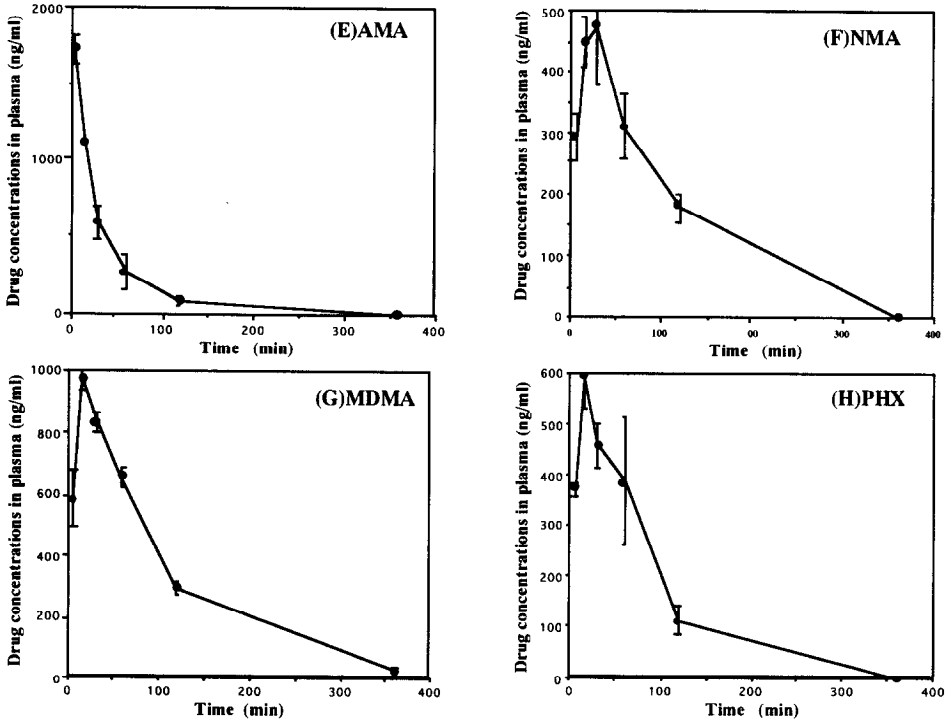


Fig. 3
The time courses(A) of rat plasma levels after the administration of (E) AMA, (F) NMA, (G) MDMA and (H) PHX. (5 mg/kg, n=3)

TABLE 1
Cmax, Tmax and AUC in the Rat Plasma after Administration of MA Analogs
(i.p. 5 mg/kg x 10 days)

	Cmax (ng/mL) mean ± SD	Tmax* (min)	AUCs (μg min/ml) mean ± SD
MA	581 ± 109	5	124.5 ± 20.8
OHMA	339 ± 31	5	9.7 ± 1.3
MOMA	920 ± 100	15	55.8 ± 13.3
BMA	263 ± 48	30	49.1 ± 6.8
AMA	1727 ± 96	5	65.9 ± 11.3
NMA	474 ± 95	30	59.4 ± 6.9
MDMA	977 ± 33	15	121.2 ± 16.0
PHX	593 ± 65	15	54.9 ± 9.7

* It shows the maximum concentrations and peak times with blood collection at 5, 15, 30, 60, 120 and 360 min after injection.

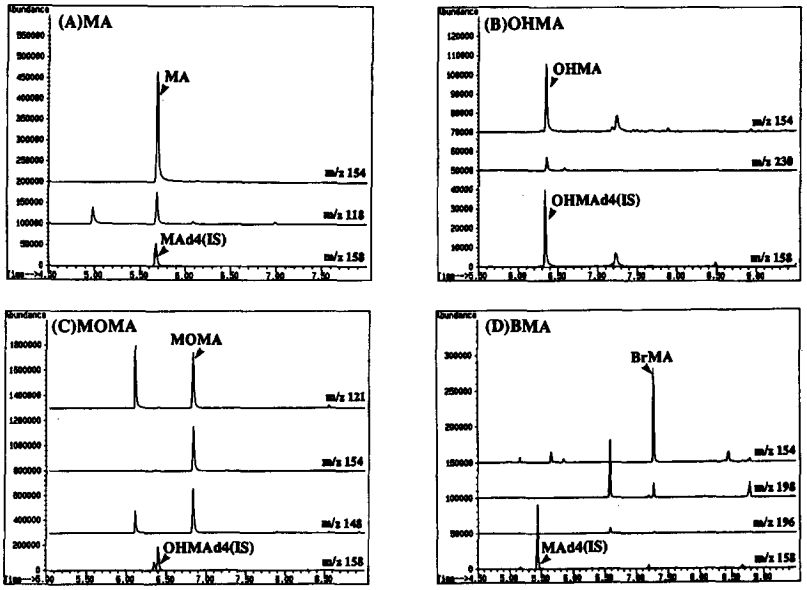


Fig. 4

The GC-MS-SIM chromatograms of the extracts from the rat hair after the administration of (A) MA, (B) OHMA, (C) MOMA and (D) BMA. (5 mg/kg, 10 doses)

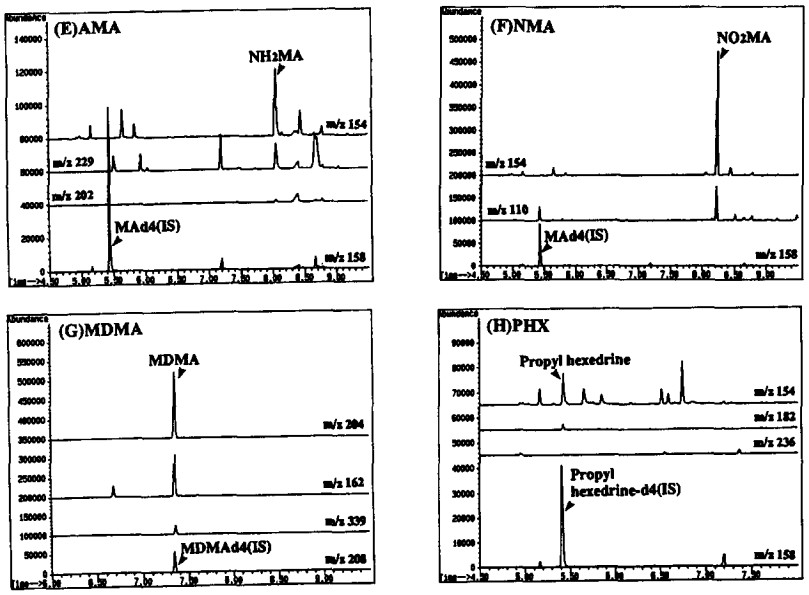


Fig. 5

The GC-MS-SIM chromatograms of the extracts from the rat hair after the administration of (E) AMA, (F) NMA, (G) MDMA and (H) PHX. (5 mg/kg, 10 doses)

TABLE 2
The Drug Incorporation Rates Calculated from AUCs and Hair Concentrations in Rats

	AUCs (μg min/ml) mean ± SD	Drug concentrations in hair (ng/mg) mean ± SD	ICR [Hair]/AUC*
MA	124.5 ± 20.8	16.3 ± 2.3	0.13
OHMA	9.7 ± 1.3	0.7 ± 0.1	0.07
MOMA	55.8 ± 13.3	22.1 ± 2.8	0.40
BMA	49.1 ± 6.8	44.6 ± 8.2	0.91
AMA	65.9 ± 11.3	23.5 ± 0.1	0.37
NMA	59.4 ± 6.9	130.4 ± 8.9	2.22
MDMA	121.2 ± 16.0	93.4 ± 10.9	0.77
PHX	54.9 ± 9.7	7.9 ± 0.5	0.13

*[Hair] / AUC means the ratio of drug concentration in hair to AUC in plasma (Drug incorporation rate into hair from plasma)

The hair was shaved on Day 28 after the first administration. The hair concentrations were determined by GC-MS-SIM analysis. Typical chromatograms of the 8 drugs are shown in Figures 4 and 5. The chromatograms showed no interfering peaks, so that the hair concentrations of each drug could be accurately determined.

It is still unclear which pharmacokinetic factors (AUC, Cmax, etc.) are associated with the hair concentrations, but in our previous study (21), it was demonstrated that AUCs were most closely associated with the hair concentrations. Therefore, it seemed reasonable to compare the ratios of hair concentrations to AUCs as an index of drug incorporation tendency into hair.
In the animal experiments, NMA had the highest hair concentration, followed by MDMA. The middle hair concentrations group contained BMA, AMA, MOMA and MA and the low hair concentrations group PHX and OHMA. There was 186 times difference between NMA and OHMA in the hair concentrations.

The ICRs calculated from AUCs and concentrations in hair for the 8 drugs are shown in Table 2. The order of AUC was MA>MDMA>AMA>NMA>MOMA>PHX>BMA >OHMA and the order of the hair concentrations NMA>MDMA>BMA>AMA>MOMA >MA>PHX>OHMA.

The ICR of NMA was calculated to be 2.22 which is a quite high value. Comparing the highest value (NMA) to the lowest one (OHMA), the ICR of NMA was 31.7 times larger than that of OHMA. On the basis of the ICR of MA which has no substitute on the benzene ring, nitro, bromo, methylenedioxy, methoxy and amino groups raised the drug incorporation into rat hair in this order. On the other hand, the hydroxy group showed a negative effect on the ICR.

TABLE 3
Comparison between melanin affinity, lipophilicity and ICR

	Melanin affinity (x 10^{-4})	Lipophilicity (log $P_{o/w}$)	I C R ([Hair]/AUC)
MA	1.25	-0.80	0.13
OHMA	0.20	-1.43	0.07
MOMA	3.74	-0.68	0.40
BMA	3.26	1.06	0.91
AMA	9.91	-0.95	0.37
NMA	10.20	-0.19	2.22
MDMA	7.55	-0.44	0.77
PHX	0.37	-0.05	0.13

Effect of substituents on melanin affinity and lipophilicity

Considering MA as a base point, nitro, amino, methylenedioxy, methoxy and bromo groups at the para position on the benzene ring increased the melanin affinity in this order, while hydroxy group decreased it (Table 3). Thus, the order of melanin affinity of the drugs was NMA>AMA>MDMA>MOMA>BMA>MA>PHX>OHMA. Comparing the melanin affinity of PHX and MA, the former was apparently lower than the latter.

Comparing the log Po/w of the 7 para-substituted drugs, bromo, nitro, methylenedioxy, and methoxy groups raised the lipophilicity in this order, while amino and hydroxy groups lowered the lipophilicity. The order of lipophilicity of the drugs was BMA > PHX > NMA > MDMA > MOMA > MA > AMA > OHMA. By comparison between benzene and cyclohexyl rings, it was suggested that cyclohexyl ring is more lipophilic than benzene ring.

Discussion

The authors have proposed(4, 16) an index of incorporation tendency of drugs into hair using the drug incorporation rate into hair (ICR) represented by the ratios of hair concentration to plasma AUC. Using the ICRs, it can easily be understood why a major drug component in blood may be different from that incorporation in hair after repeated drug administration. For example, in the hair sample of cocaine users cocaine is found in much higher concentrations than benzoylecgonine, while in their blood sample more benzoylecgonine is found. This fact could be explained by the large difference in their ICRs (cocaine: 3.60, benzoylecgonine: 0.003) (4). It is obviously suggested that these differences depend on the drug's physicochemical properties such as melanin affinity, basicity and lipophilicity.

For the ICR to be used as an index it is necessary that each drug has an almost constant value of ICR even in the case of different AUCs. In general, AUC is strongly affected by absorption,

distribution, metabolism and elimination of drugs in blood. Therefore, there were large differences between AUCs of drugs as shown in Table 2 even when the same dose was used. We previously reported(21) that there were good correlations between doses(or AUCs) and hair concentrations under the controlled animal experiments with MA, amphetamine, methoxyphenamine, cocaine, 6-acetylmorphine and morphine in the range between 1 and 6 mg/kg. Gygi et al.(22) have also found that the incorporation of codeine and morphine into hair occurred in a distinct dose-proportional manner under the controlled animal experiments. In this manner, if the relationship between AUCs and hair concentrations of each drug is almost proportional, the ICR defined as [hair]/AUC would be almost constant. For comparison of various drugs ICR might therefore be used as an index indicating the incorporation tendency into hair.

Relationship between ICR and melanin affinity/lipophilicity

Comparing between MA and para-substituted MA, the nitro group increased the melanin affinity of MA (1.25×10^{-4}) by 8.1 times, the amino group by 7.9 times, the methylenedioxy group by 6.0 times, the methoxy by 3.0 and the bromo group by 2.6 times, while the hydroxy group decreased it by a factor of 6.25 (Table 3). In particular, it was found that the melanin affinity is distinctly strengthened by the substitution of nitro, amino and methylenedioxy at the para position of the benzene ring. PHX is an analog of MA in which benzene is replaced by cyclohexane. The melanin affinity of PHX was determined as 0.37×10^{-4} which was 1/3 lower than that of MA. This implies that the benzene ring is provided more affinity to melanin than its cyclohexyl ring.

It has been mentioned in several reports (2-13) that melanin is one of the most effective factors of drug incorporation into hair. However, drug incorporation into hair from blood can not always be explained only by melanin affinity. Also in this study, AMA had the second strongest melanin affinity of the 8 drugs, but the ICR of AMA ranked 5th. On the other hand, it has been proved (1, 4, 14-16) that lipophilicity is also related to drug incorporation into hair. As shown in Fig.6, BMA and PHX both have higher ICRs than suggested from relatively low melanin affinity due to their high lipophilicity. It could be considered that drug incorporation into hair should be discussed by the combination of these two factors.

This study shows that para-substituents on the benzene ring of methamphetamine obviously affect not only the drug incorporation into hair, but also melanin affinity and lipophilicity. Also in our previous report (16), we demonstrated that the basicity of drugs is one of the most important factors of drug incorporation into hair because almost no N-acetyl MA is found in hair in spite of its high AUC after the administration of N-acetyl MA. Gygi et al (23) also found from animal experiments using phenobarbital and codeine that hair pigmentation greatly affects weak base incorporation but not weak acid incorporation into hair. These findings indicate that incorporation of basic compounds into hair is greatly affected by melanin content in hair. Thus, there are three main factors concerning drug incorporation into hair from blood, but at this point we can only predict the drug incorporation tendency with melanin affinity and lipophilicity in addition to basicity. However, basicity may be considerably overlapped with melanin affinity, since it is considered that acidic melanin molecules attract basic substances. Therefore, it would be possible to discuss drug incorporation into hair with just two factors, melanin affinity and lipophilicity.

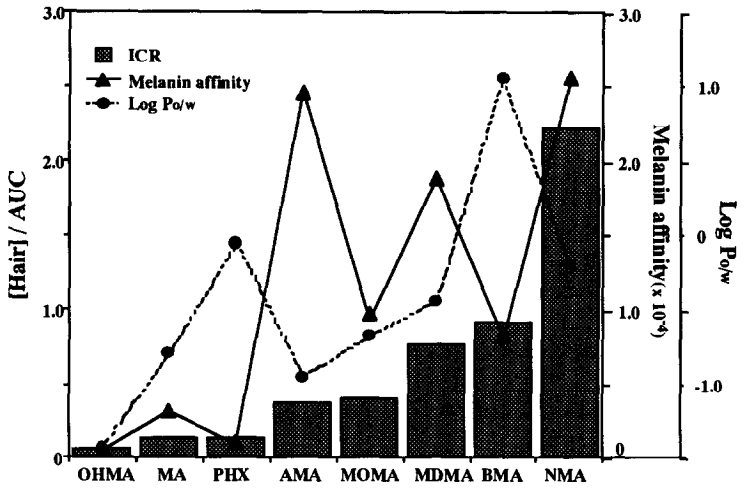


Fig. 6
The ICRs, melanin affinity and lipophilicity of 8 methamphetamines

Conclusion

Our study showed that the effect of the substituents on ICRs apparently relates to two factors, melanin affinity and lipophilicity. In comparison between PHX and MA, our data suggested that the benzene ring is more affinitive to melanin and less lipophilic than the cyclohexyl ring. As a result, PHX and MA have almost the same ICR. It is presumed that the combination of melanin affinity and lipophilicity of the drugs can determine the tendency of drug incorporation into hair.

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