

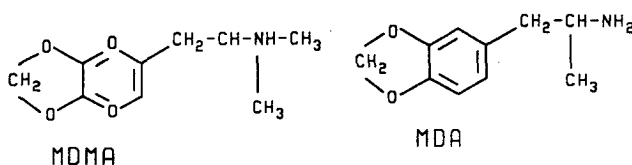
High performance liquid chromatographic assays of the illicit designer drug "Ecstasy", a modified amphetamine, with applications to stability, partitioning and plasma protein binding

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Specific, sensitive, reverse-phase high-performance liquid chromatographic (HPLC) assays of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) have been devised with analytical sensitivities as low as 2.7 ng/ml of plasma for MDMA and 1.6 ng/ml for MDA, using spectrophotometric detection at 280 nm. The assays were used to determine some properties of MDMA and MDA. Both drugs were stable in aqueous 1 M HCl, and 1 M NaOH solutions at room temperature. The half-life for MDMA was 6.6 h and for MDA was 7.1 h under the extreme conditions of 90°C and 6 M HCl. MDMA and MDA were highly stable for 28 h in plasma at 25° and 39°C. The concentrations of the drugs were unchanged in frozen plasma after 47 days. The apparent red blood cell-plasma partition coefficient determined from assayed concentrations of the drugs in plasma and erythrocytes was 1.45 for both MDMA and MDA. An equation is presented to correct drug concentration in erythrocytes for the trapped equilibrated plasma/buffer in the packed red blood cells. The fraction of MDMA and MDA bound to dog plasma proteins was determined by several methods and it is 0.34-0.40 for both drugs. The extent of protein binding was independent of the drugs' concentration.

MDMA (N, α -dimethyl-1, 3-benzodioxole-5-ethamine or 3,4-methylenedioxymethamphetamine) differs from the psychedelic MDA (α -methyl-1, 3-benzodioxole-5-ethamine or 3,4-methylenedioxymethamphetamine) by the presence of an N-methyl group. The latter has been claimed to be a metabolite of the former in the rat [1] and human [2].



Available data suggest that the actions of MDMA and MDA differ significantly [3]. Both MDMA and MDA are illicit so-called "designer drugs" with high abuse potential and at present no valid medical application [4-6]. MDMA produces effects similar to mescaline and amphetamine, *i.e.* vivid perceptual distortions and hallucinations. Possible side effects include confusion, depression, severe anxiety and sleep deprivation. However, some reports have stated [4] that, when used under controlled conditions, MDMA has few negative effects, can ease psychic trauma, and break down barriers to communication. It has been stated that "MDA appears to be a useful adjunct to psychotherapy" [3].

Data on the protein binding of MDMA and MDA in plasma do not appear in the literature. Amphetamine, which has a chemical structure close to both drugs, was found by equilibrium dialysis to be 27.1% bound to dog

plasma proteins with the extent of protein binding being independent of amphetamine concentration [7].

Although Midha *et al.* [8] have reported on a GLC assay of MDA in biological fluids, and Lim and Foltz [1, 2] on a capillary GC/MS assay under both electron ionization and chemical ionization conditions of MDA, MDMA and metabolites in biological fluids, only one paper [9] has been reported that used HPLC methods for the identification of MDMA and MDA, and that at the relatively high concentrations of 1 mg/ml in aqueous solution. This present study reports on the development of sensitive HPLC assays of MDMA and MDA in aqueous solutions and in plasma. They were used to determine an optimum solvent for extraction from biological fluids and the optimum pH values for extraction. These assays were also used to study the stabilities of the drugs in aqueous solutions and in plasma to ascertain the optimum conditions for storage in solvents and biological fluids. Red blood cell-plasma and red blood cell-plasma water partitioning, and protein binding are important values to obtain in order to predict possible dose-dependent disposition.

Experimental

Materials

The following analytical grade materials were used: MDMA hydrochloride, MDA hydrochloride (NIDA 1140 Rockville Pike, Rockville, MD 20852), glacial acetic acid, sodium acetate, 1 N sodium hydroxide, 1 N hydrochloric acid, monobasic potassium phosphate anhydrous (Fisher Scientific Co.,

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Chemical Mfg. Div., Fair Lawn, N.J. 07410), dibasic sodium phosphate anhydrous (Malinkrodt Chemical Works, St. Louis, MO, 63160). Ultrafiltration membrane cones CF50A (Amicon Division, W.R. Grace and Co., 24 Cherry Hill Drive, Danvers, MA 01923) were used. HPLC grade hexane and methanol (Fisher Scientific) and benzene (EM Science, A Division of EM Industries, Inc. Cherry Hill, N.J. 08034) were used as received.

Apparatus

A high performance liquid chromatograph equipped with fixed (at 280 nm) and variable UV absorbance detectors (Models 440 and 450 respectively), a solvent delivery system, Model M-6000, and injector WISP 710B (Waters Associates, Milford MA 01757) were used. Also used were a Zorbax CN column (DuPont Co., Medical Products Dept. Borley Mill Plaza, Wilmington, DE 19898), column C₁₈ ODS Hypersil (13 cm) and a Zorbax CN guard column. Other equipment were UV-VIS Varian large 219 Spectrophotometer (Sugarland TX), and automatic titrator (Radiometer, Copenhagen).

Determination of the UV-spectra of MDMA and MDA

The UV spectra were determined for 3.67×10^{-5} M solutions of MDMA hydrochloride and 3.48×10^{-5} M solutions of MDA hydrochloride in the following solvents: deionized water, methanol, acetonitrile, 1 M NaOH and 1 M HCl. The conditions of UV analysis were: scan rate 2 nm/sec, chart speed 10 nm/sec, range 200–400 nm, sensitivity 0.2 abs. UV spectrophotometric studies showed the same two maximal wavelengths at 234–235 and 285–286 nm for both MDMA and MDA. There were no significant effects of solvents (water, 1 M HCl, 1 M NaOH, methanol and acetonitrile) on these $\lambda_{\max}$ values. These solvents showed some variation among the specific molar absorptivities which were 3507 ± 186 for MDMA and 3302 ± 58 for MDA at 284 nm, and 4363 ± 98 for MDMA and 3807 ± 151 for MDA at 234 nm in water. Both MDMA and MDA fluoresce at 321 nm when excited at 284 nm, a fact which could be useful in HPLC analysis.

Potentiometric determination of pK_a ' values of MDMA and MDA

Aliquots (5 ml of MDMA hydrochloride, 3.67×10^{-5} M, or MDA hydrochloride, 3.48×10^{-5} M) in deionized water were acidified with 20 μ l of 1 M HCl and titrated potentiometrically with 1 M NaOH, and then back-titrated with 1 M HCl. Blank solutions without MDMA or MDA, containing 20 μ l of 1 M HCl, were titrated similarly. The pK_a ' values were estimated from the plots of the differences between the number of ml of titrant necessary to bring both blank solution and solution containing a drug to the same pH value, the Parke-Davis method [10]. The pK_a ' values so determined were 10.38 ± 0.06 (SEM) for the more basic secondary amine MDMA and 10.0 ± 0.03 for the primary amine MDA and were close to the value 9.90 reported for amphetamine [7].

HPLC-procedures

Procedure I: For aqueous solutions: The mobile phase 50:50 methanol-0.1 M acetate buffer pH 4.75 or 4.56 with UV detection at 284 or 280 nm, respectively, was used to assay aqueous solutions of MDMA and MDA containing internal standard (MDA for MDMA assay or MDMA for MDA assay).

Procedure II: Extraction procedure for MDMA and MDA in plasma: Plasma (0.95 or 1 ml) containing MDMA or MDA and internal standard was made alkaline with 150 μ l of 1 M NaOH and extracted with 4 ml of water saturated benzene by shaking (Eberbach Corp, Ann Arbor MI) at slow speed for 30 min and centrifuging for 8 min at 1240 g. The organic extract was separated and the drugs were back-extracted with 1 ml of acetate buffer pH 4.56, by shaking and centrifuging as before. The benzene layer was separated and discarded. The aqueous phase was alkalized with 150 μ l of 1 M NaOH and extracted with 4 ml fresh benzene by shaking and centrifuging as before. The organic extract was separated and evaporated under nitrogen to dryness in a laboratory hood with negative pressure. The residue was reconstituted in 0.14, 0.4 or 0.5 ml of acetate buffer pH 4.56. Then an aliquot (50–100 μ l) was injected into the chromatograph (flow rate 0.7 ml/min, 700 psi) for assay of MDMA or MDA with mobile phase 50:50 methanol-0.1 M acetate buffer pH 4.54 and UV detection at 280 nm. The same drugs were used as internal standards i.e. MDA for MDMA assay and MDMA for MDA assay. All studies with benzene were performed in a laboratory hood and with care. It may be possible to substitute non-toxic toluene. Since MDA has been claimed to be a metabolite of MDMA in various species [1, 2], another internal standard should be chosen for assay of this compound in the biological fluids of animals administered MDMA.

Typical regression coefficients ($\pm$ SE) for HPLC-assayed aqueous concentrations by HPLC Procedure I in the 250–1260 ng/ml range on peak height ratios (to the internal standard) were 817 ± 19 ($n = 7$, $r^2 = 0.998$) for MDMA and 434 ± 5.7 ($n = 8$, $r^2 = 0.999$) for MDA in the 230–1170 ng/ml range. Standard errors about regression ranged between 11 and 18 ng/ml.

Typical regression coefficients ($\pm$ SE) for HPLC-assayed plasma concentrations by HPLC procedure II in the 14–50 ng/ml range on peak height ratios were 45.6 ± 2.7 ($n = 5$, $r^2 = 0.990$, SER = 1.35 ng/ml) for MDMA and 35 ± 1.1 ($n = 6$, $r^2 = 0.990$, SER = 1.0 ng/ml) for MDA in the 15–42 ng/ml range.

Stability studies of MDMA and MDA in alkali and acid

Aliquots of aqueous stock solutions of MDMA or MDA were mixed with appropriate amounts of standard concentrates to give 6 M HCl or 1 M NaOH solutions that were 1.92×10^{-4} M in MDMA or 2×10^{-4} M in MDA. Additionally, 1 and 3 M solutions of HCl with the same concentration in MDMA were prepared. The solutions were placed in 40° or 90°C thermostated baths. Samples were withdrawn periodically and assayed by HPLC Procedure I.

Stability study of MDMA and MDA in plasma

An aliquot (0.2 ml of an aqueous solution of 1.020×10^{-5} M MDA or 1.097×10^{-5} M MDMA) was mixed with 10 ml of heparinized fresh dog plasma, thermally equilibrated at the body temperature of the dog (38.9°C) to give 2×10^{-7} M (43.1 ng/ml) or 2.15×10^{-7} M (49.4 ng/ml) solutions, respectively. Aliquots (1 ml) were removed periodically, spiked with 20 μ l of internal standard (1.020×10^{-5} M MDA for MDMA study and 1.097×10^{-5} M MDMA for MDA study) and assayed by Procedure II. Similar studies were done at 25°C and in frozen plasma. The initial concentration for MDMA was 5.377×10^{-7} M (123.5 ng/ml) and for MDA 4.915×10^{-7} M (106 ng/ml). Aliquots (0.95 ml) were withdrawn periodically, spiked with 50 μ l of internal standard (9.829×10^{-6} M MDA for MDMA study and 1.075×10^{-5} M MDMA for MDA study) and assayed by Procedure II.

Partition studies as function of pH

Partitionings between 1:1 volume ratios of water-saturated organic solvent and phosphate (pH: 7.4) or borate (pH: 7.9, 8.83, 9.81) buffer solutions as well as NaOH solutions (pH: 11.98, 12.86) were effected by shaking mixtures containing MDMA or MDA. Aliquots of the separated organic phases were evaporated under nitrogen to dryness in a laboratory hood and assayed by HPLC Procedure I. Recenstitution was effected in the same buffers as those used for the partition studies. The aqueous phases were assayed directly by HPLC Procedure I.

Plasma protein binding from red blood cell-plasma and -buffer

Whole blood and a suspension of washed red blood cells (suspended rbc's filtered 4 times) in isotonic phosphate buffer pH 7.30 were spiked with appropriate volumes (60–300 μ l) of 1.075×10^{-5} M aqueous stock solution of MDMA or 9.82×10^{-6} M MDA to give 3 ml of solution. The hematocrits of these solutions were measured (ca. 42–44). The samples were equilibrated at 38.9°C (the body temperature of the dog), centrifuged for 4 min at 3000 rpm (1240 g) and 0.95 ml of plasma or buffer in the separated phase was taken. The plasma or buffer were spiked with internal standard and assayed (C_p or C_B) by HPLC Procedure II. The packed erythrocytes (0.95 ml) after the centrifugation had experimentally determined fractions of erythrocytes ($H' = 0.82$ – 0.88) in the red blood cell concentrate. These red blood cell concentrates were mixed with 0.95 ml of deionized water and hemolyzed on a shaker for 20 min. Internal standard was added and the amount of the drug in the mixture was assayed by HPLC Procedure II and an apparent concentration, C'_{RBC} , in the volume V , of red blood cell concentrate could be determined.

The true concentration of drug in the red blood cells, C_{RBC} , can be calculated from the apparent red blood cell concentration, C'_{RBC} , by correction for plasma entrapment in the packed erythrocytes using the equation

$$C_{RBC} = \frac{C'_{RBC} - C_p(1-H')}{H'} \quad (1)$$

This equation is readily derived by rearranging the stoichiometric expression for the apparent concentration, C'_{RBC} , of drug in packed erythrocytes of an apparent H' , i.e.

$$C'_{RBC} = \frac{C_p V(1-H') + C_{RBC} V H'}{V} = C_p(1-H') + C_{RBC} H' \quad (2)$$

where the first factor in the numerator represents the amount of drug in entrapped plasma in a volume V of RBC concentrate, and the second factor in the numerator represents the true amount of drug in, or bound to, the erythrocytes.

Protein binding by ultrafiltration

Fresh ultrafiltration membrane cones were soaked for two hours in deionized water. Then a half-volume from 4 or 7 ml of 106 ng/ml solution of MDA in plasma or plasma water or isotonic phosphate buffer pH 7.30 at 38.9° was filtered through the cones with 1500 rpm centrifugation (371 g). The solutions before filtration and the subsequent filtrates were assayed by HPLC Procedure II.

The cones, pre-equilibrated by filtering a half-volume of 4 ml of isotonic phosphate buffer (pH 7.3) containing 106 ng/ml

MDA, were used to filter similar volumes of plasma or plasma water with the same concentrations of MDA, and the filtrates were assayed.

Protein binding by ultracentrifugation

Aliquots (75, 150 or 300 μ l of 1×10^{-5} M standard solution of MDMA or MDA hydrochloride in water) were mixed with fresh dog plasma (or isotonic phosphate buffer, pH 7.3) to a total of 3 ml. Plasma containing the drug was centrifuged at 35000 rpm (81415 g) for 18 hours at 4°C. Aliquots (0.95 ml) of separated plasma-water were spiked with internal standard and assayed by HPLC procedure II. Solutions of the drugs in phosphate buffer were assayed by the same HPLC procedure.

Results and discussion

Partition studies

Although ethylacetate had a higher partition coefficient for MDA and MDMA than benzene (Fig 1), the latter gave a chromatographically cleaner extract than the former and did not have the disadvantage of the ethylacetate's alkaline instability to hydrolysis which radically lowered the pH of the equilibrated buffer after partitioning. This phenomenon limited the pH values that could be used in the experimental determination of

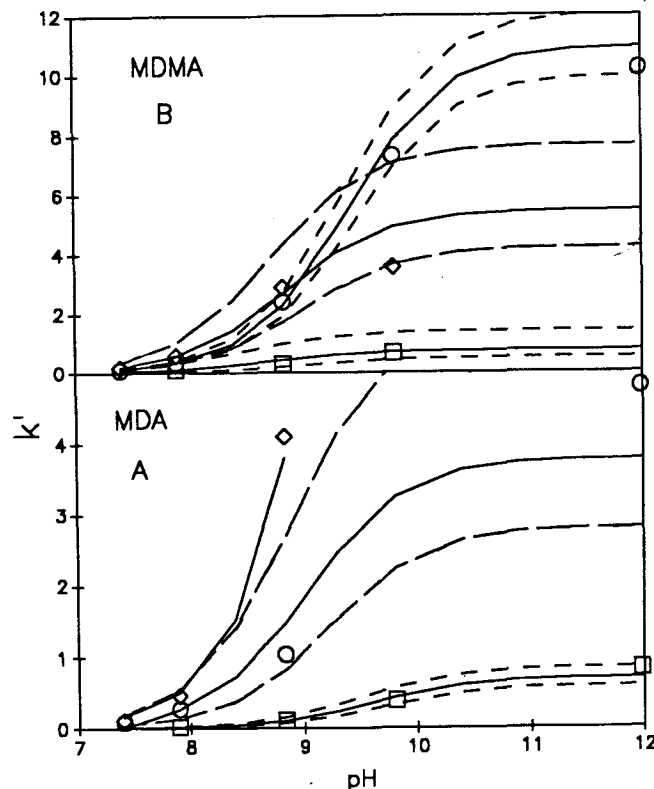


Fig. 1. Plots of apparent partition coefficients, k' , between benzene (O), hexane (□) and ethylacetate (Δ) against the buffered pH of the aqueous phase.

The lines through the data are based on the parameters given in Table 1 and the dashed lines encompass the standard errors (long dashes for benzene extractions). No standard error estimates are given for ethylacetate extractions of MDA.

the drugs' partitioning into ethyl acetate and introduced potential variability into the values obtained above pH 9.0. The pH values of equilibrated aqueous phases did not vary with the other organic solvents.

It can be shown [11] that regression of the reciprocal of the apparent partition coefficient,

$$k' = \frac{[R-NH_2]_{oil}}{[R-NH_3^+]_{aq} + [R-NH_2]_{aq}},$$

against $[H^+] = 10^{-pH}$ should have a slope of $1/k_o K'_a$ and an intercept of $1/k_o$, (the value of $1/k'$ when $[H^+] \ll K'_a$ the dissociation constant), *i.e.*

$$\frac{1}{k'} = \frac{[H^+]}{k_o K'_a} + \frac{1}{k_o} = \frac{[H^+] + K'_a}{k_o K'_a} = \frac{1}{f_{RNH_2} k_o} \quad (3)$$

when only the fraction of drug that is uncharged, f_{RNH_2} , can partition into the organic phase.

The partition coefficient,

$$k' = k_o f_{RNH_2} = k_o 10^{-pK'_a / (10 - pH)} \quad (4)$$

can be calculated at a given pH (see lines in Fig. 1) from the product of the intrinsic partition constant, k_o , and the fraction, f_{RNH_2} .

The potentiometrically determined pK'_a values of 10.38 for MDA and 10.00 for MDMA differed significantly from the pK'_a values of Table 1, estimated from eq. 3. This could possibly be rationalized if the charged fraction, $f_{RNH_3^+} = [H^+] / ([H^+] + K'_a)$, was also partitioned into the organic phase along with counter-ions from the aqueous buffers. MDMA differed significantly from the pK'_a values of Table 1, estimated from eq. 3. This could possibly be rationalized if the charged fraction, $f_{RNH_3^+} = [H^+] / ([H^+] + K'_a)$, was also partitioned into the organic phase along with counter-ions from the aqueous buffers. Buffer variation at the several pH values could also result in large errors of estimate in the partition parameters. It follows that the intrinsic partition coefficients of uncharged, $(k_o)_{RNH_2}$, and charged, $(k_o)_{RNH_3^+}$ drug could be estimated from the multiple linear regression of k' on f_{RNH_2} and $f_{RNH_3^+}$, using the potentiometrically determined values of K'_a on the premise that

$$k' = (k_o)_{RNH_2} f_{RNH_2} + (k_o)_{RNH_3^+} f_{RNH_3^+} \quad (5)$$

However, the estimated values of $(k_o)_{RNH_3^+}$ on these premises were not statistically significantly different from zero.

Table 1. Parameters of partitioning^a.

Organic Solvent	Compound	k_o	SE-range	pK'_a	SE-range
Benzene	MDA	3.8	2.8 - 5.7	9.02	8.89-9.21
	MDMA	11.0	10.0 - 12.1	9.41	9.37-9.46
Hexane	MDA	0.70	0.59- 0.85	9.58	9.49-9.69
	MDMA	0.75	0.51- 1.41	8.69	8.46-9.02
Ethyl Acetate ^b	MDA ^c	31	-	9.69	-
	MDMA	5.5	4.2 - 7.7	8.84	8.73-9.00

(SE = standard error)

^a The apparent partition coefficient at a given pH can be calculated from these parameters using eq. 4. See lines in Fig. 1.

^b Values obtained at higher buffered pH values were invalid due to pH lowering by the acidic products of alkaline hydrolysis.

^c Inadequate numbers of valid partition coefficients did not permit estimates of standard errors.

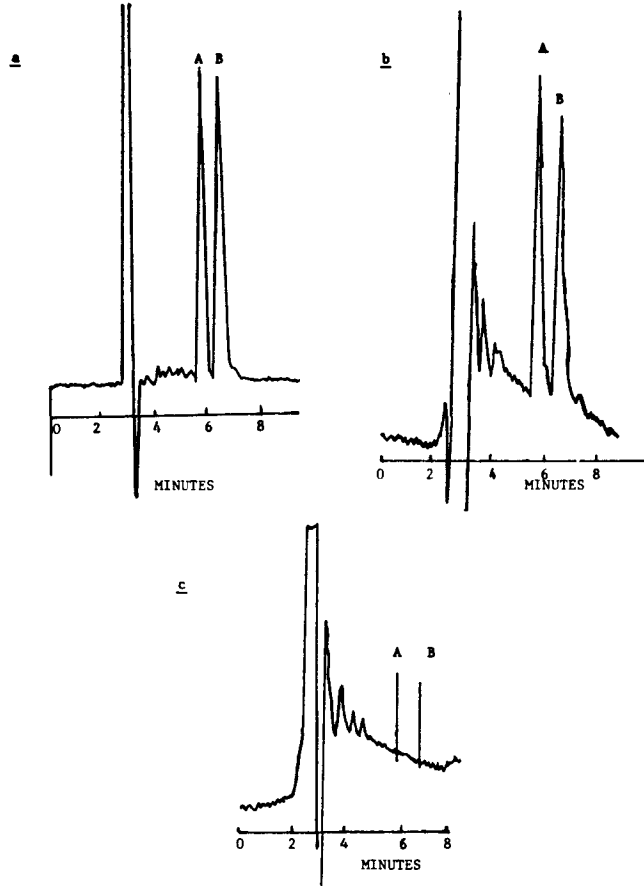


Fig. 2. Examples of reverse-phase chromatograms at 280 nm detection with 100 µl injected:

a) Aqueous solution containing (A) 586 ng/ml MDA and (B) 882 ng/ml MDMA (HPLC, Procedure I) b) Plasma (1 ml) containing 43.1 ng/ml of MDA (A) and 49.4 ng/ml of MDMA (B) after extraction with benzene (HPLC Procedure II) and evaporation of the solvent. The residue was reconstituted in 140 µl of acetate buffer pH 4.56. Retention times with guard column were: MDA, 5.83 min; MDMA, 6.69 min. c) Plasma blank after extraction, back extraction, evaporation of benzene and reconstitution in acetate buffer.

HPLC Conditions

The chosen Zorbax CN column, (pore size 5 µ, internal diameter 0.46 cm, length 15 cm, guard column 0.40x1.25 cm) and the mobile phase 50:50 methanol -0.1 M acetate buffer (pH 4.56) gave the optimal analytical sensitivity. Typical HPLC chromatograms are given in Fig. 2. The developed benzene extraction of alkaline solutions of the uncharged drug, clean-up and reconstitution (into small volumes of pH 4.56 acetate buffer) of Procedure II, well separated the two compounds from each other and from plasma components. The analytical sensitivities were (as estimated from twice the standard error about regression of concentration on peak height ratio) 3.68 ng/ml plasma for MDMA and 2.0 ng/ml for MDA.

Stabilities of MDMA and MDA in alkali, acid and plasma

MDMA and MDA showed no significant concentration change for 48 h in 1 M NaOH and 1 M HCl at 40°C.

Absorbance maxima shifted to 270 nm to indicate the production of catechol on acid hydrolysis. The stabilities of MDMA and MDA in dog plasma at 25° and 38.9°C (body temperature of the dog) were monitored up to 28 h by HPLC Procedure II and no significant changes were observed. The initial concentrations of the drugs in frozen plasma were unchanged after 47 days of freezer storage. Thus, there should be no stability problems with frozen plasma storage or an alkalization of drug solutions at room temperature.

Red blood cell partitioning and estimates of plasma protein binding [12-16]

The partition coefficients for MDMA and MDA between red blood cells and plasma (D_p) or buffer (D_B) are given in Table 2. There was no significant difference between the partitionings of MDA and MDMA between plasma and erythrocytes at the studied concentrations, $D_p = 1.45 \pm 0.05$. The fact that D_p values exceed unity implies that these drugs bind to components (e.g. membranes) of erythrocytes over and above that which would result from volume equilibration. The fractions bound to plasma proteins were 0.40 ± 0.01 (Table 3) at the respective concentrations of 123.5 and 106.0 ng/ml for MDMA and MDA.

Plasma protein binding by ultrafiltration

There was no significant difference by t-test between the concentrations of MDA in pH 7.3 phosphate buffer before and after ultrafiltration through filter cones. The initial concentration of several studies, calculated to be

Table 2. Fraction of plasma concentration of drug bound to proteins determined by drug partitioning^a between plasma or buffer and erythrocytes^b [12-16].

	MDMA	MDA
C_{p0} , ng/ml	123.5	106.0
C_p^d	75.3 $\pm$ 1.1	62.5 $\pm$ 0.8
$C_{RBC}^{e,PL}$	111.4 $\pm$ 1.3	90.7 $\pm$ 4.5
D_p^f	1.48 $\pm$ 0.04	1.45 $\pm$ 0.08
C_B^g	54.3 $\pm$ 0.7	48.9 $\pm$ 2.1
$C_{RBC}^{h,B}$	133.5 $\pm$ 1.6	119.4 $\pm$ 1.3
D_B^i	2.46 $\pm$ 0.03	2.45 $\pm$ 0.10
f_b^j	0.398 $\pm$ 0.007	0.408 $\pm$ 0.008

^a After 30 min equilibration in blood (44 hematocrit) or buffer suspension of erythrocytes (42 hematocrit) in buffer.

^b Three replicate studies of each drug were made. The values given are the means $\pm$ standard error of the means.

^c Initial concentration of drug in blood and in buffer suspension.

^d Concentration of drug in separated plasma phase of blood.

^e Concentration of drug in separated red blood cells (hematocrit of 87) as corrected for plasma entrapment of drug in red blood cell concentration.

^f The red blood cell-plasma partition coefficient of drug i.e. C_{RBC}/C_p . The ratios of the mean values of C_{RBC} and C_p were the same as the means of the ratios.

^g Concentration of drug in separated buffer phase of buffer suspension of erythrocytes.

^h Concentration of drug in red blood cells (hematocrit of 82) as corrected for buffer contamination.

ⁱ The red blood cell-buffer partition coefficient of drug i.e. C_{RBC}^{B}/C_B . The ratios of the mean values of C_{RBC}^{B} and C_B were the same as the means of the ratios.

^j As calculated from $f_b = 1 - D_p/D_B$ [12-16].

Table 3. Plasma protein binding by ultracentrifugation^a.

	C_p	C_{pw}	f_b^b
MDA	33.9 $\pm$ 1.5	22.6 $\pm$ 1.0	0.334 $\pm$ 0.005
	103.9 $\pm$ 3.3	67.4 $\pm$ 0.7	0.349 $\pm$ 0.023
	191.0 $\pm$ 8.7	114.9 $\pm$ 5.6	0.395 $\pm$ 0.046
MDMA	60.96 (n = 2)	37.6 $\pm$ 2.0	0.414 (n = 2)
	94.7 $\pm$ 2.8	63.2 $\pm$ 4.9	0.330 $\pm$ 0.065
	216.9 $\pm$ 3.1	143.0 $\pm$ 2.3	0.341 $\pm$ 0.009

^a Concentration values in plasma (C_p) and separated plasma water (C_{pw}) in ng/ml are means $\pm$ SEM of three replicate studies except for the MDMA C_p value of 60.96 ng/ml where n = 2.

^b The bound fraction of plasma drug concentration as calculated from $f_b = (C_p - C_{pw})/C_p$. The mean values for all plasma concentrations (C_p) values were 0.36 ± 0.03 for both MDA and MDMA. The average calculated f_b from the means of C_p and C_{pw} given in the table were the same as the means of the three individually calculated f_b values at a given plasma concentration.

106 ng/ml, were 104.4, 103.8, 103.5 and 98.7 ng/ml. The respective filtrates on half-filtration with 1500 rpm centrifugation of 4 or 7 ml of buffer were 103.2, 91.9, 96.7 and 103.4 ng/ml to indicate no significant binding of drug to the filter cones. However when spiked plasma water was half-filtered through filter cones (pre-equilibrated with phosphate buffer spiked with the same 108.9 ng/ml drug concentration contained in the plasma water) only 68.7% of drug concentration appeared in the filtrate, implying that 31.3% of the drug concentration was sequestered to plasma water components and unfiltered by the cones. Since the plasma ultrafiltrate concentration in the derived plasma-water was 45% of the filtered 108.9 ng/ml plasma concentration, 65% of the drug concentrate could be sequestered to plasma and plasma water components. The difference, 0.65-0.313, indicated that $f_b = 0.337$, the fraction of the plasma concentration that is bound to plasma protein. This value is close to the $f_b = 0.40$ obtained for MDA at the same concentration by the red blood cell partitioning studies.

Plasma protein binding by ultracentrifugation

The data from these studies are given in Table 3 and there were no statistically significant differences in the fractional plasma protein binding among the different plasma concentrations. The mean f_b values among all drug concentrations were 0.36 ± 0.03 for both MDA and MDMA. These values were in good agreement with the 0.34 estimated for MDA from the ultrafiltration studies and the 0.40 for MDA and MDMA estimated from the red blood cell partition studies.

These f_b values are close to the 0.27 value reported for amphetamine in dog plasma [5] by equilibrium dialysis. This moderate and non-dose dependent plasma protein binding should have no dose-dependent effects on drug disposition, at least in the dog.

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