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DETERMINATION OF PHARMACOLOGICAL LEVELS OF HARMANE, HARMINE AND HARMALINE IN MAMMALIAN BRAIN TISSUE, CEREBROSPINAL FLUID AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

Increased blood aldehyde levels, as occur in alcohol intoxication, could lead to the formation of β-carbolines such as harmane by condensation with indoleamines Endogenous β-carbolines, therefore, should occur in specific brain areas where indoleamine concentrations are high, whilst exogenous β-carbolines should exhibit an even distribution The author presents direct and sensitive methods for assaying the β-carbolines harmane, harmine and harmaline in brain tissue, cerebrospinal fluid and plasma at picogram sample concentrations using reversed-phase high-performance liquid chromatography with fluorimetric detection and minimal sample preparation Using these assay methods, it was found that the distribution of β-carbolines from a source exogenous to the brain results in a relatively even distribution within the brain tissue

INTRODUCTION

Much interest has lately developed in β-carbolines and their derivatives (Fig. 1) because of the variety of neuropharmacological and psychopharmacological actions they can invoke in humans and mammals [1]. β-Carbolines occur naturally as harmala alkaloids in *Peganum harmala* and other plant species [2] and can be produced by pyrolysis of indoles and proteins [3] and thus occur in cigarette smoke and charcoal grilled meats. The tetrahydro-β-carbolines, also known as tryptolines, can be produced in vitro by condensation of tryptamines with aldehydes [4]. Should this occur in vivo, then increased blood

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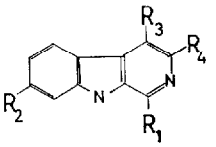

	R_1	R_2	R_3	R_4
Harmane	$-CH_3$	-H	-H	-H
Harmine	$-CH_3$	$-OCH_3$	-H	-H
Harmaline	$-CH_3$	$-OCH_3$	$-H_2$	$-H_2$
β-Carboline	-H	-H	-H	-H

Fig 1 Chemical structures of harmane, harmine, harmaline and β-carboline

acetaldehyde, as occurs in alcohol intoxication, could lead to the formation of tryptolines which would then be available for in vivo oxidation to the hallucinogenic and tremorogenic aromatic β-carbolines

Work by Bidder et al [5], Rommelspacher et al [6] and Shoemaker et al. [7] tends to support the possibility that some β-carbolines are formed in vivo and even in the brain itself. Robertson et al. [8] and Rommelspacher et al [9] have shown that 6–8 μM (1.5–2 μg/ml) solutions of the fully aromatic harmane and norharmane can displace flunitrazepam from the benzodiazepine receptor, thus making them possible contenders as natural ligands for that receptor.

Any β-carbolines found in brain tissue could be from an endogenous source in the brain, a source elsewhere in the body or from food or environmental sources. Thus before investigating the presence of harmane in brain tissue, it is first necessary to determine whether exogenous β-carbolines cross the blood–brain barrier easily and, if they do, whether they distribute evenly in the brain.

One would expect endogenous β-carbolines to occur only in those areas where indoles (e.g. serotonin) are found in high concentrations, whilst exogenous or pharmacological β-carbolines should be evenly distributed.

In order to investigate the distribution of exogenous β-carbolines into brain tissue, reliable and sensitive methods of assay are necessary.

Various methods have been described for the assay of harmala alkaloids at high concentration in aqueous media. These methods are either gravimetric and volumetric [10,11] or fluorimetric [12] and are obviously too unselective for the required assays. Neither can the paper chromatographic (PC), liquid chromatographic (LC) and thin-layer chromatographic (TLC) methods [13–16] be considered suitable when compared with gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods [6,17–26]. Following the development of the GC assay method for harmane [17] other

investigators also used GC methods of assay [18–21]. In 1980, Rommelspacher et al. [6] developed an HPLC method using UV absorption for the detection of harmane in urine and Sasse et al. [22] used reversed-phase HPLC with UV–VIS and fluorimetric detection for the determination of harmine, harmol and harmalol in cell culture extracts of *Peganum harmala*. The most recent methods [23–26] have involved the use of reversed-phase ion-pair chromatography on a home made tri-*n*-butyl phosphate column with UV and fluorimetric detection [23] and reversed-phase separation on a C_{18} column deactivated for basic compounds, with UV detection, for assay in the microgram range after tissue extraction [24].

Bosin and Faull [25] used an octadecylsilane (C_{18}) column and fluorimetric detection for the assay, in the lower nanogram range, of derivatized harmane, harmol and norharmol in rat lung extracts. However, the advantages of the simple isocratic elution used in this method are offset by the time-consuming extraction and derivatization procedures. Cavin and Rodriguez [26] used a C_{18} column for the separation of the underivatized β-carbolines in an extract of dried butter-fly tissue, but required gradient elution to elute harmaline after 40 min.

The extensive sample pretreatment used in the above assay is counter-indicated in the assay of unstable compounds, and this is particularly so in the case of β-carbolines where, apart from their instability, semi-carbazide is added to hinder their formation in vitro during extensive sample preparation.

Thus a method was sought which would give a short elution time and minimize the procedures necessary in sample preparation, but be sufficiently sensitive, selective and reproducible to use for distribution studies on fully aromatic harmane, its methoxy derivative harmine and semi-saturated harmaline The results of this investigation are reported below.

EXPERIMENTAL

Reagents and standards

All solvents used were spectroscopic grade from Burdick and Jackson (Muskegon, MI, U.S.A.) and all water was purified by the Milli-Q system (Millipore, Milford, MA, U.S.A.). Acetonitrile and methanol used in sample preparation were rendered acetaldehyde-free by glass distillation in the presence of sodium bisulphite. All other reagents were analytical reagent grade.

The internal standards sotalol and opipramol were kindly donated by Bristol-Myers Group (Braamfontein, Johannesburg, South Africa) and Geigy Pharmaceuticals (Isando, Kempton Park, South Africa), respectively. Harmane, harmine and harmaline were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample collection

Young Sprague–Dawley rats, ca. 250 g, were dosed intraperitoneally with 10 mg/kg of the relevant β-carboline. Control rats were injected with the drug vehicle only and were used for baseline values Uninjected rats were used as a source of tissue for standards.

The rats were guillotined 1 h after dosing and the brains immediately removed The relevant areas of hypothalamus, thalamus, striatum, frontal cortex and cerebellum were rapidly dissected out on a chilled glass plate on an ice-bath, folded into weighed aluminium foil and immediately frozen in liquid nitrogen for storage at −18°C until reweighed and assayed. The samples ranged in size from 5 to 20 mg wet tissue.

Sample preparation

Brain tissue Each weighed harmane- or harmine-dosed tissue sample was homogenized in a glass–PTFE homogenizer with a radial clearance of 0.25 mm; the pestle was rotated by hand. The most reproducible results were attained when using unsilanized borosilicate glass tubes. A 200-μl aliquot of a cold mixture (4°C) of methanol–0.05 *M* phosphate buffer, pH 4.6, containing 5 mg/ml semicarbazide and 1 mg/ml sotalol (1 1, v/v) was added to each tissue sample and homogenized for 1 min. In the case of harmaline-dosed tissue samples, the sotalol was replaced by 2 mg/ml opipramol The homogenate was allowed to stand in a 4°C bath for 5 min before spinning down the cell residue at 2000 *g* for 2 min. The slightly cloudy supernatant was retained and the residue vortex-mixed with 100 μl of the methanol buffer solution, again centrifuged and the supernatant retained A 300-μl aliquot of ice-cold acetonitrile was added to the combined supernatants to denature the protein, vortexed for 5 s and centrifuged at 2000 *g* for 5 min. The clear supernatant was then injected directly into the chromatograph without further extraction.

Blank and control samples, as well as standards in the 200–800 pg/ml range, were similarly homogenized and extracted, but in a methanol–buffer solution containing 50 μg/ml sotalol instead of 1 mg/ml sotalol as used for the dosed harmane and harmine samples and 200 μg/ml opipramol instead of 2 mg/ml opipramol as used for the harmaline assays.

Cerebrospinal fluid and plasma Human cerebrospinal fluid and serum samples spiked with the alkaloids gave reproducible results when 100 μl of a cold mixture (4°C) of methanol–0.05 *M* phosphate buffer containing 10 mg/ml semicarbazide and 2 mg/ml sotalol (1 1, v/v) were added to 100 μl plasma or cerebrospinal fluid and 200 ml acetonitrile were added to denature the protein. The sample was vortex-mixed for 5 s, centrifuged at 2000 *g* for 5 min and the supernatant chromatographed.

Preparation of standards

Standard solutions were prepared of each of the alkaloids (range 10 ng/ml to 10 μg/ml) in methanol–0.05 *M* phosphate buffer, pH 4.6 (1 1, v/v).

Brain tissue Blank brain tissue, ca 15 mg, was spiked with 20 μl standard solution, i.e. 200 pg to 200 ng alkaloid standard before homogenization and extraction as described for dosed and control sample tissues.

Plasma and cerebrospinal fluid A 100-μl aliquot of blank cerebrospinal fluid or plasma was spiked with 20 μl of standard solution and 100 μl of methanol–0.05 *M* phosphate buffer containing 10 mg/ml semicarbazide and 2 mg/ml sotalol (1 1, v/v), 100 μl of acetonitrile were then added and the mixture was vortexed-mixed and centrifuged as before. Standards representing 4–20 pg alkaloid were used for the blank and control sample determinations.

Chromatography

HPLC separation was performed on a Spectra-Physics 8100 liquid chromatograph fitted with a Valco autoinjector valve. A 10-μl loop was used for the dosed-sample assays and a 100-μl loop for the blank and control samples as well as for the low standards.

The same 250 mm × 4.6 mm I.D. Spherisorb S5 ODS1, singly end-capped, 5-μm column from Phase Separations (Clywd, U K) preceded by a 40 mm × 4 6 mm I.D. guard column with the same packing was used for the determination of all three alkaloids. Isocratic elution was performed with mobile phases of acetonitrile–methanol–0.02 *M* phosphate buffer, pH 7.2, in the ratio 39 13 48 (v/v) for harmane and 30 30 40 (v/v) for harmine at 2 0 ml/min and 34 °C. For harmaline, 1 ml triethylamine was added to 1 l of the buffer which was readjusted to pH 7 2 with ammonium dihydrogenphosphate. The mobile phase ratio used for harmaline was 40 30 30 (v/v) at 2 0 ml/min and column temperature was 38 °C.

A Perkin-Elmer 650-10 dual-monochromator fluorescence detector was used for sensitive and selective detection in the required concentration range. The excitation and emission wavelengths were 238 and 435 nm, 244 and 425 nm and 374 and 485 nm for harmane, harmine and harmaline, respectively.

RESULTS AND DISCUSSION

Chromatography

Previously published methods on the HPLC separation of β-carbolines in a biological matrix [22,25,26] indicated the use of octadecylsilane reversed-phase chromatography for the assay of these materials Since a method with minimal sample preparation, but with a sensitivity in the pg/mg of tissue range was being sought, UV–VIS absorption was inadequate as the means of detection. Both fluorescence and electrochemical detection give sensitivity in the required range and all alkaloids, as nitrogen-containing compounds, should be electrochemically active to a greater or lesser degree. However, dual-monochromator fluorescence, whilst less sensitive, is more selective than electrochemical detection and is particularly indicated in the case of naturally flu-

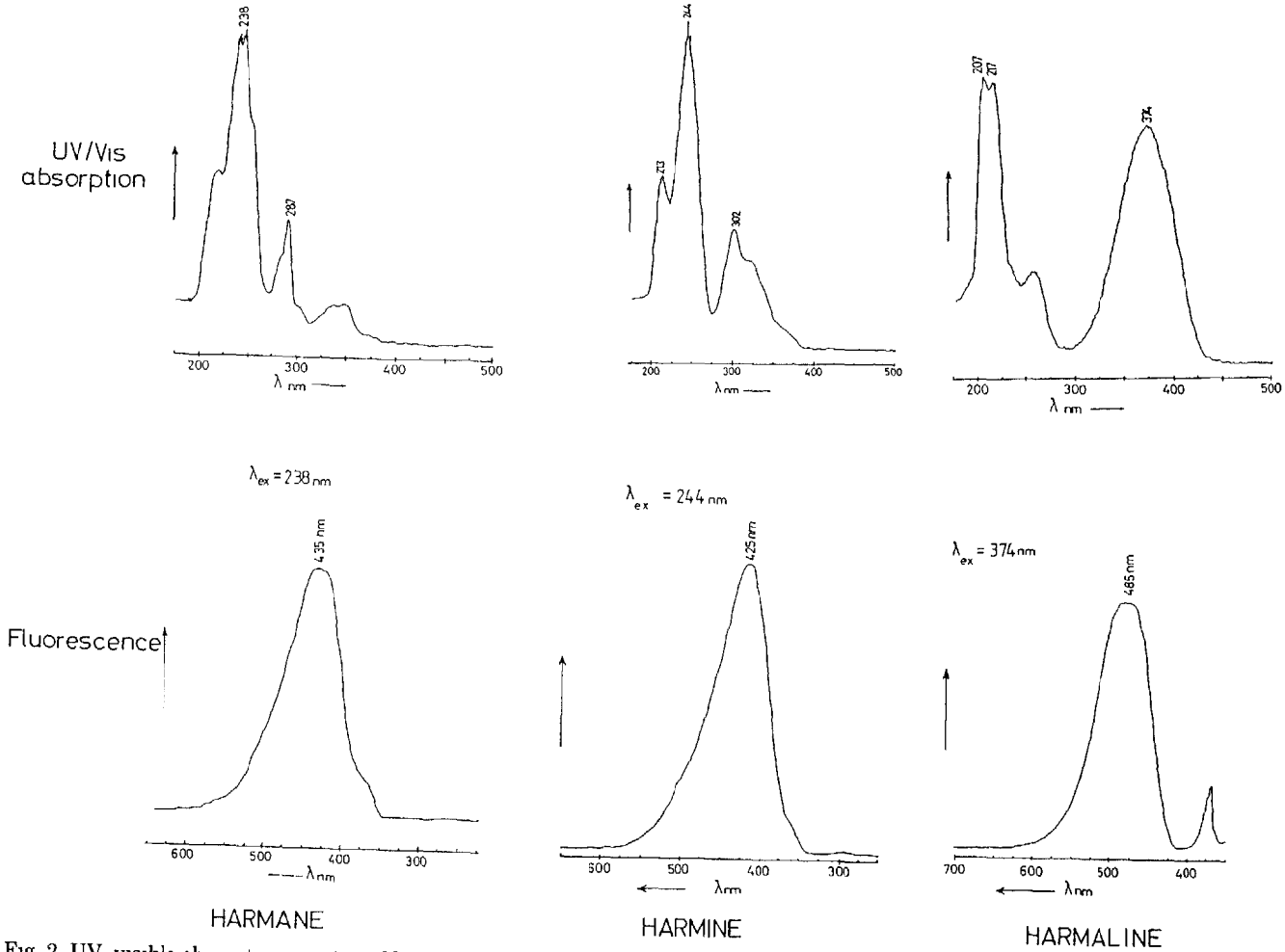

Fig 2 UV-visible absorption spectra of harmane, harmine and harmaline and fluorescence emission spectra at excitation wavelengths used for the assays

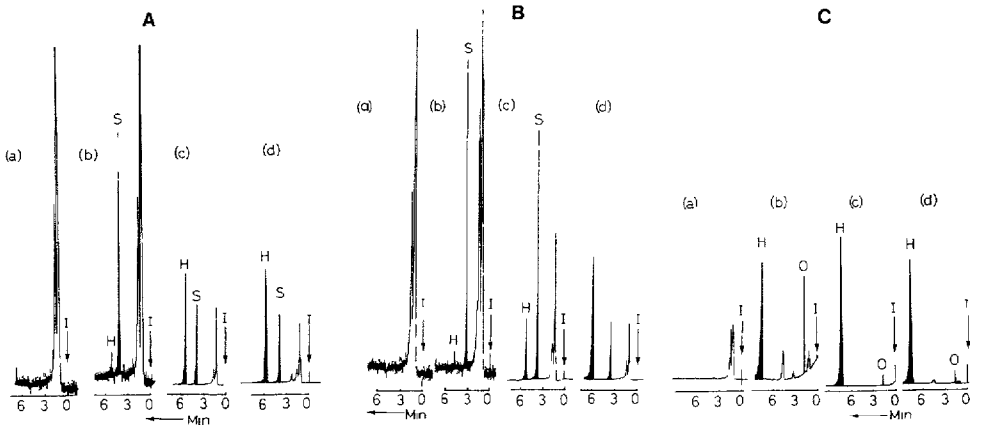

Fig 3 Chromatograms of standards and samples containing harmane, harmine and harmaline (A) Harmane $\lambda_{ex}=238$ nm, $\lambda_{em}=435$ m (a) Blank tissue, (b) blank tissue spiked with 20 pg harmane and homogenized in methanol-buffer solvent containing 50 μg/ml sotalol, (c) blank tissue spiked with 4 ng harmane and homogenized in methanol-buffer solvent containing 1 mg/ml sotalol, (d) striatal tissue after dosing with harmane, homogenized in methanol-buffer solvent containing 1 mg/ml sotalol Peaks H = harmane, S = sotalol, I = inject (B) Harmine $\lambda_{ex}=244$ nm, $\lambda_{em}=425$ nm (a) Blank tissue, (b) blank tissue spiked with 10 pg harmine and homogenized in methanol-buffer solvent containing 50 μg/ml sotalol, (c) blank tissue spiked with 1 ng harmine and homogenized in methanol-buffer solvent containing 1 mg/ml sotalol, (d) cerebellar tissue after dosing with harmine, homogenized in methanol-buffer solvent containing 1 mg/ml sotalol Peaks H = harmine, S = sotalol, I = inject (C) Harmaline $\lambda_{ex}=374$ nm, $\lambda_{em}=485$ nm (a) Blank tissue, (b) blank tissue spiked with 100 pg harmaline and homogenized in methanol-buffer solvent containing 200 μg/ml opipramol, (c) blank tissue spiked with 10 ng harmaline and homogenized in methanol-buffer solvent containing 2 mg/ml opipramol, (d) thalamic tissue after dosing with harmaline and homogenized in methanol-buffer solvent containing 2 mg/ml opipramol Peaks H = harmaline, O = opipramol, I = inject

orescent materials such as the aromatic harmala alkaloids. Thus the sample clean-up need not to be as extensive as for a less selective detector.

UV–VIS spectra of the analytes showed a λ_{max} for harmane at 238 nm with absorption ca. three times more than at the secondary peak at 287 nm (Fig 2) A similar pattern was found for harmine with λ_{max} at 244 nm. Harmaline had two major peaks at 217 and 314 nm. All three compounds showed maximum fluorescence when using λ_{max} as excitation wavelength on a scanning fluorimeter. The fluorescence detector used had a wavelength minimum of 220 nm, thus 374 nm was used as the excitation wavelength for harmaline. Difficulty was experienced in finding a suitable internal standard for the harmaline assay. Opipramol was eventually found to be visible under the fluorescence conditions used. Although its retention time of 1 1 min is rather short for internal standard purposes, the fact that no partitioning organic extraction is

used in the sample preparation means that the opipramol concentration remains representative of the amount of sample injected.

The standard curves were constructed in two ranges for each alkaloid on the basis of peak-height ratio of the alkaloid to the internal standard. All were linear in each range in brain tissue, cerebrospinal fluid and plasma.

The intra-assay coefficients of variation over ten assays were 0 30, 0.31 and 0 21% and the inter-assay coefficients of variation were 2.8, 4.6 and 2 7 for harmane, harmine and harmaline, respectively, in brain tissue and 3.5, 4 6 and 2.8% in plasma at the 50 ng/ml level (1 ng per sample). The respective extraction efficiencies were 99, 93 and 92% and the on-column detectabilities for harmane, harmine and harmaline were 10, 5 and 2 pg in brain tissue. Chromatograms of blank tissue, standards and samples under the assay conditions used are shown in Fig. 3.

Animal study

This distribution study was undertaken prior to an extensive study on endogenous harmane and norharmane formation in the brain. The object was to find if exogenous harmane would be evenly distributed in the brain and thus exhibit a sufficiently constant background value to allow endogenous formation in specific areas of the brain to be detected

Six rats were used to investigate the brain distribution of the dosed target substance, harmane To investigate the effects of alkaloid polarity on this distribution three rats were used to investigate the distribution of each of the harmane derivatives, harmine and harmaline.

TABLE I

CONCENTRATIONS OF ALKALOIDS IN THE CEREBELLUM OF RAT BRAIN AND RATIOS FOUND IN OTHER BRAIN AREAS WITH RESPECT TO THE CEREBELLUM

Concentrations were determined 1 h after dosing intraperitoneally with 10 mg/kg of the relevant alkaloid

Drug	Rat No	Concentration (pg/mg of wet tissue)		Ratio brain area to cerebellum			
		Mean ± S D of all areas	Cerebellum	Frontal cortex	Hypothalamus	Thalamus	Striatum
Harmane	1	3402 ± 838	2807	1 05	1 34	1 51	1 16
	2	2368 ± 806	2445	0 84	1 24	0 91	0 85
	3	3911 ± 753	3699	0 90	1 19	1 05	1 02
	4	2019 ± 198	1971	1 07	0 92	1 02	1 09
	5	2059 ± 201	1971	1 00	1 00	1 11	1 18
	6	1576 ± 163	1618	0 88	0 96	1 02	1 04
Harmine	7	3732 ± 228	4015	0 93	0 86	0 89	0 97
	8	2019 ± 128	1971	1 08	0 92	1 02	1 09
	9	1576 ± 144	1610	0 88	0 96	1 02	1 04
Harmaline	10	7133 ± 1510	6854	1 24	0 69	1 12	1 15
	11	5744 ± 996	5590	1 16	0 74	1 10	1 14
	12	7872 ± 899	6413	1 27	1 25	1 40	1 22

The results obtained are presented in Table I in which the amounts found in different areas of a brain are expressed as a ratio of the amount found in the cerebellum of the same brain. As can be seen from the results, the distribution to various brain areas within a single rat is very consistent in comparison with the differences in levels found for the same drug dosage between different rats.

This variation in distribution of the same alkaloid between rats could be due to the intraperitoneal route used for dosing, which would subject the drugs to possible first-pass clearance on absorption and transport in the hepatic portal system. Since hepatic and renal function may differ slightly from rat to rat of the same litter this could be one source of differences

The large differences in mean levels between the three drugs are probably due to polarity differences of the substances affecting the degree of blood–brain barrier penetration and hepatic and renal elimination. Hepatic aromatic hydroxylation will affect harmane and harmine more than the semi-saturated harmaline, whilst the more polar harmane and harmine will undergo faster renal elimination than harmaline. On the other hand, penetration of the blood–brain barrier may be easier for the more lipophilic harmaline and account for its higher levels in brain tissue.

CONCLUSION

From the results obtained, it is evident that, irrespective of the final concentrations found in each rat brain, the distribution of β-carbolines from a source exogenous to the brain results in a relatively even distribution within the brain tissue. Should a markedly higher level be found in one specific area than in another area of the brain, it can reasonably be assumed it is due to endogenous production.

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