Harmane and Harmalan Are Bioactive Components of Classical Clonidine-Displacing Substance†

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ABSTRACT: Elucidation of the structure of the endogenous ligand(s) for imidazoline binding sites, clonidine-displacing substance (CDS), has been a major goal for many years. Crude CDS from bovine lung was purified by reverse-phase high-pressure liquid chromatography. Electrospray mass spectrometry (ESMS) and nuclear magnetic resonance (^{1}H NMR) analysis revealed the presence of L-tryptophan and 1-carboxy-1-methyltetrahydro-β-carboline in the active CDS extract. Competition radioligand binding studies, however, failed to show displacement of specific [^{3}H]clonidine binding to rat brain membranes for either compound. Further purification of the bovine lung extract allowed the isolation of the β-carbolines harmane and harmalan as confirmed by ESMS, ^{1}H NMR, and comparison with synthetic standards. Both compounds exhibited a high (nanomolar) affinity for both type 1 and type 2 imidazoline binding sites, and the synthetic standards were shown to coelute with the active classical CDS extracts. We therefore propose that the β-carbolines harmane and harmalan represent active components of classical CDS. The identification of these compounds will allow us to establish clear physiological roles for CDS.

Clonidine was first synthesized by Stahle (*1*) in an attempt to develop a vasoconstricting and decongesting agent. Unexpectedly, this compound was found to lower blood pressure and heart rate and was quickly recognized as the first of a new class of potential therapeutic agents. Excitement over the potential of a panacea drug was quickly lost, however, when a number of severe side effects including dizziness, sedation, oedema, and respiratory depression were observed. The reduction of side effects in all but a minority of patients is thought to be attributable to a moderate selectivity of type I imidazoline binding sites (I-1)[1] over α_2-adrenoceptors (*2*). A drive to develop antihypertensive drugs that lack the side effects associated with clonidine has spawned the development of numerous new compounds such as moxonidine and rilmenidine. All of these clonidine-like compounds contain a common amidine moiety or close mimic of this structure (Figure 1) (*3*).

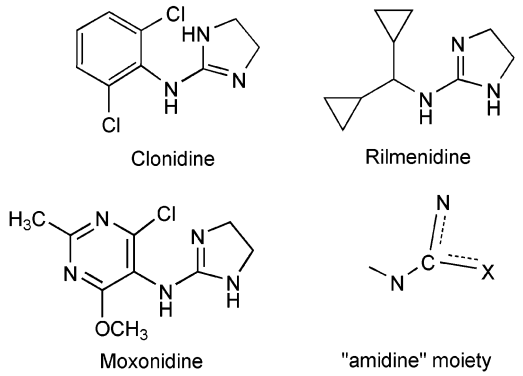

FIGURE 1: Chemical structures of imidazoline binding site ligands.

Antihypertensive drugs are hypothesized to function through the imidazoline binding sites whose subgroups are distributed over the rostroventrolateral medulla, the cardiovascular tissue, and sympathetic nerves of various species, including humans (*4*, *5*). I-1 binding sites are thought to mediate the central hypotensive actions of the clonidine-like drugs, but currently the molecular nature of these sites is unknown. Some I-2 binding sites have been shown to reside on monoamine oxidase (*6*), which may explain how I-2 site-selective drugs elevate central monoamines and are potential antidepressants. Further studies have shown that there are also I-2 binding sites on proteins distinct from monoamine oxidase. The densities of these alternate I-2 sites change in a range of human psychiatric disorders including Alzheimer's disease, Huntington's disease, opiate addiction, and depres-

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[1] Abbreviations: CDS, clonidine-displacing substance; COSY, correlated spectroscopy; ESMS, slectrospray mass spectrometry; HSQC, heteronuclear single-quantum coherence; I-1, type 1 imidazoline binding site; I-2, type 2 imidazoline binding site; NMR, nuclear magnetic resonance; RP-HPLC, reverse-phase high-pressure liquid chromatography.

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sion (*7*−*9*). Activation of a third binding site subtype, putative pancreatic islet I-3 binding sites, is thought to modulate insulin secretion (*10*).

The majority of the currently used hypotensive compounds are synthetic imidazoline derivatives and are not the naturally occurring ligands which are as yet unknown. If the endogenous ligands could be identified, they may have an improved selectivity profile (e.g., for I-1) and hence could be the starting point for the development of a new range of antihypertensive agents. One possible source of these natural ligands is classical clonidine-displacing substance or CDS. This endogenous substance, originally isolated from bovine brain (*11*), potently displaced specific [^{3}H]clonidine binding to rat brain membranes. CDS was subsequently shown to possess agonist affinity not only for α_2-adrenoceptors but also for I-1 and I-2 binding sites (*12*, *13*). CDS not only is present in central tissues but also has been discovered peripherally (*14*) and has been suggested to act as a circulating hormone (*15*).

Several candidates for classical CDS have been proposed, the most extensively studied of which is agmatine (*16*). Similar to the clonidine-derived drugs, agmatine also possesses the common amidine motif and can accordingly displace specific radioligand binding from α_2-adrenoceptors and imidazoline binding sites. Agmatine's lack of potency, however, discounts it from being the elusive component present in classical CDS.

In the past, the nonuniformity of extraction procedures has led to CDS fractions of varying compositions, a factor which has certainly resulted in confusion surrounding the true pharmacological activities of classical CDS. Microinjection of CDS into cat brainstem, for example, has been shown to increase blood pressure (*17*), yet decrease rat systemic blood pressure (*18*). Clearly, the identification of the active component(s) of CDS is necessary before the exact physiological role may be determined. Over the past few years we have partially characterized CDS from a number of tissues. High levels of CDS have previously been observed in lung tissue (*19*). As our aim was to definitively characterize the active component of CDS, we chose this source of tissue as it provides concentrations appropriate for NMR study (*20*, *21*). In this paper we conclusively identify two active components present in CDS.

MATERIALS AND METHODS

Chemicals and Reagents. [^{3}H]Clonidine (specific activity = 65 Ci mmol^{-1}) was stored at −20 °C at a concentration of 1 mCi mL^{-1} (NEN Life Science Products, U.K.). [^{3}H]-2-BFI [2-(2-benzofuranyl)-2-imidazoline] (specific activity = 69 Ci mmol^{-1}) was stored at a concentration of 0.2 mCi mL^{-1}. [^{3}H]RX821002 (specific activity 58 Ci mmol^{-1}) was stored at a concentration of 1 mCi mL^{-1} (Amersham International, U.K.). Rauwolscine, unlabeled clonidine, L-tryptophan, tryptamine (Sigma Chemical Co., U.K.), agmatine sulfate (Aldrich Chemical Co., U.K.), and BU224 [2-(4,5-dihydroimidazol-2-yl)quinoline] (Tocris Cookson, U.K.) were purchased as indicated. Harmane (1-methyl-β-carboline) was purchased from Indofine Chemical Co., Inc. (Somerville, NJ). All chemicals and reagents used were of the highest analytical grade available.

Synthesis of β-Carboline Standards. 1-Methyltetrahydro-β-carboline-1-carboxylic acid and 1-methyltetrahydro-β-carboline-3-carboxylic acid were synthesized as described previously (*22*, *23*). Harmalan (1-methyldihydro-β-carboline) was synthesized using methods described by Spath and Lederer (*24*).

Bovine Lung Extraction and RP-HPLC Purification. The initial extraction and purification method used for the classical CDS preparations was as described previously (*25*) modified from Singh et al. (*19*). For the extraction of β-carbolines, 50 g wet weight of bovine lung tissue was homogenized in 5 volumes of ice-cold 0.1 M NaOH and then centrifuged at 32000*g* for 20 min at 4 °C. The resulting supernatants were extracted into 5 volumes of ice-cold ethyl acetate. The emulsion was separated by centrifugation (32000*g* for 20 min at 4 °C), and the organic layer was collected and then evaporated to dryness using a rotary evaporator. The residue was reconstituted in 10 mL of 20% methanol (v/v), briefly spun on a microcentrifuge to remove any particulate matter, and loaded onto a C18 Sep-Pak column (J. T. Baker, Phillipsburg, NJ). The active component was eluted using 10 mL of 100% methanol and evaporated to dryness. The residue was dissolved in 0.5 mL of 0.2% TFA (v/v), sonicated for 30 min, and microcentrifuged. Aliquots of 50 μL were injected onto a reverse-phase (RP) HPLC column (C_{18}, 250 × 4.60 mm, 5 μm; Luna, Phenomenex) and eluted using an isocratic gradient of acetonitrile/TFA/water (20:0.1:78.9 v/v) (*26*) at 1 mL min^{-1} over a 20 min run. A UV scan was performed on pure harmane and harmalan, giving a maximum at 237 nm and a smaller peak at 287 nm. The ultraviolet (UV) absorbance was measured at 235 nm. Pure harmane (3 μM) and harmalan (10 μM) standards were used to calibrate the column.

Membrane Preparation for Competition Radioligand Binding Assays. Rat whole brain and kidney membranes were prepared from male Wistar rats (200−300 g) (*27*). Prior to the competition binding studies, pellets were thawed and washed twice in assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.4 at 4 °C) by repeated centrifugation (32 000*g*, 20 min at 4 °C). The membranes were resuspended in assay buffer to give 300−450 μg of protein per assay tube. The protein content of the membrane preparations was determined by the method of Bradford (*28*) utilizing Coomassie blue with bovine serum albumin as the standard.

Classical CDS Displacement Assay. Individual fractions, collected from the HPLC, were freeze-dried and reconstituted in 50 μL of distilled water prior to determining their ability to displace 3 nM [^{3}H]clonidine from rat whole brain membranes. Specific binding was defined by 10 μM rauwolscine.

I-1 Site Binding Assay. Full details of this assay are as described previously (*29*, *30*) and are briefly stated below. Rat kidney membranes prepared as above were incubated (45 min) with [^{3}H]clonidine (3 nM) in the presence of 10 μM rauwolscine to preclude binding to α_2-adrenoceptors and increasing concentrations of the displacing ligand. The specific binding component was defined using 10 μM clonidine. Under these conditions, the site labeled is a model of the central I-1 binding site (*31*). Assays were terminated by rapid filtration through Whatman GF/B filters followed by two 3 mL washes with ice-cold assay buffer. Bound radioactivity was measured using liquid scintillation counting.

I-2 Site Binding Assay. The I-2 site assay has been published (*27*). Briefly, rat whole brain membranes as

prepared above were incubated (40 min) with 1 nM [^{3}H]-2-BFI and increasing concentrations of the test ligand. The specific binding component was defined using 10 μM BU224.

α_2-Adrenoceptor Binding Assay. Rat whole brain membranes were incubated (40 min) with 1 nM [^{3}H]RX821002 and increasing concentrations of the test ligand. Specific binding was determined using 10 μM rauwolscine.

Assay Analysis. For each I-1, I-2, and α_2-adrenoceptor assay, test compounds were assessed over at least 14 concentrations covering the range 10^{-11} to 10^{-4} M and the results analyzed using iterative nonlinear regression to obtain IC_{50} values (GraphPad Prism 3.02). The IC_{50} values for I-2 binding sites and α_2-adrenoceptors were converted to K_i values using the Cheng−Prussoff equation. Results from the I-1 binding site assay were left as IC_{50} values since a K_D value for [^{3}H]clonidine at the I-1 binding sites has not been determined to date. For further details of the analysis of binding curves, conversion to IC_{50} or K_i values, and the *F*-test, see Lione et al. (*27*).

Spectroscopic Methods. ESMS analyses were performed on a VG Quattro quadrupole mass spectrometer equipped with an electrospray source. Each sample (20 μL) was directly injected into the electrospray source via a rheodyne loop injector at a solvent (water/acetonitrile, 50:50 v/v, 1% formic acid) flow rate of 20 μL min^{-1}. For MS-MS determinations of selected singly charged ions, argon was used as the collision gas, and the collision energy was set at 50 V. The machine was calibrated using appropriate standards. ^{1}H NMR spectra were recorded in CD_3OD on a Varian INOVA 600 MHz spectrometer at the Division of Biochemistry and Molecular Biology, University of Southampton. δ values are referenced to tetramethylsilane as an internal standard at 0 parts per million (ppm).

RESULTS

Identification and Preliminary Analysis of CDS from Bovine Lung. The bovine lung methanolic extract gave numerous elution peaks in the first 14 min followed by the classic "three-peak" signature at 18, 21, and 23 min as originally observed by Atlas (*14*) (Figure 2A). Displacement of radioligand binding to α_2-adrenoceptors by compounds present in the early fractions following the void volume (3−14 min) may be due to the endogenous compounds noradrenaline, adrenaline, histamine, and agmatine, which are all known to elute at this time under the experimental conditions used (data not shown). Fraction 21 exhibited over 70% displacement in the classical CDS assay (Figure 2B). ESMS (Figure 2C) of this fraction clearly identifies three signals (187.99, 204.97, and 231.05 Da). The masses at 204.94 and 187.99 Da represent tryptophan and its dehydrated form, since ESMS analysis of pure L-tryptophan showed an identical profile and RP-HPLC confirmed that L-tryptophan coeluted with fraction 21 (data not shown). Analysis of fraction 21 by ^{1}H NMR showed a clear set of peaks corresponding to a single major component, L-tryptophan, with several very minor peaks in too small a quantity to be identified (data not shown). L-Tryptophan was ineffective, however, at displacing [^{3}H]clonidine radioligand in the binding assay (Table 1), suggesting that while L-tryptophan is the major component in peak 21, it is not the active CDS component.

Agmatine (decarboxylated arginine), which has been proposed as an endogenous ligand for imidazoline receptors (*16*), displaced the bound [^{3}H]clonidine radioligand from binding to α_2-adrenoceptors present in rat whole brain membranes with an IC_{50} of 31.7 μM (Table 1). The decarboxylated metabolite of tryptophan, tryptamine, was similarly investigated as a potential CDS. Tryptamine displaced [^{3}H]clonidine from binding to α_2-adrenoceptors present in rat whole brain membranes with an IC_{50} of 14.0 μM, an affinity higher than agmatine. Tryptamine was also able to displace radioligands bound to both I-1 and I-2 binding sites with an IC_{50} value of 36.0 μM and a K_i value of 26.7 μM, respectively (Table 1). A tryptamine standard, however, eluted from the RP-HPLC column at 18 min and could therefore not be the active component of classical CDS. ESMS analysis of peak 18 of the CDS extraction showed no evidence of tryptamine, and the lack of activity in the α_2-adrenoceptor radioligand displacement assay (Figure 2B) confirms that tryptamine is not present.

In addition to tryptophan, ESMS analysis of peak 21 revealed a component of mass 231 Da. It is known that tryptophan can spontaneously condense with acetaldehyde to give 1-methyltetrahydro-β-carboline-3-carboxylic acid of molecular mass 231 Da. Alternatively, tryptamine can condense with pyruvate to give 1-methyltetrahydro-β-carboline-1-carboxylic acid, also of mass 231 Da. These β-carbolines generally arise from nonenzymic condensations of aldehyde or α-keto molecules with tryptophan or derived indolic amines (*32*, *33*). Both 1-methyltetrahydro-β-carboline-1-carboxylic acid and 1-methyltetrahydro-β-carboline-3-carboxylic acid were synthesized and analyzed by RP-HPLC, ESMS, and NMR. Both synthetic β-carboline standards coeluted with the active CDS fraction; however, the ESMS/MS fragmentation pattern of 1-methyltetrahydro-β-carboline-1-carboxylic acid (Figure 2C, inset) matched that of the 231 Da species in the classical CDS extract. It was therefore concluded that fraction 21 contained 1-methyltetrahydro-β-carboline-1-carboxylic acid. Neither 1-methyltetrahydro-β-carboline-1-carboxylic acid nor 1-methyltetrahydro-β-carboline-3-carboxylic acid displaced [^{3}H]clonidine from binding to α_2-adrenoceptors, 2-BFI from binding to I-2 binding sites, or [^{3}H]clonidine from binding to I-1 binding sites in rat kidney membranes (data not shown). Therefore, these carboxylic acid containing β-carbolines do not represent the active component of classical CDS.

Isolation and Identification of β-Carbolines from Bovine Lung Extracts. 1-Methyltetrahydro-β-carboline-1-carboxylic acid is known to be a precursor to other β-carbolines (e.g., harmane), and these have been shown to have affinity for I-1 and I-2 sites (*30*). Modification of the extraction protocol (*26*) removed excess carboxylic acid containing compounds and concentrated the nonacidic β-carboline components. Following extraction and ESMS analysis two peaks were identified (molecular masses of 185.05 and 183.01 Da, respectively; Figure 3C). Decarboxylation of 1-methyltetrahydro-β-carboline-1-carboxylic acid is known to give harmalan (expected mass 184.10 Da) while oxidation of this component would give harmane (expected mass 182.08 Da). HPLC-ESMS analysis of synthetic harmalan and harmane showed that these pure standards had the same retention times as the extracted components (8.90 and 9.20 min, respectively) and also gave molecular masses that matched those of the

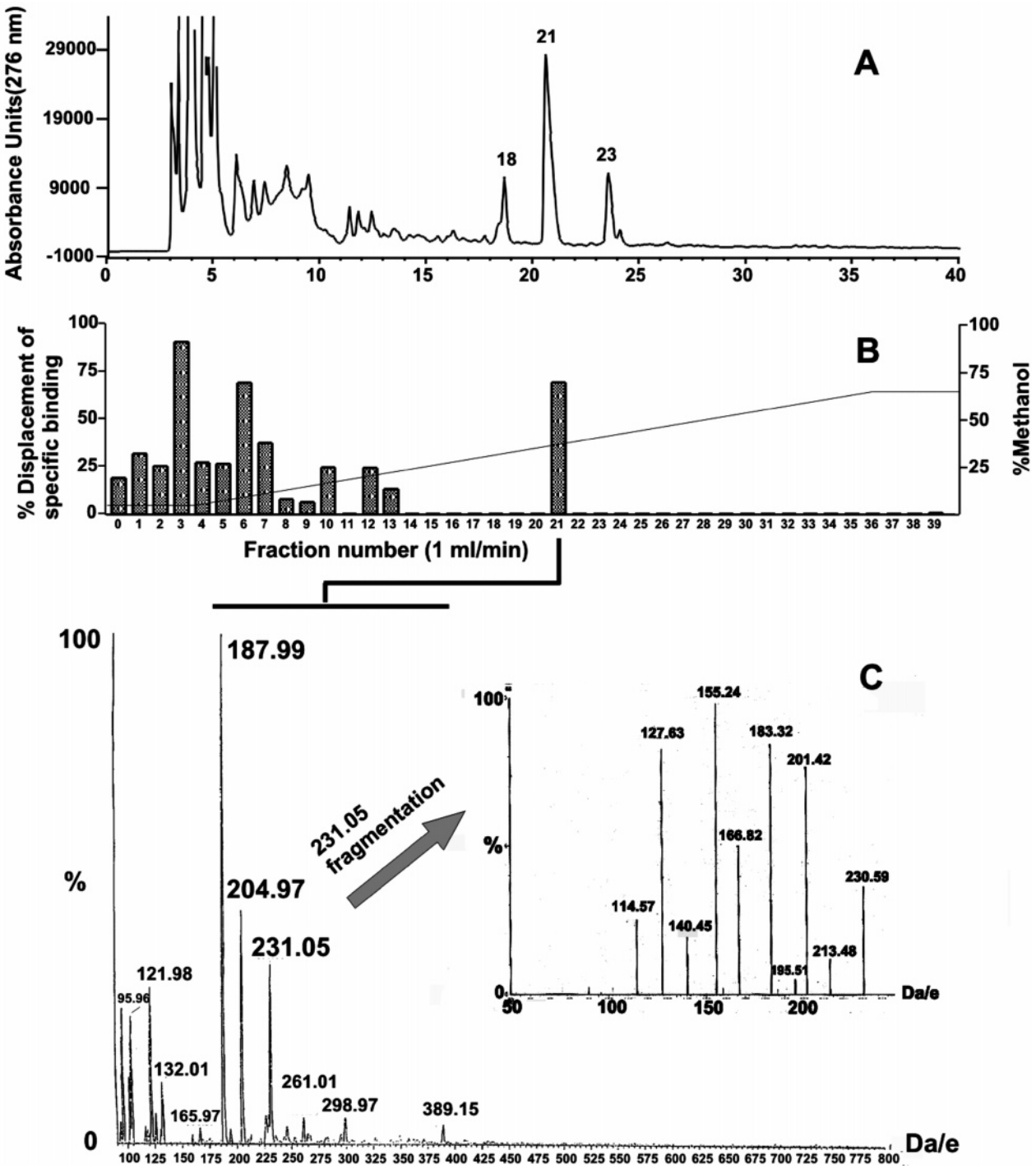

FIGURE 2: (A) RP-HPLC elution profile of bovine lung crude methanolic extract. (B) HPLC fractions (1.0 mL) were collected and assayed for their ability to displace [^{3}H]clonidine (3 nM) from binding to α_2-adrenoceptors present in rat brain membranes. Specific binding was defined by rauwolscine (10 μM). (C) HPLC pure fraction eluting at 21 min analyzed by electrospray mass spectroscopy (ESMS). (C, inset) Fragmentation pattern of peak 231 identified from the 21 min HPLC fraction.

active components in the CDS extract (Figure 3A,B). To confirm if harmane and harmalan could be present in peak 21 of the classical CDS, the pure synthetic standards were eluted from RP-HPLC using the gradient conditions described for the original CDS separation. Both harmane and harmalan coeluted with the biologically active peak 21 fraction. From the HPLC data, the concentration of harmane was determined as 1.3 ng g^{-1}, with harmalan at 0.6 ng g^{-1}. A 500 g wet weight bovine lung extraction provided sufficient material for NMR data to be collected on the CDS extract. The one-dimensional spectrum (Figure 4A), ^{1}H−^{13}C HSQC (Figure 4B,C), and ^{1}H−^{1}H COSY (data not shown) were compared with spectra of synthetic harmane and harmalan standards. The spectra of pure standards and extract matched exactly, confirming that the CDS contains a mixture of the β-carbolines harmane and harmalan present in the ratio of 70:30 (harmane:harmalan).

DISCUSSION

Elucidation of the structure and function of the endogenous clonidine-displacing substance has been the goal of several laboratories over the last 20 years. CDS has been extracted

Table 1: Affinity of Compounds for I-1 Binding Sites, I-2 Binding Sites, and α_2-Adrenoceptors[a]

compound	IC_{50} (nM) for I-1	K_i (nM) for I-2	K_i (μM) or IC_{50} (nM) for α_2-adrenoceptor
tryptamine	36000 ± 16000	26700 ± 4300	14000 ± 3000 (IC_{50})
tryptophan	>100000		>100000 (IC_{50})
agmatine	36532 ± 3080	416700 ± 118800	31700 ± 11000 (IC_{50})
harmane	30.1 ± 6.8	49.4 ± 17.4	17600 ± 2400
harmalan	46 ± 14	148 ± 50	10200 ± 2800

[a] Means ± SEM were derived from four to six experiments performed in triplicate. The values for harmane and harmalan were taken from Husbands et al. (*30*).

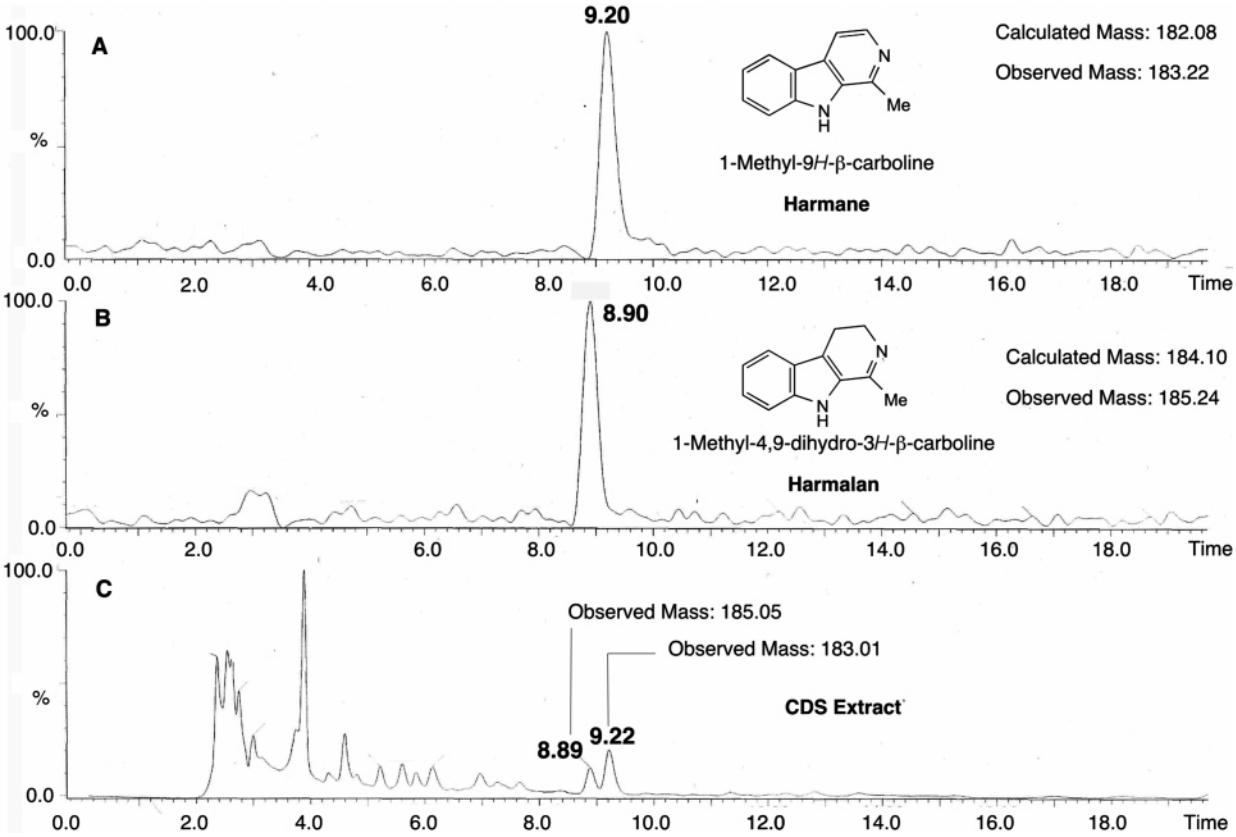

FIGURE 3: ESMS analysis of bovine lung CDS. The lower panel (C) shows a typical elution profile of the active CDS components following the modified extraction procedure (*26*). Harmalan and harmane eluted at 8.90 and 9.20 min, respectively. Elution profiles of the pure standards of harmalan (B) and harmane (A) are included.

from a number of mammalian tissues including brain (*11*), cerebrospinal fluid (*34*), serum (*35*), lung (*15*, *19*, *20*), placenta (*35*), and adrenal gland (*15*). A number of biological functions have been attributed to this compound, including arterial pressure regulation (*17*, *18*), smooth muscle contraction (*36*), and catecholamine release from adrenal chromaffin cells (*37*). In all of the studies to date, however, CDS has been poorly characterized. Polyclonal antibodies raised against *p*-aminoclonidine cross-reacted with brain CDS, suggesting that this endogenous substance might contain phenolic and imidazole rings (*38*, *39*). Atlas, in 1994, identified a CDS isolated from bovine brain as a low molecular mass substance (587 Da) which was thermostable, hydrophobic, acid resistant, unaffected by proteolytic enzymes, and devoid of primary amine groups (*14*). More recently, Grigg and co-workers analyzed purified bovine lung CDS by electrospray mass spectrometry identifying two possible components with masses of 275 and 489 Da (*40*). These workers did not fully structurally characterize the CDS but excluded the possibility that CDS was adrenaline, noradrenaline, tryptamine, GTP, GDP, or GMP on the basis of HPLC retention times. A CDS has also been purified from bovine brain (*16*) and identified as agmatine (molecular mass 130 Da). Though agmatine is capable of eliciting many of the biological responses ascribed to the CDS purified by Atlas and co-workers (*11*), agmatine does not have the potency or affinity for imidazoline binding sites or tissue distribution of CDS (*21*, *40*, *41*).

Atlas (*14*) reported a typical three-peak trace exhibited by CDS extracted from bovine brain. We have duplicated this effect with CDS extracted from bovine lung. This suggests that the overall composition of CDS from lung and brain may be similar. However, we chose bovine lung as this is known to have the highest concentrations of CDS and could potentially provide most material for NMR and ESMS analysis. The active fraction of this series of peaks was clearly a complex mixture, with tryptophan being a readily identifiable component. Tryptophan, however, does not represent the active component of classical CDS since it does not exhibit affinity for I-1 or I-2 binding sites but could

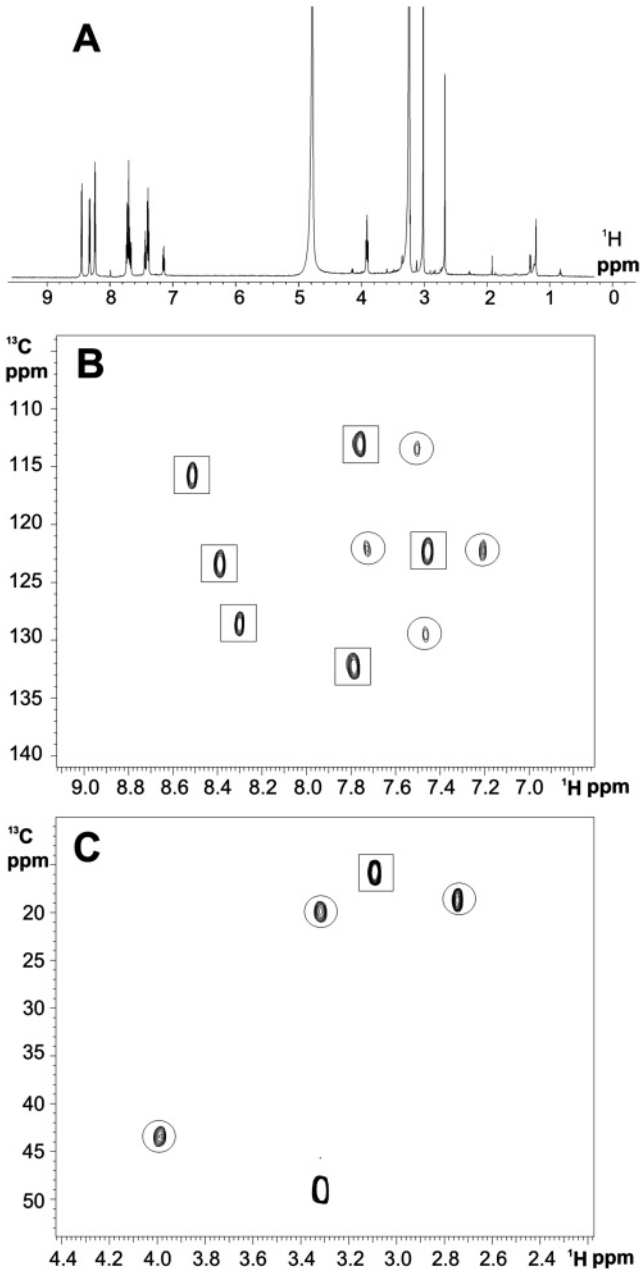

FIGURE 4: 600 MHz NMR analysis of active CDS components. (A) One-dimensional ^{1}H NMR spectrum. (B) ^{1}H−^{13}C constant time HSQC of the aromatic region of the two-dimensional spectrum and (C) ^{1}H−^{13}C constant time HSQC of the alipathic region of the two-dimensional spectrum. The peaks of the major species (harmane) are enclosed by squares and the minor species (harmalan) by circles. The data clearly demonstrate that only these two compounds were present in any significant quantity. ^{1}H NMR spectra were recorded in CD_3OD on a Varian INOVA 600 MHz spectrometer. Harmane peaks (major species): ^{1}H NMR (CD_3OD) δ 8.52 (1H, d, $J = 6.6$ Hz), 8.39 (1H, d, $J = 8.4$ Hz), 8.31 (1H, d, $J = 6.6$ Hz), 7.78−7.80 (2H, m), 7.47 (1H, m), 3.08 (3H, s); ^{13}C NMR (CD_3OD) δ 132.3, 128.6, 123.6, 122.311, 115.8, 113.2, 15.91. Harmalan peaks (minor species): ^{1}H NMR (CD_3OD) δ 7.74 (1H, d, $J = 7.8$ Hz), 7.51 (1H, d, $J = 8.4$ Hz), 7.47 (1H, m), 7.21 (1H, t, $J = 7.2$ Hz), 3.97 (2H, t, $J = 7.2$ Hz), 3.32 (2H, olp), 2.73 (3H, s); ^{13}C NMR (CD_3OD) δ 129.6, 122.2, 122.18, 113.6, 43.6, 19.9, 18.8.

conceivably serve as a precursor to the active component-(s).

Our studies confirm previous observations that agmatine does not represent classical CDS, although agmatine, among others, contributes to the radioligand displacement observed when fractions following the void volume of the column were tested. Interestingly, tryptamine, the decarboxylation product of tryptophan, was at least as potent as agmatine at ligand displacement from imidazoline binding sites. The retention time of tryptamine, however, precludes it from being the active component of classical CDS. Tryptamine is known to have a wide distribution in mammalian tissues (*42*), and specific receptors have been identified (*43*, *44*). A relatively short half-life (*45*) or conversion to other bioactive compounds may indicate that tissue levels would not be high enough to reflect the potency of classical CDS, though tryptamine should be considered at least as important a modulator of both α_2-adrenoceptors and I binding sites as agmatine.

The condensation of tryptamine with pyruvate and tryptophan with acetaldehyde forms the β-carbolines, 1-methyltetrahydro-β-carboline-1-carboxylic acid and 1-methyltetrahydro-β-carboline-3-carboxylic acid, respectively. 1-Methyltetrahydro-β-carboline-1-carboxylic acid is clearly a constituent of the lung CDS extract but, like tryptophan, does not represent an active component. Decarboxylation of this compound forms harmalan, a β-carboline previously shown to have a high affinity for I binding sites (*30*). When the extraction procedure was changed in order to remove the high concentrations of the nonactive compounds, we isolated the β-carbolines harmane and harmalan from bovine lung. Both have a high affinity for I-1 binding sites, with IC_{50} values of 30.1 and 46 nM, respectively (Table 1). The present study clearly shows that these β-carbolines are active components of classical CDS. Harmane and harmalan possess many of the functional characteristics associated with classical CDS and are endogenous ligands for imidazoline binding sites rather than the α_2-adrenoceptors (*30*). Previous studies by Musgrave and Badoer (*46*) showed that harmane, acting through an interaction with I binding sites, induced a significant decrease in blood pressure at an equivalent dose to clonidine. This effect was reversed by the mixed I-1/α_2-adrenoceptor antagonist, efaroxan. Harmane has also been shown to modulate central monoamines and reduce body temperature (*47*).

The relative concentrations of harmane and harmalan measured in this paper are of the same order of magnitude as levels seen in other mammalian tissues (*48*). The exact source of these compounds is unclear, though diet may make a significant contribution as β-carbolines have been identified in numerous foodstuffs (*49*, *50*) and, of more relevance to the bovine lung source, potentially maize silages (*51*). Pharmacokinetic studies have shown that oral administration of β-carbolines can increase plasma levels within 30 min (*52*) and that these compounds are cleared primarily by the liver over a similar time scale (*48*). It is also possible that these compounds are produced endogenously via an enzymatically catalyzed Pictet−Spengler reaction (*53*, *54*). The evidence for these enzymes is sparce, though the related salsolinol compounds (biosynthesized following a condensation of dopamine and acetaldehyde) are known to be formed enzymically, and this synthase has been purified (*55*).

The presence of naturally occurring β-carbolines in biological tissues and fluids has attracted a great deal of attention. They may act as neuroprotective agents (*56*, *57*) or at the same time can be bioactivated to give neurotoxins (*58*, *59*). They might function as neuromodulators through

effects on monoamine oxidase (*60*, *61*), affect monoamine uptake (*62*), inhibit cytochrome P450 (*63*), activate G-proteins in a receptor-independent manner (*64*), and act as comutagens or precursors to mutagens (*65*, *66*). The β-carbolines therefore exhibit a broad range of biological actions, but as yet the exact physiological role is unknown. It is clear, however, that a receptor exists that specifically binds both harmane and harmalan with nanomole affinity. This has led to speculation that one or more β-carbolines are endogenous ligands for the I-1 receptor (*67*).

The identification of the specific endogenous compounds, which make up CDS, is essential if clear functional roles are to be established and their receptors characterized. In this paper we have confirmed previous suggestions that classical CDS preparations are a complex combination of components, and therefore, the functional assays using these extracts may produce misleading results. Further purification of the classical CDS has allowed identification of the β-carbolines, harmane and harmalan, as principal active components of bovine lung CDS. Harmane and harmalan have high affinities for imidazoline receptors without having the characteristic amidine moiety, which is present in the majority of the compounds known to bind to these receptors. The β-carboline structure has already begun to be used as the basis for structure−activity studies of imidazoline lacking I_2 receptor ligands revealing that highly selective I_2 vs I_1 and α_2-adrenoceptor ligands may be achieved (*30*, *68*, *69*). It has also been shown that the β-carboline-based pyrazino-[1,2-*a*]indoles that lack both the imidazoline ring and part of the piperidine ring display very high I_2 selectivity over 5-HT_{2A} and 5-HT_{2C} serotonin receptors as well as I_1 receptors and α_2-adrenoceptors (*70*). The β-carboline structure may therefore provide us with the framework to design receptor-selective novel compounds with therapeutic desirable properties. The β-carbolines may also assist in the purification and identification of the imidazoline binding proteins.

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