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MEASUREMENT OF β-CARBOLINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A method using high-performance liquid chromatography with fluorescence detection was developed for the determination of β-carboline compounds norharman, harman, norharmol, and harmol in lung. Aqueous derivatization with acetic anhydride was used to facilitate the isolation and separation of the phenolic compounds and to reduce the fluorescence background of the biological samples. Harman was identified and quantitated in rat lung (1.88 ± 0.55 ng/g) using this method and its identity confirmed by means of gas chromatography–negative-ion chemical ionization mass spectrometry.

INTRODUCTION

The β-carbolines norharman (**1**) and harman (**2**) are found in plants [1] and have also been reported to be present in mammalian brain [2,3] and platelets [4,5]. The structures of these compounds are presented in Fig. 1. These compounds possess various pharmacological activities which include inhibition of neurotransmitter uptake [6–9], inhibition of monoamine oxidase [6,10], and antagonism of benzodiazepine receptor binding [11,12]. Recently, norharman and harman, which are not themselves mutagenic, have been shown to potentiate the mutagenicity of certain organic chemicals in the Ames' test on *Salmonella typhimurium* [13]. Furthermore, when these β-carbolines were incubated in this test with non-mutagenic aniline and *o*-toluidine, in the latter became mutagenic [14].

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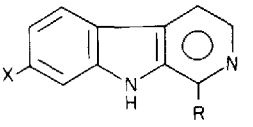

1 R=H, X=H, Norharman
2 R=CH_3, X=H, Harman
3 R=H, X=OH, Norharmol
4 R=CH_3, X=OH, Harmol
5 R=$CH_2CH_2CH_3$, X=H, Internal standard

Fig. 1. Structures of β-carboline compounds.

This activity of norharman and harman has been termed comutagenicity and has stimulated interest in the role these compounds might play in cellular injury.

The mammalian lung is exposed to numerous drugs, chemicals, and environmental pollutants, which reach it both by inhalation and the circulation. Since the lung is known to avidly accumulate and retain basic, lipophilic compounds [15], it would be expected to accumulate norharman and harman. These β-carbolines are present in significant amounts in tobacco smoke [16], beer and wine [17], and in tryptophan pyrolysates [18] which can be produced by frying tryptophan-rich foods. Thus, the mammalian lung is exposed to norharman and harman where their accumulation and comutagenic activity could result in cellular change.

In order to examine this possibility, we developed a simple, sensitive high-performance liquid chromatography (HPLC) procedure for the determination of norharman, harman and their respective phenolic metabolites norharmol (**3**) and harmol (**4**) in lung and combined it with gas chromatography–negative-ion chemical ionization mass spectrometry (GC–NICI-MS) for definitive structure proof.

EXPERIMENTAL

Materials

Norharman hydrochloride (**1**), harman hydrochloride (**2**), harmol hydrochloride (**4**), esterase, and sulfatase (type H-1) containing β-glucuronidase were purchased from Sigma (St. Louis, MO, U.S.A.). Norharmol (**3**) was isolated from the urine of rats given norharman (1 mg per 5 ml of 0.9% saline solution, orally). A 1.0-ml aliquot of the 24-h urine sample was hydrolyzed by treatment with 1.0 ml of water, 0.5 ml of 0.5 *M* sodium acetate buffer (pH 5.0), 5 mg of sulfatase/β-glucuronidase and incubated for 1.5 h at 37 °C. The unconjugated compound was isolated using the sample preparation procedure described below and pure compound was obtained by collecting the HPLC effluent corresponding to the norharmol peak. The internal standard, 1-propyl-9*H*-pyrido[3,4-*b*]indole (**5**) was synthetized from tryptophan and butyraldehyde purchased from Aldrich (Milwaukee, WI, U.S.A.), using the procedure of Jacobs and Craig [19]. Pentafluorobenzyl bromide was purchased from Pierce (Rockford, IL, U.S.A.), and glass-

distilled methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals used in this work were reagent grade.

Sample preparation

Male Sprague–Dawley rats (200–250 g) were purchased form Simonsen Lab. (Gilroy, CA, U.S.A.) and were fed Purina Rodent Chow 5012 (Ralston Purina, St. Louis, MO, U.S.A.) and water ad libitum, maintained on a 12/12 light cycle, and were housed at least five days prior to use. Animals were decapitated by a guillotine and their lungs removed and rinsed with cold 0.9% saline solution. Each lung was placed in a polypropylene tube that contained 5 volumes of ice-cold 0.4 *M* perchloric acid, 1 mg semicarbazide hydrochloride and 2 ng of the internal standard. The tissue was homogenized in an ice-bath using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.). The homogenate was centrifuged at 9000 *g* and 4 °C for 15 min. The supernatant was transferred to acid-washed (dichromate–sulfuric acid) silanized glass tubes. Silanized glassware was used throughout the sample preparation procedure. The tissue pellet was resuspended in 2 ml of cold 0.4 *M* perchloric acid, recentrifuged, and the supernatants were combined. The sample was adjusted to pH 6–7 with 10 *M* sodium hydroxide solution, treated with 25 μl of acetic anhydride, vortexed, and allowed to stand for 5 min. The sample was then adjusted to pH 10 and treated with 25 μl of acetic anhydride. After 5 min, the sample was extracted twice with 2 volumes of ethyl acetate. The combined extracts were evaporated to dryness under a stream of nitrogen gas and stored at −18 °C prior to analysis. Blank samples were carried through the entire sample preparation procedure.

Derivatization of harman with pentafluorobenzyl bromide for GC–MS

A suspension of sodium hydride in tetrahydrofuran (THF) was prepared immediately prior to use by taking 350 mg of a 60% suspension of sodium hydride in mineral oil and washing it twice with 3 ml of hexane and once with 3 ml of diethyl ether before resuspending in 2 ml of dry THF. The tissue extract was dissolved in 0.5 ml of THF, treated with 15 μl of sodium hydride suspension and sealed with a teflon-lined cap. The sample was allowed to stand for 10 min at room temperature before the addition of 1 μl pentafluorobenzyl bromide. After standing for 5 min at room temperature, the reaction mixture was evaporated to dryness under a stream of nitrogen gas, placed in an ice-bath and the excess sodium hydride hydrolyzed by the careful addition of 1 ml of cold water. The aqueous sample was extracted by vortexing with 4 ml of hexane followed by centrifugation for 10 min at 800 *g*. The hexane layer was transferred to a clean, silanized glass tube and evaporated to dryness under nitrogen gas. The dry sample was stored at −15 °C until analyzed. Samples were dissolved in 20 μl of heptane immediately prior to GC–MS analysis.

Instrumentation

High-performance liquid chromatography. Samples were dissolved in 0.01 *M* perchloric acid immediately prior to HPLC analysis. Sample background was significantly reduced by washing the 0.01 *M* perchloric acid solution with 5 vol-

umes of diethyl ether. Samples dissolved in 0.01 *M* perchloric acid were stable for at least one week when stored at −15°C.

HPLC was performed using a system which consisted of a Beckman Model 110A pump (Beckman, Irvine, CA, U.S.A.), a Rheodyne Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl loop, and a 25 cm×4.6 mm I.D. Biosphere 5-μm C_{18} reversed-phase column (Bioanalytical Systems, West Lafayette, IN, U.S.A.). Samples were eluted at a flow-rate of 1.4 ml/min using 0.5% triethylamine in methanol–water (60:40) as solvent. Compounds were detected with a Schoeffel FS-749 spectrofluorometer (Schoeffel Instruments, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. Optimal detection was achieved using 252 nm for excitation and a 430-nm cut-off filter for emission.

Gas chromatography–mass spectrometry. Typically, 1–5 μl of the heptane solution containing the pentafluorobenzyl-derivatized sample was loaded onto a dropping needle injector (Ray Allen Assoc., Boulder, CO, U.S.A.) connected to a medium-polarity, bonded-phase, fused-silica capillary column (DB-5, 15 m×0.25 mm, J&W Scientific, Folsom, CA, U.S.A.) using helium as the carrier gas. A pressure of 0.4 bar of helium was maintained in the injector port which resulted in a flow-rate of 1–2 ml/min at 20°C. The injector port and the transfer line were maintained at 250°C. The gas chromatograph oven was kept at 100°C for 1 min following injection of the sample and then increased at the rate of 20°C/min to a final temperature of 250°C. The end of the column was inserted directly into the ion source of a modified Hewlett-Packard 5985B spectrometer. The mass spectrometer was operated in the conventional electron-impact (70 eV) ionization mode for structural determination of authentic samples. For confirmation of the structural identity of derivatized harman in lung extracts, it was operated in the negative-ion chemical ionization (NICI) mode with methane as the reagent gas. The gas was delivered to the source of the mass spectrometer coaxially to the capillary column and a pressure of approximately 1 Torr was maintained.

Quantitation

A calibration curve was constructed by plotting the peak-height ratio of harman/internal standard against the concentration of harman (0.5–10 ng) for samples carried through the sample preparation procedure and HPLC analysis (vida supra). The concentration of harman was determined from the peak-height ratio of each sample by reference to the calibration curve.

RESULTS AND DISCUSSION

Several methods have been described to identify and quantitate β-carbolines in biological samples [2–5]. These procedures have typically required large sample sizes, extensive sample preparation, and have frequently used analytical techniques that lack the required specificity and sensitivity for trace level analyses. The use of HPLC with fluorescence detection in combination with GC–NICI-MS in this method has overcome some of the limitations of earlier procedures. Furthermore, this procedure utilizes aqueous derivatization with acetic anhydride to improve the isolation and chromatographic separation of norharmol and harmol

and to markedly reduce the fluorescence background of the samples. Earlier work had demonstrated the utility of aqueous derivatization in the isolation of hydrophilic amines and phenols from aqueous solution [20,21]. Derivatization of aqueous samples of β-carbolines **1–4** converted norharmol and harmol into their corresponding acetoxy derivative and gave recoveries of **1–4** (2 ng, $n=4$) of 97.3–87.8%. Acetic anhydride treatment converted other amines and phenols present in the biological sample to their corresponding neutral amides and esters, respectively. These derivatives, which were extracted with the compounds of interest, could be easily separated from the β-carbolines by dissolving the evaporated tissue extract in 0.01 *M* perchloric acid and washing with diethyl ether immediately prior to HPLC analysis. This step reduced the fluorescence background of the sample and improved the chromatographic separations and sensitivity. Derivatization of norharmol and harmol was also necessary to accomplish their reversed-phase separation. The derivatized phenols, however, had significantly reduced fluorescence intensities. A liquid chromatogram of authentic derivatized β-carbolines **1–5** is presented in Fig. 2A and demonstrates the facile separation of these compounds and the reduced fluorescence intensity of the derivatized phenols. When derivatized norharmol and harmol were treated with 1 U of esterase in phosphate buffer (pH 7.0) for 10 min at room temperature, complete hydrolysis to the parent phenol was achieved and the chromatogram peaks 1 and 2 in Fig. 2A disappeared, i.e.,moved under the solvent front. This esterase step can be used to obtain additional structural information concerning specific chromatogram peaks.

When the β-carbolines **1–4** were analyzed using this HPLC procedure (2 ng

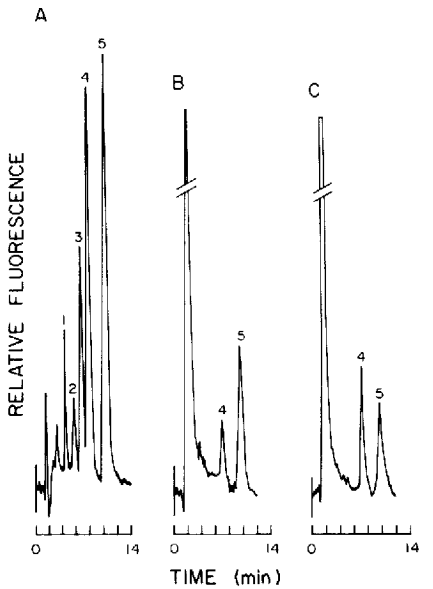

Fig. 2. Liquid chromatograms of (A) authentic compounds, (B) mouse lung extract, and (C) rat lung extract. Peaks (retention time): 1=O-acetylnorharmol (4:10); 2=O-acetylharmol (5:30); 3=norharman (6:30); 4=harman (7:35); 5=internal standard (10:10).

each, $n=4$), the following coefficients of variation and limits of sensitivity (three times background) were obtained: norharman, 1.9%, 150 pg per sample; harman, 5.6%, 100 pg per sample; norharmol, 2.1%, 750 pg per sample; and harmol, 4.6%, 500 pg per sample. Using this sample preparation and HPLC procedure, it was possible to tentatively identify harman (**4**) in the chromatograms of normal mouse (Fig. 2B) and rat (Fig. 2C) lung. Additional evidence for the identity of harman in these lung samples was obtained through co-chromatography experiments in which authentic harman was added to each sample and caused an increase in peak 4. Blank samples carried through the entire analytical procedure did not contain a peak corresponding to harman. Harman was quantitated in whole rat lung ($n=6$) and a value of 1.88 ± 0.55 ng/g was obtained. The level of harman in lung was not influenced by the presence or absence during the preparation of the sample of the aldehyde-trapping agent, semicarbazide. This indicated that the harman present in lung was not the result of artifactual formation during the

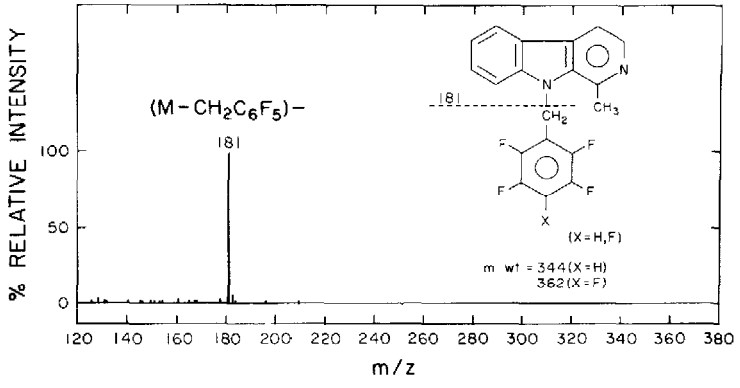

Fig. 3. Electron-capture negative-ion chemical ionization (methane) mass spectrum of the fluorobenzyl derivatives of harman. Identical spectra were obtained for both the tetra- and pentafluoro derivatives.

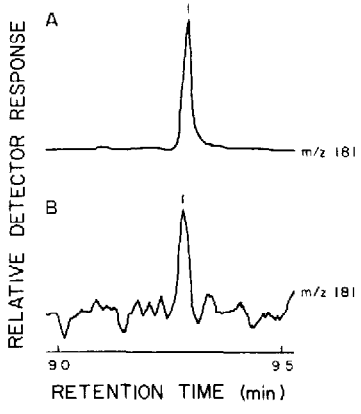

Fig. 4. Selected-ion monitoring chromatograms of the pentafluorobenzyl derivative of authentic harman (A) and rat lung extract (B). The ion at m/z 181 was monitored in the negative-ion chemical ionization mode.

sample preparation which has been a common pitfall in tetrahydro-β-carboline research [22].

The use of GC–MS with selective-ion monitoring has provided a combination of sensitivity and selectivity unequalled by other methods [23]. The recent refinements of fused-silica capillary gas chromatography [24] and electron-capture NICI mass spectrometry [25] have led to further improvements of specificity and sensitivity. However, in order to attain the enhanced sensitivity possible in the electron-capture mode, a molecule must have a positive electron affinity. In the case of harman this requirement was met by chemical derivatization of the β-carboline with an electron-capturing reagent, pentafluorobenzyl bromide. The derivative, 9-pentafluorobenzylharman, had improved GC properties and gave a simple NICI mass spectrum (Fig. 3) that consisted of a single ion at *m/z* 181 which corresponded to the indole anion. Coincidentally, *m/z* 181 was also the mass of the leaving group ($C_6F_5CH_2$). Therefore, in order to demonstrate that the ion *m/z* 181 represented the harman anion and not the pentafluorobenzyl fragment, a sample of harman was derivatized with 2,3,5,6-tetrafluorobenzyl bromide. The NICI mass spectra of this derivative also gave a base peak at *m/z* 181 which confirmed that the negative charge resided with the harman fragment.

The definitive identification of harman in rat lung was accomplished using GC–NICI-MS by selectively monitoring the *m/z* 181 ion. The rat lung chromatogram shows the presence of a peak in the *m/z* 181 ion trace at the same retention time as that of authentic derivatized harman (Fig. 4). This provides proof that peak 4 identified as harman in the liquid chromatogram of rat lung (Fig. 2C) corresponded to harman.

In conclusion, a simple, sensitive procedure has been described which utilizes HPLC with fluorescence detection in combination with GC–NICI-MS for the identification of β-carboline compounds. This method should facilitate the study of the pharmacological and toxicological properties of this important class of compounds.

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