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# DERIVATIZATION IN AQUEOUS SOLUTION, ISOLATION AND SEPARATION OF TETRAHYDRO-β-CARBOLINES AND THEIR PRECURSORS BY LIQUID CHROMATOGRAPHY

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## SUMMARY

Derivatization of indole ethylamines and 1,2,3,4-tetrahydro-β-carbolines in aqueous solution with methyl chloroformate has been used to facilitate the isolation of these compounds. The initial derivatization eliminated the potential for the artifactual formation of these compounds via the condensation of the indole ethylamine with an aldehyde or α-keto acid during the work-up procedure. The derivatized compounds possessed improved chromatographic properties which allowed for their facile separation by reversed-phase liquid chromatography and their fluorescent detection at the nanogram level.

## INTRODUCTION

The reaction of an aldehyde or α-keto acid with an indole ethylamine produces a 1,2,3,4-tetrahydro-β-carboline (THBC) via a Pictet—Spengler reaction. A generalized reaction for the formation of THBC compounds is presented in Fig. 1. Such reactions readily occur under physiological conditions [1] and produce compounds which can function as neurotransmitters [2] or neuromodulators via their inhibition of uptake [3] or their inhibition of

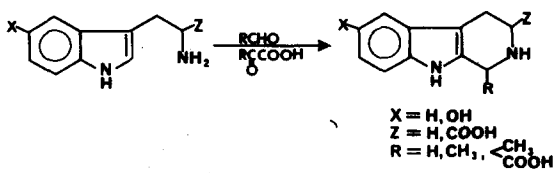


Fig. 1. Formation of 1,2,3,4-tetrahydro-β-carbolines by the condensation of an indoleamine with an aldehyde or α-keto acid.

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monoamine oxidase [4]. Furthermore, these compounds have been increasingly implicated in alcoholism. Acute and chronic administration of select THBC compounds to rats has been reported to significantly alter alcohol consumption [5, 6], and the presence of these compounds in alcoholic beverages and various foods has been confirmed [7—9]. Recently, 6-methoxy-1,2,3,4-tetrahydro-β-carboline has been identified in human pineal and proposed to function as a hormone which modulates the neuronal uptake of serotonin (5-HT) and may play an important role in the pathophysiology of depression [10].

A variety of analytical methods have been employed in attempts to identify and quantitate THBC compounds in vivo [11—18]. All of these methods suffer from limitations of simplicity, selectivity and/or sensitivity. In addition, these methods require considerable sample preparation and manipulation which make them subject to possible artifactual formation of these compounds.

In light of the pharmacological activities of THBC compounds and their possible role in alcoholism and depression, we have developed a simple, sensitive analytical procedure for the isolation, separation and identification of these compounds. In the design of this method three specific objectives were achieved: (1) the development of an analytical method which would allow for the determination of both substrate and products resulting from the reaction of indole ethylamines with aldehydes or α-keto acids; (2) the use of chemical derivatization to fix the sample and eliminate the possibility for artifactual formation; and (3) the use of chemical derivatization to facilitate the isolation of these compounds from tissues or fluids and to improve their chromatographic separation and quantitation.

## EXPERIMENTAL

### *Materials*

Tryptamine hydrochloride, α-ethyltryptamine, 5-methoxytryptamine hydrochloride, 5-hydroxytryptamine oxalate, L-tryptophan and Type II porcine liver esterase were purchased from Sigma (St. Louis, MO, U.S.A.). α-Methyl-5-hydroxytryptamine creatinine sulfate was the gift of Upjohn (Kalamazoo, MI, U.S.A.). Tetrahydro-β-carboline and 6-hydroxytetrahydro-β-carboline (6-OHTHBC) were prepared from glyoxylic acid and tryptamine or 5-hydroxytryptamine (5-HT), respectively [19]. 1-Methyltetrahydro-β-carboline (1-MeTHBC) and 6-hydroxy-1-methyltetrahydro-β-carboline (6-OH-1-MeTHBC) were synthesized from acetaldehyde and tryptamine [20] or 5-benzyloxytryptamine [21], respectively. Tetrahydro-β-carboline-3-carboxylic acid (THBC-3-COOH) and 1-methyltetrahydro-β-carboline-3-carboxylic acid (1-MeTHBC-3-COOH) were prepared from L-tryptophan by the procedures of Jacobs and Craig [22] and Snyder et al. [23], respectively. 6-Methoxytetrahydro-β-carboline-1-carboxylic acid (6-OMeTHBC-1-COOH) was prepared from 5-methoxytryptamine and glyoxylic acid under the conditions described by Vejdelek et al. [19]. Methyl chloroformate was purchased from Aldrich (Milwaukee, WI, U.S.A.). Glass-distilled methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and 0.01 *M* sodium acetate, pH 4.6, was prepared from reagent-grade sodium acetate. Solvents were vacuum-

degassed prior to use. Standard solutions (100 μg/ml) were prepared in 0.1 *M* perchloric acid and diluted to the desired concentration.

*Instrumentation*

Liquid chromatography was performed using a Varian 5020 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a universal loop injector, a 5-cm column guard packed with Vydac reversed-phase hydrocarbon (Separations Group, Hesperia, CA, U.S.A.) and a 5-μm Zorbax ODS, 25 cm × 4.6 mm I.D. column (DuPont, Wilmington, DE, U.S.A.). Samples were eluted at a flow-rate of 0.8 ml/min using one of the following solvent systems: (A) methanol—0.01 *M* sodium acetate (pH 4.6) (65:35); (B) methanol—0.01 *M* sodium acetate (pH 4.6) (45:55). Fluorescence detection was achieved using a Fluorichrom detector (Varian) equipped with a deuterium arc source and using a 200I excitation filter and a Corning 7-60 band filter (360 mm) for emission.

*Sample preparation*

The sample preparation procedure is outlined in Fig. 2. The sample (tissue homogenate or biological fluid) consisted of 0.5 ml of 0.1 *M* perchloric acid to which were added 25 μl of 1.0 *M* semicarbazide plus the appropriate internal standard. The sample was treated with 0.5 ml of 1.0 *M* dipotassium hydrogen phosphate (pH 7.2), 50 μl of methyl chloroformate, vortexed, and allowed to stand for 5 min. The sample pH was increased to 9.5 by the addition of 0.25 ml of saturated sodium carbonate (pH 11.5), 50 μl of methyl chloroformate were again added, and the sample was vortexed and allowed to stand for 10 min. Extraction of the sample with 6.0 ml of dichloromethane yielded basic precursors and tetrahydro-β-carbolines upon evaporation under nitrogen. The

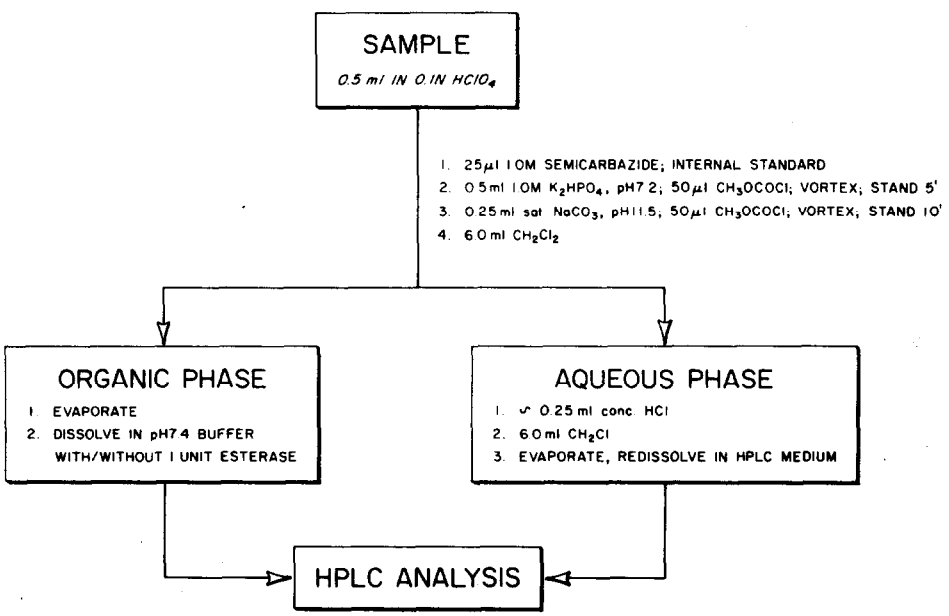



Fig. 2. Flow chart of the sample preparation procedure for the determination of 1,2,3,4-tetrahydro-β-carbolines and their precursors.

residue was dissolved in 0.5 ml of 0.1 *M* disodium hydrogen phosphate buffer (pH 7.4). In samples which were to be analyzed for 5-HT, 6-OHTHBC or 6-OH-1-MeTHBC, the buffer contained 1 U of esterase per 0.5 ml of buffer.

Acidic compounds were isolated from the remaining aqueous phase by careful acidification of the aqueous phase with concentrated hydrochloric acid (ca. 250 $\mu$l) followed by extraction with 6.0 ml of dichloromethane and evaporation under nitrogen. The samples were dissolved in solvent system B prior to analysis.

## RESULTS AND DISCUSSION

This method is based on the known chemical reactivity of alkyl chloroformates, specifically methyl chloroformate, with amines and phenols in aqueous solution to produce carbamate and carbonate derivatives, respectively [24]. Earlier work by Brooks and Horning [25] had demonstrated the potential utility of acetylation in the isolation of amines from dilute aqueous solutions. By initially derivatizing the aqueous sample with methyl chloroformate, the potential for the artifactual formation of THBC compounds is markedly decreased or eliminated, since the precursor indole ethylamine is no longer free and available to react with an aldehyde or $\alpha$-keto acid. Indeed, using this method we have demonstrated that under conditions which had previously been shown to produce substantial amounts of artifactual 6-OHTHBC in human platelets [26], initial derivatization with methyl chloroformate completely eliminated this problem and confirmed the absence of 6-OHTHBC in human platelets.

The stable, lipophilic products of this derivatization can be quantitatively extracted into a non-polar organic solvent and isolated. In addition to facilitating the isolation of these compounds from an aqueous medium, the chemical derivatization yields products which possess superior reversed-phase chromatographic properties to those of the parent compounds. The excellent chromatographic separation of tryptamine, THBC, 1-MeTHBC and the internal standard, $\alpha$-ethyltryptamine, is demonstrated in Fig. 3.

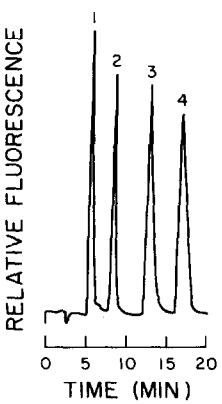



Fig. 3. Chromatogram of tryptamine (1), THBC (3), 1-MeTHBC (4) and the internal standard $\alpha$-ethyltryptamine (2) eluted with solvent system A.

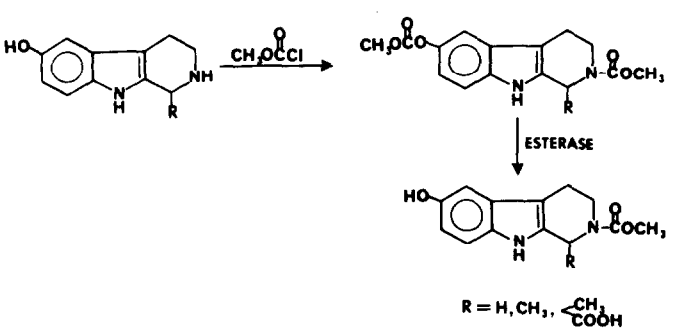
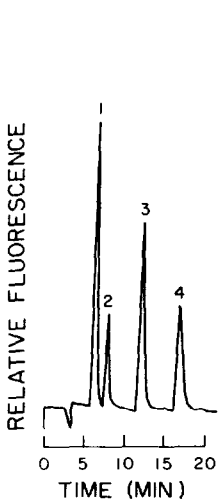



Fig. 4. The chemical derivatization reaction of a 6-OHTHBC compound.

Rapid derivatization of phenols, such as 5-HT, 6-OHTHBC and 6-OH-1-MeTHBC, occurs at low pH and this permits their protection from oxidation prior to shifting to the high pH values required for the derivatization of amines [27]. The acylation of phenolic indoles or phenolic THBC compounds results in the complete loss of their native fluorescence. As a result, this necessitates the selective hydrolysis of the carbonate function in the presence of the carbamate. This can be readily accomplished by treatment of the extraction residue with 1 U of esterase in phosphate buffer for 10 min at room temperature. The derivatization of a 6-OHTHBC compound is shown in Fig. 4. The chromatogram of these phenolic compounds and the internal standard, $\alpha$-methyl-5-HT, is shown in Fig. 5.

The requirement for the treatment of phenolic indole ethylamines and THBC compounds with esterase can be used to confirm peaks present in the chromatogram of a sample and thereby provide additional structural information. For example, Fig. 6 shows chromatograms of 5-HT and 6-OH-1-MeTHBC in the presence and absence of esterase. When the sample is chromatographed after esterase treatment, a simple chromatogram containing 5-HT and 6-OH-1-MeTHBC is obtained (Fig. 6A); however, the chromatogram obtained in the absence of esterase treatment reveals the absence of these compounds (Fig. 6B).



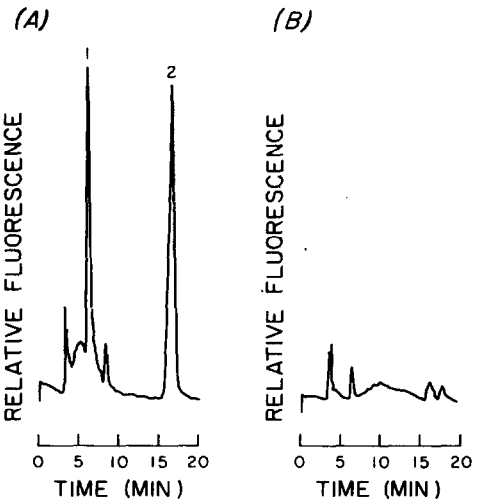




Fig. 5. Chromatogram of 5-HT (1), 6-OHTHBC (3), 6-OH-1-MeTHBC (4) and the internal standard, $\alpha$-methyl-5-HT (2) eluted with solvent system B.

Fig. 6. Chromatograms of 5-HT (1) and 6-OH-1-MeTHBC (2) with (A) and without (B) esterase treatment.

Using these phenolic compounds the limit of sensitivity and the reproducibility of the method was determined. The limit of sensitivity (three times background) for 5-HT, 6-OHTHBC and 6-OH-1-MeTHBC was found to be less than 1 ng per sample, while the reproducibility of the method (12 ng per sample, $n$ = 8) gave coefficients of variation of 1.63%, 1.75% and 1.46%, respectively.

The chromatogram of the acidic THBC compounds derived from the condensation of tryptophan with formaldehyde and acetaldehyde is shown in

Fig. 7, along with the internal standard, 6-OMeTHBC-1-COOH. The chromatographic data for these compounds are summarized in Table I.

In conclusion, a simple, sensitive procedure for the determination of tetrahydro-β-carbolines and their indolic precursors based on the aqueous derivatization with methyl chloroformate has been described. The utilization of this method, which improves the isolation and chromatographic separation of these compounds and eliminates the potential for artifactual formation, should greatly facilitate the study of THBC compounds and provide an alternate means for examining the in vivo presence of these compounds.

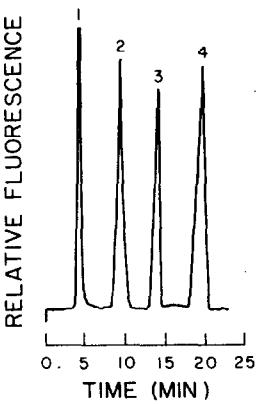



Fig. 7. Chromatogram of tryptophan (1), THBC-3-COOH (3), 1-MeTHBC-3-COOH (4) and the internal standard, 6-OMeTHBC-1-COOH (2) eluted with solvent system B.

TABLE I

CHROMATOGRAPHIC DATA FOR THBC COMPOUNDS AND PRECURSORS

See Experimental for chromatographic conditions. Samples were eluted at a flow-rate of 0.8 ml/min using one of the following solvent systems: (A) methanol—0.01 *M* sodium acetate buffer pH 4.6 (65:35); (B) methanol—0.01 *M* sodium acetate buffer pH 4.6 (45:55).

| Compound | Retention time (min) | Solvent system |
|---|---|---|
| Tryptamine | 6.03 | A |
| α-Ethyltryptamine | 8.73 | A |
| THBC | 13.59 | A |
| 1-MeTHBC | 16.86 | A |
| 5-HT | 6.15 | B |
| α-Me-5-HT | 7.73 | B |
| 6-OHTHBC | 12.01 | B |
| 6-OH-1-MeTHBC | 16.78 | B |
| Tryptophan | 4.84 | B |
| 6-OMeTHBC-1-COOH | 9.90 | B |
| THBC-3-COOH | 14.30 | B |
| 1-MeTHBC-3-COOH | 19.87 | B |

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