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STEREOSELECTIVE ENZYMATIC *O*-METHYLATION OF TETRAHYDROPAPAVEROLINE AND TETRAHYDROXYBERBINE ALKALOIDS

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Abstract—Modification of neuroamine metabolism leading to the formation of isoquinoline alkaloids has been the target of several investigations. This study describes the differential enzymatic *O*-methylation pattern of the racemates and optical isomers of tetrahydropapaveroline (THP) and 2,3,10,11-tetrahydroxyberbine (THB) by a rat liver catechol *O*-methyltransferase (COMT) preparation. Reaction products were separated and isolated by high-pressure liquid chromatography, and structural identity was confirmed by synthesis and gas chromatography/mass spectrometry. The positions of enzymatic *O*-methylations were markedly influenced by the particular optical isomeric form of substrate employed. The optical isomers and racemates of THP and THB were mainly mono-*O*-methylated with trace amounts of di-*O*-methylation. The mono-*O*-methylations of THP were contained exclusively in the isoquinoline ring at positions 6 or 7, while mono-*O*-methylations of THB occurred at the vicinal hydroxyl groups of both rings A and D. This enzymatic process was evaluated further by examining the effect of classical inhibitors of COMT on these *O*-methylations. The results presented demonstrate that vicinal hydroxyl moieties of THP and THB are mono-*O*-methylated at either of the sites and that the magnitude of the positional isomer product ratio results from the optical isomeric orientation in which the substrate binds to the enzyme.

Tetrahydropapaveroline (THP), a benzyltetrahydroisoquinoline, and 2,3,10,11-tetrahydroxyberbine (THB), a tetrahydroprotoberberine, are members of a large group of naturally occurring isoquinoline alkaloids [1]. The endogenous formation of isoquinoline alkaloids has been implicated in the neurochemical events participating in the sequelae of alcohol dependence [2–4]. A critical feature of this theory is the Pictet–Spengler condensation of amines with aldehydes to form alkaloid products [5, 6]. Formation of THP has been demonstrated *in vitro* [3, 7] and *in vivo* [8, 9]. Additionally, carboxybenzyltetrahydroisoquinolines have been detected in human urine [10].

Recent reports have indicated that chronic infusion of THP into the lateral ventricle of rat brain resulted in an increase of ethanol preference in this species [11, 12]. The half-life ($T_{\frac{1}{2}}$) of intraventricularly injected THP is approximately 17 min and is increased 3-fold in pyrogallol-treated animals [13]. The increased retention of intraventricularly injected THP produced by pyrogallol suggests that *O*-methylation of THP may be one of its potential metabolic routes. In this regard, delineation of the metabolic fate of THP or THB would be most helpful in studying the disposition of these alkaloids following intraventricular administration, as well as providing a more focused approach to the current search for the endogenous formation of neuroamine-derived alkaloids.

In vitro studies have demonstrated that radioactivity derived from *S*-adenosyl-L-[methyl-^{14}C]methionine ([^{14}C]SAM) is incorporated into the molecular structure of THP following incubation with rat liver catechol *O*-methyltransferase (COMT) preparations; however, product structural confirmation was not done [14]. Another metabolic possibility is formation of tetrahydroprotoberine alkaloids from THP or other benzyltetrahydroisoquinolines through tetracyclic ring coupling. Conversion of benzyltetrahydroisoquinolines to tetrahydroprotoberberine alkaloids by a liver enzyme preparation *in vitro* has been reported [4, 15]. Furthermore, *in vivo* experiments using rats demonstrated urinary excretion of multiple tetrahydroprotoberberine alkaloids after intraperitoneal injection of THP [4] or reticuline [16].

Assuming that *O*-methylation is a formidable biotransforming enzyme system for hydroxylated alkaloidal metabolic intermediates, *in vitro* enzymatic *O*-methylation of the optical isomers of THP and THB utilizing a partially purified catechol-*O*-methyltransferase preparation was evaluated. Product isolation and identification were accomplished by a combination of high-pressure liquid chromatography (h.p.l.c.), gas chromatography/mass spectrometry (g.c./m.s.) and radiotracer techniques. Additionally, inhibitors of COMT were employed to study further the nature of the enzymatic *O*-methylation of THP and THB alkaloids.

MATERIALS AND METHODS

Reagents. (±), R-(+) and S-(−)-1-(3,4-dihydroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide (tetrahydropapaveroline, THP); (±), R-

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(+) and S-(−)-2,3,10,11-tetrahydroxyberbine hydrobromide (THB); (±)-1-(3,4-dihydroxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (6-methoxytetrahydropapaveroline, 6-OMe-THP); (±)-1-(3,4-dihydroxybenzyl)-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (7-methoxytetrahydropapaveroline, 7-OMe-THP); (±)-1-(3-methoxy-4-hydroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (3′-methoxytetrahydropapaveroline, 3′-OMe-THP); (±)-1-(3-hydroxy-4-methoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (4′-methoxytetrahydropapaveroline, 4′-OMe-THP); (±)-2-methoxy-3,10,11-trihydroxyberbine hydrochloride (2-OMe-THB; (±)-3-methoxy-2,10,11-trihydroxyberbine hydrochloride (3-OMe-THB); (±)-10-methoxy-2,3,11-trihydroxyberbine hydrochloride (10-OMe-THB); (±)-11-methoxy-2,3,10-trihydroxyberbine hydrochloride (11-OMe-THB); (±)-1-(3-hydroxy-4-methoxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (norreticuline); and (±)-2,11-dihydroxy-3,10-dimethoxyberbine hydrochloride (coreximine) were synthesized in our laboratory using established synthetic routes [1]. The purity and identity of these compounds were determined by h.p.l.c., infrared spectroscopy, combined gas chromatography/mass spectrometry [17] and elemental analysis. The optical rotation of asymmetric compounds was determined with a Zeiss manual polarimeter (Carl Zeiss, Oberkochen, West Germany). Pyrogallol, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide were purchased from the Sigma Chemical Co., St. Louis, MO. Tropolone and *N,O*-bis-(trimethylsilyl) acetamide (BSA) were obtained from Regis Biochemicals, Morton Grove, IL. Radiolabeled *S*-adenosyl-L-[methyl-^{14}C]methionine dihydrogen sulfate (500 μCi/m-mole) was purchased from the Amersham–Searle Corp., Arlington Heights, IL. *S*-Adenosyl-L-methionine hydrogen sulfate was obtained from Boehringer–Mannheim Biochemicals, Indianapolis, IN. *N*-Methyl-bis-trifluoroacetamide (MBTFA) was obtained from the Pierce Chemical Co., Rockford, IL. Power Sil-Prep was purchased from the Applied Science Labs, State College, PA. All other chemicals were of the highest quality commercially available. Alkaloid solutions were prepared immediately before use in 0.001 N HCl containing 1 mM dithiothreitol (DTT) and all other solutions were prepared in triple distilled deionized water (16 MΩ).

Instrumentation. An ALC-202 ultraviolet detection high-pressure liquid chromatograph (Waters Associates, Milford, MA) equipped with a valve-loop injector (Glenco Scientific, Houston, TX) and a modular chromatograph consisting of a reciprocating piston pump (Laboratory Data Control, Riviera Beach, FL), a septum injector (Altex Scientific, Berkeley, CA) and an electrochemical detector (e.c.) (Bioanalytical Systems, West Lafayette, IN) were employed. The gas chromatographic instrumentation included a Barber–Colman series 5000 gas chromatograph (FID) fitted with an Infotronics model CRS-208 digital integrator and a Fisher/Victoreen series 4000 gas chromatograph (FID) equipped with an Auto Lab model 6300 digital integrator. Mass spectra were determined with a Finnigan model 4000 automated gas chromatograph/electron impact (EI)-chemical ionization (CI) mass spectrometer equipped with a model 6111 data system (Finnigan Corp., Sunnyvale, CA).

Preparation of rat liver O-*methyltransferase.* Male rats weighing 250–300 g were decapitated and the livers were quickly removed and chilled in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM DTT. Routinely, two rat livers (23 g tissue wet weight) were processed. All subsequent procedures were carried out at 0–4°. A 10% (w/v) homogenate was prepared by applying four repeated cellular disruptions (5-sec duration) with an Ivan Sorvall Omni-Mixer. The homogenate was centrifuged in a Sorvall RC2-B refrigerated centrifuge at 27,000 *g* for 30 min. The resultant pellet was discarded and the remaining supernatant fraction was retained for further purification. Solid ammonium sulfate (enzyme grade), 17.6 g, was slowly added with stirring to each 100 ml of the 27,000 *g* supernatant fraction. After the addition of the salt, the solution was allowed to equilibrate for 30 min and then was centrifuged at 15,000 *g* for 20 min. The precipitate was discarded and 19.8 g of solid ammonium sulfate/100 ml of solution was slowly added to the resultant supernatant fraction.

After the addition of the salt (60% saturation), the solution was stirred for an additional 30 min and then centrifuged at 15,000 *g* for 20 min. The resultant precipitate was dissolved in 0.01 M sodium phosphate (pH 7.4) containing 1 mM EDTA and 1 mM DTT and dialyzed overnight against 5 liters of 0.01 M sodium phosphate (pH 7.4) containing 1 mM DTT and 1 mM EDTA. Following dialysis, the protein solution was centrifuged at 40,000 *g* for 1 hr to remove insoluble protein. The 30–60% ammonium sulfate dialyzed protein solution was stored in 2-ml aliquots at −20° until use. Enzyme activity was stable for a period of at least 3 months.

Incubation system for O-*methylation.* Incubation constituents, in final concentrations, were as follows: 10 mM semicarbazide, 3.0 mM $MgCl_2$, 0.1 M sodium phosphate buffer (pH 7.4), 0.5 mM EDTA, 1.0 mM *S*-adenosyl-L-methionine (SAM), enzyme preparation (3–5 mg protein), 0.5 mM optical isomeric or racemic substrate (THP or THB), various concentrations of inhibitor compounds when indicated, and the necessary volume of water to a total reaction volume of 2.0 ml. Blank reaction mixtures contained boiled enzyme preparation (5 min at 100°). After a 30- or 60-min incubation at 37° with shaking, the reaction was terminated by the addition of 0.1 ml of concentrated perchloric acid (60%). After filtration of the deproteinized reaction mixtures (Millipore manifold, 0.45-μM filters), the filtrates were analyzed by high-pressure liquid chromatography.

Inclusion of DTT in the assay system enhances the stability of both enzyme preparation and alkaloidal substrates and products during incubation. Furthermore, the stability of the alkaloids as determined by recovery of authentic standards was unaffected during isolation, collection and identification procedures employed in this study.

High-pressure liquid chromatography. Prepacked 3.2 mm i.d. × 25 cm columns of 10 μm Vydac TP 401 SCX cation exchanger packing (The Separations Group, Hesperia, CA) were employed. Routinely, 20 to 50-μl aliquots of filtered incubation samples were injected and monitored with an ultraviolet (280 nm) or

electrochemical detector (e.c.). The mobile phase for THP and respective *O*-methylated metabolites was 0.5 M ammonium phosphate buffer (pH 4.5), whereas the mobile phase for THB and its respective *O*-methylated products was 0.5 M ammonium phosphate buffer (pH 4.5) containing 5% (v/v) 1,4-dioxane. The mobile phases were prepared in triple distilled deionized water and the flow rate was 0.5 ml/min for e.c.-h.p.l.c. and 2.0 to 3.0 ml/min for u.v.-h.p.l.c.

Quantitation and isolation of O-*methylated products.* For structure confirmation, *O*-methylated metabolites of THP and THB were isolated by collecting the effluent of individual chromatographic peaks. The effluent collections for individual metabolites were adjusted to pH 7.0 with sodium hydroxide and then dried *in vacuo*. The metabolites were dissolved in toluene–isoamyl alcohol (3:2) and back extracted into 0.1 vol. of 0.1 N HCl. Quantitation of *O*-methylated metabolites of THP and THB was achieved by h.p.l.c. utilizing authentic reference standards.

Gas chromatography/mass spectrometry structure determination. Different agents were used for derivatizing the *O*-methylated metabolites of THP and THB. Aliquots (0.5 to 1.0 ml) of the *O*-methylated THP extracts were dried *in vacuo*, reacted with 30 μl BSA, 10 μl MBTFA and 10 μl tripropylamine for 60 min at 80° to yield *N*-trifluoroacetyl, *O*-trimethylsilyl-THP derivatives. Aliquots (0.5 to 1.0 ml) of the *O*-methylated THB extracts were dried *in vacuo*, reacted with 50 μl Power Sil-Prep, a mixture of bis-trimethylsilylacetamide (BSA), trimethylsilylimidazole (TMSIM) and trimethylchlorosilane (TMCS) in a ratio of 3:3:2, for 90 min at 80° to yield trimethylsilyl (TMSI)-THB derivatives.

The silylated metabolites of THP were analyzed using glass gas chromatography columns (2 mm × 6 ft) of 3% OV-1 on 100/120 mesh Gas Chrom-Q (Applied Science Laboratories, Inc., State College, PA). Conditions were: carrier, methane at 20 ml/min; injector temperature, 260°; column temperature, 250°; and transfer line temperature, 260°. The metabolites of THB were analyzed on identical columns packed with 3% OV-17 from the above vendor. In this case, helium was used as the carrier gas, with flow nominally 20 ml/min and injector temperature 260°. The mass spectrometer was operated in the CI mode for the measurement of metabolites of THP and in the EI mode for the analysis of THB metabolites.

Incorporation of S-*adenosyl*-L[*methyl*-^{14}C]*methionine.* A radiometric procedure was employed to monitor the amount of radioactivity derived from [^{14}C]SAM enzymatically incorporated into (±)-THP or (±)-THB. The standard incubation system with the addition of *S*-adenosyl-L-[methyl-^{14}C]methionine (sp. act. 0.35 μCi/μmole) was utilized in the assay procedure. After a 30-min incubation at 37° the *O*-methylated metabolites from THP and THB reaction mixtures were isolated and collected by h.p.l.c. Aliquots (200 μl) of the resultant collected peaks were counted in 15 ml of scintillation fluid: 333 ml Triton-X-100, 667 ml of toluene, 0.2 g of 1,4-bis-{2-(5-phenyloxazolyl)}benzene (POPOP) and 4 g of 2,5-diphenyloxazole (PPO). Radioactivity was determined with a Packard TriCarb spectrometer.

Protein determination. Protein concentrations were determined by the method of Bradford [18] utilizing the Bio-Rad Protein Assay Kit. Bovine gamma globulin was used as the protein standard.

RESULTS

O-*methylation pattern of THP and THB.* The major *O*-methylated products of THP are illustrated in Fig. 1, and the h.p.l.c. elution profile is seen in Fig. 2. The four mono-*O*-methylated derivatives of THP were resolved into four distinct peaks by h.p.l.c. The incubation product peaks B and C (Fig. 2) correspond to 7-OMe-THP and 6-OMe-THP respectively. Mono-*O*-methylation of THP was contained exclusively in the isoquinoline ring of the molecule at positions 6 and 7 and there was no evidence of *O*-methylation at the 3′ or 4′ positions of the THP molecule. After a 30-min incubation at 37°

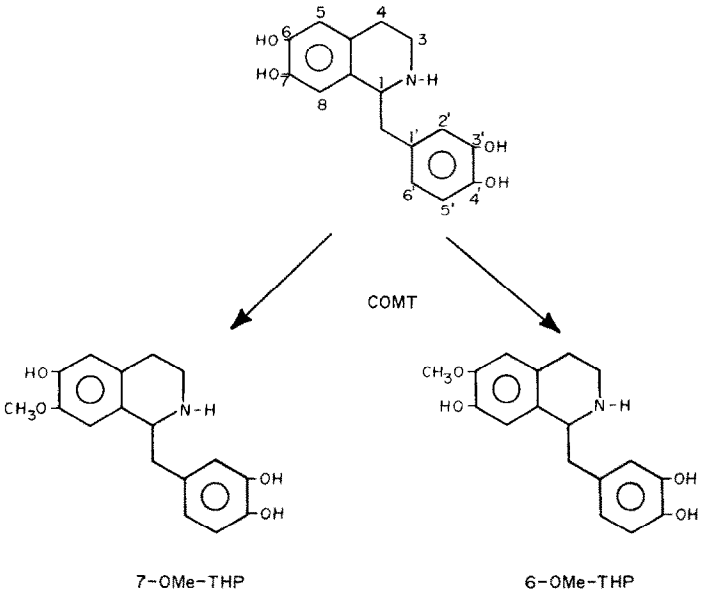

Fig. 1. *O*-Methylation of tetrahydropapaveroline (THP).

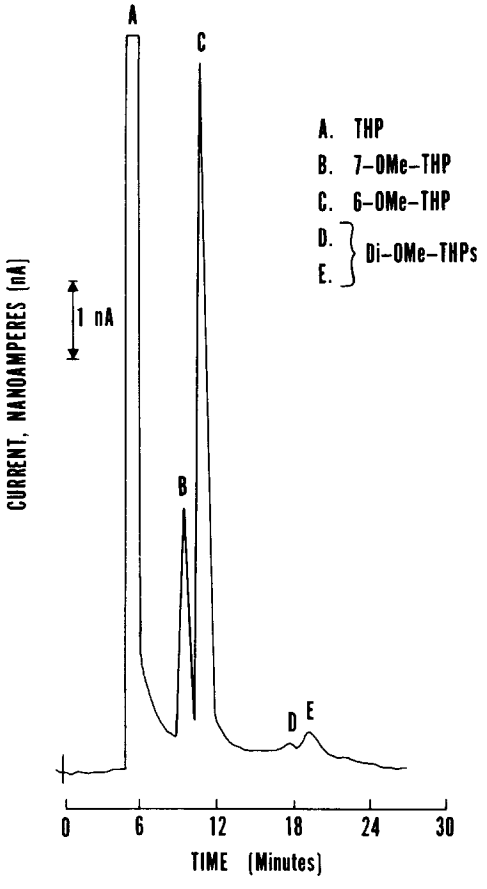

Fig. 2. High-pressure liquid chromatographic profile (e.c.-h.p.l.c.) of (±)-THP *O*-methylation. The chromatographic conditions were: mobile phase, 0.5 M ammonium phosphate (pH 4.5); flow rate, 0.5 ml/min; and working electrode setting, +0.6 V with respect to AgCl. Enzymatic reaction constituents are described in Materials and Methods and incubations were conducted at 37° for 30 min.

with (±)-THP as substrate, approximately 95 per cent of the total metabolites were identified as mono-*O*-methylated products and 5 per cent as non-vicinal di-*O*-methylated metabolites by h.p.l.c. and g.c./m.s. Longer incubation periods (60 min, data not shown) result in greater accumulation of di-*O*-methylated metabolites.

The *O*-methylation pattern of THB is illustrated in Fig. 3 and the h.p.l.c. elution profile is given in Fig. 4. The h.p.l.c. retention times of the four possible mono-*O*-methylated standards of THB were determined. The incubation product peaks B, C and D (Fig. 4) correspond to 2-OMe-THB, 10-OMe-THB/11-OMe-THB and 3-OMe-THB respectively. Separation by h.p.l.c. of 10-OMe-THB and 11-OMe-THB could not be accomplished with the present solvent system. However, separation and identification of these two positional isomers were accomplished by combined gas chromatography/mass spectrometry. Unlike THP, THB mono-*O*-methylation occurs at the vicinal hydroxyl moieties of both rings A and D (Fig. 3). With (±)-THB as substrate, approximately 95 per cent of the total metabolites were recovered as mono-*O*-methylated products and 5 per cent as non-vicinal di-*O*-methylated products by h.p.l.c. and g.c./m.s. Again, longer incubation periods (60 min, data not shown) resulted in augmented formation of di-*O*-methylated THB metabolites.

Effect of optical isomerism on O*-methylation of THP and THB.* The percentage distribution of mono-*O*-methylation of racemic, R-(+) and S-(−) isomers of THP and THB is presented in Table 1. It is evident that the magnitude of enzymatic *O*-methylation at specific positions is markedly influenced by the particular optical isomeric form of substrate employed. Thus, with racemic (±)-THP, 66 per cent of the *O*-methylated products were found as 6-OMe-THP and 28 per cent as 7-OMe-THP. With R-(+)-THP as substrate, only 26 per cent of the total *O*-methylated products was observed as 6-OMe-THP while 68 per cent was 7-OMe-THP. This pattern was reversed with S-(−)-THP as

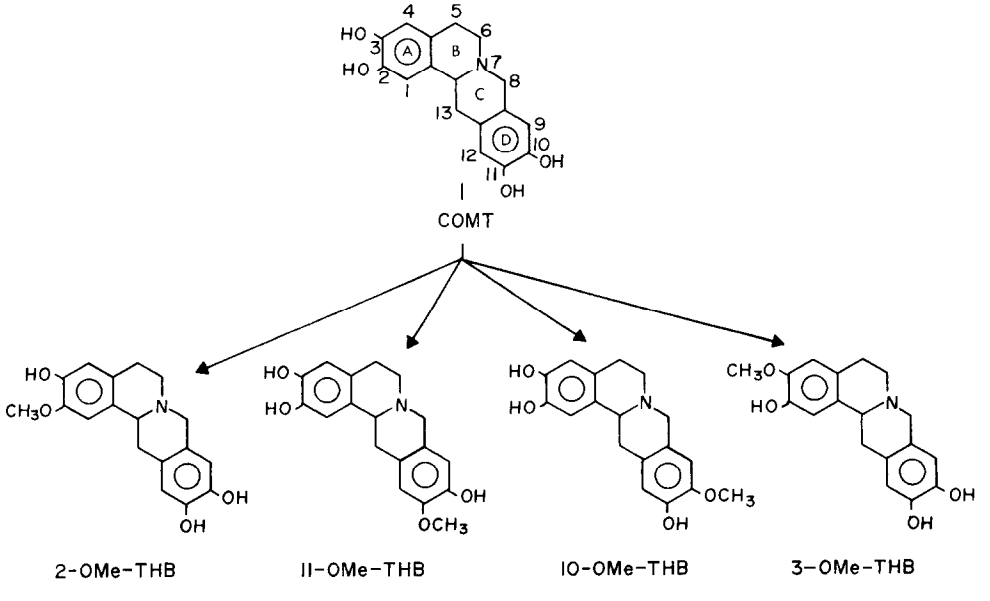

Fig. 3. *O*-Methylation of 2,3,10,11-tetrahydroxyberbine (THB).

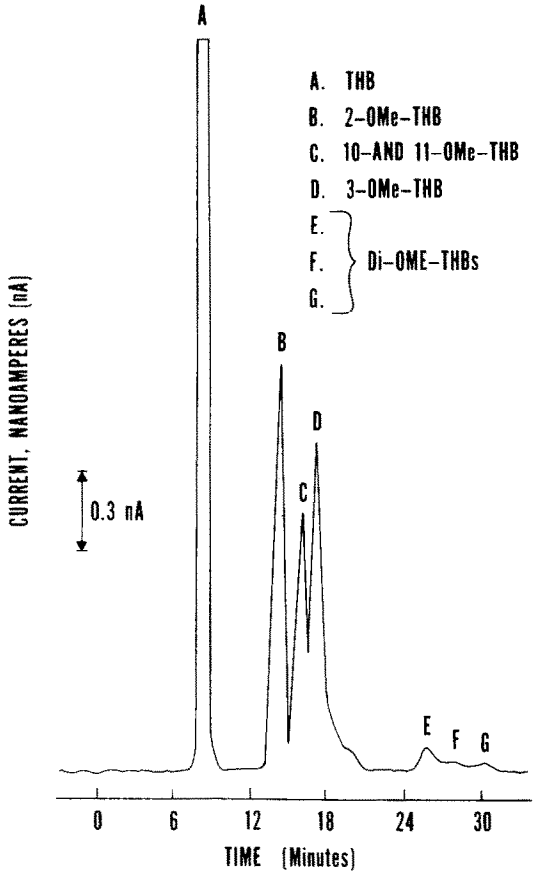

Fig. 4. High-pressure liquid chromatographic profile (e.c.-h.p.l.c.) of (±)-2,3,10,11-tetrahydroxyberbine O-methylation. The chromatographic conditions were: mobile phase, 0.5 M ammonium phosphate containing 5% (v/v) dioxane (pH 4.5); flow rate, 0.5 ml/min; and working electrode setting, +0.6 V with respect to AgCl. Enzymatic reaction constituents are described in Materials and Methods and incubations were conducted at 37° for 30 min.

substrate with 6-OMe-THP representing 79 per cent and 7-OMe-THP 14 per cent of the total O-methylated products. The 6:7 position ratio of (±)-THP, (+)-THP and (−)-THP mono-O-methylations was 2.4, 0.4 and 5.6 respectively. Although the optical isomers of THP differed markedly in the pattern of mono-O-methylation, the rate of total mono-O-methylation did not vary with respect to the optical isomeric form of the substrate [i.e. (±)-, R-(+)-, or S-(−)-THP]. With the partially purified COMT preparation employed, the specific activity was 3.98 ± 0.25 nmoles of mono-O-methylated THPs (i.e. 6-OMe- and 7-OMe-THP) formed/min/mg of protein.

As can be seen in Table 1, notable changes in the magnitude of the pattern of enzymatic O-methylation were observed at positions 2 and 3 with the different optical isomeric forms of the tetracylic protoberberine molecule as substrates. However, it was not possible to quantitate the stereochemical effects of THB substrates O-methylated at individual positions 10 and 11 of THB since resolution of 10-OMe-THB and 11-OMe-THB was not achieved in the present h.p.l.c. solvent system. As with the different optical isomeric forms of THP, the specific activity of COMT with each optical isomeric form of THB was found to be 2.99 ± 0.2 nmoles mono-O-methylated THBs (i.e. 2-OMe-, 3-OMe-, 10-OMe- and 11-OMe-THB) formed/min/mg of protein.

Incorporation of radioactivity derived from S-*adenosyl*-L-[*methyl*-^{14}C]*methionine.* As a correlative quantitative procedure and to assure that O-methylated metabolites are produced via transmethylation processes, the incorporation of radioactivity derived from S-adenosyl-L-[methyl-^{14}C]methionine into the molecular structure of (±)-THP and (±)-THB was studied. The results obtained by radiolabel incorporation exhibit close correlation with the data obtained with the O-methylation product distribution utilizing racemic substrates (Table 1). Therefore, these results verify the occurrence of a transmethylation system participating in the formation of alkaloid mono-O-methylated metabolites derived from THP and THB as well as substanti-

Table 1. Percentage distribution of mono-O-methylated metabolites* and incorporation of S-adenosyl-L-[methyl-^{14}C]methionine during mono-O-methylation of THP and THB†

Substrate	7-OMe-THP (%)	6-OMe-THP (%)	2-OMe-THB (%)	10- and 11-OMe-THB (%)	3-OMe-THB (%)
(±)-THP	28 ± 2	66 ± 4			
(±)-THP ([^{14}C]SAM)	27 ± 2	63 ± 4			
R-(+)-THP	68 ± 5	26 ± 2			
S-(−)-THP	14 ± 1	79 ± 5			
(±)-THN			39 ± 2	24 ± 2	32 ± 4
(±)-THB ([^{14}C]SAM)			40 ± 2	25 ± 2	31 ± 2
R-(+)-THB			51 ± 3	33 ± 2	15 ± 2
S-(−)-THB			23 ± 1	20 ± 2	48 ± 3

* High-pressure liquid chromatographic separation and quantitation of the mono-O-methylation of the racemates and optical isomers of THP and THB, after a 30-min incubation with a liver COMT preparation, were performed. Incubation conditions and sample preparation are described in Materials and Methods. All values are reported as mean ± S.E.M. for at least six separate determinations.

† S-Adenosyl-L-[methyl-^{14}C]methionine (sp. act. 0.35 μCi/μmole) was included in the standard O-methylation assay system with (±)-THP and (±)-THB employed as substrates. Individual radiolabeled O-methylated metabolites were isolated and collected by h.p.l.c. after a 30-min incubation, and per cent incorporation of S-adenosyl-L-[methyl-^{14}C]methionine was determined by liquid scintillation spectrometry. All values are reported as mean ± S.E.M. for at least three separate determinations.

Table 2. Effects of various COMT inhibitors on total mono-*O*-methylations of (±)-THP and (±)-THB*

Inhibitor	% Inhibition of total (±)-THP *O*-methylation	% Inhibition of total (±)-THB *O*-methylation
p-Chloromercuribenzoate		
0.1 mM	20 ± 2	19 ± 2
0.5 mM	94 ± 5	95 ± 5
N-Ethylmaleimide		
0.1 mM	27 ± 2	21 ± 3
0.5 mM	95 ± 5	81 ± 6
Tropolone		
0.1 mM	29 ± 4	20 ± 2
0.5 mM	84 ± 7	82 ± 7
Pyrogallol		
0.1 mM	17 ± 3	18 ± 3
0.5 mM	55 ± 5	71 ± 6

* High-pressure liquid chromatographic separation and quantitation of total mono-*O*-methylated metabolites of (±)-THP and (±)-THB, after incubation with liver COMT preparation, in the presence of various concentrations of inhibitor compounds were performed. Incubation conditions and sample preparations are described in Materials and Methods. All values are reported as mean ± S.E.M. for at least three separate determinations.

ating the accuracy of the magnitude of enzymatic *O*-methyl-positional isomer generation.

Effect of various COMT inhibitors on alkaloid O-methylation. The effect of sulfhydryl binding compounds, *p*-chloromercuribenzoate and *N*-ethylmaleimide, and the competitive substrate inhibitors, tropolone and pyrogallol, were studied (Table 2). These compounds (final concentrations of 0.1 and 0.5 mM) were examined for their inhibitory actions on total (±)-THP and (±)-THB *O*-methylations. At the inhibitor concentrations employed, THP and THB *O*-methylations were equally decreased.

Combined gas chromatographic/mass spectrometric analysis. Chemical ionization (CI) spectra of these fluoroacetyl–silyl compounds using methane as the reagent gas proved to be much more characteristic, with intense molecular ion regions ($M-15^+$, $M+H^+$, $M+29^+$), as well as ions resulting from loss of the benzyl portion of the molecules. As expected, 6-OMe-THP and 7-OMe-THP gave identical CI mass spectral fragmentation patterns, as did the 3′-OMe-THP and 4′-OMe-THP compounds. However, all four metabolites of THP are resolved by h.p.l.c., and utilization of a combination of preparative high-pressure liquid chromatography and gas chromatographic/mass spectral fragmentation patterns provided the means to identify and quantitate all four of these compounds.

The EI mass spectra of the silylated mono-OMe-THBs are characterized by a molecular ion and two main fragment ions representing both the top (rings A and B) and bottom (rings C and D) portions of the molecule. Mass spectra of these compounds allow no differentiation between the 2- and 3-OMe-THB or between the 10- and 11-OMe-THB positional isomers. As was the case with the tetrahydropapaveroline compounds, a combination of preparative h.p.l.c. and gas chromatographic/mass spectral fragmentation patterns was necessary for complete identification and quantitation of the various tetrahydroprotoberberines. The mass spectrometer in the multiple ion monitoring mode was programmed to respond to ions with *m/e* values of 222, 248, 280 and 306. Ions of *m/e* 222 and 306 arise only from 10- and 11-OMe-THB while the ions of 248 and 280 *m/e* values are derived only from the 2- and 3-OMe-THB. *O*-Methylation of 2,3,10,11-THB was found to occur at all four possible hydroxyl sites. The di-*O*-methylated metabolites of THP and THB gave similar h.p.l.c. and g.c. retention times and identical mass spectral fragmentation patterns to the respective authentic standards, norreticuline and coreximine, thus characterizing the multiple methylation metabolites as non-vicinal, di-*O*-methyl derivatives of the substrates.

DISCUSSION

Catechol-*O*-methyltransferase is involved in the transmethylation process providing transfer of the labile methyl group of *S*-adenosyl-L-methionine to one of the vicinal hydroxyl moieties of catechol or catechol derivatives [19]. Studies on the subcellular localization of brain COMT suggest that it is a cytoplasmic enzyme, and the proportion of enzyme found in synaptosomal fractions is accounted for by the cytoplasm being trapped (occluded) within the nerve terminals [20]. The heterogeneity of this enzyme is somewhat controversial. COMT has been reported to be a homogeneous entity [21], whereas other investigators report two electrophoretically separable bands of enzyme activity [22]. Furthermore, Inscoe *et al.* [23] reported the presence of a microsomal *O*-methyltransferase that differs from the soluble enzyme in several aspects.

Mammalian systems appear to be capable of effecting the tetracyclic protoberberine ring coupling of benzyltetrahydroisoquinolines both *in vitro* and *in vivo*. Thus, it was of interest to evaluate the *O*-methylation pattern of the stereoisomeric forms of tetrahydropapaveroline and one of its tetrahydroprotoberberine analogs, 2,3,10,11-tetrahydroxyberbine.

Although isoquinoline alkaloids have been shown to inhibit COMT *in vitro* [14, 24, 25] and *in vivo* [24, 26], these compounds are also substrates for *O*-methylations [14, 25, 27]. The structural elucidation of the *O*-methylation biotransformation products of these compounds, however, had not been accomplished

previously. Creveling *et al.* [28] have shown a significant difference in the meta:para *O*-methylation ratio of the stereoisomers of a number of vicinal hydroxyphenethylamine derivatives, which suggests enzyme recognition of substrate asymmetric centers.

The present *in vitro* enzymatic studies demonstrate that (±)-THP is predominantly mono-*O*-methylated with little di-*O*-methylation. The mono-*O*-methylations were contained exclusively in the isoquinoline ring at positions 6 or 7, sparing any *O*-methylation on the benzylic catechol moiety. In contrast, THB was mono-*O*-methylated at the vicinal hydroxyl functions of both rings A and D. Since the THP molecule maintains a freely rotating benzylic carbon—carbon bond, this portion of the molecule may have difficulty in orientating on the active catalytic site of COMT, therefore reducing the propensity of benzylic-phenolic methylation. On the other hand, the THB molecule represents a fixed analog (i.e. locked benzylic portion of molecule) of THP, thereby producing a molecule containing two identical dihydroxytetrahydroisoquinoline moieties possessing a common nitrogen. Accordingly, enzyme recognition of potential phenolic methylation sites on rings A or D would have the same probability, and, indeed, mono-*O*-methylations occurred on rings A and D at either positions 2-, 3-, 10- or 11- of the protoberberine molecule.

The recent synthesis of the optical isomers of THP and THB made it possible to evaluate the stereoselectivity of COMT for these substrates. Meshi *et al.* [29] reported that the L-isomer of trimetoquinol, a dihydroxytrimethoxybenzyltetrahydroisoquinoline, was preferentially *O*-methylated while the D-isomer was inactive as a substrate. Our studies demonstrated that both R-(+)- and S-(−)-THP and R-(+)- and S-(−)-THB exhibited equipotent substrate activity; however, the ratio of *O*-methylation at positions 6 and 7 of the THP isomers differed markedly. Likewise, differences in the magnitude of positional *O*-methylation were also observed with the optical isomers of THB. Thus, the data suggest that the optical isomeric substrate preference for the enzyme catalytic site differs and may be related to optical isomeric orientation at the active site (Table 1).

The *in vitro* *O*-methylations of THP and THB are seemingly classical methylation reactions, since inhibition of this process was curtailed by established COMT inhibitors (i.e. *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, tropolone and pyrogallol). Furthermore, there was no differential inhibitory effect produced by these COMT inhibitors for the racemates of THP and THB employed as substrates.

The application of combined g.c./m.s. analysis of products contained in various effluent peaks collected from h.p.l.c. separation afforded definitive structural confirmation of metabolites formed by enzymatic *O*-methylation reactions. The availability of authentic THP and THB reference standards in various states of phenolic methylation and optical isomerization has made it possible to utilize innovative h.p.l.c. and combined g.c./m.s. techniques for the elucidation of the phenolic *O*-methylation pathways for THP and THB *in vitro*.

At present, it is unknown whether *O*-methylated biotransformation products of THP or THB exert pharmacological effects similar to their respective tetrahydroxy parent molecules. If, indeed, such a host of isoquinoline alkaloids form *in vivo* in small quantities in select brain areas, their sequestration and/or biotransformation, i.e. *O*-methylation, oxidative phenolic coupling and tetracyclic ring coupling, and subsequent cellular interactions may participate in some of the neuropharmacologic aspects of alcohol-related disorders [3, 4, 11–13]. Consequently, this new information may greatly enhance the efforts and direction of investigations currently probing the biochemical and pharmacologic effects of neuroamine-derived alkaloids.

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REFERENCES

1. M. Shamma, in *The Isoquinoline Alkaloids: Chemistry and Pharmacology* (Eds. A. T. Blomquist and H. Wasserman), Vol. 25. Academic Press, New York (1972).
2. G. Cohen and M. Collins, *Science* **167**, 1749 (1970).
3. V. E. Davis, M. J. Walsh and Y. Yamanaka, *J. Pharmac. exp. Ther.* **174**, 401 (1970).
4. V. E. Davis, J. L. Cashaw and K. D. McMurtrey, in *Advances in Experimental Medicine and Biology* (Ed. M. M. Gross), Vol. 59, p. 65. Plenum Press, New York (1975).
5. W. M. Whaley and T. R. Govindachari, in *Organic Reactions* (Ed. R. Adams), Vol. 6, p. 151. John Wiley, New York (1951).
6. K. D. McMurtrey, L. R. Meyerson and V. E. Davis, Abstr. 190, Div. of Org. Chem., Amer. Chem. Soc., One hundred and seventy third ACS National Meeting (1977).
7. P. Holtz, K. Stock and E. Westermann, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **248**, 387 (1964).
8. A. J. Turner, K. M. Baker, S. Algeri, A. Frigerio and S. Garratini, *Life Sci.* **14**, 2247 (1974).
9. M. Sandler, S. B. Carter, K. R. Hunter and G. M. Stern, *Nature, Lond.* **241**, 439 (1973).
10. C. J. Coscia, W. Burke, G. Jamroz, J. M. Lasala, J. McFarlane, J. Mitchell, M. M. O'Toole and M. L. Wilson, *Nature, Lond.* **269**, 617 (1977).
11. R. D. Myers and C. L. Melchior, *Science* **196**, 554 (1976).
12. C. L. Melchior and R. D. Myers, *Pharmac. Biochem. Behav.* **7**, 19 (1977).
13. C. L. Melchior, A. Mueller and R. A. Deitrich, *Fedn Proc.* **37**, 420 (1978).
14. A. C. Collins, J. L. Cashaw and V. E. Davis, *Biochem. Pharmac.* **22**, 2337 (1973).
15. T. Kametani, M. Takemura, M. Ihara, K. Takahashi and K. Fukumoto, *J. Am. chem. Soc.* **98**, 1956 (1976).
16. T. Kametani, *Chem. Pharm. Bull.* **20**, 1587 (1972).
17. J. L. Cashaw, K. D. McMurtrey, L. R. Meyerson and V. E. Davis, *Analyt. Biochem.* **74**, 343 (1976).
18. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
19. J. Axelrod and R. Tomchick, *J. biol. Chem.* **233**, 702 (1958).
20. O. J. Broch, Jr. and F. Fonnum, *J. Neurochem.* **19**, 2049 (1972).
21. M. Assicot and C. Bohuon, *Eur. J. Biochem.* **12**, 490 (1970).
22. P. J. Anderson and A. D'Ioro, *Biochem. Pharmac.* **17**, 1943 (1968).
23. J. K. Inscoe, J. Daly and J. Axelrod, *Biochem. Pharmac.* **14**, 1257 (1965).

24. A. Giovine, M. Renis and A. Bertolino, *Pharmacology* **14**, 86 (1976).
25. E. E. Smissman, J. R. Reid and D. A. Walsh, *J. med. Chem.* **19**, 127 (1976).
26. A. Giovine, M. Renis and A. Bertolino, *Pharmac. Res. Commun.* **9**, 203 (1977).
27. J. A. Rubenstein and M. A. Collins, *Biochem. Pharmac.* **22**, 2928 (1973).
28. C. R. Creveling, N. Morris, H. Shimizu, H. H. Ong and J. Daly, *Molec. Pharmac.* **8**, 398 (1972).
29. T. Meshi, M. Otsuka and Y. Sato, *Biochem. Pharmac.* **19**, 2937 (1970).