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IDENTIFICATION OF CATECHOLAMINE-DERIVED ALKALOIDS IN MAMMALS BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

Tetrahydropapaveroline, the tetrahydroisoquinoline alkaloid derived from dopamine, is converted *in vivo* by rats and by rat-liver and brain preparations to tetrahydroprotoberberine alkaloids. The latter alkaloids have also been identified for the first time in the urine of parkinsonian patients receiving L-DOPA therapy. These findings suggest that man, like plants, may have the ability to elaborate several classes of alkaloids with potentially important pharmacological consequences.

INTRODUCTION

Although alcohol is the oldest and most widely abused drug known to man, a major obstacle in the prevention and treatment of alcoholism is the unresolved role that alcohol as a drug plays in the genesis of alcohol addiction. We have presented a biochemical approach postulating that drug-evoked aberrations of neuroamine metabolism may result in this endogenous formation of pharmacologically active alkaloids[1–5].

This concept is based on the premise that alcohol —through its primary metabolite acetaldehyde— as well as certain other sedative-hypnotic drugs such as chloral hydrate and barbiturates disrupt the major metabolic route for disposition of catecholamine-derived aldehydes[5,6]. This disruption increases the availability of these aldehydes for participation in a Pictet-Spengler type condensation[7] with the intact neuroamines, the products of which are the corresponding pharmacologically active benzyltetrahydroisoquinoline (THIQ) alkaloids[1–5].

Additionally, the intermediacy of THIQ alkaloids is requisite in plants to the biosynthesis of a broad array of even more complex alkaloids —*e.g.*, the protoberberine, papaverine, morphine, aporphine, benzophenanthridine, and phthalidoisoquinoline classes[8–14]. Therefore, the *in situ* formation of THIQ alkaloids in man could make these aberrant neuroamine metabolites available for further conversion to even more complex alkaloids. Thus, certain neuropharmacological effects of alcohol and pharmacologically equivalent drugs may be mediated through various aberrant alkaloid metabolites of the neuroamines.

Since tetrahydropapaveroline (THP; norlaudanosoline), the THIQ alkaloid derived from dopamine (DA), had not been demonstrated as a DA metabolite in intact animals or in man, publication of the foregoing hypothesis and its initial supporting data[1–5] generated considerable controversy[15–18]. However, subsequent identification of THP in the urine of parkinsonian patients receiving oral L-DOPA therapy[19] and in the CNS of animals after treatment with L-DOPA, DA, and ethanol[20] strengthened the credibility of the hypothesis.

Direct assessment of the total production of amine-derived THIQ alkaloids in mammalian systems is complicated by the probability that, far from being metabolic end products, they are extensively metabolized along diverse routes to even more complex alkaloids. We wish to report on further elaboration of this aspect of the hypothesis[1–5], and define the formation of tetrahydroprotoberberine (THPB) alkaloids in experimental animals and in man (Fig. 1A).

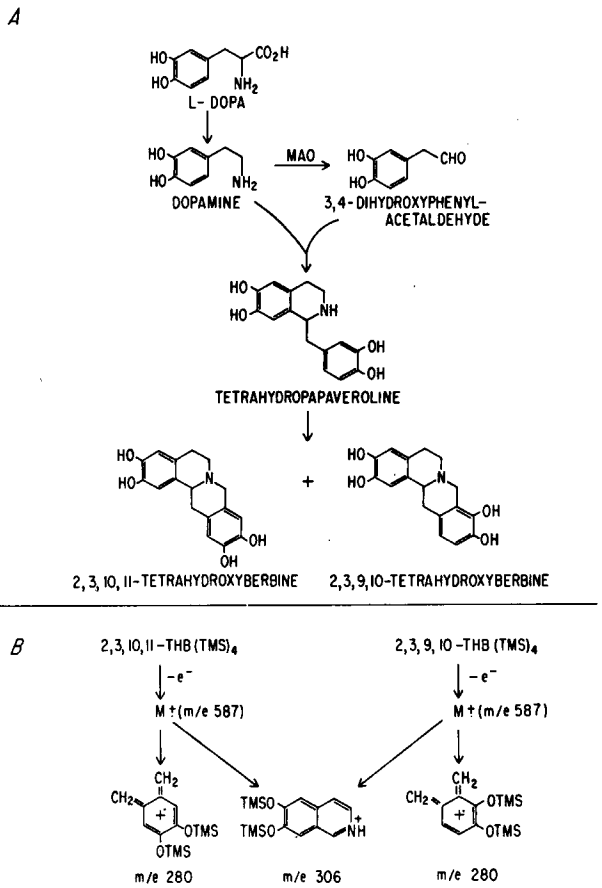

Fig. 1. (A) Reaction sequence for the formation of THPB alkaloids from catecholamines. (B) Fragmentation pattern of TMS derivatives of the isomeric 2,3,10,11-THB and 2,3,9,10-THB, indicating congruence of their mass spectra.

EXPERIMENTAL

In vivo metabolism of THP by rats

Tetrahydropapaveroline·HBr (0.5 m*M*/kg, 189 mg/kg) in 30% propylene glycol was injected intraperitoneally into male Sprague–Dawley rats weighing about 200 g each. Rats similarly injected with the vehicle served as controls. Urine was collected in 4 ml 0.1 *N* HCl for 10 h after the injection. At the close of the collection period, the urine obtained from three rats of each treatment group was pooled and diluted to 70 ml with distilled water. Ascorbic acid (35 mg), EDTA (70 mg) and one-tenth volume sodium acetate buffer (0.1 *M*, pH 5.0) were added. The mixture was then adjusted to pH 5.3 with dilute NaOH, 1 ml Glusulase (100,000 U β-glucuronidase and 50,000 U sulfatase per ml; Endo Labs., Richmond Hill, N.Y., U.S.A.) was added, and the samples were incubated at 37° for 30 h. The incubates were buffered by addition of 5 g monobasic sodium phosphate and adjusted to pH 7.0 with 5 *N* sodium hydroxide. The metabolites of THP were extracted into four volumes of toluene–isoamyl alcohol (3:2) and returned to one-tenth volume 0.1 *N* HCl. Aliquots (1 ml) of the HCl extracts were dried *in vacuo*. Trimethylsilyl (TMS) derivatives were prepared for gas chromatography (GC) by reaction of the dried extract with hexamethyldisilazane–trimethylchlorosilane (9:1), 100 μl, for 8 h at 65°. TMS derivatives were prepared from authentic compounds in a similar manner. Extraction efficiency and quantification were achieved by measuring peak area and using authentic reference compounds.

In vitro metabolism of THP by rat brain and liver

The portion of the soluble supernatant fraction —obtained by centrifuging homogenates of livers or brains from Sprague–Dawley rats at 46,000 *g* for 60 min, precipitating at 30% to 50% saturation with ammonium sulfate— was used as the enzyme source. The enzyme preparation (15 mg protein) was incubated at 37° for 1 h with 12.5 μmoles THP, 2.5 μmoles S-adenosyl-L-methionine (SAM), 1.25 μCi[^{14}C]-SAM, 1.25 μmoles $MgCl_2$, 1.25 mg ascorbic acid, and 0.5 mmoles phosphate buffer (pH 8.0) in a final volume of 6.0 ml. The reaction was terminated by adding 6 ml 0.5 *M* phosphate buffer (pH 6.0) and immediately extracting the reaction products into 50 ml toluene–isoamyl alcohol (3:2). The metabolites were then returned to one-tenth volume 0.1 *N* HCl and processed as described above.

Urinary metabolites of parkinsonian patients on L-DOPA therapy

Urine samples collected for a 24-h period from two female parkinsonian patients receiving 4 to 5 g of L-DOPA daily were processed in the same manner described for rat urine.

Gas chromatography and mass spectrometry

GC profiles were generated on a Barber–Coleman Series 5000 gas chromatograph (FID). Conditions were: column, 4 mm × 6 ft. 3% OV-1 on Gas-Chrom Q, 100–120 mesh, temperature, 260°; injector temperature, 270°; detector temperature, 290°; carrier gas, nitrogen, flow-rate, 60 ml/min. For combined GC–radioassay, the instrument was fitted with a stream-splitter (50:50), so that the radioactivity of each peak could also be monitored. Mass spectra were determined on an LKB 9000 gas chromatograph–mass spectrometer. Gas chromatographic conditions were: column,

4 mm × 9 ft. 1% SE-30 on Gas-Chrom P, 100–120 mesh; injector temperature, 260°; column temperature, 255°; carrier gas, helium, flow-rate, 30 ml/min. GC profiles obtained on SE-30 were equivalent to those on OV-1. Mass spectrometer conditions were: temperature of molecular separator, 255°; temperature of ion source, 290°; electron energy, 70 eV; trap current, 60 μA; and electron multiplier, 3.7 kV.

RESULTS AND DISCUSSION

THP is known to be an excellent substrate for catechol O-methyl-transferase *in vitro*[21]. Evidence of THP's ready conversion to O-methylated benzyltetrahydroisoquinoline derivatives by rats *in vivo* is found in the significant amounts of several O-methylated THIQ products excreted[22]. In addition, the capability of mammalian systems to effect the synthesis of the tetracyclic ring system of protoberberine alkaloids is demonstrated by the production of four THPB alkaloids (Fig. 2A).

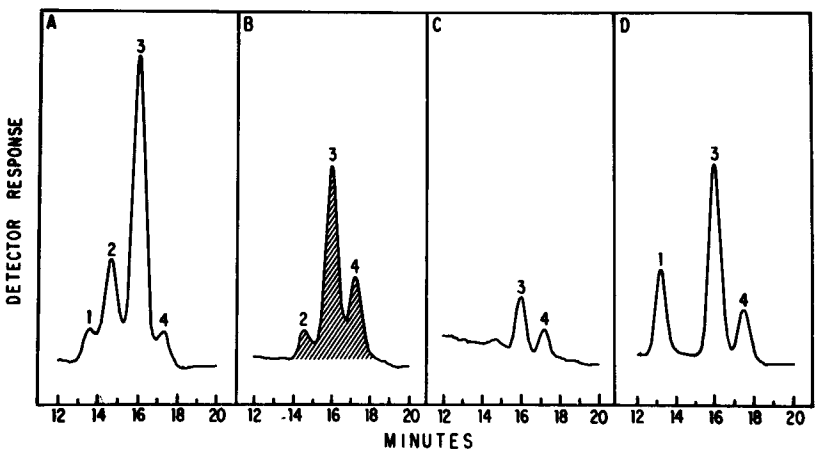

Fig. 2. Gas chromatographic separation of silylated tetrahydroprotoberberine alkaloids from (A) urine of rats pretreated with tetrahydropapaveroline; (B) an incubation mixture of the soluble fraction of rat-liver homogenates with tetrahydropapaveroline and S-adenosylmethionine; (C) urine of a parkinsonian patient receiving L-DOPA treatment; (D) a mixture of authentic reference compounds. The peaks correspond to: (1) coreximine (2,11-dihydroxy-3,10-dimethoxyberbine), (2) a 2- or 3-monomethylated derivative of tetrahydroxyberbine, (3) 2,3,10,11-tetrahydroxyberbine, and (4) 2,3,9,10-tetrahydroxyberbine. Compound No. 1 is tentatively identified as coreximine on the basis of its retention time. The structure assignments of Nos. 2, 3, and 4 are based on gas chromatographic and mass spectral characteristics.

In the 10-h period following THP administration to rats, a total of 1.23 mg of THPB alkaloids was recovered from the pooled urine. The major THPB alkaloid metabolic excreted was 2,3,10,11-tetrahydroxyberbine (64.2%). Minor constituents included 2,3,9,10-tetrahydroxyberbine (5.7%) and two methylated derivatives. The latter were tentatively identified as, first, coreximine —2,11-dihydroxy-3,10-dimethoxyberbine (6.5%)— with a retention time of 13.3 min and methylene unit value of 30.9, and, secondly, a 2- or 3-monomethylated derivative of 2,3,10,11-tetrahydroxyberbine (23.6%), with a retention time of 14.8 min and methylene unit value of 31.3 (Fig. 2A). None of these THPB alkaloids was detected in the urine of the control rats.

The mass spectra (Fig. 1B) of the silylated derivatives of two of these THP metabolites were identical and also congruent with those obtained for the authentic reference compounds —2,3,9,10-tetrahydroxyberbine and 2,3,10,11-tetrahydroxyberbine— each having an apparent molecular ion at *m/e* 587 (35%), a base peak at *m/e* 280, and a fragment ion at *m/e* 306 (23%). This fragmentation pattern is characteristic of silylated THPB alkaloids, but it does not differentiate between structural isomers. However, these isomers are resolved by GC (Fig. 2A). Comparison with authentic reference standards (Fig. 2D) established that one isomer, having a retention time of 16.1 min and methylene unit value of 31.7, was 2,3,10,11-tetrahydroxyberbine; and the other, having a retention time of 17.3 min and methylene unit value of 32.0, was 2,3,9,10-tetrahydroxyberbine.

These findings signaled the existence of a previously unrecognized enzyme system in mammals that mimics the capability of plants to form the "berberine bridge" from the benzyltetrahydroisoquinoline alkaloids. Although the enzyme systems in plants effecting THPB alkaloid biosynthesis have not been defined, it is known that the carbon atom of the "berberine bridge" can be derived from the methyl group of methionine[23]. Thus the methyl group of S-adenosylmethionine seemed a likely candidate for this role in mammalian systems. Accordingly, rat-liver or brain homogenates supplemented with [^{14}C]SAM produced three of the four THPB alkaloids detected as urinary THP products. Through combined GC–radioassay (Fig. 2B) and mass spectral analysis, the three radiolabeled THPB alkaloids were identified as 2,3,10,11-tetrahydroxyberbine, 2,3,9,10-tetrahydroxyberbine, and a 2- or 3-monomethylated derivative of tetrahydroxyberbine. The 2,3,10,11-tetrahydroxy isomer was the major THPB metabolite found both *in vitro* and *in vivo*.

Both rat-liver and brain preparations produced the same ratio of THPB products. Enzymatic mediation of this reaction is confirmed by the demonstration that these compounds are not formed in significant amounts when the tissue preparations are boiled before incubation. Further characterization of this newly discovered enzyme in mammalian systems —at present designated as tetrahydroprotoberberine synthetase— is now underway.

Two isomeric THPB alkaloids —2,3,10,11-tetrahydroxyberbine and 2,3,9,10-tetrahydroxyberbine— which were found to be THP metabolites in rats both *in vivo* and *in vitro*, were detected in the urine of two parkinsonian patients receiving L-DOPA therapy (Fig. 2C). GC profiles and mass spectral fragmentation patterns of these alkaloids were identical to the authentic reference compounds. The quantitative significance and the effects of alcohol and related drugs on the production of this class of complex alkaloids in man is presently under investigation.

Biogenic theory dictates that benzyltetrahydroisoquinoline alkaloids are obligatory intermediates in the biosynthesis of THPB alkaloids[13,14]. THP has recently been detected in the urine of parkinsonian patients receiving L-DOPA treatment[19]. Therefore, identification of THPB alkaloids as urinary metabolites in man adds further credence to the postulate that neuroamine-derived THIQ alkaloids not only can be formed by man (especially under appropriate pharmacological conditions) but also can be converted to even more complex alkaloids[1–5].

The foregoing results are in consonance with the ideas proposed[1–5]. The ability of mammalian systems to insert the one carbon unit —derived from S-adenosylmethionine— to form the "berberine bridge" thus completing the skeleton of the

THPB alkaloids is established. Furthermore, the intermediacy of THP in the biosynthesis of complex THPB alkaloids by mammalian systems is confirmed. In addition to the well known pharmacological actions of the morphine alkaloids, representatives of most other classes of complex alkaloids derived biosynthetically from the tetrahydroisoquinolines have a wide range of pharmacological activity[14,24,25]. The specific THPB alkaloids revealed by these studies as ultimately deriving from catecholamines in mammalian systems have not yet been examined for their pharmacological effects. However, derivatives closely related to them are known to produce sedation and to potentiate barbiturate hypnosis[26]. Additionally, a large number of substituted THPB alkaloids have been synthesized and patented as tranquilizers[27].

This newly demonstrated ability of mammalian systems to evoke the biosynthesis of benzyltetrahydroisoquinoline-derived alkaloids —a capability previously considered unique to plants— elects the THPB alkaloids as representative of the first class of a possible constellation of complex mammalian alkaloids elaborated from the neuroamines.

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