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Note

Method for the identification of tetrahydropapaveroline using the Pictet–Spengler condensation reaction and high-performance liquid chromatography

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Since development of the amine-derived alkaloid hypothesis [1–4], researchers have been interested in demonstrating the presence of tetrahydropapaveroline (THP) and its metabolites in mammalian systems [5–9]. A method for the determination of THP and related alkaloids, employing a multiple-stage purification procedure coupled with high-performance liquid chromatography with electrochemical detection (HPLC–ED), has recently been reported [10]. HPLC analysis does not provide unequivocal identification of a substance, therefore, a simple technique was sought for confirming the identity of THP. One well established method for obtaining support for the identity of a compound is the use of a chemical reaction. THP is especially amenable to this method of identification since the reaction of THP with formaldehyde (Pictet–Spengler condensation) results in the formation of two tetrahydroprotoberberine isomers, 2,3,9,10-tetrahydroxyberbine (2,3,9,10-THB) and 2,3,10,11-tetrahydroxyberbine (2,3,10,11-THB) [11,12]. The kinetics and product distribution of this reaction have been reported by this laboratory [13].

In this paper we describe a technique for the identification of THP in biological extracts. This method involves the reaction of THP with formaldehyde to form 2,3,9,10-THB and 2,3,10,11-THB and the subsequent separation and identification of these reaction products by HPLC–ED.

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EXPERIMENTAL

Chemicals

Tetrahydropapaveroline hydrobromide, 2,3,10,11-tetrahydroxyberbine hydrobromide, 2,3,9,10-tetrahydroxyberbine hydrobromide, and 8-methyl-2,3,10,11-tetrahydroxyberbine hydrobromide (8-Me-THB) were synthesized in this laboratory using established synthetic routes [14]. 8-Me-THB was used as the internal standard in the isolation procedure for THP and related alkaloids. All other chemicals and reagents were the highest quality commercially available.

Sample preparation

Brain extracts from rats treated with ethanol plus L-DOPA and extracts from the urine of Parkinsonian patients receiving L-DOPA therapy were used in this study. Pooled purified brain extracts from rats pretreated with ethanol (3 g/kg, intraperitoneally) 120 min and L-DOPA (200 mg/kg, intraperitoneally) 90 min before decapitation were prepared using published methodology [10,15]. An additional step was incorporated into the procedure for the preparation of purified extracts of urine obtained from Parkinsonian patients receiving L-DOPA (Sinemet) therapy. After acid hydrolysis of the urine, THP and related alkaloids were concentrated from the urine on a Bio-Rex 70 resin bed and eluted with hydrochloric acid [16]. The column eluate was subjected to the same procedure described for the isolation of alkaloids from rat brain supernatant [10].

HPLC–ED instrumentation

The HPLC–ED system consisted of a Model LC-154 LCED analyzer with an LC22A-23A temperature controller (Bioanalytical Systems, BAS, West Lafayette, IN, U.S.A.). The electrochemical detector (Model LC-4B, BAS) was equipped with a glassy carbon electrode adjusted to the fraction collection position. The chromatographic conditions were: detector potential, +0.65 V vs. Ag/AgCl; column, Supelcosil LC-18DB (25 cm×4.6 mm I.D.), 5 μm (Supelco, Bellefonte, PA, U.S.A.); column temperature, 29°C; flow-rate, 0.85 ml/min; mobile phase, 0.05 *M* ammonium dihydrogenphosphate containing 0.75 m*M* triethylamine, 0.05 m*M* sodium pentanesulfonate, and 4.6% (v/v) 1,4-dioxane, adjusted to an apparent pH of 4.5.

HPLC–ED procedure

Reference THP (6 pmol) was injected into the HPLC–ED system. Approximately 500 μl of the eluate fraction containing THP were collected in a 1-ml plastic autoanalyzer cup immediately upon exiting the glassy carbon electrode. Next, 150 μl of a formaldehyde solution (0.1 *M* HCHO in 1.0 *M* $NH_4H_2PO_4$, pH 6.0) was added to a 230-μl portion of the eluate fraction containing the collected THP. The solution was mixed well, covered with Parafilm, and held at room temperature for 20 min. The reaction was quenched by adding 10 μl of concentrated phosphoric acid and mixing well. A portion of the reaction mixture (250 μl) was rechromatographed on the HPLC–ED system and the retention time of

each reaction product was compared to that of an authentic reference standard. The same procedure was used for the identification of THP in biological extracts.

RESULTS AND DISCUSSION

The Pictet–Spengler condensation of THP with formaldehyde results in the formation of 2,3,9,10-THB and 2,3,10,11-THB (Fig. 1). Baseline resolution of THP, 2,3,9,10-THB, 2,3,10,11-THB, and 8-Me-THB (used as internal standard in sample preparations) was achieved by HPLC–ED analysis (Fig. 2A). The retention times of 2,3,10,11-THB, THP, 8-Me-THB, and 2,3,9,10-THB were 12.73, 15.06, 17.26, and 19.37 min, respectively (Fig. 2A). The products of the reaction between formaldehyde and authentic THP collected in the HPLC eluate are shown in Fig. 2B. It can be observed that only a small amount of THP remained in the reaction mixture and that the major product of the reaction was 2,3,10,11-THB. A trace amount of 2,3,9,10-THB was also detected.

The presence of THP in biological extracts was confirmed in a similar manner. The HPLC profile of pooled brain extracts from rats treated with ethanol (3 g/kg, intraperitoneally) 120 min and L-DOPA (200 mg/kg, intraperitoneally) 90 min before decapitation (also containing the internal standard, 8-Me-THB) is shown in Fig. 2C. The peak in the chromatogram identified as THP (Fig. 2C) has the identical retention time as authentic THP (Fig. 2A). The products of the reaction between formaldehyde and THP collected in the HPLC eluate from pooled rat brain extract are shown in Fig. 2D. It can be observed that the HPLC profile representing the reaction of formaldehyde with THP from rat brain extract (Fig. 2D) is indistinguishable from the profile obtained from the raction of authentic THP (Fig. 2B). In both cases, all but a trace of collected THP was converted to 2,3,10,11-THB and 2,3,9,10-THB upon reaction with formaldehyde. Identical results were also obtained (chromatograms not shown) when formal-

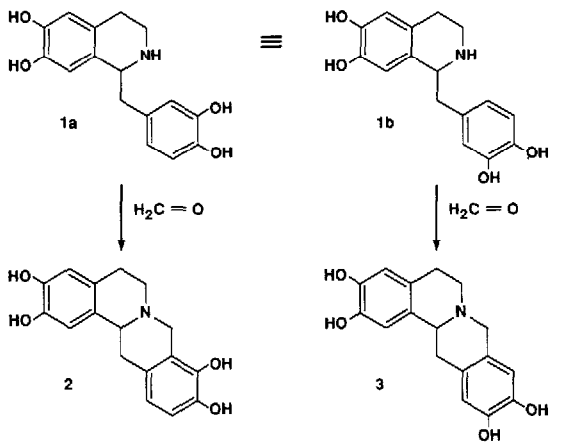

Fig. 1. Reaction depicting the Pictet–Spengler condensation of tetrahydropapaveroline (equivalent structures 1a and 1b) with formaldehyde to form 2,3,9,10-tetrahydroxyberbine (2) and 2,3,10,11-tetrahydroxyberbine (3).

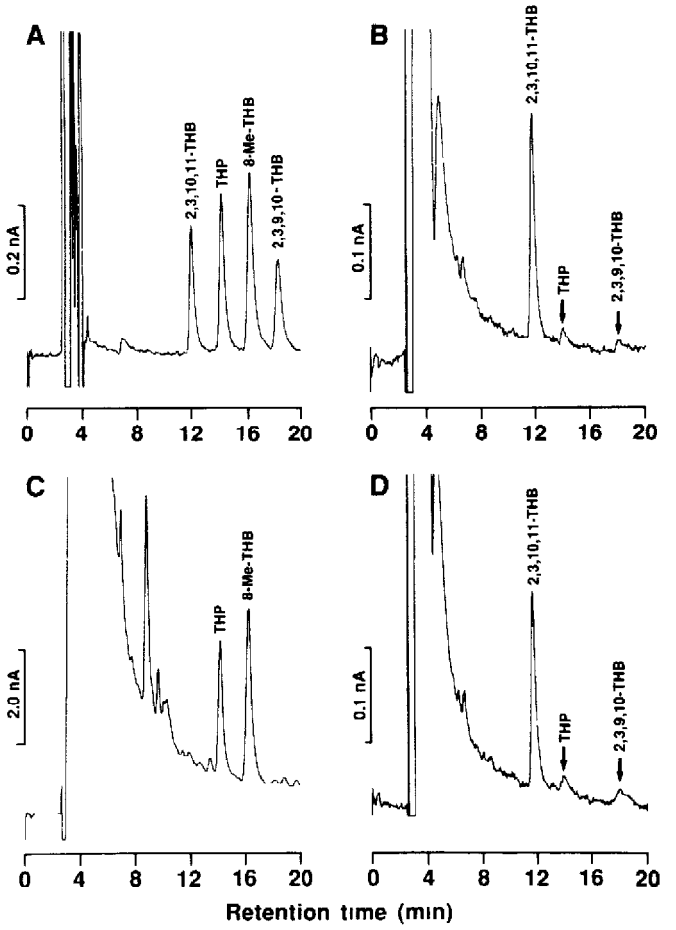

Fig. 2. Chromatographic profiles of: (A) a reference mixture of 2,3,10,11-THB (1.0 pmol), THP (0.8 pmol), 8-Me-THB (1.6 pmol), and 2,3,9,10-THB (1.6 pmol); (B) products from the reaction (pH 6.0) of formaldehyde with authentic THP collected to HPLC eluate; (C) pooled extract of brain tissue (fortified with 8-Me-THB) from rats pretreated with ethanol (3 g/kg, intraperitoneally) 120 min and L-DOPA (200 mg/kg, intraperitoneally) 90 min before decapitation; and (D) products from the reaction (pH 6.0) of formaldehyde with THP collected in HPLC eluate from the rat brain extract represented in (C).

dehyde was reacted with THP collected from the urine of Parkinsonian patients receiving L-DOPA (Sinemet) therapy.

Both the rate of condensation of THP with formaldehyde and the product distribution of the reaction are strongly pH-dependent [13]. Conducting the reaction under acidic conditions reduces the amount of the 2,3,9,10-THB isomer formed. However, the alkaloids are much less stable under alkaline conditions [10]. As a compromise, the reactions were routinely conducted at pH 6.0. It should be noted that this technique can be performed on extracts containing as little as 2–3 pmol of THP. In addition, extracts containing subpicomole quantities of THP can be pooled for identification by this method.

The multiple-step procedure used for the isolation of THP and related alkaloids from biological sources results in a highly purified extract [10]. Collection of the specific HPLC eluate fraction that contains only THP provides further purification of the alkaloid. Chemical conversion of the purified alkaloid to two unique reaction products by the Pictet–Spengler condensation with formaldehyde provides considerable evidence that the compound isolated is indeed THP.

In conclusion, a technique for the rapid identification of THP in biological extracts by use of a unique chemical reaction combined with HPLC–ED methodology has been described. This technique can be applied even when only low levels of THP are present. It is of additional importance that this technique can be accomplished using low-cost HPLC–ED instrumentation.

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