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Determination of tetrahydropapaveroline in the urine of Parkinsonian patients receiving L-dopa–carbidopa (Sinemet) therapy by high-performance liquid chromatography

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ABSTRACT

Tetrahydropapaveroline (THP) concentrations were measured in the urine of Parkinsonian patients receiving L-dopa–carbidopa (Sinemet) therapy, using a method that employs a separation scheme that selectively isolates THP from urine and utilizes the Pictet–Spengler condensation of THP with formaldehyde combined with high-performance liquid chromatography for identification and determination. The mean (± S.D.) recoveries of THP from normal urine with 0.2 pmol/ml added and from Parkinsonian patients' urines with 0.5 pmol/ml added were 48.6 ± 5.7 and 44.6 ± 3.1%, respectively. Three Parkinsonian patients who were receiving either 250, 750 or 1000 mg of L-dopa (as Sinemet) daily had 24-h urinary THP excretion levels of 989, 1017 and 1600 pmol, respectively.

INTRODUCTION

The formation of tetrahydropapaveroline (THP) through the intermediacy of dopamine has been of interest to a number of investigators [1–4]. Of particular interest to one group is the possibility that THP and related alkaloids are formed *in vivo* in Parkinsonian patients receiving L-dopa therapy [4,5]. Further, it is known that some Parkinsonian patients have an adverse reaction to the cessation of L-dopa therapy [6]. It is possible that the adverse reaction is caused by the depletion of alkaloids such as THP formed in the brains of Parkinsonian patients during L-dopa treatment. This possibility is in keeping with the original suggestion by Sourkes [5] that tetrahydroisoquinoline alkaloids may be formed during L-dopa treatment. Although the formation of aberrant alkaloid metabolites was postulated soon after the advent of L-dopa therapy, only minimal progress has been achieved in evaluating the formation of THP and related alkaloids in patients receiving L-dopa therapy [4,7,8].

This paper describes the isolation, identification and determination of THP in the urine of Parkinsonian patients receiving L-dopa–carbidopa (Sinemet) therapy (Fig. 1).

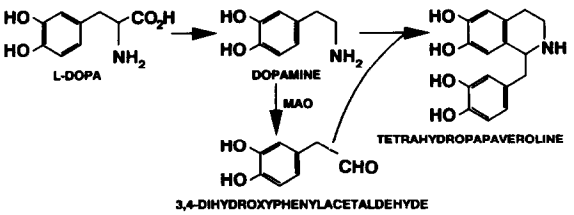

Fig. 1. Schematic representation of the formation of THP from L-dopa.

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EXPERIMENTAL

Materials

Tetrahydropapaveroline hydrobromide (THP), 2,3,9,10-tetrahydroxyberbine hydrobromide (2,3,9,10-THB) and 2,3,10,11-tetrahydroxyberbine hydrobromide (2,3,10,11-THB) were synthesized using established procedures [9]. Sinemet was obtained from Roche Labs. (Bellville, NJ, USA). Bio-Rex 70 (100-200 mesh) resin from Bio-Rad Labs. (Richmond, CA, USA) was treated and converted into the sodium form (pH 6.5) as previously described [10], with the minor modifications that EDTA was omitted from the procedure and glass-distilled water was substituted for quartz-distilled water. Disposable columns (1 and 3 ml size) packed with octadecylsilane (C_{18})-bonded silica gel (40 μm) and column reservoirs with adaptors were obtained from J. T. Baker (Phillipsburg, NJ, USA). Aluminium oxide (alumina), Woelm neutral, activity grade 1 for chromatography, was obtained from Waters (Framingham, MA, USA). The alumina was activated according to the method of Eriksson and Persson [11] and stored in a desiccator. Centrifugal microfilter units, additional receiving tubes, and regenerated cellulose membranes (0.2 μm pore size) were obtained from Bioanalytical Systems (West Lafayette, IN, USA). All other chemicals and reagents were of the highest quality commercially available. Solutions were prepared in deionized water distilled in glass. All glassware was siliconized with dilute AquaSil from Pierce (Rockford, IL, USA).

Urine collection

Urine samples (24 h) were collected from normal subjects or from Parkinsonian patients receiving L-dopa–carbidopa therapy. During the collection period, specimens were stored at 4°C in collection bottles containing 1 g of sodium metabisulfite and 10 ml of 6 *M* HCl. The sodium metabisulfite was added to react with aldehydes excreted in urine and prevents the formation of alkaloids. At the end of the collection period, the total volume of urine was recorded, an aliquot retained for various clinical tests and the remainder was stored at −5°C until analyzed for THP.

Creatinine determination

Creatinine was determined by a standard colorimetric procedure using Sigma (St. Louis, MO, USA) diagnostic kit No. 555.

Concentration and purification procedures

The scheme described here for the isolation of THP from urine is a modification of a multiple-stage procedure described previously for the isolation of the alkaloid from rat brain [12]. The isolation scheme is a repetitive process which includes (A) exchange on Bio-Rex 70 columns, (B) concentration on 3-ml octadecylsilane columns, (C) exchange on small Bio-Rex 70 columns, (D) concentration on 1-ml octadecylsilane columns and (E) adsorption on alumina.

(A) Exchange on Bio-Rex 70 columns. An aliquot of urine equivalent to 75 mg of creatinine was diluted to 150 ml with distilled water. After the addition of 100 μl of 0.12 mol/l dithiothreitol (DTT), an alkaloid-stabilizing reagent, the sample was adjusted to pH 1 with 6 *M* HCl. For the determination of recovery, 30.0 and 75.0 pmol of THP were added to 150 ml of dilute urine from normal subjects and L-dopa-treated patients, respectively. Dilution of the urine maintains consistency in the recovery of THP. All samples were aerated with nitrogen, sealed and hydrolyzed in a water-bath at 98°C for 30 min. After hydrolysis, the samples were cooled in an ice-bath, adjusted to pH 5.0 and placed on columns containing Bio-Rex 70 resin (65 mm × 10 mm I.D.). Before addition of the samples, the columns were washed with 10 ml of 0.1 *M* sodium phosphate buffer (pH 6.5) and 15 ml of distilled water. Each flask was rinsed with 5 ml of distilled water and the rinsings were added to the column. After the sample and rinse had passed through, the column was washed with 35 ml of distilled water and 5 ml of 0.4 *M* HCl. THP was eluted from the column with 30 ml of 1.4 *M* HCl.

(B) Concentration on 3-ml octadecylsilane columns. Further purification of THP was achieved by passing the acidic eluate from the Bio-Rex 70 column through 3-ml disposable columns packed with octadecylsilane (C_{18})-bonded silica gel. The C_{18} columns were preconditioned by washing

with 5 ml each of methanol, acetonitrile, and water. After placing *ca.* 2 ml of the acidic eluate on the column to prevent air locks, reservoirs with adaptors were attached to hold the remaining eluate. The columns were placed on a Baker-10 extraction system with the vacuum adjusted to provide an effluent flow-rate of 0.5 ml/min. After the effluent had passed through the column, the vacuum was released and the columns were washed with 15 ml of water. Two 1.0-ml portions of acetonitrile were added to the columns as a final wash. THP was eluted from the columns with 2.0 ml of 1.0 *M* HCl containing 50% acetonitrile. The eluates were collected in cold 3.5-ml vials containing PTFE boiling beads and 100 μl of 0.5 m*M* ascorbic acid. The samples were frozen on dry-ice, dried *in vacuo* and stored under nitrogen at −10°C.

(C) Exchange on small Bio-Rex 70 columns. In the first stage of the procedure, THP was isolated from urine on Bio-Rex 70. At this stage, further purification of the alkaloid was achieved using the same weak cation-exchange resin which selectively retains basic compounds. The dried samples were dissolved in 3.0 ml of cold 0.05 *M* sodium phosphate buffer (pH 4.7) and placed on columns containing Bio-Rex 70 resin (30 mm × 4 mm I.D.). Before addition of the samples, the columns were treated with 3.0 ml each of 0.02 *M* sodium phosphate buffer (pH 6.5) and distilled water. The vials were rinsed with 1.0 ml of 0.01 *M* sodium phosphate buffer (pH 4.7) and the rinsings were added to the columns. After the samples and rinsings had passed through, the columns were washed sequentially with 3.0 ml of 0.02 *M* sodium phosphate buffer and finally with 3.0 ml of 0.02 *M* HCl. Compounds less basic than the alkaloid were removed from the cation-exchange resin by the dilute HCl wash. THP was then eluted with 2.75 ml of 0.5 *M* HCl into chilled beakers containing 50 μl of 0.5 m*M* ascorbic acid.

(D) Concentration on 1-ml octadecylsilane columns. The alkaloid in the eluates from the Bio-Rex 70 columns was further concentrated on 1-ml C_{18} columns. The columns were conditioned with 2 ml each of methanol, acetonitrile and water before addition of the sample eluates. After the samples had passed through, the columns were washed with 2.0 ml of water followed by 0.5 ml of acetonitrile. THP was eluted with 0.75 ml of 1.0 *M* HCl containing 50% acetonitrile into 3.5-ml vials containing PTFE boiling beads and 50 μl of 0.5 m*M* ascorbic acid. The eluates were frozen, dried *in vacuo* and stored under nitrogen at −10°C.

(E) Adsorption on alumina. The alkaloid possessing catechol moieties was selectively adsorbed on alumina as a final purification step. All reagents were aerated with nitrogen. Activated alumina (50 mg) was added to each of the vials containing the dried sample eluates from the 1-ml C_{18} columns followed by 0.5 ml of 0.1 *M* sodium phosphate containing 50 m*M* Tris and 2 m*M* EDTA (pH 7.0). The vials were rotated to rehydrate the samples and then gently shaken for 15 min. The aqueous phase was removed from the alumina by aspiration and discarded. The alumina was washed successively with 1 and 0.5 ml of distilled water before it was quantitatively transferred into a centrifugal microfilter unit (Bioanalytical Systems, BAS) with 0.5 ml water. The samples were centrifuged at 650 *g* for 5 min at 4°C. After the receiving tubes had been changed, THP was desorbed from the alumina by adding 500 μl of freshly prepared 0.1 *M* HCl containing 1.0 m*M* EDTA and 0.1 m*M* ascorbic acid to the top of the filter assembly, rolling the filter units on a flat surface for 2 min and centrifuging as above. The eluates, together with 50 μl of the eluting reagent used to rinse the receiving tubes, were transferred into 3.5-ml vials. The samples were quickly frozen on dry-ice, dried *in vacuo* and then stored at −10°C.

Chromatographic analysis

Immediately before analysis by high-performance liquid chromatography with electrochemical detection (HPLC–ED), the dried eluates from the alumina were rehydrated in 500 μl of cold 5.0 m*M* HCl and filtered through centrifugal microfilters. Chromatographic analysis of THP was conducted on a Model LC-154 LCED analyzer with an LC22A-23A temperature controller (BAS, West Lafayette, IN, USA). The electro-

chemical detector (BAS, Model LC-4B) was equipped with a glassy carbon electrode. The chromatographic conditions were as follows: detector potential, +0.65 V *vs.* Ag/AgCl; column, Supelcosil LC-18DB (25 cm × 4.6 mm I.D.), 5 μm (Supelco, Bellefonte, PA, USA); column temperature, 29°C; flow-rate, 0.9 ml/min; mobile phase, 0.05 *M* ammonium phosphate containing 0.75 m*M* triethylamine and 5.0% (v/v) dioxane adjusted to an apparent pH of 3.5; and sample injection, 40 μl of urine extract or 40 μl of an authentic alkaloid standard. Typical retention times for 2,3,10,11-THB, THP and 2,3,9,10-THB were 9.7, 11.8 and 14.7 min, respectively.

The method employed for the identification of THP in HPLC eluates has been described previously [13]. Briefly, 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 leaving the glassy carbon electrode. Next, 150 μl of a formaldehyde solution (0.1 *M* in 1.0 *M* $NH_4H_2PO_4$, pH 3.0) 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 with that of an authentic reference standard. The same procedure as used for the identification of THP in urine extracts was applied.

RESULTS

The mean (± S.D.) recovery of THP from normal urine fortified with the alkaloid (0.2 pmol/ml) in six separate determinations was 48.6 ± 5.7%. The mean (± S.D.) recovery of THP (0.5 pmol/ml) from the urine of Parkinsonian patients receiving L-dopa–carbidopa (Sinemet) therapy was 44.6 ± 3.1%. A 44% recovery of THP may seem low. However, there are five separate steps in the isolation procedure and an overall recovery of 44% represents an 85% average recovery of THP for each step in the method. Thus, a 44% recovery of THP is not only reasonable but is also an indication of the stability of the alkaloid during the assay.

The Pictet–Spengler condensation of THP with formaldehyde to form 2,3,9,10- and 2,3,10,11-tetrahydroxyberbines combined with HPLC provides a convenient method for the identification and determination of THP in biological extracts [13]. The application of this methodology to the identification of THP in the urine extract from a Parkinsonian patient receiving L-dopa–carbidopa therapy is depicted in Fig. 2. Baseline resolution of authentic reference standards of 2,3,10,11-THB (retention time t_R = 9.73 min), THP (t_R = 11.8 min), 2,3,9,10-THB (t_R = 14.68 min) and DTT (the alkaloid-stabilizing agent) was achieved by HPLC (Fig. 2A). The reaction of formaldehyde at pH 3 with authentic THP collected in the HPLC eluate results in the formation of mainly 2,3,10,11-THB (Fig. 2B). The presence of THP in urine extracts from Parkinsonian patients receiving L-dopa–carbidopa therapy was confirmed in a similar manner. The HPLC profile of a urine extract from a Parkinsonian patient receiving four 250-mg doses of L-dopa (as Sinemet) daily is shown in Fig. 2C. The peak identified as THP (Fig. 2C) has a retention time identical with that of authentic THP (Fig. 2A). The compound represented by the peak that emerges several min after THP has not been identified. The products of the reaction between formaldehyde and THP collected (Fig. 2C) in the HPLC eluate from urine extracts of the patient receiving L-dopa treatment are shown in Fig. 2D. The HPLC profile representing the reaction of formaldehyde with THP from the urine of the Parkinsonian patient receiving L-dopa treatment (Fig. 2D) is indistinguishable from that obtained from the reaction of authentic THP with formaldehyde (Fig. 2B). Measurable levels of THP were not observed in HPLC profiles of urine extracts from six normal subjects (chromatograms not shown). The chromatograms in Fig. 2 (A–D) illustrate that THP in the urine of Parkinsonian patients receiving L-dopa–carbidopa therapy can

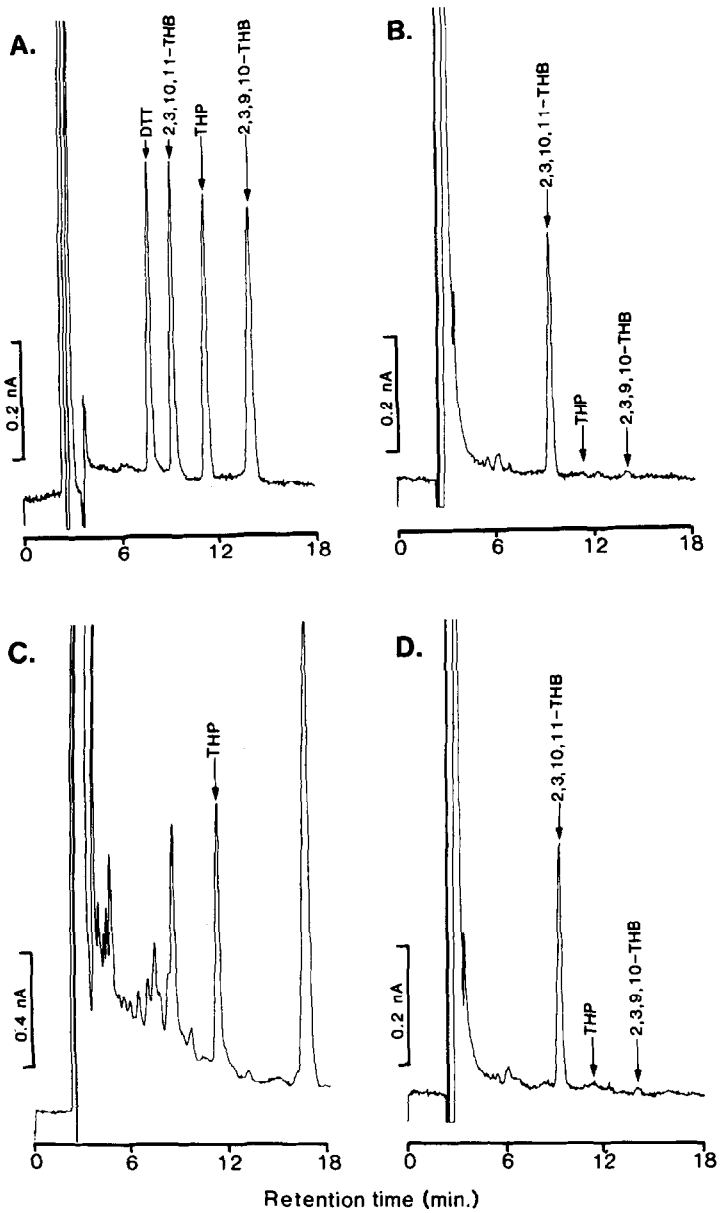

Fig. 2. Chromatographic profiles of (A) a reference mixture of 2,3,10,11-THB (1.25 pmol), THP (1.0 pmol), and 2,3,9,10-THB (2.0 pmol) with the stabilizing agent DTT, (B) products from the reaction of the collected HPLC eluate fraction containing THP from a reference standard with formaldehyde at pH 3.0, (C) extract equivalent to 5.4 ml of urine from the Parkinsonian patient receiving 1.0 g L-dopa (as Sinemet) per 24 h and (D) products from the reaction of the collected HPLC eluate fraction containing THP from an extract of a urine sample from the Parkinsonian patient receiving L-dopa (1.0 g per 24 h) with formaldehyde at pH 3.0.

be isolated and identified using the Pictet–Spengler condensation reaction in combination with HPLC.

The results obtained from an assay of the urines from three Parkinsonian patients receiving L-dopa–carbidopa therapy are given in Table I. As can be seen, each urine sample was assayed in triplicate and the variation among the values obtained for each specimen is less than 5%. These results, together with values obtained for the re-

TABLE I

URINARY EXCRETION OF THP IN THREE PARKINSONIAN PATIENTS RECEIVING L-DOPA–CARBIDOPA THERAPY

Subjects 1, 2 and 3 received the equivalent of 250, 750 and 1000 mg of L-dopa daily as Sinemet, respectively. Urine collections (24 h) were obtained and triplicate assays for THP were performed on each urine.

Subject	L-Dopa (mg per 24 h)	THP (pmol per 24 h)			
		1	2	3	Mean
1	250	998	996	972	989
2	750	1019	1032	1001	1017
3	1000	1581	1593	1626	1600

covery of authentic THP from urines, suggest that the methodology employed is satisfactory. Of equal importance, the results demonstrate that THP is present in the urine of patients receiving milligram amounts of L-dopa.

The detection limit (expressed as a signal three times the noise level) for THP in reference solutions of 0.03 pmol for a 100-μl sample injection volume was reported previously [12]. The detection limit of THP in fortified urine was not fully established. The lowest concentration of THP added to urine from normal subjects to evaluate the recovery of the alkaloid was 0.1 pmol/ml in urine and the recovery of the alkaloid remained unaltered. In practical terms, purified urine extracts were reconstituted in 500 μl of 5.0 m*M* HCl and less than 10% of the sample was used for HPLC analysis. Thus THP levels observed in the urine of Parkinsonian patients did not approach the limit of detection of the alkaloid by the method.

DISCUSSION

Unlike our method described previously for the assay of THP in rat brain supernatant [12], which used an octadecylsilane column as the first stage of the separation scheme, the present method employs the weakly acidic cation-exchange resin Bio-Rex 70 in the first stage. The advantages of using a weakly acidic cation-exchange resin in the first stage of the isolation scheme are twofold. The resin selectively separates the alkaloid from other less basic compounds such as catecholamines and amino acids and from acidic and neutral substances, and, more important, it provides a means for the rapid concentration of THP from a large volume of urine. The rapid concentration step is essential because we have observed, in previous work, that THP is extremely labile in dilute solution, *i.e.*, the alkaloid will undergo decomposition during routine sample manipulations. The rapid isolation of THP from a large volume of urine results in a concentration of the alkaloid that is less labile. The advantage of the method is apparent in that the recovery (mean ± S.D.) of THP (48.6 ± 5.7%) from normal urine is comparable to that of THP (43.4 ± 3.5%) obtained from rat brain [12], even though an additional step has been incorporated into the method.

It may be noted that although 150 ml of dilute urine (creatinine, 0.5 mg/ml) was used in the analysis, less than 10% of the final extract was required for HPLC. Hence it now appears that problems encountered by other investigators [4,8] in attempts to assay THP in the urine of Parkinsonian patients receiving L-dopa therapy were caused by the instability of THP rather than the concentration of the alkaloid. Further, the detection of THP in the urine of patients receiving milligram amounts of L-dopa daily demonstrates that man has the facility to form THP readily.

As THP is an intermediate in the formation of more pharmacologically active alkaloids [1], high levels of THP itself may not be present after any treatment regimen. THP is rapidly metabolized through O-methylation *in vitro* in rat liver prep-

aration [14] and *in vivo* in rat brain [15]. Further, the conversion of reticuline, a methylated metabolite of THP, into a morphinane alkaloid by rat liver *in vivo* and *in vitro* has been reported recently [16]. Thus, the isolation of measurable levels of THP from the urine of patients receiving L-dopa therapy may signal the presence of more pharmacologically active alkaloids which may subserve some of the clinical manifestation of Parkinsonian patients receiving long-term L-dopa therapy. In this context, the two positional isomeric alkaloids, 2,3,9,10- and 2,3,10,11-THB, which are formed through the intermediacy of THP, have been identified as urinary excretion products in Parkinsonian patients receiving L-dopa treatment [7]. It is known that the withdrawal of L-dopa from patients must be managed in a hospital under close supervision [6]. It would be of interest to know if the reaction of patients to L-dopa withdrawal is caused by the absence of L-dopa or the absence of dopamine-derived alkaloids. As L-dopa (with or without a decarboxylase inhibitor) is the mainstay for the treatment of Parkinsonism, further exploration in this area seems warranted.

In conclusion, this investigation has demonstrated that THP can be detected and determined in the urine of patients receiving L-dopa treatment. The patients' urines used in this study were obtained from the clinical laboratory and they were collected for routine clinical tests. No attempt was made to correlate THP levels with clinical symptoms of the patients. However, the demonstrated presence of THP suggest that THP and/or THP metabolites may be involved in some of the clinical manifestation of Parkinsonian patients receiving L-dopa treatment.

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