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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CARBAMAZEPINE METABOLITES EXCRETED IN RAT URINE

M.G. HORNING and K. LERTRATANANGKOON

Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030 (U.S.A.)

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SUMMARY

A procedure for the separation and isolation of the urinary metabolites of carbamazepine by reversed-phase high-performance liquid chromatography is described. After extraction from urine, the metabolites were separated on either an analytical or semi-preparative C_{18} μBondapak column by gradient elution with methanol—water—acetic acid. Following derivatization the metabolites isolated by the use of the semi-preparative column were analyzed by gas chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

Carbamazepine (5*H*-dibenzo-[*b*,*f*]azepine-5-carboxamide) is an effective agent for the control of epileptic seizures particularly those of psychomotor epilepsy. Although the drug was introduced in the early 1960's, 25—50% of the metabolites excreted in urine have not been identified. In the initial studies [1] two possible metabolites were detected in cerebrospinal fluid by thin-layer chromatography (TLC). Subsequently seven metabolites were detected by TLC in the urine of patients receiving carbamazepine (CBZ) chronically [2]. Carbamazepine 10,11-epoxide and 10,11-dihydroxy-10,11-dihydrocarbamazepine were identified by gas chromatography—mass spectrometry (GC—MS) in 1972—1973 [3, 4]. In subsequent studies, iminostilbene [5—8], *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine [9] and 1-hydroxy-, 2-hydroxy- and 3-hydroxycarbamazepine and 9-hydroxymethyl-10-carbamoylacridan [8] were identified as urinary metabolites by MS. The glucuronide metabolites of carbamazepine have also been investigated by MS. In 1976, Bauer et al. [10] isolated an N-glucuronide of carbamazepine from rat liver perfusate and more recently eight new glucuronide conjugates were identified as urinary metabolites in man [11].

The identification of metabolites of carbamazepine by GC and GC—MS is difficult because the metabolites are not resolved on packed columns and extensive rearrangement to iminostilbene and acridine derivatives occurs during GC analysis [5]. Because of the mild conditions employed, high-performance liquid chromatography (HPLC) provides another approach to this analytical problem. Methods based on HPLC have been employed for the quantification of CBZ and CBZ epoxide in plasma [12] and in saliva [13]. In this paper we describe an HPLC method for the separation and isolation of the metabolites of carbamazepine excreted in rat urine.

EXPERIMENTAL

Reagents

All reagents were analytical grade. Glass distilled methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). The HPLC grade acetic acid was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.). The 3% OV-17 column packing and bis-trimethylsilylacetamide (BSA) were purchased from Applied Science Labs. (State College, Pa., U.S.A.). Glusulase was obtained from Endo Labs. (Garden City, N.Y., U.S.A.).

Carbamazepine and carbamazepine epoxide were obtained from Ciba-Geigy (Ardsley, N.Y., U.S.A.). Iminostilbene, acridine and 10,11-dihydrocarbamazepine were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). The *cis*-10,11-dihydrodiol of carbamazepine and 10-hydroxy-10,11-dihydrocarbamazepine were a gift from Prof. A. Frigerio.

Instrumentation

High-performance liquid chromatography. C_{18} μBondapak analytical columns (3.9 mm × 30 cm) and semi-preparative columns (7.8 mm × 30 cm) were obtained from Waters Assoc. (Milford, Mass., U.S.A.). Reversed-phase HPLC analyses were carried out by gradient elution utilizing a dual solvent delivery system (Waters Assoc., Model 6000A), a solvent programmer (Waters Assoc., Model 660) and a UV-III detector (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) set at 254 nm. OmniScribe recorders (Houston Instruments, Austin, Texas, U.S.A.) were employed.

Gas chromatography and gas chromatography—mass spectrometry. GC separations were carried out on 3.7 m × 2 mm glass W columns packed with 3% OV-17. The analyses were temperature programmed from 150° at 2°/min. The MS analyses were carried out on a LKB 9000-PDP/12 analytical system using 1.85 m × 2 mm glass coil columns packed with 3% OV-17. Separations were programmed from 170° at 5°/min.

Animal procedure

Male Sprague-Dawley rats (200 g) received 20 mg of carbamazepine daily for 8 days in special rat biscuits. The biscuits were prepared by adding 22 ml of a warm 18% solution of gelatin to 9 g of ground Purina rat chow in a 3 in. square plastic weighing boat. After mixing thoroughly, 20 mg of carbamazepine was stirred into the paste which solidified on cooling. The biscuits were made daily and kept in the refrigerator until used. The rats were housed individually in

metabolism cages and 24-h urine samples were collected daily. The urine samples were stored at −20°.

Sample preparation

After enzymatic hydrolysis of the urine with glusulase at pH 4.5—4.8 for 17 h at 37°, the carbamazepine metabolites were extracted by the ammonium carbonate—ethyl acetate procedure [14]. For profiling metabolites on an analytical column, an aliquot (usually 1/10) of a diluted 24-h urine sample was used. The urine extraction was carried out in a centrifuge tube fitted with a PTFE-lined screw cap. For collection of metabolites from a semi-preparative column, an ammonium carbonate—ethyl acetate extract of a complete 24-h urine sample was used; the extraction was carried out in a separatory funnel. The extracts were taken to dryness, redissolved in methanol and transferred to Reacti-vials. The final volume of the analytical sample was 20—50 μl and the final volume of sample for analysis on the semi-preparative column was 150 μl. After centrifugation of the sample, an aliquot was injected onto the HPLC column; 1—2 μl were used with the analytical column and 20—50 μl were used with the semi-preparative column.

High-performance liquid chromatographic analysis

When the separations were carried out with an analytical column, a 40-min gradient system (gradient 6) was used with a column pressure of 1100—1200 p.s.i. and a flow-rate of 1.2 ml/min. The solvent system consisted of solvent A: methanol—water—acetic acid (20:80:0.1) and solvent B: methanol—water—acetic acid (50:50:0.1). The percentage of B varied from 10 to 90. A modified solvent system was used with a semi-preparative column and consisted of solvent A: methanol—water—acetic acid (33:67:0.1) and solvent B: methanol—water—acetic acid (50:50:0.1). A 20-min gradient system with gradient 6 was used. The flow-rate was 1.2 ml/min and the column pressure was 800 p.s.i. The time for a single gradient analysis was 50—60 min on a semi-preparative column and 60 min on an analytical column.

Gas chromatographic and gas chromatographic—mass spectrometric analysis

The individual fractions collected from a semi-preparative column (2—5 injections) were pooled and most of the solvent was removed (Rotovap). After lyophilization of the seventeen pooled fractions, the residues were transferred with methanol to Reacti-vials. An aliquot of each residue (usually 1/3) was taken to dryness under a nitrogen stream and redissolved in 10 μl of pyridine and silylated with 10 μl of BSA. After heating at 60° for 1 h, the fractions were analyzed by GC and GC—MS. Several of the fractions contained more than one metabolite and some overlap of metabolites between adjacent fractions was observed.

RESULTS

Fig. 1a shows the separation of urinary metabolites on a semi-preparative column and Fig. 1b shows the separation of reference standards on the same column. The dotted lines in Fig. 1a show where the 17 fractions were collected.

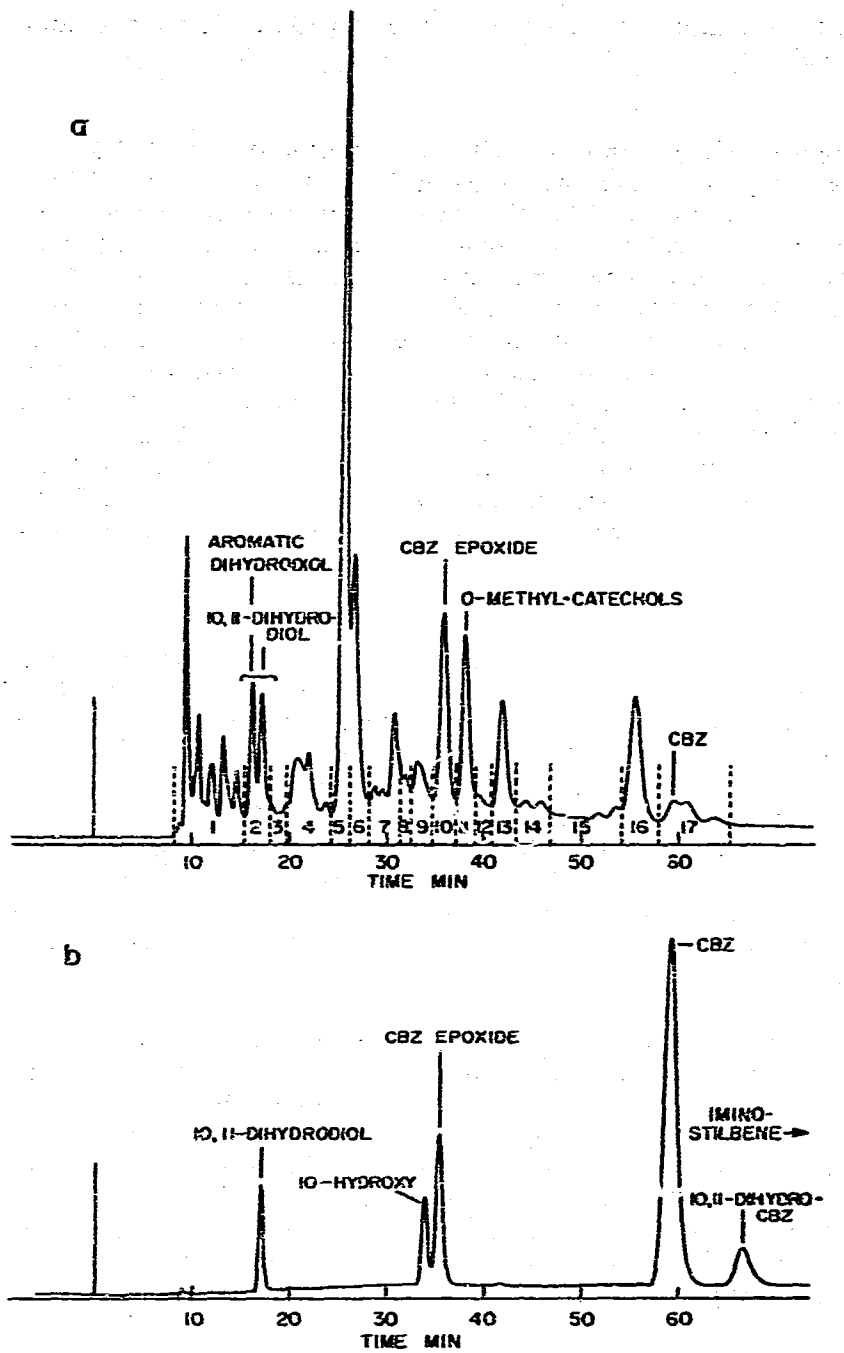

Fig. 1. HPLC separation of carbamazepine metabolites on a C_{18} μBondapak semi-preparative column by gradient elution. (a) Separation of urinary metabolites excreted by a rat after being fed carbamazepine for eight days; (b) separation of reference standards on the same column.

GC analyses of fractions 2 and 11 are shown in Fig. 2a and 2b, respectively. The major metabolite in fraction 2 was identified as a dihydrodiol of carbamazepine. This dihydrodiol had different GC and GC—MS properties from synthetic *cis*-10,11-dihydroxy-10,11-dihydrocarbamazepine [4]. The base peak in the mass spectrum of this new metabolite (TMS derivative) was observed at *m/z*

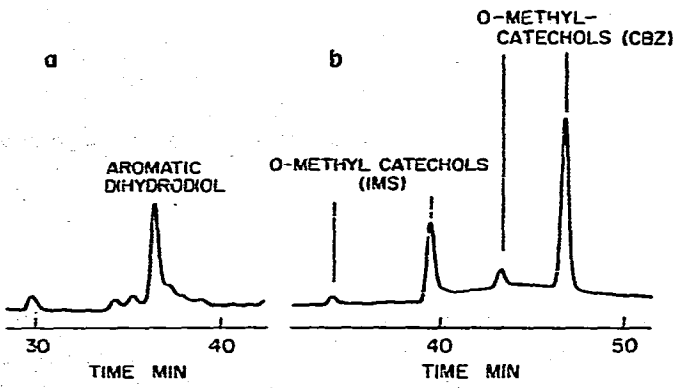

Fig. 2. GC analysis with a 3% OV-17 column of fraction 2 (a) and fraction 11 (b) collected from a semi-preparative HPLC column. The structures were determined by GC—MS analysis.

191 (Fig. 3) indicating that the dihydrodiol had been formed on one of the aromatic rings; the methylene unit (MU) of the TMS derivative was 28.0. The base peak in the mass spectrum of the *cis*-10,11-dihydrodiol of carbamazepine (TMS derivative) was observed at *m/z* 282 and the MU was 26.8. The metabolites in fraction 11 were identified by GC—MS as hydroxymethoxy derivatives of carbamazepine. The mass spectrum of one of the isomeric hydroxymethoxycarbamazepines is shown in Fig. 4. It was not possible to assign the positions of the O-methyl and hydroxyl groups from the mass spectrum, but the fragmentation indicated that the metabolite was an O-methylcatechol derivative of carbamazepine [15].

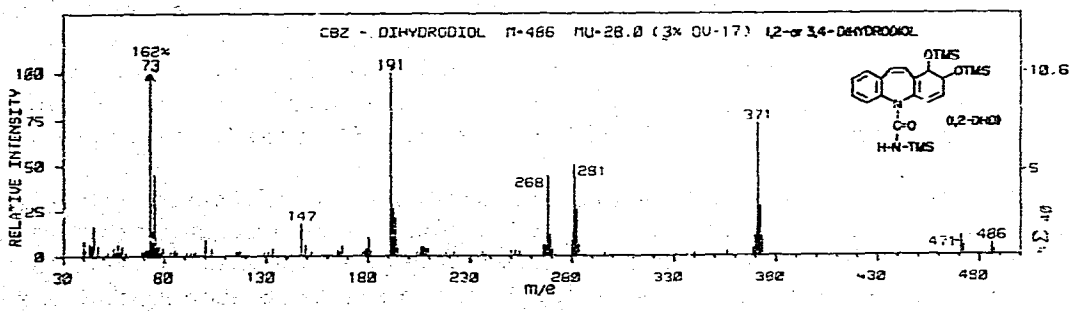

Fig. 3. Electron impact mass spectrum of the 1,2- or 3,4-dihydrodiol of carbamazepine.

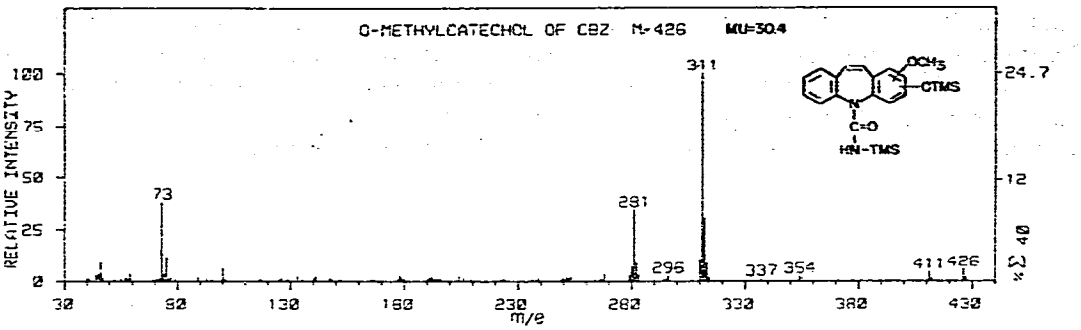

Fig. 4. Electron impact mass spectrum of one of the O-methylcatechol metabolites of carbamazepine.

DISCUSSION

The separation of metabolites of carbamazine by GC has been difficult because the metabolites are not resolved on either packed or capillary columns. However, the metabolites have been separated by TLC and the eluted metabolites analyzed by direct inlet MS [5, and references cited therein]. The structures of the metabolites have also been investigated after permethylation which permitted the GC and GC—MS analyses of the intact glucosiduronic acid derivatives as well as the unconjugated metabolites of carbamazepine [11]. In this study, we have been able to separate the metabolites by reversed-phase HPLC. The separation of the metabolites by gradient elution into seventeen or more fractions greatly facilitated the identification of the metabolites by GC and GC—MS. In addition to the dihydrodiol and hydroxymethoxy derivatives of carbamazepine already described, mono-, di-, tri- and tetrahydroxy derivatives of carbamazepine have been isolated by HPLC. The GC and GC—MS properties of these metabolites will be reported separately.

There was no evidence of degradation or rearrangement of carbamazepine or the other reference compounds during HPLC analysis; a single peak was always observed for each standard. To check that degradation had not occurred during lyophilization, *cis*-10,11-dihydroxy-10,11-dihydrocarbamazepine was collected from the semi-preparative HPLC column using the solvent system described. After removal of most of the methanol (Rotovap), the solution of the *cis*-dihydrodiol was lyophilized and the residue redissolved in methanol. When the lyophilized sample was analyzed by HPLC, a single peak with the same retention time as the *cis*-dihydrodiol was observed. No iminostilbene derivatives were formed during HPLC analysis or during the work-up of the eluted *cis*-dihydrodiol. A comparable experiment was carried out with carbamazepine epoxide. Only a single peak due to the epoxide was observed when the lyophilized sample was analyzed by HPLC.

In our laboratory iminostilbene and iminostilbene derivatives have been observed as rearrangement products during GC analysis of the free and derivatized *cis*-dihydrodiol. The rearrangement of carbamazepine metabolites to iminostilbene derivatives during GC analyses is illustrated in Fig. 2b. Since iminostilbene and its derivatives were separated from the carbamazepine analogs on the HPLC

column, it was assumed that the O-methylcatecholiminostilbenes in Fig. 2b were formed during GC analysis. This conclusion was supported by the observation that the size of the iminostilbene peaks varied during repetitive analyses of the same sample.

The HPLC procedure described was not satisfactory for quantitative analysis because some of the peaks were not symmetrical (Fig. 1a) and analysis of the individual fractions by GC—MS indicated that several of the apparently symmetrical peaks contained more than one metabolite (Fig. 2a and 2b). The separations on semi-preparative columns and on analytical columns having 10,000—12,000 theoretical plates were comparable. It is possible that a 5-μm C_{18} μBondapak column would provide the resolution necessary for quantitative analysis. The procedure, however, is very useful for isolation and identification and for comparison of urinary profiles because of the stability of the metabolites under the mild conditions of HPLC analysis.

ACKNOWLEDGEMENT

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