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SIMULTANEOUS DETERMINATION OF THE ANTICONVULSANTS, CINROMIDE (3-BROMO-N-ETHYLCINNAMAMIDE), 3-BROMOCINNAMAMIDE, AND CARBAMAZEPINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for monitoring plasma concentrations of cinromide (3-bromo-N-ethylcinnamamide) and its de-ethylated metabolite. Carbamazepine levels can be easily measured by the same technique. The N-isopropyl analogue of cinromide is used as internal standard, and all compounds are easily separated on a reversed-phase column operated at 55° with a small-diameter pre-column maintained at the same temperature. The extraction is rapid and generally applicable to plasma and urine samples that are to be analyzed by reversed-phase chromatography. Short- and long-term reproducibility studies show less than 4% relative standard deviation for replicate determinations for all drugs. Limits of quantitation are 10—20 ng/ml with an internal standard concentration of 3 μg/ml. Another metabolite of cinromide, 3-bromocinnamic acid, which may have some anticonvulsant effect, can be analyzed simultaneously by buffering the mobile phase and adding an ion-pairing reagent.

INTRODUCTION

Cinromide (3-bromo-N-ethylcinnamamide) (I) and its de-ethylated metabolite, 3-bromocinnamamide (II), exhibit strong protection against maximal electroshock convulsions, pentylenetetrazole induced convulsion and psychomotor seizures in rodents [1]. The drug is currently undergoing clinical testing for use as a broad-spectrum anticonvulsant.

During the development of a method for the determination of I and II in the plasma of epileptic patients, both gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) were investigated. Flame ioniza-

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tion GLC was excluded because of poor sensitivity. Adequate peak shape and resolution were achieved, however, with various polyester stationary phases. Because of the sensitivity required and the high absorptivity of the compounds of interest, reversed-phase HPLC with UV absorption detection was selected as the method of choice. Since carbamazepine (III) is a commonly used anticonvulsant and is functionally and chromatographically similar to II, a method was developed that allows simultaneous determination of I, II and III.

EXPERIMENTAL

Reagents

All chemicals were analytical reagent grade. Acetonitrile and iso-octane were used as purchased (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water was purified for use by passing it through an organic absorbing ion-exchange system (Bion Exchange System D, Pierce, Rockford, Ill., U.S.A.). The internal standard (3-bromo-N-isopropyl cinnamamide [IV]), I, II and 3-bromocinnamic acid were used as received (Burroughs-Wellcome, Research Triangle Park, N.C., U.S.A.). Carbamazepine was recrystallized from tablets as previously described [2].

The drug standard stock solution was made by dissolving 3 mg, 7 mg, and 12 mg of I, II and III, respectively, in absolute ethanol and diluting to 25 ml. The working drug standard solution was made daily by diluting 0.200 ml of stock solution to 2.00 ml with acetonitrile.

The internal standard stock solution was made by dissolving 1.9 mg of IV in absolute ethanol and diluting to 25 ml. The working internal standard was made fresh at least every other day by diluting 1.00 ml of stock solution to 25 ml with aqueous 0.1 *M* ascorbic acid. This solution has been used previously to increase the stability of an aqueous internal standard [2].

Apparatus

The liquid chromatograph consisted of a reciprocating piston pump with a pressurized tube pulse dampener and a fixed-wavelength UV absorbance detector (Laboratory Data Control, Riviera Beach, Fla, U.S.A.), operated at 280 nm with a static nitrogen reference. The column (250 mm × 4.6 mm I.D.) was packed with a 10-μm microparticulate C_{18} reversed phase (Reeve-Angel, Liquid Chromatography Division, Clifton, N.J. U.S.A.). A 50 mm × 1.09 mm I.D. pre-column was connected to the main column inlet fitting and dry-packed in place with a pellicular C_{18} phase (Vydac Reverse Phase; Applied Science Labs., State College, Pa., U.S.A.) by vibration for 10 min. A 1/16-in. Swagelok union was attached and the constricted portion packed with glass wool. The entire system was then connected to a sample injection valve (Model CV-6-HPax, 50-μl sample loop; Valco Instruments, Houston, Texas, U.S.A.) with a short length of 1.5 mm × 0.25 mm I.D. tubing. A water jacket was also made for the pre-column from a length of 9.5 mm I.D. × 6.4 mm wall Tygon vacuum tubing. Water ports were made by boring holes in the wall and inserting short lengths of glass tubing. The ends were sealed at the 1/16-in. Swagelok nuts with O-rings. The entire column system was operated at 55°. The mobile phase was acetonitrile—water (25:75), flowing at 120 ml/h with a pressure of 10.3 MPa (1500 p.s.i.).

Procedure

To 0.50 ml of plasma in a 13 × 100 mm disposable culture tube are added, in order, 0.50 ml of internal standard, 1 ml of acetonitrile and 2 ml of iso-octane. The acetonitrile is added while vortexing to deproteinize the plasma. After addition of the iso-octane, the tube is vortexed for 15 sec to extract lipid material. The sample is centrifuged at 1000 *g* for 3 min, the iso-octane layer is aspirated along with the small amount of solid material at the interface, and the aqueous acetonitrile supernatant is decanted into a 10 × 75 mm disposable culture tube containing about 0.7 cm of granular potassium chloride. The tube is vortexed for 10 sec to saturate the solution and salt out the acetonitrile. After centrifugation for 5 min, the acetonitrile layer is transferred to a second 10 × 75 mm tube and evaporated to dryness at 50° under an air or nitrogen stream. The residue is dissolved in 140 μl of mobile phase, and 50 μl are injected into the liquid chromatograph. Quantitation is by peak-height ratio.

RESULTS AND DISCUSSION

Chromatograms produced by extracts of plasma samples drawn from epileptic patients are shown in Fig. 1. The sample that gave trace A was drawn from a patient taking phenytoin and methsuximide. Trace B was obtained with plasma drawn from a patient receiving carbamazepine and 1200 mg/day of I in four equal doses. The sample was taken 4 h after administration of 300 mg of I. Trace C was obtained with plasma drawn from a patient 1 h after administration of a final dose of I. A sample drawn two days later from this same patient gave trace D. This patient was also receiving phenobarbital, primidone and

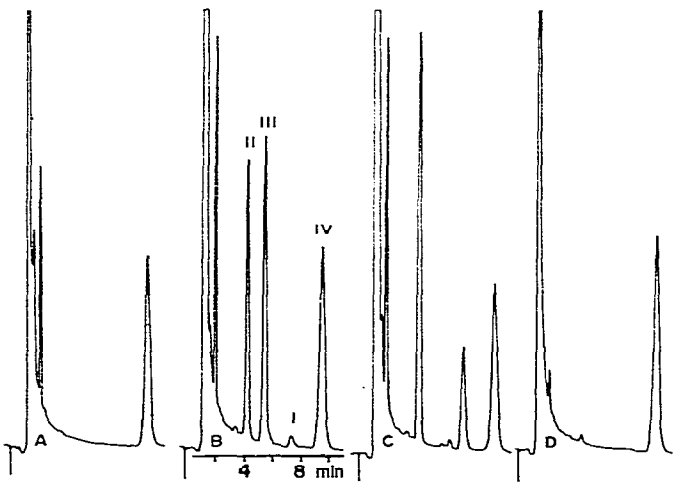

Fig. 1. Chromatograms of plasma extracts from epileptic patients. (A) Patient receiving phenytoin and methsuximide; (B) patient receiving carbamazepine (III) and cinromide (I), sample drawn 4 h after dose: (C) patient receiving phenobarbital, primidone, phenytoin and cinromide, sample drawn 1 h after final dose of cinromide; (D) same as C, sample drawn 2 days after final dose of cinromide. See text for drug concentrations. Peaks II and IV correspond to 3-bromocinnamamide and 3-bromo-N-isopropyl cinnamamide, respectively.

phenytoin. Concentrations of I and II, respectively, in traces B, C and D were 0.14 μg/ml and 1.99 μg/ml, 1.37 μg/ml and 4.39 μg/ml, and 0 μg/ml and 0.062 μg/ml, respectively. The carbamazepine concentration in trace B was 3.96 μg/ml.

The simple, clean extraction is based on the ability of acetonitrile to efficiently deproteinize plasma, its miscibility with dilute aqueous solutions and its immiscibility in potassium chloride-saturated solutions. The iso-octane wash eliminates lipid material that would be insoluble in the mobile phase but would be extractable into acetonitrile. The residue after evaporation of the acetonitrile is largely potassium chloride, which is soluble in the mobile phase and does not interfere with the analysis or contribute to degradation of the column. This procedure can also be used for efficient extraction of the barbiturate, hydantoin and succinimide anticonvulsants. Chromatographic conditions for quantitation of these compounds have been given by Adams and Vandemark [3]. Regeneration of the column system can be accomplished when necessary by successive elution with 100 ml each of acetonitrile, chloroform and acetonitrile, with injection of about 2 ml of dimethyl sulfoxide in small increments during the final acetonitrile wash.

Short-term reproducibility was assessed by extraction of six replicate samples containing 1.55 μg/ml, 2.01 μg/ml and 1.57 μg/ml of I, II and III, respectively. Relative standard deviations of peak-height ratios were 1.2%, 1.4% and 2.3%, respectively. Linearity of analytical curves and long-term reproducibility were checked by extraction of six plasma standards spiked with I, II and III to cover the concentration ranges of interest (up to 2.5 μg/ml I, 5.2 μg/ml II, and 11.1 μg/ml III). Correlation coefficients for all curves run over a 30-day period were greater than 0.999. Slopes of curves run on the 1st, 4th, 16th and 30th days had relative standard deviations of 2.8%, 3.3% and 2.9% for I, II and III, respectively.

Recoveries were assessed by comparison of extracted plasma standards with an analytical curve derived from nonextracted standards. For all samples, the internal standard was added to the final extract before evaporation. Recoveries of I, II and III were 61.7 ± 1.9%, 60 ± 5% and 61.0 ± 2.8% at concentrations of 1.0 μg/ml, 2.1 μg/ml and 4.5 μg/ml, respectively. By addition of standards at various stages of the extraction, losses at each step were estimated to be 13—15% during deproteinization; 7—11% during the iso-octane wash, aspiration and transfer; and 15—19% during the acetonitrile extraction. Recovery of all drugs can be increased by about 20% by using 0.25 ml of plasma and internal standard. When 0.5 ml of plasma and an internal standard concentration of 3 μg/ml are used, the lower limits of quantitation are 10—20 ng/ml for all drugs at a detector sensitivity of 0.016 a.u.f.s.

A dose—response relationship was determined for an epileptic female patient, weighing 51.8 kg, who was hospitalized during the study. All samples were drawn 4 h after the morning dose of I, which was given in 4 equal doses at 7 am, 12 noon, 5 pm, and 10 pm. A plot of plasma concentration (μg/ml) vs. dose (mg I per kg of body weight per day) was calculated from the average plasma levels obtained over a 3-day period, during which each dose was held constant. The correlation coefficients for I and II were 0.980 and 0.999,

respectively, and the slopes indicated plasma level changes of 0.00400 μg/ml I per mg I per kg per day and 0.0617 μg/ml II per mg I per kg per day. Preliminary pharmacokinetic data, obtained for four patients, indicated that peak concentrations of I and II were reached at 1 h and 2.5—3 h, respectively, after dosage. Half lives were about 1 h and 5—7 h for the parent drug and metabolite, respectively. More detailed clinical data are to be published in a future article.

The importance of heating the pre-column, which, in effect, only serves to pre-heat the mobile phase, is illustrated in Fig. 2. In each case a standard mix-

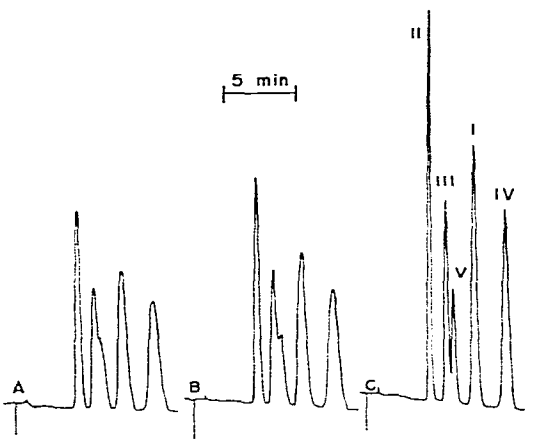

Fig. 2. Chromatograms of a standard mixture of I, II, III, IV and clonazepam (V) injected into (A) the main column at 55° with no pre-column; (B) the main column at 55° with pre-column at room temperature and (C) the main column at 55° with pre-column at 55°. Approximately 0.3 μg of each compound was injected in each case. Peak designations correspond to those of Fig. 1.

ture of the compounds used in this assay (I, II, III and IV) with the addition of clonazepam (a benzodiazepine anticonvulsant) was injected into the system. Mobile phase flow-rate was held constant. Trace A was obtained with the main column at 55° and no pre-column, trace B with the main column at 55° and the pre-colum at room temperature, and trace C with both the main column and pre-column at 55°. Clonazepam was added as a gauge of resolution because it was impossible to separate this compound from carbamazepine on the main column alone in a reasonable time with acetonitrile—water or methanol—water mobile phases. The number of theoretical plates was calcuated for each system based on the final peak (IV) from the equation: $N = 16\ (X/Y)^2$, where N is the number of the theoretical plates, X is the retention time and Y is the base width of the peak of interest. Respective efficiencies for systems A, B and C were 831, 973 and 2588 theoretical plates.

This effect does not represent a real increase in efficiency of the analytical column because if the system were run at room temperature, the higher plate count would be observed. The peak broadening in chromatograms A and B is not a characteristic of the column, but is a result of the thermal nonequilibrium that is produced when cold mobile phase enters a heated column. The analyst who does reversed-phase chromatography at increased temperatures without

pre-heating his mobile phase may be working with a serious handicap. A more detailed discussion of behavior of reversed-phase columns operated at increased temperatures is in press [4].

Preliminary data on another metabolite of cinromide, 3-bromocinnamic acid (VI), shows that it may also have some anticonvulsant effect. This compound can be analyzed by this method with only a slight change in mobile phase composition. By buffering the mobile phase to pH 4.7 with NaH_2PO_4 (0.015 mole/l) and adding tetraethylammonium perchlorate (0.005 mole/l), an ion-pairing effect is achieved that increases the retention time of the acid. Fig. 3

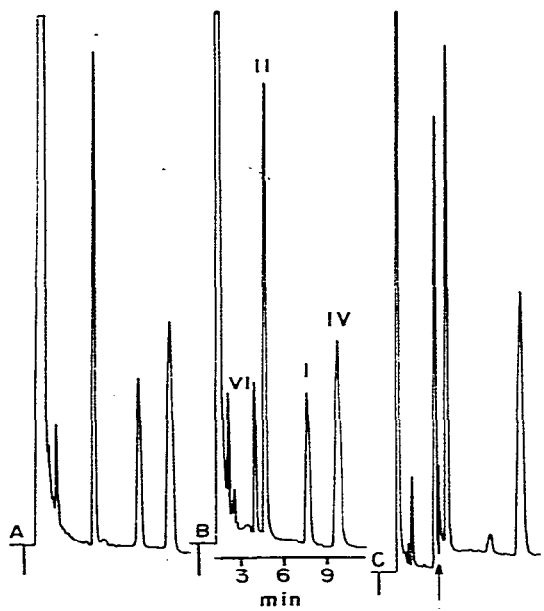

Fig. 3. Chromatograms of an extract of a plasma sample containing 1.86 μg cinromide (I) per ml, 3.50 μg 3-bromocinnamamide (II) per ml and 1.53 μg 3-bromocinnamic acid (VI) per ml run with (A) acetonitrile—water (25:75), and (B) acetonitrile—water (25:75) with NaH_2PO_4 (0.015 mole/l, pH 4.7) and tetraethylammonium perchlorate (0.005 mole/l) added. (C) Chromatogram of extract of plasma drawn from a patient taking cinromide. Arrow indicates change in detector sensitivity from 0.512 to 0.064 a.u.f.s. Concentrations are 0.21 μg/ml I, 3.01 μg/ml II and 28.4 μg/ml VI.

shows chromatograms of an extract of a plasma standard containing the parent drug and the two metabolites run with the normal mobile phase (A) and the ion-pairing mobile phase (B). In chromatogram A the acid metabolite elutes with the solvent front. Chromatogram C shows an extract of plasma drawn from a patient taking cinromide. The arrow indicates a change in sensitivity of the detector from 0.512 to 0.064 a.u.f.s. to accommodate the high concentration of acid (up to 30 μg/ml). Linearity has been confirmed up to 33 μg/ml VI with quantitation by peak-height ratio. No adjustment of peak heights is necessary as long as the ratio of the sensitivity setting for the acid to that for the internal standard is kept constant.

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