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QUANTITATION OF THE ANTICONVULSANT CINROMIDE (3-BROMO-N-ETHYLCINNAMAMIDE) AND ITS MAJOR PLASMA METABOLITES BY THIN-LAYER CHROMATOGRAPHY

R.L. DeANGELIS*, M.M. ROBINSON, A.R. BROWN, T.E. JOHNSON and R.M. WELCH

Department of Medicinal Biochemistry, Wellcome Research Laboratories, Research Triangle Park, NC 27709 (U.S.A.)

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SUMMARY

A quantitative thin-layer chromatography (TLC) procedure is described for the analysis of cinromide (3-bromo-N-ethylcinnamamide) and its two major metabolites, 3-bromocinnamamide and 3-bromocinnamic acid in plasma of the dog. These compounds were recovered from acidified plasma by extraction into benzene with a recovery of 95 ± 5%. All three compounds were quantitated directly on a TLC plate by ultraviolet absorbance densitometry at 270 nm. The linear dynamic range for the quantitation of the compounds on a TLC plate ranged between 10 and 1000 ng. The complete procedure is useful in the working range of 50 ng/ml to 100 μg/ml of plasma with a coefficient of variability of about 10%. Specificity of the method for parent drug and each of its plasma metabolites was confirmed by high-performance liquid chromatography. The method was used to determine the pharmacokinetics of cinromide and its two major plasma metabolites in dogs following a single oral dose of the drug.

INTRODUCTION

Structure activity studies on ring substituted cinnamamides have shown that these compounds have central nervous system stimulant and depressant effects in mice [1]. More recently N-ethyl-3-bromocinnamamide (cinromide) has been shown to have anticonvulsant properties in rodents [2,3] and primates [4] and is currently under clinical evaluation for the treatment of epileptic seizures in patients [5].

As with most anticonvulsant drugs, the basic pharmacokinetics, metabolism and the need for monitoring plasma levels of the drug in patients during clinical testing are important parameters in the development of this type of pharmacologic agent [6]. These factors were important considerations in developing a sensitive but rapid method for measuring cinromide in biological fluids of

animals and man. Since cinnamamides possess strong ultraviolet absorption properties through their conjugated double bond system, cinromide and its two major plasma metabolites can be quantitated by thin-layer spectrodensitometry. The present work describes a quantitative thin-layer chromatography (TLC) method for measuring cinromide and its metabolites, 3-bromocinnamic acid and 3-bromocinnamamide in plasma of animals and man. The procedure is sensitive, specific and rapid enough to accommodate multiple sample analysis. In the present report the procedure was applied to the disposition of cinromide and its metabolites in plasma of dogs.

MATERIALS AND METHODS

Preservative-free chloroform was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and high purity glacial acetic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other solvents were high-performance liquid chromatography (HPLC) grade (Burdick and Jackson Labs.). Silica gel 60 plates (20 × 20 cm, EM Laboratories, Cincinnati, OH, U.S.A.) were scored into 20 × 1 cm channels and the solvent applied mechanically to each channel using 100-μl gas–liquid syringes (Unimetrics, Anaheim, CA, U.S.A.) and an A.I.S. multispotter (Analytical Instrument Specialties, Libertyville, IL, U.S.A.). Rectangular chromatography tanks (23 × 29 cm, Brinkmann Instruments, Westbury, NJ, U.S.A.) lined with Whatman 3-mm chroma-pads (Whatman, Clifton, NJ, U.S.A.) were used for development of the plates. A standard solution for spotting contained 30 μg/ml each of cinromide and 3-bromocinnamamide (BCAM) dissolved in a chloroform–methanol (85:15) solution. A separate standard of 3-bromocinnamic acid (BCA) was prepared at 50 μg/ml in a similar manner. It is important to note that this ratio of chloroform–methanol (85:15) facilitates the competitive binding of the solvent molecules to the silica gel, thereby deactivating the silica at the point of application. This allows the total amount of solute to be chromatographed by preventing its irreversible adsorption to the silica at the origin [7,8].

Animals

Three purebred beagle dogs received cinromide orally in soft gelatin capsules containing 100 mg/kg of cinromide dissolved in Tween 80. Blood was drawn at intervals of 0.5, 1, 2, 4, 8, 12 and 24 h after the dose. Plasma was analyzed for cinromide and its metabolites as described in the text.

TLC procedure

Apparatus. Ultraviolet absorbance measurements were made by scanning the TLC plates with a single beam from a Schoeffel SD 3000 spectrodensitometer (Schoeffel Instruments, Westwood, NJ, U.S.A.) at a wavelength of 270 nm. The total, unfiltered emission from the surface of the plate was determined with a reflectance mode assembly and a Schoeffel SD 300 density computer. Peak areas for sample and reference compounds were simultaneously recorded on a Honeywell Electronic 124 recorder (Honeywell, Minneapolis, MN, U.S.A.) and integrated with an Autolab minigrator (Spectra Physics, Santa Clara, CA, U.S.A.).

Plasma extraction. Following acidification of plasma (1 ml) with 0.5 ml of 1 *N* hydrochloric acid (pH < 2), cinromide and its metabolites (BCAM and BCA) were extracted into 6 ml of benzene by shaking for 10 min. After extraction and centrifugation (1500 *g*) to separate the phases, 100 μl of the benzene layer were spotted directly onto a silica-gel plate as described below. When low amounts (<1.0 μg) of these drugs were present in plasma, an appropriate amount of the organic phase (0.5—2.0 ml) was placed into a disposable glass tube (Kimble, 12 × 75 mm) and evaporated to dryness (N-Evap, Worcester, MA, U.S.A.) under a gentle stream of nitrogen. This residue was redissolved in an appropriate volume of solvent and spotted on a TLC plate as described below. It should be noted that the extraction procedure may be applied equally well to plasma from either rat, dog or man without interference from endogenous substances.

Chromatography. The extraction residue from evaporation was redissolved in 80 μl of chloroform—methanol (85:15) solution and the entire volume drawn into a 100-μl syringe and spotted on a silica-gel plate with the aid of a TLC multispotter (Analytical Instrument Specialties, Anaheim, CA, U.S.A.). During the spotting procedure a gentle stream of warm air was blown across the surface of the plate to increase solvent evaporation. Two standard concentrations of each drug were always processed through the method to verify recovery. In addition, several standards (30—150 ng) were spotted manually on

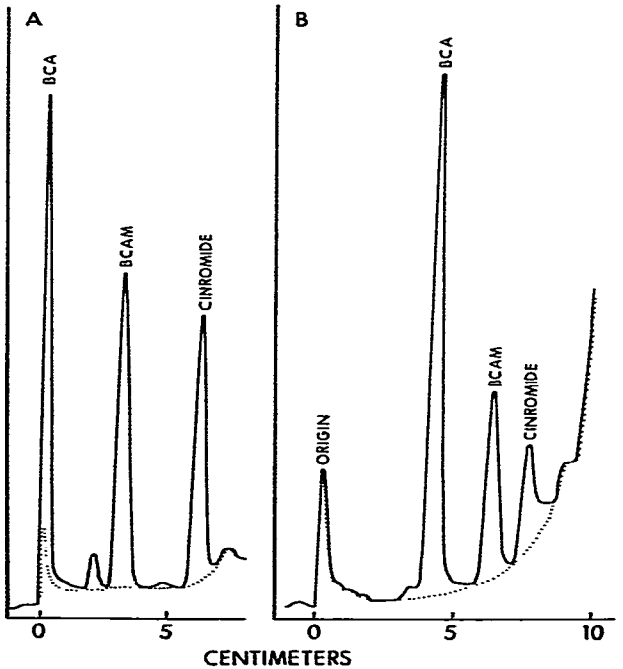

Fig. 1. Thin-layer chromatograms of dog plasma showing cinromide and its metabolites, 3-bromocinnamamide (BCAM) and 3-bromocinnamic acid (BCA), at 1 h (A) and 2 h (B) after an oral dose of 100 mg/kg of cinromide. For the quantitation of cinromide and BCAM (A) the solvent system was ethyl acetate—chloroform—ammonium hydroxide (84:15:1); quantitation of BCA (B) was accomplished in a solvent system of chloroform—methanol (85:15). The dashed line represents the dog plasma blank.

separate channels with a 10-μl Hamilton syringe. Actual chromatograms of cinromide and its metabolites extracted from plasma of a dog following an oral dose (100 mg/kg) of cinromide are shown in Fig. 1. For quantitation of cinromide and its N-dealkylated metabolite BCAM, the plate was developed to 15 cm in ethyl acetate—chloroform—ammonium hydroxide (84:15:1). In this solvent system cinromide and BCAM had R_F values of 0.4 and 0.26, respectively, which provided sufficient separation for quantitation of these two compounds (Fig. 1A). However, since BCA remained at the origin in this system, this metabolite was quantitated separately in a solvent system of chloroform—methanol (85:15) (Fig. 1B).

HPLC procedure

Apparatus. A Waters Assoc. (Milford, MA, U.S.A.) ALC/GPC-204 high-pressure liquid chromatograph equipped with a Model 440 UV detector and M6000A pump was used. A stainless-steel column (25 cm × 3.9 mm I.D.) packed with Partisil PAC (particle size 10 μm) was obtained from Whatman and fitted with a 5-cm precolumn packed with Co:Pell ODS (Whatman). Injections were made through a Waters Model U6K injection system. Peaks were recorded on a 10-mV recorder (Houston Instruments, Austin, TX, U.S.A.) and areas were quantitated with an Autolab System I integrator (Spectra Physics).

Chromatography. The extraction procedure was identical to that described for the TLC procedure. Residues from solvent evaporation were redissolved in 100 μl of chloroform and 50 μl injected onto the column. Analysis of cinromide and its metabolites (BCAM and BCA) were determined at a wavelength of 254 nm in a mobile phase of chloroform—acetic acid (95:5, v/v), at a flow-rate of 60 ml/h at 8.2 MPa (1200 p.s.i.). The column and precolumn were both operated at ambient temperature. A standard stock solution containing 100 μg/ml of cinromide, BCAM and BCA was prepared in the mobile phase and diluted as needed.

RESULTS AND DISCUSSION

Recovery of cinromide and its metabolites

Recoveries of cinromide, BCAM and BCA from human plasma, taken from several analyses over a period of several weeks, are shown in Table I. These compounds were extracted from plasma with recoveries of 96%, 88%, and 100%, respectively. The standard deviation (S.D.) for any individual assay ranged between 5—10% for each of the compounds, reflecting good reproducibility of the procedure. Essentially identical recoveries were obtained from dog plasma. Although the overall recoveries are good and reproducible above 50 ng/ml of plasma, the low polarity of the benzene extraction solvent caused the recovery to decline at low plasma levels so that at 25 ng/ml the recovery was reduced to 25%. The lowest amount of each drug which can be chromatographed and detected on a silica-gel TLC plate was 10 ng, while the upper limit of each compound which could be chromatographed and still retain linearity was about 1 μg.

TABLE I

RECOVERY OF CINROMIDE AND ITS METABOLITES FROM HUMAN PLASMA BY QUANTITATIVE TLC ANALYSIS

Various concentrations of cinromide, 3-bromocinnamamide (BCAM) and 3-bromocinnamic acid (BCA) were added to 1 ml of human plasma and analyzed as indicated in the text.

	Drug added (μg)	Drug found (μg)	S.D. (%)	Recovery (%)	Samples (n)
Cinromide	0.12	0.11	16.4	92	10
	0.24	0.22	6.8	92	9
	0.60	0.57	8.4	95	23
	1.2	1.16	7.1	97	23
	3.0	3.06	10.7	102	11
	6.0	6.01	5.6	100	5
BCAM	0.12	0.10	20.0	83	10
	0.24	0.20	9.0	83	10
	0.60	0.51	9.8	85	26
	1.2	1.00	7.1	83	25
	3.0	2.78	9.4	93	10
	6.0	5.56	4.4	93	11
BCA	1.0	0.95	2.1	95	2
	2.0	2.14	1.9	107	2
	4.0	4.02	15.9	100	4
	5.0	5.18	1.7	104	2
	8.0	8.33	8.8	104	4
	10.0	10.24	4.9	102	6
	20.0	21.02	5.9	105	4
	30.0	29.09	4.0	97	3
	40.0	37.94	3.1	95	2
	50.0	49.90	2.8	100	4

Specificity of the procedure

To verify the specificity of the TLC method, an HPLC procedure was developed to analyze for cinromide and its two plasma metabolites. Various amounts of cinromide, BCAM and BCA were extracted from plasma as described above and a portion of the extract was analyzed for the above compounds by HPLC as described under Materials and methods. Cinromide, BCAM and BCA were recovered through the method to the extent of 84.7%, 76.0% and 78.4%, respectively. It is not clear why these recoveries are lower than those achieved by the TLC method; perhaps this is a result of non-specific column adsorption. It is of interest to note that in the HPLC assay reported by Perchalski et al. [9], only a 61% recovery was obtained for cinromide and its metabolites. All experimental data were corrected for recoveries. Chromatographic profiles of cinromide and its metabolites from a standard solution containing all three compounds and from an extract of plasma containing these compounds are shown in Fig. 2. Fig. 2A shows the retention times and relative peak heights at 254 nm when a mixture containing 100 ng of each compound is separated by HPLC. Fig. 2B shows that cinromide and its two major plasma metabolites were sufficiently separated for quantitation without significant interference from substances normally present in dog plasma.

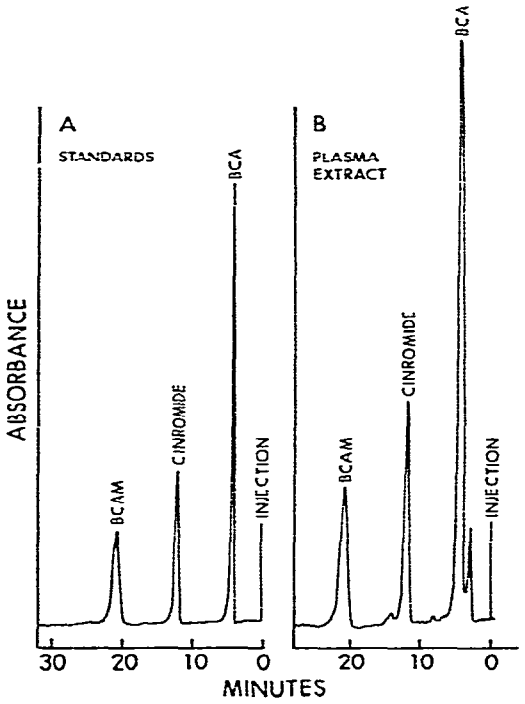

Fig. 2. HPLC chromatogram showing the separation of a standard solution of cinromide, 3-bromocinnamamide (BCAM) and 3-bromocinnamic acid (BCA); each peak represents 100 ng of compound. (B) HPLC chromatogram of a dog plasma extract at 1 h following the oral administration of 100 mg/kg of cinromide. Solvent system: chloroform-acetic acid (95:5). Column: Partisil PAC (25 cm).

TABLE II

COMPARATIVE ANALYSIS OF CINROMIDE AND ITS METABOLITES IN HUMAN PLASMA BY QUANTITATIVE TLC AND HPLC

Human subjects received 300 mg of cinromide and selected plasma samples were assayed for parent drug and metabolites by HPLC and quantitative TLC. Plasma samples selected covered the usual range of plasma concentrations normally found following a therapeutic dose of the drug. The correlation coefficient (r) for the analysis of each compound by the two methods is shown in the last column.

Compound	Plasma concentration (μg/ml)		r
	HPLC	TLC	
Cinromide	0.09	0.11	
	0.44	0.52	0.91
	1.10	0.98	
BCAM	0.46	0.59	
	1.93	1.95	0.96
	4.91	5.10	
BCA	1.10	0.96	
	4.90	5.10	0.89
	9.93	9.97	

The data in Table II show the comparative analysis of cinromide and its metabolites in human plasma by quantitative TLC and HPLC. Human subjects received 300 mg of cinromide and selected plasma samples were assayed by both procedures. The plasma samples selected covered the usual range experienced clinically following a therapeutic dose of the drug. The concentrations found for each compound, assayed by both methods, agreed well with correlation coefficients in the range of 0.89—0.96.

Recently an HPLC method has been reported for quantitating cinromide, BCAM and BCA in a subject treated with cinromide (Perchalski et al. [9]). This ion-pairing procedure requires a heated column system and a mobile phase containing a buffering system. Although this procedure is useful in quantitating cinromide and its major metabolites, the TLC procedure has a distinct advantage regarding assay time and perhaps sensitivity, since no standard curve was included in their report. Quantitative TLC allows for multiple assays of up to 36 plasma samples a day where two of three compounds can be quantitated simultaneously. In addition, the TLC method has been applied to an oral pharmacokinetic evaluation of cinromide in the dog.

Pharmacokinetics of cinromide in the dog

The quantitative TLC procedure was used to determine the pharmacokinetic profile of cinromide and its two major plasma metabolites in beagle dogs (Fig. 3). The results represent the mean curves over 24 h from three dogs following

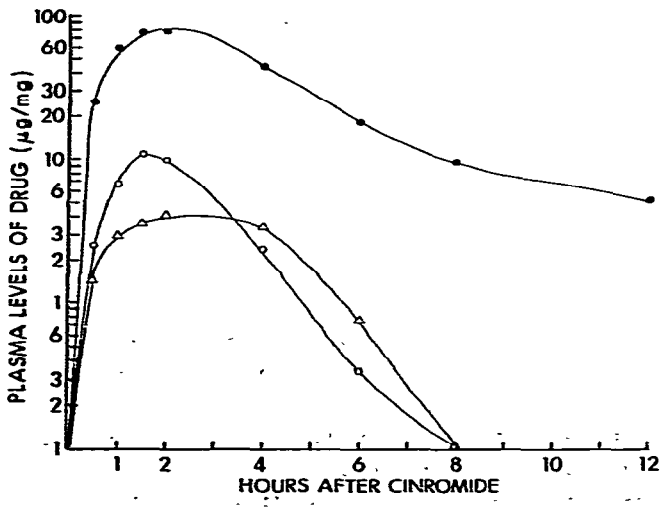

Fig. 3. Plasma levels of cinromide (○) and its metabolites, 3-bromocinnamamide (△) and 3-bromocinnamic acid (●) in beagle dogs following the oral administration of 100 mg/kg of cinromide. The data represent the mean curves from three dogs.

the oral administration of 100 mg/kg of cinromide. The drug was administered as soft gelatin capsules containing drug dissolved in Tween 80. As shown in Fig. 3 and Table III, this formulation provided rapid absorption of cinromide with peak levels of 11.0 µg/ml being achieved in 90 min. The N-dealkylated metabolite 3-bromocinnamamide, and 3-bromocinnamic acid were formed immediately, achieving peak plasma levels of 4.1 and 78.9 µg/ml respectively at

TABLE III

PHARMACOKINETIC PARAMETERS OF CINROMIDE AND ITS METABOLITES IN BEAGLE DOGS FOLLOWING THE ORAL ADMINISTRATION OF 100 mg/kg OF CINROMIDE

Three beagle dogs received 100 mg/kg of cinromide by capsule dissolved in Tween 80. The area under the plasma concentration curve (AUC) was determined by the trapezoidal rule. The results represent the mean ± S.E.

Compound	Peak level (μg/ml)	AUC after 12 h (μg/ml-h)	Half-life (min)
Cinromide	11.0 ± 3.3	29 ± 10	66
3-Bromocinnamamide	4.1 ± 0.6	18 ± 3	60
3-Bromocinnamic acid	78.9 ± 7.0	347 ± 47	132

2 h after the dose. It is of interest that the concentrations of the two plasma metabolites of cinromide are quite substantial; in fact, the area under the plasma curve for 3-bromocinnamic acid is twelve times greater than that observed for the parent drug. The rapid biotransformation of cinromide in the dog was immediately apparent by the obvious appearance of these metabolites on a TLC plate following exposure to ultraviolet light.

In the present study the development of a quantitative TLC method during the initial phase of drug development served as a very useful means for the early detection of significant plasma metabolites. In this respect the procedure not only provided a rapid quantitative tool but also allowed for the early detection and identification of major plasma metabolites of a new pharmacologic agent.

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