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ACKNOWLEDGMENTS AND ADDRESSES

Received April 5, 1976, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907.*

Accepted for publication June 17, 1976.

Supported by Grant R03 DA 00725 from the National Institute of Mental Health.

The author is indebted to Mrs. Sandra Diskin for technical assistance.

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High-Pressure Liquid Chromatographic Analysis of Drugs in Biological Fluids II: Determination of an Antiarrhythmic Drug, Tocainide, as Its Dansyl Derivative Using a Fluorescence Detector

PETER J. MEFFIN ˣ, SANDRA R. HARAPAT, and DONALD C. HARRISON

Abstract □ A sensitive and specific method is described for the determination of a new primary aliphatic amine antiarrhythmic drug, tocainide. Tocainide, together with an internal standard, is selectively extracted from plasma or blood and reacted with dansyl chloride. The highly fluorescent dansyl derivatives are separated using high-pressure liquid chromatography and measured using a fluorescence detector. The method can measure therapeutic and subtherapeutic concentrations of the drug (0.1–5.0 μg/ml of plasma) with a standard deviation of less than 2%.

Keyphrases □ Tocainide—high-pressure liquid chromatographic analysis, plasma or blood □ High-pressure liquid chromatography—analysis, tocainide, plasma or blood □ Dansyl chloride—derivatizing reagent in high-pressure liquid chromatographic analysis of tocainide □ Antiarrhythmics—tocainide, high-pressure liquid chromatographic analysis, plasma or blood

Within the last 5 years, advances in high-pressure liquid chromatography (HPLC), the commercial availability of reliable HPLC equipment, and the development of efficient columns have resulted in the application of HPLC to many analytical problems. However, the application of HPLC to drug analysis has lagged behind the increase in its general use (1, 2). Many papers reported the chromatographic conditions required to separate drugs (3–7), but only a few described the quantitative application of HPLC to the analysis of drugs in dosage forms (8–11).

A major cause of the failure to apply HPLC to the analysis of drugs in biological fluids has been the lack of suitable detectors. UV absorption detectors have been commonly used in HPLC. The microgram or nanogram per milliliter concentrations of many drugs found in plasma after therapeutic doses have confined the use of HPLC analysis to drugs having high molar absorptivities at 254 or 280 nm, the wavelengths most frequently used in these detectors (12–18).

The derivatization of pesticides with reagents yielding fluorescent derivatives and their subsequent HPLC analysis have been reported (19–22). Many drugs unsuited for analysis with UV absorption or fluorescence detectors have primary or secondary amino, phenolic or alcoholic hydroxyl, or aldehyde and keto functions suitable for the formation of fluorescent derivatives. The ability of HPLC to resolve compounds with small differences in molecular structure permits derivatization prior to injection onto the column while still achieving adequate separation of the drug of interest from other molecules reacting with the reagent.

This paper reports the use of HPLC with a commercially available fluorescence detector to determine blood and plasma concentrations of a new primary aliphatic amine antiarrhythmic drug, tocainide [2-amino-*N*-(2,6-dimethylphenyl)propanamide], after derivatization with dansyl chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride]. The described techniques should be generally applicable to drugs containing derivatizable functional groups. The use of reagents such as dansyl chloride may result in the increased utilization of HPLC for drug analysis in biological fluids, in a manner similar to that which occurred with electron-capture detection in GLC when halogenated acylating and alkylating reagents became available.

EXPERIMENTAL

Reagents—Ethyl acetate[1], hexane[1], acetone[1], and carbon tetrachloride[1] were used as received. Hexane[2], dichloromethane[2], and methanol[2] were used as chromatographic solvents. Stock solutions of tocainide[3] were prepared in 0.05 *N* HCl over the range of 0.1–10 μg/100 μl. The internal standard [2-amino-*N*-(2,6-dimethylphenyl)butanamide hydrochloride[3] (I)] was prepared in 0.05 *N* HCl at a concentration of 0.675 μg/50 μl.

A solution of dansyl chloride (1 mg/ml) was prepared in acetone and

[1] Nanograde quality, Mallinckrodt, St. Louis, Mo.
[2] SpectrAR quality, Mallinckrodt.
[3] Astra Pharmaceutical Products, Worcester, Mass.

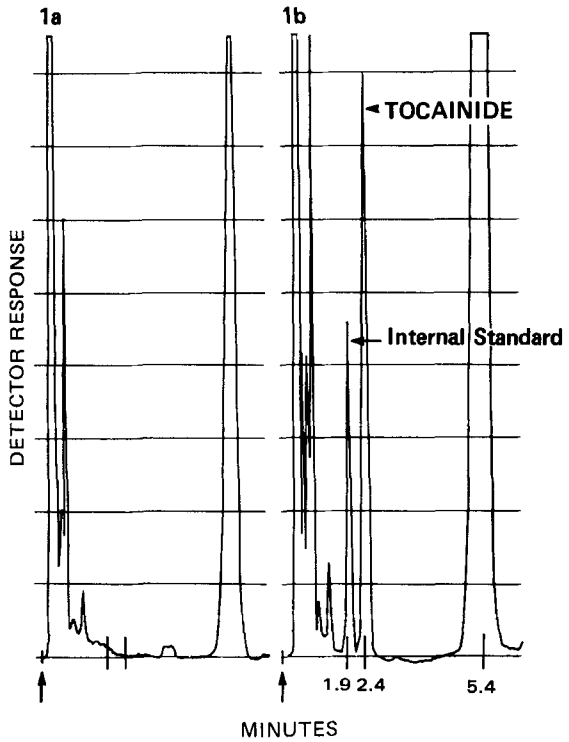


Figure 1—*Chromatograms obtained with plasma from a patient prior to receiving tocainide* (a) *and with a plasma concentration of tocainide of 1.10 μg/ml* (b). *The vertical lines in Fig. 1a correspond to the retention times of tocainide and the internal standard.*

stored in the dark under refrigeration. A fresh solution was prepared each week. Buffer (pH 6.0) was prepared by making a half-saturated solution of disodium citrate in water and adjusting the pH to 6.0 with 85% H_3PO_4. Solutions of 2 and 0.5 *N* NaOH, 0.1 *N* H_2SO_4, and 1 *M* $NaHCO_3$ were also employed.

General Procedure: Extraction and Derivatization of Tocainide—Between 0.05 and 2.0 ml of plasma or whole blood containing an estimated 0.1–5.0 μg of tocainide was transferred with a pipet[4] to a screw-capped[5] centrifuge tube (15 ml) together with 50 μl of internal standard solution containing 0.675 μg of 1, 200 μl of 2 *N* NaOH, and 3 ml of ethyl acetate. The samples were extracted by mixing, using gentle hand-tilting of the tubes for 5 min, followed by centrifugation at 2500 rpm for 10 min to separate the organic and aqueous phases.

The aqueous phase was discarded. Approximately 2 ml of the ethyl acetate phase was transferred to a second tube, which had an elongated cone at its base of nominal 0.1-ml capacity, containing 50 μl of 0.1 *N* H_2SO_4. The tube was mixed[6] for 1 min and centrifuged for 5 min to separate the phases. The ethyl acetate phase was then discarded, and hexane (1 ml) was added to the acidic extract in the tube. The mixing and centrifugation steps were then repeated. The tube was then immersed in a dry ice–acetone bath to freeze the aqueous phase; the hexane, together with the remaining ethyl acetate, was decanted and discarded.

After thawing, 50 μl of 1 *M* $NaHCO_3$ and 200 μl of dansyl chloride solution were added to the tube, which was heated[6] for 30 min at 40°. Sodium hydroxide solution (100 μl of 0.5 *N*) was then added to the tube to convert the remaining dansyl chloride to the sodium salt of 5-(dimethylamino)-1-naphthalenesulfonic acid. Most of the acetone was then removed by heating[6] the tube to 40°, at reduced pressure, for 3 min. Buffer (pH 6.0, 1 ml) was added, together with 200 μl of carbon tetrachloride. The tube was mixed for 2 min and centrifuged for 5 min to separate the aqueous and organic phases. Between 2 and 5 μl of the carbon tetrachloride phase, sampled through the aqueous phase with a 10-μl syringe, was injected into the chromatograph.

Chromatography—A high-pressure liquid chromatograph[7] fitted with a 30-cm × 4-mm i.d. column[8] was used. A fluorometer[9] having both excitation[10] and emission[11] filters was used as a detector. Pressure was maintained in the detector to prevent the formation of bubbles by a 0.5-μm porosity stainless steel frit[12], placed on the efferent side of the detector. One pump of the dual-pump gradient elution chromatograph contained hexane, and the other contained 2% methanol in dichloromethane. An isocratic 1:1 mixture of the two solvents was used in the analysis[13].

Calibration and Accuracy—Calibration curves were constructed by adding known amounts of tocainide and the internal standard to blood and plasma, which were then taken through the analytical procedure. The peak height ratio of tocainide to the internal standard was plotted *versus* the amount of tocainide added to construct the calibration curve. Replicates (10 samples) at tocainide concentrations of 0.3 and 3.0 μg were carried through the procedure to determine the reproducibility of the method.

To calibrate the method and obtain an estimate of accuracy for each particular batch of unknown samples, standards of 0.1, 1.0, and 5.0 μg of tocainide were added to 1-ml samples of blank plasma, which were assayed for tocainide at the same time as the unknown samples. The peak height ratio of each standard was divided by the amount of tocainide added to give a normalized peak height ratio. The average normalized peak height ratio was used to calculate the amount of tocainide in the unknown samples and to estimate accuracy. The effect of sample size on the method was investigated by adding 1.0 μg of tocainide to tubes containing 0.05–2.0 ml of plasma and then assaying for tocainide.

Mass Spectra—Solvent corresponding to the peaks resulting from tocainide and internal standard was collected and reduced to a small volume with a stream of air at room temperature. The mass spectra of these materials were determined by direct probe insertion into the mass spectrometer[14], which had undergone minor modifications to permit use in the chemical ionization mode (23). The reactant gas was isobutane, and the instrument was operated at 200°.

Plasma Level Study—Tocainide tablets were administered to a patient (60 years old, 67 kg) on two separate occasions after approximately 12 hr of fasting. Either 400 or 800 mg of tocainide was administered on each occasion, and food was allowed after 3 hr. Samples of venous blood (10 ml) were withdrawn by means of heparinized containers[15] at appropriate intervals for 60 hr after dosing. The blood samples were centrifuged, and the plasma was transferred to glass containers and stored at −15° until analyzed.

RESULTS AND DISCUSSION

Preliminary work showed that tocainide was satisfactorily and conveniently extracted from plasma by ethyl acetate under strongly alkaline conditions. The conditions for the reaction of tocainide with dansyl chloride were optimized for pH, reagent concentration, solvent composition, temperature, and time using published data on the reaction of the reagent with primary aliphatic amines as a guide (24). The excitation and emission maxima of the peaks corresponding to both tocainide and internal standard in the mixture of solvents used for the chromatography were at 360 and 490 nm, respectively. The excitation and emission filters used in the detector (see *Experimental*) were chosen to minimize the radiation from sources other than the fluorescence of the dansyl derivatives reaching the photomultiplier tube, consistent with a high input in the region of the excitation maxima.

With the solvents used in this analysis, the column[8] required a pressure of 120 atm to produce a flow rate of 300 ml/hr. Under these conditions, the peaks corresponding to tocainide and the internal standard had retention times of 2.4 and 1.9 min, respectively. A large peak at 5.4 min, present also in reagent blanks, prevented injection of samples into the chromatograph at intervals more frequent than 6 min. A typical chromatogram of a patient sample having 1.10 μg of tocainide/ml of plasma is shown in Fig. 1*b*. A chromatogram of blank plasma from this patient is also shown in Fig. 1*a*. In general, no peaks that interfered with the tocainide or internal standard peak, other than the partially resolved small peaks shown in Fig. 1*a*, were encountered in analysis of plasma from some 20 patients. To date, only one column[8] has been used and has shown no deterioration in performance after 6 months of use on a daily basis under the conditions described.

[4] Eppendorf, V.W.R. Scientific, San Francisco, Calif.
[5] Lined with Teflon (du Pont).
[6] Vortex-evaporator, Buchler Instruments, Fort Lee, N.J.
[7] Model 8500, Varian, Palo Alto, Calif.
[8] μBondapack NH_2, Waters, Milford, Mass.
[9] Fluoromonitor, American Instrument Co., Silver Spring, Md.
[10] Corning 7–51, Scientific Products.
[11] Wratten 8, Eastman Kodak, Rochester, N.Y.
[12] Varian, Palo Alto, Calif.
[13] Minor adjustments in the ratio of the two solvents were made each day to give the retention times reported.
[14] AEI MS 902.
[15] Vacutainers, V.W.R. Scientific, San Francisco, Calif.

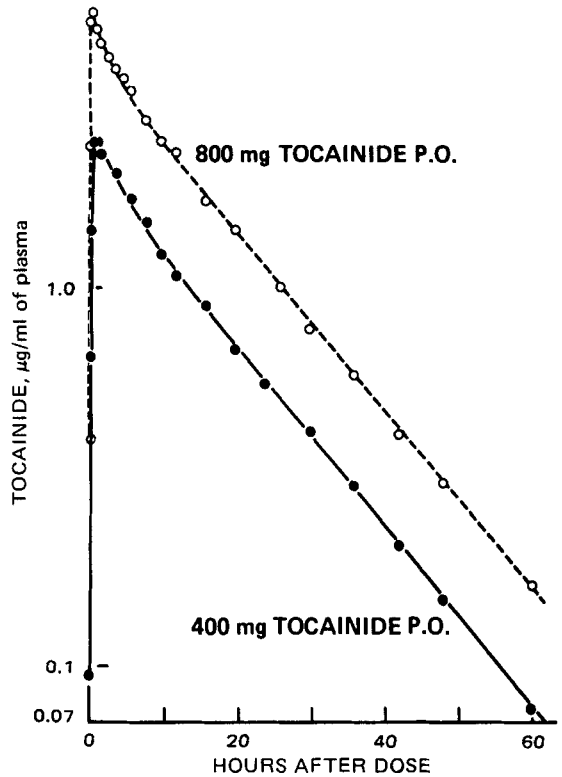


Figure 2—*Plasma concentrations of tocainide at various times in a patient receiving 400 and 800 mg po of tocainide on two separate occasions.*

Calibration curves from water, plasma, and whole blood were superimposable within the error of the method. However, the increased background observed in the chromatograms of blood extracts made the application of the method unreliable at tocainide concentrations below approximately 0.5 μg/ml of blood. The calibration curve from plasma was linear in the 0.1–5.0-μg range and passed through the origin. The average normalized peak height ratio (see *Experimental*) obtained from the daily three-point calibration curves had a mean coefficient of variation of 3.4% for 32 separate calibration curves obtained over approximately 6 months.

This estimate of accuracy probably gives a higher estimate of error than normally would be encountered during the routine application of the method to pharmacokinetic studies, since two of the three calibration points (at 0.1 and 5.0 μg of tocainide/ml of plasma) are at the maximum and minimum of the calibration curve. At the time of analysis of unknown samples, sample volumes were chosen to yield peak height ratios as close to 1.0 as possible to minimize error. Replicates of 10 samples of 3.0 and 0.3 μg of tocainide/ml of plasma had coefficients of variation of 1.1 and 1.8%, respectively.

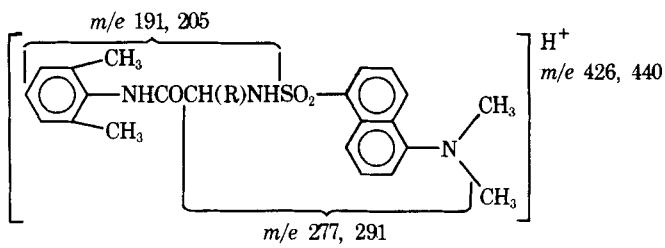
Since it was convenient to take variable volumes of plasma to determine the tocainide concentration in unknown samples, the effect of plasma volume on the peak height ratio of tocainide to the internal standard was examined. The results of this investigation indicated that the peak height ratios were independent of the volume of plasma added between 0.05 and 2.0 ml of plasma, having a coefficient of variation of 3.7% with no systematic trend apparent in the data.

The sensitivity of the method is more than adequate to measure antiarrhythmic concentrations of tocainide, which preliminary data indicate to be in the range of 4–10 μg/ml of plasma. The sensitivity of the presently described method could be increased by extracting the final reaction mixture into a volume of carbon tetrachloride less than the 0.2 ml used or by sampling a larger portion of solvent than the 1–2% injected into the chromatograph. The lower limit of sensitivity is imposed by the competition of solvent with the drug during the derivatization step. It is impractical to scale down the derivatization conditions described (total volume of 300 μl) by more than an order of magnitude. The addition of excess dansyl chloride to compensate for the low concentrations of drug gives rise to an increased solvent signal and produces no net beneficial result.

The described method was applied during the initial clinical evaluation of tocainide in some 20 patients and yielded consistent data after both single and multiple oral doses. The use of the vortex-evaporator[6] in conjunction with the rapid chromatographic analysis allowed the extraction and analysis of 30–35 samples in 8 hr. Typical data are shown in Fig. 2 for a patient who received 400 and 800 mg of tocainide on two separate occasions. Computation of the area under the plasma concentration–time curve from zero to infinite time for the low dose divided by the high dose yields a value of 0.497. This ratio is very close to 0.5, which would be expected if the product of the fraction of the dose absorbed and the clearance of the drug remained constant in this subject.

The use of dansyl chloride as a reagent for the determination of drugs and their metabolites in the biological fluids has been restricted by the lack of suitable methods for the routine separation of the compounds of interest from the many other products of the reaction. Dansyl chloride undergoes many side reactions and, as a result, the compound being assayed may be a minor constituent of the reaction mixture (24). The utility of HPLC in achieving difficult separations is evidenced in the separation of the dansyl derivative of tocainide from its internal standard. The structures of these two compounds, differing by only one methylene unit, are supported by their mass spectra.

The protonated molecular ion, *m/e* 426, was the base peak of the chemical ionization mass spectrum of the dansyl derivative to tocainide (mol. wt. 425). Peaks were also observed at *m/e* 277 (relative abundance 36%) and 191 (55), corresponding to the fragments shown in Structure II (tocainide, R = CH_3; internal standard, R = CH_2CH_3).



II

A similar series of peaks at *m/e* 440 (relative abundance 100%), 291 (30), and 205 (25), 14 amu higher than those obtained for the dansyl derivative of tocainide, was observed for the derivative of the internal standard (mol. wt. 439). All of these data are consistent with the proposed structures of the derivatives.

The ability of HPLC to separate the dansyl derivatives of two such similar compounds may be indicative of the usefulness of the combination of HPLC and fluorescence detectors. The use of similar reagents with this combination might increase significantly the number of drugs that can be measured in biological fluids and the sensitivity with which they can be measured.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 8, 1975, from the *Cardiology Division, Stanford University School of Medicine, Stanford, CA 94305.*

Accepted for publication June 22, 1976.

Supported by NIH Grant HL-5866 and Program Project Grant 1-P01-15833.

The assistance of Mr. Joseph B. Keenaghan in supplying a number of compounds for investigation as internal standards is appreciated. The authors also thank Mrs. Dorothy McCain for her editorial assistance.

[x] To whom inquiries should be directed.

NOTES

Cytotoxic and Tumor Inhibitory Agent from *Polygala macradenia* Gray (Polygalaceae): 4′-Demethyldeoxypodophyllotoxin

JOSEPH J. HOFFMANN, RICHARD M. WIEDHOPF, and JACK R. COLE [x]

Abstract □ The chloroform fraction of *Polygala macradenia* exhibited activity against the P-388 lymphocytic leukemia and human epidermoid carcinoma of the nasopharynx test systems. The constituent responsible for this activity was a lignan, 4′-demethyldeoxypodophyllotoxin ($C_{21}H_{20}O_7$). The identity was proven by elemental analysis; PMR, IR, mass spectrometric, and melting-point determinations; and preparation of a derivative.

Keyphrases □ *Polygala macradenia*—chloroform fraction of extract of aerial parts, lignan isolated, evaluated for cytotoxic and tumor inhibitory activity □ 4′-Demethyldeoxypodophyllotoxin—isolated from chloroform fraction of extract of aerial parts of *Polygala macradenia*, evaluated for cytotoxic and tumor inhibitory activity □ Cytotoxic activity—lignan isolated from chloroform fraction of extract of aerial parts of *Polygala macradenia* evaluated □ Tumor inhibitory activity—lignan isolated from chloroform fraction of extract of aerial parts of *Polygala macradenia* evaluated

In the continuing search for plants having tumor inhibitory constituents, it was found that the chloroform fraction of *Polygala macradenia* Gray (Polygalaceae)[1], obtained by partitioning the ethanol extract of the stems, leaves, flowers, and fruit between chloroform and water, showed inhibitory activity against the P-388 lymphocytic leukemia (PS) test system and the human epidermoid carcinoma of the nasopharynx (KB) test system[2].

DISCUSSION

The lignan 4′-demethyldeoxypodophyllotoxin (I), previously isolated from *Polygala paenea* L. (Polygalaceae) (1), was isolated from the chloroform fraction by column chromatography, treatment with activated charcoal, preparative TLC, and recrystallization. The structure of the lignan was confirmed by elemental analysis; PMR, IR, mass spectrometric, and melting-point determinations; and conversion to the known lignan, deoxypodophyllotoxin (II).

Compound I demonstrated an ED_{50} of 0.0012 μg/ml in the KB test system. Activity in the KB system is defined as $ED_{50} \leq 20$ μg/ml (2). In the PS test system, the lignan demonstrated activity of 132% test/control (T/C) at 2.1 mg/kg. Activity in the PS system is defined as an increase in the survival of treated animals over that of control animals resulting in a T/C ≥ 125% (3).

EXPERIMENTAL[3]

Isolation Procedure—The stems, leaves, flowers, and fruit of *P. macradenia* (16 kg) were ground and extracted exhaustively in a Lloyd-type extractor with ethanol. The air-dried residue was partitioned between chloroform (10 liters) and water (10 liters). A 200-g sample of the air-dried chloroform phase was then subjected to alumina III (2 kg) column (7 × 65 cm) chromatography. The solvents used, in increasing order of polarity, were hexane, hexane–benzene (1:1), benzene, chloroform, and methanol; elution was continued with each solvent until the eluent was colorless. The benzene fraction (1.2 g) was subjected to silica gel 60 (75 g) column (3.5 × 24 cm) chromatography, eluting with benzene–ether (4:1). Fractions 16–38 (25 ml each), consisting of one major component according to TLC, exhibited activity.

Isolation of I—The combined active fractions were treated with activated charcoal and purified by preparative TLC with a chloroform–

[1] The plant was collected in Texas in August 1974. Identification was confirmed by Dr. Robert E. Perdue, Jr., Medicinal Plant Resources Laboratory, U.S. Department of Agriculture, Bethesda, Md. A reference specimen is maintained by the U.S. Department of Agriculture.

[2] Of the Drug Evaluation Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, Md.

[3] Carbon and hydrogen analyses were performed by Chemalytics, Inc., Tempe, Ariz. PMR, IR, and mass spectra were determined using a Varian T-60 spectrophotometer, a Beckman IR-33, and a Hitachi Perkin-Elmer double-focusing spectrometer (model RMU-6E), respectively. The melting points were determined on a Kofler hot-stage apparatus and are uncorrected.