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PRE-COLUMN DERIVATIZATION WITH FLUORESCAMINE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS

APPLICATION TO TOCAINIDE

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SUMMARY

The quantitative determination of tocainide, a new antiarrhythmic agent, by high-performance liquid chromatography (HPLC) is reported. The drug and a chemically similar internal standard were extracted from blood plasma with acetonitrile under salting-out conditions obtained by saturation of the aqueous medium with sodium chloride—sodium carbonate. The organic extract, without evaporation, was treated with borate buffer (pH 8.2) and fluorescamine. The resulting derivatives were chromatographed on an ODS reversed-phase column using a methanol—phosphate buffer (pH 7.0) mixture as mobile phase and were detected fluorometrically by monitoring the emission at 485 nm, with excitation at 395 nm. The intra-assay coefficients of variation were 3.0 and 4.3% for ten replicate 0.25 and 1.00 μg/ml samples, respectively, and the inter-assay coefficient of variation was 3.6% for ten replicate 1.00 μg/ml samples. The procedure is simple, rapid, sensitive, and specific. Several other drugs and drug metabolites also were derivatized with fluorescamine and chromatographed successfully. Pre-column derivatization with fluorescamine followed by HPLC with fluorometric detection may have significant advantages in drug analysis.

INTRODUCTION

Tocainide (2-amino-2′,6′-propionoxylidide, Fig. 1), a new primary amine analogue of lidocaine, is an oral antiarrhythmic agent often effective in the treatment of chronic ventricular ectopy refractory to other modes of management [1]. Determination of the concentration of tocainide in blood serum is desirable during therapy since the therapeutic efficacy of the drug correlates with its concentration in serum [2], and is also needed in pharmacokinetic studies. Quantitative analysis of tocainide in biological fluids has been per-

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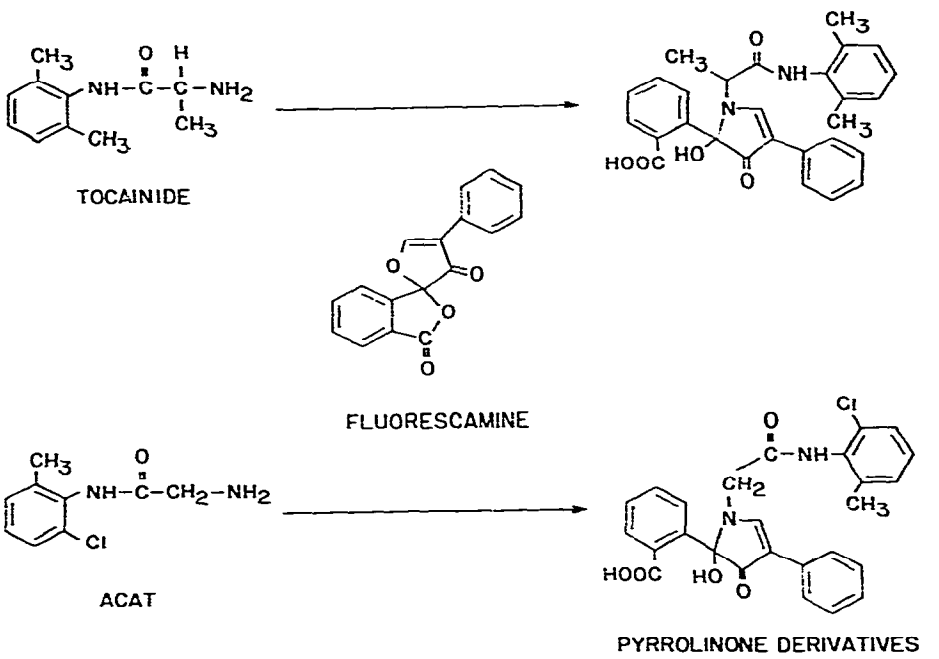

Fig. 1. The structures of tocainide and ACAT, and their reaction with fluorescamine.

formed with gas—liquid chromatography [3, 4] and with high-performance liquid chromatography (HPLC) [5—8]. The HPLC methods include the use of variable-wavelength detectors [5—7] and fluorescence detection [8].

An analytical method capable of determining the concentration of the drug at sub-therapeutic levels as low as 100 ng/ml was required for ongoing studies [9] on the disposition of tocainide. Two of the published HPLC procedures appeared to have the required sensitivity [6, 8] but neither method was suitable for our purposes: one procedure [6] required ultraviolet detection at 230 nm, not available to us, while the other [8], based on derivatization with dansyl chloride, is extremely elaborate and lengthy. Therefore we developed a new HPLC procedure for the determination of tocainide. The method is based on pre-column derivatization of the drug with fluorescamine followed by HPLC analysis with fluorescence detection. The assay is sensitive, simple, and rapid, and may have wide applicability in the analysis of drugs and drug metabolites possessing a primary amino group.

EXPERIMENTAL

Chemicals

Fluorescamine, the internal standard, 2-amino-6′-chloro-*o*-acetotoluidide (ACAT) hydrochloride, and 2,6-dimethylaniline were purchased from Aldrich (Milwaukee, WI, U.S.A.). Sulfamethoxazole, 5-hydroxytryptamine, procainamide, and *p*-chloroamphetamine were obtained from Sigma (St. Louis, MO, U.S.A.). Phentermine was purchased from Applied Science Labs. (State

College, PA, U.S.A.). The following compounds were kindly donated: tocainide (Dr. Robert Ronfeld, Astra Pharmaceuticals, Worchester, MA, U.S.A.); amphetamine and *p*-hydroxyamphetamine (Smith, Kline and French Labs., Philadelphia, PA, U.S.A.); amantadine (E.I. DuPont de Nemours Co., Wilmington, DE, U.S.A.); 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM) (Dr. Neal Castagnoli, Jr., School of Pharmacy, University of California); N-desisopropylpropranolol (Dr. John Thompson, School of Pharmacy, University of Colorado). Thyroxine was obtained from the Central Laboratory, University of Colorado Health Sciences Center, and *p*-methoxyamphetamine was synthesized as described previously [10].

Acetonitrile and methanol, distilled-in-glass grade, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents used were of analytical grade quality.

Working solutions

Fluorescamine was dissolved in acetonitrile to give a concentration of 3 mg/ml. Human plasma samples containing tocainide at several concentrations in the range of 0.1—20 μg/ml were prepared by appropriate dilutions of a 1.00 mg/ml stock solution. Other compounds to be derivatized with fluorescamine were dissolved in acetonitrile at 20—30 μg/ml. The derivatization buffer was saturated sodium borate, pH 8.2. The internal standard solution contained ACAT in acetonitrile at a concentration of 7.0 μg/ml.

Apparatus and conditions

The liquid chromatograph consisted of a Waters (Milford, MA, U.S.A.) Model 6000A pump, a Waters Model U6K injector, and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 204A spectrophotofluorometer equipped with a microflow cell.

Separations were carried out on a Beckmann Instruments (Berkeley, CA, U.S.A.) Ultrasphere ODS 5-μm 150 mm × 4.6 mm reversed-phase column. Chromatograms were recorded using a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3380A electronic integrator.

The mobile phase was prepared by diluting 425 ml of 0.067 *M* phosphate buffer, pH 7.0, to 1000 ml with absolute methanol, and was pumped at a flow-rate of 1.3 ml/min. The column effluent was monitored fluorometrically at an excitation wavelength of 395 nm and emission of 485 nm.

Solutions of tocainide and the internal standard at 5.0 μg/ml concentration in borate buffer (pH 8.2) were used to obtain the fluorescence spectra. The instrument was a Perkin-Elmer MFP-44A fluorescence spectrophotometer, operated at a sensitivity setting of 0.3 with excitation and emission slits set at 3.

Assay procedure

The plasma sample (500 μl) was placed in a 5-ml polypropylene centrifuge tube. An equal volume of internal standard solution was added, followed by ca. 200 mg of sodium chloride—sodium carbonate (4:1). The tube was capped and vortex-mixed for 60 sec, followed by centrifugation at 1700 *g* for 10 min. A 200-μl aliquot of the supernatant was transferred to another 5-ml centrifuge tube and an equal volume of borate buffer (pH 8.2) was added.

While vortex-mixing the contents of the tube, 50 μl of fluorescamine solution was added and the mixing was continued for 30 sec. A 30—50 μl aliquot of the solution was injected into the chromatograph. If a derivatized sample was not injected within 10 min after derivatization the tube was kept in ice until injection to minimize fluorophore degradation.

Quantitative evaluation of assay data

The tocainide:ACAT peak-area ratios were used to construct standard curves. The slope, intercept, and correlation coefficient were determined by linear least-squares regression analysis. The data were also analyzed by linear least-squares regression through the origin.

RESULTS

Fluorescence of derivatives

Fig. 2 shows the fluorescence spectra of the fluorescamine derivatives of tocainide and ACAT.

Chromatography

Fig. 3a shows the chromatogram of the fluorescamine derivatives of tocainide and ACAT. Analysis of a plasma sample from a patient receiving tocainide gave the chromatogram in Fig. 3b.

Recovery studies

Plasma samples containing 10.0 μg/ml tocainide were analyzed and the peak areas obtained for tocainide compared to those obtained from analysis of

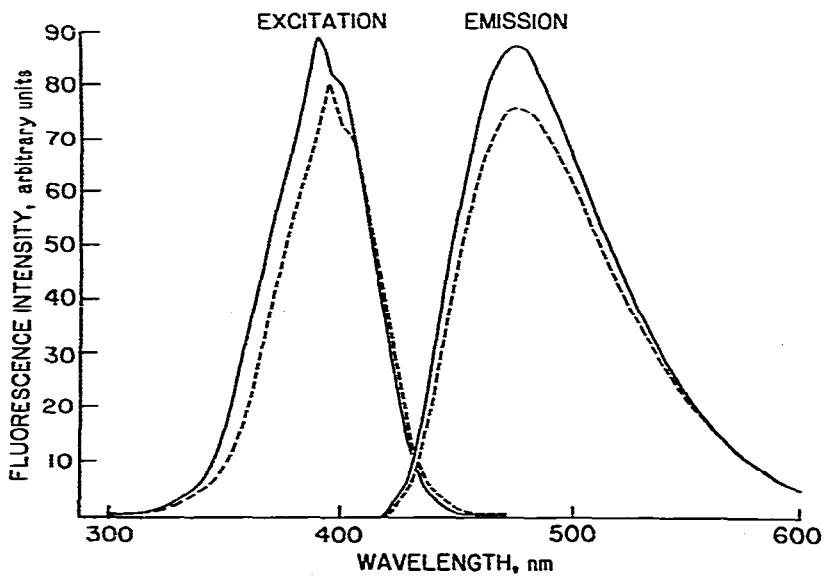

Fig. 2. Excitation—emission spectra of the fluorescamine derivatives of tocainide (——) and ACAT (– – –).

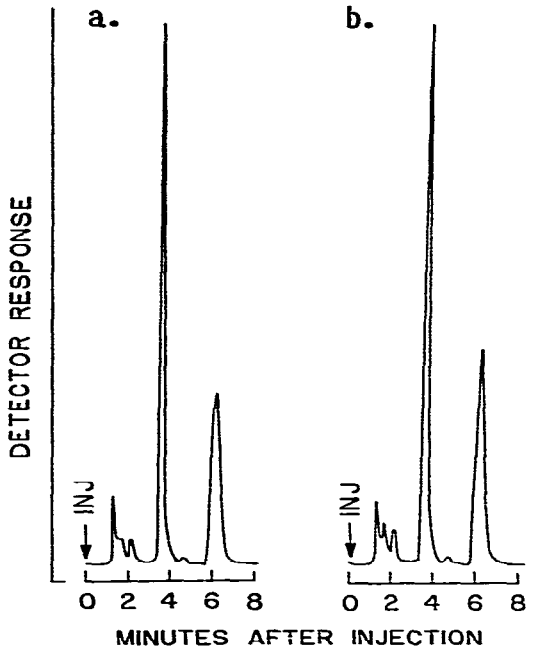

Fig. 3. Chromatograms of (a) tocainide and ACAT standards derivatized with fluorescamine; (b) derivatized extract of the serum of a patient receiving tocainide. Tocainide concentration 6.2 μg/ml. Retention times: ACAT, 3.4 min; tocainide, 6.0 min.

acetonitrile samples containing known, comparable, concentrations of the drug. The peak areas for ACAT were similarly analyzed. The recovery of both tocainide and ACAT was found to be 92 ± 5% (n=5).

TABLE I

TYPICAL CALIBRATION DATA*

Tocainide concentration (μg/ml)		Relative error (%)***
Amount added	Observed**	
0.100	0.109	+9.0
0.250	0.256	+2.4
0.500	0.514	+2.8
1.25	1.24	−0.8
2.50	2.52	+0.8
5.00	4.99	−0.2
10.0	9.85	−1.5
20.0	20.1	+0.5

*Least-squares line through origin; tocainide:ACAT peak area ratio = 168.1 (tocainide).
**From least-squares equation.
***[(observed − amount added)/amount added] × 100.

Assay linearity and precision

Standard curves were constructed by analyzing a series of plasma samples of known tocainide concentration. Each concentration was studied in triplicate. The data are given in Table I. The detector response was linear over the range of 0.1—20 μg/ml tocainide concentration. Linear least-squares regression analysis gave a line with an intercept which was not statistically different from zero. Therefore the standard curve through the origin was used in subsequent calculations.

Intra-assay precision was determined by analyzing a set of ten replicate plasma samples containing 0.25 μg/ml and a set containing 1.00 μg/ml tocainide. Inter-assay precision was determined by analyzing aliquots of a 1.0 μg/ml sample on ten separate days. The intra-assay coefficients of variation (C.V.) were 3.0 and 4.3% for the 0.25 and 1.00 μg/ml samples, respectively. The inter-assay C.V. was 3.6% for the 1.00 μg/ml samples.

Fluorescence intensity vs. pH of derivatization buffer

To determine the effect of pH in the derivatization medium plasma samples containing 1.0 μg/ml tocainide were analyzed using borate buffers of pH 7.5—10.0. The results are shown in Fig. 4. The effect of pH on the yield of the fluorescent derivatives of tocainide and ACAT is small.

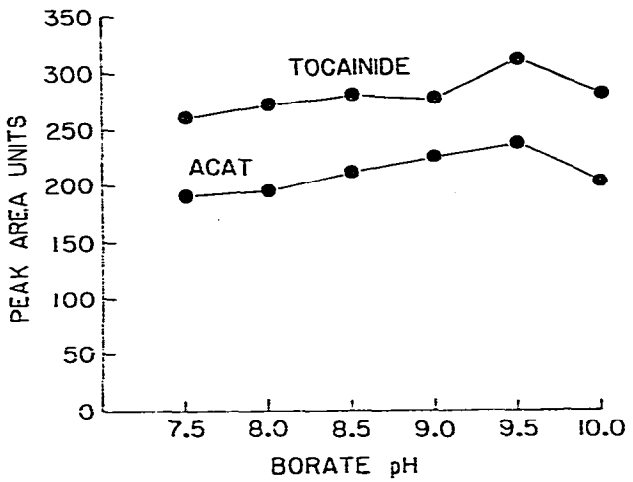

Fig. 4. Effect of pH of derivatization medium on the yield of the fluorescent derivatives of tocainide and ACAT. Each point is the mean of triplicate determinations.

Stability of fluorescence

Solutions of the fluorescamine derivatives of ACAT and tocainide were kept at room temperature and at 0°C to examine their stability. Several aliquots of each solution were injected into the chromatograph over a period of 5 h. A ca. 10% reduction in the tocainide peak area was observed at room temperature after 1 h. The rate of disappearance was slightly lower for ACAT, and increased with increasing pH for both derivatives. The decrease in peak area for both tocainide and ACAT was negligible at 0°C for 2 h.

Specificity

Serum samples containing no tocainide were carried through the analytical procedure without the addition of ACAT, and no interference from endogenous components was found. Propranolol, salicylate, and quinidine, all underivatized, gave no response. Dopamine, methyldopa, cycloserine, and tranylcypromine, all primary amines, gave no identifiable derivatives in the analysis. The hydrazine derivatives isoniazid, pheniprazine and 1,1-dibenzylhydrazine also gave no response. A series of other primary amines (Table II, see below) gave fluorescamine derivatives which did not interfere with the analysis of tocainide.

Fluorescamine derivatives of other drugs

A series of thirteen other primary amino compounds of pharmacological significance were derivatized with fluorescamine, and the derivatives were chromatographed under the conditions optimized for the derivatives of tocainide and ACAT. The results are shown in Table II. The chromatography

TABLE II

RETENTION TIMES OF THE FLUORESCAMINE DERIVATIVES OF SOME PRIMARY AMINE DRUGS AND DRUG METABOLITES

Chromatographic and derivatization conditions are given in the Experimental section.

Compound derivatized with fluorescamine (identification number)	Retention time of derivative (min)
Sulfamethoxazole (1)	1.5
5-Hydroxytryptamine (2)	2.1
Procainamide (3)	3.1
2,6-Dimethylaniline (4)	5.0
p-Hydroxyamphetamine (5)	5.6
Phentermine (6)	5.7
p-Methoxyamphetamine (7)	6.4
p-Chloroamphetamine (8)	8.8
Thyroxine (9)	8.8
N-Desisopropylpropranolol (10)	9.0
1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane (11)	9.1
Amphetamine (12)	11.8
Amantadine (13)	18.9

of the fluorescamine derivatives of N-desisopropylpropranolol (10) was also monitored at the excitation wavelength of 295 nm and emission of 350 nm, conditions suitable for the fluorometric analysis of propranolol and closely related compounds. No response was obtained.

DISCUSSION

Fluorescamine (4-phenylspiro(furan-2[3H],1'-phthalan)-3,3'-dione, Fig. 1) was synthesized ca. ten years ago [11]. This remarkable reagent, itself non-fluorescent, reacts rapidly with primary amines to give highly fluorescent

pyrrolinone derivatives (Fig. 1), and excess reagent is rapidly converted by water to non-fluorescent products [12, 13]. The reagent quickly found application in automated amino acid determinations [14, 15] and in protein [16] analysis. Subsequently, the use of fluorescamine was extended to a wide range of biophysical and biochemical applications. Fluorescamine has also been used in various analytical procedures for drugs. Interest in this area has produced detailed studies on the nature of the reaction of pharmaceuticals with fluorescamine and the factors affecting the reaction [17, 18]. It was shown [19] that conditions can be found for the selective reaction of aromatic amines with fluorescamine in the presence of aliphatic amines, and this finding prompted the development of an assay for procainamide in blood plasma [20]. Since this procedure does not involve chromatographic separation of the derivatives, interference from other aromatic amines may occur, and this was recognized by the authors [20]. Fluorescamine has been applied in the thin-layer chromatographic analysis of some therapeutic agents, drugs of abuse, and toxic substances [17, 18, 21–25].

Advances in HPLC in recent years have resulted in the use of fluorescamine to enhance the detection sensitivity of this technique. Analyses of antibiotics using post-column derivatization have been described [26—29], but pre-column derivatization of drugs followed by HPLC separation of the derivatives has received little attention. Only two reports have appeared on the pre-column derivatization and LC analysis of drugs. One of these described the quantitative determination of aminocaproic acid in serum [30], and the other concerns the analysis of the antidepressants clovoxamine and fluvoxamine in plasma [31].

For the determination of tocainide as its fluorescamine derivative by HPLC, ACAT (Fig. 1) was selected as the internal standard. This compound is a primary amine with a chemical structure closely related to that of tocainide. ACAT is readily available from a commercial source, is inexpensive, and has extraction and chromatographic properties compatible with those of tocainide.

The procedure developed for the isolation of tocainide and the internal standard from the biological fluid is based on saturation of the aqueous medium with sodium chloride—sodium carbonate and extraction into acetonitrile. This solvent is normally miscible with water, but becomes immiscible under the salting-out conditions used. This procedure, using different salt mixtures, was recently shown to have advantages in the isolation of several acidic and neutral drugs for HPLC analysis [32, 33]. With the incorporation of sodium carbonate the extraction of basic compounds was readily achieved in our procedure, and the recovery of tocainide and ACAT was nearly quantitative. The extraction procedure gave "clean" chromatograms, and appeared superior to the procedure used by De Jong [31], in which no extraction was performed and the serum samples were treated directly with fluorescamine. Significant interference from endogenous serum components was found under such conditions.

The extracts containing tocainide and ACAT were treated with fluorescamine in a procedure patterned after published derivatization methods [34, 35]. In our procedure, fluorescamine was added in acetonitrile instead of acetone [31, 34, 35]. Since the reaction between primary amines and fluores-

camine is extremely rapid [12], Schiff's base formation between the amine and acetone cannot compete with the derivatization reaction in most cases. Nevertheless, we selected acetonitrile as the solvent since it has been shown to be suitable for the derivatization [13] and since it was more convenient than acetone due to its lower volatility.

It has been suggested that pH 8—8.5 is optimal for the derivatization of aliphatic amines [13]. We examined the effects of pH in the derivatization medium on the fluorescence intensity of the tocainide and ACAT derivatives (Fig. 4). In the range of pH 7.5—10.0, the variation was small, with an apparent peak at pH 9.5. Since the decomposition of the fluorophores was more rapid at higher pH the derivatization pH selected was 8.2.

We recommend that the derivatization reaction be carried out immediately before HPLC analysis. The prepared derivatives may be stored, cooled in ice, for several hours. At room temperature the fluorescence intensity decreases significantly in 1—2 h. It has been stated that the fluorophores formed from primary amines and fluorescamine are stable for several hours [12], but a systematic study of their stability at neutral and alkaline pH has not been reported. Under sufficiently acidic conditions the fluorescence intensity of the derivatives decreases, and this has been shown to be the result of acid-catalyzed lactone formation between the hydroxy and carboxy groups of the derivatives [36].

Pre-column derivatization has significant advantages since it obviates the need for the more complex conditions and equipment required by the post-column derivatization procedures.

The fluorescamine derivatives were chromatographed on a reversed-phase ODS column, and gave good peak shapes (Fig. 3). The pH of the mobile phase was 7.0, a value compatible with both the stationary phase and the fluorescence properties of the derivatives [13]. The fluorescence spectra (Fig. 2) of the fluorescamine derivatives of tocainide and ACAT were very similar to those of the derivatives of other aliphatic primary amines [13, 17, 18]. Based on the spectra, 395 and 485 nm were selected as the excitation and emission wavelengths, respectively.

The product of the reaction of fluorescamine with racemic tocainide (Fig. 1) contains two asymmetric centers and, therefore, two diastereomeric derivatives are formed. There was no hint of separation of the diastereomers (Fig. 3). This may be due to unsuitable chromatographic conditions, or to a rapid epimerization around the carbinolamine chiral center [37].

A sample size of 0.5 ml is used in the assay procedure. If a smaller size is desired, the method may be scaled down, since only a small portion of the final derivatization mixture is injected into the HPLC. The procedure displayed good intra- and inter-assay reproducibility. Since the procedure is specific for primary amines, a variety of other, non-primary amine, drugs potentially present in patients receiving tocainide do not interfere. Amino acids are not extracted under the conditions used. Salicylic acid, a fluorescent compound, is also not extracted. Quinidine and propranolol do not interfere, since their fluorescence and chromatographic properties are different from those of the fluorescamine derivatives. In summary, the combination of specificity for primary amines, organic solvent extraction, specific fluorometry, and

chromatographic separation assures that many endogenous and exogenous compounds do not interfere. On the other hand, potential interference from primary amines must be carefully evaluated.

Therapeutically effective plasma concentrations of tocainide are in the 3.5—10 μg/ml range [2]. The lowest concentration of the drug to be measured in our pharmacokinetic studies is 100 ng/ml, and therefore the analytical procedure was not evaluated below this concentration. With appropriate modifications, e.g. concentration of the extracts, lower limits of sensitivity may be achieved. The procedure by De Jong [31], for example, is capable of determining the concentration of clovoxamine in blood plasma in the range of 10—1000 ng/ml, and has a limit of detection of 3 ng/ml. It must be noted, however, that the sensitivity attainable is also a function of the fluorescence quantum yield of the specific derivative.

Many therapeutic agents and other xenobiotic compounds are primary amines. Furthermore, many non-primary amine compounds are metabolized to primary amine derivatives via such biotransformations as N-dealkylation, reduction of azo, nitro, nitroso and hydroxylamino groups, and hydrolysis of amides, isocyanates and carbamates. Clearly, fluorescamine could play a significant role in the analysis of such metabolites. We have derivatized a series of pharmacologically significant primary amines with fluorescamine, and have chromatographed the derivatives (Table II). The chromatographic conditions were those used in the analysis of tocainide, and no attempt was made to optimize the chromatography for the compounds listed in Table II. Both aromatic (1, 3, 4) an aliphatic (2, 5—13) amines, including an amino acid (9) were studied. Several licit (5, 6, 8) and illicit (7, 11) derivatives of amphetamine (12) were included. 2,6-Dimethylaniline (4) is metabolite of lidocaine [38] formed by amide hydrolysis. N-Desisopropylpropranolol 10 is a N-dealkylated metabolite of propranolol [39]. The fluorescamine derivative of (10) was non-fluorescent at wavelengths normally used in the analysis of propranolol and its derivatives [40, 41]. It is clear that in drug disposition studies in which analytical specificity for a primary amine drug or drug metabolite is needed, precolumn derivatization with fluorescamine combined with liquid chromatography may have significant advantages.

1,1-Dibenzylhydrazine and the monosubstituted hydrazines isoniazid and pheniprazine were derivatized with fluorescamine, but derivatives could not be identified by HPLC with fluorescence detection. More unexpectedly, neither cycloserine nor tranylcypromine, both primary amines, gave an identifiable derivative. This may be due to unsuitable chromatographic conditions, although we believe this explanation is unlikely, since a wide variety of compounds gave derivatives readily chromatographed (Table II). Alternatively, the chemical structures of the two amines may give rise to derivatives with fluorescence properties different from those of other amines. It has been observed [17] that some primary amines produce non-fluorescent derivatives with fluorescamine. The catecholamines dopamine and methyldopa also failed to give identifiable derivatives. This failure was most likely due to the presence of borate in the medium, since it has been observed that borate suppresses the fluorescence of the fluorescamine derivatives of catecholamines [34].

In summary, the analytical method described possesses simplicity, speed,

sensitivity and specificity which make it very attractive for the analysis of tocainide, and, potentially, for many other primary amine drugs and drug metabolites.

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