limiting resistance to mass transport, the solution reduces to form where the mass released varies directly with time. Under these conditions, the release rate of a dispersed solute would become time independent.

The simplified model can be viewed as an extension of the familiar Higuchi model (1) for drug release from ointments and suspensions. In the region of small time, the conclusions presented here are in agreement with previous studies (12, 13). However, the application of this model is considerably simplified compared to the system presented previously (12, 13), which is more complex and results in equations that do not predict a simple relationship between the various parameters.

Experimental release-rate measurements have been conducted with monolithic systems where the particle size of the dispersed solute has been varied. The results can be adequately analyzed using the proposed mathematical model and indicate that the mass transport resistance offered by particle dissolution increases with increasing particle size. However, the release rate approaches a pseudo steady-state and becomes time independent with this increasing resistance offered to the mass transport process by particle dissolution. This information can be utilized in the design and development of controlled-release formulations.

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Determination of Enviroxime in a Variety of Biological Matrixes by Liquid Chromatography with Electrochemical Detection

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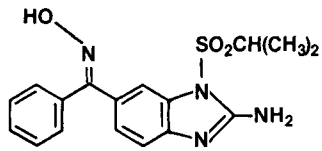
Abstract □ A simple and specific method has been developed for determination of enviroxime in biological samples. Enviroxime, a substituted benzimidazole, its coisomer zinviroxime, and the internal standard hexestrol were extracted from the samples with benzene. The benzene layer was evaporated and the residue was reconstituted and injected onto a liquid chromatograph. Reversed-phase chromatography on an octylsilane column with a 65% methanol–35% 0.14 *M* sodium acetate mobile phase separated the components. The compounds were detected electrochemically using a glassy carbon electrode held at +0.85 V. The assay could detect as little as 4 ng of enviroxime/ml of plasma, 15 ng/ml of nasal wash, and 20 ng/ml of urine or tissue homogenate. For plasma assays, the procedure was >97% accurate and had a relative standard deviation of <4%. This method has proven to be applicable and reliable for the determination of enviroxime in many types of biological samples. Several problems encountered during the routine use of electrochemical detection were explored and minimized.

Keyphrases □ Enviroxime—determination in a variety of biological matrixes by liquid chromatography with electrochemical detection □ Electrochemical detection—determination of enviroxime in a variety of biological matrixes by liquid chromatography □ Liquid chromatography—determination of enviroxime in a variety of biological matrixes with electrochemical detection

Enviroxime, anti-6-[(hydroxyimino)phenyl]methyl-1-(1-methylethyl)sulfonyl-1*H*-benzimidazole-2-amine (I), has been shown to be a highly specific inhibitor of the multiplication of rhinovirus in tissue cultures (1, 2):



I

Compound I has undergone extensive metabolic and toxicological studies in dogs and rats (3, 4) and is currently being evaluated as a treatment for the common cold (5).

In early work with I in dogs and mice, blood levels were determined using a plaque reduction assay. Plaque assays are nonspecific, since they are capable only of determining antiviral activity. To determine levels of I in the presence of the less active syn–oxime isomer, zinviroxime (II), it was necessary to develop a chemical assay. Initial experiments aimed at developing a GC assay for I and II indicated that such an approach was undesirable. Derivatization of the oxime group was required to make I and II volatile enough. However, such a derivatization eliminated the hydrogen bonding capability of this group and, thus, made the separation of I and II quite difficult. Recently, a method was reported which used high-performance liquid chromatography (HPLC) with UV detection for the determination

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of several benzimidazoles in body fluids (6). However, UV detection did not have the necessary sensitivity or specificity for the determination of I at nanogram levels in biological media.

This paper describes a sensitive and selective determination of I and II in biological fluids and tissues which is based upon HPLC with electrochemical detection. This method has proven to be extremely versatile and reliable during several years of use.

EXPERIMENTAL

Reagents—Enviroxime, *anti*-6-[(hydroxyimino)phenyl]methyl-1-(1-methylethyl)sulfonyl-1*H*-benzimidazole-2-amine (I), zinviroxime, *syn*-6-[(hydroxyimino)phenyl]methyl -1- (1-methylethyl)sulfonyl-1*H*-benzimidazole-2-amine (II), and hexestrol (III), were used as received[1]. All organic solvents were distilled in glass[2]. Distilled deionized water was used for preparation of the mobile phases. Blank plasma was separated from fresh heparinized blood of animals or human volunteers. The plasma samples were stored at 4° and used within 1 month. All other reagents were analytical grade. A stock solution of I and II was prepared fresh daily. One milligram of each isomer was weighed into a 10-ml volumetric flask and diluted to volume with methanol. These solutions were then diluted 1:100 with methanol–water (50:50) to give final concentrations of 1 μg/ml.

Equipment—The HPLC system consisted of a solvent delivery pump[3], an autoinjector[3], a guard column[3] packed with pellicular packing[4], a 250 × 4.6-mm column packed with 6-μm octylsilane reversed-phase material[5], a column temperature controller[6], and an electrochemical detector[6]. Detector response was recorded on a strip chart recorder and monitored by a central chromatographic data acquisition computer system. The detector potential was maintained at +0.85 V *versus* an Ag–AgCl–3 *M* NaCl reference electrode. The mobile phase consisted of methanol and 0.14 *M* sodium acetate (65:35) with 3 mg of disodium edetate added per liter. This mixture was filtered through a 0.2-μm pore nylon membrane filter[7] and deaerated by sonicating under vacuum. The flow rate was set at 0.9 ml/min, and the column temperature was maintained at 28°.

Some determinations required the addition of column-switching equipment. For this purpose a pneumatically actuated switching valve[8], which had a high-performance 3-cm reversed-phase precolumn[8] instead of a sample loop, was interposed between the automatic injector and the guard/analytical column. A second HPLC pump[9] was connected to this valve. Connections were made so that with the valve in one position the precolumn was in line with the main HPLC system, and in the other position it was connected to the auxiliary pump and to waste. The position of this valve was controlled by a digital timer[10] and a conventional electric solenoid. The timing was set so that the precolumn was in line for 40 sec, and then was out of line for the remainder of a chromatogram. During the latter time the auxiliary pump was turned on so that the mobile phase backflushed the precolumn at 2 ml/min.

Sample Preparation—A 1.0-ml or smaller sample of each plasma, urine, nasal washing, bile, or tissue homogenate was transferred into an individual tube. If necessary the pH was adjusted to ~7.5 by addition of dibasic potassium phosphate. Eleven and a half milliliters of benzene was added and the tubes were capped and rotated at 60 rpm for 40 min. After briefly centrifuging, as much as possible of the benzene was transferred to a second tube. The benzene layer was washed with 0.5 ml of 1 *M* potassium phosphate, pH 11.5. Ten milliliters of benzene was removed to a third tube, 0.1 ml of a 500-ng hexestrol/ml of chloroform solution was added, and the benzene was evaporated at 37° under nitrogen. The residue was reconstituted over a 15-min period with 200 μl of mobile phase. A 110-μl sample was injected onto the HPLC using injection vials equipped with microsample inserts[11]. For relatively interference free samples (plasma and nasal washings), the internal standard was added at the beginning and the pH 11.5 wash step was omitted. Conjugates of I and II were determined indirectly by overnight incubation of the samples at pH 4.0 and 37° with 10 μl of a glucuronidase–sulfatase preparation[12].

Table I—Recoveries of Drugs after Benzene Extraction

	% Recovery as Compared with Nonextracted Standards			
	Water[a]		Plasma[b]	
Compound	200 ng/ml	20 ng/ml	200 ng/ml	20 ng/ml
I	92	91	89	84
II	93	90	89	87
III	97	94	80	70

[a] $N = 5$. [b] $N = 10$.

The concentration of the drugs in the samples was calculated by comparison with calibration curves which were prepared by adding known amounts of drug to the appropriate blank media. Standards were extracted and chromatographed together with the samples. Typical calibration curves for plasma consisted of 5 points over the 20–100 ng/ml range. A linear least-squares analysis of the concentration *versus* peak height ratio (compound–internal standard) data was used to calculate the amount of I and II in the unknowns.

Electrochemistry—Cyclic voltammetry was performed using a conventional cell and electronics[13] with a glassy carbon working electrode. Chromatographically assisted hydrodynamic voltammetry was done as previously described (7).

RESULTS AND DISCUSSION

Choice of Assay Parameters—A variety of solvents were evaluated for their ability to efficiently extract I from water and plasma. Butyl chloride and hexane provided clean plasma extracts but did not extract the drug well. Ether and methylene chloride extracted I nearly quantitatively, but they also extracted a large number of endogenous components which interfered with subsequent chromatographic determination of I and II. Benzene and chloroform extracts of plasma were relatively clean, and the recovery of I and II was good. Benzene was chosen as the solvent for extractions since it is less dense than water, which eased solvent transfer, and because it extracted less of a late-eluting interference than did chloroform. It was found that toluene might be substituted for benzene, but lower recoveries are obtained and evaporation of solvent extracts requires twice as much time.

Extractions of I, II, and III from water into benzene were carried out over a wide pH range. Little or no effect of pH was noted in the 3–11.5 range. Extraction efficiency of all three compounds decreased significantly at >pH 11.5, as did the efficiency of extraction of I and II at <pH 2.0. A pH of 7.5 was used for convenience and to minimize coextraction of acidic or basic endogenous species from biological samples. The recovery of I, II, and III from both water and plasma at pH 7.5 is given in Table I. No difference in extraction efficiency was observed for I and II at 20 ng/ml *versus* 200 ng/ml. In contrast, the extraction efficiency of the internal standard (III) from plasma was lower at the lower concentration. To minimize the contribution of III to assay variability, ≥50 ng was used in this assay. Not surprisingly, the recovery of I and II from plasma was slightly lower than from water.

The good recovery obtained with a single extraction of I and II from biological matrixes allowed the addition of a backwash step to the procedure for urine and tissue extracts. Washing with pH 11.5 buffer presumably removed phenolic compounds which are coextracted at pH 7.5 and which generally are electrochemically active at the oxidation potential used. This removal of interferences more than compensates for any loss of drug during the backwash. Since the extraction efficiency for the internal standard (III) was less than for I and II, when the backwash was included, III was added after the backwash step.

The electrochemistry of I and II was explored initially by cyclic voltammetry. The cyclic voltammogram for I in mobile phase (Fig. 1) showed a nonreversible oxidation with a maximum occurring at ~ +0.8 V. The voltammogram for II was very similar to that for I. When reexamined in 67% 2-propanol–33% 0.3 *M* $HClO_4$, where wave definition is generally

[1] Synthesized by Lilly Research Laboratories, Indianapolis, Ind.
[2] Burdick and Jackson, Muskegon, Mich.
[3] Model 6000A, WISP 710B, and Guard Column, Waters Associates, Milford, Mass.
[4] CO:PELL ODS, Whatman Co., Clifton, N.J.
[5] Zorbax C-8, DuPont Instruments, Wilmington, Del.
[6] Models LC-22/23 and LC-4 with a TL-5 (glassy carbon) or TL-5A detector cell, Bioanalytical Systems, W. Lafayette, Ind.
[7] Rainin Inst. Co., Woburn, Mass.
[8] Models 7010 and 7001, and Brownlee guard column (C2-GU), Rheodyne, Cotati, Calif.
[9] Model 396, Laboratory Data Control, Riviera Beach, Fla.
[10] LC-24, Bioanalytical Systems, W. Lafayette, Ind.
[11] Kew Scientific, Columbus, Ohio.
[12] Glusulase, Endo Laboratories, Garden City, N.Y.
[13] CV-5, Bioanalytical Systems, W. Lafayette, Ind.

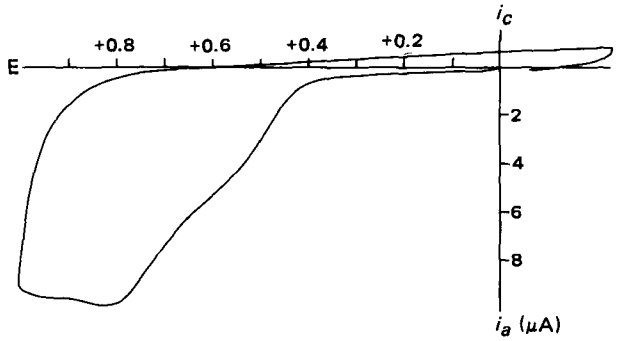

Figure 1—*Cyclic voltammogram of enviroxime. Conditions: 1 mM enviroxime in 33% 0.1 M sodium acetate–67% methanol, 3 mm diameter glassy carbon electrode, 50 mV/sec., E versus (Ag–AgCl–3 M NaCl), scan initiated positive from 0.0 V.*

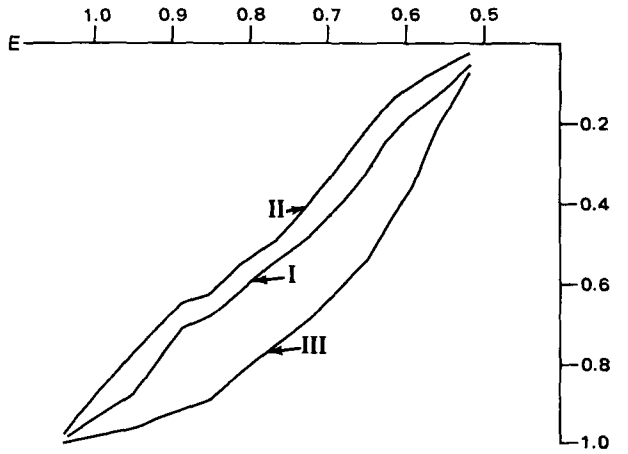

Figure 2—*Hydrodynamic voltammograms under assay conditions. Auxiliary electrode was across the channel from the working electrode. Key: (ϕ) ratio of peak height at a given potential to peak height obtained at greatest potential.*

better, both I and II showed several oxidation waves. The initial oxidation is thought to involve loss of two electrons from the extended conjugated system consisting of the oxime, benzimidazole, and the 2-amine group. The analog of I lacking the 2-amine group was not oxidizable, and the initial oxidation of the analog with a ketone instead of the oxime was 280 mV higher.

The voltammetry of I, II, and III under actual chromatographic conditions is depicted in Fig. 2. These curves were obtained with the auxiliary electrode positioned across the thin layer channel from the working electrode. This configuration minimizes uncompensated resistance between the two electrodes as well as potential gradients across the working electrode and thus allows accurate potential control under conditions of high background current. For all other work described in this paper, the auxiliary electrode was located downstream in the reference compartment. With such a configuration the hydrodynamic voltammograms began to drop off around +1.0 V. It can be seen from Fig. 2 that the voltammetric properties of III are reasonably similar to those of I and II, which is a desirable property for an internal standard. The potential used in this assay (+0.85 V) was chosen as a compromise between the desire for increased response to I and II, and the desire for low noise and a minimum of interferences from the biological matrixes.

The influence of chromatographic flow rate on the peak height of I is shown in Fig. 3. Peak heights normally increase as the flow rate is increased, due to decreased bandspreading. This is more than offset here by the decreasing efficiency of electrochemical conversion of I at higher flow rates. The flow rate chosen for use in this assay (0.9 ml/min) is a compromise between the demands of sensitivity and the need for reasonable retention times.

Assay Characteristics—The method described here was successfully applied to many different types of biological samples. Typical chromatograms from the assay of plasma, urine, and nasal turbinates are shown in Fig. 4. Compounds I and II (t_R ~ 8 and 9 min, respectively) were well resolved from each other and from major coextracted interferences. The small peak observed in the blank plasma chromatogram has a retention time close to but not identical to that of II. The chromatogram depicted represents a worse case situation, this peak not always being observed. Chromatograms of different blank tissue homogenates or urine looked different from each other and from blank plasma, so that A may not be compared against C or D. Although III elutes relatively late (t_R ~ 18 min), an alternative internal standard was not sought, because the region between 10 and 18 min occasionally contained interfering peaks.

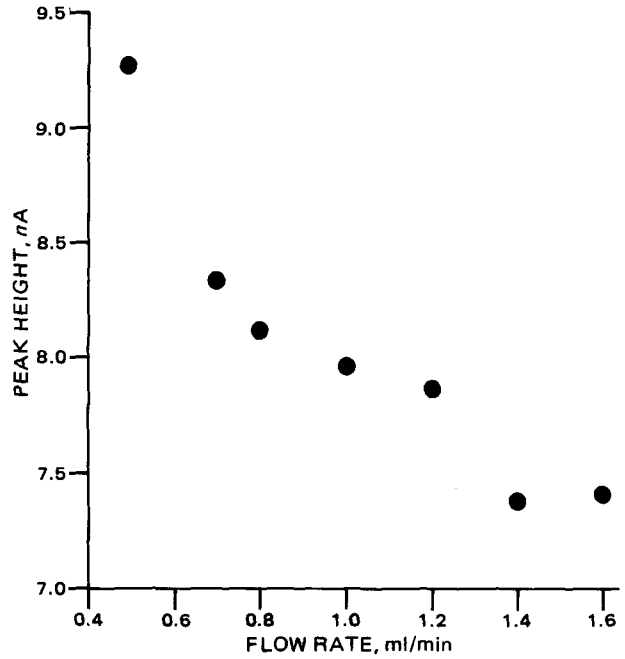

Figure 3—*Effect of flow rate on response to enviroxime. Chromatographic conditions as in text.*

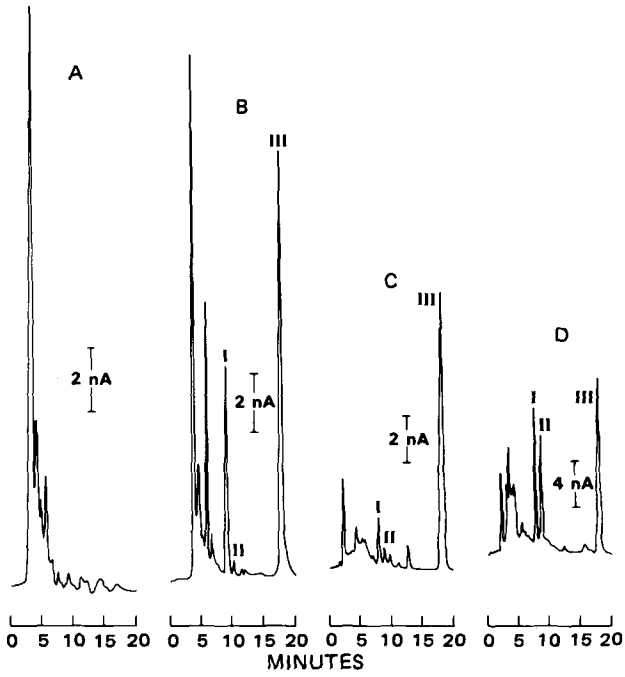

Figure 4—*Chromatograms of extracts of biological samples. Key: (A) predose dog plasma (without III added); (B) 80-min postdose plasma from dog administered 8 mg of I/kg orally, represents 43 ng of I/ml and 9 ng of II/ml; (C) homogenate of nasal tissue from dog administered 80 mg of II/kg for 90 days, represents 144 ng of I/g and 72 ng of II/g; (D) 0–2 hr urine of patient administered 25 mg of I orally, represents 1.6 μg of conjugated I/ml and 1.7 μg of conjugated II/ml.*

Table II—Precision and Accuracy of the Determination of Enviroxime in Human Plasma

Nanograms of Enviroxime per milliliter	Plasma Spiked with Enviroxime[a]		
	25	50	100
Day 1			
$\bar{x}$, ng/ml	25.1	50.4	99.8
RSD, %	1.8	2.0	1.1
Day 2			
$\bar{x}$, ng/ml	25.1	48.2	95.9
RSD, %	2.5	3.1	1.5
Day 3			
$\bar{x}$, ng/ml	24.8	48.7	95.4
RSD, %	5.6	0.6	0.6
Overall Accuracy			
$\bar{x}$, ng/ml	25.0	49.1	97.1
n	23.0	24.0	23.0
Relative Error	0.0	−1.8%	−2.9%
Overall Precision			
Between-day *RSD*, %	—	1.6	1.9
Within-day *RSD*, %	3.6	1.5	0.8
Total *RSD*, %	3.6	3.1	2.7

[a] $N = 8$.

The assay was found to be linear over the 5–800 ng range of I and II per milliliter of sample. The nonlinearity observed at >800 ng/ml was probably due to uncompensated resistance in the detector cell, a situation that occurs when large amounts of analyte are injected and/or when mobile phases of low conductivity are used. Alternative cell configurations could alleviate this problem (8). Instead, smaller volumes (<1 ml) of sample were used. This simple change kept all samples within a single sensitivity range (20nA/V), minimized electrode coating, and preserved samples.

The precision and accuracy of the determination of I in human plasma are summarized in Table II. Plasma samples spiked with 25, 50, and 100 ng of I and II per milliliter were prepared and eight aliquots were assayed on each of 3 days. The results in Table II show the precision and accuracy of the determination of I to be excellent, with both within-day and between-day variability contributing approximately equally to the overall precision. The determination of II was about one half as precise, with the largest variation observed being 7.4% *RSD* for the 25-ng/ml samples. Roughly 75% of the total *RSD* for II was attributable to between-day variability. Calibration curves were normally prepared with equal amounts of I and II, as in this characterization. However, when curves were prepared for each isomer individually, no significant interconversion of I and II was noted during the assay.

The precision of the determination of conjugated I and II in urine, which involves enzymatic cleavage of the conjugates, was estimated by assaying four aliquots of each of six urine samples on each of 3 days. The mean concentrations of total I and II ranged from 200 to 3600 ng/ml. The overall precision, as measured by the average of the *RSD*s of the individual samples was 9% for I and 11% for II. The proportion of the variability accounted for by between-day variation ranged from 68–96% for I and 50–90% for II. The reason for this day-to-day variation could not be determined, but the overall precision is quite reasonable.

The maximum sensitivity attainable with this method was not limited by detector noise. Trace levels of interferences defined detection limits in all of the many media studied. As little as 4 ng of I per milliliter of plasma, 15 ng/ml of nasal wash or 20 ng/ml of urine or tissue homogenate could be determined. Detection limits for II were always slightly higher than for I due to the lower response of the electrochemical detector to II (Fig. 2).

Improved Reliability of Electrochemical Detection—During the initial development of this assay, the background current at the glassy carbon working electrode oscillated erratically and on the whole increased continually. Some of the oscillations appeared to be very late-eluting peaks, but their locations could not be correlated with the timing of injections. Because disodium edetate is frequently used to stabilize easily oxidized species, such as penicillamine and catecholamines, it was added to the mobile phase used for I and II. Subsequently, the background current became quite stable. The mechanism of this phenomenon is as yet unknown. As a result of long experience with this method, several additional problems were encountered which could be traced to the electrochemical detector.

When samples containing high concentrations of I and II were assayed, it was noted that the peak height of III, which should remain essentially constant, decreased during the chromatography of a single set of samples. Further investigation revealed that the sensitivity of detection of I and II decreased also. Sensitivity could be regained only by repolishing the electrode, indicating that coating or fouling was involved. Phenols are known to polymerize at electrode surfaces (9), and indeed the problem was traced to the use of higher concentrations of internal standard, III, with these samples. In a separate experiment, it was found that when 1 μg of I and III was repeatedly injected, the peak height of I decreased by 14% per injection. When I alone was injected, its peak height decreased by only 0.4% per 1 μg of injection. Thus, the coating is predominantly due to the biphenolic internal standard. With a much lower amount of I, II, and III (40 ng each), the observed decrease in peak height of I and II was 0.1% per injection. To minimize this coating, the amount of III injected onto the column was subsequently kept at or below this level.

Impurities present in the mobile phase can also contribute to electrode coating. Using reagent grade methanol[14], 50% of the sensitivity of a freshly repolished electrode was lost after several days. A grade of methanol described as having been distilled in glass[15] was better but still caused decreased sensitivity when compared with the methanol actually used. This assay is probably particularly sensitive to coating effects because of the high potential required for oxidation of I and II, and because even at +0.85 V, peak heights of I and II are still increasing with increasing potential (Fig. 2). Nonetheless, caution in the preparation of mobile phases for use with electrochemical detection is indicated.

When overnight automated runs were made, the peak heights of I, II, and III in spiked samples were observed to decrease by 5–15% during the late evening and then return to normal the following day. Since this behavior paralleled the cycle of ambient temperature, it was assumed to be due to changes in chromatographic retention time. Thermostating the column eliminated changes in retention and decreased but did not eliminate changes in peak height. The majority of this remaining variability in I and half that of II was compensated for by corresponding changes in III. A thorough description of subsequent investigations of the origins and minimization of temperature-induced variability in electrochemical detection is the subject of a separate report (10).

Late-eluting peaks are strongly retained components from one injection, which elute during subsequent chromatograms. When samples are injected by hand, the time between injections varies and the source and effect of late-eluting peaks are not always obvious. However, when an autosampler is used, the time between injections is reproducible and the position of late-eluting peaks becomes constant, thus making them easier to identify. Determination of I and II in several of the biological media assayed resulted in problems with late-eluting peaks. Dog and rabbit liver homogenates and rabbit lung homogenates were particularly troublesome.

In some simpler cases it is possible, with the proper timing, to cause a late-eluting peak to elute with the solvent front or at some other noncritical time in a subsequent chromatogram. In more complex cases, one may simply delay the next injection until the late-eluting peaks are off the column, but this approach prolongs chromatographic time unduly. A slug or gradient of organic eluent can speed up their elution, but extra time is then required for re-equilibration of the column and the detector.

An alternative approach to removing late-eluting peaks is to use column switching (11). For the determination of I a 3-cm reversed-phase precolumn was positioned between the injector and the analytical column. The precolumn was in line for 40 sec after injection, which is the time required for I, II, and III to traverse the precolumn and enter the main column. The precolumn was then switched out of line for the remaining assay time. While out of line, the mobile phase was pumped through it at 2.0 ml/min to wash strongly retained components to waste. This technique produced cleaner chromatograms, which improved quantitation, and increased sample throughput as compared with the other approaches discussed above. Interestingly, when column switching was used with other relatively clean matrixes, *e.g.*, dog plasma and nasal washings, fewer trace level substances interfering with I and II were seen. The resulting improvement in detection limits was unexpected, since the interference peaks that were eliminated had been so small that they could not be distinguished as being late-eluting peaks.

Assay Applications—The method described above has been used to determine I and II in numerous biological samples arising from toxicology, pharmacology, metabolism, formulation, dose ranging, and clinical studies. One of the many types of results generated is depicted in Fig. 5. In this typical concentration–time profile for I in dog plasma, I was de-

[14] Reagent Grade ACS., MCB Manufacturing Chemists, Cincinnati, Ohio.
[15] HPLC Grade, Tedia Company, Fairfield, Ohio.

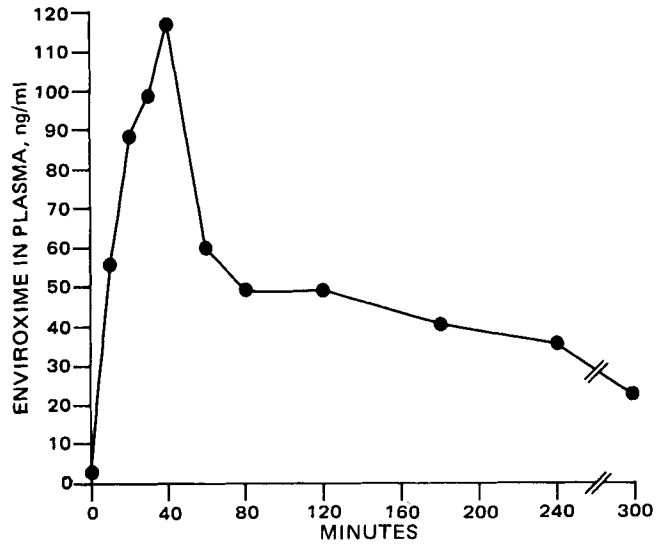

Figure 5—*Enviroxime concentration in dog plasma (ng/ml) after oral administration of an 8-mg/kg dose.*

terminable as early as 10 min after oral dosing to as long as 5–8 hr post-dose. A second example illustrates the value of a chemical assay, as compared with the original plaque reduction assay. The amount of II found in the plasma of dogs following oral dosing with I was usually very small, between 5 and 10% of I. However, when II was administered orally to dogs, I was still the major peak found in plasma. Thus, either by metabolism or by acid-catalyzed isomerization in the GI tract, the two isomers tend to form an equilibrium mixture in the plasma, in which I predominates.

The proven versatility of the methodology here points out the power of the coupling of liquid chromatography with electrochemical detection for determining oxidizable drugs in biological matrixes.

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NOTES

Antihypertensive Agents: Pyridazino(4,5-b)indole Derivatives

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Abstract □ A series of 4-hydrazino-5H-pyridazino(4,5-b)indoles (VIII) and their potential metabolites (3,4-dihydro-4-oxo-5H-pyridazino(4,5-b)indoles (V) and 11H-1,2,4-triazolo(4,3-b)pyridazino-(4,5-b)indoles (IX) were investigated for antihypertensive activity in spontaneously hypertensive rats. All compounds showed antihypertensive activity at 25 mg/kg ip. Compound VIII was the most active, and the most toxic.

Keyphrases □ Antihypertensive agents—synthesis of pyridazino-(4,5-b)indole derivatives, rats, metabolites □ Metabolites—synthesis of antihypertensive agents, pyridazino(4,5-b)indole derivatives, rats □ Pyridazino(4,5-b)indole—derivatives, synthesis of antihypertensive agents, hypertensive rats

In connection with work related to the preparation and study of new products as potential antihypertensive agents, a series of pyridazino(4,5-b)indole derivatives (1–3) related to the well-known antihypertensive agent hydralazine[1] (I), as well as its metabolites (4, 5) were synthesized. The metabolism of these compounds takes place essentially (5, 6) through N^2-acylation (formyl, acetyl) of the hydrazino group and further cyclization to triazole derivatives (II), as well as the hydrolysis of the hydrazino to give the oxo derivatives (III).

The results of a preliminary evaluation in spontaneously hypertensive rats of Compounds V, VIII, and IX (see Scheme) are reported. All of these compounds are structurally related to hydralazine (I)[1] and its metabolites.

Some preliminary data for Compound VII*a* in normotensive and hypertensive (renal fistula), anesthetized

[1] Apresoline (1-hydrazinophtalazine).

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