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Traffic Safety
Administration

Analysis for Drugs In Saliva and Breath

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16. Abstract

Collection devices for saliva and breath that involved non-invasive techniques for sample collection were evaluated. Having subjects simply spit into a specially prepared glass vial was found to be an efficient, inexpensive and simple way to collect saliva. The device for trapping drugs in breath was efficient, but limited to laboratory use.

Procedures for the extraction and analysis of secobarbital, amphetamine, chlorpromazine, diazepam, diphenhydramine and codeine from plasma and saliva were developed or validated. Procedures for the analysis of the two drugs (secobarbital and amphetamine) from those listed above that were deemed most likely to be excreted in breath were also developed.

Validation of these procedures was accomplished from samples obtained following administration of single doses of the drugs in the therapeutic or subtherapeutic range to young adult male volunteers.

No measurable quantities of drugs were found in most breath samples following administration of secobarbital or amphetamine. Correlations between concentrations in plasma and saliva of secobarbital, diazepam, diphenhydramine, and codeine were sufficiently good to permit the use of saliva samples for survey purposes, but not sufficiently precise to permit the evidential use of the results from a single sample, although calculation of approximate plasma concentrations of secobarbital from single saliva samples taken >0.5 hr after drug administration was possible. The use of the concentration of a drug in saliva to determine its concentration in plasma at some earlier time is not possible.

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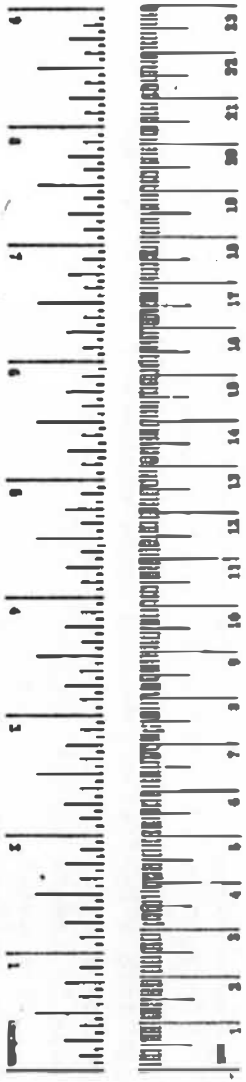
METRIC CONVERSION FACTORS

Approximate Conversions to Metric Measures

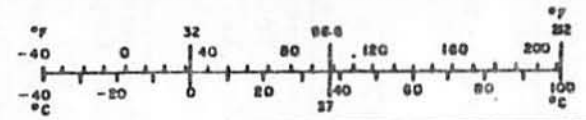
Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
in	inches	*2.5	centimeters	cm
ft	feet	30	centimeters	cm
yd	yards	0.9	meters	m
mi	miles	1.6	kilometers	km
		AREA		
in^2	square inches	6.5	square centimeters	cm^2
ft^2	square feet	0.09	square meters	m^2
yd^2	square yards	0.8	square meters	m^2
mi^2	square miles	2.6	square kilometers	km^2
	acres	0.4	hectares	ha
		MASS (weight)		
oz	ounces	28	grams	g
lb	pounds	0.45	kilograms	kg
	short tons (2000 lb)	0.9	tonnes	t
		VOLUME		
tsp	teaspoons	5	milliliters	ml
Tbsp	tablespoons	15	milliliters	ml
fl oz	fluid ounces	30	milliliters	ml
c	cups	0.24	liters	l
pt	pints	0.47	liters	l
qt	quarts	0.95	liters	l
gal	gallons	3.8	liters	l
ft^3	cubic feet	0.03	cubic meters	m^3
yd^3	cubic yards	0.76	cubic meters	m^3
		TEMPERATURE (exact)		
°F	Fahrenheit temperature	5/9 (after subtracting 32)	Celsius temperature	°C

*1 in = 2.54 (exactly). For other exact conversions and more detailed tables, see NBS Misc. Publ. 286, Units of Weights and Measures, Price $2.25, SD Catalog No. C13.10:286.



Approximate Conversions from Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
mm	millimeters	0.04	inches	in
cm	centimeters	0.4	inches	in
m	meters	3.3	feet	ft
m	meters	1.1	yards	yd
km	kilometers	0.6	miles	mi
		AREA		
cm^2	square centimeters	0.16	square inches	in^2
m^2	square meters	1.2	square yards	yd^2
km^2	square kilometers	0.4	square miles	mi^2
ha	hectares (10,000 m^2)	2.5	acres	
		MASS (weight)		
g	grams	0.035	ounces	oz
kg	kilograms	2.2	pounds	lb
t	tonnes (1000 kg)	1.1	short tons	
		VOLUME		
ml	milliliters	0.03	fluid ounces	fl oz
l	liters	2.1	pints	pt
l	liters	1.06	quarts	qt
l	liters	0.26	gallons	gal
m^3	cubic meters	35	cubic feet	ft^3
m^3	cubic meters	1.3	cubic yards	yd^3
		TEMPERATURE (exact)		
°C	Celsius temperature	9/5 (then add 32)	Fahrenheit temperature	°F



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1.0 Background Information

1.1 Binding of Drugs to Protein

The binding of small molecules by proteins has been known for many years and extensively studied. For comprehensive reviews, see Goldstein, 1949; Meyer and Guttman, 1968; Goldstein, et. al., 1974; Bridges and Wilson, 1976; Jusko and Gretch, 1976. Although drugs may be bound to extracellular macromolecular components of various organs, tissues and blood, it is the binding of drugs by plasma protein that is most prominent. By far the most important contribution to drug binding is made by albumin, the principal protein of plasma. Since protein configuration plays a key role in the binding phenomenom, there is wide variation in the extent to which drugs are bound. Some drugs, such as certain penicillin and tetracycline derivatives, are less than 10% bound, while others, such as Δ^9-tetrahydrocannabinol and diazepam, are more than 90% bound to plasma protein. The fraction of the drug that is bound to plasma protein can also change as the concentration of drug changes. One factor responsible for this is the saturation of certain protein binding sites as the concentration of drug increases. For this reason, the binding of a drug to plasma protein should be established over the drug concentration range of interest.

There is much evidence that the pharmacologic activity of drugs is a function of their free (unbound) concentration in plasma (Anton, 1960; Booker and Darcey, 1973; Yacobi and Levy, 1975; Shoeman and Azarnoff, 1975). This is also the only portion of the drug that can be secreted into saliva or undergo glomecular filtration for excretion by the kidney. The importance of knowing the binding of a drug to plasma protein in predicting the concentration of the drug in plasma from its concentration in saliva is discussed below.

1.2 Saliva

Saliva is formed from the secretions of glands in the oral cavity. These glands are the parotid, one on each side of the face below the ear, submaxillary (submandibular), principally in the floor of the mouth; sublingual, principally in the floor of the mouth; and buccal, scattered beneath the mucuous membranes of lips and cheeks. Salivary secretion is under nervous control, being reflexly initiated by mechanical, chemical, or radiant stimuli acting on taste buds in the mouth, olfactory receptors, visual receptors, or other sense organs. Secretion may also occur as a result of conditioned reflexes, as when one thinks about food.

Saliva is a complex variable mixture containing more than 99 percent water, together with polysaccharides, proteins, salivary amylase, inorganic ions, sloughed epithelial cells, disintegrating leukocytes and small organic molecules, including drugs, that are circulating in plasma. The composition of parotid saliva has been shown to vary with flow rate (Beal, 1979). Circadian rythms have also been demonstrated in parotid, submandibular and whole saliva flow rates and composition (Ferguson and Botchway, 1980; Dawes, 1972, 1975; Dawes and Ong, 1973; Ferguson et al., 1973; Ferguson and Fort, 1974). The thin, watery type of saliva is produced by serious cells in the salivary glands and the thick, viscid type by mucous cells in the same glands.

The parotid gland and the submaxillary gland are the two primary potential glandular sources for the transport of drugs into saliva cavity and thereby the oral cavity. Approximately 30-60% of the volume of saliva originates from the serous cells in these glands (this primarily represents parotid secretion).

The parotid gland (and presumably the serous cells of the submaxillary gland, cf. Atta et al., 1975) respond to three different types of physiological stimulation. Alpha-adrenergic stimulation results primarily in the secretion of potassium and water; beta-adrenergic stimulation results in secretion of a protein-rich solution containing calcium and sodium, while cholinergic stimulation results in a secretion similar in composition to that produced after alpha-adrenergic stimulation.

In a study of the secretion of ascorbic acid in human saliva, Mäkilä and Kirveskari (1969) found that the rate of secretion of ascorbic acid averaged 0.14 μg/minute for the submaxillary and sublingual glands and 0.59 μg/minute for the parotid gland. Assuming that the secretion of the drugs studied in this project is similar to that for ascorbic acid, the parotid gland is the most significant source of drug entry into the oral cavity.

In addition to diffusive transport of compounds into the oral cavity, certain compounds such as the alkali earth metals undergo specific transport phenomena. There is no evidence to suggest that any of the compounds studied in this program would be subject to active transport. However, some of the compounds may have a direct pharmacologic action on the salivary gland effecting secretion. This may be particularly true with amphetamine, which is an alpha-andrenergic agonist.

1.3 Analysis of Drugs in Saliva

Saliva samples from race horses have been analyzed for many years to determine the possible presence of illicit drugs. Only recently, however, has much attention been given to the use of this fluid for drug level determinations in man. As analytical methodology such as radioimmunoassay and electron capture-gas chromatography (GC), which provides

assay capabilities at the nanogram level and below, has become available, the use of saliva for the determination of "biologically active" drug levels has grown increasingly popular.

If diffusion into saliva is essentially a passive process (Keen 1960), then the concentration of a drug in saliva should be principally a function of relative protein binding in plasma and saliva, the pH of the two fluids, and the pKa of the drug (Rasmussen, 1964). Since the mean protein content of saliva is only about 260 mg per 100 mL, a rough estimate of the amount of an unionized drug present in saliva is the amount of free drug present in plasma. The theoretical relationship between the concentration of an ionizable drug in plasma and its concentration in saliva can be expressed mathematically in the following derivations of the Henderson-Hasselbach equation (Dvorchik and Vesell, 1976; Martin, et. al., 1976):

For acidic drugs

$$Cp \times fp = \frac{1 + 10^{(pHp-pka)}}{1 + 10^{(pHs-pka)}} \times Cs \times fs$$

For basic drugs

$$Cp \times fp = \frac{1 + 10^{(pka-pHp)}}{1 + 10^{(pka-pHs)}} \times Cs \times fs$$

Where Cs = concentration of drug in saliva

Cp = concentration of drug in plasma

pka = pka of the drug

pHs = saliva pH

pHp = plasma pH, usually assumed to be 7.4

fp = fraction of drug not bound to protein in plasma

fs = fraction of drug not bound to protein in saliva, usually assumed to be 1.0.

The relation between saliva and plasma levels of diphenylhydantoin has been determined by a number of workers (Cook et al., 1975; Bochner et al., 1974; Troupin and Friel, 1974; Conrad et al., 1974; Zion et al., 1976). Cook found that a simple linear relationship existed between plasma and saliva levels with [saliva] = 0.1 [plasma]. This ratio is close to that reported for the ratio of free to bound drug in plasma at 37°C (Lunde et al., 1971).

Similar studies have been done with phenobarbital (Cook et al., 1975; Zion et al., 1976). For this drug, the relationship was not quite linear, but over the range of most clinical interest (10-60 μg/ml plasma) the saliva concentration could be approximated by [saliva] = 0.29 [plasma]. The concentration in saliva is less than predicted on the basis of the free fraction in plasma (Waddell and Butler, 1957), but is readily explained by the effect of the pH of saliva and plasma (ca. 6.5 and 7.4, respectively) and the pKa of phenobarbital. Piraino and Di Gregorio (1977) report the correlation of saliva and plasma levels of diazepam where [saliva] = 0.03 [plasma]. This indicates that diazepam is highly bound in the plasma and compares favorably with ratios of free/bound (0.05) from plasma protein binding studies. Mucklow, et al., (1978) present data on the correlation of a number of drugs. These authors conclude that good correlations exist between plasma and saliva concentrations of drugs that are largely nonionized at normal plasma pH (e.g., phenytoin, phenobarbital and antipyrine) while correlations are usually poor unless salivary flow rate and pH can be standardized for drugs that are largely ionized at normal plasma pH (e.g., propranolol, chlorpropamide, meperidine and tolbutamide).

1.4 Analysis of Drugs in Breath

Qualitative determinations of volatiles in breath go back many years with the "analyst's" nose being the detecting device (e.g., the acetone smell of a diabetic's breath, alcohol in an intoxicated person's breath). Examples of quantitative measurements of drugs from breath samples on the other hand are quite scarce. A notable exception to this is the well-known "breathalyzer" used routinely by law enforcement officials to determine alcohol levels in the body. Due to the high volatility and therefore relatively high levels of alcohol present in the breath of drinking drivers, the technology associated with these measurements is somewhat less demanding than that required for the measurement of breath levels of therapeutic drugs.

It was recognized early that in order to measure drug levels in breath a concentration of the volatile organics was necessary. Initial attempts to collect and concentrate the volatile organics present in namogram levels were mostly based on the work of Teranishi et al. (1972), who developed a coiled tubular cold trap collection device. The cold finger trap described in a report of the University of Missouri School of Pharmacy (DOT-HS-801-660) evolved from this device. Detection instrumentation for these studies were gas chromatographs coupled with flame ionization or mass spectral detectors.

Work at the University of Missouri (DOT-HS-820-253) produced a polyethylene foam wafer device which was used in the analysis of ethchlorvynol and chloral hydrate in breath, as well as in the detection of what were thought to be marihuana constituents. On the other hand, RTI experience has been that extremely "clean" collection devices are required when working at low nanogram levels. In particular, plastics frequently cause problems.

RTI has also developed techniques for the analysis of the first exhalation from human subjects after puffing a marihuana cigarette. These samples were collected on a Cambridge filter, extracted from the filter, and analyzed by gas liquid chromatography (Wall, 1976).

2.0 Goals, Objectives, Drugs and Challenges of the Program

2.1 Goals and Objectives

As stated in the RFP for this project, the primary objective of the study was to develop methods for using breath and saliva as biological samples to detect and quantify drug concentrations in drivers, and to be able, if possible, to infer previous levels of drug concentrations. For purposes of the study, these methods need not be developed for roadside application. The product of this project would be drug detection methods that are ready for operational use in future drug incidence research studies.

Specifically, it was the purpose of this study to develop practical operational methods, procedures, and equipment for the collection, extraction, identification, and quantification of selected drugs which are considered possible highway safety hazards in breath and/or saliva and to assess the feasibility of estimating the drug concentration at the time of an accident based on samples collected some time later. It was further stated that ultimately at least the collection of these samples and possibly the analysis would be performed by operational personnel and that the products of the project should reflect this goal.

The project was divided into four major tasks, each with a particular objective. The first was the preparation of a detailed study plan. This plan would include proposed methods of breath/saliva sample collection, sample extraction, and analysis; proposed procedures for validation of the developed drug analysis methods in human subjects, and possibilities of false negatives and false positives and how these will be detected and dealt with.

The second objective was the development of methods and procedures for sample collection and analysis. The third objective was the validation of the methods and procedures in human subjects. This would involve establishing a quantitative relationship between the concentration of the drug or the suitable breakdown product in breath and/or saliva to its concentration in blood, covering a range up to the normal clinical dosage.

2.2 Selection of Drugs For This Project

Six drugs were studied as part of this contract. Criteria used for the selection of these drugs included the following:

(a) The drug must be widely used and/or abused;

(b) The drug must represent a class of drugs which are known or suspected to affect driving performance;

(c) The drugs selected must collectively possess a broad range of physical/chemical characteristics such as melting point, vapor pressure at 37°C, molecular weight, lipophyilicity/hydrophilicity, protein binding, etc.

(d) Preliminary methodology should be available in the literature for the analysis of the drug at therapeutic levels.

The six drugs for study were selected by DOT with consultation by personnel from RTI and the National Institute on Drug Abuse and were:

Secobarbital (Seconal)

Amphetamine (Benzedrine)

Chlorpromazine (Thorazine)

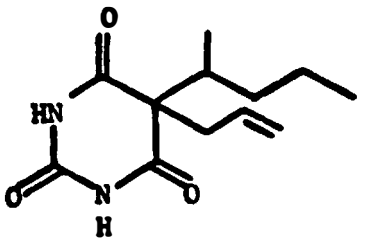
Diazepam (Valium)

Diphenhydramine (Benadryl)

Codeine

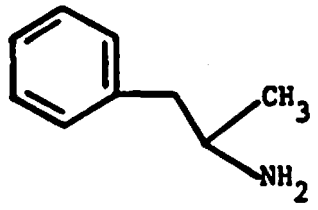
The structures and some properties of these drugs are shown in Figure 1.

Figure 1. Structures and Some Properties of Drugs Studied



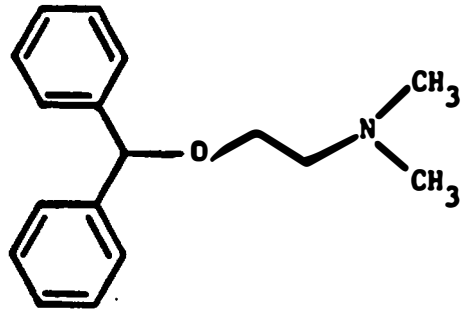
Secobarbital

Class: Barbiturate, hypnotic
Molec. Formula: $C_{12}H_{18}N_2O_3$
M.W.: 238
M.P.: 100°C
pKa: 7.90, acid
% B*: 67
Very soluble in water as its sodium salt.



Amphetamine

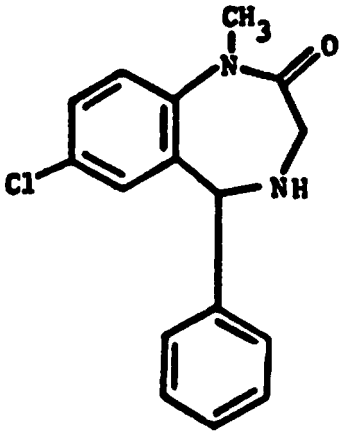
Class: CNS stimulant
Molec. Formula: $C_9H_{13}N$
M.W.: 135
B.P.: 82-85°C (13 torr); slightly volatile at room temperature
pKa: 9.95, base
% B: 13
Slightly soluble in water; soluble in organic solvents.



Diphenhydramine

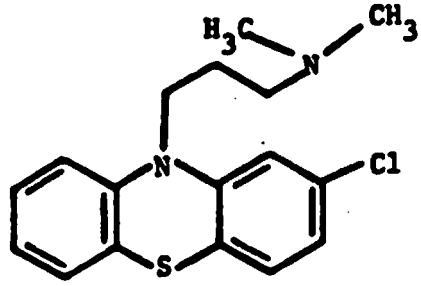
Class: Antihistamine
Molec. Formula: $C_{17}H_{21}NO$
M.W.: 255
B.P.: 150-165 (2.0 torr)
pKa: 8.3, base
% B: 72

Figure 1 (continued)



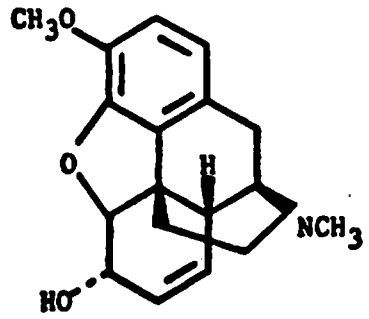
Diazepam

Class: Minor tranquilizer
Molec. Formula: $C_{16}H_{13}ClN_2O$
M.W.: 289
M.P. 125-126°C
pKa: neutral
% B: 98
Almost insoluble in water; soluble in organic solvents.



Chlorpromazine

Class: Major tranquilizer
Molec. Formula: $C_{17}H_{19}ClN_2S$
M.W.: 319
B.P.: 200-205 (0.8 torr)
pKa: 6.4
% B: ?, unstable in plasma



Codeine

Class: Narcotic analgesic
Molec. Formula: $C_{18}H_{21}NO_3$
M.W.: 299
M.P.: 154-156°C
Sublimes at 140°C/1.5 torr
pKa: 7.95
% B: 29
Moderately soluble in water; very soluble in organic solvents.

*% B = percent bound to plasma protein at therapeutic concentrations.

2.3 Challenges

The major challenge of this program was to develop analytical systems for measurement of the drugs in various biological fluids that were both sensitive and specific. The analytical systems used by most previous investigators were designed either to measure the concentrations of drugs in overdoses subjects or as a part of a clinical study where future interferences from other drugs was not encountered. The criteria used in development of our analytical systems were:

(1) The analysis had to be very sensitive so as to permit measurement of subtherapeutic concentrations of drug in both the plasma and saliva.

(2) The peaks from the chromatographic system had to be sharp so that maximum separation from other drugs that may be present in field samples could be achieved.

(3) Internal standards used in the analytical systems had to be appropriate for their purpose, but not be compounds in widespread use, as had been used in most previous studies.

(4) The procedures had to be adaptable for use for other closely related drugs.

(5) Where possible, the procedures were to utilize gas chromatography with a nitrogen-specific detector (This was requested by National Institute on Drug Abuse consultants to the DOT Scientific Project Officer).

(6) The procedures had to be usable by other investigators with current commercial equipment.

A second challenge was the development of a protocol for the human validation studies. This protocol had to be workable for the physician

and subjects involved, yet had to provide for proper controls in order to limit the number of variables in the study.

A final challenge was to try to develop methods to correlate the results of the human validation study with each drug in order to establish if a correlation existed between the concentrations of the drug in plasma, saliva and breath. Each fluid presented its own problems. One of the major problems with breath was obtaining a clean sample, uncontaminated with saliva droplets. One major problem with saliva was its variability from time to time and subject to subject.

3.0 General Methodology

3.1 Introduction - Approaches Used

Experimental work in this project was divided into three major areas: development of collection devices for breath and saliva, development of analytical methodology to measure selected drugs in these fluids, and validation of these methods by establishing a quantitative relationship between the concentration of the drugs in breath and/or saliva and their concentration in blood. Our approaches to each of these experimental areas are summarized below.

Since it was envisioned that sample collection would ultimately be performed by operational personnel, we endeavored to keep the collection of samples as simple as possible, but in manners which would not compromise the integrity of the samples. Due to the known adsorption of many drugs to plastics such as polyethylene, polystyrene, etc., the use of plastics, with the exception of teflon, was avoided. Likewise, the use of invasive techniques to collect samples of breath and saliva, such as would have been required for the collection of parotid saliva, were also avoided. For breath collection, procedures that are in common use for collecting trace organics from environmental air samples were investigated. A simple container into which subjects could spit was evaluated for the collection of saliva.

Analytical procedures should be sufficient to not only provide quantitative information concerning the concentration of drugs in saliva and breath, but also must be devised so that the investigator is provided with some measure of qualitative information, that is, am I measuring the drug that I think I am measuring? For this reason, we turned to state-of-the-art chromatographic procedures which could be reasonably

reproduced in a well-equipped laboratory. Because of the expense of mass spectrometers as detectors, we did not use them in our analytical systems. However, when used as detectors for gas chromatographs, mass spectrometers are superior to any other kind of detector for providing qualitative information on the materials present in the samples being analyzed. The use of deactivated glass and fused silica capillary gas chromatographic columns, which provide superior resolution to other types of chromatography, was pursued. This area has rapidly expanded over the past five-ten years with excellent columns now becoming commercially available. These columns were coupled to a highly sensitive nitrogen-phosphorus selective detector which a large degree of specificity as to the nature of the compounds being detected.

Validation of our methodology was accomplished with a highly selective group of subjects. These subjects were selected so as to provide a minimum of variation in the results due to sex, age, weight, presence or interaction with other drugs, or diseased states. Without information on such a restricted population, the contribution of any or all of these variables to the correlation of concentrations of drugs in saliva or breath to the concentrations in plasma (or lack of correlation) would be very difficult to determine. Likewise, it would be impossible for us to know whether our other results were being influenced by any of these factors.

3.2 Clinical Methodology

3.2.1 Subjects

At least six volunteer subjects were used for each drug. Most of these subjects were tested at two dose levels of the drug. The restrictions as to the selection of subjects were as follows:

A. All subjects were healthy.

B. Sex - male

Female subjects would introduce cyclical hormonal changes which are undesirable at this time.

C. Age - 18-35 years. No minors were used.

D. Weight - All subjects weighed 140-190 lbs.

E. Height - The heights of all subjects were within the range prescribed by age-weight-height tables for healthy individuals.

Other restrictions were placed on the subjects prior to and during the study. These are as follows:

F. No drugs, tobacco, marihuana or alcohol for one week prior to and for the duration of the experiment.

G. No caffeine (from coffee, tea, cola, cocoa, etc.) for three days prior to and for the duration of the experiment.

H. No food from 12:01 a.m. of the day of the experiment until 3 hr after administration of the drug.

I. Intake of water and other approved liquids were restricted from two hours prior to the start until 3 hr after administration of the drug.

Subjects were informed as to the purpose of the experiment, the drug to be administered and its possible side effects. They were also informed of the general protocol of the experiment and the restrictions

to be placed on them as outlined above. They were free to withdraw from the study at any time. All subjects signed the informed consent statement that had been approved by both the RTI and the University of North Carolina Committees on Protection of the Rights of Human Subjects. The weights and ages of the subjects are listed in Table 1.

3.2.2 Dose Forms of the Drugs and Method of Administration

Drugs were administered orally in a commercially available form. Two dose levels were used for each drug with the higher level being twice the lower level. Doses of secobarbital, amphetamine, chlorpromazine and diazepam were adjusted for differing body weights. The exact forms and dosages of the drugs are shown in Table 2.

3.2.3 Medical Support for Subjects

Subjects were under constant supervision of Dr. Perez-Reyes for at least the first 4 hours of the experiment. Since the doses given were within the therapeutic range, pharmacodynamic effects were noticeable for some drugs. This was especially true for chlorpromazine. Particular attention was given to the subjects until these effects disappeared and in no case were subjects released from constant supervision of the physician while pharmacodynamic effects were observable. Each subject was also seen by Dr. Perez-Reyes 6, 8, 11 and 24 hr after administration of the drug.

3.2.4 Collection of Biological Samples

3.2.4.1 Plasma (Blood)

Blood, ca. 30 mL per sample, was collected at 0, 0.5, 1.0, 1.5, 2.0, 3, 4, 6, 8, 11 and 24 hr after administration of the drug. For the first four hours these samples were collected through an indwelling

Table 1.

Ages and Weights of Subjects.

Drug	Subject	Age (yr)	Weight (kg)
Secobarbital	S1	27	70.5
	S2	26	71.4
	S3	25	67.0
	S4	24	74.8
	S5	32	79.4
	S6	22	77.1
Amphetamine	A1	27	80.7
	A2	25	68.9
	A3	24	70.3
	A4	27	68.0
	A5	26	81.2
	A6	24	83.9
Chlorpromazine	CL1	24	84.0
	CL2	25	63.0
	CL3	27	68.0
	CL4	22	70.5
	CL5	24	75.0
	CL6	24	68.0
Diazepam	D1	24	70.3
	D2	27	80.7
	D3	25	69.0
	D4	26	81.2
	D5	30	77.1
	D6	27	68.0
Diphenhydramine	DP1	24	83.0
	DP2	24	68.0
	DP3	20	71.2
	DP4	22	70.3
	DP5	24	59.0
	DP6	19	74.8
	DP7	27	68.0
Codeine	C1	24	84.0
	C2	31	78.0
	C3	28	73.0
	C4	29	68.2
	C5	31	75.0
	C6	24	66.0

Table 2.

Forms and Amounts of Drugs Administered to Subjects in the Human Validation Studies.*

Drug	Form of Administered Dose	Dosage Levels
Secobarbital	Capsules containing sodium secobarbital (Seconal)	1.22 mg and 0.61 mg of sodium secobarbital per kg body weight
Amphetamine	Capsules containing ground amphetamine sulfate tablets	0.122 mg and 0.061 mg of d,ℓ-Amphetamine per kg body weight.
Chlorpromazine	Capsules containing chlorpromazine HCl concentrate	0.312 mg and 0.624 mg chlorpromazine HCl per kg body weight.
Diazepam	Capsules containing ground Valium tablets	0.14 mg and 0.071 mg diazepam per kg body weight.
Diphenhydramine	Capsules; 50 mg each	100 mg and 50 mg of diphenhydramine·HCl per subject.
Codeine	Tablets; 15 mg each	30 mg and 15 mg codeine sulfate per subject.

*Drugs were administered as their commercially available forms except for adjusting the dose for differing body weights.

needle; thereafter by individual venipuncture. Blood was centrifuged to separate the plasma and red blood cells. The resultant plasma was frozen and stored in silylated glass vials at -20°C until analyzed.

3.2.4.2 Saliva

Mixed saliva was obtained by having the subjects spit into a 20 mL silylated scintillation vial. For the first four hours while the indwelling needle was in place for collecting blood samples, collection of saliva was initiated at the same time as collection of blood. After this time, saliva collection was initiated as soon as the blood sample had been collected. Two to five minutes were required to obtain sufficient saliva (ca. 5 mL) for analysis by gas chromatography.

In the studies with all drugs except secobarbital and diazepam, the pH of the saliva samples were measured with a pH meter immediately after collection. The saliva was then frozen and stored at -20°C until it was analyzed.

3.2.4.3 Breath

In addition to evaluating a breath collection device containing a trap composed of Tenax-GC, a trap in which the breath was bubbled through ethanol maintained at <-50°C was employed in studies to determine whether measurable quantities of drugs were being excreted in breath. We demonstrated that both devices would effectively trap the drugs being studied and that the drug could be recovered quantitatively from each trapping device.

Breath samples were collected in preliminary studies with secobarbital and amphetamine for two-minute periods at the same time that blood samples were being taken. For a number of subjects in the amphetamine study, breath collections were made until 15-17 ℓ of expired

breath were passed through the trapping device. Four to six deep exhalations were performed during this period. Breath samples were passed through plugs of silated glass wool to remove droplets of saliva. Drugs were removed from the breath by bubbling it through 10 mL of USP absolute ethanol in a glass trap maintained at -78°C by an external dry ice-ethanol bath.

3.3 Analysis of Samples

3.3.1 Selection of Internal Standards

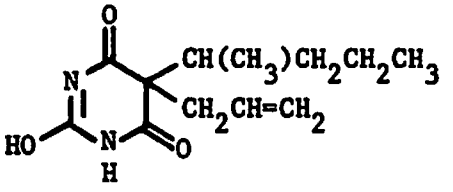
The use of internal standards in quantitative analyses of drugs by chromatographic methods is a common practice. This procedure permits the measurement of an unknown quantity of drug as a ratio of a known quantity of internal standard. Some investigators add an internal standard immediately before the chromatographic process. This procedure provides a correction only for non-reproducible aspects of the chromatographic process while, in many cases, it permits the use of compounds whose structures are totally unrelated to the drug under study. We, however, chose to find internal standards which could be added directly to the plasma or saliva before extraction of the drug. Selection of an appropriate internal standard thus permitted a correction for non-reproducibility over the entire analysis process as well as providing a carrier which could lessen adsorptive losses of very small amounts of drugs.

The internal standards were selected according to four criteria. (1) The structure of the internal standard should be very similar to that of the drug being studied. It is particularly important that the polar functions in the drug also be present in the internal standard. (2) It must have suitable chromatographic properties. The internal

standard must be separated from the drug under study by the column being used in the analysis. The retention times of the two compounds should be sufficiently close so that they can be chromatographed under identical conditions. The internal standard, as well as the drug under study, should give a sharp symetrical peak with little or no tailing. The internal standard must also be stable to the chromatographic procedures employed with no decomposition peaks occurring. The retention time of the internal standard should be different from that of common plasma or saliva interferences. It should also be different from the retention time of commonly used drugs. We selected compounds whose retention time on the column being employed was different from those of endogenous compounds in blank plasma and saliva from the subjects being studied from pooled saliva obtained from volunteers at RTI and from plasma obtained from outdated blood from the Red Cross. It was not possible in this program to do an extensive comparison of the retention times of the internal standards with those of commonly used drugs. This study, however, should be done before the assays are used with the general population. (3) The internal standard must not itself be widely used as a drug. In studies with controlled populations, e.g., hospitalized patients, where the intake of other drugs is known or can be controlled, this is not a serious concern. For example, DiGregorio et al (1978) used flurazepam as an internal standard for their analysis of diazepam. Likewise, such compounds could have been used in our study since we studied subjects who were not taking other drugs. However, extrapolation of this procedure to the general population would not be possible. Therefore, we limited our choice of internal standards to those compounds which are not in general use. (4) The internal standards should be commercially available or readily prepared.

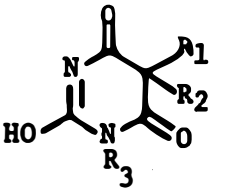
Figure 2. Structures of the Drugs Studied and the Internal Standards Employed in Their Assays

Drug



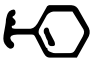
Secobarbital

Internal Standard



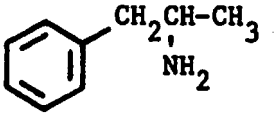
Butabarbital R_1 = $CH(CH_3)CH_2CH_3$
R_2 = CH_2CH_3
R_3 = H

RRT* = 0.72

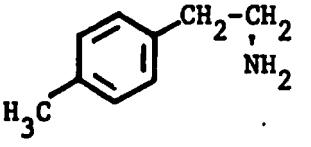


Hexobarbital R_1 =
R_2 = R_3 = CH_3

RRT = 0.56

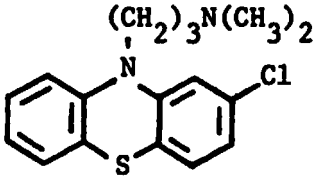


Amphetamine

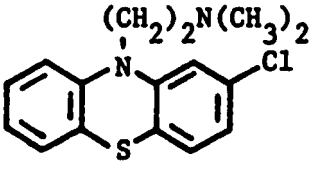


1-(4-Methylphenyl)-2-aminoethane;
2-(p-tolyl)ethylamine; TEA

RRT = 1.12



Chlorpromazine

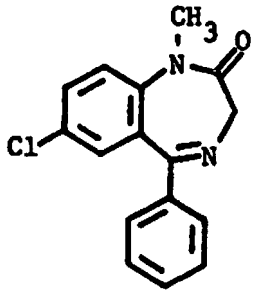


2-Chloro-N,N-dimethyl-10H-phenothiazine-10-ethanamine;
(Desmethylenechlorpromazine)

RRT = 0.91

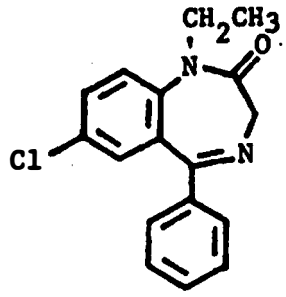
Figure 2 (continued)

Drug



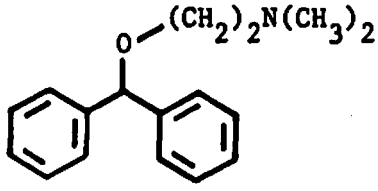
Diazepam

Internal Standard

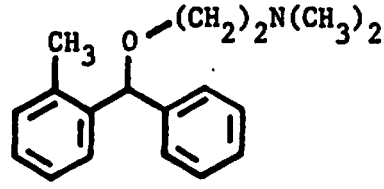


N-Ethyl Analog of Diazepam

RRT = 0.87

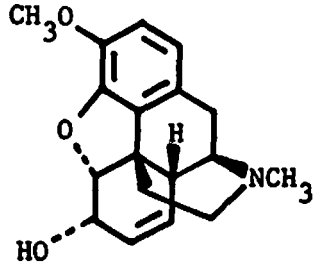


Diphenhydramine



Orphenadrine

RRT = 1.12



Codeine

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*RRT = retention time relative to the drug being analyzed under chromatographic conditions used in analyses (see Appendix A).

The structures of the compounds used for internal standards and the drugs for which they were used are shown in Figure 2. All of these internal standards meet the criteria as specified above, with the possible exception of the internal standard for secobartital. For this reason, we chose two internal standards to be used for this drug. Both are also barbiturates listed in the Merck Index. From a chromatographic standpoint, the best internal standard is butabarbital. However, this compound is also listed in the current Physicians Desk Reference, and thus may be used by a small number of people. The second internal standard, hexobarbital, is slightly inferior from a chromatographic standpoint, but is not included in the current Physicians Desk Reference as an available drug.

The internal standard chosen for amphetamines, 2-(p-tolyl)ethylamine, is available from commercial sources. Another possible internal standard for amphetamines is shown in Appendix A.2. The internal standards selected for chlorpromazine and diazepam, desmethylene chlorpromazine and the N-ethyl analog of diazepam, respectively, are not commercially available. They were prepared by alkylation of the appropriate phenothiazine or benzodiazepine respectively. An alternate internal standard for diazepam is shown in Appendix A.4. The internal standard selected for diphenhydramine is orphenadrine. While several trade names exist for this compound in the Merck Index, it is not listed as being available in the current Physicians Desk Reference. No internal standard was necessary for the assay of codeine which was done by radioimmunoassay (RIA).

3.3.2 Extraction Method

At the initiation of this project, it was hoped that a maximum of three extraction methods would suffice for all compounds studied: one for basic drugs, one for neutral drugs, and one for acidic drugs. It would, thus, be feasible to design an analytical scheme so that a number of drugs could be analyzed simultaneously. Unfortunately, due to the wide diversity of the compounds studied in this project, this was not completely possible. Codeine was analyzed without being first extracted from plasma or saliva. The extraction procedures used for the other drugs are shown in Appendix A. In general, basic drugs and diazepam were extracted with toluene or with a solution of 1-2% isoamyl alcohol in hexane from plasma and saliva that had been made basic and to which the internal standard had been added. Procedures for analysis of amphetamine and diphenhydramine call for these drugs to then be extracted into aqueous acid. The resulting amphetamine hydrochloride was concentrated and converted to its trifluoroacetamide before chromatographic analysis. In the extraction procedure for diphenhydramine, the aqueous acid was made basic and the diphenhydramine reextracted into methylene chloride. It was then ready for chromatographic analysis. Chlorpromazine and diazepam were analyzed directly from the concentrated organic extracts. The slightly acidic secobarbital was extracted from acidified saliva or plasma, after the addition of the internal standards, with chloroform. Secobarbital was then reextracted into a sodium hydroxide solution. This solution was made acidic and the secobarbital extracted once again into chloroform. The chloroform extract was concentrated and the secobarbital analyzed by gas chromatography. Silylated glassware was used throughout all extraction and analytical procedures. All solvents were "distilled in glass" quality.

For the analysis of breath, the ethanol contained in the breath traps from the subjects in the secobarbital study was made slightly basic with sodium hydroxide. It was then concentrated at 30°C. The residue was made acidic and extracted with chloroform. After evaporation of the chloroform, the residue was chromatographed. Methanolic HCL was added to the ethanol in the breath traps from the subjects in the amphetamine study in order to convert the amphetamine to the non-volatile amphetamine hydrochloride. The ethanol was then evaporated and the residue dissolved in toluene and treated with trifluoroacetic anhydride as described for the plasma and saliva.

3.3.3 Gas Chromatographic Analyses

Analyses for all drugs, except for codeine, (this analysis was done by radioimmunoassay, cf section 3.3.4) were performed on a gas liquid chromatograph equipped with capillary columns and a nitrogen-phosphorus specific (thermionic) detector. The capillary columns were employed to increase resolution and reproducibility. The columns chosen for this project had highly deactivated surfaces which allowed us to achieve quantitation at very low concentrations of the drugs. At the initiation of the project, commercial capillary columns which provided these highly deactivated surfaces were not available. The initial columns were thus prepared at RTI. The column surfaces were prepared by cleaning them with HCl gas followed by the deposition of a thin layer of barium carbonate. Next, a thin film of pyrolyzed Carbowax was formed on the surface of the barium carbonate. Finally, the desired stationary phase was introduced in the column. The inside diameter of these columns was 0.25 mm. The length of column required for the analyses ranged from 8-30 meters. Toward the end of the project, commercial columns with

satisfactory performance were available. The columns used for the analysis of diphenhydramine were purchased from Chrompack U.S.A.

The nitrogen-phosphorus specific thermionic detection further increased the specificity of the assay while providing more sensitivity than is available with flame ionization detection. This detector was also used in those cases where there was a choice between electron capture and nitrogen-phosphorus specific detectors. Since only a small portion of drugs are inherently sensitive to electron capture detection while most drugs contain nitrogen, the use of a nitrogen-phosphorus detector provides a generalized detection system for them while being insensitive to most endogenous compounds.

Samples were introduced onto the columns with a splitless injection system. Such an injection system permitted routine injection of 1 μL of the sample onto the column. (The other commonly used injection system with capillary columns is a split injection in which approximately 0.01 μL of sample actually is applied to the column.) An automatic sampler was employed. Samples to be chromatographed were dissolved in 10-20 μL of an appropriate solvent and placed in 100 μL conical vials. Two stationary phases were used in the analyses in this project: polymethyl silicone (SE-30, OV-101) and polyethylene glycol (Carbowax 20-M; CP Wax 51). Columns containing some other valuable stationary phases were not available either because techniques have not been perfected to permit the reproducible preparation of good columns (OV-17) or because the column phases contain nitrogenous substituents which slowly bleed into the nitrogen sensitive detector (e.g., OV-225).

The Carbowax stationary phase proved superior for all compounds except amphetamine. Amphetamine trifluoroacetamide and its internal

standards were not well resolved from endogenous compounds on the Carbowax column (cf. Figure 3). Much better resolution and sharper peaks were obtained using a SE-30 capillary column as is seen in Figure 4. On the other hand, excellent separation and peak shape are observed for secobarbital on a Carbowax column (Figure 5), whereas these parameters are not nearly so good when secobarbital is chromatographed on a methyl silicone (OV-101) column (cf. Figure 6). Diazepam could be chromatographed well on either column; however, the methyl silicone columns were unable to separate diazepam from any of the internal standards. This separation was readily obtained using the Carbowax column (cf. Figure 7). This column did equally well for chlorpromazine and diphenhydramine. Typical chromatograms from saliva and plasma, exact chromatographic conditions, and standard curves for assays of all compounds are given in Appendix A. All assays were run at least in duplicate. Further replicate assays were run in cases where the duplicate assays were not in close agreement.

3.3.4 Radioimmunoassay

The antiserum to codeine was obtained from Dr. John Findlay, Wellcome Research Laboratories. The procedure used for the radioimmunoassay was essentially that published by Findlay et al. (1976). This antiserum has extremely low cross reactivity with codeine-6-glucuronide and morphine, known metabolites of codeine. The assay was designed for use with 0.1 mL of plasma. The assay was adapted for use with saliva in which 10-50 μL of saliva were used per assay. Thus, the radioimmunoassay required less than 1/10 of the plasma and saliva necessary for a gas chromatographic assay. The details of the assay procedure are included in Appendix A.

Figure 3. Chromatogram of Amphetamine Extracted from Saliva Using a Carbowax 20M Capillary Column.

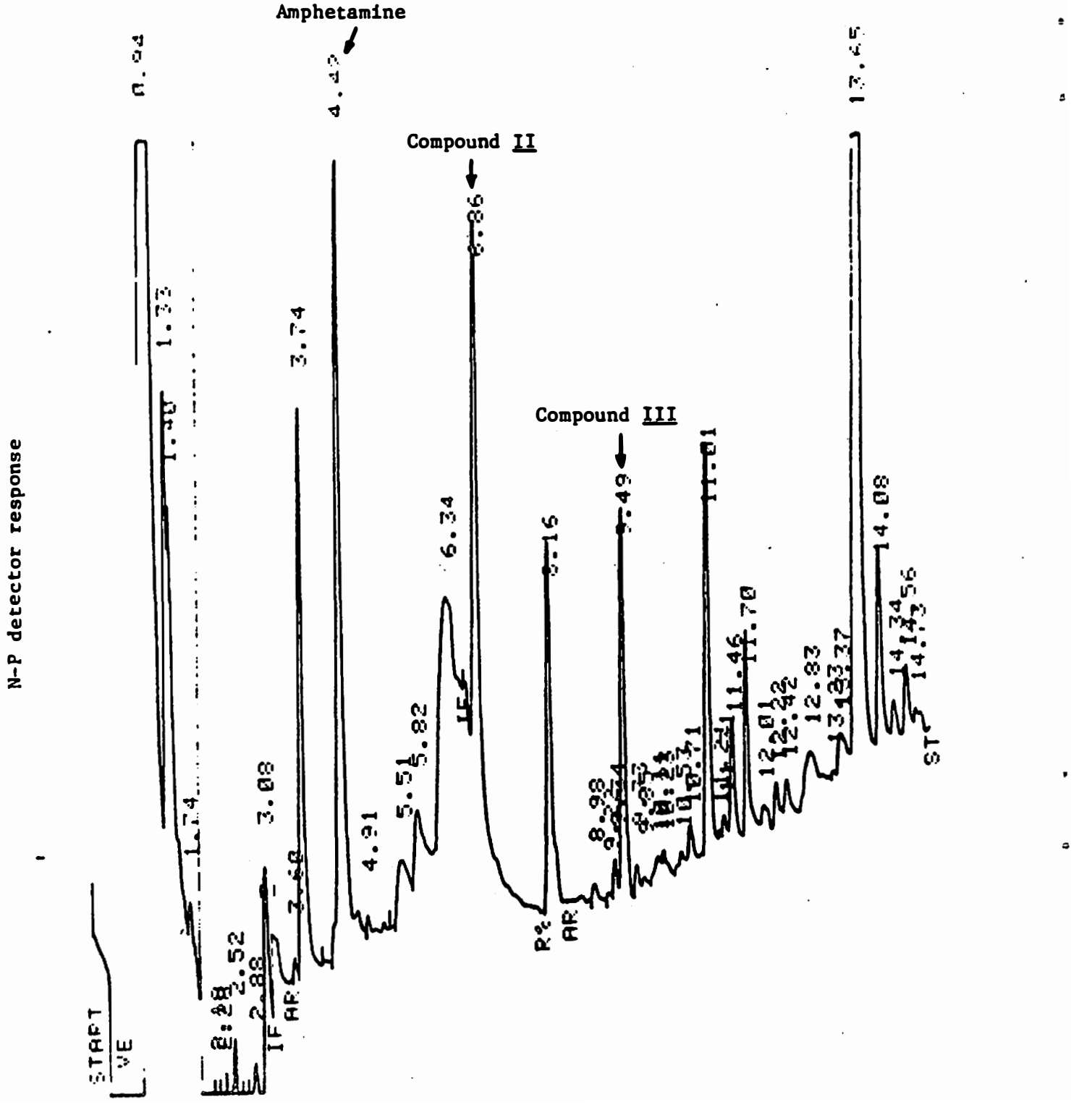


Figure 4. Chromatogram of Amphetamine Extracted from Saliva at a Concentration of 55 ng/mL on a SE-30 Capillary Column

Chromatographic conditions same as in Figure 16.

Compounds II and III are internal standards.

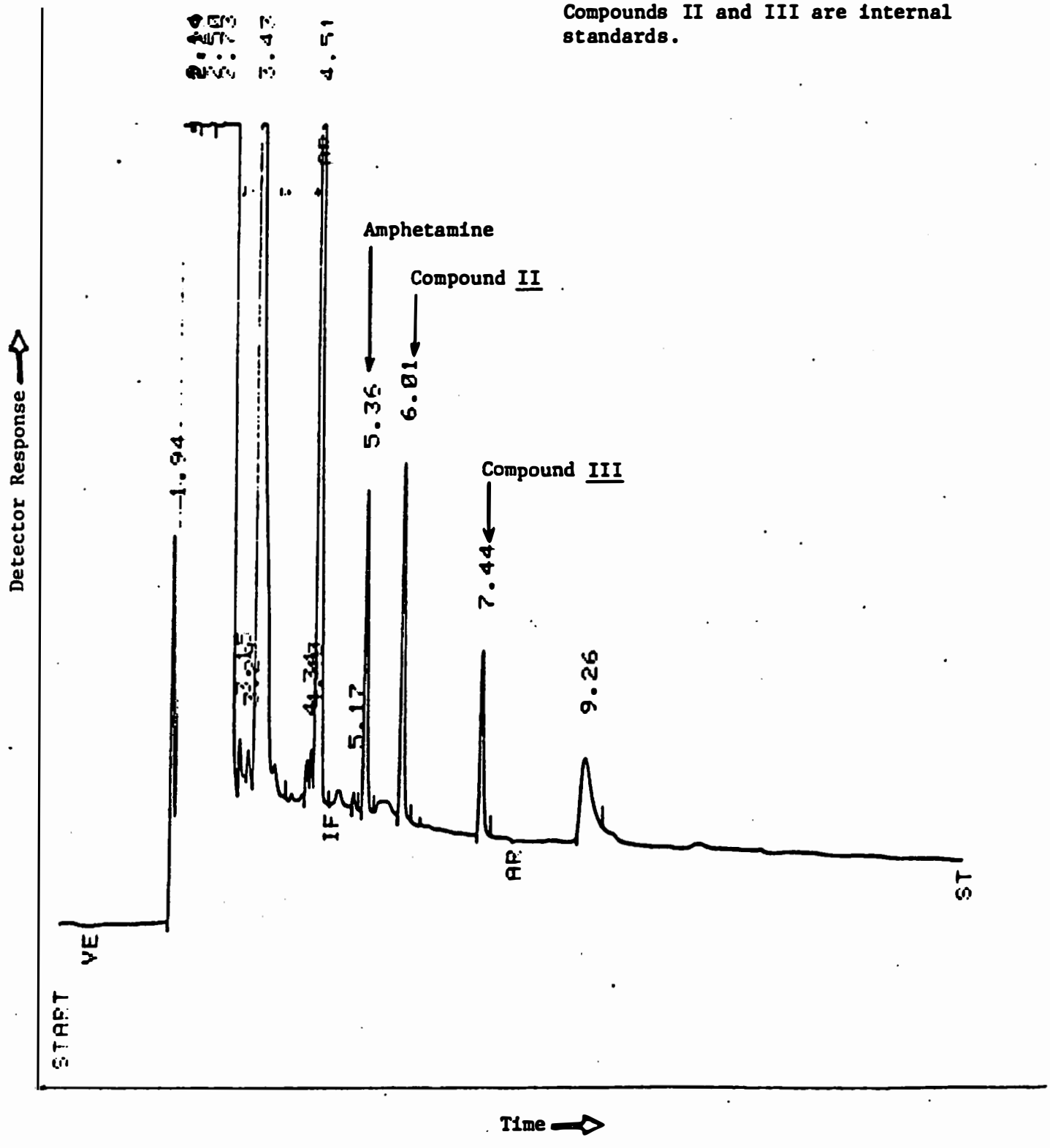


Figure 5.

Chromatogram of Plasma Extract Containing 1 µg Secobarbital (I), 0.5 µg Butabarbital (II) and 0.5 µg Hexobarbital (III) per ML Plasma. Amounts of Barbiturates Injected onto the Column Were 150 ng Secobarbital, 75 ng Butabarbital and 75 ng Hexobarbital.

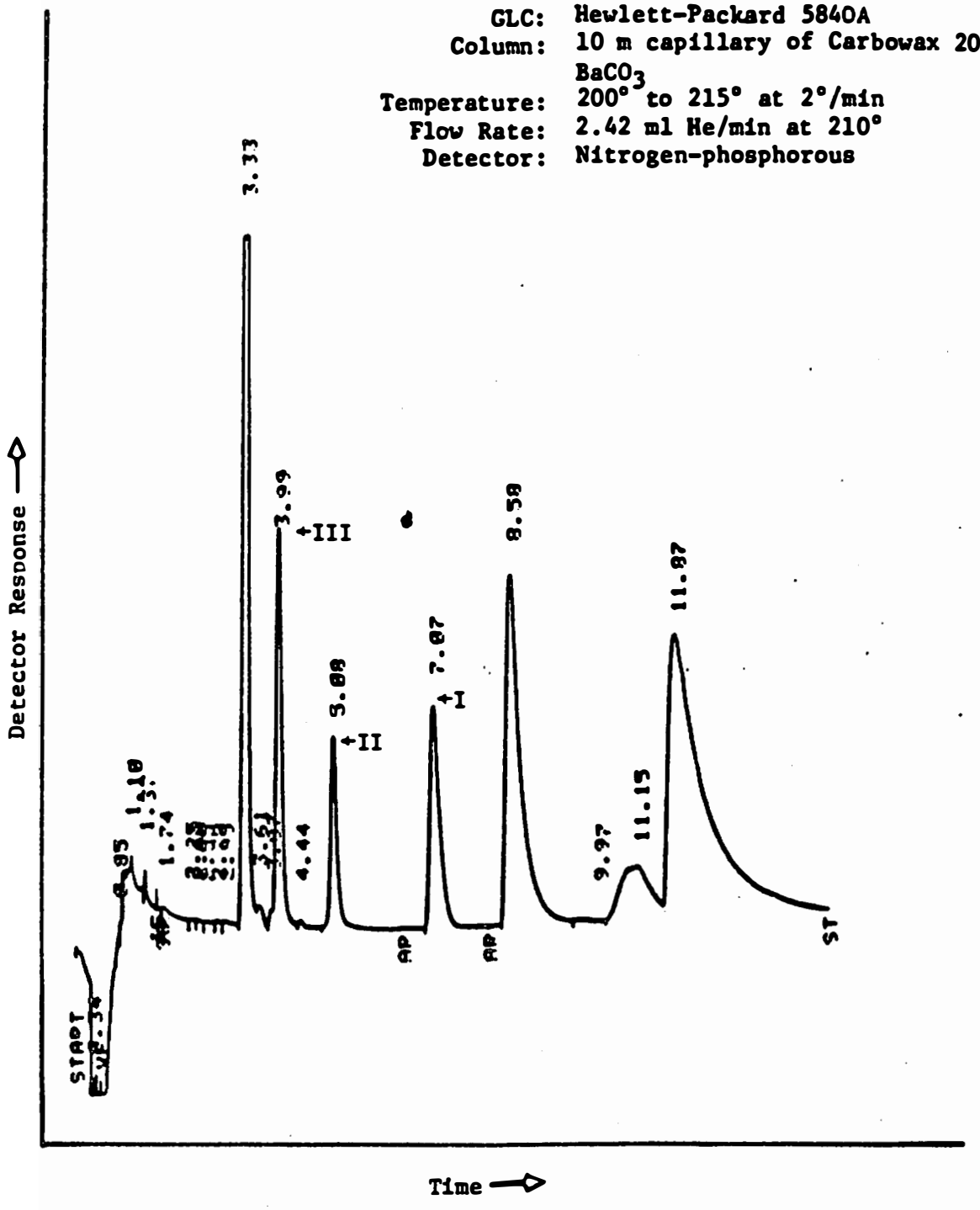


Figure 6.

Chromatogram of Plasma Extract Containing 1 µg Secobarbital (I), 0.5 µg Allylcyclopentenyl Barbituric Acid (I) and 0.5 µg Barbital (III) per ML Plasma. Amounts of Barbiturates Injected Onto the Column Were 150 ng Secobarbital, 75 ng Allylcyclopentyl Barbituric Acid and 75 ng Barbital.

GLC: Hewlett-Packard 5840A
Column: 33 m capillary of OV-101 on $BaCO_3$
Temperature: 125° to 225° at 10°/min
Flow Rate: 1.56 ml He/min at 195°
Detector: Nitrogen-phosphorous

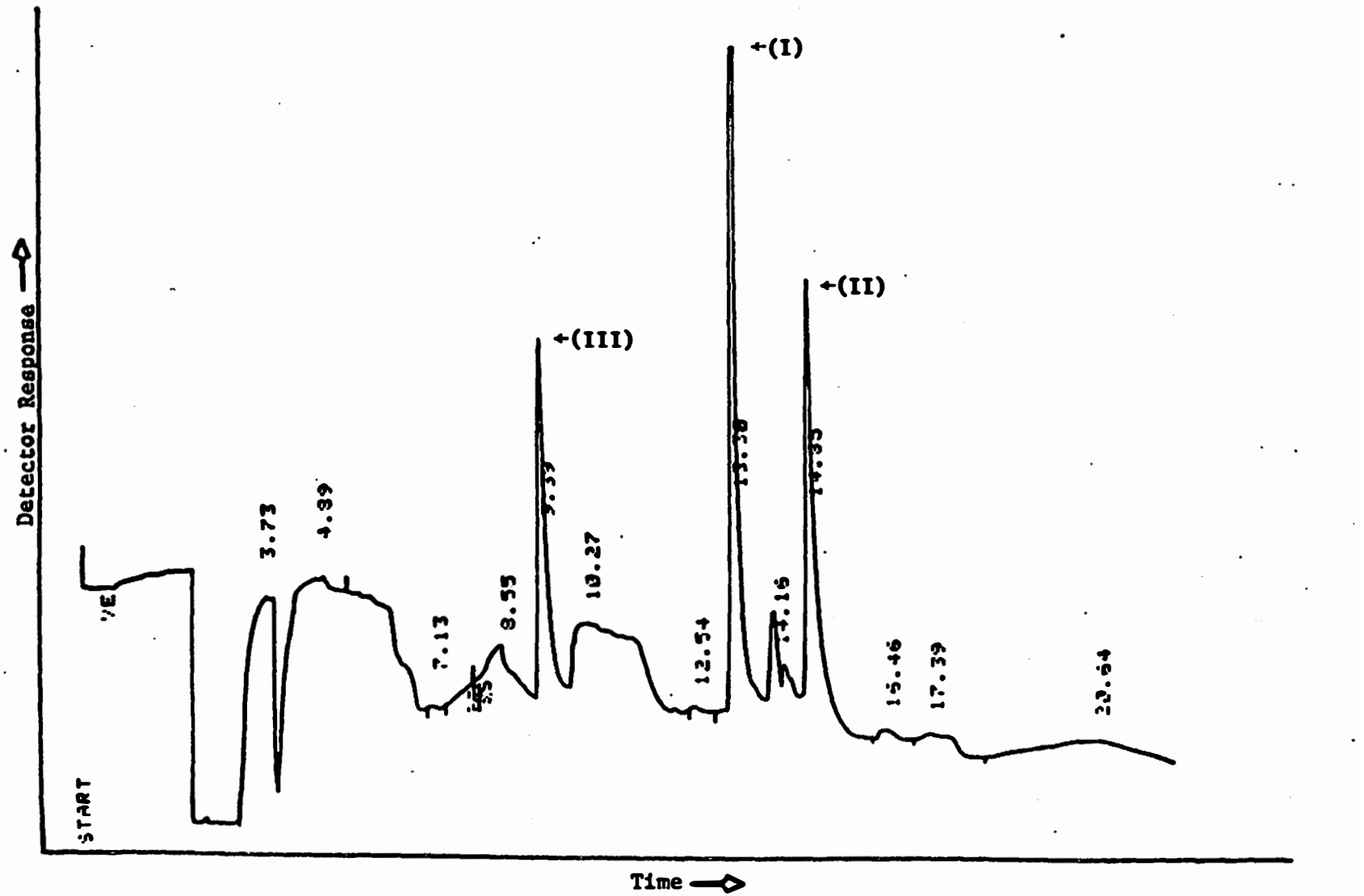
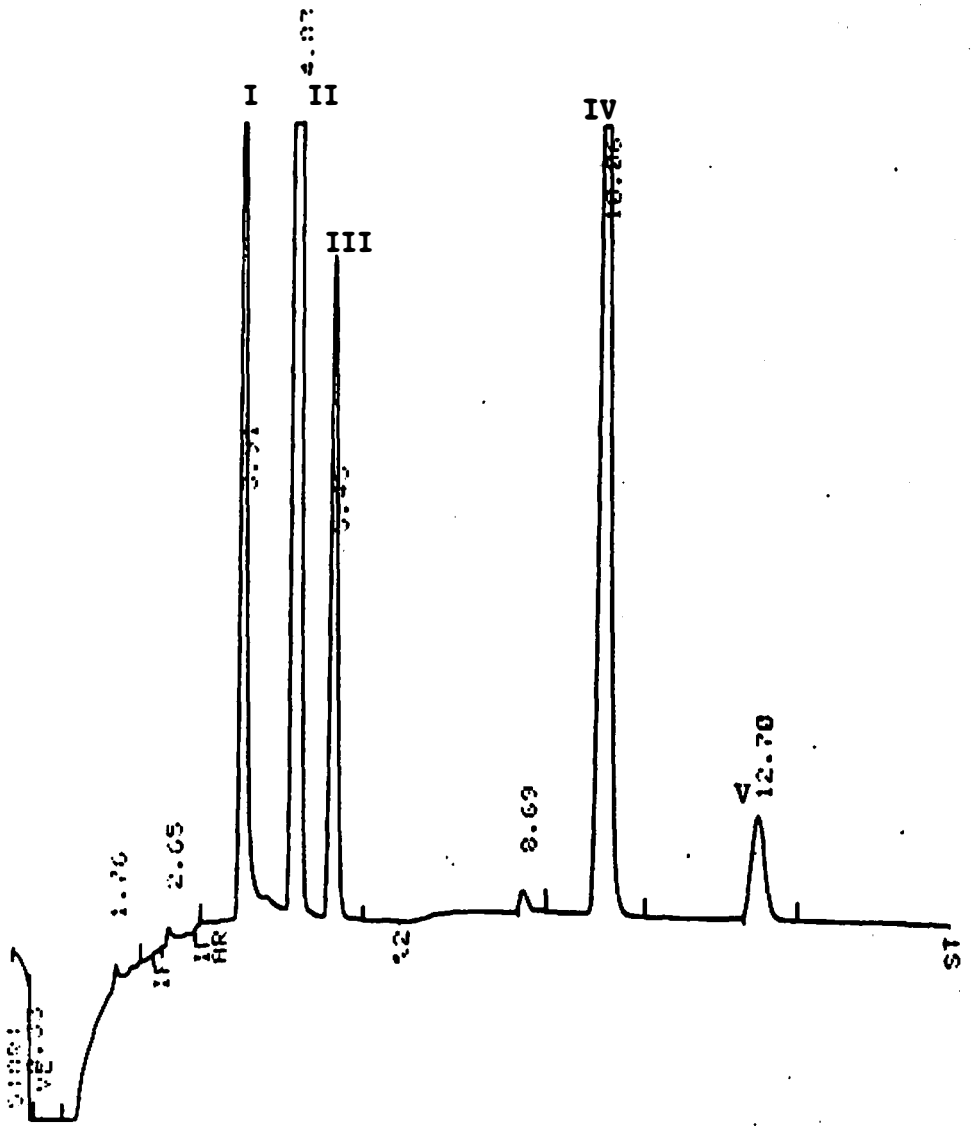


Figure 7. GC of Diazepam, Desmethyldiazepam and Possible Internal Standards on an 8 meter Carbowax-20M Capillary Column



Peak	Retention Time (min)	Compound
I	3.91	3-Methyl Diazepam
II	4.83	N-Ethyl Analog of Diazepam
III	5.45	Diazepam
IV	10.06	3-Methyl-N-Desmethyl Diazepam
V	12.70	N-Desmethyl Diazepam

3.4 Methods Used for Studying Effects of Increased and Decreased Salivary Flow

In these experiments, two subjects were each given a single 100 mg capsule of sodium secobarbital at time 0. Subjects S-1 and S-6 from the previous secobarbital studies were used in the experiments. After administration of the secobarbital, blood, breath, and saliva samples were collected at 1.5, 2.0, and 3.0 hours. Blood (10 mL) and saliva (3 mL) were collected as in the prior study. Breath samples consisted of the entire exhaled air over a 2 minute period.

After completion of the three hour sample collections, each subject was administered one drop of lemon juice to stimulate saliva production. Blood (10 mL) and saliva (3 mL) samples were then taken. The time required for saliva collection was measured. This procedure was repeated after a 15 minute interval. Immediately after the second saliva collection, atropine was administered intravenously. Fifteen minutes later, blood and saliva samples were taken as before and the time required for saliva collection was measured. After an additional 15 minutes, this collection procedure was repeated. The saliva and plasma samples collected in the study were analyzed as described in section 3.3. Breath samples were analyzed as described in section 4.5.

3.5 Methods for Analysis of Creatinine in Saliva

The possibility of using the concentration of creatinine in saliva as an "internal standard" in order to arrive at better predictions of the concentration of amphetamines in plasma from its concentration in saliva was investigated. A method for the determination of creatinine concentrations in saliva has been reported by Pu and Chiou (1979). This method involves the chromatography of deproteinized saliva by high

performance liquid chromatography (HPLC) on a strong cation-exchange column using a pH 4.8 buffer as the mobile phase with detection of the eluting creatinine achieved by measuring its UV absorption at 254 nm. When we repeated this work, we found that when creatinine spiked saliva was chromatographed, the creatinine eluted from the column as an unresolved doublet (Figure 8). By changing the ionic strength of the mobile phase, we were able to show that both peaks of the doublet were due to creatinine (Figure 9). Additional modifications of the methodology were then made in an effort to eliminate this problem by (1) changing the pH as well as the ionic strength of the buffer and (2) utilizing paired-ion chromatography. The sensitivity of the analysis using paired-ion chromatography was reduced by an interfering peak and thus could not be used to measure endogenous creatinine levels. When chromatographed on the strong cation-exchange column with a mobile phase buffered at pH 2.66, however, creatinine in saliva eluted as a single peak. The retention time of creatinine was next adjusted by changing the ionic strength of the buffer. Good resolution and separation of creatinine was achieved using the chromatographic conditions listed in Figure 10.

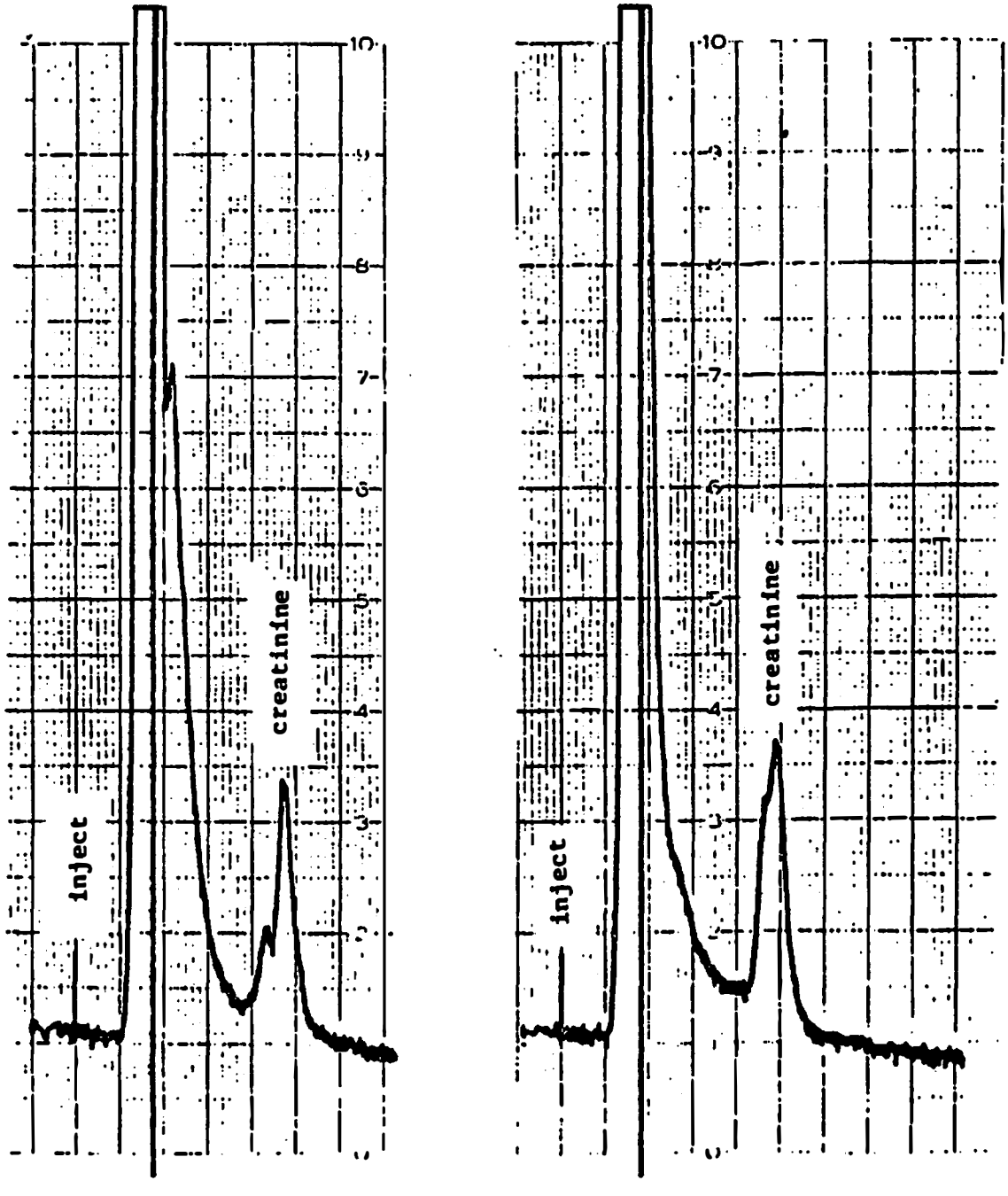
By use of these conditions and the deproteinization procedure of Pu and Chiou, a standard curve of the concentration of creatinine in saliva vs. peak area (peak height times peak width at 1/2 height) was prepared. This curve shows excellent linearity as a log-log plot (Figure 11). The lower end of the curve was limited by the endogenous concentration of creatinine in our standard saliva.

3.6 Methods for Determination of Plasma Protein Binding of Drugs

The percent of all drugs, except chlorpromazine and codeine, bound to plasma proteins was determined using the equilibrium dialysis method.

Figure 8. HPLC Chromatogram of Creatinine Using Conditions Reported by Pu and Chiou (1979).

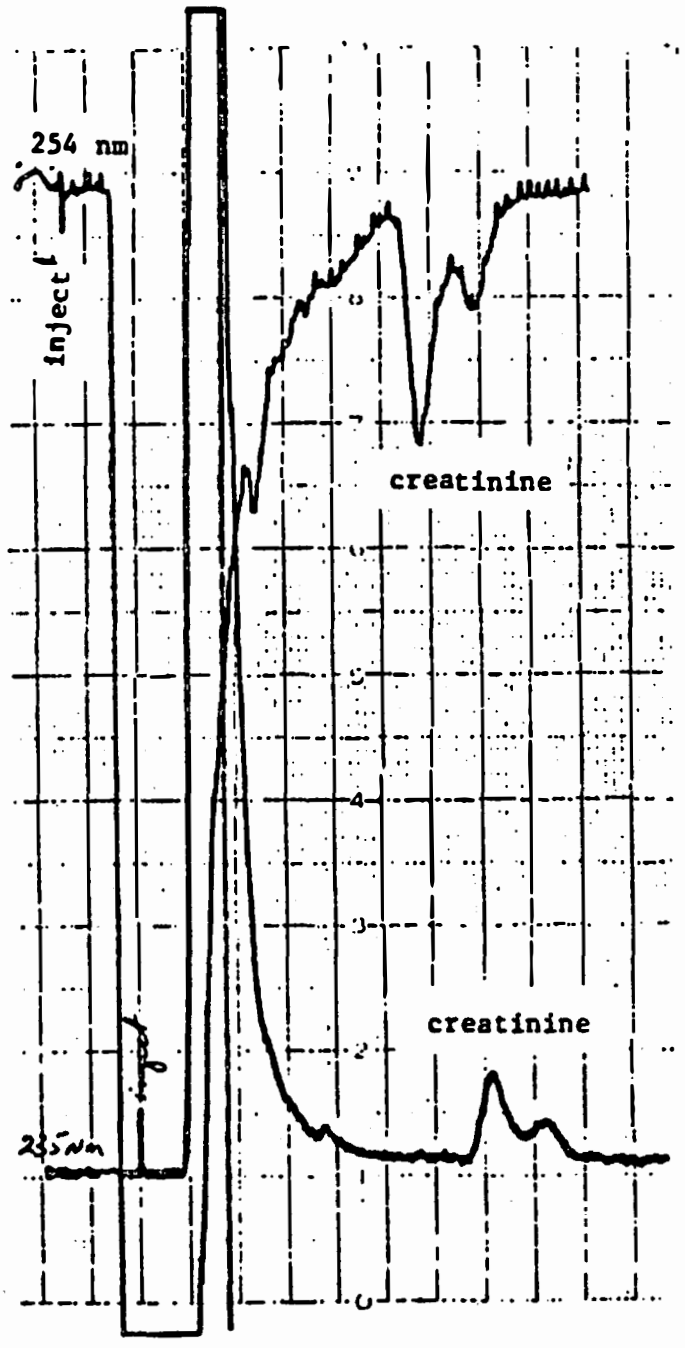
Column: Whatman PXS 10/25 SCX
Mobile Phase: 0.12 M $NH_4H_2PO_4$ (pH 4.80)
Flow Rate: 2.0 mL/min
Detector: UV at 235 nm, 0.02 AUFS
Chart Speed: 1 cm/min
Sample: a) saliva with only endogenous creatinine
b) sample in (a) spiked with 0.05 µg of creatinine



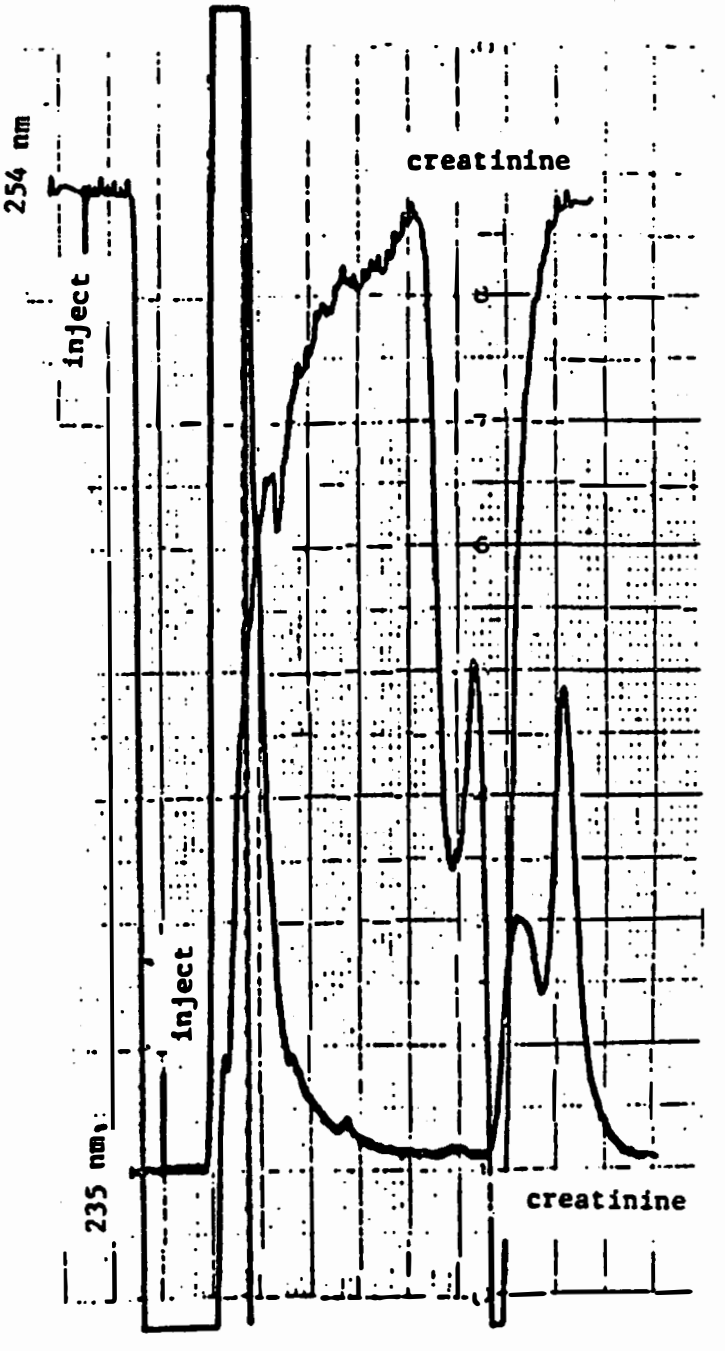
a b

Figure 9. HPLC Chromatogram of Creatinine at pH 4.80 at Lower Ionic Strength than in Figure 1.

Column: Whatman PXS 10/25 SCX
Mobile Phase: 0.0282 M $NH_4H_2PO_4$
Flow Rate: 2 mL/min
Detector: UV at 235 nm (bottom) and 254 nm (top)
Chart Speed: 1 cm/min
Sample: a) saliva with only endogenous creatinine
b) sample (a) spiked with 1 μg creatinine



a



b

Figure 10. HPLC Chromatogram of Creatinine Under Conditions Developed for Saliva Creatinine Assay.

Column: Whatman PXS 10/25 SCX
Mobile Phase: 0.055 M $NH_4H_2PO_4$ (pH 2.66)
Flow Rate: 2.0 mL/min
Detector: UV at 215 nm; 0.04 AUFS
Chart Speed: 2.0 cm/min
Sample: Subject A4; 0 time before dosing with 0.061 mg/kg amphetamine

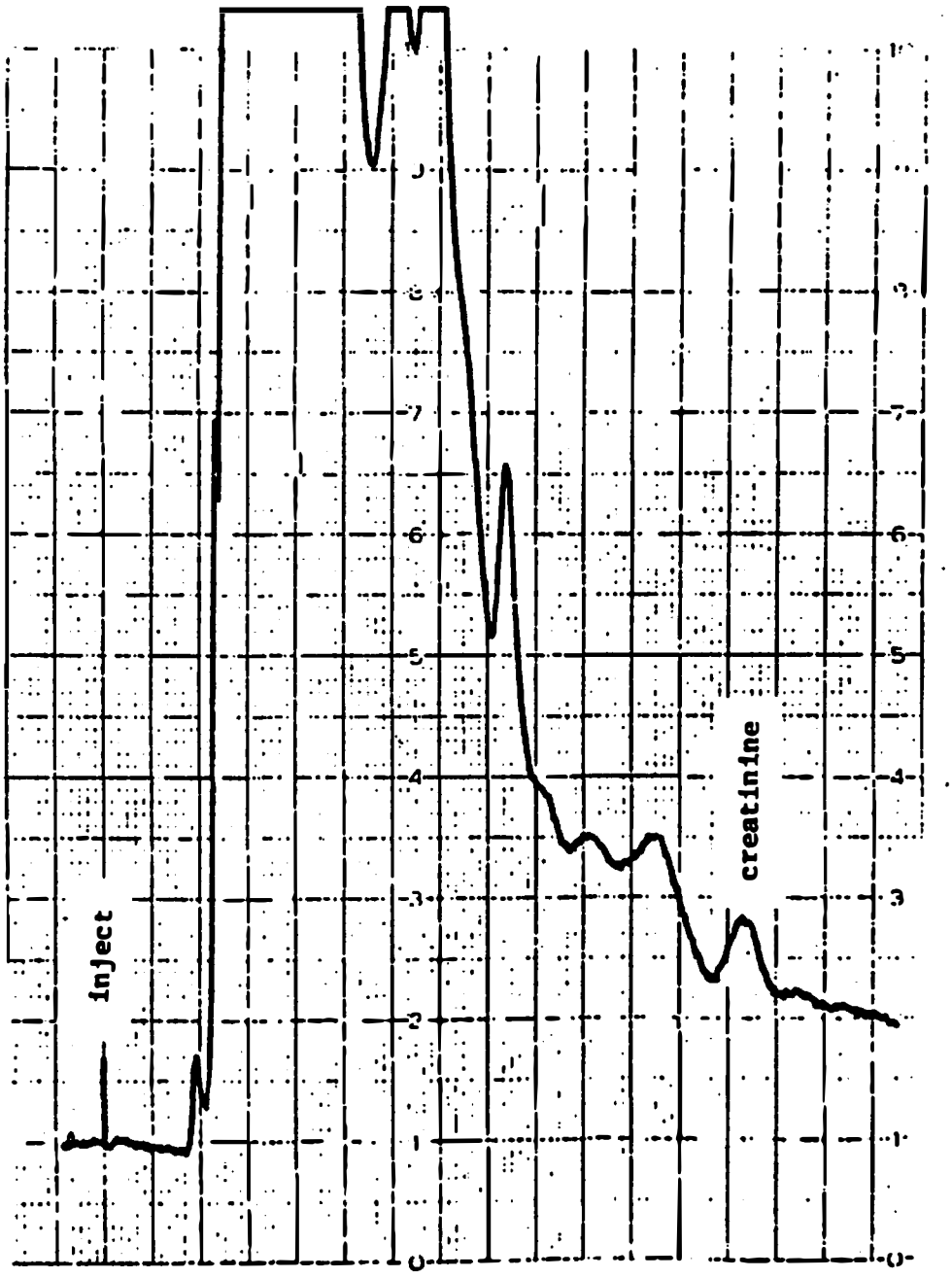
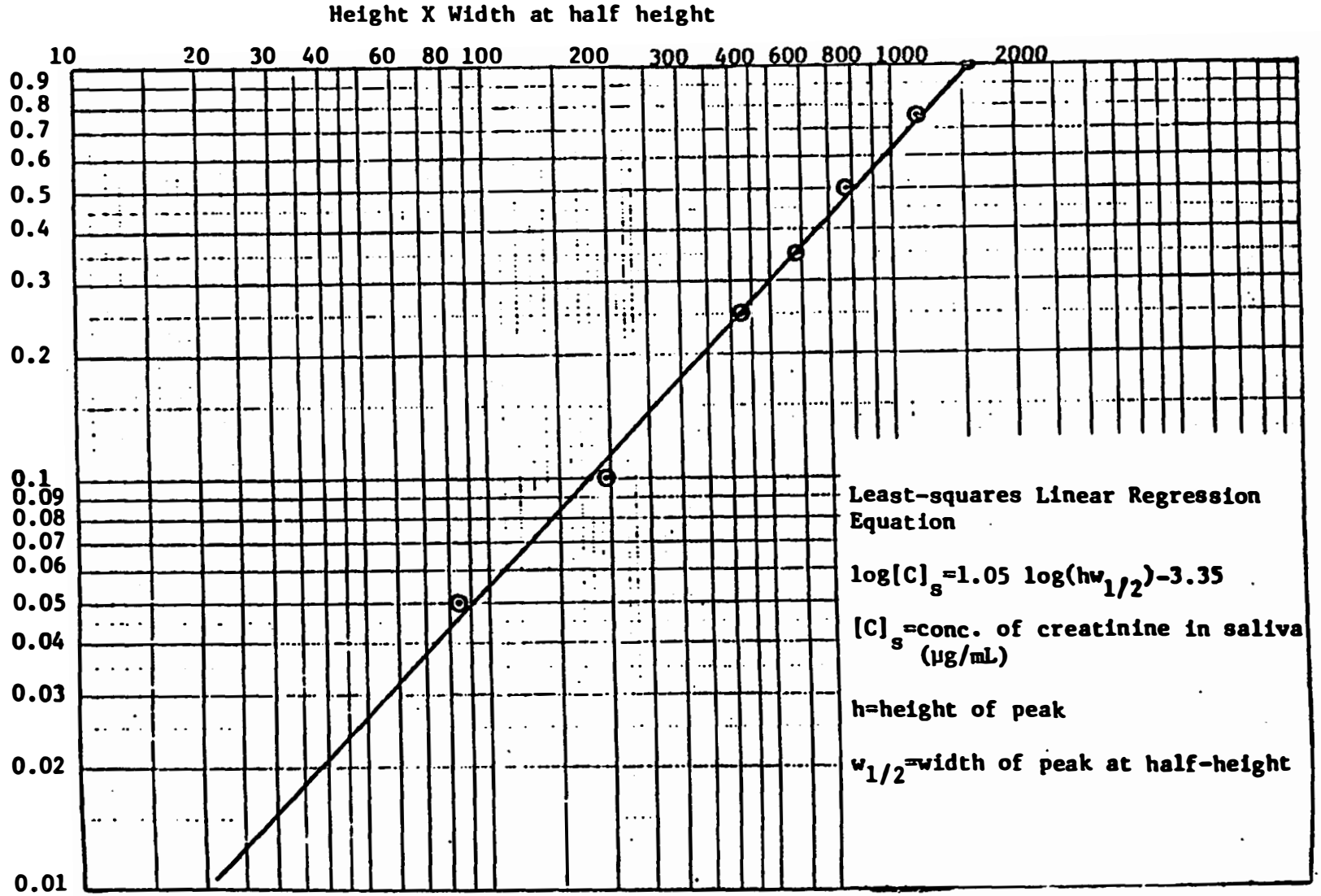


Figure 11. Calibration Curve for the Determination of Saliva Creatinine.



The binding of codeine to plasma protein has been well established (Judis, 1977); chlorpromazine is unstable in plasma.

In the equilibrium dialysis determinations, a small amount of a radiolabeled preparation of the drug to be studied was added to aliquots of plasma from subjects in the human validation study. The amount of radiolabeled drug added was less than 10% of the amount of unlabeled drug that was already present in the plasma (determined by gc analysis). The plasma was then placed on one side of a dialysis membrane, Sorensen's phosphate buffer on the other. Sorensen's buffer is a buffer whose pH, K^+ and Na^+ concentrations are approximately equal to those in plasma (Sorensen, 1909). The drug was allowed to equilibrate between the two sides of the membrane (ca 18 hr). Since free drug can pass through the membrane but protein-bound drug cannot, the difference between the concentrations of drug on the two sides is equal to the concentration of bound drug (C_B). Since the concentration of unbound drug (C_f) is the same on both sides of the membrane and is equal to the concentration of total drug in the buffer, the fraction of drug free, i.e., not bound to protein (F_f) can be calculated as follows:

$$F_f = \frac{C_f}{C_B + C_F} = \frac{\text{Concentration of drug on buffer side}}{\text{Concentration of drug on plasma side}}$$

The equilibrium dialysis procedure was carried out on plasma samples which contained a high concentration of drug and a low concentration of drug from each subject in the study. Radiolabelled drug whose purity had been established by TLC or HPLC was added to a ca 2.5 mL aliquot of plasma. The concentrations of radiolabelled drug in the

plasma and buffer compartments were measured after equilibrium had been reached by scintillation spectrometry. The total amount of radiolabelled drug in the two compartments was also measured to determine the degree of absorption of the drug to the dialysis equipment. Recovery of drug was usually >90% but in all cases was >70%.

pKa Values for the drugs being studied were also determined where these values were not available from the literature (cf. Table 3).

Table 3

pKa Values for Drugs

Drug	pKa	Type of Ionization (D = Drug Residue)	Source
Secobarbital	7.90	$DH \rightleftharpoons D^{\theta} + H^{+}$	Piraino et al., 1976
Amphetamine	9.95	$(DH)^{+} \rightleftharpoons D + H^{+}$	RTI
Chlorpromazine	6.4	$(DH)^{+} \rightleftharpoons D + H^{+}$	RTI
Diazepam	--		
Diphenhydramine	8.3	$(DH)^{+} \rightleftharpoons D + H^{+}$	RTI
Codeine	7.95	$(DH)^{+} \rightleftharpoons D + H^{+}$	Merck Index, 9th Ed.

4.0 Results

4.1 Collection Devices for Saliva and Breath

The collection device used for saliva consisted of a specially treated vial into which the subject could spit. We used 20 mL borosilicate glass scintillation vials which had been silylated by treatment at 225°C with hexamethyldisilazane. A teflon liner was inserted into the caps of these vials. The cost of the untreated vials, but with teflon lined caps, is estimated to be $250 per thousand. Equipment to silate the vials cost approximately $3,000. With this equipment, approximately 500 vials per day could be silylated with a labor commitment of 0.5 man-days per thousand vials.

The development of a collection device for breath that could be used at the roadside was not completed since we were unable to find measureable concentrations of the drugs being studied in breath. In order to collect drugs exhaled in breath, we used an ethanol bubbler trap similar to that shown in Figure 12. This device was shown to effectively trap secobarbital and amphetamine from simulated breath and is suitable for other research projects. A preliminary device which contained a cartridge of Tenax (Figure 13) was also evaluated. This device effectively trapped secobarbital and amphetamine from simulated breath. However, the Tenax usually employed for trapping trace organics in environmental samples provides two great a resistence for normal exhalations. Other groups at RTI have devised equipment to collect more volatile compounds from breath, in which the exhaled air is trapped in a large Tedlar bag and then pumped through the Tenax cartridge.

Figure 12.

Ethanol Bubler for Trapping Drugs Exhaled in Breath.

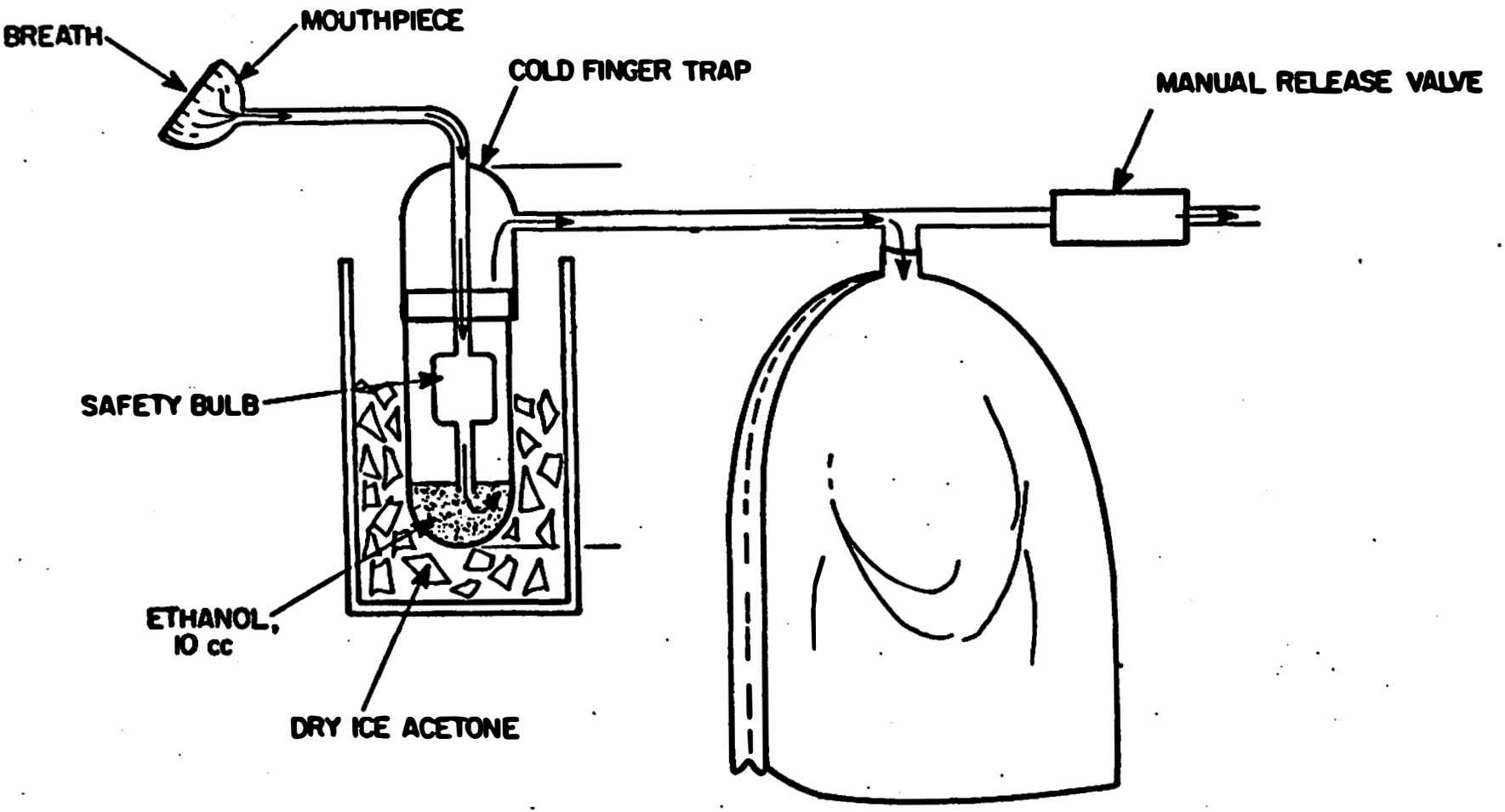
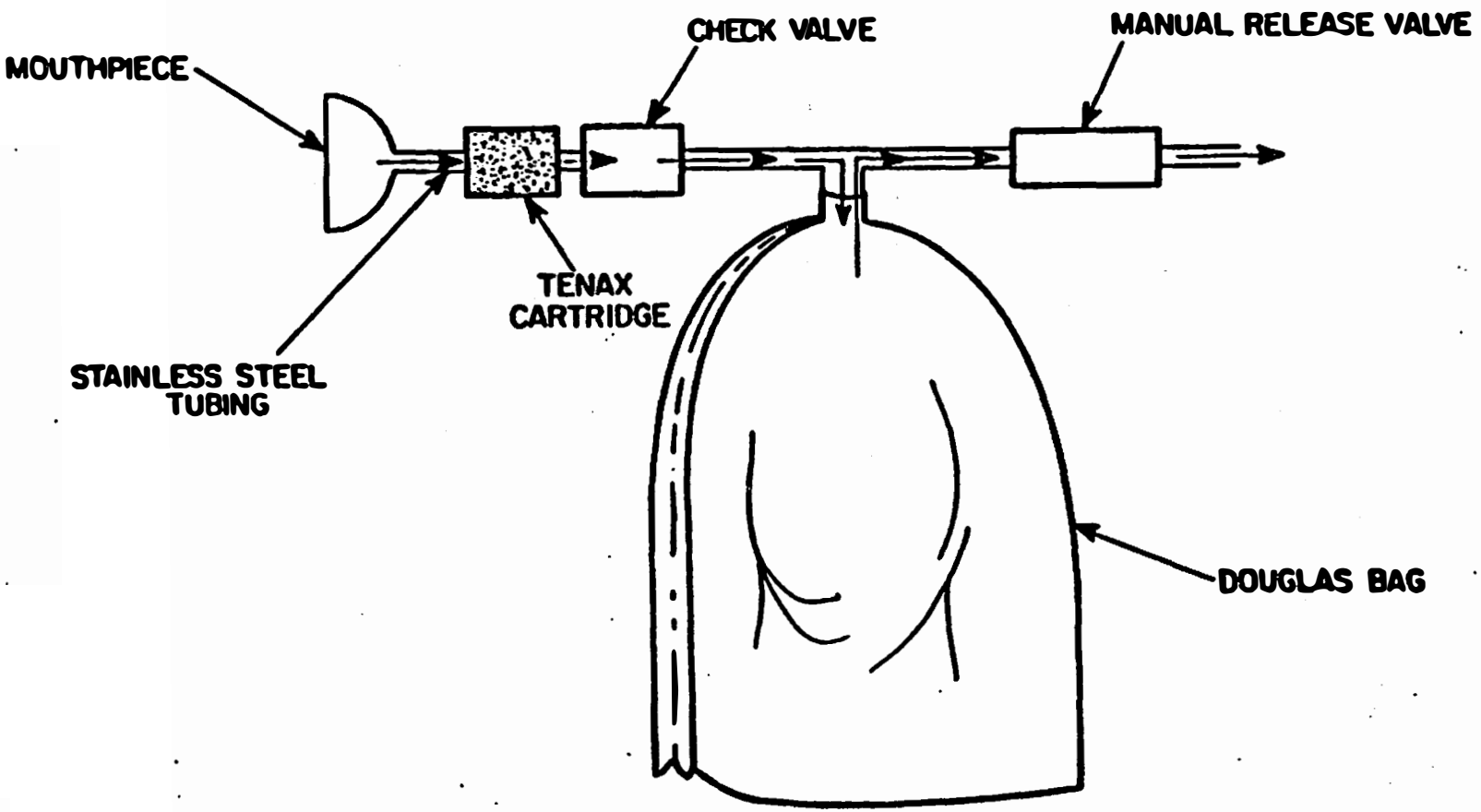


Figure 13. Breath Trap Employing Tenax Adsorbant.



4.2 Binding of Drugs to Plasma Protein

The binding of secobarbital, amphetamine, diazepam, and diphenhydramine to plasma protein was determined over the concentration ranges found after administration of therapeutic doses of these drugs. The results of our studies for the above drugs are shown in Tables 4, 5, 6 and 7, respectively. The determination of plasma protein binding of chlorpromazine using this method was not possible due to the instability of this compound in plasma. The value of the percent of codeine bound to plasma protein (29%) used in our studies is that which was reported by Judis (1977) who studied the binding of codeine, morphine, and methadone to a variety of serum proteins.

An average of 32.5 percent [coefficient of variation (CV) = 3.3%] of secobarbital in plasma was "free," i.e., not bound to plasma protein (Table 4). The percentage of "free" secobarbital was slightly higher for each subject at the higher concentration, but these differences were not significant when compared to the intersubject variability.

Amphetamine does not bind significantly to plasma protein. An average of 87 percent (CV = 2.2%) of amphetamine is plasma was "free" (Table 5). No dependence of protein binding on the concentration of amphetamine was observed over the concentration range studied.

Diazepam, however, was extensively bound to plasma protein (Table 6). An average of only 1.6 percent (CV = 17%) of diazepam in plasma was "free" (98.4 percent bound to plasma protein). The loss of radiolabeled material due to adsorption to the membrane, cell, etc. averaged 8.0% (sd = 5.4, N = 32) and was never larger than 20 percent. There is a slight, but not good, correlation between plasma concentrations of diazepam and binding. Other investigators have reported the binding of

Table 4.

Binding of Secobarbital to Plasma Protein.

Subject	Secobarbital Concentration* (μg/mL)	% of Secobarbital Not Bound to Plasma Protein
S1	1.0	33.6
	0.29	32.0
S2	0.75	34.4
	0.27	34.2
S3	0.83	31.6
	0.12	31.4
S4	0.74	32.4
	0.47	31.8
S5	0.83	32.0
	0.44	31.3
S6	0.83	33.3
	0.26	32.2
	Mean	32.5
	Coefficient of Variation	3.3

* In plasma at end of dialysis period. Binding to plasma protein was determined for each subject from the plasma sample containing the highest concentration of secobarbital and from a plasma sample containing a low concentration of secobarbital. Values reported are the averages of duplicate analyses.

Table 5.

Binding of Amphetamine to Plasma Protein.

Subject	Amphetamine Concentration* (ng/mL)	% of Amphetamine Not Bound to Plasma Protein
A1	1.6	90.0
	8.9	87.4
A2	2.6	86.0
	14.4	87.2
A3	<1	89.2
	13.4	86.6
A4	0.6	86.4
	14.1	89.1
A5	4.2	88.6
	13.4	85.5
A6	<1	86.6
	4.7	87.4
	7.6	82.4
	Mean	87.1
	Coefficient of Variation	2.2

* In plasma at end of dialysis period. Binding to plasma protein was determined for each subject from the plasma sample containing the highest concentration of amphetamine and from a plasma sample containing a low concentration of amphetamine. Values reported are the averages of duplicate analyses.

Table 6. Plasma Binding Data for Diazepam

Subject	Diazepam Concentration * (ng/mL)	% of Diazepam Not Bound to Plasma Protein
D1	25	1.54
	151	1.49
	201	1.30
D2	42	1.58
	191	1.41
	323	1.70
D3	34	2.06
	133	1.58
	450	2.09
D4	11	1.42
	201	1.42
	415	2.08
D5	10	1.60
	264	1.36
D6	45	1.28
	376	1.79
Mean		1.61
Range		1.28-2.09
Coefficient of Variation		17%

*In plasma at end of dialysis period. Binding to plasma protein was determined for each subject from the plasma sample containing the highest concentration of diazepam and from a plasma sample containing a low concentration. For some subjects, binding in a sample containing an intermediate concentration was also determined. Values reported are the averages of duplicate determinations.

Table 7.

Binding of Diphenhydramine (DPHM) to Plasma Protein.

Subject	DPHM Concentration* (ng/mL)	% of DPHM Not Bound to Plasma Protein
DP-1	4.2	17
	5.1	23
	70	23
	165	30
DP-2	4.1	19
	15.9	30
	55	36
	255	25
DP-3	12.0	34
DP-4	7.1	31
	200	32
DP-5	6.1	27
	126	33
DP-6	33	29
	319	18
DP-7	3.4	31
	149	36
Mean		28
Coefficient of Variation		22
Range		17-36

*In plasma at end of dialysis period. Binding to plasma protein was determined for each subject from the plasma sample containing the highest concentration of DPHM and from a plasma sample containing a low concentration. For some subjects, binding in sample containing intermediate concentrations was also determined. Values reported are the averages of duplicate determinations.

diazepam to plasma protein to be 95% (Sturdee, 1976), 96.8% (Klotz et al., 1976), 98% (Kanto et al., 1975) and 96.5-98% (DiGregorio et al., 1978). We took special care to purify our radiolabeled diazepam by HPLC before use, since small quantities of non-protein bound impurities would result in a significant increase in the amount of "free diazepam" calculated to be present in plasma.

The binding of diphenhydramine to plasma protein was found to be the most variable (Table 7) of any drug studied. The percent of "free" diphenhydramine in plasma ranged from 17 to 36 percent, with an average of 28 percent.

4.3 Analysis and Correlation of Concentrations of Drugs in Plasma and Saliva

4.3.1 Secobarbital

Detailed information on the extraction and analysis of secobarbital from plasma and saliva is given in Appendix A.1. Extracts from plasma and saliva could be chromatographed on either a methyl silicone (SE-30) or a polyethylene glycol (Carbowax) coated capillary GC column. Sample chromatograms obtained from an extract of plasma that had been spiked with secobarbital and internal standards are shown in Figures 6 and 5, respectively, for the two different columns. Different internal standards were used for the two analyses. Since the chromatogram from the Carbowax column is much "cleaner" in the region where secobarbital and its internal standards elute, all subsequent evaluations utilized this column. Linear calibrations (log-log scale) were established for secobarbital in plasma from 0.02 to 4.0 μg/mL and in saliva from 0.05 to 2.0 μg/mL. The relative retention times of other barbituates and their analogs on Carbowax have been reported (Berry, 1973) and are shown in Table 8.

Table 8.

Relative Retention Times of Selected Barbiturates and Barbiturate Analogs on a Packed Carbowax 20M Column, Isothermal at 205°C*

Compound	Relative Retention Time+
Allylbarbituric acid	0.73
Amylobarbitone	0.70
Barbital	0.43
Butabarbital	0.67
n-Butylallylbarbituric acid	0.87
Cyclobarbital	2.8
Glutethimide	0.42
Heptabarbital	3.7
Hexobarbital	0.48
Methaqualone	0.73
Methohexital	0.25
Nealbarbitone	0.82
Pentobarbital	0.77
Phenobarbital	4.9
Secobarbital	1.0
Thiopental	0.83

*Data taken from D. J. Berry, J. Chromatogr., 86, 89-105 (1973).

+Based on Retention time of Secobarbital = 1.0

The results of the analyses of plasma and saliva from six subjects, each dosed at two differnt levels (0.61 and 1.22 mg/kg) with sodium secobarbital, are listed in Table 9. Predicted values for the concentration of secobarbital in plasma $[S]_c$ were calculated from the concentration in saliva $[S]_s$ by the equation $[S]_c = [S]_s/fp$ where fp is the fraction of secobarbital not bound to plasma protein. In this case, additional corrections for the differences in the pH of plasma and saliva were not made. For saliva pH's of 6.5 and 7.0, $[S]_c$ would be increased by factors of 1.3 and 1.2, respectively. These corrections, however, do not result in better agreements between plasma and saliva concentrations of secobarbital.

The average ratio of the predicted to observed concentrations of secobarbital in plasma for all determinations is 1.08 (CV = 42%, n = 107). The average ratio for the 0.5 hr samples is 2.5 (CV = 40%, n = 6). Omitting these samples the average ratio of the remaining samples is 0.99 (CV = 21%n, n = 101). While the theoretical reasons for omitting these samples are uncertain, empirically, their omission results in a much better correlation of drug concentrations in plasma and saliva. Perhaps, minute drug particles still remain in the oral cavity at this time or are somehow returned to the oral cavity from the upper digestive tract. The average ratio for the 1.0 hr samples, 1.17 (CV = 28%, n = 10) is slightly higher ($P < 0.01$) than the average ratio of 0.97 (CV = 18%, n = 91%) for the samples taken after this time.

A plot of the predicted $[S]_c$ versus observed $[S]_p$ concentrations of secobarbital in plasma on a log-log scale is shown in Figure 14. The least squares linear regression of this data, expressed in ng/mL, produces a best-fit line represented by the equation:

Table 9.

Concentration of Secobarbital in Plasma and Saliva Following a Single Oral Dose

Time (hrs)	Observed [Secobarbital] in Saliva (μg/mL)	Observed [Secobarbital] in Plasma (μg/mL)	Predicted [Secobarbital] in Plasma (μg/mL)	Predicted / Observed in Plasma
	Subject 1; 0.61 mg/kg Dose			
0.5	0.16	0.31	0.49	1.6
1.0	0.23	0.71	0.71	1.0
1.5	0.17	0.67	0.52	0.78
2.0	0.15	0.53	0.46	0.87
3.0	0.13	0.43	0.40	0.93
4.0	0.12	0.41	0.37	0.90
6.0	0.11	0.36	0.34	0.94
8.0	0.10	0.34	0.31	0.91
11.0	0.086	0.32	0.26	0.81
24.0	0.060	0.22	0.18	0.82
	Subject 2; 0.61 mg/kg Dose			
0.5	0.00	sample lost	--	--
1.0	0.006	sample lost	0.02	--
1.5	0.085	0.18	0.26	1.4
2.0	0.17	0.42	0.52	1.2
3.0	0.13	0.49	0.40	0.82
4.0	0.11	0.44	0.34	0.77
6.0	0.11	0.39	0.34	0.87
8.0	0.11	0.38	0.34	0.89
11.0	0.095	sample lost	0.29	--
24.0	0.059	sample lost	0.18	--
	Subject 3; 0.61 mg/kg Dose			
0.5	0.00	0.033	--	--
1.0	0.036	0.061	0.11	1.8
1.5	0.046	0.14	0.14	1.0
2.0	a	0.32	--	--
3.0	0.12	0.37	0.37	1.0
4.0	0.14	0.38	0.43	1.1
6.0	0.12	0.39	0.37	0.95
8.0	0.13	0.36	0.40	1.1
11.0	0.12	0.28	0.37	1.3
24.0	0.046	0.11	0.14	1.3

Table 9 (continued)

Concentration of Secobarbital in Plasma and Saliva Following a Single Oral Dose

Time (hrs)	Observed [Secobarbital] in Saliva (μg/mL)	Observed [Secobarbital] in Plasma (μg/mL)	Predicted [Secobarbital] in Plasma (μg/mL)	Predicted / Observed in Plasma
	Subject 4; 0.61 mg/kg Dose			
0.5	0.11	0.11	0.34	3.1
1.0	0.23	0.48	0.71	1.5
1.5	0.15	0.48	0.46	0.96
2.0	0.15	0.51	0.46	0.90
3.0	0.14	0.44	0.43	0.98
4.0	0.14	0.52	0.43	0.83
6.0	0.10	0.37	0.31	0.84
8.0	0.10	0.35	0.31	0.89
11.0	0.096	0.33	0.30	0.91
24.0	0.062	0.18	0.19	1.1
	Subject 5; 0.61 mg/kg Dose			
0.5	0.041	0.048	0.13	2.7
1.0	0.18	0.79	0.55	0.70
1.5	0.23	0.67	0.71	1.1
2.0	0.22	0.60	0.68	1.1
3.0	0.15	0.50	0.46	0.92
4.0	0.15	0.42	0.46	1.1
6.0	0.11	0.43	0.34	0.79
8.0	0.11	0.37	0.34	0.92
11.0	0.10	0.33	0.31	0.94
24.0	0.064	0.25	0.20	0.80
	Subject 6; 0.61 mg/kg Dose			
0.5	0.095	0.089	0.29	3.3
1.0	0.23	0.53	0.71	1.3
1.5	0.20	0.56	0.62	1.1
2.0	0.18	0.59	0.55	0.93
3.0	0.14	0.50	0.43	0.86
4.0	0.14	0.43	0.43	1.0
6.0	0.13	0.43	0.40	0.93
8.0	0.12	0.42	0.73	1.7
11.0	0.10	0.35	0.31	0.89
24.0	0.046	0.19	0.14	0.74

Table 9 (continued)

Concentration of Secobarbital in Plasma and Saliva Following a Single Oral Dose

Time (hrs)	Observed [Secobarbital] in Saliva (μg/mL)	Observed [Secobarbital] in Plasma (μg/mL)	Predicted [Secobarbital] in Plasma (μg/mL)	Predicted / Observed in Plasma
	Subject 1; 1.22 mg/kg Dose			
0.5	0.00	0.088	--	--
1.0	0.38	1.1	1.2	1.1
1.5	0.51	1.4	1.6	1.1
2.0	0.40	1.2	1.2	1.0
3.0	0.29	0.99	0.89	0.90
4.0	0.25	0.72	0.77	1.1
6.0	0.23	0.75	0.71	0.95
8.0	--	0.66	--	--
11.0	0.18	0.65	0.55	0.85
24.0	0.12	0.39	0.37	0.95
	Subject 2; 1.22 mg/kg Dose			
0.5	0.00	0.024	--	--
1.0	0.22	0.7	0.68	0.97
1.5	0.41	1.1	1.3	1.2
2.0	0.38	1.3	1.2	0.93
3.0	0.34	1.0	1.0	1.0
4.0	0.29	0.78	0.89	1.1
6.0	0.27	0.71	0.83	1.2
8.0	--	0.76	--	--
11.0	0.20	0.58	0.62	1.1
24.0	0.10	0.36	0.31	0.86
	Subject 3; 1.22 mg/kg Dose			
0.5	0.00	0.004	--	--
1.0	0.00	0.028	--	--
1.5	0.32	0.79	0.98	1.2
2.0	0.42	1.1	1.3	1.2
3.0	0.27	0.95	0.83	0.87
4.0	0.22	0.78	0.68	0.87
6.0	0.18	0.72	0.55	0.76
8.0	0.18	0.74	0.55	0.74
11.0	0.12	0.48	0.37	0.77
24.0	0.068	0.16	0.19	1.2

Table 9 (continued)

Concentration of Secobarbital in Plasma and Saliva Following a Single Oral Dose

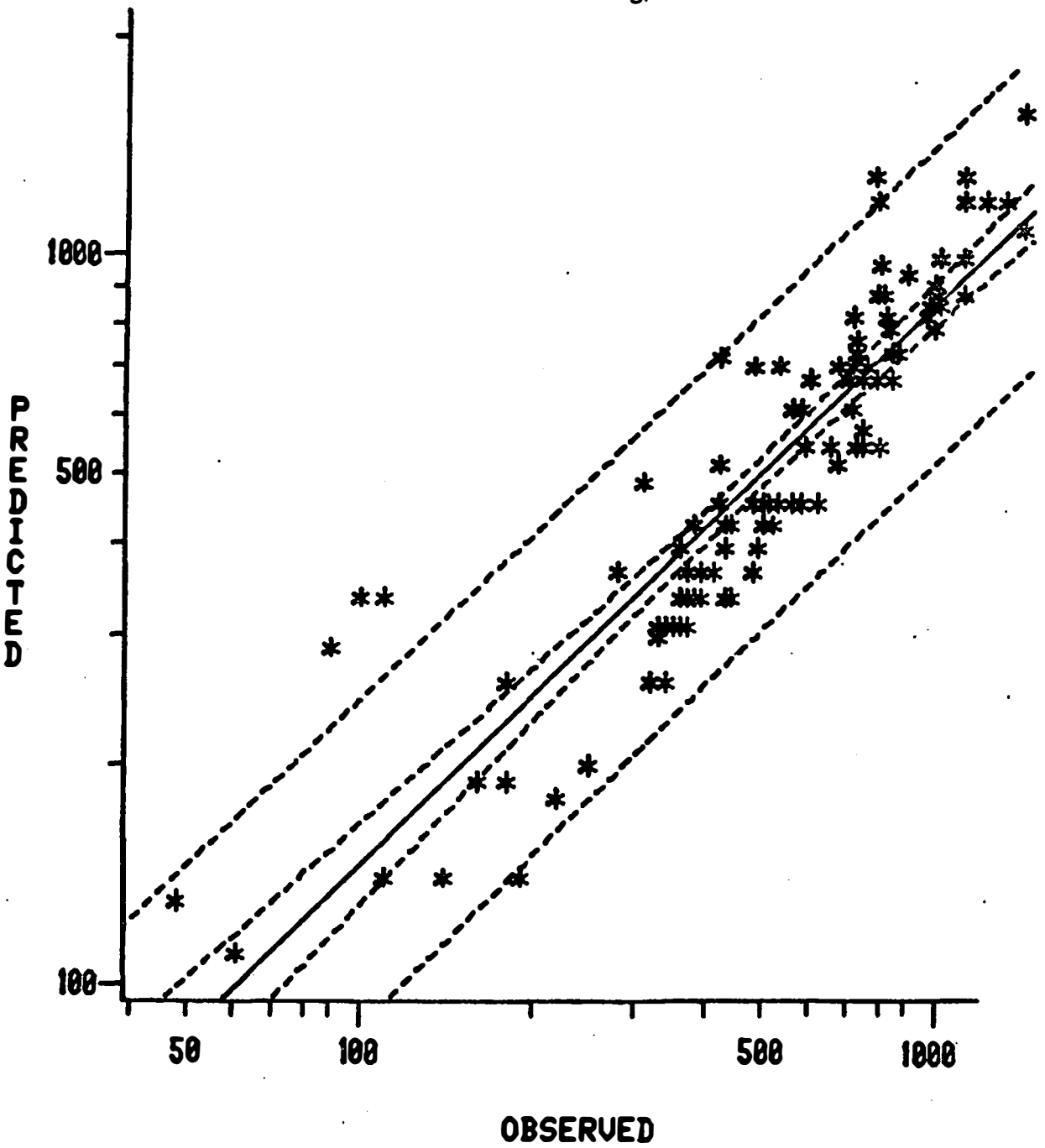
Time (hrs)	Observed [Secobarbital] in Saliva (μg/mL)	Observed [Secobarbital] in Plasma (μg/mL)	Predicted [Secobarbital] in Plasma (μg/mL)	Predicted / Observed in Plasma
	Subject 4; 1.22 mg/kg Dose			
0.5	0.00	0.00	--	--
1.0	0.38	1.2	1.2	1.0
1.5	0.30	0.98	0.92	0.94
2.0	0.28	0.96	0.86	0.90
3.0	0.28	1.0	0.86	0.86
4.0	0.24[b]	0.83	0.74	0.89
6.0	0.41[b]	0.77	1.3	1.7
8.0	0.26	0.82	0.80	0.98
11.0	0.22	0.74	0.68	0.92
24.0	0.15	0.62	0.46	0.74
	Subject 5; 1.22 mg/kg Dose			
0.5	0.26	0.98	0.80	0.82
1.0	0.36	1.4	1.1	0.79
1.5	0.38	1.1	1.2	1.1
2.0	0.34	1.1	1.0	0.91
3.0	0.29	0.80	0.89	1.1
4.0	0.27	0.81	0.83	1.0
6.0	0.24	0.85	0.74	0.87
8.0	0.22	0.83	0.68	0.82
11.0	0.15	0.58	0.46	0.79
24.0	0.20	0.58	0.62	1.1
	Subject 6; 1.22 mg/kg Dose			
0.5	0.11	0.10	0.34	3.4
1.0	0.40	0.78	1.2	1.5
1.5	0.31	0.88	0.95	1.1
2.0	0.29	1.1	0.89	0.81
3.0	0.26	0.82	0.80	0.98
4.0	0.24	0.72	0.74	1.0
6.0	0.19	0.74	0.58	0.78
8.0	0.20	0.71	0.62	0.87
11.0	0.15	0.56	0.46	0.82
24.0	0.085	0.34	0.26	0.76

[a] An interfering GC peak prevented quantitation.
[b] Sample contained a very viscous lump.

Figure 14. Correlation between the predicted and observed concentrations of Secobarbital in plasma. Predicted values are those calculated from concentrations in saliva.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.



$\log [S]_p = 1.29 \log [S]_c - 0.786$, with a correlation coefficient (r) of 0.898. This equation can be expressed in terms of the concentration of secobarbital in saliva $[S]_s$ and f_p ($f_p = 0.325$, cf section 5.2): $\log [S]_p = 1.29 \log [S]_s - 0.156$. The inner set of dashed lines in Figure 14 represent the 95% confidence interval for the line, while the outer pair of lines represent the 95% confidence interval of the individual data points. This means that one is 95% confident that the "true" line lies between the two inner dashed lines and that additional data points will fall between the two outer lines. Similar "confidence interval" lines appear on all subsequent figures showing results of linear regression analyses. Additional statistical data for this regression is in Appendix A.1. Figure 15 shows a similar plot in which the 0.5 hr samples have been omitted. The correlation coefficient for this linear regression is 0.943 which is significantly better than the correlation when the 0.5 hr samples are included. The equation for the best fit line when concentrations are expressed in ng/mL is: $\log [S]_p = 1.06 \log [S]_c - 0.148$ or $\log [S]_p = 1.06 \log [S]_s + 0.369$. Additional statistical data for the regression analysis is included in Appendix A.1.

The results of the study of the effects of stimulation and suppression of salivary flow on the correlation of secobarbital in plasma and saliva in subjects S1 and S6 are shown in Table 10. The ratios of calculated to observed concentrations in plasma do not change after suppression or stimulation of the salivary flow for either subject.

4.3.2 Amphetamine

Detailed information on the extraction and analysis of d,l-amphetamine from plasma and saliva is given in Appendix A.2. The

Figure 15. Correlation between the predicted and observed concentrations of Secobarbital at times one hour or later after administration of drug. Predicted values are those calculated from concentrations in saliva.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.

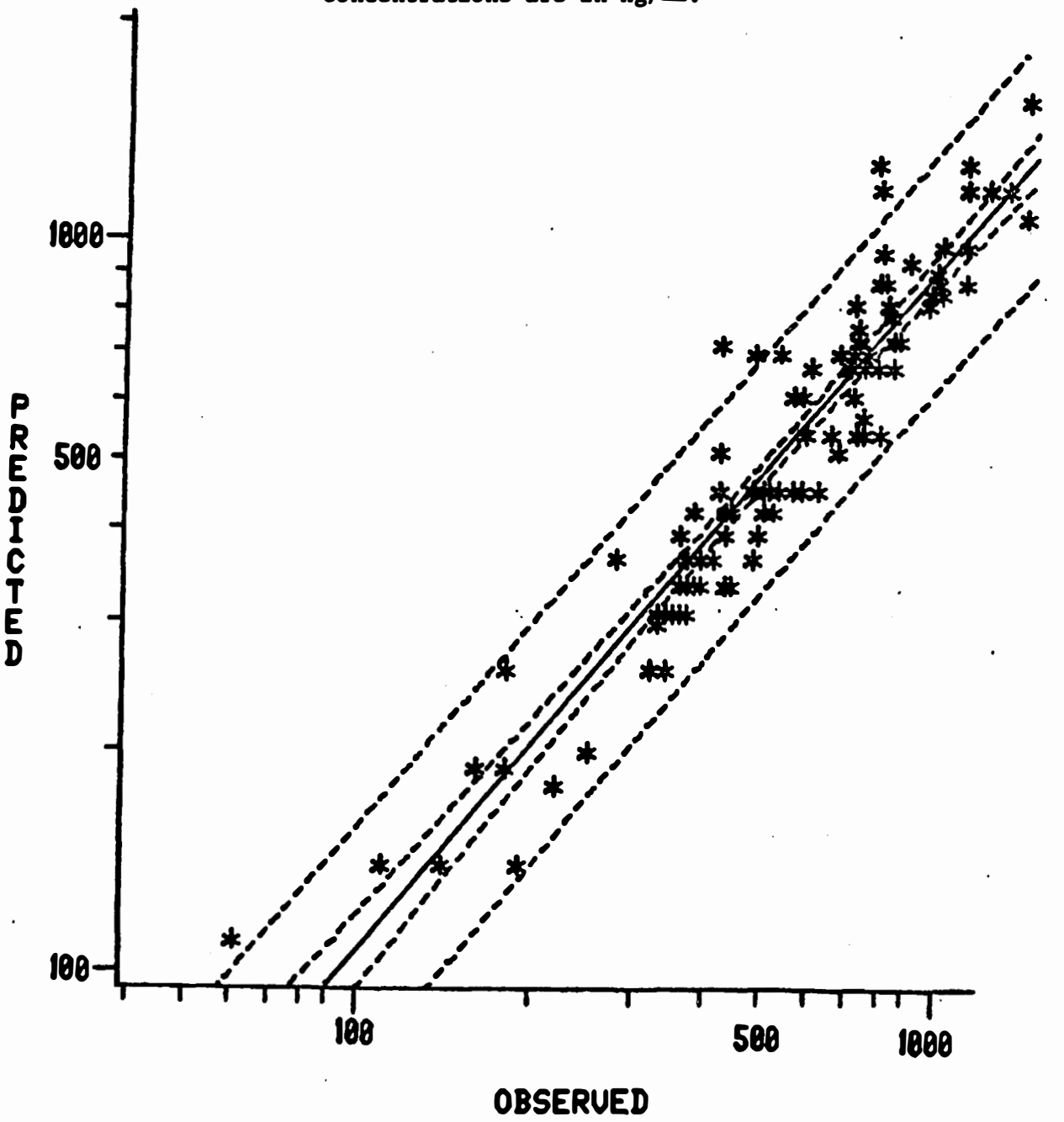
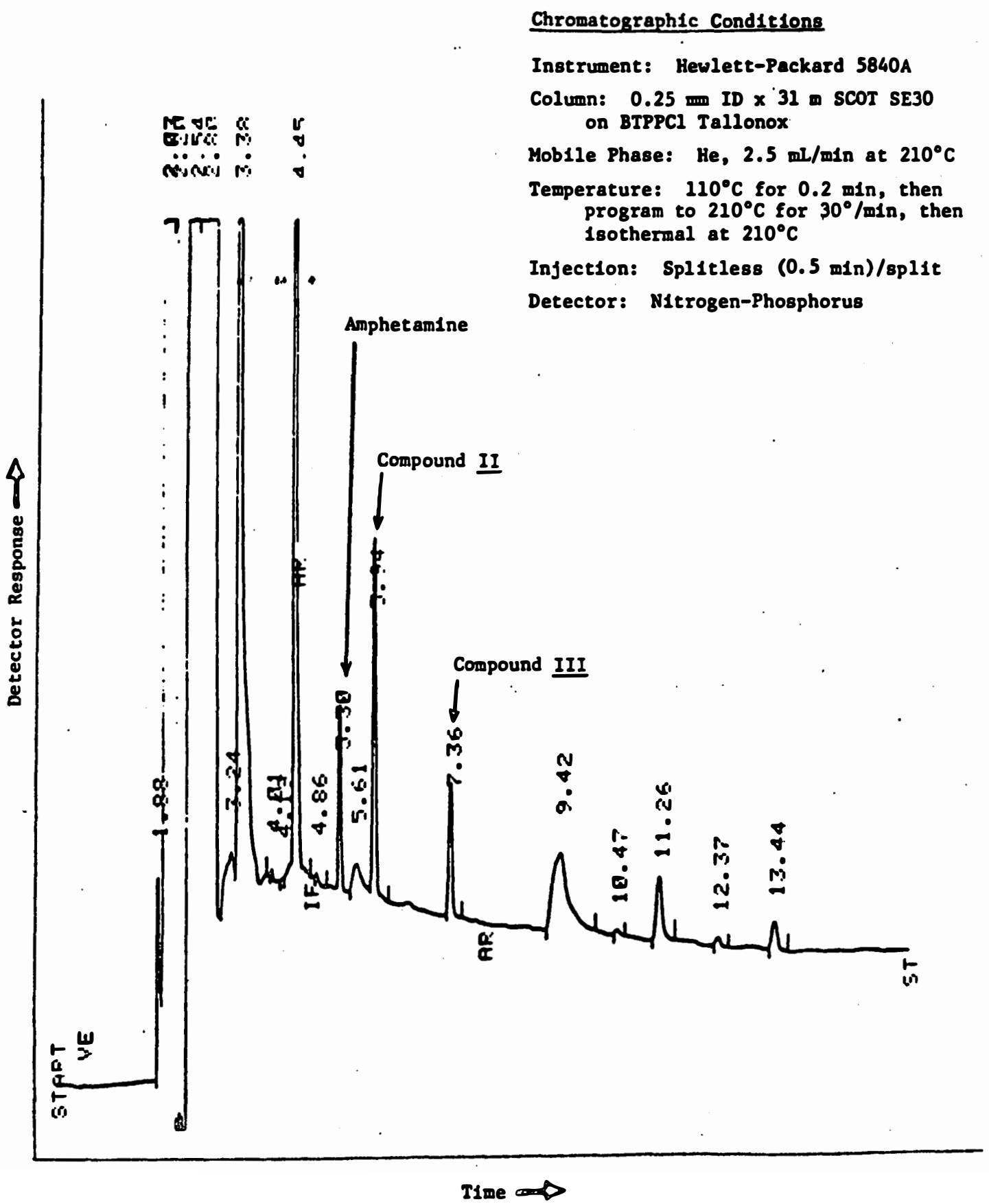


Table 10

Effect of Stimulation and Suppression of Salivary Flow on the Correlation of Concentrations of Secobarbital in Plasma and Saliva

Subject S1	Time (hr)	[Secobarbital] in Plasma (µg/mL)	[Secobarbital] in Saliva (µg/ml)	Predicted Observed in Plasma
100 mg Secobarbital given	0			
	1.5	1.64	0.41	1.3
	2.0	1.04	0.38	0.98
	3.0	0.91	0.27	1.1
Saliva Flow Stimulated →				
	3.25	0.94	0.25	1.1
	3.50	0.79	0.26	0.98
Saliva Flow Inhibited →				
	3.75	1.08	0.26	1.4
	4.0	0.93	0.26	1.2
Subject S6				
100 mg Secobarbital given	0			
	1.5	0.71	0.26	1.0
	2.0	1.04	0.30	1.1
	3.0	0.97	0.32	0.98
Saliva Flow Stimulated →				
	3.25	0.94	0.32	0.94
	3.50	0.95	0.32	0.98
Saliva Flow Inhibited →				
	3.75	0.96	0.28	1.1
	4.0	0.92	0.28	1.1

Figure 16. Chromatogram of Amphetamine Extracted from Plasma at a Concentration of 20 ng/mL on a SE-30 Capillary Column



chromatographic analysis of the small amounts of amphetamine present in plasma and saliva presents severe problems due to the adsorption of this drug to the column materials. Therefore the amphetamine was converted in the analytical procedure to its trifluoroacetamide derivative. This derivative has low adsorption and good chromatographic properties. Other investigators have similarly found it necessary to form derivatives of amphetamine in order to measure its concentration in biological fluids (Wan, et. al, 1978; Matin, et. al., 1977; O'Brien, et. al, 1972). Both methyl silicone (SE-30) and polyethylene glycol (Carbowax-20M) coated capillary GC columns were evaluated for the analysis of amphetamine in samples of plasma and saliva. Because of the much sharper peaks and the cleaner baseline in the region of chromatogram corresponding to amphetamine and its internal standards, the SE-30 column was chosen for the analysis of this drug. Figure 16 shows a typical chromatogram of amphetamine (trifluoroacetamide) extracted from plasma. Compounds II and III in the chromatogram are internal standards. Linear calibrations (log-log scale) were established for amphetamine in plasma from 1.0-100 ng/mL and in saliva from 1.0-500 ng/mL.

The results of the analyses of plasma and saliva from six subjects, each dosed at two different levels (0.061 and 0.122 mg/kg) with d,l-amphetamine, are shown in Table 11. Matin et al. (1977) reported that concentrations of amphetamine in saliva were approximately 2 1/2 times higher than the corresponding concentrations in plasma. For some of the subjects in our study and at some time periods, similar results were found. However, there appeared to be a large subject to subject variation in the concentrations of amphetamine in saliva following administration of the same amount of drug. Subject A2 is particularly "abnormal,"

Table 11.

Concentration of Amphetamine in Plasma and Saliva Following a Single Oral Dose.

Time (hr)	Saliva pH	Observed [Amphetamine] in Saliva (ng/mL)	Observed [Amphetamine] in Plasma (ng/mL)	Predicted [Amphetamine] in Plasma (ng/mL)	Predicted / Observed in Plasma
Subject A1; 0.061 mg/kg dose					
0.5	6.95	5.4	3.0	2.1	0.70
1.0	6.90	12	5.8	4.3	0.74
1.5	6.75	24	9.2	6.0	0.65
2.0	6.85	26	11	8.4	0.76
3	6.90	18	12	6.5	0.54
4	6.95	16	12	6.5	0.54
6	7.05	15	12	7.6	0.63
8	7.05	14	9.8	7.1	0.72
11	7.05	13	9.4	6.6	0.70
24	6.95	5.7	4.0	2.3	0.58
Subject A2; 0.061 mg/kg dose					
0.5	6.85	12	5.4	4.0	0.74
1.0	6.90	83	14	30	2.1
1.5	6.80	183	13	53	4.1
2.0	6.80	182	11	53	4.8
3	6.70	160	10	37	3.7
4	6.75	69	10	18	1.8
6	6.80	86	10	25	2.5
8	6.65	115	9.1	24	2.6
11	6.60	77	7.0	14	2.0
24	6.45	44	2.6	5.7	2.2
Subject A3; 0.061 mg/kg dose					
0.5	6.65	Trace	Trace	-	-
1.0	6.75	10	Trace	2.6	-
1.5	6.80	20	4.8	5.8	1.2
2.0	6.80	25	7.2	7.2	1.0
3	6.65	24	13	4.9	0.38
4	6.80	20	11	6.0	0.54
6	6.85	18	8.0	5.8	0.72
8	6.75	19	8.8	4.9	0.56
11	6.80	15	6.4	4.3	0.67
24	6.65	3.0	2.0	0.61	0.30

Table 11. Continued.

Time (hr)	Saliva pH	Observed [Amphetamine] in Saliva (ng/mL)	Observed [Amphetamine] in Plasma (ng/mL)	Predicted [Amphetamine] in Plasma (ng/mL)	Predicted / Observed in Plasma
Subject A4; 0.061 mg/kg dose					
0.5	6.60	2.1	4.8	0.38	0.079
1.0	6.55	23	9.5	3.7	0.39
1.5	6.65	30	16	6.1	0.38
2.0	6.70	36	16	8.3	0.52
3	6.65	33	17	6.8	0.40
4	6.70	22	14	5.1	0.36
6	6.75	19	13	4.9	0.38
8	6.55	29	14	4.7	0.34
11	6.55	19	13	3.1	0.24
24	6.65	9.4	6.0	1.9	0.32
Subject A5; 0.061 mg/kg dose					
0.5	7.05	0	0	0	-
1.0	6.95	20	12	8.2	0.68
1.5	7.15	28	12	18	1.5
2.0	7.05	34	11	18	1.6
3	7.10	38	15	22	1.5
4	7.05	31	16	16	1.0
6	7.10	40	19	23	1.2
8	7.10	41	18	24	1.3
11	7.05	28	15	15	1.0
24	7.05	12	5.9	6.2	1.0
Subject A6; 0.061 mg/kg dose					
0.5	6.65	11	1.3	2.3	1.8
1.0	6.45	15	5.6	1.9	0.34
1.5	6.65	45	14	9.4	0.67
2.0	6.75	37	12	9.7	0.81
3	6.70	38	15	9.0	0.60
4	6.65	22	10	4.6	0.46
6	6.85	21	14	6.9	0.49
8	6.75	25	10	6.6	0.66
11	6.75	15	8.9	4.0	0.44
24	6.70	7.8	5.3	1.8	0.34

Table 11. Continued.

Time (hr)	Saliva pH	Observed [Amphetamine] in Saliva (ng/mL)	Observed [Amphetamine] in Plasma (ng/mL)	Predicted [Amphetamine] in Plasma (ng/mL)	Predicted / Observed in Plasma
Subject A1; 0.122 mg/kg dose					
0.5	7.20	6.18	3.0	4.5	1.5
1.0	7.20	37.8	11.4	27	2.4
1.5	7.15	55.2	15.8	36	2.3
2	7.10	43.2	18.4	25	1.4
3	7.10	49.9	20.5	29	1.4
4	7.15	45.4	16.7	29	1.7
6	7.15	35.2	21.9	23	1.0
8	7.20	37.3	16.9	27	1.6
11	7.50	30.2	17.4	43	2.5
24	7.20	15.0	6.1	11	1.8
Subject A2; 0.122 mg/kg dose					
0.5	6.85	51.2	2.6	17	6.5
1.0	6.90	290	10.2	107	10.5
1.5	6.85	412	12.8	136	10.6
2.0	7.15	140	11.8	92	7.8
3	6.75	247	13.4	64	4.8
4	6.45	188	14.4	25	1.7
6	6.75	142	19.0	37	1.9
8	6.80	167	12.5	49	3.9
11	6.85	117	7.3	38	5.2
24	6.80	29.8	2.9	8.7	3.0
Subject A3; 0.122 mg/kg dose					
0.5	6.80	3.20	0	0.92	-
1.0	6.75	3.51	0	0.91	-
1.5	6.70	10.3	4.2	2.4	0.57
2.0	6.95	29.6	9.2	12	1.3
3	6.80	49.0	17.4	14	0.80
4	6.60	54.3	24.9	10	0.40
6	6.75	42.4	25.4	11	0.43
8	6.85	40.5	22.1	13	0.59
11	6.90	35.4	13.6	13	0.96
24	6.65	16.5	3.8	3.4	0.89

Table 11. Continued.

Time (hr)	Saliva pH	Observed [Amphetamine] in Saliva (ng/mL)	Observed [Amphetamine] in Plasma (ng/mL)	Predicted [Amphetamine] in Plasma (ng/mL)	Predicted / Observed in Plasma
Subject A4; 0.122 mg/kg dose					
0.5	6.62	1.2	1.1	0.2	0.18
1.0	6.85	31.4	12.3	10	0.81
1.5	6.80	66.9	23.7	19	0.80
2.0	6.85	78.0	23.8	25	1.1
3	6.50	78.9	26.3	11	0.42
4	6.61	66.1	26.7	12	0.45
6	6.60	67.1	26.3	12	0.46
8	6.55	61.0	37.4	9.9	0.26
11	6.90	42.8	29.1	16	0.55
24	-	15.4	5.2	-	-
Subject A; 0.122 mg/kg dose					
0.5	6.75	5.6	8.0	1.4	0.18
1.0	7.05	32.3	21.2	17	0.80
1.5	6.95	68.2	28.8	28	0.97
2.0	7.05	73.7	27.6	38	1.4
3	6.60	62.3	30.0	11	0.37
4	6.95	81.6	25.0	33	1.3
6	6.95	81.0	25.0	33	1.3
8	7.05	63.8	24.2	33	1.4
11	7.00	39.5	14.6	18	1.2
24	6.95	13.8	4.0	5.7	1.4
Subject A6; 0.122 mg/kg dose					
0.5	6.70	0.0	0.0	0	-
1.0	6.60	11.5	4.7	2.2	0.47
1.5	6.60	75.7	9.9	14	0.71
2.0	6.50	94.5	15.7	14	0.89
3	6.45	51.4	20.9	6.8	0.32
4	6.75	66.4	19.0	17	0.89
6	6.75	69.0	15.0	18	1.2
8	6.85	39.2	13.9	13	0.93
11	6.60	34.4	8.8	6.5	0.74
24	6.70	8.0	1.3	1.8	1.4

with concentrations of amphetamine in saliva 5-10 times as high as any of the other subjects.

Predicted concentrations of amphetamine in plasma were calculated from concentrations in saliva by the Henderson-Hasselbalch-derived equation discussed in section 2.2. The ratio of predicted to observed concentrations in plasma average 1.43 (CV = 124%, n = 113) and range from 0.18 to 10.6. The ratios for subject A2 averaged 2.6 for the lower dose of amphetamine and 5.6 for the higher dose while the corresponding ratios for subject A4 were 0.34 and 0.56, respectively. There is no significant difference in the ratios for the 0.5 hr samples and those for the later times as was observed for secobarbital.

Plots of the concentrations of amphetamine in saliva versus concentrations in plasma and of the predicted versus observed concentrations in plasma are shown in Figures 17 and 18, respectively. Correlation coefficients of the least squares linear regression (log-log scale) were 0.61 and 0.62. Thus, there is poor correlation between concentrations of amphetamine in saliva and plasma. Attempts to use an "internal standard" in saliva in order to obtain better correlations are described in section 4.4 of this report.

4.3.3 Chlorpromazine

Detailed information on the extraction and analysis of chlorpromazine from plasma and saliva, including preparation of the internal standard, is given in Appendix A.3. Extracts from plasma and saliva were chromatographed on a polyethylene glycol (Carbowax 20M) coated capillary GC column. A chromatogram obtained from an extract of saliva that had been spiked with chlorpromazine and its internal standard is shown in Figure 19. Linear calibrations (log-log scale) were established for chlorpromazine

Figure 17. Correlation between saliva and plasma concentrations of Amphetamine.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.

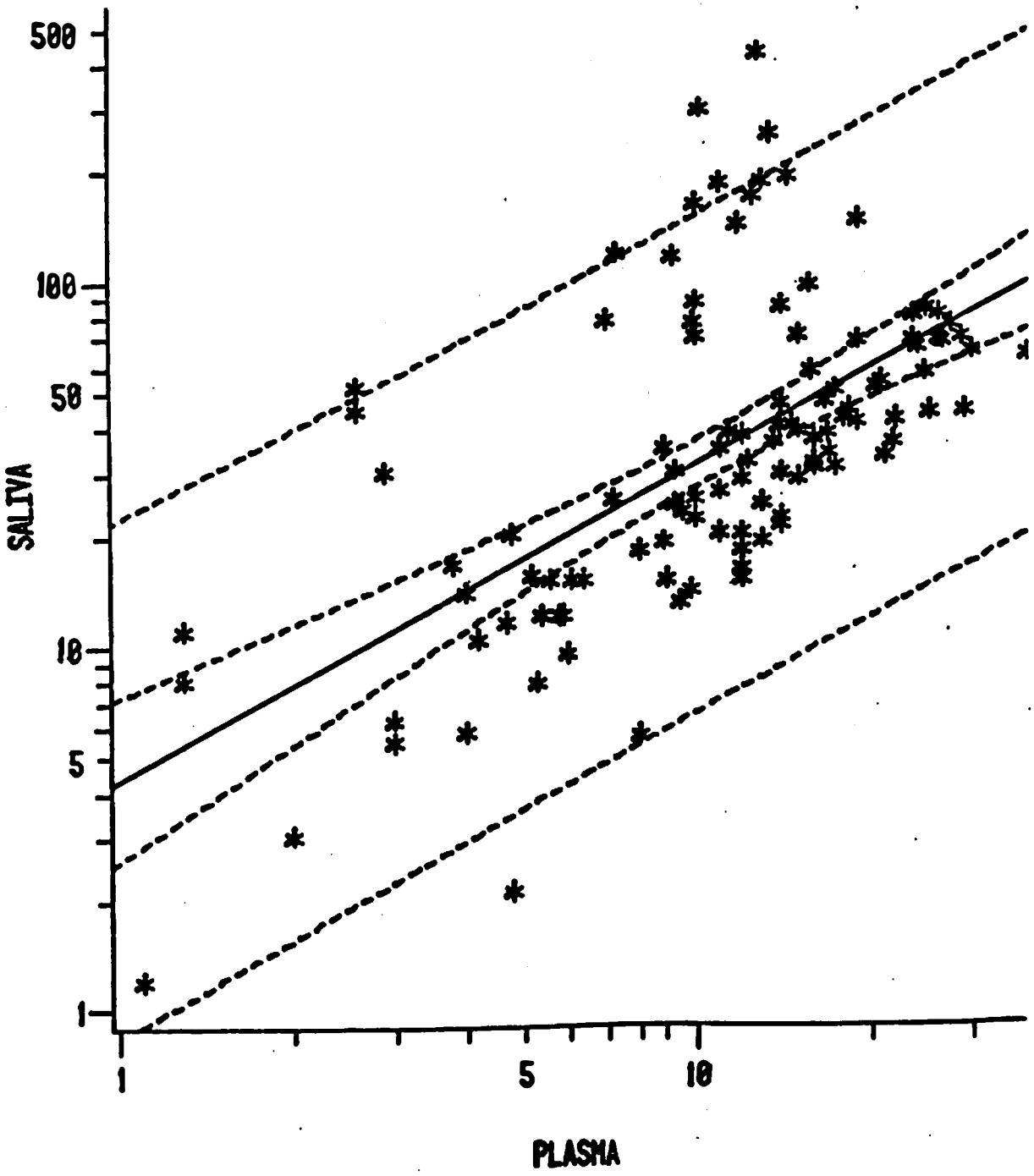


Figure 18. Correlation between predicted and observed concentrations of Amphetamine in plasma. The predicted values are those calculated from concentrations in saliva by the Henderson-Hasselbalch-derived equation.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.
Concentrations are in ng/mL.

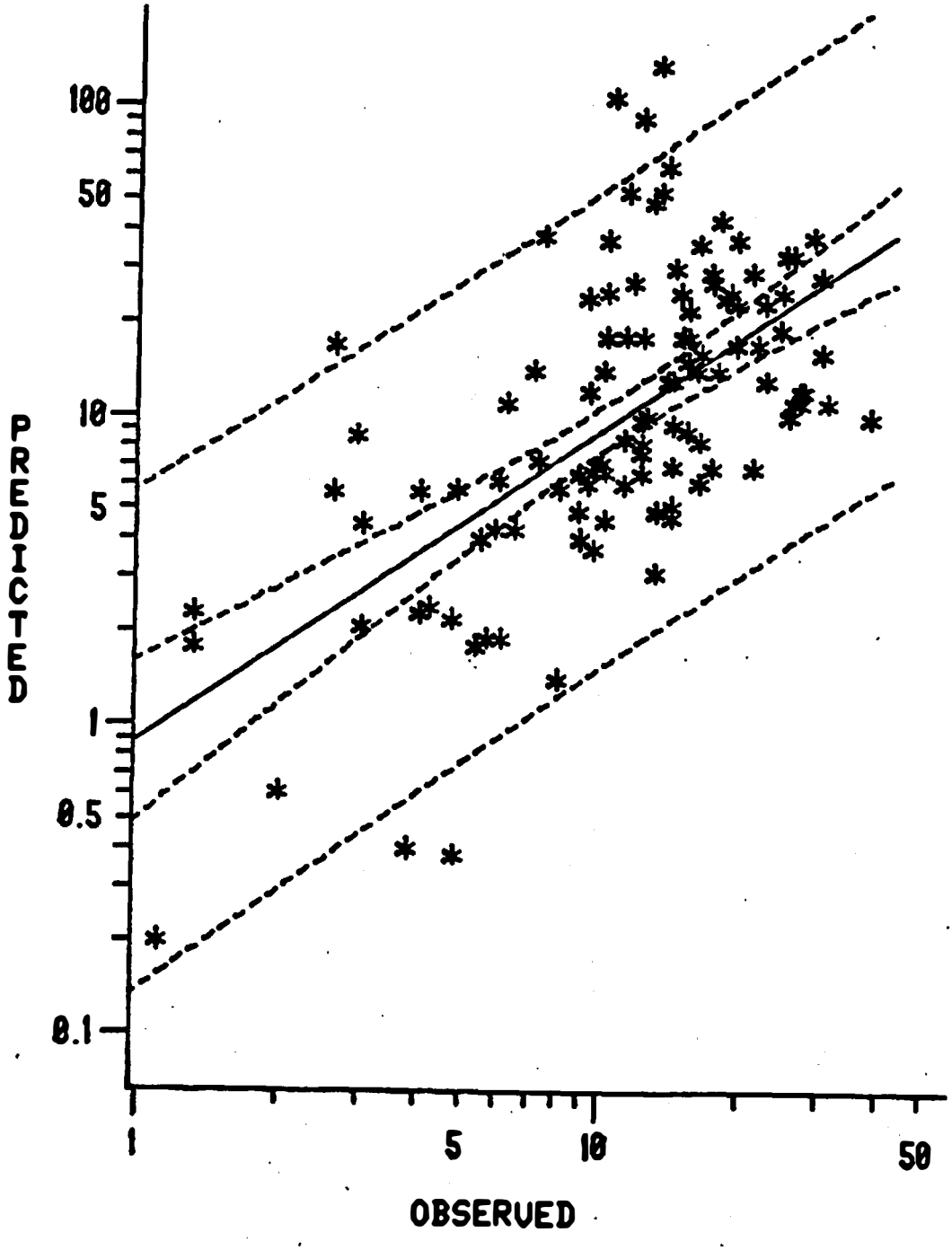
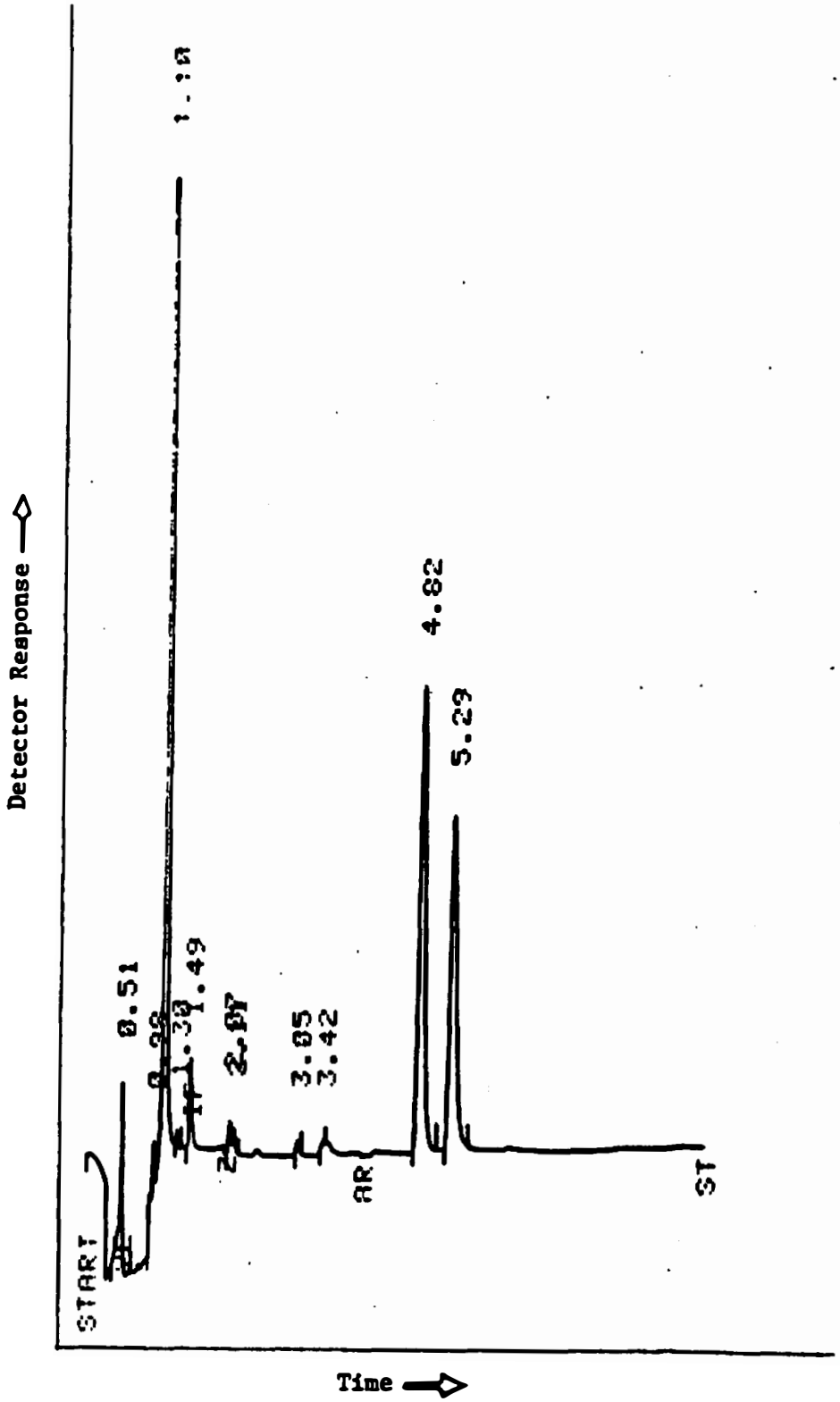


Figure 19.

Chromatogram of Chlorpromazine Extracted from Saliva.

Retention time of chlorpromazine = 5.29 min.
Retention time of internal standard = 4.82 min.

See Figure for Chromatographic Conditions.



in saliva from 20-400 ng/mL and in plasma from 5-200 ng/mL, although at concentrations of less than 20 ng/mL in plasma appreciable scatter was observed in the analytical results.

Chlorpromazine was measured in plasma from six subjects who had each been given two different levels of chlorpromazine. These samples had been stored at -20°C in the dark for several weeks before analysis. After two more weeks some of the samples were reanalyzed. The concentration of chlorpromazine in these reanalyzed samples was substantially less than was determined two weeks previously. We are therefore not confident that the values obtained for concentrations of chlorpromazine in plasma are the concentrations that were present when the blood samples were taken. Hence, comparison of concentrations of chlorpromazine in saliva and in these plasma samples is not possible.

4.3.4 Diazepam

Detailed information on the extraction and analysis of diazepam from plasma and saliva in given in Appendix A.4. Extracts from plasma and saliva could be chromatographed on either a methyl silicone (SE-30) or a polyethylene glycol (Carbowax 20M) coated capillary GC column. Diazepam elutes from the SE-30 column with a reasonable retention time and good peak shape. However, the SE-30 column was unable to separate diazepam from either of the internal standards that we have devised and therefore is not useful for the analysis of this drug unless other internal standards are found. A typical chromatogram of diazepam extracted from plasma on the Carbowax column is shown in Figure 20. Diazepam, its internal standard (the N-ethyl analog of diazepam), and 3-methyldiazepam (retention time 8.51 min), another possible internal standard, elute in sharp peaks free from interferences on this column. Linear

calibration curves (log-log scale) were established for diazepam in plasma from 10-500 ng/mL and in saliva from 1-20 ng/mL. An additional standard curve for diazepam in saliva was established between 0.1 and 5 ng/mL.

Analysis of multiple samples containing more than 25 ng/mL of diazepam was accomplished with ease on this column. Concentrations of diazepam in the plasma of six subjects who were each administered diazepam at two dose levels are shown in Table 12. However, analysis of samples containing less than 10 ng/mL of diazepam, which entailed injection of larger quantities of plasma or saliva extract, caused rapid degeneration of the chromatography column, which was being operated very close to its maximum temperature. Analysis of the diazepam in one subject, however, was completed before all of the columns which we had were destroyed. The results of these analyses are shown in Table 13.

Since diazepam is a neutral molecule, the concentration of diazepam in saliva should correspond to the concentration of "free" diazepam (i.e. that which is not bound to plasma protein) in plasma. The saliva concentrations of diazepam for subject D2 ranged from 2.1-5.6% of the concentrations in plasma with the exception of the 0.5 hr sample, which was much higher (13% of the concentration in plasma). DiGregorio et al. (1978), who reported only averaged values for nine subjects following analysis of plasma and saliva on a packed column with electron capture detection, found that mixed saliva contained an average of 2.9% as much diazepam as plasma. Using our very limited data (Table 13) and eliminating the 0.5 hr sample, we found the average predicted plasma concentration of diazepam was 2.0 times that actually observed. The concentration of diazepam in plasma predicted from the 0.5 hr sample is more than eight times that actually observed.

Table 12.

Concentrations of Diazepam in the Plasma of Subjects D1-D6 Following 0.143 and 0.0714 mg/kg Oral Doses of Diazepam. Values are in ng/mL plasma ± sd.

Time (hr)	Subject					
	D1	D2	D3	D4	D5	D6
			0.143 mg/kg/dose			
0.5	5 ± 0	4 ± 1	449 ± 2	212 ± 1	2 ± 0	280 ± 1
1.0	27 ± 1	25 ± 1	315 ± 1	414 ± 4	11 ± 6	375 ± 3
1.5	44 ± 1	168 ± 1	283 ± 1	274 ± 1	22 ± 9	294 ± 1
2.0	76 ± 3	322 ± 17	231 ± 19	124 ± 1	129 ± 5	175 ± 1
3.0	133 ± 1	261 ± 1	155 ± 1	177 ± 2	263 ± 12	127 ± 1
4.0	136 ± 3	189 ± 4	173 ± 1	143 ± 1	153 ± 3	93 ± 2
6.0	181 ± 1	131 ± 3	137 ± 7	124 ± 1	174 ± 1	121 ± 1
8.0	200 ± 0	110 ± 1	108 ± 1	251 ± 0	121 ± 1	132 ± 2
11.0	161 ± 0	104 ± 1	79 ± 0	85 ± 0	129 ± 1	90 ± 1
24.0	93 ± 0	64 ± 2	78 ± 1	60 ± 1	--	--
			0.0714 mg/kg/dose			
0.5	24 ± 1	113 ± 2	33 ± 2	>5	>5	284 ± 1
1.0	41 ± 1	189 ± 2	113 ± 1	10 ± 2	9 ± 0	176 ± 1
1.5	66 ± 2	190 ± 7	132 ± 1	189 ± 2	62 ± 0	125 ± 2
2.0	129 ± 2	159 ± 2	98 ± 1	200 ± 0	119 ± 0	94 ± 1
3.0	157 ± 1	98 ± 0	61 ± 2	134 ± 1	132 ± 1	66 ± 1
4.0	142 ± 2	91 ± 0	55 ± 2	95 ± 1	112 ± 1	59 ± 2
6.0	118 ± 2	87 ± 1	66 ± 2	70 ± 1	94 ± 1	76 ± 1
8.0	115 ± 2	74 ± 1	69 ± 1	71 ± 1	90 ± 1	66 ± 1
11.0	91 ± 2	65 ± 1	54 ± 1	63 ± 6	85 ± 1	67 ± 1
24.0	48 ± 1	41 ± 1	43 ± 1	52 ± 1	59 ± 3	44 ± 1

Table 13.

Concentrations of Diazepam in the Saliva of Subject D2 Following an Oral Dose of 0.143 mg/kg Diazepam and Comparison of These Values with Corresponding Diazepam Concentrations in Plasma.

Time (hr)	$[D]_S$* (ng/mL ± s.d.)	$[D]_S/[D]_P$†
0.5	0.48 ± 0.04Ψ	0.13
1.0	0.69 ± 0.00Ψ	0.028
1.5	9.4 ± 0.1	0.056
2.0	9.7 ± 0.2	0.030
3.0	8.1 ± 0.2	0.031
4.0	6.7 ± 0.0	0.034
6.0	3.8 ± 0.1	0.029
8.0	2.3 ± 0.1	0.021
11.0	2.5 ± 0.0	0.024
24.0	1.8 ± 0.2	0.028

*Concentration of diazepam in saliva

†Concentration of diazepam in saliva divided by concentration of diazepam in plasma

ΨRange. Two determinations were made for this analysis.

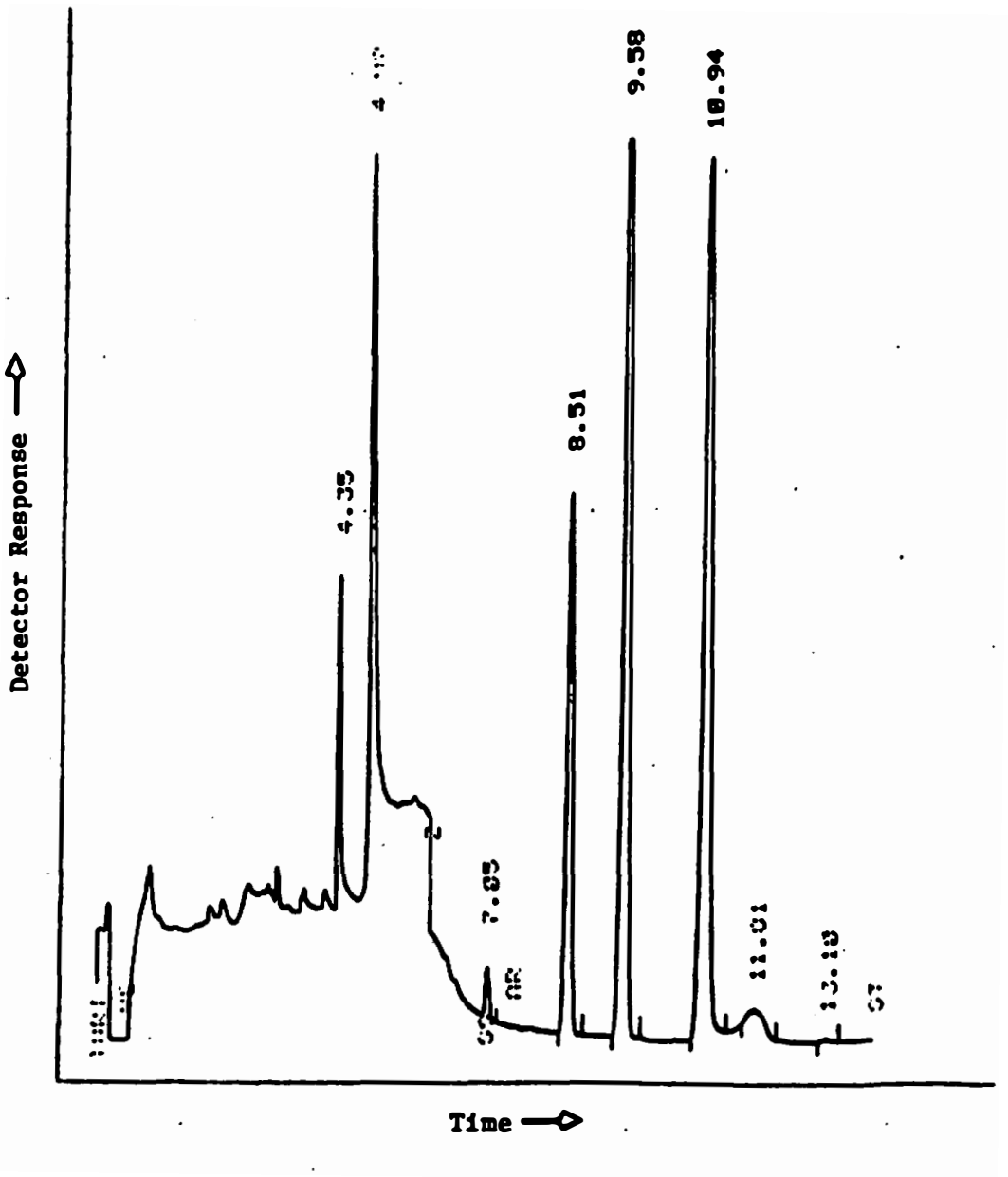
Figure 20.

Chromatogram of Diazepam Extracted from Plasma.

The retention time of diazepam = 10.94 min.
The retention time of internal standard = 9.58 min.

Chromatographic Conditions

Instrument: Hewlett-Packard 5840A
Column: 0.25 mm ID x 8m Carbowax 20M WCOT capillary
Temperature: 165°C for 1 min, then 165°C to 220°C at 20°C/min, then isothermal at 220°C
Mobile Phase: He, ca. 2 mL/min.
Detector: Nitrogen-Phosphorus
Injection: Splitless (0.5 min)/split



4.3.5 Diphenhydramine

Detailed information on the extraction and analysis of diphenhydramine from plasma and saliva is given in Appendix A.5. Plasma and saliva extracts were chromatographed on a commercial polyethylene glycol (CP Wax 51; Chrompack) glass capillary column. A sample chromatogram obtained from extracted saliva containing diphenhydramine and the internal standard, Orphenadrine, is shown in Figure 21. Linear calibration curves (log-log scale) were established for diphenhydramine in plasma and saliva over ranges of 1-200 and 15-1000 ng/mL respectively.

The results of the analysis of plasma and saliva samples from seven subjects, each of which received a 100 mg dose of diphenhydramine and two of which also received a 50 mg dose, are shown in Table 14. Predicted concentrations of diphenhydramine in plasma were calculated from concentrations in saliva by the Henderson-Hasselbalch-derived equation described in Section 2.2.

The ratios of predicted to observed concentrations of diphenhydramine in plasma average 2.3 (CV = 67%, n = 42). They range from 0.28 to 5.9, with a median value of 1.7. These ratios vary as a function of time. The maximum values of these ratios for each subject at 1.5-3 hours and in all but one case correspond to the maximum concentration of diphenhydramine in saliva. Significant variations in binding of diphenydramine to different samples of plasma from the same subject were also observed (cf Table 7).

Plots of the concentrations of diphenhydramine in saliva $[DP]_s$ versus concentrations in plasma $[DP]_p$ and of the predicted $[DP]_c$ versus

Figure 21.

Chromatogram of Diphenhydramine Extracted from Saliva.

Chromatographic conditions same as in Figure 64.
Retention Time: Diphenhydramine 9.20 min.
Orphenadrine 10.35 min.
Sample: Subject 5, 11 hr.

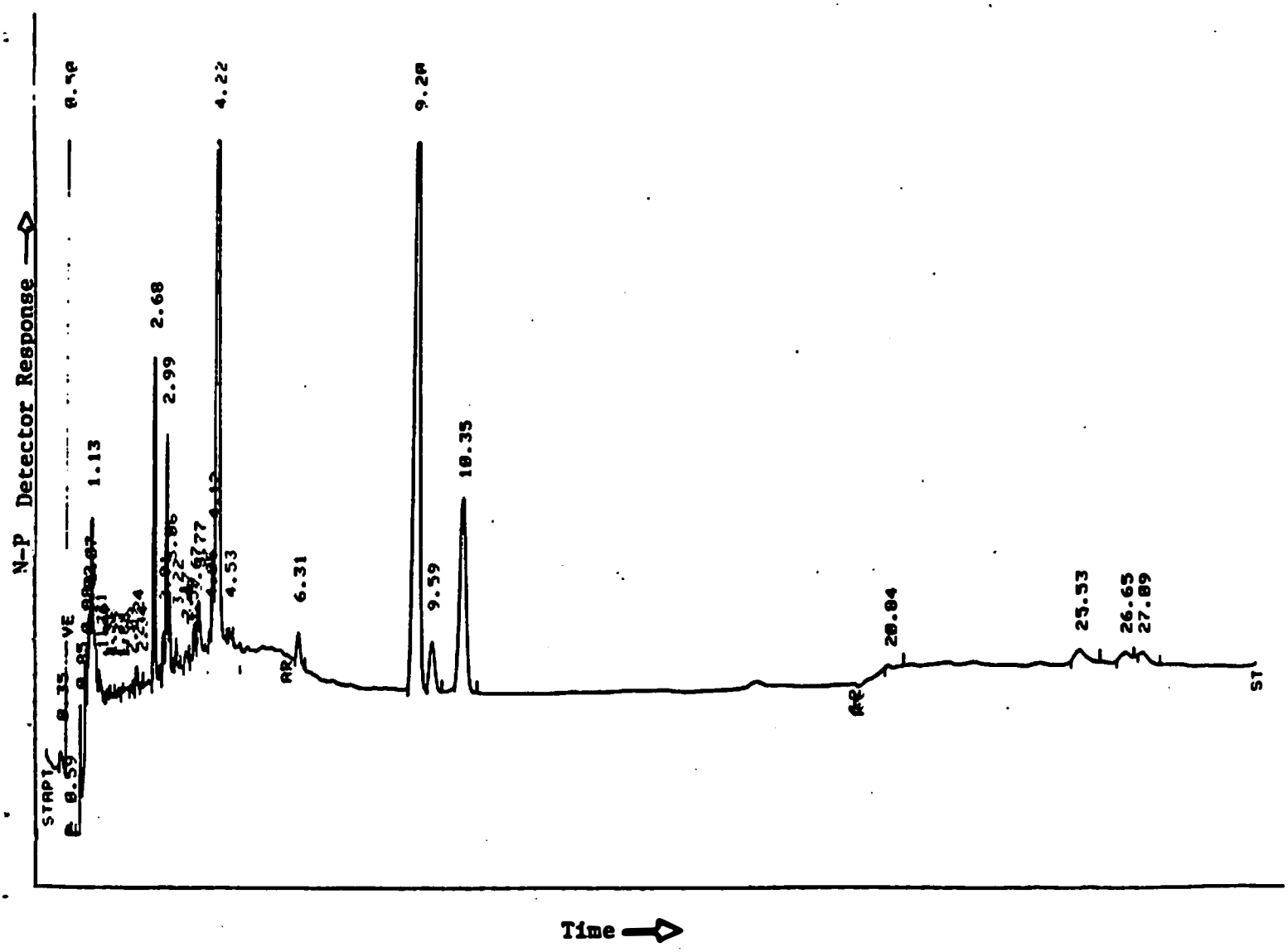


Table 14. Concentrations of Diphenhydramine (DPHM) in Plasma and Saliva following a Single Oral Dose.

Time (hr)	Saliva pH	Observed [DPHM] in Saliva (ng/mL)	Observed [DPHM] in Plasma (ng/mL)	Predicted [DPHM] in Plasma (ng/mL)	Predicted Observed (Plasma)
Subject DP-1; 50 mg Dose					
0.5	6.75	5.6	2.4	6.7	2.8
1.5	6.75	123	38	148	3.9
3.0	6.75	166	42	199	4.7
6.0	6.80	59	43	80	1.9
11.0	6.90	31	17	53	3.1
Subject DP-2; 50 mg Dose					
0.5	6.80	2.4	2.3	2.2	0.96
1.5	6.80	39	27.1	35	1.3
3.0	6.80	240	46.7	216	4.6
6.0	6.85	27	30.2	28	0.93
11.0	6.75	26	12.6	21	1.7
Subject DP-1; 100 mg Dose					
0.5	6.80	0.9	3.2	0.9	0.28
1.5	6.80	367	76	381	5.0
3.0	6.60	506	130	331	2.5
6.0	6.80	193	116	200	1.7
11.0	6.80	96	70	96	1.4
Subject DP-2; 100 mg Dose					
0.5	6.80	3.2	11.5	3.2	0.28
1.5	6.85	395	124	454	3.7
3.0	6.70	337	172	274	1.6
6.0	6.70	180	125	147	1.2
11.0	6.70	44	38	36	0.95
Subject DP-3; 100 mg Dose					
0.5	6.85	6.0	8.3	5.5	0.66
1.5	6.85	900	140	820	5.9
3.0	6.70	611	166	395	2.4
6.0	6.75	289	117	204	1.7
11.0	6.60	180	64	90	1.4

Table 14. Continued.

Time (hr)	Saliva pH	Observed [DPHM] in Saliva (ng/mL)	Observed [DPHM] in Plasma (ng/mL)	Predicted [DPHM] in Plasma (ng/mL)	Predicted Observed (Plasma)
Subject DP-4 100 mg Dose					
0.5	6.90	5.9	4.7	6.5	1.4
1.5	6.80	717	120	624	5.2
3.0	6.80	567	138	494	3.6
6.0	6.90	142	136	156	1.1
11.0	7.00	44	47	61	1.3
Subject DP-5 100 mg Dose					
0.5	6.80	S.L.	4.3	S.L.	S.L.
1.5	6.65	582	68	388	5.7
3.0	6.65	439	126	293	2.3
6.0	6.65	269	85	179	2.1
11.0	6.75	82	47	66	1.4
Subject DP-6 100 mg Dose					
0.5	6.80	14.6	33	14	0.42
1.5	6.75	673	195	557	2.8
3.0	6.80	S.L.	196	S.L.	S.L.
6.0	6.70	67	85	51	0.60
11.0	6.80	42	33	39	1.2
Subject DP-7 100 mg Dose					
0.5	6.80	S.L.	2.0	S.L.	S.L.
1.5	6.90	106	35	106	3.0
3.0	6.75	529	110	373	3.4
6.0	6.70	284	68	184	2.7
11.0	6.80	50	34	50	1.2

S.L. = Sample Lost

observed concentrations in plasma are shown in Figures 22 and 23, respectively. The best-fit line produced by the least squares linear regression of $[DP]_s$ versus $[DP]_p$ data (concentrations expressed in ng/mL) is described by the equation: $\log [DP]_p = 0.734 \log [DP]_s + 0.223$. The correlation coefficient for the data is 0.92. The correlation is not improved ($r = 0.91$) when the saliva data is corrected for differences in saliva pH. The best-fit line produced by the least squares linear regression of $[DP]_c$ versus $[DP]_p$ is described by the equation: $\log [DP]_p = 0.778 \log [DP]_c + 0.171$. Additional statistical data for these linear regression analyses are in Appendix A.5.

4.3.6 Codeine

The analytical procedures for measurement of codeine in human plasma and saliva differed markedly from those used for the other drugs in that the analysis for codeine was accomplished by radioimmunoassay (RIA) rather than by gas chromatography. Codeine had not been selected for study in this project at the time the detailed study plan was approved. After it was decided that codeine would be the sixth drug to be studied, it began to be apparent that analysis for this compound by gas chromatography following single doses of the drug at therapeutic levels would be most challenging. These concerns were not resolved with time. We therefore requested the Scientific Project Officer for permission to use a radioimmunoassay procedure developed at Wellcome Research Laboratories for the analysis of codeine in plasma and saliva. A more detailed discussion of the reasons for this request is presented in Appendix A.6. This request was granted.

Antisera, radioligand, and procedures for the radioimmunoassay were obtained from Dr. John Findlay, Wellcome Research Laboratories. This

Figure 22. Correlation between saliva and plasma concentrations of Diphenhydramine.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.

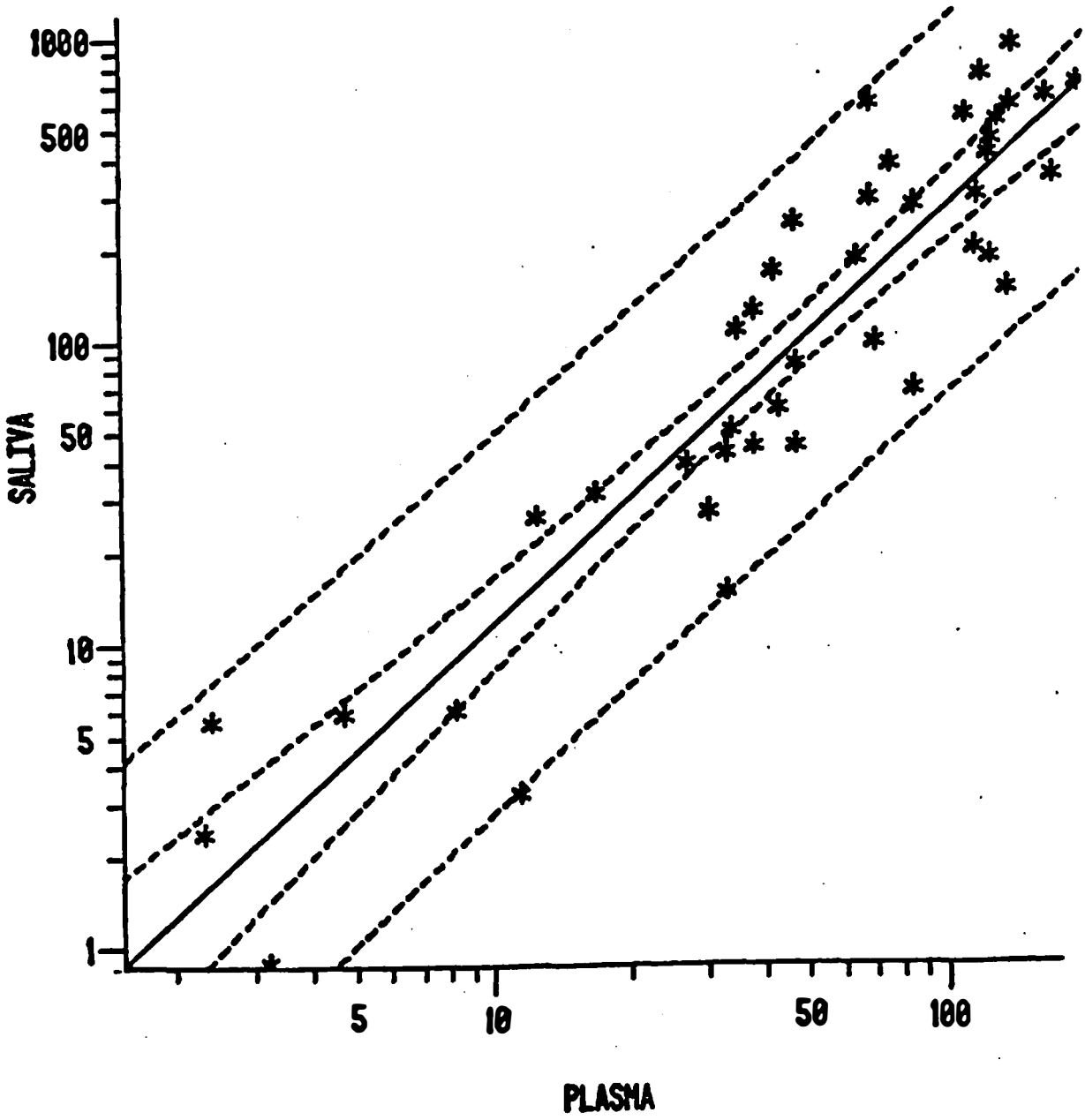
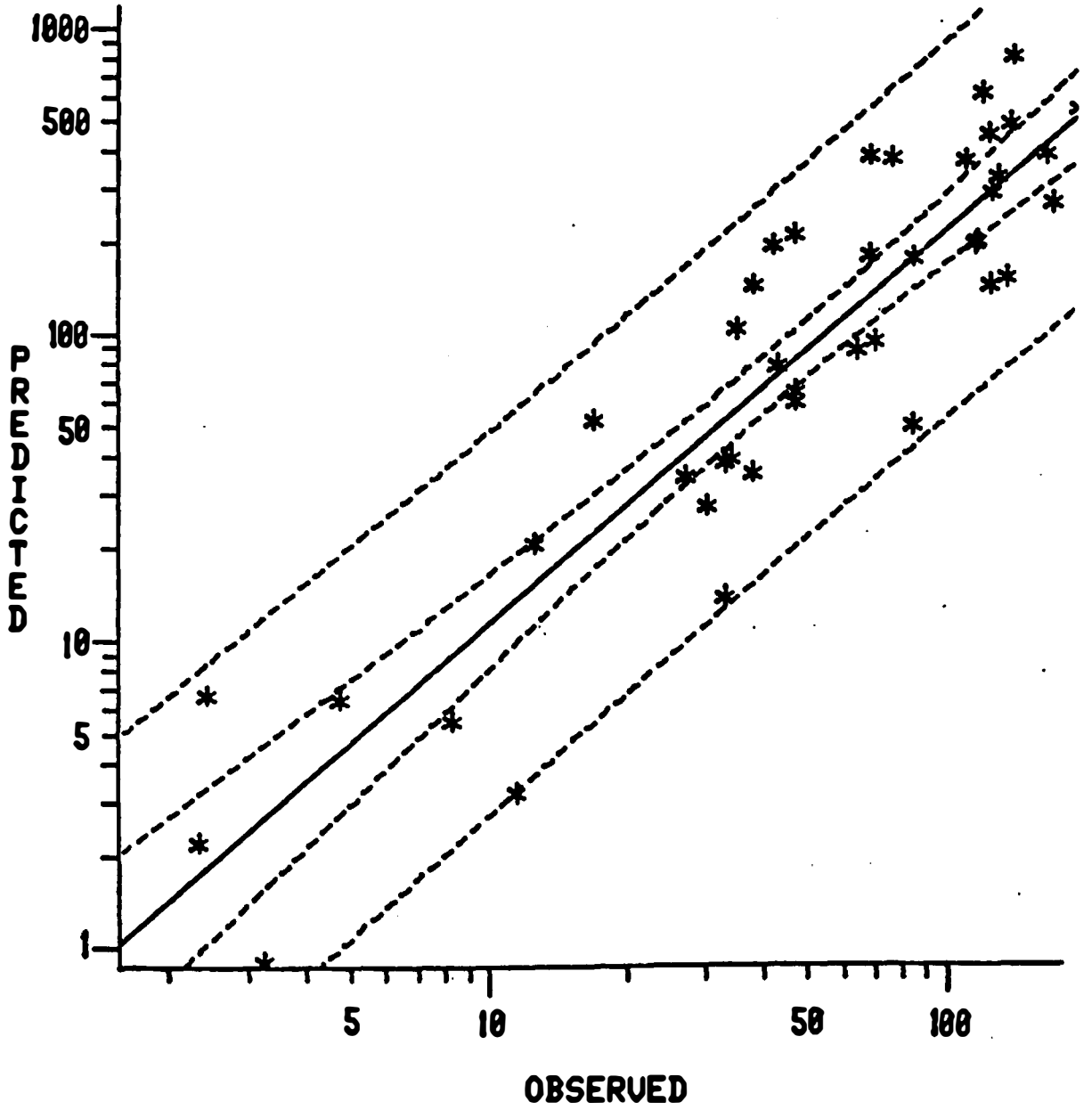


Figure 23. Correlation between predicted and observed concentrations of Diphenhydramine in plasma. The predicted values are those calculated from concentrations in saliva by the Henderson-Hasselbalch-derived equation.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.



procedure was essentially that described by Findlay et al. (1976) and is summarized in Appendix A.5. The range of the assay was 0.5-15 ng/mL. Higher concentrations of codeine in these fluids were analyzed by appropriate dilutions.

The results of the analysis of of codeine in plasma and saliva from six subjects, each dosed at two different levels (15 and 30 mg of codeine sulfate) are shown in Table 15. As was expected, concentrations of codeine in saliva were generally much higher than those observed in plasma. The predicted concentrations of codeine in plasma were calculated as from the Henderson-Hasselbalch derived equation discussed in Section 2.2.

The ratios of the predicted to observed concentrations of codeine in plasma tend to decrease with increasing time in all subjects. The average ratio is 1.87 (CV = 53%, n= 53), but declines steadily from 2.67 for the 1 hr samples to 1.19 for the 8 and 11 hr samples.

Plots of the concentrations of codeine in saliva $[C]_s$ versus concentrations in plasma $[C]_p$ and of the predicted $[C]_c$ versus observed concentrations in plasma are shown in Figure 24 and 25, respectively. The best-fit line produced by the least squares linear regression (log-log scale) of $[C]_s$ versus $[C]_p$ (expressed as ng/mL) is described by the equation: $\log [C]_p = 0.777 \log [C]_s - 0.164$, $r = 0.907$. A similar equation for the regression of $[C]_c$ vs $[C]_p$ is $\log [C]_p = 0.718 \log [C]_c + 0.194$, $r = 0.945$.

Table 15.

Concentrations of Codeine in Plasma and Saliva Following a Single Oral Dose.

Time (hrs)	Observed [Codeine] in Saliva (ng/mL)	Observed [Codeine] in Plasma (ng/mL)	Predicted [Codeine] in Plasma (ng/mL)	Predicted / Observed in Plasma
Subject 1; 12.9 mg Dose				
1	87.1	15.7	41.1	2.64
2	41.8	11.0	17.8	1.62
4	13.4	5.4	5.7	1.06
8	<2.5	2.2	-	-
11	<2.5	1.3	-	-
Subject 2; 12.9 mg Dose				
1	214	22.7	73.0	3.22
2	181	20.1	76.7	3.82
4	59.2	11.3	20.2	1.79
8	13.1	4.1	3.6	0.88
11	<2.5	2.2	-	-
Subject 3; 12.9 mg Dose				
1	292	56.5	124	2.19
2	257	30.6	121	3.95
4	161	19.6	68.2	3.48
8	47.4	9.8	18.0	1.84
11	24.3	7.7	10.3	1.34
Subject 4; 12.9 mg Dose				
1	106	30.7	68.5	2.23
2	70.1	31.5	40.9	1.30
4	43.6	18.0	18.5	1.03
8	9.1	7.5	3.9	0.52
11	4.5	4.2	1.9	0.45
Subject 5; 12.9 mg Dose				
1	198	21.2	53.8	2.54
2	83.9	13.1	22.9	1.75
4	42.0	6.3	9.2	1.46
8	5.8	2.5	1.6	0.64
11	<2.5	1.5	-	-

Table 15. Continued.

Time (hrs)	Observed [Codeine] in Saliva (ng/mL)	Observed [Codeine] in Plasma (ng/mL)	Predicted [Codeine] in Plasma (ng/mL)	Predicted / Observed in Plasma
Subject 6; 12.9 mg Dose				
1	371	42.8	126	2.94
2	136	18.7	57.7	3.08
4	32.4	8.9	17.0	1.91
8	5.8	2.9	2.7	0.93
11	9.5	1.9	4.5	2.37
Subject 1; 25.8 mg Dose				
1	76.4	23.3	40.0	1.72
2	30.4	15.7	19.6	1.25
4	9.5	10.5	6.1	0.58
8	<2.5	2.6	-	-
12	<2.5	1.4	-	-
Subject 2; 25.8 mg Dose				
1	334	57.8	195	3.37
2	138	41.2	72.3	1.75
4	78.5	24.8	50.7	2.04
8	13.8	9.3	12.1	1.30
11	8.8	5.3	4.6	0.87
Subject 3; 25.8 mg Dose				
1	613	115	321	2.79
2	444	63.3	259	4.09
4	204	43.3	107	2.47
8	152	24.2	64.4	2.66
11	30.4	12.1	15.9	1.31
Subject 4; 25.8 mg Dose				
1	202	55.4	130	2.35
2	150	54.7	78.6	1.44
4	98.0	30.4	57.1	1.88
8	36.1	15.1	21.0	1.39
11	9.4	7.2	4.9	0.68

Table 15. Continued.

Time (hrs)	Observed [Codeine] in Saliva (ng/mL)	Observed [Codeine] in Plasma (ng/mL)	Predicted [Codeine] in Plasma (ng/mL)	Predicted Observed in Plasma
Subject 5; 25.8 mg Dose				
1	457	33.8	140	4.14
2	149	26.4	50.8	1.92
4	108	15.1	26	1.72
8	<2.5	2.5	-	-
12	13.3	7.0	5.6	0.80
Subject 6; 25.8 mg Dose				
1	301	66.1	128	1.94
2	125	45.7	53.0	1.16
4	51.2	23.4	21.7	0.93
8	27.7	8.1	7.6	0.94
12	7.1	3.7	1.6	0.43

4.4 Use of Creatinine Concentrations in Saliva as an Aid in Establishing Correlations of Concentrations of Amphetamine in Plasma and Saliva

Creatinine levels in the saliva samples from subjects A2 and A6 following administration of 0.122 mg/kg of amphetamine and from subject A4 following the administration of 0.061 mg/kg of amphetamine were determined using the methods described in section 4.5. These samples were chosen because they have amphetamine concentrations from which calculations of plasma amphetamine levels were higher than (subject A2), lower than (subject A4), and approximately the same (subject A6) as that actually found.

Plots of the predicted/found amphetamine plasma levels (P-F ratio) and creatinine saliva levels for these subjects are contained in Figures 26, 27 and 28. It can be seen from the plots that subject A4, who has low P-F ratios, also has relatively low creatinine levels. Subject A2, who has high P-F ratios, also has relatively high creatinine levels. Subject A6 has intermediate values of both P-F ratios and creatine levels. Quantitative corrections of the P-F ratios using the plasma creatinine levels were, however, not possible. For instance, the saliva creatinine levels for subject A2 were lowest when the P-F ratios were the highest. At other times, the two curves were almost parallel. Thus, while some correction of the P-F ratios can be made using saliva creatinine levels, these corrections are not sufficiently good to provide quantitative relationships.

4.5 Analysis of Drugs in Breath

It was agreed by personnel at DOT, RTI, and NIDA that the drugs most likely to be excreted in breath were secobarbital and amphetamine.

Figure 24. Correlation between saliva and plasma concentrations of Codeine.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.

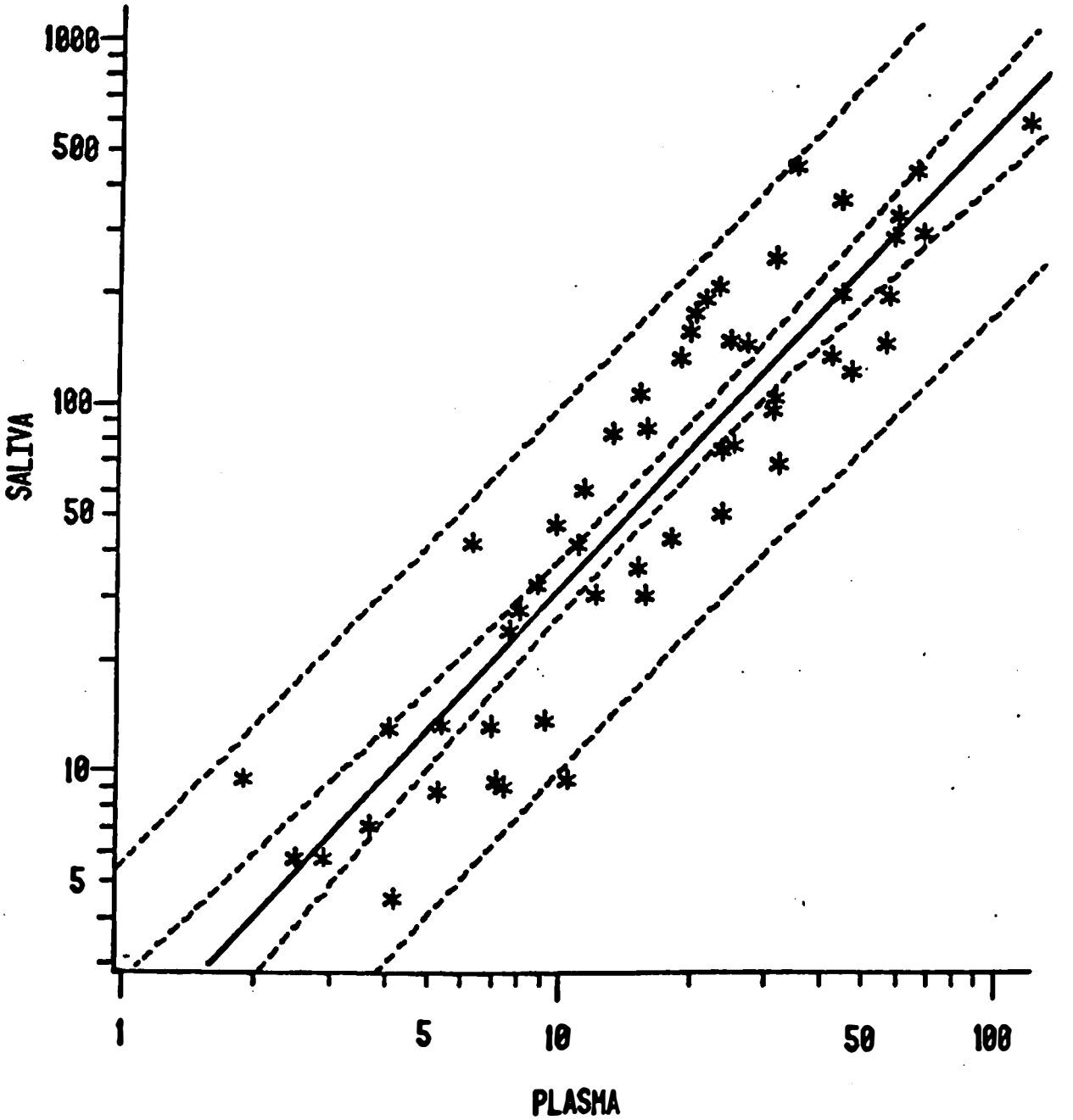


Figure 25. Correlation between predicted and observed concentrations of Codeine in plasma. The predicted values are those calculated from concentrations in saliva by the Henderson-Hasselbalch-derived equation.

The solid line is the "best-fit" by least squares regression analysis. The inner pair of dashed lines represent the 95% confidence interval for the best-fit line. The outer pair of dashed lines represent the 95% confidence interval for the data.

Concentrations are in ng/mL.

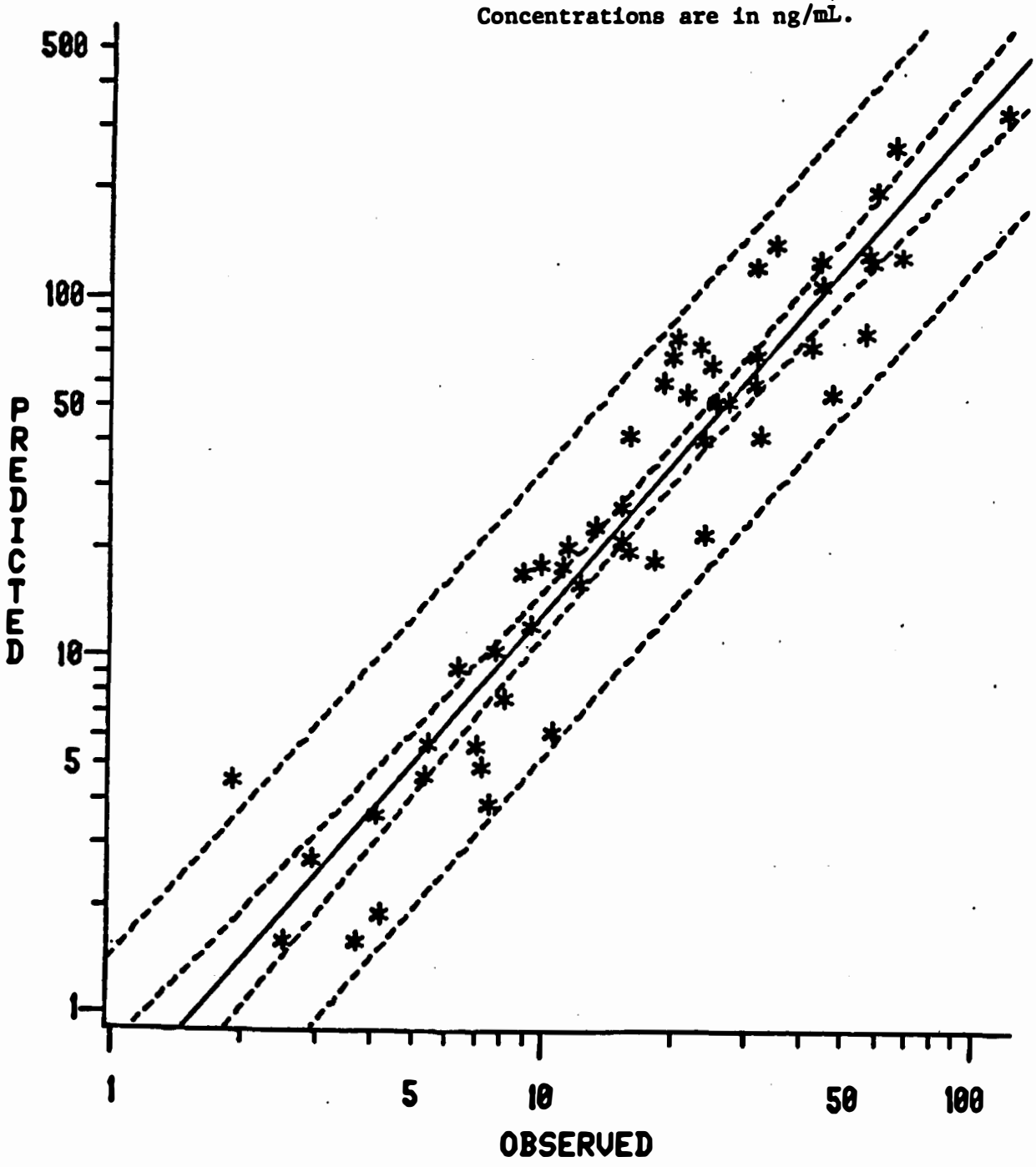


Figure 26.

Plots of the Ratios of Predicted and Found Plasma Amphetamine (P-F Ratios) and of Saliva Creatinine for Subject A2 Following Administration of 0.122 mg/kg of Amphetamine.

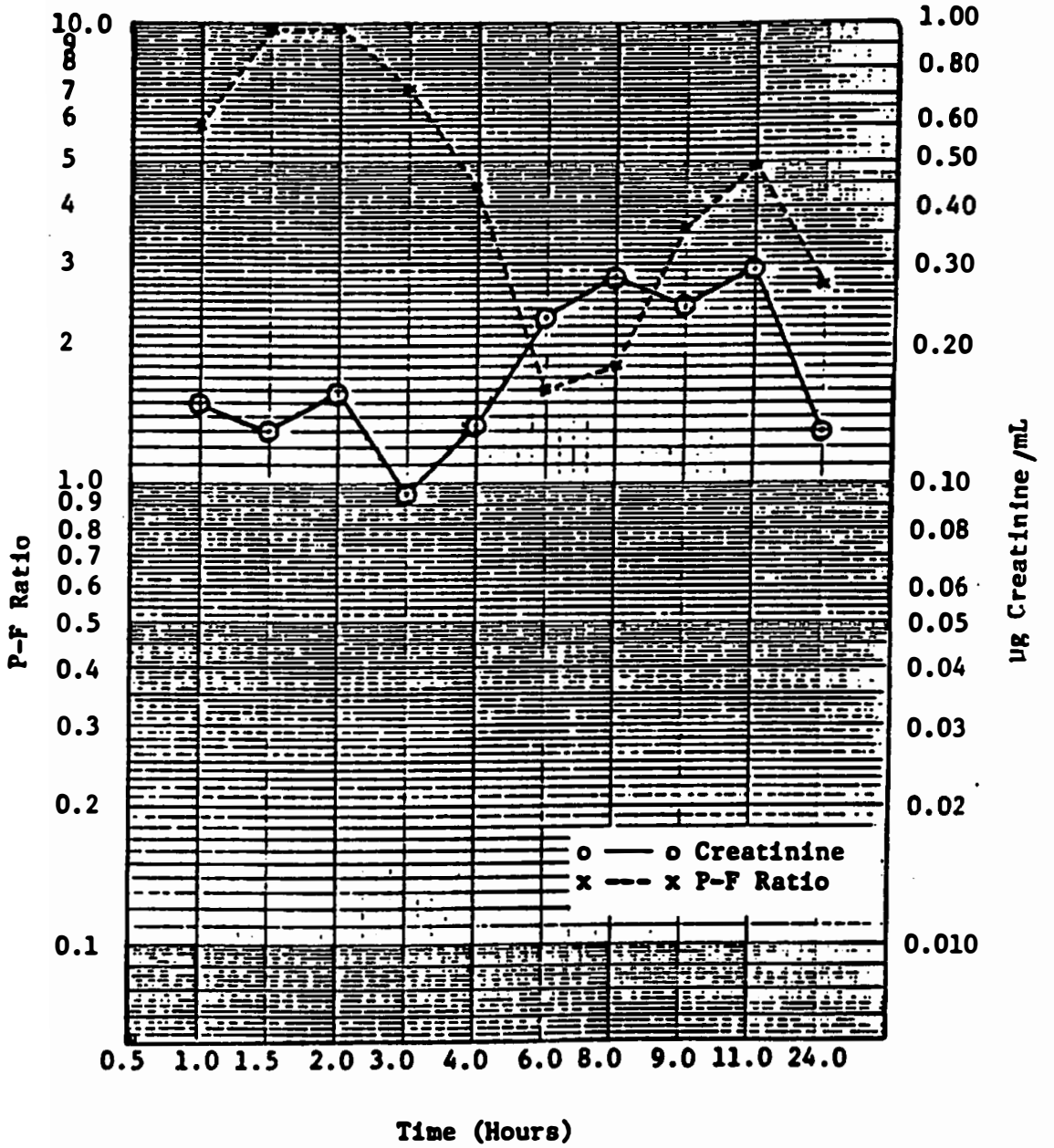


Figure 27.

Plots of the Ratios of Predicted and Found Plasma Amphetamine (P-F Ratios) and of Saliva Creatinine for Subject A4 Following Administration of 0.061 mg/kg of Amphetamine.

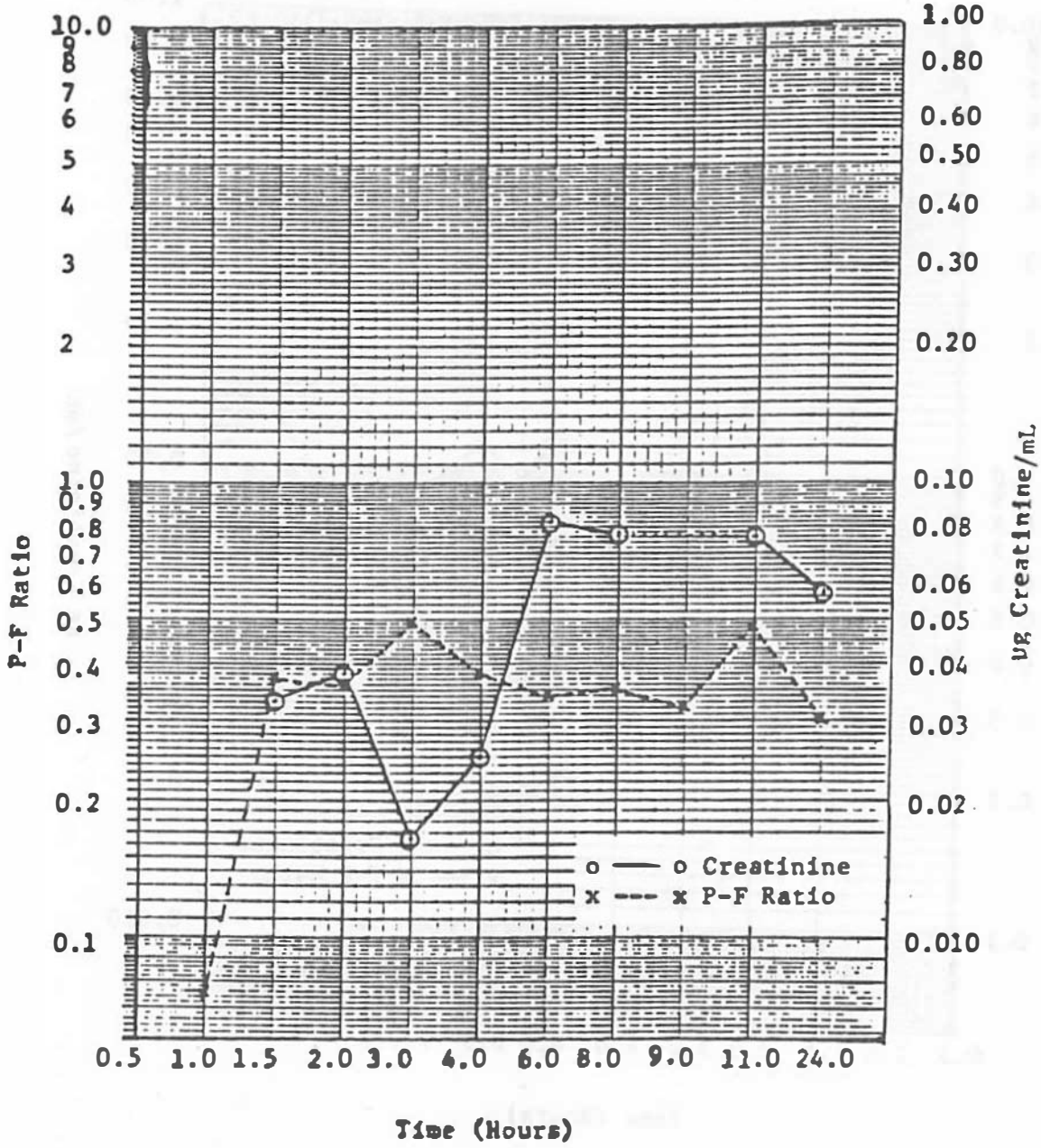
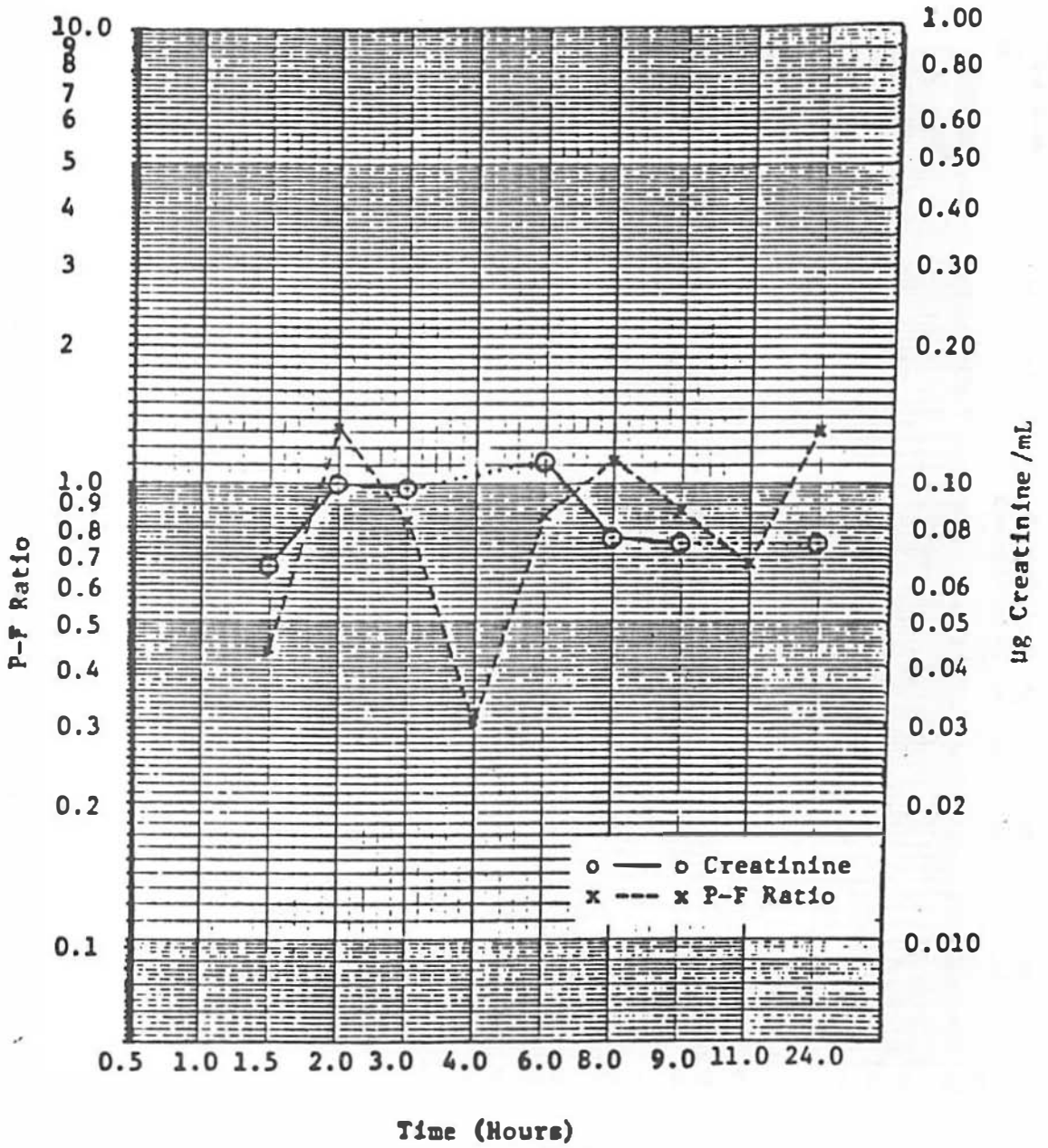


Figure 28.

Plots of the Ratios of Predicted and Found Plasma Amphetamine (P-F Ratios) and of Saliva Creatinine for Subject A6 Following Administration of 0.122 mg/kg of Amphetamine.



Secobarbital was selected because it was estimated that it should have the highest concentration of unionized drug not bound to plasma protein of any drug to be studied. Amphetamine was chosen because its volatility is higher than any of the other drugs to be studied. For that reason these drugs were studied first and breath sample from subjects who had been given the drugs were collected and analyzed. Calibration curves for the analysis of these drugs in breath were prepared by spiking drug-free breath collections with known amounts of the drug being studied. The alcohol bubbler trap was used as a collection device in all cases. We had previously shown (cf. Section 4.1) that this device would effectively remove secobarbital and amphetamine from simulated expired breath.

Breath and blood samples were taken from subjects S1 and S6 following the administration of a single oral dose of 100 mg sodium secobarbital. The results of the analysis of the samples in this study are shown in Table 16. Both subjects maintained high concentrations of secobarbital in plasma throughout the experiment. The breath samples for subject S1 contained no more than 8, 5, and 10 ng of secobarbital per 16 ℓ breath sample. This indicates a maximum concentration range of approximately 0.1-0.5 ng/ℓ of expired air. Maximum possible concentrations in the breath samples from subject S6 were somewhat higher for the 2 and 3 hr samples. Here maximum possible concentrations were 1-2 ng/ℓ of expired air. Because of peaks resulting from endogeneous materials in the breath, the peaks in the gas chromatogram corresponding to secobarbital were difficult to determine or quantitate. In the chromatogram of the two hour breath sample from subject S1 (Figure 29c), the secobarbital peak occurs at the correct retention time. For most samples, however, either the observed peaks assumed to be arising from secobarbital were

Table 16.

Concentrations of Secobarbital in Breath following a Single Oral Dose of 100 mg of Sodium Secobarbital.

Subject	Time (Hr)	Concentrations of Secobarbital in Plasma (ug/mL)	Maximum Possible Concentrations of Secobarbital in Breath (ng/16L)
S1	1.5	1.64	7.8
	2.0	1.04	4.7
	3.0	0.91	10
S2	1.5	0.71	7.8
	2.0	1.04	40
	3.0	0.97	33

very broad as in Figure 29a, or two peaks were observed (Figure 29b), one with a retention time slightly lower and the second with retention time slightly greater than that normally observed for secobarbital. The concentrations of secobarbital in breath listed in Table 16 are for the combined or larger peak in each chromatogram and thereby represent the maximum possible secobarbital concentration. The actual concentrations are probably much smaller. There does not appear to be any correlation between the maximum possible concentrations found in breath and those observed in plasma.

Breath samples for the six subjects who were administered the lower (0.061 mg/kg) dose of amphetamine were collected along with the plasma and saliva samples. For these samples, deep breaths totaling 15.5-16.5 ℓ per sample were passed through a trap that had previously been shown to efficiently trap amphetamine. The results of the analyses of these samples are shown in Table 17. The samples denoted "trace" contained a peak at the correct retention time for amphetamine which was too small to accurately quantitate. No peak was visible at all in the chromatograms from the samples which are listed as containing "0" amphetamine.

There appears to be no correlation between plasma and breath levels of amphetamine. The fact that an "amphetamine" peak appears in the zero time samples indicates that what we are measuring as amphetamine in the other breath samples may also be interferences. When the 8 hour breath sample from subject A6, which contained the largest "amphetamine" peak, was examined by GC/MS, we were not able to find any amphetamine in the sample. Thus it appears that amphetamine is excreted in breath at a rate lower than our detection limit of approximately 0.07 ng/ℓ.

Figure 29. Gas Chromatogram of Breath Samples After Administration of Secobarbital. Internal Standards are Hexobarbital and Butabarbital.

a. Subject S6;2 hour breath sample

b. Subject S1;3 hour breath sample

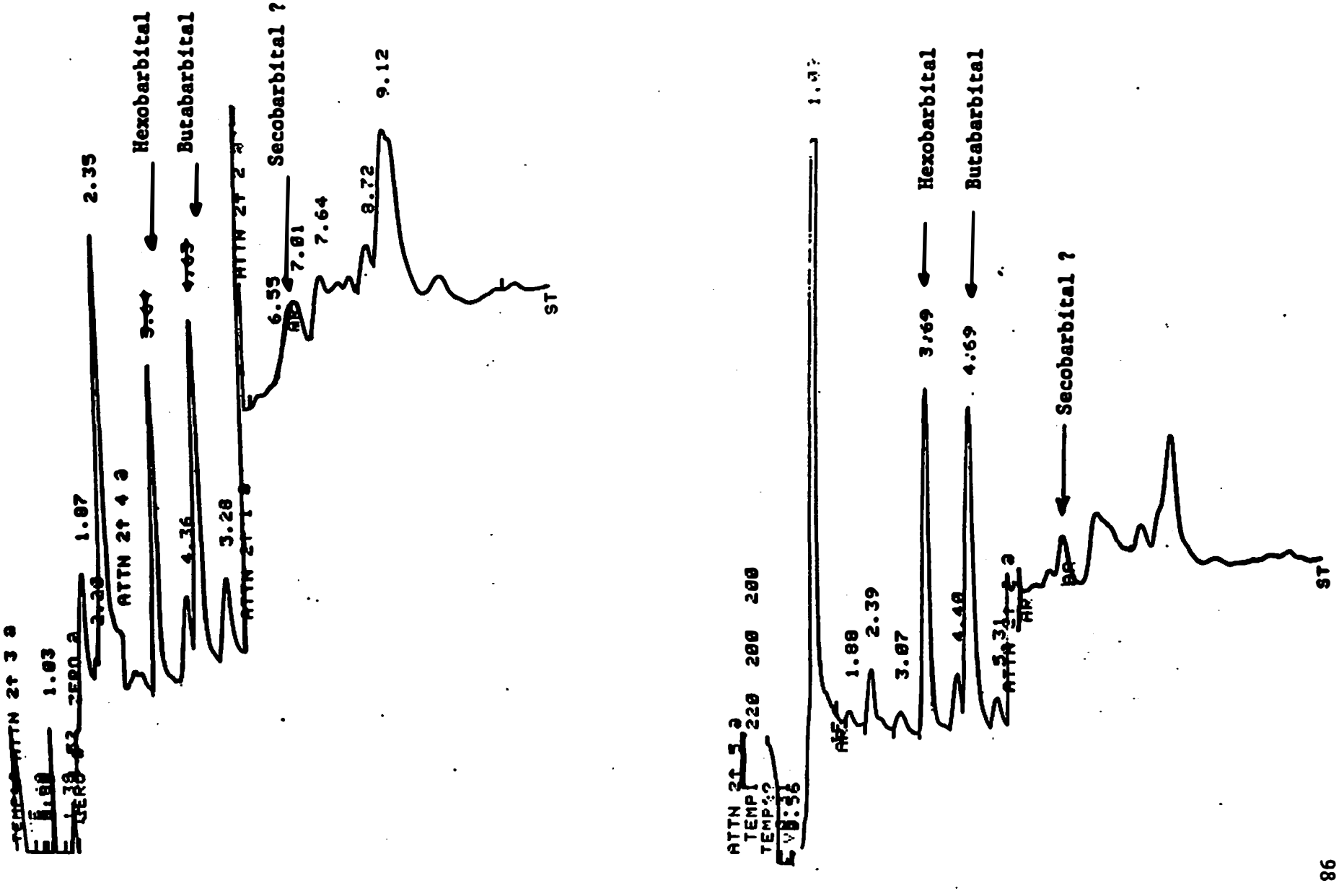


Figure 29. (cont'd.)

c. Subject S1;2 hour breath sample

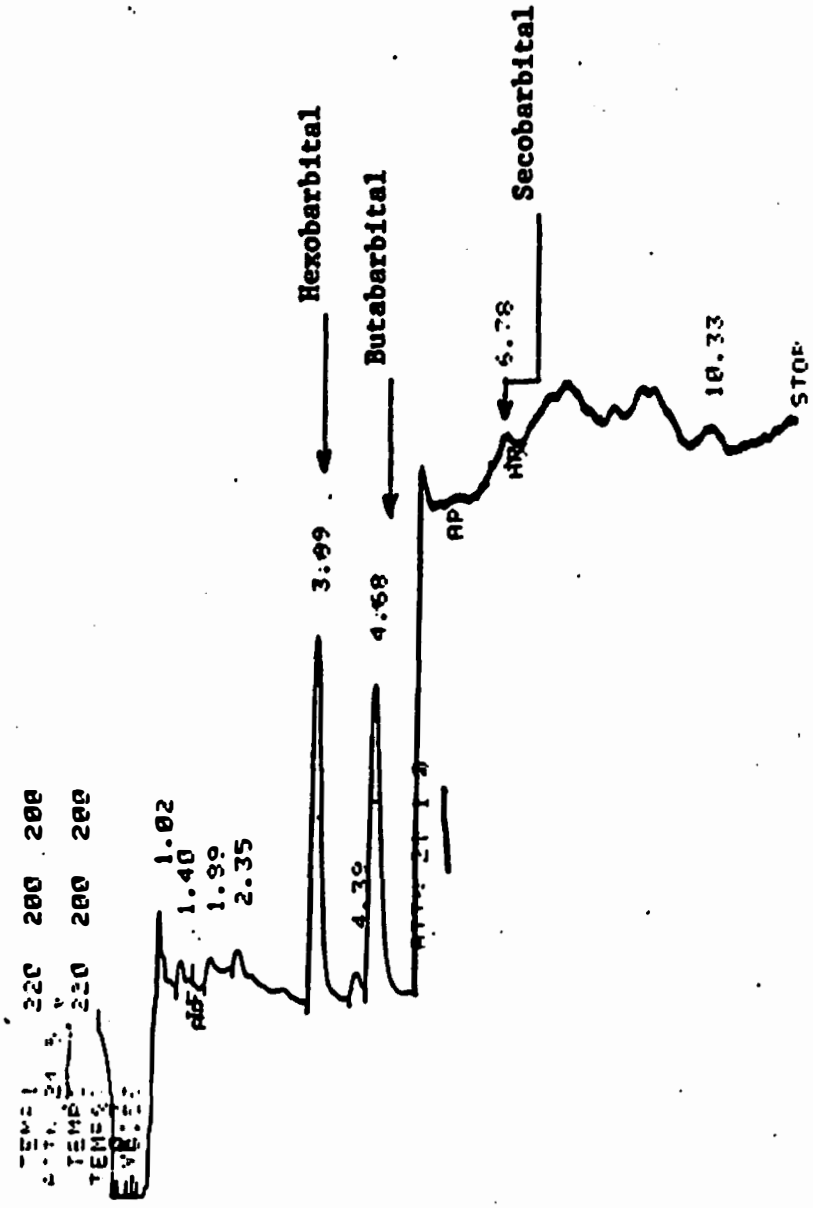


Table 17.

Maximum Concentration of Amphetamine in Breath
Following a Single Oral Dose of 0.061 mg/kg of d,ℓ-Amphetamines

Time	Subject A1		Subject A2		Subject A3	
	$[A]_p$*	$[A]_b$+	$[A]_p$	$[A]_b$	$[A]_p$	$[A]_b$
0	-	0.59	-	0	-	0.55
0.5	3.0	Tr¶	5.4	0	Tr	Tr
1.0	5.8	0	14	Tr	Tr	0.54
1.5	9.2	Tr	13	0	4.8	0
2	11	0.16	11	0	7.2	Tr
3	12	0	10	Tr	13	Tr
4	12	0	10	Tr	11	0.80
6	12	0	10	0.26	8.0	0
8	9.8	0	9.0	0	8.8	0
11	9.4	0.31	7.0	0	6.4	Tr
24	4.0	Tr	2.6	Tr	2.0	Tr

Time	Subject A4		Subject A5		Subject A6	
	$[A]_p$	$[A]_b$	$[A]_p$	$[A]_b$	$[A]_p$	$[A]_b$
0	-	0.15	-	0.27	-	0.64
0.5	4.8	0.09	0	0	1.3	0.62
1.0	9.5	0	12	0.18	5.6	0.84
1.5	16	0.17	12	0	14	0.36
2	16	Tr	11	0.15	12	Tr
3	17	0.66	15	0.14	15	Tr
4	14	Tr	16	0	10	Tr
6	13	Tr	19	0.23	14	Tr
8	14	0.26	18	1.1	10	2.5
11	13	0.13	15	0.25	8.9	0.33
24	6.0	0.30	5.9	0.17	5.3	0.23

*$[A]_p$ = Amphetamine concentration in plasma (ng/mL)

+$[A]_b$ = Amphetamine concentration in breath (ng/liter)

¶Tr = Trace

Since the presence of the drugs studied in breath was very questionable and no correlation could be established between concentrations of these drugs in plasma and the maximum possible concentrations in breath, it was decided by the Scientific Project Officer, DOT, that efforts directed toward analysis of drugs in breath in this project would be discontinued.

5.0 Discussion

5.1 Assay Methodologies

In this program, procedures which can be used for routine assays of saliva samples containing secobarbital, amphetamine, chlorpromazine, and diphenhydramine were developed. The same general chromatographic methods were used for secobarbital, chlorpromazine, diazepam, and diphenhydramine. Thus, one gas chromatograph could be set up to analyze samples for these drugs interchangeably. Since the drugs studied in this project are each members of larger classes of similar drugs the assays which were developed should easily be expanded to include many other similar compounds. This may be particularly true of the analysis of secobarbital, since several other barbiturates are also widely used.

Codeine was measured in saliva samples by an assay that is quite specific for this compound. Radioimmunoassays, while much cheaper, more specific, easier to run, and requiring much less sample, are usually developed to measure only a single compound. A radioimmunoassay, similar to the one for codeine, has been developed for measurement of morphine in plasma [Findlay et al., (1978)] and should easily be adapted for the measurement of morphine in saliva.

Measurement of diazepam in saliva is feasible by the methodology that we developed. However, routine measurements of therapeutic and subtherapeutic levels of this compound in saliva will be quite time consuming and expensive using these methods. We suggest that if large numbers of samples are to be assayed that a radioimmunoassay for this compound be considered. It is anticipated that in several years additional stationary phases will be available in capillary GC columns. Modifications of our procedures for use on these columns may considerably enhance the usefulness of the methods that were developed for diazepam.

The methodologies developed in our program provide for collection of samples at the roadside, but with analyses being conducted in a laboratory. The collection device developed for saliva (treated vial) is relatively inexpensive and simple to use. No special precautions need to be taken by the operational personnel for its use. It is necessary, however, in order to obtain adequate correlations between concentrations in saliva and in plasma of some of the drugs, for the pH of the saliva to be accurately determined. This can only be accomplished immediately after the saliva is collected, since the pH of saliva can change with storage. It was not determined in our study whether the use of pH paper would suffice for this measurement. Our general laboratory practice, which was used in this program, is to store all biological samples at -20°C. The stability of the drugs in saliva at higher temperatures was not investigated.

The laboratory procedures involve equipment that is not portable except in mobile laboratory trailers. The extractions or analyses of these samples by field personnel who are not highly trained in the procedures does not appear to be feasible.

Several problems have appeared during the collection and analysis of saliva from the subjects in this study which are likely to be more severe in less controlled populations. The first of these problems occurred during the study of the effect of stimulation and suppression of saliva flow upon the concentrations of drugs and saliva and plasma. Simply stated, when saliva flow is suppressed, it is difficult to obtain sufficient sample to analyze. Since salivary secretion is under nervous control, many potential subjects may experience a "dry mouth" when approached by highway department personnel. Although suppression of

salivary flow did not alter the relative concentrations of secobarbital in saliva and plasma, consideration should be given to methods by which the salivary flow is least suppressed. The gas chromatographic analyses for basic drugs such as diphenhydramine, amphetamine, and chlorpromazine can be performed on 1 mL of saliva. However, analyses of drugs that are not basic, and especially those that are highly bound in plasma to protein (e.g., diazepam) require several times this volume. Radioimmunoassay procedures, such as the one used for the analysis of codeine, can be performed on as little as 10-20 μL of sample.

The second problem experienced was the non-homogeneity of some saliva samples. In less controlled populations, extraneous materials from food, tobacco, cigarette smoke, lipstick and other makeup, and other drugs should be expected to be encountered. We found that centrifuging the saliva samples at 1500-2000 x g satisfactorially removed most contaminants. Studies on a much wider variety of solid contaminants should be conducted. Contamination of saliva with extraneous materials that are soluble in this fluid may present a much more serious problem. The extraction procedures employed in our analysis effectively remove those materials which would otherwise have interferred with the chromatographic determinations. These procedures should also remove most extraneous compounds.

The third problem encountered was the presence of the drug being analyzed in saliva arising from material remaining in the oral cavity from the drug tablet/capsule. This is not an actual problem in performing the analytical procedures, but rather in interpreting the results of the analysis and is an example of a false positive.

False positives are usually considered as measurements of amounts of a compound in the fluid which is not actually there. For saliva, another method of arriving at a false positive is for the compound being analyzed to be present in the saliva in the oral cavity but not having been secreted by the salivary gland. This is possible in saliva due to residues of drugs being taken orally or being inhaled remaining in the oral cavity or being refluxed from the upper gastrointestinal tract. This type of false positive can be partially dealt with by using a mouth rinse prior to the collection of saliva or by collecting two samples of saliva with a sufficiently long time interval (ca. 30-60 min) in between. False positives due to interferences in the analytical process, e.g., compounds contained in saliva which give rise to peaks in gas chromatograms at the same position as the peak corresponding to the drug under study, in somewhat the same manner as those co-eluting with the internal standard give rise to false negatives, are much more difficult to deal with. For survey purposes, analysis of saliva samples taken from a number of people which are known not to contain the drug under study should be sufficient to determine whether such interferences will be frequent. In instances where the results of a single sample are to be used, it may be necessary to analyze the sample by at least two different methods, preferably with two different modes of detection. For example, diazepam could be assayed on a Carbowax GC column with a nitrogen specific detector and on a methyl silicone capillary column with an electron capture detector. Even better would be the replacement of one of these assay procedures with a radioimmunoassay.

False positives can also occur from chance events, e.g., electronic noise peaks, dirty GC injectors, contaminated counting vials. These

false positives can be dealt with by performing each analysis at least in duplicate.

When an established extraction-analysis procedure was used, false negatives arose most easily from (1) failure to obtain a true sample of the body fluid, (2) losses occurring in sample storage and (3) interfering peaks in the chromatogram. We did not encounter false negatives (as far as can be determined) due to failure to obtain a true sample of body fluid, due to the nature of our studies. Liquids other than saliva in the oral cavity at the time of saliva collection would dilute the saliva and lead to erroneously low values for concentrations of the drug in saliva. This problem can be overcome by taking two samples of saliva in close succession from the same subject. The dilutant present in the mouth should be contained primarily in the first sample, and the second sample should contain a higher concentration of saliva. Both samples could then be analyzed in order to ascertain if the saliva in first sample had been diluted. If a sufficient volume of saliva had been collected in the first sample to rid the mouth of all of the dilutant, then the concentration of drug in the second sample could be considered valid. Alternatively, the saliva in the first sample could be routinely discarded and analysis performed only on the second sample of saliva.

False negatives due to losses occurring in sample storage will vary markedly with the drug being analyzed and the storage conditions. Chlorpromazine, for instance, is unstable in plasma but quite stable in saliva at -20°C. We experienced no losses of drugs under these storage conditions in the collection vials described in section 4.1. We have observed considerable losses of samples of some drugs stored in unsilylated or plastic containers. Possible decomposition of each drug must

be evaluated under the anticipated storage conditions. Both short-term (transporting the sample from the collection site to the analysis site) and long-term (storage of the sample at the analysis site prior to analysis) stability must be considered. We recommend that the storage conditions used in this study be employed until less stringent conditions have been evaluated.

Compounds which co-extract with the drug being analyzed and which have a retention time in the chromatographic analysis identical to that of the internal standard will make the concentration of the internal standard appear higher than it actually is and thereby produce a false negative. Before any of the chromatographic assays developed in this program are used for the general population, a study should be conducted to determine, in samples from a large number of subjects, whether such interfering peaks are to be expected. Other ways that this possibility of false negatives can be dealt with are to use multiple internal standards such as we developed for secobarbital, amphetamine, and diazepam or to chromatograph each sample on two different stationary phases. At least one manufacturer now sells an instrument that automatically injects a sample on two different columns and compares the results under microprocessor control.

5.2 Use of Saliva Concentrations of Drugs that are Mainly Non-Ionized to Predict Their Plasma Concentrations

Secretion of non-ionized drugs by the salivary glands should be independent of the relative pH's of saliva and plasma. The concentration of these drugs in saliva should then be equivalent to the concentration of "free" drug in plasma. We found that at one hour or later after administration the average concentration of secobarbital in saliva

was 0.99 times the concentration of "free" secobarbital in plasma (CV = 21%); i.e., [saliva] = 0.32 [plasma]. At 0.5 hr after administration of secobarbital (in a capsule), its concentration in saliva was 2.5 times the concentration of "free" secobarbital in plasma. Prediction of concentration of secobarbital in plasma from its concentration in saliva is thus much improved if the time following adminstration of the drug that the sample is taken is known. From the equation of the best-fit line for the linear regression analysis of our data (excluding the 0.5 hr samples), predicted secobarbital concentrations in plasma from concentrations of 10, 100, and 1000 ng/mL in saliva are 27, 308, and 3540 ng/mL respectively. Inclusion of the 0.5 hr samples in this analysis changes the predicted concentration in plasma to 14, 265, and 5180 ng/mL respectively.

The samples from 3 of the subjects in our study were also analyzed by Cook et al. (1979) who used stereoselective RIA's for secobarbital. He found relationships of [saliva] = 0.31 [plasma] and [saliva] = 0.29 [plasma] for R- and S- secobarbital, respectively. Cook et al. (1975) had previously demonstrated a similar relationship between saliva and plasma concentration of phenobarbital, where [saliva] = 0.29 [plasma].

With limited data, we found that the concentration of diazepam in saliva averaged 3.1% of that in plasma for samples taken one hr or later after administration of the drug. Almost identical results were reported by Di Gregorio et al. (1978) on samples taken from 9 subjects.

In studies with other drugs that are not appreciably ionized at plasma pH, Cook et al. (1975) found a linear relationship existed between concentrations of diphenylhydantoin in plasma and saliva where [saliva] = 0.1 [plasma]. A similar relationship, [saliva] = 0.13 [plasma], was

found by Mucklow, et al. (1978), who analyzed a single sample from each of eleven patients who were receiving regular doses of the drug.

Mucklow also reported that between 3 and 32 hr after a single dose of antipyrine to 10 subjects the relationship between concentrations of the drug in saliva and plasma was [saliva] = 0.92 [plasma].

For many drugs which are not ionized at normal plasma pH, including secobarbital and diazepam, direct relationships exist between the concentrations of the drugs in saliva and their concentrations in plasma. In surveys, where there are large number of subjects, the relationships are precise enough to permit accurate estimations of the distribution of concentrations of these drugs in plasma from analysis of saliva samples.

A calculation of the approximate concentration of secobarbital in plasma from a single sample of saliva is possible using the equation

$$\frac{[\text{plasma}]}{[\text{saliva}]} = 0.32;\ 95\%\ \text{confidence interval} = 0.19\ \text{to}\ 0.45$$

provided the sample is obtained at least 1 hr after adminstration of the drug. If the time interval between adminstration of the drug and the sample being taken is not known, then the 95% confidence interval becomes much larger.

Although the ratios of concentrations of diazepam in saliva to those in plasma varied only several percent (2.1-5.6%) in the samples from the one subject that we were able to analyze, this represents almost a 3 fold difference in the predicted concentrations of diazepam in plasma. Thus unless further data show that the results for this subject are abnormal, then a large error factor will accompany the determination of a concentration in plasma from a single sample of saliva.

The determination of the plasma concentration of either secobarbital or diazepam from the concentration of the drug in saliva at some later time is not possible. Before a drug is taken, its concentration in plasma is zero, assuming no residual drug is present from a prior administration. After the drug is taken, its concentration in plasma rises to a maximum value and then returns to zero. Thus, for every concentration of the drug in plasma (and saliva), except possibly for the maximum concentration, there are at least two time points where this concentration occurs, one before the maximum concentration is reached, the other after the maximum concentration is reached. Thus, if a certain plasma concentration were calculated (in this study based on a concentration of the drug in saliva), it would not be known if that concentration were for a time point before the maximum concentration was reached or after the maximum were reached. At some time, for instance one hour, prior to one of these time points, there could have been no drug in the plasma, while for the same time interval prior to the other, the concentration of drug could have been at its maximum value.

5.3 Use of Saliva Concentrations of Drugs that are Mainly Ionized to Predict Their Plasma Concentrations

Our results indicate that for some drugs that are mainly ionized at normal plasma pH there is a reasonable correlation between concentrations of the drug in saliva and its concentrations in plasma, while other drugs exhibit only poor correlation. Improved correlation is observed in some cases when corrections are made for the differences in the pH of saliva and plasma.

An example of a drug whose concentrations in saliva and plasma do not correlate well is amphetamine (cf. Figures 17 and 18). Attempts to

improve this correlation with an "internal indicator" (creatinine) of salivary secretion were not successful. Since amphetamine is an alpha-adrenergic agonist, it is possible that this compound interferes with its own secretion.

Much better correlations were obtained between concentrations in saliva and plasma for diphenhydramine and codeine. Correlation coefficients for the linear regression analyses of the logs of the concentrations of these drugs in plasma and saliva were greater than 0.9 for each. The correlations between concentrations in saliva and plasma were improved for codeine, but not for diphenhydramine, when corrections were made for the differences in pH of individual samples of saliva and plasma. The relation between saliva and plasma concentrations of both drugs as calculated by linear regression analysis is very concentration dependent. For example, assuming the pH of saliva is 7.0 and $f_p = 0.28$ for diphenhydramine, an observed concentration of 10 ng/mL of this drug in saliva would lead to a predicted concentration of 9.1 or 12.3 ng/mL in plasma. The latter value includes a correction for the differing pH of plasma and saliva. Similar calculations for an observed concentration in saliva of 1000 ng/mL leads to a prediction of 266 or 443 ng/mL. Approximately equal concentrations in plasma and saliva are predicted at the lower observed concentration in saliva, but less than half the saliva concentration is predicted to be in plasma at the higher concentration. In order to more fully establish whether these relationhsips really are concentration dependent, studies should be conducted where the dose of each of these drugs is varied over a wider range than was done in this project.

Mucklow et al. (1978) has reported poor correlation (linear regression analysis) between the saliva and plasma concentrations of propranolol ($r = 0.43$) and chlorpropamide ($r = 0.54$). Cook et al. (1981) found a somewhat better correlation between the observed plasma concentrations and those predicted from saliva concentrations of phencyclidine ($r = 0.76$). In this study the pH values of individual saliva samples were used in the calculations.

It does not seem likely that, at the present itme, saliva could be used for quantitative estimation of the concentrations in plasma of amphetamine or of drugs described above studied by other investigators. Saliva could possibly be used to estimate the concentrations in plasma of diphenhydramine and codeine for survey purposes. Use of single saliva samples to determine concentrations of any of these drugs in plasma does not appear feasible.

6.0 Research Conclusions

The following conclusions can be drawn from the results of this project:

(1) It is not possible at the present time to correlate the excretion of relatively non-volatile drugs in breath with their concentrations in plasma. None of the drugs examined in this study were highly volatile. If care is not taken, breath samples will be contaminated with droplets of saliva. These droplets can contain large concentrations of drug which will interfere with the accurate determination of the concentration of drug in breath.

(2) Mixed saliva can be collected noninvasively by a simple procedure.

(3) Analysis of saliva for all the drugs studied except codeine can be accomplished on capillary gas chormatographic columns with nitrogen-phosphorus specific detection.

(4) The radioimmunoassay for codeine which was developed for use with plasma can be adapted for use with saliva.

(5) Qualitative determinations of the presence of drugs in plasma from their concentrations in saliva are possible for all drugs studied. Roadside techniques for these determinations are not now available. Such roadside testing could possibly be developed from currently emerging techniques, but its development would be costly.

(6) Correlations between concentrations in plasma and saliva of the two drugs in this study that are mainly non-ionized at normal plasma pH (secobarbital and diazepam) are reasonably

good. Use of these correlations for survey purpose appears feasible. The correlations would permit semi-quantitative determinations of the concentrations of these drugs in plasma from their concentrations in a single sample of saliva one hour or more after administration of the drug. The correlations are not sufficiently good, however, to permit evidential use of the result of a single sample.

(7) Correlations between concentrations in plasma and saliva of the drugs in this study that are mainly ionized at normal plasma pH (amphetamine, diphenhydramine and codeine) are more tenuous and vary considerably from drug to drug. Calculations based on the pH of each saliva sample inprove the correlation for diphenhydramine. The correlations between plasma and saliva concentrations of diphenhydramine and codeine are sufficiently good (correlation coefficients >0.92) to permit the utilization of saliva for survey purposes.

(8) The use of the concentration of a drug in saliva to determine its concentration in plasma at some earlier time is not now possible. Knowledge of the pharmacokinetics of the metabolites of a drug and development of methods to measure these metabolites in saliva may at some future time permit the use of the concentrations of both the drug and its metabolites in saliva to make such predictions.

(9) The major problem associated with the correlation of saliva and plasma concentrations of drugs that otherwise showed good correlations is the abnormal (false positive?) values observed

for samples taken less than one hour after the drug was administered. The problem arose even though the mouth was flushed with water immediately after administration of the drug.

7.0 Names, Qualifications and Participation of Researchers

Name	Participation
Dr. M. E. Wall	Directed overall program
Dr. A. R. Jeffcoat	Directed research in program
Dr. M. Perez-Reyes	Conducted clinical experiments.

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Appendix A - Detailed Analytical Methodologies

A.1 Secobarbital

Chart 1.

Procedure for Extraction of Secobarbital from Human Plasma and Saliva.

3 mL Plasma or Saliva

Add 1.5 μg of hexobarbital and butabarbital.
Add 0.3 mL of 4 M NaH_2PO_4. Extract three times with 3 mL of $CHCl_3$; mix 1 minute with vortex mixer; centrifuge.

Chloroform Extract

Evaporate solvents.
Transfer to 1 dram vial with 3 x 0.5 mL $CHCl_3$.
Extract with 2 x 1.1 mL of 0.1 N NaOH.

NaOH Extract

Adjust to pH 3 with 2 N HCl.
Extract with 3 x 1.5 mL of $CHCl_3$.

Chloroform Extract

Evaporate solvent. Transfer to 100 μL conical vial with 4 x 50 μL $CHCl_3$. Evaporate to dryness.
Redissolve in 20 μL m-dimethoxy benzene.
Inject on gas chromatograph.

Figure 30.

Chromatogram of Plasma Extract Containing 1 µg Secobarbital (I), 0.5 µg Allylcyclopentenyl Barbituric Acid (I) and 0.5 µg Barbital (III) per ML Plasma. Amounts of Barbiturates Injected Onto the Column Were 150 ng Secobarbital, 75 ng Allylcyclopentyl Barbituric Acid and 75 ng Barbital.

GLC: Hewlett-Packard 5840A
Column: 33 m capillary of OV-101 on $BaCO_3$
Temperature: 125° to 225° at 10°/min
Flow Rate: 1.56 ml He/min at 195°
Detector: Nitrogen-phosphorous

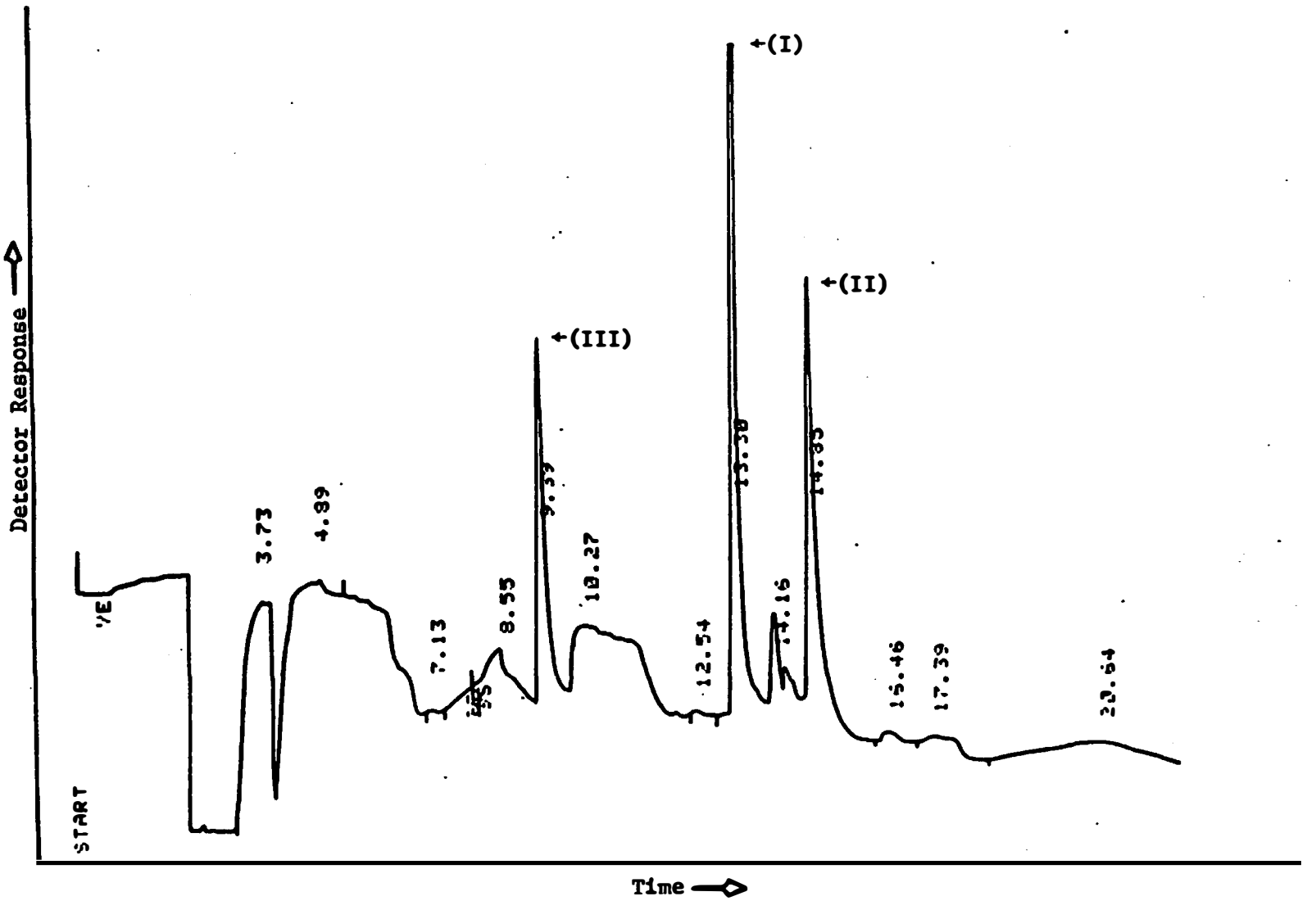


Figure 31. GLC Calibration Curve for Secobarbital in Plasma Using a OV-101/$BaCO_3$ WCOT Capillary Column

GLC: Hewlett-Packard 5840A
Column: 33 m WCOT capillary of OV-101 on $BaCO_3$
Temperature: 125° to 225° at 10°/min
Flow Rate: 1.56 ml He/min at 195°
Detector: Nitrogen-phosphorous

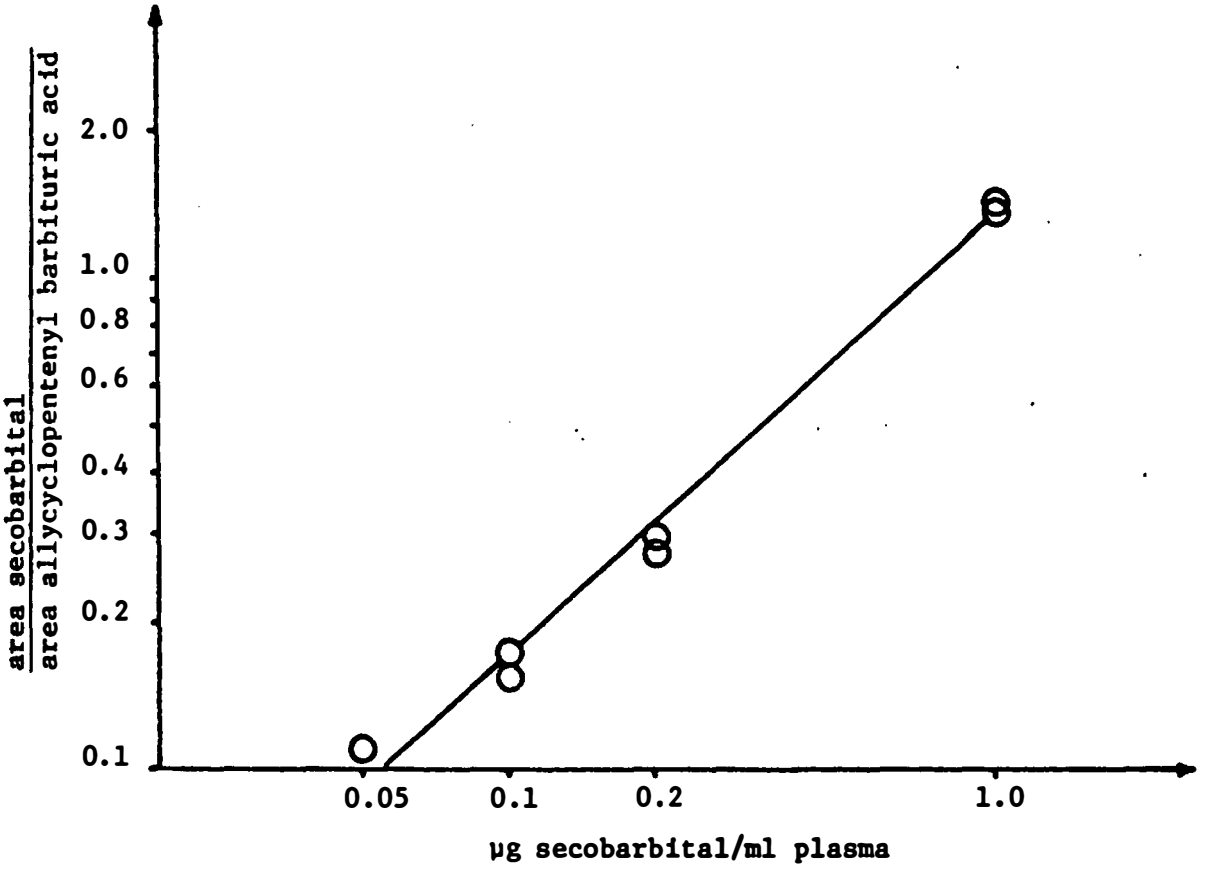


Figure 32.

Chromatogram of Plasma Extract Containing 1 µg Secobarbital (I), 0.5 µg Butabarbital (II) and 0.5 µg Hexobarbital (III) per ML Plasma. Amounts of Barbiturates Injected onto the Column Were 150 ng Secobarbital, 75 ng Butabarbital and 75 ng Hexobarbital.

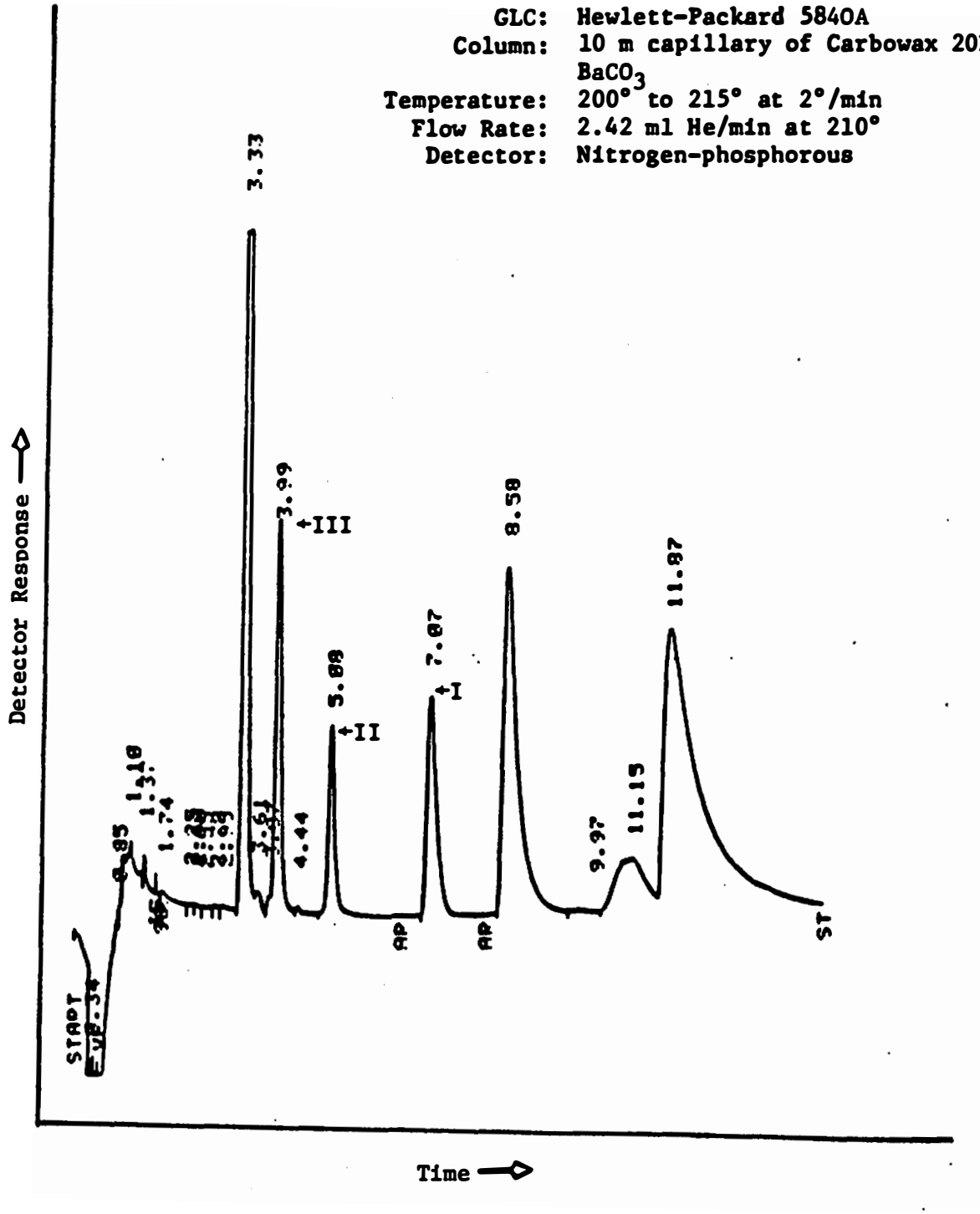


Figure 33. GLC Calibration Curve for Secobarbital in Plasma (Log-Log Plot) Using a Carbowax 20M/$BaCO_3$ WCOT Capillary Column

GLC: Hewlett-Packard 5840A
Column: 10 m WCOT capillary of Carbowax 20M on $BaCO_3$
Temperature: 200° to 215° at 2°/min
Flow Rate: 2.42 ml He/min at 210°
Detector: Nitrogen-phosphorous

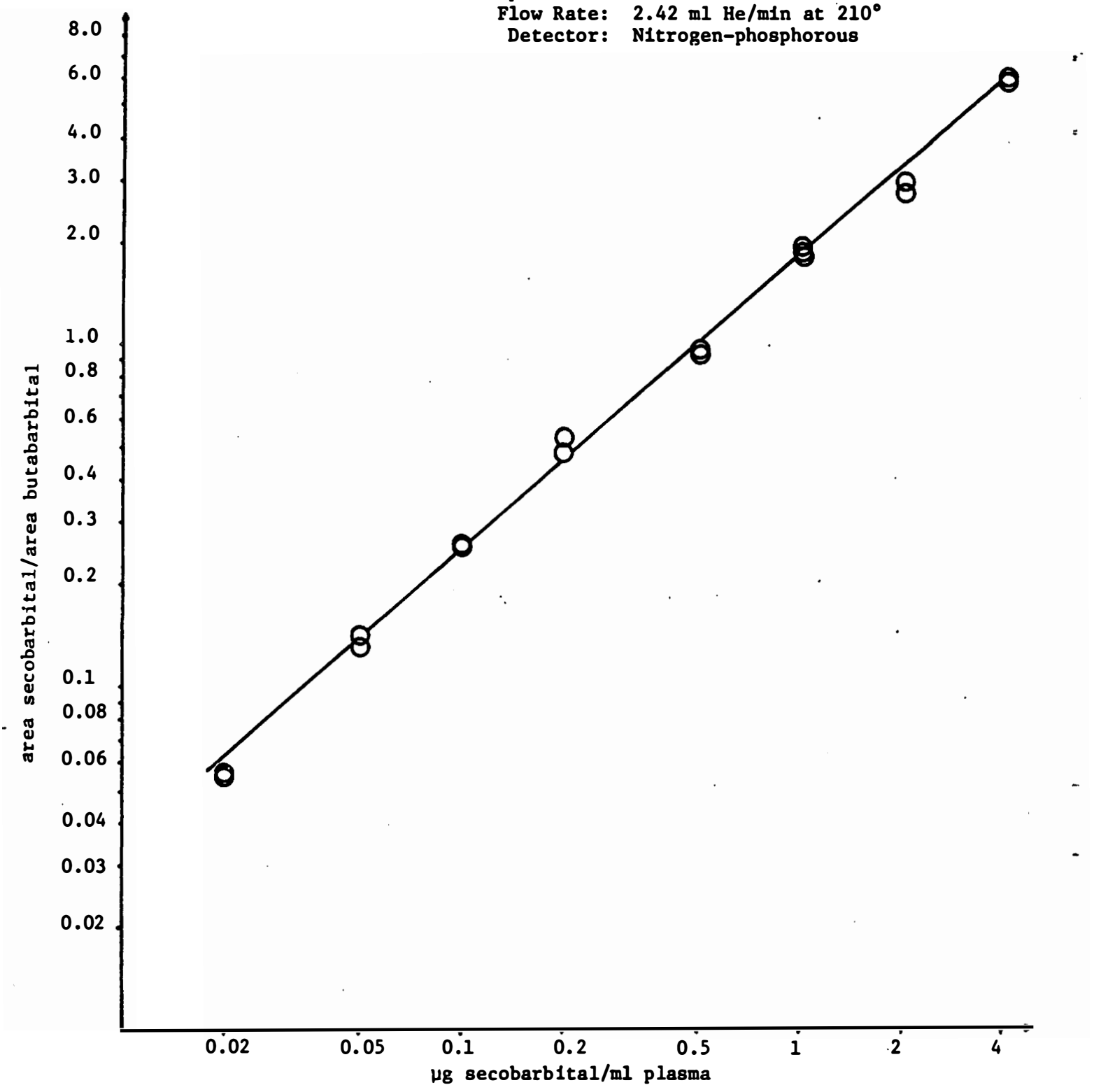


Figure 34. Chromatogram of Secobarbital Extracted From Saliva on a Carbowax 20M Capillary Column.

The retention time of secobarbital is 5.21 min.
The retention time of butabarbital is 3.7. min.
The retention time of hexabarbital is 2.86 min.

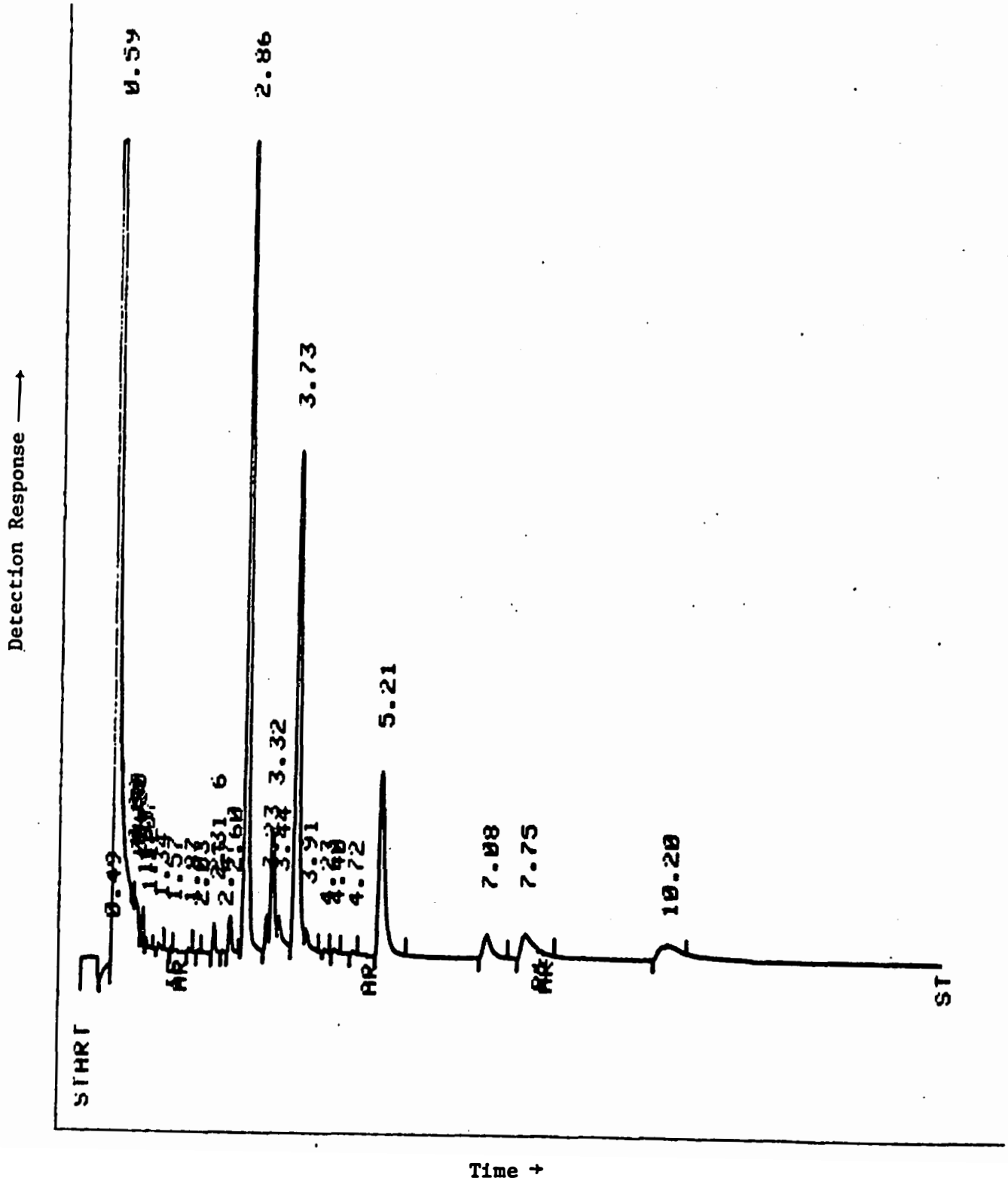


Figure 35. GLC Calibration Curve for Secobarbital in Saliva Using a Carbowax 20M/$BaCO_3$ WCOT capillary column

GLC: Hewlett-Packard 5840A
Column: 10 m capillary of Carbowax 20M on $BaCO_3$
Temperature: 200° to 215° at 2°/min
Flow Rate: 2.42 mL He/min at 210°
Detector: Nitrogen-phosphorous

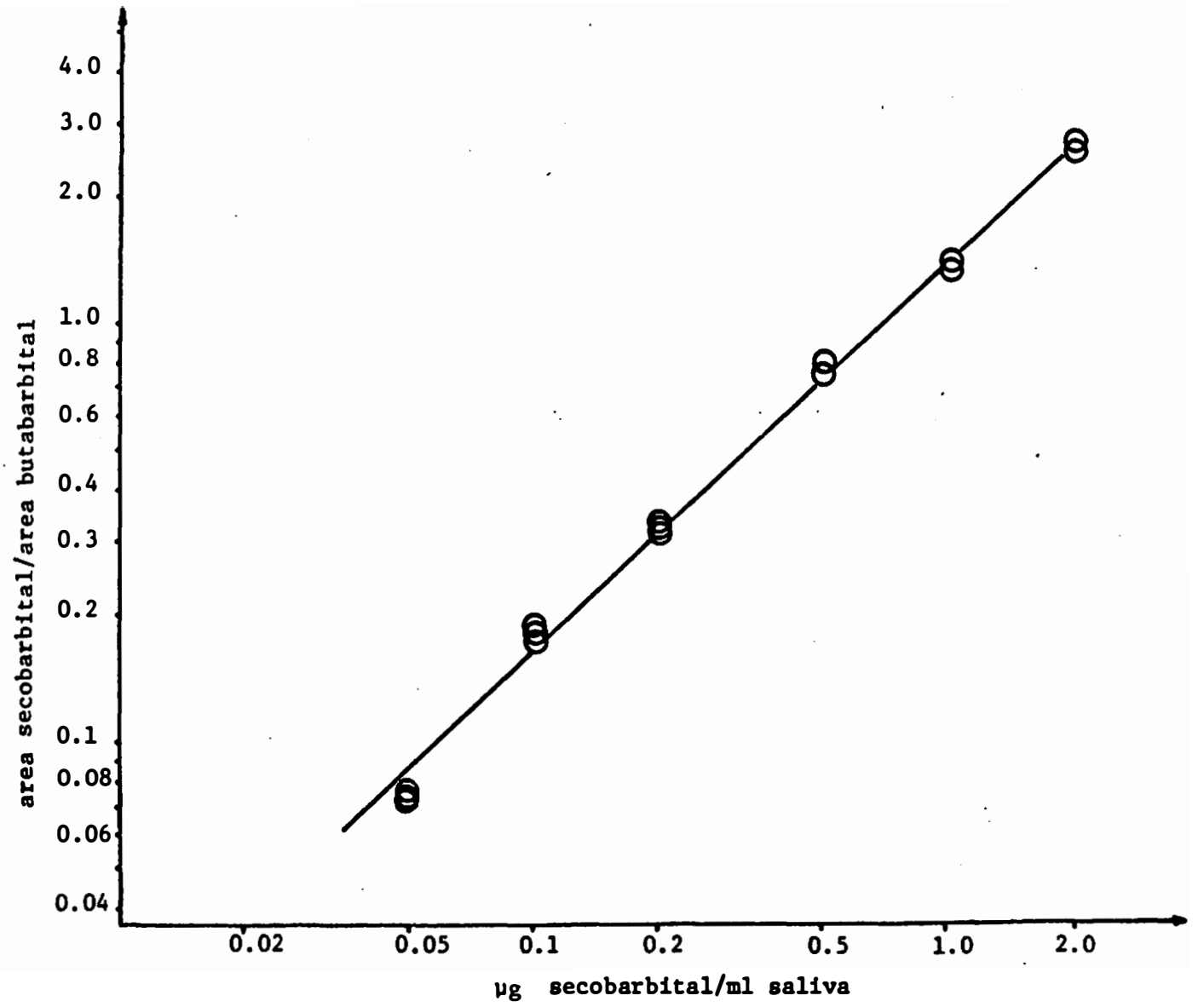
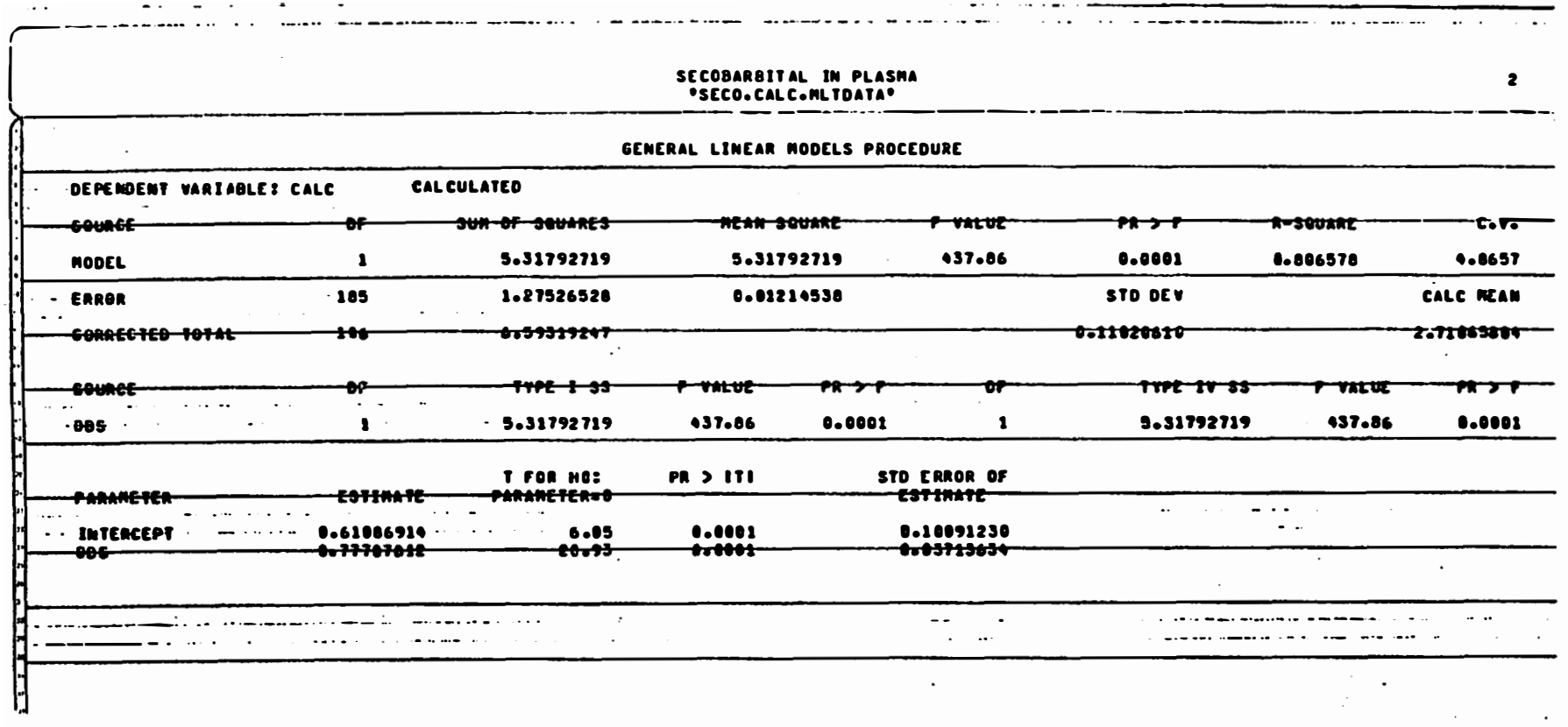


Table 18.

Accuracy and Precision of Assay for Secobarbital in Plasma and Saliva.

Fluid	Secobarbital Added (µg/mL)	Secobarbital Found by Assay (µg/mL)	Average of Assay Values (µg/mL)	C. V. (%)
Plasma	0.50	0.56 0.50 0.53 0.51 0.49 0.47	0.51	6.2
Plasma	0.20	0.22 0.21 0.23 0.24 0.26 0.22	0.23	7.8
Plasma	0.050	0.055 0.065 0.062 0.049 0.061 0.053	0.057	10.6
Saliva	0.50	0.53 0.49 0.54	0.52	5.1
Saliva	0.20	0.19 0.20 0.19	0.19	3.0
Saliva	0.05	0.038 0.036 0.038	0.037	3.1

Figure 36. Statistical Data for the Linear Regression Analysis of Predicted versus Observed Plasma Concentrations (ng/mL) of Secobarbital. All sampling times are included.



SECOBARBITAL IN PLASMA 2
'SECO.CALC.MLTDATA'

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CALC CALCULATED

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	5.31792719	5.31792719	437.86	0.0001	0.806578	4.8657
ERROR	185	1.27526528	0.01214538		STD DEV		CALC MEAN
CORRECTED TOTAL	186	6.59319247			0.11020610		2.71865884

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
DOS	1	5.31792719	437.86	0.0001	1	5.31792719	437.86	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > ITI	STD ERROR OF ESTIMATE
INTERCEPT	0.61086914	6.05	0.0001	0.10091230
DOS	0.77787012	20.93	0.0001	0.03713634

Figure 37. Statistical Data for the Linear Regression Analysis of Predicted versus Observed Plasma Concentrations (ng/mL) of Secobarbital. Samples taken at 0.5 hr have been omitted.

SECOBARBITAL IN PLASMA
'SECO.CALC.NEWDATA'

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CALC CALCULATED

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	5.39516288	5.39516288	799.42	0.0001	0.889806	3.8193
ERROR	99	0.66813728	0.00674886		STD DEV		CALC MEAN
CORRECTED TOTAL	100	6.06330016			0.00815145		2.13386756

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
OBS	1	5.39516288	799.42	0.0001	1	5.39516288	799.42	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	0.14001080	1.53	0.1298	0.09164565
OBS	0.94402561	28.27	0.0001	0.03340617

Appendix A - Detailed Analytical Methodologies

A.2 Amphetamine

Chart 2.

Procedure for Extraction of Amphetamine from Plasma and Saliva.

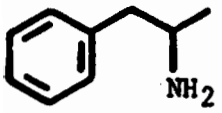
2 mL Plasma or Saliva

Add 100 ng of Internal Standard.
Add 25 µL of 2.5 N NaOH.
Extract twice with 2 mL of toluene.

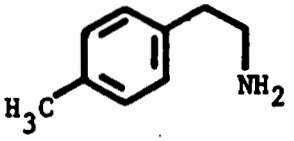
Toluene Extract

Add 15 µL of 4.8 M HCl in methanol.
Let sit for 45 min.
Carefully evaporate solvents (90°C under N_2).
Transfer to 1/2 dram vial with 2 x 100 µL EtOH. Evaporate solvents (50°C under N_2).
Add 20 µL of a 10% solution of trifluoroacetic anhydride in hexane. Add 20 µL of toluene.
Heat at 60°C for 10 min.
Keep for additional 20 min.
Transfer to 100 µL vial and chromatograph.

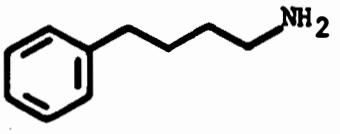
Figure 38. Structures of Amphetamine and Internal Standards



Amphetamine I
(1-phenyl-2-aminopropane)



1-(4-methylphenyl)-2-aminoethane II



1-phenyl-4-aminobutane III

Figure 39. Chromatogram of Amphetamine Extracted from Plasma at a Concentration of 10 ng/mL Using a Carbowax 20M Capillary Column.

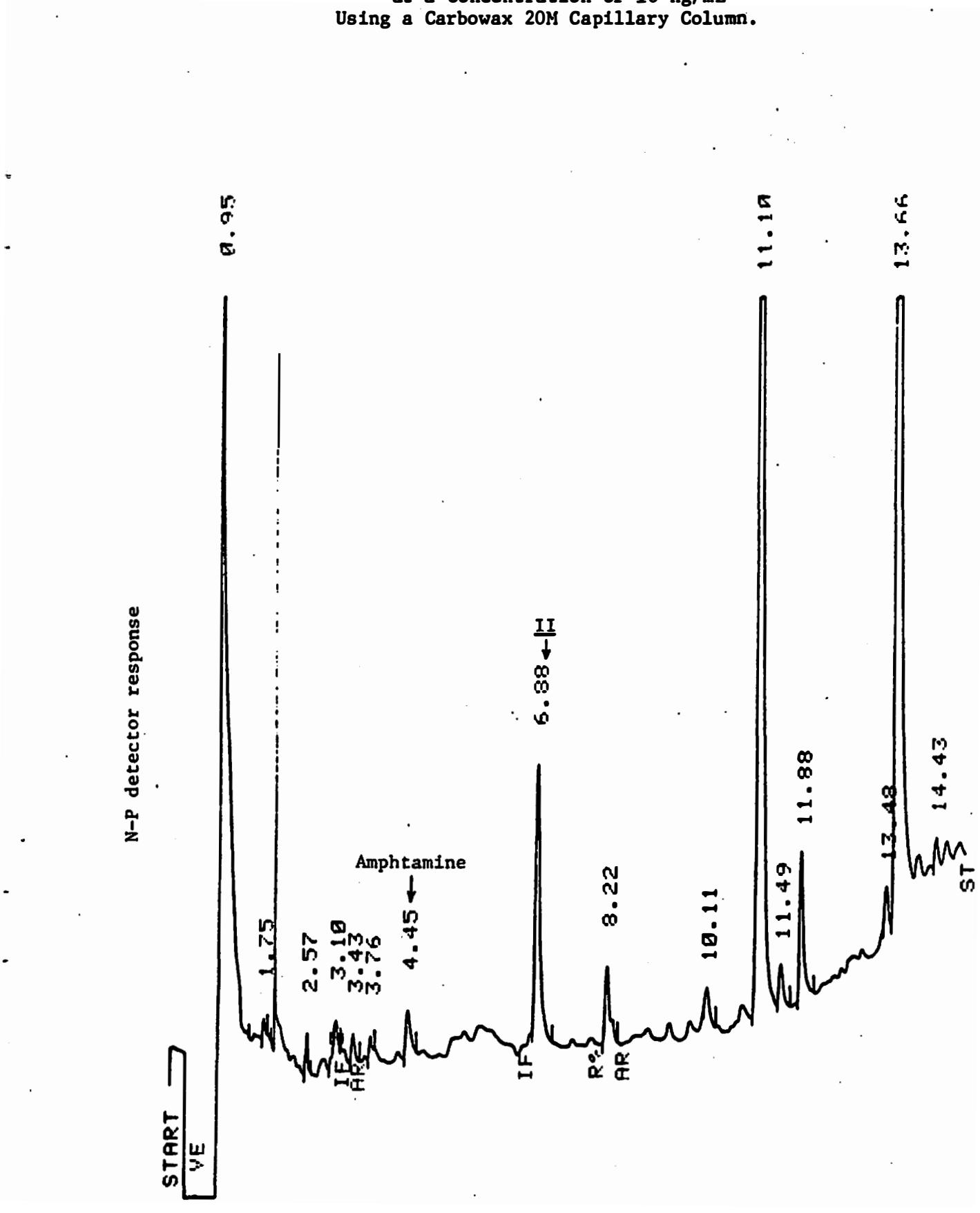


Figure 40. Chromatogram of Amphetamine Extracted from Saliva Using a Carbowax 20M Capillary Column.

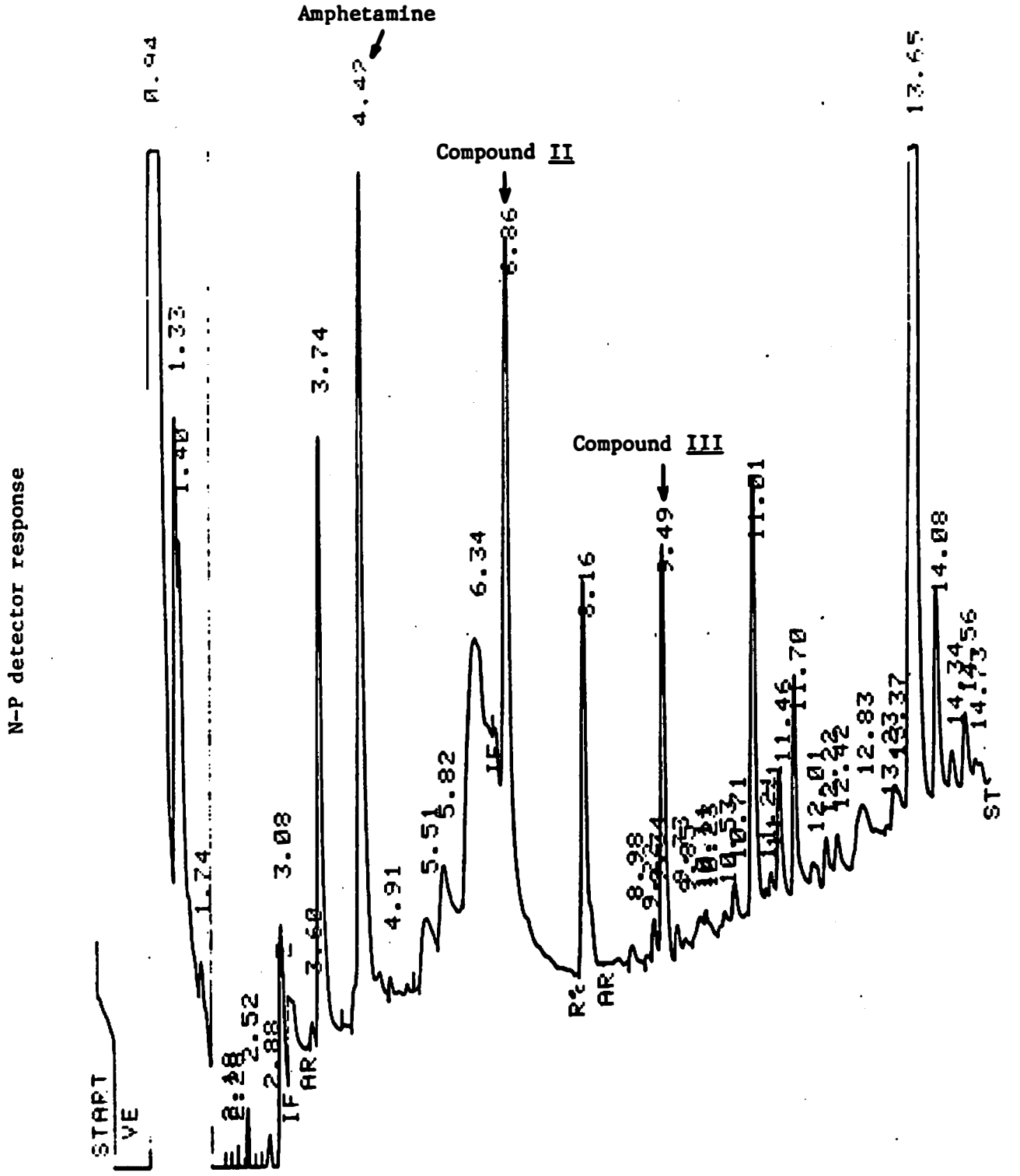


Figure 41. Standard Curve for Analysis of Amphetamine from Plasma on Carbowax 20M Capillary Using Compound II as the Internal Standard

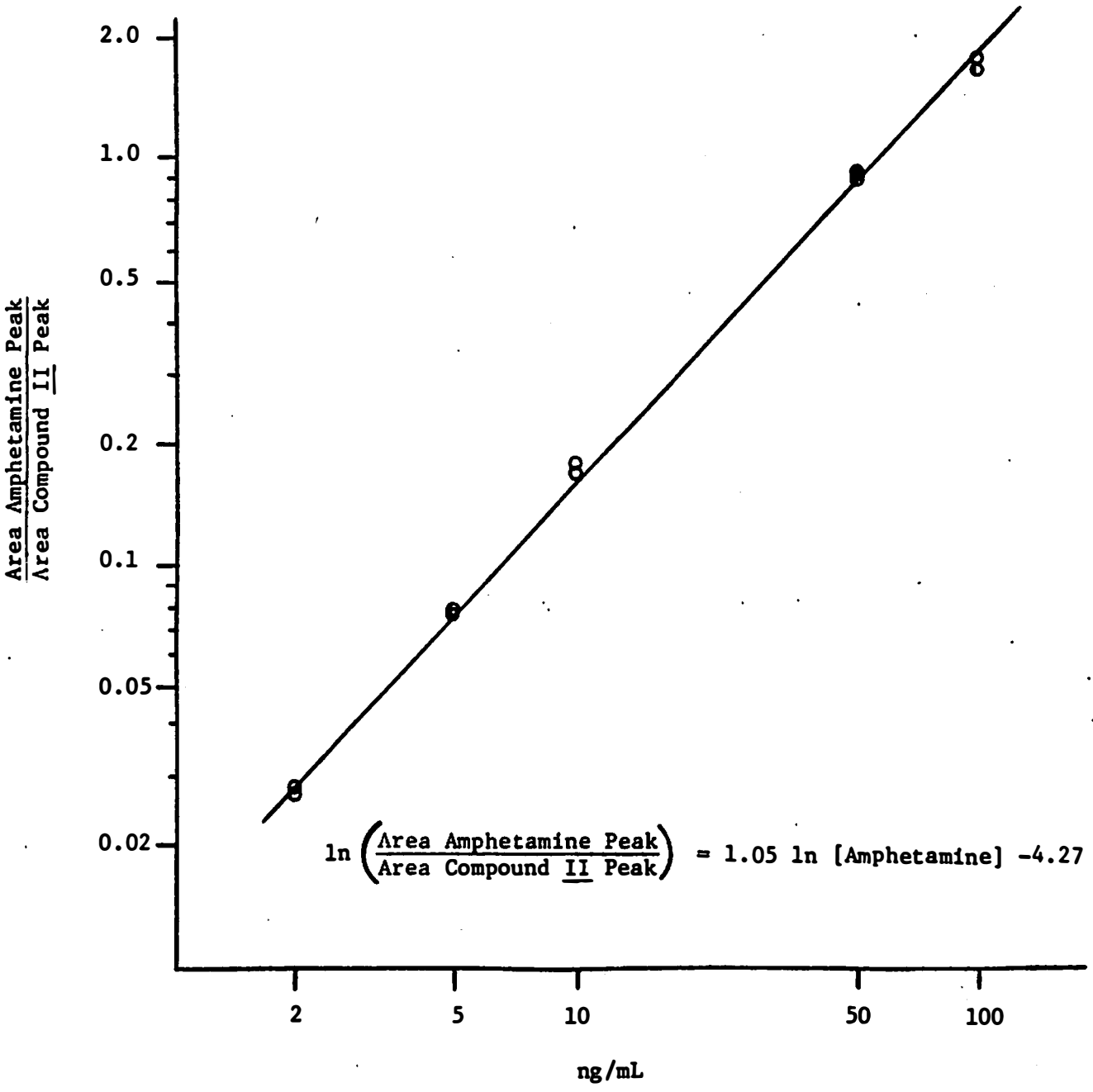


Figure 42. Chromatogram of Amphetamine Extracted from Plasma at a Concentration of 20 ng/mL on a SE-30 Capillary Column

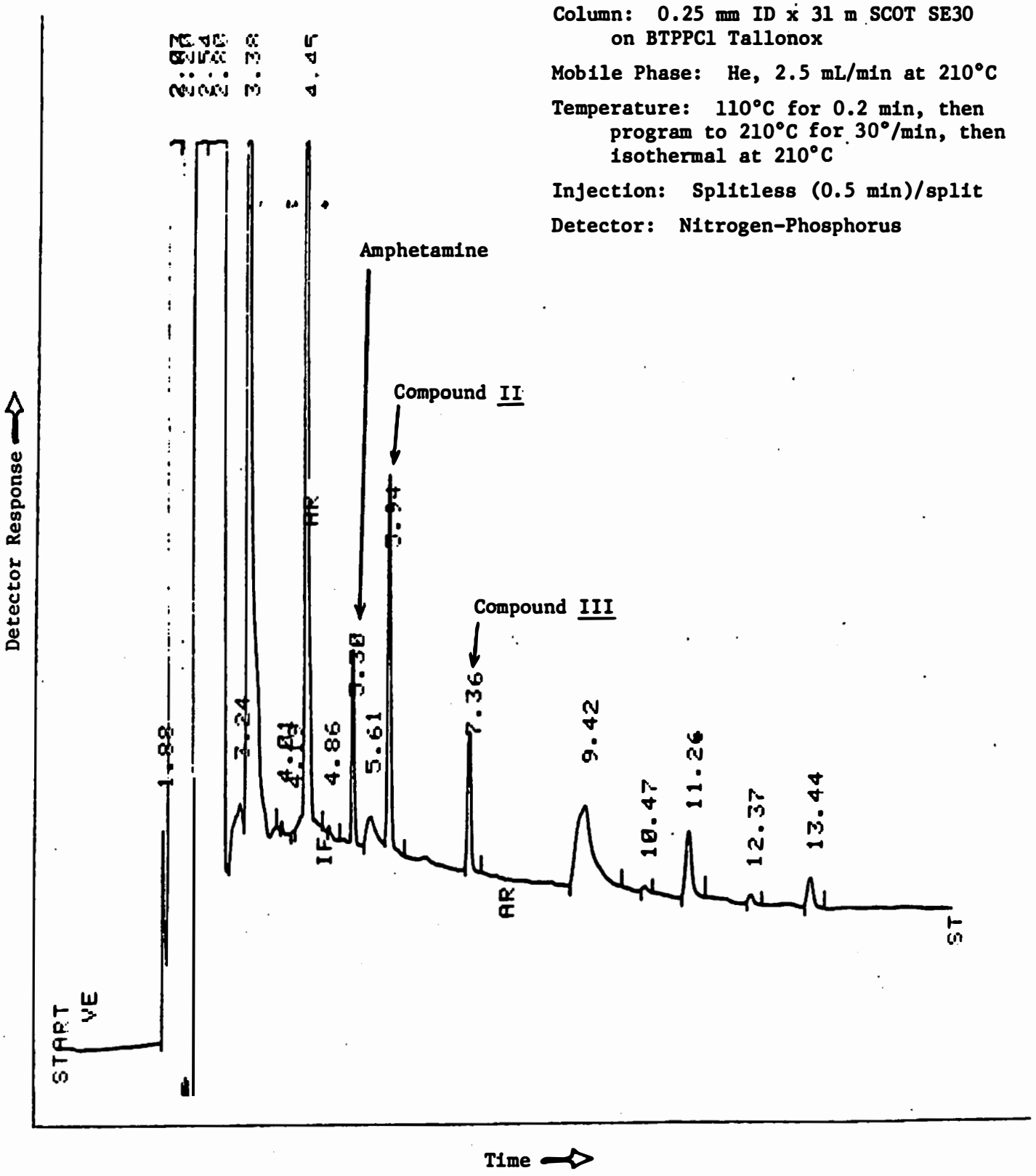


Figure 43. Chromatogram of Amphetamine Extracted from Saliva at a Concentration of 55 ng/mL on a SE-30 Capillary Column

Chromatographic conditions same as in Figure 42.

Compounds II and III are internal standards.

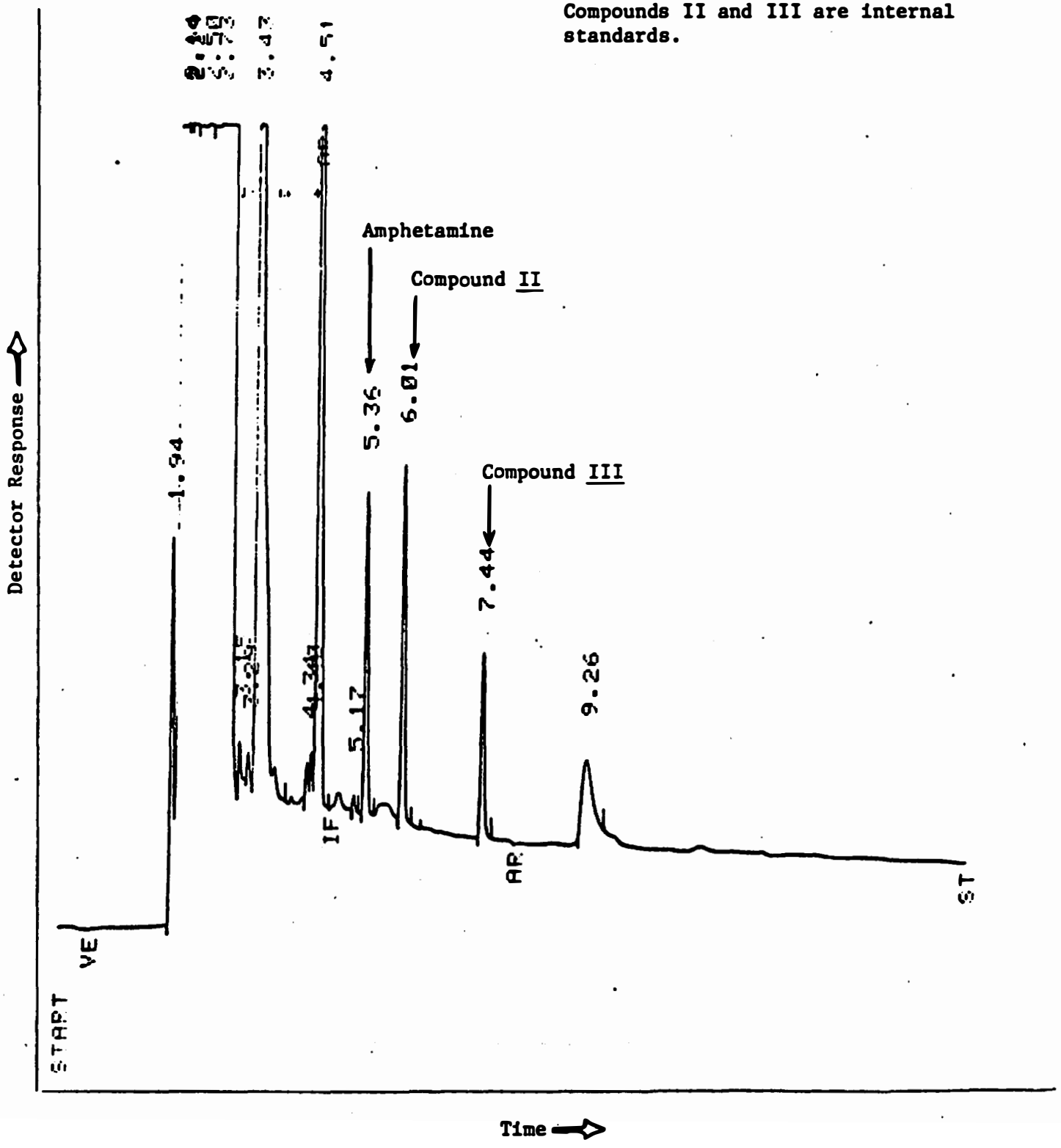


Figure 44. Standard Curves for Analysis of Amphetamine from Plasma on SE-30 Capillary

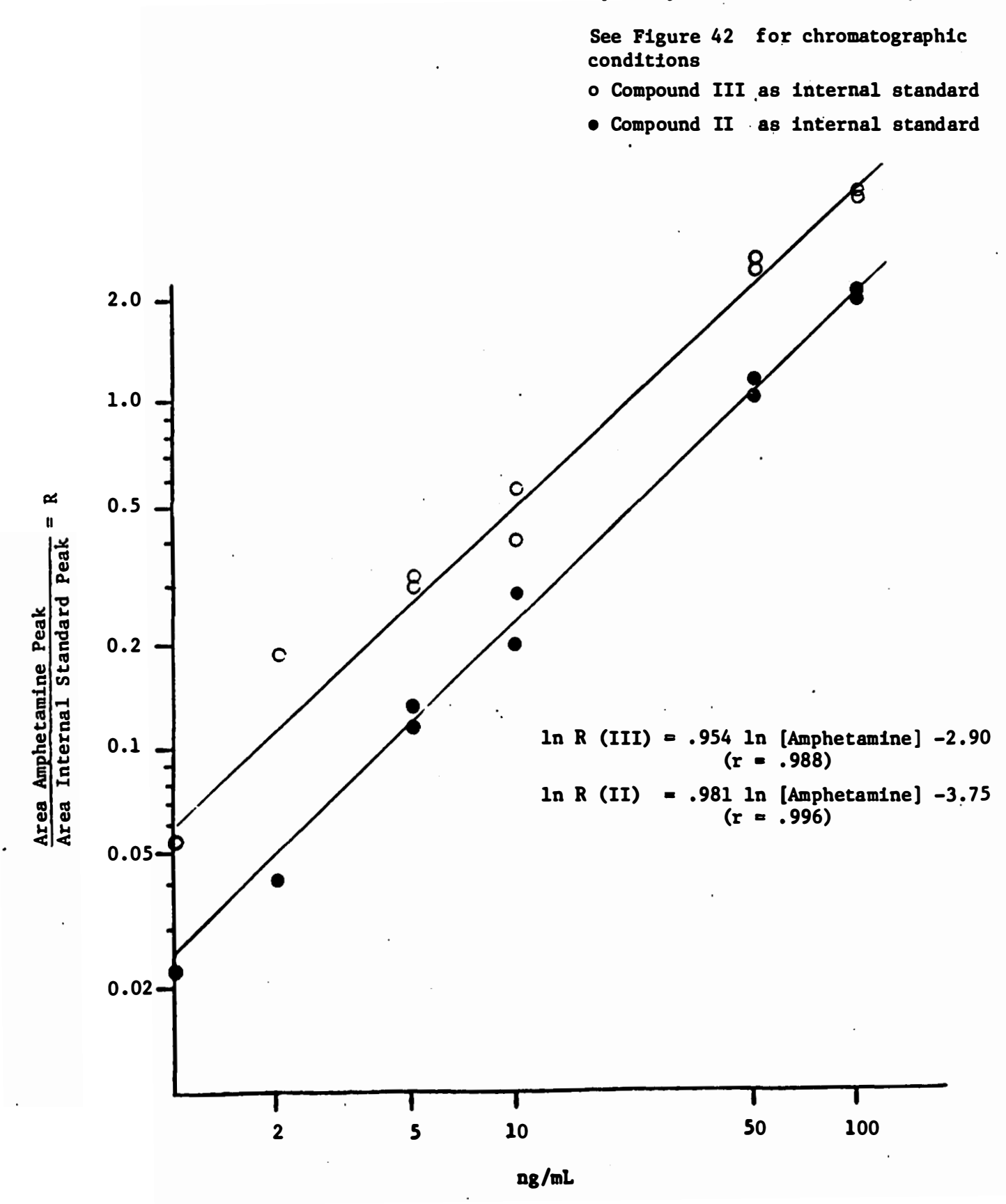


Figure 45. Standard Curves for Analysis of Amphetamine from Saliva on SE-30 Capillary

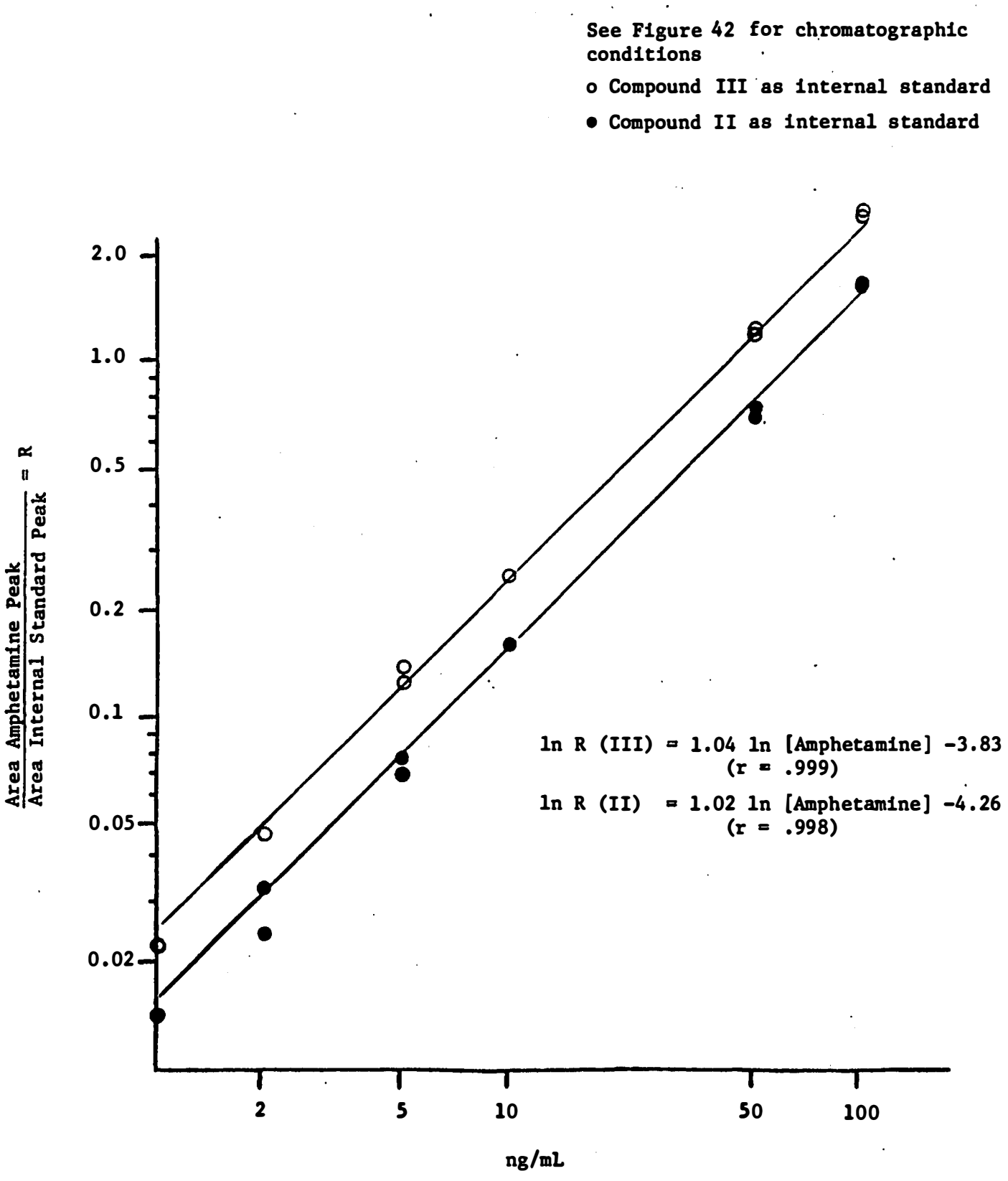


Table 19.

Accuracy and Precision of Assay for Amphetamine in Plasma and Saliva.

Fluid	Amphetamine Added (ng/mL)	Amphetamine Found by Assay (ng/mL)	Average of Assay Values (ng/mL)	C. V. (%)
Plasma	50	52 50 52 58	53	6.5
Plasma	10	9.7 9.9 13.5 9.6	10.7	17.7
Plasma	5	5.6 6.1 6.3 5.6	5.9	6.0
Saliva	100	104 105	104	
Saliva	50	47.6 47.4	47.5	
Saliva	5	4.3 4.4	4.4	
Saliva	2	1.7 1.9	1.8	

Figure 46. Statistical Data for the Linear Regression Analysis of Saliva versus Plasma Concentrations (ng/mL) of Amphetamine.

AMPHETAMINE: SALIVA VS. PLASMA 8

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: SALIVA

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	7.83111501	7.83111501	66.85	0.0001	0.373766	22.5669
ERROR	112	13.12080736	0.11715007		STD DEV		SALIVA MEAN
CORRECTED TOTAL	113	20.95192237			0.34227192		1.51672616

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
PLASMA	1	7.83111501	66.85	0.0001	1	7.83111501	66.85	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	0.63182727	5.60	0.0001	0.11287896
PLASMA	0.85935620	8.18	0.0001	0.10510725

Figure 47. Statistical Data for the Linear Regression Analysis of Predicted versus Observed Plasma Concentrations (ng/mL) of Amphetamine.

AMPHETAMINE IN PLASMA

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CALC CALCULATED

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	10.60546792	10.60546792	69.92	0.0001	0.386464	38.9888
ERROR	111	16.83688244	0.15168363		STD DEV		CALC MEAN
CORRECTED TOTAL	112	27.44235036			0.38946582		0.99912188

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
OBS	1	10.60546792	69.92	0.0001	1	10.60546792	69.92	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	-0.03830840	-0.30	0.7677	0.12936559
OBS	1.00477574	8.36	0.0001	0.12016378

Appendix A - Detailed Analytical Methodologies

A.3 Chlorpromazine

Chart 3.

Extraction of Chlorpromazine (CPZ) from Human Plasma and Saliva

3 mL Plasma or Saliva

Add internal standard.
Adjust pH to 13 with 2.5N NaOH.
Extract twice with 5 mL of hexane/isoamyl alcohol (IAA) (98.5:1.5; v/v) mixing 15 min. Centrifuge. Combine extracts.

Hexane/IAA Extract

Extract twice with 1 mL of 0.05N HCl mixing 1 min. Centrifuge. Combine extracts.

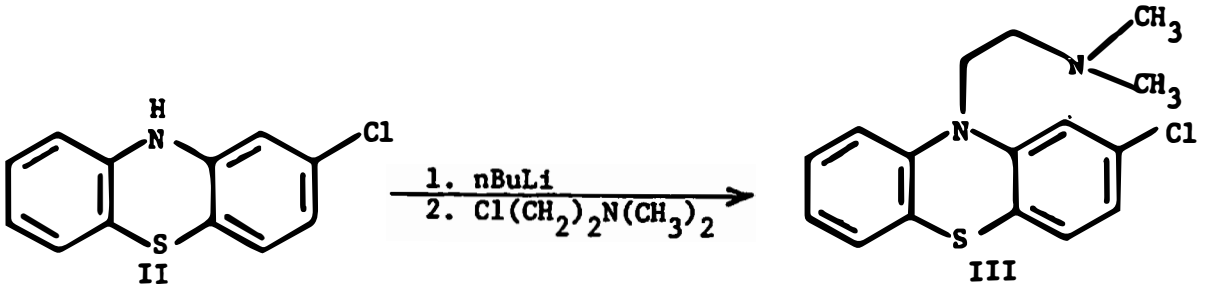
HCl Extract

Adjust pH to 9 with 2.5N NaOH.
Extract twice with 3 mL of CH_2Cl_2.
mixing 1 min. Centrifuge.
Combine extracts.

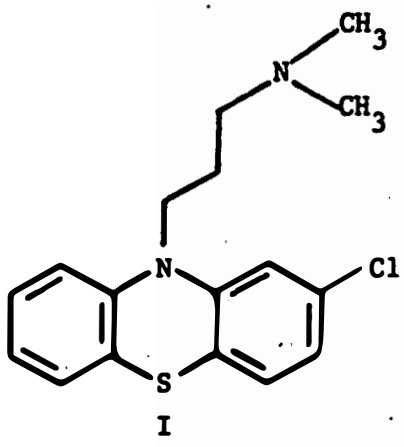
CH_2Cl_2 Extract

Evaporate solvents; redissolve in dimethoxybenzene (20 µL).
Transfer to 100 µL conical vial.
Inject on GC column.

Figure 48. Preparation of Internal Standard for Chlorpromazine Analysis.



2-Chloro-N,N-dimethyl-10H-phenothiazine-10-ethanamine



Chlorpromazine

Figure 49.

Chromatogram of Chlorpromazine Extracted from Plasma.

Retention time of chlorpromazine = 5.22 min.
Retention time of internal standard = 4.71 min.

See Figure 51 for Chromatographic Conditions.

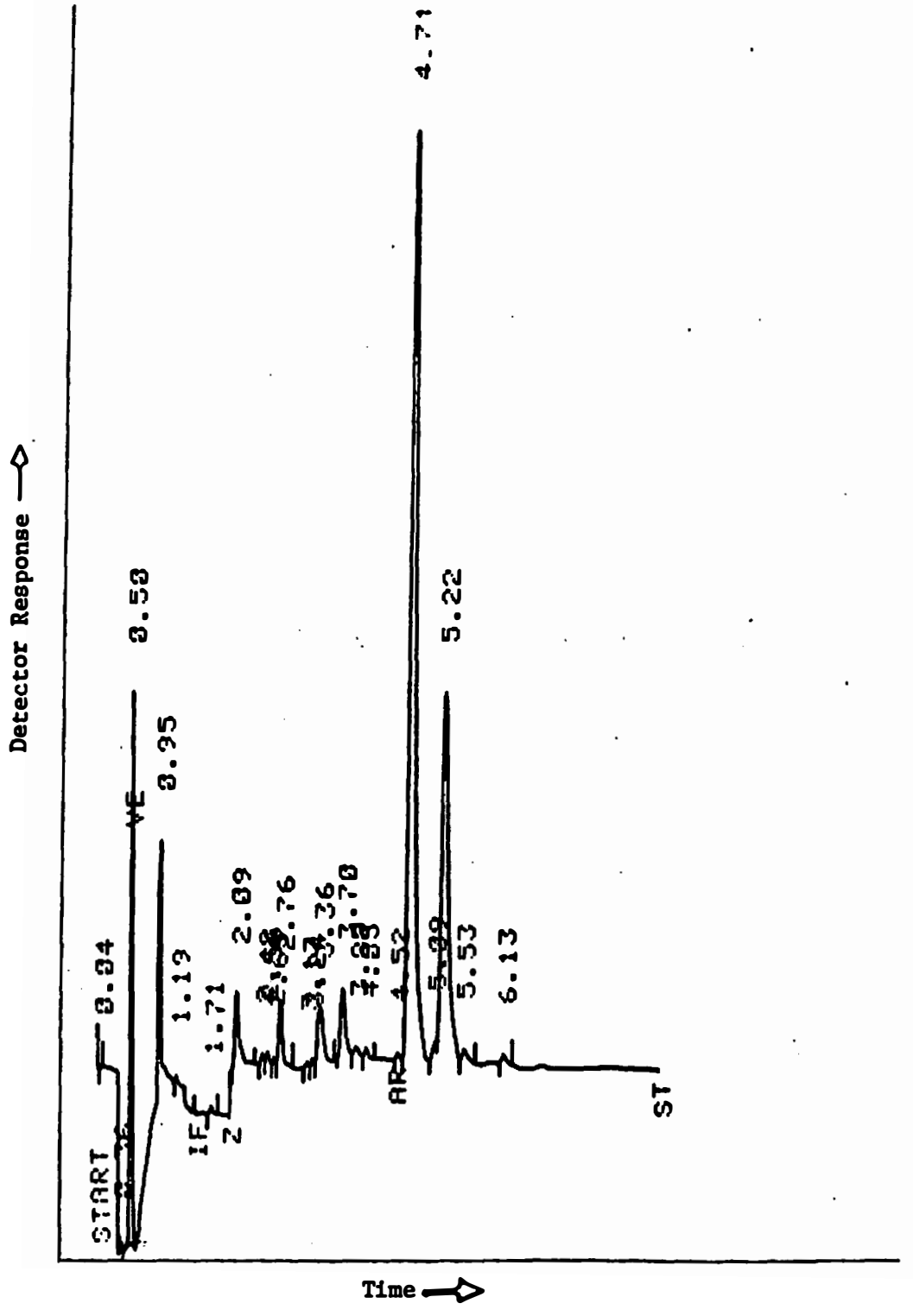


Figure 50.

Chromatogram of Chlorpromazine Extracted from Saliva.

Retention time of chlorpromazine = 5.29 min.
Retention time of internal standard = 4.82 min.

See Figure for Chromatographic Conditions.

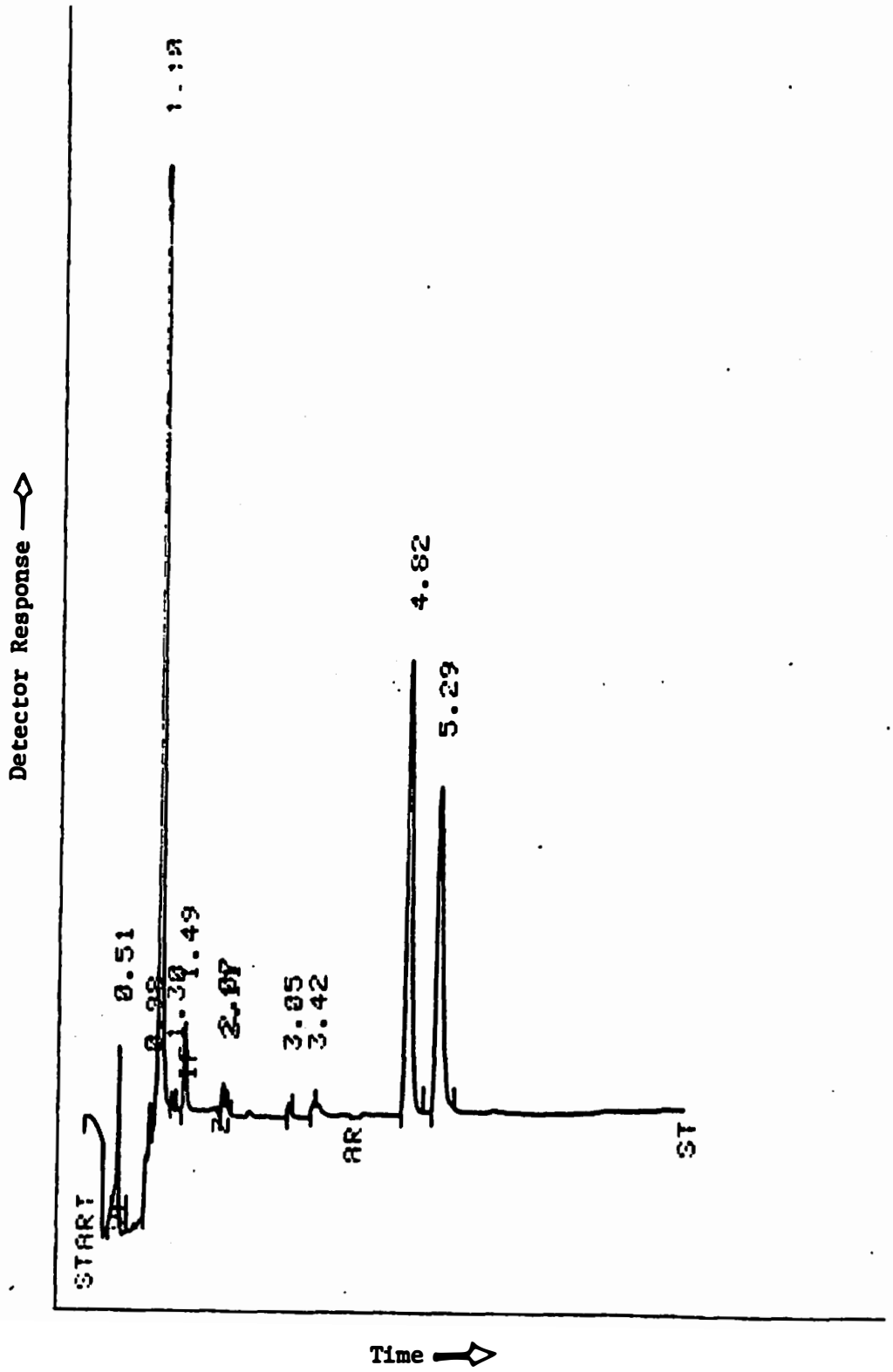


Figure 51.

Standard Curve for the Analysis of Chlorpromazine in Plasma on a Carbowax Capillary Column.

Instrument: Hewlett-Packard 5840A
Column: 0.25 mm ID x 10m WCOT Carbowax 20M Capillary
Temperature: 200°C for 0.5 min, then program to 220°C at 5°C/min, then isothermal at 220°C
Injection: Splitless (0.5 min)/split
Mobile Phase: He, ca 2.0 mL/min.
Detector: Nitrogen-Phosphorous

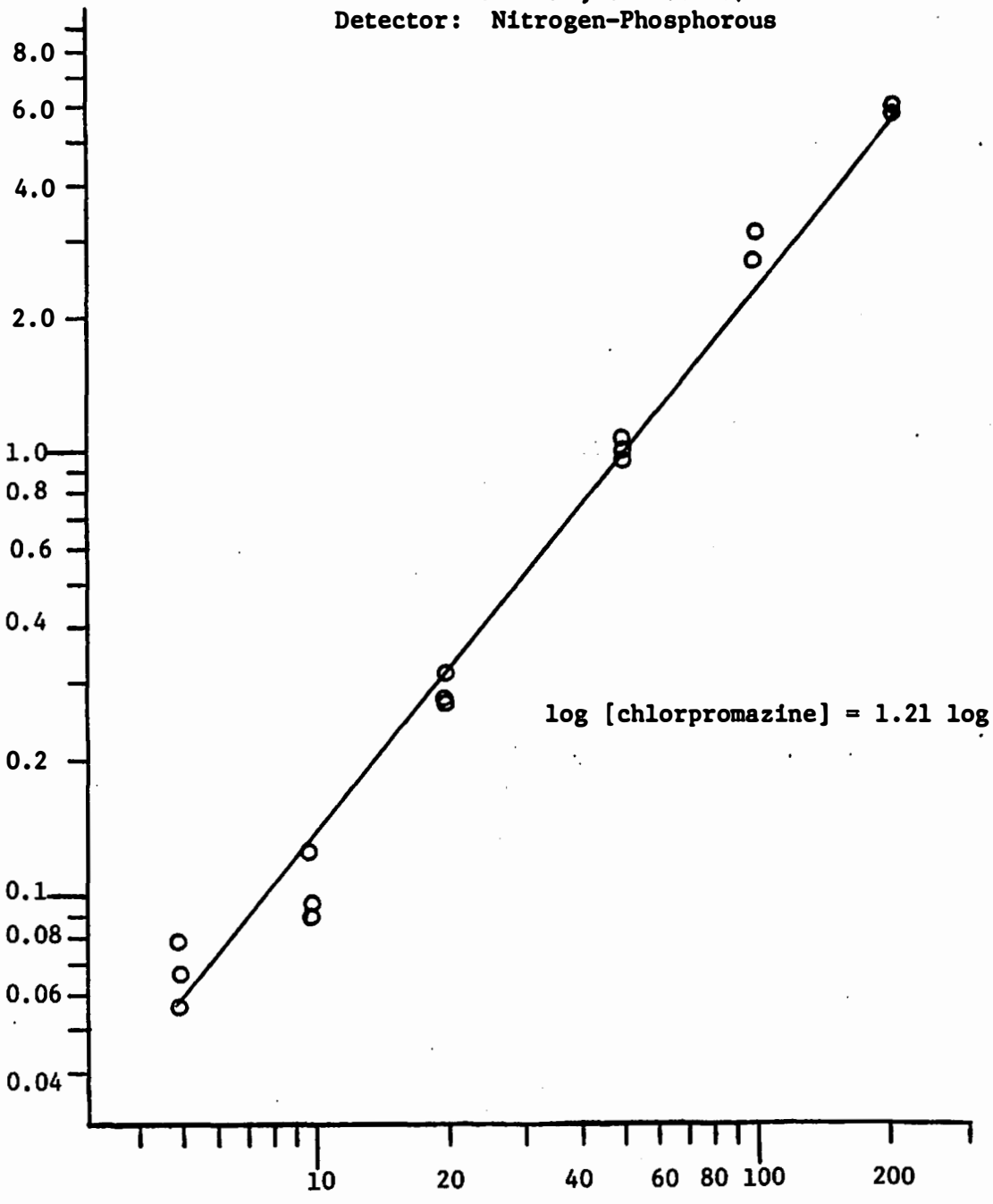


Figure 52

Standard Curve for the Analysis of Chlorpromazine in Saliva on a Carbowax Capillary Column.

Chromatographic Conditions

Instrument: Hewlett-Packard 5840A
Column: 0.25 mm ID x 10m WCOT Carbowax 20M Capillary
Temperature: 180°C for 0.5 min, then program to 220°C at 5°C/min, then isothermal at 220°C
Injection: Splitless (0.5 min)/split
Mobile Phase: He, ca. 2.0 mL/min.
Detector: Nitrogen-Phosphorus

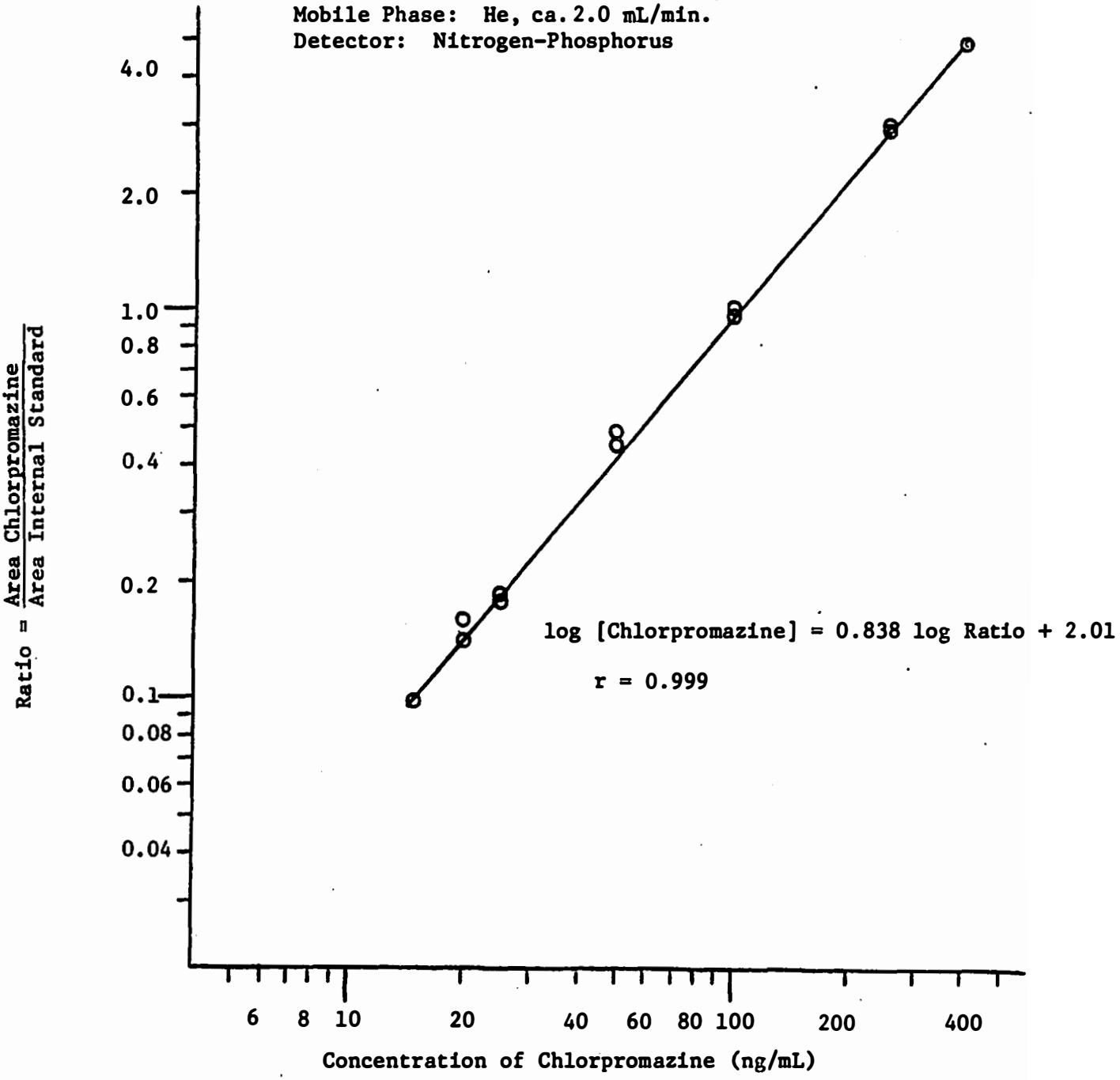


Table 20.

Accuracy and Precision of Assay for Chlorpromazine in Plasma and Saliva.

Fluid	Chlorpromazine Added (ng/mL)	Chlorpromazine Found by Assay (ng/mL)	Average of Assay Values (ng/mL)	C. V. (%)
Plasma	5.0	5.4 4.2 4.4 4.2 5.2	4.7	12.3
Plasma	20	19.7 19.2 18.1 15.8 20.6 18.1 18.1	18.5	8.3
Plasma	50	54.7 51.0 50.9	52.2	4.1
Saliva	25	24.4 23.6 23.3 23.9	23.8	2.0
Saliva	50	55.7 56.3 56.7 51.3 51.6	54.3	4.9
Saliva	100	101 100 101 102 102 103 95	101	2.6

Table 21. Reanalyses of Selected Plasma Samples for Chlorpromazine from Subject S4

Time (hr)	Chlorpromazine Concentration (ng/mL)	
	1st Analysis	2nd Analysis (13 days later)
1.5	51	10
2.0	43	6
3.0	14	6

Appendix A - Detailed Analytical Methodologies

A.4 Diazepam

Chart 4. Extraction Procedure for Diazepam from Plasma or Saliva

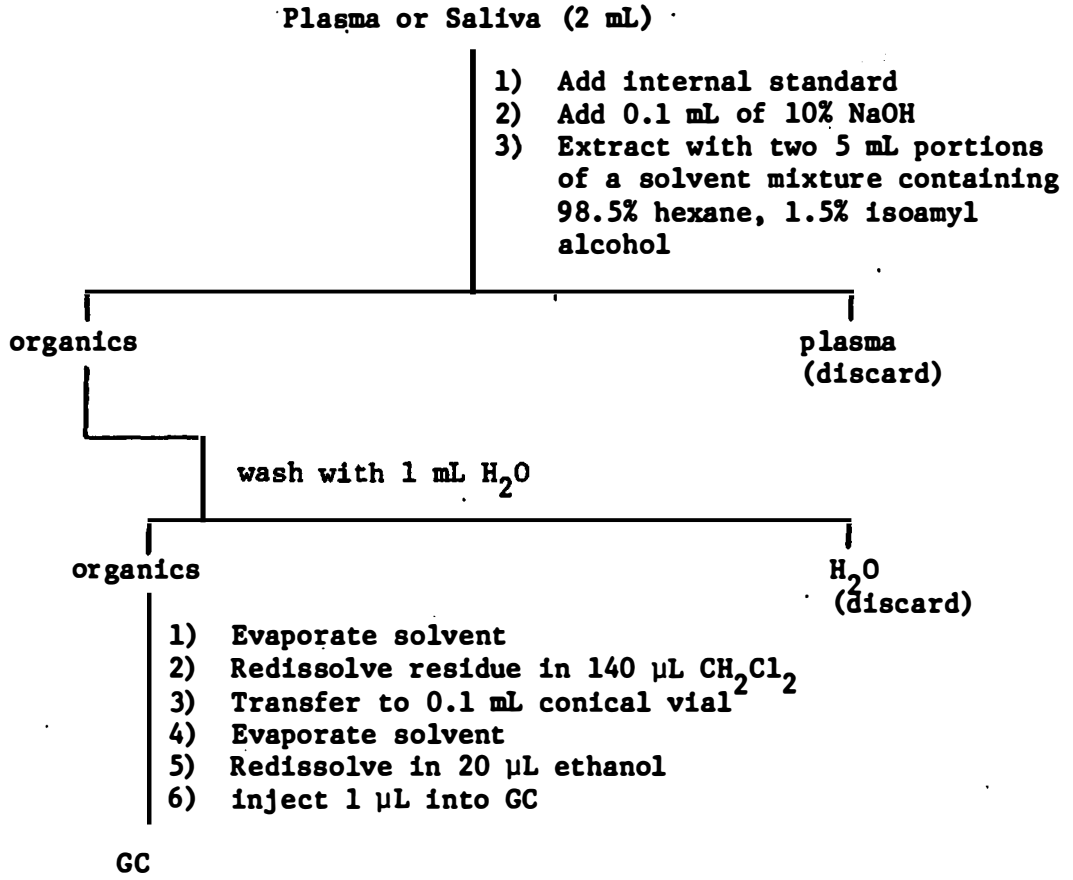
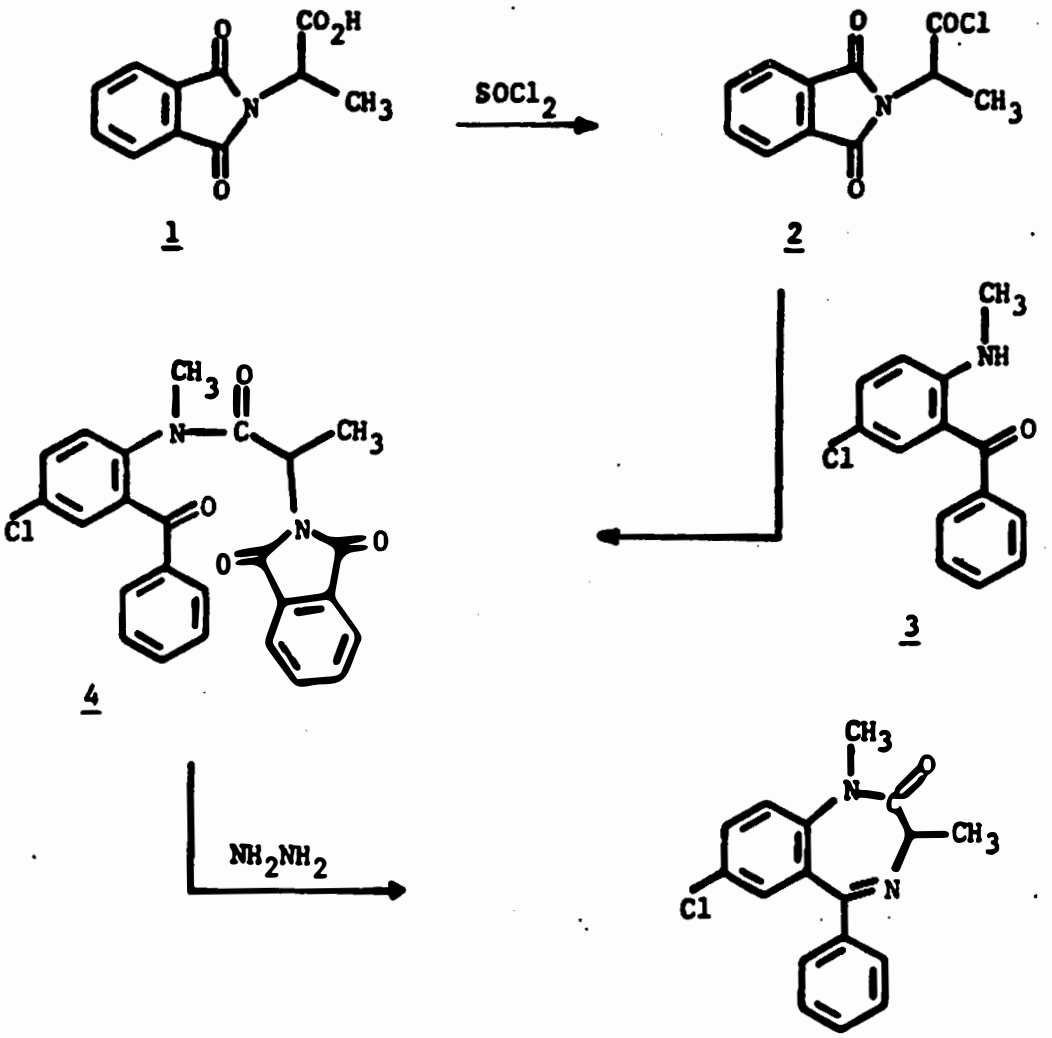
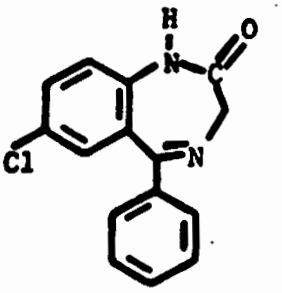


Figure 53. Synthetic Schemes for the Preparation of 3-Methyl Diazepam and the N-Ethyl Analog of Diazepam.

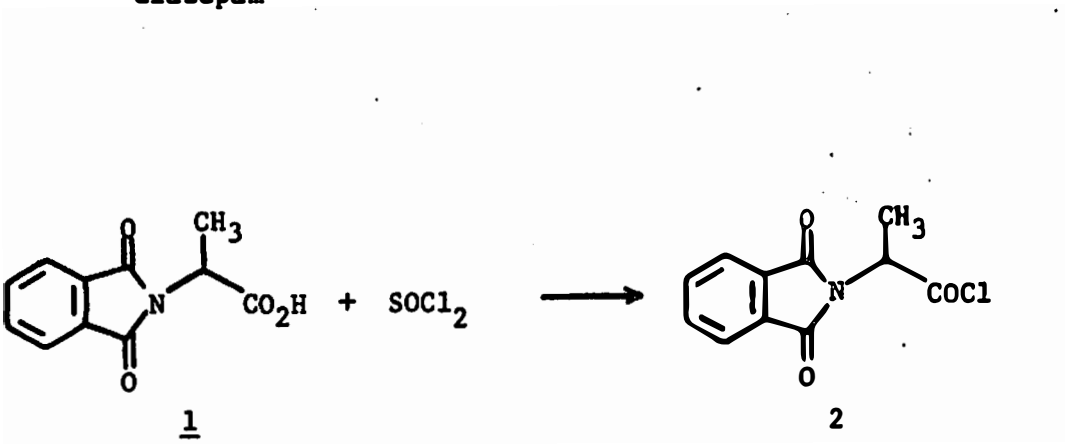


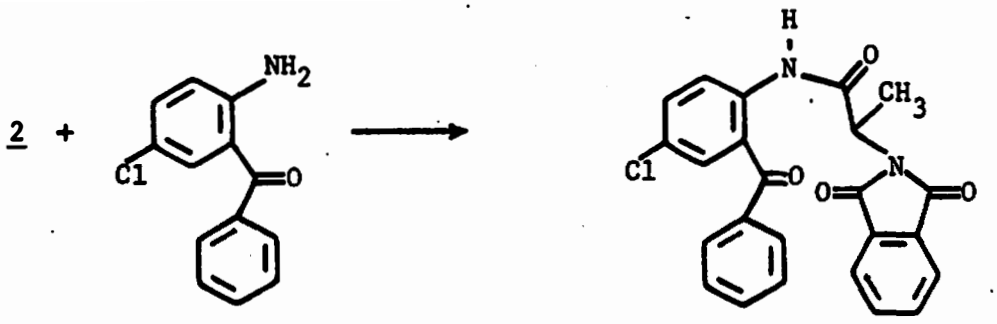
3-Methyl Diazepam



N-Ethyl Analog of Diazepam

Figure 54. Synthetic Scheme for an Internal Standard of N-Desmethyldiazepam





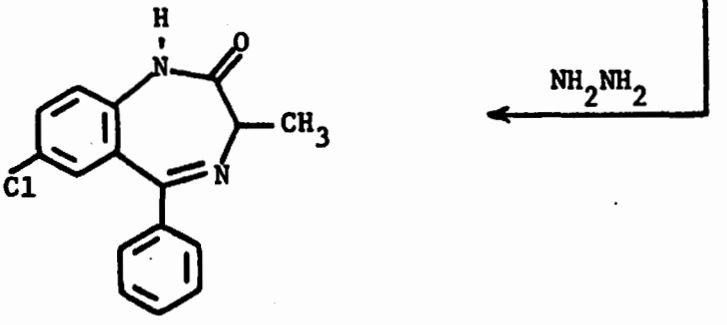
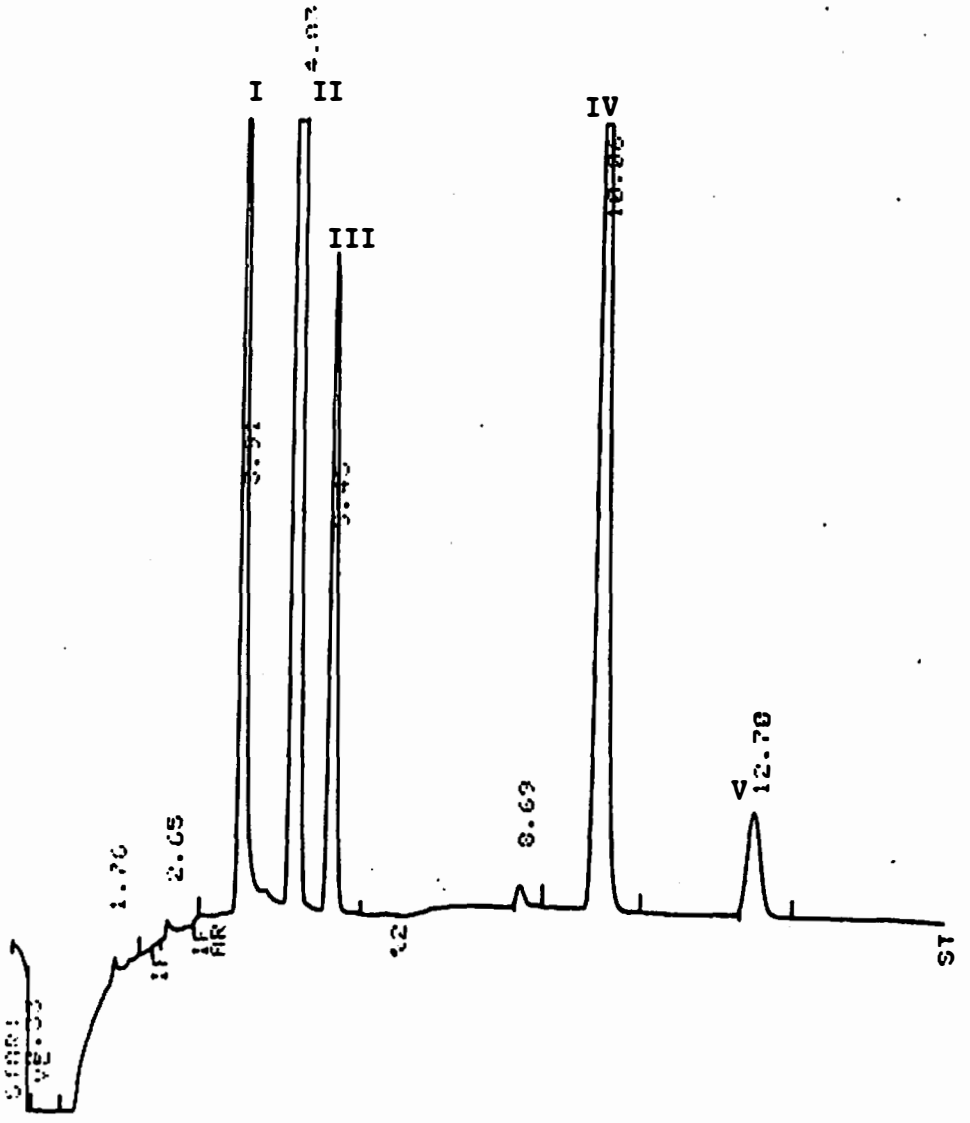


Figure 55. GC of Diazepam, Desmethyldiazepam and Possible Internal Standards on an 8 meter Carbowax-20M Capillary Column



Peak	Retention Time (min)	Compound
I	3.91	3-Methyl Diazepam
II	4.83	N-Ethyl Analog of Diazepam
III	5.45	Diazepam
IV	10.06	3-Methyl-N-Desmethyl Diazepam
V	12.70	N-Desmethyl Diazepam

Figure 56.

Chromatogram of Diazepam Extracted from Plasma.

The retention time of diazepam = 10.94 min.
The retention time of internal standard = 9.58 min.

Chromatographic Conditions

Instrument: Hewlett-Packard 5840A
Column: 0.25 mm ID x 8m Carbowax 20M WCOT capillary
Temperature: 165°C for 1 min, then 165°C to 220°C at 20°C/min, then isothermal at 220°C
Mobile Phase: He, ca. 2 mL/min.
Detector: Nitrogen-Phosphorus
Injection: Splitless (0.5 min)/split

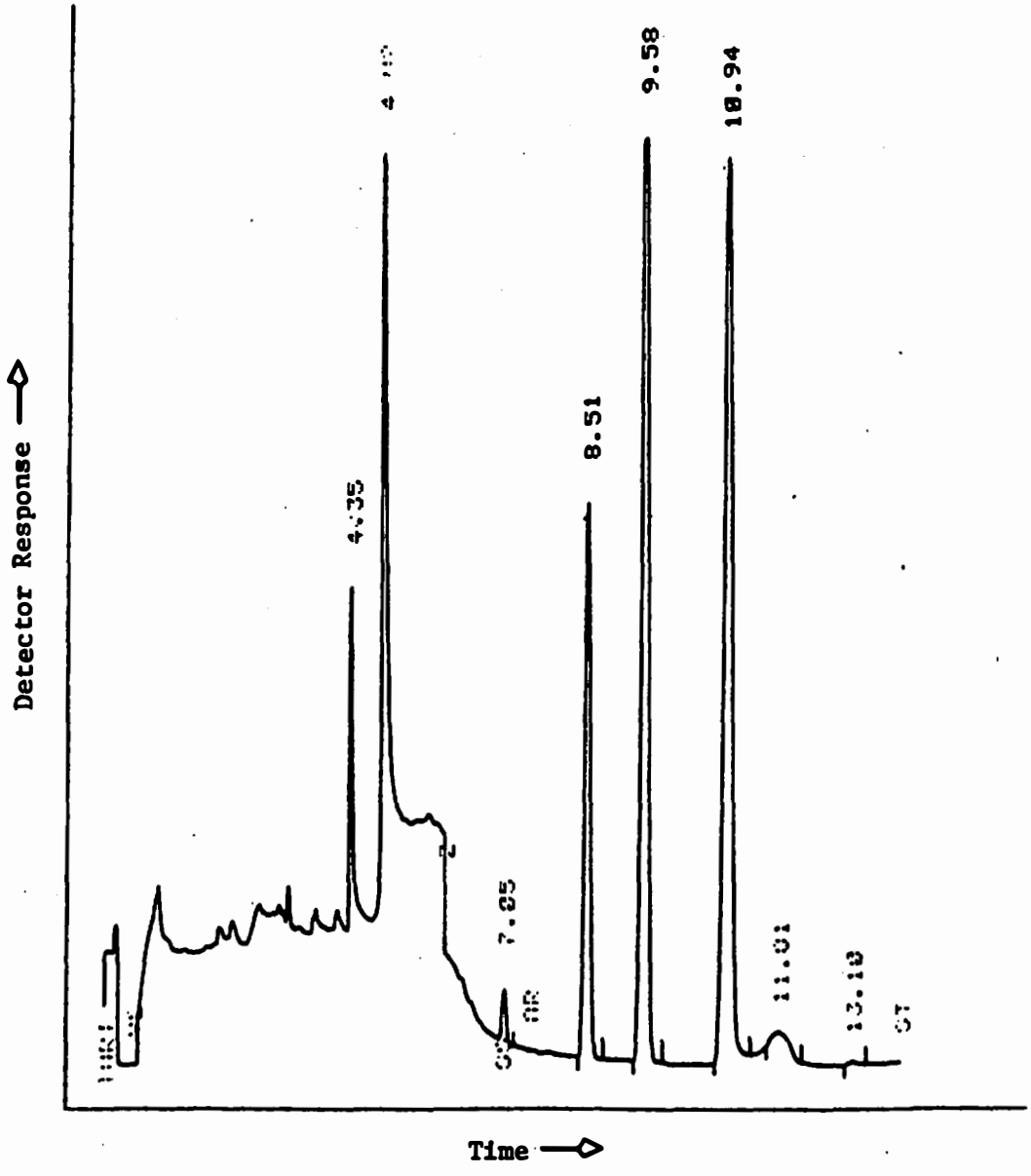


Figure 57

Chromatogram of Diazepam Extracted from Saliva.

Retention Times: Diazepam 12.12 min.
Internal Standard 10.56 min.

See Figure 56 for Chromatographic Conditions

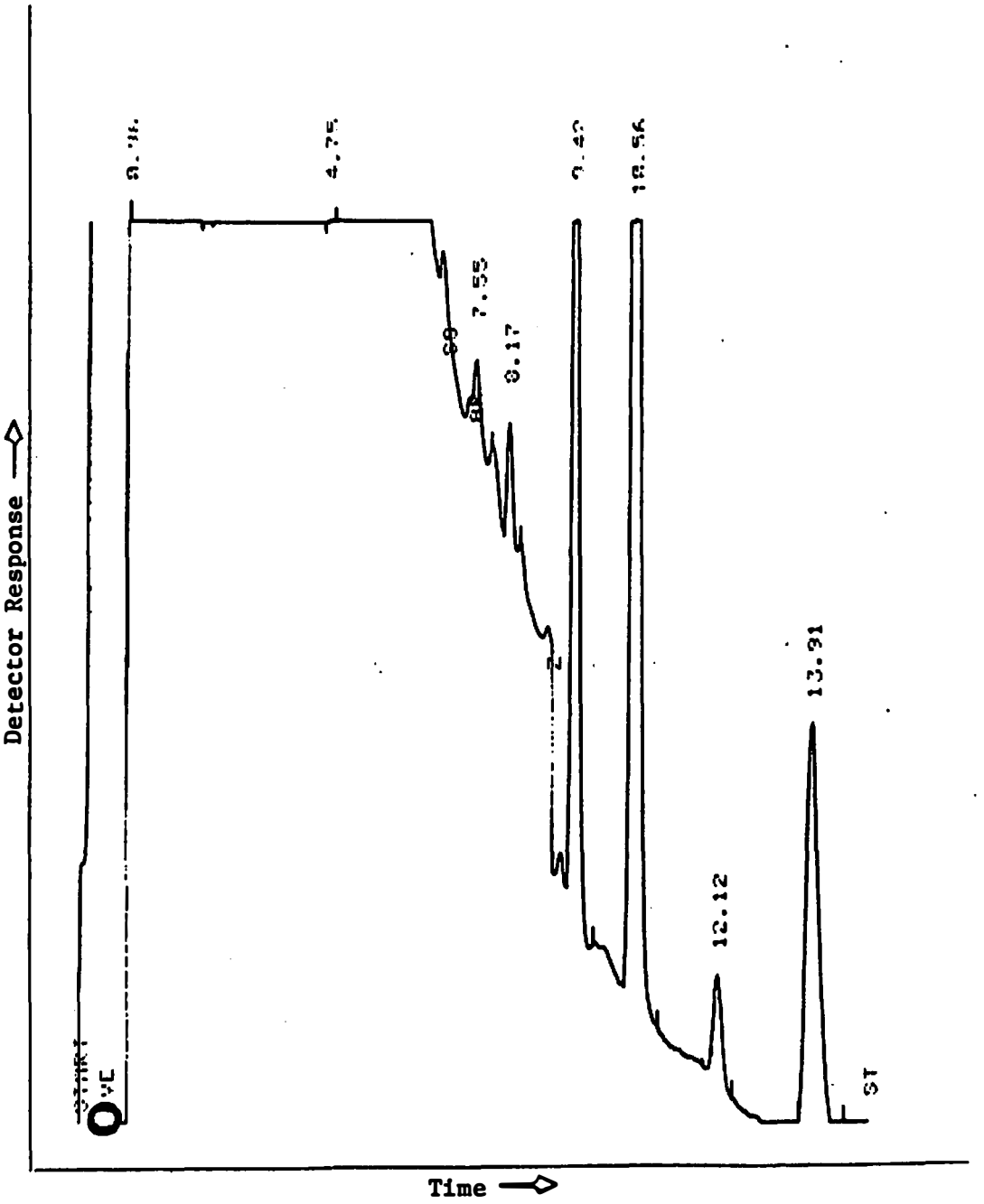


Figure 58. Standard curve for analysis of diazepam in plasma. The internal standard is the N-ethyl analog of diazepam.

See Figure 56 for chromatographic conditions.

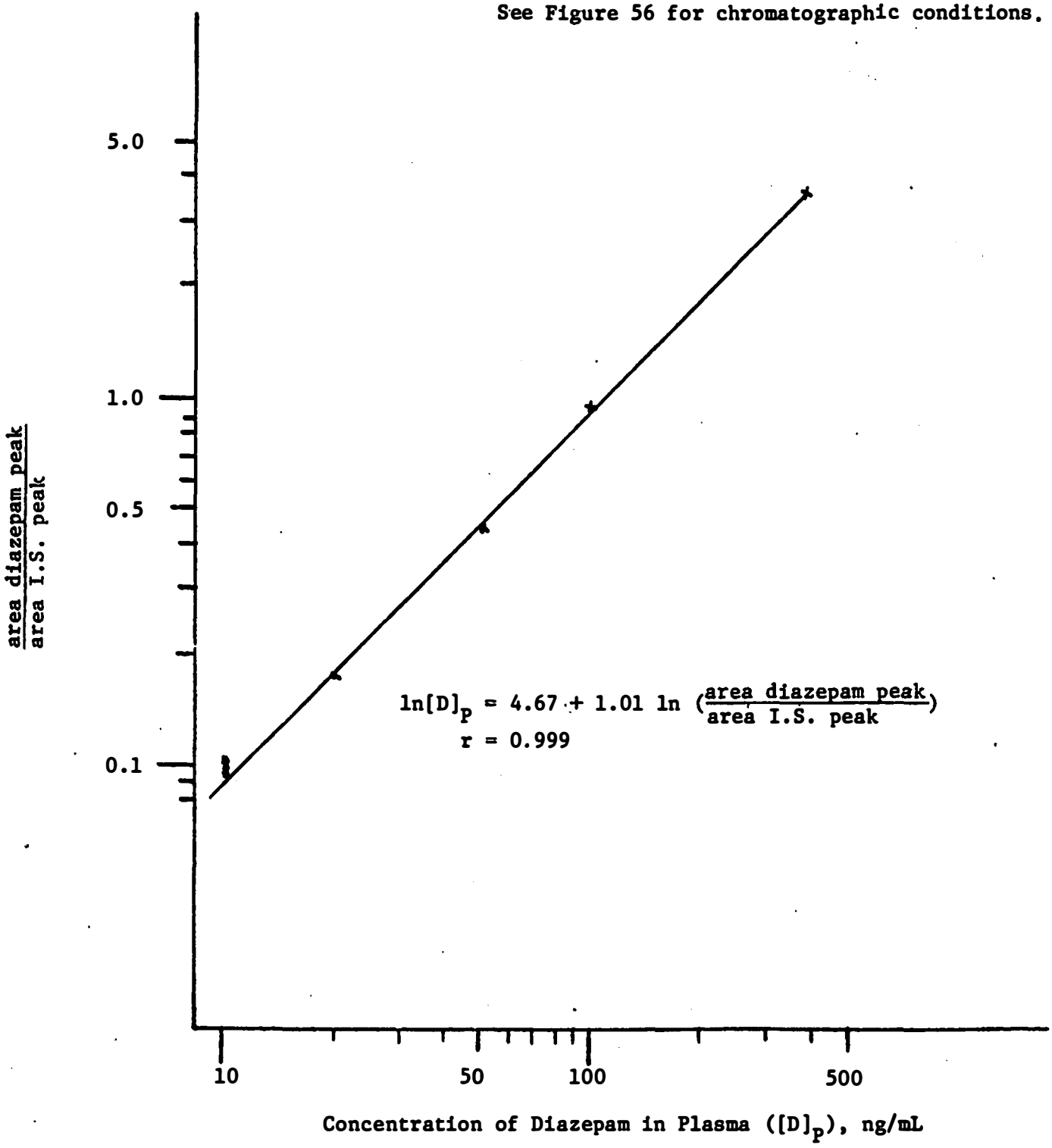


Figure 59. Standard curve for analysis of diazepam in saliva. The internal standard is the N-ethyl analog of diazepam.

See Figure 56 for chromatographic conditions.

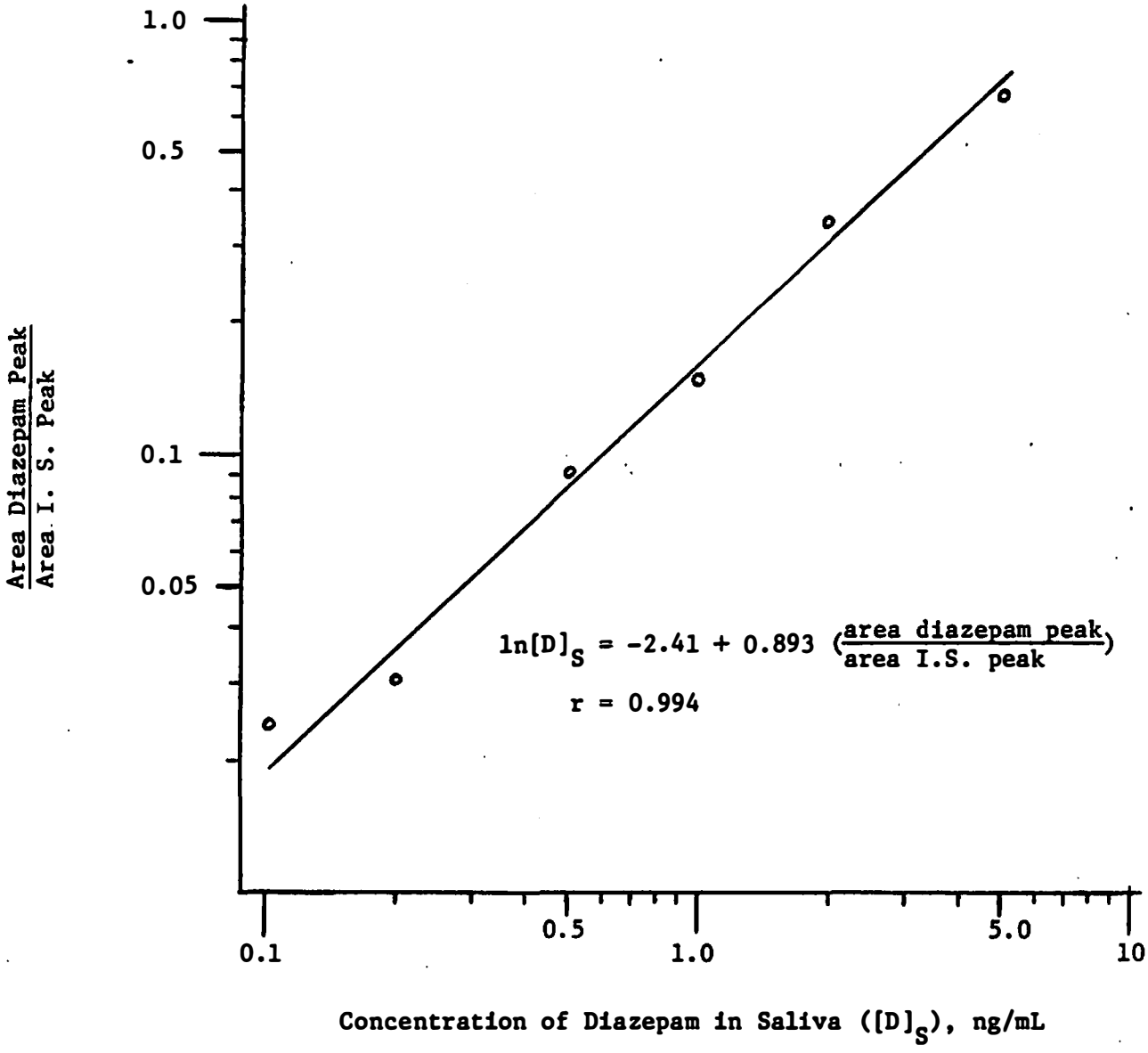


Figure 60. Standard curve for analysis of diazepam in saliva. The internal standard is the N-ethyl analog of diazepam.

See Figure 56 for chromatographic conditions.

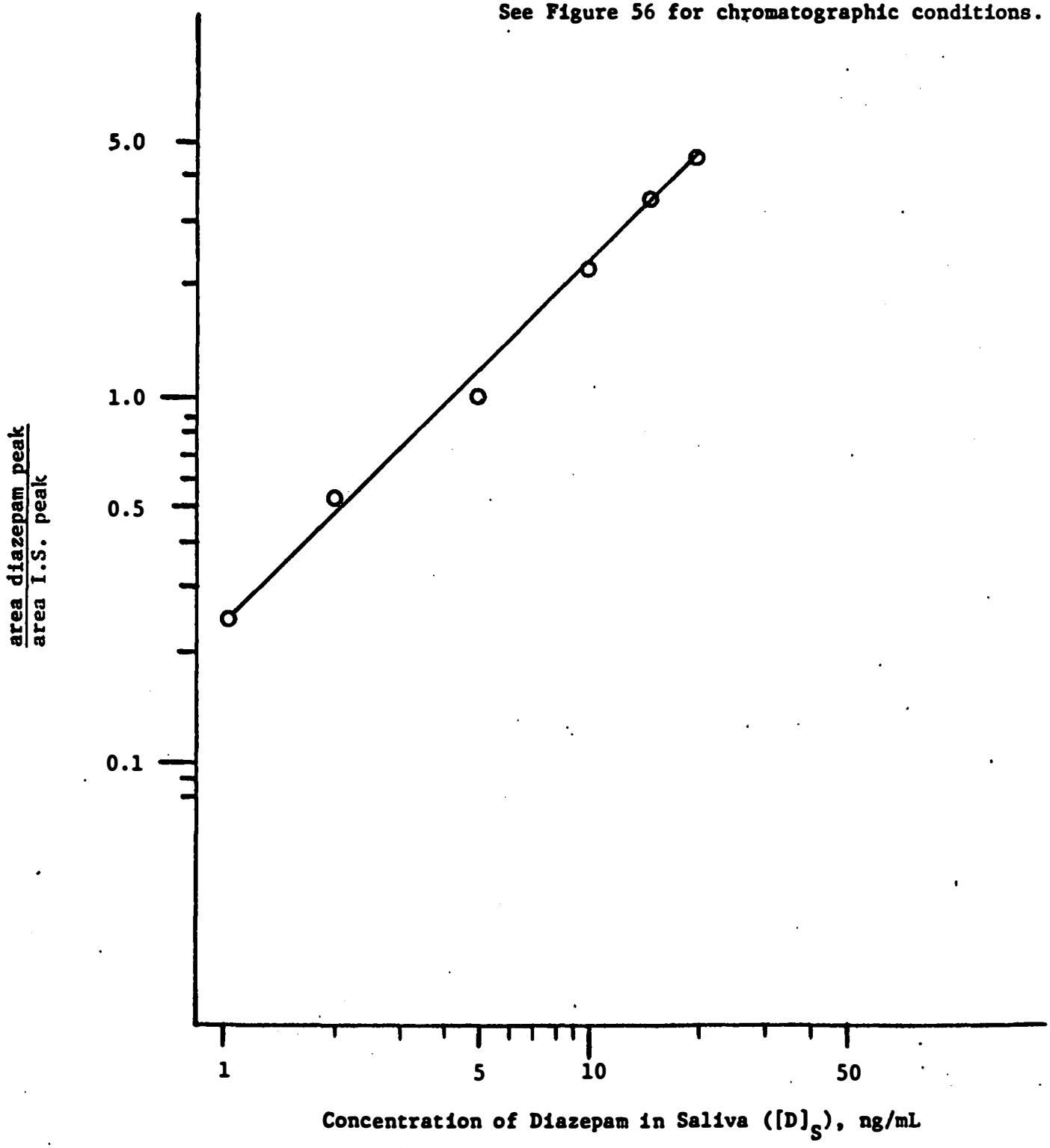


Table 22. Comparison of Two Internal Standards for Diazepam

		Ratio of Area of Diazepam Peak to Area of Internal Standard	
Internal Standard:		3-Methyl Diazepam	N-Ethyl Analog of Diazepam
Diazepam Conc.	Replicate		
400 ng/mL	1	7.85	3.72
	2	8.04	3.73
	3	8.26	3.70
20 ng/mL	1	0.384	0.185
	2	0.401	0.182
	3	0.408	0.184

Table 23.

Accuracy and Precision of Assay for Diazepam in Plasma and Saliva.

Fluid	Diazepam Added (ng/mL)	Diazepam Found by Assay (ng/mL)	Average of Assay Values (ng/mL)	C. V. (%)
Plasma	50	47.5 47.7 47.4	47.5	3.2
Plasma	200	199 197 198	198	0.5
Saliva	5.0	4.3 5.1 4.3	4.6	10
Saliva	10.0	9.8 11.4 8.9	10.0	12.6
Saliva	15.0	14.7 15.4 13.9	14.7	5.1

Appendix A - Detailed Analytical Methodologies

A.5 Diphenhydramine

Chart 5.

Procedure for Extraction of Diphenhydramine
from Human Plasma and Saliva

3 mL Plasma or Saliva

Add internal standard.
Adjust pH to 13 with 2.5 N NaOH.
Extract twice with 5 mL of toluene, mixing 15 min, then centrifuging each time. Combine extracts.

Toluene Extract

Extract twice with 1 mL of 0.05 NHCl, mixing 1 min, then centrifuge.
Combine extracts.

HCl Extract

Adjust to pH 9 with 2.5 N NaOH.
Extract twice with 3 mL of CH_2Cl_2.
Combine extracts.

CH_2Cl_2 Extract

Evaporate CH_2Cl_2 extract in a 2 dram vial until total volume is 50 to 100 µL. Transfer to 150 µL vial, rinsing 2 dram vial with 2 x 50 µL CH_2Cl_2.
Evaporate to dryness.
Dissolve residue in 20 µL of o-xylene (plasma) or isoamyl alcohol (saliva).
Inject on Chromatograph.

Figure 61

Chromatogram of Diphenhydramine Extracted from Plasma.

Chromatographic conditions same as in Figure 63.
Retention times: Diphenhydramine: 7.44 min.
Orphenadrine: 8.59 min.
Sample: Subject 2, 0.5 hr.

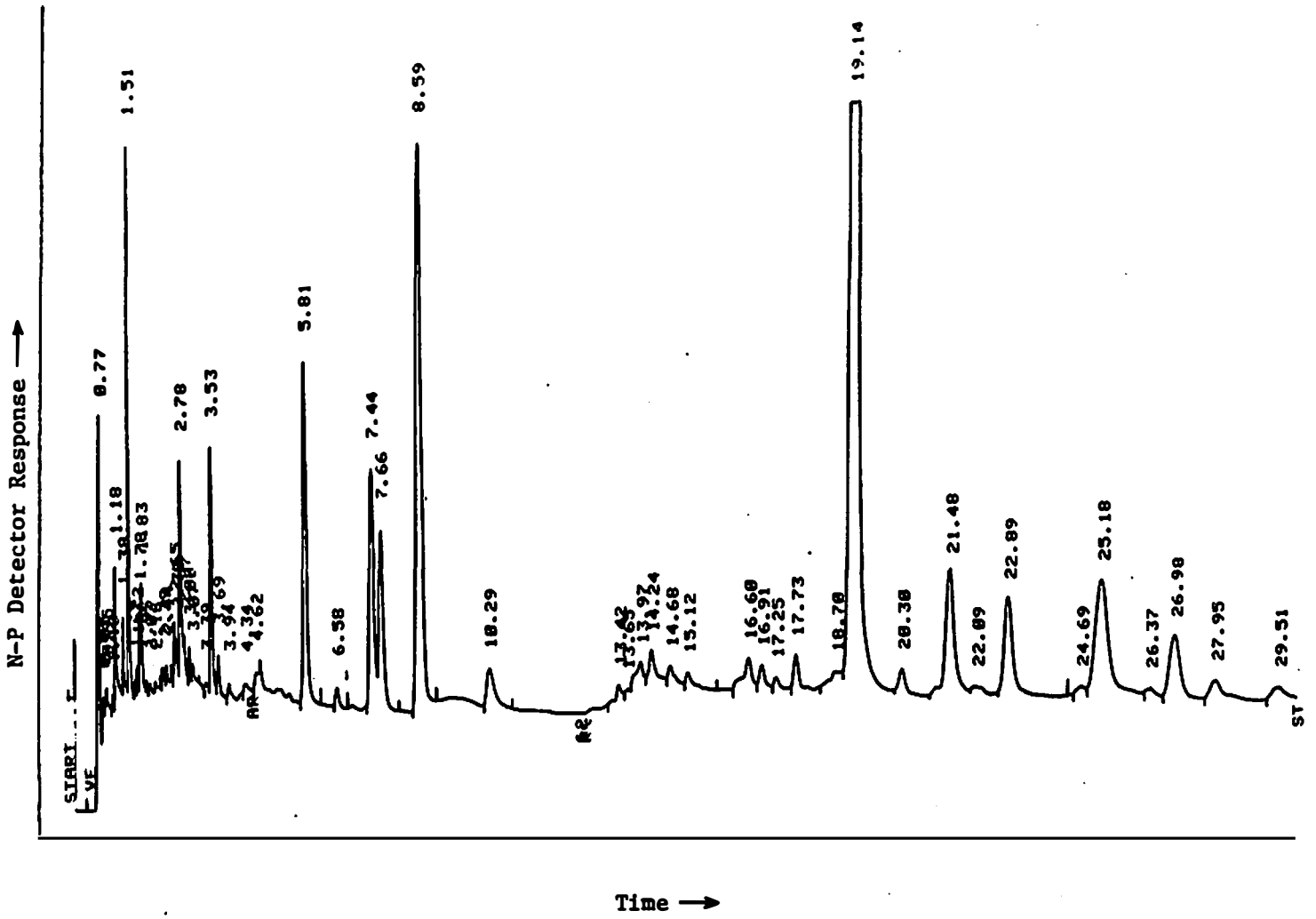


Figure 62.

Chromatogram of Diphenhydramine Extracted from Saliva.

Chromatographic conditions same as in Figure 64.
Retention Time: Diphenhydramine 9.20 min.
Orphenadrine 10.35 min.
Sample: Subject 5, 11 hr.

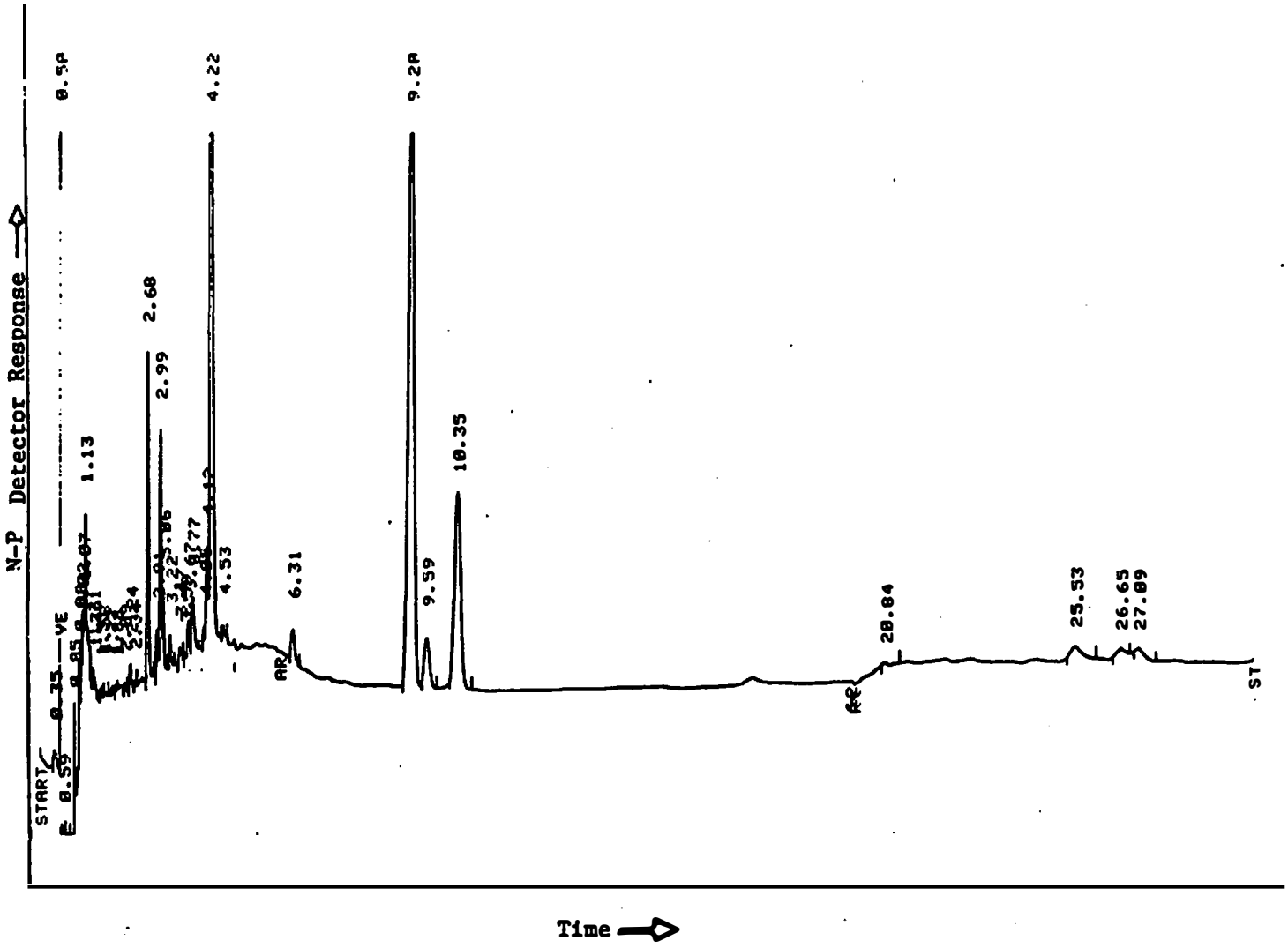


Figure 63. GLC Calibration Curve for Diphenhydramine in Plasma Using a Polyethylene Glycol WCOT Capillary Column.

Instrument: Hewlett-Packard 5840A
Column: 0.25 mm x 10 m WCOT - CP Wax 51 (Chrompack)
Temperature: 140°C for 1 min, then 140°C to 170°C at 20°C/min, then isothermal at 170°C
Flow Rate: 2.0 mL He/min at 170°C
Detector: Nitrogen - Phosphorus

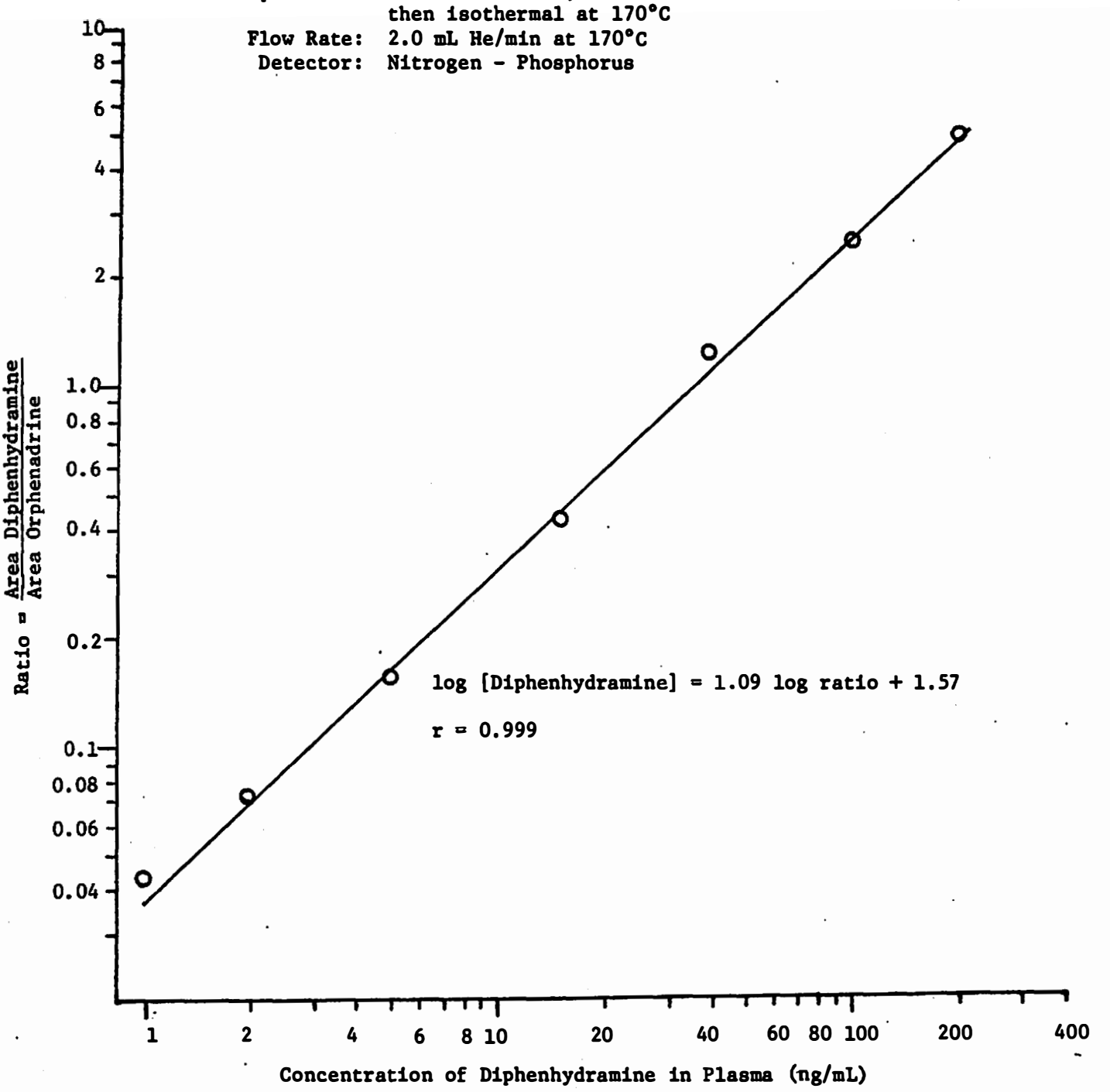


Figure 64. GLC Calibration Curve for Diphenhydramine in Saliva Using a Polyethylene Glycol WCOT Capillary Column.

Instrument: Hewlett-Packard 5840A
Column: 0.50 mm x 10 m WCOT CP Wax 51 (Chrompack)
Temperature: 128°C for 1 min, then 128°C to 180°C at 20°C/min, then isothermal at 180°C
Flow Rate: 3.0 ml/min at 180°C
Detector: Nitrogen - Phosphorus

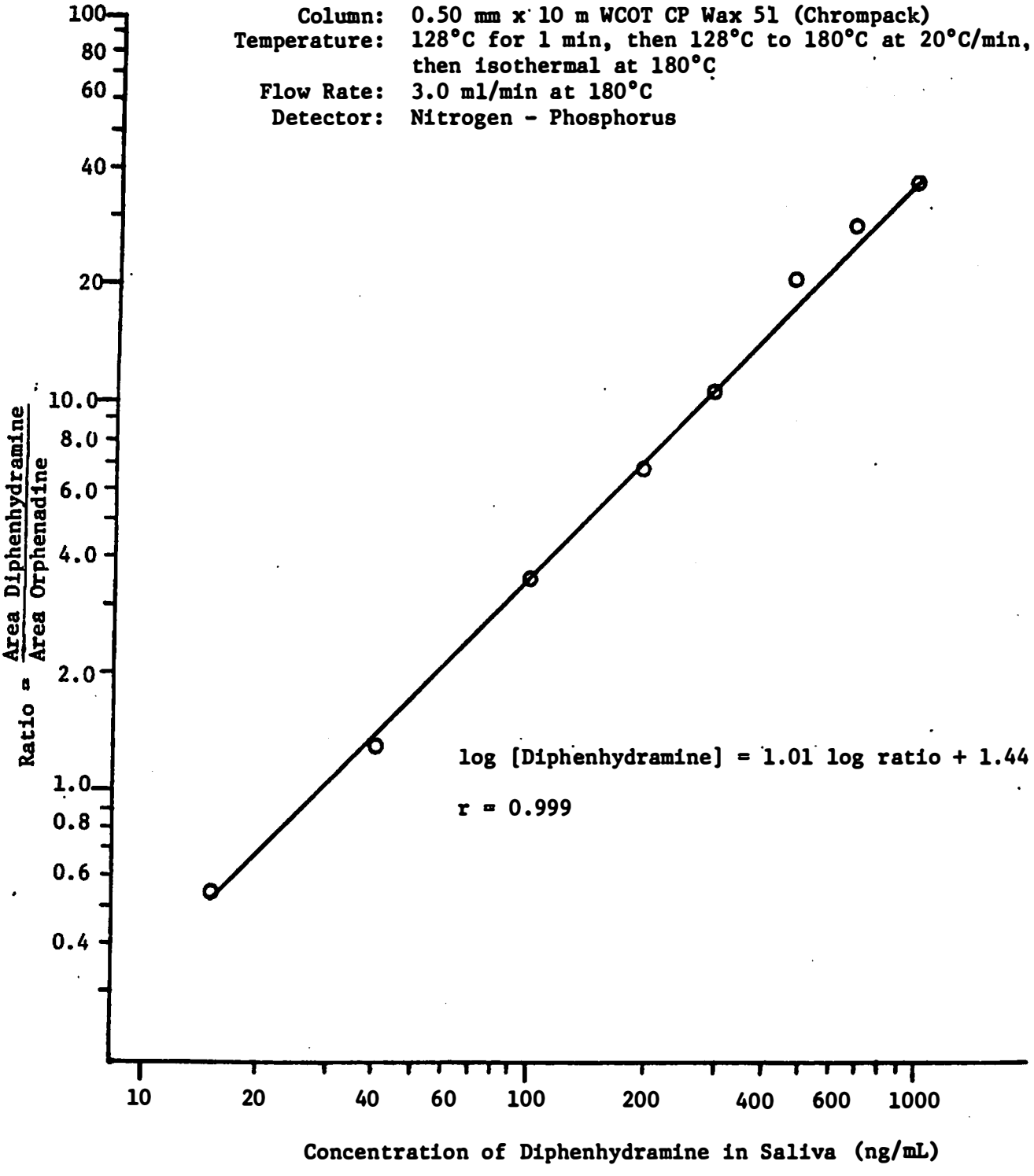


Table 24.

Accuracy and Precision of Assay for Diphenhydramine in Plasma and Saliva.

Fluid	Diphenhydramine Added (ng/mL)	Diphenhydramine Found by Assay (ng/mL)	Average of Assay Values (ng/mL)	C. V. (%)
Plasma	15.0	16.6	14.8	9.9
		14.0		
		16.3		
		14.1		
		14.7		
		12.8		
Plasma	100	113	112	
		111		
Saliva	15.0	16.0	15.6	3.8
		16.5		
		15.3		
		15.4		
		15.0		
		16.0		
		14.9		
Saliva	100	98.0	100	
		103		

Figure 65. Statistical Data for the Linear Regression Analysis of Saliva versus Plasma Concentrations (ng/mL) of Diphenhydramine.

DIPHENHYDRAMINE: SALIVA VS. PLASMA 4

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: SALIVA

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	19.83298578	19.83298578	221.57	0.0001	0.847075	15.3165
ERROR	40	3.58050167	0.08951254		STD DEV	r = 0.920	SALIVA MEAN
CORRECTED TOTAL	41	23.41348744			0.29918647		1.95336250

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
PLASMA	1	19.83298578	221.57	0.0001	1	19.83298578	221.57	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	-0.30376704	-1.92	0.0625	0.15850850
PLASMA	1.36276270	14.89	0.0001	0.09155206

Figure 66. Statistical Data for the Linear Regression Analysis of Predicted versus Observed Plasma Concentrations (ng/mL) of Diphenhydramine.

DIPHENHYDRAMINE IN PLASMA 2

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CALC CALCULATED

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	17.63265616	17.63265616	194.16	0.0001	0.829177	15.7911
ERROR	40	3.63258865	0.09081472		STD DEV		CALC MEAN
CORRECTED TOTAL	41	21.26524481			0.30135480		1.90838768

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
OBS	1	17.63265616	194.16	0.0001	1	17.63265616	194.16	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	-0.21985576	-1.38	0.1762	0.15965728
OBS	1.28494653	13.93	0.0001	0.09221558

Appendix A - Detailed Analytical Methodologies

A.6 Codeine

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CHEMISTRY AND LIFE SCIENCES GROUP

January 30, 1981

Dr. James Frank
DOT
National Highway Traffic Safety Administration
Problem Behavior Research Division (NRD42)
Massiff Building
400 7th Street SW
Washington, DC 20590

Dear Dr. Frank:

This letter is in regard to several topics that have arisen in our work on "The Analysis for Drugs in Saliva and Breath" and which we have previously discussed by phone.

The first topic concerns the analysis of codeine in plasma and saliva. As you may remember, this compound had not been selected at the time the detailed study plan was approved. After it was decided that codeine would be the sixth drug to be studied in our contract, we began looking for suitable analytical techniques for its analysis. Dr. Ruth Zweidinger of our institute had previously analyzed codeine in plasma in a project sponsored by Burroughs-Wellcome Company. At the time (1975) that she did her work, there were no good analytical procedures for analyzing codeine in plasma after single therapeutic or sub-therapeutic doses. Since that time, several procedures have been reported. I have tried to summarize these procedures below. They are divided into two classifications: gas chromatographic- and radioimmuno-assays.

(1) Gas Chromatographic Procedures

Three GC procedures have been described. All are modifications of earlier procedures but offer improvements, usually in sensitivity, to the earlier procedures.

Brunson and Nash (1975) analyzed codeine in plasma using an OV-225 stationary phase in a packed column, flame ionization detection, and papaverine as an internal standard. The published standard curve which has data points from 25 to 150 ng of codeine per ml of plasma is linear. The authors claim that "as little as 5 μg of codeine...per liter (5 ng/mL) could be measured in plasma" although no data was presented for codeine concentrations below 25 ng/mL. We have found previously that all such claims must be taken with a lot of salt. Figures showing the concentration of codeine in plasma vs. time for two subjects who were

each given a single oral does of 60 mg codeine sulfate (equivalent to 52 mg of codeine) were also presented. The peak concentrations of codeine in plasma (C_{max}) were ca 110 ng/mL. At 6 hr, the concentrations had decreased to 20-40 ng/mL. The 20 ng/mL value was lower than the lowest point on the standard curve.

A second procedure was reported by Dahlström, et. al (1977). These authors employed an OV-17 stationary phase in a packed column, derivitization of the codeine with pentafluoropropionic anhydride, and electron capture detection. The internal standard was N-ethyl morphine. The authors claim that "the method allowed determinations of codeine...with acceptable precision down to 7.5 ng per sample (0.05-1.0 mL). Data was presented for concentrations of codeine down to 20 ng per sample.

Zweidinger et al (1976) published her procedure which made use of an XE-60 stationary phase in a packed column and flame ionization detection. The limit of detection using this procedure was 5 ng/mL although below 50 ng/mL determinations were difficult.

(2) RIA Procedures

Two procedures have been developed by Findley et al. The first of these (Findley et al, 1977) required extraction of codeine from plasma and preliminary purification of the extract because of the cross-reactivity of the RIA with codeine-6-glucuronide and morphine. Large amounts of codeine-6-glucuronide are present in plasma relative to the concentration of codeine. Morphine is also present. Using this RIA procedure, the concentrations of codeine in the plasma of six subjects who had each been given a single dose of 65 mg of codeine phosphate (49 mg codeine) were followed over 22 hours. The values of C_{max} were about 100 ng/mL. Concentrations of codeine at 12 hr were 5-12 ng/mL.

The second RIA procedure (Findlay, et al, 1976) is based on a much more selective antisera. The cross-reactivities to codeine-6-glucuronide and morphine were <0.05 and 0.1 percent, respectively. Cross-reactivity with norcodeine was 16%. While norcodeine is found in the urine after administration of codeine, only a trace quantity was found in the plasma (<10% of the concentration of codeine) by gc-mass spectrometry (Brunson and Nash, 1975), and none when flame-ionization was used in place of the mass spectrometer. Using this more selective antisera, no extraction or prepurification step is required. A standard curve for the analysis of codeine in plasma over the range of 0.24-31 ng/mL was published. The authors state that "the practical limit of sensitivity is less than 1 ng/mL." Samples containing more than 31 ng/mL of codeine can be diluted.

In our studies we have plasma and saliva samples from subjects who were given 15 and 30 mg of codeine orally. Based on the results of the assays of Findlay et al and Brunson and Nash, we anticipate peak concentrations of codeine of about 35 and 65 ng/mL, respectively, for the two dose levels. Twelve hours after dosing concentrations of codeine are expected to be 5-10 percent of the peak concentrations. Concentrations of codeine in saliva will probably be higher than those in plasma.

Severe problems have been encountered in trying to adapt any of the reported GC procedures for our use. As they have been reported, all have documented lower limits much too high for our needs. The OV-225 stationary phase contains nitrile groups. The phase also "bleeds" off. Thus it is not suitable for use with nitrogen sensitive detectors and can rapidly contaminate electron capture detectors. The OV-17 stationary phase appears to be the phase of choice. Unfortunately, this phase cannot be coated evenly. At low ng/mL concentrations of most basic drugs, adsorption of the drug to uncoated or fractured support in packed columns is a major problem. The development of good OV-17 coated capillary columns has been an elusive goal, again because of the poor coating properties of this phase. Our analytical group at RTI has been unsuccessful in this regard. Several reputable commerical companies list such columns in their advertisements, but we have been unable to obtain delivery. We had one OV-17 capillary column, which was never delivered, on order for a year. Codeine fails to elute from the Carbowax coated capillary column which we are using for our other analyses at its maximum temperature (220°C). Our initial efforts using packed columns were also unsuccessful with low amounts of codeine.

We therefore propose to analyze codeine in plasma and saliva by the RIA method of Findlay, et al, (1978) using their more selective antisera. We feel that this method is the only one presently available that has the requisite selectivity and sensitivity to accurately quantitate codeine in plasma following a single sub-therapeutic dose. In using this RIA, standard curves will be prepared using plasma spiked with known amounts of codeine. Control samples will be run with each assay. If the concentration of codeine in plasma is sufficiently high, then we will attempt to analyze the codeine in a few samples both by RIA and GC. The GC will be accomplished using a packed column and OV-17 as the stationary phase.

We have already made inquiries of Drs. Findlay and Welch at Burroughs-Wellcome concerning the availability of their antisera. The Wellcome Research laboratories are less than one mile from ours and there is ongoing cooperation between the two laboratories in other areas. There were no problems anticipated in our obtaining the antisera. Dr. Findlay indicated that I should let him know when I needed it and he would make it available with no further red tape. Analysis of codeine by RIA would also be much faster and cheaper than even with a GC procedure that was already developed.

Procedure for the Radioimmunoassay for Codeine

Antisera, radioligand and procedures for the assay were obtained from Dr. John Findlay, Wellcome Research Laboratories, Research Triangle Park, NC. Purity of the radioligand (^{3}H-codeine) was established by TLC. The titer for the antiserum was determined by incubating 0.2 mL of various dilutions of the neat antiserum with 0.1 mL of drug-free plasma or diluted drug-free saliva, 0.2 mL of the radioligand (240 pg/0.2 mL), and 0.5 mL of buffer (0.05M $Na_2HPO_4 \cdot NaH_2PO_4$, 0.15M NaCl, 0.01M EDTA, 0.1% gelatin, pH 7.0). The mixture was incubated for a minimum of 2 hours at 4°C. Separation of the antibody-bound from the free radioligand was accomplished using 0.5 mL of a 5 mg/mL suspension of dextran-coated charcoal in buffer (0.25% dextran). After 30 minutes, the charcoal suspension was centrifuged at 1000xg for 15 minutes. The temperature for charcoal adsorption and centrifugation was 4°C. The resulting supernatant was decanted into 20 mL glass counting vials, and 10 mL of a toluene:Triton-X, 2:1 (v/v) counting cocktail containing 6.0 g of Omnifluor (New England Nuclear) per liter, was added. Samples were placed into a Packard Model 460 liquid scintillation counter and counted for 2 minutes.

The above procedure was used for the radioimmunoassays except that 0.2 mL of the antiserum at the titer found (1:6000 for plasma and 1:10 saliva/buffer; 1:5000 for 1:5 saliva/buffer) was used, and 0.1 mL of plasma (0.01 mL saliva) containing known (standards and controls) and unknown amounts (samples) of codeine was used instead of drug-free plasma (saliva). Since 0.1 mL of neat saliva interfered with the binding of the radiolabel, 0.1 mL of 1:10 saliva/buffer dilution (or 1:5 saliva/buffer dilution) was used in the saliva assays. For the standard

curve, 0.01 or 0.02 mL of drug-free saliva was spiked with 0.09 or 0.08 mL, respectively, of codeine phosphate standard in buffer; for the samples, 0.01 or 0.02 mL of subjects' saliva was diluted with 0.09 or 0.08 mL, respectively, of buffer.

Concentrations of codeine free base were calculated from a standard curve plot of % radioligand bound vs. codeine free base concentration on a logit-log scale. The range of the assay was 0.5-15 ng/mL. Since it was necessary to dilute saliva 1:5 with buffer before the assay, this range corresponds to initial codeine concentrations in saliva of 2.5-75 ng/mL. Plasma and saliva samples containing higher concentrations of codeine were diluted with drug-free plasma and buffer, respectively, before they were analyzed.

Figure 67. Calibration Curve for RIA of Codeine in Plasma

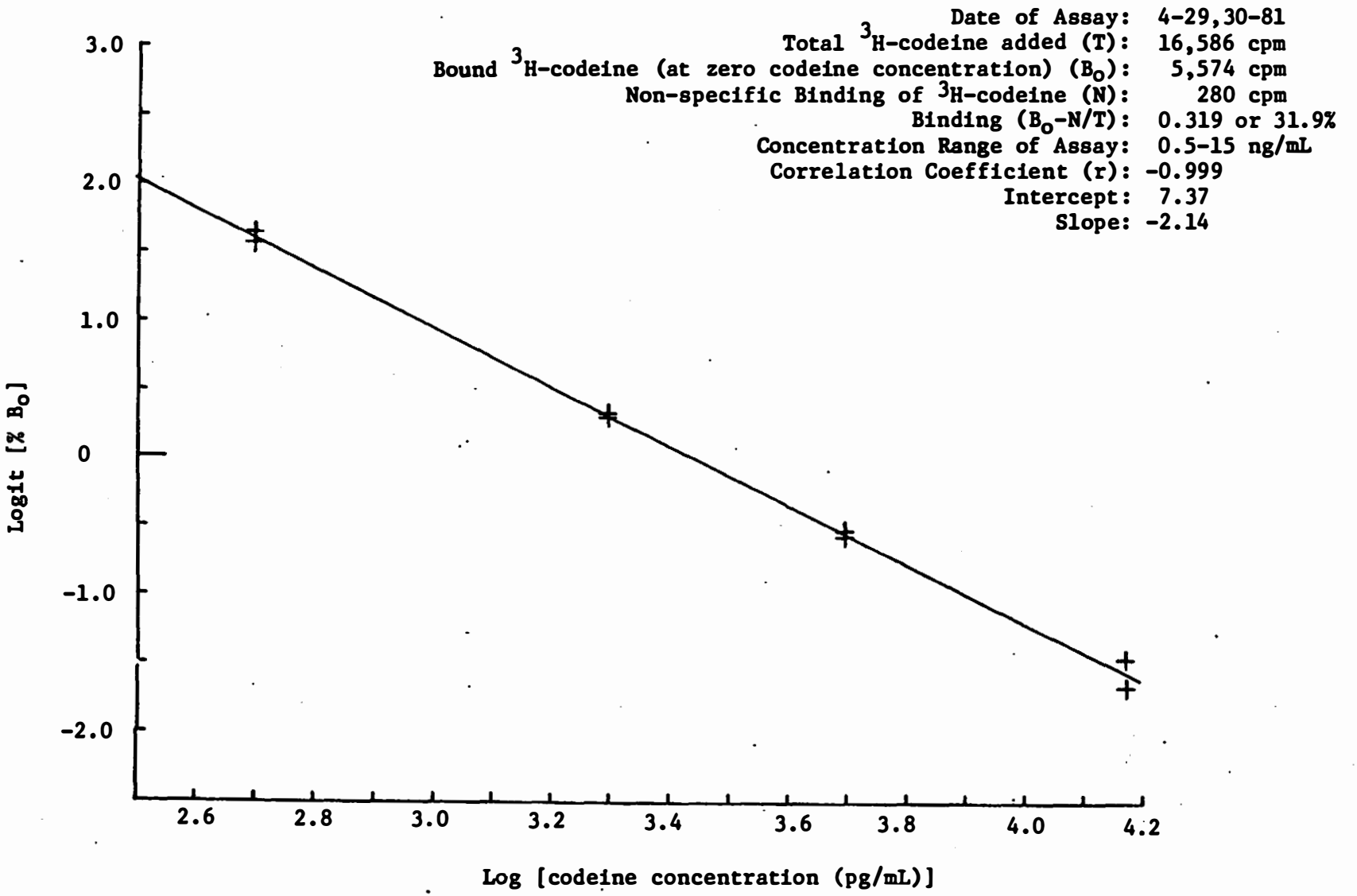

Figure 68. Calibration Curve for RIA of Codeine in 1:10 Diluted Saliva

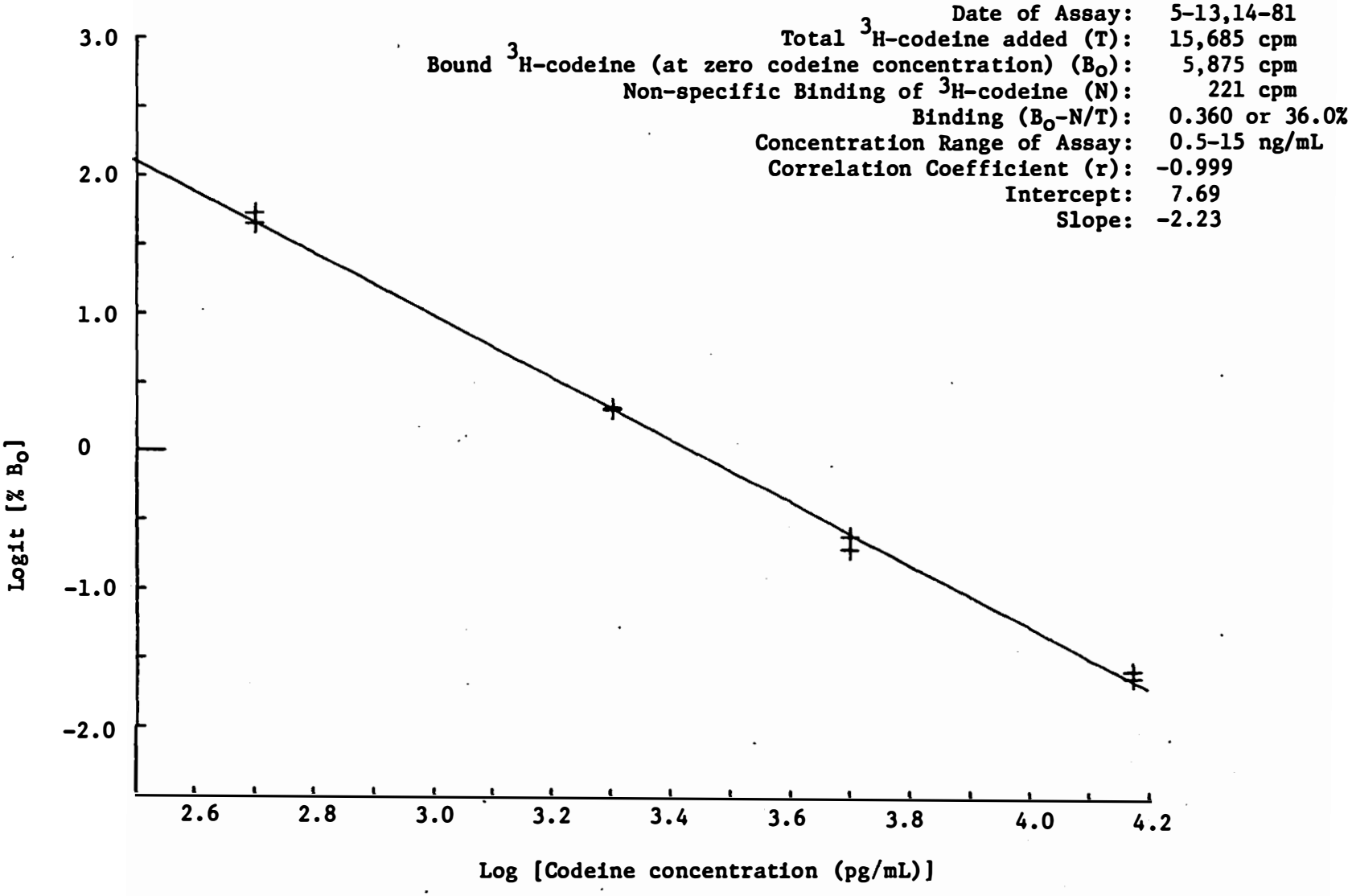

Figure 69. Calibration Curve for RIA of Codeine in 1:5 Diluted Saliva

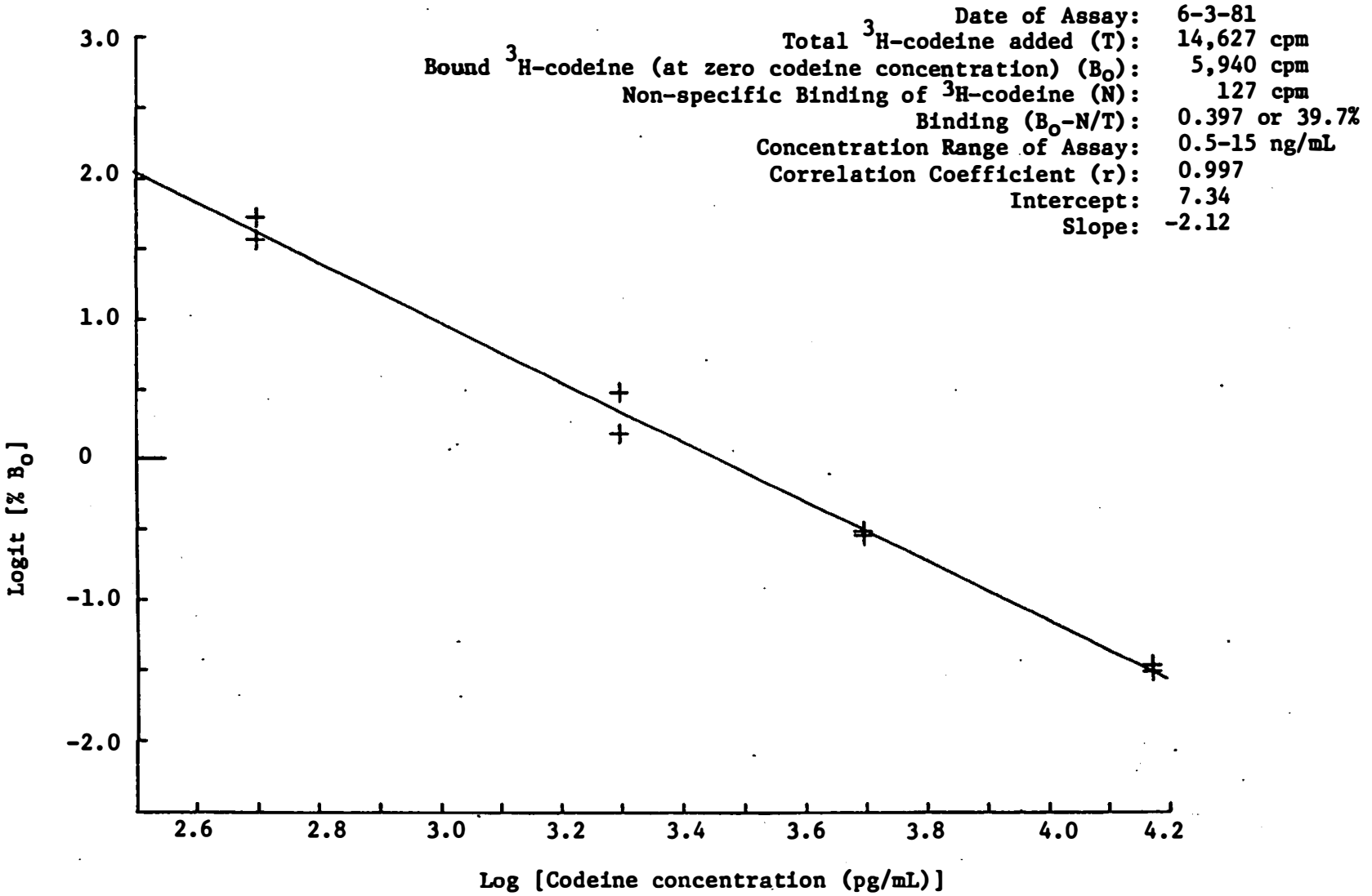

Table 25.

Cross-Reactivity Data for Codeine Antisera Bleed NC-4/8*

Compound	% Cross-Reactivity
Codeine	100
Norcodeine	44
Codeine-6-glucuronide	0.06
Morphine	0.13
Normorphine	0.01
Morphine-3-glucuronide	<0.002

*Data is from Dr. John Findlay, Wellcome Research Foundation.

Table 26.

Accuracy and Precison of Assay for Codeine in Plasma and Saliva

Fluid	Codeine Added (ng/mL)	Codeine Determined by Assay* (ng/mL)	Average Value from Assay (ng/mL)	CV
Plasma	1.0	0.970 ± 0.085 0.706 ± 0.058 0.911 ± 0.050 1.017 ± 0.039	0.901	15%
Plasma	10.0	10.79 ± 1.16 11.07 ± 0.84 10.42 ± 0.59 9.35 ± 0.62	10.41	7.2%
Saliva	1.0	1.094 ± 0.034 0.891 ± 0.214 0.864 ± 0.018 1.043 ± 0.067	0.973	11%
Saliva	10.0	10.19 ± 1.23 10.96 ± 1.13+ 10.20 ± 0.63+ 11.68 ± 0.67	10.76	6.6%

*Mean of 4 determinations ± SD.
+Mean of 2 determinations ± SD.

Figure 70. Statistical Data for the Linear Regression Analysis of Saliva versus Plasma Concentrations (ng/mL) of Codeine.

CODEINE: SALIVA VS. PLASMA 6

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: SALIVA

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	14.44422880	14.44422880	237.45	0.0001	0.823191	13.9119
ERROR	51	3.10239359	0.06083125		STD DEV		SALIVA MEAN
CORRECTED TOTAL	52	17.54662239			0.24663991		1.77286592

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
PLASMA	1	14.44422880	237.45	0.0001	1	14.44422880	237.45	0.0001

PARAMETER	ESTIMATE	T FOR HO: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	0.21237053	1.99	0.0521	0.10678610
PLASMA	1.29246242	15.41	0.0001	0.08387530

Figure 71. Statistical Data for the Linear Regression Analysis of Predicted versus Observed Plasma Concentrations (ng/mL) of Codeine.

CODEINE IN PLASMA 6

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CALC CALCULATED

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	1	16.77896632	16.77896632	423.74	0.0001	0.892574	14.0972
ERROR	51	2.01944502	0.03959696		STD DEV		CALC MEAN
CORRECTED TOTAL	52	18.79841134			0.19898985		1.41133958

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
OBS	1	16.77896632	423.74	0.0001	1	16.77896632	423.74	0.0001

PARAMETER	ESTIMATE	T FOR H0: PARAMETER=0	PR > |T|	STD ERROR OF ESTIMATE
INTERCEPT	-0.27033167	-3.14	0.0028	0.08619936
OBS	1.39300716	20.59	0.0001	0.06767086