I. QUANTITATIVE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY: DETERMINATION OF PSYCHO-PHARMACOLOGICAL DRUGS IN BLOOD SERUM

II. ANALYSIS OF VOLATILE METABOLITES IN BLOOD SERUM OF CANCER PATIENTS BY HIGH RESOLUTION GAS CHROMATOGRAPHY WITH A NITROGEN SELECTIVE DETECTOR

A Dissertation

Presented

to

the Faculty of the Department of Chemistry

College of Natural Sciences and Mathematics

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Francis Hsu

May 1978

TO MY PARENTS

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ABSTRACT

The introduction of High Performance Thin Layer Chromatography facilitated the quantitative determination of trace materials. This technique shows enhanced spot resolution and has a detection limit in the subnanogram range. Because of the highly efficient chromatographic system, the analysis time is greatly shortened.

The work described here represents the first application of HPTLC in the determination of blood levels of some psychopharmacological agents, i.e., chlorpromazine and tricyclic antidepressants. The performance of the complete system was first evaluated and found to be superior to other systems described in the literature. The serum samples were then subjected to a simple but specific solvent extraction step, dosed and chromatographed on the adsorbent layer of high efficiency. A computing integrator in the system facilitated the data collection. The quantitation was achieved via internal standards and calibration curves.

The efficiency of the extraction, the precision, accuracy and specificity of the method were carefully studied. This analytical assay is highly selective and has a low limit of detection of 5 ng/ml.

The potential of a new chromatographic system, U-chamber as an alternative means for separations was also briefly explored.

TABLE OF CONTENTS

CHAPTER			PAGE
ACKNOWLEDGMENT			i
PROBLEM I: QUANTITATIVE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY: DETERMINATION OF PSYCHOPHARMACOLOGICAL DRUGS IN BLOOD SERUM			
ABSTRACT			ii
I.	INTRODUCTION		1
II.	EXPERIMENTAL		10
	A.	Introduction	10
	B.	Chemicals and Reagents	19
	C.	Stock Solutions	20
	D.	Evaluation of the System	20
	E.	Sample Preparation	22
	F.	High Performance Thin Layer Chromatography	23
		1. The Plates	23
		2. Solvent System	25
		3. Developing Chamber	25
		4. Spotting and Separation	25
	G.	Spectrophotometer Conditions for Quantitation of HPTLC Spots	27
		1. Calibration Curves	27
		2. Extraction Recovery, Precision, and The Effect of the "Carrier"	28
		3. Accuracy	29
		4. Specificity of the Analytical Procedure	29

TABLE OF CONTENTS

CHAPTER		PAGE
	H. The U-Chamber	29
	1. Spotting and Operation	31
	2. Operating Conditions	32
III.	RESULTS AND DISCUSSION	34
	A. Selection of the Mode of Measurement	34
	B. System Performance	43
	C. Sample Preparation	51
	D. The Chromatographic System	59
	E. Photometric Quantitation in Diffuse Reflectance Spectroscopy	64
	1. Calibration Curves	67
	2. Precision Curves	70
	F. Separation on U-Chamber	76
IV.	CONCLUSION	80
PROBLEM II:	ANALYSIS OF VOLATILE METABOLITES IN BLOOD SERUM OF CANCER PATIENTS BY HIGH RESOLUTION GAS CHROMATOGRAPHY WITH A NITROGEN SELECTIVE DETECTOR	
ABSTRACT		81
V.	INTRODUCTION	82
VI.	PHYSICAL BASIS OF NITROGEN-PHOSPHORUS DETECTOR	85
VII.	EXPERIMENTAL	90
	A. Column Design	90
	B. Column Preparation	93
	1. Cleaning and Etching	93
	2. Coating	94

TABLE OF CONTENTS

CHAPTER		PAGE
	C. Optimization of Nitrogen-Phosphorus Detector	95
	D. Sampling System	96
	1. Concentration Column	97
	2. Sample Preparation	97
	3. Sample Transfer	99
	4. Serum Volatiles	101
	E. Chromatographic Conditions for Analysis	101
VIII.	RESULTS AND DISCUSSION	103
	A. Column Preparation	103
	B. Nitrogen-Phosphorus Detector	106
	C. Sampling System	111
	D. Organic Volatiles in Blood Serum	112
	1. Profiles of Normal Serum	115
	2. Profiles of Serum of Cancer Patients	117
IX.	CONCLUSION	126
X.	REFERENCES	127

LIST OF FIGURES

FIGURE		PAGE
1.	PROCEDURE FOR THE ISOLATION OF CHLORPROMAZINE AND TRICYCLIC ANTIDEPRESSANTS FROM BLOOD SERUM	24
2.	THE U-CHAMBER	30
3.	THE ABSORPTION SPECTRUM OF CHLORPROMAZINE	35
4.	THE ABSORPTION SPECTRUM OF AMITRIPTYLINE	36
5.	THE ABSORPTION SPECTRUM OF NORTRIPTYLINE	37
6.	THE ABSORPTION SPECTRUM IF IMIPERAMINE	38
7.	THE ABSORPTION SPECTRUM OF DESIPERAMINE	39
8.	THE ABSORPTION SPECTRUM OF BUTAPERAZINE, INTERNAL STANDARD	40
9.	THE ABSORPTION SPECTRUM OF LOXAPINE, INTERNAL STANDARD	41
10.	PHOTOMETRIC REPRODUCIBILITY	44
11.	INSTRUMENT REPRODUCIBILITY	46
12.	SPOTTING REPRODUCIBILITY	47-48
13.	THE METHOD REPRODUCIBILITY: EIGHT CALIBRATION CURVES COVERED TWO MONTH PERIOD	50
14.	CHROMATOGRAMS OF EXTRACTED SERUM SAMPLES SPIKED WITH CHLORPROMAZINE	53
15.	CHROMATOGRAM OF A PATIENT SAMPLE TREATED WITH CHLORPROMAZINE	54
16.	CHROMATOGRAMS OF EXTRACTED SERUM SAMPLES SPIKED WITH TRICYCLIC ANTIDEPRESSANTS	55
17.	BACKGROUNDS OF HPTLC PLATE	60
18.	REPRODUCIBILITY OF R_f VALUE	65
19.	CALIBRATION CURVES OF CHLORPROMAZINE	68

LIST OF FIGURES (cont.)

FIGURE		PAGE
20.	CALIBRATION CURVES OF CHLORPROMAZINE: SCANNING AT RIGHT-ANGLE TO THE DIRECTION OF SOLVENT FLOW.....	71
21.	A TYPICAL CHROMATOGRAM OF SCANNING PERPENDICULARLY TO THE DIRECTION OF SOLVENT FLOW.....	72
22.	CALIBRATION CURVES OF AMITRIPTYLINE AND ITS MAJOR METABOLITE, NORTRIPTYLINE.....	73
23.	CALIBRATION CURVES OF IMIPRAMINE AND ITS MAJOR METABOLITE, DESIPRAMINE.....	74
24.	CHROMATOGRAMS OF STANDARD MIXTURES OBTAINED FROM U-CHAMBER.....	79
25.	THE SCHEMATIC STRUCTURE OF NITROGEN-PHOSPHORUS DETECTOR...	86
26.	THE RECYCLING REACTION MECHANISM OF NITROGEN-PHOSPHORUS DETECTOR.....	89
27.	THE MICRO SAMPLING SYSTEM.....	98
28.	THE SAMPLE DESORPTION SYSTEM.....	100
29.	THE PROGRESS OF SIMPLEX OPTIMIZATION.....	109
30.	THE INERTNESS OF GLASS WOOL.....	113
31.	REPRODUCIBILITY OF PROFILES FROM THE SAME SERUM SAMPLE....	114
32.	REPRODUCIBILITY OF PROFILES FROM THREE DIFFERENT HEALTHY INDIVIDUALS.....	116
33.	COMPARISON OF SERUM VOLATILES BETWEEN LEUKEMIA PATIENT AND NORMAL INDIVIDUAL.....	118
34.	COMPARISON OF SERUM PROFILES FROM THE SAME LEUKEMIA PATIENT AT DIFFERENT STAGES OF DISEASE.....	119
35.	COMPARISON OF MOUSE SERUM PROFILES OF ACUTE LEUKEMIA AND CONTROL.....	121
36.	SERUM PROFILES OF BREAST CANCER.....	122

LIST OF FIGURES (cont.)

FIGURE		PAGE
37.	SERUM PROFILES OF MELANOMA-TUMOR	123
38.	SERUM PROFILES OF A PATIENT WITH LUNG CANCER	124
39.	SERUM PROFILES OF CANCER OF COLON	125

LIST OF TABLES

TABLE		PAGE
I.	COMPARISON BETWEEN HIGH PERFORMANCE AND CONVENTIONAL THIN LAYER CHROMATOGRAPHY	16
II.	THE PEAK AREA RATIO AND REGRESSION DATA OF EIGHT CALIBRATION CURVES OF TWO MONTH PERIOD	49
III.	EFFECT OF CARRIER (PERPHENAZINE) ON RECOVERY OF PSYCHOTROPIC DRUGS FROM SERUM	58
IV.	LINEAR REGRESSION DATA FOR CALIBRATION CURVES IN FIGURES 19, 20, 22 AND 23	69
V.	EFFECT OF CARRIER (PERPHENAZINE) ON REPRODUCIBILITY OF DETERMINATION OF PSYCHOTROPIC DRUGS IN SERUM	75
VI.	SINGLE BLIND DETERMINATIONS OF NANOGRAM AMOUNTS OF PSYCHOTROPIC DRUGS FROM SPIKED SERUM SAMPLES	77
VII.	R_f VALUES OF TWENTY-SIX COMMON BASIC DRUGS SCREENED FOR INTERFERENCE	78
VIII.	THE NUMERICAL DATA OF SIMPLEX MOVEMENT	110

PROBLEM I

QUANTITATIVE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY: DETERMINATION OF PSYCHO-PHARMACOLOGICAL DRUGS IN BLOOD SERUM

INTRODUCTION

INTRODUCTION

Thin-layer chromatography (TLC) has long been recognized as one of the most widely used separation techniques. It can be performed at reasonable cost and with minimum of equipment. It is a micro method for handling small quantities, at the same time it can be used for isolating and purifying substances on a preparative scale. It can be used for quantitative determination as well as for the qualitative identification of resolved materials. It is selective, simple and rapid. Indeed, TLC has gained all these merits because of its great flexibility and versatility.

Its first appearance dates back to 1889, a Dutch biologist, Beyerinck[1,2,3,4], who added a drop of hydrochloric and sulfuric acid mixture to a thin layer of gelatin. A ring of hydrochloric acid surrounding a center of sulfuric acid resulted. Hydrochloric acid was made visible by brushing with silver nitrate, while barium chloride was used on the sulfuric acid. Half a century later, Izmailov and Shraiber[5] applied a loose layer of aluminum oxide to a plate for the separation of various substances by ring development. Ten years later, Meinhard and Hall[6] introduced the application of a binder to increase the physical strength of the thin-layer. In the 1950's, Kirchner and coworkers[7,8,9] were the first to apply an adsorbent to glass strips to analyze quantitatively flavoring components in citrus juice and to adopt the linear solvent development which is widely used today. However, TLC did not become popular until the outstanding studies of Stahl[10] who invented

a convenient device for making the thin-layer and standardized the technique. Today TLC has developed into one of the most powerful research tools, and is in routine use in many fields for separation, analysis and quantitation.

In the quantitative evaluation of substances separated by TLC, two methods are commonly used:

(a) Elution, in which the layer containing the spot is scraped from the plate, the sample is extracted from the adsorbent and is then determined photometrically.[11,12] This method is usually time-consuming, laborious, less accurate and not suitable for low concentration analysis. The spot must be well separated from other components before this method can be applied. Recently, a new device has been introduced[13,14] which simplifies the elution process and gives good recovery and precision. If the study involves MS or IR analysis, this method still is of considerable importance.

(b) Direct quantitation of the samples on the TLC plates with a spectrophotometer. With the capability of a modern instrument, the quantitation can be operated in visible and ultraviolet range in transmission,[15-19] reflectance[20-25] and fluorescence[26] modes. Measurement in the fluorescence mode renders enhanced selectivity, high sensitivity and increased linearity.[27] Since transmittance measurements are limited in wavelength and suffer interference due to non-uniformity of layer thickness of the plate, reflectance measurements are generally preferred.[27-31] However, considerable controversy existed in the past

about whether the reflectance or transmittance technique was the preferred method of measurement.[16,18,20,27,28,29,32,33] After a detailed comparison between the two measuring modes, Frei[28] concluded that reflectance is superior to transmittance in many respects and the controversy may come from the specific instrument used by other workers, which had inferior design in measuring reflectance. It is thus necessary for each individual in a given laboratory to select the proper measuring mode according to his own instrument design. Jork[33] recently introduced the simultaneous reflectance and transmittance method in which the transmission and reflectance signals are added electronically at a certain ratio; therefore the baseline noise, due to non-homogenity of the layer thickness is smoothed at the same site, at the same wavelength. This operation improves the signal to noise ratio and the detection limit.

Direct scanning in situ quantitation by TLC is a well established technique for micro analysis today. It offers greater sensitivity, high precision, speed, ease of operation and reliable results with high confidence limits. It makes the quantitation at subnanogram levels possible. Some studies have been made[34,35] using a computer controlled scanning spectrophotometer for quantitation, improved performance, rapid data acquisition and automation.

Besides the progress in the instrument itself, the most recent development in TLC has centered in increasing the efficiency of the TLC plates in order to be compatible with the stringent requirements of trace analysis. One approach, for achieving high performance, Program Multiple

Development (PMD),[36] uses automatic repeated development of a TLC plate with the same solvent in the same direction for gradually increasing distances. Between developments, the TLC plate is dried by controlled evaporation which causes the solvent front to recede, while the plate remains in contact with the solvent reservoir. The net effect is spot reconcentration in the shape of an ellipse with the short axis parallel to the direction of solvent flow which leads to the improved resolution and sensitivity. The second approach,[30,37] an application of general chromatographic principle to TLC, applies sophisticated technology to the manufacture of new high performance TLC plates with small but uniform particle size and homogeneous adsorbent thickness which are empirically optimized with respect to HETP, solvent running time and distance. After the conventional development, small, concentrated spots are produced, therefore greater resolution and sensitivity are obtained. The combined use of these approaches is promising for the further improvement of separation of extremely complex mixtures.[38,39]

Today, the field which applies qualitative and quantitative TLC the most is probably that of drug analysis. In toxicology, the increasing use of a rapidly expanding group of hypnotics, sedatives, analgesics, anti-convulsants and psychiatric drugs has created many demands for chemical analysis of biological specimens. Especially, the increasing frequency of intentional and accidental poisoning by these central nervous system (CNS) drugs has become a medical and social problem of major proportions. Thin-layer chromatography is the most suitable technique and has been involved in massive screening programs for drugs of abuse. This technique

meets all the criteria of selection as a routine method for large-scale screening programs. These programs primarily emphasize the speed of analysis with yes/no or positive/negative answers. However, in clinical pharmacology, there is an increasing need for accurate determinations of drug levels in blood. In the past, instead of measuring the drug concentration in patient's serum to ascertain the adequate therapeutic drug levels, physicians generally adjusted the drug dosage by observing the patients' behavior for the onset of mild manifestations of toxicity, such as nausea, coma, etc.. Because of recent developments in pharmacology and medicine, the use of new potent therapeutic agents alone or with other potent therapeutic agents together dramatically increases the incidence of adverse reactions.[40] Also the toxic response produced is more severe. Especially with those potent agents with a low therapeutic index, their minimum effective concentration in the blood is critically close to those producing severe toxicity. If the previous approach is still being used based on behavior observation and clinical impression, dangerous situations which jeopardize patient life would often occur. Thus, therapeutic drug monitoring is thought to be able to regulate drug dosage to maintain the effective therapeutic levels more smoothly and safely. This point of view is further buttressed by:

(1) Individual variation in drug metabolism:[41,42]

It has been found that there is considerable individual variation in dosage to produce the required drug concentration in blood, mainly due to different capabilities of the individual to adsorb, redistribute, metabolize and excrete drugs. The same dosage of drug by weight as used

conventionally, may produce no response in subject A, an effective blood level in subject B and severe toxic response in subject C. If drug concentrations in the blood are monitored, especially for drugs with narrow therapeutic ranges, the best effective therapeutic level for each individual can be established according to his own rate of metabolism. The danger of overdose can be substantially reduced.

(2) Individual variation in drug interactions:[41,42]

It has been shown[43,44] that one drug taken may influence the adsorption, binding, redistribution, biotransformation and excretion of other drugs which are present. The effect exerted may shorten the duration of action of either its own or others[45] or can slow down the rate of metabolism of other drugs.[46] The latter situation is potentially more dangerous because the toxicity may result due to drug accumulation.

Since drugs have their own sites of biological action (receptor), at first glance, it seems inappropriate to use plasma levels of the drugs as an index for the degree of biological effect. Fortunately, most of the drugs attached to the receptor are in a dynamic equilibrium with the drug in the plasma water.[47]

$$\text{Drug} + \text{receptor} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \text{Drug receptor complex}$$

Therefore, the concentrations of such drugs in blood reflect their concentration within inaccessible tissues where their sites of action are located. For these reversibly-bound drugs, the measurement of blood

levels allow them to be used more effectively and efficiently with less danger to the patients.

As experience has been gained in the use of psychopharmacologic agents in the treatment of mental illness, it has become increasingly apparent that large differences in drug effect may occur from patient to patient or within an individual patient over a period of time.[48-51] By the determination of the psychotropic drug concentrations in the blood, the ambiguity of the drug effect can be cleared up and a better basis for the evaluation of the course of therapy can be obtained. The determinations need analytical procedures which will permit large numbers of samples to be processed rapidly. These drugs, however, may be present in the blood in the low nanogram per milliliter range and metabolic products of the drugs usually present closely resemble the parent compounds. Therefore, both sensitivity and selectivity are also required. Among the available techniques, chromatography is the ideal choice.

The reported blood assays utilizing gas and liquid chromatography are too numerous to mention. Chloropromazine has been determined by a number of electron capture gas chromatographic methods. Other phenothiazine and tricyclic antidepressants have been assayed in blood samples using a nitrogen sensitive detector. GC/MS and GC/Mass fragmentography are also used for these drugs blood level analysis. Liquid chromatography methods have been less prominent in blood level determinations because of the limitations in sensitivity encountered with most liquid chromatography detectors. However with the recent

advent of micro volume UV and electrochemical detectors, quite good sensitivity for phenothiazines and tricyclic antipressants in human blood serum have been reported.[53,54]

Despite the successes of gas and liquid chromatography in providing accurate assays on the rather limited number of samples found in most research studies, extension of these methods to routine clinical determinations presents problems which are related primarily to analysis time. When sequential determinations are a necessary element in an analytical procedure, the manipulation of large numbers of samples necessitates either a dramatic reduction in unit analysis time or replication of the requisite apparatus or instrumentation. Thin-layer chromatography on the other hand has many features which make it an attractive candidate for routine clinical determinations. In addition to the obvious advantage of chromatographing a number of samples at the same time, TLC permits separation of compounds which might not be possible with gas chromatography without derivatization. The principle criticism for the reported assays by TLC[54-69] has been lack of sensitivity so that large volumes of serum or plasma have to be used, and difficulties in obtaining quantitative determinations with acceptable precision. However, the introduction of High Performance Thin-Layer Chromatography (HPTLC) has done much to eliminate these weaknesses and with efficient chromatographic spectrophotometers, sensitivity and reproducibility are approaching that of the sensitive gas and liquid chromatographic assays. The present study was undertaken to examine the feasibility of utilizing HPTLC in the quantitation of representative

psychotropic agents in blood serum samples at concentrations normally encountered clinically.

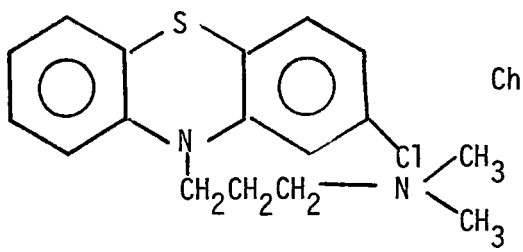
EXPERIMENTAL

EXPERIMENTAL

A. Introduction

I. Psychotropic drugs are defined as those chemical compounds that are used medically for the purpose of modifying thought, mood, and emotion. The procedures developed in this study are for the determination of

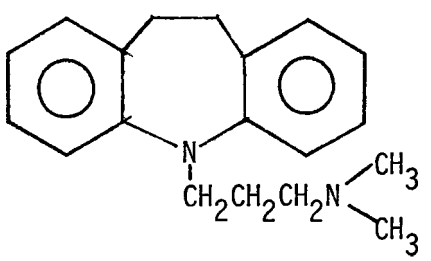
(a) Chlorpromazine: It is one of the most widely used drugs for the treatment of Schizophrenia and other mental disorders.



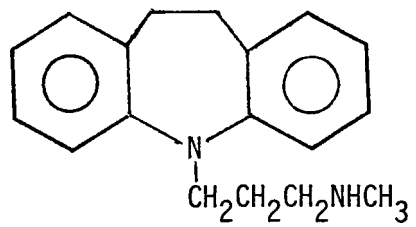
Chlorpromazine

Its metabolites, generated from hydroxylation, sulfoxidation and dealkylation reactions are also found active. The determination of chlorpromazine alone is described.

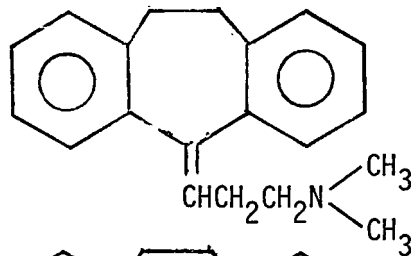
(b) Tricyclic antidepressants: Imipramine, amitriptyline and other closely related compounds are widely used in alleviating depression.



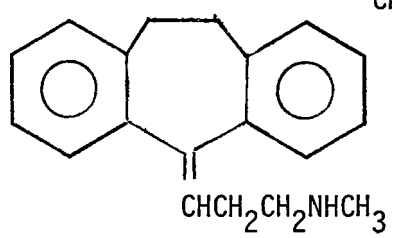
Imipramine



Desipramine



Amitriptyline



Nortriptyline

Desipramine and Nortriptyline are the principle dealkylated metabolites of Imipramine and Amitriptyline respectively. They are found also to be biologically active. Simultaneous determination of the four drugs is described.

II. In an average community in the United States today, twenty percent of the prescriptions written are for medications intended to affect mental processes, to treat abnormalities of mental function, to stimulate, sedate, or otherwise change behavior.[70] Because of a widespread desire to modify "normal" feelings, several of these drugs have become very important in the life pattern of a vast number of people in many other countries. Recently, owing to the advanced research in medicinal chemistry, it has led to the development of very potent

psychotropic drugs. When the drugs are administered as therapeutic agents, they usually display a very narrow range i.e., the blood concentration of drugs required for clinical effectiveness is limited. Below this required concentration, the desired effect cannot be produced. However, overdosage often initiates adverse reactions in the body to the point where serious toxicity may be present. Hence it is not surprising that the incidence of self-poisoning with these drugs has increased dramatically in recent years.

From the point of view of pharmacology and toxicology, there are increasing demands to establish the action mechanism, side effects, clinical outcome of the drugs, and to monitor their steady-state therapeutic levels. In pharmcology and toxicology, there are three types of analyses required:

1. The analysis of a drug to determine its composition, stability and any impurities present.

2. The determination of the concentration of a drug and/or its metabolites in body fluids or tissues.

3. The analysis of the cellular constituents to determine the effect of the drug.

The work described here belongs to the second category. Its importance has been recognized in that the blood level of the drug correlates better than dosage does for evaluation of clinical response during the course of therapy.[41]

Researchers in pharmacology and toxicology often encounter very different situations in the isolation and purification of trace amounts

of desired substance from biological fluids or tissue which may contain hundreds of other complex molecules with similar solubility properties. The most commonly used isolation methods for drug analysis are[71]

1. Solvent Extraction:

The liquid-liquid extraction technique is extensively used in the pharmaceutical field for the isolation of trace amounts of compounds of interest from complex samples. In order to be specific in the isolation which is very important in minimizing quantitative error, a "clean-up" procedure is usually included in the extraction. Immediately following the initial extraction at the proper pH value with an organic solvent, the sample is back extracted into the aqueous phase after the adjustment of pH. By this means, most of the endogeneous materials, organic or inorganic, can be eliminated. Here, the proper adjustment of pH with buffers is highly critical in order to obtain a clean sample extract.

The solvent extraction method can be used for drugs with high therapeutic levels as well as drugs with low therapeutic levels. For some cases back-extraction must be repeated several times in order to obtain a clean extract, but it is at the expense of recovery of the drugs.

2. Extraction by Solid Additives or Column Materials:

Alumina, charcoal and various ion-exchange resins, e.g. Dowex 50, Amberlite XAD-2, are examples of such solid materials. These substances are usually packed into small columns, or impregnated into the papers. After the initial adsorption of the biological fluid, e.g. plasma or urine, the desired compound may be eluted with a pure or a mixed organic

solvent at a proper pH value. "Clean-up" is achieved by elution at a specific pH value. This method is seldom used for the isolation of drugs with very narrow therapeutic ranges. This is because whatever the solid phase used, there is always a risk that the irreversible adsorption of the drugs on the solid surface may take place.

The time needed for the equilibration of the solid materials with the buffers of desired pH is quite long.

The choice of proper isolation technique, solvents and pH is dependent on the type of the compounds to be analyzed. After the initial isolation, chromatography in one form or anotheris the most common method for separation. Each form has its advantages and some intrinsic limitations. Although TLC is provided with the ability for multiple detection, it suffers from low sensitivity, poor accuracy and precision in quantitation. However, in 1976, major improvement brought the conventional quantitative TLC into a new era.

The improvements were:

1. Production of a new silica gel plate in conjunction with new technology for sample dosage with high precision and accuracy.[30,37]

2. Data acquisition with a sophisticated chromatogram spectrophotometer for in-situ quantitation[26] i.e. the concentration of a substance can be determined directly on the chromatoplate.

These factors, the plate, the dosage technique and the instrument, are the most important components which affect the precision and accuracy in quantitation. Ripphahn and Halpaap[37] introduced High Performance

Thin-Layer Chromatography (HPTLC). Based on an earlier study on the structure of silica gel,[72] it was concluded that the chromatographic properties, e.g. plate height, resolution, etc., depend mainly on the mean pore size, particle size distribution and quality of the layer. If the reproducibility of a chromatographic separation process needs to be quaranteed, the production of silica gel with narrow pore and particle size distribution in conjunction with reproducible plate-manufacturing process is a must. In the experiments undertaken to investigate the optimum plate conditions,[37] a series of plates consisting of silica gel with pore size 60 Å with different particle size, and layer thickness were used to perform the separation in the same chamber with presaturation of the layers and at the same temperature. The data collected were fed into a process control computer and the results analyzed. The plates produced are optimum with respect to particle size, layer thickness, solvent running distance and amount of substance applied. By using the advanced technology and exact quality-check, the pre-coated silica gel HPTLC for nanogram-scale analysis consists of silica gel with pore size 60 Å, particle size 7 μm ± 5%, and a layer thickness of 0.2 mm. Layer density is comparable to that of a well packed HPLC column. Under normal chromatographic conditions, it is possible to achieve a plate height of 0.012 mm [30] at a solvent running distance of 30-60 mm. Owing to the short running distance and the properties of the layer, the resolved spots are small and compact, hence it exhibits low limits of detection. The sensitivity is improved tenfold over that of a TLC. The comparison of overall performance of HPTLC and TLC is shown in Table I.[26]

TABLE I. COMPARISON BETWEEN HIGH PERFORMANCE AND CONVENTIONAL THIN LAYER CHROMATOGRAPHY

	Macro-TLC	HPTLC
Size:	20 cm x 20 cm	10 cm x 10 cm 10 cm x 20 cm
Adsorbent:	silica gel	silica gel
Plate height:	30 mm	12 mm
Dispensing volume/spot with capillaries:	1–5 µl	approx. 0.1–0.2 µl
Diameter of the spots:	approx. 3–6 mm	approx. 1–1.5 mm
Diameter of the separated spots:	approx. 6–15 mm	approx. 2–5 mm
Migration:	10–15 cm	3–6 cm
Separation time:	approx. 30–200 min	approx. 3–20 min
Substance quantity/spot		
Absorption:	approx. 0.05–5 µg	approx. 0.005–0.5 µg
Fluorescence:	approx. 1–1000 ng	approx. 0.1–100 ng
Detection limits		
Absorption:	0.005–0.05 µg	0.0005–0.005 µg
Fluorescence:	0.1–1 ng	0.01–0.1 ng
Tracks/plate	approx. 10	approx. 18 or 36
Number of samples on one plate in double analysis if three standards are used:	0.5 x 10 - 3 = 2	0.5 x 18 - 3 = 6 or 0.5 x 36 - 3 = 15

The initial spot size is always a factor which affects the chromatography power. A wide initial spot will ruin the high efficiency and resolution of HPTLC. The improved dosage technique for HPTLC is the use of a platinum-iridium metal capillary fused into a glass capillary. The Pt-Ir capillary has an internal diameter of about 0.15 mm. The desired volume, ranging from 100 nl to 1 µl, can be obtained by cutting the correct length of the capillary and calibrating it photometrically with a dye solution.[26] The Pt-Ir capillary is chemically inert, smooth on the inside surface, and most important does not show any memory effects. It is soft, and durable. The desired solution volume is drawn by capillary force into the fixed-volume capillary. Sample application can be done manually with the capillary, but it is easily clogged by the fine silica gel particles. However, this can be avoided by using the counterbalanced EVA-Chrom-TLC-Applicator (W & W Electronic and Scientific Instrument, Co., Basel, Switzerland). The capillary is held magnetically on the rocker arm and lowered down smoothly and vertically to the adsorbent layer to exert a minimum contact pressure between the capillary and the layer which ensure a good drainage of the sample solution, and no rupture of the adsorbent layer. If changing sample solution is desired, the capillary can be cleaned by simply rinsing with methanol several times.

The data acquisition and processing system is the Zeiss KM-3 chromatogram spectrophotometer with a computing integrator.[26] The KM-3 is a single-beam instrument which allows the compensation of the background at the exact point where the samples are, and is probably the

most versatile TLC scanner ever made. This system can be set for adsorption measurement (in reflectance, transmission or in simultaneous reflectance-transmission modes), for fluorescence measurement, as well as for solvent photometry in adsorption and fluorescence. The instrument can be used to scan thin-layer and paper chromatograms, transparent samples such as electrophoresis strips, film negatives, photographic plates and gel disks. Changing from one type of measurement to another is quite simple and rapid. The light sources consist of deuterium lamp (185-325 nm), tungsten lamp (325-2500 nm) and medium pressure mercury lamp with discrete emission lines. A quartz prism monochromator is employed with a spectral range of 185-2500 nm. The monochromator exit slit is imaged achromatically on the sample plate which is positioned on a scanning stage driven by a synchronous motor (1-300 mm/min) in the y direction. The scanning stage can be adjusted manually in the x direction. The slit width is able to adjust between 0.01 mm to 2 mm and the slit length, between 3.5 mm and 14 mm. The reflected radiation is detected at an angle of 45° by a photomultiplier.

The instrument is equipped with fast response, low-noise transistor electronics which allows a quick warm-up and the operation with open scanning stage regardless of the extraneous light.

Quantitative evaluation of the peak area is performed by a programmable computing integrator which ensures fast and reliable results. Since drifting baselines and partially overlapped peaks can be corrected automatically, the integrator provides excellent precision.

Based on existing data,[26,30,37] the new TLC plates in combination

with high precision sample dosage techniques and good instrumentation for quantitation, offer high sensitivity, precision and speed. In this study, the new technique is evaluated by applying it to the analysis of psychotropic drugs using the following steps:

1. Sepcific extraction of the desired drugs from serum.
2. Separation and detection of the compounds.
3. Photometric quantitation.

B. Chemicals and Reagents

Psychopharmacological agents used in this study were supplied as follows:

Chlorpromazine, Smith, Kline and French Laboratories (Philadelphia, Pa.).

Loxapine, Lederle Laboratories (Pearl River, New York)

Perphenazine, Schering Corporation (Kenilworth, New Jersey)

Butaperazine, A. H. Robins Company (Richmond, Virginia)

Amitriptyline, Merck Sharp and Dohne (West Point, Pa.)

Nortriptyline, Eli Lilly and Company (Indianapolis, Ind.)

Imipramine, Geigy Pharmaceuticals (Summit, New Jersey)

Desipramine, USV Pharmaceuticals Corp. (Tuckahoe, New York)

Extraction solvents, heptane and isoamyl alcohol, were purified by fractional distillation in all-glass systems and stored in bottles equipped with either teflon-sleeved glass stoppers or with teflon-lined screw caps. All other solvents and chemicals used were reagent-grade purity.

All glassware was silylated using hexamethyldisilazane at elevated temperature and reduced pressure.(73)

C. Stock Solutions

For the chlorpromazine assay, two stock solutions were prepared using 1.5% isoamyl alcohol in heptane. The first contained chlorpromazine at a concentration of 1 mg/ml and was used in the preparation of all calibration curves. The second contained a mixture of the internal standard, butaperazine, at a concentration of 100 µg/ml and the "carrier", perphenazine at 2 mg/ml. Small amounts of the latter solution were diluted 1:100 with 1.5% isoamyl alcohol/heptane for addition to serum samples.

For the assay of tricyclic antidepressants, 1 mg/ml solutions of each base, imipramine, desipramine, amitriptyline and nortriptyline were made using the 1.5% isoamyl alcohol/heptane solvent. A mixture of the internal standard, loxapine base, at concentration of 100 µg/ml and the perphanazine "carrier" at 2 mg/ml was made in 1.5% isoamyl alcohol/heptane to be later diluted 1:100 and added to serum samples.

All the stock solutions were stored in the freezer at 0°C.

D. Evaluation of the System

With a 1 µl Pt-Ir capillary, 10 ng/200 nl pure chlorpromazine solution and 4 HPTLC plates, each plate was dosed with 8 spots. Each spot represented 50 ng of chlorpromazine. Between each application, the capillary was aspirated with methanol. After development, the evaluation began by scanning the plates with the KM-3 in reflectance mode at 254 nm

for:

1. Photometric reproducibility:

Plate No. 1 was chosen; only one spot on this plate was scanned repeatedly 10 times without adjustment of the chromatogram track on the scanning stage.

2. Instrument reproducibility:

The same spot was chosen from Plate No. 1, and scanned repeatedly 10 times. In this case, the chromatogram track was adjusted between measurements by moving the spot out of the measuring area and manually readjusting the spot position with the precision drive on the stage using the full deflection of the recorder as an aid.

3. Spotting reproducibility:

8 spots on each plate were scanned. Each spot was scanned only once. Peak areas obtained were compared.

4. Integrator precision:

The results from photometric and instrument reproducibility are indicative of the precision of the integrated peak area for a particular spot.

5. Reproducibility of the method:

Four chlorpromazine solutions of different concentrations, 200 ng, 50 ng, 10 ng and 5 ng per µl, with 100 ng butaperazine, internal standard and 2 µg carrier, were made by a series of dilution from the stock solution.

Eight calibration curves i.e., peak area ratio (drug/internal standard) vs. concentration, were constructed once a week over a period of two months to test the reproducibility of the overall system.

E. Sample Preparation

The liquid-liquid extraction method was applied here. The same extraction procedure was used for both chlorpromazine and tricyclic antidepressants. The serum used in the calibration curve, recovery and precision studies was separated from fresh blood, free from any drug, and drawn with a sterilized syringe.

A 100 µl portion of the diluted stock solution containing the appropriate internal standard and carrier mixture was added to a 15 ml silylated screw cap culture tube (9 cm x 8 mm), with teflon liner, (Kimball Glass, Owens, Ill.), and dried under reduced pressure. To this was added 1 ml of the serum to be tested. The tube was vortexed briefly to help the dissolution of the internal standard in the serum. Together with 1 ml of 1 N NaOH (pH = 10), the basic serum was then extracted with a single 10 ml portion of the 1.5% isoamyl alcohol/heptane solvent. The tube was tightly capped. A piece of solvent-washed aluminum foil was used to prevent the solvent from touching the teflon liner. A tube rocker having gentle action (Labindustries, Berkeley, Ca.) was employed in this initial extraction from serum for a period of 30 to 45 minutes, followed by separation in a bench-top clinical centrifuge for 5 minutes. The aqueous serum layer was then frozen using an acetone-dry ice bath, and the entire organic layer decanted to another 15 ml culture tube. An acidic aqueous layer was then introduced by addition

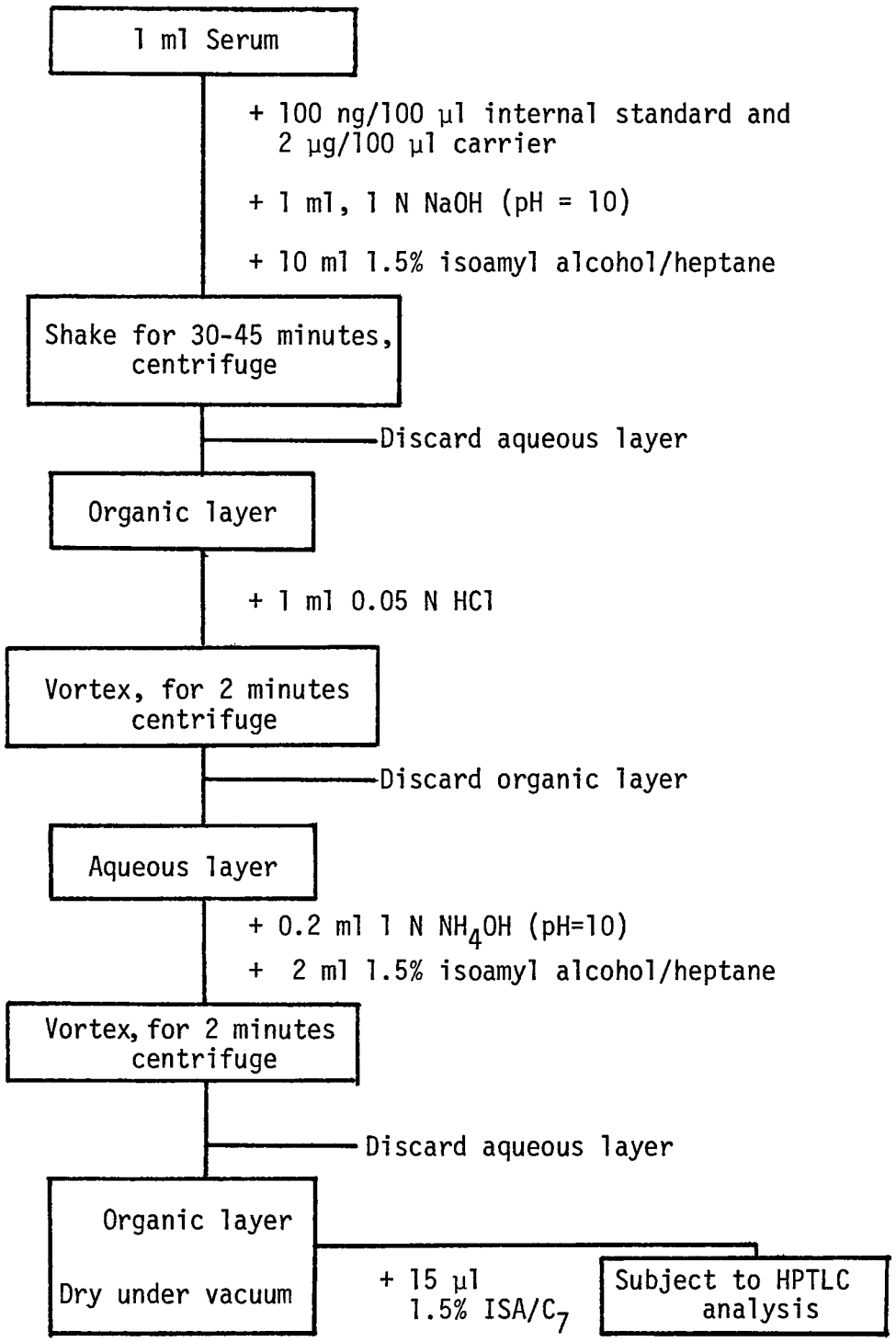
of 1 ml of 0.05 N HCl, and vortexed thoroughly for 1 to 2 minutes. After centrifugation, the organic layer was removed by aspiration, and the remaining acidic aqueous phase alkalinized to a pH of 10 by the addition of 0.2 ml of 1 N NH_4OH. This alkaline aqueous phase was then extracted with 2 ml of 1.5% isoamyl alcohol-heptane solvent by mixing thoroughly for 2 minutes and finally centrifuged. The aqueous layer was frozen with acetone-dry ice bath to enable easy and complete transfer of the organic solvent to a silylated Reactivial (Pierce Chemical Co., Rockford, Ill.). This final extract was then taken to dryness under reduced pressure and subjected to HPTLC analysis. The complete extraction procedure is schematically shown in Figure 1.

F. High Performance Thin-Layer Chromatography

1. The plates:

The precoated 10 cm x 10 cm silica gel 60 HPTLC plates without fluorescence indicator were used for all the separation (E. Merck, Darmstadt, Germany). Plates, free from small holes and scratches on the silica gel layer were chosen for chromatography and quantitation. The layer is always thicker on two sides which are easily visualized by holding the plate against the light. Samples should not be spotted on these two thick edges. In order to assure low background, UV adsorbing materials were eluted to the solvent front by performing two consecutive pre-development washes with solvent system hexane/acetone/diethylamine (80/20/3). This washing step was found necessary for quantitation at the sub-nanogram level. The plates were then dried in a vacuum oven at 80°C for one hour, and stored in a glass desiccator for later use or

FIGURE 1: PROCEDURE FOR THE ISOLATION OF CHLORPROMAZINE AND TRICYCLIC ANTIDEPRESSANTS FROM BLOOD SERUM


1 ml Serum
+ 100 ng/100 µl internal standard and 2 µg/100 µl carrier
+ 1 ml, 1 N NaOH (pH = 10)
+ 10 ml 1.5% isoamyl alcohol/heptane
Shake for 30-45 minutes, centrifuge
Discard aqueous layer
Organic layer
+ 1 ml 0.05 N HCl
Vortex, for 2 minutes centrifuge
Discard organic layer
Aqueous layer
+ 0.2 ml 1 N NH_4OH (pH=10)
+ 2 ml 1.5% isoamyl alcohol/heptane
Vortex, for 2 minutes centrifuge
Discard aqueous layer
Organic layer
Dry under vacuum
+ 15 µl 1.5% ISA/C_7
Subject to HPTLC analysis

used immediately.

2. Solvent systems:

Based on the elutropic series of Halpaap and Ripphahn[37], single pure solvents and dozens of different combinations of solvents in saturated and unsaturated chambers have been investigated. The best solvent system for chlorpromazine was hexane/acetone/diethylamine (80/20/3), single development in a saturated chamber.

The best solvent system for tricyclic antidepressants was found to be hexane/benzene/diethylamine (96/12/3), double development in a saturated chamber.

Solvent volume was measured precisely with titration burets of 50 ml, 20 ml, and measuring pipets of 3 ml. The solvent systems were stored in 250 ml glass-stoppered conical flasks.

3. Developing chamber:

The chamber was a twin-trough type (Camag AG, Muttenz, Switzerland) of 220 x 230 x 75 mm. The bottom of the tank was divided into two troughs of equal volume by means of a 2 cm high ridge running through the whole length of the chamber. The solvent volume needed to provide a saturated environment and to develop the plate was about 20 ml. Since the solvent needed was such a small amount, fresh solvent was used for every development.

4. Spotting and separation:

Extract in Reacti-vial obtained from the sample preparation was reconstituted in 15 µl, 1.5% isoamyl alcohol-heptane solvent, measured

precisely with the restricted-tip measuring pipet. The extract was applied to the HPTLC plates by means of a 1 µl Platinum-Iridium capillary (Antech, Bad Durkheim, Germany), and EVA-Chrom-TLC-Applicator. The total volume of serum extract was spotted in small portions at one time on the plate. A glass tube connected to vacuum was held beside the spot during the application procedure to remove the solvent rapidly and thereby prevent excessive enlargement of the spot. By this means the spot diameters were always kept to less than 2 mm and each spot was separated by 7 mm. After sample application, the plate was put in a vacuum desiccator for 10 minutes to ensure removal of residual isoamyl alcohol. Following this drying step the plate was allowed to stand in room atmosphere for at least five minutes before placing it in the developing chamber. This step appeared to be necessary to obtain a perfectly straight solvent front, and might be related to the equilibration of the moisture content of the plate. The plate was placed in the twin-trough chamber, one side lined with Whatmann No. 3 chromatographic paper to provide a well saturated atmosphere in the tank, for 5 minutes of solvent pre-loading. The development was started by simply tilting the chamber to allow the solvent to flow over the dividing ridge to reach the plate.

Development for chlorpromazine samples required about 5 minutes for 6 cm solvent front travel. Double development was employed with the tricyclics in which the solvent front traveled 6.5 cm in 7 minutes. The plate was dried for 2 minutes in a stream of cool air, and was re-developed after another 5 minutes solvent vapor pre-loading. After chromatographic development, the plates with the resolved drugs were dried briefly under

vacuum and were then ready for scanning without further processing.

G. Spectrophotometer Condition for Quantitation of HPTLC Spots:

In-situ quantitation of the developed HPTLC plate was accomplished utilizing the Zeiss Model KM-3 chromatogram Spectrophotometer (Carl Zeiss, New York, New York). Measurement was made in the reflectance mode for UV absorption. A deuterium light source was used with the monochromator set at 254 nm for chlorpromazine, 240 nm for amitriptyline and nortriptyline, and 275 nm for imipramine and desipramine. The slit width was 1.0 mm and slit length 3.5 mm. Scanning speed was 30 mm per minute.

The computing integrator was a Spectra-Physics Model, Minigrator which automatically printed out the integrated peak area. Peak area ratios of the drug and its internal standard were calculated on the minigrator and used exclusively for quantitation.

1. Calibration Curves

In order to simulate exact analysis conditions, the calibration data were generated by adding known amounts of the drug to be determined to fresh, pooled, drug-free serum, extracting and chromatographing as described above.

The calculated peak area ratios (drug/internal standard) were plotted against the known concentrations of the drug, added to the serum, to give the calibration curve. With the aid of a programmable calculator, the linear regression analysis was performed on the calibration data. The correlation coefficient and the linear regression equation were

obtained.

The unknown concentration of psychotropic drugs in a patient's serum were predicted using known peak area ratio (drug/internal standard) and finding graphically the corresponding concentration on the calibration curve, or more precisely, calculating with the linear regression equation.

2. Extraction Recovery, Precision and the Effect of the "Carrier"

Six sets of samples were prepared for chlorpromazine and tricyclic antidepressants, respectively. Each set in turn, consisted of 6 samples. The sample conditions were as follows:

Set 1: Serum spiked with 10 ng of drug was extracted as described in sample preparation. No perphenazine was added. The internal standard was added to the extract after the extraction step.

Set 2: As in Set 1, except perphenazine was added to the serum with the drug.

Set 3: Nonextracted samples with 10 ng drug and the same amounts of internal standard and perphenazine as in previous two sets.

Set 4, 5, 6: Serum spiked with 100 ng drug. Same preparation as sets 1, 2 and 3 respectively.

All these samples were carried through the entire procedure as described.

For extraction recovery and the effect of carrier on recovery, the information was revealed by comparing the ratio between Sets 2 and 3,

Sets 1 and 3, for the 10 ng level, and Sets 5 and 6, Sets 4 and 6 for the 100 ng level.

Information on method precision and the effect of carrier on precision, was obtained by comparison of the standard deviation between Sets 1 and 2 for the 10 ng level, Sets 4 and 5 for the 100 ng level.

3. Accuracy

The accuracy of the analytical procedure was tested by a blind study. The serum sample spiked with given amounts of various drugs (unknown to the author) were carried through the entire analysis described above. Each unknown sample had one duplicate.

4. Specificity of the Analytical Procedure

The R_f values of twenty-six commonly used psychotropic drugs, drug metabolites, and other compounds which might be present in patient blood samples and interfere with the described determination, were determined under the same analytical condition in two solvent systems. In each solvent system, the determination was repeated 3 times and the average R_f values were calculated to demonstrate the selectivity of the system.

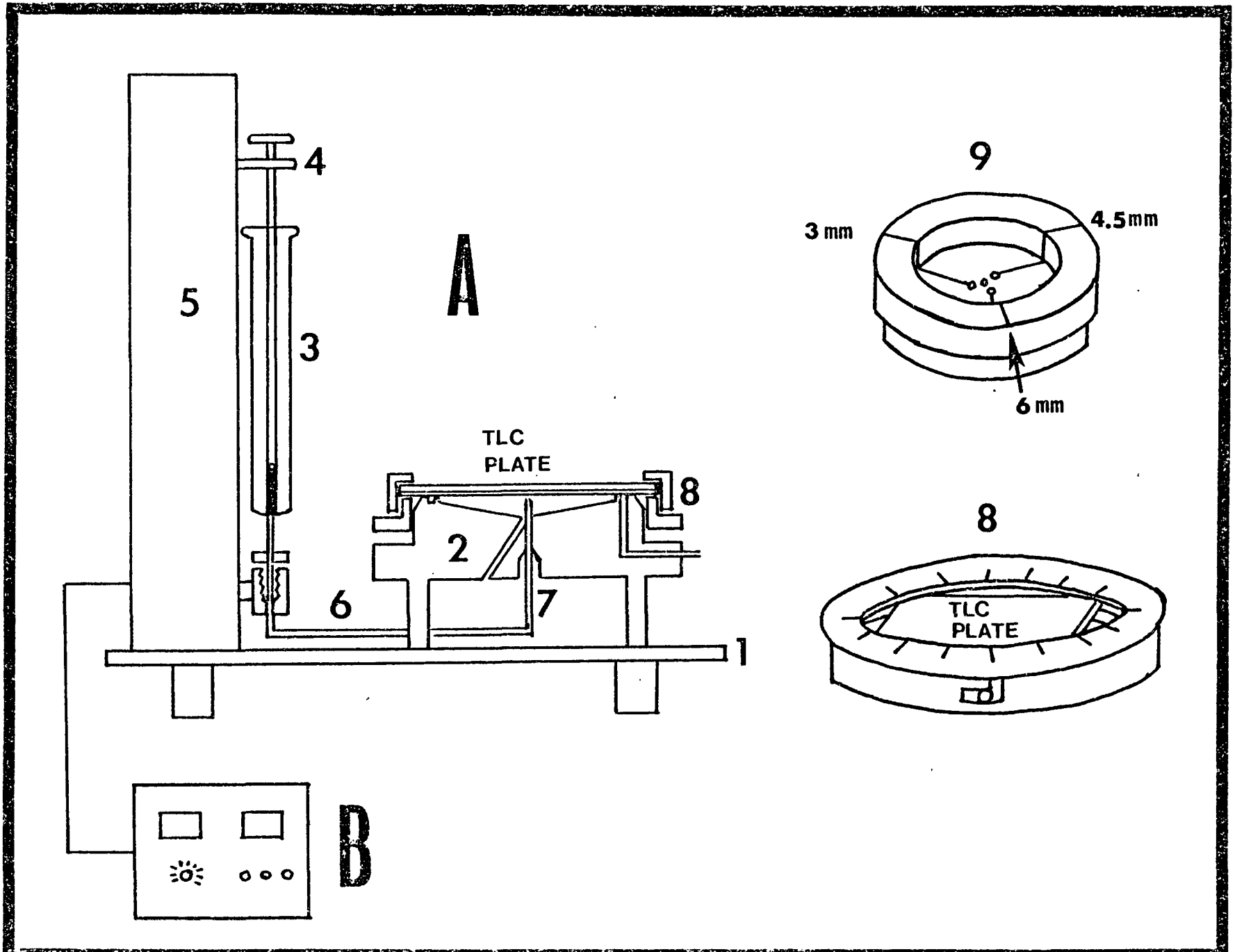
H. The U-Chamber

The entire system consisted of two parts as shown in Figure 2. Part A contained a platform (1) which positioned the chamber body (2), made of brass and a 250 µl Hamilton Syringe (3), clamped on a dosage turret (4), driven by a precision stepping motor (5). The chamber and the syringe were connected via a stainless steel union by a Platinum-Iridium capillary of 0.5 mm O.D. x 0.2 mm I.D. (6). Inside the chamber,

FIGURE 2: THE U-CHAMBER

Part A:
1 Platform
2 Chamber Body
3 250 µl Hamilton Syringe
4 Dosage Turret
5 Stepping Motor
6 Pt-Ir Capillary, 0.5 mm O.D. x 0.2 mm I.D.
7 Pt-Ir Capillary, 0.5 mm O.D. x 0.15 mm I.D.
8 Plate Holder Ring with Bayonet Lock
9 Sample Positioning Block

Part B: Control Unit


A
B
1
2
3
4
5
6
7
8
TLC
PLATE
9
3 mm
4.5mm
6 mm
8
TLC
PLATE

a small capillary 0.5 mm O.D. x 0.15 mm I.D. (7) was used to deliver the solvent to the adsorbent layer. A plate holder ring (8) with a bayonet lock was used to fix the plate, which facilitated handling, and ensured precise and repeatable positioning of the solvent entry point, and sample applications. The ring was graduated from 0 to 360 degrees. A sample positioning block (9), also made of brass, had 3 holes, 3, 4. 5 and 6 mm away from the center hole which were used for sample spotting. Part B was an electronic control device. On the control panel:

1. Total solvent volume desired to deliver to the adsorbent layer can be pre-set with a digital switch.
2. The desired solvent feeding speed (0.4-2 sec/µl) can be selected by a calibrated knob which regulates the stepping motor speed.
3. A digital display shows the volume of the solvent currently delivered. By a re-set button, the display can be set to 0.
4. A start/stop button, activates and deactivates the stepping motor drive.

1. Spotting and Operation:

All the qualitative separations were performed on 5 cm x 5 cm pre-coated HPTLC plates with fluorescence indicator (E. Merck, Darmstadt, Germany). Standard solutions were as follows:

(a) 50 ng/200 nl CPZ with internal standard, Butaperazine and carrier perphenazine.

(b) 50 ng/200 nl tricyclic antidepressants only.

For spotting, the plate was first fixed in the plate holder ring with the bayonet lock. The sample positioning block rested on the ring directly above the plate. The 200 nl Pt-Ir capillary containing the standard solution was simply inserted into the pre-selected hole on the block and the layer was touched lightly to deliver the sample. After each application, the block was turned an appropriate angle for the next spotting. Samples were applied in a circle of either 3, 4.5 or 6 mm diameter. The ring containing the plate was then laid face down on the chamber body. The capillary (0.15 mm I.D. x 0.5 mm O.D.) tip was centered on the sample circle, with a 0.1 mm clearance. The plunger of the 250 µl syringe, filled with the developing solvent, was clamped with the stepping motor. On the control panel, the desired total volume and the solvent feeding speed were selected. After pushing the start button, the development began. The operation was terminated automatically when the displayed volume equaled to the pre-set volume.

2. Operating Condition:

For Chlorpromazine:

Solvent System:	4% diethylamine in hexane
Total volume:	170 µl
Feeding speed:	1.4 sec/µl
Total running time:	4 minutes

For tricyclic antidepressants:

Triple development was necessary for baseline separation

Solvent System:	10% triethylamine in heptane
Total Volume:	170 µl x 3
Feeding speed:	1.2 sec/µl
Total running time:	10 minutes

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

A. Selection of the Mode of Measurement

The measuring method was determined by consideration of the instrument capability, the TLC plate and the nature of the compounds under study. Among the measuring methods in which the Zeiss Spectrophotometer is able to operate, i.e. fluorescence, reflectance, transmission, and simultaneous reflectance and transmission. It is the fluorescence which surmounts the other two methods, to give the highest sensitivity. However, this is restricted by the nature of the sample. According to the structure requirement of phenothiazine for fluorescence,[74] if on the C-2 carbon of the phenothiazine ring, there is no substituent or it is only substituted by chlorine, the compound does not fluoresce. The chlorpromazine has a chlorine atom at C-2 carbon, and does not fluoresce naturally. Indeed, the tricyclic antidepressants studied have not been found to have intrinsic fluorescence. Although derivatization methods [75,76] are available to convert these drugs into fluorescence materials, they are not practical because either the intensity of the individual fluorescence is too weak to meet the sensitivity requirement or the time requirement for carrying out the derivatization reactions is unfavorable. Thus, the choice is limited among reflectance, transmission, and simultaneous reflectance and transmission.

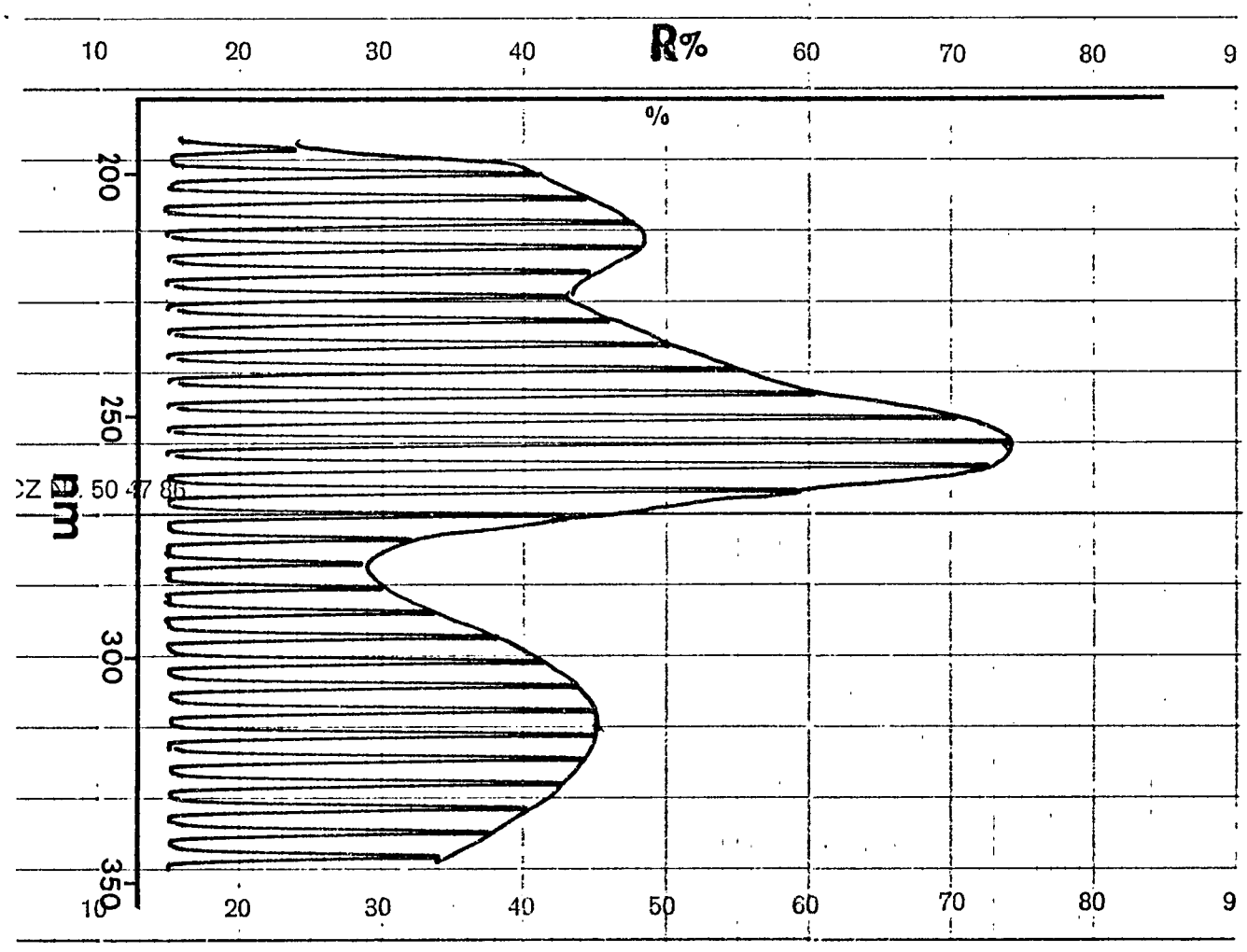
The absorption spectra of the drugs and internal standards, taken on the chromatogram in dry state, with the KM-3 (Figures 3-9) revealed

FIGURE 3: THE ABSORPTION SPECTRUM OF CHLORPROMAZINE

λ_{max} = 315 nm

= 254 nm

= 215 nm


R%
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70
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9
%
200
250
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350
nm
50
10
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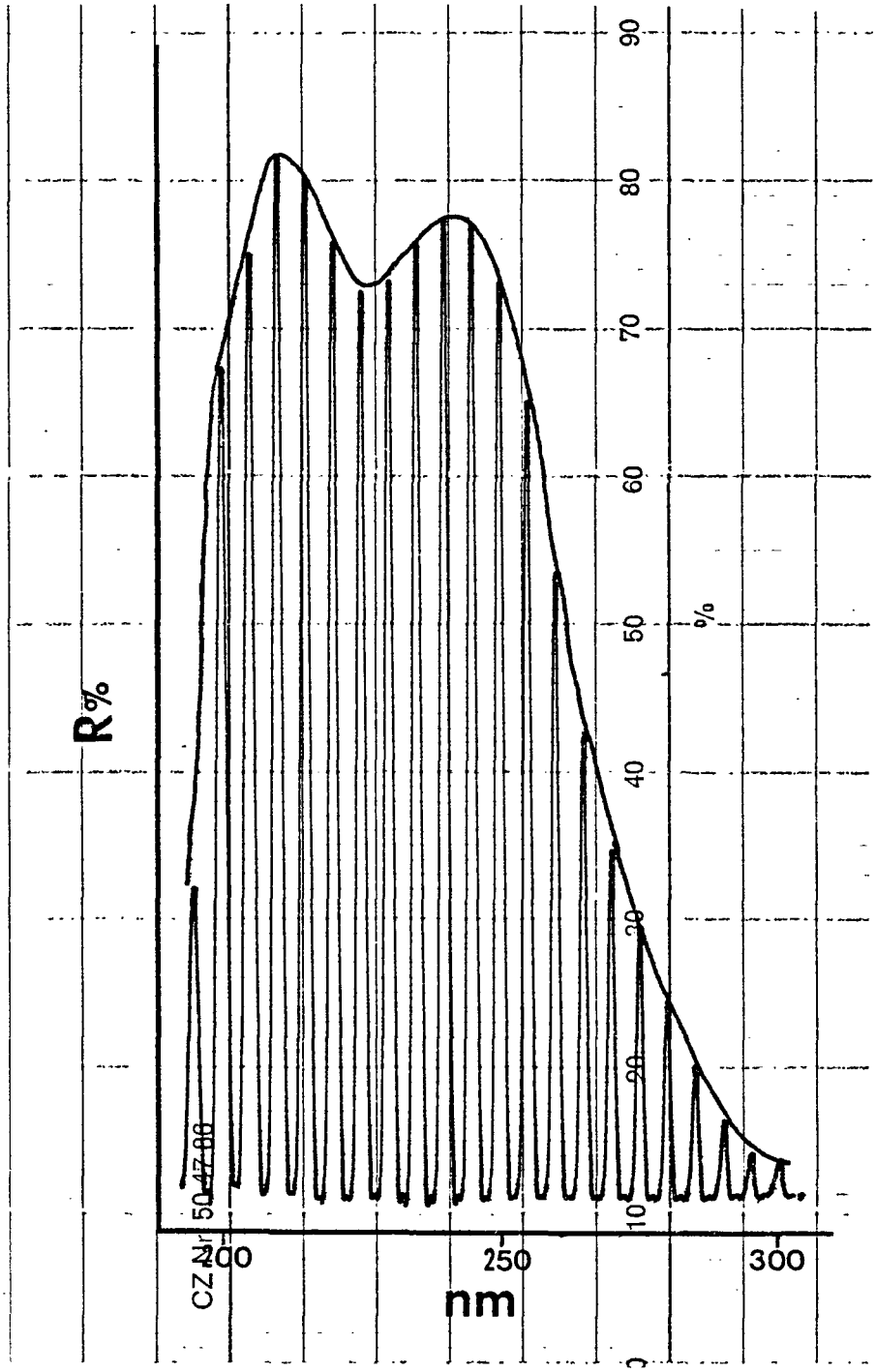
60
70
80
9

FIGURE 4: THE ABSORPTION SPECTRUM OF AMITRIPTYLINE

λ_{max} = 240 nm

= 210 nm


R%
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10
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250
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nm
CZ Nr. 50-47-66

FIGURE 5: THE ABSORPTION SPECTRUM OF NORTRIPTYLINE

λ_{max} = 240 nm

= 210 nm

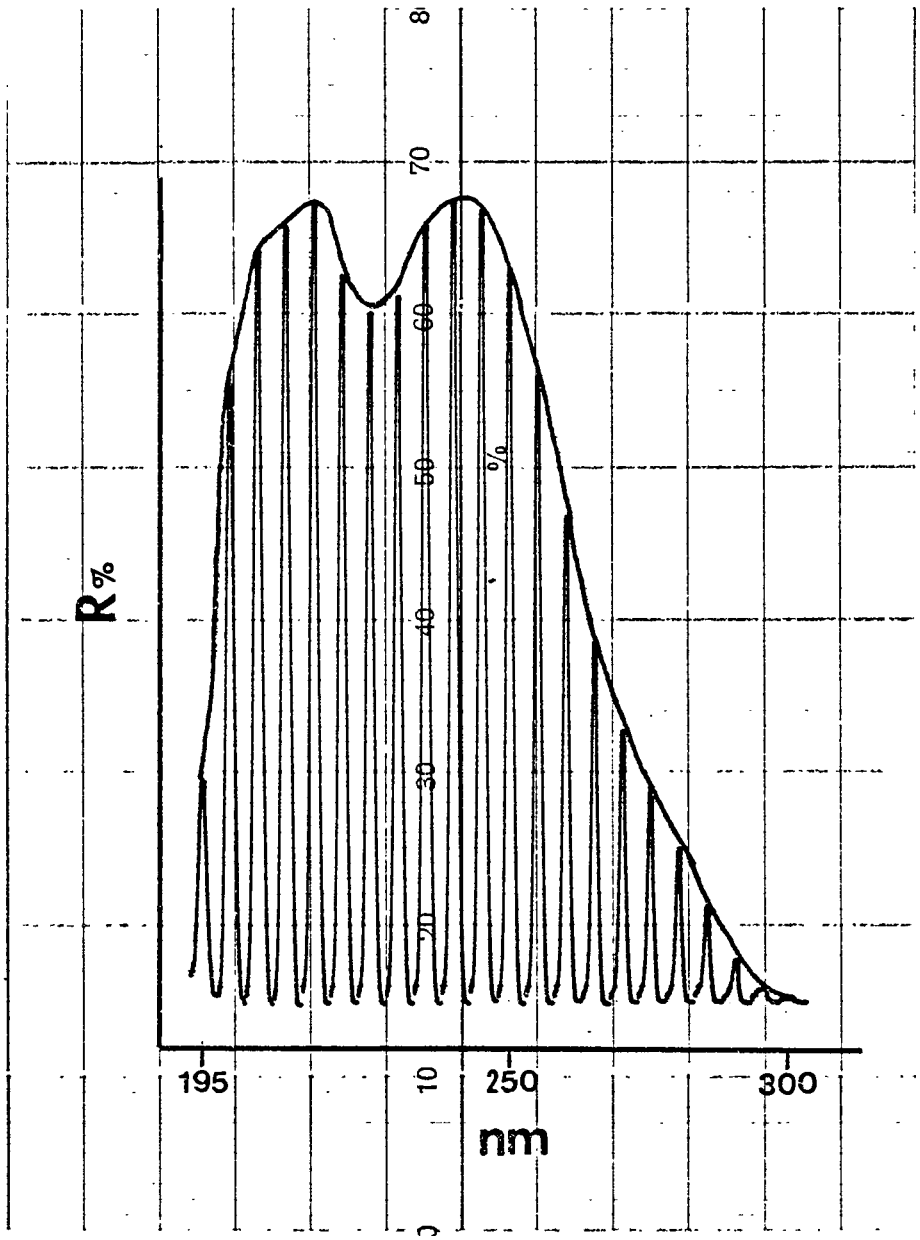

R%
80
70
60
50
40
30
20
10
0
%
195
250
300
nm

FIGURE 6: THE ABSORPTION SPECTRUM OF IMIPERAMINE

λ_{max} = 270 nm

= 215 nm

CZ Nr. 50 47 86

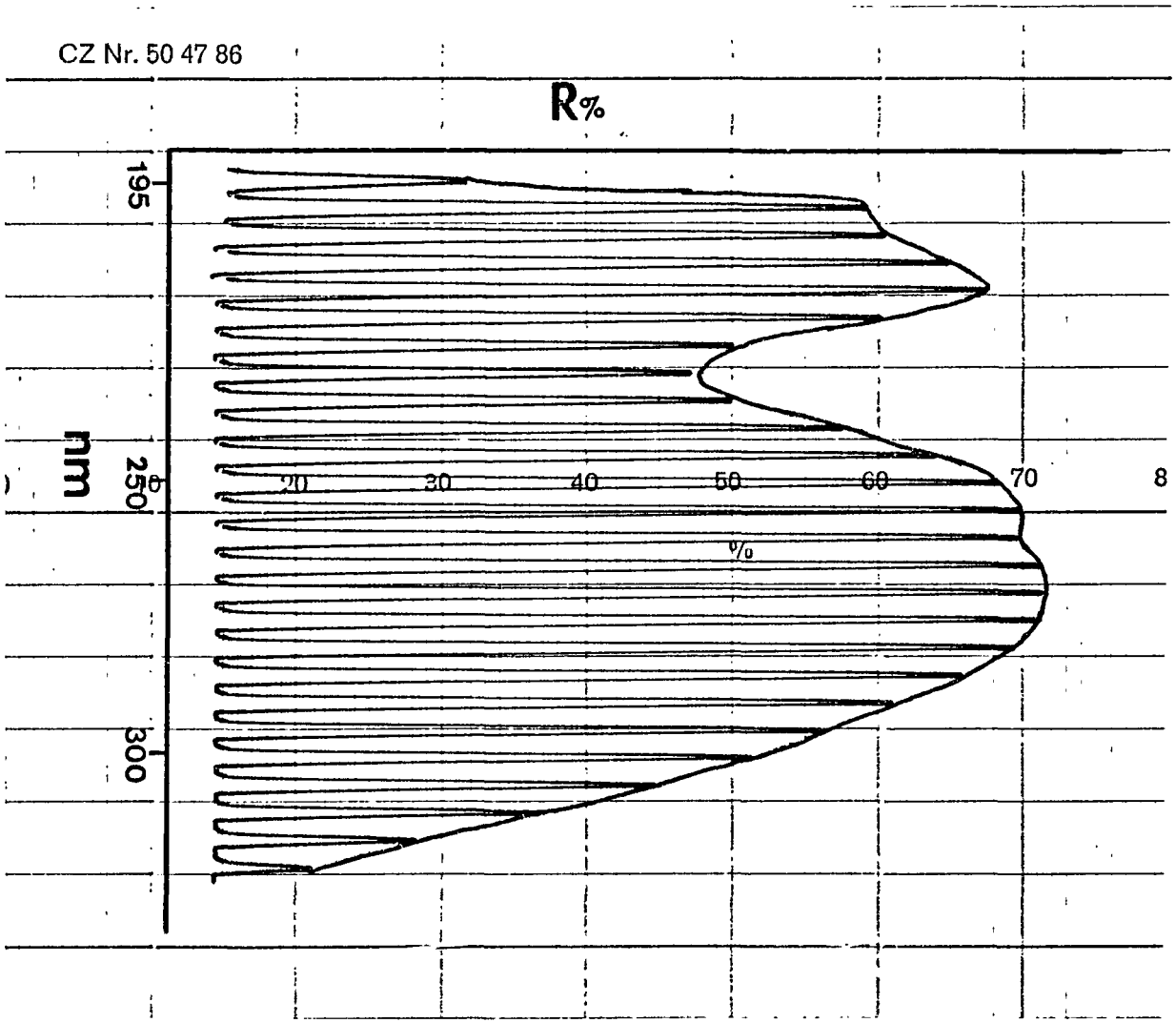


FIGURE 7: THE ABSORPTION SPECTRUM OF DESIPERAMINE

λ_{max} = 270 nm

= 215 nm

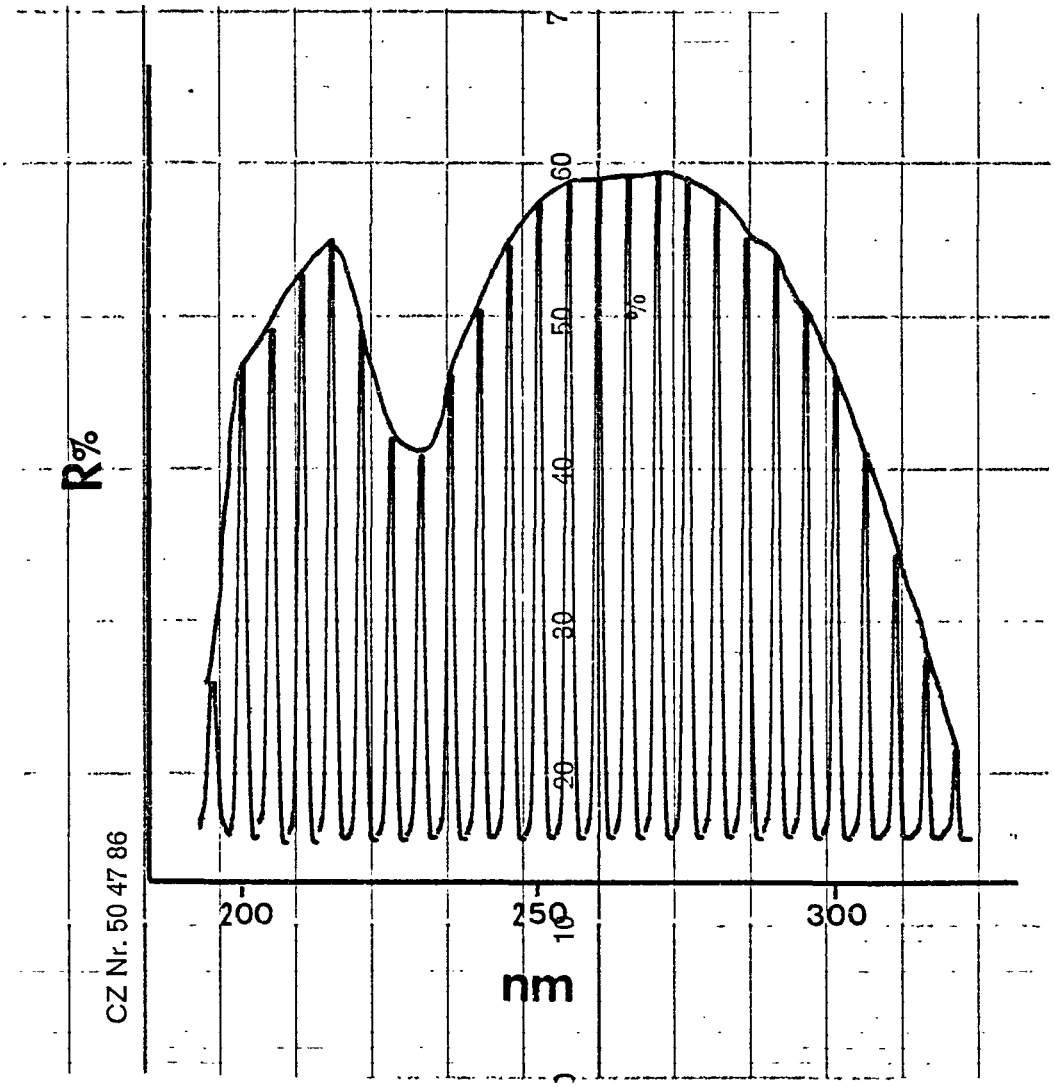
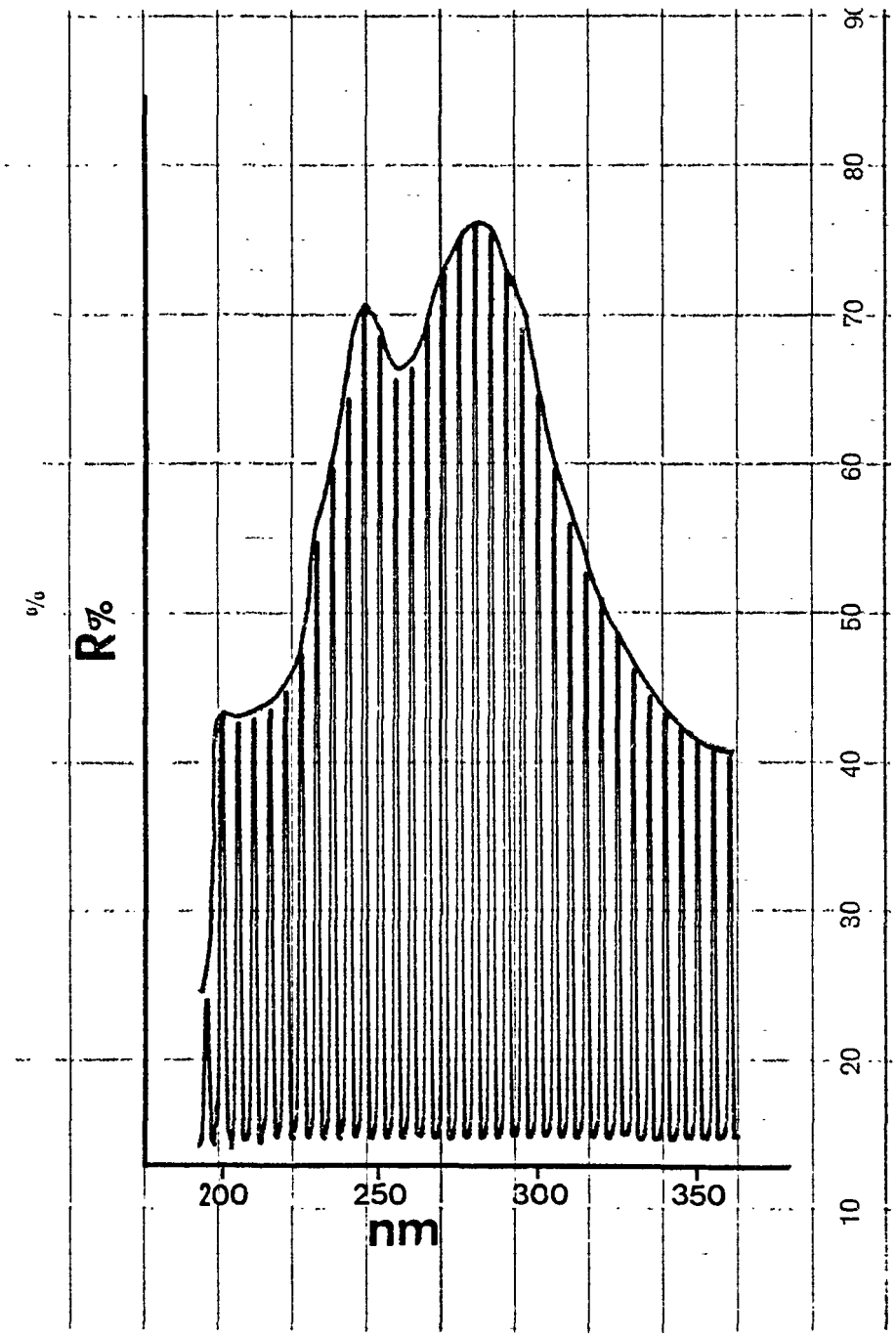

R%
70
60
50
40
30
20
10
200
250
300
nm
CZ Nr. 50 47 86

FIGURE 8: THE ABSORPTION SPECTRUM OF BUTAPERAZINE, INTERNAL STANDARD

λ_{max} = 280 nm

= 245 nm


%
R%
90
80
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60
50
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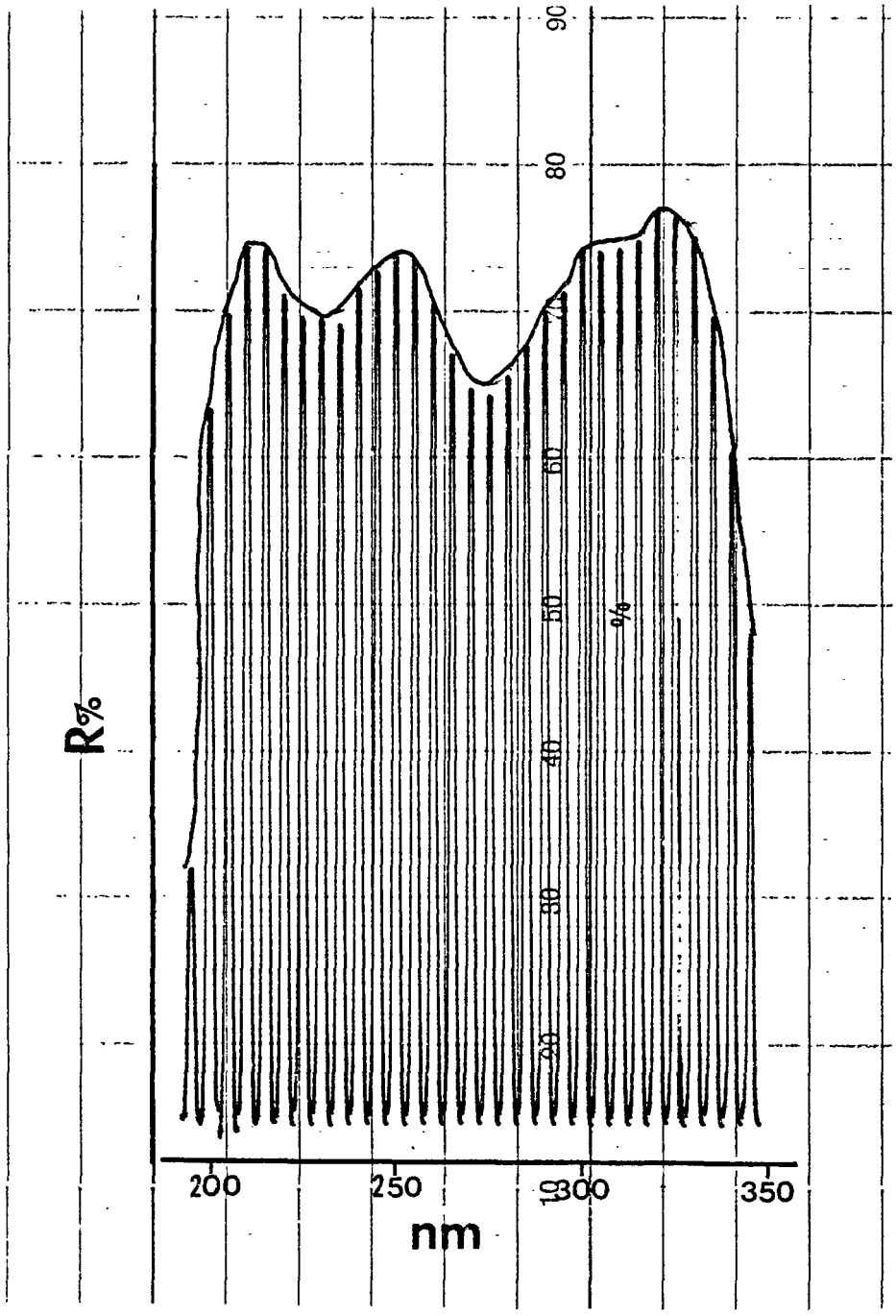
30
20
10
200
250
300
350
nm

FIGURE 9: THE ABSORPTION SPECTRUM OF LOXAPINE, INTERNAL STANDARD

λ_{max} = 320 nm

= 250 nm

= 210 nm


R%
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350
nm

their strong absorption in the ultraviolet region. In this region of the spectrum, owing to the strong absorption of the carrier materials, glass and silica gel, the transmission mode cannot be applied. In fact, transmission measurements below 325 nm wavelength is not possible unless fluorescence quenching is used.[26] The next thought was that by applying specific reactions, the spots representing the drugs on the plate could be rendered colored and absorption maxima would thus be shifted to the visible range, so that a comparison between the measuring methods could be made. Several staining reactions were tried with the following reagents:

1. Ethanol/H_2O/Conc. H_2SO_4 (80/10/10)
2. Ether/Conc. H_2SO_4 (95/5)
3. $FeCl_3$/ethanol/Conc. H_2SO_4 (0.5g/150 ml/50 ml)
4. Iodoplatinate solution
5. Dragendorff's solution

Both dipping and spraying were used to apply the reagents uniformly onto the plate. The last three reagents always gave the plate a brownish background of which the intensity changed with time. This factor affected the stability of the measurement of the samples, so they were aborted. The remaining two reagents gave a stable white background and red colored spots for the drugs. They all had absorption maxima at 550 nm as measured on the plate after derivatization. Transmission measurements at this wavelength did provide larger peak heights compared

with that from reflectance measurements at the same wavelength, but the background noise contribution from the former was also much higher. This background fluctuation came from the variation of the grain size, and the layer thickness. By consideration of the signal-to-noise ratio, instead of only the signal height, transmission measurements only gave insignificant sensitivity enhancement. Although the simultaneous reflectance and transmission mode was applied here by adjusting to 20% reflectance and 80% transmission to smooth the background fluctuation, its signal-to-noise ratio was still inferior to that of UV reflectance measurements. All this indicated that for the present study, reflectance measurement in UV was the most efficient method.

B. Evaluation of the System (System Performance)

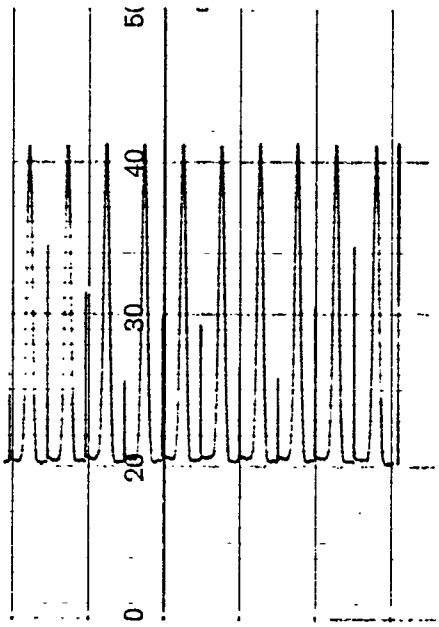
1. Photometric reproducibility:

Figure 10 demonstrates the repeated scanning of the same spot of 50 ng chlorpromazine 10 times without adjustment of the chromatogram track with the print-out of the peak area integration from the computing integrator. The calculated standard deviation and coefficient of variation are 4.8 and 0.60% respectively.

2. Instrument reproducibility:

The same spot was scanned 10 times, but between scanning, the spot was moved out of the measuring area and then moved back with the precision drive on the scanning stage with the aid of the full deflection of the recorder. The entire adjustment which was very important for reproducibility, represented the mechanical characteristics

FIGURE 10: PHOTOMETRIC REPRODUCIBILITY



# 1	ID	
	2 PW	
	500 SS	
	1 BL	
	60 TP	
	5000 MA	
	1 PL	
	1 SP	
TIME	AREA	
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783	80776	808.0
796	80127	801.0
810	80368	804.0
824	80589	806.0
839	80105	801.0
853	79914	799.0
866	79698	797.0
879	80822	808.0
893	81313	813.0

Mean 804.0

std. dev. 4.8

Relative
std. dev. 0.60%

of the scanning stage and the photometer. The result is shown in Figure 11 with the peak area printed out from the Minigrator. The calculated standard deviation and coefficient of variation are 7.2 and 0.91% respectively. The difference of the results shown in Fig. 4 and 5 represents the skill of the operator in precisely locating the sample spot under the measuring slit.

3. Spotting reproducibility:

Figure 12 shows the recorder tracings and the print-out of the integrated peak areas. From the calculated results, it can be concluded that by aspirating the capillary with solvents between spotting, there is actually no sample carry-over and a high spotting precision from plate to plate is achieved with ease and speed. The sample application for one plate with 8 samples is about 2 minutes.

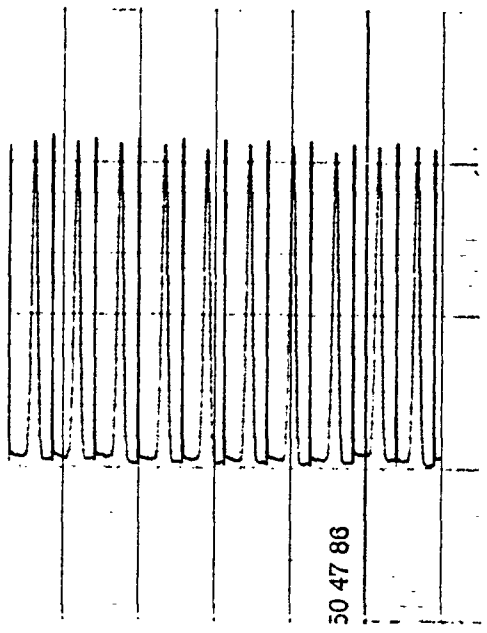
4. Integrator reproducibility:

The integrator performance can be seen from Figure 10 and 11. The repeated actual counts for the same spot is not over 5%.

5. The method reproducibility:

The numerical data obtained from the 8 calibration curves, constructed once a week over a two-month period are shown in Table II. Figure 13 shows all the curves plotted as peak area ratio (drug/internal standard) vs. concentration. By linear regression analysis, the correlation coefficients and slopes were calculated for each curve. The results indicate excellent linearity and reproducibility. The linearity represents the quality of this analytical method, while

FIGURE 11: INSTRUMENT REPRODUCIBILITY



# 2	ID	
	2 PW	808.0
	500 SS	799.0
	1 BL	796.0
	60 TP	796.0
	5000 MA	802.0
	1 PL	
	1 SP	
TIME	AREA	
955	80751	791.0
991	79897	813.0
1023	79567	807.0
1050	79639	811.0
1076	80242	807.0
1100	79061	
1130	81278	Mean 803.0
1155	80707	std. dev 7.3
1179	81052	Relative
1209	80689	std. dev. 0.91%

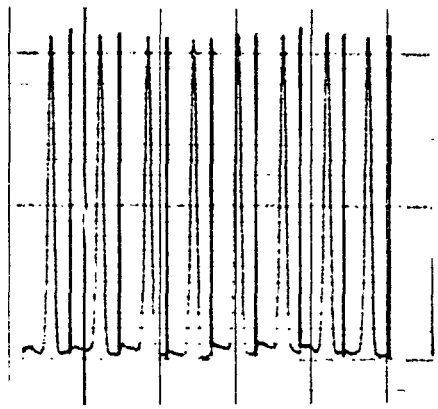
FIGURE 12: SPOTTING REPRODUCIBILITY

824.0
832.0
819.0
810.0
808.0
821.0
830.0
819.0

Mean 820.4

std. dev. 8.5

Relative
std. dev. 1.0%



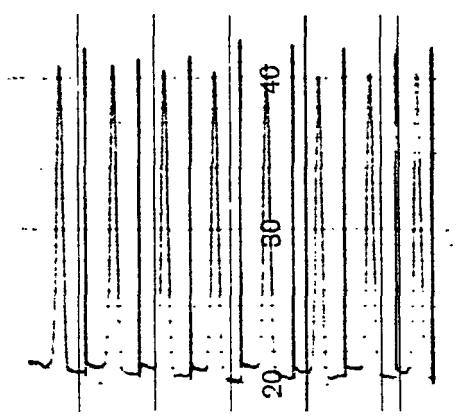
475	82364
501	83184
527	81920
559	81031
589	80752
617	82062
647	82970
680	81942

827.0
801.0
806.0
834.0
819.0
826.0
831.0
819.0

Mean 820.4

std. dev 11.7

Relative
std. dev. 1.4%



1

ID	
2	PW
500	SS
1	BL
80	TP
5000	MA
1	PL
1	SP

TIME	AREA
108	82706
141	~~77066~~
171	80089
205	80579
246	83358
279	81880
312	82617
344	83128
375	81860

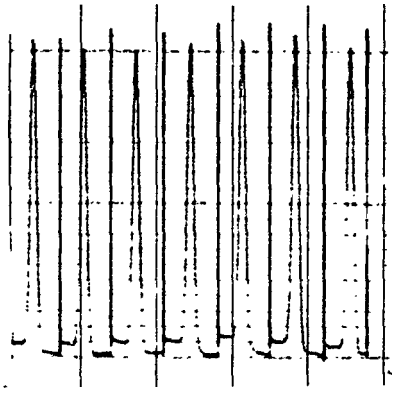
FIGURE 12 (cont): SPOTTING REPRODUCIBILITY

811.0
818.0
806.0
802.0
818.0
828.0
819.0
813.0

Mean 814.4

std. dev. 8.2

Relative
std. dev. 1.0%



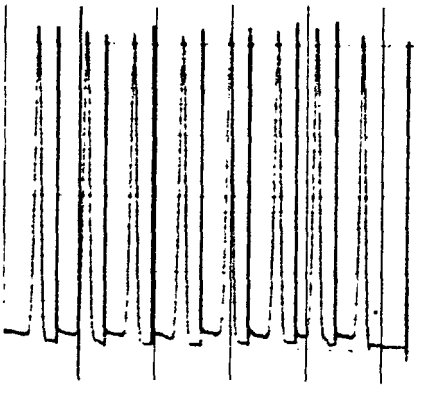
5067	81091
5098	81793
5127	80563
5157	80198
5188	81788
5217	82784
5242	81688
5268	81329

799.0
840.0
824.0
819.0
825.0
820.0
819.0
824.0

Mean 821.3

std. dev. 11.3

Relative
std. dev. 1.4%



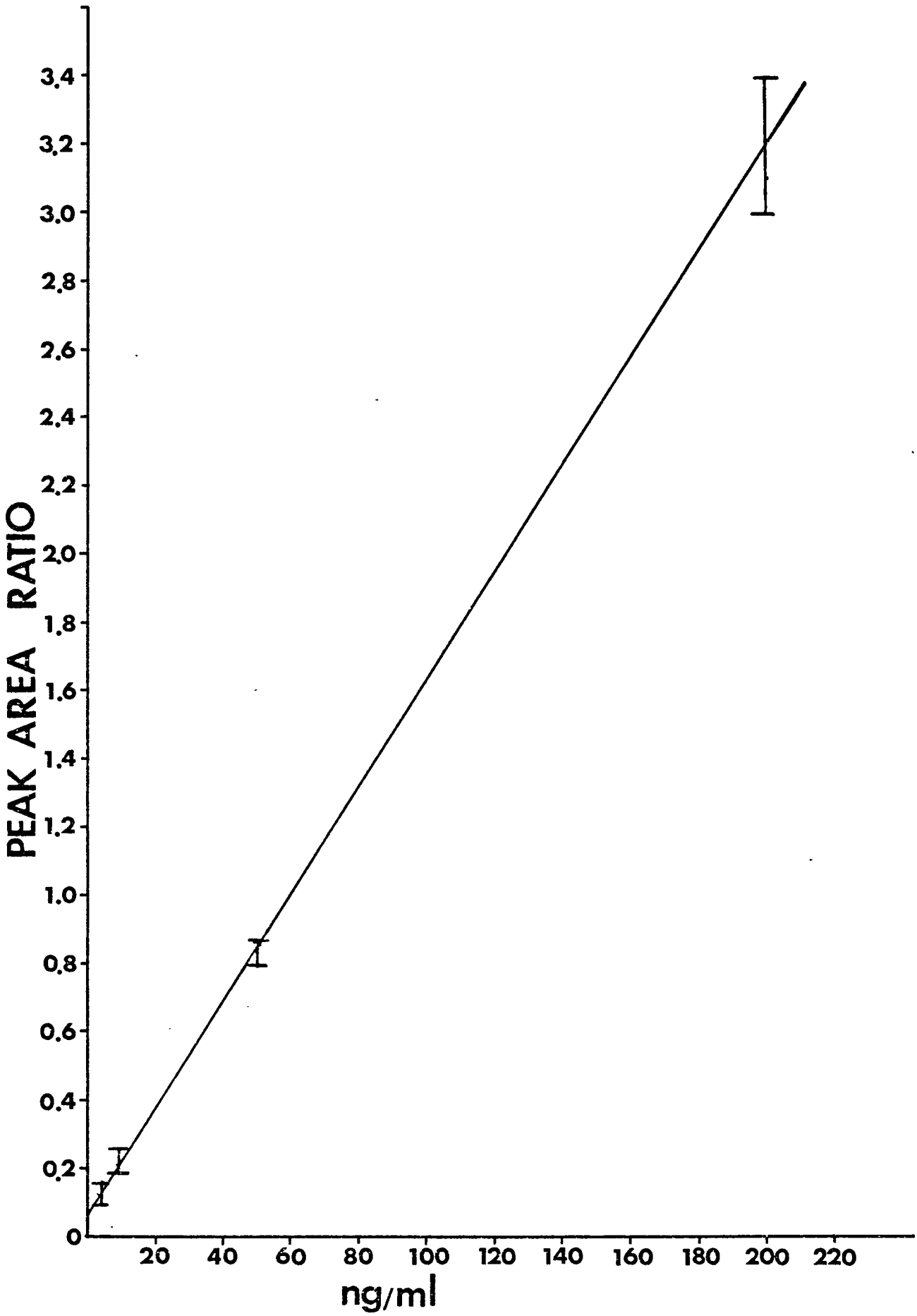
3303	79903
3332	83983
3359	82390
3402	81846
3428	82449
3460	82022
3491	81851
3522	82442

TABLE II: THE PEAK AREA RATIO AND REGRESSION DATA OF EIGHT CALIBRATION CURVES OF TWO MONTH PERIOD

Concentration ng/µl	Peak Area Ratio (CPZ/BPZ)							
200	3.10	3.21	3.00	3.40	3.25	3.20	3.31	3.26
50	0.85	0.80	0.81	0.86	0.83	0.82	0.80	0.82
10	0.23	0.21	0.19	0.25	0.24	0.20	0.25	0.23
5	0.10	0.12	0.10	0.14	0.11	0.13	0.10	0.15
Correlation Coefficient (r)	0.9998	0.9999	0.9999	0.9998	0.9999	0.9999	0.9996	0.9999
Slope (m)	0.0152	0.0158	0.0148	0.0167	0.0160	0.0159	0.0163	0.0160
Intercept (b)	0.0611	0.0364	0.0438	0.0575	0.0479	0.0403	0.0327	0.0564

	Mean	Std. dev.	Relative Std. dev.
r	0.9998	0.0001	0.011%
m	0.0159	0.0006	3.80%
b	0.0470	0.0105	22.30%

FIGURE 13: THE METHOD REPRODUCIBILITY: EIGHT CALIBRATION CURVES COVERED TWO MONTH PERIOD


PEAK AREA RATIO
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
20
40
60
80
100
120
140
160
180
200
220
ng/ml

reproducibility represents the stability of the instrument, e.g., the radiation source, photomultiplier, electronics, dosing accuracy, the quality of the plate (layer thickness, grain size), and the stability of all other chromatographic parameters such as the solvent systems, chamber saturation, temperature, etc..

C. Sample Preparation

Psychotropic drug determinations were achieved on blood serum. Although for many drugs it is possible to determine their levels by direct spotting of the serum on the TLC plate, these are limited to those with high concentration in the blood. However, for most of the drugs, in practice it is necessary to carry out an extraction procedure so that the drugs are concentrated and the effects of the interference materials are minimized.

Since psychotropic drugs have such a narrow therapeutic level in blood, accurate quantitation by TLC depends on a specific and efficient sample preparation procedure. The most important factors in the extraction procedure described, which are responsible for the extraction selectivity are first an acid-base clean up (back-extraction) which transfers the basic drugs from the organic phase into the aqueous layer and then back to the organic layer, by adjusting the pH of the aqueous solution in contact with the organic phase. The second factor is in the use of the least polar extraction solvent, which will give a reasonable recovery of the parent drug, from aqueous solution, without extracting any of the metabolites and/or extraneous materials. The extraction solvent used, 1.5% isoamyl alcohol in heptane was determined

by an empirical approach, because the nature of the impurities, except the metabolites is not completely known. Figure 14 shows the UV absorption trace of a blank serum sample with chromatograms of various concentrations of chlorpromazine recovered from 1 ml serum samples. Little or no interference is present at the position of the chlorpromazine and the internal standard. Interference from the metabolites of chlorpromazine is a possibility which must be considered in samples obtained from patients receiving the drug. Figure 15 shows a chromatogram of a blood sample obtained from a patient receiving chlorpromazine. The chlorpromazine is well isolated, and the chromatogram is very similar to those in Figure 14 from serum spiked with it. The major metabolites of chlorpromazine are its sulfoxide, hydroxylated and demethylated analogs. These substances are more polar than their parent drug. Therefore, they have lower distribution coefficients between the extraction solvent and the aqueous layer. By using this solubility difference and choosing the least polar extracting solvent, their separation was enhanced. The same extraction selectivity obtained for the tricyclic antidepressant is shown in Figure 16.

The extraction efficiency depends mainly on the strength (polarity) of the extraction solvent. Unfortunately, this is limited by the concern for extraction selectivity. Nothing much could be done in this respect. Other methods include the reduction of the transferring steps, the completeness of the transfer, and the minimization of adsorptive losses due to glassware. In the current extraction procedure, the number of transferring steps was kept at a minimum. The completness of

FIGURE 14: CHROMATOGRAMS OF EXTRACTED SERUM SAMPLES SPIKED WITH CHLORPROMAZINES

A: 100 ng/ml

B: 25 ng/ml

C: Serum blank

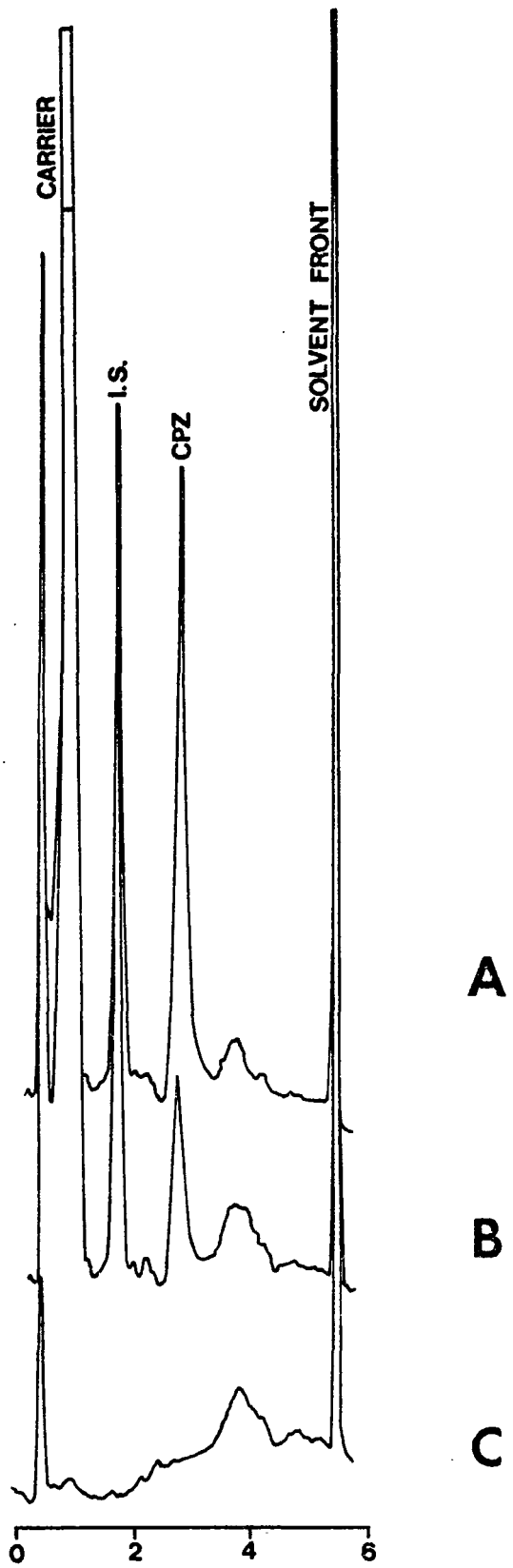

CARRIER
I.S.
CPZ
SOLVENT FRONT
A
B
C
0
2
4
6

FIGURE 15: CHROMATOGRAM OF A PATIENT SAMPLE TREATED WITH CHLORPROMAZINE

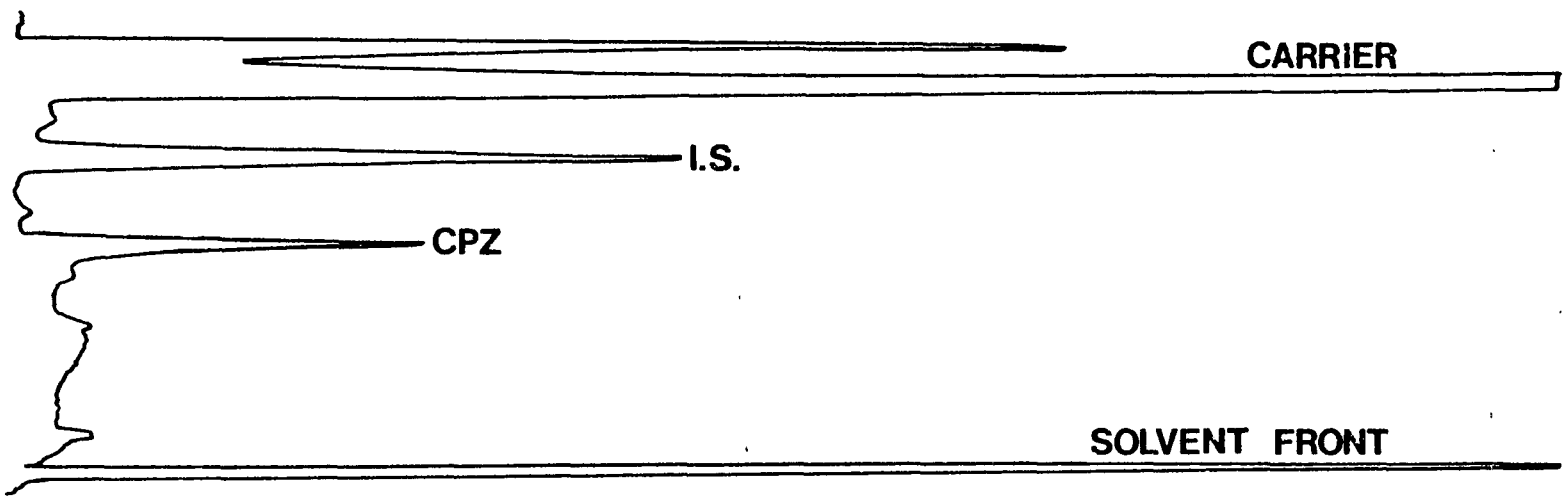
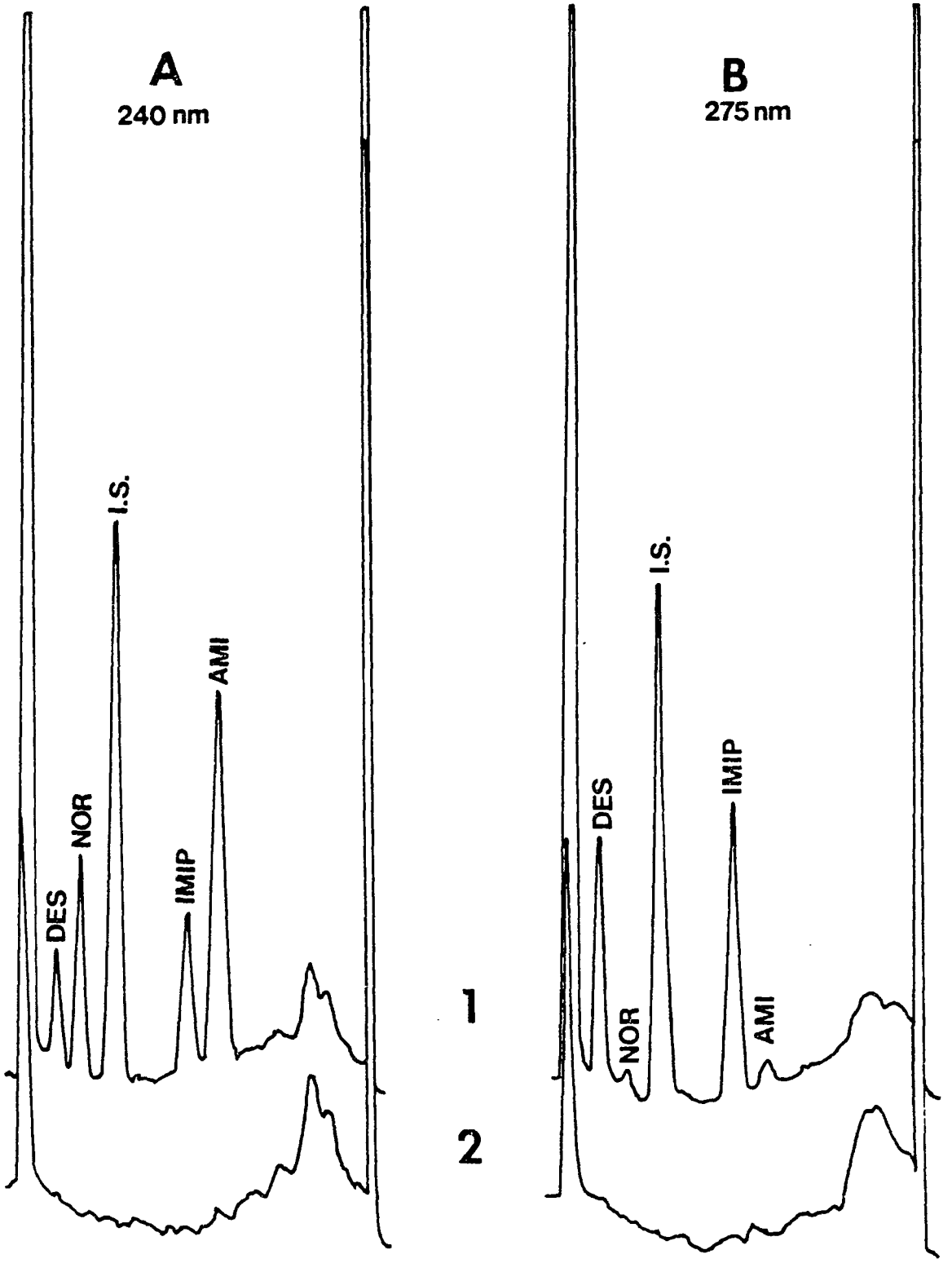

CARRIER
I.S.
CPZ
SOLVENT FRONT

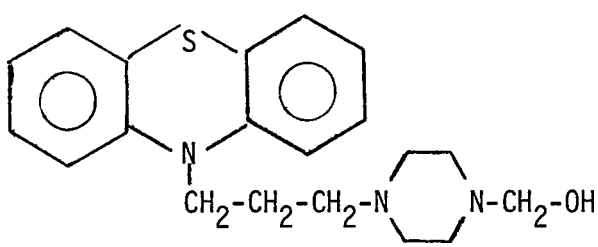
FIGURE 16: CHROMATOGRAMS OF EXTRACTED SERUM SAMPLES SPIKED WITH TRICYCLIC ANTIDEPRESSANTS

A: At 240 nm
1: 50 ng each
2: Serum blank

B: At 275 nm
1: 50 ng each
2: Serum blank


A
240 nm
DES
NOR
I.S.
IMIP
AMI
B
275 nm
DES
NOR
I.S.
IMIP
AMI
1
2

transfer was achieved by freezing the bottom aqueous layer in a dry ice-acetone bath and the top organic layer was easily and completely decanted into another extraction tube. This action not only increased the recovery, hence the sensitivity, but also saved a considerable amount of analysis time, compared with using a pipet for transferring the solvent in small portions. In order to prevent disturbing the interlayer, some organic phase was always left. Possible contamination and adsorptive losses with pipets were also a threat. Irreversible adsorption of sample materials on the glassware is a well-known problem for laboratories involved in the determination of microgram and submicrogram amounts of drugs present in biological fluids.[71] This loss can be a major contributing factor limiting the sensitivity of certain assay procedures. All glassware used in this work were silanized regularly by a vapor phase method[73] to ensure a well deactivaed surface. Besides silylation, a relatively large amount of a "carrier" compound, perphenazine was added to the serum samples.



"Carrier" Perphenazine

This compound is more polar than chlorpromazine, the four tricyclic antidepressants, and the respective internal standards. When the

perphenazine is present in large amounts, e.g. 10 to 20 times higher in concentration than the drugs and their internal standards, the remaining active sites, if any, on the glassware surface after vapor phase silylation, would show preferential interaction-irreversible adsorption with perphenazine and displace the molecules of interest from the unfavorable sites. The recovery would be improved by this two fold protection, silylation and carrier, and the loss due to irreversible adsorption can be minimized. The perphenazine was selected for this purpose, because of its polar nature and its low R_f values in the described solvent systems (Table VII). It therefore offered no interference to the compounds being measured, as can be seen from Figures 14, 15 and 16. The difference in total recovery of the various drugs from serum samples in the presence and absence of carrier is shown in Table III. The effect is to be expected, but even at 100 ng/ml concentration there is a marked difference in recovery. The calculated results also indicated the improvement of the extraction reproducibility in the presence of the carrier. In a previous study of a gas chromatography and electron capture detector procedure for the determination of chlorpromazine in blood serum,[77] the same effect of carrier was also observed.

In the quantitative determination of drugs at the subnanogram level, any contamination will destroy the desired accuracy and precision. All organic solvents and water used to make up the aqueous acid and base solvents were carefully purified. However in the initial development of the assay procedure, heavy contamination was observed

TABLE III: EFFECT OF CARRIER (PERPHENAZINE) ON RECOVERY OF PSYCHOTROPIC DRUGS FROM SERUM

	Chlorpromazine		Nortriptyline		Amitriptyline		Desipramine		Imipramine	
10 ng/ml	with	without	with	without	with	without	with	without	with	without
MEAN[1]	80.3	54.5	83.5	61.9	95.3	71.5	81.9	71.6	86.2	77.4
S.D.[2]	2.4	21.0	3.1	18.1	16.4	10.2	13.7	23.8	15.2	10.4
C.V.[3]	3.0	38.6	3.7	29.2	17.3	14.3	16.9	33.2	17.7	13.4
100 ng/ml										
MEAN	95.7	87.0	77.5	72.8	99.6	86.7	69.7	61.9	91.1	85.7
S.D.	3.2	3.4	10.2	23.1	2.3	6.4	7.2	7.1	5.2	7.3
C.V.	3.4	3.9	13.2	31.8	2.3	7.3	10.3	11.4	5.8	8.5

[1]Mean of six determinations expressed as percent of recovery.

[2]Standard deviation.

[3]Coefficient of variation.

on the TLC plate after development. Unknown fluorescence materials streaked from the origin to the solvent front and seriously affected the accurate determination. The effort in using the specific staining reactions mentioned earlier to selectively convert the drugs to colored spots has failed because the contaminants also gave the same colored reactions. Finally, after collecting the blood with a glass syringe instead of "vacutainer" tubes and using clean aluminum foil to cover the teflon liners in the screw caps of the extraction tube, the contamination disappeared.

In the previous GC-ECD study,[77] the same precaution was also taken to avoid the ghost peaks.

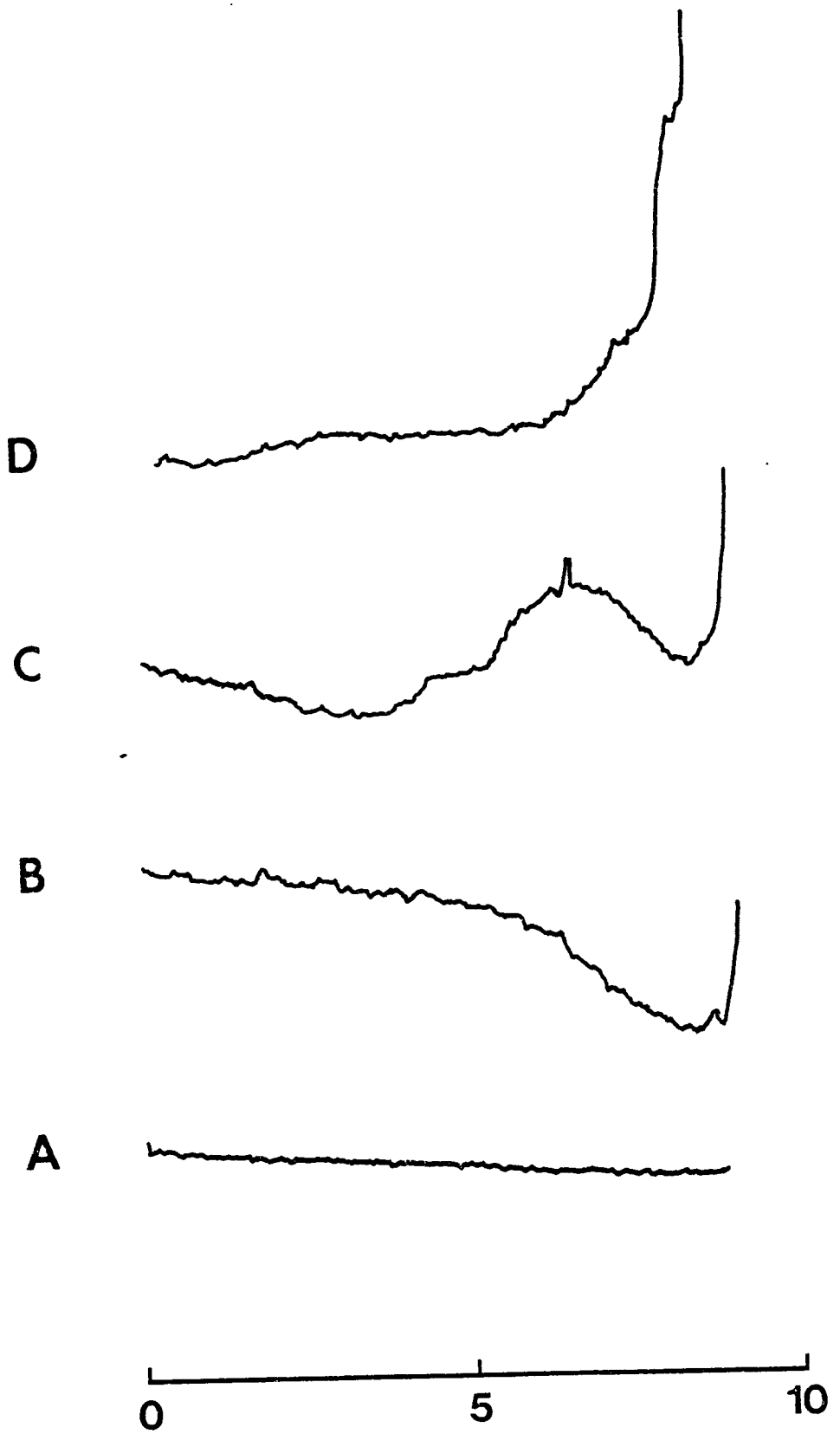
D. The Chromatographic System

The purity of the HPTLC plates is insufficient for quantitation at the nanogram range. This was also observed by other workers.[78] A large amount of UV absorbing material can be eluted to the solvent front as shown in Figure 17. After the first development with hexane/acetone/diethylamine (80/20/3), the impurities were moved to the center of the plate, while one more pre-development, moved them to the solvent front, and left a fairly good background. Plates with and without fluorescence indicator gave the same background measured in UV at 254 nm after pre-washing steps. However, plates with indicator, after being in contact with the solvent system, always show some yellow color at the edges. For this reason, plates without a fluorescence indicator were exclusively used in this work.

Three factors are responsible for the high performance, i.e. the

FIGURE 17: BACKGROUNDS OF HPTLC PLATE

A: Instrument Noise
B: Before pre-washing
C: After first pre-development
D: After second pre-development with Hexane/Acetone/Diethylamine (80/20/3)


D
C
B
A
0
5
10

plate, nanogram range concentration, and the dosage technique. The characteristics of the high performance plate are the uniform and narrow particle size, constant layer thickness and high layer density. These parameters are empirically optimized so that the layer exhibits high efficiency in a manner analogous to a perfectly coated capillary column with several hundred thousand theoretical plates. Although the solvent velocity coefficients of the new layer are about one third less than those on the conventional TLC layer,[29] the total analysis time is still shorter because the desired separation can be obtained on a short solvent running distance. The short separation time in turn reduces the opportunity for longitudinal and lateral diffusion and small, compact spots (not over 5 mm) are produced. As seen in Figures 14, 15 and 16, only about 6 cm distance is necessary for complete baseline separation for chlorpromazine, tricyclic antidepressants and their internal standards and carriers. The time needed is about 5 to 6 minutes. The base width represents the spot diameter.

The sensitivity (signal to noise ratio) improvement on the new plates is two fold:

(1) The small particle size (7 μm) results in stronger light reflectance from the layer, i.e., more diffusely reflected light is detected by the photomultiplier, thus enhancing the signal while the uniform and constant particle size and layer thickness maintain a low contribution to the background noise.

(2) Due to the small, concentrated spots, a small measuring area,

only slightly larger than the largest spot diameter on the plate, is needed (slit width 1 mm, length 3.5 mm). This reduces the background contribution which is already kept at a low value, and increases the signal strength. The sensitivity of this assay procedure is 10 ng/ml with good precision. As little as 5 ng/ml is still able to be detected but with unacceptable precision.

These micro high performance plates, considered as the reduced scale of the conventional macro-TLC, have a low sample capacity. They are suited for analysis in the nanogram range.[26] Overloading the adsorbent layer will completely ruin the high performance. These new plates are therefore primarily useful for trace analysis.

Sample application has been a major source of error in quantitative TLC. The delivery of an accurate amount of sample to the layer and the size of the spots should be kept as uniform and small as possible. This depends largely on the spotting apparatus and the skill of the operator.[79,80] In HPTLC, with the described Platinum-Iridium capillary and the rocker-arm applicator, a high dosage quality, i.e. spot size and application precision, is easily obtained. This is a prerequisite for the high performance.[81] Maintaining a good dosage quality is just as important as the sample introduction system to high resolution capillary columns. However in this analytical procedure, it is difficult to keep the solution volume in the nanoliter range which is compatible with HPTLC.[82] Instead, fifteen microliter 1.5% isoamyl alcohol in heptane was needed to reconstitute the extract. With the device described in spotting and a 1 µl Pt-Ir capillary, final spots with about 2 mm diameter

were still able to be obtained. However, care must be exercised to prevent rupture of the adsorbent surface to a minimum. Excessive rupture of the surface will cause dosage error and reduce the separation power.[82]

The solvent selection was based on the elutropic series of Halpaap.[37] Besides the polarity, other factors such as the velocity coefficient, boiling point, viscosity, surface tension and miscibility were also taken into consideration. The final solvent systems, hexane/acetone/diethylamine (80/20/3), hexane/benzene/diethylamine (96/12/3) were optimum with respect to these parameters. With the well saturated environment, fast linear speed and short running distance, the solvent demixing was believed to be at a minimum. Since the drugs determined are basic in nature, a base is needed in the solvent systems to eliminate the spot tailing. Before choosing diethylamine, several other bases were tried, e.g., pyridine, pyrrolidine, triethylamine and ammonium hydroxide. Especially worth mentioning is ammonium hydroxide. Plates after being in contact with the NH_4OH vapor always show severe baseline shift which completely prohibits the quantitation of nanogram amounts of drugs. The reason is that the ferric ion (Fe^{+3}) present as impurity in the silica gel 60 is moved by the solvent and detected.[83] Therefore, it is suggested that with HPTLC, ammonium hydroxide be employed only when no other alternative is available.

The type of chamber selected will influence the chromatographic characteristics.[37] Twin-trough chambers contribute to the reproducibility of the overall system through the following:

1. Due to the small chamber volume, a well saturated environment

is established in a short time.

2. The chamber with the divided trough allows pre-conditioning of the adsorbent with solvent vapor.

These two factors reduce the edge effect, and maintain a very straight solvent front.

3. Due to the small volume of the trough, it allows the use of fresh solvent for every development so that solvent composition is always constant.

This chamber provides for a well-defined stationary, mobile and vapor phase.

The reproducibility of the whole system can be judged by that of the R_f values of the chlorpromazine, measured over the entire period of the development, shown in Figure 18.

E. Photometric Quantitation in Diffuse Reflectance Spectroscopy

In reflectance measurement of TLC spots, the Kubelka-Munk equation[27,28,84,85] can best describe the relationship between the degree of reflection and concentration.

$$F(R_\infty) = \frac{(1-R)^2}{2R} = \frac{EC}{S} = \frac{k}{S}$$

R = relative diffuse reflectance

E = extinction coefficient

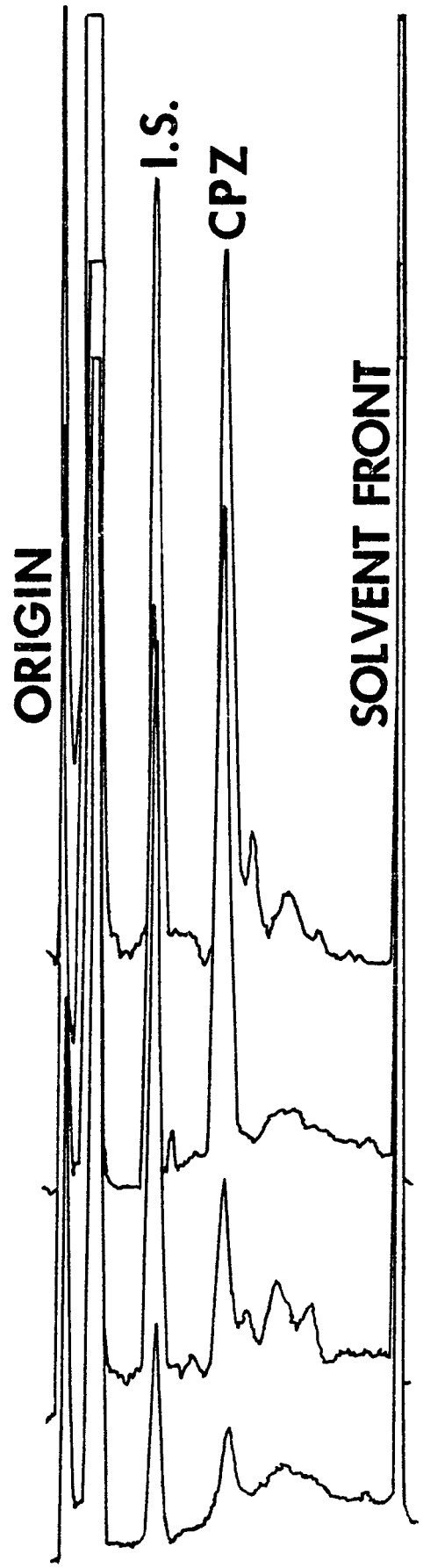
k = absorption coefficient

C = concentration

S = scattering coefficient

The equation indicates a linear correlation between $F(R_\infty)$ and C. If a

FIGURE 18: REPRODUCIBILITY OF R_f VALUE: CHROMATOGRAMS TAKEN FROM THE ENTIRE PERIOD OF METHOD DEVELOPMENT



R_f	
I.S.	CPZ
0.28	0.49
0.27	0.48
0.28	0.49
0.27	0.49

system is to adhere to the equation for linearity, its theory demands the following:

1. An infinitely thick opaque layer.
2. Homogeneous distribution of the sample within the measuring area.
3. Sufficient monochromaticity of the incident light.
4. A constant angular orientation of the light collection device.
5. Dilute absorbing substances.
6. Relatively small grain size for the absorbent layer (5-40 μ).

HPTLC plates simulate the first requirement by using silica gel of 7 μm grain size which exerts stronger light-scattering ability, hence no light penetrates the layer. From the study of actual transparencies of thin-layer chromatograms,[86] HPTLC with 200 μm layer thickness and compact layer would have less than 5% light transmission in the visible range, while in UV less than 1%. This low value in the UV region can be explained on the basis of the strong self-absorption of the substrate, silica gel and the glass support, for UV light. This factor often causes the system to deviate slightly from the Kubelka-Munk theory by manifesting the affect in a calibration curve, i.e. the curve does not go through the zero point.[20,22] This could partially explain the error in the calibration curves shown in Figures 19, 20, 22 and 23. It has been suggested that the infinite thickness and reduction of background contribution be simulated by putting two white sheets underneath the chromatograms during scanning in the visible range.[28] By scanning a blank HPTLC plate with and without backing sheets, no observable

difference in background can be found. The second requirement is accomplished by adopting a very small illuminating area in the shape of a slit whose length is adjusted to correspond to the dimensions of the spot.[22,26,27] Only a small area of the spot is measured at a time. It could then be assumed to a first approximation that no concentration gradient exists within such a small measuring area. With the Zeiss instrument, all the measurements are performed with a 1 mm slit width, 3.5 mm in length. Requirement 3 and 4 are fulfilled by the intrinsic structure of the KM-3. Generally speaking, HPTLC plates meet the demands of the Kubelka-Munk theory much more closely than TLC plates. Therefore, better quantitation accuracy and precision are anticipated from HPTLC.

1. Calibration Curves:

The internal standard method can be applied with the advantage that various changes in the basic parameters of the chromatographic process and during the sample preparation are reduced to a minimum. Any unexpected change will effect both the drug and the structurally similar internal standard to the same extent. The ratio (drug/internal standard) remains unchanged.

Calibration curves for chlorpromazine are shown in Figure 19, based on three replicate determinations of known amounts added to drug-free serum. Excellent correlation demonstrated by linear regression data in Table IV, reflects favorably both the quality of the plates and the analytical procedure.

Since the resolution (R_s) for chlorpromazine, butaperazine and the carrier is greater than 1.5, scanning at right angles to the solvent

FIGURE 19: CALIBRATION CURVES OF CHLORPROMAZINE
PEAK AREA RATIO VS. CONCENTRATION

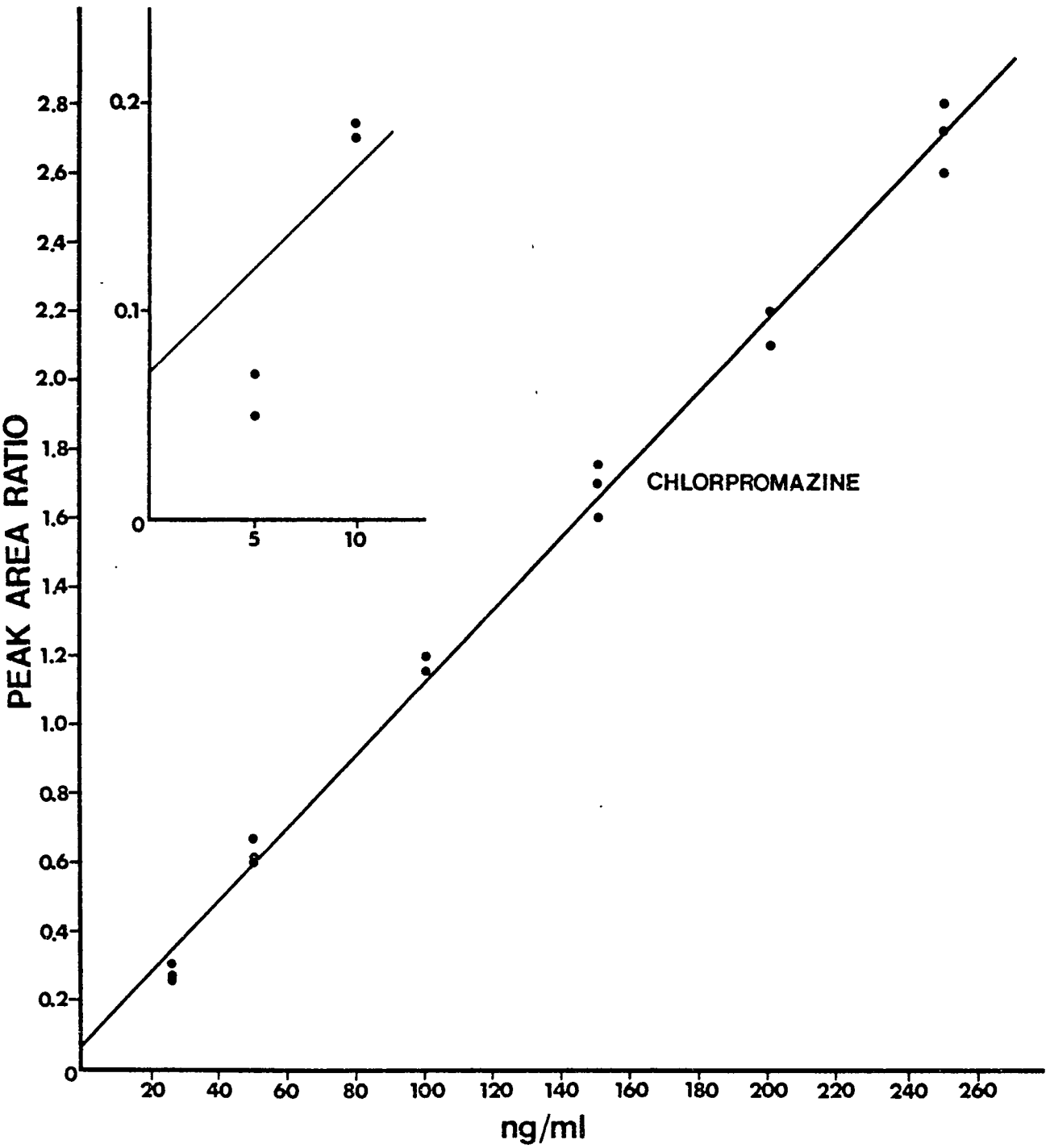

PEAK AREA RATIO
ng/ml
CHLORPROMAZINE
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
20
40
60
80
100
120
140
160
180
200
220
240
260
0.1
0.2
5
10

TABLE IV. LINEAR REGRESSION DATA FOR CALIBRATION CURVES IN FIGURES 19, 20, 22 and 23

Chlorpromazine: 0°-parallel scanning, 90° perpendicular scanning

		Correlation Coefficient	Slope	Intercept	
0°	1	0.9976	0.1089	0.4449	Fig. 19
	2	0.9982	0.1070	0.5793	
	3	0.9978	0.1037	0.6193	
90°	1	0.9979	0.1000	1.9875	Fig. 20
	2	0.9972	0.1010	1.7210	
	3	0.9964	0.0960	1.9460	

Tricyclic Antidepressants: parallel scanning only

	Correlation Coefficient	Slope	Intercept	
Ami.	0.9989	0.0136	-0.0183	Fig. 22
	0.9982	0.0141	0.0413	
	0.9998	0.0148	-0.0173	
Nor.	0.9985	0.0087	-0.0211	
	0.9989	0.0093	-0.0240	
	0.9998	0.0093	-0.0053	
Imip.	0.9996	0.0125	0.0203	Fig. 23
	0.9997	0.0120	0.0354	
	0.9999	0.0122	0.0204	
Des.	0.9975	0.0072	0.0020	
	0.9981	0.0078	-0.0046	
	0.9996	0.0074	0.0340	

flow is possible without interference from the adjacent spots. The replicate calibration curves and a typical chromatogram are shown in Figures 20 and 21. Equally good precision is obtainable compared with that of scanning in the direction of solvent flow (Table IV). Scanning perpendicularly to the solvent direction has the advantage of increased sensitivity of detection by wavelength optimization and shorter measuring time.[29,30]

Calibration curves of the tricyclic antidepressants are shown in Figure 22 and 23, again with good precision and linearity (Table IV). Because of the well saturated chamber and straight solvent front, a data-pair technique[87] was not employed in this case. This might slightly degrade the precision and linearity, but did significantly shorten the sample application time.

Due to the high reproducibility of the chromatography system, reflected from the R_f value (Figure 18), peak height ratios measured manually instead of peak area ratios, can also be used to construct the calibration curves.

2. Precision, Accuracy and Specificity:

As mentioned earlier (Table III), the presence of carrier, besides increasing the extraction efficiency, improved the degree of precision as well. The result is summarized in Table V. Again, the variance of the values is significantly less in the samples to which carrier is added. The effect is enlarged in 10 ng/ml level. This same effect was also observed in the GC/ECD determination of chlorpromazine.[77]

FIGURE 20: CALIBRATION CURVES OF CHLORPROMAZINE: SCANNING AT RIGHT ANGLE TO THE DIRECTION OF SOLVENT FLOW

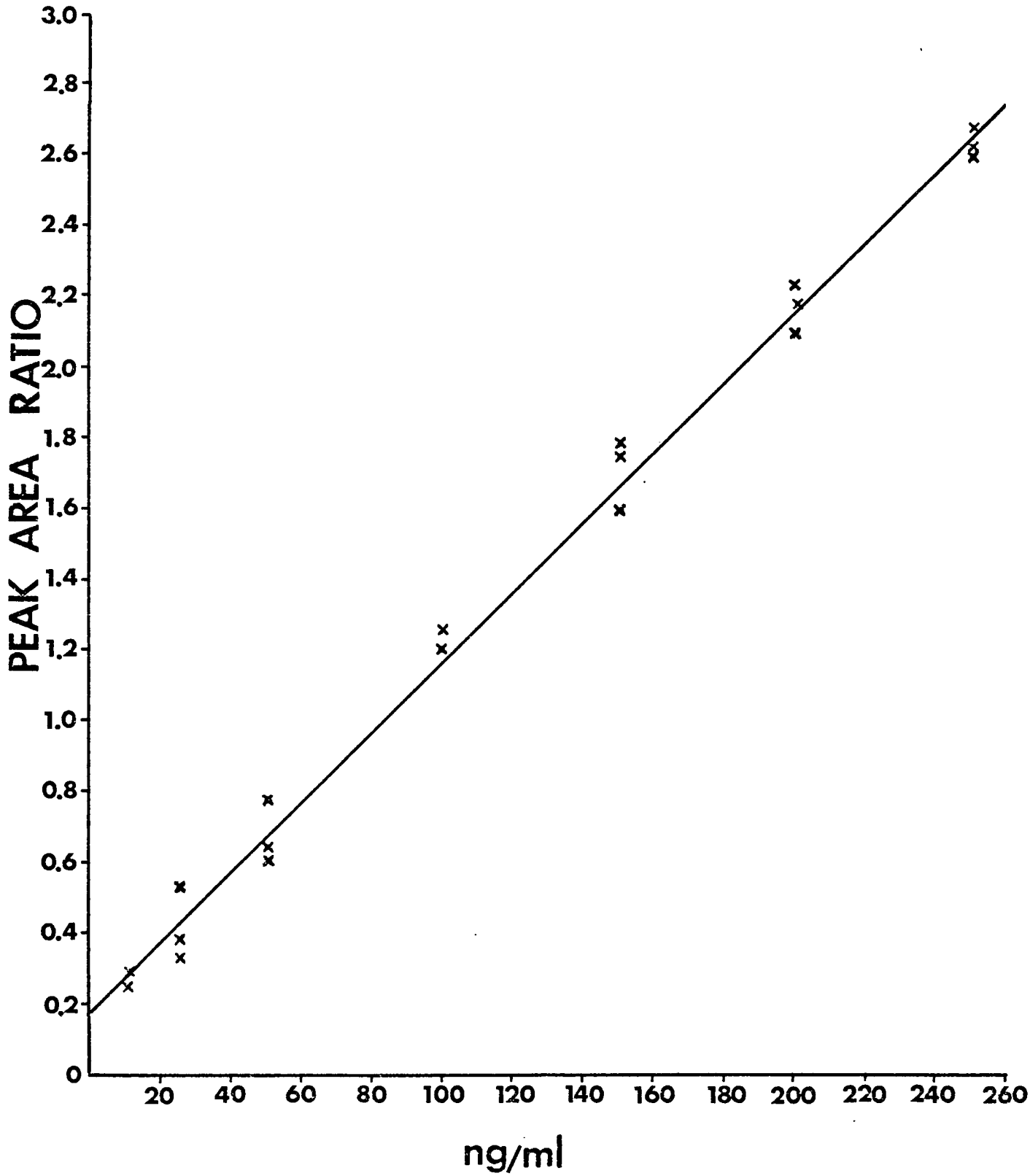
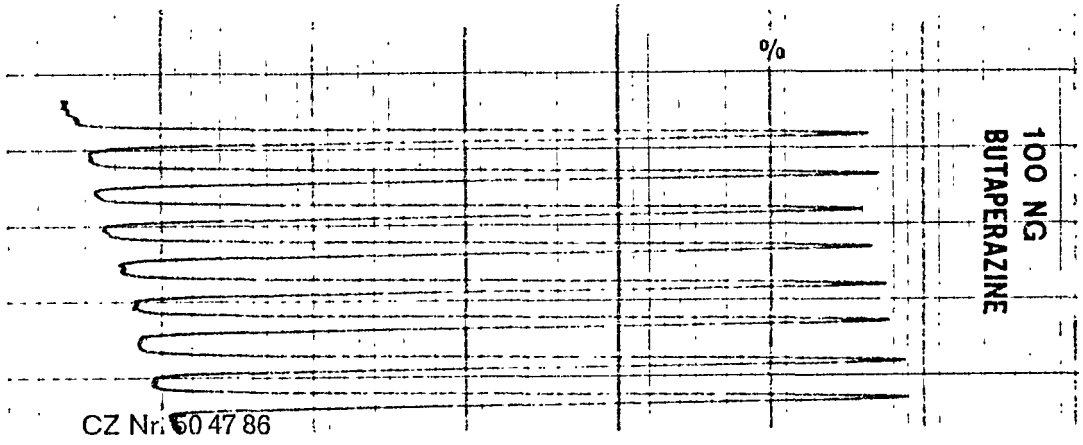

PEAK AREA RATIO
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
160
180
200
220
240
260
ng/ml

FIGURE 21: A TYPICAL CHROMATOGRAM OF CHLORPROMAZINE AND ITS INTERNAL STANDARD. BUTAPERAZINE: SCANNING PERPENDICULARLY TO THE DIRECTION OF SOLVENT FLOW


%
100 NG
BUTAPERAZINE

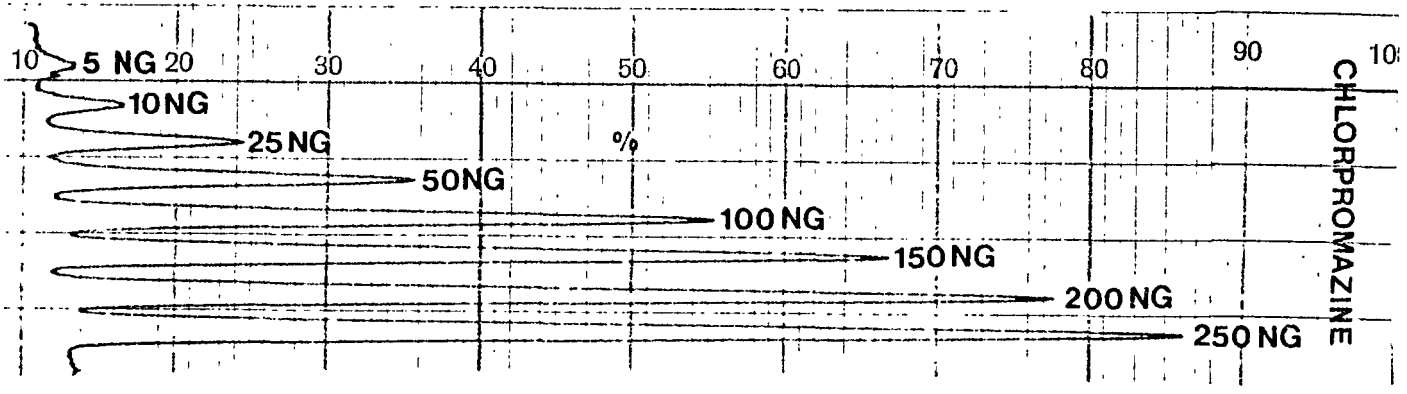

10
5 NG
20
30
40
50
60
70
80
90
10
10NG
25NG
%
50NG
100NG
150NG
200NG
250NG
CHLORPROMAZINE

FIGURE 22: CALIBRATION CURVES OF AMITRIPTYLINE AND ITS MAJOR METABOLITE, NORTRIPTYLINE

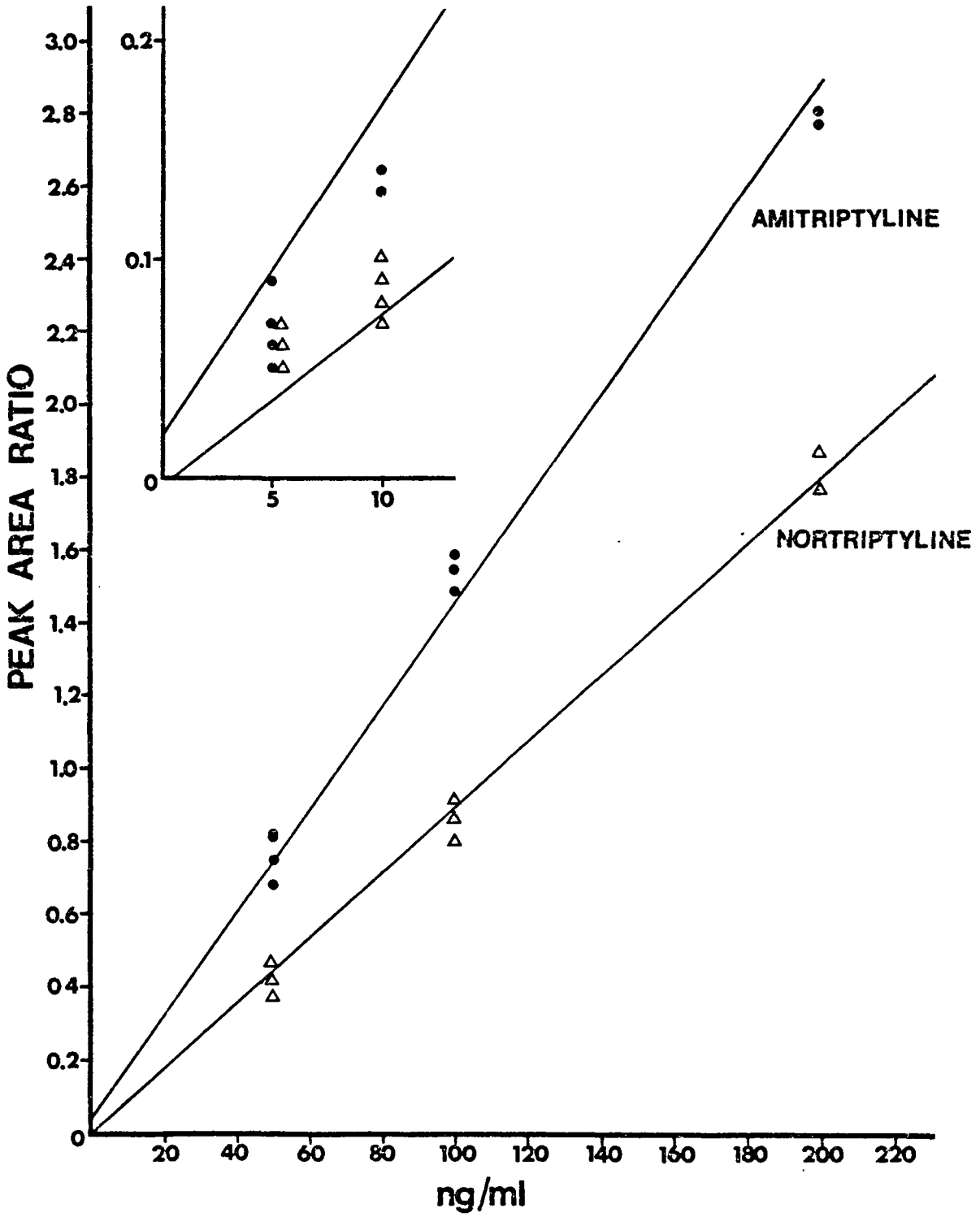

PEAK AREA RATIO
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.2
0.1
0
5
10
AMITRIPTYLINE
NORTRIPTYLINE
20
40
60
80
100
120
140
160
180
200
220
ng/ml

FIGURE 23: CALIBRATION CURVES OF IMIPRAMINE AND ITS MAJOR METABOLITE, DESIPRAMINE

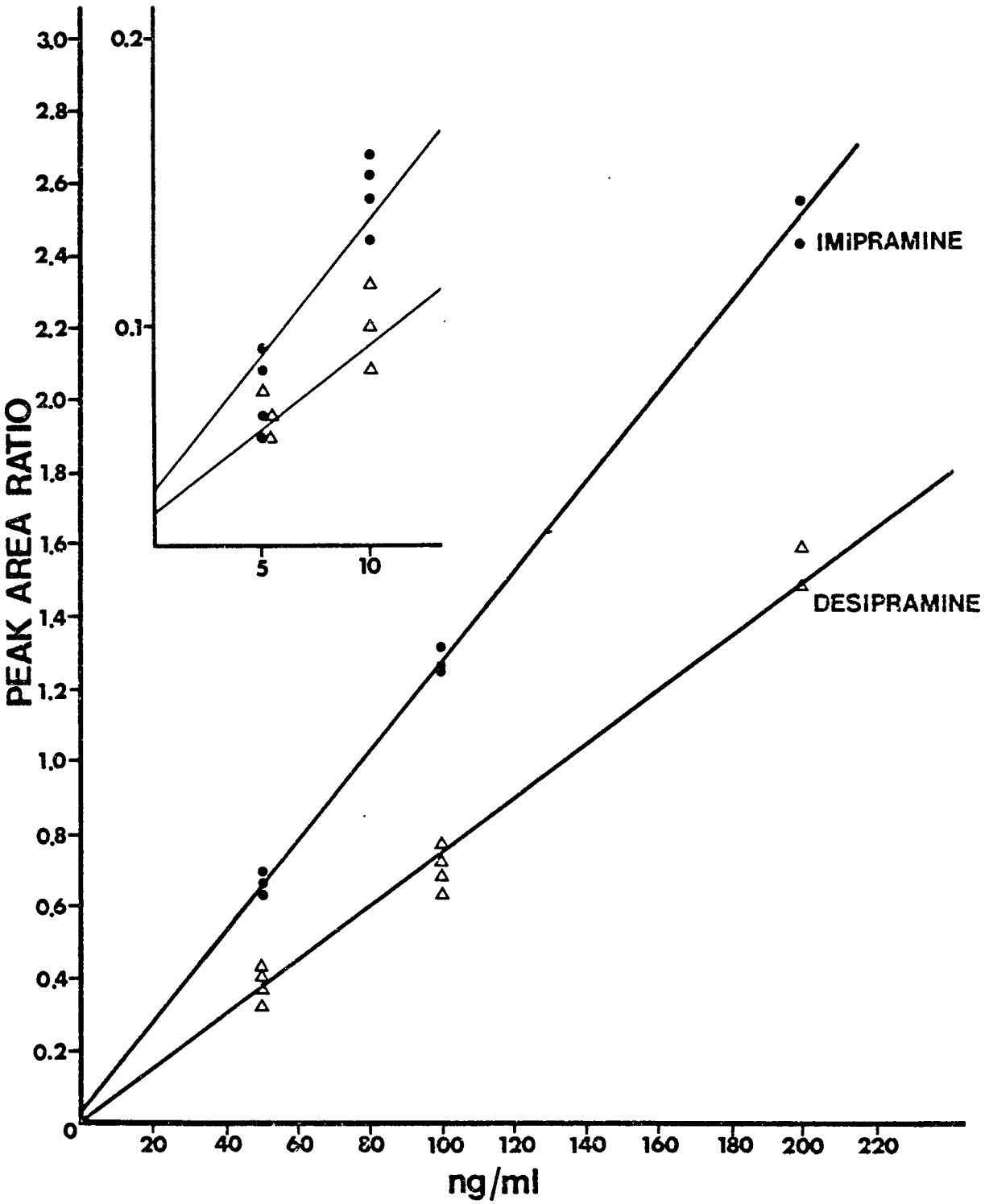

PEAK AREA RATIO
ng/ml
IMIPRAMINE
DESIPRAMINE
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
0.1
0.2
5
10
20
40
60
80
100
120
140
160
180
200
220

TABLE V: EFFECT OF CARRIER (PERPHENAZINE) ON REPRODUCIBILITY OF DETERMINATION OF PSYCHOTROPIC DRUGS IN SERUM

	Chlorpromazine		Nortriptyline		Amitriptyline		Desipramine		Imipramine	
10 ng/ml	with	without	with	without	with	without	with	without	with	without
MEAN[1]	0.088	0.059	0.083	0.064	0.160	0.121	0.079	0.062	0.180	0.158
S.D.[2]	0.007	0.020	0.007	0.012	0.013	0.014	0.009	0.011	0.008	0.018
C.V.[3]	8.0	34.2	8.5	18.5	7.8	11.3	10.7	17.7	4.2	11.3
100 ng/ml										
MEAN	1.25	1.14	0.647	0.555	1.444	1.257	0.584	0.486	1.229	1.151
S.D.	0.04	0.03	0.020	0.069	0.037	0.100	0.035	0.066	0.049	0.090
C.V.	3.3	2.6	3.1	12.4	2.6	7.9	6.0	13.5	4.0	7.9

[1]Mean of six determinations expressed as peak area ratio of drug/internal standard.

[2]Standard deviation.

[3]Coefficient of variation.

The results of the blind study for testing the accuracy are shown in Table VI. Agreement of these values are within the range usually experienced in blood serum assays, and should be of acceptable accuracy and precision for clinical use.

Table VII lists the R_f values of twenty-six basic drugs. As can be seen from these values, the possible interference is actually quite small because of the excellent selectivity of the HPTLC systems.

F. Separation on U-Chamber

The ring developed technique is applied on U-chambers. After development, the spots are in the shape of a half parenthesis with very narrow width which greatly increases the plate efficiency. Figure 24 shows theseparation of chlorpromazine and tricyclic antidepressants. The characteristics of the chromatograms are the sharpness of the peaks and the very short developing distance, therefore, the short analysis time.

In U-chambers, the feed of mobile phase is under strict control. Desired speed can be selected. In conventional TLC, this is a potential source of error. Edge effect, prevailing in linear TLC, is also eliminated because the chamber is constructed in a fully symmetrical manner. During development, the solvent front forms a perfect circle. Thus, data-pair techniques are not necessary.

TABLE VI: SINGLE BLIND DETERMINATIONS OF NG AMOUNTS OF PSYCHOTROPIC DRUGS FROM SPIKED SERUM SAMPLES

CHLORPROMAZINE			TRICYCLIC ANTIDEPRESSANTS					
Sample	Known Concentration	Observed Concentration	Sample	Known Concentration	Observed Concentration Ami	Nor	Imi	Des
1	50.0*	51.0 53.0	1	100.0	95.3 91.4	98.9 97.3	93.0 97.1	94.7 94.6
2	100.0	105.0 108.3	2	10.0	11.4 9.7	7.5 8.2	7.8 7.5	13.7 13.3
3	10.0	8.2 7.5	3	50.0	47.6 48.0	43.6 48.0	45.7 41.7	47.5 48.5

*ng/ml

TABLE VII: R_f VALUES OF TWENTY-SIX COMMON BASIC DRUGS SCREENED FOR INTERFERENCE

Drug	R_f Solvent System 1	R_f Solvent System 2
Acetophenazine	0.00	0.03
Amitriptyline	0.58	0.47
Butaperazine	0.22	0.28
Caffeine	0.04	0.09
Chlordiazepoxide	0.00	0.03
Chlorpromazine	0.54	0.48
Chlorpromazine 7-hydroxy	0.00	0.04
Chlorpromazine sulfoxide	0.02	0.10
Chlorprothixine	0.53	0.45
Clozapine	0.00	0.11
Desipramine	0.09	0.23
Diazepam	0.15	0.27
Fluphenazine	0.00	0.07
Haloperidol	0.03	0.17
Imipramine	0.49	0.48
Loxapine	0.27	0.35
Nicotine	0.35	0.40
Nortriptyline	0.17	0.18
Penfluridol	0.05	0.24
Perphenazine	0.00	0.13
Phenothiazine	0.07	0.29
Prochlorperazine	0.19	0.26
Promazine	0.26	0.40
Thioridazine	0.24	0.40
Thiothixine	0.03	0.08
Trifluroperazine	0.20	0.29

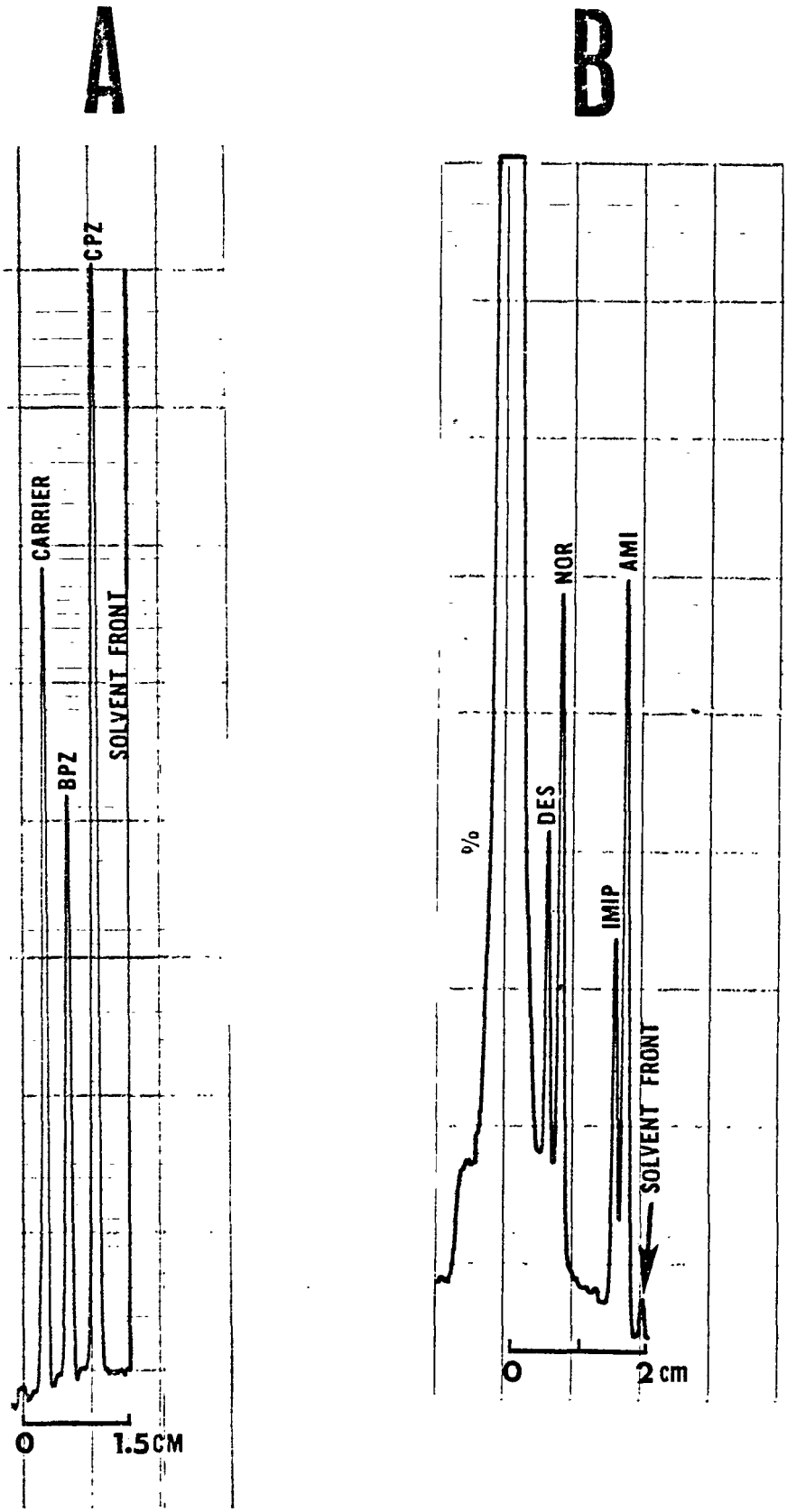
Solvent system 1: hexane/benzene/diethylamine (96/12/3)

Solvent system 2: hexane/acetone/diethylamine (80/20/3)

FIGURE 24: CHROMATOGRAMS OF STANDARD MIXTURES OBTAINED FROM U-CHAMBER

A: 50 ng Chlorpromazine

B: 50 ng each of tricyclic antidepressants


A
CPZ
CARRIER
BPZ
SOLVENT FRONT
0
1.5 CM
B
%
DES
NOR
AMI
IMIP
SOLVENT FRONT
0
2 cm

CONCLUSIONS

CONCLUSIONS

High Performance Thin Layer Chromatography was applied to the determination of the blood levels of chlorpromazine and tricyclic antidepressants to examine the feasibility of using the results to regulate the dosage so that safe and effective drug levels can be maintained for psychiatric patients.

The sensitivity of this assay procedure is at the 10 ng/ml level with a relative standard deviation of 4-10%. Excellent selectivity is also obtained by this procedure. Its performance rivals that of gas and high pressure liquid chromatography, as compared with two most recent assays.[52,58]

In the practical analysis of biological samples by HPTLC, it is difficult to keep the solution volume in the nanoliter range; large amounts must be applied to the adsorbent. Spotting becomes time-consuming and is the weakness of the described procedure. However, recent results of Halpaap[89] offer a promising solution of this difficulty.

This study clearly indicates the ability of HPTLC to be used as a routine procedure in drug analysis with reliable results.

PROBLEM II

ANALYSIS OF VOLATILE METABOLITES IN BLOOD SERUM OF CANCER PATIENTS BY HIGH RESOLUTION GAS CHROMATOGRAPHY WITH A NITROGEN SELECTIVE DETECTOR

ABSTRACT

A micro sampling procedure, parallel arrangement of a nitrogen selective and flame ionization detectors, and high resolution columns were applied to monitor the volatile metabolites in the serum as an indication of metabolic disorders, resulting from pathological conditions.

The sensitivity of the nitrogen selective detector was first optimized by a Sequential Simplex method. The detector has a detection limit in the low nanogram range and shows excellent selectivity.

The essence of the work described is in the comparison of profiles between healthy and diseased states. "Normal" profiles were established by analyzing serum samples from ten healthy individuals. Comparison of these profiles with those obtained from analysis of samples with different carcinomas indicated no excretion of new metabolites and large concentration variation of nitrogen-containing compounds.

INTRODUCTION

INTRODUCTION

At present, there is a rapidly growing interest in multi-component analysis of biological fluids and tissues. These components are the products of a large number of enzyme-catalyzed biochemical reactions controlled by precise mechanisms in the cell proceeded with great accuracy and speed. Due to in-born errors or a disease, infectious or non-infectious, developed at a later stage, some biochemical processes may become defective and produce metabolites with different concentrations or even generate extra, new abnormal metabolites. Therefore, the composition and identity of the metabolites in the body fluids are a potential source of fundamental knowledge in differentiation of normal and pathological metabolic pathways. The differences in "metabolic profiles" are thus able to function as a diagnosis and early detection for diseased states of the body.

Despite the intensive investigations, the application of the most sophisticated technology and millions of dollars, understanding cancer, its cause, prevention, or cure continues to elude us. These limitations are not the fault of the scientific community as a whole, but represent the numerous complications of the disease itself. Its multiple facets and unpredictable behavior often lead scientists into wrong interpretations. Today, cancer is an ever increasing threat to the life and health of human beings. The best approach to its containment is indicated in an old axiom that prevention of disease is better than a cure. Although prevention is slowly on the way, early diagnosis and proper treatment

still can save thousands of lives.

As early as 1925, a famous pathologist James Ewing[90] headed a National Committee on Cancer, and concluded that early diagnosis is so important that cancer detection clinics must be established in the United States. Today, however, the desirable goals of early detection have not been reached. With the recent advances in high resolution capillary GC, highly sensitive detectors, interfaced with a mass spectrometry and computers the investigation of metabolite profile seems a promising candidate for early warning of disease.[91]

The investigation of volatile metabolites was pioneered by Zlatkis and Liebich,[92] and Teranishi, et. al.[93] Numerous studies since then have appeared on volatile metabolites in human urine, blood, breath, cerebrospinal fluid and expired air. Little effort has been spent on the study of volatile metabolites in the biological fluids of cancer patients.[94]

The primary goal of the work described here focused on the comparison of the metabolic profiles of serum between various cancer patients and healthy individuals. The profiles were obtained via a micro sampling procedure,[95] developed in this laboratory, and high resolution capillary GC. The components were detected simultaneously by using a flame ionization and a nitrogen sensitive detectors. Some of the major metabolites in the serum and plasma have been identified,[96,97] but not including any nitrogen-containing compounds. However, in urine methylpyrazine, dimethylpyrazines, allylisothiocyanate, 2,3,5-trimethylpyrazine, vinylpyrazine, 2-methyl-6-ethylpyrazine, pyrrole,2-methyl-6-vinylpyrazine,

2-methylpyrrole, dimethylpyrrole, 1-butylpyrrole (tentative) have been identified by GC/MS.[98]

PHYSICAL BASIS OF NITROGEN-PHOSPHORUS DETECTOR (NPD)

PHYSICAL BASIS OF NITROGEN-PHOSPHORUS DETECTOR (NPD)[99,100]

The general background of this thermionic detector is the introduction of alkali atoms into the hydrogen flame which shows enhanced response toward molecular structures containing nitrogen and phosphorus heteroatoms. The basic structure of the NPD, as illustrated in Figure 25, consists of a modified flame ionization detector with these differences:

1. The introduction of a glass bead containing 1.5 mg non-volatile rubidium silicate ($Rb_2O \cdot SiO_2$), which is fused to a platinum wire and positioned between the jet and collector electrodes. With a power supply, the bead can be heated electrically.

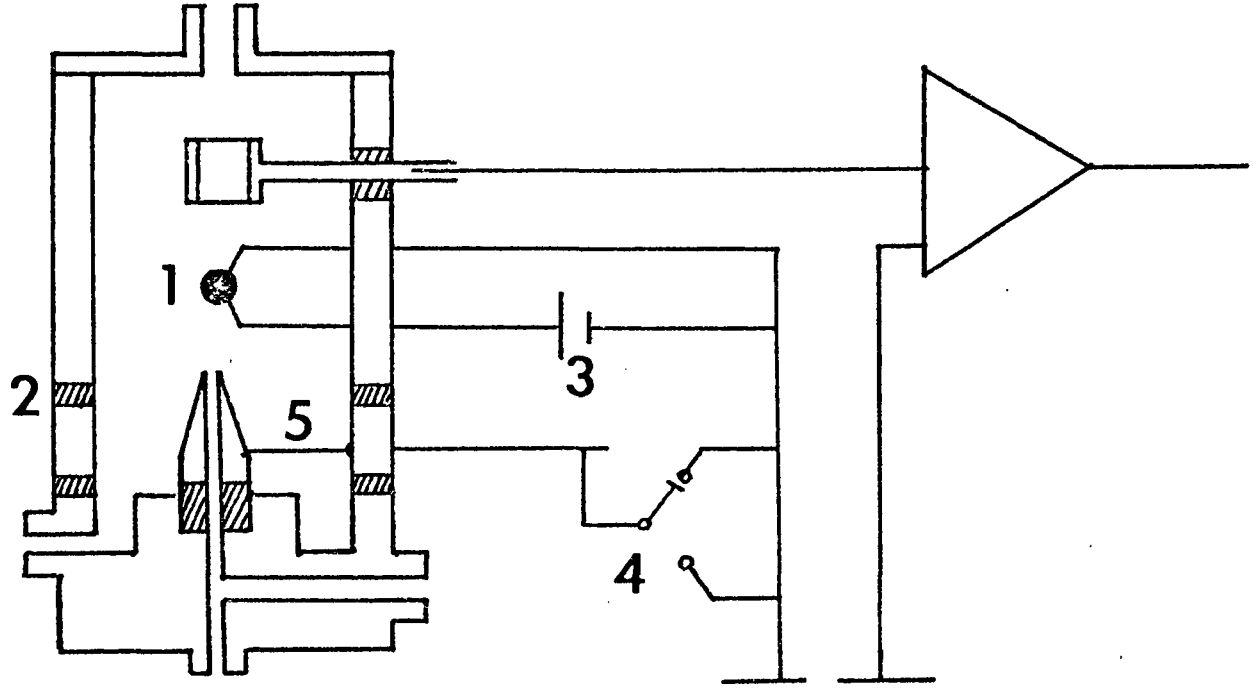
2. The detector housing is separated by an insulation disc so that the lower part containing the jet can be set to a different polarity with respect to the upper part. The complete scheme of the instrument set-up of the detector is also shown in Figure 25.

This detector can be operated in two modes, one in which nitrogen- and phosphorus-containing compounds are detected (NP mode), and the other in which only phosphorus compounds are detected (P mode). Only the NP mode will be described here.

In the NP mode, both the jet and the bead are held at a negative potential (-180 V) against the collector electrode. The bead is heated electrically to 600°C - 800°C. A cool diluted flame zone is formed around the hot bead by a 3-5% hydrogen/air mixture. At this temperature, a low, but constant background current is produced by the emission and

FIGURE 25: THE SCHEMATIC STRUCTURE OF NITROGEN PHOSPHORUS DETECTOR

1: Rubidium Silicate Bead
2: Insulation Disc
3: Bead Heating Element
4: Polarity Switch
5: Jet Polarizing Head


1
2
3
4
5

subsequent ionization of the excited neutral rubidium atoms through either thermal ionization or more likely a slow three-body process[101] with two hydrogen atoms. The ionized rubidium ion immediately returns to the bead held at negative potential.

$$H + H + Rb^{*}\cdot \rightarrow Rb^{+} + H_2 + e^{-}$$

When an organic nitrogen-containing compound emerges from the column and enters the cold flame zone around the bead, it is pyrolyzed to produce cyano radicals.

$$-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{\cdot\cdot}{N}}- \rightarrow \cdot C\equiv N :$$

These radicals can then take electrons from excited rubidium atoms, to generate negative cyanide ions and move quickly to the collector,

$$\cdot C\equiv N: + e^{-} \rightarrow [:C\equiv N:]^{-} \qquad \text{E.A.} = 3.17 \text{ eV}$$

$$Rb^{*}\cdot \rightarrow Rb^{+} + e^{-} \qquad \text{I.P.} = 2.57 \text{ eV}$$

$$Rb^{*} + \cdot C\equiv N: \rightarrow Rb^{+} + [:C\equiv N:]^{-} \qquad \Delta E = 0.60 \text{ eV}$$

while the rubidium ions again return to the bead. The reaction is spontaneous. A similar mechanism can be used for phosphorus containing compounds if PO_2 is assumed to be the reactive species. PO_2 should have a much higher electron affinity because of the stability gain from resonance structures.

$$[1\bar{O} = \bar{P} = \underline{\bar{O}}1]^- \longleftrightarrow [1\underline{\bar{O}} = \bar{P} = \bar{O}1]^-$$

This could explain the NPD's higher sensitivity for phosphorus than nitrogen compounds.

The recycling reaction mechanism is schematically shown in Figure 26.

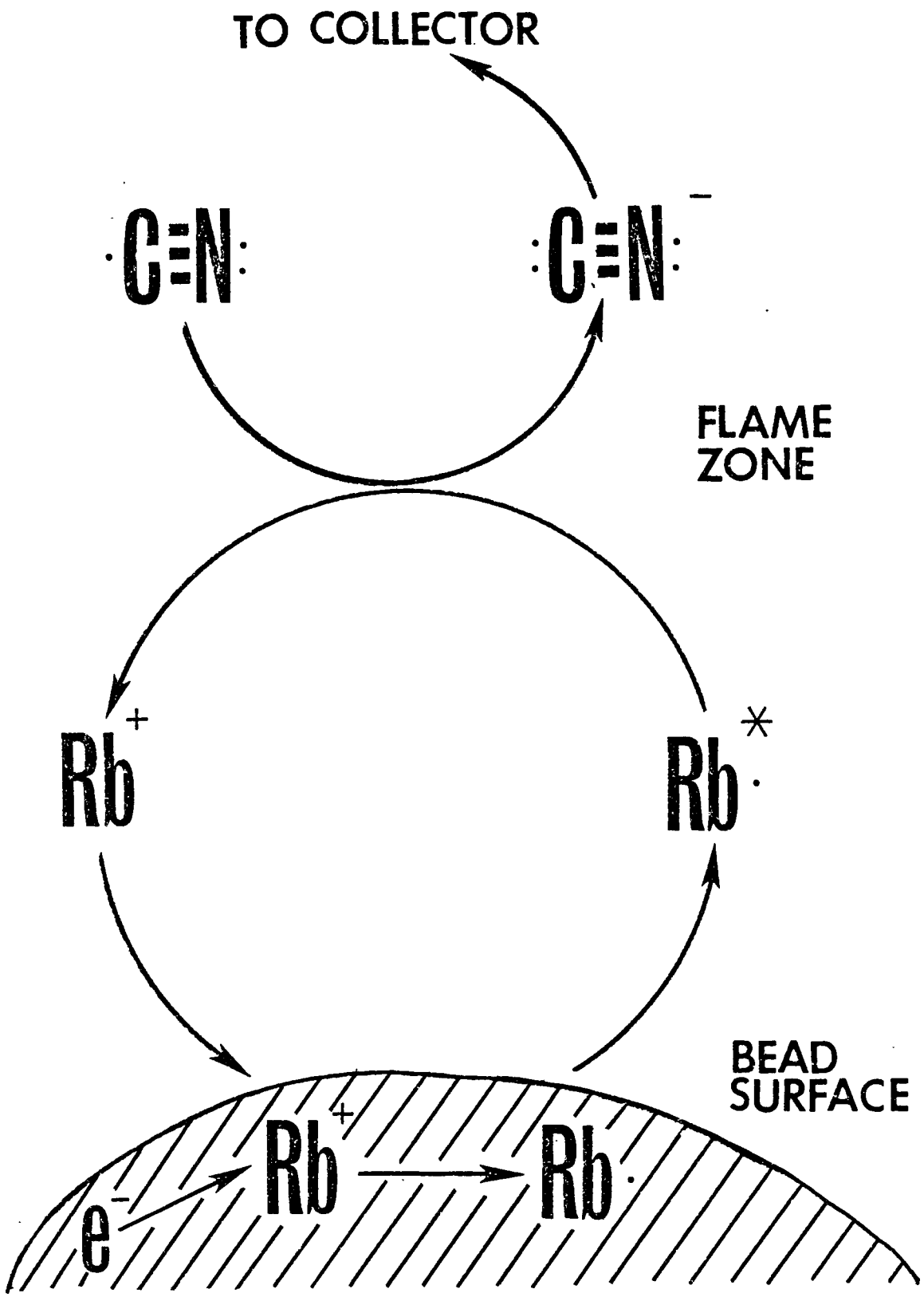
Finally, this mechanism is corroborated by:

1. Only those nitrogen-containing compounds are detected in which the nitrogen atom is covalently bonded to a carbon atom.

2. Any nitrogen-containing structure which prohibits the formation of cyano radicals shows limited or no response.

Thus the NPD cannot detect N_2, NO, NO_2, NH_3, nitrate esters, $-O-NO_2$ and shows lower sensitivity for structures such as -CO-NH-CO-, and $-CO-NH_2$, $-C-NO_2$.

FIGURE 26: THE RECYCLING REACTION MECHANISM
OF NITROGEN PHOSPHOROUS DETECTOR


TO COLLECTOR
C≡N
C≡N⁻
FLAME ZONE
Rb⁺
Rb*
BEAD SURFACE
e⁻
Rb⁺
Rb

EXPERIMENTAL

EXPERIMENTAL

A. Column Design

In the investigation of highly complexed mixtures by gas chromatography the choice of high resolution capillary columns as the means of adequate resolution is the best answer. The performance of these columns, among other factors, depends primarily on the uniformity, continuity and stability of the stationary phases coated on the inner column surface. These factors are in turn dependent upon:

1. The combining surface tension of the coating solution:

Non-polar stationary phases always exhibit lower surface tension than polar phases. The choice of stationary phases is limited by the sample type. Not much can be done with the stationary phase itself. However, a proper choice of solvent with low surface tension does offer some help.

2. The surface energy of the column surface:

On a surface with low surface energy, the liquid, instead of spreading into a homogeneous layer, will contract and form microdroplets. This explains the poor performance of columns.

Currently, several methods are used to increase the surface energy, hence helping wetting of the metal surface.

(a) Thorough cleaning of the metal surface:

A metal is classified as "high energy" surface. Under normal conditions, the surface is covered by impurities. The outermost layer

is gas and moisture adsorbed by the dirt and dust covering the surface. The deeper layer contains mainly oxides. Besides these, the drawing oil is also left on the inner surface during the manufacturing process. These impurities greatly alter the surface energy of the metal surface and makes the condition unfavorable for the stationary phase to form a homogeneous and continuous film. High purity organic solvents are usually used to flush the column to remove the drawing oil and some other organic contaminants, if present. The removal of the oxide layer is usually achieved by etching the surface with some inorganic acids. The removal of adsorbed moisture is not possible because the re-formation of a new layer of moisutre on newly exposed surface takes place instantly.

(b) Increasing the surface area of the metal:

An increase in surface area usually leads to a decrease in contact angle when a liquid is in contact with the surface. An effective and easy way of increasing the surface area is by roughing the surface. This can be achieved by:

(I) Etching the surface chemically - in order to yield a homogeneous etching surface, the inner wall of a column should be attacked slowly by a weaker acidic or basic solution instead of a strong one which gives less homogeneity and more gradient along the inside surface.

(II) Depositing inert fine powders on the inside wall physically - Silanox 101 is widely used for this

purpose. This material formed by a vapor phase reaction, is the silanized silica powder, in approximately spherical form, with a primary particle size of 7 μm. It is hydrophobic, does not self-aggregate and shows no adsorptive activity. Silanox can form stable suspensions in heavy organic solvents. After deposition on the metal surface, it forms thin but mechanically strong films which cannot be flushed away with organic solvent and with high pressure.

3. Column coating speed:

This factor directly relates to the thickness of the liquid phase. If the phase is too thin, adsorption of the sample by the wall will prevail. If the phase is too thick, the film produced has less stability and will tend to bleed off the column. By past experience, the best coating speed for a 300' x 0.02" column is 1 bubble/sec (the bubbling rate at the end of the column) which corresponds to a linear speed of 2 cm/min with a 10% (w/w) coating solution. The main requirement in this step is to maintain the coating speed as constant as possible throughout the coating process. The faster the coating speed is, the thicker the film will be. By keeping all of the above-mentioned conditions in mind, high resolution capillary columns were prepared and used in this study.

B. Column Preparation

New nickel capillary tubing (Handy & Harman Tube Co., Norisstown, Pa.) of 100 m x 0.5 mm I.D. x 0.8 mm O.D. was first checked for possible leaks by closing off one and, immersing the entire column in water, and pressurization (1000 psig). Short pieces of stainless steel tubing 1.6 mm O.D. x 1.0 mm I.D. were slipped over the ends of the column and silver soldered. Large drops of silver solder were filed off and the tubing was cut directly above the soldering connection. Nuts and ferrules, 1/16 in. (Swagelock, U.S.A.), were attached at both ends to make connections to the instrument.

1. Cleaning and Etching:

The column was connected to a stainless steel high pressure reservoir. The reservoir was in turn connected to a pre-purified Nitrogen cylinder (IWECO, Houston, Texas). The column was washed repeatedly with 50 ml portions of spectra grade methylene chloride, acetone, trichloroethylene, methanol, and distilled water under 800 psig pressure, until every portion of liquid coming out of the column was free from any particles. An etching solution glacial acetic acid/concentrated nitric acid/distilled H_2O (1:1:12 by volume), was used to fill the column with pressure. The column was then disconnected from the reservoir. By using a 1/16" stainless steel union, both ends were connected together so that no escaping of the etching solution was possible.

The etching proceeded at room temperature for at least 8 hours. At the end of the etching, the solution should come out medium green to indicate a "good" etching. The column was then flushed with H_2O until

neutral, checked by litmus paper. In order to thoroughly remove the etching reaction products, the column was further washed with 200 ml of 10% Micro cleaning solution (International Products Corp., Trenton, New Jersey) which was strongly alkaline, by connecting the column to the high pressure reservoir, immersing the entire column in a 90°C water bath and applying low pressure (200 psig). The column was again washed with H_2O until neutral, followed by acetone, methanol, methylene chloride, repeatedly and dried throughly with the flow of nitrogen.

2. Coating:

10% (w/w) solutions of Witconol LA-23 was prepared in spectra grade methylene chloride with 1% Silanox 101 (Cabot Corporation, Boston, Mass.). The suspension was then sonicated in an ultrasonic bath for 30 minutes. The homogeneity of suspension was checked by immersing a clean, stainless steel spatula into the solution, withdrawing rapidly and drying. A smooth coat with microregularity indicated sufficient dispersion. If a non-uniform granular coat resulted, the suspension was returned to the ultrasonic bath for longer dispersion. The set-up for coating consisted of a pressure source, pre-purified N_2, stainless steel needle valve to control the flow, and a coating reservoir made of teflon tube where the column was connected. The column was first fed with 4 ml spectra grade methylene chloride, immediately followed by the coating solution with silanox. The function of the methylene chloride preceding the coating solution was to wet the inner wall to lower the friction between the silanox and the surface so that no plugging would happen. In order to maintain a constant velocity throughout the coating, an empty 300' x 0.02" buffer

column was connected to the main column. The end of the buffer column was dipped into a small beaker filled with water. The coating speed was adjusted by the needle valve to give the desired 1 bubble/sec flow rate. The coating usually took 7-8 hours to complete. After all the coating solution left the column, the column was dried by slowly increasing the nitrogen flow until no methylene chloride could be detected at the end.

The column was then conditioned in a gas chromatograph with 2 psig carrier gas. The temperature was programmed from 50°C at 1/2 degree per minute, to 160°C overnight.

The quality of the column was checked by injecting the following mixtures:

(a) Amyl ether/decalins at 120°C, 10 psig, carrier gas

(b) Aromatic mixture (benzene, toluene, ethylbenzene, p- and m-xylene) at 80°C, 10 psig carrier gas.

and rated by calculating its number of theoretical plates, examining the peak sharpness, peak resolution and tailing phenomena.

C. Optimization of Nitrogen-Phosphorus Detector

Variable-size "Sequential Simplex" technique was used to change simultaneously the hydrogen and air flow rate to optimize the sensitivity. The selectivity was monitired at the same time. Details of this optimization design have been described elsewhere.[101] A standard solution of N,N-dimethylaniline (DMA) in cyclohexane was made up with a concentration of 190 µg/ml. A 1.0 µl Hamilton syringe was used to inject 0.2 µl standard solution into the column. The injector split

ratio was set at 2:1. The eluent split ratio was 1:1. Only 6.5 ng DMA reached the FID and NPD respectively.

A short nickel column 40 m x 0.5 mm I.D. coated with 10% Witconol was used. Column temperature was kept at 150°C isothermally and column flow rate at 4 ml/min helium, NPD bead temperature was 550°C. Injector temperature and detector temperature were at 200°C and 300°C respectively. All the chromatographic runs were made at nearly identical conditions as possible except for the NPD air and hydrogen flow rate which were directed by the simplex experimental design. The inlet pressure of air and hydrogen were set at 80 and 60 psig respectively. The individual gas flow was controlled by a built-in regulator and read on the pressure gauge. Air pressure was allowed to change between 20 to 70 psig, while hydrogen 5 to 50 psig. Pressure settings dictated by experimental design were rounded off to the nearest 0.1 psig.

The peak height of DMA was measured as an indication of sensitivity. The peak height ratio of DMA and solvent cyclohexane was constantly calculated as a monitor for detector selectivity. A ratio higher than 1.5 was considered as a violation of selectivity boundary condition.

D. Sampling System

The micro solvent extraction method[15] was used for volatile isolation. The system involved extraction of the sample, 100 µl serum, with distilled diethyl ether, separation of diethyl ether from the volatiles on the adsorbent, glass wool, and volatiles were thermally desorbed into a chromatographic column.

1. Concentration Columns:

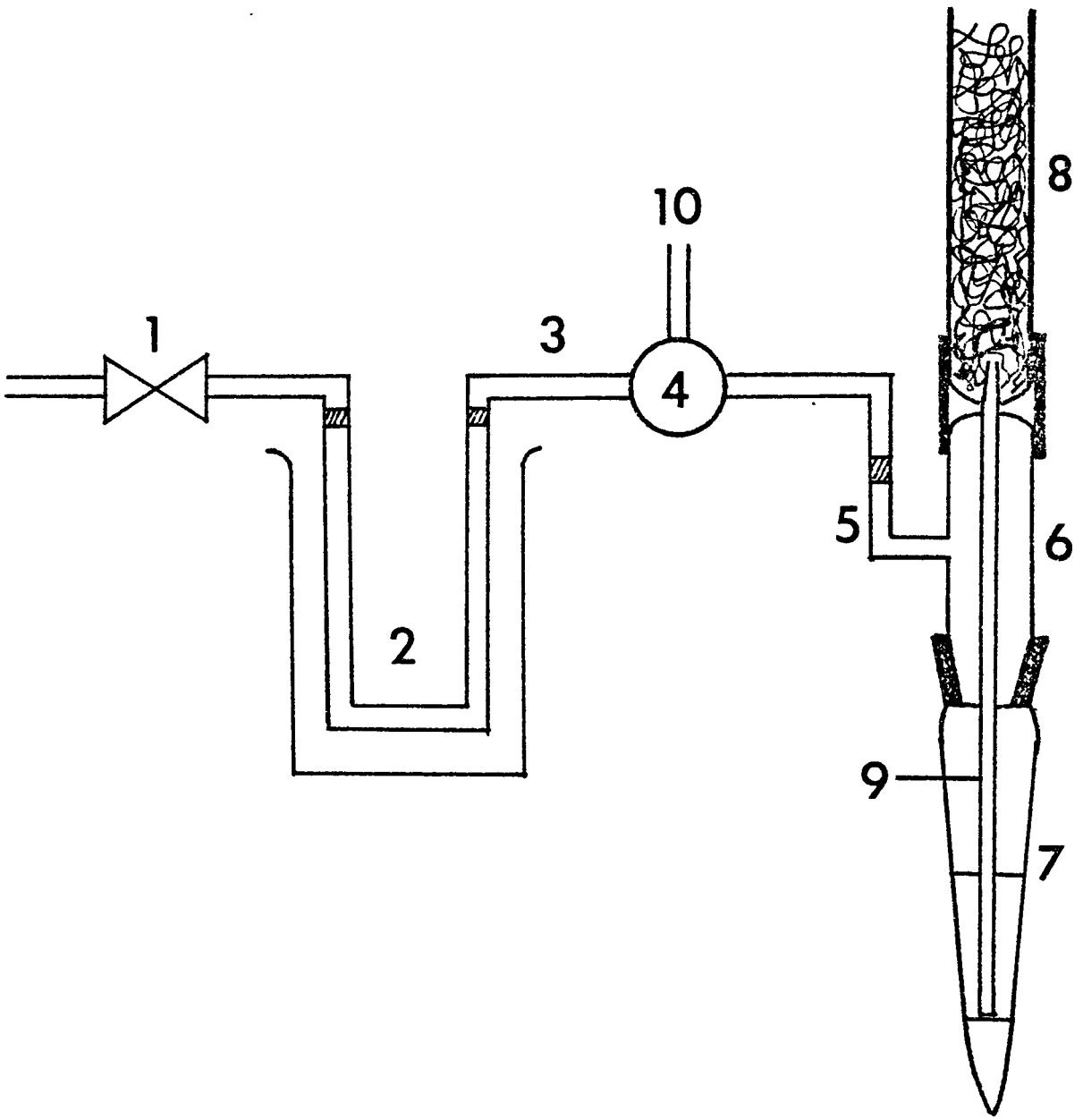
The column was a glass tube of 11 cm x 10 mm O.D. x 8 mm I.D.. The entire tube was packed with glass wool. One end of the glass tube was tapered, to provide an efficient seal in the desorption chamber. Prior to analysis, the packed concentration columns were conditioned in a stream of helium (20 ml/min) for one hour at 350°C and stored in clean culture tubes (13 mm x 10 mm) with teflon-lined screw caps.

2. Sample Preparation:

After extracting 100 µl serum, the diethyl ether extract was transferred to the concentration column via the system shown in Figure 27. High purity helium was used in the system. Before flowing into the system, it was further purified by a trap (2) of molecular sieve and charcoal at dry ice temperature. The helium was then carried through the system via a 1/8" teflon tube (3). Its flow was controlled by means of a needle valve (1). A three-way valve (4), made of teflon was used to direct the flow to either a thermal desorption chamber or to trans-evaporator. The inlet, 1/8" glass (5) tubing of the transevaporator (6) was tightly fitted into the teflon tubing. A 5 ml laboratory-made conical tip centrifuge tube with ground glass joint (Ŧ 9 pyrex) was fitted to the transevaporator (7). The concentration column (8) was connected on the top of the transevaporator by means of shrinkable PTFE tubing. Extraction was carried out in the 5 ml conical centrifuged tube with glass stopper. 100 µl serum was added to the tube via a 1 ml hypodermic syringe followed by 1 ml freshly distilled anhydrous ether

FIGURE 27: THE MICRO SAMPLING SYSTEM

1. Needle Valve
2. Gas Purification Trap
3. 1/8" Teflon Tube
4. Three way Switching Valve
5. 1/8" Inlet Glass Tube
6. Transevaporator
7. Centrifuge Tube
8. Glass wool Concentration Column
9. 1/8" Transferring Glass Tube
10. To Desorption Chamber


1
2
3
4
5
6
7
8
9
10

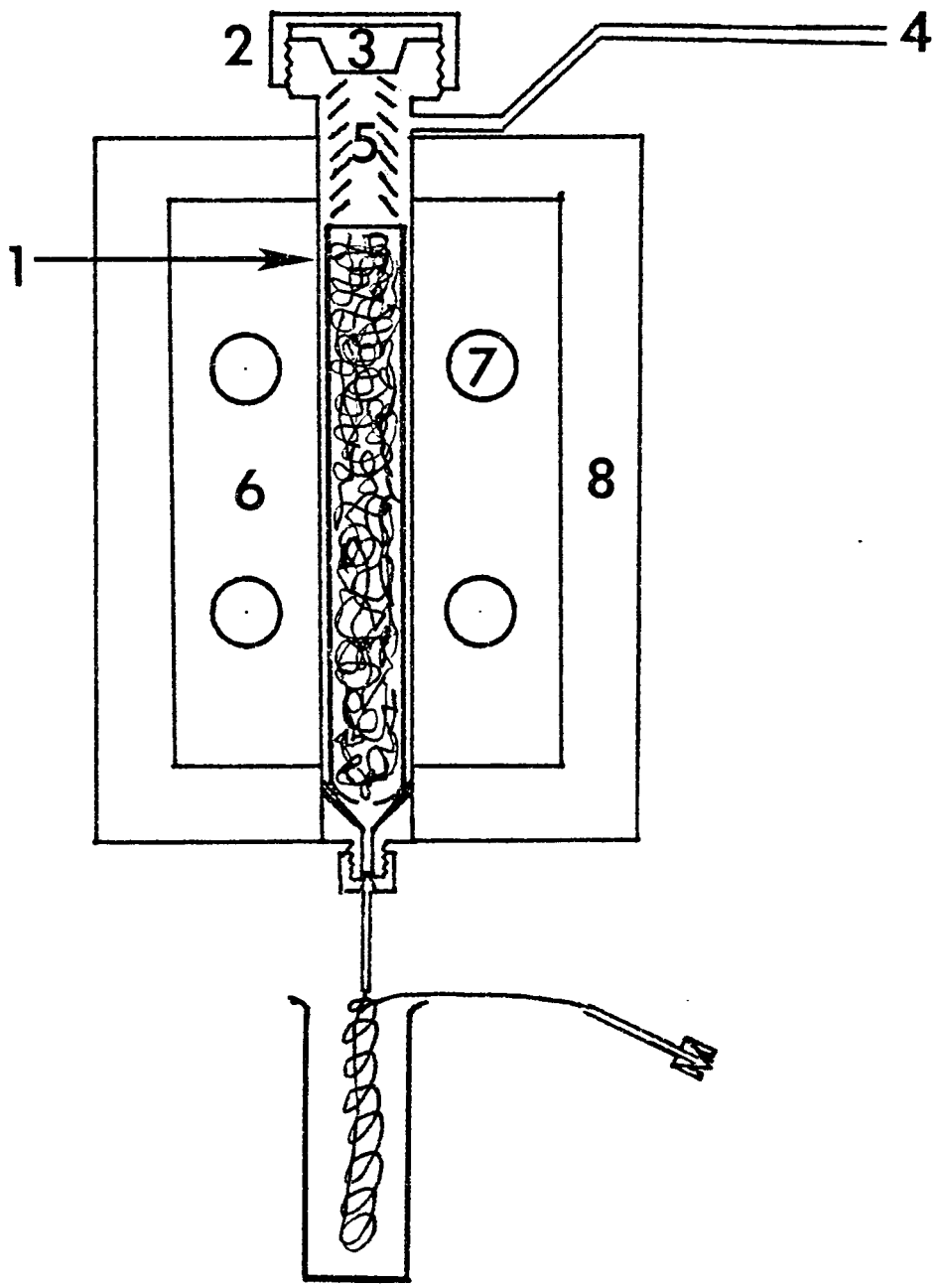
(Fisher Scientific Co., Pittsburg, Pa.). The two liquids were throughly mixed on a vortex mixer (Lab-line Instruments Inc., Melrose Park, Ill.) for 2 minutes. The mixture was then centrifuged for 3 minutes and was ready for transferring. Transferring of the ether extract was achieved via a glass tube of 1/8" O.D. (9) which extends from the transevaporator to the extraction tube to just above the serum/ether interface. The extract was forced to flow into the concentration column through the transferring tube by 20 ml/min of helium flow measured by a bubble flow meter at the top of the concentration column. The ether was stripped from the glass wool within 15 minutes. The whole system was kept at room temperature. After evaporation of the ether, the column can be subjected to thermal desorption immediately or stored in the culture tube in the freezer for later analysis.

3. Sample Transfer:

The heat desorption was performed in a chamber. It was constructed from stainless steel and resembled an injection port as shown in Figure 8. It consisted of a chamber body (1) fitted with a steel cap (2) lined with a teflon plug (3) and a helium inlet (4). Its size was designed to be able to just accomodate a concentration column. After the column was placed inside the chamber, a spring (5) was used to maintain a tight seal between the tapered column end and the bottom of the chamber. The chamber was fitted into a brass block (6) with cartridge heater (7), which in turn was surrounded by asbestos board (8) for insulation. The chamber was maintained at 290°C at all times.

FIGURE 28: THE SAMPLE DESORPTION SYSTEM

1. Chamber Body
2. Steel Cap
3. Glass-filled Teflon Plug
4. Helium Inlet
5. Spring
6. Brass Block
7. Heating Cartridge
8. Asbestos Board


2
3
4
5
1
7
6
8

4. Serum Volatiles:

The volatiles adsorbed on the glass wool were thermally released and swept by a helium stream of 10 ml/min flow, controlled by the needle valve in Figure 27, into a pre-column, connected at the outlet of the chamber and immersed in liquid nitrogen. The pre-column, 1 m x 0.75 mm I.D., was coated with the same liquid phase as the analytical column. The liquid nitrogen condensed the volatiles in the pre-column into a tiny narrow plug which is of the utmost importance for efficient use with high resolution columns. The thermal desorption process took 15 minutes to transfer the volatiles into the pre-column. The pre-column was then disconnected from the desorption chamber and placed into the gas chromatograph where it was connected to the analytical column (nickel 100 m x 0.5 mm I.D.). To start the analysis, the liquid nitrogen was removed, the oven door closed and the pre-set temperature programming was started by pushing the start button on the instrument panel.

E. Chromatographic Conditions for Analysis

The gas chromatograph used for resolving the volatiles in serum was a Perkin-Elmer, Model 3920 (Perkin-Elmer, Norwalk, Conn.) equipped with a flame ionization detector (FID) and a nitrogen-phosphorus detector (NPD). By means of a zero-dead-volume T-piece, the column eluent was equally divided (1:1) between the two detectors. To the instrument was added a make-up gas inlet between the column end and the eluent splitter to reduce the dead volume. A dual pen strip chart recorder (Leeds & Northrop, Speedomax X/L 680) was used for simultaneously recording the

output from both detectors. Operational conditions are summarized as follows:

Analysis temperature programming: Isothermal at 60°C for 8 minutes, the temperature was raised at 2°/min to 150°C and isothermal at 150°C for 64 minutes.

Carrier gas: Helium

Column flow rate: 10 psig (4 ml/min)

Make-up gas flow rate: 30 ml/min

Injection temperature: 200°C

Detector temperature: 300°C

FID: H_2: 20 psig
Air: 50 psig

NPD: Bead temperature: 550-650°C
Jet potential: 3 position (- 180 Volts potential)
H_2: 25 psig
Air: 40 psig

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

A. Column Preparation

A high resolution column in which the separation takes place is the heart of the gas chromatographic technique. Without a properly prepared column, even the best system would give poor performance. Thus, during this work special attention was paid to the preparation of highly efficient columns with the emphasis mainly on obtaining sufficient separation, long-term stability and less on analysis time. The wall-coated open-tubular (WCOT) column is apparently not the column of choice for this type are often found to deteriorate rapidly, especially at higher temperature. The possible reason is the break-up of liquid film on the column wall and the formation of micro droplets.

An ideal choice is the porous-layer open tubular (PLOT) columns. Columns of this type can be considered as a tube consisting of a thick porous wall impregnated with liquid phase with an unobstructed longitudinal gas channel and the metal tubing only functions as a protective shell for the fragile porous tube.[102]

The primary characteristic of a PLOT column is (1) the presence of a porous layer on the inside column surface which tremendously increases the total coating area and results in a higher amount of liquid phase per unit length than WCOT columns. Phase ratio (β) of PLOT columns is then reduced to that of a packed column.

(2) The liquid phase is highly dispersed in the porous layer and the film thickness is usually less than that in a WCOT column. Thus, the

PLOT columns have lower resistance to mass transfer in the stationary phase and higher efficiency than WCOT columns.

These characteristics result in the following advantages compared with WCOT columns:

(1) A shorter column can offer the same separation as a long WCOT column.

(2) Higher sample capacity and column efficiency provide better detectability.

(3) Low degree of bleeding provides higher operating temperatures and long-term stability.

The columns as designed and prepared in this work usually obtained a number of theoretical plates of 180,000. The column life was more than a year. During this time, the column was used daily for running serum extracts and conditioned up to 165°C overnight.

The exhaustive cleaning step, i.e. solvent flushing, acid etching and alkaline solution cleaning, was fairly efficient to remove the impurities from new as well as used nickel columns. Columns were always coated immediately after cleaning so that the chance of the column surface coming in contact with air was reduced to minimum. It has been observed that surface energy of solid is a property closely related to its surface cleanliness.[103] A thoroughly clean surface can easily be wetted by liquids.

The purpose of etching here was two-fold. One was for removal of the metal oxides and other impurities from the surface. The other was

for creating more surface area to help the spreading of the stationary phase.

The essential part in deposition of Silanox on the wall is to pre-wet the inside column surface with pure solvent. If this is not done properly, plugging always occurs during coating. However, if the coating suspension,thoroughly sonicated, is immediately preceded without any space by the pure solvent in the column, the coating process is usually trouble-free. Besides providing increased surface area Silanox is also thought to be able to preserve the film continuity, to eliminate the opportunity of film break-up and to reduce the column bleeding by firmly holding the stationary phase molecules. All these factors lead to longer column stability and better efficiency.

In dynamically coating PLOT columns, higher inlet pressure was needed because of the enhanced friction force between the Silanox particles and metal surface. With some practice a fair constant coating velocity can be maintained by adjusting with the fine needle valve. Although the thickness of the stationary phase inside the column is said to be controlled by the coating speed, in fact a constant coating velocity is required to obtain only a thickness homogeneity. The thickness itself is much more easier to control by changing the concentration of the coating solution instead of varying the coating velocity. In dynamic coating procedures, reproducibility is always a serious problem owing to some, but very important, factors which cannot be controlled and standardized.

Finally, it must be emphasized again that in the testing of the

column, the overall performance of a capillary column is not only dependent on a good column preparation technique, but the instrument compatibility, column connection, sample introduction technique are also equally important. If these points are neglected, a column with high performance will give disappointing and discouraging results. It is always suggested that a newly-prepared column be evaluated with several instruments, or the instrument is first checked with a known good column before evaluation of the fresh column.

B. Nitrogen-Phosphorus Detector

Accurate data on the composition of mixtures in which some components exist in very low concentration are required in many branches of science and technology. This stimulates the fast development of high resolution gas chromatography in trace analysis because of its high separating power and sensitivity. With today's high performance column technology and instrumentation, separation of hundreds of compounds from natural samples in a single run is relatively simple. As far as separation is concerned, no difficulty is encountered. However, identification of each compound especially for some interesting low concentration substances in such complex profiles, is still a complicated matter. Thus, profile simplification methods are desired. Among the available methods, e.g. reaction GC, specific extraction, selective detectors are the more refined technique. These detectors not only simplify the profile but also show enhanced signals for the specific compounds. It is particularly attractive in trace analysis when a selective detector is combined with a universal detector, such as the FID. From an FID recording, one can

see the total fingerprint while the selective detector would pick up only the compounds of interest. Today a large number of selective detectors is available for many elements in the periodical table, including metals.

The importance in trace analysis of nitrogen and phosphorus compounds is particularly emphasized in the detection of organophosphorus, carbamate pesticides in the environment, and the potent carcinogen nitrosamine in the food stuff. In the biomedical field, the appearance or absence of nitrogen compounds in a biological fluid may be used as an indication of pathological change in some metabolic pathway.

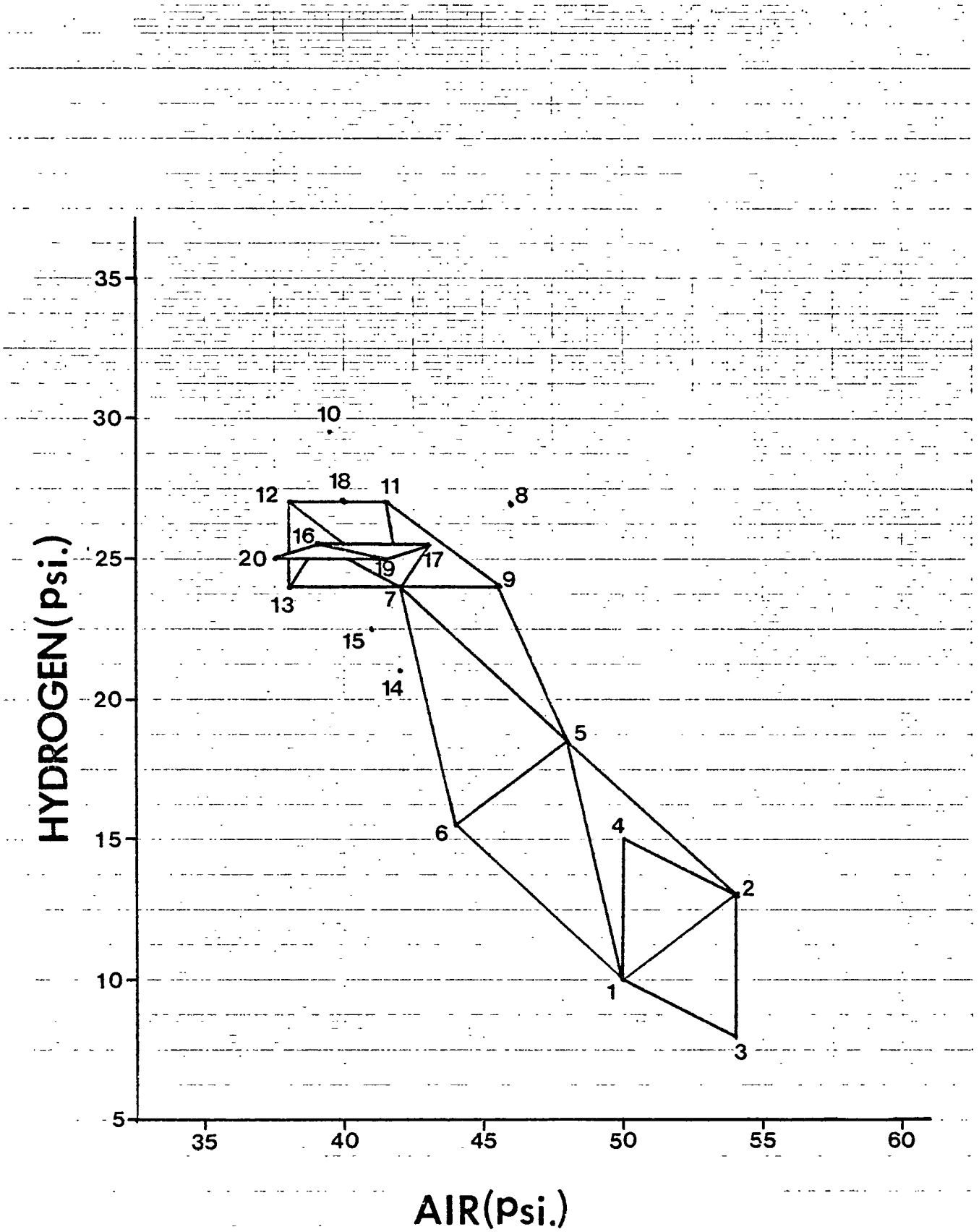
A number of nitrogen and phosphorus sensitive detectors of various design are available, but the most recently developed from Perkin-Elmer attracts attention. A survey of a number of publications using the detectors of same design reveals the different optimum operation conditions (hydrogen and air flow rate) from laboratory to laboratory possibly because the performance of each bead is different. It was therefore decided to optimize the detector sensitivity with the hope of gaining a greater understanding. Variable-size sequential simplex[101] techniques were applied here mainly because of its efficiency and capability of locating the true optimum.

The factors which are thought to effect the detector sensitivity are hydrogen, air and carrier gas flow rates, alkali source temperature, detector temperature, jet potential, relative position of jet, the bead and the collector electrode. Information from the detector manual indicates that the most critical factors in this design are carrier gas, hydrogen and air flow rate, bead temperature. The bead temperature

effects the background current which is proportional to the sensitivity. Thus, it is assumed that increasing the bead temperature would only raise the response surface, i.e. increase the sensitivity. With capillary columns, carrier flow rate can only vary within a narrow range. Hence, the bead temperature and column flow rate were fixed at 550°C and 4 ml/min respectively. Only hydrogen and air flow rate were allowed to change simultaneously. The movement of simplex is shown in Figure 29. The numerical data is summarized in Table VIII. The final optimum area is located at simplex 16, 19, 20, with hydrogen at 25 psi (3 ml/min), air at approximately 38-40 psig (90 ml/min). The detector is very sensitive toward hydrogen change and less toward air flow rate change. Compared with the response at initial simplex, the sensitivity was improved about 3 times. During the optimization, vertex 7 was retained 8 times. The response at this point was observed repeatedly in order to make sure the retaining of this vertex was not due to experimental error.

However, two weeks later, the sensitivity at vertex 16, 19, 20 were checked again under exactly the same conditions, the responses found were lower for all three points than the original values. By increasing the bead temperature to 600°C which increased the alkali atom concentration in the hydrogen flame, the original responses were approximately restored. The possible explanation is that the aging of the bead, i.e. reduction of alkali atom concentration in the flame, causes the sensitivity to drop. This indicates that if the bead temperature is kept constant, detector response is inversely proportional to time.

FIGURE 29: THE PROGRESS OF SIMPLEX OPTIMIZATION


HYDROGEN(Psi.)
AIR(Psi.)
35
30
25
20
15
10
5
35
40
45
50
55
60
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19
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TABLE VIII: THE NUMERICAL DATA OF SIMPLEX MOVEMENT

Vertex No.	Vertices Retained from Previous Simplex	Variables H_2, Air		Sensitivity (Peak Height, cm)
1		10	50	5.66
2		13	54	5.23
3		8	54	3.20
4	1,2	15	50	3.20
5	1,2	18.5	48	9.43
6	1,5	15.5	44	9.40
7	5,6	24	42	14.9
8	5,7	27	46	(1)
9	5,7	24	45.5	13.6
10	7,9	29.5	39.5	(2)
11	7,9 (3)	27.0	41.5	13.67
12	7,11 (3)	27	38	12.38 (4)
13	7,12	24	38	12.27
14		21	42	8.24
15		22.5	41	15.17
16	7,13	25.5	39	17.48
17	7,16	25.5	43	15.0
18		27	40	6.0
19	16,17	25	41.5	16.13
20	16,19	25	37.5	16.30

(1) Vertex 8 was rejected because of low selectivity.

(2) Vertex 10 was rejected because of low selectivity and high noise.

(3) Vertex 7 was re-evaluated repeatedly to verify its response (n + 1 rules).

(4) At vertex 12, negative peak was observed occasionally

The long-term stability of this detector is still in controversy. Hartigen et. al.[104] have reported a long-term stability test of this detector by repeated analysis of methamphetamine at 50 ng and 500 ng levels for a period of one month. The coefficient of variation was found to be 1.5% for both concentrations. However, Lubkowitz and coworkers[105] measured the detector background current over a period of 16 days and found the current decreasing from 51 to 0.8 picoamps. The detector sensitivity thus dropped more than 5 times. These authors suggested that strict control of a constant bead current (10 to 30 picoamps), instead of the constant dial setting for heating current was essential for obtaining long-term stability.

The observation made in this laboratory was for a newly-installed bead, the sensitivity was changed greatly during the first week of operation which was reflected from the required increasing of bead temperature to maintain the sensitivity. After a few weeks operation, the detector performance more or less approached a steady state, but required nearly maximum heating current. The long-term stability of this detector is still in question and remained to be clarified.

The detection mechanism as described in the previous section does not apply universally to other nitrogen-phosphorus detectors which have somewhat different instrumental set-up. The described theory is very useful in understanding the working parameters and their effects on the present design.

C. Sampling System

Micro solvent extraction technique[95] was used in this study mainly

because of the limited amount of serum sample, usually less than 5 ml.

Glass-distilled anhydrous ether was used as the extraction solvent because of its high volatility, low retention on the glass wool adsorbent and low affinity to water. Freshly distilled ether was stored in culture tubes with teflon-lined screw caps in the freezer. Ether blanks were checked from time to time for purity. Carefully distilled ether always gave excellent blanks in FID and NPD profiles.

Glass wool was used as an adsorbent because of its adsorptive properties for low-molecular-weight compounds, low affinity for ether and water, and inertness. Unproperly conditioned glass wool was one of the sources of contamination. Its blank was run at intervals to check the quality of the conditioning. Its inertness is demonstrated in Figure 30. Two chromatograms belong to the same serum sample. A was run immediately after sampling, while B was run after storing the glass wool tube in the freezer for one week. No appreciable profile differences could be found.

Since the essence of this work was based on profile comparison, a high precision of sampling was required. Profiles in Figure 31 are from the serum of a healthy subject, run on different days. Few variation in concentrations can be observed.

D. Organic Volatiles in Blood Serum

In this work, blood, instead of urine was used because first the composition of volatile materials in urine is effected more by external factors, such as the sampling time of the day, its total volume.[106] The blood maintains a better steady equilibrium for its composition.

FIGURE 30: THE INERTNESS OF GLASS WOOL

PROFILE A: Run Immediately after sampling

PROFILE B: Store in freezer for one week

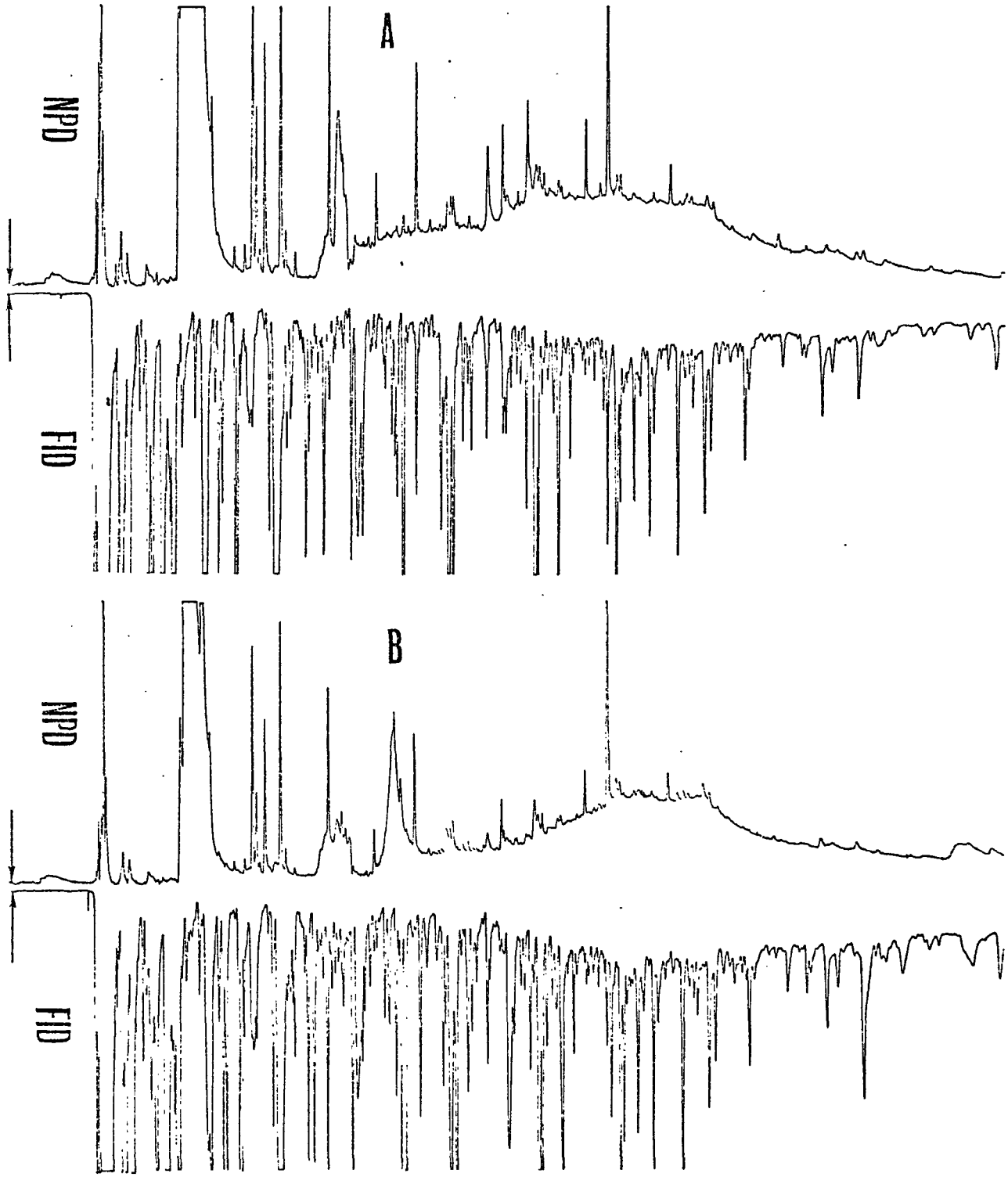

A
NPD
FID
B
NPD
FID

FIGURE 31: REPRODUCIBILITY OF PROFILES FROM THE SAME SERUM SAMPLE

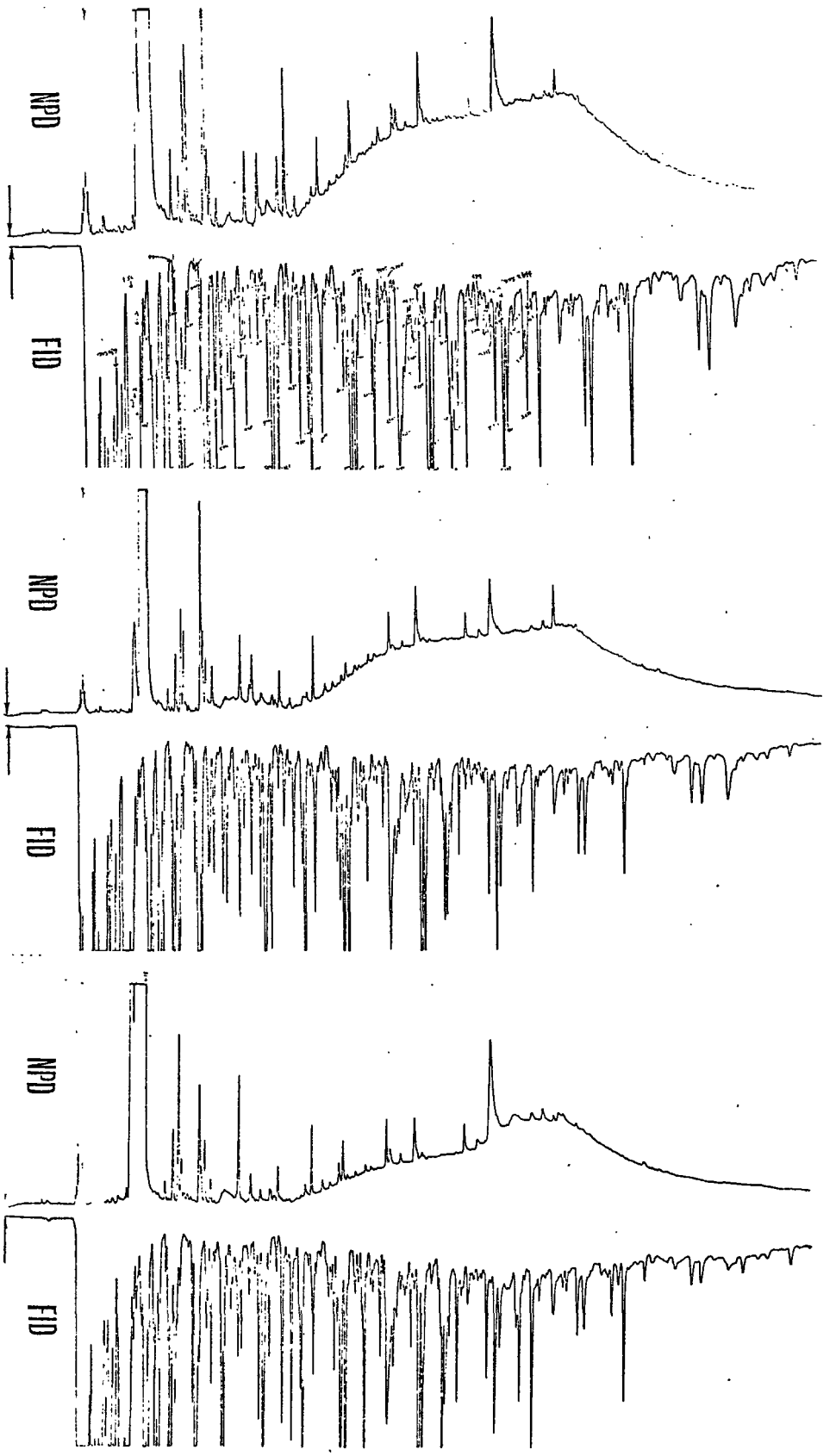
NPD
FID
NPD
FID
NPD
FID

Second, owing to the circulation of the blood in the body, it has an intimate contact with the cells in the metabolism. The constituents in the blood should reveal more information. Since cancer is a disease which often effects the entire body, blood seems to be a reasonable information source for this study. In previously reported comparisons by head-space technique,[96] GC profiles of the volatile organics in serum of normal individuals was not as complex as the urinary profile so that less total information was provided from serum. However, by the micro-solvent extraction technique, the constituent and concentration of serum as in Figure 31 are as complex as urine as is shown elsewhere.[92] Direct comparison of the profile was the backbone of this analysis; no identification of the constituents was attempted.

1. Profiles of Normal Serum:

In developing the procedure for early detection of cancer disease, conclusive information concerning the pathological change in metabolic pathways requires the establishment of reproducible GLC serum profiles of normal individuals. Serum samples of normal subjects were analyzed under strict control of sampling and chromatographic conditions. Figure 32 shows the FID/NPD profiles of three healthy individuals. The highly complex FID profiles show strong resemblance and good reproducibility with some minor differences in concentration. However, the simultaneously generated, simplified NPD profiles show more variation in concentration. The rising baseline in NPD profiles was first assumed to be the helium carrier flow rate change during temperature programming so that the

FIGURE 32: REPRODUCIBILITY OF PROFILES FROM THREE DIFFERENT HEALTHY INDIVIDUALS


NPD
FID
NPD
FID
NPD
FID

cooling effect from carrier gas was varied which in turn changed the bead temperature. But several runs of ether blanks under the same sensitivity all with straight baseline, ruled out this assumption. The only possibility is that some unknown materials were co-extracted with the volatile organics and detected by the NPD.

Profile characterization for volatile organics in normal urine has been studied extensively.[98] The volatile nitrogen-containing compounds are pyrazines in trace quantities, pyrroles, and isothiocyanate. Since some of the major volatiles in urine are known to be present in serum as well,[96,97] the nitrogen-containing compounds are also likely to exist in the serum. The presence of volatile phosphorus-containing compounds is less likely.

2. Profiles of Serum of Cancer Patients

Fifteen drug-treated leukemia samples were first carefully processed and profiles obtained were compared with those of healthy individuals. No significant profile differences could be found as shown in Figure 33, except for some minor changes in concentration for some components. Peak numbered Imp was found to be the contamination from the rubber stopper of the "vacutainer" which was used as the standard apparatus for the collection of blood in the hospitals. This peak was identified as butoxy-ethanol, a plasticizer in the rubber stopper. This peak was thus ignored. Other interesting profiles are represented in Figures 34 and 35. The three profiles in Figure 34, are from the same individual in different stages of leukemia during a 4 month period. Chromatogram A represents the initial confirmation of leukemia but not yet with drug

FIGURE 33: SERUM VOLATILE PROFILES (FID/NPD)

A: Leukemia

B: Normal

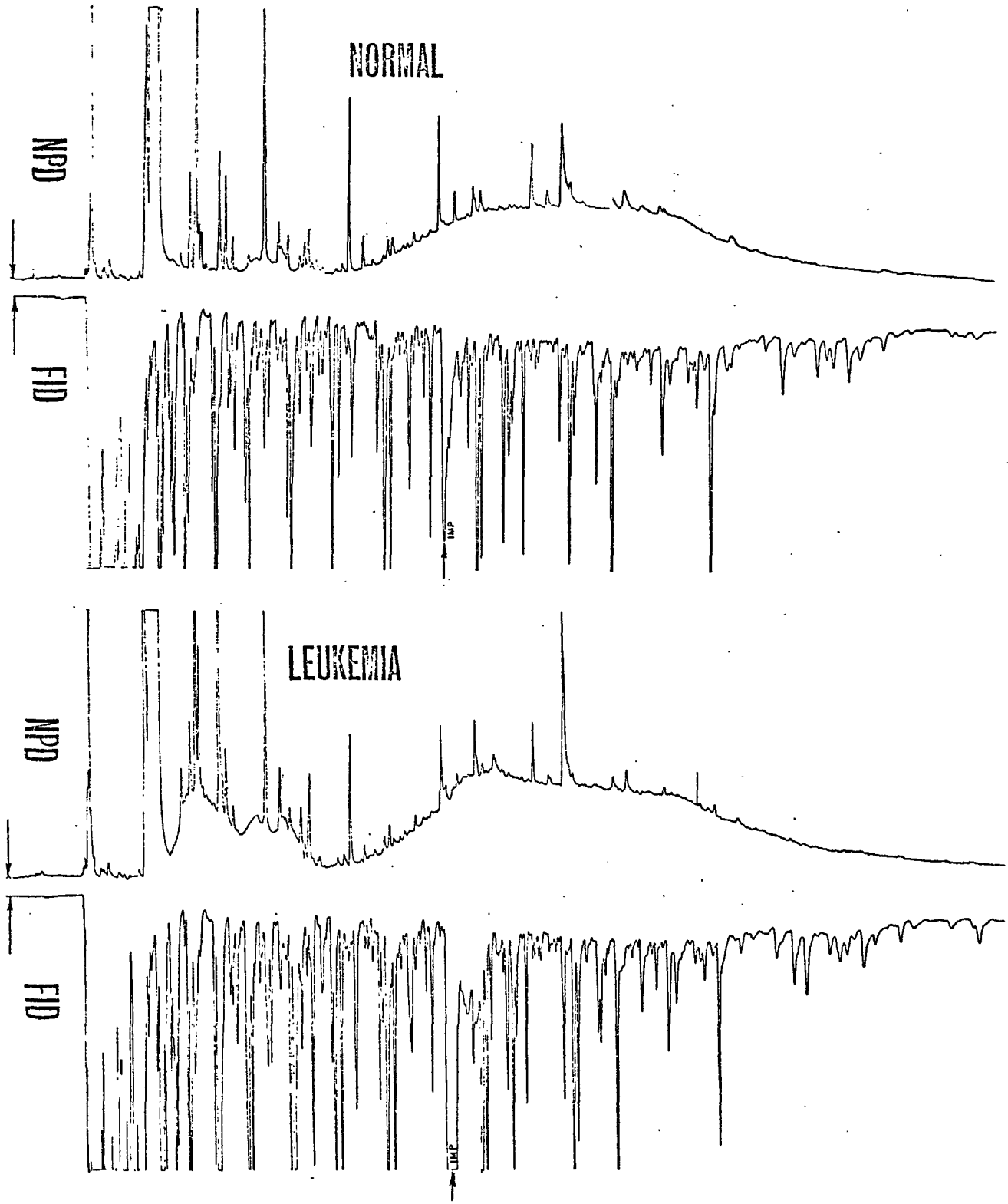
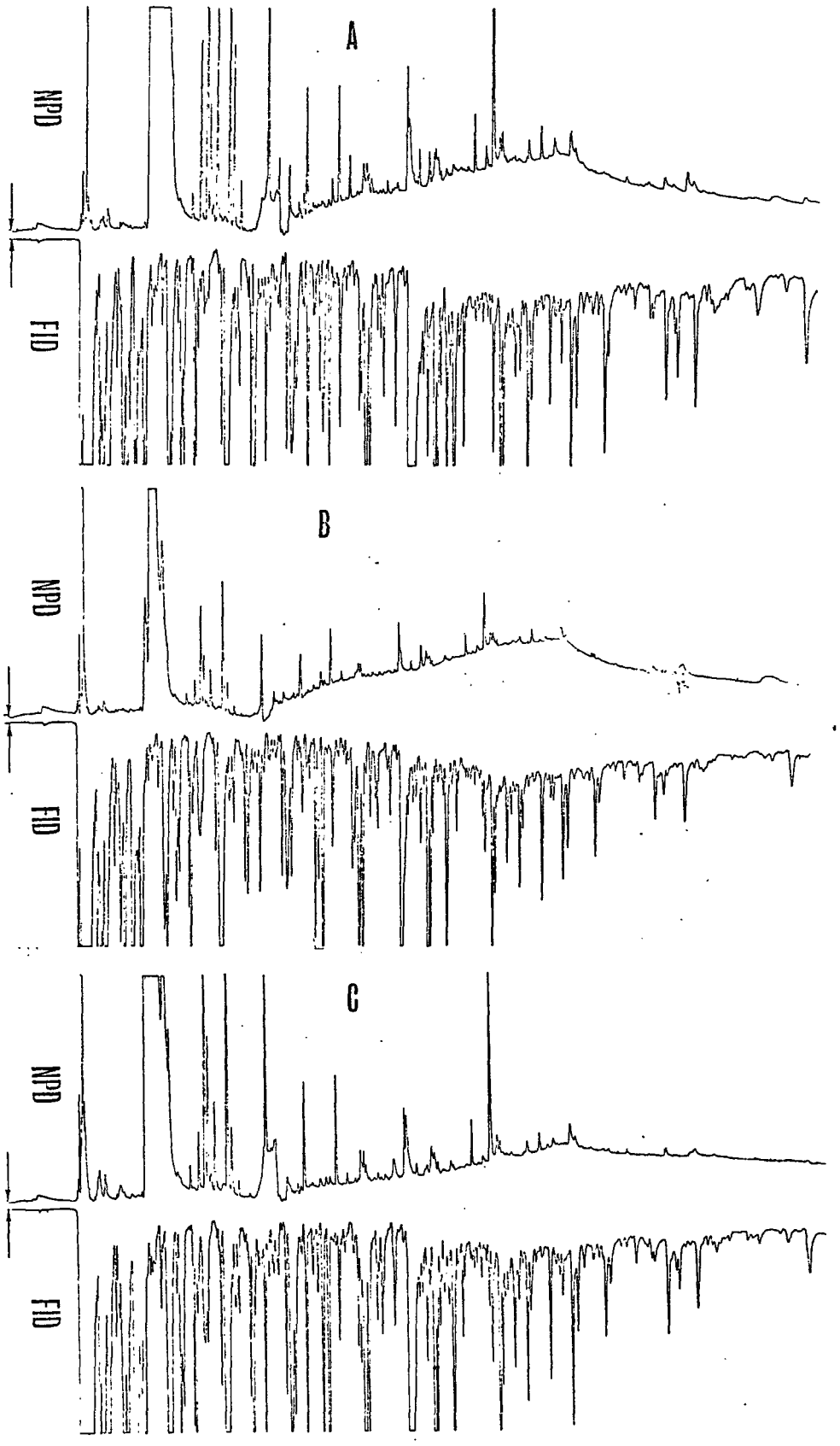

NORMAL
NPD
FID
INJP
LEUKEMIA
NPD
FID
INJP

FIGURE 34: SERUM PROFILES FROM THE SAME LEUKEMIA PATIENT

A: Before chemotherapy

B: During chemotherapy

C: In remission


A
NPD
FID
B
NPD
FID
C
NPD
FID

treatment, while chromatogram B is during the stage of chemotherapy. The effect observed is that the drug seems to surpress the excretion of volatile compounds in the metabolic pathway, since the concentration of all the peaks in profile B is lower than that in profile A, particularly those of nitrogen-containing compounds. Chromatogram C represents the patient in a remission stage, and shows increased concentration of nitrogen-containing compounds in NPD profiles.

The profile A in Figure 35 is from the serum of a mouse with acute leukemia compared with the profile of control mouse serum. Strong similarities between these profiles is observed except for some peaks with different concentrations. Owing to the amount of sample, only 50 μl serum could be used for the analysis. Profiles A and B also show no significant difference compared with those of human healthy and leukemia individuals.

Chromatograms representing other types of cancer sera are presented in Figures 36, 37, 38 and 39. The first profile is from a breast cancer patient, the second that of an individual with melanoma, the third that of a patient with lung cancer, the fourth that of a person with cancer of the colon. The most surprising and interesting result is the remarkable similarity in the FID profile between each patient and the healthy person. However, in NPD profile, of each patient, very large concentration differences were again observed.

FIGURE 35: MOUSE SERUM PROFILES

A: Control

B: Acute Leukemia

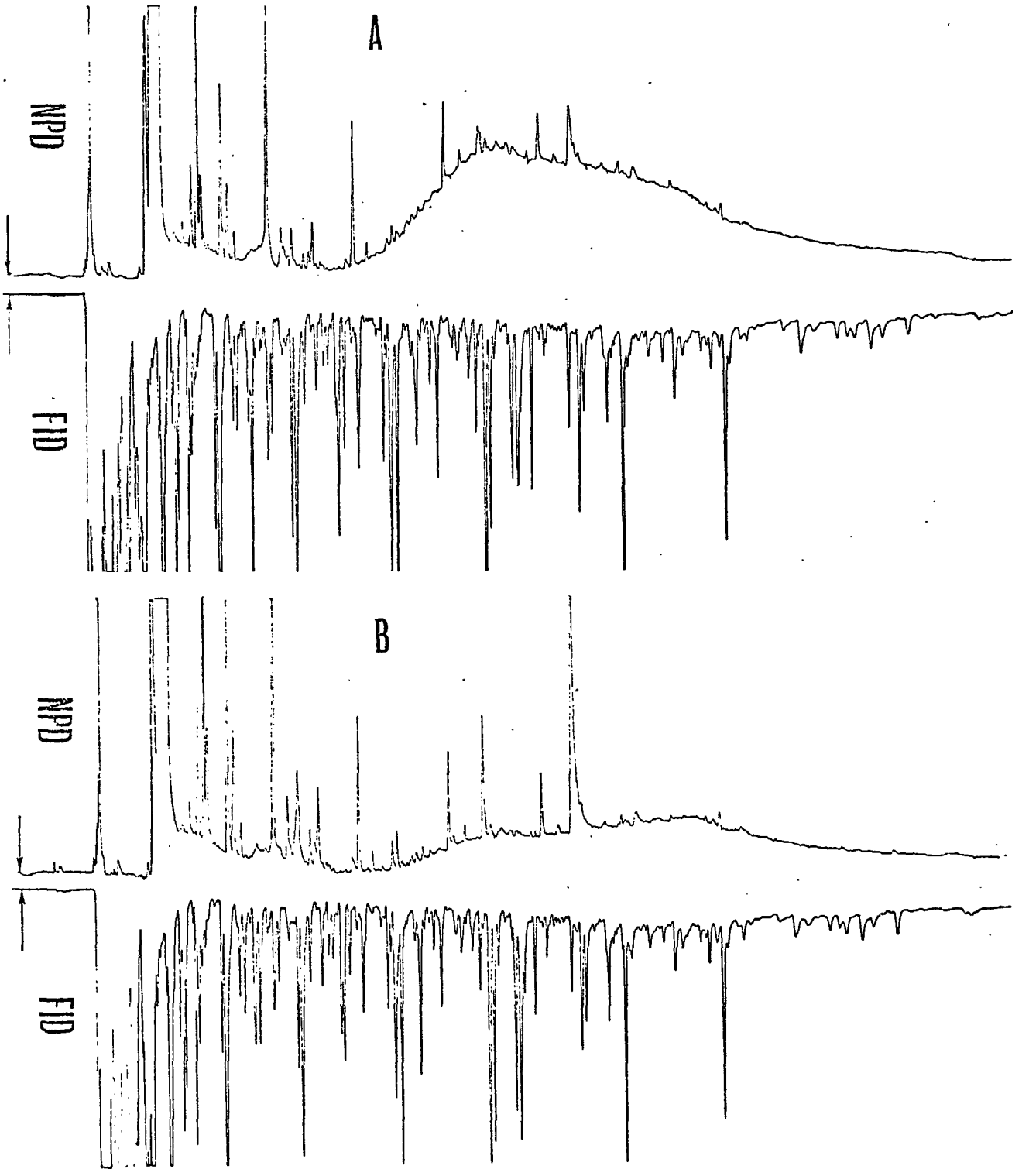

A
NPD
FID
B
NPD
FID

FIGURE 36: SERUM PROFILES OF BREAST CANCER

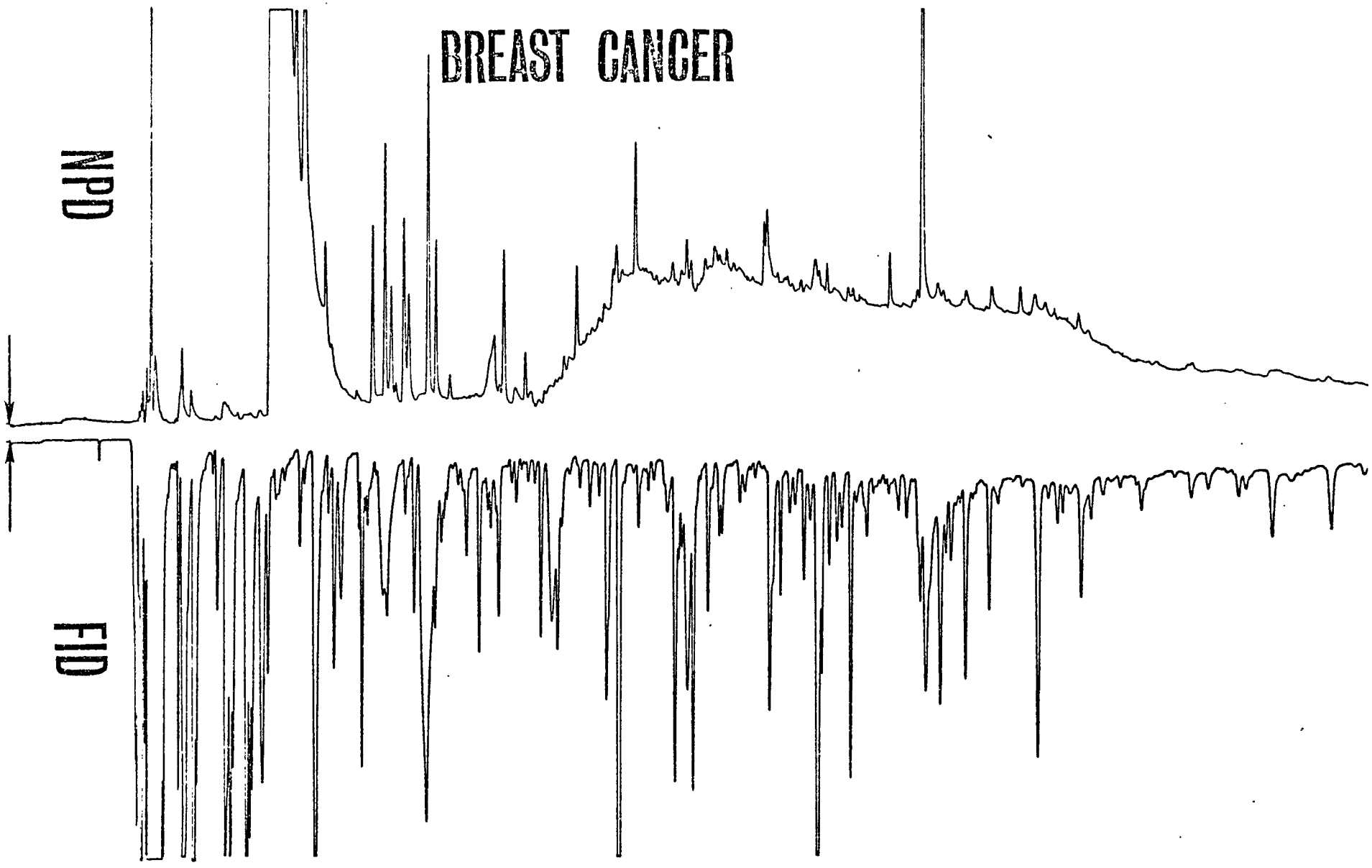

BREAST CANCER
NPD
FID

FIGURE 37: SERUM PROFILES OF MELANOMA TUMOR

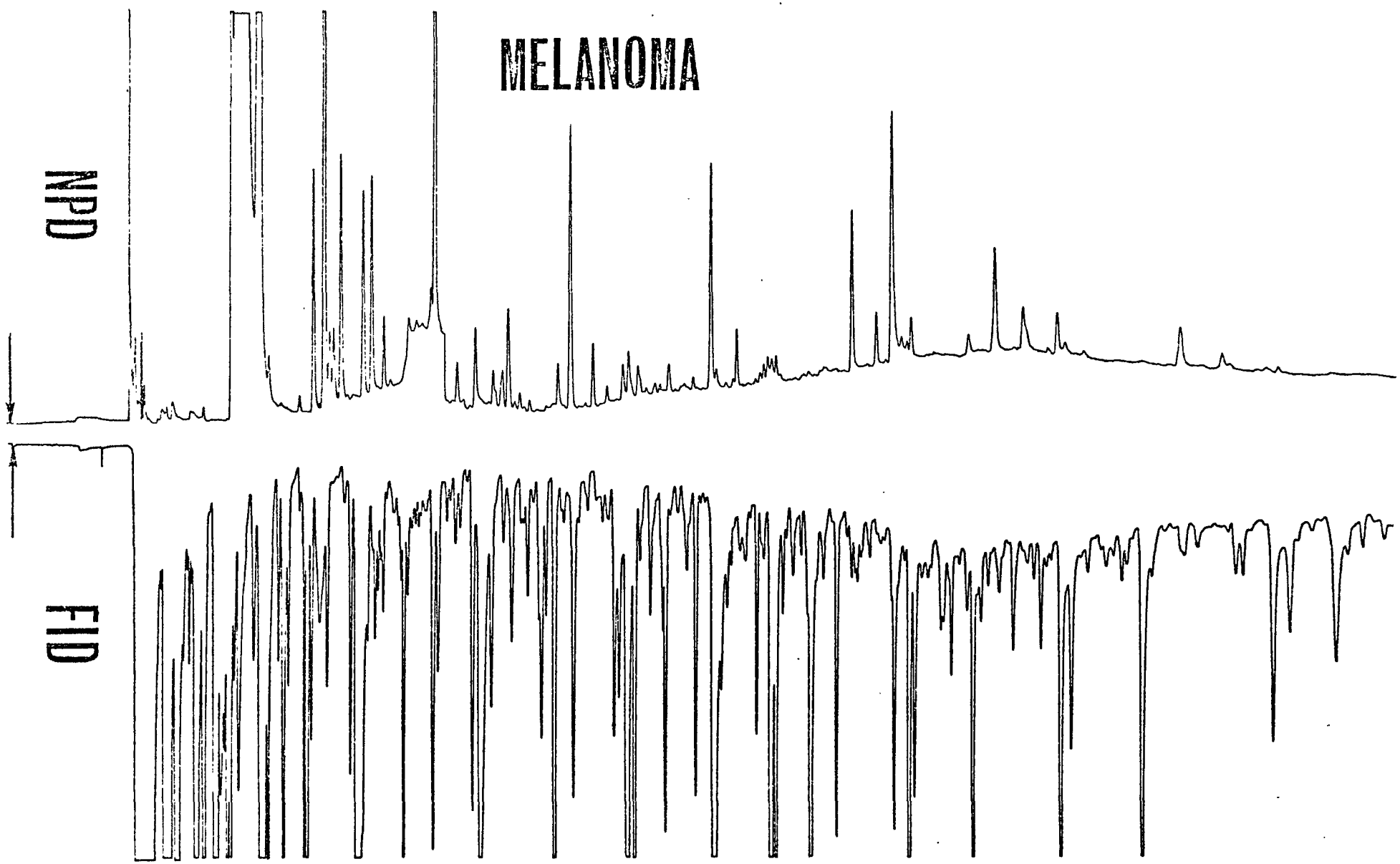

MELANOMA
NPD
FID

FIGURE 38: SERUM PROFILES OF CANCER OF LUNG

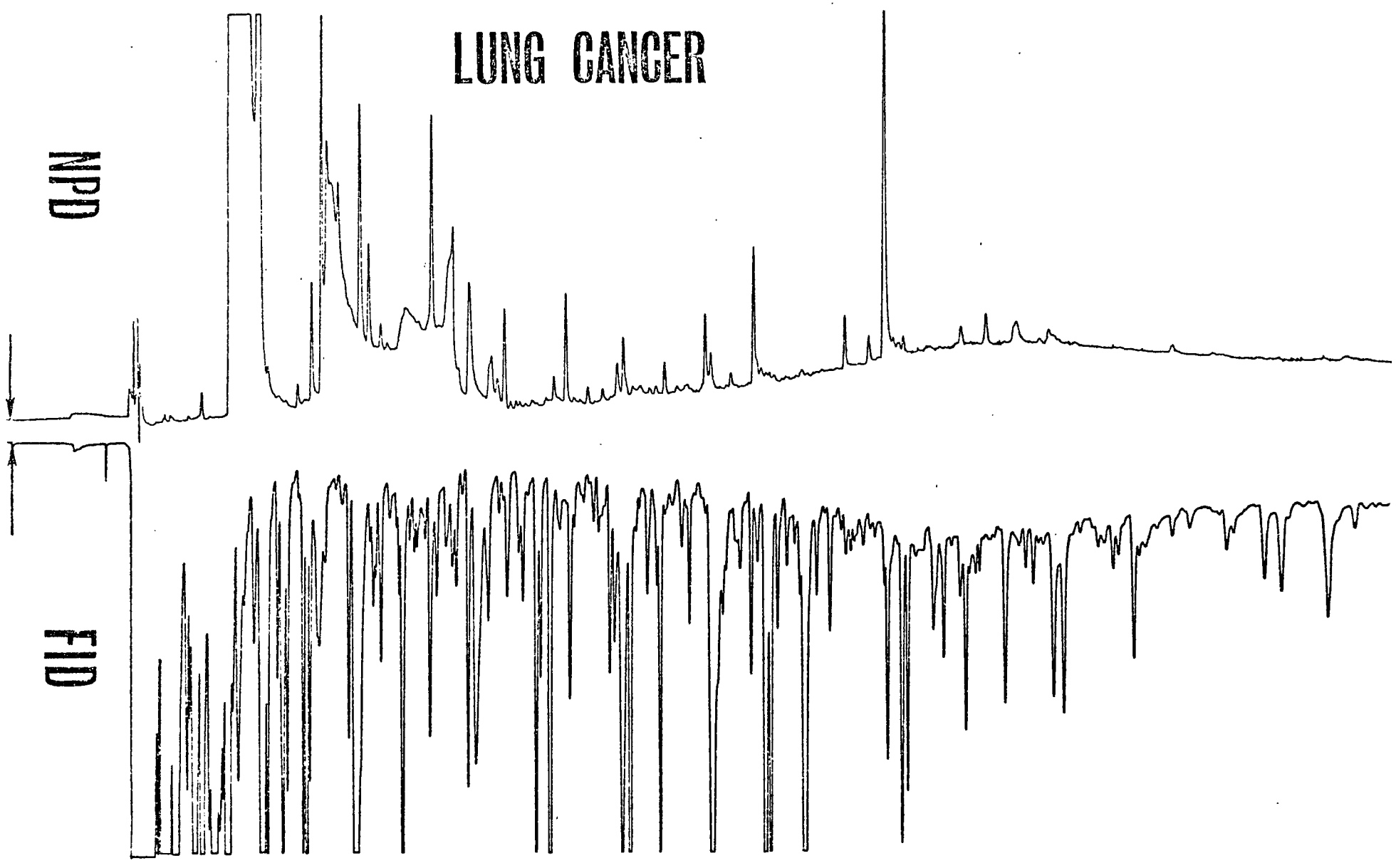
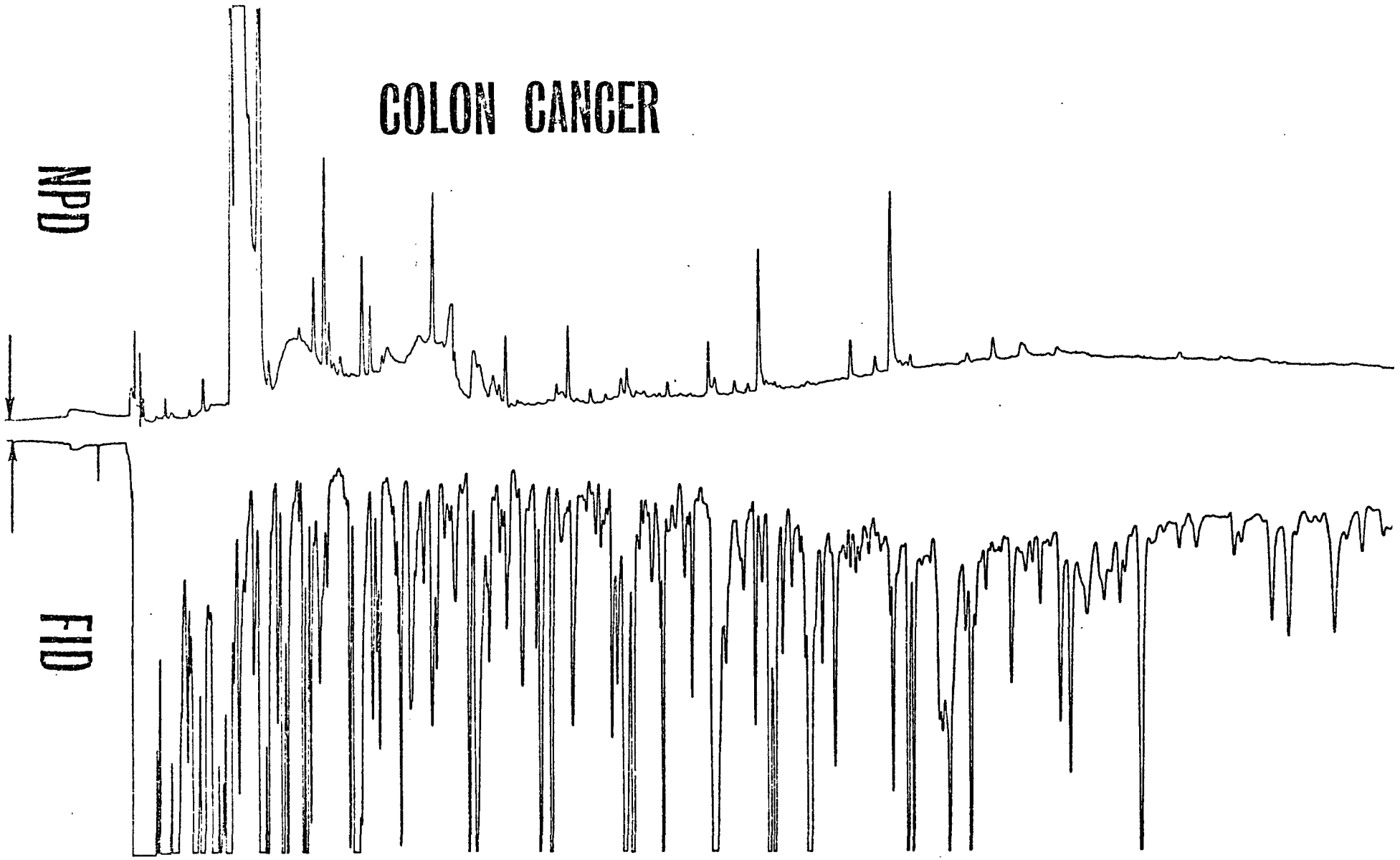

LUNG CANCER
NPD
FID

FIGURE 39: SERUM PROFILES OF CANCER OF COLON


COLON CANCER
NPD
FID

CONCLUSION

CONCLUSION

With high resolution capillary GC and a combination of the universal flame ionization detector and the specific nitrogen-phosphorus detector, serum samples representing seven different cancer disease were analyzed. By comparing profiles with those from healthy individuals, the information obtained may lead to the early detection of some types of cancer.

The nitrogen-phosphorus detector was first optimized by simultaneously changing its hydrogen and air flow rate. The results obtained clearly indicate the detector sensitivity is a function of the age of the glass bead containing the rubidium silicate as an alkali source. It was therefore necessary to use a high heating current to obtain a constant performance. This however, sacrifices the total life time of the glass bead.

By qualitative profile comparison, no obvious differences, which can be used as a positive differentiation between the healthy and the diseased state for any of the cancer sera studied, has been found. However, further studies with identification of the major compounds in both FID and NPD profiles, and the application of the pattern recognition methods, may open a new avenue for finding the significant correlation and make this procedure a screening method for early detection of disease.

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