AN ABSTRACT OF THE THESIS OF

Phyllis Anne Ericson for the Master of Science Degree

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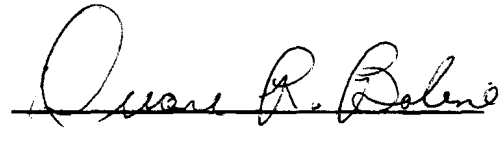
Title: Establishing a Reliable Clinical Toxicology Program for the Lincoln, Nebraska Area Hospitals

Abstract approved: 

A clinical laboratory toxicology section was established for a large midwestern pathology laboratory service. Methods of analysis of drugs of abuse and therapeutically monitored drugs in biological materials are discussed. Assay methods were established for the anticonvulsant drugs, phenytoin, primidone and phenobarbital, the cardiac drugs, digoxin and quinidine, and for theophylline, an antiasthmatic drug. A screening procedure was also developed for detecting the presence of approximately twenty drugs of abuse, such as amphetamines, barbiturates, morphine, darvon, demerol and the benzodiazepams. The procedures covered include direct screening tests, various extraction techniques, UV spectrophotometry, thin-layer chromatography, gas-chromatography and enzyme immunoassays. The main advantages and disadvantages of the methods are discussed. The organization of the request and report forms as well as other factors involved in establishing a working Toxicology Department are also included in the discussion.

ESTABLISHING A RELIABLE CLINICAL TOXICOLOGY PROGRAM FOR THE LINCOLN, NEBRASKA AREA HOSPITALS

A Thesis

Presented to

the Department of Chemistry

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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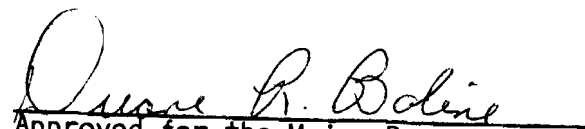
by

Phyllis A. Ericson

May 1978



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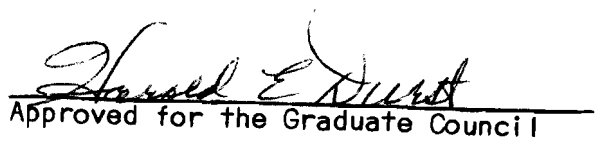

Approved for the Graduate Council

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I am also grateful to my advising professor, Duane Boline and to my husband, Ted, for their continuous encouragement throughout this study.

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INTRODUCTION

There is little doubt that one of the fastest growing areas of clinical chemistry is toxicology. Throughout the United States today there are scores of laboratories involved with drug analysis. In the hospital clinical laboratory the primary interest is general health care, whether it is monitoring therapeutic blood levels, evaluating the cause of an overdose or aiding law enforcement agents in the investigation of a suspicious death. This wide variety of situations must be considered and evaluated in proposing a method of analysis. Since they vary for each geographic area, each toxicology program is unique.

In order to establish a reliable clinical toxicology program for the Lincoln, Nebraska area hospitals, the existing drug assay procedures were evaluated and additional methods were proposed. Consideration was given not only to the specific drug assays needed but also to sensitivity requirements (depending on sample size and type of sample), intended use of data, projected workload, turn-around time and instrumentation available.

The assays available at the time the study was initiated included methods for ethanol, salicylate, carb on monoxide; phenytoin, phenobarbital and primidone by gas-liquid chromatography; digoxin and digitoxin by radioimmunoassay. These procedures were being performed by three different groups of technologists in three different laboratories. Therefore a major goal of this study was to combine all toxicology testing into one laboratory to be performed by one staff.

Since the geographic area involved included the three Lincoln, Nebraska city hospitals and approximately 35 smaller hospital laboratories in a 50 mile radius of the city, there was a definite need for a screening program for drugs of abuse. Also the Lancaster county Nebraska coroner was a staff pathologist in the laboratory which automatically involved the clinical laboratory in many cases.

It was apparent that there were two major areas of the toxicology program which needed to be developed: (1) screening for drugs of abuse and (2) monitoring therapeutic blood levels of prescribed drugs. Besides a screening method for drugs of abuse several methods for confirmation and quantitation of those drugs were investigated. Since several procedures would be involved, a logical flow-chart for analysis was also necessary.

Due to the increasing number of physician requests for antiepileptic drug levels and the need for digoxin levels to be available anytime, 24 hours a day, it was the purpose of this study to develop more rapid and efficient methods for these assays in addition to establishing methods for the analysis of quinidine and theophylline levels.

THERAPEUTIC DRUG MONITORING

METABOLISM

When the physician prescribes a drug in the treatment of his patient he usually begins by administering the drug at the recommended standard dose and then carefully monitors his patient's progress. However, he may find that a drug regimen found to be satisfactory in one patient may be ineffective or even toxic to another.[6] Pharmacologists have attributed these individual differences to many factors including (1) inherited or acquired variations in drug disposition (absorption, distribution, metabolism and excretion), (2) underlying disease processes (low renal clearance, hepatic disease, congestive heart failure), (3) noncompliance and (4) varying availability in the drug formulation used.[37]

Most drugs and other organic substances which are foreign to body chemistry undergo metabolic changes as part of the body's detoxification process. Figure 1 shows a simplified scheme of the metabolism of a drug by the body. Factors which may influence the metabolic pathway of a drug include (1) the individual's age and sex, (2) environmental conditions and (3) administration of other drugs concurrently.[14]

Absorption of a drug is the first important factor which may differ from individual to individual. A large percent of the ingested drug may pass directly through the body and be excreted. The amount that is absorbed into the blood stream is found in equilibria either as the "free" drug or that which is bound to plasma proteins. Only the "free" form of the drug is physiologically active--can pass to the cell

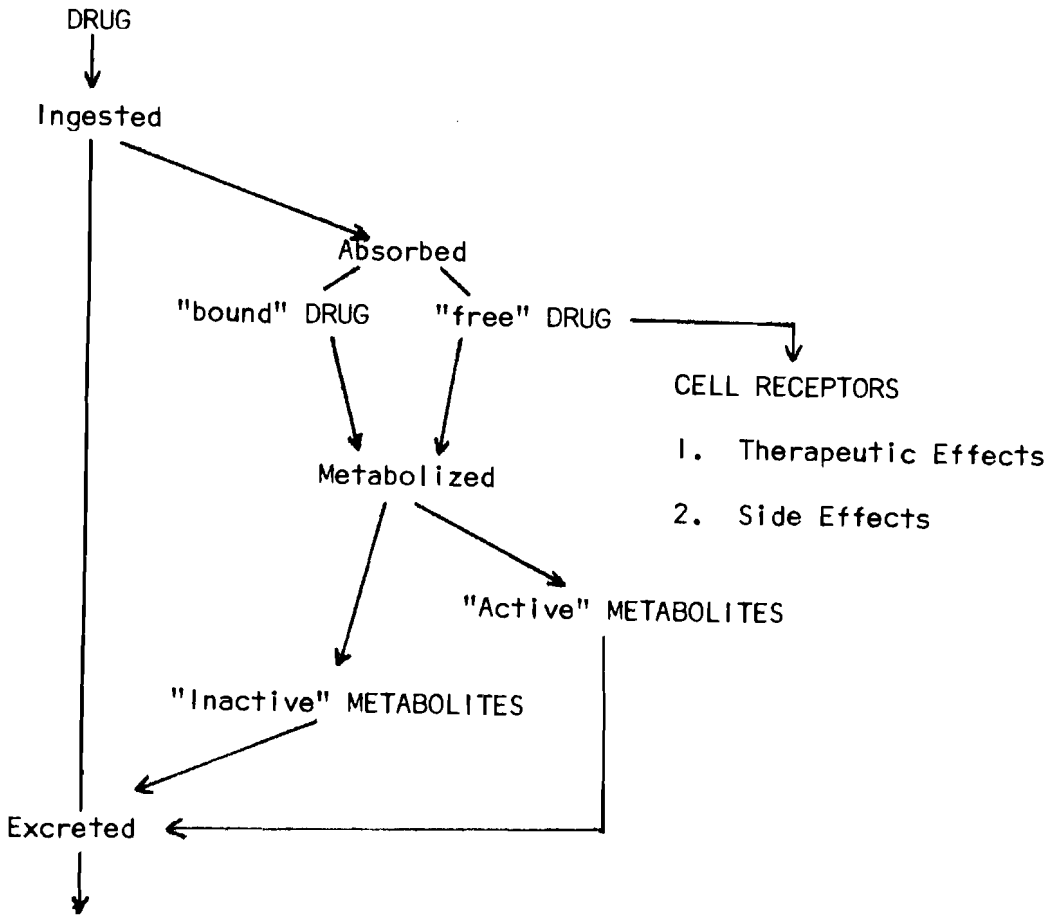
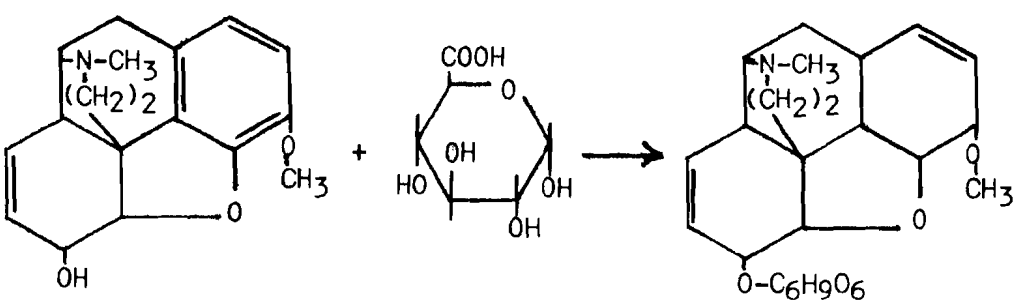


Figure 1. Metabolism of Drugs.

receptor sites and elicit a responce. Ultimately the measure of the amount of drug at the cellular level would be the ideal way of monitoring therapeutic drug levels.

The liver is the major site of drug metabolism and in general the metabolites of most organic compounds are highly polar, water-soluble substances of low toxicity. However, instances have been reported in which some drugs are converted to metabolites which are even more toxic than the parent compound.[5]

Drugs undergo many different types of chemical reactions leading to the formation of metabolites. In general there are four main types: oxidation, reduction, hydrolysis and conjugation. Conjugation may occur directly with the parent drug (in the presence of a suitable site such as hydroxyl, amino or carbonyl groups) or with one of its metabolites. Conjugation results from the reaction of β-glucuronic acid (an oxidation product of glucose) with the ingested drug and/or its metabolite to form compounds with either glucosidic or ether linkages, Equation 1.[14]



(1)

Codeine β-Glucuronic Acid ($C_6H_{10}O_7$) Codeine-6-Glucuronide

Pharmokinetics

Pharmokinetics involves the determination of the relationship, if any exists, between drug dose, blood levels and biological effect. Only those drugs demonstrating a significant correlation between blood levels and therapeutic effect are candidates for therapeutic monitoring.

In order to obtain reliable data for monitoring drug blood levels one must have a basic understanding of anticipated blood concentrations versus time. Figure 2 shows the serum concentration of a hypothetical drug versus the time in hours since the drug was administered. If an oral dose is repeated at intervals less than four-times the half-life of the drug, accumulation results. As dosage continues, the drug level gradually rises until the amount of the drug eliminated per dose interval equals the amount administered per dose. The average concentration reaches a "steady state" at this point. Therefore, the concentration achieved is directly proportional to the total amount of drug given and the drug's half-life.

Ideally the blood sample whould be obtained after steady state has been achieved (at least five half-lives after drug therapy is begun) and just prior to the next scheduled dose.[41] Additional blood levels should be obtained whenever there is a change in dosage, a change in the patient's response to drug therapy (if either symptoms of toxicity or noncompliance appear), changes in other medications being administered or when changes in the patient's physical condition occur.

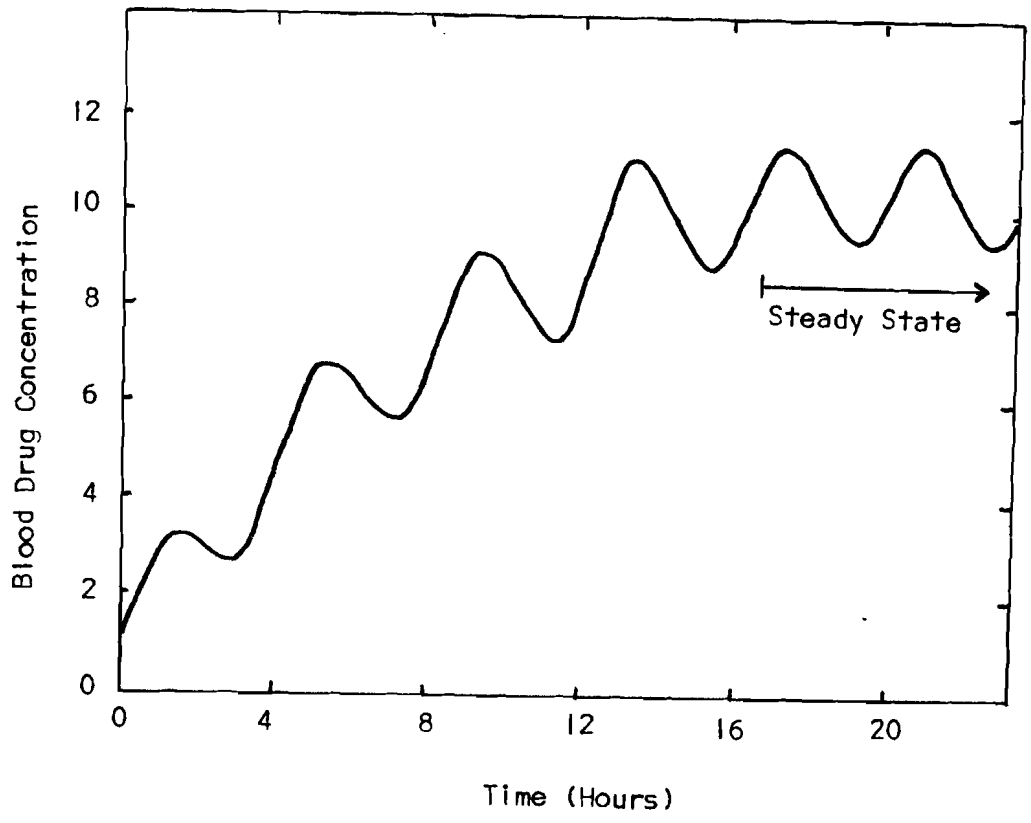


Figure 2. Blood Drug Concentration vs. Time to Establish "Steady State"[4]

THE ANTICONVULSANT DRUGS

In managing the epileptic patient, the clinician is faced with the inherent uncertainties of a disease that is episodic. It often requires powerful and potentially toxic drugs, administered chronically and frequently, often as part of a multi-drug regimen. Thus because of the many variables involved it is best to evaluate each patient individually.

Table I shows the current therapeutic and toxic ranges of the common anticonvulsant drugs.[39,41]

Phenobarbital was introduced in 1912 and is one of the most commonly used drugs in anticonvulsant therapy. It should be noted that phenobarbital metabolism in children is must more rapid than in adults, the half-life of the drug being 1 1/2 to 2 days as compared to the long half-life of 2 to 6 days in adults. Early methods for phenobarbital determinations included colorimetry, ultra-violet spectrophotometry and thin-layer chromatography.[3,9,11]

Phenytoin was introduced as an anticonvulsant drug in 1938 and is one of the most frequently prescribed drugs for the control of grand mal seizures. The earlier methods of measuring phenytoin, colorimetry and ultra-violet spectrophotometry, have been replaced by the more sensitive and specific gas chromatography and immunoassay methods.[30,31]

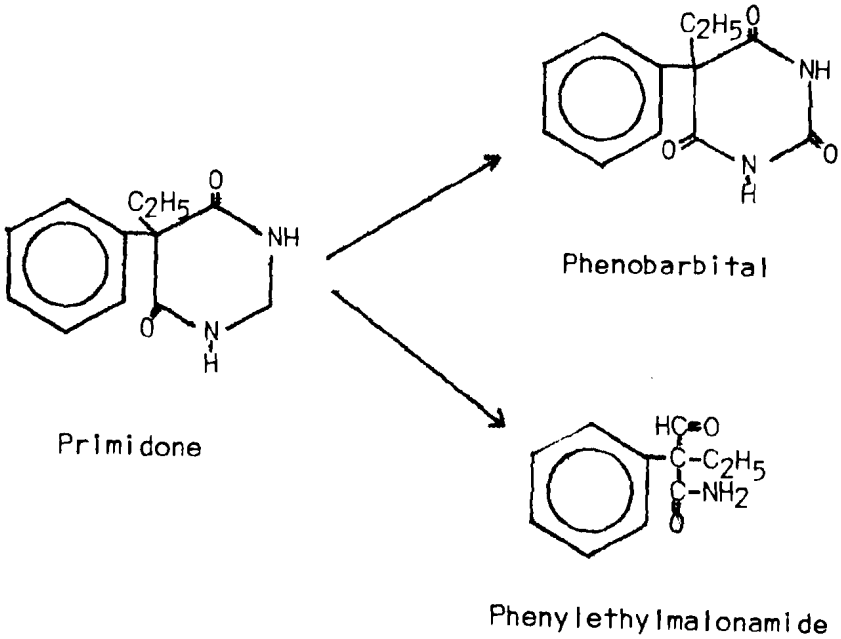
Of primary interest from Table I is that primidone is converted to two active metabolites: phenobarbital and phenylethylmalonamide (PEMA), Equation 2. Both accumulate during chronic administration, phenobarbital reaching levels up to four times higher than primidone.[22]

Table I

Characteristics of Common Anticonvulsant Drugs

Drug	Therapeutic Level	Toxic Level	Half-Life	Major Metabolites
Phenobarbital (Luminal)	15-40 ug/ml	Above 40 ug/ml	2-6 days, adults 1 1/2 - 2 days, children	p-Hydroxyphenyl Derivatives
Phenytoin (Dilantin)	10-20 ug/ml	Above 20 ug/ml	Dose Dependant	p-Hydroxyphenyl Derivatives
Primidone (Mysoline)	5-12 ug/ml	Above 12 ug/ml	3-19 hrs, adults	Phenylethylmalonamide (PEMA) Phenobarbital
Phenobarbital as a metabolite	15-40 ug/ml	Above 40 ug/ml		

Because blood levels of primidone tend to fluctuate due to a shorter half-life, greater reliance is usually placed on evaluating the phenobarbital level in patients receiving primidone.[18] Information on the other metabolite, PEMA, is incomplete at this time.



(2)

THE ANTICONVULSANT DRUG PROFILE BY GAS CHROMATOGRAPHY

Gas-liquid chromatography has been for the last eight years the method of choice for quantitative drug analysis. It has enabled the analyst to have greater reproducibility by incorporating internal standards and greater sensitivity through the use of the flame ionization detector and by derivitizing the drugs than other analytical methods could offer. It also allows the analyst to determine several drug levels simultaneously.

The original procedure for the determination of anticonvulsant drug levels by GLC was introduced in early 1973, based on the procedure published by Kupferberg.[21] However, with the following modifications the procedure has become more reliable, reproducible and more easily and rapidly performed on a routine basis, Figure 3.

1. The chloroform-toluene extractions were replaced by the adsorption of the drugs onto charcoal and elution into diethyl-ether resulting in a cleaner sample, thus prolonging the life of the GLC column.
2. It was found that phenobarbital decomposed rather rapidly in solution (even in methanol) therefore the prepared drug standard solution was not accurate after 12 hours and had to be prepared daily (a process that involved at least 30 minutes to insure complete dissolution of the sample). Preparation of a large volume of standard solution, carefully aliquoting 1.0 ml samples into test tubes and removing the methanol by quick evaporation resulted in a 3-month

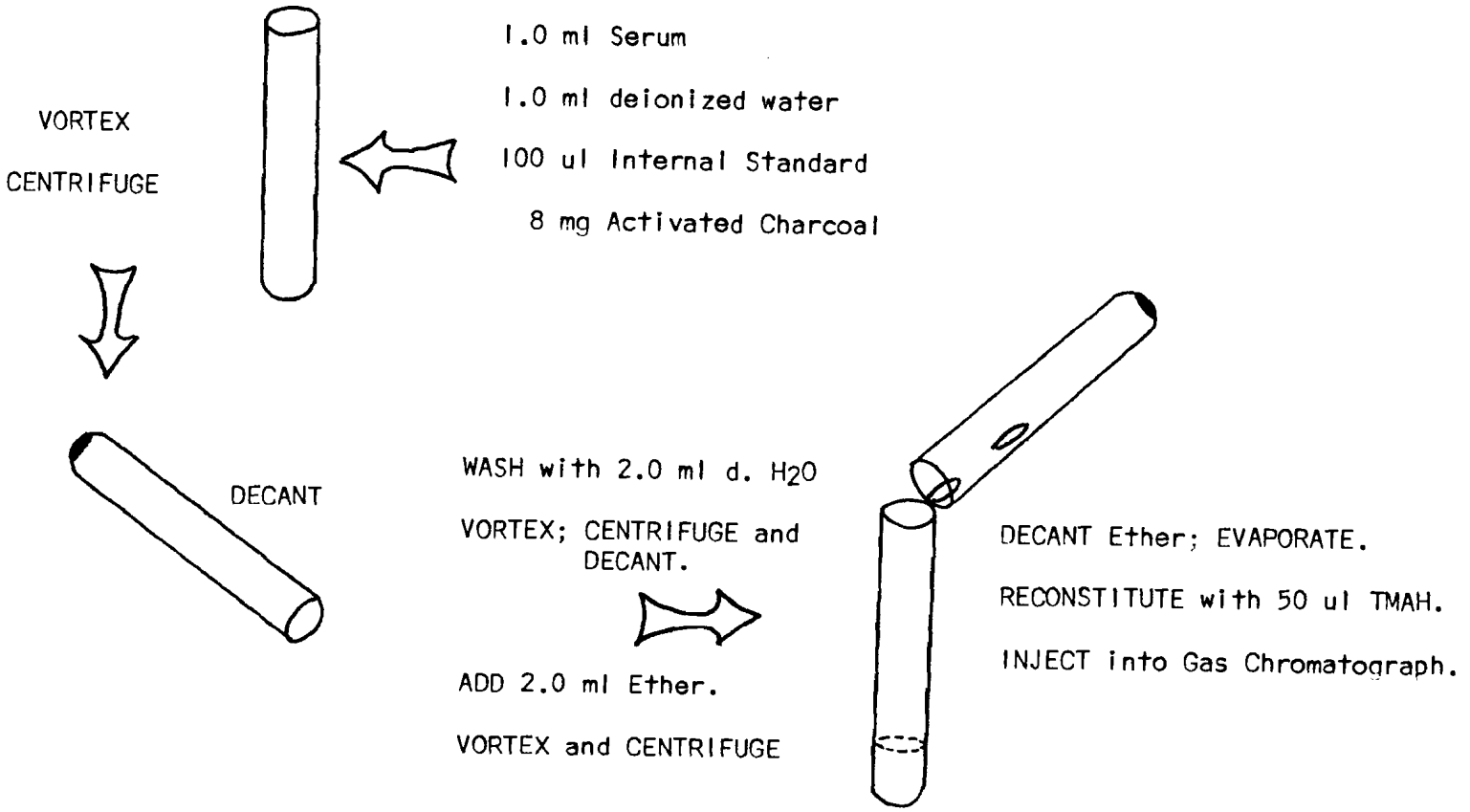


Figure 3. Anticonvulsant Drug Assay by Gas Chromatography

supply of standard with the added advantages of stability, increased reproducibility and an overall decrease in technician's time. The new stock standard once reconstituted with 1.0 ml methanol could easily be checked against the old supply for any changes in concentration so that consistency from batch to batch could be maintained.

3. Since phenobarbital rapidly decomposis in solution, each sample was reconstituted just before injection to limit the decomposition as much as possible. If however a repeat GLC analysis was desired, reinjecting the sample even 15 minutes later showed a significant decrease in the concentration present. The availability of 5-ethyl-5(p-tolyl)barbituric acid (Aldrich Chem. Co.) for use as an internal standard greatly improved the accuracy of the phenobarbital assay.
4. Since no commercial control was available for the anti-epileptic drugs, a spiked serum control was prepared which helped establish a working quality control program.

Instrumentation

The GLC analysis was performed on a Bendix, Model #2500 gas chromatograph equipped with flame ionization detector and digital integrator and recorder. The separation was achieved using a 6 ft. x 1/8" i.d. U-shaped glass column containing 3% OV-17 on Gas Chrom Q (100/120 mesh). The column was conditioned overnight at 310°C (30°C above the maximum

operating temperature) with a Nitrogen flow rate of 20 cc/minute. The following operating conditions were maintained during analysis, Table 2.

Table 2

Instrument Parameters for GLC Analysis of Anticonvulsant Drugs

		Input Pressure (psi)	Flow Rate (cc/min)
Gas Flow Rate:	Nitrogen	50	50
	Hydrogen	10	40
	Air	50	400
Temperatures:	Injection Port:	300°C	
	Detector:	300°C	
	Oven (Program):	165°C initial to 280°C final @ 10°C/min.	

Reagents

1. Ethyl ether, Anaestesia Grade (Mallinchrodt).
2. Charcoal, Norit "A" neutral, Radioimmunoassay Grade (Schwartz-Mann).
3. Trimethylphenylammonium Hydroxide, 0.2 M in Methanol, "Methelute" (Pierce Chemical Co.).
4. Drug Standard: 100 ml is prepared by weighing and diluting to volume with methanol the following drugs:

Drug	Supplying Co.	Weight	Resulting Conc.
Sodium Phenobarbital	Brinkman	40.0 mg	364 ug/ml
Primidone (Mysoline)	Ayerst Labs	20.0 mg	200 ug/ml
Phenytoin (Dilantin)	Applied Sci.	20.0 mg	200 ug/ml

One milliliter aliquots of the standard solution is dispensed into small tubes and the methanol removed quickly by evaporating in an 80°C oven equipped with ventilating fan. The tubes are then capped and refrigerated. Stable 6 months. To use, reconstitute with 1.0 ml methanol and mix well.

5. Internal Standard is prepared by weighing 40 mg 5-methylphenyl-5'-phenylhydantoin (Aldrich Chem. Co.) and 80 mg 5-ethyl-5 (p-tolyl)barbituric acid and diluting to a final volume of 200 ml with methanol. Two milliliter aliquots are prepared for storage in the same manner as the drug standard solution. Stable 6 months when refrigerated. To use, reconstitute with 2.0 ml methanol and mix well.

6. Blank Serum is prepared from a pool of hepatitis-free serum which has been centrifuged and filtered through Whatman #1 filter paper. Aliquots of 2.2 ml are put into small test tubes and frozen. Stable 6 months.

7. Serum Control: 100 ml of control is prepared by adding 10.0 ml of the Drug Standard Solution to a 100 ml volumetric flask and diluting with blank serum to volume. The solution must be mixed thoroughly before freezing in 1.2 ml aliquots. Stable 6 months.

Procedure

1. A tube of Drug Standard is reconstituted with 1.0 ml methanol and 2.0 ml of methanol is added to a tube of Internal Standard. Both tubes are vortexed 30 seconds to mix.
2. To labeled 16 x 100 mm test tubes the following is added:

	Std-1	Std-2	Control	Patient
Blank Serum	1.0 ml	1.0 ml	-	-
Control Serum	-	-	1.0 ml	-
Patient's Serum	-	-	-	1.0 ml
Deionized Water	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Standard Solution	50 ul	100 ul	-	-
Internal Standard	100 ul	100 ul	100 ul	100 ul

Vortex each tube and add approximately 8 mg charcoal to each. Vortex 30 seconds to adsorb the drugs onto the charcoal, then centrifuge 3 minutes @ 1500 rpm.

3. Decant the aqueous phase; add 2.0 ml deionized water to each tube; vortex 30 seconds and centrifuge 3 minutes @ 1500 rpm.
4. Decant the aqueous phase and carefully remove as much water as possible with an absorbant tissue. Add 2.0 ml ether to each tube. Vortex all tubes exposing as much of the surface area of the charcoal as possible to the ether. Centrifuge 3 minutes @ 1500 rpm.
5. Decant the ether phase carefully into a clean set of labeled 16 x 100 mm test tubes. Evaporate to dryness in a heating block (65°C) under a stream of nitrogen.

6. Immediately prior to the GLC analysis of each sample, the residue is dissolved in 50 ul of 0.2 M Trimethylphenylammonium hydroxide in methanol. Vortex well; inject 2 ul into the gas chromatograph using the "Program" mode.

Interpretation and Calculations

In order to determine the drug levels present it is first necessary to identify the peaks of the unknown chromatograms by comparing the relative retention times (Rel T_R) to the Rel T_R obtained from the standard chromatograms, Figure 4. (Of course the Rel T_R will vary with each column and must be determined for each new column.) Table 3 shows the Rel T_R obtained experimentally and the accepted range for accurate identification.

Table 3

Experimentally Determined Relative Retention Times

Drug	Relative Retention Time
Phenobarbital	0.59 - 0.62
p-Tolylbarbital	0.67 - 0.69
Primidone	0.79 - 0.81
Phenytoin	0.94
5-Methylphenyl-5'-phenylhydantoin	1.00

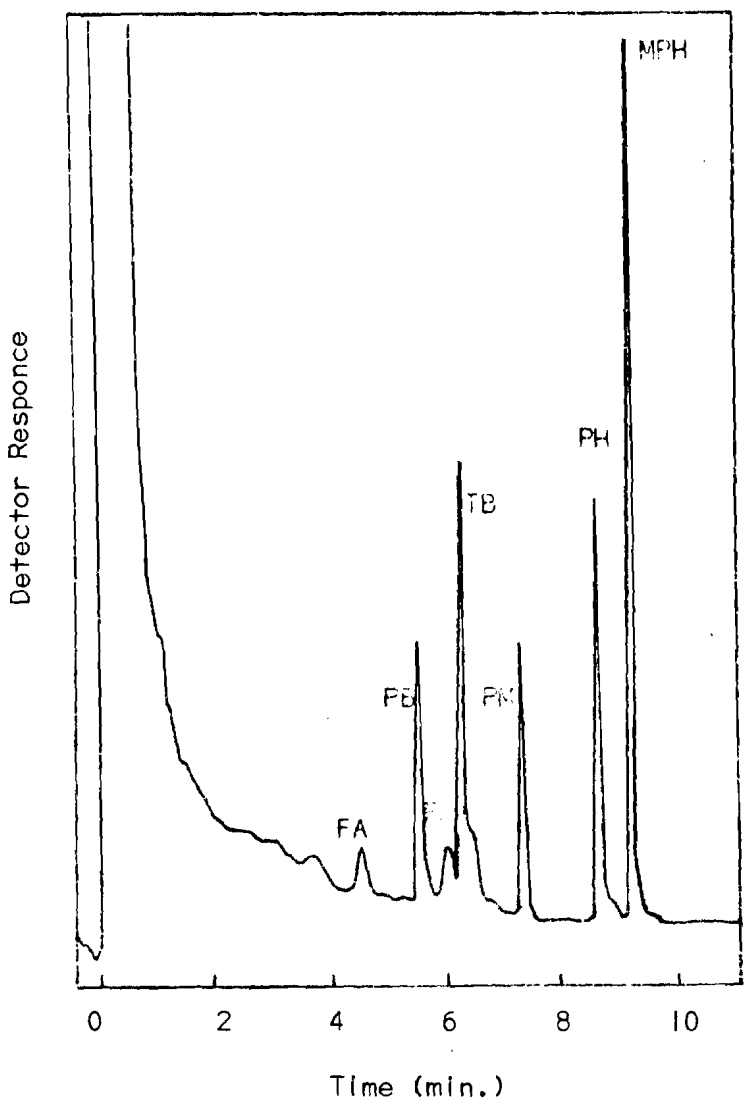


Figure 4. GLC Chromatogram of Antiepileptic Drugs in Serum

Concentration of drugs represented: 18 ug/ml Phenobarbital (PB) 10 ug/ml Primidone (PM) and 10 ug/ml Phenytoin (PH). The concentration of both Internal standards, p-tolylbarbital (TB) and 5-methyl-5'-phenylhydantoin is 20 ug/ml.
Fatty acids (FA) are often present as a natural component of serum.

The concentration of drugs present in the serum can be calculated using either the measured heights of the peaks or the areas under the curves (data from the digital integrator). The concentrations of the standard samples analyzed in ug/ml are:

	Standard-1	Standard-2
Phenobarbital	18.2	36.4
Primidone	10.0	20.0
Phenytoin	10.0	20.0

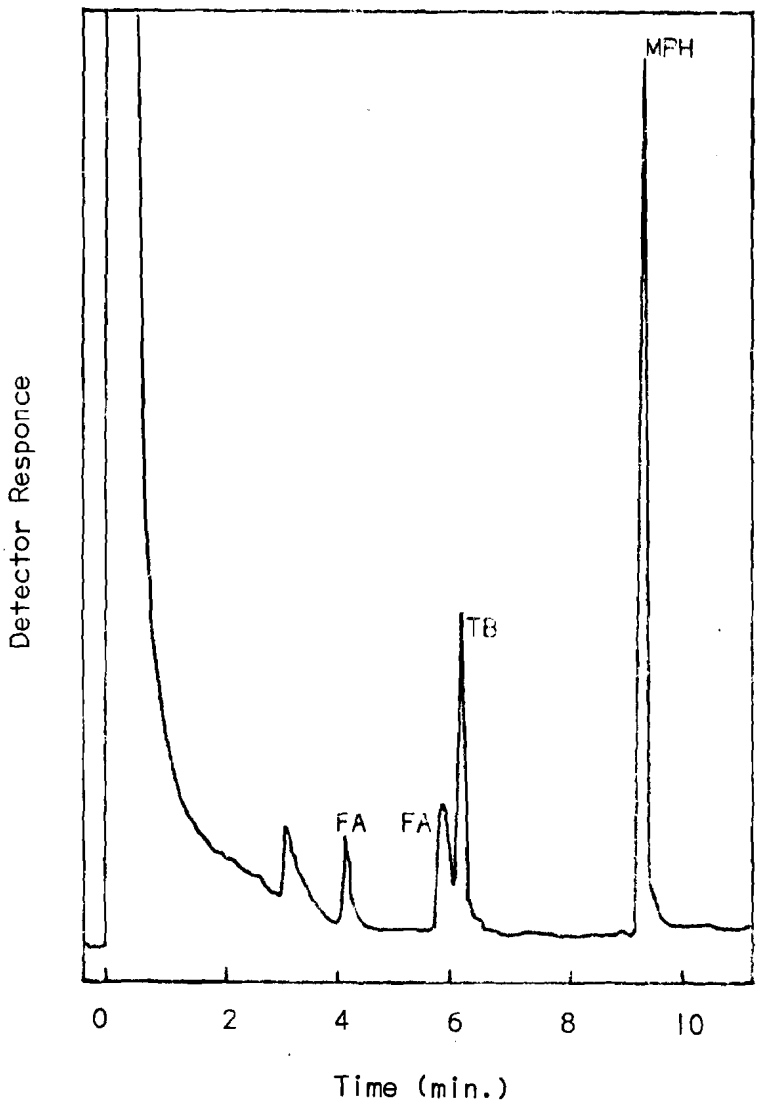


Figure 5. GLC Chromatogram of Patient's Serum

This chromatogram shows no detectable levels of antiepileptic drugs present in the patient's serum. Only the serum fatty acids (FA) and the added internal standards, p-Tolylbarbiturate (TB) and 5-methyl-5'-phenytoin (MPH), are present.

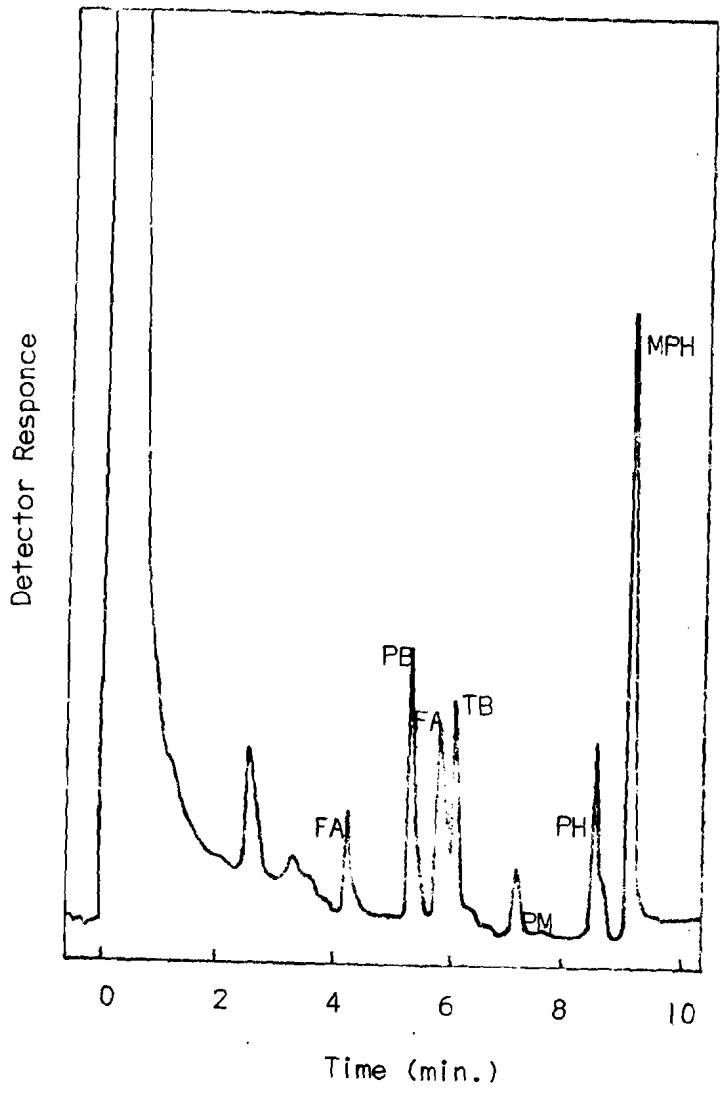
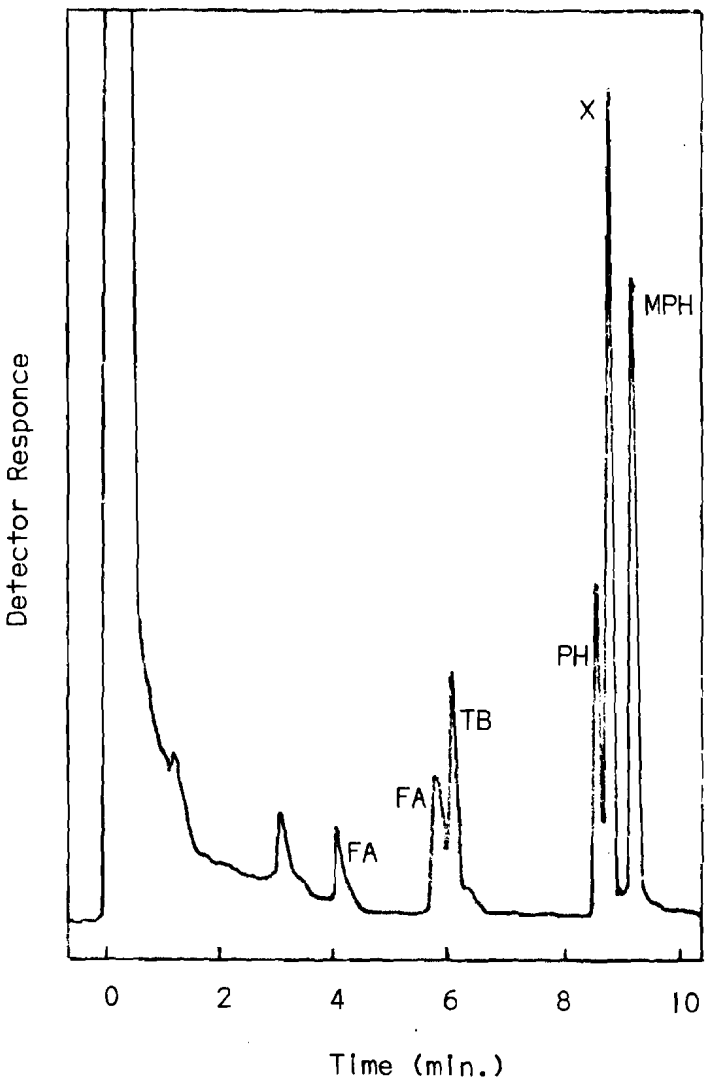


Figure 6. GLC Chromatogram of Antiepileptic Drugs in Patient's Serum

Concentration of drugs present include: 38 ug/ml Phenobarbital (PB), 7 ug/ml Primidone (PM) and 6 ug/ml Phenytoin (PH). p-Tolylbarbiturate (TB) and 5-methyl-5'-phenytoin (MPH) are the internal standards.



igure 7. GLC Chromatogram of Patient's Serum

Concentration of Phenytoin (PH) present in patient's serum was 11 ug/ml. The serum also contained an unidentified compound (X). p-Tolylbarbiturate (TB) and 5-methyl-5'-phenytoin (MPH) are the internal standards.

THE ENZYME-IMMUNOASSAY OF ANTICONVULSANT DRUGS

Although gas-liquid chromatography is the most widely used method for measuring drug levels, enzyme-immunoassay methods have specific advantages especially in the clinical laboratory. Enzyme-immunoassay is rapid, specific, reliable, sensitive and usually no sample pretreatment is required. The recently introduced Enzyme Multiplied Immunoassay Technique ("EMIT", Syva Corporation, Palo Alto, Calif.) provides an alternative to conventional GLC methods.[12] The principle disadvantage, the high cost of reagents, can be offset by using a minature centrifugal analyzer. This also saves the cost of purchasing one of the spectrophotometers specifically suggested by the company.

The serum drug levels are determined using a homogenous enzyme-immunoassay technique which involves two main steps:

1. Antibody-Antigen Reaction: the drug-specific antibody is added to the serum and binds to the drug present in the serum, Figure 8a.
2. Detection: the drug bound to the enzyme (glucose-6-phosphate dehydrogenase) is added to the system which reacts with any remaining unbound (free) antibody rendering the enzyme inactive, Figure 8b. Residual enzyme activity is directly proportional to the initial concentration of the drug in the patient's serum, Figure 8c. The enzyme activity is determined spectrophotometrically by measuring the increase in absorbance at 340 nm.

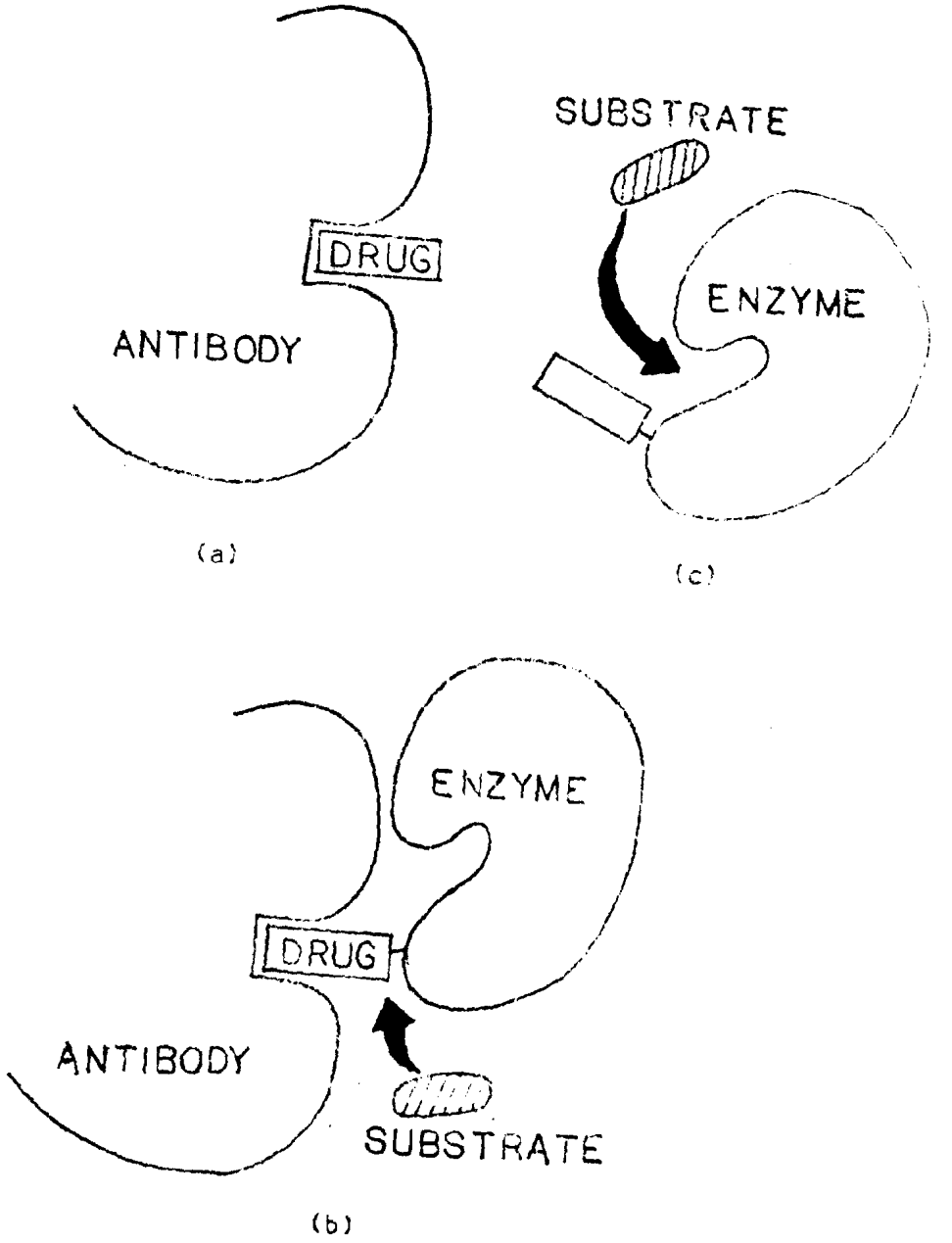


Figure 8. Homogenous Enzyme Immunoassay[27]

The following modified procedure was developed for use of the Syva "EMIT" reagents on the Centrifichem Analyzer, Union Carbide. Since the reagents were available only in kit form for the manual method, all reagent dilutions were kept the same as suggested by the manufacturer so that future changes in dilution and concentrations of reagents would not affect the overall procedure. The Centrifichem also allows for simultaneous sample analysis rather than sequential analysis, thus making the procedure even more rapid, Table 4.

Table 4

Comparison of Modified and Manual EMIT AED Assay Methods

	Manual	Modified	% of Manual
Serum Volume required	50 ul	10 ul	20 %
Serum Volume assayed	8.3 ul	3.2 ul	39 %
Amount of Reagent "A" in reaction	50 umole	16.6 umole	33 %
Amount of Reagent "B" in reaction	50 umole	19.2 umole	38 %
Amount of Buffer in reaction	43.6 umole	15.3 umole	35 %
Total Volume assayed	900 ul	335 ul	37 %
Instrument Time	2 min/test	2 min/29 tests	3 %
Total Analysis Time	6 min/test	45 min/29 tests	25 %

Instrumentation

The analysis was performed on the Centrifichem Centrifugal Analyzer (Union Carbide) equipped with automatic printer, using the following instrument parameters.

Table 5

Instrument Parameters for the Centrifichem Analysis of Antiepileptic Drugs

Centrifichem Pipettor:	Sample Size: 70 ul
	Total Volume: 99 ul
	Reagent Volume: 250 ul
Centrifichem Analyzer:	Filter #1 (340 nm, Absorbance Setting: 635)
	Temperature: 30°C
	"Auto Blank"
	"Terminal"
	"Operate"
	"Absorbance"
	T_0: 015 second ΔT: 0.5 minute
	Number of Prints: 3

Reagents

All reagents necessary for the analysis may be obtained from the Syva Corp., Palo Alto, Calif. and are included in the kit for the specific assay of interest; EMIT Phenytoin, Primidone or Phenobarbital Assays are available. The stock reagents include:

1. Buffer, 0.055 M Tris-HCl: dilute as instructed (to 150 ml total volume with distilled water). Stable at room temperature.
2. Reagent "A" (Antibody): reconstitute as directed by the manufacturer (with 6.0 ml distilled water for Phenytoin and Phenobarbital, 3.0 ml for Primidone). Swirl to dissolve and store at room temperature for 8 hours before use. Stable 12 weeks when refrigerated.
3. Reagent "B" (Drug-labeled Enzyme): reconstitute as directed by the manufacturer, the same as for Reagent "A". Stable 12 weeks when refreigerated.
4. Serum AED Calibrators: reconstitute as directed (with 3.0 ml distilled water). Swirl to dissolve and store for one hour at room temperature before use. Stable 12 weeks when refrigerated.
5. Serum AED Control: reconstitute as manufacturer instructs (with 10 ml distilled water). Swirl to dissolve and store one hour at room temperature before use. Stable 12 weeks when refrigerated.

Procedure

1. Allow all reagents, samples and materials to reach room temperature before use. The standard curve, controls and patient samples must all be run simultaneously.
2. Prepare 1:13 dilution of Reagent "B" with 0.055 M tris·HCl Buffer using the following volumes. Mix gently to avoid bubble formation.

Number of Tests	ul Reagent "B"	ml Buffer
4	200	2.4
5 - 9	300	3.6
10 - 14	400	4.8
15 - 20	500	6.0
21 - 25	600	7.2
26 - 29	700	8.4

3. With the auto pipettor-dilutor, sample 50 ul "A" and dilute with 0.1 ml 0.055 M Tris·HCl Buffer to labeled plastic (12 x 75 mm) test tubes.

   With the auto pipettor-dilutor, sample 10 ul of the standards, controls or patients and deliver with 0.050 ml 0.055 M Tris·HCl Buffer to the appropriate tubes. Mix all tubes.
4. Sample 70 ul of the samples (prepared in step 3) and dispense with 29 ul distilled water to outer wells of Centrifichem disk while dispensing 250 ul Working "B" to inner wells. Analyze on the Centrifichem, Figure 9.

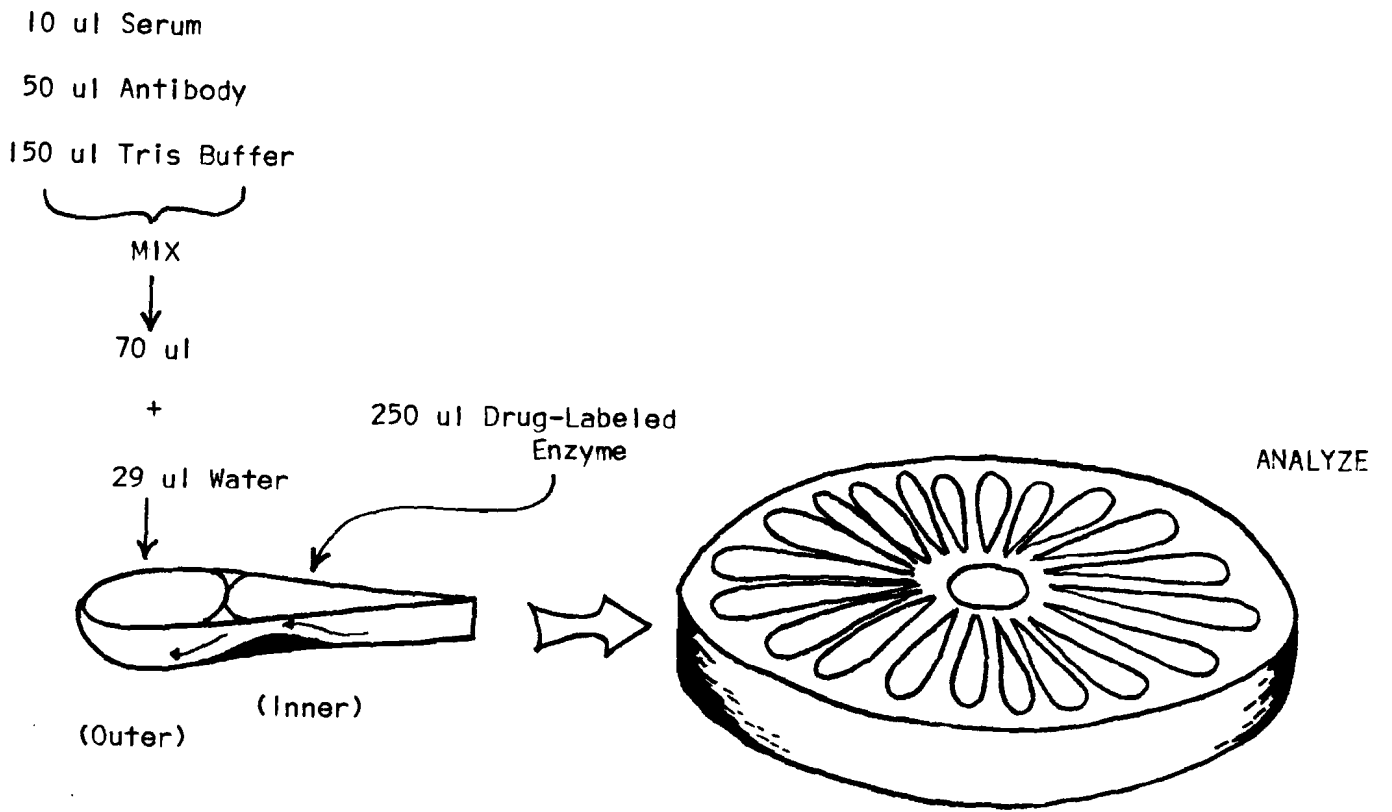


Figure 9. Anticonvulsant Drug Assay by Centrifugal Analyzer.

Interpretation and Calculations

First the difference between the initial and final absorbance readings must be calculated for each test. Then the change in absorbance for Calibrator-0 must be subtracted from all remaining calibrators, controls and patient values ($\Delta A - \Delta A_0$). Using logit-log paper plot $\Delta A - \Delta A_0$ versus the calibrator concentration and construct a best-fit line between the points for Calibrator-1 and Calibrator-5, Figure 10.

Criteria for an acceptable run includes:

1. The difference in Calibrator-0 duplicates must be less than 50 ΔA units.
2. None of the standard points may lie off of the constructed best-fit line by more than 8 ΔA units.
3. The standard curve points must meet the following specifications:

Drug	$\Delta A_1 - \Delta A_0$	$\Delta A_5 - \Delta A_1$
Phenytoin	25- 70 A units	70-115 A units
Phenobarbital	35-110 A units	115-205 A units
Primidone	25- 75 A units	75-145 A units

Using the standard curve determine the concentration of the controls and patients. If any patient gives a $\Delta A - \Delta A_0$ greater than Calibrator-5, it must be repeated with a dilution.

a. Rerun Calibrator-1 and Calibrator-4 and the controls.
b. Repeat the patient by adding 10 ul of the patient's serum and 0.05 ml Buffer to a double dilution of Reagent "A". (Dispense two volumes of "A").

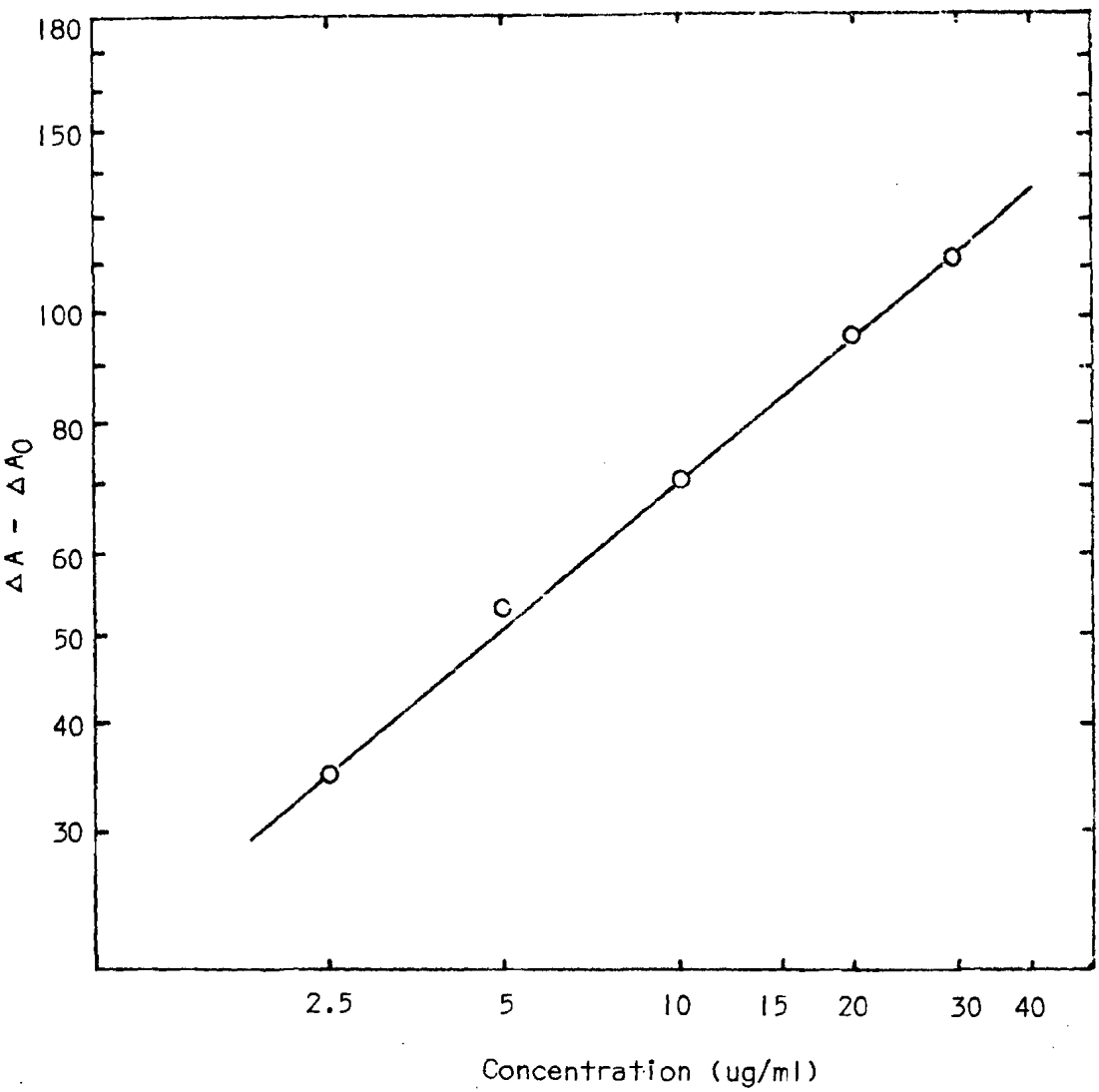


Figure 10. Representative Calibration Curve for the Analysis of Phenytoin in Serum.

c. Multiply the patient's concentration (obtained from the calibration curve) by the correct dilution factor, 1.71.

Patient serums analyzed in duplicate must give results which agree within 10% to be acceptable.

Results and Discussion

Quality control is an essential part of laboratory management. Two forms of quality control are necessary -- interlaboratory and intralaboratory controls. Interlaboratory controls consist of pooled control serum assayed with each run of standards and patient samples. The lack of reliable commercial control serum has severely hampered the toxicology program since each laboratory must establish and maintain its own spiked pool. Strict limits of 10% coefficient of variation were established for an acceptable range for the interlaboratory control.

Until May of 1975 there was no intralaboratory control sera available. At that time with funds made available by the National Epilepsy Foundation, C. E. Pippenger and associates (Columbia University) established an antiepileptic drug (AED) monitoring quality control program for clinical laboratories involved in AED testing. The results of the first tri-sample survey were startling showing a national variation of results of over 300%.[28]

Figures 11, 12, and 13 show the performance index (P. I.) achieved during routine laboratory work from May 1975 through September 1977 using the GLC method discussed. The performance index is calculated for each monthly set of three spiked samples according to Equation 3.

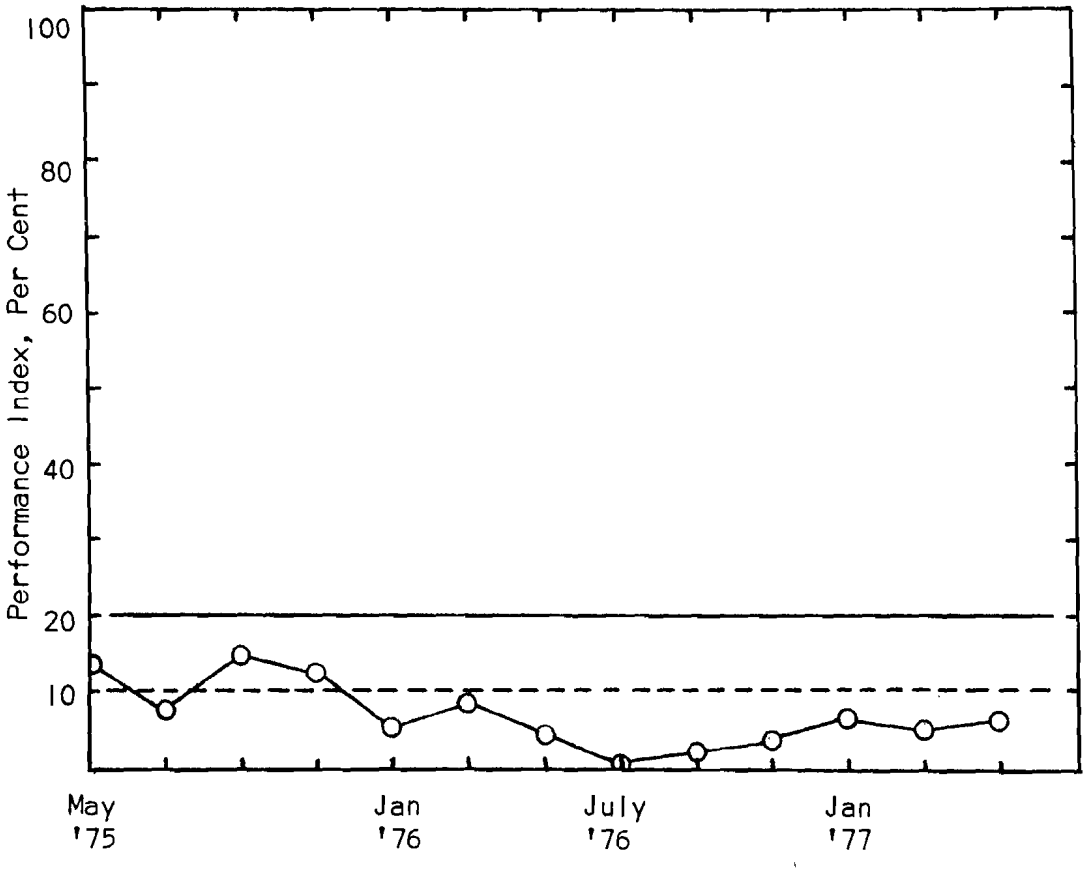


Figure 11. Performance Index Results for Phenytoin

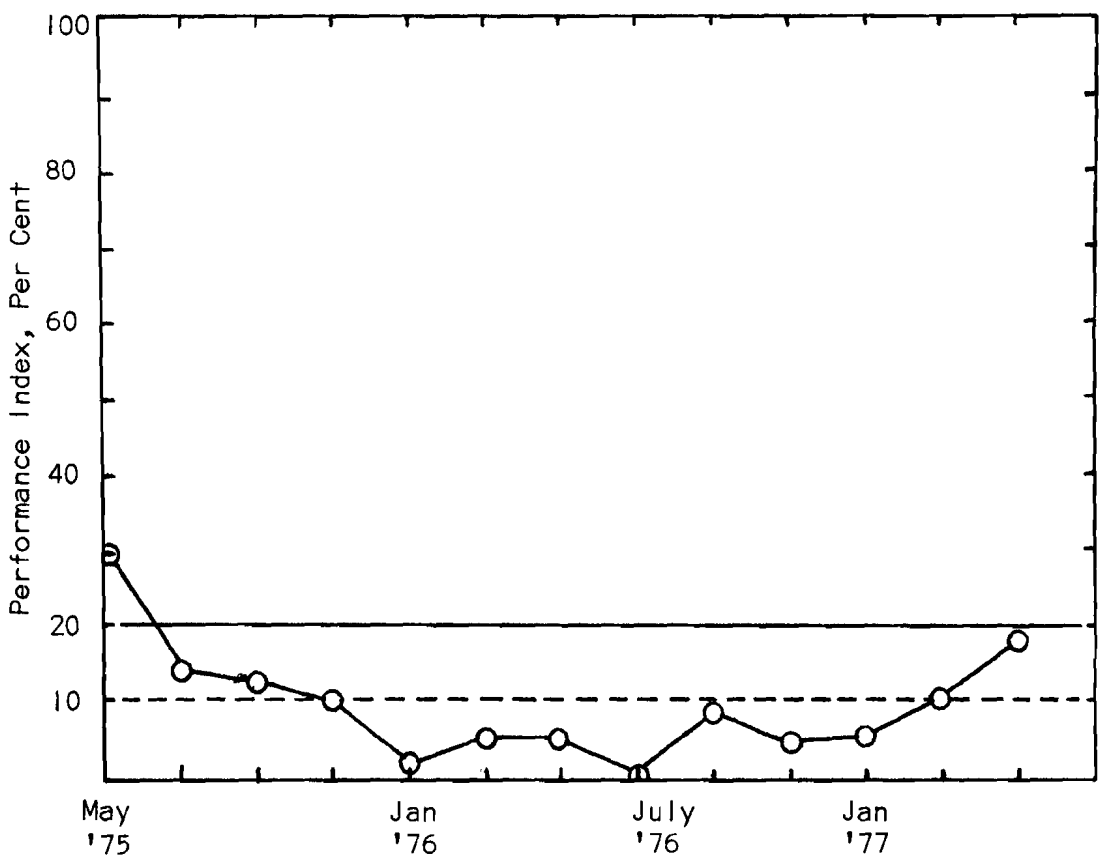


Figure 12. Performance Index Results for Primidone

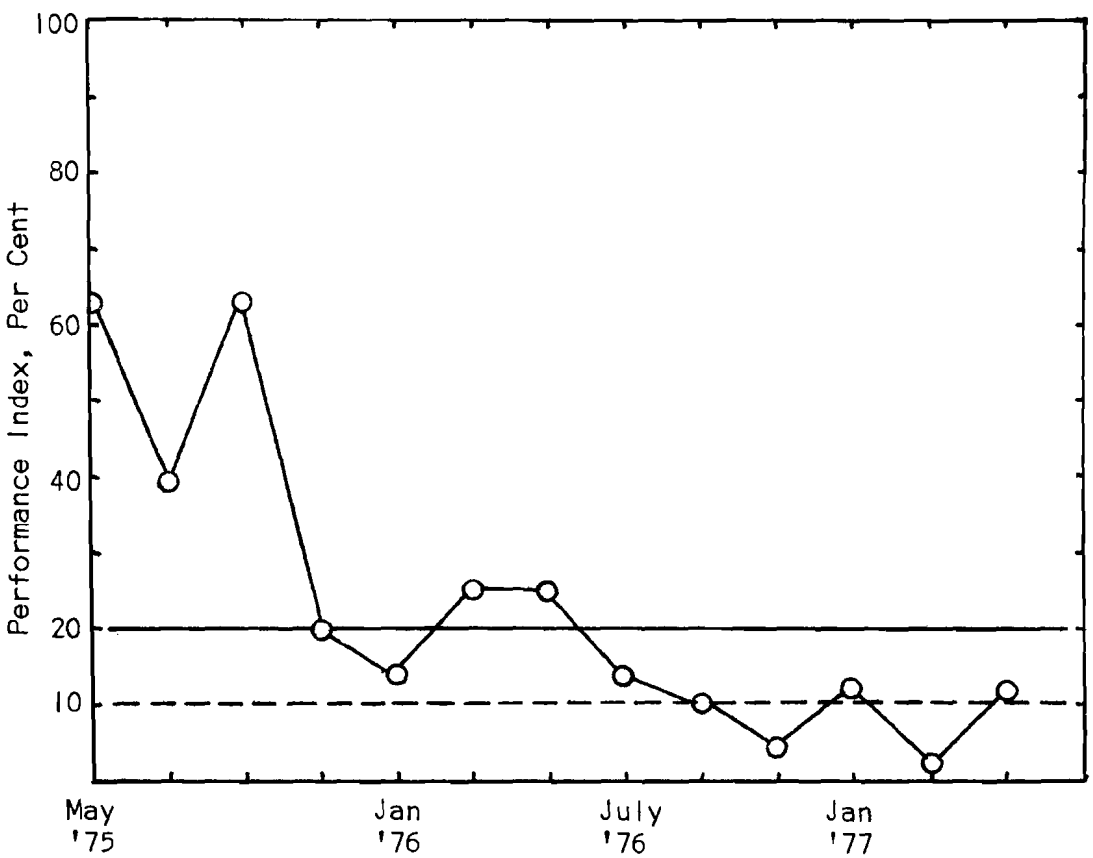


Figure 13. Performance Index Results for Phenobarbital

$$P.I. = \frac{\Sigma \left| \text{Differences between reported \& spiked values} \right|}{\Sigma \text{ Values of spiked samples}} \times 100\% \qquad (3)$$

The P. I. value is then averaged with the previous month's value and plotted.

The optimum range of variation was set by Pippenger at 10% with 10-20% as acceptable. Figure 11 shows that for phenytoin the assay has remained in the acceptable or optimum range throughout the entire study.

Figure 12 shows some initial problems with primidone analysis. This was attributed to the fact that a new GLC column had just been packed and shows the sensitivity of the primidone assay to a properly conditioned column. Towards the end of the study the beginning of another problem was again apparent. This time it was traced to the decomposition of the methylating reagent, TMAH, resulting in the incomplete methylation of primidone.[29] Primidone was found to be the most sensitive to this situation.

Figure 13 shows the extreme variation in the phenobarbital analysis through the first half of the study. Both major areas of unacceptable assays were traced to packing and conditioning new columns. At the midpoint of the study, two additional courses of action were taken, an internal standard for phenobarbital (p-tolyl barbituric acid) was incorporated into the assay method and the standard and internal standard solutions were prepared as a batch and stored instead of preparing them daily.

Comparison of GLC and EMIT Assays for the Antiepileptic Drugs

Serum samples from epileptic patients undergoing treatment and spiked serum samples supplied by the Pippinger survey were analyzed by both GLC and Enzyme Immunoassay (EMIT). The results obtained were compared by estimating the errors using least-squares parameters (slope of the least-squares line, y-intercept and standard error of estimates, S_{xy}), Table 6.

Systematic errors may be constant (expressed in concentration units) or proportional (expressed in percentage units). The constant error is reflected exactly in the intercept of the least-squares line. The exact magnitude of proportional error is quantitated by changes in the slope, m. Westgard and Hunt[32] have shown that both standard error and the standard deviation reflect the magnitude of random error and the correlation coefficient decreases as random error increases.

Phenytoin

Spiked versus GLC and modified Enzyme Immunoassay (EMIT), Figure 14: Twenty-one spiked serum samples were analyzed by GLC and 10 by EMIT methods. The slope obtained is 1.056 (GLC) and 1.005 (EMIT) indicating proportional errors of 5.6% and 0.5% respectively. The constant error is estimated at 0.75 ug/ml (GLC) and 1.26 ug/ml (EMIT) and the correlation coefficients are 0.995 and 0.994 respectively. Thus a spiked sample containing 20 ug/ml phenytoin would give an average value of 20.4 $\pm$ 2.5 ug/ml by GLC analysis and 21.4 $\pm$ 2.5 ug/ml by the modified enzyme immunoassay.

Table 6

Summary of Antiepileptic Comparison Data

	Slope (m)	Intercept (b)	Standard Error of Estimates (S_{xy})	Correlation Coefficient (r)	Number of Trials (n)
PHENYTOIN					
Spiked vs. GLC	1.055	-0.75	2.45	0.995	21
Spiked vs. EMIT	1.005	1.26	2.54	0.994	10
GLC vs. EMIT	0.824	4.48	4.36	0.963	25
PHENOBARBITAL					
Spiked vs. GLC	1.146	-2.29	3.88	0.995	21
Spiked vs. EMIT	0.869	0.86	6.46	0.971	10
GLC vs. EMIT	0.855	3.26	7.62	0.939	19
PRIMIDONE					
Spiked vs. GLC	1.062	-0.09	0.67	0.998	21
Spiked vs. EMIT	1.137	-0.07	0.82	0.996	11
GLC vs. EMIT	0.967	0.86	1.80	0.974	22

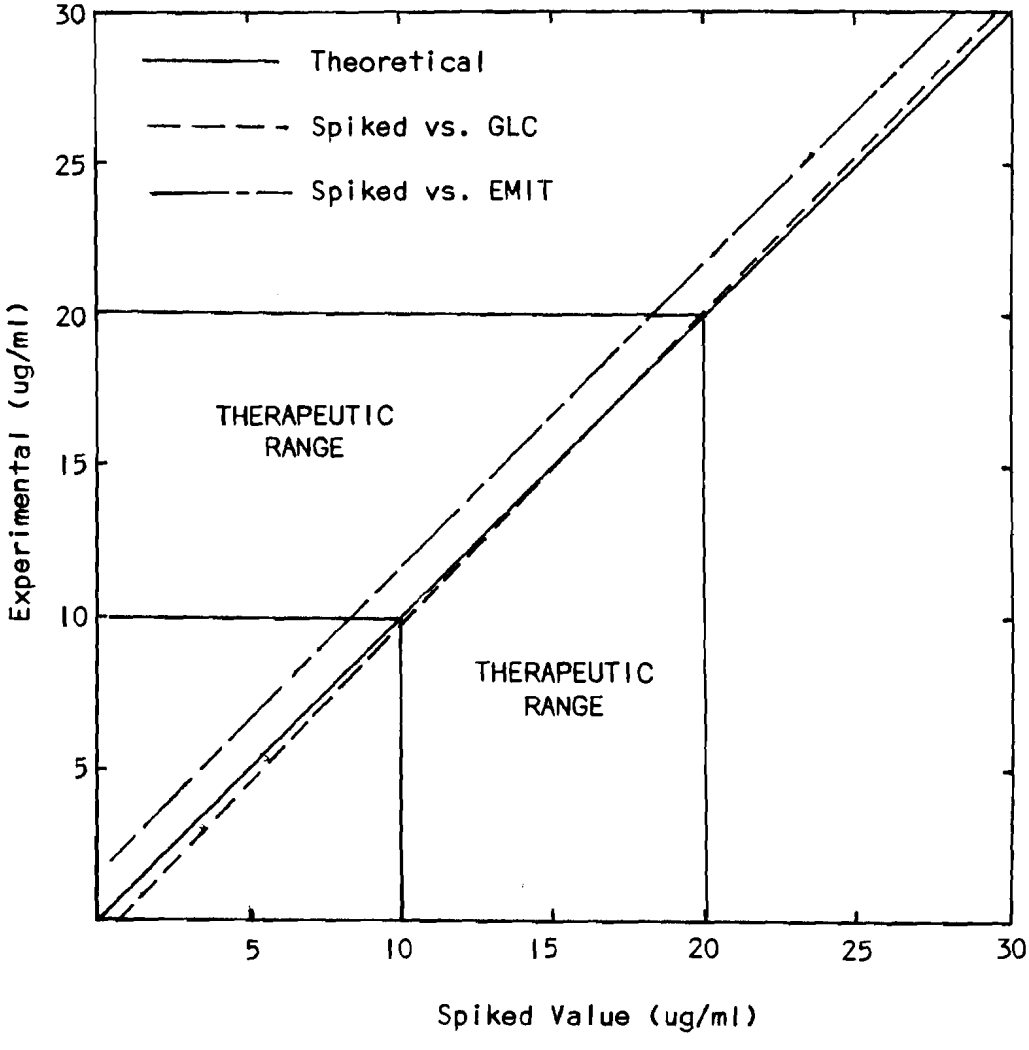


Figure 14. Spiked Values vs. Results obtained by Gas Chromatography (GLC) and Enzyme Immunoassay (EMIT) for Phenytoin.

Gas chromatography versus EMIT: Twenty-five patient samples were analyzed by both methods. The slope of the line is 0.824, which indicates a proportional error of 17.6%. The constant error is estimated at 4.48 ug/ml from the intercept. The correlation coefficient is 0.963 indicating very good correlation between the two methods. The random error estimated from the standard error is 4.4 ug/ml. Therefore a sample determined to have 20 ug/ml phenytoin by GLC would give an average value of 21.0 ± 4.4 ug/ml by the EMIT assay.

Phenobarbital

Spiked versus GLC and EMIT, Figure 15: Twenty-one spiked serum samples were analyzed by GLC and 10 were determined by the modified EMIT procedure. The slope is 1.146 by GLC and 0.869 by immunoassay, indicating a proportional error of 15% and 13% respectively. The constant error is estimated at 2.29 ug/ml (GLC) and 0.86 ug/ml (EMIT). The random error estimated from the standard error is 3.88 ug/ml by GLC and 6.46 ug/ml by EMIT. The correlation coefficients obtained were 0.971 and 0.995 respectively. Thus a spiked sample containing 40 ug/ml phenobarbital would give an average assay value of 43.6 ± 3.9 ug/ml by GLC and 35.6 ± 6.5 ug/ml by immunoassay. This indicates that values obtained from the GLC analysis may be slightly overestimated while the values from the EMIT assay are to the same degree underestimated.

Gas chromatography versus modified EMIT: Nineteen patient samples were assayed by both methods giving a slope of 0.855 indicating a proportional error of 14.5%. The constant error was estimated at 3.26 ug/ml and the correlation coefficient was 0.939. There fore a

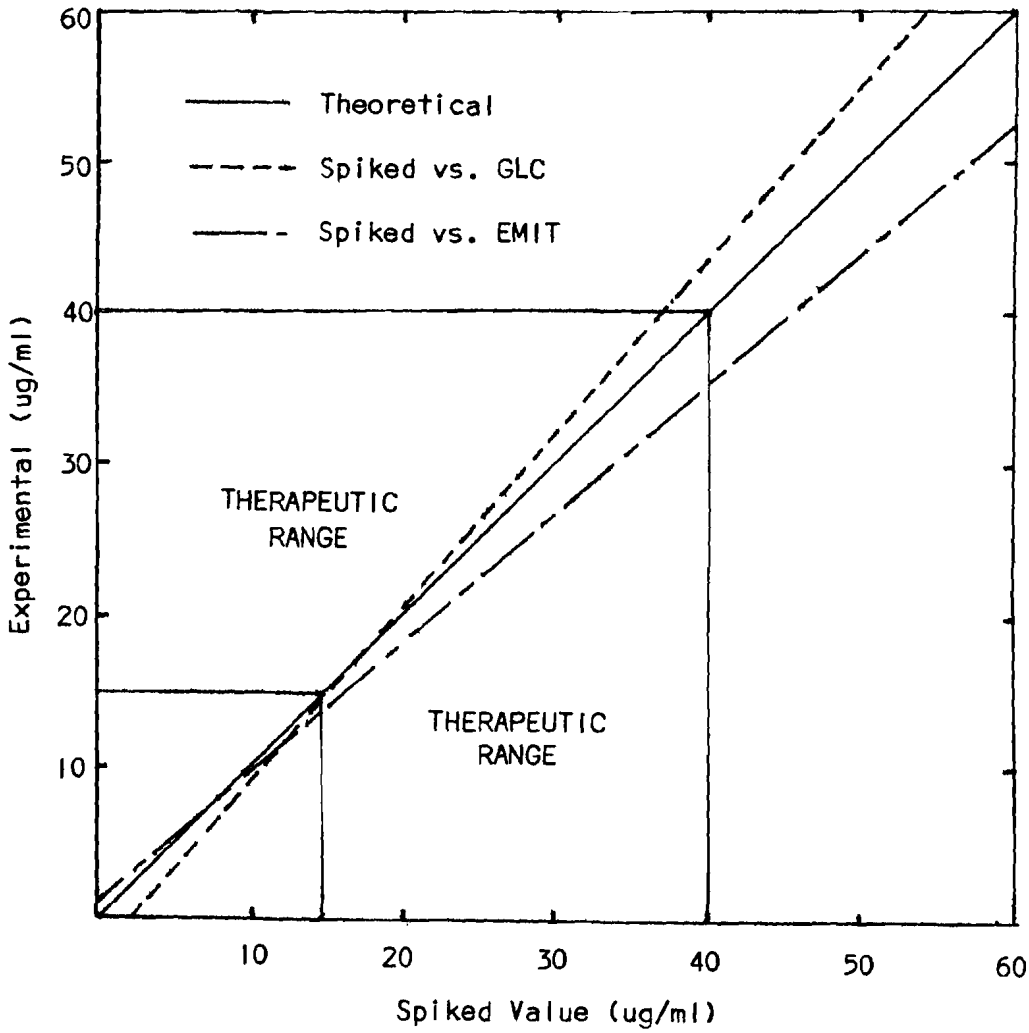


Figure 15. Spiked Values vs. Results obtained by Gas Chromatography (GLC) and Enzyme Immunoassay (EMIT) for Phenobarbital.

e assayed by GLC and giving a concentration of 40 ug/ml would give
erage assay value of 37.5 ± 7.6 ug/ml by EMIT.

done

piked versus GLC and modified EMIT, Figure 16: The slope obtained
he 21 spiked samples analyzed by GLC was 1.062 and the 11 samples
zed by immunoassay was 1.137 thus indicating proportional errors
and 14% respectively. Constant error was estimated at 0.09 ug/ml
and 0.07 ug/ml (EMIT). The random error estimates were 0.67 ug/ml
C and 0.82 ug/ml by EMIT. Thus a spiked sample containing 12 ug/ml
imidone would give an average assay value of 11.9 ± 0.7 ug/ml by
nd 13.6 ± 0.8 ug/ml by the modified immunoassay method.
as chromatography versus modified enzyme immunoassay: Twenty-two
nt samples were analyzed using both methods. A slope of 0.967
ating a proportional error of 3% and an estimate of the constant
of 0.86 ug/ml and a correlation coefficient of 0.974 was obtained.
data shows a very good correlation between the two procedures, for
le a patient sample assayed to contain a primidone concentration of
/ml by GLC would give an average value of 12.5 ± 1.8 ug/ml when
ed by the modified EMIT procedure.

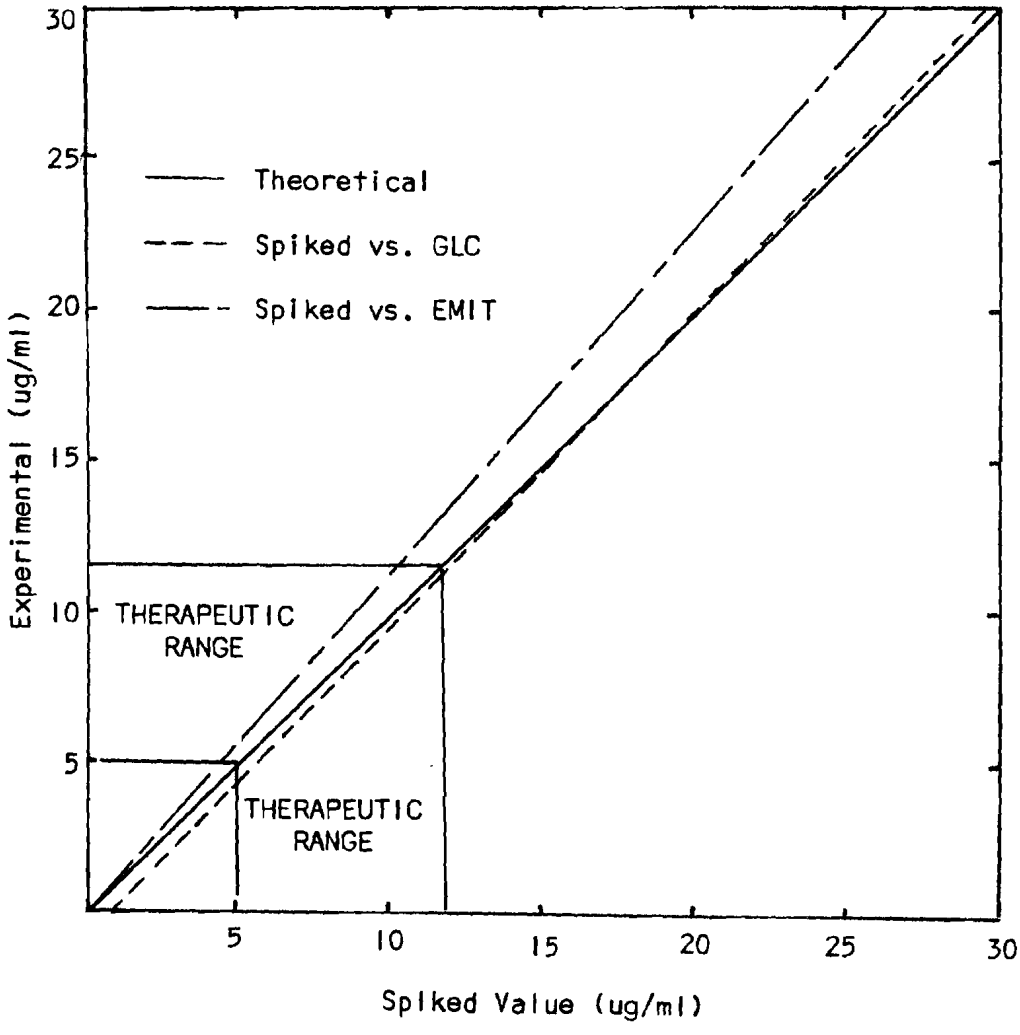


Figure 16. Spiked Values vs. Results obtained by Gas Chromatography (GLC) and Enzyme Immunoassay (EMIT) for Primidone.

THE CARDIAC DRUGS

THE ENZYME-IMMUNOASSAY OF DIGOXIN

The cardiac glycosides, derived from the foxglove plant, have been used to help manage heart disease for over 200 years. Digoxin (Lanoxin) is the main glycoside in use today and is currently being administered to several million people daily. This cardiovascular drug increases the force and the velocity of contraction in normal and failing hearts. Blood levels are necessary to monitor patients on digoxin therapy since symptoms resulting from toxic levels and subtherapeutic levels cannot be distinguished clinically. Optimal digitalis therapy is further complicated by the narrow margin of safety (less than one nanogram per milliliter of blood) between the therapeutic and toxic effects of the medication. Therefore serum levels must be available to clinicians on a 24-hour basis, thus requiring quick and accurate laboratory responce.

Until 1976 the only practical digoxin assay method was radioimmunoassay. This is a very sensitive and specific technique. However, RIA methods all have similar disadvantages inherent to all radioisotopic methods when adapted to the clinical lab: special isotopic safety considerations, licensure requirements, decay of labeled reagents and a need for specialized instrumentation.

The enzyme-immunoassay of digoxin eliminates the need for specialized instrumentation and the more rapid procedure easily fits into the more routine chemistry laboratory.

This technique is performed in three specific steps:

1. Pretreatment. Proteins in the serum sample are denatured with alkali.
2. Antibody-Antigen Reaction. The antibody specific to digoxin is added to the serum and binds to all the drug present.
3. Detection. Digoxin bound to an enzyme (glucose-6-phosphodehydrogenase) is added to the system which reacts with any remaining unbound (free) antibody rendering the enzyme inactive. Residual enzyme activity is directly proportional to the initial concentration of digoxin in the patient's serum. The enzyme activity is determined spectrophotometrically by measuring the absorbance increase at 340 nm.

The following modified procedure was developed for use of Syva "EMIT" reagents on the Centrifichem Analyzer, Union Carbide.[13] Since the reagents were available only in kit form, not separately, all reagent dilutions were kept the same as suggested by the manufacturer, except for the buffer, so that future changes in dilutions and concentrations of reagents would not affect the overall procedure. The Centrifichem also allows for simultaneous sample analysis rather than sequential analysis, thus making the procedure even more rapid, Table 7.

Instrumentation

The analysis was performed on the Centrifichem Centrifugal Analyzer (Union Carbide) equipped with automatic printer, using the following instrument parameters, Table 8.

Table 7

Comparison of Modified and Manual EMIT Digoxin Assay Methods

	Manual	Modified	% of Manual
Serum Volume required	0.5 ml	100 ul	20 %
Serum Volume assayed	200 ul	31.1 ul	16 %
Amount of NaOH in reaction	25 umole	3.8 umole	16 %
Amount of Reagent "A" in reaction	50 umole	7.8 umole	16 %
Amount of Reagent "B" in reaction	50 umole	7.6 umole	15 %
Amount of Buffer in AB-AG reaction	66 umole	9.6 umole	15 %
Amount of Buffer in Kinetic reaction	27.5 umole	6.3 umole	23 %
Total Buffer in System	93.5 umole	15.9 umole	17 %
Total Volume assayed	2.05 ml	349 ul	17 %
Instrument Time	30 min.	32 min.	same
Total Analysis Time	75 min/29 tests	45 min/29 tests	60 %

Table 8

Instrument Parameters for the Centrifichem Analysis of Digoxin

Centrifichem Pipettor:	Sample Size: 70 ul
	Total Volume: 99 ul
	Reagent Volume: 250 ul
Centrifichem Analyzer:	Filter #1, 340 nm (Absorbance Setting: 635)
	Temperature: 30°C
	"Auto Blank"
	"Terminal"
	"Operate"
	"Absorbance"
	T_0: 030 second ΔT: 8 minutes
	Number of Prints: 4

Reagents

All reagents necessary for the analysis may be obtained from the Syva Corp., Palo Alto, Calif. and are included in the EMIT Digoxin Manual Assay Kit. The stock reagents include:

1. Buffer, 0.413 M Tris·HCl: make a 1:2 dilution by diluting the 15 ml of buffer concentrate with 15 ml of deionized water. Stable at room temperature.
2. Reagent "A" (Antibody): reconstitute as directed (by adding 4.0 ml deionized water). Swirl to dissolve and store at room temperature one hour before use. Stable 12 weeks when refrigerated.

3. Reagent "B" (Drug-labeled Enzyme): reconstitute as directed (by adding 4.0 ml deionized water). Swirl to dissolve and store at room temperature one hour before use. Stable 12 weeks when refrigerated.
4. Sodium Hydroxide, 0.5 N.
5. Serum Calibrators: reconstitute as directed (with 3.0 ml deionized water). Swirl to dissolve and store for one hour at room temperature before use. Stable 12 weeks when refrigerated.
6. Serum Control: any acceptable commercial digoxin control.

Procedure

1. Allow all reagents, samples and materials to reach room temperature before use. The standard curve calibrators, controls and patient samples must all be run simultaneously.
2. Prepare a 1:33 dilution of Reagent "B" with 0.413 M Tris·HCl Buffer using the following volumes. Mix gently to avoid foaming.

Number of Tests	WORKING "B"		
	Reagent "B"	0.413 M Buffer	d. H_2O
10	100 ul	200 ul	3.0 ml
11 - 20	200 ul	400 ul	6.0 ml
21 - 30	300 ul	600 ul	9.0 ml

3. Pipet into plastic (12 x 75 mm) test tubes: 100 ul serum plus 25 ul 0.5 N NaOH. Vortex; wait 5 minutes.

4. Add 100 ul of Working "A" (1:4 dilution of Reagent "A" with 0.413 M Tris·HCl Buffer) to each test tube. Vortex; wait 15 minutes. Transfer to appropriate sample cups.
5. Sample 70 ul of the solutions prepared in Steps 3 and 4 and dispense with 29 ul deionized water to the Centrifichem disc. Dispense 250 ul Working "B" to the reagent disc.
6. Analyze on the Centrifichem.

Interpretation and Calculations

First the difference between the initial and final absorbance readings (ΔA) must be calculated for each test. Then the change in absorbance for Calibrator-0 is subtracted from all remaining calibrators, controls and patient values ($\Delta A - \Delta A_0$). Using logit-log paper plot $\Delta A - \Delta A_0$ versus the calibrator concentration and construct a best-fit line between the points for Calibrator-1 and Calibrator 4.

Criterian for an acceptable run includes:

1. The difference between Calibrator-0 duplicates must be less than 20 ΔA units.
2. The standard curve must meet the following specifications.
   a. None of the standard points can lie off of the constructed best-fit line by 10 ΔA units.
   b. $\Delta A_1 - \Delta A_0$ must be at least 20 ΔA units.
   c. $\Delta A_5 - \Delta A_1$ must be at least 85 ΔA units.

Using the standard curve the concentration of the controls and patients can be determined. Duplicate patient levels must agree within 10%.

The therapeutic range is usually 0.8 - 2.0 ng/ml. However toxicity may be evident at serum levels above 1.5 ng/ml and is frequently evident at levels greater than 2.0 ng/ml. Patients, often aged, with advanced heart and associated pulmonary disease or abnormal renal function apparently have the greatest risk of developing digitalis toxicity on levels that are not usually considered excessive.

The results of this assay cannot be interpreted properly if the patient is receiving: acetyldigitoxin (Acylanid), deslanoside (Cedilanid-D) digitoxin (Crystodigin), gitalin (Gitaligin), lanatoside C (Cedilanid) or ouabain (Strophanthin-G).[13]

Results and Discussion

The manual procedure required two absorbance measurements per test at 30-minute intervals. Samples had to be aspirated and read at 30-second intervals and then the aspirating sequence had to be repeated 30 minutes later for the final reading. The modified procedure which was developed, as part of this study, allows for simultaneous rather than sequential analysis. By adapting the procedure to the Centrifichem a substantial decrease in reagent volumes and therefore a significant cost savings is accomplished also (350 tests can be made from the amount of reagents sold by the manufacturer for 70 tests). Table 7.

In developing this procedure several important factors were noted.

1. Polyethylene (plastic) test tubes and autoanalyzer cups are essential due to the low levels of antibody being used since it is easily adsorbed onto glass surfaces.

2. The amount of buffer present to maintain the Antibody-Antigen reaction solution at the proper pH is critical.
3. The importance of the zero-calibrator cannot be emphasized enough and must be assayed in duplicate for each run.
4. An automatic micro-pipettor-dilutor (Grummond, Scientific Products) was found essential for the precise reproducible measurement of the small volumes of reagents and also as an aid in mixing the solutions adequately.
5. Linearity cannot be maintained with any kinetic reaction times less than 32 minutes. Figure 18 shows the results of the study made with kinetic reaction times of 16, 24 and 32 minutes.

Comparison of RIA and the Modified EMIT Assay for Digoxin

Serum samples from fifty-two digitalized patients were assayed by both radioimmunoassay (RIA) and the modified EMIT procedure. The RIA method used an ^{125}I-labeled digoxin tracer, sheep anti-digoxin antibody and dextran-coated charcoal as an adsorbant to separate the bound and unbound digoxin. Reagents were obtained from Schwartz-Mann, Orangeburg, N. Y., 10962.

Control sera was obtained from Ortho Diagnostics, Rariton, N. J. and the Syva Corp., Palo Alto, California.

Concentrations of digoxin found by RIA and EMIT methods were compared and errors estimated using least-squares parameters.[44]

Figure 17 shows the correlation of results for digoxin which were obtained with RIA and the modified enzyme immunoassay procedure. The

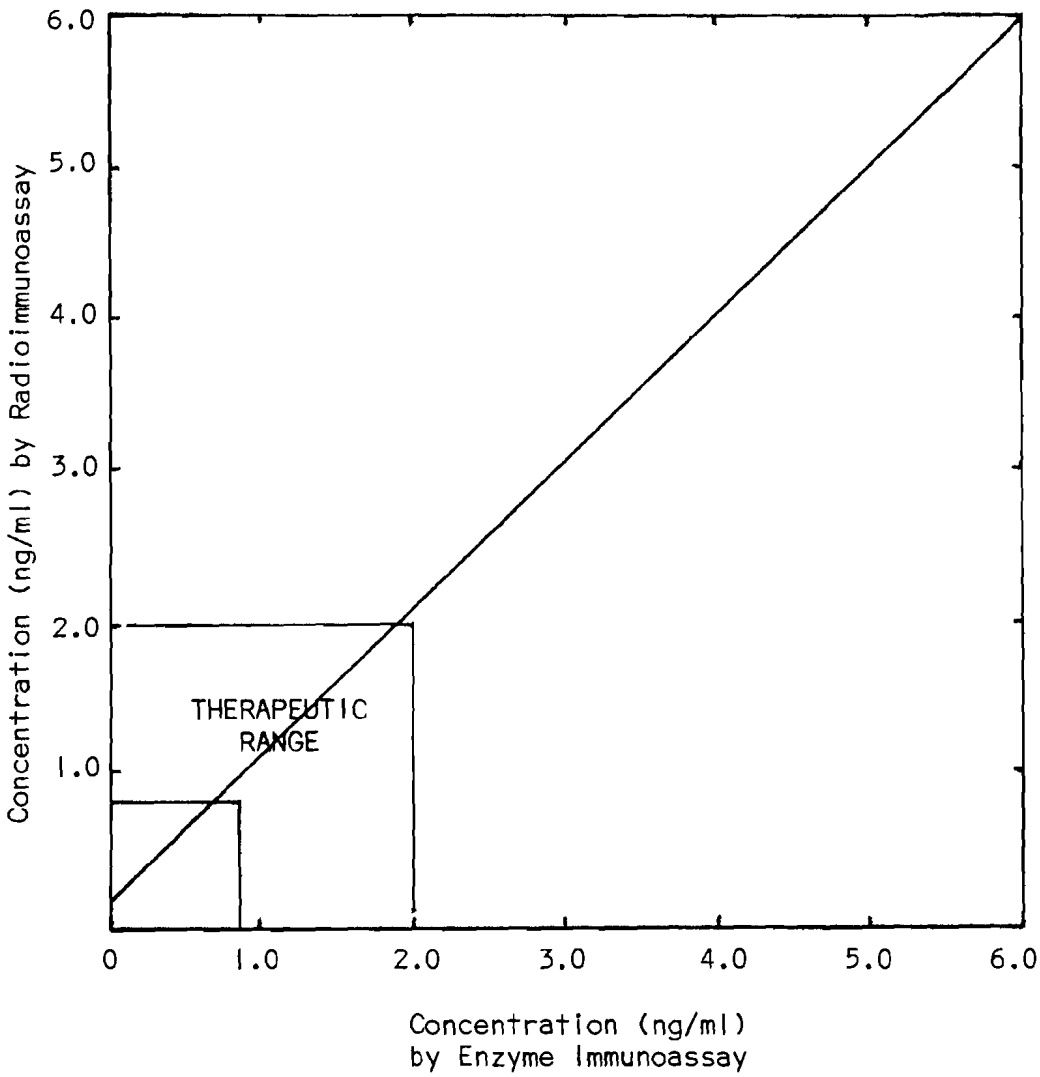


Figure 17. Correlation Between Results for Digoxin Obtained with Enzyme Immunoassay and Radioimmunoassay

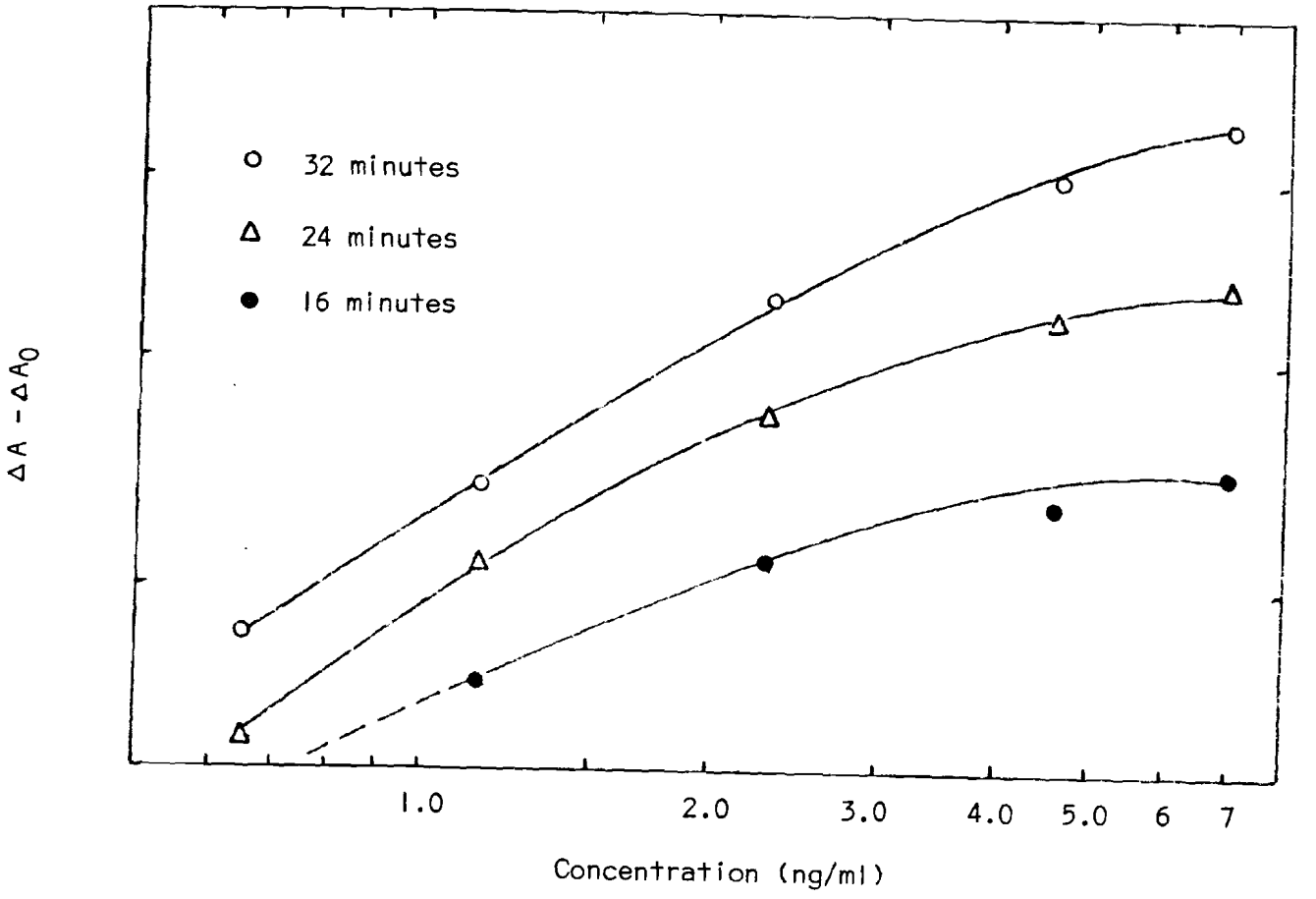


Figure 18. Effect of Varying Kinetic Reaction Times on the Calibration Curve for Digoxin

slope of the line is 0.964, which indicates a proportional error of 3.6%. The y-intercept is 0.24 which indicates a positive constant error of 0.24 ng/ml when using the modified EMIT procedure compared to the RIA values. The random error is estimated at 0.25 ng/ml from the standard error of the estimate, S_{xy}. The regression coefficient of 0.94 shows acceptable correlation between the two methods, ideal would be 1.00.

Table 9 shows the results of precision studies made using the modified EMIT procedure. The with-in run precision data was collected before the use of the automatic pipettor-dilutor and shows an expected variation of 0.2 ng/ml in the therapeutic and into the lower toxic range. The run-to-run precision data was collected from 10 daily runs, the automatic pipettor-dilutor was used for these runs and the increased precision gained is reflected in the data which shows an expected variation of 0.1 ng/ml in the therapeutic and lower toxic range. Run-to-run precision which had been obtained for the RIA method was 2.5 $\pm$ 0.2 ng/ml or 7.4% coefficient of variation and 1.3 $\pm$ 0.1 ng/ml or 5.4% C. V.

Table 9

Analytical Precision of EMIT Digoxin Assay

	Mean ± Std. Dev. (ng/ml)	Range (ng/ml)	% Coefficient of Variation	Number of Dtmns.
Inter-assay Precision:				
Serum - 1	4.3 ± 0.4	3.9 - 4.7	9.5	10
Serum - 2	3.3 ± 0.2	3.1 - 3.5	4.7	10
Serum - 4	1.1 ± 0.2	0.9 - 1.3	16	10
Intra-assay Precision:				
Serum - 2	3.5 ± 0.3	3.1 - 4.0	9.3	12
Serum - 3	2.2 ± 0.1	2.0 - 2.3	4.5	12
Serum - 4	1.1 ± 0.1	1.0 - 1.3	8.7	12

THE QUANTITATION OF QUINIDINE IN SERUM

Quinidine is one of the primary drugs used to control arrhythmias, disorders of heart rate and rhythm. In the therapeutic dosage range, about 60% of quinidine is strongly bound to plasma albumin.

The quinidine present in the serum is extracted into alkaline chloroform and then into sulfuric acid. The acid extract is scanned for quinidine and the concentration is determined by the absorbance at 250 nm. Quinidine is the optical isomer of quinine.

Instrumentation

The analysis was performed on a Beckman DB-G Spectrophotometer equipped with a 10" recorder.

Reagents

1. Potassium Hydroxide, saturated solution.
2. Quinine Stock Standard: dissolve 36.7 mg quinine(mono-)hydrochloride $C_{20}H_{24}N_2O_2 \cdot HCl \cdot 2\ H_2O$, in 100 ml of 0.1 N HCl. This gives a standard concentration of 300 ug quinine per milliliter of solution.
3. Chloroform, Reagent Grade.
4. Sulfuric Acid, 0.5 N.

Procedure

1. To labeled, screw-top 16 x 150 mm borosilicate tubes add:

	Blank	Std-1 (5 ug/ml)	Std-2 (10 ug/ml)	Patient
Distilled Water	3.0 ml	3.0 ml	3.0 ml	-
Patient's Serum	-	-	-	3.0 ml
Stock Standard	-	50 ul	100 ul	-
Saturated KOH	6 drops	6 drops	6 drops	6 drops
Chloroform	15.0 ml	15.0 ml	15.0 ml	15.0 ml

2. Shake 5 minutes. Centrifuge 3 minutes at 1500 rpm. Aspirate the upper aqueous layer from all the tubes and discard. Filter the organic layer through phase-separating filter paper into labeled 16 x 150 mm test tubes.
3. Transfer 12.0 ml of the chloroform layer to a clean set of labeled centrifuge tubes. Add 3.0 ml 0.5 N Sulfuric Acid to each tube.
4. Shake all tubes 5 minutes. Centrifuge 3 minutes at 1500 rpm.
5. Transfer 2.5 ml of the sulfuric acid layer to small test tubes and centrifuge.
6. Transfer the sulfuric acid to quartz cuvettes and scan from 400 nm to 220 nm, against the "Blank" as the reference.

terpretation and Results

The concentration of quinidine present in the sample is directly roportional to the absorbance at 250 nm, Figure 19. Since quinidine is basic drug, the presence of other basic drugs in the serum samples ould easily be extracted and interfere with the quinidine determination. herefore it is very important to compare the scan obtained for the nknown serum samples closely with those obtained for the standards to ule out any interferences.

A reliable quality control program is essential for maintaining ontrol of errors. This may be accomplished three ways: (1) running a piked control serum with each analysis (2) checking the concentration f the stock standard and (3) evaluating the extraction efficiency of he procedure.

Since no commercial control serum was available, a spiked pooled erum control was maintained to monitor interlaboratory variations.

The stock standard was checked for proper concentration periodically. ccording to the CRC Handbook of Analytical Toxicology, the absorption oefficient, $A_{cm}^{1\%}$, for quinidine at 250 nm in acid solution should be .853.[37]

The method chosen for evaluating the extraction efficiency of the rocedure was standard addition. To four patient serums which had been reviously analyzed for quinidine, 5 ug/ml of quinidine was added. The amples were again assayed and the results obtained compared, Table 10.

Therapeutic quinidine levels range from 3 - 5 ug/ml depending on the ize of the dose and the time elapsed between dose administration and pecimen collection, and can be as high as 10 ug/ml. Concentrations of

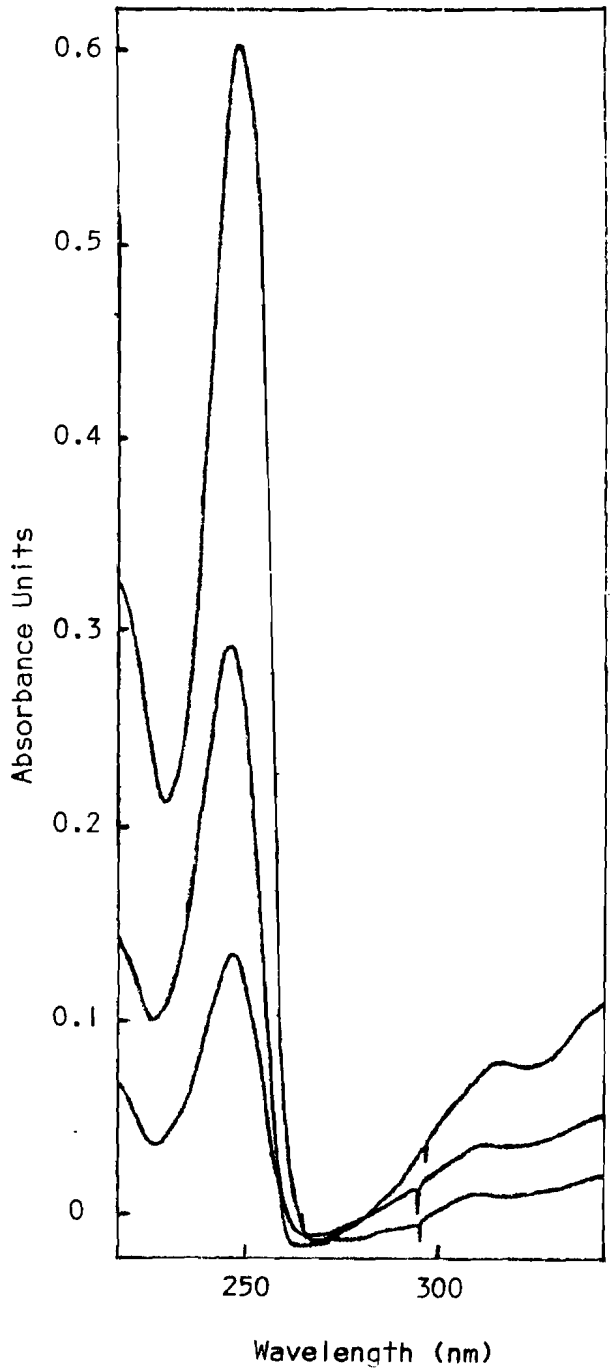


Figure 19. Absorption Spectra of Quinidine at Concentrations of 2.5, 5.0 and 10.0 ug/ml.

30 ug/ml are considered lethal.[39]

Table 10

Comparison of Quinidine Results Obtained Using
Standard Addition vs Routine Assay Analysis Method

Patient	Concentration (ug/ml)	
	Routine Method	Standard Addition
#1	0.6	0.6, 0.3
#2 (2pm)	2.7	2.4, 2.3
#2 (6pm)	1.8	1.3, 1.1
#3	4.8	5.3, 5.1

This procedure measures the actual serum level of quinidine, thus the same therapeutic range is applicable regardless whether the medication being given is quinidine sulfate or gluconate.

Papverine, chloroquine and primaquine could interfere with this determination of quinidine, although the therapeutic doses of these drugs are usually less than those for quinidine.[38]

THE QUANTITATION OF THEOPHYLLINE IN SERUM

Theophylline is a drug used in the treatment of asthma. Theophylline compounds act by dilating the bronchi.

This procedure is a modification of the Schack and Waxler method.[32] It involves extraction of the drug into chloroform/isopropanol and back extraction into sodium hydroxide. Since the presence of phenobarbital (a component of some oral theophylline preparations) in the serum will contribute to the absorption of theophylline at 274 nm at a pH of 13, the pH of the extract is adjusted to 10 before scanning. This shifts the barbiturate absorption peak to 240 nm permitting an accurate determination of theophylline at 274 nm.[19]

Standard concentrations of theophylline, added to drug-free serum, are carried through the same procedure as patient samples to minimize differences in the per cent recovery which might occur from run to run. The blood sample to be analyzed must be drawn one hour after the last oral dose. Specimens should be frozen if the assay is not performed immediately.

Instrumentation

The analysis was performed on a Beckman DB-G Spectrophotometer equipped with a 10" recorder.

Reagents

1. Phosphate Buffered Saline, pH 7.3: dilute 2.13 gm Na_2HPO_4 to 100 ml with 0.9% saline. Adjust the pH to 7.3 with concentrated HCl.
2. Theophylline Stock Standard (200 ug/ml): Theophylline standard should be dried in a 100°C oven and cooled before weighting. Weigh 10.0 mg of standard and dilute to 50 ml in a volumetric flask with buffered saline. Check the accuracy of a new standard stock solution as follows:
   a. To each of three test tubes containing 3.8 ml of 0.1 N NaOH, add 200 ul of 200 ug/ml Stock Standard and mix.
   b. Scan each from 350-250 nm and determine the absorbance at λ_{max} (274 nm). The absorbance of a 1% solution (10 ug/ml) should be 0.640.[37]
3. Drug-free Serum Pool. Freeze in 6.5 ml aliquotes.
4. Chloroform: Isopropanol (95:5).
5. HCl, 0.1 N.
6. NaOH, 0.1 N.
7. Ammonium Chloride, 2 M: Dissolve 10.7 gm NH_4Cl and dilute to 100 ml.

Procedure

1. Into disposable 16 x 150 mm screw-top borosilicate tubes add:

	Blank	Std-1 (10 ug/ml)	Std-2 (20 ug/ml)	Patient
Blank Serum	2.0 ml	2.0 ml	2.0 ml	-
Patient's Serum	-	-	-	2.0 ml
Stock Standard	-	100 ul	200 ul	-
0.1 N HCl	0.6 ml	0.6 ml	0.6 ml	0.6 ml
Chloroform: Isopropanol	15.0 ml	15.0 ml	15.0 ml	15.0 ml

2. Rotate all tubes 10 minutes at 25 rpm; centrifuge for 5 minutes at 1500 rpm. Aspirate the upper aqueous layer from all the tubes and discard.
3. Filter the organic layer through phase-separating filter paper into labeled 16 x 150 mm test tubes. Transfer 12.0 ml of the organic layer to a new set of labeled screw-top tubes. Add 3.0 ml 0.1 N NaOH to each tube and rotate them 10 minutes. Centrifuge.
4. With a Pasteur pipet transfer at least 2.5 ml of the aqueous layer to a clean set of 13 x 100 mm test tubes. Centrifuge.
5. Pipet 2.5 ml of the NaOH extract to a 1-cm quartz cuvette. Add 0.13 ml NH_4Cl and mix. Scan each extract (including the blank) from 400-220 nm using 0.1 N NaOH as the reference.

Results and Interpretation

The concentration of theophylline present in the sample is directly proportional to the net absorbance observed between the absorbance at 274 nm and 310 nm. Since theophylline is an acidic drug the presence of other acidic drugs such as barbiturates could easily be extracted and interfere with this assay. Some preparations of theophylline do in fact contain phenobarbital, which would contribute to the absorption of theophylline at 274 nm at a pH of 13. The addition of ammonium chloride adjusts the pH of the extract to 10, thus shifting the λ_{max} of any barbiturates to 240 nm so they will not interfere.

A comparison was made of results obtained by this method with those obtained by high performance liquid chromatography analysis (HPLC), Table II. The HPLC assays were done by the Regional Laboratory, Omaha, Nebraska.

Table II

Comparison of Theophylline Results Obtained for Samples Analyzed by UV Spectroscopy vs High Performance Liquid Chromatography

Patient	Concentration (ug/ml)	
	UV Spectroscopy	HPLC
#1 (3pm)	24	23.3
#1 (7pm)	22	19.1
#2	2	5.5
#3	10	10.1
#4	15	14.9

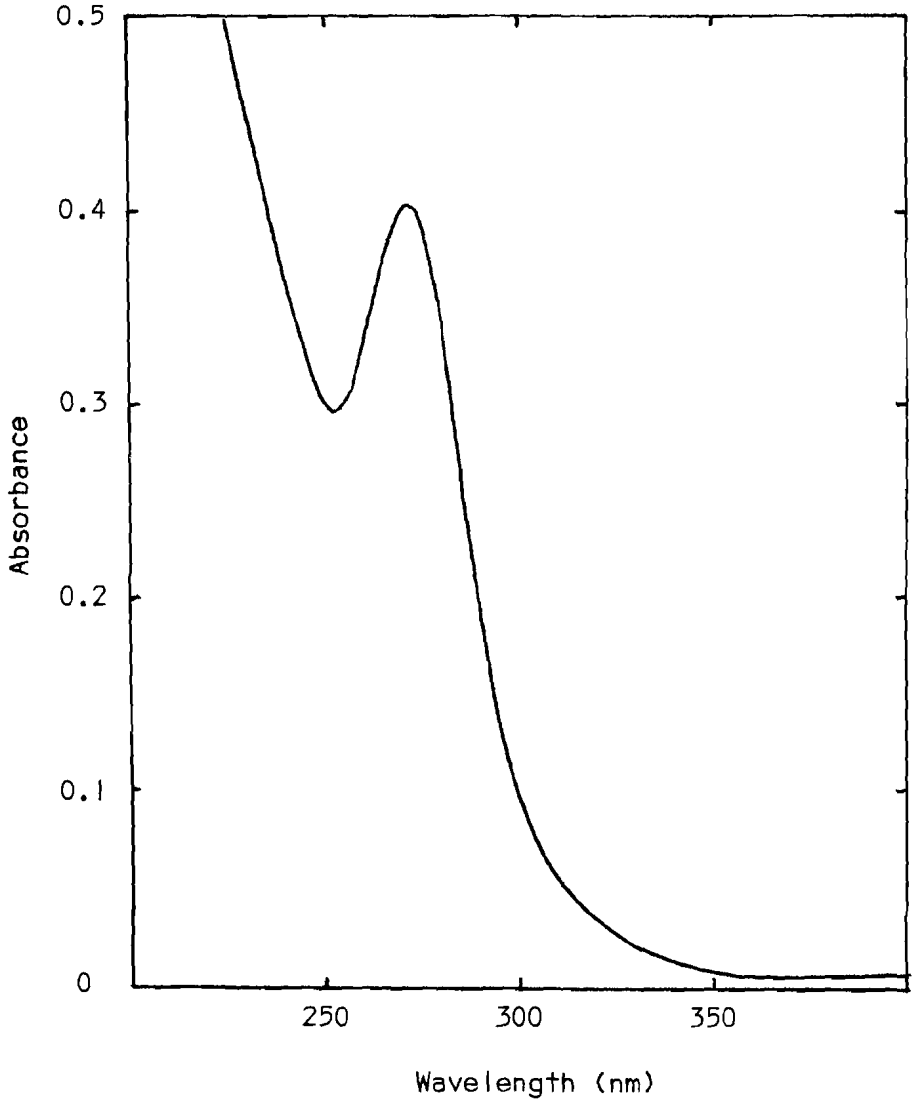


Figure 20. Absorption Spectra of Theophylline

The peak blood level of theophylline is observed 30-90 minutes after the dose. Concentrations below 5 ug/ml usually do not produce maximum therapeutic benefits while those above 20 ug/ml may produce toxic symptoms.[34]

Caffeine does not interfere with this method since it is not extracted from the solvent by the dilute NaOH. Other xanthine compounds may interfere and should be avoided. These include tea and chocolate.[39]

ESTABLISHING A RELIABLE DRUG-ABUSE PROGRAM

At the time this study was begun, in 1974, the clinical laboratory was receiving an increasing number of requests for the detection, identification and quantitation of drugs involved in cases of patient overdose. The laboratory had no drug screening procedures established except for alcohol, salicylate and carbon monoxide levels and could only respond to other drug requests by forwarding them to a reference laboratory. Results were received from 2 days to 4 weeks later--obviously of no use to the physician in his choice of patient treatment. Therefore it was necessary to establish a reliable drug-screening program for the city of Lincoln and the surrounding counties which could respond to a patient's needs on a 24-hour basis.

Obviously procedures could not be established for detecting and identifying all available drugs but would have to be limited to approximately 20 of the drugs most frequently encountered. Data obtained from the Nebraska State Crime Laboratory and other nationally established clinical toxicology programs was correlated and the drugs listed in Table 12 were determined to be the most frequently encountered abused drugs.

Since the data would be needed for the clinical treatment of the patient, turn-around-time as well as accuracy was of chief importance in the method of choice. In most cases the detection and identification of the drug or drugs present would have to be followed by a quantitative analysis. Figure 21 is a flow diagram showing the procedures used in analyzing urine in cases of suspected drug abuse and Table 13 shows the body fluid which would give the best quantitative data for specific drugs.

Table 12

Predominant Drugs of Abuse in Order of Frequency

1. ALCOHOL - Ethanol
2. MARIJUANA
3. SEDATIVES - Amobarbital, Pentobarbital, Secobarbital, Phenobarbital & Glutethemide
4. TRANQUILIZERS - Valium, Librium, Serax, Dalmane, Placidyl & Phenothiazines
5. SYNTHETIC NARCOTICS - Codeine, Demerol & Methadone
6. OPIATES - Morphine, Heroin
7. AMPHETAMINES - d-Amphetamine, Methamphetamine
8. Cocaine
9. HALLUCINOGENS - LSD, PCP
10. MISCELLANEOUS - Darvon, Salicylate & Quinine

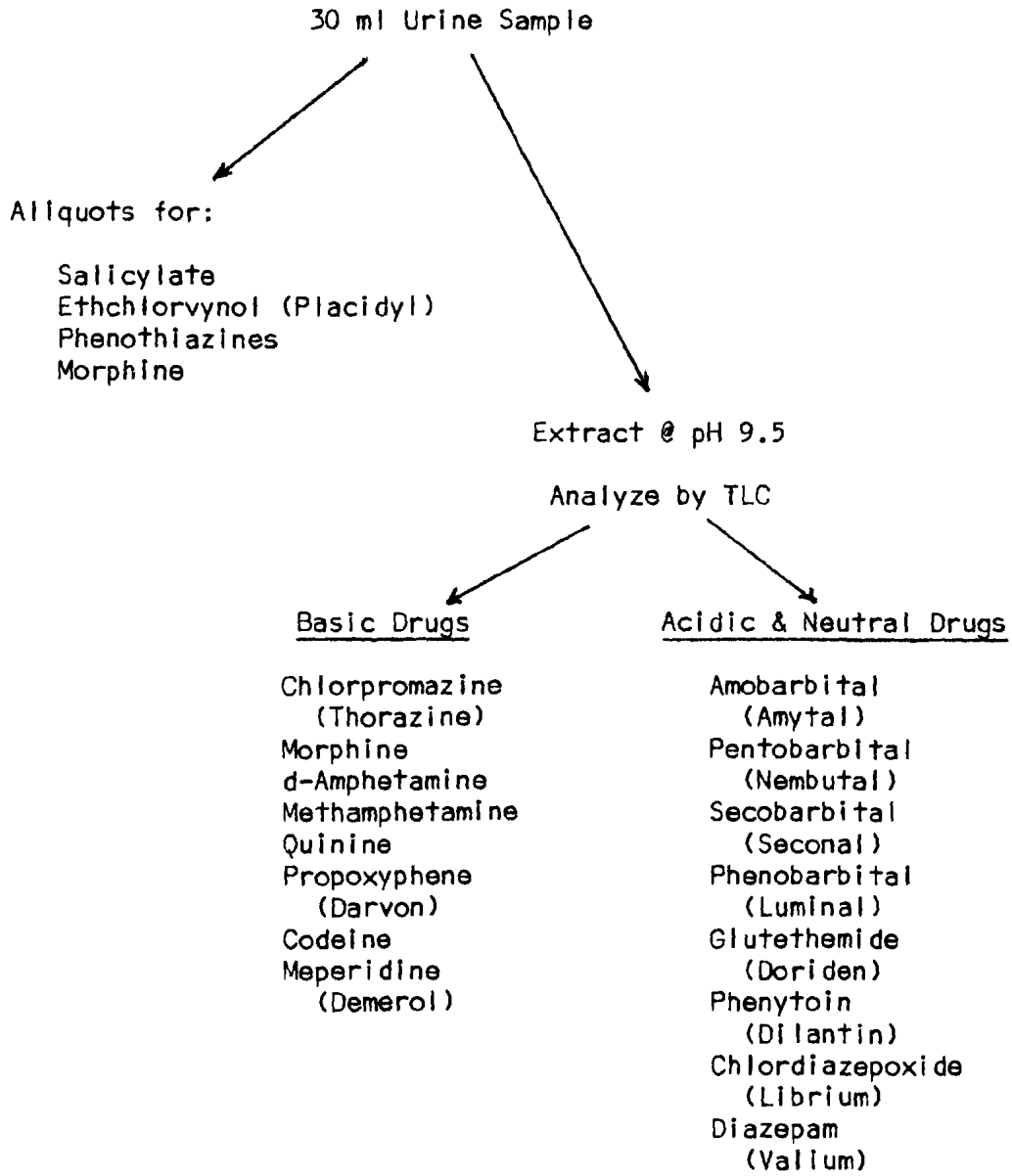


Figure 21. Flow Pattern for Analysis of Urine for Drugs of Abuse

TABLE 13

ASSAYS ESTABLISHED FOR CONFIRMATION AND QUANTITATION OF SUSPECTED DRUGS

Amobarbital - s, e

Bromide - s

Carbon Monoxide - b

Chlordiazepoxide - s, e

Diazepam - s, e

Ethanol - b, s, e

Ethchlorvynol - s, e

Meprobamate - s, e

Methemoglobin - b

Pentobarbital - s, e

Phenobarbital - s, e

Phenytoin - s, e

Primidone - s, e

Quinidine - s, e

Salicylate - s, e

Secobarbital - s, e

Theophylline - s

s = serum or plasma b = whole blood e = eye fluid

URINE SCREEN FOR COMMONLY ABUSED DRUGS BY THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography has been the method of choice by most toxicology laboratories for the rapid processing of urine samples for drug screening. Several urines can be analyzed simultaneously by TLC as opposed to individual analysis with gas chromatography. GLC analysis for the range of drugs achieved by the TLC method would involve two or more separations on two or more columns, thus lengthening the screening time. For this reason, GLC has been reserved for quantitative and confirmatory tests.

The detection of stimulants, sedative-hypnotics and narcotics in urine by thin-layer chromatogrpahy (TLC) involves only 3 steps. The separation of the drugs from the urine is accomplished by solvent extraction. Development of the TLC plates is achieved by using various developing systems in order to obtain optimal separation of the different drugs. Identification of the spots after development is accomplished by a series of chemical spray reactions, Figure 23.

Historically, the accepted method has been extraction of the drugs from the sample (usually urine) with chloroform at two or more different pH values. Drugs may be placed in three general groups, acidic, basic and neutral, based on their functional groups and the partition coefficient achieved between two immiscible solvents. For example, the acidic drugs such as phenytoin and the barbiturates may be extracted from serum, urine or tissue (aqueous phase) into an organic solvent after making the specimen slightly acidic. Conversely, in order to extract the alkaline drugs the aqueous phase must first be made more alkaline. Neutral drugs

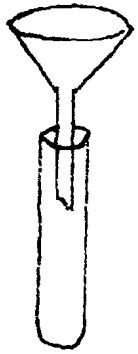
15 ml $CHCl_3$-Isopropanol

2 ml Buffer, pH 9.5

20 ml Urine

EXTRACT

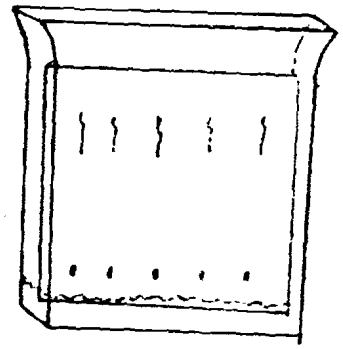
FILTER $CHCl_3$



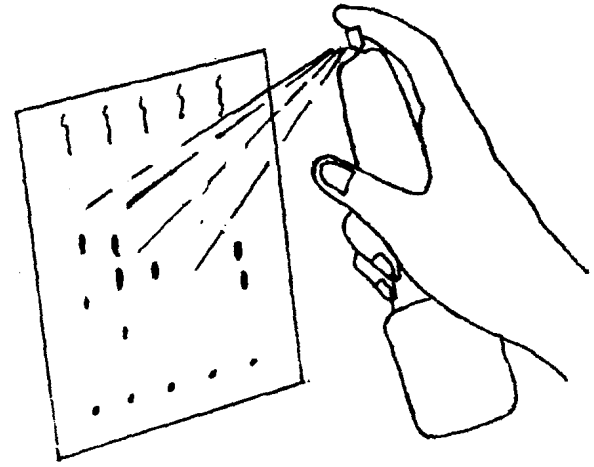
EVAPORATE

RECONSTITUTE $\bar{c}$ 30 ul MeOH

SPOT ON PLATE



DEVELOP PLATE



IDENTIFY

Figure 22. Urine Drug Screening by Thin-Layer Chromatography

such as glutethemide and meprobamate are sometimes referred to as "solvent soluble drugs" since they can be extracted into organic solvents at any pH. A few drugs, such as morphine, are amphoteric and must be extracted at a carefully controlled pH (isoelectric point) because they exhibit both acidic and alkaline properties.

Davidow simplified the procedure by showing that a single chloroform extraction at pH 9.5 will recover sufficient amounts of basic, neutral and acidic drugs into an organic solvent containing ethyl acetate:methanol: ammonium hydroxide (17:2:1).[11] A modification of his extraction procedure was developed for this procedure.

Instrumentation

The only instrumentation necessary for this procedure is an Ultraviolet Exposure and Viewing Box at two wavelengths: 254 nm and 375 nm (Gelman Co.) and a hair dryer.

Reagents

1. Extraction Solvent: 2.26% (V/V) Isopropanol in Chloroform, chromatoquality (Mallinchrodt).
2. Extraction Buffer, pH 9.5: 21 gm Ammonium Chloride in 100 ml 30% Ammonium Hydroxide.
3. Developing Solvent: Ethyl Acetate:Methanol:NH_4OH (135:10.5:4).
4. Methanol.
5. Methanolic-Sulfuric Acid: 0.5% (V/V) H_2SO_4 in Methanol.

6. Visualization Sprays: all are stable one month when refrigerated.

   Ninhydrin: 0.1 gm per 100 ml acetone.

   Diphenylcarbazone: 0.1 gm per 100 ml ethanol.

   Mercuric Sulfate: 0.25 gm in 10% (V/V) H_2SO_4.

   Iodoplatinate: 1.0 gm Platinic Chloride in 10 ml water is added to 60 gm Potassium Iodide in 200 ml water and diluted to 250 ml total volume.

   Dragendorff's Reagent: 1.3 gm Bismuth Subnitrate in 60 ml water plus 15 ml glacial acetic acid is added to 12 gm potassium iodide in 30 ml water. The mixture is then diluted with 100 ml water and 25 ml glacial acetic acid.

7. Silica Gel Thin-Layer Plates: 20 cm x 20 cm, 250 mu thick with UV indicator at 254 nm (Anal Tech).

8. Phase-Separating Filter Paper: 11 cm diameter.

9. Drug Standards: the following drug standards are available from Brinkman Instruments, Wesburg, N. Y.

   Weigh the following groups of drug standards and reconstitute each group with 2.0 ml Methanol.

   Standard-1: 8 mg Phenobarbital, 8 mg Pentobarbital, 5 mg Glutethemide.

   Standard-2: 3 mg Chlordiazepoxide (Librium), 8 mg Phenytoin (Dilantin), 8 mg Amobarbital and 8 mg Secobarbital.

   Standard-3: 3 mg Chlorpromazine, 2 mg Morphine, 5 mg d-Amphetamine, 10 mg Methadone and 1 mg Quinine.

   Standard-4: 10 mg Propoxyphene (Darvon), 2 mg Codeine, 2 mg Meperidine (Demerol) and 3 mg Diazepam (Valium).

Procedure

Developing Solvent and Activation of TLC Plate.

1. Prepare the developing solvent which will be used, directly in the developing tank. Replace the lid and shake vigorously to mix well. Line the chamber with filter paper and allow at least 30 minutes for the solvent to equilibrate within the chamber.
2. The thin-layer plates should be activated in a drying oven prior to use to ensure good results. Lay the sheet horizontally on a towel and heat for 15 minutes at 100°C. (This removes water, which could interfere with the action of the solvent.)

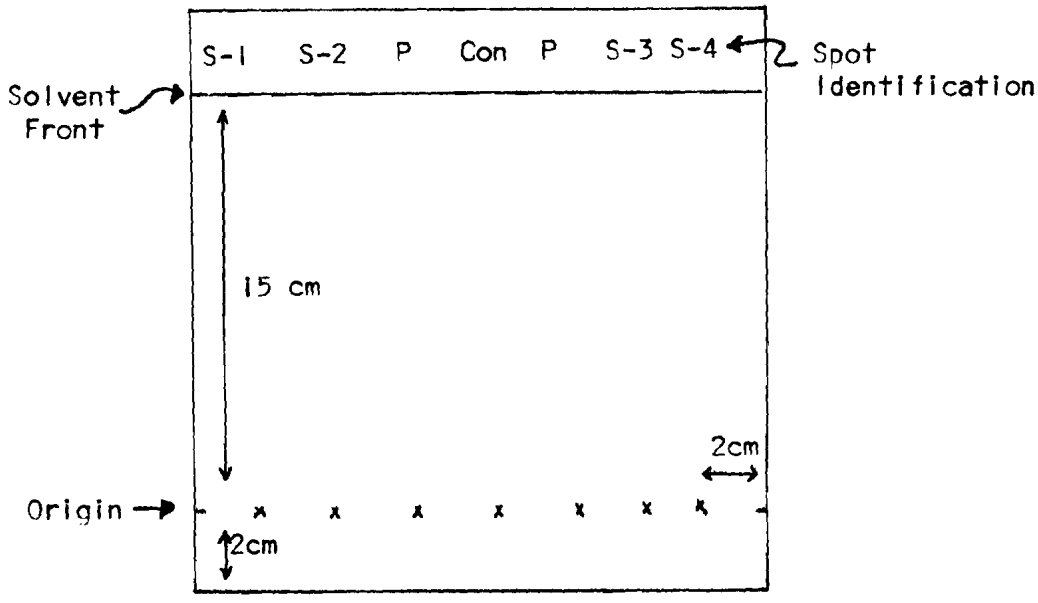
Solvent Extraction of Drugs from Urine

1. To a labeled extraction tube add: 15 ml Extraction Solvent, 2.0 ml Extraction Buffer and 15-20 ml Urine. Shake for 5 minutes. Let the phases separate, centrifuging if necessary.
2. Remove the upper aqueous layer by aspiration and discard. Decant the chloroform layer into a clean labeled test tube. Add 3 drops of Methanolic-Sulfuric Acid and evaporate to dryness in a heating block at 65°C with a stream of nitrogen.

Thin-Layer Analysis

1. While the eluate is being concentrated, the TLC plate can be marked for spotting. Standards must be spotted on each plate. Place the standards at the outside edges of the chromatogram if possible. Mark the level of the origin 2 cm from the bottom of the plate and place the spots at least 2 cm from each edge and no closer than 1.5 cm

from other spots. No matter how many spots there will be, they should be spread out equidistantly across the plate. Mark the solvent front 15 cm above the origin.



2. The extract residues should be redissolved in approximately 15 ul of Methanol. Dissolve the drugs in the bottom inch of the tube by rotating the tube. It is important to spot all of the extract onto the plate. Spot 10 ul of each Standard.
3. Allow the spotted plate to dry thoroughly on the counter for 4-5 minutes before developing the plate.
4. Place the plate into the appropriate solvent which has been sufficiently equilibrated. Allow the solvent to rise to the 15 cm mark.
5. Remove the plate from the developing chamber and allow to dry. Using the UV Viewing Box expose the plate to 254 nm UV light and locate any spots present. Place the plate in a 75°C oven and allow to dry for

10 minutes. All of the ammonia vapors must be removed before proceeding with the visualization of the chromatogram, therefore the oven should be equipped with a ventilation fan.

Visualization of the Chromatogram

1. Remove the plate from the oven and while it is still hot, spray lightly with 0.1% Ninhydrin solution over the lower half of the plate. Place under the UV light (375 nm) for 2 minutes. Remove.

   The Amphetamines will be a pink color. Metabolites of Methadone and Secobarbital will also appear as pink spots. Phenylpropanolamine and the amino acids will appear as pink spots, Figure 23a.

2. Spray with Diphenylcarbazone.

   The color of the Amphetamines will intensify.

3. Spray with Mercuric Sulfate. Start at the top and work down the plate gradually. (The background will turn purple which will clear with heating later.)

   Doriden will appear as a purple stain. Other drugs such as Meprobamate, Dilantin and the Barbiturates will show a pink-to-violet color, Figure 23b.

   Dry the plate using heat from the hair dryer.

   Chlorpromazine turns pink. Secobarbital will turn white.

   Using the hair dryer, dry with cool air. (The background will fade from purple back to white, thus making the lower concentrations of barbiturates more easily seen.)

4. Place the plate in a 75°C oven for about 2 minutes. Remove.

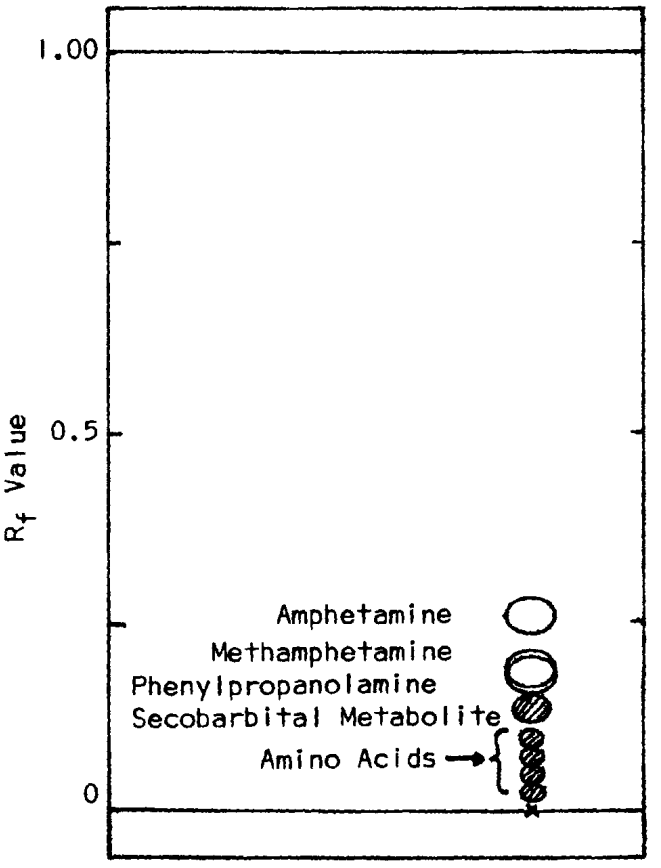


Fig. 23 a. Location of Drugs Reacting with Ninhydrin on TLC Plate.

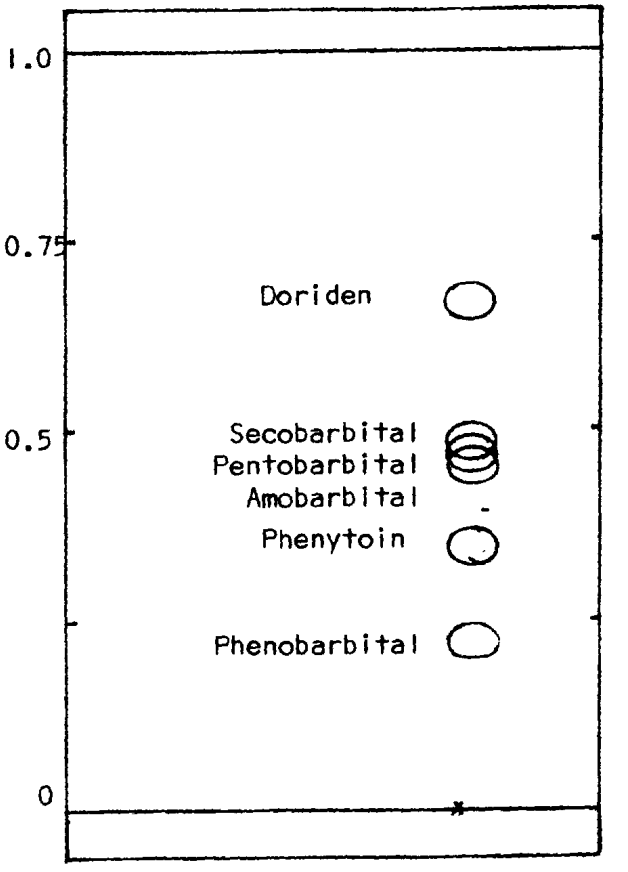


Fig. 23 b. Location of Drugs Reacting with Diphenylcarbazone/ Mercuric Sulfate on TLC Plate.

Observe the pink-colored spots for Phenothiazines and their metabolites. Most Phenothiazine tranquilizers and Antihistamines react with the H_2SO_4 in the Mercuric Sulfate spray to give colored complexes at this stage.

5. Allow the plate to cool and place under UV light (375 nm). Observe the blue fluorescent spot of Quinine and look for its metabolites in actual urine samples. Valium, Librium and their metabolites fluoresce yellow to green. Figure 24a.

6. Remove the plate and spray with Iodoplatinate. Spray generously to intensify the colors of the spots. Spray heavily towards the bottom of the plate for Morphine. Dry with the hair dryer.

   Morphine, Methadone, Meperidine (Demerol), Codeine and Quinine will all be visible. Morphine will be a blue spot. Figure 24b.

7. Spray with Dragendorff's Reagent to preserve the chromatogram.

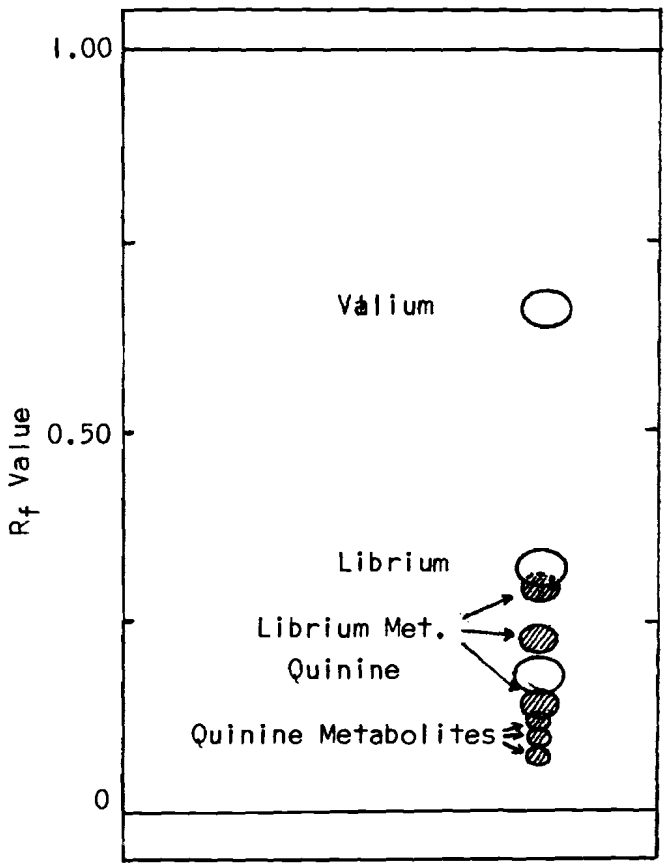


Fig. 24 a. Location of Drugs Which Fluoresce Under UV Light @ 375 nm.

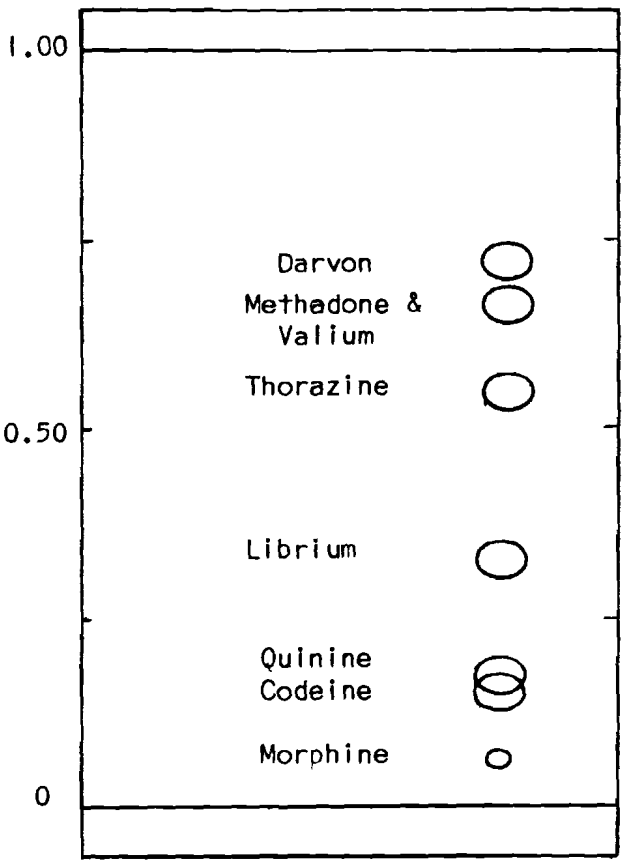


Fig. 24 b. Location of Drugs Which React with Iodoplatinate on TLC Plate.

Interpretation

One of the biggest problems encountered when interpreting thin-layer results is the presence of many metabolites in a patient's urine. Only experience can give one confidence in this area.

Tables 14 and 15 show the R_f values and color reactions of the drugs most frequently encountered in this laboratory. As a general rule, no drug should be reported as present unless the parent drug standard is actually run simultaneously with the urine samples so that R_f values and color reactions can be compared carefully. In cases of heavy overdose, known metabolite patterns of the suspected drug should also match and a second confirmatory procedure performed if possible.

Figure 25 shows the metabolite patterns of several common drugs. Data was obtained using the extraction procedure and solvent solution previously described. The fast-acting barbiturates, amobarbital, pentobarbital and secobarbital, all have a strong staining metabolite near the origin, which may be obscured at times by over-riding pigments. In addition, amobarbital and secobarbital each have a metabolite located at an R_f value of 0.37 and 0.14 respectively. Since these are fast-acting barbiturates they are sometimes almost completely metabolized by the liver, in which case a low concentration of the parent compound is present in the urine and only a very faint spot is seen on the thin-layer plate. If this occurs a screen by a more sensitive method, hemaglutination inhibition, is necessary.

Of the neutral drugs, chlordiazepoxide (librium) and diazepam (valium) both exhibit characteristic metabolite patterns with TLC analysis which

TABLE 14

TLC R_f VALUES AND COLOR REACTIONS OF ACIDIC DRUGS EXTRACTED AT pH 9.5[a]

Drug	R_f	Rel R_f[b]	Diphenylcarbazone/ Mercuric Sulfate
Amobarbital (Amytal)[c]	0.41	0.92 ± 0.02	pink
Glutethemide (Doriden)	0.67	1.50 ± 0.11	purple
Pentobarbital (Nembutal)	0.45	1.0	pink
Phenobarbital (Luminal)	0.22	0.49 ± 0.05	pink
Phenytoin (Dilantin)	0.34	0.76 ± 0.03	pink
Secobarbital (Seconal)[c]	0.47	1.04 ± 0.02	pink
Ethchlorovynol (Placidyl)	0.75	1.66	pink

[a] Solvent System: Ethylacetate:Methanol:Ammonium hydroxide (135:10.5:4.5)

[b] Rel. R_f = R_f value relative to Pentobarbital ± Standard Deviation where n=10.

[c] The metabolite of Amobarbital gave a Rel R_f = 0.82 and the metabolite of Secobarbital gave a Rel R_f of 0.30.

TABLE 15

TLC R_f VALUES AND COLOR REACTIONS OF BASIC AND NEUTRAL DRUGS EXTRACTED AT pH 9.5[a]

	R_f	Rel R_f[b]	Ninydrin & Heat	H_2SO_4 & Heat	UV Light (375 nm)	Iodoplatinate
d-Amphetamine (Dexidrine)	0.26	0.60 ± 0.02	pink	-	-	red-violet
Chlordiazepoxide (Librium)	0.32	0.75 ± 0.02	-	-	yel-grn	red-violet
Chlorpromazine (Thorazine	0.54	1.23 ± 0.04	-	pink	-	purple
Codeine	0.15	0.34 ± 0.03	-	-	-	red-violet
Diazepam (Valium)	0.66	1.52 ± 0.14	-	-	yel-grn	red-violet
Meperidine (Demerol)	0.44	1.0	-	-	-	red-violet
Methadone (Dolophine)	0.66	1.51 ± 0.12	-	-	-	red-violet
Morphine	0.06	0.13 ± 0.02	-	-	-	blue
Nicotine	0.47	1.07 ± 0.02	-	-	-	blue
Methamphetamine (Methedrine)	0.19	0.44	pink	-	-	-
Phenylpropanolamine	0.18	0.40	pink	-	-	-
d-Propoxyphene (Darvon)	0.72	1.64 ± 0.15	-	-	-	red-violet
Quinine	0.17	0.38 ± 0.08	-	-	blue	red-violet
Strychnine	0.16	0.36	-	-	-	purple
Thioridazine	0.20	0.45	-	blue	-	purple

[a] Solvent System: Ethylacetate:Methanol:Ammoniumhydroxide (135:10.5:4.5)

[b] Rel. R_f = R_f value relative to Meperidine (Demerol) ± Standard Deviation where n = 10.

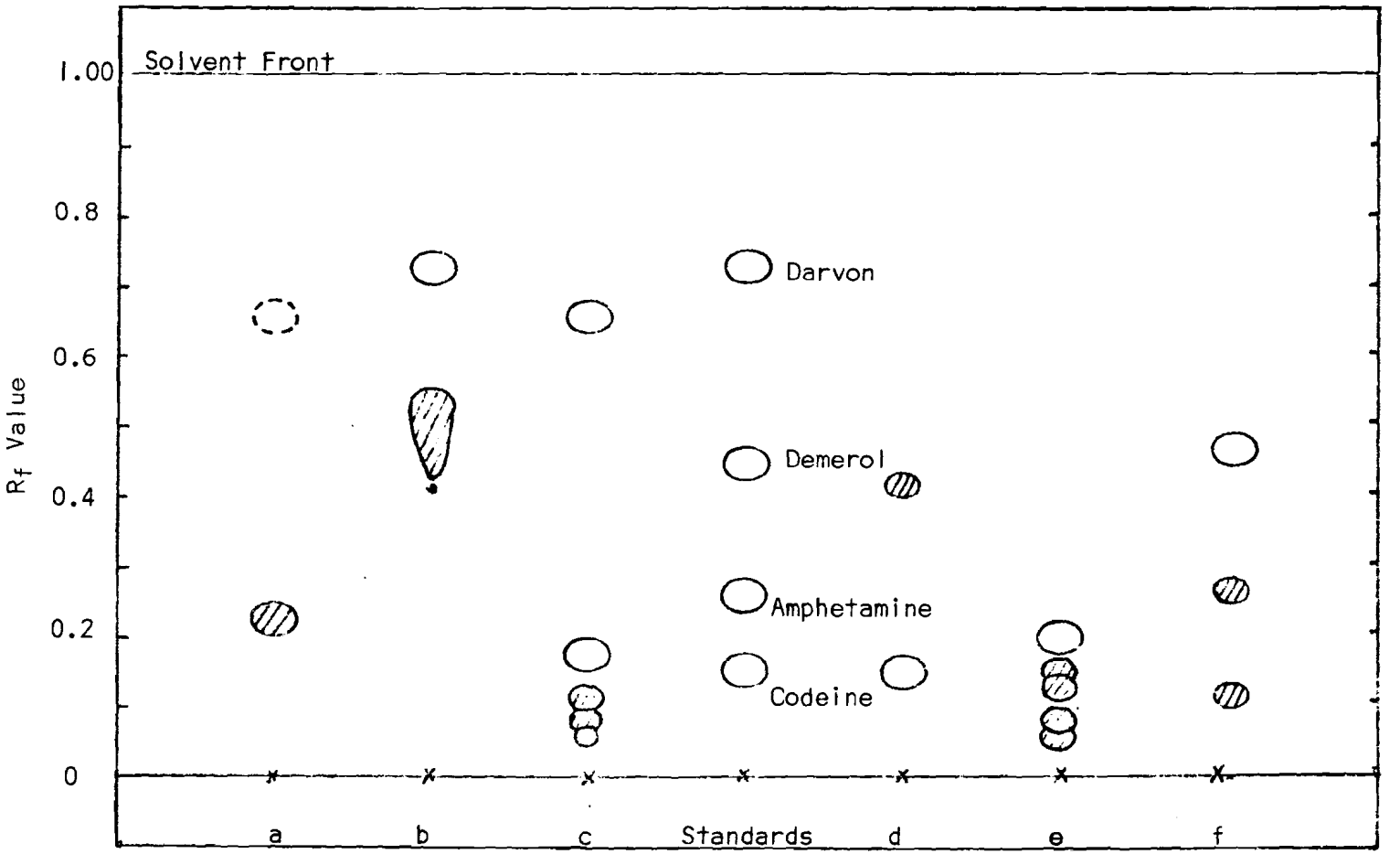


Figure 25. Thin-Layer Chromatogram Patterns of Some Common Drugs of Abuse and Their Metabolites

a. Valium & Serax
b. Darvon & metabolite
c. Methadone, Quinine & Morphine
d. Codeine
e. Mellaril & metabolites
f. Nicotine & metabolites

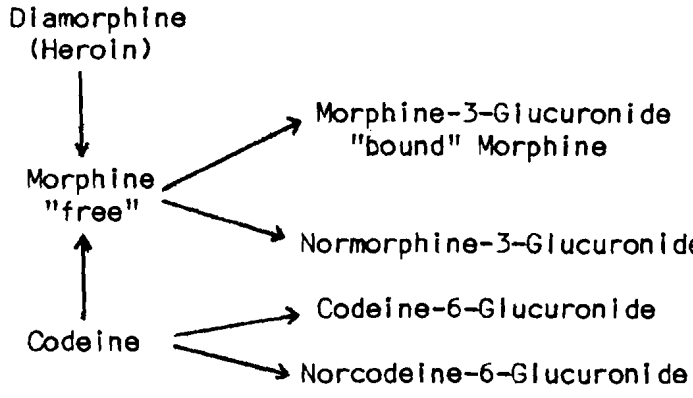
fluoresce yellow-green under UV light at 375 nm, Figure 25a. The librium metabolites have R_f values of 0.29, 0.22 and 0.13 while the metabolite of valium, oxazepam (serax), is located at an R_f value of 0.22 and reacts with iodoplatinate to give a red-violet color.

Figure 25b shows the metabolite of d-propoxyphene (darvon) which appears as a large dark purple streak with iodoplatinate. It often extends up to 2 cm in length beginning at an R_f of 0.55. With a therapeutic dose the parent drug may be barely visible but the metabolite is easily identified.

Figure 25c is a pattern which might be seen in a methadone clinic patient. Methadone, quinine and morphine are all present. Quinine, which is sometimes used to dilute heroin, can be detected for 5-14 days after a dose has been taken. It is also excreted after the ingestion of certain cold remedies or tonic (quinine) water, so if found alone is not necessarily indicative of heroin use. Quinine can be identified by the presence of its three metabolite spots which give a brilliant blue fluorescence under UV light (375 nm). Quinine metabolites also react with iodoplatinate and appear red-violet at R_f's of 0.11, 0.09 and 0.06.

Morphine has three possible drug sources to account for its presence in urine. Equation (4) shows the interrelationship of the metabolism of heroin, codeine and morphine. Since only about 7% of morphine is in the free form and can be extracted, a more sensitive technique, such as hemaglutination inhibition is necessary for detecting its presence in urine.[1]

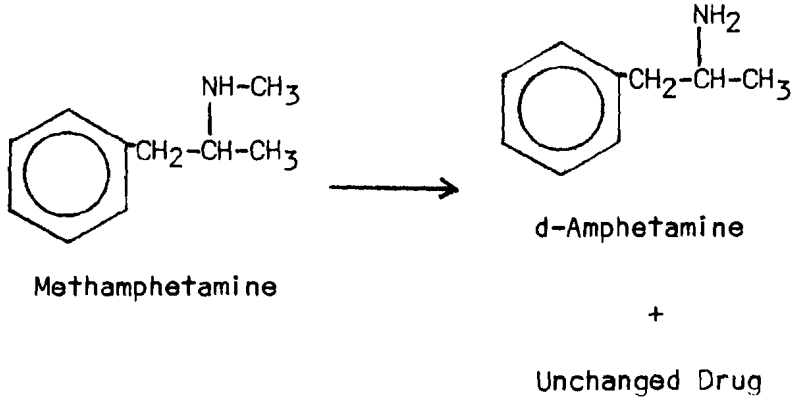
Figure 25d shows codeine and its metabolite with an R_f of 0.42, just below nicotine and demerol. Since morphine is a metabolite of codeine, a strong codeine and a weak morphine spot indicates that codeine was taken.



(4)

Two phenothiazines, chlorpromazine (thorazine) and thioridizine (mellaril) are frequently encountered. The metabolite of thorazine appears pink at an R_f of 0.20 with ninhydrin. The four metabolites of mellaril, Figure 25e, when exposed to H_2SO_4 spray and heat appear as blue, orange, purple and red spots at R_f values of 0.16, 0.13, 0.09 and 0.07 respectively.

Many normal urines may contain ninhydrin positive spots. Many compounds such as amino acids, Vitamin C and the estrogen compounds in oral contraceptives may be present. For this reason, only three ninhydrin-positive drugs are included in this screening procedure. It also must be noted that methamphetamine is demethylated to amphetamine, Equation 5, so both should be present in the case of methamphetamine abuse.



(5)

The urine of smokers will contain nicotine and its metabolites. Nicotine has an R_f value of 0.47 which may overlap demerol, R_f = 0.44, if it is present. In these cases demerol may be seen as a red-violet spot peaking out just below the blue staining nicotine spot, Figure 25f. In heavy smokers, nicotine's metabolites which are red-violet with iodoplatinate can be seen at R_f's of 0.27 and 0.12.

Sources of Error

The main sources of errors can be avoided by using good TLC techniques Table 16 summarizes some of the most frequent difficulties encountered with the method.

In general it is best to spray the plate only enough to accurately identify the spots present. Overheating or overexposing the plate to UV light will tend to oxidize some drugs and overspraying may prevent the drug from reacting further with another spray reagent applied later in the spray sequence.

In order to make a positive identification, all unknown spots must be compared to the standard drug spots on the same plate and both the R_f values and color reaction sequence must correlate.

Table 16

Summary of TLC Problems and Their Probable Causes

1.	Tailing spots.	1. Extraneous aqueous material remaining in the tube after the evaporation step. Spot on plate not thoroughly dry before placing it in the developing solvent.
2.	Distorted spots.	2. Punctured sorbent layer during spotting.
3.	Large, diffuse spots.	3. Too large of spot size.
4.	Amphetamines not appearing.	4. Residual ammonia vapors will cause the entire plate to turn pink. TLC plate should be warm when the ninhydrin is sprayed onto it.
5.	Barbiturates not appearing	5. Overspraying with ninhydrin.
6.	Alkaloids & other basic drugs not appearing.	6. Try spraying lightly with Dragendorff's first to acidify the plate, then follow with Iodoplatinate. Allow the reagents time to react.

URINE SCREEN FOR THE PRESENCE OF SALICYLATE, ETHCHLORVYNOL (PLACIDYL) AND PHENOTHIAZINES

Ethchlorovynol (Placidyl) is a mild hypnotic with a quick onset and short duration. It also has anticonvulsant and muscle-relaxing properties and may induce tolerance. Placidyl will react with diphenylamine in an acid solution to produce a pink color.[39]

In the presnece of ferric salts, acetylsalicylic acid (aspirin) develops a violet color. Death caused by toxic overdoses of aspirin in adults is uncommon; however, overdose of salicylate among children is still common and often causes death.[45]

Phenothiazines develop characteristic colors when oxidized by FPN reagent.[16]

These three quick urine screening tests are good indicators of drug overdose. Both the placidyl and salicylate reagents can be used to quantitate the serum drug levels.

Reagents

1. FPN Reagent: mix 5 ml Ferric Chloride (5 gm/dl) with 45 ml of 20% Perchloric Acid and add 50 ml 7.5 N HNO_3.
2. Trinder's Reagent: add 4 gm Ferric Nitrate $[Fe(NO_3)_3 \cdot 9\ H_2O]$ and 4 gm Mercuric Chloride ($HgCl_2$) to a 100 ml volumetric flask. Add 12.0 ml of 1 N HCl. Dissolve and dilute to 100 ml with deionized water.
3. Trichloracetic Acid, 10 gm/dl: dissolve 10 gm Trichloracetic Acid and 0.1 gm NaCl in distilled water and dilute to 100 ml.

4. Placidyl Color Reagent: dissolve 1.0 gm diphenylamine in 50 ml of conc. H_2SO_4. Carefully, while stirring constantly, add this solution to a mixture of 50 ml water and 50 ml glacial acetic acid.
5. Placidyl Control Reagent: carefully add 50 ml conc. H_2SO_4 to a mixture of 50 ml water and 50 ml glacial acetic acid, while stirring constantly.
6. Placidyl Standard, 10 mg/dl.
7. Salicylate Standard, 10 mg/dl and 30 mg/dl.
8. Phenothiazine Standard, 15 mg/dl.

Procedure and Interpretation

1. Phenothiazine Screen

To two 13 x 75 mm test tubes add 1.0 ml Phenothiazine Standard solution to the "Positive-Control" tube and 1.0 ml of the patient's urine to the "Test" tube. Add 2.0 FPN Reagent to each tube, mix and observe the intensity and color produced within 10 seconds. A blue, purple or pink color may indicate the presence of the phenothiazines.

Blue Color:	Thioridazine (Mellaril)
Orange Color:	Promazine (Sparine) Trithioperazine (Stelazine)
Pink to Purple:	Chlorpromazine (Thorazine) Prochlorperazine (Compazine) Flurophenazine (Prolixin) Promethazine (Phenegrin) Methdilazine (Tacaryl) Thiopropazate (Dartal) Perphenazine (Trilafon) Triflupromazine (Vesprin)

False Positives: Orange may indicate a false positive, where urine specimens giving blue, purple or pink colors with FPN pose no problem with false positives.

This test in some cases is not sensitive to therapeutic levels of some phenothiazines. It is a good indicator, however, of gross overdosage. Blood levels are too low to make blood a useful specimen.

2. Salicylate Screen

To two 13 x 75 mm test tubes add 1.0 ml Salicylate Standard solution to the "Positive-Control" tube and 1.0 ml of the patient's urine to the "Test" tube. Add 1.0 ml of Trinder's Reagent to each and mix. By visual comparison determine if any salicylate is present. Salicylate will form a purple complex.

If the salicylate screen is positive, salicylate may be quantitated.[46] on serum by pipetting 0.3 ml water (blank), standard (20 mg/dl), and patient's serum to three labeled 13 x 100 mm test tubes. To all of the tubes add 3.0 ml Trinder's Reagent. Mix and centrifuge all tubes 5 minutes at 1500 rpm. Transfer the clear supernatant to cuvettes and record the absorbance at 540 nm. The concentration can be calculated by using the equation:

$$\frac{\text{Absorbance of Unknown}}{\text{Absorbance of Standard}} \times 20 \text{ mg/dl} = \text{mg/dl of Salicylate}$$

For adults toxicity usually occurs above 30 mg/dl.[37]

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For adults toxicity usually occurs above 30 mg/dl.[37]

d. Mix well and allow the color to develop for 2 hours. Read at 510 nm against the appropriate blank. The color is stable for 8 hours.

Therapeutic blood concentrations range from 10-20 ug/ml. Coma will result at blood levels greater than 50 ug/ml and may cause death. When combined with small amounts of ethanol (0.05% to 0.15%), ethchlorvynol concentrations as low as 25 ug/ml can prove fatal.[4] Formaldehyde, acetaldehyde, acetone, or paraldehyde in excess of 0.1% will inhibit the color development. A chloroform extraction will overcome this. No other interferences are known.[39]

URINE SCREEN FOR MORPHINE BY HEMAGGLUTINATION INHIBITION

Hemagglutination Inhibition (HI) is an extremely sensitive technique for detecting the presence of morphine in urine which makes it a reliable test for the exclusion of drug abuse. Morphine is excreted in the urine as morphine-3-glucuronide. Only approximately 7% remains as free morphine, thus making detection by TLC difficult since only the unconjugated "free" form can be extracted. Using hemaglutination inhibition as little as 20 ng/ml morphine can be detected in the urine.[1]

In hemagglutination inhibition the diluted urine is mixed with antisera and allowed to react. If morphine is present it will be bound to and neutralize the antisera. When the sensitized red cells are added to the system there is no free antisera remaining and the cells settle to the bottom of the well--the agglutination has been inhibited.

Reagents

All reagents can be obtained from Technam, Inc., Park Forest South, Illinois.[40]

1. Morphine Antisera, lyphollzed: Reconstitute with Reagent Diluent. Shelf life of refrigerated antisera is 24-48 hours. Antisera may be frozen in small aliquotes and thawed up to three times. (Refer to package insert for reconstitution instructions.)
2. Reagent Diluent.
3. NRS 100 Diluent.
4. Morphine Standard, 1 ug/ml.

on the counter several times and then gently tapping the tray on the counter. Let stand at room temperature for 5-10 minutes.

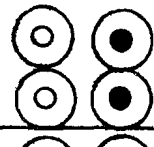
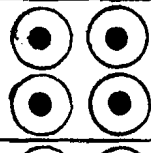
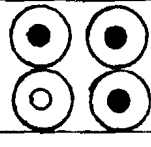
4. Prepare the red cells by gently mixing the cells well. Transfer 1.0 ml to a small test tube and centrifuge at 1700 rpm for 10 minutes. Pour off and discard the supernatant. Add 1.0 ml NRS 100 Diluent and prepare a uniform cell suspension by repeated and forceful pipeting.
5. Add two drops of washed red blood cells to all wells. Mix well; cover with a second tray to prevent evaporation and let stand at room temperature for 90 minutes.

Results and Interpretation

Table 17 shows the proper interpretations for the results which can be obtained with the morphine hemagglutination inhibition test. This procedure is very specific and sensitive for morphine in urine. Quantitation of urine levels of morphine is often desired for newborns whose mothers' are questionable heroin addicts. The level of morphine present in the baby's urine can give an indication as to the amount of heroin involved and alert the physician to possible withdrawal symptoms in the infant.

Table 17

Interpretation of Morphine Test Results Obtained by Hemagglutination Inhibition

Observation	Interpretation
	Normal urine, no morphine present.
	Positive test: equal to or greater than 300 nanograms of morphine per milliliter urine.
	Error in procedure. Tests not showing duplicate results must be repeated.

DETERMINATION OF SERUM BARBITURATE LEVEL BY GLC

The quantitation of three of the more common barbiturates, amobarbital pentobarbital and secobarbital, in serum can easily be accomplished by using the GLC procedure previously discussed for the analysis of anti-epileptic drugs. By changing the instrument parameters, slightly better GLC data was obtained for these barbiturates since they appear earlier on the chromatogram.

Instrumentation

The GLC analysis was performed on a Bendex, Model #2500 gas chromatograph equipped with flame ionization detector. Separation was achieved using a 6' x 1/8" i.d. U-shaped glass column containing 3% OV-17 on Gas Chrom Q (100-120 mesh).

Table 18

Instrument Parameters for GLC Analysis of Barbiturates

		Input Pressure (psi)	Flow Rate (cc/min)
Gas Flow Rate:	Nitrogen	50	50
	Hydrogen	10	40
	Air	50	400
Temperatures:	Injection Port:	300°C	
	Detector:	300°C	
	Oven (Program):	140°C initial to 275°C final @ 10°C/min.	

Procedure

The procedure can be carried out in the same manner as the anticonvulsant drug assay. Only one internal standard is required, 5-ethyl-5(p-tolyl) barbituric acid (Aldrich Chem. Co.) and the four barbiturate standards. To prepare 100 ml of drug standard, 20 mg of Sodium Amobarbital (Lilly), Sodium Secobarbital (Lilly) and Sodium Pentobarbital (Abbot) are weighed. Forty mg of Sodium Phenobarbital (Brinkman) is weighed and added to the 100 ml volumetric flask also. The drugs are dissolved in Methanol and diluted to volume. Figure 26 shows the barbiturate profile obtained for the drug standards.

Interpretation

Table 19 shows the toxic levels of the barbiturates.

Table 19

Toxic Levels of Some Common Barbiturates

Drug	Toxic Level
Amobarbital	Greater than 15 ug/ml
Pentobarbital	Greater than 10 ug/ml
Phenobarbital	Greater than 60 ug/ml
Secobarbital	Greater than 10 ug/ml

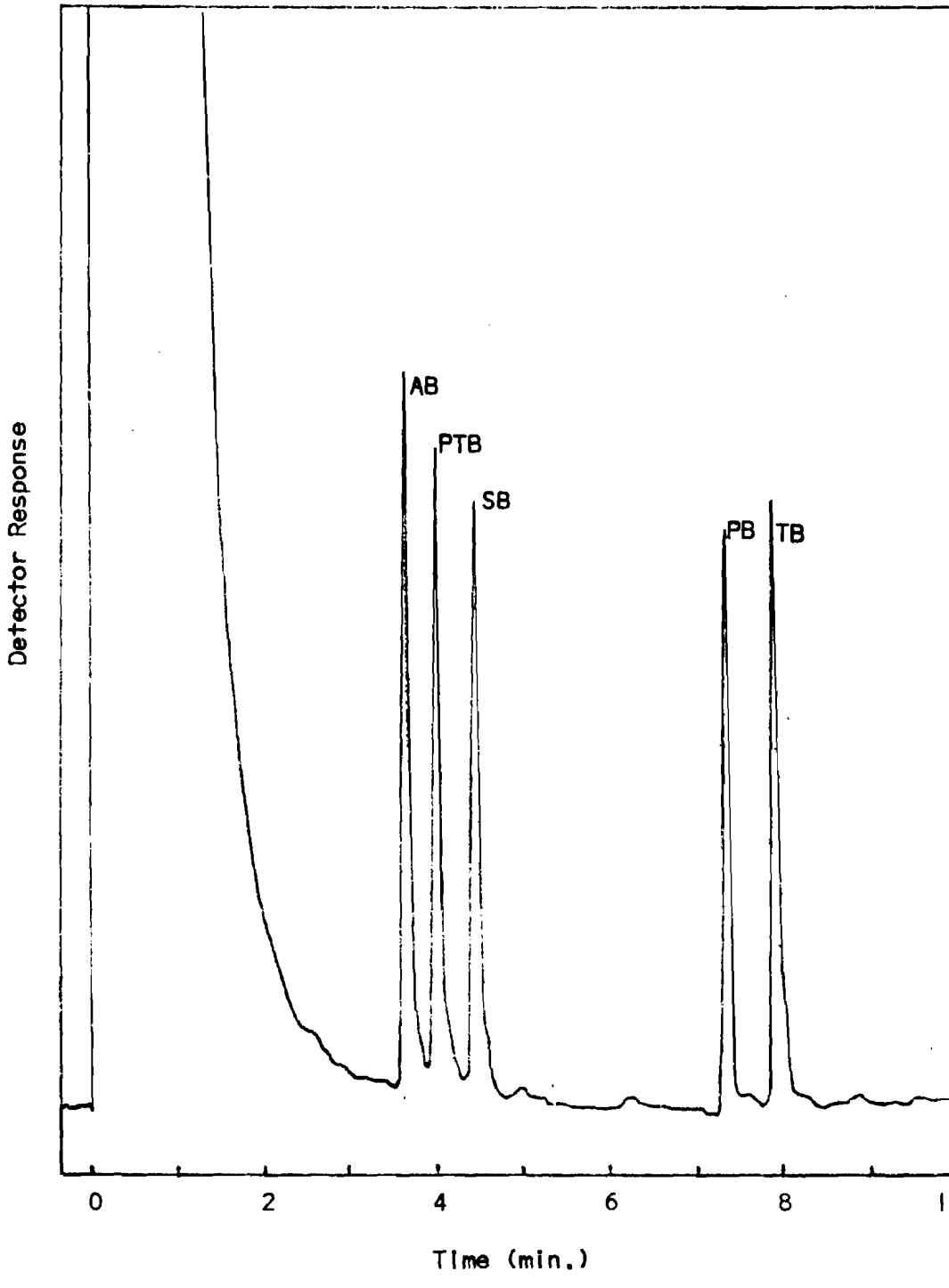


Figure 26. GLC Chromatogram of Barbiturates in Serum

Concentration of the drugs represented: 18.2 ug/ml Amobarbital (AB), 18.4 ug/ml Pentobarbital (PTB), 18.3 ug/ml Secobarbital (SB), and 36.4 ug/ml Phenobarbital (PB). The concentration of the Internal Standard, p-Tolylbarbital (TB) is 20 ug/ml.

CONFIRMATION AND QUANTITATION OF BENZODIAZEPAMS IN SERUM

This procedure can be used to screen blood, urine or gastric washings for the presence of four major benzodiazepams, Diazepam (Valium), Chlordiazepoxide (Librium), Flurazepam (Dalmane) or Oxazepam (Serax). By including the appropriate drug standards, Valium and Librium levels can be determined.

The drugs are first extracted into chloroform. The chloroform is then back-extracted with dilute acid which is scanned for Librium. After evaporating the chloroform layer to dryness, the residue is dissolved in hexane. The hexane is then extracted with acid, which is scanned for Valium, Serax and Dalmane.[39]

Reagents

1. Stock Drug Standards: 5 mg of each drug is weighed and diluted to 25 ml with distilled water.
2. Phosphate Buffer, 3 M (pH 3.6): transfer 42.4 gm of Sodium Dihydrogen Phosphate to a 100 ml volumetric flask and dilute to volume with distilled water.
3. Chloroform, spectroquality.
4. Sodium Hydroxide, 0.45 N: dissolve 18 gm of Sodium Hydroxide in water and dilute to volume with distilled water.
5. n-Hexane, spectroquality.
6. Hydrochloric Acid, 2 N: add 17 ml concentrated HCl to approximately 50 ml of distilled water and dilute to a total volume of 100 ml.

Procedure

1. To labeled, screw-top 16 x 150 mm borosilicate tubes add:

	Blank	Standard	Patient
Blank Serum	3.0 ml	3.0 ml	-
Drug Standard (20 mg/dl)	-	100 ul	-
Patient Serum	-	-	3.0 ml
Phosphate Buffer, 3 M (pH 3.6)	0.15 ml	0.15 ml	0.15 ml
Chloroform	6.0 ml	6.0 ml	6.0 ml

2. Rotate tubes 5 minutes at 25 rpm. Centrifuge 3 minutes at 1500 rpm.
3. Carefully aspirate off most of the top layer. Filter the chloroform through phase-separating filter paper into a second set of labeled screw-top tubes. Wash filter papers with a small amount of chloroform to ensure complete transfer of drugs.
4. Add 4.0 ml of 0.45 N NaOH to each tube. Rotate 5 minutes; centrifuge.
5. Remove the aqueous NaOH layer. This may be saved and scanned for barbiturates if suspected.
6. If Librium is suspected extract with 3.0 ml 0.5 N H_2SO_4. Scan the H_2SO_4 layer for Librium, Figure 27.
7. Filter the chloroform layer with phase-separating paper into a 16 x 100 mm test tube and evaporate to dryness at 65°C under a stream of nitrogen.
8. Dissolve the residue in 5.0 ml hexane; mix well. Add 3.0 ml 2 N HCl to each tube. Vortex for 30 seconds and centrifuge.
9. Aspirate the hexane and scan the HCl solution for drugs. Serax and Dalmane may be identified in this extract as well as Valium.

Results and Interpretation

The ultra-violet spectrum obtained for an unknown must first be identified by comparing it to the spectras obtained for the standards. Table 20 shows the absorbance maximas and minimas for several common benzodiazepams and Figure 27 shows the actual ultra-violet scans obtained for these drugs.

Table 20

Wavelengths of the Maximum and Minimum Absorbance For the Ultra-violet Spectras of Benzodiazepams

Drug	Wavelength (nm)		
	λ_{max}	λ'_{max}	λ_{min}
Chlordiazepoxide (Librium)	245	310	290
Diazepam (Valium)	240	285	265
Flurazepam (Dalmane)	275	-	265
Oxazepam (Serax)	230	275	265

To determine the concentrations of valium or librium present, the Net Absorbance (difference between the Absorbance value at the maximum wavelength, λ_{max}, and the value obtained at the minimum wavelength, λ_{min}, must be determined. Then using Equation 6 the level of either drug can be calculated.

$$\text{Concentration of Unknown (ug/ml)} = \frac{[\text{Net Absorbance of Unknown}]}{[\text{Net Absorbance of Standard}]} \times 6.6 \text{ ug/ml} \qquad (6)$$

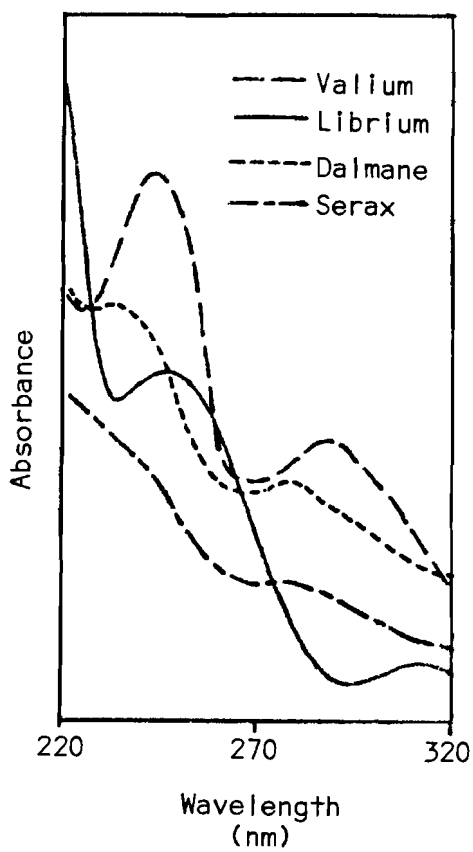


Figure 27. Absorption Spectra of Benzodiazepams

Therapeutic levels of librium and valium have been reported as 0.1-1.0 ug/ml and 1.0-2.0 ug/ml respectively. The toxic symptoms occur above 1.0 ug/ml for librium and above 2.0 ug/ml for valium, resulting in coma when levels of 10-20 ug/ml are reached with either drug.[5]

REQUESTING AND REPORTING RESULTS

In addition to establishing the various procedures for use in the toxicology department, it was necessary to develop forms for requesting and reporting specific assays and the results. Figure 28 is a copy of the request form which was made available to the physicians and nursing staff. It is divided into two major sections--therapeutic drugs and abused drug screening. It is organized to obtain other information essential for assessing the situation such as dosage and time of last dose and condition of the patient.

Figure 29 shows a facsimile of the 8 1/2 x 11" report form used by the toxicology staff. It was designed so that the "Preliminary Screens" could be performed and reported first with additional information such as the Drug Screen report or a drug level to be recorded on the same page as available. Photocopying reports has made step-by-step reporting possible which keeps the physician up-to-date as results are made available. Since several therapeutic drug levels are often necessary to follow a single patient's therapy, space was provided for reporting three consecutive levels on the same form, thus giving the physician an organized chronological report.

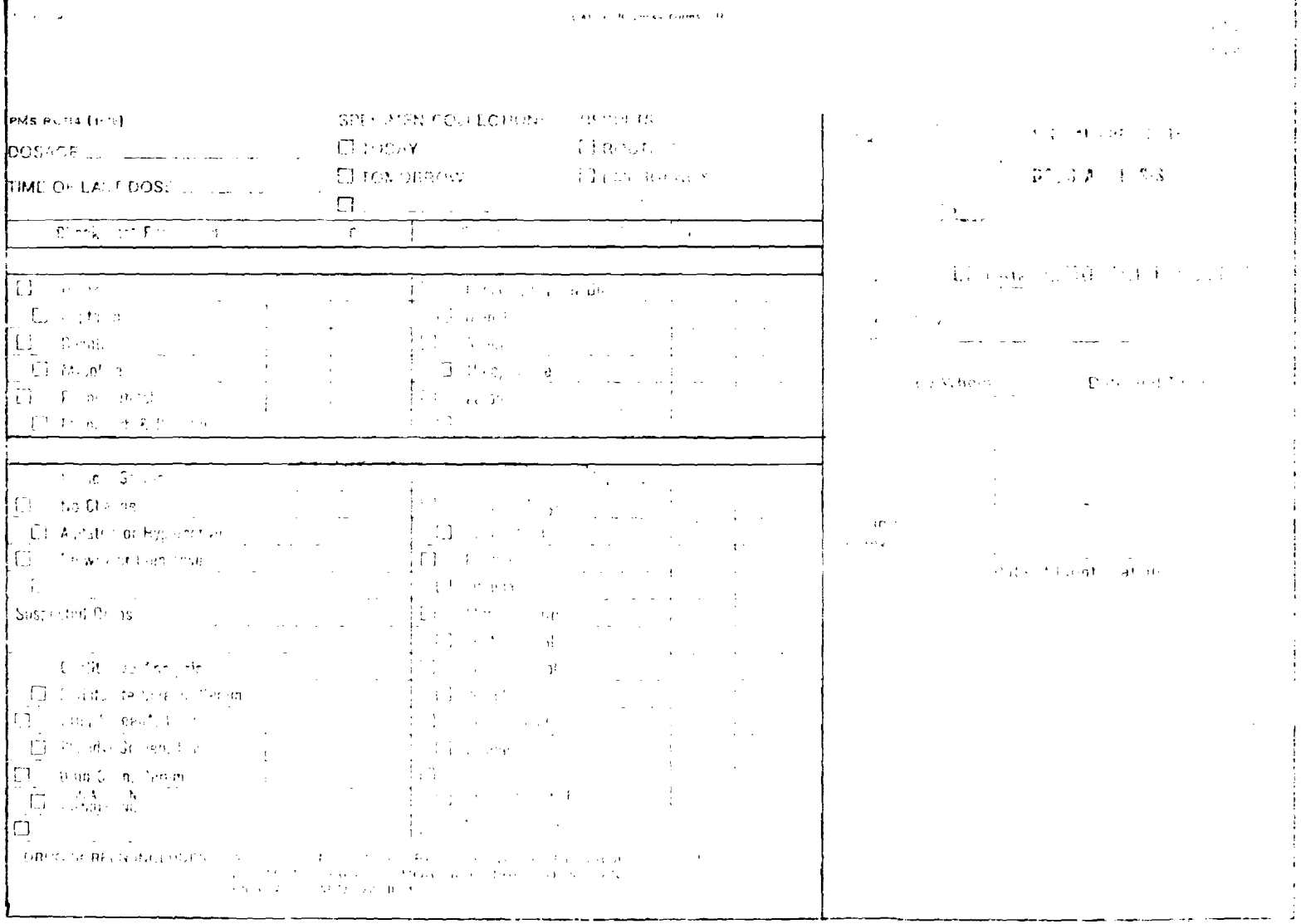


Figure 28. Drug Analysis Request Form

PATHOLOGY MEDICAL SERVICES, P.C.

Lincoln, Nebraska

CONSULTATION REPORT

DRUG ANALYSIS REPORT

Date Specimen Collected: Suspected Drugs

Specimen [ ] Blood [ ] Urine [ ] Gastric Contents [ ] Other

PRELIMINARY SCREEN (serum) Date and Time Test Done: _____ Tech: _____

Barbiturate Screen	
Alcohol, ethyl	Less than 30 mg/dl
Salicylate	Toxic: Greater than 25 mg/dl

DRUG SCREEN (urine) includes: Date and Time Test Done: _____ Tech: _____

Amobarbital	Dilantin	Pentobarbital	Salicylate
Amphetamine	Glutethemide	Phenothiazines	Secobarbital
Codeine	Librium	Phenobarbital	Valium
Darvon	Methadone	Placidyl	
Demerol	Morphine	Quinine	

QUANTITATIVE ASSAYS (serum) Date and Time Test Done: _____ Tech: _____

Amobarbital	Pentobarbital
Barbiturates – Total	Phenobarbital
Bromide	Placidyl
Librium	Secobarbital
Meprobamate	Valium

THERAPEUTIC DRUG LEVELS (serum):

DATE and TIME COLLECTED	
DATE and TIME TEST DONE	
Technologist	

DRUG	Adult Therapeutic Levels
Digoxin	0.8-2.0 ng/ml Toxicity may occur above 1.5
Digitoxin	8.0-25.0 ng/ml Toxicity may occur above 16
Dilantin	10-20 ug/ml
Mysoline	4-12 ug/ml
Phenobarbital	15-40 ug/ml
Quinidine	3-10 ug/ml
Tegretol/Imine	5-20 ug/ml 1 hr. after dose
Zarontin	40-80 ug/ml

comments:

Name:

Hosp. No.:

Age:

Doctor:

Adm. Date: Room:

SPECIAL CHEMISTRY
DRUG ANALYSIS

Figure 29. Drug Analysis Report Form

CONCLUSION

A program for drug screening and monitoring has been developed and implemented for Pathology Medical Services, P. C., in Lincoln, Nebraska. This independent laboratory service has made toxicological testing available for the three major hospitals in the city of Lincoln as well as the smaller hospitals in the surrounding counties. The procedures established required the use of gas-liquid chromatography, thin-layer chromatography, ultra-violet absorption spectroscopy, enzyme immunoassay and hemaglutination inhibition. Besides developing a working toxicology program with accurate assay methods a major goal was to establish assays which could offer same-day results. In some instances 24-hour a day coverage for the department was necessary.

Another important aspect of developing a toxicology program was to familiarize the laboratory staff and nursing staff with the drug assays which were available. This was accomplished by giving a series of hour lectures to interested personel and future medical technologists.

Organization was an important key to establishing a working department. It was necessary to set up files containing new drug assays, ordering forms, catalogs, addresses of other toxicologists. A small library of reference books was assembled. An interlaboratory quality control system was implemented for all assays and in most cases an intra-laboratory quality control program was initiated. All of the assays were then organized into a 63-page toxicology procedure manual and copies were distributed to all laboratories submitting samples.

During the last two and one half years the number of drug screens and drug levels analyzed has steadily increased. To date drug screens now average approximately 40 per month, with antiepileptic drug levels averaging 15-20 determinations per week and 10-15 digoxin requests per day. The steady increase in requests seems to support the acceptance and confidence which the physicians have placed in the toxicology program.

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