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Publication Date

1980

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SIMULTANEOUS LIQUID CHROMATOGRAPHIC DETERMINATION OF

AMITRIPTYLINE, NORTRIPTYLINE, IMIPRAMINE, DESIPRAMINE,

DOXEPIN, AND DESMETHYLDOXEPIN

by

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B.S., University of California, Berkeley 1964

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF CLINICAL LABORATORY SCIENCE

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date   University Librarian

Degree Conferred: SEP 2 4 1980

TABLE OF CONTENTS

ACKNOWLEDGEMENT

I am deeply grateful to Dr. Pokar M. Kabra, Ph. D. , my principal thesis advisor, for his kindness and guidance in the development of this method.

Many thanks to Dr. Laurence J. Marton, M.D., for his valuable suggestions in completing this manuscript, and Dr. Francis Farina , Ph. D.,for reviewing this thesis.

ABSTRACT

A method for the simultaneous analysis of amitriptyline, doxepin, and imipramine and their active metabolites nortriptyline, nordoxepin, and desipramine, respectively, in human serum or plasma is described. Reverse-phase, high-performance liquid chromatography with ultraviolet detection at 200nm is used. The drugs and the internal standard (loxapine) are extracted from 2 ml of serum with butyl-chloride at pH 14, and re-extracted into 200 ul of 0.025 N HCl. An aliquot of the aqueous acid phase is chromatographed with acetonitrile/phosphate buffer (21/79 v/v) containing 0.6 ml of n-nonylamine per liter of mobile phase; flow rate is set at 2.5 ml/minute.

Each analysis requires about 13 minutes at ambient temperature. Drug concentrations are calculated by peak height ratioing with the internal standard. A linear response is observed for each drug concentration up to 1760 ng/ml. The lower limit of detection for all drugs is about 5 ng/ml. A sensitivity of 10 ng/ml of serum for each of the drugs or metabolites is attained routinely. Recoveries for all drugs varied from 77% to 103% suggesting that serum standards should be used in the analysis. Day-to-day precision with coefficients of variation between 3.9 to 8.6% was attained. Of 33 drugs tested for possible interference, only phenacetin

interfered with the internal standard (loxapine) and propoxyphene with nortriptyline. The method correlated well with a GLC method with coefficient of correlations varying between 0.984 and 0.993 for the drugs studied.

INTRODUCTION

Mental depression is prevalent in all strata of society.

approximately 2 to 15% of the adult population is believed

to suffer from significant depressive symptoms in any given

year. Since as many as 400,000 patients require treatment

for depression in the U.S.A. annually, most of them are

treated as out-patients by family practitioners. Estimates

of successful suicide in the U.S.A. range from 26,000 known

to 75,000 possible cases; many suicide attempts are not

reported. This frequent complication of depression is the

tenth greatest cause of death among all age groups (1).

There are three major distinguishable groups of depression:

1) Reactive or secondary depression, is the most common.

This may be precipitated by an adverse life experience;

the usual key word being "loss", like the loss of a loved

one. Depression may also be secondary to various other physical

illnesses or due to the ingestion of various drugs as shown

in Table 1. It may be also secondary to other psychiatric

disorders, especially in the early stages of senile brain

disease. Reactive depression responds nonspecifically to

drugs, psychotherapy, the passage of time, or environmental

changes.

2) Endogenous depression is probably caused by a genetic

Table 1

Drugs and Illnesses Associated with Depression (2)

DRUGS   ILLNESSES

1. Stimulants and appetite suppressants,   1. Pancreatic or biliary tract disease
   Destroamphetamine, fenfluramine,
   phenmetrazine

2. Antipsychotic drugs   2. Influenza, infectious mononucleosis

3. Antihypertensives: reserpine, methyl-   3. Endocrine disorders: hypothyroidism
   dopa, quanethidine, clonidine, pro-
   panolol

4. Hormonal agents: corticosteroids,   4. Myocardial infarction
   contraceptives

5. Social drugs: alcohol, opiates,   5. Mutilating operations: mastectomy,
   lysergide   hysterectomy, amputations

6. Misc.: disulfiram, anticholinesterases

biochemical abnormality. These patients are usually more severely depressed and have lost their ability to cope. They may be afflicted at any stage of life. The characteristic symptoms are disturbances of "vital" or "vegetative" functions, such as normal rhythms of hunger and appetite, sleep, sex, and motor activity (3). Treatment with antidepressant drugs is beneficial for patients with endogenous depression. Despite remissions, endogenous depression is a relapsing disorder, and most patients will experience multiple episodes of depression after the initial attack.

3) Least common are depressions associated with manic depressive disorder. This entity is also believed to be a genetically determined biochemical abnormality. The depressed phase may be indistinguishable from that of endogenous depression. The presence of attacks of manic or hypomanic behavior is the key to its diagnosis. Treatment with lithium is preferred.

Endogenous depression are at least twice as common as those associated with manic depressive disorder, but by far the most frequent group is the reactive or secondary type. The classification of the various depressions is summarized in Table 2.

Antidepressant Drugs

Four types of drugs may be useful to various degrees

Table 2

Classification of Depressions (1)

1. Reactive or secondary depressions: most frequent
   Loss: health, money, prestige, mate, may be
   highly idiosyncratic

   Secondary to drugs, physical illness
   Secondary to other psychiatric disorders

2. Endogenous (unipolar) depressions: about 15 to
   20% of all:
   Cyclic, recurrent: may occur in any epoch of life,
   most commonly in fourth to sixth
   decades.
   Life-long: so-called "characterological"

3. Manic (bipolar) depressive disorder: least common cyclic
   mania and depression: typical cyclic mania, delayed onset
   of depression, cyclic depression, delayed onset of mania

in the treatment of different types of depressions. The general consensus is that tricyclic are the drugs of choice when drug treatment is indicated. The tricyclic antidepressants (TCA) have been used clinically for approximately 20 years and are similar to phenothiazines, both chemically and pharmacologically. Their structural formulae are presented in Figure 1 (4).

Imipramine and amitriptyline are tertiary amines, whereas desipramine and nortriptyline, respectively, are secondary amines formed by demethylation of the former two drugs. The derivatives retain the parent drug's pharmacologic activity. Doxepin and protriptyline differ by comparatively minor structural variations in the TCA ring structure (5,6).

Monoamine oxidase (MAO) (7) inhibitors are chosen for patients who fail to respond to TCA or for those who responded favorably to them.

Use of amphetamines or other sympathomimetics is currently controversial. At best they are useful only for short-term treatment of secondary depressions, such as those associated with physical illness.

Lithium is not an acceptable treatment for acute depressions unless associated with manic-depressive disorder.
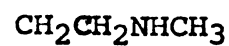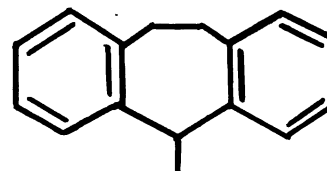
Electroconvulsive therapy (8) is indicated for depressed

Figure 1

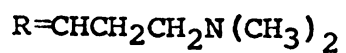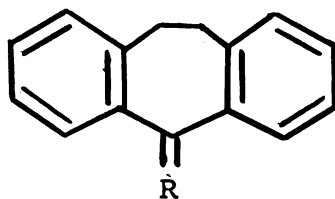Structural Formulae of the Common TCA (4)

Doxepin

Protriptyline

$CHCH_2CH_2N(CH_3)_2$

$CH_2CH_2NHCH_3$

Amitriptyline

Nortriptyline

$R=CHCH_2CH_2N(CH_3)_2$

$R=CHCH_2CH_2NCH_3$

Imipramine

Desipramine

$R=CH_2CH_2CH_2N(CH_3)_2$

$R=CH_2CH_2CH_2NHCH_3$
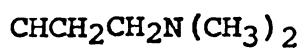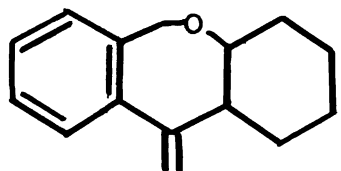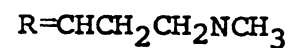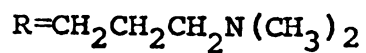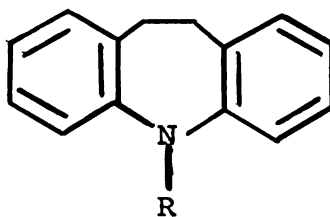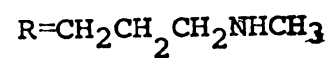
patients who are suicidal risks or for those who have failed

to respond to previous drug therapy. A combination treatment

of drugs and electroconvulsive therapy is sometimes advantageous.

Pharmacology

Tricyclic antidepressants have three major pharmacologic

actions (9,10): sedation, peripheral and central anticholinergic

action, and the blockage of the uptake of neurotransmitters

or the "amine pump" mechanism in the presynaptic nerve endings.

The sedative effect is more pronounced with the tertiary amines

and is almost absent with protriptyline. The anticholinergic

actions of tricyclic drugs (11) differ in degree, amitripty-

line being the strongest and desipramine the weakest. This

effect is considered the main cause of most of the unwanted

side effects associated with these drugs.

The blocking actions at the presynaptic nerve ending is

probably most responsible for the tricyclic's antidepressant

effect (12). This action prevents the reuptake of amine neuro-

transmitters, such as norepinephrine and serotonin, prolonging

the aminergic synaptic transmission (13). For some time endo-

genous depression has been thought to be associated with

deficient aminergic neurotransmission. Each tricyclic is

about equally effective in this regard with the exception

of doxepin (14), which is considerably less potent. The

Tricyclics differ in their effects on specific neurotransmitters. Tertiary amines inhibit the uptake of serotonin more strongly, whereas secondary amines inhibit uptake of norepinephrine. As amitriptyline is more slowly converted to its secondary amine metabolite than impramine, it is the most selective drug currently available for blocking uptake of serotonin; desipramine is most selective for norepinephrine (15). The "amine hypothesis" has been further supported by studies on the mechanism of action of various other types of antidepressant drugs. Monoamine inhibitors block a major degradative pathway for the amine neurotransmitters which presumably permits accumulation of amines presynaptically. Sympathomimetics also block the amine pump but are thought to act primarily by increasing the release of catecholinergic neurotransmitters. Thus, the three classes of antidepressant drugs might remedy a deficiency in aminergic neurotransmission albeit through someshat different mechanisms.

One of the ramifications of the "amine Hypothesis" currently under investigation is that one might be able to define, by biochemical tests, subtypes of depression and to predict the best drug for their treatment. A metabolite of norepinephrine, 3-methoxy-4-hydroxy-phenylglycol (MHPG or MOPEG) is considered to derive its effect from central adrenergic activity (16-19). Measurement of this metabolite

may be useful for patients who suffer from endogenous depression primarily due to a deficiency of noradrenergic transmission, whereas other patients with depression may be deficient in serotonergic transmission (20). This differentiation offers a potential means of objectively measuring depression, selecting appropriate drugs, and measuring the efficacy of therapy.

Pharmacokinetics

Oral administration of tricyclic drugs leads to variable bioavailability as a result of extensive first-pass metabolism by the liver (21-24). Variation depends on both individual patient metabolism and the specific drug used (25). Bioavailability may range from 27 to 79% on similar dosage (9).

Studies, in particular with nortriptyline and imipramine (26-29), have revealed that tricyclic drug are highly tissue-bound, with less than one percent of the total drug in the body present in blood. Tricyclic drugs are eliminated from the body almost exclusively by hepatic metabolism. Plasma protein binding of the drugs is usually in excess of 90%, with variation related to patient's age or pre-existing disease. Small changes in protein binding can lead to variable levels of pharmacologically active free drug. The drug is apparently sequestered in tissues because of both its lipid solubility and protein binding.

Table 3

Summary of Pharmacologic Differences Among Six TCA (9)**

Drug	Sedation	Anticholinergic Effects	Blocking of Amine Pump	
			Serotonin	Norepinephrine
Imipramine (Tofranil)	++	++	++	++
Amitriptyline (Elavil)	+++	+++	+++	+
Desipramine (Norpramin)	+	+	0	+++
Nortriptyline (Aventyl)	++	++	+	++
Doxepin (Sinequan)	+++	+++	Weak	
Protriptyline (Vivactil)			Unknown	

** 0 indicates none, + slight, ++ moderate, +++ high

The metabolism of tricyclic drugs is an important consideration in drug selection and therapeutic monitoring. The tricyclics are metabolized by alteration of the tricyclic nucleus and/or the aliphatic side chain. In the former instance, the ring is hydroxylated and subsequently subjected to glucuronide conjugation. Alteration of the aliphatic side chain involves demethylation of the tertiary amines, forming pharmacologically active secondary amines. Amitriptyline is thus converted to nortriptyline and imipramine to desipramine. When tertiary amines are used in treatment , two active drugs are present in variable ratios depending on the inherent metabolic characteristics of the patient. Inactivation occurs when the aliphatic side chain is completely demethylated or when the tricyclic nucleus is altered. There is no conclusive evidence that the therapeutic efficacy of tricyclic antidepressants is related to plasma level. Asberg et al. (1970) obtained a curvilinear relation with reduced efficacy at both lower and higher plasma levels of nortriptyline (30-35). Braithwaite et al. in 1972 (36), studied amitriptyline and found a simple positive linear correlation. However, Gruvstad, in 1973, (37) applied an alternative statistical analysis to their data and demonstrated that it supports the curvilinear result of Asberg. Burrows et al. and later Kragh-Sorensen et al. (38) did not show

such a clear relationship.

Measurements of plasma concentrations following use of either amitriptyline or imipramine must also take into account the level of their respective active secondary amines (39, 40). Therapeutic ranges given for these drugs usually represent their combined value. Table 4 summarizes the pharmacokinetic properties and therapeutic plasma concentrations of various tricyclic drugs.

Adverse Effects

In a drug surveillance program, the overall prevalence of side-effects from tricyclic drugs was 15.4%. Most reactions were minor; but toxic pychoses were observed in 4.6% (41). Side effects of tricyclic drugs are summarized in Table 5. In general, elderly patients do not tolerate the drugs well due to receptor sensitivity or to diminished plasma protein binding. In any suicide attempt these drugs cannot be overlooked. Drug overdose should be suspected if a patient shows signs of coma, convulsions and cardiac arrhythmia. Other symptoms of overdose may include hyperpyrexia, neuromuscular irritability, respiratory depression, delerium, hypotension, and bladder or lower bowel paralysis. Cardiac disturbances are most serious, and drugs such as lidocaine, phenytoin, and propanolol have been successfully employed in treating

Table 4

Pharmacokinetic Characteristics of TCA Drugs (9, 10, 46)

DRUG	Individual Variation in Metabolism	1st Pass Metabolism%	T½ Hrs.	Protein Binding%	Peak Time (post oral)	Therapeutic Window
Imipramine	30 fold	53	9-24	79-96	2-6	150-300*
Amitriptyline	10 fold	60	17-40	82-96	2-6	125-250*
Desipramine	30 fold	56-70	14-76	73-92	2-6	150-300
Nortriptyline	10 fold	39	18-93	93-95	2-6	50-150
Protriptyline	10-15 fold	15	54-198		6-12	50-150
Doxepin	10-15 fold	33	17		2-6	150-300*

* Total level of secondary and tertiary amines

Table 5

Side-Effects of Tricyclic Antidepressants (1)

	Frequent	Infrequent
Synpathomimetic	Tachycardia Sweating Tremer	Agitation Isomnia Aggravation of psychosis
Anticholinergic	Blurrd vision Constipation Urinary hesitancy Fuzzy thinking	Aggravation of glaucoma Paralytic ileus Urinary retention Delirium
Cardiovascular	Orthostatic hypotension Electrocardiogram abnormalities	Delayed cardiac condition Arrhythmias Cardiomyopathy, Sudden death
Neurological	Paresthesias Electroencephalogram Alterations	Seizures
Allergic/Toxic		Cholestatic jaundice Agranulocytosis
Metabolic, Endocrine	Weight gain Sexual disturbances	Gynecomastia Amenorrhea

them. Plasma levels of tricyclic drugs are most useful in
establishing evidence of overdose; major adverse effects are
observed in patients with a level of over 1000 ng/ml. Un-
treated overdosed patients will develop respiratory depression,
hypoxia, and subsequent respiratory arrest. Follow-up plasma
level monitoring is important to assess the continued
dissipation of the drug after the condition of patient has
improved (42,43).

Drug-Drug Interactions (44)

Such interactions are usually divided into pharmacokinetic,
in which drugs interact to alter their absorption, distribution,
metabolism, and excretion, and pharmacodynamic, in which
drugs may act with other drugs to reinforce or to cancel
pharmacological actions. Absorption of tricyclic drugs may
be altered when given with drugs that usually impair absorption
(45) such as colloidal antacids, aluminum hydroxide gel, kaolin-
containing products or cholestyramine. Drugs such as pheno-
barbital and meprobamate stimulate or induce drug metabolizing
enzymes, leading to increased rate of metabolism of tricyclic
drugs leading to a lowering of serum levels (46). Drugs like
methylphenidate (47), neuroleptics, and corticosteroids
inhibit drug-metabolizing enzymes leading to increased plasma
tricyclic drug levels. The most important drug-drug interactions
is caused by the monoamine oxidase (MAO) inhibitors (48). As

Table 6

Interactional Effects on "Steady-State" Plasma TCA Levels(49)

LOWERS	NO EFFECT	RAISES
Barbituates	Diazepam,Nitrazepam	Chlorpromazine
Chloralhydrate	Oxazepam, Flurazepam	Haloperidol
Mandrax	Chloridiazepoxide	Perphenazine
Glutethimide	L-triiodothyronine	Levomepromazine
Trihexyphenidyl	Fluphenazine	Chlorprothixene
Acid pH (Ammonium chloride)		Thioridazine
Smoking		Methylphenidate
		Fenfluramine
		Chloramphenical
		Norethindrone
		Basic pH (NaHCO$_3$)
		Aging

MAO inhibitors decrease the catabolism of biogenic pressor amines, such as norepinephrine, as well as exogenous pressor amines present in foods, such as tyramine, their use may result in acute hypertension, manifested by signs suggesting hypertensive encephalopathy, or result in subarachnoid or intercerebral hemorrhage. Additive effects may occur when tricyclic drugs are given simultaneously with sedatives such as phenothiazines, hypnotics, or with alcoholic beverages. Potentiation of cardiotoxic effects may be seen with quinidine, procainamide, and thioridazine. Tricyclic drugs are also known to reverse the antihypertensive effects of guanethidine, methyldopa, and clonidine (10).

Monitoring of TCA

Hammer and Sjoqvist in 1967 (50) reported a 36-fold difference between the lowest and highest steady-state levels of patients receiving the same dose of desipramine (51-54). This finding could be applied to other tricyclic drugs to a lesser degree. The lack of correlation between the dose administered and the resulting plasma concentration may be due to many factors, including individual differences in rate of metabolism as well as race, sex, genetic factors (55), age (56,57), concurrent medications, and the health status of the patients. Plasma levels can provide valuable clinical assistance in determining patient

compliance and regimen failures (58,59). Although there is still a controversy concerning the relationship of plasma levels and clinical efficacy of the drugs, it has been suggested that the tricyclic drugs exhibit optimal clinical effectiveness within a defined therapeutic concentration range (35,39,70). Plasma levels may also be useful in evaluating suspected side effects or toxicity (60). Monitoring plasma concentrations could be useful in treating cases of suspected overdose and/or following recovery phases of such overdoses. Monitoring the elderly or the very young can be useful, since altered protein binding (61-63) of the drugs would lead to a greater amount of pharmacologically active free drug. There is a significant time lag in attaining steady-state levels with tricyclic drugs (64), requiring 7-10 days to reach steady-state levels under normal circumstances. Significantly, plasma levels also fluctuate on a day-to-day basis, perhaps by as much as 20 percent, even after steady-state has been established. Knowledge of drug pharmacokinetics provides insight into the interpretation of clinical data, thereby resulting in better patient-drug management.

HISTORY OF ANALYTICAL METHODS

Tricyclic antidepressant drug levels have been determined by many techniques. These include photometric methods, thin layer chromatography, isotope derivative methods, gas-liquid chromatography, radioimmunoassay, and high pressure liquid chromatography.

Photometric Methods:

Photometric methods, including visible, UV spectrophotometry and fluorometry provided sensitivities ranging from 2 ng/ml to 2 ug/ml. However, these methods lacked a separating step and generally showed poor selectivity because of interferences by metabolites as well as other drugs. In 1961, the first quantitative procedure was developed by Axelrod using non specific methyl orange (65). Amundson and Manthey (66) described a quantitative ultraviolet spectrophotometric assay procedure for the determination of nortriptyline in urine. Unconjugated, free nortriptyline was read at 240 nm, and the acid-hydrolysed 10-hydroxy metabolite was read at 290 nm. This procedure again lacked the sensitivity to determine nortriptyline in specimens other than urine. In 1967, Wallace and Dahl (67) presented a quantitative UV procedure for the determination of amitriptyline and nortriptyline and their principal metabolites in biologic specimens. In this procedure, the

drugs were extracted into n-hexane and oxidized with permanganate to carbonyl derivative, which, in contrast to the original compounds, had characteristic ultraviolet absorption spectra at 250 nm. The oxidized products were stable in hexane. However, the procedure required large volume of sample and could not distinguish between amitrip-tyline and its metabolites; its lack of sensitivity permitted its use only in forensic toxicology.

More sensitive fluorometric methods were devised by Haydu et al. in 1962 (68), and Yates et al. in 1963 (69), for the determination of imipramine-like substances in the plasma of patients receiving imipramine and desipramine respectively. However, these techniques again could not differentiate imipramine from desipramine. Moody et al. (1967) (70) presented a better method for the separate estimation of imipramine and desipramine in human plasma based on the extraction of these compounds into heptane/3% amyl alcohol and separation by acetylation prior to fluorimetric analysis. Separate estimates of imipramine and other primary metabolites were obtained; desipramine concentrations could be estimated by calculating the difference. The only metabolite shown to interfere was didesmethylimipramine, though total specificity was not achieved. Fluorescence was determined

with an Aminco-Bowman spectrofluorometer at an excitation

wavelength of 285 nm and an emission wavelength of 410 nm

with a sensitivity of 20 ng/ml. Westerlund and Borg (71)

improved the sensitivity of the fluorometric method by

applying the technique of ion-pair extraction with a

counter-ion (anthracene-2-sulphonic acid) that gave the

ion pair a high fluorescence intensity. Amitriptyline as

the anthracene-2-sulphonic ion pair could be measured at

levels as low as 10 ng/ml. Moody et al., in 1073 (72),

reported a simple and sensitive method for protriptyline

analysis which utilized its fluorescent property in dilute

sulphuric acid solution. This method was sensitive enough

for 'steady-state' level monitoring, but like most other

fluorometric methods, it lacked adequate sensitivity for

measuring low therapeutic concentrations and was prone to

interference. A more sensitive assay capable of measuring

5 ug/L of amitriptyline has recently been described by

Karel et al. (73) This method was based on the reaction

of amitriptyline with alpha bromomethylacridine to form a

quarternary product that, on photolysis, yeilded stoichio-

metric fluorescence. A TLC step insured specificity, but

the method was very time consuming due to the need to form

the quarternary products.

Isotope Derivative Techniques

In this procedure, the compound to be assayed is reacted quantitatively with a radioactive reagent of known specific activity. The radioactive product is then separated and purified. The amount of activity in the pure product indicates the amount of radioactive reagent it contains; thus, from the stoichiometry of the reaction, the amount of compound present can be calculated. In 1967, Hammer and Brodie (74) developed an isotope labeling technique for the determination of therapeutic plasma concentrations of desipramine and nortriptyline. The method involved extraction of the drugs into hexane from alkalinized plasma and subsequent acylation with ^{3}H-acetic anhydride. The radioactive amide derivative thus formed, was assayed by scintillation spectrometry. The sensitivity of the method was 5 ug/L and, until recently, this was the most widely used of all published methods. However, this method suffered from the disadvantage that only secondary amines could react with ^{3}H-acetic anhydride to form the radioactive derivative; tertiary amines have no derivatizable functional groups. Sjoqvist et al. (75) applied the above method for the determination of nortriptyline. Both primary and secondary amines were found to form derivatives with

^{3}H-acetic anhydride, resulting in the over estimation by 10-15%, because of desmethylnortriptyline being present in the sample. Incorporation of a chromatographic separation step has eliminated this problem (76).

Harris et al. (77) in 1970 was able to use a ^{14}C-methyl-iodide to convert imipramine to a radiolabeled quarternary amine; thus, a method for the simultaneous determination of imipramine and desipramine using ^{14}C methyl iodide and ^{3}H-acetic anhydride respectively was presented. In 1972, Overo (78) improved the method of ^{3}H-acetic anhydride coupling for secondary amino groups. He discovered that the amount of primary amines measured as ^{3}H-amide could be reduced considerably by the addition of 0.1M salicylic aldehyde to the hexane phase in the first extraction step. Thus, increased specificity for secondary amines was achieved. The reported sensitivities for these methods varied from 5 to 20 ng/ml. The method required vigorously controlled conditions which were time-consuming. The potential interference by any compound with derivatizable functional groups (including many currently administered drugs) became a major draw back.

Thin Layer Chromatography

In 1969, Eschenhof and Rieder (79) investigated the metabolism of amitriptyline and used TLC for the separation of amitriptyline from nortriptyline and other metabolites.

They extracted the sample with benzene at pH 9 and then concentrated the organic solvent. The benzene concentrate was separated on a Kieselgel TLC plate using pure amitriptyline as a reference. The plates were immersed in 70 % perchloric acid and heated at $120^{o}C$ for 10 minutes before scanning at 350 nm. The sensitivity of this semi-quantitative method was 50 ng/ml; recovery experiments were not carried out. Later, in 1973, Nagy and Treiber (80) developed a quantitative thin layer method for imipramine and desipramine. The drugs were extracted as bases into n-heptane and then separated by thin-layer chromatography. An intense yellow color was developed by an oxidative procedure using nitrous gases. The newly designed densitometer, using reflectance and transmittance simultaneously, increased the sensitivity up to 10 fold over the earlier models which measured only reflectance or transmittance. With 5 ml of plasma, the detection limit was 10-15 ng/ml, and a quantity of 25 ng could be measured accurately.

Faber et al. (81) reported a simultaneous fluorescence TLC method using 20 % perchloric acid in ethanol to convert amitriptyline and its metabolites to fluorescent compounds. Recovery was 81 % for nortriptyline and 104 % for amitriptyline, and the sensitivity of 10 ng/ml was adequate for therapeutic monitoring. However, major drawbacks were the

three hour analysis time and the large sample size (4-5 ml of plasma) required. Interference by other concurrently administered drugs was possible because qualitative accuracy was based upon R_f values. "High-performance" thin layer chromatography has been used to assay amitriptyline, nortriptyline, imipramine and desipramine (82). Resolution and sensitivity are better than with conventional TLC plates. As little as 5ug/L from 1 ml of plasma could be detected. A similar assay with even greater sensitivity (0.5 ug/L) has been described for amitriptyline and nortriptyline (83).

Gas Liquid Chromatography

Over recent years, a large number of gas chromatographic methods have been reported, differing primary in the type of detection used. Initial studies involved flame-ionization or electron capture detection. However, the development of the alkali flame-ionization detector, which is selective to nitrogen, and the coupling of G.C. with mass spectrometry has led to the development of the most widespread methods currently in use for the complete range of tricyclic drugs.

Braithwaite and Widdop (84) presented the earliest flame-ionization detector method for amitriptyline and nortriptyline. The method required large samples (5 ml of serum) and derivatization of secondary amines to the trifluoroacetate (TFA) derivative. Reproducibility was only fair because the

internal standard was added at the end of the extraction procedure. In 1974, Hucker and Stauffer (85) improved the extraction method of Braithwaite et al. (84) and were able to provide a cleaner extract with a more stable baseline. Reproducibility was also improved by using an internal standard (protriptyline) introduced at the beginning of the procedure. Unfortunately, the flame ionization detector has limited sensitivity (detection limit = 20 ng/ml), and it did not allow monitoring of the tricyclic drugs over the entire therapeutic range.

In 1971, Walle and Ehrsson (86) described a gas chromato-graphic method, with electron capture detection, for primary and secondary amines. The method involved the acylation of these to polyfluorinated amides. The method could estimate picogram quantities of nortriptyline in plasma, but they could not apply the analysis to parent drug amitriptyline. Borga and Garle (87) developed a similar procedure and included the analysis of nortriptyline and its metabolites in plasma and urine with the growing interest in therapeutic monitoring of plasma TCA levels, Braithwaite et al. (88) observed that the combined plasma amitriptyline and nortrip-tyline levels exhibited greater correlation with clinical management of depression than did plasma levels of either drug alone. In 1975, Wallace et al. (89) reported a rapid

electron capture procedure capable of detecting 1 ng/ml or reliably quantitating 5 ng/ml of amitriptyline and nortriptyline in 0.5 ml of plasma or serum within 1 hour. The procedure involved the ceric sulfate-sulfuric acid oxidation of the drug metabolite to a polyaromatic carbonyl derivative, anthraquinone, which had the intrinsic capability to capture electrons with high efficiency. The electrophilic property of the product provided extreme sensitivity for the procedure. The inability of the method to differentiate between amitriptyline and nortriptyline limited its application to pharmacokinetic studies, but did not detract its usefulness to clinicians and toxicologists. Hartvig and colleagues (90, 91) applied column extraction techniques for the separation of amitriptyline from its metabolites before oxidation; hence, amitriptyline and nortriptyline could be determined separately with a sensitivity of 1 ug/L without metabolite interference.

A significant advance in methodology has been brought about by the introduction of nitrigen specific detectors. these very sensitive and specific detectors allow reliable estimation of drug concentrations as low as 5 ug/L in plasma samples of 2-3 ml. The technique requires lengthy sample pretreatment and derivatization (92, 95, 99-101) for acceptable sensitivity. Jorgensen (92) was the first to describe a method for the monitoring of amitriptyline and notriptyline. The

secondary amine was acetylated to improve separation with detection limit of 10-15 ng/ml. Bailey and Jatlow (93,94) published two papers in 1976 on TCA analysis describing the use of nitrogen detector. The first study involved the analysis of amitriptyline and nortriptyline, the second, imipramine and desipramine. The main difference between the two methods was the way the secondary amines were handled. Desipramine (n-demethylated metabolite of imipramine) had to go through derivatization with trifluoroacetic anhydride to enhance the sensitivity and to improve the chromatographic resolution, whereas nortriptyline (n-demethylated metabolite of amitriptyline) did not require derivatization. From the interference study, amitriptyline and trihexyphenidyl were found to interfere with the analysis of imipramine. This interference could be a problem in cases of multiple drug overdose.

Gifford et al. measure TCA and found it to be sensitive in the 1 ng/ml range. The improved detector sensitivity allowed a simple and rapid sample (4 ml serum) extraction procedure to be used without the necessity of lengthy derivatization procedures. The authors claimed that the system was equally sensitive to secondary as well as tertiary amines. However, little data relative to therapeutic monitoring was presented,

and the assay was applied only to plasma supplemented in vitro with imipramine.

Dorrity et al. (96) presented a comprehensive method for six tricyclic drugs and metabolites—amitriptyline, nortriptyline, imipramine, desipramine, doxepin, and desmethyldoxepin in plasma. All of these drug were extracted and chromatographed under identical conditions. Derivatization of secondary amines was not necessary. The lower limit of sensitivity was 10 ng/ml using only 2 ml of plasma. Analytical recoveries of tertiary and secondary amines were 100 and 80 % respectively. Between run C.V.'s for all of the drugs ranged from 5 to 7 %. However, the method did not allow simultaneous measurement of all tricyclic drugs since only one drug, its secondary metabolite, and the internal stnadard could be measured simultaneously. The chromatographic interference between various tricyclics precluded the use of the method for toxicological analysis.

Mitchel et al. (97) developed a simple and rapid extraction method for the analysis of toxic levels of imipramine, desipramine, amitriptyline, and nortriptyline. The method required only 100 ul of plasma without derivatization, while providing good recovery and precision. Dhar and Kutt (98) commented in their paper that when one used protriptyline

(a secondary amine) as the internal standard, the response given by the nitrogen sensitive detector was inadequate and often not reproducible; therefore, conversion to trifluoroacetyl derivatives was necessary.

Gas Chromatography/Mass Spectroscopic Analysis

This technique involves the use of a mass spectrometer as an ion-specific detector for the monitoring of effluent from a GLC. The mass spectrometer can be set to detect one or more characteristic fragment ions of the compound under study, therby introducing another parameter of identification in addition to the retention time in the gas chromatograph. Thus, the method combines the high resolving power of the gas chromatograph with the high sensitivity and specificity of the mass spectrometer. Selective ion monitoring allowed detection as low as 10^{-12} gram of many compounds.

In 1975, Belvedere et al. (99) presented a method for the simultaneous measurement of desipramine in 1 ml of plasma with a sensitivity of about 4 ng/ml. Derivatization was necessary for the secondary amines in order to achieve a more suitable retention time, as well as to prevent absorption onto the stationary phase. A method in which 15 samples could be analyzed per hour was presented by Biggs et al. in 1976 (100). The method used electron beam ionization GLC mass

spectrometry, employing a computer-controlled multiple-ion detector. All tertiary and secondary tricyclic antidepressants prescribed in the U.S. could be measured. Plasma levels as low as 10 ng/ml could be measured with deuterated drugs as internal standards. Dubois et al. (101) discussed a similar method for the assay of clomipramine, n-desmethylclomipramine, imipramine, and dehydroimipramine in biological fluids with a detection limit of 0.3 ug/L. Recovery exceeded 95 % and the C.V. was less than 4 % for whole blood samples supplemented with 5 to 15 ng of clomipramine hydrochloride per ml.

Jenkins et al. (102) presented a GLC chemical ionization mass spectrometry with selected ion monitoring for all the major tricyclics and their metabolites. This method enjoyed more selectivity by monitoring the compounds' characteristically abundant parent mass ions, while achieving sensitivity equivalent to electron-impact ionization. The described method gave a sensitivity of 1 ng/ml for all commonly prescribed tricyclic drugs. It has been accepted that mass spectroscopy offers the highest specificity and sensitivity , but the expense and complexity of the instrument limit its routine application.

Radioimmunoassays

Although RIA has been widely used for measurement of

many drugs, peptide proteins, and steroid hormones, only

recently has it been used for psychotropic drugs. Spector

et al. in 1975 described a method for imipramine (103).

Antisera were produced by injection of a variety of tricyclic

drugs, coupled to bovine serum albumin, into rabbits or

sheep. The extent of incorporation of the haptene into bovine

albumin was variable (104-106). Although many of the haptene-

carrier protein complexes have been less than ideal (107),

satisfactory antisera have been developed. Many antisera

showed substantial cross reactivity between the secondary

and tertiary structures, as well as with other tricyclic

drugs with similar ring structures (108). Furthermore, for

many of the antisera, the cross reactivities of the tertiary

amines and secondary amines were not identified (109). Recently

a commercial kit developed for measuring plasma concentration

of tricyclic antidepressants ("Tri-Cy", Wien Lab.,Succasunna,

N.J.) was introduced. The antiserum, raised against succinyl-NT

in rabbits, cross reacted with all secondary and tertiary

amine tricyclics. When the development of specific antisera

becomes a reality, RIA would undoubtedly be recognized as

a choice method by some clinical laboratories because of its

small serum requirement (59-500 ul) and its picogram sensitivity.

High Performance Liquid Chromatography

A. Extraction of tricyclic drugs from plasma:

Careful isolation of tricyclic drugs in necessary as they are present in ng/ml quantity in serum. There are two methods currently in use for the isolation and concentration of these drugs from serum matrix;—liquid-liquid or solvent extraction, and liquid-solid or adsorption-desorption extraction. In general, liquid-liquid extraction systems have been widely used due to their simplicity, efficiency, reproducibility, and speed for the isolation of tricyclic drugs from serum (110). These procedures involve the adjustment of serum to a pH which closely approximates the pK values of the tricyclic drugs, followed by extraction of the drugs into a suitable immiscible organic solvent. Solvents commonly used are diethyl ether, n-heptane, and hexane-isoamyl alcohol. The solvents are chosen on the basis of their polarity. As a general rule, polar compounds will partition most efficiently into polar solvents and non-polar drugs into non-polar solvents. For moderately hydrophilic compounds, solvents which form strong hydrogen bonds, such as ethylacetate, may be useful. For extremely water soluble compounds, ion-pair extraction techniques may be employed. In extraction methods where interfering endogenous compounds are co-extracted, chemical

acid-base back extraction techniques may be useful. In these instances, the basic lipophilic nature of the tricyclic drugs may be used selectively to isolate the drugs from interfering substances.

An alternative to liquid-liquid extraction is to selectively adsorb the drugs from serum onto a solid adsorptive surface such as cellulose (111,112), Celite 545, XAD-2 (113), and Clin-Elut[TM]* (114) columns. In general, the serum sample is buffered to a basic pH and then applied to the top of the extraction column. The drugs will interact with the adsorbent and are retained while serum and other endogenous compounds pass through the column. The column may then be washed to remove excess serum with subsequent desorption of the drugs by use of an organic solvent of appropriate polarity. An example of this procedure was given by Thoma et al. (114). Two ml of serum buffered to pH 10 was applied to the column. The drugs were eluted from the column using hexane followed by back-extraction into 100 ul of 0.1N HCl. The acid phase was dried and the residue was dissolved in 100 ul of the mobile phase prior to injection.

HPLC systems include four basic components: A mobile phase delivery system, injection port, column, and detector. The UV detector offers enough sensitivity generally for

* Clin-Elut[TM] (Analytical International, Lawndale, Ca.)

therapeutic drug monitoring. A 25 cm column packed with 5-10 um

particles can yield efficiencies of 8,000-12,000 theoretical

plates. Another improvement resulted from the development

of a permanently bonded phase. Hydrocarbonaceous bonded

phases are obtained by reacting silica gel with an alkyl-

trichlorosilane. A hydrocarbon chain of any length e.g. C_2,

C_8, C_{18} can be linked. Other functional groups, such as cyano

or amino can also be linked to the silica atoms.

B. Introduction to Various Modes of Separation (115):

Liquid chromatography is one of the oldest chromatographic

methods, developed over 75 years ago. In recent years, liquid

chromatography has experienced a major revival brought about

by a number of technological breakthroughs. Its high speed

and high performance were attributed to improved in-line

detection devices, efficient mobile phase delivery systems

and high efficiency column design. The technique enjoys all

the advantages of GLC in speed, specificity, and sensitivity

without the need for volatilizing samples, and extensive

sample pretreatment including complicated derivatization.

In addition, most HPLC detectors do not alter eluted samples,

so they can be saved for further analysis or use. The particular

attraction of HPLC lies in the wide variety of separation

modes available to the chromatographers. In gas chromatography,

only gas-liquid (GLC) or gas solid (GSC) chromatography is available. The mobile phase in GC is an inert gas and has no interaction with the solute or stationary phase. The gas functions only as a carrier device. In LC, there are interactions, the mobile phase not only transports the solute down the column, but also interacts with both the stationary phase and the solute. There are four basic types of LC separation modes which are characterized by the nature of the predominant interaction between the sample solute and the stationary phase.

a) Liquid-solid (adsorption) chromatography (LSC):

This mode utilizes the adsorption of analytes onto the stationary surfaces of polar solids such as silica gel, alumina, and porous glass beads. Silica gel with its acidic silanol or surface hydroxyl groups causes strong retention of polar basic compounds, whereas alumina, a basic adsorbent, will preferentially retain acidic compounds. The mobile phase is usually a relatively non-polar solvent. Therefore, nonpolar, non-ionic compounds are best separated by this mode. LSC can also be carried out by changing the mobile phase from non-polar to polar and using non-polar packing; this is generally referred to as reverse-phase LSC (RPLSC).

b) Liquid-liquid chromatography or partition chromatography:

In LSC, the stationary solid surface is coated with a

second liquid which is immiscible with the mobile liquid phase.
Thus, the partitioning equilibrium between the mobile phase
and the stationary liquid phase would determine the degree
of separation. LLC can be normal phase (polar stationary
liquid phase and non-polar mobile phase) used for polar
compounds, or reverse-phase (non-polar stationary liquid phase
and polar mobile phase) used for non-polar compounds. LLC
is generally used for compounds which exhibit solubility
differences.

c) Ion Exchange Chromatography (IEC):

In this technique, the stationary phase utilizes either
bonded silicas or resins with ionic groups on their surfaces.
This technique is generally applicable to ionic compounds,
such as organic acids or bases, and to compounds that can
interact with ionic groups (i.e. chelates and ligands). Ion
exchange resins bearing negatively charged groups are used
for exchanging cationic species, while those bearing positively
charged groups are used for anionic exchange. Strongly acidic
or basic packings are most widely used because they are ionic
at all pH values, whereas the weak packings operate over a
much smaller pH ranges, dependant upon the pK_a or pK_b of the
functional group. The separation is dependant upon the relative
affinity of the ionic solutes for the exchange surfaces.

d)   Exclusion Chromatography (EC):

This separation is strictly mechanical, based on molecular size. Solute molecules selectively diffuse into and out of the porous packing gel. Retention time depends on the size of solute molecule relative to the size of the pore. Small molecules can diffuse into all the pores and will elute last; larger molecules which are excluded from pores will elute first. Intermediate size molecules will have varying elution times.

C.   Chromatography of Tricyclic Drugs:

The tricyclic drugs are strong basic compounds. Complete ionization in the pH 2-8 range leads to poor chromatographic separations. For effective ionic suppression, a basic pH mobile phase (above or at the pK_b of the drug) is needed. However, silica columns will deteriorate rapidly when subjected to a high pH mobile phase. As a result, most LC procedures for tricyclic drug assays utilize the following chromatographic approaches:

a)   Ion-pair chromatography with bonded phase columns—the basis of this widely used technique is the formation of an ion-pair complex with a suitable counter ion of opposite charge to the solute. The neutral ion-pair then partitions into the hydrophobic packing and establishes an equilibrium with the aqueous mobile phase (116,117). A second postulated

mechanism (118) is that the hydrophobic portion of the counterion partitions into the hydrophobic stationary phase, and its ionic portion is oriented towards the hydrophilic mobile phase. Thus, an _in-situ_ dynamic ion exchanger is created. The charged solute is attracted by electrostatic force to the ionic group at the packing surface. The actual mechanism is probably more complex and probably involves both suggested mechanisms as well as other phase equilibria (122). Nevertheless, the ion-pair technique is a useful method for separating the ionic and ionizable substances by RPC. Often a long-chain alkyl ammonium compound is added to the aqueous mobile phase to prevent peak tailing, which could result from the adsorption of the basic compounds onto non-bonded silanol sites on the stationary support. The slow kinetics of these side reactions and the operation of a mixed retention mechanism cause undesirable chromatographic behavior of basic solutes. The alkyl amines added to the mobile phase would competitively inhibit the participation of the analytes, resulting in improved peak shape and a shortened retention time (123).

Ion-pair partition and liquid-solid adsorption chromatography was applied for the separation of twenty tricyclic drugs by Knox and Jurand in 1975 (124). Tricyclics were

paired with perchlorate ion, separated on a silica stationary

phase and eluted with dichloromethane and a higher aliphatic

alcohol (n-butanol or iso-amyl alcohol) mobile phase. Since

that time there has been extensive development of this analytical

approach for the quantitative determination of most of the

tricyclic antidepressants in plasma. Mellstrom et al. (125)

used ion-pair chromatography for determing chlorimipramine

and desmethylchlorimipramine in plasma. They used chloride

as the counter ion with a silica stationary phase (Diachrom

37-44 um) modified with tetraethylammonium chloride in order

to increase the separation efficiency. These authors used

similar chromatographic techniques for the separation of

amitriptyline and its metabolite nortriptyline in plasma

(126). Lagerstrom et al. (127, 128) used methanesulfonate

and perchlorate as ion-pairing reagents for the analysis of

a wide range of basic compounds in plasma and urine. They

demonstrated that the addition of a quarternary ammonium

ion-pair (tetrapentylammonium-methanesulfonate) improved

peak symmetry. This technique was also applied by Eksborg

(129) who suggested that the addition of a quarternary ammonium

ion-pair would decrease the interfacial adsorption on the

micro silica column. Later, a reverse phase column (Partisil-10

O.D.S.) was used by Brodie et al. (130) for the analysis of

amitriptyline and nortriptyline in plasma utilizing a mobile phase of acetonitrile and phosphate buffer (50/50 v/v) at pH 3.0. A sensitivity of 2 ug/L was reported by the authors.

Johansson et al. (131) demonstrated that the retention, separation and selectivity can be controlled by the concentration of counter-ions and by the type and amount of liquid stationary phase used. Several procedures utilizing high performance ion-pair chromatography for the analysis of organic ammonium compounds such as imipramine, desipramine, amitriptyline and nortriptyline with dihydrogen phosphate, bromide, cyclohexylsulphamate, dicyclohexylsulphamate or octylsulphate as counter ions and 1-pentanol as the liquid stationary phase on Li-Chrosorb-RP were reported.

Increased use of tricyclic antidepressants for the treatment of endogenous depression stimulated a need for the development of a simultaneous assay method. Proelss et al. (113), in 1978, developed a reverse phase method using pentanesulfonic acid as the ion-pair reagent, for the simultaneous quantitative analysis of doxepin, amitriptyline, nortriptyline, imipramine, and desipramine under routine clinical conditions. The sensitivity of the assay was 2-3 ug/L with ultraviolet detection at 254 nm. Unfortunately, β-naphthylamine, a known carcinogen, was used as the internal

standard. In addition, three benzodiazepines, three
phenothiazenes, and three antihistamines interfered with the
analysis of doxepin, desipramine and nortriptyline respectively.
Thoma et al. (114) reported another simultaneous assay using
a liquid-solid adsorption extraction technique with Clin-Elut™
columns. A CN-bonded column was used with phosphate as the
ion pairing reagent. Phosphate was chosen because of its
optical transparency at 210 nm. Good sensitivity to 5 ug/L
for each drug was achieved at this wavelength. However, doxepin
coeluted with amitriptyline, and desmethyldoxepin with
nortriptyline. As a result, the method was not suitable for
multiple drugs overdose analysis.

Our experience with Clin-Elut™ column extraction indicated
that this method is cumbersome and gives poor and inconsistant
recoveries for tertiary amines and their active secondary
amine metabolites.

Recently, Reece et al. (132) developed a method for
quantitation of imipramine and desipramine using a fluorescence
detector. He used a 10 nm alkyl phenyl reverse-phase column
(u Bondapak/phenyl, Waters Assoc., Milford, Mass.) with
acetonitrile and 0.015 % aqueous phosphoric acid (71:29) as
the mobile phase. Sensitivity of each drug was below 1 ug/L.
However, only the two fluorescent tricyclic drugs could be
monitored.

b)   Adsorption Chromatography:

Another common mode of tricyclic drug analysis is the
use of liquid-solid chromatography (normal phase) on a silica
column. However, tricyclic drugs are ionized at neutral or
acidic mobile phase pH; this gives rise to tailing and poor
peak symmetry. Retention and peak shape can be improved by
the addition of basic compounds like propylamine or ammonia
which suppress ionization. The use of these basic compounds
leads to early deterioration of the column due to gradual
dissolution of the silica packing at pH above 7. Additionally,
the predominantly hydrophilic endogenous plasma constituents
and hydroxylated tricyclic drug metabolites present in the
extract have a high affinity for the polar silica packing
material. This affinity causes background interference and
requires long intervals for column recovery between injections
(133). Another drawback of LSC for clinical samples is the
use of nonpolar mobile phases which are not compatible with
aqueous-based samples. Such samples need to be evaporated
or extracted into a compatible solvent. Because of these problems,
the use of reverse-phase LC is preferred for clinical analysis.

Watson and Steward, in 1975, (134) reported a separation
of three tricyclic drugs (amitriptyline, nortriptyline, and
protriptyline) on a silica column using a methanol/ammonium

solvent. A year later, Dataevernier et al. (135) were able
to separate seven tricyclic drugs on a 5 um silica gel column
with a much weaker solvent of chloromethane n-heptane (1:1)
plus 0.2 % isopropanol. A varying amount of n-propylamine
was added to the eluent to test its influence on the separation
time, capacity factor, k', and resolution. A similar method
was presented by Van Den Berg et al. in 1977 (136); the
mobile phase was ethyl acetate with 0.2 % methylamine to
suppress ionzation of the basic drugs. However, the sensitivity
was limited because ethyl actetate has a UV cut off at 260 nm.
Westenberg et al. (137) described a method for the simultaneous
determination of clomipramine and desmethylclomipramine in
plasma. The detection limits were 2 ug/L for clomipramine
and 10 ug/L for desmethylclomipramine at 250 nm, for a 1 to
4 ml plasma sample. Recoveries from plasma exceeded 95 % for
both drugs. N-hexane was used in the extraction and a ternary
mixture of n-hexane dichloromethane, and methanol (8:1:1)
was used as the mobile phase. No amine was added to the mobile
phase, but interference and precision studies were not reported.

Watson and Steward (138) developed a procedure for deter-
mining amitriptyline and nortriptyline in 2 ml of serum using
a 5 um silica gel (Micropak) column. The extraction solvent
was dichloromethane and the mobile phase consisted of dichloro-
methane-propan-2-ol-conc. ammonia (100:2:0.25); the effluent

was monitored at 240 nm. The sensitivity was 10 ug/L, but

no interference study was done. A simultaneous assay for the

determination of four tricyclic drugs (amitriptyline,

imipramine, nortriptyline, and desipramine) in 2 ml of plasma

was developed by Vandemark et al. (133). The mobile phase

consisted of acetonitrile and concentrated ammonium hydroxide

(99.3/0.7 v/v). The weaker solvent strength helped to eliminate

interfering plasma matrix substances. The column was of 5 um

silica and detection at 211 nm provided a four fold increase

in sensitivity over that obtained at 245 nm (commonly used

by many method). Drugs could be quantitated at concentrations

as low as 10 ug/L. However, doxepin and diphenhydramine

interfered with the analysis of amitriptyline and recoveries

for most drugs are quite low (ranged around 65 %) Recently,

Sutheimer (139) used similar chromatographic conditions except

for the substitution of diethylamine for ammonium hydroxide

to the mobile phase. The use of diethylamine offered greater

flexibility in the chromatographic separation and provided

complete resolution between doxepin and amitriptyline.

c) High pH Mobile Phase Chromatography:

The utilization of this technique in the assay of

tricyclic drugs is a recent development. As cited above,

the use of a mobile phase pH approximating the pK of the

drugs results in good chromatographic efficiency. However, since the tricyclic drugs have basic pK values, the use of a basic aqueous mobile phase results in premature column failure brought about by the dissolution of the silica packing. Using a silica guard column can prolong the analytical column life even with a high pH mobile phase. The mobile phase in passing through the silica guard column becomes saturated with silica, this prior saturation significantly reduces further dissolution of silica from the analytical column. Atwood et al. presented such a method (140) using C_{18} bonded phase silica column with a mobile phase of pH 10.7 at $65^{\circ}C$. With the use of a silica guard column over 400 analysis were performed on a single analytical column.

MATERIAL AND METHODS

<u>Apparatus</u>:

A Model 601 or Model Series II (Perkin Elmer Corp., Norwalk,

Conn., 06856) high performance liquid chromatograph equipped

with a variable wavelength detector (Perkin Elmer LC 55) was

used. The recorder was a Honeywell Electronic Model 194

(Honeywell Inc., Fort Washington, PA 19036). The sample was

injected into a Model 7105 vlave (Rheodynm Berkeley, Calif.

94710) mounted on the chromatograph. The pre-packed reverse-

phase column (5 um Ultrasphere O.D.S., 150 X 4.6 mm, Altex

Scientific Inc., Berkeley, Calif.) was eluted with acetonitrile/

phosphate buffer (21/79 by volume) containing 0.6 ml/L n-nonyl-

amine as the competing base, at a flow rate of 2.5 ml/min.

and ambient temperature. The column effluent was monitored

at 200 nm.

Other equipment included 50 ml and 15 ml teflon stoppered

centrifuge tubes.

<u>Reagents and Standards</u>:

All reagents were analytical (AR) grade, unless otherwise

stated.

<u>Acetonitrile</u>: Acetonitrile (ultraviolet grade), distilled

in glass (Burdick and Jackson Laboratories, Inc., Muskegon,

Mich. 49442).

<u>Phosphate Buffer (0.01 M)</u>: 1.34 grams of KH_2PO_4 to 1 liter

of distilled water (0.01 M), followed by 0.6 ml n-nonylamine,

titrate with phosphoric acid to pH 3.0.

Butylchloride: (Ultraviolet grade), distilled in glass

(Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442).

N-nonylamine: Sigma Chemical Co., St. Louis, Mo. 63178.

Mobile Phase (pH 3.0): Acetonitrile/Phosphate buffer

(0.01 M, pH 3.0, containing n-nonylamine) 21/79 by volume.

Drug Standards: All drug standards (doxepin, desmethyl-

doxepin, amitriptyline, nortriptyline, imipramine, desipramine,

and loxapine) were gifts from the Institute of Forensic

Sciences, Oakland, Calif. A stock standard mixture was prepared

as follows: 25 mg each of doxepin, desmethyldoxepin, imipramine,

desipramine, amitriptyline, and nortriptyline were dissolved

in 100 ml of methanol. The solution is stable at $4^{\circ}C$ for at

least 6 months. Working serum standards and controls were

made by spiking concentrations of these drugs into drug free

serum. Working aliquots of these standards and controls were

frozen at $-20^{\circ}C$ and found to be stable for at least 6 months.

Internal Standard: The loxapine stock standard was

prepared by adding 25 mg of loxapine to 100 ml of methanol.

The working internal standard was prepared by diluting 4 ml

of stock to 100 ml 0.1N HCl (10 ug/ml).

Procedure:

Glassware Preparation: All glassware was acid-cleaned daily before use. All centrifuge tubes were rinsed with hexane and methanol. These steps were mandatory for good recovery of tricyclic drugs.

Method: Transfer 2 ml of serum or plasma into a 12 ml glass centrifuge tube, add 100 ul of loxapine working internal standard (10 ug/ml), 200 ul of 1.5 mol/L NaOH, and 10 ml of butylchloride. Rotate-mix for 5 minutes, centrifuge for 5 minutes at 500 X g, and transfer organic phase into a 12 ml glass conical tube containing 200 ul of 0.025 mol/L HCl. Shake the mixture mechanically for 10 minutes, and centrifuge for 2 minutes at 500 X g. After discarding the top organic layer, inject 100 ul of the aqueous phase into the chromatograph using the following chromatographic conditions:

Flow rate: 2.5 ml/min.

Detector: UV, 200 nm

Sensitivity: 0.035 A full scale

Temperature: Ambient

Each drug was quantitated by measuring the ratio of the peak height of each drug to that of internal standard in the unknown sample, and comparing it with a serum of known concentration.
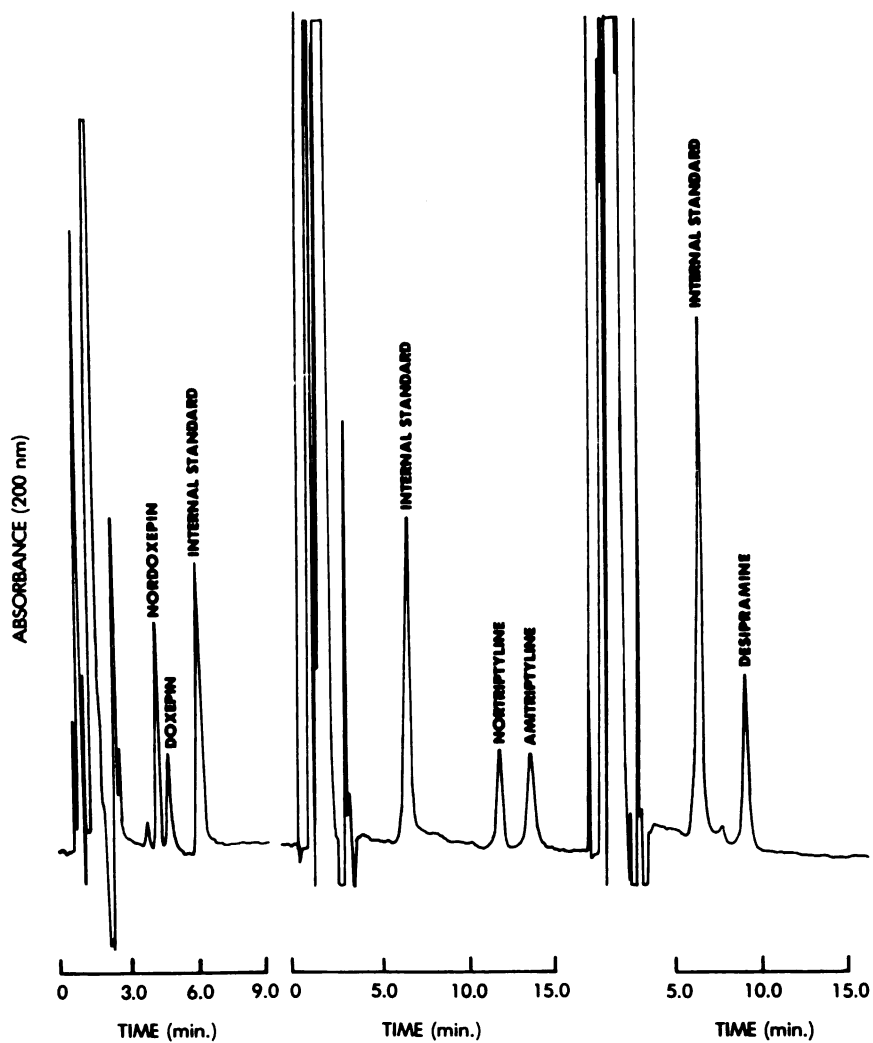
Fig 2 Left: Chromatogram from the serum of a patient

containing 140 ug/L of desmethyldoxepin,

and 300 ug/L of doxepin.

Middle: Chromatogram from the serum of a patient

containing 100 ug/L of nortriptyline and

195 ug/L of amitriptyline.

Right: Chromatogram from the serum of a patient
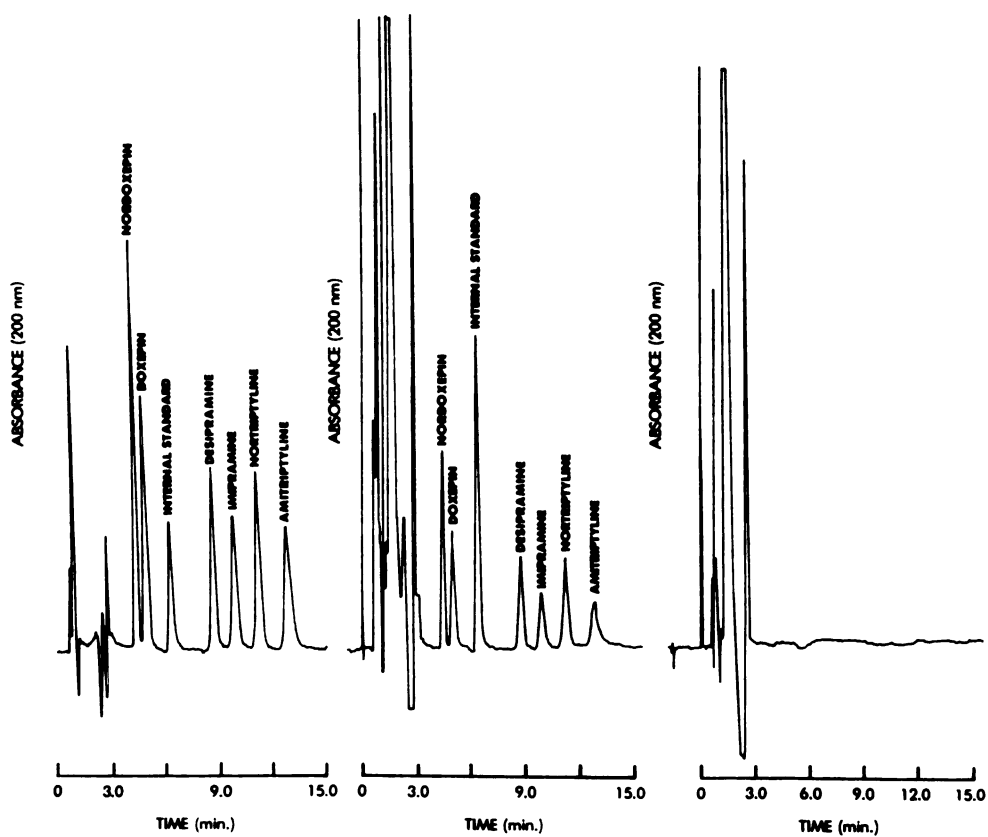
containing 120 ug/L of desipramine.

Fig. 3 Left: Chromatogram of a standard mixture of

tricyclic drugs.

Middle: Chromatogram of a serum supplemented

with approximately 80 ng of each tricyclic

drug, except for internal standrad, which

was 200 ng.

Right: Chromatogram of a drug-free serum.

RESULTS AND DISCUSSION

Analytical Variables

Chromatography: Various chromatographic conditions
were investigated by injecting drug standards in methanol.
Although tricyclic amines exhibited an average maxima at
245 nm, their absorbance again increased in the far UV region.
Analysis at 200 nm provided four fold increase in sensitivity,
which allowed for the detection of these drugs at the 10 ug/L
level in plasma or serum.

Initially, good separation was achieved using KH_2PO_4
(0.01M) as the ion-pairing reagent. The ratio of acetonitrile
to phosphate buffer was 27/73 at pH 3.0. Two ml of triethyl-
amine was added to the mobile phase as the competing base
to improve the separation and peak symmetry. A flow rate of
3 ml/min and an oven temperature of $40^{\circ}C$ were found to be
optimum for good separation. Unfortunately, the retention
time varied from run to run due to the low boiling point
of triethylamine. This undesirable property caused
inconsistancy in the mobile phase. n-Nonylamine, a non-
volatile amine was found to be an acceptable substitute.
Initially 100 ul of n-nonylamine was added to the mobile
phase; however, this did not provide adequate resolution of

desmethyldoxepin and doxepin or imipramine and nortriptyline. Lowering the temperature to ambient and reducing the concentration of acetonitrile allowed resolution of imipramine and nortriptyline. Increasing n-nonylamine to 600 ul further improved resolution and peak symmetry.

Finally, the following optimum conditions were selected for the analysis: 1) mobile phase—21 % acetonitrile and 79 % phosphate buffer with 600 ul of n-nonylamine at pH 3.0. 2) ambient temperature 3) flow rate of 2.5 ml/min. Total chromatographic time was less than 13 minutes with reproducible day to day retention times. Minor pH variations, i.e. from 2.9 to pH 3.1, did not seem to affect separations.

Spiked serum standards and plasma standards gave comparable values; therefore, both serum and plasma can be used for this analysis.

Protriptyline has been used as an internal standard for tricyclic analysis, but it interfered with our analysis of desipramine. Loxapine was chosen because of its chemical similarity and appropriate retention time.

Extraction Procedure: Various extraction procedures were tried and their efficiency, simplicity, reproducibility and speed for the isolation of tricyclic drugs were compared. The solid phase methods were generally combersome; Clin-Elut^{TM} was tested in our laboratory, and the results showed poor

and inconsistant recoveries for the tertiary amines and their active secondary metabolites.

The liquid-liquid extractions were much simpler and more suitable for routine clinical applications. However, when the procedure required solvent extraction, evaporation, and reconstitution, recoveries of the tricyclic drugs were low and highly variable. During the development of the present procedure, it was realized that butylchloride as an extraction solvent at pH 14, followed by an acid back-extraction, provided adequate and consistant recoveries of all tricyclic drugs. Furthermore, recoveries were improved by acid cleaning of all glassware and rinsing them with hexane and methanol before use.

Previous studies utilized 0.1 N HCl for the back-extraction step. Lower concentrations of HCl (0.05 N, 0.025 N) were utilized in this procedure to prolong column life. Recoveries, retention time, and sensitivity were unaffected by lowering the acid concentration to 0.025 N.

Calculations and Quantitations:

A mixture of six tricyclic drugs and the internal standard in the range of 500 ng/ml each was used to calculate the relative retention times (RRT) and response factors (RF) as follows:

$$RRT = \frac{\text{Retention time of TCA from the injection pt.}}{\text{Retention time of the I.S. from the injection pt.}}$$

$$RF = \frac{\text{Peak height of I.S.}}{\text{Peak height of TCA}}$$

Peak height measurements can be used for quantitation only when the peaks are sharp and symmetrical. The RF was used to quantitate the tricyclic drugs using the following formula:

$$\text{ug/L of TCA in serum/plasma} = \frac{\text{Peak ht. of TCA}}{\text{Peak ht. of I.S.}} \times RF \times \text{Conc. of I.S.}$$

Precicion:

Within run precision was evaluated by using aliquots of spiked sera: 88 ng/ml and 220 ng/ml. The day to day precision was evaluated on consecutive days. Precision data are shown Table 7. The standard deviation (S.D.) was calculated by the following eauation:

$$S.D. = \left[\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N} \right)^2 \right]^{\frac{1}{2}}$$

X = values in ug/L

N = no. of specimens

The coefficient of variation or (C.V.) was calculated as follows:

$$C.V. \% = \frac{S.D.}{\overline{X}}$$

$\overline{X}$ = Mean

As shown in Table 7, the C.V. for within day studies varied between 1.9 and 4.1 % for both levels, while the day to day studies varried between 3.9 and 8.6 %.

Table 7

Precision of Assays for TCA in Serum

	Day-to-day (n=12)		Within-day (n=11)	
	Range, ug/L (±SD)	CV %	Range, ug/L (±SD)	CV %
Desmethyldoxepin	88.8 ± 3.5	3.9	83.2 ± 3.4	4.1
	227.0 ± 11.1	5.2	220.0 ± 5.3	2.4
Doxepin	82.7 ± 4.1	5.0	70.8 ± 2.0	2.8
	207.0 ± 10.2	4.9	193.0 ± 3.6	1.9
Desipramine	89.0 ± 3.8	4.2	85.9 ± 2.1	2.5
	224.0 ± 10.5	4.7	226.7 ± 4.0	1.8
Imipramine	77.0 ± 4.2	5.4	69.8 ± 2.5	3.5
	188.0 ± 10.5	5.6	164.0 ± 3.8	2.3
Nortriptyline	87.4 ± 4.4	5.0	85.6 ± 1.9	2.2
	220.0 ± 11.9	5.4	222.0 ± 4.3	1.9
Amitriptyline	68.0 ± 5.5	8.1	92.0 ± 2.6	2.8
	169.0 ± 14.5	8.6	131.0 ± 5.2	4.0

Analytical Recovery:

Various amounts of the six tricyclics were added to
drug-free serum to achieve a wide range of concentrations
(22 to 1760 ug/L). These samples were run in duplicates
containing a known amount of internal standard. Analytical
recoveries ranged from 73 % to 107 % (Table 8).

Linearity:

The peak height ratio of each drug level (22 to 1760 ug/L)
was calculated and plotted against its concentration; linear
standard curves were obtained throughout this range (Fig. 4,5).

Background:

Twenty drug free serum and plasma samples were extracted
and analyzed for possible endogenous interference. As shown
in Fig. 3, there was no apparent interference and background
appeared to be negligible.

Interference:

To determine the potential usefulness of this method in
a routine clinical laboratory, an extensive interference study,
including neutral and basic drugs was carried out. Acidic
drugs were not extracted by our method. Over 33 neutral and
basic drug standards were injected under similar chromatographic
conditions, and their retention times were measured. Any drug
that eluted sufficiently close to our drugs of interest was
injected at various dilutions to evaluate its quantitative

Table 8

Relative Recovery of Drugs from Plasma

| Drug	Added ng/ml	Recovered ng/ml	Recovery %
Nordoxepin	22	22	100
	88	89	101
	220	225	102
	440	440	100
	880	892	101
	1760	1884	107
Doxepin	22	19	85
	88	81	92
	220	209	95
	440	410	93
	880	803	94
	1760	1752	99
Desipramine	22	23	103
	88	92	105
	220	220	100
	440	449	102
	880	900	102
	1760	1884	107
Imipramine	22	20	90
	88	71	80
	220	195	88
	440	386	88
	880	778	88
	1760	1760	95
Nortriptyline	22	20	90
	88	88	100
	220	219	99
	440	440	100
	880	884	100
	1760	1863	106
Amitriptyline	22	16	73
	88	68	78
	220	176	80
	440	347	79
	880	709	80
	1760	1530	87
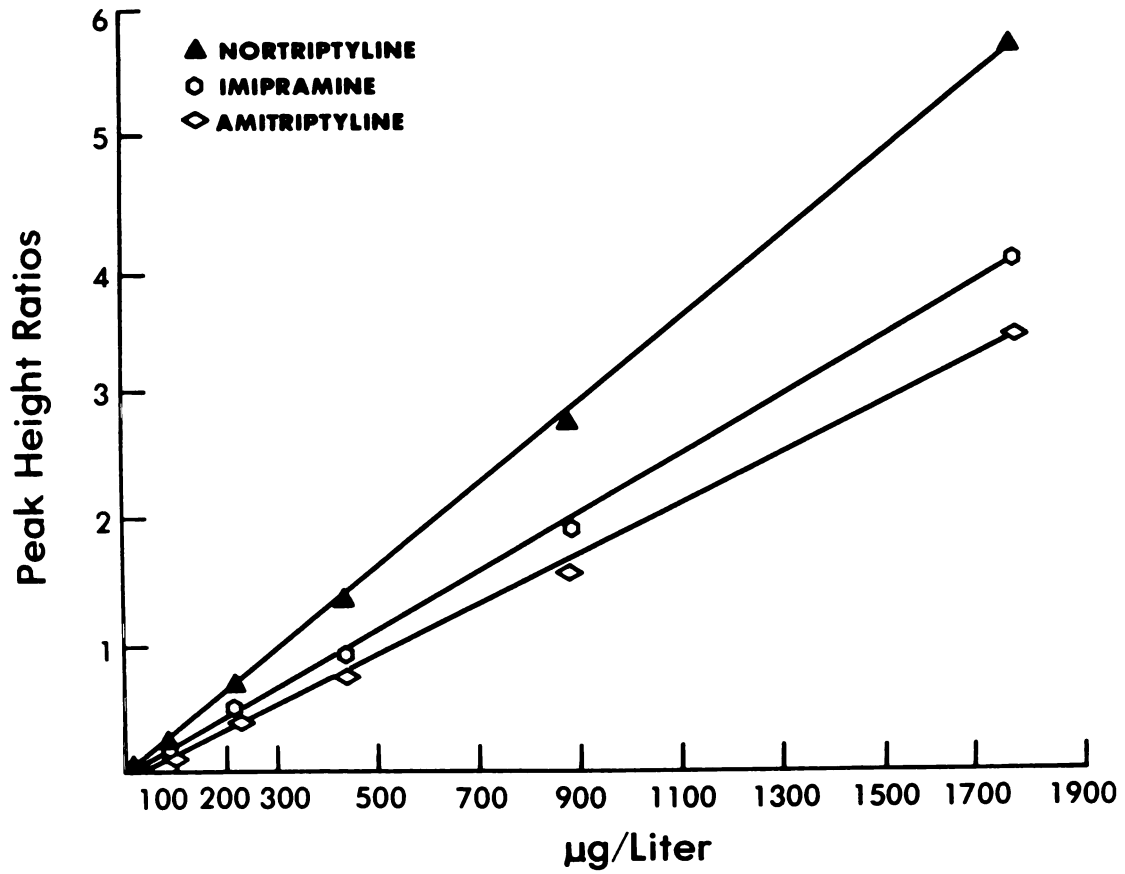
Fig. (4) Peak-height ratios (drug/internal standard)

for nortriptyline, imipramine, and amitriptyline
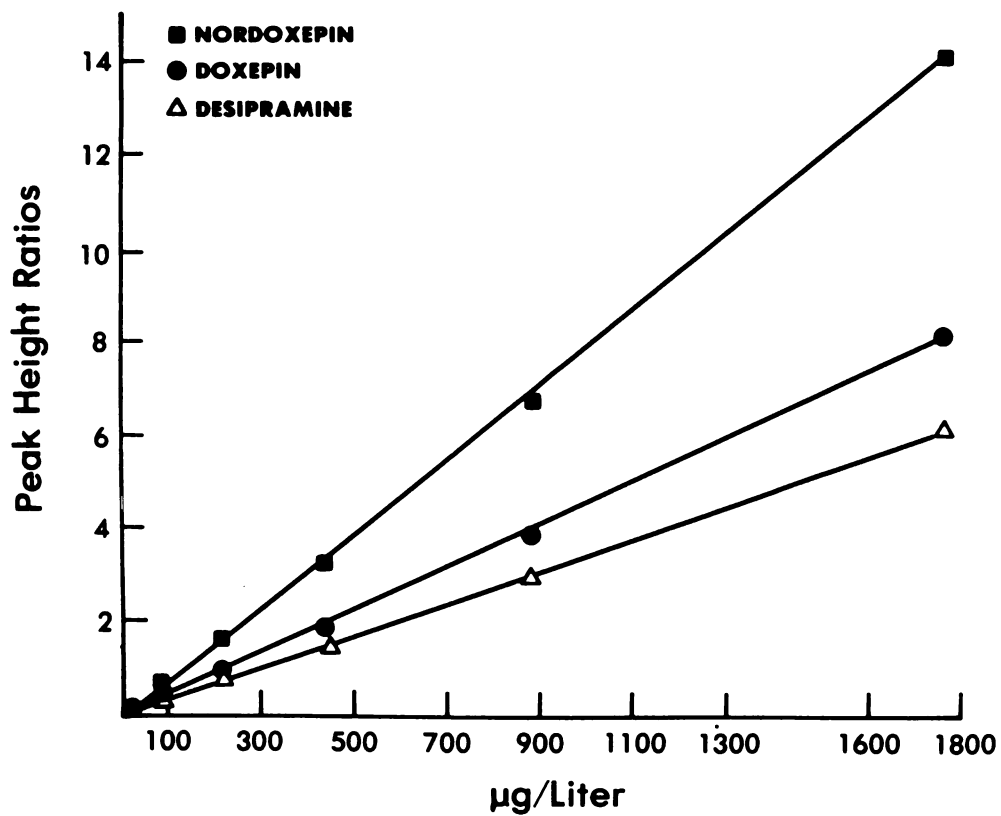
plotted vs. concentration of each drug.

Fig. (5) Peak-height ratios (drug/internal standard)

for desmethyldoxepin, doxepin, and desipramine

plotted vs. concentration of each drug.

effect. Table 9 lists the relative retention times for the

drugs studied. Of 33 drugs tested, only phenacitin coeluted

with the internal standard (loxapine). If interference is

suspected, the unknown can be run with another non-coeluting

tricyclic drug as an internal standard. Codeine and propoxyphene

eluted close to desipramine and nortriptyline, respectively.

These two drugs were not completely resolved from the

tricyclic antidepressant drugs and could give falsely elevated

results at toxic concentrations. However, these interfering

drugs could readily be recognized from the chromatogram. Most

of the commonly used benzodiazepines and phenothiazines did

not interfere.

Sensitivity and Detection Limit:

Our extraction procedure yielded a relative clean extract,

the drugs could be measured at a detector sensitivity of 0.035 A

full scale. Under the conditions of the assay, the detections

limit (signal to noise ratio exceeding 3) was about 5 ug/L

for all six tricyclic drugs. 2-3 ug/L may be detected by

increasing the injected sample volume from 100 ul to 150 ul

and the detector sensitivity to 0.025 A full sacle. The method

described above is adequately sensitive for all clinical

applications.

Correlation with GLC Method:

Accuracy was further evaluated by comparing results obtained

Table 9

Relative Retention Times (RRT) for Some Drugs

DRUGS	R.R.T.*
Promazine	0.117
Codeine	0.135
Theophylline	0.188
Mathamphetamine	0.189
Caffeine	0.2
Acetaminophen	0.22
Meperidine	0.23
Methylphenidate	0.23
Diphenhydramine	0.58
Chlordiazepoxide	0.59
Flurazepam	0.6
Desmethyldoxepin	0.66
Doxepin	0.75
Procaine	0.85
Haloperidol	0.89
Loxapine	1.0
Desipramine	1.34
Protriptyline	1.4
Imipramine	1.55
Nortriptyline	1.77
Propoxyphene	1.78
Methadone	1.85
Amitriptyline	2.06
Amphetamine	2.63
Thorazine	2.98
Diazepam	N.D.
Desmethyldiazepam	N.D.
Clomipramine	N.D.
Thioridazine	N.D.
Prochlorperazine	N.D.
Phenylpropanolamine	N.D.
Salicylates	N.D.
Fluphenazine	N.D.

N.D. Not detectable, did not elute

by gas chromatography using a nitrogen-phosphorus detector.
26 samples from patients on antidepressant drug therapy were
processed. The results of this study are summarized in
Table 10, and the comparison parameters were calculated using
the following formulae:

$$\text{Regression Coefficient (r)} = \frac{N(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[N\Sigma x^2 - (\Sigma x)][N\Sigma x^2 - (\Sigma y)^2]}}$$

x = the independent variable (GLC method)

y = the dependent varible (HPLC)

$$\text{Slope} = \frac{N\Sigma xy - \Sigma x\Sigma y}{N\Sigma x^2 - (\Sigma x)^2}$$

$$\text{Y-intercept} = \frac{\Sigma y\Sigma x^2 - \Sigma x\Sigma xy}{N\Sigma x^2 - (\Sigma x)^2}$$

A serum pool with known amounts of TCA drugs added were
obtained from the Institute of Forensic Sciences, Oakland,
California. Results are summarized in Table 11.

Table 10

Comparison of Methods: GLC verses Proposed HPLC Method

(Using patient Samples)

<u>DRUG</u>

Amitriptyline   N = 8

       r = 0.992

      Slope = 1.12

    Y-intercept = 0.013

Nortriptyline   N = 9

       r = 0.993

      Slope = 1.07

    Y-intercept =-0.005

Desipramine   N = 7

       r = 0.934

      Slope = 1.03

    Y-intercept =-0.004

Imipramine   N = 2

       r = 0.935

      Slope = 1.03

    Y-intercept = 0.004

Table 11

Comparison with Spiked Pool*

Drug	Value Obtained ng/ml (proposed method)	Amt. of Drug Added ng/ml
Desmethyldoxepin	220	200
Doxepin	224	200
Desipramine	210	200
Imipramine	205	200
Nortriptyline	202	200
Amitriptyline	203	200

* Spiked Pool sera were donated by the Institute of
Forensic Sciences, Oakland, Ca.

CONCLUSION

The relationship between plasma levels of tricyclic antidepressant drugs and their therapeutic action remains controversial, but evidence suggests that there is a therapeutic range for optimal effect. It is well documented that for a standard oral dose, a wide range of steady-state blood levels are achieved. Therefore, in the routine management of patients on tricyclic drug therapy, there is a need for an accurate, rapid and relatively easy method of analysis for the drug and its active metabolites.

Many contemporary analytical methodologies suffer from being insensitive, lengthy, comlicated, subject to interferences, or requiring highly specialized skill and instrumentation. GLC-mass spectrometry offers the required sensitivity and specificity for pharmacokinetic studies as well as therapeutic monitoring; however, this expensive and highly specialized equipment is not available in the average clinical laboratory.

The application of liquid chromatography to the determination of tricyclic drugs has had several limitations. The first was adequate sensitivity. Ultra-violet detection at 240 nm is not adequately sensitive for low therapeutic or sub-therapeutic concentrations in plasma. Fluorescence detectors offer excellent sensitivity, but are only applicable to imipramine

and desipramine analysis. The proposed method offered the required sensitivity by using the far UV range (200 nm) for detection; the fourfold increase sensitivity allows detection of about 5 ug/L in plasma.

Besides the problem of sensitivity, chromatographic separation of these basic compounds has been challenging. they are partly ionized in aqueous solutions as weak bases; therefore, adsorption or reverse-phase chromatography gives peaks that are not adequately resolved and peak tailing. Increasing the pH of the mobile phase to suppress ionization results in good separation, but silica column dissolution will begin if the pH of the mobile phase exceeds 7.0. Paired ion chromatography techniques permits good separations of these drugs without column degradation, but the reagents used for ion-pairing often have an ultraviolet cut off at about 240 nm, preventing the use of more sensitive far UV detection. The proposed method eliminated all these problems by using phosphate as an ion-pairing reagent and n-nonylamine as a competing base to improve peak shape and retention time. This mobile phase system has low UV cut-off and allows detection at 200 nm. The assay provides adequate sensitivity and precision. It is suitable for routine application in the clinical laboratory because the sample extraction is simple

and rapid, and the chromatographic time is less than 13 minutes.
In addition, the method has the advantage of simultaneous
determination of the most commonly used tricyclic antidepressants
and their active metabolites. This approach is extremely
useful in cases of drug overdose in which multiple tricyclic
drugs are ingested. Most patients treated with tricyclic
antidepressants receive other psychoactive drugs, including
sedatives and tranquillizers. Most benzodiazepine and
phenothiazine drugs do not interfere. Among the drugs tested,
only two, codeine and propoxyphene, have the potential to
interfere with desipramine and nortriptyline. Precision,
recovery, interference, and correlation studies validate
this procedure for clinical use.

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