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Distribution of Imipramine, Desipramine and Their Principal Metabolites Between Plasma, Red Blood Cells and Brain in Humans and Animal Models

Stuart Chapman Bogema Jr.
scbog@yahoo.com

DISTRIBUTION OF IMIPRAMINE, DESIPRAMINE AND THEIR PRINCIPAL METABOLITES BETWEEN PLASMA, RED BLOOD CELLS AND BRAIN IN HUMANS AND ANIMAL MODELS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

Stuart Chapman Bogema, Jr.
B.S. University of Richmond
Richmond, VA, 1974

Directors

Robert V. Blanke, Ph.D.
Professor, Departments of Pathology
and Pharmacology and Toxicology

Nedathur Narasimhachari, Ph.D.
Professor, Departments of Psychiatry
and Pharmacology and Toxicology

Virginia Commonwealth University
Richmond, Virginia
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This dissertation by STUART CHAPMAN BOGEMA, JR is accepted in its present form as satisfying the thesis requirement for the degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AUC - Area under the curve
CMP - Chlorimipramine
CNS - Central nervous system
CPL - Clinical Psychopharmacology Laboratory
CSF - Cerebrospinal fluid
C.V. - Coefficient of variation
DMD - Desmethyldesipramine (didesmethylimipramine)
DMI - Desmethylimipramine
DSM-III - Diagnostic and Statistical Manual on Mental Disorders, Third edition, American Psychiatric Association
ES - Extraction Solvent = Ethyl acetate:hexane:isopropanol: isoamylalcohol (10:9:1:0.2)
5-HT - Hydroxytryptamine (serotonin)
GC - Gas chromatography
GC-MS - Gas chromatography - mass spectrometry
HDS - Hamilton Depression Rating Score
HPLC - High performance liquid chromatography
IC_{50} - Concentration that inhibits 50% of binding
i.m. - Intramuscular
IMP - Imipramine
i.p. - Intraperitoneal
i.v. - Intravenous
K' - Column capacity factor, a measure of retention on a chromatography column for a compound
MCV - Medical College of Virginia
PAR - Peak area ratio
PHR - Peak height ratio
r - Correlation coefficient
RBC - Red blood cell
RCM - Radial compression module (Waters Associates)
RPM - Revolutions per minute
R.S.D. - Relative standard deviation
S.D. - Standard deviation
TCA - Tricyclic antidepressant
TDM - Therapeutic drug monitoring
$t_{\frac{1}{2}}$ - Half life of elimination
2HD - 2-Hydroxydesipramine
2HI - 2-Hydroxyimipramine
V_D - Volume of distribution
V_0 - Void volume of a chromatography system

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ABSTRACT

DISTRIBUTION OF IMIPRAMINE, DESIPRAMINE AND THEIR PRINCIPAL METABOLITES BETWEEN PLASMA, RED BLOOD CELLS AND BRAIN IN HUMANS AND ANIMAL MODELS

Stuart Chapman Bogema, Jr.

Medical College of Virginia - Virginia Commonwealth University, 1983.

Major Director: Robert V. Blanke, Ph.D.

An HPLC prodecure was developed for the simultaneous measurement of imipramine (IMP), desipramine (DMI), desmethyl desipramine (DMD), 2-hydroxyimipramine (2HI) and 2-hydroxydesipramine (2HD) in human, rat and rabbit blood and brain specimens. The HPLC results were validated by comparison to an established GC-MS method.

DMD is a minor metabolite of IMP and DMD in humans. 2HI is a minor metabolite of IMP in most humans. 2HD reaches appreciable concentrations in blood in humans treated with DMI and IMP. Plasma and RBC concentrations of IMP and DMI were compared to improvement in depression in patients treated with IMP. No good correlation was found. Plasma and RBC concentrations of DMI were compared to improvement in depression in patients treated with DMI. Plasma concentrations ($r = -0.750$, $p < .01$) and RBC concentrations ($r = -0.693$, $p < .01$) correlated with improvement in depression. As plasma DMI increased past 400 ng/ml, the degree of improvement declined.

In autopsy specimens from an IMP overdose, IMP, DMI, DMD, 2HI and 2HD were all measured in whole blood and brain. In autopsy specimens from two DMI overdoses, DMI, DMD and 2HD were all measured in whole blood and brain. The brain to blood ratios in all cases for IMP and DMI were

significantly higher than the brain to blood ratio of 2HD, the most polar metabolite. This finding indicates that 2HD has restricted entry into the brain compared to IMP and DMI. The distribution of 2HD between plasma and brain was studied in rats. The mean brain to plasma ratio for DMI was 5.87 and for 2HD it was 0.067. Therefore, in rats 2HD has limited access into brain from blood compared to DMI.

In rat studies, both plasma and RBC concentrations of DMI correlated well with brain DMI concentrations. In humans and rats, the plasma to RBC ratios for IMP and DMI are significantly different. IMP has greater affinity for plasma than RBCs. DMI is the reverse, having greater affinity for RBCs than plasma. As indicated, for DMI treated patients, plasma DMI concentrations correlated slightly better than RBC DMI concentrations to patient improvement in depression.

INTRODUCTION

A. Current Status of Therapeutic Monitoring of Imipramine and Desipramine.

During the last decade, there has been a continuous investigation into possible relationships between tricyclic antidepressant (TCA) concentrations in depressed patient's tissues, mostly blood, and the outcome of drug therapy. The success of therapeutic drug monitoring in many areas of pharmacotherapy, such as anticonvulsant and antiarrhythmic treatments, has been an impetus for this research. Major advances in three areas has allowed this investigation. First, there have been great improvements in the sensitivity, specificity and economy of quantitative analysis of drugs and their metabolites in body fluids and tissues so that TCA concentrations can be reliably and practically measured (1). Second, much has been learned about the fundamental pharmacology of antidepressant drugs and about different depressive syndromes. We now know that subtypes of depression exist and that these subtypes may or may not respond to a certain tricyclic antidepressant. For example, desipramine is efficacious in a large percentage of unipolar endogenous depressions but not in other subtypes (2). Third, the clinical diagnosis of depression has been improved so that these subtypes can be identified and also the extent of depression can be roughly quantified. The measurement of the extent of depression is required in order to determine the response of the patient to treatment.

Clinical response, either improvement or regression, is a parameter which must be measured if it is going to be compared to psychiatric counselling and drug treatment. Global indices and rating scales have been developed to determine the severity of the depressive syndromes. For instance, the Hamilton Depression Scale is used by psychiatric clinicians to numerically rate the severity of the patient's depression (3). By combining the advances in measuring depression, in drug treatment of depression and in quantitation of drug concentrations, researchers have tried to determine what relationships exist between the pharmacodynamics and clinical response for tricyclic antidepressants.

Drug concentrations should only be measured for practical purposes. A number of considerations should be taken into account when assessing the value of therapeutic drug monitoring (TDM). The clinical pharmacokinetics of the compounds should be understood. This knowledge is important for proper administration of the drug as well. The clinical pharmacokinetics of tricyclic antidepressants have been studied starting about fifteen years ago and sufficient knowledge exists for optimization of drug administration and for the basis of therapeutic drug monitoring (4).

Tricyclic antidepressants are principally metabolized by the liver. Oxidative demethylation of the secondary or tertiary amine on the side chain and hydroxylation of the phenyl rings of the tricyclic structure are the major phase one metabolic routes (5). As an example, the principle routes of metabolism of imipramine are given in Figure 1. Pharmacologic testing of metabolites to determine what activity they may have in relation to the parent drug is needed. For instance, it has long been known that N-demethylated metabolites of the tertiary amine

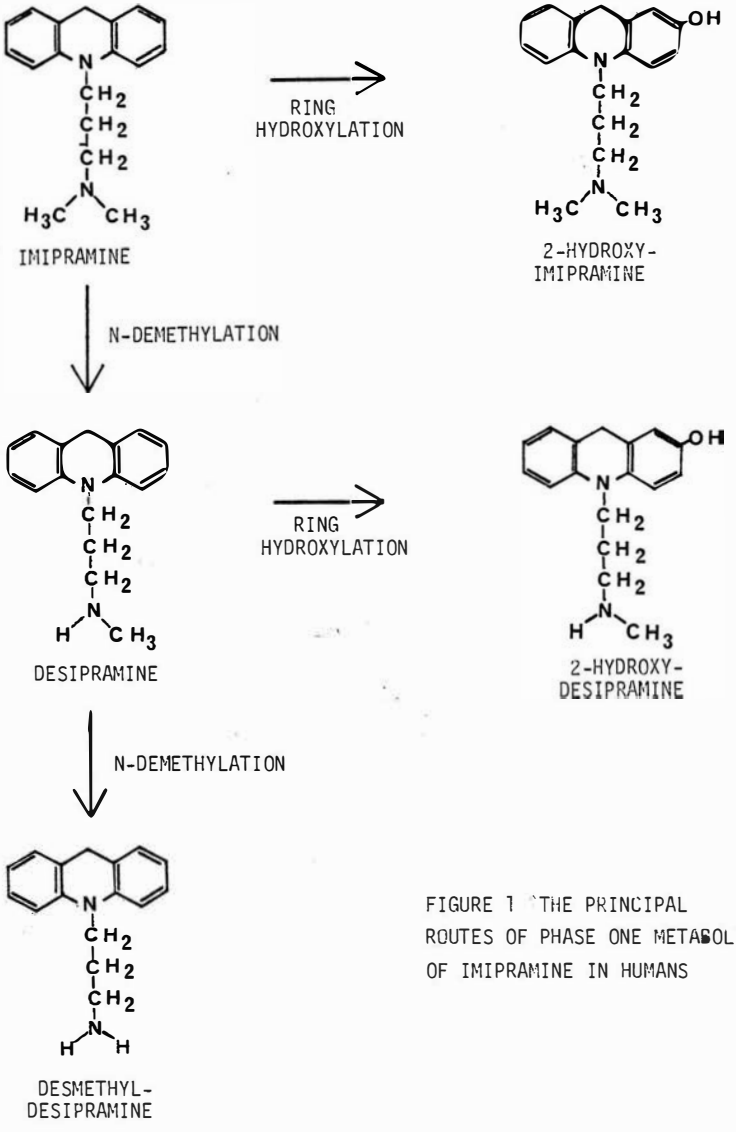


FIGURE 1 THE PRINCIPAL ROUTES OF PHASE ONE METABOLISM OF IMIPRAMINE IN HUMANS

drugs amitriptyline, doxepin and imipramine have pharmacologic activity against depression. Hydroxylated metabolites of TCA have only recently been tested for pharmacological activity. The significance of hydroxylated metabolites has not been completely elucidated.

For TDM, assay procedures for the TCAs and their active metabolites must be developed. Assay procedures have routinely measured both the tertiary amine parent drugs and their secondary amine metabolites. Most procedures for secondary amine TCAs measure them only. The measurement of these drugs and metabolites must meet sensitivity, specificity, reproducibility and economy requirements for practical use in TDM.

In the treatment of depression, the drug concentration must be maintained for a long period of time. This may be months or even years. This prophylatic therapy for depression, analogous to anticonvulsant pharmacotherapy, is benefited by periodic monitoring of drug concentration, especially during relapse, as variations in individual pharmacokinetics, or compliance, with time can increase or decrease steady state drug concentrations appreciably.

It is known that the beneficial effect of TCA treatment for depression is not usually seen for two to six weeks (6). Therefore, no immediate clinical measurement of the effect is available. There are two ways to determine the corresponding drug concentration prior to the onset of this delayed therapeutic effect. First, steady-state concentrations for most TCAs will occur approximately one week after a fixed dose regimen of drug administration is initiated. Steady-state drug concentrations are reached about five elimination half-life time periods after the start of fixed dose drug therapy. The half-life of elimination is less than a day for most patients taking TCAs (protriptyline is an

exception and many elderly patients have slower rates of elimination). Measurement of drug concentration after a week of fixed dose treatment gives this steady-state level and allows adjustment of the dose if a "therapeutic concentration" is not found. A second, better means of establishing a "therapeutic concentration" is the use of a test dose (usually 50 mg) of the TCA and the measurement of the drug in a blood sample drawn twenty-four hours later. This 24 hour test dose concentration is highly predictive of steady-state drug concentration (7, 8) so that the dose required to produce a "therapeutic concentration" can be calculated at the start of drug therapy. Therefore, a potentially therapeutic drug regimen can be initiated, although the resulting clinically observable outcome can not be seen for two to six weeks.

If the same dose given to all patients resulted in the same steady-state drug concentration for a TCA, there would be no need to measure the drug concentration. There is wide interindividual variability in TCA pharmacokinetics. For example, for the same dose of imipramine, a twelve-fold range of plasma concentrations was reported (9). A five-fold range of steady-state plasma concentrations was found for nortriptyline (10). The correlation between dose and plasma concentration is poor for the TCAs. The variability has been found to be primarily caused by differences in the rate of metabolism between patients (11). Metabolism is largely genetically controlled (12), but environmental factors such as drug interactions, disease and smoking can affect the metabolic rate. These environmental factors also influence the intrapatient, day-to-day variability of the steady-state concentration. This intrapatient variability was studied in patients on a fixed dose of certain TCAs by Ziegler et al. (13). They found

desipramine to have the largest coefficient of variation, 26 percent, in it's steady-state concentration and nortriptyline to have the lowest, 13 percent. A major axiom for therapeutic drug monitoring is that the plasma concentration reflects the tissue concentrations and hence, the relative availability of the drug at the pharmacologic receptor or site of action. For TCAs and most other drugs, the dose administered is a poor indication of the plasma concentration, so that the plasma concentration must be directly measured.

The therapeutic range of a drug is defined by a low plasma concentration limit and a high plasma concentration limit. The lower limit is a threshold value at which therapeutic effect begins and the upper limit is where beneficial effect declines or more often, where toxic manifestations or side effects become dangerous or unduly uncomfortable for the patient. If the therapeutic concentration range of a drug is large, variations in pharmacokinetics are of little concern and therapeutic drug monitoring is seldom necessary. A dose that is known to be large enough to produce a therapeutic concentration can be given without fear of adverse effects. For TCAs, this is not possible. The therapeutic range is small enough that interindividual variations in pharmacokinetics will produce a range of plasma concentrations below, within and above the therapeutic range for the same dose in a large group of patients.

The final consideration to be taken into account when assessing the value of therapeutic drug monitoring for TCAs is whether correlations between plasma concentrations and therapeutic and toxic effects have been established. This is the most important consideration. Without these correlations, the plasma concentrations are meaningless. It has

also been the most difficult consideration to be addressed for a number of reasons. The investigation of the relationship of plasma concentrations of TCA drugs and their metabolites to clinical response has been underway for over a decade. During this time many advances have been made in the three areas that have allowed this investigation, namely in measuring depression quantitatively to determine clinical response to drugs, in diagnosing depressive subtypes and determining the drugs that are efficacious for each and in quantitating the drugs and active metabolites in the patient tissues. Serious flaws have been discovered in earlier research as our understanding in each of these three areas has grown (14).

The Diagnostic and Statistical Manual of Mental Disorders, Third Edition (DMS-III) published by the American Psychiatric Association (15) contains diagnostic criteria for the major affective disorders. Appendix I contains the diagnostic criteria used for determining the type and/or subtype of affective disorders.

Patients who have depressive subtypes that are not typically responsive to TCAs, such as those with psychotic symptoms, atypical depressions or neurotic and reactive depressions (16), have been included in earlier clinical studies assessing plasma levels and response. As these subtypes which do not respond are identified and excluded, and patients who are identified as having major depressive disorder with melancholia as defined by DSM-III, are included, the correlation between plasma concentration of TCA and clinical improvement has been positive and better. On the other hand, approximately 30% of patients show substantial improvement with placebo treatment only (17). A "placebo washout" period of one to two weeks, in which the patient is

treated with placebo and counselling, should be used to identify patients who respond favorably to placebo treatment. These patients should be removed from clinical studies of TCA efficacy. Inclusion of these patients has distorted studies where there was no "placebo washout" period prior to initiating drug therapy. Improvement in patient selection for clinical studies has occurred in the last five years as knowledge of subtypes of depression and drug therapy are also improved.

The measurement of clinical outcome is still being assessed and improved. M. Asberg and F. Sjoqvist stated in 1978 (18), "compared to the increasing sophistication of the chemical methods, surprisingly little attention has been given to development of the clinical methodology. Depression rating scales may be highly reliable in the hands of well-trained raters, but their precision is less well tested. Since most of the classical scales were not originally designed for measuring treatment induced change, it seems likely that their sensitivity in this respect could be increased. " A recent review (19) of the measurement of depression in clinical trials suggests that a choice from several classes of depression measures should be used because each class has advantages and disadvantages and a combination will give a much better indication of the severity of depression. Combinations of measures have seldom been used until recently. In fact, the most commonly used scale, the Hamilton Depression Scale (HDS), is criticized as a poor measure of clinical improvement (18).

It is because of improvements in clinical studies of the last five years that reports of these studies are the most valuable. They have generally shown better correlations between plasma concentration and

clinical response for tricyclic antidepressants. I will review these studies for the two drugs of interest here, imipramine (IMP) and desipramine (DMI).

1. Imipramine Studies.

There have been more clinical studies using IMP than there have been using DMI. In IMP studies the plasma concentrations of DMI, its major psychoactive metabolite, and IMP have been compared to clinical outcome. The parent compound, IMP, and metabolite, DMI, appear to have different central neuropharmacological activities. IMP acts to block the uptake of both serotonin and norepinephrine in central synapses, while DMI acts primarily as a blocker of central uptake of norepinephrine (20). Generally, these differences in neuropharmacological activity are difficult to relate to plasma TCA concentration because the mechanism of action of TCA is still unknown. These differences in interaction with biogenic amines have not been taken into account when assessing plasma concentration versus clinical response.

Glassman et al. (21) reported a linear relationship between plasma concentrations of IMP and DMI and antidepressant efficacy. They selected a patient population suffering from endogenous depression and requiring hospitalization. They excluded patients with any evidence of schizophrenic illness, schizoaffective disease, organicity, serious medical illness or other preexisting diagnosable psychiatric illness. A one week drug-free period and one week placebo treatment period were used to screen out patients whose Hamilton Depression Scale scores dropped below 17. The patients were then placed on 3.5 mg/kg of imipramine hydrochloride per day. Actual doses varied from 100 to 300 mg/day. Nurses verified that the drug had been ingested. Treatment lasted four

weeks. Blood specimens were drawn three times each week, prior to the day's first dose. Plasma was analyzed by a spectrofluorometric technique (22) by which DMI is measured by difference following acetylation. Other metabolites of IMP, such as the 2-hydroxy compounds, would interfere. A total of sixty patients finished the study, but the investigators removed 18 unipolar delusional patients who were less responsive to IMP therapy. The remaining 42 patients consisted of 30 unipolar and 12 bipolar non-delusional endogenously depressed patients. The correlation coefficients for the log of the plasma IMP plus DMI concentration and clinical response (based on the Hamilton Depression Scale, a mood rating and a global rating but no exact definition of response is given) were 0.682 for the bipolar group and 0.435 for the unipolar group. The combined plasma steady-state concentration ranged from 50 to 1050 ng/ml with an average of 200 ng/ml ± 137 ng/ml. They noted that patients above the median (180 ng/ml) do better than those below. After four weeks of treatment, 22 of 31 responders exceded 180 ng/ml and 21 of 29 nonresponders fell below 180 ng/ml ($p<0.01$). Twenty-five (60%) of the 42 non-delusional patients responded. Thirteen of the 17 nonresponders had concentrations below 180 ng/ml. When dosage increases raised the concentrations of the nonresponders above 200 ng/ml the response rate increased to 84%. Again, this study is flawed by a nonpecific analytical method.

Reisby et al. (23) published a study involving 66 hospitalized depressed patients treated four weeks with 225 mg/day of IMP. Thirty seven patients were classified as "endogenous" depressions and 29 as "non-endogenous" depressions. A one week placebo treatment period was used to exclude placebo responders from the study. They

found no statistically significant differences in response (as measured by HDS scores) between the two diagnostic groups. They measured IMP and DMI in plasma with a quantitative thin-layer chromatography method (24). Mean IMP concentrations for each patient during the last two weeks of drug treatment ranged from 6 to 268 ng/ml while mean DMI concentrations ranged from 20 to 675 ng/ml. The plasma level to effect relationship was clearest for the endogenous group as several non-endogenous patients responded at low plasma concentrations. They found all endogenous responders (N=12) to have plasma IMP levels greater than 45 ng/ml and plasma DMI levels greater than 75 ng/ml whereas 11 of 14 endogenous nonresponders had one or both compounds below these limits. Ten out of 12 responders had IMP + DMI levels above 240 ng/ml and all nonresponders had IMP + DMI levels below 240 ng/ml. This study was flawed by the analytical technique, thin layer chromatography, which is semi-quantitative.

In a report by Muscettola et al. (25), fifteen patients in a double-blind study with a 3 week placebo washout period were administered IMP in daily doses of 125 to 300 mg. IMP and DMI were measured in plasma and spinal fluid (CSF from lumbar puncture) by a gas chromatography-mass spectrometry (GC-MS) procedure with stable isotope dilution using deuterium-labelled analogues (26). Concentrations of both drugs in CSF were approximately 10% of plasma concentrations and the concentrations in the two body fluids were highly correlated (IMP r= .92, DMI r= .83). The mean of the ratios of IMP to DMI in plasma was 1.1 but in CSF it was 0.8, meaning that DMI entry into CSF was greater than IMP entry. The number of corresponding plasma and CSF samples was sixteen. For patients showing a clear antidepressant response, the mean

plasma drug levels (IMP plus DMI, 248 ng/ml) in responders was significantly greater than the mean plasma drug levels (186 ng/ml) in nonresponders. No serious flaws are noted in this study.

A more recent report (27) of a clinical study involved 27 inpatients diagnosed as suffering from a major depressive disorder and excluding patients with schizophrenia, organic mental disorders, medical illnesses or active substance abuse. No placebo washout was included. Patients received 100 mg/day of IMP the first week and 200 mg/day the final three weeks of the study. Patients were rated by the Kellner Physician Rating Scale (28), the Zung Self-Rating Depression Scale (23) and the Clinical Global Impression scale on days 0, 7, 14, 21 and 28. Plasma IMP and DMI were analyzed by a gas chromatographic technique (30). The authors state: "Our results provide little support for a clinically useful relationship between blood levels of IMP or its metabolite, DMI and clinical improvement. Various proposed threshold levels of IMP and DMI failed to consistently differentiate responders from nonresponders. Correlations between blood levels and degree of clinical improvement yielded only a few weak associations between certain measures of outcome and certain tricyclic levels." However, these 27 patients were spread amongst five different treatment centers, no placebo washout was used and the actual drug concentrations, mean drug concentrations or range of drug concentrations are not given. They do further state: "The recent observation that the 2-hydroxy metabolites of IMP and DMI may make a substantial contribution to the overall therapeutic effects may well mean that additional measures need be incorporated into future studies."

Another recently published report concerning plasma IMP levels and response in depressed children (31) showed good correlations. Twenty prepubertal children (ages 7 to 12 years) were hospitalized with major depressive disorders (DSM-III criteria) with no diagnosis of schizophrenia, attention deficit disorder, no history of drug abuse, an IQ greater than 85 and no prior antidepressant drug treatment. Severity of depression was assessed using a Clinical Global Impression Scale, Children's Depression Rating Scale (31a) and the Childhood Depressive Inventory (CDI) (32) self-rating scale. The same study included two weeks of in-hospital treatment and counselling without drugs, 3 weeks of treatment with 75 mg IMP at bedtime for children who did not remit after the initial two weeks and three weeks of additional treatment with IMP at an altered dose for those who did not respond to the 75 mg dose. The last treatment involved dosage reduction to 50 mg for children who had shown adverse side affects or increased dose to a maximum of 5 mg/kg if there were no side effects and no response. Plasma IMP and DMI concentrations were determined by high performance liquid chromatography (HPLC) with UV detection (33). They found response to IMP to be plasma concentration dependent. Of the children who achieved total IMP plus DMI plasma levels of 125 to 225 ng/ml, 92% responded compared to 25% responding outside this range. A curvilinear relationship was found with both total (IMP + DMI) and DMI versus response using two-degree polynomial regression analysis ($r = 0.72$, $p < .01$ for total versus response and $r = 0.56$, $p < .05$ for DMI versus response). They found no correlation between IMP concentrations alone and response. They determined that the relationship between plasma drug concentration and antidepressant response was determined primarily by the DMI concentration.

There have been good studies of the relationship of IMP and its demethylated metabolite, DMI, and clinical respose and other studies flawed by methodological problems. Still, the good studies have shown that a combined IMP plus DMI plasma concentration of roughly 150 to 350 ng/ml has caused a majority of patients to improve when those patients are carefully selected as those for which IMP therapy is potentially efficacious.

2. Desipramine Studies

There are fewer clinical studies comparing DMI plasma concentrations and clinical response. Friedel et al. (34) reported on 26 symptomatic volunteers who met research diagnostic criteria for major depressive disorders according to Frighner Criteria (35) for Primary Unipolar Affective Disorder and had at least 2 of 5 predictors for positive TCA response (36). Clinical response was determined by the Hamilton Depression Rating Scale. No placebo period was used. Patients received 100 mg of DMI each night in week one, 150 mg in week two and 200 mg in week 3. Subjects were evaluated with HDS on days 0, 7, 14 and 21. Blood was drawn on the same days 10 to 12 hours after the previous nights dose. Plasma DMI concentrations were measured by a GC-MS method (37). Sixteen of the 26 subjects (62%) were deemed responders (final HDS scores of less than 7 on 17 item scale) and 10 (30%) were classified as nonresponders. The mean and median plasma DMI concentrations on day 21 were 173 ng/ml and 79.9 ng/ml respectively. In this study it was found that subjects with plasma DMI levels below the median (79.9 ng/ml) were more likely to show improvement than were those above that level. The day 21 HDS score versus day 21 plasma DMI concentration had a correlation coefficient (r) of 0.365 ($p = 0.033$).

The authors state: "These data suggest that clinical response in subjects with symptoms of major affective disorder treated with DMI is related to DMI plasma levels and that at DMI plasma levels above 160 ng/ml, clinical response decreases significantly. There are not a sufficient number of subjects in this study with low DMI plasma levels to define with confidence the minimal effective plasma concentration of DMI needed to produce a therapeutic response, although it appears to be in the region of 40 ng/ml". They continue later: "Consequently, the data reported here on DMI support the contention that monomethylated tricyclics (38, 39) appear to inhibit clinical response at elevated plasma levels by an unknown mechanism". In this study, there was no significant difference in adverse side affects between responder and nonresponder groups which reduces the possibility of adverse effects at higher DMI plasma concentrations masking clinical improvement. The authors admit to two major flaws in their study, one the lack of placebo washout periods and two, the short (three week) duration of the study which may have resulted in a lower total response rate. The correlation coefficient ($r = 0.365$) that they did find is quite low as well.

The phenomenon of a decrease in responsiveness to monomethylated TCA as the plasma concentration exceeds a particular ceiling level has been postulated to be caused by increased adverse effects corresponding to these increased plasma concentrations, as well as other explanations (40). In a study of major adverse reactions during DMI treatment, Nelson et al. (41) addressed this possibility in 84 patients ranging in age from 20 to 80 years (mean 48 ± 18.4 years). Fifty received DMI alone and 34 also received antipsychotic drugs. Side effects were classified as major adverse reactions if they required

either discontinuation of DMI therapy or dosage reduction. Fifteen patients had major adverse reactions during the three week DMI trial. Symptomatic orthostatic hypotension developed in 6 patients, delirium developed in 5 patients, severe constipation in 2 patients, urinary retention in one, and one patient had periods of intense anxiety followed in seven days by a grand mal seizure. Major side effects developed in 11 of the 28 patients over 60 years old in contrast to 4 of the 56 patients under 60 years old. Plasma DMI concentrations were measured by gas chromatography with a nitrogen detector and 2-hydroxy DMI concentrations by reverse phase liquid chromatography (42). DMI plasma concentrations ranged from 31 to 683 ng/ml. DMI plasma concentrations in patients having side effects did not differ significantly from those in patients without side effects. Steady-state DMI plasma concentrations did not increase with age although side effects did. All side effects except orthostatic hypotension occurred in patients receiving antipsychotic medication as well as DMI. The concentration of 2-hydroxy DMI, the total concentration of 2-hydroxy DMI and DMI and the ratio of 2-hydroxy DMI to DMI were not higher in 11 patients having side effects than in a comparison group without side effects. Orthostatic hypotension occurred early in therapy and at low plasma DMI concentrations.

The most recent and complete study of plasma DMI concentration versus clinical response was performed by the same group as the adverse effect investigation, namely Nelson, Jatlow, Quinlan and Bowers (43). They used the DSM-III criteria (15) for major depression with melancholia to select 30 in-patients with depression. A one week hospitalized placebo washout period was included and patients having HDS scores lower than 18 after this week were not included in the 30

patients. The 24 item HDS was used to assess the severity of depression. Drug treatment lasted three weeks and responders were defined as those with HDS scores of 9 or less at that time. Patients received 2.5 mg/kg of DMI each day (range 100 to 300 mg/day). Blood samples were drawn prior to the first dose of the day. Plasma DMI concentrations were determined by GC with a nitrogen detector (34). Steady-state plasma levels for the 30 patients completing the 3 week treatment period ranged from 29 to 454 ng/ml (mean 112 ng/ml, median 82 ng/ml). A threshold plasma concentration of 115 ng/ml best separated responders and non-responders. In contrast to the Friedel study (29), 89% of the patients with plasma concentrations above 115 ng/ml responded whereas only 19% responded who had levels below 115 ng/ml. Ten initial nonresponders were converted to responders when dosage increases raised DMI plasma concentrations to 125 ng/ml or above. The rate of response in their sample during the first three weeks was only 37%. Initial low plasma concentrations of DMI and the relatively short time period probably reduced the response rate. The authors state that 125 ng/ml is a good lower limit for the therapeutic range for plasma DMI concentration. They do not present separate analysis of or a listing of high plasma DMI concentrations. This study is the best consideration of plasma DMI concentrations and clinical response to date. It shows a low limit of the therapeutic range for DMI concentrations of about 125 ng/ml.

Finally, there is one recent case report (44) concerning a patient (30 year old woman) who had simultaneous plasma DMI determinations (45) and HDS ratings during an emergency hospitalization. The plasma DMI concentration maximum was 1150 ng/ml on 200 mg/day of DMI and later decreased to 168 ng/ml on 50 mg/day. At high plasma DMI

concentrations, the patient consistently had severe depression, while her depression improved greatly when her plasma level was reduced and maintained in the "therapeutic range" that the authors suggested to be 150 to 300 ng/ml (46).

In his review of July, 1982 (14), Friedel states: "The only other TCA (besides nortriptyline) for which a reasonably clear relationship between plasma drug and metabolite levels and clinical response has been demonstrated is IMP... with a maximal effect most often occurring above 200 ng/ml of IMP plus its demethylated metabolite, DMI." There has not been shown a clear relationship between plasma DMI concentrations and clinical response as indicated by the contradictory findings of the two DMI studies sited here. Furthermore, the possible influence of other active metabolites, namely the 2-hydroxy metabolites of IMP and DMI, has not been studied in relation to clinical response.

B. Metabolism of Imipramine and Desipramine

Bickel and Weder (47) in 1968 published the results of an investigation of the total fate of IMP in male Wistar rats. They used a single dose administered i.p. and analyzed plasma, 18 organs and tissues, and 6 excreta for IMP and 18 metabolites over the course of seven days. Thin layer chromatography was used for the analysis, which must be considered semi-quantitative at best. A very large dose (50 mg/kg) was used. In plasma, IMP, DMI, imipramine-N-oxide, 2-hydroxy IMP glucuronide and 2-hydroxy desipramine glucuronide were detected. No unconjugated phenolic metabolites could be detected. They found that 90% of the total drug is excreted via the urine (2/3) and feces (1/3) within 24 hours. The major metabolites are DMI, IMP-N-oxide, 2-hydroxy

IMP(2HI), 2-hydroxy DMI (2HD), 2HI-glucuronide and 2HD-glucuronide. Eight other metabolites are formed in minor amounts only. The percent of total drug as major metabolites in the first 24 hour urine collection were given as: IMP (0.9%), DMI (6.5%), IMP-N-oxide (7.4%), 2HI (3.2%), 2HD (10.2%), 2HI-glucuronide (9.2%) and 2HD-glucuronide (27.6%).

Later Bickel and Minder (49) studied IMP and DMI metabolism and biliary excretion in rats with bile fistulas and in perfused rat livers (all male Wistar). Rats were injected i.p. with 50 mg/kg IMP. Nearly one third of the dose is excreted into bile during the first 3 hours, mostly as glucuronides of 2-hydroxy metabolites. Unchanged IMP excreted in bile is about 5%. In the perfused rat liver experiments, after three hours 88% of administered IMP is metabolized whereas for administered DMI, 57% is metabolised in 3 hrs. This difference in rate of metabolism accounts for the longer half-life and accumulation of DMI compared to IMP. Forty percent of DMI metabolites were hydroxylated and 14% was desmethyldesipramine (DMD). A small amount of IMP was detected. Their experiments showed that metabolism in the liver of IMP and DMI accounted for almost all metabolism in these rats.

Another study (49) with male Wistar rats demonstrated that brain and plasma levels of IMP and DMI and their area under the curve (AUC) values were much lower after oral than after i.p. administration. This is a first pass liver metabolism phenomenon.

Gillette et al. (50, 51, 52) compared rat and rabbit metabolism of IMP and DMI. In their first report (50), they stated that rabbits metabolize IMP rapidly with almost complete disappearance in 6 to 8 hours. Next (51), they compared rat and rabbit liver microsome's ability to metabolize IMP. Rat microsomes were half as active and demethylate

IMP mainly to DMI and slowly transform DMI to other metabolites. In contrast, rabbit liver microsomes rapidly metabolize both IMP and DMI and little DMI accumulates. They speculate that the main product of DMI metabolism is 2HD since little DMD was formed. Their *in vivo* experiments in rats and rabbits confirmed the *in vitro* microsome experiments. Rats rapidly demethylate IMP and DMI and slowly oxidize the DMI. Rabbits rapidly metabolized both IMP and DMI. New Zealand white rabbits and Sprague-Dawley rats were used. In rabbits, they determined a plasma half-life for DMI of one hour and for IMP of two hours (52).

They also incubated IMP with homogenates of rat liver, heart, lung, brain and kidney and found that only the liver preparation metabolized IMP. Microsomal and soluble liver cell fractions when combined metabolized IMP. DMI and 2HI were isolated as metabolites, but no 2HD was detected. This system metabolized DMI much more slowly. The same preparation from rabbit liver metabolized IMP and DMI efficiently and mainly by reactions other than demethylation (52).

The metabolism of IMP has been studied by Crammer, Scott, and Rolfe (53, 54) in humans. About 95% of the radioactivity representing IMP and its metabolites after a 5 μCi oral dose of ^{14}C-IMP was extracted from human urine. By thin-layer chromatography, autoradiography and various color tests over 20 metabolites were identified. In free plus conjugated forms, 2HD totalled up to 40%, 2HI up to 25% and 2-hydroxy iminodibenzyl up to 15% of all urinary metabolite excreted. DMD and its derivatives were present in only small amounts, while free iminodibenzyl and 10-hydroxy DMI each accounted for about 3% of the total metabolite excretion. About 40% of the radioactivity appears in the urine in the

first 24 hours and about 75% within three days. Glucuronides account for at least half of the urinary metabolites.

Gram and Christiansen (55) studied the first pass metabolism of IMP in man by administering the same test doses of ^{14}C-IMP by i.v. infusion and orally. Thin-layer chromatography was used for quantitation of IMP and DMI in plasma and IMP, DMI, IMP-N-oxide, 2HI, 2HD, 2HI-glucuronide and 2HD-glucuronide. Maximum IMP plasma concentrations were higher with i.v. than oral administration whereas DMI and other metabolite concentrations were greater following the oral route. First pass metabolism ranged from 23% to 71% for IMP. Non conjugated IMP, DMI, 2HI, 2HD and IMP-N-oxide were found in urine of all three subjects. In one subject, appreciable quantities of tentatively identified 2-hydroxy didesmethylimipramine were found. They found first pass metabolism to be mediated by demethylation (DMI production) but not by hydroxylation.

The metabolism of DMI in human fetal and adult liver microsomes was investigated by von Bahr et al. (56). Both adult and fetal preparations hydroxylated DMI to form 2HD. The hydroxylation was faster in the adult liver microsomes (comparison of 10 fetal versus 6 adult liver preparations). Much lower amounts of DMD were found compared to DMI and 2HD. Semi-quantitative thin layer chromatography was used for the analysis.

In 1982, Potter et al. (57) reported substantial quantities of 2HD and 2HI in plasma and CSF of depressed patients treated with IMP and DMI. In plasma of prepubescent boys and adults the concentration of unconjugated 2HI was 15% to 25% that of IMP and the concentration of unconjugated 2HD was usually about 50% that of DMI. They identified a subpopulation of about 5% who they classified as deficient DMI hydroxylators. DMI has routinely been measured in plasma of IMP patients for

a number of years.

C. Pharmacological Activity of Hydroxylated Imipramine and Desipramine.

After the discovery of 2HD and 2HI as metabolites of DMI and IMP in rats and humans, the question of possible pharmacological activity arose. They were compared to their parent drugs, IMP and DMI, which are known to be effective antidepressants. The antidepressant activity has been hypothesized to result from the enhancement of central monoaminergic function which is regarded to be deficient in depressed patients (58). The relative potencies of IMP and DMI to inhibit the uptake of different biogenic amines are different. DMI is a more potent inhibitor of norepinephrine uptake than is IMP. IMP is a more potent inhibitor of serotonin (5-HT). Both are relatively weak inhibitors of dopamine uptake (58). Javaid, Perel and Davis (58) examined the pharmacological potencies in the central nervous system of 2HD and 2HI, as determined by their relative abilities to inhibit the reuptake of biogenic amines in purified synaptosomes isolated from rat brain (male, Sprague-Dawley) and by the relative effects of spontaneous and forced motor activity in mice. The effect of the 2-hydroxy metabolites on motor activity of mice was of the same magnitude as IMP and DMI. In the biogenic amine reuptake experiments, 2HI had very similar inhibitory activities on norepinephrine, 5-HT and dopamine as IMP. 2HD had inhibitory activities similar to DMI. Basically, "hydroxylation of IMP and DMI at position 2 affected their inhibitory activities only slightly".

Another group of investigators, Potter, Calil, Manian, Zavadil and Goodwin (59), also assessed the activity of hydroxylated tricyclic antidepressants. They found that 2HD and 2HI inhibit the reuptake of

norepinephrine and serotonin into synaptosomes to the same extent as do their parent compounds. They also employed a classic test for antidepressant screening, the reversal or prevention of reserpine-induced syndrome in rats, and found 2HD and 2HI to be active. They further suggested that 2HD will contribute to the central nervous system (CNS) activity of administered IMP because the ratios of 2HD to DMI in plasma and CSF of rats were the same. Quantitation was performed by thin-layer chromatography and GC-MS. All evidence to date indicates that 2HD and 2HI are pharmacologically active and at the approximate potency of DMI and IMP, respectively.

D. Plasma Concentrations and Pharmacokinetics of IMP, DMI, 2HI and 2HD.

There are several recent reviews of TCA plasma concentrations and pharmacokinetics (4, 60). Very little information concerning hydroxylated metabolites was available when they were written. The pharmacokinetics of the tertiary amines and secondary amines, such as IMP and DMI, are well studied. The drugs are highly bound to tissue and protein so that they have a large apparent volume of distribution (V_D). Their metabolism, as noted, occurs almost entirely in the liver with high clearance rates and large interindividual variations in elimination half-life, clearance and steady-state concentrations. Peak parent drug concentrations are reached within 2 to 10 hours after oral doses and the drugs are rapidly distributed to most tissues, including the brain. Absorption of TCA can vary markedly and the amount of first pass metabolism, as noted earlier, varies widely. Only a small percentage of the drugs will be excreted into urine unchanged. In humans, the metabolic pathways are demethylation, dealkylation, N-oxidation and ring hydroxy-

lation with glucuronide coupling.

A bi-exponential equation, indicative of a two compartment pharmacokinetic model, can be used to describe plasma concentration versus time plot following a single i.v. administration. Drug concentrations in tissues such as liver, heart and brain are much higher than plasma concentrations. The elimination rate is low even though hepatic metabolism is efficient (30% to 70% extracted and excreted during each pass through the liver) because relatively little of the total drug in the body is in the blood. There is a large percentage of plasma protein binding of drug (usually greater than 80%) with albumin, α_1-acid-glycoprotein and lipoproteins (very low density and low density) being the principle components which bind. The mean post steady-state half-life for IMP is about 15 hours and for DMI is about 20 hours.

Reisby et al. (23) administered 225 mg/day of IMP orally to 66 depressed patients for 31 days each and found a range of 6 to 300 ng/ml for IMP and 15 to 700 ng/ml for DMI in their plasma samples. In the DMI study of Nelson et al. (43), oral doses of DMI ranging from 100 to 300 mg/day resulted in steady state plasma concentrations of DMI from 29 to 454 ng/ml. In both these studies, substantial improvement in depression was noted and the doses correspond to accepted pharmacotherapy. The accepted practice is to draw blood samples 10 to 12 hours after the last dose or just prior to a first dose in the morning. This practice is required due to variations in absorption (2 to 10 hours to peak concentration) of oral doses and equilibrium of distribution is "a prerequisite for obtaining a reproducible relationship between plasma and receptor level of a drug (61)."

Potter et al. (57) have studied steady-state concentrations of hydroxylated metabolites of IMP and DMI. IMP, DMI, 2HI and 2HD were analyzed using a HPLC assay (62) in plasma and CSF. In 32 males (ages 7 to 13 years) treated with 75 mg/day of IMP the mean steady-state plasma 2HI/IMP ratio was 0.23 and the 2HD/DMI ratio was 0.38. In 7 males (also ages 7 to 13 years) treated with 75 mg/day DMI the mean steady-state plasma 2HD/DMI ratio was found to be 0.43. Adults had similar ratios. They found that within an individual the ratio of 2HD/DMI in plasma was fairly consistent for multiple time points but there was great variation among different patients. They found a high positive correlation between plasma and CSF concentrations of 2HD and DMI and postulate that "under steady-state conditions patients who have a significant plasma concentration of 2HD relative to DMI will have an even greater relative concentration in the CNS."

DeVane, Savett and Jusko (63) looked at DMI and 2HD pharmacokinetics in four normal volunteers. A single oral dose of 50 mg of DMI was taken after an overnight fast. Timed blood specimens were taken over three days. An HPLC procedure (62) was used to measure plasma DMI and 2HD concentrations. For three subjects, 2HD appeared in plasma simultaneously with DMI and with greater concentration than DMI for the first two hours following administration. Peak concentrations of DMI appeared between 3 and 6 hours and of 2HD between 2 and 4 hours. The maximum plasma concentration of 2HD was 76% to 144% of that of DMI for the four subjects. The mean AUC of 2HD was 79% of the AUC for DMI. The decline in plasma concentrations were apparently parallel for 2HD and DMI in all subjects. The authors suggest that pre-systemic elimination (that is, first pass liver metabolism) of orally administered DMI occurs, at least

in part, through formation of 2HD.

Kitanaka et al. (64) investigated the relationship of steady-state plasma concentrations of 2HD and DMI in elderly depressed patients compared to younger patients. The ratios of 2HD to DMI were higher in elderly (67 to 85 years) patients than in younger patients due to increased plasma concentrations of 2HD in the elderly. The urinary clearance of 2HD decreased with age. "The known decrease in glomerular filtration with age may explain the selective increase of 2HD concentration in the elderly." They postulate that a primary means of elimination of 2HD is through renal excretion because urinary clearance of 2HD decreased with age and 2HD reached higher concentrations in the elderly.

E. Imipramine and Metabolites in Brain.

The so-called "blood-brain barrier" at the cerebral vasculature is made up of a continuous layer of endothelial cells which are connected by tight junctions as opposed to typical endothelial cell junctions which are not tight. Close intercellular connections prevent intercellular diffusion of water-soluble non-electrolytes, ions and proteins. Vascular transport is minimal at the cerebrovascular endothelium. These three properties, tight endothelial cell junctions, minimal endothelial cell vesicular transport and close intercellular connections, provide the "blood-brain barrier" which as a whole has properties of an extended lipoid cell membrane and is selectively permeable for lipid-soluble as opposed to water-soluble substances (65). Lipid-soluble agents equilibrate rapidly between brain and blood while less lipid-soluble drugs may be restricted in blood-brain exchange and have limited intracerebral distribution (66).

Rapoport, Ohno and Pettigrew (67) determined that cerebral capillary permeability was related linearly to the octanol-water partition coefficient for a wide variety of compounds. Deviations from their linear-regression line, although small, could be affected by other factors such as size, steric and electronic parameters and specific interactions with cell membranes. From knowledge of the octanol-water partition coefficient they were able to predict brain uptake of a drug from the history of the plasma concentration in experiments with rats.

Bickel and Weder (68) studied the partitioning of IMP, DMI, DMD, 2HI and 2HD between organic solvents and isotonic buffer solutions. The partition values were presented as the percent of the compound in the organic phase after equilibration with isotonic phosphate buffer, pH 7.4.

Compound	Chloroform	Hexane	Diethylether	Dichloroethane
IMP	99.8±0.8	99.4±1.4	99.3±1.2	98.5±1.4
DMI	98.5±1.6	65.1±2.1	88.4±1.5	97.8±1.8
DMD	99.1±1.8	70.5±2.3	-	-
2HI	97.4±1.5	25.2±2.0	84.2±1.7	71.0±1.9
2HD	55.2±2.1	6.3±2.0	55.6±2.6	50.2±3.1

Although octanol-water partitioning was not studied, these data indicate that 2HD, in particular, should have limited entry into brain. Bickel and Weder also qualitatively studied these compounds passage through the blood-brain barrier in male Wistar rats. The compounds were administered in high doses (generally 50 mg) i.p. and the rats scarificed after various times. Thin layer chromatography was used to detect IMP or DMI in homogenized brain extracts. After administration of 2HI and 2HD, rats were sacrificed 5 and 15 minutes, respectively,

after administration by i.v. and i.p. routes, respectively. They stated that 2HI was detected in brain while 2HD was not.

The question of the extent of entry of IMP and metabolites into the brain is important because these are centrally acting compounds (69). Specific high affinity binding sites for IMP have been found in both rat (70) and human (71) brain and for DMI in rat brain (72). In the rat brain study, the specific binding of ^{3}H-IMP was of high affinity (Kd=4.0 nM), rapid and reversible. It was inhibited by TCAs at nanomolar concentrations and by atypical antidepressants (iprendol, viloxazine, mianserin, and pirazidol) at micromolar concentrations. These binding sites were not detected in the heart and vas deferens and were unequally distributed between various brain regions (higher in cortex and hypothalamus than corpus striatum and lowest in cerebellum). Rats chronically treated with DMI for three weeks had significantly less specific ^{3}H-IMP binding sites in cortex than did control animals. They concluded that these binding sites may be important in the study of depression and of the mechanism of action of antidepressant drugs.

In membranes prepared from human brain (71), the high affinity binding sites (Kd=1.7 nM) were found. The binding of ^{3}H-IMP was saturable, reversible and inhibited by pharmacologically active TCAs but not by other psychoactive compounds or most neurotransmitter substances in concentrations up to 10 μM. They found the hypothalamus to have greater density of sites than the cerebral and cerebellar cortex. Tertiary TCAs had IC_{50} values (IMP, 6.5 nM; amitriptyline, 22 nM; chlorimipramine, 10 nM; doxepin, 120 nM) generally lower than secondary TCAs (DMI, 130 nM; nortriptyline, 170 nM). The IC_{50} is the concentration

necessary to inhibit 50% of specifically bound ^{3}H-IMP (2 nM) to human hypothalamic membranes. 2HI had an IC_{50} value of 50 nM.

Desipramine binding was studied in rat cerebral cortex membranes by Hrdina (72). He found a high affinity component (K_d=4.5 nM) and a low affinity component (K_d=80 nM). He found a different order of potency in displacing the high affinity DMI and IMP binding by various compounds tested. Nortriptyline and nisoxetine were the most potent DMI displacers and chlorimipramine and amitriptyline showed highest potency in competing for IMP binding sites. He suggested that the high affinity binding of DMI is distinct from that of IMP and most likely associated with sites involved in neuronal up-take of norepinephrine.

Practically all theories of TCA mechanism of action involve the brain as their site of action (69). The discovery of these high affinity binding sites reinforces that premise. If the site of action of IMP and DMI is in the brain, the distribution of drug to the brain is important. In humans, the concentration of the drug has been measured most often in the plasma. The relationship between plasma concentrations and brain concentrations of CNS active drugs should be of importance.

A quantitative study in the rat of the relationship between IMP levels in brain and serum was performed by Van Wijk and Kort (73). They used male Wistar rats treated for one day or 17 days with 10 mg/kg IMP i.p. twice daily. Radioactive IMP (^{3}H-IMP) in combination with unlabelled IMP was administered in various doses i.v. and rats were sacrificed at different time intervals post injection. Rats were decapitated and blood collected from the trunk. The brains were dissected into frontal cortex, left and right striatum, occipital

cortex, cerebellum and brain stem. Radioactivity was measured by scintillation counting and HPLC was used to quantitate some specimens. They found a virtually constant ratio between serum and brain levels of IMP. They found a ratio of 25 for brain to serum concentrations at steady-state. Equilibrium between serum and brain occurred in 30 minutes or less after i.v. administration.

There was little difference in concentration of IMP in the various brain areas in either subacute or subchronic administration. They found the uptake into the brain to be nonsaturable and serum protein binding did not limit the accumulation of IMP in the brain.

In an earlier study (74), Nagy investigated blood and brain concentrations of IMP and it's metabolite DMI after oral and intramuscular administration in male Sprague-Dawley rats. IMP and DMI were estimated by thin layer chromatography with direct densitometry. Doses were a single injection of 15 mg/kg or 10 mg/kg twice daily for 8 days. They found DMI concentrations to be about ten times higher in brain than blood and IMP concentrations to be about eight times higher in brain. The IMP concentration in red blood cells was an average 26% higher than in plasma. The DMI concentration in red blood cells was on average 47% higher than in plasma. The route of administration (oral or i.m.) did not affect the brain to blood concentration ratios.

Biegon and Samuel (75) compared brain concentrations of DMI after i.p. injection in male and female Wistar rats. They found the total amount of DMI in the brains of females to be 2 to 4 times that found in the brains of males after the same dose. In females the amount of DMI was highest on the day of estrus and lowest on proestrus. They also found only small differences in regional distribution for DMI in brain

(both male and female). Maximal brain concentrations were reached 30 minutes following i.p. injection. They did not measure blood concentrations and stated that sex-dependent liver metabolism may have caused the sexual differences in brain concentrations. Hrdina and Dubas (76) studied the pharmacokinetics and regional and subcellular brain distribution of DMI in male Sprague-Dawley rats after single and repeated i.p. administration. They found twenty times the plasma concentration in brain. "No significant regional differences were found in brain distribution or disappearance half-lives of the drug." DMI concentrations in cortex, striatum, hypothalamus and cerebellum were measured. The ratio of brain to plasma concentration remained steady between one and 48 hours after the last dose.

In 1973, Christiansen and Gram (77) reported the qualitative and quantitative study of the distribution of IMP and its metabolites in 13 different regions of the brain in a fatal case of IMP intoxication. Only IMP and DMI were quantitated using a thin layer chromatographic technique. Qualitative identification of 8 identified and 8 unidentified metabolites was made. A 23 year old man committed suicide by ingestion of an unknown amount of IMP and was found dead 12 to 24 hours later. Whether IMP had been taken regularly prior to suicide was not known. The blood concentration of IMP was 39 µg/ml. A trained neuropathologist removed the various brain tissues. IMP concentrations in brain ranged from 4 to 36 µg/g and were lowest in the white substance of the frontal lobe (4 µg/g) and cortex cerebelli (5 µg/g). Much less variation occurred in the other regions: frontal cortex (18 µg/g), temporal cortex (18 µg/g), thalamus (25 µg/g), caudate (23 to 28 µg/g), striatum (25 µg/g), mesencephalon (20 µg/g), pons (13 µg/g), nucleus amygdalae

(36 µg/g) and medulla oblongata (20 µg/g). DMI concentrations were one-third to one-fourth lower than IMP concentrations. Qualitative identification of 2HI was made in most regions. 2HD was found only in heart tissue and they state "this could be due to the difference in the concentration in the tissues".

A recent report by Pentel, Bullock and DeVane (78) of an IMP overdose included postmortem concentrations of IMP, DMI, 2HI and 2HD in brain and liver. Unfortunately, postmortem blood concentrations are not reported. Serum concentrations during hemoperfusion treatment prior to death were measured. A 25 year old male was brought to the hospital 1 hour after ingesting an estimated 5000 mg of IMP. At the time of admission, the serum IMP concentration was 1440 ng/ml and the metabolites DMI, 2HI and 2HD were all present in concentrations exceeding 100 ng/ml. Hemoperfusion was initiated and continued twenty six hours until the patient was declared dead. Final concentrations in serum are estimated as IMP (150 ng/ml), DMI (300 ng/ml), 2HI (50 ng/ml) and 2HD (140 ng/ml). The authors calculated that 1.8% of the total dose was removed by hemoperfusion. Postmortem tissue concentrations (96 hours after IMP ingestion and almost three days after declared death) were reported in ng/g as follows:

Compound	Brain	Liver
IMP	91	338
DMI	407	640
2HI	23	85
2HD	97	384

It is unfortunate that no postmortem blood concentrations were determined and that three days elapsed before tissue samples were

removed for analysis. However, positive identification of 2HD was made in human brain. Quantitation was made in serum and tissues of IMP, DMI, 2HI and 2HD using hexane extraction at alkaline pH and HPLC with fluorescence detection (62).

Tricyclic antidepressants are centrally acting drugs. It has been demonstrated that drug entry into brain is determined by lipid solubility of the drug. It stands to reason that more polar metabolites of drugs may be restricted to some degree from entry into the brain. The hydroxylated metabolite of DMI, that is 2HD, has been shown to have a smaller portion partition into organic solvents from water than IMP, DMI, DMD and 2HI. This is an indication that entry of 2HD into brain may be restricted compared to IMP and DMI.

F. Red Blood Cell Versus Plasma Drug Concentration

1. Antipsychotic Studies

In 1977, Garver, Dekirmenjian, Davis, Casper and Ericksen (79) reported that red blood cell concentrations of butaperazine, a piperazine phenothiazine used as an antipsychotic, were more strongly correlated with therapeutic response than were plasma butaperazine concentrations. RBC butaperazine concentrations could be used to define a "therapeutic window" above and below which favorable response diminished. The data were preliminary in nature and consisted of the study of ten patients who met Research Diagnostic criteria (15) for schizophrenia. All were maintained drug-free for 2 to 3 weeks after admission. Various doses of butaperazine were administered orally to each patient for 15 days or more. Patients were rated by the New Haven Schizophrenic Indices conducted daily. The percentage change in score

between day 0 and day 12 was used to determine clinical outcome. Blood samples were taken on days 5, 7, 9 and 12 and separated into plasma and RBCs by centrifugation. Butaperazine was quantitated in both specimens by a fluorometric method (80). Plasma butaperazine concentrations ranged between 40 and 321 ng/ml at steady-state. RBC butaperazine concentrations ranged between 10.8 and 124 ng/ml. The ratios of RBC to plasma concentrations varied considerably from patient to patient (0.11 to 0.66). A quadratic polynomial nonlinear equation derived by least squares showed that RBC levels fit better ($r = 0.90$) than did plasma concentrations ($r = 0.58$) versus therapeutic improvement. The authors remark:

"Since physicochemical distribution factors such as affinity, capacity, lipid solubility and transport govern passage of drug across the blood-brain barrier and the localization of neuroleptic at critical central nervous system sites, it would be of considerable interest if similar distribution factors governing the accumulation of neuroleptic in or on the RBC were operative. Although such distribution factors have not been studied systematically, there is now clinical evidence indicating that RBC and functional central nervous system butaperazine levels may parallel one another". They later add: "although clinical pharmacology has generally focused on plasma levels of drugs, we suggest that RBC levels of neuroleptics may provide better correlates of therapeutic response".

These researchers later reported a more extensive study involving 24 hospitalized schizophrenic patients (81). Again, butaperazine concentrations in RBC correlated significantly with clinical improvement in an inverted U-shaped pattern (similar to the nortriptyline therapeutic

window), whereas plasma levels of butaperazine were not significantly related to clinical response. There were large interpatient variations in both plasma and RBC butaperazine concentrations. This more complete study substantiated the preliminary report.

In 1982, Dahl, Bratlid and Lingjaerde (82) reported a study of plasma and erythrocyte levels of another antipsychotic drug, methotrimeprazine and two of its nonpolar metabolites. Heparinized blood samples were separated by immediate centrifugation for 10 minutes at 1500 x g. Drug and metabolite concentrations were quantitated by gas chromatography with a nitrogen detector. The plasma/erythrocyte ratio for drug and metabolites varied widely (approximately three fold for methotrimeprazine) among five patients and five volunteers. No clinical response data were included in this study.

2. Antidepressant Study

There is one limited study comparing plasma and erythrocyte TCA concentrations in depressed patients (83). Linnoila, Dorrity and Jobson studied steady-state drug concentrations in 59 depressed inpatients. Plasma and RBC drug levels were measured by extraction and gas chromatography (84). They found the ratio of DMI to IMP to be similar in plasma and RBC (n=11). The authors stated that patients administered DMI had similar plasma versus RBC correlations for DMI as did patients administered IMP although the number of DMI treated patients was small (n=4). Independent analysis of the DMI treated patients RBC vs. plasma correlations would seem to indicate a difference from IMP treated patients for plasma DMI versus RBC DMI concentrations. There was poor correlation ($r = .38$, n=11) between plasma IMP and RBC IMP concentrations. The authors conclude: "The interindividual

variation in the RBC-plasma tricyclic level ratios was large enough to warrant further clinical studies on the relationship between efficacy and pharmacokinetics of tricyclic antidepressants".

However, since this initial study of the variation in plasma and RBC drug concentrations variation there has been no further investigation. The only related report was published in December, 1982 by Schulz and Luttrell (85). They measured the plasma to whole blood ratio (P/B) and the plasma to erythrocyte ratio (P/E), as well as the free fraction in plasma of IMP and the concentration of α_1-acid glycoprotein in four normal subjects and five cancer patients. It has been reported that the binding of IMP in plasma increases in parallel with the concentration of α_1-acid glycoprotein (86), which is often elevated in subjects who have cancer or inflammatory diseases. Only one blood sample from each subject was analyzed. ^{3}H-IMP was added to the heparinized blood samples. The free IMP fraction in cancer patients (range 1.8% to 5.3%) was lower than in normal subjects (range 5.3% to 7.0%). The free IMP fraction was negatively correlated ($r = -0.90$) to the α_1-acid glycoprotein concentration. The P/E ratio for all nine subjects ranged from 0.80 to 2.03 (mean = 1.17) and was only slightly correlated to hematocrit ($r = -0.82$).

There is some evidence for antipsychotic medications that RBC drug concentrations may better correlate with clinical response than plasma concentrations. Interindividual variation exists in TCA distribution between plasma and RBC. Investigation of plasma and RBC TCA concentrations versus clinical response has not been investigated. It is not known whether RBC TCA concentrations may correlate better to clinical response than do plasma TCA concentrations.

G. Quantitation of Imipramine and Metabolites in Plasma and Tissues.

The tertiary and secondary amine tricyclic antidepressants have been quantitated in the plasma of depressed patients for over ten years. There have been several recent reviews of the methodologies employed (87, 88, 89). Analytical techniques that have been utilized include gas chromatography with nitrogen detector, gas chromatography-mass spectrometry and high performance liquid chromatography with various detectors. In the last several years, these analytical procedures have been improved so that gas chromatography-mass spectrometry (GC-MS) (90) and HPLC (91) procedures are available that are specific, sensitive and reproducible.

Interest in the quantitation of the 2-hydroxy metabolites of IMP has increased tremendously in the last three years. In early animal metabolism studies thin layer chromatographic methods were used that could be described as semi-quantitative at best. The first good quantitative method was published by Weder and Bickel in 1968 (92). For separation and quantitation, they used a gas chromatograph equipped with a flame ionization detector and an SE-30 six foot column. Metabolites of IMP were acetylated with acetic anhydride prior to injection. The real beauty of the paper is the description of blood and tissue extractions. They determined the partition coefficients of IMP and its metabolites (including DMI, DMD, 2HI and 2HD) between various organic solvents and aqueous buffers. Optimum pH values of the aqueous phases were determined for each compound. IMP and DMI were extracted at virtually 100% yield from aqueous buffers with pH above 8.5 by diethyl ether, 1,2-dichloroethane or n-heptane. 2HD had a narrow pH range for optimum extraction by both diethyl ether and 1,2-dichloroethane: 9.0 to

11.5. In this pH range these two solvents would extract 93% and 91%, respectively, of 2HD in 5 ml of aqueous phase using two 5 ml portions of solvent. Under identical conditions, a much more nonpolar solvent, n-heptane, would extract only 14% of the 2HD in the aqueous phase. These data compare favorably with the partition values between pH 7.4, isotonic, phosphate buffer and n-hexane or chloroform for IMP and metabolites given previously (68) as the percentage of compound in the organic phase:

Compound	n-Hexane	Chloroform
IMP	99.4	99.9
DMI	65.1	98.5
DMD	70.5	99.1
2HI	25.2	97.4
2HD	6.3	55.2

Chloroform is a more polar solvent than is n-hexane. It is noted that more polar solvents (diethyl ether, 1,2-dichloroethane) extract the 2-hydroxy metabolites, especially 2HD, much more efficiently than do very non-polar solvents (n-heptane, n-hexane).

A GC-MS procedure using solvent extraction was reported in 1981 by Narasimhachari, Saady and Friedel for quantitating IMP, DMI and 2-hydroxy metabolites (93). A very polar solvent, ethylacetate, was used at pH 9.0 for an initial extraction of plasma, followed by hexane: isopropanol (9:1) extraction at pH above 11. The d_4-derivatives of IMP, DMI, 2HI and 2HD were used as internal standards. N-methyl-bis-(trifluoroacetamide) was used to derivatize DMI, 2HI and 2HD prior to injection into the GC-MS system (90). Either a 3% OV-101 or 3% OV-17 (90 cm) column was used for separation, and selected ion monitoring of

fragment ions of d_4 and d_0 compounds was used for quantitation. This procedure is the most specific and accurate method for quantitation of IMP and it's metabolites published. The use of deuterated internal standards is especially effective.

The first report of an HPLC assay of IMP, DMI, 2HI and 2HD was that of Sutfin and Jusko (62). The chromatography was performed with 5 μm particle size, silica column (0.46 x 25 cm) and mobile phase of methanol-acetonitrile (1:5) with 4 ml per liter of ammonium hydroxide. A fluorescence detector (excitation at 240 nm, emission filter of 370 nm) was utilized for detection and quantitation. They used N-desmethyl chlorimipramine as an internal standard and extracted plasma samples which were made alkaline with ammonium hydroxide. The extraction solvent was 20% n-butanol in hexane. The HPLC retention times were: IMP (6 minutes), 2HI (6.9 minutes), DMI (14.4 minutes) and 2HD (17.4 minutes). The stated sensitivity of the procedure is 1 ng per ml of plasma for each compound. There are three problems with this HPLC assay. First, silica columns are not stable at alkaline pH with low molecular weight alcohols in the mobile phase without another source of silica such as a precolumn to saturate the mobile phase. Second, the extraction of 2HD by a nonpolar solvent mixture (20% n-butanol in hexane) is inefficient. Third, one of the compounds expected in low concentrations, 2HD, eluted last from the HPLC column (17.4 minutes) so that its peak height per amount injected will be lowest and, therefore, its sensitivity will be poorest.

This procedure was later improved (64) in several ways. First, carbonate buffer, pH 10.2, was substituted for ammonium hydroxide as the plasma alkalinizer. This change brings the pH for extraction closer to

the pH optimum for the extraction of the 2-hydroxy metabolites, as found by Weder and Bickel (92). Second, they substituted a more polar extraction solvent, hexane:ether (1:1) with 5% isobutanol, for the 20% n-butanol in hexane. This more polar solvent should increase the extraction efficiency for 2HD. They also doubled the solvent to aqueous phase volume ratio to increase the extraction efficiency. The HPLC mobile phase was also modified. The methanol content was reduced and butylamine (0.5%) was substituted for ammonium hydroxide (0.4%). The solubility of the silica stationary phase is reduced in this mobile phase.

Wong, McCauley and Kramer (94) developed an HPLC procedure for the quantitation of 2HD. This procedure required 2 ml of plasma and 2HI was used as internal standard. Carbonate buffer, pH 11, was used to adjust the aqueous phase pH before extraction with 10 ml of methylene chloride: isomyl alcohol (98:2). The organic phase was back extracted into 400 µl of pH 2.5 phosphate buffer. 350 µl was injected into the HPLC system. A 10 µm particle size C_{18} column (0.46 x 25 cm) and a mobile phase of phosphate buffer, pH 4.7: acetonitrile (75:25) were the separation system. A UV absorbance detector set at 254 nm was used to detect 2HD and the internal standard, 2HI. The flow rate was 2.7 ml/min and the column was heated to 43°C. The retention time for 2HD was about 8 minutes. They reported a percentage recovery of 2HD of 68% with their extraction procedure. Their procedure did not measure DMI or IMP, however.

Sackow and Cooper (95) developed an HPLC procedure using amperometric detection for IMP, DMI, 2HI, and 2HD. Plasma was extracted at pH 9.7 into ether, back extracted into 0.1N HCl and re-extracted into ether

following alkalinization. An ion-pairing reagent, heptane sulfonate, was used in a mobile phase of acetonitrile:acetate buffer, pH 4.2 (40:60) with a 10 μm C_{18} column (30 x 0.39 cm). A model TL-4A electrochemical detector (Bioanalytical Systems, West Lafayette, Ind.) was used at a potential of + 1.05 volts versus the silver-silver chloride reference electrode. 8-hydroxyclomipramine was the internal standard. The elution order was 2HD, 2HI, internal standard, DMI and IMP. The detection limit for each was 5 ng/ml using 1 ml of plasma. The extraction procedure they employed was a significant improvement over those used previously because the initial pH for extraction was near optimum and a polar solvent, ether, was used.

Quantitation of IMP, DMI and metabolites has steadily improved. However, a simple, accurate procedure for simultaneous quantitation of these compounds can be improved still. High performance liquid chromatography (HPLC) is a promising analytical method for this purpose when it is validated by a more specific method, i.e. gas chromatography-mass spectrometry (GC-MS).

H. Statement of Purpose

The first objective of this research is to develop a procedure for the simultaneous extraction and chromatography of IMP, DMI, DMD, 2HI and 2HD. High performance liquid chromatography is to be used routinely and the results produced are to be validated by an established GC-MS method. The HPLC procedure must be shown to be practical, accurate and precise.

IMP, DMI, DMD, 2HI and 2HD are to be quantitated in patient plasma and red blood cell specimens. It is to be determined which compounds accumulate in these specimens to significant concentrations. The plasma

and RBC concentrations are to be compared statistically to the degree of improvement in the patient's depression as measured by the Hamilton Depression Ratings.

Blood concentrations of the compounds will be compared to brain concentrations in autopsy specimens from human overdose fatalities and in animal models. The relative entry into the brain of significant metabolites found in blood will be compared to the parent drugs. The relative entry into brain of metabolites should help determine the significance of these metabolites because IMP and DMI are generally considered to have their site of action in the brain.

MATERIALS

A. Standards

Reference standards of imipramine, desipramine, 2-hydroxyimipramine, 2-hydroxydesipramine and desmethyldesipramine were gifts from Dr. A. A. Manian of the National Institute of Mental Health. Chlorimipramine was manufactured by Ciba-Geigy and obtained from the Medical College of Virginia Hospitals Toxicology Laboratory. Stock standards equivalent to 10 mg/liter of free base were prepared in methanol. Various working standards were prepared by dilution of stock standards with deionized water. Working plasma based standards were prepared by diluting stock standards in salvage blood bank plasma obtained from the MCV Hospitals Blood Bank.

B. Reagents

1. Buffers

Carbonate buffer, 10% and pH 9.7 was prepared by dissolving 10 grams of sodium carbonate and 10 grams of sodium bicarbonate in about 150 ml of deionized water. The pH was raised to 9.7 with 10 N sodium hydroxide. The volume was then brought to 200 ml with additional deionized water.

Phosphate buffer, 0.6 M and pH 6.5 was prepared by dissolving 16.6 grams of sodium phosphate, monobasic, monohydrate in about 180 ml of deionized water. The pH was lowered to 6.5 with concentrated

phosphoric acid. The volume was brought to 200 ml with deionized water.

2. Chemicals

Acetonitrile, methanol, hexane, ethyl acetate, isopropanol and methylene chloride were all either Waters Associates, Fisher or Baker HPLC grades or Burdick and Jackson distilled in glass grade.

Isoamyl alcohol was obtained from Fisher Scientific (A-393).

Triethylamine 99% was obtained from Aldrich Chemical.

Concentrated phosphoric acid, hydrochloric acid and 10 N sodium hydroxide were obtained from Fisher Scientific.

N-methyl-bis-(trithioroacetamide) was obtained from Regis Chemical Co. (270091).

Salts were all ACS reagent grade.

Sodium heparin was obtained from Riker Labs, Inc. (Lot 80862, expiration date Jan. 83) and was 10,000 units/ml.

C. Instrumentation

1. HPLC

The HPLC pump was either a Waters Model 6000A or a BIO-RAD Model 1330. The HPLC detector was either a Waters 440 Absorbance Detector or a Kratos Spectroflow 773 Absorbance Detector. The strip chart recorder was Houston Instruments D5217-2. A Waters Model U6K injector was used. The HPLC column was either a Waters RCM cyanopropyl cartridge (84636) with a RCSS Guard-PAK insert (85826) in a RCM 100 module or a Altex 5 μm cyanopropyl column (Ultrasphere, 242950) 15 cm x 4.6 mm.

2. GC-MS

Either a Finnigan model 4000 or a Hewlett-Packard Model 5985 GC-MS system was used.

3. Miscellaneous

Tissue homogenization was performed with a TRI-R STIR-R Model K43 (TRI-R Instruments).

The centrifuge was a Damon/IEC HN-SII model.

The pH meter was a Corning Digital 109 equipped with an A. H. Thomas Co. electrode (#4094 L15).

A Meyer N-Evap Analytical Evaporator (Organomation Associates) was used for solvent evaporation.

D. IMP and DMI Autopsy Specimens

All blood was drawn by cardiac puncture.

IMP Fatality: A 46 year old white male was found dead. Numerous (approx. 20) empty bottles of IMP were found at the scene. Decedent last seen four days prior to discovery of body. A note dated one day prior to discovery was found at the scene. The brain specimen analyzed consisted of approximately equal portions of cortex and midbrain.

DMI Fatility-I: A 23 year old white female was found dead. She had a history of mental illness. No other information was available. The brain specimen was cortex.

DMI Fatality-II: A 38 year old woman was taken to an emergency room after ingesting approximately 100 DMI tablets (50 mg) and 100 Ativane tablets. She died 36 hours after arrival. The brain specimen consisted of cerebellum and brain stem.

METHODS

A. HUMAN STUDIES

1. Depressed patients treated with imipramine

This study was conducted by the Department of Psychiatry, MCV-VCU in conjunction with the Upjohn Company. Dr. R. O. Friedel was the Chief Investigator. Patients were "symptomatic volunteers" who responded to a newspaper ad in which a self-rated version of the Hamilton Depression Scale (HDS) was included. Inclusion criteria (96) were:

a. Age 18 to 65 inclusive.

b. A Raskin Depression Scale score of 8, a HDS score of 18 and a Cori Anxiety Scale score equal to or less than the Raskin score. These three scales are standard psychiatric tools for assessing depression and anxiety.

c. At least 3 of the 5 positive indicators of tricyclic response defined by Bielski and Friedel (36): insidious onset, anorexia, weight loss, middle and/or late insomnia, and psychomotor retardation or agitation.

d. Depressive diagnosis.

e. Voluntary participation and informed consent.

Exclusion criteria were:

a. Patients who are psychopathic, sociopathic or psychotic.

b. Patients with any form of bipolar depression, or schizo-affective depression or "involutional" depression.

c. Significant liver or kidney disorder.

d. Patients with uncontrolled cardiovascular, pulmonary, endocrinological or collagen diseases or glaucoma or conditions where IMP is contraindicated.

e. Patients with a history of urinary retention, paralytic ileus and convulsive disorders.

f. Individuals known to be sensitive to benzodiazepines or TCAs or actively abusing alcohol or other drugs.

g. Patients requiring other psychotropic medication, hypnotics or analgesics containing narcotics.

h. Patients receiving anticholinergic drugs or preparations containing sympathomimetic amines.

i. Patients receiving guanethidine, propanolol, methyldopa or thyroid medications.

j. Patients who cannot read and understand the symptoms check list.

k. Females of child-bearing potential.

Patients in this study received IMP or alprazolam. The IMP dose was gradually raised to 225 mg per day over a two week period. They remained at that dose for another four weeks unless side effects required dosage reduction to the last well-tolerated dose. Prior to the start of medication, a one week placebo period was used to determine placebo responders. If the patient's HDS dropped below 18, they were considered placebo responders and were removed from the study. At the end of the weeks 1, 2, 4 and 6, most of the patients treated with IMP finished the entire six weeks of the study. Only plasma was analyzed for IMP and metabolites in some patients. All blood samples for drug analysis were drawn in the morning prior to the first dose of the day.

Patients were evaluated by HDS and other ratings and had blood drawn for drug analysis

Patients Treated with Imipramine

Name	Steady State IMP Dose (mg/day)	Sex	Age	RBC analysis
J-1	225	F	32	+
A-1	225	F	36	+
M-1	225	F	27	+
J-1	225	F	51	+
H-1	225	F	44	
D-1	225	M	31	+
C-1	200	M	56	+
J-3	200	M	22	
H-2	225	F	40	
H-3	225	F	34	
H-4	225	M	47	
E-1	225	F	41	
D-2	225	M	24	
B-2	225	F	23	
B-3	200	F	46	
B-3	225	M	51	
W-1	200	F	38	+
R-1	100	F	45	+
K-1	225	M	33	+
H-5	200	F	47	+
C-2	225	M	32	+
C-3	225	M	32	+
T-1	200	M	45	
A-2	200	F	29	+
N=24		M=9 F=15	Mean 37.7 ± 9.4	N=12

2. Depressed patients treated with desipramine

This study was conducted by the Department of Psychiatry, MCV-VCU in conjunction with the National Institute of Mental Health. Dr. R. O. Friedel was the Chief Investigator. Again, "symptomatic volunteers" recruited via a newspaper ad were the subjects. Inclusion criteria (97) were:

a. Age 18 to 60 inclusive.

b. Patient must sign informed consent form.

c. Patient must fulfill the Research Diagnostic Criteria for depression (15) and the Bielski and Friedel criteria (36) for response to tricyclic antidepressants.

Exclusion criteria were:

a. Reported heart, kidney, liver, metabolic or other medical illness.

b. Narrow angle glaucoma or symptomatic prostatic hypertrophy.

c. Patient taking thyroid medication.

d. Patient taking MAO inhibitors within 14 days, TCAs or antipsychotic drugs within 7 days, benzodiazepines, barbiturates, opiates or psychostimulants within 3 days or meprobamate or sedative/hypnotic medications within one day of entering study.

e. Patient having received electroconvulsive therapy within 2 months.

f. Patient is a female who is pregnant, nursing, or without reliable birth control.

g. Patient is receiving psychotherapy.

h. Patient is an alcoholic who has been dry less than 2 years.

i. Patient has had one or more distinct periods with predominantly elevated, expansive or irritable mood (mania) and patient has had one week duration during which, for most of the time, at least 3 of the following have persisted (4 if mood is only irritable) and have been present to a significant degree:

1. increase in activity (either socially, at work, or sexually) or physical restlessness.
2. more talkative than usual or pressure to keep talking.
3. flight of ideas or subjective experience that thoughts are racing.
4. inflated self esteem (grandiosity, which may be delusional).
5. decreased need for sleep.
6. distractibility.
7. excessive involvement in activities that have a high potential for painful consequences which are not recognized, e.g. buying sprees, sexual indiscretions, foolish business investments, reckless driving.

The 21 item Hamilton Depression Scale (HDS) was used to rate severity of depression. Subjects chosen to begin the study were treated one week with placebo. Those whose HDS scores dropped below 18 or dropped dramatically (for example, from 42 to 22) were removed from the study and referred for further treatment. All patient's doses were raised up to 300 mg per day of desipramine over a one week period and continued with 300 mg per day for another four weeks. At this point, one-half of the patients, chosen randomly, had their dosage reduced to an amount predicted to result in a 100 ng/ml plasma concentration of DMI. This dose was calculated by the following equation:

$$\text{New Daily Dose (mg)} = \frac{300 \text{ mg}}{\text{Vis 6 (ng/ml)}} \times 100 \text{ ng/ml}$$

Where Vis 6 (ng/ml) is the plasma DMI concentration of the blood sample drawn on visit 6 (visit 6 occurs after three weeks of DMI treatment) of the patient. This new dose is continued for three additional weeks. The other one-half of the patient population were continued on 300 mg/day for those three weeks. Each week after iniation of DMI therapy, the patients were evaluated with the HDS and other rating systems to determine clinical status and they had a blood sample drawn into a green top Venoject™ 10 ml tube containing 143 units of sodium heparin. These blood samples were drawn for drug analysis. The visits to the clinic were timed so that the blood sample could be taken 12 hours after the previous nights dose of DMI. All patients except EW #10 and WK #11 had drug analysis of RBC samples as well as plasma.

Patients Treated with Desipramine

Initials and Number	Sex	Age	New Daily Dose (mg) If Applicable
EW #10	M	38	
WK #11	F	34	
MJ #16	F	51	150 mg
CA #18	F	32	
JB #19	F	30	125 mg
GW #21	M	40	100 mg
MO #23	F	34	100 mg
SJ #24	F	23	
BB #27	F	37	250 mg
EM #28	F	41	
DB #29	F	35	75 mg
EW #32	F	29	100 mg
N=12	M=2 F=10	Mean 35.3 ± 6.7	

The patient blood specimens were labelled with the visit number, date and patient initials and number. The visits correspond to weeks after start of DMI therapy as follows:

Number of Weeks After Initiation of DMI Treatment	Visit Number
1	Visit 4
2	Visit 5
3	Visit 6
4	Visit 7
5	Visit 8
6	Visit 9
7	Visit 10
8	Visit 11

Patients in both the IMP and DMI studies were allowed to smoke and to drink alcohol in moderation.

3. Clinical status versus drug concentrations

Patients were evaluated for severity of depression by experienced psychiatric clinicians using the 21 item Hamilton Depression Scale (HDS; see Appendix II). For each patient, the HDS scores prior to placebo treatment and after one week of placebo treatment were averaged to determine the Severity Index. For IMP treated patients, the HDS score from week 6 of treatment was subtracted from the Severity Index to determine the Raw Improvement score. For DMI treated patients, the HDS scores from visits 7 and 8 (weeks 4 and 5 of treatment with DMI) were averaged and substracted from the Severity Index to produce the Raw Improvement score for each subject.

For the DMI treated subjects the following Pearson correlations (98) were determined:

1. Severity Index vs. Raw Improvement
2. Raw Improvement vs. Plasma DMI, Plasma DMI + 2HD, RBC DMI and RBC DMI + 2HD.

In addition, Partial correlations (99) between Raw Improvement and each of plasma DMI, plasma DMI + 2HD, RBC DMI and RBC DMI + 2HD were made controlling for The Severity Index. Spearman's rank difference correlations (100) were calculated for Raw Improvement vs. plasma DMI and RBC DMI.

For IMP treated patients, Pearson correlations were made for:

1. Severity Index vs. Raw Improvement.
2. Raw Improvement vs. plasma IMP, plasma DMI, plasma IMP + DMI, plasma IMP/DMI, RBC IMP, RBC DMI, RBC IMP + DMI and RBC IMP/DMI.

Human blood specimens were collected via venopuncture by trained medical personnel. Blood was drawn by evacuated Venoject^{TM}, 10 ml blood collection tubes containing 143 units of sodium heparin and immediately mixed by repeated inversion. The tubes were labelled with the patients name, the date, the study name and visit number when applicable. The blood samples were then immediately transported to the Clinical Psychopharmacology Laboratory (CPL). The time interval between blood drawing and receipt at the CPL was generally less than one hour.

Immediately upon receipt at the CPL, the plasma and red blood cells were separated. The conditions of separation were consistent. The blood tubes were spun in the outer slots (mid point radius of 4 inches) of the Damon/IEC model HN-SII centrifuge for 10 minutes at 3/4 full

speed (3000 x g). The plasma was removed with a 5 inch pasteur pipet and placed in a clean 16 x 100 mm borosilicate glass test tube and tightly sealed with a double layer of Parafilm™. The upper layer (approximately one quarter inch) which consisted of the left-over plasma, the white cells and the upper red cell layer was removed with the pasteur pipet and discarded. The packed red blood cells were left in the original Venoject tube and recapped with the original green rubber stopper. The plasma and RBC specimens were stored at -20°C until analyzed.

4. Overdose Fatality Studies

Whole blood and brain tissues from the overdose fatalities were removed during autopsy at the Chief Medical Examiner's office in Richmond, Virginia. All whole blood was obtained by cardiac puncture and placed in 16 x 150 mm glass tubes and capped with a cork. Brain was placed in waxed cardboard containers which were not air or liquid tight. Both specimens were stored frozen in the Bureau of Forensic Sciences laboratory specimen freezers. They were labelled with the deceased's name, the case number and type of specimen. Portions of the brain samples (see Materials) were dissected away and transferred to clean polyethylene tubes and transported to the CPL.

B. ANIMAL STUDIES

1. Rat

a. Chronic IMP and DMI treatment

A total of twenty male Sprague-Dawley rats (175 to 200 grams each) were used in this study. Each rat was injected i.p. with a five milliliter syringe and 20 gauge needle twice each day for five

days. The rats were divided into four groups of five animals each. For the four groups, each injection contained:

Group I	5 mg/kg	IMP
Group II	10 mg/kg	IMP
Group III	5 mg/kg	DMI
Group IV	10 mg/kg	DMI

The dosing solutions were 1 milligram of drug per milliliter of deionized water. All rats were considered to be 200 grams so that 5 mg/kg injections were 1.0 milliliter and 10 mg/kg injections were 2.0 milliliters. The rats were dosed each day between 8:30 to 9:30 a.m. and 8:30 to 9:30 p.m. The last dose was at 8:30 a.m. and the animals were sacrificed that day between 5:30 and 8:00 p.m. Blood was collected with sodium heparin (143 units in green top Venoject tubes) to reduce clotting and brains were removed within three minutes of decapitation. Plasma, RBCs and whole brains were analyzed for IMP, DMI, DMD, 2HI and 2HD for the IMP treated rats and DMI, DMD and 2HD for the DMI treated rats.

b. 2HD and DMI rat distribution study

Ten male Sprague-Dawley rats, each weighing 175 to 200 grams, were injected i.p. with 7.5 mg/kg of 2HD and 12.5 mg/kg of DMI in 2.0 ml of deionized water using a 5 ml syringe with a 20 gauge needle. Five rats were sacrificed by decapitation exactly 30 minutes after injection and the other five exactly 60 minutes after injection. Immediately, whole blood was collected in sodium heparin (143 units) containing tubes and the brains were removed. Plasma and brains were

analyzed for 2HD and DMI.

c. Specimen collection

Rats were sacrificed by decapitation and blood collected immediately from the torso through a funnel into uncapped 10 ml Venoject blood collection tubes containing 143 units of sodium heparin. The tubes were shaken during collection, then immediately capped and more vigorously shaken to mix the blood and heparin to prevent clotting. Approximately 2 to 4 milliliters of blood was collected from each rat. The entire collection time from decapitation to capping the tube was ten seconds or less.

The rat skull was then opened with scalpel and scissors and the entire brain removed and placed in a standard glass scintillation counting vial and the cap screwed on. The brain sample was then placed on ice. The entire process from decapitation to placing the brain sample on ice took approximately two minutes for each rat. Specimens were collected from a group of ten animals and transported to the CPL. Brains were stored in the scintillation vials at -20°C. Blood tubes were centrifuged exactly the same as described for human blood. Plasma was transferred to 10 mm x 50 mm borosilicate glass tubes, sealed tightly with a double layer of Parafilm and labelled with the animal identification number (as were the original Venoject tubes and the brain containers). The residual plasma layer (approximately 2 millimeters) was removed from packed red blood cell specimens and discarded. To each RBC sample was added 2.0 ml of deionized water for dilution and hemolysis and the tubes vortexed. The original green rubber cap was replaced. The rat plasma and diluted RBC samples were then immediately placed upright in a -20°C freezer. The longest period of time from

sacrifice to placement of plasma and diluted RBC samples in the freezer for any animal was 70 minutes.

2. Rabbit

a. Chronic DMI treatment

Five male New Zealand white rabbits, each weighing between 4 and 5 pounds, were injected twice a day with 5 mg/kg of DMI. Each injection was 2.5 ml of a 5 mg/ml solution of DMI in deionized water. The rabbits were injected i.p. with a 5 ml syringe and 20 gauge needle. They were dosed four days with the last injection at 8 a.m. Blood was drawn at 2 p.m. by cardiac puncture into a 10 ml syringe (with 21 gauge needle) containing 150 USP units of sodium heparin (15 µl of 10,000 U/mL). Brains were removed between 4:00 and 8:00 p.m. after anesthesia with methoxyflurane and CSF sample procurement. Plasma, RBC and brain samples were analyzed for DMI, and 2HD.

b. Expired rabbit studies

Three male New Zealand white rabbits, each weighing between 4 and 5 pounds, died within 24 hours of receiving a 10 mg/kg injection of DMI. The first rabbit that died on 12-15-82 had blood collected by cardiac puncture when he was discovered dead. No brain tissue was removed. The subsequent two rabbits that were found dead on 12-16-82 and on 12-20-82 had whole blood and brain specimens taken. All whole blood and brain samples were analyzed by HPLC for DMI and 2HD.

c. 2HD and DMI distribution study

Two male New Zealand white rabbits, each weighing approximately five pounds, were injected i.p. with 1 ml of deionized water containing 7 mg of 2HD and 12.5 mg of DMI with a 5 ml syringe and 19 gauge needle. Exactly 20 minutes after each injection, 10 ml of

blood was drawn by cardiac puncture into a 10 ml syringe containing 150 USP units of sodium heparin. Immediately after each blood sample, the rabbits were killed by air injection (40 ml) into the lateral ear vein. Approximately one minute of time elapsed between blood sampling and death. Immediately after the rabbits died, their brains were removed surgically. Approximately twenty minutes elapsed between death and complete brain removal. Plasma, RBC and brain specimens were analyzed for 2HD and DMI by the HPLC method.

d. Specimen collection

All rabbits were sacrificed by the injection of at least 30 milliliters of air into a lateral ear vein with a 20 milliliter plastic syringe and a 21 gauge needle. Blood specimens from live rabbits were obtained by cardiac puncture with a 1½ inch long, 19 gauge needle connected to a 10 milliliter plastic syringe containing 150 units of sodium heparin (15 µl of 10,000 units per milliliter). Ten milliliters of blood were drawn into the syringe. Within one hour the syringe was transported to the CPL. The needle was removed and the blood transferred to a 10 x 100 mm borosilicate glass test tube. All blood samples were centrifuged and plasma and RBC specimens separated.

Post mortem blood specimens were obtained by cardiac puncture after the chest was surgically opened. Three milliliter sterile syringes with 19 gauge needles were used. Approximately 1 to 1.5 milliliters of blood were obtained. After transport to the CPL, the blood was transferred to 10 mm x 50 mm borosilicate glass test tubes, sealed with a double layer of Parafilm, labelled with the date and rabbit identification number and stored at -20°C.

Brain samples were obtained as follows: immediately following death, the skin covering the top of the skull was slit from the nose to the back of the neck and then pulled to the sides. A bone saw was used to cut through the skull from between the eyes to the base of the neck and between the ears. The skull was then chipped away with a small pair of surgical pliers to expose the brain. The brain stem and larger nerves were cut where they entered the skull and the entire brain removed in one intact piece to a sterile six ounce polyethylene specimen container. The container was labelled with the rabbit's identification number and placed on ice. The brain removal process took approximately 5 to 10 minutes for each rabbit. The brains were stored in the specimen containers at -20°C in the CPL.

The first group of five rabbits for the desipramine metabolism and distribution study were anesthetized after the cardiac puncture for blood specimen and before sacrifice so that cerebrospinal fluid could be obtained by puncture of the cisterna magna and removal by syringe. Methoxyflurane was used as the inhalation anesthetic by spraying 5 ml of the solution on the walls of the glass chamber in which the rabbit was enclosed. The rabbits were killed while still anesthetized. The time between cardiac puncture and sacrifice ranged between 2 and 4 hours for this group of rabbits.

For all other rabbit studies, the time between cardiac puncture and sacrifice was less than one minute. Post mortem blood samples were obtained within 5 minutes of brain removal.

C. *IN VITRO* STUDIES

1. Plasma-RBC equilibration

Four 10 ml Venoject tubes containing 143 units of sodium heparin were filled consecutively by venopuncture from one human volunteer. By injection with a 50 µl Hamilton syringe through the rubber stopper was added 25 µl of aqueous DMI-DMD-2HD working standard (100 ng/µl DMI, 40 ng/µl each of DMD and 2HD) for a total of 2500 ng DMI, 1000 ng DMD and 1000 ng 2HD. The test tubes were placed in a 37°C water bath-shaker and shaken throughout the incubation. One tube was incubated 5 minutes, another 10 minutes, another 20 minutes and the last 60 minutes. Each tube was immediately placed in the centrifuge after removal from the water bath and spun 10 minutes at 3000 x g. The plasma and red blood cells were separated as described earlier. To the RBC fraction of each tube was added 5.0 ml of deionized water to hemolyze the cells and the tubes restoppered and vortexed 10 seconds. All plasma and RBC samples were labelled by the incubation time (5, 10, 20 or 60 minutes) and stored at 4°C overnight. The following day they were analyzed for DMI, DMD and 2HD.

2. Equilibration from RBC to plasma

Three 10 ml Venoject tubes containing 143 units of sodium heparin were filled consecutively by venopuncture from one human volunteer. Plasma was separated from the RBC as before. To each RBC fraction was added 25 µl of the DMI-DMD-2HD working standard (total 2500 ng DMI, 1000 ng DMD and 1000 ng 2HD), the tubes were restoppered and thoroughly mixed by repeated gentle inversion. The RBC fractions were then incubated 15 minutes at 37°C and remixed each 5 minutes. After this incubation, the plasma was returned to its original RBC fraction

and the blood mixed by repeated inversion. Each blood tube was incubated in a 37°C water bath-shaker for 20 minutes. The plasma and RBC were then reseparated and 5.0 ml of deionized water added to each RBCs fraction. The tubes were labelled as either 1,2 or 3 to identify the corresponding plasma and RBC samples. All were stored overnight at 4°C. The following day each sample was analyzed for DMI, DMD and 2HD.

3. Plasma-RBC distribution over time

One 10 ml tube of heparinized blood was drawn from the same volunteer one week, two weeks, three weeks, five weeks and eight weeks after the plasma-RBC equilibration study above. The same quantity of DMI-DMD-2HD working standard was added to each tube as in the equilibration study. The blood samples were incubated at 37°C with shaking for 20 minutes. Then, the plasma and RBCs were separated, 5.0 ml of deionized water added to the RBCs and the tubes labelled. The samples were stored at -20°C. After the eighth week sample, all were thawed as was the 20 minute equilibration sample. Each was analyzed for DMI, DMD and 2HD.

4. Saturation study

Five 10 ml tubes of heparinized blood were drawn consecutively by venopuncture from the same volunteer as in the previous studies. The tubes were labelled as 250, 500, 1000, 2000 and 3000 and were injected by syringe through the stopper with 25, 50, 100, 200 and 300 µl of DMI-DMD-2HD working standard solution respectively. All were incubated together for 20 minutes in a 37°C water bath with shaking. They were immediately removed and the plasma and RBC fractions separated. 5.0 ml of deionized water was added to each RBC sample. The samples were stored at 4°C overnight and analyzed for DMI the next day.

5. Normal population plasma-RBC distribution study

Fifteen volunteers had one 10 ml tube of heparinized blood drawn by venopuncture. There were eight males and seven females, five persons age 40 or over and 10 below age 40 and all appeared healthy. Each tube of blood was injected with 30 µl of DMI-DMD-2HD working standard solution. The blood samples were incubated 20 minutes in the 37°C water bath with constant shaking. They were then removed and centrifuged 10 minutes at 3000 x g. For each sample, the RBC volume height and total blood height were measured from the bottom of the tube. The plasma and RBCs were then separated and stored at 4°C overnight. All samples were analyzed the next day for DMI, DMD and 2HD and then re-frozen. Plasma was analyzed about a week later for total protein, albumin, cholesterol and electrolytes by the Technicon SMAC continuous flow analyzer by a qualified medical technologist at MCV Hospital Clinical Chemistry Laboratories.

6. Drug and metabolite stability

To 2 ml aliquots of heparinized whole blood from a normal volunteer were added IMP, DMI, DMD, 2HI or 2HD to make concentrations of 200 ng per milliliter. The blood aliquots were mixed by vortexing five seconds and then incubated at 37°C for one hour with shaking. All samples were then stored overnight at 4°C. One aliquot containing each drug or metabolite was analyzed the next day by the HPLC procedure. Another aliquot of each was stored at -20°C for eight weeks, then thawed and analyzed in the same manner.

D. ANALYTICAL METHODS

1. Plasma Extractions

a. SEP-PAK

All solutions were manually forced through the C_{18} SEP-PAK cartridges with a 10 ml glass syringe (101). The long end of the SEP-PAK is connected to the syringe according to the package insert. The solution flow rate through the SEP-PAK was approximately one drop per second (3 ml per minute) or slower. SEP-PAKs were activated by pushing 4 ml of methanol and then 5 ml of deionized water through them.

For each solvent to be tested, 1.0 ml of blood-bank, drug-free plasma containing 100 ng per ml each of IMP, DMI, 2HI and 2HD was placed in a clean 16 x 100 mm test tube. 200 ng of chlorimipramine tube. 0.5 ml of carbonate buffer, pH 9.7, was then added to each and all tubes were vortexed 5 seconds. The plasma solution was transferred to the 10 ml syringe with a 9 inch Pasteur pipet. Each sample mixture was then pushed through a new, activated C_{18} SEP-PAK cartridge. Each SEP-PAK was then rinsed with 2 ml of deionized water. The Pasteur pipet and 10 ml syringe were rinsed with deionized water between each sample. After all the plasma samples were eluted, the 10 ml syringe was rinsed with 10 ml of deionized water and 10 ml of methanol. The following solvent combinations were used to extract separate SEP-PAKs:

A. Ethyl Acetate

B. Hexane:Isopropanol (9:1)

C. Hexane:Isoamyl Alcohol (98.5:1.5)

D. Methylene Chloride

E. Hexane:Ethyl Acetate (2:1)

F. Acetonitrile

G. Methylene Chloride:Isopropanol (9:1)

H. Methylene Chloride:Ethyl Acetate (1:1)

I. Methylene Chloride:Hexane (1:1)

J. Methylene Chloride:Ethyl Acetate: Isopropanol (5:4:1)

K. Hexane:Ethyl Acetate:Isopropanol (5:4:1)

Each contained 1% isoamylalcohol except C. 4 ml of the solvent were transferred with a 9 inch Pasteur pipet to the 10 ml syringe connected to the SEP-PAK. The syringe plunger was inserted and the solvent slowly pushed through. The first five drops of liquid from the SEP-PAK were discarded. The rest of the solvent was collected in clean 5 ml conical glass tubes, one for each SEP-PAK. All extracts were then centrifuged for 5 minutes at 3000 x g. If any aqueous phase was noted on the bottom or top of the solvent, it was carefully removed and discarded with a clean Pasteur pipet. Tube F had no aqueous phase. All extracts were dried under a stream of nitrogen nitrogen in a 40°C water bath. All SEP-PAKs were then re-eluted with 4 ml of the same extraction solvent that was originally used for that SEP-PAK. These extracts were added to the first extract and were then evaporated under a stream of nitrogen in the 40°C water bath until they contained approximately 0.5 to 1 ml of solvent. They were vortexed several seconds to rinse the wall of the tube and evaporated completely. For each sample, the second extract was evaporated just prior to injection into the HPLC. They were redissolved in 50 µl of methanol, vortexed 15 seconds and 25 µl was injected. Prior to any sample injection and interspersed in the sample injections were 5 µl injections of a solution of IMP, DMI, 2HI, 2HD (each 2 ng/µl) and CMP (4 ng/µl). These standard injections were used to determine the absorbance (by peak height) of standard amounts of the

compounds and the retention times. The Waters Radial Compression Module with 10 μm cyanopropyl cartridge was used with a flow of 1.5 ml per minute of mobile phase (acetonitrile:methanol; water: 0.6 M Na H_2 PO_4: triethylamine, 400:300:300:6:1.2, pH 7.43). The detector was always set at 254 nm and 0.005 absorbance units full scale.

The extraction efficiences were determined by calculating the peak height in millimeters per ng of standard compound, then determining the amount of each compound injected from the extractions by their peak height. This amount was doubled because 50% of each extract was injected (25 of 50 μl) and then divided by 100 ng for the drug or metabolite (100 ng/ml plasma) or 200 ng for the internal standard. This ratio was multiplied by 100 to determine the extraction efficiency in percent.

b. SEP-PAK with back extraction

Plasma based standards containing IMP, DMI, DMD, 2HI and 2HD were made with each compound in concentrations of 20, 50, 100, 200 and 400 ng per milliliter. 1.0 ml of plasma standard, 500 ng of CMP (25 μl of 20 ng per μl of deionized water) and 0.5 ml of carbonate buffer were mixed by vortexing 5 seconds in a 16 x 100 mm test tube. Each was eluted through a new activated C_{18} SEP-PAK which was then rinsed with 2 ml of deionized water. Each SEP-PAK was extracted with 4 ml of hexane:ethyl acetate:isopropanol:isoamyl alcohol (5:4:1:0.1) as described in the previous section. The solvent was evaporated and the SEP-PAK was extracted with another 4 ml of the same solvent. The tubes were evaporated to 0.5 to 1 ml remaining, vortexed several seconds and evaporated completely. 100 μl of 0.1 N hydrochloric acid:acetonitrile (50:50) were added to each tube, which were then vortexed 10 seconds.

100 µl of hexane:isoamyl alcohol (98.5:1.5) were added to each and all were vortexed 30 seconds and centrifuged 5 minutes. The upper hexane layer was carefully and completely removed with a 100 µl Hamilton syringe and discarded. 50 µl of 0.6 M NaH_2PO_4, pH 6.5, were added to each tube and they were mixed by vortexing 5 seconds. 50 or 100 µl of this solution was then injected into the HPLC. The Waters Radial Compression Module with 10 µm cyanopropyl cartridge was used. The flow rate was 1.5 ml/min. The mobile phase was acetonitrile:water:0.6 M NaH_2PO_4:triethylamine (425:375:10:2, pH 6.50).

The peak heights measured at 254 nm and 0.005 AUFS were used for quantitation. Each drug or metabolite peak height was divided by the CMP peak height to obtain the peak height ratio (PHR). A standard curve for each compound was produced by plotting the PHRs versus standard concentrations on linear graph paper. Linear regression was used to determine the best line and the correlation coefficient (r) for each drug and metabolite.

Nine patient serum samples (six DMI treated and three IMP treated) and a control serum sample were received from the Clinical Toxicology Laboratory of the MCV Hospitals after they were analyzed there by a gas chromatography-mass spectrometry procedure using a solvent extraction (90). They were analyzed in the CPL the same day by the back extraction SEP-PAK procedure detailed above. A total of fourteen IMP and DMI values were compared by the two separate procedures. No 2HI, 2HD or DMD concentrations were available from the GC-MS analysis.

Plasma based standards were extracted and chromatographed with all unknown plasma samples. Each run of 4 to 8 unknowns included

at least three standards. Comparison of the PHRs for each drug and metabolite from one run to the next was made. The mean PHR, standard deviation and relative standard deviation (S.D. ÷ mean PHR x 100) was calculated for each compound at each standard concentration (20, 50, 100, 200 and 400 ng/ml). The composite slope, y intercept and correlation coefficient were calculated by linear regression for each compound.

c. Solvent extraction

Solvent extractions were done in 16 x 150 mm glass tubes with screw-on Teflon lined caps. The final extracts were evaporated in 5 ml conical bottom glass tubes. All glassware was washed with laboratory detergent, rinsed three times with tap water and three times with deionized water. It was dried overnight in an 80°C oven. All tubes used for extractions were rinsed with extraction solvent prior to use. All mixing of aqueous solutions and organic solvents was performed by tightly capping the extraction tubes and vortexing each tube twice for 15 seconds. The tubes were then centrifuged six minutes at 3000 x g. The organic solvent phase was then transferred with a 9 inch Pasteur pipet to the evaporation tube.

The evaporation of organic extracts was done under a stream of nitrogen in a 40 to 50°C water bath. They were always dried to about 0.5 ml of volume, vortexed several seconds and then evaporated completely. They were removed from the water bath and redissolved in the HPLC mobile phase.

The Waters RCM with cyanopropyl cartridge (10 μm) was used with a mobile phase of water:acetonitrile:methanol: 0.6M NaH_2PO_4:triethylamine (200:150:150:8:1), pH 6.5. The flow rate was 1.4 ml

per minute. The detector was set at 254 nm and 0.005 AUFS.

1.0 ml each of blood bank plasma standards containing 20, 50, 200, 400 or 800 ng/ml of IMP, DMI, DMD, 2HI and 2HD was placed in extraction tubes. 200 ng of CMP (10 µl of 20 ng/µl) and 0.5 ml of carbonate buffer were added to each. Each was extracted twice with 4 ml of ethyl acetate:hexane:isopropanol:isoamyl alcohol (10:9:1:0.2) (extraction solvent, ES). These extracts were transferred to appropriately labelled extraction tubes containing 2 ml of 0.5N HCl and vortexed. The organic upper phase was discarded. 1 ml of 2N NaOH and 4 ml of the extraction solvent (ES) were added to the acid and the tubes reextracted. The solvent was transferred to 5 ml conical tubes and evaporated. 50 µl of mobile phase were used to redissolve the extracts for HPLC injection. A DMI study patient plasma sample was analyzed in triplicate along with the standards.

Extraction efficiency for the standards was determined by comparison to injected unextracted standards. A standard curve was determined by linear regression from these standards and the peak height ratios to the internal standard peak. The concentration of DMI, DMD and 2HD was determined from the standard curves for the unknown plasma.

The HPLC system was the 5 µm Altex cyanopropyl column and mobile phase of water:acetonitrile: 0.6 M NaH_2 PO_4:triethylamine (600:400:20:2, pH 6.5). The flow rate was 1.0 ml per minute. The detector was set at 254 nM and 0.005 AUFS.

This procedure was used to assay most of the plasma unknown samples. 1.0 ml of plasma was used for analysis. At least three plasma based standards were analyzed with each batch of 3 to 9 unknowns.

Within-run precision was tested using plasma from DMI study patient MO #23 visit 10). This sample was analyzed six times in one batch for DMI, DMD and 2HD. Day to day precision was checked in three assays on separate days using DMI study plasma samples for a total of ten replications. In addition, the concentrations of 2HD, DMD and DMI in two plasma standards were calculated from linear regression derived standard curves in eleven consecutive DMI study assays. For each group, the mean, standard deviation and relative standard deviation were calculated.

Nine DMI study plasma specimens and three standards were analyzed for DMI and 2HD by gas chromatography-mass spectrometry to compare with the HPLC analysis. D_4 DMI and 2HD were used as internal standards. The samples were extracted identically as for HPLC analysis. After evaporation, 4 µl of N-methyl-bis-(trifluoroacetamide) was added to each tube for derivatization. The tubes were capped, vortexed 20 seconds and placed in an 80°C oven for 15 minutes. 2 µl of this solution were used for each GC-MS injection. The ions with mass to charge ratios of 208.1 (DMI), 212.1 (d_4DMI), 320.1 (2HD) and 324.1 ($d_4$2HD) were monitored. The retention time for DMI was 1.85 minutes and for 2HD was 2.67 minutes. The peak areas of the four ions were measured and used to calculate the peak area ratios (PARs) of d_0 compound to d_4 compound for DMI and 2HD. The PARs of standards versus the standard concentrations graph was used to determine unknown plasma sample DMI and 2HD concentrations. The HPLC and GC-MS results for DMI and 2HD were compared by graph and linear regression. The mean coefficient of variation was calculated for both DMI and 2HD.

2. Red blood cell extractions

Red blood cell fractions from 10 ml of heparinized blood were diluted with 5.0 ml of deionized water. The total volume was measured prior to analysis to determine the RBC volume. The total volume minus the 5.0 ml of water equals the RBC volume. The RBC volume divided by total volume is the RBC fraction. Diluting the RBC allowed easier pipetting and hemolyzed the cells.

The RBC fractions from 10 ml heparinized blood specimens from patients on placebo treatment were used to prepare standards. The DMI-DMD-2HD working standard solution (100 ng/µl of DMI and 40 ng/µl of DMD and 2HD) was added to clean, rinsed extraction tubes in 2.5 or 5.0 µl amounts. 2.0 ml of the placebo diluted RBC was added to each to make 250-100 ng/µl and 500-200 ng/µl RBC standards, respectively. For IMP patient samples, the IMP-DMI-DMD-2HI-2HD working standard (100 ng/µl IMP and DMI and 40 ng/µl 2HI, 2HD and DMD) was used in the same way. Four standards were used for each set of eight unknown RBC determinations.

To 2.0 ml of diluted RBC standard or unknown sample was added 1.0 ml of carbonate buffer and 250 ng of CMP (50 µl of 5 ng/µl in deionized water). This mixture was extracted twice with the ES. The extracts were combined with 2 ml of 0.5N HCl and extracted. The solvent was discarded and 1 ml of 2N NaOH and 4 ml of ES added to the acid. This mixture was extracted and the solvent transferred to an evaporation tube. After evaporation, the extract was redissolved in 50 µl of mobile phase (water:acetonitrile: 0.6M NaH_2PO_4:triethylamine, 550:450:20:2, pH 6.5) which was acidified to pH 4.0 with 0.5N HCl. 25 µl were injected into the HPLC system.

All RBC assays were analyzed with the 5µm Altex cyanopropyl

column. The mobile phase flow rate was generally 0.8 to 1.2 µl/min. The detector was always set at 254 nm and 0.005 AUFS. Peak height ratios and linear regression were used to calculate unknown sample concentrations.

Within-run precision was determined by analyzing a spiked placebo RBC sample. It contained 500 ng/ml DMI, 200 ng/ml DMD and 200 ng/ml 2HD. Four replicates were analyzed along with two standards.

Eight RBC specimens were analyzed for DMI and 2HD by the HPLC procedure and by a gas chromatography-mass spectrometry method. The GC-MS method substituted d_4 DMI and d_4 2HD as the internal standards instead of CMP. D_4 DMI and d_4 2HD were prepared according to the method of Narasimhachari (93).

The samples for GC-MS analysis were extracted just as the HPLC samples. After evaporation, the GC-MS samples were dissolved in 40 µl of the N-methyl-bis- (trifluoroacetomide) by vortexing 20 seconds. They were capped and heated in the 80°C oven 15 minutes. 2 µl were injected in the GC-MS. Peak area ratios of d_0 compound to d_4 compound were used in the calculations. PAR versus standard concentration graphs were used to determine the unknown sample DMI and 2HD concentrations. HPLC values and GC-MS values were compared by graphing and by calculating the coefficient of variation for each pair of values. A mean coefficient of variation (C.V.) was determined for DMI and 2HD values.

Day to day variations in the HPLC procedure was calculated by assaying six samples on two separate days. These were DMI study samples and were analyzed for DMI, DMD and 2HD. All RBC drug and metabolite concentrations were adjusted to ng/ml of RBC by dividing by the RBC fraction.

3. Brain extraction

All brain specimens were thawed prior to homogenization. Rat brains were transferred completely to a Wheaton 15 ml glass homogenization tube. For rabbit brains, sections from cortex and midbrain were combined in roughly equal proportions to yield approximately 1.5 to 2.0 grams. Human brain samples were taken from whatever brain region was available (see Materials section) to yield approximately 2 grams of tissue. All brain samples were transferred to a clean homogenization tube that was placed on a Mettler model PB300 pan balance. The weight of brain in grams was multiplied by four to determine the volume in milliliters of deionized water which was added to the sample in the homogenization tube. This resulted in a one to five dilution of the brain sample with water. The tissues were homogenized with a TRI-R Instruments Model K43 homogenizing apparatus by setting the motor at 1000 RPM and repeatedly grinding the tissue against the bottom of the tube with the spinning Teflon rod until no sign of intact tissue remained and the solution appeared homogenous. The entire mixture was then transferred to an extraction tube, which was labelled, capped and stored at 4°C if to be analyzed within two days or at -20°C if not to be analyzed in the next two days.

For the first analysis of all brain specimens, 1.0 ml of the mixture was extracted. The mixture was mixed by vortexing prior to pipeting the aliquot to be analyzed. For those samples that required repeat analysis due to very high drug or metabolite concentrations (over 5000 ng per gram of brain), the mixture was remixed and a portion was diluted with three parts deionized water (a further one to four dilution) and one milliliter of this mixture was used for analysis.

Standards were made in the identical dilution (one to five or one to twenty) as those of the brain specimens. Brain tissue from drug free rats was used for standards for all brain (rat, rabbit or man) assays. The same working standards as those described in the RBC extraction section were added to clean extraction tubes and 1 ml of blank rat brain dilution was added. Working standards were chosen according to the drug and metabolites to be analyzed in the brain specimens. Standard concentrations were chosen to approximate the range of compound concentration expected in the specimens. For example, rats treated with DMI had their brains analyzed in conjunction with DMI-DMD-2HD working standard (100 ng DMI/µl and 40 ng each DMD and 2HD/µl). A wide range of standard concentrations was used to cover the expected brain specimen concentrations. For example, assay of brains from 5 mg/kg DMI treated rats included seven brain based standards. The two brain specimens from rats treated with 2HD and 2HI were analyzed with standards made with the 10 ng/µl solutions of 2HD and 2MI. Rabbit and rat brains from 2HD and DMI treated animals were assayed with standards made from the DMI-DMD-2HD working standard.

To 1.0 ml of each diluted brain specimen and standard was added 1 ml of carbonate buffer and 250 ng of CMP. These mixtures were each extracted twice with 4 ml of ES. The extracts were combined in another extraction tube with 2 ml of 0.5N HCl. These tubes were extracted and the solvent phase discarded. The acid phase had 1 ml of 2N NaOH added to it and was then extracted with 4 ml of ES. The solvent extract was evaporated and redissolved in 50 µl of HPLC mobile phase. 25 µl was then injected into the HPLC system.

All brain specimens were analyzed on the 5 µm Altex cyanopropyl HPLC column. The mobile phase was water:acetonitrile:0.6M NaH_2 PO_4:triethylamine (550:450:20:2, pH 6.50) with a flow rate of 1.0 ml/minute. The detector was set at 254 nm and 0.005 or 0.010 AUFS. Peak heights were used for quantitation. Peak height ratios (PHRs) of drugs or metabolites to the internal standard were compared to standard curves to calculate the unknown concentrations.

Two brain specimens, one from a rat treated with 10 mg IMP/kg (Number 10-4) and one from the rabbit treated with DMI and 2HD and sacrificed 12-22-82 and designated #3, were analyzed for 2HD by GC-MS. 1.0 ml of 1 to 5 diluted homogenized brain specimen or standard (made with DMI-DMD-2HD working standard) or blank was transferred to appropriately labelled extraction tubes. A solution of d_4 2HD prepared by the method of Narasimhachari (93) was checked for d_0 2HD contamination and d_4 2HD concentration as follows: 10 µl of unknown concentration d_4 2HD were transferred to an extraction tube. 1 ml of deionized water, 1 ml of carbonate buffer and 2 ml of ES were added and the tube extracted. The solvent was removed to an evaporation tube and dried. 40 µl of N-methyl-bis- (trifluoroacetamide) was added and the tube vortexed 20 seconds, capped and placed in an 80°C oven for 15 minutes. 2 µl were injected into the Finnigan GC-MS system. The ions for d_4 2HD (324.1) and d_0 2HD (320.1) were monitored and the peak areas determined. The same 10 µl quantity of the d_4 2HD solution was added to each brain sample aliquot and 1 ml of carbonate buffer was added to each. They were extracted identically as in the HPLC method. Evaporated samples were derivatized with N-methyl-bis- (trifluoroacetamide). Again 2 µl injections were used for GC-MS analysis. The same ions were monitored

and peak areas measured.

4. Overdose fatality whole blood and brain by GC-MS

Whole blood and brain specimens from the three overdose cases were analyzed by solvent extraction and gas chromatography-mass spectrometry. One to five brain dilutions and one to ten diluted and undiluted blood specimens were the samples used. 1.0 ml of each was extracted. The DMI overdose samples were analyzed for DMI, DMD and 2HD. The IMP overdose samples were, in addition, analyzed for IMP and 2HI. Appropriate quantities of d_4 DMI, d_4 2HI and d_4 2HD were used as internal standards. One to five diluted, homogenized blank rat brain was used to prepare standards for brain assays. Whole blood and one to ten diluted, whole blood from placebo treated patients were used to prepare standards for the blood assays. The extractions were identical to those described in the diluted RBC and brain extraction sections. Evaporated extracts were redissolved in 40 µl of N-methyl-bis- (trifluoroacetamide), vortexed 20 seconds, capped and heated fifteen minutes in the 80°C oven. Two injections of 2 µl of each sample were required to monitor all the ions required for analysis. The ions and the retention times for the compounds in question were:

Compound	Ion	Retention Time (minutes)
IMP	234.1	1.65
d_4 IMP	238.1	1.65
DMI	208.1	3.48
d_4 DMI	212.1	3.48
DMD	208.1	2.73
d_4 2HI	350.1	1.72
2HI	346.1	1.72
d_4 2HD	324.1	3.95
2HD	320.1	3.95

The area of each peak was measured. The ratio of the area of the d_0 compound to its corresponding d_4 compound area was the peak area ratio for each drug or metabolite in each sample. The peak area ratio for DMD was obtained by dividing the DMD area by the d_4 DMI area. The PARs of standards versus the standard concentration graph were used to determine drug and metabolite concentrations in the brain and whole blood overdose specimens. Concentrations were then adjusted for the dilution factor. For example, brain samples diluted one to five with deionized water had their concentrations multiplied by five to yield nanograms per gram of brain.

5. HPLC quantitation

Initially, a Waters Associates Radial Compression Module™ containing a Waters Associates 10 μm particle size 10 cm diameter cyanopropyl cartridge (No. 84636 Radial-PAK) was used for the separation of the drugs, metabolites and internal standard. A variety of mobile phase solutions were tested for their separation characteristics. These solutions contained varying proportions of methanol, acetonitrile and deionized water. They also contained 1 to 2 milliliters of triethylamine and 20 milliliters of 0.6M NaH_2 PO_4 per liter of solution. The mixtures were adjusted to pH 7.0 with 10% phosphoric acid or 2N NaOH.

A mobile phase solution containing acetonitrile:methanol:water:0.6M NaH_2 PO_4:triethylamine (400:300:300:20:1.2) was adjusted to pH 7.0 with 10% phosphoric acid using a combination electrode and pH meter. While using this mobile phase, a standard solution containing CMP, IMP, DMI, 2HI and 2HD (2 ng per μl each except CMP, 4ng per μl) was injected into the system on day 1 and day 4. The retention time and peak

height for each compound were determined.

One liter of mobile phase solution was adjusted to pH 7.52 with 10% phosphoric acid and 150 ml removed. The pH was adjusted to 7.07 with several drops of 10% phosphoric acid and again 150 ml was removed. This procedure was repeated to produce 150 ml portions of mobile phase of pHs 6.98, 6.67 and 5.82. Each aliquot of different pH was then divided into 50 ml portions. For each different pH solution, a 1.0 µg per ml solution of CMP, DMI and 2HI was made by adding 50 µl of 1 mg/ml stock standard in methanol. A Cary Model 118 UV-visible spectrophotometer was then used to measure the absorbance of each solution at 254 nm with a slit width of .24 nm. The absorbance versus the pH of solution for each compound was graphed.

A mobile phase containing acetonitrile:water:0.6M NaH_2PO_4: triethylamine (530:450:20:2) and adjusted to pH 6.50 was used because of good separation of the compounds of interest. The flow rate used was generally 1.5 ml per minute. Retention times and K' values were determined for each compound using this mobile phase and the 10 µm cyanopropyl radial compression cartridge. This high performance liquid chromatography system was used almost every day for several months. Slight variations in the proportion of acetonitrile to water and the concentration of triethylamine were made during this time as the efficiency of the cartridge decreased. A standard mixture containing 5 ng per µl each of IMP, DMI, DMD, 2HI and 2HD and 10 ng per µl of CMP was injected before each days samples. The amount injected was either 5, 7.5 or 10 µl using a 25 µl HPLC syringe. The peak height ratios of each drug and metabolite to the internal standard, CMP, were determined for thirteen injections of this mixture over a period of two weeks. The mean,

standard deviation and relative standard deviation of the peak height ratios for each drug and metabolite were calculated.

An Altex Ultrasphere™ cyanopropyl HPLC column (5 µm particle sixe, 150 x 4.6 mm, Beckman Instruments No. 242950) replaced the radial compression module several months after the study was initiated. The mobile phase which was found to produce good separation of the compounds of interest contained acetonitrile:water:0.6M NaH_2 PO_4:triethylamine (420:580:20:2) and was always adjusted to pH 6.5 ± 0.05. Again, slight variations in the acetonitrile and the triethylamine concentrations were made during the six months this column was utilized. The flow rate was 0.8 to 1.2 ml per minute. The retention times and K' values of IMP, DMI, DMD, 2HI, 2HD and CMP were determined at a flow rate of 0.8 ml per minute.

6. Gas Chromatography-Mass Spectrometry

The gas chromatography-mass spectrometry was performed on a Finnigan Model 4000 or a Hewlett-Packard Model 5985 system by Dr. N. Narasimhachari or Mr. J. Saady, respectively. The extraction and derivatization of samples are described in a previous section. The complete description of the GC/MS parameters has been published by Narashimhachari, Friedel and Saady (90, 93).

RESULTS

A. Analytical Procedures

High performance liquid chromatography (HPLC) was used to separate and quantitate imipramine, desipramine, desmethyldesipramine, 2-hydroxyimipramine and 2-hydroxydesipramine. Silica, C_{18} reverse phase and cyanopropyl reverse phase HPLC columns were evaluated for the separation of the compounds of interest. Cyanopropyl reverse phase columns gave the best resolution and the sharpest peaks for these compounds. Chlorimipramine was chosen as the internal standard because of its structural similarity to imipramine and metabolites and its separation on the HPLC system from imipramine and metabolites. UV detection at 254 nm was chosen because the instrumentation was readily available and the detection limit of 2 ng of compound injected was adequate for these studies. Gas chromatography- mass spectrometry (GC-MS) was used as necessary to validate the HPLC procedures because of its specificity and its accuracy when deuterated analogs of the compounds of interest are used as internal standards.

The extraction of the compounds from biological fluids and tissues was first performed using C_{18} SEP-PAK^{TM} cartridges from Waters Associates. Table 1 shows the extraction efficiencies determined for a number of different elution solvent mixtures. As shown in the far right column, some mixtures yielded more interfering substances than others when the extracts were chromatographed. From these data, the mixture of hexane:

TABLE 1

Extraction Efficiencies from C_{18} SEP-PAKs for Drugs and Metabolites Using Different Elution Solvents

Elution Solvent	Extraction Efficiencies (%) 2HD	2HI	DMI	IMP	Interferences
Ethyl Acetate	40.4	85.5	33.3	65.0	+
Hexane : Isopropanol (9:1)	67.0	74.6	48.3	68.3	++
Hexane : Isoamyl Alcohol (98.5:1.5)	3.2	25.4	38.3	74.9	+
Methylene Chloride	46.3	75.1	95	88.3	+++
Hexane : Ethyl Acetate (2:1)	20.2	6.4	16.7	68.3	0
Acetonitrile	16.0	74.0	16.7	58.3	+
Methylene Chloride : Isopropanol (9:1)	107	79.3	60.8	87.0	+++
Methylene Chloride : Ethyl Acetate (1:1)	12.7	32.2	51.2	89.1	+++
Methylene Chloride : Hexane (1:1)	0	50.6	60.8	91.3	++
MeCl : Ethyl Acetate : Isopropanol (5:4:1)	59.8	64.2	68.1	90.4	++
Hexane : Ethyl Acetate : Isopropanol (5:4:1)	68.4	78.2	75.3	91.1	0
Retention Time (minutes) on HPLC	8.8	9.7	12.9	14.8	
K' Value (V_0 = 1.3 min)	5.7	6.3	9.1	10.2	

[1]Scale of zero to +++, with +++ most chromatography interferences.

ethyl acetate: isopropanol (5:4:1) was chosen for further C_{18} SEP-PAK™ extractions because of good extraction efficiencies for the compounds of interest and the absence of interfering peaks in the chromatograms.

The chromatography of evaporated residues of direct SEP-PAK extracts caused deterioration of the HPLC column and a marked loss in its efficiency. This may have been due to some elution of packing material from the SEP-PAK which would then be injected into the HPLC or from non-polar plasma constituents which would be extracted and injected into the column. To remedy this situation, a back extraction procedure was developed. Table 2 shows the extraction efficiency, chromatography data and parameters of the standard curve for 2HD, 2HI, DMD, DMI, IMP and CMP using this SEP-PAK extraction with back extraction procedure. The extraction efficiencies are slightly lower with the back extraction than without it. For example, the extraction efficiency for 2HD with back extraction (Table 2) is 63.0% and without back extraction (Table 1 elution solvent hexane : ethyl acetate : isopropanol [5:4:1]) is 68.4%. Similar decreases were found for each compound. Table 2 also shows the standard curve slopes, y intercepts and correlation coefficients for 2HD, 2HI, DMD, DMI and IMP using five standards for each. All correlation coefficients (r) were greater than 0.99. The acid back extract had to be buffered with phosphate buffer because injection of an acidic sample into the buffered HPLC mobile phase changed the chromatography of the compounds. Table 3 shows the effect of the HPLC injection sample acidity on the chromatography. The stronger the acidity (the greater the number of mEq of HCl per injection) the shorter the retention times of the compounds and the larger the PHR to CMP. The pH

TABLE 2

C_{18} SEP-PAK and Back Extraction Procedure

	2HD	2HI	DMD	DMI	IMP	CMP
Extraction Efficiency (%)	63.0	76.9	79.2	70.6	83.9	82.8
Retention Time (min)	7.8	8.7	9.7	11.7	14.3	16.8
K'(V_o = 2.4)	5.9	6.7	7.5	9.3	11.6	13.8
Linear Regression Analysis of each compound's standard curve:						
y intercept	-0.02	-0.02	0.03	0.03	0.01	
Slope	0.0039	0.0040	0.0044	0.0028	0.0030	
Correlation Coefficient (r)	0.999	0.999	0.999	0.997	0.9999	
Number of Standards	5	5	5	5	5	
(20, 50, 100, 200 and 400 ng/ml)						

TABLE 3

Effect of HPLC Injection Sample Acidity on Chromatography

Sample Acidity in mEq HCl injected	2HD	2HI	DMI	IMP	CMP
A. 0 mEq					
Retention Time	9.3	10.8	15.7	18.8	23.3
Peak Height	83	72	41.5	34	39
PHR to CMP	2.13	1.85	1.06	0.87	
B. 0.001 mEq					
Retention Time	9.2	10.4	15.0	17.5	22.8
Peak Height	94	85.5	50	38.5	45.5
PHR to CMP	2.07	1.88	1.10	0.85	
C. 0.005 mEq					
Retention Time	8.8	9.9	14.2	16.3	20.7
Peak Height	86	78	44.5	36.5	38.5
PHR to CMP	2.23	2.03	1.16	0.95	
D. 0.01 mEq					
Retention Time	8.3	9.3	13.4	15.3	19.3
Peak Height	106	105	56.5	45.5	42
PHR to CMP	2.52	2.50	1.35	1.08	

Retention Time in m.m. from injection point on chart (chart speed 127 m.m./min). Peak height in m.m.

of the back extract was buffered to pH 6.5 by the addition of phosphate buffer, 0.2 M and pH 6.5 as described in Methods. This solution was then injected into the HPLC without changes in retention of the compounds when compared to aqueous standards.

The apparent acidity of the mobile phase was also important. The absorbance of the compounds changed with the pH of the mobile phase as indicated in Figure 2. The original mobile phase apparent pH was 7.0. The absorbance of all compounds tested (2HI, DMI and CMP) changes as the pH changes at 7.0. This effect was noticed in the variation in compound absorbance over a three day period as shown in Table 4. For these reasons, a more stable portion of the absorbance versus mobile phase apparent pH curve was chosen for chromatography, that is, mobile phase pH 6.50.

The SEP-PAK HPLC procedure was compared to an established GC-MS procedure for the quantitation of IMP and DMI in patient samples. Table 5 contains this comparison data. When HPLC data was plotted on the X axis and GC-MS data on the Y axis, the Y intercept of the best fit linear regression line was 4.9, the slope of the line was 0.986 and the correlation coefficient (r) was 0.976 for the 14 data points. Table 6 consists of data showing the day to day variation in this C_{18} SEP-PAK back extraction HPLC procedure. Five standards of varying concentrations (20 to 400 ng/ml) which were extracted and chromatographed over several weeks time were used to determine a composite standard curve. The mean peak height ratio (PHR) and relative standard deviation were calculated for each compound in each standard mixture. The slopes and y intercepts for the composite standard curves are given at the bottom of Table 6. The correlation coefficients for all compounds are shown to be

Figure 2. The Effect of Mobile Phase pH on the Absorbance at 254nm of 2HI, DMI, and CMP

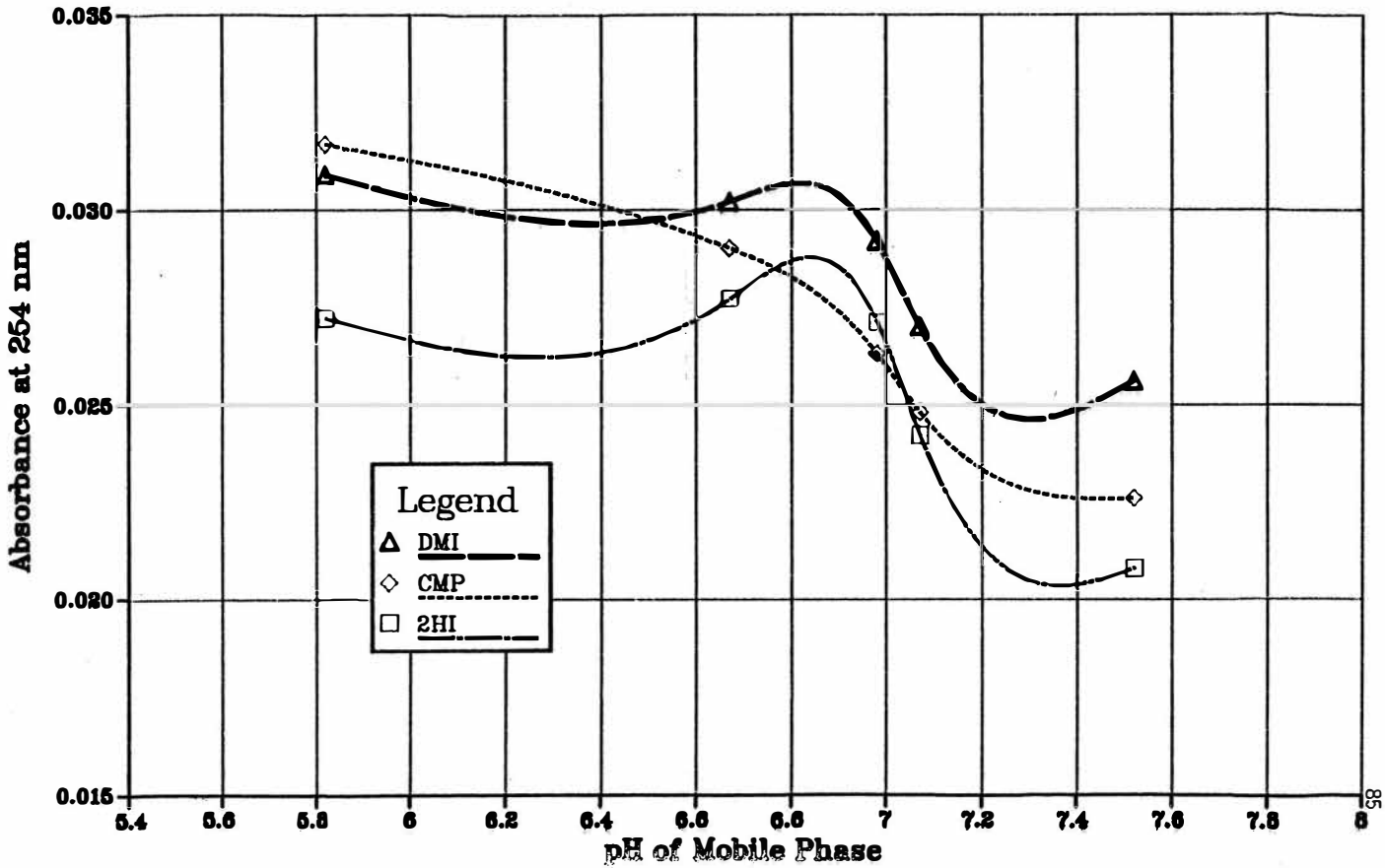


TABLE 4

Absorbance (254 nm) Variation of Standards with pH 7.0 Mobile Phase of Acetonitrile: Methanol: Water: 0.6M NaH_2PO_4: Triethylamine (400:300:300:20:1.2).

	2HD	2HI	DMI	IMP	CMP
DAY 1					
Retention Time (min)	8.3	9.1	12.3	13.7	16.0
Peak height (mm)/ng	2.37	2.17	1.52	1.39	1.82
DAY 4					
Retention Time (min)	8.2	9.0	12.0	13.8	15.7
Peak height (mm)/ng	2.25	2.10	1.25	1.225	1.19
Per Cent Change in Absorbance	5.1%	3.2%	17.8%	11.9%	34.6%

TABLE 5

SEP-PAK HPLC Versus GC-MS Quantitation of IMP and DMI in Patient Plasma Samples. All ng/ml Plasma

Sample	SEP-PAK HPLC		GC-MS	
	IMP	DMI	IMP	DMI
Clin. Tox. #157	137	306	126	334
Cin. Tox. #149		66		60
Clin. Tox. #759		165		182
C.C.C. 3/8/82	39	62	44	99
TCA Control	103	92	100	90
Clin. Tox. #758		49		65
Props		95		121
Clin. Tox. #442	210	281	188	270
Clin. Tox. #393		121		180
Clin. Tox. #339		64		47

Correlation Coefficient (r) for IMP and DMI (N = 14)	0.976
y intercept (GC-MS data as y axis)	4.9
slope	0.986

TABLE 6

Day to Day Variation in C_{18} SEP-PAK Back Extraction HPLC Procedure

	2HD	2HI	DMD	DMI	IMP
20 ng/ml std. (N)	9	9	9	9	9
Mean PHR	.044	.065	.080	.081	.071
Relative S.D. (%)	37%	35%	21%	31%	27%
50 ng/ml std. (N)	12	12	12	12	12
Mean PHR	.153	.172	.238	.180	.172
R.S.D. (%)	43%	30%	24%	28%	15%
100 ng/ml std. (N)	12	12	12	12	12
Mean PHR	.316	.400	.496	.347	.349
R.S.D. (%)	38%	40%	20%	23%	19%
200 ng/ml std. (N)	13	13	13	13	13
Mean PHR	.669	.759	.928	.664	.639
R.S.D. (%)	27%	21%	11%	14%	10%
400 ng/ml std. (N)	8	8	8	8	8
Mean PHR	1.37	1.56	1.73	1.19	1.21
R.S.D. (%)	14%	14%	8%	13%	7%
LINEAR REGRESSION					
Std. curve data					
y intercept	-.02	-.01	.02	.03	.02
slope	.0035	.0039	.0043	.0030	.0030
Correlation coefficient (r)	.9998	.9998	.9988	.9982	.9992

greater than 0.99. There were problems in the C_{18} SEP-PAK back extraction procedure. The cartridges are expensive and the extraction of chlorimipramine was variable, especially with a later lot number of cartridges. Several procedures were attempted to improve CMP extraction reproducibility, including double extraction at two buffer pHs, without total success. At this point, a three step solvent extraction procedure was developed. Plasma samples were made alkaline with 10% carbonate buffer, pH 9.7 and extracted twice with 4 ml of ethyl acetate : hexane : isopropanol : isoamyl alcohol (10:9:1:0.2). These extracts were backed extracted into 2 ml of 0.5 N hydrochloric acid. The acid portion was made alkaline with 1 ml of 2 N sodium hydroxide and then extracted with 4 ml of the extraction solvent. The solvent extract was then evaporated. The residue was redissolved in HPLC mobile phase for injection into the HPLC system.

Table 7 shows the HPLC retention times for the compounds on the 5 µm Altex cyanopropyl column with the mobile phase listed, the extraction efficiency for each compound using the 3 step solvent extraction, the slopes and y intercepts for the standard curves for each compound and the mean concentration with relative standard deviation (S.D.) of the compounds assayed in triplicate in a patient sample. The standard curve data are comparable to that found using the C_{18} SEP-PAK back extraction procedure. Again, all r values exceed 0.99. The patient sample assayed in triplicate was reproducible for 2HD (relative S.D. of 7.6%) and DMI (relative S.D. of 4.1%). The reproducibility of the DMD value (relative S.D. of 26.6%) was poor due to the low concentration of DMD (13 ng/ml). This analysis procedure on HPLC was the basic method used to analyze fluids and tissues in the studies documented here.

TABLE 7

Three Step Solvent Extraction and Chromatography on 5 μm Altex Ultrasphere Cyanopropyl Column.
Extraction Solvent: Ethyl Acetate : Hexane : Isopropanol : Isoamyl Alcohol (10:9:1:0.2)

	2HD	2HI	DMD	DMI	IMP	CMP
Retention Time (min)	5.9	7.1	8.0	9.6	11.8	15.6
Extraction Efficiencies (%)						
50 ng/ml plasma std.	56	70	66	85	86	106
200 ng/ml plasma std.	78	85	86	94	89	95
400 ng/ml plasma std.	79	80	83	94	81	97
Mean	71	78	78	91	85	99
Linear Regression Data for these standards						
y intercept	-.074	-.008	-.034	-.028	.027	
slope	.0103	.0093	.0102	.0098	.0068	
Correlation Coefficient (r)	.9989	.998	.9985	.9996	.9976	
CA #18 5-27-78 Plasma Sample analyzed in triplicate						
Mean (ng/ml)	80	Not	13	321	Not	
Relative S.D. (%)	7.6%	Detected	26.6%	4.1%	Detected	

Table 8 contains the precision data (intra-run and inter-run) for plasma analysis. The Methods section describes the studies in detail. The intra-run precision for 2HD and DMI (C.V. values of 8.7% and 11.9% respectively) was approximately equal to the inter-run precision (C.V. values of 10.8% and 10.0%, respectively). Standard curves were run with every assay. The mean values for two of the plasma based standard mixtures are presented in Table 8 also. Table 9 compares HPLC and GC-MS results for DMI and 2HD. The comparison of DMI results (C.V. of 5.55% and r = 0.980) is better than that for 2HD results (C.V. of 14.2% and r = 0.941). It was found that the quantitation of 2HD, 2HI and DMD was not as precise as that of IMP and DMI. Lower concentrations and lower extraction efficiencies of 2HD, 2HI and DMD may cause this phenomenon.

The same 3 step solvent extraction and chromatography on the Altex 5 μm cyanopropyl column were used for RBC analysis as well. Table 10 contains the precision data and standard curve data for RBC analysis. Table 11 contains the HPLC versus GC-MS quantitation results comparison of DMI and 2HD in RBC specimens. The precision data is good, with all coefficient of variations below 6% for 2HD and DMI. HPLC and GC-MS compared very well as the correlation coefficients for DMI and 2HD were 0.972 and 0.969, respectively.

The chromatography system reproducibility was checked over a two week time period by injection of a standard mixture. The peak height ratio of each compound to the internal standard (CMP) was calculated. Table 12 consists of these data. The chromatography was found to be consistent as indicated by the low relative standard deviations. The Altex 5 μm column was used instead of the Waters radial compression module (RCM) because it was more efficient as indicated in Table 13.

TABLE 8

Precision Data: 3 Step Solvent Extraction and
HPLC Analysis with 5 µm Altex Ultrasphere Cyanopropyl Column

	2HD	DMD	DMI
Intra-Run Precision	52	0	48
MO #23 Visit 10 (N = 6)	54	6	74
ng/ml	51	5.5	66
	38	3.4	54
	47	4	64
	51	8	67
Mean	48.8	4.5	62.2
Standard Deviation	5.8	2.5	8.6
Coefficient of Variation	8.7%		11.9%
Inter-Run Precision			
DMI Study Plasma (N = 10)			
Coefficient of Variation	10.8%		10.0%
DMI Study Assays (N = 10)			
100-40 ng/ml STD Mean	37.6	41.8	106.6
Standard Deviation	4.5	5.5	8.1
Relative S.D. (%)	12%	13.2%	7.6%
400-160 ng/ml STD Mean	156.1	160.8	401.7
Standard Deviation	8.8	14.0	19.9
Relative S.D. (%)	5.6%	8.7%	5.0%

TABLE 9

Comparison of HPLC and GC-MS Results for DMI and 2HD.
Both Three Step Solvent Extractions. All ng/ml Plasma.

S A M P L E		DESIPRAMINE HPLC	DESIPRAMINE GC-MS	2-HYDROXYDESIPRAMINE HPLC	2-HYDROXYDESIPRAMINE GC-MS
MO	Visit 7	198	224	62	65
BB	Visit 5	227	197	119	81
GW	Visit 9	570	555	10	13
EM	Visit 5	290	263	108	119
SJ	Visit 10	399	340	141	160
SJ	Visit 9	372	308	208	180
MO	Visit 6	283	253	140	107
MO	Visit 11	67	66	28	37
SJ	Visit 11	338	341	Not measured	
Coefficient of Variation (%)		5.55%		14.2%	
Correlation Coefficient (r)		0.980		0.941	

TABLE 10

HPLC Quantitation of RBC Samples from DMI Study: Precision Data.

	2HD	DMD	DMI
Inter-run Variation DMI Study Samples (N=12) (Coefficient of Variation (%)	5.72%	10.8%	5.54%
Intra-run Variation DMI Study Control Coefficient of Variation (%)	2.65%	2.15%	3.04%
Mean Linear Regression Data for Standard Curves for all RBC DMI Assays (N=20)			
y intercept	-.007±.040	.0036±.0034	.0318±.0578
slope of regression line	.0031±.0012	.0043±.0009	.0050±.0009
Correlation Coefficient (r)	.9924±.0081	.9975±.0031	.9977±.0042

TABLE 11

Comparison of HPLC and GC-MS Results for DMI and 2HD in RBC Specimens (ng/ml)

S A M P L E	DESIPRAMINE HPLC	DESIPRAMINE GC-MS	2-HYDROXYDESIPRAMINE HPLC	2-HYDROXYDESIPRAMINE GC-MS
SJ Visit 3	53	48	16	15
SJ Visit 4	103	114	61	54
SJ Visit 5	299	287	371	341
SJ Visit 6	302	278	126	138
SJ Visit 7	402	380	192	287
SJ Visit 8	394	462	398	384
SJ Visit 9	424	369	382	376
SJ Visit 10	448	449	339	365
Coefficient of Variation (%)	4.25%		5.52%	
Correlation Coefficient (r)	0.972		0.969	

TABLE 12

Peak Height Ratios (PHRs) of Standard Mixture Injections into HPLC Over Two Week Period

	2HD	2HI	DMD	DMI	IMP
	.687	.540	.632	.531	.436
	.645	.544	.560	.520	.446
	.677	.567	.589	.521	.440
	.629	.510	.609	.500	.406
	.707	.610	.638	.568	.477
	.644	.537	.660	.534	.430
	.673	.572	.644	.557	.456
	.645	.555	.632	.529	.439
	.665	.567	.629	.545	.455
	.654	.560	.626	.526	.438
	.637	.541	.619	.513	.421
	.630	.536	.607	.509	.421
	.626	.526	.607	.493	.404
Mean	.6553	.5512	.6194	.5266	.4361
S.D.	.0249	.0250	.0257	.0213	.0204
R.SD %	3.80%	4.54%	4.15%	4.05%	4.67%

TABLE 13

Comparison of Column Efficiency : Radial Compression Module (RCM) Versus 5 μm Altex Column

RCM	2HD	2HI	DMD	DMI	IMP	CMP
V_1	12.4	14.8	17.0	19.8	24.2	28.7
W	2.3	3.3	3.5	3.8	4.0	5.0
N	465	322	377	434	586	527
HETP	0.215	0.311	0.265	0.230	0.171	0.190
Altex						
V_1	12.7	14.2	17.4	20.8	24.6	33
W	1.4	1.5	1.5	1.5	1.5	1.8
N	1317	1434	2153	3077	4303	5370
HETP	0.114	0.105	0.070	0.049	0.035	0.028

V_1 = Peak retention in mm; W = peak width in mm; N = number of theoretical plates; HETP = height equivalent of a theoretical plate.

The number of theoretical plates (N) was substantially higher with the Altex column for the compounds of interest. For example, N was 586 for IMP on the Waters RCM and 4303 on the Altex column.

B. *In Vitro* Studies

These *in vitro* studies were performed to standardize techniques to be performed on patient samples and to investigate the distribution of DMI, DMD and 2HD between plasma and RBC. The first study, as documented in Table 14, investigated the time necessary for DMI, DMD and 2HD to equilibrate between plasma and RBC after their addition to blood. It is evident that equilibrium is reached quickly and that 10 minute incubation is sufficient. All other distribution studies used 20 minute incubations.

It was then investigated whether equilibration from RBC into plasma differed from equilibration from plasma to RBC. The compounds were added to RBC first and then the plasma was returned to the RBC. Table 15 shows the distribution in RBC and plasma in this study. The mean recovery in percent expresses the amount of compound found in the RBC and plasma specimens compared to the total added to the blood originally. The ratio of plasma to RBC concentrations (P/RBC) is given in Tables 14, 15 and 16 to allow direct comparison of the data. Comparison of the mean P/RBC values in Tables 14 and 15 show that little difference was found for DMI (0.664 and 0.691) and DMD (0.554 and 0.591). There was a greater difference for 2HD when 2HD was added to whole blood (0.569) or to RBCs alone first (0.410). It appears that more 2HD associates with RBCs when it is added to the RBCs first.

TABLE 14

Equilibration of DMI, DMD and 2HD Between Plasma and RBC with Time.

Specimen	DMI ng/ml	DMI P/RBC	DMD ng/ml	DMD P/RBC	2HD ng/ml	2HD P/RBC
5 minute plasma	202		65		54.5	
5 minute RBC	269.5	0.750	102	0.637	115	0.474
10 minute plasma	182		52		65	
10 minute RBC	296	0.615	104	0.500	105	0.619
20 minute plasma	190		55		64	
20 minute RBC	285	0.667	90	0.611	99	0.647
60 minute plasma	185		49		59	
60 minute RBC	297.5	0.622	105	0.467	110	0.536
Mean P/RBC		0.664		0.554		0.569
Mean Recovery (%)	96.9± 1.1		79.5±3.9		85.8±1.7	

TABLE 15

Distribution of DMI, DMD and 2HD from RBC to Plasma

Specimen	DMI ng/ml	DMI P/RBC	DMD ng/ml	DMD P/RBC	2HD ng/ml	2HD P/RBC
Tube 1 Plasma	202		49		52	
Tube 1 RBC	273	0.740	86	0.570	115	0.452
Tube 2 Plasma	187		47		44	
Rube 2 RBC	287	0.652	72	0.653	117	0.376
Tube 3 Plasma	194		49		49	
Tube 3 RBC	283	0.686	87	0.563	121	0.405
Mean Plasma	194.3		48.3		48.3	
Mean RBC	281.0	0.691	81.7	0.591	117.7	0.410
Mean Recovery (%)	96.4		66.3		85.8	

Next, the equilibrium of DMI, DMD and 2HD between plasma and RBC was studied in blood samples drawn from the same individual over an eight week period. The purpose was to obtain an indication of the variability in distribution over a time period equal to that used in the clinical trials. DMI, DMD and 2HD were incubated in whole blood for 20 minutes prior to separation of RBCs and plasma. As noted in Table 16, there are some weekly variations. Week two DMI concentrations had a P/RBC of 0.827 which is substantially higher than the other weeks.

As rather large variations in drug and metabolite concentrations occur in different patients taking the same dose of TCA, the distribution between RBC and plasma of a range of concentration of DMI from 250 to 3000 ng/mL in whole blood was studied. Table 17 shows that the plasma to RBC ration of DMI concentration was consistent throughout this concentration range. This ratio ranged from 0.598 to 0.613.

The distribution of DMI, DMD and 2HD between plasma and RBC of blood samples from 15 "normal" persons was investigated to determine the variability in a population of these distributions. In all samples used for *in vitro* studies, the hematocrit is an estimation calculated by measuring the height of the RBC volume in the Venoject tube and dividing it by the total blood volume height after the sample has been centrifuged under standard conditions noted in the methods section. Tables 18 and 19 contain the normal population plasma - RBC distribution data. The plasma to RBC ratios are included. The DMI ratio ranged from 0.390 to 0.732 with a mean of 0.526. No significant difference was found between males and females or those over 40 and under 40. No correlation to the hematocrit was found either. The mean ratio for 2HD was higher (0.619) than for DMI (0.526) while the ratio for DMD was lower (0.429). Again,

TABLE 16

Eight Week Plasma-RBC Distribution Study of DMI, DMD and 2HD. (Equilibration Time of 20 Minutes)

Specimen		DMI ng/ml	DMI P/RBC	DMD ng/ml	DMD P/RBC	2HD ng/ml	2HD P/RBC
Week one	Plasma	190		55		64	
Week one	RBC	285	0.667	90	0.611	99	0.646
Week two	Plasma	215		61		64	
Week two	RBC	260	0.827	125	0.488	101	0.634
Week three	Plasma	198		60		67	
Week three	RBC	296	0.669	109	0.480	105	0.638
Week five	Plasma	181		48		57	
Week five	RBC	288	0.628	90	0.533	112	0.509
Week eight	Plasma	185		62		57	
Week eight	RBC	291	0.636	112	0.554	116	0.491
Mean Plasma ± S.D.		193.8±12.0		57.2± 5.2		61.8±4.1	
Mean RBC ± S.D.		284.0±12.5		105.2±13.5		106.6±6.5	
Mean P/RBC		0.682		0.544		0.580	
Mean Recovery (%)		97.0		83.1		86.0	

TABLE 17

Plasma - RBC Distribution Saturation Study.
All Concentrations are ng/ml

Concentration In Blood	Plasma DMI	RBC DMI	Total DMI Recovered	Plasma to RBC Ratio
250	164.5	273	90.1%	0.603
500	293	480	79.5%	0.610
1000	629	1051	86.5%	0.598
2000	1247.5	2035	84.4%	0.613
3000	1855	3041	84.0%	0.610

Mean Recovery 84.9% ± 3.5%

Mean Plasma to RBC DMI Concentration 0.607 ± 0.005

TABLE 18

Normal Population Plasma - RBC Distribution of DMI. All Concentrations ng/ml

Subject	Sex	Hematocrit	Plasma DMI	RBC DMI	Plasma to RBC Ratio
PH	F	0.394	203	473	0.429
CM	M	0.490	173	433	0.400
RB	M	0.455	160	355	0.451
SB	M	0.496	240	407	0.590
SC	F	0.479	212	380	0.558
FF	F	0.465	190	325	0.585
PB	F	0.433	193	393	0.491
LF	M	0.503	147	377	0.390
GJ	M	0.468	180	371	0.485
JL	F	0.414	205	351	0.584
DM	M	0.559	203	352.5	0.576
LT	M	0.528	227	310	0.732
NL	F	0.426	155	347	0.447
RG	M	0.519	189	297.5	0.635
BV	F	0.423	206	381.5	0.540

All mean ± S.D. 0.526 ± 0.092
Male mean ± S.D. 0.532 ± 0.113
Female mean ± S.D. 0.519 ± 0.059

TABLE 19

Normal Population Plasma - RBC Distribution of 2HD and DMD. All Concentrations ng/ml

Subject	Plasma 2HD	RBC 2HD	Plasma to RBC Ratio	Plasma DMD	RBC DMD	Plasma to RBC Ratio
PH	54	78	0.692	70	178	0.393
CM	48	100	0.480	57	152	0.375
RB	53	95	0.558	53	147.5	0.359
SB	70	95	0.737	86	199	0.432
SC	39	102	0.382	64.5	150	0.430
FF	60	81.5	0.736	61	137	0.445
PB	61	77	0.792	63	194	0.325
LF	46	102	0.451	47.5	151	0.315
GJ	54	85	0.635	58	173	0.335
JL	66	80	0.825	63	164	0.384
DM	71	88	0.807	63	130	0.485
LT	85.5	98	0.872	73	104	0.702
NL	49	123	0.398	47	133	0.353
RG	57.5	101	0.569	62	103	0.602
BV	51	146	0.349	66	130	0.508
Mean ± S.D.			0.619 ± 0.171			0.429 ± 0.104

TABLE 20

Drug and Metabolite Stability in Whole Blood Stored at -20°C for Eight Weeks.
All Concentrations are ng/ml

Compound	24 Hour Concentration	8 Week Concentration	Per Cent Change
IMP	215	197	-8.4%
DMI	189	201	+6.3%
DMD	168	181	+7.7%
2HI	206	185	-10.2%
2HD	195	180	-7.7%

there was a wide range in the distribution ratio (plasma to RBC) for 2HD (0.349 to 0.872) and for DMD (0.315 to 0.702). There were no good correlations found between the plasma to RBC ratios of these compounds and plasma total protein, albumin, cholesterol and triglycerides.

The final *in vitro* study performed investigated the stability of the compounds of interest (IMP, DMI, DMD, 2HI and 2HD) in whole blood when stored at -20°C for eight weeks. Table 20 indicates that the concentrations at 24 hours and 8 weeks did not differ significantly, especially when the overall precision of the assay is considered. The largest change was a decrease of 10.2% in the 2HI concentration.

C. Human Studies

1. Imipramine clinical study.

The patients in the imipramine clinical study had their daily dose raised during the first two weeks of the study to 225 mg. If this dose was not well tolerated, the dose was decreased to the last well tolerated amount. This was an outpatient study so that compliance could not be completely verified. For the most part, a daily dose of 200 to 225 mg was achieved and continued through the end of the six week study. Blood samples were drawn at the end of the first, second, fourth and sixth weeks but there were exceptions. Some patients did not finish the study and week four was their last blood sample. Others missed week six but came in a week later. The steady state drug and metabolite concentrations for imipramine clinical study patients were considered to be week four and week six. If patients came in and had blood drawn both weeks, the average of the two values was considered their steady state

concentrations. If one or the other blood sample was drawn, its concentrations were considered steady state. All IMP, DMI, DMD, 2HI and 2HD concentrations were measured by HPLC. Table 21 contains this steady state concentration data. One patient, R-1, had a maximum daily dose of 100 mg. All others had maximum daily doses of 200 to 225 mg. Only the 23 patients with daily doses of 200 to 225 mg were included in the mean data analysis. Table 21 includes the IMP and DMI steady state plasma concentrations in ng per ml and the total of IMP plus DMI. Also in Table 21 is the DMI to IMP ratio and the ratios of DMD, 2HI and 2HD to the total (IMP plus DMI).

The ratios of DMD, 2HI and 2HD to the total of IMP plus DMI show the relative amounts of these metabolites (DMD, 2HI and 2HD) that are found in the plasma in relation to the parent drug, IMP, and principle metabolite, DMI. IMP and DMI plasma concentrations have been measured at MCV for several years and the total of the two concentrations is what is generally used clinically (16). Total means the IMP concentration plus DMI concentration sum. The mean DMD to total ratio in the 23 patients is 0.051 (± 0.047) with a range from zero to 0.15. Five of 23 patients had ratios over 0.10. The mean 2HI to total ratio is 0.091(± 0.090) with a range from zero to 0.43. Only one patient's 2HI/total ratio was over 0.20 while 7 of 23 (30%) had ratios greater than 0.10. The mean 2HD to total ratio was 0.20 (± 0.12). The range of 2HD/total ratios was from zero to 0.43. Twelve of 23 patients had ratios over 0.20 and 16 of 23 patients' ratios exceeded 0.10. 2HD was the most significant metabolite of the three.

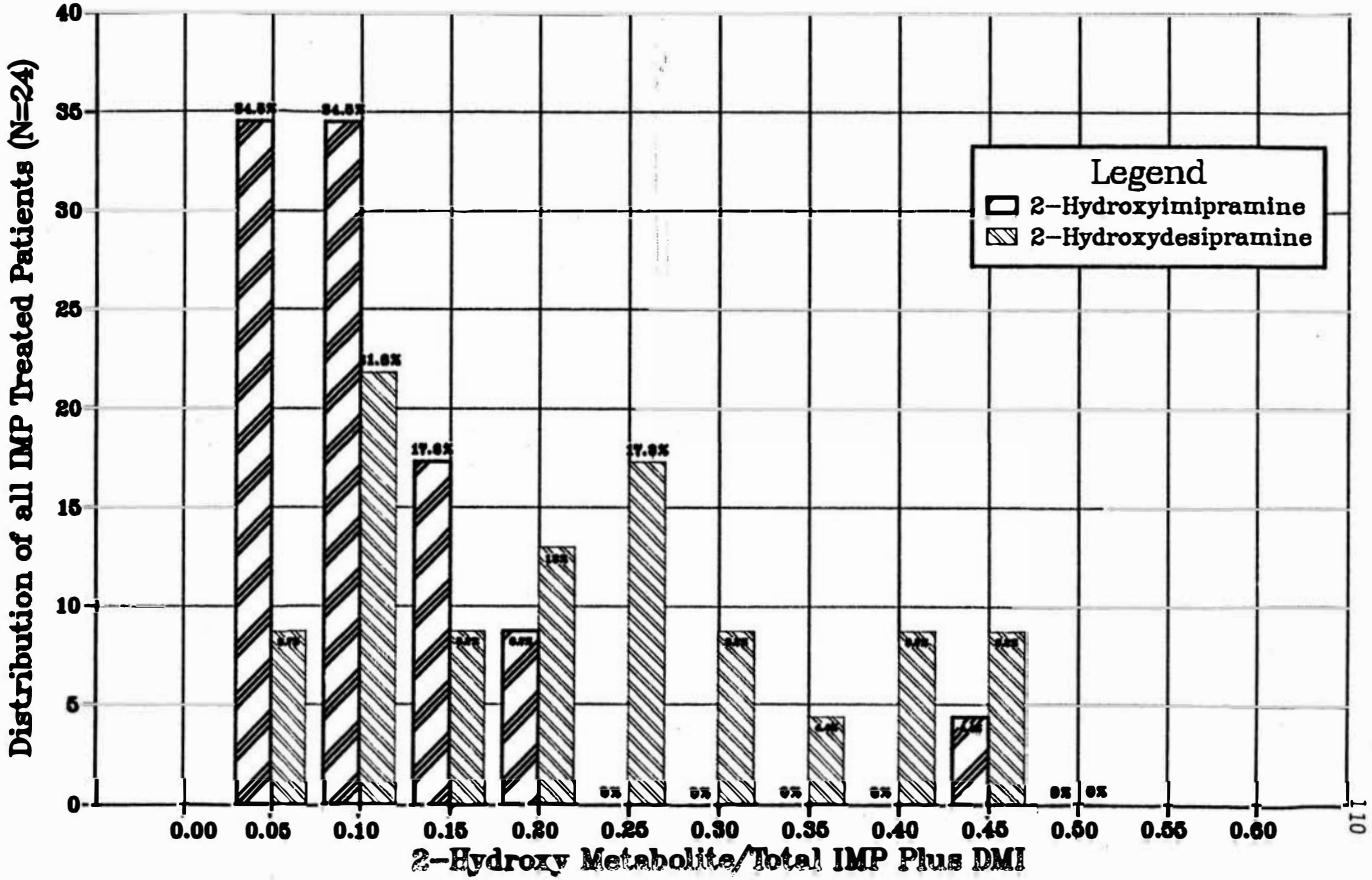
Figure 3 shows the percent of all IMP treated patients with particular values for the ratio of both 2HI and 2HD to their total IMP

TABLE 21

Imipramine Treated Patients Steady State Plasma Drug Contentrations at 200 to 225 mg/day of IMP. IMP and DMI Concentrations are ng/ml.

Patient	IMP	DMI	DMI/IMP	Total	DMD/T	2HI/T	2HD/T
M-1	58	39	0.67	96	0.066	0.070	0.380
A-1	43	37	0.86	80	0.150	0.100	0.125
J-2	110	89.5	0.81	200	0.135	0.185	0.430
C-2	311	220	0.70	531	0.020	0.080	0.094
J-2	97	126	1.30	223	0.022	0.180	0.305
W-1	64	580	9.06	644	0.110	0.014	0.062
C-1	169	124	0.73	293	0.046	0.034	0.246
R-1 (100 mg)	70	38	0.54	108	0.092	0.079	0.167
K-1	35	223	6.37	258	0.039	-	0.300
H-5	395	84	0.21	479	0.125	0.019	0.097
D-1	34	103	3.04	137	0.073	-	0.383
A-2	54	29	0.54	83	-	0.430	0.230
H-1	161	76	0.47	237	-	0.140	0.156
J-3	24	67	2.79	91	0.027	-	0.406
E-1	171	285	1.67	456	0.015	0.061	0.207
H-4	49	83	1.69	132	-	0.038	0.136
H-2	266	388	1.46	654	0.000	0.035	-
D-2	151	133	0.88	284	0.025	0.077	-
B-3	145	155	1.07	300	0.027	0.077	0.093
C-3	279	154	0.55	433	0.053	0.140	0.225
H-3	78	895	11.50	973	0.121	0.000	0.000
B-2	72	158	2.19	230	0.017	0.104	0.230
T-1	111	148	1.33	259	0.008	0.127	0.260
B-1	165	131	0.79	296	0.014	0.094	0.074

Figure 3. Distribution of 2–Hydroxy Metabolites Relative to Total IMP Plus DMI Concentration in Plasma



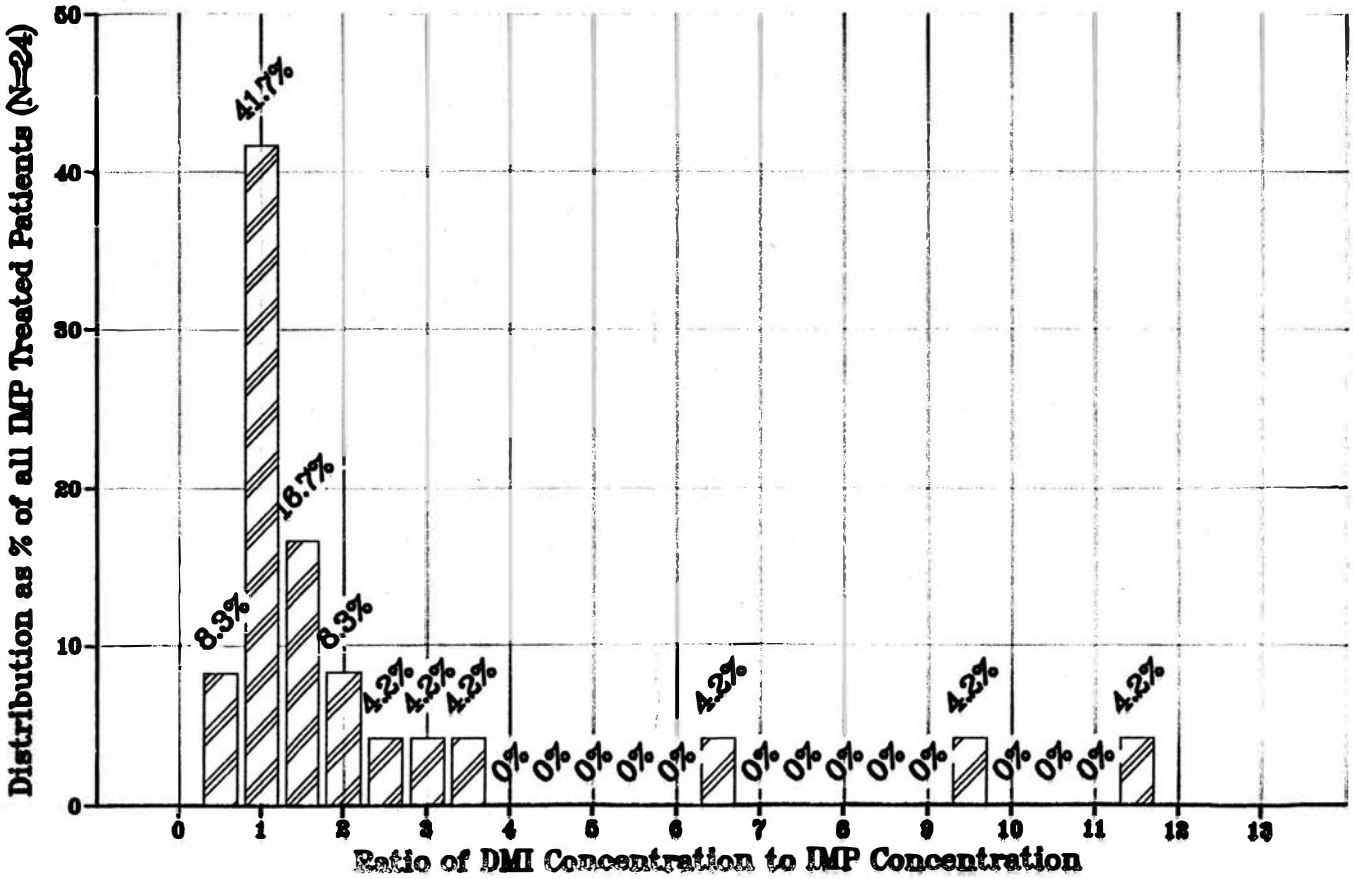
plus DMI concentration. The 2HI to total ratios are low (most less than 0.20) while the 2HD to total ratios are spread more evenly from zero to 0.45. Again, these ratios of metabolites 2HD, 2HI and DMD to IMP plus DMI show the relative amounts of these metabolites in the plasma. A low ratio indicates that little metabolite is present in plasma compared to the amount of IMP plus DMI.

Table 21, as noted, contains the DMI/IMP ratio. The value of this ratio ranges from 0.21 to 11.5. This indicates that there is a large variation in the relative proportions of DMI and IMP in these patients plasma at steady state. Figure 4 displays the distribution of all 24 patients plasma DMI to IMP ratios. Most patient DMI/IMP ratios are below three and half are below one.

DMI is the demethylated metabolite of IMP. The degree of demethylation may be seen in the relative proportions of IMP and DMI measured in the plasma at steady state. Arbitrarily, slow demethylators have been designated as those patients with DMI/IMP ratios less than one. Fast demethylators are designated as those with DMI/IMP ratios greater than one, that is, with more DMI than IMP in the plasma. In this study, there are twelve each of fast and slow demethylators.

Again, arbitrarily, these patients can be divided into fast and slow hydroxylators on the basis of their plasma drug and metabolite concentrations. 2HI and 2HD are the hydroxylated metabolites of IMP. If the patients 2HI/total plus 2HD/total sum is greater than 0.20 they are designated as fast hydroxylators. This means that the hydroxy metabolites in plasma reach 20% of the value of the IMP plus DMI concentration. Slow hydroxylators have a 2HI/total plus 2HD/total ratio less than 0.20. Of these 24 patients, nine were considered slow hydroxylators

Figure 4. Distribution of IMP Treated Patients by Their Plasma DMI to IMP Ratios.



and fifteen as fast hydroxylators. Table 22 helps illustrate the hydroxylator and demethylator designations. The steady state plasma IMP plus DMI concentrations are divided up first into slow or fast demethylator categories. Within demethylator category, the concentrations are separated into their hydroxylator type, either fast or slow. The number of patients in each group and the mean plasma IMP plus DMI concentration with the standard deviation of each group are included in Table 22. It can be seen that within both demethylator categories the slow hydroxylators have a mean steady state plasma IMP plus DMI concentration double that of the fast hydroxylators. Fast demethylators have mean IMP plus DMI concentrations greater than slow demethylators in both hydroxylation groups.

The concentrations of drug and metabolites in the plasma during the course of the six week study in two patients are shown in Figures 5 and 6. These graphs help to show the relative amounts of metabolites in these two examples. In Figure 5 the data for patient K-1 is given. This patient is designated as a fast demethylator and the DMI concentrations are greater than IMP concentrations during the entire study. Patient C-1 in Figure 6 is an example of a slow demethylator. In both cases, the concentration of 2HD is greater than 2HI at all time points.

For twelve imipramine patients, drug and metabolite concentrations were measured in red blood cells (RBCs) as well as plasma. The IMP and DMI concentrations in plasma and RBCs are compared in Table 23. The data for all samples is included and the IMP plus DMI and the DMI to IMP ratio for both plasma and RBCs are listed. The mean IMP plus DMI concentration in plasma is 234.5 (± 175). The mean IMP plus DMI

TABLE 22

Steady-State Plasma IMP Plus DMI
Concentrations (ng/ml) of Metabolism Groups

	SLOW DEMETHYLATORS		FAST DEMETHYLATORS	
	Slow Hydroxylators	Fast Hydroxylators	Slow Hydroxylators	Fast Hydroxylators
	531	96	644	223
	479	200	132	258
	284	80	654	137
	296	293	300	91
		108	973	456
		83		230
		237		259
		433		
N	4	8	5	7
Mean	397.5	191.2	540.6	236.2
Standard Deviation	109	118	295	107
% S.D.	27%	62%	55%	45%

Figure 5. Time Course of IMP and Metabolites
Plasma Concentrations in PATIENT K–1

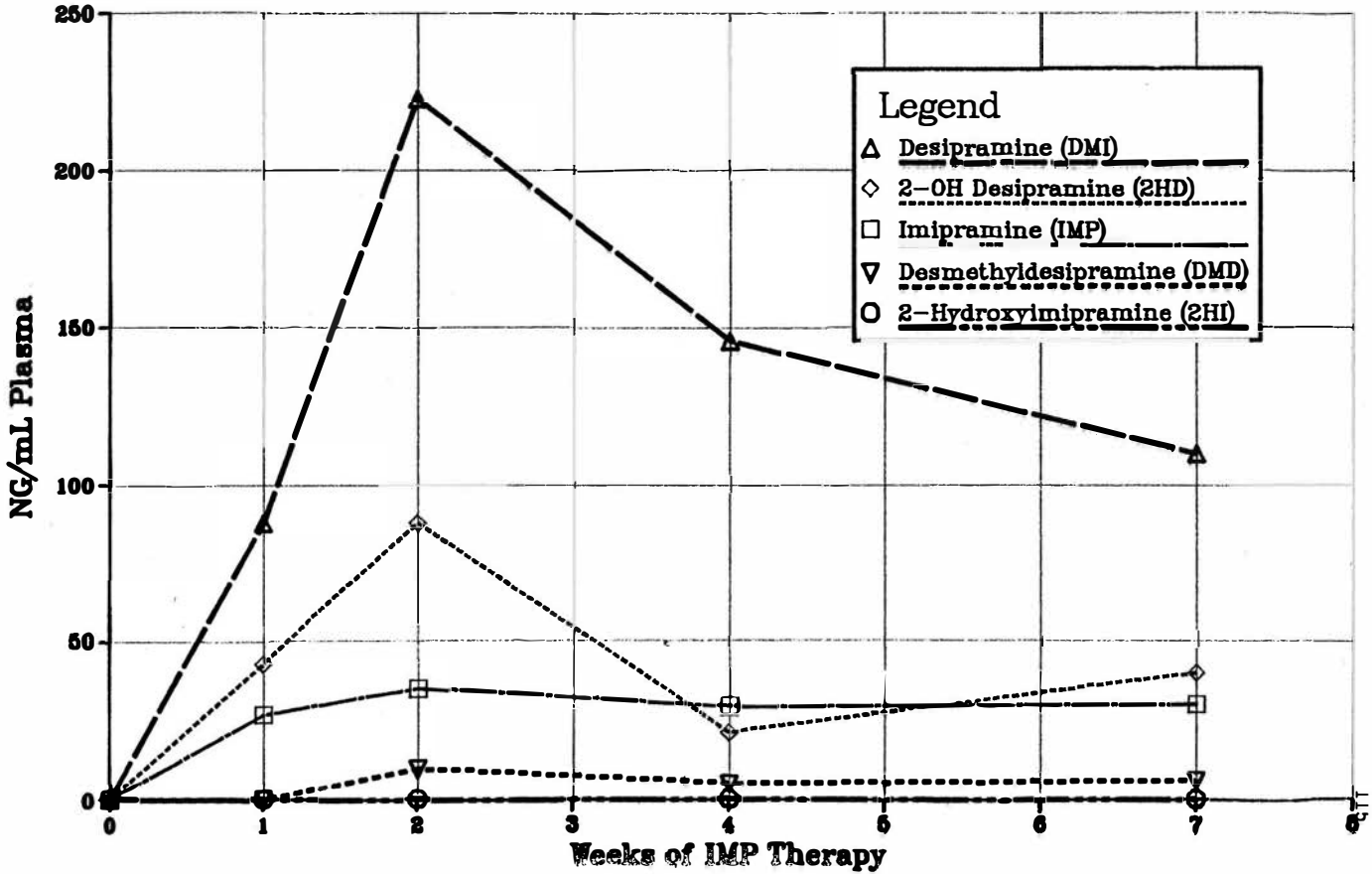


Figure 6. Time Course of IMP and Metabolites Plasma Concentrations in PATIENT C–1

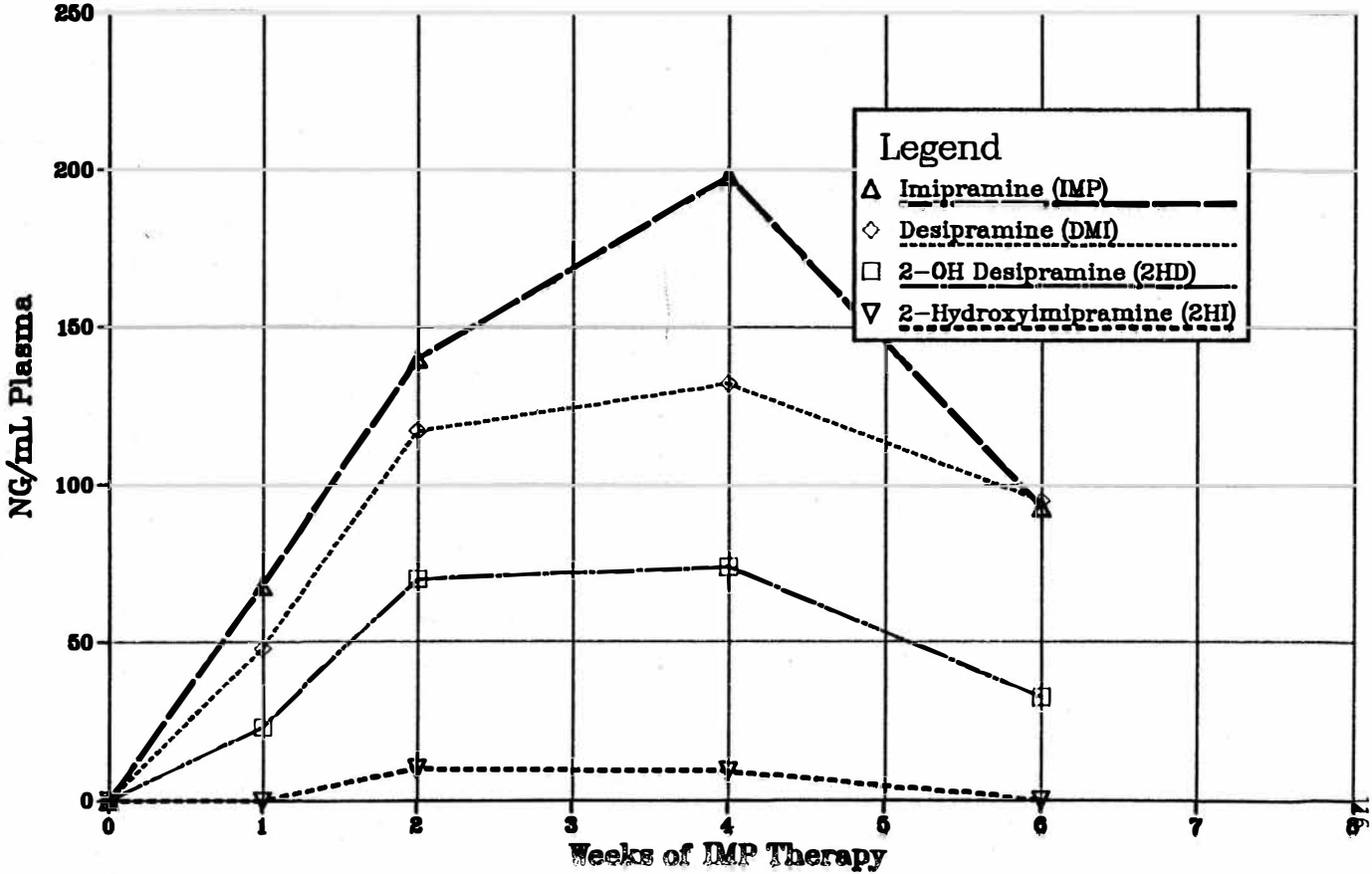


TABLE 23

Comparison of Plasma and RBC Concentrations of IMP Plus DMI (ng/ml) and DMI/IMP

		PLASMA		RBC	
Patient	Week	IMP + DMI	DMI/IMP	IMP + DMI	DMI/IMP
J-1	1	77	0.54	85	1.08
	2	166	0.73	185	1.56
	4	233	0.88	205	1.59
M-1	1	47	0.34	43	0.95
	2	100	0.67	101	1.15
A-1	1	44	1.45	63	0.85
C-2	4	650	0.81	437	1.64
	6	408	0.84	179	1.43
J-2	1	68	0.89	81	2.70
	2	199	0.92	246	5.56
	4	198	1.35	230	4.76
	6	280	1.59	265	4.55
W-1	1	336	3.33	518	9.09
	2	383	6.25	1074	16.7
	4	676	8.33	964	14.3
	6	542	9.09	523	14.3
C-1	2	257	0.83	370	4.35
	4	329	0.66	259	1.79
	6	188	1.02	182	3.85
R-1	1	138	0.41	261	2.38
	2	79	0.88	115	3.25
	4	51	1.04	59	5.00
K-1	1	116	3.33	233	12.5
	2	257	6.25	512	20.0
	4	132	4.55	422	5.88
	6	135	4.00	369	5.26
H-5	1	195	0.26	190	0.16
	2	423	0.20	234	0.71
	4	448	0.27	419	1.92
	6	602	0.21	220	0.44
D-1	2	138	4.17	333	10.0
	4	125	2.13	305	10.0
	6	148	3.33	322	7.14
A-2	1	145	0.83	90	3.70
	2	74	0.40	44	1.92
	6	55	0.37	43	2.56

concentration in RBCs is 283 (± 226). The DMI to IMP ratio differs considerably between plasma and RBCs. For these 36 blood samples, the mean ratio in plasma was 2.03 (± 2.30) and the mean ratio in RBCs was 5.14 (± 4.97). Figure 7 illustrates this difference in the DMI/IMP ratio. It is a plot of the plasma ratios versus RBC ratios. If the DMI/IMP was the same for plasma and RBCs the line slope would be near 1. The actual slope is 1.90. These data indicate DMI and IMP distribute differently between plasma and RBCs. The correlation coefficient is 0.878, meaning that there is a significant ($p \leq .005$) relationship between DMI/IMP in plasma and RBCs for these patients.

The plasma and RBC IMP and DMI steady state concentrations were compared to the Severity Index and Raw Improvement derived from Hamilton Depression Rating scores. The Methods section describes how the Severity Index (S.I.) and Raw Improvement (R.I.) values were calculated for each patient. Table 24 is a compilation of this data with plasma drug concentrations for 24 patients. Table 25 contains RBC drug concentrations, S.I.s, and R.I.s for the twelve patients whose RBC concentrations were measured. All of these data were compared in order to ascertain the degree of correlation, if any, between drug concentration and clinical response. The Pearson correlation (98) was used to compare these factors. Table 26 contains the results of these correlations. The parameter used on the X axis and the parameter used on the Y axis are given with the line parameters for each correlation. The best correlation was between Raw Improvement and the Severity Index. That correlation coefficient (r) was 0.653 ($p<.01$). The best correlation of drug concentration to improvement was for RBC DMI to IMP ratio versus Raw Improvement. However, its correlation coefficient of -0.395 was not

Figure 7. IMP Treated Patients
DMI/IMP Ratios in Plasma versus RBCs

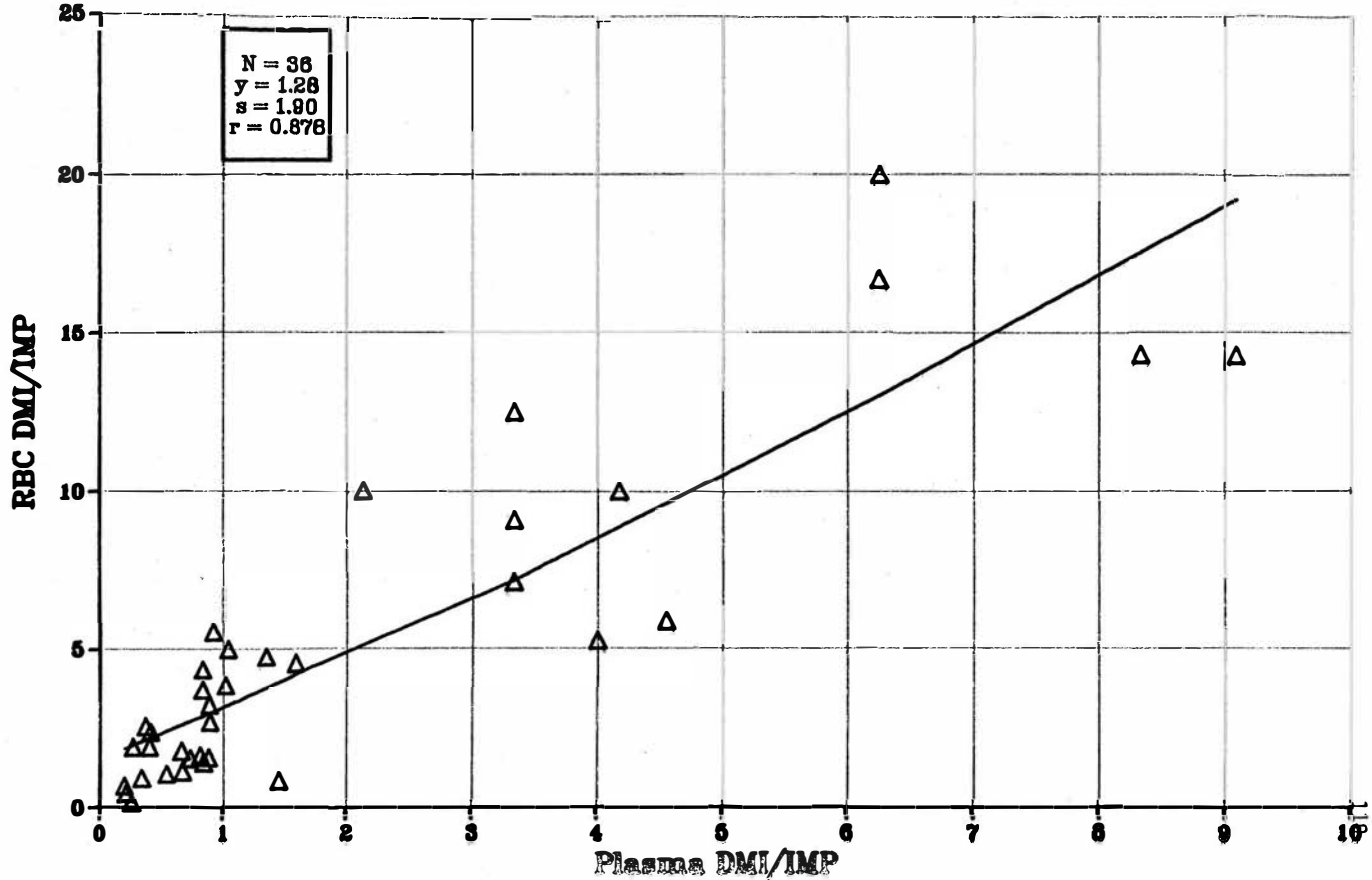


TABLE 24

IMP Patient Depression Ratings and Plasma Drug Concentrations (ng/ml)

Patient	S.I.	R.I.	IMP	DMI	IMP + DMI	DMI/IMP
M-1	32	22	58	39	96	0.67
A-1	22	16	43	37	80	0.86
J-1	20.5	13.5	110	90	200	0.81
H-1	32.5	22.5	161	76	237	0.47
J-2	20.5	4.5	97	126	223	1.30
D-1	25	19	34	103	137	3.04
C-1	30.5	16.5	169	124	293	0.73
D-2	27	8	151	133	284	0.88
J-3	27	0	24	67	91	2.79
H-2	36	22	266	388	654	1.46
H-3	33.5	25.5	78	895	973	11.5
H-4	30.5	15.5	49	83	132	1.69
E-1	19.5	7.5	171	285	456	1.67
B-1	33	28	165	131	296	0.79
B-2	24	5	72	158	230	2.19
B-3	28	18	145	155	300	1.07
W-1	18.5	3.5	64	580	644	9.06
R-1	23	1	70	38	108	0.54
K-1	31.5	15.5	35	223	258	6.37
H-5	19.5	6.5	395	84	479	0.21
C-Z	25	23	311	220	531	0.71
C-3	26.5	18.5	279	154	433	0.55
T-1	23	20	111	148	259	1.33
A-2	27.5	21.5	54	29	83	0.54

S.I. = Severity Index
R.I. = Raw Improvement

TABLE 25

IMP Patient Depression Ratings and RBC Drug Concentrations (ng/ml)

Patient	S.I.	R.I.	IMP	DMI	IMP + DMI	DMI/IMP
J-1	20.5	13.5	79	126	205	1.59
M-1	32	22	47	54	101	1.15
C-2	25	23	165	272	437	1.65
J-2	20.5	4.5	47	218	265	4.64
W-1	18.5	3.5	64	900	964	14.1
C-1	30.5	16.5	93	166	259	1.78
R-1	23	1	27	88	115	3.26
K-1	31.5	15.5	62	360	422	5.81
H-5	19.5	6.5	148	147	295	0.99
D-1	25	19	40	282	322	7.05
A-2	27.5	21.5	15	30	45	2.00

S.I. = Severity Index
R.I. = Raw Improvement

TABLE 26

IMP Patient Pearson Correlations

X Axis	Y Axis	y	s	r
Raw Improvement	Severity Index	20.5	0.406	0.653
Plasma [IMP]	Raw Improvement	13.0	0.013	0.160
Plasma [DMI]	Raw Improvement	15.8	0.001	0.016
Plasma [IMP + DMI]	Raw Improvement	12.4	0.007	0.200
Plasma [DMI/IMP]	Raw Improvement	14.8	-0.039	-0.014
RBC [IMP]	Raw Improvement	12.0	0.018	0.108
RBC [DMI]	Raw Improvement	16.0	-0.011	-0.336
RBC [IMP + DMI]	Raw Improvement	16.4	-0.009	-0.304
RBC [DMI/IMP]	Raw Improvement	16.6	-0.819	-0.395

y = y intercept of correlation line
s = line slope
r = correlation coefficient

significant. Other r values approached zero, indicating that there is no significant correlation between that data.

2. Desipramine clinical studies.

In the desipramine clinical study, the patients were adjusted upward to a maximum daily dose of 300 mg during the first week of drug therapy. The blood samples drawn at visits 5, 6, 7 and 8 (corresponding to 2, 3, 4 and 5 weeks after initiation of DMI therapy) were considered to contain steady state concentrations of DMI, DMD and 2HD. All DMI, DMD and 2HD concentrations were measured by HPLC.

Table 27 contains the plasma steady state concentrations. Drug and metabolite concentrations from visits 5, 6, 7 and 8 were averaged to obtain these values. In several cases a visit was missed so that it could not be included. Table 27 also contains the plasma DMD to DMI ratio and plasma 2HD to DMI ratio. Figure 8 shows the distribution of plasma DMI steady state concentrations for the twelve patients. The mean value is 408 (± 278) ng/mL. Patient EW #32 had the highest steady state plasma DMI concentration, 1123 ng/ml, and WK #11 had the lowest, 165 ng/ml.

The variation in the steady state DMI concentrations for each patient was determined by calculating the relative standard deviation of each mean steady state concentration. The relative standard deviation is the standard deviation divided by the mean and multiplied by 100 to yield a percent standard deviation. The overall relative standard deviation for DMI for all 12 patients was 18.2% (± 5.1%). It ranged from 8.8% to 29.3%.

TABLE 27

DMI Treated Patients (300 mg per day): Steady-state Plasma Concentrations of DMI, DMD and 2HD (Mean of Visits 5, 6, 7 and 8). All Concentrations in ng/ml.

Patient	DMI	DMD	DMD/DMI	2HD	2HD/DMI
EW #10	424	9.5	0.022	136	0.321
WK #11	165	11	0.067	15	0.091
MJ #16	608	56	0.092	190	0.312
CA #18	291	15	0.052	96	0.330
JB #19	289	31	0.107	88	0.304
GW #21	786	46	0.059	19	0.024
MO #23	254	22	0.087	104	0.409
SJ #24	300	22	0.073	200	0.667
BB #27	180	8	0.044	99	0.550
EM #28	224	13	0.058	98	0.438
DB #29	249	13	0.052	81	0.325
EW #32	1123	101	0.090	88	0.078

The relative standard deviation in the steady state 2HD concentrations was 36.7% (± 22.1%). It ranged from 20.7% to 102%. The 2HD concentration therefore varied more from week to week than did the DMI concentration.

The DMD/DMI ratio for plasma steady state concentrations was low. The mean was 0.067 (± 0.023) and it ranged from 0.022 to 0.107. That means that in only one patient did the DMD concentration reach 10% of the DMI concentration.

The mean 2HD/DMI ratio for plasma steady state concentrations was 0.321 (± 0.181). It ranged from 0.024 to 0.667. In 8 of the 12 patients this ratio was greater than 0.300, meaning that in those patients the 2HD concentration was at least 30% of the DMI concentration. The 2HD plasma concentration at steady state never exceeded the DMI plasma concentration.

For 10 of the 12 desipramine patients, red blood cell (RBC) drug and metabolite concentrations were measured. Table 28 contains the RBC steady state DMI, DMD and 2HD concentrations. Again, DMD to DMI and 2HD to DMI ratios are included. As can be seen, the DMD/DMI ratio is low (mean of 0.099 ± 0.017) and the 2HD/DMI ratio is significantly higher (mean of 0.478 ± 0.307). In both RBCs and plasma, 2HD reaches significantly greater concentrations than does DMD.

A comparison of plasma and RBC drug and metabolite concentrations is made in Table 29. For DMI, DMD and 2HD, the ratio of plasma to RBC concentrations at steady state are shown. The mean value, the standard deviation and relative standard deviation were calculated and included as well. For DMI and its two metabolites, the mean plasma concentration is less than the RBC concentration. That is, the mean plasma

TABLE 28

DMI Treated Patients (300 mg per day): Steady-State RBC Concentrations of DMI, DMD and 2HD (mean of Visits 5, 6, 7 and 8). All Concentrations in ng/ml.

Patient	DMI	DMD	DMD/DMI	2HD	2HD/DMI
MJ #16	368	36.5	0.099	79	0.215
CA #18	352.5	30.5	0.087	258.5	0.733
JB #19	393	38	0.097	97.5	0.248
GW #21	1738	174	0.100	96	0.055
MO #23	434	43	0.099	284	0.654
SJ #24	349	36	0.103	272	0.779
BB #27	298.5	42	0.141	257	0.861
EM #28	294	28	0.095	245	0.833
DB #29	483	34	0.070	157	0.325
EW #32	2655	251.5	0.095	204	0.077

TABLE 29

Steady State Plasma Concentration to RBC Concentration Ratios for DMI, DMD and 2HD

Patient	DMI	DMD	2HD
MJ #16	1.65	1.53	2.41
CA #18	0.83	0.49	0.37
JB #19	0.74	0.82	0.90
DW #21	0.45	0.26	0.20
MO #23	0.59	0.51	0.37
SJ #24	0.86	0.61	0.74
BB #27	0.60	0.19	0.39
EM #28	0.76	0.46	0.40
DB #29	0.52	0.38	0.52
EW #32	0.42	0.40	0.43
Mean	0.742	0.565	0.673
SD	0.336	0.362	0.610
% S.D.	45%	64%	91%

to RBC ratio for all three is less than one. However, the variation as expressed by the relative standard deviation is high for all three.

For comparison to clinical response, only drug and metabolite concentrations at visit 7 and 8 were used. The visit 7 and 8 Hamilton Depression Rating scores were used to calculate the patient's Raw Improvement values (R.I.s). Visits 7 and 8 are 4 and 5 weeks after the initiation of drug therapy and should allow time for DMI treatment to take effect (6). The DMI and DMI plus 2HD concentrations were compared to the Severity Index (S.I.) and Raw Improvement (R.I.). All the plasma data used is in Table 30 and all the RBC data used is in Table 31. The visit 7 and 8 average Hamilton Depression Rating scores (HDS) are also included. The Methods section describes S.I. and R.I. value calculations.

Table 32 contains the DMI patient Pearson correlations derived from the data in Tables 30 and 31. Both plasma DMI and RBC DMI concentrations give good correlations versus Raw Improvement. ($r = -0.750$ and $r = -0.693$, both $p<0.01$). The combined DMI plus 2HD concentrations in both plasma and RBCs also correlated well with Raw Improvement ($r = -0.752$ and $r = -0.655$). These correlations are all negative and indicate an inverse relationship between drug concentrations in this range and Raw Improvement. Figure 9 is a plot of the plasma DMI concentration versus Raw Improvement which illustrates this relationship.

Rank order correlations and partial correlations are statistical manipulations which may increase the correlation coefficients for some data sets. Rank order correlations often are helpful when some extreme values or outliers are in the data. Partial correlations are

TABLE 30

DMI Patient Depression Ratings and Plasma Drug Concentrations (ng/ml)

Patient	S.I.	Vis 7+8 Ave HDS	R.I.	R.I. Rank	DMI	DMI Rank	DMI+2HD
EW #10	36.5	28.5	8	10	399	9	523.5
WK #11	30	12.5	17.5	4	201.5	4	232
MJ #16	28	15	13	7.5	582.5	10	707.5
CA #18	25	10	15	6	286	6	401
JB #19	39	19.5	19.5	3	291.5	7	395.5
GW #21	31	30	1	12	906.5	11	931.5
MO #23	32.5	8	24.5	1	198	3	272
SJ #24	35.5	22.5	13	7.5	324	8	568.5
BB #27	35	18.5	16.5	5	148.5	1	230.5
EM #28	30	6.5	23.5	2	172.5	2	251.5
DB #29	34.5	24	10.5	9	246	5	316
EW #32	44	36.5	7.5	11	1062	12	1125.5

S.I. = Severity Index
HDS = Hamilton Depression Score
R.I. = Raw Improvement

TABLE 31

DMI Patient Depression Ratings and RBC Drug Concentrations (ng/ml)

Patient	S.I.	Vis 7+8 Ave HDS	R.I.	R.I. Rank	DMI	DMI Rank	DMI+2HD
MJ #16	28	15	13	6.5	394.5	4	492.5
CA #18	25	10	15	5	352.5	3	611
JB #19	39	19.5	19.5	3	515.5	8	670.5
GW #21	31	30	1	10	1892	9	1982
MO #23	32.5	8	24.5	1	430	7	700
SJ #24	35.5	22.5	13	6.5	398	5	693
BB #27	35	18.5	16.5	4	298.5	2	555.5
EM #28	30	6.5	23.5	2	253.5	1	529
DB #29	34.5	24	10.5	8	410.5	6	521.5
EW #32	44	36.5	7.5	9	2567	10	2708

S.I. = Severity Index
HDS = Hamilton Depression Score
R.I. = Raw Improvement

TABLE 32

DMI Patient Pearson Correlations

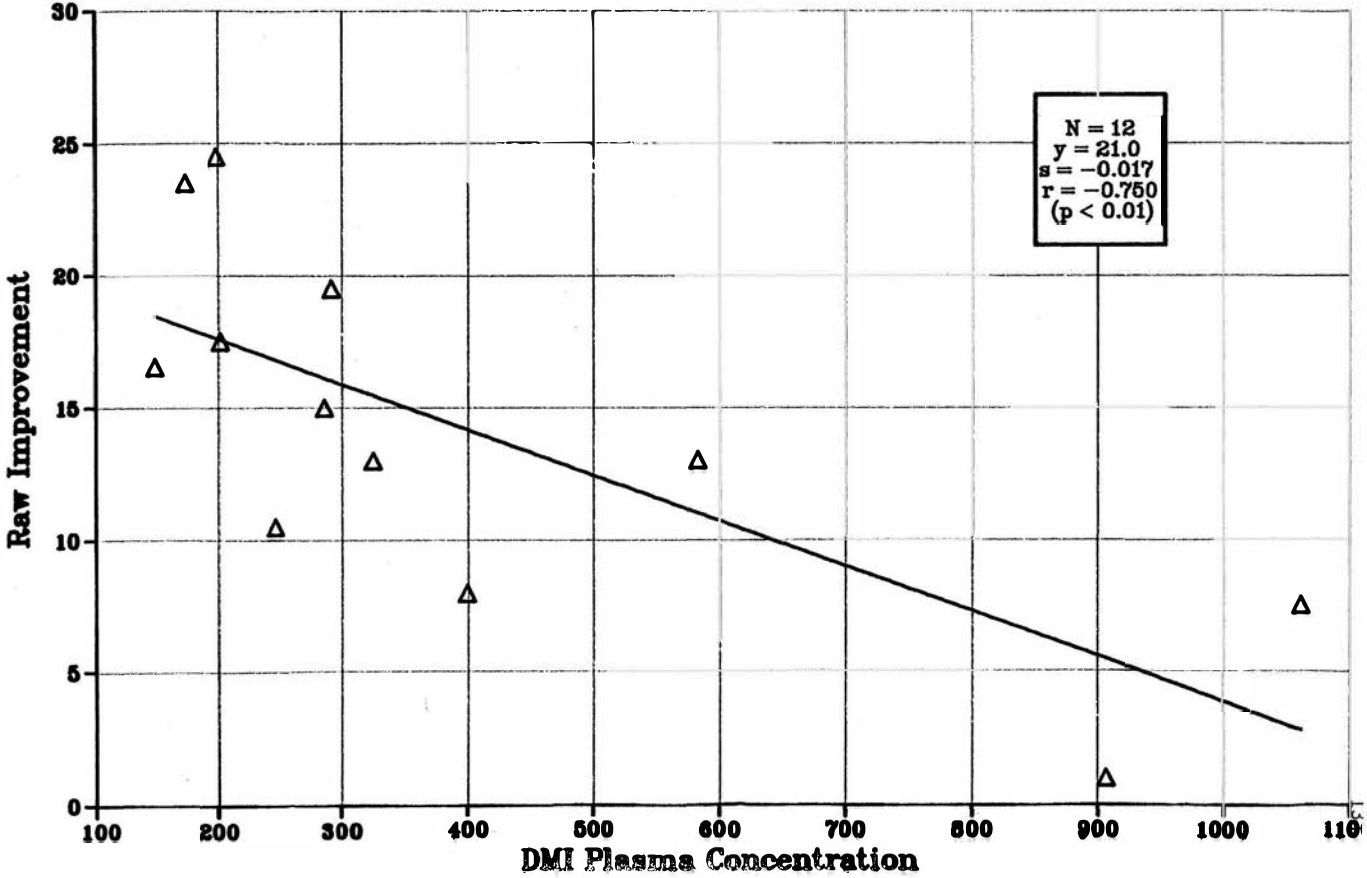
X Axis	Y Axis	y	s	r
Raw Improvement	Severity Index	23.8	-0.289	-0.218
Plasma [DMI]	Raw Improvement	21.0	-0.017	-0.750
Plasma ([DMI]	Severity Index	30.9	0.0062	0.359
Plasma [DMI + 2HD]	Raw Improvement	22.8	-0.0176	-0.752
Plasma [DMI + 2HD]	Severity Index	30.1	0.0067	0.379
RBC [DMI]	Raw Improvement	19.1	-0.0062	-0.693
RBC [DMI]	Severity Index	30.7	0.0036	0.524
RBC [DMI + 2HD]	Raw Improvement	20.2	-0.0062	-0.655
RBC [DMI + 2HD]	Severity Index	29.8	0.0038	0.532

y = y intercept of correlation line

s = line slope

r = correlation coefficient

Figure 9. DMI Plasma Concentration
Compared to Raw Improvement



often helpful when a third variable, in this case the Severity Index, may have an effect on the comparison of two variables, in this case drug concentration and Raw Improvement. For the DMI data, rank order correlations and partial correlations controlling for S.I. did not significantly improve correlation coefficient values over those calculated by The Pearson correlation. The rank for R.I. was from most improvement (number 1) to least improvement (highest number). The rank for DMI concentration was from lowest (number 1) to highest concentration. These ranks are included in Tables 30 and 31 for plasma and RBC data, respectively. The rank order correlation of plasma DMI concentration versus R.I. yielded a correlation coefficient of 0.785 ($p<0.01$). The rank order correlation of RBC DMI concentration versus R.I. yielded a correlation coefficient of 0.488. Partial correlations of plasma and RBC DMI concentration versus R.I. were made controlling for the initial severity of depression (as measured by the Severity Index). The partial correlation coefficient value for plasma DMI versus R.I. controlling for S.I. was -0.738. The corresponding RBC value was -0.697.

From these different ways of measuring correlations, it does appear that drug concentrations correlate well with Raw Improvement in this study. As the drug concentrations increased, the degree of patient improvement decreased.

3. Overdose fatality studies.

The specimens analyzed in the overdose fatality studies came from three Medical Examiner's cases. Two cases were shown to involve DMI ingestion and one was an IMP ingestion. Whole blood and brain

specimens were analyzed by both HPLC and GC-MS as described in Methods. There was a time lapse of several months between HPLC and GC-MS analyses. These samples contained more interfering peaks on HPLC than did clinical and animal specimens.

The concentrations in blood and brain for the three cases measured by HPLC and GC-MS are shown in Table 33. In the IMP fatality, IMP, DMI, DMD, 2HI and 2HD were found in both blood and brain. In the two DMI fatalities, DMI, DMD and 2HD were found in all blood and brain specimens. One interesting finding in DMI fatality-II was IMP in brain by both HPLC and GC-MS. It is noted that the concentration of IMP (0.34 μg/g by GC-MS) is much lower than that of DMI (51.9 μg/g by GC-MS) and is 0.65% of the DMI concentration.

The concentrations measured by GC-MS were used to derive brain to blood ratios of the parent drugs and metabolites. These data are shown in Table 34. Generally, the brain to blood ratios decrease from parent compounds to the more polar metabolites. The brain to blood ratio for DMI divided by the brain to blood ratio for 2HD in IMP fatality, DMI-fatality-I and DMI fatality-II, respectively, are 13.3, 5.86 and 6.15. Therefore, the concentration in brain compared to blood in these autopsy cases is much higher for DMI than for 2HD. In the one IMP fatality, the brain to blood ratio for IMP divided by the brain to blood ratio for 2HI is 2.22. Thus to a lesser extent, the concentration in brain compared to blood is higher for IMP than for 2HI in this one case. The DMI and 2HI brain to blood ratios are similar in the IMP case, 5.31 and 4.59, respectively. The quantities of IMP and DMI in the two tissues are greater than for the metabolites.

TABLE 33

Overdose Fatality Drug and Metabolite Concentrations
Determined by HPLC and GC-MS

	IMP	DMI	DMD	2HI	2HD
IMP FATALITY					
Blood by HPLC	3.88	3.27	INT	1.55	INT
Blood by GC-MS	2.87	2.77	0.076	1.54	1.23
Brain by HPLC	47.7	20.4	INT	8.34	INT
Brain by GC-MS	29.3	14.7	0.072	7.07	0.49
DMI FATALITY-I					
Blood by HPLC	-	42.5	0.96	-	6.50
Blood by GC-MS	-	35.0	0.66	-	3.96
Brain by HPLC	-	50.7	0.87	-	0.97
Brain by GC-MS	-	59.0	0.88	-	0.87
DMI FATALITY-II					
Blood by HPLC	-	5.56	1.95	-	1.87
Blood by GC-MS	-	5.90	1.70	-	1.67
Brain by HPLC	1.27	62.7	4.80	-	3.85
Brain by GC-MS	0.34	51.9	2.68	-	2.40

Blood concentrations in μg/ml
Brain concentrations in μg/g
INT - unable to measure due to interfering substances

TABLE 34

Overdose Fatality Brain to Blood Ratios
for Drug and Metabolites

	IMP	DMI	DMD	2HI	2HD
IMP Fatality	10.2	5.31	0.95	4.59	0.40
DMI Fatality-I	--	1.69	0.69	--	0.22
DMI Fatality-II	--	8.80	1.58	--	1.43

All concentrations used to calculate ratios were determined by GC-MS.

D. Animal Studies

1. Rabbit

Rabbits were used as an experimental model because they had been found to hydroxylate IMP and DMI as a major route of Phase I metabolism (52). The first rabbit experiment consisted of five male New Zealand white animals injected twice daily with 5 mg/kg of DMI. Blood was drawn by cardiac puncture, CSF from cisterna magna puncture and brain removed by surgery. Plasma, RBCs and brain were analyzed by HPLC for DMI and 2HD. In all specimens, no 2HD was detected at a detection limit of 5 ng per ml or g. Table 35 shows the DMI concentrations found in plasma, RBCs and brain in these five animals. Also included in Table 35 are the plasma to brain and RBC to brain ratios.

The dose of DMI was raised to 10 mg/kg twice per day in order to increase the blood and brain concentrations and to try to cause accumulation of 2HD. Three animals died within 24 hours of receiving the dose. Two of these rabbits had brain tissue removed after death and all three had whole blood samples removed by cardiac puncture. Table 36 contains the DMI and 2HD concentrations found in these specimens.

Two rabbits were injected with 2HD (7 mg each) and DMI (12.5 mg each) and sacrificed 20 minutes later. Plasma, RBCs and brain were analyzed for 2HD and DMI. One rabbit had low concentrations in all specimens (130 ng/g DMI in brain), possibly due to a poor i.p. injection. The other had DMI concentrations of 489 ng/ml in plasma, 1378 ng/ml in RBCs and 2350 ng/g in brain. The 2HD concentrations were 400 ng/ml in plasma, 1248 ng/ml in RBCs and none detected in brain. This rabbits brain specimen was also analyzed by GC-MS for 2HD. Again, none was detected.

TABLE 35

DMI Concentrations in Plasma, RBCs and Brain in Rabbits Treated Chronically with DMI

Animal	Plasma	RBCs	Brain	Plasma/ Brain	RBC/ Brain
1	47	22.5	285	0.165	0.079
2	32	96	360	0.089	0.267
3	64	16	189	0.339	0.085
4	237	106	550	0.431	0.193
5	89	109	175	0.509	0.623
Mean	93.8	69.9	312	0.307	0.249
S.D.	74.1	41.6	13.7	0.158	0.200
% S.D.	79%	60%	44%	51%	80%

Plasma and RBC concentrations in ng/ml.
Brain concentrations in ng/g.

TABLE 36

DMI and 2HD Concentrations in Whole Blood and Brain from Expired Rabbits

Animal Identification by Date of Death	D M I		2 H D	
	Plasma	Brain	Plasma	Brain
12-15-82	1154	N.M.	564	N.M.
12-16-82	228	535	348	N.D.
12-20-82	22	165	N.D.	N.D.

N.M. = Not measured
N.D. = None detected
Blood concentrations in ng/ml
Brain concentrations in ng/g

2. Rat

Rats were used in a chronic metabolism and distribution study. Four groups of five animals were administered IMP or DMI twice daily for five days. Plasma, RBCs and brain were analyzed for IMP, DMI, DMD, 2HI and 2HD by HPLC. No 2HI or 2HD was detected in any specimen (detection limit 5 ng/ml). Table 37 contains the IMP, DMI and DMD concentrations found in plasma, RBCs and brain of rats treated with 5 or 10 mg/kg of IMP. Table 38 contains the calculated plasma to RBC ratios for IMP, DMI and DMD in these animals. Table 39 contains the brain to plasma and brain to RBC ratios of the IMP concentrations calculated for these IMP treated rats. Generally, the DMI concentrations are higher than IMP concentrations in all specimens. DMD concentrations are the lowest.

DMI and DMD were measured in the DMI treated rats. These concentrations in plasma, RBCs and brain are listed in Table 40. The plasma to RBC ratios for DMI and DMD for these DMI treated rats are in Table 41. The brain to plasma and brain to RBC ratios of DMI concentrations for both IMP and DMI treated animals are listed in Table 42. The overall mean, and the mean for DMI treated and IMP treated rats is included.

In order to further study the relationship of brain DMI concentrations to plasma and RBC DMI concentrations these concentrations were compared in Figures 10, 11, 12 and 13. Brain DMI concentrations (y axis) are compared to plasma and RBC concentrations for both groups, IMP and DMI treated rats. In each comparison, linear regression was used to examine what correlation exists for the data. Included on each figure is the y intercept and slope of the best fit line and the correlation coefficient. For comparison, they are also listed in Table 43.

TABLE 37

IMP, DMI and DMD Concentrations in IMP Treated Rats

		PLASMA			RBC			BRAIN		
Animal	Dose	IMP	DMI	DMD	IMP	DMI	DMD	IMP	DMI	DMD
1	5	54	223	24	39	305	22	50	1505	165
2	5	83	347	104	69	763	46	155	2845	645
3	5	27	186	75	79	611	30	100	1080	460
4	5	50	34	16	14	42	0	615	195	57
5	5	22	406	36	291	347	0	140	1950	185
6	10	288	512	147	36	325	80	185	2340	685
7	10	38	410	69	35	366	6	38	2320	383
8	10	144	1076	396	40	366	278	115	2660	2810
9	10	109	1872	131	46	1215	30	400	7460	490
10	10	51	448	311	100	376	260	295	2000	1430

Dose in mg/kg B.I.D.
All concentrations in ng/ml except brain, ng/g.

TABLE 38

Plasma to RBC Ratios of IMP, DMI and DMD
in IMP Treated Rats

Animal	Dose	IMP	DMI	DMD
1	5	1.38	0.731	1.09
2	5	1.20	0.455	2.26
3	5	0.342	0.304	2.50
4	5	3.57	0.810	-
5	5	0.076	1.17	-
6	10	8.00	1.58	1.84
7	10	1.09	1.12	11.5
8	10	3.60	2.94	1.42
9	10	2.37	1.54	4.37
10	10	0.51	1.19	1.20
Mean		2.21	1.18	3.29
Standard Deviation		2.26	0.71	3.26
Relative S.D. (%)		102%	60%	100%

TABLE 39

Brain to Plasma and Brain to RBC IMP Concentration Ratios in IMP Treated Rats

Animal	Dose	Brain/Plasma	Brain/RBC
1	5	0.93	1.28
2	5	1.87	2.25
3	5	3.70	1.27
4	5	12.3	43.5
5	5	6.37	0.48
6	10	0.64	5.13
7	10	0.74	0.80
8	10	0.80	2.88
9	10	3.67	8.70
10	10	5.78	2.95
Mean ± Standard Deviation		3.68 ± 3.51	6.92 ± 12.4

Dose in mg/kg B.I.D.

TABLE 40

DMI and DMD Concentrations (ng/ml) in DMI Treated Rats

		PLASMA		RBC		BRAIN	
Animal	Dose	DMI	DMD	DMI	DMD	DMI	DMD
1	5	376	76	597	5	4275	460
2	5	279	91	572	59	4,050	1,035
3	5	266	51	403	0	4,855	510
4	5	193	161	356	142	2,685	1,760
5	5	707	61	766	16	4,765	315
6	10	803	324	1,551	194	7,200	2,120
7	10	949	319	1,366	245	8,560	2,040
8	10	962	607	1,439	579	10,370	3,900
9	10	1,189	251	1,632	99	14,320	1.530
10	10	1,352	211	1,789	135	12,760	1,340

Dose in mg/kg B.I.D.

TABLE 41

Plasma to RBC Ratios of DMI and DMD
in DMI Treated Rats

Animal	Dose	DMI	DMD
1	5	0.630	15.2
2	5	0.489	1.54
3	5	0.663	--
4	5	0.541	1.13
5	5	0.919	3.81
6	10	0.521	1.67
7	10	0.694	1.30
8	10	0.669	1.05
9	10	0.728	2.54
10	10	0.757	1.56
Overall mean		0.661	3.31
Standard Deviation		± 0.121	± 4.28
5 ng/kg mean		0.648	5.42
Standard Deviation		± 0.149	± 5.74
10 ng/kg mean		0.674	1.62
Standard Deviation		± 0.082	± 0.51

TABLE 42

Brain to Plasma and Brain to RBC DMI Concentration Ratios in IMP and DMI Treated Rats

Animal	Dose	Brain/Plasma	Brain/RBC
IMP-1	5	6.76	4.93
IMP-2	5	11.5	3.73
IMP-3	5	5.81	1.77
IMP-4	5	5.75	4.65
IMP-5	5	4.81	5.62
IMP-6	10	4.56	7.19
IMP-7	10	5.65	6.33
IMP-8	10	2.47	7.25
IMP-9	10	3.98	6.13
IMP-10	10	4.46	5.32
DMI-1	5	11.4	7.14
DMI-2	5	14.5	7.09
DMI-3	5	18.2	12.0
DMI-4	5	13.9	7.52
DMI-5	5	6.76	6.21
DMI-6	10	8.98	4.65
DMI-7	10	9.01	6.25
DMI-8	10	10.7	7.19
DMI-9	10	12.0	8.77
DMI-10	10	9.43	7.14
Overall mean ± S.D.		8.53 ± 4.04	6.34 ± 2.00
IMP mean ± S.D.		5.57 ± 2.27	5.29 ± 1.57
DMI mean ± S.D.		11.5 ± 3.2	7.40 ± 1.83

Figure 10. Plasma versus Brain DMI Concentrations in IMP Treated Rats

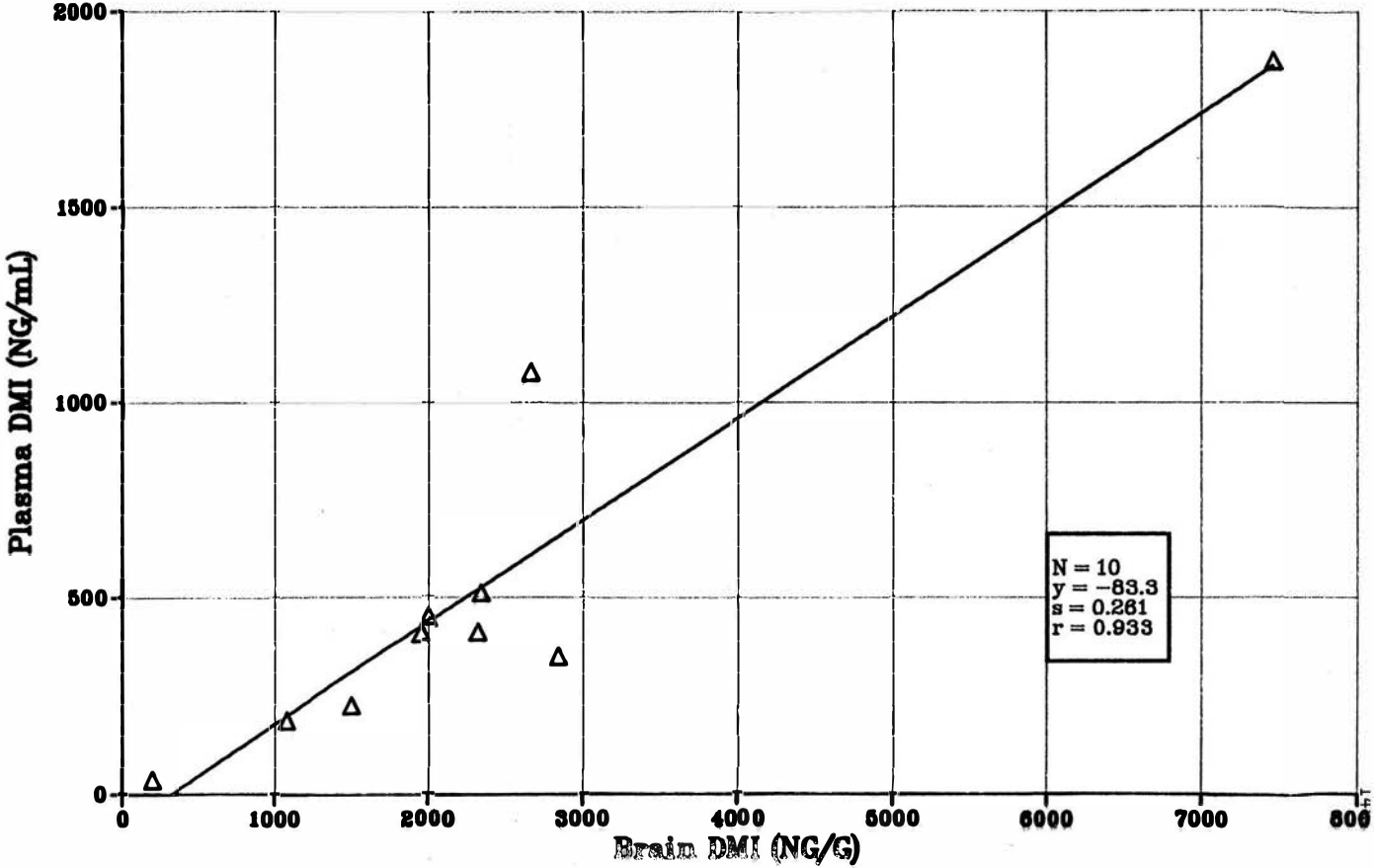


Figure 11. RBC versus Brain DMI
Concentrations in IMP Treated Rats

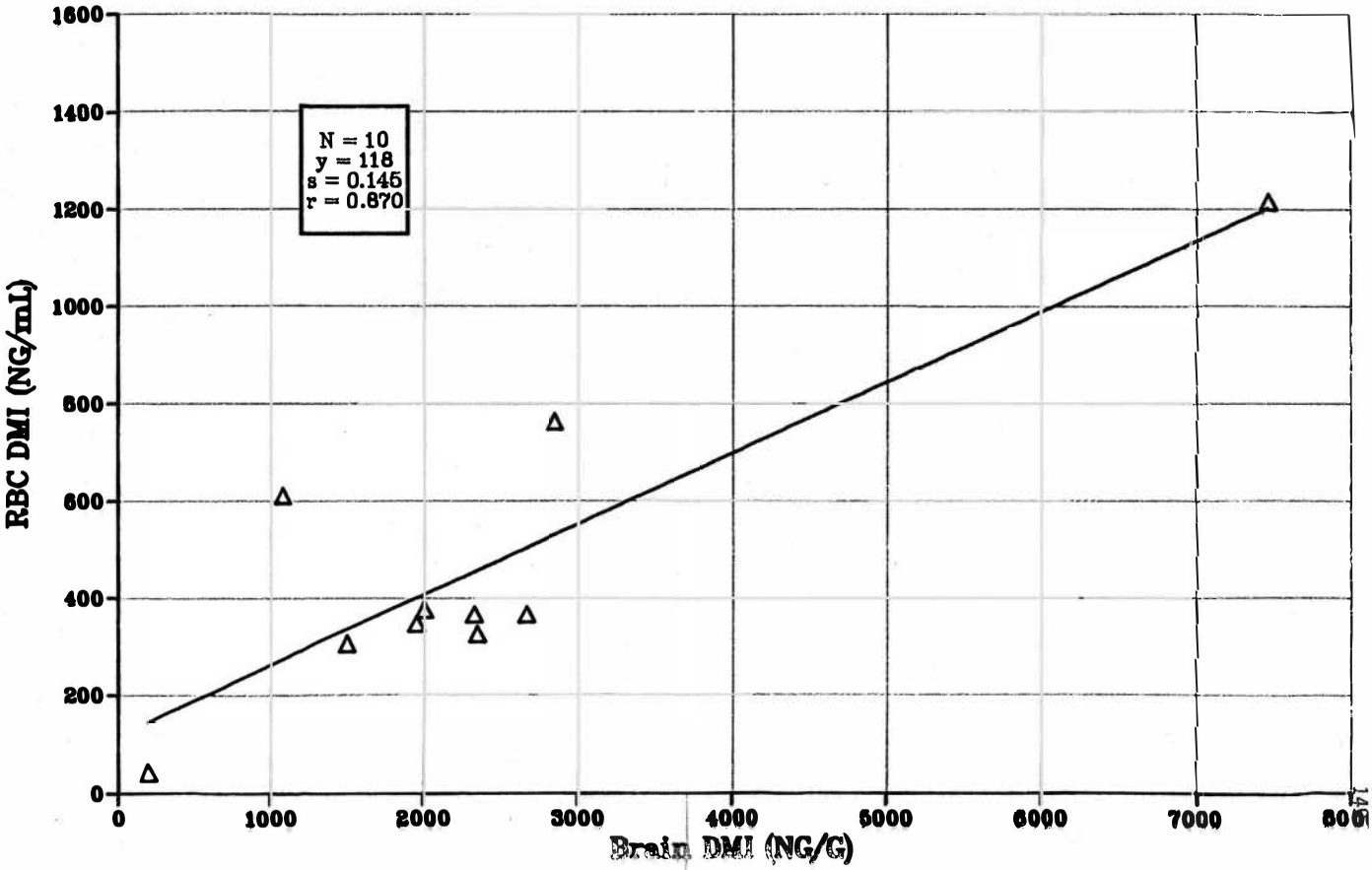


Figure 12. Plasma versus Brain DMI Concentrations in DMI Treated Rats

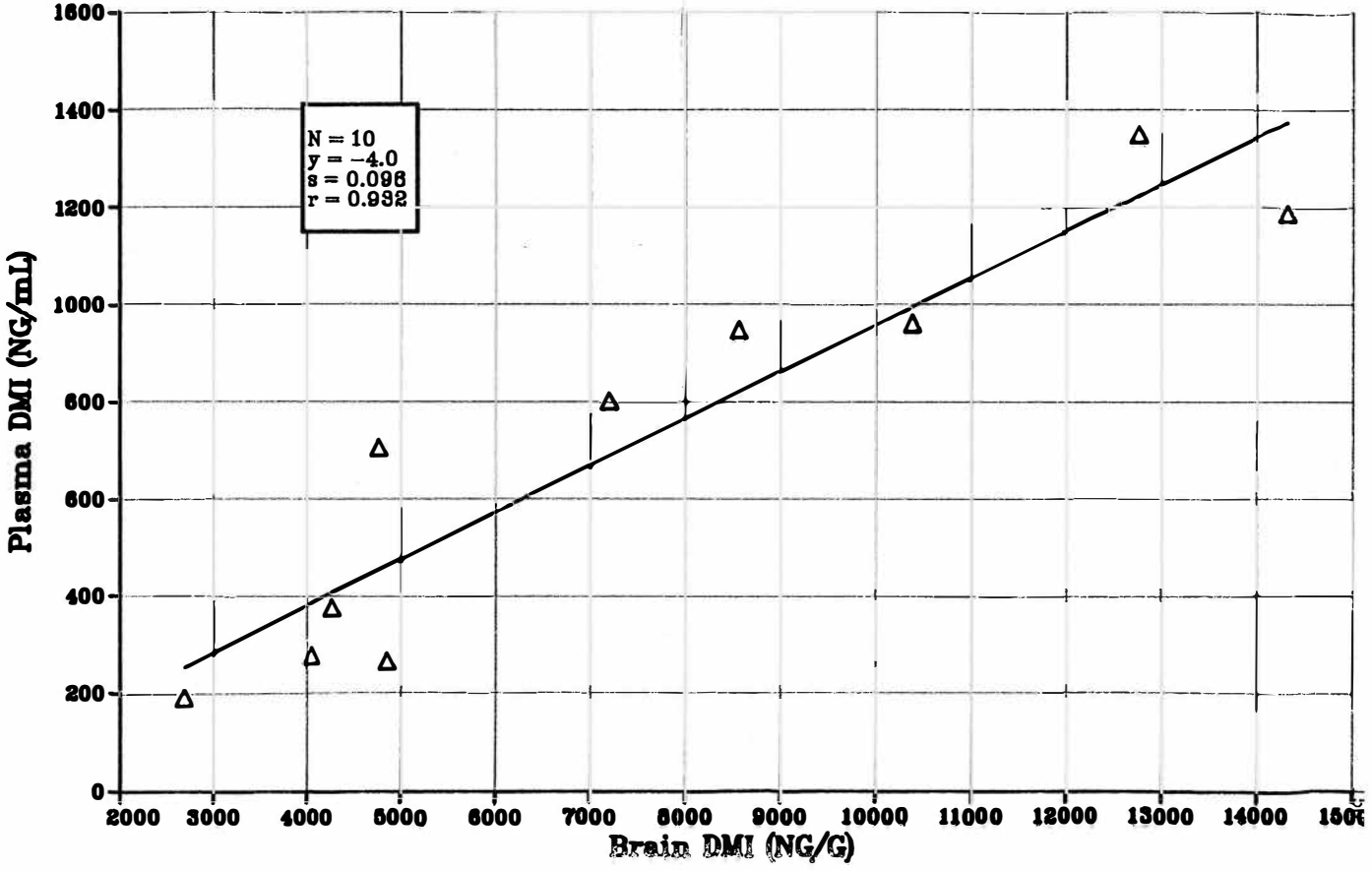


Figure 13. RBC versus Brain DMI
Concentrations in DMI Treated Rats

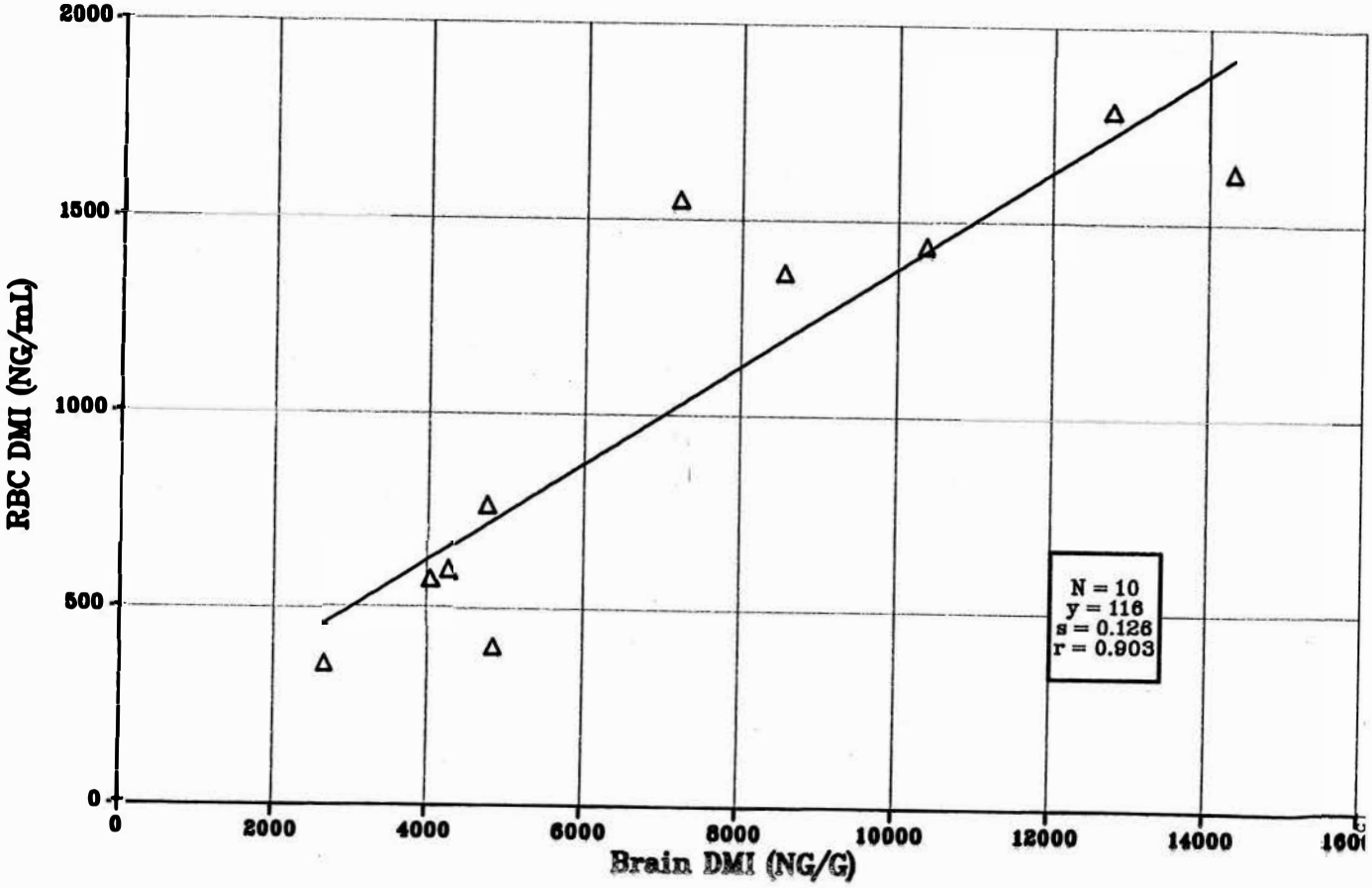


TABLE 43

Linear Regression Data for Brain DMI Concentrations Versus Plasma or RBC DMI Concentrations Comparisons in IMP or DMI Treated Rats

Comparison	y	s	r
Brain vs. plasma in IMP treated (Fig. 10)	-83.3	0.261	0.933
Brain vs. RBC in IMP treated (Fig. 11)	118	0.145	0.870
Brain vs. plasma in DMI treated (Fig. 12)	-4.0	0.096	0.932
Brain vs. RBC in DMI treated (Fig. 13)	116	0.126	0.903

y = y intercept of regression line

s = line slope

r = correlation coefficient

A group of ten rats were injected with 2HD and DMI to study the distribution of the two compounds. Five animals were sacrificed 30 minutes after their injections and the other five were sacrificed 60 minutes after the injections. Plasma and brains were analyzed for 2HD and DMI by HPLC. Table 44 contains these concentrations. The brain to plasma ratios of the DMI and 2HD concentrations were calculated and placed in Table 45. Also included are values in a column labelled: 2HD Entry to Brain Relative to DMI. These values for each animal represent the 2HD to DMI ratio in brain if the plasma concentrations of the two were equal and the same brain to plasma ratios for 2HD and DMI existed as found experimentally. These values were calculated by dividing the 2HD brain to plasma concentration ratio by the DMI brain to plasma concentration ratio for each animal as listed in Table 45. The purpose of the 2HD Entry to Brain Relative to DMI value is to show the relative accessibility of 2HD and DMI from blood into brain found in this experiment. Also included in Table 45 are the overall mean for 10 animals and the means for the 0.5 and 1 hour groups for the brain to plasma ratios and the 2HD Entry to Brain Relative to DMI data.

The brain specimen from a rat treated with 10 mg/kg B.I.D. for five days (animal No. 9 from Table 37) was analyzed for 2HD by GC-MS. This rat was chosen because of the high concentration of DMI found in the brain (7460 ng/g). By GC-MS, no 2HD was found above the level of d_0 2HD present as contamination in the d_4 2HD internal standard.

TABLE 44

DMI and 2HD Concentrations in Plasma and Brain
in DMI-2HD Treated Rats

		DMI		2HD	
Animal	Time	Plasma	Brain	Plasma	Brain
1	0.5 hr	132	624	122	17
2	0.5 hr	80	376	64	15
3	0.5 hr	24	92	21	15
4	0.5 hr	154	403	96	17
5	0.5 hr	171	372	76	17
6	1 hr	48	433	53	30
7	1 hr	49	460	42	10
8	1 hr	85	557	76	23
9	1 hr	69	547	58	37
10	1 hr	77	641	51	6

Plasma concentrations are ng/ml.
Brain concentrations are ng/g.

TABLE 45

Brain to Plasma Ratios for DMI and 2HD Concentrations in DMI-2HD Treated Rats

Animal	Time	D M I Brain/Plasma	2 H D Brain/Plasma	2HD Entry to Brain Relative to DMI
1	0.5 hr	4.73	0.14	0.030
2	0.5 hr	4.70	0.23	0.049
3	0.5 hr	3.83	0.71	0.185
4	0.5 hr	2.62	0.18	0.069
5	0.5 hr	2.17	0.22	0.101
6	1 hr	9.02	0.57	0.063
7	1 hr	9.39	0.24	0.025
8	1 hr	6.55	0.30	0.046
9	1 hr	7.39	0.64	0.087
10	1 hr	8.32	0.12	0.014
Overall mean ± S.D.		5.87 ± 2.49	0.33 ± 0.21	0.067 ± 0.047
0.5 hour mean ± S.D.		3.61 ± 1.05	0.30 ± 0.21	0.087 ± 0.054
1 hour mean ± S.D.		8.13 ± 1.04	0.37 ± 0.20	0.047 ± 0.026

DISCUSSION

A. Metabolism

1. Rat

The metabolism of IMP and DMI as shown in the chronic administration rat studies in this research supports the findings of earlier research. IMP is rapidly metabolized via demethylation to DMI, which then accumulates in the blood. Table 37 shows that the DMI concentration in plasma, RBCs and brain exceeds the IMP concentration in IMP treated rats in all instances except for animal Number 4. There is demethylation of DMI, also, to DMD. This demethylation of DMI does not appear to occur as rapidly as IMP demethylation. In no tissue of IMP treated rats (Table 37) or in DMI treated rats (Table 40) is the DMD concentration equal to or greater than the DMI concentration. This supports the findings of Gillette et al. (50, 51) who noted that IMP is more rapidly demethylated than DMI in rat microsome preparations.

Hydroxylation of IMP and DMI has been found to be a minor metabolic pathway in rats (50, 51, 52). In the chronic administration studies presented here, very little free 2HI or 2HD could be detected in plasma, RBCs and brain. The amounts present could not be differentiated from background noise on the HPLC and if they were present they could only be small amounts in relation to IMP, DMI, and DMD.

The metabolism of IMP and DMI in the Sprague-Dawley rats used here caused rapid disappearance of IMP from blood and brain with

appearance of DMI. The clearance of DMI was slower and DMD appeared as the principle metabolite. No accumulation of free 2-hydroxy metabolites was found.

2. Rabbit

As documented by Gillette et al. (52), rabbits hydroxylate IMP and DMI to a greater extent than do rats. They also demethylate IMP and DMI. It was found here that the overall metabolism of DMI was much faster in the rabbit than the rat. Chronic treatment with 5 mg/kg of DMI in rabbit gave only one animal of five with DMI concentration in plasma over 100 ng/ml (Table 35) while the same dose regimen in rats caused DMI concentrations over 100 ng/ml in all five animals (Table 40). The clearance of DMI and 2HD from rabbits was rapid enough so that little 2HD accumulated in plasma when the DMI was administered twice daily and blood drawn six hours after the last dose. However, 2HD was found in rabbits injected with 10 mg/kg of DMI who expired sometime in the next 12 hours as shown in Table 36. The presence of 2HD in the plasma of two of these three rabbits is evidence of hydroxylation. The ratio of 2HD to DMI in these plasma specimens was 0.49 and 1.53 (rabbits expiring 12-15-82 and 12-16-82, respectively), which indicate a substantial production of 2HD.

Rabbits clear DMI from their blood more rapidly than do rats. They produce 2HD which reaches their blood in much greater concentrations than 2HD that reaches the blood of the rats of these studies. Clearance of DMI was too rapid to allow practical distribution studies of 2HD in these rabbits. The distribution studies of 2HD in both rat and rabbit was performed by administering 2HD and DMI together.

3. Human

In the studies of rat, rabbit and man the concentrations of parent drug and metabolites in the blood were used as an indication of the type (i.e. demethylation and hydroxylation) and degree (as indicated by the relative concentrations) of metabolism of IMP and DMI which occurs to the greatest extent in the liver (55). Therefore, the actual types and rates of metabolism were not studied. It is the concentrations of the drugs and metabolites in the tissues, in this case plasma, RBCs and brain, that is of interest in this research. The concentrations in the brain (where the receptor sites are thought to be) and in the blood, which may be in equilibrium with the receptor sites, were determined in order to find the relative amounts of parent drug and metabolites.

In rat and rabbit it was found that qualitatively the same metabolites are present in blood and brain with one exception. The exception was 2HD in the rabbit which was present in plasma but not detected in brain.

The metabolism of IMP and DMI in human, as indicated by plasma and RBC concentrations of IMP, DMI, DMD, 2HI and 2HD, does not directly compare to metabolism in rat or rabbit. In patients treated with IMP, all or some of these compounds accumulated in the plasma. These results compare well to earlier studies (53-57, 77) which have found the same metabolites. Table 21 shows the steady state concentrations of IMP and DMI and the relative amounts of DMD, 2HI and 2HD. As noted, all metabolites were not detected in all patients. For example, patients A-2, H-1 and H-4 had no DMD detectable and patients K-1, D-1 and J-3 had

no 2HI detectable. All patients had IMP and DMI concentrations greater than the detection limit of 5 ng/ml.

There was variation in the degree of demethylation of IMP to DMI. Figure 4 shows the range of the DMI to IMP ratio in plasma. This ratio ranged from below 1 to over 10. Half of the 24 patients had more DMI than IMP in plasma at steady state and the other half more IMP than DMI. This variation in the degree of demethylation of IMP has been reported by other investigators (7, 9, 55).

There was variation in the relative amounts of hydroxylated metabolites found in plasma at steady state also. Examination of Table 21 points this out. There was relatively more 2HD than 2HI in IMP treated patients. Figure 3 shows the distribution of the 2HI and 2HD to total IMP plus DMI ratios. More patients had ratios greater than 0.20 for 2HD to total IMP plus DMI than for 2HI to total IMP plus DMI. In these patients, 2HD accumulates in plasma to a greater extent than does 2HI. In fact, 2HI is a minor metabolite in plasma as only one patient in 24 had a 2HI concentration greater than 20% of the combined IMP and DMI concentrations. 2HD is a more prominent metabolite in plasma as 25% of patients had 2HD concentrations of 30% or greater of the combined IMP and DMI concentrations.

From Table 22, it will appear that patients who accumulate hydroxylated metabolites (designated as fast hydroxylators) will clear IMP and DMI more quickly than patients who do not accumulate 2HI and 2HD. Table 22 shows that "fast hydroxylators" have less than half the combined IMP plus DMI concentrations than do "slow hydroxylators" without regard to the DMI to IMP ratio (the factor used for selecting patients as fast or slow demethylators). It appears that the rate of

hydroxylation may determine the rate of clearance of IMP and DMI in humans.

In patients in this study taking IMP, both demethylation and hydroxylation is evident. The degree of demethylation of DMI appears to be low, as little DMD accumulates in plasma (Table 21). The largest DMD to total IMP plus DMI ratio is 0.150 and the mean is 0.051. These studies are the first to investigate the significance of DMD as a metabolite of IMP and DMI. In both cases, DMD is a minor metabolite. These plasma concentrations would indicate that based on relative concentrations, IMP and DMI are the most prominent compounds and 2HD reaches appreciable concentrations in some patients.

In patients in the DMI study, DMI, DMD and 2HD were detected in plasma (Table 27). At a steady state, DMD is a minor metabolite of DMI in plasma. The highest DMD to DMI ratio is in patient JB#19 and was 0.107. The mean DMD/DMI ratio was 0.067. The variation in this ratio was not great as it ranged from 0.022 to 0.107.

On the other hand, steady state plasma concentrations indicate 2HD is a major metabolite of DMI. In the 12 patients studied the mean 2HD to DMI ratio was 0.321 and 8 patients (67%) had ratios greater than 0.300. In no instance, however, was the steady state 2HD concentration greater than the DMI concentration. Other investigators have reported 2HD plasma concentrations in humans. Potter et al. (57) found mean 2HD to DMI ratios of 0.38, 0.23, 0.43 and 0.45 in four study groups. These mean values compare very well to the value of 0.321 found here. They, however, found ratios greater than one in some patients. Their patient population age range (7 to 13 years) was lower than the age range used in this DMI study (23 to 51). Perhaps adolescents accumulate more 2HD

in plasma due to its increased production or reduced elimination compared to adults. Devane, Savett and Jusko measured 2HD and DMI in four subjects. Only a single 50 mg dose was administered so no steady state concentrations were measured. They found the maximum 2HD plasma concentration to be 76% to 144% of the DMI concentration and the mean AUC of 2HD to be 79% of the AUC of DMI. Although not direct comparisons with the present study, this data is consistent with the present study. The degree of hydroxylation does seem to effect the rate of clearance of DMI just as it appears to effect the rate of clearance of IMP. The two patients with the highest steady state plasma DMI concentrations, GW#21 (786 ng/ml) and EW#32 (1123 ng/ml), had the lowest 2HD to DMI ratios (0.024 and 0.078, respectively). Low relative accumulation of 2HD in plasma may indicate low rates of hydroxylation and low rates of clearance of DMI.

In summary, humans show demethylation and hydroxylation of IMP and DMI. There is variation from patient to patient in both demethylation and hydroxylation. DMD and 2HI are minor metabolites of IMP and DMD is a minor metabolite of DMI. 2HD reaches appreciable concentrations in some IMP and a majority of DMI treated patients in these studies. The rate of clearance of both IMP and DMI is effected by hydroxylation and patients with greater relative concentrations of hydroxylated metabolites had lower IMP plus DMI concentrations or lower DMI concentrations depending on IMP or DMI treatment, respectively.

B. Plasma Versus RBC Concentrations

In twelve IMP patients, ten DMI patients and in 10 rats each treated with IMP or DMI, the red blood cells were analyzed for parent drug and

some metabolites as well as plasma. Some *in vitro* studies of DMI, DMD and 2HD distribution between plasma and RBCs were performed also. The purpose was to determine how the compounds equilibrated between plasma and RBC as a basis for comparison of plasma and RBC concentrations versus clinical response studies.

The data indicate that the equilibrium ratios for IMP between plasma and RBC's differs from the equilibrium ratios for DMI between plasma and RBCs in humans. Table 23 contains the DMI/IMP ratios for both plasma and RBCs in IMP patients. The mean RBC DMI/IMP ratio was 5.14 and the mean DMI/IMP ratio in plasma was 2.03. The mean combined IMP plus DMI concentrations in plasma (234.5 ng/ml) and RBCs (283 ng/ml) did not differ significantly. The mean plasma IMP concentration to RBC IMP concentration was 1.80 (± 0.80) for the twelve IMP patients. The DMI concentration plasma to RBC ratio mean was 0.678 (± 0.285) for the same patients. Therefore, in blood the IMP concentration in plasma is greater than in RBCs while the DMI concentration in plasma is less than in RBCs in these patients.

The mean steady state plasma concentration to RBC concentration ratio for DMI in DMI treated patients (Table 29) was similar to that found in IMP treated patients. The mean ratio in DMI treated patients was 0.742 (± 0.336) and in IMP patients it was, again, 0.678 (± 0.285). The difference in the two ratios is not significant ($p<.001$). Linnoila, Dorrity and Jobson (83) found similar plasma to RBC ratios for DMI concentrations. The mean value for 15 samples was 0.50, which is within the range of values found in the present study. The difference in the plasma to RBC ratios for IMP versus DMI in IMP patients was significant ($p<.005$). In these patients, IMP and DMI distribute

significantly different between plasma and RBCs. DMI has a greater affinity for RBCs.

From Table 29 it can be seen that the plasma to RBC ratios for DMD (0.556 ± 0.362) and 2HD (0.673 ± 0.610) do not differ much from that for DMI (0.742 ± 0.336). DMI, DMD and 2HD distribute similarly between plasma and RBCs in these patients.

In the *in vitro* studies of 2HD, DMD and 2HD, it was shown that equilibrium between plasma and RBCs was reached in ten minutes or less (Table 14). If DMI was added to RBCs first rather than to whole blood, the mean plasma and RBC concentrations of DMI did not differ significantly (Tables 14 and 15). There were slight variations in those plasma and RBC concentrations from week to week (Table 16). It was found that the plasma to RBC DMI concentration ratio was constant over a range of 250 to 3000 ng/ml of DMI in whole blood (Table 17). This means there was no saturation of RBCs or plasma in this range causing a shift in the ratio.

The distribution of DMI between plasma and RBCs for 15 normal persons is shown in Table 18. The mean plasma to RBC ratio was 0.526 ± 0.092. No difference for males (0.532) and females (0.519) was noted. This mean plasma to RBC DMI concentration ratio of 0.526 is lower than that found in DMI patients (0.742) and IMP patients (0.678). For this number of patients (15 normals, 10 DMI patients and 12 IMP patients) the difference in plasma to RBC ratios for DMI concentrations may be significant but more patients would need study to confirm it. It is interesting that Linnoila et al. (83) found a ratio of 0.50 in an *in vivo* study of normal volunteers. That ratio, 0.50, is very close to the *in vitro* normal population value found here of 0.526.

In rats, the IMP plasma to RBC ratio was greater than the DMI plasma to RBC ratio as it was in humans. From Table 38, the IMP ratio is 2.21 (± 2.26) and the DMI ratio (for IMP treated rats) is 1.18 (± 0.71). The DMI ratio for DMI treated rats (Table 41) is 0.661 (± 0.121). For DMI treated rats the dose of DMI did not effect the DMI plasma to RBC ratios (mean for 5 mg/kg dose is 0.648 and the mean for 10 mg/kg dose is 0.674). The DMI plasma to RBC ratio means for IMP treated rats (1.18) differed from that for DMI treated rats (0.661). However, the large variation in the IMP treated group's ratios (standard deviation of 0.71) make the difference not significant.

It appears that IMP and DMI distribute differently between plasma and RBCs in both man and rat. In both species, IMP has a greater affinity for plasma than RBCs. Also in both species, DMI has a greater affinity for RBCs than plasma.

C. Drug Concentrations Versus Clinical Response

1. Imipramine

No good correlation between clinical response as measured by Hamilton Depression Rating Scores and plasma or RBC drug and metabolite concentrations was found for IMP treated patients. Tables 24 and 25 contain all the data used to calculate the Pearson correlation data in Table 26. Raw Improvement Scores indicate the difference in HDS before therapy and during therapy. The Severity Index is the HDS at the start of therapy and is a measure of the extent of depression. The higher the score the worse is the depression rating at the time of testing. Both plasma and RBC values for IMP and DMI concentrations,the total IMP plus DMI concentrations and the DMI to IMP ratios were compared by Pearson

correlation to raw improvement. Table 26 shows that the correlation coefficients (r) for all these comparisons were low and no significant ($p < .01$) correlation was found. The highest value for r was a negative 0.395. The $r = -0.395$ was found for RBC DMI/IMP ratio versus raw improvement and would indicate that there was greater improvement for patients with low DMI/IMP ratios than those with higher ratios. Again, this correlation was not statistically significant.

This data indicates that for this patient population (24 patients for plasma studies and 12 for RBC studies) in this particular study protocol no clear correlation between plasma and RBC drug concentration and clinical response was found. Other studies using IMP have found good correlations (21, 31), while others have not (23, 27). The present study of IMP treated patients and clinical response was well planned and executed. A placebo washout period was included and IMP was administered long enough to be efficacous (6). Compliance with dose regimen could not be certain due to the use of out-patients.

2. Desipramine

There were some good correlations found between plasma and RBC drug concentrations and Raw Improvement in the patients treated with DMI. Tables 30 and 31 contain Severity Index Scores, raw improvement scores and plasma and RBC drug concentrations for these patients. Table 32 contains the results of Pearson correlations for this data. The correlations of Raw Improvement versus plasma [DMI] ($r = -0.750$), plasma [DMI + 2HD] ($r = -0.752$), RBC [DMI] ($r = -0.693$) and RBC [DMI + 2HD] ($r = -0.655$) are all significant (at least $p < 0.05$). Figure 9 is a graph example of one of these comparisons. In all instances, these correlations indicate that Raw Improvement declines as drug concentrations

increase. These patients were treated with a high dose of DMI (300 mg per day) so that high concentrations in blood would generally occur. The purpose was to study clinical response at high concentrations. The data indicate that response declines (that is, less improvement in depression occurs) at high DMI concentrations in blood. There is little apparent difference between using RBC and plasma DMI concentrations to check correlation to Raw Improvement. The correlation coefficient for RBC [DMI] of -0.693 and for plasma [DMI] of -0.750 are not significantly different for the number of patients used. The inclusion of 2HD concentrations did not significantly change the correlation coefficients for the plasma comparison (-0.750 without 2HD and -0.752 with 2HD) or for the RBC comparison (-0.693 without 2HD and -0.655 with 2HD).

In this study using high dose DMI therapy, a negative correlation between drug concentration in blood and clinical response was found. At high plasma and RBC DMI concentrations there was less improvement in the patient's depression syndrome. This is the first report of a decrease in improvement at high steady state DMI concentrations. This phenomenon has been reported for nortriptyline (102) and protriptyline (103).

D. Drug and Metabolite Distribution to Brain

As noted earlier, IMP patients were found to have IMP, DMI, DMD, 2HI and 2HD in their plasma. All metabolites were not found in all these patients. DMI treated patients had DMI, DMD and 2HD in their plasma. Three sets of autopsy specimens consisting of whole blood and brain were analyzed for these compounds. Two fatalities were associated with DMI ingestion and one with IMP ingestion. In both blood and brain,

IMP, DMI, DMD, 2HI and 2HD were detected in specimens from the IMP fatality (Table 33). Blood and brain specimens from both DMI fatalities were shown to contain DMI, DMD and 2HD. Therefore, there was evidence that all metabolites found in clinical patient's plasma could be found in human brain after overdose with IMP and DMI.

The relative amounts of the drugs and metabolites that reach the brain from the blood are important when considering relative importance of drug and metabolites in the pharmacology of IMP and DMI. IMP and DMI are considered to be centrally acting drugs (58). The site of action or receptors are thought to be in brain (71). The concentrations of the drugs and metabolites at the receptors may influence the degree of pharmacological response.

From the plasma concentrations, 2HI and DMD were considered to be minor metabolites. 2HD was found in appreciable concentrations in both DMI and some IMP treated patients. The relative amount of 2HD that reaches the brain may determine whether 2HD is indeed a significant metabolite which should then be considered along with DMI in DMI treated patients and perhaps with IMP and DMI in IMP treated patients.

The brain to blood ratios listed in Table 34 for the overdose fatality cases are an indication of the entry of each drug and metabolite into brain from blood. The degree of entry into brain has been theorized to depend on the polarity of a compound (65). The more polar the compound, the less the degree of entry into brain. If this theory is correct, the more polar metabolites of IMP and DMI would cross the "blood-brain barrier" to a lesser amount than IMP and DMI.

In Table 34, the brain to blood ratio for 2HD is always less than the brain to blood ratio for IMP and DMI. In the IMP fatality, the

IMP ratio is 10.2, the DMI ratio is 5.31 and the 2HD ratio is 0.40. That means ten times as much IMP was found in brain than blood, 5 times as much DMI was found in brain than blood but less than half as much 2HD was found in brain than blood.

The brain to blood ratios for 2HD in the two DMI fatalities were significantly lower than the brain to blood ratios for DMI. In both instances, it is indicated that 2HD enters the brain to a lesser extent than does DMI. The overdose fatality data must be viewed carefully. The conditions of drug administration were not controlled and the specimens not collected promptly at time of death.

A controlled study using rats was used to better study the relative entry of 2HD and DMI from blood to brain. Both DMI and 2HD were administered to ten rats. Five were sacrificed at 30 minutes post-injection and five after 60 minutes. The brain to blood ratio of DMI concentrations was used to judge when distribution had reached equilibrium. In the chronic DMI administration study, the brain to plasma DMI ratio had a mean value of 11.5 (± 3.2) as shown in Table 42. These are equilibrium values because this was a chronic study and animals were sacrificed at least 10 hours after the last dose. In comparison, the brain to plasma ratio for DMI concentrations in the 30 minute group of rats was 3.16 (± 1.05) and in the 60 minute group was 8.13 (± 1.04) as shown in Table 45. Therefore the 60 minute group was approaching equilibrium for distribution of DMI to brain from blood.

Table 44 contains all the 2HD and DMI concentrations in plasma and brain from rats administered 2HD and DMI. Table 45 contains all the brain to plasma concentration ratios. As distribution approached equilibrium at 60 minutes, the brain to plasma 2HD ratio was 0.37 (± 1.20)

and the brain to plasma DMI ratio was 8.13 (± 1.04). These ratios show that 2HD entry into brain is much less than entry for DMI. The DMI concentration in brain was an average 8 times the plasma concentration. On the other hand, 2HD brain concentration was on average less than one-half the plasma 2HD concentration.

In Table 45, a column labelled 2HD Entry to Brain Relative to DMI shows the relative access of 2HD to brain compared to DMI. If blood concentrations were equal for 2HD and DMI, the 2HD concentration in brain would be an average of 4.7% of the brain DMI concentration. This experiment shows that in the rat, here used as a model, 2HD has poor access to brain compared to DMI and 2HD blood concentrations would have to be much higher than DMI blood concentrations to yield similar brain concentrations. The DMI clinical patient data showed no steady state plasma samples to have 2HD concentrations equal to or higher than DMI concentrations.

Rats were also used as a model to compare plasma and RBC DMI concentrations to brain DMI concentrations. The Pearson Correlation data are shown in Table 43. As can be seen in the correlation coefficients (r), the plasma and RBC DMI concentrations correlated well to brain DMI concentrations for both IMP treated and DMI treated animals. All r values were 0.870 or greater ($p < .01$). Figures 10 through 13 are graphs of each correlation. The brain versus RBC correlations for both IMP and DMI treated rats were very similar. IMP treated animals had $r = 0.870$ versus $r = 0.903$ for DMI treated animals. More importantly, the y intercepts (118 and 116) and slopes (0.145 and 0.126) of the linear regression line were very similar, indicating that the relationship of brain to RBC DMI concentrations was the same for both groups, IMP and

DMI treated animals. It made no difference in these comparisons whether the animals were treated with IMP or DMI.

However, the plasma versus brain DMI concentration comparisons differed for IMP treated versus DMI treated rats. Both had good correlation coefficients ($r = 0.933$ and $r = 0.932$, respectively), in fact, better than the RBC to brain comparisons. The slopes of the regression lines differed substantially. The IMP treated group had a slope of 0.261 while the DMI treated group's slope was 0.096. The y intercepts were -83.3 and -4.0, respectively. These data indicate that the relationship between the plasma DMI concentration and the brain DMI concentration may be different if the animal is administered IMP rather than DMI. IMP in the tissues appears to shift the ratio of plasma to brain DMI concentrations but not the ratio of RBC to brain DMI concentrations. If other drugs also shifted the plasma to brain DMI ratio but not the RBC to brain DMI ratio, RBC concentrations might be a more reliable indicator of brain DMI concentrations. However, as noted, in all instances plasma and RBC DMI concentrations correlated well with brain DMI concentrations in these rat studies. Further investigation in the rat of these relationships might be fruitful.

CONCLUSION

A three step extraction procedure for isolating IMP, DMI, DMD, 2HI and 2HD from plasma, red blood cells and brain has been developed. An HPLC method has been developed to separate the compounds and allow quantitation by peak height compared to an internal standard. These procedures have been used to accurately and precisely quantitate these compounds in human, rat and rabbit blood and brain specimens. Accuracy has been shown by comparison to an established GC-MS procedure.

DMD is a minor metabolite of IMP and DMI. DMD does not accumulate in patient plasma samples to above 20% of the combined IMP and DMI concentrations in IMP treated patients or to above 20% of the DMI concentration in DMI treated patients. For therapeutic drug monitoring, the DMD concentration is probably insignificant.

2HI is a minor metabolite of IMP. It infrequently accumulates to over 20% of the combined IMP plus DMI concentration in plasma. At steady state, 2HI was found to be less than 10% of the IMP plus DMI concentration in the majority of patients. Measurement of 2HI may be useful in pharmacokinetic and metabolism studies but not for general therapeutic drug monitoring.

2HD is a major metabolite of DMI in plasma of patients at steady state. It also reaches appreciable quantities (defined here as greater than 20% of IMP plus DMI concentrations) in IMP treated patients. 2HD

concentrations reach values large enough in plasma to be potentially significant therapeutically in both DMI and some IMP treated patients.

The accumulation of hydroxylated metabolites is associated with increased clearance of IMP and DMI compared to patients who have lower relative concentrations of hydroxylated metabolites. Patients designated fast hydroxylators have significantly lower plasma IMP plus DMI concentrations than do slow hydroxylators. Hydroxylation may be the rate limiting route of metabolism in man for IMP and DMI.

IMP, DMI, DMD, 2HI and 2HD have been quantitated in the brain of an IMP overdose fatality. DMI, DMD and 2HD have been quantitated in the brains of two DMI overdose fatalities. The brain to blood ratio of 2HD concentrations is much less than the brain to blood ratios of IMP and DMI concentrations. This is an indication that 2HD cannot enter the brain as easily as IMP and DMI.

Rats and rabbits were used as models to study IMP, DMI and 2HD entry into brain. In rabbits, no 2HD could be detected in brain despite significant amounts in the blood. A larger study in rats showed that 2HD is, indeed, restricted from entering brain compared to DMI. The relative ability of 2HD and DMI to enter brain shows that substantially higher blood concentrations of 2HD compared to DMI would be necessary to produce equal concentrations in brain. The combined rat data and human overdose fatality data indicate that 2HD probably does not reach concentrations in brain to be significant pharmacologically compared to DMI.

It has been shown that IMP and DMI distribute differently between plasma and red blood cells. DMI has a greater affinity for RBCs than plasma. IMP is the reverse. It has higher concentrations in plasma than RBCs. The distribution for both is similar in humans and rats.

Both RBC and plasma DMI concentrations correlated well with brain concentrations in the rats. However, the presence of IMP changed the relationship between plasma DMI and brain DMI concentrations but not RBC DMI and brain DMI concentrations. Because IMP shows a greater affinity for plasma than RBCs, it may shift the DMI equilibrium between plasma and RBCs.

These quantitative drug studies were performed in conjunction with clinical evaluation of patients. Clinical improvement could not be correlated to drug and metabolite concentrations for IMP treated patients. Some similar clinical studies have found reasonably good correlations between the two while other studies have not.

There were found to be good correlations between both plasma and RBC DMI concentrations and improvement in the patient's depressive syndrome. The DMI study was designed to study the effect of high DMI concentrations and clinical response. As the DMI concentration increased in this study, the degree of improvement in depression decreased. That is, patients with high plasma DMI concentrations (generally, 400 ng/ml or greater) showed less improvement than patients with lower plasma DMI concentrations (less than 400 ng/ml). Combining the 2HD concentration and the DMI concentration did not change the correlations to improvement significantly.

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APPENDIX 1

American Psychiatric Association; Quick Reference to the Diagnostic criteria from Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Washington, D. C., APA, 1980, pages 117 to 130.

MAJOR AFFECTIVE DISORDERS

Manic Episode

Differential diagnosis. Organic Affective Syndromes; Schizophrenia, Paranoid Type; Schizoaffective Disorder; Cyclothymic Disorder.

Diagnostic criteria.

A. One or more distinct periods with a predominantly elevated, expansive or irritable mood. The elevated or irritable mood must be a prominent part of the illness and relatively persistent, although it may alternate or intermingle with depressive mood.

B. Duration of at least one week (or any duration if hospitalization is necessary), during which, for most of the time, at least three of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree:

(1) increase in activity (either socially, at work, or sexually) or physical restlessness
(2) more talkative than usual or pressure to keep talking.
(3) flight of ideas or subjective experience that thoughts are racing
(4) inflated self-esteem (grandiosity, which may be delusional)
(5) decreased need for sleep
(6) distractibility, i.e. attention too easily drawn to unimportant or irrelevant external stimuli
(7) excessive involvement in activities that have a high potential for painful consequences which is not recognized, e.g., buying sprees, sexual indiscretions, foolish business investments, reckless driving

C. Neither of the following dominate the clinical picture when an affective syndrome (i.e., criteria A and B above) is not present, that is, before it developed or after it has remitted:

(1) preoccupation with a mood-incongruent delusion or hallucination (see definition below)
(2) bizarre behavior

D. Not superimposed on either Schizophrenia, Schizophreniform Disorder, or a Paranoid Disorder.

E. Not due to any Organic Mental Disorder, such as Substance Intoxication.

(Note: A hypomanic episode is a pathological disturbance similar to, but not as severe as, a manic episode).

Fifth-digit code numbers and criteria for subclassification of manic episode:

6-In Remission. This fifth-digit category should be used when in the past the individual met the full criteria for a manic episode but now is essentially free of manic symptoms or has some signs of the disorder but does not meet the full criteria. The differentiation of this diagnosis from no mental disorder requires consideration of the period of time since the last episode, the number of previous episodes, and the need for continued evaluation or prophylactic treatment.

4-With Psychotic Features. This fifth-digit category should be used when there apparently is gross impairment in reality testing, as when there are delusions or hallucinations or grossly bizarre behavior. When possible specify whether the psychotic features are mood-congruent or mood-incongruent.

Mood-congrent Psychotic Features: Delusions or hallucinations whose content is entirely consistent with the themes of inflated worth, power, knowledge, identity, or special relationship to a deity or famous person; flight of ideas without apparent awareness by the individual that the speech is not understandable.

Mood-incongruent Psychotic Features. Either (a) or (b):

(a) Delusions or hallucinations whose content does not involve themes of either inflated worth, power, knowledge, identity, or special relationship to a deity or famous person. Included are such symptoms as persecutory delusions, thought insertion, and delusions of being controlled, whose content has no apparent relationship to any of the themes noted above.

(b) Any of the following catatonic symptoms: stupor, mutism, negativism, posturing.

2-Without Psychotic Features. Meets the criteria for manic episode, but no psychotic features are present.

0-Unspecified.

Major depressive episode
Differential Diagnosis. Organic Affective Syndrome, Primary Degenerative Dementia, Multi-infarct Dementia, psychological reaction to functional impairment associated with a physical illness, Schizophrenia, Schizoaffective Disorder, Dysthymic Disorder, Cyclothymic Disorder, other chronic mental disorders associated with depressive symptoms, Separation Anxiety Disorder, Uncomplicated Bereavement.

Diagnostic criteria.
A. Dysphoric mood or loss of interest or pleasure in all or almost all usual activities and pastimes. The dysphoric mood is characterized by symptoms such as the following: depressed, sad, blue, hopeless, low, down in the dumps, irritable. The mood disturbance must be prominent and relatively persistent, but not necessarily the most dominant symptoms, and does not include momentary shifts from one dysphoric mood to another dysphoric mood, e.g., anxiety to depression to anger, such as are seen in states of acute psychotic turmoil. (For children under sex, dysphoric mood may have to be inferred from a persistently sad facial expression.)

B. At least four of the following symptoms have each been present nearly every day for a period of at least two weeks (in children under six, at least three of the first four):

(1) poor appetite or significant weight loss (when not dieting) or increased appetite or significant weight gain (in children under six consider failure to make expected weight gains)
(2) insomnia or hypersomnia
(3) psychomotor agitation or retardation (but not merely subjective feelings of restlessness or being slowed down) (in children under six, hypoactivity)
(4) loss of interest or pleasure in usual activities, or decrease in sexual drive not limited to a period when delusional or hallucinating (in children under six, signs of apathy)
(5) loss of energy; fatigue
(6) feelings of worthlessness, self-reproach, or excessive or inappropriate guilt (either may be delusional)
(7) complaints or evidence of diminished ability to think or concentrate, such as slowed thinking, or indecisiveness not associated with marked loosening of associations or incoherence
(8) recurrent thoughts of death, suicidal ideation, wishes to be dead, or suicide attempt

C. Neither of the following dominate the clinical picture when an affective syndrome (i.e., criteria A and B above) is not present, that is, before it developed or after it has remitted:

(1) preoccupation with a mood-incongruent delusion or hallucination (see definition below)
(2) bizarre behavior

D. Not superimposed on either Schizophrenia, Schizophreniform Disorder, or a Paranoid Disorder.

E. Not due to any Organic Mental Disorder or Uncomplicated Bereavement.

Fifth-digit code numbers and criteria for subclassification of major depressive episode:
(When psychotic features and melancholia are present the coding system requires that the clinician record the single most clinically significant characteristic.)

6-In Remision. This fifth-digit category should be used when in the past the individual met the full criteria for a major depressive episode but now is essentially free of depressive symptoms or has some signs of the disorder but does not meet the full criteria.

4-With Psychotic Features. This fifth-digit category should be used when there apparently is gross impairment in reality testing, as when there are delusions or hallucinations, or depressive stupor (the individual is mute and unresponsive). When possible specify whether the psychotic features are mood-congruent or mood-incongruent.

Mood-congruent Psychotic Features. Delusions or hallucinations whose content is entirely consistent with the themes of either personal inadequacy, guilt, disease, death, nihilism, or deserved punishment; depressive stupor (the individual is mute and unresponsive).

Mood-incongruent Psychotic Features. Delusions or hallucinations whose content does not involve themes of either personal inadequacy, guilt, disease, death, nihilism, or deserved punishment. Included here are such symptoms as persecutory delusions, thought insertion, thought broadcasting, and delusions of control, whose content has no apparent relationship to any of the themes noted above.

3-With Melancholia.
A. Loss of pleasure in all or almost all activities.

B. Lack of reactivity to usually pleasurable stimuli (doesn't feel much better, even temporarily, when something good happens).

C. At least three of the following:
(a) distinct quality of depressed mood, i.e., the depressed mood is perceived as distinctly different from the kind of feeling experienced following the death of a loved one
(b) the depression is regularly worse in the morning

(c) early morning awakening (at least two hours before usual time of awakening)
(d) marked psychomotor retardation or agitation
(e) significant anorexia or weight loss
(f) excessive or inappropriate guilt

2-Without Melancholia

0-Unspecified

BIPOLAR DISORDER

296.6x Bipolar Disorder, Mixed
Diagnostic criteria.
Use fifth-digit coding for manic episode.
A. Current (or most recent) episode involves the full symptomatic picture of both manic and major depressive episodes intermixed or rapidly alternating every few days.

B. Depressive symptoms are prominent and last at least a full day.

296.5x Bipolar Disorder, Depressed
Diagnostic criterion.
Currently (or most recently) in a manic episode. (If there has been a previous manic episode, the current episode need not meet the full criteria for a manic episode.)

296.5x Bipolar Disorder, Depressed
Diagnostic criteria.
A. Has had one or more manic episodes.

B. Currently (or most recently) in a major depressive episode. (If there has been a previous major depressive episode, the current episode of depression need not meet the full criteria for a major depressive episode.)

MAJOR DEPRESSION

296.2x Major Depression, Single Episode

296.3x Major Depression, Recurrent

Diagnostic criteria.
A. One or more major depressive episodes.
B. Has never had a manic episode or hypomanic episode.

OTHER SPECIFIC AFFECTIVE DISORDERS

301.13 Cyclothymic Disorder
Differential Diagnosis. Bipolar Disorder, major depressive episode, manic episode.

Diagnostic criteria:
A. During the past two years, numerous periods during which some symptoms characteristic of both the depressive and the manic syndromes were present but were not of sufficient severity and duration to meet the criteria for a major depressive or manic episode.

B. The depressive periods and hypomanic periods may be separated by periods of normal mood lasting as long as months at a time, they may be intermixed, or they may alternate.

C. During depressive periods there is depressed mood or loss of interest or pleasure in all, or almost all, usual activities and pastimes, and at least three of the following:	During hypomanic periods there is an elevated, expansive, or irritable mood and at least three of the following:
(1) insomnia or hypersomnia	decreased need for sleep
(2) low energy or chronic fatigue	more energy than usual
(3) feelings of inadequacy	inflated self-esteem
(4) decreased effectiveness or productivity at school, work, or home	increased productivity, often associated with unusual and self-imposed working hours
(5) decreased attention, concentration, or ability to think clearly	sharpened and unusually creative thinking
(6) social withdrawal	uninhibited people-seeking (extreme gregariousness)
(7) loss of interest in or enjoyment of sex	hypersexuality without recognition of possibility of painful consequences

(8) restriction of involvement in pleasurable activities; guilt over past activities	excessive involvement in pleasurable activities with lack of concern for the high potential for painful consequences, e.g., buying sprees, foolish business investments, reckless driving
(9) feeling slowed down	physical restlessness
(10) less talkative than usual	more talkative than usual
(11) pessimistic attitude toward the future, or brooding about past events	overoptimism or exaggeration of past achievements
(12) tearfulness or crying	inappropriate laughing, joking, punning

D. Absence of psychotic features duch as delusions, hallucinations, incoherence, or loosening of associations.

E. Not due to any other mental disorder, such as partial remission of Bipolar Disorder. However, Cyclothymic Disorder may precede Bipolar Disorder.

300.40 Dysthymic Disorder (or Depressive Neurosis)
Differetial Diagnosis. Major Depression; normal fluctuations of mood; chronic mental disorders, such as Obsessive Compulsive Disorder or Alcohol Dependence, when associated with depressive symptoms.

Diagnostic criteria.
A. During the past two years (or one year for children and adolescents) the individual has been bothered most or all of the time by symptoms characteristic of the depressive syndrome that are not of sufficient severity and duration to meet the criteria for a major depressive epidode.

B. The manifestations of the depressive syndrome may be relatively persistent or separated by periods of normal mood lasting a few days to a few weeks, but no more than a few months at a time.

C. During the depressive periods there is either prominent depressed mood (e.g., sad, blue, down in the dumps, low) or marked loss of interest or pleasure in all, or almost all, usual activities and pastimes.

D. During the depressive periods at least three of the following symptoms are present:

(1) insomnia or hypersomnia
(2) low energy level or chronic tiredness
(3) feelings of inadequacy, loss of self-esteem, or self-deprecation
(4) decreased effectiveness or productivity at school, work, or home
(5) decreased attention, concentration, or ability to think clearly
(6) social withdrawal
(7) loss of interest in or enjoyment of pleasurable activities
(8) irritability or excessive anger (in children, expressed toward parents or caretakers)
(9) inability to respond with apparent pleasure to praise or rewards
(10) less active or talkative than usual, or feels slowed down or restless
(11) pessimistic attitude toward the future, brooding about past events, or feeling sorry for self
(12) tearfulness or crying
(13) recurrent thoughts of death or suicide

E. There are no psychotic features, such as delusions, hallucinations, or incoherence.

F. If the disturbance is superimposed on another mental disorder or a preexisting mental disorder, such as Obsessive Compulsive Disorder or Alcohol Dependence, the depressed mood, by virtue of its intensity or effect on functioning, can be clearly distinguished from the individual's usual mood.

ATYPICAL AFFECTIVE DISORDERS

296.70 Atypical Bipolar Disorder
This is a residual category for individuals with manic features that cannot be classified as Bipolar Disorder or a Cyclothymic Disorder. For example, an individual who previously had a major depressive episode, now has an episode of illness with some manic features (hypomanic episode), but not of sufficient severity and duration to meet the criteria for a manic episode. Such cases have been referred to as "BipolarII."

296.82 Atypical Depression
This is a residual category for individuals with depressive symptoms who cannot be diagnosed as having Major or Other Specific Affective Disorder or Adjustment Disorder. Examples include the following:

(1) A distinct and sustained episode of the full depressive syndrome in an individual with Schizophrenia, Residual Type, that develops without an activation of the psychotic symptoms.
(2) A disorder that fulfills the criteria for Dysthymic Disorder; however, there have been intermittent periods of normal mood lasting more than a few months.
(3) A brief episode of depression that does not meet the criteria for a Major Affective Disorder and that is apparently not reactive to psychosocial stress, so that it cannot be classified as an Adjustment Disorder.

NOTE: five-digit codes, such as 296.2x Major Depression, Single Episode, are used as a classification scheme for the diagnosis of patients.

HAMILTON DEPRESSION SCALE - PART 1

Treatment Period (Check One): Screen (001) ☐ Baseline (002) ☐ End of Week 1 (003) ☐ 2 (004) ☐ 3 (006) ☐ 4 (008) ☐

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ORD NO.	DRUG U-NO.	CIS NO.	MONITOR	MONITOR I.D. NO.	PROTOCOL NO.
4 (1–2)	U / 0 3 1 8 8 9 / b b (3–11)	5 0 2 (12–14)	R. P. PURPURA, M.D.	1 0 7 (15–17)	4 5 0 6 (18–21)

STIGATOR'S NAME	INVES. I.D. NO.	PATIENT'S NAME	PATIENT'S I.D. NO.
R. O. FRIEDEL, M.D.	7 3 1 1 (22–25)		— — (26–29)

0	SEQ. NO.	DATE OF THIS REPORT	MO.	DAY	YR.
	— (31–33)		— (34)	/ — — (35–36)	/ — (37–39)

FOR EACH ITEM CHECK THE BOX NEXT TO THE RESPONSE THAT BEST CHARACTERIZES THE PATIENT

1. DEPRESSED MOOD (Sadness, hopeless, helpless, worthless) (40)

0 ☐ Absent
1 ☐ These feeling states indicated only on questioning.
2 ☐ These feeling states spontaneously reported verbally.
3 ☐ Communicates feeling states non-verbally – i.e., through facial expression, posture, voice and tendency to weep.
4 ☐ Patient reports VIRTUALLY ONLY these feeling states in his spontaneous verbal and non-verbal communication.

2. FEELINGS OF GUILT (41)

0 ☐ Absent
1 ☐ Self-reproach, feels he has let people down.
2 ☐ Ideas of guilt or rumination over past errors or sinful deeds.
3 ☐ Present illness is a punishment. Delusions of guilt.
4 ☐ Hears accusatory or denunciatory voices and/or experiences threatening visual hallucinations.

3. SUICIDE (42)

0 ☐ Absent
1 ☐ Feels life is not worth living.
2 ☐ Wishes he were dead or any thoughts of possible death to self.
3 ☐ Suicide ideas or gesture.
4 ☐ Attempts at suicide *(only serious attempt rates 4).*

I. INSOMNIA EARLY (43)

0 ☐ No difficulty falling asleep.
1 ☐ Complains of occasional difficulty falling asleep – i.e., more than 1/2 hour.
2 ☐ Complains of nightly difficulty falling asleep.

. INSOMNIA MIDDLE (44)

0 ☐ No difficulty.
1 ☐ Patient complains of being restless and disturbed during the night.
2 ☐ Waking during the night – any getting out of bed rates 2 *(except for purposes of voiding).*

. INSOMNIA LATE (45)

0 ☐ No difficulty.
1 ☐ Waking in early hours of the morning but goes back to sleep.
2 ☐ Unable to fall asleep again if gets out of bed.

WORK AND ACTIVITIES (46)

0 ☐ No difficulty.
1 ☐ Thoughts and feelings of incapacity, fatigue or weakness related to activities; work or hobbies.
2 ☐ Loss of interest in activity; hobbies or work – either directly reported by patient, or indirect in listlessness, indecision and vacillation *(feels he has to push self to work or activities).*
3 ☐ Decrease in actual time spent in activities or decrease in productivity. In hospital, rate 3 if patient does not spend at least three hours a day in activities *(hospital job or hobbies),* exclusive of ward chores.
4 ☐ Stopped working because of present illness. In hospital, rate 4 if patient engages in no activities except ward chores, or if patient fails to perform ward chores unassisted.

RETARDATION (Slowness of thought and speech; impaired ability to concentrate; decreased motor activity) (47)

0 ☐ Normal speech and thought.
1 ☐ Slight retardation at interview.
2 ☐ Obvious retardation at interview.
3 ☐ Interview difficult.
4 ☐ Complete stupor.

AGITATION (48)

0 ☐ None
1 ☐ "Playing with" hands, hair, etc.
2 ☐ Hand-wringing, nail-biting, hair-pulling, biting of lips.

ANXIETY PSYCHIC (49)

0 ☐ No difficulty.
1 ☐ Subjective tension and irritability.
2 ☐ Worrying about minor matters.
3 ☐ Apprehensive attitude apparent in face or speech.
4 ☐ Fears expressed without questioning.

Upjohn **HAMILTON DEPRESSION SCALE - PART 2**

1-780 8/80

Treatment Period (Check One): Screen ☐ (001) | Base-line ☐ (002) | End of Week 1 ☐ (003) | 2 ☐ (004) | 3 ☐ (006) | 4 ☐ (008)

PROTOCOL NO. 5 0 6 | PATIENT'S INITIALS | PATIENT'S I.D. NO. ___ ___ ___ ___

FOR EACH ITEM CHECK THE BOX NEXT TO THE RESPONSE THAT BEST CHARACTERIZES THE PATIENT

11. ANXIETY SOMATIC (50)
0 ☐ Absent
1 ☐ Mild
2 ☐ Moderate
3 ☐ Severe
4 ☐ Incapacitating
Physiological concomitants of anxiety, such as:
Gastro-intestinal – dry mouth, wind, indigestion, diarrhea, cramps, belching
Cardio-vascular – palpitations, headaches
Respiratory – hyperventilation, sighing
Urinary frequency
Sweating

12. SOMATIC SYMPTOMS GASTRO-INTESTINAL (51)
0 ☐ None
1 ☐ Loss of appetite but eating without staff encouragement. Heavy feelings in abdomen.
2 ☐ Difficulty eating without staff urging. Requests or requires laxatives or medication for bowels or medication for G.I. symptoms.

13. SOMATIC SYPMTOMS GENERAL (52)
0 ☐ None
1 ☐ Heaviness in limbs, back or head. Backaches, headache, muscle aches. Loss of energy or fatigua-bility.
2 ☐ Any clear-cut symptom rates 2.

14. GENITAL SYMPTOMS (53)
0 ☐ Absent
1 ☐ Mild
2 ☐ Severe
Symptoms such as: Loss of libido
Menstrual disturbances

15. HYPOCHON-DRIASIS (54)
0 ☐ Not present
1 ☐ Self-absorption (bodily)
2 ☐ Preoccupation with health.
3 ☐ Frequent complaints, requests for help, etc.
4 ☐ Hypochondrical delusions.

16. LOSS OF WEIGHT (Answer only A or B)

A. WHEN RATING BY HISTORY: (55)
0 ☐ No weight loss.
1 ☐ Probable weight loss associated with present illness.
2 ☐ Definite (according to patient) weight loss.

B. ON WEEKLY RATINGS BY WARD PSYCHIATRIST, WHEN ACTUAL WEIGHT CHANGES ARE MEASURED: (56)
0 ☐ Less than 1 lb. weight loss in week.
1 ☐ Greater that 1 lb. weight loss in week.
2 ☐ Greater that 2 lb. weight loss in week.

17. INSIGHT (57)
0 ☐ Acknowledges being depressed and ill.
1 ☐ Acknowledges illness but attributes cause to bad food, climate, overwork, virus, need for rest, etc.
2 ☐ Denies being ill at all.

18. DIURNAL VARIATION

(58) AM	(59) PM	
0 ☐	0 ☐	Absent
1 ☐	1 ☐	Mild
2 ☐	2 ☐	Severe

If symptoms are worse in the morning or evening note which it is and rate severity of variation.

19. DEPERSONAL-IZATION AND DEREALIZA-TION (60)
0 ☐ Absent
1 ☐ Mild
2 ☐ Moderate
3 ☐ Severe
4 ☐ Incapacitating
Such as: Feelings of unreality
Nihilistic ideas

20. PARANOID SYMPTOMS (61)
0 ☐ None
1 ☐ Suspicious
2 ☐ Ideas of reference
3 ☐ Delusions of reference and persecution

21. OBSESSIONAL AND COMPULSIVE SYMPTOMS (62)
0 ☐ Absent
1 ☐ Mild
2 ☐ Severe

TIGATOR'S SIGNATURE:

VITA