SIMULTANEOUS QUANTITATION OF PHENYTOIN, ITS MAJOR METABOLITES, AND THEIR STABLE ISOTOPE LABELLED ANALOGS IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHIC MASS SPECTROMETRY.

by

Agnes Van Langenhove

Submitted in Partial Fulfillment

of the Requirements for the

Degree of Doctor of Philosophy

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October 1980

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Department of Chemistry, Oct. 28, 1980

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To Hank

Simultaneous Quantitation of Phenytoin, its Major Metabolites, and their Stable Isotope Labelled Analogs in Biological Fluids by Gas Chromatographic Mass Spectrometry.

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ABSTRACT

A gas chromatographic-mass spectrometric (GC-MS) method has been developed for the determination of 5,5-diphenylhydantoin (phenytoin, PHT), its major metabolite [5-(4-hydroxyphenyl)-5-phenylhydantoin], and, simultaneously, their stable isotope labelled analogs (5,5-diphenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin and 5-(4-hydroxyphenyl)- 5-phenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin) in biological fluids. The ($^{13}C^{15}N_2$)-labelled phenytoin will be administered 'in vivo' to patients, in order to study the pharmacokinetics of phenytoin in man. Accuracy in quantitation is achieved by use of 5,5-di(pentadeuterophenyl)hydantoin and 5-(4-hydroxy-3,5-dideuterophenyl)-5-phenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin as internal standards. The chemical work-up procedure (processing) of serum or plasma (1.0ml) and urine (0.5ml) involves acid hydrolysis, extraction at pH 7.4 and

permethylation of drug and metabolite analogs by extractive methylation. The mass spectrometric measurement technique consists of repetitive scanning over the molecular ion region of the permethylated derivatives of the phenytoin and metabolite analogs as they elute from the gas chromatograph. The data are processed by the computer and ratios of molecular ion abundances of drug and metabolite to internal standard are computed. Selectivity, reproducibility and linearity are discussed for serum or plasma phenytoin levels of 0.1-30.0 µg/ml, serum or plasma metabolite levels of 0.1-10.0 µg/ml and urine metabolite levels of 5.0-200.0 µg/ml. At all levels, the coefficient of variation remains below 5.5% (N=10). The pharmacological equivalence of labelled and unlabelled phenytoin was demonstrated in dogs and human volunteers. The method was therefore extended to measurement of the meta hydroxylated metabolite, because this is the major metabolite in dogs. The results are discussed in the light of the 'pulse dosing' applications of the method.

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List of Schemes

Abbreviations frequently used in the text

1. Reference compounds and chemicals

PHT	5,5-diphenylhydantoin
p-HPPH	5(4-hydroxyphenyl)-5-phenylhydantoin
m-HPPH	5(3-hydroxyphenyl)-5-phenylhydantoin
($^{13}C^{15}N_2$)-	2-^{13}C-1,3-$^{15}N_2$-hydantoin labelled analog
d-	derivatized (permethylated) analog
D	deuterium (H)
MIBK	methylisobutylketone
$TBA^+HSO_4^-$	tetrabutylammonium hydrogen sulfate
TBA^+I^-	tetrabutylammonium iodide
CH_2Cl_2	methylene chloride
CH_3I	methyl iodide

2. Instrumentation

GC	gas chromatography
MS	mass spectrometry
GC-MS	gas chromatographic mass spectrometry
amu	atomic mass unit
m/z	mass to charge ratio
FID	flame ionization detector
M^+	molecular ion
TIP	total ionization plot
CRT	cathode ray tube
S/N	signal to noise ratio

Abbreviations frequently used in the text (cont.)

3. Statistics

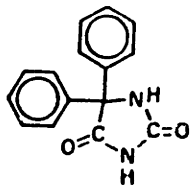
SD	standard deviation
CV	coefficient of variation
r	correlation coefficient
N	number of observations
LRA	linear regression analysis

4. Pharmacokinetic parameters

V1	volume of the central compartment
Vd	totl volume of distribution
$T_{\frac{1}{2}\alpha}$	initial distribution half-life
$T_{\frac{1}{2}\pi}$	intermediate distribution half-life
$T_{\frac{1}{2}\beta}$	elimination half-life
K_{Ia}	rate constant for the hydroxylation of Ia
K_{Ib}	rate constant for the hydroxylation of Ib

Chapter I. INTRODUCTION

Phenytoin[a] (PHT, Ia) is one of the most effective and extensively used drugs in the treatment of epilepsy.



Ia

Epilepsy is a collective term for a class of chronic convulsive disorders having in common the occurrence of brief episodes of loss or disturbance of consciousness (seizures); these are usually, but not always, associated with characteristic body movements (convulsions) and are always correlated with abnormal and excessive electro encephalographic (EEG) discharges. The aim of treatment of epilepsy with phenytoin is to prevent the occurance of seizures without causing significant side effects. Phenytoin must therefore be administered daily to maintain a steady state level of the drug in the serum. Since epilepsy is a chronic disorder, many of its victims are forced to take phenytoin for life. The exact mechanism of the anti-convulsant effect of phenytoin is unknown, but it

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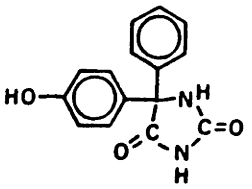
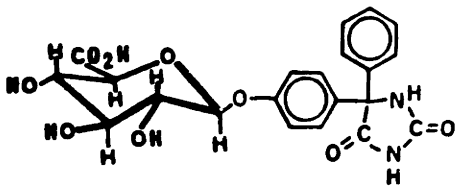
[a] Phenytoin is the international nonproprietary name for 5,5-diphenylhydantoin, Parke-Davis Trade name: Dilantin^{R}.

is most probably related to its ability to prevent the spread of abnormal or excessive neuronal discharge in the central nervous system.

The major metabolite of phenytoin in man is the para-hydroxylated derivative, 5-(4-hydroxyphenyl)-5-phenyl hydantoin (p-HPPH, IIa) (1). It does not exhibit anticonvulsant activity and it is excreted in the urine mainly as a conjugate with glucuronic acid (GlcUA-p-HPPH, GlcUA-IIa) (2).

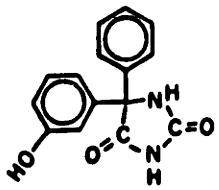


IIa

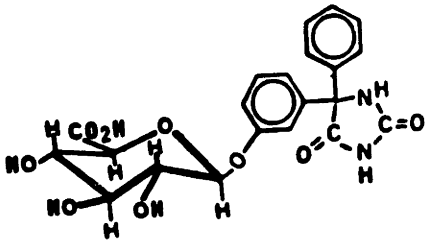


GlcUA-IIa

A meta-hydroxylated derivative, 5-(3-hydroxyphenyl) -5-phenylhydantoin (m-HPPH, IIIa), is a major metabolite in dogs and is also excreted in the urine as a glucuronide (GlcUA-m-HPPH, GlcUA-IIIa); in dogs the ratio of m-HPPH/p-HPPH is 3/1 (3). In man, none or very little m-HPPH is produced (4).



IIIa



GlcUA-IIIa

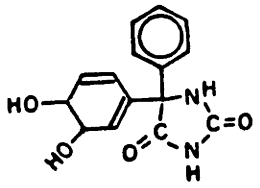
Other minor metabolites which are formed from phenytoin are: the dihydrodiol [5-(3,4-dihydroxy-1,5-cyclo

hexadien-1-yl)-5-phenylhydantoin, IVa] (5,6), a series of catechols [5-(3,4-dihydroxyphenyl)-5-phenylhydantoin, Va (7,8), 5-(2,4-dihydroxyphenyl)-5-phenylhydantoin, VIIa (8) and 5-(4-hydroxy-3-methoxy)-5-phenylhydantoin, VIIIa (8)], and diphenylhydantoic acid (VIIIa) (9). The presence of diphenylglycine (IXa) has also been reported (9), but this could not be confirmed by other investigators. Less than 1% of the administered dose of phenytoin is excreted unchanged in the urine. The structures of the minor metabolites of phenytoin are listed in Figure I-1.

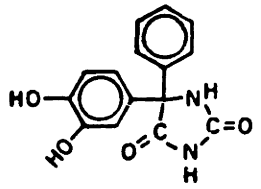
For each individual, the serum level of phenytoin and the degree of clinical response are directly related. Most patients will achieve maximum seizure control when the serum steady state level is between 10 and 20 µg/ml. However, at concentrations lower than 10 µg/ml seizures may not be suppressed, while at concentrations larger than 20 µg/ml adverse side effects may occur. This relatively low therapeutic index is a serious problem in the clinical use of phenytoin. The situation is further complicated by the dose-dependent kinetics of phenytoin and by the fact that large variations in serum levels can result from the same dose administered to different subjects. Of major importance in the process of treatment with phenytoin is the fact that as the serum level approaches the lower limits of the optimum therapeutic range, the slope of the dose-serum level curve changes markedly. This change reflects the dose-dependent kinetics of phenytoin and

Figure I-1. Structures of the minor metabolites of PHT.

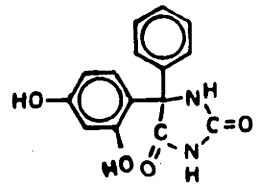
Figure I-1



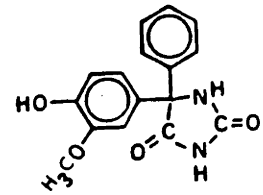
IVa



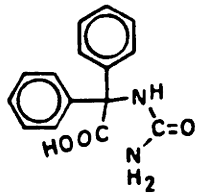
Va



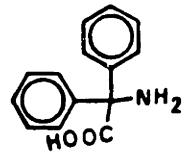
VIa



VIIa



VIIIa



IXa

occurs when elimination changes from first-order to zero-order. At the point of change, small increases in dose result in large increases in the serum level (e.g. an increase in dose from 200 to 300 mg/day can cause a rise in the serum level from a steady state around 5 µg/ml to a new steady state above 30 µg/ml). This results in significant overkill in which the patient is exposed to potentially severe toxicity. In addition, the patient and/or physician may conclude that phenytoin is an ineffective drug based on observations that the previous dose did not control seizures whereas the next dose caused significant toxicity. Daily doses of 200-500 mg of phenytoin are commonly needed to maintain the usual steady state conditions, but small modifications in drug disposition rate often result in pronounced changes in serum concentrations (other drugs that are taken at the same time, e.g. phenobarbital, or impaired liver or kidney function can alter phenytoin metabolism and alter the usual dosage requirements). Therefore, to obtain maximum efficiency and benefit from treatment with phenytoin, serum level determinations are invaluable if not imperative; they will indicate when and how to adjust the dose so that subtherapeutic or toxic concentrations are avoided.

However, theoretically, continuous drug monitoring should not be necessary because it should bepossible to calculate steady state levels for a particular patient from pharmacokinetic data. Pharmacokinetics is concerned with

the translation of the many individual factors that determine drug absorption, distribution, biotransformation, and excretion into succinct mathematical expressions that allow calculation of drug concentrations in serum (plasma), or in other body fluids or tissues.

The application of pharmacokinetics to optimize individual dosage requires the formulation of a model that describes disposition with drug administration. Individual pharmacokinetic parameters (e.g. elimination and distribution half-life, volume of distribution and clearance) which are used as estimators of the system are calculated from a limited number of measurements of serum drug levels. These periodic determinations are then used to correlate with and predict the probability of therapeutic and adverse pharmacological response, and to correlate with secondary variables related to the properties of the drug and physiological variables related to the patient and the disease state.

Pharmacokinetic analysis of drugs that follow exponential (first-order) kinetics is relatively simple; however, for phenytoin, the relationship between dose and serum level in individual patients is non-linear (the kinetics are dose-dependent) and multi-compartment models are necessary to study the drug. When first-order kinetics are operative, most parameter values can be obtained from the measurement of the serum concentration of the drug, but to investigate dose-dependent kinetics, the concentration

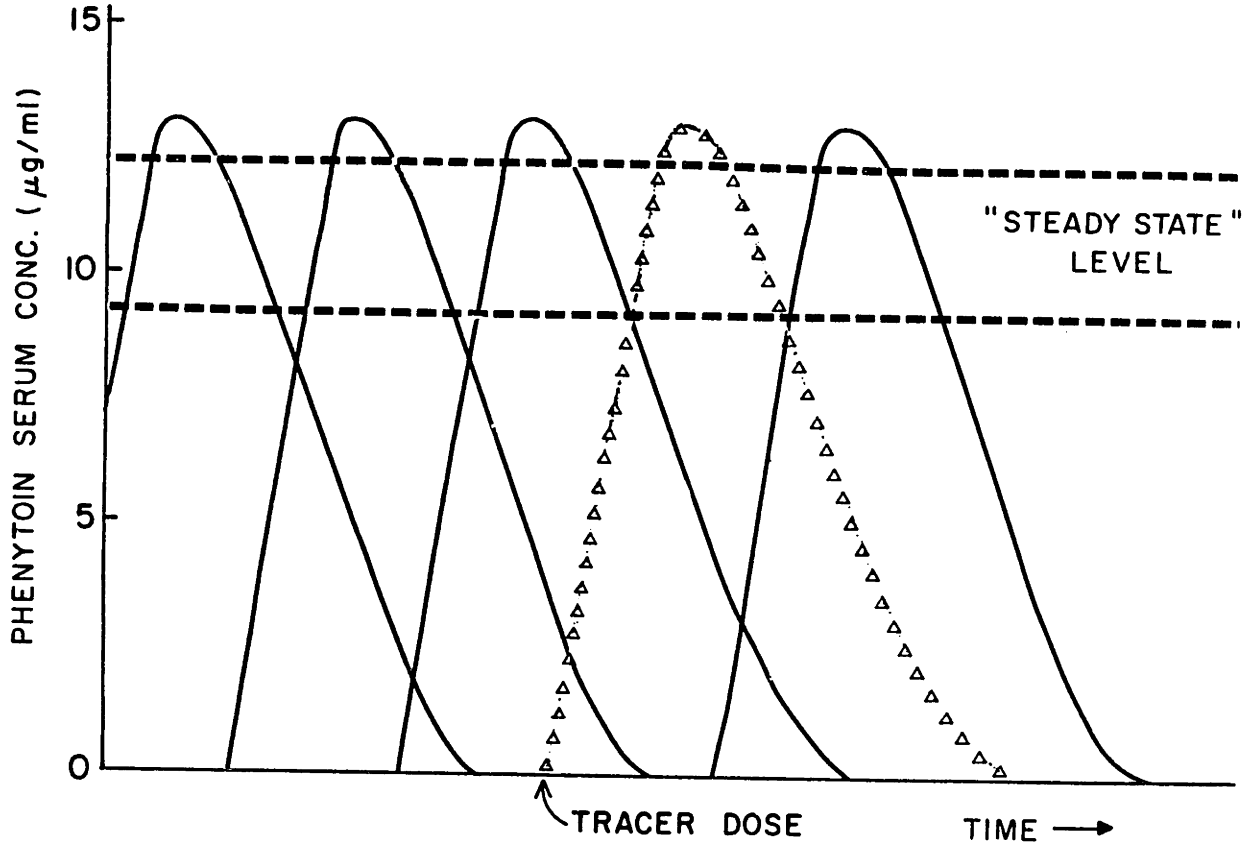
of the major metabolite is often required (e.g. to evaluate product inhibition of drug metabolism by major metabolites).

For phenytoin, many unanswered question concerning its pharmacokinetics remain and different hypotheses have been proposed to explain the dose-dependent elimination (10-12). The lack of a safe technique to measure the pharmaco kinetics of the drug at steady state in humans has been a major obstacle in the study of phenytoin metabolism and has added to the complexity of the problem. Investigations in which the drug is discontinued and its rate of disappearance from the plasma is monitored carry the risk of increased seizure frequency because a therapeutic serum concentration has to be maintained to prevent seizures. These measurements are also not fully representative of the true 'steady state' conditions, because the plasma concentration of the drug is constantly falling. Studies which involve the use of radioactive tracers expose the patient to radiation. In addition, the measurement of radioactivity by liquid scintillation counting is unspecific because the amount of radioactive isotope present in the sample is measured without regard to its chemical or molecular form.

An alternative is 'pulse dosing' with stable isotope labelled tracers, and independent monitoring of the tracer. This is illustrated in Figure 1-2 where the serum concentration of phenytoin is plotted as a function of time

Figure I-2. Serum concentration of PHT as a function of time after repeated administration of fixed doses of the drug.

Figure I-2

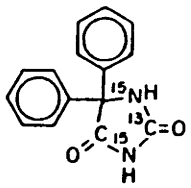

15
10
5
0
PHENYTOIN SERUM CONC. (μg/ml)
"STEADY STATE"
LEVEL
TRACER DOSE
TIME

after repeated administration of fixed doses of the drug. If a labelled dose is substituted for a normal dose of the drug, its specific elimination pattern can be followed. In contrast to an unspecific radiation assay, gas chromatographic mass spectrometric (GC-MS) detection systems are highly specific for the stable isotope labelled compounds under investigation. Volume of distribution, distribution and elimination half-life and clearance of the labelled drug would hereby reliably be determined without interrupting the dosage regimen. It would also be possible to measure the apparent rate of formation and elimination of the major metabolites of the drug by measuring their concentration in plasma and urine over time. In addition, eventual redistribution of the unlabelled drug could be verified.

Pure isotopes and molecules labelled with stable isotopes for use in biological and medical research have not always been available. Only in the 1960's did the production of drugs enriched in stable heavy isotopes of hydrogen, ^{13}C and ^{15}N receive increased interest. At this time alternatives were sought to radioactive techniques and the potential of mass spectrometry was realized (in spite of the availability of radioactive labelled molecules and the existence of sensitive measuring equipment, the use of radioactive tracers was often limited to animal studies). Today, rather pure isotopes and molecules labelled with stable isotopes are becoming increasingly available and

mass spectrometric techniques, using stable isotope dilution, allow accurate and selective quantification of these labelled analogs. Reports in the literature demonstrate that the use of stable isotope labelled drugs makes it possible to distinguish doses of a drug administered at different times or in different forms, and to study the specific pharmacokinetics of the different doses or formulations (13-16).

In an effort to gain insight into the pharmacokinetics of phenytoin (Ia), we chose 5,5-diphenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin [($^{13}C^{15}N_2$)-PHT, Ib] as a stable isotope labelled analog for 'pulse dosing' studies in humans.



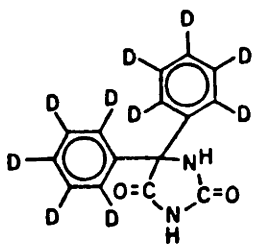
Ib

The position of the label was carefully chosen so that it would be retained during hydroxylation of the phenyl ring (major pathway of metabolism), that there is no risk that the label could be lost either 'in vivo' or in the course of sample preparation and analysis, and that there would be no conflicts in the later selection of stable isotope labelled analogs to be used as internal standards for quantitative analysis.

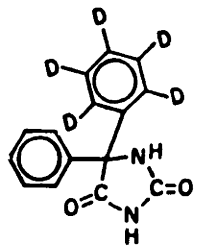
Apparently there is concern in the medical community about 'in vivo' isotope effects especially when deuterium

is used (17). However, 1. the pharmacokinetic parameters of a deuterium labelled drug will only change if the label is placed at the site of metabolism and if cleavage of the bond involving the stable isotope is the rate limiting step in the enzymatic reaction, and 2. labelled metabolites will be indistinguishable from unlabelled ones only if the entire label is placed at a position where it is lost.

For PHT (Ia), deuterium should therefore not be placed in the p-position only because p-hydroxylation is the major pathway of Ia metabolism in man. On the other hand, if several deuterium atoms were substituted in the phenyl ring [e.g. as in 5,5-di(pentadeuterophenyl)hydantoin, (D_{10})-PHT, Ic or 5-(pentadeuterophenyl)-5-phenylhydantoin, (D_5)-PHT, Id],



Ic



Id

the loss of the p-deuterium atoms would result in a metabolite that contains 9, 5 or 4 deuterium atoms and thus easily distinguished from the unlabelled drug. If no other isotope effects involving the rate or mechanism of metabolism are operative, these compounds could be administered for 'in vivo' in pharmacokinetic studies.

Andresen (for Id) (18) and Tomaszewski (for Ic) (19) demonstrated that the cleavage of the para C-D bond is not

the rate limiting step in the enzymatic hydroxylation (they found no significant isotope effects in the rate of formation of p-HPPH). A fully labelled phenyl ring also eliminated complications arising from the NIH shift (i.e. a substituent originally present at the site of para-hydroxylation may be partially transferred to an adjacent carbon atom), so that there should be no problems associated with the use of Ic and Id for 'in vivo' pharmacokinetic studies in man. It was kept in mind however that during the work-up of the samples, the metabolite has to be cleaved from its glucuronide. This is best accomplished by acid hydrolysis, which would lead to the loss of deuterium by exchange at the position adjacent to the phenolic hydroxyl group. Therefore, deuterium labelled compounds were not used in our studies for 'in vivo' administration, but were employed as internal standards (see Chapter II-A). For 'in vivo' adminis tration, we preferred the ($^{13}C^{15}N_2$) label in the hydantoin ring, at positions that are not at all involved in the metabolism (with the exception of the very minor metabolite VIIIa).

In general, metabolism studies require the quantification only of one species and mass spectrometric methods for such measurements using a stable isotope labelled internal standard are widely used (8,20,21). These reports describe methods to quantify Ia and/or IIa and IIIa in plasma and/or urine so that metabolic profiles

of subjects who have reached steady state plasma levels of Ia or whose plasma level is falling from the steady state level can be studied or interpreted. Hoppel et al. (20) described their procedure using 5-(4-hydroxyphenyl)-5-pentadeuterophenylhydantoin as internal standard for analysis of Ia, IIa and IIIa in plasma. Baty and Robinson (21) used pentadeuterophenyl-5-phenylhydantoin and 5-(4-hydroxyphenyl)-5-pentadeuterophenylhydantoin as internal standards, but determined Ia and IIa separately (Ia was analyzed as the permethyl derivative; IIa as the persilyl derivative). Egger et al (8) mention the use of 5-(4-hydroxyphenyl)-5-pentadeuterophenylhydantoin as internal standards but report only semi-quantitative results.

On the other hand, for the 'pulse dosing' approach, one has to measure simultaneously the pharmacokinetics of two substances: the unlabelled drug with which the 'steady state' was achieved and the labelled drug which represents the 'pulse dose'. At the same time, a pair of metabolites -unlabelled and labelled- also should be measured. The quantification then requires a second, differently labelled analog to be used as the internal standard for the analysis.

Therefore, the immediate objective of this study was the development and evaluation of a method to quantify unlabelled and stable isotope labelled phenytoin (Ia and Ib) and their major metabolites in man (IIa and IIb) in appropriate biological fluids, and, in addition, the

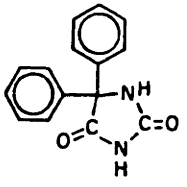
verification of the pharmacological equivalence of unlabelled and ($^{13}C^{15}N_2$)-labelled PHT (Ia and Ib). One would anticipate that the replacement of C and N in the hydantoin ring system by the heavy isotopes would not lead to an isotope effect in the hydroxylation at the p-position of one of the phenyl groups, i.e. the rate constants (K) for the hydroxylation of Ia and Ib would be the same ($K_{Ia}/K_{Ib} = 1$). However, because of the general concerns about isotope effects in the medical community (17), it was felt prudent to demonstrate the absence of 'in vivo' isotope effects experimentally and thus lay all these doubts to rest once and for all.

Ultimately, tracer dose studies can then be used to determine if phenytoin (Ia) induces its own metabolism (by measuring its elimination before and during therapy) and to determine how administration of other drugs influences its elimination (by measuring its elimination before and after administration of other drugs).

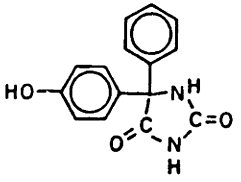
The structures of the different sets of analogs for which measurement is required in these studies are listed in Figure I-3. The studies to demonstrate the absence of 'in vivo' isotope effects were first carried out on dogs; therefore, an extension of the method to measure the m-HPPH analogs (IIIa and IIIb) was necessary, because as mentioned before, m-HPPH is the major metabolite in dogs (4). As discussed in Chapter II, Section A, either Id or Ic was used as internal standard to quantitate Ia and its

Figure I-3. Structures of the PHT analogs (Ia, Ib, Ic, Id) and HPPH analogs (IIa, IIb, IIc, IIIa, IIIb) that are measured in these studies.

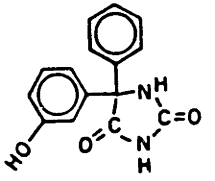
Figure I-3



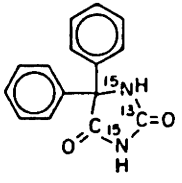
Ia



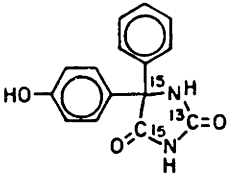
IIa



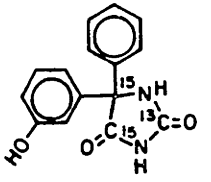
IIIa



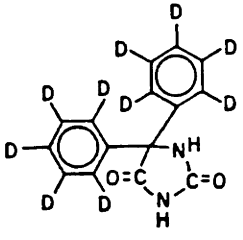
Ib



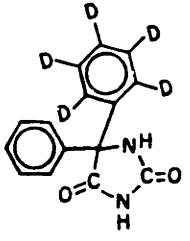
IIb



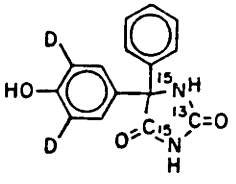
IIIb



Ic



Id



IIc

PHT analogs

p-HPPH analogs

m-HPPH analogs

($^{13}C^{15}N_2$)-labelled analog (Ib); 5-(4-hydroxy-3,5-dideuterophenyl)-5-phenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin [($^{13}C^{15}N_2D_2$)-p-HPPH, IIc] was used to quantitate IIa and IIIa and their respective ($^{13}C^{15}N_2$)-labelled analogs (IIb, IIIb). It should also be noted that 1. during the development and evaluation of the method and during the studies with human volunteers, no m-HPPH analogs (IIIa-IIIb) were measured; they were only monitored during the studies on dogs, and 2. IIIb was not availabe as reference compound; IIb was only available as reference compound during the studies with human volunteers.

Chapter II. DEVELOPMENT OF THE METHOD

A. APPROACH

Development of a method to measure PHT (Ia) and p-HPPH (IIa) and their ($^{13}C\,^{15}N_2$)-labelled analogs (Ib and IIb) in serum and urine involved careful study of all aspects of the chemical work-up of the samples and of the design of the GC-MS measurement technique. The chemical work-up of the samples involved cleavage of the p-HPPH-glucuronide bond and extraction and derivatization of the compounds of interest; the GC-MS measurement involved the gas chromatographic separation of the PHT analogs and the p-HPPH analogs from each other and from endogenous components, and their measurement by mass spectrometry.

We chose a stable isotope dilution technique (i.e. the use of stable isotope labelled analogs as internal standards) so that the recovery of Ia, IIa, and their ($^{13}C^{15}N_2$)-labelled analogs would be corrected for, and so that most systematic error would be eliminated.

However, stable isotope dilution only guarantees accuracy if no extraneous substances contribute to the particular ion current measured. Unlike other studies where only Ia, IIa and an internal standard were monitored (8, 20, 21), for our work several sets of analogs had to be measured in the same serum or urine sample. Since a preferential extraction of these analogs from serum and urine would still coextract endogenous substances, it was

very important to design the instrumental analysis step in such a way that interfering substances could be identified and their contributions eliminated or at least minimized.

We therefore chose to repetitively scan over a small mass spectral region of interest rather than to monitor selected ions only. The molecular ion region was chosen to be monitored because it is definitely characteristic of the analytes and retains the complete isotopic information for all the analogs that are monitored (see also Chapter II, Section B-2a). The full selectivity of the mass spectral measurements was hereby maintained.

As mentioned in Chapter I, the method was developed using Ia, Ib, Ic or Id, IIa and IIc as reference compounds. It was found that for good gas chromatographic separation of the available PHT analogs (Ia, Ib, Ic or Id) from the p-HPPH analogs (IIa, IIc) and for separation of both (Ia, Ib, Ic or Id) and (IIa, IIc) from endogenous components, it was necessary to convert them to more volatile compounds (22,23). As discussed later in this Section, a number of methylation and silylation procedures were therefore evaluated (8, 20-27); the extractive methylation procedure of Hoppel et al. (20) was finally selected because it gave the most reproducible derivatization.

Gas chromatographic conditions were chosen such that the permethylated derivatives of Ia, Ib, Ic or Id were coeluting, and that also the permethylated derivatives of IIa and IIc were coeluting. The structures of the

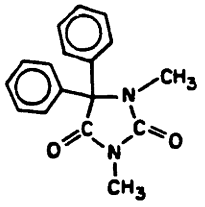
permethylated derivatives of the PHT analogs and HPPH analogs for which measurement was required during these studies (i.e. d-Ia, d-IIa, d-IIIa and their respective stable isotope labelled analogs) are shown in Figure II-1. We are referring to the permethylated derivatives of the studied PHT and HPPH analogs by the abbreviation "d-" (this indicates that the compounds are derivatized); the abbrevation "d-" should not be confused with "deuterium", for which in this work, we use the abbreviation "D" (see List of Abbreviations, p.21). It should be noted again that 1. during the development and evaluation of the method and during the studies with human volunteers, no m-HPPH analogs (d-IIIa and d-IIIb) were measured and 2. that d-IIb was only available as reference compound during the studies with human volunteers; that d-IIIb was not available as reference compound.

Acid hydrolysis of the glucuronides with 10 N HCl was preferred over enzymatic hydrolysis because the latter is very time consuming and precision has been reported to be worse (18). Addition of the internal standard [($^{13}C^{15}N_2D_2$)-p-HPPH, IIc] to the sample after the hydrolysis step prevented the possibility of acid catalyzed exchange of the deuterium atoms in ortho position of the phenolic hydroxyl group.

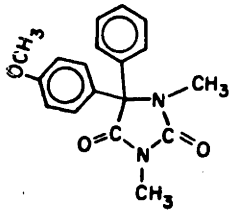
We recorded full mass spectra of the permethylated derivatives of the reference compounds that were available at this time. The mass spectra of the permethylated

Figure II-1. Structures of the permethylated derivatives of the PHT analogs (d-Ia, d-Ib, d-Ic, d-Id) and HPPH analogs (d-IIa, d-IIb, d-IIc, d-IIIa, d-IIIb) that are measured in these studies.

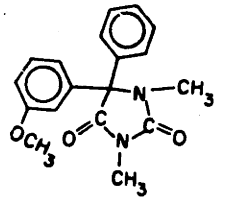
Figure II-1



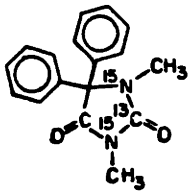
d-Ia



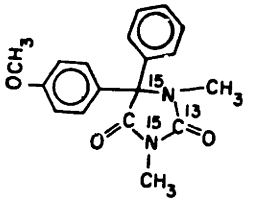
d-IIa



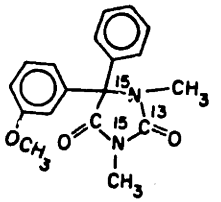
d-IIIa



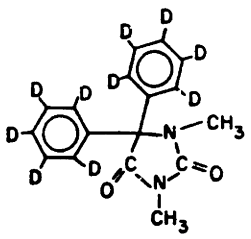
d-Ib



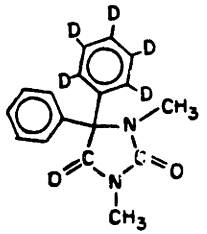
d-IIb



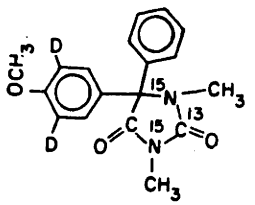
d-IIIb



d-Ic



d-Id



d-IIc

permethylated derivatives of the PHT analogs

permethylated derivatives of the p-HPPH analogs

permethylated derivatives of the m-HPPH analogs

derivatives of PHT (d-Ia) and the available stable isotope labelled analogs (d-Ib, d-Ic and d-Id) are shown in the Appendix, Figure A-1, a-d. The mass spectra of the permethylated derivates of the p-hydroxylated metabolite (d-IIa) and its available stable isotope labelled analog (d-IIc) are shown in Figure A-2, a and d. In Figure A-2, b and c, the mass spectra of the permethylated derivatives of the reference compounds IIIa and IIb are also shown; they were recorded during the studies described in Chapter IV and were included in this Figure so that their fragmentation pattern could be compared with that of the other HPPH analogs. In these mass spectra, the major fragment ions have been labelled; the fragmentation pattern is explained in the text.

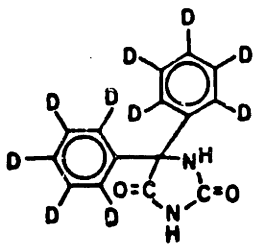
We also determined the actual level of incorporation of the stable isotope label in the molecule for all isotopically labelled substances. The isotopic purity and the presence of overlapping species could so be verified (see Chapter VI).

As expected, the deuterium labelled substances contained a larger percentage of fully labelled material because deuterium is available at 99+% isotopic purity, while available ^{13}C and ^{15}N have enrichment factors of 90 % and 99 % respectively. For (D_{10})-PHT (Ic) and $(^{13}C\,^{15}N_2)$-PHT (Ib), no impurities were found that would interfere with molecular ions (M^{+}) of any of the PHT analogs (Ia, Ib, Ic or Id). There is, of course, also no

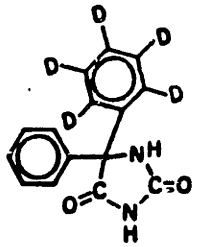
overlap of the natural isotope distribution of Ia to Ib and Ic, or of Ib to Ic because the molecular ions are separated by sufficient amu. (Natural abundance ions from Ib at m/z $(M+2)^+$ will of course overlap with the molecular ion from Id, but as discussed further, Id was only used during the initial measurement to quantitate Ia and in this case no overlap occurred.) However, 3 % trilabelled material was found in ($^{13}C\,^{15}N_2D_2$)-p-HPPH (IIc). This contributes to the ion abundances measured for ($^{13}C\,^{15}N_2$)-p-HPPH (IIb) and therefore has to be corrected for. In addition, IIc is only two amu heavier than IIb. Natural abundance ions from IIb at m/z $(M+2)^+$ will therefore overlap with M^+ ions from IIc. We did not have IIb available as reference compound during the method development and could therefore not determine to what extent these overlapping ion abundances would influence the measurements (see further Chapter III, Section E-3; it should be noted here that by permethylation, the increase in mass is the same for all analogs, so that the same interferences will occur).

We developed our method using serum and urine samples to which PHT (Ia), ($^{13}C\,^{15}N_2$)-PHT (Ib) and p-HPPH (IIa) had been added in known concentrations (i.e. serum and urine standard solutions, see Chapter VI). For serum, we used standard solutions containing 1.0-30.0 µg/ml of each Ia and Ib, and 0.5-10.0 g/ml of IIa; for urine, we used standard solutions containing 5.0-100.0 µg/ml of IIa. These ranges were chosen because, for serum, they represent the

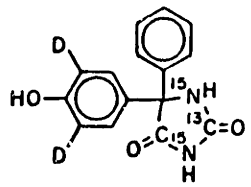
therapeutic concentration ranges of Ia under steady state conditions and for urine, they represent the excretion of IIa under these conditions. Initially, (D_5)-PHT (Id) and ($^{13}C^{15}N_2D_2$)-p-HPPH (IIc) were used as internal standards to develop the GC-MS measurement and the hydrolysis-, extraction- and derivatization procedure.



Ie



Id



IIc

Id had been previously synthesized in this laboratory by Dr. Brian Andresen and was available in sufficient quantities. It was not used in the later analyses because it is only two amu heavier than Ib (for the simultaneous measurement of Ia and Ib, Ic was always used as internal standard).

During the initial measurements, the data were recorded with a conventional oscillographic recorder so that peak heights of ion abundances could be measured manually. When the software for computerized data acquisition and processing became available, the data were recorded on tape and processing was done by the computer. Both height and area measurement of ion abundances was possible in this mode.

For the statistical interpretation of the data during the development of the method it was kept in mind that only

a limited number of experiments were done. The main purpose was to demonstrate the usefulness of the method under the existing operating conditions so that experiments for the further evaluation of the method could be designed (see further Chapter III).

B. GAS CHROMATOGRAPHIC MASS SPECTROMETRY

1. Gas chromatography

1a. Choice of the gas chromatographic derivatives

In order to maintain accuracy during the gas chromatographic measurements, well shaped gas chromato graphic peaks at reasonable retention times are required. Therefore, study of the gas chromatographic behaviour of the available PHT analogs (Ia, Ib, Ic or Id) and p-HPPH analogs (IIa, IIc) was an important first step in the development of the method. Initially, standard solutions of Ia and IIa were used; the analogous behaviour of the more expensive stable isotope labelled reference compounds was verified once the gas chromatography of Ia and IIa themselves was found satisfactory.

Gas chromatography of underivatized Ia on 3% OV-17 or 3% OV-101 column packings on 80-100 mesh Supelcoport was unsatisfactory. It was found that in order to obtain reproducible peak heights (measured off the flame ionization detector (FID) trace), the active sites of the column had to be saturated with at least 10 µg of Ia. The phenolic metabolite (IIa) did not chromatograph at all;

interaction between its polar functional group and the chromatographic support caused severe adsorption.

Silylation of the labile hydrogens of Ia and IIa with BSTFA or Sylon BFT (8, 21, 23) did not go to completion unless pyridine or acetonitrile were added as solvent. Even then, irreproducible results were obtained, possibly due to the fact that in the presence of moisture, the TMS derivatives are hydrolyzed. Drying of the solvent extracts (i.e. with anhydrous Na_2SO_4) and storing them in a desiccator increased the stability over a longer period of time, but also increased the analysis time substantially.

On column methylation (also referred to as flash-heater methylation) (23) produced excessive column bleed, and resulted in a high background for the mass spectrometric measurements. In addition, it shortened the column life considerably. The alkalinity of the ammonium hydroxides that were used, in combination with the high temperature required for methylation caused also the formation of a small amount of the O-methyl isomers. Incomplete permethylation was noticed when using low injector temperatures.

Extractive methylation (20, 24-27) of Ia and IIa gave the most satisfactory results. The procedure has been described for the analysis of Ia, IIa and IIIa in serum by Hoppel et al. (20). These authors developed optimum conditions, especially for the extractive permethylation of IIa. We applied the extractive methylation procedure (as

described in Chapter VI) to a solution of 50 µg of each Ia, Id and IIa and did not observe any partially methylated or degraded compounds. In addition, the permethylation was found to be reproducible (judged from peak heights in the gas chromatogram). Dry extracts of the permethylated derivatives were stable for at least 12 months when stored at 4°C. The analysis of randomly chosen extracts from serum or urine in later analyses (as discussed in Chapters III and IV), always showed complete permethylation of all compounds of interest.

Extractive alkylation has also been referred to as phase transfer catalysis (26). Acids are extracted as ion pairs (through the use of a bulky quaternary cation) from an alkaline solution into an organic phase, which contains the alkylating agent. [In our case the ion pairs consisted of Ia^-TBA^+, IIa^-TBA^+, etc.; they were extracted into methylene chloride (CH_2Cl_2) which contained methyl iodide (CH_3I)]. The alkylation takes place in the organic phase (selected to have a poor solvating ability) by a nucleo philic displacement reaction, as illustrated for Ia:

$$\begin{array}{lll} [TBA^+OH^-] + PHT^- \rightleftharpoons [TBA^+PHT^-] + OH^- & & \text{aqueous phase} \\ \hline \downarrow\uparrow \qquad\qquad\qquad \downarrow\uparrow & & \text{organic phase} \\ [TBA^+I^-] + PHT\text{-}CH_3 \longleftarrow [TBA^+PHT^-] + CH_3I & & \end{array}$$

A high concentration of counter ion increases the efficiency of the extraction. The rate of alkylation is

determined by the concentration of alkylating reagent and the reaction temperature.

The final dry residue, obtained after evaporation of the organic phase, contains the methylated acids and the tetrabutylammonium iodide (TBA^-I^+), which is formed as byproduct in the extractive alkylation reaction. This final dry residue is then dissolved in a suitable solvent for GC-MS analysis.

TBA^-I^+ decomposes in the injector port of the gas chromatograph to tributylamine (27). If present in high concentrations, this trialkylamine gives a considerable column background. It was found that the amount of TBA^-I^+ in the supernatant could be reduced by dissolving the final dry residue in toluene or benzene instead of methanol.

1b. Choice of gas chromatographic conditions

On a 3 ft OV-17 column, the permethylated derivatives of PHT (d-Ia) and p-HPPH (d-IIa) eluted in two sharp gas chromatographic peaks, when temperature programming from 180-310°C at 12°/min. The permethylated derivatives of the available ($^{13}C\,^{15}N_2$)-labelled analogs (d-Ib and d-IIc) eluted at the same time as the respective unlabelled analogs (d-Ia and d-IIa). The permethylated derivatives of (D_5)-PHT and (D_{10})-PHT (d-Id, d-Ic) eluted one and two seconds, respectively, earlier than their unlabelled analog (d-Ia). This did not lead to a separation in the gas chromatographic peak profile, it merely broadened the peak

slightly. In summary, this resulted in two gas chromatographically resolved sets of compounds consisting of (d-Ia, d-Ib, d-Ic or d-Ib) and (d-IIa, d-IIc) respectively. This is further discussed in Section B-2b of this Chapter, which discusses the mass spectrometric measurements and is also illustrated in Figure II-2a (see explanation in Section B-2b), which shows a total ionization plot (TIP) constructed during analysis of a serum sample. (A total ionization plot is a summation of the recorded ion currents and can be interpreted in the same way as an FID trace.)

Methyldocosanoate (X) coeluted with the permethylated

$$CH_3(CH_2)_{20}COOCH_3 \qquad (X)$$

derivatives of (Ia, Ib, Ic or Id) (it appeared at the tail end of the gas chromatographic peak) but did not interfere with their analysis at the levels used for the development of the method. (For lower levels of Ia or Ib encountered in the analysis of serum samples, the contribution of X, if substantial, must be corrected for as will be described in Section 3).

During the mass spectrometric measurements, the gas chromatographic behaviour of the analytes was constantly monitored by diverting 20-30% of the effluent from the column to the FID of the gas chromatograph (the remaining portion of the effluent was diverted to the mass spectrometer, see Chapter VI, Section C).

2. Mass spectrometric measurements

2a. Choice of the monitored ions

The molecular ion region was chosen to be monitored because it is definitely characteristic of the analytes and, in addition, it retains the complete isotopic information for all the analogs that are monitored. For the stable isotope labelled analogs, this information is partly lost in the major fragment ions because the part of the molecule bearing the stable isotope label has been lost. This is further discussed in the Appendix, e.g. for d-Ib (Figure A-1b), the ion abundances at m/z 195 arise from losses of $CH_3{}^{15}NCO$ and $^{13}CO,H^{\cdot}$ from the $M^{+\cdot}$; for d-Ic (Figure A-1c), the ion abundances at m/z 208 arise from loss of $C_6D_5^{\cdot}$ from the $M^{+\cdot}$.

Also, analysis of an extract of blank serum or urine (i.e. a drug-free serum or urine sample to which no internal standard has been added, see Chapter VI) showed only a low level of background at m/z 280, 283, 285, 290, 310, 313, 315, at the respective gas chromatographic retention times of d-Ia, d-Ib, d-Id, d-Ic, d-IIa, d-IIb and d-IIc (see also further in this Chapter, Section B-3). A constant background at m/z 315 was due to OV-17 column bleed, but was corrected for consistently in all analyses.

2b. Measurement of ion currents

The measurements of the molecular ion abundances of (d-Ia, d-Ib, d-Ic or d-Id) and (d-IIa, d-IIc) required

monitoring of two gas chromatographically resolved sets of compounds (as discussed previously in Section B-1b). We chose to repetitively scan over the molecular ion region of the analogs, rather than to step from one m/z value to the other (so called multiple ion monitoring), because the m/z values of the ions of which the abundances were to be measured during the emergence of each GC peak differ only by a few mass units (a maximum of 10). It should be noted that this approach is not related to the limited mass monitoring technique (LMM) described by Murphy et al. (28), where selected short mass ranges scattered over the entire spectrum and/or the gas chromatographic run are scanned to provide more qualitative information.

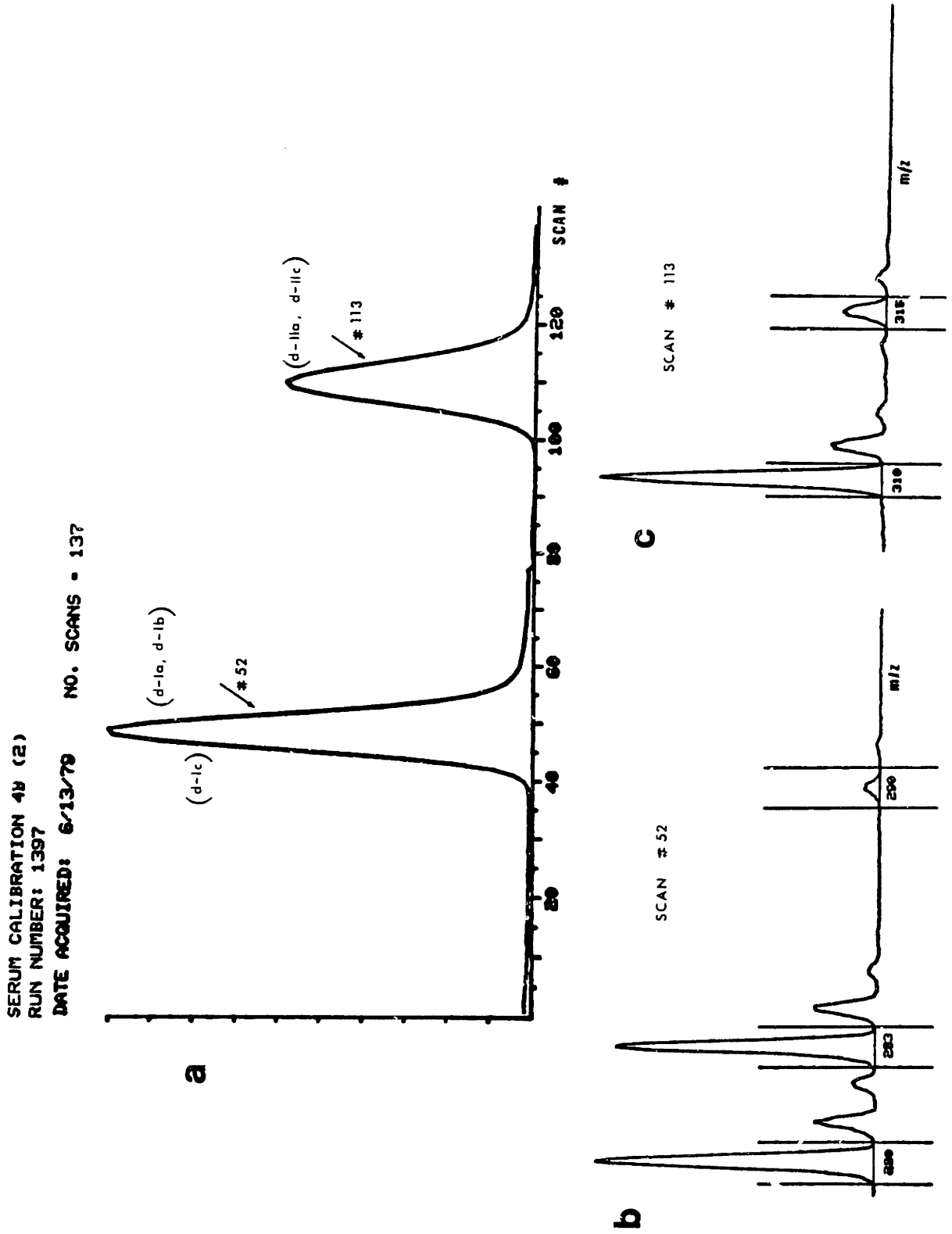
Although single or multiple ion monitoring usually leads to higher sensitivity because of the higher duty cycle, scanning through a short mass range does not waste that much more time. On the other hand, it makes it possible to display (eventually in real time), evaluate and utilize the complete mass spectral peak profile and this has many advantages: it assures that the top of the peak is always reliably measured -one of the major risks in the stepping method where peak tops are defined by hardware adjustments and jumping between preset voltages can cause inaccuracies-, that one can spot the presence of interferences from distortion of peak shapes or appearance of peaks which do not belong to the mass spectrum of the analyte, and that the baseline is always accurately

established for each scan.

With the preliminary instrument modifications, a linear mass range of m/z 278-293 was scanned in 4 s during the elution of the PHT analogs (d-Ia, d-Ib, d-Ic or d-Id) and a linear mass range of m/z 309-317 was scanned in 2 s during the elution of the p-HPPH analogs (d-IIa, d-IIc). This is illustrated in Figure II-2: in (a) a display of the total ionization plot (TIP) constructed during the analysis of a standard serum sample containing 10.0 µg/ml of Ia and Ib, 5.0 µg of II a and 1.0 µg of the respective internal standards (Ic and IIc) is presented; in (b) the mass range scanned for the measurement of the molecular ion abundances of (d-Ia, d-Ib, d-Ic), i.e. scan #52 of from the TIP , in (c) the mass range scanned for the measurement of the molecular ion abundances of (d-IIa, d-IIc), i.e. scan #113 from the TIP. The horizontal and vertical cursors allow exact definition of height and area of each individual preselected peak in the scanned mass range (see Chapter VI, Section C). Noise and interference from neighbouring peaks such as natural isotopic abundance peaks (m/z 281, 284, 291, 311, 314, 315) and isotopic impurity peaks (m/z 282, 313, 314) can thus be minimized. Due to the partial separation of d-Ic and (d-Ia, d-Ib) under the described gas chromatographic conditions, no information about the exact ratio of drug to internal standard is obtained from one specific scan (i.e. in scan #52 -the one shown in Figure II-2b most of d-Ic had eluted); the

Figure II-2. Display of (a) the total ionization plot (TIP) constructed during analysis of a serum standard sample (b) the mass range scanned for the measurement of molecular ion abundances of (d-Ia, d-Ib, d-Ic), as illustrated by scan # 52 from the TIP (b) the mass range scanned for the measurement of molecular ion abundances of (d-IIa, d-IIb), as illustrated by scan # 113 from the TIP.

Figure II-2


SERUM CALIBRATION 4β (2)
RUN NUMBER: 1397
DATE ACQUIRED: 6/13/79
NO. SCANS = 137
(d-Ic)
(d-Ia, d-Ib)
#52
(d-IIa, d-IIc)
#113
SCAN #
a
SCAN #52
SCAN #113
m/z
b
c

correct ratio is obtained by averaging all the scans selected from the total ionization plot.

3. Interferences

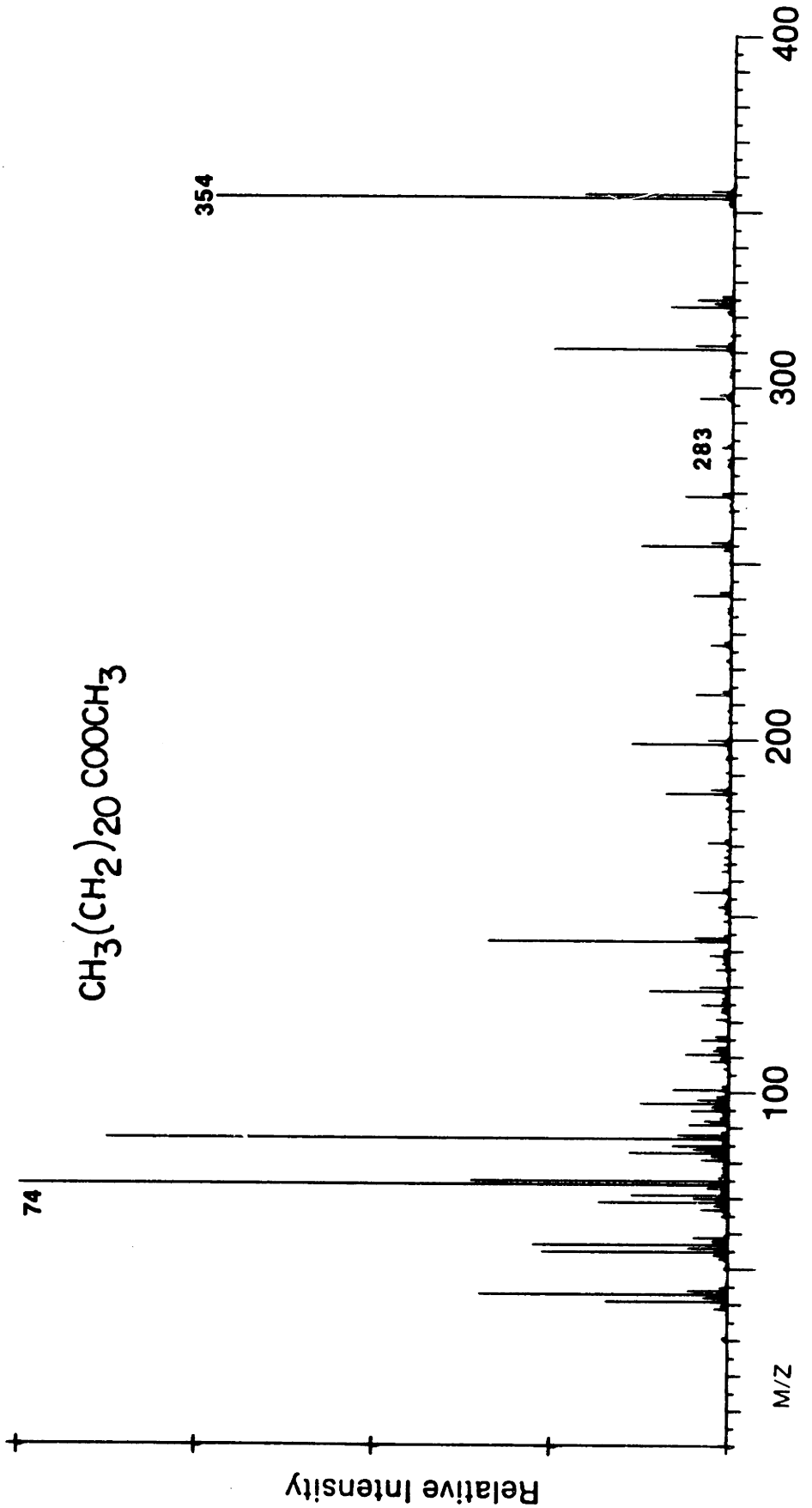
Interferences at the selected ions can come from many sources: there can be background interference from the sample matrix or solvents and reagents (i.e. a compound can have the same gas chromatographic retention time and have the same specific ion in its mass spectrum as the ion that is being measured); there can be instrumental background (e.g. arising from a contaminated ion source, column bleed, memory between succesive samples, or electronic noise); and there can be a significant contri bution from the labelled internal standard to the analyte and vice versa.

Although under the described gas chromatographic conditions, methyldocosanoate (X) coeluted with (d-1a, d-1b, d-1c or d-1d) (it appeared at the tail of the gas chromatographic peak), the contribution of its ion abundances to the ion abundances that were measured for the phenytoin analogs remained insignificant at the initially expected serum phenytoin levels (1.0-30.0 µg/ml). No corrections were therefore necessary at this point. A mass spectrum of methyldocosanoate (X) is presented in Figure II-3.

Instrumental background was found to be insignificant under normal operating conditions because the concentra

Figure II-3. Mass spectrum of methyl docosanoate

Figure II-3


$CH_3(CH_2)_{20}COOCH_3$
Relative Intensity
M/Z
74
283
354
100
200
300
400

tions of the analytes that were measured during the development of the method were well above the detection limits of the assay.

No interferences arising from residual unlabelled analyte present in the stable isotope labelled compounds, or overlapping natural abundance ions from these analogs had to be taken into account during these initial studies (see Section A).

In all cases, the significance of the interferences was assessed by interpretation of the calibration curves. If the calibration curve was linear through the lowest point, the interference was not considered to be significant (this is discussed in Chapter III, Section E-3).

C. EXTRACTION AND HYDROLYSIS

1. Choice of the procedure for processing of the samples

The processing of the samples (i.e chemical work-up of the samples to obtain extracts suitable for GC-MS analysis) is based on the one described by Hoppel et al. (20) and includes the extractive methylation procedure, as previously described. The serum and urine samples are hydrolyzed in acid, extracted at pH 7.4 with MIBK, back extracted into NaOH and permethylated with CH_3I (see Chapter VI for more details).

However, during the acid hydrolysis step, the dihydrodiol metabolite (IVa) is converted to equal amounts

of IIa and IIIa (6, 20). The measured values of IIa in samples from patients will thus be higher than the actual value, but this bias will be the same for unlabelled and ($^{13}C^{15}N_2$)-labelled p-HPPH (IIa and IIb) in samples from the same patients. IVa accounts for 7-11 % of the urinary metabolite of Ia in man (6). Under the reasonable assumption that no or very little IIIa is produced in man (4), this excess IIa could be corrected for by measuring the amount of IIIa present and subtracting it from the total IIa that is found.

During acid hydrolysis, the diphenylhydantoic acid metabolite (VIIIa) is reconverted to Ia (29). As we are not analyzing for Ia in urine, this does not affect our measurements.

D. INITIAL STATISTICAL ANALYSIS

1. Precision of the GC-MS measurement

The precision of the GC-MS measurement was initially evaluated by making repeated injections of a standard solution containing equal amounts (1 µg/ml) of d-Ia and d-Id in benzene. The CV for the measurement of the molecular ion abundance ratios (m/z 280/285) was 0.62% for N=4 (Peak heights measured manually off the oscillographic trace, N is the number of replicate injections). Magnet drift was minimized by monitoring the room temperature and was, if necessary, corrected for (as judged from the position of the peaks as they were displayed on the

oscilloscope of the mass spectrometer).

However, the precision of this instrumental step depends on the amount of material injected, the nature of the internal standard, and the ratio of analyte to internal standard. In addition, variations in mass spectrometric operating conditions have a significant influence on the measurement. This is especially important when the analysis is performed over a wide range of isotope ratios and over a long period of time (long-term precision) (30-34). To evaluate these factors, replicate analyses of selected extracts were carried out during the further development, evaluation and applications of the method so that the precision of the measurements under the prevailing conditions was always known (see further Chapter III, Section E-3).

2. Precision of the extraction and derivatization procedure, followed by GC-MS analysis.

Initially, the precision of the extraction and derivatization followed by GC-MS analysis, was evaluated only for the analysis of Ia in serum and Id was used as internal standard.

Samples (1.0 ml) of three albumin serum substitute standard solutions, which respectively contained 30.0 µg/ml, 10.0 µg/ml and 1.0 µg/ml of Ia, were processed and analyzed by GC-MS (replicate samples were processed for each of the serum standard solutions, and each extract was

analyzed once by GC-MS). Human drug free serum was not available at this time and therefore albumin serum substitute was substituted. The amount of Id that was added to each sample was equal to its concentration of Ia, so that ratios of molecular ion abundances of 1:1 would result and oscillographic recording would be easy. Due to excessive instrumental instability (i.e. magnet drift), which displaced the m/z values of the ions that were monitored and affected the peak shape, some of the data had to be discarded. Only these GC-MS runs for which the measured ion abundances were within the linear range were used for statistical interpretation. The CV for measurement of the molecular ion abundance ratios of d-Ia/d-Id (m/z 280/285) in samples of the 30.0 µg/ml standard solution was 1.34 % (N=7); for analysis of samples of the 10.0 µg/ml standard solution, the CV was 1.20 % (N=8); for analysis of samples of the 1.0 µg/ml standard solution, the CV was 0.74 % (N=6) (Peak heights measured manually off the oscillographic trace). A better estimate would have been obtained here by closer attention to the magnet drift (i.e. the scanned mass range) during the runs. In further analyses, magnet drift was minimized by keeping the room temperature below 78°C and was, if necessary, corrected for in real time (judging from the appearance of the peak on the oscilloscope of the mass spectrometer).

Samples (1.0 ml) of a human serum standard solution

containing 10.0 µg/ml of Ia (and which had been kept frozen) were processed and analyzed by GC-MS (12 samples of the serum solution were worked-up, each extract was analyzed once by GC-MS). To each sample, 10.0 µg of Id was added after thawing. For N=12, the CV for measurement of the molecular ion abundance ratios of d-Ia/d-Id (m/z 280/285) was 1.35 %. The stability of Ia in these frozen biological samples was also monitored over a period of six months. No concentration changes were found. These results confirmed findings described elsewhere in the literature (35).

As expected, the experiments indicated that the error introduced during the work-up of the samples remained insignificant (most systematic errors during sample work-up are eliminated by the use of appropriate internal standards; the main source of inaccuracy is pipetting error). However, during further development and evaluation of the method, more replicate and duplicate analyses of the samples were carried out so that the precision of the method under the different conditions was known.

3. Precision and effectiveness of the hydrolysis procedure.

It was first verified that the hydrolysis conditions did not affect the previously determined precision for Ia (i.e. that Ia was stable under these acid hydrolysis conditions). Samples (1.0 ml) of a human serum standard

solution, containing 10.0 µg/ml of Ia, were subjected to the acid treatment, 10.0 µg of internal standard (Ic) was added, and they were extracted and analyzed by GC-MS while control samples were analyzed simultaneously. No significant differences were found between molecular ion abundance ratios of d-Ia/d-Ic (m/z 280/290) of hydrolyzed and non- hydrolyzed samples (peak heights measured manually off the oscillographic trace). This confirmed that Ia is stable during acid hydrolysis (29).

The usefulness of IIc as internal standard for the measurement of IIa was then evaluated by analysis of samples (1.0 ml) of a urine standard solution, containing 25.0 µg/ml of IIa. The samples (N=8) were subjected to the acid treatment, 10.0 µg of IIc was added, they were extracted, derivatized and analyzed by GC-MS. The molecular ion abundance ratios of d-IIa/d-IIc (m/z 310/315) were measured and for N=8, the CV was 1.97 % (peak heights measured manually off the oscillographic trace). This low CV ruled out any deuterium exchange during sample work-up under these conditions.

The effectiveness of the hydrolysis procedure was verified on serum and urine samples from patients on phenytoin (Ia) therapy, because serum and urine standard solutions, as prepared by us, only contained the free metabolite (IIa) and no glucuronide (GlcUA-IIa). A series of 0.5 ml of urine samples from the same patient were therefore hydrolyzed in 10 N HCl at 96 °C for different time

periods up to 90 min (25.0 µg of the internal standard were added). Duplicate samples were processed at each point, followed by a single GC-MS analysis for each extract. The molecular ion abundance ratios (heights) of d-II a/d-IIc (m/z 310/315) at each time point are reported in Table II-1, part A. These data show that the hydrolysis was complete after 45 min of acid treatment at 96°C and that longer acid treatment (up to 90 min) did not affect the stability of IIa. In a second experiment, a series of 1.0 ml of serum and urine samples from different patients were hydrolyzed for 1 h. To the serum samples, 10.0 µg of Ic and 5.0 µg of IIc were added; to the urine samples, 10.0 µg of IIc were added. The mean of the molecular ion abundance ratios of d-Ia/d-Ic (m/z 280/290) and d-IIa/d-IIc (m/z 310/315) and the SD and CV of the measurements are listed in Table II-1, part B. The precision of the analysis was hereby confirmed and, in addition, it was shown again that Ia is stable during acid hydrolysis. The CV (4.1 % for Ia and 5.3 % for IIa; N=2 or 3) was within the precision that could be estimated for the overall analysis under the prevailing conditions. The precision was not as good as in the previous measurements, because unexpectedly large amounts of IIa were found in the urine samples so that carry-over from one injection into the other occurred. As explained earlier (see Section C-1) these samples from patients also contained small amounts of IIIa, which was not fully resolved from IIa under the (at

TABLE II-1

ANALYSIS OF SERUM AND URINE SAMPLES FROM PATIENTS ON PHT THERAPY (A) EFFECTIVENESS OF THE HYDROLYSIS PRODECURE [To 0.5 ml of urine, 25.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standard.] (B) PRECISION [To 1.0 ml of serum, 10.0 µg of (D_{10})-PHT and 5.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards; to 1.0 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)p-HPPH were added as internal standard.]

(A)

Time Point	Height Ratio m/z 310/315	
	Sample 1	Sample 2
15 min	3.91	4.18
30 min	9.02	9.04
45 min	9.96	9.86
60 min	9.91	9.86
90 min	10.13	10.18

(B)

Sample	N	PHT Analysis Height Ratio m/z 280/290 Mean ± SD	CV(%)	p-HPPH Analysis Height Ratio m/z 310/315 Mean ± SD	CV (%)
Patient 1, serum	2	1.37 ± 0.06	4.13	0.581 ± 0.011	1.95
Patient 2, serum	3	0.386 ± 0.017	4.33	0.514 ± 0.021	4.12
Patient 3, urine	3			23.89 ± 1.25	5.25
Patient 4, urine	3			10.56 ± 0.52	4.89

this time) employed gas chromatographic conditions. (In the urine standard solutions which were prepared for the evaluation of the method no IIIa was present and the amount of material that was injected into the gas chromatograph was better controlled.)

4. Linearity and accuracy

Samples (1.0 ml) of four human serum standard solutions, which respectively contained 1.0, 5.0, 10.0 and 25.0 µg/ml of Ia, were extracted, derivatized, and analyzed by GC-MS. For each serum standard solution, a single sample was processed (so that one extract was obtained); for each extract, duplicate GC-MS analyses were carried out. To each sample, 10.0 µg of Id was added. No hydrolysis step was included. Linearity was demonstrated by linear regression analysis (LRA) of the mean values of the molecular ion abundance ratios of d-Ia/d-Id (m/z 280/285) on the concentrations of Ia. Table II-2 lists the measured ion ratios (heights) at each concentration point and the results from the LRA.

Samples (1.0 ml) of four urine standard solutions, which respectively contained 5.0, 25.0, 50.0 and 100.0 µg/ml of IIa, were hydrolyzed, extracted, derivatized, and analyzed by GC-MS. For each urine standard solution, four samples were processed and for each extract, a single GC-MS analysis was carried out. To each sample, 10.0 µg of IIc was added. Linearity was again demonstrated by linear

TABLE II-2

LINEARITY OF THE METHOD. Sample: 1.0-25.0 µg of PHT/ml serum. [To 1.0 ml of serum, 10.0 µg of (D_5)-PHT were added as internal standards]

Concentration (µg/ml)	Height Ratio m/z 280/290	
	Sample 1	Sample 2
1.0	0.107	0.097
5.0	0.505	0.494
10.0	1.06	1.07
25.0	2.56	2.54

LINEAR REGRESSION ANALYSIS

$y = 0.10\ x + 0.01$
$r = 0.9997$

regression analysis of the mean value of the molecular ion abundance ratios of d-IIa/d-IIc (m/z 310/315) on the concentrations. Table II-3 lists the mean values of the ion abundance ratios (heights) for each standard sample, the SD and CV of the measurements, and the results of the LRA.

For the measurements reported in this Chapter, linearity was demonstrated by LRA of the data without weighting (see Chapter III, Section E-3). It should be kept in mind however that linearity can also be shown graphically by plotting the isotope ratios against the concentrations. This is not illustrated in this Chapter, but examples are presented for selected data obtained during the evaluation of the method (see Chapter III).

The accuracy of the method was verified for the determination of Ia in serum. Serum samples from patients on phenytoin (Ia) therapy were analyzed and the results compared with values obtained by an established gas chromatographic method, in which 5-(4-methylphenyl)-5-phenylhydantoin is used as internal standard (36). We used the previously obtained calibration data for Ia in serum (Table II-2). Only one sample per patient was analyzed. The results are reported in Table II-4. As is obvious from inspection, the values that we obtained are close to the values obtained by the on column alkylation technique (V.A. Hospital Laboratory). We calculated 95% confidence limits for small sample estimates based on the range for the calibration curve values (N=2). Confidence limits for the

TABLE II-3

PRECISION AND LINEARITY OF THE METHOD. Sample: 5.0-100.0 µg of p-HPPH/ml urine. [To 1.0 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standard.]

Concentration (µg/ml)	N	Height Ratio m/z 310/315 Mean ± SD	CV (%)
5.0	4	0.664 ± 0.035	5.29
25.0	4	2.85 ± 0.045	1.60
50.0	4	5.60 ± 0.016	2.85
100.0	4	10.49 ± 0.32	3.08

Linear Regression Analysis

$y = 0.10 x + 0.25$

$r = 0.9995$

TABLE II-4

ANALYSIS OF PHT IN SERUM SAMPLES FROM PATIENTS: COMPARISON OF TWO DIFFERENT METHODS. [To 1.0 ml of serum, 10.0 μg of (D_{10})-PHT were added as internal standard.]

Sample	Extractive methylation and GCMS (our laboratory)	On column hexylation and GC (VA Hospital laboratory)
Patient 1	3.06 ± 0.64*	3.2 **
Patient 2	9.71 ± 0.64	9.3
Patient 3	11.76 ± 0.70	11.6
Patient 4	3.72 ± 0.64	3.3

* Confidence limits (95%) for small sample estimates based on the range (see text).

** No statistical data on VA analysis available.

unknowns were determined by extrapolation. It is clear that in this experiment the 95 % confidence range will be large because it is only based on two observations. (No statistical data were available on the V.A. Analysis).

Chapter III. EVALUATION OF THE METHOD

A. APPROACH

In order to have confidence in the continued use of the method, its performance characteristics were evaluated.

We determined the within-sample and between-sample precision for analysis of samples from selected serum or urine standard solutions which contained low, normal and above-normal concentrations of PHT (Ia), ($^{13}C^{15}N_2$)-PHT (Ib) and p-HPPH (IIa). For the within-sample measurements, a single sample was processed for each of the serum or urine standard solutions so that one extract per sample was obtained, and for each extract, replicate GC-MS analyses were then carried out; for the between-sample measurements, replicate samples were processed for each of the serum or urine standard solutions, and each extract was analyzed once by GC-MS. Appropriate isotopic dilutions were always employed.

The day-to-day variation of the method was determined for analysis of serum and urine standard solutions containing therapeutic levels of Ia, Ib and IIa. A limited number of replicate analyses were done and the SD and CV for the between-day measurements of ion abundance ratios was calculated. The data were not subjected to further statistical analysis because it is evident that the appropriate use of the stable isotope dilution technique, as described in Chapter II, eliminates significant

day-to-day variation. (Day-to-day variation studies are only important in methods where the concentration of analytes in (frozen) specimens can change, where no internal standards are used, or where calibration data are not consistent between days. A meaningful analysis of variance (ANOVA) of the within-day and the between-day measurements then requires more experimental data.)

Additional evaluation of the method involved construction of standard curves and interpretation of calibration data covering the appropriate concentration ranges of Ia, Ib and IIa so that it could be verified that the relationship between ion abundance ratios and concentrations remained linear. Finally, the short-term and long-term precision of the GC-MS measurements under various isotopic dilution conditions was evaluated and the effect of interferences on the measurement of the analytes was determined.

For the simultaneous determination of unlabelled PHT (Ia) and ($^{13}C^{15}N_2$)-labelled PHT (Ib) in serum, (D_{10})-PHT (Ic) was always used as internal standard. Additional evaluations for the simultaneous measurement of unlabelled and ($^{13}C^{15}N_2$)-labeled p-HPPH (IIa and IIb) were not carried out because the chemical behaviour of IIb was assumed to be identical to that of IIa (which had been proven to be correct for Ia and Ib).

Precision and linearity were first evaluated for the simultaneous determination of Ia (1.0-30.0 µg/ml), Ib

(1.0-30.0 µg/ml) and IIa (0.5-10.0 µg/ml) in serum (i.e. at concentrations under steady state conditions in most patients).

Day-to-day variation was determined for analysis of Ia and Ib (both at levels of 1.0, 15.0 and 30.0 µg/ml), and IIa (at levels of 0.5, 5.0 and 10.0 µg/ml) in serum; for IIa (at levels of 5.0, 25.0, 50.0, 100.0 and 200.0 µg/ml) in urine. The extra point (200.0 µg/ml of IIa) for analysis of urine samples was included because in a previous experiment (see Chapter II, Section D-3 and Table II-1, part B) it was found that concentrations of IIa in urine of 238 µg/ml could occur (the initially obtained calibration data only covered a range of 5.0-100.0 µg of IIa/ml urine, see Table II-3). It was not possible to extend the calibration further upwards (i.e. to obtain points for concentrations of IIa higher than 200.0 µg/ml urine) because no accurate urine standard solutions containg high concentrations of IIa could be made (e.g. when it was attempted to prepare a urine standard solution containing 250 µg of IIa/ml, IIa did not seem to dissolve completely.) If in future analyses, urine samples containing IIa at levels higher than 200.0 µg/ml are to be analyzed, the concentrations of IIa will have to be determined by extrapolation of the calibration data.

Measurement of serum levels of 1.0 µg/ml of Ia and 0.5 µg/ml of IIa under the prevailing conditions (i.e. in most cases only part of the extract was injected and gas

chromatographic mass spectrometric operating conditions were often not optimized) gave adequate precision. It was therefore assumed that measurements at lower concentrations would be possible by more careful analysis, and in particular, by improving the scanning conditions (see further Section E-1). Reliable measurements of lower levels of Ia, IIa and their ($^{13}C^{15}N_2$)-labelled analogs would have the obvious advantage that less drug would have to be administered in the studies with human volunteers and the chance of accidental overdosing of a particularly sensitive individual would be eliminated. Measurements as low as 0.1 µg/ml of Ia, Ib and IIa were therefore evaluated. A downward extension of the calibration data for both Ia and Ib to the range of 0.1-30.0 µg/ml; for IIa to 0.1-10.0 µg/ml was accomplished by adjusting the isotopic dilution.

B. ANALYSIS OF SERUM STANDARD SOLUTIONS CONTAINING 1.0-30.0 µG/ML OF EACH PHT (Ia) AND ($^{13}C^{15}N_2$)-PHT (Ib) AND 0.5-10.0 µG/ML OF p-HPPH (IIa).

1. Precision and linearity

Samples (1.0 ml) of three serum standard solutions containing Ia, Ib and IIa in increasing concentrations (standard solution #1: 1.0 µg of each Ia and Ib, 0.5 µg of IIa; standard solution #2: 15.0 µg of each Ia and Ib, 5.0 µg of IIa; standard solution #3: 30.0 µg of each Ia and Ib, 10.0 µg of IIa) were analyzed in quadruplicate (i.e.

for each standard solution, four samples were processed, and each extract was analyzed once by GC-MS). To each sample, 10.0 µg of Ic and 5.0 µg of IIc were added. The mean of the molecular ion abundance ratios of d-Ia/d-Ic (m/z 280/290), d-Ib/d-Ic (m/z 283/290) and d-IIa/d-IIc (m/z 310/315), and the SD and CV of the measurements (N=4) are listed in Table III-1. Both height and area measurements are reported. During the processing of the samples of solution #1, two samples were lost. During the GC-MS analysis of the extracts obtained by processing of samples of standard solution #3, in two instances samples were injected which were so large that the signals at m/z 280 and 283 were off scale and could thus not be used.

Linearity was demonstrated by plotting the mean values of the ion abundance ratios (as listed in Table III-1) against the respective concentrations (this is illustrated for the area meaurements in Figure III-1; in (a) the area ratios of m/z 280/290 and m/z 283/290 are plotted against the respective concentrations of Ia and Ib, in (b) the area ratios of 310/315 is plotted against the concentration of IIa), or by linear regression analysis (LRA) of the data (see Table III-1).

2. Day-to-day variation

Samples (1.0 ml) of three serum standard solutions containing Ia, Ib and IIa in increasing concentrations (solution #1: 1.0 µg/ml of each Ia and Ib, 0.5 µg/ml of

Figure III-1. Calibration curves for analysis of (a) Ia and Ib (b) IIa, in serum samples. Sample: 1.0-30.0 µg/ml of each Ia and Ib, and 0.5-10.0 µg/ml of IIa.

The difference in slope of the calibration curves of Ia and Ib is explained in the text (p. 108)

Figure III-1

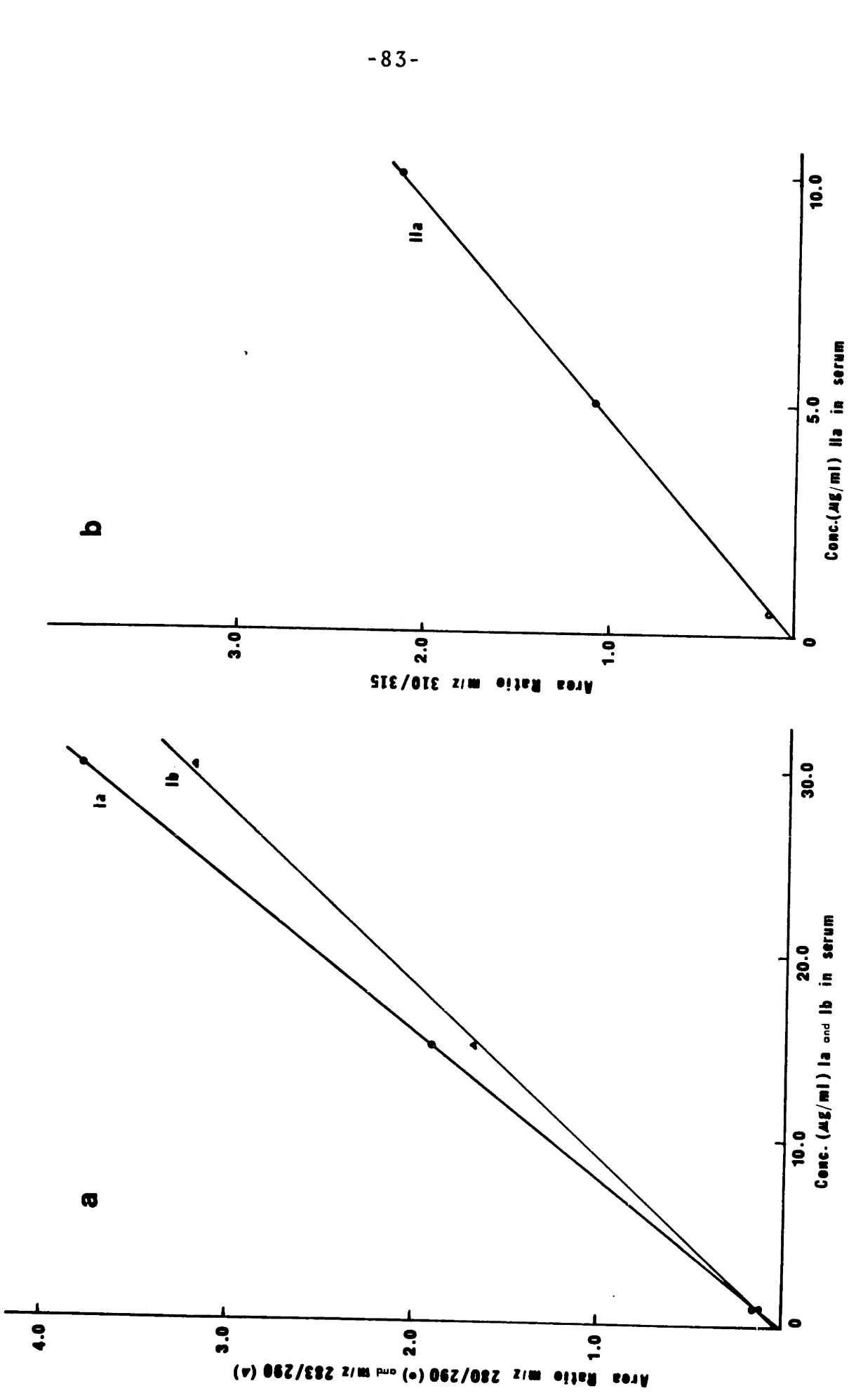

a
Area Ratio m/z 280/290 (•) and m/z 283/290 (▲)
4.0
3.0
2.0
1.0
0
10.0
20.0
30.0
Conc. (μg/ml) Ia and Ib in serum
Ia
Ib
b
Area Ratio m/z 310/315
3.0
2.0
1.0
0
5.0
10.0
Conc.(μg/ml) IIa in serum
IIa

TABLE III-1

PRECISION AND LINEARITY OF THE METHOD. Sample: 1.0-30.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5-10.0 µg of p-HPPH/ml serum. [To 1.0 ml of serum, 10.0 µg of (D_{10})-PHT and 5.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

PHT

Concentration (µg/ml)	N	Height Ratio m/z 280/290 Mean ± SD	CV (%)	Area Ratio m/z 280/290 Mean ± SD	CV (%)
1.0	2	0.162 ± 0.005	3.06	0.154 ± 0.008	5.51
15.0	4	1.76 ± 0.04	2.04	1.92 ± 0.01	0.74
30.0	2	3.35 ± 0.14	4.22	3.81 ± 0.06	1.51

($^{13}C^{15}N_2$)-PHT

Concentration (µg/ml)	N	Height Ratio m/z 283/290 Mean ± SD	CV (%)	Area Ratio m/z 283/290 Mean ± SD	CV (%)
1.0	2	0.150 ± 0.005	3.31	0.137 ± 0.008	5.70
15.0	4	1.61 ± 0.01	0.78	1.69 ± 0.01	0.30
30.0	2	2.97 ± 0.11	3.81	3.20 ± 0.06	1.99

p-HPPH

Concentration (µg/ml)	N	Height Ratio m/z 310/315 Mean ± SD	CV (%)	Area Ratio m/z 310/315 Mean ± SD	CV (%)
0.5	2	0.158 ± 0.005	3.14	0.122 ± 0.003	2.32
5.0	4	1.17 ± 0.02	1.76	1.09 ± 0.02	2.04
10.0	4	2.22 ± 0.04	1.67	2.14 ± 0.04	1.77

LINEAR REGRESSION ANALYSIS

PHT	$y = 0.11 x + 0.07$, $r = 0.9998$	$y = 0.13 x + 0.03$, $r = 0.9999999$
	$\log y = 0.89 \log x - 0.79$, $r = 0.99996$	$\log y = 0.94 \log x - 0.81$, $r = 0.9999$
($^{13}C^{15}N_2$)-PHT	$y = 0.10 x + 0.09$, $r = 0.9992$	$y = 0.11 x + 0.06$, $r = 0.9996$
	$\log y = 0.88 \log x - 0.82$, $r = 0.999999$	$\log y = 0.93 \log x - 0.86$, $r = 0.999999$
p-HPPH	$y = 0.22 x + 0.06$, $r = 0.9998$	$y = 0.21 x + 0.02$, $r = 0.99998$
	$\log y = 0.88 \log x - 0.54$, $r = 0.9999$	$\log y = 0.95 \log x - 0.63$, $r = 0.99999$

IIa; solution #2: 15.0 µg/ml of each Ia and Ib, 5.0 µg/ml of IIa; solution #3: 30.0 µg/ml of Ia and Ib, 10.0 µg/ml of IIa) were analyzed in duplicate on three consecutive days (i.e. each day, two samples were processed for each standard serum solution and for each extract, a single GC-MS analysis was carried out). To each sample, 10.0 µg of Ic and 5.0 µg of IIc were added. The molecular ion abundance ratios (height and areas) of d-Ia/d-Ic (m/z 280/290), d-Ib/d-Ic (m/z 283/290) and d-IIa/d-IIc (m/z 310/315) are listed in Table III-2. It is obvious from inspection that there are no significant differences between the within-day and between-day measurements. For each day, the mean of the ion abundance ratios was calculated, and an estimate for the day-to-day variation was obtained by comparing the results for the three consecutive days (see Table III-3 for the statistical analysis; Mean, SD and CV are listed). These data were also used to demonstrate linearity: the results from the LRA of the ion abundance ratios on the concentrations are also reported in Table III-3.

C. ANALYSIS OF STANDARD URINE SOLUTIONS CONTAINING 5.0-200.0 µG/ML OF p-HPPH (IIa).

1. Precision and linearity

The precision and linearity for measurement of IIa in urine (5.0-100.0 µg/ml) has been reported in Table III-3 (Development of the method). Since IIb was not available,

TABLE III-2

PRECISION OF THE METHOD, DETERMINED ON THREE CONSECUTIVE DAYS. Sample: 1.0, 15.0, and 30.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5, 5.0 and 10.0 µg of p-HPPH/ml serum.
[To 1.0 ml of serum, 10.0 µg of (D_{10})-PHT and 5.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

	Concentration (µg/ml)	Day 1		Day 2		Day 3	
		Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
PHT							
		Height Ratio m/z 280/290					
	1.0	0.136	0.128	0.128	0.118	0.135	0.123
	15.0	1.66	1.69	1.64	*	1.71	1.67
	30.0	3.20	3.33	3.26	3.39	3.30	3.28
		Area Ratio m/z 280/290					
	1.0	0.115	0.107	0.108	0.109	0.124	0.115
	15.0	1.73	1.76	1.71	*	1.76	1.75
	30.0	3.33	3.49	3.48	3.44	3.49	3.52
($^{13}C^{15}N_2$)-PHT							
		Height Ratio m/z 283/290					
	1.0	0.141	0.128	0.124	0.122	0.134	0.137
	15.0	1.49	1.52	1.54	*	1.53	1.53
	30.0	2.84	2.97	2.95	2.96	2.95	2.96
		Area Ratio m/z 283/290					
	1.0	0.112	0.107	0.106	0.105	0.119	0.119
	15.0	1.55	1.57	1.55	*	1.58	1.58
	30.0	2.97	3.16	3.06	3.04	3.06	3.10
p-HPPH							
		Height Ratio m/z 310/315					
	0.5	0.163	0.142	0.150	0.139	0.150	0.136
	5.0	1.12	1.16	1.14	*	1.15	1.21
	10.0	2.31	*	2.26	*	2.22	*
		Area Ratio m/z 310/315					
	0.5	0.120	0.116	0.109	0.117	0.125	0.113
	5.0	1.08	1.10	1.06	*	1.10	1.11
	10.0	2.15	*	2.19	*	2.17	*

* Data were lost because of computer hardware problems.

TABLE III-3

DAY-TO-DAY VARIATION AND LINEARITY OF THE METHOD. Sample: 1.0-30.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5-10.0 µg of p-HPPH/ml serum.

[To 1.0 ml of serum, 10.0 µg of (D_{10})-PHT and 5.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

DAY TO DAY VARIATION (N=3)

PHT

Concentration (µg/ml)	Height Ratio m/z 280/290 Mean ± SD	CV (%)	Area Ratio m/z 280/290 Mean ± SD	CV (%)
1.0	0.128 ± 0.005	3.58	0.113 ± 0.006	5.17
15.0	1.67 ± 0.03	1.58	1.74 ± 0.03	1.52
30.0	3.28 ± 0.01	0.30	3.46 ± 0.05	1.45

($^{13}C^{15}N_2$)-PHT

Concentration (µg/ml)	Height Ratio m/z 283/290 Mean ± SD	CV (%)	Area Ratio m/z 283/290 Mean ± SD	CV (%)
1.0	0.131 ± 0.007	5.51	0.112 ± 0.007	5.96
15.0	1.53 ± 0.02	1.00	1.56 ± 0.02	0.98
30.0	2.94 ± 0.03	0.98	3.07 ± 0.02	0.50

p-HPPH

Concentration (µg/ml)	Height Ratio m/z 310/315 Mean ± SD	CV (%)	Area Ratio m/z 310/315 Mean ± SD	CV (%)
0.5	0.147 ± 0.005	3.60	0.117 ± 0.003	2.76
5.0	1.15 ± 0.02	2.00	1.09 ± 0.03	3.32
10.0	2.26 ± 0.05	1.99	2.17 ± 0.02	0.92

LINEAR REGRESSION ANALYSIS

PHT

$y = 0.11 x + 0.03$, $r = 0.99997$

$\log y = 0.95 \log x - 0.89$, $r = 0.99999$

$y = 0.12 x + 0.001$, $r = 0.99999$

$\log y = 1.01 \log x - 0.95$, $r = 0.999996$

($^{13}C^{15}N_2$)-PHT

$y = 0.10 x + 0.05$, $r = 0.9998$

$\log y = 0.91 \log x - 0.88$, $r = 0.99998$

$y = 0.10 x + 0.02$, $r = 0.99997$

$\log y = 0.97 \log x - 0.95$, $r = 0.9999998$

p-HPPH

$y = 0.22 x + 0.04$, $r = 0.999999$

$\log y = 0.91 \log x - 0.56$, $r = 0.9999$

$y = 0.22 x + 0.01$, $r = 0.99999996$

$\log y = 0.97 \log x - 0.64$, $r = 0.99999$

no additional experiments were carried out. Further information concerning precision and linearity for measurement of concentrations of IIIa of 5.0-200.0 µg/ml was obtained by interpretation of the data in the day-to-day variation studies.

2. Day-to-day variation

Samples (0.5 ml) of five urine standard solutions, which respectively contained 5.0, 25.0, 50.0, 100.0 and 200.0 µg/ml of IIa, were analyzed in duplicate on three consecutive days (i.e. each day, two samples were processed for each urine standard solution and for each extract, a single GC-MS analysis was carried out). To each sample, 10.0 µg of IIc were added. The measured ion abundance ratios (height and area measurements) are reported in Table III-4. It is obvious from inspection that there are no significant differences between the within-day and between-day measurements. For each day, the mean of the ion abundance ratios was again calculated, and an estimate of the day-to-day variation was obtained by comparing the results for the three consecutive days (see Table III-5 for the statistical analysis; Mean, SD and CV are listed). These data were used to demonstrate linearity: plotting the ion abundance ratios against the respective concentrations resulted in straight lines (as illustrated in Figure III-2 for area measurement of the isotope ratios); the slope and intercept of these lines

TABLE III-4

PRECISION OF THE METHOD, DETERMINED ON THREE CONSECUTIVE DAYS. Sample: 5.0, 25.0, 50.0, 100.0 and 200.0 µg of p-HPPH/ml urine.
[To 0.5 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.

Concentration (µg/ml)	DAY 1		DAY 2		DAY 3	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
			Height Ratio m/z 310/315			
5.0	0.288	0.298	0.292	0.298	0.287	0.287
25.0	1.42	1.43	1.42	1.46	1.44	1.47
50.0	2.83	2.87	2.81	2.83	2.91	2.91
100.0	5.55	5.75	5.69	5.59	5.67	5.70
200.0	10.56	10.84	11.11	10.57	10.49	10.71
			Area Ratio m/z 310/315			
5.0	0.270	0.279	0.276	0.278	0.268	0.276
25.0	1.39	1.38	1.39	1.39	1.40	1.41
50.0	2.77	2.79	2.82	2.85	2.81	2.81
100.0	5.46	5.60	5.57	5.53	5.62	5.58
200.0	10.45	11.01	11.02	10.58	10.49	10.82

TABLE III-5

DAY-TO-DAY VARIATION AND LINEARITY OF THE METHOD.

Sample: 5.0 - 200.0 µg of p-HPPH/ml urine.

[To 0.5 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

DAY TO DAY VARIATION (N=3)

Concentration (µg p-HPPH/ml)	Height Ratio m/z 310/315 Mean ± SD	CV (%)	Area Ratio m/z 310/315 Mean ± SD	CV (%)
5.0	0.292 ± 0.004	1.43	0.275 ± 0.003	0.92
25.0	1.44 ± 0.02	1.06	1.40 ± 0.01	0.83
50.0	2.86 ± 0.05	1.60	2.81 ± 0.03	1.07
100.0	5.66 ± 0.03	0.47	5.56 ± 0.04	0.65
200.0	10.71 ± 0.12	1.13	10.73 ± 0.07	0.65

LINEAR REGRESSION ANALYSIS

$y = 0.11 x + 0.14$
$r = 0.9996$

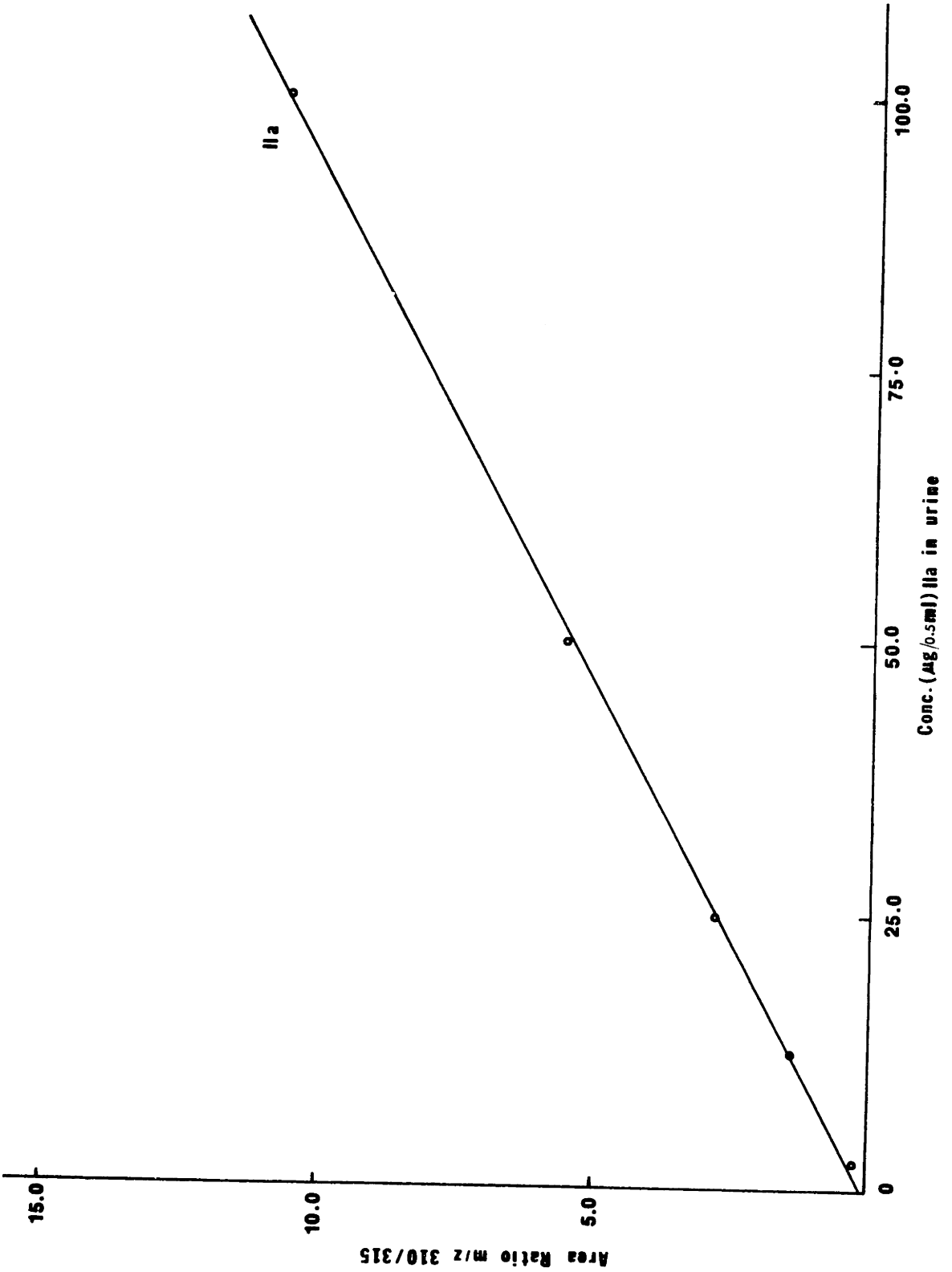
$y = 0.11 x + 0.08$
$r = 0.9998$

$\log y = 0.98 \log x - 0.92$
$r = 0.9999$

$\log y = 1.00 \log x - 0.95$
$r = 0.9999$

Figure III-2. Calibration curve for analysis of IIa in urine samples. Sample: 5.0-200.0 µg/ml of IIa.

Figure III-2


IIa
15.0
10.0
5.0
0
25.0
50.0
75.0
100.0
Area Ratio m/z 310/315
Conc.(μg/0.5ml) IIa in urine

were accurately defined by LRA of the data (as shown in Table III-5).

D. ANALYSIS OF SERUM STANDARD SOLUTIONS CONTAINING 0.1-30.0 µG/ML OF EACH PHT (Ia) AND ($^{13}C^{15}N_2$)-PHT (Ib), AND 0.1-10.0 µG/ML OF p-HPPH (IIa).

1. Precision

The precision of the method was evaluated for serum levels of 0.1, 0.5 and 30.0 µg/ml of each Ia and Ib, and 0.1, 0.5 and 10.0 µg/ml of IIa (extreme points of the calibration data) by replicate analyses (N) of the appropriate serum standard solutions. (1.0 ml samples of the standard solutions were processed and 1.0 µg of the respective internal standard was added). The results are summarized in Table III-6.

In addition, the precision of the GC-MS measurement was evaluated by duplicate GC-MS analyses of extracts containing increasing amounts of d-Ia, d-Ib and d-IIa and a constant amount of d-Ic and d-IIc. These extracts had been obtained from the following serum standard solutions: solution #1: 0.1 µg/ml of each Ia, Ib and IIa; solution #2: 0.5 µg/ml of each Ia, Ib and IIa; solution #3: 1.0 µg/ml of each Ia, Ib and IIa; solution #4: 10.0 µg/ml of each Ia and Ib, and 5.0 µg/ml of IIa; solution #5: 30.0 µg/ml of each Ia and Ib, and 10.0 µg/ml of IIa). The measured ion abundance ratios are listed in Table III-7. Some of the samples containing 30.0 µg/ml of Ia and Ib and

TABLE III-6

PRECISION OF THE METHOD. Sample: 0.1, 0.5 and 30.0 μg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.1, 0.5 and 10.0 μg of p-HPPH/ml serum.

[To 1.0 ml of serum, 1.0 μg of each (D_{10})-PHT and ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

PHT

Concentration (μg/ml)	N	Height Ratio Mean ± SD	m/z 280/290 CV (%)	Area Ratio Mean ± SD	m/z 280/290 CV (%)
0.1	10	0.133 ± 0.007	5.11	0.077 ± 0.006	7.16
0.5	9	0.570 ± 0.018	3.10	0.523 ± 0.029	5.47
30.0	10	34.22 ± 0.81	2.35	39.38 ± 1.57	3.98

($^{13}C^{15}N_2$)-PHT

Concentration (μg/ml)	N	Height Ratio Mean ± SD	m/z 283/290 CV (%)	Area Ratio Mean ± SD	m/z 283/290 CV (%)
0.1	10	0.153 ± 0.010	6.34	0.096 ± 0.011	11.76
0.5	9	0.550 ± 0.016	2.85	0.495 ± 0.030	6.03
30.0	10	30.80 ± 0.66	2.16	35.42 ± 1.39	3.94

p-HPPH

Concentration (μg/ml)	N	Height Ratio Mean ± SD	m/z 310/315 CV (%)	Area Ratio Mean ± SD	m/z 310/315 CV (%)
0.1	10	0.127 ± 0.004	3.05	0.112 ± 0.005	4.64
0.5	10	0.597 ± 0.015	2.57	0.590 ± 0.018	2.98
10.0	10	10.55 ± 0.32	3.03	12.86 ± 0.37	2.91

TABLE III-7. PRECISION OF THE GC-MS MEASUREMENTS. Sample: 0.1-30.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.1-10.0 µg of p-HPPH/ml serum. [To 1.0 ml of serum, 1.0 µg of each (D_{10})-PHT and ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

		Sample #1		Sample #2		Sample #3		Sample #1		Sample #2		Sample #3	
		Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2
PHT Concentration (µg/ml)	N	Height Ratio m/z 280/290						Area Ratio m/z 280/290					
0.1	3	0.118	0.108	0.114	0.112	0.117	0.116	0.099	0.094	0.097	0.093	0.096	0.099
0.5	3	0.542	0.537	0.547	0.543	0.[illegible]	0.520	0.515	0.509	0.519	0.505	0.503	0.490
1.0	2	1.07	1.10	1.07	1.07			1.04	1.05	1.05	1.03		
10.0	2	9.72	10.09	9.12	9.80			10.47	10.82	10.43	10.48		
30.0	2	30.70	*	*	*			32.46	*	*	*		
** 30.0	2	30.58	30.47	30.5[illegible]	30.24			35.98	35.74	34.84	36.06		
($^{13}C^{15}N_2$)-PHT Concentration (µg/ml)	N	Height Ratio m/z 283/290						Area Ratio m/z 283/290					
0.1	3	0.130	0.115	0.113	0.114	0.113	0.117	0.107	0.098	0.097	0.096	0.096	0.103
0.5	3	0.507	0.491	0.529	0.510	0.503	0.492	0.496	0.484	0.515	0.489	0.486	0.471
1.0	2	0.975	0.967	0.974	0.943			0.972	0.979	0.971	0.946		
10.0	2	8.62	8.60	8.42	9.11			9.34	9.46	9.14	9.44		
30.0	2	27.36	*	*	*			29.41	*	*	*		
** 30.0	2	27.40	27.78	27.71	27.48			33.12	32.46	31.67	32.90		
p-HPPH Concentration (µg/ml)		Height Ratio m/z 310/315						Area Ratio m/z 310/315					
0.1	3	0.129	0.131	0.123	0.121	0.120	0.127	0.103	0.111	0.100	0.102	0.101	0.112
0.5	3	0.566	0.563	0.576	0.564	0.583	0.598	0.549	0.539	0.552	0.564	0.551	0.555
1.0	2	1.18	1.14	1.11	1.09			1.12	1.12	1.13	1.10		
5.0	2	5.59	6.03	5.34	5.94			6.03	6.11	5.92	5.62		
10.0	2	10.26	*	10.19	10.31			11.30	*	11.18	10.91		
** 10.0	2	9.12	9.20	9.35	9.68			11.28	11.48	11.36	11.19		

* Failure to adjust electron multiplier gain resulted in peaks going off scale
** Re-injection of the extracts at a later date

10.0 µg of IIa were injected in too high a concentration causing the signal to go off scale and the data could therefore not be used. They were re-injected a few days later, under improper instrumental conditions (see Section E-4).

2. Linearity

Linearity was demonstrated using the data listed in Table III-7. A linear regression analysis of the mean values of the ion abundance ratios (for N determinations at each point of the calibration graph) on the concentrations was carried out. Table III-8 lists these mean ion abundance ratios, the SD and CV of the measurements and the results of the LRA.

Linearity was also demonstrated graphically by plotting the mean values of the ion abundance ratios against the concentrations on logaritmic paper, as illustrated in Figure III-3 for area measurement of the isotope ratios.

E. COMMENTS

1. GC-MS measurement

It was found that small gas chromatographic peaks of the PHT analogs (d-Ia, d-Ib, d-Ic) and the p-HPPH analogs (d-IIa, d-IIc) were not well defined by the use of scan cycles of 4 s and 2 s respectively. The accuracy of the measurements at low levels of analytes was therefore

TABLE III-8

PRECISION AND LINEARITY OF THE METHOD. Sample: 0.1-30.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.1-10.0 µg of p-HPPH/ml serum. [To 1.0 ml of serum, 1.0 µg of each (D_{10})-PHT and ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.

PHT

Concentration (µg/ml)	N	Height Ratio m/z 280/290 Mean ± SD	Area Ratio m/z 280/290 Mean ± SD
0.1	3	0.114 ± 0.002	0.097 ± 0.002
0.5	3	0.538 ± 0.009	0.507 ± 0.009
1.0	2	1.08 ± 0.01	1.05 ± 0.01
10.0	2	9.69 ± 0.32	10.56 ± 0.13
30.0	1	30.70	32.46

($^{13}C^{15}N_2$)-PHT

Concentration (µg/ml)	N	Height Ratio m/z 283/290 Mean ± SD	Area Ratio m/z 283/290 Mean ± SD
0.1	3	0.117 ± 0.005	0.100 ± 0.003
0.5	3	0.506 ± 0.012	0.490 ± 0.012
1.0	2	0.965 ± 0.008	0.968 ± 0.012
10.0	2	8.69 ± 0.11	9.35 ± 0.08
30.0	1	27.36	29.41

p-HPPH

Concentration (µg/ml)	N	Height Ratio m/z 310/315 Mean ± SD	Area Ratio m/z 310/315 Mean ± SD
0.1	3	0.125 ± 0.004	0.105 ± 0.003
0.5	3	0.575 ± 0.014	0.552 ± 0.007
1.0	2	1.13 ± 0.04	1.12 ± 0
5.0	2	5.73 ± 0.12	5.92 ± 0.21
10.0	2	10.26 ± 0.01	11.18 ± 0.18

LINEAR REGRESSION ANALYSIS

PHT	$y = 1.02\ x - 0.07$ $r = 0.9998$	$y = 1.08\ x - 0.07$ $r = 0.99997$
	$\log y = 0.98 \log x + 0.03$ $r = 0.9999$	$\log y = 1.02 \log x + 0.01$ $r = 0.99998$
($^{13}C^{15}N_2$)-PHT	$y = 0.91\ x - 0.04$ $r = 0.9998$	$y = 0.98\ x - 0.08$ $r = 0.9999$
	$\log y = 0.96 \log x + 0.002$ $r = 0.9998$	$\log y = 0.99 \log x - 0.01$ $r = 0.99996$
p-HPPH	$y = 1.03\ x + 0.13$ $r = 0.998$	$y = 1.13\ x + 0.04$ $r = 0.9995$
	$\log y = 0.97 \log x + 0.06$ $r = 0.9998$	$\log y = 1.02 \log x + 0.05$ $r = 0.9999$

Figure III-3. Calibration curves for analysis of (a) Ia and Ib (b) IIa, in serum samples. Sample: 0.1-30.0 µg/ml of each Ia and Ib, and 0.1-10.0 µg/ml of IIa.

The difference in slope of the calibration curves of Ia and Ib is explained in the text (p. 108)

Figure III-3

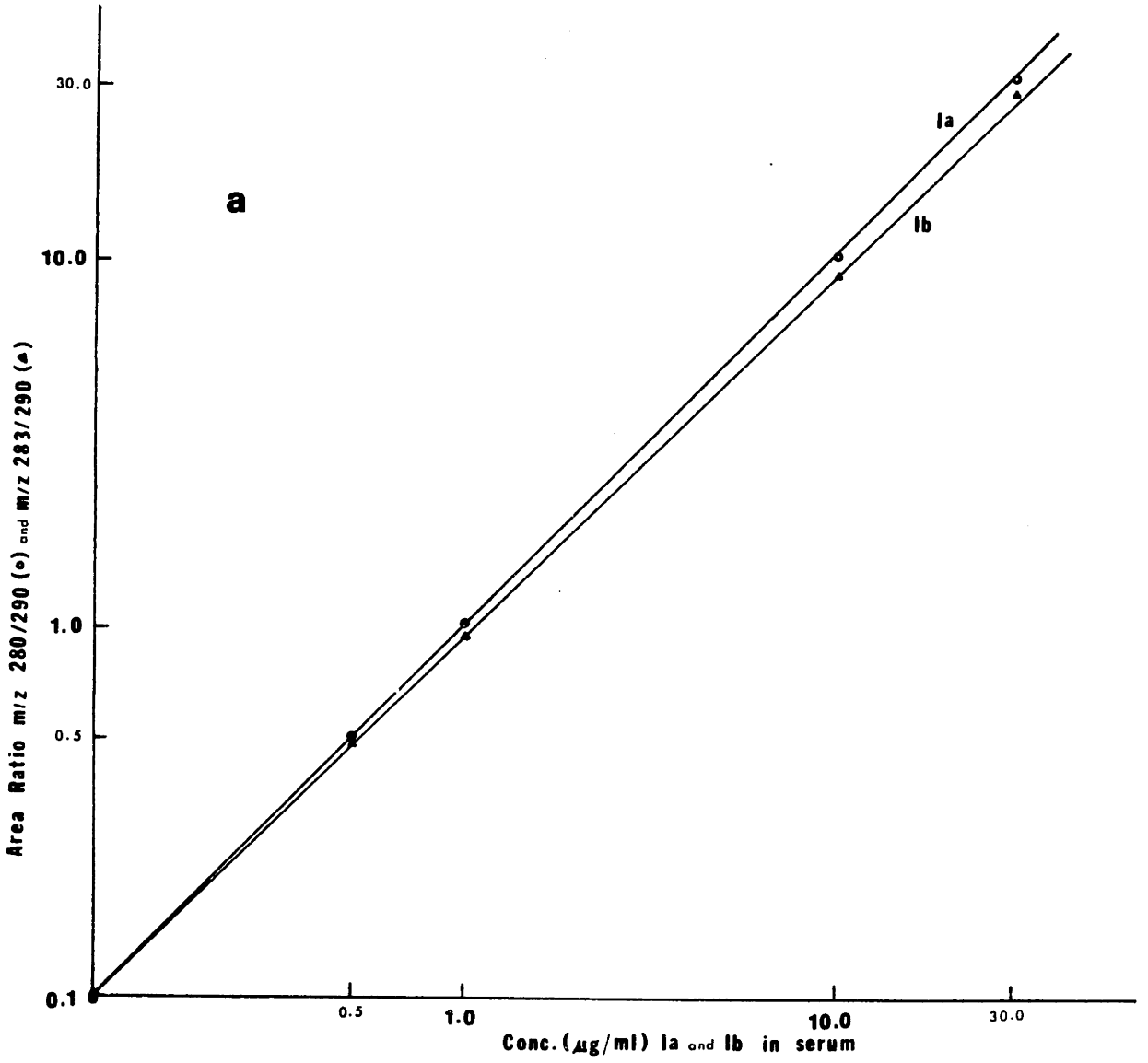
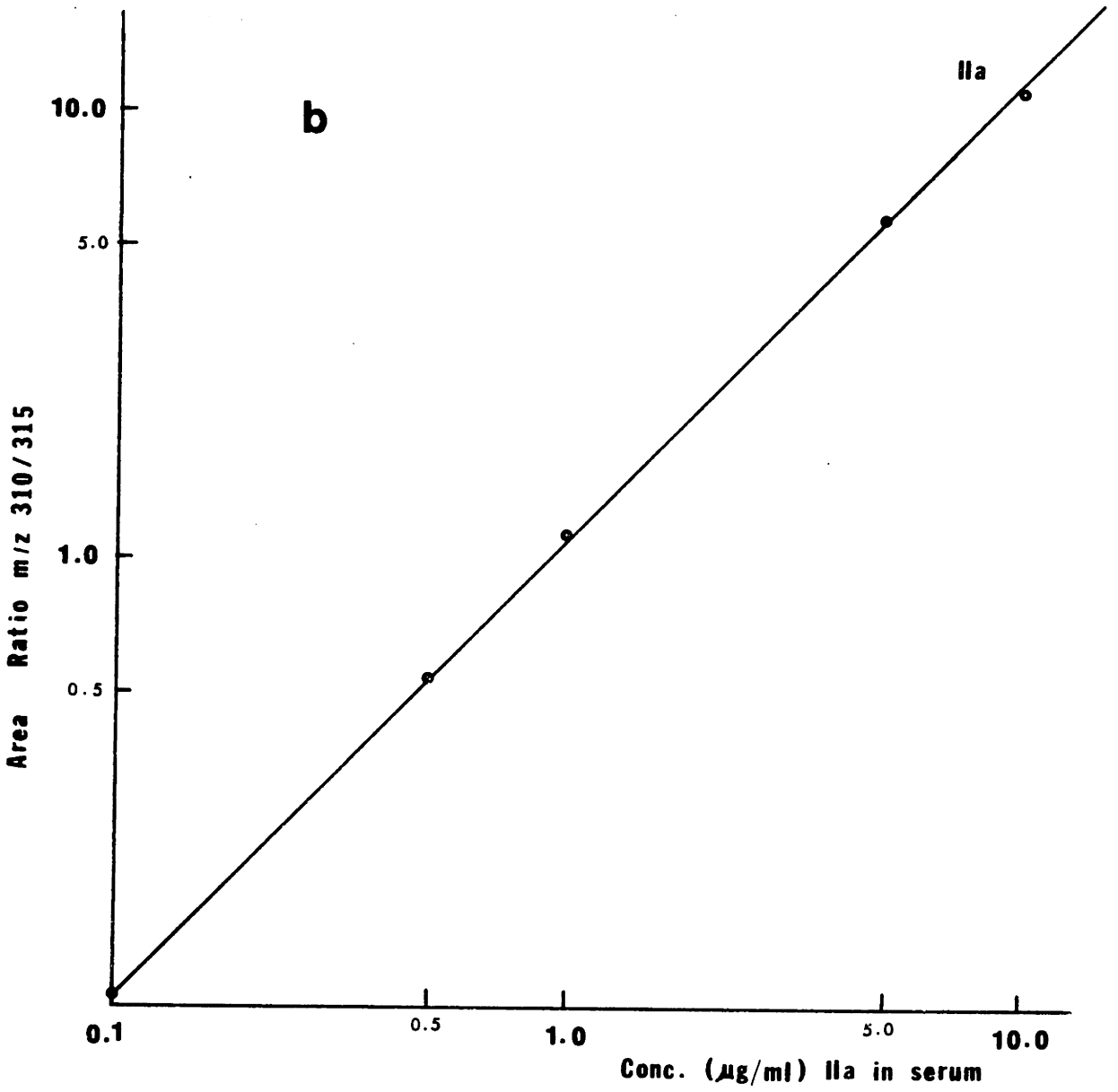

a
Ia
Ib
30.0
10.0
1.0
0.5
0.1
Area Ratio m/z 280/290 (•) and m/z 283/290 (▲)
0.5
1.0
10.0
30.0
Conc. (μg/ml) Ia and Ib in serum

Figure III-3


b
IIa
10.0
5.0
1.0
0.5
Area Ratio m/z 310/315
0.1
0.5
1.0
5.0
10.0
Conc. (μg/ml) IIa in serum

reduced because the statistical variation of ion currents is determined by both the mass spectrometric conditions (formation, acceleration and separation of the ions) and the partitioning process in the gas chromatographic column. There are two quantities being measured: ion currents (direct measurement in real time) and the gas chromatographic elution profile (indirect measurement). It is therefore not only necessary to monitor the ion currents for a sufficient time duration; one must also have enough sampling points to define the gas chromatographic peak.

For more sensitive measurements (as discussed in Section D), the initially installed power supply was therefore replaced with a more sophisticated one which allowed faster scanning of the accelerating voltage. It was found that one second scan cycles guaranteed accuracy of both the gas chromatographic and mass spectrometric measurements of Ia, Ib and IIa at levels of 0.1 µg/ml.

2. Isotopic dilution

It was always kept in mind that optimum precision of the GC-MS measurements is obtained when the ratios of analyte to internal standard are close to unity; therefore, whenever possible, the amount of internal standard was adjusted in regard to the concentration range to be measured.

3. Linear Regression Analysis

Calculation of the best straight line by linear regression analysis requires that the data conform to four assumed relationships beween the variables x (concentrations) and y (ion abundance ratios) (37-39). Only then can the regression line be used as a calibration line for determining the concentration of unknowns (reverse linear regression analysis). First, there should be no (or as little as possible) error in the x values. Second, for each x value, there exists a population of y values that is independent of all other y populations. Third, there is a linear relationship between the x values and the mean of each population of y values; fourth, the SD of each population of y values are equal. The regression line is therefore meaningful only if there is a significant correlation between ion abundance ratios and concentrations. This correlation is usually indicated by the correlation coefficient (r). Statistical Tables give values for r for different degrees of freedom (N-2) and confidence levels (37, 40). The calculated value of r must be closer to one than the theoretical value from the Table if there is to be a significant correlation.

For our studies, we did not anticipate lare errors due to preparation of the standard solutions so that the conditions to uphold the first assumption were met; therefore a regression of the isotope ratios on the concentrations could be done. (Concentrations of standard

solutions are not known with perfect accuracy but typically exhibit a small variance that results from variations in volumetric glassware. However, this variance is usually small compared to that of the isotope ratios and can be controlled by careful sample manipulation.)

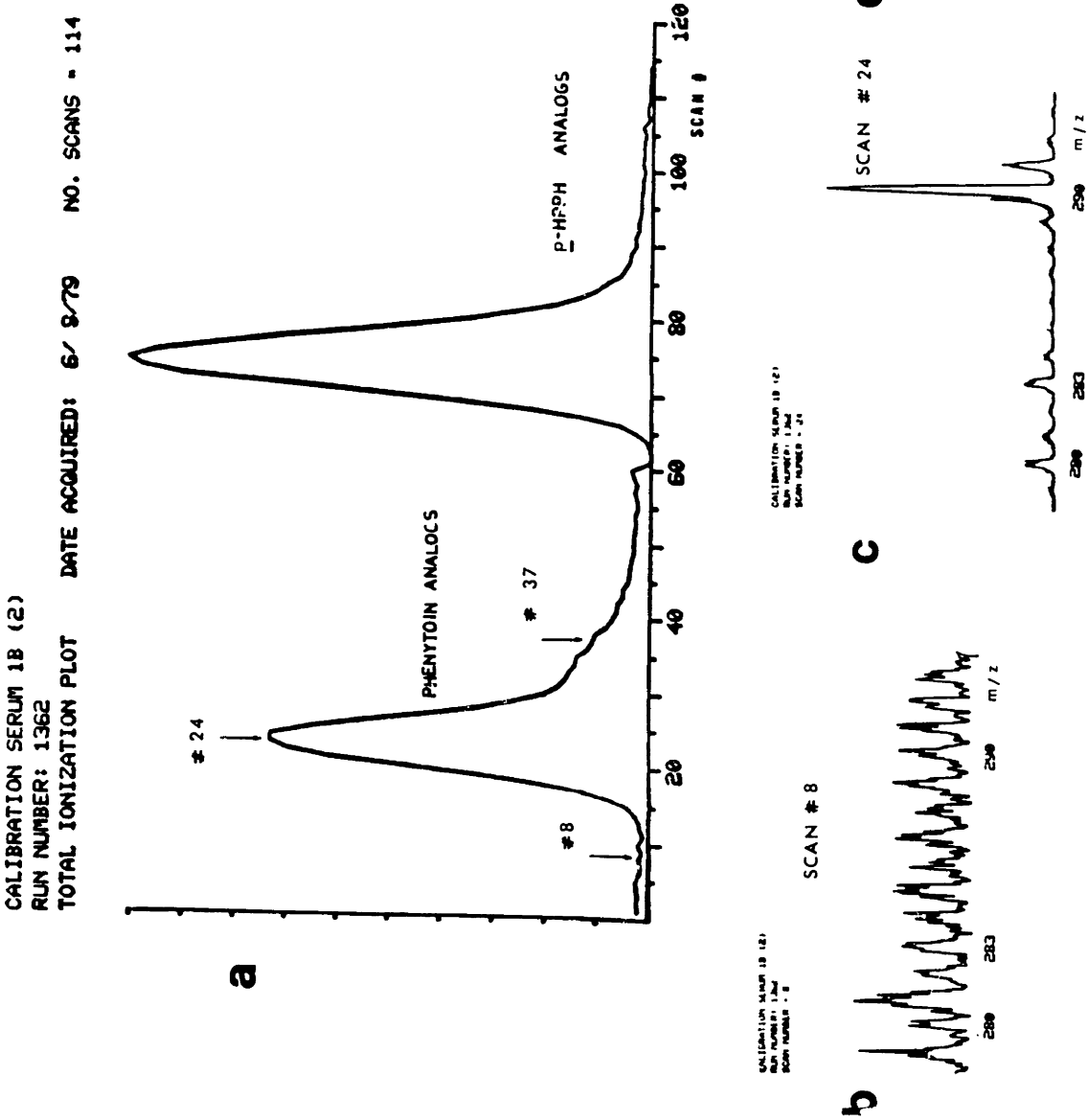
The second assumption was only violated when a memory effect in the gas chromatograph or mass spectrometer caused each isotope ratio to be influenced by the previous ratio. Significant memory effects caused departure from linearity because a linear relationship between concentration and response ratios is only obtained if no significant contribution to the response ratio of the compound to be measured is made by interfering substances.

Therefore, to conform to the third assumption, linearity, all interfering substances had to be corrected for either in real time by background subtraction (use of cursors) or in later data reduction and manipulation (correction for overlapping ion abundances). The nature of possible interferences in our studies has been discussed above (see Chapter II-B 3). Methyldocosanoate began to interfere with the analysis of Ib at levels of 0.1 µg/ml because of its contribution to the ion abundance at m/z 283, the molecular ion for d-Ib. The magnitude of the interference remained small and reproducible, and good precision (CV$\leq$5%) and linearity were obtained by excluding from the measurements these scans where the ester began to appear. In these analyses, the samples were all prepared

from the same batch of drug-free serum; the fatty acid content was therefore the same. Interferences were easily detected by inspection of the total ionization plot (as illustrated in Figure III-4), which would show unsymmetrical gas chromatographic peaks. Figure III-4a is a display of the total ionization plot constructed during analysis of a serum sample containing 0.1 µg/ml of Ia, Ib and IIa and 1.0 µg of the appropriate internal standards. A substantial amount of fatty acids was noticed in the corresponding FID trace. Scan #8, #24 and #37 are displayed in Figure III-4, part (b), (c) and (d) respecively. The peak at m/z 283 in scan #37 (Figure III-4d) is due to methyldocosanoate (see mass spectrum in Figure II-3), and obviously not to Ib because the signal at m/z 283 had already disappeared. Interferences caused by isotopic impurities or natural isotopic abundance ions of the monitored PHT or HPPH analogs (d-Ia, d-Ib, d-Ic, d-IIa and d-IIc) were not encountered at this point (see Chapter II, Section A; no interferences of such kind exist when (d-Ia, d-Ib, d-Ic) and (d-IIa,d-IIc) are monitored). Overlapping ion abundances would lead to a departure of linearity when very high or low ratios of d-IIb to d-IIc would be measured i.e. the extremes of calibration curves. A correction for the contribution of the trilabelled contaminant of d-IIc to m/z 313 and for the natural isotopic contribution of d-IIb to m/z 315 would then restore linearity (see further Chapter IV).

Figure III-4. Display of (a) the total ionization plot (TIP) constructed during analysis of a serum sample containing low levels of Ia and Ib and a substantial amount of fatty acids (b) scan #8 from the TIP (c) scan #24 from the TIP (d) scan #37 from the TIP.

Figure III-4


CALIBRATION SERUM 1B (2)
RUN NUMBER: 1362
TOTAL IONIZATION PLOT
DATE ACQUIRED: 6/ 8/79
NO. SCANS = 114
a
#24
#8
#37
PHENYTOIN ANALOGS
p-HPPH ANALOGS
SCAN #
b
SCAN #8
c
SCAN #24
d
SCAN #37
m/z

The fourth assumption (i.e. that the standard deviations of small isotope ratios and large isotope ratios are equal) was usually not upheld because of ion statistics: small isotope ratios (although having a larger relative or percentage error) have a smaller SD than large isotope ratios. Thus the former are inherently defined with greater precision and should be given greater numerical emphasis through weighting. Otherwise an isotope calibration would result that is inaccurate near the origin. A better estimate for the regression line (i.e. slope and intercept) can therefore be obtained by e.g. logarithmic transformation of the data which eliminates the effect of concentration on the standard deviation (33,34); this is especially important when the calibration curve extends over a wide range of concentrations and small concentrations of unknowns are calculated from the regression line (see furter Chapter IV).

It is also important to consider the long-term precision and linearity of the GC-MS measurements, especially when the analysis is performed over a wide range of isotope ratios. Ideally a series of standard samples should be run with each set of unknowns. As discussed before (see Chapter II, Section D-1), variations in mass spectrometric operating conditions have a significant influence on the observed isotope ratios, and this is both instrument and operator dependent. A change in resolution e.g. alters the degree of cross talk of ions between

adjacent masses. A loss of resolution decreases the relative intensity of major ions and increases the relative intensity of minor ions that have neighbouring ions of greater intensity. Mass discrimination reduces the yield of ions collected at the detector as a function of mass. The degree of discrimination is altered by source tuning and will therefore, vary from one month to the next. Any increase in the discrimination against higher masses will reduce the response measured for the labelled species. This will reduce the measured isotope ratio and it will appear that there is less of the labelled species in each mixture. In addition, despite the case of extreme caution, contamination of the reference compounds can occur during storage and handling. If the contamination is minimal, it might go undetected until several analyses have been performed.

The difference in slope for the regression lines of Ia and Ib is attributed to the different isotopic purity of the reference compounds. Ib was found to be 94 % trilabelled (see Chapter VI, Section A); the ratio of the molecular ion abundances of equal amounts by weight of Ib to Ia will therefore be 0.94. However, non-linear scanning conditions or interferences at the measured m/z values can cause this value to be slightly different (see further Chapter IV).

4. Height and area measurements

Both height and area measurements of ion abundances were evaluated. Inspection of the Tables III-1 through III-8 indicates that they can both be used with about the same precision to measure the listed levels of analytes. However, when ion abundance ratios differ by as much as a factor of 30, height measurement shows better precision because it avoids integrating the noise which then contributes more to the small signal than to the large one (e.g. Table III-7). Peak heights are also superior to peak areas when the instrumental background is high and the level of analyte is low (e.g. Tables III-1, III-7). Under these conditions, definition of the range over which the area is to be integrated is less precise than the measurement of the peak height.

Most of the data presented show precision and linearity under short-term conditions (i.e. GC-MS measurements of the samples on the same day or under the same instrumental conditions) during which changes in peak shape and width (e.g. by magnet drift, source defocusing, electron multiplier gain) can be avoided or kept constant. Between days or weeks however, operating conditions can significantly change (see Section E-3). Depending on the kind of variation in mass spectrometric conditions, either height or area measurements will be most precise: when distorted or slightly saturated peaks are recorded, area measurements will give a better long-term precision; when

the resolution changes (especially when large ion intensity ratios are measured) height measurements will be more precise. This can be illustrated for the data reported in Table III-7 for analysis of the extracts containing 30.0 µg/ml of Ia and Ib and 10.0 µg/ml of IIa. These extracts were re-injected on a different day. For the measurements of d-Ia and d-Ib, the long-term precision of the height measurements was found best (the ratio of ion intensities approaches 30 and a slight difference in peak shape changed the baseline resolution). For measurements of d-IIa, the long-term precision of the area measurements was better (defocusing of the ion source resulted in distorted peak shapes and electrical noise resulted in split peak tops).

Because of time constraints, it is impossible to evaluate the relative importance of height and area measurements for every series of samples that is analyzed. Therefore, the decision on whether to measure heights or areas must be based on inspection of peak profiles to maintain the best consistency of the specific set of measurements.

Chapter IV. APPLICATIONS: DEMONSTRATION OF THE ABSENCE OF 'IN VIVO' ISOTOPE EFFECTS IN THE METABOLISM OF ($^{13}C^{15}N_2$)-PHT (Ib) IN DOGS AND HUMAN VOLUNTEERS.

A. APPROACH

To demonstrate the absence of 'in vivo' isotope effects in dogs and human volunteers, a mixture of Ia and Ib was administered intravenously to dogs and human volunteers. Ib had been formulated for intravenous injection by Warner-lambert/Parke Davis in a manner similar to their marketed Dilantin^R ("Phenytoin sodium injection", containing 50 mg/ml of the sodium salt of Ia). Equal volumes of solutions containing the same concentrations of Ia and Ib (as sodium salts) were therefore mixed in sterile syringes of 10 ml (estimated accuracy: 0.2 ml); these mixtures were diluted with normal saline to a concentration of 10 mg/ml of total phenytoin sodium and infused as described in Chapter VI. Serial blood samples were then drawn and urine samples collected and the concentrations of Ia and Ib and their respective metabolites were determined by GC-MS. The ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolites could so be followed throughout the serial collections, and the pharmacokinetic parameters for Ia and Ib could be computed. A constant ratio of unlabelled to ($^{13}C^{15}N_2$)- labelled drug and metabolites, and identical pharmacokinetic parameters would indicate that

there is no isotope effect in the the 'in vivo' metabolism of Ib. The ratio of the two drugs infused was not exactly 1.00 because of differences between batches of drug and the difficulties in accurately measuring the volume using ordinary syringes. For this reason, the exact ratio of Ia to Ib in the mixture to be infused was determined by GC-MS in the studies with human volunteers.

In the studies with dogs, serum samples were obtained; for the studies on human subjects, plasma samples were preferred because the preparation of plasma samples has the advantage that the risk of haemolysis is reduced and that the sample can be centrifuged immediately after adding the anticoagulant. (Serum is obtained from blood which has has not been made unclottable by the addition of anticoagulants; the blood is allowed to clot spontaneously in the centrifuge tubes at room temperature; to obtain plasma, an anticoagulant is added to the blood samples before centrifugation.)

In dog samples, both m-HPPH analogs (IIIa and IIIb) and p-HPPH analogs (IIa and IIb) were measured, because the m-HPPH is the major metabolite (ratio m-HPPH/p-HPPH is 3:1) (3). The permethylated derivatives of IIIa and IIIb (d-IIIa and d-IIIb) appeared fully separated from the permethylated derivatives of IIa and IIb (d-IIa and d-IIb) on a 6 ft OV-17 column, programmed from 210-320 °C at 8 °/min (on a 3 ft OV-17 column they were not completely resolved, so that separate measurement of the m-HPPH analogs and the

p-HPPH analogs was not possible). Because, initially, the method had only been evaluated for the determination of the p-hydroxylated metabolites (by use of serum and urine standard solutions containing known amounts of II a, see Chapter III), a re-evaluation was carried out for the simultaneous determination of the m-hydroxylated and the p-hydroxylated metabolites in the same serum or urine sample (for these experiments, serum and urine standard solutions containing known amounts of IIIa and IIa were used).

The concentation of unlabelled and ($^{13}C^{15}N_2$)-labelled drug and respective metabolites in the serum (plasma) and urine samples from dogs and human subjects was calculated by the use of the slope and intercept of corresponding regression lines. These regression lines were obtained by analysis of a series of serum, plasma or urine standard solutions (see Chapter III E-3). As will be discussed in Section B-1a of this Chapter, regression lines obtained by LRA of the molecular ion abundance ratios of d-IIIb/d-IIc and d-IIb/d-IIc on the respective concentrations of IIIb and IIb, were not available during the studies with dogs; therefore, the regression lines obtained by LRA of the ion abundance ratios of d-IIIa/d-IIc on the concentrations of IIIa, and by LRA of the ion abundance ratios of d-II a/d-IIc on the concentrations of IIa were used to calculate the concentrations of IIIb and IIb respectively (i.e. the ($^{13}C^{15}N_2$)-labelled analogs). For the studies on human

subjects, ($^{13}C^{15}N_2$)-labelled p-HPPH (IIb) was synthesized because it was anticipated that the serum samples from the human subjects would contain low levels of metabolites and the availability of regression lines relating the measured isotope ratios of d-IIb/d-IIc to the concentrations of IIb would certainly increase the accuracy of the measurements.

During analysis of the samples of the first human subject, significant irreproducible interferences from methyldocosanoate (X), interfering with the measurement of d-Ib, were noticed on a 3ft OV-17 column (apparently the serum fatty acid content was high and not constant over longer periods of time). Attempts to obtain better gas chromatographic resolution were therefore undertaken because excluding from the measurements the scans where the interference started to appear (as discussed in Chapter III, Section E-3) did not guarantee good accuracy and precision anymore. (Pre-extraction with hexane had not given satisfactory results.) A 6 ft OV-17 column resulted in better separation between the gas chromatographic peaks of (d-Ia, d-Ib, d-Ic) and (X) but was still not adequate to accurately measure low levels of Ib in the presence of high levels of (X). The (d-Ia, d-Ib, d-Ic) peak appeared gas chromatographically resolved from (X) on a 6 ft OV-101 column, temperature programmed from 180-310°C at 12 °/min; for the further analysis of samples from human subjects, we therefore substituted an OV-101 column for the previously used OV-17 columns.

B. STUDIES WITH DOGS

1. Data acquisition and processing

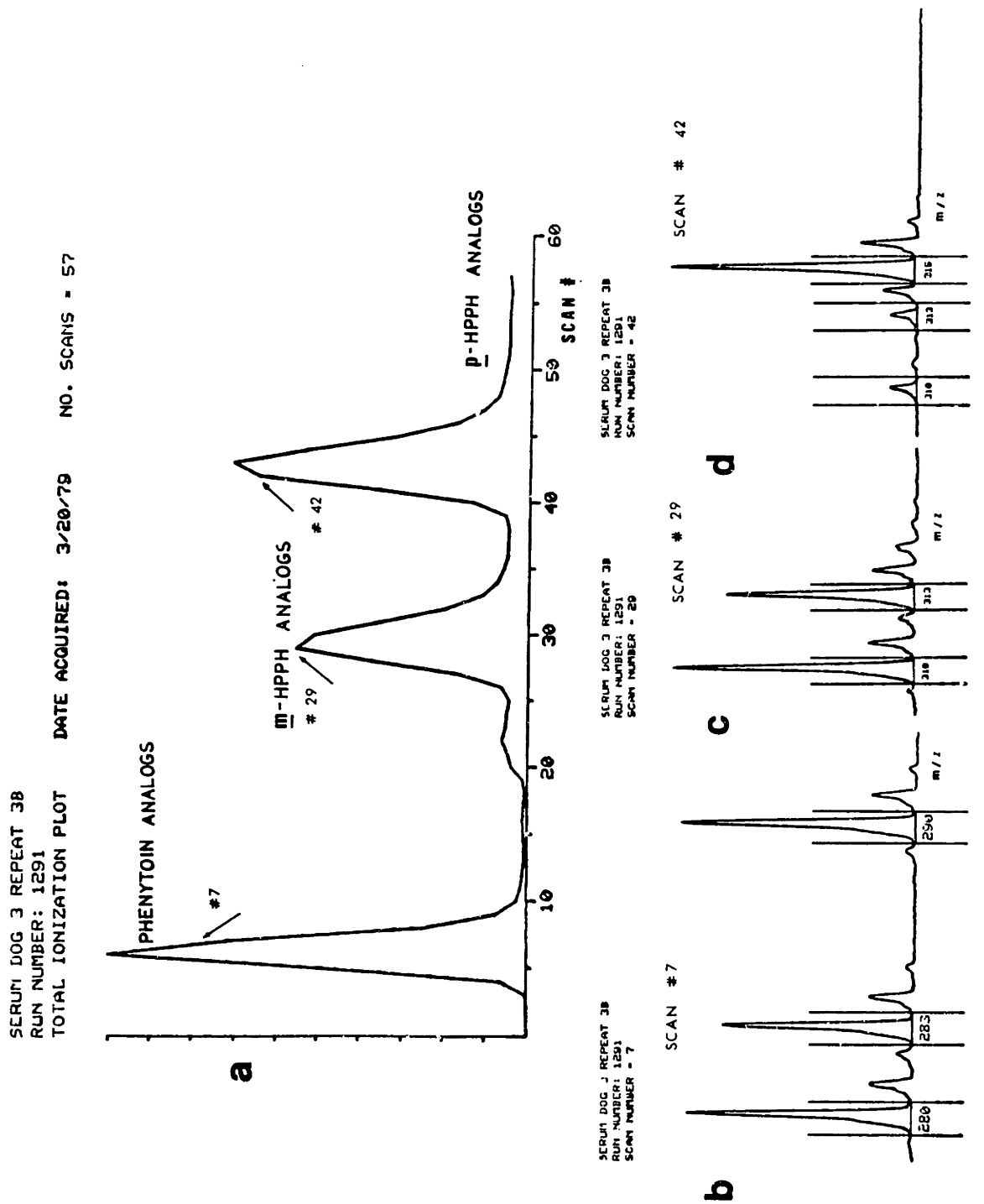
1a. Gas chromatographic mass spectrometric measurement

Under the in Section A described gas chromatographic conditions for the analysis of samples from dogs, the permethylated derivatives of the PHT analogs (d-Ia, d-Ib, d-Ic) were not separated; they eluted in a sharp gas chromatographic peak. Measurement of ion currents proceeded as outlined in Chapter VI, i.e. isotope ratios of unknown to internal standard were calculated for every scan taken during the elution of (d-Ia, d-Ib, d-Ic) and then averaged over a selected range of scans.

The permethylated derivatives of the m-HPPH analogs (d-IIIa, d-IIIb) were gas chromatographically resolved from these of the p-HPPH analogs (d-IIa, d-IIb, d-IIc) and measurement was accomplished as follows: for every scan taken during elution of (d-IIa, d-IIb, d-IIc), molecular ion abundances at m/z 310, 313 and 315 were measured; for every scan taken during elution of (d-IIIa, d-III b), molecular ion abundances at m/z 310 and 313 were measured. The amount of IIIa and IIIb was determined as the ratio of the summed ion abundances at respectively m/z 310 and 313, measured during the elution of (d-IIIa, d-IIIb), and the summed ion abundances at m/z 315 measured during the elution of (d-IIa, d-IIb, d-IIc); the amount of IIa and IIb was determined as the ratio of the summed ion abundances at respectively m/z 310 or 313, measured during

Figure IV-1. Display of (a) the total ionization plot (TIP) constructed during analysis of a serum sample from a dog (b) scan #7 from the TIP (c) scan #29 from the TIP (d) scan #42 from the TIP.

Figure IV-1


SERUM DOG 3 REPEAT 3B
RUN NUMBER: 1291
TOTAL IONIZATION PLOT
DATE ACQUIRED: 3/20/79
NO. SCANS = 57
PHENYTOIN ANALOGS
#7
m-HPPH ANALOGS
29
42
p-HPPH ANALOGS
SCAN #
a
SERUM DOG 3 REPEAT 3B
RUN NUMBER: 1291
SCAN NUMBER = 7
SERUM DOG 3 REPEAT 3B
RUN NUMBER: 1291
SCAN NUMBER = 29
SERUM DOG 3 REPEAT 3B
RUN NUMBER: 1291
SCAN NUMBER = 42
SCAN #7
SCAN # 29
SCAN # 42
b
c
d
280
283
290
310
313
316
m/z

elution of the p-HPPH analogs and the summed ion abundances at m/z 315, measured during elution of the (d-IIa, d-IIb, d-IIc). Figure IV-1 shows in (a) a display of the total ionization plot (TIP) constructed during analysis of a serum sample from a dog and in (b), (c) and (d), respectively, the mass ranges scanned for the measurement of the molecular ion abundances of the permethylated PHT analogs (d-Ia, d-Ib, d-Ic) (b) the permethylated m-HPPH analogs (d-IIIa, d-IIIb) and the permethylated p-HPPH analogs (d-IIa, d-IIb, d-IIc). As indicated by the arrows on the TIP presented in (a), the displays in (b), (c) and (d) represent scans #7, #29 and #42, respectively. When these data were taken, the region of m/z 280-290 was scanned every 4 s (for the monitoring of the PHT analogs); the region of m/z 310-315 was scanned in 2 s scan cycles (for the monitoring of the m-HPPH and p-HPPH analogs).

The mass spectra of d-IIIa and d-IIa are presented and discussed in the Appendix, Figure A-2, b and a (see also Ref. 3). It can be seen that the relative abundances of the molecular ions are identical so that no systematic error is introduced in our measurements (only in the further fragmentation, differences in ion abundances are important because of favored fragmentation pathways).

1b. Linearity and Precision

Linearity was demonstrated for measurement of Ia (1.0-30.0 µg/ml), Ib (1.0-30.0 µg/ml), IIa (0.5-5.0 µg/ml)

and IIIa (0.5-5.0 µg/ml) in serum; for measurement of IIa (5.0-200.0 µg/ml) and IIIa (5.0-200.0 µg/ml) in urine. For this experiment, 1.0 ml serum standard solutions containing increasing concentrations of Ia, Ib, IIa and IIIa (10.0 µg of Ic and 2.0 µg of IIc were added as internal standards), and 0.5 ml urine standard solutions containing increasing concentrations of IIIa and IIa (10.0 µg of IIc were added as internal standard) were processed. For each standard solution, duplicate samples were worked up. The urine samples from the dogs (0.5 ml) were processed along with the urine standard samples; the extracts were stored in the freezer at -4 °C and analyzed later by GC-MS (see further Section B-1c of this Chapter).

Each extract from the serum standard solutions was analyzed in duplicate by GC-MS; for the extracts of the urine standard solutions, duplicate GC-MS measurements were carried out for each extract obtained from standard solutions containing 5.0, 25.0 and 200.0 µg/ml; a single injection was done for the remaining extracts. Tables IV-1 and IV-2 list the ratios of the molecular ion abundances (areas) of d-Ia/d-Ic, d-Ib/d-Ic, d-IIIa/d-IIc, d-IIa/d-IIc (m/z 280/290, m/z 283/290, m/z 310_m/315, m/z 310_p/315, respectively) for each injection and the mean value for each sample ($\overline{\#1,\#2}$). It should be kept in mind that during the experiments involving the analysis of samples from dogs, ion currents at m/z 310 and 313 were measured for both m-HPPH analogs and p-HPPH analogs. The ion current at

TABLE IV-1

PRECISION AND LINEARITY OF THE METHOD. Sample: 1.0-30.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5-5.0 µg of each m-HPPH and p-HPPH/ml serum. [To 1.0 ml of serum, 10.0 µg of (D_{10})-PHT and 2.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

Concentration	N	Sample #1 Inj. 1	Sample #1 Inj. 2	Sample #2 Inj. 1	Sample #2 Inj. 2	$\overline{\#1}$	$\overline{\#2}$	$\overline{\#1,\#2}$ ± SD	Linear Regression Analysis
µg PHT/ml					Area Ratio m/z 280/290				
1.0	2	0.101	*	0.111	0.101	0.101	0.106	0.104 ± 0.004	y = 0.12 x - 0.03
15.0	2	1.71	1.71	1.68	*	1.71	1.68	1.70 ± 0.02	r = 0.99995
30.0	2	3.40	*	3.56	3.51	3.40	3.54	3.47 ± 0.10	
µg ($^{13}C^{15}N_2$)-PHT/ml					Area Ratio m/z 283/290				
1.0	2	0.092	*	0.093	0.086	0.092	0.090	0.091 ± 0.001	y = 0.10 x - 0.04
15.0	2	1.49	1.44	1.45	*	1.47	1.45	1.46 ± 0.01	r = 0.9996
30.0	2	3.00	*	3.17	3.10	3.00	3.14	3.07 ± 0.10	
µg m-HPPH/ml					Area Ratio m/z 310_m/315				
0.5	2	0.236	0.178	0.194	0.214	0.207	0.204	0.206 ± 0.002	y = 0.42 x - 0.01
1.0	2	0.408	0.381	0.380	0.402	0.395	0.391	0.393 ± 0.003	r = 0.9999
5.0	2	2.12	2.04	2.03	2.09	2.08	2.06	2.07 ± 0.014	
µg p-HPPH/ml					Area Ratio m/z 310_p/315				
0.5	2	0.203	0.179	0.188	0.205	0.191	0.197	0.194 ± 0.004	y = 0.43 x - 0.03
1.0	2	0.373	0.377	0.373	0.411	0.375	0.392	0.384 ± 0.012	r = 0.9999
5.0	2	2.22	2.13	2.11	2.04	2.17	2.07	2.12 ± 0.07	

* Data were lost by computer hardware problems.

TABLE IV-2

PRECISION AND LINEARITY OF THE METHOD. Sample: 5.0-200.0 µg of each m-HPPH and p-HPPH/ml urine. [To 0.5 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standard.]

Concentration	N	Sample #1 Inj. 1	Sample #1 Inj. 2	Sample #2 Inj. 1	Sample #2 Inj. 2	$\overline{\#1}$	$\overline{\#2}$	$\overline{\#1,\#2}$ ± SD	Linear Regression Analysis
µg m-HPPH/ml					Area Ratio m/z $310_m/315$				
5.0	2	0.422	0.420	0.396	0.398	0.421	0.397	0.409 ± 0.017	
25.0	2	1.91	2.07	1.95	1.78	1.99	1.87	1.93 ± 0.08	
50.0	2	3.88	*	3.54	*	3.88	3.54	3.71 ± 0.24	y = 0.15 x + 0.09
100.0	2	7.41	*	7.49	*	7.41	7.49	7.45 ± 0.06	r = 0.99995
200.0	2	14.05	15.76	14.41	14.13	14.91	14.27	14.59 ± 0.45	
µg p-HPPH/ml					Area Ratio m/z $310_p/315$				
5.0	2	0.347	0.347	0.332	0.328	0.347	0.330	0.339 ± 0.012	
25.0	2	1.32	1.40	1.37	1.35	1.36	1.36	1.36 ± 0	
50.0	2	3.41	*	3.39	*	3.41	3.39	3.40 ± 0.01	y = 0.14 x - 0.10
100.0	2	6.85	*	6.94	*	6.85	6.94	6.90 ± 0.06	r = 0.9995
200.0	2	14.40	13.19	13.36	13.17	13.80	13.27	13.54 ± 0.37	

* For these samples, only one injection was made.

m/z 310 and 313 measured during elution of the m-HPPH analogs is therefore referred to in the text as m/z 310_m and m/z 313_m; the ion current at m/z 310 and 313 measured during elution of the p-HPPH analogs is referred to as m/z 310_p and 313_p. This convention is also followed in Tables IV-1 through IV-12 (analysis of samples from dogs). For each serum standard solution, i.e. at each concentration point, the mean value of the ion abundance ratios ($\overline{\#1,\#2}$) and the SD of the measurements were then calculated and regression of these mean values on the corresponding concentrations defined the best straight line and correlation coefficient. For the analysis of Ia and Ib in serum, only three GC-MS measurements are reported because data were lost by computer hardware problems.

1c. Analysis of the dog samples

For each serial urine and serum collection taken from the dogs (for protocol see Chapter VI, Section D; the samples were numbered in the order of collection) duplicate samples were worked up, and each extract was analyzed once by GC-MS. For serum, 1.0 ml samples were extracted and 10.0 µg of Ic and 5.0 µg of IIc were added as internal standards. For urine, 0.5 ml samples were extracted and 10.0 µg of IIc were added as internal standards. Blanks (i.e. serum and urine samples collected before infusion of Ia and Ib, and to which no internal standard was added) were always included.

The serum samples were processed on three different days; each day, a corresponding series of serum standard solutions containing increasing concentrations of Ia, Ib, IIIa and IIa was included. Along with the processing of the samples from Dog #1 and Dog #3, the serum standard solutions were worked up once; along with the processing of the samples from Dog #2, the serum standard solutions were worked up in duplicate. The calibration data are summarized in Table IV-3. LRA of the listed ratios of molecular ion abundances (areas) of d-Ia/d-Ic, d-Ib/d-Ic, d-IIIa/d-IIc and d-IIa/d-IIc (m/z 280/290, m/z 283/290, m/z 310_m/315, m/z 310_p/315, respectively) on the corresponding concentrations of Ia, Ib, IIIa and IIa, defined slope and intercept of the best straight lines. Attempts were made to analyze the extracts from the serum samples and the respective standard samples by GC-MS on the same day or under the same instrumental conditions.

All the urine samples from the dogs were processed during the same day, along with the previously described series of urine standard solutions. When the urine extracts from the dogs were analyzed by GC-MS, the validity of the previously obtained regression lines (Table IV-2) was verified in two separate instances by replicate GC-MS analysis of selected extracts from these previously processed series of standards, and statistical comparison of the, on the different occasions, measured ion abundance ratios. Extracts from the urine standard solutions

TABLE IV-3

ANALYSIS OF SERUM SAMPLES FROM DOGS: CALIBRATION DATA FOR ANALYSIS OF SAMPLES FROM (A) Dog 1; (B) Dog 2; (C) Dog 3. Sample: 1.0-30.0 μg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5-5.0 μg of each m-HPPH and p-HPPH/ml serum. [To 1.0 ml of serum, 10.0 μg of (D_{10})-PHT and 5.0 μg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

(A)

Concentration μg PHT/ml	N	Area Ratio m/z 280/290	Linear Regression Analysis
1.0	1	0.114	y = 0.12 x + 0.01
15.0	1	1.79	r = 0.9999
30.0	1	3.49	

μg ($^{13}C^{15}N_2$)-PHT/ml		Area Ratio m/z 283/290	
1.0	1	0.109	y = 0.11 x + 0.01
15.0	1	1.59	r = 0.999995
30.0	1	3.16	

μg m-HPPH/ml		Area Ratio m/z 310_m/315	
0.5	1	0.126	y = 0.28 x - 0.03
1.0	1	0.227	r = 0.9996
5.0	1	1.364	

μg p-HPPH/ml		Area Ratio m/z 310_p/315	
0.5	1	0.131	y = 0.29 x - 0.03
1.0	1	0.243	r = 0.9997
5.0	1	1.434	

TABLE IV-3, continued

(B)

Concentration	N	Sample #1	Sample #2	$\overline{\#1,\#2}$	Linear Regression Analysis
µg PHT/ml		Area Ratio m/z 280/290			
1.0	2	0.113	0.115	0.114	$y = 0.11 x + 0.01$
15.0	2	1.65	1.61	1.63	$r = 0.99997$
30.0	2	3.21	3.21	3.21	
µg ($^{13}C^{15}N_2$)-PHT/ml		Area Ratio m/z 283/290			
1.0	2	0.103	0.099	0.101	$y = 0.09 x + 0.005$
15.0	2	1.42	1.42	1.42	$r = 0.999999$
30.0	2	2.82	2.85	2.84	
µg m-HPPH/ml		Area Ratio m/z 310_m/315			
0.5	2	0.096	0.089	0.093	$y = 0.22 x - 0.02$
1.0	2	0.183	0.210	0.197	$r = 0.99997$
5.0	2	1.058	1.144	1.101	
µg p-HPPH/ml		Area Ratio m/z 310_p/315			
0.5	2	0.112	0.113	0.113	$y = 0.22 x - 0.01$
1.0	2	0.180	0.190	0.185	$r = 0.9994$
5.0	2	1.089	1.071	1.080	

TABLE IV-3, continued

(C)

Concentration µg PHT/ml	N	Area Ratio m/z 280/290	Linear Regression Analysis
1.0	1	0.110	y = 0.11 x - 0.005
15.0	1	1.57	r = 0.99995
30.0	1	3.19	
µg ($^{13}C^{15}N_2$)-PHT/ml		Area Ratio m/z 283/290	
1.0	1	0.102	y = 0.10 x - 0.005
15.0	1	1.40	r = 0.9999
30.0	1	2.86	
µg m-HPPH/ml		Area Ratio m/z 310_m/315	
0.5	1	0.131	y = 0.29 x - 0.01
1.0	1	0.279	r = 0.999999
5.0	1	1.448	
µg p-HPPH/ml		Area Ratio m/z 310_p/315	
0.5	1	0.128	y = 0.25 x - 0.01
1.0	1	0.227	r = 0.99976
5.0	1	1.255	

containing 50.0 and 100.0 µg/ml of each IIIa and IIa were re-injected; they contained 25.0 and 50.0 µg of each IIIa and IIa (0.5 ml of urine had been extracted), and 10.0 µg of IIc. These experiments were carried out when the urine samples from Dog 1 and Dog 3 were analyzed. Table IV-4 part A, lists the ratios of molecular ion abundances of d-IIIa/d-IIc (m/z 310 /315) and d-IIa/d-IIc (m/z 310 /315), measured in the extracts from the urine standard solutions under the same conditions as the isotope ratios in samples from Dog #1 and Dog #3 were measured. The ratios were compared (t-test, Table IV-4 part B) with the previously obtained values (Table IV-2) and in all cases, the difference in the ratios was not considered to be significant at the 95 % confidence level because the calculated values of t were smaller than the tabulated values for t (P=0.95) (37).

Table IV-5 lists the ratios of molecular ion abundances (areas) of d-Ia/d-Ic, d-Ib/d-Ic, d-IIIa/d-IIc, d-IIIb/d-IIc, d-IIa/d-IIc and d-IIb/d-IIc for the serial serum collections from the three dogs. Table IV-6 lists the ratios of the molecular ion abundances (areas) of d-IIIa/d-IIc, d-IIIb/d-IIc, d-IIa/d-IIc, d-IIb/d-IIc for the serial urine collections from the three dogs. For some extracts only one useful measurement was obtained because either computer hardware problems led to loss of the data, or inappropriately set electron multiplier voltages caused the ion current to go off scale. The appropriate

TABLE IV-4

ANALYSIS OF URINE SAMPLES FROM DOGS: VALIDITY OF PREVIOUSLY OBTAINED CALIBRATION DATA (A) REPLICATE INJECTIONS OF STANDARD URINE EXTRACTS CONTAINING 25.0 AND 50.0 µg OF EACH m-HPPH and p-HPPH, AND 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH (B) t-TEST.

(A)

	Concentration (µg p-HPPH and m-HPPH)	N	Sample #1	Sample #2	$\overline{\#1,\#2 \pm SD}$	Sample #1	Sample #2	$\overline{\#1,\#2 \pm SD}$
			Area Ratio m/z $310_m/315$			Area Ratio m/z $310_p/315$		
**	25.0	2	3.55	3.63	3.59 ± 0.06	3.44	3.47	3.46 ± 0.02
	50.0	1	6.84	*	6.84	6.67	*	6.67
***	25.0	1	3.47	*	3.47	3.37	*	3.37
	50.0	1	7.09	*	7.09	6.77	*	6.77
****	25.0	2	3.88	3.54	3.71 ± 0.24	3.41	3.39	3.40 ± 0.01
	50.0	2	7.41	7.49	7.45 ± 0.06	6.85	6.94	6.90 ± 0.06

(B)

	Concentration (µg p-HPPH and m-HPPH)	Degrees of Freedom (D.f)	t-test for area ratios m/z $310_m/315$	t-test for area ratios m/z $310_p/315$
**	25.0	2	0.71	3.79
	50.0	1	8.30	3.29
***	25.0	1	1.21	2.45
	50.0	1	4.90	1.77

Critical Tabulated Values for t (P = 0.95) (Ref. 37) 1 D.f 12.71; 2 D.f 4.70

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S_p \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

** Injection of the standard extracts when the samples from dog 3 were analyzed.

*** Injection of the standard extracts when the samples from dog 1 were analyzed.

**** Ratios from Table IV-2.

TABLE IV-5. ANALYSIS OF SERUM SAMPLES FROM DOGS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED PHT, m-HPPH and p-HPPH TO THEIR RESPECTIVE INTERNAL STANDARD. [To 1.0 ml of serum, 10.0 µg of (D_{10})-PHT and 5.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

	N	Sample#1 Area Ratio m/z 280/290	Sample#2 Area Ratio m/z 280/290	Sample#1 Area Ratio m/z 283/290	Sample#2 Area Ratio m/z 283/290	Sample#1 Area Ratio m/z 310_m/315	Sample#2 Area Ratio m/z 310_m/315	Sample#1 Area Ratio m/z 313_m/315	Sample#2 Area Ratio m/z 313_m/315	Sample#1 Area Ratio m/z 310_p/315	Sample#2 Area Ratio m/z 310_p/315	Sample#1 Area Ratio m/z 313_p/315	Sample#2 Area Ratio m/z 313_p/315
Dog 1													
#1	2	1.291	1.206	1.102	1.052	0.450	0.474	0.407	0.428	0.235	0.253	0.207	0.238
#2	2	1.000	*	0.886	*	0.694	0.620	0.606	0.553	0.386	0.314	0.325	0.289
#3	2	0.737	0.759	0.651	0.650	0.975	1.007	0.875	0.934	0.503	0.462	0.465	0.436
#4	2	0.698	0.689	0.644	0.636	0.987	0.938	0.792	0.759	0.487	0.474	0.391	0.371
#5	2	0.463	0.506	0.428	0.445	0.857	*	0.745	*	0.433	*	0.376	*
#6	2	0.314	0.318	0.284	0.270	0.821	0.786	0.699	0.665	0.429	0.419	0.380	0.362
Dog 2													
#1	2	0.840	0.826	0.736	0.737	0.185	0.166	0.170	0.157	0.047	0.049	0.046	0.048
#2	2	0.775	0.805	0.702	0.708	0.232	0.252	0.227	0.232	0.064	0.063	0.056	0.056
#3	2	0.779	*	0.694	*	0.508	*	0.471	*	0.112	*	0.104	*
#4	2	0.599	0.590	0.536	0.534	0.476	0.501	0.473	0.503	0.121	0.116	0.117	0.113
#5	2	0.557	0.528	0.504	0.474	0.521	0.555	0.508	0.539	0.124	0.130	0.124	0.118
#6	2	0.432	0.436	0.374	0.393	0.460	0.464	0.457	0.460	0.129	0.127	0.119	0.121
Dog 3													
#1	2	0.781	0.785	0.654	0.647	0.298	0.300	0.259	0.260	0.072	0.059	0.060	0.050
#2	2	0.739	0.699	0.616	0.588	0.435	0.440	0.372	0.373	0.102	0.107	0.086	0.090
#3	2	0.597	0.601	0.509	0.510	0.554	0.595	0.474	0.513	0.126	0.129	0.107	0.121
#4	2	0.505	0.515	0.431	0.434	0.613	0.597	0.525	0.512	0.144	0.138	0.115	0.120
#5	2	0.411	0.418	0.351	0.358	0.624	0.675	0.518	0.557	0.140	0.155	0.125	0.118
#6	2	0.315	0.321	0.269	0.277	0.554	0.541	0.481	0.467	0.141	0.131	0.129	0.116

* No useful measurements were obtained because of either computer hardware problems or inappropriately set electron multiplier voltages.

TABLE IV-6

ANALYSIS OF URINE SAMPLES FROM DOGS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED m-HPPH AND p-HPPH TO THEIR RESPECTIVE INTERNAL STANDARDS. To 0.5 ml of urine, 10 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standard.

	N	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2
		Area Ratio m/z $310_m/315$		Area Ratio m/z $313_m/315$		Area Ratio m/z $310_p/315$		Area Ratio m/z $313_p/315$	
Dog 1									
#1	2	7.32	7.23	6.64	6.59	2.70	2.49	2.46	2.33
#2	2	29.15	*	26.75	*	10.29	*	9.49	*
#3	2	32.27	*	29.51	*	12.16	*	10.94	*
#4	2	41.83	42.17	38.16	38.45	15.59	16.17	14.16	14.71
#5	2	21.93	*	19.94	*	7.90	*	7.20	*
Dog 2									
#1	2	9.34	*	8.54	*	2.54	*	2.31	*
#2	2	21.34	18.69	20.14	17.38	6.59	5.75	6.35	5.33
#3	2	27.41	22.19	25.96	20.80	8.72	7.35	8.46	6.93
#4	2	26.67	28.61	24.61	26.61	8.83	9.45	8.15	8.84
#5	2	26.94	34.98	24.94	32.30	8.87	9.43	8.24	8.83
Dog 3									
#1	2	3.88	4.17	3.20	3.46	1.16	1.30	0.99	1.08
#2	2	13.47	*	11.21	*	3.85	*	3.18	*
#3	2	24.34	*	20.43	*	6.43	*	5.38	*
#4	2	23.99	*	20.15	*	6.80	*	5.75	*
#5	2	6.53	6.84	5.39	5.59	1.94	1.96	1.62	1.66

* No useful measurements were obtained because of either computer hardware problems or inappropriately set electron multiplier voltages.

regression lines were used to calculate the concentrations of Ia, Ib, IIIa, IIIb, IIa and IIb in serum (µg/ml) and IIIa, IIIb, IIa and IIb in urine (µg/0.5 ml); the concentrations (Mean and SD for the duplicate analyses) are listed in Tables IV-7 (serum values) and IV-8 (urine values).

1d. Comments

The chemical work-up of serum samples was found to be more time-consuming than that of the urine samples; two internal standards had to be added to the serum samples (Ic before the hydrolysis, IIc after the hydrolysis), and separation of layers after extraction of the serum samples with MIBK required longer centrifugation and had to be done more carefully due to the formation of a rather stable emulsion. It was therefore not possible to extract all the serum samples from the three dogs (in duplicate) and the appropriate standard samples during a single day.

For the LRA, the logarithmic transformation of the data was omitted because for the small concentration range monitored for the serum samples, the accuracy of the results would not be improved significantly by weighting of the regression line. For urine, the concentrations of IIIa, IIIb, IIa and IIb in the urine samples from dogs were very high so that the upper part of the calibration curve was most representative to calculate the concentrations of unknowns. Again, weighting the smaller ion abundances

TABLE IV-7

ANALYSIS OF SERUM SAMPLES FROM DOGS: CONCENTRATIONS OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED PHT, m-HPPH and p-HPPH (µg/ml).

	N	PHT Mean ± SD	($^{13}C^{15}N_2$)-PHT Mean ± SD	m-HPPH Mean ± SD	($^{13}C^{15}N_2$)-m-HPPH Mean ± SD	p-HPPH Mean ± SD	($^{13}C^{15}N_2$)-p-HPPH Mean ± SD
Dog 1							
#1	2	10.62 ± 0.52	10.18 ± 0.33	1.77 ± 0.06	1.71 ± 0.06	0.94 ± 0.04	0.92 ± 0.08
#2	2	8.48 ± *	8.36 ± *	2.47 ± 0.18	2.33 ± 0.14	1.31 ± 0.18	1.23 ± 0.08
#3	2	6.29 ± 0.10	6.13 ± 0	3.67 ± 0.08	3.56 ± 0.16	1.76 ± 0.10	1.75 ± 0.07
#4	2	5.84 ± 0.06	6.02 ± 0.06	3.57 ± 0.12	3.08 ± 0.09	1.75 ± 0.03	1.50 ± 0.05
#5	2	4.05 ± 0.26	4.09 ± 0.11	3.19 ± *	2.96 ± *	1.59 ± *	1.47 ± *
#6	2	2.60 ± 0.03	2.57 ± 0.10	3.00 ± 0.09	2.72 ± 0.09	1.56 ± 0.02	1.46 ± 0.05
Dog 2							
#1	2	7.67 ± 0.09	7.75 ± 0.01	0.89 ± 0.06	0.88 ± 0.04	0.29 ± 0.01	0.30 ± 0.01
#2	2	7.26 ± 0.20	7.41 ± 0.04	1.18 ± 0.06	1.19 ± 0.02	0.36 ± 0.01	0.34 ± 0
#3	2	7.16 ± *	7.29 ± *	2.36 ± *	2.33 ± *	0.58 ± *	0.57 ± *
#4	2	5.43 ± 0.06	5.61 ± 0.01	2.28 ± 0.08	2.41 ± 0.10	0.61 ± 0.02	0.63 ± 0.02
#5	2	4.95 ± 0.19	5.12 ± 0.23	2.50 ± 0.11	2.58 ± 0.11	0.65 ± 0.02	0.66 ± 0.02
#6	2	3.93 ± 0.03	4.00 ± 0.14	2.16 ± 0.01	2.28 ± 0.01	0.65 ± 0.01	0.66 ± 0.01
Dog 3							
#1	2	7.42 ± 0.02	6.89 ± 0.05	1.07 ± 0	1.00 ± 0.01	0.31 ± 0.04	0.28 ± 0.03
#2	2	6.82 ± 0.27	6.38 ± 0.21	1.55 ± 0.01	1.40 ± 0	0.46 ± 0.01	0.42 ± 0.01
#3	2	5.69 ± 0.03	5.41 ± 0.01	2.01 ± 0.10	1.84 ± 0.10	0.55 ± 0.01	0.53 ± 0.04
#4	2	4.86 ± 0.06	4.60 ± 0.02	2.12 ± 0.04	1.93 ± 0.03	0.60 ± 0.01	0.54 ± 0.01
#5	2	3.96 ± 0.05	3.78 ± 0.05	2.27 ± 0.13	2.00 ± 0.10	0.63 ± 0.04	0.56 ± 0.02
#6	2	3.05 ± 0.04	2.92 ± 0.06	1.92 ± 0.03	1.77 ± 0.03	0.58 ± 0.03	0.56 ± 0.04

* Only one useful measurement was obtained (see Table IV-5).

TABLE IV-8

ANALYSIS OF URINE SAMPLES FROM DOGS: CONCENTRATIONS OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED m-HPPH AND p-HPPH (µg/0.5 ml).

	N	m-HPPH Mean ± SD	($^{13}C^{15}N_2$)-m-HPPH Mean ± SD	p-HPPH Mean ± SD	($^{13}C^{15}N_2$)-p-HPPH Mean ± SD
Dog 1					
#1	2	49.4 ± 0.4	47.6 ± 0.3	19.7 ± 1.1	19.3 ± 0.7
#2	2	199.9 ± *	194.4 ± *	75.9 ± *	74.2 ± *
#3	2	221.4 ± *	214.5 ± *	89.5 ± *	85.5 ± *
#4	2	288.3 ± 1.7	278.6 ± 1.5	116.7 ± 3.0	112.5 ± 3.0
#5	2	150.2 ± *	144.7 ± *	58.4 ± *	56.5 ± *
Dog 2					
#1	2	63.6 ± *	61.6 ± *	19.3 ± *	18.6 ± *
#2	2	137.1 ± 12.9	136.1 ± 14.2	45.8 ± 4.3	46.0 ± 5.6
#3	2	170.0 ± 25.4	169.8 ± 26.6	59.4 ± 7.1	60.3 ± 8.4
#4	2	189.5 ± 9.4	186.1 ± 10.3	67.5 ± 3.2	66.5 ± 3.8
#5	2	212.3 ± 39.1	208.0 ± 37.9	67.5 ± 2.9	66.8 ± 3.2
Dog 3					
#1	2	27.1 ± 1.4	23.6 ± 1.3	9.7 ± 0.7	8.8 ± 0.5
#2	2	92.0 ± *	81.1 ± *	28.8 ± *	25.4 ± *
#3	2	166.8 ± *	148.3 ± *	47.7 ± *	42.4 ± *
#4	2	164.4 ± *	146.3 ± *	50.4 ± *	45.3 ± *
#5	2	45.4 ± 1.5	39.4 ± 1.2	15.0 ± 0.1	13.5 ± 0.2

* Only one useful measurement was obtained (see Table IV-6).

would not significantly improve the accuracy of the results. In addition, for these studies, the ratios of unlabelled to ($^{13}C^{15}N_2$)-labelled analogs was close to unity so that a slightly different value for slope and intercept of the regression lines would introduce the same bias in the concentration of unlabelled and ($^{13}C^{15}N_2$)-labelled analogs. This would, therefore, not change the ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolites and would also not result in different pharmacokinetic parameters.

The regression lines of the ratios of the molecular ion abundances of d-IIIa/d-IIc and d-IIa/d-IIc on the concentrations of IIIa and IIa, respectively, were used to calculate the concentrations of the respective ($^{13}C^{15}N_2$)-labelled analogs (IIIb and IIb). The values obtained for IIIb and IIb were therefore multiplied by 1.06, because the administered Ia (and therefore also the resulting metabolites) were only 94 % trilabelled, i.e. the ratio of Ia/Ib is 1.06.

Because of time constraints, it was impossible to evaluate the relative importance of height and area measurements for every set of samples that was analyzed. Area measurements were chosen because it was anticipated that these studies with dogs would require a few weeks of analysis time under possibly changing instrumental conditions that could affect the peak shape (see Chapter III, Section E-4).

The high SD for the measurement of IIa and IIIa at concentrations of 200.0 µg/ml in the urine standard samples (see Table IV-2) was attributed to memory effects in the GC-MS system.

Several comments on the GC-MS analysis of the urine and serum extracts are in order.

1. For measurement of d-IIb in serum samples from dogs, the measured ion abundances at m/z 313 were corrected for the contribution of the trilabelled contaminant of the internal standard (d-IIc) at this value. This was necessary because low isotope ratios (m/z 313/315) were measured. For each series of GC-MS measurements, the exact correction factor was determined by measuring the ion abundances at m/z 313 when an extract from the standard samples was injected. These extracts did not contain IIb; therefore, the measured ion abundances at m/z 313 indicated the magnitude of the interference under the instrumental conditions employed. For d-IIIb, no such corrections were necessary because d-IIIb and d-IIc were gas chromatographically resolved. No corrections were made for the contribution of natural isotope abundance from d-IIb to the measured ion abundances at m/z 315 for the internal standard (d-IIc) because the amount of IIb in the samples was always small compared to the amount of IIc that was added (i.e. low isotope ratios of m/z 313/315 were measured).

2. The relatively large variability of the within-sample measurements in serum (duplicate analysis of the same

sample) was mainly due to the fact that the serum samples contained low levels of IIIa, IIIb, IIa and IIb so that the elution profile of (d-IIIa, d-IIIb) and (d-IIa, d-IIb, d-IIc) could not well be defined with 2 sec scans. The mass spectrometric measurements required a high electron multiplier gain and some of the measured ion currents were close to the S/N limit. Small changes in the gas chromatographic or mass spectrometric conditions had a profound effect on the isotope ratios ratios. Slight changes in gas chromatographic conditions resulted in a worse precision for measurement of d-IIIa and d-IIIb, while a light increase in column background at m/z 315 made it difficult to define the abundances of the molecular ions of d-IIc at m/z 315. In the latter case, a correction of the ion abundances at m/z 313 , measured for d-IIb was also difficult (for these corrections, a percentage of the ion abundance at m/z 315 was subtracted from the ion abundance at m/z 313; the determination of this correction factor has been discussed on the previous page).

3. For measurement of d-IIb in urine samples from dogs, the contribution of interfering ion abundances from the internal standard (d-IIc) to the measured m/z 313 values was neglible because of the high levels of metabclites, and corrections at m/z 313 were unnecessary.

4. The calibration data for the analysis of urine samples covered concentrations of IIa and IIIa ranging from 5.0-200.0 µg/ml, because these levels were expected in the

samples from the dogs. To 0.5 ml of urine, 10.0 µg of IIc were added so that isotope ratios close to unity would result. The standard samples also contained equal amounts of IIa and IIIa. Gas chromatographically, d-IIIa and (d-IIa, d-IIc) appeared fully resolved and the d-IIIa peak was always smaller than the (d-IIa, d-IIc) peak, because d-IIc is a p-HPPH analog. However, in the urine samples from the dogs, roughly three times as much IIIa and IIIb as IIa and IIb were present and both in very high concentrations (for measurement of the m-HPPH analogs, ratios of d-IIIa/d-IIc and d-IIIb/d-IIc as high as 30 were measured when 10.0 µg of IIc had been added as internal standard to 0.5 ml samples, see Table IV-6). These high concentrations were unexpected; they were later attributed to the fact that the air-conditioning was not working in the cages where the dogs were kept, resulting in extraordinarily small urine volumes. In the urine samples from the dogs, the gas chromatographic peak of (d-IIIa, d-IIIb) was always larger than than the gas chromatographic peak of (d-IIa, d-IIb, d-IIc). In most cases, too much material was injected onto the gas chromatograph (in order to measure the 10.0 µg internal standard) so that overloading of the column occurred and the permethylated derivatives of the m-HPPH analogs tailed into these of the p-HPPH analogs. This broadened elution profile of (d-IIIa, d-IIIb) became even more visible in the TIP, and it was difficult to define exactly the elution profiles of

(d-IIIa, d-IIIb) and (d-IIa, d-IIb, d-IIc). In addition, overloading of the column resulted in high memory effects, so that the within-sample and between-sample precision of the GC-MS measurements was worse than the previously defined 5 % CV.

5. Attempts were made to calculate the concentrations of IIIa, IIIb, IIa and IIb in these urine samples from dogs by use of the appropriate regression lines, but it is evident that the systematic error in the definition of the elution profiles reduced the accuracy of the results. In addition, ion ratios larger than 15 (measured for d-IIIa/d-IIc and d-IIIb/d-IIc, see Table IV-6) were beyond the range of the regression lines and the extrapolated concentrations were inherently less accurate. Also, no corrections were done at m/z 315 for overlapping natural abundance ions from high levels of d-IIb (The natural abundance of C18 H18 O3 N2 at $(M+2)^+$ is 2.62 %, Ref. 41). The reported values for the concentrations of IIIa and IIa and their ($^{13}C^{15}N_2$)-labelled analogs (IIIb and IIb) are therefore slightly lower than the real values. However, this bias is the same for unlabelled and ($^{13}C^{15}N_2$)-labelled analogs because the error is introduced in the measurement of the internal standard (at m/z 315) and the ratio of unlabelled and ($^{13}C^{15}N_2$)-labelled metabolites is close to unity so that LRA again introduces the same bias (see also p 131).

2. Interpretation of the results

As outlined in section A of this Chapter, demonstration of the absence of 'in vivo' isotope effects in the metabolism of Ib in man could be shown in two ways: it could be verified 1. that the pharmacokinetic parameters for Ia and Ib were identical, and 2. that the ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolites remained constant throughout the collection periods.

Pharmacokinetic analyis involved calculation of the slope (β) and intercept (B) of plots of concentration of Ia and Ib in plasma versus time. These parameters were calculated from the measured serum concentrations of Ia and Ib (see Table IV-7). The elimination half-life ($t_{\frac{1}{2}}$), volume of distribution usingthe area method (Vd) and total clearance were then calculated as described by Greenblatt et al. (42). The pharmacokinetic parameters for Ia and Ib are summarized in Table IV-9. The parameters are virtually identical for every parameter for each dog. There was no trend toward any significant difference in distribution, elimination or clearance of the two forms of phenytoin.

Calculation of the ratios of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolites was approached in two ways: both the ratios of the concentrations of unlabelled to ($^{13}C^{15}N_2$)-labelled analogs (Ia/Ib, IIIa/IIIb, IIa/IIb), and the ratios of molecular ion abundances of the monitored permethylated derivatives of the unlabelled to the ($^{13}C^{15}N_2$)-labelled analogs (d-Ia/d-Ib, d-IIIa/d-IIIb,

TABLE IV-9

PHARMACOKINETIC PARAMETERS FOR PHT AND ($^{13}C^{15}N_2$)-PHT IN THE STUDIED DOGS.

	$T_{½}$* hours	V_d* liters/kg	Clearance ml/min/kg
Dog 1			
PHT	2.43	1.93	4.88
($^{13}C^{15}N_2$)-PHT	2.49	1.06	4.93
Dog 2			
PHT	4.72	1.65	4.04
($^{13}C^{15}N_2$)-PHT	4.70	1.63	4.00
Dog 3			
PHT	3.60	1.51	4.86
($^{13}C^{15}N_2$)-PHT	3.76	1.62	4.98

* For abbreviations, see text (Chapter IV, Section B-2).

d-IIa/d-IIb) were calculated.

Table IV-10 lists the ratios of the concentrations (Mean and SD for the duplicate analyses) of unlabelled to ($^{13}C^{15}N_2$)-labelled analogs in serum and urine. The tabulations demonstrate the constancy of the ratio of Ia/Ib, IIIa/IIIb and IIa/IIb throughout the serial serum collections, and of IIIa/IIIb and IIa/IIb throughout the serial urine collections. As discussed previously, interferences were corrected for by use of corresponding calibration data (i.e. for Ia, regression lines of the molecular ion abundance ratios of d-Ia/d-Ic on the respective concentrations of Ia; for Ib, regression lines of the molecular ion abundance ratios of d-Ib/d-Ic on the respective concentrations of Ib). It was hereby assumed that the amount of interference was the same for standard solutions and unknowns. Calibration data were also available for the analysis of IIIa and IIa, but for the analysis of IIIb and IIb, no regression lines of the molecular ion abundance ratios of d-IIIb/d- IIc and d-IIb/d-IIc on the respective concentrations of IIIb and IIb, were available. Use of the regression lines of molecular ion abundance ratios of d-IIIa/d-IIc and d-IIa/d-IIc for calculation of IIIb and IIb, respectively, gave less accurate results (especially for serum samples containing low levels of IIIb and IIb).

Table IV-11 lists the measured ion abundance ratios (Mean and SD for the duplicate analyses) for unlabelled to

TABLE IV-10

ANALYSIS OF SERUM AND URINE SAMPLES FROM DOGS: RATIOS OF CONCENTRATIONS OF UNLABELLED PHT, p-HPPH and m-HPPH TO THEIR RESPECTIVE ($^{13}C^{15}N_2$)-LABELLED ANALOG.

		SERUM			URINE	
		PHT/ ($^{13}C^{15}N_2$)-PHT	m-HPPH/ ($^{13}C^{15}N_2$)-m-HPPH	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH	m-HPPH/ ($^{13}C^{15}N_2$)-m-HPPH	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH
	N	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Dog 1						
#1	2	1.04 ± 0.01	1.04 ± 0.01	1.03 ± 0.05	1.04 ± 0	1.02 ± 0.01
#2	2	1.01 *	1.06 ± 0.01	1.06 ± 0.07	1.03 *	1.02 *
#3	2	1.03 ± 0.02	1.04 ± 0.02	1.01 ± 0.02	1.03 *	1.05 *
#4	2	0.97 ± 0	1.16 ± 0	1.17 ± 0.01	1.03 ± 0	1.04 ± 0
#5	2	0.99 ± 0.04	1.08 *	1.08 *	1.04 *	1.10 *
#6	2	1.01 ± 0.05	1.11 ± 0.01	1.07 ± 0.02		
Dog 2						
#1	2	0.99 ± 0.01	1.01 ± 0.02	0.97 ± 0	1.03 *	1.03 *
#2	2	0.98 ± 0.02	0.99 ± 0.03	1.05 ± 0.02	1.02 ± 0.01	1.00 ± 0.03
#3	2	0.98 *	1.01 *	1.02 *	1.01 ± 0.01	0.99 ± 0.02
#4	2	0.97 ± 0.01	0.95 ± 0.01	0.97 ± 0	1.02 ± 0.01	1.02 ± 0
#5	2	0.97 ± 0.01	0.97 ± 0	0.98 ± 0.06	1.02 ± 0	1.01 ± 0
#6	2	0.98 ± 0.01	0.95 ± 0	0.98 ± 0.02		
Dog 3						
#1	2	1.08 ± 0.01	1.08 ± 0.01	1.11 ± 0.01	1.15 ± 0.01	1.11 ± 0.02
#2	2	1.07 ± 0.01	1.11 ± 0.01	1.11 ± 0.06	1.14 *	1.14 *
#3	2	1.05 ± 0	1.10 ± 0.01	1.05 ± 0.04	1.12 *	1.12 *
#4	2	1.06 ± 0.01	1.10 ± 0.01	1.11 ± 0.06	1.12 *	1.11 *
#5	2	1.05 ± 0	1.14 ± 0.01	1.13 ± 0.12	1.16 ± 0.01	1.11 ± 0.01
#6	2	1.05 ± 0.01	1.09 ± 0.01	1.04 ± 0.03		

* Only one useful measurement was obtained (see Tables IV-5 and IV-6).

TABLE IV-11

ANALYSIS OF SERUM AND URINE SAMPLES FROM DOGS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF UNLABELLED PHT, p-HPPH and m-HPPH TO THEIR RESPECTIVE ($^{13}C^{15}N_2$)-LABELLED ANALOG.

		SERUM			URINE	
		m/z 280/283	m/z $310_m/313_m$	m/z $310_p/313_p$	m/z $310_m/313_m$	m/z $310_p/313_p$
	N	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Dog 1						
#1	2	1.16 ± 0.01	1.11 ± 0.01	1.10 ± 0.05	1.10 ± 0	1.09 ± 0.02
#2	2	1.13 *	1.14 ± 0.02	1.14 ± 0.08	1.09 *	1.09 *
#3	2	1.15 ± 0.03	1.10 ± 0.02	1.07 ± 0.01	1.09 *	1.11 *
#4	2	1.08 ± 0	1.28 ± 0.04	1.27 ± 0.02	1.10 ± 0	1.10 ± 0
#5	2	1.15 ± 0.05	1.15 *	1.15 *	1.10 *	1.10 *
#6	2	1.11 ± 0.04	1.18 ± 0.01	1.15 ± 0.02		
Dog 2						
#1	2	1.13 ± 0.01	1.08 ± 0.02	1.02 ± 0.01	1.09 *	1.10 *
#2	2	1.12 ± 0.02	1.06 ± 0.05	1.13 ± 0	1.07 ± 0.01	1.06 ± 0.03
#3	2	1.12 *	1.08 *	1.07 *	1.07 ± 0.01	1.05 ± 0.03
#4	2	1.12 ± 0.01	1.00 ± 0.01	1.04 ± 0.01	1.08 ± 0	1.08 ± 0
#5	2	1.12 ± 0.01	1.03 ± 0.01	1.05 ± 0.07	1.08 ± 0	1.08 ± 0.01
#6	2	1.13 ± 0.01	1.01 ± 0	1.07 ± 0.03		
Dog 3						
#1	2	1.20 ± 0.01	1.15 ± 0	1.18 ± 0.01	1.21 ± 0.03	1.19 ± 0.03
#2	2	1.20 ± 0.01	1.18 ± 0.01	1.19 ± 0.01	1.20 *	1.21 *
#3	2	1.18 ± 0.01	1.17 ± 0.01	1.13 ± 0.08	1.19 *	1.19 *
#4	2	1.18 ± 0.01	1.17 ± 0	1.20 ± 0.07	1.19 *	1.18 *
#5	2	1.17 ± 0.01	1.21 ± 0.01	1.22 ± 0.13	1.22 ± 0.01	1.19 ± 0.01
#6	2	1.17 ± 0.01	1.16 ± 0.01	1.11 ± 0.03		

* Only one useful measurement was obtained (see Tables IV-5 and IV-6).

($^{13}C^{15}N_2$)-labelled analogs in the serial serum and urine samples. Again, the constancy of the ratio of the molecular ion abundances of d-Ia/d-Ib, d-IIa/d-IIb and d-IIIa/d-IIIb throughout the serial serum collections, and for d-IIa/d-IIb and d-IIIa/d-IIIb throughout the serial urine collections was demonstrated. It should be noted that the SD of the measurements of the molecular ion abundance ratios of d-Ia/d-Ib, d-IIIa/d-IIIb and d-IIa/d-IIb is very low; this is attributed to the fact that the ratio remained close to unity (approximately equal amounts of Ia and Ib had been administered).

A summary of the data of Table IV-10 is presented in Table IV-12, part A. For each dog, the mean ratio of Ia/Ib, IIIa/IIIb and IIa/IIb in serum, and IIIa/IIIb and IIa/IIb in urine was calculated by averaging the ratios obtained for the serial collections. Similarly, a summary of the data of Table IV-11 is presented in Table IV-12, part B. For each dog, the mean ratio of the molecular ion abundances of d-Ia/d-Ib, d-IIIa/d-IIIb and d-IIa/d-IIb in serum, and d-IIIa/d-IIIb and d-IIa/d-IIb in urine was calculated by averaging the ratios obtained for the serial collections.

The difference in ratios of d-Ia/d-Ib, d-IIa/d-IIb and d-IIIa/d-IIIb in serum and d-IIIa/d-IIc and d-IIa/d-IIc in urine from the same dog (e.g. for Dog # 2, serum: d-Ia/d-Ib, 1.12; d-IIIa/d-IIIb, 1.04; d-IIa/d-IIb, 1.06; urine: d-IIIa/d-IIIb, 1.08; d-IIa/d-IIb 1.07) was

TABLE IV-12

ANALYSIS OF SERUM AND HUMAN SAMPLES FROM DOGS: SUMMARY OF THE DATA PRESENTED IN TABLES IV-10 AND IV-11. (A) RATIOS OF CONCENTRATIONS (B) RATIOS OF ION ABUNDANCES.

(A)

	SERUM				URINE			OVERALL RATIO		
	PHT/ $(^{13}C^{15}N_2)$-PHT	m-HPPH $(^{13}C^{15}N_2)$-m-HPPH	p-HPPH $(^{13}C^{15}N_2)$-p-HPPH		m-HPPH $(^{13}C^{15}N_2)$-m-HPPH	p-HPPH $(^{13}C^{15}N_2)$-p-HPPH		unlabelled/ $(^{13}C^{15}N_2)$-labelled analog	
	N Mean ± SD	Mean ± SD	Mean ± SD	N	Mean ± SD	Mean ± SD	N	Mean ± SD	CV (%)
Dog 1	6 1.01 ± 0.03	1.08 ± 0.05	1.07 ± 0.06	5	1.03 ± 0.01	1.05 ± 0.03	5	1.05 ± 0.03	2.44
Dog 2	6 0.98 ± 0.01	0.98 ± 0.03	1.00 ± 0.04	5	1.02 ± 0.01	1.01 ± 0.02	5	1.00 ± 0.02	1.79
Dog 3	6 1.06 ± 0.01	1.10 ± 0.02	1.09 ± 0.04	5	1.14 ± 0.02	1.12 ± 0.01	5	1.10 ± 0.03	2.75

(B)

	SERUM			URINE			OVERALL RATIO		
	m/z 280/283	m/z $310_m/313_m$	m/z $310_p/313_p$	m/z $310_m/313_m$	m/z $310_p/313_p$		m/z $M^+/(M+3)^+$		
	N Mean ± SD	Mean ± SD	Mean ± SD	N	Mean ± SD	Mean ± SD	N	Mean ± SD	CV (%)
Dog 1	6 1.13 ± 0.03	1.16 ± 0.07	1.15 ± 0.07	5	1.10 ± 0.01	1.10 ± 0.01	5	1.13 ± 0.03	2.46
Dog 2	6 1.12 ± 0.01	1.04 ± 0.04	1.06 ± 0.04	5	1.08 ± 0.01	1.07 ± 0.02	5	1.07 ± 0.03	2.76
Dog 3	6 1.18 ± 0.01	1.17 ± 0.02	1.17 ± 0.04	5	1.20 ± 0.01	1.19 ± 0.01	5	1.18 ± 0.01	1.10

attributed to the fact that the error introduced when measuring the ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled analogs was different for the PHT analogs and the HPPH analogs, and was also different for the serum and urine samples. In serum samples, low levels were measured and it was noticed that the ratios of ion abundances of unlabelled to ($^{13}C^{15}N_2$)-labeled analogs showed a large variability for each consecutive scan taken within the gas chromatographic elution profiles (i.e. the defined profile from the TIP). It was therefore concluded that these low levels of analytes were not accurately measured by 4 s or 2 s scan cycles (PHT analogs and HPPH analogs respectively), i.e. not enough sampling points were taken to reveal the presence of interferences and to determine which scans were representative for the analytes. In urine samples, high levels of metabolites were present and no significant differences in ratios were noticed for the consecutive scans within the gas chromatographic elution profiles of the analytes, so that the inability to define the elution profile did not affect the validity of the listed ratios (there is no gas chromatographic separation between the unlabelled and ($^{13}C^{15}N_2$)-labelled analogs). Unfortunately, the ratio of Ia to Ib in the drug mixture infused was not determined and the direction of the error could therefore not be made. The ratios found in the urine would certainly approach best the real ratio, because these measurements refer to high levels and are therefore much less

susceptible to interferences.

Because of the isotopic impurity of Ib, a different value was obtained for the ratio of concentrations (Table IV-12, part A) and the ratio of the ion abundances (Table IV-12, part B). The molecular ion abundances of the ($^{13}C^{15}N_2$)-labelled analogs (d-Ib, d-IIIb and d-IIb) only account for 94 % of the total amount of, respectively, d-Ib, d-IIIb and d-IIb (Ib and the resulting metabolites are only 94 % trilabelled). Therefore, ratios of the molecular ion abundances of d-Ia/d-Ib, d-IIIa/d-IIIb and d-IIa/d-IIb will show this discrepancy (as discussed in Chapter III, Section E-3), while the use of appropriate calibration data to calculate concentrations eliminates this difference in isotopic purity.

As shown in Table IV-12, the CV of the ratios of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolites within each dog remained within 3 %. We could therefore rule out that in dogs the metabolism of Ib was different from that of Ia.

C. STUDIES WITH HUMAN VOLUNTEERS

1. Data acquisition and processing

1a. Gas chromatographic mass spectrometric measurement

Under the descibed gas chromatographic conditions (see Section A of this Chapter), the permethylated derivatives of the PHT analogs (d-Ia, d-Ib, d-Ic) were separated from these of the p-HPPH analogs (d-IIa, d-IIb,

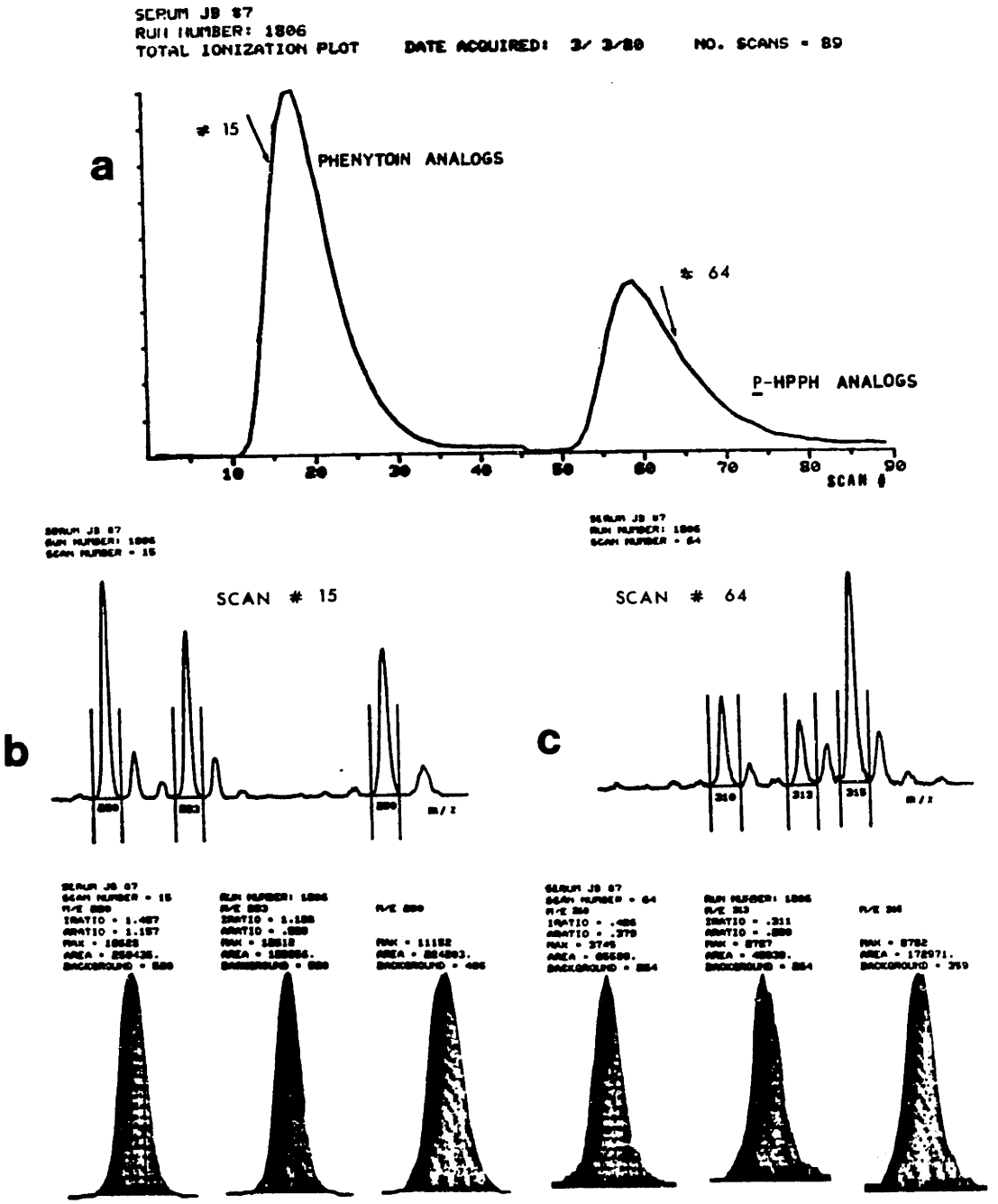
d-IIc) and both sets of analogs eluted in well defined gas chromatographic peaks. Measurement proceeded as outlined in Chapter VI, Section C: isotope ratios were computed for every scan during elution of (d-Ia, d-Ib, d-Ic) and for every scan during elution of (d-IIa, d-IIb, d-IIc) and were then averaged over all the defined scans. One second scan cycles were used for monitoring both the PHT and p-HPPH analogs. Figure IV-2 shows in (a) a display of the total ionization plot (TIP) constructed during analysis of a plasma sample from a human volunteer, and in (b) the mass range scanned for the measurement of the molecular ion abundances of (d-Ia, d-Ib, d-Ic), as shown by scan #15 from the TIP, in (c) the mass range scanned for the measurement of the molecular ion abundances of (d-IIa, d-IIb, d-IIc), as shown by scan # 64 from the TIP. In addition, the integration profile of the ion currents at the preselected m/z values is shown in the expanded mode.

1b. Linearity and Precision

No additional experiments were carried out to re-evaluate the linearity and precision as such, but with each series of plasma and urine samples from the volunteers, plasma or urine standard solutions were analyzed in order to obtain meaningful calibration data.

Figure IV-2. Display of (a) the total ionization plot (TIP) constructed during analysis of a serum sample from a human volunteer (b) scan #15 from the TIP (c) scan #64 from the TIP.

Figure IV-2


SERUM JB 87
RUN NUMBER: 1806
TOTAL IONIZATION PLOT
DATE ACQUIRED: 3/ 3/80
NO. SCANS = 89
a
15
PHENYTOIN ANALOGS
64
P-HPPH ANALOGS
10
20
30
40
50
60
70
80
90
SCAN #
SCAN # 15
b
SCAN # 64
c
m/z

1c. Analysis of samples from human volunteers

For each serial urine and plasma collection from the human subjects (for protocol see Chapter VI, the samples were numbered in the order of collection) one sample was processed. For plasma, 1.0 ml samples were worked up and 1.0 µg of each Ic and IIc were added as internal standards. For urine, 0.5 ml samples were processed and 10.0 µg of IIc were added as internal standards. The urine samples were centrifuged before pipetting. Blanks (i.e. plasma and urine samples collected before infusion of Ia and Ib, and to which no internal standards were added) were always included.

The plasma samples were processed on three different days; each day a corresponding series of plasma standard solutions containing Ia, Ib, IIa and IIb in the appropriate concentration ranges was included (0.1-10.0 µg/ml of each Ia and Ib; 0.1-5.0 µg/ml of each IIa and IIb). For Subject 2, two samples of each of the plasma standard solutions were worked up; for Subjects 1 and 3, a single sample of each of the plasma standard solutions was processed. Attempts were made to analyze the extracts from the plasma samples from the human subjects and the corresponding standard samples by GC-MS on the same day or under the same instrumental conditions. For the final GC-MS analysis of the samples from Subject 1, no such calibration data were obtained because the corresponding set of extracts of the standard samples had been used up in

previous analyses. (The series of plasma extracts from Subject 1, who was the first human subject, and the corresponding standard extracts had initially been analyzed on a 3 ft OV-17 column; these data could not be used because of the fatty acid interference. The extracts from the standard samples were therefore used to select better gas chromatographic conditions; the extracts from plasma samples from Subject 1 with high levels of Ia and Ib (#1-#6) were then re-injected on a 6 ft OV-17 column, the remaining extracts on a 6 ft OV-101 column.) For Subject 1, therefore, the regression lines prepared for the analysis of the plasma samples of Subject 2 were used to calculate the concentrations of Ia, IIa and their ($^{13}C^{15}N_2$)-labelled analogs (Ib and IIb) in the extracts that had been injected on an OV-101 column (these samples were analyzed under the same conditions as the samples from Subject 1), and previously presented regression lines for the determination of Ia and Ib that were obtained by injection of serum extracts on an OV-17 column (see Table III-8) were used as reference for the remaining extracts which had been injected on an OV-17 column.

The calibration data for analysis of plasma samples from Subject 2 are listed in Table IV-13. For the extracts from the plasma standard samples extracts containing 0.1 µg/ml of Ia, Ib, IIa and IIb, duplicate GC-MS measurements were made; the remaining extracts were injected once. The Table lists the molecular ion abundance ratios (areas) of

TABLE IV-13

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: CALIBRATION DATA FOR ANALYSIS OF SAMPLES FROM SUBJECT 2. Sample: 0.1-10.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5-5.0 µg of each p-HPPH and ($^{13}C^{15}N_2$)-p-HPPH/ml plasma. [To 1.0 ml of plasma, 1.0 µg of each (D_{10})-PHT and ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.]

Concentration	N	Sample #1 Inj. 1	Sample #1 Inj. 2	Sample #2 Inj. 1	Sample #2 Inj. 2	$\overline{\#1}$	$\overline{\#2}$	$\overline{\#1,\#2}$ ± SD	Linear Regression Analysis
µg PHT/ml				Area Ratio m/z 280/290					
0.1	2	0.102	0.098	0.096	0.090	0.100	0.093	0.097 ± 0.005	
0.5	2	0.474	*	0.464	*	0.474	0.464	0.469 ± 0.007	log y = 0.99 log x - 0.02
1.0	2	0.958	*	0.936	*	0.958	0.936	0.947 ± 0.16	r = 0.99998
10.0	2	9.52	*	9.27	*	9.52	9.27	9.40 ± 0.18	
µg ($^{13}C^{15}N_2$)-PHT/ml				Area Ratio m/z 283/290					
0.1	2	0.104	0.100	0.097	0.098	0.102	0.098	0.100 ± 0.003	
0.5	2	0.459	*	0.440	*	0.459	0.440	0.450 ± 0.013	log y = 0.97 log x - 0.04
1.0	2	0.895	*	0.896	*	0.895	0.896	0.896 ± 0.001	r = 0.9999
10.0	2	8.61	*	8.42	*	8.61	8.42	8.52 ± 0.13	
µg p-HPPH/ml				Height Ratio m/z 310/315					
0.1	2	0.120	0.116	0.110	0.108	0.118	0.109	0.114 ± 0.006	
0.5	2	0.530	*	0.530	*	0.530	0.530	0.530 ± 0	log y = 0.97 log x + 0.02
1.0	2	1.07	*	1.05	*	1.07	1.05	1.06 ± 0.01	r = 0.9999
5.0 **	2	4.77	*	4.71	*	4.77	4.71	4.74 ± 0.04	
µg ($^{13}C^{15}N_2$)-p-HPPH/ml ***				Height Ratio m/z 313/315					
0.081	2	0.101	0.104	0.104	0.094	0.103	0.099	0.101 ± 0.003	
0.403	2	0.419	*	0.411	*	0.419	0.411	0.415 ± 0.005	log y = 0.89 log x - 0.02
0.806	2	0.793	*	0.790	*	0.793	0.790	0.792 ± 0.02	r = 0.9999
4.03	2	3.49	*	3.38	*	3.49	3.38	3.43 ± 0.07	

* For these extracts, only a single injection was made.

** Calibration point not included in the LRA.

*** The labelling of the x-axis was done such that the listed concentrations represented 94% trilabelled ($^{13}C^{15}N_2$)-p-HPPH (see text).

d-Ia/d-Ic, d-Ib/d-Ic, and the molecular ion abundance ratios (heights) of d-IIa/d-IIc and d-IIb/d-IIc, for each of the plasma standard solutions; for the samples containing 0.1 µg/ml of Ia, Ib, IIa and IIb, the mean ratio for the duplicate measurements of each extract ($\overline{\#1, \#2}$) is also reported. For the LRA, the averaged ratios, obtained for the two samples that had been processed for the each plasma standard solution ($\overline{\overline{\#1, \#2}}$) were used. Only three points were used to calculate the linear regression lines for measurement of IIa and IIb because the molecular ion abundance ratios of d-IIa/d-IIc and d-IIb/d-IIc, measured for Subject 2 were within the range of 0.1-1.0. Appropriate corrections were done for the points on the x-axis (=concentrations) of IIb because the reference compound of IIb was not chemically pure and had a different isotopic purity than the IIb isolated from samples from the human subjects (see also Section C-1d of this Chapter and Chapter VI). The weight of IIb for preparation of the calibration data was therefore adjusted so that the listed concentrations on the x-axis represented only the 94 % of trilabelled IIb.

The calibration data for analysis of the plasma samples from Subject 3 are listed in Table IV-14. Each extract was analyzed once by GC-MS.

The calibration data that were used for the analysis of the plasma samples from Subject 1 are reported in Table IV-15.

TABLE IV-14

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: CALIBRATION DATA FOR ANALYSIS OF SAMPLES FROM SUBJECT 3. Sample: 0.1-10.0 µg of PHT and ($^{13}C^{15}N_2$)-PHT, and 0.5-1.0 µg of p-HPPH and ($^{13}C^{15}N_2$)-p-HPPH/ml plasma. [To 1.0 ml of plasma, 1.0 µg of each (D_{10})-PHT and ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.

Concentration (µg/ml)	N		Linear Regression Analysis
PHT		Area Ratio m/z 280/290	
0.1	1	0.089	
0.5	1	0.474	log y = 1.03 log x - 0.02
1.0	1	0.944	r = 0.99999
10.0	1	10.07	
($^{13}C^{15}N_2$)-PHT		Area Ratio m/z 283/290	
0.1	1	0.096	
0.5	1	0.455	log y = 0.99 log x - 0.04
1.0	1	0.887	r = 0.9999
10.0	1	9.03	
p-HPPH		Height Ratio m/z 310/315	
0.1	1	0.117	
0.5	1	0.553	log y = 0.96 log x + 0.03
1.0	1	1.08	r = 0.99999999
($^{13}C^{15}N_2$)-p-HPPH *		Height Ratio m/z 313/315	
0.079	1	0.109	
0.397	1	0.434	log y = 0.87 log x - 0.005
0.794	1	0.820	r = 0.9997

* The labelling of the x-axis was done such that the listed concentrations represented 94% trilabelled ($^{13}C^{15}N_2$)-p-HPPH.

TABLE IV-15

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: CALIBRATION DATA USED IN THE ANALYSIS OF SAMPLES FROM SUBJECT 1. Sample: 0.1-10.0 µg of each PHT and ($^{13}C^{15}N_2$)-PHT, and 0.1-5.0 µg of p-HPPH and ($^{13}C^{15}N_2$)-p-HPPH/ml plasma. [To 1.0 ml of plasma, 1.0 µg of (D_{10})-PHT and ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standards.

OV-17 (see also Table III-8)

Concentration (µg/ml)		Linear Regression Analysis
PHT	Area Ratio m/z 280/290	
0.1	0.097	
0.5	0.507	log y = 1.02 log x + 0.01
1.0	1.05	r = 0.99996
10.0	10.56	
($^{13}C^{15}N_2$)-PHT	Area Ratio m/z 283/290	
0.1	0.100	
0.5	0.490	log y = 0.99 log x - 0.01
1.0	0.968	r = 0.9999997
10.0	9.35	

TABLE IV-15, continued

OV-101 (see also Table IV-13)

Concentration (g/ml)		Linear Regression Analysis
PHT	Area Ratio m/z 280/290	
0.1	0.097	
0.5	0.469	log y = 0.99 log x - 0.02
1.0	0.947	r = 0.99998
10.0	9.40	
($^{13}C^{15}N_2$)-PHT	Area Ratio m/z 283/290	
0.1	0.100	
0.5	0.450	log y = 0.97 log x - 0.04
1.0	0.896	r = 0.9999
10.0	8.52	
p-HPPH	Height Ratio m/z 310/315	
0.1	0.114	
0.5	0.530	log y = 0.95 log x + 0.01
1.0	1.06	r = 0.9999
5.0	4.74	
($^{13}C^{15}N_2$)-* p-HPPH	Height Ratio m/z 313/315	
0.081	0.101	
0.403	0.415	log y = 0.90 log x - 0.02
0.806	0.792	r = 0.9999
4.03	3.43	

* The labelling of the x-axis was done such that the listed concentrations represented 94% trilabelled ($^{13}C^{15}N_2$)-p-HPPH.

The urine samples were processed on one day, together with a series of urine standard solutions containing 5.0-100.0 µg/ml of each IIa and IIb. Three samples of the urine standard solutions containing 5.0 and 10.0 µg/ml of IIa and IIb were worked up, and two samples of the remaining urine standard solutions were processed. The extracts from the urine samples from the human subjects and these from the standard samples were analyzed by GC-MS on the same day. It was found that the last urine collection from each subject contained levels of IIa and IIb which were substantially lower than 5.0 µg/ml, i.e. the lowest point of the calibration data (these data are presented in Table IV-19 and are discussed further; the analysis of the last urine collection resulted in ratios of d-IIa/d-IIc and d-IIb/d-IIc smaller than 0.5 when 10.0 µg of internal standard was added to 0.5 ml of urine). Additional urine standard solutions containing 1.0 and 2.0 µg/ml of each IIa and IIb were therefore worked up in duplicate. At the same time, urine samples containing these low levels of metabolites were processed again and both series were analyzed by GC-MS on the same day. The calibration data for urine are listed in Table IV-16. Each extract was analyzed once by GC-MS. In part A of table IV-16, the mean molecular ion abundance ratios (heights) of d-IIa/d-IIc (m/z 310/315) and d-IIb/d-IIc (m/z 313/315), measured for each of the urine standard solutions, which respectively contained 5.0, 10.0, 20.0 and 100.0 µg/ml of IIa and IIb,

TABLE IV-16

ANALYSIS OF URINE SAMPLES FROM HUMAN VOLUNTEERS: CALIBRATION DATA FOR ANALYSIS OF SAMPLES FROM SUBJECTS 1, 2 AND 3. Sample: (A) 5.0-100.0 µg of each p-HPPH and ($^{13}C^{15}N_2$)-p-HPPH/ml urine (B) 1.0 and 2.0 µg of each p-HPPH and ($^{13}C^{15}N_2$)-p-HPPH/ml urine. [To 0.5 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standard.]

(A)

Concentration (µg/ml)	N		Linear Regression Analysis
p-HPPH		Height Ratio m/z 310/315 Mean ± SD	
5.0	3	0.288 ± 0.006	
10.0	3	0.568 ± 0.007	log y = 0.96 log x - 0.92
20.0	2	1.15 ± 0.014	r = 0.9998
100.0	2	5.11 ± 0.014	
($^{13}C^{15}N_2$)-p-HPPH *		Height Ratio m/z 313/315 Mean ± SD	
3.90	3	0.221 ± 0.002	
7.82	3	0.431 ± 0.003	log y = 0.94 log x - 0.92
15.62	2	0.855 ± 0.007	r = 0.9999
78.12	2	3.75 ± 0.021	

(B)

p-HPPH		Height Ratio m/z 310/315 Mean ± SD	
1.0	2	0.064 ± 0.002	
2.0	2	0.116 ± 0.004	
($^{13}C^{15}N_2$)-p-HPPH *		Height Ratio m/z 313/315 Mean ± SD	
0.78	2	0.057 ± 0.001	
1.56	2	0.096 ± 0.004	

* The labelling of the x-axis was done such that the listed concentrations represented 94% trilabelled ($^{13}C^{15}N_2$)-p-HPPH (see text).

and the SD of the measurements, are listed. In part B of Table IV-16, the mean molecular ion abundance ratios (heights) of d-IIa/d-IIc and d-IIb/d-IIc, measured for the standard solutions containing 1.0 and 2.0 µg/ml of IIa and IIb are listed.

The measured isotope ratios in the serial plasma and urine collections from the human volunteers are listed in Tables IV-17, IV-18 and IV-19. Table IV-17 lists the molecular ion abundance ratios (areas) of d-Ia/d-Ic and d-Ib/d-Ic in plasma; Table IV-18 lists the molecular ion abundance ratios (heights) of d-IIa/d-IIc and d-IIb/d-IIc in plasma. Table IV-19 lists the molecular ion abundance ratios (heights) of d-IIa/d-IIc and d-IIb/d-IIc for the serial urine collections. A single GC-MS analysis was carried out for each extract of the plasma and urine samples that had been processed. The corresponding calibration data, i.e. regression lines (Table IV-16 for the urine analyses, Tables IV-13, IV-14 and IV-15 for the plasma analyses) were used to calculate the concentration of Ia, Ib, IIa and IIb in plasma (µg/ml) and IIa and IIb urine (µg/0.5 ml). (For the urine samples containing levels of IIa and IIb smaller than 5.0 µg/ml, the concentrations were calculated from the bracketed measurements of the urine standard solutions containing 0.1 and 0.5 µg/0.5 ml of IIa and IIb (Table IV-16, part B).) The concentrations are listed in Tables IV-20, IV-21 (plasma values), and IV-22 (urine values).

TABLE IV-17

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED PHT TO THE INTERNAL STANDARD. [To 1.0 ml of plasma, 1.0 µg of (D_{10})-PHT was added as internal standard.]

	Subject 1**			Subject 2			Subject 3		
N		Area Ratio m/z 280/290	Area Ratio m/z 283/290		Area Ratio m/z 280/290	Area Ratio m/z 283/290		Area Ratio m/z 280/290	Area Ratio m/z 283/290
1	#1	4.96	4.30	#1	4.81	3.56	#1	3.23	2.88
1	#2	3.70	3.32	#2	4.34	3.21	#2	2.86	2.60
1	#3	2.89	2.63	#3	3.62	2.67	#3	2.87	2.58
1	#4	2.14	1.90	#4	2.75	2.05	#4	2.75	2.48
1	#5	1.98	1.75	#5	2.17	1.61	#5	3.54	3.24
1	#6	1.75	1.56	#6	2.01	1.48	#6	3.37	3.04
1	#7	1.66	1.51	#7	1.99	1.48	#7	3.21	2.92
1	#8	1.56	1.43	#8	1.80	1.34	#8	3.00	2.73
1	#9	1.46	1.34	#9	1.69	1.28	#9	2.73	2.51
1	#10	1.44	1.32	#10	1.62	1.21	#10	2.55	2.37
1	#11	1.31	1.20	#11	1.51	1.13	#11	2.36	2.18
1	#12	1.20	1.12	#12	1.37	1.04	#12	2.28	2.09
1	#13	1.17	1.07	#13	1.42	1.07	#13	2.21	2.03
1	#14	1.08	0.986	#14	1.27	0.953	#14	1.52	1.42
1	#15	0.739	0.678	#15	0.948	0.721	#15	0.759	0.712
1	#16	0.391	0.373	#16	0.866	0.664	#16	0.578	0.552
1	#17	0.296	0.283	#17	0.458	0.356	#17	0.468	0.454
1	#18	0.139	0.135	#18	0.320	0.250	#18	0.149	0.153
1	#19	*	*	#19	0.226	0.184	#19	*	*
1				#20	0.105	*			
				#21	*	*			

* Ratio not determined (smaller than lowest calibration point).

** Ion intensity ratios from extracts #1-#6 were determined on an OV-17 column.

TABLE IV-18

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED p-HPPH TO THE INTERNAL STANDARD. [To 1.0 ml of plasma, 1.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH was added as internal standard.]

	Subject 1			Subject 2			Subject 3		
N		Height Ratio m/z 310/315	Height Ratio m/z 313/315		Height Ratio m/z 310/315	Height Ratio m/z 313/315		Height Ratio m/z 310/315	Height Ratio m/z 313/315
1	#1	0.135	0.141	#1	*	*	#1	*	*
1	#2	0.176	0.183	#2	*	*	#2	*	*
1	#3	0.264	0.258	#3	0.141	0.123	#3	0.184	0.197
1	#4	0.362	0.348	#4	0.243	0.200	#4	0.287	0.291
1	#5	0.428	0.406	#5	0.311	0.251	#5	0.437	0.416
1	#6	0.572	0.536	#6	0.352	0.285	#6	0.466	0.444
1	#7	0.672	0.619	#7	0.383	0.298	#7	0.621	0.583
1	#8	0.667	0.624	#8	0.444	0.343	#8	0.795	0.725
1	#9	0.654	0.627	#9	0.511	0.406	#9	0.794	0.724
1	#10	0.720	0.667	#10	0.566	0.439	#10	0.983	0.895
1	#11	0.609	0.571	#11	0.602	0.454	#11	1.04	0.938
1	#12	0.836	0.767	#12	0.672	0.514	#12	1.05	0.980
1	#13	0.755	0.705	#13	0.807	0.611	#13	1.26	1.17
1	#14	1.30	1.18	#14	0.877	0.667	#14	1.22	1.11
1	#15	0.759	0.695	#15	0.764	0.584	#15	0.806	0.724
1	#16	0.528	0.499	#16	0.767	0.579	#16	0.657	0.619
1	#17	0.413	0.395	#17	0.508	0.389	#17	0.547	0.537
1	#18	0.180	0.176	#18	0.419	0.327	#18	0.255	0.258
1	#19	0.136	0.139	#19	0.337	0.272	#19	0.162	0.176
1				#20	0.187	0.161			
1				#21	*	*			

* Ratio not determined (smaller than lowest calibration point).

TABLE IV-19

ANALYSIS OF URINE SAMPLES FROM HUMAN VOLUNTEERS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF UNLABELLED AND ($^{13}C^{15}N_2$)-LABELLED p-HPPH TO THE INTERNAL STANDARD. [To 0.5 ml of urine, 10.0 µg of ($^{13}C^{15}N_2D_2$)-p-HPPH were added as internal standard.]

N	Subject 1	Height Ratio m/z 310/315	Height Ratio m/z 313/315
1	#1	3.68	3.36
1	#2	4.22	3.83
1	#3	1.59	1.47
1	#4	1.13	1.06
1	#5	0.401	0.382
1	#6	0.103	0.108
	Subject 2		
1	#1	1.54	1.15
1	#2	4.10	3.05
1	#3	4.34	3.22
1	#4	2.78	2.08
1	#5	1.41	1.07
1	#6	0.465	0.357
1	#7	0.073	0.066
	Subject 3		
1	#1	1.43	1.30
1	#2	1.44	1.31
1	#3	3.12	2.85
1	#4	2.57	2.36
1	#5	0.586	0.550
1	#6	0.952	0.888
1	#7	0.056	0.064

TABLE IV-20

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: CONCENTRATIONS OF PHT AND ($^{13}C^{15}N_2$)-PHT (μg/ml).

	Subject 1			Subject 2			Subject 3		
N		PHT	($^{13}C^{15}N_2$)-PHT		PHT	($^{13}C^{15}N_2$)-PHT		PHT	($^{13}C^{15}N_2$)-PHT
1	#1	4.70	4.54	#1	5.13	4.13	#1	3.30	3.20
1	#2	3.53	3.49	#2	4.63	3.71	#2	2.93	2.89
1	#3	2.77	2.76	#3	3.85	3.06	#3	2.94	2.86
1	#4	2.06	1.98	#4	2.92	2.33	#4	2.82	2.75
1	#5	1.91	1.82	#5	2.30	1.82	#5	3.60	3.61
1	#6	1.69	1.62	#6	2.13	1.66	#6	3.44	3.38
1	#7	1.76	1.70	#7	2.11	1.66	#7	3.28	3.25
1	#8	1.65	1.61	#8	1.91	1.50	#8	3.07	3.03
1	#9	1.55	1.50	#9	1.79	1.43	#9	2.80	2.79
1	#10	1.52	1.48	#10	1.72	1.35	#10	2.62	2.63
1	#11	1.39	1.34	#11	1.60	1.26	#11	2.43	2.42
1	#12	1.27	1.25	#12	1.45	1.16	#12	2.35	2.31
1	#13	1.24	1.19	#13	1.50	1.19	#13	2.28	2.25
1	#14	1.14	1.09	#14	1.34	1.06	#14	1.58	1.57
1	#15	0.779	0.742	#15	1.00	0.791	#15	0.804	0.778
1	#16	0.411	0.400	#16	0.914	0.727	#16	0.616	0.601
1	#17	0.310	0.301	#17	0.482	0.381	#17	0.502	0.493
1	#18	0.145	0.140	#18	0.336	0.265	#18	0.164	0.164
1	#19	<0.1	<0.1	#19	0.237	0.193	#19	<0.1	<0.1
1				#20	0.109	<0.1			
1				#21	<0.1	<0.1			

TABLE IV-21

ANALYSIS OF PLASMA SAMPLES FROM HUMAN VOLUNTEERS: CONCENTRATIONS OF p-HPPH AND ($^{13}C^{15}N_2$)-p-HPPH.

	Subject 1			Subject 2			Subject 3		
N		p-HPPH	($^{13}C^{15}N_2$)-p-HPPH		p-HPPH	($^{13}C^{15}N_2$)-p-HPPH		p-HPPH	($^{13}C^{15}N_2$)-p-HPPH
1	#1	0.119	0.119	#1	<0.1	<0.1	#1	<0.1	<0.1
1	#2	0.157	0.159	#2	<0.1	<0.1	#2	<0.1	<0.1
1	#3	0.240	0.232	#3	0.125	0.101	#3	0.160	0.157
1	#4	0.334	0.323	#4	0.220	0.175	#4	0.254	0.246
1	#5	0.398	0.384	#5	0.284	0.225	#5	0.392	0.370
1	#6	0.539	0.522	#6	0.323	0.260	#6	0.419	0.399
1	#7	0.638	0.612	#7	0.353	0.273	#7	0.564	0.545
1	#8	0.633	0.618	#8	0.411	0.320	#8	0.729	0.700
1	#9	0.620	0.621	#9	0.475	0.386	#9	0.728	0.699
1	#10	0.686	0.665	#10	0.528	0.421	#10	0.908	0.892
1	#11	0.575	0.560	#11	0.563	0.437	#11	0.963	0.941
1	#12	0.802	0.776	#12	0.631	0.503	#12	0.973	0.990
	#13	0.721	0.707	#13	0.763	0.610	#13	1.18	1.21
1	#14	1.27	1.25	#14	0.832	0.673	#14	1.14	1.14
1	#15	0.725	0.696	#15	0.721	0.580	#15	0.740	0.699
1	#16	0.495	0.482	#16	0.724	0.574	#16	0.598	0.584
1	#17	0.383	0.372	#17	0.472	0.368	#17	0.495	0.496
1	#18	0.160	0.152	#18	0.387	0.303	#18	0.224	0.214
1	#19	0.120	0.117	#19	0.309	0.247	#19	0.140	0.138
1				#20	0.168	0.137			
1				#21	<0.1	<0.1			

TABLE IV-22

ANALYSIS OF URINE SAMPLES FROM HUMAN VOLUNTEERS: CONCENTRATIONS OF p-HPPH and ($^{13}C^{15}N_2$)-p-HPPH.
(A) µg/0.5 ml (B) Total urinary excretion (mg)

N	(A)	p-HPPH	($^{13}C^{15}N_2$)-p-HPPH	(B)	p-HPPH	($^{13}C^{15}N_2$)-p-HPPH
	Subject 1			Subject 1		
1	#1	34.92	34.30	#1	9.78	9.60
1	#2	40.27	39.40	#2	9.10	8.90
1	#3	14.56	14.29	#3	18.49	18.15
1	#4	10.20	10.11	#4	29.27	29.02
1	#5	3.47	3.43	#5	7.70	7.61
1	#6	0.888	0.878	#6	2.67	2.64
	Subject 2			Subject 2		
1	#1	14.09	11.02	#1	7.66	5.99
1	#2	39.08	30.96	#2	11.18	8.85
1	#3	41.47	32.79	#3	11.61	9.18
1	#4	26.07	20.64	#4	22.68	17.96
1	#5	12.85	10.21	#5	35.59	28.28
1	#6	4.05	3.19	#6	7.57	5.97
1	#7	0.570	0.452	#7	1.60	1.27
	Subject 3			Subject 3		
1	#1	13.04	12.55	#1	9.52	9.16
1	#2	13.14	12.65	#2	13.14	12.65
1	#3	29.40	28.82	#3	18.52	18.16
1	#4	24.02	23.60	#4	20.18	19.82
1	#5	5.15	5.05	#5	12.51	12.27
1	#6	8.53	8.38	#6	11.69	11.48
1	#7	0.438	0.438	#7	1.29	1.29

1d. Comments

As discussed in the studies with dogs, it was again not possible to extract the plasma samples from the three human subjects and the corresponding plasma standard samples in one day. In addition, 19-21 samples had to be processed for each subject. Therefore, only a single plasma sample was worked up for each collection (the respective plasma standard solutions were still worked up in duplicate). One analysis was considered adequate in these studies, because, as discussed before, the error introduced during the work-up of the samples is mainly due to pipetting (and if an error is made in pipetting, the ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolites will not be affected so that interpretation of the results will pose no problems).

A difference in slope for the regression lines of the unlabelled and ($^{13}C^{15}N_2$)-labelled analogs was noticed again and this was attributed to the different isotopic purity of the reference materials (see Chapter VI; Ib was found to be 94 % trilabelled, IIb was found to be 93 % trilabelled). Analysis of a mixture of equal amounts by weight of Ia and Ib, resulted indeed in a ratio of molecular ion abundances of d-Ib/d-Ia (m/z 283/280) of 0.94. However, analysis of a mixture of equal amounts by weight of IIa and IIb, did result in a ratio of molecular ion abundances of d-IIb/d-IIa (m/z 313/310) of 0.76. This substantial difference in values (0.93 versus 0.76) led us to assume

that the IIb, purchased as reference material, was not 100 % chemically pure. The weight of IIb in the calibration data was therefore adjusted so that the concentrations used in the LRA calculations would correspond to 100 % chemically pure trilabelled material. The correction factor was determined by preparation of a mixture of equal amounts by weight of IIa and the reference IIb, and measurement of the molecular ion abundance ratios of the, after derivatization, obtained d-IIb and d-IIa (m/z 313/310). For the final calculations of the regression lines for the determination of IIb in plasma or urine (i.e. regression of molecular ion abundance ratios of d-IIb/d-IIc, measured for the urine or plasma standard solutions, on the concentrations of IIb present in the standard solutions), this ratio was multiplied by 0.94, because IIb that is formed by 'in vivo' metabolism of the administered Ib contains only 94% trilabelled material. In order to maintain accuracy, this correction factor was determined for each new set of experiments and/or instrumental conditions.

For the measurement of ($^{13}C^{15}N_2$)-p-HPPH at m/z 313, no corrections were applied for the contribution of the trilabelled contaminant of d-IIc at this value: the magnitude of the overlapping ion abundances was the same for standard samples and unknowns and no extremely low ratios of d-IIb/d-IIc had to be measured. Similarly, the contribution of the natural isotopic abundance ions from

d-IIb to the measured ion abundance ratios at m/z 315 for d-IIc could be neglected because no extremely high ratios of d-IIb/d-IIc were measured. No significant departure of linearity was found over the concentration ranges that were monitored; however, the tendency towards non-linearity at the extremes of the calibration curves is noticable by meticulous examination of the reported ion abundance ratios (Tables IV-13 through IV-16).

For measurement of the molecular ion currents of (d-Ia, d-Ib and d-Ic) in plasma, area measurements were preferred because a large number of samples had to be analyzed for each subject (in addition to the corresponding plasma standard samples). The possibility that more than one day would be needed to analyze all these samples did therefore exist; different instrumental conditions could thus affect the peak shape, and in this case area measurements are most precise (see Chapter III, Section E-4). However, for the measurement of the ion currents of d-IIa, d-IIb and d-IIc in serum, it was taken into consideration that the measured levels were very low, and it was anticipated that this would affect the measurement of the isotope ratios more significantly than changes in peak shape (see again Chapter III, Section E-3), so that for this case it was decided to use height measurements. For the urine samples, a limited number of samples had to be analyzed, and this could easily be done in one day. Height measurements were used, because both high and low

levels of IIa and IIb were measured.

2. Interpretation of the results

The plasma concentrations of Ia and Ib were analyzed by iterative nonlinear least square regression techniques as described by Greenblatt et al. (42). Data points were fitted by a computer to a linear sum of three exponential terms. After correction for the infusion period, coefficients and exponents from the fitted function were used to calculate the following kinetic variables: volume of the central compartment (V1), total volume of distribution using the area method (Vd), initial distribution half-life ($t_{\frac{1}{2}}\ \alpha$), intermediate distribution half-life ($t_{\frac{1}{2}}\ \pi$), elimination half-life ($t_{\frac{1}{2}}\ \beta$), and clearance. The pharmacokinetic parameters for Ia and Ib are summarized in Table IV-23. The parameters are virtually identical for every parameter for every volunteer. There was no trend toward any difference in distribution, elimination or clearance of the two forms of PHT. In addition, the reported values agree with previousiy published pharmacokinetic parameters from PHT (10-12).

Again, the absence of 'in vivo' isotope effects in the metabolism of Ib was also demonstrated by the constancy of the ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled drug and metabolite throughout the collection periods. Table IV-24 lists the ratios of concentrations of unlabelled to

TABLE IV-23

PHARMACOKINETIC PARAMETERS FOR PHT AND ($^{13}C^{15}N_2$)-PHT IN THE STUDIED HUMAN VOLUNTEERS

		$T_{½\alpha}$* minutes	$T_{½\pi}$* hours	$T_{½\beta}$* hours	V_1* liters/kg	V_d* liters/kg	CLEARANCE ml/min/kg
Subject 1							
	PHT	2.5	0.64	12.5	0.13	0.85	0.779
	($^{13}C^{15}N_2$)-PHT	3.0	1.01	12.7	0.15	0.88	0.800
Subject 2							
	PHT	5.4	0.42	12.0	0.27	0.99	0.958
	($^{13}C^{15}N_2$)-PHT	5.2	0.39	12.2	0.25	0.95	0.895
Subject 3							
	PHT	**	**	11.6	**	0.56	0.562
	($^{13}C^{15}N_2$)-PHT	**	**	11.4	**	0.57	0.575

* Abbreviations are explained in text (see Chapter IV, Section C-2).

** Not calculated because data showed best fit with 1 compartment model.

($^{13}C^{15}N_2$)-labelled analogs in plasma and urine. It is obvious from inspection of the listed values that the ratios remain constant throughout the serial collections and are not significantly different from the ratio of Ia/Ib in the administered drug mixture (see also Table IV-26). In analogy with the studies in dogs, the ratios of ion abundances of unlabelled to ($^{13}C^{15}N_2$)-labelled analogs in plasma and urine were also calculated. The results are listed in Table IV-25. Here the ratios do not appear to be very constant throughout the collection periods; the variability is due to the fact that the concentration of Ia, IIa, and their ($^{13}C^{15}N_2$)-labelled analogs varies greatly, being low at the beginning and towards the end of the collection period. (When small amounts of Ia, IIa or the ($^{13}C^{15}N_2$)-labelled analogs are measured, background interference becomes significant and under these conditions, accurate measurements are only obtained by the use of calibration data for which the same amount of interferences have been measured.)

A summary of the data presented in Tables IV-24 and IV-25 is presented in Table IV-26, parts A and B respectively. For each subject, the mean ratio of Ia/Ib and IIa/IIb in plasma, and IIa/IIb in urine was calculated by averaging the ratios obtained for the serial collections, as listed in Table IV-24; similarly, the mean ratio of the molecular ion abundances of d-Ia/d-Ib and d-IIa/d-IIb were obtained by averaging the ratios obtained

TABLE IV-24

ANALYSIS OF PLASMA AND URINE SAMPLES FROM HUMAN VOLUNTEERS: RATIOS OF CONCENTRATIONS OF UNLABELLED PHT AND p-HPPH TO THEIR RESPECTIVE ($^{13}C^{15}N_2$)-LABELLED ANALOGS.

N	Subject 1			Subject 2			Subject 3		
		PHT/ ($^{13}C^{15}N_2$)-PHT	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH		PHT/ ($^{13}C^{15}N_2$)-PHT	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH		PHT/ ($^{13}C^{15}N_2$)-PHT	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH
	SERUM								
1	#1	1.04	1.00	#1	1.24	-	#1	1.03	-
1	#2	1.01	0.99	#2	1.25	-	#2	1.01	-
1	#3	1.00	1.03	#3	1.26	1.24	#3	1.03	1.02
1	#4	1.04	1.03	#4	1.25	1.26	#4	1.03	1.03
1	#5	1.05	1.04	#5	1.26	1.26	#5	1.00	1.06
1	#6	1.04	1.03	#6	1.28	1.24	#6	1.02	1.05
1	#7	1.04	1.04	#7	1.27	1.29	#7	1.01	1.03
1	#8	1.02	1.02	#8	1.27	1.28	#8	1.01	1.04
1	#9	1.03	1.00	#9	1.25	1.23	#9	1.00	1.04
1	#10	1.03	1.03	#10	1.27	1.25	#10	1.00	1.02
1	#11	1.04	1.03	#11	1.27	1.29	#11	1.00	1.02
1	#12	1.02	1.03	#12	1.25	1.25	#12	1.02	0.98
1	#13	1.04	1.02	#13	1.26	1.25	#13	1.01	0.98
1	#14	1.05	1.02	#14	1.26	1.24	#14	1.01	1.00
1	#15	1.05	1.04	#15	1.26	1.24	#15	1.03	1.06
1	#16	1.03	1.03	#16	1.26	1.26	#16	1.02	1.02
1	#17	1.03	1.03	#17	1.27	1.28	#17	1.02	1.00
1	#18	1.04	1.05	#18	1.27	1.28	#18	1.00	1.05
1	#19	-	1.03	#19	1.23	1.25	#19	-	1.01
1				#20	-	1.23			
1				#21	-	-			
	URINE								
1	#1		1.02	#1		1.28	#1		1.04
1	#2		1.02	#2		1.26	#2		1.04
1	#3		1.02	#3		1.26	#3		1.02
1	#4		1.01	#4		1.26	#4		1.02
1	#5		1.01	#5		1.26	#5		1.02
1	#6		1.01	#6		1.27	#6		1.02
1				#7		1.26	#7		1.00

TABLE IV-25

ANALYSIS OF PLASMA AND URINE SAMPLES FROM HUMAN VOLUNTEERS: RATIOS OF MOLECULAR ION ABUNDANCES OF THE PERMETHYLATED DERIVATIVES OF PHT AND p-HPPH TO THEIR RESPECTIVE ($^{13}C^{15}N_2$)-LABELLED ANALOG.

N	Subject 1			Subject 2			Subject 3		
		m/z 280/283	m/z 310/313		m/z 280/283	m/z 310/313		m/z 280/283	m/z 310/313
	SERUM								
1	#1	1.15	0.957	#1	1.35	-	#1	1.12	-
1	#2	1.11	0.962	#2	1.35	-	#2	1.10	-
1	#3	1.10	1.02	#3	1.34	1.15	#3	1.11	0.934
1	#4	1.13	1.04	#4	1.34	1.22	#4	1.11	0.986
1	#5	1.13	1.05	#5	1.35	1.24	#5	1.09	1.05
1	#6	1.12	1.07	#6	1.36	1.24	#6	1.11	1.05
1	#7	1.10	1.09	#7	1.34	1.29	#7	1.10	1.07
1	#8	1.09	1.07	#8	1.34	1.29	#8	1.10	1.10
1	#9	1.09	1.04	#9	1.32	1.26	#9	1.09	1.10
1	#10	1.09	1.08	#10	1.34	1.29	#10	1.08	1.10
1	#11	1.09	1.07	#11	1.34	1.33	#11	1.08	1.11
1	#12	1.07	1.09	#12	1.32	1.31	#12	1.09	1.07
1	#13	1.09	1.07	#13	1.33	1.32	#13	1.09	1.08
1	#14	1.10	1.10	#14	1.33	1.31	#14	1.07	1.10
1	#15	1.09	1.09	#15	1.31	1.31	#15	1.07	1.11
1	#16	1.05	1.06	#16	1.30	1.32	#16	1.05	1.06
1	#17	1.05	1.05	#17	1.29	1.31	#17	1.03	1.02
1	#18	1.03	1.02	#18	1.28	1.28	#18	0.974	0.988
1	#19	-	0.978	#19	1.23	1.24	#19	-	0.920
1				#20	-	1.16			
1				#21	-	-			
	URINE								
1	#1		1.10	#1		1.34	#1		1.10
1	#2		1.10	#2		1.34	#2		1.10
1	#3		1.08	#3		1.35	#3		1.09
1	#4		1.07	#4		1.34	#4		1.09
1	#5		1.05	#5		1.32	#5		1.07
1	#6		0.954	#6		1.30	#6		1.07
1				#7		1.11	#7		0.875

TABLE IV-26

ANALYSIS OF PLASMA AND URINE SAMPLES FROM HUMAN VOLUNTEERS: SUMMARY OF THE DATA PRESENTED IN TABLES IV-24 AND IV-25 (A) RATIOS OF CONCENTRATIONS (B) RATIOS OF ION ABUNDANCES.

(A)

	SERUM				URINE		OVERALL RATIO			RATIO	
	N	PHT/ ($^{13}C^{15}N_2$)-PHT Mean ± SD	N	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH Mean ± SD	N	p-HPPH/ ($^{13}C^{15}N_2$)-p-HPPH Mean ± SD	N	unlabelled/ ($^{13}C^{15}N_2$)-p-HPPH Mean ± SD	CV(%)	N	m/z 280/283 in infused drug mixture x 0.94
Subject 1	18	1.03 ± 0.01	19	1.03 ± 0.02	6	1.02 ± 0.01	3	1.03 ± 0.01	0.56	1	1.02
Subject 2	19	1.26 ± 0.01	18	1.26 ± 0.02	7	1.26 ± 0.01	3	1.26 ± 0	0	1	1.26
Subject 3	18	1.01 ± 0.01	17	1.02 ± 0.02	7	1.02 ± 0.01	3	1.02 ± 0.01	0.57	1	1.02

(B)

	SERUM				URINE		OVERALL RATIO			RATIO	
	N	m/z 280/283 Mean ± SD	N	m/z 310/313 Mean ± SD	N	m/z 310/313 Mean ± SD	N	m/z $M^+/(M+3)^+$ Mean ± SD	CV(%)	N	m/z 280/283 in infused drug mixture
Subject 1	18	1.09 ± 0.03	19	1.05 ± 0.04	6	1.06 ± 0.05	3	1.07 ± 0.02	1.95	1	1.08
Subject 2	19	1.32 ± 0.03	18	1.27 ± 0.05	7	1.30 ± 0.09	3	1.30 ± 0.03	1.94	1	1.34
Subject 3	18	1.08 ± 0.03	17	1.05 ± 0.06	7	1.06 ± 0.08	3	1.06 ± 0.02	1.44	1	1.08

for the serial collections, as listed in Table IV-25. The values for the ion abundance ratios (part B of Table IV-26) are not considered to be be accurate (as discussed previously), because they include the incorrectly measured ratios at low concentrations. However, no inaccuracies were introduced by summarizing the individual ratios of the concentrations (part A of Table IV-26) so that these data can be interpreted with confidence. The ratios of the concentrations of Ia/Ib and IIa/IIb in plasma, and IIa/IIb in urine (part A of Table IV-26) are virtually identical. In addition, they are not significantly different from the ratio of Ia/Ib in the administered drug mixture. These data therefore support the previously described pharmacokinetic data (see Table IV-23), which demonstrated that 'in vivo' isotope effects in the p-hydroxylation of Ib are not operative (i.e. K_{Ia}/K_{Ib} is 1.00 with an estimated experimental error of $\pm$ 0.01).

We know of no other report on the metabolic effects of stable isotope labelling in the hydantoin ring of PHT, but our studies indicate clearly that isotope effects in the metabolism of compounds of such kind can conclusively be ruled out.

Chapter V. CONCLUSION

It was demonstrated that it is possible to determine simultaneously and accurately in the same sample:

1. in plasma samples from human subjects, at both therapeutic and sub-therapeutic levels: phenytoin (PHT, Ia), its major metabolite in man (p-HPPH, IIa), and their ($^{13}C^{15}N_2$)-labeled analogs (Ib and IIb),
2. in serum samples from dogs: phenytoin (PHT, Ia), its major metabolites in dogs (m-HPPH, IIIa; p-HPPH, IIa), and their ($^{13}C^{15}N_2$)-labelled analogs (Ib, IIIb and IIb),
3. in the corresponding urine samples: the previously mentioned metabolites (IIa and IIb in man; IIIa, IIIb, IIa and IIb in dogs).

In addition, the absence of 'in vivo' isotope effects in the metabolism of Ib was shown in dogs and human volunteers: the ratio of unlabelled to ($^{13}C^{15}N_2$)-labelled drug as well as unlabelled to ($^{13}C^{15}N2$)-labelled metabolites remained constant throughout the collection periods, and the pharmacokinetic parameters for Ia and Ib were not significantly different.

The further application of the method will be the pulse dosing studies, and here low levels of ($^{13}C^{15}N_2$)-labelled analogs and high levels of unlabelled analogs will be present in the samples (see Chapter I, Figure I-2). It is not anticipated that major modifications of the analytical method will be required for

these studies. However, based on the acquired experience, a few comments should be made:

1. The samples from the patients must be processed in duplicate so that it is possible to trace the pipetting errors, and so that accurate measurements are obtained for the samples containing low or high amounts of drug and/or metabolites, i.e. for samples where ratios of unknown to internal standard larger than 10 are to be measured (large ion intensity ratios are measured with worse precision than ratios close to unity, see Chapter II, Section D-1, Chapter III, Sections E-2 and E-3, and Figure 6 in Reference 34; so that it is recommended to average the results of replicate measurements).

2. For each new series of plasma, serum or urine samples from patients, calibration data must be included. Plasma, serum and urine standard solutions containing known amounts of Ia, Ib, IIa and IIb for plasma or serum and IIa and IIb for urine can be prepared and kept frozen until use (i.e. the processing of the samples from patients). The calibration data should cover the expected concentration ranges of drug and metabolites; these ranges will be different for the unlabelled and ($^{13}C^{15}N_2$)-labelled analogs.

3. For each new batch of stable isotope labelled reference compounds, the identity and isotopic and chemical purity must be verified. Ideally, the same batch of Ib used to prepare the plasma or urine standard solutions should be

administered to the patients. For IIb, extra caution will have to be exercised because the isotopic purity of IIb isolated from patients will probably be different from that of the reference IIb, because IIb that is formed by 'in vivo' metabolism of Ib will have the same isotopic purity as this Ib (and the isotopic purity of Ib and IIb reference material will most likely be different). If necessary, any differences can be corrected for, either by adjusting the amount of reference material used, or by calculating the regression lines using adjusted values for the concentration of the standards.

4. If the calibration data cover a wide range of concentrations, logarithmic linear regression analysis of the data is strongly recommended. In addition, for the measurement of the molecular ion intensities of the permethylated derivatives of the p-HPPH analogs (d-IIb and d-IIc), the ion intensities at m/z 313 (for d-IIb) and m/z 315 (for d-IIc) must be corrected for overlapping isotopic impurities.

5. Interferences must always be looked for, because a variety of other drugs are usually administered simultaneously with phenytoin to patients suffering from epilepsy.

The method can also be extended to the analysis of phenobarbital and its major metabolites in man, (1-(β-D-glucopyranosyl)phenobarbital and 4-hydroxypheno barbital) (43). As in the case of phenytoin, 4-hydroxy

phenobarbital is excreted mainly as a glucuronide so that acid hydrolysis will have to be included in the sample processing. During acid hydrolysis, the N-glucopyranoside metabolite will release phenobarbital; therefore, the measurement of phenobarbital and 4-hydroxyphenobarbital in separately processed acid hydrolyzed samples will allow determination of the amount of the respective metabolites.

Initial work has shown that no major modifications of the analytical technique are necessary to measure phenobarbital , 4-hydroxyphenobarbital and their ($^{13}C^{15}N2$)-labelled analogs in serum or urine standard solutions when using ($^{13}CD_4$)-phenobarbital and ($^{13}C^{15}N_2D_2$)-hydroxy phenobarbital as internal standards. In this way, the stable isotope methodology will allow study of the interactive pharmacokinetics of phenytoin and pheno barbital.

Chapter VI. EXPERIMENTAL

A. REFERENCE COMPOUNDS, STANDARD SAMPLES AND REAGENTS

Compounds Ia and IIa were purchased from Aldrich. Compound IIIa was a gift from Parke Davis. Compound Id was previously synthesized in this laboratory by Dr. B. Andresen. Reference compounds Ib, IIb, Ic and IIc were purchased from KOR Istopes, Cambridge, MA. The heavy isotope contents (atom %) were stated by the manufacturer as follows: Ib, 2-^{13}C 90%, 1,3-$^{15}N_2$ 99%; Ic, ring-D_{10} 99%; IIb, 2-^{13}C 90%, 1,3-$^{15}N_2$ 99%; IIc, 2-^{13}C 90%, 1,3-$^{15}N_2$ 99%, D_2 95%. The actual level of incorporation of the stable isotope in the molecule was: for Ib, 94% trilabelled and 6% dilabelled; Ic, 96% decalabelled and 4% nonalabelled; Id, 97% pentalabelled, 3% tetralabelled; IIb, 93% trilabelled, 6% dilabelled and 1% unlabelled, IIc, 81% pentalabelled, 16% tetralabelled and 3% trilabelled. Ib was formulated for intravenous injection by Warner-Lambert/Parke Davis in a manner similar to their marketed Dilantin^R ("phenytoin sodium injection", containing 50 mg/ml of the sodium salt of Ia).

Mass spectra of the permethylated derivatives of the reference compounds are presented in Figures A-1 and A-2.

Ethanolic stock solutions containing 1.0 mg/ml of these reference compounds and an additional methanolic stock solution of 10.0 mg/ml of IIa were prepared. Serum and urine standard solutions containing known amounts of

Ia, IIa, and Ib for serum and IIa and IIb for urine were prepared by adding aliquots of the respective alcoholic stock solutions to drug free serum or urine ("spiking"). This was always done at room temperature and one hour of equilibration time was allowed before further sample manipulations. All alcoholic stock solutions were kept in the freezer at -4°C in teflon lined screw capped centrifuge tubes.

Methylisobutylketone (MIBK) (Burdick and Jackson), toluene (Burdick and Jackson), methylene chloride (CH_2Cl_2) (Mallinckrodt) and methanol (Mallinckrodt) were nanograde reagents, distilled in glass. Ethanol (200 proof) was bottled by IMC, Chemical Group. Methyl iodide (CH_3I) (Aldrich) was anaytical grade 99%, stabilized with copper, and was used without further purification. Tetrabutyl ammonium hydrogen sulfate ($TBA^+HSO_4^-$) (Aldrich) was prepared as a 1M solution in 0.2 N NaOH and extracted once with methylene chloride to remove impurities. The Tris-HCl buffer was prepared as a 3M solution with a pH adjusted to 7.4 with HCl. All reagents were tested for purity by gas chromatography either directly, or as a CH_2Cl_2 extract. All glassware was acid washed and rinsed with methanol and distilled water.

B. PROCESSING OF THE SAMPLES

The processing of the samples (i.e. the chemical work-up of the serum and urine samples to obtain extracts

for GC-MS analysis) was based on the extractive methylation technique described by Hoppel et al. (20) for the generation of permethylated derivatives. The procedure is described below for the analysis of 1.0 ml of serum, plasma or urine. For 0.5 ml samples, the only adjustment to be made was the use of only 0.5 ml of 10N HCl in the hydrolysis step.

To 1.0 ml sample in a 15 ml screw cap centrifuge tube, the appropriate amount of Ic (or Id) was added. After mixing and equilibration for 20 min, 1.0 ml 10 N HCl was added, the mixture was vortexed and placed in an oven at 96± 2°C for 1 h. After cooling in an ice bath, the HCl was neutralized with 10N NaOH, 1.0 ml Tris buffer (pH 7.4) was added, and the mixture was vortexed. The required amount of IIc was added, the solutions were mixed and after equilibration for 20 min, 5 ml MIBK was added. The tubes were mechanically shaken for 25 min at room temperature and centrifuged. The MIBK layer was transferred into a tube containing 0.25 ml of 1N NaOH, the solutions were mixed for 15 min and the tubes were centrifuged. The MIBK layer was carefully removed with a Pasteur pipette and discarded. To the aqueous layer remaining in the tube, 50 µl of a solution of TBA-HSO_4 (1M in 0.2N NaOH was added. The mixture was vortexed and 2.0 ml of a solution of CH_3I in CH_2Cl_2 (1:10, v:v) was added. The tube was shaken for 30 min and centrifuged. The organic layer was transferred to a 3 ml conical tipped test tube. The tube was covered

lightly with aluminum foil, and the solvent was allowed to evaporate at room temperature. To the dry extract, 25-50 µl of toluene was added; the tube was vortexed for 30 s, sonicated for 10 min and centrifuged. For the GC-MS analysis, 2-4 µl of the clear supernatant solution were injected into the gas chromatograph.

C. GAS CHROMATOGRAPHIC MASS SPECTROMETRIC MEASUREMENT TECHNIQUE

Gas chromatography was carried out on a Perkin Elmer 990 gas chromatograph with flame ionization detector. Initially, for method development and evaluation, a 1m x 2 mm i.d. glass column packed with 3% OV-17 on 80/100 mesh Supelcoport was used. For analysis of serum and urine samples from dogs and human subjects -in the later applications of the method-, a 2m x 2mm i.d. column was substituted. An OV-17 column was used for the dog samples and an OV-101 column for the human samples. The injector temperature was maintained at 250 °C and the gas chromatograph was temperature programmed (3 ft OV-17 column: 180-310 °C at 12 °C/min; 6 ft OV-17 column: 210-320 °C at 8 °C/min; 6 ft OV-101 column: 180-310 °C at 12 °C/min). Helium was used as carrier gas.

The effluent from the gas chromatograph was passed through a valve such that 70-80 % of the effluent was diverted into an Hitachi RMU-6L mass spectrometer, while the remaining portion was monitored by the FID of the gas

chromatograph. A jet separator was used as interface between the gas chromatograph and mass spectrometer. The entire interface was usually kept at 250 °C. The mass spectrometer was directly coupled to an IBM 1800 computer system. A detailed description of the mass spectrometer and its computer aided data acquisition system can be found elsewhere (44,45).

Full mass spectra were recorded by scanning of the magnet at 70 eV of ionization voltage and an ion source temperature of 230 °C. A mass spectrum was taken repetitively every 4 s. After processing by the IBM computer, the data (mass spectra and mass chromatograms) were filmed on 16 mm microfilm and also stored on magnetic tape (46).

Partial mass spectra of selected m/z values were recorded by linear scanning of the accelerating voltage. The conventional accelerating voltage supply of the previously described mass spectrometer was therefore replaced with a highly stable, programmable power supply and a digital scan controller which controls the accelerating voltage over the range selected at whatever scan speed is appropriate for the experiment. (Two different mass ranges can be selected and scanned alternately (if compounds elute at about the same time) or consecutively (if they are well separated gas chromatographically).) With the preliminary instrument modifications, a linear mass range of m/z 278-293 was

scanned in 4 s to monitor the molecular ion currents of the permethylated derivatives of the phenytoin analogs (d-Ia, d-Ib, d-Ic or d-Id) when they eluted from the gas chromatograph, and a linear mass range of m/z 309-317 was scanned in a 2 s during the elution of the HPPH analogs in order the measure the molecular ion currents of IIa, IIb, IIc, IIIa and IIIb. For more sensitive measurements, the installation of a more sophisticated power supply and scan electronics allowed faster scanning of the accelerating voltage so that 1 s scan cycles could be used. The data were acquired on magnetic tape and transferred to a PDP 11/45 computer system with a CRT terminal. This system is much faster and has a larger disk storage capability (presently 300 Mbytes) than the IBM 1800; it allows interactive dialogue and display capabilities. For each set of our data, a total ionization plot (TIP) was displayed on a CRT screen, so that the boundaries of the elution profile of the compounds of interest could be manually selected by the use of cursors. For every scan within this defined window, mass spectral peak profiles are displayed on the CRT screen so that ion current profiles of preselected ions can be defined. This was accomplished by the use of horizontal and vertical cursors and both height and areas were automatically computed. For every scan the ratio of ion intensities of unknown to internal standard was computed and these ratios were then averaged over the previously defined gas chromatographic elution profile of

either the PHT or HPPH analogs. The ratio for every individual scan as well as the averaged ratio were printed out. If desired, data could also be recorded on a conventional oscillograph recorder and peak heights of ion abundances measured manually.

A Varian 731 mass spectrometer was used to determine the actual level of incorporation of the stable isotope in the labelled reference materials. Repetitive scans of the molecular ion region were recorded with an oscillograph and ion abundances at appropriate m/z values were measured manually from this record.

D. PROTOCOL FOR ADMINISTRATION OF PHT AND ($^{13}C^{15}N_2$)-PHT TO DOGS AND HUMAN VOLUNTEERS AND FOR COLLECTION OF SERUM (PLASMA) AND URINE SAMPLES.

1. Studies with dogs

Three mongrel male dogs (10.9-12.7 kg) were anesthesized with fentanyl and droperidol (Inovar^R) and infused intravenously with a mixture of Ia and Ib (both as sodium salts) at a rate of 25 mg total phenytoin sodium/min (15 mg/kg/body weight of each of the sodium salts of Ia and Ib were given). Blood specimens (5-10 ml) were drawn before the infusion started ("blank") and 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 h after the end of the infusion. After clotting the samples were centrifuged and the serum was stored in a -10 °C freezer. Total urine was collected hourly for 5 hours after infusion and the total urine

volume for each collection recorded (Dog 1: 0-1 h, 40.0 ml; 1-2 h, 6.3 ml; 2-3 h, 9.9 ml; 3-4 h, 6.9ml; 4-5 h, 27.8 ml; Dog 2: 0-1 h, 12.2 ml; 1-2 h, 18.4 ml; 2-3 h, 16.3 ml; 3-4 h, 13.6 ml; 4-5 h, 18 ml; Dog 3: 0-1 h, 40.0 ml; 1-2 h, 23 ml; 2-3 h, 16.4 ml; 3-4 h, 6.1 ml; 4-5 h, 42.8 ml). A urine sample (10 ml) was also collected before the infusion ("blank"). All samples were numbered in the order that they were collected (urine: blank, #1-#5; serum: blank, #1-#6) and kept in the freezer at -10°C.

2. Studies on human volunteers

Three healthy adult males taking no medications and with no history of prior hydantoin use or cardiac, renal, or hepatic disease were selected. A mixture of Ia and Ib (both as sodium salts) was infused at a rate of 25 mg total phenytoin sodium/min. To Subjects 1 and 3, 150 mg of each of the sodium salts of Ia and Ib were administered; to Subject 2, 200 mg of the sodium salt of Ia and 150 mg of the sodium salt of Ib were given. After infusion, the syringe was rinsed with ethanol and the exact ratio of Ia to Ib in this rinse was determined by GC-MS. Blood specimens (5-10 ml) were drawn before the infusion started ("blank") and at selected times after the end of the infusion (Table VI-1). Plasma was decanted and immediately put in a -10 °C freezer. Consecutive complete urine specimens were obtained at selected times after the end of the infusion and the total urine volume was recorded (Table

VI-2). A urine sample (10 ml) was also collected before the infusion ("blank"). All samples were numbered in the order that they were collected (see Tables VI-1 and VI-2) and kept in the freezer at -10°C.

TABLE VI-1

TIME POINTS FOR COLLECTION OF BLOOD SAMPLES FROM HUMAN VOLUNTEERS

Subject 1		Subject 2		Subject 3	
Assigned #	Time after infusion	Assigned #	Time after infusion	Assigned #	Time after infusion
	(min)		(min)		(min)
1	0	1	0	1	2
2	2	2	2	2	5
3	5	3	5	3	15
4	15	4	15	4	30
5	30	5	30	5	45
6	45	6	45		(h)
	(h)		(h)	6	1
7	1	7	1	7	1.5
8	1.5	8	1.5	8	2.5
9	2	9	2	9	3
10	2.5	10	2.5	10	4
11	3	11	3	11	5
12	4	12	4	12	6
13	6	13	6	13	8
14	8	14	8	14	12
15	12	15	12	15	24
16	24	16	16	16	30
17	30	17	24	17	36
18	48	18	30	18	48
19	60	19	36	19	60
		20	48		
		21	60		

TABLE VI-2

COLLECTION PERIODS AND VOLUMES OF URINE SAMPLES FROM HUMAN VOLUNTEERS.

Subject 1			Subject 2			Subject 3		
Assigned #	Collection Period (h after infusion)	Vol. (ml)	Assigned #	Collection Period (h after infusion)	Vol. (ml)	Assigned #	Collection Period (h after infusion)	Vol. (ml)
1	4 - 8	113	1	0 - 4	272	1	4 - 8	365
2	8 - 12	140	2	4 - 8	143	2	8 - 14	500
3	12 - 24	635	3	8 - 12	140	3	14 - 24	315
4	24 - 48	1435	4	12 - 24	435	4	24 - 33	420
5	48 - 72	1110	5	24 - 48	1385	5	33 - 48	1215
6	72 - 96	1506	6	48 - 72	935	6	48 - 72	685
			7	72 - 96	1400	7	72 - 96	1470

APPENDIX. DISCUSSION OF THE MASS SPECTRAL FRAGMENTATION OF THE PERMETHYLATED DERIVATIVES OF THE ANALOGS.

The mass spectra the permethylated derivatives of phenytoin (d-Ia) and its stable isotope labelled analogs (d-Ib, d-Ic and d-Id) are shown in Figure A-1, a-d. The mass spectra of the permethylated derivatives of the hydroxylated metabolites (d-IIa and d-IIIa) and the stable isotope labelled analogs of d-IIa (d-IIb and d-IIc) are shown in Figure A-2, a-d. The major fragment ions have been labelled by the letters A-G (for the PHT analogs) and A-I (for the HPPH analogs); their assignments are tabulated in Table A-1. The mass spectrum of d-Ia served as a basis to designate the shifted peaks in the mass spectra of the analogs: the same capital letter was used to indicate a fragment ion in the mass spectrum of d-Ia, as for the corresponding shifted ion (or cluster of ions) in the mass spectra of the permethylated analogs. The structural assignments in Table A-1 were made after study of the fragmentation mechanism, as discussed in the following.

The mass spectrum of d-Id was also shown by Baty et. al (21) and the mass spectrum of d-Ia is included in most mass spectral data collections (e.g 47, 48). These published mass spectra agree well with these obtained by us.

Figure A-1. Mass spectra of the permethylated derivatives of PHT and the stable isotope labelled analogs: (a) mass spectrum of d-Ia (b) mass spectrum of d-Ib (c) mass spectrum of d-Ic (d) mass spectrum of d-Id.

Figure A-2. Mass spectra of the permethylated derivatives of the hydroxylated metabolites of PHT and the stable isotope labelled analogs: (a) mass spectrum of d-IIa (b) mass spectrum of d-IIIa (c) mass spectrum of d-IIb (d) mass spectrum of d-IIc.

Figure A-1

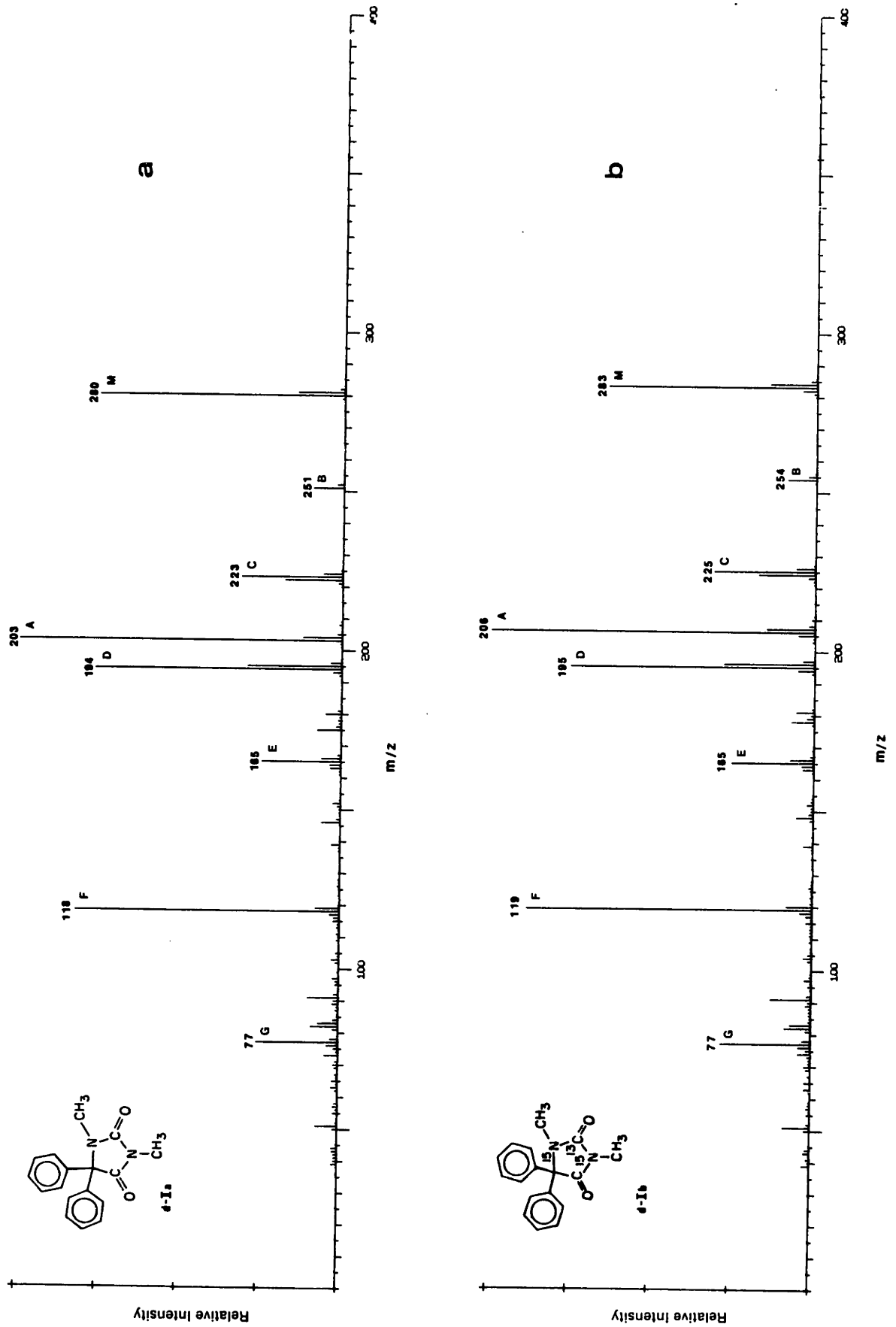

a
Relative Intensity
m/z
280 M
251 B
223 C
203 A
194 D
185 E
118 F
77 G
4-Ia
b
Relative Intensity
m/z
283 M
254 B
225 C
206 A
195 D
185 E
118 F
77 G
4-Ib

Figure A-1

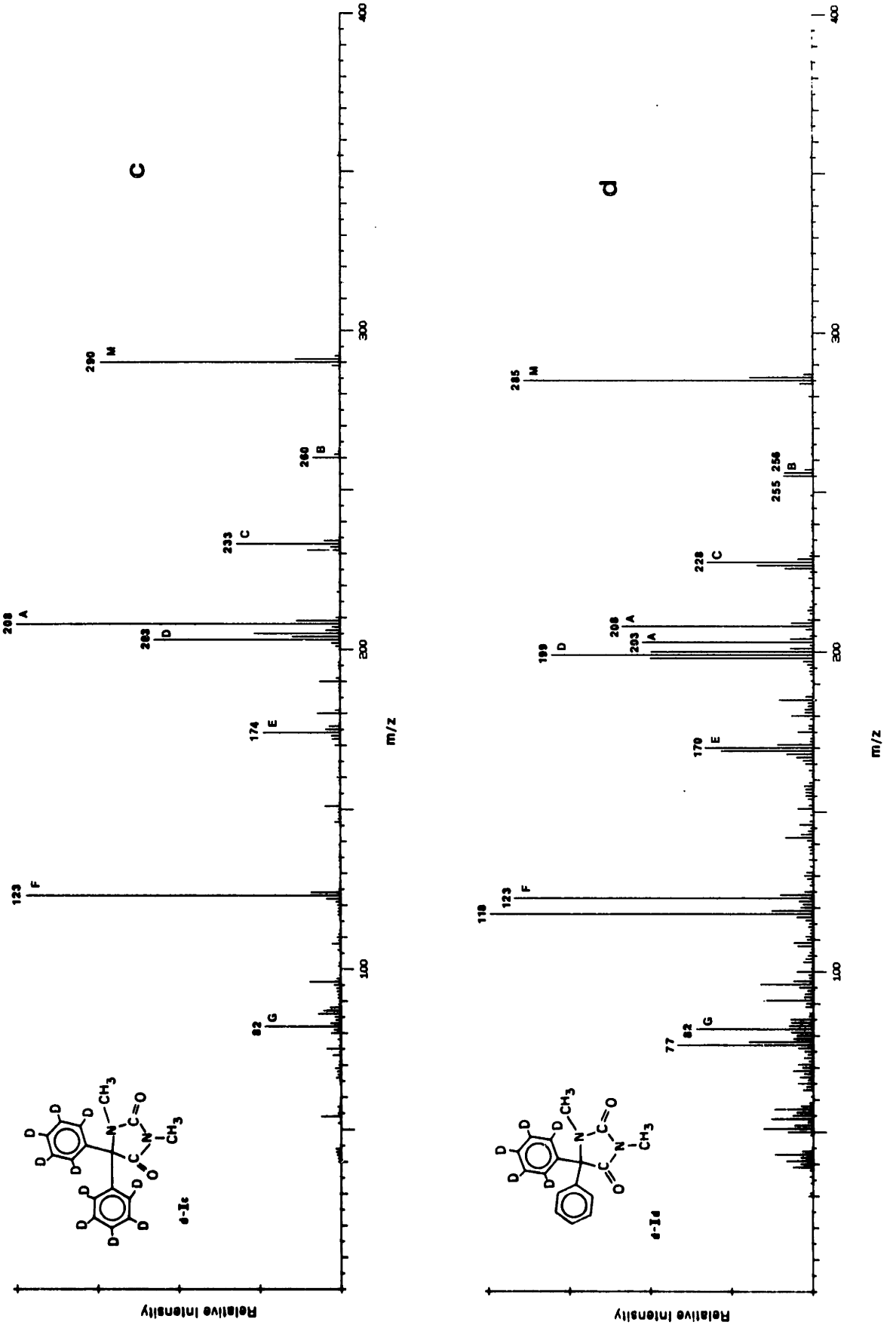

c
d
Relative Intensity
m/z
100
200
300
400
290 M
260 B
233 C
208 A
203 D
174 E
123 F
82 G
285 M
255 256 B
228 C
208 A
203 A
198 D
170 E
118
123 F
77
82 G

Figure A-2

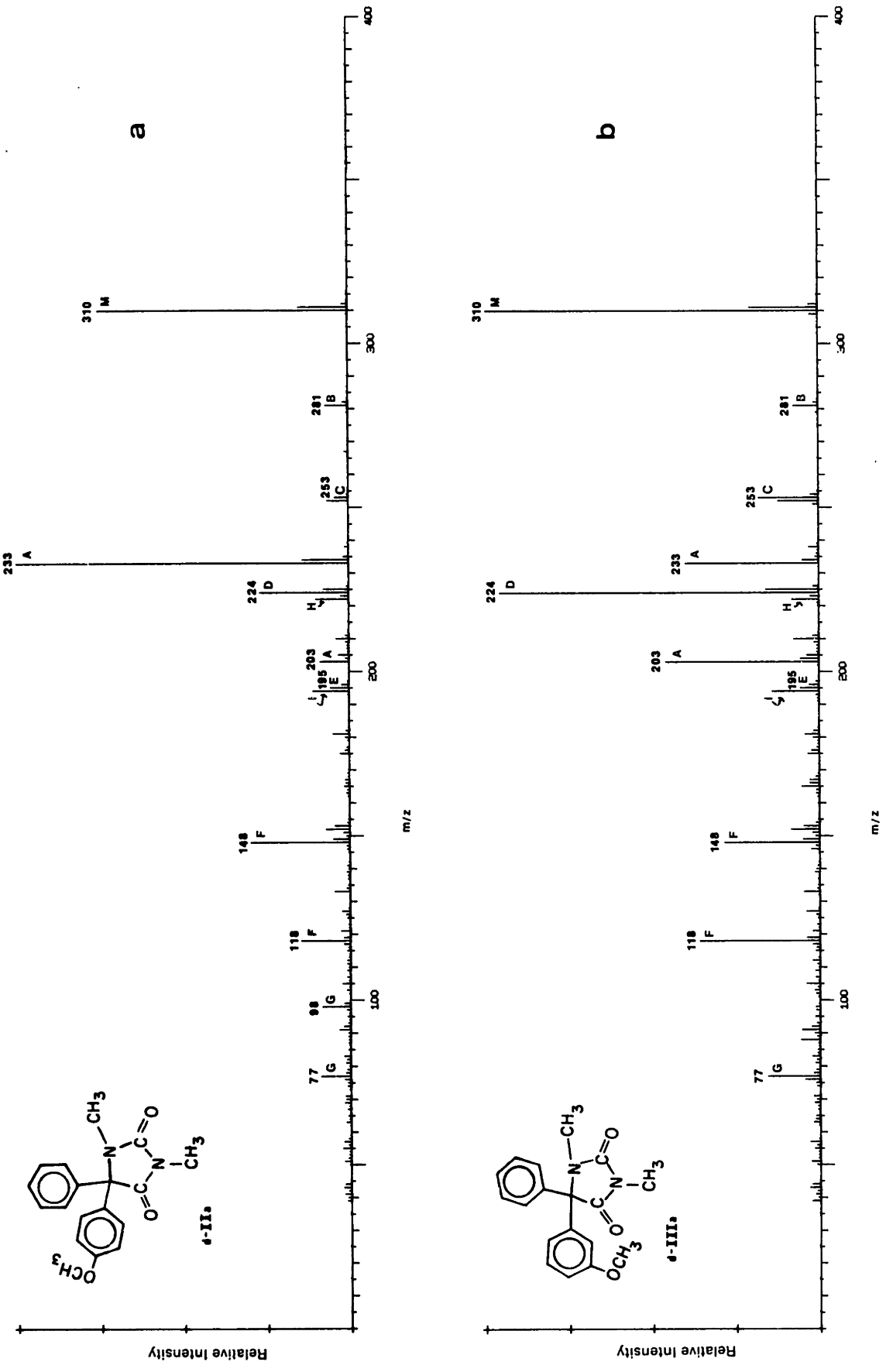

a
b
Relative Intensity
m/z
100
200
300
400
310 M
281 B
253 C
233 A
224 D
203 A
195 E
148 F
118 F
98 G
77 G
4-II
4-III

Figure A-2

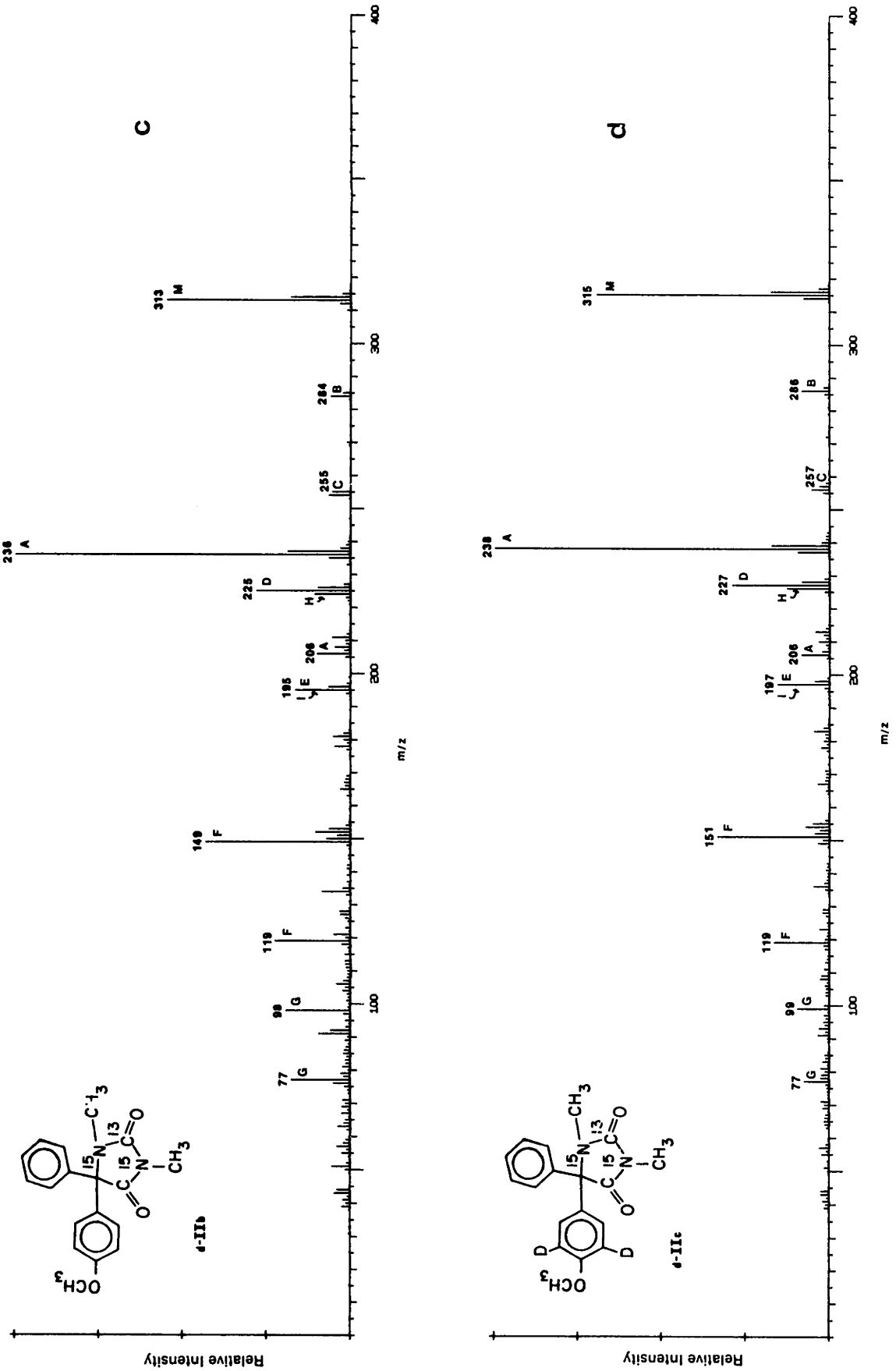


TABLE A-1. ASSIGNMENT OF THE FRAGMENT IONS IN THE MASS SPECTRA OF THE PERMETHYLATED DERIVATIVES OF THE ANALOGS.

Compound	M^+ m/z	CLUSTER B Loss	m/z	CLUSTER C Loss	m/z	CLUSTER D** Loss	m/z	CLUSTER E Loss	m/z	FRAGMENTS F Structure	m/z	FRAGMENTS G Structure	m/z	FRAGMENTS A Loss	m/z	FRAGMENT H Loss	m/z	FRAGMENT I Loss	m/z
d-Ia	280	M^+-CO	252	M^+-CH_3NCO	223	*C^+-CO	195	C^+-CH_3NCO	166	CH_3-$N^+\equiv C$-⬡	118	⬡$]^+$	77	M^+-⬡$]^\cdot$	203				
		M^+-CO,H$^\cdot$	251	M^+-CH_3NCO,H$^\cdot$	222	C^+-CO,H$^\cdot$	194	C^+-CH_3NCO,H$^\cdot$	165										
d-Ib	283	M^+-CO	255	M^+-$CH_3{}^{15}NCO$	225	C^+-^{13}CO	196	C^+-$CH_3{}^{15}N^{13}CO$	166	CH_3-$^{15}N^+\equiv C$-⬡	119	⬡$]^+$	77	M^+-⬡$]^\cdot$	206				
		M^+-CO,H$^\cdot$	254	M^+-$CH_3{}^{15}NCO$,H$^\cdot$	224	C^+-^{13}CO,H$^\cdot$	195	C^+-$CH_3{}^{15}N^{13}CO$,H$^\cdot$	165										
d-Ic	290	M^+-CO	262	M^+-CH_3NCO	233	C^+-CO	205	C^+-CH_3NCO	176	CH_3-$N^+\equiv C$-⬡(D)	123	(D)⬡$]^+$	82	M^+-(D)⬡$]^\cdot$	208				
		M^+-CO,D$^\cdot$	260	M^+-CH_3NCO,D$^\cdot$	231	C^+-CO,D$^\cdot$	203	C^+-CH_3NCO,D$^\cdot$	174										
d-Id	285	M^+-CO	257	M^+-CH_3NCO	228	C^+-CO	200	C^+-CH_3NCO	171	CH_3-$N^+\equiv C$-⬡	123	⬡$]^+$	77	M^+-⬡$]^\cdot$	208				
		M^+-CO,H$^\cdot$	256	M^+-CH_3NCO,H$^\cdot$	227	C^+-CO,H$^\cdot$	199	C^+-CH_3NCO,H$^\cdot$	170	CH_3-$N^+\equiv C$-⬡(D)	118	(D)⬡$]^+$	82	M^+-(D)⬡$]^\cdot$	203				
		M^+-CO,D$^\cdot$	255	M^+-CH_3NCO,D$^\cdot$	226	C^+-CO,D$^\cdot$	198	C^+-CH_3NCO,D.	169										
d-IIa	310	M^+-CO	282	M^+-CH_3NCO	253	C^+-CO	225	C^+-CH_3NCO	196	CH_3-$N^+\equiv C$-⬡	118	⬡$]^+$	77	M^+-⬡$]^\cdot$	233	C^+-$OCH_3^\cdot$	222	H^+-CO	194
		M^+-CO,H$^\cdot$	281	M^+-CH_3NCO,H$^\cdot$	252	C^+-CO,H$^\cdot$	224	C^+-CH_3NCO,H$^\cdot$	195	CH_3-$N^+\equiv C$-⬡-OCH_3	148			M^+-⬡-$OCH_3]^\cdot$	203					
d-IIIa	310	M^+-CO	282	M^+-CH_3NCO	253	C^+-CO	225	C^+-CH_3NCO	196	CH_3-$N^+\equiv C$-⬡	118	⬡$]^+$	77	M^+-⬡$]^\cdot$	233	C^+-$OCH_3^\cdot$	222	H^+-CO	194
		M^+-CO,H$^\cdot$	281	M^+-CH_3NCO,H$^\cdot$	252	C^+-CO,H$^\cdot$	224	C^+-CH_3NCO,H$^\cdot$	195	CH_3-$N^+\equiv C$-⬡ (OCH_3 at ortho)	148			M^+-⬡$]^\cdot$ (OCH_3 at ortho)	203					
d-IIb	313	M^+-CO	285	M^+-$CH_3{}^{15}NCO$	255	C^+-^{13}CO	226	C^+-$CH_3{}^{15}N^{13}CO$	196	CH_3-$^{15}N^+\equiv C$-⬡	119	⬡$]^+$	77	M^+-⬡$]^\cdot$	236	C^+-$OCH_3^\cdot$	224	H^+-^{13}CO	195
		M^+-CO,H$^\cdot$	284	M^+-$CH_3{}^{15}NCO$,H$^\cdot$	254	C^+-^{13}CO,H$^\cdot$	225	C^+-$CH_3{}^{15}N^{13}CO$,H$^\cdot$	195	CH_3-$^{15}N^+\equiv C$-⬡-OCH_3	149			M^+-⬡-$OCH_3]^\cdot$	206				
d-IIc	315	M^+-CO	287	M^+-$CH_3{}^{15}NCO$	257	C^+-^{13}CO	228	C^+-$CH_3{}^{15}N^{13}CO$	198	CH_3-$^{15}N^+\equiv C$-⬡(D)	119	⬡$]^+$	77	M^+-(D)⬡$]^\cdot$	238	C^+-$OCH_3^\cdot$	226	H^+-^{13}CO	197
		M^+-CO,H$^\cdot$	286	M^+-$CH_3{}^{15}NCO$,H$^\cdot$	256	C^+-^{13}CO,H$^\cdot$	227	C^+-$CH_3{}^{15}N^{13}CO$,H$^\cdot$	197	CH_3-$^{15}N^+\equiv C$-⬡(D)-OCH_3	151			M^+-(D)⬡-$OCH_3]^\cdot$	206				
		M^+-CO,D$^\cdot$	285	M^+-$CH_3{}^{15}NCO$,D$^\cdot$	255	C^+-^{13}CO,D$^\cdot$	226	C^+-$CH_3{}^{15}N^{13}CO$,D$^\cdot$	196										

*The ion designated as C^+ is the ion from the cluster C that has not lost H$^\cdot$ or D$^\cdot$

**Shown here are the transitions C^+-CO (or ^{13}CO); an analogous tabulation can be made for B^+-CH_3NCO (or $CH_3{}^{15}N^{13}CO$)

Mass spectra of the unlabelled and underivatized analogs have been documented more extensively in the literature. Atkinson et al. (3) showed the mass spectra of Ia, IIa and IIIa and, in addition, indicated the structure of the major fragment ions; Locock and Coutts (49) studied the fragmentation mechanism of Ia; Andresen (18) studied the fragmentation mechanism of Id; the mass spectral data collections that were consulted by us (47, 48) contain the mass spectra of Ia and IIa, and also mass spectra of a variety of alkylated and silylated derivatives.

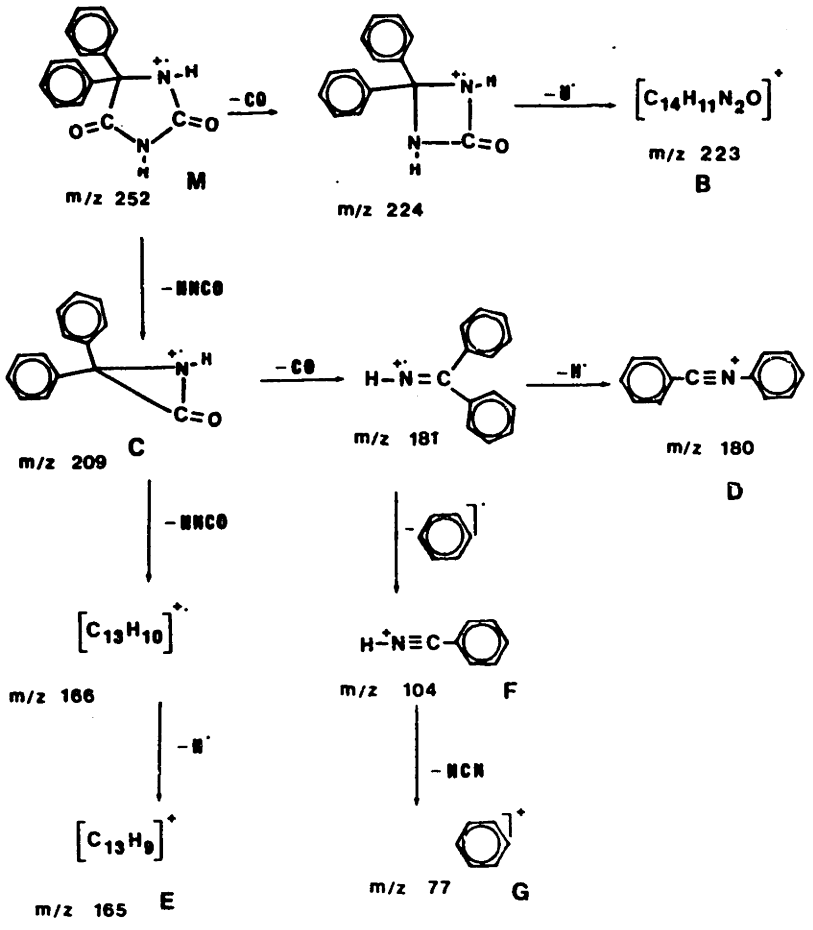
The schemes of Locock and Coutts, and of Andresen, have been reproduced in part A of, Schemes A-1 and A-2, respectively (part B will be discussed later). These schemes deal specifically with the fragmentation processes involving the cleavage of the hydantoin ring. The ion resulting from the loss of a phenyl group from the molecular ion (or for Id, the ion cluster, resulting from the loss of both a phenyl group and a phenyl-d_5 group from the molecular ion) are, of course, self-explanatory and have been omitted from these schemes to retain clarity. (In the mass spectra presented in Figures A-1 and A-2, these ions have been labelled A, and they have also been included in Table A-1).

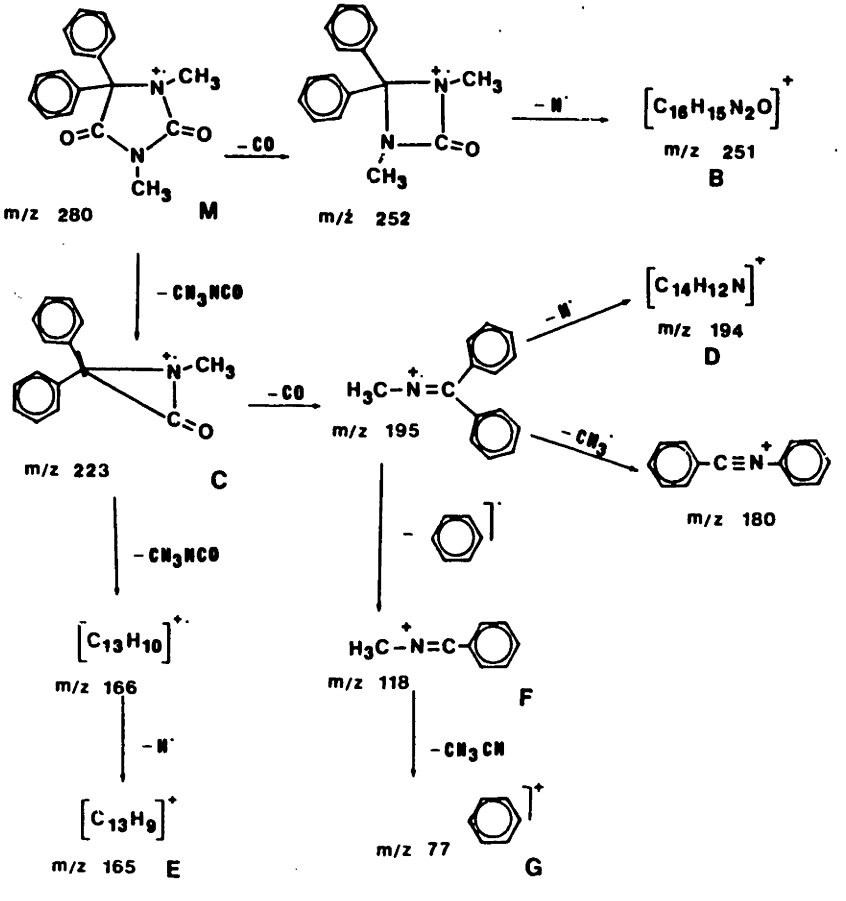
The fragmentation mechanism proposed by Andresen involved a modification of the mechanism suggested by Locock and Coutts. Andresen showed that that the initial

Scheme A-1. Fragmentation mechanism proposed by Locock and Coutts (49): Part A, for Ia; part B, applied to d-Ia.

Scheme A-2. Modification as described by Andresen (18) of the fragmentation mechanism proposed by Locock and Coutts: Part A, for Id; Part B, applied to d-Id.

Scheme A-1


-CO
-H·
[C14H11N2O]+
m/z 223
B
m/z 252
M
m/z 224
-HNCO
-CO
-H·
m/z 209
C
m/z 181
m/z 180
D
-HNCO
-
[C13H10]+·
m/z 166
m/z 104
F
-H·
-HCN
[C13H9]+
m/z 165
E
m/z 77
G


-CO
-H·
[C16H15N2O]+
m/z 251
B
m/z 280
M
m/z 252
-CH3NCO
[C14H12N]+
m/z 194
D
-CO
-H·
m/z 223
C
m/z 195
-CH3·
m/z 180
-CH3NCO
-
[C13H10]+·
m/z 166
H3C-N+=C
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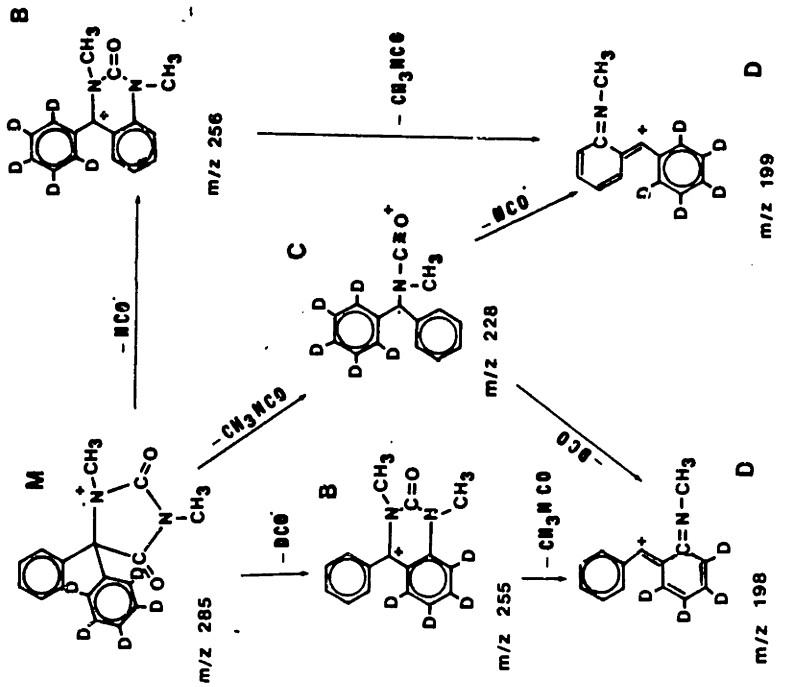
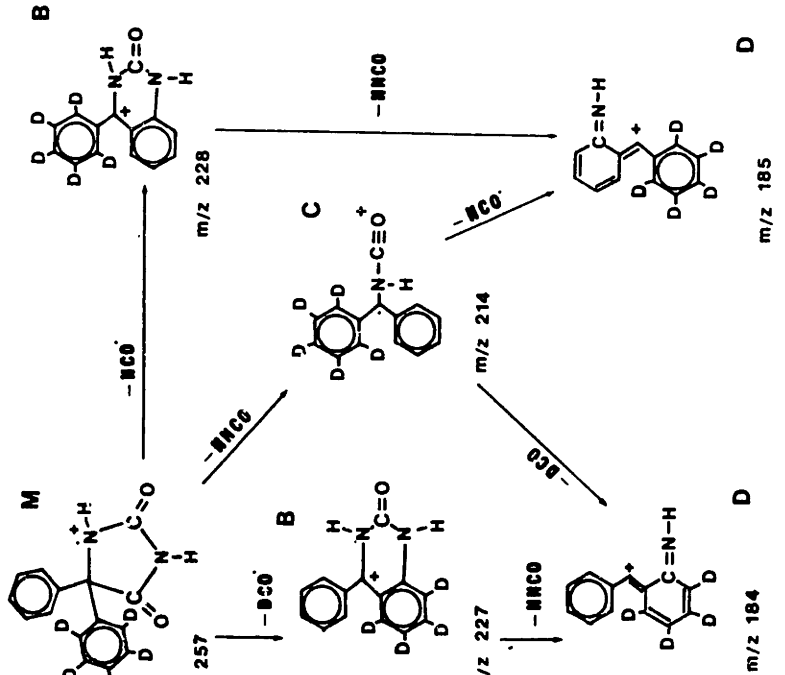
F
-H·
-CH3CN
[C13H9]+
m/z 165
E
m/z 77
G

Scheme A-2


B
m/z 256
- CH3NCO
D
m/z 199
- NCO·
C
m/z 228
- CH3NCO
M
m/z 285
- NCO·
- BCO·
B
m/z 255
- CH3NCO
D
m/z 198


B
m/z 228
- HNCO
D
m/z 185
- NCO·
C
m/z 214
- HNCO
M
m/z 257
- NCO·
- BCO·
B
m/z 227
- HNCO
D
m/z 184

loss of HCO from the molecular ion (transition M→B) involved hydrogen abstraction from either one of the phenyl rings.

As is shown in Scheme A-1, part A (for Ia), Locock assumed that in the transition C→D, the hydrogen is solely lost from nitrogen (to give the ion at m/z 180). If this assumption would have been correct, a single abundant ion at m/z 185 would result in the mass spectrum of Id, from the corresponding transition C→D (the d5-labelled phenyl ring would retain all five deuterium atoms). However, Andresen found ions at m/z 184 and 185 in the mass spectrum of Id (as indicated in Scheme A-2, part A), and this clearly indicated that the labelled phenyl ring had somehow been involved in the transition. Also, the ion at m/z 223, observed by Coutts in the spectrum of Ia, shifted to a doublet at m/z 227 and 228 in the spectrum of Id, and this also revealed that the phenyl groups (labelled and deuterium labelled) were participating in the transition.

It is evident that permethylation of Ia and Id (and, of course, also of the other analogs) will not affect the mechanism of the fragmentation. Applying the fragmentation mechanism as proposed by Locock and Coutts to d-Ia (as shown in Scheme A-1, part B), again showed that in the transition C→D, loss of the substituent on nitrogen (in this case a CH_3 group) did not account for this transition (loss of CH_3 from m/z 195 would result in an ion at m/z 180; this ion is of very low intensity in the mass

spectrum of d-Id, see Figure A-1 d). However, the ion at m/z 194 (resulting from the loss of a hydrogen from the ion at m/z 195) was very abundant, and this led us to assume that the modification proposed by Andresen (i.e. that the phenyl rings are involved in the transition C⟶D) was correct.

Study of the fragment ions in the mass spectra of the permethylated derivatives of the other analogs (see Figure II-1 for their structure, Figures A-1 and A-2 for their mass spectra) confirmed this assumption: in the mass spectra from the ring-deuterated compounds, a cluster of ions at the corresponding shifted m/z values resulted and in the mass spectra from the analogs that did not contain deuterium on the phenyl ring, a single abundant ion resulted (see also Table A-1). This is illustrated for d-Id in Scheme A-2, part B. The structural assignment of the fragment ions was based on the mechanism suggested by Andresen for Ia itself (as shown in part A of scheme A-2).

However, conclusive evidence for the fact that the initial loss of HCO from the $M^{\cdot+}$(transition M⟶B), involves abstraction of a hydrogen, from the ortho position of the phenyl ring without prior scrambling (as shown in Scheme A-1, part A), can only be obtained by verifying the fragment ions of an analog of Ia that is specifically labelled, in the o-position of the phenylring. If e.g. a deutrium would be substituted in o-position of the phenyl ring, this deuterium would be lost in the transtion M⟶B if

the theory of Andresen holds; if, on the other hand, the substituents on the phenyl ring are scrambled (before they are lost), the deuterium would be only partially lost. The only ring deuterated analog availabe to us that could give some indication on eventual scrambling was d-IIc, which has two deuterium atoms on the phenolic ring, in m-position with regard to the hydantoin ring. In the event of scrambling, the loss of deuterium would only produce a weak fragment ion, because there are only two deuteriums but a total of seven hydrogens on the two phenyl rings. The mass spectrum of d-IIc (Figure A-2, d) does show an ion of very low abundance at m/z 285 (which would be the m/z value of the ion resulting from the loss of DCO from the molecular ion of d-Id). It would therefore be of interest to further investigate the possibility of scrambling of the phenyl hydrogens in this transition by studying the mass spectra of different labelled analogs that can provide conclusive evidence (i.e. Ia with deuterium in the o-positions).

It appears that in the transition C→D, scrambling of the hydrogens occurs (before abstraction), because in all spectra studied by us, an ion was observed at that m/z value corresponding to a fragment that still contained the hydrogen or deuterium (for example, in the spectrum of d-Ia, the ion at m/z 195; in the spectrum of d-Id, the ion at m/z 200).

The scheme suggested by Andresen for the transition M→C (initial loss of HCNO from the molecular ion) would

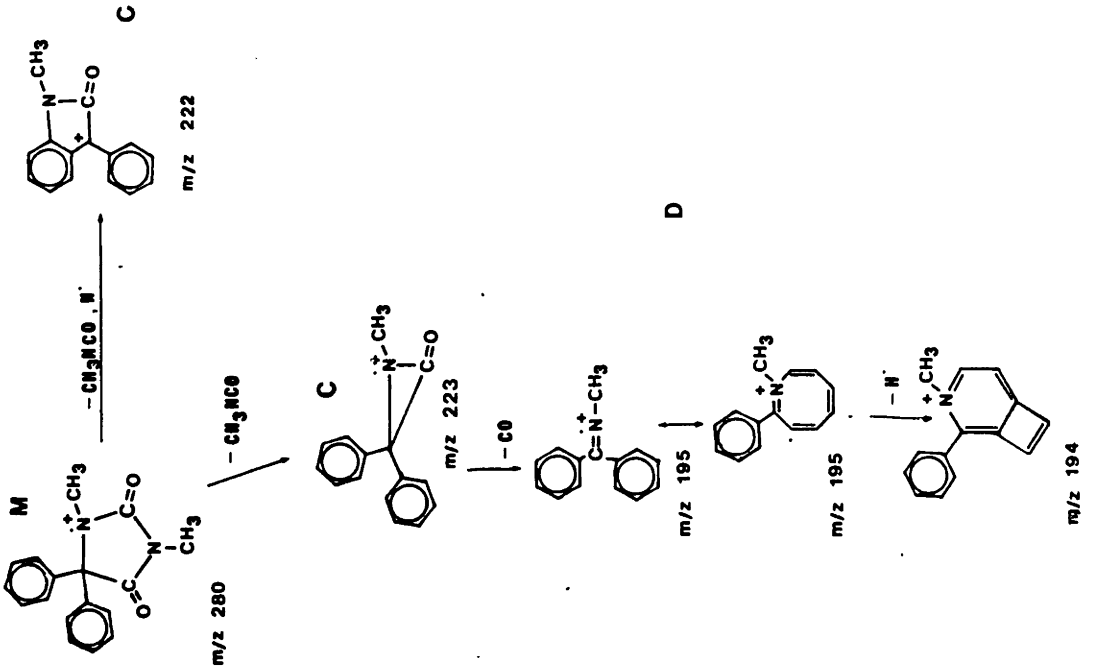
predict a single ion in the spectra of the compounds studied (e.g. for Ia at m/z 214, as indicated in Scheme A-1, part A). However, in the mass spectra of the permethylated derivatives studied by us, a cluster of ions in this region was observed (ion cluster C in the mass spectra shown in Figures A-1 and A-2). The participation of a phenyl hydrogen (or deuterium) could be inferred from the cluster of fragment ions, spaced two amu apart for the phenyl ring substituted analogs (e.g. for d-Id, where both phenyl rings contain deuterium, see Figure A-1 c, ions at m/z 231 and 233), and spaced only 1 amu apart for the non-deuterium labelled analogs.

We therefore suggest (see Scheme A-3) a modification of the fragmentation processes proposed by Andresen to better explain the transitions M→C and C→D

As mentioned in the beginning of this Chapter, the information in Table A-1 was based on the discussed observations. The fragment ions at lower mass that have not been discussed (i.e. for the base spectrum of d-Ia, at m/z 166, 165, 118 and 77) are self-explanatory and follow the fragmentation intially suggested by Locock and Coutts. The fragment ions designated by H and I are only present in the mass spectra of the permethylated HPPH analogs, but fit in the suggested scheme.

Scheme A-3. Modification as suggested by us of the fragmentation mechanism proposed by Andresen: Part A, for Id; Part B, for d-Id.

Scheme A-3


M
m/z 280
C
m/z 222
m/z 223
m/z 195
m/z 194
D
m/z 200
m/z 199
m/z 198
m/z 285
m/z 228
m/z 227
m/z 226

References

1. T.C. Butler, J. Pharmacol. Exp. Ther., 119, 1 (1957).
2. E.W. Maynert, J. Pharmacol. Exp. Ther., 130, 275 (1960).
3. A.J. Atkinson et al., Biochem. Pharmacol., 19, 2483 (1970).
4. T. Chang et al., Epilepsia, 18, 291 (1977).
5. M.G. Horning et al., Anal. Lett., 4 (8), 537 (1971).
6. T. Chang et al., Biochem. Biophys. Res. Commun., 38 (3), 444 (1970).
7. O. Borga et al., Pharmacology, 7, 129 (1972).
8. H.-J. Egger et al., In "Quantitative Mass Spectrometry in Life Sciences-II", A.P. De Leenheer et al., Eds., Elsevier Scientific Publishing Co., Amsterdam, 1978, pp. 303-314.
9. F.L. Kozelka and C.H. Hine, J. Pharmacol. Exp. Ther., 77, 175 (1943).
10. W.J. Jusko, In "Quantitative Analytic Studies of Epilepsy", P. Kellaway and I. Peterson, Eds., Raven Press, New York, 1976, pp. 115-136.
11. A.J. Glazko et al., Clin. Pharmacol. Ther., 10 (4), 498 (1969).
12. K.S. Albert et al., Res. Comm. Chem. Pathol. Pharmacol., 9 (3), 463 (1974).
13. E.R. Klein, and P.D. Klein, Biomed. Mass Spectrom.,

5 (2), 91 (1978); Ibid, 5 (5), 321 (1978); Ibid., 5 (6), 373 (1978); Ibid., 5 (7), 425 (1978); Ibid., 6 (12), 515 (1979).

14. W.D. Lehman and H.-R. Schulten, Angew. Chem. Int. Ed. Engl., 17 (4), 221 (1978).
15. G.H. Draffan, In "Stable Isotopes: Applications in Pharmacology, Toxicology and Clinical Research", Chapter 3, .T.A. Baillie, Ed., University Park Press, London, 1978, pp. 27-42.
16. H.R. Sullivan and R.E. McMahon, In "Quantitative Mass Spectrometry in Life Sciences -I", A.P. De Leenheer and R.R. Roncucci, Eds., Elsevier Scientific Publishing Co., Amsterdam, 1977, pp. 83-93.
17. D.J. Jenden and A.K. Cho, Biochem. Pharmacol. 28, 705 (1979).
18. B.D. Andresen, Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA, 1974, pp. 144, 151.
19. J. E. Tomaszewski et al., Biochemistry, 14, 2024 (1975).
20. C. Hoppel et al., J. Chromat., 116, 53 (1976).
21. J.D. Baty and P.R. Robinson, Biomed. Mass Spectrom., 4 (1), 36 (1977).
22. B. Rambeck, In "Antiepileptic Therapy: Advances in Drug Monitoring", S.I. Johannessen et al., Eds., Raven Press, New York, 1980, pp. 365-372.
23. H.J. Kupferberg, In "Antiepileptic Drugs: Quantitative Analysis and Interpretation", Chapter 2,

C.E. Pippinger et al., Eds., Raven Press, New York, 1978, pp. 11-12.

24. R. De Sagher, J. Chromat., 156, 43 (1978).
25. J.H.G. Jonkman, Pharm. Weekblad, 110, 649 (1975).
26. R.A. Jones, Aldrichim. Acta, 9 (3), 35 (1976).
27. H. Ehrsson, Anal. Chem., 46 (7), 922 (1974).
28. R.C. Murphy et al., Biomed. Mass Spectrom., 5 (7), 444 (1978).
29. A.J. Glazko, In "Antiepileptic Drugs", D.M. Woodbury et al., Eds., Raven Press, New York, . 1972, pp. 103-123.
30. I. Bjorkhem et al., Clin. Chem., 22 (11), 1789 (1976).
31. L. Eldjarn, In "Quantitative Mass Spectrometry in Life Sciences -II", A.P. De Leenheer et al., Eds., Elsevier Scientific Publishing Co., Amsterdam, 1978, pp. 151-159.
32. M. Claeys et al., Biomed. Mass Spectrom., 4 (2), 122 (1977).
33. B.J. Millard, In "Quantitative Mass Spectrometry in Life Sciences -II" , A.P. De Leenheer et al., Eds., Elsevier Scientific Publishing Co., Amsterdam, 1978, pp. 83-102.
34. D.A. Schoeller, paper presented at the Third International Symposium on Quantitative Mass Spectrometry in Life Sciences, Ghent, Belgium, 1980. To be published in Biomed. Mass Spectrom.

35. A.J. Wilensky, Clin. Chem., 24 (4), 722 (1978).

36. T.J. Giovannielli, and J. Pecci, Clin. Chim. Acta, 67, 7 (1976).

37. B.J. Millard, in "Quantitative Mass Spectrometry", Heyden, London, 1978.

38. C.E. Pippinger and H. Kutt, In "Antiepileptic Drugs: Quantitative Analysis and Interpretation", Chapter 18, C.E. Pippinger et al., Eds., Raven Press, New York, 1978, pp. 199-218.

39. D.A. Schoeller, Biomed. Mass Spectrom., . 3, 265 (1976).

40. B.W. Brown and M. Hollander, "Statistics: A Biomedical Introduction", Wiley and Sons, New York, 1977.

41. J.H. Beynon and A.E. Williams, "Mass and Abundance Tables for use in Mass Spectrometry", Elsevier Publishing Company, N.Y., 1963.

42. D.J. Greenblatt et al., J. Pharmacol. Exp. Ther., 202, 365 (1977).

43. B.K. Tang et al., Drug Metab. Dispos., 7 (5), 315 (1979).

44. K. Biemann and J.E. Biller, In "Biochemical Applications of Mass Spectrometry", First Supplementary Volume, G.R. Waller and O.C. Dermer, Eds., Wiley Interscience, N.Y., 1980.

45. R.A. Hites and K. Biemann, Anal. Chem., 40, 1217 (1968).

46. R.A. Hites and K. Biemann, Anal. Chem., 42, 855 (1970).

47. "Mass Spectra of Drugs", Ed., K. Biemann, M.I.T. Dept. of Chemistry, Cambridge, MA, 1971.

48. "EPA/NIH Mass Spectral Data Base", S.R. Heller and G.W.A. Milne. Eds., National Standard Reference Data system, U.S. Dept of Commerce, December, 1978.

49. R.A. Locock and R.T. Coutts, Org. Mass Spectrom., 3, 735 (1970).

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Biographical Note

Agnes Van Langenhove, daughter of Mr. and Mrs. Paul and Elza Van Langenhove-Michiels, was born in Opwijk, Belgium, on June 29, 1952. She attended primary and secondary schools in Opwijk and graduated from the Maria Immaculata Instituut Highschool with the Greatest Distinction. She entered the School of Pharmacy at the University of Ghent in September 1969 and obtained the Degree of Pharmacist in June 1974 with the Greatest Distinction. She also received degrees in Phytopharmacy and Advanced Toxicology. She was assistant at the Toxicological Institute of the University of Ghent from August 1974-September 1975. In October 1974, she received the annual prize from the Royal Society of Pharmacists of East Flanders (K.O.V.A.G.) for her work entitled: "Toxicological Investigation of a Fatal Cantharidin Intoxication in Man". She specialized in Forensic Toxicology and Clinical Biology and obtained a license to perform clinical analyses in June 1975.

She received a CRB Fellowship from the Belgian American Educational Foundation in September 1975 for study in the U.S.A. She was guest worker at the National Institutes of Health, in Bethesda Md., from October 1975-July 1976, under supervision of Dr. H. Fales. She worked on biomedical applications of mass spectrometry.

In September 1976, she entered the Chemistry

Department of the Massachusetts Institute of Technology as a Doctoral Candidate. Here she received support as a Teaching Assistant (1976-77) and Research Assistant (1977-80).

The author is a member of the American Chemical Society, the American Association for Advancement of Science, the American Society for Mass Spectrometry, the International Association of Forensic Toxicologists and the Cercle des Alumni Universitaires de Belgique. She has presented or co-authored the following publications:

"A Gas Chromatographic Mass Spectrometric Method for the Simultaneous Quantitation of 5,5-Diphenylhydantoin (Phenytoin), its Para-hydroxylated Metabolite and their Stable Isotope Labelled Analogs", A. Van Langenhove. C.E. Costello, J.E. Biiler, K. Biemann, and T.R. Browne, submitted to Clinica Chimica Acta.

"A Mass Spectrometric Method for the Determination of Stable Isotope Labelled Phenytoin suitable for Pulse Dosing Studies", A. Van Langenhove, C.E. Costello, J.E. Biller, K. Biemann and T.R. Browne, presented at the Third International Symposium on Quantitative Mass Spectrometry in Life Sciences, held in Ghent, Belgium, 1980. Proceedings to be published in Biomedical Mass Spectrometry.

"Equivalence of Pharmacokinetic Properties of Stable

Isotope Labelled and Unlabelled Phenytoin in Dog and Man", T.R. Browne, A. Van Langenhove, C.E. Costello, K. Biemann, D.J. Greenblatt, submitted to Neurology.