AFRL-HE-WP-TR-2004-0002

# United States Air Force Research Laboratory

# Pharmacokinetic Modeling of JP-8 Jet Fuel Components: II. A Conceptual Framework


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December 2003
Final Report – March 1997 – December 2003


20061212231


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# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | December 2003 | Final Report - March 1997 - December 2003 |

**4. TITLE AND SUBTITLE**

Pharmacokinetic Modeling of JP-8 Jet Fuel Components: II. A Conceptual Framework

**5. FUNDING NUMBERS**

PE 62202F
PR 1710
TA 1710D4
WU 1710D410

**6. AUTHOR(S)**

Robinson, Peter J.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

ManTech - GEO Centers Joint Venture
P O Box 31009
Dayton OH 45437-0009

**8. PERFORMING ORGANIZATION REPORT NUMBER**

AFRL-HE-WP-TR-2004-0002

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

Air Force Research Laboratory, Human Effectiveness Directorate
Biosciences and Protection Division
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-5707

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION AVAILABILITY STATEMENT**

Approved for public release; distribution is unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

Complex mixtures such as the widely used jet fuel JP-8 consist of hundreds of individual, though often chemically similar, components. To facilitate the pharmacokinetic and toxicokinetic analyses of such complex mixtures, we made a number of simplifying assumptions. First, the interconnection of PBPK models of individual substances, via binary mechanisms, is enough to predict the interactions present in the mixture. Secondly, complex mixtures can often be approximated as pseudo-binary systems, consisting of the compound of interest plus a single interacting complex vehicle with well-defined, composite properties. This conceptual approach is applicable to a number of different kinds of potential interaction mechanisms, including substrate metabolism and dermal absorption. This approach has been previously explored for dermal absorption of JP-8. In the current report we show that in a complex mixture with components that interact via competitive metabolic inhibition of a specific enzyme, the metabolic rate of each component can be described as if it were part of a binary mixture, with the total mixture concentration replacing that of the single competitor, and the effective inhibition constant given by the (concentration-weighted) harmonic mean of the individual (binary) inhibition constants.

**14. SUBJECT TERMS**

JP-8 JP-8+100 hydrodesulferized keronsene jet fuel toxicity

**15. NUMBER OF PAGES**

39

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| UNCLASSIFIED | UNCLASSIFIED | UNCLASSIFIED | UL |

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## PREFACE

This is the second in a series of technical reports describing the pharmacokinetic modeling of JP-8 fuel components. Specifically, this report outlines a conceptual approach for using physiologically-based pharmacokinetic (PBPK) modeling to explore the toxicokinetics of complex mixtures. Some of this work was presented at the Society of Toxicology Annual Meeting, San Francisco, 2001 and at the *Conference on Application of Technology to Chemical Mixtures Research*, Colorado State University, 2001.

Part 1 of this series was published as: Robinson, PJ. *Pharmacokinetic modeling of JP-8 jet fuel components I. Nonane and C9 –C12 aliphatic components.* Air Force Research Laboratory, Operational Toxicology Branch, AFRL-HE-WP-TR-2000-0046, 2000

This research was accomplished at the Operational Toxicology Branch, Human Effectiveness Directorate of the Air Force Research Laboratory (AFRL/HEST) at Wright Patterson Air Force Base, OH (Dr. Richard Stotts, Program Manager). The work was sponsored in part by AFIERA. This Technical Report constitutes the Final Report for AFRL/HEST Workunit 1710D410.

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# PHARMACOKINETIC MODELING OF JP-8 JET FUEL COMPONENTS: II. A CONCEPTUAL FRAMEWORK

## INTRODUCTION

The objective of the present study is to develop a conceptual framework in which to consider the effects of component interactions in complex mixtures (consisting of more than a dozen, possibly hundreds or thousands of components) such as JP-8, in which all the individual interactions cannot be fully characterized.

JP-8 is a kerosene based fuel consisting of a complex mixture of hundreds of components from a number of hydrocarbon classes, including straight chain alkanes, branched chain alkanes, cycloalkanes, diaromatics and naphthalenes (Potter and Simmons, 1998). Human exposures to JP-8 in the vapor, aerosol and liquid forms all have the potential to be harmful. Situations where fuel becomes aerosolized have the greatest potential to be a hazard via inhalation (Pleil *et al.*, 2000). Both aerosol and liquid forms of JP-8 have the potential to cause local and systemic effects with prolonged or repeated skin contact; JP-8 exposure has been shown to result from aircraft engine starts at low ambient temperatures as a result of incomplete combustion inhalation (Pleil *et al.*, 2000). It may be inhaled, irritate the eyes or result in skin irritation and absorption. Potential JP-8 dermal exposure scenarios are:

1) prolonged contact with the skin of ground personnel through soaked clothing
2) splashes during refueling or fuel handling,
3) handling engine parts which are coated with fuel,
4) coming in contact with sides of fuel tank during fuel tank maintenance operations, and
5) coming in contact with fuel leaks on the underside of the aircraft or on the ramp.

Many JP-8 components interact in a number of ways, such as competing for the same metabolic enzymes, or modifying each others' partitioning into various tissues of the body. In order to fully characterize the behavior of any one component, one must simultaneously characterize the effects of all others. One way to explore these component interactions is to develop



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physiologically-based pharmacokinetic (PBPK) models of individual chemical components (Robinson, 2000) and then model these interactions. However, with the large number of components, this becomes an impossibly complex undertaking. For example, a single type of (simple) binary interaction between 300 components would require the specification of some $10^5$ interaction coefficients.

One proposed solution to this problem is to "lump" the components into a fewer number of groups consisting of "similar" chemicals according to some well defined chemical properties or behaviors (such as equivalent carbon number, molecular weight, water solubility, lipid partitioning, vapor pressure, etc.) (US EPA, 1999). Each group can then be characterized by examining in detail a chosen representative compound of that group; interactions between groups can be examined by studying interactions between these representative compounds. This approach has been advocated, for example, by the Total Petroleum Hydrocarbon (TPH) Criteria Working Group, which has classified petroleum hydrocarbon fractions into six aliphatic and seven aromatic classes (Gustafson *et al.*, 1997).

Although it is useful as perhaps a first approximation (Kenyon *et al.*, 1996), there are a number of difficulties with this lumping approach, including the problem that suitable categorization criteria may be suitable for one type of behavior (such as blood-air partitioning), but may not be suitable for another (such as blood-tissue distribution). In addition, the composition of a group may change with time so that the properties of the group of chemicals as a whole may no longer be reflected by those of the originally chosen representative compound. We therefore propose an alternative approach that retains some of the advantages of the "lumping" process, while allowing the changing properties of the mixture to be mathematically characterized.

PBPK models for a number of individual components of JP-8, such as benzene, xylene, toluene and nonane, have been developed, and in addition, models of the interactions of up to five component mixtures of chemicals have been studied (Haddad *et al.*, 1999; Tardif *et al.*, 1997). A key result of these studies is that a complete description of the interactive processes can be obtained by simultaneously tracking all the binary interactions in the mixture (i.e., interactions of

one chemical with another). Higher order interactions (i.e. interactions between three or more chemicals) are automatically taken into account in this way.

The present approach to developing a quantitative framework for assessing tissue exposure to specific components of a complex mixture, in the presence of all the other components, is suggested by the observed behavior of JP-8 components as they penetrate the skin following dermal exposure to JP-8 (Robinson and McDougal, 2000). It was shown that skin penetration of JP-8 constituents was determined by their specific chemical properties (in this case, octanol water partition coefficients), together with the properties of the JP-8 vehicle, in the same way as has been previously observed from other vehicles such as water (Wilschut *et al.*, 1995). The JP-8 vehicle could be considered essentially the same for all components because each contributed only a small fraction (less than a couple of percent) of the total.

In the approach to be presented here for each interactive process, each component is assumed to interact with a single "vehicle", where the vehicle is characterized as some kind of well-defined "average" of all the other components of the mixture. The problem is to determine both qualitatively and quantitatively how to characterize the interactions of the component with this vehicle. This involves answering two questions:

- What are the appropriate parameters that characterize the mixture (vehicle) as a whole in relation to the interaction of interest?
- What is the appropriate way to average these parameters over the components of the mixture?

Once a particular mixture is characterized in terms of a set of parameters representing a particular interactive process, changes in the mixture composition (as a result, for example, of kinetic processes in the body, or of weathering in the environment) can be conveniently tracked in terms of specific changes in these calculated (average) parameter values with time. In this way, relevant properties of different mixtures can be distilled, visualized and compared.

## VEHICLE EFFECTS ON DERMAL ABSORPTION

We begin with a summary of our results on the dermal penetration of JP-8 (Robinson and McDougal, 2000). Briefly, static diffusion cells with 4.9 $cm^2$ skin exposure area were used to determine flux and skin concentrations of JP-8 and its components. The receptor compartment was filled with a solution of 6% Volpo 20 (polyethylene glycol-20 oleyl ether, Croda, Mill Hill, PA) in physiological saline. A Volpo/saline receptor solution was chosen to assure that the solubility of JP-8 components in the receptor solution would not be a limiting factor in determining penetration. The receptor solution was sampled at half-hour intervals for four hours. Chemical concentration in the 20 μL samples was determined by headspace analysis using gas chromatography with flame ionization detection. Flux (mass/area-time) was determined from the slope of the plot of cumulative chemical mass per unit area in the receptor solution over time. Time points before chemical was detected in the receptor solution were not used in the determination of slope. Flux was determined for each diffusion cell and reported with standard deviation. Permeability coefficients (distance/time) from JP-8 were determined for each component by dividing individual fluxes by the concentration of the component in JP-8.

The log of the dermal penetration coefficient $K_p$ was observed to have a linear dependence on lipophilicity as estimated from the the log-octanol-water partition coefficient $K_{o/w}$ (with slight dependence on molecular weight, $MW$) (see Figure 1):

$$\log K_p = -2.69 - 0.471 \log K_{o/w} + 0.00716\, MW \qquad (1)$$

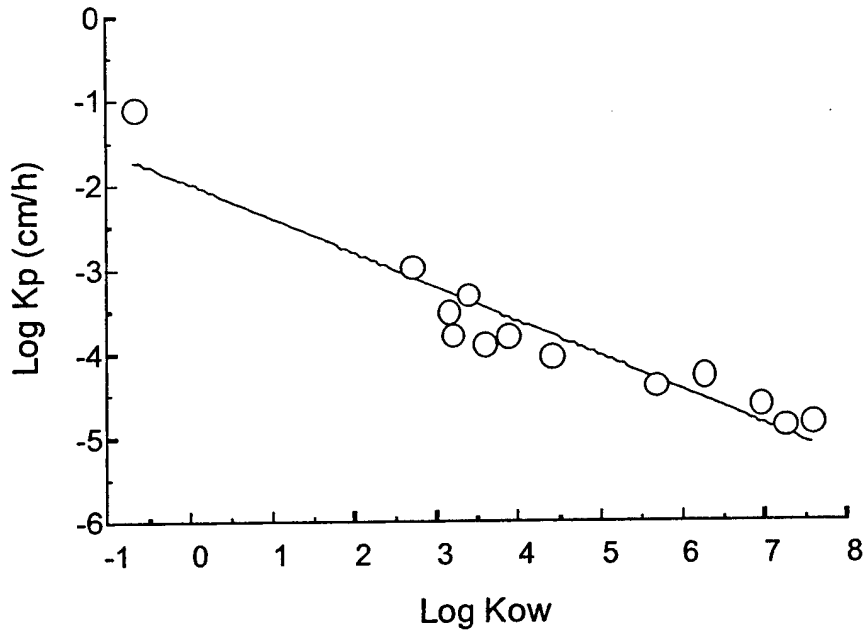


*Figure 1. Log plot of skin penetration coefficient (log Kp) against log octanol-water partition coefficient (log Kow) for 13 components of JP-8 measured with rat skin in static diffusion cells (McDougal et al., 2000). The linear regression is given by equation 1 in the text.*

This semi-empirical relation can be used to predict (estimate) $K_p$ values for other JP-8 components (McDougal and Robinson, 2002). In contrast to the penetration of compounds from an aqueous vehicle (Potts and Guy, 1992), in which skin permeability generally increased with the compound's lipophilicity, in the case of JP-8 vehicle, the more lipophilic compounds penetrated the skin at a lower rate. This is consistent with other results of more hydrophilic compounds preferentiallypartitioning into the stratum corneum from a more lipophilic (rather than hydrophilic) vehicle. Nevertheless, the behaviors are similar in that there was an observed linear dependence of $K_p$ on log $K_{o/w}$, and that the nature of the vehicle in each case determines this dependence. To a first approximation at least, JP-8 can be regarded as a simple vehicle, without taking into account its individual components and their separate interactions. Similar results are obtained for silastic sheeting, suggesting the possibility of using this membrane as a skin surrogate for predicting vehicle effects on dermal penetration.

The key here is that there are a sufficiently large number of components in JP-8 and each of them makes up a very small fraction of the total (1% or 2% at most). Thus even though the vehicle for each component is lightly different (total JP-8 minus that component), to a first approximation the vehicle remains the same.

This conceptual approach is also applicable to different kinds of potential interactions, including metabolism (competitive inhibition, non-competitive inhibition, uncompetitive inhibition, enzyme induction), distribution (competition for protein binding sites, alterations of blood flow) and absorption (vehicle effects in dermal absorption, alterations of skin permeability, alteration of pulmonary ventilation rate). In the remainder of this report, we will focus on competitive metabolic inhibition of JP-8 components.

## INTERACTIONS VIA COMPETITIVE INHIBITION

In a recent series of studies, Tardif and colleagues have explored the pharmacokinetic interactions of up to five related compounds, based on the notion that PBPK modeling provides a unique framework that can account for higher order pharmacokinetic interactions based on information from binary mixtures (Haddad *et al.,* 1999; Tardif *et al.,* 1997). In particular, they studied the metabolic interactions between benzene (B), toluene (T), m-xylene (X), ethylbenzene (E) (all minor components of JP-8) and dichloromethane (D). Analysis of the blood kinetic data suggested that competitive metabolic inhibition of P450 2E1 was the most likely interaction mechanism for these compounds. The metabolic inhibition constants $K_i$ for each binary interaction was determined and the values are summarized in Figures 2 and 3 and in Table 2 below.

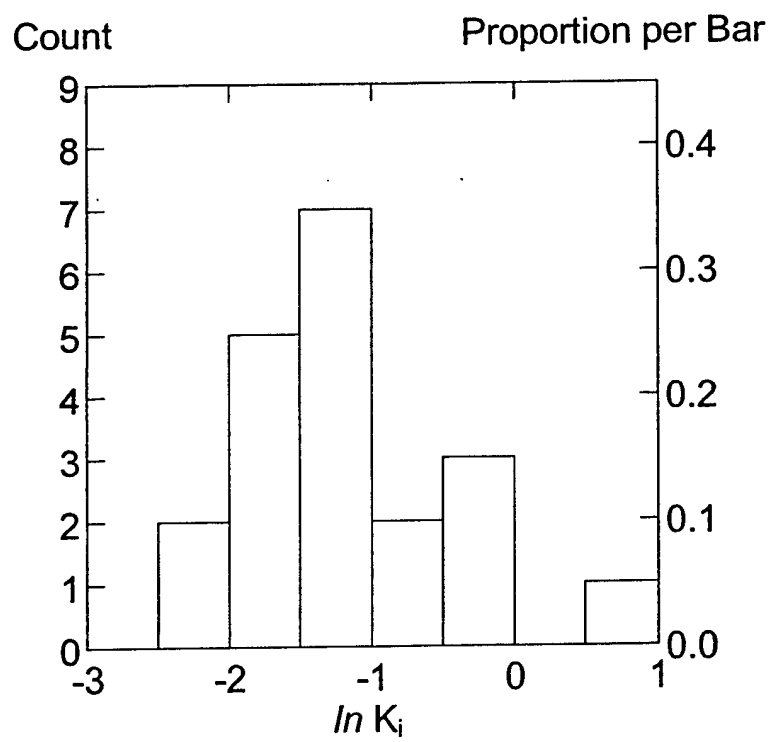


*Figure 2. Statistical distribution of the measured inhibition constants (Ki) for binary interactions between five compounds (data from Tardiff et al., 2000)*

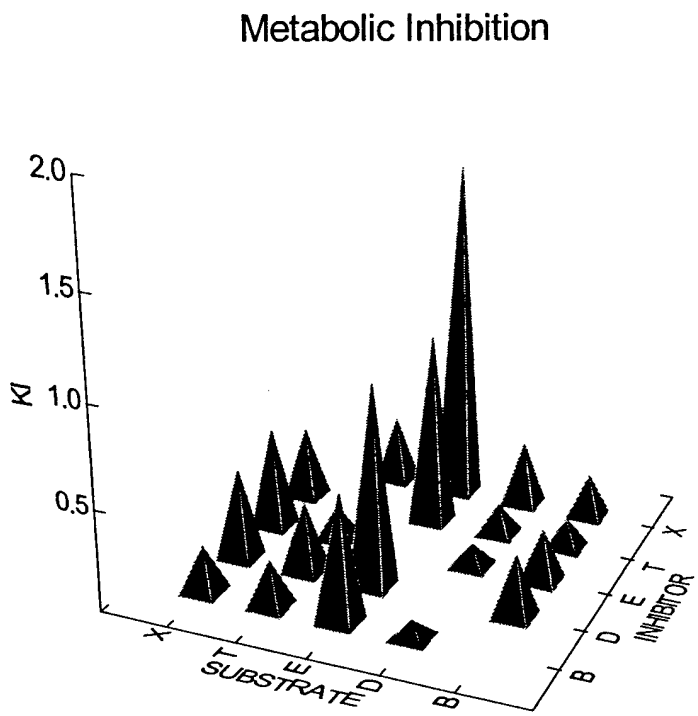


*Figure 3. Distributions of measured pairwise inhibition constants (Ki) as a function of both the substrate and the inhibitor (data from Tardiff et al., 2000)*

*Table 1. Chemical characteristics and log of the measured permeability coefficients (Kp) for each of the chemical components identified in the receptor solutions from JP-8 exposures (from McDougal et al., 2000). Note that the more negative the log Kp value, the slower the rate of penetration through the skin.*

| Component | Molecular Weight | Log $K_{ow}$ | Log Kp (cm/hr) |
|---|---|---|---|
| Diethylene glycol monomethyl ether | 120.2 | -0.68 | -1.09691 |
| Methyl benzene (toluene) | 92.1 | 2.69 | -2.95861 |
| Naphthalene | 128.2 | 3.37 | -3.29243 |
| Ethyl benzene | 106.2 | 3.13 | -3.50864 |
| Dimethyl benzene (xylene) | 106.2 | 3.18 | -3.76955 |
| Methyl naphthalenes | 142.2 | 3.87 | -3.79588 |
| Trimethyl benzene | 120.2 | 3.58 | -3.88606 |
| Dimethyl naphthalenes | 156.2 | 4.38 | -4.03152 |
| Decane | 142.3 | 6.25 | -4.25964 |
| Nonane | 128.3 | 5.65 | -4.37675 |
| Undecane | 156.3 | 6.94 | -4.60206 |
| Tridecane | 185.4 | 7.57 | -4.82391 |
| Dodecane | 170.3 | 7.24 | -4.85387 |

Most components of JP-8 are metabolized by cytochrome P-450 2E-1 as part of the Phase 1 detoxification process. Individual components are thus subject to competitive metabolic inhibition. Based on these observations, in order to describe the pharmacokinetic behavior of JP-8 we need to at least take into account metabolic interactions between the components, specifically competitive metabolic inhibition of P450 2E1.

We thus make a number of general hypotheses for competitive enzyme inhibition in sufficiently complex mixtures of metabolically similar components.

- The interconnection of PBPK models of individual substances, via the mechanisms of binary interactions, is enough to predict the higher order interactions present in the mixture.

- Complex mixtures can often be approximated as pseudo-binary systems, consisting of the compound of interest plus a single interacting complex "vehicle" with well-defined, composite properties.

Generalizing the results of Tardif *et al.* (1997) and Haddad *et al.* (1999) to a complex mixture of hydrocarbon components such as JP-8, with competitive metabolic inhibition between each pair of components, the metabolic rate for a particular component $x$ in a mixture of an arbitrary number of other components $i$ can be written as:

$$RAM_x = \frac{V_{\max}^x c_x}{K_m^x\left(1+\sum_{i\neq x}\frac{c_i}{K_{ix}}\right)+c_x} \qquad (2)$$

where $V_{\max}^x$ is the maximal metabolic rate of $x$, $c_x$ is the concentration of $x$, $K_m^x$ is the (true) Michaelis-Menten constant for $x$, and $c_i$ and $K_{ix}$ are the concentrations and inhibition constants of each component $i$.

Thus,

$$RAM_x = \frac{V_{\max}^x c_x}{K_m^x\left(1+\frac{c_{tot}}{\overline{K_x}}\right)+c_x} \qquad (3)$$

where $c_{tot}$ is the total concentration of the mixture in the blood (excluding component $x$, which for many complex mixtures may make a negligible contribution to the total), and

$$\overline{K_x} = \frac{c_{tot}}{\sum_{i\neq x}\frac{c_i}{K_{ix}}} = \frac{1}{\sum_{i\neq x}\frac{f_i}{K_{ix}}} \qquad (4)$$

is an effective inhibition constant for compound $x$ in the presence of other mixture components $i$, and where $f_i$ is the fraction of the mixture with an inhibition constant $K_{ix}$.

Note that $\overline{K}_x = 1\Big/\sum_{i\neq x}\frac{f_i}{K_{ix}}$ is the *harmonic mean* of the individual inhibition constants for the rest of the mixture (weighted according to relative abundances in the mixture). Note also that equation (3) is identical in form to that which describes the interactions of just two components in a binary mixture.

Thus, in a complex mixture with interactions via competitive metabolic inhibition, the metabolic rate of each component can be described as if it were part of a binary combination. *The total mixture concentration replaces that of the single competitor, and the inhibition constant is given by the harmonic mean of the individual (binary) inhibition constants.*

Note that, in general, the harmonic mean $\overline{K}_x$ changes as the relative composition of the mixture changes (i.e., as each $f_i$ changes). For example, as JP-8 is metabolized, some components may be metabolized to a larger extent than others, and will find that ~~see~~ their relative fraction fall (and vice versa). (However, if there happens to be no correlation between the rate of metabolism and inhibition $K_{ix}$, then metabolism will result in no systematic change in the *average* value $\overline{K}_x$).

Because of the large number of components involved, it is impossible in practice to characterize each pairwise interaction in a complex mixture in terms of its individual inhibition constant. An alternative approach can be suggested ~~itself~~ from an examination of Figure 2, which shows the distribution of inhibition constants given in Table 2. The distribution ~~here~~ found has a mean of 0.4 mg/L and a harmonic mean of 0.25 mg/L. If we assume the same harmonic mean is maintained for the entire JP-8 mixture, we can estimate $RAM_x$ from Equation 3 for each component of JP-8 in the presence of the others. Note that $K_m$ is replaced by a (larger) effective $K_m$ that depends on the total JP-8 concentration, relative to the (harmonic) mean of the pairwise inhibition constants.

*Table 2. Values of inhibition constants ($K_i$) obtained by fitting PBPK model simulations to data on blood concentrations of parent chemicals observed following exposure to binary mixtures of benzene (B) and toluene (T), ethylbenzene (E), or m-xylene (X) according to the hypothesis of competitive inhibition*

| Inhibitor | Substrate | $K_i$ (mg/L) |
|---|---|---|
| B | T | 0.223 |
| B | E | 0.626 |
| B | X | 0.226 |
| T | B | 0.144 |
| T | E | 0.948 |
| T | X | 0.357 |
| E | B | 0.256 |
| E | T | 0.168 |
| E | X | 0.505 |
| X | B | 0.216 |
| X | T | 0.328 |
| X | E | 1.667 |

*From Haddad et al. (1999)*

Certain limiting conditions can be examined to explore the implications of Equation 3. For compound $x$ and a constant fraction $\alpha$ of $c_{tot}$, Equation 3 becomes:

$$RAM_x = \frac{V_{\max}^x \, \alpha \, c_{tot}}{K_m^x \left(1 + \frac{c_{tot}}{\overline{K}_x}\right) + \alpha \, c_{tot}}$$

$$\xrightarrow[large\, c_{tot}]{} \quad \frac{V_{\max}^x}{\left(1 + \frac{K_m^x}{\alpha \overline{K}_x}\right)} = \beta V_{\max}^x \leq V_{\max}^x \tag{5}$$

If the limit in which the total mixture concentration is much less than the harmonic mean of the inhibition constants ($c_{tot} << \overline{K}_x$), the metabolic rate becomes (from Equation 3):

$$RAM_x = \frac{V_{\max} c_x}{K_m^x + c_x} \tag{6}$$

In this limit, the pharmacokinetics of each of the components are the same as if other chemicals were not present; interactions between components become negligible. Figure 4 shows schematically the effect of competitive inhibition on metabolism of component *x* by all others in the mixture, as a function of the total mixture concentration.

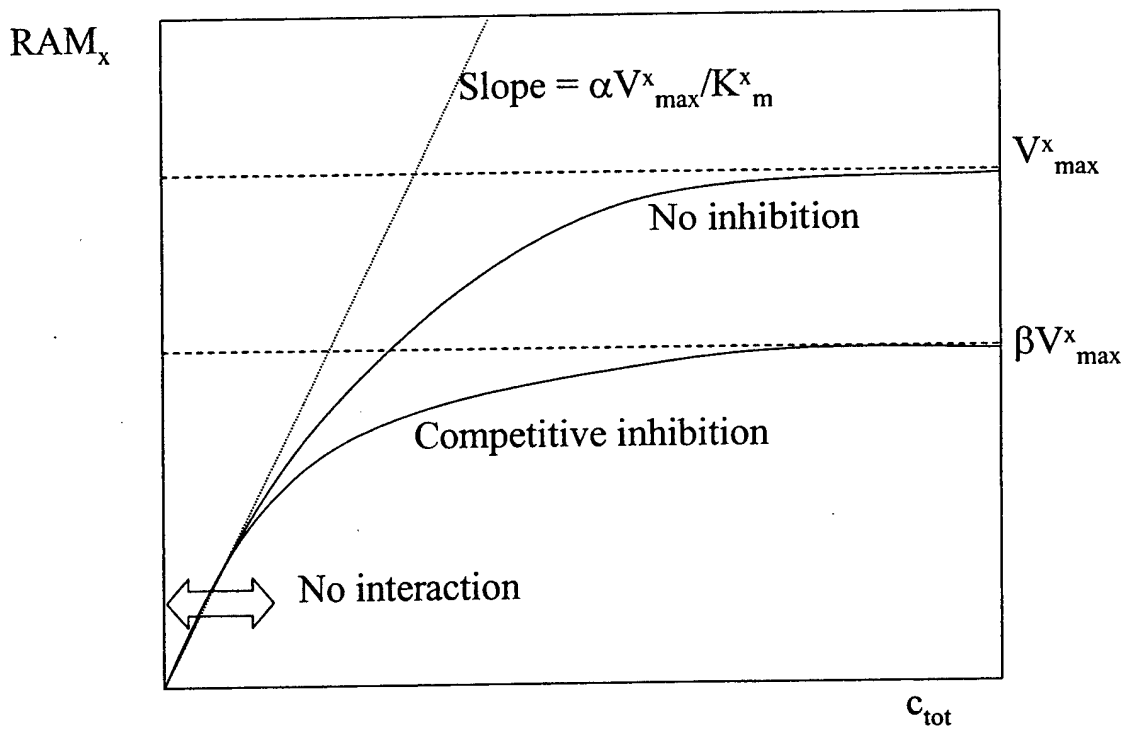


*Figure 4. Schematic representation of the effect of competitive inhibition on metabolism of component x ($RAM_x$) by all others in the mixture, as a function of the total mixture concentration $c_{tot}$. The initial slope of the curve is obtained from the first part of equation (5) in the limit of small $c_{tot}$ values, while the asymptote is given by the second part of equation (5). The result in the absence of competitive inhibition (upper curve) is given for comparison.*

Note that these considerations can be extended to cases in which there is more than one component of toxicological interest. For example, when two compounds are of interest, the mixture can be viewed as being made up of three potentially interacting components. Similarly, a vehicle may consist of more than one group of similar compounds that could be characterized

separately. (This may be appropriate in some cases for JP-8, as its major components are aliphatics and aromatics).

## APPLICATIONS AND VALIDATION

We know from the results of Tardif *et al.* (1997), Haddad *et al.* (1999) and Tardif *et al.* (2000) that this approach works for mixtures of up to five components (BTEX plus dichloromethane) where the values for the individual inhibition constants are known (and where the concentration-time profiles of each component can be modeled). For mixtures of hundreds of components, such as JP-8, it is not feasible to expect measured values for all components and possible combinations thereof. Therefore, we must make some assumptions about the likely values of these parameters.

Equation 3 describes the rate of metabolism ($RAM_x$)of a single compound $x$ in a mixture in which components interact via competitive metabolic inhibition. Essentially, $RAM_x$ is reduced by the interaction as a result of an increased effective Michaelis-Menten constant (decreased affinity) by a factor $(1 + c_{tot}/\overline{K}_x)$. Thus the effect of the interaction on metabolism can be predicted provided we know the total mixture concentration $c_{tot}$ and the (fraction weighted) harmonic mean $\overline{K}_x$ of the relevant inhibition constants. This helps somewhat, but it is still not clear in general how to estimate values for these parameters in a specific situation, since both $c_{tot}$ and $\overline{K}_x$ change with time as components of the mixture are subject to differential effects of pharmacokinetic processes.

In particular (and in contrast to the case in which there are just a few components), $c_{tot}$ cannot be modeled directly (since it is a mixture of hundreds of components, each one of which would have to be simulated). It can, however, be either controlled (kept approximately constant) or measured experimentally.

The model can be tested (verified, validated) experimentally, although this has not been done at this stage. The Appendix outlines a proposed protocol to validate the present model using male Fisher F-344 rats. The rats will be dosed intraperitoneally by means of surgically implanted ALZET™ osmotic pumps, available in a variety of sizes and pumping rates. This method of exposure is chosen because:

- It is resource efficient and convenient.

- The focus of the studies is on the kinetics of the compounds, particularly metabolism. Specific route of entry effects are not of interest at this stage. Intraperitoneal implantation is preferred over sub-cutaneous exposure in order to maximize the concentrations circulated to the liver before exhalation in the lungs.
- We are not at this stage attempting to mimic real-life exposure scenarios (which may be inhalation and/or dermal) ~~and~~ or the modifications in relative composition of the fuel due to selective absorption.

Concentrations in both blood and tissue will be able to be maintained at or near steady-state for extended periods of time. Dosing solutions will be as follows:

- Neat component: nonane or benzene: 0, 50, 100 mg/kg; 5 dosing groups
- Mixture: nonane with benzene spiked at 0, 50, 100 mg/kg each in JP-8 (750 mg/kg); 5 dosing groups

Both the neat JP-8 and the mixtures will be emulsified (5% w/w) in a 6% solution of Volpo20 in physiological saline, to minimize the potential interaction of the JP-8 with pump and the rat tissue.

Blood samples (100 μL) will be collected from the tail vein following dosing at 1, 2, 4, 8, 12 and 24 h (and, if necessary, daily thereafter), and analyzed for total jet fuel and specific components by gas chromatography/flame ionization detection and headspace analysis. Tissue samples will include fat, liver, brain and muscle. Serial tissue samples (1h and 24h) will be taken in the JP-8 dosed groups. In the neat nonane and benzene groups, tissue samples will be taken at the end of the experiment (24 h).

Data from these experiments can be compared with the model predictions (Equations 3 and 4) for model validation purposes. Experimental data may also ~~suggest ways in which the model structure can be improved~~ be valuable to aid in improvement of model structure.

## DISCUSSION AND CONCLUSIONS

Based on Tardiff *et al.* (1997, 2000) and Haddad *et al.* (1999), we have fitted $K_i$ values to pairwise metabolic interactions independently of each component's $K_m$ value. $K_i$ values were allowed to vary depending on the other substrate with which it was competing. In general, however, $K_i$ values tended to be very strongly correlated with $K_m$ (i.e., a good substrate for competitive metabolism by a particular enzyme is also generally a good inhibitor). It is therefore also possible to assume that $K_i$ values are the same as the $K_m$ values for the substrate (and not dependent on what other substrate it ~~is~~ may be inhibiting). This approach has been used to model gasoline kinetics (Dennison *et al.*, 2003). If this were indeed the case, then as more easily metabolized components (and presumably more effective inhibitors) are removed from the mixture, the composition of the mixture will change in a systematic way. The mixture as a whole will drift towards having higher average $K_m$ and $K_i$ values and the kinetics will change (slow down) accordingly.

Although we have treated the complex JP-8 mixture as essentially a single lumped compartment, which ~~(~~interacting with a specific component of concern~~)~~, as more information becomes available, it may be useful to further refine the analysis by considering more than one hydrocarbon "lump". For example, the aliphatic and aromatic components of the fuel behave sufficiently differently in a number of ways (lipophilicity, volatility, etc.) that they could be treated separately. This difference in behavior between aliphatics and aromatics was noticed, for example, in our earlier skin penetration experiments, in which the JP-8 components formed two distinct clusters based on their calculated dermal penetration coefficients (as a function of molecular weight and octanol-water partition coefficient) (McDougal and Robinson, 2002; Robinson and McDougal, 2000).

The development of pharmacokinetic models for complex mixtures such as JP-8 presents a specific set of problems due to the large number of interacting components involved. PBPK models of individual components are critically dependent on values for their blood-air and tissue blood coefficients. These may be difficult to measure experimentally due to the large number of chemicals involved, and so algorithms for predicting partition coefficients from more readily

available structural and chemical properties have been developed (e.g., Poulin and Krishnan, 1996), and we are looking at their usefulness for predicting blood-air and tissue-blood partition coefficients for JP-8 components in our model (Sterner *et al.*, in preparation). Although this approach is promising in that it obviates the need to experimentally determine blood and tissue partition coefficients for each component in a complex mixture, so far, the results for JP-8 components have been disappointing. It did not work...

Additives, since they are often designed to alter the overall performance characteristics of a fuel, have a relatively large effect on JP-8 properties, particularly in relation to their typically small contribution to the fuel's overall composition. Also, additives (such as the relatively hydrophilic diethylene glycol monomethyl ether, DiEGME) tend to be chemically quite different from the bulk of the fuel. In light of these considerations, additives may ultimately need to be treated separately, rather than lumped with the hydrocarbon components of a fuel.

As with traditional PBPK models of single chemicals, extended PBPK models for complex mixtures (such as the one described here), once appropriately validated, are useful for target site dosimetry, extrapolation to different species and routes of exposure, and to examine the consequences of real life exposure scenarios. For example, our previously developed nonane model allowed predictions of brain concentrations under occupational JP-8 exposure conditions to be made (albeit in the absence of any component interactions). Once the present mixtures model is validated, these predictions can be modified to take competitive metabolic inhibition into account, resulting in more accurate and reliable safety and health risk assessments.

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# APPENDIX

## COMPLEX MIXTURES EXPERIMENTAL PROTOCOL:

**Validation of a PBPK Model for Component Interactions via Competitive Metabolic Inhibition in the Complex Fet Fuel Mixture JP-8 in the Rat**

***(Rattus norvegicus)***

PROTOCOL COVER SHEET

**PROTOCOL TITLE: Validation of a PBPK model for component interactions via competitive metabolic inhibition in the complex jet fuel mixture JP-8 in the rat (*Rattus norvegicus*)**

**PRINCIPAL INVESTIGATOR:**

Peter J. Robinson, Ph.D.
AFRL/HEST
2856 G Street
WPAFB, OH 45433-7400
(937) 255-5150 ext. 3143

**SCIENTIFIC REVIEW:** Signature verifies that this proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Richard R. Stotts, DVM, Ph.D., D.A.B.T.
**Chief, Operational Toxicology Branch**
2856 G St, Bldg 79, WPAFB, OH 45433
Voice: 937-255-5150

**ATTENDING/CONSULTING VETERINARIAN:** The attending/consulting veterinarian has reviewed the protocol and was consulted in the planning of procedures that require veterinary input. In addition, the veterinarian/veterinary medicine department has assisted with coordination for veterinary support to the protocol.

Shannon A. Stutler, DVM, MPH, DACVPM
MAJ, VC, USA
Operational Toxicology Branch
Attending Veterinarian, Vivarium Support Function
AFRL/HEST, 2760 Q St., Bldg. 838, WPAFB, OH 45433
Voice: 937-255-7120

**STATISTICAL REVIEW:** A person knowledgeable in statistics has reviewed the experimental design.

Jeffery M. Gearhart, Ph.D., D.A.B.T.
Research Manager, AFRL/HEST

**PROTOCOL TITLE: Validation of a PBPK model for component interactions via competitive metabolic inhibition in the complex jet fuel mixture JP-8 in the rat (*Rattus norvegicus*)**

**PRINCIPAL INVESTIGATOR:** Dr Peter J. Robinson (937) 255-5150 ext 3143

**CO-INVESTIGATOR(S):** Dr. Jeffery M. Gearhart

## I. NON-TECHNICAL SYNOPSIS:

Complex mixtures such as the widely used jet fuel JP-8 often consist of hundreds of individual, though often chemically similar, components. Developing physiologically-based pharmacokinetic (PBPK) models of such complex mixtures by describing each component individually is not feasible due to the large number of components. However, the mixture can often be approximated as a pseudo-binary system, consisting of the compound of interest (that is modeled in detail) plus a single interacting complex vehicle with well-defined, composite properties. We have developed such a model of complex mixtures in which interactions are via competitive metabolic inhibition of a specific enzyme activity. According to this model, the metabolic rate of each component can be described as if it were part of a binary mixture, with the total mixture concentration replacing that of the single competitor, and the effective inhibition constant given by the (concentration-weighted) harmonic mean of the individual (binary) inhibition constants. The purpose of this protocol is to validate this mathematical mixture model for JP-8 with experimental kinetic animal exposures.

## II. BACKGROUND:

### A. Background:

JP-8 is a kerosene based fuel consisting of a complex mixture of hundreds of components from a number of hydrocarbon classes, including straight chain alkanes, branched chain alkanes, cycloalkanes, diaromatics and napthalenes (Potter and Simmons, 1998). Human exposures to JP-8 in the vapor, aerosol and liquid forms all have the potential to be harmful. Situations where fuel becomes aerosolized have the greatest potential to be a hazard via inhalation. Both aerosol and liquid forms of JP-8 have the potential to cause local and systemic effects with prolonged or repeated skin contact. JP-8 exposure has been shown to result from aircraft engine starts at low ambient temperatures as a result of incomplete combustion. It may be inhaled, irritate the eyes or soak clothing and come into prolonged contact with the skin of ground personnel.

Many JP-8 components interact in a number of ways, such as competing for the same metabolic enzymes, or modifying each others' partitioning into various tissues of the body. In order to fully characterize the behavior of any one component, one must simultaneously characterize the effects of all the others. One way to explore these component interactions is to develop physiologically-based pharmacokinetic (PBPK) models of individual chemical components and then model these interactions. However, with the large number of components, this becomes an impossibly complex undertaking.

In a recent series of studies, Tardif and colleagues have explored the pharmacokinetic interactions of up to five related compounds, based on the notion that PBPK modeling provides a unique framework that can account for higher order pharmacokinetic interactions based on information from binary mixtures (Tardif *et al.*, 1997; Haddad *et al.*, 1999). In particular, they have studied the metabolic interactions between benzene (B), toluene (T), m-xylene (X), ethylbenzene (E) (all components of JP-8) and dichloromethane (D). Analysis of the blood kinetic data suggested that competitive metabolic inhibition of P450 2E1 was the most likely interaction mechanism for these compounds.

Generalizing the results of Tardif *et al.* (1997) and Haddad *et al.* (1999) to a complex mixture of hydrocarbon components such as JP-8, with competitive metabolic inhibition between each pair of components, the metabolic rate for a particular component $x$ in a mixture of an arbitrary number of other components $i$ can be written as:

$$RAM_x = \frac{V_{\max}^x c_x}{K_m^x\left(1+\frac{c_{tot}}{\overline{K_x}}\right)+c_x} \qquad (2)$$

where $V_{\max}^x$ is the maximal metabolic rate of $x$, $c_x$ is the concentration of $x$, $K_m^x$ is the (true) Michaelis-Menten constant for $x$, $c_i$ and $K_{ix}$ are the concentrations and inhibition constants of each component $I$, $c_{tot}$ is the total concentration of the mixture in the blood (excluding component $x$, which for many complex mixtures may make a negligible contribution to the total), and

$$\overline{K_x} = \frac{c_{tot}}{\sum_{i\neq x}\frac{c_i}{K_{ix}}} = \frac{1}{\sum_{i\neq x}\frac{f_i}{K_{ix}}} \qquad (3)$$

is an effective inhibition constant for compound x in the presence of other mixture components i, and where $f_i$ is the fraction of the mixture with an inhibition constant $K_{ix}$.

Note that $\overline{K}_x = 1\Big/\sum_{i\neq x}\frac{f_i}{K_{ix}}$ is the *harmonic mean* of the individual inhibition constants of the rest of the mixture (weighted according to their relative abundances in the mixture) (Robinson, 2001a, b).

In particular (and in contrast to simple mixtures in which there are just a few components) $c_{tot}$ cannot be modeled directly (since it is of course a mixture of hundreds of components, each one of which would have to be simulated). It can, however, be either controlled (e.g. kept approximately constant) or measured experimentally. The approach we take in the present protocol is to measure $c_{tot}$ as the total hydrocarbon content of the blood as estimated by the total area under appropriate GC curves (without identifying individual peaks).

**B. Literature Search**:

1. **Literature Source(s) Searched**: MEDLINE, TOXLINE, BRD, EMBASE, BIOSIS, AGRICOLA, Environline & FEDRIP.

2. **Date and Number of Search**: 6-28-02 A2002008

3. **Key Words of Search**: biologically based models, PBPK, toxicokinetics, rat, complex mixtures, jet fuel, JP-8, BTEX, nonane, benzene, naphthalene, xylene, competitive metabolic inhibition.

4. **Results of Search**: Duplication of research efforts proposed for this project were not observed.

## III. OBJECTIVE/HYPOTHESIS:

The purpose of this protocol is to validate a specific mathematical mixture model for JP-8 in which the components interact via competitive metabolic inhibition of cytochrome P450 2E1 enzyme in the liver.

## IV. MILITARY RELEVANCE:

JP-8 is the battlefield fuel for DoD and NATO countries. It is the standard military fuel for all types of vehicles, including the U.S. Air Force Inventory, and in addition, similar fuels are used extensively in the commercial aviation industry. Human exposures to JP-8 in the vapor, aerosol and liquid forms all have the potential to be harmful. Situations where fuel becomes aerosolized have the greatest potential to be a hazard via inhalation. Both aerosol and liquid forms of JP-8 have the potential to cause local and systemic effects with prolonged or repeated skin contact. JP-8 exposure has been shown to result from aircraft engine starts at low ambient temperatures as a result of incomplete combustion. It may be inhaled, irritate the eyes or soak clothing and come into prolonged contact with the skin of ground personnel. Other potential dermal exposures to JP-8 are:

1) splashes during refueling or fuel handling,
2) handling engine parts which are coated with fuel,
3) coming in contact with sides of fuel tank during fuel tank maintenance operations, or
4) coming in contact with fuel leaks on the underside of the aircraft or on the ramp.

## V. MATERIALS AND METHODS:

### A. Experimental Design and General Procedures:

This animal protocol will outline studies that are required to address the above stated objective. The studies described below will be implemented using IPRLs. For all the experiments, male Fisher F-344 rats (Charles River Laboratories) will be ordered to arrive 150-200 g at receipt. All animals have free access to food and water. All animal studies described in this study are conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

*Dosing:*
The rats will be dosed intraperitonealy by means of implanted ALZET™ osmotic pumps, available in variety of sizes, pumping rates. The pumps will be tested *in vitro* to make sure that their function is unaffected by the presence of JP-8 and its components. An initial *in vivo* pilot (rangefinding) study will be conducted (n=5) to make sure that enough JP-8 can be delivered by this method to provide meaningful tissue and blood measurements of the chemicals of interest. The initial choice of pump will have a 2 mL reservoir and provide an infusion rate of 10 μL/h for a period of 7 days. A smaller pump may be chosen if possible.

This method of exposure is chosen because:

- It is resource efficient and convenient
- The focus of the studies is on the kinetics of the compounds, particularly metabolism, and specific route of entry effects are not of interest at this stage. Intrperioneal implantation is preferred over sub-cutaneous exposure in order to maximize the concentrations seen by the liver before exhalation in the lungs.
- We are not at this stage attempting to mimic real-life exposure scenarios (which may be inhalation and/or dermal) and the modifications in relative composition of the fuel due to selective absorption

- Concentrations in both blood and tissue will be able to be maintained at or near steady-state for extended periods of time

Dosing solutions will be as follows:

- *Neat component:* nonane, benzene: 0, 50, 100 mg/kg: 5 dosing groups
- *Mixture*: nonane, benzene spiked at 0, 50, 100 mg/kg each in JP-8 (750 mg/kg): 5 dosing groups

Both the neat JP-8 and the mixtures will be emulsified (5% w/w) in a 6% solution of Volpo20 (polyethylene glycol-20 oleyl ether, Croda, Mill Hill PA) in physiological saline, to minimize the potential interaction of the JP-8 with pump and the rat tissue.

*Surgical Implantation of Alzet Osmotic Minipump:.*

Sterile Alzet Mini-Osmotic pumps will be obtained from the Alza Company, Palo Alto, CA. Prior to implantation of osmotic pumps a small area on the abdominal area is clipped and cleaned with betadine solution veterinary, Povidone-iodine(titratable iodine, 0.5%) is distributed by the Purdue Frederick company of Norwalk, CT. Sterile equipment will be used at all times (sterilized using steam autoclaving). The pumps will be incubated in 0.9%NaCl overnight in a water bath at body temperature. After the pumps are incubated, the rats will be anesthetized by inhalation of 50%$CO_2$/50%$O_2$ gas mixture from the supplier. A small incision will be made at the prepared site, the pumps will be inserted into the abdomen and the incision closed with surgical staples.

After recover from surgery, each animal will be placed in a whole body metabolism cage for the collection of urine and feces during the course of the study.

Blood samples (100μL) will be collected from the tail vein following dosing at 1, 2, 4, 8, 12 and 24 h (and, if necessary, daily thereafter), and analyzed for total jet fuel and specific components (see *Analytical Methods* below).
Tissue samples will include fat, liver, brain and muscle. Serial tissue samples (1h, 24h) will be taken in the JP-8 dosed groups. In the neat nonane and benzene groups, tissue samples will be taken at the end of the experiment (24h). Serial tissue sampling will increase the number of groups by 10.

*Analytical Methods:*

A Hewlett-Packard 5890 gas chromatograph with a 5971 series mass spectrometric detector will be used for liquid JP-8 separation and identification work with a 0.25mm X 60m SPB-1 column. JP-8 is a mixture of hundreds of components. Identities will be determined for the components with the larger concentrations. Identities of the smaller concentration components will be found only if they are well separated.

Liquid injections of JP-8 will also be run on a Gas Chromatograph/Flame Ionization Detector for JP-8 separation and quantification. A similar 0.20mm X 60m SPB-1 column will be used. JP-8 components of interest will be separated and quantified from the average of several injections. JP-8 and spiked JP-8 samples will be separated and quantified. FID gives similar area/wt response to unsubstituted hydrocarbons. This was checked with six unsubstituted hydrocarbons and confirmed. Therefore, areas will be used to determine the component and total JP-8 sample weights.

A small blood or tissue sample from a JP-8 exposed rat will be placed in a 20 mL headspace vial. A Tekmar 7000/7050 Headspace Sampler will be used for injection from a headspace vial at 140 °C. The sample will separated and quantified on a Varian 3700 Gas Chromatograph /Flame Ionization Detector with a 0.53mm X 30m SPB-1. JP-8 standards will be prepared in headspace vials with control blood or tissue.

*Data Analysis:*
The data collected in the present study will be combined with data previously collected in our laboratory (nonane kinetics, Robinson, 2000) and available in the literature in order to further develop and validate a model for mixtures interaction. The mathematical structure of the model, based on competitive inhibition of P-450 metabolism has already been outlined (Robinson, 2001a, b). As shown in the *Background*, this model describes JP-8 as a "lumped" chemical with a total concentration $c_{tot}$ and inhibition constants $K_x = K_n$ and $K_b$ for nonane and benzene metabolism respectively (equations 2 and 3). The present data will be used to validate this model and to estimate values for $K_n$ and $K_b$.

For PBPK modeling, ACSL software (AEgis Technologies, Huntsville, AL) or equivalent will be used.

**Animals required**

Table 1. Six animals will be used for each dose group:

| **Experiment** | **Rats (#)** |
|---|---|
| Pilot range-finding study | 5 |
| Neat component (5 dose groups) | 30 |
| Mixture with JP-8 (5 dose groups) | 30 |
| Mixture with JP-8, serial tissue sampling (1h, 24h) (10 groups) | 60 |
| TOTAL | 125 |

**B. Laboratory Animals Required and Justifications:**

**1. Non-Animal Alternatives Considered:**

**2. Animal Model and Species Justification:** Rats were chosen for several reasons:

(1) Extensive data bases on rat kinetics for hydrocarbon components of fuel.
(2) Surgical procedures are more easily performed on rats than smaller animals.
(3) The higher yield of tissue from rats allows for more information to be obtained from fewer numbers of animals.
(4) Rats are used as a standard test species for toxicological studies.

**3. Laboratory Animals:**

**a. Genus & Species:** *Rattus norvegicus*

**b. Strain/Stock:** CDF$^R$(F344)/CrlBR

**c. Source/Vendor:** Charles River Laboratories

**d. Weight:** 150-200g at receipt.

**e. Sex:** Male

f. **Special Considerations**: N/A

g. **Other**: N/A

4. **Total Number of Animals Required:** Rats - 125

5. **Refinement, Reduction, Replacement:**

a. **Refinement**: The use of anesthesia and euthanasia

b. **Reduction**: This study will result in the development of a predictive mathematical model for component interactions in JP-8. Such a model, while requiring a significant number of animals to develop and validate, will significantly reduce animal use in the long-term by replacing animal studies with model predictions under a wide variety of exposure conditions.

c. **Replacement**:

C. **Technical Methods**

1. **Pain**

a. **USDA (Form 18-3) Pain Category:**

(1) **No Pain __(#) ____% (Column C)**

(2) **Alleviated Pain _125 (#) __100___% (Column D)**

(3) **Unalleviated Pain or Distress ._(#) ._% (Column E)**

b. **Pain Alleviation**

(1) **Anesthesia/Analgesia/Tranquilization**: Anesthesia (prior to surgery): the rats will be anesthetized by inhalation of 50%$CO_2$/50%$O_2$ gas mixture from the supplier. This procedure will be performed by Mr. Dick Godfrey.

(2) **Paralytics**: N/A

c. **Alternatives to Painful Procedures**:

(1) **Source(s) Searched**: AWIC, ARICOLA, CAAT, MEDLINE, TOXLINE.

(2) **Date and Number of Search**: Dec. 16, 2002, A2003001

(3) **Key Words of Search**: Pain, analgesia, surgery, rat

(4) **Result of Search:** The literature search found no less invasive procedures which allowed the controlled delivery of the chemicals described in this protocol, while studying their kinetics.

d. **Painful Procedure Justification**: Surgery is required to implant the osmotic mini-pump.

2. **Prolonged Restraint**: N/A

3. **Surgery**

**b. Pre- and Postoperative Provisions:**

Pre-surgery, all rats are cared for by the vivarium staff and maintained in accredited animal rooms.

Post-operative care: animals will be allowed to recover from the surgical implant of the ALZET osmotic minipump. Following surgery, animals will be housed separately in room 18, building 838, and allowed to recover from anesthesia. Recovering animals will be placed on a heating pad and monitored continuously until conscious. Food and water will be provided ad libitum. The animals will be monitored by Tim Bausman/Dick Godfrey, the attending veterinarian, and the VSF staff. A "Research Rodent Surgical Report", and "Clinical Observations Form" will be filled out for each individual rodent by the technician assigned to protocol. Recovered animals will then be returned to room 157 in building 79.

If the animal appears to be experiencing pain or distress that cannot be relieved, it will be euthanized by $CO_2$ overdose under the supervision of the attending veterinarian. The veterinarian will inform the PI or CO-PI of a decision to carry out euthanasia before killing the animals. The attending veterinarian will have the final authority for ordering euthanasia to be carried out for sick or distressed animals that cannot be relieved through medication or other courses of action. Once the animal has been euthanized, it will be placed in buffered formalin until necropsy can be performed on the animal.

**c. Location:** Rat surgeries under anesthesia and recovery will be completed in Building 838, room 18. Subsequent experimentation (blood sampling and euthenasia) will take place in Bldg. 79, room 157.

**d. Multiple Survival Surgery Procedures**: N/A

**(1) Procedures**: N/A

**(2) Scientific Justification**: N/A

4. **Animal Manipulations:**

**a. Injections:** Anesthesia (see Sect C1b(1) above)

**b. Biosamples:** Blood samples (tail vein); tissue samples (brain, muscle, liver, fat) following euthanasia

**c. Animal Identifications:** cage cards

**d. Behavioral Studies**: N/A

**e. Other procedures**: euthanasia (see below)

5. **Adjuvants**: N/A

6. **Study Endpoint**: The study endpoint for the animals is euthanasia.

7. **Euthanasia**:

Rats will be euthanized by exsanguination following anesthesia.

All procedures will be conducted by Mr. Dick Godfrey.

**D. Veterinary Care:**

**1. Husbandry Considerations**:

Receipt, quarantine, and general husbandry will be performed by vivarium personnel in accordance with Vivarium Support Function SOP's and the National Research Council's "Guide for the Care and Use of Laboratory Animals". Upon receipt at Bldg. 838 the rats will be housed individually with each lot segregated from all other rodents for a 7-10 day quarantine/acclimation period. All animals will be observed at least twice daily by vivarium personnel for general condition and any signs of illness will be recorded. A polycarbonate shoebox caging system with cellulose fiber contact bedding (Cell-Sorb Plus, A.W. Products, Inc., New Philadelphia, OH) will be the primary housing unit. Rats will be changed into freshly bedded and sanitized cages at least once per week. Rodent chow (#5008, Purina Mills, Inc., St. Louis, MO) and fresh conditioned (reverse osmosis) water will be available *ad libitum*. Prior to surgery all rats will be kept in sanitized animal holding rooms designed to provide 10-15 complete fresh air changes per hour. Room air temperature and humidity will be maintained between 21-26$^{o}$ C and 35-65%. Rodent cage racks will remain inside Bio-Clean mass air displacement units, which provide a constant supply of HEPA filtered air. Electronically controlled full spectrum fluorescent light will be provided on a 12:12 hour light:dark cycle.

**2. Attending Veterinary Care**: NA

**3. Enrichment Strategy**:

**a. Dogs**: NA

**a. Nonhuman Primates**: NA

**b. Rodents**: Nylabone bones will be provided for environmental enrichment.

**E. Data Analysis:**

**F. Investigator & Technician Qualifications/Training:**

Mr. Dick Godfrey
Procedures: anesthesia, euthanasia
Qualifications: Mr. Godfrey has over 20 years experience administering anesthesia, euthanasia and conducting various animal handling techniques.

Dr. Jeffery M. Gearhart
Procedures: Consultant for all procedures
Qualifications: Dr. Gearhart is a Research Manager at AFRL/HEST. Dr. Gearhart has over 25 years experience in toxicology research while at Toxic Hazards Division (Armstrong Laboratory), Lovelace Inhalation Toxicology Research Institute, and New York University.

All personnel performing experimental animal manipulations are technically competent and have been properly trained. All personnel have completed the Investigators Training course provided and have a copy of the "Manual for the Care and Use of Research Animals on Wright-Patterson Air Force Base".

## VI. BIOHAZARD/SAFETY:

Chemicals planned for use in this study that are biohazardous include: Benzene

Personnel will take proper safety precautions to protect from biohazards, such as the use of laboratory protective gear (lab coats, gloves, safety glasses, etc.). All of these chemicals will be used in a hood, and properly stored and disposed according to Wright-Patterson AFB hazardous material/hazardous waste management guidelines.

## VII. ASSURANCE:

As the Primary Investigator of this protocol I provide the following assurance:

**A. Animal Use**: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a deviation is specifically approved by the IACUC.

**B. Duplication of Effort**: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

**C. Statistical Assurance**: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

**D. Biohazard/Safety**: I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, etc., in the preparation of this protocol, recombinant issues, etc., in the preparation of this protocol.

**E. Training**: I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused because of the procedures/manipulations.

**F. Responsibility**: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" which the DoD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible and conducting humane and lawful research.

_____

Peter J. Robinson

**G. Painful Procedures**: I am conducting biomedical experiments that may potentially cause more than momentary or slight pain or distress to animals that will be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of the proposed experiment.

_____

Peter J. Robinson

## VIII. Enclosures:

A. References
B. WPAFB Animal Procotol Form
C. Material Data Safety Sheets

## REFERENCES


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