The Use of Liver Microsome *In-Vitro* Methods to Study Toxicant Metabolism and Predict Species Efficacy

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ABSTRACT: Liver microsomes are used extensively in human pharmaceutical development to study the metabolism of compounds of interest; however, they are rarely part of the development of toxicants for the control of wildlife. Liver microsome samples from wildlife of interest would likely be harvested in the field without access to laboratory perfusion equipment. Therefore, the metabolic activity of microsomes from perfused and non-perfused livers was compared. There was no significant difference in diphacinone metabolism by liver microsomes from both perfused and non-perfused Wistar rat livers, although chlorophacinone metabolism was significantly different. There are often significant differences in metabolism between species that can be utilized to increase toxicant specificity. In this study bobwhite quail liver microsomes metabolized more diphacinone and chlorophacinone than both Wistar and brown rats. This information can be used during toxicant development to help determine the most sensitive species to toxicants of interest. Additionally, the effect of incubation time on toxicant metabolism was examined to determine optimal experimental conditions. The data from these experiments support the use of liver microsomes as a tool to be used during toxicant development to provide information that can be incorporated into whole animal studies.

KEY WORDS: chlorophacinone, cytochrome p450, diphacinone, liver microsomes, metabolism, rodenticides, toxicant development

INTRODUCTION

Many of the toxicants used to control wildlife, such as chlorophacinone, diphacinone, and zinc phosphide, are formulated for oral delivery. Although this method is convenient and offers numerous benefits, drugs that are absorbed through the GI tract will, most often, pass through the portal circulation into the liver prior to reaching the site of action. The liver is the major organ responsible for biotransformation and metabolism. This metabolism serves to detoxify and ultimately eliminate chemicals from the body. Efficient metabolism and elimination of toxicants serves as an evolutionary advantage, but poses a major hurdle in wildlife toxicant development.

Toxicant metabolism is characterized by two types of enzymatic reactions, namely phase I and phase II. Phase I reactions involve oxidation, reduction, or hydrolysis and are performed by a class of enzymes called cytochrome p450 (Klassen 2001). These enzymes are located in the endoplasmic reticulum of many tissues but occur in very high density in the liver. Metabolic reactions catalyzed by cytochrome p450 enzymes can reduce the toxicity of a compound by increasing its water solubility, thereby enhancing excretion. Phase I reactions may also produce metabolites with greater toxicity than the parent compound. These processes can be examined through the isolation of and experimentation using liver microsomes.

Early knowledge regarding metabolic fate of potential toxicants for the control of wildlife population helps in the development process by allowing for the selection of the most promising candidates, based on criteria such as their efficacy, duration of action, or threat to non-target species. By isolating liver microsomes, multiple *in-vitro* experiments can be performed to determine the extent of metabolism and possible interactions with other chemicals that have affinity for the same enzymes (Pelkonen et al. 1998, Wrighton et al. 1993).

In addition to helping in the toxicant development process, liver microsomes can be used to assist in the determination of possible risks to non-target species. Often the metabolic profiles of species will differ, which, while it poses a problem in veterinary medicine, can be exploited during toxicant development (Lewis et al. 1998, Fink-Gremmels 2008). If metabolic differences between target and a non-target species are identified, it remains possible to modify toxicants to increase toxicity to the species of interest, while decreasing risks to non-target species.

Although much research has been done in the human health sciences using liver microsomes and metabolic determination, this line of research is just beginning to be used in wildlife management. Unlike conventional laboratory settings, harvesting livers for microsome isolation from wildlife species often occurs in the field with limited availability to instrumentation, limiting the tissue preparation that could be done. To test the viability of microsomes isolated using field techniques, a series of experiments were conducted to compare saline perfused and non-perfused microsomes. Also, differences in metabolism between Wistar rats, brown rats, and bobwhite quail were investigated. Finally, the effects of microsomal incubation time on total metabolism were characterized.

METHODS

Study Animals

Livers were harvested from Wistar and brown rats (*Rattus norvegicus*) and bobwhite quail (*Colinus virginianus*). Following euthanasia by CO_2, a longitudinal incision was made in the abdomen, and the liver was removed and weighed. The tissue was rinsed thoroughly

and either perfused with ice-cold 0.9% NaCl or immediately frozen in liquid nitrogen. The perfusion was continued until the liver appeared blanched; the perfused tissue was then snap-frozen in liquid nitrogen. The frozen samples were stored at -80°C until further processing.

Test Solutions

All test fortification formulations consisted of diphacinone or chlorophacinone solutions at approximately 40 ppm in phosphate (0.010 M) solutions buffered at a pH of 7.4. Solutions of magnesium chloride, phosphate buffer, and NADH were all 0.010 M. Homogenization buffer was as follows: sucrose 250 mM, HEPES 50 mM, KCl 25 mM, $MgCl_2$ 5 mM, EDTA 0.1 mM, adjust pH to 7.4. Cofactor solution was made by adding 11.5 mg NADP sodium salt, 5.2 mg glucose-6-phosphate, and 50 μL glucose-6-phosphate dehydrogenase to 950 μL 0.01 M MgCl in phosphate buffer.

Liver Microsome Preparation

Livers microsomes were isolated using differential centrifugation according to the method by Pelkonen et al. (1974) with minor alterations. Frozen liver samples were minced, weighed, and transferred to a Teflon pestle/glass homogenizer with 2 volumes (w/v) homogenization buffer with the addition of phenylmethanesulfonyl fluoride (Sigma) 2.5 μL/ mL homogenization buffer. The tissue was homogenized with 6 passes of the Teflon pestle homogenizer (Wheaton Overhead Stirrer, Wheaton Science Products, Millville, NJ). The homogenates were centrifuged at 10,000 g for 10 min at 4°C (Beckman Coulter Avanti J-301, Beckman Coulter Inc., Brea, CA). The supernatants were transferred to a clean centrifuge tube and spun at 15,000 g for 20 minutes at 4°C. The supernatant was then transferred to ultracentrifuge tubes and spun at 105,000 g for 60 minutes at 4°C. The pellets were then washed with approximately 1 mL homogenization buffer, transferred to the Teflon pestle/glass homogenizer and resuspended in homogenization buffer (1 g w/ 0.8 mL vol), and spun at 105,000 g for 60 minutes at 4°C. The supernatants were discarded, and the remaining pellets were resuspended in homogenization buffer using Teflon pestle/glass homogenizer (1:1, w:v) and frozen at -80°C.

Microsome Incubation

Microsome incubations were performed using 50 μL microsome extract, 50 μL cofactor solution, the analyte, and 0.01 M phosphate buffer added to bring volume to 500 μL. These incubations contained 2.4 ppm of either diphacinone or chlorophacinone. Incubations were done at 37°C for 60 minutes unless otherwise noted.

Residue Determination

Chlorophacinone and diphacinone residue determination was completed by quenching 0.400 mL of the incubation solution with 0.600 mL of methanol containing 5 mM tetrabutylammonium phosphate and vortex mixing. These samples are filtered with 0.45 μm Teflon syringe filters prior to analysis by reverse phase ion-pairing high performance liquid chromatography (HPLC) using the following parameters: 55:45 methanol:

water w/5 mM tetrabutylammonium phosphate, 10 mM phosphate buffer @ pH = 8.5, octadecyl silane column 150 mm × 3.0 mm particle size 3 μm at 0.300 mL/min at 35°C, UV detection at 325 nm with UV spectral confirmation using an Agilent 1100 with ChemStation software (Agilent Technologies Inc., Santa Clara, CA).

Statistical Methods

Data are representative of 4 samples per group and are expressed as mean ± SEM. Data were tested for homogeneity of variance and normality. The differences between groups were analyzed using Student's t-test.

RESULTS

Metabolic activity of microsomes from both perfused and non-perfused Wistar rat livers was examined using both diphacinone and chlorophacinone as substrates (Figure 1). Perfusion did not significantly affect the percent of diphacinone metabolized, although there was a significant difference in the amount of chlorophacinone metabolized between microsomes from the perfused livers (60% chlorophacinone metabolized) and non-perfused livers (52% metabolized) (p = 0.05). Comparing perfused liver metabolism of chlorophacinone and diphacinone showed that more diphacinone is metabolized than chlorophacinone. Microsomes extracted from perfused livers metabolized 95% of diphacinone and only 60% of chlorophacinone in the 60-minute incubation (p < 0.01). The same pattern occurred in microsomes from non-perfused livers, with 91% of diphacinone being metabolized and 52% of chlorophacinone (p < 0.01).
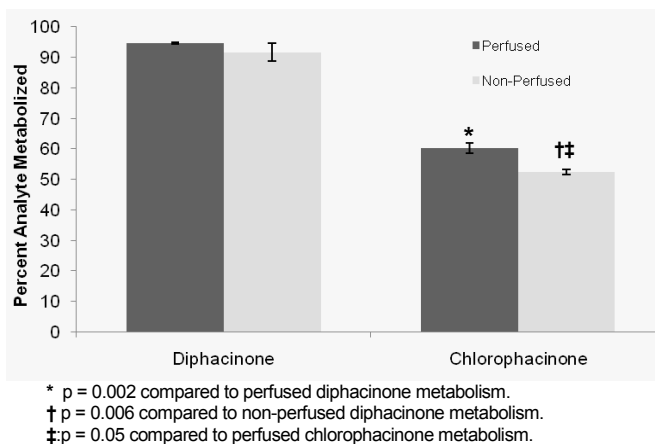

* p = 0.002 compared to perfused diphacinone metabolism.
† p = 0.006 compared to non-perfused diphacinone metabolism.
‡:p = 0.05 compared to perfused chlorophacinone metabolism.

Figure 1. Percent diphacinone metabolized (average ± SEM) in incubations using liver microsomes from perfused and non-perfused Wistar rat livers.

To investigate species differences in metabolism, liver microsomes from Wistar rats, brown rats, and bobwhite quail were incubated with diphacinone and chlorophacinone (Figure 2). Wistar rats metabolized significantly less chlorophacinone than both brown rats and bobwhite quail (53%, 66%, and 71% respectively, p < 0.05). The same trend was true for diphacinone, although the difference in metabolism is only significant between Wistar rats and bobwhite quail, with Wistar rats metabolizing 78% of the diphacinone, brown rats metabolizing 86%, and bobwhite quail metabolizing 93% (p < 0.05).

In each species tested, the metabolism of diphacinone was significantly higher than that of chlorophacinone. Wistar rats had a difference in metabolism between diphacinone and chlorophacinone of 25%, in brown rats the difference was 20%, and in bobwhite quail the difference was 22% (p < 0.05).
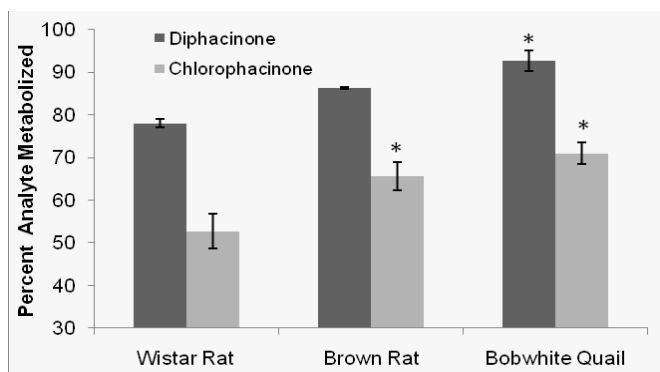
$*$ p < 0.05 compared to Wistar rat metabolism

Figure 2. Percent diphacinone and chlorophacinone metabolized by liver microsomes from Wistar rats, brown rats, and bobwhite quail.
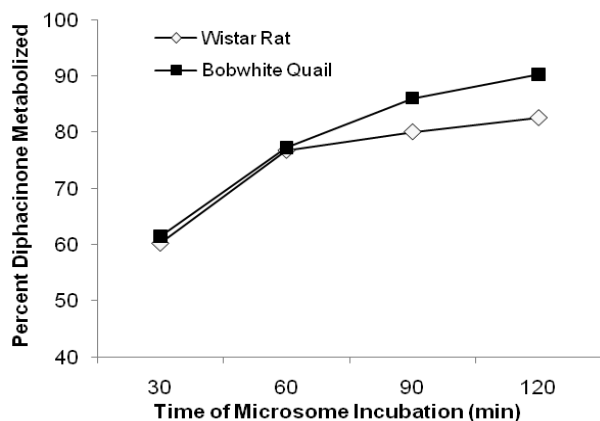

Figure 3. Time course study comparing percent of diphacinone metabolized by Wistar rat and bobwhite quail microsomes following 30, 60, 90, and 120 minutes of incubation.

The effect of incubation time on amount of diphacinone metabolized was investigated using both Wistar rats and bobwhite quail. As incubation time increased so did the percent of diphacinone metabolized, from 60% after 30 minutes of incubation to 83% following 120 minutes of incubation. The bobwhite quail data showed the same increase in percent metabolized with increasing incubation time. After 30 minutes of incubation, bobwhite quail metabolized 61% of the diphacinone, increasing to 90% with 120 minutes of incubation (Figure 3).

DISCUSSION

For liver microsomes to be a beneficial tool used in the development of compounds for the control of wildlife, livers must often be harvested in the field where access to laboratory equipment is limited. Specifically, it would be beneficial if livers could be harvested in the field without the burden of tissue perfusion with saline. Therefore, the metabolic activity of microsomes from non-perfused livers was compared with those harvested using the traditional perfusion methodology. There was not a significant difference in diphacinone metabolism between the two groups, although there was a significant difference in chlorophacinone metabolism. The important information from microsome experiments used in toxicant development is the relative amount of metabolism of various compounds. There was no significant difference in the ratios of chlorophacinone to diphacinone metabolized between perfused and non-perfused liver microsomes (data not shown). Since comparisons of the metabolism of chemicals used for control would, in theory, be performed with non-perfused livers, the difference in metabolism between perfused and non-perfused would not affect results. Use of microsomes from non-perfused livers will still enable researchers to screen possible toxicants to streamline the development process. By eliminating the perfusion step, harvesting livers for subsequent microsome extraction becomes a technique that can realistically be done by technicians in the field with little additional training.

It is possible to study the metabolic profiles of a wide variety of animals through the use of liver microsomes. These *in-vitro* experiments enable researchers to study multiple compounds of interest without the burden of numerous liver animal studies. An example of this is the series of experiments comparing the metabolism of diphacinone and chlorophacinone in Wistar rats, brown rats, and bobwhite quail. It is evident that more diphacinone than chlorophacinone was metabolized under the same conditions in all of the species studies. Although there are wide variations in the published toxicity data, these results are congruent, with the Wistar rat LD_{50} for diphacinone (7.0 mg/kg) being higher than that for chlorophacinone (3.15 mg/kg) (Fishel 2005, reviewed in Eisemann and Swift 2006). These differences in toxicity are what would be predicted using only the liver microsome data. Since more of the diphacinone than chlorophacinone is metabolized, it follows that diphacinone would be less toxic.

In addition to mirroring the differences between *in-vivo* toxicity of diphacinone and chlorophacinone, liver microsome data also demonstrate differences in species toxicity. In the liver microsome experiments, bobwhite quail metabolized 15% more diphacinone and 18% more chlorophacinone that Wistar rats, with brown rat microsome metabolism at an intermediate level between the two. These *in-vitro* data indicate that both diphacinone and chlorophacinone should be less toxic to bobwhite quail than rats; this is the trend that is found in whole animal toxicity tests that report an LD_{50} for bobwhite quail between 400 and 2,000 mg/kg, with rat LD_{50} being 10 to 100 times higher (US EPA 1998, reviewed in Eisemann and Swift 2006). The ability to compare relative toxicities between species is important during toxicant development, as this information can be used to direct future whole animal toxicity tests to include the more sensitive species.

One of the variables that must be considered when

conducting microsome incubations is the amount of time the microsomes are incubated with the compound of interest. If the incubation time it too short, possible differences in metabolic activity may not be evident (Figure 3). To determine the differences in metabolism between Wistar rats and bobwhite quail, it is important to allow the microsomes to metabolize the diphacinone for more than 60 minutes to detect differences. If the microsome experiments were terminated after 30 minutes, information about the distinct metabolic profiles would be lost.

Advances are being made to use data from liver microsome experiments to estimate metabolism and therefore *in-vivo* doses. Most of the work to date has been aimed at predicting drug clearance in humans, although many of these studies use preclinical animal data (Riley et al. 2005, Boxenbaum 1982). Many papers have been published concerning the allometric scaling of data from microsome studies to *in-vivo* dosing and clearance (Obach 1999). Although little work has been done to scale microsome data to whole animal drug clearance and toxicity in wildlife, this area of research shows promise in helping scientists choose compounds for further development. The ability to estimate starting doses for *in-vivo* toxicity tests will not only save time and money for investigators, but it also could decrease the number of experimental animals used to determine toxicity levels.

ACKNOWLEDGEMENTS

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