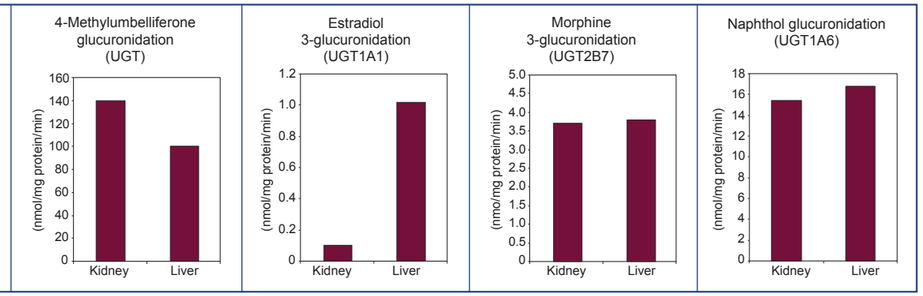
Figure 5: Comparison of UGT activities in human kidney and liver microsomes



Results

Pooled samples of kidney S9 and microsomes were prepared from Sprague Dawley rat, Beagle dog, Cynomolgus monkey and human, and assayed for NADPH-cytochrome *c* reductase, CYP4A, CYP3A, FMO and UGT activity. The results are shown in **Table 2** and **Figures 1-2.**

Individual samples of human renal S9 and microsomes were assayed for lauric acid 12-hydroxylase activity to assess the inter-individual variation in CYP4A expression. Lauric acid 12-hydroxylase activity varied about four fold in S9 (from 43.5 to 192 pmol/mg protein/min) and about seven fold in microsomes (from 119 to 843 pmol/mg protein/min) from one individual to the next, as shown in **Figure 4.**

UGT activity in human kidney microsomes was assessed and compared to human liver microsome activity. Human kidney UGT activity equals liver in terms of umbelliferone glucuronidation, UGT1A6 and UGT2B7; but kidney has substantially less UGT1A1 activity, as shown in **Figure 5.**

To assess the stability of human and animal renal subcellular fractions, pooled samples were subjected to several freeze/thaw cycles, and analyzed for lauric acid 12-hydroxylation, umbelliferone glucuronidation and NADPH-cytochrome *c* reduction. As shown in **Figure 3**, renal samples prepared by XenoTech's subcellular fractionation protocol can be repeatedly frozen and thawed at least ten times without significant loss of CYP4A activity. Similar results were obtained for umbelliferone glucuronidation and NADPH-cytochrome *c* reduction (results not shown).

To assess the long-term stability of renal S9 and microsomes, samples were stored for 6 months at -70^{o}C or below, and analyzed intermittently for lauric acid 12-hydroxylase, UDP-glucuronosyltransferase, and NADPH-cytochrome *c* reductase activity. Renal subcellular fractions prepared by XenoTech's subcellular fractionation protocol can be stored for at least 6 months without significant loss of enzymatic activity (results not shown).

Conclusions

This study describes a method to prepare subcellular fractions (S9 and microsomes) from human and animal kidneys. The subcellular fractions have high levels of anticipated drug-metabolizing enzyme activity (CYP, FMO, UGT) and are stable both to repeated freezing/thawing and to long-term (>6 months) storage.

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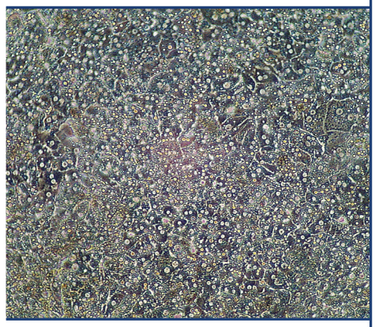

Microsomes and S9 Prepared from Renal Tissue Yield High CYP, FMO and UGT Activities that are Stable over Multiple Freeze/Thaw Cycles

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Abstract

We have developed procedures to prepare microsomes and S9 fraction from the kidneys of humans, Cynomolgus monkeys, Beagle dogs and Sprague Dawley rats, and we have assessed selected CYP, FMO and UGT activity in pooled and, in the case of humans, individual preparations. These microsomal samples were analyzed for their ability to catalyze the 12-hydroxylation of lauric acid (a marker of CYP4A activity), the *N*-oxygenation of benzydamine (a marker of FMO activity), the *N*-demethylation of benzydamine (a marker of CYP3A activity) and the glucuronidation of 4-methylumbelliferone (a reaction catalyzed by several forms of UDP-glucuronosyltransferase). We have also established that renal microsomes and S9 fraction can be subjected to as many as 10 freeze/thaw cycles with little or no loss of CYP4A, UGT, or NADPH-cytochrome *c* reductase activity. These results suggest that our method for processing kidney tissue is well suited to preserving the enzymatic activity of renal microsomes and S9 fraction, such that these subcellular fractions can be used to assess the Phase 1 and Phase 2 metabolism of drugs in the kidney.

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Introduction

Drug-metabolizing enzymes in the kidney play a significant role in the metabolism of many xenobiotics. Human liver microsomes contain multiple CYP enzymes belonging to the CYP1A, 2B, 2C, 2D, 2E, 3A and 4A subfamilies, whereas human kidney microsomes contain relatively high levels of CYP4A and CYP4B enzymes. The human kidney also expresses high levels of certain Phase II drug-metabolizing enzymes including UGT1A6, UGT1A9/10 and 2B7 as well as SULT1C2, DT-diaphorase (NQO1) and the efflux transporter p-glycoprotein (MDR1).

Kidneys have many metabolic responsibilities including the hydroxylation of fatty acids and their derivatives, which is one of the functions of the CYP4A and CYP4B enzymes expressed in renal microsomes. There is increasing interest in renal metabolism due to the significant role that kidneys play in the metabolism of drugs and the fact that the kidney is a major site of drug-induced toxicity.

The focus of this study was to prepare renal S9 and microsomes from multiple species and to characterize the enzymatic activities of the subcellular fractions. Drug-metabolizing enzymes are unevenly distributed throughout the kidney medulla and cortex. For the purpose of this project, we used the whole kidney to prepare subcellular fractions with the aim of supporting *in vitro* studies of renal drug metabolism. We also determined the stability of the enzymes when renal subcellular fractions were subjected to repeat freeze/thaw cycles and long-term storage at -70ºC or below.

Methods and Materials

In this study, renal S9 (post-mitochondrial supernatant) and microsomes were prepared by procedures originally developed for processing liver samples (Pearce *et al.*,1996).

Renal S9 and microsomes were prepared from Sprague Dawley rats, Beagle dog, Cynomolgus monkey and human. All laboratory animals were from reputable AAALAC-accredited and FDA-regulated facilities. Only sexually mature laboratory animals were used for this project. The pool of human S9 and microsomes were prepared from five males and three females.

Preparation of S9

Rodents were anesthetized, decapitated and the organs were perfused with a buffered solution (50 mM Tris HCl pH 7.4 at 4ºC, 150 mM KCl, 2 mM EDTA). The kidneys were removed, snap-frozen on liquid nitrogen and stored frozen (at or below -70ºC) until processing. Dog and primate tissue arrived frozen on dry ice and was stored frozen (at or below -70ºC) until processing. Non-transplantable human kidneys were obtained with informed consent from donors harboring no known infectious diseases. The tissues were mechanically disrupted in homogenization buffer, as described above, and homogenized with a Teflon pestle as described by Pearce *et al.* (1996). The homogenates were subjected to centrifugation at 12,000 g_{max} at 0-8ºC for 20 minutes to prepare the postmitochondrial (S9) fraction. This procedure was conducted on both animal and human kidneys.

Preparation of microsomes

Microsomes were prepared by ultracentrifugation of the postmitochondrial (S9) fraction at 104,000 g_{max} at 4ºC for 60 minutes. The initial microsomal pellet was resuspended in wash buffer (10 mM EDTA pH 7.4 containing 150 mM KCl) and homogenized with a motor-driven Teflon pestle. The washed microsomes were then re-isolated by the same ultracentrifugation process, resuspended in 250 mM sucrose and stored at or below -70ºC.

Enzymatic assays

Renal S9 and microsomes were assayed for various Phase I and Phase II drug-metabolizing enzymes. The 12-hydroxylation of lauric acid was measured based on a - of cytochrome *c*, a measure of NADPH-cytochrome P450 reductase, was measured based on a method described by Phillips and Langdon (1962). The glucuronidation of 4-methylumbelliferone, a measure of UPD-glucurono-
syltransferase activity, was measured based on a method described by Burchell *et al.* (1995). The *N*-oxygenation of benzydamine, a measure of flavin monooxygenase (FMO) activity, and the *N*-demethylation of benzydamine, a measure of CYP3A activity, were both measured based on the method described by Rettie *et al.* (1995).

The freeze/thaw cycles were conducted by placing the S9 and microsome vials in a room temperature water bath until the fraction was visibly thawed (~10 minutes). The vials were then placed at -70ºC or below for an hour to refreeze the sample. This process was repeated until the desired numbers of cycles were completed for each sample.

Tables and Figures

Table 1: Donor demographics for human renal pools

Donor	Gender	Age (Yrs)	Race	Cause of Death	Chronic Medications	Alcohol use	Tobacco use	Donor	Gender	Age (Yrs)	Race	Cause of Death	Chronic Medications	Alcohol use	Tobacco use
1	Female	63	Caucasian	CVA	None	No recent use	1-2 PPD	5	Male	56	Caucasian	ICH	None listed	No recent use	1 PPD
2	Male	58	Caucasian	ICH	None	Occasional use	1 PPD	6	Female	63	Caucasian	ICH	None listed	Occasional use	None
3	Male	60	Caucasian	CHI	None listed	Occasional use	2 PPD	7	Male	39	Caucasian	CVA	None	Occasional use	None
4	Male	26	Caucassian	MVA	None listed	None	None	8	Female	69	Caucassian	CVA	Prilosec, Phenobarbital, Coumadin	None	None

CVA: Cerebrovascular aneurysm ICH: Intracranial hemorrhage CHI: Closed head injury MVA: Motor vehicle accident PPD: Packs of cigarettes per day

Figure 1: Species differences in renal microsomal Phase I drug-metabolizing enzymes

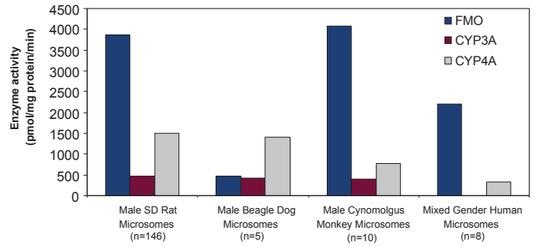

Figure 2: Species differences in renal microsomal Phase II drug-metabolizing enzymes (logarithmic scale)

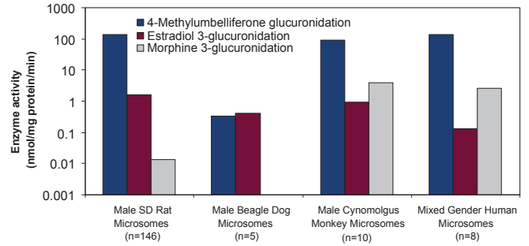

Figure 3: Species differences and stability of CYP4A (lauric acid 12-hydroxylase) activity in renal S9 and microsomes

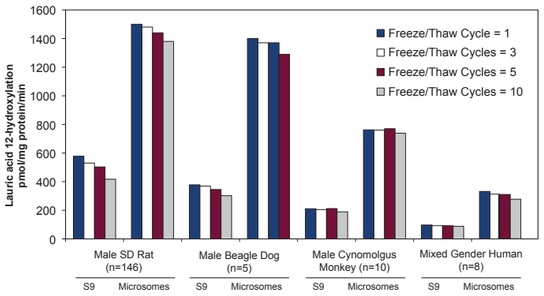

Figure 4: Inter-individual differences in human renal S9 and microsomal CYP4A activity as determined by lauric acid 12-hydroxylation

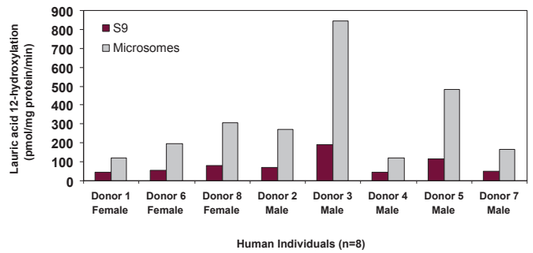

Table 2: Enzymatic activities in renal microsomes

Species[a]	NADPH-cytochrome *c* reductase (nmol/mg protein/min)		Lauric acid 12-hydroxylation (CYP4A) (pmol/mg protein/min)		Benzydamine *N*-demethylation (CYP3A) (pmol/mg protein/min)		Benzydamine *N*-oxygenation (FMO) (pmol/mg protein/min)		4-Methyl-umbelliferone glucuronidation (UGT) (nmol/mg protein/min)		Morphine 3-glucuronidation (UGT2B) (nmol/mg protein/min)		Estradiol 3-glucuronidation (UGT1A) (nmol/mg protein/min)	
	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver
Sprague Dawley Rat, Male (n=146)	33	180	1500	1830	460	2350	3860	6620	140	242	0.013	10	1.6	3.4
Beagle Dog, Male (n=5)	27	890	1400	NP	420	4770	480	7820	0.3	NP	0.001	16	0.4	10
Cynomolgus Monkey, Male (n=10)	28	200	760	NP	400	6780	4090	4880	90	NP	3.8	15	0.9	2.6
Human, Mixed Gender (n=8)	37	180	330	1570	0	1630	2210	1010	140	100	3.7	3.8	0.1	1

NP: Assay not performed
[a] Values in parenthesis are the number of individuals used to prepare the pooled kidney sample.