Human Liver S9 Fractions Stored at -70°C Maintain High Phase I and Phase II Enzymatic Activities Over Multiple Freeze/Thaw Cycles and for at Least 10 Years

Clayton J.M. Otwell, Zell Woodworth, and David Buckley
XenoTech, LLC, Lenexa, KS, USA

Introduction

Expanded interest in the biotransformation of xenobiotics by phase II enzymes and other various cytosolic enzymes has led to an increase in the use of S9 fractions in metabolic stability, clearance, and phenotyping studies. To ensure the integrity of an *in vitro* test system, it is important to understand the stability of metabolic enzymes during long-term storage and throughout multiple freeze/thaw cycles. Data regarding the stability of human liver microsomes have been previously published by multiple authors (Pearce *et al*, 1996, Yamazaki *et al*, 1996). However, metabolic enzyme stability for S9 stored for long periods and/or subjected to multiple freeze/thaw cycles has not been thoroughly investigated.

The objectives of this study were to evaluate the effects of long-term storage at -70°C or below and multiple freeze/thaw cycles on the enzymatic activities in human liver S9 fractions. Nine lots of pooled (n = 16 to 200) human liver S9 samples were prepared over a ten year period and stored at -70°C or below for up to 10 years. These S9 fractions were analyzed for their ability to catalyze reactions for various phase I and phase II enzymes including cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), glutathione S-transferase (GST), sulfotransferase (SULT), aldehyde oxidase (AO), and *N*-acetyltransferase (NAT). Additionally, a single lot of pooled (n = 200) human liver S9 samples were subjected to up to 10 freeze/thaw cycles and further evaluated for potential loss of enzymatic activities with increasing freeze/thaw cycles.

Materials and Methods

Human Liver S9:

Pools (n=16 to 200) of human liver S9 from non-transplantable donated tissue were prepared by XenoTech from 2001 through 2011. Frozen tissue samples were thawed in homogenization buffer (50 mM Tris.HCl, pH 7.4 at 4°C containing 150 mM KCl and 2 mM EDTA), and homogenized with a Polytron and motor-driven Teflon pestle and mortar. The homogenate was subjected to centrifugation at 12,000-13,000 g_{max} for 20±1 minutes at 0-8°C to prepare a postmitochondrial supernatant (*S9*) fraction. Each pool of human liver S9 was diluted with homogenization buffer and stored at -70°C or below. The sample ID and general description of each pool is summarized in **Table 1**.

Enzymatic Assays:

Comparison of multiple marker reactions to determine phase I and phase II enzyme stability was carried out with marker substrates (with [substrate]>K_m in all cases) according to the conditions summarized in **Table 2**.

Samples prepared in the years 2001, 2003, 2004, 2005, 2006, 2007, 2008, 2009, and 2011 were characterized at the time of preparation for 7-ethoxycoumarin O-dealkylation, 4-methylumbelliferone glucuronidation and 1-chloro-2,4-dinitrobenzine-glutathione conjugation. In this study, these data are referred to as ORIGINAL DATA in **Figures 1-3**. These samples were stored at -70°C or below and re-analyzed for the same marker reactions in 2011 to monitor the effects of long-term storage on CYP, UGT, and GST enzymes. The enzyme activity data determined in 2011 for each pool are referred to as RECENT DATA in this study. These nine pools were also analyzed for phthalazine oxidation, p-aminobenzoic acid acetylation, and sulfamethazine acetylation following 0-10 years in storage at -70°C or below.

To further assess the effects of storage conditions, a single lot of pooled (n=200) human liver S9 was subjected to 0, 2, 4, 6, 8 and 10 freeze/thaw cycles (freezing at -70°C or below followed by thawing in a room temperature water bath) and evaluated for potential loss of enzymatic activities. Following each freeze/thaw cycle the S9 pool was analyzed for its ability to catalyze reactions for CYP, UGT, GST, SULT, AO and NAT enzymes.

Table 1. Pooled Human Liver S9 Samples

Sample ID	Description
2001	Mixed gender human liver S9, pool of 16, prepared in 2001
2003	Mixed gender human liver S9, pool of 50, prepared in 2003
2004	Mixed gender human liver S9, pool of 50, prepared in 2004
2005	Mixed gender human liver S9, pool of 50, prepared in 2005
2006	Mixed gender human liver S9, pool of 50, prepared in 2006
2007	Mixed gender human liver S9, pool of 50, prepared in 2007
2008	Mixed gender human liver S9, pool of 50, prepared in 2008
2009	Mixed gender human liver S9, pool of 50, prepared in 2009
2011	Mixed gender human liver S9, pool of 200, prepared in 2011

Table 2. Summary of incubation conditions for measuring phase I and phase II enzme activities

Enzyme	Substrate	Substrate Concentration (µM)	Protein Concentration (mg/mL)	$KP0_4$ (mM)	$MgCl_2$ (mM)	EDTA (mM)	Tris (mM)	Time (min)	Method of Analysis	Temperature
CYP3A4/5	Midazolam	30	0.05	50	3	1	NA	10	LC/MS/MS or HPLC	37°C
CYP	7-Ethoxycoumarin	500	0.2	50	3	1	NA	10	LC/MS/MS or HPLC	
SULT	7-Hydroxycoumarin	500	0.2	50	3	1	NA	10	LC/MS/MS	
GST	1-Chloro-2,4-dinitrobenzene-glutathione (CDNB)	1	0.02	100*	NA	NA	NA	5	UV spectroscopy	
Multiple UGT enzymes	4-Methylumbelliferone	1	0.1	NA	10	NA	100	10	LC/MS/MS or HPLC	
UGT1A1	17ß-Estradiol	100	0.1	NA	10	NA	100	10	LC/MS/MS	
UGT1A9	Propofol	50	0.05	NA	10	NA	100	10	LC/MS/MS	
UGT2B7	Morphine	100	0.05	NA	10	NA	100	10	LC/MS/MS	
UGT1A6	1-Naphthol	500	0.01	NA	10	NA	100	10	LC/MS/MS	
UGT1A4	Trifluoperazine	250	0.25	NA	10	NA	100	5	LC/MS/MS	
AO	Phthalazine	20	0.05	50	NA	NA	NA	10	LC/MS/MS	
NAT1	*p*- aminobenzoic acid (PABA)	150	0.5	50	3	1	NA	10	LC/MS/MS	
NAT2	Sulfamethazine	600	0.5	50	3	1	NA	10	LC/MS/MS	

* pH 6.5

Results

Figure 1 shows the effects of long-term storage at -70°C or below over a period of up to 10 years on glutathione S-transferase (GST) activity in various pools of human liver S9. In pools stored from 0 to 10 years there was no significant decrease in the ability of human liver S9 to catalyze 1-chloro-2,4-dinitrobenzene glutathione conjugation. The greatest apparent decrease in GST activity (-24%) occurred in sample 2007, which was stored for four years prior to reanalysis. This decrease in measured enzyme activity is likely attributed to year-by-year fluctuations in standard values, which artificially introduces increases or decreases in enzymatic rates, rather to an actual loss of enzyme activity.

Figure 2 illustrates the effects of long-term storage at -70°C or below over a period of up to 10 years on UDP-glucuronosyltransferase (UGT) activity in pools of human liver S9. In pools stored from 0 to 10 years there was no significant decrease in the ability of human liver S9 to catalyze 4-methylumbelliferone glucuronidation.

Figure 3 shows the effects of long-term storage at -70°C or below over a period of up to 10 years on cytochrome P450 (CYP) activity in pools of human liver S9. In pools stored from 0 to 10 years there was no significant decrease in the ability of human liver S9 to catalyze 7-ethoxycoumarin O-dealkylation, as evidenced by comparing the Recent Data from all pools.

Figures 2-3, in which enzyme activity data analyzed at the time of preparation of the S9 fractions (Original Data) are compared to data analyzed following 0 - 10 years in storage (Recent data), consideration must be given to the methods of analysis. The Original Data for S9 pools 2001, 2003, 2004, 2005, and 2006 were analyzed with HPLC. The Original Data for the remaining pools and all Recent Data were analyzed with LC/MS/MS. Continuous improvements in analytical methods and assay incubation methods (manual vs. automated) can cause artificial differences between the Original Data and Recent Data. When comparing Original Data and Recent Data in samples 2007, 2008, 2009 and 2011 in which the same analytical methods were used the percent difference is smaller than in the samples prepared prior to 2007.

Figure 4 shows the effects of long-term storage at -70°C or below over a period of up to 10 years on aldehyde oxidase (AO) and *N*-acetyltransferase activities in various pools of human liver S9.

Figure 5 illustrates the effect of subjecting a pool (n=200) of human liver S9 to multiple freeze/thaw cycles and analyzing its ability to catalyze midazolam 1´-hydroxylation, glucuronidation of estradiol, propofol, naphthol, morphine and trifluoperazine, p-vanillin oxidation, 1-chloro-2,4-dinitrobenzene-glutathione conjugation and the acetylation of *p*-aminobenzoic acid and sulfamethazine. Freezing and thawing human liver S9 for up to ten cycles did not significantly decrease the activities of CYP, UGT, GST, SULT, AO, or NAT (typically less than 10%). In the worst case, GST activity decreased by 13% after 10 freeze/thaw cycles.

Figure 1. Effects of long-term storage on 1-chloro-2, 4-dinitrobenzene-glutathione conjugation as a measure of glutathione S-transferase in mixed gender human liver S9 pools

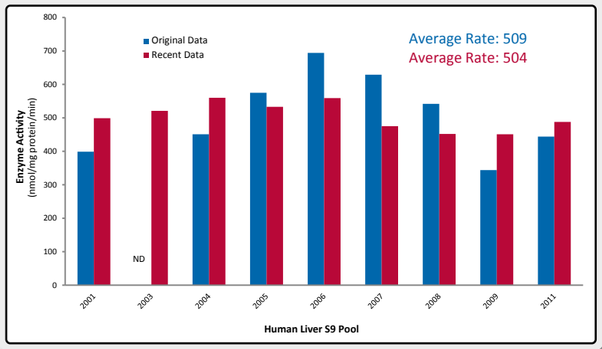


Figure 2. Effects of long-term storage on 4-methylumbelliferone glucuronidation as a measure of UDP-glucuronosyltransferase in mixed gender human liver S9 pools

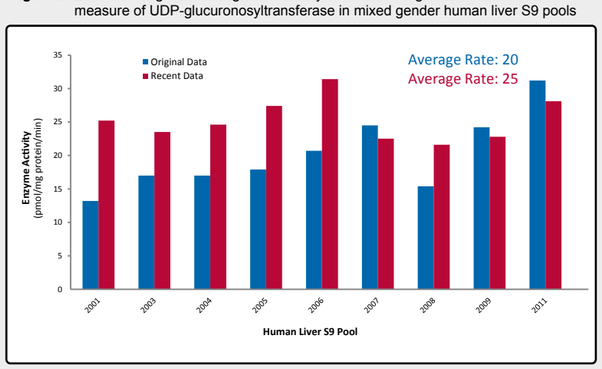


Figure 3. Effects of long-term storage on 7-ethoxycoumarin O-dealkylation as a measure of CYP activity in mixed gender human liver S9 pools

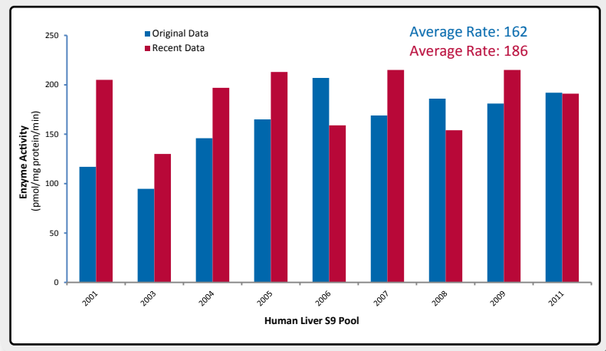


Figure 4. Effects of long-term storage on aldehyde oxidase and *N*-acetyltransferase activities in mixed gender human liver S9 pools

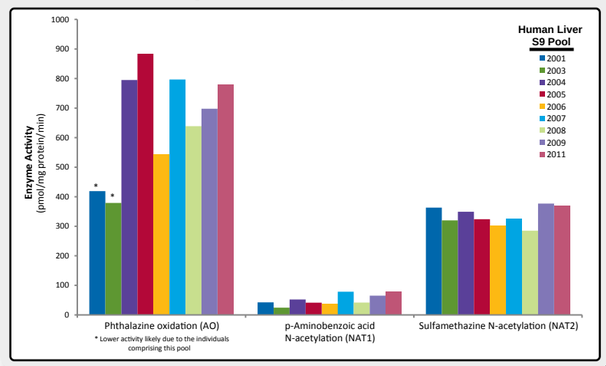
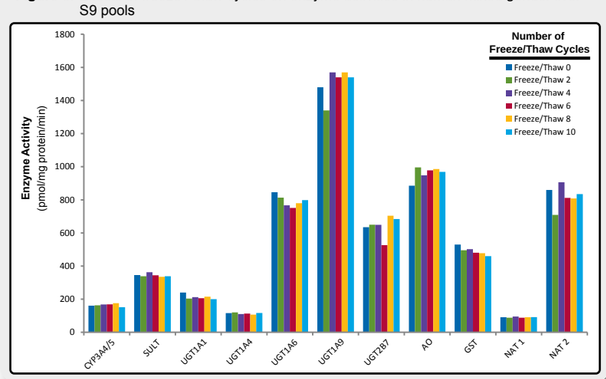


Figure 5. Effects of freeze / thaw cycles on enzyme activities in human mixed gender liver S9 pools



Conclusion

- There was little to no effect of long-term storage at -70°C or below on the enzyme activities tested. Sample-to-sample variation in the activity of CYP, GST, UGT, NAT and AO activities was nominally effected by long-term storage of liver S9 at -70°C.
- The observed variation in enzyme activities from lots prepared over numerous years was within the typical lot-to-lot variation observed as donors are replaced in pooled subcellular fractions and analytical methods are changed.
- Similarly, CYP, UGT, GST, SULT, AO, or NAT enzyme activities in liver S9 samples subjected to up to 10 freeze/thaw cycles did not exhibit significant loss (typically less than 10%).
- These results suggest that our methods for preparation and storage of human liver S9 fractions are well suited to preserving enzymatic activities for up to 10 freeze/thaw cycles and for at least 10 years.

References

1 Pearce RE et al. (1996) *Arch Biochem Biophys* **331**: 145-169.

2 Yamazaki H et al. (1996) *Drug Metab and Dispo* **25**:168-174.