181

MICROSOMES PREPARED FROM ELUTED ENTEROCYTES YIELD HIGH CYTOCHROME P450 AND UGT ACTIVITIES THAT ARE STABLE OVER MULTIPLE FREEZE/THAW CYCLES

Zell W. Woodworth*, Landon Watkins, Terry D. Graves II, Brian D. Smith, Lisa A. Collins, I. Rochelle Riley, Amy E. Gipson, L. Anne Dwyer, Tiffin M. Ramsey*, Kathleen M. Carroll*, and Andrew Parkinson*

XENOTECH
16825 W 116th Street Lenexa, KS 66219

ABSTRACT

Enterocytes in the upper region of the small intestine play a significant role in the first-pass metabolism (pre-systemic clearance) of many orally ingested xenobiotics. For this reason, functionally active and stable intestinal subcellular fractions are required to assess the first-pass metabolism of drugs by cytochromes P450, UDP-glucuronosyltransferases and other drug-metabolizing enzymes. The present study summarizes enzymatic activity data from individual and pooled human and animal intestinal microsomes that were prepared from fresh duodenum/jejunum based on an enterocyte elution method with EDTA and various protease inhibitors. All samples were analyzed for their ability to catalyze testosterone 6ß-hydroxylation, 4-methylumbelliferone glucuronidation, and NADPH-cytochrome *c* reduction. Microsomes prepared from chemically eluted enterocytes had substantially greater CYP3A activity than those prepared from small intestinal samples subjected to mechanical scraping. Freezing/thawing small intestinal microsomes for up to 5 cycles did not cause significant loss of CYP3A, NADPH-cytochrome *c* reductase or UDP-glucuronosyltransferases (UGTs) activity. These results suggest that our elution method for processing small intestines is well suited to preserving microsomal enzymatic activities.

INTRODUCTION

Metabolic enzymes (Phase I and II) within the mature enterocytes of the small intestine play a significant role in the metabolism of many orally administered xenobiotics. Therefore, it is important to consider the potential for intestinal metabolism during the development of orally administered drugs.

The majority of drug-metabolizing enzymes reside in the mature enterocytes of the villus tips. Immature enterocytes and crypt cells contain little to no cytochrome P450 activity. It is apparent that the majority of metabolic enzymes are located in the duodenum and jejunum and that CYP content dramatically decreases distal to the jejunum. It is, therefore, important to ensure that the majority of enterocytes that are processed to microsomes and other metabolically suitable subcellular fractions are derived from the upper portions of the duodenal and jejunal villi.

Traditionally, intestinal microsomes are prepared from enterocytes that have been collected by one of two general methods, namely mechanical scraping of the intestinal lumen or elution of enterocytes.

Scraping Method

- Intestine is cut longitudinally and the lumen is exposed
- A gel knife or glass slide is used to scrape the mucosa and dislodge the enterocytes
  - · This leads to contamination with unwanted cell types or immature enterocytes
  - · Quality can vary greatly between preparations
  - · Many of the enterocytes are lysed and exposed to digestive enzymes found in the lumen
- Harvesting is quick and total protein yield is high, but the specific activity of CYP enzymes and other drug-metabolizing enzymes is low

Elution Method

- Intestine is cut into manageable sections (8-12 inches)
- Enterocytes are harvested from the intestinal lumen by an elution buffer (typically chemically-based with protease inhibitors)
- Harvesting is time-consuming and protein yield is low, but the specific activity of CYP enzymes and other drug-metabolizing enzymes is high

MATERIALS AND METHODS

In this study, microsomes were prepared with an elution procedure with EDTA and various protease inhibitors. For comparative purposes, intestinal microsomes were also prepared by a scraping method with or without protease inhibitors.

Enterocyte harvest

Enterocytes were harvested from fresh small intestinal (duodenum and jejunum) tissue from various species. An ethylenediaminetetraacetic acid (EDTA)-based elution method with various protease inhibitors, including phenylmethylsulfonyl fluoride, was used to separate mature enterocytes from the underlying lamina propria.

Preparation of microsomes

The eluates were subjected to low-speed centrifugation to concentrate and wash the enterocytes. The concentrated enterocyte pellets were suspended in homogenization buffer containing protease inhibitors and mechanically lysed with a motor-driven Teflon pestle. Centrifugation of the homogenates resulted in preparation of S9 fractions, which were subjected to ultracentrifugation to isolate the microsomal fraction. Microsomes were resuspended in 250 mM sucrose and stored at -70°C or below.

Note: These microsomes were prepared with buffers containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), which irreversibly inhibits carboxylesterases.

Enzymatic assays

Small intestinal microsomes from various species were incubated with testosterone, 4-methylumbelliferone, or cytochrome *c* to measure the activity of CYP3A, UDP-glucuronyltransferase (UGT), and NADPH-cytochrome *c* reductase, respectively; essentially as described in Pearce *et al*.

Figure 1:
A comparison of two methods of preparing intestinal microsomes from various species: Effect on CYP3A activity

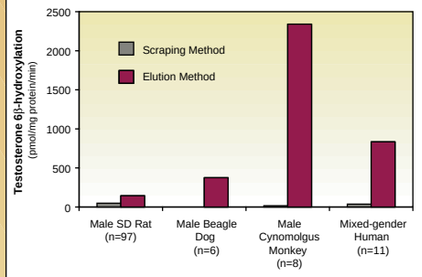


Figure 2:
A comparison of two methods of preparing intestinal microsomes from various species: Effect on UGT activity

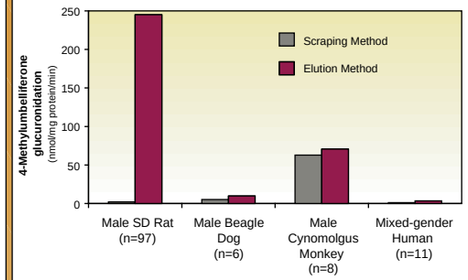


Figure 3:
Effect of repetitive freezing and thawing of human intestinal microsomes on enzymatic activity
Samples prepared by XenoTech elution method with protease inhibitors

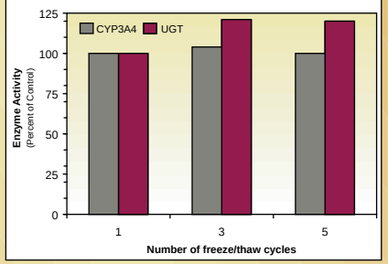


Figure 4:
Effect of long-term storage (at -70°C or below) of human intestinal microsomes on testosterone 6β-hydroxylase (CYP3A4) activity
Samples prepared by XenoTech elution method with protease inhibitors

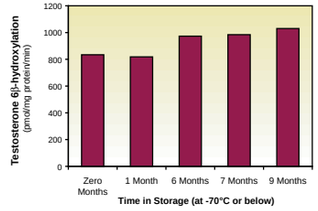
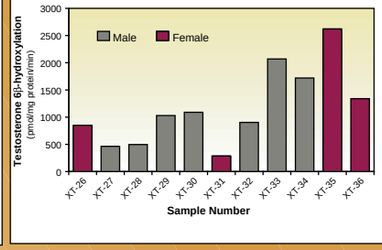


Figure 5:
Testosterone 6β-hydroxylation by individual samples of human small intestinal microsomes
Samples prepared by XenoTech elution method with protease inhibitors



RESULTS

Microsomes were prepared by mechanical scraping and chemical elution of intestinal samples from Sprague-Dawley rat, Beagle dog, Cynomolgus monkey, and human, and assayed for CYP3A and UGT activity. The results are shown in Figs. 1-2. In all species, CYP3A and UGT activity was considerably greater in intestinal microsomes prepared by the chemical elution method than by the mechanical scraping method.

To assess the stability of CYP3A4 in human intestinal microsomes, a pooled sample of human intestinal microsomes was repeatedly thawed and frozen (at -70°C or below), and analyzed for testosterone 6ß-hydroxylase activity. As shown in Fig. 3, human intestinal microsomes prepared by XenoTech's elution procedure can be repeatedly frozen and thawed at least five times without loss of CYP3A4 activity. The same results were obtained for UGT activity.

To assess the stability of CYP3A4 in human intestinal microsomes, a pooled sample of human intestinal microsomes was stored for nine months at -70°C or below, and was analyzed intermittently for testosterone 6ß-hydroxylase activity. As shown in Fig. 4, human intestinal microsomes prepared by XenoTech's elution procedure can be stored at -70°C or below for at least nine months without any loss of CYP3A4 activity.

Individual samples of human intestinal microsomes were assayed for testosterone 6ß-hydroxylase activity to assess the inter-individual variation in CYP3A4 expression. Testosterone 6ß-hydroxylase activity varied about 10-fold from one sample to the next (from 285 to 2620 pmol/mg protein/min), as shown in Fig. 5.

CONCLUSIONS

The results of this study demonstrate that microsomes prepared from various species using an EDTA-based elution method with various protease inhibitors possess systematically greater CYP3A and UGT activity compared with microsomes prepared by mechanical scraping of the intestine. Intestinal microsomes prepared by XenoTech's elution method are stable over multiple freeze/thaw cycles and for at least 9 months when stored at -70°C or below.

Enzymatic activities in intestinal microsomes

Species	Testosterone 6β-hydroxylation (CYP3A4 activity) pmol/mg protein/min	4-Methylumbelliferone glucuronidation (UGT activity) nmol/mg protein/min	NADPH-cytochrome *c* reductase nmol/mg protein/min
Male Sprague-Dawley Rat (n=97)	144	245	65
Male Beagle Dog (n=6)	375	10	47
Male Cynomolgus Monkey (n=8)	2340	71	111
Human (n=11)	834	4	38

REFERENCES

Cotreau MM, Von Moltke LL, Beinfeld MC, Greenblatt DJ: Methodologies to study the induction of rat hepatic and intestinal cytochrome P450 3A at the mRNA, protein, and catalytic activity level. *Journal of Pharmacological and Toxicological Methods*, **43:** 41-54, 2000.

Fasco MJ, Silkworth JB, Dunbar DA, Kaminsky LS: Rat small intestinal cytochromes P450 probed by warfarin metabolism. *Molecular Pharmacology*, **43:** 226-233, 1993.

Kaminsky LS, Fasco MJ: Small Intestinal Cytochromes P450. *Critical Reviews in Toxicology*, **21(6):** 407-422, 1992.

Mohri K, Uesawa Y: Enzymatic activities in the microsomes prepared from rat small intestinal epithelial cells by differential procedures. *Pharmaceutical Research*, **18(8):** 1232-1236, 2001.

Pearce RE, McIntyre CJ, Madan A, Sanzgiri U, Draper AJ, Bullock PL, Cook DC, Burton LA, Latham J, Nevins C, Parkinson A: Effects of freezing, thawing and storing human liver microsomes on cytochrome P450 activity. *Archives of Biochemistry and Biophysics*, **331:** 145-169, 1996.

2004