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Development and Validation of a Standardized Workflow for Multi-Species Liver Microsome Preparation

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Abstract

Liver microsomes are widely used to assess metabolic stability and intrinsic clearance, yet substantial variation exists in laboratory preparation protocols, and the detailed manufacturing processes of commercial reference products are often undisclosed, together limiting methodological transparency and cross-study reproducibility. Metabolic stability was assessed using testosterone, verapamil, raloxifene, and ketanserin. The CD-1 mouse and Sprague-Dawley rat microsomes were compared with the corresponding XenoTech reference microsomes. Beagle dog microsomes were evaluated using the same probe substrates, but no commercial beagle dog reference microsomes were included. The results provide a preliminary functional characterization of the workflow. For some probe substrates, the depletion profiles of the in-house mouse and rat preparations appeared broadly similar to those of their corresponding XenoTech reference microsomes. These descriptive observations do not establish equivalence or superiority. Reproducibility and suitability for routine application require further validation.

Keywords: Liver microsomes; Drug metabolism; Probe substrates; Functional reverse optimization; Reproducibility

1. Introduction

Liver microsomes are among the most widely used in vitro models in drug metabolism research. Owing to their enrichment in endoplasmic reticulum-associated metabolizing enzymes, they are particularly well suited for assessing phase I metabolic stability (Almazroo et al., 2017), conducting enzyme phenotyping (Kong et al., 2020), and estimating intrinsic clearance across species (Tess et al., 2022). Compared with whole liver tissue or primary hepatocytes, the microsomal system offers several advantages, including relatively simple preparation, well-controlled experimental conditions, and suitability for high-throughput analysis, making it an important tool in drug discovery and nonclinical evaluation.

Commercial liver microsome products (Wang et al., 2025), such as XenoTech reference microsomes, are widely used for method development and experimental controls because of their stable functionality and relatively good batch-to-batch consistency. However, a long-standing and often undisclosed issue is that their certificates of analysis (COAs) typically disclose only the composition of the final storage medium, such as the commonly used 250 mM sucrose solution (Pearce et al., 1996), while details of the pre-preparation buffer system, tissue homogenization conditions, and differential centrifugation procedures are not provided. Even when the final storage formulation is known, it remains difficult to reproduce the upstream preparation process. It is also difficult to recapitulate the functional characteristics of the commercial products. This lack of methodological transparency limits the reproducibility of commercial reference materials and hinders functional benchmarking

between in-house prepared liver microsomes and commercial products.

An increasing number of investigators prepare liver microsomes directly from experimental animals to assess enzyme activity in their own model systems and to support drug metabolism studies (White et al., 1995). What is needed at present is not merely evidence that microsomes can be successfully prepared, but rather a reverse-optimization strategy guided by the functional characteristics of commercial reference products, with the goal of generating locally prepared liver microsomes whose metabolic behavior is comparable to that of commercial reference products.

Based on this, the present study focused on the preparation and evaluation of animal-derived liver microsomes from CD-1 mice, SD rats, and beagle dogs. The CD-1 mouse and SD rat preparations were compared with the corresponding XenoTech reference microsomes. Beagle dog microsomes were functionally characterized using the same probe substrates, but were not compared with a commercial beagle dog reference microsome. The aim was to establish a standardized workflow for preparing multi-species liver microsomes with reproducible and comparable functional properties.

2. Materials and Methods

2.1 Chemicals and Reagents

Reagents and materials. Sucrose, testosterone, verapamil, raloxifene, ketanserin, bovine serum albumin (BSA), dipotassium hydrogen phosphate, sodium phosphate monobasic, sodium chloride, disodium ethylenediaminetetraacetic acid, formic acid, and acetonitrile were purchased from Beijing InnoChem Science & Technology Co., Ltd. The BCA protein assay kit was obtained from Beyotime Biotechnology Co., Ltd.

NADPH was purchased from Solarbio. XenoTech CD-1 mouse liver microsomes (product no. 2510159) and XenoTech Sprague-Dawley rat liver microsomes (product no. 1310030) were used as species-matched reference materials for the mouse and rat preparations, respectively. No commercial beagle dog liver microsome reference was included. Ultrapure water was prepared using a Milli-Q system. Pentobarbital was purchased from InnoChem. An IKA homogenizer (model 05011572; IKA, Germany), a pH meter (Mettler Toledo, Switzerland), and a glass tissue homogenizer were used. LC-MS/MS analysis was performed on an AB Sciex 7500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA).

2.2 Animals

Male CD-1 mice (6–8 weeks old, 20–25 g) and Sprague–Dawley (SD) rats (6–8 weeks old, 180–220 g) were purchased from Viton Lihua Laboratory Animal Technology Co., Ltd. Beagle dogs (8 months old, approximately 8.0 kg) were purchased from Shanghai Xingang Lab-Animal Farm. Each species group comprised three male animals. Animals were housed under standard conditions with free access to food and water, and their health was monitored throughout the study. All animals were fasted for 12 h before the procedures, with free access to water. No cynomolgus monkeys were used in this study.

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Laisen Bio-Origin Science Co., Ltd. (CD-1 mice, protocol no. SOP-MAE-006-079; SD rats, protocol no. SOP-MAE-006-078; beagle dogs, protocol no. SOP-MAE-006-077; approved on September 12, 2026). All methods

were performed in accordance with relevant guidelines and regulations, and the study was reported in accordance with the ARRIVE guidelines.

CD-1 mice and SD rats were followed by cervical dislocation. Their livers were then perfused in situ and collected as described in Section 2.3. In beagle dogs, the liver was collected under pentobarbital anesthesia and perfused ex vivo; the dogs were then euthanized by intravenous administration of pentobarbital sodium at 100 mg/kg. Death was confirmed by sustained cessation of respiration and heartbeat. No animals were returned to housing after the procedures.

2.3 Preparation of liver microsomes

Following euthanasia by cervical dislocation under isoflurane anesthesia, the livers of CD-1 mice and SD rats were perfused in situ with ice-cold normal saline at a flow rate of 10 mL/min until the effluent ran clear and the livers changed in color from dark red to tan. The livers were then excised (Watanabe et al., 2019). Beagle dogs were anesthetized with intravenous pentobarbital sodium (25 mg/kg), and their livers were excised and immediately perfused ex vivo with ice-cold normal saline at the same flow rate. After liver collection and perfusion, the dogs were euthanized with intravenous pentobarbital sodium (100 mg/kg), as described in Section 2.2. All subsequent tissue-processing steps were performed on ice to minimize protein degradation and preserve enzyme activity.

After rinsing with ice-cold normal saline, blotting dry, and weighing, the tissues were first coarsely disrupted using a mechanical homogenizer (5000 rpm, 1 min per cycle, five cycles) and then further homogenized on ice with a glass homogenizer. The

homogenization time was adjusted according to liver size to ensure uniform sample preparation and minimize large tissue debris. The homogenization buffer was prepared with four volumes of ice-cold 0.1 mol/L potassium phosphate buffer (pH 7.4), 250 mM sucrose, and 1 mM EDTA-Na_2. The homogenate was then centrifuged at 10,000 × g for 20 min at 4 ℃ to remove cell debris, nuclei, and insoluble impurities (Eriksson et al., 1978).

The resulting supernatant was subjected to two rounds of ultracentrifugation at 105,000 × g for 60 min each. After each centrifugation step, the supernatant was discarded, and the pellet was resuspended in an appropriate volume of buffer to obtain the microsomal fraction and minimize residual contaminants such as hemoglobin. During ultracentrifugation, the upper white lipid layer was carefully removed with a pipette. In some cases, a dark brown, transparent material, possibly DNA, appeared at the bottom of the centrifuge tube and was resuspended together with the pellet. The pellet was then washed and ultracentrifuged again to further improve purity. Finally, the microsomal pellet was resuspended in 250 mM sucrose, adjusted to the desired concentration with preservation buffer, aliquoted, and stored at −80 °C. Before use, samples were rapidly thawed in a 37 ℃ water bath, and no more than three freeze–thaw cycles were allowed. The final liver microsomal preparation typically appeared pale red to pale yellow. **Fig. 1** shows the workflow for liver microsome preparation.

2.4 Protein quantification

Microsomal protein concentration was determined using the BCA assay (Zhang et al., 2021), using BSA as the standard to construct a calibration curve over a

concentration range of 0–2 mg/mL in PBS. Microsomal samples were appropriately diluted with PBS, mixed with the assay reagent, and incubated at 37 ℃ for 30 min prior to absorbance measurement. Protein content was then calculated from the absorbance at 562 nm.

2.5 Probe substrate metabolism assay

Testosterone, verapamil, raloxifene, and ketanserin were selected as probe substrates. Stock solutions were prepared in DMSO, and the final substrate concentration in the incubation system was 1 μmol/L. The final DMSO concentration did not exceed 1% (v/v) of the total reaction volume. The reaction mixture contained microsomal protein (0.5 mg/mL), probe substrate (1 μmol/L), and 100 mmol/L potassium phosphate buffer (pH 7.4) in a total volume of 200 μL. After preincubation at 37 ℃ for 5 min, the reaction was initiated by adding NADPH to a final concentration of 1 mmol/L. Incubations were carried out at 37 ℃ with gentle shaking, and aliquots were collected at 0, 5, 15, 30, and 60 min. Reactions were terminated by adding three volumes of ice-cold acetonitrile containing the internal standard (tolbutamide). Blank control and NADPH-free control groups were included. For subsequent calculations, the compound remaining rate at 0 min was set as 100%, and the remaining rates at 5, 15, 30, and 60 min were expressed relative to the 0 min value.

2.6 LC-MS/MS analysis

After termination, the samples were vortex-mixed and centrifuged at 14,000 × g for 10 min, and the supernatant was collected for LC-MS/MS analysis (Attwa et al., 2023). Chromatographic separation was performed on a C18 column (2.1 × 50 mm, 1.7

μm) maintained at 40 °C with an injection volume of 5 μL. The mobile phases consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.3 mL/min. The gradient elution program was as follows: 0–0.3 min, 10% B; 0.3–1.8 min, 10–85% B; 1.8–2.4 min, 85–95% B; 2.4–2.8 min, 95% B; 2.8–2.9 min, 95–10% B; and 2.9 – 4.0 min, 10% B (re-equilibration). Mass spectrometric analysis was carried out on an AB Sciex 7500 triple quadrupole mass spectrometer in positive ion mode using multiple reaction monitoring (MRM). The source settings were as follows: ion spray voltage, 5500 V; source temperature, 500 °C; gas 1, 50 psi; gas 2, 50 psi; curtain gas, 35 psi; and collision gas, medium. The optimized precursor/product ion pairs were m/z 289.2→97.1 for testosterone, 455.3→165.1 for verapamil, 474.3→112.1 for raloxifene, and 314.2→58.1 for ketanserin. The corresponding declustering potentials were 80, 85, 95, and 70 V, respectively, and the collision energies were 28, 35, 32, and 25 eV, respectively. The dwell time was 50 ms for each transition.

2.7 Metabolic evaluation

The functional activity of the domestically prepared liver microsomes was evaluated by comparing the remaining percentage of probe substrates in the prepared microsomes and the reference liver microsomes. Parameters including the residual rate, clearance rate, elimination rate constant, half-life, and intrinsic clearance were analyzed. The elimination rate constant (kel) was obtained by linear regression of the natural logarithm of the percentage of remaining substrate versus incubation time during the initial linear phase of depletion.

196 Formulas: Half-life

197 $$t_{1/2} = \frac{0.693}{k_{el}} \quad (1)$$

198 where k_{el} is the elimination rate constant, usually expressed in min^{-1}.

199 Intrinsic clearance (CL_{int}) was calculated according to the following equation:

200 $$CL_{int} = \frac{0.693 \times V}{t_{1/2} \times P} \quad (2)$$

201 where V is the total volume of the reaction system, and P is the microsomal protein
202 amount. The equation is based on first-order depletion kinetics under linear microsomal
203 incubation conditions.

204 ***2.8 Statistical analysis***

205 Data are presented as mean ± standard deviation (SD) of three biological replicates.
206 Time-dependent substrate depletion was analyzed by plotting the natural logarithm of
207 the percentage of substrate remaining against incubation time. The elimination rate
208 constant (k_{el}) was calculated as the negative slope of the linear regression fitted to the
209 initial linear phase of substrate depletion. The half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$.

210 # 3. Results

211 ***3.1 Determination of the elimination profiles of four probe substrates in liver***
212 ***microsomes***

213 Under the tested incubation conditions, the in-house CD-1 mouse and SD rat
214 microsomes were compared with the corresponding XenoTech reference microsomes.
215 Beagle dog microsomes were assessed using the same probe substrates, but no
216 commercial beagle dog reference was available for comparison. As shown in **Fig. 2**, all
217 four probe substrates exhibited clear time-dependent depletion in both microsomal

systems, indicating that both systems retained certain metabolic activity.

Compared with the XenoTech reference microsomes, the in-house microsomes showed a similar depletion pattern for testosterone and verapamil. Overall, the metabolic rate of testosterone in the domestically prepared liver microsomes varied across species in the following order: SD rat > CD-1 mouse > beagle dog. For verapamil, the metabolic rate followed the order: SD rat > CD-1 mouse > beagle dog. CD-1 mouse liver microsomes metabolize ketanserin faster than raloxifene. Testosterone and verapamil showed closely overlapping depletion profiles between the two microsomal systems, whereas raloxifene and ketanserin exhibited modest divergence at later time points (30 and 60 min). The calculated CL_{int} values ranged from 73.72 to 107.44 μL/min/mg, suggesting comparable metabolic competence across the two microsomal systems.

Overall, the elimination curves of testosterone and verapamil were highly consistent between the two types of microsomes, showed comparable depletion profiles. Raloxifene and ketanserin also showed similar overall trends, indicating that the domestically prepared microsomes showed broadly similar metabolic behavior to the commercial reference products. Although slight differences were observed for some substrates at later time points, they did not alter the overall functional consistency between the two sample types. Overall, testosterone and verapamil exhibited highly comparable depletion profiles between the two microsomal systems. Their calculated CL_{int} values were also broadly consistent, ranging from 293.64 to 561.13 μL/min/mg for testosterone and from 246.62 to 523.02 μL/min/mg for verapamil. Raloxifene

showed very similar kinetics across groups, with CLint values clustered around 99.71–107.44 μL/min/mg. Ketanserin displayed modest inter-group differences, particularly at later time points (30 and 60 min), but the overall metabolic trends remained comparable.

3.2 Protein quantification and linearity.

Microsomal protein concentrations were determined using the BCA assay to ensure accurate normalization in subsequent incubation experiments. As shown in **Fig. 3g**, the BSA calibration curve exhibited good linearity over the range of 0–2 mg/mL, with an R^2 value of 0.9920, meeting the requirements for microsomal protein quantification. The measured protein concentrations of the in-house and reference microsomes were within a comparable range (13.5–37.7 mg/mL), and the COA for XenoTech CD-1 mouse liver microsomes reported a protein concentration of approximately 26 mg/mL (**Fig. 3h**), indicating that both microsomal preparations were suitable for subsequent metabolic stability analysis. Under standardized incubation conditions, the domestically prepared microsomes and the reference microsomes showed consistent functional performance across the two probe substrates (**Fig. 3a–e**). The residual curves and the overall extent of change were highly similar between the two systems, suggesting that the established preparation process effectively preserved microsomal functional integrity.

3.3 Precision and inter-batch reproducibility of in-house microsomes.

Further evaluation demonstrated good analytical performance of the domestically prepared microsomes. Minimal variation was observed within a single analytical run

(**Fig. 3a–d**). In addition, three independent batches showed only small inter-batch differences (**Fig. 3g–h**), further confirming the reproducibility and stability of the preparation process.

Overall, the domestically prepared liver microsomes showed satisfactory metabolic stability, protein quantification reliability, functional activity, indicating their suitability for routine in vitro drug metabolism studies. In commercial testing, three replicates are commonly used; therefore, to remain consistent with commercial practice and improve cost-effectiveness, three replicates were selected in this study. Given that the sample size in each group was n=3, these findings are more suitable for methodological exploration and preliminary validation, and larger sample sizes will be needed for further confirmation.

4. Discussion

This study was not merely intended to determine whether in-house prepared microsomes could replace commercial products, but rather to address a more methodologically meaningful question: whether a reproducible and standardized preparation workflow with performance comparable to commercial microsomes could be established through functional reverse optimization, despite the incomplete disclosure of pretreatment procedures for commercial reference microsomes. This question is particularly relevant for laboratory-prepared microsomes, because preparation methods vary widely across studies, whereas the pretreatment details of commercial products are often not fully disclosed.

The present results provide a preliminary, descriptive assessment of the prepared

284 microsomes. The in-house CD-1 mouse and SD rat preparations were compared with
285 their corresponding XenoTech reference microsomes. For some probe substrates, their
286 observed depletion profiles appeared broadly similar to those of the corresponding
287 references. However, these comparisons were not designed as formal equivalence or
288 superiority tests and should not be interpreted as evidence that the in-house preparations
289 are equivalent or superior to the commercial products. Beagle dog microsomes were
290 assessed using the selected probe substrates, but no commercial beagle dog reference
291 microsome was included; therefore, the beagle dog results describe the in-house
292 preparations only. The present data also do not establish why depletion profiles differed
293 among preparations or substrates.

294 Commercial reference microsomes provided a comparator for the CD-1 mouse and
295 SD rat preparations in this study. The workflow described here may serve as a starting
296 point for laboratories interested in preparing liver microsomes in-house. Its
297 transferability to other laboratories, consistency across independently prepared batches,
298 and performance relative to additional species-matched commercial references remain
299 to be evaluated.

300 Protein concentration was measured using the BCA assay to normalize
301 microsomal protein input in the metabolic incubations. The BSA calibration curve
302 showed linearity over the tested concentration range (R^2= 0.9920). This result supports
303 the use of the assay for protein quantification under the conditions tested, but does not
304 by itself establish assay precision or stability.

305 This study has several limitations. First, the sample size was limited to three

animals per species; therefore, the findings should be interpreted as preliminary. Second, functional assessment was restricted to four probe substrates and may not represent the full range of microsomal enzyme activities. Third, microsomal composition and enzyme abundance were not characterized, so the mechanisms underlying the observed differences could not be determined. Fourth, formal intra-day, inter-day, and independent batch-to-batch precision studies were not performed; accordingly, reproducibility across analytical runs, days, or independently prepared batches cannot be concluded from the present data. Finally, the commercial reference comparisons were limited to the CD-1 mouse and SD rat preparations; no commercial beagle dog reference microsome was included. Further studies using larger sample sizes, additional probe substrates, mechanistic characterization, and independent preparations are needed.

In summary, this study describes a workflow for preparing liver microsomes from CD-1 mice, SD rats, and beagle dogs and provides preliminary functional characterization using four probe substrates. The mouse and rat preparations were compared with their corresponding XenoTech references, whereas the beagle dog preparations were evaluated without a commercial beagle dog comparator. Some depletion profiles appeared broadly similar between the in-house mouse or rat preparations and their corresponding references; these descriptive observations do not establish equivalence or superiority. Because formal intra-day, inter-day, and independent batch-to-batch validation was not performed, reproducibility across runs or batches remains to be determined. Further validation is required before the workflow’

s broader transferability or routine applicability can be established.

5. Conclusion

This study describes a workflow for preparing liver microsomes from CD-1 mice, SD rats, and beagle dogs and provides preliminary functional characterization using four probe substrates. The mouse and rat preparations were compared with their corresponding XenoTech reference microsomes, whereas the beagle dog preparations were assessed without a commercial beagle dog comparator. Some observed depletion profiles appeared broadly similar for selected substrates, but these descriptive observations do not establish equivalence or superiority. Formal intra-day, inter-day, and independent batch-to-batch validation was not performed; reproducibility across runs or batches therefore remains to be determined. Further studies are needed to evaluate the workflow’s broader transferability and applicability.

CRediT authorship contribution statement：

Yaqi Wang: Writing - review & editing, Investigation, Validation, Data curation, Formal analysis, Conceptualization. **Guoshuai Li**: Methodology, Investigation, Data curation, Formal analysis. **Wenbin Gui**: Writing - review & editing, Methodology, Investigation, Formal analysis. **Chi Chen**: Writing - review & editing, Supervision, Project administration, Resources, Conceptualization. **Peng Tang**: Writing - original draft & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Yibo Hu**: Investigation, Validation, Data curation. **Shuang Chen**: Methodology, Investigation, Validation. **Jingyao Li**: Writing - review & editing,

Formal analysis, Visualization. **Junjuan Wang**: Investigation, Data curation, Formal analysis.

Declaration of competing interest：

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement：

The data supporting the findings of this study are available from the corresponding author upon reasonable request. They are not publicly available because they contain commercially sensitive information and relate to ongoing intellectual property considerations concerning the proprietary workflow. Access may be subject to appropriate permissions.

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Figure legends:

Fig. 1 Schematic diagram of liver microsome preparation.

Fig. 2. Time-dependent depletion of four probe substrates in domestically prepared and XenoTech reference liver microsomes. Testosterone, verapamil, raloxifene, and ketanserin were incubated in parallel with domestically prepared liver microsomes and commercial reference liver microsomes, and substrate residual levels were monitored at different time points to evaluate metabolic stability. Data are presented as mean ± SD, n=3. FACD1, FACD2: in-house prepared CD-1 mouse liver microsomes; XeCD: XenoTech CD-1 mouse liver microsomes; FASD1, FASD2: in-house prepared SD rat liver microsomes; XeSD: XenoTech SD rat liver microsomes; FADOG1, FADOG2: in-house prepared beagle dog liver microsomes.

Fig. 3 Liver microsome protein quantification and functional evaluation. (a) BSA standard curve used for microsomal protein quantification. (b) Measured protein concentrations of domestically prepared and reference liver microsomes. (c–h) Comparison of functional performance between domestically prepared liver microsomes and XenoTech reference liver microsomes under standardized conditions. Data are presented as mean ± SD, n=3.

Fig. 4 LC–MS chromatograms of FACD1 liver microsomes for raloxifene, ketanserin, testosterone, and verapamil at 0 min and 60 min.

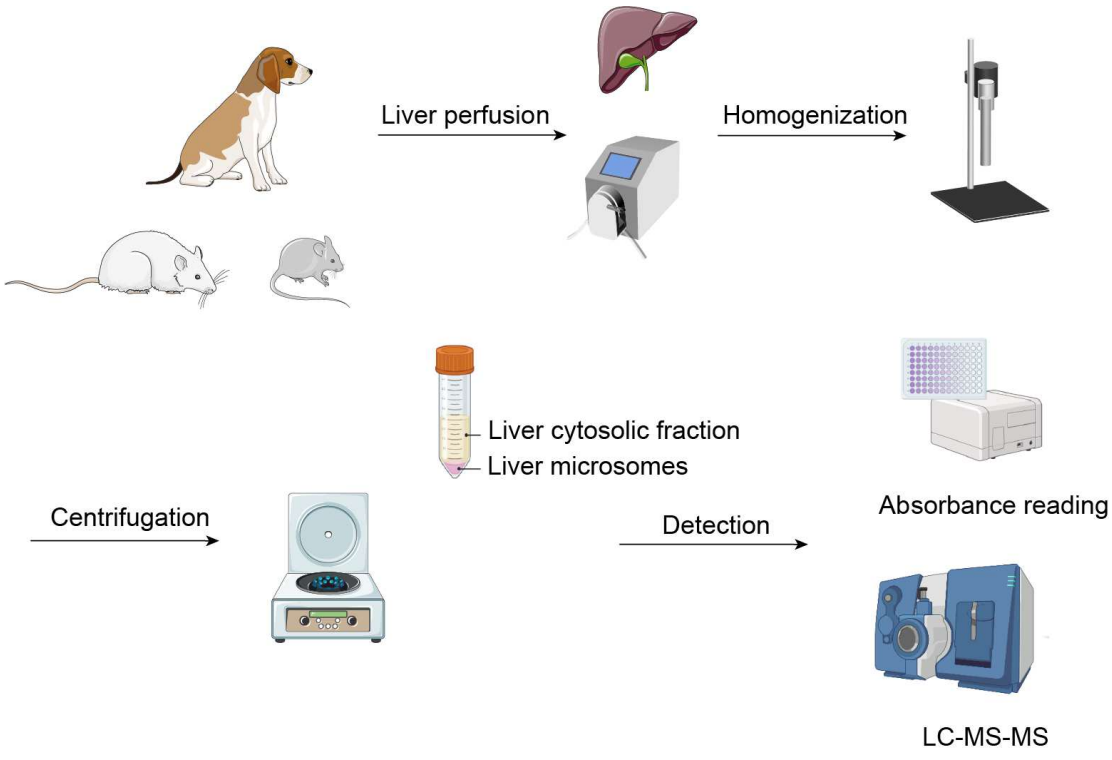


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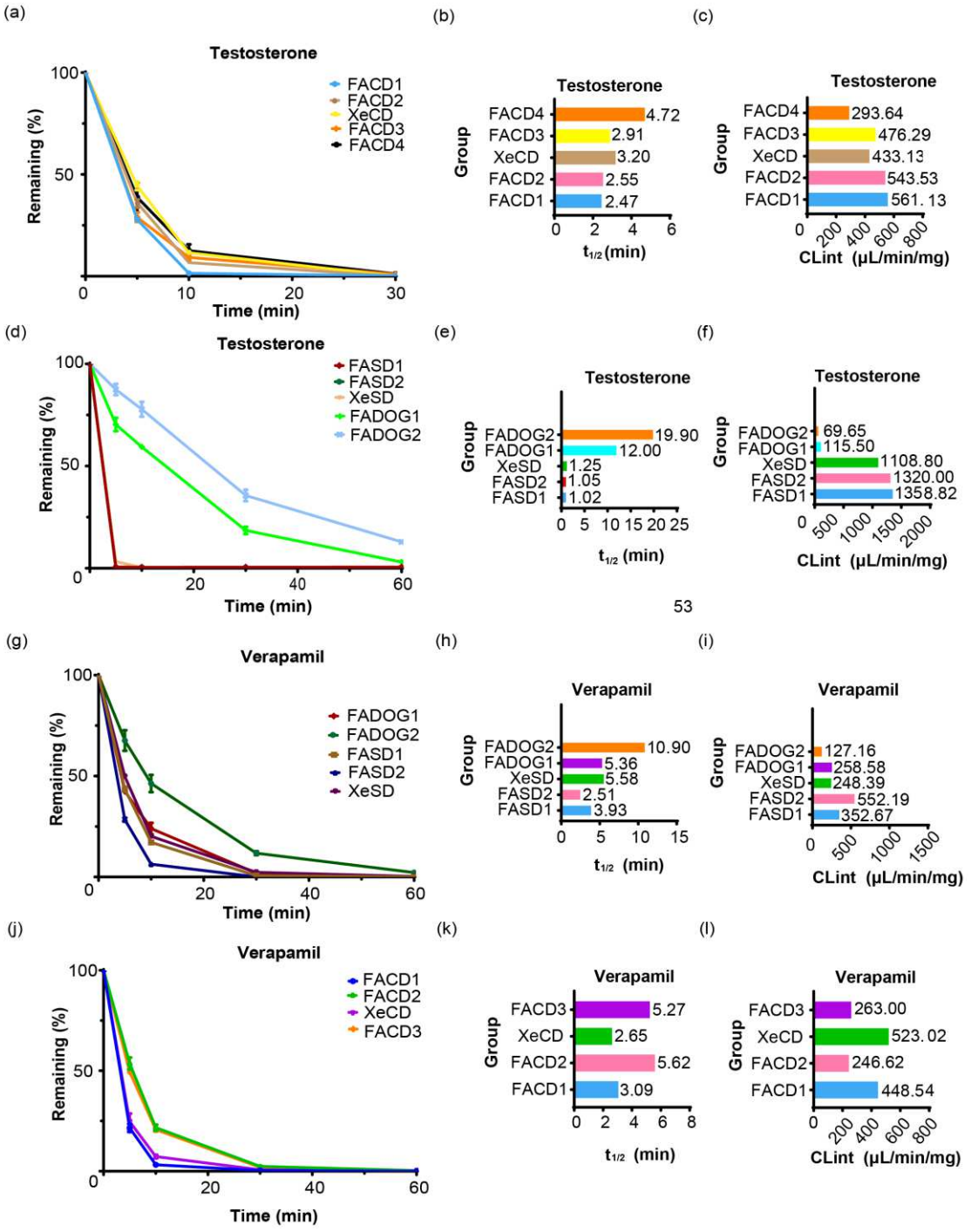


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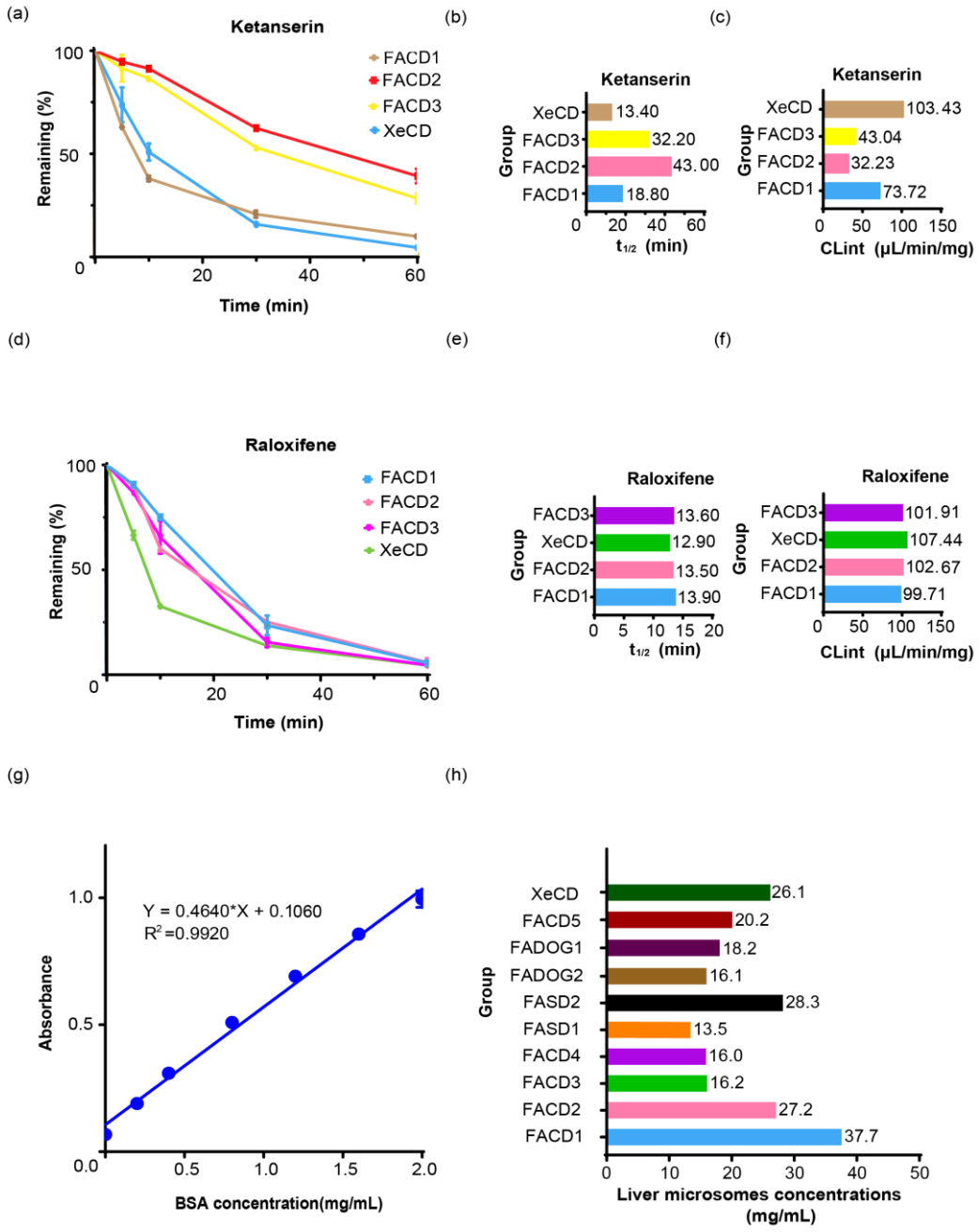
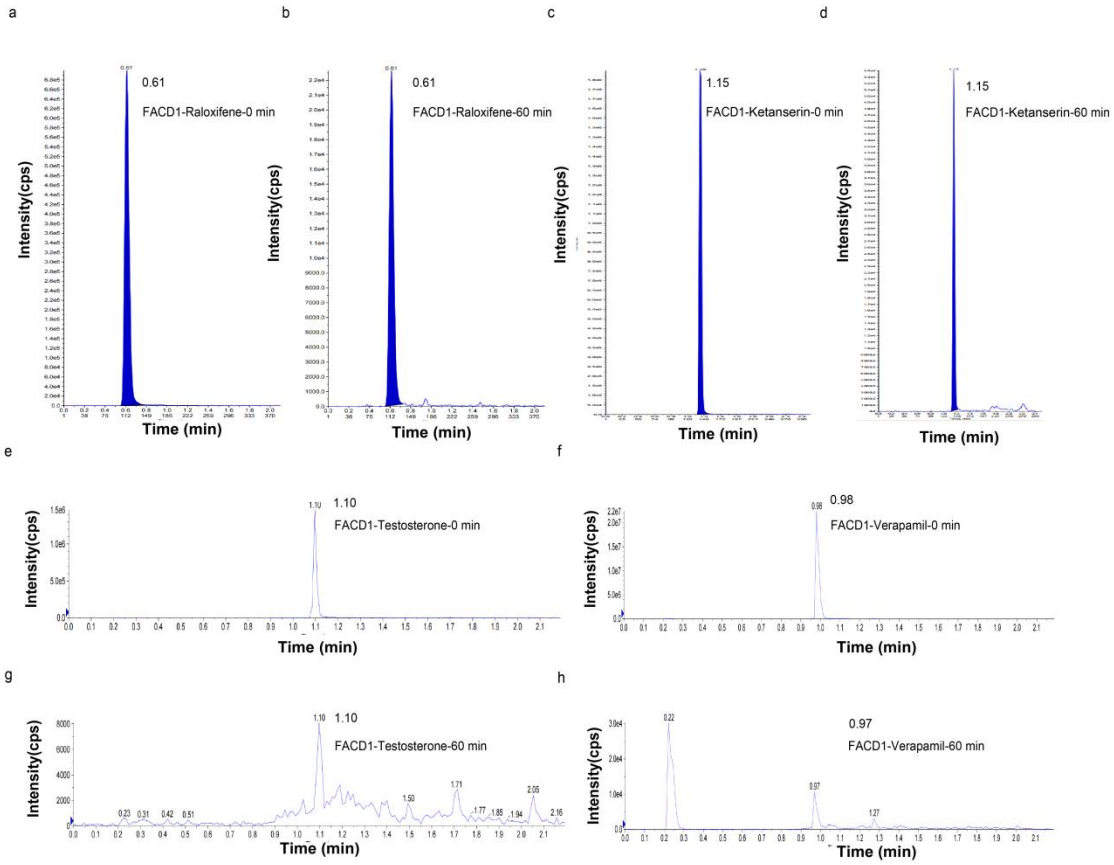


Fig. 3 Protein quantification and functional assessment of liver microsomes. (a) Time-dependent depletion of ketanserin. (b) Half-life ($t_{1/2}$) of ketanserin. (c) Intrinsic clearance (CL_{int}) of ketanserin. (d–f) Corresponding depletion, $t_{1/2}$, and CLint results for raloxifene. (g) BSA standard curve used for microsomal protein quantification. (h) Protein concentrations of the prepared and reference liver microsomes. In panels (a–c), XeCD denotes the XenoTech CD-1 mouse liver microsome reference.



444

445 **Fig. 4** LC–MS chromatograms of FACD1 liver microsomes for raloxifene, ketanserin,

446 testosterone, and verapamil at 0 min and 60 min.

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