1-P-73 Comparison of two hepatic uptake assay methods, plated and oil layer methods, using human and preclinical species cryopreserved hepatocytes

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Introduction

The prediction of *in vivo* human hepatic clearance (CL) of drug from *in vitro* data is important for drug development. Primary human cryopreserved hepatocytes are widely used as an *in vitro* tool for the prediction of *in vivo* CL since a variety of hepatic uptake transporters are expressing in them. The oil layer method is a current major hepatic uptake assay method. On the other hand, the plated method has come to be used to evaluate hepatic uptake recently. In this study, we compared the difference between the plated method and the oil layer method using human cryopreserved hepatocytes in order to show the usefulness of the plated method. Also, we examined whether or not the plated method is useful for the evaluation of species difference of hepatic uptake using preclinical species (monkey, dog, and rat) cryopreserved hepatocytes.

Method

<Preparation of hepatocytes>

Cryopreserved hepatocytes were purchased from Sekisui XenoTech (Human: HPCH10+/1610260, pool of 10 donors, Monkey: P2000.H15B+/1510189, Dog: D1000.H15+/1710018, Rat: R1000.H15+/1610409). The hepatocytes were purified using K8000 OptiThaw Hepatocyte Kit (Sekisui XenoTech). For the oil layer method, the purified hepatocytes were suspended in InVitro GRO KHB (KHB)(Bioreclamation IVT). For the plated method, the purified hepatocytes were suspended in K8200 OptiPlate Hepatocyte Media (Sekisui XenoTech) and seeded to BioCoat™ 24-well plates (Corning) (seeding density: 0.3×10^6 cells/well). The plates were cultured in a CO_2 incubator (37℃, CO_2: 5%) for 5 hr (human, monkey, dog) or 24 hr (rat).

< Uptake study >

[Oil layer method]

The suspended hepatocytes were pre-incubated at 37 ℃ for 2 min. After the pre-incubation, an equal volume of incubation buffer (KHB containing probe substrates) was added to the suspended hepatocytes (final hepatocyte concentration: 1×10^6 cells/mL). After the incubation for the designated time, 80 µL of the incubation mixture was transferred to cell-separation tubes (Hepatocyte Transporter Suspension Assay Kit, BD Biosciences), in which 50 µL of 2 mol/L NaOH was overlaid with 80 µL of filtration oil layer. The tubes were immediately centrifuged, and transfer of the cells to the lowest layer was confirmed. The centrifuged tubes were stored overnight at -80 ℃. The top layer (for initial concentration) and bottom layer (for uptake concentration) were cut with a slicer and collected in separate glass vials. The collected samples were mixed with the scintillator Hionic-Fluor (PerkinElmer) to measure the radioactivity using a liquid scintillation counter (LSC). The remaining hepatocyte suspension was used to determine the protein concentration by BCA Protein Assay Kit (Thermo Fisher Scientific).

[Plated method]

All medium in the plates was removed and replaced with 1 mL of KHB. The buffer was replaced with 0.3 mL of KHB, and the plates were pre-incubated at 37 ℃ for 15 min. After the pre-incubation, the buffer was replaced with 0.3 mL of incubation buffer (KHB containing probe substrates). After the incubation for the designated time, each solution was removed, and the cells were washed once with 1 mL of ice-cold 0.2% BSA-PBS and twice with 1 mL of ice-cold PBS. The PBS was removed, and the cells were dissolved in 0.5 mL of 0.1 mol/L NaOH with pipetting. 0.3 mL of the solution was collected in a glass vial and mixed the scintillator Hionic-Fluor to measure the radioactivity using a LSC. The remaining sample was used to determine the protein concentration by BCA Protein Assay Kit.

<Calculation of uptake volume, uptake CL, K_m, and V_{max} values>

Uptake volume (µL/mg protein)
= Uptake amount (dpm) / [Protein amount (mg protein) × Initial concentration (dpm/µL)]

Uptake CL (µL/mg protein/min) for plated method
= Uptake volume (µL/mg protein) / Incubation time (min)
Incubation time: 0.5 min (E3S) or 2 min (CCK-8, MPP+, TCA)

Uptake CL (µL/mg protein/min)) for oil layer method
= [Uptake volume for 2 min incubation (µL/mg protein) – Uptake volume for 0.5 min incubation (µL/mg protein)] / [2 (min) – 0.5 (min)]

K_m and V_{max} values were calculated from the relationship between the uptake velocity and concentration of the probe substrate according to the equation shown below.

$V = V_{max} \times S / [(K_m + S)] + P_{dif} \times S$

V_{max} : Maximum uptake velocity (pmol/mg protein/min)
K_m : Michaels constant (µmol/L)
S: Concentration (µmol/L)
P_{dif} : Passive diffusion (µL/mg protein/min)

Results

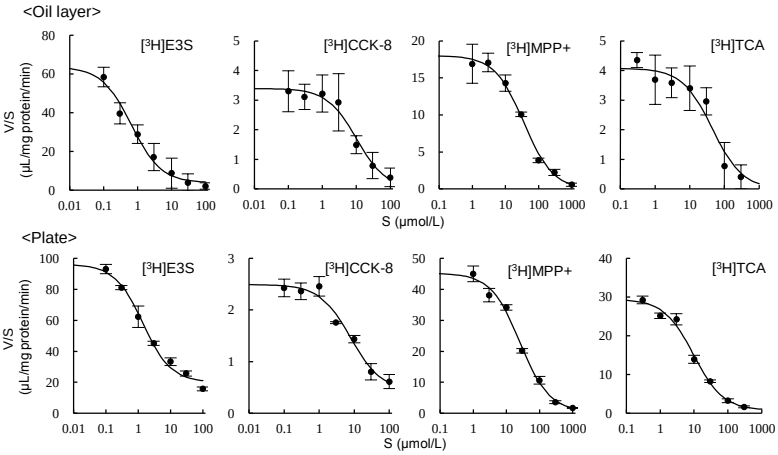

Figure 1 Comparison of concentration dependent uptake of probe substrates into human hepatocytes between two hepatic uptake assay methods.
Concentration dependent uptake of probe substrates (estrone 3-sulfate (E3S) for OATP1B1, cholecystokinin 8 (CCK-8) for OATP1B3, 1-methyl-4-phenylpyridinium (MPP+) for OCTs, taurocholic acid (TCA) for NTCP) into human hepatocytes was observed in both plated and oil layer method.

Table 1 Comparison of kinetic parameter of probe substrates between two hepatic uptake assay methods using human hepatocytes.

Substrate	Method	Uptake CL (µL/mg protein/min)	K_m (µmol/L)	V_{max} (pmol/mg protein/min)
[3H]E3S	Oil layer	58.5	0.652	39.0
	Plate	93.1	1.40	107
[3H]CCK-8	Oil layer	3.30	10.1	34.4
	Plate	2.43	7.76	15.9
[3H]MPP+	Oil layer	16.9	35.5	639
	Plate	45.0	25.6	1141
[3H]TCA	Oil layer	4.35	44.8	182
	Plate	29.3	9.71	278

The uptake characteristics of E3S, CCK-8, MPP+ in plated method were comparable to those in oil layer method. On the other hand, uptake CL and K_m value of TCA in oil layer method was 6.7-fold lower and 4.6-fold higher than those in plated method, respectively.

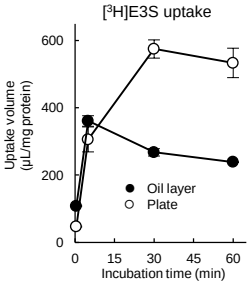

Figure 2 Comparison of long term incubation effect on E3S uptake into human hepatocytes between two hepatic uptake assay methods.
The uptake activity of E3S into human hepatocytes in plated method was maintained longer than that in oil layer method.

Table 2 Comparison of kinetic parameter of probe substrates among plated monkey, dog, and human hepatocytes.

<[3H]E3S>

Species	Uptake CL (µL/mg protein/min)	K_m (µmol/L)	V_{max} (pmol/mg protein/min)
Monkey	142	1.50	196
Dog	55.2	25.1	1019
Human	58.5	0.652	39.0
Human (expressing system)	-	0.23 ~ 12.5	-

<[3H]CCK-8>

Species	Uptake CL (µL/mg protein/min)	K_m (µmol/L)	V_{max} (pmol/mg protein/min)
Monkey	4.83	4.84	22.6
Dog	0.525	10.4	3.69
Human	2.43	7.76	15.9
Human (expressing system)	-	3.82 ~ 16.5	-

<[3H]MPP+>

Species	Uptake CL (µL/mg protein/min)	K_m (µmol/L)	V_{max} (pmol/mg protein/min)
Monkey	32.0	76.7	3018
Dog	56.8	8.93	554
Human	45.0	25.6	1141
Human (expressing system)	-	50 ($K_{0.5}$)	-

<[3H]TCA>

Species	Uptake CL (µL/mg protein/min)	K_m (µmol/L)	V_{max} (pmol/mg protein/min)
Monkey	32.3	12.2	399
Dog	74.8	21.2	1449
Human	29.3	9.71	278
Human (expressing system)	-	2.1 ~ 7.9	-

We examined the species difference of hepatic uptake characteristics of probe substrates using plated monkey, dog, and human hepatocytes. The uptake characteristics of probe substrates in monkey hepatocytes were comparable to those in human. On the other hand, there was species difference of uptake characteristics between dog and human. The K_m values of probe substrates in human plated hepatocytes were consistent with those in human transporter expressing system (apparent affinity constant ($K_{0.5}$) value of MPP+ in OCT1-expressing system was referred instead of the K_m value). We could not succeed in the uptake assay using rat hepatocytes because of the poor adhesion to the plates.

References

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Discussion

At first, we compared two hepatic uptake assay methods (plated and oil layer method) using human cryopreserved hepatocytes. The concentration dependent uptake of each probe substrate (E3S, CCK-8, TCA, and MPP+) into human hepatocytes was observed in both methods (Figure 1). The uptake characteristics of each probe substrate in the plated method were comparable to those in the oil layer method (Table 1). On the other hand, uptake CL and K_m value of TCA in the oil layer method were 6.7-fold lower and 4.6-fold higher than those in the plated method, respectively. Regarding this difference, further consideration will be needed. Comparing other indexes between two methods, the throughput of assay in the plated method was higher than that in the oil layer method because simultaneous multi-well assay was available in 24-well plate. In addition, the variation of uptake CL of each probe substrate in the plated method was smaller than that in the oil layer method. From these results, we conclude that the plated method is a useful assay method to evaluate hepatic uptake.
Second, we investigated a long term incubation effect on E3S uptake into human hepatocytes (Figure 2). Comparing the plated and oil layer methods, the uptake activity of E3S into human hepatocytes in the plated method was maintained longer than that in the oil layer method. This result suggests that the plated method is more useful than the oil layer method for the experiments that need long term incubation (e.g., evaluation of long-lasting inhibitory effect of compounds on OATPs, calculation of unbound drug concentration ratio between media and hepatocyte ($K_{p,uu}$), and so on).
Third, we examined the species difference of hepatic uptake of probe substrates using plated monkey, dog, and human hepatocytes (Table 2). The uptake characteristics of probe substrates in monkey hepatocytes were comparable to those in human hepatocytes. On the other hand, there was a species difference of uptake characteristics between dog and human. From these results, we conclude that the plated method is useful to evaluate species difference of hepatic uptake.
In summary, this study indicates that the plated method is a useful assay method for the evaluation of human and preclinical species (monkey and dog) hepatic uptake and for the other experiments that need long term incubation.

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