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Research Strategy of Chiral PROTACs in Discovery Stage Based on Analysis Platforms of UPCC-MS/MS and UPLC-MS/MS

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PURPOSE

Proteolysis targeting chimerics (PROTACs) are known as heterobifunctional molecules that degrade specific endogenous proteins through the E3 ubiquitin ligase pathway 1. The potential advantages of PROTAC technology may compensate for the shortcomings of traditional drug therapy1. Chiral compounds such as thalidomide and pomalidomide are often used as the ligand of E3 ubiquitin ligase. Due to the chiral structures of these ligands, the *in vivo* chiral characteristics of PROTACs should be evaluated during the early stage. To understand the chiral PROTACs pharmacokinetics, efficacy, and toxicity properties. , it is critical to quantify the enantiomers in the systemic circulation and different tissues.

The enantiomers analysis of chiral PROTACs was rarely reported. Analysis of the enantiomers in the biological samples faces two major challenges: complete separation of the enantiomers and good sensitivity to detect the low concentration of enantiomers.

Both the liquid chromatography (LC) and the convergence chromatography (CC) are used for the quick separation of enantiomers for chiral PROTACs, while the mass spectrometer is used to detect enantiomers. We developed rapid and sensitive quantitation methods for chiral PROTACs in biological matrix using the ultrahigh performance convergence chromatography coupled with mass spectrometer (UPCC-MS/MS) and the ultrahigh performance liquid chromatography coupled with mass spectrometer (UPLC-MS/MS). The stability and conversion characteristics of enantiomers of chiral PROTACs in animal plasma was investigated with these methods. The result well supported the early discovery studies of chiral PROTACs .

METHOD(S)

Samples for stability and conversion assay of the enantiomer B of chiral PROTACs

Chiral PROTAC Compound 1 has one chiral center, and two enantiomers A and B. 2μM enantiomer B was incubated in the CD-1 mouse plasma (K2-EDTA) at 37°C for 0, 10, 30, 60, 120 minutes, then the samples were precipitated with acetonitrile which contained the internal standard (IS), mixed well, and centrifuged at 4000 rpm and 4°C for 20 minutes. The supernatant was collected and the enantiomer A and B of the Compound 1 were analyzed with the instruments (Table1).

Platform - UPLC-MS/MS and UPCC-MS/MS conditions

Waters ACQUITY UPLC was coupled with a triple-quadrupole mass spectrometry AB Sciex 6500 plus. Waters ACQUITY UPCC was coupled with a triple-quadrupole mass spectrometry AB Sciex 6500 plus or Sciex 4000. Separation was conducted on chiral columns (Daicel, Japan).

RESULT(S)

It took 6 minutes to separate the two enantiomers of the chiral PROTAC Compound 1 by UPLC, while only 3.5 minutes were needed for UPCC when using the same column (Figure 1a and 1b, Figure2). Since the supercritical fluid CO2 was used as the mobile phase for UPCC, whose diffusion coefficient and viscosity are close to gas, then a quicker separation of the enantiomers can be achieved.

The result of the stability and conversion test of enantiomer B showed that, 69.1% of enantiomer B remained in the mouse plasma after 120 minutes incubation at 37°C, and 18.4% enantiomer A was observed. The change of enantiomer A and B in mouse plasma showed a direct correlation with the chiral conversion between the enantiomers (Figure 1c, Table 2).

The conversion of enantiomer A and B was observed in mouse plasma by using UPLC-MS/MS. Basing the result, the stabilizer was investigated. By adding citric acid in samples immediately after collection, the *ex vivo* conversion was avoided in the samples. The actual conversion and ratio of the enantiomer A and B in the animal's body was investigated in the pharmacokinetics (PK) study. After the dominant enantiomer was found out, it was focused on during the efficacy and safety studies.

The lower limit of quantitation (LLOQ) of 1 ng/mL for the enantiomer was achieved in dog plasma by using Sciex 4000 (Figure 1d) mass spectrometry, and this quantitation method was applied to sample analysis of a dog PK study. When the Sciex 6500 plus or Sciex 7500 was used, lower LLOQ can be obtained.

Table 1. *UPLC and UPCC conditions for compound 1.*

	UPLC	UPCC
Column	IH-3 (2.1 mm, 100 mm, 3 μm)	
Mobile phase	A: 0.1% formic acid and 2mM ammonium acetate in ACN: H_2O (v: v, 5:95) B: 0.1% formic acid and 2mM ammonium acetate in ACN: H_2O (v: v, 95:5)	A: CO_2 B: Methanol and dichloromethane (DCM) mixture

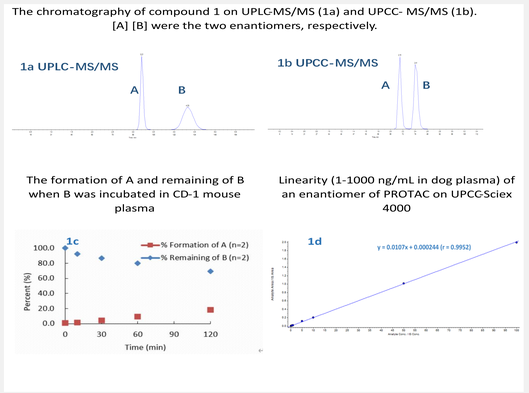

Figure 1. *The chromatography of compound 1 on UPLC-MS/MS (1a) and UPCC-MS/MS(1b). [A][B] were the two enantiomers, respectively.*

Table 2. *The formation A and remaining B when B was incubated in CD-1 mouse plasma.*

a: The formation of A when B was incubated in CD-1mouse plasma

The Formation of A from B in Mouse Plasma						
Incubated compound	Analyte	Time (min)	Analyte Peak Area	IS Peak Area	Area(analyte) /Area (IS)	% Formation of A (n=2)
B	A	0	4.06E+05	5.60E+06	0.0725	0.7
B	A		4.11E+05	5.67E+06	0.0725	
B	A	10	8.15E+05	5.58E+06	0.1461	1.6
B	A		9.26E+05	5.70E+06	0.1625	
B	A	30	2.26E+06	5.61E+06	0.4029	4.3
B	A		2.50E+06	5.67E+06	0.4409	
B	A	60	5.52E+06	5.95E+06	0.9277	9.4
B	A		5.27E+06	5.86E+06	0.8993	
B	A	120	1.06E+07	5.93E+06	1.7875	18.4
B	A		1.06E+07	5.93E+06	1.7875	
A	A	0	5.53E+07	5.59E+06	9.893	100.0
A	A		5.26E+07	5.50E+06	9.564	

The % formation of A after incubation in plasma was calculated using following equation:
% Remaining= 100 x (PAR of analyte A at appointed incubation time / PAR of analyte A at T0 time)
where PAR is the peak area ratio of analyte versus internal standard (IS)
The appointed incubation time points are T0 (0 min), Tn (n=0, 10, 30, 60, 120 min)

b: The remaining of B when B was incubated in CD-1mouse plasma

The Remaining of B in Mouse Plasma						
Incubated compound	Analyte	Time (min)	Analyte Peak Area	IS Peak Area	Area(analyte) /Area (IS)	% Remaining of B (n=2)
B	B	0	3.97E+07	5.67E+06	7.0018	100.0
B	B		3.96E+07	5.60E+06	7.0714	
B	B	10	3.78E+07	5.70E+06	6.6316	92.2
B	B		3.54E+07	5.58E+06	6.3441	
B	B	30	3.49E+07	5.67E+06	6.1552	86.5
B	B		3.38E+07	5.61E+06	6.0250	
B	B	60	3.37E+07	5.86E+06	5.7509	80.2
B	B		3.29E+07	5.95E+06	5.5294	
B	B	120	2.94E+07	5.93E+06	4.9578	69.1
B	B		2.83E+07	5.93E+06	4.7723	

The % remaining of B after incubation in plasma was calculated using following equation:
% Remaining= 100 x (PAR of B at appointed incubation time / PAR of B at T0 time)
where PAR is the peak area ratio of analyte versus internal standard (IS)
The appointed incubation time points are T0 (0 min), Tn (n=0, 10, 30, 60, 120 min)

CONCLUSION(S)

Both UPLC and UPCC can be applied to separate the enantiomers of chiral PROTACs, and UPCC can significantly reduce 50-90% of the separation time comparing to UPLC, especially for the PROTACs with multiple chiral centers. The LLOQ was achieved for sample analysis by utilizing the UPLC coupled with triple-quadrupole mass spectrometry, which could support the low concentration samples from PK, distribution, and safety studies.

The conversion of the enantiomers and the ratio of the enantiomers in the target tissues or systemic circulation samples is critical for PROTAC early discovery. The corresponding assay is suggested to be investigated at the first step. We developed a rapid and sensitive UPCC-MS/MS and UPLC-MS/MS method for quantitation of chiral PROATCs in biomatrix to support the pharmacokinetics and pharmacodynamics for chiral PROTAC drugs.

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REFERENCE

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