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Development of high speed CYP cocktail inhibition assay using UHPLC-MS/MS

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1. Introduction

Pharmacokinetics is one of most important evaluations for New Chemical Entity (NCE). Unwilling Drug – Drug Interaction (DDI) that may cause serious side effect to patients must be strictly excluded before the new drugs being into the market. DDI is mainly resulted from Cytochrome P450 (CYP), which hydroxides water-insoluble toxic substances and promote excretion of such compounds. Most drugs are chemically designed considered the function of this enzyme, however, when the activity of this enzyme declines for some reason, drugs may work too much more than expected and some of them may be harmful to our body. Therefore, it is quite important to clarify whether a NCE affects the function of CYP.

CYP cocktail assay is one of the experiments that evaluate function of CYP. Some compounds that could be substances of CYP are incubated with human liver microsome for a fixed time, and modified substances are quantified with LC or LCMS.
We introduce an application of our Nexera and LCMS-8080 for this CYP cocktail assay. Although our conventional method takes 7 min for one analysis, now we have developed a new 1 min method including column wash and equilibrium. Moreover, no crucial difference from conventional method in LOQ, linearity and repeatability has been identified. Nexera and LCMS-8080 may have opened the new era of ultra-high performance CYP cocktail assay.

2. Material and Methods

2-1. Substances and Metabolites

Substances and their metabolites are shown in Table 1. These compounds are normally utilized as substances of CYP.

Substance	Metabolite
Resorufin Ethyl Ether	Resorufin
Bufuralol Hydrochloride	1′-Hydroxy Bufuralol
(S) - Mephenytoin	(+/-)-4′-Hydroxy Mephenytoin
Nifedipine	Oxidized Nifedipine
Tolbutamide	Hydroxy Tolbutamide

Table 1 Substances and Their Metabolites

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2-2. Sample Preparation

The flow chart of sample preparation is described below. All procedure are performed under the aseptic environment.

Human liver microsome (0.1 mg/mL)
Potassium phosphate buffer (pH 7.4) (50 mmol/L)
Magnesium Chloride (5 mmol/L)
Isocitric acid (5 mmol/L)
Isocitric acid dehydrogenase (1 unit/mL)
Substances (near Km concentration)

Diluted in 500 µL
ultra purified water

↓

Preincubate for 10 min in 37℃

↓

Add NADPH (1 mmol/L)

↓

Incubate for 10 min in 37℃

↓

Add 1 mL Acetonitirile (Stop the reaction)

↓

Centrifuge with 1000 rpm for 3 min

↓

Take a surface layer as sample

Fig. 1 Nexera and LCMS-8080 System

2-3. LC/MS/MS analysis

Table 2 Analytical conditions

The flow chart of sample preparation is described below. All procedure are performed under the aseptic environment.

UHPLC conditions (Nexera UHPLC system)

Column : Shim-pack XR-ODSII (30 mm x 1.5 mm I.D., 2.2 um)
Mobile phase : A – 0.05% formic acid – water B – Acetonitrile
Gradient program : 5%B (0 min) – 35%B (0.50 min) – 80%B (0.51 min) – 80%B (0.60 min) – 5%B (0.61 min) – 5%B (1.0 min)
Flow rate : 0.8 mL / min.
Column temperature : 50℃

MS conditions (LCMS-8080)

Ionization : ESI (Positive / Negative)

MRM transitions

Analytes	Polarity	Precursor *m/z*	Product *m/z*
Resorufin	+	214.10	186.05
1′-Hydroxy Bufuralol	+	278.15	186.20
(+/-)-4′-Hydroxy Mephenytoin	+	235.10	150.20
Oxidized Nifedipine	+	345.05	284.15
Hydroxy Tolbutamide	-	285.15	186.15

3. Result

3-1. Improvement of throughput

In conventional method using Shim-pack XR-ODSII (flow rate at 0.2 mL/min), all compounds were eluted within 4.5 min (the cycle time was 7 min). In UHPLC method, the cycle time was successfully reduced within 60 seconds. UHPLC-MS/MS improves the throughput 7 times compared with conventional system.

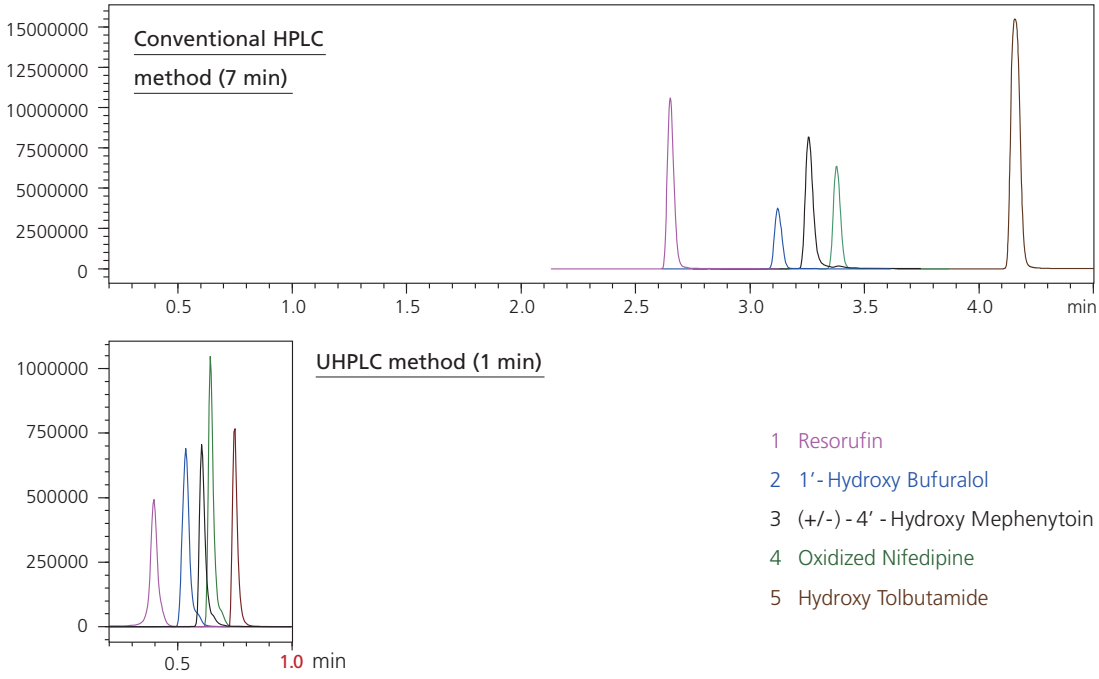

Fig. 2 Chromatograms of conventional HPLC method and UHPLC method

3-2. Calibration Curves

Each calibration curve had sufficient dynamic range, linearity and repeatability. Table 3 shows dynamic ranges and r^2 values of each compound. All the r^2 values achieve over 0.99. Table 4 shows each % RSD at n = 6 and all of them is below 10%. Fig. 3 shows the chromatograms of each compound at the lowest concentration. They maintained good shape of peak at the LOQ concentration.

Analytes	Dynamic Range(nmol/L)	r^2
Resorufin	0.6-300	0.999
1'-Hydroxy Bufuralol	0.6-300	0.998
(+/-)-4'-Hydroxy Mephenytoin	0.6-300	0.996
Oxidized Nifedipine	0.6-1000	0.998
Hydroxy Tolbutamide	0.6-300	0.999

Table 3 Information of Matrix Matched Calibration curves

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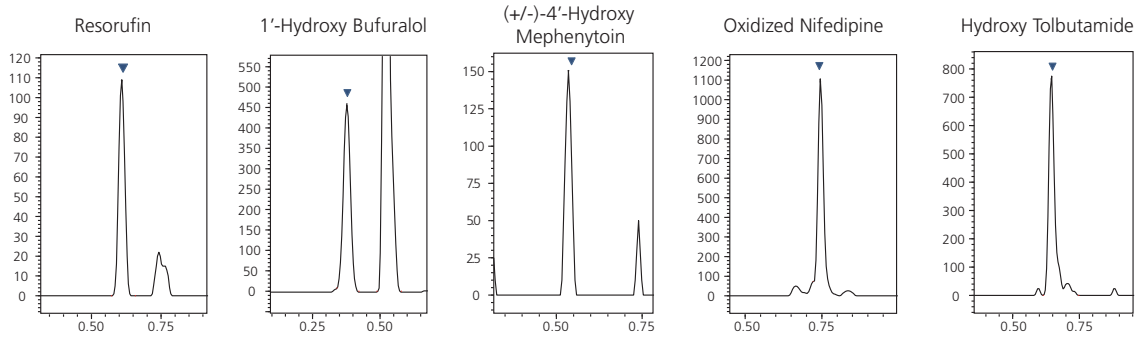

Fig. 3 Chromatograms of the lowest concentration

Table 4 Area % RSD of each analytes in the lowest concentration

Analytes	%RSD in the lowest concentration (n = 6)
Resorufin	9.97%
1'-Hydroxy Bufuralol	9.80%
(+/-)-4'-Hydroxy Mephenytoin	8.75%
Oxidized Nifedipine	8.04%
Hydroxy Tolbutamide	8.57%

3-2. Quantification of sample

The real sample was quantified with obtained calibration curves. The result is described below.

Table 5 Quantitation result of the real sample

Analytes	Concentration in the sample solution
Resorufin	9.43 nmol/L
1'-Hydroxy Bufuralol	83.22 nmol/L
(+/-)-4'-Hydroxy Mephenytoin	1.84 nmol/L
Oxidized Nifedipine	2829.24 nmol/L *1
Hydroxy Tolbutamide	69.93 nmol/L

*1 : The sample was diluted 10 times for analysis and the obtained value was increased by 10 .

4. Conclusion

Total run time for a single analysis of CYP cocktail assay has been considerably shortened with Nexera and LCMS-8080 system. 1 minute analysis has been accomplished.

The new prompt method doesn't deteriorate the quality of data; peak separation, LOQ, linearity and repeatability of each analytes doesn' t crucially deteriorate compared with conventional method.

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