

Supplementary Table 1 Accuracy and precision in the intra-assay and inter-assay for LC-MS/MS determinations of brexpiprazole and its metabolite, DM-3411, in rat and monkey plasmas

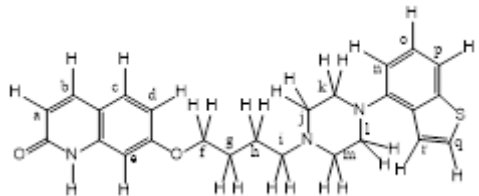
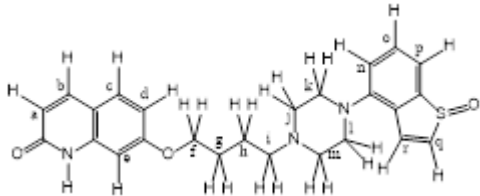
Species	Analyte	MRM ( <i>m/z</i> )	Calibration		Intra-assay *				Inter-assay **			
			Concentration (ng/mL)	Correlation coefficient	Accuracy		Precision		Accuracy		Precision	
Rat	Brexpiprazole	434→273	1 - 300	> 0.9949 (8) ***	97.2%	- 110.8%	< 10.5%		103.3%	- 107.3%	< 7.1%	
	Brexpiprazole	434→98	1 - 100	> 0.9968 (12)	100.6%	- 111.0%	< 9.0%		102.5%	- 104.0%	< 9.6%	
	M2: DM-3411	450→289	1 - 100	> 0.9970 (12)	101.0%	- 118.0%	< 8.5%		103.8%	- 104.6%	< 13.5%	
Monkey	Brexpiprazole	434→273	1 - 300	> 0.9966 (9)	91.8%	- 113.1%	< 14.4%		95.8%	- 105.5%	< 4.7%	
	Brexpiprazole	434→98	1 - 100	> 0.9979 (10)	95.0%	- 108.0%	< 8.4%		100.4%	- 104.0%	< 6.9%	
	M2: DM-3411	450→289	1 - 100	> 0.9956 (10)	96.0%	- 115.0%	< 7.3%		103.6%	- 110.0%	< 6.9%	

\* : Intra-assay represents the range of data from 4 different concentrations (*n*=5) in the calibration range, including LLOQ (*n*=5~6).

\*\* : Inter-assay represents the range of data from 3 different concentrations (*n*=15) in the calibration range.

\*\*\* : Value in the parenthesis represents the number of determinations

Supplementary Table 2 Chemical shift assignments of brexpiprazole and its metabolite, DM-3411, in [<sup>1</sup>H]NMR spectra

Structure	Brexpiprazole				M2: DM-3411			
								
Position	$\delta_H$ (ppm)	proton	m	( <i>J</i> in Hz)	$\delta_H$ (ppm)	proton	m	( <i>J</i> in Hz)
a	6.29	1H	d	9.4	6.29	1H	d	9.5
b	7.80	1H	d	9.4	7.80	1H	d	9.5
c	7.55-7.56	1H	m	-	7.54-7.56	1H	m	-
d, e	6.79-6.80	2H	m	-	6.79-6.80	2H	m	-
f	4.06	2H	t	6.3	4.05	2H	t	6.3
g	1.80	2H	tt	7.3, 6.3	1.76-1.82	2H	m	-
h	1.64	2H	tt	7.3, 7.3	1.61-1.66	2H	m	-
i	2.44	2H	t	7.3	2.42	2H	t	7.2
j, m	2.62	4H	br	-	2.58	4H	br,t	5.0
k, l	3.06	4H	br	-	3.02	4H	br,t	5.0
n	6.88	1H	dd	7.7, 0.8	7.17	1H	dd	7.7, 0.8
o	7.27	1H	t	7.7	7.45	1H	t	7.7
p	7.61	1H	d	7.7	7.56	1H	d	7.7
q	7.69	1H	d	5.5	7.35	1H	d	6.5
r	7.40	1H	dd	5.5, 0.8	7.43	1H	dd	6.5, 0.8
-NH	11.59	1H	s	-	11.58	1H	s	-

[<sup>1</sup>H]NMR spectrum was measured in DMSO-d<sub>6</sub> using a JNM-ECA500 NMR spectrometer (500 MHz, JEOL, Tokyo, Japan) at room temperature with tetramethylsilane as the internal standard.

s: singlet; d: doublet; dd: doublet of doublet; t: triplet; tt: triplet of triplet; br: broad; br,t: broad triplet; m: multiplet

Supplementary Table 3 Inhibition of CYP inhibitors to metabolic reactions of brexpiprazole by human liver microsomes

Inhibitor (CYPs)	Metabolites	% of control				
		Inhibitor concentration (μmol/L)				
		0.1	0.3	1	3	10
Furafylline (CYP1A1/1A2)	M2: DM-3411	95.7	98.2	100.1	96.7	96.5
	M3	107.6	109.6	103.6	105.1	111.1
	M6	100.4	87.9	95.5	90.0	93.0
		0.5	1.5	5	15	50
Orphenadrine (CYP2B6)	M2: DM-3411	98.5	100.0	97.4	94.5	84.5
	M3	98.5	92.5	101.7	94.1	96.7
	M6	104.3	104.1	96.8	97.0	100.4
		0.1	0.3	1	3	10
Sulfaphenazole (CYP2C9)	M2: DM-3411	93.8	95.3	101.8	98.8	92.9
	M3	107.0	104.1	98.5	95.4	99.9
	M6	97.3	98.0	98.0	92.6	99.1
		0.5	1.5	5	15	50
Tranylcypromine (CYP2C19)	M2: DM-3411	109.0	102.7	100.0	86.8	71.6
	M3	94.9	97.4	98.5	91.9	73.2
	M6	98.6	99.3	103.2	95.5	80.7
		0.1	0.3	1	3	10
Quinidine (CYP2D6)	M2: DM-3411	97.4	96.4	90.5	91.2	84.8
	M3	102.1	95.9	98.2	104.8	87.6
	M6	104.3	102.5	96.4	101.5	97.2
		0.1	0.3	1	3	10
Ketoconazole (CYP3A4)	M2: DM-3411	62.0	38.8	31.5	28.0	20.6
	M3	27.5	20.8	10.0	9.4	10.0
	M6	45.4	28.8	20.0	24.9	18.0

The metabolic reaction was performed at 20  $\mu\text{mol/L}$  of [ $^{14}\text{C}$ ]brexpiprazole, 2.5 mmol/L of  $\beta\text{-NADPH}$  and  $\beta\text{-NADH}$ , and 0.4 mg/mL of microsomal protein for 30 minutes at 37°C.

[ $^{14}\text{C}$ ]Brexiprazole and inhibitors for CYPs were preincubated with microsomes without  $\beta\text{-NADPH}/\beta\text{-NADH}$  solution at 37°C for 5 minutes, and  $\beta\text{-NADPH}/\beta\text{-NADH}$  solution was added to initiate the reaction. In the case of furafylline, the reaction mixture with  $\beta\text{-NADPH}/\beta\text{-NADH}$  solution was preincubated at 37°C for 15 minutes, and [ $^{14}\text{C}$ ]brexpiprazole was added to initiate the reaction.

Each value represents the mean of 2 determinations.

Supplementary Table 4 Inhibition of anti-CYP antibody to the metabolic reactions of brexpiprazole by human liver microsomes

CYP antibody	Metabolites	% of control		
		Anti-CYP antibody concentration (%)		
		1	2	4
CYP1A1/1A2	M2: DM-3411	124.4	122.2	111.8
	M3	101.6	96.1	100.3
	M6	106.5	107.5	99.4
CYP2B6	M2: DM-3411	99.9	98.1	99.0
	M3	101.1	105.2	101.7
	M6	109.5	102.4	100.5
CYP2C9	M2: DM-3411	110.9	114.3	107.1
	M3	106.9	108.2	105.2
	M6	93.2	99.7	95.5
CYP2C19	M2: DM-3411	111.9	104.6	109.5
	M3	96.9	98.0	97.8
	M6	97.1	94.1	88.5
CYP2D6	M2: DM-3411	89.4	91.6	90.3
	M3	109.9	105.0	103.4
	M6	99.9	101.8	102.7
CYP3A4	M2: DM-3411	68.9	49.5	46.0
	M3	41.6	27.4	20.5
	M6	55.5	38.8	21.3

The metabolic reaction was performed at 20  $\mu$ mol/L of brexpiprazole, 2.5 mmol/L of  $\beta$ -NADPH and  $\beta$ -NADH, and 0.4 mg/mL of microsomal protein for 30 minutes at 37°C.

The antibody was preincubated with microsomes for 10 minutes at room temperature (for 15 minutes and on ice in the case of CYP2B6), and [<sup>14</sup>C]brexiprazole and β-NADPH/β-NADH were added to initiate the reaction.

Each value represents mean of 2 determinations.

Supplementary Table 5 Serum protein binding of q-[<sup>14</sup>C]brexpiprazole and its metabolite, DM-3411 by an equilibrium dialysis in the mouse, rat, monkey and human

Animal	Protein binding (%)					
	q-[ <sup>14</sup> C]Brexiprazole			M2: DM-3411		
	0.05 µg/mL	0.5 µg/mL	5 µg/mL	0.05 µg/mL	0.5 µg/mL	5 µg/mL
Mouse	N.D.	99.6 ± 0.0	99.4 ± 0.0	93.4 ± 0.6	93.3 ± 0.8	93.5 ± 0.5
Rat	N.D.	99.7 ± 0.1	99.4 ± 0.1	93.1 ± 0.6	92.8 ± 1.2	92.6 ± 0.8
Monkey	N.D.	99.5 ± 0.1	99.5 ± 0.1	94.2 ± 0.0	94.3 ± 0.3	89.8 ± 0.4
Human	N.D.	99.8 ± 0.0	99.8 ± 0.0	96.9 ± 0.2	97.0 ± 0.2	95.5 ± 0.3

Each data represent mean ± S.D. of 3 determination.

N.D.: Radioactivity was not detected in the free fraction compartment.



**Supplementary Figure 1** ESI positive spectra of brexpiprazole and its metabolite, M2 (DM-3411)

(A)-1: Full scan mass spectrum of brexpiprazole; (A)-2: Product ion spectrum of brexpiprazole

(B)-1: Full scan mass spectrum of M2; (B)-2: Product ion spectrum of M2

Spectral analysis was conducted using the 4000QTRAP LC-MS/MS system (SCIEX) equipped with an electrospray ionization source.

Fragment ions, such as those originating from the piperazine ring, are just structures proposed from the ion masses.

Proposed molecular formula of fragment ion with reference by Thakkar and Kate (2019):  $m/z$  289:  $C_{16}H_{21}N_2O_1S_1^+$ ; 273:  $C_{16}H_{21}N_2S_1^+$ ; 261:  $C_{14}H_{17}N_2O_1S_1^+$ ; 231:  $C_{13}H_{15}N_2S_1^+$ ; 216:  $C_{12}H_{12}N_2S_1^+$ ; 188:  $C_{11}H_{10}NS_1^+$ ; 174:  $C_{10}H_8N_1O_2^+$ ; 98:  $C_6H_{12}N_1^+$ .

**Supplementary Figure 2** ESI positive spectra of M3 and M4

(A)-1: Full scan mass spectrum of M3; (A)-2: Product ion spectrum of M3

(B)-1: Full scan mass spectrum of M4; (B)-2: Product ion spectrum of M4

MS/MS analysis was conducted using the 4000 QTRAP LC-MS/MS system (Applied Biosystems/MDS SCIEX) equipped with an electrospray ionization source.

Fragment ions, such as those originating from the piperazine ring, are just structures proposed from the ion masses.

Proposed molecular formula of fragment ion with reference by Thakkar and Kate (2019):  $m/z$  305:  $C_{16}H_{21}N_2O_2S_1^+$ ; 174:  $C_{10}H_8N_1S_1^+$ ; 147:  $C_8H_5N_1S_1^+$ ; 134:  $C_8H_6S_1^+$ .

**Supplementary Figure 3** ESI positive spectra of M5 and M6

(A)-1: Full scan mass spectrum of M5; (A)-2: Product ion spectrum of M5

(B)-1: Full scan mass spectrum of M6; (B)-2: Product ion spectrum of M6

Spectral analysis was conducted using the 4000QTRAP LC-MS/MS system (SCIEX) equipped with an electrospray ionization source.

Fragment ions, such as those originating from the piperazine ring, are just structures proposed from the ion masses.

Proposed molecular formula of fragment ion with reference by Thakkar and Kate (2019):  $m/z$

216 :  $C_{12}H_{12}N_2S_1^+$ ; 176 :  $C_{10}H_{10}N_1S_1^+$ ; 162 :  $C_9H_8N_1O_2^+$ ; 144 :  $C_9H_6N_1O_1^+$ .

#### **Supplementary Figure 4** ESI positive spectra of M7 and M7-Glucuronide

(A)-1: Full scan mass spectrum of M7; (A)-2: Product ion spectrum of M7

(B)-1: Full scan mass spectrum of M7-Glucuronide; (B)-2: Product ion spectrum of M7-Glucuronide

Spectral analysis was conducted using the 4000QTRAP LC-MS/MS system (SCIEX) equipped with an electrospray ionization source.

Proposed molecular formula of fragment ion with reference by Thakkar and Kate (2019):  $m/z$

289:  $C_{16}H_{21}N_2O_1S_1^+$ .

#### **Supplementary Figure 5** ESI positive spectra of metabolite in monkey plasma and synthesized *N*-oxide

(A)-1: Full scan mass spectrum of metabolite in monkey plasma; (A)-2: Product ion of metabolite in monkey plasma

(B)-1: Full scan mass spectrum of synthesized *N*-oxide; (B)-2: Product ion spectrum of synthesized *N*-oxide

Spectral analysis was conducted using the 4000QTRAP LC-MS/MS system (SCIEX) equipped with an electrospray ionization source.

Proposed molecular formula of fragment ion with reference by Thakkar and Kate (2019):  $m/z$  289:  $C_{16}H_{21}N_2O_1S_1^+$ ; 273:  $C_{16}H_{21}N_2S_1^+$ ; 231:  $C_{13}H_{15}N_2S_1^+$ .

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### Supplementary Table 2

Chemical shift assignments of brexpiprazole and its metabolite, DM-3411, in  $[^1H]$ NMR spectra

$[^1H]$ NMR spectrum was measured in DMSO- $d_6$  using a JNM-ECA500 NMR spectrometer (500 MHz, JEOL, Tokyo, Japan) at room temperature with tetramethylsilane as the internal standard.

s: singlet; d: doublet; dd: doublet of doublet; t: triplet; tt: triplet of triplet; br: broad; br,t: broad triplet; m: multiplet

### Supplementary Table 3

Inhibition of CYP inhibitors to metabolic reactions of brexpiprazole by human liver microsomes

The metabolic reaction was performed at 20  $\mu\text{mol/L}$  of [ $^{14}\text{C}$ ]brexpiprazole, 2.5 mmol/L of  $\beta\text{-NADPH}$  and  $\beta\text{-NADH}$ , and 0.4 mg/mL of microsomal protein for 30 minutes at 37°C.

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Each value represents the mean of 2 determinations.

#### **Supplementary Table 4**

Inhibition of anti-CYP antibody to the metabolic reactions of brexpiprazole by human liver microsome

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The antibody was preincubated with microsomes for 10 minutes at room temperature (for 15 minutes and on ice in the case of CYP2B6), and [ $^{14}\text{C}$ ]brexpiprazole and  $\beta\text{-NADPH}/\beta\text{-NADH}$  were added to initiate the reaction.

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#### **Supplementary Table 5**

Serum protein binding of q-[ $^{14}\text{C}$ ]brexpiprazole and its metabolite, DM-3411 by an equilibrium dialysis in the mouse, rat, monkey and human

Each data represent mean  $\pm$  S.D. of 3 determination.

N.D.: Radioactivity was not detected in the free fraction compartment.