Is the Direct or Indirect Method the Ideal One for Evaluation of Blood to Plasma Ratio?

Jie Wang[1], Yan Liu[2], Yuan Hu[2], Chunhong Lu[1], Mengying Li[1], Duo Zhang, Cheng Chen[1], Xiangling Wang[1], Liang Shen[1] , Genfu Chen[1]

1. DMPK Department, WuXi AppTec, 31 Yiwei Rd., Waigaoqiao Free Trade Zone, Shanghai, China
2. DMPK Department, WuXi AppTec, 71 Longshan North Road, Pukou District, Nanjing, China

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Abstract

In vitro blood to plasma ratio (BPR) assays are important for understanding drug distribution into red blood cells (RBCs), thus informing a rational choice of appropriate biological fluid for pharmacokinetic analysis. Two methods, namely direct method and indirect method, are routinely performed in the industry. However, our preliminary data showed there are false negative blood partition results occasionally in monkey for a few compounds that should show positive blood partition. In order to evaluate whether the two methods are able to provide similar results qualitatively and quantitatively, a set of 16 compounds with reported *in vitro* or *in vivo* BPR values were selected for comparison of the two methods in both human and monkey blood. Moreover, matrix effect and extraction recovery were further evaluated for 5 of the compounds that showed larger differences between two methods. In blood sample lysis, methods like Triton X-100 (0.1–0.5%), sonication, 1% SDS, or high-concentration denaturants provided slight enhancements over simple water treatment. However, for certain compounds—primarily strong carbonic anhydrase inhibitors and those exhibiting reversible covalent binding to hemoglobin—the BPR values were differed by more than 2-fold. While both methods show comparable results for most compounds, the indirect method demonstrates superior accuracy for certain analytes by overcoming the limitations of incomplete hemolysis and extraction variability inherent to direct blood analysis.

Methods

Items	Name	Vender
Compound	Acetazolamide, Chlorthalidone, Forosemide, Indapamide, Metformin, Rapamycin, Tacrolimus, Carboplatin, Tucaresol, Dorzolamide, Voxelotor, Brinzolamide, Afatinib, Osimertinib, Chloroquine and Methazolamide	MedChemExpress
Matrix	Human Blood and Human Plasma	The Second People's Hospital of Liaocheng
	Monkey Blood and Monkey Plasma	WuXi AppTec
Instrument	Liquid Chromatography	Waters
	Mass Spectrometry	Sciex

- **Indirect method:** Apart from typical blood incubations, additional incubation of the drug candidate in plasma is performed (reference plasma). Following incubation, an aliquot of blood is centrifuged to obtain the plasma (test plasma). Both test and reference plasma samples are processed using the appropriate protein precipitation method, and the supernatants obtained are analyzed using LC–MS/MS.
- **Direct method:** Test plasma is obtained following a similar procedure as described above, and levels of the drug candidate in both test blood and test plasma are measured.
- For the same compound, experiments of direct and indirect method were performed using the same lot of blood.

Key Findings and Conclusion

- Quantitatively, 5 out of 16 compounds had BPR values over 1.5-fold after 1 or 3 hours in human blood, and 6 out of 16 compounds had BPR values over 1.5-fold in monkey blood. The indirect method typically provides higher values, which agree better with literature data.
- Except for Brinzolamide in human and monkey blood, which exhibited significant matrix effect after protein precipitation, the other compounds showed neglected matrix effect in blood or plasma for both species in standard protein precipitation procedure.
- Four compounds from 5 of the compounds that showed larger differences between two methods showed very low extraction (<30%) from blood samples, but much higher recovery in plasma was observed.
- In blood sample lysis, methods like Triton X-100 (0.1–0.5%), sonication, Urea (6–8 M) and 1%SDS provided slight enhancements over simple water treatment.
- While both methods show comparable results for most compounds, the indirect method demonstrates superior accuracy for certain analytes by overcoming the limitations of incomplete hemolysis and extraction variability inherent to direct blood analysis.

References

Please contact us to obtain detailed references due to limited space.

Results

Table 1 The comparison of $K_{B/P}$ values of 16 compounds in human and monkey blood with direct and indirect methods.

Quantitatively, 5 out of 16 compounds had BPR values over 1.5-fold after 1 or 3 hours in human blood, and 6 out of 16 compounds had BPR values over 1.5-fold in monkey blood. Most of the $K_{B/P}$ were the mean values of triplicate measurements, while a few data points were derived from duplicate samples. The %Recovery of the direct method ranged between 70.0 and 130.0.

Compound	Human Blood			Monkey Blood			Literature $K_{B/P}$ Values*	Comment on in-house assay conditions
	$K_{B/P}$ [a] Direct	$K_{B/P}$ Indirect	Indirect/ Direct [b]	$K_{B/P}$ Direct	$K_{B/P}$ Indirect	Indirect/ Direct		
Acetazolamide	0.83 ± 0.14	0.80 ± 0.05	0.96	1.07 ± 0.05	1.31 ± 0.08	1.22	2.9; 1.5	1 µM, 1 hr
Chlorthalidone	1.92 ± 0.28	2.42 ± 0.11	1.26	17.60 ± 0.50	21.32 ± 1.32	1.21	22.1	1 µM, 1 hr
Forosemide	0.74 ± 0.07	0.84 ± 0.04	1.15	1.44 ± 0.13	1.56 ± 0.05	1.09	0.775 - 0.85	1 µM, 1 hr
Indapamide	4.20 ± 0.24	4.25 ± 0.33	1.01	8.18 ± 1.47	9.83 ± 0.99	1.20	3.44	1 µM, 1 hr
Metformin	0.59 ± 0.05	0.71 ± 0.04	1.20	0.67 ± 0.04	0.69 ± 0.04	1.02	0.8 - 1.4	1 µM, 1 hr
Rapamycin	6.23 ± 1.23	11.65 ± 0.81	1.87	6.14 ± 0.91	20.77 ± 1.83	3.39	0.894; 1.5; 11.1	0.1 µM, 1 hr
Tacrolimus	18.58 ± 3.61	22.55	1.21	17.55 ± 1.12	13.70 ± 0.54	0.78	9.86; 13 - 114; 15	0.1 µM, 1 hr
Carboplatin	0.43 ± 0.06	0.36 ± 0.03	0.85	0.87 ± 0.04	0.80 ± 0.12	0.92	0.9 - 1.1	1 µM, 1 hr
Tucaresol	1.60 ± 0.09	1.93 ± 0.12	1.21	2.27 ± 0.29	2.08 ± 0.10	0.92	5.05 - 6.40	1 µM, 1 hr
Dorzolamide	22.84 ± 5.58	59.57	2.61	14.25	78.44	5.50	28.4	1 µM, 1 hr
	20.13 ± 1.67	39.81 ± 6.39	1.98	37.52 ± 9.63	130.08 ± 14.94	3.47	75.9	1 µM, 3 hr
Voxelotor	1.45	3.88 ± 0.28	2.67	1.53 ± 0.30	4.84	3.17	9 ± 2 - 46 ± 69	1 µM, 1 hr
	0.89 ± 0.08	5.51 ± 0.37	6.19	0.92 ± 0.09	12.18	13.18		1 µM, 3 hr
Brinzolamide	14.92 ± 3.24	28.23 ± 4.61	1.89	10.58	46.60 ± 3.85	4.41	66.7 - 100	1 µM, 1 hr
	15.18 ± 0.71	55.48 ± 8.05	3.65	23.28	144.04	6.19		1 µM, 3 hr
Afatinib	1.74 ± 0.05	2.06 ± 0.14	1.19	1.85 ± 0.21	2.14 ± 0.10	1.15	2.21; 1.02	1 µM, 1 hr
	1.75 ± 0.05	2.03 ± 0.14	1.16	1.34 ± 0.20	2.41 ± 0.07	1.80		1 µM, 3 hr
Osimertinib	1.51± 0.07	1.06 ± 0.04	0.70	2.33 ± 0.27	1.92 ± 0.07	0.82	1.57	1 µM, 1 hr
	1.50 ± 0.16	1.39 ± 0.08	0.93	2.03 ± 0.10	1.45 ± 0.02	0.72		1 µM, 3 hr
Chloroquine	3.01 ± 0.29	3.43 ± 0.09	1.14	4.03 ± 0.47	4.49 ± 0.18	1.11	3.91	1 µM, 1 hr
	2.70 ± 0.12	3.22 ± 0.10	1.19	3.68 ± 0.06	4.19 ± 0.31	1.14		1 µM, 3 hr
Methazolamide	37.60 ± 2.37	47.82 ± 0.62	1.27	7.39 ± 1.38	16.69 ± 0.99	2.26	35.9	1 µM, 1 hr
	11.77	50.03	4.25	55.52 ± 6.65	64.62	1.16	52.9	1 µM, 3 hr

[a] $K_{B/P}$ = The concentration ratio of test compound in erythrocytes to plasma concentration.
[b] Indirect / Direct = The $K_{B/P}$ ratio of test compound with indirect to direct method.
* Please contact us to obtain more detailed references due to limited space.

Table 2 The evaluation of matrix effect and extraction recovery for 5 compounds in blood and plasma from human and monkeys.

Except for Brinzolamide in blood, which exhibited significant matrix effect, the other compounds showed neglected matrix effect in blood or plasma for both species. Four compounds from 5 of the compounds showed very low extraction from blood samples, but much higher recovery in plasma was observed. The data represents the mean of triplicate measurements.

Compound	Matrix Effect [a]				Extraction Recovery [b]			
	Human Blood	Monkey Blood	Human Plasma	Monkey Plasma	Human Blood	Monkey Blood	Human Plasma	Monkey Plasma
Afatinib	94.86 ± 0.01	120.29 ± 0.06	109.72 ± 0.02	162.46 ± 0.03	96.65 ± 0.09	97.81 ± 0.12	100.54 ± 0.05	86.58 ± 0.07
Brinzolamide	57.07 ± 0.01	68.21 ± 0.02	81.45 ± 0.02	80.50 ± 0.002	26.67 ± 0.05	18.18 ± 0.01	119.21 ± 0.01	95.84 ± 0.01
Dorzolamide	70.29 ± 0.06	/	84.23 ± 0.03	93.64 ± 0.03	29.55 ± 0.03	/	104.45 ± 0.05	95.69 ± 0.05
Methazolamide	81.93 ± 0.004	102.66 ± 0.03	100.93 ± 0.02	125.42 ± 0.02	29.38 ± 0.01	8.11 ± 0.004	99.75 ± 0.06	85.04 ± 0.07
Voxelotor	119.49 ± 0.02	119.18 ± 0.02	121.02 ± 0.02	116.00 ± 0.03	1.58 ± 0.001	1.13 ± 0.001	11.74 ± 0.01	16.35 ± 0.01

[a] Matrix Effect = AT_0-BA / Neat * 100; AT_0-BA = The peak area ratio of analyte and internal standard in the supernatant of inactive matrix at time zero; Neat = The peak area of analyte in Water: ACN (1: 3, v: v) or Water: ACN (1: 6, v: v).
[b] Extraction Recovery = T_0 / AT_0-BA * 100; T_0 = The peak area ratio of analyte and internal standard in the loading matrix sample at time zero.

Table 3 The methods to improve extraction recovery of 4 compounds in monkey blood.

Using methods like Triton X-100 (0.1–0.5%), sonication, Urea (6–8 M) and 1%SDS provided slight enhancements for extraction recovery over simple water treatment (which is the standard protocol). The data represents the mean values of triplicate measurements.

Methods	Extraction Recovery [a]			
	Brinzolamide	Dorzolamide	Methazolamide	Voxelotor
Three freeze-thaw cycles	4.08 ± 0.20	4.15 ± 0.19	3.09 ± 0. 17	0.81 ± 0.04
One freeze-thaw cycle	10.95 ± 1.12	11.64 ± 0.78	6.49 ± 0.59	1.46 ± 0.13
Sonication	69.68 ± 7.94	59.14 ± 6.27	21.63 ± 2. 78	3.54 ± 0.24
0.5%Triton X-100	22.49 ± 2.03	30.53 ± 5.56	9.56 ± 1.13	2.48 ± 0.17
0.1%Triton X-100	42.43 ± 3.51	44.04 ± 2.24	13.46 ± 0.71	2.38 ± 0.17
8 M Urea	63.02 ± 2.28	66.69 ± 5.73	85.72 ± 7.19	11.01 ± 1.93
6 M Urea	69.79 ± 5.40	72.07 ± 5.89	97.13 ± 5.18	8.40 ± 2.95
1%SDS	39.00 ± 3.64	42.41 ± 4.67	17.09 ± 0.87	3.71 ± 0.18

[a] Extraction Recovery = T_0 / AT_0-BA * 100; T_0 = The peak area ratio of analyte and internal standard in the loading matrix sample at time zero; AT_0-BA = The peak area ratio of analyte and internal standard in the supernatant of inactive matrix at time zero;

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