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Impact of hemolysis during sample collection: How different is drug concentration in hemolyzed plasma from that of normal plasma?

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ABSTRACT

Hemolysis is a common phenomenon in clinical studies. Despite the growing interest in hemolysis matrix effect, how hemolysis impacts the representability of hemolyzed plasma samples was rarely evaluated. The purpose of this research is to perform such an evaluation by theoretical consideration and experiment. A formula for estimating the impact is proposed, which includes the degree of hemolysis and the drug's red blood cell (RBC): plasma concentration ratio. The impact of hemolysis on the representability of hemolyzed plasma samples is compound-dependant. Given the same degree of hemolysis, the stronger a drug binds to RBCs, the more significant the impact of hemolysis. For a drug with high affinity to RBCs, the results of hemolyzed plasma samples may not be useful even though they are accurate. There is an overall agreement between theoretical predication and experimental results. Among the ten different drug compounds tested, only methazolamide, which binds strongly to RBCs, showed significant change in plasma concentration due to hemolysis.

1. Introduction

Hemolysis (lysis of red blood cells, RBCs) occurs quite often during blood sample collection and handling in clinical studies, which results in a pink to red discoloration of the subsequently harvested plasma samples because the blood pigments (e.g. hemoglobin) in RBCs are released into the plasma. Since additional components, which are normally absent in plasma, are released into the plasma during hemolysis, it is therefore very important to evaluate the associated matrix effect, hemolysis matrix effect, during bioanalytical method development and validation to ensure that hemolyzed plasma samples can be accurately analyzed [1–5]. In some cases, it is even necessary to evaluate the impact of hemolysis on drug stability in hemolyzed samples [6].

Despite the importance of assessing hemolysis matrix effect and drug stability in hemolyzed samples, one must foremost evaluate whether hemolysis during sample collection and handling could significantly impact the representability of the harvested plasma samples. In other words, is the drug concentration in hemolyzed plasma still representative of that of normal plasma without hemolysis? After all, if hemolysis has dramatically affected the concentration of a drug in hemolyzed plasma, the accuracy of the reported concentrations for the hemolyzed samples would not make much difference anymore, because they have to be excluded from the intended pharmacokinetic evaluation, i.e. not compatible with those of non-hemolyzed plasma samples.

Unfortunately, such an important evaluation is lacking in the literature, yet there is no shortage of reports regarding drug erythrocyte-to-plasma distribution ratio [7–9], a related but different topic. It was the purpose of this research to assess the impact of hemolysis on the sequestration of drug compounds into harvested plasma during sample collection and handling first by theoretical calculation and then followed by experimental testing of ten different drug components (Fig. 1). The findings would fill in the information gap and provide much needed confidence in utilizing the reported concentrations for hemolyzed study samples.

2. Experimental

2.1. Blood sample processing procedure for post-spiking hemolysis test

Fresh whole blood was collected in EDTA K_2 tubes from 11 donors (7 females and 4 males, about 30 ml per subject) and pooled. Then, each drug (except for donepezil which had already been tested during the preliminary experiment) was spiked in a 12 ml aliquot of this pooled blood (spiking ratio: 1 part of working solution to 99 parts of blood). The spiked blood aliquot was subsequently split into two and processed as shown in Fig. 2. The resulting comparison and hemolyzed plasma samples were all stored in a −80 °C freezer until being retrieved for analysis.

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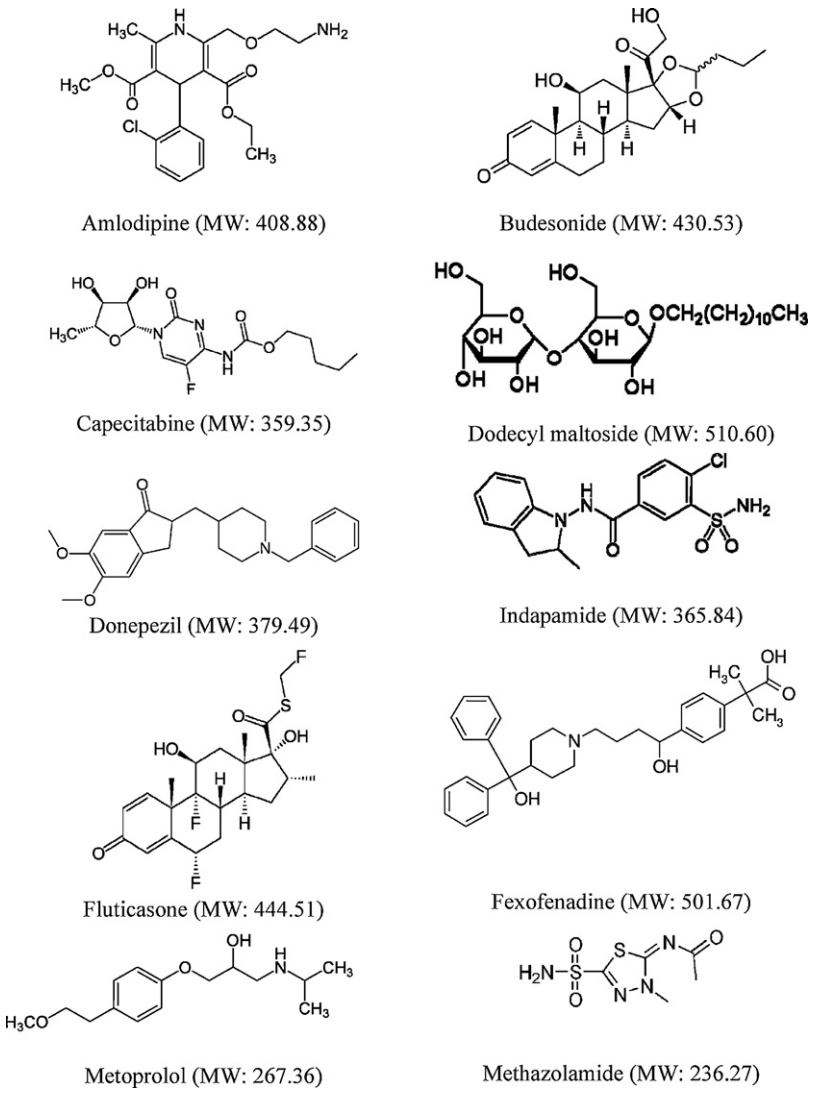

Fig. 1. Chemical structures of the tested drug compounds.

2.2. Blood sample processing procedure for different percentage of hemolysis test

Fresh whole blood was collected in EDTA K_2 tubes from a male donor and was aliquoted (3 ml per aliquot). One of the aliquots was flash-frozen in a dry ice/methanol bath and then thawed for complete hemolysis. Then, this hemolyzed whole blood was added to fresh whole blood aliquots at different percentages, i.e. 1%, 2%, 5%, and 10%. These hemolyzed blood aliquots as well as one aliquot without the addition of hemolyzed blood were spiked individually with donepezil and methazolamide at the concentrations of 300 pg/ml and 3000 ng/ml, respectively. These spiked blood samples were then incubated at 4 °C for about 30 min and centrifuged (3000 rpm, 4 °C) for 5 min to separate plasmas. The harvested plasma samples were then analyzed by the respective validated analytical methods in three replicates each.

2.3. Analysis of comparison and hemolyzed plasma samples for post-spiking hemolysis test

The comparison and hemolyzed samples associated with a drug compound were analyzed in three replicates each together with a calibration curve and six replicates each of low, medium, and high quality control samples using a validated analytical method. In addition, these comparison and hemolyzed samples were also analyzed after being diluted fivefold in the same run (batch) to reduce the potential impact of hemolysis on the quantitation though the methods had been validated for lack of hemolysis matrix effect at 2% level.

2.4. Analytical methods

Due to the large number of analytical methods used and considering that the analytical methods are not the main topic in this research, the details of the analytical methods are not presented here. Only the key features of the analytical methods involved are summarized in Table 1. Each method was validated for lack of hemolysis matrix effect at 2% hemolysis level by analyzing three replicates of hemolyzed blank plasma and six replicates each of low and high quality control samples containing 2% of hemolyzed whole blood [5].

2.5. Evaluation of hemolysis level

A visual reference scale was prepared by adding 1%, 2%, 5%, and 10% of hemolyzed blood into normal non-hemolyzed pooled control (blank) plasma. Then, a hemolyzed sample was visually

Table 1
Summary of analytical methods.

Name of drug	Conc.[a] range	Sample vol. (ml)	Extraction method	Internal standard	Retention time (min)	MS detection
Amlodipine	50–10,000 pg/ml	0.5	SPE[b]	Amlodipine-d_4	1.20 (1.19)[h]	ESI+[e]; 408.9 → 238.1 (412.9 → 238.1)[h]
Budesonide	2–1000 pg/ml	0.3	LLE[c]	Budesonide-d_8	1.16 (1.12)	ESI−[f]; 489.1 → 357.2 (497.1 → 357.2)
Capecitabine	10–5000 ng/ml	0.05	PP[d]	Capecitabine-d_{11}	0.56 (0.54)	ESI+; 360.3 → 244.2 (371.3 → 255.2)
Dodecyl Maltoside	0.5–2500 ng/ml	0.12	LLE	Decyl-β-D-maltoside	1.62 (1.05)	ESI+; 528.4 → 325.2& 349.4 (500.4 → 325.2)
Donepezil	0.1–50 ng/ml	0.2	SPE	Donepezil-d_4	1.20 (1.19)	ESI+; 380.5 → 91.1 (384.5 → 93.1)
Fexofenadine	0.5–500 ng/ml	0.05	SPE	Fexofenadine-d_{10}	1.01 (0.95)	APCI+[g]; 502.5 → 466.4 (512.5 → 476.4)
Fluticasone	1–500 pg/ml	0.5	LLE	Fluticasone-d_5	1.74 (1.71)	APCI+; 501.2 → 293.2 (506.3 → 293.3)
Indapamide	0.25–125 ng/ml	0.1	LLE	Indapamide-d_3	0.52 (0.51)	ESI+; 366.1 → 132.1 (369.2 → 135.1)
Methazolamide	5–10,000 ng/ml	0.05	PP	Methazolamide-d_3	0.60 (0.58)	ESI+; 237.1 → 194.9 (240.1 → 195.9)
Metoprolol	0.25–100 ng/ml	0.2	SPE	Metoprolol-d_7	0.79 (0.77)	ESI+; 268.2 → 133.1 (275.2 → 121.1)

[a] Conc.: concentration.
[b] SPE: solid-phase extraction.
[c] LLE: liquid–liquid extraction.
[d] PP: protein precipitation.
[e] ESI+: electrospray ionization in positive mode.
[f] ESI−: electrospray ionization in negative mode.
[g] APCI+: atmospheric pressure chemical ionization in positive mode.
[h] The mass transitions and retention times in brackets are for the internal standards.

compared with this reference scale to determine its level of hemolysis.

3. Results and discussion

3.1. Theoretical consideration

Drug blood concentration (C_b), drug red blood cell concentration (C_r), and drug plasma concentration (C_p) can be related to each other through hematocrit (H) as in Eq. (1) [10,11]:

$$C_b = H \times C_r + (1 - H) \times C_p \quad (1)$$

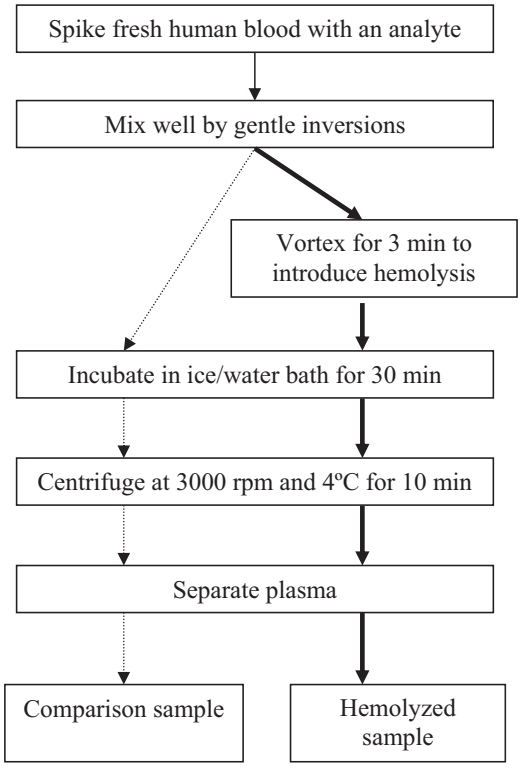

Fig. 2. Blood sample processing procedure for post-spiking hemolysis test.

Accordingly, drug plasma concentration can be calculated as:

$$C_p = \frac{C_b - H \times C_r}{1 - H} \quad (2)$$

When hemolysis occurs, the amount of a drug that normally associates with RBCs is released into plasma together with the contents of lysed RBCs, accompanied with a slight increase in volume for the harvested hemolyzed plasma. Hence with γ % of hemolysis, the concentration of the drug in hemolyzed plasma (C_p') can be estimated according to Eq. (3).

$$C_p' = \frac{C_b - H \times C_r + 0.01\gamma \times H \times C_r}{1 - H + 0.01\gamma \times H} \quad (3)$$

The percentage of change in drug concentration (δ(%)), i.e. hemolyzed plasma versus normal non-hemolyzed plasma can be defined as in Eq. (4).

$$\delta(\%) = \frac{C_p' - C_p}{C_p} \times 100 \quad (4)$$

Assuming drug RBC: plasma concentration ratio (α) as:

$$\alpha = \frac{C_r}{C_p} \quad (5)$$

And by combining Eqs. (2)–(4), the following formula (Eq. (6)) for estimating hemolysis effect on drug plasma concentration change is obtained.

$$\delta(\%) = \frac{\gamma \times H \times (\alpha - 1)}{1 - (1 - 0.01\gamma) \times H} \quad (6)$$

Since the hematocrit value (H) for a given subject (donor) should be constant though it varies among individuals (age and sex, etc.) [12]; the change of concentration is therefore mainly related to the degree of hemolysis (γ) and drug RBC: plasma concentration ratio (α). Depending on different scenarios, hemolysis could cause an increase, decrease or no change of drug concentration in hemolyzed plasma samples (versus non-hemolyzed plasma samples) (Table 2). For example, when a drug is mainly sequestrated in RBCs, therefore, hemolysis can increases drug concentration for hemolyzed plasma in relation to non-hemolyzed plasma. A special situation is that a drug is not sequestrated in plasma at all, and then any tiny amount of hemolysis should cause significant change in drug plasma concentration, i.e. % change being infinitively large. On the other hand,

Table 2
How hemolysis would impact drug plasma concentration in different scenarios.

If	Then	Signification
$\gamma = 0$	$\delta = 0$	No hemolysis, no change in drug plasma concentration.
$\alpha < 1$ ($C_r < C_p$)	$\delta < 0$	Hemolysis decreases drug plasma concentration.
$\alpha = 1$ ($C_r = C_p$)	$\delta = 0$	Hemolysis does not affect drug plasma concentration.
$\alpha > 1$ ($C_r > C_p$)	$\delta > 0$	Hemolysis increases drug plasma concentration.

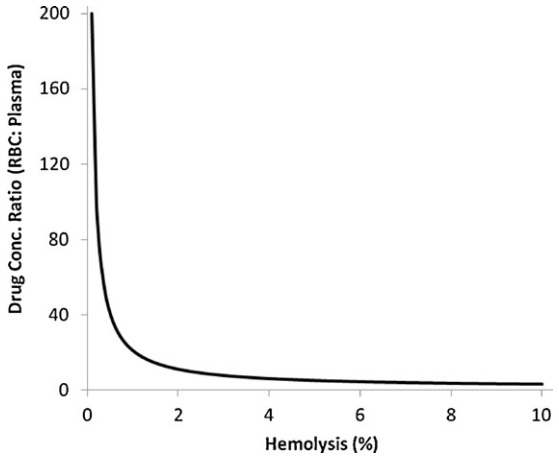

Fig. 3. Maximum allowable drug RBC: plasma concentration ratio versus degree of hemolysis to avoid hemolysis impact on drug plasma concentration (no significant impact below the curve). RBC: red blood cell; Conc.: concentration.

if a drug is 100% sequestrated in plasma, then hemolysis can cause a decrease in drug plasma concentration due to an increase in volume for hemolyzed plasma and no extra amount of drug brought in to the hemolyzed plasma. Finally, when α happens to be one ($C_r = C_p$), then there should be no change of drug plasma concentration at all no matter how severe the hemolysis is.

Furthermore, for a given degree of hemolysis, a maximum allowable drug RBC: plasma concentration ratio can be calculated from Eq. (6) (Fig. 3 and Table 3) by using the same acceptance criterion of 15% as in stability evaluation [13] for concentration change and using an average hematocrit value of 0.43 (the average of hematocrit values for adult males and females [12]). If the RBC: plasma concentration ratio of a drug is less than the maximum allowable value, the impact of the corresponding degree of hemolysis on drug plasma concentration would be negligible, i.e. less than 15% change in drug plasma concentration. For example, if the RBC: plasma concentration ratio for a drug is less than 11.1, then no significant change in concentration would be observed when the percentage of hemolysis is equal to or less than 2%.

On the other hand, though hemolysis could cause a decrease in drug plasma concentration for hemolyzed samples when α is less than one, it is practically not a concern because the percentage of change in drug plasma concentration associated with the lowest possible drug RBC: plasma concentration ratio (i.e. $\alpha = 0$) is only −7% at the hemolysis level of 10% (a very unlikely severity of hemolysis).

3.2. Effectiveness of different approaches to introduce hemolysis

To introduce hemolysis, various approaches were tested, including vortexing, sonication, and addition of methanol or citric acid. Though the addition of methanol or citric acid was quite effective, it was not selected due to unwanted side effects, such as pH change and/or protein precipitation. Sonication was not as effective as expected. After 5 min of sonication, less than 1% of hemolysis was produced. Vortexing was finally chosen for post-spiking hemolysis test due to its efficiency in producing hemolysis as well as the absence of unwanted side effects. For 3 min of maximum speed vortexing, approximately 2% of hemolysis can be produced.

Moreover, flash-freezing and thawing was tested and it was found very effective, nearly 100% hemolysis. However, it was difficult to introduce limited hemolysis level required for post-spiking hemolysis test by this approach. Therefore, it was used only in the test of different percentages of hemolysis.

3.3. Selection of drug compounds

Based on the theoretical consideration mentioned above, the impact of hemolysis on drug sequestration in plasma should be compound-dependent because different drugs have different plasma sequestration ratios. Therefore, it is necessary to test a wide variety of drug compounds. However, to avoid extensive extra experimental work (e.g. preparation of drug stock and reagent solutions) and due to the difficulty in finding drug plasma sequestration ratios, the selection of drug components were limited to on-going studies in our laboratory and it was random with the exception of indapamide and methazolamide, which were deliberately chosen due to their relatively high affinity to erythrocytes [10,11,14,15].

For methazolamide, Pradhan et al. mentioned that more than 95% of methazolamide in blood is strongly bound to RBCs [10]. Based on this, a drug RBC: plasma concentration ratio of 25 can be obtained, i.e. $(95/5) \times (0.57/0.43)$. Hence, even 1% or 2% of hemolysis could cause significant change in plasma concentration for hemolyzed plasma samples (Table 3). For indapamide, it was reported that the radioactivity uptake ratio between red cell and plasma is approximately 4:1 [15]. If assuming a drug RBC: plasma concentration ratio of 5.3, i.e. $(4/1) \times (0.57/0.43)$, then 2% of hemolysis should not impact significantly indapamide plasma concentration for hemolyzed plasma samples despite its low plasma sequestration ratio.

3.4. Hemolysis impact on sequestration of different drug compounds

The estimated hemolysis level for hemolyzed plasma samples was approximately 2%. At this hemolysis level, out of the nine drug compounds tested in post-spiking hemolysis test, only methazolamide showed significant plasma concentration change due to hemolysis (Table 4). As mentioned earlier, this was expected because of the high affinity of methazolamide to RBCs. For other

Table 3
Maximum allowable drug RBC: plasma concentration ratio at a given degree of hemolysis to avoid significant change in drug plasma concentration.

	Hemolysis (%)					
	0.25	0.5	1	2	5	10
Maximum RBC: plasma concentration ratio[a]	80.1	40.9	21.0	11.1	5.13	3.14

[a] A drug plasma conc. change outside 15% is considered significant and a hematocrit value of 0.43 is assumed.

Table 4
Hemolysis impact on plasma concentrations of different drug compounds.

Name of drug	Spiked conc. in blood	Conc.[a] unit	Conc. in comparison sample		Conc. in hemolyzed plasma		% change	Estimated drug RBC: plasma conc. ratio (α)[b]
			Mean	CV (%)	Mean	CV (%)		
Amlodipine	4048.00	pg/ml	3396.38	1.4	3352.20	0.8	−1.30	1.45
Budesonide	151.20	pg/ml	200.68	3.2	194.41	2.2	−3.12	0.43
Capecitabine	500.00	ng/ml	499.73	6.7	522.83	3.2	4.62	1.00
Dodecyl Maltoside	126.70	ng/ml	212.53	0.5	212.59	2.1	0.03	0.06
Fexofenadine	100.08	ng/ml	145.41	6.5	141.48	5.7	−2.70	0.28
Fluticasone	99.20	pg/ml	168.31	3.5	168.30	1.7	−0.01	0.05
Indapamide	25.08	ng/ml	12.56	4.1	11.56	3.2	−7.96	3.32
Methazolamide	1000.00	ng/ml	78.94	7.7	91.67	5.1	16.13	28.13
Metoprolol	19.92	ng/ml	21.04	1.2	20.71	0.8	−1.57	0.88

[a] Conc. = concentration.
[b] $\alpha = \frac{C_b-(1-H)\times C_p}{H\times C_p}$, where C_b and C_p are the spiked conc. in blood and the conc. in comparison sample, respectively. In addition, a hematocrit value of 0.43 is assumed.

drug compounds, including the aforementioned indapamide, their estimated RBC: plasma concentration ratios are all lower than 11.1 (the maximum allowable drug RBC: plasma concentration ratio to avoid significant impact at 2% of hemolysis level, Table 3). Therefore, no significant hemolysis impact on drug plasma sequestration was observed.

Despite the overall agreement of theoretical calculations and experimental results, a discrepancy was noted between the measured % of concentration change for methazolamide (16.13%) and its calculated value by using Eq. (6). Specifically, assuming a hematocrit value of 0.43 and the drug RBC: plasma concentration ratio of 25, the % of change in plasma concentration should be around 36% at the hemolysis level of 2%. This discrepancy could be due to several reasons. Firstly, the assumed hematocrit of 0.43 might be different from the actual hematocrit value for this particular pool of fresh blood. Secondly, the 2% of hemolysis was based on visual inspection, i.e. not with sufficient accuracy. In addition, there was slight hemolysis due to the organic solvent of working solution during spiking. Last but not least, 30 min of incubation might not be enough for methazolamide.

Finally, it should be mentioned that results from the analyses after fivefold dilution match well with those reported above, which corroborate the previous validation results for lack of hemolysis matrix effect at 2% level (data not shown).

3.5. Drug plasma sequestration versus different degrees of hemolysis

To see how different degrees of hemolysis impact drug plasma concentration, methazolamide and donepezil were used as representatives to evaluate their concentration changes in the presence of 1%, 2%, 5%, and 10% of hemolysis in comparison with 0% of hemolysis. As shown in Table 5, for donepezil, no substantial change in drug plasma concentration was observed even at 10% of hemolysis because its estimated RBC: plasma concentration ratio, 2.88, is lower than the corresponding maximum allowable ratio listed in Table 3, i.e. 3.14 for 10% of hemolysis.

On the other hand, the concentration of methazolamide in hemolyzed plasma samples increased significantly due to hemolysis and the severer the hemolysis, the higher the percentage of concentration change is. The overall trend of calculated changes is in agreement with that of measured concentrations. However, the % changes from experiment are lower than those from calculation. In addition to the reasons mentioned in Section 3.4, another reason could be the difference in binding ability between freshly lyzed RBCs and those lysed by freezing and thawing. Furthermore, the frozen-and-thawed whole blood might not be 100% hemolyzed though it appeared to be.

3.6. Some thoughts on future work

Though some meaningful results and useful guideline have been obtained, further experiments are necessary to rigorously test the proposed formulae using improved experimental design and with more drugs of varying affinities to RBCs. To this end, a more accurate method for the measurement of hemolysis level as well as a more controllable/predictable approach to introduce hemolysis should be used. In addition, the hematocrit value of the whole blood lot used needs to be measured. Moreover, the spiking associated hemolysis should be reduced, such as using water based working solution, mixing whole blood with drug powder, etc. To mimic the real situation, whole blood samples from dosed subjects can be used and aliquoted, with one aliquot as comparison and the others subject to controllable introduction of hemolysis. After accurate measurement of hemolysis levels, plasmas can be

Table 5
How different degrees of hemolysis affect drug plasma concentration for methazolamide and donepezil.

Hemolysis (%)	Donepezil			Methazolamide		
	Plasma concentration[a] (pg/ml)	Measured change (%)	Calculated change (%)	Plasma concentration (ng/ml)	Measured change (%)[c]	Calculated change (%)[d]
0	165.84	N/AP[b]	N/AP	263.96	N/AP	N/AP
1	162.65	−1.9	1	284.72	7.9	18
2	159.82	−3.6	3	316.07	19.7	36
5	172.54	4.0	7	406.65	54.1	88
10	172.28	3.9	13	558.03	111.4	169

[a] Conc. = concentration.
[b] N/AP = not applicable.
[c] Measured change = (Conc. of hemolyzed sample/Conc. of comparison non-hemolyzed sample − 1) × 100.
[d] Calculated changes were calculated based on the formulae using 0.43 as hematocrit value and the estimated drug RBC: plasma concentration ratios of 2.88 and 25.1 for donepezil and methazolamide, respectively.

harvested and analyzed for the evaluation of hemolysis impact on the representability of hemolyzed plasma samples.

4. Conclusions

Despite wide awareness of hemolysis matrix effect, how hemolysis would impact the representability of hemolyzed plasma samples (drug concentration difference between hemolyzed and non-hemolyzed plasmas) was rarely evaluated. For the first time, a formula for estimating the impact was proposed and tested using ten different drug compounds. Just as hemolysis matrix effect is usually method-specific, the effect of hemolysis on drug plasma concentration is compound-dependant. The two most important factors are the drug compound's inherent RBC: plasma concentration ratio and the degree of hemolysis. The stronger the affinity of a drug to red blood cells is and the severer the hemolysis is, the more significant the impact would be.

Since blood matrix instead of plasma would be chosen for a drug compound that has strong affinity to RBCs [16], so normally, hemolysis (even severe one, e.g. 2% or 5%) would not be a concern because the total concentration is measured. However, should both whole blood and plasma matrices are used for a compound with high affinity to RBCs [10], careful evaluation should be performed as whether to include the reported drug plasma concentrations of severely hemolyzed samples in the pharmacokinetic evaluation despite the good accuracy of the reported concentrations, i.e. no hemolysis matrix effect.

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