Integrated hemolysis monitoring for bottom-up protein bioanalysis

Frédérique L. van Holthoon, William D. van Dongen, Anne J. Kleinnijenhuis
Triskelion, Zeist, The Netherlands

Highlights

- Triskelion developed an LC-MS method module to quantify hemolysis.
- Analyte protein and hemoglobin are analyzed simultaneously, which saves time and costs and requires no additional sample volume.
- The specific module is applicable in direct digestion approach and after non-selective protein purification.
- The basic concept is widely applicable.
- Comparison of the results obtained after visual inspection, UV-VIS detection and LC-MS of two tryptic hemoglobin peptide targets.

Keywords

Hemolysis, UPLC-MS, bioanalysis, hemoglobin, therapeutic proteins

Triskelion B.V.
Utrechtseweg 48
P.O. Box 844
3700 AV Zeist
The Netherlands
T +31 88 866 2800
E info@triskelion.nl
www.triskelion.nl

Introduction

For clinical development of biopharmaceuticals it is required to investigate their *in vivo* behavior using validated bioanalytical methods. In guidances for bioanalytical method validation[1-3] it is strongly recommended to investigate matrix effects for the analyte(s) in hemolyzed plasma or serum, in addition to clean matrix. During hemolysis, the contents of red blood cells, mainly hemoglobin, will be released into the surrounding matrix. When UPLC-MS (ultra-performance liquid chromatography - mass spectrometry) is applied, hemolysis can affect analyte suppression or enhancement and the extraction efficiency[4] or analyte stability.[5]

Figure 1: plasma samples exhibiting different degrees of hemolysis.

There is no strict definition of when a sample is hemolyzed, but there is a consensus that plasma containing >2% hemolyzed whole blood is considered to be hemolyzed.[3,6] Therefore it makes sense to at least include a 2% hemolyzed sample in the validation set. When it is demonstrated that hemolysis does not affect the quantitative results there will be no analytical issues, but what to do when there is an effect or even a gradual effect already below 2% hemolysis? How will you assess the sample results when they exhibit varying degrees of hemolysis (see Figure 1) and how should the hemolysis be quantified?

While visual inspection of samples is simple and cost-effective, it is very subjective. A color chart could improve results but is still not completely objective. Determination of hemolysis using UV-VIS is quick and easy, but only feasible when there is sufficient sample material available. It would be preferable to systematically provide samples with an objective value for hemolysis[7] without using additional sample volume, whilst saving time and costs. Therefore Triskelion developed an UPLC-MS module to meet these preferences. In this approach a tryptic peptide from hemoglobin, which causes the red color of blood through the porphyrin rings in the heme groups, is targeted. The LC-MS results are compared with UV-VIS detection and visual inspection. The LC-MS method module can be applied when simultaneously therapeutic proteins are quantified in biological matrix after non-selective protein purification and/or direct digestion. The basic concept can be implemented in several other applications and can be integrated with existing quantitative protein LC-MS methods, but needs to be modified considering the sample purification steps and analytical requirements for the analyte of interest.

Target selection

Tryptic peptide targets were selected from hemoglobin, see Table 1. Besides applying typical theoretical and experimental target selection criteria, special attention was given to the generic applicability of the target to matrices obtained from the most common (laboratory) animals. The tryptic peptide LLVVYPWTQR has only few variations considering the summarized animals in Table 1. However, LC and MS/MS settings, see the Experimental section, should be adapted to implement a suitable module when a target variation is analyzed.

Table 1: tryptic targets from hemoglobin.

Uniprot entry	(Laboratory) animal	Aligned sequence
sp|P02091|HBB1_RAT	Rat	...KVNPDDVGGEALGRLLVVYPWTQR...
sp|P02088|HBB1_MOUSE	Mouse	...KVNSDEVGGEALGRLLVVYPWTQR...
sp|P02089|HBB2_MOUSE	Mouse	...KVNPDEVGGEALGRLLVVYPWTQR...
sp|P02095|HBB_CAVPO	Guinea pig	...KVNVGEIGAEALGRLLVVYPWTQR...
sp|P02094|HBB_MESAU	Golden hamster	...KVNADAVGAEALGRLLVVYPWTQR...
sp|P02057|HBB_RABIT	Rabbit	...KVNVEEVGGEALGRLLVVYPWTQR...
sp|P68223|HBB_MACFA	Crab-eating macaque	...KVNVDEVGGEALGRLLVVYPWTQR...
sp|P02026|HBB_MACMU	Rhesus macaque	...KVNVDEVGGEALGRLLLVYPWTQR...
sp|P68873|HBB_PANTR	Chimpanzee	...KVNVDEVGGEALGRLLVVYPWTQR...
sp|P68871|HBB_HUMAN	Human	...KVNVDEVGGEALGRLLVVYPWTQR...
sp|P02070|HBB_BOVIN	Cow	...KVKVDEVGGEALGRLLVVYPWTQR...
sp|P02075|HBB_SHEEP	Sheep	...KVKVDEVGAEALGRLLVVYPWTQR...
sp|P02067|HBB_PIG	Pig	...KVNVDEVGGEALGRLLVVYPWTQR...
sp|P68231|HBB_CAMDR	Dromedary	...KVKVDEVGGEALGRLLVVYPWTRR...
sp|P68226|HBB_LAMGL	Llama	...KVKVDEVGGEALGRLLVVYPWTRR...
sp|P02062|HBB_HORSE	Horse	...KVNEEEVGGEALGRLLVVYPWTQR...
sp|P07412|HBB_FELCA	Cat	...KVNVDEVGGEALGRLLVVYPWTQR...
sp|P60524|HBB_CANLF	Dog	...KVNVDEVGGEALGRLLIVYPWTQR...
sp|P68044|HBB_MUSPF	Ferret	...KVNVDEVGGETLGRLLVVYPWTQR...
sp|P02112|HBB_CHICK	Chicken	...KVNVAECGAEALARLLIVYPWTQR...

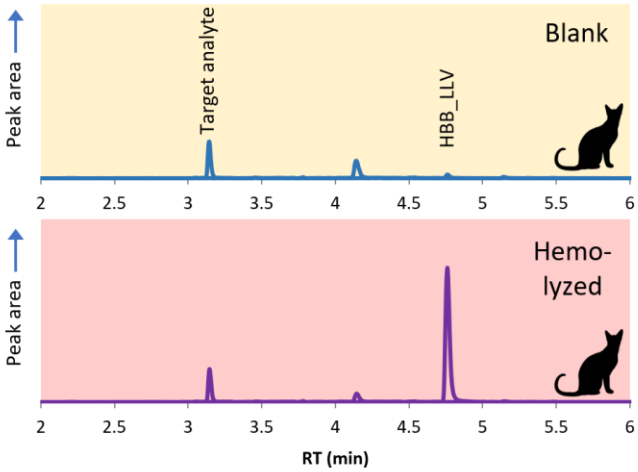
Experimental

A set of serum samples, with different degrees of hemolysis, was visually inspected, the UV-VIS absorbance was measured and the peptides LLVVYPWTQR and VNVDEVGGEALGR were determined after tryptic digestion and UPLC-MS/MS. Visually, samples were classified as 0, 0.05 or 0.1 (no, little, heavy hemolysis). UV absorbance was measured at 570 nm (reference 655 nm)[8] using 50 µl sample with a SpectraMax M5 (Molecular devices). Samples were digested with trypsin and analyzed using an HSS T3 column (100 x 2.1 mm) on a Waters Xevo TQ-S. The LLVVYPWTQR precursor $[M+2H]^{2+}$ ions had m/z 637.85 and the quantifier and qualifier ions were either the y_4^+ or the y_5^+ product ions at m/z 590.30 and 687.35. The VNVDEVGGEALGR precursor $[M+2H]^{2+}$ ions had m/z 657.85 and the quantifier and qualifier ions were the y_7^+ and y_8^+ product ions at m/z 659.35 and 758.40, respectively.

Results and discussion

Depending on the animal species of interest, LC-MS/MS targets can be selected according to Table 1. These targets are either generic or slightly different between animal species. As an example, in Figure 2 extracted chromatograms are provided for non-hemolyzed feline serum (blank) and hemolyzed feline serum, which show the signals for a digested analyte protein and for a hemoglobin target, after having applied a direct digestion approach.

Figure 2: Extracted chromatograms of analyte protein target and LLV hemoglobin target in (hemolyzed) feline serum.



The selected part of the hemoglobin sequence is identical between cat, human, cynomolgus monkey and others. In Figure 3 the results of a comparative analysis are shown for a set of cynomolgus monkey serum samples. The serum samples were visually inspected (indicated by the size and color of the spheres), their UV-VIS absorbance was determined (x-axis) and the hemoglobin LLV target was analyzed using LC-MS (y-axis). In Figure 4 the results obtained for the same cynomolgus serum samples are shown in a histogram, including the results for the VNV target and a second visual inspection.

Figure 3: Results of visual inspection, UV-VIS absorbance and LC-MS analysis (LLV) of a set of cynomolgus monkey serum samples.

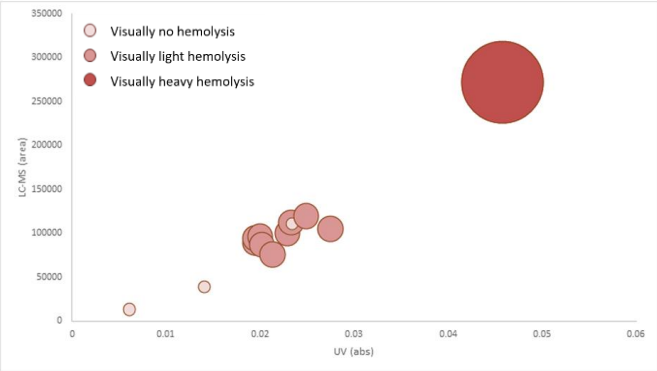
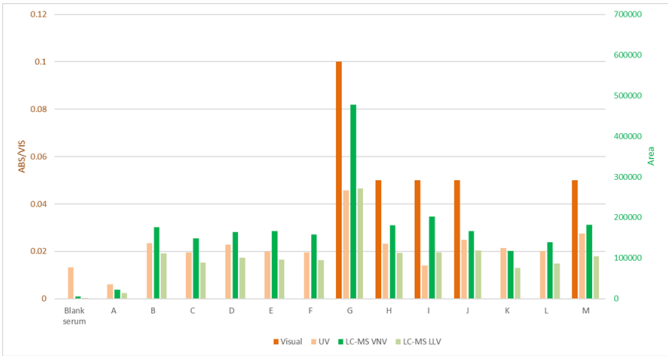


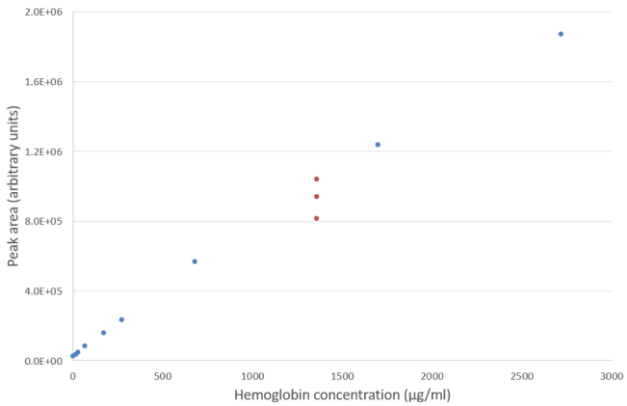
Figure 4: Histogram of the results of visual inspection, UV-VIS absorbance and LC-MS analysis (LLV and VNV) of a set of cynomolgus monkey serum samples.



Figures 3 and 4 illustrate that the UV-VIS and LC-MS results correlated quite well in the analyzed samples. The visual inspection however, most probably due to its subjectivity, could lead to wrong assignment of the extent of hemolysis. Typically, hemolysis is visible in serum or plasma starting at a hemoglobin concentration of 200 mg/L[9] or 300 mg/L[10]. The marker protein used to determine hemolysis in this study was hemoglobin. The normal hemoglobin levels in whole blood (WHO reference values) are 13.8-17.2 g/dL for adult males and 12.1-15.1 g/dL for adult (non-pregnant) females.

A calibration curve was prepared from human hemoglobin (Sigma-Aldrich, product no. H7379) in pooled gender human plasma in the 17-2717 µg/ml concentration range and the results are shown in Figure 5 (blue points). Spikes of human hemoglobin were prepared at 1358 µg/ml in cynomolgus monkey, feline and human serum (orange points), which corresponds to approximately 1% hemolysis. The mean obtained recovery was 89% (RSD 15%), showing acceptable method performance.

Figure 5: Calibration curve of human hemoglobin in pooled gender human plasma in the 17-2717 µg/ml concentration range (blue) and spikes of human hemoglobin in cynomolgus monkey, feline and human serum (orange), LLV hemoglobin target.



Finally, three 2% human hemolyzed serum and plasma samples were purchased and analyzed using the presented method. These samples were found to contain 2.2 mg/ml (female serum), 3.1 mg/ml (female plasma) and 3.1 mg/ml (pooled plasma) hemoglobin. Although detailed information about the donors and preparation of the hemolysate was not available, these concentration are close to the normal hemoglobin levels. As the presence of bilirubin (icteric samples) or lipemia/turbidity may impair visual inspection of samples[9] and UV-VIS measurements, LC-MS analysis of hemoglobin targets might provide more accurate and objective values for the extent of hemolysis.

Conclusions and future outlook

In the present study the principle of quantifying the extent of hemolysis using an LC-MS method module was shown. The basic methodology and approach can also be applied for parallel assessment of other parameters in particular sample types, such as hyperlipidemic samples (after assignment of representative markers) or icteric samples (bilirubin). It should of course be noted that the sample purification procedure for the analyte and the parameter assessed in parallel need to be (made) compatible to obtain accurate results.

References

[1]EMA. Guideline on Bioanalytical Method Validation. EMA, Committee for Medicinal Products for Human Use (CHMP), London, UK (2011).

[2]US FDA. Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Rockville, MD, USA (2018).

[3]ICH. Bioanalytical Method Validation M10. ICH Harmonized Guideline (draft version, endorsed on 26 February 2019).

[4]Hughes NC, Bajaj N, Fan J, Wong EYK. Assessing the matrix effects of hemolyzed samples in bioanalysis. *Bioanalysis* 2009; **1**: 1057–1066.

[5]Kleinnijenhuis AJ, Staal YCM, Duistermaat E, Engel R, Woutersen RA. The determination of exogenous formaldehyde in blood of rats during and after inhalation exposure. *Food and Chemical Toxicology* 2013; **52**: 105-112.

[6]Ingelse B, et al. European Bioanalysis Forum: recommendation on dealing with hemolyzed and hyperlipidemic matrices (White paper). *Bioanalysis* 2014; **6**: 3113-3120.

[7]Lippi G, Cadamuro J, Von Meyer A, Simundic A. Practical recommendations for managing hemolyzed samples in clinical chemistry testing. *Clinical Chemistry and Laboratory Medicine* 2018; **56**: 718-727.

[8]Saha K, Moyana DF, Rotello VM. Protein coronas suppress the hemolytic activity of hydrophilic and hydrophobic nanoparticles. *Materials Horizons* 2014; [1]: 102–105.

[9]Dolci A, Panteghini M. Harmonization of automated hemolysis index assessment and use: Is it possible? *Clinica Chimica Acta* 2014; **432**: 38-43.

[10]Thomas L. Haemolysis as Influence & Interference Factor. *Journal of the International Federation of Clinical Chemistry and Laboratory Medicine* 2002; **13**: 95-98.