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A fit-for-purpose LC–MS/MS method for the simultaneous quantitation of ATP and 2,3-DPG in human K_2EDTA whole blood

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ABSTRACT

Many hemolytic anemias results in major metabolic abnormalities: two common metabolite abnormalities include increased levels of 2,3-diphosphoglycerate (2,3-DPG) and decreased levels of adenosine triphosphate (ATP). To better monitor the concentration changes of these metabolites, the development of a reliable LC–MS/MS method to quantitatively profile the concentrations of 2, 3-DPG and ATP in whole blood is essential to understand the effects of investigational therapeutics. Accurate quantification of both compounds imposes great challenges to bioanalytical scientists due to their polar, ionic and endogenous nature. Here we present an LC–MS/MS method for the reliable quantification of 2,3-DPG and ATP from K_2EDTA human whole blood (WB) simultaneously. Whole blood samples were spiked with stable isotope labeled internal standards, processed by protein precipitation extraction, and analyzed using zwitterionic ion chromatography-hydrophilic interaction chromatography (ZIC-HILIC) coupled with tandem mass spectrometry. The linear analytical range of the assay was 50–3000 µg/mL. The fit-for-purpose method demonstrated excellent accuracy and precision. The overall accuracy was within ± 10.5% (%RE) for both analytes and the intra- and inter-assay precision (%CV) were less than 6.7% and 6.2% for both analytes, respectively. ATP and 2,3-DPG were found to be stable in human K_2EDTA blood for at least 8 h at 4 °C, 96 days when stored at −70 °C and after three freeze/thaw cycles. The assay has been successfully applied to K_2EDTA human whole blood samples to support clinical studies.

1. Introduction

2, 3-DPG and ATP levels have been well documented to be altered in many hemolytic anemia patients. [1–4] Anemia patients with low blood ATP levels leads to increased red blood cell (RBC) dehydration through potassium efflux driven by increased activity of the Gardos channel. This can cause a decrease in membrane integrity which may result in increased hemolysis during RBC stress [5,6]. 2, 3-DPG acts as an allosteric effector increasing release of oxygen from hemoglobin allowing oxygenation to tissues. In certain anemias such as Sickle cell anemia this shift to the deoxyhemoglobin increases the aggregation of hemoglobin. Inherited PK deficiency results in hemolytic anemia and is the most common cause of hereditary nonspherocytic anemia, where red blood cells assume non-spherical shapes. Pyruvate kinase (PK) is a key regulatory enzyme in glycolysis, catalyzing the irreversible conversion of phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP). Patients with hemolytic anemia may have a range of symptoms from mild to severe. Severe cases in infancy can be life-threatening, requiring regular blood transfusions to survive. The presence of a PK deficiency results in two major metabolic abnormalities: increased levels of 2, 3-DPG and decreased levels of ATP. [7] Hence, the development of a reliable method to quantitatively profile the concentrations of 2, 3-DPG and ATP in whole blood is essential for understanding the effects of investigational therapeutics.

Classical measurements of these metabolites individually are either luminescent, spectrophotometric or fluorescent enzyme assays. These classic systems are not direct measurements of the metabolites. Indirect measurements have a potential to incorrectly measure absolute levels. There is also a ^{31}P NMR method published that can directly measure both ATP and 2, 3-DPG in intact red blood cells [8]. This NMR method is expensive, uses fresh whole blood or purified RBC and not routinely accessible to clinical labs. LC–MS/MS has become the gold standard for almost all small molecule bioanalysis. Therefore, an LC–MS/MS method that can directly measure 2,3-DPG and ATP in frozen stored samples of whole blood is highly desirable. Accurate measurement of these two

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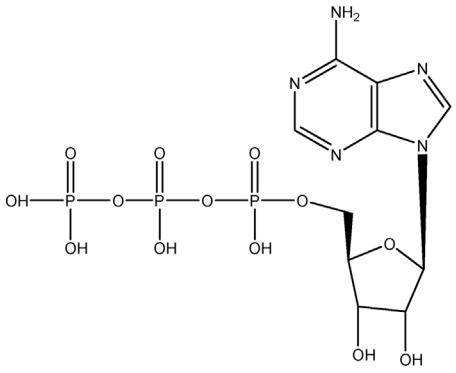
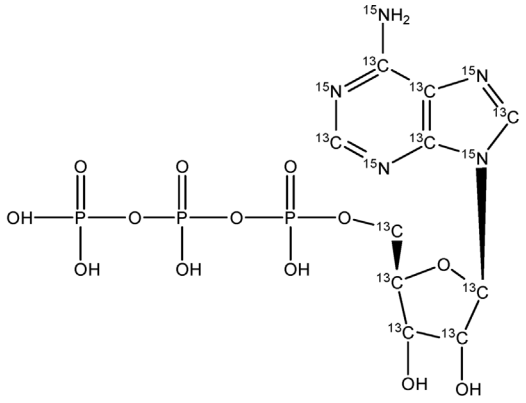
metabolites will offer good biomarkers for assessing the treatment of relevant hemolytic anemias and other indications where tracking changes in 2, 3- DPG and/or ATP concentration is important.

It is well known that analysis of highly polar, acidic metabolites using LC–MS/MS is a bioanalytical challenge. As shown in Fig. 1, both 2, 3-DPG and ATP are polyphosphorylated, and will be negatively charged at pH > 2 resulting in poor or no retention on conventional reversed phase LC columns. Theoretically, ion exchange chromatography (IEX) can be used to resolve anionic species where the separation is based on ionic interactions with a charged stationary phase. However, this approach typically utilizes buffer types and in high concentrations not amenable to long term MS application. An alternative approach which has gained popularity in many metabolomic workflows has been the use of ion pairing reagents, such as tri-butylamine (TBA) buffered with acetic acid. The separation in this case is based on the formation of a hydrophobic ion pair between the negatively charged phosphate groups and the positively charged ion pair mobile phase resulting in retention on a reversed phase column. Although this methodology has proved successful in similar applications, TBA is extremely persistent in the LC hardware and its presence can hinder the usefulness of the LC–MS/MS instrument in other assays, causing unwanted and oftentimes prohibitive ion suppression. To this end, we investigated the use of zwitterionic ion chromatography-hydrophilic interaction chromatography (ZIC-HILIC), whereby MS compatible mobile phase additives such as ammonium acetate can be used to effect separations of highly polar and charged analytes in a similar fashion to that of IEX, whilst maintaining favorable electrospray characteristics. Our objective is to develop a fit-for-purpose, simple, fast and scientifically sound bioanalytical method to simultaneous quantify 2, 3-DPG and ATP in human whole blood. To the best of our knowledge, there is not such a method reported to date.

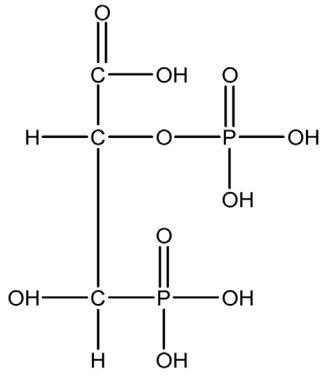
Another challenge related to 2, 3-DPG and ATP quantitation in biological matrix is that both are endogenous compounds and present at very high levels at basal conditions in human whole blood. It is well known that there is well defined guidance [9,10] and best practices [11] across the pharmaceutical industry on the requirements of a validated bioanalytical method. In general, these guidance have a main focus on drug candidates, which usually are exogenous compounds and could impose significant safety risk to patients/healthy volunteers if not monitoring accurately. When it comes to endogenous biomarkers, fit-for-purpose (scientific validation) method validation is usually recommended [12], depending on the role of the biomarkers in the specific clinical trials. Specific to our case, 2,3-DPG and ATP are present at several hundred μg/mL in whole blood due to high intra-cellular



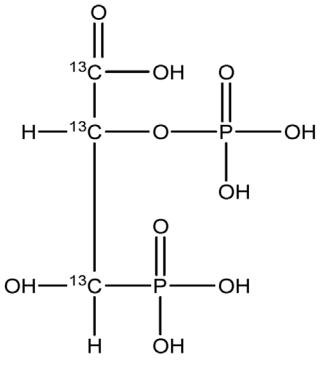
ATP
Molecular formula: $C_{10}H_{16}N_5O_{13}P_3$
Monoisotopic mass: 507.00 Da



$^{13}C_{10}{}^{15}N_5$-ATP
Molecular formula: $^{13}C_{10}H_{16}{}^{15}N_5O_{13}P_3$
Monoisotopic mass: 522.02 Da



2, 3-DPG
Molecular formula: $C_3H_8O_{10}P_2$
Monoisotopic mass: 265.96 Da



$^{13}C_3$-2, 3-DPG
Molecular formula: $^{13}C_3H_8O_{10}P_2$
Monoisotopic mass: 268.97 Da

Fig. 1. Chemical structure for ATP, 2, 3-DPG and their internal standards.