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Facts about High-Energy Phosphates Estimation in Heart

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ABSTRACT

Energy utilization in the heart is an important regulatory mechanism that exists in cardiomyocytes, which provides energy for calcium regulation and contraction. The high-energy phosphates such as adenine tri-phosphate (ATP), adenine di-phosphate (ADP), adenine mono-phosphate (AMP) and creatine phosphate (CrP) are the energy-supplying pathways for the energy requiring process such as myocardium contraction and calcium regulation in the heart. Hydrolysis of ATP is finely balanced by the rate of ATP production in order to maintain a stable steady state of cardiac performance. The myocardial energy metabolism gets altered in heart under various pathological conditions. To understand these metabolic alterations, it is important to estimate energy status in the heart. In past, various techniques have been used for the estimation of these compounds such as high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy and capillary electrophoresis. In addition, various indirect methods have been developed to assess energy status of myocardium using different enzyme assay.

Keywords: Adenine tri-phosphate, cardiomyocytes, myocardial energy, Nuclear Magnetic Resonance Spectroscopy, High performance liquid chromatography.

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INTRODUCTION

ATP is a multifunctional nucleotide and plays an important role in cell biology and intracellular energy transfer. ATP transports chemical energy within cells for metabolism. The energy in the form of ATP is utilized by the cardiomyocytes by controlled hydrolysis of ATP to ADP and phosphate (Pi) by various adenosine tri phosphatases (ATPases) to perform cardiac functions. Thus, estimation of high energy phosphates levels are of immense importance to access the myocardium energy status as these are life line for myocardium contraction [1]. In addition, the measurement of high-energy phosphates in myocardial tissue is essential clinical tools to evaluate the biochemical mechanism of cellular injury and degradation of myocardial function. Thereby, selection of a reliable technique to estimate high-energy phosphates is an important objective of current research. Tremendous efforts have been made to develop various techniques to estimate high energy phosphates, that include high performance liquid chromatography (HPLC), capillary electrophoresis [2] and NMR [3, 4, 5]. Among these developed techniques, MRS is used to access the energy status in live animals [6], while HPLC is method of choice for estimation in myocardial biopsies [7]. In addition, estimation of lactate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase and enzymes of electron transport chain including Creatine kinase, indirectly reflect the energy state of myocardium. This review discussed various presently employed and recently developed experimental techniques to estimate high-energy phosphates estimation in heart.

High Performance Liquid Chromatography (HPLC)

HPLC utilizes a column that holds chromatographic packing material called stationary phase, a pump that moves the mobile phases through the column, and a detector that shows the retention times of the molecules. This technique is most widely used in biochemistry and analytical chemistry to separate, identify, and quantify compounds. The high energy phosphates are highly polar molecules due to the presence of multiple phosphate groups that may interfere with chemical determination. These high energy phosphates due to their extremely high polarity are not retained on column under conventional reversed-phase chromatographic conditions. Therefore, ion-suppression HPLC and ion-pairing HPLC [8-11], ion exchange [12] and reverse phase HPLC [13, 14] have been developed and used for the analyses of high energy phosphates estimation and their analogues.

Mobile Phase

The mobile phase used by different analyst is either isocratic or gradient of high ionic strength have been used in order to have baseline resolution of ATP and ADP [13]. Isocratic mobile phase is most commonly used in various studies is phosphate buffer with various pH, some has added with tetrabutylammonium hydrogen sulfate (TBAH) [10, 14, 16], while gradient mobile have also been tried in which column was equilibrated with 0.2M KH_2PO_4 with pH adjusted to 5.0 by KOH one minute after injection a linear gradient was started from 1% of mixture of water, acetonitrile and methanol (50/25/25) to 35% at 4 minutes [13]. In addition, column that has been most commonly used is silica based reverse phase column C-18. Creatine phosphate and Creatine, relative to ATP and ADP, are smaller and less negatively charged molecules and are poorly retained on the column specially the creatine phosphate, so interference by un-retained compound in column cannot be ruled out [13]. In some of the early reports another alternative has been tried to avoid this problem by converting creatine phosphate to ATP by use of enzyme creatine kinase but this method is not good because some of the creatine phosphates may still remains in matrix after the use of enzyme.

Detection Methods

The various detection methods tried for analyzing these compounds are UV spectroscopy and fluorimetry. Among the most sensitive methods are the immunosorbent assays with femtomole detection limits but these methods require nucleotides to be derivatized and to be attached to a carrier protein in order to become immunogenic. Mass spectrometric detection of these compounds has also been tried because of the development of various ionization techniques [17]. The other techniques tried for these nucleotides are fast-atom bombardment, matrix-assisted laser desorption ionization and electrospray ionization**.** These generated nucleosides are analyzed by using LC/UV at 210, 220 and 254nm in various reports but mainly by 210 nm [10, 11, 13, 14, 18], radioimmunoassay, and LC–MS/MS [9, 19]. In addition fluorometric detection have been used by derivatizing these high energy phosphates to 1,N6-ethenoderivatives of ATP (epsilon-ATP), ADP (epsilon-ADP), AMP (epsilon-AMP), and adenosine (epsilon-Ado), formed by reaction with chloro-acetaldehyde which strongly fluorescent at an emission wavelength of 280 nm [20] which have a detection limit in picomole. Mass spectrometry (MS) combined with HPLC is also other important technique for the analyses of these high energy phosphates because of its high sensitivity compared to other detection methods. LC–MS with electro-spray ionization (ESI) has also been used for analyzing these adenine nucleotides [17, 21].

Ion Pairing

In ion-pairing HPLC, positively charged ammonium salts (tri-alkyl and tetra-alkyl ammonium) form ion-pairs with the negatively charged nucleotides. Which make them more lipophilic, and hence retained on the reverse phase HPLC column for their easy separation. The ammonium salts tried for this purpose includes tetra-ethyl ammonium salts, tetra-butyl ammonium salts and tri-propyl ammonium salts [10, 16]. These ion pairs provide excellent chromatographic resolution due to better retention of these high energy phosphates, by the formation of ion-pairs between the positively charged ion-pairing agent and the negatively charged phosphate group of high energy phosphates, which make them more hydrophobic and hence better retained on reverse phase column. In addition more the aqueous nature of the mobile phase more easily these compounds will be retained on reversed-phase column and hence better will be their resolution. The problem that may be faced if we are using electro spray ionization mass spectrometry (ESI-MS) due to their relatively non-volatile nature and the high concentration needed for their separation. This problem can be solved by use of dimethylhexylamine (DMHA) [19].

Column

These nucleotide phosphates due to their extremely high polarity are not retained on column under conventional reversed-phase chromatographic conditions. Column that has been most commonly used is silica based reverse phase column C-18 with various diameters. Analytical methods using small-diameter HPLC columns have been used with better results [13] due to its strong chromatographic resolving power, compared to a larger-diameter HPLC column.

Method validation

The main purpose of method validation is to develop a precise, accurate, rugged and reproducible method for world wide acceptance of results obtained by this method. Method validation means weather our method is valid or not and the process of analytical method's validation should demonstrate that the method is fit for its purpose such as estimation of high energy phosphates. For analyzing these compounds on HPLC, method should be properly validated for assay range, limit of quantitation and detection, linearity and standardization and should be checked for accuracy, precision and robustness.

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

NMR spectroscopy is a non invasive technique to evaluate changes in high energy phosphates in heart with different cardiac pathologies. This is a promising technique due to its ability to provide insight information about myocardial metabolism in live animal gives it a unique role for investigation of various cardio-myopathies. Two type of NMR has been used 31P-nuclear magnetic resonance (31P-NMR) and Proton Nuclear Magnetic Resonance (1H NMR) Spectroscopy. Both of these approaches are useful in the assessment of metabolite reduction of myocardium [22]. Applying ^{31}P MRS with commonly-available two-dimensional chemical shift imaging (2D-CSI) sequences is a valuable technique to evaluate various heart problems such as ischemic injury and dilated cardiomyopathy by determining PCr/ATP ratios non-invasively [23].

Principle

This technique is based on the fact as described by Schaefer [24] that within a uniform magnetic field, the tissue is excited by a radio frequency pulse near the resonant frequency of the nuclei of interest. After excitation nuclei reorient them selves back in to the axis of the magnetic field, giving off energy and hence emitting a signal that decreases exponentially with time called a free induction decay (FID), After this detection, fourier transformation is performed to define the intensity of different frequency component of FID, which is reflected in the form of peak, whose intensity is proportional to the strength of signal. The presence of species with different resonant frequencies in the spectrum is due to phenomena called chemical shift. In addition to the main magnetic field each nucleus in a molecule is subjected to a small magnetic field produced by the electron surrounding it. This local electron induced field changes the resonant frequency of that nucleus slightly, this change is termed as chemical shift. This chemical shift allows detection of different peaks in a spectrum. The β phosphate of ATP has a resonant frequency that differs from the phosphates in creatine phosphate and allows their identification. It is important to mention here that α and γ resonance of ATP overlaps the resonance of ADP. So it is β resonance, which helps in identification of ATP from ADP.

Magnetic resonance spectroscopy is a valuable tool for measuring cardiac high energy phosphates non-invasively in live animal [25, 26]. From various studies reduced PCr/ATP ratios observed in dilated cardio-myopathies and various ischemic injuries, which point out that these pathological condition results from impaired cardiac energy metabolism [23,27]**.** In addition, several spectroscopic studies obtained contradictory results on the reduction of PCr/ATP ratio in the myocardium of patients with dilated cardio-myopathies. Some studies found significant reductions in PCr/ATP ratio [23, 27, and 28] while others reported no significant changes (Schaefer *et al.,* 1990b). New techniques have been developed to include signal improvements by the nuclear overhauser effect (NOE) and spatial localisation with optimal pointspread function (SLOOP) techniques [29]. SLOOP is able to match the size and the shape of the sensitive volumes to the anatomical structures to minimize partial volume effects.

These special techniques are currently not in widespread use due to the sophisticated hardware or software required. Taken together, this techniques helps in determining PCr/ATP ratios non-invasively and may be correlated with structural impairment of the myocardium in live animal. The decreased PCr/ATP ratio as observed by various NMR spectroscopic studies directly reflects that there is some problem with cardiomyocytes energy metabolism in dilated cardiomyopathies. So, we can say like that, the degree of reduction of high energy phosphates seems to be a direct indicator to structural abnormalities in the failing heart in patients with various ischemic injuries and dilated cardio-myopathies. Hansch *et al.,* (2005) demonstrate that the PCr/-ATP ratios are linearly correlated with reduced left ventricular ejection fraction, which is an important predictor of heart failure. Thus, PCr/ATP ratios may also serve as a predictor of disease severity; In addition reduction in the PCr/ATP ratio by itself is a risk factor for heart failure [23]. However, to evaluate the results by one and two-dimensional chemical shift imaging measurements of the heart muscle using NMR have several limitations that needs to be considered. Since chest muscles have significantly higher PCr/ATP may can create some error by motion artifacts (e.g. due to respiration).These effects may explain the contradictory results reported in the literature on the reduction of PCr/ATP ratio in the myocardium of patients with dilated cardio-myopathies.

COMPARISON OF HPLC AND NMR

A primary advantage of P-NMR spectroscopy is the capability of detecting and quantifying sequential changes in the level of high energy phosphates compounds under progressive heart failure in live animal. Some authors reported that the ATP content in the heart is partially detectable by P31 NMR [30]. The presence of NMR-invisible ATP might depend on the fact that this compound is sequestered in mitochondria and bound to macromolecules such as myosin, the most abundant protein in myocytes. Some authors maintain that the NMR-invisible pool of ATP exists under all conditions. Two pool of ATP has been identified by Zahler *et al.,* [31) such as NMR-visible (NMR-VIS) and another that is NMR-invisible (NMR-INVIS) using 31P nuclear magnetic resonance saturation transfer technique. But in some assay it was observed that this level is similar to as observed by biochemical assays [32]. However, it is important to mention here that the area of the phosphorus resonance is proportional to the amount of metabolites, so quantitative interpretation of P-31 NMR spectra of tissue should represent the same values as measured by other techniques.

Capillary Electrophoresis

Capillary electrophoresis (CE) is conducted in an open capillary to resolve species according to their different migration rates resulting from an applied electric field. Here one of the advantages of this method is that the sample volume required is only a few nanoliters. This technique has been used for determining various nucleotides [2, 33, 34]. Capillary electrophoretic methods for the determination of adenine nucleotides and for their derivatives have been reported [35]. The capillary electrophoresis method is very simple, fast and inexpensive as compared to the previously reported cumbersome and expensive HPLC and NRM spectroscopy but capillary electrophoresis results in a low yield and low reproducibility [33, 34, 36]. Here one of the problems is with their detection, such as UV and fluorescence, due to the minute sample volume injected. This problem can be avoided by use of stacking methods which can increase the concentration sensitivity of this technique. Out of which, whole capillary stacking can increase the sensitivity up to many folds [37]. Whole capillary stacking involves three steps. In the first, the capillary is filled with sample. In the second step voltage is applied to this sample, which results in positively charged and neutral compounds to be removed with the sample matrix. Which results negatively charged nucleotides stack at the buffer sample interface. In the third step: the electro osmotic flow is reversed by reversing the voltage, which results in the separation of nucleotides on the basis of their relative mobility. This capillary stacking has many advantages for the analysis of nucleotides. Cost of analyses is less. The amount of sample required is minimal. Detection limits can be lowered without any sample pre-concentration or derivatization. Finally, sample preparation time and cost are minimized because the same capillary is used for both the concentration and analysis steps [35]. Due to low yield and low reproducibility this method is not good for analyzing these compounds.

INDIRECT METHODS

In addition to the above described techniques some of the indirect method has also been used by measuring the level NADH and some of the enzymes, which are important in energy producing pathways such as Lactate dehydrogenase, isocitrate dehydrogenase and α-Ketoglutarate Dehydrogenase and enzymes of electron transport chain, which indirectly reflect the energy state of mycardium. Further, Creatine kinase is also impotent enzyme in myocardium which can affect the level of ATP and Creatine phosphate [38]. Moreover, direct relation is postulated between reduced PCr/ATP ratios and reduced protein level of mitochondrial creatine kinase in myocytes due to an altered mitochondrial oxidative phosphorylation regulation [38]. This reduction of mitochondrial creatine kinase may cause accumulation of reactive oxygen species, which may open the mitochondrial permeable transition pore and can induce myocardial apoptosis [23]. This injury to myocardium may leads to loss of mitochondrial enzymes required in tri-carboxylic acid (TCA) and electron transport chain (ETC) cycle and hence leads to lowering of energy status by decreasing their production. But these enzymes cannot determine the exact energy state of the myocardium because one enzyme may be at normal level but another enzyme in the next step may be less which can affect the energy state of the myocardium because these methods can not exactly determine the energy state of myocardium.

CONCLUSION

Taken together, it may be concluded that each method possesses its own merits and demerits but it is worth mentioning that there is no alternative for NMR spectroscopy for measuring high energy phosphates levels in live animals, whereas HPLC has been recommended for estimation of high energy phosphates in myocardial biopsies. In addition, the enzymatic assays may be very useful in combination with the above methods from which we can pin point where is the exact problem.

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