Creatine Assay for Use on Bench Top Chemistry Analyzer: Quick Analysis of Creatine in Human Serum Smaples

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Creatine Assay for Use on Bench Top Chemistry Analyzer: Quick Analysis of Creatine in Human Serum Samples.

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A Thesis in the Field of Biotechnology

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Abstract

Creatine (Cr) was initially discovered in the 1830s by a Frenchman named Chevreul. In subsequent years, it was discovered that creatine was utilized by the musculatory system, which fueled research on its overall use as a metabolite in the human body. Investigation into the role of this chemical in the human body led to its current use as a sports supplement and potential treatment option for individuals with musculatory disorders such as Parkinson’s and Huntington’s. With creatine’s increased use as a sports supplement there is also a strong interest in its long term effects, as well as its potential as a treatment option. There is need for further investigation as well as a means for dosage determination, and a method that will allow for accurate concentration determination. The current methods used to determine creatine concentration are time consuming and complicated analytical methods such as high pressure liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GS-MS). A single reagent enzymatic assay (Cr assay) that can be used on any bench top chemistry analyzer for accurate and precise measurement of Cr in human serum and aqueous samples was developed and is summarized here. The assay can measure samples in 10 minutes, is linear from 0-15mg/dL of Cr, the reagent has a shelf life of 45 days refrigerated. The Cr assay is within 98% accuracy, run to run CV from 1.6-5.3% for clinical samples and a sample-to-sample CV of less than 0.5%. This assay can easily be adapted for any bench top chemistry analyzer that has the capability of a programmable channel, and can read clinical samples with accuracy, precision and reproducibility.
# Table of Contents

List of Tables ........................................................................................................ vi

List of Figures .........................................................................................................viii

I. Introduction ........................................................................................................... 1

   Potential Beneficial Impacts of Cr ................................................................. 2

   Current Assays for Cr Detection ................................................................. 3

   Assay Requirements ................................................................................... 5

   Proposed Enzymatic Creatine Assay from Adapting Enzymatic Creatinine
   Assay ........................................................................................................ 6

   Goals for Creatinine Assay ......................................................................... 8

II. Materials and Methods .................................................................................. 9

   Participants .................................................................................................. 9

   Measures ...................................................................................................... 10

   Reagents ...................................................................................................... 10

   Reference Method ..................................................................................... 11

   Data Processing ......................................................................................... 12
List of Tables

Table 1. Cr assay parameters programmed on the open channel option on the Roche Cobas C311 .......................................................... 25

Table 2. Results of Precision study of new Cr assay vs commercial Roche crea assay on Roche Cobas C311 .............................................. 26

Table 3. Summary data of Cr recovery from plasma samples ............................ 27

Table 4. Summary of Goals and achievements for Cr assay .............................. 28
List of Figures

Figure 1. Enzymatic Cascade from creatinine to hydrogen peroxide.......................... 29

Figure 2a. UV Absorption spectra of enzymatic cr reagent................................. 30

Figure 2b. Absorbance versus time profile for enzymatic single cr assay reagent................................................................. 31

Figure 3. Comparison of two calibration standard prep methods.......................... 32

Figure 4. Linearity of Cr assay ............................................................................. 33

Figure 5. Study of single Reagent shelf life stability............................................. 34

Figure 6a. Summary of all variability for Cr assay precision study.......................... 35

Figure 6b. Summary of all variability for Crea precision study.............................. 36

Figure 7a. Day to Day, Run to run and sample to sample variability of Cr assay................................................................. 37

Figure 7b. Day to Day, Run to run and sample to sample variability of Crea assay................................................................. 38

Figure 8a. Cr recovery from plasma samples
(enzymatic assay on Cobas C311 vs IDMS)…………………………………… 39

Figure 8b. Average Cr recovery from plasma samples

(enzymatic assay on Cobas C311 vs IDMS)…………………………………… 40

Figure 9. Distribution of cr values from 60 clinical patient samples run on

the Cobas C311 and then correct using IDMS correction………………………… 41
Creatine (Cr) was initially discovered in the 1830s by a French man named Chevreul (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994). Studies of this chemical have been ongoing ever since, the first of which was done in foxes by Lieberg in 1847, who noted that foxes killed during a fox chase had much higher concentrations of Cr than those killed in captivity (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994). With the discovery of creatinine (crea) in urine by Heintz and Pettenkofer speculations began that the crea was derived by the cr stored in muscles (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994).

This eventually led to studies on cr ingestion, which showed that not all cr ingested could be recovered in urine, indicating that it was being utilized by the body. Although there have been a multitude of studies completed to investigate the metabolism of cr, few investigated the effect and role of cr supplementation on exercise and performance in humans. Only recently have these studies been gaining popularity, and with the gain in interest in cr, the scientific community has experienced an increased need
for a method to detect Cr concentration in the human circulatory and musculatory systems that is quick and reliable. (Schilling 2001).

Potential Beneficial Impacts of Cr

As the knowledge of the musculatory system and its function increases so are the possible treatment options for patients with muscular dysfunctions. The role of Cr in the musculatory system has made it a key target for treatment of disorders such as Parkinson’s, Huntington’s disease (HD) and sclerosis (Ferrante 2000; Andrew 1993; Taylor 1995; Bender 2006). HD is a genetic neurodegenerative disease that has a very late onset of mid to late life (Ferrante 2000). Symptoms of the disease include but are not limited to loss of motor function, mental function and behavioral symptoms (Ferrante 2000; Andrew 1993). Ferrante tested Cr supplementation in an HD mouse model in 2000; the data suggested that Cr could be used as a novel therapeutic agent (Ferrante 2000). Testing showed that Cr supplementation in the HD mouse model significantly increased survival, slowed the development of brain atrophy, and delayed atrophy of striatal neurons and the formation of huntingtin-positive aggregates, therefore delaying the onset and severity of symptoms of HD (Ferrante 2000). Parkinson’s disease is also a degenerative disorder but impacts the central nervous system, which in turn affects motor function (Taylor 1995). In 2006, a randomized placebo-control study was conducted at the University of Munich by Bender et. al. using 60 Parkinson’s disease patients to determine the effects of Cr supplementation on the progression of the disease symptoms (Bender 2006). Although the study results did not prove promising for the motor function of the patient population, patients did experience an increase in positive feelings,
outlook and overall mood, allowing for the reduction of dopaminergic therapy dosage (Bender 2006). A summary of the potential benefits of Cr supplementation was published by Tarnopolski in 2000 which indicates clear evidence that Cr monohydrate has potential neuroprotective effects in animal models of Parkinson’s disease, Alzheimer’s disease, after ischemia and amyotrophic lateral sclerosis (Tarnopolsky 2000). These are only a few examples of the current research being conducted to determine a beneficial impact for Cr supplementation. As many researches mentioned in their publications, there is an increasing need for further research in the impact of Cr as a supplement (Becque 2000; Farquhar 2002; Yoshizumi 2004). In addition to investigation on beneficial implication of Cr, there is also ongoing investigation on the long-term impact of Cr supplementation on healthy individuals (Becque 2000; Farquhar 2002; Yoshizumi 2004).

Current Assays for Cr Detection

Currently, research on the effects of Cr supplementation on kidney function relies on more complicated analytical methods such as HPLC and GC-MS (Yoshimuzi 2004). In this growing research field there is a need for a method to measure Cr with high throughput and without complicated separation techniques. Arias, et al. published a paper in 2006 explaining the current and very complicated procedure of Cr detection (Arias 2006). High pressure liquid chromatography-tandem mass spectrometry (HPLC/MS) is a process which combines the capabilities of liquid chromatography to physically separate chemical compounds and the capability of mass spectrometry to detect the mass of a compound (Aebersold 2003; Jonsson 2004). A sample that potentially contains Cr is passed through a liquid chromatography column filled with adsorbent material, and
chemical components that are in the sample each react differently with the adsorbent material. The time it takes for each compound to pass through the column is different based on that interaction (Aebersold 2003). Each chemical passes through the column at different rates allowing for the separation and identification of each chemical based on the interaction with the adsorbent material (Aebersold 2003). A very complicated method that also has other limitations, such as interfering substances such as crea, and other metabolites such as glucose which will not allow for proper separation with Cr molecule and therefore will give incorrect or no results at all (Aebersold 2003). Mass spectrometry enables identification of amount and type of chemicals present in a solution (Schilling 2001). This is done by separating the chemicals based on the mass to charge ratio in their gas-phase (Jonsson 2004). The second method described by Arias is gas chromatography mass spectroscopy (GS/MS). Similar to liquid chromatography, gas chromatography separates chemicals, but does so by vaporization, and is then combined with mass spectrometry to detect the presence and concentration of each chemical (Arias 2006; Rawson 2011). As Arias et. al. concludes, in comparing Cr to Crea ratios in patient samples there is a constant and proportional error and therefore the Cr to Crea ration should be evaluated with caution, especially when using GC/MS (Arias 2006). Finally BioVision (Milpitas, CA) has a colorometric/fluorometric assay kit, which uses a colorometric or fluorometric intensity change to detect the concentration of cr in samples (Rawson 2011). The assay is relatively easy to use according to the manufacturer’s instructions – but the reaction requires 1 hour for incubation and no separation is required. However, the assay is for research purposes only and is not traceable to
standard methods so it cannot be used for clinical testing. The methods currently available to test for cr in clinical samples are either very complicated to use such as GC/MS and HPLC/MS or not approved for analysis of patient samples. There is an increasing need for the ability to quickly analyze cr in patient samples to be able to evaluate cr deficiency syndroms (Arias 2006). There is also an increased need for evaluation of long-term cr supplementation (Yoshizumi 2004).

Assay Requirements

To be able to detect an unknown concentration of a chemical, the assay must fulfill some basic requirements. The method of detection must be sensitive enough to respond to the lowest concentrations that are to be measured. The detection method must be accurately and easily calibrated against a known standard concentration that is within the range in which is to be detected, and the reaction must be linear in response to the concentration ranges of measurement. For clinical sample analysis, the impact of biological sample matrix on accuracy and precision should be part of the critical criteria of the assay. This is applicable to any measurement system that is sensitive to a specific chemical in any background matrix. Currently to calculate accurate estimated glomerular filtration rate (eGFR) hospitals must have a system that can accurately measure concentrations of crea in either blood or plasm over a period of time from the same patient (Levey 1999). Therefore a hospital must have an instrument that can accurately measure the Crea level from a patient’s blood or serum. Instruments that are utilized for these purposes in hospitals are either large, slow bench top chemistry analyzers, or
critical care instruments which are smaller and easier with much quicker throughput. These instruments are capable of measuring crea using enzymatic methods via detection of colorimetric changes or current changes (Pamidi 2010). Pamidi explains that by taking advantage of the natural breakdown process of crea via enzymatic methods, one can develop a sensor that has the capability to detect the final breakdown product, hydrogen peroxide, which can be detected by a spectrophotometer or by a current detector. There has not been a method developed that utilizes a similar concept for the accurate detection of Cr in human blood.

Proposed Enzymatic Creatine Assay from Adapting Enzymatic Creatinine Assay

For the measurement of crea in clinical samples, a quick assay has been developed utilizing creatinine amidohydrolase (creatininase), creatine amidohydrolase (creatinase) and sarcosine oxidase as can be seen in Figure 1 to convert crea to cr then creatine to urea and sarcosine. Sarcosine is then converted to formaldehyde and hydrogen peroxide. Hydrogen peroxide will interact with a specific dye or chromophore to give a colorimetric change, which can be detected by a spectrophotometer. This assay has been made available by several different manufacturers such as Roche, Sekisui, and Horriba. The available assays are two-step, two reagent assays, in which reagent one contains creatinase, which converts cr to sarcosine and urea. This is done to eliminate all cr present in the sample as it causes interference with the crea detection. Sarcosine oxidase is in turn needed to convert the produced sarcosine to glycine, formaldehyde and hydrogen peroxide. The time delay between the addition of the first and second reagent
allows for the degradation of hydrogen peroxide which is determined by the concentration of the enzymes present in the reagent. The second reagent contains creatininase which converts Crea to Cr and the creatinase that is still present in reagent one converts Cr to sarcosine and the same cascade reaction will follow until hydrogen peroxide is formed. The hydrogen peroxide will interact with the 4-aminoantipyrine dye in reagent two, which is catalyzed by horse radish peroxidase to give a color change that can be detected by photospectrometry. We are proposing a similar enzymatic based method to allow for the breakdown of Cr to hydrogen peroxide. The enzymatic method that we are suggesting only requires a single reagent as the Cr molecule can be directly converted to sarcosine by the creatinase enzyme that is in reagent one of crea assay and the produced sarcosine is converted to hydrogen peroxide, which will react with 4-aminoantipyrine in the presence of horse radish peroxidase to allow for detection via uv-vis on any photospectrometer. All chemicals can be maintained in one reagent as no time delay is needed.

Having a reference material such as National Institute of Standard and Technology (NIST) traceable standard allows for accurate clinical assay development and is recommended by FDA regulations. Unlike crea, there is no NIST traceable Cr standard available making the assay development more challenging. As there are very limited testing assays currently available on the market for Cr and most of them are for laboratory research purposes only, there is a need for a method with quicker results, which can be utilized for clinical sample measurement.
Goals for Creatine Assay

Our ultimate goal is to create an accurate and convenient creatine assay, which is quick and easy to use. It is based on a single part reagent via enzymatic conversion of creatine with colorimetric end point detection of 4-aminoantipyrine at 547 nm. In contrast to the purchasable manual microtiter plate based assay, our assay incubation time is much faster so it can be fully automated by easily adapting it to any clinical chemistry analyzer which provides customizable assay channels. The cr in clinical samples can be analyzed simultaneously with other commercially available assays like crea, glucose, lactate, etc.

Using a bench top chemistry analyzer, the goal is to develop a cr assay through optimizing assay conditions and procedures with a specific small (<300uL) sample and reagent volume, and an incubation time of less than 20 minutes. Methods and procedures will be developed for calibration standards and reagents that can measure the cr concentrations in clinical samples. The assay will achieve linearity of 0-15mg/dL, accuracy within 2%, recovery within +/- 3% from gravimetric values. In addition, coefficient of variation (CV), which is a standardized measure of dispersion of a probability distribution or frequency distribution, will be applied to analyze variability from samples and runs. It is defined as the ratio of the standard deviation to the mean. The target set for run to run is CV of less than 2% and sample to sample of 0.5%.
Chapter II

Materials and Methods

Materials and methods utilized for the development of the enzymatic creatine assay include participants who provided human blood and serum samples. To measure the outputs of our assay instruments such as a benchtop chemistry analyze and a spectrophotometer were utilized. Reagents, calibration material and standard (control) material were developed along with parameters for specific use on bench top chemistry analyzers with an open channel feature. Finally data processing and statistical outputs were utilized to determine the success of our enzymatic creatine assay.

Participants

Participants are individuals that have in some form contributed to the development on the cr enzymatic assay; individuals from who blood and serum were used are participants. For the purposes of our study human biological samples such as blood and serum were needed to test the capability of the enzymatic cr assay. Human blood and serum were chosen as there are easy samples to collect from patients and in addition the enzymatic cr assay must have the capability to detect cr concentrations regardless of matrix and specifically in serum samples. These patient samples were collected by Lahey Hospital and Medical Center in Burlington Massachusetts. These samples were collected as de-identified pathologic discard whole blood and serum samples.
Measure

To aid in the development of the enzymatic cr reagent a spectrophotometer was used to detect the optimal absorption value and the optimal incubation time. A bench top chemistry analyzer was used to conduct detailed evaluation of the parameters selected for the assay along with over all evaluation of the assay reagent, and calibration solutions. The instruments used are as follows.

UV-vis spectrometer, Evolution 6000 (Hitachi Ltd, Chiyoda, Japan) with quartz cells for optimization of wavelength for assay conditions. The spectrometer was used to determine the optimal absorption value for the photophor selected which was the 4-aminoantiparine, as well as to determine the optimal incubation time for the assay. Data shown in Figures 2a and 2b.

Bench top chemistry analyser, Roche Cobas C311 analyzer (Roche Diagnostics, Indianapolis, IN, USA), with programmable channel capability (open channel). Using the open channel function for programmability this instrument allowed for the programming of the optimized parameters for the enzymatic cr assay and all optimization and final analysis was completed using this instrument.

Reagent

To develop a enzymatic cr reagent to use in the analysis of the enzymatic cr assay a commercially available assay for crea (Sekisui Diagnostics LLC, Lexington MA) was obtained and modified by adding 4-amynoantipyrine and horse radish peroxidase to
reagent one of the crea assay. Enzymatic Cr reagent includes the following chemicals and enzymes: buffer (pH 7.5) >12000 U/L creatine amidohydrolase, > 4000 U/L sarcosine oxidase, > 2 U/mL horse radish peroxidase, > 0.24 mmol/L N-ethyl-N-sulfopropyl-m-toluidine, > 0.16mg/mL 4-aminoantipyrine, ascorbate oxidase, stabilizers, surfactants, and preservatives. (22)

To determine whether the reagent would be satisfactory, a predetermined concentration of 4-aminoantipyrin and horse radish peroxidase were added to the Sekisui reagent, for a total of 250mL of reagent. Based on the parameters of the Sekisui Creatinine assay, a ratio of one part sample to five parts reagent was tested. Total volumes tested were 1.5mL of reagent and 0.3mL of sample. The sample that was chosen was a 5mg/dL concentration of cr in deionized water. The reagent and sample were mixed and allowed to incubate for 5 and 30 minutes, at which point absorption spectra was taken using a UV-Vis spectrometer (the Evolution 6000) with quartz cell. A blank was taken as well which included only the reagent with deionized water (Fig. 2).

Reference method

HPLC analysis was conducted by an outside laboratory (Organix Inc, Woburn MA, USA) as an independent check to verify the correct concentrations of our prepared standards S1, S2, S3 and S4.

Isotope Dilution Mass Spectroscopy was conducted to measure cr values in plasma samples.
Data Process and Statistical Analysis

Microsoft excel and Minitab were used for statistical analysis along with figure and table generation.

Calibration Material

The cr assay is calibrated using a two point calibration at concentrations 0mg/dL and 5mg/dL of cr. The 0mg/dL concentration also serves as a blank measurement for the reagent. A 5mg/dL standard was prepared to calibrate the assay. Initially the standard was prepared using the gravimetric procedure by dissolving 5mg/dL of cr in deionized water at pH 11.0. As part of optimization another procedure was found to be more suitable as it showed less variability. This procedure required preparing a high concentration of cr, 1000mg/dL, and the standard was then prepared by diluting to 5mg/dL.

As seen in Figure 3, the gravimetric method was conducted by weighing out 44.0mg and 58.7mg of creatine monohydrate and then dissolving in 1L of deionized water to make a 3.9mg/dL and 5.2mg/dL cr solution respectively. A 1000mg/dL Cr stock solution was prepared by weighing 1.131g of creatine monohydrate and dissolving it in 100mL of deionized water. This stock solution was then used to make the two lower concentration solutions. The 5.2mg/dL solution was made by adding 52uL of the 1000mg/dL solution to 10mL of deionized water and the 3.9mg/dL solution was made by adding 39uL of the 1000mg/dL solution to 10mL deionized water.
Standard material (Control Standards)

For the development of standards and calibration materials for the enzymatic creatinine assay, samples of known concentration were used to ensure proper function of assay and assay calibration. These samples were expected to recover the same value over time as they are stable materials and are stored in a refrigerator (2-8°C). These materials were prepared and values were determined independently so these materials can be used as quality control material, HPLC analysis was conducted to assign their value by an outside lab, Organix. The target value of cr is 5.2mg/dL.

When developing calibration standard and procedures these Control Standards were used as calibration standards, and other investigational test solutions were tested as samples on the cr assay.

Assay Parameters

As shown in Table 1, the parameters for this assay are easily programmable for any bench top chemistry analyzer that has the capability of an open channel. The required sample volume is 30µL, the reagent volume is 150µL, an incubation time of 10 minutes and a wavelength of 546nm are required to read the final absorbance.
Chapter III

Results

To determine the proper enzymatic Cr reagent formulation, the reagent was first formulated. Reagent formulation as described in Chapter II was executed by modifying a crea reagent purchased by Sekisui, modification was completed by adding HRP and 4-aminoantipyrine. The enzymatic Cr reagent was then tested on a spectrometer to determine optimal absorption values and incubation times.

To determine the sensitivity of the proposed enzymatic reagent we measured the absorbance change of the reagent in the presence of 5mg/dL Cr using UV-Vis spectrophotometry. Measurements were taken at various incubation times from 0 to 30 minutes. The data in Figure 2 shows that the proposed reagent was undergoing a chemical change in the presence of Cr which was detected via UV-Vis at absorbance peak of 540nm. As shown in Figure 2 the dye selected was the proper dye as the interaction with hydrogen peroxide in the presence of horseradish peroxidase caused a peak in the spectral scan at 540nm. Furthermore the presence of a peak indicated that the concentrations of enzymes and the dye were appropriate. The absorbance data also showed that the reaction was fully completed within 10 minutes from the plateau, so the incubation time was then set for 10 minutes for the assay.
Calibration Solution Development

To develop a precise, accurate and stable calibration standard solution, two methods of preparation were tested. These calibration solutions were run as samples on the Cobas C311 (Roche Diagnostics) using the single reagent enzymatic Cr assay after it was calibrated with an independent calibration solution. Standards were prepared via dilution method and gravimetric method. Gravimetric method was conducted by weighing out Cr to directly make a 3.9mg/dL and 5.0mg/dL solution, while the dilution method was conducted by weighing out Cr to first make a 1000mg/dL Cr stock solution, then diluting that solution to 3.9mg/dL and 5.0mg/dL. As seen in Figure 3 the data shows that there is less variability in the preparation of the calibration solution by following the dilution method.

Linearity

For proper performance of the assay linearity to substrate must be established and determined, if assay is non-linear a mathematical formulation must be established to correct to non-linearity.

To test for linearity of the assay, concentrations of 0, 1, 2.5, 5, 10 and 15mg/dL of Cr were prepared via serial dilution. The samples were run on the Cobas C311 using the single reagent Cr assay after it was calibrated. As seen in Figure 4 the $R^2$ value is > 0.999 indicating that the assay is linear within the range of 0-15mg/dL Cr.
Reagent Stability

To evaluate the stability of the enzymatic Cr assay reagent over its shelf life, reagent was prepared and stored under refrigeration (2-4°C) and tested after 1, 5, 10, 20, 30 and 45 days of storage. The stability of the reagent was evaluated by completing a three point calibration using 0mg/dL, 3.9mg/dL and 5.2mg/dL of Cr, and correlate the absorbance values of each calibration sample versus gravimetric Cr concentrations. The absorbance value increases as the reagent ages but the linearity of the calibration does not change, indicating that the reagent can maintain functionality after 45 days of refrigerated storage, giving the assay a shelf life of 45 days (Figure 5).

Precision and Accuracy

To determine the accuracy and precision of the assay, four individual samples were prepared and run after three separate calibrations. Samples included crea as well as Cr to allow for the comparison of the two enzymatic reagent methods which were both run simultaneously on the Cobas C311. These Cr results were compared to crea data to ensure comparability. As seen in Figure 6a the total variability of recovery for the Cr assay is minimal and comparable to the crea assay as shown in Figure 6b. As shown in Table 2 the crea assay is capable of precision within 1.3% and accuracy within 1.7% and 0.5% and 1.2% respectively for the enzymatic Cr assay, proving that the Cr assay is comparable to a commercially available crea assay. As can be seen in Figures 7a and 7b, day to day, run to run and sample to sample variability are shown for each individual sample Cr and crea assay respectively.
Creatine Assay Performance in Plasma Samples

To complete determination of successful enzymatic cr assay clinical samples were tested and evaluated using all optimized parameters, reagents (assay reagent and calibration samples).

To establish the cr assay capability for clinical sample recovery, plasma samples of known cr concentration were measured on the Cobas C311 using the cr assay. This was done to determine the linearity, accuracy and precision of the method in the clinical sample matrix, as opposed to aqueous samples. As can be seen in Figure 8a samples were run over a 4 day period and for each day data were used to establish correlation between the Isotope Dilution Mass Spectrometry (IDMS) measured values and the cr recovery using the cr assay. Data are summarized in Figure 8b and a single formula was calculated to show correlation of the two methods. The formula for correlation of Cr assay vs. IDMS (to correct the matrix effect) was then used to correct subsequent clinical samples as shown in Figure 9 that were run on the Cobas C311 to calculate cr recovery using the cr assay
Cr has been investigated since the 1830s and research has revealed its role in the proper functioning of the musculatory system (Hunter, 1928; Lundsgaard, E. 1930; Balsom 1994). With this knowledge the use of Cr as a potential drug for treatment of muscular disorders such as Parkinson’s, Huntington’s disease and sclerosis, along with other musculatory system disorders are subjects of research (Ferrante 2000; Andrew 1993; Taylor 1995; Bender 2006). The sport nutrition/supplementation market quickly realized that Cr can be used as a supplement and as such can increase strength, fat free mass, and muscle morphology in humans (Cooper 2012). It increases crea storage, which in turn increases muscle mass and promotes a faster regeneration of ATP between high intensity exercises (Cooper 2012). These improved outcomes will increase performance and promote greater training adaptations (Cooper 2012). Although short term use of Cr is fairly well understood and considered safe and ethical in the sports world, the perception of safety cannot be assumed, that it is safe and ethical especially in long term users from diverse population background. To summarize there is an increased need for investigation of the impact of Cr on potentially beneficial implications as well as long term impact of its use as a supplement (Cooper 2012; Yoshizumi 2004).

Currently to determine the concentration of Cr researchers must rely on methods such as HLPC and GC-MS, which are complicated to conduct and require a high level of
expertise (Arias 2006; Aebersold 2003; Jonsson 2004; Rawson 2011). Furthermore as discussed by Arias et. al. there are also issues with using human serum with such techniques that do not allow for accurate measurements due to interference from other components that are present in serum (Arias 2006). There is an increasing need for the capability of quick analysis of Cr in patient samples to be able to evaluate long term impact of supplementation or potential dosage management for use of cr as a drug (Arias 2006).

Cr Assay Parameter Optimization

An accurate and convenient enzymatic based Cr assay was developed, which is easy to use and reports results much quicker than the current methods. The single part reagent uses enzymatic conversion of cr with a colorimetric end point detection of 4-aminoantipyrine at 547nm. The method has been optimized and demonstrated on a bench top chemistry analyzer and has a run time of 10 minutes, using only 30uL of sample.

The enzymatic reagent is adapted from a commercially available Creatinine assay from Sekisui which is then modified by adding 4-aminoantipyrine and horse radish peroxidase. To determine whether the reagent would be satisfactory a predetermined concertation of 4-aminoantipyrin and horse radish peroxidase were added to the Sekisui reagent. Based on the parameters of the Sekisui Creatinine assay and optimization a final formulation was reached along with appropriate assay parameters adaptable to a bench top chemistry analyzer.

Using a Roche Cobas C311 analyzer, which is a bench top chemistry analyzer typically used in hospitals for measurements of electrolytes, and metabolites such as crea,
the assay and its parameters were evaluated. The assay parameters were programmed into
the instrument’s open channel, which allows for user programmable assays. The assay
parameters are simple and easy to program as can be seen in Table 1. For assay
development the proposed assay was initially calibrated with a 5mg/dL cr in deionized
water.

Stability of the Reagent

To then study the stability of the regent as it ages a fresh calibration material was
made and a three point calibration was executed to determine the linearity at multiple test
points of the reagent shelf life. Testing was executed on the day that the reagent was
prepared and 5,10,20,30 and 45 days after that with the reagent being stored in a
refrigerator (2-4 C). As can be seen in Figure 5, all data points show linearity and more
importantly very similar slopes indicating that the linearity of the assay has not been
changed over time. This data shows that the cr reagent is stable for at least 45 days when
stored in the refrigerator.

Optimize Procedure for Calibration Standards

To develop a precise, accurate and stable assay, an equally as accurate and
reproducible calibration solution is necessary. To achieve this, two different methods of
making calibration solutions were evaluated. Initial experiments tested concentrations of
3.9 and 5.2 mg/dL cr. These two standard concentrations were prepared by two different
methods. The gravimetric method in which the appropriate concentration of Cr is
weighed out and dissolved in deionized water. The dilution method, by which a high
concentration of cr stock solution is prepared and then diluted down to the final concentration was the other method tested. Based on calibration standard preparation optimization the dilution method was determined to be the most reproducible and accurate preparation method as can be seen in Figure 3.

Linearity of Assay

With optimized assay parameters and calibration standards, we then evaluated the linear range of the assay. To do so a high concentration sample of 1000mg/dL cr was prepared using creatine monohydrate. This sample was then diluted to 1, 2.5, 5, 10 and 15mg/dL cr. All samples were tested in triplicate on the Roche Cobas C311 using the Cr assay. The data in Figure 3 shows an $R^2 > 0.999$; therefore our assay shows linearity within the range of 0-15mg/dL.

Accuracy and Precision

To determine precision and accuracy of the cr assay, three samples from four individual cr test solutions were run in triplicate. The test was repeated three times with three separate calibrations, giving a total of 27 measurements. Detailed data is presented in Figures 6a & 7a. These data were compared to a commercially available Roche crea assay to ensure comparability (Figures 6b & 6b). As can be seen in Figure 6a the accuracy of the cr assay is comparable to that of the crea assay shown in Figure 6b. This experiment summarized replicate to replicate variability, sample to sample variability and run to run variability. Also shown in Figures 7a and 7b, no outlier was observed. Statistical analysis is shown in Table 2, which indicates that the CV is consistently better
for the cr assay as opposed to the crea assay with CVs of 0.28-0.49% and 0.74-1.26% respectively. In addition the analysis indicated better accuracy with the cr assay ranging from 0.4-1.2% as compared to the crea assay ranging from 0.8-1.7%. The data also show that there is no interference from crea as all solutions recover very close to their gravimetric values with and without the presence of crea. Samples S1, S3 and S4 are mixtures of both crea and cr and the presence of these chemicals together does not impact the recovery of the cr assay, as seen in Table 2. Furthermore, the data shown in table 2 indicated that the cr assay gives both better precision and accuracy than the commercially available crea assay. This could be explained by the volume ratio of sample to assay reagent as the commercially available crea assay has a ratio of 1:32 in comparison to that of the cr assay of 1:5. The higher sample volume that the cr assay has could explain the improvement in the accuracy and precision over the commercially available crea assay.

Recovery in Clinical Samples

Performance of the cr assay on the Cobas C311 in plasma samples was evaluated by testing plasma samples with cr values determined by IDMS, as seen in Figure 8. Three levels of plasma samples were tested at 0.5mg/dl, 1.8mg/dL and 4.5mg/dL. All samples were tested in duplicates over four separate days under four separate calibrations. The data was correlated against the IDMS measured values as can be seen in Figures 8a and b, showed good linearity $R^2 > 0.99$ for samples between 0.5-4.5mg/dL cr. These data show that regardless of the sample matrix the cr assay developed on the Cobas C311 has
good linearity (R² > 0.99). The bias at these three levels and the CV from run to run are well within our original goals, as can be seen in Table 3 and Table 4.

There was a bias of +0.2479 as can be seen in Figure 8b against the IDMS which is likely due to plasma sample matrix effect versus aqueous calibration. The effect can be corrected if sample measurements are normalized against IDMS serum samples. As can be seen in Figure 9 clinical samples that were tested on the Cobas C311 using the cr assay and then normalized versus IDMS fall within the described clinical ranges (0.2-1.7 mg/dL +/- 0.3) with some high samples that were spiked to increase the cr concentration. Clinical samples tested using the enzymatic Cr show recovery of 0.26 -1.26 mg/dL +/- 0.24 (excluding the spiked samples), showing that the cr assay developed is capable of measuring cr in clinical samples (Straseski 2011).

Based on the data presented, a enzymatic cr assay that can be used to measure clinical samples on a bench top chemistry analyzer was developed and successfully met all of the requirements that we set out in Table 4. The assay is a single part reagent that uses enzymatic conversion of cr with colorimetric end point detection of 4-aminoantipyrine at 547nm. The assay uses a small sample size of 30uL, and takes 10 minutes (Table 2 and 4). Furthermore the enzymatic cr assay has proven linearity from 0-15mg/dL, accuracy within 2%, run to run CV from 1.6-5.3% for clinical samples and finally sample to sample CV of less than 0.5%. This assay can easily be adapted for any bench top chemistry analyzer that has the capability of a programmable channel, and can analyze clinical samples with accuracy, precision and reproducibility. In comparison to other methods of cr detection such as HPLC and GS/MS the enzymatic cr assay has less
interference from sample matrix and the accuracy and precision is comparable to both methods, with the enzymatic cr assay having precision and accuracy within 2%, recovery of 0.5% as compared to HPLC which has better precision and accuracy which is as low as 0.5% but has many interference issues from samples matrix, GS/MS has similar issues and recovery. This enzymatic cr assay could have future application for the potential beneficial implication of cr, as well as investigation on the long term impact of cr supplementation of healthy individuals.
Appendix 1. Tables

Table 1. Cr assay parameters programmed on the open channel option on the Roche Cobas C311.

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Table 2: Results of Precision study of new Cr assay vs commercial Roche Crea assay on Roche Cobas C311

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<td>Crea</td>
<td>Cr</td>
<td>Crea</td>
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<tr>
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<td>0.02</td>
<td>0.02</td>
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<tr>
<td>Precision (%CV)</td>
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Table 3: Summary data of Cr recovery from plasma samples

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<td>IDMS Value (mg/dL)</td>
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Table 4: Summary of Goals and performance for Cr assay

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Appendix 2. Figures

Figure 1. Enzymatic Cascade from creatinine to hydrogen peroxide.

Pictorial description of the change that occurs for the Crea enzymatic reaction for the conversion of creatinine to hydrogen peroxide. Which allows for the interaction of hydrogen peroxide with 4-aminoantipyrine to give a colorimetric change allowing for the detection of Crea concentration.
Figure 2a. UV Absorption spectra of enzymatic cr reagent.

Uv-vis spectrometer: Evolution 6000 with quartz cells. Sekisui Reagent RI modified with 2U/mL HRP and 16 mg/dL 4-AMP, 2.5 mL. Sample: 5 mg/dL Cr solution, 0.5 mL. Blank: DI water. Absorbance collected at 0, 5, 10, 20, and 30 minutes of incubation time.
Figure 2b. Absorbance versus time profile for enzymatic single Cr assay reagent.

Uv-vis spectrometer: Evolution 6000 with quartz cells. Sekisui Reagent RI modified with 2U/mL HRP and 16 mg/dL 4-AMP, 2.5 mL. Sample: 5 mg/dL Cr solution, 0.5 mL. Blank: DI water. Absorbance collected at 0, 5, 10, 20, and 30 minutes of incubation time at 547nm.
Figure 3. Comparison of two calibration standard prep methods.

Two different concentrations, 3.9mg/dL and 5.2mg/dL were tested by both methods.

*Individual standard deviations are used to calculate the intervals.*
Figure 4. Linearity of Cr assay.

Total of 6 samples at gravimetric values of 0, 1.5, 2.5, 5, 10 and 15mg/dL cr were run in replicated of five on the Roche Cobas C311 using the single reagent cr assay. Gravimetric Values of Cr solution against the reported cr values by the cr assay adapted to the Cobas C311.

\[ y = 0.9957x + 0.0148 \]

\[ R^2 = 1 \]
Figure 5. Study of single Reagent shelf life stability.

Scatterplot for cr assay linearity of fresh standards in miliabsorbance over a period of 45 days of reagent shelf life. Each data point represent duplicated samples of each concentration point of each calibration.
Figure 6a. Summary of all variability for Cr assay.

Summary of all variability on four different test solutions for Cr results by cr assay tested on the Cobas C311. The average along with the 95% confidence interval showed the overall variability of the assay. Source of variability includes calibration to calibration, and sample imprecision. Data point representing each sample includes the average and 95% confidence interval of a total of 27 measurements. Test were completed on three different days, on each day a fresh calibration using fresh calibration standards was conducted, three different preparations of Samples 1-4 were made and tested in triplicated using the cr assay on the Roche Cobas C311.
Figure 6b. Summary of all variability for Crea precision study

Summary of all variability on four different test solutions for Crea results by Roche crea assay tested on the Cobas C311. The average, shown with the 95% confidence interval showed the overall variability of the assay. Source of variability includes calibration to calibration, and sample imprecision. Data point representing each sample includes the average and 95% confidence interval of a total of 27 measurements. Tests were completed on three different days. On each test day a fresh calibration solution was prepared and used to calibrate the instrument, this was repeated twice. Samples 1-4 were made and tested in triplicated using the crea assay on the Roche Cobas C311.
Figure 7a. Day to Day, Run to run and sample to sample variability of Cr assay precision study.

All individual data points are presented to show the overall change when presented with different variables. Variability of run to run, sample to sample and replicate to replicate for Cr recovery on Roche C311 Cr assay. These are all individual measurements shown summarized in Fig 6a.
Figure 7b. Day to Day, Run to run and sample to sample variability of Crea assay.

All individual data points are presented to show the overall change in value when presented with different variables. Variability of run to run, sample to sample and replicate to replicate for Crea on Roche C311crea assay. These are all individual data points shown summarized in Fig 6b.
Figure 8a. Cr recovery from plasma samples (enzymatic assay on Cobas C311 vs IDMS)

Plasma samples were tested on the Cobas C311 to determine Cr recovery. Data was correlated to the Isotope Dilution Mass Spectrometry (IDMS) measured values to determine the correlation. Data were collected in duplicate over four different days to account for run to run variations.
Figure 8b. Average Cr recovery from plasma samples (enzymatic assay on Cobas C311 vs IDMS)

Using all data collected in duplicate over a period of four days a single formula was calculated to determine the bias of the two methods - the cr assay on the Cobas C311 vs IDMS.
Figure 9. Distribution of cr values from 60 clinical patient samples run on the Cobas C311 with normalization to IDMS samples.

Dot plot of Clinical sample recovery using the cr assay after correction of bias the calculated formula from IDMS samples.
Appendix 3. Raw Data

Absorbance Data

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44
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| 517 | 0.007 | 517 | 0.039 | 517 | 0.373 | 517 | 0.56  |
| 518 | 0.007 | 518 | 0.04  | 518 | 0.379 | 518 | 0.569 |
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Bibliography


Fekete, S., Ganzler, K., & Fekete, J. (2010). Facts and myths about columns packed with sub-3\(\mu\)m and sub-2\(\mu\)m particles. *Journal of pharmaceutical and biomedical analysis, 51*(1), 56-64.


