Accuracy Matters: Enzymatic Assays for Creatinine Biomarker Measurement during Drug Development

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Abstract

Background

Glomerular filtration rate and staging of chronic kidney disease (CKD) is typically assessed by measuring the endogenous concentrations of creatinine. Bioanalytical use of creatinine is also increasing for drug development leading to greater FDA scrutiny. Creatinine is commonly measured by the rapid and inexpensive colorimetric Jaffe reaction. However, the Jaffe reaction is notoriously inaccurate and very small shifts in creatinine cause large miscalculations in CKD staging. While attempts have been made to improve Jaffe reaction assays, an enzymatic assay for creatinine is more accurate and more stable. We review substances found in many common disease states that create chromo gens that interfere is the Jaffe reaction but do not affect the enzymatic assay. This includes cephalosporin antibiotics, glucose, and bilirubin. We also provide a framework for modifying creatinine commercial kits for drug development studies. Most creatinine kits, whether using Jaffe or enzymatic chemistry, are based on the Clinical and Laboratory Standard Institute (CLSI) validation approach, which is designed to distinguish diseased from healthy, is not suitable for use in drug development. The benefits of enzymatic creatinine assays have been demonstrated for over a decade yet the Jaffe reaction continues to be the primary method of measurement demonstrating the need for continued education and nuanced recommendations. For clinical lab use, we highlight a two-phase reflexive method that balances cost and accuracy. For critical decisions during a drug development study, the data strongly supports the use of the enzymatic creatinine assay in combination with bioanalytical method validation for the high accuracy needed in these settings.

Keywords: Creatinine; Enzymatic methods; Biomarker validation; Kidney disease

Abbreviations: CKD: Chronic Kidney Disease; GFR: Glomerular Filtration Rate estimation (GFR); ECr: Enzymatic Reaction Creatinine Assays; JCr: Jaffe Assays

Introduction

The real world clinical and drug development applications of biomarkers discovered through over a decade of basic science biomarker research have been limited. Accordingly, regulatory bodies such as the FDA have started biomarker qualification programs to ensure biomarkers are appropriately used during clinical studies [1]. A primary objective of this process is to reduce "measurement errors that could result in biases and affect the biomarker's predictive accuracy thus [limiting] its utility as a valuable drug development tool" [2]. In regard to endogenous renal biomarkers, this program has resulted in the qualification of both novel and existing renal biomarkers, including creatinine, for monitoring kidney function in drug development [3-4]. In this article, we evaluate the analytical performance of creatinine assays to review guidelines for its use in drug development.

Accurate creatinine measurements are critical for efforts to improve the diagnosis and treatment of CKD and other bioanalytical uses of creatinine. The benefits of enzymatic creatinine assays (ECr) over standard kinetic Jaffe assays (JCr) have been argued for over 10 years [5-6]. Despite these recommendations JCr assays are still commonly used. In a recent study at a German hospital center, 15% of patients diagnosed with acute kidney injury were detected with JCr assays alone [7]. Use of JCr assays alone are estimated to result in a 4% miscalculation rate of serum creatinine levels overall [8]. This error rate may be acceptable in a clinical lab screening setting due to the increased cost of ECr assays and the intrinsic biological variability of creatinine concentrations. However, studies have shown that minimal shifts in creatinine results can cause major alterations in GFR calculations and

patient classification [6-9]. In drug development, precise determination of kidney function with biomarkers with the lowest analytical and biological variability is necessary to determine therapeutic efficacy and obtain approval by regulatory bodies.

The National Kidney Disease Education Program (NKDEP), in collaboration with the International Federation of Clinical Chemistry and Laboratory Medicine and the European Federation of Clinical Chemistry and Laboratory Medicine, created the Creatinine Standardization Program (CSP) in an effort to improve and standardize creatinine measurement. As discussed in this review, CSP's recommendations include making creatinine methods traceable to an isotope dilution mass spectrometry (IDMS) reference measurement procedure. Standardization of method calibration reduces inter-laboratory bias in results and enables more consistent measurement of creatinine in serum and urine. Implementation of IDMS traceable reference materials does not, however, solve the issues of analytical non-specificity common in alkaline picrate-based methods, including "compensated" Jaffe methods. Enzymatic assays for creatinine are known to be more specific for creatinine and provide more reliable results. Due to these reasons as discussed further in this review, they are strongly recommended for drug development settings.
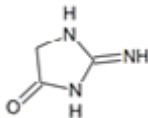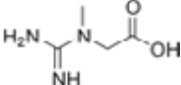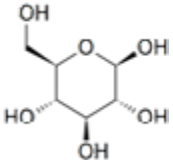
Uses of Creatinine in Drug Development

Serum creatinine measurement is the gold standard endogenous biomarker for determination of GFR. Creatinine is a breakdown product of creatine phosphate from muscles produced at a fairly constant rate and removed by the kidneys [10-12]. The vast majority is removed by glomerular filtration with nearly no tubular reabsorption. Serum creatinine levels rise as glomerular filtration decreases and GFR estimating equations are used to correlate the serum concentration to GFR and determine the stage of CKD. Since many therapeutic compounds are eliminated through the kidneys, renal function can control drug clearance, efficacy, and safety. Therefore, regulatory agencies such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA) have issued detailed guidelines for the requirements to determine GFR in new drug applications [13].
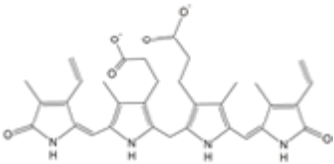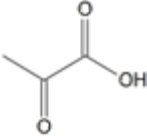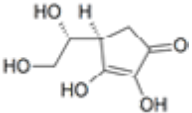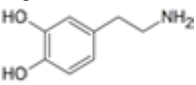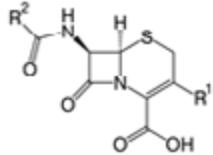
While many of these studies use clinical lab assays to determine creatinine, the uses of creatinine requiring bioanalytical validation and compliance are increasing. Clinical are only designed to distinguish healthy from abnormal values and thus therapeutics that require regulatory approval demonstrating improved renal function should follow bioanalytical guidelines. Measurement of urine creatinine is also a common assay used to normalize biomarkers of exposure, toxicity, and efficacy in urine [14]. This requires an accurate and validated creatinine assay rather than clinical lab measurement of creatinine. New biomarkers for metabolism, oncology, muscle mass also require bioanalytical measurement of creatinine in matrices such as urine, saliva, and serum [15-16].

Disadvantages of Conventional Assays for Creatinine

The Jaffe reaction (JCr) to measure creatinine originated in 1886 as one of the first clinical laboratory tests. Though it has evolved over the years, the basic reaction involves the reaction of creatinine with NaOH and picric acid in an alkaline solution to produce a colorimetric compound. The assay became automated in the 1960s into a kinetic assay where development of the chromogen is tracked over time [5]. The simple and cost-effective JCr assay has persisted to this day despite its significant shortcomings.

The primary concern with JCr assays is poor analytical specificity. Many substances commonly found in the serum of patients with many types of pathological conditions falsely elevate or decrease serum creatinine values leading to significant false positives and false negatives of kidney dysfunction. These compounds containing ketone, carboxyl, or amide groups similar to creatinine react with alkaline picrate to generate similar chromo gens. These compounds, summarized in Table 1 produce large changes in readings of creatinine and occur in large populations of patients such as those with liver disease, neonates, and those taking antibiotics. The interferences are especially important because many of the conditions that interfere with the assay, such as diabetes with elevated glucose and acetoacetates, are particularly prone to developing kidney disease [17-18].

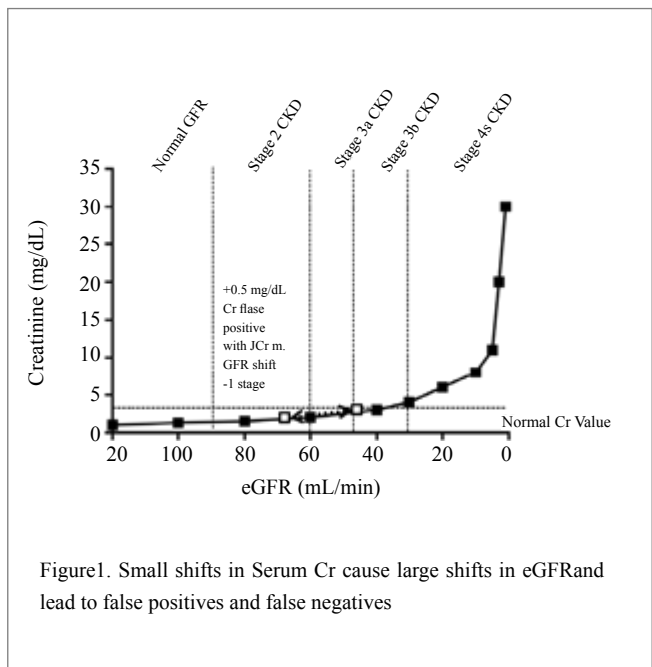| Compound added to serum creatinine (condition)	JCr	Expected Interference (mg/dL)
		ECr
Creatinine		+0.80 (baseline)
Creatine	+0.18	0
Albumin	+0.24	0
Glucose & Acetoacetate (diabetes)	+0.21	0

Compound		
Bilirubin (liver disease)	-0.20	-0.12
Fetal Hemoglobin (hemolyzed samples in neonates)	-0.75	0
Pyruvate (delay in sample processing)	+0.35	0
Vitamin C/ Ascorbate	+0.41	-0.15
Dopamine/ Dobutamine (from IV infusion)	-0.17	-0.68
Cephalosporins (Cefpirome, Cefoxitin, Ceftriaxone)	+0.22 to +2.41	0
IgM (Waldenstrom'smacroglobulinemia)	+0.7	+1.53

Data obtained from [5-27].
Table1. Interfering Compounds in Creatinine Measurement

A primary contributor for interference in JCr assays is cephalosporin antibiotics such as cefoxitin and cephalothin. The reaction product of alkaline picrate with creatinine and cephalosporin antibiotics has an overlapping molar absorptivity producing interferences as large as 213 μmol/L of creatinine in the JCr assay depending on the form of antibiotic used [5-19]. This is especially troublesome in developing countries where it is estimated that a staggering 44-97% of hospitalized patients are given antibiotics [20] and many individuals inappropriately self-medicate with antibiotics [20-21]. While newer forms of cephalosporin antibiotics have lower interference with JCr assays due to changing the equilibrium constants of the reaction with alkaline picrate, enzymatic assays see no interference with antibiotics [19].

It is important to note that even minimal shifts in creatinine results can cause major alterations in eGFR calculations and the number of subjects classified as having different grades of reduced kidney function. As shown in Figure1, creatinine values do not increase past normal range until stage 3 CKD [22]. Therefore, small 0.05 mg/dL shifts in creatinine due to an inaccurate assay can cause an up to 2-stage shift in CKD leading to large numbers of false negatives and positives. For example, Klee et al. showed that a positive shift of 0.23 mg/dL (20μmol/L) creatinine tripled the number of individuals with an eGFR value less than 60 mL/min in a typical outpatient population [9].

Figure1. Small shifts in Serum Cr cause large shifts in eGFRand lead to false positives and false negatives
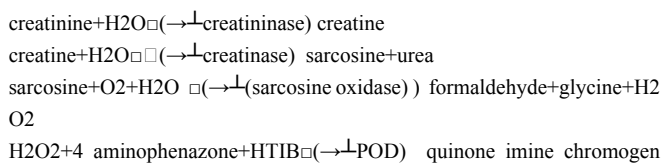
Disadvantages of Modified and Compensated Jcr Methods

To improve the analytical performance of JCr assays, many agencies and vendors have modified the assay and launched efforts to improve and standardize these assays. First, international agencies have led efforts to standardize the reference material used in these assays to higher order creatinine reference material to improve precision of the assay across laboratories around the world. Most vendors have now calibrated their JCr assays to isotype dilution mass spectrometry (IDMS) reference methods [23]. However, IDMS and reference material traceability does not improve specificity of the assay and only ensures consistent precision between laboratories. Vendors have also introduced "compensated" methods to address some of these specificity concerns that adjust calibrator concentrations. For example, a negative offset value can be introduced to compensate for positive interference caused by serum proteins. However this methodology is flawed because it determines an offset from an average interference from correlation studies and assumes that protein interference is constant across samples and linear across the calibration range. It does not account for changes in serum protein and creatinine ratios that occur in children, pregnancy, and the elderly. Methods have also been introduced to correct for bilirubin interference in JCr assays by reacting the bilirubin prior to analysis or "rate-blanking" where a correction factor is determined by reacting the serum with NaOH alone after sample analysis [24-25]. However, this linear correction method does not account for the non-linearity of oxidation of bilirubin and thus does not fully correct for interference.

Advantages of Enzymatic Creatinine Assays

The enzymatic assay (ECr) originated in Australia to address growing concerns of false values from the JCr assays. Creatinine in serum is incubated with creatininase and creatinase to form hydrogen peroxide which can be measured spectrophotometrically as shown in the reaction below [26].

creatinine+H2O□(→⊥creatininase) creatine
creatine+H2O□□(→⊥creatinase) sarcosine+urea
sarcosine+O2+H2O □(→⊥(sarcosine oxidase)) formaldehyde+glycine+H2O2
H2O2+4 aminophenazone+HTIB□(→⊥POD) quinone imine chromogen

This enzymatic cascade adds significant specificity to the formation of the chromogen and removes interference of the majority of compounds and physiological conditions that interfere with JCr assays. Although the enzymatic methods have been reported to have generally fewer interferences than the Jaffe methods, there have been reports of various substances that do interfere such as serum IgM and intravenously infused dopamine/dobutamine [5,27-29]. Nevertheless, the growing body of evidence that ECr assays eliminate interference from many disease groups such as liver disease, diabetes, and bacterial infections supports their suitability for replacement of JCr assays in clinical and drug development settings.

In addition, and largely due to this improved specificity, the chemistry of the ECr assay allows improved accuracy. In a 2007 study, several laboratories determined that ECr had a median 0.1% deviation from nominal creatinine values while JCr assays showed a 16% median deviation from the nominal concentration [30]. This indicates that in situations where accuracy is crucial, such as when making dialysis or transplant decisions, ECr assays must be used when determining GFR. In addition, the deviation was found to be greatest at low concentrations of creatinine where early CKD is indicated (60 mL/min GFR) lending support to the belief that ECr assays should be used especially near the when GFR is expected to be near the 60 mL/min GFR early CKD decision limit or in drug development when detection of the early signs of CKD is crucial.

Finally, with the bioanalysis of any biomarker, increased emphasis is now placed on monitoring and controlling sample handling and collection as biological processes can have major effects on endogenous compounds [31]. To this effect, in addition to increased specificity and precision with ECr assays, the enzymatic reaction significantly improves variability from sample handling and collection [6]. In one study, delays in sample centrifugation built up chromogenic metabolites such as pyruvate and caused false increases in measured creatinine by three JCr assays of on average 50% and significantly changed CKD staging which did not occur affect ECr assays [32].

Disadvantages of ECr Assays

Despite the benefits of ECr, some disadvantages exist for ECr and the measurement of creatinine overall. In spite of the increase in vendors offering ECr assays, the cost is relatively higher than traditional methods ($0.50 per test for ECr vs. $0.10 per sample for JCr).

In addition, the benefits of using the ECr assay is limited primarily at concentrations of creatinine near the 60 mL/min GFR cutoff for kidney impairment where JCr is most inaccurate [8]. In addition, the difference in miscalculation due to use of JCr assays is often less than the biological variability, discussed below, which reduces the ultimate clinical diagnostic miscalculation resulting from using JCr assays [8]. Nonetheless, the accuracy benefits are expected to outweigh the increase in cost, especially in drug development and critical clinical diagnostic situations, because the increase in accuracy is near the critical decision threshold for the first stages of CKD.

Creatinine Biological Variability

The biological variability of creatinine is a result of the dynamics of creatinine production and elimination from the body. Specifically, muscle mass, which varies by age, ethnicity, and gender, significantly alters baseline levels of creatinine. While GFR estimating equations such as the Modification of Diet in Renal Disease (MDRD) equation account for these subgroups, the magnitude of change in muscle mass with these groups varies among populations and does not account for other physiological changes such diet, illness, inflammation, and deconditioning that affect muscle mass. To this effect, regulatory bodies and kidney disease agencies have started evaluating other renal biomarkers such as cystatin C, a small protease produced in all nucleated cells, as an eventual replacement for creatinine measurement [4]. Many comparison studies have shown cystatin C to outperform creatinine as an estimation of GFR and it is beginning to be used in clinical studies [33-34].

Recommendations for use of Creatinine in Clinical and Drug Development Settings

Even with newer biomarkers being validated for determination of GFR, creatinine will continue to be used for the foreseeable future. However, JCrassays continue to be widely used lending uncertainty to patient care,

patient safety, and drug development decisions. Continued education of clinical laboratories and drug development clinical study coordinators is necessary to demonstrate the availability and benefits of the more specific biomarkers for renal function. It is also possible that more nuanced recommendations for specific use cases of each assay will aid in balancing cost and accuracy.

Based on the studies demonstrating that the JCrassay is most inaccurate around the 60 mL/min eGFR decision limit, we propose the following process for determining which assay should be used as shown in Figure2 [8]. For screening of kidney function in an clinical setting the JCr assay can be used initially. If results fall within the 45-60 mL/min, they should be repeated with ECr. Results outside of this zone can be used and reported with the original JCr result. However, for critical decisions where high accuracy is required (e.g. regulatory claim for improved kidney function, normalization or urine biomarkers) the use of ECr is highly recommended.
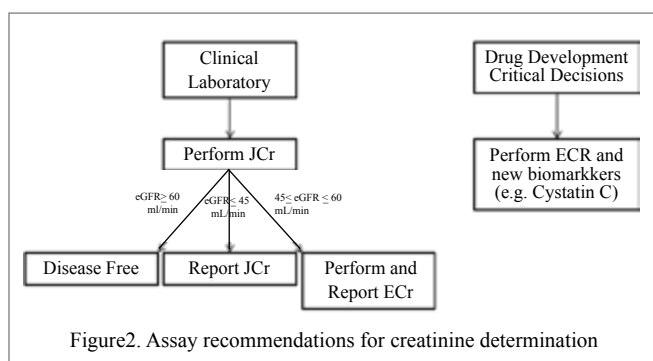

Figure2. Assay recommendations for creatinine determination

A number of alternative analytical methods have been developed for drug development use including GC or LC/MS for measurement of creatinine several matrices including in serum, urine, saliva, and dried blood spots [35] [16][36]. However the ECr assay remains a high throughput and accurate assay applicable to bioanalytical data. Comparison of a BMV validated LC-MS/MS method for serum creatinine with an ECr assay showed reported concentrations were near 1% of the LC-MS method while the JCr assay showed a bias of 12% [37]. In addition, ECr assays can be adapted to fully meet bioanalytical method validation criteria as shown below.

Bioanalytical Considerations to Support Drug Development Studies

Most assays for the measurement of creatinine are run in a clinical laboratory or in a laboratory using the principle of clinical laboratory testing. The primary purpose of a diagnostic assay is to distinguish diseased patients from healthy patients. We find that the assay design implemented to serve diagnostic purposes is not suitable for the measurement of eGFR in drug development, where priority is placed on accuracy across a large quantitation range, precision, and specificity with robust and consistent data across time and laboratories to determine the safety, mechanism of action, and pharmacokinetics.

Therefore, for drug development studies, most ECr assays acquired as research use only or in vitro diagnostic use commercial kits must be adapted to meet FDA bioanalytical validation guidelines and recent guidelines for biomarker validation [38-39]. The major differences in validation approaches used for drug development purposes following the FDA Bioanalytical Guidances vs. clinical lab validation approach is outlined in Table 2 which should be followed for adapting creatinine commercial kits.

To illustrate the concepts in Table 2, we validated an ECr assay using a commercially available kit from Somru BioScience Inc. The kit was developed using the principle of FDA bioanalytical method validation (BMV) guidelines. The method uses eight non-zero calibration curve points as shown in Figure 3. The calibration curve was run using a surrogate matrix, and the QCs were in the intended matrix (urine). The BMV compliant validation demonstrates the appropriateness of the use of the surrogate matrix, showing an acceptable recovery of creatinine during accuracy and precision. Five levels of QCs across the entire range of the assay were utilized for determining accuracy and precision. Accuracy was shown to range from -4.3% to 0% bias, and precision was shown to range from 3.9% to 5.6%. The lower limit of quantitation was reliably shown to be 30μg/mL. Both long-term and short term stability bracketing the time period between samples collection and sample analysis was demonstrated. In addition, incurred sample re-analysis (ISR), as required by FDA BMV guidelines was performed on various studies and results were reproducible for greater than 90% of samples.

Purpose	IVD Biomarker Assay	Drug Development Biomarker Assay
Calibrators/ Standards	Patient care - distinguish healthy from diseased	Safety, mechanism of action, drug dosing
Accuracy & Precision	Often <6 calibrators	>6 calibrators levels
Quality Controls (QCs)	One run per day over 5 days	3-6 runs over multiple days needed
Sensitivity	QCs often in surrogate matrix only	3-5 QC levels recommended including QCs in study matrix
Stability Testing	Only Limit of Detection typically defined	Lower Limit of Quantitation must be evaluated for study samples
Regulatory and Compliance Requirements	Reagent Stability	Analyte stability needed
Purpose	Methods validated following CLIA and CLSI guidelines	FDA BMV 2013 draft guidelines for biomarkers, Critical Path Institute and industry white papers

Table 2. Adaptations for validation of a biomarker assay from a commercial kit

patient safety, and drug development decisions. Continued education of clinical laboratories and drug development clinical study coordinators is necessary to demonstrate the availability and benefits of the more specific biomarkers for renal function. It is also possible that more nuanced recommendations for specific use cases of each assay will aid in balancing cost and accuracy.

Based on the studies demonstrating that the JCrassay is most inaccurate around the 60 mL/min eGFR decision limit, we propose the following process for determining which assay should be used as shown in Figure2 [8]. For screening of kidney function in an clinical setting the JCr assay can be used initially. If results fall within the 45-60 mL/min, they should be repeated with ECr. Results outside of this zone can be used and reported with the original JCr result. However, for critical decisions where high accuracy is required (e.g. regulatory claim for improved kidney function, normalization or urine biomarkers) the use of ECr is highly recommended.
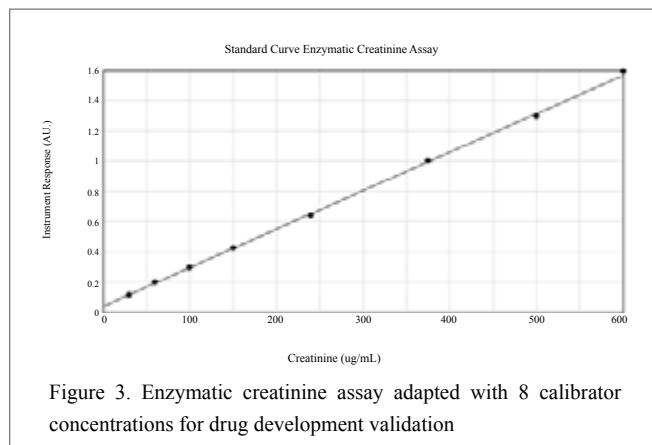

Figure 3. Enzymatic creatinine assay adapted with 8 calibrator concentrations for drug development validation

The data indicates that the validation of a commercial kit for bioanalytical purposes can be achieved. However due diligence must be done and the kit should be chosen carefully. The development of the assay and its historical performance should be carefully considered so that a successful context of use validation can be performed. In addition, use of an ECr method improves assay specificity, accuracy, and analyte stability to meet BMV criteria.

Conclusion

The enzymatic assay for creatinine achieves greater specificity, accuracy, precision, and stability compared to older JCr assays. Since creatinine values do not significantly rise until nearly half of kidney function is absent, small changes in reported creatinine concentrations causes large shifts in GFR calculations and risks missing detection of early kidney disease. The enzymatic assay attains the level of accuracy needed for regulatory and drug development decisions. For clinical lab settings, a two-step protocol using ECr assays when GFR is near the 60 mL/min achieves highest accuracy while managing costs.

Acknowledgements

Executive Summary

Creatinine in drug development. Creatinine assays to measure GFR is typically measured using the picric acid based Jaffe reaction which is subject to interference from many substances commonly found in blood. These assays are not suitable for bioanalytical data.

Small shifts in creatinine cause large changes in calculated GFR which is especially problematic in situations where accurate reporting and tracking of GFR is necessary for therapeutic claims. The enzymatic assay for creatinine does not show interference and leads to improved accuracy. Adaptations of Creatinine Kits for Bioanalytical Data Research Use Only and In Vitro Diagnostic commercial kits that measure creatinine using the enzymatic reaction can be adapted to conform to FDA bioanalytical guidelines.

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