ORIGINAL ARTICLE

Spectrophotometric assay of creatinine in human serum sample

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Abstract   A new spectrophotometric method for the analysis of creatinine concentration in human serum samples is developed. The method explores the oxidation of p-methylamino phenol sulfate (Metol) in the presence of copper sulfate and creatinine which yields an intense violet colored species with maximum absorbance at 530 nm. The calibration graph of creatinine by fixed time assay ranged from 4.4 to 620 μM. Recovery of creatinine in human serum samples varied from 101% to 106%. Limit of detection and limit of quantification were 0.145 μM and 0.487 μM respectively. Sandell's sensitivity was 0.112 μg cm^{-2} and molar absorptivity was 0.101×10^4 L mol^{-1} cm^{-1}. Within day precision was 2.5–4.8% and day-to-day precision range was 3.2–7.8%. The robustness and ruggedness of the method expressed in RSD values ranged from 0.78% to 2.12% and 1.32% to 3.46% respectively, suggesting that the developed method was rugged. This method provides good sensitivity and is comparable to standard Jaffe's method with comparatively less interference from foreign substances.

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1. Introduction

Creatinine a break-down product of creatine phosphate is a nontoxic biomolecule with no significance in biological metabolism. Filtration of creatinine in blood is done by the kidney without any reabsorption. Any significant decrease of the efficiency of filtration by the kidney is identified by an increase in the concentration of creatinine in the blood. Thus, creatinine levels in the blood and urine are used to calculate the creatinine clearance which reflects the glomerular filtration rate (GFR). Estimation of GFR is the most widely used test for renal function in clinical practice (Zamora et al., 2007).

Jaffe's reaction involving the alkaline sodium picrate method is the widely accepted for creatinine measurement (Parvesh et al., 1981). This method lacks in sensitivity, reproducibility, and precision due to endogenous and exogenous interferants (Weber and Zanten, 1991). Several modifications were effected to Jaffe's method to eliminate the drawbacks. These included the specific adsorption of creatinine, removal of interfering compounds, dialysis, varying the pH, and kinetic measurements. But, none of these modifications could successfully eliminate the interferants present in varying concentrations in biological samples.

Jaffe's method has been modified using multienzyme systems to improve the specificity of creatinine determination. Under optimum conditions this enzymatic method gives accurate results but they are less precise and also of high cost. The use of multi-enzyme systems requires caution, as the risk of interference by enzymes increases with the use of more number of enzymes (Thompson et al., 1974; Moss et al., 1975).

There are other methods for the assay of creatinine which include the use of reagents such as 3,5-dinitrobenzoic acid (Langley and Evans, 1936; Benedict and Behre, 1936), 3,5-dinitrobenzoyl chloride (Parekh et al., 1976; Critique et al., 1977), methyl-3,5-dinitrobenzoate in a mixture of dimethyl sulfoxide, methanol, and tetramethyl ammonium hydroxide (Sims and Parekh, 1977), 1,4-naphthoquinone-2-sulfonate (Sullivan and Irreverre, 1958; Cooper and Bigga, 1961; Conn, 1960), Sakaguchi's color reaction of creatinine with o-nitrobenzaldehyde (Pilsum et al., 1956; Pilsum, 1959) and mass fragmentography (Bjorkhem et al., 1977).

We here propose a new spectrophotometric assay method for the estimation of creatinine. The main objective in developing this method was to obtain better sensitivity, more convenience and simultaneously overcoming the need for costly equipment and reducing the time gap. The reagent metol used in this study is readily water soluble, economic and is less toxic when compared to other aromatic reagents reported for the creatinine assay. Metol can be selectively oxidized to yield a chromogenic product (Allinson, 1943). Using metol in the assay simplifies the estimation of creatinine in the human serum sample, as it is a single reagent method. During this experimental study we performed linearity, precision, accuracy, standard deviation and interference tests, applicability of the method is carried out in serum samples and the related data are compared with the routine pathological kit method.

2. Materials and methods

2.1. Apparatus and reagent preparation

A JASCO model UVIDEC – 610 PC spectrophotometer with 1 cm matched quartz cells was used for all measurements. pH of the solution was maintained using chemlabs (Nairobi, Kenya) pH meter. All chemicals used in the assay were of analytical grade. Reagents were freshly prepared using double distilled water and stored in amber colored standard flasks and refrigerated at −4 °C until use. Metol solution (Himedia. Ltd, India) (58 mM) was prepared by dissolving 200 mg in 10 ml distilled water. Copper solution (Himedia) (2.06 mM) as copper (II) sulfate pentahydrate was prepared by dissolving 5 mg in 10 ml of distilled water.

Creatinine was purchased from S.D Fine Laboratory, Mumbai, India and the required concentration was prepared by dissolving in double distilled water. Quality control material (QCM), Lot No. 179800, used as calibrator in Roche/Hitachi Systems used in modified Jaffe's method was purchased from Cobas, UK, and diluted to the required concentration at the time of experimental study. A stock solution of 10 mM acetate buffer of pH 5.4 was prepared and used throughout the study.

2.2. Analytical method development

Different media were investigated to develop a suitable spectrophotometric method for the analysis of creatinine concentration. For selection of media the criteria employed were sensitivity of the method in a particular pH, ease of sample preparation, interference of excipients, cost of reagents and applicability of the method. Absorbance of reaction product in the selected medium at respective wavelength was determined, apparent molar absorptivity; Sandell's sensitivity, robustness and ruggedness were calculated according to the standard formulae

2.3. General procedure for the estimation of creatinine

Calibration graph for the estimation of creatinine was carried out in a final 3 ml reagent mixture containing 1.93 mM Metol, 68.6 μM copper and 1 mM acetic acid/sodium acetate buffer of pH 5.4. The reaction was initiated by adding 100 μL of various concentrations of creatinine. The reaction mixture was allowed to stand for 30 min at room temperature. Absorbance of the colored solution was recorded at 530 nm.

2.4. Precision studies

Precision studies were carried out to evaluate the magnitude of total precision of the proposed method. A final 3 ml solution containing optimized reagent concentration along with varying amounts of creatinine within the linearity range was employed for the precision studies. Three different concentrations of creatinine of 88, 265 and 442 μM were selected to analyze the precision of the proposed method. The study included 10 runs in a day with a time interval of 1 h for within day precision and 20 days run for day-to-day precision with a time interval of 24–25 h. All solutions were prepared freshly every day.

2.5. Sandell's sensitivity (S)

Sandell's sensitivity (Bode, 1991), $S = 10^{-3}/\varepsilon_s = \mu g\, cm^{-2}$

where ε_s is the specific absorptivity and its value (in $mL\, g^{-1}\, cm^{-1}$) corresponds to the determinant in a cuvette with an optical length of 1 cm.

Also, $\varepsilon_s = (\varepsilon/\text{molecular weight of creatinine}) \times 1000$, where ε = molar absorptivity = A/Cd, where C is the molar concentration of the determinant and d = 1 cm path length.

3. Results and discussion

3.1. Absorption spectra

The reagents for measuring absorbance spectra were prepared as described in the general procedure. The absorption spectra

of the reaction were investigated using three different concentrations of creatinine in the wavelength range of 400–700 nm. One absorbance spectra was obtained from the scanning spectrophotometer for the creatinine concentration of 265 μM at a scan rate of 2 nm/s after incubating the reaction mixture for 30 min. The result obtained showing maximum absorbance at 530 nm is shown in Fig. 1. Spectral interference of the reagent was eliminated by measuring the colored solution against a reagent blank containing all reagents except creatinine.

3.2. Temperature sensitivity

Effect of temperature on the sensitivity of the assay was determined by preincubating 1.93, 68.66 and 342.2 μM of metol, copper and creatinine respectively in 1 mM acetate/acetic acid buffer of pH 5.4 at various temperatures (0–60 °C). The absorbance of the colored solution was registered with respect to temperature. There was an almost constant value of absorbance at 530 nm in the temperature range of 20–35 °C. Any further increase in the temperature beyond 35 °C resulted in a decrease in the absorbance due to the color development of the blank, and decrease in temperature below 20 °C resulted in decrease in absorbance due to the slow development of color. The results were analyzed for reproducibility and observed to be reproducible. Hence all analysis were carried out at room temperature (27 ± 5 °C) which was found to be optimum.

3.3. Study of pH effect

The effect of pH on the absorption maxima at 530 nm was studied for solutions containing a fixed concentration of creatinine and prepared as described in the general procedure in the pH range of 3.6–8.0. The different buffers used at 0.1–100 mM included, citric acid/potassium citrate at pH 3.6–5.6, acetate/acetic acid at pH 3.6–5.6, potassium dihydrogen phosphate/

sodium hydroxide at pH 6.0–8.0, and potassium dihydrogen orthophosphate/dipotassium hydrogen orthophosphate at pH 6.0–7.8. Increase in absorbance of the reagent mixture was observed in acetate/acetic acid buffer. The results obtained showed a gradual increase in absorbance of the colored solution by increasing the pH up to 5.4, above this pH showed a decrease in the absorbance up to pH 6 and thereafter the color was not formed. Therefore, total concentration of 1 mM acetate/acetic acid buffer of pH 5.4 was selected as optimum pH for further work. The obtained results are graphically represented in Fig. 2.

3.4. Effect of time

The optimum reaction time was determined by following the color development spectrophotometrically at optimized temperature. It was found that, complete color development was attained after 30 min. Also the color remained stable for 6 h using these reagents. The obtained results are graphically represented in the inset of Fig. 2.

3.5. Effect of reagent concentrations

The effect of varying concentrations of metol and copper was studied under optimum experimental conditions with 3 ml solution by varying the concentration of one at each experiment. The results showed an increase in the absorbance with an increase in each reagent concentration, but it remained constant beyond 1.93 and 68.66 μM for metol and copper respectively. Hence the final optimal concentration was fixed at the same level for all further experiments.

3.6. Robustness and ruggedness

Robustness is described as the reproducibility of the analytical method in different circumstances without the occurrence of
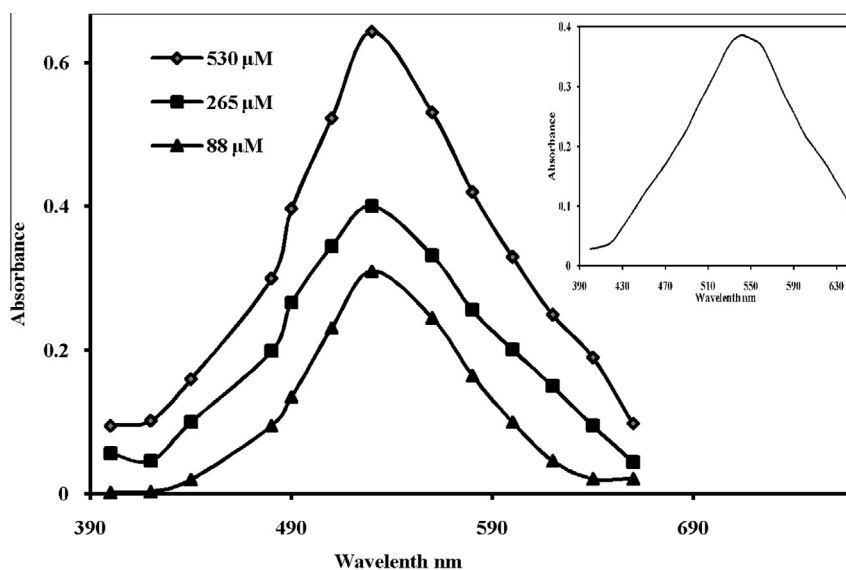
Figure 1 Absorption spectra for three different concentrations of creatinine and the inset shows the absorption spectra obtained by the scanning spectrophotometer for 265 μM concentration of creatinine. The spectrum was recorded at a scan rate of 2 nm/s after incubating the reaction mixture at 30 °C.
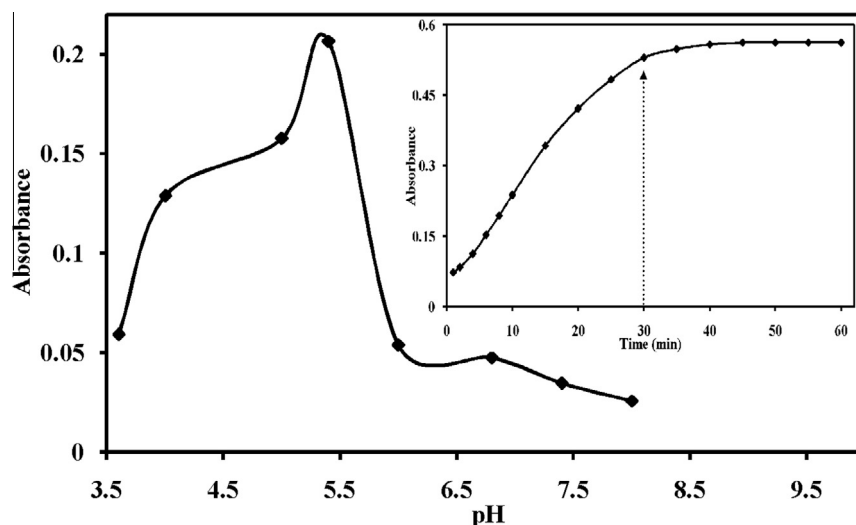
Figure 2 Effect of buffer on the color development and the inset shows the time taken for the stabilization of color.

$y = 0.00101x + 0.05918$
$R^2 = 0.99646$
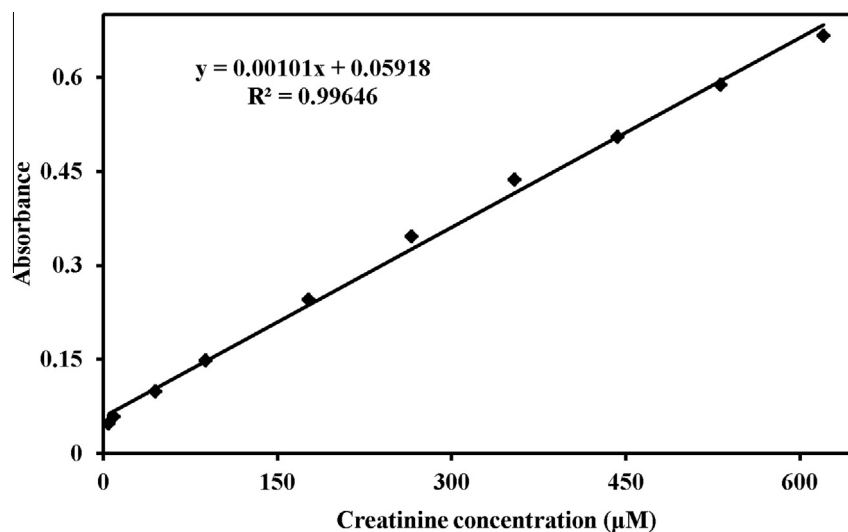
Figure 3 Calibration graph for the estimation of creatinine.

Table 1 Precision studies.

Within day precision[a]				Day-to-day precision[a]			
X μM	SD	% RSD	n	X μM	SD	% RSD	n
88	0.0072	4.85	10	88	0.011	7.83	20
265	0.0115	3.01	10	265	0.016	4.45	20
442	0.0149	2.49	10	442	0.019	3.25	20

Note: n = number of runs; SD = standard deviation; %RSD = percentage relative standard deviation; X = creatinine concentration.
  [a] Duplicate measurements.

unexpected differences in the obtained result(s) and ruggedness is defined as the degree of reproducibility of the test results obtained under a variety of normal test conditions (Heyden et al., 2001). For the evaluation of the method robustness, an important experimental variable, reaction time was slightly varied deliberately. The analysis was performed at ±2 min of the optimum reaction time by taking three different concentrations of creatinine and found to remain unaffected as shown by the RSD values in the range of 0.78–2.12%. Method ruggedness was expressed as the RSD of the same procedure applied by four different analysts as well as using three different instruments. The inter-analysts RSD were within

Table 2 Study of t-test and F-test by the proposed and standard methods in human serum samples.

Sample number	Creatinine concentration* (μM)		t-Test	F-test
	Proposed method	Standard method		
1	64.98	59.15	1.38	1.24
2	73.72	71.68	0.93	0.89
3	75.37	72.32	1.09	2.92
4	75.97	76.70	0.61	2.36
5	152.02	144.78	1.03	0.99

Tabulated t-value for 10 df at $P(0.95)$ is 2.228; Tabulated F-value for (5,5) df at $P(0.95)$ is 5.05.
* Average of six measurements.

Table 3 Interference studies.

Interferants	Tolerance ratio[a]
Nickel, Calcium, Iron $+2, +3$, Phosphate	15
Nitrite, Amoxycyllin	30
Hemoglobin, Ascorbic acid	35
Ammonia	70
Bicarbonate, Uric acid	190
EDTA	200
Glucose	310
Nitrate, Sodium, Potassium	380
Magnesium, Aluminum	510
Sulfamic acid	950
Carbonate	980
Chloride, Creatine	1650
Urea	39,176

[a] Note: Tolerance ratio corresponds to the ratio of the limit of inhibiting species concentration to that of 176 μM creatinine used.

was recorded and plotted against the concentration of creatinine as shown in Fig. 3. Absorbance of the colored solution increased with an increase in the concentration of creatinine. The calibration graph of creatinine showed excellent linearity over the concentration range of 4.4–620 μM. The linearity graph yielded a regression equation as, absorbance $= 0.001 C_{cr} + 0.059$, $R^2 = 0.996$. The molar absorptivity and Sandell's sensitivity were 0.101×10^4 L mol^{-1} cm^{-1} and 0.112 μg cm^{-2}, respectively and the RSD was 0.0301% ($n = 10$). Limit of detection and limit of quantification were 0.145 μM and 0.487 μM, respectively. Precision studies were carried out by analyzing solutions containing known amounts of creatinine within the Beer's law range and the results are shown in Table 1. Within day precision range was 2.5–4.8% ($n = 10$) and the day-to-day precision range was 3.2–7.8% ($n = 20$). Student's t-test and F-test were carried out for the estimation of creatinine by the proposed and reference methods. For all t tests and F tests, significances were evaluated at $P < 0.05$ probability. The F test and Student's t test values confirmed that the 'null hypothesis' was valid at the 95% confidence level and that there were no significant differences between the precisions (variances) of the proposed and reference methods. The results are tabulated in Table 2.

0.92% whereas the inter-instruments RSD for the same creatinine concentrations ranged from 1.32% to 3.46% suggesting that the developed method was rugged.

3.7. Analytical parameters

The calibration graph for creatinine was carried out by the fixed time assay method in 3 ml of solution containing 1.9 mM metol, and 68.6 μM copper in 1 mM acetic acid/sodium acetate buffer of pH 5.4. Varying concentrations of creatinine were added and allowed to stand for 30 min at optimized temperature. The absorbance of the colored product

3.8. Interference studies

Interference study was performed to assess selectivity of the method for creatinine. Spectral and chemical interferences may occur because of reagents and matrix effects during the analytical procedure. Selected potential interference compounds were chosen according to matrix composition and added reagents. Known quantities of many chemical species

Table 4 Recovery of creatinine from the human serum sample by the proposed method.

Sample number	Creatinine μM		Added μM	Found μM	Recovered μM	Recovery[b]%
	Proposed method[a]	Standard method				
1	52.1	54.9	38	92.4	40.3	106
			305	373	320.9	105.2
2	71.8	75.0	76	156.7	84.9	111.7
			190.6	265.1	193.3	101.4
3	111.5	114.1	38	152.2	40.7	107.1
			228.8	351.3	239.8	104.8

[a] Mean of duplicate measurements.
[b] [(Final concentration−initial concentration)/added concentration] × 100.
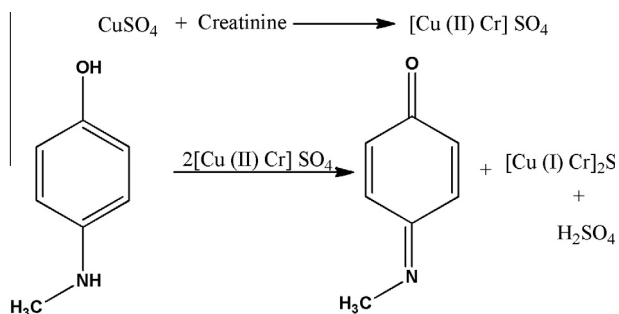
Scheme 1   Probable reaction mechanism.

were added to the solution containing 176 μM of creatinine. A deviation of ±3% of the original value in the absorbance reading was considered tolerable. The resultant tolerance ratio is summarized in Table 3. It can be clearly observed that some compounds like creatine, glucose, ammonia, nitrate, urea and common inorganic ions showed a reliable tolerance under the given conditions, whereas hemoglobin, ascorbic acid, Fe (II), (III), nitrite and some drugs showed a low tolerance.

3.9. Recovery and applicability

Recovery-experiments were performed in order to prove the specificity and the accuracy of our method in pathological creatinine concentration ranges. Serum samples previously analyzed by modified jaffe's kit method in the range of 54.9–114.1 μM were analyzed by the proposed method. The obtained results indicated a linear correlation with the diagnostic kit method. Analytical recovery was evaluated by adding stock creatinine solution to serum samples and analyzing the concentration recovered. The mean analytical recovery was 106%. There was minimal interference and good reproducibility of the assay procedure with the creatinine recovery of 101.4–111.7% in the serum sample. The results obtained are summarized in Table 4.

4. Probable reaction mechanism

In the proposed reaction, copper sulfate forms a complex with creatinine giving [copper(II)creatinine]sulfate (Birdsall and Weber, 1990), This [copper(II)creatinine]sulfate selectively oxidizes the metol leading to the formation of the imine group which provides an intense purple colored product, during which [copper(II)creatinine]sulfate gets reduced to [copper(I)creatinine]sulfate. The amount of [copper(II)creatinine]sulfate reduced to [copper(I)creatinine]sulfate is directly proportional to the amount of imine groups formed. The probable reaction mechanism is as shown in Scheme 1.

5. Conclusion

Estimation of creatinine by direct spectrophotometric procedure has been attempted. In the optimization of different parameters for the proposed method, the reaction pH and the concentration of all components were taken into account; optimal conditions were considered to be those affording maximum absorbance, greatest possible color stability and

maximum linearity. The problems encountered in setting up the procedure involved several aspects of the reaction, namely, the assay of creatinine, test sensitivity, and competition by some interfering substances with the chromogenic system, reflecting the relative non specificity of the proposed system.

This study is the first to report on the estimation of creatinine by the oxidation of metol in the presence of copper and creatinine. The reagents used for the assay are versatile and economical, the oxidized product has maximum absorbance wavelength in the visible region. The reaction can be carried out at room temperature. Buffer studies helped to attain the best absorbance with selective assay thereby avoiding the addition of strong base or acids which results in multiple interferences caused by precipitation.

The oxidation of metol in the presence of copper and creatinine allowed the spectrophotometric determination of creatinine within the range of 4.4–620 μM. Fixed time assay provides the ease of manual analysis with negligible errors. Interference by foreign substances is negligible when compared to other spectrophotometric methods. The LOD and LOQ data indicated that microgram quantities of creatinine can be accurately determined. The applicability of the proposed method was investigated through the recovery test in serum samples by adding known concentrations of the analyte. The recovery values showed good reproducibility, accuracy, independent of matrix effects, and is not affected by the sample matrix. These outcomes of the assay show that the proposed method is suitable for the routine estimation of creatinine in the serum sample. Results of within day and day to day precision indicate that the method is highly reproducible. The ease in reagent preparation and relatively low instrumentation cost make the proposed method potentially feasible and more attractive.

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