Assay of Urea with *p*-Dimethylaminobenzaldehyde

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Summary: *p*-Dimethylaminobenzaldehyde and urea in acid media give yellow colouration. The chromogen responsible for the yellow colour is Schiff-base(I). The structure of chromogen is illustrated with chemical and spectroscopic means. The yellow colour absorbs at λ_{max} 420 nm. The fluctuation of yellow colour absorbance depends upon the strength of acid. Several mineral acids and their mixtures with different concentrations in methanol have been tried as reaction media, but, the CH_3OH-H_2SO_4 mixture appears to be the best and yield good results. The optimum amount of H_2SO_4 (36N) 0.2 – 0,3 ml in CH_3OH is found to be the best reaction media where reactants and products do not get precipitated. In it, the yellow colour remains stable and is used as an analytical tool for the determination of urea present in biological fluids. The % age of urea is calculated using *p*-dimethylaminobenzaldehyde, diacetylemono-oxime and urease (enzymatic) methods.

Introduction

Urea is one of the end products of the reactions that are occurring in a human body. It is also a chief nitrogen containing end product of protein metabolism. For diagnosis of various diseases, connected with variation of urea level in blood and urine serum, exact determination of urea in biological fluids is required. Early method [1-5] for urea estimation rely on the specific hydrolysis of urea to ammonia and carbon dioxide by the enzyme urease, produced in moulds, bacteria and plants (i.e Jack bean) followed by measurement of one of these end-products by nesslerisation or by the Berthelot reaction. The optimum parameters in the Berthelot reaction have been suggested by various workers [5-10]. More recently it has been superceded by the glutamate dehydrogenase method, currently one of the most commonly used urea method.

However, disadvantages in the urease method arise from the facts that ammonia from other sources may already be present in plasma, and that urease is subjected to inhibition or inactivation. The presence of fluoride ions in serum further aggravate analytical procedure.

Direct chemical estimation is relatively new and is rather less specific than the enzymic one. Urea may be measured without conversion to ammonia with some α-diketones and other chemicals. They yield various colorations with urea. These colours have been used for the colorimetric determination of urea concentration in biological fluids for the last many year [10-12]. Of three α-diketones, diacetyl (butane-2,3-dione) has been extensively used and is often preferred to the urease method.

As stated above, the analytical procedure for the determination of urea in biological fluids depends upon the colour that forms and the nature of the coloured products has remained a mystery for the last many years. Recently, we have been involved in the investigation of the reactions of urea with α-diketones. Also the merits and demerits of the chemicals that are being used in hospital and private linical laboratories have been studies [13-16].

Several workers [17-33] made use of p-dimethylaminobenzaldehyde as a diagnostic reagent for the estimation of urea and other compounds present in biological as well as non-biological fluids and compared results with diacetylmono-oxime and Berthelot methods. They could not generalise analytical procedure. Moreover, they were unable to determine the exact parameters and role of interfering substances involved in this reaction.

Recently [17], we have isolated all possible chromogenes resulting from the reactions of *p*-dimethylaminobenzaldehyde with urea, thiourea and their alkyl/aryl derivatives. This reaction appears to be acid sensitive and the yellow colour results from the reaction of *p*-dimethylaminobenzaldehyde with urea absorbs at λ_{max} 420 nm and obeys Beer's law at a definite pH. We have used this yellow colour as an analytical tool for the estimation of urea present in urine serum. The results seem to be very encourging

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when compared with diacetytmono-oxime and urease methods.

Results and Discussion

When equimolar quantities of urea and aldehyde suspended in benzene containing TFA were stirred at room temperature, a yellow solid was obtained. It showed molecular ion peak at 191 and elemental analysis corresponded to an emperical formula of $C_{10}H_{13}N_3O$. In proton NMR spectra it gave two doublets at δ 6.83 and 7.73 ppm. They were assigned to protons Ha and Hb. The amide NH and CH = N- protons absorbed at δ 8.72 and 9.74 ppm respectively. The integration ratio of absorption peaks well matched with the number of protons present in the compound (**1**). Hence, we suggested structure (**1**) to $C_{10}H_{13}N_3O$. It is soluble in water and imparts yellow colour. In UV/Visible spectra it absorbed at λ_{max} 420 nm. The yeliow colour disappeared when treated with an acid or base. It also decolourized $KMnO_4$ and bromine water suggesting the presence of C = N in the compound. Moreover, its infrared spectra confirmed the presence of C=O and C=N groups in compound (**1**). After establishing the structure of chromogen responsible for yellow colour, we have diverted our attention to find the exact parameters of colour-development and intends to use the colour as an analystical tool for the estimation of urea present in biological fluids. Honsey and Finney [27] have used *p*-dimethylamino-benzaldehdye for the determinaion of urea in acid media, but they could not reach at final conclusion. We have tried our best to find out the optimum parameters of this reaction, where the yellow colour should remain stable, sensititve and specific. We have discovered that the yellow colour is highly sensitive and fluctuates with acid strength. It should be possible that degree of protonation of the chromogene is the cause of absorbance fluctuation. In addition, we have also focused our attention to study the pH of the reaction media where the yellow colour remains stable and helps in shifting the equilibria from left to right. We believe that actual chromogen which is responsible for the yellow colour is Schiff-base (**1**). The formation of the Schiff-base in acidic media depends upon the acid strength. A reacion mechanism as shown in Scheme-1 is suggested.

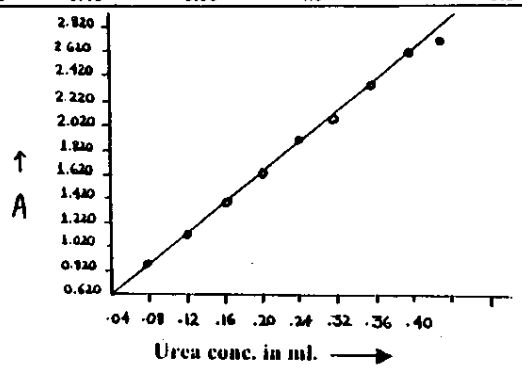
Schiff-base with quinoid structure (**1a**) has light obsorbing property and it absorbs at λ_{max} 420 nm. The decrease and increase in the absorption intensity depend upon the quantity of compound (**I**) and amount of the acid used. Greater the formation of compound (**I**), greater the absorption intensity. We have tried CH_3OH - HCl, CH_3OH - H_3PO_4, CH_3OH - - H_2SO_4 - H_3PO_4 and CH_2OH N_2SO_4 mixtures in the development of yellow colour. The CH_3OH - HSO_4 mixture appears to be the best reaction media which we report here. In the study reactants and products remain in solution and do not get precipitated at any stage. The absorbance of standard solutions of urea are recorded using reference solutions as given in Tables (I-III). In these experiments we have used CH_3OH – H_2SO_4 and *Blank solutions (without urea)* as references solutions. In this way, the absorbance of yellow colour if at any stage, produced by *p*-dimethyl aminobenzaldehyde in the absence of urea is itself electronically subtracted. *p*-Dimethylamino-benzal-dehyde in methanol absorbs at λ_{max} 320 nm and in acid media its absorbance is further suppressed. Hence, the fear of its interference with yellow colour (λ_{max} 420 nm) solution resulting from urea and *p*-DMAB is itself eliminated.

We have focused our attention to find a suitable amount of H_2SO_4 which can be used in urea *p*-DMAB reaction. It is observed that as the concentration of sulphuric acid is increased, the abosrbances of the resulting chromogen are decreased. Two to ten ml H_2SO_4 (36N) in 70 ml methanol has been used. The pH of the reaction found to be below zero. In these cases, the absorbances range is found to be 0.519 to 0.909. It reflects that chromogen (**I**) which is responsible for the yellow colour is heavily protonated resulting in the depression of the absorbances in each case. Contrarily, when H_2SO_4 ranging from 1.0 ml to 0.25 ml in 70 ml CH_3OH is used, the absorbances of yellow solution are considerably increased, i.e., from 1.725 to 2.832. Thereafter, when the amount of H_2SO_4 ranging from 0.2 to 0.05 ml in 70 ml CH_3OH is used, the absorbances of yellow colour starts decreasing.

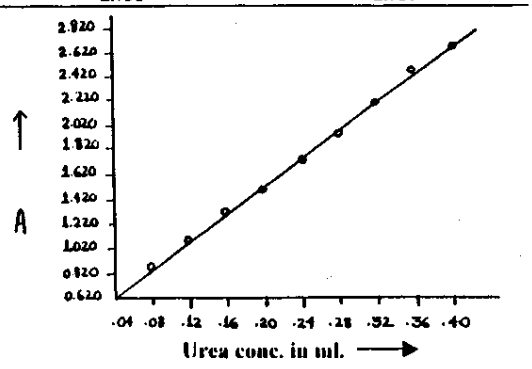
After thorough studies of the reacion of *p*-dimethylaminobenzaldehyde and urea, it has seen that 0.2 to 0.3 ml H_3SO_4 (36N) in 70 ml CH_3OH is a suitable quantity where reliable results can be achieved. Moreover, a brief look at Tables (I-III) reflects that 0.25 ml H_2SO_4 in 70 ml H_2SO_4 is the most favourable amount for the reaction to proceed where the protonation of the reactants and reacton products are productive, do not precipitate and exist in a very congenial environment. All the absorbance points in Graphs (I-III) fall in a straight line but the Graph II appears to be the best and reliable as compare to other graphs.

Table 1: Absorbances using CH_3OH-H_2SO (70:0.2ml v/v) solution at 420 nm.

Sample No.	Vol. of urea soln. 1 ml =10 mg (ml)	Vol. of water (ml)	Vol. of *p*-Dimethyl-amino-benzaldehyde 1 ml = 20 mg (ml)	Volume of CH_3OH-H_2SO_4 soln. (70:0,2 v/v) (ml)	Absorbances after heating for 20 minutes At 50°C Reference	
					CH_3OH-H_2SO_4 (70:0.2 v/v)	Blank solution
1	0.04	0.36	4.0	5.6	0.623	0.620
2	0.08	0.32	4.0	5.6	0.93	0.921
3	0.12	0.28	4.0	5.6	1.141	1.160
4	0.16	0.24	4.0	5.6	1.356	1.388
5	0.20	0.20	4.0	5.6	1.613	1.551
6	0.24	0.16	4.0	5.6	1.818	1.758
7	0.28	0-12	4.0	5.6	2.135	2.020
8	0.32	0.08	4.0	5.6	2.273	2.220
9	0.36	0.04	4.0	5.6	2.560	2.514
10	0.40	0.00	4.0	5.6	2.731	2.710



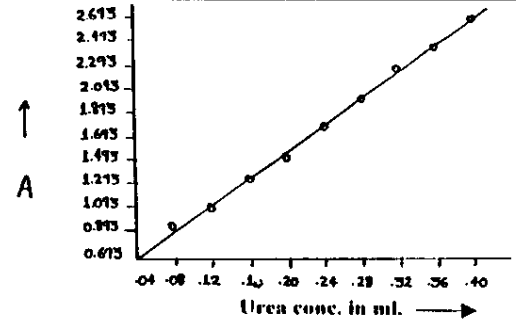
Graph IA: Asborbances using CH_3OH-H_2SO_4 (70:0.2 V/V) soln. References: CH_3OH-H_2SO_4 soln



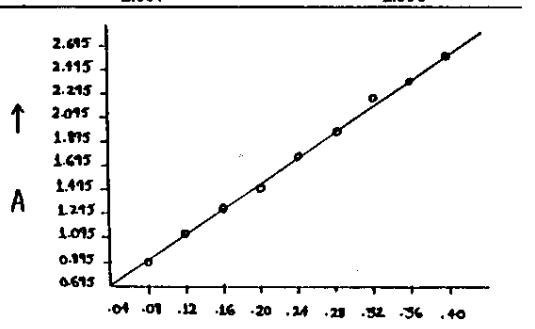
Graph IB: Asborbances using CH_3OH-H_2SO_4 (70:0.2 V/V) soln. References: Blank soln

Table II Absorbances using CH_2OH-H_2SO_4 (70 : 0.25ml v/v) solution at 420 nm.

Sample No.	Vol. of urea soln. 1 ml= 10 mg (ml)	Vol. of water (ml)	Vol. of *p*-Dimethylamino-benzaldehyde 1 ml = 20 mg (ml)	Volume of CH_3OH-H_2SO_4 soln. (70:0.2 v/v) (ml)	Absorbances after heating for 20 minutes at 50°C Reference	
					CH_3OH-H_2SO_4 (70:0.2 v/v)	Blank solution
1	0.04	0.36	4.0	5.6	0.693	0.695
2	0.08	0.32	4.0	5.6	0.955	0.924
3	0.12	0-28	4.0	5.6	1.112	1.114
4	0.16	0.24	4.0	5.6	1.324	1.311
5	0.20	0.20	4.0	5.6	1.457	1.472
6	0.24	0.16	4.0	5.6	1.754	1.756
7	0.28	0.12	4.0	5.6	2.951	1.945
8	0.32	0.08	4.0	5.6	2.253	2.231
9	0.36	0.04	4.0	5.6	2.398	2.332
10	0.40	0.00	4.0	5.6	2.607	2.550



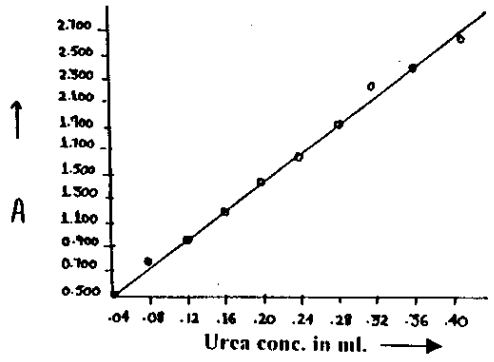
Graph IIA: Asborbances using CH_3OH-H_2SO_4 (70:0.25 V/V) soln. References: CH_3OH-H_2SO_4 soln



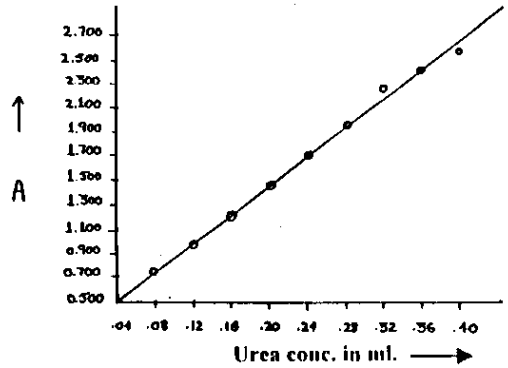
Graph IIB: Asborbances using CH_3OH-H_2SO_4 (70:0.2 V/V) soln. References: Blank soln

Table-III: Absorbances using CH_3OH-H_2SO_4 (70:0.3 ml v/v) solution at 420 nm

Sample No.	Vol. of urea soln. 1 ml=10 mg (ml)	Vol. of water (ml)	Vol. of p-Dimethyl-amino-benzaldehyde 1 ml = 20 mg(ml)	Volume of CH_3OH-H_2SO_4 soln. (70:0.2 v/v) (ml)	Absorbances After Heating for 20 Minutes At 50°C Reference	
					CH_3OH-H_2SO_4 (70:0.2 v/v)	Blank solution
1	0.04	0.36	4.0	5.6	0.501	0.500
2	0.08	0.32	4.0	5.6	0.838	0.720
3	0.12	0.28	4.0	5.6	1.013	1.000
4	0.16	0.24	4.0	5.6	1.321	1.301
5	0.20	0.20	4.0	5.6	1.540	1.562
6	0.24	0.16	4.0	5.6	1.732	1.803
7	0.28	0.12	4.0	5.6	2.070	1-069
8	0.32	0,08	4.0	5.6	2.440	2.409
9	0.36	0.04	4.0	5.6	2.567	2.549
10	0.40	0.00	4.0	5.6	2.711	2.598



Graph IIIA: Asborbances using CH_3OH-H_2SO_4 (70:3 V/V) soln. References: CH_3OH-H_2SO_4 soln



Graph IIIB: Asborbances using CH_3OH-H_2SO_4 (70:0.3 V/V) soln. References: Blank soln

In addition, it was also found that time is needed for the reacion to complete. The absorbances of the yellow colour after immediate mixing of *p*-DMAB and urea do not yield a straight line. On the other hand, when the yellow solution was heated for 20 minutes at 50°C, all the absorbances points come up in one line. Hence, it was concluded that this reaction is not instantaneous but needs time for its completion. In the present work we used our findings in the determinaton of urea present in biologuical samples. In this regard, patient samples sustaining various diseases were examined and compared with other existing methods.

Diacetylmono-oxime (DAM) and urease (enzymatic) methods [1-4,10] are being widely used in clinical laboratories for the estimation of urea. The urea concentrations obtained by *p*-dimethylamino-benzaldehyde are found to be high and in some cases low as compare to DAM and urease methods. But, in some cases they are very close to each other. (Table IV). The chemistry of DAM method has been thoroughly discovered by Butler and Hussain [14,16]. Keeping in view the background of DAM and urease methods one can say that *p*-dimethylamino-benzaldehyde method seems to be a better method.

It was also found that *p*-dimethylamino-benzaldehyde did not react with any of commonly biological substances present in urine except those which contain free NH_2 group. It is reported [4, 27] that tryptophane, indoles, kynurenine, citrulline, indoxyl sulfate, phorphobilinogen and urobilinogen impart coloration with *p*-dimethylaminobenzaldehyde and absorb at different wavelengths as compared to *p*-dimethytaminobenzaldehyde-urea reaction.

It is reported that urobilinogen does not have free amino (NH_2) group, but gives red coloration with *p*-dimethylaminobenzaldehyde [4]. The red color absorbs at λ_{max} 530 and this technique is being used for the determination of urobilinogen present in blood. The chemistry of this reaction appears to be

Table-IV: Comparison of results using patient's urine samples sustainning various diseases

Sample Nos.	AMOUNT OF UREA (mg/dl)			
	p-DMAB Method	Dam Method	Urease Method	Disease
1	1013	1050	1440	Right Ureter caculus
2	2125	2040	1985	B.P.H.
3	250	300	220	Haematuria
4	256	225	140	Chronic renal failure
5	400	340	280	Renal calculi
6	260	206	280	Obstructive Uropathy
7	675	752	680	B.P.H.
8	2938	2365	3540	B.P.H.
9	250	352	220	Bladder Outlet obstruction
10	275	254	280	B.P.H,
11	400	434	540	Renal calculi
12	1425	1622	1420	Renal Stone
13	1320	1280	1200	Renal stone
14	2379	2264	1480	B.P.H.
15	2250	2066	1560	Left Ureter Obstruction
16	1087	920	1080	Renal Calculi
17	300	265	220	B.P.H.
18	838	875	900	Renal Stone
19	800	780	1000	Visicle Calculi
20	840	800	1060	Renal Stone
21	925	880	1000	B.P.H.
22	250	240	220	Left Ureter Obstruction
23	1900	1325	1980	Rental Stone
24	380	186	300	Chronic renal failure
25	1850	1850	1820	B.P.H.
26	120	117	140	Renal Calculi
27	140	150	120	Haematuna
28	256	150	300	Renal Calculi
29	2786	2792	2660	Renal Calculi
30	975	1540	1000	B.P.H.
31	350	385	340	Haematuna
32	630	640	580	Right Ureter Obstruction
33	250	344	200	Renal Calculi
34	2150	2140	2320	B.P.H.
35	2610	2720	2525	Renal Calculi
36	1400	1428	1580	Tramantic Urethra Obstruction
37	300	265	210	Carcinoma Urinary Bladder
38	850	875	900	B.P.H.+ Renal failure
39	300	328	340	B.P.H.
40	4825	4975	5325	B.P.H.
41	263	250	300	Chronic Renal failure
42	375	360	400	Stricture Urethra

ambigous. It is feared that urobilinogen as well as porphobilinogen may not interfere in the determination of blood urea although the reactions products of urea-dimethylaminobenzaldehyde and urobilinogen-*p*-dimethylaminobenzaldehyde absorb at different λ_{max}. i.e. 420 nm and 530 nm. Some drugs contain free NH_2 group or aminoderivatives. The effects of unconsumed medicines and their break-down products present in biological fluids are to be examined. Here, initially results are reported and it is intended to apply the findings extensively on biological fluids of patients suffering from various kinds of diseases taking different kinds of medicines.

Experimental

General Procedure for the Isolation of Chromogen

p-Dimethylaminobenzatdehyde (0.02M) and urea (0.02M) were suspended in benzene (50 ml) containing TFA (3 ml). This mixture was stirred at room temperature for 3 hrs. A yellow solid settled down. Benzene was removed by decantation and the remaining solid washed with benzene, ether, cold water, acetone and finally, with ether to give *p*-dimethylaminobenzaldehyde -- urea product (I).

(yield, 80%), m.p 155°C; Mass: M/z 191 (M^+)

IR (KBr): 3744, 3356 cm^{-1} (NH_2), 1751cm^{-1} (C=O), 1665 cm^{-1} (C=N), ^{1}H-NMR (DMSO-d_6): δ 3.1 ($N(CH_3)_2$ s), 6.83(2Ha, J = 9Hz, d), 7.73 (2Hb J = 9Hz, d), 8.72 (NH_2, broad), 9.74 (CH=N, s). Found: C, 62.86; H, 6.87; N, 22.04.$C_{10}H_{13}N_3O$ requires C, 62.84; H, 6.86; N, 21.99%.

Preparation of Stock Solution.

a) Urea

Anhydrous Urea (1.0 g) was dissolved in distilled water and made up the volume 100 ml.

1 ml of soln. = 10 mg. urea.

b) p-Dimethylaminobenzaldenvde: (DMAB) 2.0 g was dissolved in methanol and diluted to 100 ml.

1 ml of soln. = 20 mg. of *p*-DMAB.

c) Methanol-H_2SO_4

Methanol (70 ml) was taken in a beaker and different volumes of acid (H_2SO_4, 36N) ranging from 0.05 to 10 ml were added. The whole mixture was thoroughly stirred and pH of the solution in each case was noted.

d) Blank Solution

In a 10 ml measuring flask, distilled water (0.4 ml) and *p*-dimethylaminobenzalehyde (4 ml=80 mg) were added. The volume was then made upto the mark with methanol-H_2SO_4 solution.

Absorbance Recording Procedure

Ten measuring flask of each 10 ml capacity were taken. Varying quantities of urea ranging from

0.4 to 4.0 mg. were added in each flask. Keeping in view that each flask should contain 0.4 ml water, an additional amount of water was adjusted in such a fashion that each flask must contain 0.4 ml water. Constant quantities of *p*-dimethylaminobenzaldehyde (4 ml = 80 mg) were added in each flask. The way of addition of chemicals is shown in Tables (I-III). The volume of each flask was made upto the mark by adding Methanol - H_2SO_4 solutions. Yellow colour developed in each flask. The absorbances of these solutions were noted at λ_{max} 420 nm before and after heating at 50°C for 20 minutes using references methanol-acid and blank solutions.

Preparation of Calibration Curves Using Different Quantities of Methanol-H_2SO_4 Solutions.

The way of mxing chemicals was shown in their respective Tables (1-111). The absorbances of yellow solution were rccorded using methanol - H_2SO_4 and blank solutions as reference solvents, at interval time of 30, 60 and 90 minutes. The absorbances were also noted after heating at 50°C, for 20 minutes. Graphs were drawn using absorbances against urea concentration,

Determination of Urea Present in Biological Samples by p-Dimethylaminobenzaldehvde.

First of all, urine samples were centrifuged and 1.0 ml of supernatant liquid was taken and diluted with 1.5 ml distilled water. Total volume of urine solution became *2.5 ml* Now, 0.4 ml of urine solution was mixed with 4.0 ml stock solution of *p*-dimethylaminobenzatdehyde in a measuring flask of 10 ml capacity. The volume was made upto the mark with CH_3OH-H_2SO_4 (70:0.25 v/v) solution. Absorbance of yellow colour solution was recorded at λ_{max} 420 nm after heating 20 minutes at 50°C using blank solution as reference solvent. The percentage of urea amount was calculated using standard graph IIB.

Calculations

2.5 ml urine solution contains = 1.0 ml urine sample,

0.4 ml urine solution contains = 0.4/2.5= 0.16 ml urine sample

Urea concentration in urine solution. taken from graph = x mg urea. Therefore, 0.16 ml urine sample contains = x mg urea

100 ml urine sample contains x/0.16 x 100 = y% mg urea.

All other samples were treated in the same manner and results were reported in Table (IV).

Determination of Urea Present in Biological Samples using Diacetylmono-oxime Method.

MERCK KIT for determination of urea and directions given in this regard were used.

Three test tubes were marked as T_1, T_2 and T_3. In each test tube distilled water (3.3 ml) was added. To test tube T_1 sample (0.1 ml) and to test tube T_2 standard solution (0.1 ml, 40 mg/100) were added. Now in all test tubes sodium tungstate solution (0.3 ml, 10% and sulphuric acid 0.4 ml, 2/3 N) were added and mixed thoroughly. They were centrifuged at 3000 rpm for 5 minutes.

T_1 = Test solution

T_2 = Standard Solution

T_3 = Blank solution

One ml of supernatant liquid from each test tube, T_1, T_2 and T_3 was withdrawn and mixed with distilled water (1.0 ml, acid mixture (1.6 ml, H_2SO_4-H_3PO_4-H_2O) and diacetylmono-oxime (0.4 ml, 2% in acetic acid.) Heated the test tubes for half an hour. Yellow colour developed in all cases.

Absorbance were recorded at 480 nm using blank solution as reference. The amount of urea was calculated using the equation:

Concentration of urea = T/S x cone. of standard= urea in mg/100

T= Test solution absorbance

S = Standard solution absorbance

All other urine samples were treated in the same manner and their results are recorded in Table No.(IV).

Determination of Urea Present in Biological Samples Using Enzymatic (Urease) Method

Medical kit and directions given in this regard for the determinaton of urea are followed.

Principle

This method is based on the following reaction:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

Salicylate and hypochlorite in the reagent react with the ammonium ions to form a green complex (2,2 dicarboxylindophenol).

Reagents

A.	Urease	50,000 µ/lit
B	Phosphate buffer	120 m mol/lit, pH = 7.0
	Sodium salicylate	62.5 m mol/lit
	Sodium nitroprusside	5.0 m mol/lit
	EDTA	1.48m mol/lit
C.	Sodium hypochlorite	18 m mol/lit
	Sodium hydroxide	450 m mol/lit
	Standard	50 mg/100
	Urine sample	distilled water (1:100)

Procedure

First of all, the reagent "B" was dissolved in 100 ml water and reagent "A" was mixed in it. It was called "working reagent".

Three test tubes were marked as T_1, T_2 and T_3. In test tube T_1 urine solution (10 µL) and in test tube T_2 standard solution (10 µL) were added, In each test tube "working reagent" (1.0 ml) was added. Incubated at 37°C for 3 minutes.

T_1 = Test solution

T_2 = Standard solution

T_3 = Blank solution

In each test tube reagent "C" (200 µL) was added. Mixed thoroughly and incubated at 37°C for 5 minutes. Bluish green colour developed in all cases. Absorbances were recorded at λ_{max} 600 nm, using blank solution as reference. The amount of urea was calculated as:

$$\text{Concentration of urea (mg/100ml)} = \frac{\text{Absorbance of Test soln.} \times 50}{\text{Absorbance of standard soln.}}$$

Note: All other urine samples were treated in the same manner and their results are recorded in Table-IV.

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