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Simple and Accurate Determination of Urinary Glucose Excretion with Anthrone Reagent

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Summary. The use of anthrone reagent for the quantitative determination of glucose in urine is described. Glucose added to urine was completely recovered on sufficient dilution (1 to 500) of the urine samples, whereas the normal constituents of urine did not interfere with the recovery of glucose. The method allows a rapid and accurate determination of glucose in large numbers of samples with widely diverging glucose concentrations (0.1%–12.5%). In alloxan-diabetic rats the urinary glucose concentration appeared to be remarkably constant (104±0.13 mg/ml) irrespective of the severity of their diabetes. In these animals the urine volume per 24 h was practically linearly proportional to the glucose excretion.

Détermination simple et exacte de l'excrétion urinaire de glucose avec le réactif à l'anthrone

Résumé. La détermination quantitative du glucose urinaire est effectuée par le réactif à l'anthrone. Le glucose ajouté à l'urine est recouvré complètement après dilution suffisante (1 à 500) des échantillons d'urine, alors que les éléments normaux de l'urine ne perturbent pas la détermination du glucose. Des concentrations en glucose très divergentes peuvent être mesurées en grand nombre d'une façon rapide et exacte par cette méthode. La concentration en glucose dans l'urine des rats rendus diabétiques par l'alloxane était remarquablement constante (104 ± 0.13 mg/ml) et indépendante de la gravité du diabète. La quantité d'urine secrétée en 24 h est à peu près en relation linéaire avec l'excrétion urinaire de glucose chez ces animaux.

Einfache und zuverlässige Bestimmung der Glucose-Ausscheidung im Harn mit dem Anthronreagenz

Zusammenfassung. Das Anthronreagenz wurde benutzt zur Bestimmung von Glucose im Harn. Dem Harn zugefügte bekannte Glucosemengen wurden nach fünfhundertfacher Verdünnung quantitativ wiedergefunden, wobei die normalen Harnbestandteile die Bestimmung nicht stören. Die Methode ist schnell und zuverlässig und eignet sich für die serienweise Bestimmung in Harnproben mit sehr verschiedenem Glucosegehalt. Bei alloxandiabetischen Ratten war der Glucosegehalt des Harns sehr konstant (104 ± 0.13 mg/ml) und unabhängig von der Schwere des Diabetes. Das Harnvolumen pro 24 Std war nahezu direkt proportional der Glucose-Ausscheidung im Harn bei diesen Tieren.

Key-words: Glucose determination in urine, anthrone reagent, urinary glucose concentration, urinary glucose excretion, alloxan-diabetic rats, reaction of anthrone with different sugars.

In the past, various methods have been devised for the quantitative determination of glucose in urine. The titrimetric method of Benedict, based on the reduction of cupric sulphate in alkaline solution, has been used on a large scale for this purpose. However, the method is laborious, sensitive to reducing substances in general and its accuracy has been criticized. Similar objections may be raised against a number of other methods which are based on the reduction of a metallic ion. The glucose oxidase peroxidase method on the other hand ist of great specificity as far as its initial step is concerned. However, reducing substances (notably uric acid, ascorbic acid and creatinine) interfere with the peroxidase step of the procedure. Previous purification of the sample by means of ion exchange resins, charcoal etc. is required to obtain sufficient specificity [5, 8].

In 1946 Dreywood [2] introduced anthrone as a qualitative and specific reagent for carbohydrates. Its use was subsequently adapted for the quantitative determination of glycogen in liver and muscle by Seifter et al. [9], of glucose in blood and spinal fluid by Roe [6] and of glucose in tissue incubation media by Bouman and Dermer [1].

In the present report the adaptation of the anthrone method to the determination of urinary glucose is described. In particular, the question was investigated whether urine samples from animals with widely diverging degrees of glycosuria could be determined by one single procedure. This problem is of special importance in studies on patterns of urinary glucose excretion in diabetic animals.

Material and Methods

Glucose determinations. The anthrone method of Seifter et al. [9] was used with slight modifications as earlier described by Bouman and Dermer [1]. The anthrone reagent is freshly prepared for each series of determinations by dissolving 200 mg of anthrone (Merck Co., analytical grade) in 100 ml of 80% (w/v) sulphuric acid (analytical grade). To 1 ml samples of diluted urine 5 ml of anthrone reagent is slowly added while cooling the test tubes in ice water. Subsequently the mixture is heated in a water-bath at 100°C for 6 min. Appropriate blanks and standard solutions containing 0.2 mg/ml of glucose are included. After cooling, extinctions are measured colorimetrically.

The hexose-anthrone complex shows maximum extinction at 625 mμ. However, extinction measurements carried out at any wavelength between 560 and

640 mμ yield linear values over a range of glucose concentrations from 0—0.25 mg/ml.

Urine samples. Rat urines were collected over a 24 h period in flasks containing 0.5 ml of 0.1 N sulphuric acid, which were placed under metabolic cages. Since rat urine appeared to behave identically to human urine the latter was used at later stages of this work. For the same reason no distinction will be made between these two media in the presentation of the data. All glucose determinations were carried out after diluting 0.1 ml of filtered urine to 50 ml with distilled water.

Results

Results of experiments on the recovery of glucose in urine are given in Table 1. Weighed amounts of glucose were added to give concentrations of 100, 50 and 25 mg/ml. As in shown by the table the recovery at these concentrations was complete both in urine and distilled water.

Table 1. *Recovery of glucose in distilled water and urine at four different concentration levels. Each value is the mean ± S.E.M. of four separate determinations in duplicate*

glucose added mg/ml	recovery mg/ml in water	in urine
100	100.8 ± 0.08	101.0 ± 0.06
75	75.9 ± 0.04	76.5 ± 0.02
50	50.9 ± 0.04	50.9 ± 0.02
25	25.1 ± 0.03	25.0 ± 0.02

Table 2. *Recovery in mg/ml of different sugars added to urine as determined against a glucose standard curve. The number of separate determinations in duplicate is given in brackets*

Sugars added	100 mg/ml	50 mg/ml	25 mg/ml
glucose (4)	100.8 ± 0.09	50.3 ± 0.04	25.1 ± 0.04
fructose (6)	108.0 ± 0.8	54.2 ± 0.6	27.0 ± 0.4
galactose (3)	59.1 ± 0.8	29.5 ± 0.6	14.7 ± 0.5
rhamnose (6)	100.5 ± 0.6	49.9 ± 0.5	24.9 ± 0.3
maltose (6)	104.5 ± 0.6	52.0 ± 0.7	25.5 ± 0.4
lactose (6)	83.0 ± 0.8	41.4 ± 0.7	20.7 ± 0.2
sucrose (6)	110.7 ± 0.8	55.9 ± 0.4	26.1 ± 0.4
arabinose (4)	4.5 ± 0.03	2.2 ± 0.01	1.1 ± 0.00
ribose (3)	5.3 ± 0.2	2.4 ± 0.2	0.6 ± 0.2
xylose (3)	6.6 ± 1.3	2.9 ± 1.3	0.8 ± 0.7

Since it has been demonstrated that a variety of sugars react with anthrone [4, 5, 7], various hexoses, pentoses and disaccharides were added to urine to give concentrations of 100 mg/ml. From these solutions two-fold dilutions were made with urine to obtain concentrations of 50 and 25 mg/ml, after which the samples were tested with the anthrone method against a glucose standard curve. The measured recoveries for these substances in urine, as given in Table 2, are in good agreement with those found in literature concerning other solvents than urine.

In order to test the possibility that anthronating a mixture of hexoses and pentoses yields a recovery different from that expected on the basis of their individual recoveries, glucose and arabinose were added simultaneously to normal urine to give concentrations of 100 and 50 mg/ml respectively. Subsequent two-fold dilutions were made as in the preceding experiment. After a further 500-fold dilution the various samples were tested with the anthrone method in the usual manner. The measured recoveries given in Table 3 are based on readings against a glucose standard curve. The expected recovery was obtained by addition of the individual recoveries for glucose and arabinose determined separately in the experiments reported in Table 2. It can be seen from Table 3 that the expected and actual recovery of this mixture of anthrone-sensitive sugars was identical at the three different concentration levels. Apparently the simultaneous presence of these two sugars does not interfere with their individual recoveries.

Table 3. *Recovery of a mixture of glucose and arabinose based on readings against a glucose standard curve. Expected values were obtained by summation of individual recoveries listed in Table 2*

Sugars added mg/ml		Recovery expressed as glucose mg/ml	
glucose	arabinose	expected	measured
100	50	103.0	102.9 ± 0.04
50	25	51.4	51.2 ± 0.03
25	12.5	25.6	25.6 ± 0.03

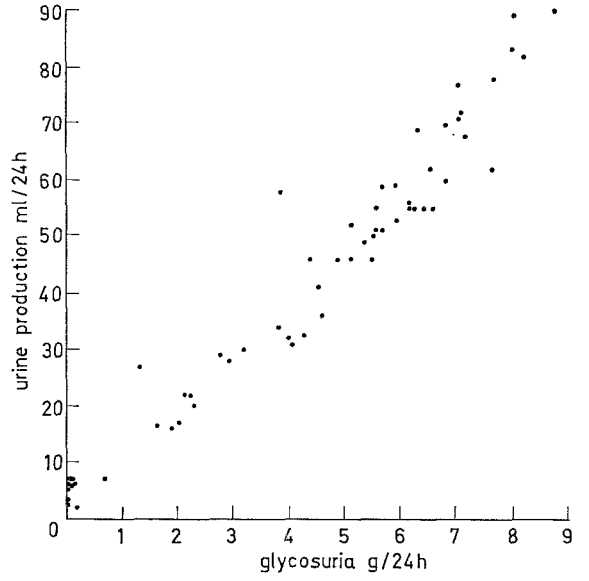

Fig. 1. Glucose excretion of 60 alloxan-diabetic rats plotted against the urine production per 24 h

The application of the method for the determination of widely diverging degrees of glycosuria in rats is demonstrated in Fig. 1. The glucose excretion in g/24 h of 60 alloxan-diabetic rats of 150—170 g bodyweight was plotted against the volume of urine excreted over a 24 h period. From the graph it is apparent that the volume of urine excreted is linearly propor-

tional to the degree of glycosuria (correlation coefficient = 0.99). Obviously the glucose concentration of the different samples remains constant within narrow limits. In the samples under consideration the average glucose concentration (± S.E.M.) amounted to 104 ± 0.13 mg/ml, which is well within the range of the anthrone method after 500-fold dilution. The highest urinary glucose concentration observed in approximately 1000 determinations was 143 mg/ml.

Discussion

The results of this study indicate that anthrone reagent can be used for highly accurate, quantitative determinations of glucose in diabetic urines. Glucose added to urine was completely recovered when the determinations were performed in urine samples diluted 500-fold. Hence, the influence of interfering substances, if present in urine, is completely lost after such a dilution.

As a reagent for the determination of glucose in biological materials anthrone has the distinct advantage that, unlike many other reagents, it is not sensitive to reducing substances in general. In the sulphuric acid medium glucose undergoes dehydration and ring formation resulting in furfural derivatives. These in turn react with anthrone to form a blue-coloured compound, which is determined colorimetrically [7].

A possible disadvantage might be that anthrone also reacts with various other hexoses, pentoses and disaccharides [4, 5, 7]. This is also shown by the present investigation, in which a variety of sugars were added to urine. Hexoses of distinct biochemical significance such as fructose and galactose yielded extinctions identical to or closely approaching those obtained with glucose. Theoretically, these substances may therefore contribute to the extinction measured in urine samples. However, significant amounts of these hexoses are only excreted in cases of essential fructosuria, hereditary galactosaemia and severe impairment of liver function. The presence of disaccharides. which also strongly react with anthrone reagent, may occur by contamination of urine with spilled dietary substances and can be avoided. Compared with hexoses and disaccharides, pentoses like arabinose, ribose and xylose, appear to react only slightly with anthrone reagent. Their significance as a disturbing factor in urinary glucose determination with anthrone, is further limited by the fact that pentoses only occur in urine under exceptional conditions, such as hereditary xylosuria and alimentary pentosuria.

It should be noted, that the ambiguous results on anthronating a mixture of hexose (glucose) and pentose (arabinose) as observed by Johanson [3], did not occur with our adaptation of the anthrone method after adding both sugars to urine. On the whole, the recovery of single sugars in urine closely approximates the values reported by various authors using solutions in water referred to by Reinauer and Hollmann [5]. Again, this indicates that urine diluted 500-fold may be regarded as a true aqueous solution in this respect.

Of particular interest is the remarkable constancy of the glucose concentration (104 ± 0.13 mg/ml) in the urine of alloxan-diabetic rats of all degrees of diabetes intensity. Apparently, in glycosuric rats urinary osmolarity is kept at a constant level by adjustment of water intake and excretion to the load of glucose which escapes tubular reabsorption. On the other hand, widely diverging glucose concentrations as may occur in diabetic humans, can be determined equally well after one single dilution step. Extinctions obtained with the anthrone method follow Beer's law over a concentration range up to 0.25 mg/ml at least. Therefore, a single 500-fold dilution of any glycosuric sample of urine which contains between 0.1 and 12.5% of glucose will fall within the range of the method. This eliminates the neccessity of pilot determinations as may be needed with Benedict's method, which is still widely used and far more laborious than the anthrone technique.

In conclusion it can be stated that the anthrone method is very well suited to the accurate determination of glucose in the urine of diabetic animals and humans. The rapidity and simplicity of the method allows large series of samples to be determined, even when the 24-h glucose excertion and the urinary glucose concentration are widely diverging.

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