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An Improved Enzymatic Assay for Glucose Determination in Blood Serum Using a 1,1′-Dimethylferricinium Dye

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ABSTRACT

1,1′-dimethylferricinium ($DMFe^+$), a stable and pH-insensitive blue dye, was prepared via enzymatic oxidation of a 1,1′-dimethylferrocene (DMFe):2-hydroxypropyl-β-cyclodextrin (HPCD) water-soluble inclusion complex, using bilirubin oxidase immobilized onto porous aminopropyl glass beads via glutaraldehyde activation. In the presence of glucose, $DMFe^+$ was reduced to DMFe by reacting with the reduced glucose oxidase ($FADH_2$), and the absorbance decrease was followed at 650 nm. In acetate pH 5.2 buffer, the response to glucose in blood serum was nonlinear, especially in the low concentration range, because of a competition for the reduced glucose oxidase between the $DMFe^+$ dye and oxygen. At this pH, endogenous ceruloplasmin was also observed to oxidize residual DMFe (16%) in the dye preparation, causing an increase in absorbance at 650 nm. An assay protocol was then developed using maleate buffer, pH 6.5, to overcome these interferences as well as mutarotation of α-D-glucose. The results obtained for glucose in the blood serum samples agreed well with those of the reference hexokinase/glucose-6-phosphate dehydrogenase method.

Index Entries: Glucose; 1,1′-dimethylferricinium ($DMFe^+$); 1,1′-dimethylferrocene (DMFe); 2-hydroxypropyl-β-cyclodextrin (HPCD); inclusion complex; colorimetric method; blood serum.

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INTRODUCTION

The determination of glucose levels in biological samples is an indispensable test for the diagnosis and therapy of diabetes mellitus, hypoglycemia, and suspected meningitis (*1*). Blood normally has a glucose concentration in the range of 4.4–6.6 m*M* (*2*); the pathological value may increase up to 10-fold. Diabetic patients control their glucose levels by insulin injection; however, this results in daily fluctuations of hyperglycemia (up to 15–20 m*M*) and hypoglycemia (as low as 3 m*M*). Continuous blood glucose monitoring is thus critical in diabetes therapy and abnormalities of carbohydrate metabolism.

The reference method for glucose determination uses a coupled-enzyme assay containing hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH), along with the necessary cofactors ATP and either NAD or NADP. The absorbance of NADH or NADPH formation is monitored at 340 nm (*1*). Although highly accurate and precise, the method is time-consuming for routine clinical procedures, since deproteinization steps are required to remove blanks arising with serum or plasma samples. Alternatively, the reaction can be applied directly to the samples, but a specimen blank is required. Standards are also required to ensure the adequacy of the enzyme reagent, since most of the components are unstable. Indicators can be added to the system so that measurements will be in the visible range, but this complicates the method further and the reagents need to be refrigerated and protected from light. Another popular coupled enzyme assay procedure for glucose determination involves glucose oxidase and peroxidase in the presence of a chromogenic oxygen acceptor such as *o*-dianisidine, aminoantipyrine/phenol, and so on. The main drawback of this procedure is that various substances in the blood samples, such as uric acid, ascorbic acid, bilirubin, and glutathione, interfere by competing with the chromogen for the hydrogen peroxide released by the glucose oxidase reaction. Also, *o*-dianisidine and phenol are toxic compounds (*1*).

In this study, the ability of 2-hydroxypropyl-β-cyclodextrin (HPCD) to enclose water-insoluble 1,1′-dimethylferrocene (DMFe) to form an inclusion complex was exploited to develop a rapid spectrophotometric assay for the detection of glucose in blood serum samples. The cyclodextrin forms an inclusion complex by incorporating the guest molecule into its hydrophobic cavity (*3*). DMFe has been studied extensively to develop mediated biosensors, because of its electron-exchange properties with various enzymes (*4,5*). The yellow inclusion complex, with two absorbance peaks at 325 and 435 nm, was oxidized by immobilized bilirubin oxidase to its oxidized form $DMFe^+$ to produce a blue dye with an absorbance peak at 650 nm (*6*). This colorimetric reagent is pH insensitive and can be reduced by various reducing agents, such as ascorbic acid, uric acid, and sulfite (*7*), as well as being used for the detection of many

analytes, including glucose in the presence of their corresponding oxidase enzymes. During the reaction, the $DMFe^+$ is reduced to DMFe, but since this redox couple has different UV-vis spectra no interference is observed and the absorbance decrease at 650 nm can be correlated to the glucose concentration.

MATERIALS AND METHODS

Materials

Glucose oxidase (EC 1.1.3.4, type X-S from *Aspergillus niger*), bilirubin oxidase (EC 1.3.3.5 from *Myrothecium verrucaria*), hexokinase type VI, glucose-6-phosphate dehydrogenase type XXIV (EC 1.1.1.49 from *Leuconostoc mesenteroides*), human ceruloplasmin, α-amylase, NADH peroxidase, NAD, NADH, ATP, glutaraldehyde (25% w/v), bilirubin, hemoglobin, and porous aminopropyl glass beads (pore size 70 nm) were purchased from Sigma (St. Louis, MO). 2-hydroxypropyl-β-cyclodextrin (HPCD, average MW 1500) and 1,1′-dimethylferrocene (DMFe) were obtained from Aldrich (Milwaukee, WI). D-glucose and all other chemicals were obtained from Anachemia (Montreal, Quebec, Canada). Whole blood samples (supplied from Dr. Alan Pollard of Mount Sinai Hospital, Toronto, Ontario, Canada) were centrifuged and the serum supernatant was used for all experiments.

Preparation of 1,1′-Dimethylferricinium Using Immobilized Bilirubin Oxidase

1,1′-dimethylferricinium ($DMFe^+$) was prepared by the method of Male and Luong (*6*) with some modifications. In brief, a solution of DMFe (40 mL, ca. 85 m*M*) solubilized in HPCD (250 m*M* in 200 m*M* acetate, pH 4.5) was oxidized by bilirubin oxidase (50 unit, 1.6 mg protein) immobilized on aminopropyl glass beads (500 mg). The yellow solution was stirred and bubbled with pure oxygen (0.09 L/min) for 90 min until the reaction was complete, as indicated by no further increase in the absorbance of the resulting blue solution as measured at 650 nm. Using the absorption coefficient of 325/cm·*M* (*7*) the concentration of the $DMFe^+$ was determined to be 71 m*M*, representing a conversion yield of 84%. The $DMFe^+$ was separated from the beads by centrifugation, and after extensive washing the beads were stored at 4°C until reuse. The $DMFe^+$ was stable for at least 6 mo when stored at 4°C.

Determination of Glucose in Blood Serum

$DMFe^+$ (100 μL in a final assay volume of 1 mL, ca. 6.5–7.5 m*M*) was used with glucose oxidase (10 IU) to determine the concentration of β-D-glucose by following the reduction of $DMFe^+$ to DMFe at 650 nm for

10–30 min. The assays were performed in plastic cuvets using a Beckman DU-640 spectrophotometer equipped with a kinetics software package. Optimization regarding pH (5.0–7.5) as well as buffer type was necessary to overcome interference problems encountered with the serum samples. Serum (50–400 μL) was added to 100 μL of $DMFe^+$ and the final assay volume was made up to 1 mL with buffer. The effect on the assay of known serum components, such as ceruloplasmin, α-amylase, hemoglobin, and bilirubin, were also evaluated. It should be noted that two ferricinium molecules are reduced for each glucose molecule oxidized by the enzyme, so that 1 m*M* β-D-glucose effects an absorbance change of 0.65. Glucose levels in the blood serum were determined using 200 m*M* maleate buffer, pH 6.5, and the results were compared to the standard spectrophotometric procedure, using HK/G6PDH as well as the HPLC method (*8*). In calculating glucose concentrations, it should be remembered that D-glucose contains 64% β-D-glucose and 36% α-D-glucose (*1*), and, therefore, when comparing results to the HK/G6PDH or HPLC methods, the values obtained from the $DMFe^+$ assay must be multiplied by 1.56 to obtain the value for the total D-glucose pool.

RESULTS AND DISCUSSION

$DMFe^+$ Assay with Serum β-D-Glucose and Removal of Oxygen Interference

Seven serum samples of known glucose concentration (4–20 m*M*, diluted to give a range of concentrations) were tested for β-D-glucose, using the standard $DMFe^+$ dye assay (250 m*M* acetate buffer, pH 5.2) previously reported (*6*). The measured absorbance decrease (650 nm) in the presence of oxygen was underestimated by 10–40%, with the highest error being observed at the lowest serum β-D-glucose concentration, in which the samples were most diluted (Fig. 1). The relation was nonlinear, rather than an expected straight line, with a slope of 0.65 OD/m*M*. Similar results for β-D-glucose standards were also reported (*7*) and such behavior was attributed to a competition between $DMFe^+$ and oxygen for the reduced glucose oxidase. The oxygen interference was not completely unexpected, since the K_m (Michaelis-Menten constant) of $DMFe^+$ for the glucose reaction using 250 m*M* acetate buffer was reported (*6*) to be quite high (4.9 m*M*) when compared to other oxidase enzymes utilizing $DMFe^+$, in which oxygen interference was not encountered. Increasing the serum sample volume in the assay (total 1 mL) improved the accuracy of the assay procedure as the estimated glucose concentration followed by monitoring absorbance decrease at 650 nm appeared to agree well with the expected value. However, for clinical applications, it is more desirable to minimize the serum volume. Ideally, the determination of glucose concentration must be independent of the serum volume analyzed.

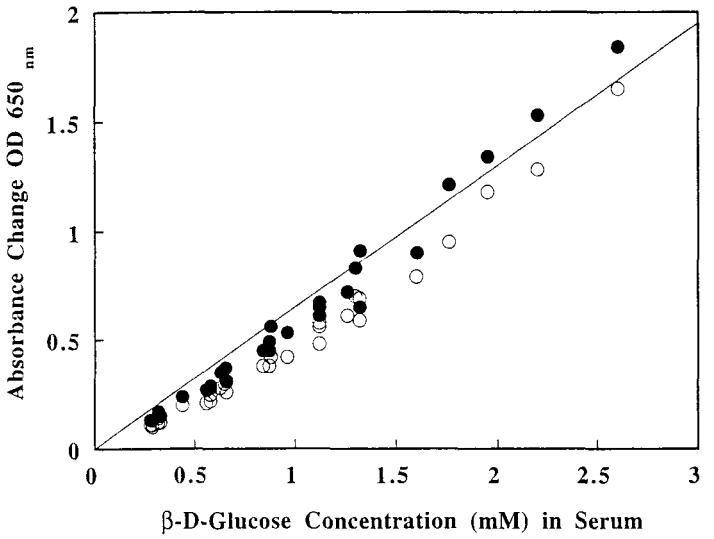

Fig. 1. Measurement of β-D-glucose in seven different serum samples of known glucose concentration (4–20 m*M*) diluted to give a range of desired concentrations using 250 m*M* acetate buffer, pH 5.2. Assay time was 10 min and the straight line represents the expected slope of 0.65 OD/m*M*. (○) Oxygen present; the slope was determined to be 0.53 OD/m*M*, correlation coefficient = 0.95, which reflected the nonlinearity of the experimental data. (●) Addition of 0.4 m*M* Na_2SO_3 to remove dissolved oxygen; the slope was determined to be 0.63 OD/m*M*, correlation coefficient = 0.96.

As previously reported (7), oxygen removal by treatment of the sample with sodium sulfite (0.4 m*M*) before the addition of glucose oxidase will completely overcome this interference. When sodium sulfite was added to the assay of serum samples, the results obtained improved when compared to the samples with oxygen present (Fig. 1). However, there was still some underestimation of the glucose concentration in the range of most interest (<2 m*M*), indicating that there was some further interference other than oxygen. The removal of the oxygen interference by sodium sulfite addition is not considered very practical for a routine clincial procedure, because excess sulfite must be added to ensure complete oxygen removal. Undoubtedly, residual sulfite will reduce a small portion of the $DMFe^+$ to DMFe to effect an absorbance decrease. As a result, glucose oxidase cannot be added until a time lapse of 10–15 min. It should be noted that the sodium sulfite reaction with oxygen was much faster than its reaction with $DMFe^+$. Alternative methods were considered and, interestingly, changing the buffer from acetate to phosphate prevented the oxygen interference and results similar to the addition of sodium sulfite could be obtained. Although such behavior was not understood, the maximal response was obtained at 200 m*M* phosphate and this concentration was used for all further experiments.

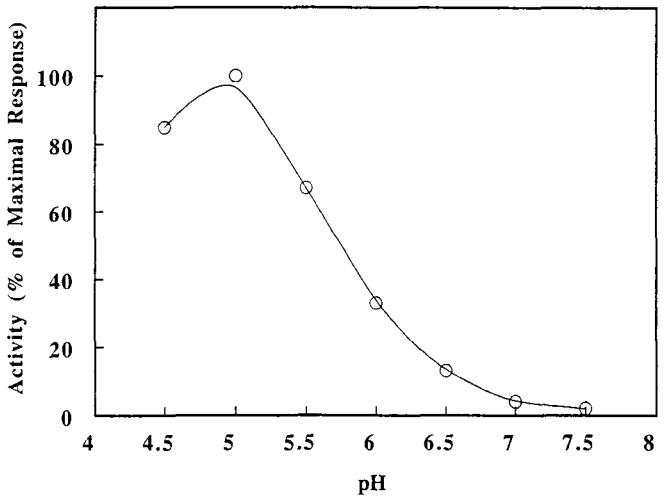

Fig. 2. Effect of phosphate pH on human ceruloplasmin activity (20 units) with DMFe (1 m*M*) as substrate. The activity was measured by following the absorbance increase at 650 nm caused by the oxidation of DMFe to $DMFe^+$.

Elimination of Interference Caused by Ceruloplasmin

As mentioned in the previous section, the $DMFe^+$ assay underestimated the real glucose levels for the serum samples tested even if the oxygen interference was overcome. It was noticed that the absorbance drifted upwards after the assay seemed completed (10 min), so that the true endpoint of the assay could not really be defined. Controls were performed in the absence of glucose oxidase and it was found that the absorbance at 650 nm increased linearly with time when serum samples were added to the $DMFe^+$ dye, and that the rate of the absorbance increase was different, depending on the sample. If not corrected, this would account for an appreciable underestimation of the glucose levels, since endogenous chemicals/enzymes in the samples must be partially oxidizing the residual DMFe (16%) in the dye preparation. Bovine ceruloplasmin has been reported to oxidize DMFe to $DMFe^+$ (*7*), so it is logically anticipated that ceruloplasmin in the serum samples may be causing this interference. Ceruloplasmin levels vary in serum with the normal range being 0.15–0.60 mg/mL, although the levels are elevated during exercise, pregnancy, and estrogen administration, or reduced in the case of Wilson's disease (*9*).

Commercially available human ceruloplasmin (20 Sigma U, 120 U/mg) was observed to oxidize DMFe (1.0 m*M*) at significant rates, with a twofold higher rate (0.02 OD/min) observed using acetate buffer compared to phosphate (0.01 OD/min) at pH 5.0. The rate was maximal between pH 4.5–5.5 and then decreased rapidly, so that no activity was detected above pH 7.0, while the rate at pH 6.5 was only about 10% (Fig. 2). The pattern observed using $DMFe^+$ (6.5 m*M*) as the starting substrate was similar. The rate observed for serum samples tested at pH 5.0 phosphate

Table 1
The Effect of Endogenous Ceruloplasmin
and Mutarotation on the Determination of Glucose in Serum Samples

	Glucose concentration (m*M*)				
	DMFe+ Assay (10 min)				
Serum sample	Acetate pH 5.2	Phosphate pH 6.0	Phosphate pH 6.5	HPLC	HK/G6PDH
1	3.5	5.5	6.1	5.6	5.6
2	4.0	5.9	6.4	5.8	5.8
3	3.0	5.3	5.0	4.8	5.4
4	3.0	4.4	4.8	3.7	4.3
5	3.6	5.3	5.3	5.1	5.1
6	3.0	4.9	4.7	4.7	5.0

buffer ranged from 0.002–0.007 OD/min (the equivalent of about 4–14 Sigma U, or 0.03–0.12 mg) and this rate continued even after the reaction between glucose and the glucose oxidase was completed, making it virtually impossible to establish an endpoint. The sample volumes were about 200 μL, which would represent 0.03–0.12 mg of ceruloplasmin, as mentioned previously (*9*), and therefore the rate observed was consistent with a ceruloplasmin presence. Treatment of the sample with trichloroacetic acid to denature protein, followed by pH neutralization, resulted in the removal of the interference, and provided further evidence that the artifact was protein-linked and probably ceruloplasmin. Alternatively, increasing the phosphate buffer pH to above 6.5 eliminated the interference caused by ceruloplasmin by at least 90–95%. Other components of blood, such as hemoglobin, bilirubin, and α-amylase, were tested and found not to interfere with the DMFe+ dye assay.

Effect of α-D-Glucose Mutarotation on DMFe+ Assay

Increasing the pH of the buffer from 5.0 to 6.0–6.5 improved the accuracy of the glucose determination in six serum samples tested (Table 1). However, a trend developed with increasing pH, as glucose values, which had been underestimated because of the ceruloplasmin problem (acetate, pH 5.2), became overestimated (phosphate, pH 6.5) as a result of a new interference. At higher pHs, it was noted that the absorbance decrease did not reach a plateau, but actually drifted downwards slowly after an initial large decrease. The data in Table 1 were recorded after 10 min; however, if the DMFe+ assay was monitored after 30 min in the case of sample 5 the value of glucose increased from 5.3 to 6.4 m*M* at pH 6.0. Closer examination of the problem using standard β-D-glucose (0.5 m*M*), rather than serum samples, revealed similar behavior. As demonstrated in Table 2, at pHs above 5.0 the expected absorbance change of 0.32 was

Table 2
The Effect of pH on the Measurement of β-D-Glucose Using the $DMFe^+$ Blue Dye

Phosphate pH	Absorbance change 640 nm (0.5 m*M* β-D-Glucose)		
	10 min	20 min	30 min
5.0	0.26	0.29	0.31
5.5	0.34	0.38	0.40
6.0	0.39	0.44	0.44
6.5	0.44	0.48	0.48
7.0	0.47	0.49	0.49
7.5	0.45	0.47	0.47

Table 3
The Effect of pH on the Measurement of Glucose in a Serum Sample

pH, buffer	Glucose concentration (m*M*) via $DMFe^+$ assay of serum sample #2 (5.8 m*M* expected)		
	10 min	20 min	30 min
pH 5.0, acetate	3.6	3.1	2.9
pH 5.0, phosphate	4.9	5.2	5.0
pH 5.5, phosphate	5.8	5.9	5.6
pH 6.0, phosphate	6.6	6.6	6.3
pH 6.5, phosphate	7.3	7.3	7.0
pH 7.0, phosphate	7.9	7.9	7.9

always overestimated. The expected absorbance change (0.32) was 65% of the maximum absorbance change (0.49) obtained at pHs greater than 6.5, leading to the hypothesis that mutarotation of the α-D-glucose to β-D-glucose may be occurring, since D-glucose contains 64% of the β-D-glucose form (*1*). As the β-D-glucose is consumed by the glucose oxidase reaction (pH > 5.5), the α-D-glucose will mutarotate to reestablish equilibrium, until eventually the entire D-glucose pool will be measured. Sample 2 (5.8 m*M* D-glucose) from Table 1 was tested at different pHs and the same phenomenon was observed as the level was overestimated at higher pHs (Table 3). Using phosphate buffer at the ideal pH condition (> 6.5), in which the ceruloplasmin interference was negligible, resulted in poor reproducibility, as indicated by the values for sample 2 in Table 1 (6.4 m*M*) and 3 (7.3 m*M*) performed under the same condition (10 min). The inaccuracy could be a result of many factors, including the pH control and glucose oxidase concentration. Ideally, the best assay method should be independent of these two parameters. As well, the assay time should not affect the determined glucose concentration once the endpoint has been defined.

Undoubtedly, the mutarotational effect will cause severe problems in determining an accurate value for glucose, since pH control cannot be guaranteed and, ideally, a pH of at least 6.5 is desirable to eliminate the ceruloplasmin effect. The values correlated well at pH 5.5 for the six serum samples tested (data not shown), although this occurs just by coincidence, since the two interfering effects cancel each other out. It should be noted that the mutarotational effect was not a result of the $DMFe^+$ assay, as similar pH effects were obtained if glucose oxidase was coupled to the spectrophotometric assay using NADH peroxidase and NADH. Other buffers were tested and it was found that imidazole buffer behaved much like phosphate buffer regarding mutarotation. However, the mutarotation of α-D-glucose did not occur with maleate buffer (pH 6–7). With the $DMFe^+$ assay and 0.5 m*M* β-D-glucose, the expected absorbance change of 0.32 was obtained regardless of pH (6.0–7.0); the assay was complete after 10 min and a steady plateau was observed up to 30 min. These results are in sharp contrast to those reported in Table 2 using phosphate buffer and the mode of action of maleate in countering this mutarotational effect remains unclear. Similar to phosphate buffer or sodium sulfite addition, maleate buffer at pH 6.5 did not show any evidence of oxygen interference when determining β-D-glucose concentrations (0.125–2.0 m*M*). The relationship was linear, with a slope of 0.64 OD/m*M*, as would be expected from the absorption coefficient (0.325 OD/m*M*) of $DMFe^+$. These desirable assay conditions were then tested on serum samples.

Measurement of Glucose in Serum Samples

Sample 1 from Table 1 was tested and the determined glucose concentration was consistent, regardless of the maleate buffer pH (6.0–7.0) or the serum volume added (50–400 μL). This finding is important since it implies the result will not be dependent on the serum volume added (i.e., variable glucose concentrations), which is critical for flexibility in clinical applications. The optimal assay protocol using maleate buffer at pH 6.5 was tested on samples 1, 2, 5, and 6 from Table 1 and the glucose concentrations (m*M*) determined were 5.3, 5.7, 5.0, and 5.0, respectively. The results were in good agreement with the standard HPLC (5.6, 5.8, 5.1, and 4.7) or HK/G6PDH (5.6, 5.8, 5.1, and 5.0) assay (Table 1). The reproducibility of the response was verified by seven repeated analyses of sample 1. The resulting value was 5.3 ± 0.42, representing ±8% error at a 95% confidence interval. The concentrations of glucose were then determined in a number of serum samples by the dye method (using maleate buffer, pH 6.5) and compared with the HK/G6PDH method. The values of the dye method plotted against those of HK/G6PDH resulted in a straight line with a slope of 0.96 and a correlation coefficient of 0.99 ($n = 29$) (Fig. 3). Such agreement thus validated the applicability of the $DMFe^+$ method for measurement of glucose levels in blood serum.

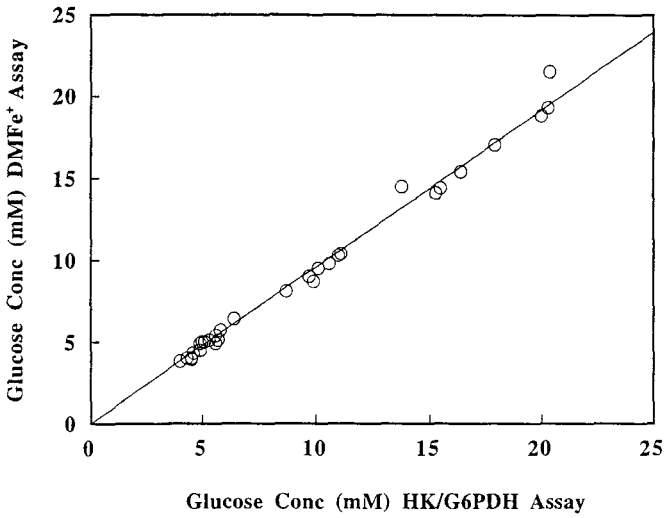

Fig. 3. D-Glucose concentration in serum samples determined by the hexokinase/glucose-6-phosphate dehydrogenase assay C_{Hexo} and the $DMFe^+$ assay C_{DMFe^+}. Assay time was 10 min. $C_{Hexo} = 0.96\ C_{DMFe^+}$, $R^2 = 0.999$, $n = 29$.

In brief, a rapid and reliable colorimetric assay based on a $DMFe^+$ dye has been successfully developed for the determination of glucose levels in blood serum samples. The main advantage of this protocol as a colorimetric method for glucose determination is its simplicity, since only glucose oxidase is required, rather than the two enzymes and other reagents necessary in the coupled systems. Also, the measured wavelength (650 nm) of the assay will prevent interferences that can occur at shorter wavelengths. Although the absorption coefficient of $DMFe^+$ is lower (325/cm·*M*) than other colorimetric reagents (4000–7000/cm·*M*) the detection limit for glucose (0.1 m*M*) using the $DMFe^+$ blue dye is well within the range of several important clinical applications.

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