Influence of Pigments and pH of Urine on the Determination of *N*-Acetyl- β -D-Glucosaminidase Activity With 2-Methoxy-4-(2'-Nitrovinyl)-Phenyl-*N*-Acetyl- β -D-Glucosaminide

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The influence of urinary pigments and urine pH on the spectrophotometric determination of *N*-acetyl- β -D-glucosaminidase (NAG; EC 3.2.1.30) activity with 2-methoxy-4-(2'-nitrovinyl)-phenyl-*N*-acetyl- β -D-glucosaminide as a substrate was studied. The investigation was performed with human and rabbit urine samples. It was found that alkaline urine pH values influenced NAG activity in two ways: 1) NAG activity decreased due to enzyme instability with pH increase, and 2) NAG activity increased because of the contribution of urinary pigments to absorbance of 2-methoxy-4-(2'-nitrovinyl)-phenol (MNP) at 505 nm. It was shown that besides the maximum (I) in the range of 350–360 nm of the absorption

spectra of alkaline urine, there was a maximum (II) in the range of 380–460 nm. With the increase of pH, maximum II was shifted toward higher wavelengths and contributed to MNP absorption (5–90%). On the other hand, the maximum of MNP absorption was shifted toward lower wavelengths (495–400 nm) with increasing pH. Two procedures to eliminate the influence of urinary pigments are presented. The justification of applying a correction to the values of NAG activity in human and rabbit urine (a model system for studying the toxic effects of cadmium) was discussed. J. Clin. Lab. Anal. 19:260–266, 2005. © 2005 Wiley-Liss, Inc.

Key words: alkaline urine; urinary pigments; *N*-acetyl- β -D-glucosaminidase (NAG) spectrophotometric determination

INTRODUCTION

The activity of the lysosomal enzyme *N*-acetyl- β -D-glucosaminidase (NAG) in urine is a sensitive indicator of early damage to the renal proximal tubules (1,2). Increased urinary NAG activity originates in pathological states (e.g., diabetes (3–5)) and renal damage during treatment with medications (6), organic solvents (7), metals (cadmium (8), mercury (9), and lead and manganese (10), and during the rejection of kidney transplants (11). In experiments investigating the nephrotoxicity of various substances with animal models (rabbits (12,13), rats (14), and hamsters (15)), NAG activity was determined as an indicator of damage to the renal tubules.

The most common substrates applied for the spectrophotometric determination of NAG activity in clinical and toxicological laboratories are p-nitrophenyl-*N*-acetyl-beta-D-glucosaminide (pNP-NAG) (16), 2-chloro-4-nitrophenyl-*N*-acetyl-beta-D-glucosaminide

(CNP-NAG) (17), 2-methoxy-4-(2'-nitrovinyl)-phenyl-*N*-acetyl- β -D-glucosaminide (MNP-NAG) (18), and ammonium 5-[4-(2-acetamido-2-deoxy-beta-D-glucopyranosyloxy)-3-methoxyphenylmethylene]-2-thioxothiazolidin-4-one-3-ethanoate (VRA-NAG) (19). The influence of the yellow color of urine samples on the determination of NAG activity decreases by

Abbreviations: MNP-NAG, 2-methoxy-4-(2'-nitrovinyl)-phenyl-*N*-acetyl- β -D-glucosaminide; MNP, 2-methoxy-4-(2'-nitrovinyl)-phenol; NAG, *N*-acetyl- β -D-glucosaminidase

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applying MNP-NAcGlc, i.e., by measuring the absorbance at 505 nm (18).

Investigations of the influence of pH on NAG activity showed that NAG inactivation occurs under alkaline conditions (pH > 8) (20). The pigments present in urine, especially at alkaline pH, give urine an intensive color (yellow, orange, yellow-brown, and brown). The urine of patients who have pyelonephritis or bacterial infections, or are taking medications (e.g., metotreksat, ranitidin, and kinolon) is often alkaline and may also be intensively colored. Rabbit urine (a model system used in toxicological investigations) is also alkaline and strongly colored (often brown). The pigments and alkaline pH values of the urine could make the determination of NAG activity unreliable.

Consequently, we investigated the influence of pigments and pH on the spectrophotometric determination of the total NAG activity with MNP-NAcGlc in human and rabbit urine, as well as the possibility of correcting for the obtained results.

MATERIALS AND METHODS

Chemicals and Instrumentation

2-Methoxy-4-(2'-nitrovinyl)-phenyl-*N*-acetyl- β -D-glucosaminide was used as a substrate for the determination of NAG activity. The required chemicals (sodium dihydrogen phosphate monohydrate, sodium hydrogen phosphate dihydrate, sodium carbonate, sodium bicarbonate, sodium hydroxide, and citric acid) were purchased from Merck GmbH (Darmstadt, Germany). Spectrophotometric measurements were performed using a Beckman DU-50 spectrophotometer (CA).

Urine Samples

Freshly collected urine samples from healthy human subjects were used to study the influence of pigments and pH on the determination of NAG activity. The initial pH values of the urine ranged from 5.0 to 6.6. The total NAG activity in the urine samples was found to range from 0.8 to 1.8 IU/L. The urine was then made alkaline to defined pH values (in the range of 7.0–10) by the addition of a 6 mol/L sodium hydroxide solution, and the NAG activity was redetermined.

The rabbits were orally administered 10 mg of Cd/kg of body mass as a CdCl₂ solution daily for 4 weeks. Urine samples were taken after 0, 10, 15, 17, 19, 21, 23, 25, and 28 days and kept frozen until they were assayed. All of the rabbit urine samples were alkaline.

NAG Enzyme Assay

The activity of NAG was assayed by adding 100 μ L of urine sample to 1,000 μ L of 1.5 mmol/L substrate

solution and 500 μ L of 0.1 mol/L citrate buffer, pH 4.5. After 30 min at 37°C the enzyme reaction was stopped by adding 500 μ L of 0.1 mol/L carbonate buffer, pH 9.5, and the absorbance was read at 505 nm. Reagent blanks were performed for each determination. The blank run contained all of the above-mentioned substances in the same ratios, except that distilled water was used instead of urine. The calibration curve was recorded with 2-methoxy-4-(2'-nitrovinyl)-phenol (MNP) as the standard in the concentration range of 0.025–0.200 mmol/L ($y = 1.275x - 0.00089$, $r = 0.9996$). The unit of enzymatic activity (IU) is defined as the amount of enzymes that hydrolyze 1 μ mol of substrate in 1 min at 37°C.

RESULTS

Investigation of the Influence of Pigments and pH on the Determination of NAG Activity in Human Urine

To assess the influence of urinary pigments on the determination of NAG activity, the absorption spectra of human weak acidic and alkaline urines, as well as the absorption spectra of the colored product MNP (obtained by incubating the same urines with MNP-NAcGlc) were recorded. The obtained spectra in the wavelength range of 350–700 nm are presented in Fig. 1.

Analysis of the absorption spectra of 18 human urine samples of various pH values and pigments (Table 1, Fig. 1a) indicates that the urine color changes from light yellow to yellow-brown with increasing pH. All of the urine samples had maximum absorption (max I) in the wavelength range of 350–360 nm. In the case of alkaline samples of yellow-brown color and, sometimes, of weak acidic urines of the same color, there is another maximum (max II). With increasing pH, max II shifts considerably toward higher wavelengths (from 379 to 407 nm). When the enzyme activity is determined, as stated above, the absorbance of MNP, evolved by the hydrolysis of the substrate in the presence of NAG, is measured at 505 nm. This wavelength (at the end of the broad maximum in normal weak acidic urine; Fig. 1b) was selected to avoid the influence of urine color. With increasing pH of urine the absorption maximum of the colored product MNP shifts toward lower wavelengths (490–459 nm; Table 1, Fig. 1b).

The denoted shifts of the absorption maxima of urinary pigments contribute to an increase in MNP absorption. Table 2 shows the values of the contributions of urinary pigments to MNP absorbance at 505 nm. In the case of normal, weakly acidic urine, it ranges from 5% to 22%. In the case of alkaline urine (the pH values of which were adjusted between 7 and 8), the range was from 10% to 20%. In the case of alkaline

Fig. 1. Absorption spectra from (a) human urine samples with various pigments and pH values (— light yellow, pH 5; ···· intensive yellow, pH 5.5; – – yellow-brown, pH 9) and (b) MNP that was obtained with the same urine.

TABLE 1. Characteristics of human urine absorption spectra

Color of urine (number of samples)	pH ^a	Max I (nm)	Max II (nm)	Max MNP (nm)
Light yellow (3)	5	350	—	480–505
Yellow (4)	5–5.9	355–359	—	468–489
Yellow-brown (2)	5.5–6.6	359–360	382–385	488–489
Yellow-brown (4)	7.5–8.5	358–360	379–393	488–490
Yellow-brown (4)	8.9–9.5	359–360	382–407	459–490
Light yellow (1)	9	350	—	480

^aThe alkaline values of the pH were adjusted by adding sodium hydroxide solution.

TABLE 2. Contribution of pigments to the absorbance of MNP in human urines of various pH

Urine pH ^a	Color of urine	A ₅₀₅ MNP	A ₅₀₅ of urine	A ₅₀₅ of urine ^b	Contribution of pigments (%)
5	Light yellow	0.046	0.104	0.005	11
5	Yellow	0.098	0.113	0.005	5
5.5	Yellow brown	0.061	0.177	0.008	13
5.9	Yellow	0.054	0.258	0.012	22
6.6	Yellow brown	0.131	0.419	0.020	15
7.5	Yellow brown	0.048	0.209	0.010	21
8	Yellow brown	0.058	0.121	0.006	10
8	Yellow brown	0.113	0.403	0.019	17
8.5	Yellow brown	0.117	0.380	0.018	15
8.9	Yellow brown	0.032	0.142	0.007	22
9	Yellow brown	0.015	0.125	0.006	40
9	Yellow brown	0.025	0.181	0.009	36
9.5	Yellow brown	0.034	0.366	0.017	50

^aThe alkaline values of the pH were adjusted by adding sodium hydroxide solution.

^bThe absorption was corrected with the dilution factor (21 times) under the conditions used to determine NAG activity.

urine (with pH > 8), the contribution of the pigments was greater and found to range from 36% to 50%. The MNP absorbances at 505 nm in these urine samples were considerably lower compared to the acidic ones. The increased values of the contributions of urinary pig-

ments in them are therefore the consequence of low NAG activity, i.e., NAG instability under alkaline conditions. Thus, the influence of pH on NAG determination in urine with pH > 8 is more significant than the influence of pigments.

Investigation of the Influence of Pigments and pH on NAG Determination in Rabbit Urine

The absorption spectra of rabbit alkaline urine and MNP obtained by incubating the same urine with MNP-NAcGlc are presented in Fig. 2. Although all of the urine samples ($n = 50$) were alkaline, their color varied from light yellow, via intensive yellow to yellow-brown and brown. One (max I in urine with yellow color) or two (max I and max II in urine with yellow-brown and brown color) maxima appear in the absorption spectra of rabbit urine (Table 3, Fig. 2a). Similarly to human urine, with increasing pH, max I slightly shifts from 350 to 360 nm ($r = 0.669$). The shift of max II (from 387 to 458 nm) with increasing pH ($r = 0.530$) is more expressed compared to human urine. The values of absorbance at 505 nm in yellow-brown and brown urine are therefore considerably greater than the values for human urine (Table 3). With increasing urine pH, the MNP absorption maximum shifts to lower wavelengths ($r = 0.486$; from 495 to 400 nm). Since the rabbit urines were alkaline (pH = 7.5–9.5) and had low NAG activity, the contribution of urinary pigments to MNP absorbance at 505 nm was very high (40–98%).

Correction of the NAG Activity Values for the Influence of Urinary Pigments

The results of the analysis of the absorption spectra of urine and MNP indicate that the contribution of urinary pigments, especially max II, in alkaline and sometimes weakly acidic urine (yellow-brown color) requires a correction of the MNP absorbance at 505 nm. The correction was done in two ways, as described below:

First, the MNP absorbance value at 505 nm was subtracted by the absorbance of urine at the same wavelength. The dilution of urine, which was performed during the determination of NAG activity (dilution factor of 21), was taken into consideration:

$$A_{505\text{corr}} = A_{505\text{MNP}} - A_{505\text{urine}}/21$$

Second, the urinary NAG activity was determined against the blank run, which (as opposed to the standard determination conditions) contained urine instead of distilled water (sample blank), and the absorbance was measured at zero time of incubation ($A_{5050'}$). The absorption spectrum of the incubation mixture at zero time (Fig. 3) indicates that urinary pigments, under the conditions at which the NAG activity is determined,

Fig. 2. Absorption spectra from (a) rabbit urine samples with various pigments and pH values (— light yellow, pH 7.5; -- intensive yellow, pH 8; ···· yellow-brown, pH 8; - · - brown, pH 9) and (b) MNP that was obtained with the same urine.

TABLE 3. Characteristics of rabbit urine absorption spectra

Urine color (number of samples)	pH	Max I (nm)	Max II (nm)	Max MNP (nm)	A_{505} of urine	Contribution of pigments (%)
Light yellow(4)	7.5–8.75	350	—	457–494	0.100–0.220	39–95
Intensive yellow (15)	7.85–9.43	350–359	—	400–476	0.260–0.330	39–95
Yellow-brown (13)	7.5–9.0	358–359	387–413	400–463	0.370–0.494	42–98
Brown (18)	8.98–9.31	358–359	390–458	400–450	0.591–1.198	44–98

have an absorption maximum at 460 nm. If the NAG activity in the sample is low and the urine color is intensive (yellow, brown), the absorption spectrum of the incubation mixture after 30 min of incubation (i.e., the absorption spectrum of MNP) will be deformed (a shift of the maximum toward lower wavelengths will be registered). The contribution of the pigments is eliminated

Fig. 3. Determination of NAG activity against the blank-incubation mixture at zero time (the second correction process). Shown are the absorption spectrum of the incubation mixture at zero incubation time (· · · ·), recorded against the blank run prepared in the standard way; absorption spectrum of the incubation mixture after 30 min of incubation (— · —), recorded against the blank run prepared in the standard way; and absorption spectrum of the incubation mixture after 30 min of incubation (—), recorded against the sample blank-incubation mixture at zero time.

by measuring MNP absorbance at 505 nm against the blank run $A_{5050'}$ (Fig. 3).

No statistically significant difference between the results obtained by applying both correction procedures was found ($P < 0.0001$). A correlation of the NAG activity values that were determined by both procedures for 19 human samples shows good agreement between the results ($r = 0.997$).

Therefore, the influence of pigments in the case of alkaline urine (especially of yellow-brown color) may be successfully eliminated by applying both procedures. Since the first procedure requires only the measurement of urine absorbance at 505 nm, and less time is required for the determination compared to the other procedure, the application of the former in clinical practice is proposed.

In addition to the described correction procedures, it was interesting to analyze whether urinary pigments (especially max II, which appears in alkaline urine) could be removed during urine dialysis. Two weakly acidic human urine samples were dialyzed against a 0.01 mol/L phosphate buffer, pH 7.0, for 2 hr at $+4^{\circ}\text{C}$. The buffer was changed after 1 hr of dialysis. Aliquots of urine samples were taken every 15 min and the absorption spectra were recorded. During the dialysis of urine 1, max II disappeared after 45 min (Fig. 4a), while during the dialysis of urine 2 (pH 9) it disappeared after 15 min (Fig. 4b). Max I was not removed even after 2 hr of dialysis. A longer dialysis time was not investigated, since long analyses are not appropriate for clinical practice. A comparison of the values of urine absorbance at 505 nm before and after removing max II indicated that the contribution of max II to the total pigments was 10% in urine 1 and 37% in urine 2.

Fig. 4. The influence of dialysis on the removal of urinary pigments as a function of dialysis time. **a:** Absorption spectra of urine 1 (pH 6.6, color yellow-brown): · · · · 0 min, — · — 45 min, — 2 hr. **b:** Absorption spectra of urine 2 (pH 5.5, color yellow): 0 min (· · · ·) before and (— · —) after adjusting the pH to 9 (yellow-brown), · · · · 15 min, — 2 hr.

Fig. 5. Dependence of urinary NAG activity on the time of cadmium intake (— without and - - with the correction for pigment influence ($A_{505\text{corr}}$)). **a:** Rabbit 1. All the investigated urine samples were light yellow and yellow, except for the urine samples from days 19 and 25, which were yellow-brown and brown. **b:** Rabbit 2. The urine samples were brown and yellow-brown.

Precision and Accuracy

We investigated the precision of determining the urinary NAG activity by repeating ($n = 16$) the determination in the same urine sample, and applying both procedures of correction for the influence of the pigments ($A_{505\text{corr}}$ and $A_{5050'}$). The variation coefficients were 3.63% and 2.54%, respectively. The accuracy of the method was tested by the standard addition method. The average recovery and standard deviation were $100.73 \pm 1.98\%$.

Application of the Correction during the Determination of NAG Activity

The determination of urinary NAG activity as a parameter for monitoring renal damage is often applied when the nephrotoxic effect of various substances on experimental animals is investigated. All of the rabbit urine samples (the model system in this study), to which, over a period of 4 weeks, 10 mg of Cd/kg of body mass were orally administered daily, were alkaline. In 45 urine samples the pH was >8 . The urine was often brown colored. The absorbance values of MNP at 505 nm were low, i.e., the NAG activities were low. Because of the low absorbance of MNP, the contribution of pigments was very high (40–98%). The determination of NAG activity under these conditions was very unreliable, as was, consequently, the monitoring of renal changes under the influence of cadmium as a function of time. Figure 5 shows the curves of the change in urinary NAG activity with time of cadmium application for two rabbits without and with application of the correction ($A_{505\text{corr}}$) for pigment influence on MNP absorbance. After the correction, the shape of the curve of the

dependence of NAG activity on the time of cadmium application, was changed.

DISCUSSION

The accuracy and reliability of NAG activity determination depend on the stability of the enzyme in urine in vivo from release from the cells to emptying of the bladder, as well as on the influence of urinary pigments and pH of the urine. Investigation of the influence of urine pH on NAG activity showed that a pH change from 6.0 to 8.5 led to a gradual decrease of the total NAG activity by about 20%, which made this determination unreliable (21). It was also shown that the NAG activity in urine, the adjusted pH values of which were higher than 8.5, drastically decreased (by 40% at pH 9, i.e., by 68% at pH 9.8). The obtained results were in agreement with the results of Jung et al. (20), who indicated that enzymes were unstable under alkaline conditions.

On the basis of these results, it may be concluded that alkaline pH values of urine influence NAG activity in two ways: 1) NAG activity decreases because of enzyme instability in alkaline medium, and 2) pigments contribute to the increase of the MNP absorbance at 505 nm (the appearance and shift of max II in alkaline urine), and therefore to the increase of NAG activity value.

In human urine the correction of the value of NAG activity for the influence of pigments (applying either of the correction procedures) should be performed when determining the NAG activity in weak acidic urine of intensive color (yellow-brown, orange). In alkaline urine (pH = 7–8), the increase in NAG activity due to the appearance of max II (up to 20%) and the decrease in activity due to enzyme instability (up to 20%) is often

evened out. In alkaline urine ($\text{pH} > 8$), which is often yellow-brown and brown in color, the enzyme is very unstable and NAG activity distinctly decreases. Therefore, the determination, with or without this correction for pigment influence, is unreliable.

In the urine of rabbits exposed to cadmium, which is often brown colored and alkaline, the contribution of pigments was very high (40–98%). The influence of urinary pigments can be eliminated by correcting for the absorbance, but the influence of pH on total NAG activity cannot be removed. However, the correction is reasonable because the obtained corrected results (curves) give a more reliable presentation of changes in NAG activity with time for rabbits exposed to toxic actions of metal.

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