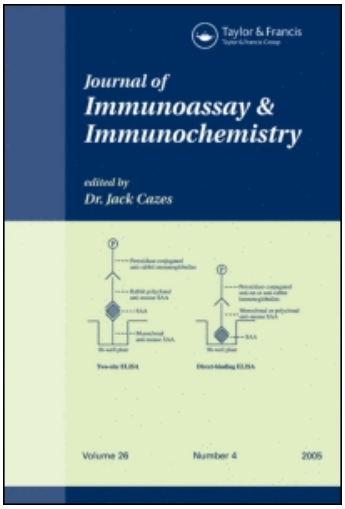

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COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR A SELECTIVE AND SENSITIVE DETERMINATION OF DOPAMINE IN THE PRESENCE OF ASCORBIC ACID AND URIC ACID

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□ *Dopamine (DA) is one of the most important catecholamine neurotransmitter molecules in the central nervous system (CNS). An abnormal level of DA in vivo can cause CNS diseases such as Parkinsonism and Schizophrenia. Thus, it is essential to develop an accurate and easy to use method for determining the level of DA in biological fluids such as urine and serum as a tool for clinical diagnostics as well as for pathological research. This work is the first ELISA application to detect DA in the presence of a high concentration of ascorbic acid (AA) and uric acid (UA) which are endogenous in urine. The LOD value of* 1.26×10^{-9} *M and dynamic ranges of* 3.16×10^{-3} *M to* 3.16×10^{-7} *M were observed. It shows a good sensitivity with a broad dynamic range. Also, the competitive ELISA method for DA developed here showed no interference effect due to AA and UA, which are found in urine. The presence of AA and UA caused interference for DA determination by an electrochemical method because UA, AA, and DA have similar oxidation potential. Also, this selective and sensitive ELISA method can be made into an ELISA test kit which can be used to quantify the level of DA with ease and simplicity in clinics and laboratories.*

Keywords ascorbic acid, catecholamine, dopamine, enzyme-immunoassay, HRP, uric acid

INTRODUCTION

Dopamine (DA) is a neurotransmitter molecule member of the catecholamine family and is a precursor of adrenaline and noradrenaline neurotransmitters in the biosynthetic pathway. It plays very critical roles in the functioning of mammalian central nervous systems;[1] it is related to the leading role in "pleasure and motivation system" in certain activities such as sports, and shortage of DA is a known cause of some nervous diseases, such as Parkinsonism and attention-deficit hyperactivity disorder (ADHD). Thus, it is essential to develop a method for quantitation of DA

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in biological fluids such as urine and serum[2–6] for clinical diagnostics as well as for pathological research. DA usually coexists with uric acid (UA) and ascorbic acid (AA) in biological samples. UA is the primary end product of purine metabolism and AA is the agent which participates in several biological reactions. In biological systems, the concentration of DA is 10^{-8} to 10^{-6} mol L^{-1}, while that of AA is around 10^{-4} mol L^{-1} and it is almost four orders higher than DA.[6–8] Thus, the analytical method needs to be selective for DA in the presence of AA and UA and sensitive due to the low concentration of the substrate in biological fluids.

The analysis of the easily oxidizable compound, DA, using various electrochemical methods[2,3,5,9] based on anodic oxidation are commonly performed. However, the major problem for these methods is that the presence of endogenous AA in urine samples at much higher concentration also oxidizes at nearly same oxidation potential with common electrode materials, thus causing interference in the test.[5,9–11] As a result, poor selectivity and sensitivity results due to the accumulation of oxidized products on the electrode surface. Thus, much work was done for modification of an electrode to overcome these problems.[12–18] Recently, polymer film modified electrodes have attracted great attention because of its good stability and reproducibility gained from the polymer film homogeneity. Zhang et al.[19] reported the poly(styrene sulfonic acid) sodium salt/single-wall carbon nanotube film modified glassy carbon electrode and gave the anodic peak current proportional to the concentration of DA in the range of 1.6×10^{-8} to 6×10^{-4} M, and detection limit (LOD) of 8×10^{-9} M. Lin et al.[20] employed a polymer film of *p*-nitrobenzenazo resorcinol to modified glassy carbon electrode; it shows good electrocatalytic activity for the oxidation of DA. The linear range of 5.0×10^{-6} to 2.5×10^{-5} M and LOD of 3.0×10^{-7} M were observed. It provided to be selective for the quantification of DA in the presence of AA and UA, along with well-defined voltammetric peaks at potentials around 0.390, 0.195, and 0.559 V for DA, AA, and UA, respectively. Other analytical methods that were explored include high performance liquid chromatography and other liquid chromatography methods[4,21–24] and spectrophotometric detection.[25,26] These methods have an advantage of determining individual DA analogues with good sensitivity; however, they have disadvantages of requiring time-consuming, lengthy sample preparation and exhibit low specificity.

Enzyme-Linked Immunosorbent Assay (ELISA) is an immunological technique based on specific interaction of antigens (analyte) and antibodies (binding protein). It has been known as a simple, sensitive, and powerful tool for the selective detection of substances at trace levels in a complex biomatrix. Furthermore, it can be applied to develop test kits in order to evaluate target molecules in a sample matrix effectively in the workplace without lengthy sample preparation.

Recently, we developed a competitive ELISA method to detect and quantify DA in serum with good validation for the first time.[27] A dose-response curve was constructed with an LOD of 1.0×10^{-9} M and a dynamic range of 3.2×10^{-8} to 3.2×10^{-3} M in serum assay. The cross-reactivity study was performed using six cross-reactants which are structurally similar to DA as oxidation products and metabolites of DA: epinephrine, norepinephrine, serotonin, 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid. The results demonstrated that epinephrine and 3-methoxytyramine showed cross-reactivity (18.9%), whereas norepinephrine, serotonin, 3,4-dihydroxyphenylacetic acid, and homovanillic acid showed less than 1% cross-reactivity. However, there is no literature report on the development of a DA immunoassay in the presence of AA and UA.

In this paper, we describe a competitive ELISA method for determination of DA in the presence of AA and UA in a urine matrix for the first time, with high sensitivity and selectivity. Urine consists of several organic and inorganic constituents. Although the urine matrix is complicated, this ELISA method can detect DA in the presence of AA and UA in the urine matrix successfully without sample pre-treatment. Also, this method provides an excellent LOD and a dynamic range of DA in urine with a good reproducibility.

EXPERIMENTAL

Reagents

Dopamine hydrochloride (DA), L-ascorbic acid (AA), uric acid (UA), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal to DA (ab8888) was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP) conjugated goat IgG fraction to rabbit IgG (55676) was supplied from Cappel (Aurora, OH, USA). DA conjugated to BSA was obtained from US Biological (Swampscott, MA, USA). 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) and phosphate–citrate buffer with sodium perborate were purchased from Sigma. Urea, sodium chloride, magnesium sulfate, calcium chloride, sodium carbonate, sodium bicarbonate, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Duksan Pure Chemical (Ansan, Kyonggido, Korea). All chemicals were of analytical-reagent grade or better.

Instrumentation

Enzymatic activity was measured using an E_{MAX}® precision microplate reader (Molecular Device Co., Sunnyvale, CA, USA) at 450 nm. ELISA was

performed in Immulon 4 HBX Flat bottom microtiter plates (high binding) (Dynex technologies Inc., USA) and the 96-well plates were washed using Multiwasher III (Tricontinent, Grass valley, CA, USA). The pH values were measured with an Orion pH meter (Thermo Electron Corporation, Beverly, MA, USA).

Buffers and Artificial Urine

All solutions were made using triple deionized distilled water (Milli-Q water purification system, Millipore, Billerica, MA, USA). The coating buffer was a 50 mM bicarbonate buffer (pH 9.6). A 10 mM phosphate buffered saline (PBS) containing 0.01% Tween 20® (pH 7.2) was used as the assay buffer. The wash buffer was a 10 mM PBS containing 0.05% Tween 20® (pH 7.2). The blocking solution was 3% BSA in 10 mM PBS. The substrate buffer was a 50 mM phosphate-citrate buffer (pH 5.0). The substrate solution was prepared by adding a TMB tablet to 10 mL phosphate-citrate buffer. The quenching solution was 2 M H_2SO_4. All buffers were filtered through a 0.20 μm pore size filter before use.

Artificial urine solution (pH 6.3) is prepared, consisting of 1.94% urea, 0.80% NaCl, 0.11% $MgSO_4 \cdot 7H_2O$ and 0.06% $CaCl_2$ (% w/w).[28]

Dose-Response Curve for DA in Artificial Urine

The sensitivity of the competitive assay is regulated by concentrations of primary antibody and competitor immobilized on the plate. Optimization was performed by varying concentrations of both. After optimization, 5 μg mL^{-1} BSA-DA conjugate and 1/7500 dilution anti-DA antibody were selected as the competitor and the primary antibody, respectively. A 100 μL of BSA-DA conjugate was coated onto a plate well for 2 h. Then, 300 μL of blocking buffer was added followed by incubation for an additional 30 min. The anti-DA-antibody was diluted with the assay buffer. Using the artificial urine, a stock solution of DA standard (2×10^{-2} M) was prepared and serially diluted to range in concentration from 2×10^{-2} to 2×10^{-12} M. 50 μL of various concentrations of standard DA solution and anti-DA antibody solution were added to the wells, followed by incubation for 1 h. 100 μL of HRP-goat rabbit IgG conjugate (1/5000 of total protein 13.7 mg mL^{-1}) in assay buffer was added to each well, followed by incubation for 30 min. 100 μL of substrate solution was added to each well and incubated for 10 min. The color development was stopped by adding 50 μL of the quenching solution. Then, the absorbance was measured at 450 nm. All absorbance intensities reported are the average of a minimum of triplicates and have been corrected for the contribution of the blank.

Between incubation steps, the assays were performed at RT with constant shaking and wells were washed three times with 300 µL wash buffer.

Specificity Study

A cross-reactivity study was performed with the AA and UA in the same manner as the studies conducted for DA assay, in artificial urine. The interference effect was also investigated for DA in the presence of AA and UA, respectively, in artificial urine, using UA (1.0×10^{-4} M) and three different concentrations of AA (0.5×10^{-4}, 1.0×10^{-4} and 2.0×10^{-4} M) in artificial urine.

Recovery Study

A recovery study was performed using urine samples spiked with known amounts of DA. Free DA (19.0 µg mL^{-1}, 1.90 µg mL^{-1}, 0.19 µg mL^{-1} and 0.060 µg mL^{-1}) was spiked to artificial urine, and percent recovery was calculated as follows; % recovery = (concentration obtained from assay)/(spiked concentration) × 100.

RESULTS AND DISCUSSION

The chemical structure of DA (Fig. 1) is 4-(2-aminoethyl)benzene-1,2-diol and it has three ionization states[29] in an aqueous environment; The first deprotonation step ($pKa_1 = 8.61$) takes place at C_2-OH, generating the zwitterions with negatively charged and positively charged species.

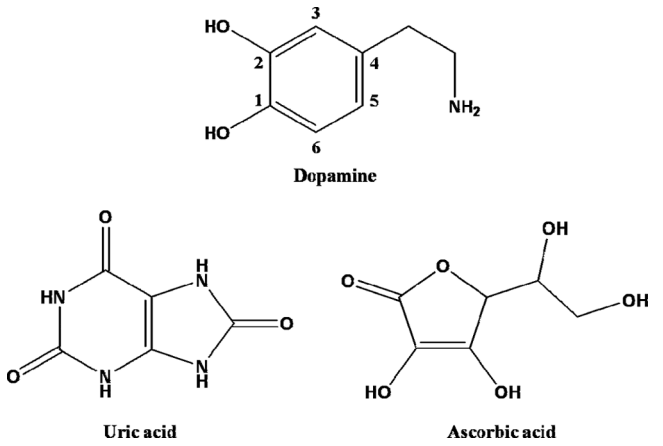

FIGURE 1 Chemical structures of dopamine, uric acid, and ascorbic acid.

The second one ($pKa_2 = 9.95$) occurs at C_4-NH_3. The third deprotonation ($pKa_3 = 12.04$) step takes place at C_1-OH, generating a di-anion, and it finally results in a quinoid form. Thus, free DA is unstable and deteriorates in alkaline solution. The increased pH value is critical for DA oxidation in addition to the presence of oxygen and light. As mentioned in our previous work,[27] the immunoassay conditions for DA were optimized; competitive assay was employed to reduce the assay time, and the assay was performed under acidic to neutral pH value and dark environment to prevent DA oxidation. Also, HRP was employed as a signal-generator along with TMB substrate, which was prepared in phosphate-citrate buffer at pH 5.0.

This competitive immunoassay was performed using various concentrations of BSA-DA conjugate ($3\,\mu g\ mL^{-1}$, $5\,\mu g\ mL^{-1}$, and $10\,\mu g\ mL^{-1}$) which was immobilized on the plates and primary antibody (1/5000, 1/6000, 1/7500, and 1/10000 dilution) because the assay sensitivity is dependent on two major variants, i.e., concentrations of competitor and antibody. Based on the results of the optimization work, the concentrations of BSA-DA conjugate and anti-DA antibody were selected as $5\,\mu g\ mL^{-1}$ and 1/7500 dilution, respectively. With a serial dilution of DA in artificial urine, the dose-response curve and calibration curve were constructed. Figure 2 shows the dose-response curve and calibration curve obtained for DA in artificial urine. The LOD and the dynamic range were $1.26 \times 10^{-9}\,M$ ($0.24\,\mu g\ L^{-1}$) and $3.16 \times 10^{-7}\,M - 3.16 \times 10^{-3}\,M$, respectively. LOD as defined here is the concentration of the absorbance value corresponding to the three times the standard deviations of the mean absorbance value. The calibration curve in urine was calculated; it indicated linearity in the dynamic range. The least-squares regression of this section gives the following relationship: Absorbance $= -0.0897 \log[DA] + 0.0221$, $R^2 = 0.9917$.

It is important to assure the absence of a variety of interferences caused by endogenous components existing in biological fluids, in order to have a useful assay technique to determine a target-molecule from an actual sample. As a part of the evaluation of the immunoassay, the cross-reactivity study for antibody was performed. Assay specificity indicates the ability of an antibody to generate a measurable target molecule. It is known that AA is usually present at $10^{-4}\,M$ concentration, while DA is at $10^{-8} - 10^{-6}\,M$ level[16] in biological fluids. Here, a study was performed using two known interferences, UA and AA, for DA assay in urine. (Fig. 3) The cross-reactivity studies were carried out by a competitive ELISA by adding various free cross-reactants at different concentrations to compete with antigen coated on the surface, to bind with the antibody. Their 50% B/B_0 values were estimated and then the individual percent cross-reactivity was calculated: % cross-reactivity = (concentration of DA giving 50% B/B_0)/(concentration of cross-reactant giving 50% B/B_0). B/B_0 is the ratio of response B, to the maximum response when no analyte is present (B_0).

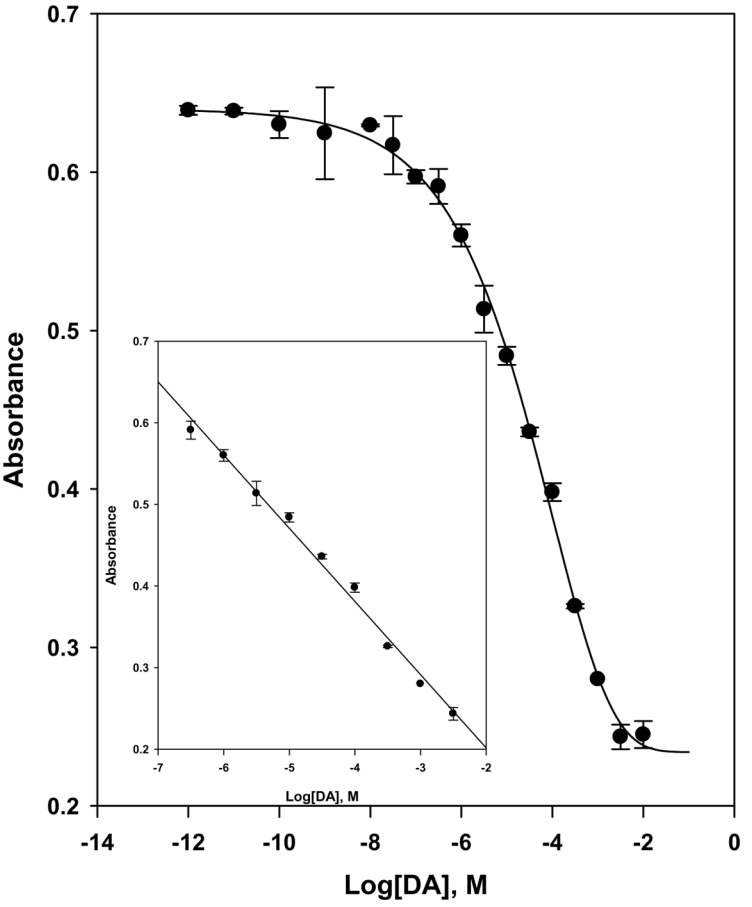

FIGURE 2 Dose-response curve and calibration curve (inset) for DA in urine. Absorbance $= -0.0897$ log[DA] $+ 0.0221$ ($R^2 = 0.9917$). Data points are the average plus $\pm$ one standard deviation ($n = 5$).

In the case of UA (▼), the absorbance of UA did not change for all concentrations of UA tested, from which we concluded that the anti-dopamine antibody used fails to recognize UA. A cross-reactivity study was also performed for AA (O), and it showed that the signal declined as the concentration of AA increased. This result demonstrated less than zero percent cross-reactivity with DA according to the 50% B/B_0 value. Because of the interaction between anti-dopamine antibody and competitor, BSA-DA immobilized in the well was obstructed by AA at higher concentrations. Therefore, an experiment was performed to determine how the coexisting AA influenced the DA assay. Thus, an interference study was performed for DA in urine containing three different concentrations of AA (0.5×10^{-4}, 1.0×10^{-4} and 2.0×10^{-4} M). Figure 3 shows the dose-response curve for DA with 1.0×10^{-4} M of AA (△). As seen in Fig. 3, dose-response curves

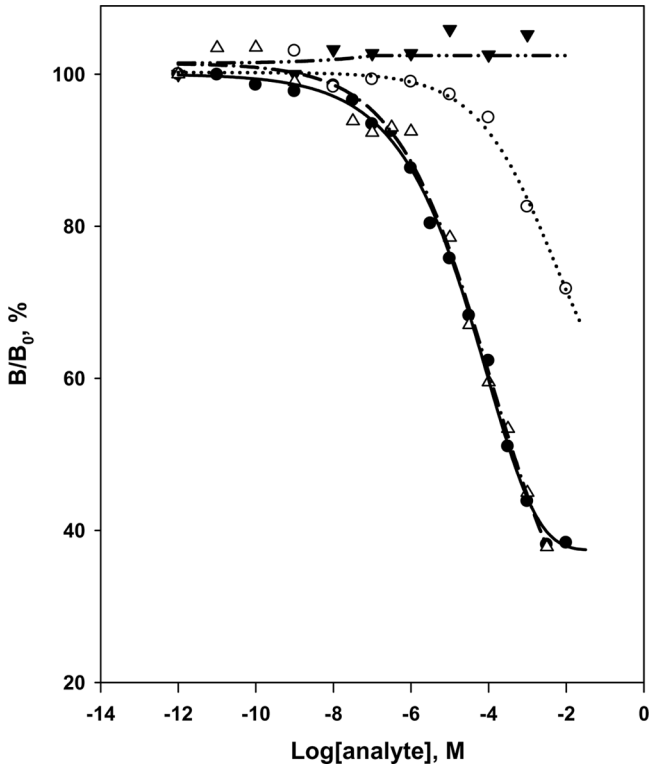

FIGURE 3 Interference study for interference-reactants: dopamine (●), ascorbic acid (○), uric acid (▼) and dopamine containing 1.0×10^{-4} M ascorbic acid (△).

showed no difference between • (for DA assay) and △ (for DA assay with 1.0×10^{-4} M of AA). Assay sensitivity, LOD and dynamic range showed the same values, respectively.

Using other concentrations of AA (0.5×10^{-4} and 2.0×10^{-4} M), an identical trend of dose-response curve was observed for the 1.0×10^{-4} M AA case. This meant that the coexisting high-concentration of AA had no

TABLE 1 Percent Recovery of Dopamine in Urine

Spiked Concentrations (μg mL^{-1})	Measured Concentrations (μg mL^{-1})	Recovery (%)[a]
19.0	19.9 ± 1.62	104
1.90	1.81 ± 0.10	95
0.19	0.18 ± 0.010	95
0.060	0.064 ± 0.0050	106

[a] % recovery is calculated as follows: (Concentration obtained from immunoassay)/(spiked concentration) × 100%, (n = 10).

TABLE 2 Analytical Data for Dopamine Compared with Other Studies

Method	LOD	Linear Range	Matrix	Correlation Coefficient	Reference
Electrochemical method	0.5 nM	1.0×10^{-9} M ~ 5.0×10^{-4} M	serum, urine	–	[2]
Electrochemical detection	1.4 μM	2.0×10^{-6} M ~ 7.0×10^{-5} M	urine	0.9926	[5]
Carbon ionic liquid electrode (CILE) detection	1.0 μM	2.0×10^{-6} M ~ 1.5×10^{-3} M	serum, urine	–	[8]
Electrochemical detection	50 nM	5.0×10^{-6} M ~ 3.0×10^{-5} M	serum	0.9998	[15]
SPE-LC-ECD	24 μg L^{-1}	50 μg L^{-1} ~ 500 μg L^{-1}	urine	0.9965	[18]
HPLC-fluorescence	70 nM	1.0×10^{-6} M ~ 1.0×10^{-4} M	urine	0.998	[22]
Methanol plug assisted sweeping-MEKC	4.7 nM	5.0×10^{-7} M ~ 0 M	urine	0.9997	[24]
Spectrophotometry	0.12 mg L^{-1}	0.16 mg L^{-1} ~ 40 mg L^{-1}	urine	0.9993	[25]
Indirect chemiluminescence detection	0.18 μM	2.6×10^{-7} M ~ 5.3×10^{-5} M	urine	0.9698	[26]
ELISA	1.26 nM (0.24 μg L^{-1})	3.16×10^{-7} M ~ 3.16×10^{-3} M	urine	0.9917	This work

effect on detection of DA. In other words, DA antibody interacted with only dopamine, specifically. Therefore, the competitive ELISA method developed here to detect the DA can apply without any interference effect due to AA and UA, which caused big interferences to DA analysis by electrochemical methods.

The accuracy of the method was measured by the % recovery of spiked samples. The recovery study was performed by spiking four different concentrations (19.0, 1.90, 0.19, and 0.060 μg mL^{-1}) of DA in artificial urine solution. Mean percent recoveries were analyzed and the percent recovery was calculated. As seen in Table 1, the results indicated 95%–106% recovery, which was satisfactory.

In summary, the competitive enzyme linked immunosorbent assay method showed a selective and sensitive detection of DA in the presence of high concentrations of AA and UA in urine samples without any interference. An LOD value of 1.26×10^{-9} M and a wide dynamic range (3.16×10^{-3} M to 3.16×10^{-7} M) was observed. Table 2 represents the comparison of analytical data obtained for DA with other methods in biological fluids. An electrochemical method based on a gold nanoparticle modified ITO (Au/ITO) electrode detecting DA in urine was described by Goyal et al.[2] They obtained an LOD of 0.5 nM and a working range from 1.0×10^{-9} M to 5.0×10^{-4} M. Feng et al.[15] also applied electrochemical detection based upon a glassy carbon electrode modified by the hybridization adduct of Fc-SWNTs and obtained an LOD of 50 nM and a dynamic range of 5.0×10^{-6} M – 3.0×10^{-5} M. This work was the first ELISA application to detect DA in the presence of high concentrations of AA and UA in urine. It showed a good sensitivity with broad dynamic range, with no interference from AA and UA which coexist in urine and, thus, causes interferences for DA determination by electrochemical methods. Also, this ELISA method can apply to the ELISA test kit with simplicity and ease of used for determination of DA in therapeutic approach and quantitative analysis of DA.

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