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Determination of berberine in pharmaceutical preparations using acidic hydrogen peroxide–nitrite chemiluminescence system

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A stronger chemiluminescence (CL) was observed when hydrogen peroxide was mixed with nitrite and berberine in sulfuric acid solution. The stronger CL originated from peroxidation of berberine by peroxynitrous acid that was synthesized online by the mixing of acidic hydrogen peroxide solution with nitrite solution in a flow system. The emitting species was excited state oxyberberine, a peroxidized product of berberine. Based on the stronger CL, a flow injection CL method for the determination of berberine was proposed. Under optimum experimental conditions, the stronger CL intensity was linearly related to the concentration of berberine over the range of 2.0×10^{-7}–2.0×10^{-5} mol L^{-1}. The limit of detection ($s/n = 3$) was 6.2×10^{-8} mol L^{-1}. The proposed method has been evaluated by analyzing berberine in pharmaceutical preparations.

Keywords: chemiluminescence; flow-injection; peroxynitrous acid; berberine

Introduction

Berberine is an isoquinoline derivative alkaloid isolated from a variety of medicinal plants such as *Coptis chinensis* Franch and *Phellodendron amurense* Rupr. It is widely used as medicine for stomach and bowel ailments[1,2] and can inhibit the multiplication of bacteria, fungi, and viruses.[3] Several methods have been reported for the quantitative determination of berberine. Spectrophotometric method was based on the formation of a binary ion-associate complex between berberine and bromophenol blue[4] or a ternary ion associate complex among berberine, cinchona-alkaloid, and eosin.[5] The formed binary and ternary ion-associate complexes were subsequently extracted into organic solvents such as *o*-nitrophenyl octyl ether and 1, 2-dichloroethane and measured at 610 and 536 nm, respectively. Spectrofluorimetric method has been extensively utilized in biological samples and dosage forms. It was based on berberine intrinsic fluorescence enhanced by the formation of an inclusion complex of berberine with butylated-β-cyclodextrin or cucurbit,[6,7] or based on quenching of the fluorescence of thioglycolic acid-capped CdTe quantum dots (TGA-CdTe QDs) by berberine in aqueous solution.[8] Liquid chromatography using tandem mass spectrometry (MS/MS) or electrospray ionization–mass spectrometry (LC–ESI–MS) as detector was used in rat or human plasma.[9,10] Capillary zone electrophoresis (CZE) using ultraviolet absorption at 254 nm can be utilized in the extract of the traditional Chinese medicinal herb.[11] All these methods, however, require expensive instruments or lack sensitivity.

Compared with the methods mentioned above, chemiluminescence (CL) methods have the advantages of high sensitivity, linear dynamic range, and simple instrumentation.[12] But up to now, only two CL methods for the determination of berberine were reported. One was based on berberine inhibition on the CL reaction of luminol with ferricyanide in alkaline solution.[13] The other was based on chemical oxidation of berberine by acidic permanganate.[14] These two methods suffer from high background luminescence from chemical oxidation of luminol by ferricyanide, or overlapping between the CL band and the absorption band of acidic permanganate itself.[15]

The weak CL reaction of nitrite with hydrogen peroxide in acidic medium has been studied in detail. The reaction of hydrogen peroxide with nitrite first forms *cis*-peroxynitrous acid (*cis*-ONOOH),[16] and then part of the formed *cis*-ONOOH convert into *trans*-peroxynitrous acid (*trans*-ONOOH).[17] The *trans*-ONOOH can further isomerize to excited state peroxynitrous acid (ONOOH*).[17] A weak CL is emitted during the isomerization of ONOOH* into nitrate.[18] As peroxynitrous acid (including *cis*-ONOOH, *trans*-ONOOH and ONOOH*) possesses both strong oxidizing and peroxidizing ability and ONOOH* has sufficiently high energy, the acidic hydrogen peroxide–nitrite weak CL system has potential application in pharmaceutical and biomedical analysis.[19,20] To date, reports on its application in CL analysis are scarce.[21–24]

In this work, a stronger CL was observed when berberine was present in acidic hydrogen peroxide–nitrite weak CL system. The stronger CL mechanism was discussed in detail, and moreover a flow injection CL method was proposed for the determination of berberine. The method has been evaluated by analyzing berberine in pharmaceutical preparations.

Materials and methods

Chemicals and materials

All solutions were prepared from analytical reagent grade reagents. 1.00×10^{-3} mol L^{-1} berberine stock solution was prepared by

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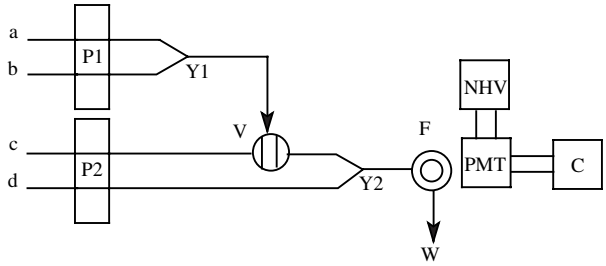


Figure 1. Schematic diagram of the flow injection CL manifold used for the determination of berberine: P1 and P2, peristaltic pump; Y1 and Y2, three-way pipe; V, six-way valve; F, flow cell; W, waste; NHV, negative high voltage; PMT, photomultiplier tube; C, computer; a, berberine solution; b, 0.13 mol L^{-1} nitrite solution; c, 0.10 mol L^{-1} sulfuric acid carrier solution; d, 0.25 mol L^{-1} hydrogen peroxide solution.

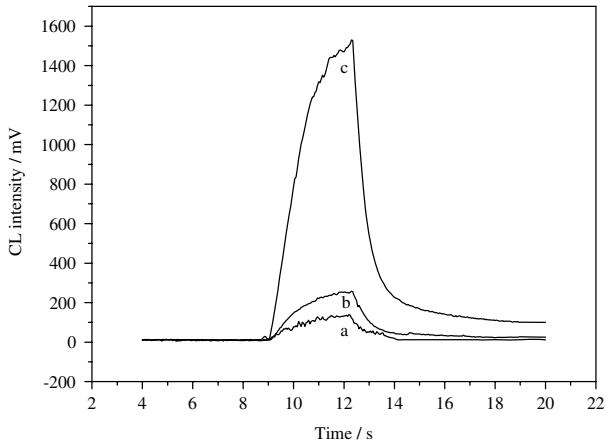


Figure 2. CL dynamic response curves of reaction of 0.25 mol L^{-1} hydrogen peroxide in 0.10 mol L^{-1} sulfuric acid medium with (a) 0.13 mol L^{-1} nitrite, (b) 1.0×10^{-6} mol L^{-1} berberine and (c) 0.13 mol L^{-1} nitrite and 1.0×10^{-6} mol L^{-1} berberine.

dissolving 0.0408 g berberine hydrochloride (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) in hot water and diluting to 100 ml with water. The stock solution was stored at about 4 °C in a dark bottle. Other standard working solutions of berberine were obtained by diluting the stock solution with water. The hydrogen peroxide used was prepared from a 30% H_2O_2 solution (Xi'an Chemical Reagent Plant, Xi'an, China). The 0.25 mol L^{-1} H_2O_2 solution was standardized with standard potassium permanganate. The 0.13 mol L^{-1} nitrite solution was prepared by dissolving $NaNO_2$ (Beijing Chemical Reagent Company, Beijing, China) in water. Twice distilled water was used throughout the experiments.

Apparatus and manifold

The flow injection system used (Figure 1) consisted of two peristaltic pumps and a six-way injection valve. PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. One peristaltic pump was used to deliver nitrite and berberine solutions. The nitrite solution was mixed with berberine solution in the first Y-shaped mixing element (Y1), and then the mixing solution was injected into carrier solution using a six-way valve equipped with an 80 µl sample loop. The other peristaltic pump was used to deliver the carrier (sulfuric acid) and hydrogen peroxide solutions. A mixing coil (glass tubing, 100 mm × 1 mm i.d.) after the second Y-shaped mixing element (Y2) was used as flow cell, and was positioned in front of a photomultiplier tube (PMT) (Model R105UH, Hamamatsu, Japan). The CL signal produced in the flow cell was collected with the PMT and recorded with a computer equipped with CL analysis system software (Xi'an Remax Electronic Science-Tech Co. Ltd., China). The PMT was operated at −850 V. The fluorescence spectra were monitored using RF-540 fluorescence spectrometer (Shimadzu, Japan).

General procedure

As shown in Figure 1, flow lines were inserted into berberine solution, nitrite solution, sulfuric acid solution, and hydrogen peroxide solution, respectively. By keeping the valve in washing position, sulfuric acid, and hydrogen peroxide solutions were continuously pumped into the manifold until the baseline was established on the recorder. Berberine and nitrite solutions were mixed via Y1 and injected into sulfuric acid carrier stream through a six-way injection valve. The carrier stream was then merged with a stream of hydrogen peroxide in Y2 before flow cell. When the mixed solution flowed into the cell, CL reaction took place. The CL signal produced was measured. The concentration of berberine was quantified by measuring the CL intensity. The flow rates of P1 and P2 were set at 2.0 and 5.5 ml min^{-1}, respectively.

Procedure for CL kinetic profiles and CL spectrum

The CL kinetic profiles were obtained using batch method. 5.0 ml hydrogen peroxide–sulfuric acid solution was added to a reaction cell in front of the PMT. Then 5.0 ml of berberine solution, nitrite-berberine solution or nitrite solution was separately injected into the reaction cell through a fill orifice by an injector. The CL signal produced was recorded.

The CL spectrum in the range of 400–680 nm was achieved with a set of interference filters. The filters were set between the mixing coil and the PMT. Other procedures described as general procedure were used to obtain the CL emission at different wavelength bands.

Results and discussion

Characteristics of CL

Experiment showed that a weak CL phenomenon was observed (Figure 2, line a) when nitrite solution was injected into hydrogen peroxide/sulfuric acid solution. This weak CL resulted from excited state peroxynitrous acid.[16] When nitrite was substituted with berberine, another weak CL phenomenon appeared (Figure 2, line b). However, on injection of berberine/nitrite solution into hydrogen peroxide/sulfuric acid solution, a stronger CL was recorded (Figure 2, line c). Obviously, this stronger CL resulted from the interaction between berberine and peroxynitrous acid synthesized online.

In order to obtain more information about the stronger CL of berberine in hydrogen peroxide-nitrite-sulfuric acid solution, two kinds of experiments were carried out. On the one hand, various oxidants were used instead of peroxynitrous acid to check the CL behaviour of berberine. Experiment showed that the mixing of periodate, peroxydisulfate or cerium (IV) solution with berberine/sulfuric acid solution didn't give CL. While hydrogen peroxide was mixed with periodate/berberine/sulfuric acid solution, a mediate CL was observed. The mediate CL was due to the interaction of berberine with singlet oxygen ($^1O_2$) that

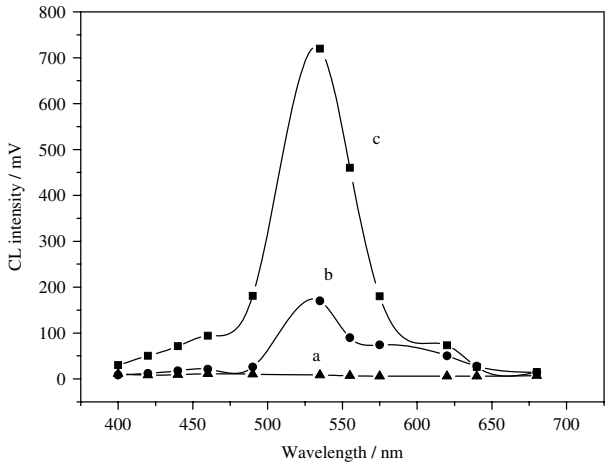
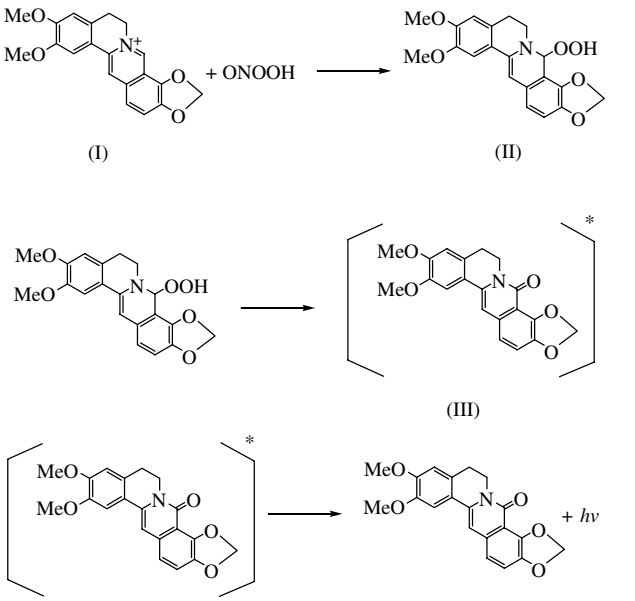


Figure 3. CL spectra in 0.25 mol L^{-1} hydrogen peroxide–0.13 mol L^{-1} nitrite–0.10 mol L^{-1} sulfuric acid solution (a), in 0.25 mol L^{-1} hydrogen peroxide–5.0 × 10^{-6} mol L^{-1} berberine–0.10 mol L^{-1} sulfuric acid solution (b) and in 0.25 mol l^{-1} hydrogen peroxide–0.13 mol L^{-1} nitrite–5.0 × 10^{-6} mol L^{-1} berberine–0.10 mol L^{-1} sulfuric acid solution (c).



Scheme 1. The proposed CL reaction mechanism of berberine in hydrogen peroxide/nitrite/sulfuric acid solution.

was produced by the reaction of acidic periodate with hydrogen peroxide.[25,26] From chemical properties of hydrogen peroxide, singlet oxygen and peroxynitrous acid,[27–29] it is deduced that all the CL phenomena mentioned above are due to the peroxidation of beberine by these peroxidants.

On the other hand, the fluorescence and CL spectra were examined. The fluorescence spectra were recorded in the range of 400–700 nm. Two fluorescence peaks (λ_{max} = 460, 530 nm) were observed in berberine/sulfuric acid solution. The fluorescence peaks were from berberine itself.[30] After hydrogen peroxide solution was added into the berberine/sulfuric acid solution, the fluorescence spectrum hardly changed while the peak intensity at 460 and 530 nm decreased slightly. Moreover, after adding a small amount of nitrite solution into berberine-hydrogen peroxide-sulfuric acid solution, the two fluorescence peaks almost disappeared and no new fluorescence emission was observed. This is because peroxidation of berberine by peroxynitrous acid for a long time (several minutes) in the static test results in the decomposition of berberine.[22]

The CL spectra were recorded in range of 400–680 nm. When berberine was absent, no CL spectrum from hydrogen peroxide/nitrite/sulfuric acid solution was observed (Figure 3, line a). The reason is that the CL from ONOOH* is too weak to penetrate the interference filters. In the presence of berberine, the CL spectrum of berberine in hydrogen peroxide/sulfuric acid solution (Figure 3, line b) shows one CL peak band at 460–575 nm, which is identical with that in hydrogen peroxide–nitrite–sulfuric acid solution (Figure 3, line c). The CL peak band (460–575 nm) is in good agreement with the CL spectra of excited state isoquinolinone (λ_{max} = ~526 nm),[31] excited state oxypalmatine (λ_{max} = ~512 nm) that is very similar to oxyberberine in molecular structure and excited state organic molecules containing carbonyl group (>C═O)* (λ_{max} = ~520 nm).[32,33] These results indicate that the excimer of berberine in both hydrogen peroxide/sulfuric acid and hydrogen peroxide/nitrite/sulfuric acid solutions is excited state oxyberberine (oxyberberine*).

Based on above results, the CL reaction mechanism of berberine in hydrogen peroxide/sulfuric acid and hydrogen peroxide/nitrite/sulfuric acid solutions was proposed as follows. Hydrogen peroxide or peroxynitrous acid peroxidized berberine (I) to a peroxide (II),[34] which subsequently decomposed to oxyberberine* (III).[35] A CL was observed when oxyberberine* went back to its ground state. Because peroxynitrous acid possesses much stronger peroxidizing ability than hydrogen peroxide, thus more light was emitted when hydrogen peroxide was replaced with peroxynitrous acid. The possible CL reaction mechanism of berberine in hydrogen peroxide–nitrite–sulfuric acid solution is shown in Scheme 1.

Optimization of experimental variables

Based on the stronger CL of berberine in hydrogen peroxide/nitrite/sulfuric acid solution, a flow injection CL method for the determination of berberine was proposed. The analytical conditions were optimized using 1.0 × 10^{-6} mol L^{-1} berberine standard solution. The parameters optimized included selection of carrier solution, nitrite, and hydrogen peroxide concentrations, and the flow rate for the flow injection system.

Selection of carrier solution

The formation of peroxynitrous acid through the reaction of nitrite with hydrogen peroxide needs the presence of acid as catalyst.[36] Therefore, in such strong inorganic acid solution as HCl, H_2SO_4, HNO_3 and H_3PO_4, the stronger CL of berberine was recorded. When sulfuric acid was used as carrier solution, not only was the maximum ratio of the stronger CL signal of berberine to the background CL signal from the reaction of nitrite with hydrogen peroxide obtained, but also good reproducibility of the stronger CL signal of berberine was achieved. The effect of sulfuric acid concentration on the CL intensity in the presence of 1.0 × 10^{-6} mol L^{-1} berberine was examined in the range 0.02–0.20 mol L^{-1} sulfuric acid. The strongest CL intensity was obtained at the concentration of 0.10 mol L^{-1} sulfuric acid. Therefore, 0.10 mol L^{-1} sulfuric acid was chosen in further experiments.

Effect of hydrogen peroxide concentration

The effect of hydrogen peroxide concentration on the CL intensity in the presence of 1.0×10^{-6} mol L^{-1} berberine was investigated over the range of 0.10–0.40 mol L^{-1}. The CL intensity increased with increasing hydrogen peroxide concentration in the range of 0.04–0.25 mol L^{-1}. Above 0.25 mol L^{-1} hydrogen peroxide, the CL intensity decreased because higher hydrogen peroxide concentration caused decay of the synthesized peroxynitrous acid.[37] Therefore, 0.25 mol L^{-1} hydrogen peroxide was chosen for the following experiments.

Effect of nitrite concentration

The effect of nitrite concentration on the CL intensity was examined by using 1.0×10^{-6} mol L^{-1} berberine standard solution. The CL intensity rose with the increasing of nitrite concentration from 0.02–0.13 mol L^{-1} and reached its maximum value at 0.13 mol L^{-1}. The raising of nitrite concentration over 0.13 mol L^{-1} caused the decrease of the CL intensity. The decrease resulted from the fast reaction of nitrite with hydroxyl radical (·OH) radical ($k = 1 \times 10^{10}$ mol^{-1} L s^{-1}, rate constant) that was the decomposition product of *trans*-ONOOH.[38] Therefore, 0.13 mol L^{-1} nitrite was chosen for the following experiments.

Effect of flow rate

Pump P2 was used to deliver the carrier (sulfuric acid) and hydrogen peroxide solutions. Hydrogen peroxide solution and sulfuric acid carrier solution that contained nitrite and sample were first mixed at the second Y-shaped mixing element (Y2), and then the mixed solution was delivered by pump P2 to the flow cell placed in front of the PMT. The longer the distance between Y2 and the flow cell is, the less the peroxynitrous acid reaches to the flow cell because the produced peroxynitrous acid is a short-lived species ($t_{1/2}$ *ca.* 1 s).[39,40] Except that the distance between Y2 and the flow cell is shorten as possible, the raising of the flow rate of pump P2 is favourable to increase the amount of peroxynitrous acid reaching to the flow cell. The effect of its flow rate on the CL intensity was examined in the range of 1.5 to 7.0 ml min^{-1}. The results showed that CL signal increased sharply with the increasing of flow rate in the range of 1.5 to 5.1 ml min^{-1}, and reached a maximum value at a higher flow rate. Thus a flow rate of 5.5 ml min^{-1} was selected for pump 2.

Interferences study

In order to assess the possible analytical application of the proposed CL method, the interference of commonly used excipients and additives, co-existing ions and compounds was examined. The solutions used for this purpose contained 1.0×10^{-6} mol L^{-1} berberine and increasing amounts of interfering species. A substance was considered no interference if the variation of the CL intensity was within ±5%. The results of interference were shown in Table 1. As showed in Table 1, 5-fold Fe^{2+}, Fe^{3+}, Co^{2+} and Cu^{2+} cause positive inference, which may be from the reaction of berberine with hydroxyl radical that was produced from the reaction of hydrogen peroxide with these ions mentioned above in acid solution.[41]

Analytical characteristics

Under the selected experimental conditions, the linear range was 2.0×10^{-7} to 2.0×10^{-5} mol L^{-1}. The regression equation was $I = 15.2 + 12.8 \times 10^{7}\ C$ (where I is CL intensity and C is berberine concentration, units are mV and mol L^{-1}, respectively) with a correlation coefficient of 0.9996 ($n = 11$). The reproducibility of the proposed method was good, shown by RSD of 0.9% for nine replicate determinations of 1.0×10^{-6} mol L^{-1} berberine standard solution. The detection limit was 6.2×10^{-8} mol L^{-1} which was calculated according to IUPAC definition that is three times of standard deviation of blank value.[42] The sample measurement frequency was calculated about 50 samples h^{-1}.

Table 1. The tolerable concentration ratios with respect to 1.0×10^{-6} mol L^{-1} berberine for some interfering species

Substance	Tolerable concentration ratio
Cation	
Zn^{2+}, K^{+}, Na^{+}, NH_4^{+}	1000
Pb^{2+}, Mg^{2+}, Al^{3+}	500
Mn^{2+}, Ni^{2+}, Ca^{2+}, Cd^{2+}, Ag^{+}	100
Cu^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}	5
Anion	
Cl^{-}, SO_4^{2-}, PO_4^{3-}, NO_3^{-}, Ac^{-}	1000
Vitamin	
Thiamine hydrochloride (vitamin B_1)	1000
Riboflavin (vitamin B_2), folic acid (vitamin Bc)	100
Ascorbic acid (vitamin C)	50
Amino acid	
L-Threonine, L-serine, L-arginine, L-glutamic acid,	1000
L-Valine, L-cystine	500
L-Histidine, L-tyrosine, L-lysine	100
Others	
Oxalic acid, starch, urea, uric acid	1000
Glucose, sucrose, trimethoprim	100
Polyethylene glycol 6000, sodium lauryl sulfate	50

Analytical application

The proposed method was applied to the determination of berberine in capsules. The sample solutions were prepared by dissolving amount of the powder equivalent to about 200 mg berberine in hot water from the well-proportioned mixture of ten berberine capsules. The resulting mixture was filtered and diluted to 100 ml with water. An appropriate of the sample solution was further diluted with water so that the final berberine concentration was in the working range. Other measurements were completed as general procedure mentioned above. The results were shown in Table 2, which agreed well with those obtained by potassium dichromate titrimetry (Pharmacopoeia method).[43] Moreover, recovery studies were also carried out on each sample solution to which known amounts of berberine standard solution were added. Each recovery was calculated by comparing the results obtained before and after the addition. As shown in Table 2, the recoveries were between 95 and 105%.

Conclusions

A new flow injection CL method for the determination of berberine was described, which was based on the peroxidation of berberine

Table 2. Results for the determination of berberine hydrochloride in capsules

Sample	Labelled (mg)	Proposed method[a] (mg)	Official method[b] (mg)	Added ($\times 10^{-6}$ mol L^{-1})	Found[c] ($\times 10^{-6}$ mol L^{-1})	Average recovery (%)
1	100	98.8 ± 0.8	99.7 ± 0.6	0.60	0.63 ± 0.02	105
				2.00	1.90 ± 0.02	95
				8.00	8.16 ± 0.11	102
2	100	97.1 ± 1.1	97.7 ± 0.7	0.60	0.58 ± 0.02	97
				2.00	1.98 ± 0.01	99
				8.00	8.31 ± 0.14	104

[a,b,c] Mean value ± S.D. ($n = 5$).

by peroxynitrous acid in sulfuric acid solution. As compared to the CL methods,[13,14] the proposed method is simpler and more selective. In addition, it could also be applied to simultaneous determination of protoberberine class of isoquinoline alkaloids such as palmatine and berberine in natural medical plants or traditional Chinese medicines if it was used as the detector in capillary electrophoresis or HPLC.

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