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Determination of Boronophenylalanine in Biological Samples Using Precolumn *o*-Phthalaldehyde Derivatization and Reversed-Phase High-Performance Liquid Chromatography[1]

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A reversed-phase high-performance liquid chromatographic method for the detection of boronophenylalanine is described. Determination was obtained by precolumn reaction of *o*-phthalaldehyde with a mixture of standard amino acids containing boronophenylalanine and separating the corresponding *o*-phthalaldehyde derivatives, using a Kromasil C-18, 250 × 4.6 mm, 5-μm particle size column, a step gradient with two buffers, a flow rate of 1.2 ml/min, a column temperature of 23°C, and fluorimetric detection (excitation and emission wavelengths of 330 and 430 nm, respectively). The use of such a method for assaying boronophenylalanine in biological samples was tested in neutralized perchloric acid blood and cerebral tissue extracts of rats treated with intracarotid administration of 300 mg/kg of body weight boronophenylalanine. Results of these experiments showed that the present HPLC method represents a valid alternative to currently available analytical techniques for assaying boronophenylalanine based on boron determination in terms of reproducibility, recovery, or sensitivity. Therefore, it is suggested that the present method may routinely be used in all preclinical and clinical studies in which quantification of circulating and tissue concentrations of boronophenylalanine is critical for the application of boron neutron capture therapy.

Key Words: **boronophenylalanine; HPLC; boron neutron capture therapy; brain tumors; cerebral tissue.**

Neurosurgical interventions for removing malignant brain tumors strictly depend on their cerebral localization. Brain tumors that cannot surgically be removed are complicated to treat pharmacologically since most anticancer drugs do not easily permeate the blood–brain barrier. In this light, a therapeutic approach characterized by boronophenylalanine (BPA)[3] administration has recently been set up in animal models of induced cerebral tumors (1–3) and it is currently under evaluation in various clinical trials (4–6). The method is based on the principle that this modified amino acid is able to cross the blood–brain barrier and to accumulate preferentially within tumoral cells. The subsequent step is to submit BPA-enriched cancerous tissues to low-energy neutron bombardment, which, through a nuclear scission, should cause the selective disruption and death of malignant cells (the so-called boron neutron capture therapy, BNCT). To obtain positive results, it is critical that the level of BPA inside the tumor be much higher than that present not only in healthy cerebral cells but also in other tissues and in the bloodstream (3, 6).

The concentration of BPA in different tissues has been determined up to now by measuring boron levels

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[3] Abbreviations used: BNCT, boron neutron capture therapy; BPA, boronophenylalanine; GABA, γ-aminobutyric acid; OPA, *o*-phthalaldehyde.

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mainly by direct-current plasma atomic emission spectroscopy (3, 7). Even though this spectroscopic method is highly selective, it is not equally sensitive, it has a high instrument cost (another main disadvantage), and thus a relatively low number of laboratories are equipped with such an instrument. Since we are interested in this new promising therapeutic strategy for cerebral tumor treatment, we wished to introduce a different analytical method for BPA determination based on one of the most common analytical techniques, i.e., HPLC. This study reports an HPLC method for the separation and quantification of BPA in biological samples using precolumn derivatization with *o*-phthalaldehyde (OPA) and spectrofluorometric detection. Data referring to BPA concentration in the brain and blood, after intracarotid infusion of BPA in the rat, are presented.

MATERIALS AND METHODS

Chemicals

DL-*p*-Boronophenylalanine was obtained from Boron Biologicals, Inc. (Raleigh, NC). *o*-Phthalaldehyde and ultrapure standard amino acids were purchased from Sigma (St. Louis, MO). HPLC-grade tetrahydrofuran and methanol were obtained from Merck (Darmstadt, Germany) and Carlo Erba (Milan, Italy), respectively. All other reagents were of the highest purity available from commercial sources.

HPLC Apparatus and Chromatographic Conditions

A ThermoQuest Constametric 3500 dual-pump system (ThermoQuest Italia, Rodano, Milan, Italy) was connected to a Kromasil C-18 (Eka Chemicals, AB, Bohus, Sweden), 250 × 4.6 mm, 5-μm particle size column equipped with its own guard column (2-cm length). The detector was an FL3000 spectrofluorimeter (ThermoQuest Italia, Rodano, Milan, Italy) set up at 330-nm excitation and 430-nm emission wavelengths. It was connected to a PC for data acquisition and analysis using the software package (ChromQuest) supplied by HPLC manufacturers. Separation of BPA was carried out after precolumn derivatization with OPA. Briefly, 50 μl of standard mixture (containing BPA and 19 amino acids) was allowed to react for 60 s at 37°C with 100 μl of a solution composed of CH_3OH, 1 M sodium borate, pH 10.5, 3-mercaptopropionic acid, and water (10:50:1:39; v:v:v:v), containing 26 mM OPA. After OPA derivatization, the standard mixture was diluted to 1 ml with the starting eluent (Buffer A) and 100 μl was then used for the analysis. The HPLC column was previously equilibrated with Buffer A having the following composition: 50 mM CH_3COONa, pH 7.4, 50 mM Na_2HPO_4, pH 7.4, CH_3OH, tetrahydrofuran (48:48:2:2; v:v:v:v). Separation was obtained by using a step gradient with a second buffer (Buffer B) consisting of CH_3OH:H_2O (65:35; v:v). The gradient was as follows: 3 min at up to 80% Buffer A; 12 min at up to 70% Buffer A; 15 min at up to 50% Buffer A; 10 min at up to 45% Buffer A; 10 min at up to 20% Buffer A; 5 min at up to 15% Buffer A; 3 min at up to 10% Buffer A; 2 min at up to 0% Buffer A and hold for an additional 15 min; the flow rate was 1.2 ml/min, at a constant column temperature of 23°C. Assignment of BPA in blood and brain tissue extracts was carried out on the basis of retention time comparison and cochromatograms performed by the addition of a known concentration of standard BPA to each biological sample. BPA quantification was calculated by comparing the peak area of unknown samples with that of a BPA standard with a known concentration.

BPA Administration and Sample Processing

Male Wistar rats weighing 300–350 g were fed with a standard laboratory diet and water *ad libitum* in a controlled environment. To increase BPA solubility, a sterile solution containing 0.85 M BPA and 0.85 M fructose, pH 8.8, was prepared immediately before use. After induction of anesthesia with intraperitoneal injection of 50 mg/kg of body weight ketamine, the right common carotid arteries were exposed in the neck through a midline incision. Intracarotid drug infusion was performed in 10 rats according to Yang *et al.* (3), with a cumulative BPA dose of 300 mg/kg of body weight administered in 3 min using a constant-flow pump (Harvard Apparatus, Inc., Cambridge, MA). After 30, 60, and 120 min from the injection, 100 μl of blood was withdrawn from the femoral vein and immediately deproteinized by adding 200 μl of ice-cold 1.2 M $HClO_4$. Immediately after the last blood withdrawal, rats were sacrificed by decapitation, and the brain was quickly removed, dropped in liquid nitrogen, weighed, and finally homogenized in ice-cold 1.2 M $HClO_4$ (1:10; w:v), as previously described (8). Tissue homogenates and deproteinized blood were centrifuged at 20,190*g* for 15 min at 4°C, and supernatants were collected, neutralized with 5 M K_2CO_3 in the cold, centrifuged again, and extracted with HPLC-grade chloroform. Fifty microliters of each sample (both blood and brain extracts) were derivatized with OPA as described above, diluted to 1 ml with the starting buffer, and then loaded onto the HPLC column (100 μl) for BPA determination. Six sham-operated rats, subjected to the same anesthetic protocol and tissue sample preparation, but receiving an intracarotid infusion of fructose only, were used as our control group.

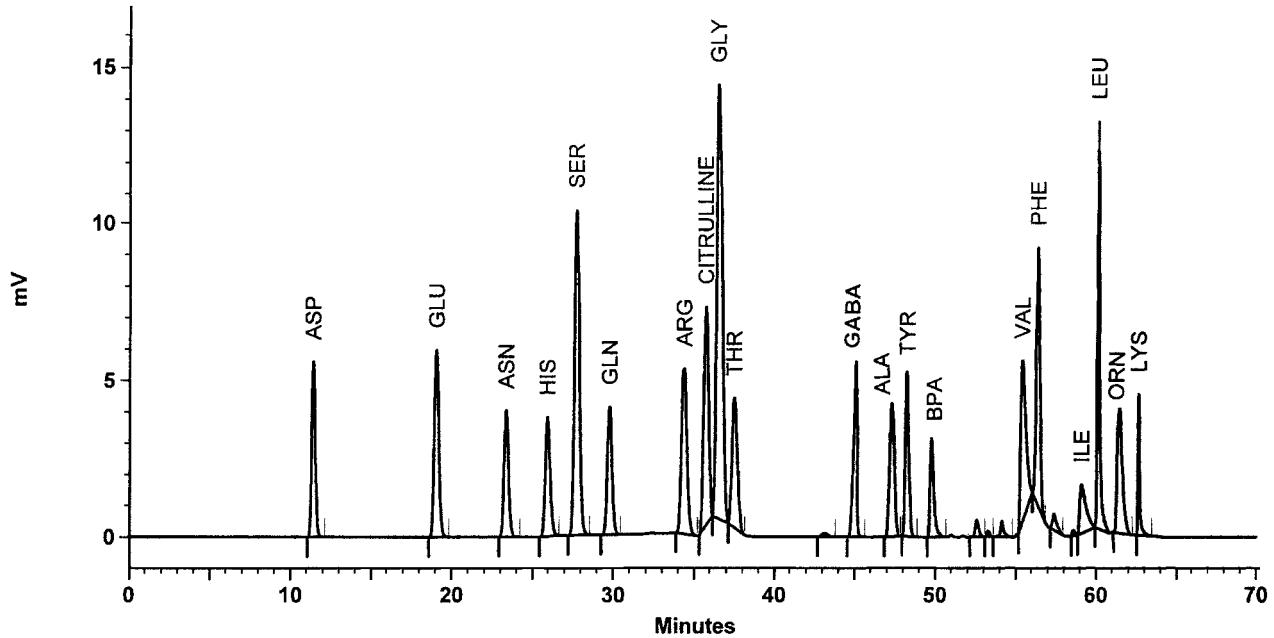

FIG. 1. Separation by HPLC, after precolumn derivatization with OPA, of a standard mixture containing 1 μM BPA (100 pmol/100 μl injected) and 0.25–1.25 μM of 19 amino acids commonly present in various tissues. Fluorimetric detection was performed using excitation and emission wavelengths of 330 and 430 nm, respectively. The apparatus and chromatographic conditions are described in detail under Materials and Methods.

RESULTS AND DISCUSSION

HPLC Separation of BPA-Containing Standard Mixture

Figure 1 reports the separation of a standard mixture containing 1 μM BPA and 0.25–1.25 μM of 19 amino acids (Asp, Glu, Asn, His, Ser, Gln, Arg, citrulline, Gly, Thr, GABA, Ala, Tyr, Val, Phe, Ile, Leu, Orn, and Lys). BPA was eluted with a $k' = 23.90$ (k' = retention factor = $(V - V_0)/V_0$, where V is the peak elution volume and V_0 is the void volume of the system) and it was positioned in the chromatographic run after Tyr, in a relatively uncrowded area of the chromatogram. The lowest limit of detection, recorded by setting the spectrofluorimeter full scale to 0.1 mV, was 5 nM (corresponding to 0.5 pmol/100 μl injected), while linearity (determined by calculating the area under the BPA peak) was maintained at up to 4 μM BPA (400 pmol/100 μl injected) with a correlation coefficient $r = 0.993$ ($P < 0.001$). Variability of the retention time and concentration of repeated injections ($n = 6$) of the same BPA-containing standard mixture was 1.05% (SD = 0.18) and 2.24% (SD = 0.15), respectively.

HPLC Determination of BPA in Biological Samples

Figure 2 shows representative chromatograms of a cerebral tissue extract of a rat sacrificed 120 min after the intracarotid administration of 300 mg/kg of body weight BPA (A) and of a control rat (B). As is clearly evident, in the tissue extract of the BPA-treated animal, as well as in all other cerebral samples of BPA-treated rats, a peak with the same k' value of standard BPA was recorded. This peak was absent in any chromatogram of cerebral tissue extract of control rats. A cochromatogram effected with the addition of BPA with known concentration as an internal standard and retention factor comparison with a chromatographic run of standard BPA allowed us to attribute the peak with $k' = 23.90$ to BPA in blood and cerebral tissue samples. Cerebral BPA concentrations in rats sacrificed 120 min after the intracarotid administration of 300 mg/kg of body weight BPA were 528.70 nmol/g wet weight (SD = 275.95), which, expressed in terms of boron concentration, gave 5.71 μg/g wet weight (SD = 2.98), i.e., a BPA value very close to that previously obtained under similar experimental conditions and by determining BPA using direct-current plasma atomic emission spectroscopy (3). To assess the recovery of this HPLC method for BPA determination, 16 additional anesthetized rats were sacrificed and brains (divided into four groups) were homogenized with 1.2 M $HClO_4$ supplemented with 0.01, 0.1, 1, or 10 μM BPA just before homogenization. Recovery of 88.25 (SD = 10.38), 94.11 (SD = 12.44), 90.84 (SD = 7.73), and 91.36 (SD = 13.65), respectively, was found in the four groups of exogenously BPA-supplemented brain samples. Results of this experiment showed that BPA recovery did not depend on how much BPA was added to

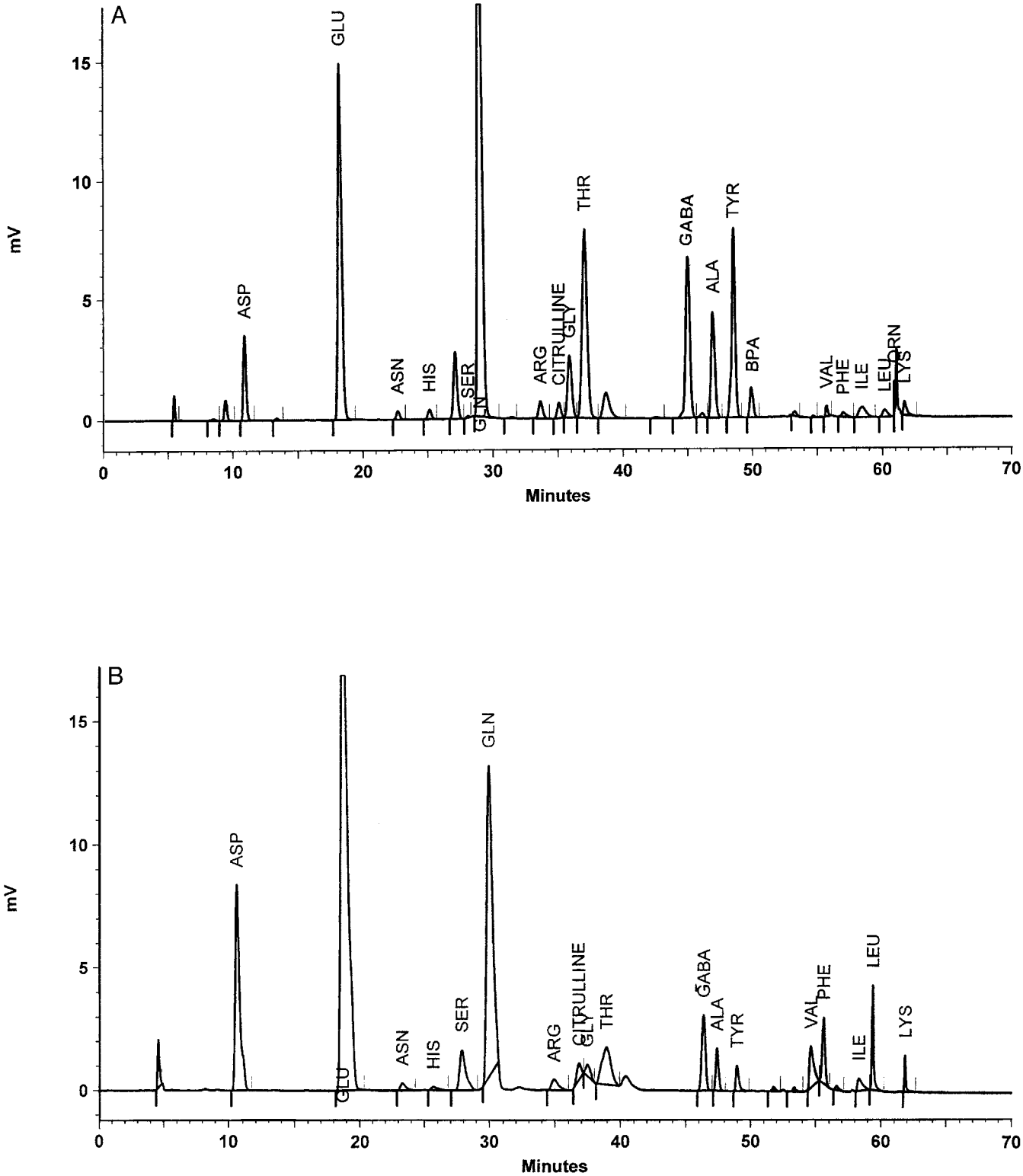

FIG. 2. Representative HPLC separation of BPA (using precolumn derivatization with OPA) of a perchloric acid cerebral tissue extract of a rat sacrificed 120 min after the intracarotid infusion of 300 mg/kg of body weight BPA (A). A representative chromatogram of a perchloric acid cerebral tissue of a control rat is also reported (B). BPA concentration in this sample was 634 nmol/g wet weight (i.e., about 6.3 μg of ^{10}B/g), corresponding to 31.7 pmol/100 μl injected. The BPA administration protocol, apparatus, and chromatographic conditions are fully described under Materials and Methods.

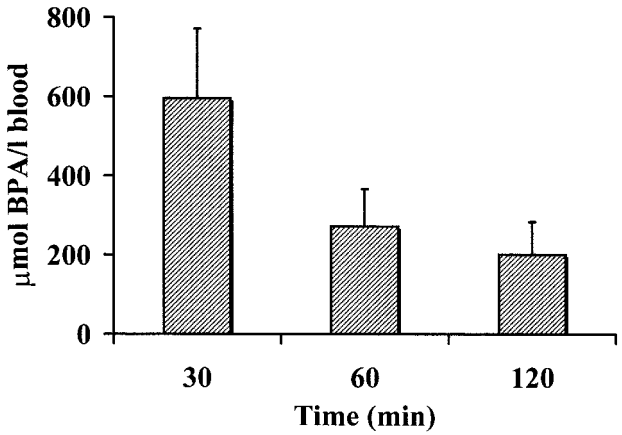

FIG. 3. BPA concentration in rat blood after 30, 60, and 120 min from the intracarotid infusion of 300 mg/kg of body weight BPA. Determination of BPA was carried out by HPLC as described in detail under Materials and Methods. Each column is the mean of blood samples from 10 different rats and is expressed as μmol of BPA/liter of blood. Standard deviations are represented by vertical bars.

brain samples, thus indicating a fair efficiency of sample processing (at least in the range of BPA concentrations tested) and supporting the suitability of the present HPLC assay for BPA quantification.

In Fig. 3 are reported BPA blood concentrations of rats treated with intracarotid injection of 300 mg/kg of body weight BPA. Time course decrease of blood BPA indicated that, after 120 min from the injection, 200.98 μmol/liter blood BPA (corresponding to about 2.1 μg of ^{10}B/g) was still present in the bloodstream. Our BPA blood concentration was comparable to values previously reported and obtained under similar experimental conditions (3). It is worth emphasizing that either blood or brain tissue values of BPA were well above the sensitivity of this HPLC assay.

BPA is not the sole boron-containing compound utilized in BNCT. At present, the determination of these substances for the bioavailability, tissue distribution, pharmacokinetics, etc. was effected through boron assay using different analytical techniques having some inconvenience or limitation. For instance, spectrophotometric analysis using different boron complexing reagents has a modest sensitivity, has a relatively low specificity, and is also time-consuming (9, 10). Nuclear techniques require a thermal neutron source (such as a nuclear reactor or a neutron-emitting radioisotope) (11, 12); hence, the relative scarcity of instruments did not render such methods very common. The last group of methods is represented by atomic absorption or emission spectroscopy. Atomic absorption spectroscopy suffers from the presence of several interferants and from low sensitivity (7). Therefore, inductively coupled plasma atomic emission spectroscopy has alternatively been used, even though sample processing is not simple and samples with high salt concentration cannot be assayed by this technique (7, 13). For these reasons, direct-current plasma atomic emission spectroscopy for boron determination was subsequently developed (7) and it was successfully applied for quantifying BPA in biological samples (3), so that it is now routinely used in preclinical and clinical studies regarding BPA application for BNCT (1–6). By this very specific method, which is capable of measuring total boron, a minimum of about 50 mg of tissue and 16 nmol of boron/sample are, however, necessary.

The HPLC method described in the present study, using BPA derivatization with OPA and a spectrofluorimetric detector, has a lowest limit of detection of 0.5 pmol of BPA injected; i.e., it is about 32,000 times more sensitive than direct-current plasma atomic emission spectroscopy. This implies that the present HPLC method might be successfully applied for BPA determination when very low amount of tissue are available, such as in the case of needle biopsies. Although it has been reported that BPA concentration during BNCT, both in tumor and in normal tissues, is well above the detection limit of this new HPLC technique (6), it is conceivable that its application might allow determination of BPA values that are currently indicated as "not detectable" (6).

Therefore, the broad availability of HPLC instruments (which can nowadays be considered as a "basic technique" for the majority of laboratories), coupled with its relatively moderate cost, might render the present method for BPA detection certainly useful not only in preclinical studies but also in the routine practice of clinical laboratories involved in BNCT application and suggests that BPA detection by HPLC is a valid alternative to other currently available techniques.

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