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Journal of Chromatography B, 682 (1996) 225–231

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simple and rapid procedure for the purification of lipoprotein(a)

Giuseppe Lippi, Claudia Lo Cascio, Orazio Ruzzenente, Giovanni Poli, Claudio Brentegani, Giancesare Guidi*

Laboratorio di Chimica Clinica, C.O.C. Valeggio s/M, Istituto di Chimica e Microscopia Clinica, Università di Verona, Verona, Italy

Received 1 December 1995; revised 9 February 1996; accepted 20 February 1996

Abstract

Lipoprotein(a) [Lp(a)] is a low-density lipoprotein-like particle displaying strong athero-thrombotic properties. Highly purified Lp(a) is increasingly requested for standardization of Lp(a) measurements and for biological studies. Several procedures have been described for Lp(a) separation and purification but none of them appear completely suitable. We present here a procedure for Lp(a) purification based on sequential elutions after lysine–Sepharose affinity chromatography. We were able to identify four distinct subspecies of Lp(a) showing different affinity to ε-amino groups of lysine–Sepharose, simply by modifying molarity and pH of the eluents; the fraction obtained in highly purified state represented the major form and could be eluted with 0.5 *M* sodium phosphate buffer (pH 4.4). Advantages of this procedure are represented by simplicity, rapidity and final yield.

Keywords: Lipoprotein(a); Proteins

1. Introduction

Lipoprotein(a) [Lp(a)] is a cholesterol-rich plasma lipoprotein discovered by Kåre Berg in 1963 [1]. In Lp(a), the highly polymorphic and glycosylated apolipoprotein(a) [apo(a)] is covalently linked by a single disulfide bridge to apolipoprotein B_{100} (apo B_{100}), the main protein moiety of low-density lipoproteins (LDL).

The structure of apo(a) is similar to the zymogen plasminogen; the amino acid sequence of the two proteins shows almost 80% homology [2]. The two proteins mainly differ in the absence of plasminogen kringle I–kringle III domains and the presence of multiple tandem repeats of plasminogen kringle IV in apo(a) in agreement with the identification of 34 or more alleles in apo(a) locus [3] and 34 or more isoforms in human plasma [4,5].

Several other plasma proteins contain kringle-like sequences, such as hepatocytes growth factor, urokinase, coagulation factor XII, prothrombin and tissue-type plasminogen activator [6]. The role of the kringle domains seem to be essential for the activation of fibrinolysis. The supposed binding between kringle-containing proteins and the carboxyl-terminal lysine residual of the stabilized fibrin involves a highly hydrophobic pocket in the kringle domain, called the "lysine binding site". The affinity of different kringles for the ε-amino group of lysine can be affected by subtle amino acid substitutions or conformational changes of the lysine binding site. In particular, the apo(a) lysine-binding properties seem to reside chiefly on kringle VI type 10 and is

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00098-9

somehow affected by the number of kringle IV type 2 repeats [7].

Increased values of Lp(a) in plasma have been related to atherosclerosis in patients suffering from different artery diseases [8–12]. Moreover, the established homology between apo(a) and plasminogen provides a further powerful linkage between atherosclerosis and thrombosis [13].

Although it has been reported that Lp(a) in plasma can be present in at least two subspecies heterogeneous in respect of their ability in binding to lysine–Sepharose and previously identified as Lys^+ and Lys^- [14], further studies proved that only the former retains athero-thrombotic properties [15,16].

Isolated and purified Lp(a) is increasingly needed for use as a standard to overcome most of the problems in the standardization of Lp(a) measurements [17] and for in vitro biological studies. Several methods have been proposed for Lp(a) purification over the past decade but none of them appear completely suitable in terms of yield, purity and practicability [18–21]. In the present paper we present a new and rapid procedure for purification of Lp(a).

2. Experimental

2.1. Lp(a) purification

Dry lysine–Sepharose (15 g) (Pharmacia, Uppsala, Sweden) was swollen in distilled water, packed in a refrigerated 40×2.6 cm I.D. chromatographic column and extensively washed with 500 ml of 50 m*M* $(NH_4)HCO_3$, pH 7.95. Lysine is bound to the matrix (with no spacer arm) by its α-amino group, leaving ε-amino group free to interact with kringle-containing proteins.

Blood was collected from healthy volunteers and sera were obtained after centrifugation at 3000 *g* for 10 min. Pooled sera were filtered through a Whathman 4 filter paper.

After stabilizing the flux at 80 ml/h by a peristaltic pump, 100 ml of pooled sera were injected in the column. The eluate following the pool injection was collected, the absorbance read at 280 nm and Lp(a) positive fractions [Lp(a) Lys^-] were identified using a semi-quantitative manual latex method [22]. The gel was then washed with 500 ml of 50 m*M* $(NH_4)HCO_3$, pH 7.95 until the 280 nm absorbance of the effluent returned to baseline.

The release of the Lp(a) bound to the ε-amino group of lysine–Sepharose was obtained employing three sequential elution steps:

(1) Continuous linear gradient from 0 to 0.5 *M* sodium phosphate buffer, pH 7.4: 100 ml of a linear gradient between 0 and 0.5 *M* sodium phosphate buffer (pH 7.4) was flowed through the column, the eluate was collected in 2.5-ml fractions, read at 280 nm and tested for Lp(a) positivity throughout all the elutions.

(2) 0.5 *M* sodium phosphate buffer, pH 4.4: 50 ml 0.5 *M* sodium phosphate buffer (pH 4.4) was injected onto the column and the eluate was collected.

(3) 0.5 *M* sodium phosphate buffer–0.2 *M* ε-aminocaproic acid (ε-ACA), pH 4.4: 50 ml 0.5 *M* sodium phosphate buffer–0.2 *M* ε-ACA (pH 4.4) was injected onto the column and the eluate was collected. ε-ACA was added to the buffer since it acts as a strong competitor for the elution of high-affinity proteins.

All pH adjustments were performed by mixing 0.5 *M* NaH_2PO_4 and 0.5 *M* Na_2HPO_4 in appropriate amounts. Following each elution step, the gel was washed with 50 ml of 50 m*M* $(NH_4)HCO_3$, pH 7.95. The whole procedure was performed at 8°C.

2.2. Lp(a) characterization

Total proteins were measured according to the method described by Lowry et al. [23]. Total Lp(a) immunoreactivity was measured in each Lp(a) positive fraction with three commercial kits. Macra Lp(a) (Strategic Diagnostic, Newark, NJ, USA) uses monoclonal anti-apo(a) antibody coated plates for capturing the Lp(a) particles and horseradish peroxidase (HRP)-conjugated anti-apo(a) polyclonal antibody for detection. Intra- and inter-assay coefficient of variations (C.V. values) were 1.7 and 3.8%, respectively. Innotest Lp(a) (Byk-Sangtec Diagnostica, Dietzenbach, Germany) uses monoclonal anti-apo(a) antibody coated plates for capturing the Lp(a) particles and a second HRP-conjugated polyclonal anti-apolipoprotein B (apo B) antibody for detection. Intra- and inter-assay C.V. values were respectively 2.8% and 4.2%. N-Lp(a) nephelometric assay

(Behringwerke, Marburg, Germany) employs an antiserum to human Lp(a). Intra- and inter-assay C.V. values were 1.6 and 2.2%, respectively. Each assay was calibrated using a common calibrator (Behringwerke) to reduce the bias due to use of different calibrators among kits; final results were expressed as mean of values obtained with the three assays. Total cholesterol, triglycerides and phospholipids were analyzed using enzymatic (Trinder) assays (Alifax Diagnostici, Padova, Italy).

Apolipoprotein A-I (apo A-I) and B measurements were performed on a Behring nephelometer analyzer (BNA) using nephelometric anti-apo A-I and anti-apo B antisera (Behringwerke). Plasminogen activity was measured on an ACL 3000 Plus (Instrumentation Laboratory, Milan, Italy).

Protein electrophoresis was performed on agarose gel employing a commercial kit (Paragon, Beckman Analytical, Milan, Italy) followed by Coomassie brilliant blue staining. Immunofixation after protein electrophoresis on agarose gel (Paragon) was performed using an antiserum to human Lp(a) (Behringwerke). Lipoprotein electrophoresis was performed on agarose gel employing a commercial kit (Paragon) followed by Sudan Black B staining. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 5% acrylamide, either stained with Coomassie brilliant blue R 250 (Bio-Rad, Milan, Italy) or immunoblotted employing a specific anti-apo(a) HRP-conjugated antibody (Strategic Diagnostic) were performed as previously described [21].

3. Results

A first Lp(a) positive specie unable to bind to lysine–Sepharose could be collected in the first unretained eluate following the injection of the pooled sera. This fraction has been previously described as Lp(a) Lys^{-}, since it has little or no affinity for lysine–Sepharose [14]. The amount of this fraction in our experience is almost constant employing pooled sera from the same donors and ranged from 22 to 26% of Lp(a) injected (Table 1).

The elution profile employing continuous linear gradient from 0 to 0.5 *M* sodium phosphate buffer (pH 7.4) is shown in Fig. 1a. A significant amount of protein was eluted, reaching a maximum absorbance of 1.9 at 280 nm. Few Lp(a) positive fractions [Lp(a) Lys^{+1}] could be identified at the end of the peak, reaching a maximum value of 50.2 mg/l. These Lp(a) positive fractions represent less than 5.4% of the total Lp(a) injected (Table 1).

Fig. 1b reports the 0.5 *M* sodium phosphate buffer (pH 4.4) elution profile. A second proteic peak could be observed, reaching a maximum absorbance of 0.73 at 280 nm. The whole proteic peak exhibited strong Lp(a) immunoreactivity (reaching a maximum value of 882 mg/l) and coincided with the peak of Lp(a) suggesting a complete identity between eluted proteins and Lp(a). These Lp(a) positive fractions [Lp(a) Lys^{+2}] are representative of 49.4% of the total amount of Lp(a) injected (Table 1). Plasminogen contamination could be ruled out by the absence of plasminogen activity in the Lp(a) positive fractions.

A third elution profile after injection of 0.5 *M* sodium phosphate buffer–0.2 *M* ε-ACA (pH 4.4) is reported in Fig. 1c. This latest peak, reaching maximum absorbance of 2.1, showed only weak immunoreactivity for Lp(a) [Lp(a) Lys^{+3}] and, in agreement with a previous report [21], contained large amounts of plasminogen as established by plasminogen activity quantification.

The electrophoretic pattern followed by Coomassie blue staining of the four distinct Lp(a) species is reported in Fig. 2a. No significant differences could

Table 1
Relative yields of Lp(a) fractions following each sequential elution after lysine–Sepharose affinity chromatography

	Immunoreactive Lp(a) mass (mg)	Relative value (%)
Starting pool	41.5	100
Lp(a) Lys^{-}	10.6	25.5
Lp(a) Lys^{+1}	2.24	5.4
Lp(a) Lys^{+2}	20.5	49.4
Lp(a) Lys^{+3}	2.25	5.4

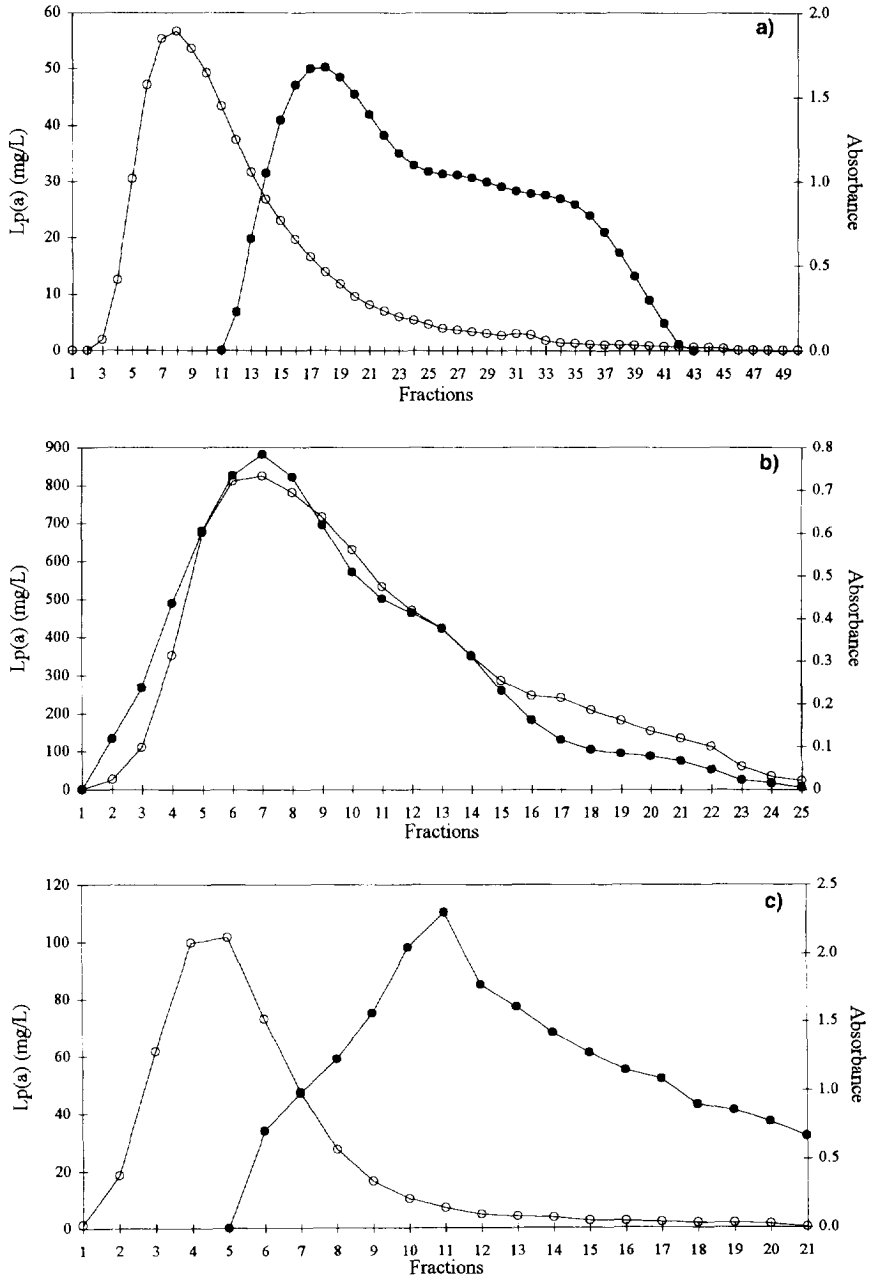

Fig. 1. Sequential elution profiles (●=Lp(a) manual latex assay immunoreactivity and ○=280 nm absorbance) of Lp(a) after lysine–Sepharose affinity chromatography employing: (a) continuous linear gradient from 0 to 0.5 *M* sodium phosphate buffer, pH 7.4; (b) 0.5 *M* sodium phosphate buffer, pH 4.4 and (c) 0.5 *M* sodium phosphate buffer and 0.2 *M* ε-ACA, pH 4.4.

be observed between the pool and the Lp(a) Lys^-, though a single band with pre-β mobility was clearly present in fractions from Lp(a) Lys^{+1}–Lp(a) Lys^{+3}. The immunofixation employing a polyclonal anti-apo(a) antibody following the agarose gel electrophoresis proved that these bands were almost completely attributable to Lp(a) (Fig. 2b). The lipoprotein electrophoresis on agarose gel (Fig. 2c) showed an

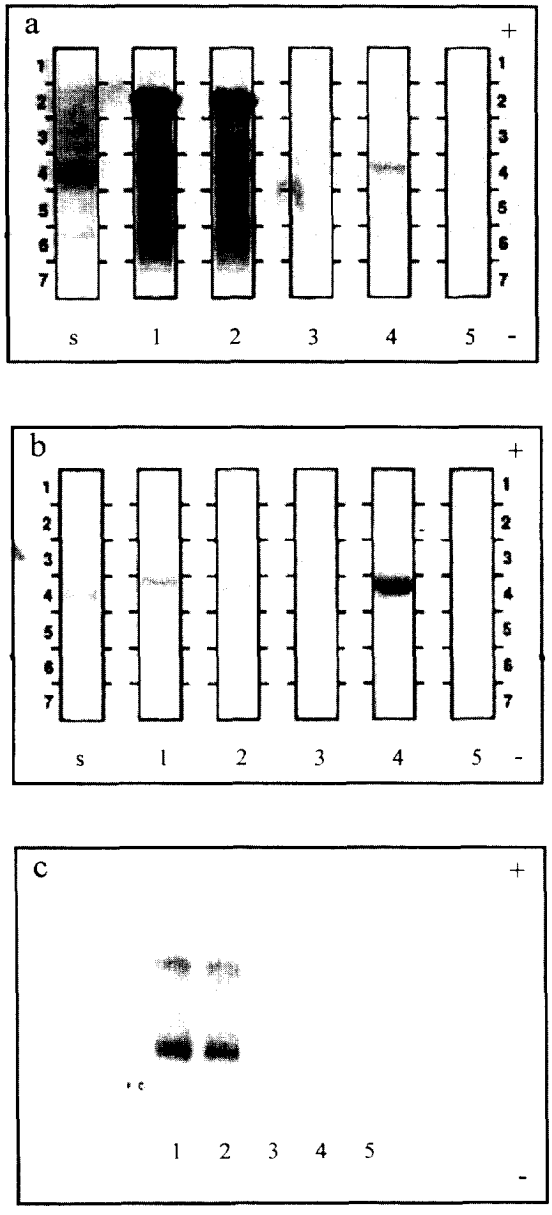

Fig. 2. Electrophoretic patterns on agarose gel of: (s) standard control serum, (1) pooled sera, (2) Lp(a) Lys^-, (3) Lp(a) Lys^{+1}, (4) Lp(a) Lys^{+2} and (5) Lp(a) Lys^{+3}. Electrophoresis followed by Coomassie blue staining is shown in (a), immunofixation employing an anti-apo(a) antibody in (b) and lipoprotein electrophoresis followed by Sudan Black B staining in (c).

elective pre-β mobility of the purified Lp(a) in agreement with previous reports; no other bands could be observed in Lp(a) Lys^{+1}–Lp(a) Lys^{+3} electrophoretic courses, excluding the presence of other lipoproteins than Lp(a).

The comparison between SDS-PAGE, either stained with Coomassie blue or followed by immunoblot analysis with an anti apo(a) HRP-conjugated antibody demonstrated the presence of a single band at high molecular mass in the Lp(a) Lys^{+2} fractions, almost completely attributable to Lp(a) (Fig. 3).

The integrity of the disulfide bond that links apo(a) to apo B in the intact Lp(a) particle could be established by the concordance of Lp(a) immunoreactivities as measured either with anti-apo(a) or anti-apo B HRP-conjugated antibodies. Furthermore, no Lp(a) residual immunoreactivity could be found in the supernatant of the Lp(a) Lys^{+2} fractions after immunoprecipitation with a commercial anti apo B antiserum. The main properties of the purified Lp(a) Lys^{+2} are reported in Table 2 and are in agreement with previous reports [14].

4. Discussion

Several procedures have been proposed for Lp(a) purification. Most of them require a preliminary ultracentrifugation step in NaBr or KBr. Ultracentrifugation is a time-consuming procedure and allows the purification of a narrow Lp(a) fraction, floating in a limited density gradient whereas Lp(a) can be recovered in buoyant densities ranging from less than 1.006 kg/l to more than 1.12 kg/l considering free apo(a). Moreover, isolated Lp(a) recovered in the 1.05–1.12 kg/l density requires further steps to eliminate high-density lipoproteins and LDL, leading to relatively poor yields [18–20].

Alternative procedures employ affinity chromatography, particularly on lysine–Sepharose, but eventually require further steps, such as gel permeation, to eliminate plasminogen co-eluting with apo(a) when employing ε-ACA [14,21].

In this paper we describe a new, simple and rapid procedure for Lp(a) Lys^- purification based on further differential elutions after a single affinity chromatography on lysine–Sepharose. The clinical significance of Lp(a) species unretained by lysine–Sepharose is still uncertain. Lp(a) Lys^- appears not to be related either to the incidence or to the severity

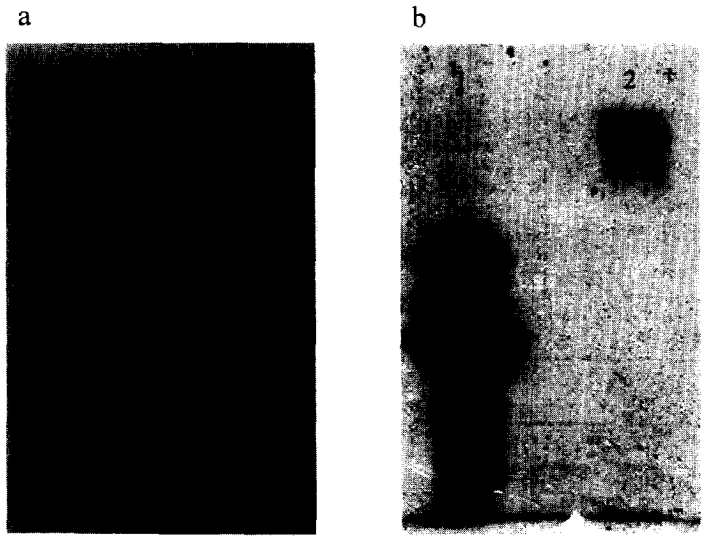

Fig. 3. SDS-PAGE in 5% acrylamide of (1) pooled sera and (2) Lp(a) Lys^{-2} followed by (a) immunoblot with a polyclonal anti-apo(a) HRP-conjugated antibody and (b) Coomassie blue staining.

of coronary artery diseases in the general population [15]; moreover the biological activity in the inhibition of in vitro fibrinolysis seem to reside only in Lp(a) Lys^{+}, not in Lp(a) Lys^{-} since it is mainly dependent on the capability of apo(a) to bind to the carboxyl terminal lysine residual of stabilized fibrin [16]. At present we have not identified the nature of the Lp(a) Lys^{-}; in a previous report, Armstrong et al. thoroughly analyzed this fraction observing that Lp(a) Lys^{-} retains all the main characteristics and properties of native Lp(a) [14].

In the present study we firstly identified four Lp(a) subspecies heterogeneous in binding to lysine–Sepharose that could be released by slightly varying molarity or pH of the eluent.

Table 2
Main characteristics of purified Lp(a) Lys^{-2}

	Relative mass (%)
Lipoprotein(a)	100
Proteins	21.1
Total cholesterol	47.2
Phospolipids	24.5
Triglycerides	4.0
Apolipoportein B	12.2
Apolipoprotein A-1	Not detectable
Plasminogen	Not detectable

Comparison of the X-ray crystallographic structure of each apo(a) kringle and plasminogen kringle IV revealed that apo(a) kringle IV type 10 is the principal domain capable of binding lysine in the apo(a) molecule. It has been reported that subtle amino acid substitutions in the lysine binding site can justify differences in the specificity of the binding as proved by the evidence that distinct kringle domains show dissimilar properties in this respect [7]. Thus, the structure of each kringle in apo(a) may provide insight into the physiological role of Lp(a) and further define the athero-thrombotic properties of the lipoprotein [24]. We suggest that the observed heterogeneity in lysine binding may be due to a wide cluster of modifications (oxidation, glycation, proteolytic fragmentation) of the apo(a) domain, mainly occurring in the ligand binding site (that binds) to lysine. The catabolism of Lp(a) significantly differs from LDL; the linkage between apo(a) and apo B in Lp(a) reduces the affinity for the LDL receptor and increases the half-life of the lipoprotein in vivo [25]. Moreover, the reduced antioxidant content in Lp(a) in respect of LDL [26], together with the extended half-life, contribute to increase the susceptibility of the particle to oxidative modifications that potentially alter properties and functions of the whole particle.

The main advantages of the present method are the simplicity and the rapidity; the whole procedure can be completed in less that 9 h compared to almost 20 h when using a prior ultracentrifugation process.

The global yield of the procedure is satisfactory. In repeated experiments the total amount of isolated and purified Lp(a) Lys^{+2} ranged from about 50 to 60%; it may be noted that Lp(a) Lys^{-1} and Lys^{+3} show negligible amounts when compared to Lp(a) $Lys^{+}2$ (Table 1). The purity of the material, as assessed by our investigation, is also satisfactory.

At present, there is no standardization for Lp(a) measurements; one of the main problem to overcome is the lack of an universally agreed primary standard to calibrate secondary standards and thus reach uniformity of results among different assays. Additionally, time-consuming purification procedures can alter structure, properties and functions of the particles and therefore influence the results of biological studies. We believe that this method may represent a potential solution.

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