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Zinc Determination in Pleural Fluid

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In this study, an enzymatic zinc determination method was applied to pleural fluid, the basis of which was the regaining of the activity of apo carbonic anhydrase by the zinc present in the sample. The method was used for pleural fluid zinc determination in order to show the application to body fluids other than serum. For this purpose, pleural fluids were obtained from 20 patients and zinc concentrations were determined.

Carbonic anhydrase was purified by affinity chromatography from bovine erythrocytes. The zinc present in its structure was removed by dialysis against dipicolinic acid, resulting in apoenzyme obtained at a ratio of 100%. The activity of the enzyme was determined by the esterase action on p-nitrophenyl acetate. For comparison, the same samples were analyzed in atomic absorption. The results obtained were evaluated statistically. (>0.05 t-test, <0.001 Zn^{2+} (AA)-Zn^{2+} (apoCA), <0.005 Zn^{2+} (apoCA)-Total protein).

Key Words: Pleural fluid, Zinc determination, Apo Carbonic Anhydrase

Introduction

Carbonic anhydrase (E. C. 4.2.1.1) (CA) is a zinc-containing metallo-enzyme, which catalyzes hydration of CO_2 and dehydration of H_2CO_3. When the zinc, covalently bound to the active site, is removed, the apo carbonic anhydrase (apoCA) is obtained, resulting in no enzymatic activity[1]. The apoCA can show activity when the Zn^{2+} is added to the reaction medium, which is proportional to the Zn^{2+} added; this is the basic principle of the method. This method was first tried by Kobayashi et al.[2]. They used the Zn^{2+} in fruit juices and water for the reactivation of apoCA and determined the activity by means of esterase action. However, they used 1,10-phenanthroline as the chelating agent and could not test the enzyme in a high concentration of Zn^{2+}, since they could not obtain the apoenzyme at a ratio of 100%, i.e., they could not entirely remove the native Zn^{2+} present on the enzyme. Küfrevioğlu and Keha[3] tried a different chelating agent, dipicolinic acid (pyridine-1,6-dicarboxylic acid), and achieved more purified enzyme (97%) in a short time (3 h). They used urine, cerebrospinal fluid and serum as samples and inactivated the CA present in serum by boiling it.

Recently, Demir et al.[4] obtained the enzyme at a high purity (100%) by extending the dialysis time to 5 h and they tried the method in different samples obtained from patients with diabetes mellitus and cirrhosis.

A healthy subject has only a limited amount of fluid in his/her pleural space. The surface of the pleura is humidified. In diseases such as cardiac failure, cirrhosis, and nephritis, the pleural fluid is increased; on the other hand, knowledge of the pleural fluid composition some diseases is limited.

In order to show the usability of a new, enzymatic method for zinc determination in pleural fluid, the present study was conducted, which shows the usage of the method for every body fluid material.

Materials and Methods

Preparation of apoCA from Bovine Erythrocytes

Bovine blood was anticoagulated with 15 ml of ACD solution (22 g of sodium citrate dihydrate, 8 g of citric acid and 24 g of dextrose per liter) per 100 ml of blood, and red blood cells were obtained and washed with saline. The cells were lysed with distilled water. The ghosts were removed by centrifugation. CA was purified (400-fold) by affinity chromatography[5]. The purified CA was concentrated Sephadex G-25 in a batchwise process and was dialyzed first against distilled water and then against Tris-H_2SO_4 (0.05 M, pH 7.4). The resultant enzyme (100 mg) was dialyzed against 0.075 M pyridine-2,6-dicarboxycilic acid in 0.2 M phosphate buffer (pH 7.4) for 5 h in order to remove the Zn^{2+} from the enzyme[6].

Five ml of apoenzyme reagent can be prepared by using 100 mg of CA. The resulting reagent may be sufficient for about 100 determinations. In addition, CA is a rather stable enzyme and has a long reconstitution life. For instance, only 5% activity loss is detected after one year standing.

CA activity in eluates obtained during purification was determined by the method of Wilbur and Anderson[7] as modified by Rickli et al.[8] For the preparation of a standard curve in serum Zn^{2+} determinations, the esterase action of CA was used[9]. In this method, 4-nitrophenyl acetate is hydrolyzed to 4-nitrophenyl by CA and the absorbance of the product is measured at 348 nm. Reaction mixtures in 3 ml cuvettes contained 0.1 ml apoenzyme solution, 1.0 ml Tris-H_2SO_4 (0.05 M, pH 7.4), 0.4 ml serum (or standard) and 1.5 ml substrate. Three minutes later, the absorbances of the sample and blank cuvettes (distilled water instead of sample) were measured at 348 nm and 25°C. The 4-nitrophenyl acetate solution was prepared by dissolving 27.2 mg of the ester in 1 ml of acetone and then adding this to 49 ml of stirred distilled water drop by drop.

Protein Determination

The protein content of chromatographic eluates was measured specrophotometrically at 280 nm and by the Coomassie brilliant blue method[10].

Zinc Determination in Pleura

In order to show the applicability of this method to Zn^{2+} determination in pleural fluid, measurements were made on 20 samples obtained from pleural patients. Since high esterase activity is observed in pleural fluid, the fluids were heated in a boiling water bath for 1 h in capped tubes and then centrifuged. By this procedure the esterase activities of the fluids were eliminated. The previous method[4] measured the Zn^{2+} contents of supernatant solutions.

Zn determination was also made by atomic absorption in pleural fluid samples. For this purpose, the samples were diluted (1/4) with deionized water and they were deproteinized with trichloroacetic acid (TCA) (sample/TCA: 1/1). The resulting contents were centrifuged (for 15 min, 1500xg). The supernatants were used for zinc determination with AAS[11].

Results and Discussion

The Table 1 shows the statistical analysis of the results of ten pleural fluid samples.

Table 1. Statistical evaluation of the results.

t-test		
Zn^{2+} (μg/dL) with AAS method X± (n=20)	Zn^{2+} (μg/dL) with ApoCA method X± (n=20)	P
90.0 ± 22	88.4 ± 22	> 0.5
Correlation		
	r	**P**
Zn^{2+} (AAS)-(apoCA)	0.990	< 0.001
Zn^{2+} (AAS)-(apoCA)	0.610	0.005
Apo CA: Apo Carbonic Anhydrase	AAS: Atomic Absorption Spectrometry	

When one compares the results of the AA and apoCA methods it is easily seen from the r and P values that there is a good correlation between the two methods. In addition, the insignificant t-test result shows the significant correlation.

In conclusion, the enzymatic method can easily be used in pleural fluid zinc determination. This method is cheap and can be used in each laboratory. On the other hand, studies are continuing on its application to autoanalytic equipment and routine use.

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