Original Paper

# Sodium Ion Effect on Separation Of Butyrylcholinesterase from Plasma by Ion-Exchange Chromatography

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**ABSTRACT:** Purpose of the paper was the optimization of mobile phase in natrium chloride gradient in the chromatographic separation of butyrylcholinesterase from human plasma. Materials/Methods. Butyrylcholinesterase (BuChE) was isolated from human plasma using a diethylamminoethyl-cellulose column, by elution with 0.02M acetate buffer pH=4.0, gradually increasing NaCl percentage from 10% to 80%. The procedure lasted approximately 28 days. Results. Absorbance of the successive collected fractions at 280 nm presented a maximum at 60 % NaCl concentration. Activity of obtained BuChE was maximum at the same concentration. Another observed effect of NaCl was the decrease of resistance of the column to flow of the elution fluid. In the absence of NaCl the flow rate was 7 mL/h. Increasing of NaCl concentration induced a continuous increase of the flow to a value of 21 mL/h at 60% NaCl solution. After this concentration the flow remained practically constant. The effect on the ionic exchange is essentially an effect on chromatographic partition coefficient, leading, as a rule, to a peak having a Gaussian form. Fitting separately of ascending and descending parts of the apparent peak, led to practically the same exponential coefficient. Conclusions. Separation of proteins and particularly of BuChE on a chromatographic DEAE Cellulose column can be considered as a method for separation and purification of BuChE from human plasma. Optimum concentration of NaCl is 60 %. Exponential fittings in the neighbourhood of maximum indicated a prevalence of effects of NaCl on the chromatographic partition face to effects on gel-sol equilibrium of stationary phase.

***KEYWORDS:*** *butyrylcholinesterase, DEAE Cellulose, ionic exchange, human plasma*

## Introduction

Butyrylcholinesterase (BuChE) is a stoichiometric bioscavenger for organophosphorous (OP) toxics and is a soluble globular tetrameric form of cholinesterase that is present in the circulation of all vertebrates including man [1]. It is a glycoprotein with a molecular weight of 340000 Da [2].

Organophosphorous nerve agents such as soman, GB, VX and tabun exert their toxicity by inhibiting acetylcholinesterese (AChE) in the central nervous system (CNS) leading to the increase in acetylcholine levels at cholinergic synapses, triggering an acute cholinergic crisis [3].

The physiological function of BuChE is not yet established, but it was discovered that exogenously administered BuChE can sequest OP, thus preventing them to inhibit AchE in the central nervous system and protecting animals from these nerve agents.[4-10].

Obtaining and characterization of BuChE from human blood become, in this context, an important step in improvement the emergency treatment of intoxications with organophosphorous compounds.

Separation of cholinesterase isoenzymes was performed some fifty years ago by P. K. Das and J. Liddell which isolated four isoenzymes by a three-stage procedure involving chromatography on diethylaminoethylcellulose (DEAE-cellulose) at pH 4.0, an electrofocussing technique and gel filtration on Sephadex G-200. The final product was concentrated more than ten thousand fold and the purity was checked by polyacrylamide-disc electrophoresis. [11]. Ashima Saxena et al developed a procedure for preparation of gram quantities of BuChE from outdated human plasma or Cohn Fraction IV-4. The isolation and purification of Human BuChE was accomplished by absorption on procainamide-Sepharose-CL-4B affinity gel followed by ion-exchange chromatography on DEAE-Sepharose column and chromatography on analytical procainamide affinity column.[12] Oksana Lockridge et al developed a large scale isolation and purification method for human BuChE from human plasma with precautions to minimize endotoxin content, by the following sequence of procedures: dialysis in pH 4.0 buffer, ion exchange chromatography at pH 4.0, affinity chromatography on procainamide-Sepharose, followed by HPLC ion exchange at pH 7.4. [13]. Subsequently technical efforts have been made to reduce the cost, such as large-scale production

from Cohn fraction IV [14], and recombinant production in milk of transgenic goats (Protexia, PharmAthene, Annapolis, MD, USA) [15] or tobacco [16].

The aim of this study was to isolate BuChE from human plasma using gel filtration-ion exchange chromatography and to find the optimum concentration of NaCl and chromatographic parameters for obtaining a maximum yield of extraction.

## Materials and methods

### Materials

Human plasma was provided by the Army Center for Transfusional Hematology. Diethylaminoethylcellulose (DEAE Cellulose) was purchased from Serva and was of analytical grade. Sodium acetate, sodium chloride and PEG 600 were obtained from Merck and were of analytical grade. For the separation process a glass column with 2.8 cm internal diameter, 50 cm length and provided with an inferior stopcock was used. The dialysis sack with 20 cm internal diameter and 30 m length was purchased from Union Carbide Corporation. Prior to its use, it was washed with pH=4.0 acetate buffer 0.02 M.

For measuring the absorbance of samples it was used a Lambda 40 UV/VIS Spectrometer (Perkin Elmer producer).

### Methods

Before being introduced in the column, the DEAE Cellulose anion exchanger was processed as following: 125 g anion exchanger was homogenized using 2L of a 0.5 M solution of hydrochloric acid for acidification, followed by a 20-30 minutes stirring with a magnetic bar. The suspension was then rinsed with distillated water 2 or 3 times using a Buchner funnel, followed by the addition of a 0.5 M sodium hydroxide solution up to pH=6.5. The suspension was allowed to settle and the supernatant liquid was discarded. Finally, the DEAE Cellulose was suspended in the pH=4.0 0.02 M acetate elution buffer.

The DEAE Cellulose and elution buffer mixture was gently stirred and allowed to stand overnight at 4ºC. Hence the suspension was allowed to settle, the supernatant liquid was discarded and the procedure was repeated in order to complete the ions hydration and to discard the gel fine particles.

The 50 x 2.8 cm column filling was carried out using successive anionic suspension volumes, after the valve of the column was closed. The addition of the gel was carried out cautiously in order to prevent air bubble intake. Between additions the buffer drained in order to allow the gel to settle in the column. Finally the gel layer settled to a constant height of 42-44 cm.

In order to defibrinate the human plasma, the biological specimen was processed as following: 1 mL of a 1M calcium chloride solution was added to 100 mL of human plasma, followed by cautiously heating the mixture on water bath at 37ºC, for approximately 2 hours, until fibrin clots were completed. The fibrin clots were discarded using a Pasteur pipette. The obtained serum specimen was then dialyzed using a dialyze sack, using the same elution acetate buffer, at 4ºC, for approximately 12 hours. If in the dialyzed container forms a precipitate, the supernatant was separated after centrifugation at 5000 G for 10 minutes .

The serum specimen obtained as described above was added (100 mL) to the chromatographic column. The specimen was then eluted with 0.02 M, pH=4.0 acetate buffer and there were collected 5 mL aliquots/test tube.

The elution of human BuChE was carried out using a continuous gradient of NaCl, gradually increasing the NaCl percentage in the acetate buffer from 10% to 80 %.

The aliquots collected as described above were tested for total protein concentration by measuring their absorbance at $\lambda$=280 nm (Lambda 40 UV/Vis Spectrometer-Perkin Elmer producer). Farther the elution was performed with the same buffer until the absorbance values drop below 0.02. Starting with the 145th aliquot, the proteins were eluted using the gradient procedure, using different NaCl concentration (varying from 10% to 80%) in 0.02 M acetate buffer pH=4.0. The used volumes of acetate buffer for every NaCl concentration varied from 150 to 300 mL. 8 mL /test tube aliquots were collected, following by continuous protein concentration monitoring. The total number of aliquot sample collected was 420. The aliquots with high absorbance values were tested for enzymatic activity.

The specific BuChE enzymatic activity from the collected aliquots was carried out using a Lambda 40 UV/VIS Spectrometer – UV KinLab Software Module, following Worek procedure, at 436 nm. [17].

The mixed aliquots sample were concentrated through dialyze on PEG 6000**.** For the enzymatic activity assay was used a volume of 500 µL of each mixed aliquot sample .

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## Statistical methods

Parameters of theoretical exponential model fitting the experimental data were established by a least square algoritm included in the "trendline" command in Microsoft Excel. Correlation coefficient concerned the two raws: experimental values and calculated interpolation of the model. Exponential were compared applying t-test for parallelism between straight lines obtained with logarithmic transformed data.

## Results

The absorbance of the successive separated samples at 280 nm is presented in Fig. 1.

Numbers of aliquots represent represpectively:173-203 the 10% NaCl elution; 204-218 the 20% NaCl elution; 219-255 the 30% NaCl elution; 256-292 the 40% NaCl elution; 293-330 the 50% NaCl elution; 331-368 the 60% NaCl elution; 369-403 the 70% NaCl elution and 404-420 the 80% NaCl elution;

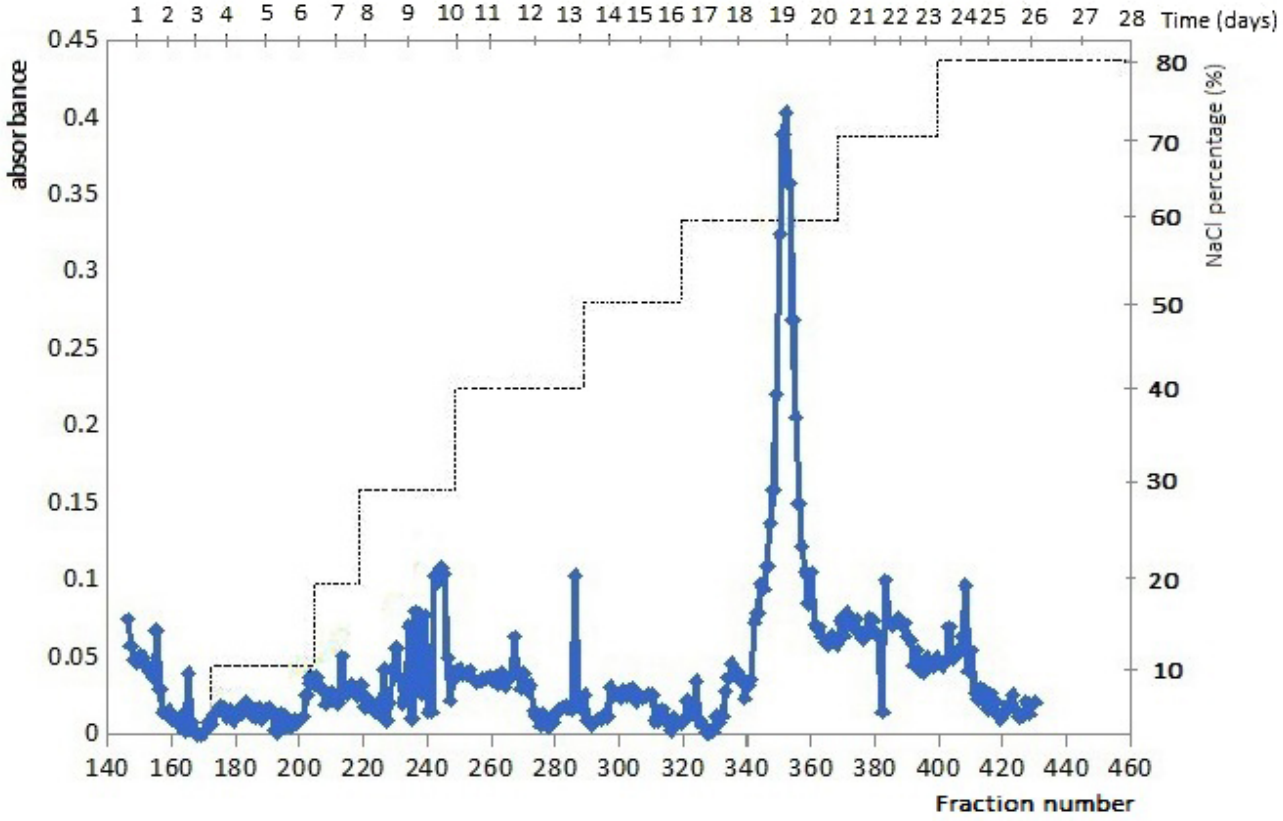


***Fig.1. Absorbance of the successive collected fractions at 280 nm***

The absorbance - number of sample curve, is in fact an absorbance - time curve for an approximately five weeks interval. The first part of chromatogram ( samples 1 – 360 ) seems to represent a constant absorbance. Dependence in the interval 340 – 360 presents the characteristics of a chromatographic peak. Other single points maxima are outliers, most probably experimental errors.

In the 225 – 250 interval significant fluctuations were recorded, whereas the 360 – 420 interval appears as a tail of the "340 – 360 peak".

Dependence of the absorbance on NaCl concentration for the samples before the appearance of the chromatographic peak was not linear but a two phase effect, increasing followed by decreasing as can be seen in Fig. 2.

Dependence of the enzymatic activity of eluted BuChE, cumulated samples corresponding to different NaCl concentrations, determined by method of Worek was quantified finally by absorbance at 436 nm and is presented in Fig.2 (right hand scale). It appears that before 40 % NaCl BuChE was not detected , at 60 % reached a maximum concentration and decreased slowly at 70 and 80 % .

Another observed effect of NaCl was the decrease of resistance of the column to flow of the elution fluid. In the absence of NaCl the flow rate was 7 mL/h. Increasing of NaCl concentration induced a continuous increase of the flow to a value of 21 mL/h at 60% NaCl

solution. After this concentration the flow remained practically constant.

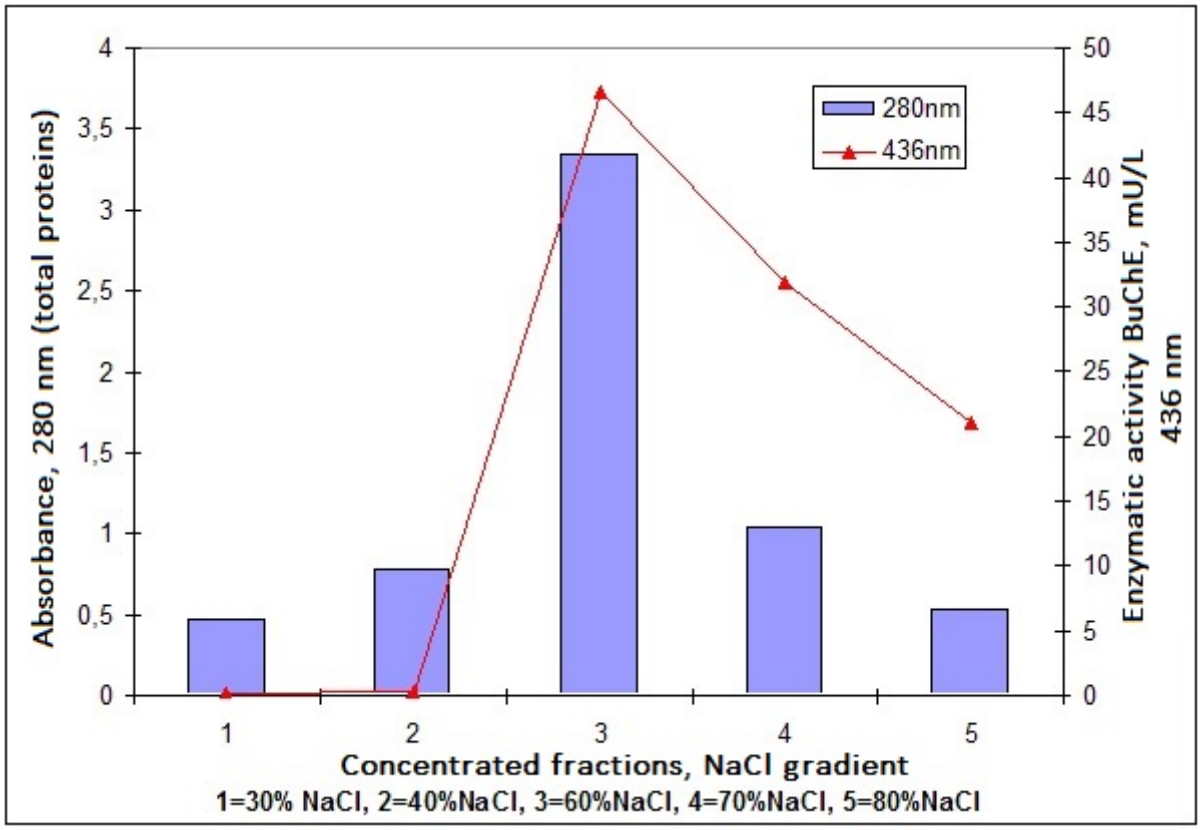


***Fig.2 Dependence of the absorbance on the concentration of NaCl for samples before the appearance of the chromatographic peak -cumulated data.***

## Discussion

Introduction of a protein solution into a chromatographic column filled with ion-exchange resins establishes electrostatic bonds between the negative charges of proteins and the positive charges of the resin. The proteins are further eluted using a solution containing an ion ( acetate ) with greater affinity for the stationary phase than the protein in condition of osmotic pressure created by high concentrations of sodium ion. In case of BuChE the mechanism can be imagined like in Fig.3.

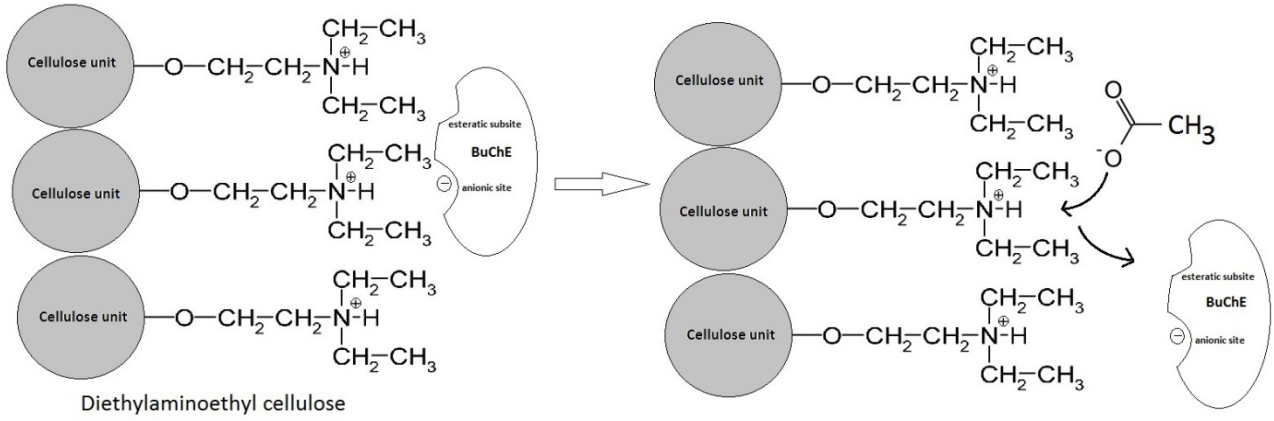


***Fig 3. Ion exchange process between DEAE-Cellulose column and Butyrylcholinesterase serum specimen followed by elution with acetate buffer***

Effect of charged molecules on the ionic exchange and separation could appear by different mechanisms: directly on the reactions, effect on solvation of ions, effect on the


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structure of electrical double layers and consequently on repulsion between cellulose units etc. Natrium chloride was used, in this study to increase the elution of BuChE because has not absorption at 280 nm, is not inhibitory of activity of enzymes and is inexpensive.

It is not possible to weight and make an hierarchy of these mechanisms . At a global level, it were observed the resultant effects on the flow and on the concentration of BuChE in eluent fluid .

Fundamental aspects of the ionic interactions and their effect on the particle-particle interactions and sol-gel equilibrium were early explained in the classical Derjaghin Landau Verwey Overbeeck theory [18] .

More recently, theory was extended to include, beside ionic, steric, elastic [19] and osmotic forces [20-21] in so called extended DLVO theory (XDLVO). These interactions include farther in vitro and in vivo protein interactions inside and outside cells. Mate Hermansson reviewed where the DLVO theory has been used to explain adhesion of microorganisms, bacteria, some viruses and yeast [22-23].

The effect of charged molecules on the electrical double layers at interphases is , as a general rule, biphasic: increasing of electrophoretic potential at small concentrations and decreasing at high concentrations. In our case, the increase respectively decrease of repelling potential are translated in distances between cellulose units and dimensions of pores in the structure of pores and resistance to flow of fluid and BuChE across gel as is illustrated in Fig 4 and represent in fact the sol-gel equilibrium.

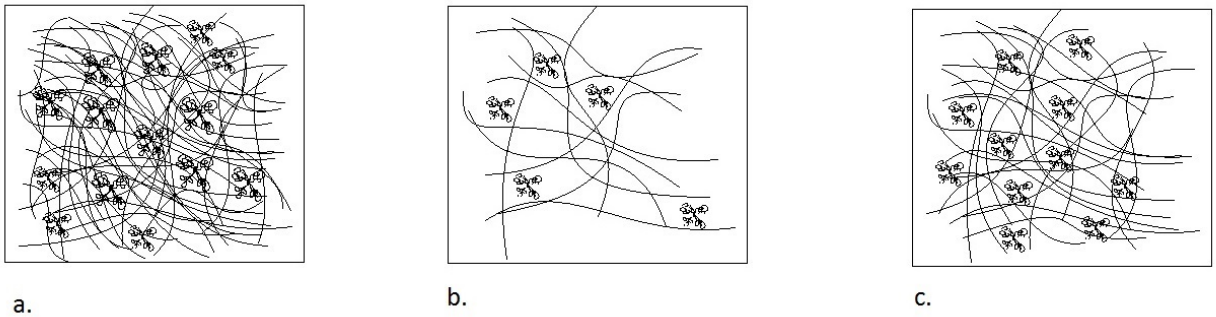


***Fig. 4. The fluid flow through porous media at different NaCl concentrations***

***a.under 60% NaCl-compact structure; b.60% NaCl – loose structure; c.80% NaCl intermediate structure.***

The effect on the ionic exchange is essentially an effect on chromatographic partition coefficient, leading, as a rule, to a peak having a Gaussian form.

Fitting separately of ascending and descending parts of the apparent peak in the 320 – 360 interval, led (Fig.5) to practically the same exponential coefficient 0.239 and 0.240 .

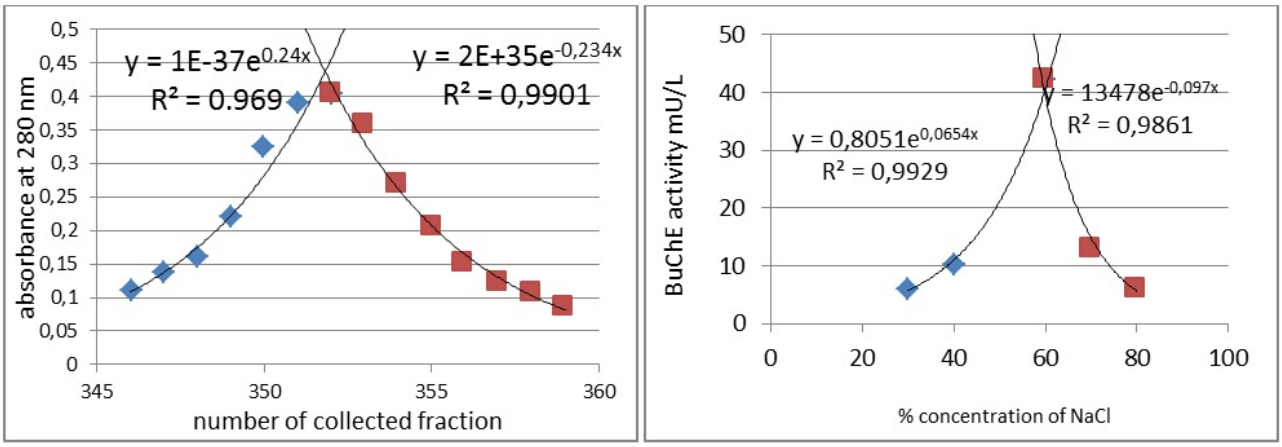


***Fig.5 Exponential fitting of pre and post maximum data. a. absorbance at 280 nm; b. butyrylcholinesterase activity***

This symmetry of effects and exponential dependences are characteristic of effects on the chromatographic partitions between stationary and mobile phases. The decreasing of absorbance in the second part of the chromatographic peak is rather an effect resulting from complete depletion of adsorbed analyte , in our case BuChE. The effect on the sol-gel equilibrium would end by complete destructuration of gel, which phenomenon didn't appeared in our experiment.

The symmetric exponential behaviour of both absorbance at 280 nm and BuChE activity are solid arguments for chromatographic exchange as mechanism prevailing on the effect on structure of solid phase in the neighbourhood of 60 % NaCl concentration.

## Conclusions

Separation of proteins and particularly of BuChE on a chromatographic DEAE Cellulose column in NaCl gradient can be considered as a method for separation and purification of BuChE from human plasma.

Dependence of eluted amount of total proteins absorbing at 280 nm on NaCl concentration was very poor until 60 % and significant at high concentrations (greater than 50 %).

Both increasing and decreasing of absorbance at the two wavelength corresponding to proteins and to BuChE followed an exponential model. The exponents were practically identical, proving a real Gaussian behavior determined by effect on ionic exchange rather than effect on the sol-gel equilibrium of stationary phase.

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