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Short communication

Capillary electrophoretic analysis of recombinant human apolipoprotein E
Calibration mode of a protein reference material

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Abstract

Actual lack of standardization of serum apolipoprotein (apo) E measurements prevents intensive studies on the significance of apoE concentration in clinical chemistry. For this purpose the use of standards calibrated against a common reference material is essential in order to obtain reliable results. The assignment of a certified value to this reference material involves the use of an accurate and non immunological technique. We demonstrate here that capillary electrophoresis in sodium dodecyl sulfate containing gel (SDS-CGE) can be a method of choice. ApoE quantification was performed according to the peak area and a standard solution of purified apoAI. We obtained very close results to that obtained by HPLC of phenylalanine apoE content measurement i.e., 81.6 mg/l versus 84.6 mg/l and concluded that the SDS-CGE analysis is an accurate and reliable method for the certification of an apoE reference material to be used in serum apoE concentration measurements. The SDS-CGE combines advantages of SDS–polyacrylamide gel electrophoresis (high resolving power, rapidity and tolerance of complex sample) with that of quantitative HPLC analysis (accuracy, precision, linearity and speed).

Keywords: Lipoproteins; Recombinant proteins; Apolipoproteins

1. Introduction

Apolipoprotein E (apoE) is a 299 amino acid, lipid transport protein of 34.2 kD. ApoE is a protein component of several classes of lipoproteins including chylomicrons, very low density lipoproteins (VLDLs) and their remnants, intermediate density lipoproteins (IDL) and several subclasses of high density lipoproteins (HDL_1, preβ_2-HDL and γLpE) [1,2]. The major site of apoE synthesis and secretion is in liver parenchyma cells, but it is also highly expressed in astrocytes, peripheral nerves and mature macrophages. In the plasma compartment, apoE plays a major role in lipoprotein metabolism through its ability to mediate specific binding of lipoproteins to several hepatic and extrahepatic receptors and to modulate activity of enzymes involved in lipoprotein structural modifications. These metabolic processes are significantly influenced by apoE concentration which has been strongly related to its polymorphism [3]. ApoE is present in humans as three major isoforms named apoE2, E3 and E4. The importance

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of apoE polymorphism in lipid metabolism and in the development of cardiovascular diseases is well-established [4]. At this date it is difficult to establish clinical interest of apoE concentration measurement, particularly due to a lack of standardization in the various methods developed for its measurement as well as in calibration of the assays, which contribute to the wide range of normal values reported in healthy subjects (30–250 mg/l) [5].

The basis of standardization will be the use of high-quality secondary reference standards calibrated against a common primary reference material that permits us to compare results between laboratories and ensure continuing accurate and precise results. ApoE purified from human plasma VLDL has initially been suggested as a primary standard. However, it revealed inherent problems in maintaining the stability of stored material. We thus turned our efforts to develop and validate commutable reference material utilizing tools of recombinant DNA technology to make stable molecule apoE3 for the primary standard [6].

One critical part in the validation of the recombinant reference material was to provide consensus mass concentration for the recombinant apoE3. In order to avoid problems linked to the antigen/antibody reaction, we considered non immunological methods such as colorimetric Lowry's analysis and quantification of the phenylalanine (Phe) content by HPLC.

We proposed here capillary electrophoresis as a faster and easier method to assess recombinant apoE3 concentration with a good level of accuracy. Capillary electrophoresis in sodium dodecyl sulfate containing gel (SDS-CGE) has been demonstrated to be a powerful and sensitive method for the qualitative and quantitative microanalysis of biotechnology-derived proteins [7,8].

2. Experimental

2.1. Chemicals

Ammonium hydrogencarbonate, caprilic acid and bovine serum albumine (BSA) were obtained from Sigma Aldrich (St. Quentin Fallavier, France); Tris base and sucrose from Merck (Lyon, France); sodium chloride (NaCl) and hydrochloric acid (HCl) were obtained from Prolabo (Nogent sur Seine, France); sodium dodecyl sulfate (SDS) from Serva (Heidelberg, Germany). β-Mercaptoethanol, pure reagent for electrophoresis, was provided by Bio-Rad (Ivry sur Seine, France) and Orange G was obtained from the eCAP SDS 14-200 kit (Beckman Coulter, Fullerton, CA, USA).

2.2. Proteins

The recombinant human apoE3 (apoE3R) was produced and purified as described earlier [9]. It was then added to a matrix of 50 m*M* ammonium hydrogencarbonate, (50 g/l) sucrose, 100 m*M* caprilic acid and 154 m*M* of NaCl. The preparation was lyophilized and stored at −80°C. Immediately before SDS-CGE analysis it was reconstituted in 50 m*M* of ammonium hydrogencarbonate. Plasma apoE3 was purified from human plasma VLDL [10].

ApoAI was purified from human plasma HDL as described previously [11]. The stock solution was obtained by reconstitution of lyophilized apoAI protein in 50 m*M* ammonium hydrogencarbonate at a concentration of 2.71 g/l.

2.3. Preparation of the samples

Apolipoprotein samples were mixed with an equal volume of 60 m*M* of Tris–HCl (pH 6.8), 2% SDS and 5% β-mercaptoethanol, containing orange G as the front migration marker. The mixture was heated at 95°C for 5 min and cooled on ice before injection.

2.4. SDS-CGE separation

Capillary SDS gel electrophoresis was performed on a P/ACE system 5510 controlled by the system Gold software (version 8.0) and equipped with a diode array detector and a coated fused-silica capillary [eCAP SDS, 37 cm (effective length 30 cm)×50 μm I.D.]. All capillary electrophoresis apparatus were supplied by Beckman Coulter (Fullerton, CA, USA).

Each sample was injected by pressure into a capillary which was thermostated at +20°C and filled with a non polyacrylamide, SDS containing linear gel buffer (eCAP SDS 14-200 gel buffer,

Beckman Coulter) replaceable between each run. Separations were run in reversed polarity and at constant voltage (300 V/cm) during 25 min. UV detection was performed at 214 nm. First, molecular mass marker (eCAP Molecular Weight, Beckman Coulter) was coinjected by pressure with apoE3R solution (15 and 30 s respectively) in order to generate a calibration curve and identify the apoE3R specific peak according to its calculated molecular weight based on its relative migration time (RMT= specific peak migration time/orange G migration time). Then a multistep external calibration was established by injection of a series of standard solutions obtained from the stock solution of purified apoAI diluted in the apoE3R matrix (271, 181.7, 135.5 and 67.8 mg/l). Quantification was based on apolipoprotein peak area response at 214 nm by plotting apoAI concentration (mg/l) versus corrected peak area (peak area/migration time ratio) and interpolation of apoE3R concentration from the standard curve.

2.5. Colorimetric analysis

The method of Lowry [12] was carried out on the apoE material using bovine serum albumin (BSA) as a standard.

2.6. Estimation of protein concentration by hplc quantification of phenylalanine

Recombinant apoE3 material was hydrolyzed in 6 *M* HCl at 110°C for 20 h. The protein hydrolysate was fractionated by HPLC [13] in order to quantify its phenylalanine content. Phenylalanine was used as an external standard and the detection was performed by measuring the UV absorbance at 254 nm. The protein concentration was recalculated based upon the mass fraction of phenylalanine in human apoE3.

3. Results and discussion

3.1. Identification of the recombinant apoE3 material in SDS-CGE

As shown in Fig. 1, apoE3R was detected within 6 min of the front marker orange G, and was characterized by a very reproducible RMT (RSD<0.002%). No supplementary peak was detected in the recombinant material solution, in agreement with its high purity (>96%). The apparent molecular mass of apoE3R calculated was greater than that of human apoE3 analyzed in the same conditions. In both cases apoE3 molecular masses were overestimated in comparison with the theoretically calculated values (Table 1). The SDS-CGE separation relies upon the assumption that protein binds to SDS with the same weight ratio of 1.4:1 [14]. However, amphipathic apolipoproteins were shown to bind to detergent molecules with a higher affinity than other proteins [15] and may exhibit abnormal charge to mass ratio and migration in the SDS polymer solution. We adjusted molecular mass values by using the Ferguson plot (log 1/RMT versus % gel buffer). Slopes of linear curve fits obtained for each protein marker, for apoE3R and for purified plasma apoE were used to calculate their retardation coefficient (K_r). The linear semi logarithmic plot of molecular weights against K_r square roots of protein markers ($y = 56.52x + 2.42$; $r = 0.99$) was used to estimate molecular weights of apoE3R and purified plasma apoE3. The molecular mass of the plasma apoE3 obtained was reasonably close to the theoretical value, while that of apoE3R remained higher (Table 1). This result may be due to the fusion peptide added to the *N*-terminal amino acid residue of the recombinant protein for fast purification [9]. However, we can not exclude a concomitant effect of the matrix used in the lyophilized apoE3R material on the molecular sieving process as well.

3.2. Quantitative analysis of the recombinant apoE3 material

In order to overcome the unexpected influence of peak migration on quantification accuracy, the concentration of apoE3R was determined based on the corrected peak area (peak area/migration time). The method involved preparation of a series of standard solutions that approximated the composition of apoE. Thus we used highly purified apoAI solution, showing similar behavior in SDS polymer solution and similar molar absorbance coefficient (ϵ) to apoE ($\epsilon = 1.15$ for apoAI vs 1.24 for apoE). A significant linear correlation was observed within the range of

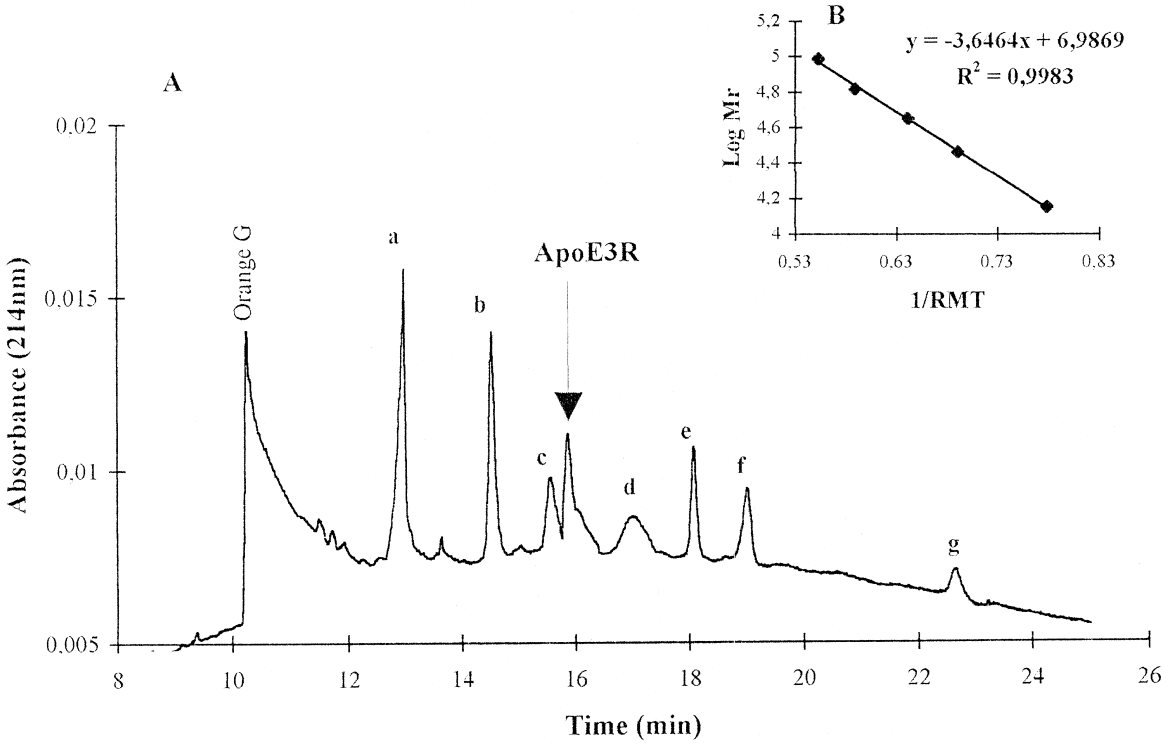

Fig. 1. Internal molecular mass calibration of the apoE3R by coinjection of molecular mass markers and apoE3R sample. Relative migration time of each protein peak was calculated according to the Orange G peak, which was used as the front reference peak of the migration. Molecular mass marker kit was constituted of seven proteins (a = α-lactalbumin, b = carbonic anhydrase, c = ovalbumin, d = bovine serum albumin (BSA), e = phosphorylase b, f = β-galactosidase and g = myosin) ranging from M_r 14 200 to 205 000.

67.8 to 271 mg/l ($y = 58.26x + 39.82$; $r = 0.99$). By interpolation from the standard plot, we estimated the apoE3R concentration to be 84.6 mg/l with a satisfactory reproducibility (RSD < 2.2%). Values obtained for measurement of the apoE3R reference material using three different non immunological methods ranged from 81.6 to 90.7 mg/l (Table 2). Assessment of phenylalanine in the apoE3R by HPLC and its measurement using capillary SDS gel electrophoresis gave very close results, i.e. 81.6 vs. 84.6 mg/l (3.6% difference). They were slightly lower than the result obtained by Lowry's method, which may lack specificity and accuracy. As a matter of fact Lowry's method is based on the reaction of chromatogene with a few amino acids of the peptide chain and on the use of standard protein, i.e. bovine

Table 1
Estimation of apoE3R molecular mass by SDS-CGE in comparison with that of apoE3 purified from human VLDL and theoretical molecular mass values

ApoE	Molecular mass (M_r) (D)		
	Theoretical	SDS-CGE analysis	
		Log M_r vs. 1/RMT[a]	Ferguson plot
Plasma apoE3	34 200	37 000	35 600
Recombinant apoE3	39 000	46 600	44 800

[a] RMT = Relative migration time.

Table 2
Determination of the apoE content of the material by quantitative SDS-CGE and comparison with colorimetric and amino acid assays

Analysis methods	ApoE3R concentration (mg/l)		
	Mean	SD	RSD (%)[a]
Lowry method	90.7	3.34 ($n=12$)	3.7
Phenylalanine quantification by HPLC	81.6	2.58 ($n=6$)	3.2
SDS-CGE	84.6	1.86 ($n=6$)	2.2

[a] RSD = Relative standard deviation.

serum albumin, with an amino acid composition obviously different than that of apoE. On the contrary capillary electrophoresis overcomes these biases by proceeding to on-line specific detection of the recombinant apoE3 content in the lyophilized material without staining and by using a calibrating material, i.e. apoAI, which shows significant similarity with apoE in the amino acid composition. Moreover in contrast to HPLC, the SDS-CGE procedure is faster and does not require tedious preceding preparative steps.

In conclusion, SDS-CGE appears to be well suited to qualitative and quantitative analysis of recombinant proteins. It is automated and rapid, requires only a small volume of samples and buffers and shows highly reproducible separation, and specific and selective detection, ensuring accurate quantification of the targeted protein. By using certified apoAI as calibrator (CRM393 EC, Brussels, Belgium), the value measured by capillary electrophoresis will be traceable to completely admitted reference material. This may ensure continuing accurate and reproducible measurements of plasma apoE concentration among all laboratories and help to understand its pathophysiological role.

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