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Capillary electrophoresis, a rapid and sensitive method for routine analysis of apolipoprotein A-I in clinical samples

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Abstract

A method for the determination of apolipoprotein A-I, the major protein compartment of HDL, in human serum is described. Rapid and easy serum sample preparation, well separated Apo A-I peaks in the human serum electropherogram and good linearity of the peak area vs. concentration plot, covering the range of the clinically relevant Apo A-I serum contents, suggest the introduction of this method routinely in clinical laboratories.

1. Introduction

Minimum sample and buffer requirements in combination with rapid and efficient separation have made capillary electrophoresis (CE) [1–5] one of the most attractive tools for the analysis of biopolymers such as peptides [6–10], proteins [11–16], glycoproteins [17,18] and oligonucleotides [19–21]. Recently, pharmaceutical and clinical analysis laboratories have started to develop routine CE methods for purity testing [22], determinations of the formulation contents [23–25], chiral analysis [26], monitoring of drugs in body fluids [27,28] and reliable and precise analyses of blood serum and its fractions [29–31]. Apolipoprotein A-I (Apo A-I) is the major protein constituent of human high-density lipoprotein (HDL). Decreased Apo A-I levels in human serum are indicative of arteriosclerotic processes [32], acute hepatitis and hepatic cirrhosis [33]. The median serum concentrations for Apo A-I were determined to be 145 mg/dl for men and 160 mg/dl for women [34]. Today, immunonephelometric assay (INA) is the most common method applied in clinical laboratories for the routine determination of Apo A-I concentrations in human serum [35]; other methodologies used include radial immunodiffusion (RID), radioimmunoassay (RIA), electroimmunoassay (EIA), enzyme-linked immunosorbent assay (ELISA) and immunoturbidimetric assay (ITA). The major drawbacks of immunological methods for the determination Apo A-I are the inhomogeneity of HDL [36] and that masking lipids prevent antigenic sites being expressed [37,38]. Further, a serious problem of any INA or ITA is that hyperlipaemic samples may disturb the Apo A-I determination. A

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reliable Apo A-I assay to be performed via a simple serum sample work-up procedure and applying the advantages of CE is therefore of great interest for clinical chemists and such a method is presented in this paper.

2. Experimental

2.1. Materials and reagents

Fused-silica capillaries (50 cm × 50 μm I.D.) (Grom, Herrenberg, Germany) were used for all electrophoretic runs and pretreated with 1 *M* NaOH for 15 min, followed by 15 min rinsing with water before the electrophoretic separations. Using 30 m*M* borate buffer (pH 10), the capillary was rinsed for 60 s with water, followed by 120 s with 0.1 *M* NaOH, 120 s with water and finally 60 s with buffer after each run. After separations, performed with sodium dodecyl sulphate (SDS)-containing Bio-Rad (Munich, Germany) evaluation LLV buffer, the following rinsing sequence was applied by pressure 6.89 · 105 Pa (100 psi): water (60 s), LLV buffer (120 s), water (120 s) and LLV buffer (60 s). The Apo A-I standard sample was purchased from Sigma (St. Louis, MO, USA). The serum samples were prepared from blood from normal fasting male donors or clinical patients and allowed to clot. The serum was separated by low-speed centrifugation at 2000 *g* for 7 min and used immediately after recovery or stored at −70°C until used.

2.2. Electrophoretic apparatus

All separations were performed on a Bio-Rad BioFocus 3000 capillary electrophoresis system, equipped with an automatic constant-volume sample injection system, a temperature-control system for the capillary, sample and fraction collection compartment, a high sensitivity fast-scanning UV–Vis detector with wavelength programming and a dedicated computer system with a Microsoft Windows interface. During all runs, the capillary and the sample compartment was cooled to 15°C.

2.3. Immunonephelometric assay (INA)

For the INA we used a Behring nephelometer (Behringwerke, Marburg, Germany) and fixed-time kinetic analysis. The Apo A-I nephelometric determinations were performed according to the procedures provided by the manufacturers. After adding an aliquot of diluted sample (100 μl) and antibody (40 μl) to a cuvette containing the reaction buffer (80 μl), a background reading (zero time) was taken. After 6 min, the net increase in scattered light was calculated from a second reading. The scattered light value was compared with those on a calibration graph and the concentration was calculated. The manufacturer stated an assayable range from 18 to 580 mg/dl for Apo A-I, using a plasma or serum dilution of 1:20.

3. Results and discussion

As mentioned in the Introduction, INA is the most commonly used method for determining the content of Apo A-I in human serum in routine clinical laboratories. INA allows automated and rapid analysis, but nephelometric and turbidimetric methods are susceptible to factors that interfere with light transmission such as lipoproteins, dust and other plasma proteins [35]. Moreover, immunological procedures for Apo A-I determination in serum are complicated by the fact that it is a component of a large, heterogeneous particle, and some of the antigenetic sites are masked by lipids [37,38]. Consequently, the results are strongly influenced by variations in the specificity of antisera, the standardization procedure and the methodology used in a particular assay system. Therefore, our intention was to establish a CE method for determining the Apo A-I concentration in human serum which can be used conveniently in every routine clinical laboratory.

The feasibility of the routine analysis of human serum protein fractions by CE has been demonstrated [31,39–41], mostly using phosphate or borate buffers. Hence, our first attempt

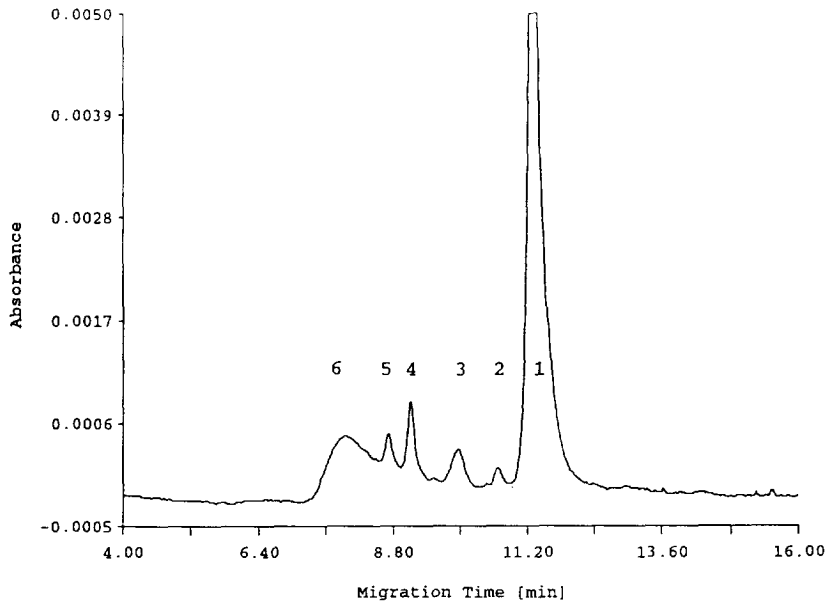

Fig. 1. Capillary electropherogram of a normal human serum sample (dilution: 1:30). Electrophoretic conditions: capillary, fused-silica (50 cm × 50 μm I.D.); loading, $2.75 \cdot 10^7$ Pa s; running conditions, 15 kV; buffer, 30 m*M* borate (pH 10); detection, UV at 220 nm; equipment, Bio-Rad CE 3000, BioFocus 3000. Peaks: 1 = albumin; 2 = α_1-globulin; 3 = α_2-globulin; 4 = β_1-globulin; 5 = β_2-globulin; 6 = γ-globulin.

to separate HDL, with Apo A-I as the main protein constituent, started with borate buffer (pH 10). Fig. 1 shows a capillary electropherogram of a diluted (1:30) serum sample obtained with a 50 cm × 50 μm I.D. fused-silica capillary and 30 m*M* borate running buffer (pH 10), demonstrating the well known separation profile of six fractions. Even using capillaries with different lengths and diameters and different buffer molarities, no lipoprotein fractions, in particular no specific HDL peak for quantitative evaluation, could be detected from normal human serum samples or HDL-spiked samples.

To determine Apo A-I concentrations in serum directly, a series of buffer systems with different additives were tested and it was found that the Bio-Rad LLV buffer allows the specific determination of Apo A-I in blood samples. Fig. 2 shows electropherograms of a diluted serum sample (1:30) spiked with 0.25 mg/ml of Apo A-I, recorded at wavelengths from 220 to 195 nm and using Bio-Rad LLV buffer. By adding an Apo A-I standard to the normal serum sample, the well separated peak at a migration time of 21.10 min could be identified. As the sensitivity could be drastically increased by using a recording wavelength of 195 nm instead of 200–220 nm, which is commonly used, all further runs were performed at the lowest wavelength. Fig. 3 demonstrates that the electrophoretic conditions discussed above allow a clear separation of Apo A-I from serum proteins and quantitative peak evaluation even in highly lipaemic serum samples, whereas nephelometry gives doubtful results (see above).

The electropherogram of a patient's serum sample with an elevated level of Apo A-I is presented in Fig. 4, showing a major and an additional minor Apo A-I peak with a shorter migration time. Different polymorphic Apo A-I forms are responsible for this separation pattern, as revealed by analytical isoelectric focusing [42,43] and recently also by capillary electrophoresis [31,44]. Therefore, for Apo A-I determination the area of both peaks must be considered.

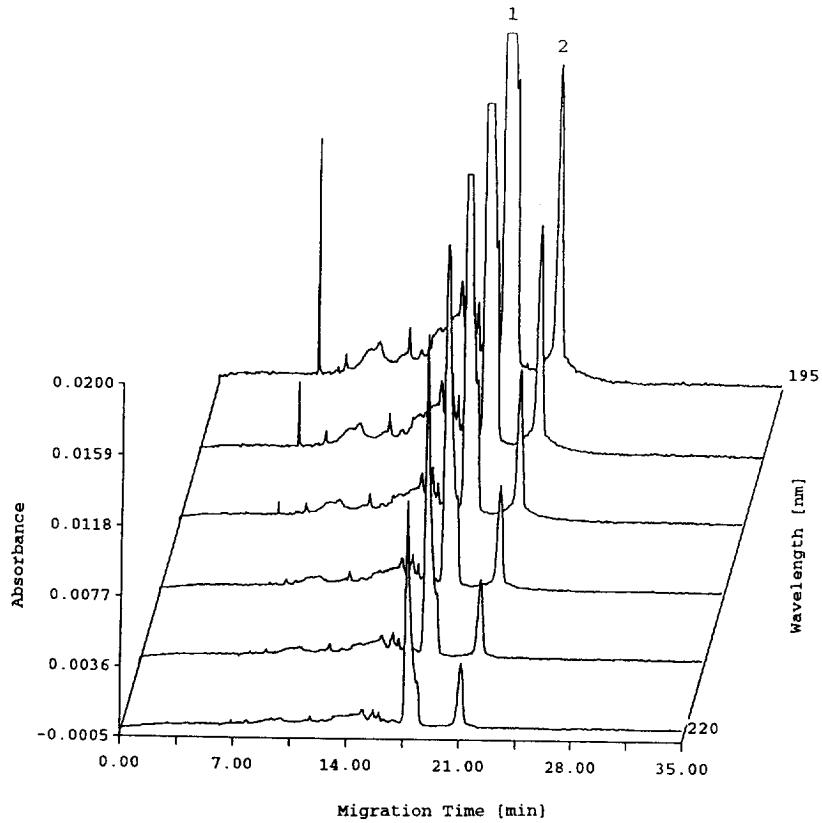

Fig. 2. Multi-wavelength electropherogram of a normal human serum sample (dilution 1:30) spiked with 0.25 mg/ml of Apo A-I. Electrophoretic conditions: capillary, fused-silica (50 cm × 50 μm I.D.); loading, $2.75 \cdot 10^7$ Pa s; running conditions, 20 kV; buffer, Bio-Rad evaluation LLV buffer; equipment, Bio-Rad CE 3000, BioFocus 3000. Peaks: 1 = albumin; 2 = Apo A-I.

Fig. 5 shows a linear plot of relative peak area vs. amount of Apo A-I standard (co-injected with a serum sample) obtained under the same conditions as in Fig. 2. The peak areas at each concentration were determined four times. As clinically relevant Apo A-I serum concentrations are 5–300 mg/dl, the detection limit and range of the CE method are suitable for routine analysis.

It is well known from the literature that protein sticking to the fused-silica capillary wall surface and basic washing buffers influence the migration time and peak areas [45–47], and both are undesirable in routine clinical analysis. However, when LLV buffer was used for washing between runs for 50 consecutively measured serum electropherograms, a relative standard deviation of 1.8% for the migration time (Fig. 6) and of 8.7% for the relative peak area (Fig. 7) were found, which are acceptable for routine analysis. The slightly increasing migration time of Apo A-I observed is a phenomenon well known for proteins separated with fused-silica capillary columns [15,39]. The slightly decreasing peak areas might well be a consequence of irreversible adsorption on the detector window, or protein adsorption could affect the efficiency of non-adsorbing species by setting up complex flow patterns within the capillary [48].

In Table 1, nephelometrically and capillary electrophoretically determined Apo A-I concentrations in sera from different patients are compared. If only the main Apo A-I peak fraction is considered, the concentrations determined by

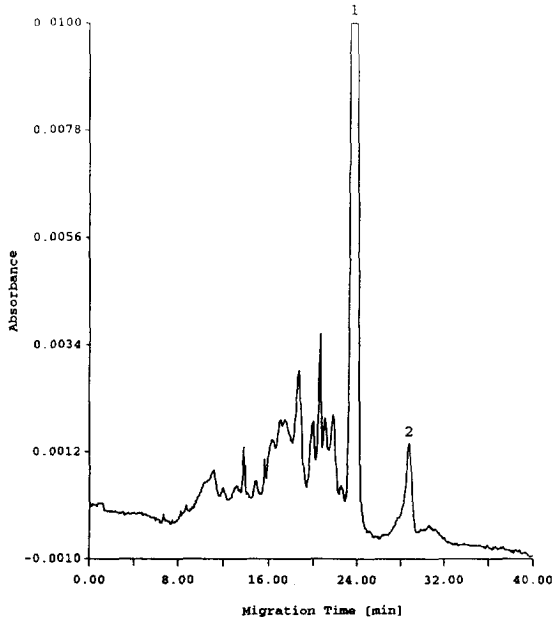

Fig. 3. Electropherogram of a lipaemic serum sample from a patient (triglycerides, 967 mg/dl; cholesterol, 298 mg/dl). Separation conditions as in Fig. 2; detection, UV at 195 nm. Peaks: 1 = albumin; 2 = Apo A-I. Results of determination of Apo A-I: CE, 167 mg/dl; nephelometry, 147 mg/dl.

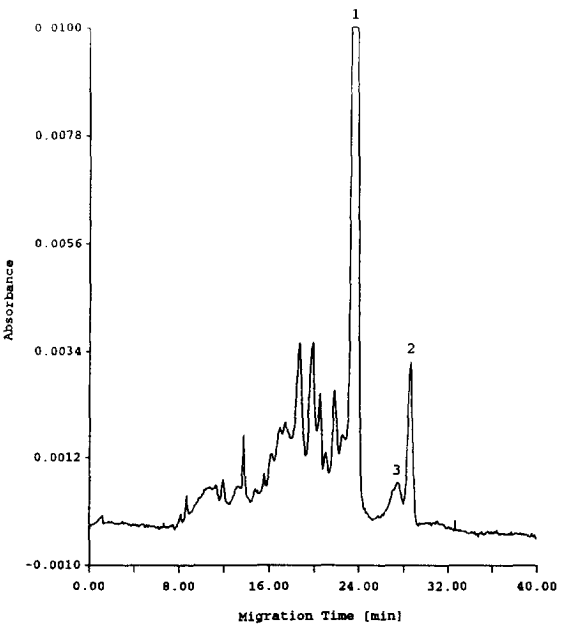

Fig. 4. Electropherogram of a patient's serum sample with an elevated level of Apo A-I. Separation conditions as in Fig. 2; detection, UV at 195 nm. Peaks: 1 = albumin; 2 + 3 = Apo A-I. Results of determination of Apo A-I: CE, 301 mg/dl; nephelometry, 244 mg/dl.

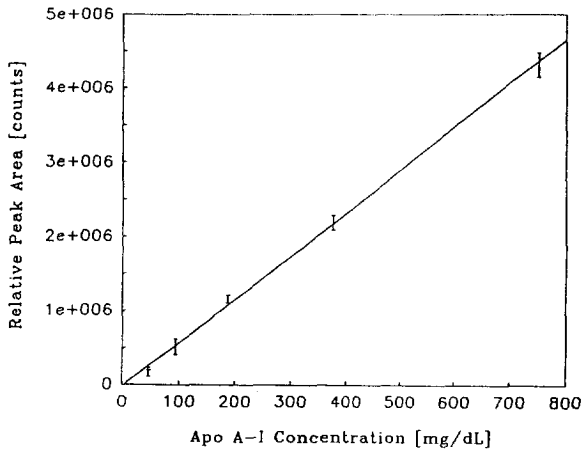

Fig. 5. Plot of relative peak area vs. amount of separated Apo A-I standard (co-injected with a serum sample). Electrophoretic conditions as in Fig. 2. All samples were analysed four times.

CE are about 50% lower than the nephelometric results. If the peak areas of the polymorphic Apo A-I forms are also included, there is a good agreement between nephelometry and CE, although on average about 10% higher values were found with CE, which may be caused by the above-mentioned drawbacks from which nephelometry suffers.

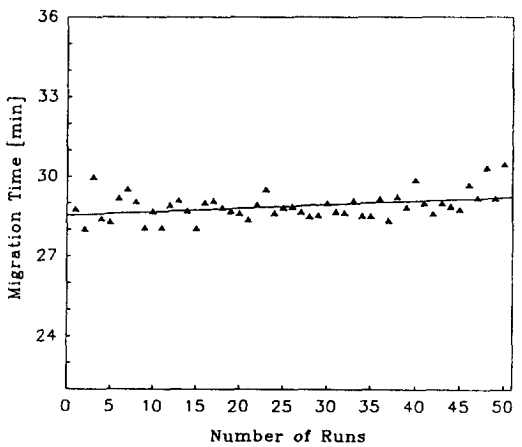

Fig. 6. Reproducibility of the migration time (50 runs) of the Apo A-I peak (intra-assay precision) for the electropherogram illustrated in Fig. 4. Mean, 28.88 min; S.D., 0.53 min; R.S.D., 1.84%.

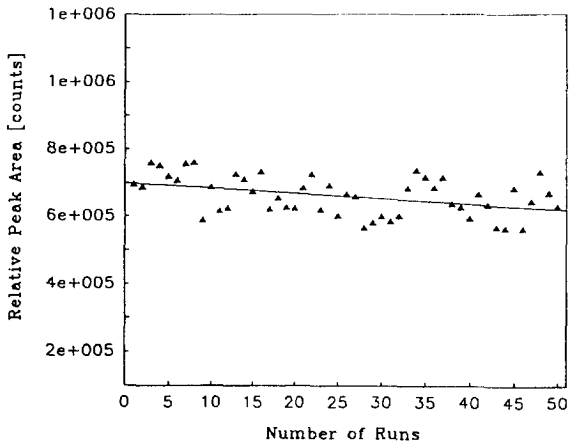

Fig. 7. Reproducibility of the relative peak area (50 runs) of the Apo A-I peak (intra-assay precision) for the electropherogram illustrated in Fig. 4. Mean, 660 441 counts; S.D., 57 812 counts; R.S.D., 8.75%.

4. Conclusions

The described buffer system makes possible the direct determination of Apo A-I concentrations in human serum samples. In contrast to nephelometry, CE also allows the detection of different polymorphic Apo A-I forms, the clinical relevance for which can now be investigated with our method. The ease of serum sample preparation, the good linearity of relative peak area vs. Apo A-I concentration, automation of the technique and low-cost reagents are advantages for application in routine clinical laboratories.

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Table 1
Comparison Apo A-I concentrations (mg/dl) in sera from different patients determined by nephelometry and capillary electrophoresis (conditions as in Fig. 2).

Patent	Nephelometry	CE[a]	
		Main peak	Both peaks
1	128	83 (−54%)	131 (+2%)
2 (lipaemic)	147	97 (−52%)	167 (+12%)
3	149	92 (−63%)	173 (+14%)
4	139	85 (−63%)	136 (−2%)
5	244	170 (−43%)	301 (+19%)

[a] The CE values are calculated on the basis of the peak area vs. concentration plot (Fig. 5).

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