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ORIGINAL RESEARCH

Identification of κ and λ chains of the major immunoglobulin G subclasses by capillary zone electrophoresis

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ABSTRACT: Immunoglobulins are present in most tissues and plasma and play crucial role in immune system. Alteration of the levels of the immunoglobulin G (IgG) subclasses (IgG1, IgG2, IgG3 and IgG4) is an indication of a disturbed immunological response. The aim of the present study was the development of a capillary electrophoresis (CE) method for the analysis of IgG subclasses in respect to their variable κ and λ chains. Various analytical conditions and CE modes, including capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF) and micellar electrokinetic capillary chromatography (MECC) have been thoroughly studied. CZE was found to be the most convenient way to separate IgG subclasses. Three of the human IgG subclasses were resolved using uncoated fused-silica and 50 mM phosphate, pH = 9.3, as operating buffer at 20 kV and detection at 214 nm. IgG1κ was completely separated from IgG2κ and IgG3κ, whereas IgG2κ co-migrated with IgG4κ, which is the minor IgG subclass. Under the same conditions IgG4λ was completely separated from IgG1λ, IgG2λ and IgG3λ, enabling the identification of the various λ chains. The developed CE method is rapid and can be applied to the identification of the major immunoglobulin G subclasses in respect to their variable κ and λ chains. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Human immunoglobulin G (IgG) belongs to the family of glycoproteins. IgG is the most abundant among the immunoglobulin classes. It is the antibody against foreign antigens, such as viruses, bacteria and antitoxins, found in most tissues and plasma and plays a unique role in immune response. The IgG classes are further differentiated to subclasses, adding another level of complexity to antibody structure and function. In humans, IgG antibodies comprise four IgG subclasses: IgG1, IgG2, IgG3 and IgG4. The heterogeneity of IgG, which is due to variations in the amino acid sequence, post-translational modifications and degradation, is considered the most critical point in antibody function and effectiveness. Immunoglobulin deficiency syndromes are a group of immunodeficiency disorders in which patients have reduced amounts or lack of antibodies. Deficiency in IgG1 is associated with recurrent infections and chronic fatigue syndrome, whereas low levels of IgG2 is associated with reduced response to bacterial infections, lung inflammation, pneumonia and influenza type B. Deficiency in IgG3 is related to recurrent infections resulting in chronic respiratory diseases and in IgG4 with respiratory infections in children. Since several immune-related diseases are associated with decreased or increased levels of IgG subclasses, the alterations in the amounts of IgG subclasses may well indicate a disturbed immune response (Kahaly, 1996; Lamari *et al.*, 2000).

Analysis of IgG subclasses is widely used in the daily laboratory practice for diagnosis and/or monitoring of several diseases, such as autoimmune diseases, multiple sclerosis, bacterial infections, viruses and asthma. Membrane, polyacrylamide and agarose gel electrophoresis have all been used for the detection of immunoglobulin G subclasses and screening of monoclonal antibody variants as well as for studying oligoclonal bands in multiple sclerosis and other neurological diseases (Grimaldi *et al.*, 1986). Capillary electrophoresis (CE) is a modern analytical technique of high resolving power and efficiency. Its wide applicability is due to the short separation times and the requirement for ultra low amounts of biologic samples and toxic organic solvents (for a review see Lamari and Karamanos, 1999). The

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Abbreviations used: CE, capillary electrophoresis; CZE, capillary zone electrophoresis; CIEF, capillary isoelectric focusing; IgG, human immunoglobulin G; MECC, micellar electrokinetic capillary chromatography; PEG, polyethyleneglycol.

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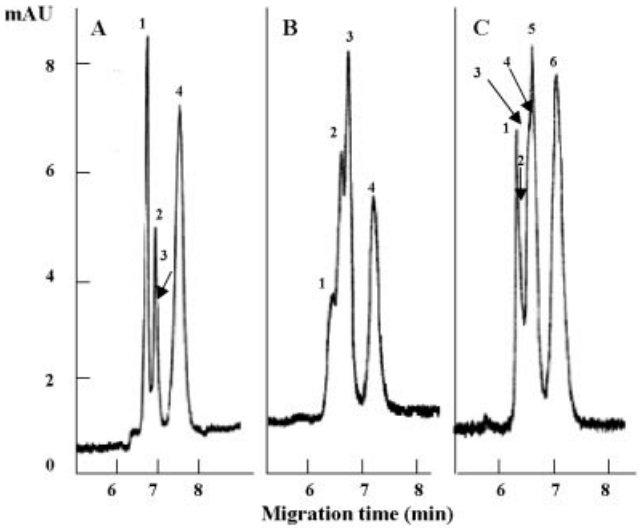


Figure 1. Typical electrophoretic profiles of κ and λ of the IgG subclasses. CZE of IgGs (0.1 μg mL^{-1}) was performed on an uncoated fused-silica with 50 mM phosphate (pH = 9.3), as operating buffer, using normal polarity at 20 kV and detection of the migrated species at 214 nm. Sample injections were performed under pressure 100 mbar s. (A) and (B) resolution of IgG κ and λ chains, respectively and (C), electrophoretic profile obtained for an equimolar mixture of the major κ and λ chains of IgGs. Identity of peaks: (A) **1**, IgG3κ; **2**, IgG2κ; **3**, IgG4κ; **4**, IgG1κ. (B) **1**, IgG2λ; **2**, IgG3λ; **3**, IgG1λ; and **4**, IgG4λ; (C) **1**, IgG3κ; **2**, IgG2λ; **3**, IgG2κ; **4**, IgG3λ; **5**, IgG1λ; and **6**, IgG1κ.

various CE operation modes have been successfully used for the analysis of proteins/glycoproteins (Karamanos and Lamari, 2001). Recently, CE has also been used for the detection of immunoglobulin subclasses (Kelly and Lee, 1997; Wong and Aw, 1998; Sanders *et al.*, 1999). Kelly and Lee (1997) reported a successful method for the resolution of IgG subclasses and monoclonal antibody variants. Although the post-capillary affinity detection utilized provides high sensitivity and selective identification of IgGs, only some of the IgG subclasses (IgG1λ, 2λ and 3κ) were completely resolved. Capillary zone electrophoresis (CZE) is the simplest mode of CE. However, various operation modes, such as capillary isoelectric focusing (CIEF) and micellar electrokinetic capillary electrophoresis (MECC) can also be useful for protein analysis (Kuhn and Hoffsteller-Kuhn, 1993; Karamanos and Lamari, 2001). The aim of the present study was therefore to examine the various CE operation modes and to develop a simple CE method for the separation and identification of the major IgG subclasses in respect to their variable κ and λ chains.

EXPERIMENTAL

Biologic material and chemicals. High-purity light chains of IgG subclasses of human origin (IgG1κ, IgG2κ, IgG3κ, IgG4κ and IgG1λ, IgG2λ, IgG3λ and IgG4λ) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Uncoated fused-silica capillary and polyvinyl-alcohol (PVA) coated capillary were obtained form Agilent Technologies (Waldbronn, Germany). Operating buffers used for CE analysis were first filtrated through a 2 μm membrane (Millipore GmbH, Eschborn, Germany). All other chemicals used were of the best commercially available grade.

Capillary electrophoresis conditions. CE analysis was performed in a HP^{3D}CE system (Agilent Technologies) with a built-in high voltage power supply and a UV-diode array detection unit connected with a data collection and processing device. CZE and MECC were performed on uncoated fused-silica capillaries (50 μm i.d.) with total length of 64.5 cm and effective length up to the optical window of 56 cm. Electrophoresis was performed at 25°C and detection of migrated species at 214 nm. Voltage was selected following monitoring of the migration profiles from 10 to 25 kV. Sample introduction was performed using the pressurized injection mode and depending on the sample concentration it ranged from 100 to 500 mbar s. Before each analysis the capillary was washed with 0.1 M NaOH for 2 min, followed by another washing with 2× distilled water for 2 min and finally with the operating buffer for 4 min. The operating buffers examined in CZE were phosphate at final concentrations of 15, 30, 50, 80 and 100 mM buffered at various pH (3.0, 7.2, 8.0, 8.2, 8.4, 8.6, 8.8, 9.3 and 9.5) as well as 20 mM borate buffered at pH 9.3. The effects of various buffer additives and organic modifiers, such as 20% (v/v) acetonitrile, 0.2% (v/v) polyethylene glycol (PEG), 0.5% (v/v) Triton X-100 and 0.1% SDS (w/v) were examined. The operating buffers examined in MECC were 20 and 50 mM borate buffered at pH values of 8.2, 8.6 and 9.3, in the presence of 0.1% (w/v) SDS. CIEF was performed on a PVA coated capillary (50 μm i.d. and effective length 56 cm). The ampholytes used to create the pH gradient in the capillary were 3.3 mM phosphoric acid (anode) and 22.5 mM NaOH (cathode). Following sample injection and focusing for 6 min, mobilization of proteins was achieved by applying pressure of 25 mbar.

Identification of the migrated IgG κ and λ chains was achieved comparing the migration times with those obtained using reference compounds.

Sample treatment. Immunoglobulin G subclasses were obtained as a freeze-dried material derived from a phosphate solution buffered at pH 7.3. The material was dissolved in 2× distilled water to a final concentration 0.1 mg mL^{-1}. In order to proceed to CE analysis, solvent exchange with the operating buffer was performed following centrifugation through a centricon-3 membrane with cut-off of 3-kDa (Amicon, GmbH, Witten, Germany) at 11 000 ***g*** for 5 min.

RESULTS AND DISCUSSION

In order to obtain a capillary electrophoresis profile suitable for the resolution and identification of the major IgG subclasses, various operating buffers at normal and reversed polarity were examined. Attempts to separate the IgG subclasses by CIEF on PVA coated capillary showed that the solutes co-migrate without any sig-

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nificant separation between them. This is mainly due to IgG κ and λ chains having almost identical isoelectric point (pI). Examining whether CZE and MECC are suitable methods for the analysis of IgGs, the ionic strength and the pH value of the operating buffer, factors that greatly affect the migration of the analytes, were taken into consideration. High ionic strength values, such as 100 and 80 mM resulted in reduced electroosmotic flow (EOF), increased current (150–200 mA) and Joule heating, whereas low values (15 and 30 mM) resulted in low resolution of the analytes mainly due to the adsorption of immunoglobulins into the capillary wall. On the other hand, pH is a crucial parameter since at high values, where pH is higher than pI, immunoglobulins are negatively charged, whereas at pH lower than pI the analytes are positively charged. pH values of 3.0, 7.2, 8.0, 8.2, 8.4 and 8.6 resulted in peak tailing and low resolution. The best electrophoretic profiles of all individual κ and λ chains of IgG subclasses tested, i.e. sharp and homogeneous peaks, were obtained following CZE analysis with 50 mM phosphate, pH 9.3, as operating buffer. At this pH, EOF overwhelms the electrophoretic mobility (EM) of IgG analytes, which drives the molecules to the opposite direction anode, and finally they migrate according to the apparent mobility EOF-EM.

Following voltage application the molecules migrate through the capillary at different rates. Among the various voltage values examined, 20 kV was the selected one since it resulted in the faster migration of analytes without any effect on resolution. Electrophoretic profiles were highly repeatable and migration times were reproducible with a relative standard deviation lower than 3.2%. The electropherograms showing the separation of κ chains of IgG subclasses, the respective λ chains as well as a mixture of κ and λ chains are given in Fig. 1(A–C), respectively. As shown in Fig. 1(A), IgG1κ (peak 4) was completely separated from IgG2κ (peak 2) and IgG3κ (peak 1). Although IgG4κ (peak 3), which is the minor IgG subclass, migrated close to IgG2κ, it can also be identified. As shown in Fig. 1(B), IgG4λ (peak 4) was completely separated from IgG1λ (peak 3), IgG2λ (peak 1) and IgG3λ (peak 2). Although the separation of IgG1λ, IgG2λ and IgG3λ was not complete, these IgG subclasses can be well identified. A typical CE profile, obtained following analysis of an equimolar mixture of the major IgG κ and λ chains, i.e. IgG1, 2 and 3, is given in Fig. 1(C). IgG3κ (peak 1) and IgG1κ (peak 6) were completely resolved and identified. Although IgG2λ (peak 2), IgG2κ (peak 3), IgG3λ (peak 4) and IgG1λ (peak 5) were not completely resolved, these IgG subclasses can also be distinguished and identified.

In spite of the great heterogeneity of IgG subclasses, using the developed CE method the major immunoglobulin G subclasses can be identified in respect to their κ and λ chains. Further experiments in respect to the application of the method in several immunological diseases are under progress in our laboratories.

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