Journal of Chromatography A, 1216 (2009) 2717–2724

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Isolation of cell-free DNA from plasma by chromatography on short monolithic columns and quantification of non-apoptotic fragments by real-time polymerase chain reaction

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ARTICLE INFO

Article history:
Available online 30 October 2008

Keywords:
Cell-free DNA
DNA isolation
Chromatography
Plasma
Convective interaction media (CIM)
Quantitative PCR

ABSTRACT

Human plasma is an important medical substance and a raw material for production of various therapeutics. During blood sampling, storage and processing, genomic DNA is released into plasma from nucleated blood cells that are damaged in the course of the procedure. In order to determine the concentration of contaminating DNA in plasma, we developed a method for DNA isolation by using anion-exchange chromatography on a BIA Separations CIM (convective interaction media) diethylaminoethyl column. DNA was quantified by SYBR Green based real-time polymerase chain reaction. The concentration of cell-free, non-apoptotic DNA in plasma ranged between 0.06 and 22.5 ng/ml. As substantial volumes of plasma or whole blood are administered directly into the vascular system, a recipient is exposed to high amounts of cell-free DNA, several orders of magnitude higher than the amount found in other biologicals.

1. Introduction

Although the presence of extracellular, circulating DNA in human plasma was initially detected already in 1948 [1], reported ranges of its concentration in healthy individuals vary. Generally, the upper value is estimated to be around 50 ng/ml [2]. Primary source of the DNA in plasma are apoptotic cells, and therefore only small fragments (smaller than 220 nucleotides) are predominantly detected [3,4]. Spontaneous and active release of DNA by activated lymphocytes [5] and neutrophils [6] has also been described.

Many diagnostic applications based on circulating DNA have been developed because its level is elevated in patients with several autoimmune disorders [7,8]. Elevated concentration of DNA can also be detected in the plasma of patients with various types of cancer [9], especially in patients with metastases [10]. Furthermore, a correlation has been found between the plasma DNA level and the severity of injury in acute disorders such as trauma [11] and stroke [12]. Fetus-derived DNA is also found in maternal plasma [13] and can be used for non-invasive prenatal diagnosis [14] and monitoring of pregnancy-associated disorders [15].

In the bloodstream, extracellular DNA is under pressure from factors influencing its clearance [16]. Besides neutral deoxyribonuclease I, which is responsible for 90% of the deoxyribonuclease activity of plasma [17], deoxyribonuclease II [18], phosphodiesterase I [19], DNA-hydrolyzing autoantibodies [20] and lactoferrin [21] are also involved. The total DNase activity is dependent on the complex combination of the concentration and composition of the DNA degrading enzymes and factors influencing their activity [22].

In transfusion medicine and quality control of plasma-derived medicinal products, genomic cell-free DNA is not considered to be a safety issue. The potential risk of residual contaminating DNA to cause a neoplastic event in a recipient has been debated for a long time for vaccines and biological products manufactured in continuous cell lines (CCLs) or cells derived from tumours [23], but not for plasma products or for products manufactured in primary or diploid cell lines which have been used successfully and safely for many years for the production of viral vaccines [24]. In 1986, a WHO study group concluded that the risk is negligible when the amount of CCLs' residual DNA is 100 pg per vaccine/biological dose [24]. In 1998 the acceptable levels were increased up to 10 ng per dose [24]. DNA oncogenicity has been considered to arise mainly through the introduction of a dominant oncogene while the oncogenesis arising through insertional mutagenesis has been regarded as improbable [23]. Still, this level of tolerated residual DNA is several orders of magnitude lower than the amount of DNA in a unit of human blood for transfusion [25].

Besides the cell-free DNA that is normally present in plasma, during blood storage and processing plasma is additionally contaminated with the DNA released from damaged nucleated blood cells [25]. Strong influence of blood sampling and handling, as well

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doi:10.1016/j.chroma.2008.10.087

as plasma preparation on DNA yield and quality has been shown [26–28]. Furthermore, the frequency of major chromosomal aberrations such as intra- and inter-chromosomal exchanges, severe fragmentations of chromatids and acentric fragments increase with storage time of human blood [29]. Another potential safety concern associated with larger DNA fragments is the risk that the genome of an infectious virus is present in the DNA, either integrated or as an extrachromosomal element [23].

Circulating DNA can be isolated from plasma by different manual and automated DNA extraction methods [2,30]. DNA yield obtained by various methods varies substantially [30] and the extraction procedure seems the most likely factor confounding the results [31]. Most commercial kits are based on chromatographic separations, but their limitation is the small volume of the starting material that can be applied. This restriction can be circumvented by the usage of CIM (convective interaction media) short monolithic columns. It has already been shown that they could be used for isolation of genomic DNA from various biological suspensions [32,33]. CIM monoliths are characterised by large flow-through pores, they exhibit low diffusional resistance and low pressure drop [34,35]. These properties enable the usage of monoliths in purifications of high molecular weight DNA [36] that is very vulnerable to hydrodynamic shearing.

The goal of the research presented in this paper was to establish a chromatographic method for isolation of DNA from human plasma using CIM monolithic column and to determine the concentration of contaminating, non-apoptotic DNA by quantitative polymerase chain reaction (PCR). The developed method was based on the facts that (a) pores in the monolithic columns are large enough to harbour nanoparticles [37]; and (b) high sensitivity of detection can be achieved by using quantitative PCR [23].

2. Experimental

2.1. Chemicals

Trishydroxymethylmethylamine (Tris), sodium chloride, boric acid, 3-(N-morpholino)propanesulfonic acid (MOPS), ethanol and agarose were obtained from Merck (Darmstadt, Germany). Chloroform:isoamyl alcohol (24:1), isopropanol and ethidium bromide were from Sigma (St. Louis, MO, USA). Chloroform and sodium acetate were purchased from Amresco (Solon, OH, USA). Phenol (pH ≥8.0), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), RNase, ΦX-174 RF DNA–HaeIII digest size maker (containing DNA fragments of 1535, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 nucleotides), *Taq* DNA polymerase, PCR buffer and dNTPs were obtained from GE Healthcare (Amersham, UK). Power SYBR Green PCR master mix was from Applied Biosystems (Foster City, CA, USA). Filters with 0.45 μm pore size were purchased from Millipore (Billerica, MA, USA).

2.2. Instrumentation

All anion-exchange chromatography experiments were performed using a Waters HPLC System: Waters 600 System Controller, Waters 600 Pump, Waters In-Line Degasser AF, 2996 photodiode array detection (DAD) system, Empower Software (Milford, MA, USA). When the sample volume exceeded 2 ml, the application was performed using P50 peristaltic pump (GE Healthcare).

Conventional PCR reactions were carried out in 9600 GeneAmp PCR System; quantitative PCR was performed with 7500 Real Time PCR System and the data was collected and analysed using 7500 System SDS software (all from Applied Biosystems).

Electrophoresis was carried out using the electrophoretic unit HE 33 (Hoefer, San Francisco, CA, USA). Agarose gels were illuminated and photographed using Kodak digital science Image Station 440 CF (Eastman Kodak, Rochester, NY, USA).

Spectrophotometric determination of DNA concentration was performed by Biophotometer (Eppendorf, Hamburg, Germany).

2.3. Chromatographic media and buffers

CIM monolithic columns bearing weak (diethylaminoethyl) anion-exchange groups were from BIA Separations (Ljubljana, Slovenia). CIM monolithic column consists of a 3 mm × 12 mm disk-shaped highly porous polyglycidyl methacrylate-*co*-ethylene dimethacrylate matrix that is seated in a non-porous self-sealing fitting ring. The bed volume of one disk is 0.34 ml, and the porosity is 62%. Average pore radius is 634 nm. The disk-shaped matrix was inserted into a commercially available housing (BIA Separations) and connected to the HPLC system. The binding buffer used for the separations was 25 mM MOPS, 0.5 M NaCl, pH 7.0, containing 15% (v/v) isopropanol and the elution buffer was 25 mM MOPS, 1.5 M NaCl, pH 7.0, containing 15% (v/v) isopropanol. For removal of substances residually bound after chromatography, buffer containing 25 mM MOPS, 2 M NaCl, 15% (v/v) isopropanol, pH 7.0 (buffer 3) was used.

2.4. Plasma samples

Plasma pools containing plasma from 400 to 4500 individual healthy donors were used. Plasma was obtained from whole blood donations from various Croatian transfusion centres as well as by plasmapheresis. Plasma was stored at −20 °C.

Prior to chromatography, plasma samples were thawed and centrifuged for 5 min at 1200 × *g*. Plasma was filtered through filter of 0.45 μm pore size and mixed with the same volume of binding buffer. The volume of applied plasma samples ranged from 0.5 to 10 ml.

2.5. Plasmid DNA

Plasmid pGEM-3Zf(+) which has 3197 base pairs, was purchased from Applied Biosystems. Plasmid DNA purity and concentration were determined spectrophotometrically.

2.6. Preparation of DNA standard

Genomic DNA used as standard for quantitative PCR was isolated from the MRC-5 continuous diploid cell line (human lung fibroblasts, obtained from European Collection of Cell Cultures, Porton Down, Salisbury, UK). DNA was isolated from 0.5×10^6 cells.

Cell pellet was resuspended in 500 μl of lysis buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0). Fifty microgram of RNase and 5 μl of 10% SDS were added and the samples were incubated at 37 °C for 1.5 h. Afterwards, 50 μg of proteinase K was added and the samples were incubated at 56 °C for 3 h. Genomic DNA was isolated with 2 extractions: (a) 500 μl of phenol (pH 8.0)/chloroform (1:1) was added and samples were centrifuged at 20 817 × *g* for 5 min; (b) aqueous phase was taken and 2 volumes of chloroform:isoamyl alcohol (24:1) were added. Samples were centrifuged at 20 817 × *g* for 5 min. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol. After incubation at −20 °C overnight, samples were centrifuged at 20 817 × *g*. Pellets were washed by adding 400 μl of 70% ethanol and were centrifuged at 20 817 × *g* for 10 min. All traces of alcohol were carefully removed, pellets were dried and resuspended with water. DNA purity and concentration were determined spectrophotometrically.