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Reversed phase monolithic analytical columns for the determination of HA1 subunit of influenza virus haemagglutinin

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ABSTRACT

Monoliths are chromatographic stationary phases, which were specially designed for efficient purification of large biomolecules, like proteins, viruses and DNA. In this work, the small scale monolithic butyl (C4) and styrene-divinyl benzene (SDVB) columns were applied for reversed phase analyses of various degraded influenza viruses. The binding of the HA1 subunit of haemagglutinin to the monolithic columns was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Western blot. The working linear range was determined as 1.60×10^{10} viral particles/mL to at least 1.64×10^{11} viral particles/mL, the limit of detection was found to be 2.56×10^{9} virus particles/mL and the limit of quantification was 5.12×10^{9} virus particles/mL. The analytical HPLC method developed with the H1N1 virus was also applicable for the analytics of the HA1 subunit of H3N2 influenza virus and the influenza B virus.

1. Introduction

Monoliths are continuous stationary phases, cast as homogeneous columns of various dimensions in a single piece [1]. Monolithic chromatographic supports are known for their ability to effectively purify large molecules in a short time. Structural and hydrodynamic characteristics of monoliths make them beneficial for purification of proteins, DNA and viruses [2,3]. Convective Interaction Media (CIM) ion-exchange monoliths have been applied for the concentration or purification of plant viruses [4–6], phages [7,8], rotaviruses [6], hepatitis A virus and feline calicivirus [9], as well as for measles and mumps virus [10]. According to the preliminary results, they have also shown to be applicable for the purification of the influenza virus [2]. Besides ion-exchange monoliths, pseudo-affinity methacrylate monoliths have been reported for the purification of the influenza A virus as well [11].

Influenza virus is an enveloped virus. Single stranded RNA is stored inside of a lipid membrane, which contains two main glycoproteins on the outside layer; haemagglutinin (HA) and neuraminidase (NA) [12]. For the preparation of influenza vaccines various purification steps are employed. Virus titers are determined for different samples, which are collected from various process steps (in-process control) as well as in the final vaccine (final control). Methods applied are either based on the infectivity of the virus, like the tissue culture infectious dose ($TCID_{50}$) [13,14], or applicable for determining virus particles like the haemagglutination assay [14,15] or single radial immunodiffusion (SRID) [16,17]. The latter is highly sensitive however it is labor intensive and mostly suitable for relatively pure vaccine products [18]. It also requires the production of reference antigens and specific antibodies, which is costly and time-consuming [19]. For this reason various chromatographic methods for determining the concentration of the influenza virus and profiling of its proteins started to emerge. Phelan and Cohen managed to separate the major proteins of the detergent-disrupted influenza virus by RP-HPLC on conventional porous media, however, individual protein recoveries were low [20], as was the case with the ion-exchange chromatography [21]. Size exclusion chromatography showed higher potential to separate viral proteins with recoveries >90% [21]. A 2D HPLC method was reported for quantitative detection of virus proteins in vaccines, utilizing SEC and a non-porous silica RP column [22]. Non-porous C18 silica-based column gave satisfactory results in terms of sensitivity, protein recovery and sensibility as opposed to conventional porous silica particle based C4 column [23]. Kapteyn et al. developed an RP-HPLC method using a perfusion particle-based POROS column. The method is based on measuring the peak area of HA1, the hydrophilic subunit of HA, which is proportional to the amount of the analyzed HA [24,25].

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In this work, we examined the applicability of reversed phase monolithic columns for influenza virus analytics. Initial screenings were performed on commercially available monolithic butyl (C4) and styrene-divinyl benzene (SDVB) columns. Both were examined for the selectivity of binding the HA1 subunit of haemagglutinin. Optimal chemistry was chosen and an analytical SDVB column optimized in terms of sensitivity and resolution was examined. Gradient conditions were optimized to achieve optimal resolution for HA1 from other sample components. Basic analytical parameters like linearity of the method, limit of detection and quantification were determined; the applicability of the column was tested for various egg-based influenza A strains, as well as for influenza B.

2. Experimental

2.1. Chromatographic equipment

All chromatographic experiments were carried out using a gradient chromatography workstation, consisting of two pumps, an autosampler with various sample loop volumes and a UV detector (Knauer, Berlin, Germany) set to 214 nm. For data acquisition and control, ChromGate 3.1.6 software (Knauer) was used.

2.2. Chemicals

Chemicals for buffer preparations (Tris, NaCl, NaOH, HCl) were obtained from Merck (Darmstadt, Germany). Acetonitrile (gradient grade), trypsin agarose beads and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA), dithiothreitol (DTT) and iodoacetamide (IAA) from GE Healthcare (Uppsala, Sweden) and Zwittergent 3-14 from Calbiochem (San Diego, CA, USA).

2.3. Columns

CIM butyl (C4) and styrene divinyl benzene (SDVB) monolithic disks (12 mm I.D × 3 mm: *V*: 0.34 mL) were provided by BIA Separations (Ljubljana, Slovenia) as was the SDVB prototype analytical column of the following dimensions: 5.2 mm I.D. × 15 mm (*V*: 0.312 mL).

For buffer exchange of the samples PD 10 columns (GE Healthcare) were applied.

2.4. Method development

All method development was performed using the following mobile phases: 5% acetonitrile with 0.1% TFA in water was mobile phase A, and 90% acetonitrile with 0.1% TFA in water was mobile phase B. Injected influenza samples were degraded (see Section 2.6.) prior to HPLC analyses. Applied gradients are depicted on the respective chromatograms. Unless stated otherwise, experiments were performed using the A/Solomon Islands/3/2006 (H1N1) sample.

2.5. Influenza virus samples

Method development was performed using egg-based influenza samples that were purified with zonal centrifugation on a sucrose gradient and inactivated by treatment with β-propriolactone. The following strains were tested: A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004. A pooled split antigen concentrate of A/Solomon Islands/3/2006 (H1N1) was also examined. All samples were supplied by Novartis (Speke Liverpool, UK).

2.6. Sample preparations for RP HPLC analysis

Samples were pre-treated according to a modified procedure by Kapteyn et al. [24,25] before they were loaded onto the columns. Influenza samples were first buffer exchanged using a PD 10 column (GE Healthcare) to a final 150 mM Tris buffer, pH 8.0. Zwittergent was added to a final concentration of 1% and the solution was incubated for 10 min at ambient temperature, in order to disintegrate the virus. After the disintegration step samples were treated with trypsin, conjugated to agarose beads (Sigma–Aldrich) at a final concentration of 1.3 mU/mL for 30 min in a water bath at 30 °C. This step enabled the cleavage of HA into HA1 and HA2 subunits. After the incubation step samples were centrifuged at 7000 × *g* for 5 min in order to remove the trypsin beads, and the supernatant was treated with DTT (final concentration in the sample was 25 mM) for 10 min at 90 °C to reduce the disulphide bonds. After cooling to room temperature, HA1 and HA2 subunits were alkylated with IAA (final concentration was 100 mM) at 37 °C in the dark for 10 min in order to prevent the re-association of proteins. The final step was the re-addition of 25 mM DTT to neutralize the remaining IAA.

2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis

Collected HPLC fractions were first partially evaporated to remove most of the acetonitrile from the samples and then concentrated using the Microcon centrifugal filtering devices (YM-10, cut off 10 kDa; Millipore, Billerica, MA, USA).

SDS-PAGE was carried out with a Mini-Protean II electrophoresis Cell (Bio-Rad, Hercules, CA, USA) using 4–20% gradient gels under non-reducing conditions according to the manufacturer's instructions (Cambrex, Rockland, ME, USA). The gels were run at 200 V for 60 min using a discontinuous Tris-glycine buffering system. Protein bands were visualized by silver staining (GE Healthcare). A 10–200 kDa molecular weight standard was used (Fermentas Life Sciences, Burlington, Canada).

Electrophoretically separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad) and incubated in a blocking buffer. After 1 h influenza specific antibodies (Rabbit polyclonal to HA1 (H1N1) A/California/14/2009 (Abcam, Cambridge, UK)) were added to the blocking buffer in the 1:1000 ratio and overnight incubation at 4 °C was performed. After that secondary Goat polyclonal to Rabbit Ig (Abcam) antibodies as alkaline phosphatase conjugate were added in the 1: 1000 ratio and 1 h incubation followed. The membrane was dried and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate (Perkin Elmer, Waltham, MA, USA) was added on the membrane for the development of bands.

2.8. Haemagglutination assay

Haemagglutination assay was performed to determine the amount of haemagglutinating virus particles per mL by using a 0.5% solution of chicken red blood cells (RBCs) as described by Wacheck et al. [13]. A 0.5% solution is defined as having 4×10^7 RBCs per mL. Two-fold serial dilutions of viral suspension were mixed with an equal volume of RBCs and wells were observed for the presence of lattice. The end point of the titration was determined as the last dilution showing agglutination [13]. The concentration of hemagglutinating virus particles was calculated according to Eq. (1):

$$C[\text{particles/mL}] = C_{HA} \times 4 \times 10^7 \quad (1)$$

where *C* stands for the concentration of the haemagglutinatin virus particles and C_{HA} is the concentration of virus particles determined by the hemagglutinating assay [HA units].

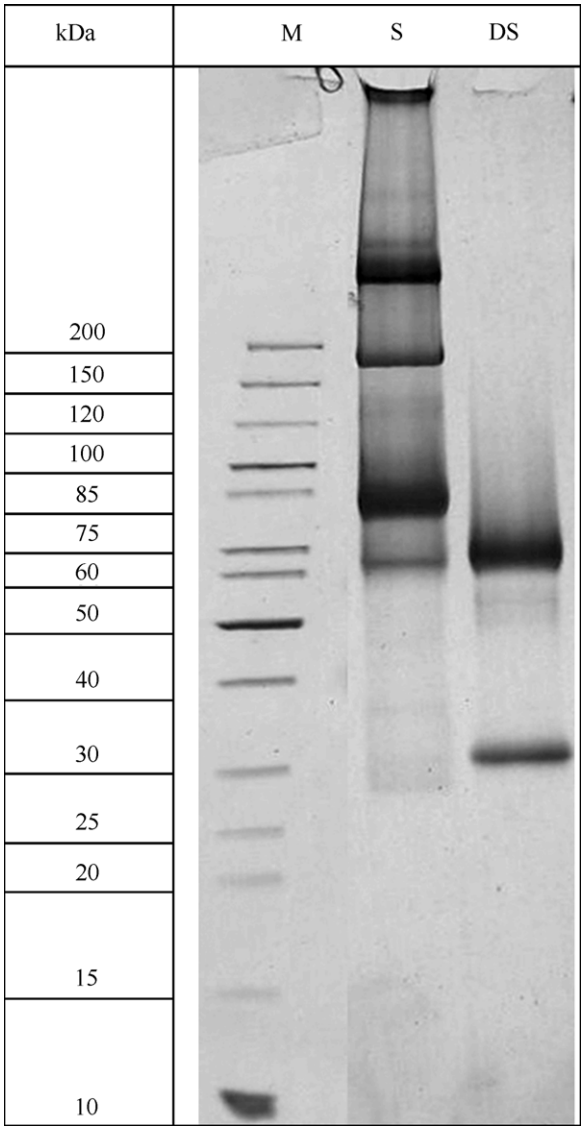

Fig. 1. Monitoring of virus degradation by SDS-PAGE; M: protein standards, S: virus sample; DS: degraded virus sample.

3. Results and discussion

3.1. Screening of monolithic supports for the selectivity of HA1 binding

In order to develop an HPLC analytical monolithic column for the HA quantification, different monolithic supports were screened for their selectivity and specificity. Influenza samples were degraded and examined by SDS PAGE to establish the degree of degradation. The HA molecule has a molecular mass of 225 kDa and consists of three monomeric units of 75–80 kDa [11]. Each monomeric unit is composed of two subunits; HA1 (55 kDa) and HA2 (20–25 kDa), linked by a disulphide bond [11]. After degradation the separated subunits are visible on the SDS PAGE gel (Fig. 1).

Initial screenings with the degraded virus sample were performed on C4 and SDVB CIM disks. The separation on a C4 disk resulted in broader elution peaks when compared to the elution profile from the SDVB disk (Fig. 2A and B). To confirm the presence of the HA1 subunit, larger volumes of the sample were first injected on the SDVB column. With increasing the injection volume the peak area and peak height were not proportional to the injected volume

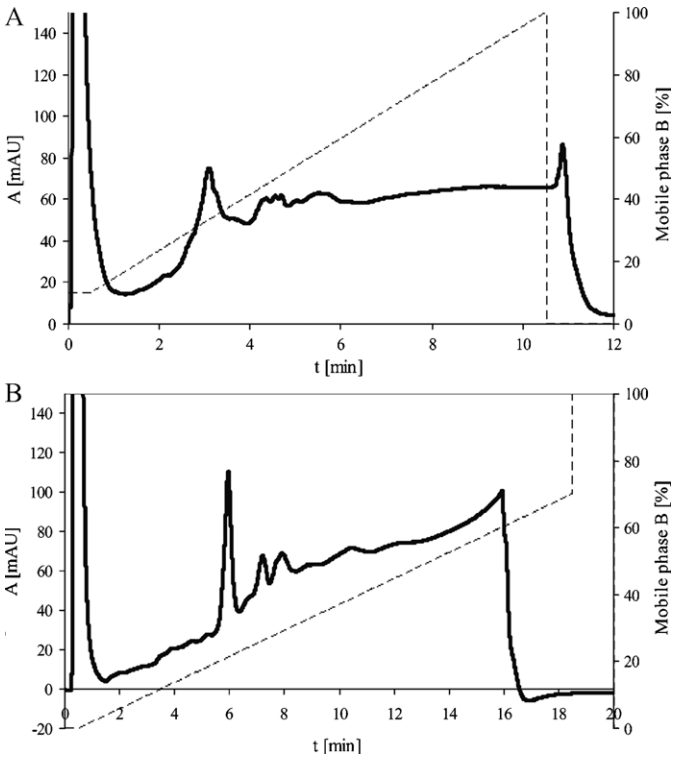

Fig. 2. Injection of a degraded influenza sample on reversed phased monoliths. (A) C4 disk: elution profile of A/Solomon Islands/3/2006 (H1N1); injection volume: 50 μL, flow rate: 1 mL/min, gradient as depicted on the chromatogram. (B) SDVB disk elution profile of A/Solomon Islands/3/2006 (H1N1); injection volume: 50 μL, flow rate: 1 mL/min, gradient as depicted on the chromatogram.

(Fig. 3), most likely due to volume overload problems. Before loaded on the column, the influenza sample was degraded, and therefore contained many additives and was diluted in 150 mM Tris buffer, pH 8. The composition of the sample was different from the mobile phase used for the equilibration of the column, which was water containing acetonitrile. For this reason with higher loadings equilibrium on the column was not achieved and the response was not linear to the injected sample volume. To obtain the appropriate peak shape, despite a higher loading volume, three monolithic disks were put together in one housing [26]. In this way the absolute capacity of the column was increased since three times more

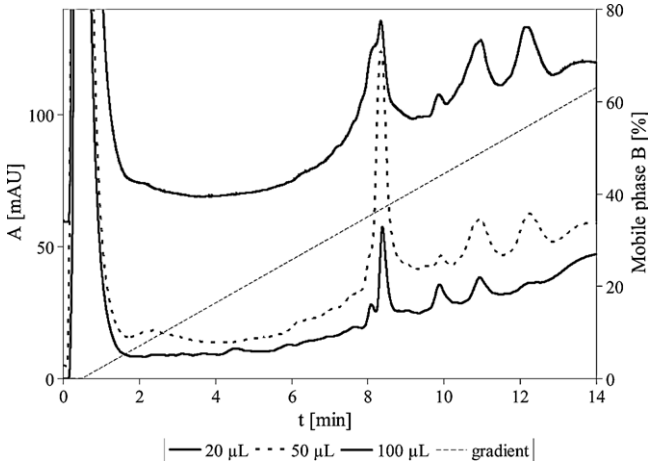

Fig. 3. Volume overload effects; injections of various volumes of A/Solomon Islands/3/2006 (H1N1) on a SDVB column; flow rate 1 mL/min, gradient as depicted on the chromatogram.

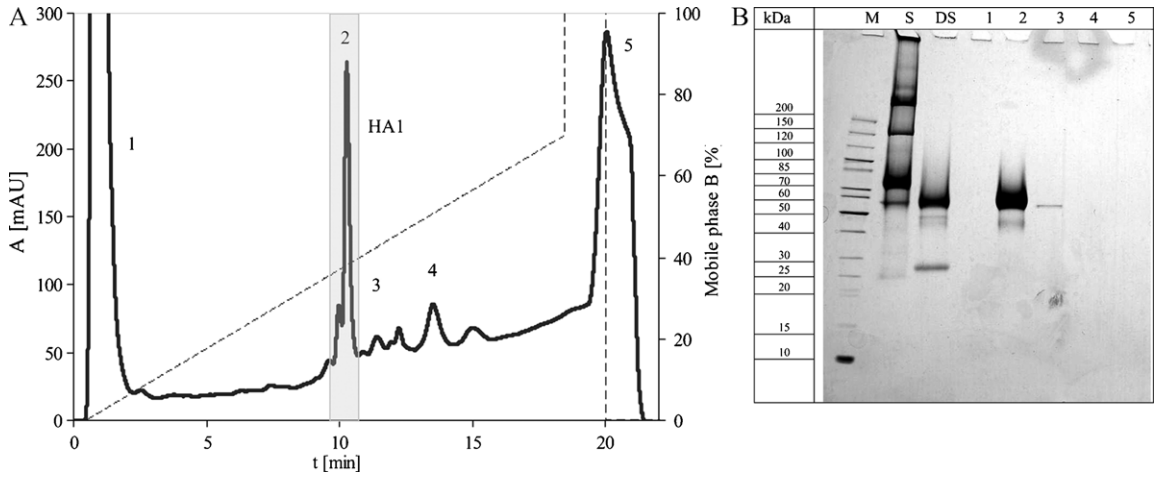

Fig. 4. Elution profile of A/Solomon Islands/3/2006 (H1N1) from a column made of three SDVB disks (A); injection volume: 100 μL, flow rate: 1 mL/min, gradient as depicted on the chromatogram (B) SDS-PAGE analyses of collected fractions; S: starting virus sample, DS: degraded virus sample loaded on the SDVB column, 1–5: collected fraction.

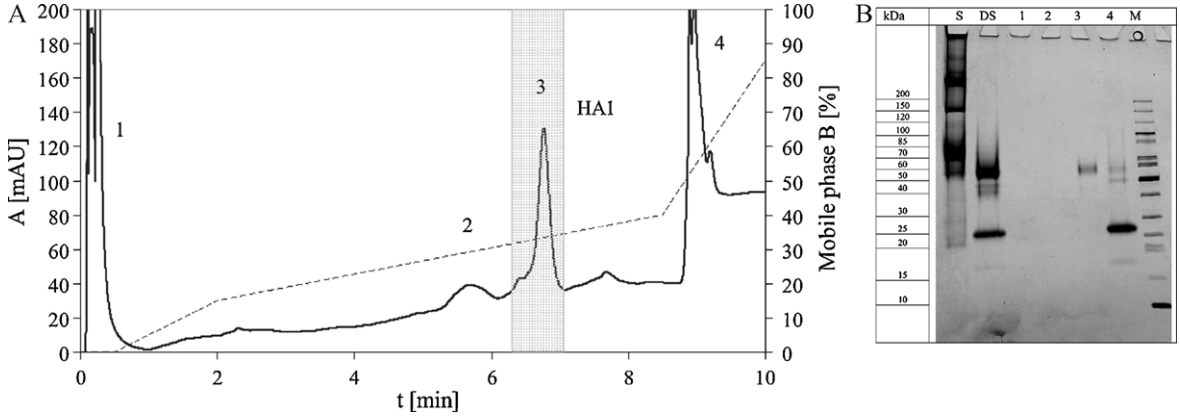

Fig. 5. Elution profile of a pooled split concentrate of A/Solomon Islands/3/2006 (H1N1) from a column made of three C4 disks (A); injection volume: 20 μL, flow rate: 1 mL/min, gradient as depicted on the chromatogram (B) SDS-PAGE analyses of collected fractions from three C4 disks; S: starting virus sample, DS: degraded virus sample loaded on the SDVB column, 1–4: collected fraction.

active sites were available for binding. Consequently, the volume overload problem was less pronounced. The influenza sample was injected on the column and fractions collected, concentrated and analyzed by SDS-PAGE (Fig. 4A and B). The HA1 eluted in a single peak (Fig. 4B, fraction 2) and it was not detectable in the flow-through fraction. The same experiment was performed with the C4 disks; three disks were put in one housing [26] and larger sample volumes were injected (Fig. 5A). HA1 mainly eluted in one peak [Fig. 5B, fraction 3], however, the volume overload was much more pronounced on the C4 column (Fig. 6). By injecting only 30 μL of the influenza sample, the HA1 peak was already broad and the response was no longer linear to the amount of the influenza sample injected.

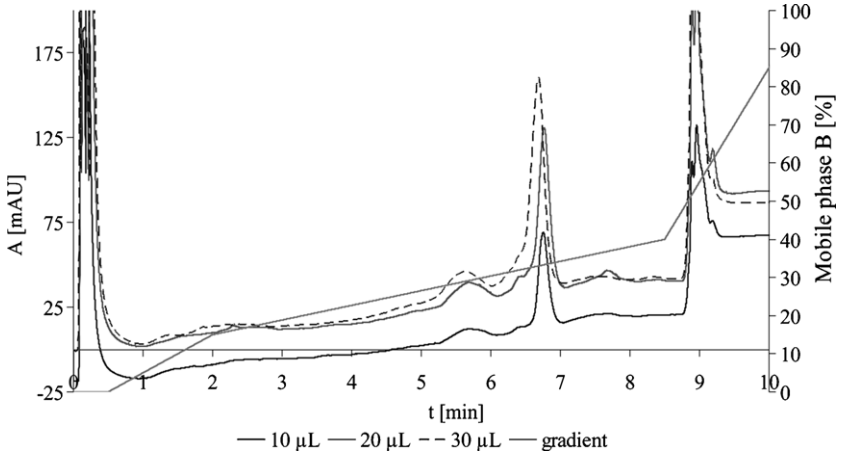

Fig. 6. Volume overload effects; injections of various volumes of a pooled split concentrate of A/Solomon Islands/3/2006 (H1N1) on three C4 disks; flow rate 1 mL/min, gradient as depicted on the chromatogram.

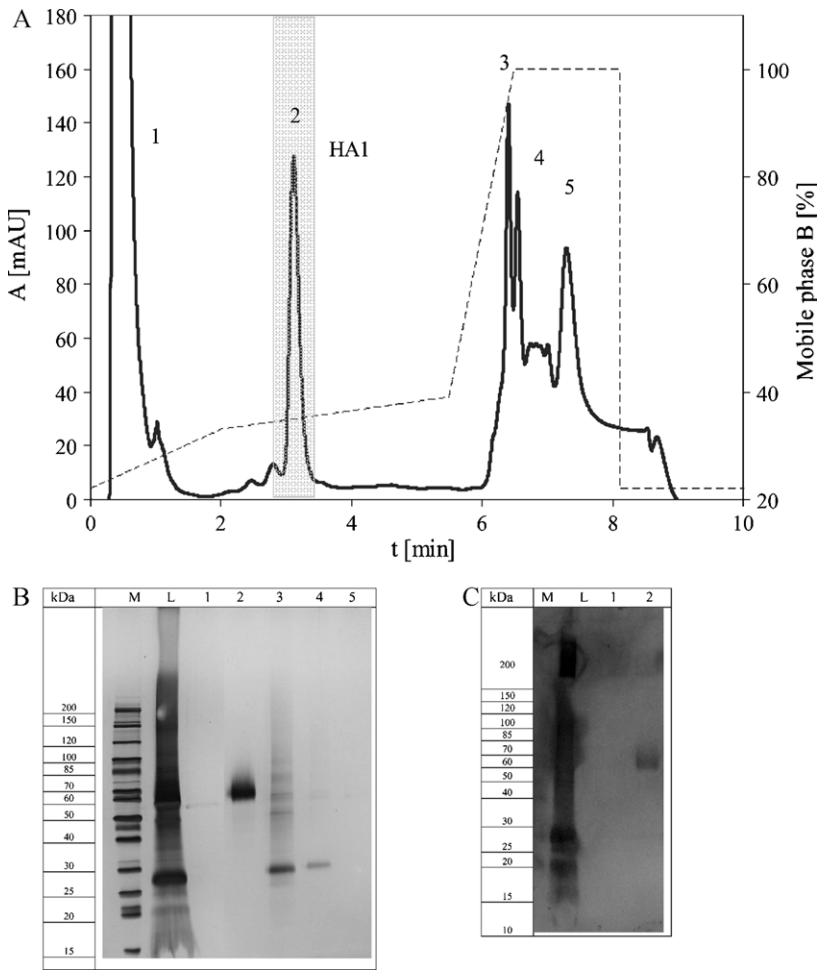

Fig. 7. SDVB analytical column (5.2 mm I.D. × 15 mm). (A) Elution profile of A/Solomon Islands/3/2006 (H1N1); injection volume: 30 μL, flow rate: 0.8 mL/min, gradient as depicted on the chromatogram (B) SDS-PAGE analyses of collected fractions; L: degraded virus sample loaded on the SDVB column, 1–5: collected fractions. (C) Western blot analysis of collected fractions; L: degraded virus sample loaded on the SDVB column, 1–2: collected fraction.

This was the main reason for choosing the SDVB column for further analyses.

3.2. The SDVB analytical column

The design of the conventional SDVB disk-shaped column was not optimal for analytics. We therefore switched to a longer monolithic column with a smaller diameter. For this purpose, a 15 mm long monolith with the diameter of 5.2 mm was placed into a designated stainless steel housing. This type of column was better for analytics in terms of sensitivity and resolution. After switching from the disk to the analytical column, first the gradient was optimized, so that the baseline was stable and the HA1 peak was better resolved from other sample components (Fig. 7A as opposed to Fig. 2B or Fig. 4A). After gradient optimization, the degraded influenza sample was injected on the SDVB analytical column and fractions were collected and analyzed. It was shown that HA1 eluted in one peak (Fig. 7A and B fraction 2), the eluted fraction was pure according to SDS-PAGE. The identity of HA1 was confirmed by the Western blot analysis using specific antibodies (Fig. 7C).

3.3. Comparison of the SDVB assay to the haemagglutination assay

The virus titer of the influenza A virus sample was first determined by the haemagglutination assay. This gave us the information about the amount of the hemagglutinating virus particles present in the sample A/Solomon Islands/3/2006 (H1N1). Various dilutions of the sample were prepared; diluted samples as well as the non diluted sample were degraded as described in section 2.6. and injected in duplicate on the SDVB column. Linearity was determined by plotting the virus titer as determined by the haemagglutination assay as a function of peak area. The correlation coefficient (r^2) was calculated to estimate the linearity of the standard curve ($r^2 = 0.9984$). The working linear range was determined as 400 HA units to at least 4096 HA units which accounts for 1.60×10^{10} viral particles/mL to at least 1.64×10^{11} viral particles/mL (Fig. 8). The concentration of virus particles was calculated from HA units as described in Section 2.8. LOQ and LOD were determined empirically by injecting progressively more diluted samples on the column and measuring the response. They were measured using the signal to noise approach as recommended by the CPMP/ICH/281/95 (ICH Topic Q2B) [27], where the LOD was defined as 3 times S/N and the LOQ 10 times S/N. LOD was found to be 2.56×10^9 virus particles/mL and LOQ was 5.12×10^9 virus particles/mL.

3.4. Applicability of the column for different virus strains

Various influenza virus strains were degraded and examined with the SDVB monolithic analytical column (Fig. 9). Retention times of A/Solomon Islands/3/2006 (H1N1) (Fig. 9A, t_R: 3.12 min), a pooled split antigen concentrate of A/Solomon Islands/3/2006

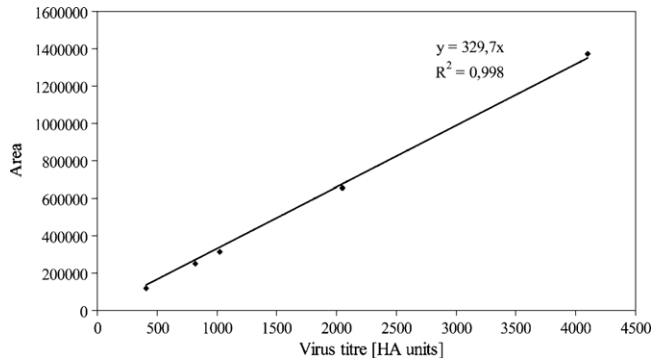

Fig. 8. Calibration curve; virus titer measured with the haemagglutination assay, chromatographic conditions as described in Fig. 7A.

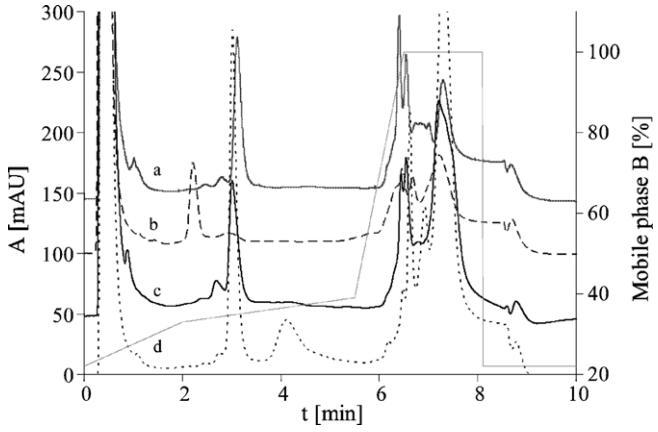

Fig. 9. Elution profiles of (a) A/Solomon Islands/3/2006 (H1N1), (b) B/Malaysia/2506/2004, (c) A/Wisconsin/67/2005 (H3N2), and (d) pooled split antigen concentrate of A/Solomon Islands/3/2006 (H1N1). Conditions: CIMac SDVB analytical column (5.2 mm I.D × 15 mm), injection volume: 30 μL, flow rate: 0.8 mL/min, gradient as depicted on the chromatogram.

(H1N1) (Fig. 9D, t_R: 3.02 min) and A/Wisconsin/67/2005 (H3N2) (Fig. 9C, t_R: 3.04 min) were very similar. Influenza B, strain B/Malaysia/2506/2004 eluted sooner in the gradient (Fig. 9B: t_R: 2.23 min). The SDVB column and the applied method were appropriate for all samples examined.

4. Conclusions

In this paper two different reversed phased monolithic columns were used for the analytical separation of the influenza virus. Butyl (C4) and styrene divinyl benzene (SDVB) were found to efficiently bind HA1 – the hydrophilic subunit of haemagglutinin under the applied chromatographic conditions. The elution fractions containing HA1 were pure according to SDS-PAGE analyses. However, due to pronounced volume overload effects on the C4 column, the SDVB chemistry was chosen for further experiments and optimization. An SDVB analytical column of 5.2 mm I.D. and 15 mm length was examined with the influenza virus. After additional gradient optimization the HA1 peak was well resolved from other sample components. This was confirmed by SDS-PAGE and Western blot analyses. The linear working range was determined as 400 HA units to at least 4096 HA units which accounts for 1.60×10^{10} viral particles/mL to at least 1.64×10^{11} viral particles/mL. LOD was 2.56×10^{9} virus particles/mL and LOQ was 5.12×10^{9} virus particles/mL. Additionally, the column proved to be appropriate for several influenza strains that were examined.

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