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review

Development of Medical Products Based on Human Plasma*

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Summary

Product development and process validation are shown in the case of several products obtained from human plasma. These are virus inactivated plasma, intravenous immunoglobulins and the clotting factors VIII and IX. Different analytical methods are presented, which are used for product development, quality control and in-process control.

The introduction of fast chromatographic methods allows in-process analyses to be carried out within two or three minutes only. Analytical methods with high resolution such as 2D-electrophoresis will lead to corresponding results even in the case of components with high molecular masses such as clotting factor VIII and von Willebrand factor. These methods have recently been introduced into the process.

For the production of virus inactivated human plasma a down-scale protocol is presented, allowing a simulation of the production on a laboratory scale. Virus validation has shown that the reduction of transfusion-relevant viruses in the process was higher than 6 log units. Determination of leachables from the RP-column, which was used in this production, proved that they appear in the final product in quantities below the detection limits only. It was also shown that the chemicals used for virus inactivation could be quantitatively removed from the product.

For the isolation of other products, like intravenous gamma globulins and the clotting factors VIII and IX, similar validation steps had to be taken. In the case of clotting factor VIII the following data were determined: the reduction of viruses, the amount of leachables from the column and the residues of chemicals from the solvent/detergent treatment for virus inactivation. Virus reduction was successfully performed as well as the removal of chemicals used for virus inactivation. The amount of leachables from the columns used for chromatographic purification was found to be far below the permissible levels.

Key words: virus inactivated human plasma, intravenous γ-globulins, clothing factors VIII and IX, chromatographic separation

Introduction

Medical products based on human plasma belong to a group of substances, which all carry the name »biologicals«. Biologicals are physiologically active products and almost always obtained from complex biological materials *(1)*. Many of these substances, *e.g.* human serum albumin *(2)*, have been used therapeutically for years. Biologicals are isolated from natural raw materials, often involving highly complicated down-stream processing (*3*).

The fast development of biotechnology in many cases has paved the way for a special production process. In recent years more and more complex substances have been produced by the use of recombinant DNA technology. Nowadays, highly complex (glyco-)proteins such as antithrombin III (*4*), tissue plasminogen activator (*5*), clotting factors VII (*6*), VIII (*7*) and IX (*8*) or von Willebrand factor (vWF) (*9*) are produced through gene-technologically manipulated mammalian cells.

These successes in biotechnology were accompanied by considerable progress in separation technology (*10–12*) and analytical biotechnology (*13–15*). It was necessary to analyse and define the new products and to test their safety. The requirements laid down with regard to products obtained gene-technologically are increasingly

* Dedicated to Professor Pavao Mildner for his 80th birdthday

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becoming a standard of quality also for those biologicals that are derived from other sources (*16,17*). This leads to much stricter criteria for the licencing of biologicals. All the basic requirements are still valid, namely

- effectiveness of the product,
- immunological safety, and
- virus safety of the product.

In this paper some aspects of product development and process validation are shown in the case of several products obtained from human plasma. Some examples will be presented and discussed, concerning the production of virus inactivated human plasma, intravenous immunoglobulins and plasma-derived human clotting factors VIII and IX (FVIII and FIX). The chief characteristics of these products are that they derive from complex biological materials and that they have to meet special requirements concerning product safety (*18–21*).

The risk of virus transmission (mainly hepatitis B and C, and HIV), which threatens the recipients of human blood, plasma or plasma products, is well known (*18–22*). In order to eliminate the risk, methods have been developed that allow the inactivation of transfusion-relevant viruses. In the case of human plasma only two methods for virus inactivation have so far been widely used: the so-called solvent/detergent (S/D) treatment (*20, 21*), and UV light irradiation in the presence of methylene blue (*22*).

It is an advantage of the S/D method that it can be used for treating large amounts of human plasma. The virucidic reagents, that is solvent and detergent, are subsequently removed from the plasma by oil extraction and subsequent solid-phase extraction using an C–18 modified silicagel (*20, 21, 23, 24*). Virus inactivation by UV light irradiation can be carried out in single plasma bags (*22*). However, in this case the methylene blue cannot be removed afterwards. In both alternatives the production process is relatively simple as far as virus inactivated human plasma is concerned. However, as mentioned above, the reproducibility of the process as well as the safety and quality of the product have to be guaranteed for every batch.

With other plasma products the production process is much more complicated. If possible, more than one step for virus inactivation or removal is provided for (*18*). However, what all processes have in common is at least

- one step for virus inactivation, and
- one chromatographic step (*19*).

For products which are isolated from human plasma by complicated purification procedures, accompanying analytical methods with high resolution are required. The most important methods that are used both in product development and quality control are

- electrophoretical methods such as SDS-PAGE, isoelectric focusing, 2D-electrophoresis and capillary electrophoretic methods, (*13–15,25*)
- chromatographic methods, above all HPLC, (*14, 15,25,26*)
- immunochemical methods (*25*), and
- mass spectrometry (*27*).

This review shows the aspects of product development and its quality control by using high resolution separation methods. Besides, other aspects are shown, including virus validation of the production processes as well as removal of S/D reagents and possible contaminations from the reagents. It also deals with the materials which are used in the production process. The analytical methods which have been developed, are the basis for subsequent controls, of the chromatographic supports before they are used and of the final product at the end, before it is released.

Analytical Methods

In biotechnology, monitoring of the production process and appropriate quality control are basic requirements of optimization. Exact data are needed, not only to optimize the processes themselves, but also to satisfy the demands of regulatory authorities with regard to registration of the product as a drug. For in-process control the time factor is of vital importance. The results of any analyses carried out during production have to be available within a very short period of time in order to allow adjustments, should the process deviate from its course.

Electrophoretic as well as chromatographic analyses are too slow to meet the above-mentioned requirements. Only the development of particularly fast chromatographic media has made it possible to obtain reliable results within a few minutes (*26,28*), Fig. 1 shows that the use of the so-called convective interaction media (CIM) allows within about 90 seconds the determination of a complex protein such as clotting factor IX. By optimizing the separation method, the period of time required for analysis can be less than one minute *(26,28)*.

As far as the reduction of the virus load is concerned, specific regulations exist for virus validation in the production process of drugs derived from human plasma. However, studies on the immunogenicity of the preparations are limited to a few specialized laboratories *(29,30)*. Such studies are essential, *e.g.* in the case of FVIII-concentrates. They include immunochemical studies with polyclonal and monoclonal antibodies, which reveal different reactivity of FVIII preparations. Experiments with the corresponding HCh and LCh peptide fragments have subsequently shown the specificity of the inhibitorial antibodies already formed *(29,30)*. The development of a biochemical method for the identification of immunogenic preparations has proved more difficult than expected. This is chiefly due to the fact that purified FVIII is an extremely sensitive protein. The commercially available preparations are consequently stabilized with large amounts of human serum albumin (HSA) or vWF. These proteins, which are found in large excess, are an obstacle to analysis *(25)*.

In this review, additional chromatographic and electrophoretic methods for further analysis of FVIII polypeptides are presented. Such analysis is essential in process development and will allow improved product control, especially with regard to immunological safety.

As is seen in Figs. 2a and 2b, SE-HPLC of Octavi SDP, a FVIII product with large amounts of vWF, results

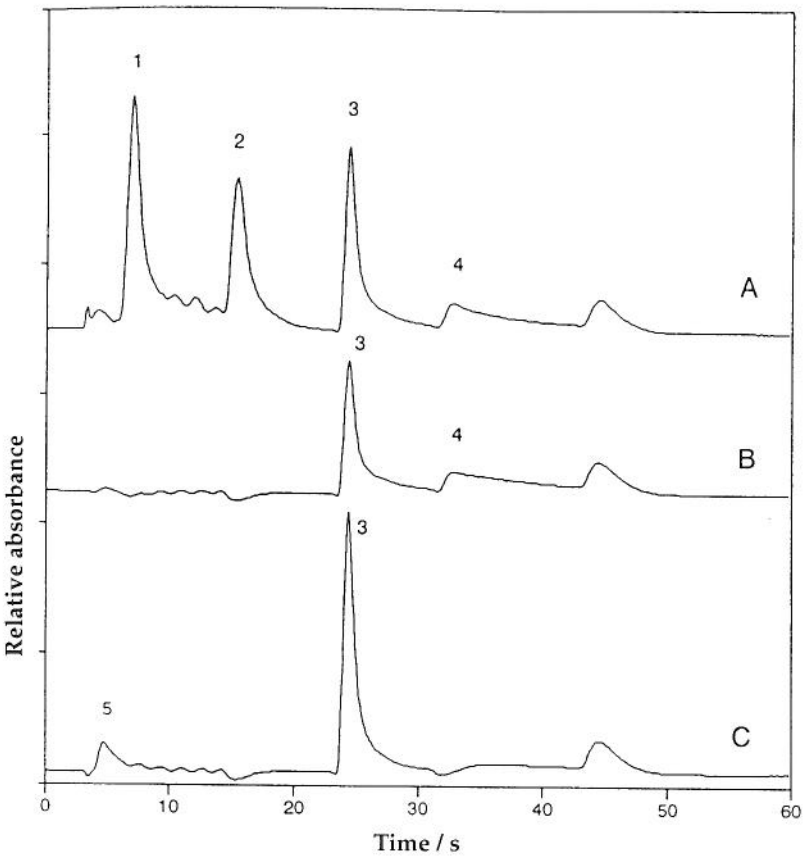

Fig. 1. In-process analysis, anion-exchange (AE) chromatography of samples taken from the production process of clotting factor IX (FIX). (A) Sample after anion-exchange chromatography, containing impurities (cf. ref *6*). Chromatographic conditions: separation unit, a QA-GMA-EDMA-CIM disk with 10 mm diameter and 3 mm thickness; buffer A, 10 mM Tris-HCl, pH=7.4; buffer B, 1 M NaCl in buffer A; gradient time, 10 % buffer B, in 1 s, 10 s isocratic step with 10 % buffer B, then in 1 s 30 % buffer B, followed by 7 s isocratic step with 60 % buffer B, and finally in 1 s to 100 % buffer B, and 11 s isocratic step with 100 % buffer B; flowrate, 5 mL/min; back pressure, 0.5 MPa; UV-detection, at 280 nm. Sample: 10 µg of Tf, 10 µg of IgG (peak 1), and 40 µg of HSA (peak 2) as impurities and 10 IU (about 40 µg) of FIX (peak 3) in 20 µL of buffer A. (B) For chromatographic conditions, see (A). Sample: FIX preparation Octanyne (Octapharma), containing 10 IU of FIX (peak 3) and some impurities with apparent molecular weights of about 180 000 in SDS-PAGE (peak 4) in 20 µL of buffer A. (C) For chromatographic conditions, see (A) Sample: FIX preparation (Mononine), purified by monoclonal antibodies, containing 10 IU of FIX (peak 3) and some smaller polypeptides (peak 5) in µL of buffer A (reprinted from ref. *26* with permission).

in a picture that corresponds to the distribution of identical polypeptide bands in SDS-PAGE under non-reducing conditions. Immunoblot with the monoclonal anti-FVIII antibody no. 530p (Fig. 2c), which reacts against HCh, shows that most reactive polypeptide bands are found in the range of high molecular masses, above 80 kDa *(29,30)*. The reason for this is that even under these separation conditions, FVIII polypeptides are still partly associated with vWF, a protein with a high molecular mass. Apparently this complex cannot be quantitatively dissociated by SDS. The complexes formed by FVIII and vWF are so large that to some extent they are unable to migrate into the gel under non-reducing conditions (Peak 1 in SE-HPLC and the corresponding picture in SDS-PAGE). Only by separation in SDS-PAGE under reducing conditions (Fig. 2b, lane 2) can these complexes dissociate and penetrate into the separating gel, yielding a clear picture of FVIII polypeptides in immunoblot, as it is known in literature *(30)*.

All FVIII preparations which contain vWF but no HSA show a similar picture in SE-HPLC as well as in SDS-PAGE under reducing or non-reducing conditions *(31)*. Experiments carried out with the aim to use these two methods for quality control of plasma derived FVIII (pd FVIII) products have shown that it was only the existence of a peak with an apparent molecular mass of 40 kDa in SE-HPLC (Peak 4 in Fig. 2a) and of a corresponding polypeptide in SDS-PAGE (see arrows in Figs. 2a and 2c) which may be indicative of proteolysis of FVIII in commercial products. The pasteurized preparations, which contained such a polypeptide, were also associated with possible immunogenicity of single FVIII batches. No other differences between single batches or between different commercial products from this group were found with this method *(29)*.

The methods described above, SDS-PAGE and SE-HPLC, allow adequate quality control with regard to potential immunogenicity of single batches. However, the polypeptide with an apparent molecular mass of 40 kD, which was detected in this case, was not proven to be directly immunogenic *(29)*. To get more information 2D-electrophoresis presents itself as a method with high

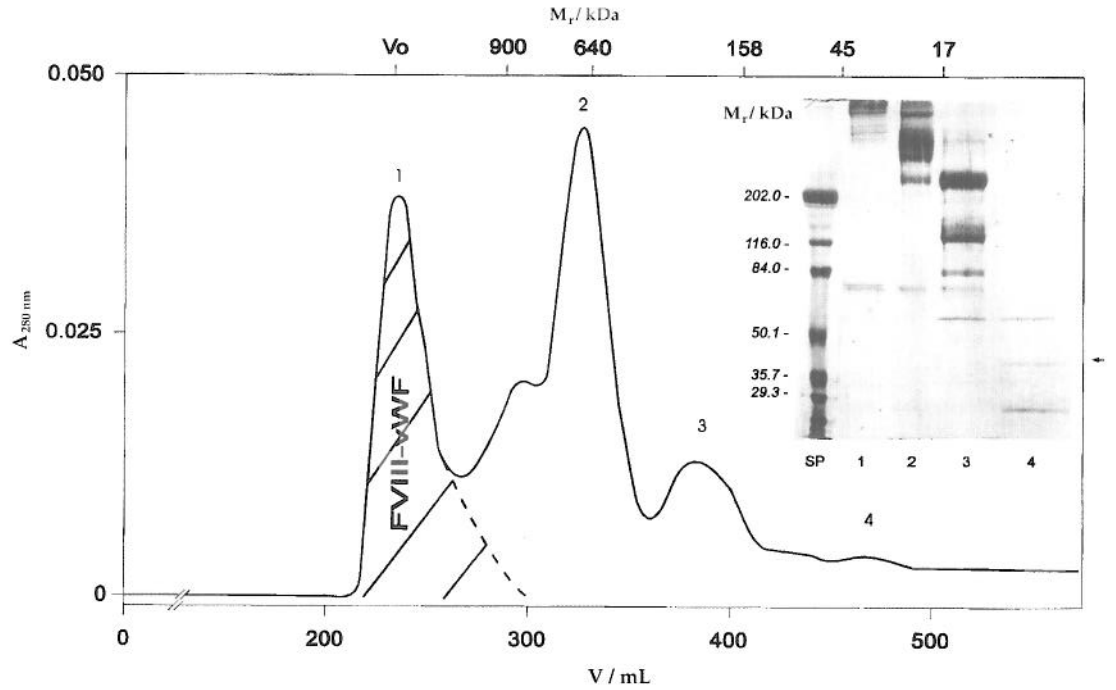

Fig. 2a. Size exclusion chromatography of a FVIII concentrate vWF (Octavi SDP). Only in the fraction with high molecular mass containing FVIII-vWF clotting activity could be detected (peak 1). SDS-PAGE of collected peaks under non-reducing condition is shown in the upper right part of the figure. For separating conditions see text

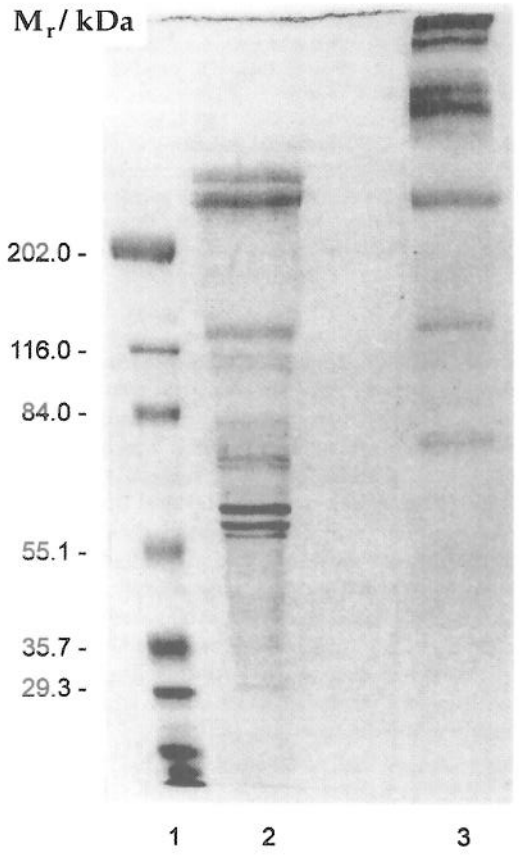

Fig. 2b. SDS-PAGE of the FVIII concentrate Octavi SDP used for chromatographic separation (cf. (a) and reducing (lane 2) and non-reducing conditions (lane 3))

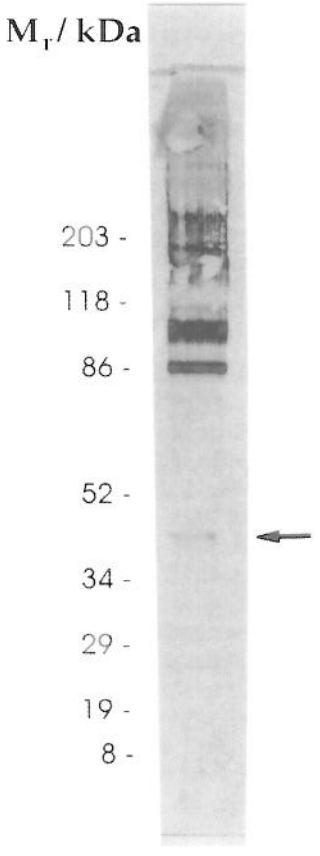

Fig. 2c Immunoblot of the FVIII concentrate Octavi SDP with the monoclonal antibody 530p (anti-FVIII HCh). The band with an apparent molecular mass of 40 kDa is marked with an arrow (reprinted from ref. 25 with permission)

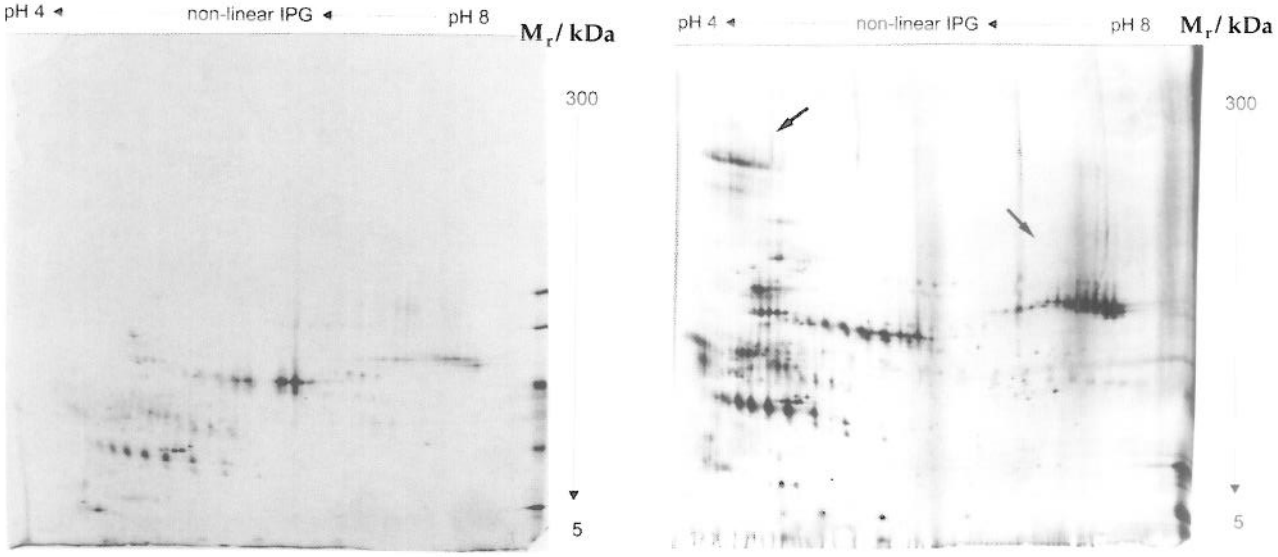

Fig. 3a. 2D-electrophoresis of a recombinant FVIII concentrate. IEF with non-linear 3–10 IPG, in 8 M urea, 2 % CHAPS, detection with silver staining. Fig. 3b. 2D-electrophoresis of a recombinant FVIII concentrate. IEF with a non-linear 3–10 IPG, in 5 M urea, 2 M thiourea, 4 % CHAPS, detection with silver staining. The arrows indicate some of the additional spots due to focusing in the presence of thiourea (reprinted from ref. *25* with permission)

resolution. It can detect small changes of the polypeptide chain *(25)*.

With a molecular mass of over 200 kDa, FVIII is a very large glycoprotein. It has several additional posttranslation modifications on its polypeptides with apparent molecular masses above 100 kDa in 2D-electrophoresis *(25)*. In Fig. 3a 2D-electrophoresis is shown according to the protocol previously used. It is shown in Fig. 3b that by 2D analysis many more spots in the same rFVIII preparation were detected, when the concentration of detergent (CHAPS) was increased and much more thiourea was added during isoelectric focusing. This included spots with high molecular masses (above 100 kDa).

The optimization of 2D-separation of FVIII preparations allows much better analysis by immunoblot. In this figure, immunoblots of pdFVIII samples, which contain vWF are shown. As is it seen in Fig. 4a, it is possible to detect vWF in the range of high molecular masses with polyclonal antibodies. The FVIII immunoblot with a monoclonal LCh antibody (Fig. 4b) shows two groups of spots in the range of apparent molecular masses of 50 and 70 kDa. The microheterogeneity shown here has to be attributed to possible posttranslational modifications,

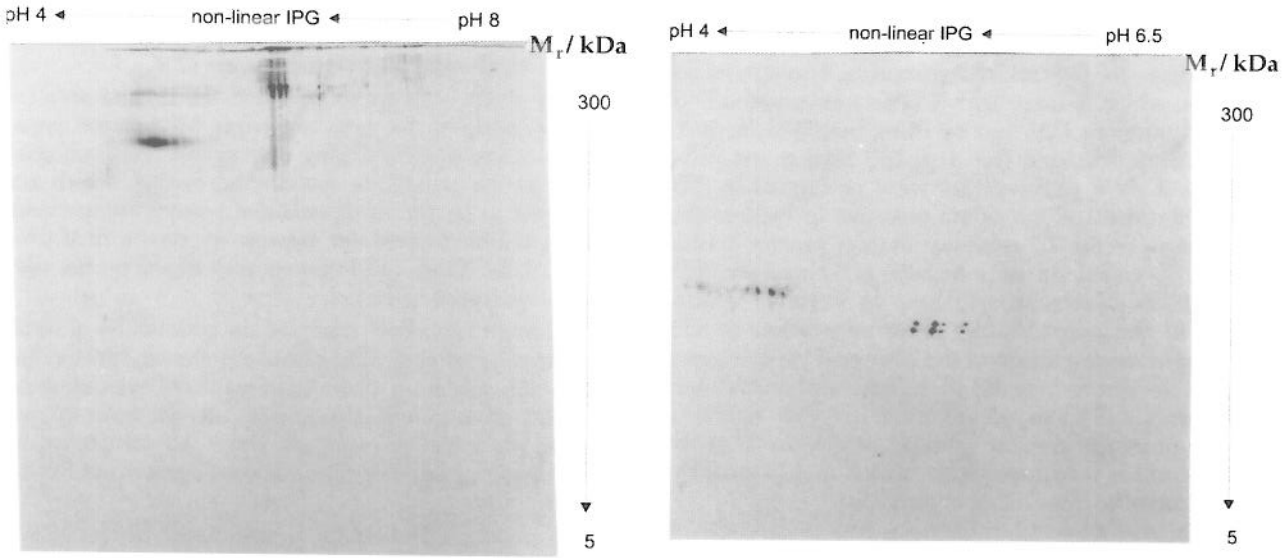

Fig. 4a. 2D-electrophoresis and blot of a plasma derived FVIII concentrate (Octavi SDP) IEF with a non-linear pH=3–10 IPG, immunodetection by probing with a polyclonal rabbit anti human vWF antibody and diaminobenzidine staining of the peroxidase conjugated secondary antibody. Fig. 4b. 2D electrophoresis and blot of a plasma derived FVIII concentrate (Octavi SDP) IEF with a non-linear pH=4–7 IPG. Immunodetection by probing with a mouse monoclonal anti-FVIII antibody and NBT/BCIP staining of the alkaline phosphatase conjugated secondary antibody (reprinted from ref. *25* with permission)

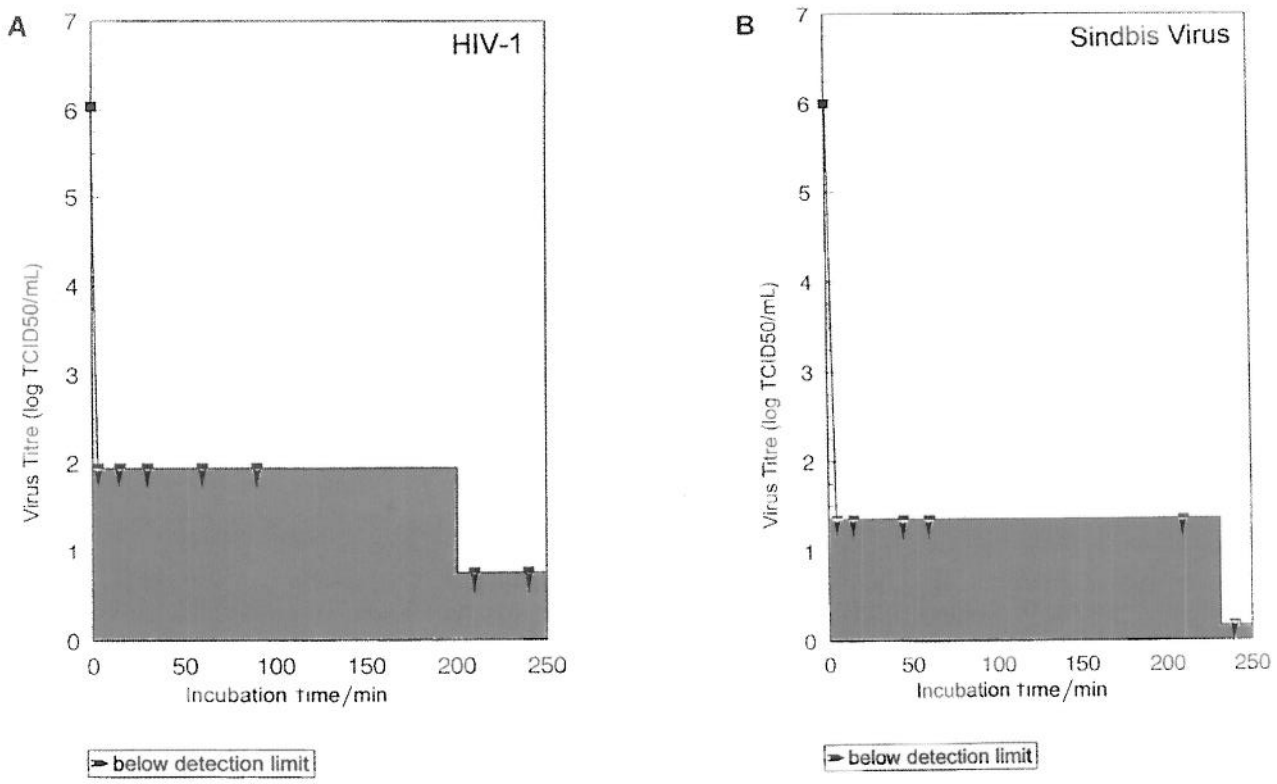

Fig. 5. Kinetics of destruction by solvent/detergent treatment (1 % TnBP and 1 % Triton X–100) of lipid-enveloped viruses in human plasma A – (HIV–1), B – Sindbis virus (as a model virus for the hepatitis C virus) (reprinted from ref. *35* with permission)

above all N- and O-glycosylation. However, other posttranslational modifications such as sulfation and also some degradation during production process are possible *(30,31)*.

Until now FVIII has not been described in the 2D electrophoretic reference map of human proteins *(32)*. Apart from its low concentrations in the plasma, the behaviour of this complex glycoprotein as shown in Figs. 3 and 4, forming many spots in 2D-electrophoresis, may be the reason for the lack of any reports. Through its enrichment, which is more than 4 orders of magnitude in FVIII preparations *(33)*, and by using modified methods for isoelectric focusing (see Fig. 3b), further extensive analyses of FVIII polypeptides were performed by 2D-electrophoresis. It is the prime objective to include this glycoprotein in the 2D reference map of human plasma proteins. A second aim is to develop a 2D map for FVIII preparations. This map will help to improve quality control of the preparations. After separation by 2D, screening and comparison of the pictures by a computer, possible variations will be identified, and inadequate preparations will be sorted out, *e.g.* those with an immunogenic potential. Similar models already exist in the plasma protein map mentioned above, and also in the map of proteins from other organs *(32)*.

Scale-up/Scale-down Strategy

According to Sofer *(17)* and Chew *(34)* »a validation study is actually the final step in development«. Scale-up of a production process is a first step to transfer a process from the laboratory to a production plant. In order to validate the single steps of a process, it has to be transformed back into small scale. This means that a scale-down protocol has to be developed along with the scale-up protocol. For the first step in process validation it is usually necessary to prove that the small scale production is an equivalent of the large scale production in the production plant. Therefore, the most important process parameters have to be measured and compared both in large scale and in small scale *(20)*.

The down-scale protocol allows the control on a laboratory scale of the most important biochemical parameters of the plasma during production. This concerns above all the activity of the clotting factors, which are supposed to be the most sensitive plasma components and therefore markers for the quality of the final product *(20,31)*. Virus validation is also based on an adequate down-scale protocol.

In Table 1 the flow chart for the production of virus inactivated plasma (OCTAPLAS®) is shown. On the basis of this scheme a down-scale protocol was established. It is important to demonstrate that the most important biochemical parameters show no difference in either the large-scale or the small-scale process in the laboratory *(20)*.

Virus Validation

Virus safety of plasma products plays a key role in their safe use. Therefore, the development of a product has to concentrate on virus inactivation and validation

Table 1. Flow scheme of production of solvent/detergent-treated human plasma (OCTAPLAS®) (reprinted from ref. 20 with permission).

Step no.	Sample no.	Description	Mass kg		Scale factor
			Large scale	Small scale	
1		Selection of appropriate plasma according to blood groups. Haemolytic and lipemic units are discarded.			
2		Thawing and pooling of 1530 plasma units achieving a final amount of 384 kg.			
3	1	Addition of buffer ingredients and filtration across 1 μm at room temperature.	384		-
		Withdrawn of 3 × 3 kg for down-scaling experiments	382	3.06	125
4	2	Virus inactivation: Addition of TnBP and Triton X–100, each to 1 %. Heating to 30 °C and stirring for 4 h.			
5	3	Addition of castor oil (5 %). Establishing an emulsion by stirring for 1 h at 30 °C. Cooling down to 20 °C and waiting for phase separation. 0.45 μm filtration of plasma layer.	345	2.7	128
6	4	Removal of detergent: Reversed phase chromatography using μBondapak C18 resin.	365	0.52	702
7	5	Addition of amino acids, pH adjustment, sterile filtration	365	0.1	3650
8		Aliquoting in 0.2 kg bags and flash freezing	365	–	–

of inactivation processes. In this paper some aspects of virus validation are discussed, concerning the production of virus inactivated human plasma, intravenous immunoglobulins and clotting factor IX.

Solvent/Detergent Virus Inactivation

The data contained in Table 1 were the basis for designing a protocol for virus validation of virus inactivated human plasma *(20)*. The studies were designed and executed in accordance with EU-guidelines *(21,35)*.

By adding the solvent TnBP and the non-ionic detergent Triton X–100, the lipid envelopes of the coated viruses are destroyed, killing the viruses *(21)*. In other pharmaceutical products other detergents such as Tween 80 are used instead of Triton X–100. The concentration of the solvent TnBP may vary in the different products *(21,36)*. It has to be pointed out that viruses without lipid envelopes are not or not entirely inactivated by this method *(21,35)*.

The model viruses, which are discussed in this paper, were recommended by several authors for validation purposes of plasma products *(37–42)*. Fig. 5 shows the kinetics of destruction by S/D-treatment of two lipid-enveloped viruses, *i.e.* HIV and Sindbis virus, the latter being a model virus for the hepatitis C virus (HCV). It is seen that both viruses are inactivated below detection level in less than one hour. The reduction factors for some model viruses are summarized in Table 2.

By S/D-treatment lipid-enveloped viruses are inactivated *(21)*. The transfusion-relevant viruses without lipid envelope can be inactivated by immuno neutralization. The presence of neutralizing antibodies is explained by the pooling of plasma from different donors *(35)*. Subsequent solid phase extraction with the RP 18 support, which is primarily used for the removal of Triton X–100, leads to further virus reduction in the product *(21)*. The S/D method for virus inactivation of other plasma products allows the use of different methods for the removal of virucidal reagents. The simplest way is to include a chromatographic step, in which the S/D reagents do no bind to the column. If the substance which is to be isolated binds to the column, solvent and detergent will appear in flow-through, and are therefore separated in an elegant manner *(43,44)*. Consequently the development of processes must provide not only for a virus inactivation step, but also for suitable methods which remove the virucidal reagents (see below).

Table 2. Inactivation of lipid enveloped viruses in solvent/detergent treatment of human plasma

Virus	Reduction factor ($\log_{10}$) SD treatment
HBV*	6.0
HCV*	5.0
Sindbis	≥ 5.73 ± 0.26
HIV	≥ 5.20 ± 0.42
PRV	≥ 7.37 ± 0.32
VSV	≥ 7.5
HSV–2	6.0

* Animal studies

For virus validation, the influence of several critical factors has to be investigated. They can occur as a consequence of variations in the production process or through differences in the starting materials. In the case of human plasma, the contents of protein and lipid may vary, depending on the technique used for donation (plasmapheresis or whole blood plasma, cf. Ref. *18*) and also on the country of origin. As is seen in Fig. 6, the variations in the protein and lipid contents are not critical for virus inactivation. However, a critical point proved to be the concentration of virus inactivating chemicals. Fig. 7a shows that total virus inactivation is no longer

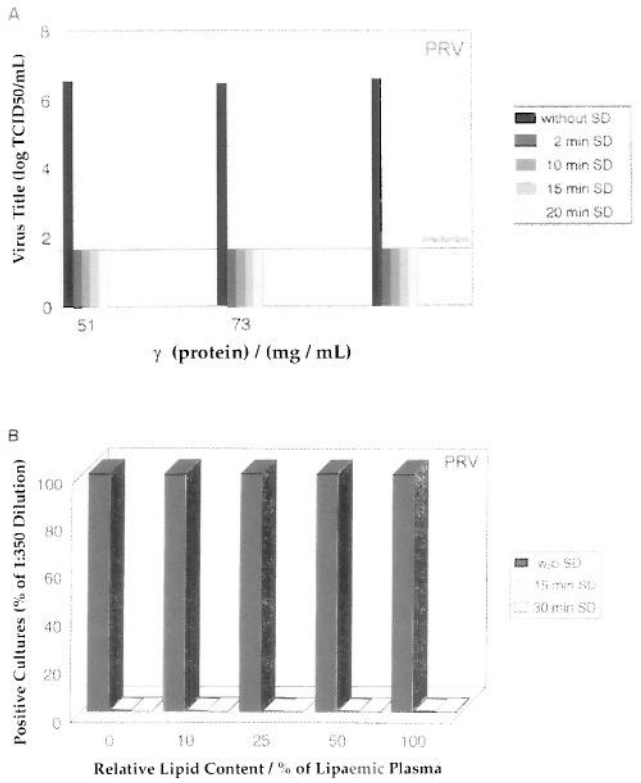

Fig. 6. Influence of protein (A) and lipid (B) content in plasma on virus inactivation by the solvent/detergent method in human plasma. S/D concentration was kept constant at 1 % TnBP and 1 % Triton X–100; pseudorabies virus (PRV) was taken as a model virus (reprinted from ref. 35 with permission)

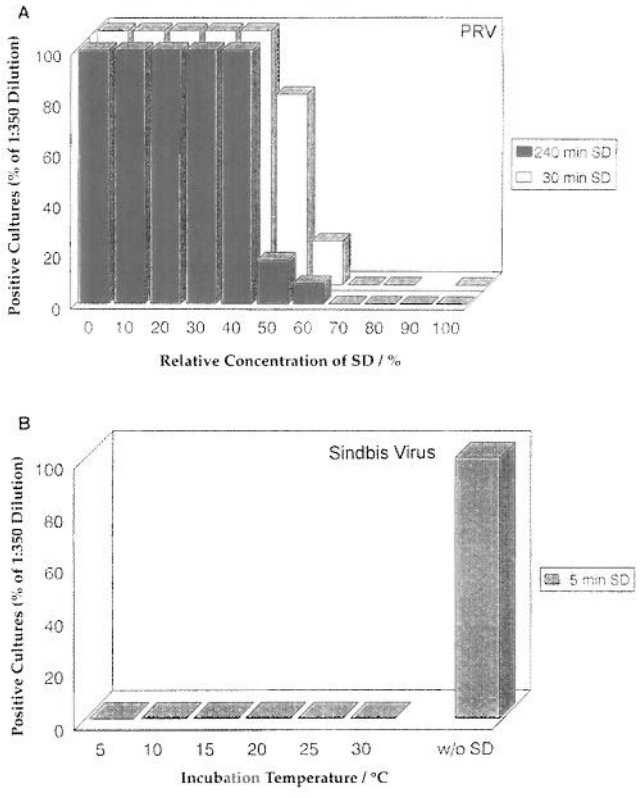

Fig. 7. Influence of relative solvent/detergent concentration on (A) the inactivation of PRV and influence of temperature on inactivation of Sindbdis virus (B) in human plasma (reprinted from ref. 35 with permission)

guaranteed, if the concentration of S/D falls below 0.8 %. Temperature plays no important part in this experiment (not shown here). However, the role of the matrix, that is the surroundings in which virus inactivation takes place, should always be taken into account. This means that the investigations shown here should be carried out for every single product *(23,39)*.

The investigations show that the different steps rely on developments that take place in other fields. The determination of the critical S/D concentration (cf. Fig. 7 a) is not possible without appropriate down-scale and in-process analysis of S/D reagents (see below).

Other Methods for Virus Inactivation and Virus Removal

The International Association of Biological Standardization (IABS) recommends that at least two independent steps for virus inactivation be carried out *(42)*. In most cases the S/D method is combined with pasteurization at 60 or 63 °C, in the presence of stabilizers *(41, 43,44)*. Pasteurization at 63 °C ensures inactivation of a wide range of the S/D inactivation resistent viruses without lipid envelopes *(43)*.

In the case of some less sensitive plasma proteins such as immunoglobulin G, treatment at low pH can be applied. In Fig. 8 the kinetics of the inactivation of HIV–1 is shown. In combination with the solvent/detergent method this virus inactivation step increases the safety of the product *(35)*. Proof of virus safety has for five years now been emphasized for immunoglobulin products in particular. Before, several cases of transmission of the hepatitis C virus had been reported, due to inadequately virus inactivated immunoglobulin G products, which were intravenously administered *(35,45–47)*. However, in the case of human plasma, a very complex and sensitive protein mixture, such rather agressive methods for virus inactivation cannot be used.

Apart from methods of virus inactivation, the removal of viruses from the protein mixture by filtration has been carried out successfully *(18,39,40)*. This technique employs practically all the common types of filter, including cross-flow and dead-end filtration units. All units are based on the same principle, the separation of the larger virus particles, which are held back by the filters, from the smaller protein molecules, which pass through the unit. This makes the size of the particles concerned a crucial point for the viability of the method. If the molecular mass of the proteins is below 100 kDa, separation is easy *(18,48,49)*. However, in the case of larger proteins such as concentrated clotting factor VIII, which occurs in a complex with the von Willebrand factor, virus filtration can no longer be applied, as no sufficient difference in size exists between the proteins and the viruses (Stadler, Josić, unpublished data).

Virus filtration was used successfully for the removal of viruses from the FIX and FXI concentrates and the concentrate of the vitamin K dependent clotting factors (factor II, VII, IX and X, so-called PPSB) *(18,48–50)*. Experiments indicate that the filter with an exclusion limit of 70 Å (Viresolve 70) is much more efficient than that with an exclusion limit of 180 Å (Viresolve 180), especially for the removal of smaller viruses. These pass

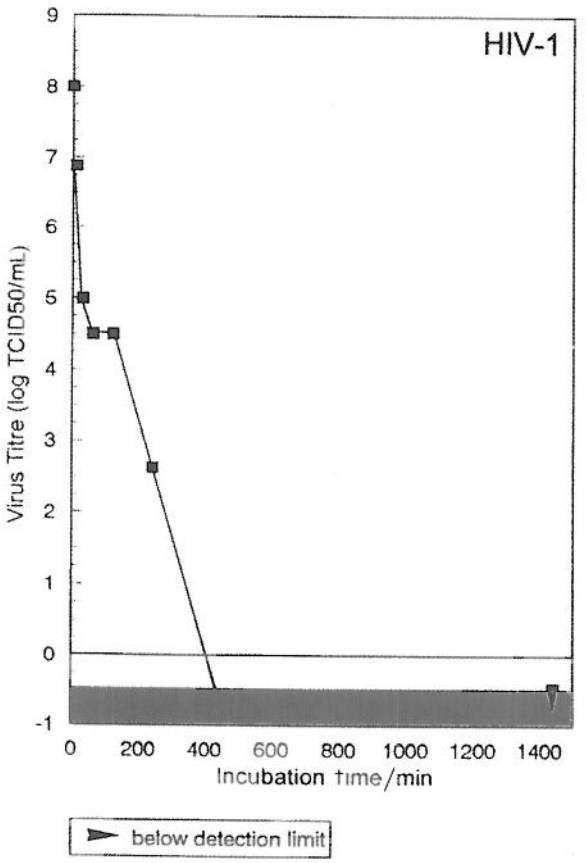

Fig. 8. Kinetics of the inactivation of model virus in human immunoglobulin G concentrate (OCTAGAM®) at pH=4.0. As a model, HIV–1 was chosen (reprinted from ref. *35* with permission)

through the larger pores and consequently are not separated from FIX (not shown here).

In many cases, however, virus filtration has to be treated with care. As the filters hold back the larger proteins, it may change the composition of the product *(18)*. This can have serious consequences, *e.g.* a request for new registration. Choosing the filter for virus removal and optimizing the separation conditions often requires extensive experimental work. In the case of the FIX solution, a tangential-flow filter proved to be adequate *(18)*. For the same step with a PPSB solution the use of a dead-end filter was required *(49)*.

Removal of Virus Inactivating Reagents

The chromatographic steps, above all ion-exchange and affinity chromatography, are simple and elegant methods for the removal of virus inactivating reagents. They exploit the fact that the active component interacts with the column material and therefore binds to the column, whereas both solvent and detergent run through the column without interaction. The remaining reagents are removed together with any other contaminants in a subsequent washing step *(19,44,51,52)*.

Such a chromatographic step cannot be used for the removal of solvent and detergent from plasma. The solvent TnBP is removed from the plasma chiefly by ex-

traction along with the castor oil, step no. 5 *(20)*. The remainder of the solvent is then removed through solid phase extraction with the RP 18 material. However, the extraction of the oil hardly affects the Triton X–100 in the plasma at all. The detergent is removed only in the subsequent step, solid phase extraction, below the detection limit of 1 ppm. For the determination of Triton X–100 the time-consuming, but very sensitive method had to be used in this case. It includes complicated pretreatment of the sample *(23)*. The same method has been used for determining the Triton X–100 content in the final product. No detectable level of Triton X–100 was found in any of the batches under investigation (not shown here).

The silica-based C-18 material, which is used for removing the Triton from the plasma, has the disadvantage of being unstable under alkaline conditions. Consequently, sanitization with 0.5 M NaOH is impossible. The alternative polymer-based RP 18 supports, stable at high pH, have a far too low capacity for Triton X–100. Therefore it cannot be used for this purpose *(23)*.

The silica-based RP 18 column used for the removal of Triton X–100 from virus-inactivated plasma and human IgG concentrate, has to be regenerated and equilibrated after each run. For this purpose the following data have to be determined *(53,56)*:

– how often can the column be used?

– in which regeneration steps is the detergent removed?

– what causes the decline in column performance after a certain number of runs?

In order to answer these questions, the complete process for regeneration and equilibration of the column has to be down-scaled to laboratory standard *(20)*.

Products from Hydrolysis (Leachables) of the Column Materials

General Aspects

As discussed above in connection with impaired column performance in the case of the C 18 support, several reasons can be responsible for a decrease in binding capacity or changes in elution behaviour after extensive use in production. These may be mechanical damage to the support, hydrolytic cleavage of the ligand or contamination of the surface of the support by components from the sample. In the case of mechanical damage to the gel bed, an increase in back pressure and plugging of the column would have occurred as well. Hydrolytic cleavage of the ligand, as well as contamination of the surface of the chromatographic material by components of the sample, can influence severely both capacity and binding characteristics of the column. If the components of the sample are not completely removed from the column, an additional risk exists of cross-contamination of the product from components of the preceding batch. Substances from hydrolysis of the support and from the filter materials used can also contaminate the product *(53–56)*.

In this section the problem of leachables from the column supports is given special attention. Two types of chromatographic materials were investigated:

– a silica-based, reversed phase support (C 18) for solid phase extraction of Triton X–100 from plasma and from human IgG concentrates *(20,21,23,35)*,

– a synthetic polymer-based anion exchange support (DEAE), used for the purification of FVIII from human plasma *(19,34,43,44)*.

The most important characteristic of each separation material which is to be used in the production and purification of biologicals, is its chemical stability *(44,47,48)*. Degradation of chromatographic materials is not only related to the respective lifetime of the column. It can also cause the release of different compounds, which are usually called leachables (monomers coming from polymer resin, ligands, hydrolysis products). These in turn can contaminate the product. The acceptable level of leachables depends on the toxicological and genotoxicological dangers of the respective compounds. Usually the acceptable concentrations lie in the range of 1 to 100 ng per mg of biologicals. The amount of about 10 ng per mg is usually considered an average value *(47,53,54)*.

The risk of a toxic contamination of biologicals during chromatography is small. The concentration of leachables is usually quite low, and the materials chosen for chromatographic purposes have to be non-toxic. Still, because of the remaining danger the chromatographic materials used in the production process of biologicals must be validated as a precaution.

Experimental Strategies for the Determination of Leachables

As there are still no generally agreed upon rules concerning the validation of chromatographic resins with regard to leachables, different experimental strategies can be considered. Three different strategies are used for this purpose:

– **static experiment**, in which leachables are determined by resin extraction with different solutes;

– **final product experiment**, in which leachables are determined in the final product, selected at random from the production process;

– **on-column experiment**, in which leachables are determined at different steps of the production process, simulated in the laboratory on a down-scale column *(47, 53,54)*.

Static Experiments

In the static experiments the influence of different solutes on the chromatographic resin or other materials used for separation is studied. For this purpose the chromatographic resin is incubated in aqueous solutions at an extreme pH for different periods of time and at different temperatures. The same static experiment can be performed with different organic solvents. In the case of static experiments, decomposition of chromatographic resin and the concentration of leachables under extreme conditions are studied *(47,53,54)*.

Determination of Leachables in the Final Product

When the reversed-phase packing C-18 gel is used, the possible products of hydrolysis, which may appear in the final product, are octadecylmethyl silanol (C-18 monomer) and dioctadecylmethyl disiloxane (C-18 di-

mer). The amount of C 18 monomer and dimer was determined by GC/FID-analysis in 6 final containers of different production batches of virus inactivated human plasma (OCTAPLAS®). The data on the identification of possible leachables and the method of determination are taken from the master file of the producer. The C 18 monomer and the C 18 dimer were found after spiking of non treated plasma, in quantities of 1 ppm monomer and 2 ppm dimer, respectively. However, the amount of C 18 monomer and dimer in the final product OCTAPLAS® was below the detection limit. The amount of C--18 monomer or dimer did not exceed detection in any of the 6 batches under investigation (not shown here)

On-column Experiments

On-column experiments are carried out after the static experiments and the investigation of the products of hydrolysis (leachables) in the final product. In on-column experiments the behaviour of the column in all its functions in a production process is investigated, that is during sample application, washing and elution, during sanitization, regeneration and re-equilibration *(20,47, 53–57)*. For this purpose all these steps are simulated.

The on-column experiments provide detailed data about the behaviour of the chromatographic materials during its use in production. The data are specific for every production process. However, it has to be pointed out that the static experiments which are carried out by the column producers, form the basis for further investigations. The first step in the static experiments is the identification of possible leachables of the chromatographic material. Then a method is chosen for their detection *(53–56)*.

The investigation of leachables of the polymer-based anion exchange material (DEAE support) is discussed in on-column experiments. It is used in this case for purification of the plasma-derived FVIII-vWF complex. The anion exchange material is used for two different functions in the production process, the separation of the FVIII-vWF complex from contaminating proteins and the removal of virus inactivating reagents, namely solvent and detergent (see also above). The separation mechanism is based on the fact that most contaminating proteins and S/D either do not bind at all or bind only weakly to the anion exchange material, and therefore are washed out by a buffer with a relatively low salt concentration. The active component consisting of a FVIII-vWF complex binds strongly to the column and is eluted by a step gradient at higher salt concentrations *(19,33,43)*.

The possible leachables from the chromatographic material are according to the supplier of the resin 2-(diethylamino)ethanol (DEAE), diethyleneglycol (DEG), glycidyl methacrylate (GMA) and ethyleneglycol dimethacrylate (EGDMA). In order to detect these substances, the method of GC-FID was recommended.

In order to determine the parameters for the detection of leachables by the on-column experiments, a down-scale protocol was made to simulate the application of the column in the laboratory. It was shown that most leachables stayed below detection limit after 150 h. The exception was DEAE. This ligand was detected in the sample after treatment with 25 % ethanol, distilled water and 0.1 M HCl.

In all the on-column experiments the amounts of extracted products of hydrolysis were below the detection limit. This is not surprising, as treatment in the on-column experiments is carried out for much shorter periods of time compared with the static experiments.

Conclusions

The statement by Jungbauer and Boschetti *(16)*, made in 1994 with regard to the production of recombinant proteins, is valid for plasma derived proteins also: »Exact protocol of the production and purification process is part of the definition of the compound. This approach provides consistency and safe production methods, but it also inhibits the rapid development of novel processes.« We may add that the behaviour of the chromatographic material in every phase of its use has to be known as well. For products from human plasma, virus safety is another important aspect. This means that virus validation plays a key role in process development.

Quality control of the final product becomes increasingly strict and extensive. Analysis of biologicals includes not only chromatographic and immunochemical methods, but also mass spectrometry, capillary electrophoresis and other methods of high resolution. For virus safety of the product, polymerase chain reaction (PCR) has increasingly become an important standard *(35,57)*. This calls for multidisciplinary knowledge and corresponding collaboration between work groups specialized in different fields. This is true for the development of a new product as well as for the dialogue between the producer and the regulatory authorities.

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Razvoj medicinskih proizvoda iz humane plazme

Sažetak

U radu je prikazan razvoj i provjera procesa pri dobivanju nekoliko biološki aktivnih supstancija iz humane plazme. To su plazma u kojoj je provedena inaktivacija virusa, intravenozni imunoglobulini i koagulacijski faktori VIII i IX. Opisani su različiti analitički postupci primijenjeni za razvoj i kontrolu kakvoće proizvoda i »in-line« praćenje procesa.

Uvođenjem brzih kromatografskih postupaka, što se mogu provesti unutar samo 2–3 minute, omogućeno je analitičko praćenje proizvodnog procesa. Analitičkim postupcima s velikom mogućnosti razlučivanja (dvodimen-

zionalna elektroforeza) dobiveni su odgovarajući rezultati, čak i u prisutnosti sastojaka velike molekularne mase kao što su faktor grušanja VIII (F VIII) i faktor von Willebrand (vWF). Ti su postupci nedavno uvedeni u procesnu kontrolu.

Prikazan je postupak proizvodnje humane plazme u kojoj je provedena inaktivacija virusa što je omogućio simuliranje proizvodnog procesa u laboratorijskom mjerilu. Provjerom procesa ustanovljeno je da je smanjenje količine virusa, relevantnih za transfuziju, veće od 6 logaritamskih jedinica. Određivanje hidrolizom nastalih tvari iz kolone za procesnu kromatografiju, upotrijebljene u proizvodnji, uočeno je da se te tvari pojavljuju u gotovu proizvodu u količinama ispod granica detekcije. Isto je tako pokazano da se kemikalije upotrijebljene za virusnu inaktivaciju mogu kvantitativno ukloniti iz gotova proizvoda.

U postupku izolacije drugih proizvoda, intravenoznih γ-globulina i kogulacijskih faktora VIII i IX, mogu se primijeniti slične metode. Pri određivanju koagulacijskog faktora VIII praćena je i određivana količina virusa, zatim količina produkata hidrolize iz kromatografskog stupca te preostale kemikalije upotrijebljene za inaktivaciju virusa. Dokazano je da je inaktivacja virusa uspješno provedena te da su kvantitativno uklonjene kemikalije za njihovu inaktivaciju. Količina mogućih produkata hidrolize iz kolone, primijenjene za procesnu kromatografiju, bila je kudikamo ispod dopuštene razine.