Review

Issues in the development of medical products based on human plasma[1]

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Abstract

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 control and in-process control. 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 six log steps. 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, here 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, 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.   © 1997 Elsevier Science B.V.

Keywords: Plasma-based medical products; Virus-inactivated plasma; Immunoglobulins; Factor VIII; Factor IX

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1. 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 [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 gene technologically obtained products are increasingly becoming a standard of quality also for those biologicals which are derived from other sources [16,17]. This leads to much stricter criteria for the licensing of biologicals. All the basic requirements are still valid, namely (i) effectiveness of the product, (ii) virus safety of the product and (iii) lack of side-effects.

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 virus 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 which 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 [21], and UV light irradiation in the presence of methylene blue (MB) [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 RP-18 modified silica gel [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 (i) one step for virus inactivation and (ii) one chromatographic step [19].

This review shows the aspects of virus validation of the production processes, the 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.

2. Scale-up/scale-down strategy

According to Sofer [17] and Chew [25] "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 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 to control on a laboratory scale 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. Virus validation is also based on an adequate down-scale protocol.

In Fig. 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. Table 1 shows the behaviour of the clotting factors I (fibrinogen), V, VII, VIII and XI as well as that of the aPTT values in the production process, in large scale (l) and in small scale (s). As can be seen in Table 1, the most important biochemi-

Step no.	Sample no.	Description	Volume [kg] large scale	Volume [kg] small scale	scale factor
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		-
		Withdrawal of 3 x 3 kg for down-scaling experiments.			
4	2	Virus inactivation: Addition of TnBP and Triton X-100, each to 1%. Heating to 30°C and stirring for 4 h.	382	3.06	125
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
.a)		Aliquoting in 0.2 kg bags and flash freezing.	365	-	-

a) For large scale only

Fig. 1. Flow scheme of production of solvent/detergent-treated human plasma (Octaplas). Reprinted from Ref. [19] with permission.

cal parameters show no difference in either the large-scale or the small-scale process in the laboratory.

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

3.1. Solvent/detergent virus inactivation

The data contained in Tables 1 and 2 and in Fig. 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 III/8115/89-EN and III/8973/EN-89 [26–28].

Table 1
Comparison of biological parameters in virus-inactivated plasma; large scale (l) versus small scale (s) process

Parameter	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5 (final product)	
	l	s±S.D.	l	s±S.D.	l	s±S.D.	l	s±S.D.	l	s±S.D.
Fibrinogen (mg/ml)	2.9	2.9±0.3	n.a.	n.a.	2.6	2.5±0.3	2.7	2.6±0.3	2.6	2.6±0.3
Factor V (U/ml)	0.9	0.9±0.2	n.a.	n.a.	0.5	0.5±0.2	0.8	0.7±0.2	0.8	0.7±0.2
Factor VII (U/ml)	1.0	1.0±0.2	n.a.	n.a.	0.9	0.7±0.2	1.2	1.0±0.2	1.2	1.0±0.2
Factor VIII (U/ml)	1.0	0.9±0.2	n.a.	n.a.	0.8	0.7±0.2	0.8	0.7±0.2	0.8	0.7±0.2
Factor XI (U/ml)	0.9	0.8±0.1	n.a.	n.a.	0.9	0.8±0.1	0.9	0.8±0.1	0.9	0.9±0.1
aPTT (s)	37	38±4	n.a.	n.a.	n.a.	n.a.	38	39±4	38	40±4
Total protein (mg/ml)	59	62±4	n.a.	n.a.	63	64.4±4	64	60±4	60	58±4

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S.D.=Standard deviation.
n.a.=Not assessible due to high detergent concentration.

By adding the solvent TnBP and the non-ionic detergent Triton X-100, the lipid envelopes of the coated viruses are destroyed, inactivating 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 [26].

The model viruses, which are discussed in this paper, were recommended by several authors for validation purposes of plasma products [29–33]. Fig. 2 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 15 min. The reduction factors for some model viruses are summarized in Table 3.

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 [26]. 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 [34,35]. 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 Section 3.2).

For virus validation, the influence of several critical factors has to be investigated. They can occur as a consequence of variations in the production

Table 2
Comparison of chemical parameters in virus-inactivated plasma (content of virus-inactivating agents Triton X-100 and TnBP), large scale (l) versus small scale (s) process

Parameter	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5 (final product)	
	l	s±S.D.	l	s±S.D.	l	s±S.D.	l	s±S.D.	l	s±S.D.
Triton X-100 (μg/ml)	–	–	9800	9600±1000	7300	6500±1000	<1	<1	<1	<1
TnBP (μg/ml)	–	–	9900	9500±1000	500	600±200	<0.5	<0.5	<0.5	<0.5

Reprinted from Ref. [19] with permission.
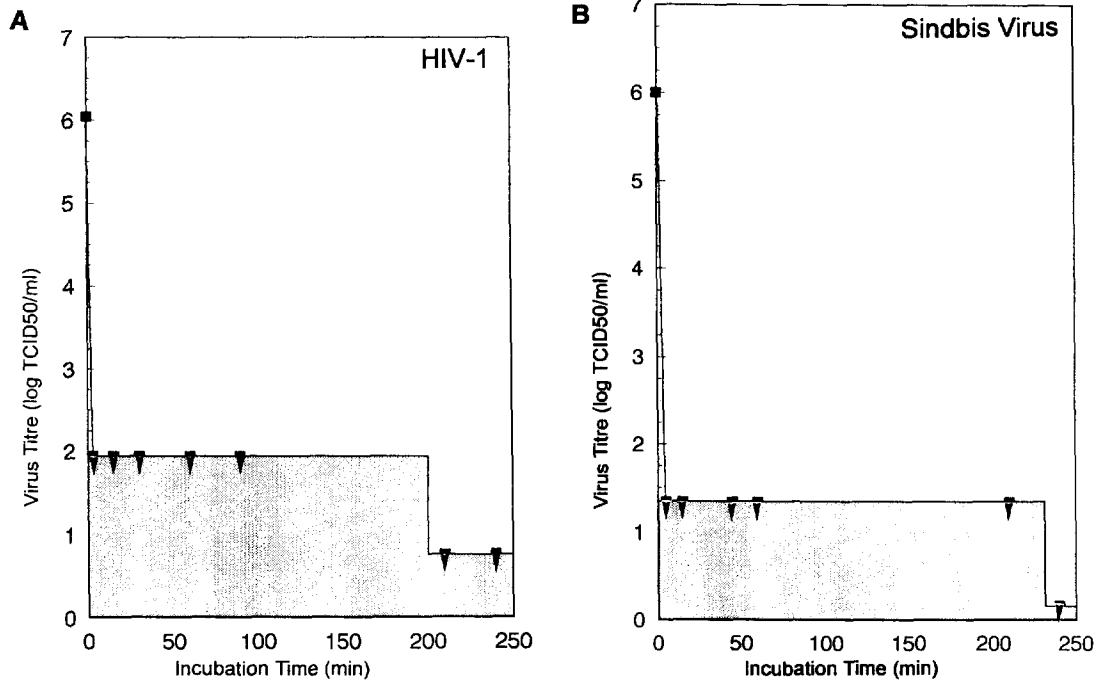S.D.=Standard deviation.

Fig. 2. Kinetics of destruction by solvent/detergent treatment (1% TnBP and 1% Triton X-100) of two lipid-enveloped viruses in human plasma. (A) HIV-1, (B) Sindbis virus (as a model virus for the hepatitis C virus).

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. 3, 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.

Table 3
Inactivation of lipid enveloped viruses in solvent/detergent treatment of human plasma (Octaplas)

Virus	Reduction factor (log 10) S/D treatment
HBV[a]	≥6.0
HCV[a]	≥5.0
Sindbis	≥5.73±0.26
HIV	≥5.20±0.42
PRV	≥7.37±0.32
VSV	≥7.5
HSV-2	≥6.0

[a] Animal studies.

Fig. 4A shows that total virus inactivation is no longer guaranteed, if the concentration of S/D falls below 0.8%. The incubation temperature plays no important part in this (Fig. 4B). 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 [23,30].

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. 4A) is not possible without appropriate down-scale and in-process analysis of S/D reagents (see Section 3.2).

3.2. Other methods for virus inactivation and virus removal

The International Association of Biological Standardization (IABS) recommends that at least two
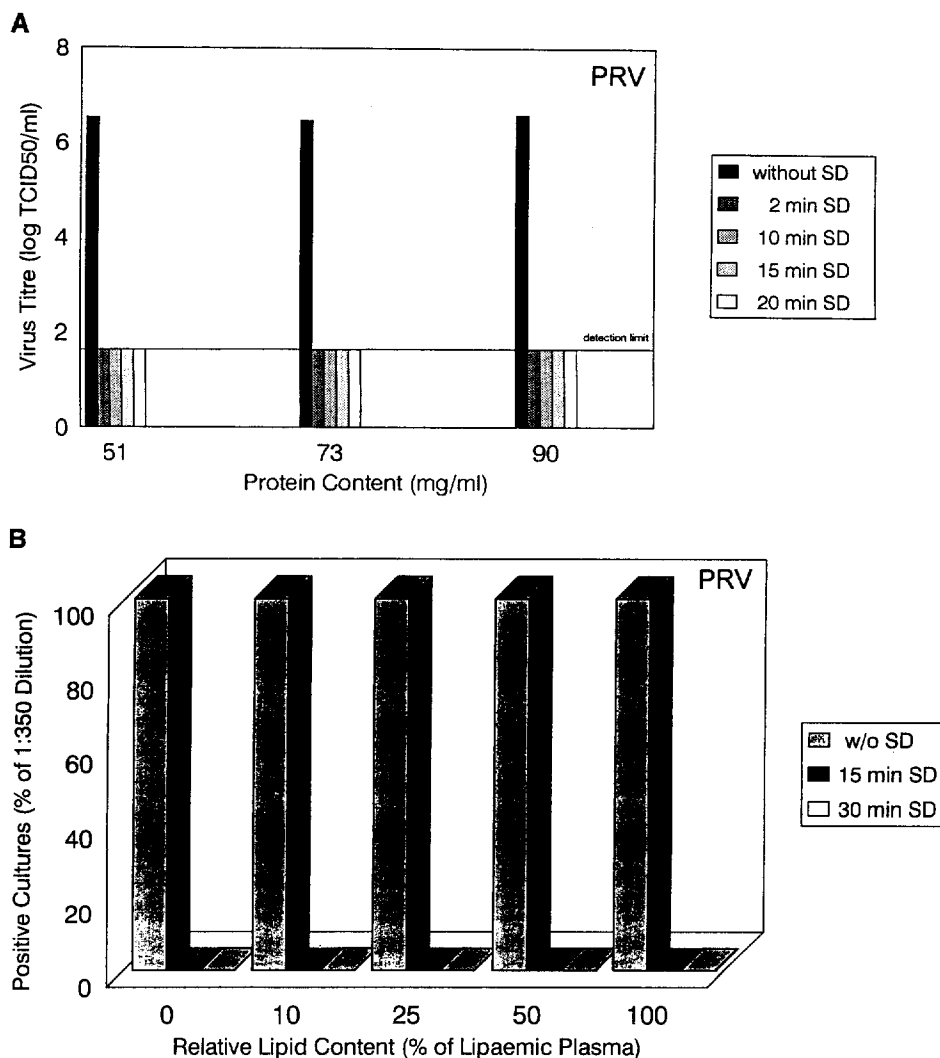
A

B

Fig. 3. 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.

independent steps for virus inactivation are carried out [33]. In most cases the S/D method is combined with pasteurization at 60°C or 63°C, in the presence of stabilizers [32,34,35]. Pasteurization at 63°C ensures inactivation of a wide range of the S/D inactivation resistant viruses without lipid envelopes [34].

In the case of some less sensitive plasma proteins such as immunoglobulin G, treatment at low pH can be applied. In Fig. 5A,B the kinetics of the inactiva-

tion of two model viruses are shown, HIV-1 and pseudorabies virus (PRV). In combination with the solvent/detergent method this virus inactivation step increases the safety of the product [26]. 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 [26,36–38]. However, in
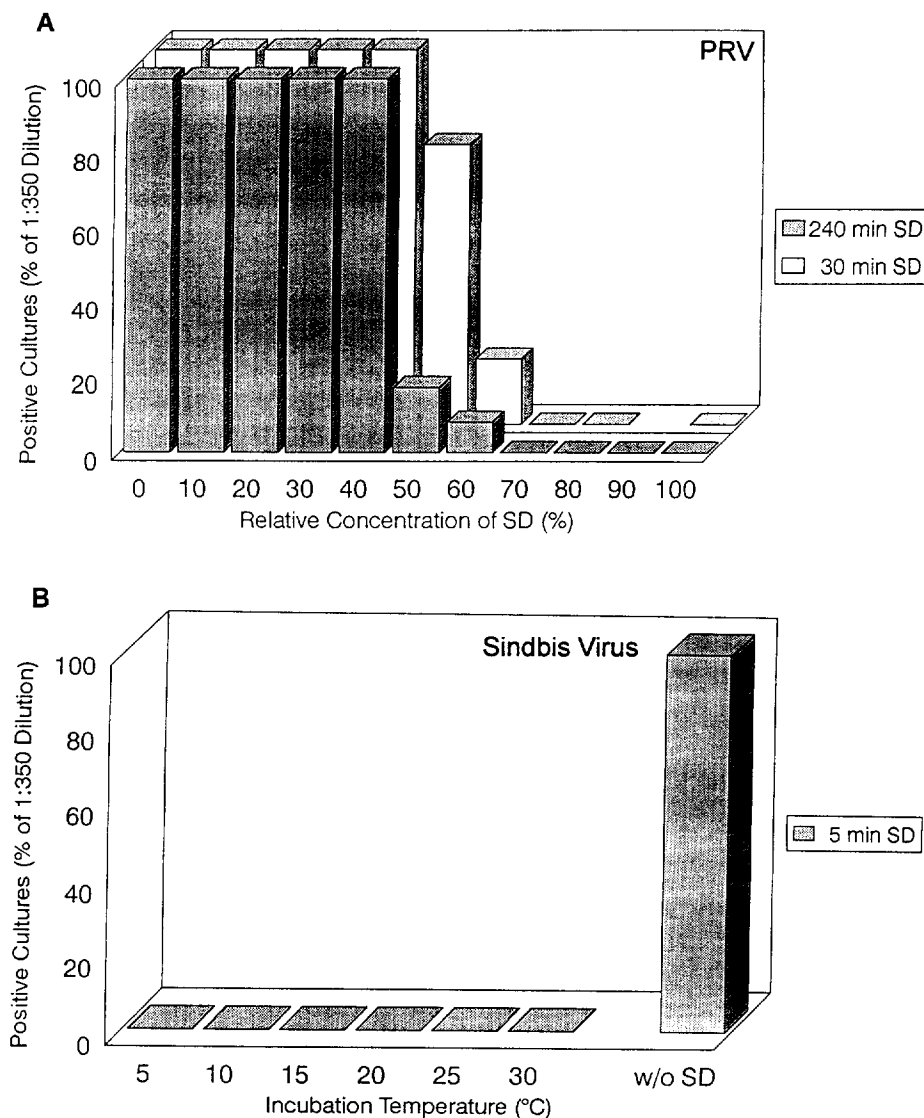
Fig. 4. Influence of relative solvent/detergent concentration (A) on the inactivation of PRV and influence of temperature on inactivation of Sindbis virus (B) in human plasma.

the case of human plasma, a very complex and sensitive protein mixture, such rather aggressive 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 100 000 Da, separation is easy [18,39,40]. However, in the case of larger proteins such as concentrated clotting factor VIII, which occurs in a complex with the von
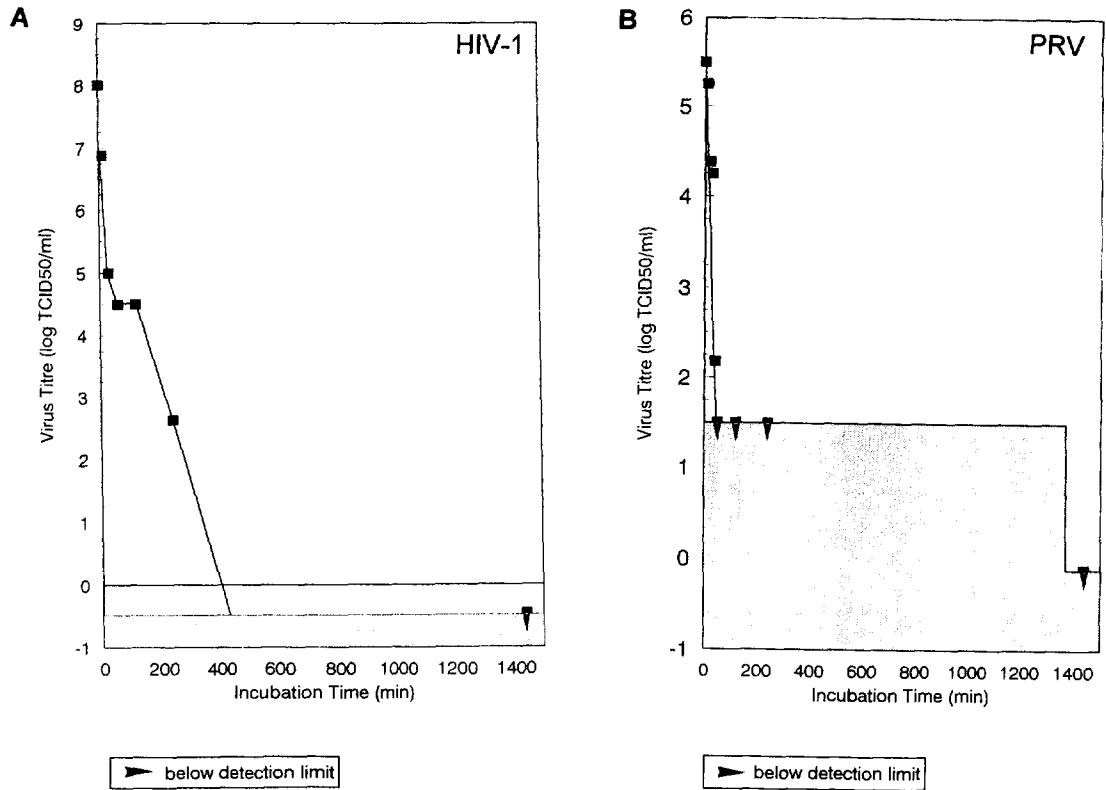
Fig. 5. Kinetics of the inactivation of two model viruses in human immunoglobulin G concentrate (Octagam) at pH 4.0. As a model, HIV-1 (A) and pseudorabies virus (B) were chosen. Reprinted from Ref. [26] with permission.

Willebrand factor (vWF), virus filtration can no longer be applied, as no sufficient difference in size exists between the proteins and the viruses (Stadler and 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) [18,39–41]. Tables 4 and 5 show the data for the removal of model viruses from a factor IX product. The results indicate that the filter with an exclusion limit of 70 Å (Viresolve 70, Millipore, Bedford, MA, USA; cf., Ref. [18]) is much more efficient than that with an exclusion limit of 180 Å,

Table 4
Virus removal by filtration of a factor IX concentrate using Viresolve 180 filter (S.D. standard deviation)

Virus	Reduction factor (log $10\pm$S.D.)
SV40	2.87 ± 0.40
Reovirus-3	4.65 ± 0.22
Coxsackie-B6	3.08 ± 0.38

Table 5
Virus removal by filtration of a factor IX concentrate using Viresolve 70 (exclusion limit 70 Å)

Virus	Reduction factor (log $10\pm$S.D.)
Sindbis	7.28 ± 0.28
Reovirus-3	$>6.49\pm0.24$
Coxsackie-B6	4.81 ± 0.36
HSV-1	$>8.30\pm0.23$
HIV-1	$>4.96\pm0.21$

especially for the removal of smaller viruses. These pass through the larger pores and consequently are not separated from FIX.

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]. As is seen in Fig. 6, filtration with Viresolve 70 separates not only the viruses (cf., Table 5), but also some substances with high molecular masses. The specific activity of the final product is increased, but a change in its composition has taken place. 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 [41].

4. Validation of Triton X-100 distribution

Apart from the biochemical factors which indicate that the product has retained its biological activity, its virus safety is of utmost importance. Therefore, the presence of reagents for virus inactivation as well as their removal has to be given special attention.

Table 2 shows the behaviour of the virus-inactivating reagents Triton X-100 and tri-n-butyl phosphate (TnBP) during the production process. It is shown that these substances, which are added in the second step, are present in sufficient quantities during virus inactivation both in large scale and in small scale. As expected, they are removed in the subsequent production steps. These are extraction with castor oil and solid-phase extraction, using a RP-18 resin (cf., Table 2 and Refs. [20,21]).

In order to monitor the presence of Triton X-100 and its distribution in the plasma during virus inactivation, an adequate method had to be developed for determining this reagent in the plasma. The method of routine, sample preparation through solid-phase extraction, is very sensitive and reproducible, but also rather slow [23]. In Fig. 7 a fast method is shown for Triton X-100. This method allows direct injection without sample preparation,

reducing the time required for analysis to about 15 min.

5. 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 [19,35,42,43].

Such a chromatographic step cannot be used for the removal of solvent and detergent from plasma. Table 2 and Fig. 1 show that the solvent TnBP is removed from the plasma chiefly by extraction along with the castor oil, step No. 5 (sample No. 3). 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 (Table 2 and Ref. [23]). For the determination of Triton X-100 the time-consuming, but very sensitive method had to be used in this case. It includes complicated pre-treatment of the sample [23]. The same method has been used for determining the Triton X-100 content in the final product. As can be seen in Table 6, no detectable level of Triton X-100 was found in any of the batches under investigation.

The RP-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 [23]. Therefore they cannot be used for this purpose.

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, cf., the scheme shown in Table 7. For this purpose the
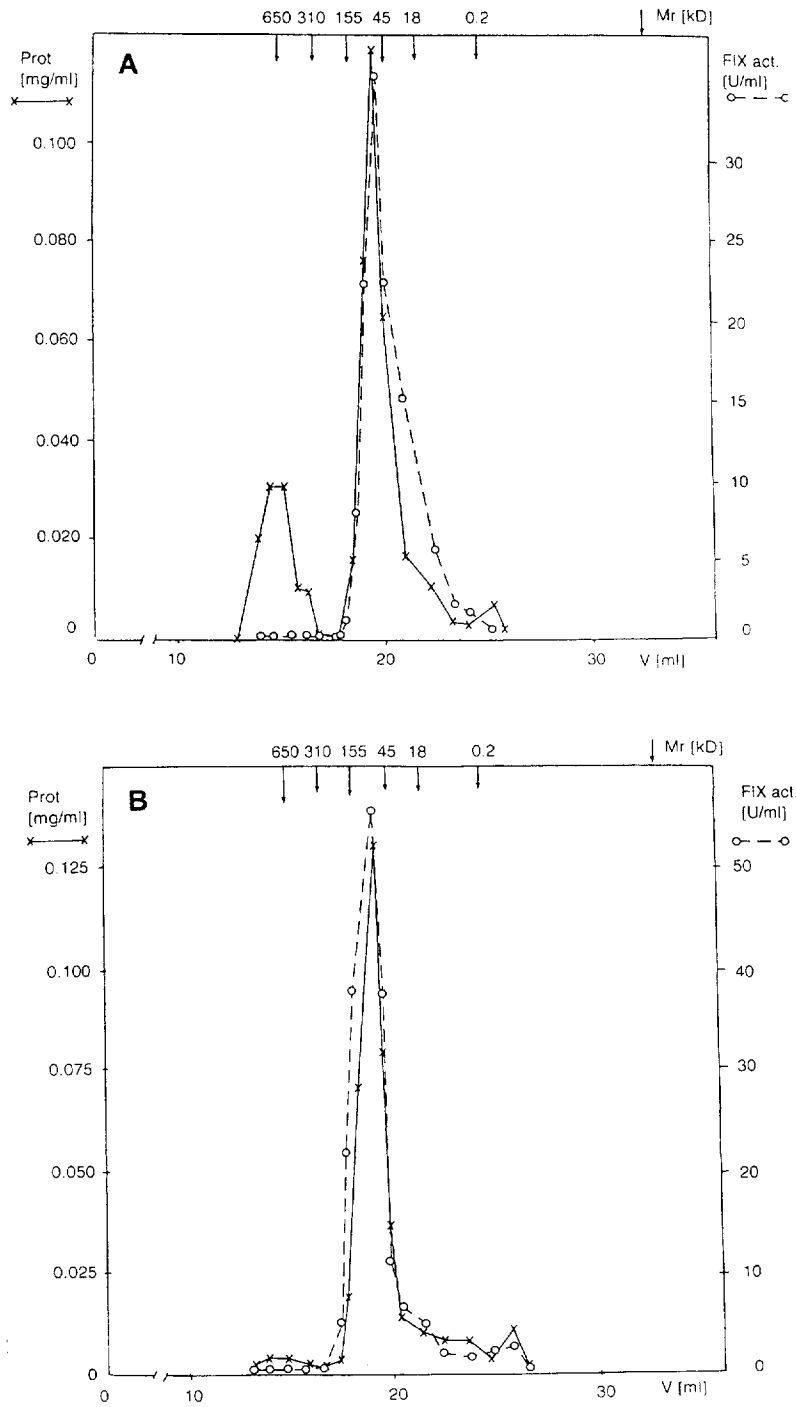
Fig. 6. Size-exclusion HPLC of factor IX preparation before (A) and after virus removal by nanofiltration (B). In both analyses a 500-μl aliquot of sample containing 100 IU of factor IX was applied to a size-exclusion HPLC column tandem containing a Zorbax GF-250 and a Zorbax GF-450 (6 μm, 250×9.4 mm I.D., Säulentechnik Knauer, Berlin, Germany). Reprinted from Ref. [20] with permission.
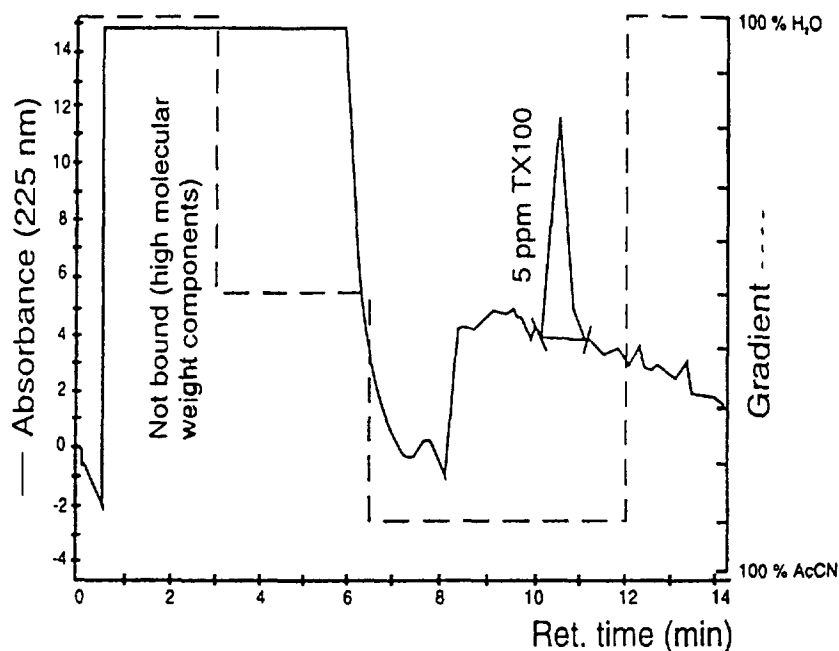
Fig. 7. Reversed-phase HPLC determination of Triton X-100 in human plasma without sample pretreatment. A 100 μl volume of plasma after virus inactiviation and removal of solvent and detergent was spiked with 5 ppm Triton X-100 and injected into a Ultrabiosep column (5 μm, 250×4.6 mm I.D.) with a pre-column (25×4.6 mm I.D., both from Bischoff, Analysentechnik, Leonberg, Germany). The gradient (acetonitrile–water) is shown by the dashed line. Reprinted from Ref. [24] with permission.

following data have to be determined [44,45]: (i) how often can the column be used, (ii) in which regeneration steps is the detergent removed and (iii) what causes the decline in the performance of the column 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]. As is seen in Table 7, the chief amount of Triton X-100 is removed in the step with 50–75% EtOH. The results obtained in the large scale pro-

Table 6
Determination of Triton X-100 in different batches of Octaplas final product

Octaplas final products batch No.	Triton X-100 (μg/ml)
548127950	<5
603007950	<5
603006950	<5
548126950	<5
600309950	<5
603008950	<5

cesses agree with those from the small scale ones. After 60 cycles in the laboratory, the concentrations of Triton X-100 in the plasma began to increase with every new cycle. The same investigations are carried out with the column used for the removal of Triton X-100 from IgG products (not shown here). This points to a slow decrease in the binding capacity of the column. For safety reasons it was therefore decided to replace the column in the production process of virus-inactivated plasma after usage for 40 cycles.

6. Products from hydrolysis (leachables) of the column materials

6.1. 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

Table 7
Determination of Triton X-100 in human plasma during production of Octaplas (sample 1, 2, 4 and 5) and in different eluents during column regeneration

Sample	Triton X-100 slow method (ppm)	Triton X-100 fast method (ppm)
Sample 1 (plasmapool prior to Triton X-100 addition)	0.1	
Sample 2 (after addition of S/D-reagents)	8 769	
Sample 4 (after oil extraction)	8 070	
Sample 5 (after Triton X-100 removal)	0.2	
Water A	0.5	<1.0
Water M	0.3	1.3
Water E	0.3	1.3
25% Ethanol A	<0.1	1.6
25% Ethanol M	<0.1	1.5
25% Ethanol E	<0.1	<1.0
50% Ethanol A	<2.0	2.1
50% Ethanol M	<3.0	<1.0
50% Ethanol E	21 380	23 400
75% Ethanol A	29 640	20 060
75% Ethanol M	10 020	10 760
75% Ethanol E	5 610	5 850
93.6% Ethanol A	18	36.2
93.6% Ethanol M	66	51.6
93.6% Ethanol E	4	6.4
2-Propanol A	1.4	6.7
2-Propanol M	0.3	<1.0
2-Propanol E	0.3	<1.0

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 [44–48].

In this section the problem of leachables from the column supports is given special attention. Two

types of chromatographic materials were investigated, Bondapak C_{18}, a silica-based, reversed-phase support for solid-phase extraction of Triton X-100 from plasma and from human IgG concentrates [20,21,23,26] and Toyopearl DEAE, a synthetic polymer-based anion-exchange support, used for the purification of FVIII from human plasma [19,34,35,42].

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 main reasons for leakage from chromatographic materials are partial matrix hydrolysis, hydrolysis of the bonds where the ligand is coupled to

the matrix, dissociation of non-covalently bound ligands, partial hydrolysis of the ligand molecule and degradation of covalent bonds due to redox reactions or nucleophilic attacks.

The leakage from chromatographic materials is influenced by outside factors, such as changes in pH and ionic strength, changes in temperature and pressure, influence of different solutes and influence of biological material (enzymatic hydrolysis of polysaccharide material, e.g., dextran-based sorbents, cellulose fibres).

The acceptable level of leachables depends on the toxicological and dangers of the respective compounds. Usually the acceptable concentrations lie in the range of 1 to 100 ng per mg of biologicals. An amount of about 10 ng per mg is usually considered as an average value [47,49].

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.

6.2. Experimental strategies for the determination of leachables

As there are still no generally agreed 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 and 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].

6.2.1. 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 [47,48].

These experiments are always carried out by the manufacturers of the resin. They provide a general picture about the stability of the resin. The data are listed in the so-called drug master files. Older data only present the quantities of extracted organic carbon. More recent data provide information about chemical composition and amounts of the leachables, which have been hydrolysed under the given conditions. These data help to identify possible products of hydrolysis and to develop methods of analysis for their determination in each process.

However, since the behaviour of every resin depends on the actual chromatographic conditions, the producer of biologicals usually must carry out additional, individual investigations, depending on the kind of production process [47].

6.2.2. Determination of leachables in the final product

When the reversed-phase packing Bondapak C_{18} 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} dimer). The amount of C_{18} monomer and dimer was determined by GC–FID analysis in six 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 (Waters, Milford, MA, USA). As is seen in Fig. 8C, both the C_{18} monomer and the C_{18} dimer were found after spiking of non treated plasma, 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 (Fig. 8D). It is seen in Table 8 that the amount of C_{18} monomer or dimer did not exceed detection in any of the six batches under investigation.

Table 9 shows the results of the determination of leachables in the final product, the freeze-dried factor
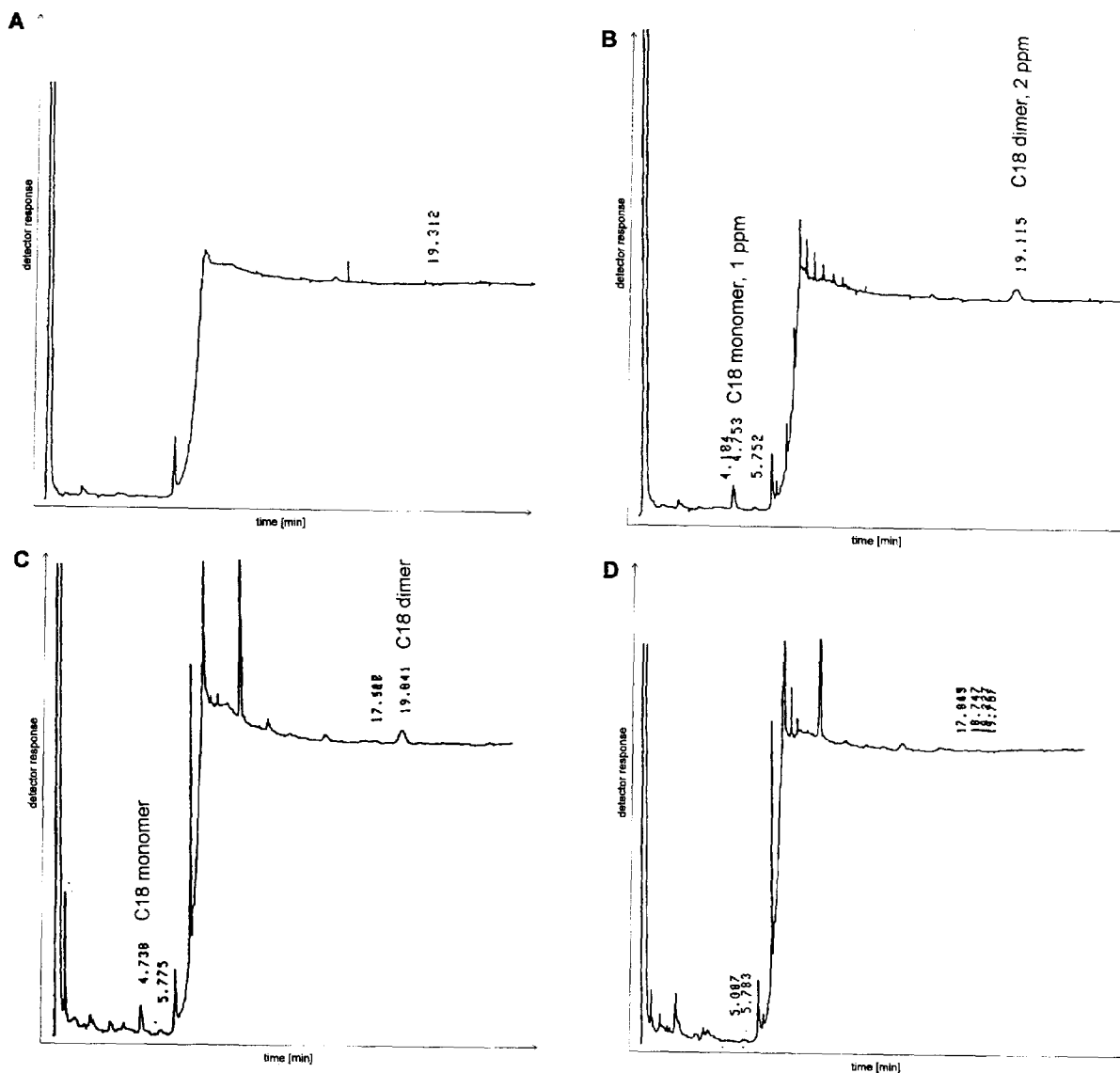
A

B

C

D

Fig. 8. Determination of possible leachables from the C_{18} resin in OCTAPLAS final containers by GC–FID. (A) shows a blank gradient, (B) a chromatogram of a standard solution containing 1 ppm C_{18} monomer and 2 ppm C_{18} dimer, respectivley. The chromatogramm (C) represents the analysis of a sample of Octaplas, final container, spiked with 1 ppm C_{18} and 2 ppm C_{18} dimer. The analysis of Octaplas, final container (D) showed, that the amount of the possible leachables of the RP-18 resin C_{18} monomer and C_{18} dimer are below the detection limit.

VIII. No detectable amounts of leachables were found in any of the samples under investigation, although their limit of detection in the lyophilized product is much lower than in an aqueous sample (cf., also Fig. 9). The possible peak with the retention time corresponding to EGDMA could not be distinguished from the base line noise under the given experimental conditions.

It can be concluded from these experiments that the amounts of leachables from the resin, which are applied to the patient along with the FVIII injection, are below the detection limit of the applied method

Table 8
Determination of leachables from reversed-phase column (C_{18} monomer and C_{18} dimer) in virus-inactivated plasma (Octaplas)

Octaplas final products batch No.	C_{18} monomer (ppm)	C_{18} dimer (ppm)
548127950	<1	<2
603007950	<1	<2
603006950	<1	<2
548126950	<1	<2
600309950	<1	<2
603008950	<1	<2

Table 9
Residual monomers DEAE, DEG, GMA and EGDMA in final containers of a FVIII concentrate

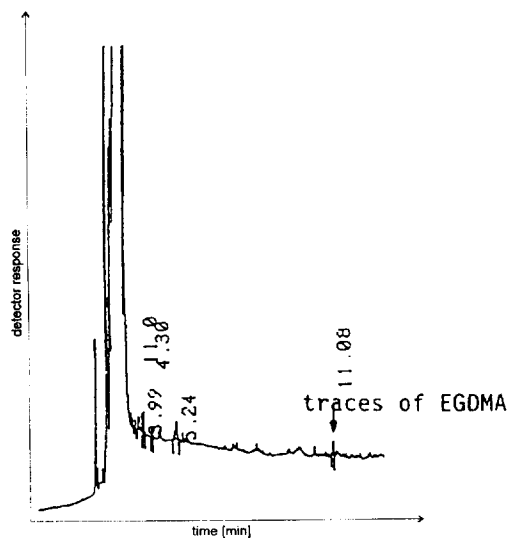| Batch No.	IU FVIII per vial	DEAE (ppm)	DEG (ppm)	GMA (ppm)	EGDMA (ppm)
42101519/U	970	<3	<5	<2	<2*
41100719/B	255	<3	<5	<2	<2
41200919/U	880	<3	<5	<2	<2*
42301719/U	1080	<3	<5	<2	<2*
41901419/U	1060	<3	<5	<2	<2*
41201019/U	1060	<3	<5	<2	<2*
41301219/U	1020	<3	<5	<2	<2*
42301619/U	1090	<3	<5	<2	<2
41601319/U	850	<3	<5	<2	<2

Fig. 9. Determination of traces of EGDMA by GC–FID in sample 41901419/U containing 1060 units of factor VIII per vial.

(see Table 9). However, if the column is stored in distilled water, diluted hydrochlorid acid or in 25% ethanol for longer periods of time, a certain loss of DEAE ligands is possible as well as hydrolysis of components from the matrix (see Table 10).

6.2.3. 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 reequilibration [45,47]. For this purpose all these steps are simulated. The first step in simulation is the development of an adequate scale-down protocol, in which all steps are taken into account. The column is first packed with unused resin. Subsequently samples are taken after each step that is carried out, and they are analysed for products of hydrolysis (leachables). The number of simulated cycles in the laboratory should exceed the expected number of cycles in the production by at least 10%. Usually between 5 and 20 additional cycles are run in the laboratory in such experiments, in order to observe the behaviour of the "ageing" gel. The behaviour of the column is not only investigated with regard to leachables, but also to capacity and separation performance.

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 static experiments show the behaviour of the chromatographic supports

Table 10
Leachables in the resin Toyopearl 650 M after treatment with different solutes for 150 h

Solute	DEAE (mg/ml)	DEG (mg/ml)	GMA (mg/ml)	EGDMA (mg/ml)
25% Ethanol	10	<20	<5	<5
H_2O	13	<20	<5	<5
1 M NaCl	<10	<20	<5	<5
0.1 M HCl	14	<20	<5	<5
0.5 M NaOH	<10	<20	<5	<5

under extreme conditions, concerning the mobile phase and the solutions used for sanitization and regeneration. 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 [47,48].

The investigation of leachables of the Toyopearl DEAE support (TosoHaas, Stuttgart, Germany), an anion-exchange material on a polymer basis 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 [19,34,35,39].

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 use 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. The protocol is shown in Fig. 10. The parameters chosen from the protocol and the corresponding results of the static experiments for leachables from the chromatographic support Toyopearl DEAE are shown in Table 10. 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 (cf., Table 9).

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

Step no.	Volume used [ml]	description	solution
1	300	wash and storage	25% EtOH
2	300	wash	H_2O
3	300	sanitisation	0.5 M NaOH
4	300	wash	buffer A or H_2O
5	300	sample-application	sample
6	300	wash	buffer A
7	270	wash	buffer B
8	37	elution	buffer C
9	210	wash	buffer D
10	300	wash	H_2O

Fig. 10. Schematic presentation of down-scaling protocol for DEAE-chromatography, applied in factor VIII isolation process (cf., Ref. [35]).

the on-column experiments is carried out for much shorter periods of time compared with the static experiments.

7. 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 [26,50].

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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