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Extraction of Triton X-100 and its determination in virus-inactivated human plasma by the solvent–detergent method

Ales Strancar

BIA d.o.o., Adamiceva 4, 61000 Ljubljana (Slovenia)

Peter Raspor

Biotechnical Faculty, Food Science and Technology Department, Chair of Biotechnology, Jamnikarjeva 101, 61000 Ljubljana (Slovenia)

Horst Schwinn, Raimund Schütz and Djuro Josic*

Octapharma Produktionsgesellschaft mbH, Oberlaaer Strasse 235, A-1100 Vienna (Austria)

ABSTRACT

For inactivation of lipid-enveloped viruses during the production of fresh frozen and lyophilized human plasma, the solvent–detergent method was applied. In this process, the solvent tri-*n*-butyl phosphate is removed by extraction with castor oil. The removal of the non-ionic detergent Triton X-100 is performed by solid-phase extraction using reversed-phase supports. For this purpose, different polymer- and silica-based supports were tested. The highest capacity for Triton X-100 was achieved with C_{18} silica gels. These supports can bind more than 0.1 ml of Triton X-100 per ml of support. None of the proteins, *e.g.*, clotting factors, bind to the support and therefore they pass through the column and their biological activity is hardly affected. The determination of detergent during the production process was also studied. The application of special columns allowing direct sample injection was introduced. This is a simple method for the rapid in-process determination of Triton X-100 in human plasma by reversed-phase chromatography under isocratic conditions. Using the method developed here, less than 1.0 ppm of Triton X-100 can be detected in less than 12 min without any sample pretreatment.

INTRODUCTION

Virus inactivation plays a key role in the production of medicaments made from pooled human plasma. The most dangerous viruses that can contaminate the plasma and the products made from plasma, such as HIV, hepatitis B and hepatitis C (HBV and HCV), are lipid-enveloped. These viruses can be inactivated by the solvent–detergent (S/D) procedure developed by Horowitz *et al.* [1]. It requires treatment of the protein solution with a combination of mild detergents such as Triton X-100, Tween 80 and other, mostly non-ionic, surfactants. A solvent, usually tri-*n*-butyl phosphate (TNBP), is also applied. The treatment takes several hours and destroys the lipid envelope of the viruses, whereas the biologically active plasma proteins remain

* Corresponding author.

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unaffected. As regards the plasma proteins which are purified chromatographically, such as the clotting factors VIII and IX [2,3] and α_1-antitrypsin [4], the solvent and the non-ionic detergents can be removed in steps involving anion-exchange or affinity chromatography. However, as far as the other products are concerned, *e.g.*, the pooled, virus-inactivated plasma or preparations of immunoglobulin G, the production scheme does not provide for such chromatographic steps. In these cases the solvent is removed by oil extraction and the detergent is removed by subsequent solid-phase extraction with a reversed-phase support. The material most often used for solid-phase extraction has so far been octadecylsilica gel, which binds up to 13% (v/v) Triton X-100. None of the other proteins, *e.g.*, clotting factors, bind to the support and therefore they pass through the column and their biological activity is hardly affected. All important plasma proteins retain their biological activity, even after the removal of the detergent [1,5].

The removal of the detergent is a critical step in the production process, especially in the case of virus-inactivated plasma. Solid-phase extraction has to be carried out at pH >7.0. At this level, however, the chemical stability of the silica gel is no longer guaranteed and consequently the column used in the production process has to be constantly monitored for its efficiency and has to be exchanged fairly often. Hence a rapid and effective in-process analysis for monitoring the residual Triton X-100 concentration in plasma had to be developed.

This paper deals with the solid-phase extraction of Triton X-100 from plasma and the in-process determination of Triton X-100.

EXPERIMENTAL

Plasma products and chemicals

Human plasma was obtained from Octapharma (Vienna, Austria). Detergents and other chemicals were purchased from Merck (Darmstadt, Germany), Sigma (Munich, Germany) or Serva (Heidelberg, Germany).

Chromatographic equipment

A BioPilot chromatographic system (Pharmacia–LKB, Vienna, Austria) was used for preparative experiments. The HPLC system, which is controlled by a microprocessor, consists of two pumps, a mixer, an injection valve and a UV spectrophotometer (Knauer, Berlin, Germany). A Frac 100 fraction collector was obtained from Pharmacia–LKB.

Materials

All preparative and semi-preparative glass columns were purchased from Pharmacia. The C_{18} cartridges for solid-phase extraction of Triton X-100 were obtained from Millipore–Waters (Vienna, Austria).

For solid-phase extraction of Triton X-100 the following reversed-phase silica gels were used: Prep C_{18}, particle size 55–105 μm, pore size 300 Å (Millipore–Waters), Eurospher Bioselect C_8 and C_{18}, particle size 60 μm, pore size 300 Å (Eurochrom, Berlin, Germany), Kromasil C_{18}, particle size 10 μm, pore size 100 Å (Eka Nobel, Surte, Sweden) and Ultrasep C_8 and C_{18}, particle size 40 μm, pore size 300 Å (Bischoff Analysentechnik, Leonberg, Germany). Of the available polymer-based supports the following materials were used: PI gel, particle size 50 μm, pore size 50 Å (Polymer Labs., Church Stretton, UK), Bio-Gel PRP, particle size 170–10 μm, pore size 300 Å (Bio-Rad, Vienna), Eurochrom Poly C_{18}, particle size 100–300 μm, pore size 300 Å (Eurochrom), TSK gel OD-4PW, particle size 40 μm, pore size 1000 Å (Tosohaas, Stuttgart, Germany) and MP-1, reversed-phase polymer, particle size 10 μm, pore size 200 Å (Interaction Chemicals, Mountain View, CA, USA).

Chromatographic methods

The determination of Triton X-100 in plasma products was based on solid-phase extraction with a C_{18} cartridge for sample preparation, with subsequent reversed-phase HPLC on a C_8 or C_{18} column, as described by Horowitz *et al.* [1].

A C_{18} cartridge was first rinsed with 5 ml of 2-propanol and the latter was then removed by rinsing three times with 5 ml of distilled water. In a subsequent step, a total of 5 ml of sample was applied in fractions of 0.1 ml each. The

cartridge was rinsed with 5 ml of water and 5 ml of 2-propanol–water (50:50, v/v). The Triton X-100 was eluted with 5 ml of 2-propanol into a pointed glass tube. The 2-propanol was then removed by creating an airstream in the tube. The dried sample was dissolved in 100 or 500 μl of water.

The determination of Triton X-100 was effected by reversed-phase chromatography with a gradient consisting of either water–2-propanol or water–acetonitrile (from 0% to 100% of organic component in 45 min). The sample volume injected was 200 μl. For this a Hypersil C_8 column (120 × 4.0 mm I.D.) was used, particle size 10 μm, pore size 60 Å (Säulentechnik Knauer).

In an isocratic run for the determination of Triton X-100, 50 μl of sample from a total of 100 μl of concentrated eluate (corresponding to 2.5 ml of plasma sample) were injected and eluted with methanol–water (90:10, v/v). The column used for this experiment was Hypersil C_8 (120 × 4.0 mm I.D. or 250 × 4.0 mm I.D.) (Säulentechnik Knauer).

For the in-process determination of Triton X-100 a Ultrabiosep column (250 × 4.6 mm I.D.) was used, particle size 10 μl, with a precolumn (Bischoff Analysentechnik, Leonberg, Germany). The column was first rinsed with water and subsequently with acetonitrile–water (50:50, v/v). Elution was carried out with acetonitrile–water (90:10, v/v). Other chromatographic parameters are given in the figure captions.

Other analytical methods

The determination of clotting factors and immunochemical determination of plasma proteins have been described previously [1,3,5]. The protein content of the samples was determined by using the method of Lowry *et al.* [6]. The remaining amount of TNBP was determined by gas chromatography, as described previously [1].

Virus inactivation

Human plasma was virus-inactivated by treatment with 1% (w/v) Triton X-100 and 1% (w/v) TNBP at 30°C for 4 h. Subsequently, the solvent was extracted with 5% (v/v) castor oil and the removal of the detergent was carried out out by solid-phase extraction with octadecylsilica gel or other reversed-phase supports. At least 10 kg of C_{18} silica gel has to be applied for 100 kg of plasma, containing 1 kg of Triton X-100 after virus inactivation. The column was subsequently rinsed with water and Triton X-100 was eluted with ethanol or 2-propanol. The procedure for virus inactivation has been described in detail previously [1,5].

RESULTS AND DISCUSSION

Solid-phase extraction of Triton-X-100 from plasma

The treatment of human plasma with solvent and detergent, in this case 1% each of Triton X-100 and TNBP, inactivates the lipid-coated viruses within the first hour. For safety, the treatment is extended to 4 h at 30°C [1]. In the production of plasma proteins, *e.g.*, of the clotting factors VIII and IX, the virus-inactivating reagents are removed in the isolation process, usually during anion-exchange chromatography or heparin affinity chromatography [2,3]. However, chromatographic methods are not used in the production of human virus-inactivated plasma and a number of other products such as immunoglobulin G. In these production processes the solvent TNBP is removed by extraction with castor oil. The non-ionic detergent Triton X-100 cannot be removed in this step. Consequently, Triton X-100 is extracted in the next step, by solid-phase extraction with a reversed-phase support. This step is particularly critical in terms of capacity, effectiveness and stability of the support. Several types of reversed-phase materials were tested in order to find the most efficient support for solid-phase extraction. In most instances supports with octyl (C_8) or octadecyl (C_{18}) groups, covalently bound to silica gel or to a polymer matrix, are used.

Fig. 1 shows the behaviour of different reversed-phase materials in a model experiment with a 1% Triton X-100 solution in phosphate-buffered saline (PBS). It can be seen that Triton X-100 binds to all the supports. The supports based on a polymer usually have a 2–8 times lower capacity than the supports based on silica

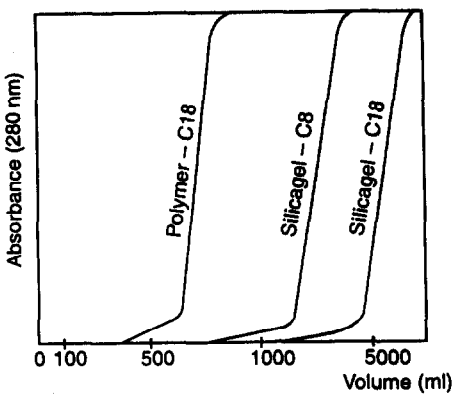

Fig. 1. Removal of Triton X-100 from human plasma by solid-phase extraction using different reversed-phase supports. Model experiments with 1% (v/v) Triton X-100 in PBS. A 1% (v/v) solution of Triton X-100 in PBS was pumped through the column packed with different reversed-phase supports: Eurochrom Poly C_{18} (polymer-C_{18}), Eurochrom Bioselect C_8 (silica gel-C_8) and Prep C_{18} (silica gel-C_{18}). The Triton X-100 concentration was determined by measuring the absorbance at 280 nm. Chromatographic conditions: column, 190 × 26 mm I.D. (100-ml bed volume); flow-rate, 15 ml/min; pressure, maximum 5 bar; room temperature. The experiments were performed on a BioPilot chromatographic system.

gel. The capacity of octadecylsilica gel was slightly higher than that of octylsilica gel. The Triton X-100 breakthrough observed in all the experiments is not steep. As shown in Fig. 1, a sigmoidal increase in absorbance is seen at 280 nm. Parallel experiments with human plasma, to which 1% Triton X-100 was added, gave similar results. Triton X-100 binds to all supports, the supports based on silica gel again showing a higher capacity than those based on a polymer. The results are given in Table I. The broad ranges in Table I occur because the capacity of the support for Triton X-100 also depends on the plasma quality. This means that plasma with a higher lipid content allows a lower binding capacity than lipid-rich plasma. In contrast, when plasma with constant quality was used, the silica-based C_{18} support has the highest capacity, followed by the C_8 silica-based support. The support Prep C_{18} is currently used in our production process. With a capacity of 0.09–0.13 ml of Triton X-100 per ml of support, Prep C_{18} is the best among the commercially available supports based on silica gel (*cf.*, Table I).

TABLE I

SUITABILITY OF SOME SILICA- AND POLYMER-BASED REVERSED-PHASE SUPPORTS FOR SOLID-PHASE EXTRACTION OF TRITON X-100 FROM VIRUS-INACTIVATED HUMAN PLASMA

More data for Prep C_{18} silica gel and methods for determination of plasma proteins are given in Ref. 5.

Support	Capacity (ml Triton X-100 per ml)	Residual activity in human plasma after virus inactivation and solid-phase extraction (%)									Total protein (%)
		Factor II	Factor V	Factor VII	Factor VIII	Factor IX	Factor XI	Protein C	Protein S	Antithrombin III	
Prep C_{18} silica gel	0.09–0.13	90–100	85–100	90–100	70–95	90–100	80–90	60–90	50–80	90–100	*ca.* 100
Eurospher Bioselect C_8 silica gel	0.06–0.08	*ca.* 90[a]	80–100	90–100	80–90	90–100	*ca.* 90	70–80	50–70	90–100	*ca.* 100
Eurospher Bioselect C_{18} silica gel	0.09–0.11	*ca.* 90	80–100	*ca.* 95	70–90	*ca.* 100	*ca.* 95	60–90	50–70	90–100	*ca.* 100
Eurochrom Poly C_{18} polymer	0.03–0.06	80–100	*ca.* 90	*ca.* 90	60–90	*ca.* 100	*ca.* 90	50–70	50–70	*ca.* 95	*ca.* 100
TSK gel OD-4PW polymer[b]	*ca.* 0.05	*ca.* 80	*ca.* 95	*ca.* 90	*ca.* 70	*ca.* 100	*ca.* 90	*ca.* 60	*ca.* 55	*ca.* 100	*ca.* 100

[a] The accuracy of the method for determination of clotting factor activity is ±20%.
[b] Only one test was done for the TSK gel OD-4PW support.

It is well known that Triton X-100 and other detergents of the Triton group bind to silica gel supports [7]. The interaction between reversed-phase silica gel and Triton X-100 is apparently stronger than the interaction between protein and detergent, which is why Triton X-100 can be separated from the plasma proteins by solid-phase extraction. The fact that the silica gel matrix itself binds Triton X-100 [7] may also be responsible for the much higher capacity of reversed-phase supports based on silica gel than the corresponding polymer-based supports. There are strong indications that interaction with Triton X-100 is not caused by the functional alkyl groups on the surface of the support alone, but that the surface of the support itself is also involved. The silanol groups probably play an important part in this. However, the lower capacity of C_8 compared with C_{18} silica gels indicates that the hydrophobic functional groups are the cause of the interaction, at least to some extent. The binding of Triton X-100 probably occurs in several layers on the surface of the reversed-phase silica gel. Such a mechanism would explain the extraordinarily high capacity of such supports for Triton X-100, which may be as high as 13% of the support volume (see Fig. 1 and Table I).

All supports listed in Table I, especially C_{18} silica gel, have a much higher affinity to Triton X-100 than to plasma proteins. Therefore, all the important plasma proteins remain unaffected by this kind of solid-phase extraction, retaining their activity almost completely [1,5]. The activities of some of the most important plasma proteins after virus inactivation and solvent–detergent removal are also given in Table I.

Despite the above-mentioned positive characteristics of silica gel, it is not an ideal medium for separating Triton X-100 from virus-inactivated human plasma. As the support is not stable at pH >7.5, sanitation of the column with 0.5 *M* sodium hydroxide solution is impossible, and consequently the removal of pyrogenic substances that the column may contain at this point cannot be achieved. Also, the lifetime of the column cannot be predicted with certainty, which necessitates careful in-process analysis (see below). However, in this kind of production process, polymer-based supports are not a practical alternative to C_{18} silica gels at present, as their capacity is up to eight times lower, and they are up to ten times more expensive.

Determination of Triton X-100 by reversed-phase HPLC

Triton X-100 can easily be separated from other plasma components by reversed-phase HPLC with a C_8 or C_{18} support, as shown in Fig. 2. A problem arises from the fact that direct UV detection of Triton X-100 in plasma is impossible at concentrations between 0.1 and 5 ppm, which is the concentration usually found in plasma after removal of detergent by solid-phase extraction. Triton X-100 has an indistinct absorption maximum at 280 nm. However, the sensitivity of detection at this wavelength is far too weak. For this reason, detection has to be carried out at lower wavelengths, usually between 210 and 220 nm (absorption spectrum not shown). As many other plasma components absorb in this range, interferences may occur. Therefore, the method requires a complicated sample treatment, involving pre-extraction from 5 ml of plasma (see Experimental).

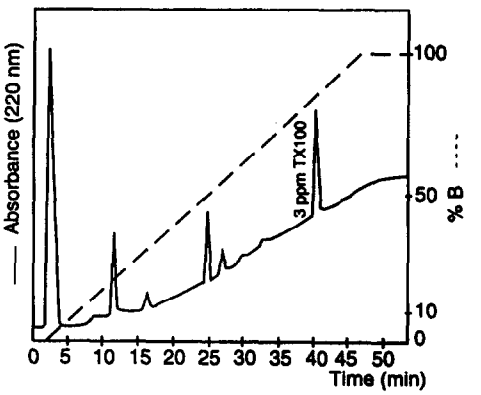

Fig. 2. Reversed-phase HPLC determination of residual Triton X-100 in human plasma using gradient elution with sample pretreatment. A 200-μl volume of sample after sample treatment (see Experimental; 40% of material extracted from 5 ml of plasma) was injected into the column. Chromatographic conditions: column, Hypersil C_8 (120 × 4.0 mm I.D.); flow-rate, 1.0 ml/min; pressure, 60–90 bar; room temperature. Solvent A = water and solvent B = acetonitrile, with a gradient as shown by the dashed line. A Knauer HPLC system was employed.

The sample pretreatment has two objectives: to remove a 10–20-fold concentration of Triton X-100 from 5 ml of sample in the sample preparation cartridge, and to remove less hydrophobic, interfering components, *e.g.*, proteins, by rinsing the cartridge with a 2-propanol–water (50:50, v/v).

The time required for the determination of Triton X-100 with gradient elution can be decreased from 45 to about 8 min by a simple isocratic method, as shown in Fig. 3. Under the conditions used to obtain Fig. 3, Triton X-100 could be separated from other components, thereby preventing distortion of the results. This can happen through components of the plasma which co-elute with Triton X-100, forming a single peak. A control experiment with a plasma sample that did not contain Triton X-100 showed that no distortion occurred.

The detection limit for Triton X-100 is high, and detection in the UV range between 210 and 220 nm is both relatively insensitive and non-specific. Therefore, the methods mentioned above require complicated sample pretreatment. When supports made of silica gel are used to remove Triton X-100 from the plasma by solid-phase extraction, the average column lifetime in the production process is 30–40 runs. When the residual content of Triton X-100 in the sample after solid-phase extraction increases steadily with each new batch, reaching 3–4 ppm, the column has to be replaced with a new one. If a batch volume of 60 l is chosen, a column with 8 l of C_{18} support is needed. If the amount of plasma undergoing virus inactivation is increased to 200 l, the volume of the column has to be about 27 l in order to maintain identical working conditions. The capacity of the support is not fully used under such conditions for safety reasons. With 60 l batches, the amount of silica gel is not a large cost factor. However, if the process is scaled up to 200 l, the economic aspects of providing the support have to be taken into account. These also include the considerable cost of the disposal of used silica gel. Therefore, it is imperative to exploit fully both the lifetime and capacity of the material. With virus-inactivated plasma, a column with about 22 l of C_{18} support has sufficient capacity to purify 200 l (see Table I). Under normal conditions the column can be used at least 40 times. However, these conditions require a rapid in-process analysis. An increase in the amount of Triton X-100 in the plasma has to be detected at once, in order to allow immediate replacement of the column to avoid a failure of the production process.

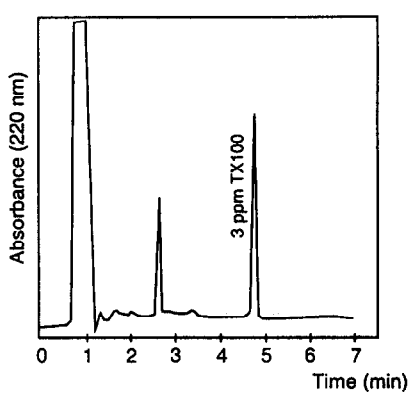

Fig. 3. Reversed-phase HPLC determination of Triton X-100 in human plasma using isocratic elution with sample pretreatment. A 50-μl volume of sample after sample pretreatment (see Experimental; 50% of material extracted from 5 ml of plasma) was injected into the column and eluted isocratically with methanol–water (90:10, v/v). Chromatographic conditions: column, Hypersil C_8 (250 × 4.0 mm I.D.); flow-rate, 1.0 ml/min; pressure, 90–100 bar; room temperature. A Knauer HPLC system was employed.

The direct detection of Triton X-100 in the plasma at concentrations below 20 ppm requires sample pretreatment, if carried out "conventionally" by RP-HPLC. Direct injection of the plasma sample into the RP column, followed by either isocratic elution or separation with a gradient (Figs. 2 and 3) does not yield satisfactory results unless concentration of Triton X-100 in the sample is achieved and interfering substances are removed.

With direct injection without sample pretreatment, the interfering substances can have the same retention times as Triton X-100 and distort the results (chromatograms from experiments carried out under such conditions are not shown). These substances can bind irreversibly to the column, reducing its lifetime. The problem can be solved, however, by using a new type of support which carries a kind of hydrophilic network on its surface, through which only small

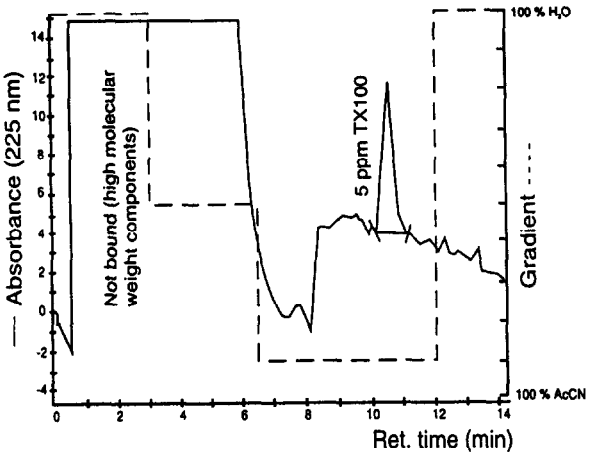

Fig. 4. Reversed-phase HPLC determination of Triton X-100 in human plasma without sample pretreatment. A 100-μl volume of plasma after virus inactivation and removal of solvent and detergent was injected into a Ultrabiosep column with precolumn. The column was rinsed with 3 ml water and subsequently with 3.5 ml of acetonitrile–water (50:50, v/v) to remove non-bound and weakly bound components. Triton X-100 was eluted with acetonitrile–water (90:10, v/v). Chromatographic conditions: column, 250 × 4.6 mm I.D. with a 25 × 4.6 mm I.D. precolumn; flow-rate, 1 ml/min; pressure, 60 bar; room temperature. The gradient is shown by the dashed line. A Knauer HPLC system was used. Separation of 5 ppm of Triton X-100.

molecules can pass. Large molecules never reach the hydrophobic chains, which are bound directly to the surface of the matrix. The mechanism works like a kind of molecular sieve. The small Triton X-100 molecules with molecular mass between 7000 and 8000 penetrate the net and bind to the alkyl groups on the surface. However, the hydrophobic components from the plasma, *e.g.*, lipoproteins, are too large to penetrate and flow through the column without retention. Such a column allows the determination of Triton X-100 in less than 10 min without sample pretreatment, as shown in Fig. 4. The calibration graph for Triton X-100 determined by this method is linear in the range between 0 and 35 ppm and can be described by the equation $y = 20.9x + 40$, where x = Triton X-100 concentration (ppm) and y = peak area (integration units). The correlation coefficient for the equation determined from twelve runs was 0.995.

The sensitivity of analysis is about 1 ppm, which is 5–10 times lower than that of the methods that need sample treatment as mentioned previously. However, with this sensitivity it is possible to achieve rapid analyses. If the removal of Triton X-100 during solid-phase extraction is found to be inadequate, leaving residual amounts higher than 3 ppm, appropriate steps can be taken to correct the production process, *e.g.*, replacing the column for solid-phase extraction.

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