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Journal of Chromatography A, 946 (2002) 163–168

JOURNAL OF CHROMATOGRAPHY A

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Determination of Triton X-100 in plasma-derived coagulation factor VIII and factor IX products by reversed-phase high-performance liquid chromatography

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Received 8 June 2001; received in revised form 23 November 2001; accepted 23 November 2001

Abstract

Plasma protein pools are often virus-inactivated by the solvent–detergent method, using tri-*n*-butyl phosphate and Triton X-100, followed by removal and determination of these compounds. We used reversed-phase high-performance liquid chromatography for the determination of Triton X-100 in coagulation factor VIII and factor IX products, Octonativ-M and Nanotiv, respectively (Pharmacia, Stockholm, Sweden). The chromatographic system included a C_{18} silica column and a linear acetonitrile gradient. The advantage of this method is the low detection limit (0.3 μg/ml) combined with detection at 280 nm, which gives a more stable baseline and has less interference from other compounds. As compared to other methods, where shorter wavelengths are used.

Keywords: Pharmaceutical analysis; Triton X-100; Factor VIII; Factor IX; Nanotiv; Octonativ-M; Surfactants

1. Introduction

Virus inactivation is a crucial step in the production of protein drugs derived from pooled human plasma. Pathogenic viruses that can contaminate plasma pools include human immunodeficiency virus (HIV), hepatitis B and hepatitis C, all of which are lipid-enveloped. These types of viruses can be inactivated by the solvent–detergent (S/D) procedure, developed by Horowitz et al., which includes treatment of the protein by an ionic detergent, e.g. sodium cholate, or more often, a nonionic detergent, e.g. from the Triton X-series or Tween 80, together with an organic solvent, often tri-*n*-butyl phosphate (TNBP) [1–3]. To avoid toxic reactions in vivo, added chemicals must be removed, usually by extraction and chromatography. After removal of these substances, an accurate determination must then be performed to demonstrate that the concentrations are below the specified limits. In several studies, Triton X-100 has been analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) [2,4–8]. For higher concentrations of Triton X-100 (>10 μg/ml) absorbance at 280 nm is employed for quantification [8,9], while at lower levels (down to 1 μg/ml), absorbance at 200–230 nm is used to obtain a stronger response [2,4–7]. Because many other compounds absorb at these shorter wavelengths, a sample treatment, e.g. solid-phase extraction, is usually performed before the analysis [4].

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01565-5

In this study, we used RP-HPLC for the determination of Triton X-100 in coagulation factor VIII and IX products, using an elevated temperature, high injection volume, and steep acetonitrile gradient. By this method we could detect Triton X-100 down to 0.3 μg/ml by absorbance at 280 nm, which is advantageous in avoiding interference from many other compounds.

2. Materials and methods

The commercial coagulation factor VIII product, Octonativ-M 500 IU (containing 20 mg albumin, about 50 μg factor VIII, 70 mg sodium chloride, 16 mg L-histidine, 2 mg polyethylene glycol, and 1 mg calcium chloride) and factor IX product, Nanotiv 1000 IU (containing 4 mg protein, 84 mg sodium chloride, 20 mg L-lysine monohydrochloride, and 17 mg sodium citrate) were obtained from Pharmacia, Stockholm, Sweden. Methanol (HPLC grade), and acetonitrile (Far-UV), were obtained from Labscan (Dublin, Ireland). Ethanol (99.5%) was from Kemetyl (Haninge, Sweden) and Triton X-100 (laboratory grade) was from Sigma (Munich, Germany). Acetyltryptophan and sodium caprylate (pharmaceutical quality) were obtained from Ajinomoto (Tokyo, Japan) and Napp Technologies (Lodi, NJ, USA), respectively, and tri-*n*-butyl phosphate (TNBP) (analytical-reagent grade) was from Fluka (Ronkonkoma, NY, USA).

We used an HP 1090 chromatographic system, controlled by the Chemstation software, both from Hewlett-Packard (Palo Alto, CA, USA), equipped with a Brownlee Spheri-5 RP-18 (5 μm, 80 Å, 100×4.6 mm I.D.), analytical column with a Brownlee NewGuard RP-18 precolumn (7 μm, 15×3.2 mm I.D.), from Perkin-Elmer (Norwalk, CT, USA).

For elution, the mobile phases were 60% (v/v) acetonitrile in water (mobile phase A), and 100% acetonitrile (mobile phase B). A linear gradient was used (0–2 min 0% B, 2–3 min 0–90% B, 3–4 min 90% B, 4–7 min 0% B). The injection volume was 250 μl, and flow-rate was 2.0 ml/min at 55 °C. Detection was carried out by measuring the absorbance at 280 nm.

The Octonativ-M product was reconstituted in water, spiked with Triton X-100, and diluted to a final concentration of 100 IU/ml. Prior to the analysis, the major part of the protein in the samples was precipitated by adding one volume of methanol, followed by centrifugation at 10 000 *g* for 20 min at 4 °C. Triton X-100 standard samples (1, 2, 5 and 10 μg/ml, $n=4$) were prepared with the addition of methanol in the same way, but were not centrifuged.

The Nanotiv product was treated and analyzed in the same way, with some exceptions. Because of the low protein content in Nanotiv, compared to Octonativ-M, precipitation of the protein was not performed prior to the analysis, i.e. no methanol was added to the samples or the standard samples, and a

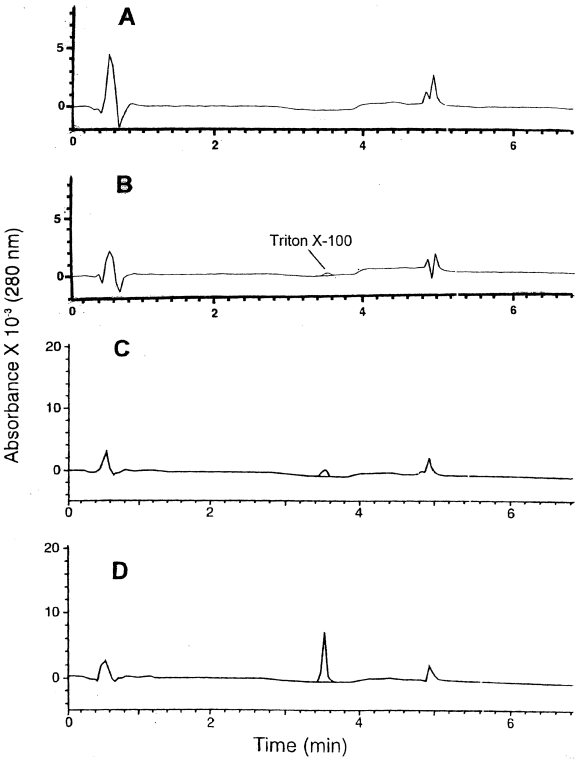

Fig. 1. Reversed-phase HPLC of Triton X-100. A Brownlee C_{18} column equilibrated with 60% (v/v) acetonitrile in water was used at a flow-rate of 2.0 ml/min and temperature of 55 °C. A linear gradient, 60–96% acetonitrile was used for 2–3 min. Injection volume was 250 μl and detection was performed by absorbance at 280 nm. Methanol was added to the samples prior to the analysis. (A) Water blank, (B) Triton X-100, 0.3 μg/ml, (C) Triton X-100, 1 μg/ml, (D) Triton X-100, 5 μg/ml. Different scales for the response axis were used in A–B respective C–D. The baseline disturbance at about 5 min is due to the gradient. See the Materials and methods section for further details.

wider range of the standard curve was also used, 2.5–100 μg/ml Triton X-100 (2.5, 5.0, 10, 50 and 100 μg/ml, $n=5$).

3. Results and discussion

The chemicals used in the virus inactivation procedure of plasma protein preparations can be removed by a variety of methods. TNBP can be extracted by soybean or castor oil [2,4], while Triton X-100 is washed away from the proteins that are bound to ion-exchange or heparin-affinity chromatography columns [10,11] or reversed-phase chromatography on a solid-phase extraction column [2,4]. Typically, about 0.1–5 μg/ml Triton X-100 remains after this step. If a person with a body mass of 70 kg were to receive 2 l of S/D-treated plasma, containing 5 μg/ml Triton X-100, the dose would be 0.14 mg/kg, which is more than two orders of magnitude lower than the reported lowest effective toxic dose on intravenous injection into mice [12]. These findings were confirmed by more recent studies of acute toxicity levels of Triton X-100 in mice and rats (unpublished results from a study at the Laboratory of Pharmacology and Toxicology (Hamburg, Germany) 1991, cited from Ref. [2]).

The concentration of Triton X-100 has been determined after S/D treatment by extracting it from the plasma protein sample by a C_{18} solid-phase extraction cartridge, followed by concentration, and final analysis on C_8 or C_{18} columns by RP-HPLC, as described by Horowitz et al. [2] and Strancar et al. [4]. In these works, as well as in other publications [5–7], a detection wavelength of 200–230 nm was used to achieve sufficient absorbance to measure the concentration of Triton X-100 down to about 1 μg/ml. Triton X-100 gives a much higher absorbance at 220 nm, compared to the response obtained at 280 nm, so that these lower wavelengths were used for quantification at levels below 10 μg/ml. Because many compounds absorb at 200–230 nm the specificity at these lower wavelengths is generally lower than at 280 nm. Therefore, detection of Triton X-100 at 280 nm with a quantification range down to about 1 μg/ml should improve the specificity and also simplify the RP-HPLC analysis.

Triton X-100 is composed of a mixture of octylphenol ethoxylates, with an average of about 9–10 ethylene oxide units per molecule. These oligomers can be separated on a reversed-phase chromatographic system to give a Gaussean distribution pattern [7]. The elution order of the oligomers has also been shown to be influenced by the nature of the alkyl-bonded support [13]. In the present work, the analytical method was optimized to achieve a single sharp peak containing the Triton X-100 oligomers.

As seen in Fig. 1, a distinct peak was obtained by detecting the absorbance at 280 nm from 1 μg/ml Triton X-100 eluting at a retention time of about 3.5

Table 1
Reversed-phase HPLC analyses of Triton X-100 in spiked Octonativ-M samples

Analysis	Linearity	Precision		Recovery			
		Sample 1 (μg/ml)	Sample 2 (μg/ml)	2.5 μg/ml	Recovery (%)	5.0 μg/ml	Recovery (%)
1	0.998	2.23	4.87	2.14	85.6	4.38	87.6
2	0.999	2.33	4.85	2.17	86.8	4.23	84.6
3	0.999	2.31	5.01	2.17	86.8	4.71	94.2
4	0.999	2.32	4.96	2.09	83.6	4.49	89.8
5	0.999	2.23	4.81	2.01	80.4	4.45	89.0
6	0.999	2.29	4.83	1.95	78.0	4.55	91.0
Mean		2.3	4.9	2.1	83.5	4.5	89.4
RSD (%)		2.0	1.6		4.4		3.6

Linearity of the standard curve (1, 2, 5 and 10 μg/ml, $n=4$) expressed as correlation coefficient (r). Recovery was measured after spiking of Octonativ-M samples with Triton X-100. Most of the protein in the samples was precipitated by methanol and removed prior to the analysis. The nominal concentrations of Sample 1 and Sample 2 were 2.5 and 5.0 μg/ml, respectively. Experimental conditions are as described in the Materials and methods section.

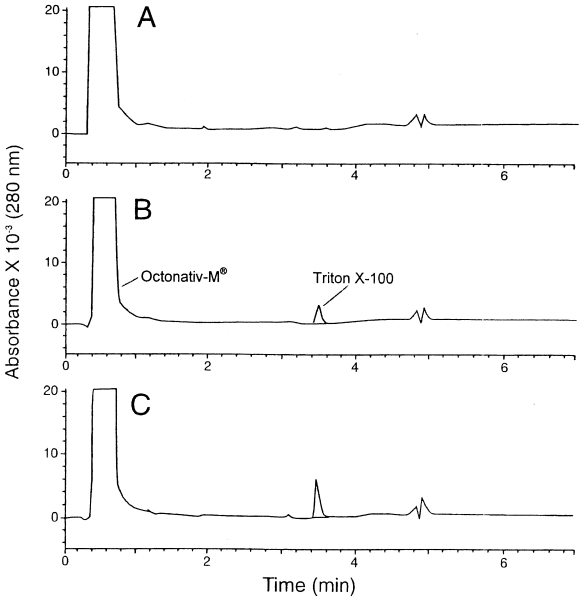

Fig. 2. Reversed-phase HPLC of Octonativ-M (factor VIII) samples, spiked with Triton X-100. (A) Octonativ-M, (B) Octonativ-M, spiked with 2.5 μg/ml Triton X-100, (C) Octonativ-M, spiked with 5 μg/ml Triton X-100. Most of the protein in the sample was precipitated by methanol and removed prior to the analysis.

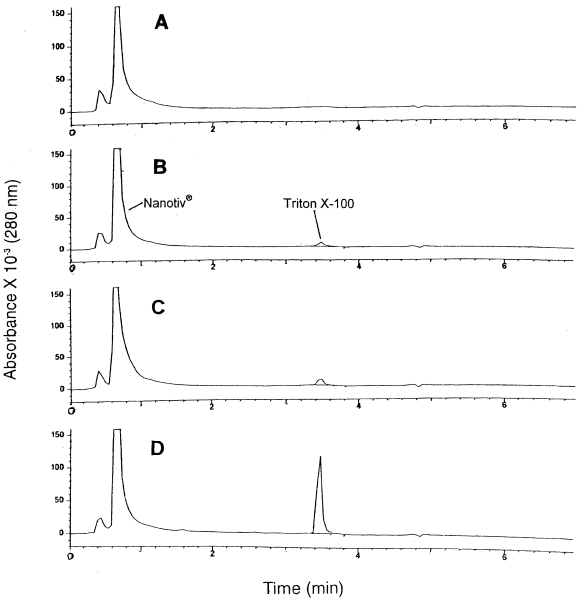

Fig. 3. Reversed-phase HPLC of Nanotiv (factor IX) samples, spiked with Triton X-100. (A) Nanotiv, (B) Nanotiv, spiked with 2.5 μg/ml Triton X-100, (C) Nanotiv, spiked with 5 μg/ml Triton X-100 and (D) Nanotiv, spiked with 50 μg/ml Triton-X-100. No sample preparation was performed prior to the analysis.

min, corresponding to approximately 80% acetonitrile. The combination of an elevated temperature, steep acetonitrile gradient and high injection volume made it possible to achieve sharp and relative high peaks, which resulted in a low detection limit (0.3 μg/ml; with a signal-to-noise ratio of 3) at 280 nm (Fig. 1). In the analysis, we chose a column temperature of 55 °C because of the improved sharpness of the Triton X-100 peak, and the reduction in the system back-pressure, allowing for a high flow-rate. According to the manufacturer, Perkin-Elmer, a maximum temperature of 70 °C is acceptable for the column. At the end of the gradient, the maximal acetonitrile concentration of 96% was maintained for 1 min to wash off strongly bound hydrophobic substances from the column.

Table 2
Reversed-phase HPLC analyses of Triton X-100 in spiked Nanotiv samples

Analysis	Linearity	Precision	Recovery					
		CS (μg/ml)	2.5 μg/ml	Recovery (%)	5.0 μg/ml	Recovery (%)	50 μg/ml	Recovery (%)
1	0.9999	39.91	2.60	104.0	4.89	97.8	50.03	100.1
2	0.9999	39.08	2.41	96.4	5.00	100.0	50.07	100.1
3	0.9997	38.10	2.70	108.0	4.83	96.6	48.33	96.7
Mean		39.0	2.6	102.8	4.9	98.1	49.5	99.0
RSD (%)		2.3		5.7		1.8		2.0

Linearity of the standard curve (2.5, 5.0, 10, 50 and 100 μg/ml, $n=5$) expressed as correlation coefficient (r). Recovery was measured after spiking of Nanotiv samples with Triton X-100. No sample preparation was performed. The control sample (CS) was a diluted process sample with a nominal concentration of 40 μg/ml.

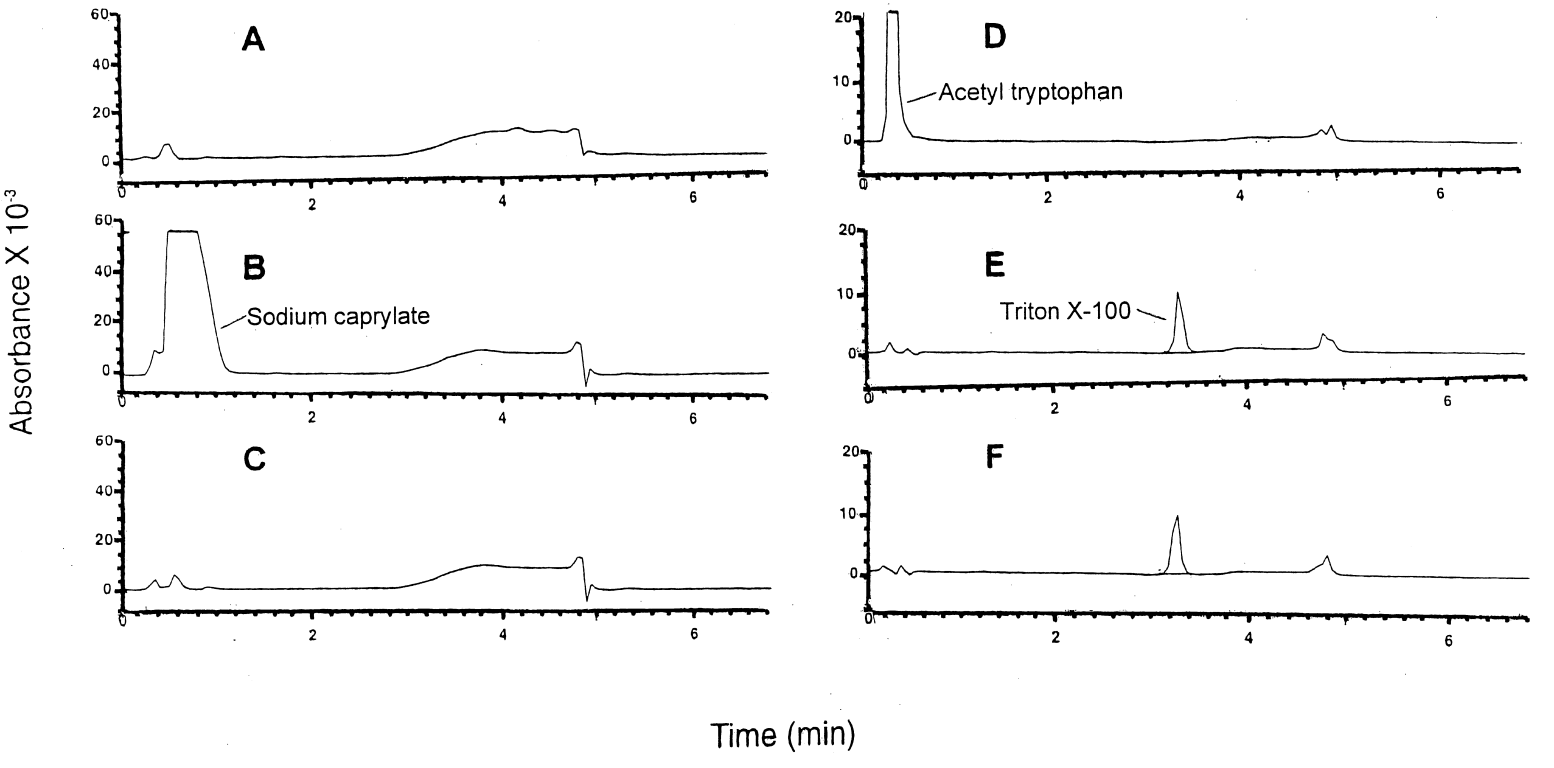

Fig. 4. Test of specificity for the reversed-phase HPLC method. (A) Water, (B) sodium caprylate 250 μg, (C) TNBP 125 μg, (D) acetyltryptophan 1 μg, (E) Triton X-100 1.2 μg, (F) Triton X-100 1.2 μg, spiked with 5 μg tri-*n*-butyl phosphate (TNBP). Injection volume was 25 μl and all samples were injected in 50% aqueous methanol, except sample C, which was injected in 50% aqueous ethanol. Detection by absorbance at 210 nm was used in chromatograms A–C and 280 nm was used for D–F. The integrated area was 60 (mAU s) for the Triton X-100 peak in both chromatogram E and F. None of the analyzed compounds, in addition to Triton X-100, shows interference at the retention time for Triton X-100.

For six separate analyses, the intermediate precision was 2.0 and 1.6% RSD at 2.5 and 5 μg/ml Triton X-100, respectively (Table 1). As shown in Figs. 2 and 3, the Triton X-100 peak is well separated from the remaining Octonativ-M, and Nanotiv proteins that both elute in, or close to, the void peak. The recoveries were 89 and 84% in the Octonativ-M sample that was spiked with 5 and 2.5 μg/ml Triton X-100, respectively (Table 1), while recovery values for Nanotiv were 98–103% for 2.5–50 μg/ml Triton X-100 (Table 2). The higher recovery for Nanotiv was likely because no addition/precipitation with methanol was performed prior to analysis of this product, and hence, no losses of Triton X-100 occurred due to the binding between Triton X-100 and protein in the precipitation step. Because the Nanotiv samples were not diluted with methanol, twice as much Triton X-100 was injected on the column as in the Octonativ-M method. In the analysis of Nanotiv, the precipitation step was omitted because this product contains a low concentration of protein, about 0.4 mg/ml, and it was also not possible to precipitate the protein with 1:1 addition of methanol. In the analysis of Nanotiv a wider Triton X-100 standard curve was used (2.5–100 μg/ml), to permit the analysis of both final product and moderately diluted process samples. No detectable level of Triton X-100 was found in the original Octonativ-M and Nanotiv samples (Figs. 2 and 3). The Triton X-100 standard curves used in the analysis of both Octonativ-M and Nanotiv (range 1–10 and 2.5–100 μg/ml Triton X-100, respectively) showed a high degree of linearity, $r>0.998$ (Tables 1 and 2). The analysis time was 7 min, which allows for rapid analyses.

In this method, the elution of Triton X-100 was specific and not interfered with the present proteins or the formulation components in Octonativ-M and Nanotiv. To further investigate the specificity, high concentration samples of TNBP, acetyl tryptophan and sodium caprylate were analyzed, using both 280 and 210 nm for detection. As shown in Fig. 4, acetyltryptophan and sodium caprylate, both used as stabilizers for some proteins in the heat-treatment for virus inactivation, elutes in the void. TNBP could not be detected at 210 nm (Fig. 4C) and consequently, Triton X-100 was spiked with TNBP with a 4-fold amount; and the Triton peak was then not affected by added TNBP so that the Triton X-100 peak gave the same integrated area and shape as that in the non-spiked Triton X-100 sample (Fig. 4D–E). These results indicate that the specificity of the elution of Triton X-100 is sufficient, at least for the two products analyzed.

Because the detection is at 280 nm, the method is limited to the analysis of Triton X-100, which has an absorbing aromatic group. Other detergents, such as the Tween type of detergents would therefore not be possible for analysis by this method. The major advantage of this method is the low detection limit (0.3 μg/ml) combined with detection at 280 nm. Compared to other methods, where shorter wavelengths are used, the detection at 280 nm gives a more stable baseline and shows less interference from other compounds. In conclusion, this RP-HPLC method is suitable for the determination of low levels of Triton X-100 in pharmaceutical final products, or in process samples of plasma protein preparations. The method may also be used in other applications.

References

[1] M.P. Piet, S. Chin, A.M. Prince, B. Brotman, A.M. Cundell, B. Horowitz, Transfusion 30 (1990) 591.
[2] B. Horowitz, R. Bonomo, A.M. Prince, S.N. Chin, B. Brotman, R.W. Shulman, Blood 79 (1992) 826.
[3] A.M. Prince, B. Horowitz, B. Brotman, Lancet i (1986) 706.
[4] A. Strancar, P. Raspor, H. Schwinn, R. Schütz, D. Josic, J. Chromatogr. A 658 (1994) 475.
[5] D. Josic, P. Schulz, L. Biesert, L. Hoffer, H. Schwinn, M. Kordis-Krapez, A. Strancar, J. Chromatogr. B 694 (1997) 253.
[6] Y. Piquet, G. Janvier, P. Selosse, C. Doutremepuich, J. Jouneau, G. Nicolle, D. Platel, G. Vezon, Vox Sang. 63 (1992) 251.
[7] K. Heinig, C. Vogt, Fresenius J. Anal. Chem. 359 (1997) 202.
[8] T. Kamiusuki, T. Monde, F. Nemoto, T. Konakahara, Y. Takahashi, J. Chromatogr. A 852 (1999) 475.
[9] K. Pardue, D. Williams, BioTechniques 14 (1993) 580.
[10] H. Schwinn, A. Smith, D. Wolter, Arzneimittelforschung 39 (1989) 1302.
[11] D. Josic, F. Bal, H. Schwinn, J. Chromatogr. 632 (1993) 1.
[12] J.W. Cornforth, P. D'Arcy Hart, R.J.W. Rees, J.A. Stock, Nature 168 (1951) 150.
[13] P. Chaimbault, C. Elfakir, M. Lafosse, J. Chromatogr. A 797 (1998) 83.