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Study to the Repeated Use of Commercial Immunoaffinity Columns

Key words: Ochratoxin A, Immunoaffinity column, Renaturation, Recovery, Clean up

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Introduction

In recent years immunoaffinity columns (IAC) containing immobilized monoclonal antibodies providing selective enrichment of analytes became a powerful analytical tool for the isolation, concentration and cleanup of mycotoxins in feed, food and body fluids (1–8). However, the commercial availability of IAC for many mycotoxins of interest is limited to date and their prizes are relatively high. In addition, the manufacturers do generally not recommend their re-use.

In two previous studies we described the successful application of a commercial IAC (Biocode) for the clean up of ochratoxin A (OA) containing extracts of human blood serum and milk, total-diet, coffee brew, beer, wine and grape-juice (7, 8). In these studies, it was outlined that the columns could be re-used up to 30 times, at least at OA sample concentrations in the ng/g-range, and apparently a crucial point for acceptable OA-recoveries of re-used IAC was the time period elapsed between various uses (8). To our knowledge no study dealing with this subject, addressed to analytical chemists, has been published, although the re-use of self-prepared IAC for aflatoxins, OA and other analytes was mentioned on various occasions (1, 2, 9, 10). The aims of this study were first to substantiate this potential critical time factor by experimental results and second to compare the repeatabilities of OA-recoveries from IAC obtained from three manufacturers.

Experimental

The method used to measure OA, such as the extraction procedure as well as all instrumental and chemical details is given in a previous publication (7); concise experimental details are given below.

[1] Retired since 31 August 1996

The ochratoxin A EASI-EXTRACT ™ columns were supplied by Biocode (Heslington, York, UK), since March, 1995 distributed by Rhône-Poulenc Diagnostics Limited (Coring-System GbR [Gernsheim/Rhine, Germany]), the OchraTest ™ by Vicam (Watertown MA, USA), represented in Switzerland by Fakola AG (Basle), and the RIDA ® ochratoxin A column by R-Biopharm (Darmstadt, Germany). The following abbreviations of the various columns were used in this study: Biocode, Vicam and Biopharm.

The chemicals to prepare the phosphate buffered saline (PBS) solution (Diagnostics N^0 1000-3) were obtained from Sigma (St. Louis, MO, USA). To the PBS solution 0.5 g/l of sodium azide was added; the solution was stored in the refrigerator. All other reagents except methanol (Mallinckrodt, Nanograde, Oryx Pharmazeutika AG, Zurich, Switzerland) were from Merck (Darmstadt, Germany) and p.a. grade, they were used without further purification. However, the reagent blanks were checked regularly. Deionized water was used throughout this study.

All columns were stored in the refrigerator as prescribed by the manufacturers and used within the given shelf-life, but not always according to their loading, washing or eluting instructions. The procedures used are summarized in table 1; they are based on one of the first protocols of Biocode. The temperature of the IAC, as well as the solutions, and solvents given in table 1 were equilibrated to ambient temperature before their use.

No additional experiments to optimize the loading and/or washing procedure (e.g. by increasing or decreasing the time of contact), the elution pattern of OA from the column, e.g. with other solvents or mixtures (having a less denaturing effect than methanol) or the renaturation process were made.

The sample extracts or standards were loaded onto the IAC (ca. 1.3 ml/min) using an adapted 10 ml-syringe. After washing with deionized water (2 ml/min) and passing air (syringe) through the column (drying) the OA was eluted with methanol (0.2–0.3 ml/min) and air was again passed through the column to collect all the eluate. The eluate was evaporated to dryness (40–45 °C, N_2-stream), the

Table 1. Clean up procedure for OA with various IAC (one use)

IAC[a]	Rinsing[b]	Loading[c]	Washing	Elution	Rinsing
Biocode (0.6)	20 ml PBS[d]	4 x 5 ml PBS[e]	10 ml water	3 ml methanol	20 ml PBS
Biopharm (0.1)	10 ml PBS	4 x 5 ml PBS[f]	10 ml water	1 ml methanol	10 ml PBS
Vicam (0.2)	10 ml PBS	4 x 5 ml PBS[e]	10 ml water	1 ml methanol	10 ml PBS

[a] type of IAC; in parenthesis: approximate volume of column-filling (ml)
[b] only applied with new IAC or those that were stored between the various uses (at least 1–2 days, refrigerator)
[c] including rinsing of the extract-containing vial
[d] PBS: 120 *mmol/l* NaCl, 2.7 *mmol/l* KCl, 10 *mmol/l* phosphate buffer pH 7.4, ambient temperature
[e] containing 15% (v/v) methanol
[f] containing 10% (v/v) methanol

residue dissolved in 50 µl of the HPLC mobile phase (methanol and water containing 9% [v/v] glacial acetic acid, volume ratio 18:7) and a volume of 20 µl was injected into the HPLC-system (ODS1, 50 °C; fluorescence detection at pH 9). Each IAC was immediately rinsed with PBS solution after use, and stored (filled with PBS) in the refrigerator at 2–8 °C (generally 4 °C) until its next use. The whole procedure according table 1 lasted about 35 min (one use).

For Biocode IAC the manufacturer claimed that a minimum of 2 µg OA will be bound when 5 µg are loaded in 25 ml of 15% (v/v) methanol in PBS; likewise the Biopharm type has a maximum capacity of 0.2 µg. No such information was available for Vicam IAC (but the recovery of each lot was tested by the manufacturer with 150 ng OA) and no attempt was made to determine its capacity. In our experiments the total OA amounts loaded onto the columns were about 0.3 ng, sometimes reaching a maximum of some ng. The former amount corresponds to OA concentrations in human serum of about 0.3 ng/g (1 g sample), in the order of the mean of Swiss adults (7).

Results and Discussion

Figure 1 shows the HPLC chromatograms of human serum extracts containing 0.3 ng OA/g. The two extracts of the same sample were prepared according to the previous publication (7), and for the cleanup either Biocode or Vicam IAC were used. Independent of the type of extract (including reagent blanks), and even after use of up to 30 times (total volumes: ca. 1.2 l PBS, 0.3 l water and 0.1 l methanol) the Biocode IAC showed still more coeluting fluorescent impurities (affinity gel,

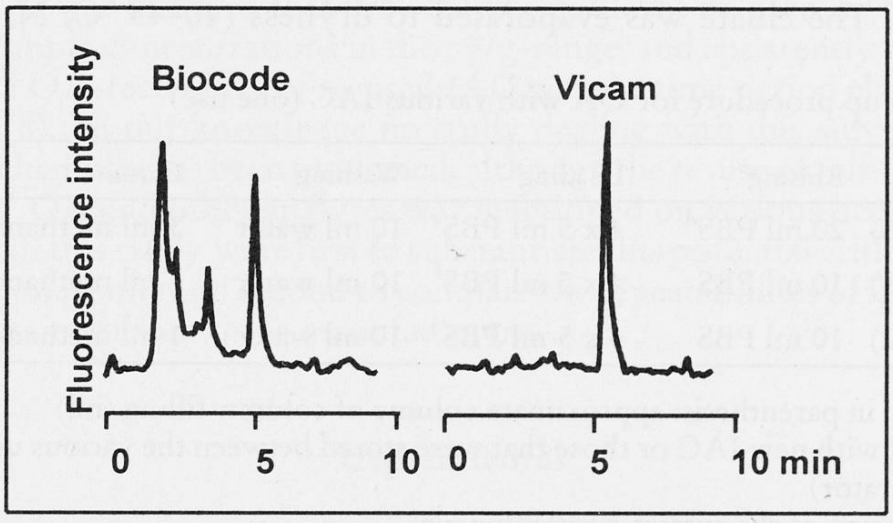

Fig. 1. HPLC-chromatograms (fluorescence detection at pH 9) of a sample of human blood serum naturally contaminated with OA. The cleanup was made either with a Biocode or a Vicam IAC. The injected amount of extract is equivalent to 0.2 g of serum; the OA concentration in the serum is 0.3 ng/g.

plastic material?) than the Vicam (or Biopharm) IAC. However, these impurities did not interfere with the OA peak and its methyl or ethyl ester (7, 8).

The results given in table 2 represent an overview of the mean OA-recoveries obtained with new columns according to the procedures given in table 1. Satisfactory mean OA-recoveries within a range of 78.9 to 98.1% resulted with all tested types of columns. The standard deviations of the mean recovery (proportional to the repeatability limit) appear to decrease in the following order (first use): Biocode $\simeq$ Biopharm > Vicam, if the data with the Biocode prototype and the batch N° Bio-5ID-0597 are excluded (see next paragraph). Concerning the handling of the IAC in the laboratory, the Biocode type was the most suitable because of its larger size. In addition the following decreasing tendency of the IAC to clog up with fine particles were observed: Biopharm > Vicam > Biocode.

It is interesting to note (table 2), that the individual column with the lowest recovery of 57.4% in the first use (batch N° Bio-5ID-0597) resulted in a value of 96.2% in the second one (time period of rest 48 h). On the other hand another column yielded 89.1% when first used, however only 59.6% at the second use (time period of rest 24 h). Such a behavior of the Biocode IAC was in our experience not generally observed with other batches[1] (see also table 3).

The mean overall recovery (first use) obtained with Biocode IAC of 84.5 ± 10.0% (± standard deviation; n = 22, from table 2) using an OA-standard is in good accordance with the mean overall recovery of OA from human serum, milk and alcoholic (7, 8). The reason for the lower mean OA-recovery as well as for the higher repeatability limit achieved with Biocode IAC than with Vicam or Biopharm appears to be the washing step with water (table 1), which results in variable OA losses, at the mean of about 10%. Similar results for Biocode IAC were obtained by others, e.g. from the analysis of radioactive OA in human serum (11). This might indicate that the antibodies of the Biocode IAC may have lower strength of interaction with OA (affinity of the antibody to the antigen) than the other IAC types (9). The overall mean OA-recovery with new Vicam IAC amounts to 97.3 ± 2.6% (± standard deviation; n = 30) and corresponds well with the published overall OA-recovery from beer (12).

The effects of consecutive uses of IAC according to the procedures given in table 1 (but rinsed only one time with PBS), *and without any time period of rest between the various uses,* on the OA-recovery are summarized in table 3. From the first use of new Vicam column to its fourth use the mean OA-recovery was reduced approximately by a factor of 10 and its coefficient of variation increased from about 3% to 60%. A separate trial with a Vicam column previously used four consecutive times demonstrated that OA was in fact practically no longer retained in its fifth use. Hence, the possibility can be excluded that OA was retained but for unknown reasons no more eluted. The reduction of the mean OA-recoveries and the increase

[1] – Therefore these results might be explained by a change in the production process or the storage conditions applied by the distributors.
– In spring 1996 Biocode IAC from Rhône-Poulenc Diagnostics were no more available, but in summer 1996 a new type was offered (OCHRAPREP™).

Table 2. Recovery of 275 pg OA-standard from various IAC types

Type of IAC[a]		First use[b] (%)			Second use[b] (%)					
		n[c]			n[c]	24 h[d]		n[c]	48 h[d]	
Biocode										
4BL-1193 (prototype)	(1992)	8	78.9 ± 10.9	(61.2–98.0)	–	–	–	–	–	–
4CT-0794	(1993)	4	90.9 ± 5.1	(84.5–96.7)	–	–	–	–	–	–
Bio-51D-0597	(1995)	10	86.4 ± 11.6	(57.4–98.2)	5	78.7 ± 13.3	(59.6–91.5)	5	84.2 ± 16.5	(55.4–96.2)
Biopharm										
91046	(1995)	10	93.8 ± 4.4	(83.3–97.8)	5	94.0 ± 5.6	(84.8–100.0)	5	97.5 ± 2.7	(93.8–100.0)
Vicam										
163	(1995)	–	–	–	4	98.0 ± 1.7[e]	(96.4–100.1)	–	–	–
164	(1996)	25	97.1 ± 2.8	(87.0–100.0)	5	95.5 ± 2.6[f]	(93.9–98.0)	5	96.2 ± 3.2[g]	(91.3–100.0)
165	(1996)	5	98.1 ± 1.4	(96.1–100.0)	–	–	–	–	–	–

[a] lot (or batch) number and year of purchase in parenthesis
[b] arithmetic mean of the recovery and standard deviation; in parenthesis range
[c] number of IAC used
[d] number of hours stored at 4 °C after the first use before re-use
[e] 3–5 days instead of 24 h; used at the first use with extracts (1 wine, 2 serum, 1 reagent blank) instead of standard
[f] 96 h instead of 24 h
[g] 2 months instead of 48 h

Table 3. Effect of the consecutive use of IAC, without any time period of rest between the various uses, on the recovery of 275 pg OA-standard

Type of IAC[a]		Previous use[b]	n^c	Mean recovery ± standard deviation (%)			
				first use	second use	third use	fourth use
Biocode							
Bio-51D-0597 (Rhône-Poulenc)	(1995)	zero	1	98.1[d] } e	70.9[d] } e	73.2[d] } e	55.6[d] } e
		1 x with ST	1	97.8[d]	62.6[d]	37.4[d]	27.4[d]
		5 x with ST	1	98.0	79.1	50.0	56.2
Bio-51A-0296	(1994)	18 x with J, 6 x with O	1	100.6[d]	86.3[d]	59.2[d]	45.0[d]
Biopharm							
91046	(1995)	2 x with ST	5	85.3 ± 11.9	64.4 ± 2.3	49.2 ± 12.5	44.3 ± 5.3
Vicam							
164	(1996)	zero	10	96.7 ± 3.8	71.1 ± 11.9[f]	18.8 ± 8.8	7.4 ± 4.8
		1 x with ST	5	96.2 ± 3.2	53.3 ± 7.6[f]	13.9 ± 4.0	7.6 ± 4.7

[a] lot(or batch) number and year of purchase in parenthesis

[b] – number of uses before this experiments: zero or 1 x or...x; with... ST = standard, J = grape-juice extract, O = other extracts (serum and/or wine)
– time period of rest in the refrigerator at 4 °C of at least 3 days before use in this experiment

[c] number of IAC used

[d] grape-juice extract with a «natural» OA concentration of 358 ± 8 pg/ml (n = 3) instead of OA-standard; absolute amount of OA loaded ca. 0.8 ng

[e] arithmetic mean ± standard deviation: first use 98.6 ± 1.3%; second use 74.7 ± 10.2%; third use 55.0 ± 15.1%; fourth use 46.1 ± 13.5%

[f] the means are probably significantly different (t-test $p = 0.01$), but not the standard deviations (F-test, $p > 0.05$)

of its coefficient of variation during the consecutive use of the Biocode and Biopharm IAC are much less pronounced than in the case of Vicam columns (table 3). This could be explained by a higher number of antigen binding sites or a faster renaturation rate of the biological activity after use of these IAC types. Taking into account the manufacturers' information as well as our results the OA-capacity of the IAC might decrease in the order of Biocode > Biopharm > Vicam.

In order to study the renaturation kinetics of previously used IAC, first new Vicam types (lots 164, 165) were used four times consecutively with 275 pg OA-standard (as described above), second, used in the same manner as new ones (table 1) but after various time periods of rest (t) at 4 °C and 23 °C (T). Vicam IAC's were used for these trials because their recoveries were much stronger dependent on consecutive uses than the other types (table 3). The results are presented in table 4, indicating an increase of the recoveries according to increasing time periods of rest and an increase of the rate of renaturation at higher storage temperatures. If instead of new Vicam IAC previously used ones (only one time with standard) were applied in these experiments, apparently lower recoveries resulted at time periods of rest of less than 15 hours in comparison to new ones (table 4 and table 3, footnote f). However, the repeatability of these single measurements may be considerable (table 3). In addition, no attempt was made to thermostatize the IAC during the time period of rest. After longer time periods of rest no significant differences concerning the OA-recoveries were seen between new and previously used columns (table 2 and 4).

Table 4. Effect of time period of rest (t) and temperature (T) on the recovery of 275 pg OA-standard from previous consecutively used (4 x) Vicam IAC (t = 0)

t (h)	Recovery (%)			
	T = 23 °C		T = 4 °C	
	A[a]	B[b]	A[a]	B[b]
0	(1.3)	–[c]	–	–
1	15.6	–	–	–
1.5	37.1	–	4.0	–
2	38.8	–	–	–
3	68.6	57.5	35.6	18.8
6	76.0	60.4	57.1	31.1
13	90.0	–	54.2	–
18	–	80.9	73.1	–
24	95.7	–	–	82.2
48	91.9	–	81.1	93.0[d]
120	–	–	92.9	–

[a] new IAC
[b] used 1 x, then stored at least 10 days at 4 °C before this experiment
[c] not studied
[d] used 4 x, then stored 15 days at 4 °C before this experiment

If it is assumed that the OA recovery is, within a certain range, proportional to the fraction of biologically active antibodies of the IAC and that the initial rate of the renaturation process of consecutively used Vicam IAC may formally be described by a unimolecular first-order reaction kinetics (13, 14), it follows that $ln\ [(100\text{-}x) / 100] = k \cdot t$; x = OA-recovery, t = time period of rest, k = reaction constant. Taking into account the time periods of rest of less than 14 h only (table 4), apparent reaction constants for the renaturation process were estimated at 0.195 ± 0.018 h^{-1} (n = 8), and 0.075 ± 0.014 h^{-1} (n = 6) for the temperatures of 23 °C and 4 °C, respectively (± standard error; regression line through zero; details not given). These reaction rate constants correspond to half-times of about 4 h (23 °C) and 9 h (4 °C), which means that after these time periods 50% of the maximal OA-recovery will be re-achieved. According the *Arrhenius* equation the apparent energy of activation of the renaturation process was estimated at 35 ± 9 kJ/mole.

It appears, that the nature of one of the important interactions to bind the antigen to the antibody is hydrophobic. The application of polarity-lowering agents such as methanol, as used in our study, favors the dissociation of the antigen-antibody complex (9, 10). In our experiments the dissociation was virtually completed within about 5 minutes. This treatment could change the conformations of the antibodies fixed in the IAC resulting in a loss of their biological activities. A possible explanation is that methanol replaces, at least partially, the water molecules at the interior as well as on the surface of the proteins. On the other hand, hydrophobic interactions also appear to be a key factor in the stability of (folded), biologically active proteins. To restore the biological activity of the antibody the denaturing environment has to be removed and the protein refolded to its biologically active conformation. The rates of folding reactions in dissolved proteins can vary from submilliseconds to several hours, depending on its structure and probably on the nature of the denaturing environments to that they were exposed before (9, 10, 13, 14).

If it is assumed that the renaturation kinetics of IAC is dominated by the rate of protein folding, then the estimated half-times may indicate the existence of a high kinetic barrier, although the apparent energy of activation amounts to only about half of that measured for the known slow *cis-trans* isomerization of proline in folding proteins (14). It was also suggested that e.g. the forming of native polar interactions in proteins in a relatively hydrophobic environment is an inherently difficult step which slows down folding (15).

However, it has also to be taken into account that the proteins of the IAC do not exist as dissolved entities but are immobilized onto a stationary phase. In addition, the properties of the stationary phase itself might also be affected by the denaturing solvent. Besides protein folding, restricted diffusion and/or desorption processes, probably to replace methanol in and on the proteins by water, may also be a possible explanation for the observed half-times of several hours and the considerable apparent energy of activation of 25–45 kJ/mole (16). Half-times of 1.5–3 h at 21 °C were e.g. measured for the equilibration of dry ion-selective, highly plasticized poly (vinyl chloride)-based membranes with water (17, 18).

Concluding remarks

That IAC after treatment with chaotropic agents and exposure to a native environment can be re-used is well known as well as the possibility to recover the biological activity of denatured proteins (e.g. enzymes). In general, the most gentle conditions, under which effective elution is still possible should be chosen; this will maximize the chance of reestablishing the biological activity of the IAC (9, 10, 13, 14). Self prepared IAC for aflatoxins and OA could e.g. be re-used more than 100 and 30 times, respectively (1, 2). Recently, it was shown that even for other types of immunoassays (e.g. ELISA), where immobilized antibodies or enzymes are involved, repeated use was possible (19–23). However, in most studies with IAC no or only very vague information about the necessary time period of rest of the active entity before its re-use was given (24).

This study clearly demonstrates that for the successful re-use of commercial OA retaining IAC a time period of rest of at least 2 days in the refrigerator and in contact with PBS is obligatory. This fact was not taken into account in our previous study (7). As a first approximation we assumed that mainly the number of exposure of the IAC to coextractives (e.g. protein, fat) and/or eluting solvent (protein denaturing) was the limiting factor in its re-use (7, 24). Probably the nature of the eluting solvent, the time period of rest and the number of exposure as well as the original capacity of the IAC including the applied antigen concentrations may be important parameters in the potential re-use of IAC.

Although the results of this study were predominantly obtained with OA-standard we dare, based on our laboratory experiences, to generalize them for various types of extracts. Under our conditions (0.1–10 ng OA) the IAC of Biocode could be successfully re-used (recoveries > 80%) more than 30 times, Vicam more than 10 times and Biopharm more than 5 times. In principal, our results should also be valuable for any other type of IAC and analyte. Apparently the main limiting factor in their re-use is clogging up with fine particles, depending on the type of extract and its pre-filtration step.

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Summary

The recoveries and repeatabilities of the mycotoxin ochratoxin A (OA) from immunoaffinity columns (IAC) of three manufacturers were determined with satisfactory results using about 0.3 ng OA-standard. Renaturation studies of the IAC clearly demonstrated that all tested types can be successfully re-used if the time period elapsed between the various uses was at least 2 days. During this time the columns were in contact with buffer solution and

were stored in the refrigerator. The apparent energy of activation of this renaturation process was estimated at 35 ± 9 kJ/mole.

Zusammenfassung

Mit Ochratoxin-A-Standardlösungen (0,3 ng) wurden Wiederfindungen (und Wiederholbarkeiten) von Immunoaffinitätssäulen (IAC) dreier verschiedener Hersteller mit befriedigenden Ergebnissen bestimmt. Weitere Untersuchungen an zuvor gebrauchten IAC zeigten, dass diese wiederverwendbar sind, sofern sie vor der nächsten Verwendung während mindestens 2 Tagen im Kühlschrank, in Kontakt mit Pufferlösung, gelagert werden. Die scheinbare Aktivierungsenergie dieses Renaturierungsprozesses wurde auf 35 ± 9 kJ/Mol geschätzt.

Résumé

Les taux de récupération et de répétabilité ont été déterminés avec des solutions d'étalonnage d'ochratoxine A (0,3 ng) en utilisant des colonnes d'immunoaffinité (IAC) de trois différents fournisseurs. D'autres analyses ont démontré que les colonnes IAC peuvent être réutilisées, à condition qu'elles soient stockées au frigidaire dans une solution tampon et ceci pendant au moins deux jours avant une réutilisation. L'énergie d'activation apparente de ce processus de régénération a été estimée à 35 ± 9 kJ/mole.

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