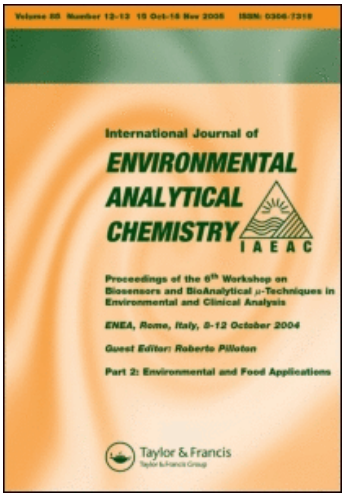

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Non-Selective Desorption of Immuno Precolumns Coupled On-Line with Column Liquid Chromatography: Determination of Aflatoxins

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NON-SELECTIVE DESORPTION OF IMMUNO PRECOLUMNS COUPLED ON-LINE WITH COLUMN LIQUID CHROMATOGRAPHY: DETERMINATION OF AFLATOXINS

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Immuno precolumns, i.e. short columns packed with immobilized antibodies, are used for selective sample pretreatment coupled on-line to column liquid chromatography (LC). A column-switching system with an anti-aflatoxin immuno precolumn as a model system is described, which allows on-line transfer of the analytes from the immuno precolumn to the LC separation column. After selective preconcentration on the immuno precolumn the analytes are desorbed non-selectively with methanol-water (70:30, v/v), diluted on-line with water, subsequently reconcentrated on a second, reversed-phase precolumn and finally transferred to the LC separation column. Aflatoxin M1 can be preconcentrated from 30-ml samples with recoveries of 70%. The detection limit is 10 ng/l and the calibration plot is linear up to 1 μg/l. The analysis time of the fully automated procedure is 45 min. The selectivity of the system is demonstrated with direct injections of spiked urine and milk samples. The regeneration and the stability of the immuno precolumn and the general applicability of non-selective desorption for on-line coupled immuno precolumn/LC systems are discussed.

KEY WORDS: Column liquid chromatography, immuno affinity clean-up, aflatoxins, milk.

INTRODUCTION

Precolumns packed with immobilized antibodies, so-called immuno precolumns, are an effective tool to enhance the performance of sample pretreatment. Due to the selectivity and strength of antibody-antigen interactions, a high degree of sample clean-up and trace enrichment can be achieved in a single step. Various matrices such as urine, plasma and tissue homogenates, which often cause problems in conventional sample pretreatment, have successfully been analyzed with this still relatively novel technique[1–37]. Moreover, complete automation can easily be achieved by coupling the immuno precolumn to an LC system by means of a column-switching set-up[6,10,12,21,22,29]. Sorption of the analytes of interest on the immuno precolumn seems to be no problem with such systems. The critical step in an on-line system is the desorption of the analyte from the immuno precolumn. This can be done in several ways, which are shortly discussed below.

The technically most simple approach is to desorb the immuno precolumn directly

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with the LC eluent. This is done by switching the precolumn on-line with the separation column[10,29]. When the organic modifier content of the eluent is high enough, the immobilized antibodies will be denatured and the analytes will be desorbed. In most cases denaturation is reversible and, after proper renaturation, the immuno precolumn can be reused. An inherent drawback of the method is that the organic modifier content of the LC eluent may be too low for quantitative desorption of the analytes from the immuno precolumn. In most cases analysis of large sample volumes will not be possible at all with this technique, since desorption of the broad analyte zone in a sufficiently small band will be difficult[10,29].

Another on-line desorption technique circumvents the band broadening problem by using a refocussing effect during transfer of the analytes from the immuno precolumn to the reversed-phase (RP) LC separation column. The analytes are desorbed from the immuno precolumn with a relatively weak mobile phase (low percentage of organic modifier) and reconcentrated on the RP–LC column. In contrast with the first method, this set-up allows the use of relatively large volumes of desorption solvent (mixture). This solvent mixture should meet two requirements: (i) it must effect rapid dissociation of the antibody-antigen (analyte) complex, and (ii) it must be sufficiently 'weak' to allow reconcentration of the desorbed analytes on a RP-type separation column or second precolumn (cf. below). If the analyte—i.e. the antigen—is a macromolecular compound, this can easily be achieved with aqueous solutions of low or high pH or by using so-called chaotropic ions, which are able to alter the structure of the antigen. To quote a few examples, bovine serum albumin[6], human haemoglobin[6] and lysozyme[22] have been desorbed on-line with a glycine-hydrochloric acid buffer of pH 2.0. Aqueous solutions containing 0.1–0.2% of trifluoroacetic acid were used for the on-line desorption of interferon[21] and lysozyme[22]. However, for small molecules the chaotropic desorption will be more difficult, because such solutes normally lack structural features (e.g. ternary and quaternary structure) which are susceptible to this mechanism. Therefore, only a few examples are known where *small* molecules have been desorbed from an immuno precolumn using such aqueous solutions. The only real on-line desorption was reported by Reh[12], who used an aqueous solution containing 1% hydrochloric acid for the desorption of digoxin. In several other studies chaotropic solutions were found to be ineffective for the desorption of small molecules[4,38,39].

A further alternative is to use immunoselective desorption[39–41]. Desorption is now performed with an aqueous solution containing a solute which possesses high affinity for the immobilized antibody and consequently can act as a displacer. In principle immunoselective desorption can be used with any type of analyte, no matter whether it is a large or a small molecule. However, the technique has its limitations[41]. The displacer, which has to be used in a rather large excess, will in most cases produce a large peak in the chromatogram and consequently limit the separation window for the analytes. Besides, stringent demands have to be made with regard to aspects such as purity, stability, retention time, non-toxicity, price and affinity for the immobilized antibodies. The selection of a suitable displacer will, therefore, often be very time-consuming.

In the present study, a method is developed that can be used as a general tool for the desorption of analytes from an immuno precolumn in on-line immunoaffinity/RP-

LC systems. Desorption is done with an aqueous solution containing a high percentage of an organic modifier. Subsequently the eluate is diluted with water and the analytes are reconcentrated on a C-18 precolumn. Finally the analytes are transferred from the C-18 precolumn to the RP-LC separation column and separated. An anti-aflatoxin immunosorbent, which has recently become commercially available, is used as a model system in the present study.

EXPERIMENTAL

Apparatus and set-up

The set-up of the LC system is shown in Figure 1. It consisted of two Gilson (Villiers-le-Bel, France) Model 302 pumps—one for the separation column (pump 3) and one for the water dilution step (pump 2)—an Applied Biosystems (Ramsey, NJ, U.S.A.) Spectroflow 400 pump (pump 1), a Spark (Emmen, The Netherlands) PROSPEKT 1.0 programmer containing the valve switching units and the solvent selection valve, a Perkin–Elmer (Beaconsfield, UK) LS-2 fluorescence detector (λ_{ex} = 360 nm, λ_{em} = 440 nm; slit widths, 10 nm) and a Kipp & Zonen (Delft, The Netherlands) BD 40 recorder. The separation column was a home-packed 100 mm × 3.0 mm I.D. glass column filled with 5 μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.), protected with a 10 mm × 2.0 mm I.D. guard column packed with the same material.

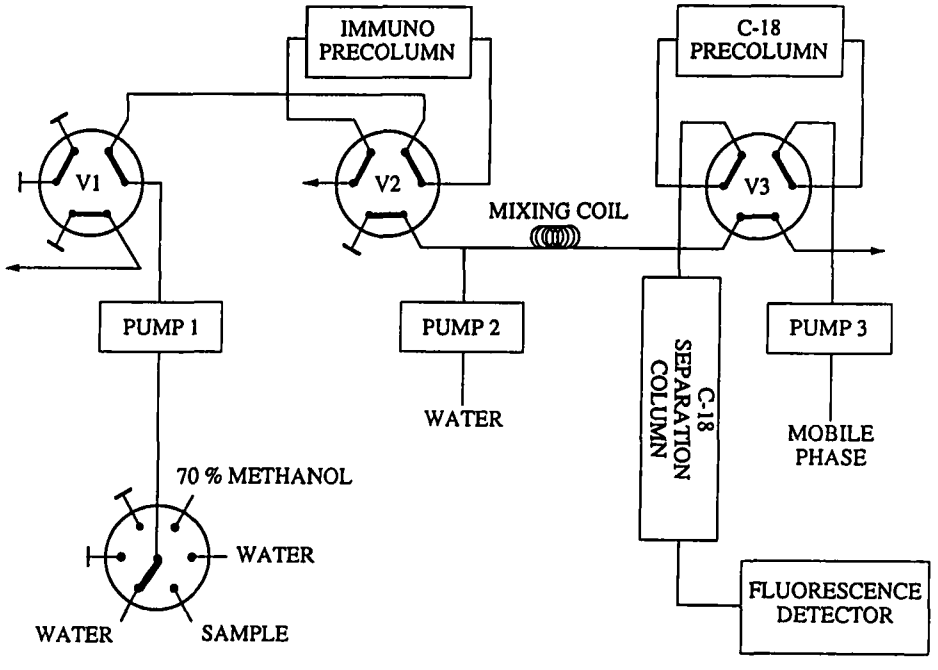

Figure 1 Set-up of automated sample pretreatment system for the LC determination of aflatoxins. Valves V1–V3 are all shown in position A (cf. Table 1). The system is described in the Experimental section.

The 10 mm × 2.0 mm I.D. stainless-steel C-18 precolumn was packed with 40 μm Baker (Deventer, The Netherlands) C-18 Bonded Phase. The mixing coil was a stainless-steel LC capillary with an I.D. of 1.1 mm and an internal volume of 1 ml.

Chromatography was performed at ambient temperature using acetonitrile-methanol-water (16:24:60 v/v) as the mobile phase. The flow rate was 0.5 ml/min. The immuno precolumns were home-made 10 mm × 10 mm I.D. or 10 mm × 4.0 mm I.D. stainless-steel columns equipped with stainless-steel screens and PTFE rings as the column inlet and outlet. The packing procedure of the immuno precolumns has been described elsewhere[39].

Chemicals

Sodium dodecyl sulphate (SDS) was obtained from BDH (Poole, UK), hexamethyltrimethylammonium chloride (cetrimide) was from Kodak (Rochester, NY, U.S.A.). The aflatoxins M1, B2 and G2 were a gift from RIKILT (Wageningen, The Netherlands). LC-grade acetonitrile and methanol were purchased from Baker. LC-grade water was prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system with subsequent filtration over a 500 mm × 10 mm I.D. LC column filled with 40 μm Baker C-18 Bonded Phase. The LC eluents were degassed under vacuum in an ultrasonic bath. Plastic precolumns containing immobilized monoclonal anti-aflatoxin antibodies were obtained from Koopman & Koek (Rijswijk, The Netherlands). These precolumns were used as such (off-line procedure) or, alternatively, the material was removed and repacked into stainless-steel precolumns. The immuno precolumns were stored in methanol-water (70:30, v/v).

Analytical procedure

The time schedule of the analytical procedure when using the 10 mm × 4.0 mm I.D. immuno precolumn is given in Table 1. First the immuno precolumn is preconditioned with 5.2 ml of water using pump 1. Then the sample is loaded via the same pump. The immuno precolumn is flushed with 5.2 ml of water (pump 1) to displace the remaining sample and to remove non-specifically bound impurities. Simultaneously the C-18 precolumn is preconditioned with 4.7 ml of water via pump 2. Subsequently, the immuno precolumn and the C-18 precolumn are switched in series and the immuno precolumn is desorbed with 520 μl of methanol-water (70:30, v/v) in the backflush mode using pump 1. The methanol-water eluate is diluted with water to a methanol content of 3.7% by pump 2 in order to allow reconcentration of the aflatoxins on the C-18 precolumn. After transfer of the analytes to the C-18 precolumn, the actual separation is started by switching the C-18 precolumn on-line with the separation column, using pump 3 for the eluent. Finally, the immuno precolumn is flushed with 650 μl of methanol-water (70:30, v/v).

If the 10 mm × 10 mm I.D. immuno precolumn was used, the regeneration of the immuno precolumn (step 1 in Table 1) was performed by first flushing with 5 ml of water during 1 min and subsequently with an additional 5.2 ml of water during 20

Table 1 Schedule of the on-line analytical procedure using the set-up according to Figure 1.

Step	*Event*	*Valve positions**		
		Valve 1	*Valve 2*	*Valve 3*
1	Flushing immuno precolumn with water (20 min, 5.2 ml)	A	A	A
2	Flushing capillaries with sample	B	A	A
3	Flushing immuno precolumn with sample (4 min, 10.4 ml)	A	A	A
4	Flushing capillaries with water	B	A	A
5	Flushing immuno precolumn with water via pump 1 (2 min, 5.2 ml) and simultaneously flushing C precolumn with water via pump 2 (1 min, 4.7 ml)	A	A	B
6	Flushing capillaries with methanol-water (70:30, v/v)	B	A	B
7	Analyte desorption from immuno precolumn with methanol-water (2 min, 0.52 ml) with subsequent dilution with water (2 min, 9.4 ml) and analyte reconcentration on the C-18 precolumn	A	B	B
8	Desorbing C-18 precolumn by switching on-line with separation column and flushing immuno precolumn with additional methanol-water (0.25 min, 0.65 ml)	A	B	A
9	Flushing capillaries with water	B	A	A

* Position A corresponds with the valve positions shown in Figure 1.

min. Desorption of the immuno precolumn (step 7 in Table 1) was performed with 2080 µl instead of 520 µl of methanol-water (70:30, v/v) by increasing the desorption time from 2 to 8 min.

Calibration plots and capacity determinations

Calibration plots were obtained by preconcentrating the selected aflatoxin from 10 ml of an aqueous solution on a 10 mm × 4.0 mm I.D. immuno precolumn and performing the analysis according to the analytical procedure.

The capacity of the 10 mm × 4.0 mm I.D. immuno precolumn was determined by preconcentrating 10 ml of an aqueous solution containing 500 ng of aflatoxin (overloading) and performing the analysis according to the analytical procedure.

RESULTS AND DISCUSSION

In a study with immuno precolumns coupled on-line with (reversed-phase) LC, one has to consider aspects such as analyte sorption on and desorption from the immuno precolumn and analyte reconcentration on a C-18 precolumn or separation column. In addition, the stability and lifetime of the immuno precolumn and its ease of regeneration are of importance, because they help determine the practicability of the analytical method. These subjects are discussed in the present paper, in which a commercial immunosorbent containing an immobilized monoclonal antibody against the aflatoxins B1, B2, G1, G2 and M1 was used. Because of the relatively low detectability of the aflatoxins B1 and G1, and the high price of aflatoxin M1, the aflatoxins B2 and G2 were selected for initial characterization of the system.

Preliminary off-line experiments

According to the manufacturer, aflatoxins preconcentrated on the prepacked plastic immuno precolumns can be desorbed by flushing with aqueous solutions containing a high percentage (80–100%, v/v) of organic modifier. To evaluate the efficiency of the desorption, a (10 mm × 8.0 mm I.D.) plastic immuno precolumn was overloaded by syringe action with 20 ml of a standard solution containing 100 ng of aflatoxin B2. After flushing with 20 ml of water, the immuno precolumn was first desorbed with 4 ml of methanol-water (30:70, v/v), followed by the same amount of methanol-water (50:50, v/v) and finally by 4 ml of methanol-water (70:30, v/v). Each 4-ml eluate was divided into two 2-ml fractions, and the aflatoxin B2 content was determined by RP-LC. The total aflatoxin B2 recovery was 72%. The individual fractions contained 2% and 3% (methanol-water [30:70, v/v]), 16% and 18% (methanol-water [50:50, v/v]) and 32% and 1% (methanol-water [70:30, v/v]) of the analyte. As expected, the desorption efficiency improved with higher methanol percentages. Using a percentage of 70% methanol allowed us to desorb the aflatoxin almost quantitatively in (the first) 2 ml of solvent.

After these experiments the immuno precolumn was flushed with 20 ml of water, in order to attempt regeneration of the denatured antibodies, and the loading/desorption cycle was repeated, except that now desorption was performed directly with two 2-ml flushes of methanol-water (70:30, v/v). In both the second and the third run, the immuno precolumn still showed considerable activity, although lower than before (aflatoxin B2 recovery, 30–40%). All aflatoxin was found in the first 2-ml fraction.

The preliminary experiments showed that the immobilized antibodies partly regain their biological activity upon regeneration by flushing with water. Efficient desorption of the analyte is also possible, viz. with a few milliliters of methanol-water (70:30, v/v). In other words, the immuno precolumn can, in principle, be re-used. All further work on the evaluation of the analytical potential of the immunoaffinity sample pretreatment procedure was therefore, carried out in a system coupled on-line to RP-LC.

Optimization of the automated desorption-dilution-reconcentration procedure

Since separation of aflatoxins on a RP-LC column is performed with a mobile phase containing 16% of acetonitrile and 24% of methanol, it is obvious that the analytes can not be directly injected onto the RP-LC separation column dissolved in 2 ml of methanol-water (70:30, v/v), the eluate of the immuno precolumn. Therefore, the eluate is diluted with a large excess of water, via a T-piece (Figure 1), before the analytes are introduced onto the RP-LC separation column. Due to the reduced solvent strength after dilution, the analytes can now effectively be reconcentrated. The reconcentration can in principle be performed directly on top of the separation column. However, because of the large volumes to be reconcentrated and the low flow rate (0.5 ml/min) on the separation column, this would be a time-consuming step. Therefore, this step was performed on a C-18 precolumn, which allows higher flow rates. Transfer of the analytes to the separation column can be performed by on-line switching.

With such a system, two aspects should be considered with regard to the volume of aqueous methanol used for desorption of the immuno precolumn; (i) quantitative desorption of the immuno precolumn requires a certain minimum volume of aqueous methanol and (ii) the use of a large volume of aqueous methanol, combined with the strong dilution with water may well cause breakthrough on the C-18 precolumn and result in a decreased overall recovery. In order to determine the optimum conditions, the breakthrough characteristics of the aflatoxins on the C-18 precolumn were studied to determine the maximum volume of methanol-water (70:30, v/v) that can be used for reconcentration on this column. Secondly, the volume of methanol-water (70:30, v/v) required for quantitative desorption of the immuno precolumn, was determined.

Efficiency of dilution-(p)reconcentration on C-18 precolumn. A methanol-water (70:30, v/v) solution containing 0.5 μg/l of each aflatoxin B2 and aflatoxin G2 was pumped directly to the T-piece with pump 1 (0.26 ml/min; pump 1 was connected to the free port V2, cf. Figure 1) and mixed with water from pump 2 (4.7 ml/min). The combined mixture—now containing 3.7% of methanol—was, after passage of the mixing coil, preconcentrated on the C-18 precolumn (10 mm × 2.0 mm I.D.). After switching the C-18 precolumn on-line with the separation column the analytes were desorbed, separated and quantitated. Different sample volumes (analyte dissolved in methanol-water [70:30, v/v]) were loaded onto the system. The dilution factor was kept constant with the consequence that, with increasing sample volume, increasing volumes of the diluted 3.7% methanol solution were loaded on the precolumn. With a sample volume of 0.5 ml, a recovery of 100% was achieved for both aflatoxins. For larger volumes the recovery slowly decreased, due to breakthrough. At a sample volume of 7 ml, recoveries of 90% and 75% were achieved for the aflatoxins B2 and G2, respectively.

As was to be expected, band broadening was no problem. For example, when preconcentrating aflatoxin B2 from up to 5 ml of methanol-water (70:30, v/v)—while diluting, self-evidently, with water to 3.7% methanol—the peaks were even sharper than with a direct 8 μl loop injection of aflatoxin B2 in pure methanol.

Desorption of the immuno precolumn. Next, using the same degree of dilution as in the previous experiment (70% to 3.7% methanol), the total analytical procedure was performed: the immunosorbent was packed into a 10 mm × 4.0 mm I.D. stainless-steel precolumn, 500 ng of aflatoxin B2 were loaded on the immuno precolumn and desorbed with different volumes of methanol-water (70:30, v/v). The amount of water used for dilution was adapted each time in order to have the same degree of dilution in all experiments. Except for regeneration of the immuno precolumn, which was performed with 5.2 ml of water, all other steps corresponded with the procedure described in the Experimental section. The recovery was calculated by comparison with a loop injection. The same experiments were performed for aflatoxin G2 and aflatoxin M1. All work was done with a single immuno precolumn, containing an immunosorbent that had already been subjected to five sorption/desorption cycles in off-line experiments. As can be seen in Figure 2, the highest aflatoxin recoveries

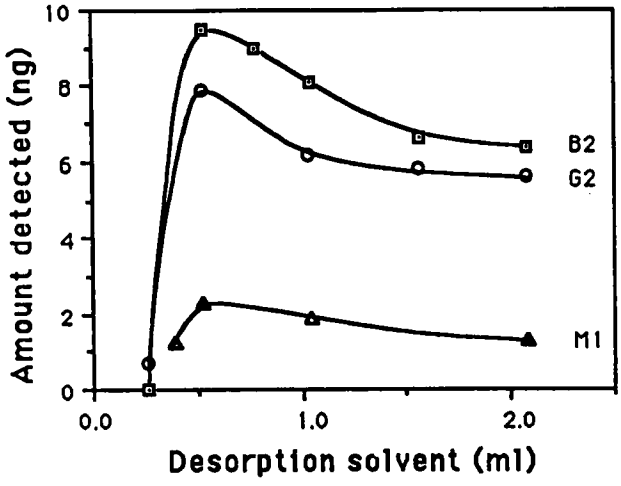

Figure 2 Recovery of the aflatoxins B2, G2 and M1 as a function of the amount of methanol-water (70:30, v/v) used for desorption of the 10 mm × 4.0 mm I.D. immuno precolumn. For conditions, see text.

are achieved with desorption volumes of 520 μl of methanol-water (70:30, v/v). The plot in Figure 2 is the result of two effects. The recovery increase due to a better desorption of the immuno precolumn with increasing desorption volume—a function which rather quickly reaches a plateau value—is superimposed on a recovery decrease due to breakthrough on the C-18 precolumn.

Stability of the immuno precolumn

The previous experiments already indicated that repeated use of the immuno precolumn is possible. In the next set of experiments the stability of the column in

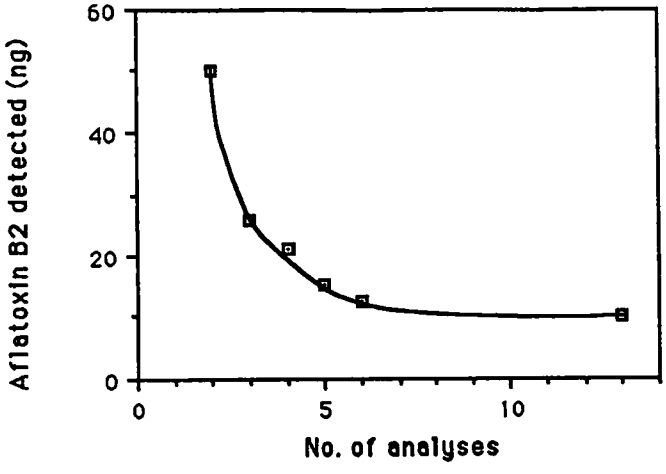

Figure 3 Capacity of 10 mm × 4.0 mm ID. immuno precolumn as a function of the number of analyses. Analyte, aflatoxin B2. For conditions, see text.

the automated system was evaluated as a function of the number of standard analyses performed.

A fresh 10 × 4.0 mm I.D. immuno precolumn was repeatedly overloaded with 500 ng of aflatoxin B2. The analyses were performed according to the procedure described in the previous section, using 520 µl of methanol-water (70:30, v/v) for desorption. During the first runs a capacity decrease of over 90% was observed (Figure 3). However, after five to six analyses the capacity stabilized at a value of about 10 ng. A fast loss of capacity during early analyses with subsequent stabilization, has been observed earlier with other polyclonal antibodies[39,41]. However, in those cases the initial capacity drop did not exceed 50%. Obviously, under the experimental conditions used, the anti-aflatoxin immuno precolumn is highly susceptible to denaturation and/or bleeding of the immobilized antibody. In view of the low amounts of aflatoxins present in real samples, the recovery should be satisfactory, even with the low capacity observed after stabilization.

Regeneration of the immuno precolumn

Desorption of the immuno precolumn with methanol-water (70:30, v/v) causes denaturation of the immobilized antibodies. Consequently, the antibodies have to be regenerated before the next analysis. This can be performed by restoring the physiological conditions, the simplest solution being flushing with water—with the volume and pH of the flushing solution being important parameters. The influence of the pH was investigated by flushing a 'stabilized' 10 mm × 4.0 mm I.D. immuno precolumn with different volumes of 0.05 M phosphate buffers of pH 7.0 and 8.0 (step 1 in Table 1), before overloading the immuno precolumn with an aqueous solution containing 500 ng of aflatoxin G2; pH 7.0, which is closer to the physiological pH, gave somewhat better results than pH 8.0 (Figure 4). No significant difference

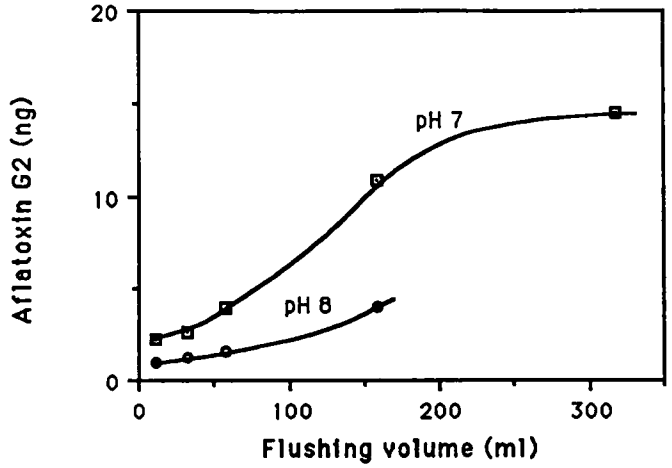

Figure 4 Dependence of 10 mm × 4.0 mm I.D. immuno precolumn capacity on volume and pH of solution used for regeneration prior to sample loading (step 1 in Table 1) Analyte, aflatoxin G2. For conditions see text.

was found when flushing the immuno precolumn with pure water instead of pH 7.0 buffer.

In all cases the immuno precolumn capacity—i.e., regeneration—improved when using a larger flushing volume. It is interesting to know, however, whether the improved performance of the immuno precolumn is due to an increased volume of the flushing solvent or to an increased contact time. To investigate this aspect, a constant amount of pure water (10.5 ml) was used, but the contact time was varied between 2 and 80 min by using different flow rates. The data in Figure 5 show that regeneration of the immuno precolumn primarily depends on the contact time and not on the amount of water used. Obviously, regeneration of the biological activity is a relatively slow process. (It is important to note that with a polyclonal anti-estrogen antibody which was tested under the same conditions, no such time dependence was found[42].) As a result, regeneration should be fairly prolonged in order to re-establish most of the activity of the immunosorbent. In order to maintain an acceptable analysis time, a regeneration time of 20 min was chosen in all subsequent experiments.

Calibration plots

The calibration plots of the aflatoxins B2 and M1 (Figure 6), recorded using a 'stabilized' 10 mm × 4.0 mm I.D. immuno precolumn, show the saturation effects, which are typical for precolumns with limited capacity: for high amounts pre-concentrated the absolute recovery approaches a constant value. With aflatoxin B2 this value was 25–30 pmol (7.8–9.4 ng), while for aflatoxin M1 a value of 6–8 pmol (2.0–2.6 ng) was found. The differences in capacity values are unexpected, because an immuno precolumn containing a monoclonal antibody should exhibit the same

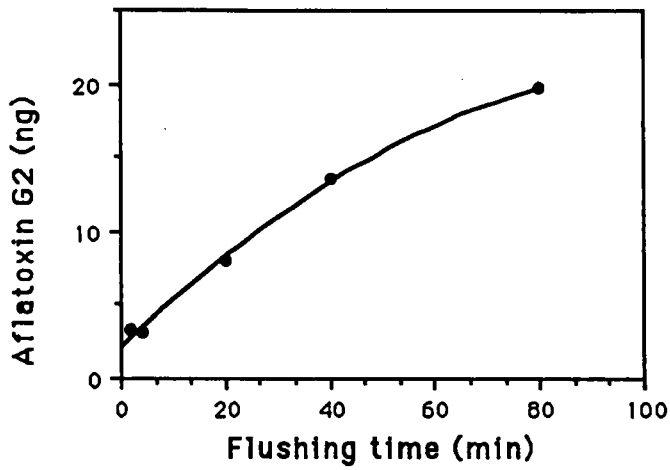

Figure 5 Dependence of 10 mm × 4.0 mm I.D. immuno precolumn capacity on water flushing time (step 1 in Table 1). 10.5 ml of water were used in all experiments. Analyte, aflatoxin G2. For conditions, see text.

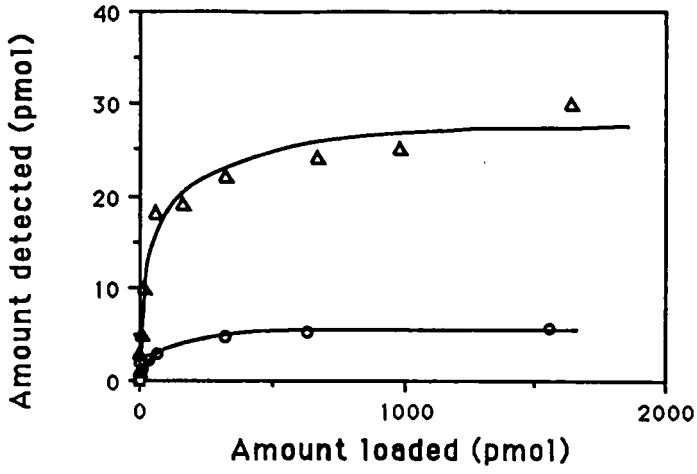

Figure 6 Calibration plot for aflatoxin B2 (△) and M1 (○), recorded using a 10 mm × 4.0 mm I.D. immuno precolumn. For conditions, see text.

number of active sites for all analytes. The most simple explanation is that more than one clone is present in the immuno precolumn.

For both aflatoxins tested the calibration curves showed a linear dynamic range up to 15 pmol (amount loaded). The recovery in this range was 80% for aflatoxin B2, but only 9% for aflatoxin M1, which is the main analyte to be determined in milk. The recovery could be significantly improved by using a 10 mm × 10 mm I.D. instead of the 10 mm × 4.0 mm I.D. immuno precolumn. With the larger immuno precolumn (the adapted analytical procedure is given in the Experimental section), the linear dynamic range improved up to at least 30 pmol (10 ng) of aflatoxin M1 with a recovery of 70%. The 'stabilized' capacity for aflatoxin M1 was 15–20 ng. Over 15 analyses with 10-ml aqueous standard samples were performed with the same immuno precolumn without a loss of recovery being observed. No breakthrough occurred if up to 30 ml of standard sample containing 5 ng of aflatoxin M1 were loaded.

Loading flow rate

The flow rate used to load the analyte on the immuno precolumn may be an important parameter. In this study, no influence on the recovery was found for flow rates of between 5 and 19 ml/min. Obviously the kinetics of the antibody-antigen interaction and of the diffusion processes is not critical in the indicated flow rate range.

Analysis of spiked urine and milk samples

In order to study the selectivity of the immunoaffinity sample pretreatment, biological samples were spiked with aflatoxins and analyzed according to the procedure in

Table 1. Figure 7 shows a chromatogram of urine spiked with 200 ng/l of aflatoxin B2 and the corresponding blank. The only off-line sample pretreatment performed was dilution of 5 ml of urine with an equal amount of water before loading it on a fresh 10 mm × 4.0 mm ID. immuno precolumn. By comparison with a loop injection, the recovery was found to be 95%. Figure 7 nicely demonstrates the high selectivity that is achieved when directly injecting a biological sample. Figure 8 shows the chromatogram of a 10-ml sample of raw milk; the peak area for aflatoxin M1 corresponds with a concentration of about 90 ng/l, compared with spiked aqueous standard samples. The detection limit for aflatoxin M1 in milk was about 10 ng/l. Since the determination of aflatoxin M1 in milk samples is the primary goal of this study, repeated analysis of spiked samples was attempted. Unfortunately, the repeatability was rather poor. Contrary to what was found with aqueous samples, no stabilization occurred: the capacity kept decreasing with each further analysis, while the recovery varied rather strongly. Therefore, a fresh immuno precolumn had to be used for every analysis.

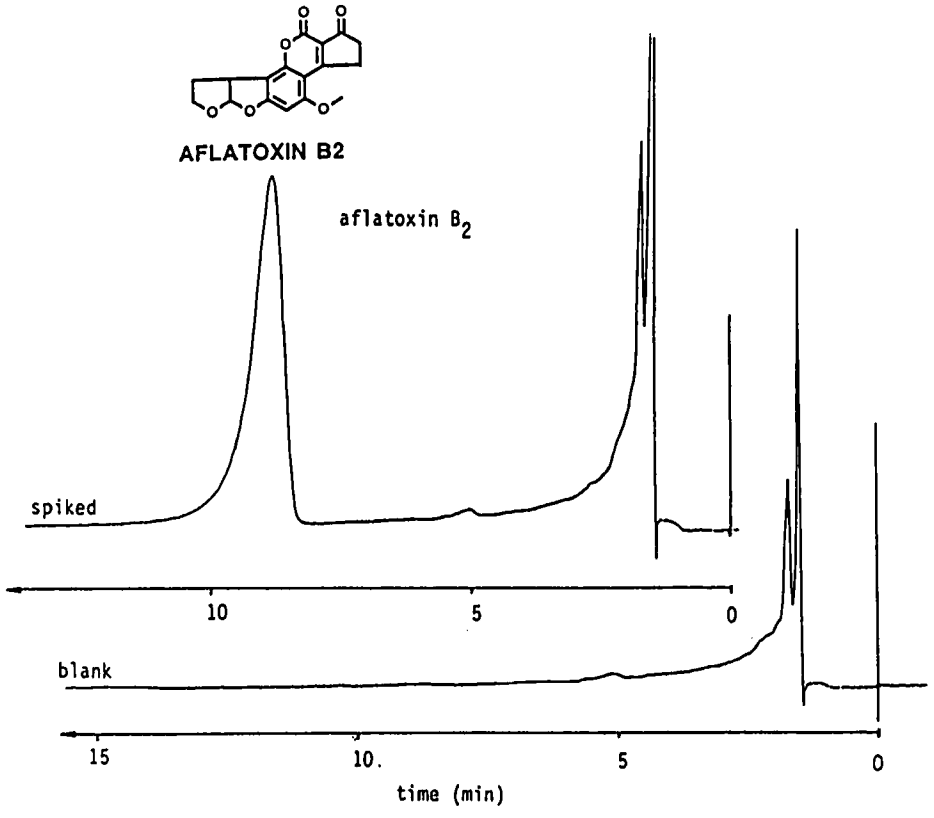

Figure 7 Chromatogram of a urine sample spiked with 200 ng/l of aflatoxin B2, and of the corresponding blank urine. The samples were filtered and diluted with an equal amount of water. An aliquot of 10 ml (containing 5 ml urine) was loaded on a fresh 10 mm × 4.0 mm I.D. immuno precolumn and processed according to the analytical procedure.

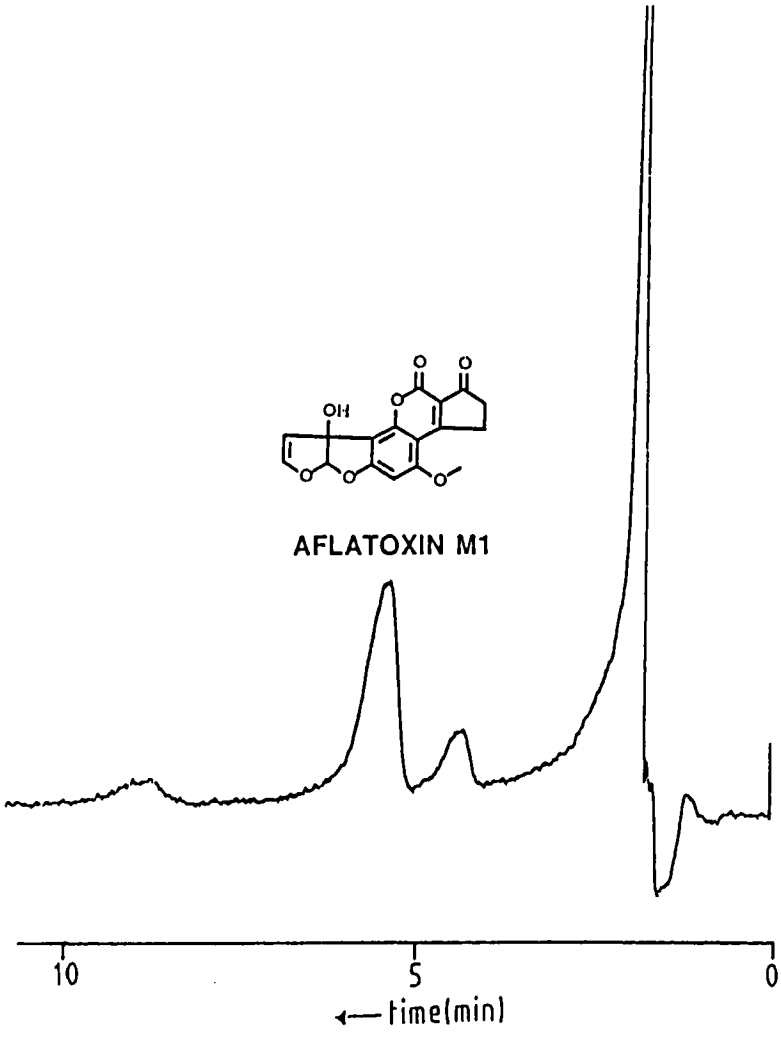

Figure 8 Chromatogram of a crude milk sample. 10 ml of untreated milk were loaded on a fresh 10 mm × 4.0 mm ID. immuno precolumn and processed according to the analytical procedure.

CONCLUSIONS

A column-switching system containing an anti-aflatoxin immunoaffinity precolumn was designed for automated sample pretreatment coupled on-line to LC. The critical desorption step was carried out with an aqueous solution containing 70% methanol, this step being combined with subsequent on-line dilution and reconcentration on a C-18 precolumn. The non-selective desorption can, in principle, be used with any other immunosorbent because such high percentage of organic modifiers will cause denaturation of the antibodies and induce the release of the trapped analytes.

The monoclonal antibodies used in this study could be regenerated after desorption by flushing with water. The efficiency of the regeneration primarily depended on the contact time with water. Although the immuno precolumn lost about 90% of its initial capacity during the first 5–6 analyses, the remaining capacity (15–20 ng of aflatoxin M1 for a 10 mm × 10 mm I.D. precolumn), was constant for at least 15 analyses, and was sufficient to allow recoveries of 70% with a linear dynamic range from 10 to 1000 ng/l for 10-ml standard samples of aflatoxin M1. The overall analysis time was 45 min, and the detection limit was 10 ng/l of aflatoxin M1 in milk. The method shows good selectivity with biological samples, which nicely confirms the earlier results of Mortimer *et al.*[2]. However, if milk samples were analyzed, a new immuno precolumn had to be used for every analysis, probably because milk components deteriorate the immunosorbent. In future work, the rapid deterioration of the immunosorbent with milk samples will be investigated in order to find conditions allowing repeated use of the immunosorbent. Besides, the characteristics of other anti-aflatoxin antibodies will be studied.

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