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On-line immunochemical detection in liquid chromatography using fluorescein-labelled antibodies

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ABSTRACT

A postcolumn immunochemical detection system for on-line coupling to HPLC is described. The effluent from a reversed-phase LC column is mixed with fluorescein-labelled antibodies that are added via a mixing union. Antigenic analytes react with the antibodies to form strongly fluorescent immunocomplexes. In a second step, free antibodies are removed prior to fluorescence detection via passage through a small column packed with an antigen-bound support. The performance of the immunochemical reaction system was investigated using digoxin and its metabolites as analytes and fluorescein-labelled Fab fragments of polyclonal anti-digoxigenin as immunoreagent. This system tolerates up to 95% methanol or 45% acetonitrile in the LC eluent, allowing the separation of digoxin and its metabolites. The immunoreaction sequence is in equilibrium after *ca.* 1 min resulting in peak broadening comparable to that in standard postcolumn derivatization systems. The detection limits obtained for digoxin and digoxigenin after separation on a C_{18} column are 200 and 50 fmol, respectively. The applicability of the method is demonstrated for the bioanalysis of digoxin and digoxigenin. Owing to the high selectivity of the immunodetection system, sample pretreatment can be reduced to deproteination and dilution of plasma and urine samples. Detection limits in both matrices (100-μl injections) are $1 \cdot 10^{-9}$ *M* for digoxigenin and $4 \cdot 10^{-9}$ *M* for digoxin.

INTRODUCTION

The inherent selectivity of biospecific interactions is widely exploited in affinity chromatographic separations and immunoassays [1,2]. Immunoaffinity sorbents have found application in the selective preconcentration of low- and high-molecular-mass compounds [3–10] in combination with liquid chromatography. Immunoassays, on the other hand, have revolutionized many fields of clinical chemistry and biochemistry especially in the form of radioimmunoassays (RIA). Although highly sensitive, immunoassays suffer from cross-reactivity, *i.e.*, the reactions of the antibodies with more than one analyte, leading to erroneous results. Consequently, HPLC is frequently employed in a fractionation step prior to the immunoassay [11–14].

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The on-line coupling of liquid chromatography with an immunoassay would overcome this problem in an elegant way by combining the high separation power and ease of automation of HPLC with the selectivity and sensitivity of immunoassays. Several approaches have been described to perform continuous-flow immunoassays in the form of a postcolumn reaction detection system, most of them being based on sequential addition immunoassay (SAIA). Cassidy *et al.* [15] developed a kinetically controlled immunoassay based on the sequential addition of antibody, sample and label on a protein A column, performing immunoassays for albumin and transferrin in under 1 min. Another continuous-flow competitive assay involving enzyme-labelled antibodies was described by Mattiasson *et al.* [16]. This system was coupled to a size-exclusion column allowing the monitoring of horseradish peroxidase. A different approach was used

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by Kusterbeck *et al.* [17], who developed a detection technique based on a displacement reaction between ^{125}I- or fluorescein-labelled 2,4-dinitrophenyl (DNP)-insulin and DNP-containing analytes.

The sequential nature makes SAIA techniques unsuitable candidates for on-line coupling to liquid chromatographic systems, since it does not allow the continuous monitoring of the LC effluent. In this paper we report the on-line coupling of reversed-phase liquid chromatography with a fluoroimmunoassay using fluorescein-labelled antibodies. The immunoassay is performed in a postcolumn derivatization system and is entirely based on association reactions of antibodies with analytes (antigens) eluting from the analytical column. Owing to the generally high reaction rate of association reactions between antibodies and antigens, the peak broadening obtained in this system is comparable to that with standard postcolumn derivatization systems in liquid chromatography. By using antibodies labelled with fluorescein, detection limits in the nanomolar range are obtained. The selectivity of the method is demonstrated by the determination of antigens in virtually untreated plasma and urine samples.

EXPERIMENTAL

Chemicals and supports

Digoxin, digoxigenin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA), sodium metaperiodate, sodium tetrahydroborate, sodium hydrogensulphite and Tween 20 from Merck (Darmstadt, Germany) and all organic solvents (analytical-reagent grade) from J.T. Baker (Deventer, Netherlands). Immunoaffinity-purified fluorescein-labelled Fab fragments of polyclonal anti-digoxigenin (Fab-DIG) and blocking solution consisting of a casein hydrolysate were obtained from Boehringer Mannheim (Mannheim, Germany). The immunoreagent solution was prepared by dissolving Fab-DIG ($1.3 \cdot 10^{-9} M$) in phosphate-buffered saline (PBS) (pH 7.5) and adding 0.5% of Tween 20.

Amino-silica was synthesized according to a procedure described by Ernst-Cabrera and Wilchek [18] using Nucleosil Si-100 (10 nm pore size) (Macherey–Nagel, Düren, Germany). Affi-Prep HZ and aminoethyl Bio-Gel P-100 were purchased from Bio-Rad Labs. (Richmond, CA, USA), Fractogel TSK AF from Merck and Carbolink hydrazide from Pierce (Rockford, IL, USA).

Synthesis of antigen-bound supports

Digoxin (100 mg) was suspended in 10 ml of ethanol and 10 ml of 0.1 *M* sodium metaperiodate solution were dropwise added in the dark with stirring. After 30 min the excess of metaperiodate was titrated with 1 *M* sodium hydrogensulphite solution until disappearance of the brown colour. After addition of 5 ml of saturated ammonium sulphate solution, the ethanol phase was recovered and used without further treatment for the synthesis of digoxin-bound supports.

Binding of digoxin to amino supports. Amounts of 200 mg of the respective amino supports (amino-silica, aminoethyl Bio-Gel P-100, Fractogel TSK AF) were suspended in 10 ml of potassium carbonate buffer (100 m*M*, pH 9.5), 10 ml of the ethanolic solution of oxidized digoxin were added dropwise and the mixture was stirred for 2 h at room temperature. Then 10 ml of sodium tetrahydroborate solution (15 mg/ml) were added and the reaction was allowed to proceed for 1 h. The digoxin support obtained was washed with 200 ml of dimethyl sulphoxide–water (50:50, v/v), 500 ml of distilled water and 200 ml of PBS and stored at 4°C.

Binding of digoxin to hydrazide supports. Affi-Prep HZ and Carbolink hydrazide-activated supports were suspended in 10 ml of acetate buffer (100 m*M*, pH 5.5), 10 ml of the ethanolic solution of oxidized digoxin were added dropwise and the mixture was stirred for 2 h at room temperature. No reduction of the hydrazone was carried out. The digoxin supports produced in this way were washed according the procedure described for amino supports.

Blocking of the derivatized supports with a casein hydrolysate in order to reduce non-specific binding was carried out according to the manufacturer's procedure. In the final system, Tween 20 was added to the immunoreagent solution to prevent non-specific binding.

HPLC system

All experiments were carried out in an LC system consisting of a Kratos-ABI (Ramsey, NJ, USA) Spectroflow 400 pump, a Kontron (Zürich, Swit-

zerland) MSI 660 autosampler, a 100 × 3.0 mm I.D. stainless-steel separation column packed with 5-μm Nucleosil ODS (Macherey–Nagel) and a Kontron SFM 23 fluorescence detector (excitation wavelength 488 nm, emission wavelength 514 nm). The LC mobile phase was acetonitrile–water (30:70, v/v) pumped at a flow-rate of 0.25 ml/min. The immunoreagent pump was a Pharmacia (Uppsala, Sweden) P3500 used at a flow-rate of 0.5 ml/min. Mixing of the eluent with the immunoreagent solution was performed by using an inverted Y-type low-dead volume mixing union. The reaction coil (volume 600 μl) consisted of 0.3 mm I.D. PTFE tubing; the reaction was performed at 20°C. A 10 × 3.0 mm I.D. column slurry packed with Carbolink hydrazide-coupled digoxin was used to bind free antibodies.

Pretreatment of urine and plasma samples

Blank and spiked urine samples were diluted 1:1 with water and filtered through a 2-μm membrane filter. The filtrate was injected into the LC system. Blank and spiked plasma samples (1 ml) were deproteinated with acetonitrile (1.5 ml). After centrifugation for 15 min the supernatant was injected into the LC system.

RESULTS AND DISCUSSION

Design of the postcolumn immunodetection system

In contrast to immunoassays, where the immunoreaction is usually carried out in batch, on-line coupling to an LC system requires fast reaction times in order to minimize extra-column band broadening. Consequently, only those immunoassays that have reaction times of the order of minutes rather than hours should be considered. Therefore, all assays that are based partly or entirely on the dissociation of immunocomplexes can be excluded. The postcolumn immunodetection system (for the principle, see Fig. 1) presented here is based on the kinetically fast association of fluorescein-labelled antibodies with antigenic analytes. In a first step, the antibodies are added to the LC effluent via a mixing union. Antigenic analytes react with the antibodies to form strongly fluorescent immunocomplexes in concentrations proportional to the analyte concentration. In a second step the excess of free antibodies is removed prior to fluorescence detection via passage

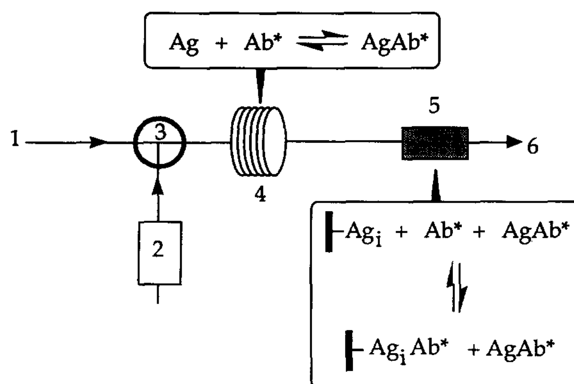

Fig. 1. Scheme of the immunodetection system. 1 = Effluent from analytical column; 2 = immunoreagent pump; 3 = mixing union; 4 = reaction coil; 5 = immobilized-antigen column; 6 = to fluorescence detector. Ag = antigen; Ab* = fluorescein-labelled antibody; AgAb* = immunocomplex; Ag_i = immobilized antigen.

through a small column packed with an antigen-bound support (see Fig. 1).

We investigated the performance of such a reaction scheme using the fluorescein-labelled Fab fragments of anti-digoxigenin antibodies (Fab-DIG). The Fab fragments which have a molecular mass of *ca.* 45 000 are labelled with 2–3 mol of fluorescein per mol of Fab fragment according to manufacturer's specifications. These Fab-DIG antibodies are highly cross-reactive with all compounds possessing the genin moiety of the original immunogenic cardenolide, *e.g.*, digoxin (for structure, see Fig. 2) and its metabolites [19].

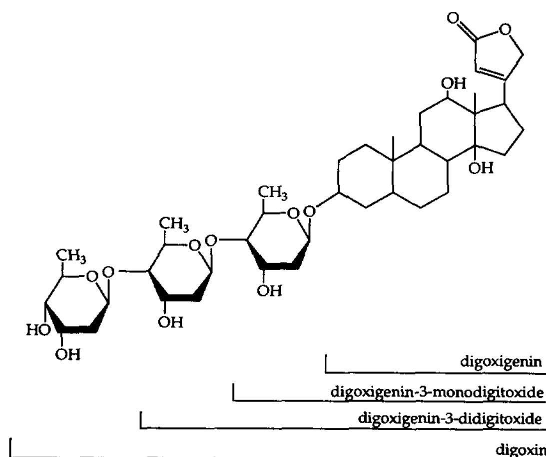

Fig. 2. Structure of digoxin and its metabolites.

Synthesis of an antigen-bound support

The separation step of the postcolumn immunoassay prior to fluorescence detection can be performed by exploiting the physico-chemical differences of free and bound antibodies. A possible difference in molecular mass or charge between free and bound antibodies could be exploited by membrane-based separation systems or ion exchangers, respectively. The most universal means of separation, however, is the implementation of a solid support or a membrane to which the antigen is immobilized while still being reactive towards the antibodies. The synthesis of such an immunoaffinity support or membrane for low-molecular-mass antigens can be performed in a similar manner to the way in which the hapten is bound to a carrier protein to induce the production of antibodies.

With anti-digoxigenin antibodies, digoxin instead of digoxigenin was bound to silica gel, agarose or polymeric supports. Digoxin is highly cross-reactive towards anti-digoxigenin antibodies compared with digoxigenin (manufacturer's specification) and provides an ideal spacer group if it is bound via its hydrophilic carbohydrate moiety. After oxidation of the vicinal OH groups with sodium metaperiodate, the resulting aldehyde groups were bound to supports functionalized with either amino or hydrazide groups. Binding to amino supports requires the subsequent reduction of the Schiff base with sodium tetrahydroborate to obtain a stable bonding. The hydrazones formed in the reaction with hydrazide supports are stable even without further reduction. The highest surface loading of digoxin was obtained for Affigel-Hz, Carbolink hydrazide and aminoethyl Bio-Gel P-100 and ranged from 5 to 10 μmol/g dry support.

In addition to a high surface loading with digoxin, a low degree of non-specific binding is a crucial requirement since non-specific binding would result in the removal of both free antibodies and immunocomplexes and, thus, in an increase in detection limits. All the supports investigated except Carbolink hydrazide exhibited a high degree of non-specific binding (higher than 60%) that could not be suppressed by blocking of the supports with casein hydrolysate or Tween 20. The agarose-based Carbolink hydrazide, however, provided virtually no non-specific binding (lower than 2%) whereas 75% of the fluorescent antibodies present in the polyclonal antiserum were retained. Changes in the flow-rate did not influence the binding of the antibodies, indicating that the background fluorescence is caused by either inactive antibodies or other protein impurities and not by slow association kinetics.

Optimization of parameters affecting the immunoreaction

Organic modifier and pH. Most reversed-phase liquid chromatographic eluents contain organic modifiers such as methanol or acetonitrile and separations are carried out mostly at pH values between 2 and 8. As immunoreactions normally take place in buffered aqueous media at approximately neutral pH, the effects of different concentrations of methanol or acetonitrile and different pH values on the detector response were tested. All experiments were carried out using flow-injection analysis (FIA) at a mixing ratio of 1:2 between the LC eluent and immunoreagent solution.

Fig. 3 (representing organic modifier concentrations after mixing) shows that methanol and acetonitrile influence the immunoreaction in different ways. Whereas up to 95% methanol could be tolerated in the LC eluent (corresponding to *ca.* 32% after mixing), acetonitrile concentrations higher than 45% (15% after mixing) caused a considerable decrease in antibody binding to the immobilized-digoxin support. However, with these maximum concentrations of organic modifier most com-

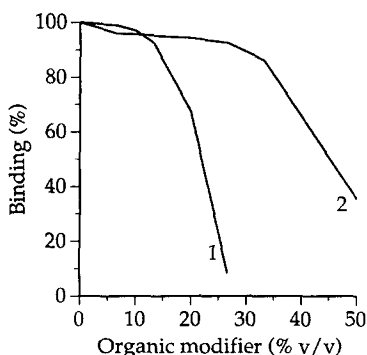

Fig. 3. Influence of organic modifier on the binding to the immobilized-digoxin column. 1 = Acetonitrile; 2 = methanol. 100% response refers to the binding obtained with PBS in both LC eluent and immunoreagent solution. All data (n = 3) were acquired in the flow-injection mode. For other conditions, see Experimental.

pounds of interest can still be chromatographed on a C_{18} stationary phase. Further, it was found that the immunoreaction was unaffected by pH changes in the range 5–8. By employing a strong buffer in the immunoreagent solution (usually PBS of pH 7.5) it is therefore possible to operate the LC column at pH values ranging from 2 to 11 without affecting the immunoreaction.

Reaction time and concentration of Fab-DIG. The reaction rate of the association reaction between Fab-DIG and analytes and the dimensions of the postcolumn reaction system are the most important factors that contribute to extra-column band broadening and therefore determine the sensitivity of the analytical method. The amount of immunocomplex formed during the immunoreaction depends on the reaction rate of the association, the concentration of Fab-DIG employed and the mass transport of the reaction detection system. As the reaction rate constant for the association reaction of digoxin with anti-digoxin, $k_a = 10^7$ l $mol^{-1}s^{-1}$ [20], is extremely high, the latter two parameters mainly determine the performance of the postcolumn immunodetection system. Fig. 4 shows the dependence of the detector response on the reaction time using a Fab-DIG concentration of $1.3 \cdot 10^{-9}$ *M*. The stage of equilibrium is reached after *ca.* 1 min, which demonstrates that immunoreactions of this type are well suited for postcolumn reaction detection systems even at extremely low antibody concentrations. At higher Fab-DIG concentrations the gain in response was counterweighted by the increase in noise caused by the non-binding fraction of Fab-DIG.

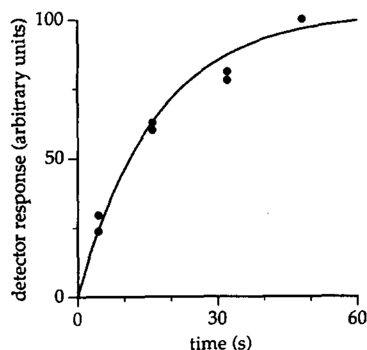

Fig. 4. Influence of the reaction time on detector response. All data ($n = 3$) were acquired in the flow-injection mode. For other conditions, see Experimental.

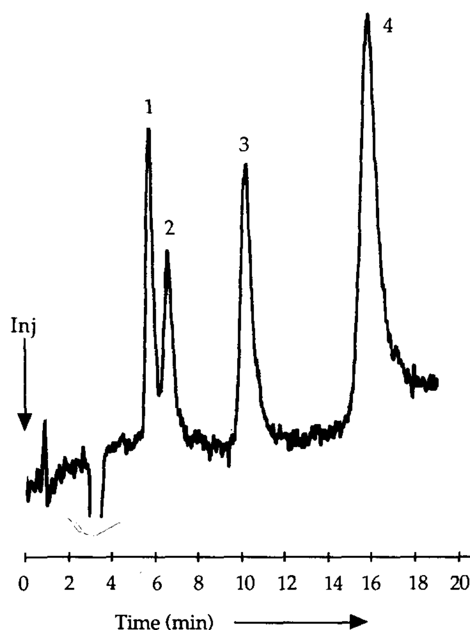

Fig. 5. Chromatogram obtained from the hydrolysis of digoxin (10^{-8} *M*) after 30 min at 37°C and pH 3. 1 = Digoxigenin; 2 = digoxigenin-3-monodigitoxide; 3 = digoxigenin-3-didigitoxide; 4 = digoxin. Injection volume, 100 μl; for other conditions, see Experimental.

Coupling to a reversed-phase LC system

Fig. 5 shows a chromatogram obtained from a hydrolysate mixture of digoxin using fluorescence detection after postcolumn immunochemical reaction. The hydrolysis was carried under the conditions described by Sonobe *et al.* [21]. All degradation products of digoxin, digoxigenin-3-didigitoxide, digoxigenin-3-monodigitoxide and digoxigenin are antigenic towards Fab-DIG and can therefore be monitored with the immunoreaction system. The additional band broadening of the digoxin peak caused by the introduction of a mixing piece, reaction coil and immobilized-digoxin column (reaction coil volume 600 μl; column dimensions 10 × 3.0 mm I.D.) prior to the fluorescence detector amounted to $\sigma^2 = 111$ s^2 compared to $\sigma^2 = 270$ s^2 for the same chromatographic system using UV detection. The separation efficiency in terms of plate numbers decreases from $N = 2610$ for a direct detection system to $N = 1850$ for the postcolumn reaction system. As the reaction kinetics of the antibody–antigen reaction are very fast (see Fig. 4), the band

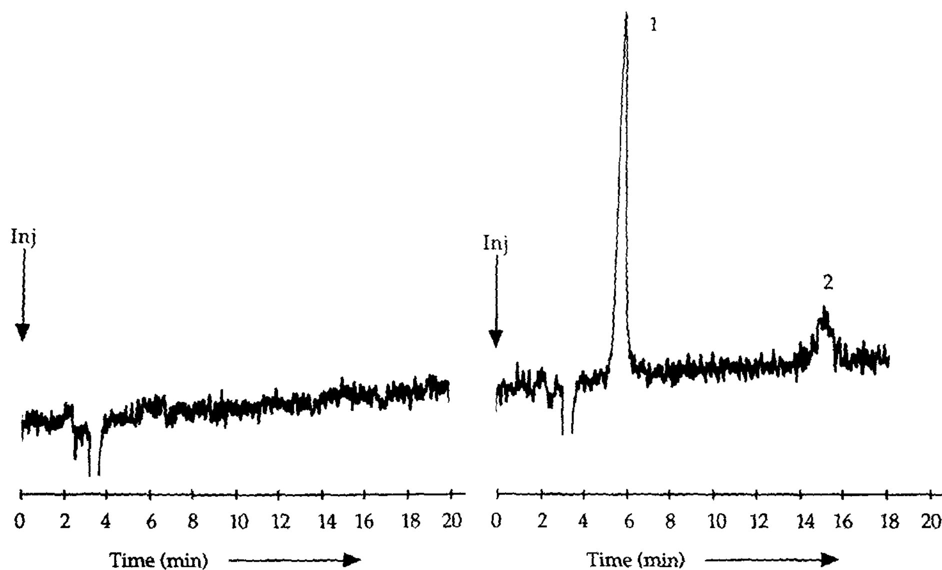

Fig. 6. Chromatograms of (left) blank plasma and (right) plasma spiked with (1) $4 \cdot 10^{-9}$ *M* digoxigenin and (2) $4 \cdot 10^{-9}$ *M* digoxin. Injection volume, 100 μl; for other conditions, see Experimental.

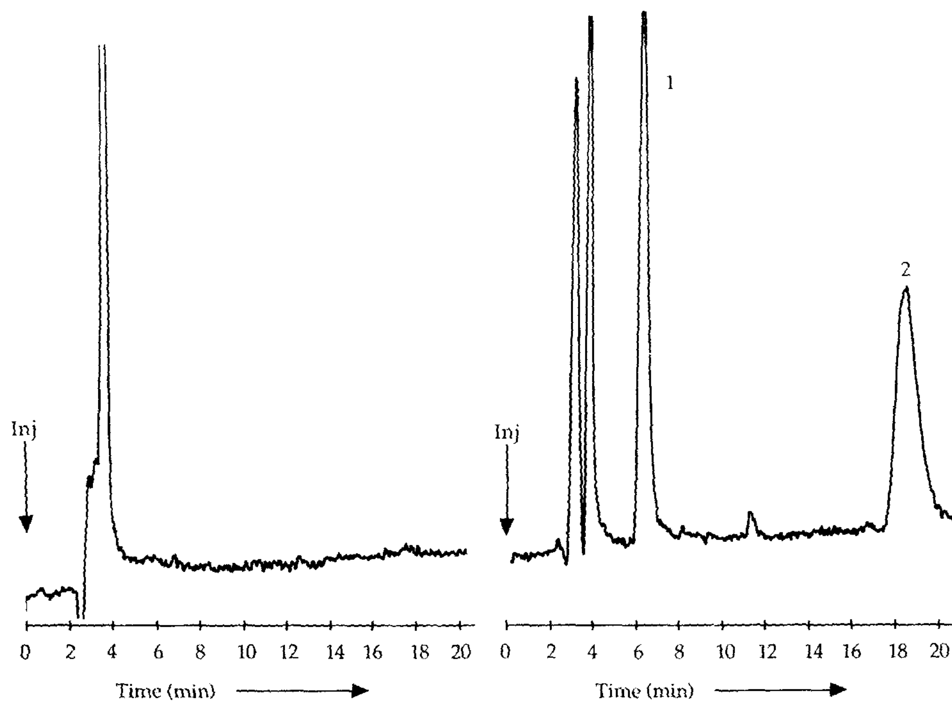

Fig. 7. Chromatograms of (left) blank urine and (right) urine spiked with (1) 10^{-8} *M* digoxigenin and (2) 10^{-8} *M* digoxin. Injection volume, 100 μl; for other conditions, see Experimental.

broadening mainly originates from the design of the reaction detection system. This decrease in separation efficiency, however, is compensated for by the enormous gain in selectivity as the analysis of plasma and urine samples demonstrates (see below). The organic modifier content of the LC mobile phase, *i.e.*, 30% acetonitrile, caused no loss in sensitivity as it is well below the critical value of 45%.

Analytical data

The detection limits (signal-to-noise ratio = 3) for digoxin and digoxigenin amounted to 200 and 50 fmol, respectively (Fab-DIG concentration 1.3 n*M*; injection volume 100 μl). Although these detection limits are higher than those obtained with RIA, they compare favourably with all those methods that determine both compounds simultaneously. The relative standard deviation for 20-μl injections of 100 fmol of digoxigenin was less than 2.0% (n = 7). The detector response was linear (r = 0.998, n = 4) between 50 and 1000 fmol of digoxigenin. The relatively small linear range can be attributed to the fact that the Fab-DIG concentration used is in the same order of magnitude as the analyte concentration (which is usually the case in most immunoassays). Owing to this rather low Fab-DIG concentration, the immobilized-digoxin column could be used without regeneration for at least 500 injections before breakthrough of Fab-DIG occurred.

Applicability to the determination of digoxin and digoxigenin in urine and plasma

The selectivity of the immunochemical reaction detection system was tested by determining digoxin and digoxigenin in biological matrices such as plasma or urine. Sample pretreatment consisted of filtration and 1:1 dilution for urine and deproteination using acetonitrile and centrifugation for plasma. The chromatograms in Figs. 6 and 7 representing blank and spiked plasma and urine, respectively, demonstrate the high selectivity and sensitivity of immunochemical reaction detection. Virtually no interferences were observed in the analysis of plasma samples, whereas chromatograms deriving from urine samples exhibited a single peak eluting with the void volume which possibly derives from cross-reactive steroid components of urine. Both digoxin and digoxigenin can be determined in urine and plasma with detection limits of $4 \cdot 10^{-9}$ and $1 \cdot 10^{-9}$ M, respectively (injection volume 100 μl).

CONCLUSIONS

The incorporation of an immunochemical reaction using fluorescein-labelled antibodies in a post-column reaction detection system is a promising way to improve both the selectivity and sensitivity of liquid chromatographic systems. Long incubation times characteristic of immunoassays can be avoided by employing solely association reactions. In this way the benefits of immunoassays, high sensitivity and selectivity, can be combined with the separation power of LC, avoiding the tedious collection of fractions characteristic for off-line systems.

The method presented here requires that (preferably fluorescent) labelled antibodies and an immobilized-antigen support are available. The labelling of antibodies with fluorescent labels has been extensively described [22]. The production of an antigen-bound support requires synthetic work which, in the case of low-molecular-mass antigens, can be performed in a similar way as the synthesis of hapten carriers used for the production of antibodies. High-molecular-mass antigens such as peptides or proteins might be coupled directly to activated supports via free amino groups. In order to reduce background fluorescence, non-binding labelled antibodies should be removed as far as possible. The immunoaffinity purification of polyclonal antisera using the antigen-bound support as chromatographic stationary phase is currently under investigation. In addition to regenerating the immobilized antigen column, this procedure should lead to the recovery of a homogeneous antibody fraction with high specificity.

The choice of antibodies, *i.e.*, monoclonal *vs.* polyclonal, obviously influences the selectivity and sensitivity of the analytical method in addition to the range of compounds that can be determined. Highly specific monoclonal antibodies with high binding constants are preferable if only a small number of structurally similar antigens are to be determined. Polyclonal antibodies may find application in metabolic screening procedures for drugs, for example, thus helping to identify antigenic metabolites.

The selectivity of the immunochemical detection system was demonstrated by the determination of digoxin and digoxigenin in virtually untreated bi-

ological matrices. Currently the coupling of the present method with on-line solid-phase extraction is being investigated, which should lead to a further decrease in detection limits.

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