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# High-performance liquid chromatography with on-line post-column immunoreaction detection of digoxin and its metabolites based on fluorescence energy transfer in the far-red spectral region

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**Abstract**

The combination of immunoassays with separation techniques such as chromatography can result in enhanced selectivity and sensitivity. This paper describes an on-line chromatography with immunochemical post-column fluorescence energy transfer detection for digoxin and its metabolites. *R*-phycoerythrin (PE) was used as the donor and an indodicarbocyanine dye (Cy5) as the acceptor label. These labels allow the detection in the far-red spectral region, which is more selective for biological samples. Hence, digoxin was labeled with PE using the activated digoxigenin-NHS-ester and monoclonal anti-digoxin antibody was labeled with Cy5. Digoxin and its metabolites was injected into the HPLC system followed by post-column injection of *R*-phycoerythrin labeled digoxin and by Cy5 labeled anti-digoxin antibody. Incubation time was provided using an open tubular reactor coil at room temperature. The detection was performed by measurement of the sensitized emission of Cy5 at 670 nm due to fluorescence energy transfer from PE labeled with digoxin. The system was optimized with regard to the concentrations of the used post-column reagents as well as incubation time and temperature. The dynamic range of digoxin spiked in 0.01 *M* phosphate buffer (pH 7.4) was 0.05 to 10 ng/ml with a correlation coefficient of 0.989. The limit of detection was 33 pg/ml. The precision of two controls, 0.4 and 4 ng/ml, was found to be 2.2 and 8.7% RSD, respectively, accuracy was 10.7 and 20.3% ($n=6$ in each case). 

*Keywords:* Hapten conjugation; Fluorescence energy transfer; Digoxin; Phycoerythrin; Cy5

## 1. Introduction

Fluorescence energy transfer involves non radiative energy transfer between a donor and an acceptor label, which leads to a measurable decrease in the intensity and lifetime of the donor fluorescence and an enhancement of the acceptor fluorescence [1]. In the present study digoxin labeled with *R*-phycoerythrin (PE) was used as the donor labeled antigen and monoclonal anti-digoxin antibody labeled with a cyanine dye (Cy5) as the acceptor labeled antibody. The use of these labels was already established by Shahdeo and Karnes using biotin as a model analyte [2].

Phycobiliproteins are a family of reasonably stable and highly fluorescent proteins derived from cyano-

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bacteria and eukaryotic algae. Due to their very high quantum yield and high extinction coefficients, these fluorescent proteins provide high sensitivity. Moreover, the sensitivity of PE conjugated antibodies is 5–10-times greater than that of the corresponding fluorescein conjugate [3–5]. Cy5 dyes have been shown to be useful as fluorescent labels for biological compounds and produce an intense signal in the far-red region of the spectrum where background fluorescence from biological matrices is very low [6].

The principle of the developed homogeneous competitive assay is described in Fig. 1: there is a competition of labeled and unlabeled antigen with the antibody. Due to binding of the labeled antigen and antibody, there is sufficient overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor for efficient fluorescence energy transfer to take place. PE was excited at 488 nm and the energy transfer emission (''sensitized emission'') could be measured at 670 nm which corresponds to the maximum of fluorescence emission. If no antigen is present in the sample, the antibody reacts with the labeled antigen, and thus maximum energy transfer takes place. The presence of antigen in the sample decreases the amount of antibody reacting with the labeled antigen resulting in inhibition of energy transfer.

Immunoassays are analytical methods based on the interaction of antibodies and antigens, which provide high sensitivity and selectivity. The selectivity is not complete, since antibodies are able to recognize an epitope of the antigen and not the entire antigen [7]. Hence, antigens having similar epitopes may interact with the antibody, which leads to reduced selectivity, especially regarding the determination of compounds

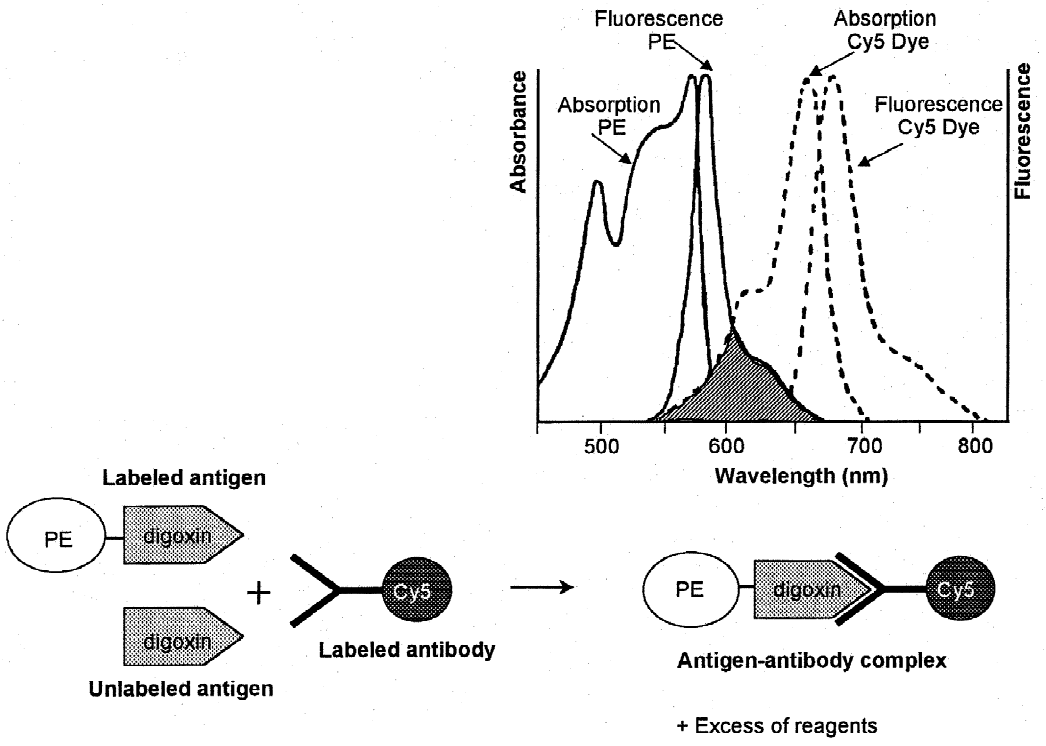


Fig. 1. Principle of the developed homogeneous competitive immunoassay based on fluorescence energy transfer detection: there is a competition of unlabeled (digoxin) and labeled antigen (digoxigenin-PE) with the labeled antibody (anti-digoxin-Cy5). Due to the binding of the labeled antigen and labeled antibody, there is sufficient overlap (marked area) of the emission spectrum of the digoxigenin-PE and the absorption spectrum of anti-digoxin-Cy5 for efficient fluorescence energy transfer to take place. In absence of antigen maximum energy transfer will take place. The presence of antigen will inhibit fluorescence energy transfer.

of similar structure such as metabolites of drugs. The combination of immunoassays with separation techniques such as chromatography can result in enhanced selectivity and sensitivity by separating the compounds of similar epitopes prior to the immunoassay [8,9]. Several immunodetection systems for digoxin and its metabolites in combination with high-performance liquid chromatography (HPLC) have been reported in literature, most of which are in the off-line mode [10–13]. In these cases, fractions eluting from the HPLC column are manually collected and then analyzed off-line with the help of immunoassays, which is very time consuming. Oosterkamp and co-workers reported an on-line immunochemical detection system for digoxin and metabolites following liquid chromatography using fluorescein labeled antibodies [14,15]. The separation of bound and unbound antibody immunocomplexes was conducted using immobilized antigen affinity columns prior to detection.

The common use of digitalis-glycosides, e.g., digoxin, for the treatment of congestive heart failure over two centuries indicates the importance of these compounds [16]. Digoxin is the most widely used form of digitalis and it is the second most commonly prescribed drug in the USA. Due to the low therapeutic index of digoxin, dosing is important and therefore accurate measurement of digoxin concentrations is required. The very low clinical concentrations of digoxin in human plasma (0.5–2.0 ng/ml), which are not measurable using conventional HPLC methods, require very sensitive analytical methods. The aim of the present study was to develop an on-line post-column homogeneous competitive immunoassay for digoxin and its metabolites based on fluorescence energy transfer. This system obviated the need for a separation step to eliminate excess labeled reagent and a regeneration of the affinity column.

## 2. Experimental

### 2.1. Chemicals

Digoxigenin-3-*O*-methylcarbonyl-[ϵ]-aminocaproic acid-*N*-hydroxysuccinimide ester (digoxigenin-NHS-ester) was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). Digoxin and digoxigenin were obtained from Sigma–Aldrich (St. Louis, MO, USA); digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and dihydrodigoxin were donated by GlaxoWellcome (Research Triangle Park, NC, USA). Affinity-purified monoclonal anti-digoxin antibody (subclass IgG1) was obtained from Biodesign (Kennebunk, ME, USA) and the Fluorolink MabCy5 labeling kit was purchased from Amersham Life Sciences (Pittsburgh, PA, USA). *R*-Phycoerythrin was obtained from Molecular Probes (Eugene, OR, USA). All organic solvents were purchased from Baxter (B&J Brand, Muskegon, MI, USA) and were of analytical grade. Dibasic sodium phosphate and monosodium phosphate were from Fisher (Midlothian, VA, USA), sodium chloride and potassium chloride were from Mallinkrodt (Paris, KY, USA).

### 2.2. Equipment

The principle of the post-column system had already been described by Shahdeo and Karnes [2]. The HPLC system consisted of two Gilson pumps Model 302 with a Model 802B manometric module (Gilson Medical Electronics, Middleton, WI, USA). The HPLC column used was a Zorbax ODS column (150 mm×4.6 mm I.D., 5 μm particle size, Macmod, Chadds Ford, PA, USA). All mobile phases were filtered through a 0.45-μm nylon filter (Alltech, Deerfield, IL, USA) and were helium sparged prior to use. Two metal-free static mixing tees and a polyether ether ketone (PEEK) Y connector (flow splitter) were purchased from Upchurch Scientific (Oak Harbor, WA, USA). The required incubation time for the immunoreaction was warranted by using a PTFE knitted open tubular reactor coil (KOT) (10 m×1.58 mm O.D.×0.5 mm I.D., 2.0 ml final volume) from Supelco (Bellefonte, PA, USA). A Rheodyne Model 7125 manual injector equipped with a 20-μl loop for HPLC and two injectors with 50-μl loops were used for reagent injection (Rheodyne, Rohnert Park, CA, USA). Injections were performed manually using Hamilton syringes (Reno, NV, USA).

Fluorescence detection was performed using a Hitachi F-1080 (12-μl flow cell) fluorescence detector (Hitachi, Tokyo, Japan) with excitation at 488 nm and emission at 670 nm. Data were acquired with a

Hewlett-Packard 3392A integrator (Hewlett-Packard, Avondale, PA, USA). Initial UV detection for digoxin, digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and dihydrodigoxin was accomplished using a Shimadzu SPD-6A UV spectrometric detector (Shimadzu, Kyoto, Japan) at 220 nm.

### 2.3. Sample preparation

#### 2.3.1. Labeling of digoxin with R-phycoerythrin

Following the instructions of the product information, 0.5 ml *R*-phycoerythrin (4 mg/ml) was centrifuged and the resulting pellet was reconstituted in phosphate-buffered saline (PBS) buffer, pH 7.0 [5]. In order to remove salts, and provide an environment that would support the labeling procedure the solvent was changed by retaining *R*-phycoerythrin on a Nap-10 column (Pharmacia Biotech, Piscataway, NJ, USA) followed by elution with PBS buffer, pH 7.0. The final volume was approximately 3 ml.

A 5-mg amount of digoxigenin-NHS-ester was dissolved in 5 ml dimethylformamide of which 400 μl (1 mg/ml) was allowed to react with the solution of *R*-phycoerythrin (Fig. 2a). The mixture was stirred slowly at 4°C overnight and was then dialyzed against PBS buffer, pH 7.0. Measurement of the final concentration of digoxigenin labeled *R*-phycoerythrin (*R*-PE) was performed on a Perkin-Elmer UV–Vis spectrophotometer Lambda 2S ($\lambda_{max}$=565 nm, $\epsilon$=1 960 000 $cm^{-1}$ $M^{-1}$) [17].

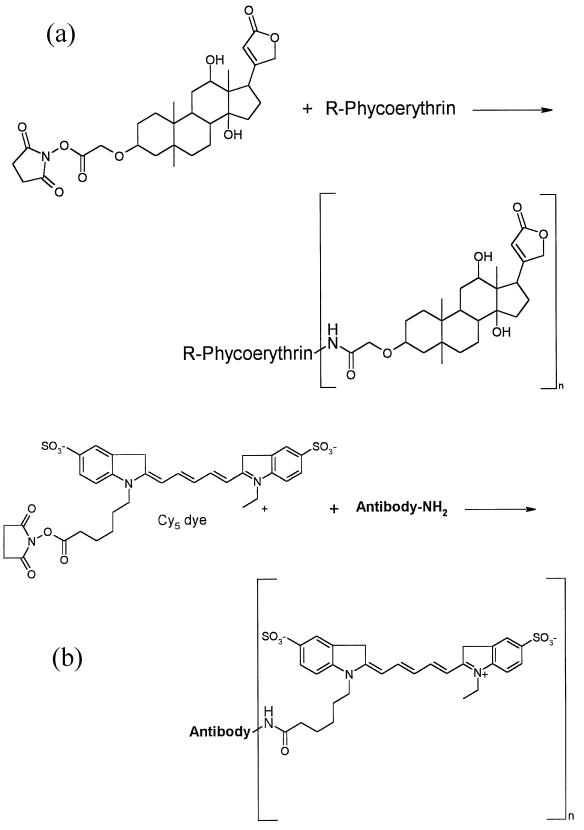


Fig. 2. (a) Synthetic scheme for labeling of *R*-phycoerythrin with digoxigenin-NHS-ester. (b) Structure of Cy5 dye, which was provided as a monofunctional *N*-hydroxysuccinimide ester for labeling with monoclonal anti-digoxin antibody.

#### 2.3.2. Labeling of monoclonal anti-digoxin antibody with Cy5

Labeling of the monoclonal anti-digoxin antibody was performed with the help of the Fluorolink Mab Cy5 Labeling Kit [6]. Cy5 was provided as a monofunctional-NHS-ester (Fig. 2b). Monoclonal anti-digoxin antibody was dissolved at 1 mg/ml in 50 m*M* PBS buffer, pH 7.0, 5 μl coupling buffer (1 *M* sodium phosphate buffer, pH 9.3) was added to 100 μl of the protein solution. The mixture was mixed thoroughly by gentle vortexing and transferred to the reactive dye vial, again mixed thoroughly, and incubated at room temperature for 30 min with additional mixing approximately every 10 min. The separation of protein from free dye was performed using a gel filtration column. The final dye/protein ratio was determined on a Perkin-Elmer UV–Vis spectrophotometer Lambda 2S (Cy5 dye: $\lambda_{max}$=650 nm, $\epsilon$=250 000 $cm^{-1}$ $M^{-1}$; protein: $\lambda_{max}$=280 nm, $\epsilon$=224 000 $cm^{-1}$ $M^{-1}$). The final ratio was approximately 10 mol dye per mol protein.

### 2.4. Off-line fluorescence energy transfer assay

Off-line experiments were performed to determine the optimal concentration of Cy5 labeled monoclonal anti-digoxin antibody (Mab-Cy5), incubation time and temperature for fluorescence energy transfer. Various concentrations of the reagents were mixed thoroughly by gentle vortexing to prevent foaming of the protein solution. The concentration of *R*-phycoerythrin digoxigenin (digoxigenin-PE) was kept at

$6.4 \cdot 10^{-10}$ *M* (0.5 ng/ml) since therapeutic plasma concentrations of digoxin are in this range. For the optimization of reagent concentrations, the mixture was incubated at room temperature for 30 min to ensure complete binding ($n=3$). The solution was injected manually using the 20-μl loop, which was directly connected to the Hitachi F-1080 fluorescence detector with excitation at 488 nm and emission at 670 nm. All solutions were prepared in 0.1 *M* phosphate buffer, pH 7.4, which was also used as carrier buffer.

Experiments concerning incubation time and temperature were based on the optimal reagent concentrations. Digoxigenin-PE and Mab-Cy5 were mixed by gentle vortexing and incubated at room temperature as well as at 37°C for 5, 10, 15, 20, 25 and 30 min ($n=3$). Incubation at 37°C was carried out using a Pierce Reacti-Therm heating block. The possible effect on energy transfer was then observed in the fluorescence detector.

The concentration of Mab-Cy5 was $1.28 \cdot 10^{-9}$ *M* and the digoxigenin-PE concentration was kept at $6.4 \cdot 10^{-10}$ *M*. The Inhibition of the energy transfer by digoxin was investigated using the optimized reagent concentrations and conditions at digoxin concentrations of $1.28 \cdot 10^{-11}$, $6.4 \cdot 10^{-11}$, $1.28 \cdot 10^{-10}$, $6.4 \cdot 10^{-10}$, $1.28 \cdot 10^{-9}$, $6.4 \cdot 10^{-9}$ and $1.28 \cdot 10^{-8}$ *M* ($n=3$). The dynamic range for digoxin was determined and the limit of detection (LOD) was calculated.

### *2.5. On-line HPLC–post-column reaction detection*

The HPLC system for digoxin alone consists of methanol–water (60:40) as mobile phase, the flow-rate was set to 1.2 ml/min. The second pump delivered 0.01 *M* phosphate buffer, pH 7.4 at 0.5 ml/min. For the separation of digoxin and its metabolites and for the determination of their response factors, the mobile phase consisted of tetrahydrofuran–water (20:80) [18]. The HPLC column was first connected to the UV detector and a mixture of $10.3 \cdot 10^{-9}$ *M* digoxigenin (DG), $7.7 \cdot 10^{-9}$ *M* digoxigenin monodigitoxoside (DM), $6.2 \cdot 10^{-9}$ *M* digoxigenin bisdigitoxoside (DB), $6.4 \cdot 10^{-4}$ *M* dihydrodigoxin (DHD) and $5.1 \cdot 10^{-9}$ *M* digoxin (D) was injected in order to determine the peak retention times. Resolution studies for the cardiac glycosides had to be carried out with relatively high quantities (nmol) of drug on column. DHD required at least 125-fold higher concentrations than digoxin to obtain an adequate signal for ultraviolet detectability due to the low molar absorptivity of DHD relative to digoxin and other metabolites. Then, the UV detector was removed and the column was reconnected to the on-line system where mixing of digoxin or its metabolites with digoxigenin-PE took place, followed by the injection of Mab-Cy5, incubation in the KOT and finally detection (see Fig. 3 for a schematic diagram).

### *2.6. Detectability of the on-line HPLC–post-column detection reaction*

Digoxin was injected (20 μl) on column in concentrations of 0, $6.4 \cdot 10^{-11}$, $1.28 \cdot 10^{-10}$, $6.4 \cdot 10^{-10}$, $1.28 \cdot 10^{-9}$, $6.4 \cdot 10^{-9}$ and $1.28 \cdot 10^{-8}$ *M*. Since the peak elution time of digoxin was 6 min and 43 s, 50 μl of $6.4 \cdot 10^{-10}$ *M* digoxigenin-PE was injected at 6.48 min, since it took approximately 5 s for digoxigenin-PE to reach the mixer. For the same reason, $1.28 \cdot 10^{-9}$ *M* Mab-Cy5 (50 μl) was injected immediately after the injection of digoxigenin-PE. The fluorescence energy transfer peak was detected at 670 nm. The LOD was calculated at the blank ($n=6$).

## 3. Results and discussion

### *3.1. Off-line fluorescence energy transfer assay*

As mentioned above, therapeutic plasma concentrations of digoxin are in the range of 0.5–2 ng/ml, and therefore the concentration of digoxigenin-PE was kept at $6.4 \cdot 10^{-10}$ *M* (0.5 ng/ml). Several concentrations of Mab-Cy5 ($3.20 \cdot 10^{-10}$, $6.40 \cdot 10^{-10}$, $1.28 \cdot 10^{-9}$, $2.56 \cdot 10^{-9}$, $3.84 \cdot 10^{-9}$, $5.12 \cdot 10^{-9}$ *M*) were tested and the energy transfer was observed (Fig. 4a). The fluorescence energy transfer was greater at higher concentrations, which indicates that antigen–antibody reaction still takes place. Up to a molar concentration of $3.84 \cdot 10^{-9}$ *M* an increase of fluorescence response was noticed. Above the molar concentrations of $3.84 \cdot 10^{-9}$ *M* a saturation effect was indicated. But due to the principle of the used competitive assay, a lower concentration of Mab-

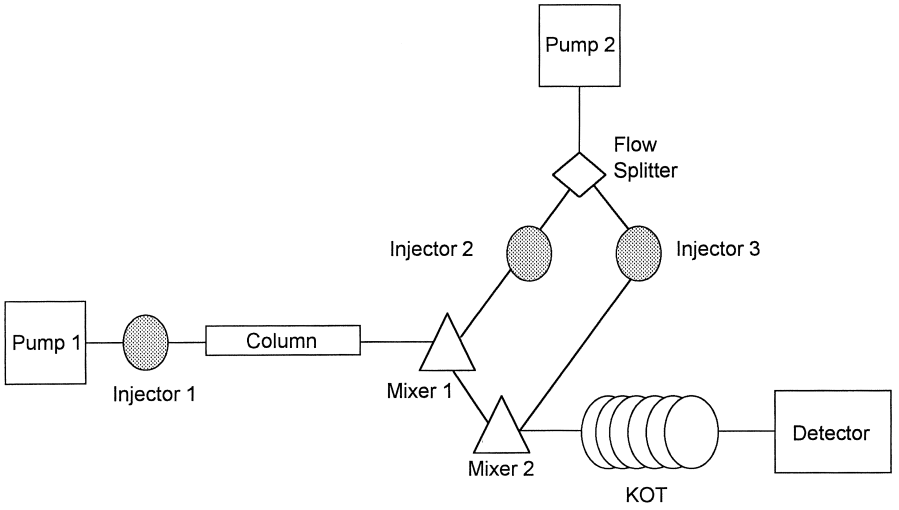


Fig. 3. Scheme of on-line HPLC system combined with post-column fluorescence energy transfer reaction detection.

Cy5 would be more suitable for the detection of digoxin. Therefore, the optimum concentrations of the reagents represent a compromise between the detectability of digoxigenin-PE and the corresponding lowest concentration of Mab-Cy5 at which fluorescence energy transfer takes place.

Additional experiments were carried out without addition of digoxigenin-PE to the Mab-Cy5 solutions to exclude a possible emission component from Cy5 itself due to direct excitation. There was direct excitation of Mab-Cy5 at concentrations of $2.56 \cdot 10^{-9}$ *M* and higher with the detector set at 488 nm excitation and 670 nm emission. Therefore, the optimum concentration of Mab-Cy5 was found to be $1.28 \cdot 10^{-9}$ *M* and was chosen for further experiments.

Fig. 4b shows the graph for the optimization of incubation time at room temperature and at 37°C. At room temperature it was observed that the response had already reached a plateau within 5 to 10 min and that there was a slight decrease with longer incubation time. Therefore, an incubation time between 5 to 10 min was considered to be the optimum time to ensure complete binding. When incubation at 37°C was carried out, no plateau was observed. The energy transfer peak was negligibly larger as compared to room temperature and the slight difference did not justify incubation at 37°C. Moreover, a strong decrease was found with incubation times longer than 5 min, which might have an influence on the feasibility of the method, because it would make it difficult to find out the optimum time for the injection of the post-column reagents. Hence incubation was carried out at room temperature and a KOT providing a 7-min incubation time was used for further experiments.

Fig. 4c shows the inhibition of fluorescence energy transfer in the presence of digoxin. A calibration curve was plotted with injection of digoxin, digoxigenin-PE, and Mab-Cy5 off-line and was found to be dynamic in the range of 0.01 ng/ml to 10 ng/ml. The correlation coefficient was 0.997 using a log-linear fit. The LOD was 6.9 pg/ml, calculated as 2×SD of the blank ($n=6$).

### *3.2. On-line HPLC–post-column reaction detection*

Fig. 3 shows a schematic illustration of the used HPLC system which was already established by Shahdeo and Karnes [2]. The system consisted of two pumps, three injectors, a column, two mixers, a flow splitter, a KOT and a detector. Pump 1 was set to a flow-rate of 1.2 ml/min. For the quantification of digoxin methanol–water (60:40) and for the separation of digoxin and its metabolites tetrahydrofuran–water (20:80) served as mobile phase, respectively. Pump 2 was set to a flow-rate of 0.5 ml/min delivering 0.01 *M* phosphate buffer to provide a

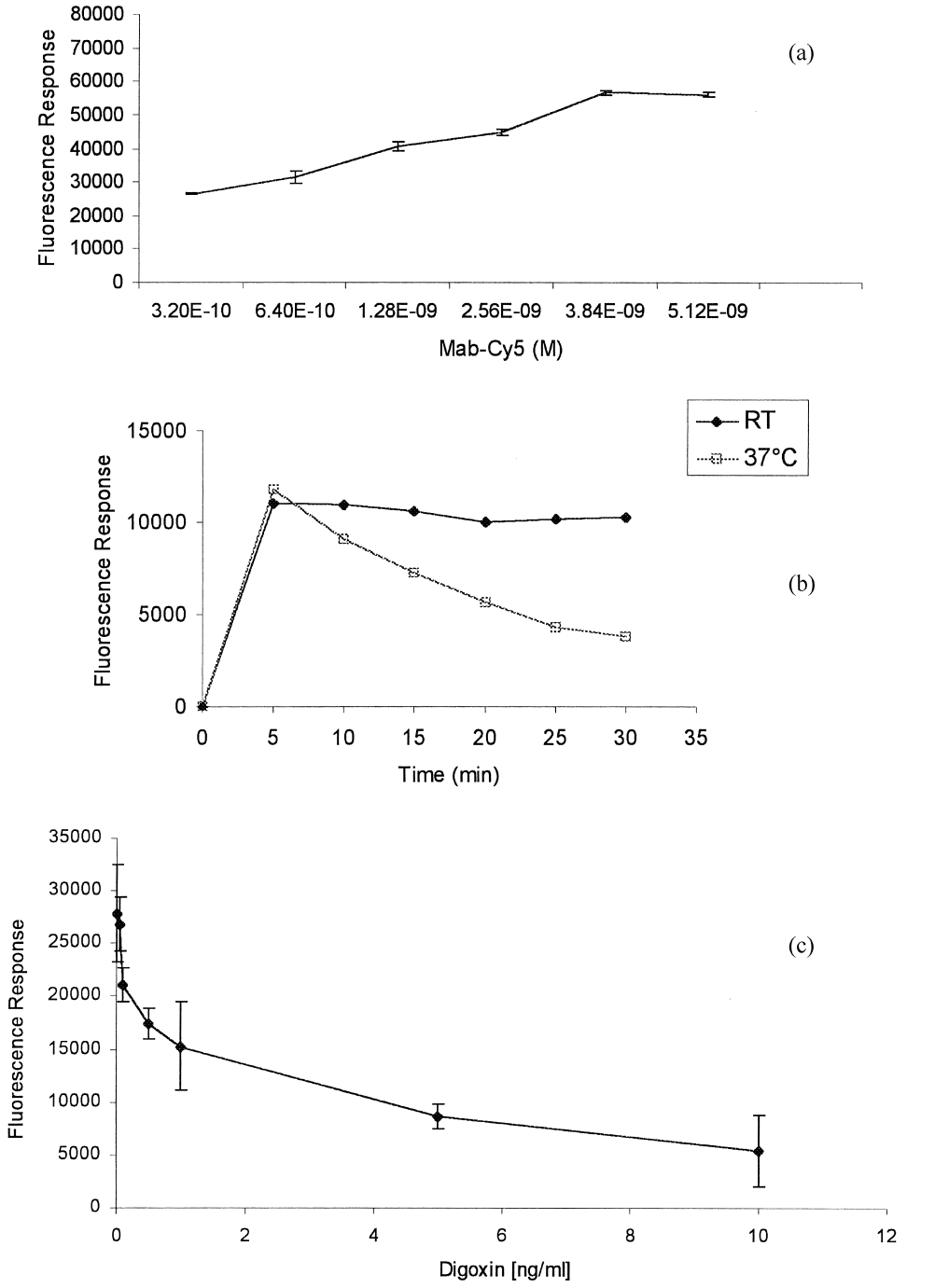


Fig. 4. (a) Off-line optimization of Cy5 labeled anti-digoxin antibody (Mab-Cy5) with *R*-phycoerythrin labeled digoxin (digoxigenin-PE). The concentration of digoxigenin-PE was kept constant at $6.4 \cdot 10^{-10}$ *M*, fluorescence energy transfer was detected at 670 nm. (b) Off-line optimization of the incubation time at room temperature and 37°C. (c) Inhibition of fluorescence energy transfer by digoxin in the off-line mode: non-linear calibration curve. Cy5 labeled anti-digoxin antibody (Mab-Cy5) concentration was $1.28 \cdot 10^{-9}$ *M* and *R*-phycoerythrin labeled digoxin (digoxigenin-PE) concentration was $6.4 \cdot 10^{-10}$ *M*. Fluorescence energy transfer was measured at 670 nm.

suitable environment for the immunoreaction. With the help of a flow splitter the flow coming through pump 2 was split into two equal channels, one of which was connected to injector 2 and one to injector 3. Digoxin and its metabolites were injected onto the analytical column using injector 1. Approximately 5 s after to the time of peak elution, digoxigenin-PE was injected using injector 2. The optimal time for the injection of digoxigenin-PE was determined by calculation and experiments. Based on the calculation including the volume of the tubing from injector 2 to the mixer 1 and the flow-rate, the maximum inhibition should be obtained with the injection of digoxigenin-PE 1 s prior to the time of peak elution. Additional experiments were carried out by injecting digoxigenin-PE at different times prior to and after the time of peak appearance and the maximum inhibition was obtained by the injection of digoxigenin-PE 5 s after the time of peak elution. Immediately after the injection of digoxigenin-PE, Mab-Cy5 was injected using injector 3 and mixing of the reagents took place in the mixer 2. After incubation in the KOT the fluorescence energy transfer emission at 670 nm was detected. The assay was completed within 15 min for each injection of digoxin.

Regarding fluorescence energy transfer, it is very important to consider a possible contamination of the sensitized emission peak by emission of either the donor or the acceptor due to direct excitation. There was background fluorescence from digoxigenin-PE at 670 nm with the detector set at 488 nm. As already shown in the experiments with respect to optimum concentrations of digoxigenin-PE and Mab-Cy5, there was no background emission from Cy5 due to direct excitation at a concentration of $1.28 \cdot 10^{-9}$ *M*. Hence, the relatively small working range was determined by the fluorescence energy transfer and the background fluorescence from digoxigenin-PE.

### *3.3. Linearity of the on-line HPLC–post-column reaction detection*

The assay was found to be linear from 0.05 ng/ml to 10 ng/ml with a correlation coefficient of 0.989. The limit of detection was 33 pg/ml calculated as two times the standard deviation of the blank ($n=6$). The precision of the assay observed at the blank ($n=6$) was 2.1%. The precision of two controls, 0.4 and 4 ng/ml, were found to be 2.2 and 8.7% RSD, respectively; accuracy was 18.7 and 20.3%, respectively ($n=6$ in each case).

The developed system includes three manual injection steps. Hence, precision and accuracy can be improved by automation of the system.

### *3.4. Consideration of cross-reactive compounds*

Anti-digoxin antibodies are known to cross-react with analogous substances, e.g., metabolites, other steroid-like compounds, and digoxin-like immunoreactive substances. Theoretically, compounds having a binding constant similar to digoxin should be detectable with the same sensitivity. Therefore, a separation of digoxin and its metabolites was carried out and the response factors of digoxin and its metabolites as potentially cross-reactive compounds were determined. A chromatogram representing the separation of digoxin, digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and dihydrodigoxin is shown in Fig. 5. With the present chromatographic system tetrahydrofuran–water

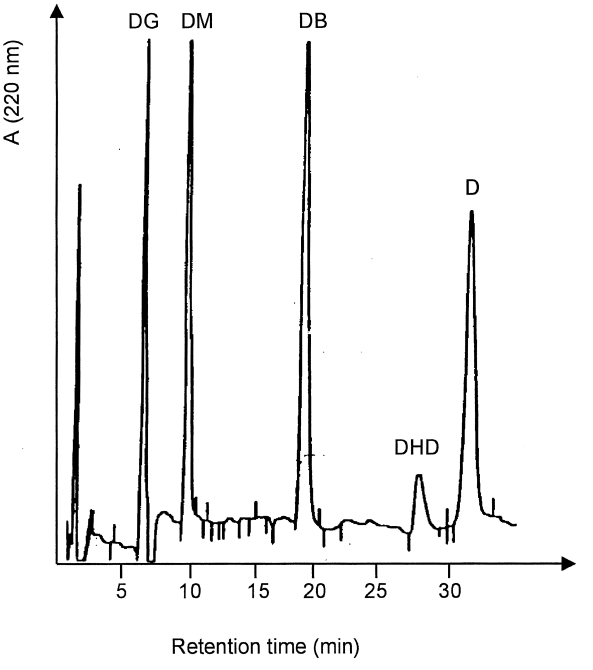


Fig. 5. Chromatogram of digoxin and digoxin metabolites: digoxigenin (DG), digoxigenin monodigitoxoside (DM), digoxigenin bisdigitoxoside (DB), dihydrodigoxin (DHD), and digoxin (D) (solid phase: Zorbax ODS; mobile phase: tetrahydrofuran–water, 20:80; flow-rate 1.2 ml/min; UV detection at 220 nm).

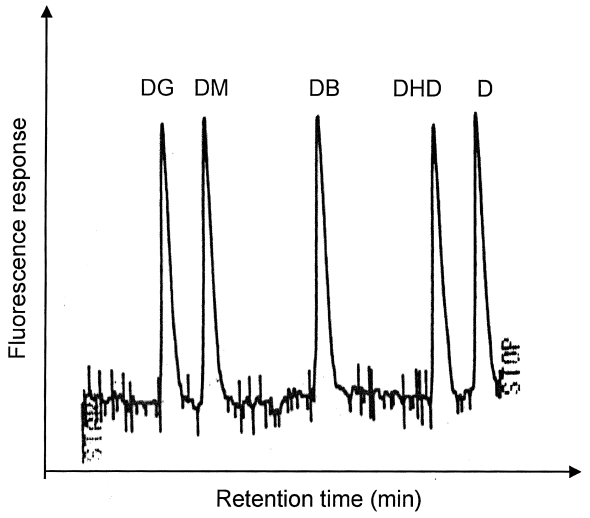


Fig. 6. Chromatogram showing inhibition of energy transfer by digoxigenin (DG), digoxigenin monodigitoxoside (DM), digoxigenin bisdigitoxoside (DB), dihydrodigoxin (DHD) and digoxin (D). *R*-Phycoerythrin labeled digoxin (digoxigenin-PE) concentration was $6.4 \cdot 10^{-10}$ *M* and Cy5 labeled anti-digoxin antibody (Mab-Cy5) was $1.28 \cdot 10^{-9}$ *M*. Fluorescence energy transfer (sensitized emission) was measured at 670 nm.

(20:80) digoxin and its pharmacologically inactive dihydrodigoxin were separated. Commonly used mobile phases, e.g., methanol–water (60:40), are not able to resolve these two compounds. The error due to dihydrodigoxin was reported to be negligible, because the response factor of dihydrodigoxin is five-fold lower than that of digoxin and since the amount of dihydrodigoxin formed by metabolism is only 10–15% [15]. Since in some cases metabolism up to 50% was described [19] and since the presence of an endogenous form of a dihydrodigoxin-like immunoreactive factor, a separation is required [20].

The retention times at 220 nm were determined by UV detection: digoxigenin (7:18 min), digoxigenin monodigitoxoside (10:38 min), digoxigenin bisdigitoxoside (19:19 min), dihydrodigoxin (27:48 min) and digoxin (31:31 min). The column was reconnected to the on-line system and injection of the $6.4 \cdot 10^{-10}$ *M* digoxigenin-PE was carried out at 7:23, 10:43, 19:24, 27:53 and 31:36 min using injector 2. Each injection of digoxigenin-PE was followed immediately by the injection of $1.28 \cdot 10^{-9}$ *M* Mab-Cy5 using injector 3. After incubation into the KOT the fluorescence energy transfer emission at 670 nm was detected (Fig. 6).

A calibration curve for digoxin and each metabolite was plotted in the range from 0.05 ng/ml to 10 ng/ml, the resulting correlation coefficients, slopes and LODs, calculated as 2×SD of the blank, are shown in Table 1. As can be seen, all metabolites have response factors similar to digoxin which means that their affinity for the used anti-digoxin antibody is in the same range. In contrast to cases reported in literature, DHD has a response factor similar to digoxin, although the double bond at the card-20(22)-enolide ring is reduced. Apparently an intact D-ring system is no essential epitope of the used anti-digoxin antibody. The developed method with the used monoclonal anti-digoxin antibody provides the simultaneous determination of digoxin and all metabolites with the same sensitivity.

## 4. Conclusions

An on-line post-column homogeneous competitive immunoassay for digoxin and its metabolites based on fluorescence energy transfer has been established to investigate digoxin and its metabolites. The method was used for the quantification of digoxin and its

Table 1
Determination of response factors of digoxin and its metabolites: log-linear calibration curves [$y = a \cdot \lg(x) + b$] for digoxin and each metabolite were plotted in the range from 0.05 to 10 ng/ml and the resulting correlation coefficients (*R*), slopes (*a*), intercepts (*b*) and LODs were determined

| Compound | *R* | Slope | Intercept | LOD (pg/ml) |
|---|---|---|---|---|
| Digoxigenin | −0.93 | −7646 | 27 037 | 24 |
| Digoxigenin monodigitoxoside | −0.97 | −8548 | 28 198 | 26 |
| Digoxigenin bisdigitoxoside | −0.95 | −8050 | 29 686 | 24 |
| Dihydrodigoxin | −0.92 | −6679 | 28 088 | 40 |
| Digoxin | −0.95 | −8663 | 23 716 | 33 |

metabolites in phosphate buffer and the LOD obtained was in the pg/ml range, indicating potential application in measuring digoxin (0.5–2.0 ng/ml) and digoxin metabolites in serum provided adequate selectivity can be obtained. It can be automated and employed for high throughput screening if motorized switching valves with timed triggers are successfully employed.

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