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Post-column reaction detection based on fluorescence energy transfer in the far red spectral region

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Abstract

Post-column reaction detection may result in enhanced analytical sensitivity and selectivity. This paper describes an on-line HPLC with post-column fluorescence energy transfer assay using biotin as a model analyte. Biotin labeled with *R*-phycoerythrin was used as the donor labeled ligand and streptavidin labeled with an indodicarbocyanine dye (Cy5), the acceptor labeled binder protein. The use of these labels provided a detection wavelength in the far red spectral region which is more selective for biological samples. In the on-line system, biotin was injected into the HPLC system followed by Cy5 labeled streptavidin and *R*-phycoerythrin labeled biotin, post-column. The mixture was incubated on-line in an open tubular reactor coil maintained at 37°C. The measured response was the sensitized emission of Cy5 due to fluorescence energy transfer from *R*-phycoerythrin labeled biotin measured at 670 nm. Excitation was at 488 nm, which provided a large Stokes shift for reduction of scatter interference. The system was optimized with regard to the post-column reagents to obtain the minimum detectable concentration while maintaining appropriate dynamic range for the analysis of biotin. Biotin spiked in 0.01 M phosphate buffer, pH 7.4, showed a dynamic range of 304.0 pg/ml–122.20 ng/ml with a correlation coefficient of 0.993. The limit of detection for this assay was 304.0 pg/ml. The precision calculated at the blank ($n = 6$) was 4.14%.

Keywords: Post-column; HPLC; Immunoassay; Fluorescence energy transfer assay; Cy5; Phycoerythrin; Biotin; Immunoreactor

1. Introduction

Immunoassays involve interaction of antigen and antibody. The antibody binds to specific sites on the antigen and hence immunoassays are very selective. However the antibody fails to discriminate antigens with similar binding sites, which leads to reduced selectivity, especially when individual compounds of similar structure such as metabolites of drugs need to be analyzed. In such cases combining a separation technique such as HPLC with immunoassay enhances the selectivity of the assay overall by separating the cross-reactive components prior to immunoassay [1–3].

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Several post-column immunodetection systems following HPLC have been reported in the literature [4–12]. Most of these immunoassays, combined post-column with HPLC, are in the heterogeneous mode. Heterogeneous assays involve separation of the bound and unbound label, using a solid phase immunoreactor to bring about this separation. Homogeneous assays are much easier to perform, as they do not require a separation step. Oosterkamp et al. [6,7] performed post-column homogeneous assays using labels in which the fluorescence is enhanced on binding to antibodies. Though this provides for simple assays it is not universally applicable and non-specific binding by serum proteins can lead to undesired enhancement and quenching of fluorescence. If binding to an antibody leads to partial quenching of the signal, labeling the antibody with a quencher, as in fluorescence energy transfer, can lead to a greater quenching effect [13].

This paper describes a post-column homogeneous binding assay based on fluorescence energy transfer with biotin as the model analyte. Fluorescence energy transfer involves non-radiative energy transfer from one induced dipole to another [14]. Energy transfer between the donor and acceptor label can lead to a measurable decrease in the intensity and lifetime of the donor fluorescence and enhancement of fluorescence of the acceptor. The efficiency of energy transfer depends on the distance between the donor and acceptor as shown in the Forster equation below:

$$E = 1/(1 + R^6/R_o^6)$$

where R is the distance between the donor and acceptor and R_o is the distance at which 50% energy transfer takes place and is expressed as:

$$R_o = (8.79 \times 10^{-5} J q_D n^{-4} \kappa^2)^{1/6} \quad \text{(in A°)}$$

where J is the integrated spectral overlap of the donor emission and acceptor absorption, q_D is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium and κ, the orientation factor, is related to the angle between the donor and acceptor dipoles [14]. Binding of a labeled antigen and antibody brings the labels close enough for energy transfer to occur. Fluorescence energy transfer immunoassay has been employed for both small molecules and proteins [15].

In choosing the donor and acceptor labels certain criteria should be met for efficient energy transfer. High extinction coefficient and high quantum yield are important characteristics for the donor. It should have good water solubility and be able to be linked without effecting the binding properties of the ligand or binder as well as the properties of the donor label. Interference from biological matrix components is a problem at shorter wavelengths and a donor with longer wavelength absorbance and emission is preferred. In addition, donor labels should not be sensitive to their environment [15]. Phycobiliproteins are naturally occuring molecules that have several advantages when employed as labels for fluorescence energy transfer (FET). They have high extinction coefficients in the order of 2.4×10^6, very high quantum yields reaching upto 0.98. They are water soluble and not easily effected by the environment [16–19].

The acceptor should have an absorption band at a wavelength that ensures good spectral overlap with the donor emission in order to effect fluorescence energy transfer. It should have a high extinction coefficient such that R_o is large. It should have negligible fluorescence when excited at the donor absorption maximum, ensuring low background due to direct excitation of the acceptor, and it should be water soluble [13,15]. Cy5, a fluorescent cyanine dye is a superior label for fluorescence energy transfer. Cy5 dyes are photostable and highly water soluble. They do not change the physical characteristics of a dye-protein conjugate since they are relatively small molecules with high water solubility. Cy5 dyes emit and absorb in the far-red region of the spectrum where background fluorescence from biological matrices is very low [20].

In this study, R-phycoerythrin was used in the fluorescence energy transfer assay as the donor and was conjugated to the ligand, biotin. An indodicarbocyanine moiety (Cy5) was used as the acceptor and was labeled to the binding protein, streptavidin. R-phycoerythrin has absorption maxima between 530 and 565 nm and around 480–495 nm with extinction coefficient of $1.96 \times$

10^6 M^{-1} cm^{-1} at 565 nm with a quantum yield of 0.82. When excited at the argon ion laser line of 488 nm the extinction coefficient is 1.28×10^6 M^{-1} cm^{-1}. *R*-Phycoerythrin is 14.5 times more fluorescent than an equimolar solution of fluorescein when excited at 488 nm [19]. Cy5 works well as an acceptor with an extinction coefficient of 2×10^5 M^{-1} cm^{-1} yielding a large R_o for energy transfer [21]. There is sufficient overlap of the emission spectrum of the donor and the absorption spectrum of the acceptor for efficient energy transfer to take place. *R*-Phycoerythrin is excited at 488 nm and the energy transfer emission, 'sensitized emission' can be measured at 670 nm. When fluorescent labels are employed in the far red spectral region, additional selectivity with regard to interfering substances from biologic samples is realized. Another advantage in using this label combination is that phycoerythrin is maximally excited at 488 nm and Cy5 fluoresces at 670 nm which leads to a large stokes shift, that can reduce interference from scattered light [22]. The excitation wavelength used was 488 nm, for *R*-phycoerythrin as this corresponds with a strong emission line of an argon ion laser which provides the possibility of enhanced sensitivity. The reaction sequence for fluorescence energy transfer is as follows:

1. Biotin + $Sa[Cy5]_5$ (excess) $\leftrightarrow$ Biotin-$Sa[Cy5]_5$
2. $Sa[Cy5]_5$ (excess from step 1) + $B_{10}[PE] \leftrightarrow Sa[Cy5]_5$-$B_{10}[PE]$ + $B_{10}[PE]$
3. Excitation at 488 nm $\rightarrow$ Energy transfer emission ($\lambda = 670$ nm) inversely related to [Biotin]

$Sa[Cy5]_5$ = Cy5 labeled streptavidin, label/protein = 5

$B_{10}[PE]$ = *R*-phycoerythrin biotin conjugate, biotin/label = 10

Fluorescence energy transfer assays have potential for automation, and can be used on-line post-column with HPLC to provide the added selectivity and sensitivity without requiring prolonged assay times. This paper describes an on-line post-column fluorescence energy transfer competitive binding assay coupled to HPLC using biotin as the model analyte.

2. Experimental

2.1. Materials

Biotin was purchased from Sigma–Aldrich (St. Louis, MO). *R*-Phycoerythrin, biotin-xx conjugate (B-PE) was obtained from Molecular Probes (Eugene, OR) and Fluorolink Cy5 labeled streptavidin (Sa-Cy5) from Amersham Life Sciences (Pittsburgh, PA). Dibasic sodium phosphate and monobasic sodium phosphate from Mallinckrodt (Paris, KY). Methanol, analytical grade was from Baxter (B&J brand, Muskegon, MI). All solutions were prepared in deionized distilled water (Corning Mega Pure).

2.2. Instrumentation

All mobile phases were filtered through a 0.45-micron nylon filter (Alltech, Deerfield, IL) and helium sparged prior to use. The HPLC system consisted of two Gilson pumps Model 302 with a model 802B manometric module (Gilson Medical Electronics, Middleton, WI). The HPLC column used was a Supelcosil ABZ^+ Plus C_{18} column (150 mm × 2.1 mm i.d., 5 μm particle size) from Supelco (Bellefonte, PA). A PEEK Y connector (flow splitter) used with the second pump and two metal-free static mixing tees were purchased from Upchurch Scientific (Oak Harbor, WA). A knitted open tubular reactor coil (KOT) was knitted in the laboratory using TFE teflon tubing, 1.58 mm o.d. × 0.5 mm i.d., 10 m length, 2.0 ml final volume (Supelco, Bellefonte, PA). Initial UV detection for biotin was accomplished using a Shimadzu SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan). Fluorescence detection was performed using a Hitachi F-1080 fluorescence detector (Hitachi, Tokyo, Japan) with excitation at 488 nm and emission at 670 nm. Data was acquired with a Hewlett–Packard 3396A integrator (Hewlett–Packard, Avondale, PA). Off-line experiments were carried out using a Perkin Elmer Luminescence Spectrometer Model LS 50 (Perkin Elmer, Norwalk, CT). 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 (Cotati, CA). In-

jections were performed manually with Hamilton syringes (Reno, Nevada).

2.3. Off-line fluorescence energy transfer assay

Experiments were carried out to optimize the *R*-phycoerythrin biotin-xx conjugate (B-PE) concentration, Cy5 labeled streptavidin (Sa-Cy5) concentration, incubation time and temperature for fluorescence energy transfer. All solutions were prepared in 0.1 M Phosphate buffer, pH 7.4. Various concentrations of the reagents were mixed and incubated at room temperature for 30 min to ensure complete binding ($n = 3$), for optimization of reagent concentration. The solution was then scanned in the Perkin Elmer Luminescence Spectrophotometer through 500–800 nm.

Incubation time was optimized using optimal reagent concentrations. Sa-Cy5 and B-PE were mixed by vortexing and incubated at room temperature for 0, 5, 10, 15, 20, 25 and 30 min ($n = 3$). A comparison was done with and without incubation after addition of Sa-Cy5 to biotin, to observe whether or not incubation was required after addition of Sa-Cy5 to biotin (8.25×10^{-8} M). Incubation time was later optimized at the optimal temperature ($n = 3$).

Incubation was carried out at room temperature and 37°C using a Pierce Reacti-Therm heating block to observe the effect of temperature on fluorescence energy transfer. Sa-Cy5 and B-PE were mixed by vortexing and incubated for 30 min at the respective temperature ($n = 3$). The fluorescence energy transfer peak was then observed in the fluorescence spectrometer.

Inhibition of fluorescence energy transfer by biotin was carried out using the optimized conditions and reagent concentrations, for biotin concentrations of 1.65×10^{-7} M, 4.13×10^{-7} M, 8.25×10^{-7} M, 1.24×10^{-6} M, 1.65×10^{-6} M, 4.13×10^{-6} M and 6.20×10^{-6} M ($n = 3$).

2.4. Flow injection fluorescence energy transfer assay

Flow injection experiments were carried out to assess the feasibility of the assay on-line. Phosphate buffer (pH 7.4) 0.01 M was used for the on-line experiments. The incubation time was optimized based on the knitted open tubular reactor coil (KOT) size. The residence times observed were 0 (no KOT), 3, 7 and 10 min (5, 10 and 15 m tubing length). Sa-Cy5 1.66×10^{-8} M and B-PE 1.65×10^{-8} M were injected simultaneously into a static mixer on-line. It was incubated in the KOT which was maintained at 37°C by immersion in a constant temperature water bath ($n = 5$).

Inhibition of fluorescence energy transfer (sensitized emission) by biotin with off-line mixing was observed for the flow injection system. Biotin concentrations were 0, 1.65×10^{-9} M, 1.65×10^{-8} M, 1.65×10^{-7} M and 1.65×10^{-6} M. Quenching of B-PE fluorescence at 578 nm was also observed in the flow injection mode. The concentration of Sa-Cy5 was 1.66×10^{-8} M and the B-PE concentration was kept at 1.65×10^{-9} M. The biotin concentrations were in the same range as used for the sensitized emission measurement.

The photomultiplier tube in the fluorescence detector was changed from R3788 to R928 to increase the signal output. The concentrations of the reagents were decreased to 4.15×10^{-9} M Sa-Cy5 and 1.65×10^{-9} M B-PE and inhibition of fluorescence energy transfer by biotin was observed for flow injection assay ($n = 3$). The dynamic range for biotin detection was determined and the limit of detection was calculated.

2.5. On-line HPLC-post-column reaction detection

The HPLC system for biotin consisted of 5:95 methanol: 0.01 M phosphate buffer, pH 7.4 as the mobile phase (flow rate 0.2 ml/min). The second pump delivered 0.01 M phosphate buffer, pH 7.4 at 0.2 ml/min. The HPLC column was first connected to the UV detector and 1.0×10^{-4} M biotin was injected to observe the peak retention time. The UV detector was then removed and the column was connected to a static mixer on-line that mixed biotin with Sa-Cy5 followed by injection of B-PE, incubation in the KOT and detection.

2.6. Optimization of the on-line HPLC-post-column reaction detection

Biotin concentrations, 0, 1.0×10^{-9} M, 2.5×10^{-9} M, 3.13×10^{-9} M, 1.0×10^{-8} M, 1.0×10^{-7} M and 5.0×10^{-7} M in buffer were injected (20 μl) on the column. Since the retention time was 2 min 55 s, the Sa-Cy5 4.15×10^{-9} M (50 μl) was injected at 2 min 52 s, since it took approximately 3 s for Sa-Cy5 to reach the static mixer. Following this, B-PE (1.65×10^{-9} M, 50 μl injection volume) was injected at 3 min and 24 s, as the time gap between injection of Sa-Cy5 and B-PE which provided optimum mixing was found to be 32 s. The fluorescence energy transfer peak was then detected at 670 nm. The limit of detection was calculated at the blank ($n = 6$).

3. Results and discussion

3.1. Off-line fluorescence energy transfer assay

Fig. 1(a, b) shows the graph for optimization of reagent concentration. The optimum concentration of Sa-Cy5 was found to be 1.66×10^{-8} M. The fluorescence energy transfer emission is greater at higher concentrations but the difference was not determined to be enough to justify use of higher concentration of reagent. The lower concentration would be more suitable for lower concentration detection of analyte since it is a competitive inhibition assay. Maximum energy transfer emission was observed with a B-PE concentration of 1.65×10^{-8} M and this was chosen as the optimum concentration for further experiments. The graph for the optimization of incubation time at room temperature is shown in Fig. 2(a). It was observed that the response demonstrated a plateau within 25 min and 30 min was considered the optimum time for incubation to ensure complete binding. It was found that there was no significant difference between the responses when incubated for 30 min after addition of Sa-Cy5 to biotin and when not incubated following addition of Sa-Cy5 to biotin, data not shown. When incubation was carried out at 37°C the plateau was seen earlier, within 10 min (Fig. 2(b)).

The energy transfer emission peak was larger when incubated at 37°C as compared to the peak for room temperature incubation. The mean area under the energy transfer emission peak was $3.64 \times 10^3 \pm 3.61$ ($n = 3$) at room temperature (25°C) and $7.10 \times 10^3 \pm 0.80$ ($n = 3$) at 37°C. Fig. 3 shows the inhibition of fluorescence energy transfer in the presence of biotin. The range in which the inhibition was observed was 40.30 ng/ml–1.51 μg/ml.

3.2. Flow injection fluorescence energy transfer assay

Fig. 4 shows a graph of the incubation time in minutes based on the KOT size and the fluores-

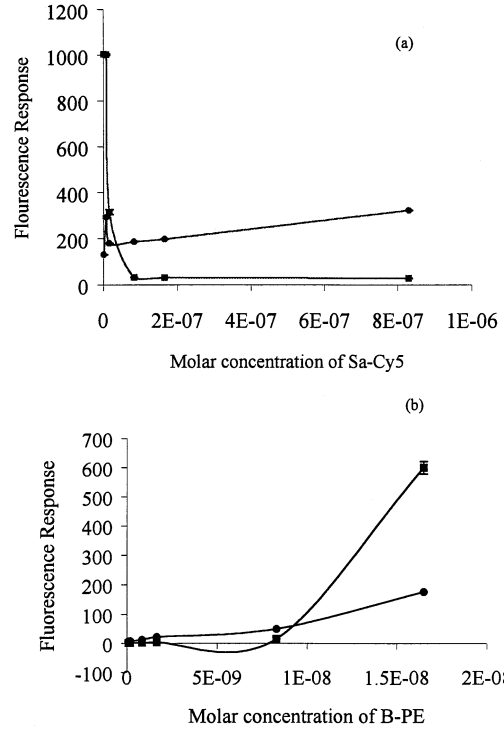

Fig. 1. (a) Off-line optimization of Cy5 labeled streptavidin (Sa-Cy5) concentration with *R*-phycoerythrin labeled biotin (B-PE) concentration kept constant at 1.65×10^{-8}M, B-PE emission at 578 nm (?), fluorescence energy transfer peak at 670 nm (?). Optimization of *R*-phycoerythrin labeled biotin concentration with Cy5 labeled streptavidin concentration at 1.66×10^{-8}M, B-PE emission at 578 nm (?), fluorescence energy transfer peak at 670 nm (?).

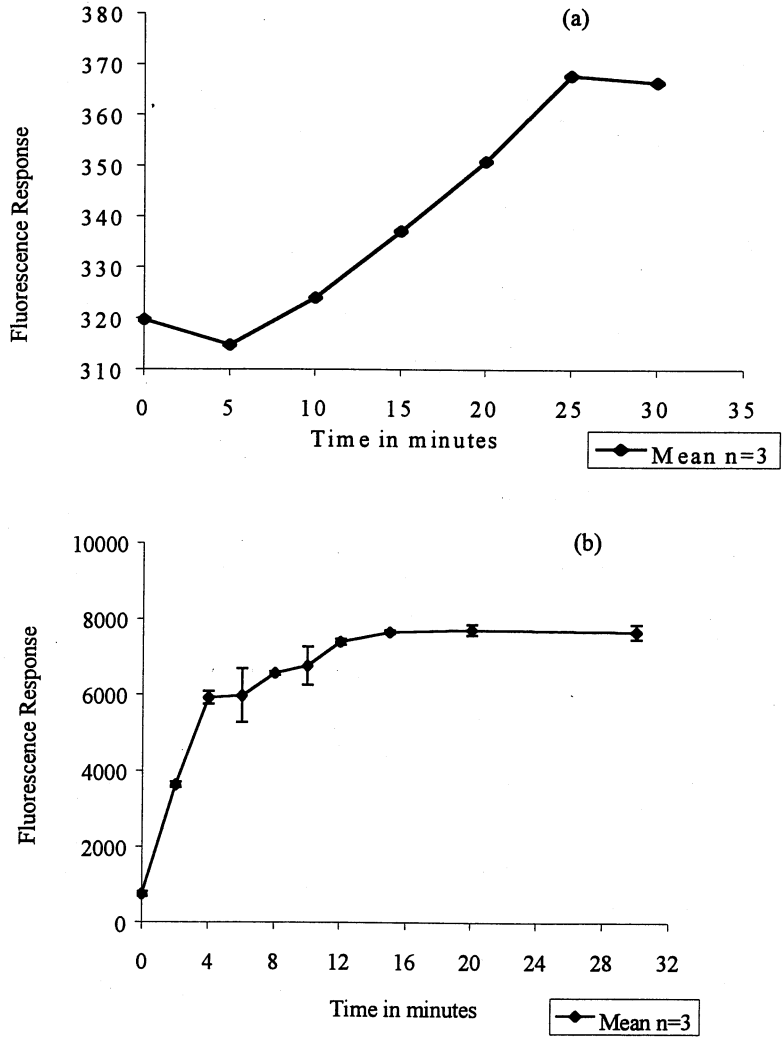

Fig. 2. Optimization of incubation time at room temperature (a), at 37°C (b).

cence response. Based on instructions by Selavka et al. [23], two different sized KOTs were constructed to provide different incubation times. The incubation times in the KOTs were 3 and 7 min. In the third case the two KOTs were combined to provide an incubation time of 10 min. As can be seen from the graph there was no significant difference between the 7- and 10-min incubation times, hence the KOT that provided a 7-min incubation time was used for further experiments.

In the flow injection mode both fluorescence energy transfer emission of Cy5 at 670 nm and quenching of the B-PE fluorescence at 578 nm by the binding of B-PE and Sa-Cy5 was observed. The range in which inhibition was observed was 40.3–403.0 ng/ml in both cases.

The degree of labeling of the protein with acceptor label influences the extent of the quenching of the donor fluorescence and the intensity of fluorescence energy transfer [24]. This could be due to the fact that a large number of label molecules on the protein may increase the probability of acceptor labels being closer to the ligand binding sites. This decreases the distance between

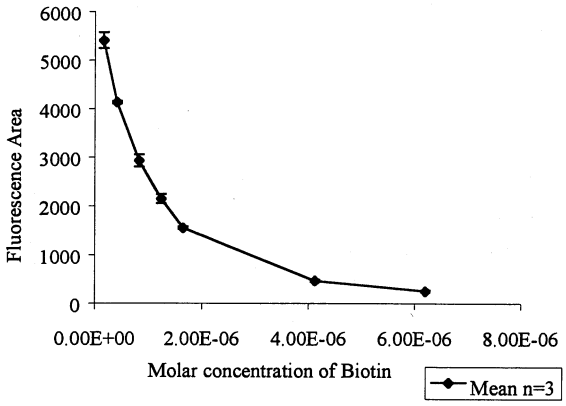

Fig. 3. Inhibition of fluorescence energy transfer by biotin in the off-line mode. Cy5 labeled streptavidin concentration was 1.66×10^{-8} M and *R*-phycoerythrin labeled biotin concentration, 1.65×10^{-8} M. Sensitized emission was measured at 670 nm.

donor and acceptor labels and in turn effects efficient energy transfer [25]. Excess label can lead to decreased solubility of the binder protein. In the case of Cy5 labeled streptavidin, the label/protein ratio was fixed by the supplier and could not be adjusted to provide more suitable ratios. The efficiency of energy transfer can be increased if this ratio is increased.

The degree of donor labeling is also an important consideration. It has been shown by Lim et al. [25], that at higher label to protein ratio, albumin labeled with fluorescein showed less quenching by energy transfer than that with a lower ratio. The explanation was that the fluorescein to fluorescein energy transfer competes with the donor to acceptor (in this case rhodamine) energy transfer when the label to protein ratio is higher. In this case there was one donor label for ten biotin molecules and hence quenching within the donor label was not a problem. However a one is to one ratio of biotin to PE would have been a more suitable conjugate but this ratio was determined by the supplier and hence it could not be changed.

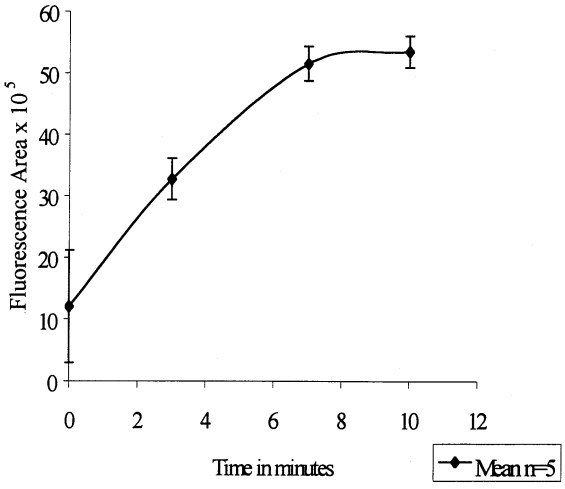

Fig. 4. Effect of incubation time (based on knitted open tubular reactor coil size) on fluorescence energy transfer, on-line.

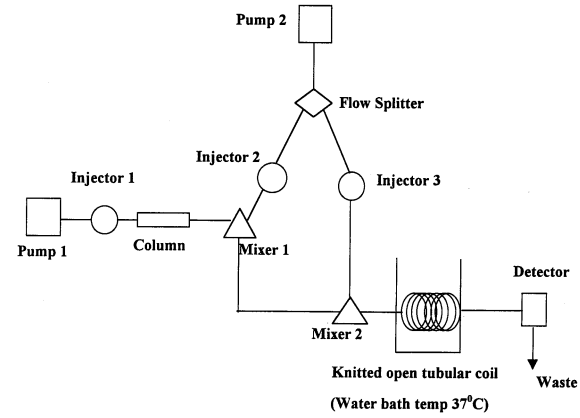

Fig. 5. Schematic diagram of on-line HPLC and post-column fluorescence energy transfer reaction detection.

A calibration curve was plotted with injection of biotin and Sa-Cy5 mixed off-line. It was found to be linear in the range 183.22 pg/ml–122.15 ng/ml. The correlation coefficient was 0.998 using a power fit. The LOD was 142.0 pg/ml, calculated as $2 \times$ SD of the blank ($n = 6$). Using a quadratic fit with log molar concentration of biotin on the *x*-axis the dynamic range was 122.15 pg/ml–2.4 ng/ml with a correlation coefficient of 0.988.

3.3. On-line HPLC-post-column reaction detection

A schematic of the on-line HPLC-post-column reaction detection system is shown in Fig. 5. Pump 1 was set at a flow rate of 0.2 ml/min

pumping mobile phase 5% methanol/95% 0.01 M phosphate buffer (pH 7.4). Pump 2 was set at the same flow rate but pumping only buffer as biochemical assays function well in an aqueous environment. The flow coming through pump 2 was split into two channels using a flow splitter. One tubing from the splitter was connected to injector 2 and the other to injector 3.

Biotin in buffer was injected onto the HPLC column using injector 1. Approximately 3 s prior to the time of peak elution, Sa-Cy5 was injected using injector 2. Three seconds was calculated based on the volume of the tubing from injector 2 to static mixer 1 and the flow rate in that tubing, as well as optimized based on the maximum inhibition obtained when Sa-Cy5 was injected at different times prior to the time of peak appearance. Following this, B-PE was injected using injector 3, mixing took place in static mixer 2. The mixed solution then passed onto the knitted open tubular reactor coil (KOT), which was immersed in a water bath in order to maintain the temperature at 37°C. 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 biotin. In this system timing of analyte elution as well as reagent addition is a very important consideration. It is required that the time of elution of the analyte peak be known beforehand, which in this case was accomplished by the help of a UV detector. However, once the time is known it works like any other on-line post-column system. Once the timings are established the entire system has the potential for automation.

Fig. 6(a) shows the emission peak for B-PE at 670 nm and Fig. 6(b) shows the fluorescence energy transfer (sensitized) peak at 670 nm. As can be seen from the chromatograms there is background fluorescence, which is the B-PE fluorescence at 670 nm. The working range is therefore between the fluorescence energy transfer peak and the background. Since this difference is relatively small, the working concentration range is limited. This background could be decreased by use of time resolved fluorescence such that fluorescence due to donor emission (relatively short-lived) could be resolved from energy transfer fluorescence (relatively long-lived).

The B-PE peak at 578 nm, which is the emission maximum for phycoerythrin is quenched upon addition of Sa-Cy5. This assay could also be quantified by measuring quenching at 578 nm. The benefit of a large Stokes shift is sacrificed however and the fluorescence energy transfer emission peak at 670 nm (sensitized emission) was considered the optimal response for the assay.

An important consideration when measuring sensitized emission of an acceptor is that the sensitized emission peak is contaminated by the emission of the acceptor by direct excitation. This is due to absorption of the excitation light necessary for excitation of the donor. This adds to the background present from the donor emission observed at the emission of the acceptor [26].

The Cy5 acceptor and *R*-phycoerythrin donor overcomes this problem, since PE is excited at 488 nm and the Cy5 excitation is red shifted thus preventing it from adding to the background emission by direct excitation. In the on-line mode when Sa-Cy5 was injected into the system with the detector set at 488 nm excitation and 670 nm emission, there was no emission component from Cy5 by direct excitation thus resulting in reduced background at 670 nm, the background is from B-PE emission only.

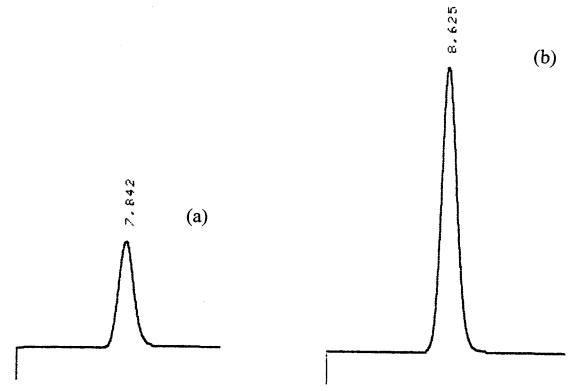

Fig. 6. Chromatogram showing *R*-phycoerythrin labeled biotin emission (background) at 670 nm. Area 30.0×10^5 for *R*-phycoerythrin labeled biotin concentration of 1.65×10^{-9} M (a). Energy transfer (sensitized emission) peak at 670 nm, area 82.10×10^5 with Cy5 labeled streptavidin concentration 8.3×10^{-9} M and *R*-phycoerythrin labeled biotin concentration 1.65×10^{-9} M (b).

The large apparent Stokes shift of 182 nm using this dye pair also reduces background due to scattered light. The R_o value calculated for B-Phycoerythrin and Cy5 was 72 A° which shows that the efficiency of energy transfer is 50% at a donor acceptor distance of 72 A°, a considerably long distance [21]. For a hypothetically perfect dye pair R_o has been calculated to be 84 A° [15].

3.4. Optimization of the on-line HPLC-post-column reaction detection

The assay was found to be linear from 304.0 pg/ml to122.15 ng/ml with a correlation coefficient of 0.993. The limit of detection was 304 pg/ml calculated as twice the standard deviation of the blank ($n = 6$). The precision of the assay observed at the blank ($n = 6$) was 4.14%. The reference range of biotin in human blood serum is 200–700 pg/ml with a mean reported at 400 pg/ml [27]. This system can potentially be optimized for measuring biotin in serum.

4. Conclusions

This study is the first report of an on-line post-column fluorescence energy transfer binding assay coupled to HPLC in which both ligand and protein are labeled. Liquid chromatography with post-column addition of lanthanide chelates for luminescence detection of organic compounds has been studied [28–30]. These are based on the triplet state emission of an organic compound matching and transferring the energy to the excited state of a lanthanide ion. The energy transfer that occurs can be an intermolecular process based on collision or intramolecular based on bonding of the lanthanide ion with proper functional groups on the organic compound. This method can only be applied to specific compounds, which have appropriate energy levels that match with the lanthanide ions for energy transfer to occur.

The assay described in this paper can be used for the determination of both small molecules and proteins and any ligand/protein pair can be labeled with a donor and acceptor label. It can be employed for the high throughput screening of combinatorial chemical libraries using appropriate binder proteins. An advantage of the method is that it can be potentially used with any binder and is not limited to antibodies. It does not require derivatization at low concentration, as the analyte to be measured is not labeled.

This method was used for the analysis of biotin in phosphate buffer and the limit of detection obtained was in the pg/ml range. Biotin, a water soluble vitamin, was chosen as the model analyte. It has high binding affinity for streptavidin/avidin (K_D, 10^{-15} M). Conjugates with phycoerythrin as well as streptavidin labeled with Cy5 were commercially available. The metabolites of biotin can bind to avidin/streptavidin, therefore it is a good model to demonstrate the need for HPLC prior to reaction detection. The detector used in this study was a conventional fluorescence detector. An argon ion laser line of 488 nm can be used for exciting *R*-phycoerythrin resulting in higher intensity of fluorescence at lower concentrations than that brought about by excitation at 488 nm with a conventional source.

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