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Theoretical concepts of on-line liquid chromatographic-biochemical detection systems
I. Detection systems based on labelled ligands

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Abstract

A theoretical foundation for on-line coupling of liquid chromatography (LC) with fluororeceptor assays based on fluorescent ligands is presented. Using a recently developed LC–receptor affinity detection (RAD) system as a model, equations are derived which describe the detector response and the signal-to-noise ratio as a function of important biochemical and instrumental parameters. The effect of ligand and label affinity, and receptor concentrations on the detector performance were investigated. It was found that the response of the RAD system is correlated with the affinity of the injected compounds and that the relative affinity order of binding ligands is maintained in the RAD system. © 1997 Elsevier Science B.V.

Keywords: Biochemical detection; Detection, LC; Receptor affinity detection; Affinity detection; Steroids; Estrogens; Estradiol

1. Introduction

Biological assays find widespread use in biological sciences as an analytical tool characterised by high selectivity and sensitivity. Implemented in robotics system bioassays, such as immuno- or receptor assays, they are performed with high-throughput allowing the screening of thousands of samples within a week. One of the major drawbacks of bioassays stems from the fact that the bioaffinity molecules involved, antibodies or receptors, for example, in many cases are not specific for a single compound, but exhibit cross-reactivity to a series of structurally related compounds. In the presence of several cross-reactive compounds, the response provided by the bioassay reflects the sum of the concentrations of the individual compounds and their affinity for the affinity protein. Moreover, matrix components present in far higher concentrations than the analyte(s) might interfere leading to false-positive or false-negative results.

Whenever the concentrations of individual compounds are required, the sample has to undergo a considerable amount of sample pretreatment followed by liquid chromatographic separation and fractionation. The bioassay is then performed in the

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fractions containing the analyte(s). To overcome the laborious steps involved in this procedure analytical system, integration of the bioassay with liquid chromatography, in the form of a post-column reaction detection system, has been developed. By using suitable, mostly fluorescent labels attached to either the receptor [1–5] or the ligand [6–10], sensitivities in the order of 10^{-10} mol/l were obtained in LC–biochemical detection (BCD). Moreover, receptor assays similar to those employed in drug discovery were performed in a continuous-flow mode and coupled on-line to liquid chromatography [11]. A fluorescent ligand was used to monitor the interaction of analytes with the receptor. In on-line LC–receptor affinity detection (RAD) biologically active compounds present in a complex matrix, for example in a plant extract used in drug discovery, are separated from matrix components prior to detection. In this way false-negative or false-positive results due to matrix interferences can be avoided and, moreover, active compounds can be isolated based on their response in the RAD system.

The response in a continuous-flow RAD system using a fluorescent ligand for detection is influenced by several instrumental and biochemical parameters. Similar to chemical post-column reaction detection systems, dispersion in the reaction coil(s), the concentration of receptor and label, and the affinity constants of both analyte–receptor and label–receptor complexes influence the detection response. We have developed a theoretical model describing the detector response for high-affinity ligands in an LC–BCD system based on labelled ligands as a function of these parameters [6]. In this paper we extend this theoretical model to continuous-flow RAD systems based on labelled ligands covering a wider range of affinity constants. The human estrogen receptor is used as model affinity protein since affinity constants for a large number of estrogenic steroids measured by radioreceptor assay are reported allowing the independent verification of the theoretical model.

2. Theory

The present LC–RAD system is based on two sequential affinity reactions which are performed in an open tubular reactor (scheme, see Fig. 1). In reaction (1), receptor (R) is added to the LC effluent to react with ligands (L) eluting from the analytical column. Receptor–ligand complexes (RL) are formed in concentrations proportional to the ligand concentration:

$$R + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL \qquad \text{reaction (1)}$$

Subsequently, a labelled ligand (L*), in the following denoted as label, is added via a second mixing union to the reaction mixture. The label reacts primarily with remaining free receptor binding sites (reaction (2)) to form fluorescent receptor–label complexes (RL*) which are detected by a fluorescence detector. Simultaneously, a competitive reaction between the ligand and the label for the receptor binding sites will take place:

$$\begin{array}{l} R + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL \\ + \\ L^* \\ k_{-2} \uparrow\downarrow k_{+2} \\ RL^* \end{array} \qquad \text{reaction(2)}$$

In analogy to competitive receptor assays, the bound fluorescent receptor–label fraction after reaction (2) can be monitored by a fluorescence detector. Thus, injection of ligands will decrease the concentration

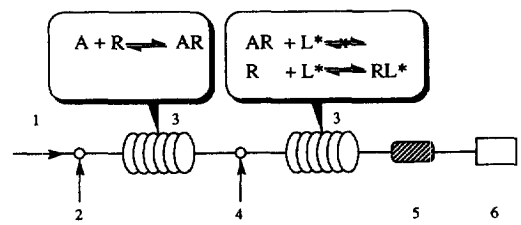

Fig. 1. Scheme of the RAD system. 1, from injector or HPLC column; 2, reagent pump for estrogen receptor solution; 3, reaction coil; 4, reagent pump for coumestrol solution; 5, optional column packed with C_{18}-silica restricted-access support; 6, fluorescence detector. R, receptor; A, analyte; L*, labelled ligand.

of receptor-bound fluorescent labels, resulting in negative peaks.

We have shown that the reaction mixture after reaction (2) can directly be monitored by a fluorescence detector (homogeneous set-up) if a significant difference exists in the fluorescent properties [7,11]. In cases where no difference in detection characteristics can be observed free and receptor-bound label have to be separated prior to detection (heterogeneous set-up) by employing, for example, a restricted-access column which retains only the low-molecular-mass free label [6,7,11]. The theoretical model in the present paper has been developed for the homogeneous set-up but can easily be extended to the heterogeneous set-up by accounting for the additional dispersion caused by the separations step.

The reactions of the receptor with the ligand and the label are described by second-order kinetics, involving both association and dissociation rate constants. The reaction of the receptor with the ligand (1) and label (2) are described by:

$$\frac{d[RL]}{dt} = k_1[R][L] - k_1[RL] \quad (1)$$

$$\frac{d[RL^*]}{dt} = k_2[R][L^*] - k_2[RL^*] \quad (2)$$

in which k_{+1} and k_{+2} are the association rate constants and k_{-1} and k_{-2} the dissociation rate constants of the receptor–ligand and the receptor–label complexes, respectively.

The association rate constants are, in general, diffusion controlled and range from 10^7 to 10^8 l mol^{-1} s^{-1} [12,13]. The dissociation rate constants, in contrast, are dependent on the affinity of the ligand and range from 10^{-5} s^{-1} for high-affinity ligands to 10^3 s^{-1} for low-affinity ligands.

2.1. Reaction 1

In reaction coil (1), a bimolecular reaction takes place between the ligand injected and the receptor. Initially, the concentrations of the ligand and receptor are $[L]_0$ and $[R]_0$, respectively. The concentration of receptor/ligand complex after reaction (1) ($[RL]_{t=1}$) can be related to $[L]_0$, $[R]_0$ and their affinity by integrating Eq. (1) [14]:

$$k_{+1}t = \frac{[RL]_e}{[R]_0[L]_0 - [RL]_e^2} \cdot \left(\ln \frac{[RL]_e([L]_0 - [RL]_{t=1}[RL]_e/[R]_0)}{[L]_0([RL] - [RL]_e)} \right) \quad (3)$$

in which $[R\text{-}L]_e$ is the concentration of a receptor–ligand complex when the reaction reaches equilibrium. $[RL]_e$ is dependent on the affinity of the receptor and its ligand, defined by the dissociation affinity constant, K_D. The $[RL]_e$ is calculated as described earlier [6]:

$$[RL]_e = \frac{[R]_0 + [L]_0 + K_D - \sqrt{([R]_0 + [L]_0 + K_D)^2 - 4[R]_0[L]_0}}{2} \quad (4)$$

Eq. (3) can be rewritten to

$$[RL]_{t=1} = [RL]_e[R][L] \cdot \left(\frac{1 - e^{\alpha \cdot k_{+1} \cdot t}}{[RL]_e^2 - [R][L]\, e^{\alpha \cdot k_{+1} \cdot t}} \right) \quad (5)$$

in which:

$$\alpha = \frac{[R][L]}{[RL]_e} - [RL]_e \quad (5a)$$

2.2. Reaction 2

In a second step, a defined concentration of fluorescent label, $[L^*]_0$ is added to react with the free receptor binding sites. Next to the formation of receptor–label complexes, the bound receptor–ligand complex can dissociate and the ligand be replaced by the label, resulting in a lower concentration of $[RL]_{t=1}$, as predicted by Eq. (5). The degree of dissociation is dependent on the stability of the receptor–ligand complex. The concentration of formed receptor–label complex during reaction (2), $[RL^*]_{t=2}$, can be calculated by integrating the two differential Eqs. (1) and (2). If the concentrations of the ligand and the label do not change drastically during the time course of the second reaction, i.e., only a small fraction of the label and the ligand binds to the receptor (<10%), these equations can be

solved according to Motulsky and Mahan [15] with, as boundary condition, $t=0$, $[RL]=[RL]_{t=1}$:

$$[RL^*]_{t=2} = \frac{[R]_0[L^*]_0 k_2}{K_F - K_S} \cdot \left(\frac{k_1 - (K_F - K_S)}{K_F K_S} + \frac{k_1 - K_F \cdot \left(1 - \frac{[RL]_{t=1}}{[R]_0}\right)}{K_F} \cdot \exp^{-K_F t_2} - \frac{k_1 - K_S \cdot \left(1 - \frac{[RL]_{t=1}}{[R]_0}\right)}{K_S} \cdot \exp^{-K_S t_2} \right) \quad (6)$$

in which t_2 is the reaction time of reaction (2) and K_A, K_B, K_F and K_S are constants defined by the affinity constants and ligand and label concentrations:

$$K_A = k_2[L^*]_0 + k_2 \quad (6a)$$

$$K_B = k_1[L]_0 + k_1 \quad (6b)$$

$$K_F = 0.5K_A + K_B + \sqrt{(K_A - K_B)^2 + 4k_1k_2[L]_0[L^*]_0} \quad (6c)$$

$$K_S = 0.5K_A + K_B - \sqrt{(K_A - K_B)^2 + 4k_1k_2[L]_0[L^*]_0} \quad (6d)$$

The boundary conditions are only met when the concentration of ligand and label are high in comparison with the receptor concentration or their respective affinity is low. If this is not the case, the system can only be modelled using numerical methods.

2.3. Detection

The detector noise, N, is calculated by a function consisting of a constant part, i.e., the instrument noise in absence of any fluorescence signal (shot noise, n_s) and a variable part, which depends linearly on the concentration of fluorescence (flicker noise, n_f) [16]:

$$N = n_f S + n_s \quad (7)$$

in which S is the amount of background fluorescence. An inherent fluorescent background is present due to the receptor–label complex. Thus, the S term is linearly dependent on the concentration of receptor–label complex formed:

$$N = n_f r[R\text{-}L^*] + n_s \quad (8)$$

In which r is the response factor for the receptor–ligand complex. If homogeneous assays are used, the background fluorescence is also influenced by the fluorescence of the free fluorescence label. Thus,

$$N = n_f(r_{bound}[R\text{-}L^*] + r_{free}[L^*]) + n_s \quad (9)$$

r_{bound} and r_{free} being the response factors of the bound and the free label, respectively.

2.4. Combined system

To calculate the response of the RAD system, the several parameters that influence detector response have to be combined in one model. Several authors have discussed the complexity of modelling reaction kinetics in post-column detection systems [17,18]. Under first-order reaction kinetics, it was found that by first approximation the dispersion and reaction kinetics can be modelled separately.

In the RAD system, however, second-order reaction kinetics are dominating. Initial experiments showed that the influence of the dispersion of the post-column RAD system compared to the dispersion of the total LC–RAD set-up is relatively small, i.e., <30%. Therefore, and in order to simplify the modeling in the flow-injection RAD system as used below, the dispersion is considered to take place before the reaction and assumed to be constant. Thus, by adjusting the concentrations of the reagents and the ligands after every mixing step for the dilution of the merging fluids, the detector response can be predicted by sequential calculation of dispersion, reaction efficiency, as defined by Eq. (6), and the response factor of the fluorescence detector.

3. Experimental

3.1. Materials

Coumestrol was obtained from Eastman Kodak (Rochester, USA). 17β-Estradiol, ethinyl-estradiol and estriol (Fig. 2) were purchased from Sigma (Bornem, Belgium). Zeranol, equol and genistein

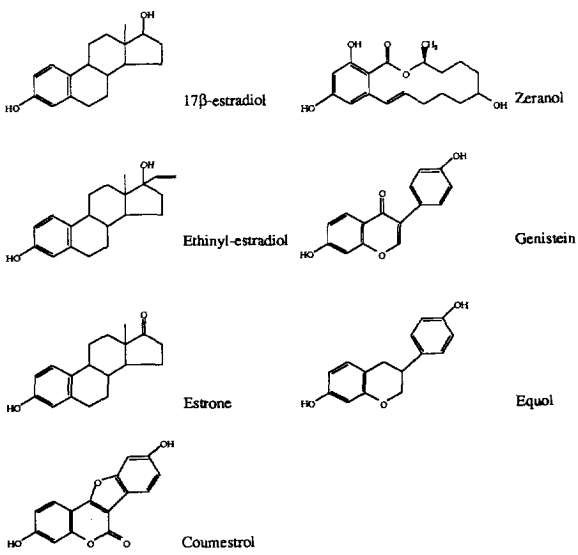

Fig. 2. Structurally related steroids.

were kindly donated by TNO (Zeist, The Netherlands). A yeast extract containing the human estrogen receptor ligand binding domain was a gift from KaroBio (Huddinge, Sweden) [19]. Potassium phosphate was from Merck (Darmstadt, Germany). Acetonitrile and methanol were obtained from Rathburn (Walkerburn, UK). All other organic solvents were purchased from Baker (Deventer, The Netherlands) and were of analytical grade. The binding buffer consisted of potassium phosphate (200 mmol/l, pH 7.4).

3.2. Apparatus

The receptor affinity detection system based on the estrogen receptor was similar to the detection system as described in [6] with a few modifications. The flow injection (FI)– and LC–RAD system (scheme, see Fig. 1) consisted of two Kratos-ABI (Ramsey, NJ, USA) Spectroflow 400 pumps and a Pharmacia (Uppsala, Sweden) P3500 pump used to deliver the mobile phase, coumestrol and the estrogen receptor solution, respectively. Sample handling and injection was performed using a Gilson (Villiers-le-Bel, France) ASPEC XL equipped with a Rheodyne six-port injection valve (injection loop, 20 μl). A Merck-Hitachi (Darmstadt, Germany) 1080 fluorescence detector (excitation wavelength, 340 nm; emission wavelength, 410 nm) was used for detection. The FI carrier solution consisted of binding buffer and was pumped at a flow-rate of 0.3 ml/min. The estrogen receptor solutions were prepared in binding buffer and added the FI carrier solution via inverted Y-type mixing unions and was pumped at a flow-rate of 0.3 ml/min. The coumestrol solution, at a concentration of 30 nmol/l, was added to the carrier stream via an inverted Y-type mixing union at a flow-rate of 0.3 ml/min. Knitted 0.3-mm I.D. PTFE reaction coils were used for reaction detection. The reaction was performed at ambient temperature. The analytical system was controlled by Gilson 719 Pascal software.

3.3. Calculations

Association and dissociation rate constants of 8×10^{6} l mol^{-1} s^{-1} and 8×10^{-3} s^{-1}, respectively for 17β-estradiol were used for the theoretical calculations [19–21]. The binding constant for the coumestrol–ER complex is 20% of the 17β-estradiol–ER complex. We therefore assumed a dissociation rate constant of 0.04 s^{-1} for the coumestrol receptor in all subsequent calculations. The dilution factor due to dispersion was measured by injecting an inert dye, potassium iodide, into the RAD system. It was found that the original analyte concentration was diluted four times in the RAD system. In all calculations, the analyte concentrations were therefore corrected by a factor of 4.

All theoretical calculations were performed in Turbo Pascal (Borland, Sunnyvale, CA, USA). The theoretical detector response for an analyte was calculated by first calculating the analyte concentration after dispersion in the RAD system. Subsequently, the concentrations of analyte, receptor and label were corrected for dilution due to merging of the analyte solution with receptor and label solution in reaction mixing unions 1 and 2. Finally, the ligand–receptor and label–receptor complex concentrations after reactions (1) and (2) were calculated using Eqs. (5) and (6), respectively.

Limits of detection (LODs) were determined by first calculating the detector noise using Eq. (8). Subsequently the detector response under the same conditions was calculated as described above. Finally the LOD was determined by an iteration procedure in

which the analyte concentration was varied by increments of 10^{-10} mol/l until a signal-to-noise ratio of 3 was obtained.

4. Results and discussion

4.1. Set-up

The RAD system is based on the steroid binding domain of the estrogen receptor (hereafter, estrogen receptor) and a fluorescent estrogen, coumestrol [11]. Upon binding to the estrogen receptor, coumestrol exhibits a significant blue shift in emission wavelength, while the fluorescence intensity increases as well. Therefore the RAD system was operated in the homogeneous mode omitting the separation step between free and receptor-bound label.

4.2. Reaction kinetics

The detector response of the post-column RAD system is dependent on the reaction time and the concentration of both reagents, i.e., the estrogen receptor and coumestrol. Fig. 3 shows the predicted values of the detector response, calculated using Eqs. (5)–(7), and the measured values at several reaction times for reaction (1). The relative peak height is the normalised negative height of the peaks. At $t=0$, the reaction is practically identical to a competitive assay were the receptor and the label are added simultaneously to the carrier solution. Theoretically, this situation has been described by Motulsky and Mahan [17]. In a sequential set-up, i.e., when the receptor is incubated with the ligand prior to the addition of the label, better sensitivities are obtained (see Fig. 3). These observations are in agreement with the results of Rodbard, who proved theoretically as well as experimentally that delayed addition of the label results in better sensitivity [22].

Fig. 4 depicts the RAD response when the receptor concentration is varied (1.5–15 nmol/l; both reaction times, 30 s). At high receptor concentrations, the reaction is close to complete, resulting in almost linear calibration curves for 17β-estradiol from 1 to 100 nmol/l (results not shown). At lower receptor concentrations, however, the reaction kinetics become rate limiting, resulting in non-linear response curves.

In Fig. 5 the theoretical detector response for 17β-estradiol is determined at three label concentrations varying from 3 to 300 nmol/l. Due to the higher concentration of the label, more receptor–label complex is formed during reaction (2). Thus, the absolute signal of label and receptor increases. When a homogeneous set-up is used, the high label

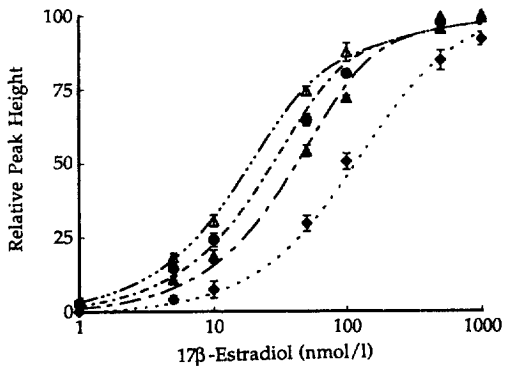

Fig. 3. Detector response vs. 17β-estradiol concentration at four reaction times for reaction (1), i.e., (♦) 0, (▲) 15, (●) 30 and (△) 60 s. Estrogen receptor=5 nmol/l. The points indicate measured data and the lines calculated values. For further details, see Section 3.

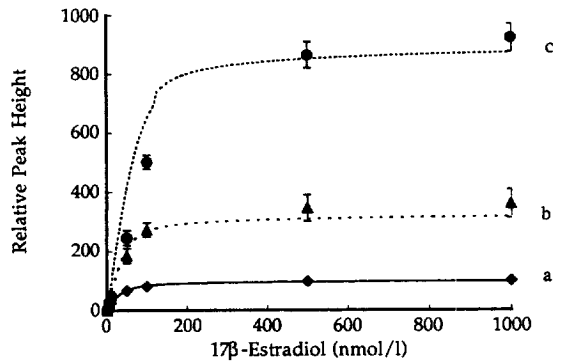

Fig. 4. Detector response vs. 17β-estradiol concentration at three receptor concentrations, i.e., (a) 1.5, (b) 5, (c) 15 nmol/l. The points indicate measured data and the lines calculated values.

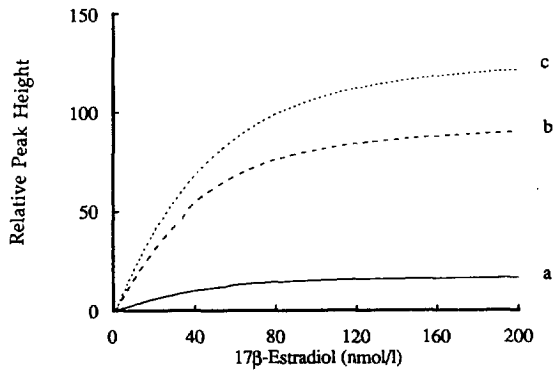

Fig. 5. Theoretical detector response vs. 17β-estradiol concentration at three coumestrol concentrations, i.e., (a) 3, (b) 30 and (c) 300 nmol/l.

concentrations also result in a high fluorescent background and, consequently, in a higher noise level.

4.3. Detector response vs. affinity

Previous experiments indicated that the response of the RAD system is dependent on the affinity of the injected ligands [11]. In Fig. 6, the calculated and measured detector response is depicted for six estrogenic compounds ranging in affinity from 1 to 200 nmol/l [23]. The calculated response was manually fitted, by keeping the association rate constant fixed and modifying the dissociation rate constant. We assumed that the association rate constants were equal and the differences in affinity were only due to a difference in the dissociation rate constants. Most of the response curves for the ligands tested exhibited a good fit when compared with the calculated response. Only genistein exhibited a deviation of the predicted values.

The concentration of estrogenic compounds in which 50% of maximum signal is obtained, EC_{50}, was compared the IC_{50} values of the batch receptor assay values of Arts et al. [23] (see Fig. 7). It appeared that the measured EC_{50} values of the RAD system are higher by a factor of 20 than the IC_{50} values of the batch radioreceptor assay, which is due to dilution caused by dispersion and mixing and to incomplete reaction of the receptor and the ligands. Thus, good correlation is observed between the values obtained by the RAD data and the batch receptor assays ($r^2 = 0.95$).

By iteration of the ligand concentration at a fixed affinity constant, a theoretical EC_{50} could be calculated (Fig. 7, solid line). A good correlation is observed between the measured and the calculated EC_{50} values.

For high-affinity interactions, the EC_{50} values deviate from the linear relationship between affinity and response. Whereas the response for lower affinity

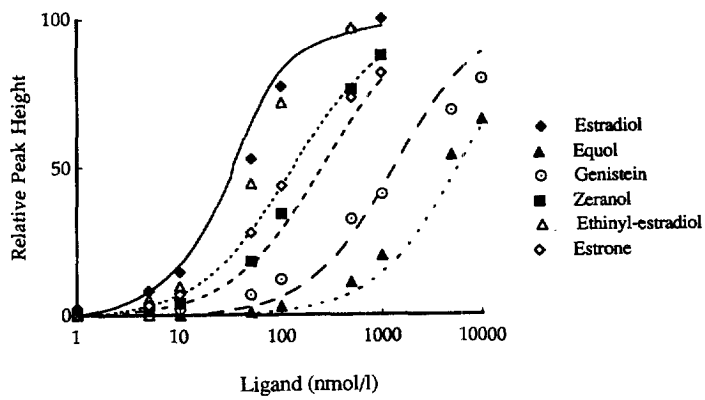

Fig. 6. Detector response vs. ligand concentration of six estrogens. The points indicate measured data and the lines calculated values. The lines for 17β-estradiol and ethinyl-estradiol do not differ significantly.

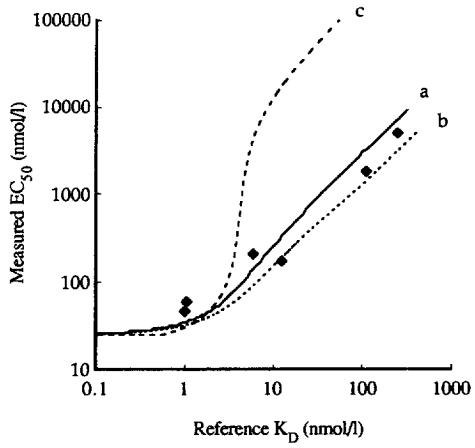

Fig. 7. Correlation of the EC_{50} values as measured by the RAD system and the affinity as given in Ref. [21]. The solid line (a) describes the expected affinity as calculated by the theoretical model. (b) As (a), only with a label concentration of 3 nmol/l; and (c) as (a), 3000 nmol/l coumestrol.

compounds is mainly determined by the dissociation rate constant, at high affinity the dissociation of the receptor–ligand complex is negligible. Thus, the response is only dependent on the association rate of the affinity interaction which is constant. Consequently, an identical response for compounds with different dissociation rates is expected. These findings are supported by earlier findings using avidin and biotin ($K_D = 1 \times 10^{-15}$ mol/l, $EC_{50} = 100$ nmol/l) [6], in which a similar response is measured as for the 17β-estradiol in the current set-up.

Fig. 8 shows LC–RAD chromatograms demonstrating the influence of affinity constants and concentrations injected on the response of the LC–RAD system. A 200-fold higher concentration of equol has to be injected to obtain a similar response as 17β-estradiol (10 nmol/l).

4.4. Label affinity and concentration

The performance of the RAD system is strongly dependent on the label used. The amount of label that binds to the receptor in absence of ligand is dependent on the affinity and concentration of the label, comparable to the receptor–ligand binding in reaction (1). Thus, when a low-affinity label is used a high concentration of label is necessary to obtain similar receptor–label binding as when a high-affinity label is used.

We also calculated the effect of the label concentration on the receptor–ligand complexes (Fig. 7). In the second reaction, the label can compete with the bound ligand for the receptor binding site resulting in lower receptor–ligand complex concentrations than expected at the end of reaction (1). An increase in the label concentration will result in a higher degree of dissociation of the receptor–ligand complexes and, thus, in higher EC_{50} values. Due to the limited reaction time and the slow dissociation rate constant of high-affinity ligands, these are less affected than low-affinity ligands.

The described effect is in accordance with earlier results reported by Cheng and Prusoff, who derived an equation for the influence of the label affinity and concentration on the receptor–ligand binding under equilibrium conditions [24]:

$$EC_{50} = \frac{K_D}{1 + [L^*]/K_L} \tag{10}$$

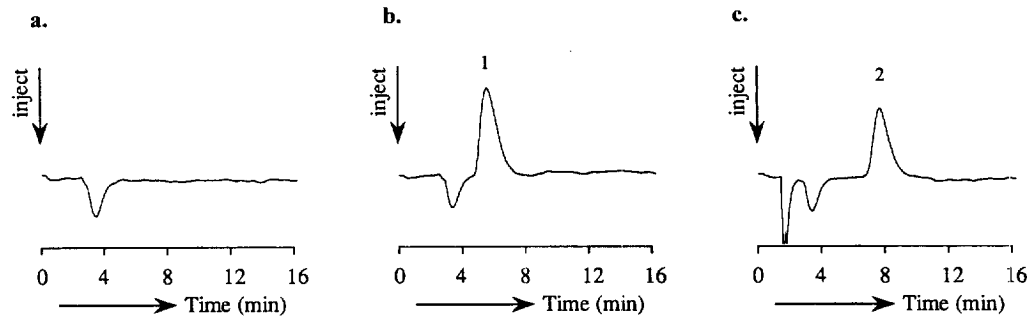

Fig. 8. (a) LC–RAD of a blank sample, (b) LC–RAD of equol (2×10^{-6} mol/l) and (c) 17β-estradiol (1×10^{-8} mol/l).

K_D being the dissociation affinity constant of the ligand and K_L the dissociation affinity constant of the label.

5. Conclusions

In this paper, a model has been developed which relates the detector response of an RAD system to the most important instrumental and biochemical parameters of the reaction detection system. The predicted detector response correlates very well with measured data of an RAD system based on the human estrogen receptor. These findings strengthen the assumption that the effect of dispersion in RAD systems on the reaction efficiency is a constant factor.

A strong relationship between the affinities as measured by a batch radioreceptor assay and the EC_{50} values of the RAD system was observed for affinities from 1 to 100 nmol/l. This proves that the characteristics of batch receptor assays are maintained in a RAD configuration. The RAD system can, thus, in principle be used to estimate the affinity of novel ligands. When combined with a structure-elucidating detector, e.g., mass spectrometer, the analysis of complex mixtures on affinity and structure of binding ligands is simplified to one liquid chromatographic run.

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