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Journal of Chromatography A, 744 (1996) 195–203

JOURNAL OF CHROMATOGRAPHY A

Critical evaluation of the applicability of capillary zone electrophoresis for the study of hapten–antibody complex formation

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Abstract

The applicability of capillary zone electrophoresis (CZE) for the determination of association constants of an hapten–antibody complex with values as high as 10^7 $(mol/l)^{-1}$ was investigated (dissociation constant in the nmol range). As a reference method the well known, enzyme-linked immunosorbent assay (ELISA) was selected. The study describes the optimisation of the experimental conditions of the CZE technique. The CZE measurements were optimised according to an experimental design. The results of the CZE and ELISA methods are compared giving consideration to the reproducibility (repeatability) of the two methods.

Keywords: Affinity capillary electrophoresis; Association constants; Frontal analysis; Antibodies; Haptens; Affinity capillary electrophoresis

1. Introduction

Various analytical separation techniques have been applied to determine either the unbound (free) drug or the bound drug concentration in solution without disturbing the equilibrium. Of these methods, the techniques whereby the unbound drug and the protein–drug complex are separated are nowadays frequently applied [1]. An alternative separation technique used to study protein–drug interactions is capillary zone electrophoresis (CZE). It is a very simple and extremely efficient one-phase separation technique capable of separating e.g. a drug and its protein–drug complex according to their net charge and size difference. The small amount of sample required to establish an equilibrium constant is another advantageous feature of capillary electrophoresis. The promising potential of CZE to study protein binding is now recognized and the number of applications is increasing steadily. As far as we know, the free drug concentration has either been estimated indirectly from a shift in migration time due to binding [2–6] or measured as the UV signal of the free drug [7].

In this paper, we present the results of an investigation in which CZE is used for the determination of the binding parameters of a hapten–antibody system. These (catalytic) antibodies have been used to accelerate a Diels–Alder reaction. This reaction is a versatile tool in organic chemistry for the synthesis of six-membered ring compounds. Meekel et al. [8] reported an example of an antibody-catalysed hetero-Diels–Alder reaction between a 1,3-diene and an arylnitroso dienophile.

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0021-9673/96/$15.00
PII S0021-9673(96)00275-0

In this paper, the applicability of CZE for the determination of the association constants of two hapten–antibody complexes was investigated and compared to the enzyme-linked immunosorbent assay (ELISA) method [9]. Because only a very limited sample quantity was available, the frontal analysis method [7] was used.

2. Theoretical

2.1. Binding isotherm

In describing binding processes the following terminology is adopted:

D (Drug): the interactant whose UV absorption is measured; in this study the "hapten".
P (Protein): the interactant whose concentration is fixed; in this study the "antibody".

It has been shown that IgG antibodies have two identical but independent binding sites [10]. The binding isotherm for such a complexation reaction with two independent binding sites can be expressed as:

$$r = \frac{[D_b]}{[P_t]} = \frac{2K_{Ass}[D_f]}{1 + K_{Ass}[D_f]} \quad (1)$$

where: $[D_b]$ is the bound hapten concentration; $[D_f]$ is the free hapten concentration; $[P_t]$ is the total antibody concentration and K_{Ass} is the association constant.

The determination of the binding parameters using CZE is based on the measurement of the concentration of the free hapten $[D_f]$ as a function of the total concentration of hapten $[D_t]$ added and constructing the binding isotherm according to Eq. 1.

In the present CZE and ELISA measurements two ways were used to estimate the association constant and the confidence interval: (i) using Eq. 1, subsequently this will be called estimation from the r–$[D_f]$ plot; (ii) the binding isotherm was rewritten as a direct relationship between the free hapten concentration, $[D_f]$, and the total hapten concentration, $[D_t]$, $[D_f] = f([D_t])$. This is subsequently called a $[D_f]$–$[D_t]$ plot. From this relationship, the association constant and the number of binding sites can also be estimated. Compared to an estimation using a r–$[D_f]$ plot or an estimation from the commonly used Scatchard plot, $r/[D_f] = f(r)$, [11], this $[D_f]$–$[D_t]$ relationship has the advantage that measurement errors do not affect the x-values of the plot, which gives a clearer statistical interpretation. An explicit expression for $[D_t]$ can be derived from $[D_t] = [D_f] + [D_b]$ and Eq. 1.

$$[D_f] = \frac{-[P_t]n - \frac{1}{K_{Ass}} + [D_t]}{2} + \frac{1}{2}\sqrt{\left([P_t]n + \frac{1}{K_{Ass}} - [D_t]\right)^2 + \frac{4[D_t]}{K_{Ass}}} \quad (2)$$

2.2. Experimental design

An experimental design was used to select those hapten concentrations whereby the optimum precision for the estimated association constant is obtained. This selection was made by first expressing the standard deviation of K_{Ass} as a function, f, of the D_t concentration, the value of K_{Ass} and the value of n, the number of binding sites

$$\sigma_{K_{Ass}} = f(K_{Ass}, n, D_t)$$

Subsequently, a minimisation of this function f, with respect to D_t, yields the optimum concentration, $[D_t]_{opt}$, for making measurements. This derivation can be applied when the stoichiometric model is adequate and a real value of K_{Ass} is given. The value of K_{Ass} and the number of binding sites used in the derivation can be determined from another set of measurements. In order to allow for the fact that this estimated value of K_{Ass} might change, two D_t concentrations on both sides of $[D_t]_{opt}$ were chosen, so that they were optimal should the value of K_{Ass} deviate from the set value by 10%. From computer simulations it was found that the experimental design is applicable for both the r–$[D_f]$ plot and the $[D_f]$–$[D_t]$ plot.

3. Experimental

3.1. CZE

3.1.1. Materials

The antibody, indicated as 290-4B10 ⟨4B10⟩, and the hapten 5 ⟨H6A⟩ and 6 ⟨H6B⟩ were prepared as described in Meekel et al. [8]. Stock solutions of the haptens were made in dimethylsulfoxide (DMSO): H6A $3.51 \cdot 10^{-2}$ mol/l and H6B $3.74 \cdot 10^{-2}$ mol/l. The 0.1 *M* phosphate buffer was prepared by dissolving $KH_2PO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany) in deionized water; The pH was set to 9.4.

Hydrochloric acid and potassium hydroxide were also obtained from Merck. DMSO used to dissolve the haptens was purchased from Janssen Chimica (Geel, Belgium).

Phosphate-buffered saline (PBS) pH=7.4, was prepared by dissolving: 1.435 g $Na_2HPO_4 \cdot 2H_2O$ + 0.2 g KH_2PO_4 + 0.2 g KCl + 8 g NaCl (per litre).

Deionized water for the preparations of solutions was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA) and filtered through a Millipore filter (Type HV, 0.45 μm) before use.

3.1.2. Apparatus

Electrophoresis was performed at 25°C with a laboratory-built system as described by Kraak et al. [7]. The operating voltage was 10 kV. Electrokinetic sample introduction was used (90 s, 10 kV). Before each run and subsequently, the capillary was rinsed for 2 min with 0.03 *M* hydrochloric acid solution, 1 *M* potassium hydroxide solution, and finally with the running buffer (0.1 *M* phosphate buffer, pH=9.4). Fused-silica capillary tubing of 50 μm I.D. (365 μm O.D.) was purchased from Polymicro Technologies (Phoenix, AZ, USA); effective length 31.70 cm, total length 58.70 cm. The optical window was prepared by burning off the outer polyimide coating. The bubble-cell capillary was constructed in the glass-works of the University of Amsterdam, effective length 31.75 cm, total length 58.65 cm. UV detection was carried out at 282 nm, with a sensitivity setting of 0.005 AUFS. Electropherograms were recorded with a BD41 chart recorder (Kipp and Zonen, Delft, Netherlands) at a chart speed of 10 mm/min.

3.1.3. Procedures

In frontal analysis, the capillary is filled with buffer. A large sample plug, containing buffer + hapten + antibody, is injected into the capillary. Before injection, the monoclonal antibody is incubated in solution with the hapten until equilibrium is reached. To accomplish this, a solution of the haptens (40 μl) in phosphate buffer at varying concentrations, (H6A 2.78 to 10.4 μmol/l, H6B 3.40 to 7.40 μmol/l) was mixed with 40 μl of a solution of the antibody (4B10 2.07 μmol/l) and equilibrated for 30 min. Since the mobilities of the hapten ($2.22 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}) and the antibody ($1.95 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}) differ slightly, the free hapten leaks out of the plug at the front edge and a plateau is formed. The plateau at the rear end of the elution profile is related to the hapten–antibody complex that is supposed to be still in equilibrium with free antibody and free hapten. It has to be remarked that the mobility of the free antibody equals the mobility of the hapten–antibody complex. This is a pre-requisite for the technique in the present form. The height of the plateau at the front edge reflects the free hapten concentration. In Fig. 1 a scheme of the frontal analysis CZE method is presented, accompanied by a representative electropherogram. For more details, see [7].

3.2. ELISA

3.2.1. Materials

Apparatus: Titertek Multiskan Plus (Eflab, Finland); ABTS and gelatine from Sigma (St. Louis, MO, USA); low affinity microtiter plate from Greiner (Frickenhausen, Germany); ELISA plate from Costar (Cambridge, MA, USA); Tween 20 from Merck–Schuhardt (Hohenbrunn, Germany) and goat anti-mouse IgG coupled with horseradish peroxidase from Pierce (Rockford, IL, USA).

3.2.2. Procedures

Following a previously described procedure [9,12], the association constants were determined by means of indirect ELISA. Briefly the method involves incubating the antibody, 4B10, at constant concentration with haptens H6A and H6B at various

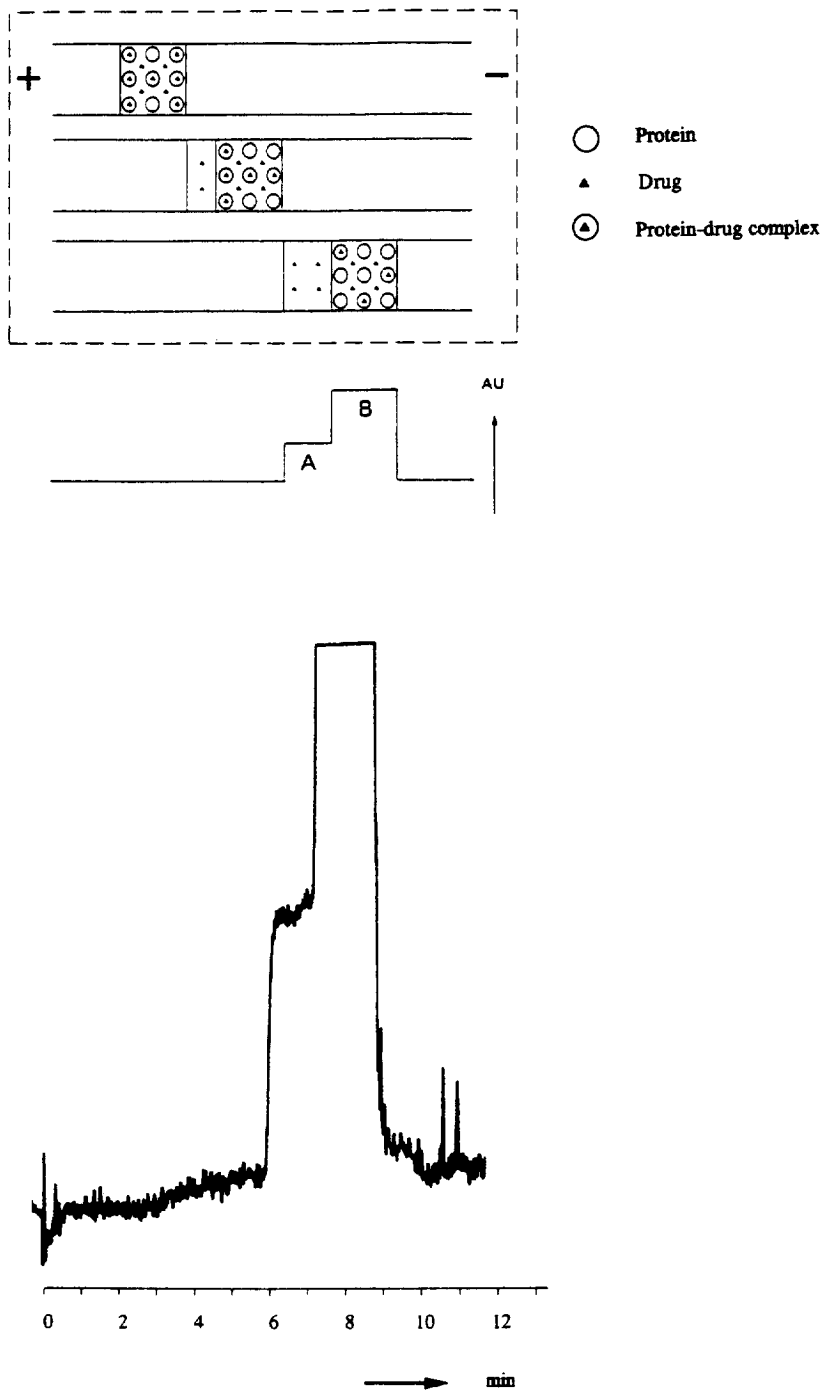

Fig. 1. Schematic representation of the frontal analysis CZE method and a representative electropherogram.

concentrations until equilibrium is reached. Then the concentration of the free antibody was determined by indirect ELISA.

4. Results and discussion

4.1. CZE

4.1.1. Optimization of the experimental conditions

The experimental work in CZE was initially done with PBS pH 7.4, the same buffer as used in the ELISA experiments, and in a 50 μm I.D. fused-silica capillary. However, with this running buffer unreproducible migration times were found, most probably due to the low buffering capacity of the PBS solution. Replacing the PBS solution by a phosphate buffer, 0.1 *M* pH=7.4, results in stable migration times but the separation was poor. In order to create sufficient difference in migration time between the hapten and the hapten–antibody complex, the pH of the phosphate buffer had to be increased to 9.4. Fig. 2 shows a typical binding isotherm as measured under these conditions. As can be seen the data points scatter largely. This scattering of the data points can be attributed to the limited detector sensitivity due to the short optical path length. This limitation excludes the precise measurements of the small plateau heights. In fact, due to the rather high detection limit, only a small part of the curve could be measured properly. Therefore it was not meaningful to fit a Langmuir curve to this data.

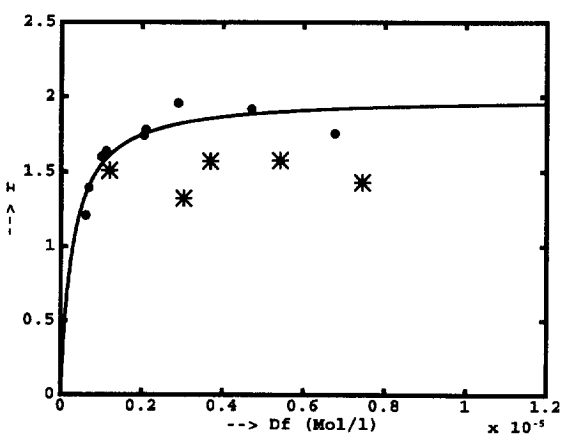

Fig. 2. The r–[D_t] plot obtained with a normal 50 μm I.D. capillary (*) and with a bubble-cell capillary (●). Hapten–antibody system: H6A–4B10.

In order to decrease the limit of detection, the optical pathlength had to be extended. For this purpose, we replaced the 50 μm I.D. capillary by a 50 μm I.D. laboratory made bubble-cell capillary. The bubble-cell capillary offers a unique method to extend the pathway with nearly no degradation of the separation efficiency. In this way, we were able to extend the path length by a factor of five. The improved signal-to-noise ratio allowed us to measure hapten concentrations as low as $6 \cdot 10^{-7}$ mol/l, and thus a larger part of the isotherm could be measured. Besides an improvement in the limit of detection, the precision in the determination of the small plateau heights was considerably improved by using the bubble-cell capillary. All further experiments were therefore carried out with the bubble-cell capillary.

4.1.2. Matrix effects

The free hapten concentration is determined from the plateau occurring when the free hapten leaks out of the sample plug and of the reference plateau, when injecting only the hapten, according to:

$$[D_f] = \frac{A}{B}[D_t] \quad (3)$$

where: A is the height of the hapten plateau in the sample; B is the height of the hapten plateau in the reference; [D_t] is the total hapten concentration.

When matrix compounds also leak out of the sample plug and show a response to the detector, a systematic error in the plateau height is introduced. The magnitude of the effect of co-eluting substances on the plateau height was determined by injecting a plug of the separate antibody and haptens. From these experiments, it appeared that DMSO, always present in the hapten solution, and a matrix compound from the protein solution is co-eluted with (free) hapten and heightens the plateau of the hapten. Since the DMSO content in the hapten solutions and the amount of protein is the same in all experiments a constant height (AU) has to be subtracted from the hapten plateau in the sample and the reference plateau according to:

$$[D_f] = \frac{A - C}{B - D}[D_t] \quad (4)$$

where: C is the height of a matrix component in the antibody solution; D is the height of the DMSO plateau present in the sample.

The order of magnitude of C and D are approximately $1.4 \cdot 10^{-04}$ AU and $3.8 \cdot 10^{-05}$ AU.

4.1.3. Determination of the binding parameters

With the optimized experimental set-up, the binding isotherms of different hapten–antibody systems were measured. The K_{Ass} and the number of binding sites, n, were extracted from the experimental data using a nonlinear regression procedure [13]. The confidence intervals of K_{Ass} were determined by performing Monte Carlo simulations [14].

Fig. 3 show the typical r–$[D_f]$ and $[D_f]$–$[D_t]$ plots as obtained with CZE. As can be seen, only a limited part of the isotherm can be determined.

The antibody quantity is usually very small, (500 μl), and with this volume about 25 measurements can be performed. In order to calculate a reliable association constant from the limited part of the binding isotherm, it is important to select the right hapten concentration range. To achieve this, we decided first to measure an isotherm with 8–12 data points and to use this data set to select the optimal hapten concentrations for the measurement of the second final isotherm, via the experimental design approach. The calculated association constants and the number of binding sites, including the confidence intervals of the two runs are given in Table 1. From the first run, the validity of the 2:1 stoichiometric model can be checked by examining whether any of the n values are within the 95% confidence interval. As can be seen from Table 1, for hapten H6A this is indeed the case but for H6B, n is only slightly out of the confidence limits. Therefore we can conclude that the selected stoichiometric model is the most adequate for the measured data. This is in agreement with Stryer [10] who reported that IgG antibodies have two binding sites.

From the data set of run 1 and supposing the 2:1 model, the optimal hapten concentrations for making

Table 1
The calculated association constants (K_{Ass}) and the 95% confidence intervals (C.I.) using the r–$[D_f]$ and $[D_f]$–$[D_t]$ plots for the initial run (run 1) and the second run in which the conditions are optimized by means of an experimental design assuming two binding sites (run 2)

Method	K_{Ass} $(\mu mol/l)^{-1}$	95% C.I.	n	95% C.I.	Run
$r-[D_f]$ plot					
H6A	3.1	1.7–8.1	2.02	1.86–2.17	1
	3.5	2.8–5.4	-		2
H6B	29	21–59	1.89	1.84–1.93	1
	25	21–38	-		2
$[D_f]-[D_t]$ plot					
H6A	4.1	2.4–8.5	1.97	1.87–2.09	1
	3.5	2.7–4.6	-		2
H6B	49	33–82	1.87	1.84–1.90	1
	24	21–28	-		2

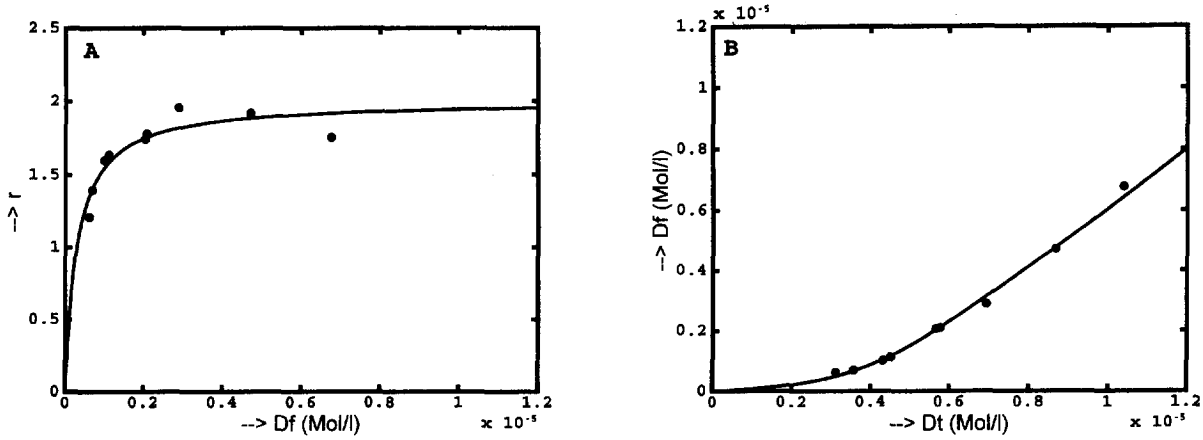

Fig. 3. Typical plots of r–$[D_f]$ (A) and $[D_f]$–$[D_t]$ (B) obtained with CZE using the bubble-cell capillary. Hapten–antibody system: H6A-4B10.

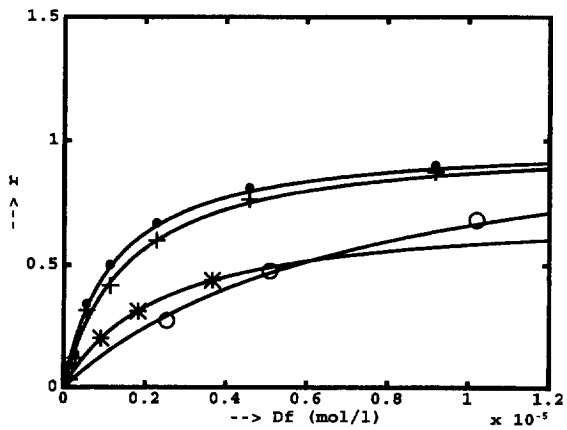

Fig. 4. Typical plots of r–$[D_f]$ measured on different days with ELISA. Hapten–antibody system: H6A–4B10. r–$[D_f]$ plots of the data in Table 2 indicated by: (●) 1#A1; (○) 2#C1; (+) 3#D1; (*) 4@F1.

measurements can be determined by the experimental design and computer simulations. It appears that the measurements can be best performed at three hapten concentrations and in fourfold. Under these conditions, an improvement of the standard deviation by a factor of 5.5 compared to the first run can be expected. The established improvement is reflected in significantly smaller confidence intervals (about a factor 2–2.5) as can be seen from Table 1.

Table 1 and Fig. 3 also show that processing of the data using the $[D_f]$–$[D_t]$ curve leads to a further decrease of the size of the confidence intervals, compared to the r–$[D_f]$ plot. For both haptens, the new confidence intervals are largely overlapping with the original ones. This indicates that the reproducibility of the two runs is good. It appears that the reproducibility for hapten H6A is somewhat better than for hapten H6B.

4.2. ELISA

Fig. 4 shows the r–$[D_f]$ plots of the same hapten–antibody system as obtained at different days. In contrast to CZE, the whole binding isotherm can be accurately measured. However, the difference in the shape of the binding isotherm as determined on different days is striking.

The association constants were calculated using the r–$[D_f]$ plot and are listed in Table 2. The experiments were performed by the workers indicated by # and by @. On each plate, the measurements were performed in duplicate. In the ELISA technique, the free antibody concentration is not directly measured. Therefore, it is not possible to confirm the number of binding sites. However, the estimate of the association constant does not depend on the value of n. The same association constants were found when using the $[D_f]$–$[D_t]$ plot for the calculation.

From Table 2, it can be seen that the repeatability of the association constant on the same plate is good. The repeatability as measured on different plates but on the same day is quite acceptable. However, the

Table 2
The association constants (K_{Ass}) and the 95% confidence intervals (C.I.) as found with the ELISA method using the r–$[D_f]$ plot. The letter in the code indicates the plate, the last number the duplicate and the first number indicates a different day

Run	K_{Ass} $(\mu mol/l)^{-1}$ H6A–4B10	95% C.I.	K_{Ass} $(\mu mol/l)^{-1}$ H6B–4B10	95% C.I.
1#A1	0.85	0.79–0.91	8.5	7.88–9.24
1#A2	0.87	0.82–0.94	10.3	9.49–11.2
1#B1	0.81	0.72–0.92	8.95	8.77–9.13
1#B2	1.07	0.95–1.22	8.92	8.75–9.10
2#C1	0.13	0.10–0.18	2.13	1.82–2.51
2#C2	0.14	0.11–0.19	2.68	2.29–3.17
3#D1	0.66	0.61–0.70	8.04	7.66–8.46
3#D2	0.73	0.68–0.78	7.92	7.54–8.33
3#E1	0.80	0.72–0.91	13.1	12.3–13.9
3#E2	1.03	0.92–1.16	15.6	14.6–16.7
4@F1	0.41	0.09–1.46	3.70	3.32–4.12
4@F2	0.43	0.14–1.20	3.87	3.48–4.32

value of the association constants as found on different days appear to differ significantly.

4.3. Average value of the association constants

An average value for the association constant as found with CZE was determined using all available CZE data. The 95% confidence interval was again established by Monte Carlo simulations.

The calculation of an overall association constant with ELISA is more complicated. The 95% confidence intervals in Table 3 are within-run estimates. Because of the poor reproducibility from run to run these intervals cannot be used for the derivation of an estimate of the 95% confidence interval for the average association constant from all the ELISA experiments. Therefore we took the average of all the measured association constants as the final estimate of the association constant. For the lower limit of the confidence interval, the smallest lower limit of all the confidence intervals was taken. The same was done for the upper limit of the confidence interval. The average association constants calculated with CZE and ELISA are given in Table 3. As can be seen, the association constants obtained with CZE are a factor of 3–5 larger than those found with the ELISA method. Our former results with this method for the bovine serum albumin–warfarin system indicated that the results acquired with the same CZE method were in agreement with the results obtained with other experimental methods [7]. Furthermore, the method showed an excellent reproducibility (robustness) for the measurements on this test-system (unpublished results). Therefore, we concluded that the frontal analysis method seems to be a good experimental method for these kind of measurements. It is known that differences in environmental conditions may lead to measurably different association constants [4]. In the ELISA and CZE method, the type of buffer and pH differ and this may partly explain the differences in association constants. Which value approaches the true value most closely can never be known and a discussion of the method of choice in that respect has little meaning. Therefore, we choose to discuss the advantages and disadvantages of the CZE and the ELISA method.

From the confidence intervals, it can be concluded that the precision of the ELISA measurements is comparable to that of the CZE measurements when the best ELISA runs are selected (indicated by ELISA/SEL in Table 3). It should be noted that the precision of the final ELISA results is adversely influenced by the poor run to run reproducibility.

Table 3
The average association constant (K_{ass}) and the 95% confidence intervals (C.I.) as estimated. In the case of the CZE measurements all data points of run 1 and 2 were used. For the ELISA method, the addition of ALL means all available data were used, the addition SEL means that the best runs (1#A1/A2, 1#B1/B2, 3#D1/B3, 3#E1/E2) were used in the calculations.

Method/plot	System	K_{Ass} (μmol/l)$^{-1}$	95% C.I.
CZE			
r–[D_f]	H6A–4B10	3.43	2.83–4.66
	H6B–4B10	22.3	19.5–27.9
[D_t]–[D_f]	H6A–4B10	3.60	2.98–4.48
	H6B–4B10	21.4	19.1–24.2
ELISA			
r–[D_f] ALL	H6A–4B10	0.66	0.09–1.46
	H6B–4B10	7.81	1.82–16.7
r–[D_f] SEL	H6A–4B10	0.85	0.61–1.22
	H6B–4B10	10.2	7.54–16.7

4.4. Comparison of the CZE and the ELISA method

One of the strong points of the CZE technique is the fact that the free hapten concentration is measured directly. This allows the confirmation of the stoichiometric model. Furthermore, the CZE measurements are simple and can be fully automated. The reproducibility of the association constants from run to run is good.

A weak point of the CZE measurements is the poor concentration sensitivity of the UV detection. This not only hampers the collection of data points over the full [D_f] range, but results also in a relatively large uncertainty in the measurement of [D_f]. This is mainly a limitation of concern for high association constants, because the higher the association constant the lower the free drug concentration will be. The application of an extended optical path length such as the bubble-cell is necessary to obtain reliable binding parameters under such conditions. Furthermore, the presence of matrix interferences might complicate the measurements and diminish the

reliability of the data points. The amount of antibody solution needed to measure an isotherm with CZE is about 500 μl. In contrast, with the ELISA method a complete binding isotherm can be obtained with 10 μl.

The ELISA method has several advantages. It is a fast method, because the samples are processed simultaneously. This gives the opportunity to establish isotherms for several antibodies and/or several haptens in one day. In contrast, with CZE it takes approximately one day to construct the isotherm for one hapten. Another advantage is that ELISA measurements can be made at very low free antibody concentrations. This offers the opportunity to measure the complete isotherm for the haptens–antibody systems.

However, there are some disadvantages connected to the ELISA method. The stoichiometric model cannot be confirmed with the method, because in this type of experiment the free antibody concentration is not directly measured. The free hapten concentration is calculated assuming that two haptens or only one has been bound per antibody molecule. Another drawback could be that ELISA measurements are difficult to automate. Finally, it seems that the reproducibility of the method from run to run is rather low, as is apparent in Table 2.

5. Conclusions

The selected hapten–antibody system can be considered as a test case to determine the application range of CZE for binding studies. Apart from the limited sample quantity, additional difficulties could be expected due to interferences by matrix compounds, inevitable in such systems. The results of the present work clearly shows that under optimized experimental conditions and with a strict measurement protocol, statistically reliable binding parameters can be obtained with CZE. An advantage of the frontal analysis CZE compared to ELISA is the possibility of checking the stoichiometric model.

Acknowledgments

The authors thank H.J. van Groen for his excellent technical contribution. Furthermore we wish to express our gratitude to Professor Dr. R. van Driel and Dr. J. Kragten for their helpful discussions. Astra Hässle, Sweden, is acknowledged for financial support.

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