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Immobilization of antibodies as a versatile tool in hybridized capillary electrophoresis

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Abstract

Hybridization of capillary electrophoresis (CE) and immunoassays (IA) can theoretically lead to highly sensitive and selective assays. Immobilization of antibodies in the capillaries employed for CE can be achieved either by adsorption to the capillary wall, which was coated prior to use in order to improve the adsorption, or by covalent binding to modified capillaries. For the evaluation of the concept, a fluoroimmunoassay for the herbicide atrazine was used. Antibodies were immobilized by adsorption, and the specificity of the binding of the labeled ligand was confirmed by saturation and competition experiments. For this particular assay the use of a C_8-modified capillary was shown to be preferable over C_{18}- and mercaptodimethylsilane-modified capillaries. The first part of the C_8 capillary wall was partially covered by antibodies and the remainder was covered by adsorbed bovine serum albumin to eliminate non-specific binding of the labeled ligand. In the present approach the antibody-bound fraction of the labeled ligand was quantitated, which means that after removal of the free fraction of the labeled ligand from the capillary, the binding of the labeled ligand and the analyte to the antibodies, should be broken. By changing the chemical environment such as pH, salts and organic solvents, this dissociation process can be facilitated. Addition of 25% methanol to the assay buffer increased the dissociation rate by 50% without inactivation or mobilization of the antibodies. On the other hand, these chemical tools should not interfere with the requirements for CE and fluorescence detection. Moreover, the methanol caused stacking of fluorescein-labeled atrazine (FA) in the sample plug by a factor of 30, which was very advantageous for the quantitation of FA. The results of this study imply that combination of antibodies and fluorescent labels with CE opens the way to multi-analyte immunoassays and forms a valuable tool for the selective preconcentration of analytes originating from complex biological matrices.

Keywords: Capillary electrophoresis; Immunoassay; Antibodies; Atrazine; Laser-induced fluorescence; Stacking

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1. Introduction

Immunochemical methods are being used extensively for qualitative and quantitative analyses of drugs and hormones present in biological matrices at nM–pM concentrations [1–3]. Clean-up steps are often not required, because most endogenous compounds do not interfere with the antigen–antibody binding. An antibody only recognizes a small part of the antigen molecule, the so-called epitope. Any molecule containing such an epitope and accessible to the antibody will bind as if it is the analyte of interest. This interaction is called cross-reactivity and the impact is largely dependent on the relative affinity to the antibody and the relative concentration in comparison with the affinity and concentration of the analyte. In practice, this means that the measured concentration of the analyte of interest is higher than its actual concentration. The cross-reactivity in antigen–antibody binding of structural analogs cannot always be controlled in a manner such that only a single analyte will interact with the antibody. This can be explained by the fact that structural changes do not always substantially change the epitope. On the other hand, cross-reactivity is an advantage if one wishes to screen for the presence or absence of a group of analytes belonging to the same class. Another positive effect of cross-reactivity of antibodies is that they are nowadays often employed for the preconcentration of analytes either off-line or on-line with chromatographic and/or spectroscopic procedures [1–3].

Separation of analytes can also be achieved by capillary electrophoresis (CE), which is based on size and charge, whereas liquid chromatography (LC) is mainly based on hydrophobic interaction with the stationary phase. It is therefore obvious to expect that an on-line combination of immunoaffinity electrophoresis and CE can offer a different type of selectivity in comparison with immunoaffinity chromatography. The resolving power of CE is enormous in comparison with LC, while the utilization of other physico-chemical properties for the separation can in many cases contribute to the selectivity of the analytical system [4]. The advantage of negligible consumption of sample and reagents is counteracted by the generally much lower concentration sensitivity in CE in comparison with HPLC. An important side effect of the selective, immunoaffinity extraction of analytes from a biological matrix is that at the same time substantial concentration of analytes is obtained.

Various researchers have tried to use ligand-binding species, such as proteins, antibodies and receptors, in combination with CE, calling it affinity capillary electrophoresis (ACE). The ligand-binding species can be dissolved in the separation buffer used or immobilized. Birnbaum and Nilsson [5] cross-linked bovine serum albumin with glutaraldehyde in the capillary. The protein–gel formed allowed the separation of optical enantiomers of tryptophan. The applicability of this procedure to other proteins and to enantiomeric separations is dependent on the effects of cross-linking on the actual binding sites of the protein [5]. Barker et al. [6] showed that the separation of the enantiomers of leucovorin could be achieved by the addition of BSA to the separation buffer. Coating of the capillary wall with polyethylene glycol was essential to reduce protein adsorption and subsequent peak broadening. ACE can also be used for the determination of affinity constants of peptides to low molecular weight receptors or enzymes. In that case, the binding protein is dissolved in the separation buffer and the change in the migration time of the receptor complex with a charged and labeled ligand is determined as a function of the concentration of the unlabeled peptides [7,8]. The use of antibodies in combination with CE was described by Guzman et al. [9]. They used aminopropyltriethoxysilyl-derivatized glass beads and coupled monoclonal antibodies after modification of the glass beads with phenylene-1,4-diisothiocyanate. The glass beads were installed in the capillaries between two glass frits [9].

When antibodies are to be immobilized in a capillary, this should not affect the ruggedness of the CE system or its general applicability. Filling capillaries with glass beads dramatically increases the chance of blocking. Moreover, the binding of the antibody to the antigen should be uniform, providing identical association and dissociation

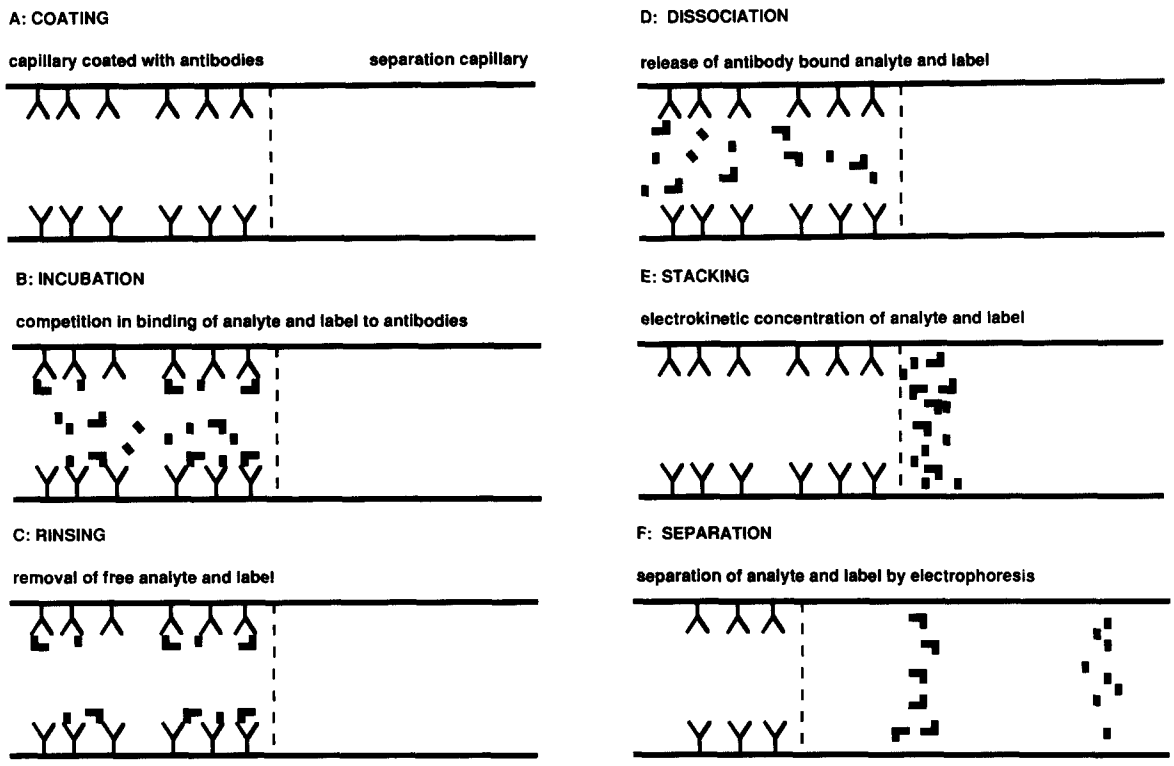


Fig. 1. Schematic representation of an on-line immunoassay in capillary electrophoresis.

kinetics irrespective of the place of attachment of the antibodies in the capillary. Differences in mass transport of the analyte molecules from the solution to the antibody, giving rise to peak broadening, should be avoided. Therefore, antibodies should preferably be attached to the capillary wall, either by adsorption or by covalent binding. Adsorptive binding is generally easier to achieve and theoretically opens up possibilities for the regeneration of the immunoaffinity part of the capillary. In principle, a limited length of the capillary wall should be occupied by antibodies, so that an adequate length of the capillary remains for the actual electrophoretic separation of the analyte(s) after release from the antibodies. Preferably, a well defined length of the capillary should be coated with antibodies in order to allow the selection of matching IA and CE conditions that provide maximum sensitivity and selectivity. A fixed length of capillary coated with antibodies can be obtained by controlled injection of reagents, which modify a limited length of the capillary wall.

2. The concept of coupled IA–CE

This concept is depicted in Fig. 1 for a single class of antibodies, one analyte and one label. Antibodies are bound to a limited length of the capillary wall at the injection side of the capillary (Fig. 1A). Then, a mixture of the analyte and a fixed concentration of the labeled ligand is injected so that these can react with the antibodies. With increasing concentration of the analyte, the amount of the label bound to the antibodies will decrease. When the incubation time is adequate, an equilibrium will be reached between the bound and free fractions of the labeled ligand and the analyte (Fig. 1B). With shorter incubation times, dependent on the relative association and dissociation rates of the compounds, the bound fraction

of the label will be different, although still dependent on the concentration of the analyte. As long as standardized assay conditions are maintained, non-equilibrium conditions can be used, which may sometimes drastically improve the sensitivity of an assay [10]. With a rinsing procedure, the unbound fractions of the label and analyte can be removed from the capillary (Fig. 1C). In the next step, the antibody-bound fraction of the label and analyte can be released and after dissociation these molecules diffuse mainly in the radial direction of the capillary, avoiding reassociation. By injection of a chaotropic agent, e.g., a salt solution, an organic solvent or a buffer solution with a high or low pH, the dissociation rate can be increased and reassociation diminished (Fig. 1D). However, chaotropic agents often disturb the tertiary structure of the antibody, which may sometimes be irreversible. Because it is desired to use the antibody-coated capillaries for multiple assays, reversibility of binding is essential.

The next step is the separation of the labeled ligand and the analyte by electrophoresis. The separation efficiency in CE is strongly dependent on the size of the injected sample plug. In the present situation the analytes are spread out over the length of the antibody coating. If the conductivity of the zone with the chaotropic agent and the analytes is lower than the conductivity of the separation buffer, the analyte is concentrated owing to the higher electric field, assuming that the analyte molecules are charged under these conditions (Fig. 1E). This stacking effect is essential for suitable quantitation of the label and for the separation efficiency (Fig. 1F). The latter does not play a major role when only one antibody and one labeled ligand, which can be selectively detected, are present, but for multi-analyte assays employing different antibodies and labels this is very important.

In this work, the potential of immobilizing antibodies to capillary walls was investigated, paying special interest to the modalities of the coating procedures, the non-specific binding of the labeled ligand and the electron-osmotic flow in the capillary.

3. Materials and methods

3.1. Chemicals

A ready-to-use 20 mM sodium tetraborate buffer (pH 8.0) (BB8) was obtained from Fluka (Buchs, Switzerland) and a ready-to-use 69 mM sodium–potassium phosphate buffer (pH 7.0) (PB7) was provided by Ciba-Geigy (Basel, Switzerland). Methanol and toluene of chemical grade (Ciba-Geigy) and Milli-Q water were used throughout all experiments.

Atrazine (A) (2-ethylamino-4-chloro-6-isopropylamino-1,3,5-triazine), monoclonal antibodies against atrazine and fluorescein-labeled atrazine (FA) were a gift from Ciba-Geigy. Bovine serum albumin (BSA) was obtained from Fluka and used without further purification. Mercaptomethyldimethylethoxysilane (MDS) was obtained from Fluka.

3.2. Instrumentation

A P/ACE 2100 CE instrument equipped with a fluorescence detector and a UV detector was used (Beckman Instruments, Fullerton, CA, USA). A 15 mW argon ion laser (Spectra-Physics, Mountain View, CA, USA) operating at 488 nm and a custom-built optical system, delivering 5 mW at the end of the optical fiber positioned on the detection window of the capillary, were used for excitation of the fluorescein-labeled ligand. Electrophoretic separations were made by applying 20 or 30 kV over the capillary, which was kept at 30°C; injections were made by pressure. The injection volume was proportional to the injection time.

The mobilities of the analyte and the label were determined, in addition to the electro-osmotic flow in the C_8 capillary coated with antibodies and BSA. Because mesityl oxide and atrazine do not have any fluorescence, the capillary was removed from the special LIF cartridge and installed in a UV cartridge compatible with the Beckman instrument.

3.3. Columns

Uncoated, open-tubular fused-silica capillaries (50 or 75 μm i.d., 360 μm o.d.) were obtained

from Polymicro Technologies (Phoenix, AZ, USA) and C_8- and C_{18}-coated fused-silica capillaries (50 μm i.d., 360 μm o.d.; CElect-H150 and CElect H250) from Supelco (Bellefonte, PA, USA).

3.4. Coating procedures

Coated capillaries were custom made after cleaning fused-silica capillaries with 1 M KOH for 2 h, rinsing with water for 10 min and rinsing with 0.1 N HCl for 10 min and drying for 3 h at 200°C during, which the capillary was flushed with nitrogen. Coating with MDS was done by filling the capillary with this reagent and placing it for 18 h in an oven at 200°C under vacuum in order to obtain a thin layer on the capillary wall.

3.5. Antibody immobilization

Coating of capillaries with antibodies was achieved by filling the capillaries with a mixture of the antibody solution (10 μg protein ml^{-1}) and PB7 (1:1 v/v) by means of capillary coupling or by pressure injection for 30 or 60 s by means of the CE instrument. In the latter case the capillary-containing cartridge was removed from the electropherograph after injection and laid horizontally in order to avoid siphoning of the antibodies through the capillaries during the 3 h of incubation at room temperature. Capillaries were filled with a solution of BSA in PB7 (1–2 mg ml^{-1}) and incubated for another 3 h at room temperature. The effect of BSA coating on the reduction of non-specific binding of the labeled ligand was studied.

3.6. Incubation conditions

After rinsing the capillary for 1 min with BB8, a solution containing FA with or without A was injected with pressure for 15–60 s and incubated for 10 min. By means of a 1 min backward rinse with BB8, unbound FA and A were removed from the capillary. Then a mixture of methanol and BB8 (1:3, v/v) was injected for 15–60 s by pressure. After a 5 min incubation during which the bound fraction of FA and A was released from the antibodies, the actual separation process was started by applying 20 kV over the capillary for 5–30 min, depending on the retention time of FA in the particular system. These steps correspond to parts B–F in Fig. 1.

Saturation experiments were conducted with FA concentrations ranging from 1 to 100 nM and competition experiments were conducted with a fixed concentration of FA (10 nM) and concentrations A ranging from 0.1 to 1000 nM. A series of experiments was run to establish the reproducibility and stability of binding with the C_8- and MDS-coated capillaries.

4. Results and discussion

4.1. Incubation conditions

For on-line IA–CE it is important to limit the total analysis time in order to obtain an acceptable sample throughput. For that reason, the migration times in a fused-silica capillary with different buffers were determined, which revealed that the migration times were almost constant between pH 7 and 9. The migration times were substantially shorter when 20 mM borate buffer was used instead of 20 mM phosphate buffer. Because at higher pH the fluorescence of fluorescein is higher and modified silicas degrade at pH > 8, it was decided to conduct all further experiments in a borate buffer of pH 8, irrespective of the coating of the capillaries.

4.2. C_8-coated capillaries

Antigen–antibody association

In a series of on-line immunoassays employing the C_8 capillary coated with antibodies, the capillary was rinsed (low-rinse setting of the CE instrument, 10 mbar) with the analyte and label-containing solutions for 10 min. This was done to avoid diffusion-dependent mass transport to the antibodies coated on the wall, which might seriously affect the association rates to the antibodies [11]. A 40-fold increase in rinse rate (high-rinse setting of the CE instrument) or rinsing with the electro-osmotic flow, generated with a 20 kV

voltage drop over the capillary did not have a detectable effect on the amount of bound label. These observations are in line with previous observations of Oroszlan and co-workers [12,13] with an immuno-sensor for atrazine that the association half-life is about 5 min at room temperature, which rules out the possibility that the association rate is limited by mass transport of label and analyte to the wall. It was therefore decided to inject a constant volume of the samples containing the label and analyte, instead of using a rinsing procedure. The reduction in sample consumption obtained in this way might be an advantage.

In these experiments it was predicted that equilibrium conditions would not be reached within an acceptable time, because with a half-life association rate for FA of 5 min equilibration would take about 1 h. However, after 10 min about 75% of the maximum binding was obtained, which was adequate and reproducible when the temperature in the capillary was kept constant.

Antigen–antibody dissociation

Within 1 min, 40% of the bound fraction FA dissociated from the immobilized antibodies when the capillary was refilled with the chaotropic agent, 25% methanol in BB8. Reassociation was negligible owing to the sink conditions created. After 5 min of incubation with 25% methanol in BB8, 90% of the bound label was released from the antibodies. The presence of methanol increased the dissociation rates by about 50% compared with borate buffer alone, but this effect was not completely understood. Higher methanol concentrations gave rise to denaturation and/or mobilization of the antibodies.

Non-specific binding

Non-specific binding of the label and analyte in other parts of the capillary was avoided during the incubation when a fixed sample volume was injected into the capillary and removed by backward rinsing. The fact that adsorption of the label during the actual electrophoretic separation might be a serious problem was confirmed with a number of control experiments. With a completely new, untreated C_8 capillary, the migration time of FA increased five-fold in comparison with normal fused-silica capillaries. This is mainly due to the strong interaction with the wall of the C_8 column, which resulted in plate numbers as low as 300. It was observed that coating of the capillary can be of the utmost importance, although is strongly dependent on the physico-chemical properties of the labeled ligand. Coating of the C_8 capillary with antibodies and BSA reduced the migration times of FA and increased the plate numbers to $>50\,000$.

Assay specificity

A number of experiments were performed to establish the specificity of the assay employing the C_8-coated capillary. This coating is required for the adsorption of both the antibodies and BSA, which is based on hydrophobic interactions. When such a capillary was coated only with BSA without antibodies, conduction of a pseudo-immunoassay with concentrations of FA up to 100 nM revealed that no peaks were detected. This implies that after rinsing no label was present in the capillary that could be released afterwards by the chaotropic agent. A second C_8 capillary, but now coated with antibodies and BSA, was used for saturation and competition experiments and the resulting curves are depicted in Fig. 2A and B, respectively. The affinity of FA to the antibodies expressed as an equilibrium dissociation constant (free concentration of FA corresponding to 50% occupation of binding sites) was estimated from Fig. 2A and found to be approximately 5 nM, which is in good agreement with the observations of Oroszlan and co-workers [12,13]. Also, the IC_{50} value for atrazine, with a fixed label concentration of 10 nM, was close to the value reported in the literature [12] and estimated to be between 5 and 10 nM (Fig. 2B). When 10% inhibition of FA binding can be determined, a limit of quantitation of 1 nM can be achieved.

Migration rates

The electro-osmotic flow in the C_8 capillary, determined with mesityl oxide was found to $61.3 \times 10^{-5}\,cm^2\,V^{-1}\,s^{-1}$. Atrazine, which is uncharged at pH 8, moved through the capillary with the electro-osmotic flow. Fluorescein-labeled atrazine has a negative charge at pH 8 and an

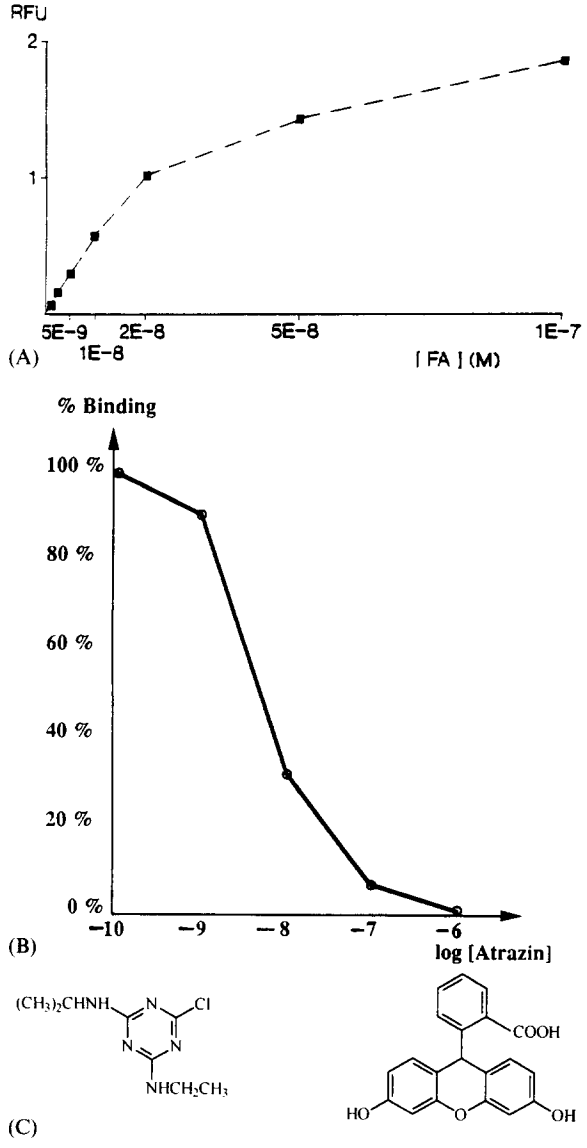


Fig. 2. Characterization of the binding of atrazine (A) and fluorescein-labeled atrazin (FA) to monoclonal antibodies. (A) Saturation of the binding of FA; [FA] (M) is the concentration of FA in M; B (RFU) is the amount antibody bound FA in relative fluorescence units. (B) Competition between A and FA for binding to monoclonal antibodies; % binding reflects the amount antibody-bound FA; [atrazine] is added concentration of A in M. (C) Structures of atrazine and fluorescein.

electrokinetic mobility of -31.9×10^{-5} cm$_2$ V^{-1} s^{-1}. The negative charge is favorable for the stacking process in that the combination of a large sample plug relative to the length of the capillary and the high electro-osmotic flow limits the time available for complete stacking.

Length of antibody coating

The length of the capillary antibody coating equals the injected plug of antibody solution and can be calculated when the column dimensions, the viscosity of the medium and the applied pressure are known [14]. In the present case, the lengths of the antibody coatings were estimated by determining the relation between the length of the capillary and the breakthrough time of a continuous injection of an aqueous FA solution. Making the assumption that the viscosity of the antibody solution (5 μg ml^{-1}) does not differ from that of other aqueous solutions, the plug length injected can be calculated on the basis of the injection time. With a 27 cm × 75 μm i.d. MDS-coated capillary the break-through time was 72 s, corresponding to 20 cm of capillary; with a 15 s injection of antibodies about 4 cm of the capillary was exposed to the antibody-containing solution. With two identical C_8 capillaries of 37 cm × 50 μm i.d., after coating with antibodies and BSA or with BSA alone the breakthrough times were 372 and 348 s, respectively, corresponding to about 30 cm of capillary. A 30 s injection therefore corresponds to about 2.5 cm of antibody-coated surface.

The difference in maximum binding capacity, reflected in the amount of antibody-bound FA, for different capillaries can be explained mainly by differences in the coating, capillary diameter and length of the coating. The actual length of capillary filled with label and analyte and the actual length of the capillary filled with the chaotropic agent which causes dissociation of the antigen–antibody complex determine the actual binding capacity from run to run.

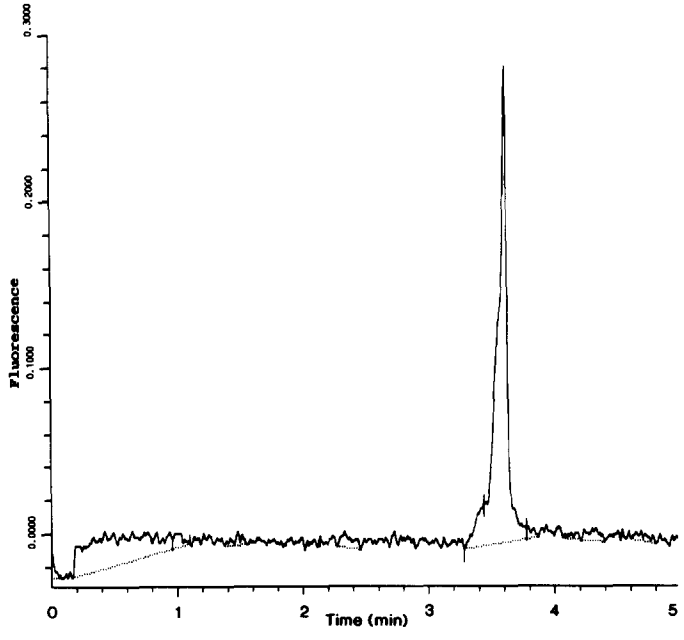
Stability of immobilized antibodies

The stability of the immobilized antibodies was determined by repetitive incubations with FA and assessment of the bound fraction. Experiments with the C_8 capillaries coated with antibodies and BSA showed good stability during four series of nine runs completed in 16 h. The peak areas for FA obtained for the four series were 24.2 ± 8.1, 18.7 ± 5.2, 17.3 ± 4.4 and 18.1 ± 1.5 (mean ± SD).

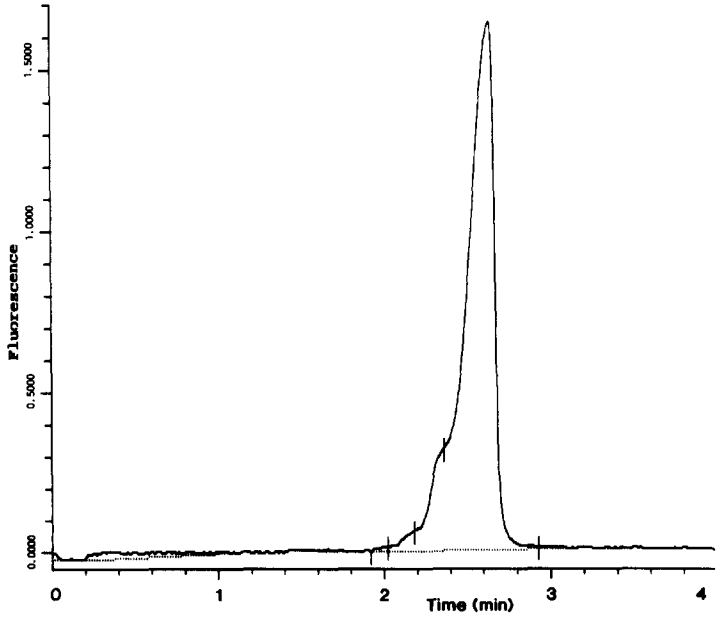
The variability in the binding of FA was higher than expected, but may be due to variations in temperature and injected volumes of label and chaotropic agent.

Stacking

One of the most important features of the combination of IA and CE is the sample stacking which can be achieved. When one considers that

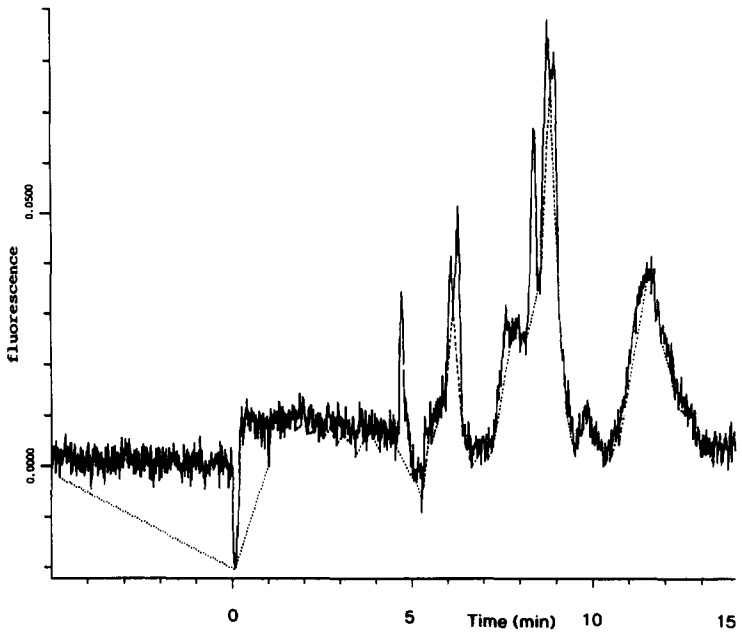


(A)



(B)

Fig. 3. (A), (B).



(C)

Fig. 3. Electropherograms obtained after on-line immunoassays with different capillaries coated with antibodies: (A) C_8 capillary, 50 μm i.d., coated with antibodies and BSA; (B) MDS capillary, 75 μm i.d., coated with antibodies; (C) C_{18} capillary, 75 μm i.d., coated with antibodies and BSA.

between 10 and 25% of the length of the capillaries is coated with antibodies and incubated with label and analyte, one would expect peak widths that are 0.1–0.25 times the retention time of the label peak in the electropherogram. In the stability test a control experiment with a direct 1 s injection of a 10 nM FA solution was made to determine not only the migration time but also the plate numbers in comparison with those obtained in the actual assay, where a 30 times longer analyte-containing sample plug is created. The plate numbers were only slightly different, 53 000 for a direct injection and 46 000 in case of the immunoassays, indicating an enormous stacking effect. The presence of 25% methanol slightly reduces the conductivity of the borate buffer, namely from 121 to 90 μS cm^{-1}, and will result in a 30% increase in field strength, which has a very small stacking effect [15,16]. Most probably, the ionization of FA is strongly affected by the methanol. Alcohols have a differentiating effect on the pK_a of fluorescein (HA), boric acid (HA^-) and atrazine (BH^+ and BH^{2+}). The net result is an increase in the ionization in the sample zone. Hence the electrophoretic mobility is increased and concentration will take place at the borderline between the borate buffer and the chaotropic agent.

Attempts to increase the stacking further by replacing the 20 mM borate buffer (pH 8) by 100 mM borate buffer were unsuccessful and resulted in substantial peak broadening. Attempts with phosphate buffer or the addition of sodium chloride to the separation buffer did not have the expected effects. Moreover, the latter had a detrimental effect on the binding properties of the immobilized antibodies.

4.3. Alternative capillary coatings

MDS

The MDS-coated capillary has excellent binding properties for the antibodies, partly based on the formation of disulfide bridges. This is reflected in the amount FA bound in comparison with the C_8 capillary, as shown in Fig. 3B. The binding

characteristics were in line with those observed for the C_8 capillary. With the MDS-coated capillaries, the stability of binding was determined by repetition of incubation experiments with 10 nM FA. These experiments revealed that the binding to the MDS capillary coated with antibodies was reduced by about 5% per run until a constant value was reached, which implies that a certain amount of the label is non-specifically bound and not released by the rinsing procedure.

C_{18}

Experiments with a C_{18} capillary, in which part of the capillary was coated with antibodies and the remainder with BSA, resulted in multiple peak electropherograms as depicted in Fig. 3C, when an on-line immunoassay was conducted. This can be explained by non-specific binding of the label in the capillary and mobilization of antibodies with one or two FA molecules bound. In these experiments, the label-containing solution was flushed through the capillary. This implies that by a controlled injection of the label- and analyte-containing solution and backward rinsing, the non-specific binding can be partially reduced. These results show that the modes of interaction of C_8 and C_{18} capillaries with antibodies are rather different, which is generally not observed in chromatographic applications.

With all capillaries, control experiments were made: injections of label without incubations and direct application of the electric field revealed the effective migration time of the label and possible interactions with the wall. With the MDS-coated capillary, coating of the entire capillary with BSA resulted in long migration times for FA and severe peak broadening. This observation differs from those with the C_8 capillary and may be explained by a difference in interaction between BSA and either hydrophobic or hydrophilic surfaces. This means that adsorption of the label to the capillary wall is minimal or of very low affinity when the surface is hydrophilic so that only reversed-phase coatings should be treated afterwards with BSA in order to obtain a hydrophilic surface as well. It should be pointed out that this observation may be label and antibody dependent.

5. Conclusions

These experiments have confirmed that it is feasible to combine immunoassays with capillary electrophoresis. Because the atrazine assay employed here was only used as a test case, no further optimization experiments were done. The stability and binding properties of antibodies were virtually unaffected by the immobilization and the electric field applied in this experimental set-up. The stacking of the label employed was facilitated by the presence of methanol in the chaotropic agent, causing a change in the ionization of the label. Immobilization of different antibodies, mixed or in separate zones in the capillary, will allow the development of multi-analyte assays.

On the basis of this study, it is concluded that a label which keeps its fluorescent properties over the entire pH range and which also has a permanent positive or negative charge offers better possibilities for optimization of electrophoretic stacking and separation. The latter is essential for multi-analyte approaches.

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