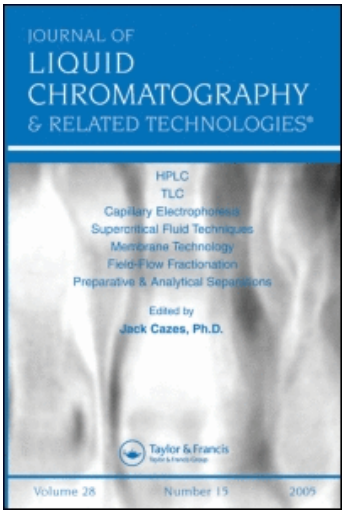

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Thomas, Chadwick V. , Cater, Amy C. and Wheeler, John J.(1993) 'HPCE as an Analytical Probe for Assessing Irreversible Ligand/Macromolecule Binding Interactions', Journal of Liquid Chromatography & Related Technologies, 16: 9, 1903 — 1921
To link to this Article: DOI: 10.1080/10826079308019905
URL: http://dx.doi.org/10.1080/10826079308019905

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(9&10), 1903-1921 (1993)

HPCE AS AN ANALYTICAL PROBE FOR ASSESSING IRREVERSIBLE LIGAND/MACROMOLECULE BINDING INTERACTIONS

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ABSTRACT

High Performance Capillary Electrophoresis (HPCE) is rapidly becoming recognized as a valuable tool for clinical and biomedical analysis. In this paper, we present a novel application of this technique for performing ligand binding analysis for drug/protein systems. Specifically, metabolites of the antiarrhythmic procainamide have been studied with respect to their irreversible binding to hemoglobin and histone proteins. Using Hughes-Klotz analysis, free zone HPCE is shown to give comparable binding constants to FIA-EC. Further, information regarding the nature of the binding interaction is provided by analyzing the electrophoretic profiles associated with the protein complex. Advantages of this

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format include broad applicability to samples of limited volume and the ability to consider binding of a single ligand to several different macromolecules in a single analysis. Improvements in deleterious wall interactions may ultimately permit independent quantitation of free and bound protein as well as free and bound ligand in some systems.

INTRODUCTION

As the fundamental development of high performance capillary electrophoresis (HPCE) has blossomed over the last few years, applications outside the specialized field of analytical chemistry have become increasingly popular. Areas that hold a great deal of promise for potential HPCE use are clinical and biomedical analyses. The number of publications incorporating HPCE has dramatically increased over the last two years (1), and comprehensive reviews of the technique have been published in forums such as *Clinical Chemistry* in the recent past (2). As just one example of the possible applications of HPCE to problems of biomedical relevance, Whitesides published an interesting paper in the *Journal of Medicine* recently (3) in which the so-called technique "affinity capillary electrophoresis" (ACE) was used to assess binding in an ideal system, that of carbonic anhydrase interacting with 4-alkylbezenesulfonamides. Essentially, by measuring the change in migration time of the anhydrase protein as a function of varied concentrations of the sulfonamide ligand(s), Scatchard analysis was performed. This permitted a direct determination of the relevant thermodynamic binding constants for this protein/ligand system. A few other such systems were investigated, however, the typical

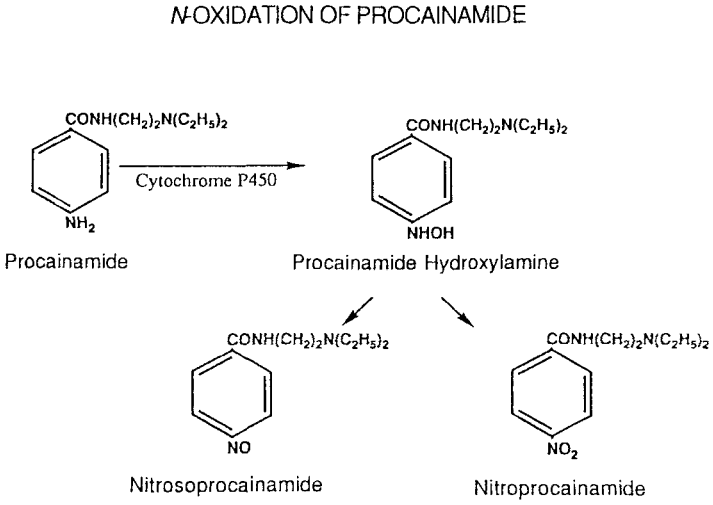

Figure 1: Schematic diagram of Procainamide and the N-oxidized metabolites

problems associated with adsorption of proteins to the capillary wall were described as a considerable obstacle in certain cases.

We have been interested for some time in one particular drug/protein system with ramifications in the autoimmune condition known as Drug-Related Lupus (DRL) (4-10). Specifically, it has been postulated that the mechanism of autoantibody production associated with procainamide(PA)-induced DRL may be a direct result of irreversible binding between N-oxidized metabolites of this anti-arrhythmic aromatic amine and selected proteins (see Figure 1). Among those proteins to which autoantibodies have been observed in PA-induced DRL are hemoglobin and nucleohistone (7-10). We have also been interested in the usefulness of HPCE for

performing irreversible binding determinations, since this technique is ideally suited for the separation of macromolecules from traditional organosolutes in a small volume format. In this paper, we present evidence that HPCE may indeed be used for reliable estimation of both the thermodynamic association constant, K_{ass}, and the number of binding sites, *n*, in a well-studied but non-ideal system. In addition to analyzing the concentration of free ligand to deduce concentration of bound ligand as in traditional binding analyses, we are able to examine the peak profile of free versus bound protein to obtain relevant information about the specific nature of the bound product. The major advantages of our approach include applicability to analyses using the ultrasmall volumes typically associated with binding measurements, but what is more important, the ability to analyze mixtures of different proteins with a common binding ligand. Our ultimate goal is to quantitate the bound ligand/protein complex independent of the free ligand determination. Although protein adsorption to the capillary wall has remained a difficulty in these measurements, we believe that with proper pretreatment such problems may be minimized to the extent that separation of free and bound protein may be possible.

EXPERIMENTAL

Reagents

Procainamide (PA) hydrochloride, N-ethylmaleimide (N-EMI), chromatographically pure hemoglobin A_0 stabilized to ferrohemoglobin, and purified calf-thymus histone subfractions Type V-S, VI-S, VII-S and VIII-S were obtained from Sigma Chemical (St.

Louis, MO) and were used as received. Nitroprocainamide (NPA) was prepared from nitrobenzoyl chloride as previously reported (9), and procainamide hydroxylamine (PAHA) was prepared by zinc reduction of nitroprocainamide also as previously reported (9). Nitrosoprocainamide (NOPA) was prepared by the coulometric oxidative electrolysis of PAHA at an applied potential of +0.700 V vs Ag/AgCl using a packed carbon bed flow cell constructed in house (9-10). The purity of the synthetically prepared PA metabolites was assayed as > 99% by gradient reversed phase HPLC. PAHA and to a much lesser extent NOPA are sensitive to oxidative decomposition in air (9); these metabolites were stored refrigerated under argon until use, and aqueous mixtures were degassed prior to solution preparation. Ultrapure sodium phosphate and sodium tetraborate salts were obtained from Aldrich Chemical (Milwaukee, WI) and were used as received. All water used for the preparation of chromatographic and electrophoretic buffers was obtained from a Barnstead Nanopure System at a resistivity > 18.1 Mohm·cm.

Flow-Injection Analysis with EC Detection

For the determination of PA metabolite binding without a separation step, flow-injection analysis with electrochemical detection (FIA-EC) was used. A Bioanalytical Systems (West Lafayette, IN) BAS 400 liquid chromatograph without an analytical separation column was used with a dual glassy carbon thin-layer cell operated in the series configuration. The applied potentials were -0.400 V vs Ag/AgCl for the upstream electrode, and +0.500 V vs Ag/AgCl for the downstream electrode. In this format, signal contributions from the presence of dissolved oxygen was

conveniently eliminated by selectively monitoring the downstream response, since the upstream reduction of O_2 is electrochemically irreversible under these conditions. A mobile phase consisting of 0.1 M sodium phosphate (pH 7.4) was used for these studies at a flow rate of 1.0 mL/min. Sample injection was achieved using a 20μL fixed loop.

Electrophoresis

All electropherograms were obtained using a Spectra-Physics Analytical (Fremont, CA) Model 500 Capillary Electrophoresis System. Fused-silica capillary tubing of 75 μm i.d. was obtained from Polymicro Technologies (Phoenix, AZ). All capillaries used in this study were cut to a total length of 44 cm, with a 2 mm long optical window placed 36 cm from the positive (cathode) end. Capillaries were initially conditioned using 1.0 M NaOH prior to use. In all cases, a 10-minute wash with 0.1 M phosphoric acid was used between successive runs to reduce reproducibility problems associated with protein adsorption to the capillary walls. All injections were performed hydrodynamically for 2.0 seconds, and the run temperature was maintained at 25° C. All buffers and samples were filtered prior to use through a 0.20 μm membrane (Fisher Scientific, Atlanta, GA).

Incubations

All incubations were carried out at ambient temperature. Hemoglobin concentrations were determined spectrophotometrically. Free ligand concentration as a function of incubation time was initially monitored by FIA-EC, and it was determined that

equilibrium was achieved in approximately 25 minutes for hemoglobin binding and in less than 20 minutes for histone binding. All incubations were therefore carried out for at least 30 minutes prior to binding analysis. Each point in the Hughes-Klotz analyses represents the mean of three injections.

RESULTS AND DISCUSSION

In order for HPCE to provide a practical alternative for ligand/protein binding analysis following incubations performed *a priori*, the binding process must be essentially irreversible; thus, separating *free* ligand from *bound* ligand must not disturb the equilibrium associated with the ligand/protein complex. In the case of the binding of PAHA and NOPA to hemoglobin and histone proteins, the specific nature of the interaction has to this point remained unknown. As a means of comparing the effect of separating the free and bound ligand versus quantitating free ligand in the presence of the bound product, FIA-EC was investigated to study these systems as previously described (11).

Figure 2 shows downstream electrode responses obtained for one sample incubation in the binding of NOPA to ferrohemoglobin. By performing sets of these four measurements for an entire range of ligand concentrations incubated with a fixed concentration of protein, a Hughes-Klotz plot can be constructed as demonstrated in Figure 3 (11). Thus, from the Hughes-Klotz equation,

$$[ML]/[M] + [ML] = 1/n + Kd/n[L]$$

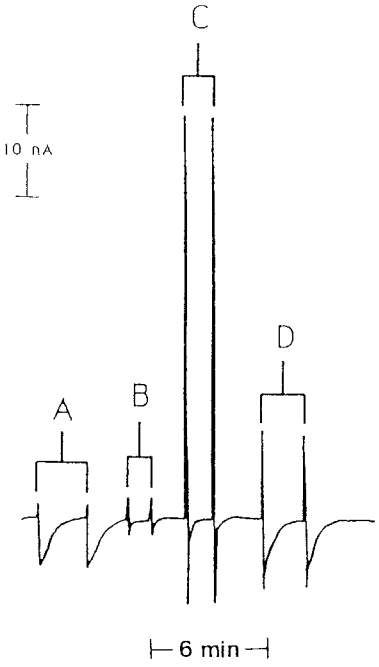

Figure 2: Flow-injection analysis downstream electrode responses for the injection of **A)** 10 μM ferrohemoglobin standard; **B)** phosphate buffer blank; **C)** 15 μM NOPA standard; **D)** 10 μM ferrohemoglobin + 15 μM NOPA following 30 minute incubation.

it is possible to determine the number of binding sites (n) on the macromolecule **M** from the y-intercept of the regression line and the association constant (*Kass* = 1/Kd) for the complex **ML** from the slope of the regression. From the FIA-EC experiment for the binding of NOPA to ferrohemoglobin, we observe an n value of 2.3 binding sites per hemoglobin molecule with an apparent binding constant of 1×10^5. It is worth pointing out that FIA-EC analysis does, in fact, give a reliable estimation of the relevant binding parameters, and

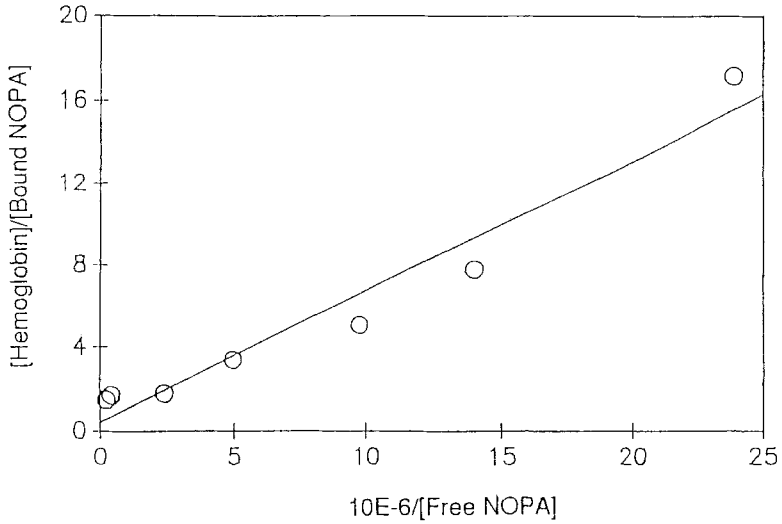

Figure 3: Hughes-Klotz plot for the binding of NOPA to human ferrohemoglobin as determined by FIA-EC. Incubations performed in 0.1 M sodium phosphate, pH 7.4 for 30 min. Coefficient of correlation from linear regression = 0.991; *Kass* = 1 x 10^5; n = 2.3.

that problems associated with non-specific binding inherent with dialysis membranes are eliminated. However, since only response from the free ligand is quantitated, we learn no information about the specific nature of the interaction with the protein, and protein mixtures cannot be independently evaluated. Further, since only a relatively small percentage of drug metabolites are electroactive, a much more versatile approach that incorporates conventional UV detection after *separation* allows quantitation of free ligand for virtually any system (*i.e.*, HPCE).

Figure 4 shows the response obtained for an incubation of hemoglobin with NOPA following separation by free zone

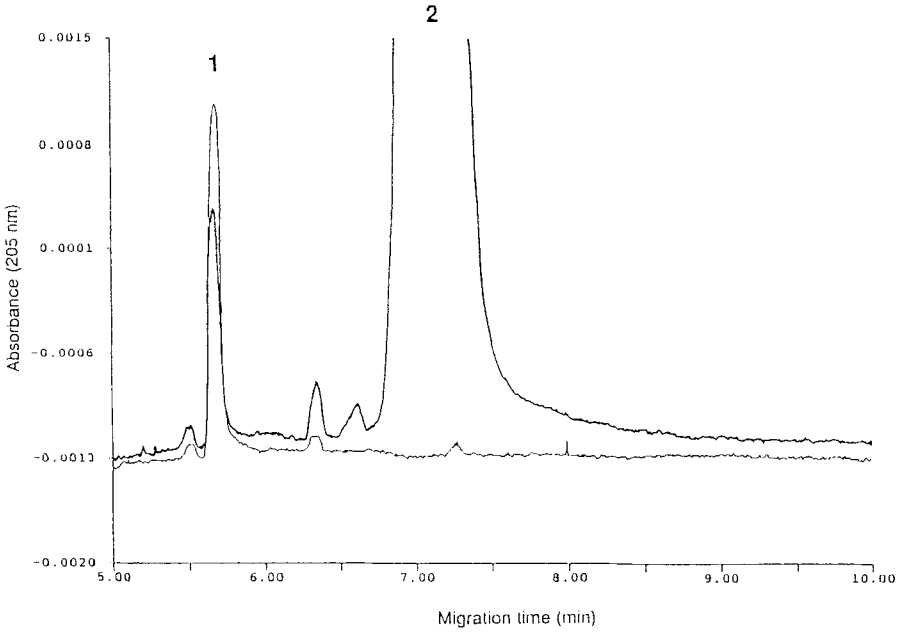

Figure 4: Electropherograms obtained for the injection of (—) 10μM NOPA and (—) 10μM NOPA + 10 μM human ferrohemoglobin PEAK 1 is NOPA response; PEAK 2 is ferrohemoglobin response at 205 nm. Incubation performed for 30 min. in 0.1 M sodium phosphate, pH 7.4. Electrophoretic run buffer was 60 mM sodium tetraborate, pH 8.5; Voltage was ramped from 2 kV to 12 kV over 5 minutes, then maintained at 12 kV for the remainder of the run.

electrophoresis. It is immediately apparent that the molar extinction coefficient for hemoglobin at the monitored wavelength is considerably greater than that for NOPA, giving rise to an inherently larger UV response. This makes quantitation of the bound product difficult for this particular system, because the complex formed has a similar electrophoretic mobility to that of the free

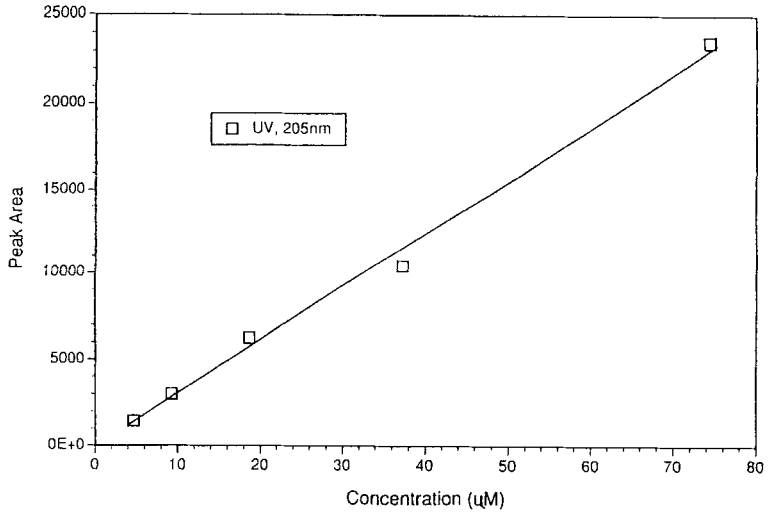

Figure 5: Calibration plot for NOPA standards as determined from peak area. All buffer and run conditions as in Figure 4. Coefficient of correlation from linear regression = 0.995.

hemoglobin (*vide infra*). Aside from this observation, however, separation of free NOPA from bound NOPA has been achieved, and we are able to quantitate the free NOPA that remains even though the NOPA metabolite is electrically neutral. Figure 5 shows a representative calibration plot for NOPA standards used in the concentration range of the binding analysis, and Figure 6 provides the Hughes-Klotz results obtained based on the determination of free ligand using the calibration plot from Figure 5. From this plot, we observe that the apparent binding constant (K_{ass} = 1 x 10^5) and the number of binding sites per hemoglobin molecule (n = 2.1) are consistent with the earlier FIA-EC study. We may conclude for the present system that HPCE provides a reasonable alternative for analyzing free ligand in a small volume format. This in itself is an

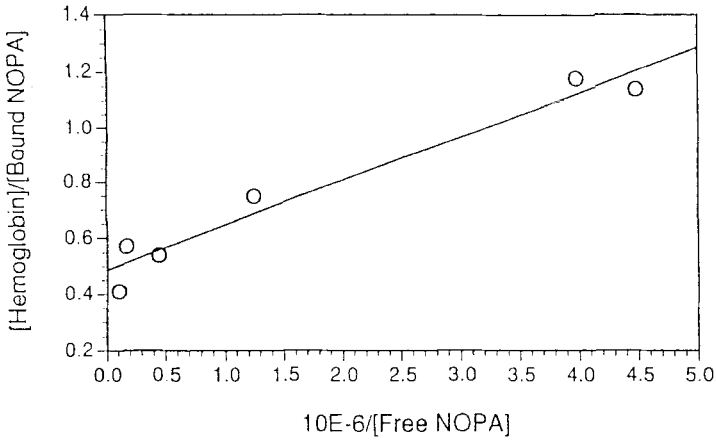

Figure 6: Hughes-Klotz plot for the binding of NOPA to human ferrohemoglobin as determined by free zone electrophoresis. All incuabation conditions as in Figure 3. Coefficient of correlation from linear regression = 0.985; K*ass* = 1 x 10^5; n = 2.1.

attractive advantage for frequently used techniques that utilize ultra-small volumes such as microdialysis and ultrafiltration. However, we are ultimately interested in obtaining more specific information regarding the NOPA/hemoglobin complex; thus, careful analysis of the electrophoretic profile associated with the NOPA/hemoglobin product is useful.

In this system, the overall electrophoretic mobility associated with hemoglobin is not appreciably altered upon binding. This is perhaps not unexpected, since NOPA itself is in fact a neutral molecule. Additionally, as is evidenced by the broad electrophoretic profile, the ferrohemoglobin Ao preparation is neither homogeneous nor efficiently focused. We have confirmed by isoelectric focusing

that two forms of hemoglobin are present in roughly equal concentration that have been preliminarily identified as oxy and deoxy forms. Further, although protein adsorption is minimized for hemoglobin at this particular operating pH, the capillary has in no way been permanently treated to eliminate deleterious wall interactions.. It is anticipated that this problem may be reduced by taking advantage of capillary pretreatment (12-14), although *in situ* additives must be used with care since they have the potential to alter the protein/ligand equilibrium. While bound and free protein are not separated in this example, we still observe considerable changes in the electrophoretic profile as a function of ligand (NOPA) concentration. Figure 7 shows the electropherograms obtained for two different molar ratios of NOPA added to the ferrohemoglobin preparation. It is apparent that as more NOPA binds to the protein, the peak associated with hemoglobin and the NOPA/hemoglobin complex becomes much wider and shorter, consistent with a change in the heterogeneity of the mixture. Since nitroso agents have been suggested to undergo sulfhydryl binding through two available cysteine residues in the β domain of hemoglobin in other reports (15-16), we used a known sulfhydryl complexing agent for electrophoretic comparison. Figure 8 shows the electropherogram obtained following the incubation of N-EMI with the hemoglobin mixture. In contrast to the NOPA work, there is very little change observed in the electrophoretic profile for hemoglobin, although a decrease in the N-EMI response does indicate appreciable binding is taking place (data not shown). In other studies, when hemoglobin was pre-incubated with N-EMI prior to NOPA introduction, only a moderate decrease in extent of NOPA binding was observed, and the

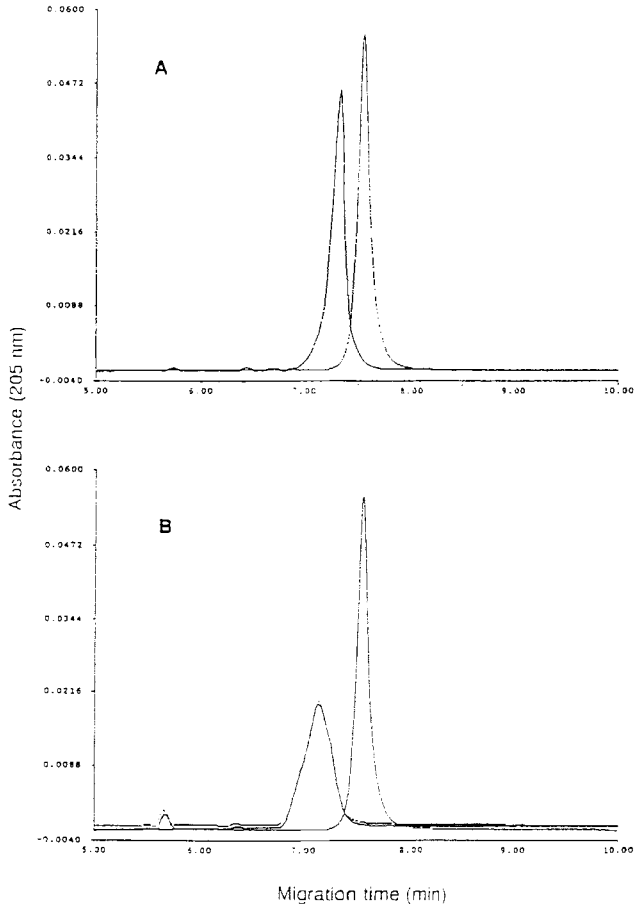

Figure 7: Electropherograms obtained for (—) 10 μM ferrohemoglobin standard and (—) 10 uM ferrohemoglobin incubated with NOPA. Figure **A**, NOPA/hemoglobin = 2:1 M/M ratio; Figure **B**, NOPA/hemoglobin = 6:1 M/M ratio. All buffer and run conditions as in Figure 4.

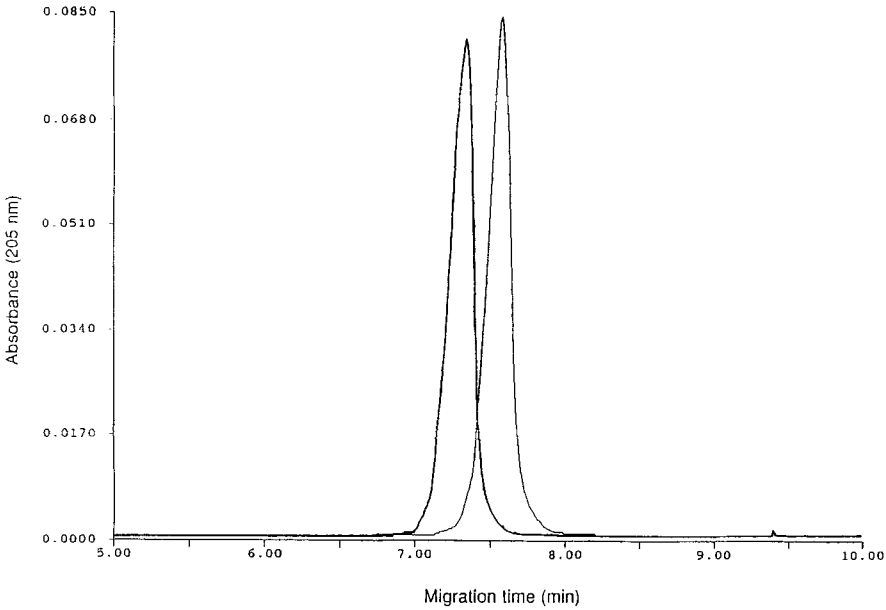

Figure 8: Electropherograms for (—) 10 μM hemoblobin standard and (—) 10 μM hemoglobin standard incubated for 1.0 hour with *N*-EMI (6:1 M/M ratio of N-EMI/hemoglobin) in 0.1 M sodium phosphate, pH 7.4. All buffer and run conditions as in Figure 4.

characteristic change in the hemoglobin response to a short, wide peak persisted (Figure 9). Isoelectropherograms indicate that NOPA preferentially binds to only one of the two forms of hemoglobin present, whereas N-EMI was observed to bind equally well to both forms. Although the specific interaction is not fully elucidated by HPCE, these results provide sufficient evidence to assume that the interaction between NOPA and hemoglobin is not a straightforward sulfhydryl interaction as might otherwise have been anticipated.

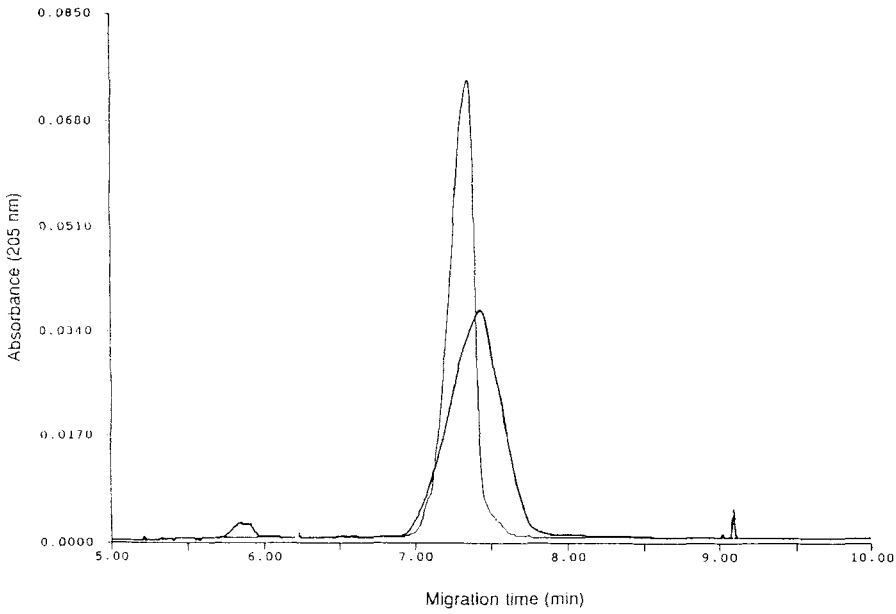

Figure 9: Electropherograms for (—) 10 μM hemoglobin standard and (—) 10 μM hemoglobin standard pre-incubated 1.0 hour with N-EMI prior to 30 min. incubation with NOPA. Incubation ratios: 1:1 M/M *N*-EMI to hemoglobin; 6:1 M/M NOPA/hemoglobin. All buffer and run conditions as in Figure 4.

In addition to the NOPA/hemoglobin system, several other binding reactions have been considered using the free zone approach. PAHA was also observed to undergo irreversible binding with hemoglobin, although the significant susceptibility of this compound to oxidative decomposition has made Hughes-Klotz analysis more difficult in this instance. As controls, both NPA and PA were incubated with hemoglobin under various concentration ranges, and neither the free ligand response nor the protein response were

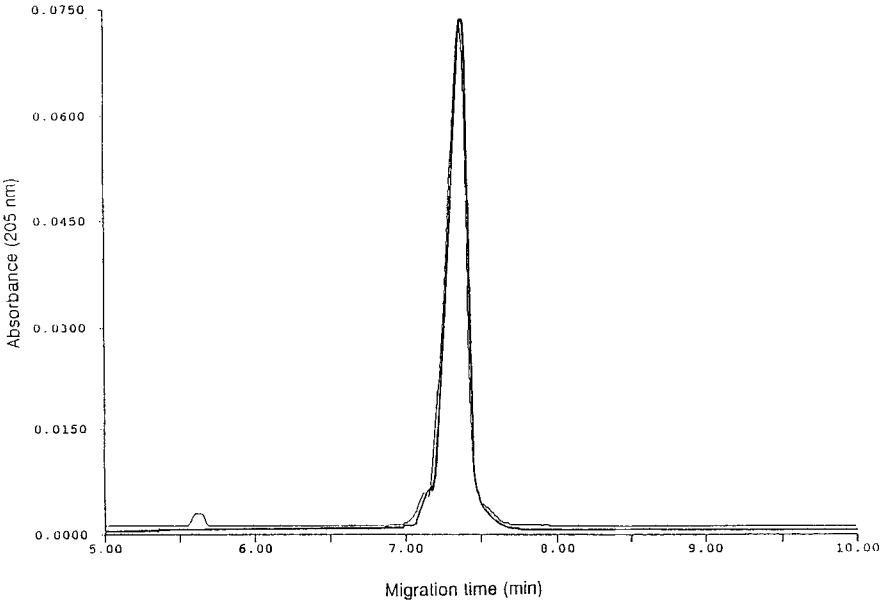

Figure 10: Electropherograms for (—) 10 μM hemoglobin standard and (—) 10 μM hemoglobin incubated for 30 min. with 6:1 M/M procainamide. All incubation, buffer and run conditions as in Figure 4.

shown to measurably change (Figure 10). This result is consistent with earlier observations that these molecules are not involved in irreversible binding (9-10). Histone subfractions have also been used in binding studies with each of the PA metabolites, and irreversible binding is observed to a lesser degree than for hemoglobin with both PAHA and NOPA. In this case, it is possible to examine binding with multiple histone subfractions simultaneously. Further results obtained with isoelectric focusing and a more complete analysis of an improved separation for histone

subfractions using free zone HPCE will be the subjects of later reports in this area.

As the availability and popularity of HPCE continues to expand to areas outside of the analytical realm, systems in which actual physicochemical measurements can be made will become increasingly important. Affinity capillary electrophoresis and ligand/macromolecule binding assessment for irreversible complexes such as those presented here represent one significant step in that direction. The potential applications are enormous, and we are hopeful that investigators from a variety of biomedical disciplines will attempt to benefit from these approaches.

ACKNOWLEDGEMENTS

The authors wish to gratefully acknowledge Research Corporation, the National Science Foundation and Furman University for their financial support of this project, and Program Resources, Inc. for its continued support of the Frederick Conference. We also wish to thank Daniel B. Meetze for his assistance with the isoelectric focusing and Spectra-Physics Analytical for providing instrumental support.

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