11-1-2006

Analysis of Free Drug Fractions Using Near-Infrared Fluorescent Labels and an Ultrafast Immunoextraction/Displacement Assay

Corey M. Ohnmacht
University of Nebraska - Lincoln

John E. Schiel
University of Nebraska-Lincoln

David S. Hage
University of Nebraska-Lincoln, dhage1@unl.edu

Published in final edited form as:
Anal Chem. 2006 November 1; 78(21): 7547–7556. doi:10.1021/ac061215f.

ANALYSIS OF FREE DRUG FRACTIONS USING NEAR-INFRARED FLUORESCENT LABELS AND AN ULTRAFAST IMMUNOEXTRACTION/DISPLACEMENT ASSAY

Corey M. Ohnmacht, **John E. Schiel**, and **David S. Hage***
Department of Chemistry University of Nebraska Lincoln, Nebraska 68588−0304

Abstract

A chromatographic method was developed for measuring free drug fractions based on the use of an ultrafast immunoextraction/displacement assay (UFIDA) with near-infrared (NIR) fluorescent labels. This approach was evaluated by using it to determine the free fraction of phenytoin in serum or samples containing the binding protein human serum albumin (HSA). Items considered in the design of this method included the dissociation rate of HSA-bound phenytoin, the rate of capture of free phenytoin by immunoextraction microcolumns, the behavior of NIR fluorescent labels in a displacement format, and the overall response and stability of the resulting assay. In the final UFIDA method, the free fraction of phenytoin was extracted in approximately 100 ms by a microcolumn containing a small layer of anti-phenytoin antibodies. This gave a displacement peak for a NIR-fluorescent labeled analog of phenytoin that appeared within 2−3 min of sample injection, creating a signal proportional to the amount of free phenytoin in the sample. The UFIDA method provided results within 1−5% of those determined by ultrafiltration for reference samples. The lower limit of detection was 570 pM and the linear range extended up to 10 μM. This approach is not limited to phenytoin but can be adapted for other analytes through the use of appropriate antibodies and labeled analogs.

INTRODUCTION

Many drugs exist in two forms in the circulation: a free fraction and a fraction which is reversibly bound to serum proteins or other agents in blood [1,2]. The free fraction of a drug is generally thought to represent its active form, since this is the form that can cross cell membranes or bind to receptors [3]. The binding of drugs to blood or serum components is important in drug delivery [4]. However, the extent of this binding can be affected by various factors which can lead to individual variations in free drug fractions [5]. For instance, the extent of a drug's binding with serum proteins can vary as a result of illness, trauma, surgery or age [2]. This can make it difficult to correlate the total concentration of a drug with its free fraction and has created a need for new methods that can routinely measure free drug fractions.

Phenytoin is an anti-epileptic drug that has significant binding in blood [6,7]. This drug is mainly bound in blood to the protein human serum albumin (HSA), with approximately 90% of phenytoin being complexed at therapeutic levels in adults [8-14]. Two clinical situations in which the bound fraction of phenytoin might decrease include infants with jaundice (due to the competition of bilirubin with phenytoin for HSA) and patients that have low HSA levels following trauma or surgery [9]. It is also possible for a patient to have an increased bound

*Author for correspondence. Phone: (402) 472−2744; FAX: (402) 472−9402; Email: dhage@unlserve.unl.edu.

fraction of phenytoin, as can occur in individuals with high HSA concentrations (i.e., hyperalbuminemia) [9].

There have been numerous methods developed for the measurement of phenytoin's free fraction in blood, plasma or serum [6,10,12,15-22]. Examples include techniques based on equilibrium dialysis, ultrafiltration, and restricted access media (RAM) columns [6,10,14,15,18,19,21]. Other techniques such as CE can also be used to examine drug-protein interactions in standard solutions [23-26], but many of these methods are not applicable for work with the small free fractions of drugs or the complex matrices that are found in clinical samples. Some previous methods for free phenytoin measurements suffer from long analysis times (e.g., equilibrium dialysis) or non-specific adsorption to membranes (e.g., ultrafiltration) [19]. Other techniques (e.g., RAM) give only an incomplete separation of phenytoin's free and bound forms, making these methods difficult to use with real clinical samples [1,2,16,27].

This paper describes an alternative chromatographic-based approach for measuring free drug fractions based on an ultrafast immunoextraction/displacement assay (UFIDA), as illustrated in Figure 1 [28]. This approach uses an immunoextraction microcolumn that can bind a measurable amount of the free analyte (e.g., phenytoin) on a time scale that is sufficiently small to avoid dissociation of this analyte from its binding agents in the sample (e.g., HSA). At the beginning of this assay, shown in the upper left of Figure 1(a), an excess of a labeled analog of the analyte is injected onto the immunoextraction microcolumn in the presence of an application buffer. Some of this labeled analog will bind to antibodies in the column while the remainder will be washed away prior to sample injection. When a sample is later passed through the column, the free fraction of the analyte will compete with any labeled analog that is momentarily dissociated from the immobilized antibodies. This results in a displacement peak for the labeled analog, as shown in Figure 1(b), and gives a signal that is proportional to the analyte's free fraction. The retained analyte and labeled analog can later be removed from the column by using an elution buffer. The column is then allowed to regenerate and the entire process is repeated for another sample.

Ultrafast immunoextraction and a displacement assay have recently been used with chemiluminescence detection for the measurement of free thyroxine in serum [1]. However, this past work involved the use of a complex postcolumn reaction and required serum blanks to correct for matrix effects on the chemiluminescence signal [1]. This current study will examine an alternative approach using near-infrared (NIR) fluorescent cyanine dyes as labels (see Figure 2). A major advantage of these labels is they do not require any postcolumn reaction for detection. In addition, these dyes have low background signals for many biological samples and can provide detection limits in the femtomole range [29]. These dyes have been used in DNA sequencing, traditional immunoassays and capillary electrophoresis [29,30], but they have not yet been used in either chromatographic immunoassays or in the measurement of free drug fractions. Although more traditional fluorescent labels have been employed in some types of chromatographic-based immunoassays [31-34], these other labels have also not been used in UFIDA methods or free drug assays.

This current study will explore the combined use of NIR fluorescent dyes and UFIDA for measuring free phenytoin fractions. One parameter that will be examined in this study is the development of immunoextraction microcolumns that can bind free phenytoin on the millisecond time scale. The creation and use of NIR fluorescent labeled analogs of phenytoin for displacement assays will also be considered. The final UFIDA method will be evaluated in terms of its accuracy, precision and speed when analyzing both drug-protein mixtures and serum samples. The advantages and limitations of this method will then be considered, as well as its possible extension to other analytes.

EXPERIMENTAL SECTION

Reagents

The phenytoin (99% pure), mouse IgG, human plasma (lyophilized, pooled) and HSA (Cohn fraction V, 99% fatty acid free) were from Sigma-Aldrich (St. Louis, MO). The IRDye 800 CW dye (*N*-hydroxysuccinimide, or NHS ester) was donated by LI-COR Biosciences (Lincoln, NE). The monoclonal anti-phenytoin antibodies (clone P0825.1, purified from ascites fluid; stored in pH 7.2 phosphate buffered saline plus 0.05% sodium azide) were from Accurate Chemical (Westbury, NY). Nucleosil Si-300 (7 μm particle size, 300 Å pore size) was purchased from P.J. Cobert (St. Louis, MO). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL). The phenytoin calibration standards, free phenytoin calibrators and control serum samples (pooled serum containing known amounts of phenytoin) were from Abbott Laboratories (Chicago, IL). Other chemicals were reagent grade or better. All aqueous solutions were prepared using deionized water from a Nanopure water system (Barnstead, Dubuque, IA).

Apparatus

Mass spectra of phenytoin and its conjugates were obtained using electron ionization mass spectrometry (EI-MS) performed on a VG AutoSpec mass spectrometer from Fisons (now Waters/Micromass) (Milford, MA). ^{1}H NMR spectra were acquired for phenytoin and its conjugates on a Bruker DRX Avance 500 MHz spectrometer (Billerica, MA) using samples dissolved in deuterated dimethylsulfoxide (DMSO). Ultrafiltration was performed using Centrifree Micropartition Devices (MW cutoff, 30 kDa; sample capacity, 0.15−1.0 ml) from Amicon (Danvers, MA), along with a fixed rotor basket centrifuge from Dynac (Parsippany, NJ). The temperature during the ultrafiltration experiments was controlled by placing the centrifuge in a Precision P3 incubation cabinet from Expotech (Houston, TX). Samples for the BCA protein assay were analyzed using a Shimadzu UV160U absorbance spectrophotometer (Kyoto, Japan). The fluorescence properties of the labeled phenytoin and serum samples were examined using a QuantaMaster spectrofluorometer from Photon Technology International (Rockwood, TN), using an excitation wavelength of 770 nm and an emission wavelength range of 780−900 nm.

The HPLC system used to analyze free phenytoin fractions after ultrafiltration consisted of a Jasco PU980 pump (Easton, MD), an Alltech water jacket, and a LDC Analytical 3100 UV detector (Riviera Beach, FL), along with a 2.1 mm I.D. × 4.5 cm HSA column (prepared as described previously) [8,35]. Samples were injected onto this system using a 20 μL sample loop and a Thermoseparations AS3000 autosampler (Schaumberg, IL). The same chromatographic system was used to determine the extraction efficiency of the anti-phenytoin immunoextraction microcolumns, with these columns or their controls being used in place of the HSA column. The immunoextraction microcolumns were packed according to a previous method [36,37].

The chromatographic system used for the UFIDA method was similar to that described in the previous paragraph but included a second Jasco PU980 pump and a Rheodyne model EV700 six port switching valve (Cotati, CA) for alternating passage of the application and elution buffers through the anti-phenytoin immunoextraction microcolumn. The labeled phenytoin was injected onto this system in volumes ranging from 5 to 100 μL, followed by 5 μL injections of the sample. Detection of the labeled phenytoin was performed using a custom-built HPLC NIR fluorescence detector from LI-COR. This detector was constructed with a 25 μL flow cell positioned at the interface of the laser and detector focus, using the same optical system as described in Ref. [38], with the laser diode source and microscope detector being positioned 90° with respect to the flow cell. The emitted wavelength of the laser diode was 785 nm.

Excitation wavelengths were selected using a 20 nm bandpass filter centered at 820 nm. The temperature of the immunoextraction microcolumn or control column was maintained by using water jackets (Alltech) and a Brinkmann circulating water bath (Westbury, MA). All chromatographic data were collected using programs written in Labview 5.1 (National Instruments, Austin, TX). Retention times and areas of the resulting chromatographic peaks were calculated using PeakFit 4.12 (Systat Software, Richmond, CA).

Preparation of labeled phenytoin

Phenytoin was converted into an amine derivative (i.e., 3-*N*-amino-5,5-diphenylhydantoin, or ADPH) by the reaction scheme shown in Figure 2 [39-42]. This involved heating a mixture of 2.50 g phenytoin (0.01 mol) with 3.90 mL hydrazine hydrate (0.08 mol) at 135°C for 5 h; no other solvent was required for this reaction. ADPH was recrystallized by placing 1 g of the solid product in approximately 15 mL ethanol. Deionized water was then added and this mixture was allowed to stand undisturbed at 4°C for two days. The resulting crystals were filtered and dried at 80°C under vacuum for 8 h. When analyzed by EI-MS, the product of this reaction gave a molecular ion with a mass-to-charge ratio of 267.1019 (mass accuracy, 4.2 ppm) and fragment ions with masses in agreement with those previously reported for ADPH [39]. A ^{1}H NMR spectrum for this substance also agreed with that expected for ADPH. Some byproduct (i.e., 5,5-diphenyl-1,2,4-triazine-3,6-dione) was also noted in this preparation, which was estimated to make up approximately 40−50% of the final product.

ADPH was conjugated to NHS-activated IRDye 800 CW dye by using an approach similar to that shown in Figure 2 [43]. This involved dissolving 9 μmol of NHS-activated IRDye 800 CW dye in 0.5 mL DMSO, followed by the slow addition of 20 μmol ADPH in 0.5 mL DMSO and 1 mL of pH 8.0, 0.10 M potassium phosphate buffer. The resulting mixture was slowly stirred with a magnetic stir bar for 4 h in an ice water bath. This mixture was then dried to remove all solvent, with the remaining residue being dissolved in 10 mL of pH 7.4, 0.067 M potassium phosphate buffer. This product was extracted three times with 10 mL portions of ethyl acetate to remove any unconjugated reactants. The remaining aqueous fraction was dried to remove any ethyl acetate; this gave a residue containing the phenytoin-dye conjugate and unconjugated dye. No further purification of the final labeled-phenytoin dye conjugate (i.e., “labeled phenytoin”) was required for this study.

The purity of the labeled phenytoin in the final product was estimated to be 62%. This purity was sufficient for use in this study, since an excess of labeled phenytoin was used in the displacement assay. It was found later that the 38% of unconjugated dye in this preparation did not create any noticeable interference due to background signal or nonspecific binding in the final UFIDA assay. The purity of this labeled phenytoin was measured by comparing the NIR fluorescence for the retained peak (i.e., the labeled phenytoin) and non-retained peaks (containing the unconjugated dye) when a 5 μL sample of this preparation was injected in pH 7.4, 0.067 M potassium phosphate buffer at 0.5 ml/min onto a 2.1 mm I.D. × 4.5 cm immobilized HSA column, with pH 7.4, 0.067 M potassium phosphate buffer also being used as the mobile phase. This HSA column was prepared as described earlier [8,35,44,45] and had a protein content of 28 (± 2 mg) HSA/g silica (± 1 SD). As has been noted for phenytoin [8, 35], this HSA column was found to retain the labeled phenytoin while the free acid form of the NIR fluorescent dye eluted near the column void volume of this column.

A stock solution was made for the labeled phenytoin in pH 7.4, 0.067 M phosphate buffer. The concentration of labeled phenytoin in this stock solution was estimated to be 558 (± 20) μM after a correction was made for unconjugated dye in the final product. This concentration was determined by HPLC, as described in the previous paragraph, using both this stock solution and standards containing known concentrations of the NIR fluorescent dye. When not in use,

this stock solution was stored in the dark in an amber vial at 4°C. This stock solution was used over the course of approximately four months during this study.

Preparation of immunoextraction microcolumn

Prior to immobilization, the anti-phenytoin antibodies were transferred from their original solution (pH 7.2 phosphate buffered saline containing 0.05% sodium azide) into pH 6.0, 0.10 M potassium phosphate buffer. This transfer was accomplished by applying a 3.0 mL sample of these antibodies to a 10 mL Econo-PAC 10DG column (exclusion limit, 6,000 Da) from BioRad (Hercules, CA) using pH 6.0, 0.10 M potassium phosphate buffer as the mobile phase. The collected antibodies were stored at 4°C in the same pH 6.0, 0.10 M phosphate buffer.

Prior to its use in immobilization, Nucleosil Si-300 silica was converted into a diol-bonded form according to a previous method [44]. The final diol coverage of this material was 306 (± 3) μmol/g silica, as determined in triplicate by an iodometric capillary electrophoresis assay [45]. The anti-phenytoin antibodies in pH 6.0, 0.10 M potassium phosphate buffer were immobilized onto this diol-bonded silica by the Schiff base method [46]. An inert control support was prepared in an identical manner but with no antibodies being added during the immobilization step (note: this type of support was found to be an adequate control for this work because of the specificity of the anti-phenytoin antibodies and relatively low nonspecific binding of the analytes or labeled analogs to the control support; however, a control support containing immobilized nonspecific antibodies could also be used). After immobilization, the anti-phenytoin support was washed three times with pH 7.4, 0.067 M potassium phosphate buffer and stored at 4°C until use. A portion of this support was washed several times with deionized water, dried, and analyzed in triplicate by a BCA protein assay [47]. This assay gave a protein coverage of 29 (± 7) mg antibodies/g silica when using mouse IgG as the standard and the control support as the blank.

The anti-phenytoin support was used to prepare immunoextraction microcolumns [2,37] which had an inner diameter of 2.1 mm and a total length of 0.5 cm. The central layer of these columns was approximately 1 mm thick (940 μm) and contained the anti-phenytoin support, while the remainder of the columns contained the inert control support. These columns were prepared by making fifteen 50 μL injections at 3 mL/min of a 4.2 mg/mL slurry of the control support to one end of the column in the presence of pH 7.4, 0.067 M phosphate buffer. This was followed by application of the same buffer at 5 ml/min for 5 min to stabilize this layer to a thickness of approximately 0.20 cm. A small layer of the anti-phenytoin support was next placed within this column by making fourteen, 50 μL injections of a 2.1 mg/mL slurry of this material in pH 7.4, 0.067 M phosphate buffer and at 3 mL/min; this layer was also stabilized by later increasing the flow rate to 5 mL/min for 5 min. The remainder of this column was filled in the same manner with the inert control support. Each immunoextraction microcolumn was stored in pH 7.4, 0.067 M phosphate buffer at 4°C when not in use. The typical backpressure of these columns at 0.5 to 1.6 mL/min was 130 to 420 psi (0.9 to 2.9 MPa) and increased by only 19% over four months of regular use.

Characterization of immunoextraction microcolumn

The amount of active anti-phenytoin antibodies in the immunoextraction microcolumns was determined by frontal analysis [47,48]. This was performed using solutions that contained 2 to 40 μM phenytoin in pH 7.4, 0.067 M phosphate buffer and that were applied at 1.2 mL/min. The breakthrough curves for phenytoin were monitored at 205 nm, with all runs being conducted in triplicate at 37°C. Elution of the retained phenytoin was accomplished by applying pH 2.5, 0.067 M phosphate buffer to the immunoextraction microcolumns. Sample application and column regeneration were performed by using pH 7.4, 0.067 M phosphate buffer as the mobile phase. Corrections for the system void time and non-specific binding of

phenytoin to the column were made by conducting identical experiments using a column with the same dimensions as the immunoextraction microcolumn but containing only control support. The extent of phenytoin binding to this control support was less than 8% of the total binding measured for the immunoextraction microcolumn [8,35].

The extraction efficiency of the immunoextraction microcolumn was determined by making 20 μL injections of a 6 μM phenytoin standard at 37°C in pH 7.4, 0.067 M phosphate buffer; these injections were made onto both a column containing only the control support and onto the immunoextraction microcolumn. The amount of non-retained phenytoin was measured on each column at 205 nm and at flow rates ranging from 0.6 to 1.6 mL/min. Similar experiments were conducted when no column was present. The difference between the total peak areas measured with the control column and with no column present was less than 3%.

Ultrafiltration of phenytoin samples

Ultrafiltration was used as a reference method for validating the free fraction measurements made for phenytoin in this study [4,19,21,49]. This was performed on control serum samples containing known concentrations of phenytoin and on various HSA/phenytoin mixtures in pH 7.4, 0.067 M potassium phosphate buffer. Each of these samples was centrifuged in the presence of an ultrafiltration membrane at 37°C for 45 min at 1,500 × g. After centrifugation, approximately 0.5 mL of the filtrate was collected and stored at 4°C until further use. The concentration of free phenytoin in the filtrate was measured by using HPLC along with the same HSA column described earlier for examining the purity of the labeled phenytoin. Samples containing 20 μL of the filtrate were injected onto this column in triplicate at 0.5 mL/min and at room temperature. The elution of phenytoin was monitored at 205 nm and gave a retention factor of 2.3 on this column. No peaks from other sample components were noted in the vicinity of the retention time for phenytoin. A linear response was obtained on this column for phenytoin standards containing 2 to 15 μM phenytoin in pH 7.4, 0.067 M phosphate buffer (correlation coefficient, 0.9998 for $n = 5$).

Non-specific binding by phenytoin in the ultrafiltration device was measured by using a series of standards that contained phenytoin in pH 7.4, 0.067 M phosphate buffer. The amount of phenytoin in the recovered filtrate was then determined by HPLC, as described previously. These experiments indicated that phenytoin had 7.6 (± 0.1)% nonspecific binding to ultrafiltration membrane, in agreement with earlier studies [19]. All phenytoin results obtained by ultrafiltration were corrected for nonspecific binding based on this value.

Ultrafast immunoextraction/displacement assay

The labeled phenytoin and immunoextraction microcolumn developed in this work were used in an UFIDA method according to the scheme given earlier in Figure 1(a). At the beginning of this assay, 20 μL of a 55.8 μM sample of the labeled phenytoin was applied to the immunoextraction microcolumn at 0.8 mL/min using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. Once a baseline had been established, the flow rate was increased to 1.2 mL/min. A 5 μL sample containing a phenytoin/HSA mixture or phenytoin in human serum was injected at 6 min after application of the labeled phenytoin. Once the resulting displacement peak had eluted, the retained phenytoin and any remaining labeled phenytoin were removed from the column by applying a pH 2.5, 0.067 M phosphate solution as the elution buffer at 1.2 mL/min for 5 min. The column was then regenerated by applying pH 7.4, 0.067 M phosphate buffer at 0.8 mL/min for 5 min prior to the next application of the labeled phenytoin. All experiments measuring free phenytoin concentrations on this system were performed in triplicate at 37°C. Further details on how the conditions for this final method were selected can be found later in this study.

RESULTS AND DISCUSSION

Selection of conditions for ultrafast immunoextraction

An underlying requirement for free drug measurements by ultrafast immunoextraction is that this method must be able to extract a representative portion of a drug's free fraction while avoiding any appreciable release of the same drug from its bound form in the sample. The timescale needed for this can be estimated by using the dissociation rate constant of a drug from its carrier agents in a sample along with the extent of this binding in typical samples [1, 2,36]. Several previous reports have examined the binding of phenytoin to HSA [8,35]. Two specific regions on HSA that are involved in this binding are the indole-benzodiazepine site (Sudlow site II) and the digitoxin site of HSA. These sites have been reported to have association equilibrium constants for phenytoin of 1.04×10^4 and 6.5×10^3 M^{-1}, respectively, at pH 7.4 and 37°C. It has also been found that phenytoin has allosteric plus possible direct interactions with the warfarin-azapropazone site (Sudlow site I) and tamoxifen site of HSA [8,35].

Through previous kinetic measurements using HSA columns, the overall dissociation rate constant for phenytoin from these binding sites of HSA has been estimated to be 10.8 (± 0.05) s^{-1} at pH 7.4 and 37°C [50]. Based on this result and previous work performed in the ultrafast immunoextraction of warfarin and thyroxine [1,2], it was originally estimated that the analysis of free phenytoin by UFIDA would require a sample residence time in an immunoextraction microcolumn of less than 200 ms. To obtain these conditions, a sandwich microcolumn was used that contained a 2.1 mm I.D. × 940 μm thick layer of an anti-phenytoin support. This gave an expected residence time for samples in the immunoextraction layer of roughly 100 ms at a flow rate of 1.2 mL/min.

The binding capacity of the immunoextraction microcolumn was estimated by frontal analysis to be 67 (± 3) pmol phenytoin at pH 7.4 and 37°C. It was further found from protein assays that this column contained approximately 290 (± 90) pmol antibodies. This gave an effective concentration of active antibody binding sites in the immunoextraction layer of 3.4 (± 0.2) μM and a relative activity of the antibodies for phenytoin of 12 (± 3)% (assuming there were two accessible binding sites per antibody). Although this relative activity was low, the total column binding capacity was sufficient for this work since it was roughly two times higher than the amount of free phenytoin expected at the high end of this drug's therapeutic range in a 5 μL sample (i.e., based on a 90% bound fraction and a total phenytoin concentration of 80 μM). If desired, this relative activity and binding capacity could be improved by employing more site-selective techniques for antibody immobilization; examples include the use of hydrazide-activated supports plus antibodies that have been oxidized in their carbohydrate regions, or the use of biotin tags in these same regions along with avidin or avidin-containing supports [51-53].

The association equilibrium constant for phenytoin with the immobilized anti-phenytoin antibodies was determined by frontal analysis to be 5.4 (± 0.3) × 10^8 M^{-1} at pH 7.4 and 37°C (note: no significant change in this value was noted at lower flow rates). Although this result represents reasonably strong binding, this association equilibrium constant is approximately 20-fold lower than a solution-phase equilibrium constant of 1×10^{10} M^{-1} that was provided by the manufacturer of these antibodies. This difference is not surprising because immobilized antibodies can often have lower binding constants than their soluble forms [54,55]. Using this equilibrium constant and the binding capacity of the immunoextraction microcolumn, the retention factor for phenytoin on this column at pH 7.4 and 37°C was estimated to be 348 (± 26). This value was calculated by using the relationship $k = K_A\, m_L/V_M$, where K_A is the association equilibrium constant for phenytoin with the antibodies, m_L is the moles of active antibodies in the column, V_M is the void volume of the immunoextraction layer, and (m_L/

V_M) is the effective concentration of active antibodies in this layer [48]. Further examination of the frontal results, as described in [28], gave a measured association rate constant (k_a) for phenytoin with the immobilized anti-phenytoin antibodies of 2.4 (± 0.4) × 10^6 M^{-1} s^{-1} at pH 7.4 and 37°C. From the relationship $K_A = k_a/k_d$, the dissociation rate constant (k_d) for phenytoin from the immobilized antibodies was also determined, giving a value of 4.4 (± 0.8) × 10^{-3} s^{-1}. All of these results indicated that any phenytoin extracted by the immobilized antibodies would bind tightly to this column and only slowly dissociate from these antibodies in the presence of a pH 7.4 buffer.

The ability of this microcolumn to extract phenytoin was measured by comparing injections of phenytoin standards made onto this column versus injections made onto an inert control column. Table 1 summarizes the results that were obtained. The concentration and quantity of phenytoin that was injected (20 μL of a 6 μM solution, or 120 pmol phenytoin) corresponded to the free amount of this drug that would be expected in serum at typical therapeutic levels. It was found that 95% or more of the phenytoin was extracted when using residence times of 100 ms or greater in the immunoextraction layer, with 98% being extracted at residence times of 150 ms or greater. These results are in agreement with previous observations made for the ultrafast immunoextraction of thyroxine and warfarin [1,2].

The immunoextraction microcolumn was also used in studies examining the effect of flow rate and sample residence on the apparent free fraction that was obtained for phenytoin in a displacement assay. It was found that a flow rate of 1.2 mL/min or greater (i.e., a sample residence time in the immunoextraction layer of 103 ms or less) gave a consistent measured free phenytoin fraction of 15.8% for the test mixture in Table 1. However, slower flow rates and longer sample residence times resulted in a greater apparent free fraction (1.9% higher at 0.8 mL/min and 5.7% higher at 0.6 mL/min). As indicated in previous simulations performed for warfarin and thyroxine [1,2], this increase in the apparent free fraction is believed to reflect dissociation of the analyte from proteins or other binding agents in the sample. Thus, as a compromise between extraction efficiency and accuracy, an injection flow rate of 1.2 mL/min (i.e., a sample residence time of roughly 100 ms in the immunoextraction layer) was used in all later experiments for free phenytoin measurements.

Behavior of NIR fluorescent label under assay conditions

The next item considered was the signal intensity and behavior of the labeled phenytoin under the conditions to be used for ultrafast immunoextraction. It was found in pH 7.4, 0.067 M phosphate buffer that the NIR fluorescence of the labeled conjugate gave a linear response over a broad range of concentrations. This linear range extended from approximately 1.7 fmol to 2.1 pmol (i.e., 0.33 to 412 nM for a 5 μL injection, with a lower limit of detection of 1.4 nM at a signal-to-noise ratio of three). Although this amount is less than the free phenytoin levels in 5 μL of serum (i.e., 20 to 40 pmol or 4 to 8 μM), this UFIDA format only requires that a small, representative amount of the labeled phenytoin be displaced by an injected analyte. Thus, as will be seen later, the response for the NIR fluorescent label was more than adequate for analyzing the displacement peaks that were created during the detection of free phenytoin fractions in such samples.

Another item examined was the effect of biological samples on the fluorescence of the labeled phenytoin. This was of interest since it has been noted in previous work with chemiluminescent labels that up to a 30% change in signal can be seen for a labeled analyte in the presence of serum versus buffer [1]. To study this effect, the emission spectrum of the labeled phenytoin was obtained in the presence and absence of human plasma (i.e., serum plus clotting factors), as shown in Figure 3(a). It was found that there was no appreciable reduction in signal intensity (i.e., less than 3% change) between a buffered standard and plasma containing labeled phenytoin. Similar results were obtained when comparing plasma and buffered solutions

containing 0.33 to 412 nM labeled phenytoin, which gave less than a 17% difference in signal at all concentrations examined. The similarity of the plasma and buffer results indicated that there was either only a small amount of binding between the labeled phenytoin and HSA in plasma or that this binding did not have any appreciable affect on the NIR fluorescence of the dye in this conjugate.

The actual extent of interferences from the sample matrix would be expected to be even smaller than 3 to 17% when the labeled phenytoin is used in a displacement assay, since the peak for the displaced conjugate elutes slightly after the non-retained fraction of the sample. This is demonstrated in Figure 3(b), where even the highest concentration standards gave displacement peaks with a mean elution time that occurred 1 min after the non-retained components of a serum sample (as represented by HSA). The overlap of the sample and displacement peaks was estimated to be less than 5% for this assay, which would further reduce any effects of the sample matrix on the fluorescent signal of the NIR dye used in the labeled phenytoin.

The emission spectrum for a sample of human plasma with no NIR dye added was acquired to see what type of background signal could be expected from such a sample. This spectrum is also shown in Figure 3(a) and gave no detectable signal at the emission wavelengths that were monitored. From this result, as well as the difference in sample and displacement peak elution times noted in Figure 3(b), it was concluded that no significant background signal should have been present under the conditions used in this study for detection of the labeled phenytoin in the displacement peaks.

Optimization of UFIDA method

After the initial conditions for ultrafast immunoextraction and detection of the labeled phenytoin had been selected, these components were combined and optimized for use in an UFIDA assay for measuring free phenytoin fractions. One item considered was the effect of varying the amount of labeled phenytoin that was applied to the system for analyte detection. This item was studied by applying 5 to 100 μL of a 0.558 to 55.8 μM solution of the labeled phenytoin (i.e., 2.8 pmol to 5.6 nmol) at 0.8 ml/min to the immunoextraction microcolumn prior to the injection of a 5 μL sample of 35 μM phenytoin (i.e., a typical therapeutic concentration expected for free phenytoin in serum). The amount of labeled phenytoin used under these conditions ranged from 0.04- to 84-times the binding capacity of the immunoextraction microcolumn. The area of the displaced peak gave less than a 6% change when the amount of labeled phenytoin was at least 110 pmol (e.g., a 20 μL injection of 5.58 μM labeled phenytoin), or conditions in which the amount of the labeled phenytoin was present in more than a 1.6-fold excess versus the column binding capacity.

The only change noted when using larger amounts of labeled phenytoin was a slight increase in the time it took to wash the excess, non-retained labeled conjugate from the column. When using small amounts of this conjugate, it took approximately 3 min to remove 99.9% of the non-retained labeled phenytoin from the column. However, it took around 5.5 min to wash off the excess conjugate when using the highest amounts of labeled phenytoin that were examined in this work. Based on these results, a 20 μL injection of a 5.58 μM preparation of the labeled phenytoin (i.e., 110 pmol) was used along with a wash time of 3 min in the final UFIDA method as a compromise between assay speed and response.

Another item considered in developing the UFIDA method was the effect of varying the time between injection of the labeled phenytoin and the injection of a sample. This was examined to see if overlap of the sample peak with the remaining non-retained labeled phenytoin (at small injection times) or loss of the retained labeled phenytoin (at long injection times) gave a significant change in response for the displacement peak. The results of these studies are shown in Figure 4(a). Using the displacement peak obtained with sample injection at 2 min as the

reference, the changes in area noted for displacement peaks measured after sample injections at 4, 6 or 12 min were 1.7, 1.9, and 4.8% respectively. It was concluded from these data that sample injection should be performed in 2 to 6 min after application of the labeled phenytoin to give less than 2% variation in the displacement peak's size. These conditions minimized loss of the retained labeled phenytoin while also allowing sufficient time for excess labeled conjugate (and any associated contaminants) to be washed from the column.

The next study considered the use of sequential sample injections during the UFIDA method. This was done to determine if it was possible to perform more than one analysis per column loading of the labeled phenytoin. As shown in Figure 4(b), the use of two sequential sample injections gave less than a 5% change in displacement peak area and less than a 3% change in peak height. The intensity of the displacement peaks then began to decrease with further sample injections, giving signals that were 36 and 93% lower (versus the first sample) for the third and fourth injections. In the remainder of this study only one sample injection was performed after each application of the labeled phenytoin. However, the findings in Figure 4(b) indicate that multiple injections could be used in such an assay to further increase sample throughput in the UFIDA method.

Elution of the retained phenytoin and labeled phenytoin in the UFIDA method was accomplished by using a pH 2.5, 0.067 M phosphate buffer applied at 1.2 mL/min for 5 min. Regeneration of the immunoextraction microcolumn was conducted by applying pH 7.4, 0.067 M phosphate buffer for at least 5 min prior to a new injection of labeled phenytoin. The UFIDA method was found to be quite stable under these conditions, allowing a reproducible response to be obtained on a single column over at least 250 injections and four months of regular use.

Validation of UFIDA method

The final conditions used in the UFIDA assay for free phenytoin measurements are summarized in the Experimental Section. An example of a typical run obtained under such conditions is given in Figure 1(b). The displacement peak for this assay occurred within 2−3 min of sample injection, and the total assay time (i.e., one injection, elution and regeneration cycle) was 20 min. However, it was found that the total time to detect and analyze free phenytoin fractions could be reduced to less than 10 min per sample by using multiple sample injections after each application of the labeled phenytoin (see previous section).

A typical calibration curve obtained for the UFIDA method is shown in Figure 5. The lower limit of detection for this method was 570 pM phenytoin (S/N = 3) for a 5 μL sample (i.e., 2.9 fmol). The linear range (i.e., the range of analyte concentrations giving a response within 10% of the best-fit line) extended from the lower limit of detection up to approximately 10 μM phenytoin (approximately 50 pmol). This linear response covered the entire range of free phenytoin concentrations that would be expected at normal therapeutic levels of this drug. The calibration curve for this assay leveled off as phenytoin concentrations above 10 μM were injected. This behavior is probably due to saturation of the immunoextraction microcolumn, since these high sample concentrations gave rise to amounts of phenytoin that approached or exceeded the binding capacity of this system (i.e., 50 pmol phenytoin in 5 μL of a 10 μM sample versus a 67 pmol binding capacity for the column).

The precision of this assay was determined by making replicate injections of standards, phenytoin/HSA mixtures and spiked control serum samples. A relative standard deviation of ±0.5% or less was seen for standards containing free phenytoin concentrations of 1.24 nM to 2.02 μM. As will be shown later, the precision was ! 2 to 5% for serum and HSA samples that contained phenytoin at typical therapeutic concentrations.

The accuracy of the UFIDA assay was first assessed by comparing it to ultrafiltration in the analysis of phenytoin/HSA mixtures that had been prepared in pH 7.4, 0.067 M potassium phosphate buffer (see Table 2). The sample concentrations used in this study were chosen to match the levels of HSA and phenytoin that would be expected in serum samples containing typical therapeutic concentrations of phenytoin. These samples gave average free phenytoin fractions of 15.9, 13.7 and 12.0% at HSA concentrations of 550, 650 and 750 μM, respectively (i.e., results similar to literature values reported for free phenytoin fractions in serum) [11, 14]. All of the twenty-five phenytoin/HSA samples that were examined gave statistically identical results at the 95% confidence level for UFIDA versus ultrafiltration. In addition, all of the UFIDA and ultrafiltration results overlapped within 1 SD of their values and had differences in these values of only 1.6 to 5.4% (average, 2.9%). Although there was a small apparent bias in the ultrafiltration results compared to UFIDA, the size of this bias was strongly linked with experimental uncertainly in the nonspecific binding measured for the ultrafiltration membranes.

Another comparison was made between UFIDA and ultrafiltration in terms of their ability to determine free phenytoin concentrations in human serum. This was conducted with standards and samples used in a commercial immunoassay kit for free phenytoin [12,15,21]. The four standards supplied with this kit (total phenytoin concentration, 1.98 to 12 μM in phosphate buffer) gave a linear response for the UFIDA method, with a best fit line of $y = 4.85 (\pm 0.04) \times + 2.9 (\pm 3.0)$ and a correlation coefficient of 0.9932 (n = 4). The results for control serum samples are shown in Table 3. Comparison of the UFIDA and ultrafiltration results for these samples again resulted in statistically identical values for the measured free fractions of phenytoin. When using ultrafiltration, control sera containing 10 to 40 μM concentrations of total phenytoin gave free phenytoin concentrations of 1.32 to 6.11 μM (or 13.4 to 15.4% free fractions), while analysis of the same samples using UFIDA gave free phenytoin concentrations of 1.27 to 5.99 μM (or free fractions of 12.2 to 15.1%). The difference between the results of these two assays was less than 2 to 5%, with all differences being within 1 SD of the measurements.

Comparison of UFIDA and ultrafiltration

Although UFIDA and ultrafiltration gave comparable results for the samples in Tables 2 and 3, they did differ in terms of their precision and speed. For instance, the relative precision of the UFIDA results in Table 2 was 2.4 to 4.8%, while the relative precision of the ultrafiltration results for the same samples was over two-fold larger, ranging from 8.1 to 9.7%. A similar trend was noted for the control serum results in Table 3. The worse precision of the ultrafiltration results is thought to be the combined result of 1) the multiple, manual steps that are involved in this method and 2) the random experimental variations that were noted in nonspecific binding of phenytoin to the ultrafiltration membrane. In contrast to this, the UFIDA method was performed as an automated system that did not require any sample pretreatment steps, which probably contributed to the better precision of this method versus ultrafiltration.

In terms of speed, UFIDA gave results for each sample within 2−3 min of injection, with a total run time of 20 min per cycle. For ultrafiltration, the minimum time required for one sample was approximately 1 h, which included 45 min to perform the ultrafiltration and 15 min to conduct the HPLC analysis of the filtrate's free phenytoin content. For UFIDA, a total of 15 h was required for a triplicate analysis of all the samples and standards used to generate the data in Table 2. Although ultrafiltration has a much higher analysis time per sample, up to 15 samples could be centrifuged simultaneously in this approach, giving it an overall sample throughput comparable to that of the UFIDA method (i.e., 14 h for analysis of all samples and standards used in Table 2). However, it should be kept in mind that the throughput of the UFIDA method could be increased by almost two-fold by using sequential injections of

samples after each application of the labeled phenytoin, as demonstrated earlier in Figure 4(b). This throughput could be improved even further through the use of multiple immunoextraction columns and a multi-port valve for flow-splitting. Thus, UFIDA has the capability of not only providing a faster analysis per sample than ultrafiltration but can also provide higher sample throughput in some applications.

CONCLUSIONS

This study examined the development and validation of NIR fluorescent labels and an ultrafast immunoextraction/displacement assay for free fraction measurements of the drug phenytoin. The final system used an immunoextraction microcolumn that allowed for the extraction and analysis of free phenytoin when using a sample residence time as low as 100 ms. Phenytoin that had been labeled with a NIR fluorescent dye was then combined with this microcolumn to provide detection limits for phenytoin down to the picomolar range without any observable background signals or matrix effects from serum samples.

The UFIDA method developed in this study gave good correlation versus ultrafiltration for the measurement of free phenytoin fractions in serum and phenytoin/HSA samples. This new method allowed free phenytoin fractions to be measured within 2−3 min of sample injection, while giving a precision and analysis time per sample that was much better than that obtained for ultrafiltration. This method is also much faster than the technique of equilibrium dialysis and, unlike methods based on capillary electrophoresis and RAM columns, can easily be used for free analyte measurements in real clinical samples. Although this method does require appropriate antibodies and labeled analogs for the drug or analyte of interest, this is not a major limitation since these reagents can be obtained or modified from those used for the same analytes in other types of immunoassays. These properties should make the UFIDA method useful in a variety of areas, including therapeutic drug monitoring, pharmacological studies, and measurements of drug-protein binding [1,2,5,6,13-15,20,21,27,56,57].

ACKNOWLEDGMENTS

The authors thank Sara Basiaga and Dipanjan Nag for their help with the NMR studies and Carita Kordik for assisting with the mass spectrometric analysis. The authors also thank LI-COR Biosciences for the donation of the NIR dyes and detector and Chuck Prescott and Dan Draney for their helpful discussions. This work was supported by the National Institutes of Health under grant R01 GM044931 and was conducted in facilities that were renovated under NIH grant RR015468-01.

REFERENCES

1. Clarke W, Schiel JE, Moser A, Hage DS. Anal. Chem 2005;77:1859. [PubMed: 15762597]
2. Clarke W, Chowdhuri AR, Hage DS. Anal. Chem 2001;73:2157. [PubMed: 11393835]
3. Lunde PKM, Rane A, Yaffe SJ. Clin. Pharmacol. Ther 1970;11:846. [PubMed: 4394754]
4. Barre J, Delion F, Tillement JP. Therapeut. Drug Monitor 1988;10:133.
5. Burt M, Anderson DC, Kloss J, Apple FS. Clin. Chem 2000;46:1132. [PubMed: 10926893]
6. Bereczki A, Horvath V. Anal. Chim. Acta 1999;391:9.
7. Hara S, Hagiwara F, Ono N, Kuroda T. Anal. Sci 1999;15:371.
8. Chen J, Ohnmacht CM, Hage DS. J. Chromatogr. B 2004;809:137.
9. P., CTSU. Drug Information for the Health Care Professional. 17 ed.. Rand McNally; Tauton: 1997.
10. Johansen K, Krogh M, Andresen AT, Christophersen AS, Lehne G, Rasmussen KE. J. Chromatogr. B 1995;669:281.
11. Kodama H, Kodama Y, Shinozawa S, Kanemaru R, Todaka K, Mitsuyama Y. J. Clin. Pharm. Therapeut 1998;23:361.

12. Roberts WL, Annesley TM, De BK, Moulton L, Juenke JM, Moyer T. Therapeut. Drug Monitor 2001;23:148.
13. Warner A, Privitera M, Bates D. Clin. Chem 1998;44:1085. [PubMed: 9590393]
14. Gurley BJ, Marx M, Olsen K. J. Chromatogr. B 1995;670:358.
15. Troupin AS, Shaw LM. Epilepsia 1985;26:455. [PubMed: 4043014]
16. Jack L, Cunningham C, Watson ID, Stewart M. Ann. Clin. Biochem 1986;23:603. [PubMed: 3532923]
17. Lu-Steffes M, Pittluck GW, Jolley ME, Panas HN, Olive DL, Wang C-HJ, Nystrom DD, Keegan CL, David TP, Stroupe SD. Clin. Chem 1982;28:2278. [PubMed: 6751601]
18. McGregor AR, Crookall-Greening JO, Landon J, Smith DS. Clin. Chim. Acta 1978;83:161. [PubMed: 340082]
19. Melten JW, Wittebrood AJ, Hubb JJ, Faber GH, Wemer J, Faber DB. J. Pharmaceut. Sci 1985;74:692.
20. Miller TD, Pinkerton TC. Anal. Chim. Acta 1985;170:295.
21. Oellerich M, Mueller-Vahl H. Clin. Pharmacokin 1984;9:61.
22. Popelka SR, Miller DM, Holen JT, Kelso DM. Clin. Chem 1981;27:1198. [PubMed: 7016373]
23. Bertucci C, Bartolini M, Andrisano GV. J. Chromatogr. B 2003;797:111.
24. Rippel G, Corstjens H, Billiet HA, Frank J. Electrophoresis 1997;18:2175. [PubMed: 9456032]
25. Heegaard NHH. J. Mol. Recogn 1998;11:141.
26. Heegaard NHH. Electrophoresis 2003;24:3879. [PubMed: 14661223]
27. Wang H, Zou H, Zhang Y. Anal. Chem 1998;70:373. [PubMed: 9450364]
28. Nelson MA, Reiter WS, Hage DS. Biomed. Chromatogr 2003;17:188. [PubMed: 12717809]
29. Reng X, Draney DR. LabPlus Int 2004;18:8.
30. Sowell J, Parihar R, Patonay G. J. Chromatogr. B 2001;752:1.
31. Miki S, Kaneta T, Imasaka T. J. Chromatogr. A 2005;1066:197. [PubMed: 15794571]
32. Castle PE, Phillips TM, Hildesheim A, Herrero R, Bratti CM, Rodriguez AC, Morera LA, Pfeiffer R, Hutchinson ML, Pinto LA, Schiffman M. J. Biochem. Biophys. Methods 2003;12:1449.
33. Dodge A, Fluri K, Verpoorte E, de Rooij NF. Anal. Chem 2001;73:3400. [PubMed: 11476241]
34. Phillips TM. J. Biochem. Biophys. Methods 2001;49:253. [PubMed: 11694283]
35. Ohnmacht CM, Chen S, Tong Z. J. Chromatogr. B 2006;836:83.
36. Clarke W, Beckwith JD, Jackson A, Reynolds B, Karle EM, Hage DS. J. Chromatogr. A 2000;888:13. [PubMed: 10949468]
37. Clarke W, Hage DS. Anal. Chem 2001;73:1366. [PubMed: 11305676]
38. Middendorf, L.; Amen, J.; Bruce, R.; Draney, D.; DeGraff, D.; Gewecke, J.; Grone, D.; Humphrey, P.; Little, G.; Lugade, A.; Narayanan, N.; Oommen, A.; Osterman, H.; Peterson, R.; Rada, J.; Raghavachari, R.; Roemer, S. Near-infrared dyes for high technology applications. Daehne, S., editor. Kluwer Academic; Netherlands: 1998. p. 21
39. Kiec-Kononowicz K, Zejc A, Byrtus H. Pol. J. Chem 1984;58:585.
40. Khan MN. J. Org. Chem 1996;61:8063. [PubMed: 11667789]
41. Lauer R, Zenchoff G. J. Heterocyclic Chem 1979;16:339.
42. Shaffer JW, Sheasley R, Winstead MB. J . Med. Chem 1967;10:739. [PubMed: 5341213]
43. Hermanson, GT. Bioconjugate Techniques. Academic Press, Inc.; San Diego: 1996.
44. Ruhn PF, Garver S, Hage DS. J. Chromatogr 1994;669:9.
45. Chattopadhyay A, Hage DS. J. Chromatogr 1997;758:255.
46. Larsson PO. Methods Enzymol 1984;104:2121.
47. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Anal. Biochem 1985;150:76. [PubMed: 3843705]
48. Hage DS. J. Chromatogr. B 2002;768:3.
49. Argyle CJ, Kinniburgh DW, Costa R, Jennison T. Therapeut. Drug Monitor 1984;6:117.
50. Ohnmacht, CM. Ph.D. Dissertation. University of Nebraska-Lincoln; Lincoln: 2006.
51. Kim, HS.; Hage, DS. Handbook of Affinity Chromatography. 2nd Ed.. CRC Press; Boca Raton, FL: 2005. Chap. 3

52. Hermanson, GT.; Mallia, AK.; Smith, PK. Immobilized Affinity Ligand Techniques. Academic Press; New York: 1992.
53. Hage, DS.; Phillips, TM. Handbook of Affinity Chromatography. 2nd Ed. CRC Press; Boca Raton, FL: 2005. Chap. 6
54. Van Regenmortel, MHV. Structure of Antigens. 1. CRC Press; Boca Raton, FL: 1992.
55. Butler, JE. Immunochemistry of Solid-Phase Immunoassay. CRC Press; Boca Raton, FL: 1991.
56. Beck DE, Farringer JA, Ravis WR, Robinson CA. Clin. Pharm 1987;6:888. [PubMed: 3677566]
57. Oates MR, Clarke W, Zimlich AI, Hage DS. Anal. Chim. Acta 2002;470:37.

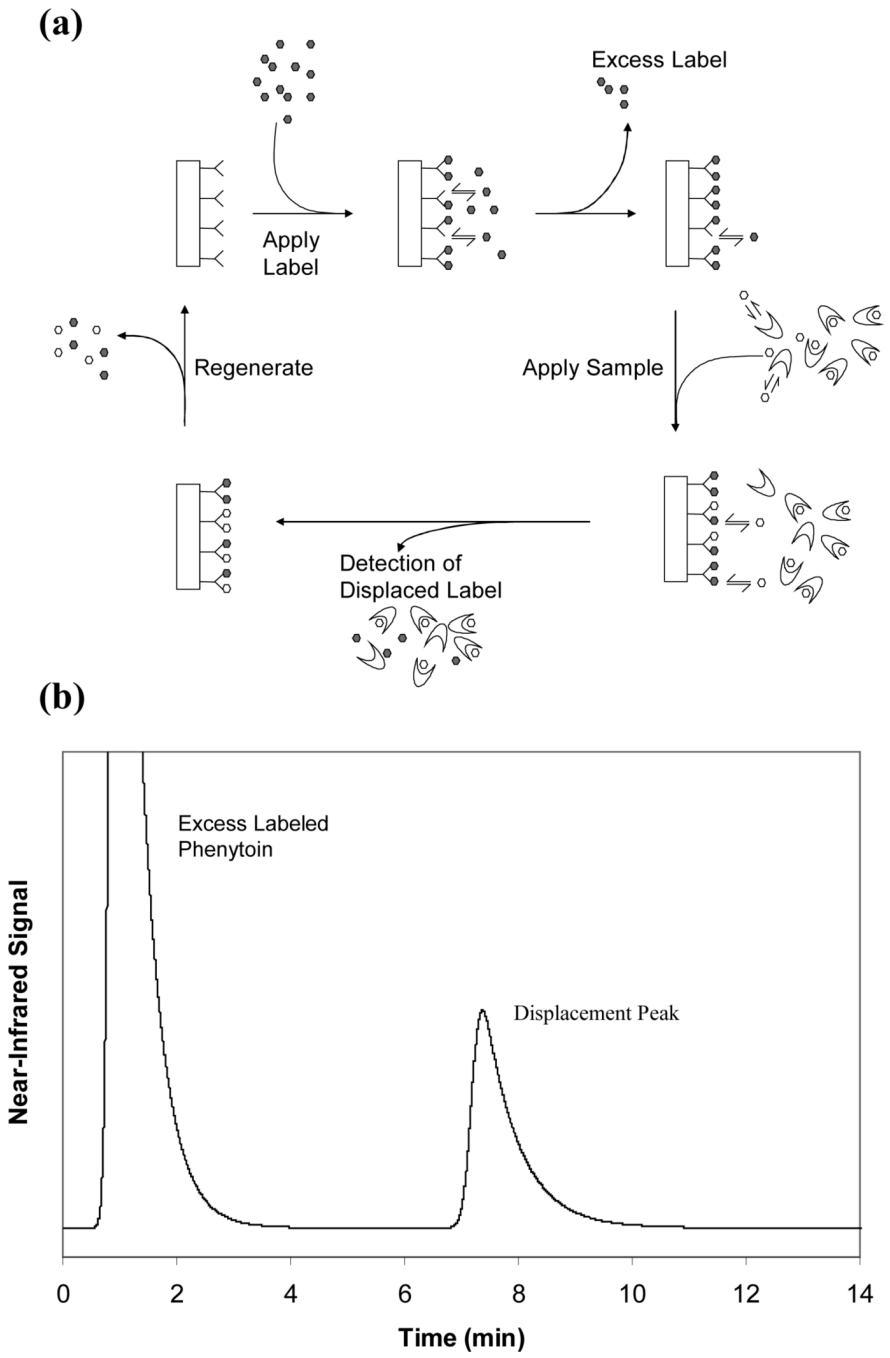

Figure 1.

(a) General scheme for an ultrafast immunoextraction/displacement assay (UFIDA) and (b) a typical chromatogram for such an assay. The example in (b) is based on the phenytoin system described in the Experimental Section. In (b) the excess labeled phenytoin was injected at 0 min and the sample containing the unlabeled phenytoin was injected at 6 min. Symbols: (•), labeled analog of analyte; (○), unlabeled analyte from sample; (⊃), binding agent in a sample for the analyte; (⌒), immobilized antibody.

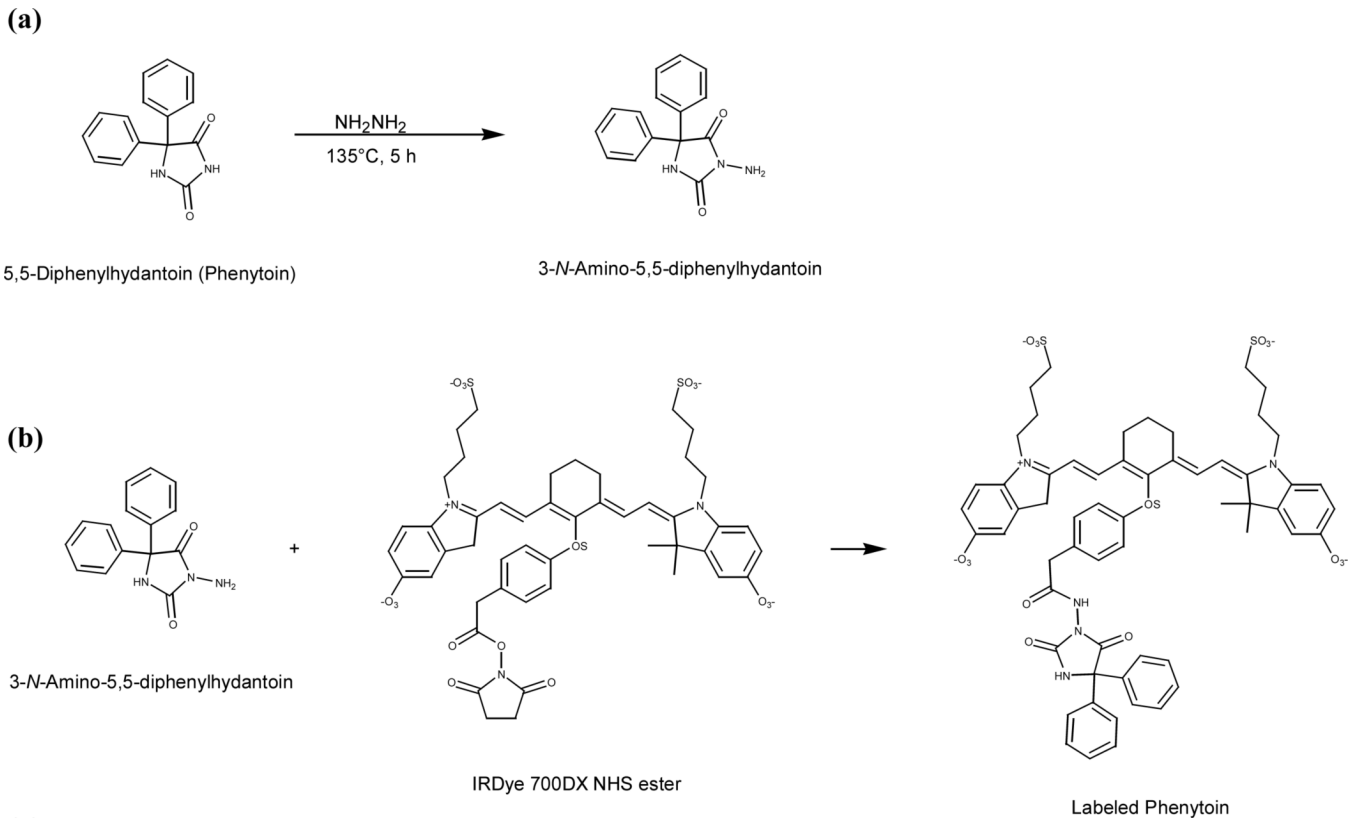

Figure 2.

Scheme for the synthesis of a phenytoin conjugate containing a label based on a near-IR fluorescent dye. The first reaction (a) shows the preparation of 3-*N*-amino-5,5-diphenylhydantoin (ADPH), while the second reaction (b) shows how ADPH was used to prepare the final labeled phenytoin. The dye shown in this example is NHS-activated IRDye 700DX from LI-COR Biosciences, which is similar to the proprietary dye used in this current study [29].

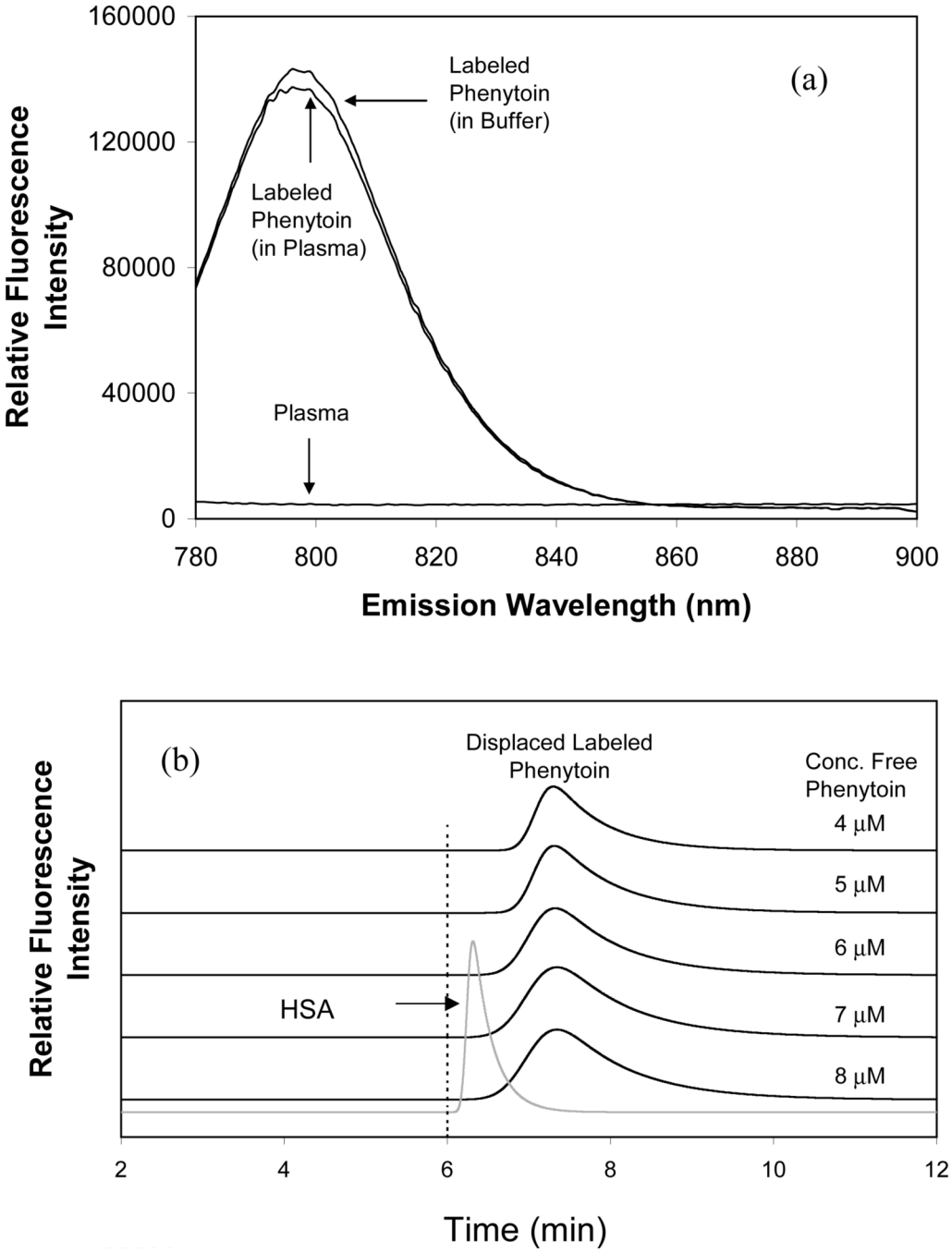

Figure 3.

(a) Emission spectrum for human plasma and labeled phenytoin in the presence of plasma or pH 7.4, 0.067 M phosphate buffer, and (b) chromatograms for a UFIDA method performed for samples containing free phenytoin concentration of 4 to 8 μM injected at 6 min after the application of labeled phenytoin. A chromatogram is also shown (b) that was obtained under the same conditions for a 5 μL, 550 μM sample of HSA (representing the main protein of serum or plasma and a non-retained component in the UFIDA method), as monitored using absorbance detection at 205 nm. The emission spectra in (a) were obtained using a 100 μM solution of labeled phenytoin in plasma or buffer at an excitation wavelength of 770 nm.

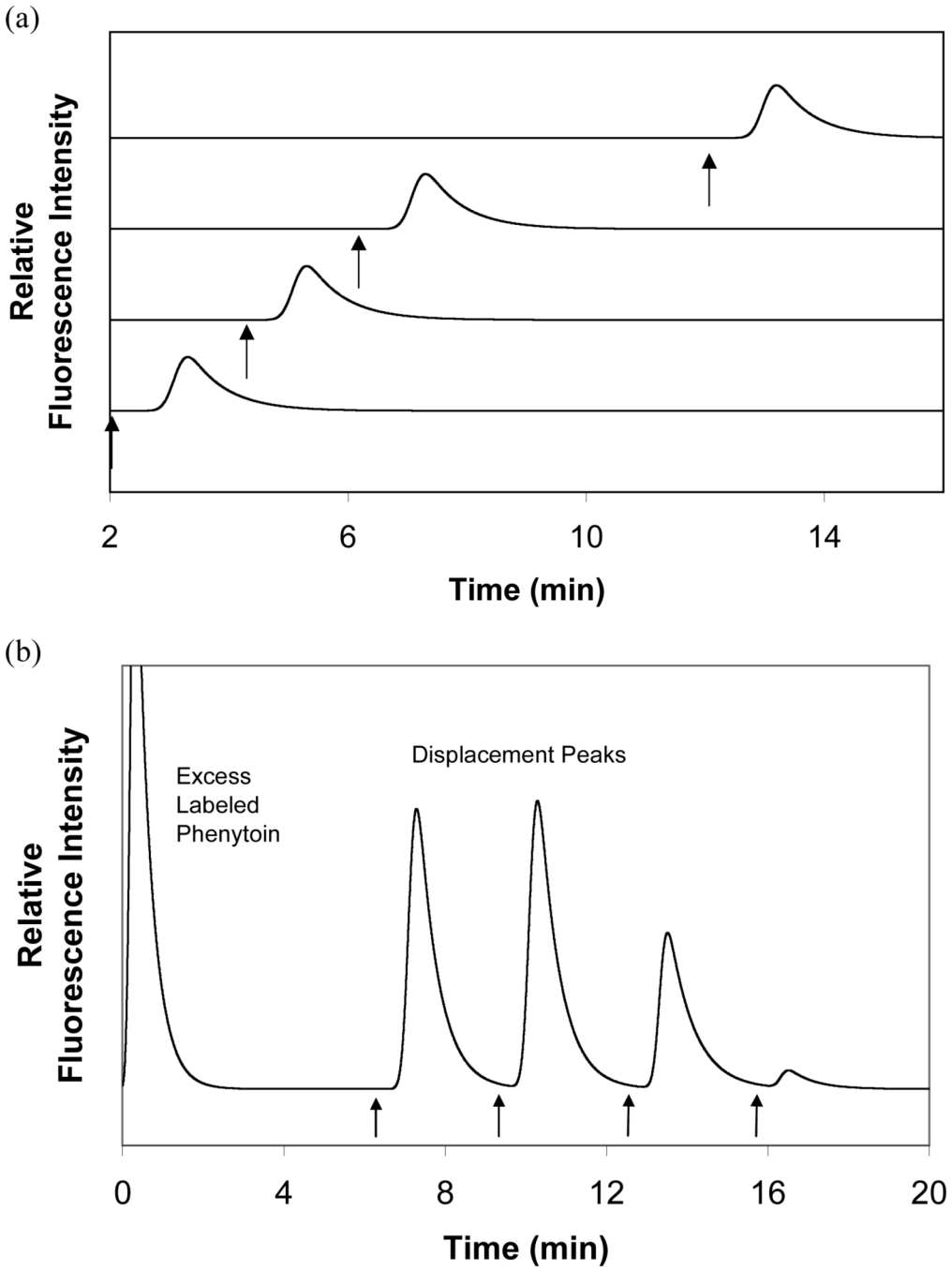

Figure 4.

Effect of (a) injection time on the application of phenytoin samples to the UFIDA system and (b) the use of sequential sample injections in this assay. The experiments with the injection time were performed using a 5 μL, 4 μM phenytoin sample. The sequential injection studies were performed using a sample containing 30 μM phenytoin and 550 μM HSA. In both (a) and (b), the column was loaded with 20 μL of a 5.58 μM solution of labeled phenytoin at 0.8 mL/min, with the flow rate being changed to 1.2 mL/min after 1.5 min into each run and before sample injection.

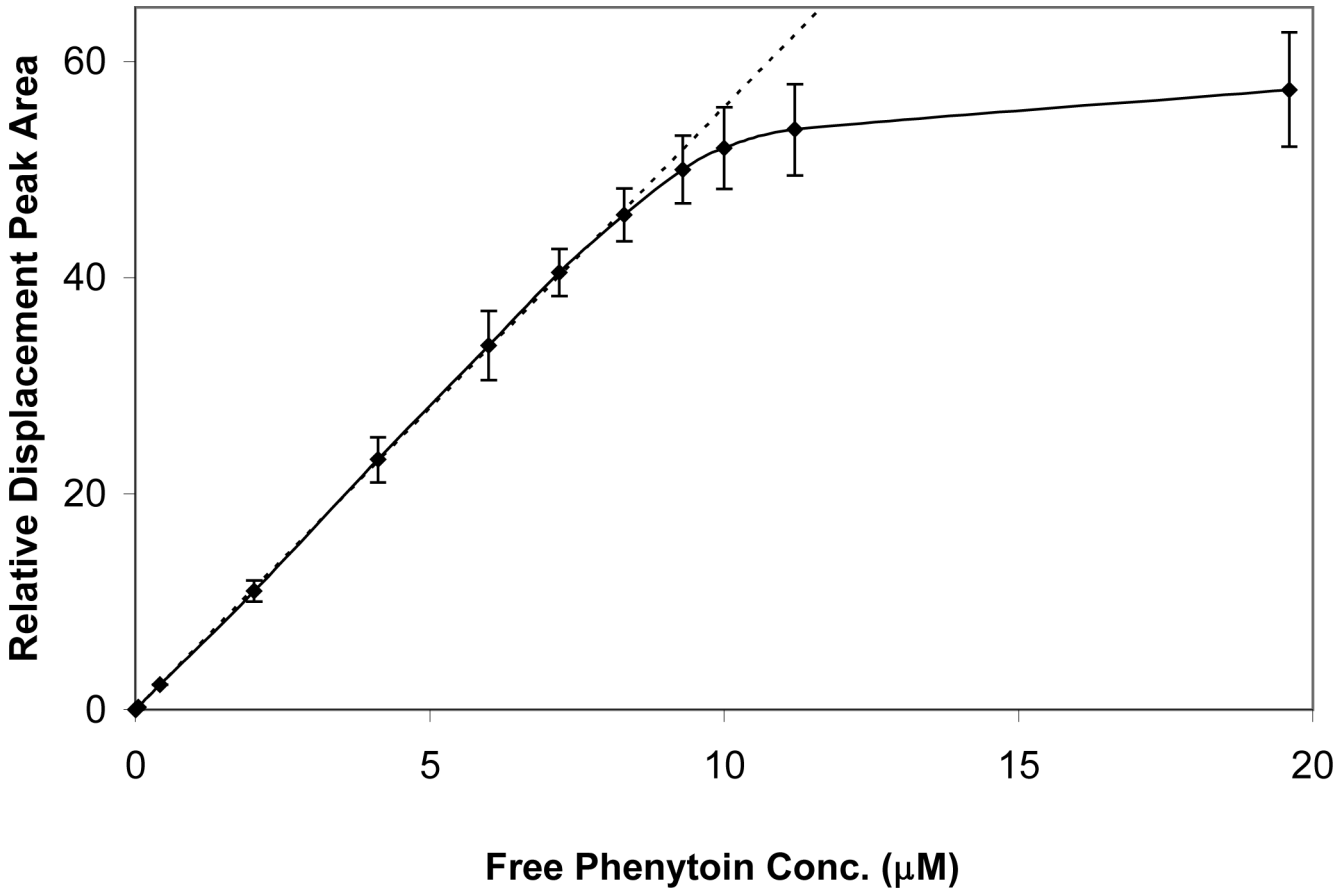

Figure 5.
(a) Calibration curve based on displacement peak area for phenytoin in the UFIDA method. The experimental conditions are given in the text. The best-fit line over the linear range in (a) was y = 5.58 (± 0.02) × + 0.010 (± 0.106), with a correlation coefficient of 0.9999 (n = 10).

Table 1

Ultrafast immunoextraction of phenytoin at various flow rates in a sample containing a fixed total amount of phenytoin and HSA[a]

Flow rate (mL/min)	Residence time in antibody layer of immunoextraction microcolumn (ms)	Extraction efficiency (%)	Measured free fraction phenytoin (%)
0.6	207	100 (± 10)	16.7 (± 0.6)
0.8	155	98 (± 2)	16.1 (± 0.5)
1.2	103	95 (± 3)	15.8 (± 0.5)
1.4	89	92 (± 3)	15.8 (± 0.4)
1.6	78	87 (± 5)	15.8 (± 0.2)

[a]These values were determined using sample that contained 550 μM HSA and 35.0 μM phenytoin. The numbers in parentheses represent a range of ±1 SD.

Table 2

Determination of free phenytoin fractions in phenytoin/HSA mixtures by ultrafiltration or a UFIDA method[a]

Total conc. HSA (μM)	Total conc. phenytoin (μM)	Measured free phenytoin conc. (μM)	
		Ultrafiltration	UFIDA
550	30	4.9 (± 0.4)	4.7 (± 0.2)
550	35	5.7 (± 0.5)	5.5 (± 0.2)
550	40	6.6 (± 0.6)	6.4 (± 0.2)
550	45	7.5 (± 0.7)	7.2 (± 0.2)
550	50	8.3 (± 0.7)	8.1 (± 0.2)
650	30	4.2 (± 0.4)	4.1 (± 0.1)
650	35	4.9 (± 0.4)	4.8 (± 0.1)
650	40	5.6 (± 0.5)	5.5 (± 0.1)
650	45	6.4 (± 0.6)	6.2 (± 0.2)
650	50	7.1 (± 0.6)	6.9 (± 0.2)
750	30	3.7 (± 0.3)	3.5 (± 0.1)
750	35	4.3 (± 0.4)	4.2 (± 0.2)
750	40	4.9 (± 0.4)	4.8 (± 0.2)
750	45	5.6 (± 0.5)	5.4 (± 0.2)
750	50	6.2 (± 0.6)	6.1 (± 0.2)

[a] All measurements were performed at 37°C in pH 7.4, 0.067 M phosphate buffer. The values for ultrafiltration have been corrected for nonspecific binding to the membrane. The numbers in parentheses represent a range of ± 1 SD.

Table 3

Determination of free phenytoin fractions in serum samples by ultrafiltration or a UFIDA method[a]

Total conc. phenytoin (μM)	Measured free phenytoin conc. (μM)	
	Ultrafiltration	UFIDA
10	1.32 (± 0.34)	1.27 (± 0.05)
20	2.81 (± 0.42)	2.70 (± 0.12)
40	6.11 (± 0.44)	5.99 (± 0.14)

[a] All measurements were performed in pH 7.4, 0.067 M potassium phosphate buffer and 37°C. The values for ultrafiltration have been corrected for nonspecific binding to the membrane. The values in parentheses represent! 1 SD.