Anal. Chem. **2002,** *74,* 5993−5997

Negligible Depletion Solid-Phase Microextraction with Radiolabeled Analytes To Study Free Concentrations and Protein Binding: An Example with [^{3}H]Estradiol

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A new method is presented that enables sensitive measurement of free concentrations of radiolabeled ligands. Additionally, protein binding of radiochemicals in complex matrixes can be determined with this new technique that combines negligible depletion solid-phase microextraction (nd-SPME) with liquid scintillation counting (LSC) as detection. [^{3}H]Estradiol was taken as an example compound. Possible matrix effects of protein on fiber uptake kinetics were studied. No matrix effect was found, either by fouling of the fiber, or by changed uptake kinetics. The validity of the method was shown in the determination of the affinity constant (K_a) of estradiol for human serum albumin (HSA). The K_a was estimated at 8.9×10^4 M^{-1}, which corresponds well with literature values. This study shows that nd-SPME is suitable to study the free concentration and protein binding of [^{3}H]-estradiol. The method described in this paper combines the advantages of nd-SPME with the advantages of radiolabeled analytes, creating a timesaving, simple, and sensitive analytical tool that will be particularly useful in complex matrixes containing many potential interferences for chromatographic methods.

The free concentration of compounds is toxicologically and pharmacologically of eminent importance, because generally, only the freely dissolved molecules can pass through cell membranes and thereby reach their target sites.[1] Protein binding of a compound diminishes its free concentration and can therefore reduce a compound's biological and pharmacological activity.[2−7]

Several methods have been developed to measure the free concentration of compounds, most of which involve the physical separation of free and (protein-) bound fractions followed by a conventional analysis step. Examples of separation techniques are equilibrium dialysis, ultrafiltration, and gel filtration. These techniques are usually time-consuming, can suffer loss of analyte to membranes, or can create a shift in the binding equilibrium during the separation.[8−10] The charcoal assay is a separation technique used frequently in receptor-binding studies and measures the bound fraction of analytes after removing the free fraction via charcoal adsorption. Here, the binding equilibrium may shift as well, making the separation poorly defined and highly dependent on the experimental circumstances (e.g., temperature, time, concentrations) and materials.

In 1990, Pawliszyn and Arthur introduced solid-phase microextraction (SPME) as a new, solvent-free extraction technique.[11] Since then, SPME has been applied in various research areas from environmental chemistry to biomedical analysis.[12−16] In short, SPME uses optical fibers with an organic polymer coating around a glass core. The polymer acts as a hydrophobic extracting phase (similar to an organic solvent) from which the analyte can be desorbed by, for example, thermal desorption in a GC. The main advantages of SPME are the combination of a separation and sampling step in one, hence reducing time and labor, reduction in use of environmentally polluting organic solvents, its effortlessness, simplicity, and low cost.

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10.1021/ac0204552 CCC: $22.00
Published on Web 11/01/2002

Vaes et al.[17] introduced nd-SPME (negligible depletion-SPME) as a simple method to measure the free concentration of a compound in various matrixes. In nd-SPME, a negligible amount of the freely dissolved fraction of the analyte partitions to the fiber coating, leading to a negligible depletion of the freely dissolved fraction and thus a negligible shift in the equilibrium between the bound and the free fraction. The concentration of analyte in the fiber coating (C_f) in time is then related to the freely dissolved concentration in the aqueous phase ($C_{aq,free}$) through a first-order one-compartment model.[17]

$$C_f = \frac{k_1}{k_2} C_{aq,free}(1 - e^{-k_2 t}) \quad (1)$$

In this equation, k_1 and k_2 are the uptake and release rate constants between fiber and aqueous phase, respectively, and t is the time of exposure of the fiber.

The basic assumptions behind nd-SPME lead to certain conditions that need to be met for a correct analysis:[17]

(1) there must be equilibrium between the free and matrix-bound fraction of the analyte,

(2) the fiber should extract only a negligible amount of the free fraction, and

(3) the matrix (e.g., protein) should not influence the uptake kinetics or adsorb to the fiber.

This last condition is still a subject of discussion. Some studies have reported fouling of the SPME fiber by proteins,[15,18] while others have not.[17,19,20] Effects of proteins on the uptake kinetics of an analyte into an SPME fiber have been reported as well.[21,22]

Nd-SPME does not have the disadvantages of the mentioned traditional separation techniques: it needs much less time, it suffers no loss of analyte by adsorption to membranes, and it leaves the binding equilibrium intact continuously. Therefore, nd-SPME is a promising new way to measure protein binding.

Originally, only the commercially available fibers from Supelco, which are very suitable for automated GC and manual HPLC analyses, were used for nd-SPME. Recently, however, Mayer et al. rediscovered disposable SPME fibers for the analysis of persistent and bioaccumulative pollutants in sediment.[23] The advantage of these fibers is their low cost, the flexibility in selection of the length of fiber and in the experimental setup, and the possibility they create to run many analyses simultaneously.

This study describes how these disposable fibers are applied for nd-SPME analysis to measure free concentrations and study protein binding of radiolabeled analytes. Radiolabeling of a compound makes its analysis more sensitive and more simple, because it does not require separation from other compounds prior to detection. Therefore, radiolabeling is especially useful in the areas of biochemistry, pharmacology, toxicology, etc., where matrixes are usually complex, containing many different (interfering) compounds. As discussed above, the free concentration of a compound is an important entity in these fields of research. Therefore, the combination of nd-SPME with radiolabeled analytes can provide very interesting new possibilities for the analysis of compounds in biological matrixes. Although Yuan et al.[24] used radiolabeled theophylline to test their immunoaffinity SPME fibers, performing total extractions, to date, no reports have focused on measuring free concentrations of radiolabeled analytes using commercially available SPME fibers.

Because of the recently increased interest in hormones due to the endocrine disruption issue, one radiolabeled compound that is studied intensively and for which free concentration measurements are of interest, is [^{3}H]estradiol. This compound is often studied in its radiolabeled form, because it is present in biological matrixes at very low concentrations. Therefore, we have chosen it as our test compound. Estradiol is a nonvolatile compound, so headspace SPME is not straightforward, and direct-immersion SPME is recommended.

The matrix effects mentioned before are important if the application of nd-SPME is expanded to the biochemical field, such as in this paper. Therefore, specific attention was paid to the influence of matrix (in this case, a solution of BSA) on the kinetics of uptake of estradiol to the fiber and on a potential influence of protein on the estimation of freely dissolved concentrations and binding affinities. To finally prove the validity of the method and demonstrate how it can be used to study protein binding, the affinity constant (K_a) of estradiol for human serum albumin (HSA) was determined and compared to literature values.

EXPERIMENTAL SECTION

Apparatus and Reagents. A length of 50 m of SPME fiber with a coating of 7 μm of polyacrylate (PA) was purchased from Supelco (Bellefonte, PA). [2,4,6,7-^{3}H]17β-estradiol (2627 GBq/mmol, 37 MBq/mL) was purchased from New England Nuclear (Boston, MA) and used within two months to ensure radiochemical purity (>97%). BSA and HSA were purchased from Sigma Chemical Co. (St. Louis, MO). Charcoal (Norit SA-3) and dextran for the stripping slurry were purchased from Boom (Meppel, The Netherlands) and Sigma Chemical Co. (St. Louis, MO), respectively. Ultima GOLD scintillation fluid was purchased from Packard Bioscience Co. (Meriden, CT). SPME samples were shaken at maximum speed on a KS 125 basic of IKA (Staufen, Germany), which was equipped with a board with clamps for the vials attached to it. All samples were analyzed on a Minaxi Tricarb 4000 liquid scintillation counter of Packard Bioscience Co. (Meriden, CT).

Safety Considerations. Tritiated compounds should be handled with care, because tritium can cause radiation damage when present on or in the body, possibly leading to cancer. National guidelines on working with and disposing of radionuclides should be followed.

Estradiol is a powerful hormone, uptake of which can deregulate many processes in the body, including reproduction. It is also a suspected carcinogen. Therefore, ingestion of estradiol should be prevented by working hygienically and uptake via the skin, by wearing gloves.

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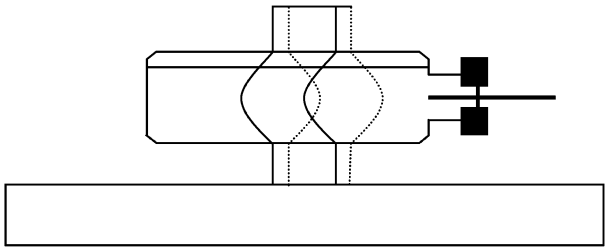

Figure 1. SPME setup: the fiber is pierced through the septum and the vial is clamped horizontally on a shaking device.

General SPME Procedure. A length of 7-μm PA fiber was cut into pieces ~1.5 cm long and pierced through the septa of the lids of 2-mL glass vials with the help of a syringe needle. The length of fiber sticking into the vial was set manually to 0.5 cm. The standard deviation in loads of individual fibers, which were exposed exactly the same, was found to be 4.6%. This variation was partly due to the error in fiber length.

Sample volumes were 1.6 mL throughout the study, and the top of the vial was kept clear of sample to ensure the fiber was not exposed already when screwing on the lid. At time zero, the vial was flipped horizontally and clamped to the shaking device (Figure 1), which was set to maximum speed. After a certain absorption time, the vial was taken off, and the fiber was pulled out of the septum and transferred wholly into a scintillation vial. It was left for desorption with 3.8 mL Ultima GOLD for at least 3 h and then vortexed before counting radioactivity. All experiments were carried out at room temperature (22 ± 2 °C). Disintegrations per minute (dpm) was calibrated with a series of different volumes of the same solution that was used to prepare the samples.

The depletion by the 7-μm fiber was 4.2% (calculated via a mass balance), which was considered as negligible. The detection limit (10 times background activity) of the method was 1.5×10^{-11} M of [2,4,6,7-^{3}H]17β-estradiol freely dissolved in an aqueous phase. Analyte loss due to sorption to the vial walls or the Teflon septum was found to be around 8 ± 4% in buffer and absent in the presence of protein.

Absorption Profiles and Effect of Protein. To determine whether protein affects the uptake of estradiol into the fiber, absorption profiles of estradiol were measured at different bovine serum albumin (BSA) concentrations with 7-μm PA fibers. Concentrations were 7.1×10^{-9} M [^{3}H]estradiol, and 0, 6.5×10^{-6}, 1.6×10^{-5}, 6.4×10^{-5}, and 1.0×10^{-3} M BSA, all in 50 mM Tris buffer (pH 7.4). Mixtures were left to incubate for 10 min at 20 °C, which had been shown to be sufficient to obtain binding equilibrium in the literature[17] and in a preliminary experiment. Absorption times were 2, 5, 15, 30, 60, 90, 120, 180, 300, and 420 min, in duplicate. Curves were fitted separately through the data points using eq 1, but yielding the total concentration of estradiol in the water phase (C_{tot}) instead of $C_{aq,free}$, because the latter was unknown. Therefore, instead of k_1, ff × k_1 was determined, where ff is the free fraction of estradiol in the water phase, and thus, $C_{aq,free} = ff \times C_{tot}$.

DCC Stripping of Protein. To be sure that no endogenous compounds were bound to the protein in the binding assay, HSA was stripped with dextran-coated charcoal (DCC). A DCC slurry with 0.5% charcoal and 0.05% dextran in 50 mM Tris buffer (pH 7.4) was prepared and left to stir overnight at 4 °C. A volume of slurry similar to the volume of protein solution to be stripped was centrifuged 10 min at 1500*g*. The supernatant was discarded, and the protein solution was added to the remaining pellet. This slurry was shaken for 4 h at 4 °C at 500 rpm on an IKA-Schüttler MTS 4 (Staufen, Germany), and subsequently centrifuged for 20 min at 1500*g*. The supernatant was filtered over a 0.2-μm filter, and the filtrate was used for the assays. This procedure was found to remove >96% of the endogenous estradiol in a sample.

Determination of an Affinity Constant. To prove the feasibility of SPME in studying protein binding of tritiated compounds, the method was used to determine the affinity constant of estradiol for HSA. Samples with 0.89×10^{-9} M [^{3}H]estradiol and 2.2×10^{-6}, 9.3×10^{-6}, 2.0×10^{-5}, 6.1×10^{-5}, 1.5×10^{-4}, 3.0×10^{-4}, or 6.1×10^{-4} M charcoal-stripped HSA in 50 mM Tris buffer (pH 7.4) were prepared in triplicate and were extracted with fibers for 3 h, as described above. The fiber concentrations (C_f) were calculated by calibrating fiber activity with standards of [^{3}H]-estradiol in ethanol and dividing the resulting amount in the fiber by the coating volume (14.8 nL). Fiber concentrations were plotted against the protein concentrations (P_t), and the K_a was determined by fitting eq 2 through the data points (adapted from Rowland & Tozer[25]).

$$C_f = \frac{C_{f,0}}{1 + K_a f_{up} P_t} \quad (2)$$

In this equation, $C_{f,0}$ is the fiber concentration at a protein concentration of 0, determined by the fit, and f_{up} is the unoccupied fraction of protein. In our experiment, $f_{up} \approx 1$ because the total protein concentration was much higher than the total ligand concentration. This experiment was repeated two times to study the reproducibility.

To verify the effect of protein on the determination of K_a, this value was also calculated at each protein concentration individually using eq 2 and the fitted value for $C_{f,0}$. The K_a values of the triplicates of all three experiments together were averaged ($n = 9$).

RESULTS AND DISCUSSION

Absorption Profiles and Effect of Protein. Figure 2 shows the absorption curves measured at the different BSA concentrations. It can be seen that higher protein concentrations do not change the shape of the absorption profile, but only affect the maximum concentration of estradiol in the fiber. This maximum is related to the free concentration in the solution, which is, of course, lower at higher protein concentrations. These observations are supported by the values of the kinetic rate constants (Table 1): ff × k_1 is lower at higher protein concentrations because ff decreases, but k_2, which describes the time to equilibrium, remains constant. If the protein had affected the uptake kinetics of estradiol into the fiber, k_2 would have been influenced. The k_2 values at the two higher protein concentrations are somewhat higher than at the lower protein concentrations, but a one-way ANOVA with a Tukey multiple comparison test[26] showed this does not concern a significant difference ($P > 0.05$). Therefore, the presence of

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Table 1. Kinetic Constants of the Absorption Profiles of [^{3}H]Estradiol in the Presence of Various Concentrations of Protein

	BSA concn (M)				
	0	6.5×10^{-6}	1.6×10^{-5}	6.4×10^{-5}	1.0×10^{-3}
ff $\times$ k_1 (min^{-1})[a]	64	45	31	16	1.3
std statistical error, ff $\times$ k_1 (min^{-1})	3	2	2	1	0.1
95% confidence limits ff $\times$ k_1	57−71	40−50	27−35	13−18	1.0−1.5
k_2 (min^{-1})[b]	0.014	0.014	0.013	0.016	0.016
std statistical error, k_2 (min^{-1})	0.001	0.001	0.001	0.002	0.002
n	19	22	22	21	22
95% confidence limits k_2	0.012−0.016	0.012−0.016	0.011−0.015	0.013−0.020	0.012−0.020
r^2	0.984	0.981	0.977	0.961	0.933

[a] ff = free fraction, k_1 = uptake rate constant. [b] k_2 = release rate constant.

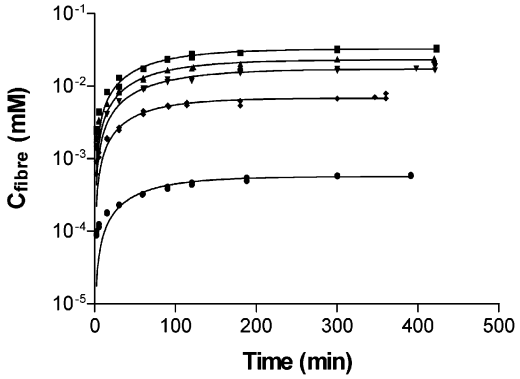

Figure 2. Absorption profiles of [^{3}H]estradiol to the 7 μm polyacrylate fiber in the presence of various concentrations of BSA: none (■), 6.5×10^{-6} (▲), 1.6×10^{-5} (▼), 6.4×10^{-5} (◆), and 1.0×10^{-3} M (●).

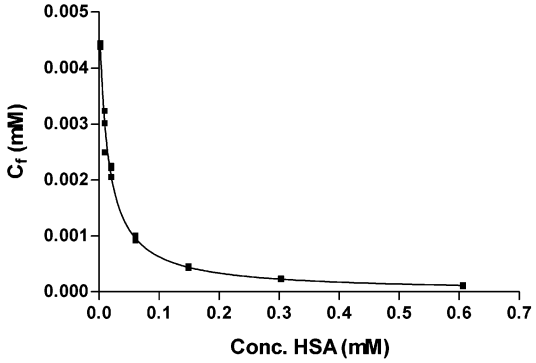

Figure 3. Typical results for the determination of an affinity constant of [^{3}H]estradiol for human serum albumin (HSA): measured fiber concentrations at different HSA concentrations.

protein does not seem to have an effect on the uptake kinetics of this particular compound into this type of fiber.

The observation that the loss of estradiol by binding to the glass wall is different in the presence or absence of protein, can raise the question whether this has affected the outcome of this experiment. The $C_{aq,free}$ will have been overestimated for the curve measured in the absence of protein, and not for the other curves, possibly leading to a difference in the k_2 calculation. However, we have found that raising or lowering the $C_{aq,free}$ by 10% does not change the calculated k_2 value. This should not be the case theoretically either, because the k_2 value is related to the time that is needed to reach equilibrium, and this is not sensitive to the actual concentration of the analyte.

The observation that there is no influence of protein on the uptake kinetics supports the hypothesis that the presence of protein does not disturb the free concentration measurement. Such a disturbance was suggested by Oomen et al.[21] for the measurement of freely dissolved concentrations of hydrophobic PCB's in a soil−chyme matrix. They discussed that net desorption of analyte from the proteins present in the unstirred water layer around the fiber might occur when the free fraction in this layer is significantly depleted. In that case, the uptake of analyte in a sample with protein is higher than in a sample without protein, except when equilibrium has been reached between the fiber and the solution. Indeed, Jeannot and Cantwell[22] found an increased extraction rate constant in the presence of BSA when using solvent microextraction to measure free progesterone. Vaes et al.,[17] however, did not observe such phenomena.

Oomen et al.[21] have argued that matrix effects can occur when (1) the rate-limiting step of the uptake is the diffusion through the unstirred water layer around the fiber, and (2) the analyte desorbs from the matrix fast enough to replenish the depleted free concentration in this layer. It is likely that for estradiol, diffusion in the fiber itself is the rate-limiting step (unpublished results), and therefore, the first condition is not met. In the study from Vaes et al.,[27] this was also the case. These workers used polyacrylate fiber for several organic compounds with log K_{ow} ranging from 0.83 to 4.78 and showed that these compounds are not hydrophobic enough to let the diffusion through the unstirred water layer become rate-limiting. Estradiol has a log K_{ow} of 4.01 (as reported in Kowwin from Syracuse Research Cooperation, Syracuse, NY), so in comparison with the data from Vaes et al., estradiol should not be hydrophobic enough for the first condition to be met, either. In general, compounds with relatively low hydrophobicity are unlikely to show matrix effects.

Nevertheless, in using nd-SPME to measure free concentrations of other compounds in the presence of a matrix, one should be aware of the possibility that matrix effects can occur when one is measuring in the kinetic phase, that is, before equilibrium is reached, especially in the case of very hydrophobic analytes. In the case of volatile analytes, measuring in the headspace of samples can avoid these problems.[15,21]

Determination of an Affinity Constant. Figure 3 shows one of the measured free fraction curves. The mean value for K_a from the three experiments is 8.9×10^4 M^{-1}, with a standard deviation

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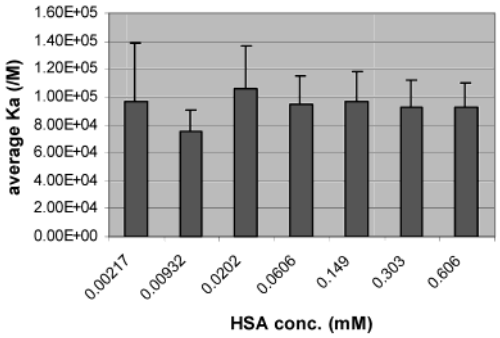

Figure 4. Effect of protein concentration on the determination of the K_a of [^{3}H]estradiol for HSA. Averaged K_a values are shown with their standard deviation ($n = 9$).

of 1.6×10^4 M^{-1} (18% of mean), and $r^2 > 0.97$ for all three fits. The mean K_a corresponds well with the literature value of 7.1×10^4 M^{-1} (at 20 °C), taking into consideration that this was determined with another method.[28] The reproducibility (18%) is satisfactory, considering that this was determined from three independent experiments and that the calculation assumed normal distribution, whereas most authors assume log-normal distribution.

Figure 4 shows the average K_a values calculated for each individual protein concentration. It is clear that the protein concentration has no significant effect on the determination of K_a, and thus, fouling of the fiber is unlikely.

These observations are in contrast to the report of Poon et al.[18] and observations discussed by Ulrich,[15] but in agreement with the observations of Abdel-Rehim et al.,[19] Okeyo et al.,[20] Vaes et al.,[17] and Yuan and Pawliszyn,[10] who do not report fouling. It is unclear how such a difference in observations has arisen. One should, therefore, always be aware of a fouling potential of fibers and experimentally investigate it.

In this study, however, the satisfactory reproducibility, the similarity to literature values, and the absence of fouling show that SPME is suitable for the determination of K_a values by direct immersion in protein solutions.

Prospect. For measuring absolute free concentrations of [^{3}H]-estradiol for analytical purposes, special attention should be paid to the calibration, because a difference exists in analyte loss for solutions with and without protein. A similar calibration issue has also been addressed by Yuan et al.[29] Usually SPME-calibration curves are made by measuring the amount of analyte in the fiber at different nominal concentrations of the analyte in buffer solutions (i.e., containing no matrix). This may lead to inaccurate measurements, and it is strongly recommended that the total concentrations of the analyte in the calibration standards be measured. Attention should be paid to the sample volumes used as well, because Górecki et al.[30] have shown that small sample volumes can lead to aggravation of analyte loss by sorption to glass. These workers have therefore advised the use of the same volumes for calibration standards and samples.

The nd-SPME method presented here is intended for radiolabeled compounds. Of course, the use of radioactivity should be prevented whenever possible to prevent the production of radioactive waste. For analyzing endogenous compounds in biological samples, radiolabeling is, of course, also not an option. Other analytical tools such as LC/MS or GC/MS in combination with nd-SPME represent promising techniques in those cases.

However, in complex mixtures containing many different chemicals that are difficult to separate, radiolabeling is still advantageous. Additionally, in the case that the protein studied is not available in pure form, radiolabeled analytes are ideal, because they enable the distinction between specific and nonspecific binding. Last, for competitive binding assays, the use of a radiolabeled compound will always be easiest as well, because one needs to distinguish between binding of the natural ligand and that of the competitor.

It is clear that radiolabeled compounds will remain useful in many cases. The combination with nd-SPME, as presented in this paper, broadens the possibilities in analytical chemistry. Free concentrations and protein binding can now be measured for compounds in very low concentrations, in inseparable mixtures of compounds, and in biological matrixes. As the disposable fibers make it possible to analyze many samples simultaneously, future developments similar to the GC-autosampler would enable the method presented here to be eligible for high-throughput screening, for example in the pharmaceutical industry.

ACKNOWLEDGMENT

We thank Jan C. H. van Eijkeren and Wout Slob of the National Institute for Public Health and the Environment (RIVM) in The Netherlands for their critical comments on this work.

Received for review July 15, 2002. Accepted September 14, 2002.

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