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REVIEW

Binding of low molecular mass compounds to proteins studied by liquid chromatographic techniques

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ABSTRACT: The newest achievements in the application of miscellaneous liquid chromatographic techniques such as size-exclusion, ion-exchange and reversed-phase high-performance liquid chromatography, and thin-layer chromatography for the elucidation of the various aspects of the binding of ligands to proteins are compiled and briefly discussed. Examples of employment in pharmaceutical and clinical chemistry, drug design, enzyme kinetic studies and environmental protection are presented.

KEYWORDS: ion-exchange chromatography; reversed-phase high-performance liquid chromatography; size-exclusion chromatography; thin-layer chromatography

INTRODUCTION

Interactions between bioactive compounds cause the modification of the structure of the interacting species, frequently resulting in modified biological activity as well. Interactions play a considerable role in many important biological processes such as the regulation of muscle contraction (Medley *et al.*, 1992), change of enzymatic activity (Kauzmann, 1959; Trias and Nikaido, 1990), peptide-MHC recognition (Bergethon and Simons, 1990), virus infection (Ando *et al.*, 1993), binding of bioactive peptide to receptors (Boehncke *et al.*, 1993), determining peptide configuration (Madden *et al.*, 1992), etc. Interactions between amino acids, peptides and proteins have paramount importance in many biochemical, biological and biotechnological processes. They influence protein structure (Spadaccini *et al.*, 2001), alter biological efficacy (Chen *et al.*, 2000), and enzyme activity (Fulop *et al.*, 2001), participate in ligand recognition (Cordier *et al.*, 2000), modulate the efficacy of active sites of proteinase (Strisovsky *et al.*, 2000), etc.

The character of such types of interactions has been vigorously discussed, the conclusions highly depending on the chemical structure of the interacting molecules. Thus, the involvement of electrostatic forces (hydrogen bonds) in the interaction of amino acids (Cox, 2000), in the determination of multiple turns in proteins (Guruprasad *et al.*, 2000), in the modification of protein (Arnold and Oldfield, 2000) and peptide structure (Kasim and Swenson, 2000), in the aggregate formation of peptides (Mong *et al.*, 2001), in protein–protein interaction (Tanahashi and Tabira, 2000; Matern *et al.*, 2000) and in the association of proteins (Wang *et al.*, 2000) has been demonstrated. Furthermore, the interaction between the aromatic ring of phenyalanine side chain has been observed (Gorbitz, 2000). The simultaneous occurrence of hydrophobic binding forces and hydrogen bonds has also been reported (De Beer *et al.*, 2000; Wu *et al.*, 2000). It has been illustrated that both hydrophobic and electrostatic interactions may influence peptide–protein binding (Liu *et al.*, 2000) and protein–protein associations (Nusrat *et al.*, 2000).

In recent decades a great number of biochemical and biophysical methods have been applied to study the molecular interactions between various molecules such as peptides (Garone and Steiner, 1990), proteins (Cserháti and Szögyi, 1995), and amino acids (Soltés *et al.*, 1985). Because of the high separation capacity and sensitivity chromatographic methods have been frequently applied in the determination of molecular interactions (Cserháti and Valkó, 1993). These techniques have been some advantages: they are rapid and

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Abbreviations used: Aβ, amiloid β-peptide; AChe, acetylcholinesterase; ACN, acetonitrile; AOT, sulfonic acid *bis*-(2-ethylhexyl) ester; AP50, medium chain μ2 subunit of the clathrin-associated adapter protein complex 2; CaM KII, Ca^{2+}–calmodulin-dependent protein kinase II; CoA, coenzyme; DEAE, diethylaminoethyl; DTT, dithiothreitol; FABP, fatty acid binding protein; GSH, glutathione [*N*-(*N*-L-τ-glutamyl-L-cysteinyl)glycine]; GTPτS, guanosine 5′-(3-*O*-thio)-triphosphate; HSA, human serum albumin; MCP-1, monocyte chemoattractant protein-1; ODS, octadecyl bonded silica; SEC, size-exclusion chromatography; rSPR, rat sepiapterin reductase; TFA, trifluoroacetic acid; TTR, transthyretin.

the compounds to be studied need not to be very pure because their impurities separate under the chromatographic process.

The objectives of this review were the compilation of the newest results obtained in the study of the interaction of proteins with different low molecular mass solutes using various liquid chromatographic techniques such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) and the critical evaluation of the advantages and disadvantages of the methods.

BIOCHEMISTRY AND BIOPHYSICS

Numerous liquid chromatographic procedures have been developed and successfully applied for the elucidation of the biochemical and biophysical aspects of the interaction of proteins with other compounds. These techniques have been employed for the prepurification of the interacting proteins or other molecules the complex being characterized by other methods, for the separation and identification of the products of interaction, and for the assessment of the molecular mass of these complexes. Because of the large molecular mass of proteins involved in the investigations, the overwhelming majority of separation procedures consist of size-exclusion (SEC) or ion-exchange chromatography. Reversed-phase HPLC (RP-HPLC) techniques have also been used, mainly for the separation of free and bonded ligands. The complexity of the potein–ligand interaction frequently requires the use of more than one chromatographic method for the elucidation of the various aspects of interaction. Because of the high water-solubility of protein complexes the overwhelming majority of measurements have been carried out in aqueous mobile phases (reversed-phase separation mode).

Size-exclusion gel chromatography

The application of SEC for the study of antibody–antigen binding has been recently reviewed (Sanny, 2002). Combined chromatographic methods including SEC have been employed for the purification and characterization of nuclear type II [^{3}H] estradiol binding sites from rat uterus. Binding sites were solubilized from rat uterine nuclei, and digested with DNAase and RNAase. Prepurification was performed on single-stranded calf thymus DNA cellulose column equilibrated in 50 mM Tris, 1.5 mM EDTA and 0.1 M NaCl (pH 8.0). Fractions were eluted with a NaCl gradient from 0.1 to 2 M, collected and tested for type II binding activity. Active fractions were further separated by affinity chromatography using 2,6-*bis* [(3-methoxy-4-hydroxyphenyl) methylene]-cyclo-hexanone ligand bonded to epoxy sepharose as stationary phase. Fractions were eluted with a gradient of guanidine hydrochloride (0.1–3.0 M) and tested again for type II binding activity. Size exclusion HPLC was applied for the measurement of the approximate molecular mass of type II binding site. Elution was carried out isocratically on a TosoHaas G3000PW$_{XL}$ column (300 × 7.8 mm i.d.; particle size, 4 μm) using 30 mM Tris, 0.4 M ammonium sulfate, pH 7.4). The results suggested that the molecular mass of the binding site is between 10 and 15 kDa (Markarevich *et al.*, 2001). The separation and purity tests of the medium chain μ2 subunit (AP50) of the clathrin-associated adapter protein complex 2 for the study of its interaction with cytotoxic T-lymphocyte antigen 4 have been also carried out by HPLC. Analysis was carried out on a Superdex 75 column, the isocratic mobile phase being 50 mM Tris–HCl (pH 7.9), 0.5 M NaCl and 1 mM dithiothreitol. Fractions were detected at 280 nm. It was concluded from the measurements that a large hydrophobic residue is required for the binding but other residues are also important in the interaction (Follows *et al.*, 2001).

SEC has found application in the study of the effect of multimerization of monocyte chemoattractant protein-1 (MCP-1) on its capacity to interact with glycosaminoglycans. The state of multimerization was assessed on a Superdex 75 column using a Hanks balanced salt solution, 10 mM Hepes at pH 7.4. Analytes were detected at 280 nm. The measurements illustrated that multimerization is not a prerequisite for the biological activity of MCP-1 (Ali *et al.*, 2001). Native talin, vinculin and actin were also purified by SEC using a Superdex-200 column for the study of their interaction (Bass *et al.*, 2002). The insulin-free and insulin-bound human insulin receptor ectodomain and recombinant membrane-anchored ectodomain complexes were studied by gel chromatography using 50 mM Tris–HCl–150 mM NaCl–0.1% Triton X-100 mobile phase (pH 7.4) and the binding of the recombinant membrane-anchored ectodomain to insulin was demonstrated (Flörke *et al.*, 2001). The binding of the yeast Hsp-40 Sis1 heat-shock protein to the yeast Hsp70 Ssal protein has been demonstrated by using gel filtration chromatography. A Superdex 200 column was equilibrated with 10 mM Tris buffer (pH 7.9)–50 mM NaCl and the elution of individual proteins and their mixture was performed in the same buffer at a flow-rate of 1 mL/min. The decreased retention time of complex indicated direct interaction. Because the complex formation cannot be assessed in buffers of higher inoic strength, the polar character of the binding has been supposed (Quian *et al.*, 2002).

Gel filtration chromatography has been used for the examination of the complex formation between the GTPase domains of *Thermus aquaticus* Ffh and FtsY. Mixtures of Ffh and FtsY were incubated under various conditions and the reaction mixtures were eluted on a Superdex 200 HR 10/30 column equilibrated with 50 mM HEPES (pH 7.5), 2 mM $MgCl_2$ and 50 mM NaCl. Changes in the retention time demonstrated complex

formation. The dissociation of the complex was monitored on an anion-exchange column using a linear gradient of 1 M NaCl in 50 mM Tris (pH 8.0). Analytes were detected in both chromatographic systems at 280 nm. The measurements indicated that the dissociation of the complex was fairly slow (Shepotinovskaya and Freymann, 2002).

SEC unambiguously proved the existence of direct interaction between plasminogen and a bacterial lipopeptide compound, surfactin C. Measurements were performed on a TSK-Gel G-3000SW column (600 × 7.5 mM i.d.) using 50 mM Tris–HCl and 100 mM NaCl with 0.001% Zwittergent as mobile phase. The column was not thermostated. The flow-rate was 1 mL/min and proteins were detected at 280 nm. The retention time of plasminogen decreased in the presence of surfactin C (Kikuchi and Hasumi, 2002).

A combined method consisting of spin SEC followed by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy has been developed for the rapid screening of small molecules for their capacity to interact with target proteins. Sephadex G25 and polyacrylamide Bio-Gel P6 column were used in the experiments. Mixtures of proteins and interacting compounds were incubated and the reaction mixtures were eluted using centrifugation. The bonded compounds were identified by MS, and the structure of the complex was elucidated by NMR. The method has been proposed for drug discovery and design programs (Moy *et al.*, 2001).

Ion-exchange chromatography

Both anion exchange and SEC have been used for the study of the binding of linoleic, arachidonic and docosahexanioc acids and their hydroperoxides to partially purified retinal fatty acid binding protein (FABP). Retinal cytosolic proteins were incubated with arachidonic acid and with arachidonic acid hydroperoxide, and the mixtures were separated on a Sephadex G75 (400 × 30 mm i.d.) at 4°C. Fractions were eluted with 10 mM Tris–HCl buffer (pH 7.4) containing 0.005% NaN_3 at a flow rate of 80 mL/h. Proteins were detected at 280 nm. The low molecular mass fraction showing binding capacity was collected and applied to a DEAE-cellulose column (85 × 15 mm i.d.). FABP was eluted with 30 mM Tris–HCl buffer (pH 8.5) containing 0.3 M NaCl. The flow-rate was 1 mL/min, separation was carried out at 20°C, and FABP was detected at 280 nm. The results indicated that FABP reduces lipid peroxidation on rod outer segment membranes (Guajardo *et al.*, 2002).

Anion-exchange chromatography was employed for the purification of inner-arm dynein species of *Chlamydomonas*. Separation was carried out on a Mono Q column, the mobile phase consisting of 10 mM Hepes, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM EGTA containing 1 mM phenylmethylsulfonyl fluoride. Analytes were eluted with a KCl gradient and monitored at 280 nm. It was established that actin associates with the light chains in *Chlamydomonas* flagellar inner-arm dyneins (Yanagisawa and Kamiya, 2001).

Purification of guanosine 5′-(3-*O*-thio) triphosphate (GTPτS) binding proteins from membranes of porcine brain has been performed on a DEAE column. Proteins were separated by a gradient of NaCl (0–2 M) dissolved in 20 mM Tris–HCl, 1 mM EDTA and 1 mM DTT, pH 8). Solutes were detected at 280 nm (Bavec *et al.*, 2000).

Reversed-phase high-performance liquid chromatography

The elucidation of influence of the length of the fatty acid bonded to the Ca^{2+}-binding protein neurocalcin on its capacity to interact with membranes has also applied liquid chromatographic methods for the control of the purity of preparation. Bovine neurocalcin δ was acylated with lauryl, myristoil and palmitoyl acids in the presence of sulfonic acid bis [2-ethylhexyl ester (AOT). The residues of AOT in the protein preparation was checked with TLC, and the purity of proteins was determined by RP-HPLC using a C_{18} grafted silica column. Proteins were detected at 280 nm. It was established that each protein preparation binds to phospholipid vesicles in a calcium-dependent manner, proving that the binding is not restricted to myristate (Béven *et al.*, 2001).

The peptide profile of the protein interacting with the intracellular part of the β_5 integrin receptor subunit was determined by RP-HPLC after trypsin digestion. Separation was performed on a C_2/C_{18} column using gradient elution (0–80% aceto-nitrile in 0.1% TFA). Peptides were detected at 214 nm. RP-HPLC facilitated the indentification of the protein as bovine annexin-V (Andersen *et al.*, 2002).

The iron-binding state of human serum transferrins was studied by HPLC using a stationary phase consisting of *N*-methylpyridinium polymer crosslinked with ethylene glycol dimethacrylate. Transferrins were eluted from the column (250 × 4 mm i.d.) with a 30 min linear gradient from 5 to 30% B. Mobile phase A was 0.05 M Tris–acetic acid (pH 7.0), and mobile phase B was mobile phase A containing 0.5 M sodium acetate. The flow-rate was 0.5 mL/min and the detection wavelength was 280 nm. Monoferric transferrin bound to the C-site, and holotransferrin, apotransferrin and monoferric transferrin bound to the N-site were well separated. It was stated that the method is suitable for the easy detection and separation of iron binding protein fractions in human sera (Harada *et al.*, 2002).

RP-HPLC has been employed for the analysis of inhibitors of the mammalian peptide transporter PEPT1 in extracellular uptake medium, flux studies and in cells. Inhibitors (derivatives of the dipeptide H–Lys–Pro–OH) were analyzed on an ODS column (125 × 4.5 mm i.d.)

with the mobile phase 30% acetonitrile–0.1% TFA. The flow-rate was 0.8 mL/min, and peptide derivatives were monitored at 220 nm. The results indicated that these compounds bind to the transporter and inhibit its function (Knütter *et al.*, 2001).

Another RP-HPLC technique has been used for the purification of biologically active human progastrin$_{6–80}$ prior to study its capacity to bind calcium ion. Purification was performed on an ODS column (100 × 8 mm i.d.) using gradient elution (20–50% acetonitrile in 50 mM ammonium bicarbonate). The flow-rate was 1 mL/min, and solutes were detected at 214 nm (Baldwin *et al.*, 2001).

An RP-HPLC technique has been developed for the evaluation of the binding selectivity of 15 transthyretin (TTR) amiloid fibril inhibitors in blood plasma. TTR incubated with nonsteroidal anti-inflammatory drugs and other amyloid inhibitors was analysed on an ODS column. TTR and small interactive compounds were separated with different gradients containing acetonitrile and water in 0.2% TFA. Solutes were monitored at 280 nm and by MS. The method was proposed for the determination of the binding selectivity of small molecules to any protein in biological fluids (Purkey *et al.*, 2001).

RP-HPLC has been used for the separation and identification of the glutathione (GSH) conjugate of the reactive metabolite of terbinafine 7,7-dimethylhept-2-ene-4-ynal. Separation was performed on an ODS column (100 × 2 mm i.d.; particle size, 5 μm). Initial mobile phase composition was 5% aqueous methanol in 1% of acetic acid and 2 mM ammonium acetate. The concentration of methanol was increased to 35% in 2 min, held for 15 min then increased again to 60% methanol in 5 min, and held for 10 min. The flow-rate was 0.2 mL/min, and solutes were monitored by MS. The unbonded metabolite and the GSH conjugate were well separated under these chromatographic conditions (Iverson and Uetrecht, 2001).

The interaction of 16 peptides with the corn protein zein has been studied by RP-HPLC using zein-coated silica stationary phase. Peptides were eluted from a column of 150 × 4 mm i.d. with bidistilled water and were detected at 210 nm. Peptides eluted with different retention times, proving that the strength of peptide–zein interaction markedly depends on the chemical structure of the peptide. Calculations indicated that lipophilicity and steric correspondence account for the strength of protein–peptide interaction (Szikszay and Forgács, 1997).

Thin-layer chromatography

A combined high performance TLC–gas chromatographic technique has been developed for the analysis of the monosialoganglioside GM3 in human lymphocytes and its association with the phosphorylation protein Zap-70 was assessed by immunofluorescence and scanning confocal microscopy (Garofalo *et al.*, 2002).

Charge transfer reversed-phase TLC (RP-TLC) has been employed for the elucidation of the role of various physicochemical parameters on the binding of some free amino acids and peptides to bovine serum albumin. L-amino acids (Ala, Gly, Leu, Phe, Tyr, Val) and L-dipeptides (Met–Ala, Met–Gly, Met–Leu, Met–Phe, Met–Tyr, Met–Val) were spotted onto ready made RP-TLC plates (RP-18W/UV$_{254}$) and the plates were developed with water containing bovine serum albumin in the concentration range of 0–40 mg/mL. After development analytes were detected by the ninhydrin reagent. The slope of the plot retention vs serum concentration was considered as quantitative indicator of the strength of protein–amino acid and protein–dipeptide interaction. It was found that only Ala, Leu, Phe and Met-Tyr interacted with the bovine serum albumin and the strength of interaction significantly depended on the bulkiness of the interacting amino acids and peptide (Cserháti and Forgács, 2000).

A similar method has been employed for the assessment of the binding of eight commercial anticancer drugs to human serum albumin (HSA) and the effect of acidic (0.16 M of acetic acid), basic (0.16 M of sodium acetate) and ionic environments (0.16 M of NaCl and $CaCl_2$) on the strength of interaction was determined. The concentration range of HSA was 0–50 mg/mL, and the anticancer drugs were monitored by UV absorption and with iodine vapour. It was established that drugs readily bind to HSA the strength of interaction depending on the chemical structure of the drug and on both the pH and ionic environment. Calculations indicated that the sterical parameters of drugs have a considerable impact on their capacity to bind to HSA (Cserháti and Forgács, 1995a).

The association of 13 antibiotics with HSA has been studied by applying the same chromatographic procedure. Principal component analysis of the retention data suggested that the strength of interaction markedly depended on the chemical structure of the antibiotic, the pH and the type of ion present in the mobile phase (Cserháti and Forgács, 1997).

Other liquid chromatographic techniques

Two nuclear high-mobility-group proteins and a sulfoglucuronyl carbohydrate binding protein were purified by heparine-sepharose and sulfatide-octyl-sepharose chromatography prior the investigation of their capacity to bind sulfated glycolipids, sulfoglucuronylglycolipids, sulfatide and seminolipid. Heparin-sepharose chromatography was carried out on 2 mL of stationary phase equilibrated with 50 mM Tris–HCl (pH 8.5). After injection the protein mixture the column was washed with 20 bed volume of equilibrating buffer and proteins were eluted with 0.7 M NaCl in the same buffer. The sulfatide–octyl–sepharose chromatographic step used

1 mL of stationary phase in 50 mM Tris–HCl (pH 7.2). Solutes were eluted with 0.25 M NaCl in the buffer. Purity check of proteins was performed on an ODS column (250 × 1 mm i.d.; particle size 3.5 μm) using gradient elution (from 0.1% aqueous TFA to 65% acetonitrile in 0.1% TFA in 70 min). The flow rate was 60 μL/min, proteins were detected by mass spectrometry (Chou *et al.*, 2001).

Heparin agarose chromatography has been used for the purification of chloroplast proteins (37–38 kDa) from barley. Protein fractions precipitated by various concentrations of ammonium sulfate were separated on heparin–agarose columns of 2.5 mL. Columns were washed with five column volumes of buffer (10 mM Hepes–KOH pH 8, 40 mM KCl, 10 mM $MgCl_2$, 0.05 mM EDTA, 1 mM DTT, 8.5% glycerol), and proteins were removed by 0.2 and 0.5 M KCl dissolved in the same buffer. The binding of these purified proteins to the *in vitro* transcribed chloroplast *psb*A mRNA was proved by UV cross-linking assay and SDS-PAGE electrophoresis (Memon and Aktoprakligil, 2002).

STUDIES ON ENZYME KINETICS

Various liquid chromatographic methods have frequently been used in the study of numerous enzyme kinetic processes. Thus, the mechanism of phosphorylation of rat sepiapterin reductase (rSPR) by Ca^{2+}–calmodulin-dependent protein kinase II (CaM KII) was assessed by RP-HPLC and TLC. After the phosphorylation step rSPR was digested with the endoprotease Lys-C and the hydrolysis products were separated on a Sephasil protein column (250 × 4.6 mm i.d.) using a linear gradient of 0–72 vol% ACN in 0.6% TFA. The flow rate was 1 mL/min, and peptides were detected at 215 nm. Phosphorylated (τ-^{32}P) amino acids were separated by TLC. The results proved that only serine residues in rSPR were phosphorylated (Fujimoto *et al.*, 2002).

Hydroxylapatite column was used for the isolation of native and modified lecithin:colesterol acyltransferase and the peptides resulting form their trypsin digestion were separated by RP-HPLC. The hydroxylapatite column was washed with 1 mM phosphate buffer, proteins were eluted with 6 mM phopshate buffer (pH 6.8) followed by 39.2 mM phosphate buffer (pH 7.2). Proteins were detected at 280 nm. RP-HPLC analysis of peptides were carried out on an ODS column (250 × 4.6 mm i.d.) using gradient elution (acetonitrile in 0.1% TFA). The flow-rate was 0.4 mL/min, detection wavelength was 210 nm. The purified enzyme preparations were employed for the study of their capacity to bind the oxidation products of low-density lipoprotein. The binding required the availability of the sulhydryl group of cystein residue and the hydroxyl group of serine residue(Howlader *et al.*, 2002).

Acyl-CoA thioesters released from human glutaryl–CoA dehydrogenase were separated by RP-HPLC in the study of the binding, hydration and decarboxylation of the reaction intermediate glutaconyl–CoA by human glutaryl-CoA dehyrogenase. Analysis was performed on an ODS column (250 × 4.5 mm i.d.). Gradient elution was carried out with 0.1 M potassium phosphate (pH 5.0) and acetonitrile, and analytes were monitored at 260 nm. The elution order was: CoASH, glutaconyl–CoA, glutaconyl–CoA *trans*-3-isomer, acetyl–CoA, glutaryl–CoA, 3-hydroxyglutaryl–CoA, 3-hydroxybutyryl–CoA, flavin adenine dinucleotide, and crotonyl–CoA (Westover *et al.*, 2001).

RP-HPLC was employed for the assessment of the diversity in the mechanism of substrate oxidation by cytochrome P450 2D6. The oxidation products of the β-adrenergic blocker metoprolol {1-[4-(2-methoxyethyl) amino]-2-propanol} were separated on an ODS column (150 × 4.6 mm i.d., paricle size, 5 μm) using 20 mM $NaClO_4$ (pH 2.5)–acetonitrile (3:2, v/v) as mobile phase. The flow-rate was 2 mL/min and solutes were detected fluorimetrically excitation and emmission wavelengths being 222 and 300 nm, respectively. Bufuralol oxidation products were analyzed on an ODS column (250 × 4.6 mm i.d., paricle size, 5 μm) using a linear gradient. Initial composition of the mobile phase was 50% 20 mM $NaClO_4$ (pH 2.5)–acetonitrile (9:1, v/v; buffer A) and 50% 20 mM $NaClO_4$ (pH 2.5)–acetonitrile (3:2, v/v; buffer B). The ratio of B was enhanced to 100% in 8 min and held constant for 5 min. The flow-rate was 2 mL/min and solutes were monitored at 254 nm. It was concluded from the data that oxidation follows a similar but different chemical mechanism (Hanna *et al.*, 2001).

The strength of interaction between the heme prothetic group and various P450 enzymes (mammalian CYP4B, CYP4F and CYP4A subfamilies) has been determined by RP-HPLC. Analyses were performed on a POROS R2 column (150 × 2.1 mm i.d.). Elution began with 20% acetonitrile in 0.05% TFA at a flow-rate of 1 mL/min, after 3 min it was decrease to 0.2 mL/min and the concentration of acetonitrile was enhanced to 85% in 20 min. Polar heme and free heme eluted at about 10 and 12 min, respectively; the retention time of proteins was 14–16 min. Detection wavelengths were 214 (protein) and 400 nm (heme). Proteins with strong binding capacity eluted together with the heme while weaker complexes were entirely dissociated (Henne *et al.*, 2001).

The association of the acetylcholinesterase (AChe) derived peptides of *Torpedo california* with the amiloid β-peptide (Aβ) has been determined by RP-HPLC. Peptides alone and their mixtures were eluted from an ODS column (150 × 3.9 mm i.d.) with a linear gradient of 0–80% (v/v) acetonitrile in 0.1% TFA in 50 min. Solutes were detected at 214 nm. The data proved the complex formation and indicated that hydrophobic binding forces are involved in the interaction (De Ferrari *et al.*, 2001).

The biodegradation rate of debrisoquine [3,4-dihydro-2(1H)-isoquinolinecarboximidamide], an antihypertensive by cytochromes P450, was measured in the presence of human serum albumin, α1-acid glycoprotein, and α- and τ-globulins. The 4-hydroxylation of debrisoquine was followed by a radio-TLC method. Analyses were carried out on silica gel plates using chloroform–methanol (100:1, v.v) mobile phase. The binding of debrosoquine to proteins was measured with an ultrafiltration method. It was established that the inhibitory effect of proteins cannot be explained by their capacity to bind the antihypertensive, it was supposed that plasma proteins interact with the enzyme (Ishii *et al.*, 2001).

RP-HPLC was employed for the study of interactions between the apo form of horse cytochrome *c*, its fragments and heme in the presence and absence of proteinase K. Measurements were carried out on an ODS column (150 × 4.6 mm i.d.) using gradient elution (5–50%, v/v acetonitrile in 0.05% TFA in 30 min). Solutes were monitored at 226 nm. The results suggested that protein interactions may lead to conformational changes of the interacting protein components (Spolaore *et al.*, 2001).

BINDING OF ENVIRONMENTAL POLLUTANTS

Because of their wide spread applications in various industrial processes (Hankey *et al.*, 2001), phenol and ring-substituted phenol derivatives are of potential environmental pollutants occurring in wastewater (Gonzalez *et al.*, 2001), environmental waters (Mishra *et al.*, 2001), and soils (Bastos *et al.*, 2001). Phenols can exert manyfold toxic effects (Powley and Carlson, 2001), they impair the viability of primary colonic epithelial cells (Pedersen *et al.*, 2002), are hematotoxic (Van Den Heuvel *et al.*, 2001), and influence enzyme production (Pessione *et al.*, 2001). The noxious effect of phenols is mainly attributed to their binding to proteins; however, their interaction with DNA (Skarfstad *et al.*, 2000) has also been demonstrated. The association of phenols with bovine serum albumin (Bartolome *et al.*, 2000), and arginase (Legaz *et al.*, 2001) has recently been established.

The character of the binding forces involved in the interaction of phenols with proteins has been vigorously investigated. Because of the high variety of proteins and phenols the results considerable depended on the protein–phenol pair under examination. It has been established that ionic bonding is predominant in the binding of phenolic compounds to canola proteins (Xu and Diosady, 2000), and hydrogen bonds play a decisive role in the binding of phenolic molecules to horseradish peroxidase (Henriksen *et al.*, 1999).

The binding of 22 ring-substituted phenol and 23 ring-substituted aniline derivatives to the wheat protein gliadin was determined using various amount of gliadin physicosorbed on silica surface as stationary phases. Bidistilled water was used as mobile phase and the environmental pollutants were monitored at 254 nm. It was assumed that higher retention time indicates a higher affinity of solutes to the protein, and it can be employed as a quantitative indicator of the strength of the binding of pollutants to gliadin. It was established that the strength of interaction considerably depended on the chemical structure of the solute the binding being stronger with higher number of substituents and with the higher amount of physicosorbed gliadin in the stationary phase. Principal component analysis suggested that the sterical parameters exert the highest influence on the strength of interaction and the role of polarity character of the pollutants is of secondary importance (Cserháti *et al.*, 1999).

A similar method has been employed for the measurement of the interaction 12 ring-substituted phenol, three aminophenol and four ring-substituted aniline derivatives to the corn protein zein physicosorbed on silica and alumina supports in 2.5 and 10% (w/w). As in the previous experiments bidistilled water was used as mobile phase and the pollutants were detected at 254 nm. The data demonstrated that the steric and electronic parameters of solutes strongly influence the strength of interaction while the effect of hydrophobic interactive forces is negligible (Cserháti *et al.*, 2001a).

The interaction of 12 phenoxyacetic acid derivatives with human and bovine serum albumin and with egg albumin was also elucidated by using charge transfer chromatography carried out on ready-made RP-TLC plates. The concentration of albumins in the aqueous mobile phase varied between 0 and 49 mg/mL, and solutes were detected by their UV spectra. It was established that phenoxyacetic acid derivatives do not bind to egg albumin and the lipophilicity influences their affinity to bind to serum albumins. It was concluded from the results that the interaction of these herbicides with serum albumins involves hydrophobic binding forces occurring between the amino acid side chains of proteins and the apolar substructures of these herbicides (Cserháti *et al.*, 2001b).

The non-covalent binding of 28 other commercial pesticides to human and bovine serum albumin and to egg albumin was also investigated using the same charge transfer chromatographic procedure the concentration of albumins in the aqueous mobile phase being between 0 and 1 mM. Pesticides were detected by their UV spectra. It was found that only the herbicide fuberidazole [2-(furyl)benzimidazole] showed significant interaction with egg albumin and the majority of other pesticides bonded readily to serum albumins. Similarly to the behavior of phenoxy acetic acid derivatives, stepwise regresssion analysis proved that the association of pesticides is also governed by their lipophilicity (Cserháti and Forgács, 1995b).

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