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Review

High-performance liquid chromatography packing materials for the analysis of small molecules in biological matrices by direct injection

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ABSTRACT

The increasing demand on high-performance liquid chromatography to resolve mixtures of closely related components in complex biological matrices in less time with higher precision has led to the development of a variety of new high-performance liquid chromatography columns, which eliminate the need for sample preparation. These packings isolate small molecules from biological macromolecules on direct sample injection by exerting two separation mechanisms. They allow elution of all sample macromolecules with high recovery in one peak at the extraparticulate void, because of size-exclusion interactions with hydrophilic outer particulate surfaces. Simultaneously, these packings allow permeation and partitioning of small molecules on bonded-phases which are protected from contamination by macromolecules. The names given to these new packings include "internal surface reversed-phase", "shielded hydrophobic phase", "semipermeable surface", "dual zone material" and "mixed-functional phases". The fundamental principles behind each of the design concepts are reviewed, and applications are cited.

CONTENTS

1. INTRODUCTION

The isolation and quantification of small molecules from complex macromolecular matrices has presented significant challenges to the field of liquid

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chromatography. In particular, the analysis of drugs, metabolites and peptides in biological fluids has historically posed problems because of the need to remove proteins prior to high-performance liquid chromatography (HPLC) in order to avoid damage to chromatographic columns. Various approaches have been taken to deal with unwanted sample proteins. Conventional sample preparation procedures involve precipitation of proteins, followed by extraction and preconcentration of analytes. In many cases, sample preparation is disadvantageous because of labile components, low extraction yields, difficulties with reproducibility, and consumption of analysis time. In order to streamline sample preparation, many automated schemes have been developed. Another approach has been to eliminate sample preparation entirely by developing methods which allow direct sample injection [1–3]. These direct injection techniques have included the use of column switching, the use of micellar mobile phases with conventional reversed-phase columns, or the use of HPLC columns which are specifically designed to partition small molecules but elute proteins in one peak with high recovery.

The development of HPLC packing materials for the direct injection of biological samples is the subject of this review. The packing concepts described are quite unique in their ability to remain innocuous to proteins, while separating small molecules with high resolution. These HPLC packings protect partitioning bonded phases from protein contamination by preventing access to the bonded phases through size-exclusion mechanisms which allow proteins to interact only with hydrophilic, non-adsorptive layers on the outer packing surfaces. Small molecules, on the other hand, penetrate the porous silica packings and gain full access to partitioning phases. Small analytes are dynamically extracted from directly injected samples and separated from matrix macromolecular components. The terms associated with the new packings, designed to act in this fashion, included "internal phase", "internal surface", "shielded", "dual-zone", "semipermeable", and "mixed-functional". These packing materials have also been referred to as "restricted access media", because the packings are so designed to prevent the macromolecules for accessing the bonded phase. The "restricted access" term, however, also refers to packing material produced with hydrophilically coated bonded phases on large-pore silica, where macromolecules are not completely size-excluded. Reviewed here are only those packing designs which utilized small pore silica supports with hydrophilic outer surfaces and partitioning inner surfaces, thus the packings function in two modes: (i) complete non-adsorptive size-exclusion of macromolecules and (ii) dynamic partitioning of small molecules. Because of the dual mechanisms of these packings, they have been called "bimodal" [4]. These packings also fall into a more general classification now referred to by many as "mixed-mode" packings. This latter term has been associated to any HPLC column with a packing which is designed to concomitantly function with more than one separation mechanism (*i.e.*, size-exclusion, reversed-phase, ion-exchange, etc.).

HPLC packing materials, designed for the assay of small molecules in biological samples by direct injection, should accommodate the resolution of small analytes from all species with the elution and complete recovery of all macromolecules. Ideally, such a packing should possess the following characteristics.

(1) The partitioning bonded-phase should be completely protected from irreversible contamination by macromolecules (*e.g.*, proteins, nucleic acids, etc.).

(2) The packing support should be rendered non-adsorptive to macromolecules,

and macromolecules should be recovered from a column in excess of 98% under chromatographic conditions suitable for separation and recovery of small molecules.

(3) The packing should enable the elution of unwanted macromolecules in one peak, away from the small analytes of interest.

(4) The packing should allow partitioning of small molecules with the protected bonded phase with good diffusional mass transport.

(5) The bonded-phase should have sufficiently high selectivity or efficiency to resolve analytes from related compounds or interfering endogenous small molecules.

(6) The bonded-phase strength should be high enough to retain the analytes of interest, but low enough to allow the use of mobile phases that will not induce the coalescence of sample macromolecules.

Since the characteristics of an HPLC packing designed for direct sample injection will be governed in part by the nature of the sample matrix and the analytes being quantified, it is clear that many such packings could be developed to meet particular application needs. Currently, the driving force behind the development of these columns has been the HPLC analysis of drugs in blood serum or plasma by direct injection for use in therapeutic drug monitoring [4]. However, these specialty columns have also been used to assay for endogenous metabolites in blood serum [5], to quantify peptide toxins in cyanobacterium [6], to determine extraneous substances in human salvia [7], to study exogenous molecules in insect or crustacean hemolymph [8], and to conduct protein-binding studies [9–15]. Conceivably, other applications could broaden the use of these types of columns. Such uses might include the purification of biotechnology products, the isolation of components from fermentation broths, the assay of therapeutic or endogenous species in other biological fluids, the analysis of contaminates in food products, or the isolation of small molecules from nucleic acid mixtures.

In order to contribute to a better understanding of this growing area of HPLC packing development, six different design concepts are reviewed. With any of these packings, when a untreated sample is injected on-column, the macromolecules (*e.g.*, proteins, etc.) are excluded and eluted in the column interstitial void, while the small molecules are dynamically extracted and separated because of their ability to penetrate the packing particulates and interact with a partitioning phase. The six design concepts reviewed here include the protein-coated ODS phases, the internal surface reversed-phase (ISRP) supports, the shielded hydrophobic phases (SHP), the semipermeable surface (SPS) phases, the dual zone material (DZM) and the mixed-functional phases (MFP). Each is presented in chronological order. Particular emphasis is given to ISRP packings. The ISRP columns were the first commercial columns specifically designed for the analysis of substances in blood serum or plasma by direct injection. The greatest amount of data has been published on the use of the ISRP columns. Only limited information is available on the other design concepts.

2. PROTEIN-COATED ODS COLUMNS

Historically, the predominate means of assaying for drugs in biological fluids has been by reversed-phase HPLC. To facilitate more rapid analysis early investigators simply injected untreated plasma samples onto reversed-phase columns. Under conditions typical of reversed-phase separations with octadecylsilane (ODS) columns,

plasma proteins denatured on the alkyl bonded-phases, accumulated in the interstitial packing space, and clogged the columns. To circumvent these difficulties Yoshida and co-workers [16,17], attempted to coat the outer surfaces of large particulate (20–30 μm) ODS silica, with 120 Å pore diameters, by purging packed ODS columns with bovine serum albumin (BSA) or rabbit plasma at pH 3 in methanol. Subsequently, the columns were washed repeatedly with methanol to remove denatured, unabsorbed protein. Presumably, the protein, denatured by the methanol, coated the external surfaces of the ODS silica, and provided a means of attenuating the adsorption of injected sample proteins, while allowing small drug analytes to penetrate the pores. The access of the internal ODS bonded phase was restricted to small molecules. Although this concept was applied to the direct injection of plasma samples onto protein-coated 5 μm diameter ODS packings, the small particulate columns exhibited considerable decrease in efficiencies and were short lived [18]. The protein-coated ODS packings have been most successful with precolumn deproteinazation in coordination with column switching [19,20].

3. INTERNAL SURFACE REVERSED-PHASE COLUMNS

In 1985 Hagestam and Pinkerton [21] introduced the ISRP concept. The ISRP packings were specifically designed for the analysis of drugs in blood serum or plasma by direct injection. High-performance ISRP packings are produced by first bonding a high coverage (300 μmol/g) hydrophilic phase to small pore (80 Å), 5-μm diameter silica [22]. This can be done with either glycerylpropyl (diol) groups [21,22] or aminopropyl groups, capped with glycidol [23]. To these hydrophilic layers are attached various partitioning phases. The bulk packing is then treated with enzymes to remove the partitioning moieties only from the outer surfaces of the packing particulates. The enzymes cannot penetrate the porous supports and reach a partitioning phase inside the silica particulates. Since most of the surface area is inside the packing only a small percentage of capacity is lost (*e.g.* 14%). The physical characterization of ISRP supports is detailed elsewhere [22].

A resulting ISRP packing has a hydrophobic or ion-exchange partitioning phase on the internal surface and a hydrophilic diol phase on the external surface. When a serum sample is directly injected onto an ISRP column, the serum proteins are excluded and not adsorbed, while the small molecules penetrate and undergo partitioning (Fig. 1). A variety of molecular entities have been investigated as potential partitioning phases for ISRP packings. Polypeptide phases include glyceryl-propyl-glycyl-L-phenylalanine (diol-GF) [21], glycerylpropyl-glycyl-L-phenylalanyl-L-phenylalanine (diol-GFF) [21,22], glycerylpropyl-glycyl-L-phenylalanyl-L-phenyl-alanyl-L-phenylalanine (diol-GFFF) [24], which are treated with carboxypeptidase A. Additional investigative ISRP partitioning moieties, which have been bound through carboxyl groups to aminopropyl spacers and cleaved by chymotrypsin, include N-*tert.*-butoxycarbonyl-L-phenylalanine, N-carbobenzoxy-L-valyl-L-phenylalanine, N-acetyl-L-phenylalanine, N-benzoyl-L-phenylalanine and phenylpropionic acid [23,25]. In a similar manner, Haginaka *et al.* [26] has more recently demonstrated the synthesis of an aminopropyl-N-octane phase with cleavage of the external octyl phase using polymyxin acylase. In both of these latter cases, after enzyme cleavage, the residual aminopropyl phase is capped with glycidol to ensure the external surface is

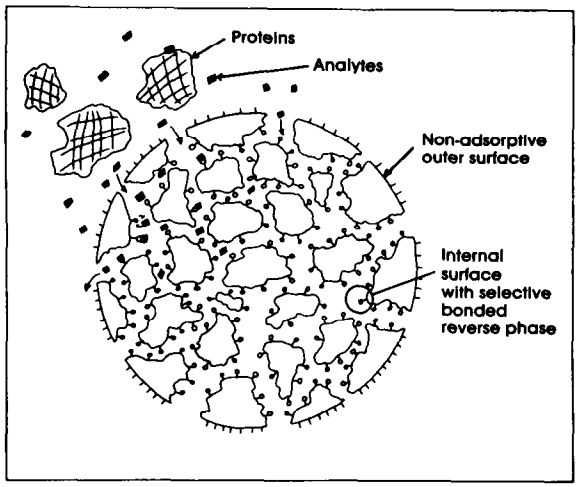

Fig. 1. Schematic of enzyme cleaved, peptide bonded-phase ISRP packing particulate.

hydrophilic and non-adsorptive to proteins. These investigation have demonstrated that many different types of bonded-phase moieties can be attached to silica and enzymatically cleaved from outer surfaces. In view of this, the ISRP concept offers a method unlimited in scope for producing biocompatible bonded-phases with either alkyl, phenyl or ion-exchange partitioning.

The phenyl and alkyl ISRP phases made with the aminopropyl spacer are high capacity, neutral, and exhibit their best protein recovery at a low pH [26]. These neutral ISRP phases are most suitable for very hydrophilic drugs, such as cephaloporins [26]. On the other hand, the diol-GFF phase is lower capacity, negatively charged, and exhibits high protein recovery in slightly acidic or neutral pH [21,22,27]. The non-adsorptivity of each of these ISRP packing designs is due to the high external diol coverage. The protein recovery from diol-GFF, 5 μm packing has been shown to be $99 \pm 3\%$ when injecting human serum into a mobile phase of 0.1 *M* phosphate buffer (pH 6.8) containing less than 16% organic solvent [27].

In contrast to the neutral alkyl phases, the diol-Gly-Phe-Phe (diol-GFF) phase exhibits very high, versatile selectivity. Phenylalanine provides reversed-phase partitioning, while the terminal carboxylate enables weak cation-exchange interactions [28]. The low capacity of the diol-GFF phase also allows hydrophobic analytes to be eluted with small amounts of organic solvent in a mobile phase. For the direct injection of serum or plasma, irrespective of the chromatographic packing, it is advisable to keep the organic content of a mobile phase low (*i.e.*, $< 20\%$ v/v) in order to prevent coalescence of proteins in the mobile phase [27]. Organic solvents which have been used in organic/aqueous mobile phases for ISRP assays have included methanol, isopropanol, *n*-propanol, acetonitrile and tetrahydrofuran.

One ISRP column type, the diol-GFF phase, is commercially available from Regis (Morton Grove, IL, U.S.A.). Currently, this is the most widely used specialty column for the HPLC analysis of drugs in serum or plasma by direct injection. The retention characteristics of hundreds of drug substances and metabolites have been

evaluated on the GFF ISRP columns by investigators worldwide. The columns have been used in clinical laboratories in conjunction with therapeutic drug monitoring, in veterinarian laboratories for abuse drug screening, in agricultural laboratories for assay of toxins in plant samples, in pharmaceutical laboratories for drug metabolism studies, and in research laboratories for fundamental protein binding studies. Some human clinical studies which have used validated ISRP assays include the monitoring of theophylline and caffeine in the serum of 53 patients [29]; the quantification of acetaminophen and salicylate in the serum of 160 patients [30]; and the determination of indoxyl sulfate in uremic serum of 80 patients [31]. In these studies the ISRP assays exhibited precision ranging from 2 to 5% relative standard deviation (R.S.D.), excellent drug recoveries, and good linear correlations with other methods. A typical column efficiency obtainable with an ISRP column is 35 000 plates/m. In one case, the ISRP column performance is reported to have remained unchanged after an excess of 2000 injections of serum diluted 1:10 with internal standard and injected into a 0.1 *M* phosphate buffered (pH 6.8) mobile phase containing 5% methanol [29]. A guard column was routinely replaced after every 400 injections. In addition to column ruggedness, the surprisingly high selectivity of the GFF tripeptide phase has meant a low coincidence of interference from other substances. This phenomena was recently demonstrated with the testing of 76 drugs for potential coelution in an ISRP assay of the theophylline analogs dyphylline and doxoyfylline [32]. None of the drugs were found to interfere with the ISRP assay.

Many other direct injection ISRP assays have been developed and validated for similar uses, such as phenylalanine in human plasma for diagnosis and treatment of phenylketonuria [33]; metronidazole and phenylbutazone in human serum for therapeutic drug monitoring [34]; oxyphenbutazone, phenylbutazone and furosemide in horse plasma [35]; cefpiramide in human plasma [36]; propofol in human plasma [37]; probinecid and lidocane in human plasma [28]; and carbovir (CBV) and its metabolites in mouse plasma and urine [38]. Also using column-switching techniques the ISRP columns have been used in conjunction with chiral BSA or α-acid glycoprotein (AGP) columns to quantify warfarin enantiomers in human serum [39] and in conjunction with β-cyclodextrin columns to assay for dideoxycytidine (DDC) and azidodeoxythymidine (AZT) in infected feline plasma [40].

In addition to the analysis of drug in serum or plasma, the unique selectivity and mixed-mode reversed-phase/ion-exchange partitioning has allowed the peptide bonded-phase ISRP columns to be used in high resolution of peptide mixtures [41]. Also, because of the unique bimodal size-exclusion and small molecule partitioning abilities, the ISRP columns have been used to study protein binding phenomena. In several cases, it has been demonstrated that the "free" and total drug concentrations in serum can be estimated simultaneously with ISRP columns by either direct injection [9–11,42] or frontal [12–14] techniques. Also, ISRP columns have been used with a modified Hummel–Dreyer HPLC method to determine protein binding parameters [15].

Although the diol-GFF peptide bonded-phase ISRP columns have been highly successful for a wide variety of applications, improvements in performance would be advantageous in selected cases. The current diol-GFF columns exhibit capacities and efficiencies lower than theory. As a result, in order to facilitate many applications, three column lengths (*i.e.*, 5, 15 and 25 cm) are commercially available. If packing

capacity and efficiency were increased, one column length would suffice for more uses. Although greater bonded-phase capacity has been demonstrated with ISRP supports synthesized with aminopropyl spacers, these ISRP packings have not been commercialized. However, technological advances have been made in improving the synthesis of the diol-GFF phase to produce a second generation peptide bonded-phase ISRP columns with increased retention and higher efficiencies. It has been demonstrated that the second generation of ISRP columns, called GFFII, exhibit efficiencies of 63 000 plates/m compared to 35 000 plates/m of the first-generation material. A further discussion of this new ISRP packing can be found in this issue [43].

4. SHIELDED HYDROPHOBIC PHASE COLUMNS

In 1988, Gisch and co-workers introduced another means of protecting partitioning phase moieties from protein adsorption with a packing referred to as the shielded hydrophobic phase (SHP) [44,45]. This material was manufactured by synthesizing a hydrophilic polymer through the bonding of polyethylene oxide to porous silica with hydrophobic groups "embedded" in the polymer. In principle, this is purported to create a polyoxyethylene (or polyethylene glycol, PEG) network with a phenyl phase enclaved within the polymer mesh (see Fig. 1 of ref. 44). The hydrophilic PEG network is said to "shield" the hydrophobic phenyl groups, thus preventing proteins from reaching the phenyl partitioning phase. The packing is produced with 5-μm diameter silica with 100 Å pores, so a size-exclusion mechanism prohibits proteins from reaching the inner regions of the packing, where the "embedded" phenyl groups should also be found.

Although only a few articles have been published on this packing, it is commercially available from Supelco as "Hisep SHP". The capacity factors for about 50 drug substances have been measured under mobile phase conditions typically required for direct injection of serum or plasma. The SHP has a high affinity for analytes with phenyl groups such as phenytoin. With an acetonitrile–0.18 *M* ammonium acetate (10:90) mobile phase phenytoin could not be eluted from a 15-cm SHP column [45]. The strong retention necessitated a programmed mobile phase change to pH 2.5 to elute the phenytoin. Although the exact nature of the phenyl partitioning moieties has not been published, their selective discrimination appears to be based mainly on aryl interactions. Resolution of a series of barbiturates required the use of an ion-pairing agent [45]. The high hydrophobic retentive capabilities of the SHP packing could be advantageous in assays of very hydrophilic compounds; however, the greater capacity results in retention of many endogenous components from human serum (see Fig. 2 of ref. 44), which could interfere with lesser retained analytes. The developers indicate that the "bulk of the protein matrix" elutes from the SHP columns, but protein recovery data has not been reported. The performance of the SHP columns for the analysis of drugs in serum or plasma by direct injection will become more evident when the packing is evaluated by users.

5. SEMIPERMEABLE SURFACE COLUMNS

Another concept which also utilizes polyethylene glycol as a hydrophilic layer grew out of work by Desilets and Regnier [46] from research involving the coating of

alkyl bonded-phases with surfactants. Using 300 Å porous silica bound with C_8 and C_{18}, these investigators demonstrated that alkyl bonded-phases could be coated with Tween and Brij non-ionic surfactants. The hydrocarbon tails of these surfactants were believed to associate with the alkyl bonded phases of the packing while the PEG heads provided a layer above the bonded phase that prevented proteins from adsorbing irreversibly to the packing. Since the silica had large pores, proteins were not excluded from the inner reaches of the porous particulates. The serum protein recovery from these surfactant–coated alkyl supports varied according the alkyl phase, the degree of surfactant coating, and the age of the column. As the packing was used with organic–aqueous mobile phases, the surfactant molecules were washed off and the protein recovery, from injected serum samples, decreased. With a surfactant-coated C_8 columns after 44 h of flow with a 0.05-*M* phosphate (pH 6.5)–*n*-propanol (97:3) mobile phase, approximately 55% of the surfactant had been removed and the protein recovery had dropped to 92% [46]. Surfactant-coated C_8 columns were evaluated for retention properties with a series of benzene derivatives. The retention of the solutes with polar functional groups was a function of the degree of surfactant coating, while benzene derivatives with only hydrophobic groups were not affected by the surfactant coating.

As an extension of the surfactant-coating work, similar surfactants were covalently bound to 300 Å pore silica in an attempt to maintain a constant coverage [47]. The covalently bound surfactant alkyl phases exhibited properties similar to the surfactant coated packings having the same ratio of reversed phase to PEG coverage; however, lower than desirable protein recoveries could be achieved.

The concept has been carried further by Glunz *et al.* [48] by covalently bonding PEG with alkyl phases bound to 5-μm diameter silica with 100 Å pores using a modified, proprietary synthesis. A distinct difference here is that size-exclusion of proteins will be invoked because of the small pores. The PEG SPS partitioning phases created in this fashion have included C_4, C_8, C_{18}, CN and phenyl. The SPS phases appear to have efficiencies comparable to conventional alkyl bonded phases. The serum protein recovery from the SPS columns has been stated to be $97 \pm 3\%$ [48]. This is less than the $99 \pm 3\%$ protein recovery reported for the ISRP columns using the same protein assay method [27]. When put into use, the SPS columns may not prove to be as long lived or as highly selective as the GFF peptide bonded-phase ISRP columns, but the advantages of the SPS columns should include lower manufacturing cost and ease of adaptation to reversed-phase separations, previously developed with conventional alkyl bonded phases.

6. DUAL ZONE MATERIAL

Williams and Kabra [49] have recently introduced an additional method for preparing HPLC packing from porous silica which possess a hydrophilic, non-adsorptive phase on the outside of the packing particulates but an alkyl bonded phase on the inside of the packing. The discrimination is brought about by differential reaction kinetics. A hydrophilic perfluorobutyethylene dimethylsilyl (PFB) phase is bound to the outer surfaces of the silica by using the "ultrafast" leaving group N-methylacetamidyl. It is purported that the reaction is faster than the time required for diffusion into the pores, thus limiting the PFB phase to the external surface of the

silica particulate. A C_{18} phase is then bound to the remainder of the porous silica's innermost regions by the use of chloride as a slow leaving group. The packing is made with 10-μm diameter silica with 60 Å pores and results with a hydrophilic PFB on the external surface and C_{18} bonded-phase on the internal surface. As with the other packing concepts described here complete size-exclusion is used as a mechanism to prevent proteins from reaching the bonded-phase. The packing material, referred to as dual zone material, has only recently been introduced and its performance has not been fully evaluated.

7. MIXED-FUNCTIONAL PHASE COLUMNS

Haginaka *et al.* [50] have taken the approach of producing what is termed "mixed-functional phase" silica by using a multistep synthesis which mixes the silica-bonded hydrophobic phase in among a diol phase. Very-small-pore silica (55 Å diameter) is used to ensure that protein is size-excluded from the pores. A low-coverage 3-glycidoxypropyl phase is bonded to the silica followed by the attachment of a hydrophobic phase onto remaining silanols by means of either phenyl-, butyl- or octyltrimethoxysilane. The oxirane ring of the former is then hydrolyzed to produce a diol phase among the hydrophobic phase. When protein containing samples are injected onto this packing, the protein is not recovered from the first few injections, thus the outer surfaces of packing are coated with adsorbed protein. This initially prepared "mixed-functional phase" packing behaves much like the protein-coated phases of Yoshida and co-workers (see above). The columns in this form could be used for the assay of drugs in serum by direct injection; however, periodic washing with a strong eluent was required to remove the accumulation of residual proteinaceous substances. To improve the protein recovery, the packing was further treated with 3-glycidoxypropyltrimethoxy silane in aqueous acid. Under these reaction conditions polymerization would likely occur creating a hydrophilic network over the outer surface. This was evidenced by closing of the pore diameter to 39 Å. Good protein recovery could be obtained on this packing; however, column efficiency had been decreased to 12 000 plates/m by the additional reaction process. It was demonstrated that this level of performance could be maintained for as much as 500 serum injections.

8. CONCLUSION

It is evident from the above discriptions that considerable effort has been made to develop HPLC packing materials that will remain innocuous to macromolecular substances in sample matrices yet extract small molecules of interest and separate them with high resolution from related and endogenous components. The success of the enzyme cleaved, peptide bonded-phase ISRP columns demonstrates that the scope of applications possible with such surface discriminatory, mixed-mode types of HPLC packings goes beyond the analysis of drugs in blood serum or plasma by direct injection. With a strong need to simplify a wide variety of assays through the elimination of sample preparations, it is anticipated that the field will continue grow in order to facilitate the HPLC analysis of a broad spectrum of analytes with greater ease.

REFERENCES

1 D. Westerlund, *Chromatographia*, 24 (1987) 155.
2 Z. K. Shibabi, *J. Liq. Chromatogr.*, 11 (1988) 1579.
3 T. C. Pinkerton, *Am. Clin. Lab.*, 8 (1989) 32.
4 S. H. Y. Wong, *J. Pharm. Biomed. Anal.*, 7 (1989) 1011.
5 N. Takeda, T. Niwa, K. Maeda, M. Shibata and A. Tatematsu, *J. Chromatogr.*, 431 (1988) 418.
6 J. A. O. Meriluoto and J. E. Eriksson, *J. Chromatogr.*, 438 (1988) 93.
7 R. L. Smith, presented at the *American Association of Pharmaceutical Scientists National Meeting, Atlanta, GA, September 29, 1988*, abstract AP115.
8 J. C. Cherton, D. Ladjama and D. Soyez, *Analysis*, 18 (1990) 71–77.
9 T. C. Pinkerton, T. D. Miller and L. J. Janis, *Anal. Chem.*, 61 (1989) 1171.
10 A. Shibukawa, T. Nakagawa, M. Miyake and H. Tanaka, *Chem. Pharm. Bull.*, 36 (1988) 1930.
11 A. Shibukawa, T. Nakagawa, M. Miyake, N. Nishimura and H. Tanaka, *Chem. Pharm. Bull.*, 37 (1989) 1311.
12 A. Shibukawa, T. Nakagawa, N. Nishimura, M. Miyake and H. Tanaka, *Chem. Pharm. Bull.*, 37 (1989) 702.
13 A. Shibukawa, N. Nishimura, K. Nomura, Y. Kuroda and T. Nakagawa, *Chem. Pharm. Bull.*, 38 (1990) 443.
14 A. Shibukawa, M. Nago, Y. Kuroda and T. Nakagawa, *Anal. Chem.*, 62 (1990) 62.
15 T. C. Pinkerton and K. A. Koeplinger, *Anal. Chem.*, 62 (1990) 2114.
16 H. Yoshida, I. Morita, T. Masujima and H. Imai, *Chem. Pharm. Bull.*, 30 (1982) 2287.
17 Y. Yoshida, I. Morita, G. Tamai, T. Masujima, T. Tsuru, N. Takai and H. Imai, *Chromatographia*, 19 (1985) 466.
18 H. Yoshida, K. Takano, I. Morita, T. Masujima and H. Imai, *Jpn. J. Clin. Chem.*, 12 (1983).
19 K. Matsumoto, H. Kikuchi, S. Kano, H. Iri, H. Takahashi and M. Umino, *Clin. Chem.*, 34 (1988) 141.
20 I. Morita, T. Masujima, H. Yoshida and H. Imai, *Anal. Biochem.*, 151 (1985) 358.
21 I. H. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757.
22 S. E. Cook and T. C. Pinkerton, *J. Chromatogr.*, 368 (1986) 233.
23 I. H. Hagestam and T. C. Pinkerton, *J. Chromatogr.*, 351 (1986) 239.
24 S. E. Cook, *Ph.D. Thesis*, Purdue University, West Lafayette, IN, December 1988.
25 I. H. Hagestam and T. C. Pinkerton, *J. Chromatogr.*, 368 (1986) 77.
26 J. Haginaka, N. Yasuda, J. Wakai, H. Matsunaga, H. Yasuda and Y. Kimura, *Anal. Chem.*, 61 (1989) 2445.
27 T. C. Pinkerton, T. D. Miller, S. E. Cook, J. A. Perry, J. D. Rateike and T. J. Szczerba, *Biochromatogr.*, 1 (1986) 96.
28 T. Nakagawa, A. Shibukawa, N. Shimono, T. Kawashima and H. Tanaka, *J. Chromatogr.*, 420 (1987) 297.
29 S. J. Rainbow, C. D. Dawson and T. R. Tickner, *Ann. Clin. Biochem.*, 26 (1989) 527.
30 C. M. Dawson, T. W. M. Wang, S. J. Rainbow and T. R. Tickner, *Ann. Clin. Biochem.*, 25 (1988) 661.
31 T. Niwa, N. Takeda, A. Tatematsu and K. Maeda, *Clin. Chem.*, 34 (1988) 2264.
32 F. Tagliaro, R. Dorizzi, A. Frigerio and M. Marigo, *Clin. Chem.*, 36 (1990) 113.
33 N. D. Atherton, *Clin. Chem.*, 35 (1989) 975.
34 L. T. Karolak, R. Farinotti, M. J. Bossant and A. Dauphin, *J. Pharm. Clin.*, 7 (1988) 543.
35 T. C. Pinkerton, J. A. Perry and J. D. Rateike, *J. Chromatogr.*, 367 (1986) 412.
36 T. Ohsnima, I. Johno, T. Hasegawa and S. Kitazawa, *J. Liq. Chromatogr.*, 11 (1988) 3457.
37 R. H. Pullen, C. M. Kennedy and M. A. Curtis, *J. Chromatogr.*, 434 (1988) 271.
38 L. M. Rose, A. A. Beaty, S. M. El Dareer, K. F. Tillery and D. L. Hill, presented at the *14th International Symposium on Column Liquid Chromatography, Boston, MA, May 20–25, 1990*, poster 626.
39 Y. Q. Chu and I. W. Wainer, *Pharm. Res.*, 5 (1988) 680.
40 L. E. Mathes, G. Muschik, L. Demby, P. Polas, D. W. Mellini, H. J. Issaq and R. Sams, *J. Chromatogr.*, 432 (1988) 346.
41 T. C. Pinkerton and K. A. Koeplinger, *J. Chromatogr.*, 458 (1988) 129.
42 T. Ohshima, I. Johno, T. Hasegawa and S. Kitazawa, *J. Pharm. Sci.*, 79 (1990) 77.
43 J. A. Perry, B. Invergo, H. Wagner, T. J. Szczerba and J. D. Rateika, *J. Chromatogr.*, in press.
44 D. J. Gisch, B. T. Hunter and B. Feibush, *J. Chromatogr.*, 433 (1988) 264.
45 D. J. Gisch, B. Feibush, B. T. Hunter and T. L. Ascah, *Biochromatogr.*, 4 (1989) 206.

46 C. P. Desilets and F. Regnier, presented at the *12th. International Symposium on Column Liquid Chromatography, Washington, DC, June 19–24, 1988.*
47 C. P. Desilets, *Ph.D. Thesis*, Purdue University, West Lafayette, IN, December 1988.
48 L. J. Glunz, J. A. Perry, B. J. Invergo, H. Wagner, T. J. Szczerba and J. D. Rateika, presented at the *Pittsburgh Conference on Analytical Chemistry, New York, NY, March 1990*, paper 490.
49 D. E. Williams and P. M. Kabra, *Anal. Chem.*, 62 (1990) 807.
50 J. Haginaka, J. Wakai and H. Yasuda, presented at the *14th International Symposium on Column Liquid Chromatography, Boston, MA, May 20–25, 1990*, poster 422.