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INTERNAL SURFACE REVERSED-PHASE SILICA SUPPORT PREPARED WITH CHYMOTRYPSIN

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SUMMARY

A silica bonded phase packing material specifically designed to accommodate the direct injection of serum or plasma samples is described. The support represents the second in a series of packing materials derived from a new concept in liquid chromatography referred to as internal surface reversed-phase (ISRP) chromatography. In this alternative synthesis method to the prototype ISRP material, the silica based supports are first modified with an amine silane and butoxy-L-phenylalanine (Boc-L-Phe). The Boc-L-Phe partitioning phase is then cleaved from the external surface of the silica particulates with α-chymotrypsin. The remaining amine groups are capped with hydrophilic glycidol. The anticonvulsant drugs phenytoin, carbamazepine, and phenobarbital in serum are successfully analyzed with the new ISRP packing material.

INTRODUCTION

Analyses of drugs in serum or plasma by high-performance liquid chromatography (HPLC) has for years been plagued with sample pretreatment prior to injection. Not only are conventional sample clean-up procedures (involving protein precipitation and extractions) time consuming and labor intensive, but often they present problems of low recoveries of drugs and metabolites.

With the difficulties associated with the automation of sample clean-up, many investigators have been searching for ways to directly inject serum or plasma samples into HPLC systems. Serum samples, of course, cannot be directly injected onto conventional small particulate (*i.e.* < 10 μm) reversed-phase columns, because proteins denature at the partitioning phase interface and accumulate in high performance chromatographic columns. This accumulation closes down the pores of the silica particulates, inhibits analyte diffusional mass transport, and decreases column efficiency[1,2]. After a relatively small number of serum injections, HPLC columns are rendered useless due to clogging of the interparticulate space.

In lieu of being able to inject serum samples directly onto reversed-phase HPLC analytical columns, many investigators have demonstrated the use of precolumns packed with large particulate (30–50 μm), alkyl bonded phase silicas, placed

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in-line with analytical columns. Along with various switching valve arrangements, drugs are first loaded onto the pre-columns by direct injection of serum samples, followed by attempts to wash away matrix components before the drugs are back-flushed with stronger solvents from precolumns to analytical columns.

In 1980 Frei[3], reviewed the use of pre-columns for trace enrichment and suggested their use for serum sample clean-up. In the same year De Jong[4] demonstrated the HPLC analysis of several drugs by direct injection of serum with the use of large particulate (*i.e.*, 32 μm) C_2 pre-columns. The process was hampered by the need to perform a purge wash of the pre-columns after each injection, and the replacement of precolumn packing after five to ten injections. With this arrangement the breakthrough of proteinaceous material deteriorated analytical columns after several weeks. One major problem appears to have been the high percentage of methanol used in the mobile phase which is known to precipitate proteins. Subsequently, Werkhoven-Goewie *et al.*[5] suggested the use of enzymatic hydrolysis of blood samples prior to injection onto CN bonded silica pre-columns with aqueous dioxane mobile phases for better sample through-put. This, however, reintroduces sample pretreatment of which it is the intent to eliminate.

In 1981 Roth *et al.*[6] placed in tandem two pre-columns packed with large particulate (*i.e.*, 37–50 μm) C_{18} supports and demonstrated an "alternating pre-column enrichment" technique with the direct injection of plasma onto the precolumns followed by backflushing to analytical columns. Although the technique encompassed a time consuming multi-tasking operation involving equilibration, adsorption, purge wash, and chromatography phases, multiple analyses by direct injection of plasma were demonstrated. An important aspect realized by Roth was that direct injection eliminates the need for an internal standard, commonly required with a concentional off-line sample clean-up procedure. Two years later Roth[7] used the pre-column technique to analyze for sulmazole and its metobolites in plasma by direct injection. He pointed out in that study the importance of using sieves at the top of a pre-column in place of stainless steel frits to reduce clogging.

In intervening years a variety of investigators demonstrated the use of pre-columns for the on-line clean-up of serum and plasma samples. Voelter *et al.*[8] simply placed a large particulate (*i.e.*, 25–40 μm) C_{18} pre-column in the position of the sample loop. Hux *et al.*[9] demonstrated the use of Amberlite XAD-2 in pre-columns for the analysis of methaqualone in plasma by direct injection. More recently, Arvidsson *et al.*[1], Juergens[2], and Nazareth *et al.*[10] each demonstrated the use of alkyl bonded silica pre-columns in conjunction with analytical columns for the analysis of drugs in serum or plasma by direct injection.

All of the above mentioned on-line pre-column clean-up procedures have the same inherent limitations when applied to the analysis of drugs in serum or plasma by direct injection. First and foremost, the pre-column techniques with alkyl bonded phases work only with large particulates. This means that band broadening is introduced to system. The use of small particulates (*i.e.*, < 10 μm) leads to rapid clogging of the interstitial space due to protein accumulation. Second, since proteinaceous materials accumulate in alkyl bonded silica pre-columns, the packing must be periodically replaced. Third, the alkyl-bonded phases inherently require higher concentrations of organic modifiers to remove the drug analyte from the pre-columns. This increases the probability of protein precipitation in the chromatographic system.

Fourth, since protein precipitation in a mobile phase is also a function of its concentration, sample sizes are generally limited to less than 200 μl. Fifth, although a pre-column method is easier to automate than a conventional off-line clean-up procedure, the pre-column switching with equilibration times and purges washes is still time consuming.

In order to eliminate the problem of protein accumulation in chromatographic systems on direct injection of serum samples, Hagestam and Pinkerton[11,12] introduced a new concept in reversed-phase silica packing referred to as "internal surface reversed-phase" (ISRP) supports. The packings incorporate a hydrophobic polypeptide phase which is located only on the internal surface of the porous particulates. The external surface of the packing consists of a non-adsorptive, hydrophilic glycerylpropyl bonded phase. Since the pores of the supports are intentionally kept small, the proteins see only the non-adsorptive external surface and elute in the external void volume with very high recoveries. The drugs, on the other hand, being small penetrate the supports, partitition with the internal polypeptide phase. The ISRP supports can be used either as extraction pre-columns or high performance analytical columns. The primary advantage of the ISRP supports over the alkyl bonded precolumn methods, is that the ISRP supports allow for the complete passage of proteins. Secondly, the ISRP supports work successfully with small particulates (*e.g.* 5 μm), thus allowing direct injection of serum or plasma samples onto the high performance ISRP separation columns[11]. The use of ISRP HPLC columns is normally desired under circumstances where preconcentration is not required. When preconcentration is necessary, the ISRP precolumns can be used with small particulate C_{18} pre-columns and analytical columns giving each a lifetime comparable to that experiences with the injection drug alone, without loss of efficiency[11]. Thirdly, the ISRP supports are advantageous because the internal polypeptide partitioning phase is deliberately designed to be weaker by comparison to a C_8 or C_{18} phase. This enables the use of a lower percentage of organic modifier in the mobile phase to elute drugs, thus ensuring against protein precipitation. Typically, the total organic modifier concentration can be less than 20% of isopropanol, tetrahydrofuran or acetonitrile, used alone or in combination.

The first internal surface reversed-phase supports were prepared by derivatizing controlled pore silica with glycerylpropyl silane followed by the attachment of glycine-L-phenylalanine or glycine-L-phenylalanine-L-phenylalanine to a given fraction of the glycerlylpropyl groups with carbonydiimidazole as a coupling reagent[11,12]. The phenylalanine moieties were then removed from the external surface of the silica particulates by treatment with carboxypeptidase. In the following work, an alternative approach is described for the preparation of ISRP supports. Rather than modifying the silica first with a glycerylpropyl phase, through which the polypeptides are bound, the hydrophobic partitioning phase is bound to the silica via an amine spacer. The hydrophobic partitioning moieties are then cleaved from the external surface with chymotrypsin leaving the attached amine spacer. The external surface is then rendered non-adsorption to proteins by attaching glycol groups to the freed amine spacers. With this approach greater partitioning phase capacity can be achieved due to the high yield of silanization reactions.

EXPERIMENTAL

Internal phase synthesis

Attachment of amine phase. The entire ISRP synthesis procedure is outlined in Fig. 1. The first step in the derivatization is the attachment of an amine phase to the silica supports in a manner similar to that described elsewhere[13]. The silica supports employed in this work consisted of either low performance packing ("controlled pore glass"; particle size, 37–47 μm; initial pore size, 86 Å; Electro-Nucleonic, Fairfield, NJ, U.S.A.) or high performance packing (Hypersil; particle size, 5 μm; initial pore size, 123 Å; Shandon, Chesire, U.K.).

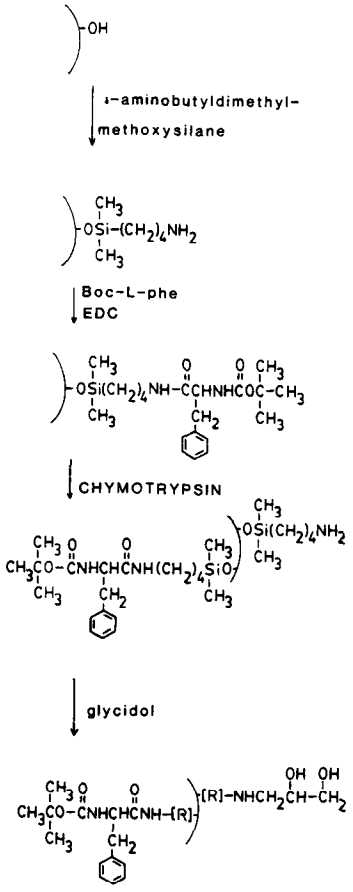


Fig. 1. Synthetic scheme outlining ISRP modification.

Typically, 4.5 g of the unmodified "controlled pore glass" (surface area 153 m^2/g) is heated to 80°C with 0.4 *M* hydrochloric acid, washed with distilled water, and dried at 190°C. The support is then dispersed in 20 ml of dry toluene. Two times excess of aminobutyldimethylmethoxy silane compared to the maximum theoretical

silanol group content, is added to the toluene mixture. The slurry is refluxed at 108°C for 5 h, keeping the reflux condenser at 65°C to allow the escape of methanol generated. After the reaction, the support is rinsed with toluene and methanol. To confirm the presence of the amine phase, several drops of an ethanolic solution of salicylaldehyde are combined with a small amount of the support. A bright yellow color confirms the presence of amine[14].

Attachment of hydrophobic phase. Next, the hydrophobic peptide partitioning phase is bound to the amine spacer. Boc-L-phenylalanine serves as the partitioning moiety since it can be easily cleaved from an external silica surface with α-chymotrypsin. The Boc-L-Phe is attached to the amine with the 1,1-ethyl-3-(3-diaminopropyl)carbodiimide (EDC) coupling catalyst[15]. Typically, 1.5 g of the amine support is combined with 1.5 mmol (398 mg) of Boc-L-Phe in 6 ml of methanol and 1.5 mmol (288 mg) of EDC in 2 ml of water. The resulting pH of the reaction mixture of 5.5, thus no pH adjustment is required. The reaction is allowed to procedure in a shaker overnight at room temperature. The derivatized support is subsequently washed with methanol and distilled water. The amount of Boc-L-Phe attached to the support is removed by acid hydrolysis and subsequently quantified by reversed-phase HPLC.

Enzyme cleavage. In order to remove the hydrophobic partitioning Boc-L-Phe phase from the external surface of the silica, the particulates are treated with chymotrypsin. Typically, 25 mg of chymotrypsin is dissolved in 1 ml of 0.001 *M* hydrochloric acid and diluted to 10 ml with 0.08 *M* Tris–0.1 *M* calcium chloride buffer (pH 7.8) as normally recommended for optimum enzyme activity[16]. The combined support and enzyme mixture are allowed to react for several hours, whereupon an additional 25 mg of chymotrypsin is added, then the reaction is allowed to incubate overnight with gentle shaking at 37°C. The enzyme is subsequently washed from the support by filtration and the ISRP particulates sequentially rinsed with 0.1 *M* acetic acid (pH 4.0), methanol, and distilled water.

Attachment of glycol groups. The last step in the production of this type of ISRP material is to cap the external surface residual amine phase with glycerol groups, as illustrated in Fig. 1. Usually, 1 g of the support is allowed to react at room temperature with shaking overnight with 3 ml of glycidol (2,3-epoxy-1-propanol). The derivatized packing material is subsequently washed with water. The amount of glycol groups attached to the silica is quantified by means of oxidation with paraperiodate, conversion of excess paraperiodate and iodite to form triiodide, and titration of the triiodide with standardized thiosulfate, as described by Siggia[17].

It should be noted, that the production of ISRP supports by various methods, either as outlined in Fig. 1 or as indicated in previous publications[11,12], is protected under U.S. patent[12]. The Purdue Research Foundation has granted a license to the Regis Chemical Company of Morton Grove (IL, U.S.A.) to manufacture and distribute the ISRP supports.

Reagents

Boc-L-Phe was purchased from Chemical Dynamics Corporation. EDC and α-chymotrypsin (three times crystallized, salt free, lot 92F-8205) were obtained from Sigma. Human plasma was obtained from a local blood bank. Phenytoin (*i.e.*, 5,5-diphenylhydantoin) (purity > 99%) and glycidol were obtained from Aldrich. The δ-aminobutyldimethylmethoxy silane was supplied by Silar.

Chromatographic procedures

The low performance, large particulate ISRP supports were manually packed with a slurry into a 10 cm × 3 mm I.D. glass column, supplied by Altex. The column was incorporated into a standard liquid chromatographic system consisting of a single piston Milton Roy pump (Model 396), a Tefzel 30 μl injection valve, and a Beckman Model 153 254-nm single-wavelength detector equipped with a 100-μl flow cell. Experiments were conducted at a flow-rate of 1.5 ml/min with a mobile phase of 0.1 *M* sodium sulfate–0.1 *M* phosphate buffer (pH 6.0). The performance of the large particulate columns was evaluated with the injection phenytoin and human plasma.

The high performance, small particulate 5-μm ISRP supports were slurry packed under pressure in a standard fashion into a 15 cm × 4.6 mm I.D. stainless-steel column. The chromatographic system was modified with a Rheodyne Model 7010 injection valve, a 20-μl loop, and a 8-μl flow cell in the UV-vis detector, to accommodate the HPLC column. Chromatographic elution of standard anticonvulsant drugs in human plasma off the ISRP 5 μm column was conducted at a flow-rate of 1 ml/min with a mobile phase of acetonitrile–0.1 *M* phosphate buffer (pH 6.0) (20:80).

RESULTS AND DISCUSSION

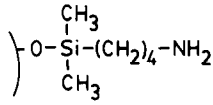
Synthesis of ISRP supports using chymotrypsin

The amine spacer is required for the attachment of hydrophobic partitioning moieties which are susceptible to enzyme cleavage with α-chymotrypsin. The chymotrypsin, an endopeptidase, must have enough space to reach and conform around the susceptible amide linkage. A short spacer promotes adverse steric effects and surface repulsion between the enzyme and the silica backbone. It is desired that the amine spacer be as hydrophilic as possible, because it will remain on the external surface after glycidol addition (Fig. 1). Since such ideal spacers are not commercially available, preferences is given to the alkyl chain length and monomethoxy functionality of the silane in order to favor a high monolayer coverage of amine spacer of sufficient length to accommodate enzyme cleavage. The monofunctional δ-aminobutyldimethylmethoxysilane was chosen over the commonly used γ-aminopropyltriethoxysilane because of the longer alkyl chain and the monomethoxysilane's ability to provide a higher, more homogeneous coverage with a minimum number of residual silanols[14]. Obviously, the greater amine coverage will result in an increased capacity of the internal hydrophobic peptide phase, thus resulting in an increased retention of drug analytes. The greater capacity factors are desired for ISRP precolumn applications so more time is available for shunting proteins to waste prior to channeling drug fractions from the ISRP precolumn to a conventional C_{18} analytical column[11].

The EDC coupling catalyst was selected over other carbodiimide derivatives, such as the dicyclohexylcarboiimide (DCC), because the urea produced is easily washed from the hydrophilic support with aqueous buffers and methanol[18]. In contrast, the DCC reagent requires a methylene chloride wash. Packing support exposed to methylene chloride have shown more interaction with albumin than supports rinsed with methanol[19].

Evaluation of large particulate Boc-L-Phe ISRP supports

The injection of human plasma (60–80 g/l) onto the modified amine phase support is illustrated in Fig. 2. The peak heights and peak tailing indicate that plasma proteins adsorb to the support. Since the aqueous mobile phase contained 0.2 *M* sodium sulfate, it is unlikely that the adsorption results from an ion-exchange mechanism. It is surmised that the hydrophobic nature gives rise to the protein adsorption. Similar behavior has been observed by Kiselev *et al.*[20] with silica-bonded amine phases produced with γ-aminopropyltriethoxy silane. Although the plasma proteins adsorb onto the amine phase support, the drug phenytoin exhibits only slight retention with a capacity factor of 1.3.



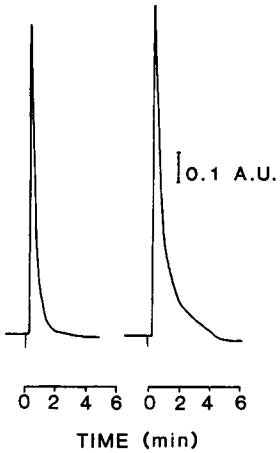
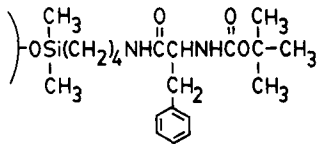


Fig. 2. Elution of human plasma on the amine modified silica support. Column dimensions: 10 cm × 3 mm I.D. Mobile phase: 0.2 *M* sodium sulfate–0.1 *M* phosphate (pH 6.0). Flow-rate: 1.5 ml/min. Detection: optical at 254 nm. Injection loop: 30 μl. Concentration: about 70 g/l of human serum albumin.

After attachment of the Boc-L-Phe, hydrolysis and quantification of the phenylalanine yielded a coverage of 360 μol of phenylalanine per gram of support (2.4 μmol/m^2). A chromatogram illustrating the elution of human plasma from the Boc-L-Phe derivatized support is shown in Fig. 3. The small peak height and severe tailing indicate adsorption of the protein onto the external surface Boc-L-Phe moieties.

After cleavage of the Boc-L-Phe groups from the external surface of the silica with α-chymotrypsin, the supports contained 332 μmol of phenylalanine per gram of support. This meant that 28 μmol/g had been removed from the external surface. Since the external surface of porous silica comprises only a small fraction of the total surface area[21], the 8% cleavage is not unexpected. The enzyme treated support was



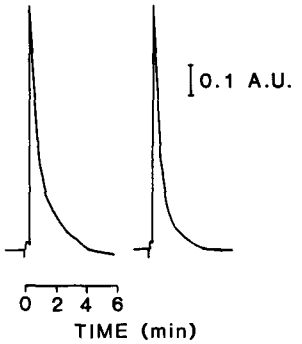


Fig. 3. Elution of human plasma on the Boc-L-Phe modified column (non-enzyme treated). Mobile phase: 0.1 *M* sodium sulfate–0.1 *M* phosphate (pH 6.0). Other conditions are the same as in Fig. 2.

then reacted with glycidol which capped the residual amine groups on the external surface with hydrophilic glycol groups.

The elution of human plasma from the Boc-L-Phe ISRP is illustrated in Fig. 4. The greater peak height and less tailing indicates significantly less protein interaction compared to the non-enzyme treated material. The plasma proteins interact only with the phase attached to the external surface of the silica particulates because of the small pore diameters. With derivatization of the supports the nominal pore diameter is less than the initial 86 Å. Human serum albumin, the smallest and most abundant serum protein, has a molecular weight of *ca.* 65 600 daltons[22], consequently the proteins cannot gain access to the internal surface region.

The analyte phenytoin when injected onto the Boc-L-Phe ISRP low performance material exhibited an apparent infinite retention with an aqueous mobile phase. With a mobile phase of acetonitrile–methanol–0.1 *M* phosphate (pH 6.0) (20:33:47) the phenytoin could be eluted with a capacity factor of 1.0.

High-performance Boc-L-Phe ISRP supports

Small silica particulates (5 μm) were derivatized with the alkylamine and the Boc-L-phe reagent. The material was then enzyme treated and capped with glycol groups as described above. A human serum sample containing anticonvulsant drugs was injected onto the Boc-L-Phe ISRP column. The resulting chromatogram is illustrated in Fig. 5. Phenobarbital is eluted first followed by carbamazepine and phenytoin. The plasma proteins elute in the void volume. This particular separation was carried out with a mobile phase comprising acetonitrile–0.1 *M* phosphate (pH 6.0) (20:80).

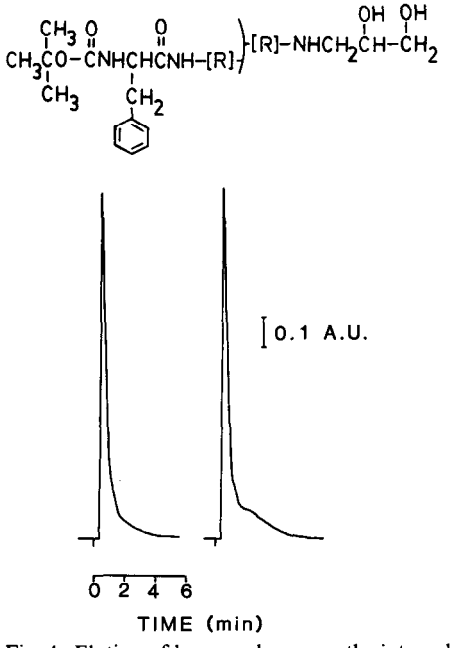


Fig. 4. Elution of human plasma on the internal surface reversed-phase Boc-L-Phe silica support. Experimental conditions are the same as in Fig. 3.

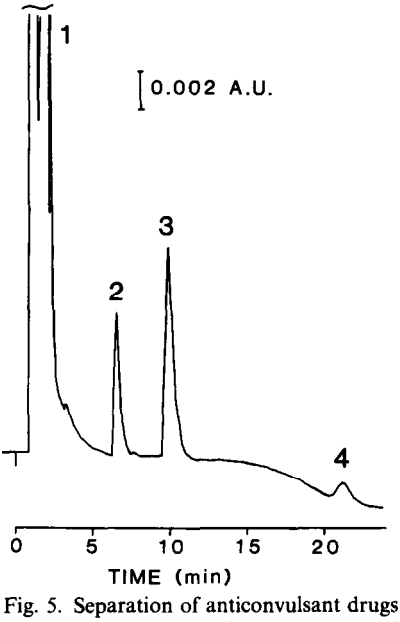


Fig. 5. Separation of anticonvulsant drugs in human serum with the Boc-L-Phe ISRP high performance column. Column dimensions: 15 cm × 4.6 mm I.D. Injection loop: 20 μl. Mobile phase: acetonitrile–0.1 *M* phosphate (pH 6.0) (20:80). Flow-rate: 1.0 ml/min. Detection: optical at 254 nm. Peaks: 1 = human serum proteins; 2 = phenobarbital (56.4 mg/l); 3 = carbamazepine (16.8 mg/l); 4 = phenytoin (26.3 mg/l).

A similar chromatographic separation using the ISRP gly-L-phe-L-phe tripeptide silica support in a 25 cm × 4.6 mm I.D. column under identical mobile phase conditions is illustrated in Fig. 5 of ref. 11. A comparison of eluted profiles demonstrates that the Boc-L-Phe butylamine ISRP material yields greater capacity factors compared to the Gly-L-Phe-L-Phe ISRP supports. The increased retention results from a higher phase coverage achieved with the amine spacer method. The technique yielded 332 μmol/g for the Boc-L-Phe support compared to the typical 45–65 μmol/g for the Gly-L-Phe-L-Phe phase accomplished with direct attachment through the glycerylpropyl bonded phase[11].

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