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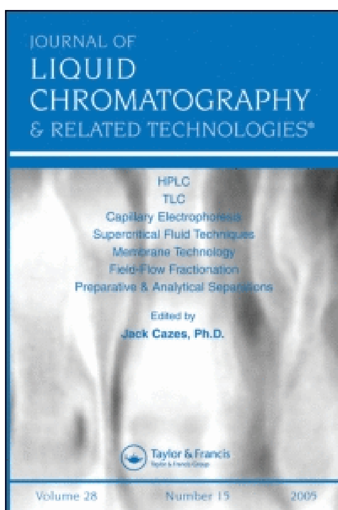

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AN IMPROVED INTERNAL SURFACE REVERSED PHASE

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ABSTRACT

Invented by Dr. Thomas C. Pinkerton, the Internal Surface Reversed Phase (ISRP) allows the direct analysis of serum and body fluids without requiring the prior removal of protein. The first ISRP, based on glycine - phenylalanine - phenylalanine (GFF) bonded to 80A spherical silica, proved to have excellent column life, phenomenal selectivity, and further capabilities not originally perceived. However, its original synthesis yields a material that, being partially polymeric, is difficult to reproduce. Moreover, chromatographically the original ISRP shows poor efficiency and retentivity. Each is lower than either expectable or desirable. A revised synthesis has yielded an inherently monomeric GFF much easier to reproduce. Although

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the carbon load of the new ISRP is lower than that of its predecessor, its efficiency and retentivity are now adequate. For carbamazepine, the efficiency is now 60,000 plates per meter (up from 25,000 to 35,000); and the retention has more than doubled. These characteristics suggest that the molecular elements of the new stationary phase are used more efficiently than those of the old.

INTRODUCTION

Invented by Thomas C. Pinkerton, the Internal Surface Reversed Phase (ISRP)(1) allows the direct analysis of serum and body fluids without requiring the prior removal of protein (see the original paper, ref. 2; and reviews, for instance refs. 3 and 4).

For the first ISRP, Dr. Pinkerton bonded glycine - phenylalanine -phenylalanine (GFF) to 80A-pore spherical silica. In high pressure liquid chromatography (HPLC), proteins pass right through ISRP columns. Serum proteins are size-excluded from the pores of ISRP packings (4,5) and are not retained by the hydrophilic ISRP outer surfaces. With normal 10- to 20-microliter injections, ISRP-guarded ISRP columns routinely receive well over 500 hundred injections (see, for example, Meriluoto, ref. 6). ISRP columns have proven to be amenable to automation; ISRP column life, quite satisfactory.

In ISRP synthesis, the GFF is first amine-bonded to all surfaces. The phenylalanines are then enzymatically removed from only the outer surfaces (1,2), which thus carry only glycine. On all surfaces, free carboxylic acid groups face the

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mobile phase, the proteins, and the analytes. With regard to its selectivity, GFF is not only a multifunctional tripeptide but also a pH-variable weak cation exchanger. In consequence, GFF selectivity has been found surprisingly wide (7), experimentally variable by control of pH (4), and generally more than adequate.

GFF has been shown highly effective in separating peptides (8)--an unexpected but welcome capability.

The ISRP concept and the original GFF packing have been extended to analyzing on a given injection both the free and the bound forms of drugs and, when relevant, the different bound forms (9-13).

Inadequate retention of many hydrophilic drugs has limited the application of the original GFF to the analysis of them. Its poor retention of hydrophilic substances has prevented not only its straightforward application to the analysis of them but also any substantial increase of the sensitivity attendant on its use, for instance by the injection of larger-than-normal amounts. Correspondingly, a general increase in GFF retentivity would expand both GFF applicability and the sensitivity attainable with GFF ISRP columns.

The original GFF has always displayed only a mediocre 25,000 to 35,000 plates per meter. GFF has a complex structure that underlies not only its highly useful selectivity but also, it has been suggested, its low efficiency. This suggestion,

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that several concurrently acting GFF retention mechanisms inevitably cause poor GFF efficiency, could be disproven only by the bringing-about of the much higher efficiencies that are to be expected in modern HPLC. With the original GFF, this was never found possible, so the suggestion could not be gainsaid.

For quality control in synthesis of the original GFF, standard multicomponent mixtures were devised. With these, however, large and apparently mutually unrelated retention differences between successive lots of the original GFF could always be found. This was unacceptable. For some analyses, off-the-shelf ISRP columns were and are required to be exact replicates of each other, to be used as is, without inspection or adjustment of analytical conditions. The inability of the original GFF to satisfy this criterion was considered the one most necessary to correct.

The achievement of much improved reproducibility was taken as the prime objective. The extreme reproducibility achieved at Regis in synthesizing other monomeric stationary phases suggested that a similar approach to GFF synthesis might best improve GFF reproducibility. Accordingly, this was taken as top objective in the overall ISRP research and development program.

Among subsidiary targets of the research, once achievement of the primary objective was assured, the GFF-related ISRP materials glycine - phenylalanine (GF) and glycine -

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(phenylalanine)$_4$ (GF_4) were to be synthesized and characterized.

MATERIALS

The 3-glycidyloxypropyldimethylethoxysilane used for the revised ISRP synthesis was purchased from Huls America, Piscataway, New Jersey; the 70% perchloric acid, A.C.S. reagent grade, from Fisher Scientific Company, Fairlawn, N. J.

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METHODS

Except for the change to be described, the synthesis of the monomeric glycine - phenylalanine - phenylalanine was performed essentially as described earlier (1). In this change, the monoethoxysilane (mentioned under Materials) and related bonding technique were substituted for the trimethoxysilane and its bonding technique; the monoethoxysilane was bonded to silica by refluxing in heptane for 48 hours.

RESULTS AND DISCUSSION

Analysis of GFF chemical synthesis suggested possible sources of variability. Several enter with the first step, the bonding of gamma-glycidoxypropyltrimethoxysilane (I) to silica.

With reagent I, the first methoxy-silanol reaction leaves two reactive $-OCH_3$ leaving groups still available. Reaction of

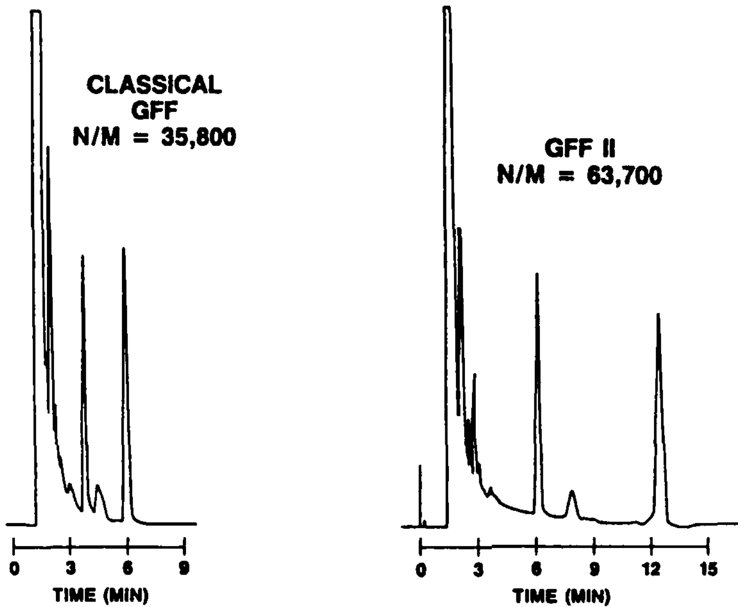

FIGURE 1. The monomeric GFFII increases chromatographic retention and efficiency over the earlier, apparently partly polymeric GFF. The retention of the carbamazepine (the second peak) has more than doubled and the chromatographic efficiency for that peak has increased from 35,000 to about 63,000 plates per meter. Analytes: phenobarbital and carbamazepine in human serum. Sample volume: 10 microliters. Mobile phase: 20/80 acetonitrile/0.1M KH_2PO_4 (pH 6.8) v/v, at 0.6 mL/min. Columns: 5 micron ISRP, 15 cm x 4.6 mm I.D. Detection: 254 nm.

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either with water produces a silanol on the now-bound reagent and leads to a polymerization that is difficult to control. The resultant stationary phase is not reproducible, nor are its properties. Polymerization can be completely avoided by using a monofunctional reagent, rather than a polyfunctional.

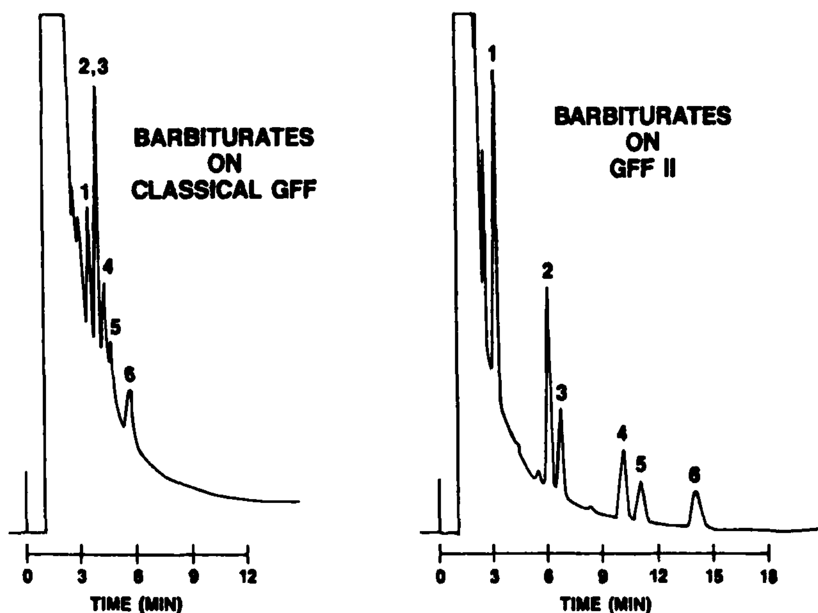

FIGURE 2. With regard to both retention and efficiency, the new GFFII performs dramatically better than the earlier GFF. Analytes: a standard mix of six barbiturates in human serum. 1: Barbital; 2. Phenobarbital; 3: Butabarbital; 4: Amobarbital; 5: Pentobarbital; 6: Secobarbital. Sample volume: 10 microliters. Mobile phase: 5/95 methanol/0.1M KH_2PO_4 (pH 7.5) v/v, at 1.0 mL/min. Columns: 5 micron ISRP, 15 cm x 4.6 mm I.D. Detection: 240 nm.

The commercially available 3-glycidoxypropyl-dimethylethyl-oxysilane is a monofunctional reagent usable for this purpose. Having the same type of alkoxy leaving group as the trifunctional, it was found to have similar bonding conditions. A now-consistent chromatographic behavior and coverage then provided a reliable basis for optimizing the various parameters associated with the overall synthesis.

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DISCUSSION

The change in coverage provided something of a surprise. The apparent coverage, as assessed by carbon elemental analysis, was a consistent 4.9% C from run to run, lower than the approximately 6.0% generally observed in use of the trimethoxy reagent. Usually, lower carbon results in less retention, but not in this case.

Despite its lower carbon content, the new material--called GFFII (the second GFF, not GFF plus isoleucine)--not only retains solutes more than GFF but also shows higher chromatographic efficiency. This is strikingly evident in Figures 1 and 2 (14). For carbamazepine as a test solute, GFFII produces twice the retention of GFF and also yields 63,000 plates per meter rather than the 35,000 found with GFF (14).

Possibly the improved retention and efficiency result from the constructive participation, in the monomeric GFFII, of each stationary phase molecule. In contrast, some of the molecules in the partly polymeric GFF on the one hand apparently hinder mass transfer and thus decrease chromatographic efficiency, and on the other do not participate fully in retaining solutes.

The retentions of the monomomeric GF, GFFII, and GF_4 increase in order of chain length--the number of phenylalanines--and also all exceed that of the original GFF. The chromatographic efficiency of the monomeric GF is similar

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to that of the monomeric GFF, i.e., GFFII; but that of the GF_4 is only mediocre, about 40,000 plates per meter.

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