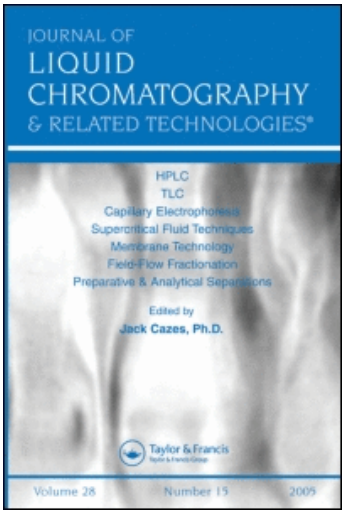

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The Internal Surface Reverse Phase. Concepts and Applications

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Review

THE INTERNAL SURFACE REVERSE PHASE. CONCEPTS AND APPLICATIONS

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ABSTRACT

Patented in 1985, introduced commercially in 1986, internal-surface reversed phase (ISRP) supports have attracted wide attention. ISRP supports allow the analysis of serum and plasma samples by high pressure liquid chromatography (HPLC) without requiring the prior removal of protein. Proteins cannot enter the pores of ISRP supports and are not adsorbed by ISRP outer surfaces; proteins pass right through ISRP HPLC columns. Therefore, the number of serum injections that given ISRP-guarded ISRP columns can receive runs into the thousands; ISRP columns nicely lend themselves to automation. ISRP indifference to proteins is complemented by the remarkable selectivity toward drugs of its stationary phase, glycine-phenylalanine-phenylalanine (GFF), a selectivity that recently has been shown to extend to peptides. More recently still, it has been shown that ISRP columns can be used to analyze both the free and the bound forms of drugs, even distinguishing among different bound forms. A potential new intrinsically monomeric GFF shows improved retention and surprisingly high chromatographic efficiency.

INTRODUCTION

The internal-surface reversed-phase particle was invented at Purdue University by Dr. Thomas C. Pinkerton (1,2; for reviews, see 3-5).

Internal-surface reversed-phase (ISRP) particles have two surfaces: outer, and inner. As suggested diagrammatically in Figure 1 and described presently in more detail, proteins are neither adsorbed nor denatured by ISRP outer surfaces, cannot reach the inner surfaces. In consequence, proteins pass right through ISRP columns. Functionally, therefore, ISRP stands for the direct HPLC determination of drugs in plasma or serum without prior removal of protein.

In this paper, ISRP concepts and applications are scanned thoroughly enough to lend insight into ISRP history, function, and synthesis; the growing range of ISRP applications; factors that affect ISRP selectivity, with application to method development; and some characteristics of potential new ISRP packings.

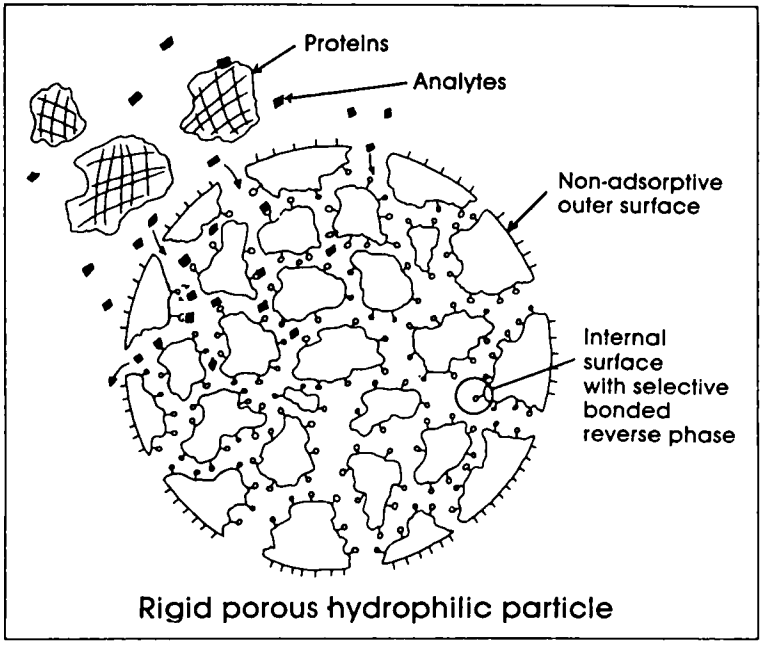


FIGURE 1. The ISRP particle has a hydrophilic outer surface that does not adsorb proteins, an inner surface that proteins cannot reach. Proteins pass right through ISRP columns. Analytes, however, can reach and become separated by the internal-surface reversed phase.

WHY AN ISRP?

Serum must be analyzed for drugs and drug metabolites. Such analysis is required for substances that may become drugs and for drugs that have been submitted for certification. The analysis occurs when the concentration levels of drugs must be either detected or monitored in either humans or animals. The need is absolute, continuing, and vast.

A principal method for the analysis of drugs in serum or plasma is high pressure liquid chromatography (HPLC). The number of such analyses is phenomenal: Just one pharmaceutical company,

for instance, was recently running about 30,000 HPLC analyses per year (6). Before ISRP, protein had to be removed from each potential HPLC serum or plasma sample before it could be injected.

The usual stationary phase for the drug-analyzing HPLC column is the paraffinic octadecylsilyl (ODS). Protein-containing fluids such as serum and plasma may not be directly injected into such a column because proteins denature on the ODS surface, adsorb onto the porous silica to which the ODS is bonded, and thus accumulate within the column. Initially, such accumulation clogs the pores, inhibiting diffusion of the analytes into the pores (7,8) (within which chromatographic separations primarily take place) and decreasing the column capacity (9). Continuing protein accumulation narrows interparticle channels, constrains the flow of mobile phase, and raises the "back pressure"--the pressure required to maintain a given flow rate of mobile phase through the column--to an increasingly unacceptable level. Soon, the column becomes unusable.

If the HPLC column is not to become ruined in this way, the sample must first undergo "clean-up": one way or another, the analytes must be first separated and isolated from the protein matrix (8,10-14). In one clean-up method (10), the proteins are precipitated, the analytes extracted from the supernatant, the extraction solvent evaporated, and the analytes reconstituted in the mobile phase. Inaccurate--the protein-coprecipitated analytes are not fully recovered from the supernatant--and slow, this method nevertheless removes endogenous species and preconcentrates the analytes. In another method (7,8,11,12,15), the analytes are first extracted onto disposable, large-particle, bonded-phase silica supports--a costly approach that requires one disposable column per sample.

In a third clean-up method, the samples are injected into short packed precolumns connected through a switching valve with the HPLC analytical columns. The precolumns are packed with either coarse (30-50 microns diameter) reversed-phase silica particles (8,11-13) or ion exchange materials (14). The analytes, strongly retained on the precolumn, are then backflushed onto the analytical columns. Proteins retained on the precolumns must be and are removed every 3 injections by purge washes, but the expensive precolumns must nevertheless be replaced after perhaps 100 injections. Although in this method the analytes are preconcentrated, the backflushes, purge washes, and precolumn replacements all consume time.

ISRP PRINCIPLES

It was to obviate the difficulties invariably associated with these protein clean-up procedures that Hagestam and Pinkerton introduced a new concept in HPLC: the Internal Surface Reversed-Phase (ISRP)(1,2). As will now be explained in some detail, columns that contain ISRP packings do not retain proteins, but do retain and separate analytes of interest such as drugs and metabolites.

The external surfaces of ISRP supports are hydrophilic and do not adsorb proteins; moreover, these supports are chosen to have pores too small -- with the ISRP in place, 5.2 nm diameter -- to admit proteins. ISRP supports do not retain proteins.

The hydrophobic partitioning phase of an ISRP support, confined exclusively to the internal surface of the porous support, is accessible to the usual analytes, which are small. ISRP supports do not retain proteins but do separate analytes such as drugs.

Protein exclusion: Albumin has a molecular weight of 65,600 daltons; a prolate ellipsoid shape, 15 nm by 3.8 nm; and a radius of gyration of 3.11 nm (16). With regard to its exclusion from pores, albumin may be considered to have an effective "solid sphere" radius of 4.0 nm (17). Because spherical silica supports are made up of agglomerated silica microspheres, the pores can be modeled as squares in cross-section (18). A molecule is completely excluded from a pore when its radius equals that of the pore (17). Thus the crucial albumin, the most abundant of the proteins found in human plasma, is excluded from spherical silica pores that have diameters less than 8.0 nm.

Protein recovery: Proteins are not retained by porous spherical silicas if they are 1) size-excluded from the pores (we have just seen the conditions for this) and 2) not adsorbed onto the external surfaces. But proteins are not adsorbed onto silica surfaces to which a glycerylpropyl group has been bonded (19), given a mobile phase of appropriate pH and ionic strength.

Analyte retention and separation: The analytes, which can penetrate into the small pores from which the proteins are excluded, can be retained and separated within the pores if the surfaces there are adequate and suitable. They are adequate: The internal surface areas of typical spherical silicas are 150 to 400 square meters per gram (whereas the external surface area of a 5-micron spherical silica is only 0.5 to 1% as much: about 2 square meters per gram (20). The internal silica surface can be made suitable by bonding to it a hydrophobic partitioning phase. How this has been done for the present ISRP packings will now be described.

BASICS OF ISRP SYNTHESIS

For ISRP synthesis, a glycerylpropyl phase is first bonded to a silica particle (or glass bead) of suitably small pore diameter--as mentioned earlier, 5.2 nm after the ISRP has been affixed. A chosen polypeptide is then bonded to this glyceryl surface. By the action of an enzyme too large to penetrate the pores, the polypeptide is then removed from only the external surface (1). We now take up these treatments in more detail.

The silica or glass bead is first modified with the glycerylpropyl phase (21), then activated with carbonyldiimidazole (22), and finally coated with peptide--either the dipeptide glycine-L-phenylalanine or the tripeptide glycine-L-phenylalanine-L-phenylalanine. The external surface is then treated with the enzyme, carboxypeptidase A (23).

Carboxypeptidase acts specifically on free carboxyl groups (22), strongly selectively toward aromatic amino acids. The phenylalanines are therefore removed sequentially and quantitatively, but not the glycine.

Weighing 35,000 daltons, carboxypeptidase A has an approximate "solid sphere" radius of 3.1 nm, thus is excluded from pores smaller than 6.0 nm in diameter. Smaller than the 65,600-dalton 4.0-nm-radius albumin, this enzyme has somewhat greater access to the larger pores than do the serum proteins. Therefore serum proteins cannot reach whatever ISRP peptides may remain after enzyme treatment.

THE NATURE OF THE INTERNAL SURFACE REVERSED PHASE

Surface Chemistry

The Outer Surface

The present ISRP outer surface is glycine, attached through an amide link to the rest of the ISRP stationary phase molecule,

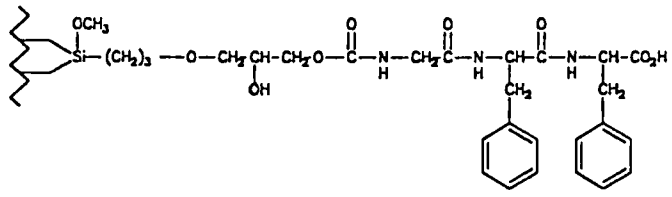


FIGURE 2. The structure of the complete GFF internal surface stationary phase is complex and sizeable.

its free carboxylic acid group displayed to the mobile phase. This outer ISRP surface does not interact with or denature proteins. However, it is similar to the inner surface in retention and ion-exchange capability--the latter to be considered presently.

The Inner Surface

The present ISRP inner surface is glycine-phenylalanine-phenylalanine (GFF), attached through the glycine amide to the link to silica (Figure 2), and like the outer surface displaying the free carboxylic acid group of the outer phenylalanine to the mobile phase. The GFF ISRP is highly selective not only to drugs--see Table I for some illustrative retentions--but also to peptides (24). Presently, we shall show that this already initially promising selectivity can be further fitted to the case at hand.

Size Exclusion Characteristics

The size exclusion characteristics of the present ISRP GFF particles are suggested in Figure 3 (25). Of six separately injected proteins of known molecular weight, two of weights below 15,000 were retained, showing penetration into the pores.

TABLE I

SOME ILLUSTRATIVE ISRP DRUG RETENTION CHARACTERISTICS

CARDIAC DRUGS/METABOLITES	RETENTION, MINUTES	k'
PROCAINAMIDE	3.5	1.1
LIIDOCAINE	4.3	1.5
2-NAPHTHOXY ACETIC ACID	5.2	2.1
NIFEDIPINE	15.	7.9
QUINIDINE	18.	9.5
PROPRANOLOL	22.	12.
alpha-NAPHTHOL	22.	12.
VERAPAMIL	28.	14.
ANTIBACTERIAL DRUGS		
SULFAPYRIDINE	3.6	1.0
SULFAMETHAZOLE	3.8	1.1
TRIMETHOPRIM	6.0	2.4
SULFASALAZINE	23.	12.
ANTIHYPERTENSIVE DRUGS		
CHLORTHALIDONE	6.4	2.6
HYDROCHLOROTHIAZIDE	7.0	3.1
FUROSEMIDE	9.2	4.4
ANTIDEPRESSANT DRUGS		
IMIPRAMINE	38.	21.
TRIMIPRAMINE	45.	25.
AMITRIPTYLINE	47.	26.
ANTIINFLAMMATORY/ANALGESIC DRUGS		
ACETYLSALICYLIC ACID	1.7	0.0
ACETAMINOPHEN	2.5	0.47
SALICYLIC ACID	3.1	0.85
IBUPROFEN	4.4	1.6
PHENYLBUTAZONE	8.2	3.9
SULFINPYRAZONE	8.4	4.0
INDOMETHACIN	14.	7.0
ANTICONVULSANT DRUGS		
ETHOSUXIMIDE	2.2	0.27
THEOPHYLLINE	2.4	0.43
PRIMIDONE	3.0	0.78
PHENOBARBITAL	4.9	1.9
CARBAMAZEPINE	7.4	3.3
PHENYTOIN	13.	6.4

Mobile phase: 0.1M phosphate/isopropanol/tetrahydrofuran 84/10/6.
Flow rate: 1.0 mL/min. Column: 15 cm x 4.6 mm ID.

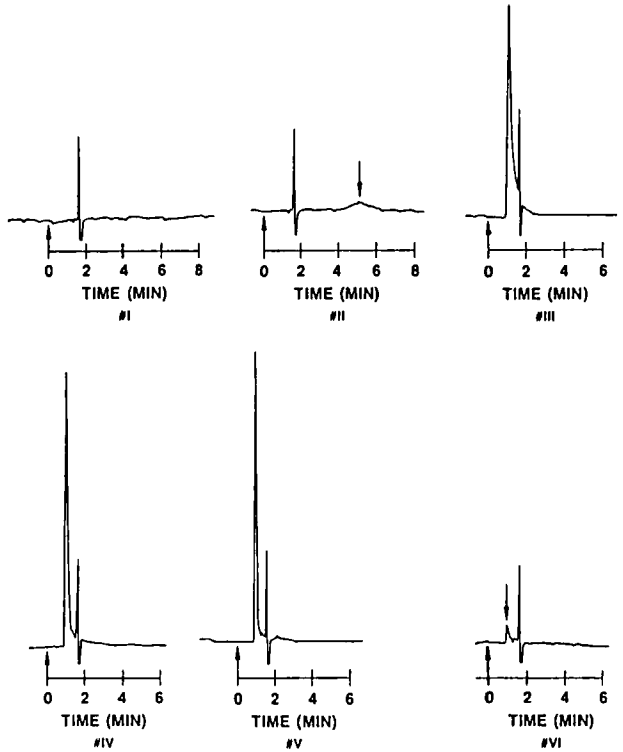


FIGURE 3. As ISRP theory suggests, the size-excluded proteins are not retained, being detected earlier than the "dead-volume" signal. The 5.2-nm pores apparently exclude proteins heavier than about 20,000-25,000 daltons--for instance, the 29,000-dalton Carbonic Anhydrase. The 12,400-dalton Cytochrome C and 6,500-dalton Aprotinin, however, enter the pores and are retained the Aprotinin, retained so long it could not be detected). Analytes: #I: Aprotinin (MW=6,500); #II: Cytochrome C (MW=12,400)(top arrow); #III: Carbonic Anhydrase (MW=29,000); #IV: Globulin (MW=55,000); #V: Bovine serum albumin (MW=66,000); #VI: Blue Dextran (MW=2,000,000)(top arrow). Sample matrix: 2.5 mg/mL of 0.1 M KH2PO4. Sample filtered through 0.2 micron membrane prior to injection. Sample size: 20 microliters. Mobile phase: 90% 0.1 M KH2PO4 (pH 6.8), 10% acetonitrile (HPLC-grade reagents). Flow rate: 1.0 mL/min. Detection: 254 nm, 0.16 AUFS. Column packings: 5-micron GFF ISRP. Column dimensions: guard, 1 cm x 3.0 mm ID; analytical, 15 cm x 4.6 mm ID.

However, the proteins of weights of 29,000 or greater were eluted within the dead volume marker, showing that they were excluded from the pores. (This is as expected. In the final step in ISRP preparation (1,2), the 36,000-dalton enzyme Carboxypeptidase A cleaves the phenylalanine residues from the GFF available to it, leaving only glycine on the outer surface.) Apparently the molecular weight cutoff of the present ISRP is between 15,000 and 25,000.

ISRP APPLICATIONS

Protein Recovery: Column Life

In ISRP functionality, "protein recovery" has just one meaning: column life. If the protein injected into the ISRP column is retained there, continuing serum injections quickly destroy the column. The first evidence of this is increase in "back pressure": the pressure required to maintain the given flow rate increases. Fairly quickly, that required pressure becomes intolerably high; the column is ruined. Meanwhile, the column efficiency also plummets.

Adequate ISRP column life--given adequate sample filtering through 0.2-micron filters before injection--is achieved in two ways: adequately functional ISRP packing, and ISRP guard columns. A proper ISRP packing retains essentially no protein. Whether the ISRP back pressure increase eventually seen with injection of enough hundreds of serum samples is caused by serum protein or by other serum components has never been determined. But there is an eventual increase in back pressure. The ISRP guard column resolves this difficulty.

The ISRP guard column can receive hundreds--over 500, generally--of the usual 10- or 20-microliter injections of serum

before it must be replaced. Meanwhile, the ISRP analytical column remains essentially unchanged (26).

Non-Drug Analyses

Toxins

Meriluoto and Erikson have recently applied the combined size exclusion and peptide-selective characteristics of the present GFF ISRP to analyze for 1,000-dalton toxins in cyanobacteria-containing environmental waters (27). As they summarized, "Use of the ISRP column drastically simplifies sample cleanup and thus shortens the total analysis time. Detection limits are good [about 1,000 micrograms per gram], and several hundred samples could be injected before it was necessary to install a new guard column."

Peptides

A new applicability of the extreme selectivity of GFF has recently been demonstrated by Pinkerton and Koeplinger (24): the separation of peptides, as shown in Figure 4.

Drugs: Free, Bound, and Whole

In serum, a drug exists in equilibrium; the bound drug is bound to protein; the free drug is not. The sum of the bound and the free drugs is the whole drug. Most drugs tend to exist nearly wholly in the bound form, i.e., to be tightly bound.

Analyses for Whole Drugs

Whole Drug Recoverability

Usually, a drug is tightly bound. Nevertheless, as has been repeatedly established and as we shall now see, when the size of

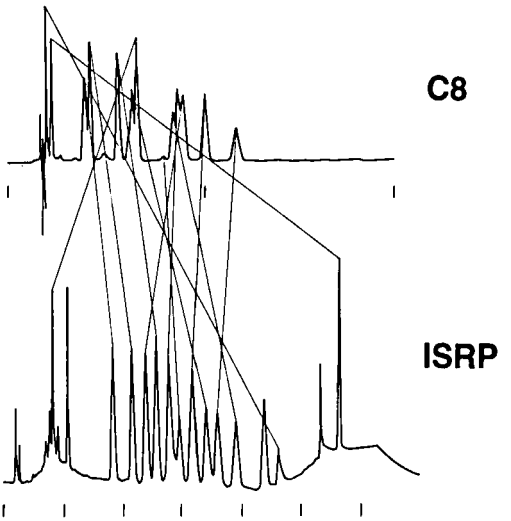


FIGURE 4. For this mixture of peptides, GFF selectivity is different from and compares favorably with that of C8. (Figure adapted from Figures 7 (for C8) and 8 (for GFF) of ref (24).)

the injected serum sample is the usual 10- or 20-microliters, the concentration of the whole drug is found by ISRP analysis.

In calibrating for the ISRP analysis of phenylbutazone in horse plasma, for instance, Sams found that "Water and plasma standards agree" (28)--the whole drug is found. This, although phenylbutazone is 98% bound.

Similarly, phenytoin is over 90% bound, but in ISRP analysis recovery is 98%--the whole drug was found (2). And further: probenecid is 83-94% bound, but 98% ISRP-recovered; and lidocaine is 65-77% bound, but 100% ISRP-recovered (29).

These conclusions are typical: Given the usual 10- or 20-microliter ISRP injection, the finding has been for the whole drug. In this matter, the following studies of Nakagawa et al. were particularly pertinent.

Organic solvent speeds drug release. In the analysis for cefpiramide in human plasma, Nakagawa et al. found that 2.5% organic solvent in the buffered mobile phase improved the chromatographic efficiency of the ISRP column, the precision of the analysis, and the degree of recovery (from 70% to 80% without the 2.5% solvent to about 100% with): "These results suggest that the organic solvent accelerates the rate of [drug] release from plasma proteins..." (29).

Variables That Affect Selectivity

Organic Modifier As just noted (29), a small mobile phase concentration of organic solvent benefits ISRP analysis in several ways. Should they be indicated, larger concentrations are also admissible.

ISRP mobile phases may contain up to 20% by volume of any of four organic solvents: acetonitrile, isopropanol, methanol, or tetrahydrofuran. Any combination may also comprise the allowable 20%. As shown below (Method Development), each of these affects selectivity in a different way.

Like the 6.0-7.5 allowable pH range, the solvent concentration maximum is set by protein, not by the ISRP column. While protein is present, it must not be denatured. Once it has been swept from the column, the mobile phase composition restrictions become no more restrictive than they are for any silica-based column.

pH (Buffered mobile phases) In Figure 5 are shown the structures of 7 drugs chosen for dissimilarity with regard to acidity-basicity, and the coding (solid line, dotted line, etc.) for retention representation of these drugs (30). In Figure 6 are

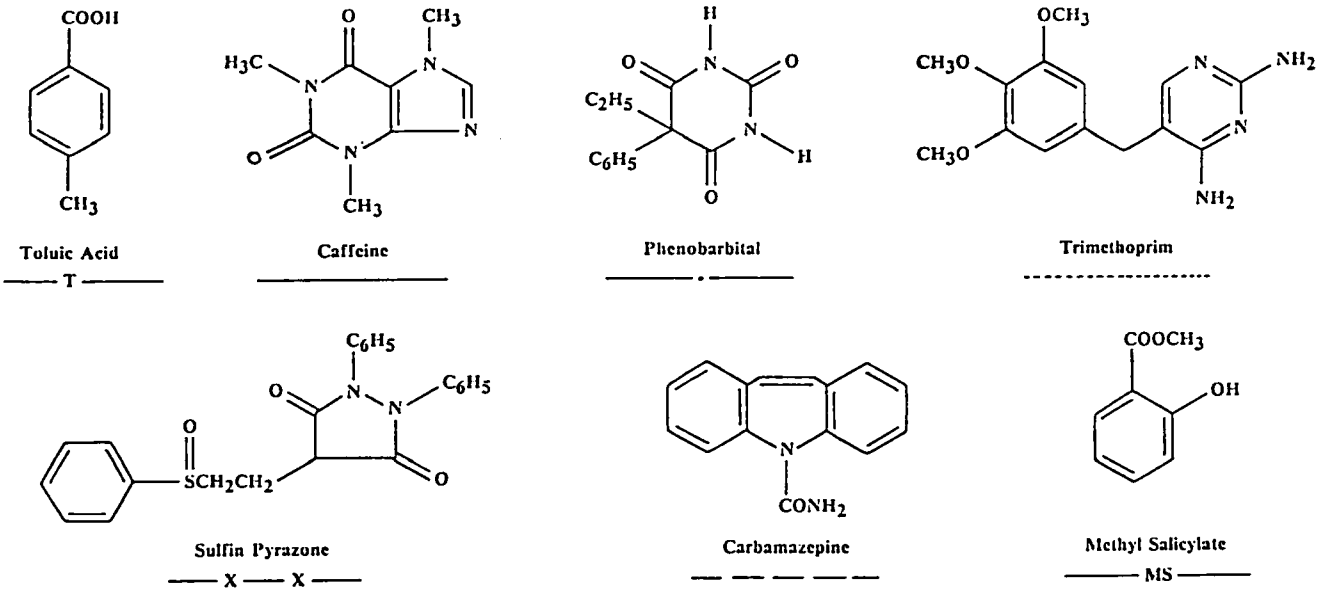


FIGURE 5. The drugs in this mixture were chosen for acid-base dissimilarity. See the ISRP behavior of them in Figure 6.

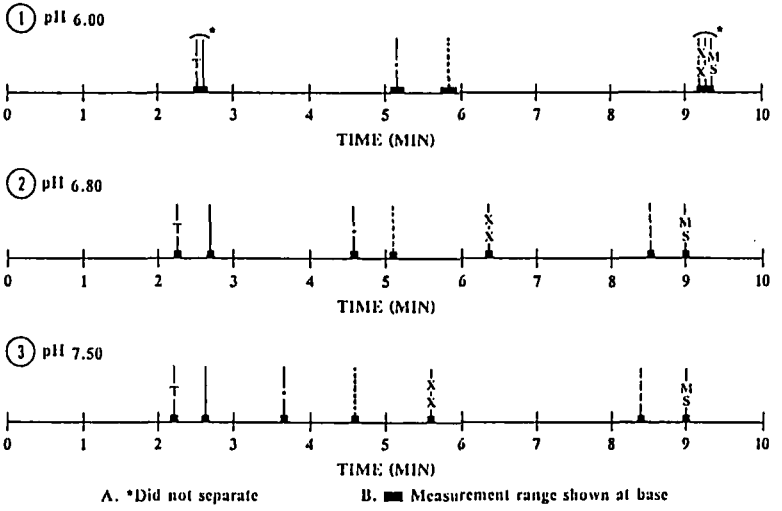


FIGURE 6. The retentions of acid-base-dissimilar drugs, the identities and structures of which are shown in Figure 5, nicely illustrate the increase of GFF selectivity with increasing pH. Mobile phase: 80% 0.1 M KH2PO4 (pH's 6.0, 6.8, 7.5), 20% acetonitrile (HPLC-grade reagents). Mobile phase flow rate: 1.0 ml/min. Sample size: 200 microliters. Column dimensions: 15 cm x 4.6 mm ID. Packing: 5-micron GFF ISRP.

shown the retentions of these drugs at 3 pH's: 6.0, 6.8, and 7.5 (30).

It can be seen that selectivity increases strongly with increasing pH. (For 21 drugs studied at pH 7.5 by Sams and Evec, ISRP GFF selectivity was found to be more than twice that of ODS. (31). As can also be seen, retention varies inversely with pH, although relatively weakly.

The decrease of GFF selectivity with decreasing pH can be explained. The ISRP GFF presents a free carboxylic acid group to the usually buffered, aqueous mobile phase. With increasing acidity, the ionization of that carboxylic acid group tends increasingly to be suppressed, and with it, GFF capability of participating in ion exchange.

Achieving reproducible retentions with GFF requires equilibration. Equilibration should not be considered initially assured until no further drift in retentions is seen after 3 successive injections of a given sample.

(Non-buffered mobile phases) As a mobile phase, simple water may reflect whatever pH happened to exist previously in the column. The retention of theophylline demonstrates these points. With buffered mobile phases, the retention of theophylline does not change as a function of pH. However, with only straight water as mobile phase, theophylline retention varies over a factor of 3, depending on the pH of the mobile phase used before the water! This unexpected behavior is shown in Figure 7. Obviously, ISRP mobile phases should be buffered.

Temperature In Table II, the retentions of 4 drugs are shown for column temperatures 10o C. and 26o C. Decreasing the temperature tends to increase GFF retention. In Table III, the ratios of these retentions for these temperatures show that GFF selectivity increases strongly with decrease in column temperature.

Method Development

An early ISRP brochure showed several separations all made with a mobile phase of only a single composition. However, as the following study in method development illustrates, a wide variety of mobile phase compositions is usable.

In Figure 8 are shown the structures of 3 drugs--furosemide, phenylbutazone, and oxyphenbutazone--that are commonly administered to and must be analyzed for in racehorses. The intimidating complexity of these structures need not be considered in method development. In a straightforward, empirical

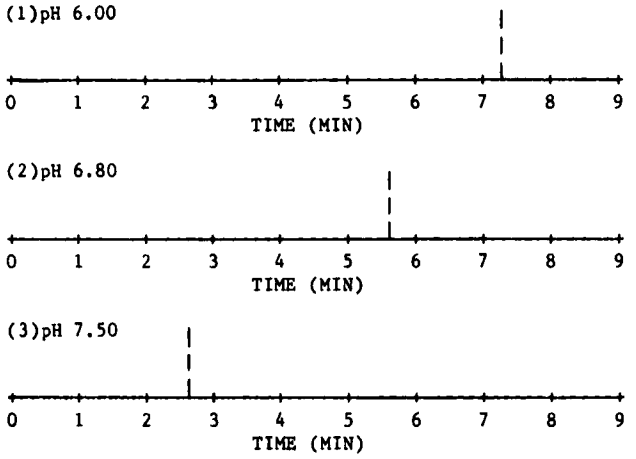


FIGURE 7. The retention of theophylline by GFF does not change with pH when the mobile phase is buffered. However, as shown here in diagram, if the mobile phase is straight water, then the theophylline retention reflects the pH of the previous mobile phase and changes radically with that pH. GFF mobile phases should always be buffered. Mobile phase: water (HPLC grade). Mobile phase that preceded water: 80% 0.1 M KH_2PO_4 (pH's 6.0, 6.8, 7.5), 20% acetonitrile (HPLC-grade reagents). Mobile phase flow rate: 1.0 ml/min. Sample size: 20 microliters. Column dimensions: 15 cm x 4.6 mm ID. Packing: 5-micron GFF ISRP.

TABLE II

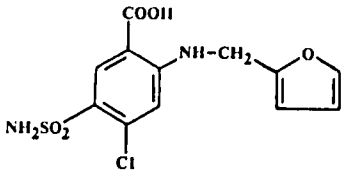
DECREASING COLUMN TEMPERATURE INCREASES ISRP RETENTION

Drugs	Retention, minutes	
	Column temperature	
	26o C.	10o C.
Caffeine	2.63	2.65
Phenobarbital	3.76	4.35
Sulfinpyrazone	4.87	6.34
Carbamazepine	5.58	7.58

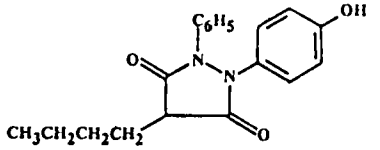
TABLE III

DECREASING COLUMN TEMPERATURE
INCREASES ISRP SELECTIVITY

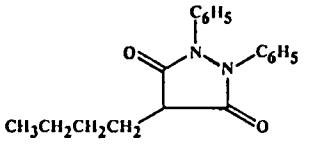
Drugs	Separation factor	
	Column temperature 26o C.	10o C.
Phenobarbital / Caffeine	1.43	1.64
Sulfinpyrazone / Phenobarbital	1.30	1.47
Carbamazepine / Sulfinpyrazone	1.15	1.20



FUROSEMIDE
(Lasix)



OXYPHENBUTAZONE
(Oxalid)



PHENYLBUTAZONE
(Azolid)

FIGURE 8. The structures of three drugs--furosemide, phenylbutazone, and oxyphenbutazone--to be separated from each other and from serum. In method development, use straightforward empiricism. Methodically determine the mobile phase composition that works, rather than try to predict it from these structures and that of the stationary phase GFF (see Figure 2).

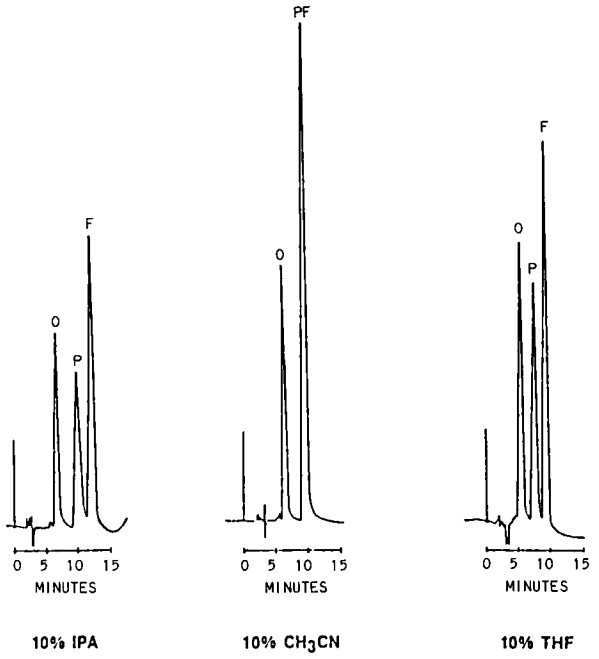


FIGURE 9. Which organic modifier should be used? Immediately, separations show that tetrahydrofuran is the one of choice. GFF ISRP column length: 15 cm. Mobile phase composition: 0.1 M KH2PO4 buffer/10% organic modifier; pH 6.8. Flow rate: 1.0 mL/min.

way that recommends itself, John David Rateike of our laboratories developed an ISRP separation for these, as follows.

ISRP selectivity was optimized for this separation by altering the mobile phase. Organic modifiers were tested first, then pH. Four organic modifiers are usable, each up to as much as 20% by volume: acetonitrile (MeCN), isopropanol (IPA), methanol (MeOH), and tetrahydrofuran (THF). The separations that resulted from using 10% of three of these is shown in Figure 9 (methanol was not tested because at the time of this work the use of it was mistakenly thought prohibited). Obviously, THF was here the modifier of choice.

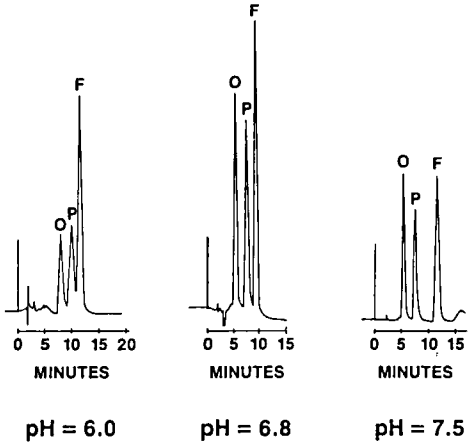


FIGURE 10. Varying the pH of the mobile phase in this separation of furosemide, phenylbutazone, and oxyphenbutazone brings out a general truth: between pH's 6.0 and 7.5, GFF selectivity increases with pH. Experimental conditions as stated in Figure 9, with tetrahydrofuran as buffer.

Next, pH was varied between 6.0 and 7.5. In this case, the retentions varied as shown in Figure 10. The quite general conclusion--already noted: Increasing pH increases selectivity; decreasing pH increases retention somewhat but decreases selectivity. In this case, the best separation occurs at pH 7.5. (Again, note that the pH range is limited not by the ISRP column but by protein, which must not be denatured but remain globular and pass right through the column. Once the protein has left the ISRP column, pH may be varied at will, over the full range available to any silica-based column.)

The final separation of the drugs from horse plasma and from each other is shown in Figure 11 (32; see also 33).

Analyses for Free and Bound Drugs

In a lecture presented in 1987 in Japan (34), Dr. Pinkerton described the discovery in his laboratories of the ISRP

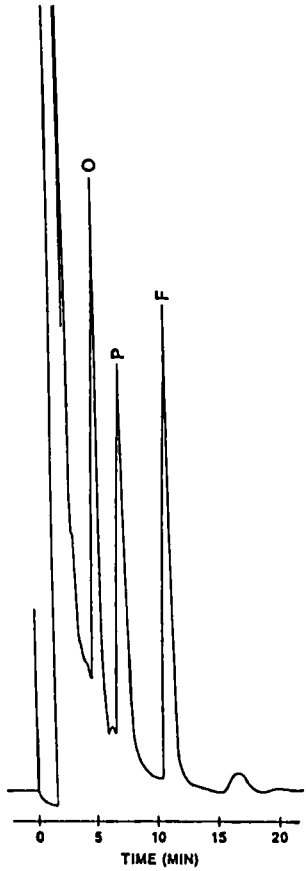


FIGURE 11. The optimized 12-min separation of furosemide, phenylbutazone, and oxyphenbutazone from each other and from serum. GFF ISRP column, 15 cm x 4.6 mm ID. Mobile phase composition: 0.1 M KH2PO4 buffer/10% organic modifier; pH 7.5. Flow rate: 1.0 mL/min.

capability for separating the free drug forms from the bound, and its elucidation. In 1989, this description became available in Analytical Chemistry (35): "When [imirestat-containing] serum samples in excess of 200 (microliters) were injected onto a 5-cm ISRP column...the imirestat eluted...unexpectedly... as two peaks...The same [phenomenon]...was observed with [a separate, similar injection of] phenytoin. [See Figure 12.]

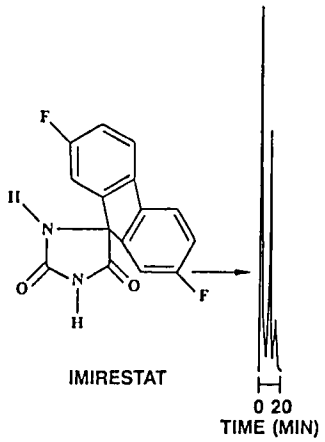


FIGURE 12. "When [imirestat-containing] serum samples in excess of 200 (microliters) were injected onto a 5-cm ISRP column...the imirestat eluted...unexpectedly...as two peaks...The same [phenomenon]...was observed with [a separate, similar injection of] phenytoin." (35) From this observation was developed the ability to determine, from one injection into an ISRP column, the drug distribution between bound and free states, and even between different bound states. In the imirestat chromatogram shown, the first peak is serum, the second peak is the "bound" drug--the drug that had been bound to protein at the time of injection--and the third and last peak is the "free" drug. For this work, the column was a GFF ISRP, 5 cm x 4.6 mm ID; the mobile phase, 0.01 M phosphate (pH 6.8); the flow rate, 1.0 mL/min. Imirestat at 20 micrograms/mL: 200 microliters injected; phenytoin at 51 micrograms/ml: 500 microliters injected.

"...it was surmised that the peaks were fractional parts of the drug, which had been split by an on-column effect.

"[Further study indicated that] the split-peak phenomenon was uniquely dependent on sample size...[and] required...a serum matrix...the on-column presence of drug and serum proteins.

"...the area of the first peak should approximate the drug bound to the protein prior to injection...the second peak should represent the free drug and any drug released immediately on introduction to the column.

"The splitting of the chromatographic drug fraction into two peaks is facilitated when the following conditions are met: the

sample contains binding proteins, the sample size is [equal to or greater than] 200 [microliters], and the mobile phase is weak.

"Subsequent work by investigators at Kyoto University (39) using warfarin, bovine serum albumin (BSA), a 4.6 mm X 25 cm ISRP column, and a 200-[microliter] sample size has demonstrated the same type of peak-splitting phenomenon (35)." (See also 36.)

Shibukawa et al. reported further investigations of this ISRP capability. Extending it to frontal analysis, they were able to determine the free drug concentrations of warfarin and of indometacin (37). Later, through the two warfarin peaks eluted from an ISRP column, they evaluated the warfarin-albumin interactions for both strong and weak drug-protein binding; in this case, the free warfarin (1%) was evaluated by ultrafiltration (38). (See also 39.)

In summation, Dr. Pinkerton wrote: "...drugs [that] are strongly bound to proteins may elute as two peaks when a serum sample is directly injected onto an ISRP column, under very select conditions. [The] drug molecules bound to a strong primary binding site experience a delayed release, [whereas] drugs bound to weaker secondary binding sites are released immediately on introduction to the mobile phase. As a result of the exclusion of proteins from the packing, the drug [that] had been bound to the primary site elutes in a major peak ahead of a second smaller peak, which contains both the drug bound initially to secondary sites and the original free drug. The peak splitting [depends markedly] on column length, sample size, and...mobile phase...The peak positions can be controlled with mobile phase ionic strength, pH, and organic modifier concentration. The peaks can be easily merged into one peak if desired. The phenomenon has been observed for phenytoin and its derivatives in human serum

(34,35,40), and warfarin combined with bovine serum albumin(BSA)(39,41). If the drug is combined with pure protein and the free drug concentration is measured by an independent method (e.g., ultrafiltration), then the concentration of the drug bound to the secondary site can be determined from the second peak by difference. With this and a knowledge of the number of moles bound per site, the binding constants for each binding site can be directly calculated (41)."

It becomes apparent that another powerful clinical and research avenue has been opened by ISRP.

POTENTIAL NEW ISRP'S

In a silica-based packing, at the base of each stationary phase molecule is a silicon through which the molecule is attached to the silica particle. If to this silicon are attached two methyl groups, that stationary phase is intrinsically monomeric. We have made such intrinsically monomeric stationary phases for glycine-phenylalanine (GF), glycine-phenylalanine-phenylalanine (GFF), and glycine-phenylalanine-phenylalanine-phenylalanine-phenylalanine (GF4).

In Figure 13 the retentions and chromatographic efficiencies of "classical" (present) GFF, monomeric GF, and monomeric GFF are presented for intercomparison. In Figure 13A, the chromatograms are shown; in Figure 13B, the retentions are shown in diagram, and the corresponding chromatographic efficiencies.

Both the GF and GFF monomeric materials show greater retentions and efficiencies than the "classical" GFF. Indeed, the monomeric GF and GFF efficiencies reach 55,000 plates per meter: reasonably efficient by current HPLC standards. The relatively low efficiency of "classical" GFF apparently has been imposed by

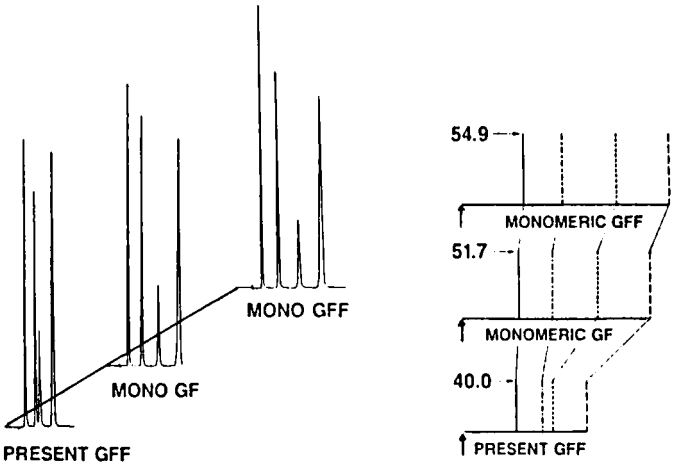


FIGURE 13. The retention, selectivity, and efficiency of "classical" GFF, monomeric GF, and monomeric GFF are here intercompared in chromatographic and diagrammatic forms. On all counts, the monomeric materials are improvements over the classical. They also demonstrate that GFF is not inherently chromatographically inefficient. In the diagrams, the numbers show thousands of theoretical plates per meter. Column: 15 cm x 4.6 mm ID. Sample volumes: 10 microliters. Solutes, in order of appearance: caffeine, phenobarbital, trimethoprim, and carbamazepine. Mobile phase composition: 0.1 M KH_2PO_4/acetonitrile, 80/20 v/v. Flow rates: 1.0 mL/min. Detection: 254 nm, 0.1 AUFS.

its physical form rather than by its multiplicity of retention mechanisms.

The monomeric GF4 shows retentions even greater than the monomeric GFF, but its efficiency is only a relatively low 25,000 plates per meter.

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