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Note

Separation of furosemide, phenylbutazone and oxyphenbutazone in plasma by direct injection onto internal surface reversed-phase columns with systematic optimization of selectivity

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Furosemide and phenylbutazone are common drugs of abuse in veterinary medicine. Furosemide is a potent diuretic which causes rapid fluid loss and improved muscle tone in livestock. Phenylbutazone and its primary metabolite oxyphenbutazone exhibit antipyretic and analgesic properties. The molecular structure of each is illustrated in Fig. 1. A variety of liquid chromatographic methods have been developed to quantify these drugs in mammalian plasma[1–5]. All of these methods involve the use of a sample clean-up procedures to remove proteins prior to injection onto high-performance liquid chromatography (HPLC) columns. These clean-up procedures are time consuming and loss of analytes during extraction may occur. Since rapid plasma level monitoring of these substances is desired, direct injection of the plasma onto an HPLC system is preferred; however, this is impractical with conventional reversed-phase columns.

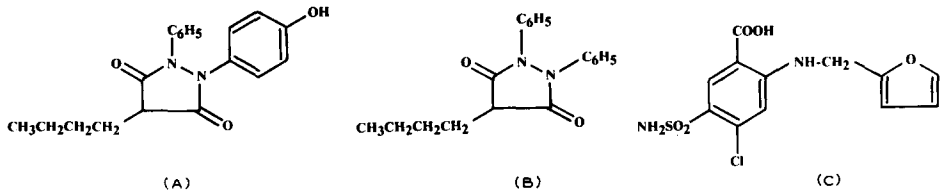

Fig. 1. Structures of (A) oxyphenbutazone, (B) phenylbutazone, and (C) furosemide.

Historically the quantitative determination of drugs in serum and plasma by HPLC has required laborious sample clean-up procedures to remove proteins before injection onto small particulate HPLC columns. Recently, the introduction of internal surface reversed-phase (ISRP) supports[6–8], has enabled the production of small particulate (5 μm) HPLC columns which can sustain the direct injection of serum and plasma samples without significant loss of performance[9].

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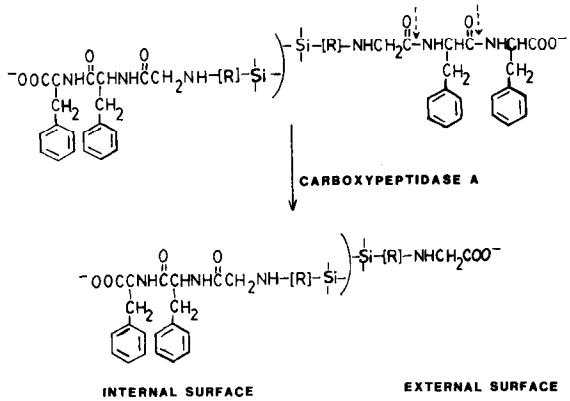

Fig. 2. Structure of internal surface reversed-phase, where Si[R]- is Si-$OCH_2CH_2CH_2O$-$CH_2CH(OH)CH_2OOC$-.

The ISRP concept is based upon size exclusion and internal surface partitioning. The ISRP supports have a median pore diameter of 52 Å (ref. 10), thus confining proteins to the interstitial space. The external surfaces of the spherical silica supports are rendered non-adsorptive to the proteins via a glycerylpropyl-bonded phase, so proteins elute in the interstitial void volume. Drugs and metabolites, on the other hand, penetrate the packing and interact with a glycine–phenylalanine–phenylalanine partitioning phase (Fig. 2). Prior to packing, the phenylalanine moities are removed from the external surface by enzyme cleavage, so the external surface of the supports remains non-adsorptive to proteins[8].

In developing an HPLC separation it is common practice to select an initial mobile phase condition based upon the analyte molecular structure and an intuitive knowledge of the retention characteristics of the bonded phase in use. Invariably the first separation will yield overlapping peaks. One then proceeds to improve resolution by trial and error or by systematically optimizing capacity factors, selectivity, and efficiency.

In this paper, a systematic approach to optimizing the selectivity of furosemide, phenylbutazone, and oxyphenbutazone on ISRP columns is demonstrated. The results illustrate the unique molecular interactions of the solutes with the glycine–phenylalanine–phenylalanine partitioning phase of the ISRP packing.

EXPERIMENTAL

Apparatus

The chromatographic separations were carried out by the Regis Chemical Company. Separations were conducted on either a 25 cm × 6 mm I.D. or 15 cm × 4.6 mm I.D. internal surface reversed-phase column (GFF-S5-80) manufactured by the Regis Chemical Company, Morton Grove, U.S.A. The internal surface reversed-phase supports were produced with 5-μm spherical silica by synthetic procedures described elsewhere[8]. The chromatographic system consisted of an Altex/Beckman Model 110A pump, a Rheodyne Model 70-10 injection valve, an ISRP column, and a Kratos 757 Spectroflow variable-wavelength UV detector set at 241 nm.

Materials

The drugs furosemide, phenylbutazone and oxyphenbutazone were purchased from Sigma. The HPLC grade water, isopropanol, acetonitrile, and tetrahydrofuran were obtained from Baker. The HPLC grade potassium dihydrogen phosphate utilized in the preparation of aqueous buffers was purchased from Fisher Scientific. The horse plasma was kindly furnished by Dr. Robert McKenzie of the Michigan Department of Agriculture, East Lansing, MI, U.S.A.

Chromatographic procedure

Prior to the direct sample injection, the horse plasma was filtered simultaneously through two Gelman filters: first, a Type A/E glass fiber filter, and second, a Turryn™ membrane filter of 0.2 μm porosity. The drugs were present in each sample at a concentration of 20 μg/ml. Unless otherwise stated, the sample size was 10 μl; the flow-rate was 0.6 ml/min; the detector attenuation was 0.05 a.u.f.s.; and the temperature was ambient.

RESULTS AND DISCUSSION

Selectivity of ISRP supports

The glycine–L-phenylalanine–L-phenylalanine internal partitioning phase of the ISRP columns interacts most readily with aromatic solutes. The capacity factors of 36 drugs substances have been measured[9] with 0.1 *M* phosphate–isopropanol–tetrahydrofuran (84:10:6), pH 6.8 as mobile phase. The oxopurines, pyrimidines, single ring heterocyclics, sulfonamides, and single ring aromatics yield capacity factors ranging from 0 to 2. Substituted compounds with two aromatic rings exhibit capacity factors from 2 to 12. Substances with 3 aromatic rings or diaryl substituted compounds with extended aliphatic chains produce capacity factors from 14 to 26.

Tetrahydrofuran, isopropanol, and acetonitrile have been selected as organic modifiers for use with ISRP columns. Serum proteins are found not to precipitate in the presence of these solvents when maintained at concentrations less than 10%, 20%, and 25% (by vol.), respectively. These solvents, when combined with water,

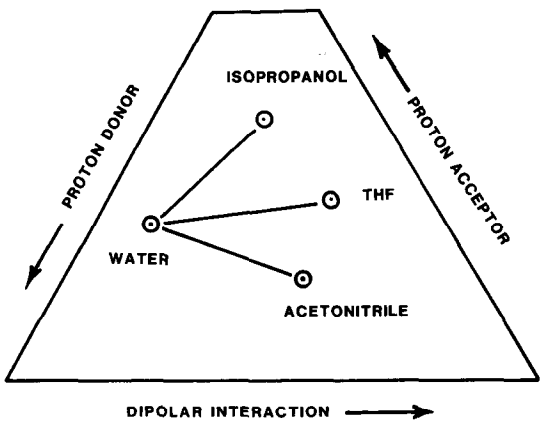

Fig. 3. Synder's solvent selectivity triangle.

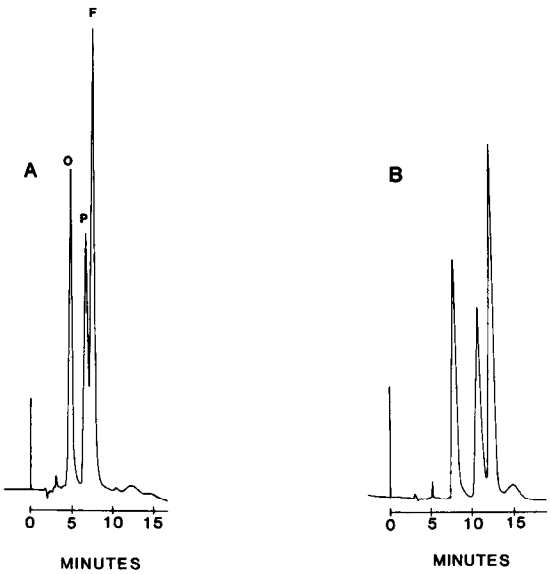

Fig. 4. Separation of a standard mixture of oxyphenbutazone (O), phenylbutazone (P), and furosemide (F) on ISRP columns of 15 cm (A) and 25 cm (B) in length with 0.1 *M* phosphate–isopropanol–tetrahydrofuran (84:10:6) (pH = 6.8) as mobile phase.

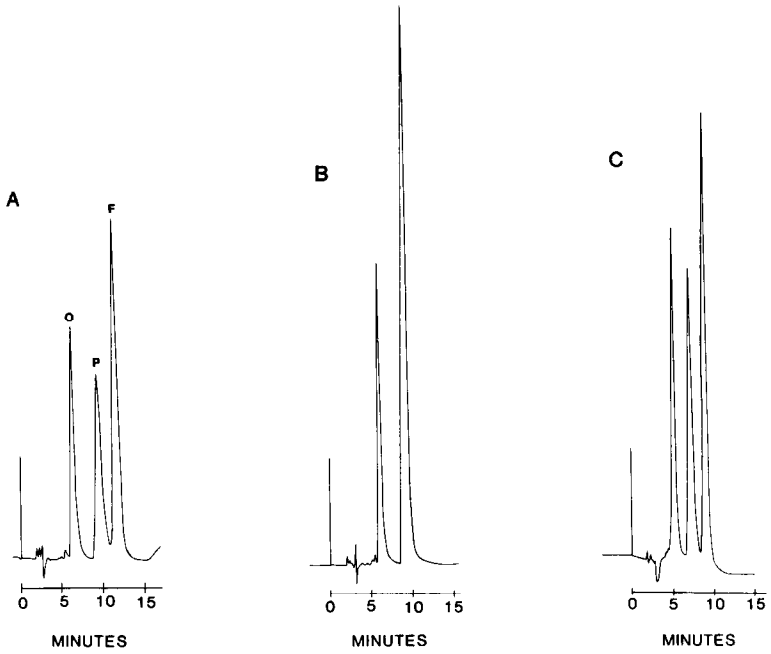

Fig. 5. Separation of the drug mixture on a 15-cm ISRP column with systematic variation in organic modifier. Mobile phase compositions: (A) 0.1 *M* phosphate–isopropanol (90:10); (B) 0.1 *M* phosphate–acetonitrile (90:10); (C) 0.1 *M* phosphate–tetrahydrofuran (90:10).

afford a broad range of selectivity control in reversed-phase chromatography. As illustrated by Synder's triangle (Fig. 3), water acts as the proton donor, while the remaining modifiers vary in proton acceptor and dipolar interactions[11].

Developing an ISRP separation

A mixture of furosemide, phenylbutazone, and oxyphenbutazone in water was chromatographed with 0.1 *M* phosphate–isopropanol–tetrahydrofuran (84:10:6), pH 6.8, as mobile phase. The drugs eluted in the order oxyphenbutazone, phenylbutazone, and furosemide with the latter two being unresolved on a 15-cm ISRP column (Fig. 4A). Increasing the column length to 25 cm does not achieve baseline resolution (Fig. 4B).

In order to evaluate the selectivities of the solutes with regard to each organic modifier, the drug mixture was separated with three separate mobile phases consisting of 90% 0.1 *M* phosphate buffer and 10% of each modifier, respectively. The elution order remained the same in each case. With isopropanol the analytes were retained longer with only slight improvement in the resolution of phenylbutazone and furosemide (Fig. 5A). With acetonitrile the oxyphenbutazone was retained to the same degree but phenylbutazone and furosemide remained unresolved (Fig. 5B). With tet-

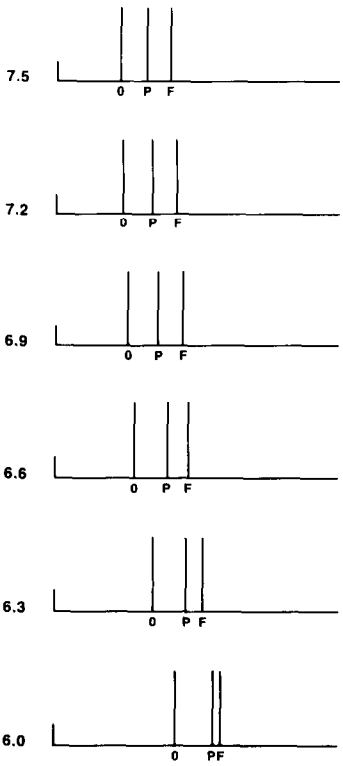

Fig. 6. Illustration of changes in relative retention of oxyphenbutazone (O), phenylbutazone (P), and furosemide (F) as a function of pH.

rahydrofuran all of the drugs eluted more rapidly with a greater degree of resolution. The latter mobile phase was obviously the preferred.

Using 0.1 *M* phosphate–tetrahydrofuran (90:10) as mobile phase the pH was varied from 6.0 to 7.5. The results illustrated in Fig. 6 indicate a decrease in retention for all the drugs from pH 6.0 to 6.6, and a selective decrease in the retention of phenylbutazone from 6.6 to 7.5. This is logical since the drugs are basic and would be protonated to some degree under acidic conditions. This indicates a weak ion-exchange interaction with the terminal carboxylic groups of the partitioning phase (Fig. 2). The relative changes in these shifts merely reflect the differences in acid dissociation constants of the protonated drugs.

In sum, one can optimize the selectivities of an ISRP separation in a manner similar to that followed with other types reversed-phase columns. Separate the mixture with each organic modifier present in 10% (by vol.). If separation is achieved, adjust the pH between 6.0 and 7.5 to further improve resolution. If the mixture is not separated with a binary mobile containing one modifier, prepare a ternary mobile phase consisting of buffer and two modifiers. Using knowledge of solute–solvent interactions gained from the initial separations, modifier concentrations can be change to achieve desired resolution[11]. In this case, optimum resolution is achieved with 0.1 *M* phosphate–tetrahydrofuran (90:10) at pH 7.5. A separation of the drugs in horse plasma under these optimum mobile phase conditions is seen in Fig. 7. Baseline resolution is accomplished on either a 15-cm or 25-cm ISRP column.

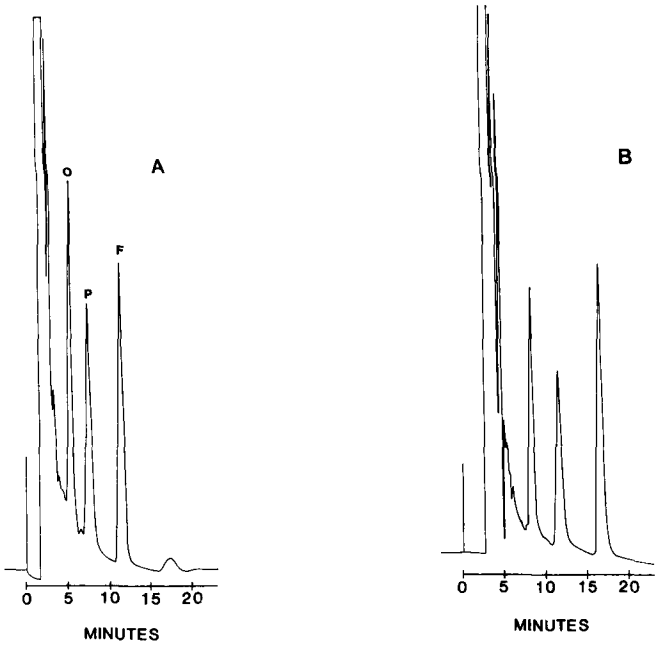

Fig. 7. Separation of drugs in horse plasma by direct injection onto ISRP columns under the optimum mobile phase conditions of 0.1 *M* phosphate–tetrahydrofuran (90:10) (pH = 7.5). The columns lengths are (A) 15 cm and (B) 25 cm.

Solute–solvent interaction

Understanding the solute–solvent intermolecular interactions which control the reversed-phase separation mechanism on the ISRP supports is not straightforward. Clearly each solute is sufficiently aromatic to interact with the phenylalanines of the partitioning phase. Also, it is not surprising that the oxyphenbutazone metabolite elutes first, being the least hydrophobic. With isopropanol and tetrahydrofuran having comparable "polarity indices"[11] and proton donor characteristics, the greater retention with the isopropanol compared to the tetrahydrofuran implies that the solutes are responding to the proton donor. The reversal of this trend with the acetonitrile, which has a greater "polarity index", along with the increase in relative retention of phenylbutazone to a point of overlapping with the furosemide (Fig. 4B), indicates that the greater polarity does not favor the elution of the phenylbutazone, which is less polar. The results suggest that elution of the drugs with optimum resolution is favoured at low polarity with sufficient proton donor–solvent interaction. This implies that the glycine–phenylalanine–phenylalanine phase, in addition to its primary hydrophobic interaction and weak ion-exchange interaction, may be exhibiting some hydrogen bonding. This would not be surprising considering the partitioning phase is a polypeptide.

CONCLUSION

Although the solute–solvent interactions on the ISRP columns appear complex, the selectivities are predictable and consistent with other reversed-phase separations. Resolution of solutes can be affected by exploiting solvent interactions of organic modifiers as systematically varied in the standard fashion, along with pH optimization.

ACKNOWLEDGEMENT

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