

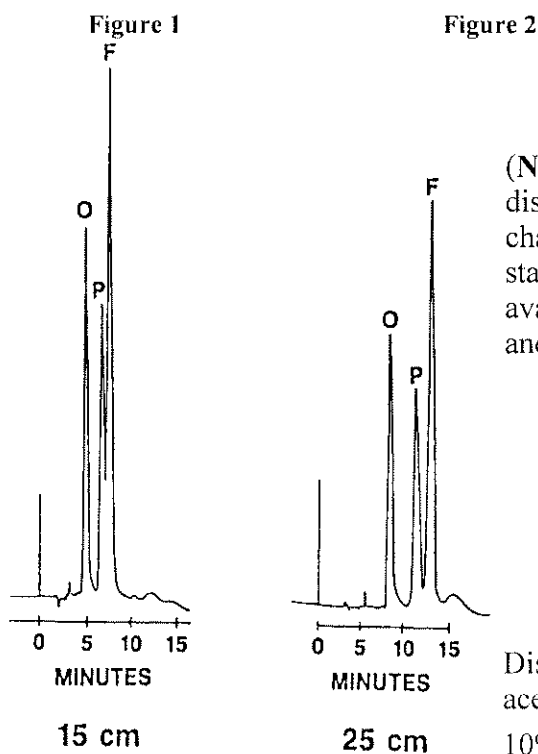
NOTE NO. 24 - July 23 1987

## OPTIMIZING SELECTIVITY WITH INTERNAL-SURFACE REVERSED-PHASE HPLC COLUMNS

Discussion: At the 1987 Pittsburgh Convention, Regis presented four papers. One was on optimizing ISRP selectivity. From the many questions we have received from those who heard the papers and also from those who did and have not, we have realized that the information in them should be as quickly and widely disseminated as we can arrange. As a beginning, we shall summarize the paper as Application Note No. 24. Herewith follows the gist of the paper on selectivity:

The retention data listed in the ISRP brochure constitute merely one set among many sets that are perfectly usable. The brochure data hold only for one set of conditions--one modifier, its concentration, one pH. The statement of those conditions originally constituted and does now constitute neither a recommendation nor a directive. The user should experiment: Varying and optimizing conditions usually results in a satisfactory separation. Such experimentation is properly empirical, phenomenological: just observe what happens as conditions are systematically varied, and do what the data suggest.

Consider the separation from each other and from horse plasma of furosemide (F), phenylbutazone (P), and oxyphenbutazone (O). The "suggested" mobile phase of the ISRP brochure does not produce a good separation (Figure 1), and lengthening the column from 15 cm to 25 cm (Figure 2) does not help enough. We proceed, then, as just suggested, in a systematic but empirical way: vary the modifiers, then the pH.



(Note: We do not try to predict results. As a rule we do not attempt to disentangle, much less to predict, the interactions between the various partially charged functional groups of the glycine-phenylalanine-phenylalanine stationary phase, the functionalities of the organic modifiers, and the protons available at the various pH's. Separations are best explained only if necessary and then only after having been achieved.)

Discussion (continued): First, we tried each of 3 organic modifiers separately: acetonitrile ( $\text{CH}_3\text{CN}$ ), isopropanol (IPA), and tetrahydrofuran (THF), each at 10% concentration--see Figure 3. Obviously, THF did best. Therefore, with THF as the 10% organic modifier, the pH was varied within the permissible 6.0-7.5 range (Fig. 4). Obviously, again, pH 7.5 was best.

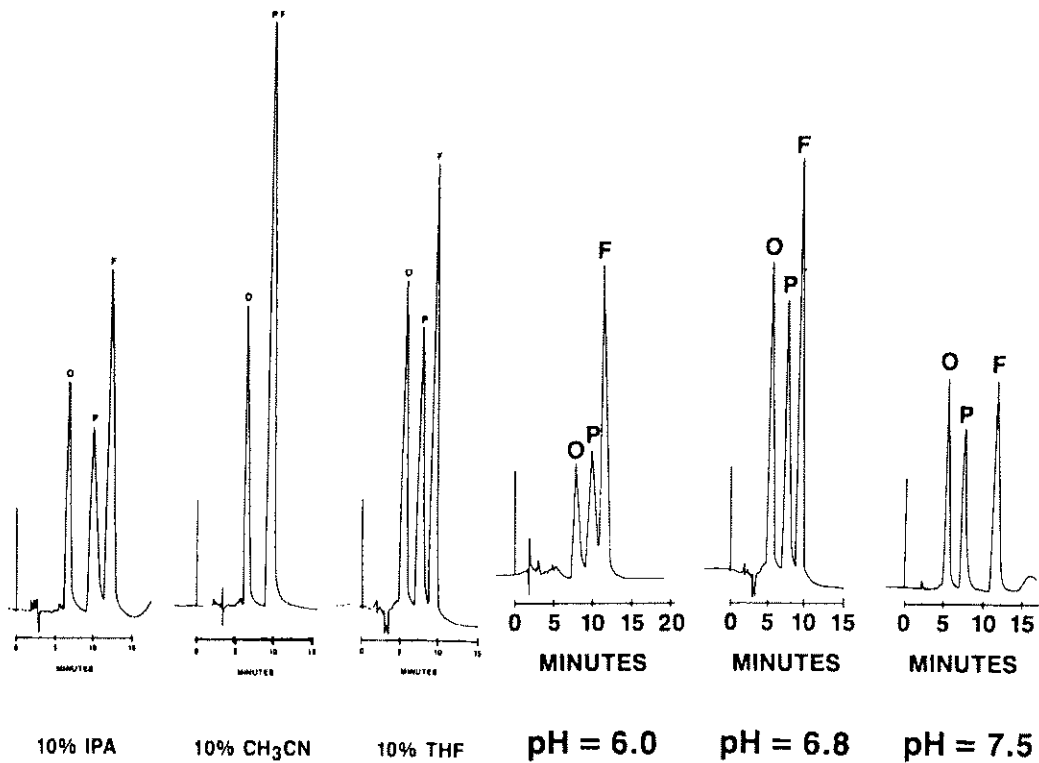


Figure 3

Figure 4

**Discussion (continued):** In Figure 5, the six retention patterns observed between 6.0 and 7.5 are diagrammatically presented: any pH between 6.8 and 7.5 would have done. The final result, achieved with 10% THF, pH 7.5, and a 15-cm column, is shown in Figure 6.

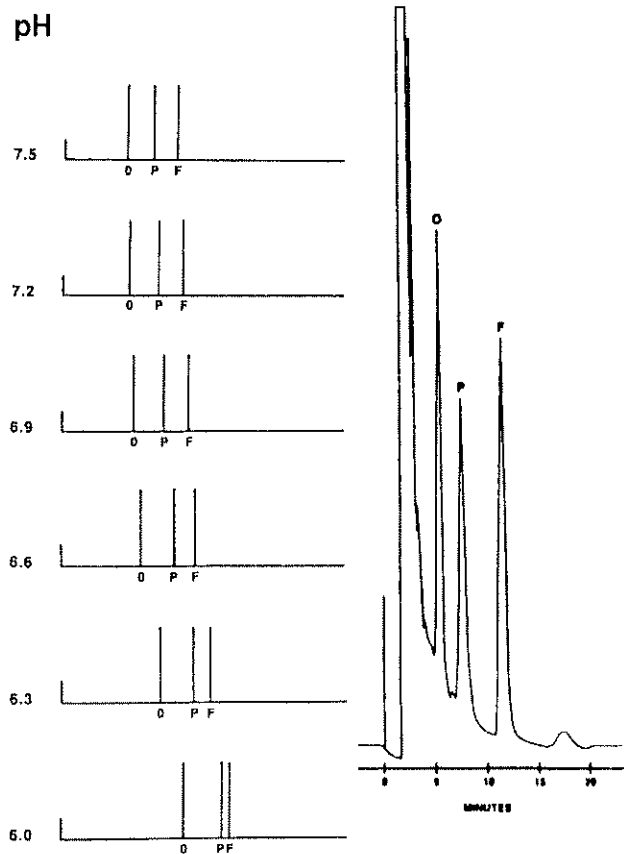


Figure 5

Figure 6

## Discussion (continued):

MOBILE PHASE: 20% CH<sub>3</sub>OH

0.1M KH<sub>2</sub>PO<sub>4</sub>

$\alpha = 1.11$

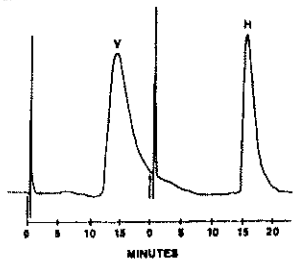


Figure 7

MOBILE PHASE: 20% IPA

0.1M KH<sub>2</sub>PO<sub>4</sub>

$\alpha = 1.36$

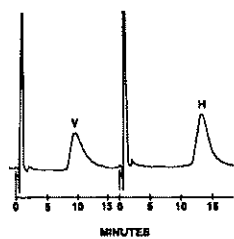


Figure 8

Figure 9

### SEPARATION OF HYDROXYZINE AND VERAPAMIL

MOBILE PHASE:

20% CH<sub>3</sub>CN

0.1M KH<sub>2</sub>PO<sub>4</sub> pH = 6.8

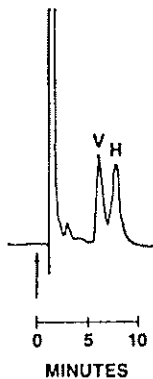
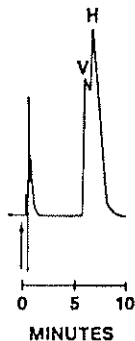
$\alpha = 1.16$

MOBILE PHASE:

20% THF

0.1M KH<sub>2</sub>PO<sub>4</sub> pH = 6.8

$\alpha = 1.31$



**Next example:** Verapamil (V) is not well separated from hydroxyzine (H) with the mobile phase and column length "suggested" in the brochure: the substances are retained too long, the peaks are broad, the absorptivities at 254 nm are too low. During method development, a 5-cm column and a faster-than-normal 1.0 ml/min flow rate were used to get the peaks to come out quicker. Also, to make the results easier to observe during the development, unusually high concentrations, 1.0 mg/ml, were used. Then development proper began with modifier variation: in 0.1M buffer, 20% each of MeOH (Figure 7), IPA (Figure 8), and CH<sub>3</sub>CN and THF (Figure 9). (Notice that at first--Figures 7 and 8--the verapamil and hydroxyzine were injected separately, making for easier observation until retention order was securely established.)

The results to this point: poor peak shapes with MeOH and IPA, but improved and usable relative retention (1.36) with IPA; good peak shapes with CH<sub>3</sub>CN and THF, poor relative retention again with CH<sub>3</sub>CN, but reasonably good

relative retention with THF. The IPA/THF ratio was then varied and tested to see if good peak shape could be retained while relative retention was improved. It could. Figure 10 shows the separation with buffer/IPA/THF 80/15/5, wherein relative retention has also become a very usable 1.51. Some further tests produced the optimum, shown in Figure 11: buffer/IPA/THF 80/12.5/7.5; relative retention 1.53. Finally, to improve detectability, the detection wavelength was moved to 230 nm. The final and optimized separation is shown in Figure 12.

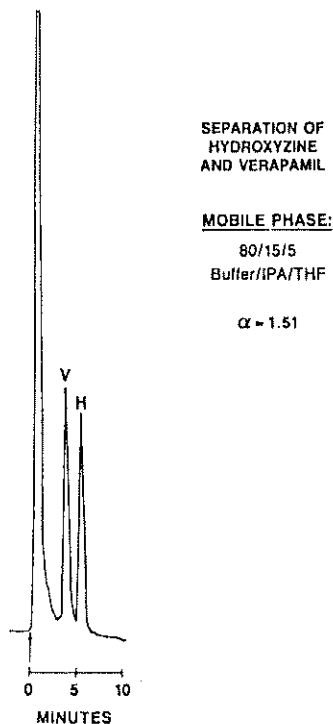


Figure 10

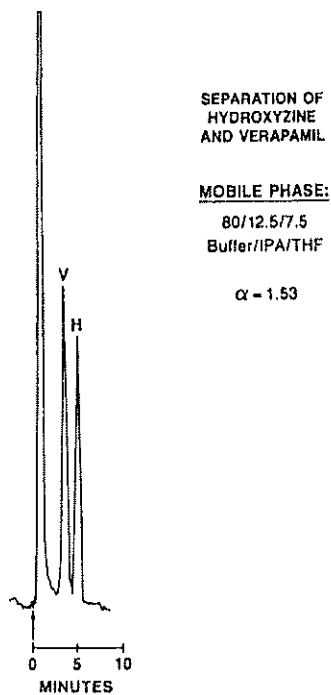


Figure 11

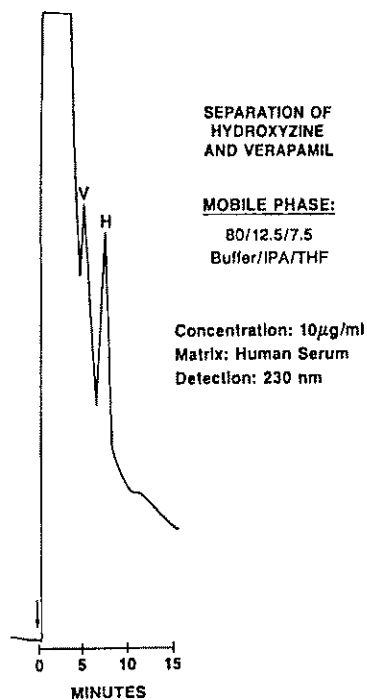


Figure 12

**Discussion (continued):** Finally, we present a separation of 3,4-diaminopyridine from human serum (see Application Note No. 17). There were 3 concurrent objectives: optimize the separation of the drug from serum, maximize the detectability of the drug, and minimize the time per determination. The variables used in the method were buffer concentration and column length. In the first 3 tries, buffer concentration was reduced from 0.020 M (Figure 13) to 0.015 M (Figure 14) and then to 0.010 M (Figure 15). The drug/serum separation improved nicely as the increasing acidity caused increasing retention of the dual primary amine by the tripeptide. To use this improved separation, the column length was decreased from 15 to 5 cm (Figure 16), shortening the analysis from over 13 minutes to about 9, and tripling peak height and thus detectability.

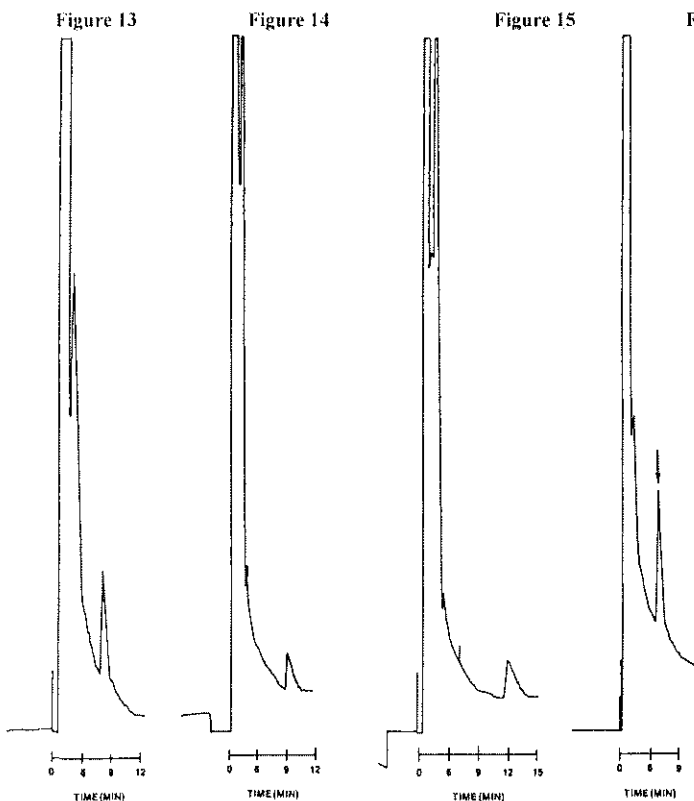


Figure 16

**Summary:** To improve a given separation, the analyst should take an active, frankly experimental attitude. There are four allowable organic modifiers: acetonitrile, isopropanol, methanol, and tetrahydrofuran, each variable up to 20% concentration in the mobile phase. At a given pH, test each separately. The pH may be varied between 6.8 and 7.5: with the best modifier, test the separation at each of several pH's. Mixtures of the modifiers may be explored, given that the total modifier concentration does not exceed 20%. The buffer strength may also be considered a variable, the column length, and the flow rate (especially with the shorter columns).

We have found most separations quite amenable to these approaches.

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