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# PRACTICAL ASPECTS OF SPEED IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF PHARMACEUTICAL PREPARATIONS*

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## SUMMARY

For many years, improvement of the separation power (number of theoretical plates) was the most important goal in chromatography. In high-performance liquid chromatography (HPLC) however, where selectivity plays an important role, adequate separation systems with fewer than 1000 plates solve many problems in routine analysis. Very high speed and super speed liquid chromatography, with retention times of unretained peaks of less than 1 sec, are playing an increasing role in LC laboratories.

The experience gained in our laboratories in recent years has shown that fast HPLC can be implemented successfully in the routine analysis of pharmaceutical preparations. In these analyses, especially for content uniformity, dissolution rate and stability tests, rapid separation is advantageous as large numbers of samples are involved. The separation speed also plays an important role in the total time of method development.

Fast HPLC can lead to new methods of investigating the kinetics of fast reactions. Results for the determination of the reaction rate of a pre-column derivatization are presented.

Practical aspects of fast HPLC are discussed in terms of chromatographic theory, instrument requirements and practical needs for typical HPLC users. Examples of fast HPLC separations of pharmaceutical preparation are shown.

## INTRODUCTION

In recent years high-performance liquid chromatography (HPLC) has undergone a dramatic evolution. Improvements in separations (theoretical plate number and selectivity) have been accompanied by the enhancement of chromatographic performance (efficiency per unit time). Because of the unique role of selectivity in

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HPLC, fewer than 1000 theoretical plates are needed for many routine separations. However, for these practical applications the time involved for the separation may have an important impact on laboratory productivity. The limits of speed in HPLC have been discussed previously[1]. It is the purpose of this paper to discuss practical aspects related to speed with emphasis on instrumental limitations under routine conditions and to report on experiences in the application of fast HPLC in the pharmaceutical industry.

Various definitions of fast HPLC have been given in the literature. We believe that the classification should be based on the chromatographic time base, which is the chromatographic dead time. Definitions as used in this paper are given in Table I.

TABLE I

DEFINITIONS OF FAST HPLC

| *HPLC technique* | *Dead time* | *Analysis time* |
|---|---|---|
| Conventional HPLC | *ca.* 1 min | *ca.* 10 min |
| Fast HPLC | | |
| Very high speed | *ca.* 10 sec | *ca.* 100 sec |
| Super speed | *ca.* 1 sec | *ca.* 10 sec |

## EQUIPMENT

### *Columns*

Instrumental requirements have been discussed by different workers from different viewpoints[2–6]. Chromatographic theory clearly suggests the use of short columns with small particles (5 $\mu$m, 3 $\mu$m or even smaller) and operation at the minimum of the $H/u$ curve[1]. However, even when operating columns under far from optimum conditions, it can be shown that the shortest possible column leads to the fastest separation. Columns for fast HPLC should have a flat profile of plate height *versus* flow-rate ($H/u$ curve), which means that only a small reduction in the theoretical plate number should occur at high flow-rates.

To accommodate the various separation problems, columns with different lengths should be available. In order to keep the flow-rate in a normal range of up to 10 ml/min and to enhance the sensitivity, small-bore columns are desirable. Good column permeability is also necessary.

The columns used in this work were packed with 5 $\mu$m Spherisorb (RP-8, RP-18), and were 100 or 30 mm long with I.D. 4.6 or 2.1 mm (Brownlee Labs., Santa Clara, CA, U.S.A.).

### *Detector*

Because of the small dead volumes of the columns required, the detector for fast HPLC must have a cell with good hydrodynamic flow characteristics, small cell volume and very short response time in order to minimize external band broadening in volume and time units. The influence of the detector time constant on band broad-

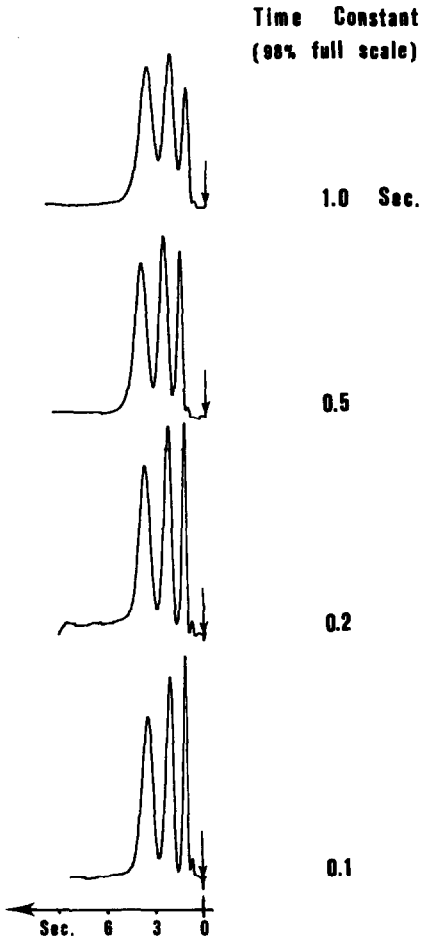


Fig. 1. Influence of time constants on resolution.

ening, especially for fast eluting peaks, is shown in Fig. 1. Special attention should be given to low volume connections from the column to the cell.

A variable-wavelength UV–visible detector with a 2.8 $\mu$l flow cell of 10 mm path length and a response time constant of 0.1 sec (98% full-scale) was used (Uvikon 720 LC; Kontron, Switzerland).

As the volume of short columns with a small inner diameter is low, the lag volume in the tubing and connections can play a significant role and needs to be minimized. This volume must be known, and was determined by measuring the peak volume of a sample injected directly (without a column) into the detector at a low flow-rate. The peak volume at peak half-height of up to 50 $\mu$l was measured in normal HPLC instruments. For small-bore columns this external band broadening is too large. To minimize extra-column effects, a dead volume free assembly for the detector was developed, which is now commercially available (Kontron, Kat. 91-00362). With this setup it is possible to place the column cartridges of different length directly

between the loop injector (Valco Instruments, Houston, TX, U.S.A.) and the flow cell and to reduce the peak volume to 9.5 $\mu$l at peak half-height.

*Injector*

It is necessary for the injection system to have a minimal dead volume and good rinsing characteristics. The rinsing can contribute to peak broadening and adversely affects the separation performance of good columns. The injection system should not interrupt the flow of the mobile phase to the extent that high-pressure pulses are generated, which shorten the column life. To reduce the pressure jump by shortening the switching time of the loop, 1/4 in. instead of 1/8 in. tubing was used for the connection to the air actuator. With this modification the pressure jump during injection could be reduced and the column life maintained as for conventional HPLC. Automatic injectors must be able to make a sample injection every 10–20 sec. An ASI 45 automatic sample injector (Kontron) provided a minimum cycle time, including rinsing of the loop, of 18 sec. An example with a cycle time of 24 sec is shown in Fig. 2.

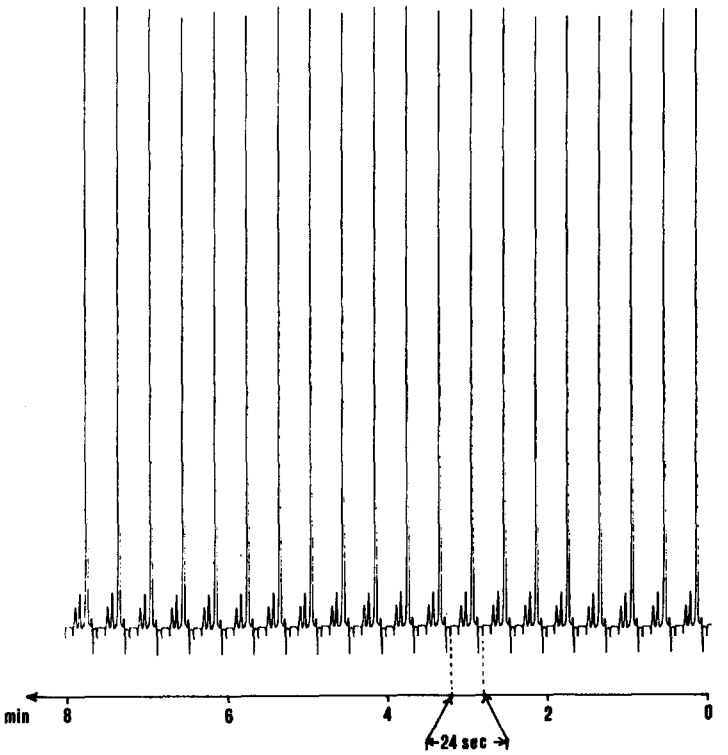


Fig. 2. Repetitive injections of a three-component drug mixture (chromatographic conditions as in Fig. 5a).

*Pump*

The pump system should be as pulse-free as possible. Double-piston pumps need a pulse damper so that the pressure pulses from the injection valve are absorbed

to prevent adverse influences on the column life. An Altex Model 100 dual-piston pump (Altex Scientific, Berkeley, CA, U.S.A.) or a Perkin-Elmer Model 3B (Perkin-Elmer, Norwalk, CT, U.S.A.) was used. Additional pulse dampers were used with both pumps at high flow-rates to eliminate the pulses during injection.

*Integrator and recorder*

The peak shape of fast-eluting peaks can be influenced by inadequate detector and recorder time constants. Therefore, the limiting peak width (in time units) of the detection and recording system was determined. On increasing the flow-rate to a maximum and using the same installation as for measuring the detector volume, the peak width will reach a final value, which cannot be lowered. For the system and with the lowest time constant possible for the detector of 0.1 sec, the limiting peak width was determined to be 0.18 sec.

The output of the reports from the integration system must be fast or buffered to avoid an unnecessary waiting time between injections. A Hewlett-Packard Model 3357 Laboratory System and a Uvikon Model 21 recorder (Kontron) were used.

## APPLICATIONS

Routine applications of fast HPLC require that the HPLC system gives reproducible retention times, resolution and peak response, as well as good quantitative results with adequate reproducibility and a linear detector response. Several multicomponent formulations were studied to demonstrate the applicability of fast HPLC to the analysis of pharmaceutical preparations.

*Three-component analgesic*

Prior to the use of fast HPLC, a conventional HPLC method for the simultaneous determination of caffeine, acetylsalicylic acid and propyphenazone using a 25 cm column packed with 10 $\mu$m particles was used. The chromatographic run time, including recycling of the gradient, was about 20 min. With the use of a high-performance column packed with 5 $\mu$m particles (30 × 4.6 mm I.D.) and a high flow-rate of the mobile phase it was possible to separate and quantify the three drug substances and the degradation product salicylic acid within 40 sec (see Fig. 3). The enhanced sensitivity, allowing the assay and purity test to be completed in one run, is a result of the higher concentration in the reduced elution volume.

A disadvantage in this application is the high flow-rate of 8.0 ml/min, as it is near the upper limit of commercially available HPLC pumps. This can be overcome by the use of small-bore columns of *e.g.*, 2.1 mm I.D., which reduces (for the same linear flow-rate) the flow-rate by a factor equivalent to the reduction of the square of column radial diameter (Fig. 3). A reduction in the flow-rate from 8.0 to 1.7 ml/min with a change in column from 4.6 to 2.1 mm I.D. represents a similar linear flow. The use of the columns of smaller I.D. enhances the sensitivity due to the reduction in elution volume (see Fig. 3).

*Three-component antihypertensive*

Fast HPLC was used with Brinerdine, an antihypertensive containing clopamide, dihydroergocristine and reserpine as active drug substances (Fig. 4). The sepa-

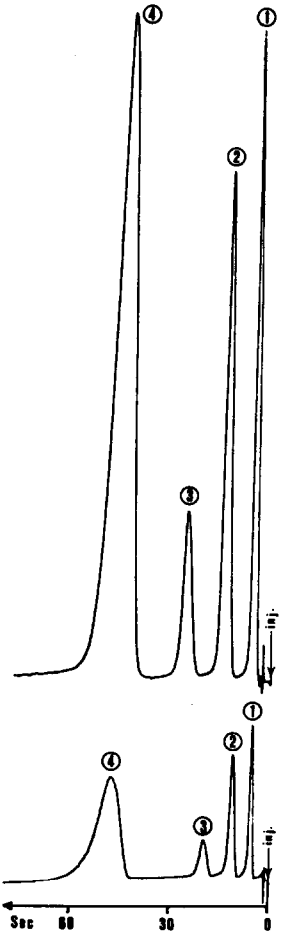


Fig. 3. Advantages of small-bore columns. Mobile phase, methanol–water–concentrated phosphoric acid (30:70:3); stationary phase, RP-8, 5 μm; detection, 295 nm; sample, one tablet extracted in 100 ml; injection volume, 3 μl. Above: small-bore column (30 × 2.1 mm I.D.); flow-rate, 1.7 ml/min. Below: conventional column (30 × 4.6 mm I.D.); flow-rate, 8.0 ml/min. Peaks: 1 = caffeine; 2 = acetylsalicylic acid; 3 = salicylic acid; 4 = propyphenazone.

ration in Fig. 5 shows a fast HPLC application with this drug combination. The components have significant differences in dose levels (a factor of 50 between clopamide and reserpine), polarity (clopamide polar, reserpine non-polar) and absorption spectra. With the previously used conventional HPLC method the chromatographic run time was 20 min. With fast HPLC, separation was achieved in less than 20 sec (Fig. 5a). This short chromatographic run time is especially advantageous for handling large numbers of samples, as is required in the content uniformity and dissolution rate tests prescribed in many pharmacopoeias. For the release of a batch, the content uniformity of up to 30 individual tablets must be analysed. Hence the reduction of the chromatographic run time contributes substantially to increased productivity and more economical analyses (Table II).

TABLE II

BENEFITS OF FAST HPLC FOR CONTENT UNIFORMITY TESTING

| *HPLC technique* | *Chromatographic run time* | *Flow-rate (ml/min)* | *Number to be analysed** | *Chromatographic analysis time* | *Solvent consumption (ml)* |
|---|---|---|---|---|---|
| Conventional HPLC | 20 min | 2.0 | 42 | 14 h | 1680 |
| Fast HPLC | 30 sec | 3.0 | 42 | 21 min | 63 |

* Samples: 30 tablets. Standard solutions: 4 × 3 = 12 (four groups with 80, 100 and 120% of the declared drug amount).

Fast HPLC not only allows the analyst to monitor and control the separation efficiently but also contributes substantially to lower solvent consumption and overall cost. A fast analysis is also advantageous when the drug is not stable for long periods in the media used to extract or dilute the sample. In the application described here, for example, it was necessary to analyse reserpine within 6 h.

Fig. 2 is an example of chromatograms showing the reproducibility of repeated injections of a Brinerdine sample and Table III summarizes the precision, linearity and detection limits observed for the content uniformity method.

TABLE III

VALIDATION OF THE FAST HPLC METHOD FOR CONTENT UNIFORMITY TESTING OF BRINERDINE TABLETS

| *Parameter* | *Drug substance* | | |
|---|---|---|---|
| | *Clopamide* | *Dihydroergocristine* | *Reserpine* |
| Amount injected (μg)* | 1.5 | 0.15 | 0.03 |
| Retention time (sec) | 7.5 | 13.5 | 16.5 |
| Capacity ratio | 0.79 | 2.21 | 2.93 |
| Peak symmetry** | 2.70 | 1.6 | 1.3 |
| Precision, $S_{rel}$ (%)*** | 0.15 | 1.1 | 1.4 |
| Linearity§ | 0.9993 | 0.9998 | 0.9992 |
| Detection limit (ng)§§ | 1.5 | 3.0 | 0.9 |

* Equivalent to the concentration of one tablet extract.
** At 5% peak height.
*** Determined by 20 injections of a tablet extract.
§ Correlation coefficient calculated up to 100% of the declared drug amount.
§§ Signal-to-noise ratio = 3:1.

For the purity tests and the determination of degradation products during stability tests, a longer run time of about 2 min was required to ensure resolution of the intact drug substances from possible degradation products (Fig. 5b). This still represented a 10-fold decrease in the chromatographic run time over the previous conventional HPLC method.

Resolution must be maintained for the analysis of all samples over the whole

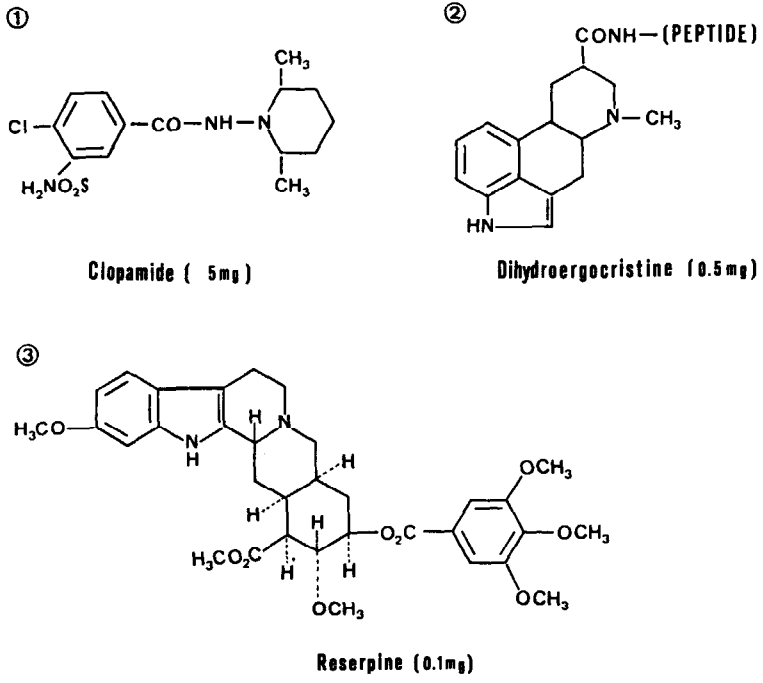


Fig. 4. Components of Brinerdine tablets.

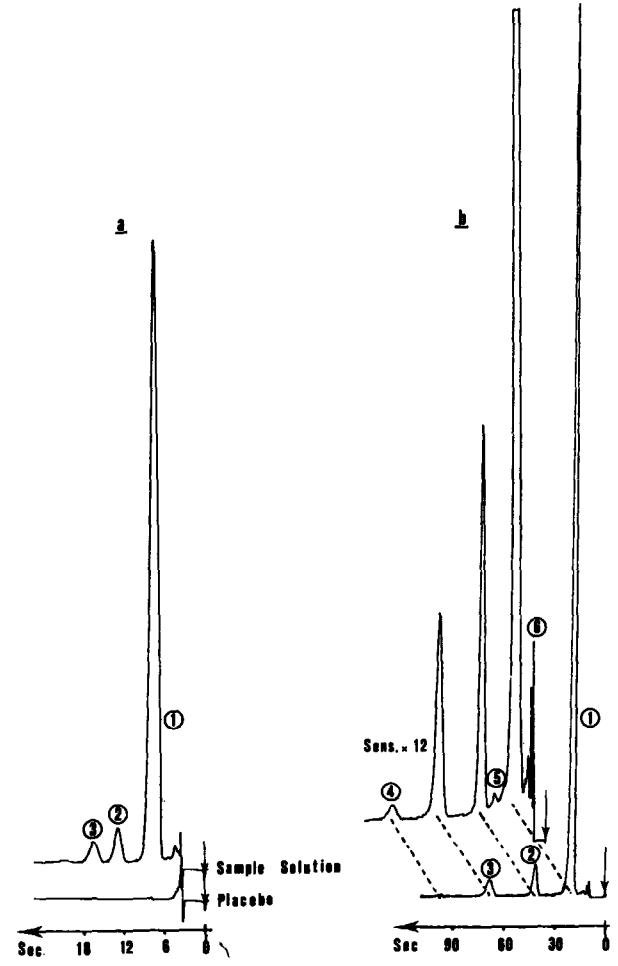


Fig. 5. Fast HPLC of Brinerdine tablets. Column, 100 × 2.1 mm I.D.; stationary phase, RP-18, 5 μm; detection, 260 nm. (a) Content uniformity testing: one tablet extracted in 10 ml; mobile phase, acetonitrile–0.4 *M* phosphoric acid (425:75); flow-rate, 3.0 ml/min; injection volume, 3 μl. (b) Stability testing of a stressed Brinerdine sample: one tablet extracted in 10 ml; mobile phase, acetonitrile–0.2 *M* phosphoric acid (325:175); flow-rate, 1.0 ml/min; injection volume, 3 μl. Peaks: 1 = clopamide; 2 = dihydroergocristine; 3 = reserpine; 4 = 3,4-dehydroreserpine; 5 = unknown degradation product; 6 = excipients.

column lifetime. Therefore, repetitive injections were made with a standard solution and, as can be observed from Fig. 6, even after 1100 injections with a cycle time of 30 sec the resolution was not affected. With the high linear flow-rate of more than 20 mm/sec, it was feared that frictional heating of the mobile phase would lead to a reduced column efficiency and a decrease in resolution. Fig. 6 shows that the resolution remained constant during the testing period and that this effect can be neglected.

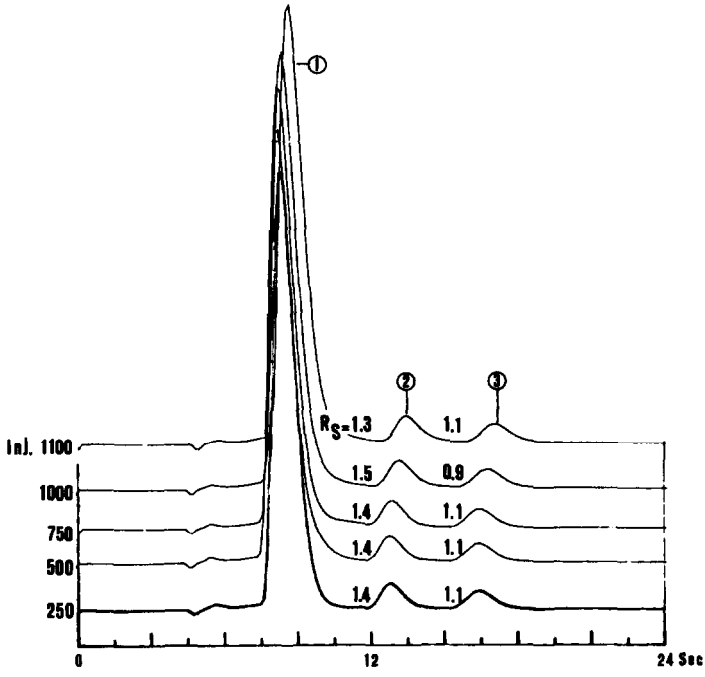


Fig. 6. Ruggedness of fast HPLC. Chromatographic conditions as in Fig. 5a. Peaks: 1, clopamide; 2, dihydroergocristine; 3, reserpine.

Peak symmetry is also important for good resolution and depends on the substance being investigated and the separation system used. From Table III it can be seen that especially the first peak shows tailing (peak symmetry = 2.7) and therefore the speed of separation is limited. Experimentally it was found that peak tailing is not influenced by the flow-rate and therefore is not an effect inherent in fast HPLC.

Our experience shows that column lifetime (total number of injections on a column) is similar to that in conventional HPLC.

The results presented show that fast separation is feasible for the routine analysis of pharmaceutical preparations, and the method has been employed in our laboratories for more than 2 years also for other separation problems.

To show the extent to which the separation time can be reduced, an artificial test mixture with the drug substances as for Brinerdine was separated on short columns (30 mm × 2.1 or 4.6 mm I.D.). The flow-rate was increased so that the retention time of non-retarded substance was less than 1 sec. To obtain an equivalent detector

signal, a solution containing 25 mg of dihydroergocristine and 10 mg of clopamide or reserpine in 100 ml was injected. For the microbore column this solution had to be diluted 4-fold prior to injection. As Fig. 7 shows, with both columns the three substances are eluted in less than 4 sec under super speed HPLC (SS-HPLC) conditions as defined in Table I. The resolution with the small-bore column was reduced, indicating that external band broadening originating from the detector and injector begins to play a role.

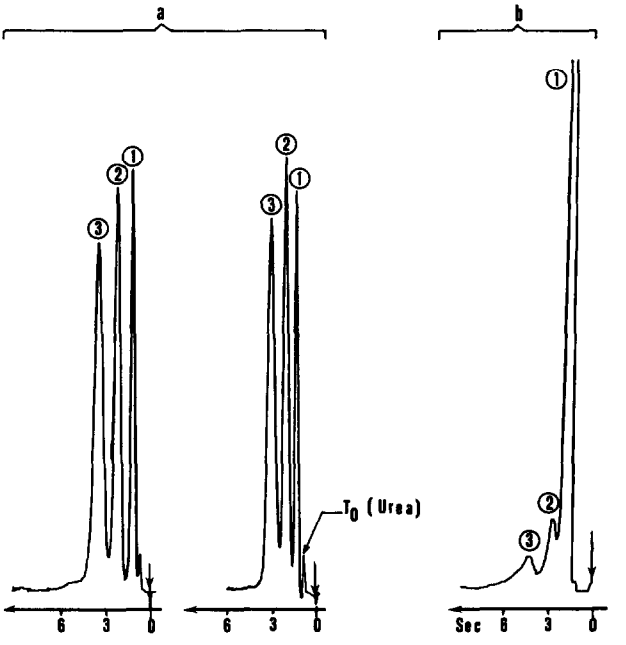


Fig. 7. Super speed HPLC of Brinerdine tablets. Chromatographic conditions as in Fig. 5b; sample, see text, (a) Influence of column diameter: flow-rate with 3 × 0.46 cm I.D. column, 30 ml/min; flow-rate with 3 × 0.21 cm I.D. column, 5 ml/min. (b) Influence of the amounts applied. Peaks: 1, clopamide; 2, dihydroergocristine; 3, reserpine.

With the proportion of active ingredients as found in Brinerdine, SS-HPLC is less suitable because of the large amount of clopamide (Fig. 7b). The tailing of the first peak (clopamide) complicates the separation of the 10- and 50-fold lower dosed drug substances. To obtain baseline separation a separation time of at least 10 sec is required. For the routine analysis the system as in Fig. 5a is recommended. The example presented here demonstrates that the ratios of the peak responses can reduce the acceptable speed in HPLC for quantitative work.

*Investigation of a fast reaction for pre-column derivatization*

Endralazine is the active agent of the antihypertensive preparation Miretilan available from Sandoz and its structure is shown in Fig. 8. In the analyses of En-

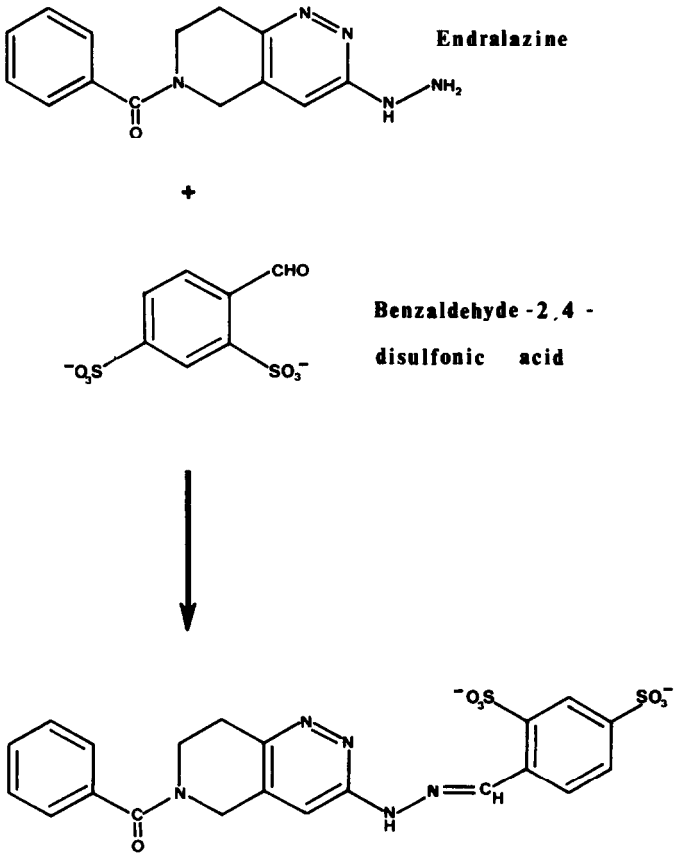


Fig. 8. Reaction scheme of the precolumn hydrazone formation.

dralazine, the active ingredient is unstable in solution because of the hydrazine substituent. Therefore, it is converted into its hydrazone by a pre-column derivatization before injection into the HPLC system. This pre-column derivatization had to be optimized and it was found that with a 10-fold excess of reagent the reaction is completed almost instantaneously (in 0.1 *N* hydrochloric acid at room temperature). This procedure is used in routine analysis and for validation of the method the reaction rate was evaluated, which was possible using SS-HPLC. For the determination of the reaction rate, benzaldehyde-2,4-disulphonic acid solution was placed in a beaker equipped with a magnetic bar. The Endralazine solution was added and the reaction solution was pumped with continuous stirring into the injector loop, which was controlled by the automatic sample injector. SS-HPLC was carried out, using a 4.6 mm I.D. column for better resolution and a flow-rate of the mobile phase of 12 ml/min. This allowed all the components to be separated within 4 sec, and hence an injection cycle of 6 sec was chosen. As can be seen from Fig. 9, the increase in the hydrazone derivative (dashed peak) and the decrease in Endralazine can be followed precisely and the reaction is completed within 40 sec. A delay between the first and

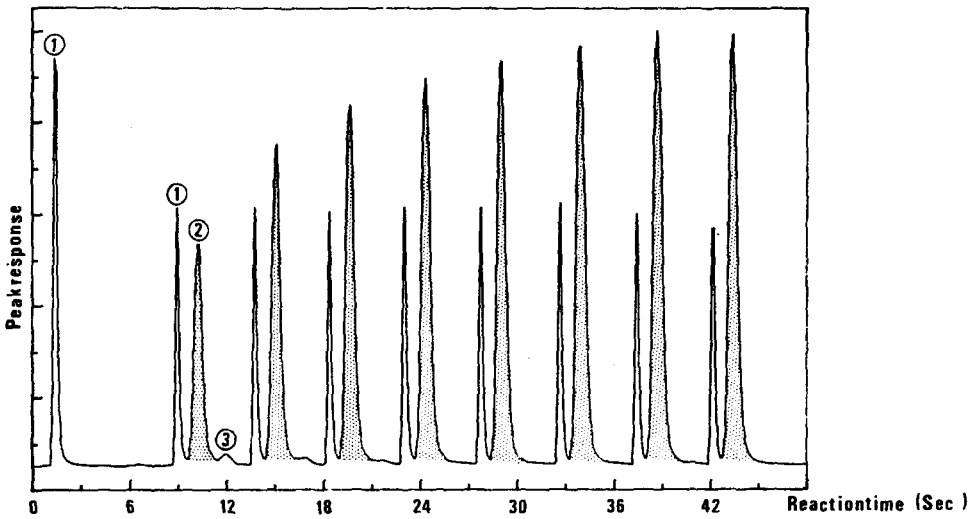


Fig. 9. Kinetic study of a pre-column derivatization. Mobile phase, acetonitrile–water–0.1 *M* $KH_2PO_4$–concentrated phosphoric acid (200:500:150:1); flow-rate, 12 ml/min; column, 3 × 0.46 cm I.D.; stationary phase, RP-18, 5 μm; detection, 295 nm; injection volume, 3 μl. Peaks: 1, reagent; 2, hydrazone derivative; 3, unreached Endralazine.

second injections of about 2 sec was introduced to ensure complete mixing of the reaction solution.

The advantages of super speed HPLC for the investigation of the kinetics presented are accuracy, because the products are separated and therefore the reaction rate can be determined precisely, performance, as the reaction parameters (concentration, temperature, pH, etc.) can be quickly checked, and flexibility, as the observation points can be adapted to the speed of reaction or for the detection of intermediates with a short lifetime.

The example demonstrates that fast HPLC and especially super speed HPLC offers new posibilities for the investigation of fast reactions (kinetics, reaction mechanism), for reaction optimization and for process control.

## CONCLUSIONS

The above examples and experience gained over more than 2 years have shown that fast HPLC can be successfully implemented for routine work. Chromatographic separations with run times of less than 1 min can be achieved with conventional HPLC equipment. For faster separations, especially super speed separations, detection systems with very fast responses are necessary.

The time constant of the detector largely determines the maximum chromatographic speed. For slightly retained peaks, the detector time constant was shown to have a negative influence on resolution, but for very large capacity ratios (*e.g.*, $k' > 20$) the time constant is a less restrictive parameter.

For small-bore columns with high-performance packings and lengths of only a few centimetres, microflow cells and low dead volume connections of the tubing are needed in order to maintain resolution of peaks with small capacity ratios.

Experience has shown that the lifetimes of the columns are not adversely affected by the high flow-rates and pressure pulses of the injections, and the same total number of injections per column can be made with fast HPLC as with conventional HPLC.

Speed in HPLC can be used advantageously if the analysis time is limited by the chromatographic time. This is the case when simple sample preparation possibilities exist or no sample preparation is required as, for example, with drink solutions, injectables and dissolution rate samples.

The examples shown demonstrate that fast HPLC has good potential for content uniformity tests, dissolution rate tests and stability analyses where a large number of samples must be handled. Another important application is the investigation of fast reactions, as could be shown with a pre-column derivatization, where information about the reaction could be generated with a simple experiment in real time.

An additional advantage of speed in HPLC is the ability to optimize an HPLC separation in a faster time. The results of the optimization trials are immediately available and permit "on-line optimization". Also worth noting is the possibility of better utilization of the equipment, which reduces the analytical costs.

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