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Automatization for development of HPLC methods

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Abstract Within the frame of inprocess analytics of the synthesis of pharmaceutical drugs a lot of HPLC methods are required for checking the quality of intermediates and drug substances. The methods have to be developed in terms of optimal selectivity and low limit of detection, minimum running time and chromatographic robustness. The goal was to shorten the method development process. Therefore, the screening of stationary phases was automated by means of switching modules equipped with 12 HPLC columns. Mobile phase and temperature could be optimized by using Drylab® after evaluating chromatograms of gradient elutions performed automatically. The column switching module was applied for more than three dozens of substances, e.g. steroidal intermediates. Resolution (especially of isomeres), peak shape and number of peaks turned out to be the criteria for selection of the appropriate stationary phase. On the basis of the "best" column the composition of the "best" eluent was usually defined rapidly and with less effort. This approach leads to savings in manpower by more than one third. Overnight, impurity profiles of the intermediates were obtained yielding robust HPLC methods with high selectivity and minimized elution time.

Introduction

Developing a synthesis for a drug substance is a time-consuming process. The synthesis continues from drug research into the development phase, where it is optimized and then subjected to trials. Here, the first quantities (kg) are determined for toxicological studies, preformulation trials and clinical studies. Finally, the synthesis is then approved and released for production.

Numerous HPLC methods are necessary for in-process controls during development as well as production, in order to enable a critical evaluation of the quality of each synthesis phase. Therefore, all impurities along with the main substance must be able to be determined. On the one hand, the results of the synthesis optimization analysis should provide for rapid orientation with regard to the statistically designed trials for investigation of influential factors such as yield, content and impurity profile [1, 2]. Concise methods allowing high throughput in a short amount of time are required here. On the other hand, refined methods which fulfill the highest requirements with respect to selectivity, robustness and transferability to other laboratories must be perfected before the synthesis can be transferred to production.

In the early development stage it is necessary that the analytical lab is able to offer the internal client flexible and quick solutions to the many often unexpected problems associated with pharmaceutical development. The results must provide reliable and clear information. With this in mind it is our goal to develop HPLC methods, currently the most frequently used methods for solving problems, for use with a minimum of manpower in the shortest amount of time. They should also demonstrate the maximum selectivity and robustness in order to be successfully transferred to production analysis.

In order to fulfill these high demands, we are proceeding as follows. Following the evaluation of all available data on structure, detection and chromatographic behavior, and after deciding on HPLC as the best method, a few orientational HPLC tests and/or thin layer chromatography tests are conducted in order to assess the polarity spectrum of the substances to be separated.

First a suitable stationary phase is selected for the preliminary trial. An initial chromatogram for orientation is attained in combination with, for example diode array detection. Problems are seldom solved with the first stationary phase selected. The time-tested method in our lab consists of a standardized set of twelve stationary phases [3–5]. This transpires overnight or at the weekend, in other words, without supervision.

Following selection of the "best" column, the mobile phase and the column temperature where needed, can be systematically optimized with Drylab® and/or Turbo-Method Development® [7, 8].

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Materials and methods

Instrumentation. An HPLC unit consisting of an autosampler and pump (Perkin Elmer series 200, Norwalk, USA) and an UV detector (Waters model 2487, Milford, Massachusetts, USA) were used for the experiments. A set of 12 columns was located in a column thermostat (Mistral Type 880, Spark, Netherlands), equipped with two 12-position valves (Model E12, VICI Valco Instruments Inc., USA). The dead volume of the equipment amounted to 97 µL, the dwell volume was 1.7 mL.

Materials. The stationary phases were, e.g., Hypersil ODS 5 µm (250 × 4.6 mm), YMC ODS AQ 3 µm (150 × 4.6 mm), YMC Pro C18 5 µm (250 × 4.0 mm), Spherisorb ODS II 5 µm (250 × 4.0 mm), Ultrasep ES RP18e Pharm 5 µm (250 × 4.6 mm), Hypersil Hi Purity Elite C18 5 µm (250 × 4.6 mm), Inertsil ODS-2 5 µm (250 × 4.6 mm), Luna C-18 5 µm (250 × 4.6 mm), Megapharm ODS 5 µm (250 × 4.6 mm), Alltima C18 5 µm (250 × 4.6 mm), Ultrasep ES100 Phenyl 5 µm (250 × 4.0 mm), YMC ODS H80 4 µm (250 × 4.0 mm). The choice of stationary phases depends upon the class of the substances. Novel phases are taken into account in order to test selective performance.

Columns were selected with the dimensions 250 mm × 4.0–4.6 mm. These large-scale columns were shown to be superior in cases where separation performance, pollution by sample matrix and transferability to other laboratories cannot be compromised.

Where, however, the aim of the method development was high sample throughput, columns of the dimensions in the range of 33–50 mm × 4.0 mm were used combined with 8–10 min gradient elutions. Selectivity was almost suboptimal, whereas total running time of such "fast" HPLC methods was in the range of 10 to 20 mins.

Stationary phase optimization. The mobile phase was able to be conducted manually through the 12 columns by means of an external switch or automatically by means of control software (Turbochrom™ release 6.1.1, Perkin Elmer) (Fig. 1). That was done by means of the integrated relais of the link interface. Following appropriate column equilibration and injection of an aliquot of the test solution the mobile phase gradient started at, e.g., a 60/40 (v/v) mixture of water and acetonitrile. Within 45 min the final composition of 10% water and 90% acetonitrile was reached followed by a 5 min isocratic elution. During the next elution step the portion of organic solvent decreased to initial conditions. The column was equilibrated for another 5 min. Subsequently, the two valves were switched to the next position, i.e. the next column. After a 20 min equilibration an aliquot of the sample solution was injected and chromatographed as described above. In this way the 12 columns were tested one after the other and a series of 12 chromatograms of the same sample on different columns was generated.

45-min gradients with large-scale columns were normally selected for the elution in order to generate all the chromatograms in one night. Initial and final composition of the gradients were oriented towards the characteristics of the analysis and were established as by an orientational chromatography. Suitability was verified with one sample run. This usually occurred in the first column during the column test. The chromatograms were compared and evaluated visually. Peak resolution, peak shape and number of peaks were the criteria for assessment.

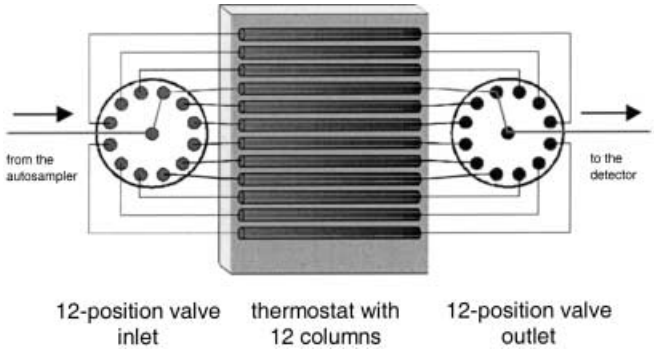

Fig. 1 Diagram of column switch module with 12 HPLC columns

Mobile phase optimization. After selecting the "best" column mobile phase and column temperature were optimized. That was done by using Drylab® (LC Resources Inc., CA, USA). Regarding the example shown in Fig. 6 the sample solution was chromatographed at two temperatures (15 °C and 50 °C). At each temperature a short gradient (30 min) and a long gradient (90 min) were performed. Retention data of the peaks of interest were processed with Drylab® in order to find acceptable resolutions ($R_s \geq 1.5$) for the relevant peaks. The two-dimensional resolutions maps (column temperature versus % B) supported to find optimal chromatographic conditions.

The example chromatograms shown in Figs. 2–7 were based upon processing steroidal compounds. However, the technique had been applied for other substance classes and is considered applicable in general.

Results

The column switch is used routinely in the in-process control analysis for the development of HPLC methods. The machine was shown to be very useful for rapid screening of stationary phases for conventional and "fast" HPLC methods. The advantages should be elucidated with respect to improved selectivity and time savings.

The example in Fig. 2 shows how different a normal steroid sample was separated by six reversed phase columns. Peak samples resulted, which differed from each other in number and elution sequence. Column A provided the "best separation" here, in other words the most suitable peak profile for further development. It demonstrated the highest number of peaks, i.e. a dozen. It can be assumed that the greater the number of tested columns the lower the probability of an oversight with respect to impurities in an unknown sample.

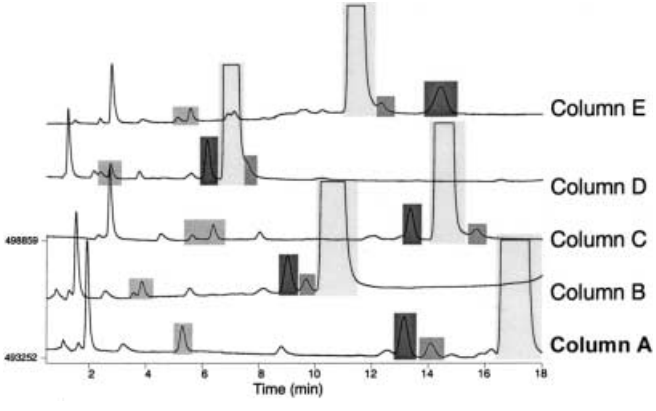

Fig. 2 Chromatograms of an artificial mixture of a steroid and impurities with five stationary phases
A: Ultrasep ES 100 RP18e Pharm 5 µm (250 × 4.6 mm)
B: EncaPharm 100 RP18 5 µm (125 × 4.6 mm)
C: Inertsil ODS-2 5 µm (250 × 4.6 mm)
D: Symmetry C18 5 µm (150 × 3.9 mm)
E: Hypersil ODS 5 µm (250 × 4.6 mm)

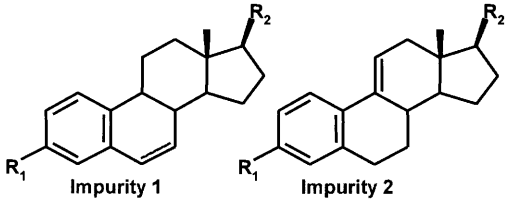

Fig. 3 Structural formulae of two isomeric impurities

The results from other method developments showed that peaks, much more sharply defined peaks were produced in the one column as compared to the other 11 columns. All the major peaks then had such a good resolution that the running time could be shortened by varying the mobile phase.

Difficult separation problems, such as for example the separation of double-bonded isomeric compounds as seen in Fig. 3, were able to be solved easily. Figure 4 shows the first six of the twelve column chromatograms. In column nos. 4 and 5 the isomers are completely separated from each other. In the case of eight other, no separation was achieved at all, while a partial separation was observed in two other columns.

The time-consumptive manual operations such as the switching out of columns and operating pumps, as well as the technicians' waiting periods during equilibration times, are all omitted. The use of sequence templates for the control of the HPLC unit reduces the programming time to a minimum. The time savings are obvious. The preparation of the unit and manufacture of the injection solution require a maximum of two hours. The visual in-

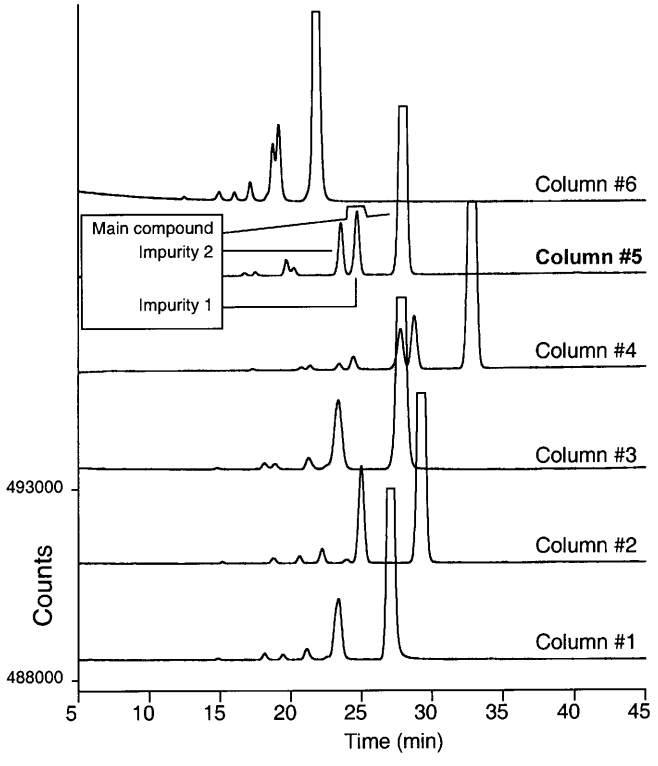

Fig. 4 Chromatogram of an impure steroid with six stationary phases
1: Hypersil ODS 5 µm (250 × 4.6 mm)
2: YMC ODS AQ 3 µm (150 × 4.6 mm)
3: YMC Pro C18 5 µm (250 × 4.6 mm)
4: YMC Pro C8 5 µm (250 × 4.6 mm)
5: YMC Pro C4 5 µm (250 × 4.6 mm)
6: Hypersil High Purity Elite C18 5 µm (250 × 4.6 mm)
(Isocratic elution with 50/50 v/v water/acetonitrile, flow rate: 1.5 mL/min, column temperature: 25 °C, UV-detection at 220 nm)
Only the chromatograms of six stationary phases with optimal resolution are shown.

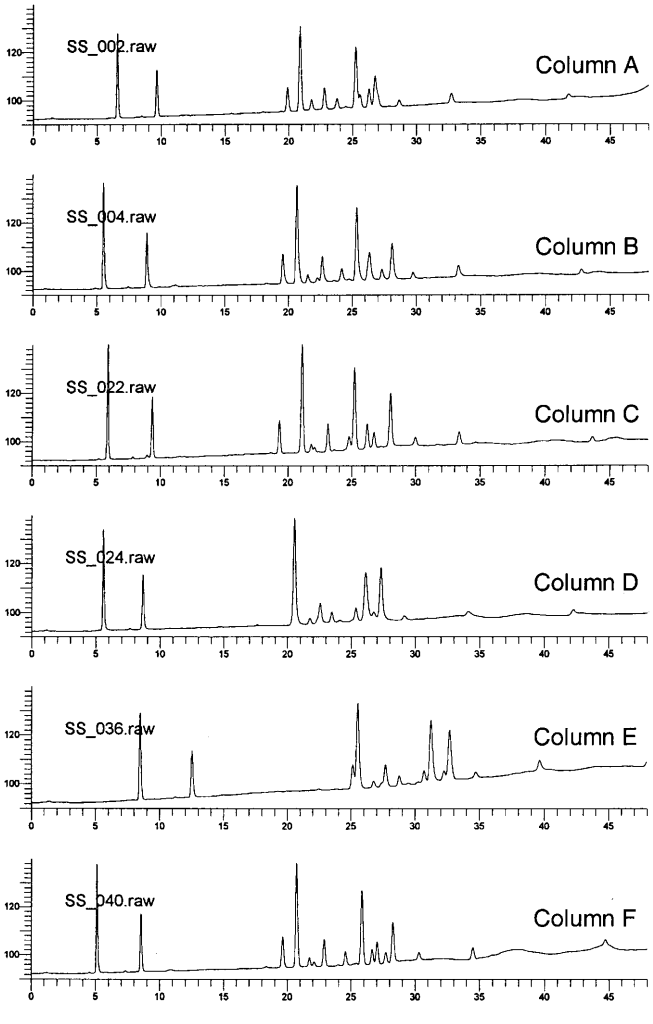

Fig. 5 Chromatograms of a synthetic mixture of a steroid and of its impurities with six stationary phases
A: Hypersil ODS 5 µm (250 × 4.6 mm)
B: YMC ODS AQ 3 µm (150 × 4.6mm)
C: YMC Pro C18 5 µm (250 × 4.6 mm)
D: Spherisorb ODS-2 5 µm (250 × 4.0 mm)
E: Alltima C18 5 µm (250 × 4.6 mm)
F: YMC ODS-H80 4 µm (250 × 4.0 mm)
The column was equilibrated for 30 min at 40% acetonitrile. Thereafter, % B increased up to 90% within 60 min. (Flow rate: 1.0 mL/min, column temperature: 15 °C, UV-detection at 215 nm).
Only the chromatograms of six stationary phases with optimal resolution are shown.

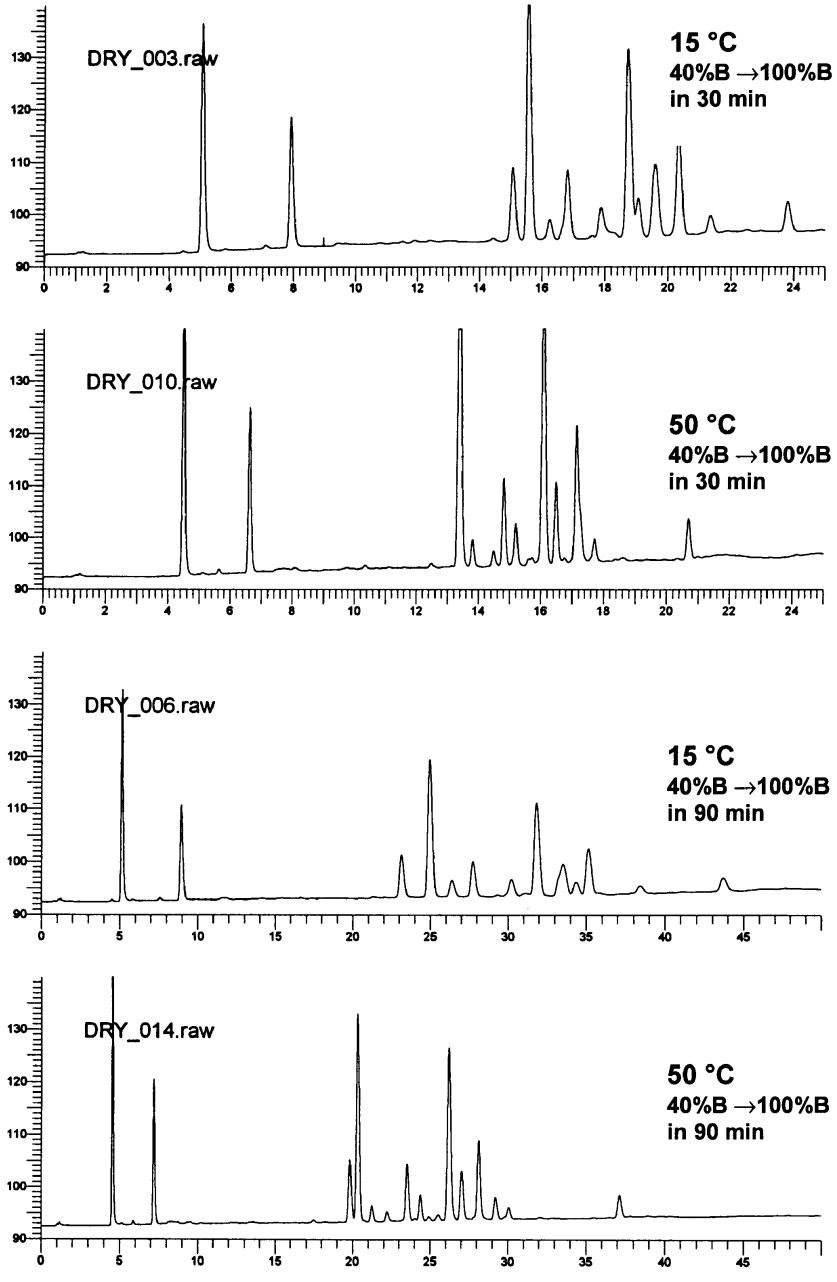

Fig. 6 Four chromatographic runs for Drylab® optimization of column temperature and % B (acetonitrile). Elution conditions (gradient and column temperature) are given in the chromatograms. Flow rate was 1.0 mL/min. UV-detection at 215 nm

spection of the chromatogram series was completed in one hour.

For a 12-column run with conventional HPLC normally requiring 12–16 h only 3–4 h are necessary with "rapid" HPLC methods. If the weekend is used for column screening then HPLC methods can be processed for five intermediates of the synthesis of a drug substance on the one hand, while on the other hand the impurities can be classified, or the basis runs for a Drylab® optimization can be chromatographed with temperature taken into consideration. The technique has been shown to be reliable and robust for these types of "projects".

Another example is shown in Figs. 5–7. The first step was to find the "best" stationary phase again. The chromatogram of column F (Fig. 5) is considered to be superior, because all peaks are separated from each other very well. Especially, the small peak at approx. 19 min is separated by column D not at all and very poorly by column E. The basic chromatograms for Drylab® optimization indicate that the good resolution between that peak and the following depends on column temperature as shown in Fig. 6. At 15 °C, it was separated totally, but not at 50 °C. Furthermore, optimal separation for most of the peaks can be reached by gradients resulting in slow increase of the organic moiety.

As a result of that, the influence of solvent composition, gradient time, column temperature and flow rate on the separation of the analytes could be checked in a sim-

ple manner, because both the resulting chromatograms were simulated and the R_s values were calculated immediately. Optimum separation was found at a gradient of 45% to 60% acetonitrile within 20 min followed by an increase to 90% acetonitrile in 15 min at a flow rate of 1.25 mL/min and at a column temperature of 30 °C. A running time optimized simulated chromatogram is shown in the upper part of Fig 7. The software indicated that all relevant peaks could be separated from each other. The aim to limit the running time to approx. half an hour should be reachable. The HPLC equipment was set up with the conditions found. The chromatogram of the verified HPLC run is also given in the lower part of Fig. 7. The peaks in the retention range of 12 to 17 min were actually separated completely. The running time resulted in 30 min, equilibration time excluded.

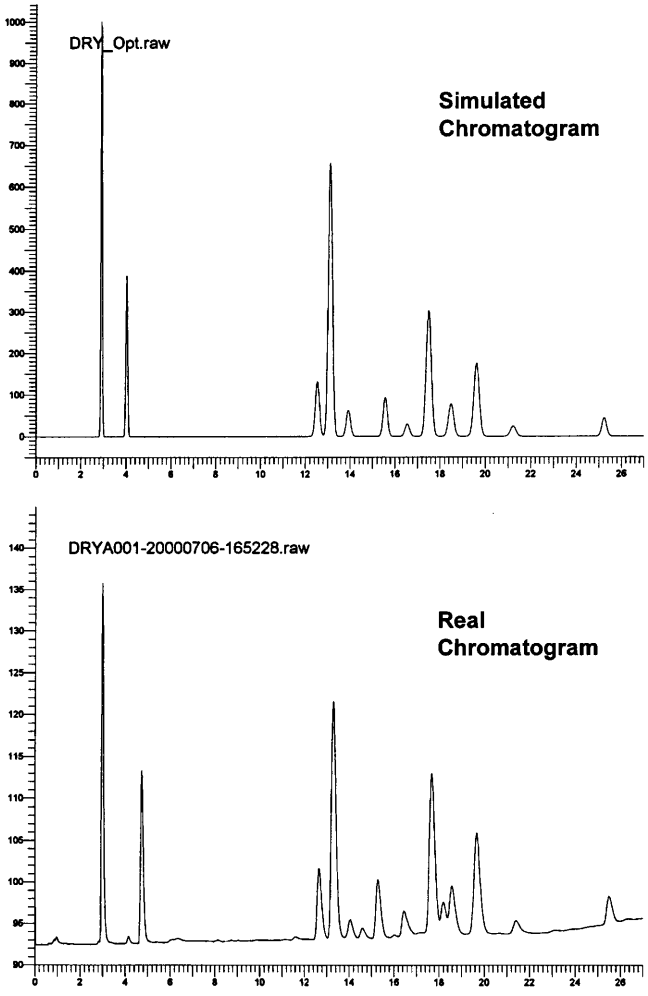

Fig. 7 Optimization of the HPLC separation of a synthetic mixture of a steroid and its impurities
Simulated Chromatogram:
The separation was optimized regarding chromatographic resolution, running time and column temperature.
Real Chromatogram:
The conditions found during Drylab® processing were tested. The elution conditions were as follows. 55/45 to 40/60 water acetonitrile (v/v) within 20 min, followed by 10/90 (v/v) in 15 min. (Flow rate: 1.25 mL/min; column temperature: 30 °C, detection of the UV absorption at 215 nm)

Since its establishment the column setup has been used for several dozen examples. In most cases a stationary phase was found immediately which became the basis for a final method. It was also demonstrated that the same column doesn't always "come in first". Stationary phases were able to be selected upon which important minor components eluted without tailing compared to other columns. Thus, it was possible to do away with the addition of substances to the mobile phase, such as buffers etc., or complex mobile phase mixtures. It was not necessary to optimize the selectivity of the separation system via the mobile phase. In almost all cases basic mobile phase systems – mostly based on acetonitrile/water mixtures – were found which resulted in low limits of detection and quantitation.

Conclusion

The switch technique was shown to be of multifaceted usefulness for the development of HPLC methods. The resulting method development strategy presented in Fig. 8 has been proven to be effective, that means saving of costs and time. This technique opens up other automatisation possibilities for the validation and use of HPLC methods. These are summarized in Table 1. The advantages are given in Table 2.

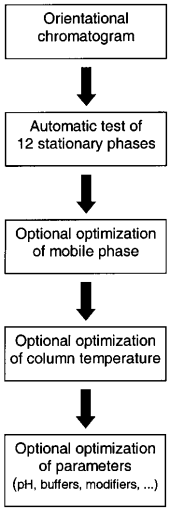

Fig. 8 General development run for HPLC methods

Table 1 Possible use of column switching technique

- Screening of stationary phase during method development in HPLC
- Screening of different column batches (different manufacturers, refillers, column age etc.) during method validation
- Analysis of a sample on different columns
- Performance of different analyses with different columns and mobile phases
- Control of purity of reference substances

Table 2 Advantages of the column switching technique

- Time savings by omission of manual operations and waiting times
- Complete determination of impurity profiles
- Influence of characteristics of stationary phases on separation, such as silanol activity and hydrophobicity, become clear.
- No need for (amine) modifiers, buffers etc. and complex solvent mixtures
- Improved robustness of HPLC methods
- Improved limits of detection and quantitation by using simpler solvent mixtures
- Time savings by shortening the optimization of the mobile phase
- Increased sample throughput

Altogether it is now possible to perform the method developments much more rapidly, because manual operations and waiting times are omitted and the optimization of the mobile phase has been shown to be unnecessary in many cases. The time advantage was especially positive in providing smart solutions to difficult problems. Qualitatively, with respect to the resolution and number of peaks and the chromatographic robustness, the current HPLC methods were significantly improved.

In our opinion, the secret to the strategy described above is that a maximum of selectivity can be attained by finding the "right" stationary phase for the "right" sample (i.e. chemical structure of the analyte) in combination with the "right" mobile phase. In other words, stationary and mobile phase must be brought into agreement with the analyte in order to ensure optimal interferences. Once the best combination of the three factors (analyte, stationary and mobile phase) has been found, sharply focused peaks are attained and the results achieved with fewer experiments meet even the toughest demands.

This proposal presents a very effective alternative for the development of methods for HPLC. The column switching technique used is robust and reliable; it has already become widely accepted in our lab and in the analytical labs of research, development and quality control.

The future work will be to automate the finding of appropriate stationary and mobile phases for the analytes. Efforts are being made to develop a tool employing multifactorial statistical methods. That tool will help us to find the best chromatographic separation, i.e. the global maximum in resolution, obtained from testing a reasonable number of columns, mobile phases and other chromatographic factors. That will help us to avoid a tremendous number of chromatograms in order to check each chromatographic factor in a systematic sequential manner. Finally, this tool should set up the HPLC equipment by the chromatographic parameters found. In summary, the development of HPLC methods would be performed fully automated.

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