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SELECTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS IN PHARMACEUTICAL ANALYSIS

IV[a]. SELECTION OF MOST APPLICABLE SEPARATION SYSTEM

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SUMMARY

Different analytical tasks in the pharmaceutical analysis can be classified according to the separation problems into three main groups: trace analysis, assay methods and separation of closely related compounds including isomers. The most important requirements of high-performance liquid chromatographic (HPLC) methods with respect of the separation problems are summarized. Considerations and recommendations for the selection of the most applicable HPLC system to solve particular analytical problems are discussed. HPLC methods can be compared on the basis of the system resolution (SR) and system selectivity (SS). Criteria developed for the characterization of HPLC methods considering the difficulties created by the different analytical problems are established. The principles of the selection of the most applicable separation systems are demonstrated through some practical examples in pharmaceutical analysis.

INTRODUCTION

As discussed in Parts I and II[1,2], two HPLC systems (normal- and reversed-phase) were developed and optimized in order to minimize the possibility of band overlap and failure to recognize the presence of some unknown species. Based on validation data, the systems can be compared[3]. To establish the suitability of any high-performance liquid chromatographic (HPLC) method in pharmaceutical analysis, further information about the quality of the separations considering the difficulties created by the analytical problems to be solved may be necessary. Method validation data provide important information about the separation systems and criteria can be formulated to express directly the quality of the separation. These criteria are the system resolution (SR) and system selectivity (SS) and relate to the applicability of a separation system. As a continuation of our previous work[1–3], the

[a] For Part III, see ref. 3.

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basic principles used in our laboratory to compare different separation systems considering the most important features of the individual analytical tasks are discussed in this paper.

EXPERIMENTAL

The following experimental conditions were used.

Steroid separation

The same instrumentation (HP 1090A) and conditions were used as in Parts I and II[1,2].

Stability test on sulfinpyrazone tablets

A Varian 8500 liquid chromatograph equipped with a loop-type injector, variable-wavelength UV detector (both from Labor MIM) and a Hewlett-Packard HP 3392A electronic integrator was used. Separations were performed on the same types of column (Nucleosil C_{18}, 10 μm; LiChrosorb Si 60, 5 μm) as used in steroid investigations[1,2].

Purity test on ergotamine tartrate

A Liquochrom 2010 liquid chromatograph equipped with a loop-type injector, variable-wavelength UV detector (all from Labor MIM) and HP 3392A electronic integrator was used. Separations were performed on the same types of column as used in steroid separations[1,2].

RESULTS AND DISCUSSION

Most important analytical tasks in pharmaceutical analysis (connection between method used and analytical problem to be solved)

The most important analytical tasks in pharmaceutical analysis and their characteristics are collected in Table I. Considering the chromatographic separation problems, the analytical tasks can be further classified as follows.

Trace analysis. Several of the analytical tasks indicated in Table I may occur which necessitate the separation of compounds present at low concentrations in the sample in the presence of large amounts of unknown (A-1, A-2, A-3 and E) or known (B-1, C-1, C-4, D-2 and D-5) components. Three main types of trace analytical problems can be distinguished:

(a) A limited number of components are of interest and can be separated from each other and from the unknown background materials (A-1–A-3 and E), which occur at high concentrations. The most important considerations can be summarized as follows:

(i) A high selectivity and resolution of the HPLC method are required in order to achieve adequate separation of the compounds from many unidentified background materials.

(ii) Detectability of the trace components requires their early elution. However, in most instances the unknown materials also elute with short retention times, resulting in several unidentified peaks on the chromatograms. Late retention of trace

TABLE I

CHARACTERIZATION OF THE MOST IMPORTANT ANALYTICAL TASKS IN PHARMACEUTICAL ANALYSIS

Analytical task	*Symbol*	*Characteristics*[a]				
		Recommended resolution			*Peak of interest*	*Aim of analysis*
		b	*a*	$R_{s,min}$		
Investigation of starting raw materials:	A					
Plant extracts	A-1	1.5	1.8	1.0	L	T
Extracts of animal organs	A-2	1.5	1.8	1.0	L	T
Fermentation mixture	A-3	1.2	1.5	1.0	L	T
Investigation of intermediates and crude products:	B					
Intermediates and crude products	B-1	1.3	1.5	0.8	E	T
Reaction mixture	B-2	1.2	1.5	0.8	E	A
Mother liquors and secondary products	B-3	1.3	1.8	0.8	L	T
Investigation of active ingredients:	C					
Purity test	C-1	1.8	2.0	1.2	E	T
Assay	C-2	1.2	1.2	0.7	L	A
Separation of closely related compounds	C-3	1.5	1.8	1.0	E	T
Stability test	C-4	2.0	2.5	1.2	E	A+T
Investigation of formulated products:	D					
Assay	D-1	1.2	1.2	0.8	L	A
Purity test	D-2	1.7	2.0	1.0	E	T
Content uniformity test	D-3	1.2	1.2	1.0	L	A
Dissolution test	D-4	1.2	1.2	1.0	L	A
Stability test	D-5	2.5	3.0	1.2	E	A+T
Pharmacokinetic and metabolic studies	E	1.5	1.8	1.0	L	T

[a] Symbols: *b* and *a*, recommended values of R_{sb} and R_{sa}; L, limited number of peaks of interest; E, equal importance of peaks; T, trace analysis; A, assay.

compounds may result in their easier separation from the matrix materials, but their detectability may be more difficult. A good compromise can be found between chromatographic resolution and detectability.

(iii) Precise and accurate sample preparation involving optimization of extraction, clean-up and sample concentration procedures are necessary.

(iv) The use of selective and sensitive detection is an important factor including pre- or post-column derivatizations, if necessary.

(b) Separation and quantitative determination of a known number of essentially known impurities at low concentrations in the sample (B-1, C-1 and D-2). Depending on the aim of the analysis, the impurities may be present in the concentration range 0.01–1% in the sample. A sufficiently high selectivity of the separation and suitable sensitivity of the detection are the most relevant factors. One of the essential considerations is the order of elution of the separated peaks. It can be expressed by the system selectivity, (SS), as discussed in Part III[3]. The minimum resolution measured between the worst separated pair of peaks at any place in the chromatogram ($R_{s,min}$)

and the resolution between the peak of the main component and the preceding (R_{sb}) and following (R_{sa}) peaks can characterize the separation power of the HPLC system. The effect of these three parameters on the separation of trace components is expressed by the system resolution term[3] and it is recommended that it be considered when an appropriate HPLC system is selected.

(c) Stability-indicating methods (C-4 and D-5). A stability test is a specific case of trace analysis, and the method development requires suitable skill and expertise. As discussed in Part III[3], special demands arise with regard to the HPLC system as impurities with similar chromatographic properties can be separated and identified. Investigation of the peak purity of the main component to recognize incomplete resolution (discussed in detail in Part III[3]) by the absorbance-ratio method, plotting the ratio of the absorbances at two (or more) preselected wavelengths as a function of time using samples subjected to various stress conditions in order to produce real degradation products has been recommended[3].

The accuracy and precision of the methods are not critical factors. A relative standard deviation of 5% is adequate.

Assay methods (B-1, B-3, C-2, D-1, D-3 and partly C-4 and D-5). When the analytical task is to determine the active content of bulk drug substances, the selectivity and efficiency of the separation are not as critical as in other instances. The aim is to separate the main components from the impurities, but the separation of impurities from each other is not required. The accuracy and precision of the method are of more importance.The relative standard deviation should not exceed 2%.

Separation of closely related compounds and isomers. Almost every group of analytical problems requires the separation of closely related compounds and isomers. This task involves different degrees of difficulty during the method development. In the separation of related compounds, greater structural differences are mostly sufficient for their easy separation by reversed-phase or normal-phase chromatography. Method development for the separation of isomers, except the separation of optical isomers, requires more time and expertise, but in most instances the separations can be solved without using special HPLC techniques.

The most difficult problem is the separation of enantiomeric forms of pharmaceutically important compounds. At present no universal method is available for solving this analytical problem. According to literature data the methods suitable for enantiomeric separations can be divided into four groups: (a) separation in the form of diastereomeric derivatives using a chiral reagent for pre-column derivatization; (b) separation on dynamically coated or chemically bonded chiral stationary phases; (c) separation on conventional stationary phases (bare silica or chemically bonded phases) using a chiral eluent; and (d) separation by inclusion complex formation. The different separation possibilities have been excellently reviewed by Souter[4], Lindner and Petterson[5], Armstrong[6] and Wainer[7].

To establish finally the suitability of any HPLC method in pharmaceutical analysis, further information about the quality of separations considering the difficulties created by the analytical problems to be solved may be necessary. Table I contains data for the different characteristics of various analytical tasks (recommended resolutions, peak of interest, aim of analysis). Method validation data provide important information about the separation systems[3] and criteria (SR and SS) can be formulated in order to express the quality of the separation. The system resolution

TABLE II

DATA ELEMENTS RECOMMENDED FOR METHOD VALIDATION

Category I = trace analysis: LN, limited number of peaks of interest; a, in the presence of unknown materials (A-1, A-2, A-3, E); b, known number of essentially known components (B-3, D-2); EN, all peaks are of equal importance (B-1, B-2, C-1). Category II = assay methods (B-2, C-2, D-1, D-3, D-4). Category III = separation of closely related compounds (C-3, C-4, D-5). Symbols: +, recommended (importance is expressed by the number of + signs); (+), depending on the analytical tasks; −, determination is not necessary.

Analytical performance parameter	*Category I*			*Category II*	*Category III*
	LN		*EN*		
	a	*b*			
Accuracy	++	+	+	+++	++
Precision	+	++	+	+++	++
Linearity	+	+	+	++	++
Range	+	+	+	+	++
Lowest detectable quantity (LDQ)	+++	++	++	(+)	+++
Recovery	+++	(+)	(+)	(+)	(+)
Ruggedness	++	++	++	++	++
$R_{s,min}$	+++	++	++	−	+++
R_{sb}	−	−	++	−	+++
R_{sa}	−	−	++	−	+++
SR	−	−	++	−	+++
SS	−	−	++	−	+++

TABLE III

CRITERIA FOR HPLC METHODS

Symbol	*SR*	*SS*[a]	*Sample preparation*	*R.S.D.*[b] *(%)*	*Desired number of peaks*	*Recovery (%)*	*LDQ*[a]
A-1	2.0	n.d.	Problematic	⩽5	2–3	Min. 95	1–5 ng
A-2	2.0	n.d.	Problematic	⩽5	2–3	Min. 95	1–5 ng
A-3	2.0	n.i.	Problematic	⩽5	2–3	Min. 95	1–5 ng
B-1	1.8	+	Easy	⩽5	2–5	100	0.1%
B-2	1.8	n.i.	Easy	⩽2	2–3	100	n.i.
B-3	1.8	+	Easy	⩽5	2–5	100	0.1%
C-1	2.2	+	Easy	⩽2	2–10	100	0.01%
C-2	1.7	n.i.	Easy	⩽1.5	2–3	100	n.d.
C-3	2.0	+	Easy	⩽2	2–5	100	0.01%
C-4	2.2	+	Easy	⩽2	2–10	100	0.01%
D-1	1.8	n.i.	Should be problematic	⩽2	1–2	Min. 99	n.d.
D-2	2.0	+	No problem	⩽5	2–5	Min. 98	0.01%
D-3	1.8	n.i.	Problematic	⩽2	2–3	Min. 98	n.d.
D-4	1.8	n.i.	n.d.	⩽5	2–3	n.d.	1–5 ng
D-5	2.2	+	No problem	⩽2	5–15	Min. 99	0.1%
E	2.0	n.d.	Problematic	⩽5	2–10	Min. 90	100 pg

[a] n.d., not defined; n.i., not important; +, elution order is important, should be as positive as possible.

[b] Relative standard deviation.

(SR) and system selectivity (SS) correlate closely with the applicability of a separation system.

The variety of analytical tasks require the application of different validation schemes. Recommended validation data elements for various analytical tasks are given in Table II.

Considering the difficulties created by the various analytical problems, criteria to be formulated for HPLC methods can be established (Table III).

Selection of the most applicable separation system

Based on the data in Table III, the most applicable separation system for solving the particular analytical problem is selected. The following scheme is used in the authors' laboratory, assuming that two HPLC methods are available to solve the separation problems. The first decision is based on the SR values obtained for the two systems. Higher SR values provide better separation conditions.

When the systems possess similar SR values (both HPLC methods can solve the analytical tasks) the SS values are compared. The recommended separation system can be selected on the basis of the numerical value of SS; a higher value means a more advantageous elution order and enhanced selectivity. Finally, the validation data are taken into consideration in method selection.

The principles used for the selection of a recommended separation system are now demonstrated with some practical examples. The first example (which is theoretical) is based on the experiments described in Parts I and II[1,2]. Six different steroids were used as model compounds (for the names and symbols, see Table IV) for the experiments. The chromatograms obtained with the optimized systems were presented in Parts I and II[1,2]. Norgestrel (NG) is assumed to be the main component and the others are impurities. The criteria for both normal- and reversed-phase systems were calculated and are given in Table IV.

The data in Table IV indicate the more advantageous properties of reversed-phase chromatographic separation owing to its higher separation power (SR). In a normal-phase system the minimum recommended value of SR cannot be achieved.

Another example is when various steroids at different concentrations can be

TABLE IV

CALCULATED DATA FOR STEROIDS IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (PURITY TEST)

System A: column, Nucleosil C_{18} (10 μm) (250 × 4.6 mm I.D.); eluent, acetonitrile–tetrahydrofuran–water (12.9:22.4:64.7). System B: column, LiChrosorb Si 60 (5 μm) (250 × 4.6 mm I.D.); eluent, hexane–dioxane–isopropanol (95:3:2). Compounds: main component, norgestrel (NG); others, traces.

Parameter	*System A*	*System B*
Desired number of peaks (n)	6	6
Number of peaks before the main component (z)	2	3
Number of peaks after the main component (v)	4	3
System resolution (SR)	3.05	2.17
System selectivity (SS)	−0.094	+0.077
Running time (min)	40	25

TABLE V

CALCULATED DATA FOR STEROIDS IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (STEROID ASSAY)

Systems A and B: as in Table IV. System C: (ref. 8) column, silica; eluent, cyclohexane-isopropanol (97:39). System D: (ref. 9) column, octadecylsilica; eluent, methanol–10 m*M* buffer (4:1). Compounds: northehindrone (N), ethinylestradiol (E), norgestrel (NG), mestranol (M).

Parameter	*Composition*							
	NG–E		*N–E*		*N–M*			
	A	*B*	*A*	*B*	*A*	*B*	*C*	*D*
Desired number of peaks (*n*)	2	2	2	2	2	2	2	2
System resolution (SR)	3.27	3.66	10.52	2.31	18.8	9.26	6.85	6.28
System selectivity (SS)	−0.099	−0.229	−0.418	+0.138	−0.781	+0.712	+0.216	−0.233
Running time (min)	18	15	18	25	40	25	10	10

determined in oral contraceptives. Calculated data for the criteria are shown in Table V. For the N + E combination, the use of a reversed-phase system (system A) can be recommended owing to its higher SR value. For NG + E tablets, both systems provide the same elution order. The values of SR are close to each other but a higher SS value is obtained with system A. This result supports the use of system A for steroid assay. With the N + M combination, high SR values were obtained. Based on the significantly better SS value obtained with the normal-phase system (system B), it can be recommended for steroid assay. Similar conclusions can be drawn from examples taken from the literature[8,9], where the higher SS value obtained with the normal-phase system (system C) provides better detection for small amounts of M.

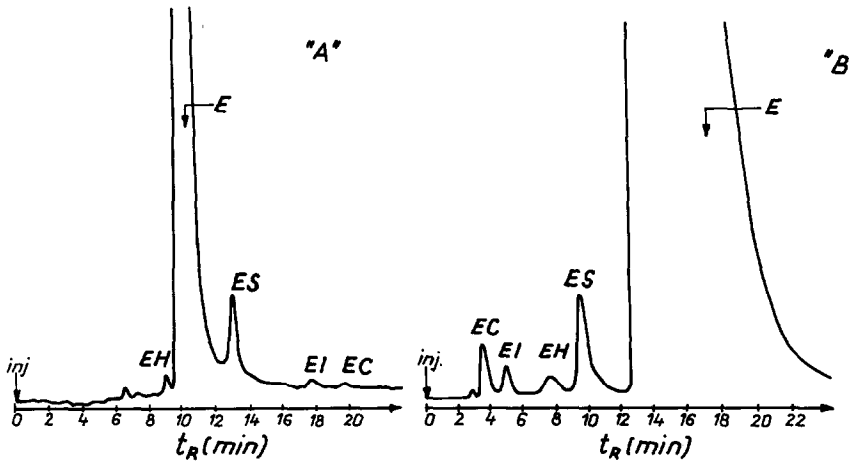

Fig. 1. Separation of ergotamine tartrate with (A) reversed-phase and (B) normal-phase systems. Conditions as in Table VI.

TABLE VI

CALCULATED DATA FOR ERGOTAMINE TARTRATE IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (PURITY TEST)

System A: column, Nucleosil C_{18} (10 μm) (250 × 4.6 mm I.D.); eluent, acetonitrile–10 m*M* ammonium carbonate solution (1:1). System B: column, Micropack SI-10 (10 μm) (250 × 2 mm I.D.); eluent, chloroform–methanol (95:5). Compounds: ergotamine (E), ergocristine (EC), ergosine (ES), 8-hydroxyergotamine (EH).

Parameter	*System A*	*System B*
Desired number of peaks (*n*)	5	5
Number of peaks before the main component (*z*)	2	5
Number of peaks after the main component (*v*)	3	–
System resolution (SR)	2.30	3.01
System selectivity (SS)	+0.031	+0.363
Running time (min)	25	30

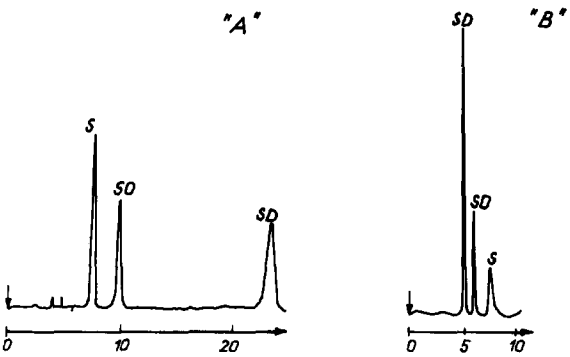

Fig. 2. Separation of sulfinpyrazone and its impurities. (A) Reversed-phase system; (B) normal-phase system. Conditions as in Table VII.

TABLE VII

CALCULATED DATA FOR SULFINPYRAZONE IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (STABILITY TEST, PRELIMINARY INVESTIGATIONS)

System A: column, Nucleosil C_{18} (10 μm) (250 × 4.6 mm I.D.); eluent, acetonitrile–water (1:1), pH 3. System B: LiChrosorb Si 60 (5 μm) (250 × 4.6 mm I.D.); eluent, hexane–tetrahydrofuran–methanol–glacial acetic acid (40:50:4:6). Compounds: sulfinpyrazone (S), 1,2-diphenyl-4-(2-phenylsulphonylethyl)pyrazolidine-3,5-dione (SO); 1,2-diphenyl-4-(2-phenylthioethyl)pyrazolidine-3,5-dione (SD); others unknown.

Parameter	*System A*	*System B*
Desired number of peaks (*n*)	3	3
Number of peaks before the main component (*z*)	–	2
Number of peaks after the main component (*v*)	2	–
System resolution (SR)	4.53	3.28
System selectivity (SS)	−0.529	+0.152
Running time (min)	25	10

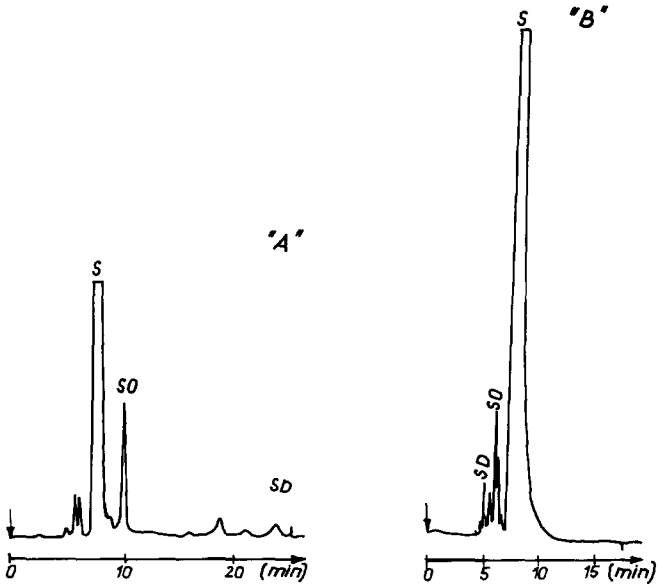

Fig. 3. Chromatograms of tablets subjected to heat treatment. Conditions as in Table VII.

The next example relates to the selection of an HPLC system suitable for purity testing of ergotamine tartrate[10,11].

Chromatograms obtained with reversed- and normal-phase systems are shown in Fig. 1 and the data are given in Table VI. High SR values are obtained by using both HPLC methods, but owing to the significantly higher SS value obtained with the normal-phase system (system B), it can be recommended for routine analysis.

The last example is connected with the stability testing of sulfinpyrazone tablets (a reversed-phase HPLC method has recently been published[12]). Chromatograms obtained with reversed- and normal-phase systems for model compounds are shown in Fig. 2 and the data are given in Table VII. Based on these data, system B was selected

TABLE VIII

CALCULATED DATA FOR SULFINPYRAZONE IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (STABILITY TEST, CORRECTED DATA ON THE BASIS OF STABILITY INVESTIGATIONS)

Conditions as in Table VII.

Parameter	*System A*	*System B*
Desired number of peaks (n)	10	7
Number of peaks before the main component (z)	3	6
Number of peaks after the main component (v)	6	–
System resolution (SR)	1.84	1.45
System selectivity (SS)	−0.136	+0.079
Running time (min)	30	15

for stability testing owing to its significantly higher SS value (the SR values are similar to each other).

When the first experimental runs with the tablets subjected to various stress conditions were performed and the criteria were re-calculated (chromatograms are shown in Fig. 3 and the calculated data are given in Table VIII), the application of system A (reversed-phase system) for stability testing can be recommended, as it provides higher SR and $R_{s,min}$ values than system B.

CONCLUSIONS

As the analytical aims in pharmaceutical analyses may vary widely, the effects of several factors on the method development have to be taken into consideration. The examples presented here indicate the advantageous characterization of HPLC systems with SR and SS criteria for the selection of the most applicable separation systems.

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