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APPLICATION OF OPTIMIZATION PROCEDURES FOR THE SEPARATION OF ANABOLIC COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple systematic approach is presented for optimizing high-performance liquid chromatographic separations of anabolics with multi-component isocratic mobile phases. A computer program was obtained and adjusted for use with an IBM-compatible XT personal computer. The program requires experimental retention data with three quaternary solvent mixtures to calculate the optimum solvent composition using a geometric model of a prism. For each possible composition of the mobile phase the set of retention data can be calculated. Applications are shown for mixtures of anabolic compounds using a mobile phase composed of methanol, tetrahydrofuran and acetonitrile. The predicted retention data agreed very well with the experimental data.

INTRODUCTION

In the analysis of anabolic compounds, high-performance liquid chromatography (HPLC) has proved to be an excellent separation, purification and detection technique [1–7]. Especially reversed-phase HPLC has been applied in a variety of analytical procedures. As anabolic preparations usually contain a combination of several anabolics, multi-component analysis is required in order to detect all the relevant compounds present in the sample. The high resolving power of the technique has been applied successfully to the detection of several anabolic compounds simultaneously [8]. Specific detection techniques such as the use of diode-array detectors no longer require a complete separation for unambiguous detection or identification of anabolic compounds [9]. For confirmation purposes, however, with other techniques such as thin-layer chromatography [10–12], mass spectrometry [13,14] and immunochemical detection [15–17], a separation and purification step using semi-preparative HPLC is often an indispensable part of the analytical procedure.

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To increase the selectivity of the whole analytical confirmation procedure, all anabolic compounds that are present in the sample must be separated completely. One of the important techniques for obtaining an optimum separation is variation of the solvent composition. In addition to the method of trial and error, more systematic approaches have been described for optimizing isocratic HPLC separations using empirical models [18,19], graphical procedures [20] or statistical methods such as sequential simplex calculations [21,22]. Often a large number of experiments must be carried out or complex calculations must be performed in order to obtain the desired optimization.

Amongst others, applications have been published for the separation of sulphonamides [23] and steroids [24]. A comprehensive survey of the optimization of chromatographic separations has been published recently [25]. In this paper, a simple, systematic approach is described for optimizing separations in isocratic reversed-phase HPLC with a multi-component mobile phase composition. The data of only a few experiments are needed to calculate the optimum separation with the use of a computer program. Applications to separations of mixtures of anabolic compounds are presented.

EXPERIMENTAL

HPLC equipment

The HPLC equipment consisted of the following components: a Model 231 automatic injector (Gilson, Meyvis, Bergen op Zoom, The Netherlands) equipped with a sample loop of 250 μl, two LKB Model 2150 solvent delivery systems (Pharmacia-LKB, Woerden, The Netherlands) controlled by an LKB Model 2152 gradient controller (Pharmacia-LKB), Model 1040A diode-array detection system (Hewlett-Packard, Amstelveen, The Netherlands), an LDC/Milton Roy Model CI-10B computing integration system (Interscience, Breda, The Netherlands) and a Multitech Model MPF 700 personal computer (Interscience) for data storage with integration software from Wico Electronics (Utrecht, The Netherlands; Take Five program, version 3). The column (150 mm$\times$4.6 mm I.D.) with 2-μm frits and Valco fittings (Chrompack, Middelburg, The Netherlands) was filled with Hypersil ODS (particle size 5 μm) (Shandon, Astmoor, U.K.) using a column packing instrument (Shandon) according to the manufacturer's instructions.

Computer program

The computer progam for optimization of HPLC retention times [26] was kindly provided by Dr. Ch. Gertz (Hagen, F.R.G.). The Basic program was adjusted to the requirements of an IBM-compatible personal computer. The following steps are needed in order to obtain data for optimization: (1) Determination of the methanol–water (A) composition of the mobile phase where the retention time of the last-eluting component does not exceed 20 min. (2) Choice of two other organic modifiers, e.g. tetrahydrofuran and acetonitrile, and the calculation of the volume percentages of these modifiers in water (B and C). (3) Determination of the retention times of all components in the test mixture with one of

the following combinations of three mobile phases: AB–AC–BC, AB–BC–A, AC–BC–C or AB–BC–B, where AB is a mobile phase composed of A–B (1:1), BC is B–C (1:1) and AC is A–C (1:1). In fact, only three solvent compositions have to be used. In principle, the experiment with three solvent compositions must be performed for each analyte in the mixture unless multi-wavelength or diode-array detection can be used. (4) Calculation of the retention times of all components for a number of compositions of A, B and C and/or calculation by choice of the optimum values of the chromatographic optimization function [18] or the chromatographic response function [18,27,28] with the computer program.

Reagents

Methanol, tetrahydrofuran and acetonitrile (J.T. Baker, Deventer, The Netherlands) were used as organic modifiers for optimization of anabolic compounds.

RESULTS AND DISCUSSION

Separation of stilbene compounds

Stilbenes include a number of anabolic compounds with proven carcinogenic and teratogenic properties, such as diethylstilbestrol (DES), the two stereoisomers (*Z,Z*)-dienestrol (Z-DE) and (*E,E*)-dienestrol (E-DE) and hexestrol (HEX). These compounds can be separated completely by isocratic reversed-phase HPLC using a methanol–water mixture [4]. Optimization of the separation of these compounds leads to the unexpected observation that when tetrahydrofuran (solvent B) was present in the mobile phase the retention of the stilbenes was increased substantially. As is shown in Fig. 1, the selectivity can also be changed by variation of the percentage of tetrahydrofuran. One composition (AB–BC–A, 10:10:80) was selected as giving one of the optimum separations, taking the time of analysis into account. The experimental data agreed very well with the calculated values (Table I).

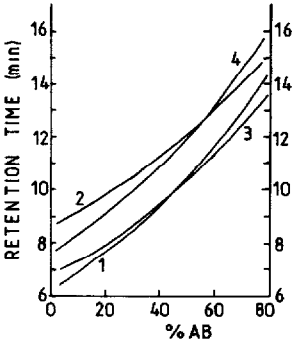

Fig. 1. One-dimensional representation of calculated elution behaviour of (1) DES, (2) Z-DE, (3) E-DE and (4) HEX as a function of the percentage of AB in the quaternary solvent system AB–BC–A. The contribution of BC was kept constant (20%). The compositions of A, B and C are described in the text.

TABLE I

EXPERIMENTAL AND CALCULATED RETENTION TIMES OF SEVERAL ANABOLICS WITH A QUATERNARY SOLVENT SYSTEM

A = methanol–water (60:40); B = tetrahydrofuran–water (39.3:60.7); C = acetonitrile–water (45.2:54.8). The values in parentheses are corrected retention times (see text).

Compound	Retention time (min)				
	AB	BC	A	AB–BC–A (10:10:80)	
				Experimental	Calculated
DES	15.92	10.26	5.54	6.66	6.49
Z-DE	16.80	10.28	8.14	8.84	8.93
E-DE	15.01	9.81	6.08	7.05	6.94
HEX	17.91	10.87	6.88	7.77	7.88
α-Zearalanol	6.11	4.13	4.74	4.44	4.79 (4.39)
β-Zearalanol	4.08	3.10	3.02	2.88	3.11 (2.85)
α-Zearalenol	7.00	4.46	5.30	5.08	5.35 (4.91)
β-Zearalenol	4.52	3.21	3.52	3.30	3.57 (3.28)
Zearalanone	7.80	6.00	5.44	5.13	5.69 (5.22)
Zearalenone	8.32	6.22	5.94	5.58	6.17 (5.66)

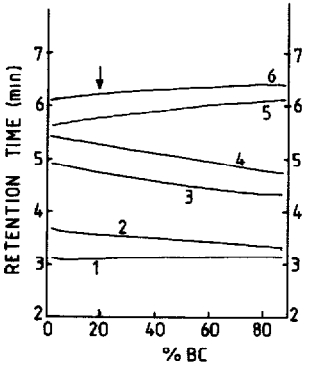

Fig. 2. One-dimensional representation of calculated elution behaviour of (1) β-zearalanol, (2) β-zearalenol, (3) α-zearalanol, (4) α-zearalenol, (5) zearalanone and (6) zearalenone as a function of the percentage of BC in the quaternary solvent system AB–BC–A. The contribution of AB was kept constant (10%). The compositions of A, B and C are described in the text.

Separation of resorcylic acid lactones

The major representative of this class of anabolic compound is zeranol (α-zearalanol). Other mainly metabolic products are β-zearalanol, zearalanone, α-zearalenol, β-zearalenol and the f2-toxin zearalenone [29]. Optimization of the HPLC separation yields a number of retention data from which a suitable mobile phase composition can be selected or calculated. As an example one set of data are plotted in Fig. 2 in a one-dimensional plot. The optimization program gave the solvent composition indicated with an arrow as producing the best separation. The calculated optimum separation of the six closely related compounds is shown

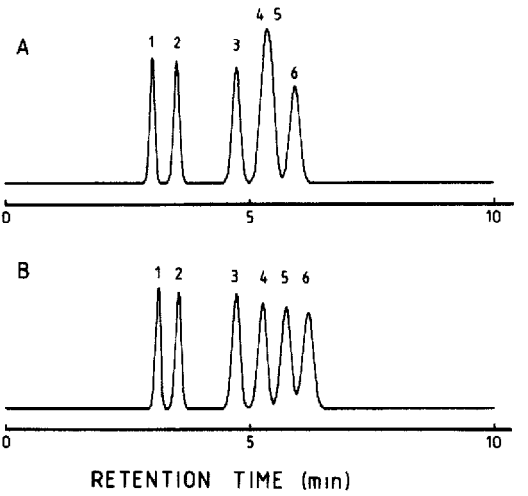

Fig. 3. Schematic HPLC traces of the separation of metabolites of zeranol. (A) HPLC separation with the starting solvent system A (methanol–water, 60:40). (B) Optimum separation as calculated with the program. Solvent composition: AB–BC–A (10:20:70). The identification of the compounds is given in Fig. 2. The compositions of A, B and C are described in the text.

in Fig. 3B and the separation obtained with the starting conditions is shown in Fig. 3A. As can be seen from Fig. 3B, an almost equidistant separation is obtained, and also between components 4 and 5. In Table I calculated and experimental data are compared for the solvent composition AB–BC–A (10:10:80). The two sets of data do not agree as well as expected. On regression analysis it appeared that an average factor of 1.16 existed between the two sets of data. This general deviation for the resorcylic acid lactones probably occurs from small variations in the solvent composition or a slightly different elution behaviour for this class of compounds. Correction leads to very good agreement (Table I) between the corrected calculated retention times (in parentheses) and experimental data. Only the separation between α-zearalenol and zearalanone was not as good as expected from the calculated data.

Separation of several anabolics

In practice, especially in tissues in which injection has been performed (application or injection sites), a variety of anabolic agents can be found mainly as esters. Before analysis the parent compounds are liberated by hydrolysis. The parent anabolic compounds frequently found in application sites are 19-nortestosterone (NT) and medroxyprogesterone (MP). Compounds that might be expected are the stilbenes, zeranol (ZER), trenbolone (TB) and 17α-methyltestosterone (MT). Also the natural steroids testosterone and estradiol have frequently been found. Optimisation of the HPLC separation of the eight exogenic compounds again gave interesting information about the retention behaviour. In Fig. 4 an example is shown of a one-dimensional representation for a selected quaternary solvent composition. If necessary all components can be sep-

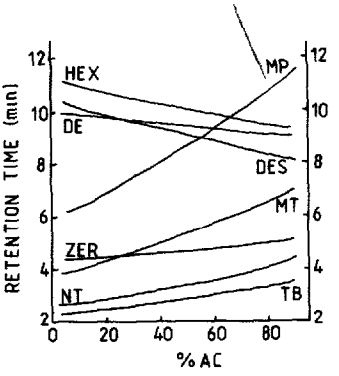

Fig. 4. One-dimensional representation of calculated elution behaviour of TB, NT, MT, MP, DES, DE, HEX and ZER as a function of the percentage of AC in the quaternary solvent system AB–AC–BC. The contribution of AB was kept constant (5%). The compositions of A, B and C and the abbreviations of the compounds are described in the text.

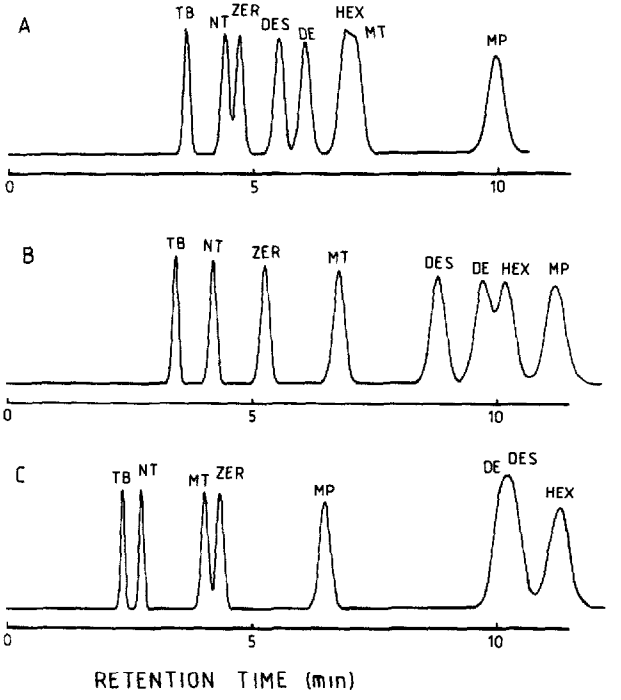

Fig. 5. Schematic HPLC traces of the separation of several anabolics. (A) HPLC separation with the starting solvent system (methanol–water, 60:40). (B) Calculated separation with the solvent system AB–AC (20:80). (C) Calculated separation with the solvent system AB–AC–BC (10:10:80). The compositions of A, B and C are described in the text.

arated almost completely or other conditions can be chosen which might be preferred from a practical point of view. This is illustrated in Fig. 5.

Fig. 5A shows the separation under normal standard conditions (methanol–water, 60:40) Fig. 5B and C show further optimized separations, with an almost

complete base-to-base separation of all eight components (Fig. 5B). In Fig. 5C a separation is shown in which all stilbenes have been shifted to longer retention times and can be collected in one fraction. Especially for the analysis of samples of bovine urine it may be useful to create such elution conditions in order to avoid the majority of the matrix components that elute in the first part of the chromatogram.

Possibilities of the optimization method

The optimization progam presented here constitutes a simple and fast method for obtaining an optimum HPLC separation of a number of components by variation of the composition of the mobile phase. Several organic modifiers can be used. For complete analysis, data from only four HPLC experiments are required. All solvent compositions for the separations can be calculated and the resulting chromatograms are shown on the screen or the retention times can be listed afterwards. Although only a limited number of calculated data have been checked experimentally in this study, the good agreement between these sets of values and those from other studies [26] allows the conclusion that the program will be very helpful in the optimization of HPLC separations. Probably the most important advantage of the method is a better understanding of the separation phenomena for complex mixtures of compounds. Especially in the field of anabolics, the optimum separation is not always the most useful one from a practical point of view. The program supplies extensive systematic listing of all possible elution conditions as a function of the percentages of the three solvents by interpolation. From this listing the optimum separation for each problem can be chosen. An example is the group-specific elution behaviour of the stilbenes. With increasing concentration of tetrahydrofuran the stilbenes are shifted selectively to longer retention times, where they can be collected in one fraction without matrix components of the sample. The different components can be identified subsequently by multicomponent mass spectrometry.

Further evaluations will be performed in the near future with other classes of compounds such as derivatized amino acids. In addition, possibilities will be investigated for calculating HPLC separations using normal-phase columns.

A listing of the program is available from the authors on request.

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