Journal of Chromatography, 415 (1987) 261–274
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3503

SIMULTANEOUS LIQUID CHROMATOGRAPHIC DETERMINATION OF SEVENTEEN OF THE MAJOR MONOAMINE NEUROTRANSMITTERS, PRECURSORS AND METABOLITES

I. OPTIMIZATION OF THE MOBILE PHASE USING FACTORIAL DESIGNS AND A COMPUTER PROGRAM TO PREDICT CHROMATOGRAMS

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(First received August 25th, 1986; revised manuscript received November 13th, 1986)

SUMMARY

Utilizing reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection and optimization of the mobile phase using factorial designs and a constructed computer program to predict chromatograms, it has been possible to obtain a satisfactory resolution of seventeen of the major monoamine neurotransmitters, precursors and metabolites. A rapid (<25 min) isocratic system for the simultaneous determination of 3,4-dihydroxyphenylalanine, dopamine, dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic acid, norepinephrine, normetanephrine, 3,4-dihydroxyphenylethylene glycol, 3-methoxy-4-hydroxyphenylethylene glycol, epinephrine, metanephrine, vanillylmandelic acid, 5-hydroxytryptophan, serotonin, 5-hydroxytryptophol and 5-hydroxyindoleacetic acid in addition to the internal standard isoproterenol is presented. The optimization strategy included (a) selection of variables to optimize by a reduced factorial design (b) a detailed study of these variables by a complete factorial design, (c) theoretical predictions of chromatograms by a constructed computer program and (d) test on the HPLC system. This optimization strategy can easily be applied to any problem of solute separation by liquid chromatography.

0378-4347/87/$03.50

INTRODUCTION

Reversed-phase chromatography in the ion-pair mode, pioneered by Eksborg and Schill [1], is a very powerful technique for the separation of polar substances. Ion-pair chromatography has frequently been used for the separation of catecholamines, indoleamines and their precursors and metabolites [2]. Much work has been carried out in order to study the effects of the variables controlling these separations (e.g. ref. 3). However, in most methods presented so far these variables have been studied separately and interaction effects between them, i.e., if the effect of one variable depends on the value of another variable, have therefore not be seen (e.g., refs. 4–7). In addition, it is well known that reversed-phase C_{18} column materials from different commercial suppliers can show large differences in chromatographic behaviour. Thus, the surface area of the base silicas, pore size, surface coverage, monomeric or polymeric phases (i.e., how derivatization of the C_{18} material is performed) are all factors that affect the chemistry of the C_{18} packing material [8]. There have also been reports concerning difficulties in reproducing complex reversed-phase separations when changing batches of C_{18} material from the same commercial supplier [9].

Optimization of complex reversed-phase ion-pair separations by a trial and error approach is time-consuming and likely to fail owing to the many variables involved and their interactions. Therefore, there has been increasing interest in finding a simplified and systematic means of optimizing high-performance liquid chromatographic (HPLC) separations. Many different methods and optimization criteria have been used, such as solvent triangles [10], overlapping resolution mapping (ORM), chromatographic optimization functions (COF) [11], factorial designs [12–14], window diagrams [15,16] and directed search techniques such as the sequential simplex procedure and modifications thereof [17,18]. A crucial but difficult step in all optimization procedures is to choose suitable quality criteria for the separation [19–23]. For recent reviews of optimization procedures, see refs. 21–23.

The purpose of this study was to develop an easy and therefore useful optimization strategy to be applied to the mobile phase variables in order to simultaneously separate and detect seventeen of the major monoamine neurotransmitters, precursors and metabolites. A computer program was composed, based on the data achieved in two-level factorial designs, which made systematically theoretical predictions of chromatograms, leading to an optimized mobile phase.

EXPERIMENTAL

Chromatography

The liquid chromatographic system included a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.); a pulse damper consisting of a 16-ml 1-m PTFE tube double wound with a stainless-steel wire, burst pressure 860 bar (HPLC-Teknik, Robertsfors, Sweden); a stainless-steel presaturator column, 50×8 mm I.D. (HPLC-Teknik), packed with the same packing material as the analytical column, connected between the pump and the injector; a cou-

lometric guard cell (Environmental Sciences Assoc., Bedford, MA, U.S.A.); a Model 7025 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 10-, 20- or 50-μl loop; stainless-steel guard columns and columns (HPLC-Teknik), the column temperature being controlled by means of a plastic jacket (HPLC-Teknik) coupled to a circulating water thermostat; a Coulochem ESA detector (Environmental Sciences Assoc.) with a Model 7021 serial coupled analytical cell [the first cell consisting of a porous graphite working electrode (coulometric) and the second cell being designed amperometrically in order to achieve an optimal signal-to-noise ratio, which is due to a smaller and thinner porous graphite working electrode and therefore oxidizing approximately 50–60% of oxidizable compounds]; both of these electrodes have modified palladium as reference systems; and a Model CI10 integrator (Laboratory Data Control).

Reagents

All reference and internal standards were obtained from Sigma (St. Louis, MO, U.S.A.). Citric acid monohydrate (citrate) (p.a. grade), Titriplex III disodium ethylenediaminetetraacetate (Na_2EDTA) (p.a. grade) and sodium octylhydrogensulphate (octyl sulphate, OSA) for surfactant tests were purchased from Merck (Darmstadt, F.R.G.), anhydrous sodium acetate (reagent grade) and 85% orthophosphoric acid (reagent grade) from Riedel-de Haën (Seelze-Hannover, F.R.G.), acetonitrile (HPLC grade) from Rathburn (Walkerburn, U.K.), diethylamine (DEA (p.a. grade) from May and Baker (Dagenham, U.K.) and sodium hydroxide (p.a. grade) from EKA (Bohus, Sweden). All chemicals were used as received.

The column packing materials were Spherisorb ODS II, 3 μm (batch No. 19/179) from Phase Separations (Queensferry, U.K.), Hypersil C_{18}, 5 μm (batch No. 1/1255) from Shandon (London, U.K.) and Nucleosil C_{18}, 5 μm (batch No. 4042) from Macherey-Nagel (Düren, F.R.G.).

The mobile phase was filtered under vacuum through a 0.22-μm GSWP filter (Millipore, Bedford, MA, U.S.A.). Acetonitrile was filtered separately through a 0.22-μm GVWP filter (Millipore).

Factorial design

In order to optimize the mobile phase variables, two-level factorial designs were used as described by Box et al. [12] and Massart et al. [13]. Briefly, two values denoted by + (the upper level) and − (the lower level) are given for each variable, defining the experimental domain. For n variables, 2^n experiments have to be performed for a complete factorial design. This allows the calculation of the magnitude of the effects of the variables on the retention times of the different solutes. The numerical value (β) is calculated from Yates' algorithm [12]. The β value represents the effect of a variable on a substance and is obtained by subtracting the responses (retention times) at the minus level from the responses at the plus level and dividing this by the number of experiments conducted (2^n). In addition, a reduced factorial design [12] was used with six variables and eight experiments to study the main effects of the variables. The β values were calcu-

TABLE I

CHROMATOGRAPHIC DATA FOR SOME COMMERCIAL STATIONARY PHASES

Mobile phase: acetate (50 mmol/l)-citrate (25 mmol/l) buffer (pH 4.45).

Column	Substance	k'	N	Peak asymmetry*
Nucleosil C_{18} (5 μm)	MHPG	1.06	1230	1.71
(150×4.6 mm I.D., flow-rate 1.5 ml/min)	DA	3.98	4310	1.30
	5-HIAA	6.14	4550	1.40
	5-HTP	9.02	5880	1.26
Hypersil C_{18} (5 μm)	MHPG	0.82	840	1.35
(150×4.0 mm I.D., flow-rate 1.0 ml/min)	DA	6.11	3700	1.67
	5-HIAA	5.16	4630	1.53
	5-HTP	9.61	4440	1.38
Spherisorb ODS II (3 μm)	MHPG	0.80	840	3.10
(100×4.6 mm I.D., flow-rate 1.5 ml/min)	DA	3.90	4900	2.19
	5-HIAA	5.06	5470	1.60
	5-HTP	10.2	7090	1.78

*Peak asymmetry was measured at 10% of the peak height.

lated in the same way as in the complete factorial design. All experiments were performed in a random order.

Computer program

A computer program was constructed in Pascal (see Fig. 1) to predict systematically retention times and resolutions of all solutes when systematically changing the mobile phase variables (see below). The complete program documentation and a program list can be obtained from the authors on request.

RESULTS AND DISCUSSION

Choice of chromatographic column packing material

Commercial supports may vary in chromatographic performance for different compounds [4,24]. In order to find a suitable packing material for the optimization procedure, three different supports were tested in screening experiments; 3-methoxy-4-hydroxyphenylethylene glycol (MHPG, a neutral glycol), dopamine (DA, an amine), 5-hydroxyindoleacetic acid (5-HIAA, an acid) and 5-hydroxytryptophan (5-HTP, a zwitterion) were chosen as test compounds. Spherisorb ODS II (3 μm) had the highest efficiency and gave the shortest analysis time, but gave asymmetric peaks, especially for MHPG (Table I). In addition, not more than 20 μl could be injected, as larger injection volumes caused peak broadening. Hypersil C_{18} (5 μm) gave better peak symmetry, and Nucleosil C_{18} (5 μm) showed the best compromise of peak symmetry and efficiency (Table I). Nucleosil C_{18} (5 μm) was also favoured in a similar separation problem in comparison with some other reversed-phase materials using serotonin (5-HT)

as the test compound [4]. In addition, in this study Nucleosil gave the best retention (k') for MHPG, which was of special importance as the chromatographic method was intended to be applied to tissue and fluid assessment (Wester et al. [25]) and previous studies had indicated difficulties in separating MHPG from early eluting unknown compounds [2,7,26–31]. Therefore, Nucleosil C_{18} (5 μm) was chosen for further optimization studies.

Optimization of the mobile phase

A large number of additives have been used in mobile phases for the separation of different catecholamines, indoleamines and their precursors and metabolites. The choice and concentration of buffers, ion-pair reagents and organic modifiers and the pH and temperature are all discrete and continuous variables that affect the separation. As interaction effects between the different variables are commonly encountered in HPLC, factorial designs are especially useful. Factorial designs also have the advantage of requiring few analyses per variable to be investigated, if not studying a large number of variables. In that event, reduced factorial designs [12] may be of great value for screening the main from the less important variables. However, in comparison with, e.g., simplex optimization, the experimental domain of the variables needs to be chosen carefully [21]. An a priori knowledge from the literature and personal HPLC experience is of great importance, but screening experiments will often have to be conducted before starting the factorial design. The approach used in this study was to select the most powerful and suitable mobile phase variables and to study these in detail. Therefore, a four-step optimization procedure was designed. First, a reduced factorial design [12] with many different variables was made to study the main effects of these variables in order to select the most suitable parameters to optimize. Second, the selected variables were studied in detail by means of a complete factorial design [12,13]. Third, a computer program was written to predict retention times and peak resolutions for the substances on the basis of the data obtained in the complete factorial design. Fourth, this theoretically optimized mobile phase was then tested on the HPLC system.

Reduced factorial design. Eight experiments with broad variable intervals were carried out to test the influence of the six mobile phase components as chosen (see experimental presentation, Table II). A citrate–acetate buffer was used as this buffer had been used successfully in an earlier HPLC separation [32]. In Table III, a positive β value indicates an increased retention time at the + (high) level of the mobile phase variable, whereas a negative β value indicates a longer retention time at the − (low) level. The figures are relative, but indicate the influence of the different variables on the retention. In general, the effect of a variable is greater for a substance having a long average retention time (β_0). Because of the factorial design being reduced, interaction effects of different variables are hidden within the major effects. As can be seen in Table III, temperature, acetonitrile and octylsulphate concentrations were the main variables affecting the retention. The effect of increased acetonitrile concentration was similar to that of increased temperature, i.e., both decreased the retention in general. Therefore, in the complete factorial design (see below), acetonitrile was

TABLE II

REDUCED FACTORIAL DESIGN: EXPERIMENTAL PRESENTATION

Mobile phase: acetate (100 ml/l)-citrate (50 mmol/l) buffer at a flow-rate of 1.2 ml/min. The pH was adjusted by addition of 85% phosphoric acid or 10 mol/l sodium hydroxide.

X_1 Acetonitrile (CH_3CN)	+ = 6.0% (v/v)	− = 0.1% (v/v)
X_2 Octylsulphate (OSA)	+ = 0.50 mmol/l	− = 0.10 mmol/l
X_3 pH	+ = 4.6	− = 2.7
X_4 Temperature (T)	+ = +30°C	− = +20°C
X_5 Na_2EDTA	+ = 54 μmol/l	− = 0 μmol/l
X_6 Diethylamine (DEA)	+ = 0.1% (v/v)	− = 0% (v/v)

Eluent No.	Mobile phase variable					
	CH_3CN	OSA	pH	T	Na_2EDTA	DEA
1	−	−	−	+	+	−
2	+	−	−	−	+	+
3	−	+	−	+	−	+
4	+	+	−	−	−	−
5	−	−	+	−	−	+
6	+	−	+	+	−	−
7	−	+	+	−	+	−
8	+	+	+	+	+	+

further studied whereas the temperature was kept constant at an intermediate level. An increased concentration of the octylsulphate counter ion increased the retention of the protonated amines but had only a small or no effect on the neutral substances, whereas the acids were influenced slightly in the opposite direction to the amines. pH had, in general, a varying effect on retention, as can be expected using the ion-pair chromatographic mode for a mixture of basic, neutral, acidic and zwitterionic substances. Therefore, both octylsulphate and pH were studied further in the complete factorial design (see below). Na_2EDTA is often routinely added to the mobile phase when using amperometric and coulometric LC detectors. Its metal-chelating properties are believed to prevent traces of metal ions (especially Fe^{2+}) originating from the stainless-steel material in the equipment from causing detector interferences. However, Na_2EDTA did not show any major effect on the selectivity (Table III), although the different effects on ISOP and 5-HT versus 5-HTP may be of importance. Na_2EDTA was therefore at first excluded from the complete factorial design. However, as a spurious peak appeared in the chromatogram when it was removed, the Na_2EDTA concentration was held constant at the lowest level that abolished this spurious peak. Diethylamine (DEA) was added to the mobile phase mainly to see if it had any effect on peak symmetry. No such effect was seen in this study (data not shown). However, it was observed that an increased DEA concentration either decreased the retention or had no effect. Those substances that increased their retention most with increasing octylsulphate concentration (NE, E, NM, DA, MET, ISOP and 5-HT) also showed the most significant decrease when the DEA level was increased.

TABLE III

REDUCED FACTORIAL DESIGN: CALCULATED MEAN EFFECTS (β VALUES) ON THE RETENTION TIMES OF THE SUBSTANCES WHEN CHANGING THE MOBILE PHASE PARAMETERS FROM + TO − ACCORDING TO TABLE II

Substance*	Mobile phase variable						
	β_0**	CH_3CN: β_1	OSA: β_2	pH: β_3	T: β_4	Na_2EDTA: β_5	DEA: β_6
DHPG	0.97	−0.05	−0.01	+0.02	−0.03	0	0
VMA	1.04	−0.05	−0.05	−0.34	+0.03	+0.03	0
L-DOPA	1.43	−0.10	+0.18	−0.52	+0.06	−0.17	−0.04
DOPAC	1.53	−0.13	+0.06	−0.30	−0.08	+0.02	−0.01
NE	1.54	−0.19	+0.37	+0.06	−0.14	+0.6	−0.14
MHPG	1.59	−0.20	−0.05	+0.05	−0.07	+0.02	−0.02
E	2.17	−0.40	+0.63	+0.14	−0.29	+0.13	−0.26
NM	3.10	−0.68	+1.00	+0.21	−0.42	+0.23	−0.41
5-HTP	3.88	−0.51	+0.81	−2.18	+0.07	−0.71	−0.24
DA	3.98	−1.05	+1.43	+0.40	−0.74	+0.36	−0.63
MET	4.93	−1.36	+1.77	+0.47	−0.86	+0.49	−0.85
5-HIAA	4.95	−0.85	−0.18	−0.90	−0.52	+0.17	−0.10
5-HTOL	5.10	−1.06	−0.17	+0.36	−0.68	+0.06	−0.06
HVA	6.38	−1.17	−0.39	−1.87	−0.29	+0.37	−0.24
ISOP	7.80	−2.60	+3.04	+0.99	−1.59	+0.95	−1.47
5-HT	12.6	−4.24	+5.17	+1.80	−3.33	+1.49	−2.59

*Abbreviations: DHPG = 3,4-dihydroxyphenylethylene glycol; VMA = vanillylmandelic acid; DOPA = 3,4-dihydroxyphenylalanine; DOPAC = dihydroxyphenylacetic acid; NE = norepinephrine; MHPG = 3-methoxy-4-hydroxyphenylethylene glycol; E = epinephrine; NM = normetanephrine; 5-HTP = 5-hydroxytryptophan; DA = dopamine; MET = metanephrine; 5-HIAA = 5-hydroxyindoleacetic acid; 5-HTOL = 5-hydroxytryptophol; HVA = homovanillic acid; ISOP = isoproterenol (internal standard); 5-HT = serotonin.

**β_0 is the average retention time.

This indicates a competing role of DEA in the ion-pair retention mechanism. As DEA was not considered to be a major variable, it was not investigated further.

Complete factorial design. Acetonitrile, octylsulphate and pH were chosen as variables for a complete factorial design study (for experimental presentation, see Table IV). The variable intervals were set more narrowly than in the reduced factorial design after some screening experiments. The retention times obtained for the eluites are presented in Table V and calculated mean effects and interaction effects are given in Table VI. Similar conclusions as in the reduced factorial design could now be established. Thus, an increased octylsulphate concentration lengthened the retention for the amines. Also, an increased pH generally decreased the retention for most substances but DHPG, VMA, MHPG, DOPAC, 5-HTOL and HVA were not affected. The effect of pH was most pronounced for DOPA, 5-HTP and 5-HT, although the experimental domain only included a difference of 0.2 pH units. A significant interaction effect between acetonitrile and octylsulphate concentration (β_{12}) was seen for DOPA, MET, 5-HTP, 3-MT and 5-HT. Acetonitrile concentration and pH interaction (β_{13}), and also octylsulphate con-

TABLE IV

COMPLETE FACTORIAL DESIGN: EXPERIMENTAL PRESENTATION

Mobile phase: citrate buffer (100 mmol/l) containing 0.3 mmol/l Na_2EDTA at a flow-rate of 1.2 ml/min. The temperature was kept at +25°C. The pH was adjusted by addition of 10 mol/l sodium hydroxide.

X_1 Acetonitrile (CH_3CN)	+ = 7.5% (v/v)	− = 5.5% (v/v)
X_2 Octylsulphate (OSA)	+ = 0.430 mmol/l	− = 0.215 mmol/l
X_3 pH	+ = 2.45	− = 2.25

Eluent No.	Mobile phase variable		
	CH_3CN	OSA	pH
1	−	−	−
2	+	−	−
3	−	+	−
4	+	+	−
5	−	−	+
6	+	−	+
7	−	+	+
8	+	+	+

centration and pH interaction (β_{23}), were found to be especially important for the retention behaviour of ISOP, 5-HTP, 3-MT and 5-HT. For ISOP and 3-MT, the acetonitrile, octylsulphate and pH interaction term β_{123} also have to be considered. It is therefore obvious that trying to optimize a separation of the above-mentioned substances and predict their retention behaviour using a simple one-variable step approach is likely to fail.

Computer-aided separation. A model equation to predict retention times for each solute was formulated:

$$t(X_1X_2X_3) = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3 \quad (1)$$

where t describes the predicted retention time, X_{1-3} the mobile phase variables (Table IV) that have been transformed to a range from −1 to +1, β_0 is the average retention time, β_1X_1, β_2X_2 and β_3X_3 represent the main effects of the variables and $\beta_{12}X_1X_2$, $\beta_{13}X_1X_3$, $\beta_{23}X_2X_3$ and $\beta_{123}X_1X_2X_3$ represent interaction effects.

A computer program was then constructed (Fig. 1) using eqn. 1 to predict retention times and resolutions for each substance when systematically altering the mobile phase variables within a chosen domain. The predicted retention times between adjacent peaks, irrespective of elution order, were tested according to the following standard resolution criteria [33]:

$$R_s = \frac{T_j - T_i}{0.5(W_j + W_i)} \quad (2)$$

TABLE V

COMPLETE FACTORIAL DESIGN: RETENTION TIMES (IN MINUTES) WHEN CHANGING THE MOBILE PHASE ACCORDING TO TABLE IV

For abbreviations see Table III; 3-MT = 3-methoxytyramine.

Expt. No.	DHPG	VMA	MHPG	DOPAC	NE	E	L-DOPA	NM	DA	5-HTOL	MET	5-HIAA	ISOP	HVA	5-HTP	3-MT	5-HT
1	2.3	3.2	3.7	4.2	4.4	5.3	7.0	7.9	8.9	10.2	10.4	12.0	14.3	16.3	22.2	21.6	24.5
2	2.2	3.1	3.3	3.5	3.3	4.0	5.0	5.3	5.3	8.0	6.8	9.2	6.4	12.2	12.3	9.3	13.8
3	2.4	3.0	3.6	4.0	5.6	7.3	9.6	10.2	12.2	10.0	15.0	11.7	19.9	15.9	30.2	30.6	34.0
4	2.2	3.0	3.1	3.4	4.6	5.7	6.6	7.7	8 7	7.7	10.2	9.0	13.2	11.6	18.4	19.1	21.6
5	2.3	3.3	3.6	4.0	3.9	4.9	5.3	6.5	7 8	10.2	9.4	11.7	12.4	16.5	16.3	18.8	21.2
6	2.2	3.0	3.1	3.5	3.2	3.8	4.2	4.8	5.4	7.7	6.3	9.1	7.8	11.8	9.6	11.1	12.5
7	2.2	3.4	3.6	4.1	5.0	6.4	7.3	9.1	11.1	10.2	13.2	11.7	18.3	15.9	22.1	28.2	29.7
8	2.2	2.9	3.1	3.5	4.2	5.1	5.1	6.8	7.7	7.8	9.0	8.6	11.7	11.7	13.3	16.9	18.4

TABLE VI

COMPLETE FACTORIAL DESIGN: CALCULATED MEAN EFFECTS (β VALUES) ON THE RETENTION TIMES OF THE SUBSTANCES WHEN CHANGING THE MOBILE PHASE FROM + TO − ACCORDING TO TABLE IV

β_0 is the average retention time and β_{12}, β_{13}, β_{23} and β_{123} represent the different interplay effects between the variables. For abbreviations, see Table III.

Substance	Mobile phase variable							
	β_0	CH_3CN: β_1	OSA: β_1	pH: β_3	β_{12}	β_{13}	β_{23}	β_{123}
DHPG	2.25	−0.05	0	−0.03	0	+0.03	−0.03	+0.03
VMA	3.11	−0.11	−0.04	+0.04	−0 01	−0.09	+0.04	−0.04
MHPG	3.39	−0 24	−0.04	−0.04	−0.02	−0.02	+0.04	+0.01
DOPAC	3.77	−0.30	−0.03	0	0	+0.02	+0.05	+0.03
NE	4.27	−0.46	+0.57	−0.21	−0.01	+0.08	−0.05	−0 03
E	5.31	−0.67	+0 81	−0.27	−0.07	+0 06	−0.12	+0.01
L-DOPA	6.26	−1.04	+0.89	−0.79	−0.27	+0.21	−0.17	−0 02
NM	7.29	−1.14	+1 16	−0.49	−0.07	+0 14	−0.15	−0 09
DA	8.39	−1.61	+1.55	−0.38	−0.13	+0.15	−0 15	−0.13
5-HTOL	8.98	−1.18	−0.06	−0.01	0	−0.05	+0.08	+0 03
MET	10.0	−1.97	+1.81	−0.57	−0.29	+0 14	−0.19	+0.01
5-HIAA	10.4	−1.41	−0.13	−0 11	−0.05	−0.03	0	−0.08
ISOP	13 0	−3 23	+2.77	−0 46	−0.11	+0.43	−0.33	−0.40
HVA	14.0	−2.17	−0.22	−0 02	+0 04	−0 07	+0 04	+0.09
5-HTP	18.0	−4.66	+2.95	−2 73	−0.51	+0.78	−0.78	−0.03
3-MT	19 4	−5 36	+4.25	−0 71	−0.36	+0.60	−0.46	−0.56
5-HT	22 0	−5 39	+3.96	−1.52	−0 54	+0.39	−0.37	−0.12

where T is the retention time and W is the peak width at the inflection points of curves i and j. The peak width (W) at different retention times was determined experimentally from test chromatograms obtained with the analytical column to be used. Thus, a linear function describing the relationship between peak width and retention time was calculated; linear regression analysis showed a positive correlation ($r = +0.997$). This function was then used for interpolation of peak widths for the retention times calculated by the computer program. It was thus assumed that the number of plates was constant and the same for all solutes. This assumption was made because the resolution is proportional to the square root of the plate number ($R_s \propto \sqrt{N}$) [9] and the solutes studied have similar molecular weights and are chemically related.

The retention times for all substances as measured in the complete factorial design (Table V) were entered into the computer where a RAW FILE was created. This file was then used in the PROGRAM RUN where the program automatically and systematically searched through the variable domain as chosen. In the PROGRAM RUN the operator specified: (A) the start point, end point and number of steps for each mobile phase variable; (B) maximum total retention time; (C) high and low limits of desired resolution, i.e., the program started by testing all variable combinations with the high limit of desired resolution; any substance having a lower resolution was considered as not resolved; after all combinations had been tested, the desired resolution was automatically decreased by

Fig. 1. JSP diagram over factorial design program. Abbreviations: var. = variables; subst. = substances; t_r = retention time, R_s = resolution. (1) This procedure produces a matrix, called multiplication scheme, later used to calculate β values and retention times. If three variables were used in the experiment, the matrix would hold 1, 2, 3, 12, 13, 23, 123. (2) Limits for mobile phase variables and specific search conditions.

0.1 and another test of all combinations was performed, and this procedure was repeated until the low limit was reached and searched; (D) maximum number of optima required.

If all resolutions and the total chromatographic time were found to be satisfactory, the theoretical eluent was stored for later output and thereafter displayed on the computer screen or written out.

Test on the HPLC system. From the above, several alternatives with good resolution between peaks and short chromatographic time were presented on the computer. The choice of mobile phase was based on an a priori knowledge of the concentrations of these transmitters in brain tissue and cerebrospinal fluid. Therefore, an alternative could be chosen with satisfactory resolution, short total chromatographic time and with complete resolution of the internal standard and other substances known to appear in high concentrations from their adjacent peaks. The predicted optimal mobile phase was tested on the HPLC system and was found to be satisfactory (Fig. 2). The predicted and actual retention times were found to be in good agreement, with a difference of 1–3% (Table VII).

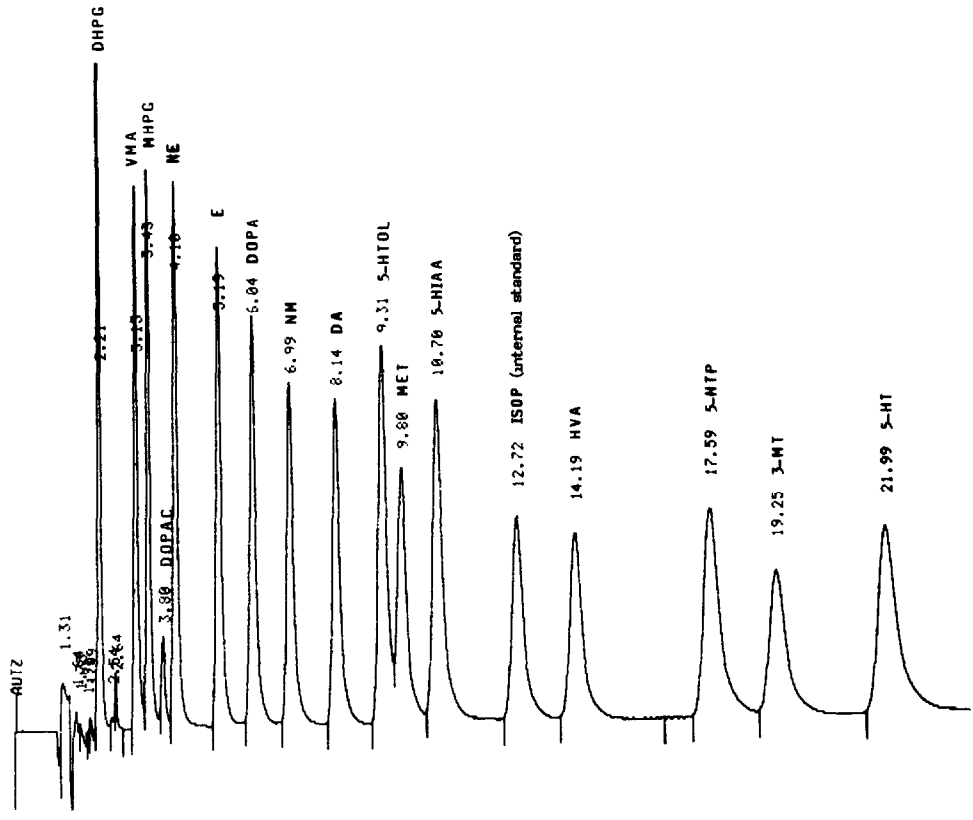

Fig. 2. Chromatogram of 15 pmol of standards; 20 μl were injected. The mobile phase (optimized according to the procedure described under Results and discussion) consisted of a 0.1 mol/l citrate buffer containing 0.3 mmol/l Na_2EDTA, 0.293 mmol/l octylsulphate and acetonitrile–water (6.3:93.7, v/v) at a pH of 2.35. The guard cell was set at +1.20 V, the first detector cell at +0.20 V and the second detector cell (coupled to the integrator) at +0.80 V. The flow-rate was 1.2 ml/min and the temperature was kept at 25 °C. For abbreviations, see Table III.

CONCLUSION

In conclusion, a rapid technique for the simultaneous separation of seventeen of the major monoamine neurotransmitters, precursors and metabolites has been developed. Our approach using a reduced and then a complete two-level factorial design leading to the theoretical prediction of an optimal mobile phase (by using the computer program) could be applied to any HPLC separation problem.

ACKNOWLEDGEMENTS

The authors thank Dr. John Hardy, Dr. Anne-Marie O'Carroll, Dr. Olle Magnusson and Dr. Cora O'Neil for help with the manuscript. This work was supported by the Swedish Medical Research Council (Grant No. 12X-5664), Centrala försöksdjursnämnden in Sweden, Nordiska Samfundet mot Plågsamma djurförsök and Stiftelsen för Gamla Tjänarinnor, Sweden.

TABLE VII

PREDICTED AND ACTUAL RETENTION TIMES AND RESOLUTION

The theoretical optimized mobile phase showed the following levels of the variables: $CH_3CN = 6.3\%$ (v/v); OSA = 0.293 m*M*; pH = 2.35. For abbreviations, see Table III.

Substance	Predicted retention time (min)	Actual retention time (min)	Predicted resolution
DHPG	2.21	2.26	2.85
VMA	3.14	3.15	0.83
MHPG	3.44	3.43	1.01
DOPAC	3.84	3.80	0.85
NE	4.22	4.10	2.09
E	5.20	5.19	1.75
L-DOPA	6.20	6.04	1.50
NM	7.20	6.99	1.44
DA	8.25	8.14	1.17
5-HTOL	9.22	9.31	0.76
MET	9.90	9.80	0.81
5-HIAA	10.7	10.7	2.00
ISOP	12.9	12.7	1.30
HVA	14.5	14.2	2.48
5-HTP	18.1	17.6	0.70
3-MT	19.3	19.2	1.41
5-HT	21.9	22.0	

REFERENCES

1 S. Eksborg and G. Schill, Anal. Chem., 45 (1973) 2092.
2 S.M. Lasley, A. Michaelson, R.D. Greenland and P.M. McGinnis, J. Chromatogr., 305 (1984) 27.
3 M. Johansson, Thesis, Department of Analytical Pharmaceutical Chemistry, Biomedical Centre, Uppsala.
4 O. Magnusson, L.B. Nilsson and D. Westerlund, J. Chromatogr., 221 (1980) 237.
5 L. Semerdjian-Rouquier, L. Bossi and B. Scatton, J. Chromatogr., 218 (1981) 663.
6 C.D. Kilts, G.R. Breese and R.B. Mailman, J. Chromatogr., 225 (1981) 347.
7 C. van Valkenburg, U. Tjaden, J. van der Krogt and B. van der Leden, J. Neurochem., 39 (1982) 990.
8 K.K. Unger, Porous Silica–Its Properties and Use as Support in Column Liquid Chromatography, Elsevier, Amsterdam, 1979.
9 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979, p. 183.
10 L.R. Snyder, J. Chromatogr. Sci., 16 (1978) 223.
11 J.L. Glajch, J.J. Kirkland, K.M. Squire and J.M. Minor, J. Chromatogr., 199 (1980) 57.
12 G.E.P. Box, W.G. Hunter and J.S. Hunter, Statistics for Experiments. An Introduction to Design, Data Analysis and Model Building, Wiley, New York, 1978.
13 D.L. Massart, A. Dijkstra and L. Kaufman, Evaluation and Optimisation of Laboratory Methods and Analytical Procedures, Elsevier, Amsterdam, 1978.
14 W. Lindberg, E. Johansson and K. Johansson, J. Chromatogr., 211 (1981) 201.
15 S.N. Deming and M.L.H. Turoff, Anal. Chem., 50 (1978) 546.
16 M. Otto and W. Wegscheider, J. Liq. Chromatogr., 6 (1983) 685.

17 J.A. Nelder and R. Mead, Comput. J., 7 (1965) 308.
18 J.C. Berridge and E.G. Morrissey, J. Chromatogr., 316 (1984) 69.
19 H.J.G. Debets, B.L. Bajema and D.A. Doornbos, Anal. Chim. Acta, 151 (1983) 131.
20 H.J.G. Debets, J.W. Weyland and D.A. Doornbos, Anal. Chim. Acta, 150 (1983) 259.
21 J.C. Berridge, Techniques for the Automated Optimization of HPLC Separations, Wiley-Interscience, Chichester, 1985.
22 G. D'Agostino, L. Castagnetta, F. Mitchell and M.J. O'Hare, J. Chromatogr., 338 (1985) 1.
23 W. Wegscheider, E.P. Lankmayr and M. Otto, Anal. Chim. Acta, 150 (1983) 87.
24 N.H.C. Cooke, B.G. Archer, K. Olsen and A. Berick, Anal. Chem., 54 (1982) 2277.
25 P. Wester, J. Gottfries and B. Winblad, J. Chromatogr., 415 (1987) 275.
26 J. Wagner, P. Vitali, M.G. Palfreyman, M. Zraika and S. Hout, J. Neurochem., 38 (1982) 1241.
27 C.F. Saller and A.I. Salama, J. Chromatogr., 309 (1984) 287.
28 J.J. Warsh, A. Chiu and D.D. Godse, J. Chromatogr., 228 (1982) 131.
29 R.J. Martin, B.A. Bailey and R.G.H. Downer, J. Chromatogr., 278 (1983) 265.
30 G.S. Mayer and R.E. Shoup, J. Chromatogr, 255 (1983) 533.
31 I.C. Kilpatrick, M.W. Jones and O.T. Phillipson, J. Neurochem., 46 (1986) 1865.
32 P. Wester, J.A. Hardy, J. Marcusson, P. Nyberg and B. Winblad, Neurobiol. Aging, 5 (1984) 199.
33 P.R. Brown and A.M. Krstulovic, Anal. Biochem., 99 (1979) 1.