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Two-parameter mobile phase optimization for the simultaneous high-performance liquid chromatographic determination of dopamine, serotonin and related compounds in microdissected rat brain nuclei

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ABSTRACT

A new high-pressure liquid chromatography method with electrochemical detection is described that allows the simultaneous determination of dopamine, serotonin, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 5-hydroxytryptophan and 5-hydroxyindoleacetic acid in microdissected nuclei from individual rat brains. No sample pre-treatment steps are required. Resolution and analysis time were optimized by a simple limited optimization procedure, involving two-parameter factorial design.

INTRODUCTION

A great number of methods based on high-performance liquid chromatography with electrochemical detection (HPLC–ED), especially in the ion-pair mode (IP), have been developed, successfully dealing with the quantitative analysis of endogenous catecholaminergic and indolaminergic compounds [1–11]. However, laborious sample preparation or large analysis times are often required that could lead to a loss of sensitivity for later-eluted substances. In addition, differences in instrumentation and experimental conditions can make it difficult to reproduce a reported separation, and so the need for formal optimization strategies arises. In this way, two-parameter optimization has proved to be a practical approach in the improvement of chromatographic separations [12,13].

The aim of this work is to develop a selective chromatographic method for simultaneous measurement in microdissected rat brain nuclei of the aminergic neurotransmitters dopamine (DA) and serotonin (5HT), their major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA), 5-hydroxyindoleacetic acid (5HIAA), and the serotonin precursor 5-hydroxytryptophan (5HTP). For this purpose, an optimization strategy was followed that involved the use of factorial design coupled with computer-aided chromatogram prediction and evaluation. From the mobile phase parameters that can be

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used for controlling selectivity for ionized solutes [14–16], pH and organic modifier content were selected, in order to optimize both resolution and analysis time. An evaluation procedure that allowed us to relate variations in separation performance to variations in mobile phase composition was used, based on the multi-criteria decision making method [17]. Compounds expected to be present in biological samples as major interfering peaks, norepinephrine (NE) and its metabolite 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), have been also considered. Thus, a limited optimization approach [18] was undertaken, resolution between NE and MHPG not being taken into account.

EXPERIMENTAL

Reagents

Citric acid monohydrate, sodium octyl sulphate (SOS), EDTA disodium salt and glacial acetic acid were from Scharlau (Barcelona, Spain). Anhydrous sodium acetate, sodium metabisulphite, perchloric acid (PCA) and methanol (HPLC grade) were from Merck (Darmstadt, Germany). All standards were obtained from Sigma (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore).

Chromatographic system

The HPLC system consisted on a solvent delivery pump (Model 420, Kontron, Zürich, Zwitzerland) equipped with a pulse damper, a six-port rotary valve (Model 7125, Rheodyne, Berkeley, CA, USA) and a 20-μl sample loop. The amperometric detector was a BAS LC-4B (West Lafayette, IN, USA) coupled with a TL-5A glassy carbon electrode, and the recorder was a Hewlett-Packard HP-3390 A integrator. The detector potential was set at +0.7 V *vs.* Ag/AgCl. A LiChrospher RP-18 125 mm × 4 mm 5 μm (Merck, Darmstadt, Germany) analytical column operated at a flow-rate of 0.9 ml/min was used. All separations were performed at room temperature (20–22°C).

Mobile phases and standards

The mobile phases consisted of a 0.1 *M* sodium acetate–0.02 *M* citric acid buffer containing 0.1 m*M* Na_2EDTA and 0.69 m*M* SOS, mixed with methanol to the desired volume ratio. The pH was adjusted by adding glacial acetic acid to the methanol–buffer mixture. After pH adjustment, mobile phases were filtered through 0.2-μm nylon filters (Alltech Assoc.) and degassed under vacuum. Stock solutions of standards (100 μg/ml) were prepared in 0.1 *M* PCA containing 0.1 m*M* EDTA and 0.2 m*M* sodium metabisulphite, and stored at −80°C. Working standards (10 ng/ml) were prepared daily making appropriate dilutions of the stock solution with mobile phase.

Experimental design and calculations

A two parameters–two levels (2 × 2) factorial design was used to measure the effects of mobile phase pH and methanol content over the capacity factors (k') of the compounds to be separated, following the methods decribed by Box *et al.* [19]. Upper and lower levels have to be established for both variables, defining a bidimensional, rectangle-shaped, factor space. Chromatograms of standards must be obtained at the four mobile phase compositions corresponding to the rectangle vertices. These were

assayed in a random order during four consecutive days, allowing the system to be stabilized between successive trials. Chromatographic data were recorded at the same hour every day, in order to avoid daily variations in experimental conditions.

A model equation [20] was fitted for each compound from the factorial design retention data:

$$\ln k' = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 \quad (1)$$

where the variables X_1 (pH) and X_2 (methanol) were transformed ranging from -1 to $+1$; β_1 and β_2 are the main effects of each variable on the ln k' of compounds, β_{12} the term reflecting interaction between both variables, and β_0 the independent term. The β coefficients were obtained through Yate's algorithm (see ref. 19).

Chromatographic resolution (R_s) was calculated by the expression:

$$R_s = \tfrac{1}{4} N^{0.5} (\alpha - 1/\alpha) (k'_2/k'_2 + 1) \quad (2)$$

where $\alpha = k'_2/k'_1$ and the indices for k' indicate elution order of two adjacent peaks. The column plate number (N) was thus assumed to remain constant, and a very conservative value ($N = 1500$) was used.

Software. All calculations were performed using a standard spreadsheet program (Symphony, Lotus Development). The program has built-in simulation facilities which allowed easy implementation of all required formulae and data structure definition (for details, contact the authors). In all, the time required for the design and implementation of mathematic and logic statements, enter basic data and obtain the desired output was about 4 h of interactive work.

Preparation of tissue samples

Male Sprague–Dawley rats weighing 250–300 g. were used. After decapitation, brains were quickly removed, frozen and stored at -80°C until analysis (less than 1 week). The brain was sliced into 300-μm thick coronal sections using a cryo-cut (American Optical) with a chamber temperature of -10°C. A micropunch technique [21] was used to dissect out from the unfixed, frozen brain sections the following nuclei: accumbens (A), caudate putamen (CP), olfactory tubercle (TUL) and suprachiasmatic (SQ). From consecutive brain slices, the nuclei were bilaterally (CP, TUL, A) or unilaterally (SQ) micropunched, using 800- or 900-μm stainless-steel needles. A total of two (SQ), four (A, TUL) or six (CP) punches/brain were taken using the atlas of König and Klippel [22] as a guide. The frozen samples were pushed into 500-μl polypropylene microcentrifuge tubes and homogenized by ultrasonic disruption in 35 μl (SQ), 100 μl (A), 150 μl (TUL) or 200 μl (CP) of chilled mobile phase. Following centrifugation (12 000 g for 10 min at 4°C), 20 μl of the supernatant were injected into the chromatographic system.

RESULTS AND DISCUSSION

In order to find the methanol content interval giving a suitable k' range for the considered compounds, values of 5, 10 and 15% (v/v) were first tested (data not shown). The pH interval was set on the basis of previous experience with the buffer

TABLE I

FACTORIAL DESIGN. EXPERIMENTAL DESIGN: pH AND METHANOL LEVELS, TESTED MOBILE PHASE COMPOSITIONS, TRIAL ORDER AND RESULTING CAPACITY FACTORS

Fitted model for each compound: β_1 (pH main effect), β_2 (methanol main effect), β_{12} (interaction of pH and methanol) and β_0 (independent term).

Trial order	pH	Methanol content (%, v/v)	NE	MHPG	DA	DOPAC	HVA	5HTP	5HT	5HIAA
			Capacity factors (k')							
1	3.90	12.0	0.67	0.81	2.19	1.69	4.77	1.08	6.08	3.30
2	4.90	10.0	1.26	1.55	5.25	1.08	5.55	1.59	16.82	3.06
3	4.90	12.0	1.04	1.22	3.96	0.90	3.94	1.24	11.85	2.45
4	3.90	10.0	0.82	1.00	2.92	2.04	6.71	1.39	8.62	4.28
			β coefficients							
		β_1	0.438	0.422	0.588	−0.632	−0.191	0.137	0.669	−0.315
		β_2	−0.196	−0.227	−0.284	−0.185	−0.342	−0.254	−0.350	−0.240
		β_{12}	0.001	−0.014	0.003	0.006	−0.001	0.003	−0.001	0.019
		β_0	−0.084	0.104	1.223	0.301	1.637	0.271	2.313	1.166

used. Levels for the factorial design are shown in Table I, together with the k' found for each compound with the four tested mobile phase compositions and the estimated β coefficients. Methanol content exerts greater effects over later eluting compounds. The major factor affecting both retention and selectivity appeared to be pH, which had opposite effects on retention of basic and acidic compounds. This result agrees well with current theory, weak bases being expected to be more ionized and then less retained as pH decreased, and the opposite occurring for weak acids [23]. The mobile phase pH had a positive and small effect on retention of 5HTP, a zwitterionic species. All interaction terms were negligible with respect to main effects.

Although additional experiments could be made at this point to further improve the fitted model, it was decided to proceed with the optimization procedure by making use of the available data. A grid of 0.05 pH units and 0.1 methanol content units was used for systematic chromatogram prediction within the factor space; further diminishing interval size was thought to reflect only minor changes in separation performance. A matrix containing predicted k' and resolutions (eqns. 1 and 2) for the resulting 441 mobile phase compositions was obtained. Resolution between NE and MHPG was never computed.

Resolution for the critical pair (minimum resolution, or $R_{s_{min}}$) and analysis time were chosen as the major optimization criteria. The retention time of the last eluted substance (t_{max}) was taken as a measure of analysis time. In order to avoid interferences with the sample front an additional, exclusive criterion was introduced. So, chromatograms showing peaks, other than NE or MHPG, with a predicted retention time lower than 2.5 min were discarded. To optimize simultaneously both resolution and analysis time, the multi-criteria decision making (MCDM) method suggested by Smilde *et al.* [17] was applied. Briefly, a two-dimensional graph is made, plotting the predicted $R_{s_{min}}$ values against the corresponding t_{max} values. The points constituting

the edge of the obtained scatter plot are named as the pareto-optimal (PO) points, which represent the best possible combinations of both criteria.

Criticisms made to the MCDM approach lie, mostly, on the fact that MCDM plots do not illustrate the relation between the behaviour of the optimization criteria and the variations in mobile phase composition [18]. Attempts to circumvent this problem have been made by mapping the PO points over a diagrammatic representation of the factor space [17,24]. In the present report the MCDM plot shows not only the PO points, but all those having a predicted $R_{s_{min}}$ value of 1.0 or higher (Fig. 1). The employed software allowed rapid identification of these points, which defined a discrete region of the factor space (pH from 3.90 to 4.15 and methanol content from 10 to 12%). Points corresponding either to the same eluent pH or to the same methanol content were linked by continuous and discontinuous lines, respectively. All points with the same pH value fell in a smooth line, and adjacent points on this line differed by 0.1% methanol content. At least for this region, it is clear from Fig. 1 that the pay-off between $R_{s_{min}}$ and t_{max} was closely related to variations in eluent composition. As was seen later, the analysis time was predicted to be determined by a same peak (5HT) for all points in the plot. An inflexion appeared when plotting $R_{s_{min}}$ versus t_{max} for pH values of 4.10 and 4.15, showing that a variation in methanol content could change the pair of peaks determining $R_{s_{min}}$. However, for pH values from 3.90 to 4.05, a same pair of peaks (MHPG and 5HTP) showed the minimal predicted resolution over the entire methanol range.

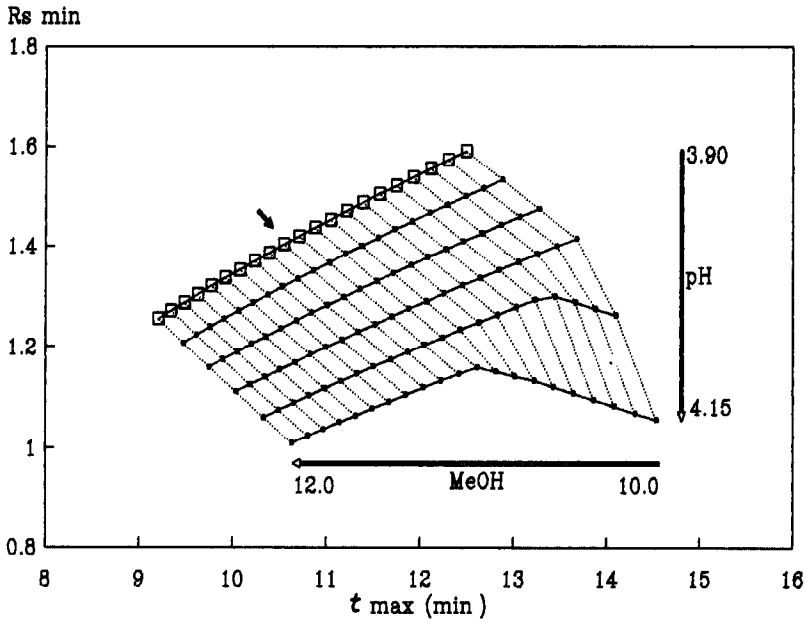

Fig. 1. Multi-criteria decision making plot. Points corresponding to the same eluent pH are linked by solid lines, contiguous lines differing by 0.05 pH units. Points for a same methanol (MeOH) content are linked by dotted lines, contiguous lines differing by 0.1% methanol content. The pareto-optimal points (squares) correspond to the eluents of pH 3.90. The small arrow points to the selected pareto-optimal mobile phase composition: pH 3.90, 11.1% (v/v) methanol content.

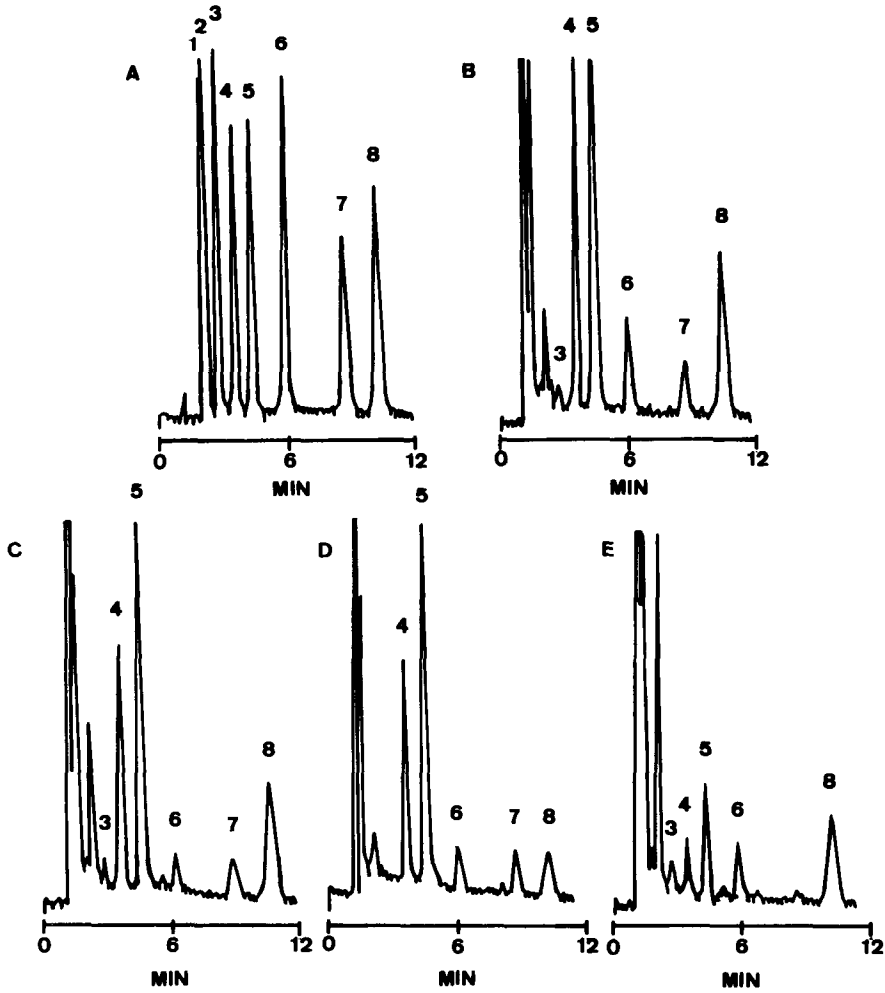

Fig. 2. Chromatograms recorded at the selected pareto-optimal mobile phase composition (pH 3.90, 11.1% (v/v) methanol content). (A) Chromatogram of a standard mixture containing 200 pg/20 μl injection of each compound. Chromatograms of microdissected nuclei from a single rat brain: (B) olfactory tubercle; (C) accumbens; (D) caudate putamen; (E) suprachiasmatic. Peaks: 1 = NE; 2 = MPHG; 3 = 5HTP; 4 = DOPAC; 5 = DA; 6 = 5HIAA; 7 = HVA; 8 = 5HT. Sensitivity 0.5 nA full scale. See text for other chromatographic conditions.

The PO points corresponded to the eluents with a pH value of 3.90. The PO point for 11.1% methanol content was selected for checking in the HPLC system. These conditions gave a predicted $R_{s\min}$ value higher than 1.4, which we considered appropriate for quantitative analysis. The predicted analysis time was less than 10.5 min. The chromatogram obtained is presented (Fig. 2A) and was considered entirely satisfactory for the initial purposes. The predicted and actual retention times were found to be in good agreement, with a difference of 0.27% to 5.43% (Table II). Although separation quality is increased towards the lower pH limit, it was discarded

TABLE II

PREDICTED AND ACTUAL RETENTION TIMES FOR THE SELECTED PARETO-OPTIMAL MOBILE PHASE COMPOSITION (pH 3.90, 11.1% (V/V) METHANOL CONTENT)

	Compound							
	NE	MHPG	DA	DOPAC	HVA	5HTP	5HT	5HIAA
Retention time (min)								
Predicted	2.25	2.46	4.55	3.69	8.53	2.87	10.54	6.12
Actual	2.23	2.33	4.50	3.70	8.94	2.88	10.50	6.10
Difference (%)	0.89	5.43	1.10	0.27	4.69	0.35	0.38	0.33

to explore more acidic pH values because too low capacity factors were expected for 5HTP. The optimization procedure was consequently stopped at this point.

The validity of the method was tested with microdissected rat brain nuclei. Chromatograms from TUL, A, CP and SQ obtained from a single rat brain are presented (Fig. 2B–E). No interference with unknown peaks was seen for the determination of the compounds of interest. The mean within-assay coefficient of variation, calculated by making 15 consecutive injections of the same working standard solution, was about 5%. The limit of detection, calculated at a signal-to-noise ratio of 2:1, ranged from 6 pg for 5HTP to 14 pg for HVA.

In summary, an HPLC method has been described that is ideally suited to the simultaneous determination of compounds of the dopaminergic and serotoninergic pathways in micropunches from individual rat brains. The employed optimization procedure is simple and could be reliable in solving similar separation problems. Furthermore, knowledge of the followed methodology can aid in fitting this HPLC method to different experimental conditions and/or analytical purposes.

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REFERENCES

1 I. N. Mefford and J. D. Barchas, *J. Chromatogr.*, 181 (1980) 187.
2 I. N. Mefford, M. Gilberg and J. D. Barchas, *Anal. Biochem.*, 104 (1980) 469.
3 P. T. Kissinger, C. S. Bruntlett and R. E. Shoup, *Life Sci.*, 28 (1981) 455.
4 I. N. Mefford, *J. Neurosci. Meth.*, 3 (1981) 207.
5 A. M. Krstulovic, *J. Chromatogr.*, 229 (1982) 1.
6 E. Shohami, M. Segal and D. M. Jacobowitz, *J. Neurosci. Meth.*, 8 (1983) 275.
7 P. Kontur, R. Dawson and A. Monjan, *J. Neurosci. Meth.*, 11 (1984) 5.
8 S. M. Lasley, I. A. Michaelson, R. D. Greenland and E. M. Mcginnis, *J. Chromatogr.*, 305 (1984) 27.
9 K. J. Renner and V. N. Luine, *Life Sci.*, 34 (1984) 2193.
10 C. D. Kilts and C. M. Anderson, *Neurochem. Int.*, 9 (1986) 437.
11 A. Jakubovic, D. Fu and H. C. Fibiger, *J. Pharmacol. Meth.*, 17 (1987) 1.
12 H. A. H. Billier, J. Vuik, J. K. Strasters and L. de Galan, *J. Chromatogr.*, 384 (1987) 153.

13 F. Szokoli, Zs. Németh and J. Inczédy, *Chromatographia*, 29 (1990) 265.
14 I. Molnár and Cs. Horváth, *Clin. Chem.*, 22 (1976) 1497.
15 Cs. Horváth, W. Melander, I. Molnár and P. Molnár, *Anal. Chem.*, 49 (1977) 2295.
16 H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 485 (1989) 27.
17 A. K. Smilde, A. Knevelman and P. M. J. Coenegracht, *J. Chromatogr.*, 369 (1986) 1.
18 A. Bartha, H. A. H. Billiet and L. de Galan, *J. Liq. Chromatogr.*, 12 (1989) 173.
19 G. E. P. Box, W. G. Hunter and J. S. Hunter, *Statistics for experimenters. An Introduction to Design, Data Analysis and Model Building*, Wiley, New York, 1978.
20 P. Wester, J. Gottfries, K. Johansson, F. Klintebäck and B. Winblad, *J. Chromatogr.*, 415 (1987) 261.
21 M. Palkovits and M. J. Brownstein, in A. C. Cuello (Editor), *Brain Microdissection techniques*, Wiley, New York, 1983, p. 1.
22 J. K. König and R. A. Klippel, *The Rat Brain: a Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem*, R. E. Krieger, New York, 1967.
23 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 145 (1978) 371.
24 A. K. Smilde, C. H. P. Bruins and D. A. Doornbos, *J. Chromatogr.*, 410 (1987) 1.