Journal of Chromatography, 449 (1988) 191–205
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 667

PERFLUORINATED ACIDS AS ION-PAIRING AGENTS IN THE DETERMINATION OF MONOAMINE TRANSMITTERS AND SOME PROMINENT METABOLITES IN RAT BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

MIKLÓS PATTHY* and RÓZSA GYENGE
Institute for Drug Research, Szabadságharcosok útja 47–49, 1045 Budapest (Hungary)
(First received March 14th, 1988; revised manuscript received May 24th, 1988)

SUMMARY

The behaviour of trifluoroacetate and heptafluorobutyrate as pairing ions for the reversed-phase ion-pair separation of monoamine transmitters and related metabolites was studied. The performance of systems with the perfluorinated acids was compared with that of systems containing sodium octyl sulphonate and was found to be better in terms of peak resolution combined with total analysis time, day-to-day reproducibility and the time required for attaining initial chromatographic equilibrium. Rat brain samples were deproteinized in the acidified mobile phase, injected directly on to a high-performance liquid chromatographic column and quantitated using an amperometric detector. Sample run times were 6–8 min, at a relatively low flow-rate. The detection limits achieved are fairly uncommon with conventional bore columns. The two perfluorinated acids studied differ in the dominant mechanisms of ion-pair formation and show selectivity differences as a result.

INTRODUCTION

High-performance liquid chromatography (HPLC) with amperometric detection is now a method of choice for the determination of catecholamines, indoleamines and related metabolites in the central nervous system. Reversed-phase ion-pair HPLC has frequently been used for the separation of these compounds[1–8]. Much work has been carried out in order to study the effects of the variables that control the separations, using systematic sequential methods[9–14] and computer-aided optimization strategies[15]. Optimal conditions can be selected in terms of peak resolution combined with total analysis time by a systematic study of the influence of some mobile phase variables (pH, nature and concentration of the ion-pairing agent, percentage of the organic modifier) and by examining a variety of reversed-phase packing materials and column dimensions.

Several ion-pairing agents have been described that give satisfactory results in the analysis of monoamine transmitters and related metabolites. Although some workers have used trichloroacetate as a pairing ion with remarkable success[10,16], ion-pairing agents belonging to the alkanesulphonate (or sulphate) class have been in

0021-9673/88/$03.50

common use since the late 1970s[9,17]. However, most of the methods using these pairing ions [of which sodium octyl sulphonate (SOS) has been the most popular] require fairly long sample run times if both catecholamines and indoles are to be resolved in the same chromatographic run. Also, as equilibrium is attained in 12–16 h, the "start-up" time for an assay procedure may be unacceptably long with the larger alkylsulphonates in the eluent[10,14].

The excellent resolving power of perfluorinated acids in the ion-pair separation of proteins and peptides[18] and aminoglycoside antibiotics[19] and the isolation of peptide hormones[20] has been well documented. In this work, we investigated the behaviour of some perfluorinated carboxylic acids [trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA)] as providers of pairing ions for the separation of biogenic amines [noradrenaline (NA), adrenaline (A), dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT)] and some of their metabolites [3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA)] using reversed-phase HPLC systems combined with amperometric detection.

Mobile phase conditions for systems containing TFA, HFBA or SOS (for comparison) as ion-pairing agents were optimized in a known, systematic way. Three types of reversed-phase packing material and columns of three lengths were examined. Comparing the peak resolutions combined with total analysis times obtained in optimized systems with TFA, HFBA or SOS in the eluent and the same chromatographic column we found that the performance of the TFA and HFBA systems compares favourably with that of the SOS systems. Using TFA or HFBA as the pairing ion, baseline resolution of all analytes of interest was achieved within 6–8 min, even at a relatively low flow-rate. Low background noise and k' values together resulted in sensitivities that are fairly uncommon with conventional bore columns. Optimized TFA and HFBA systems were used for the determination of monoamine transmitters and their metabolites in rat brain striatum and hypothalamus.

EXPERIMENTAL

Chromatography

The HPLC system included a Varian 8500 pulse-free pump (Varian, Palo Alto, CA, U.S.A.), a Rheodyne 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 200-μl loop, a Model LC-4A thin-layer amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with graphite paste (CP-S) as the working electrode operated at 0.75 V *vs.* an Ag–AgCl reference electrode and a Type OH-814/1 strip-chart recorder (Radelkis, Budapest, Hungary).

Chromatographic supports were packed in stainless-steel columns (Bio-Separation Technologies, Budapest, Hungary). The chromatographic columns and the compositions of the mobile phases are specified in the figure legends. The mobile phase was sonicated and degassed under vacuum for 1 min before use. All separations were carried out at ambient temperature.

Chemicals

Materials and their sources were as follows. NA bitartrate, A hydrochloride, DA hydrochloride, isoproterenol hydrochloride and DOPAC were obtained from Sigma

(St. Louis, MO, U.S.A.) and α-methyldopamine (α-MDA) hydrobromide from Merck, Sharp and Dohme (West Point, PA, U.S.A.). HVA and the Servachrom packing material [Polyol, RP-18 (5 μm)] were purchased from Serva (Heidelberg, F.R.G.), the Vydac 218 TPB C_{18} (10 μm) packing material from The Separations Group (Hesperia, CA, U.S.A.) and the Nucleosil 5 C_{18} (5 μm) and Nucleosil 3 C_{18} (3 μm) packing materials from Macherey, Nagel & Co. (Düren, F.R.G.). The 5-HT creatinine sulphate complex, 5-HIAA, dipotassium hydrogen orthophosphate (K_2HPO_4), the disodium salt of ethylenediaminetetraacetic acid (EDTA) and trichloroacetic acid (TCA) were obtained from Reanal (Budapest, Hungary). Sodium hydrogensulphite ($NaHSO_3$) and methanesulphonic acid (MSA), which we purified on a charcoal column, were supplied by Fluka (Buchs, Switzerland), acetonitrile (LiChrosolv) and sodium perchlorate by Merck (Darmstadt, F.R.G.) and SOS by Supelco (Bellefonte, PA, U.S.A.). The perfluorinated acids (Sequanal quality), TFA and HFBA, were obtained from Pierce (Rockford, IL, U.S.A.). All other chemicals used were of analytical-reagent grade.

All solutions were prepared with deionized, glass-distilled water. Stock solutions of the analytes of interest were prepared at a concentration of 0.1 mg/ml in the respective mobile phase containing $4 \cdot 10^{-4}$ *M* $NaHSO_3$ as antioxidant. Standard solutions were prepared every 2 weeks from a portion of the stock solutions after appropriate dilution in the same solvent. All solutions were stored at −20°C.

Sample preparation

Adult male OFA rats weighing 200–250 g were killed by decapitation. The brain was removed within 30 s and dissected on ice. The regions of interest were immediately cooled with dry-ice and stored at −20°C until analysed. For analysis, pieces of tissue (4–50 mg) were weighed in conical 1.5-ml test-tubes and a solution (200–500 μl) consisting of the respective mobile phase (the pH of which was previously adjusted to 3 with 42.5% orthophosphoric acid), $4 \cdot 10^{-4}$ *M* $NaHSO_3$ and the internal standard (α-MDA · HBr) (1.0 and 0.2 μg/ml for examination of the striatum and hypothalamus, respectively) was pipetted into the tubes. The mixture was sonicated at 200 W for about 5 s while kept on ice, and the homogenate was centrifuged for 10 min at 15 000 *g* and 4°C. An aliquot of the supernatant was injected on to the chromatographic column.

Quantitations were performed from calibration graphs of peak-height ratio relative to the internal standard against concentration.

RESULTS AND DISCUSSION

Choice of the reversed-phase column packing material

Reversed-phase supports from different suppliers can show large differences in chromatographic behaviour[11,15]. In order to find a suitable packing material for our studies, three different supports were tested. NA, 5-HT and HVA were chosen as test compounds. As shown in Table I, both Nucleosil C_{18} (5 μm) and Servachrom RP-18 (5μm) packings gave good retention for NA. The capacity factor (k') was calculated using the equation $k' = (t_R - t_0)/t_0$, where t_0 and t_R are the retention times of an unretained solute and the solute in question, respectively. All three packing materials exhibited remarkable chromatographic efficiency (N) for the acid metabolite, HVA; N was calculated using the equation $N = 5.54(t_R/w_{1/2})^2$, where t_R is the retention time

TABLE I

CHROMATOGRAPHIC DATA FOR SOME COMMERCIAL COLUMN PACKING MATERIALS

Column (and main eluent variables)*	*Test compound*	*k'*		*N/m*		*Peak asymmetry***	
		SOS	*HFBA*	*SOS*	*HFBA*	*SOS*	*HFBA*
Servachrom RP-18 (5 lm) (170 (4.0 mm I.D.)	NA	0.87	1.27	6129	17 600	1.83	1.28
(A) 0.2 m*M* SOS, 6% acetonitrile, pH 4.68	5-HT	8.56	12.90	4879	10 794	2.33	1.48
(B) 15 m*M* HFBA, 6% acetonitrile, pH 4.15 0.83 ml/min (both)	HVA	4.97	8.08	19 100	18 867	1.16	1.18
Vydac 218TPB C_{18} (10 μm) (220 × 4.0 mm I.D.)	NA	0.49	0.60	14 073	22 950	1.50	1.08
(A) 0.4 m*M* SOS, 6% acetonitrile, pH 4.15	5-HT	3.79	4.40	12 568	19 527	1.59	1.12
(B) 15 m*M* HFBA, 6% acetonitrile, pH 4.05 0.83 ml/min (both)	HVA	2.79	3.07	16 564	20 018	1.25	1.06
Nucleosil 5 C-18 (5 μm) (120 × 4.0 mm I.D.)	NA	1.79	1.29	47 408	60 210	1.08	1.03
(A) 0.75 m*M* SOS, 6% acetonitrile, pH 4.32	5-HT	18.0	13.10	47 958	54 760	1.03	1.05
(B) 15 m*M* HFBA, 6% acetonitrile, pH 4.20 0.83 ml/min (both)	HVA	7.92	7.19	52 533	70 420	1.02	1.02

* The eluent was a mixture of a 0.05 *M* phosphate buffer and acetonitrile containing the pairing ion.
** Peak asymmetry was measured at 10% of the peak height and a chart speed of 2.5 cm/min.

of the solute in question and $w_{1/2}$ is the width of the peak at half-height. Based on the excellent peak symmetry values and overall chromatographic performance, the Nucleosil support was chosen for further optimization studies. For some separations the Vydac column was also used.

Dependence of k' on the nature and concentration of the pairing ion

Both TFA and HFBA are fully dissociated and available for ion pairing at pH higher than 2.0 (their pK_a values are 0.23 and 0.17, respectively). The capacity factors of the analytes of interest are plotted as a function of the concentration of TFA and HFBA in Figs. 1 and 2, respectively. Apart from the nature of the pairing ion, other chromatographic variables were identical in both systems. As shown in Fig. 1, there was a 2-fold increase in the retention of the amines when the TFA concentration was raised from 0 to 50 m*M*. The same increase in HFBA concentration resulted in an average 8-fold increase in the retention of the amines (Fig. 2). The amine curve shapes are similar to those obtained for C_4–C_8 alkyl sulphates[17] and TCA[10]. Whereas the capacity factors for the acids (DOPAC, HVA and 5-HIAA) are unaffected by the presence of TFA in the eluent (Fig. 1), there is a sharp concave downward turn in the *k' vs.* [HFBA] plots for these acids when the HFBA concentration is raised from 0 to 2 m*M* (Fig. 2), indicating a higher retention for acids in eluents containing TFA and a marked difference in the ion-interaction mechanisms of TFA and HFBA in reversed-phase systems in general.

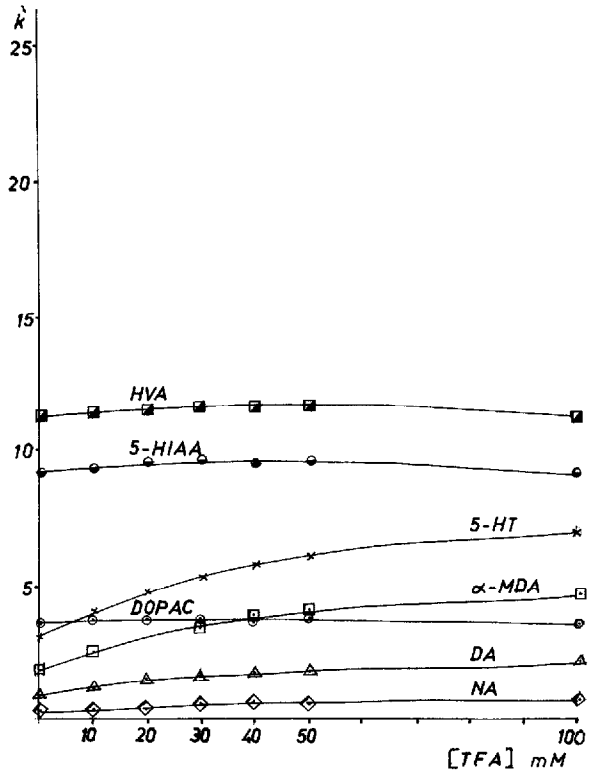

Fig. 1. Dependence of k' on the concentration of TFA in the eluent. Column: Nucleosil C_{18} (5 μm), 100 × 4.0 mm I.D. Eluent: aqueous buffer (pH: 4.15)–acetonitrile (94:6); the aqueous buffer consisted of 0.05 M K_2HPO_4, 0.1 mM EDTA and 0–100 mM TFA (TFA concentrations: 0, 10, 20, 30, 40, 50 or 100 mM). The pH of the aqueous buffer was adjusted to 4.15 using 42.5% (w/v) orthophosphoric acid or 5 M potassium hydroxide solution. Flow-rate: 0.83 ml/min.

Dependence of k' on mobile phase pH and acetonitrile admixture

The mobile phase pH greatly affects the k' values for the acidic metabolites. The decrease in k' for the acids with increasing pH is dramatic in both TFA and HFBA systems and the k' values are indirectly related to the degree of ionization of the metabolite of interest at a given pH (see Figs. 3 and 4). The retention of the acidic metabolites is most sensitive to a change in mobile phase pH between pH 3 and 5.5, as expected for acids with $pK_a \approx 4.7$. In accordance with the difference between the two pairing ions observed in Figs. 1 and 2, the retention of an acid of interest is lower (at each pH) in an HFBA system than in systems containing TFA.

As their pK_a values are higher than 6.0, the amines carry the same charge over the pH range examined and their retention is relatively unaffected by changes in the mobile phase pH.

The addition of acetonitrile produced a decrease in k' for all compounds examined without major changes in the relative retentions either in TFA systems (Fig. 5) or in those prepared with HFBA. For acetonitrile concentrations between 0 and 6% the log k' values fall on straight lines in both systems.

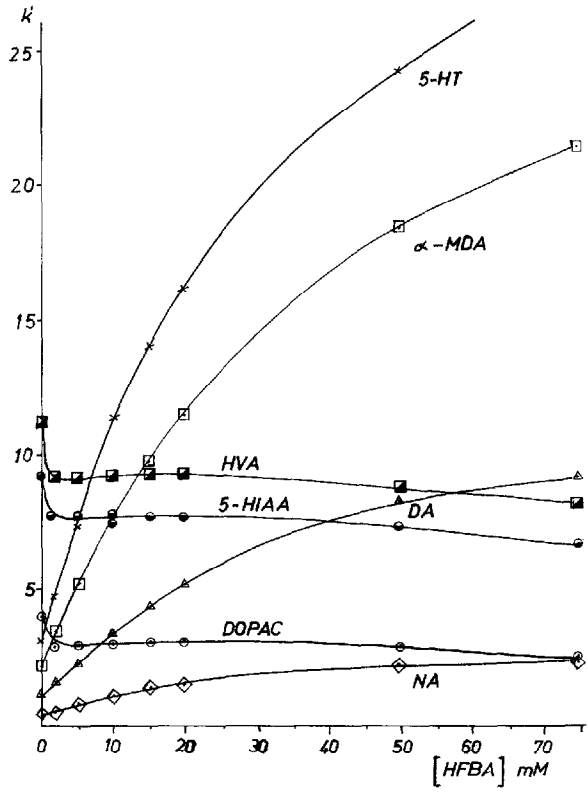

Fig. 2. Dependence of k' on the concentration of HFBA in the eluent. HFBA concentrations in the aqueous buffer: 0, 2, 5, 10, 15, 20, 50 or 75 mM. Other details as in Fig. 1.

Comparison of TFA and HFBA with pairing ions of different nature

In order to position TFA and HFBA in the group of anionic ion-pairing agents, their influence on the retentions of some monoamines and acidic metabolites was compared with those of some pairing ions of different nature. The pairing ions studied were used in equimolar concentrations (except SOS) and without salt control, but under otherwise identical chromatographic conditions. As shown in Table II, TFA behaves much like perchlorate, an inorganic pairing ion, whereas HFBA is very similar in character to TCA and SOS. The difference between the two groups lies in the fact that, under the conditions of the study, perchlorate and trifluoroacetate ions cannot bind to the hydrophobic surface of the support (they cannot form a primary ion layer on the surface) and, as a result, they exercise no electrostatic repulsion toward molecules of identical charge (DOPAC, 5-HIAA, HVA) binding to the stationary phase. Hence, increasing the concentration of TFA in the eluent causes no decrease in the retention of the acidic metabolites, as was shown in Fig. 1. On the other hand, the negative retention shifts ($k'_p - k'_o$) of DOPAC, 5-HIAA and HVA caused by TCA, HFBA or SOS in the eluent (see Table II) indicate a fairly strong binding of these pairing ions to the lipophilic stationary phase. These pairing ions do form, on the support surface, a negatively charged primary ion layer, expelling the acidic

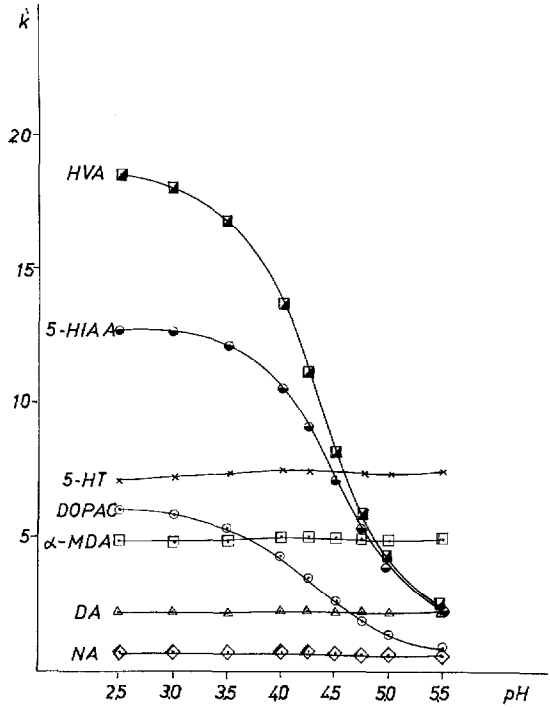

Fig. 3. Dependence of k' on eluent pH in a TFA system. TFA concentration in the aqueous buffer: 100 mM. The pH of the aqueous buffer varied between 2.0 and 6.0. Capacity factors were measured at pH 2.5, 3.0, 3.5, 4.0, 4.25, 4.5, 4.75, 5.0 and 5.5. Other details as in Fig. 1.

metabolites from the layer on the support even at concentrations below 2 mM (see Fig. 2).

Selectivity

Inspection of the above data shows that, under the conditions of the study, there is a marked difference in the chromatographic behaviour of TFA and HFBA in ion-pair reversed-phase systems. Whereas TFA can bind to the hydrophobic surface of the support in the form of a neutral ion pair only (in association with a sufficiently hydrophobic cationic counter ion), HFBA can bind to the surface as a solvated anion and also as a neutral ion pair. Consequently, the dominant mechanism of the chromatographic process with TFA is ion-pair formation in the mobile phase and binding of the neutral complex to the non-polar stationary phase. Hence the so-called dynamic ion-exchange model[21] and the ion-interaction model[22] cannot be the underlying mechanisms in ion-pair chromatography with TFA. With HFBA, however, all three of the proposed models can more or less explain the chromatographic process.

At a mobile phase pH of 4.20, which appears to be the optimal pH in both TFA and HFBA systems (see Figs. 3 and 4), the elution orders with TFA and HFBA are NA, DA, DOPAC, α-MDA, 5-HT, 5-HIAA, HVA and NA, DOPAC, DA, 5-HIAA, HVA, α-MDA, 5-HT, respectively. The remarkable difference in these elution orders

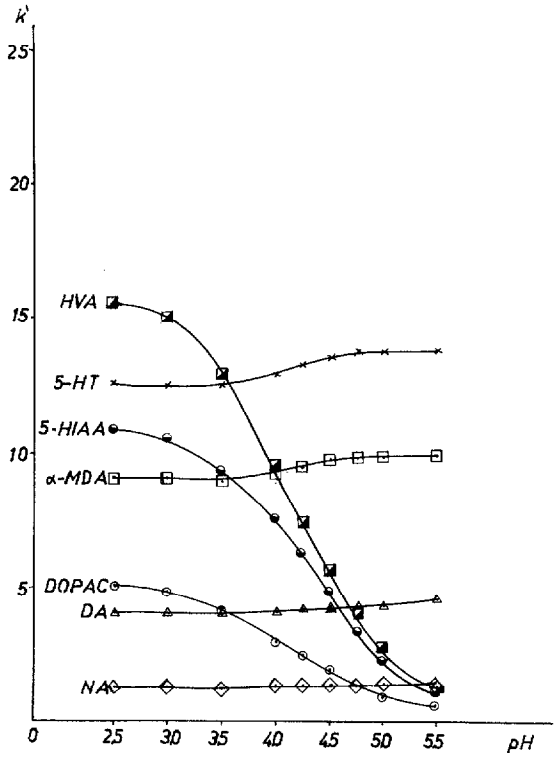

Fig. 4. Dependence of k' on eluent pH in an HFBA system. HFBA concentration in the aqueous buffer: 15 mM. Other details as in Figs. 1 and 3.

clearly shows that TFA systems are more retentive for the acids, and HFBA systems (like SOS systems) are more retentive for the amines. Our results demonstrate that in the perfluorinated carboxylic acids series special selectivity effects are obtained as a function of the chain length. This is in agreement with the conclusion of a recent study on the ion-pair separation of aminoglycoside antibiotics[19]. On the other hand, the retention shift data with MSA and SOS in Table II seem to support earlier suggestions that no special selectivity effects can be expected on changing the chain length of alkylsulphonate pairing ions[23]. We have no simple explanation of the difference in chromatographic behaviour between MSA and TFA.

Optimization of the TFA and HFBA chromatographic systems

Our aim was to separate the compounds under study in the minimal time with maximal resolution, and also to achieve as low detection limits as possible with conventional bore columns. Maximum sensitivity (combined with low residual current and background noise) can be expected from carbon paste working electrodes[24,25]. With careful handling, the long-term stability of pre-tested carbon-paste electrodes is much better than that of glassy carbon electrodes (which may need frequent repolishing) if the aqueous eluent contains not more than 8% of acetonitrile. Eluents containing more than 8% of acetonitrile are not practical anyway, as the resolution of

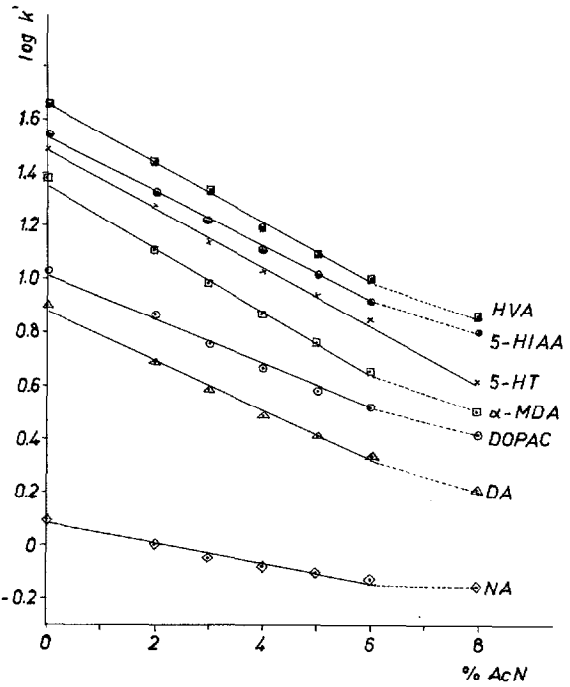

Fig. 5. Dependence of k' on acetonitrile (ACN) content in a TFA system. TFA concentration in the aqueous buffer: 100 mM. pH of aqueous buffer: 4.15. The acetonitrile concentration in the eluent was varied between 0 and 10% (v/v). Capacity factors were measured at acetonitrile concentrations of 0, 2, 3, 4, 5, 6 and 8%. Other details as in Fig. 1.

TABLE II

INFLUENCE OF VARIOUS PAIRING IONS ON THE RETENTION OF MONOAMINES AND ACIDIC METABOLITES

$k'_o = k'$ value for an analyte of interest obtained in a system without pairing ion (eluent buffer only); $k'_p = k'$ value for an analyte of interest obtained in the eluent buffer for k'_o containing 20 mM of the specified pairing ion in addition.

Compound examined	k'_o *(buffer* only)*	$k'_p - k'_o$					
		*MSA***	*$HClO_4$***	*TFA***	*TCA***	*HFBA***	*SOS****
NA	0.31	0.02	0.14	0.17	0.45	1.15	2.14
DA	0.81	0.05	0.33	0.49	1.33	4.05	6.79
α-MDA	1.57	0.10	0.69	1.05	2.95	9.38	15.65
5-HT	2.43	0.14	1.19	1.57	4.22	12.05	20.45
DOPAC	2.85	−0.01	0.05	0.02	−0.23	−0.61	−0.40
5-HIAA	6.93	−0.03	0.06	0.05	−0.28	−1.31	−1.17
HVA	8.56	−0.13	0.16	0.06	−0.68	−1.80	−1.97

* The eluent buffer was a mixture of 0.05 M phosphate (pH: 4.25) and 6% acetonitrile. The column was Nucleosil 5 C_{18} (100 × 4.0 mm I.D.).

** Pairing ion, 20 mM.

*** 2.0 mM (instead of 20 mM) sodium octylsulphate in the buffer.

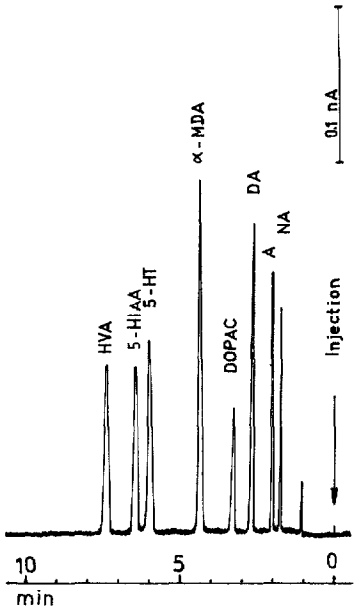

Fig. 6. Separation of standards in an optimized TFA system. Column: Nucleosil C_{18} (5 μm), 100 × 4.0 mm I.D. Eluent: aqueous buffer (pH 4.20)–acetonitrile (92:8); TFA concentration in the aqueous buffer: 100 m*M*; other components in the aqueous buffer as in Fig. 1. Flow-rate: 0.83 ml/min.

the analytes of interest deteriorates quickly with higher concentrations of the organic modifier (see Fig. 5).

The optimal concentration of acetonitrile in both the TFA and HFBA systems is between 5.5 and 8%. The optimal pH range for the separation of the compounds studied is 4.05–4.35 in both systems (see Figs. 3 and 4), and the most advantageous pairing ion concentrations appear to be 100 m*M* for TFA and 12–16 m*M* for HFBA (see Figs. 1 and 2), the concentrations being determined in part by the requirement that a k' value of 0.65–0.70 is necessary in our methods for NA to be well resolved from early eluting unknown compounds in a sample. Fig. 6 shows a chromatogram of standards obtained with a Nucleosil 5 C_{18} column (100 × 4.0 mm I.D., without a pre-column) in a highly optimized TFA system. In this system plate counts (N per metre) of over 100 000 were generated, allowing a separation to take place in less than 8 min even at a relatively low flow-rate (0.83 ml/min).

Some further improvement in speed can be achieved with a shorter column and a packing material of smaller particle size (in order to maintain a stable baseline and high sensitivity, we did not increase the flow-rate). Fig. 7 shows a chromatogram of standards obtained on a Nucleosil 3 C_{18} column (30 × 4.0 mm I.D. with a 20 × 4.0 mm I.D. pre-column) in an optimized TFA system. A representative chromatogram of a sample of rat striatum in the same system is shown in Fig. 8. Fig. 9 shows a chromatogram of standards obtained in an optimized HFBA system and Fig. 10 presents a representative chromatogram of a sample of rat hypothalamus in the same system. In both instances the column was the same as in Fig. 7. The average plate counts in Figs. 7–10 are lower than expected ($N \approx 93\ 000$/m), presumably owing to the

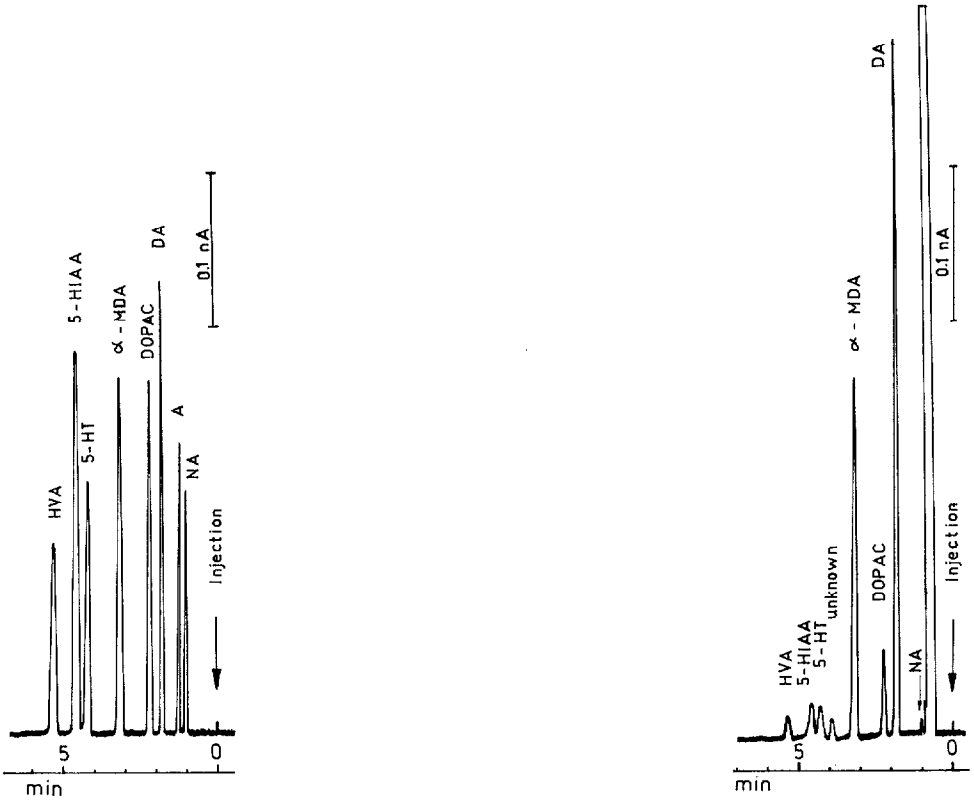

Fig. 7. Separation of standards in an optimized TFA system. Column: Nucleosil C_{18} (3 μm), 30 × 4.0 mm I.D., with a 20 × 4.0 mm I.D. pre-column. Eluent: aqueous buffer (pH 4.30)–acetonitrile (94:6). Amounts injected: 25 pg (Na, A); 50 pg (DA, DOPAC, 5-HT), 75 pg (5-HIAA) and 200 pg (HVA). Other details as in Fig. 6.

Fig. 8. Representative chromatogram of striatal tissue (untreated rat) after direct injection of centrifuged homogenate. Column and eluent as in Fig. 7.

pre-column, but the resolutions are still good. No chromatographic interference was found.

For fast analyses and high sensitivity it is essential that the k' values are as low as possible, which in turn also means that the solvent fronts should be as "clean" as possible. If homogenization is carried out with the acidified mobile phase, even direct injection of the extracts causes a narrow solvent front eluting earlier than $k' = 0.6$, indicating that the broad solvent fronts in earlier papers were the consequence of a chromatographic imbalance after the injection of perchloric acid-containing homogenates rather than of an excessive amount of impurities in the sample. At proper homogenization buffer-to-tissue ratios the mobile phase is at least as effective as 0.1 M perchloric acid in extraction efficiency and in precipitating tissue proteins[13,14].

The sensitivity of this assay is more than sufficient to determine each of the amines and the metabolites studied in an extract derived from about 0.5 mg of tissue.

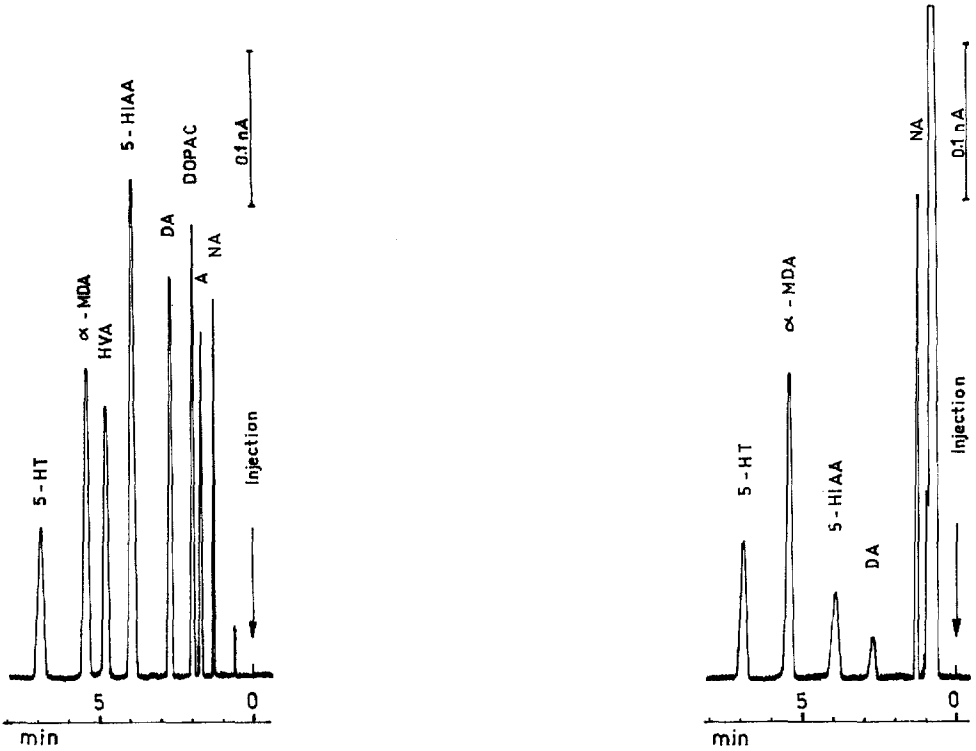

Fig. 9. Separation of standards in an optimized HFBA system. Column as in Fig. 7. Eluent: aqueous buffer (pH 4.35)–acetonitrile (94:6). HFBA concentration in the aqueous buffer: 12 m*M*. Other details as in Fig. 1.

Fig. 10. Representative chromatogram of hypothalamic tissue (untreated rat) after direct injection of centrifuged homogenate. Column and eluent were as in Fig. 9.

The detection limits for these compounds (amount injected, twice the noise) in the system shown in Fig. 7 were as follows: 1.25 pg for NA, 1.05 pg for A, 1.30 pg for DA, 1.70 pg for DOPAC, 2.35 pg for 5-HT, 2.35 pg for 5-HIAA and 12.5 pg for HVA. Table III shows the control levels of the amines and metabolites studied in rat striatum and hypothalamus, as measured under the conditions in Fig. 9. Although A is essentially missing in most regions of the vertebrate brain, the HPLC systems presented here also resolve this compound. The resolution with the short column used under the conditions in Figs. 7 and 9 was not impaired provided that the sample volume injected was kept below 30 μl. For larger loads the Vydac column was used. A typical chromatogram of standards obtained from this conventional column (220 × 4.0 mm I.D., 10 μm particle size) in an HFBA system is shown in Fig. 11. The often used internal standards isoproterenol and α-MDA are very similar in their chromatographic properties, but in our systems the use of the latter was slightly more advantageous.

Comparison of optimized systems

For comparison, we also optimized SOS-containing systems with all three

TABLE III

CONTROL LEVELS OF AMINES AND METABOLITES IN RAT STRIATUM AND HYPOTHALAMUS

Results are expressed in ng/g wet tissue ± S.E.M. (n = 5).

Brain area	*NA*	*A*	*DA*	*DOPAC*	*HVA*	*5-HT*	*5-HIAA*
Striatum	55 ± 11	ND*	9123 ± 381	1520 ± 145	878 ± 129	418 ± 42	717 ± 35
Hypothalamus	1570 ± 118	ND*	140 ± 35	ND*	ND*	1210 ± 210	705 ± 30

* ND, not detectable.

columns indicated in Figs. 6, 7 and 11, and compared the separation speeds (the reciprocal of the time needed for a complete chromatographic run) obtained in optimized SOS systems with those shown in Figs. 6, 7, 9 and 11. Other criteria of the comparisons were $k'_{NA} \geqslant 0.6$ and $R_{s\,i,j} \geqslant 1.3$, where $R_{s\,i,j}$ is the resolution of adjacent peaks of compounds i and j. Resolution was calculated using the equation $R_s = 2(t_{R_2} - t_{R_1})/(w_1 + w_2)$, where t_{R_1} is the retention time of compound i, t_{R_2} is the retention time of compound j, w_1 is the peak width (at the base) of compound i and w_2 is the peak width of compound j, both expressed in time units. We found that at identical k'_{NA} values and sufficient resolution the separation speed of optimized SOS systems was, on average, 20% lower than that of TFA systems and 5–10% lower than that of HFBA systems. Selectivity differences and the lower plate counts achieved with

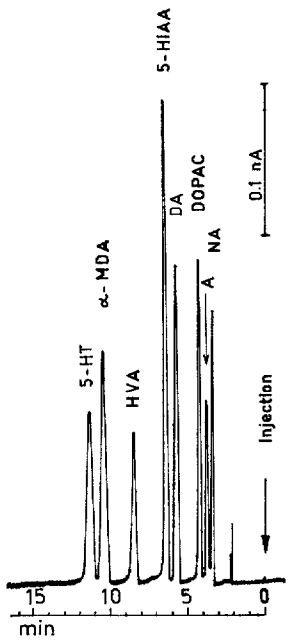

Fig. 11. Separation of standards on a conventional column in an optimized HFBA system. Column: Vydac 218 TPB C_{18} (10 μm), 200 × 4.0 mm I.D., with a 20 × 4.0 mm I.D. pre-column. Eluent: aqueous buffer (pH 4.05)–acetonitrile (94:6). HFBA concentration in the aqueous buffer: 15 mM. Other details as in Fig. 1.

SOS systems (see Table I) may explain the difference in the separation speeds attainable.

Other areas of comparison were the time needed to achieve equilibration and the day-to-day reproducibility of the separations. As was shown in an earlier paper, the equilibration time is directly related to the size of the pairing ion employed[26]. In the equilibration studies we measured the retention times of HVA and 5-HT. By definition, initial equilibrium conditions were reached when the changes in the retention times of HVA and 5-HT in optimized TFA, HFBA or SOS systems were within ± 1 s (the injections considered were 30 min apart and the temperature and flow-rate were constant). Equilibrium in SOS, HFBA and TFA systems was reached in 13, 2.5 and 2.0 h, respectively.

The day-to-day reproducibility of the separations was poorer with SOS systems than with HFBA or TFA systems. With SOS systems the concentration of the pairing ion must be held constant within narrow limits (the optimal concentration is around 0.5 m*M*). As a consequence, a slight change in ambient temperature or in the conditions of eluent degassing may induce unwanted changes in the separation patterns. With HFBA and TFA systems these concentration limits are 25 and 200 times wider, respectively, allowing better day-to-day reproducibility.

CONCLUSIONS

The results show that short-chain perfluorinated carboxylic acids are a very attractive alternative to alkylsulphonates in the reversed-phase ion-pair separation of monoamine transmitters and related metabolites. TFA and HFBA are fully compatible with amperometric detection.

The sample analysis time of 6–8 min is a significant improvement over most published procedures (higher separation speeds were achieved at much higher flow-rates and column back-pressures[27]). The short, conventional bore Nucleosil columns used provide an excellent compromise for maintaining a high sample throughput, low back-pressures and detection limits approaching those achieved in microbore systems[28,29].

Selectivity differences between TFA and HFBA systems have been explained by the suggestion that TFA and HFBA differ in the dominant mechanisms of ion-pair formation.

ACKNOWLEDGEMENTS

The authors thank Mrs. E. Blazsek for excellent technical assistance and Mrs. P. Fazekas for typing the manuscript. Special thanks are due to S. Juhász of B.S.T. (Budapest) for packing the chromatographic columns.

REFERENCES

1 J. Wagner, P. Vitali, M. G. Palfreyman, M. Zraika and S. Huot, *J. Neurochem.*, 38 (1982) 1241–1254.
2 G. S. Mayer and R. E. Shoup, *J. Chromatogr.*, 255 (1983) 533–544.
3 S. M. Lasley, I. A. Michaelson, R. D. Greenland and P. M. McGinnis, *J. Chromatogr.*, 305 (1984) 27–42.
4 M. G. Hadfield, C. Milio and N. Narasimhachari, *J. Chromatogr.*, 369 (1986) 449–453.

5 I. C. Kilpatrick, M. W. Jones and O. T. Phillipson, *J. Neurochem.*, 46 (1986) 1865–1876.
6 R. F. Seegal, K. O. Brosch and B. Bush, *J. Chromatogr.*, 377 (1986) 131–144.
7 C. Kim, M. B. Speisky and S. N. Kharouba, *J. Chromatogr.*, 386 (1987) 25–35.
8 P. Herregodts, Y. Michotte and G. Ebinger, *J. Chromatogr.*, 421 (1987) 51–60.
9 T. P. Moyer and N.-S. Jiang, *J. Chromatogr.*, 153 (1978) 365–372.
10 P. A. Asmus and C. R. Freed, *J. Chromatogr.*, 169 (1979) 303–311.
11 O. Magnusson, L. B. Nilsson and D. Westerlund, *J. Chromatogr.*, 221 (1980) 237–247.
12 R. L. Michaud, M. J. Bannon and R. H. Roth, *J. Chromatogr.*, 225 (1981) 335–345.
13 C. D. Kilts, G. R. Breese and R. B. Mailman, *J. Chromatogr.*, 225 (1981) 347–357.
14 C. F. Saller and A. I. Salama, *J. Chromatogr.*, 309 (1984) 287–298.
15 P. Wester, J. Gottfries, K. Johansson, F. Klintebäck and B. Winblad, *J. Chromatogr.*, 415 (1987) 261–274.
16 B. H. C. Westerink, *J. Liq. Chromatogr.*, 6 (1983) 2337–2351.
17 C. Horváth, W. Melander, I. Molnár and P. Molnár, *Anal. Chem.*, 49 (1977) 2295–2305.
18 W. C. Mahoney, *Biochim. Biophys. Acta*, 704 (1982) 284–289.
19 G. Inchauspe, P. Delrieu, P. Dupin, M. Laurent and D. Samain, *J. Chromatogr.*, 404 (1987) 53–66.
20 M. Patthy, D. H. Schlesinger, J. Horváth, M. Mason-Garcia, B. Szoke and A. V. Schally, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 2969–2973.
21 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671–688.
22 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525–539.
23 Á. Bartha, Gy. Vigh, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 303 (1984) 29–38.
24 C. A. Marsden, *Methodol. Surv. Biochem. Anal.*, 14 (1984) 319–330.
25 M. Patthy, R. Gyenge and J. Salát, *J. Chromatogr.*, 241 (1982) 131–139.
26 R. Gloor and E. L. Johnson, *J. Chromatogr. Sci.*, 15 (1977) 413–423.
27 P. Y. T. Lin, M. C. Bulawa, P. Wong, L. Lin, J. Scott and C. L. Blank, *J. Liq. Chromatogr.*, 7 (1984) 509–538.
28 T. A. Durkin, E. J. Caliguri, I. N. Mefford, D. M. Lake, I. A. Macdonald, E. Sundstrom and G. Jonsson, *Life Sci.*, 37 (1985) 1803–1810.
29 A. Carlsson, T. Sharp, T. Zetterström and U. Ungerstedt, *J. Chromatogr.*, 368 (1986) 299–308.