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Preparation of an optimum mobile phase for the simultaneous determination of neurochemicals in mouse brain tissues by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A systematic method is described for the optimization of a mobile phase for the simultaneous determination of 24 neurochemicals consisting of catecholamine, serotonin, their precursors and metabolites and related materials. This mobile phase contained sodium acetate (0.04 *M*), citric acid (0.01 *M*), sodium chloride (0.0126 *M*), sodium octyl sulfate (91 mg/l), tetrasodium EDTA (50 mg/l) and 10% (v/v) methanol. When this optimum mobile phase was applied to the analysis of brain tissues of the Swiss male mouse, twelve neurochemicals were quantified in the free state: tyrosine, L-β-3,4-dihydroxyphenylanine, dopamine, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, norepinephrine, 3-methoxy-4-hydroxyphenylglycol, DL-3,4-dihydroxymandelic acid, DL-4-hydroxy-3-methoxymandelic acid, serotonin, L-tryptophan, 5-hydroxyindole-3-acetic acid and DL-synephrine and normetanephrine, appearing as a fused peak. This fused peak was present on the chromatogram tracings of all the mouse brain tissues. The separable neurochemicals not found by this procedure in the Swiss male mouse tissues were DL-3,4-dihydroxyphenylglycol, 5-hydroxytryptophan, epinephrine, DL-octopamine, metanephrine, deoxyepinephrine, homovanillyl alcohol, N-acetylserotonin, tyramine and 3-methyltyramine.

INTRODUCTION

Catecholamine and serotonin, their precursors and metabolites are important materials in neuroscience [1–5] and many procedures have been developed for the study of these substances [6–17]. In recent years, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been widely used for the measurement of these neurochemicals, and some reviews have been published on the theory and techniques employed [18–22]. However, many analytical procedures involve one or more steps of prior separation [13,23–30] and others the use of multiple electrodes [31,32].

The general controlling factors for the separation of neurochemicals by HPLC–ED procedure are well understood [18], and there are many variations [16,24,29–35] of the method [8]. Moreover, it has been applied by researchers in many disciplines to investigations involving neurochemicals [13,29,32,36–45]. The conventional method is first used to separate the analytes into groups (acids, amines and neutral compounds) before HPLC separation [27]. This preliminary separation is useful, because it reduces the number of compounds in each group and hence reduces the chances of co-elution. After this preliminary separation, three optimum mobile phases are used to determine the compounds in three chromatographic steps. In addition to the correction for the recovery of the preliminary separa-

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tion, other corrections are necessary for each of the three subsequent steps. Therefore, to obtain meaningful comparisons of the original concentration of the analytes in the native state of the tissue, appropriate normalization among the three measurements must be made. A single-step analysis eliminates the need for these corrections.

Optimized mobile phases for the simultaneous analysis of a large number of neurochemicals including amines, acids and neutral molecules in brain tissues are desirable but rare. Recently, a report on the distribution of biogenic amines in the cricket nervous system showed that it was possible to separate 21 chemicals simultaneously in a synthetic mixture by using a monochloroacetic acid system [42]. This one-step analysis of a large number of neurochemicals in advantageous over multi-step procedures. In this paper, we report the preparation of an optimized mobile phase that permits the simultaneous separation of 24 neurochemicals. Application of this optimum mobile phase to the analysis of three mouse brain tissues (olfactory bulbs, pituitary gland and hypothalamus) is described.

EXPERIMENTAL

The HPLC system was fabricated as described in ref. 36. It consisted of a Milton Roy pump (Model 396), a pulse damper, a Rheodyne rotary injection valve (Model 7125) with a 50-μl injection loop, a precolumn filter, a 5-μm Ultrasphere ODS reversed-phase column (250 mm × 4.6 mm I.D.) (Beckman) fitted with a water-jacket for temperature control, an amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) fitted with a flow cell (Model LC-3) using a carbon paste (CP-W), a Ag/AgCl reference electrode (RE-1) and a Varian Recorder (Model A-4223 A). The operational isocratic chromatographic conditions for this HPLC system were set as follows: column temperature, 25°C; flow-rate, 1.1 ml/min; working potential, 0.75 V *vs.* Ag/AgCl reference potential. When tissue samples were measured, the detector or recorder sensitivity settings were momentarily adjusted to accommodate peak heights on the recording chart.

Mobile phase

The optimum mobile phase contained 0.04 *M* sodium acetate, 0.01 *M* citric acid, 91 mg/l sodium octyl sulfate, 50 mg/l tetrasodium EDTA, 0.013 *M* sodium chloride and 10% (v/v) methanol. It was degassed before use.

Reagents

Chemicals were purchased from Sigma (St. Louis, MO, USA), except for sodium octyl sulfate (Research Plus, Bayonne, NJ, USA), methanol (Baxter, Hayward, CA, USA) and 4-hydroxy-3-methoxybenzylamine hydrochloride (Aldrich, Milwaukee, WI, USA).

Reference materials

A standard solution of each component was prepared in 0.01 *M* HCl and stored at 4°C. The compounds and their abbreviations are listed in Table I according to their retention times on the optimum chromatogram (Fig. 1, trace G). A calculated aliquot of each was delivered into a 25-ml plastic tube and chilled-dilute HCl was added to make up the desired concentration. A 100-μl mixture was delivered into a batch of 1.5-ml centrifugal tubes and stored at −76°C until use. A thawed sample (sonicated for the same period of time as a tissue sample, see next section) was used as a reference for each determination. A more concentrated synthetic mixture was used for the optimization procedure.

Animals and tissues

Seven-week-old male Swiss Webster mice (Simonsen Labs., Gilroy, CA, USA), after acclimatization for one week with a 12-h light and dark cycle, were killed with carbon dioxide during the light-up period. The brains were quickly removed from the skulls and dissected, according to ref. 46 with the modification described below, on an inverted watch-glass (covered with a bibulous paper soaked with isotonic saline) over ice. The entirety of the olfactory bulbs to their borders with the cerebral hemispheres were collected. Then the pituitary gland was removed, followed by the hypothalamus. The boundaries of these cuts were performed according to the ventral view and sag-

ittal section drawings in ref. 47. The tissues were weighed into tared 1.5-ml centrifugal tubes and stored at −76°C until analysis. The tissues were homogenized in chilled 0.3 *M* HCl containing the internal reference, 0.044 ng/μl 3,4-dihydroxybenzylamine hydrobromide (DHBA) (100 μl for the olfactory bulbs or hypothalamus and 50 μl for the pituitary gland) by sonication (Heat System Ultrasonic, Farmingdale, NY, USA). The homogenates were centrifuged at 10 000 *g* for 30 min at 4°C (Model J2-21 centrifuge, Beckman Instruments, Palo Alto, CA, USA). The supernatant was transferred by a Pasteur pipette to a 0.22-μm filter unit (Cat. No. UFC3 OGV 00; Millipore, Bedford, MA, USA) and centrifuged briefly. The filtrates were stored at −45°C until analyzed later on the same day.

Calculation

The amount of a substance was obtained by the measurement of the ratio of the peak height of that substance to the peak height of the internal standard and its amount on the sample chromatogram, and the concentration ratio (according to peak heights) of that substance to the internal standard on the reference chromatogram.

RESULTS AND DISCUSSION

During the early stage of development of the HPLC–ED method, only NE and DA in brain tissues were analyzed in one experiment [8,33,34]; the metabolites of catecholamine or indoleamine were measured by alternative experiments [15,26,28,48]. Later, methods were developed to determine complex mixtures containing multiple neurochemicals [27,30,32,35,40–44,49–52]. Many mobile phases were designed to study a small number of electroactive neurochemicals and to avoid co-elution and at the same time to shorten the chromatogram. As is known, to optimize a mobile phase for HPLC–ED, one has to consider the choice of stationary phase material, the particle size, the flow-rate, the organic modifier, the ion-pair reagent, the buffer system, the pH, the ionic strength and the column temperature. Changing any one of this set of variables may affect the retention time of all the components in a sample. As a result, extensive co-elution is expected to give rise to complex chromatograms unless the mobile phase is carefully designed.

A mobile phase containing sodium acetate and citric acid has been established for the analysis of catecholamines, or indoleamines, or together with their metabolites [13,15,25,28]. Obviously, as this type of mobile phase has the dual capacity for the determination of both the parent biogenic amines and their metabolites, its optimization to include a larger number of neurochemicals for a simultaneous determination is highly feasible. The compounds we selected to be analyzed simultaneously are listed in Table I [with DHBA as internal standard and also 4-hydroxy-3-methoxybenzylamine hydrochloride (HMBA) as another possible internal standard], because these neurochemicals might possibly be found and be quantifiable in mouse brain tissue by HPLC–ED procedure.

The systematic optimization of this mobile phase is illustrated by eight selected mobile phase compositions (Table II) and their chromatograms (Fig. 1). The utilization of the optimum mobile phase is demonstrated by the analyses of three different brain tissues (olfactory bulbs, pituitary gland and hypothalamus; Table III and Fig. 2) of the Swiss male mouse.

First, the working potential of the detector electrode was adjusted until each component of the synthetic mixture became detectable. Next, the flow-rate was adjusted to maximize peak heights and minimize peak distortion and noise and also to improve separation of the peaks. Composition A (Table II) can be used for the analysis of DOPA, NE, EPI and DA with DHBA as an internal reference. It is similar to a mobile phase [13] used together with the alumina extraction method [23]. When it was used to separate the 26 chemicals in our synthetic mixture by the direct injection method, we found six fused peaks (Fig. 1, trace A; for the identity and labeling of the chromatogram peaks, see Table I). When we increased the percentage of methanol in composition A, the retention time of the components de-

TABLE I

SENSITIVITY, RETENTION TIMES AT 25°C, ABBREVIATIONS AND CHROMATOGRAM LABELING OF THE BIOGENIC AMINES, THEIR PRECURSORS, METABOLITES AND RELATED MATERIALS

Label	Constituent form of the compound	Abbreviation	Sensitivity[a]	Retention time[b] (min)
a	DL-3,4-Dihydroxymandelic acid	DOMA	5	1.86
b	DL-4-Hydroxy-3-methoxymandelic acid	VMA	9	2.02
c	L-β-3,4-Dihydroxyphenylalanine	DOPA	7	2.82
d	DL-3,4-Dihydroxyphenylglycol	DOPEG	5	3.10
e	L-Tyrosine hydrochloride	Tyrosine	440	3.43
f	3,4-Dihydroxyphenylacetic acid	DOPAC	5	4.72
g	3-Methoxy-4-hydroxyphenylglycol hemipiperazium salt	MOPEG	18	5.25
h	5-Hydroxytryptophan	5-HTP	4	5.72
i	Norepinephrine hydrochloride	NE	7	6.08
j	Epinephrine hydrochloride	EPI	7	8.42
k	DL-Octopamine hydrochloride	OCTO	600	8.95
l	5-Hydroxyindole-3-acetic acid	5-HIAA	6	9.57
m	4-Hydroxy-3-methoxyphenylacetic acid	HVA	10	11.05
n	3,4-Dihydroxybenzylamine hydrobromide	DHBA	8	11.17
o	DL-Synephrine	SYNE	440	13.17
p	Normetanephrine hydrochloride	NMN	40	13.18
q	L-Tryptophan	TRP	1000	14.13
r	Dopamine hydrochloride	DA	12	19.00
s	Metanephrine hydrochloride	NM	28	20.03
t	Deoxyepinephrine hydrochloride (epinine)	EPIN	17	13.03
★	Homovanillyl alcohol	HVL	19	24.30
u	N-Acetylserotononin	NAS	20	29.77
v	4-Hydroxy-3-methoxybenzylamine hydrochloride	HMBA	40	32.85
w	Tyramine hydrochloride	TYRAM	300	33.73
x	3-Methyltyramine hydrochloride	MTA	50	58.45
y	Serotonin creatine sulfate complex	5-HT	30	59.93

[a] Sensitivity: defined as the amount of material in picograms giving a peak twice as high as the baseline fluctuation.

[b] Retention time measured in minutes from the point of injection. Chromatographic conditions: flow-rate, 1.1 ml/min; working potential, 0.75 V *vs.* Ag/AgCl reference electrode; column temperature, 25°C; mobile phase containing 0.04 *M* sodium acetate, 0.01 *M* citric acid, 91 mg/l sodium octyl sulfate, 50 mg/l tetrasodium EDTA, 0.013 *M* sodium chloride and 10% (v/v) methanol.

creased. However, the higher methanol content led to more fused peaks on the chromatogram (not shown), so we temporarily kept the methanol concentration at 15%. After a serial adjustment of first the concentration of the citric acid, then that of the sodium acetate from composition A (to minimize co-elution), at composition B (Table II), peak c was clearly separated from peak d, but peak f was now fused with peaks g and h, leaving the chromatogram with four fused peaks (Fig. 1, trace B). The best combination of sodium acetate and citric acid appeared to be 0.04 and 0.01 *M*, respectively (Table II, composition C), and the number of fused peaks was reduced to three (Fig. 1, trace C). Any increase or decrease in the sodium acetate or citric acid concentration from composition C would give more fused peaks.

After this, we reduced the content of methanol from 15% to 12% (v/v) (Table II, composition D), and peaks g and h became separable, leaving two fused peaks. Still further reduction of the concentration of methanol to 10% (v/v) (composition E) led to the separation of peaks r and s but now the fused peak jl slightly overlapped with peak k (Fig. 1, trace E). Although both composi-

TABLE II

COMPOSITION OF EIGHT MOBILE PHASES[a] AND THEIR COELUTION PATTERN

Mobile phase[a]	Temperature (°C)	Sodium acetate (*M*)	Citric acid (*M*)	Methanol (%,v/v)	NaCl (*M*)	Fused peaks[b]
A	Ambient[c]	0.05	0.01	15	–	cde; gh; jk; lm; op; rs
B	Ambient	0.035	0.11	15	–	fgh; kl; op; rs
C	Ambient	0.04	0.01	15	–	gh; op; rs
D	Ambient	0.04	0.01	12	–	opq; rs
E	Ambient	0.04	0.01	10	–	jlk[d]; op
F	25	0.04	0.01	10	–	jl; op
G	25	0.04	0.01	10	0.0126	op
H	25	0.04	0.01	10	0.1470	ief; kn; op; rs; tu

[a] All mobile phases contained 100 mg/l sodium octyl sulfate and 50 mg/l tetrasodium EDTA.
[b] For peak labeling, see Table I.
[c] 20–22°C.
[d] The fused peak jl slightly overlapped with peak k.

tions D and E gave two fused peaks and composition D gave a shorter chromatogram, for further development composition E is preferred to D because peaks r and s are less temperature-sensitive than peaks j, k and l. It has been reported that the retention time of peak l (5-HIAA) is sensitive to column temperature [35]; consequently, it would be more difficult to separate peaks r and s (if at all) than to separate peaks, j, l and k by controlling the column temperature. Therefore, we increased the column temperature from 20–22°C (ambient) to 25°C; composition E became condition F (Table II). This successfully allowed the separation of peak k from peaks j and l.

Although we used a reversed-phase column (see Experimental), and ionic strength has been considered to have very little utility in reversed-phase chromatography [18], we found that ionic strength did achieve the optimization objective for this mobile phase. It has been described that by reversed-phase ion-pair chromatography, the migration rate of the sample is easily controlled by the concentration of the counter ions in the mobile phase [53]. The principle lies in the adduct formation of ion pairs. On the other hand, it has been known since early discoveries on the theory of ionic reactions and equilibrium constants that the activity coefficients of ionic species vary with the ionic strength of the solution [54–57]. The interaction between members of the analytes, molecules of the mobile phase and those partitioned on the stationary phase materials can be considered as a moving network of equilibrium. The chromatographic retention mechanism has been assumed to be that ion pairs are already formed in the mobile phase [22] or ion exchange occurs between solute ions and counter ions adsorbed at the interface [58]. Whatever the retention mechanism may be, as the analyte travels along the analytical column, the kinetics and thermodynamic properties of this network of equilibrium are expected to follow the rules of the Debye–Hückel theory and the principles of ionic reaction equilibrium. Hence the ionic strength of the solution also is important. Indeed, it also has been pointed out to be important in an aqueous buffer [58].

As most of the compounds of the synthetic reference mixture are hydrochlorides and most of the reagents in the mobile phase are sodium salts, we used sodium chloride to increase the ionic strength of the mobile phase. A gradual increase in the ionic strength of the mobile phase by adding sodium chloride (at 0.0126 *M*) led to the separation of peaks j and l without affecting the apparent relative retention time of the other components of the mixture. Now the 24 components were separated, leaving only one fused peak (o

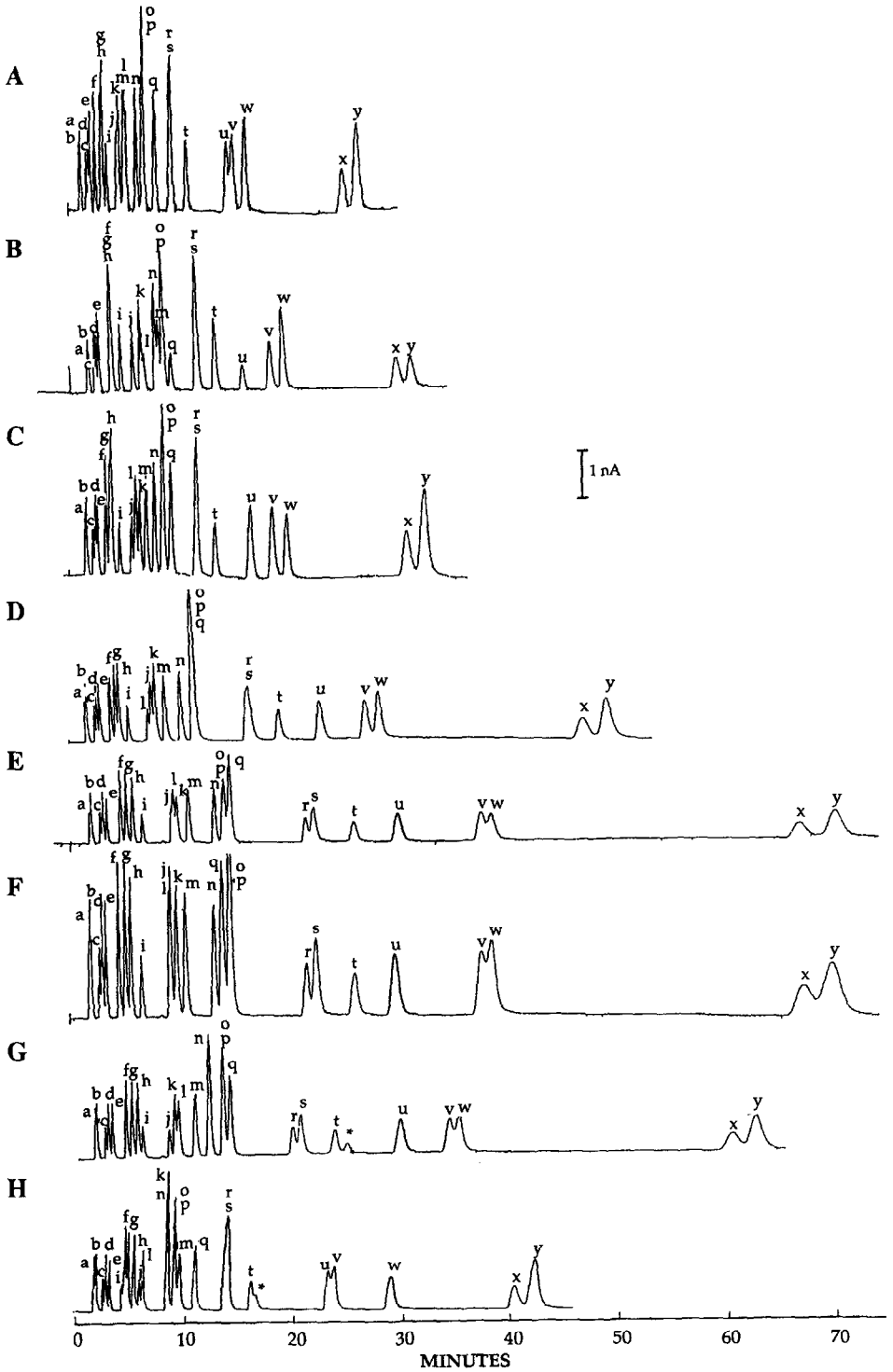

Fig. 1. Chromatograms of a standard mixture containing 24 neurochemicals and related substances. All traces were recorded at a flow-rate of 1.1 ml/min and a working potential of 0.75 V *vs.* Ag/AgCl reference electrode. For peak labelings, see Table I. For solvent compositions and column temperature, see Table II.

TABLE III

FREE FORMS[a] OF DOPAMINE, NOREPINEPHRINE, SEROTONIN, THEIR PRECURSORS AND METABOLITES IN SWISS MALE MOUSE OLFACTORY BULBS, PITUITARY GLANDS AND HYPOTHALAMUS MEASURED IN ONE STEP[b]

Group	Compound	Found (mean ± S.D.) (μg/g)		
		Olfactory bulbs	Pituitary glands	Hypothalamus
Dopamine precursors	Tyrosine	29.1 ± 8.0	17.6 ± 5.1	31.9 ± 15.6
	DOPA	2.77 ± 0.79	1.50 ± 0.62	2.21 ± 1.73
Dopamine and metabolites	DA	0.135 ± 0.036	0.606 ± 0.222	0.283 ± 0.038
	DOPAC	0.237 ± 0.099	1.061 ± 0.387	0.231 ± 0.115
	HVA	0.093 ± 0.021	0.355 ± 0.069	0.171 ± 0.036
Norepinephrine and metabolites	NE	0.217 ± 0.059	0.522 ± 0.209	0.935 ± 0.082
	MOPEG	0.197 ± 0.055	0.289 ± 0.133	0.269 ± 0.264
	DOMA	1.7 ± 0.3	4.8 ± 0.1	2.1 ± 0.3
	VMA	13.6 ± 3.1	19.9 ± 9.7	6.4 ± 0.7
Serotonin precursor	TRP	6.9 ± 2.2	3.1 ± 0.3	3.6 ± 1.4
Serotonin and metabolites	5-HT	0.398 ± 0.171	0.641 ± 0.538	1.70 ± 0.434
	5-HIAA	0.382 ± 0.152	1.22 ± 0.712	0.896 ± 0.235

[a] Average of three determinations, concentrations in μg/g wet tissue; the number of significant figures after the decimal point is in accordance with the sensitivity of measurement (see Table I) and the attenuation of detector and recorder settings.

[b] For chromatographic conditions, see footnote to Table I.

and p). The difficulty in separating the fused o and p peak (SYNE and NMN) is not unusual, as it has been reported as a fused peak in a monochloroacetate system [42]. At this juncture, we added HVL to the synthetic mixture and again to composition H. We found that HVL did not co-elute with any of the other neurochemicals of the synthetic mixture (Fig. 1, trace G). Because HVL was not found in the mouse brain tissues in this investigation, to improve clarity we did not include HVL in the other selected chromatographic traces (Fig. 1, traces A–F).

It can be seen from Fig. 1 (traces F, G and H) that increasing the ionic strength reduces the overall retention time. In an attempt to decrease further the time needed to run one chromatogram, we increased the sodium chloride content. Unfortunately, when the ionic strength was increased over the critical composition G, new fused peaks appeared (*e.g.*, Fig. 1, trace H).

Hence the procedure outlined above established the optimum mobile phase as containing 0.04 *M* sodium acetate, 0.01 *M* citric acid, 0.0126 *M* sodium chloride, 100 mg/l sodium octyl sulfate, 50 mg/l tetrasodium EDTA and 10% (v/v) methanol. The operating conditions for this HPLC system (see Experimental) were column temperature 25°C, flow-rate 1.1 ml/min and working potential 0.75 V *vs.* Ag/AgCl reference electrode.

The utilization of this mobile phase is demonstrated by the analysis of three mouse brain tissues (Fig. 2 and Table III). The preparation of the mouse brain tissues was described under Experimental. Many other neurochemicals were not included, because they are not detectable by HPLC–ED. However, the hydroxylphenylacetic acids are detectable by this method. When the optimum mobile phase was used, we found that *o*- and *p*-hydroxylphenylacetic acid co-eluted between NE and EPI (peaks i and j) and *m*-hydroxylphenylacetic acid eluted between 5-HIAA and HVA (peaks l and m) without interference with the other peaks of the synthetic mixture. As seen in the chromatograms of the mouse brain tissues (Fig. 2A, B and C), these hydroxylphenylacetic acids were absent at those positions or were at concentrations too low to be detectable by this procedure. Therefore, the hydroxylphenylacetic acids were not included in the reference mixture.

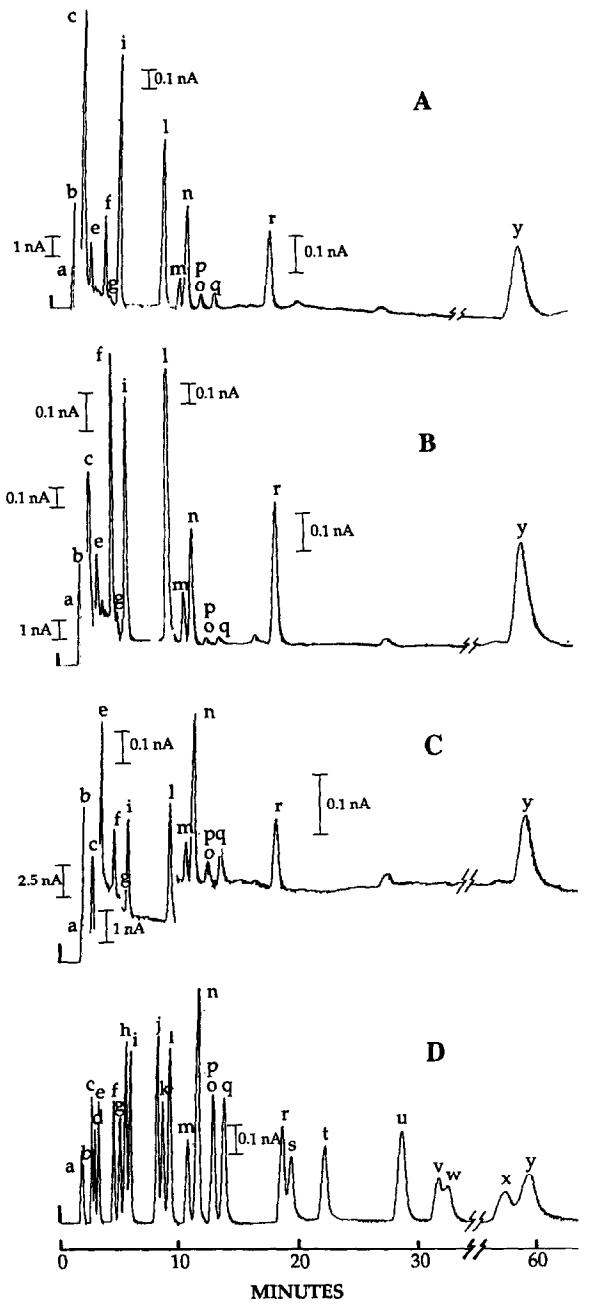

Fig. 2. Typical chromatograms of biogenic amines, their precursors and related metabolites in mouse brain tissues, and related substances in a standard mixture. (A) Hypothalmus; (B) pituitary gland; (C) olfactory bulbs; (D) standard reference mixture. All traces were recorded at a column temperature of 25°C, a flow-rate of 1.1 ml/ml and a working potential of 0.75 V *vs.* Ag/AgCl reference electrode; the mobile phase contained 0.04 *M* sodium acetate, 0.01 *M* citric acid, 0.0126 *M* sodium chloride, 91 mg/l sodium octyl sulfate, 50 mg/l tetrasodium EDTA and 10% (v/v) methanol.

The sensitivity reported here (defined as the amount of material giving a peak twice as high as the baseline fluctuation) agrees with other reported sensitivity values [13,26,32,40]. Under the present experimental conditions, the sensitivity of the compounds investigated (see Table I) can be classified into five groups: (a) between 5 and 10 pg (DOMA, VMA, DOPA, DOPEG, DOPAC, 5-HTP, NE, EPI, 5-HIAA and DHBA); (b) between 10 and 20 pg (MOPEG, HVA, DA, EPIN and NAS); (c) between 20 and 40 pg (NMN, NM, HVL and 5-HT); (d) between 40 and 70 pg (HMBA and MTA); and (e) those with lower sensitivity [TYRAM (300 pg), tyrosine and SYNE (440 pg), OCTO (600 pg) and TRP (1000 pg)]. The whole tissue weight of the two olfactory bulbs, pituitary gland and hypothalamus were 10, 1–2 and 10 mg, respectively. Among these three tissues, the pituitary gland contains the highest concentration of these neurochemicals (Table III). Consequently, one determination requires only half the supernatant of the olfactory bulbs or the pituitary gland, or only one quarter of the hypothalamus supernatant.

Twelve neurochemicals in their free state were identified (Fig. 2) and determined (Table III) in each of these three mouse brain tissues. In addition to identifying these neurochemicals by their retention times (Fig. 2), they were further identified by the addition method. These neurochemicals may be divided into five groups: (a) two dopamine precursors (tyrosine and DOPA); (b) dopamine and two of its metabolites (DA, DOPA and HVA); (c) norepinephrine and three of its metabolites (NE, MOPEG, DOMA and VMA); (d) one serotonin precursor (TRP); and (e) serotonin and one of its metabolites (5-HT and 5-HIAA). There was also a fused peak, possibly that of SYNE and NMN (if both neurochemicals were present), which was found in all three tissues. The other ten separable electroactive neurochemicals were not found (*i.e.*, DOPEG, MN, HVL, MTA, 5-HTP, NAS, EPI, EPIN, OCTO and TYRAM). Alternatively, their concentrations might be too low to be detected by the present method.

By examining the results obtained for the hy-

pothalamus (not many data on neurochemical analysis of the olfactory bulbs or the pituitary gland are available in the literature) in Table III, we concluded that our results are comparable to those obtained by other procedures, including analysis of the catecholamines [34], catecholamines and DOPAC [13], catecholamines, DOPAC and DOPA [29], analysis of tryptophan and metabolites [15], analysis of the metabolites of dopamine and serotonin [39], and analysis of multiple neurochemicals by various extraction procedures [40,41,43]. As is known, these biogenic amines and their metabolites are subject to oxidation in solution and need to be handled with extreme care [20]. Therefore, it is beneficial to omit the extraction steps, because they may unevenly destroy portions of the compounds of interest. In addition, for the conventional extraction method, as there is a time lag between HPLC analysis of the preliminary fractions obtained (from the prior separation), the kinetics of decomposition of the unstable compounds in each fraction may make it difficult to determine accurately all the substances of interest. On the other hand, to analyze the three fractions simultaneously, one would require three HPLC systems with three optimum mobile phases.

The advantage of using the present mobile phase is that it simplifies the procedure for the determination of neurochemicals in mouse brain tissues, including the calculations. Other sample preparation steps are no longer necessary, as in the aluminum absorption [23,33], gel absorption [51], ion-exchange chromatographic [40] or solvent extraction [30,59,60] methods. Further, here the sample preparation is by sonication with the internal standard, followed by centrifugation and filtration, all in one concerted step. There is no absorption or desorption involved; therefore, no correction of recovery of the chemicals was performed. Correction for recovery is an essential step in other extraction procedures.

Although the chromatogram is long, the advantage of using this mobile phase is that one chromatogram (see Fig. 2) from one sample provides the information in three conventional chromatograms. The benefits obtained by a longer separation time for collecting a chromatogram outweigh the time consumed in the elaborate preliminary separation of the sample into acidic, alkaline and neutral fractions, subsequent HPLC separation of each of the three fractions, and the normalization of the three different chromatograms obtained under different conditions. Further, having a common internal standard on the same chromatogram simplifies the calculation.

This paper illustrates that optimization of a mobile phase based on sodium acetate–citric acid is possible, and perhaps optimization of other mobile phase systems would also be relatively simple. When such mobile phase systems are applied to analyze biological samples by a probe which does not detect non-probe-sensitive compounds, the analysis becomes relatively simple, and a larger number of probe-sensitive compounds could be determined simultaneously. Here, we have an optimum mobile phase suitable for the analysis of some 24 neurochemicals related to electroactive biogenic amine metabolism in the mouse brain by HPLC–ED. It involves only one concerted step of weighing, homogenization, ultracentrifugation, brief centrifugal filtration and direct injection, followed by calculation. Optimum mobile phases may simplify analytical procedures, enhance coherence in interpretations of data, eliminate recovery or inter-procedural corrections, and make the various extraction steps redundant.

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