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Glassy carbon pre-column for direct determination of acetylcholine and choline in biological samples using liquid chromatography with electrochemical detection

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ABSTRACT

The determination of acetylcholine and choline has been quite successfully accomplished using liquid chromatography with electrochemical detection following the original reports of Potter *et al.* [*J. Neurochem.*, 41 (1984) 188]. A post-column reactor containing acetylcholinesterase and choline oxidase allows conversion of the desired species into hydrogen peroxide, an electrochemically active substance. However, the direct injection of tissue homogenates and other biological samples into such a system exhibits quite large solvent fronts and unidentified peaks. Using a pre-column packed with glassy carbon particles, we were able to dramatically decrease the size of the solvent front for such injections and tentatively identify the unknown peaks to be caused, at least in part, by common catecholamines. The glassy carbon pre-column, in addition to increasing the selectivity of the results, allowed the required chromatographic time per sample to be decreased from 20 to 10 min.

INTRODUCTION

Since the original reports by Potter *et al.* [1] describing the determination of acetylcholine (ACh) and choline (Ch) using liquid chromatography (LC) with electrochemical detection (ED), multiple subsequent papers have clearly demonstrated the usefulness of this general approach [2–8]. The desired ACh and Ch are first separated on an analytical column. Upon emerging from the analytical column, the eluents are exposed to both acetylcholinesterase (AChE) and choline oxidase (ChO), which convert the desired species and the internal standard, ethylhomocholine

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(EHC), into hydrogen peroxide. The hydrogen peroxide is then detected by oxidation at a platinum electrode. While the two enzymes were previously mixed with the eluent in a mixing coil prior to the electrochemical detector, most systems today employ a post-column reactor containing the immobilized enzymes for the necessary transformation to hydrogen peroxide. This general approach works quite well if the sample is relatively pure and/or subjected to appropriate pretreatment prior to injection into the LC–ED system. For example, rat brain tissue homogenates subjected to precipitation of the desired analytes with a Reinicke salt [1] or KI–I_2 [2] or solvent extraction [9] have provided adequate results. However, such sample purification is time-consuming and may lead to losses of the desired species due to incomplete recoveries. Thus, a more direct injection of the tissue homogenates and other biologically related samples is clearly desirable. But, an initial attempt by ourselves at the more direct analysis of a perchloric acid homogenate of rat brain tissue, purified only by centrifugation and filtration, exhibited quite large solvent fronts and some unidentified peaks.

Previous alternative analyses of such tissue homogenates caused us to initially suspect catecholamines, indoleamines, and/or related substances to possibly be the unidentified peaks in the attempted direct analysis of ACh and Ch. Quite accidentally, in an unrelated investigation, we discovered that considerable amounts of catecholamines were adsorbed onto the surface of a cylindrical glassy carbon rod when solutions containing these species were simply passed through the rod. Thus, we constructed and tested a pre-column of glassy carbon particles to assess its possibly applicability to the more direct LC–ED determination of ACh and Ch.

EXPERIMENTAL

Animals

Male Wistar rats, weighing 240–250 g and obtained from the Institute of Experimental Animal Research at Gunma university, were used in all the studies employing animals. The rats were housed individually and allowed access to water and standard laboratory chow (Oriental Yeast, Tokyo, Japan) *ad libitum*. The animals were housed in a facility having a temperature of 23 ± 1°C, a relative humidity of 55 ± 5%, and controlled lighting, with the lights on from 07:00 to 19:00 h daily.

Reagents

All chemical reagents employed, with the exception of EHC, were obtained from commercially available sources in the highest possible purity and used without further purification. Of the analyte species, Ch, ACh, dopamine (DA), homovanillic acid (HVA), 3,4-dihydroxybenzylamine (DHBA), 5-hydroxyindoleacetic acid (5-HIAA), and 5-hydrodytryptamine (5-HT) were purchased from Sigma (St. Louis, MO, USA); 3,4-dihydroxyphenylacetic acid (DOPAC) and N_ω-methyl-5-hydroxytryptamine (nMET) were obtained from Aldrich (Milwaukee, WI, USA). The N,N-dimethyl-N-ethyl-3-amino-1-propanol, better known as ethylhomocholine, was prepared from iodoethane and 3-dimethylamino-1-propanol using previously described procedures [1].

Microwave irradiator

Animals were sacrificed by exposure to 9.0 kW of 2450 MHz microwave irradiation for 0.95 s from a Model NJE-2603-10kW microwave irradiator from New Japan Radio (Saitama, Japan). Providing rapid inactivation of the enzymes associated with, particularly, ACh metabolism, such a unit is essential to obtaining results for these endogenous neurochemicals which are not disturbed by post mortem degradation [10]. This unit elicits an optimal heat distribution and rapid inactivation of pertinent enzymes by employing a predominantly magnetic field distribution rather than the usual electric field distribution, a rise to peak power in only 10–15 ms, an integrated tuning system to optimize irradiation for individual animals, and a water-jacketted head region in the animal holder.

Liquid chromatographic systems

Glassy carbon pre-column. Glassy carbon par-

ticles, labelled as IRICA Type CP-2250, were obtained from the Analytical Laboratories of IRICA Instruments (Kyoto, Japan). These particles were derived from a glass-like, vitreous hard carbon material which exhibited an electrical resistivity of $4.5 \cdot 10^{-3}$ Ω/cm, a specific gravity of 1.52, a thermal expansion coefficient of $2.2 \cdot 10^{-6}$, and less than 0.08% impurities. Four separate stainless-steel columns were prepared from the particles using conventional slurry packing procedures to be used as pre-columns in the LC–ED analysis of ACh and Ch. The four columns were 3 mm × 3 mm I.D., 3.4 mm × 4 mm I.D., 5.1 mm × 4 mm I.D., and 10 mm × 4 mm I.D.

LC–ED system for determination of ACh and Ch. The liquid chromatographic system employed for the determination of ACh and Ch consisted of an LC100P pump from Yokogawa (Tokyo, Japan), an LC100S injector with a 20-μl sample loop from the same supplier, an Acetylcholine Separation analytical column (60 mm × 4 mm I.D., 3 μm, polymeric styrene-based packing material) from BAS (Bioanalytical Systems, West Lafayette, IN, USA), an LC22A temperature controller, maintained at 35°C, from BAS, and an LC-4A amperometric detector with a dual platinum electrode from BAS. The post-column, containing immobilized AChE and ChO, was a 5 mm × 4 mm I.D. unit also obtained from BAS; enzyme immobilization was accomplished similar to that described in a previous report [11]. The data were monitored with a Model LC100W/F-PC workstation from Yokogawa. The potential of the platinum electrode was maintained at +0.50 V *vs.* the Ag/AgCl reference electrode. The eluting solvent for this system had a pH of 8.4 and was composed of 0.05 *M* phosphate containing 1.0 m*M* Na_2EDTA and 0.40 m*M* sodium 1-octanesulfonate. The flow-rate was typically 0.80 ml/min. When employed, the glassy carbon pre-column was inserted between the injection port and the analytical column in the flow path of the eluting solvent.

LC–ED system for determination of catecholamines, indoleamines, and related metabolites. The LC–ED system employed for the determination of catecholamines, indoleamines and related metabolites [12,13] used many of the components already described for the ACh/Ch system. The notable differences were as follows. This system incorporated a BioPhase ODS II (3 μm, 150 mm × 4.6 mm I.D.) analytical column and a glassy carbon working electrode; both of these components were obtained from BAS. The mobile phase was a 0.1 *M* acetate–citrate buffer, pH 3.2, containing 1.0 m*M* sodium 1-octanesulfonate and 0.50 m*M* Na_2EDTA. The potential of the working electrode was maintained at +0.70 V *vs.* Ag/AgCl. And, as with the ACh/Ch system, the temperature was maintained at 35°C, and the flow-rate was typically 0.80 ml/min.

Tissue sample preparation

After sacrifice by microwave irradiation, the brains were quickly removed from the skull of the animals and dissected into seven brain regions according to the method of Glowinsky and Iversen [14]. The seven regions were, in order of dissection: cerebellum, medulla-pons, hypothalamus, midbrain-thalamus, hippocampus, striatum, and cortex. Each region was then placed, separately, into 1.0 ml of a 0.050 *M* perchloric acid solution containing 1.0 nmol of DHBA, the internal standard for catecholamine determinations, 1.0 nmol nMET, the internal standard for indoleamine determinations, and 10.0 nmol EHC, the internal standard for ACh/Ch determinations. Homogenization of the tissue was accomplished by a Model US-300T ultrasonic cell disruptor from Nissei (Tokyo, Japan), set at 300 W and 20 KHz for 60 s. The homogenate was centrifuged at 20 000 *g* and 4°C for 15 min to remove cellular debris and macromolecules. Finally, the homogenate was purified by passage through a 0.45-μm Millipore filter. Aliquots, typically 10 μl, of the filtrate were injected into the appropriate LC–ED system for the determinations of catecholamine-, indoleamine-, and acetylcholine-related substances.

RESULTS AND DISCUSSION

In an investigation concerned with the levels of catecholamines, indoleamines, ACh, and related

TABLE I

NEUROCHEMICALS IN RAT STRIATUM AND CORRESPONDING TISSUE HOMOGENATES

Each value represents the mean derived from seven animals. The mean ± S.D. tissue weight was 135 ± 18 mg. Each striatum was homogenized in 1.0 ml of perchloric acid containing 1 nmol DHBA, 1 nmol nMET, and 10 nmol EHC.

Compound	Striatal concentration		Amount in 10 μl LC–ED injection (pmol)
	nmol/g tissue	nmol/striatum	
DA	46.66	6.29	62.9
DOPAC	4.89	0.66	6.6
HVA	2.93	0.39	3.9
NE	2.09	0.28	2.8
DHBA	–	–	10.0
5-HT	4.04	0.55	5.5
5-HIAA	2.96	0.40	4.0
nMET	–	–	10.0
ACh	76.80	10.37	103.7
Ch	25.10	3.39	33.9
EHC	–	–	100.0

TABLE II

NEUROCHEMICALS IN RAT HYPOTHALAMUS AND CORRESPONDING TISSUE HOMOGENATES

Each value represents the mean derived from seven animals. The mean ± S.D. tissue weight was 87 ± 4 mg. Each hypothalamus was homogenized in 1.0 ml of perchloric acid containing 1 nmol DHBA, 1 nmol nMET, and 10 nmol EHC. N.D. for the HVA results indicates that this compound was not detected.

Compound	Hypothalamus concentration		Amount in 10 μl LC–EC injection (pmol)
	nmol/g tissue	nmol/ hypothalamus	
DA	3.03	0.26	2.6
DOPAC	0.85	0.07	0.7
HVA	N.D.	N.D.	N.D.
NE	6.72	0.59	5.9
DHBA	–	–	10.0
5-HT	6.40	0.56	5.6
5-HIAA	4.21	0.37	3.7
nMET	–	–	10.0
ACh	30.10	2.61	26.1
Ch	21.20	1.84	18.4
EHC	–	–	100.0

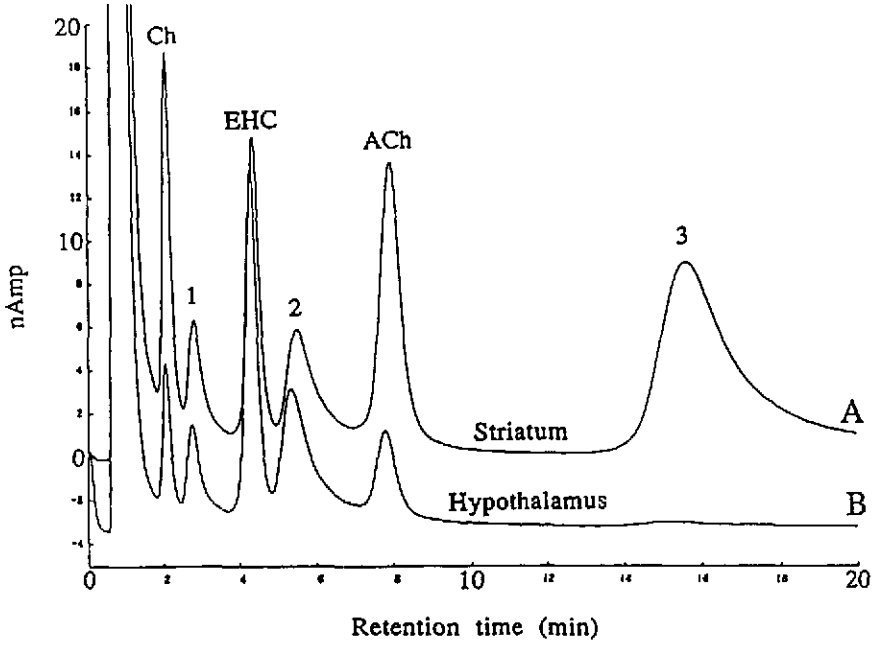

Fig. 1. Typical chromatograms obtained from the direct injection of 10 μl of rat striatal (A) and hypothalamus (B) tissue homogenates into the LC–ED system designed for the determination of ACh and Ch. Peaks *1*, 2, and 3 were initially unidentified (vide infra).

substances in rat brain tissues, we decided to attempt to employ a single perchloric acid homogenate preparation for both of the LC–ED systems employed. A 10-μl aliquot of the homogenate, when injected into the chromatographic system designed for the determination of catecholamines, indoleamines, and related substances, yielded the expected chromatograms, and analysis of the data indicated levels of compounds appropriate to previous results. Typical results are shown for the striatum and hypothalamus, respectively, in Tables I and II. However, direct injection of a 10-μl aliquot of the homogenate into the LC–ED system designed for the determination of ACh and Ch without any pre-column yielded a chromatogram with substantially more than the three peaks expected for the two endogenous substances and the internal standard, EHC. Such chromatograms showed a substantially large solvent front as well as three unidentified peaks. As seen in Fig. 1, the three unknown peaks appeared at retention times of 2.60, 5.68, and 15.70 min, respectively, while the expected peaks for Ch, EHC, and ACh appeared at their normally observed retention times of 2.04, 4.07 and 7.80 min. The results for individual brain regions consistently exhibited the three known and the three unknown peaks, although the relative peak areas of each noticeably changed from region to region. In all regions examined, extension of the chromatogram to as much as 60 min revealed no further peaks eluting after the third unknown, which appeared at a retention time of 15.70 min.

Suspecting, as previously indicated by Potter *et al.* [1], that the unknown peaks in the chromatograms might represent dopamine-like substances, we prepared a standard mixture containing catecholamines, indoleamines, and related substances in amounts corresponding to the striatum tissue values shown in Table I. Injections of 10 μl of this mixture into the LC–ED system designed for ACh and Ch, without any pre-column, are shown in Fig. 2; as can be seen, the chromato-

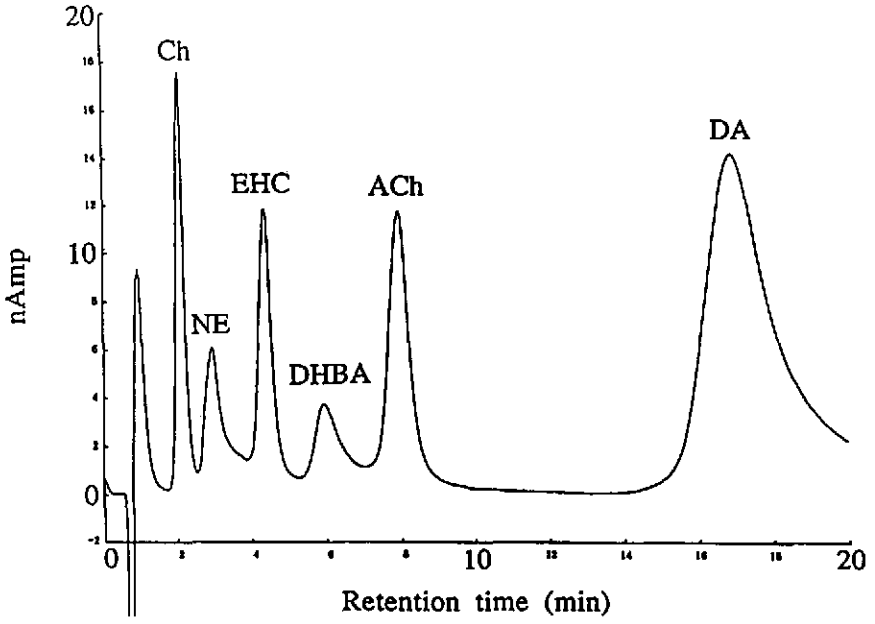

Fig. 2. Chromatogram obtained from the injection of a 10-μl aliquot of a mixture containing, all in 1.0 ml of 0.05 *M* perchloric acid, the following: 100 pmol DA, 10 pmol DOPAC, 5 pmol HVA, 4 pmol NE, 10 pmol DHBA, 6 pmol 5-HT, 5 pmol 5-HIAA, 10 pmol nMET, 100 pmol ACh, 50 pmol Ch, and 100 pmol EHC. The LC–ED system employed was that designed for the determination of ACh and Ch. The concentrations of the individual species approximately reflects those obtained in a rat striatal homogenate, as described in Table I.

gram obtained was virtually identical to that found for the tissue extracts. The three unknown peaks appear along with the three known peaks in approximately the same relative amounts with all six peaks revealing identical retention times to those found for the striatal extracts. Separate injections of the individual constituents of the mixture allowed a tentative identification of the unknown peaks to be norepinephrine (NE), DHBA, and DA, respectively. Verification of the identity of these peaks is currently underway using LC with mass spectrometry [15].

We next inserted a 3 mm × 3 mm I.D. glassy carbon pre-column into the flow path of the LC–ED system for the determination of ACh and Ch between the injection port and the analytical column. Injection of a 10-μl sample of the perchloric acid extract taken from striatum, identical to that previously used for Fig. 1A, yielded the chromatogram shown in Fig. 3B. Insertion of the pre-column clearly eliminated the three unknown peaks from the previous attempts; however, there were notably no changes in the peaks obtained for the three targeted species (Ch, EHC, and ACh) or their associated, calculated tissue levels after addition of this pre-column.

Since the glassy carbon pre-column obviously provided an improvement in the results, we subsequently attempted to increase the amount of packing material in the pre-column primarily to see if we could extend the usefulness of such a device. In these experiments, we successively employed pre-columns having dimensions of 3 mm × 3 mm I.D., 3.4 mm × 4 mm I.D., 5.1 mm × 4 mm I.D., and 10 mm × 4 mm I.D. These approximately contain one, two, three and six times, respectively, as much packing material as the originally employed pre-column. Using each of these separately as a pre-column for the ACh/Ch system, individual 10-μl aliquots of the standard striatal homogenate, prepared as described above, was repeatedly injected into the system until peaks for NE, DHBA, and DA appeared in the chromatograms. The adsorption capacity of each pre-column for each of these species, shown in Table III, was then calculated considering the number of injections prior to appearance of the corresponding peak and the known injection

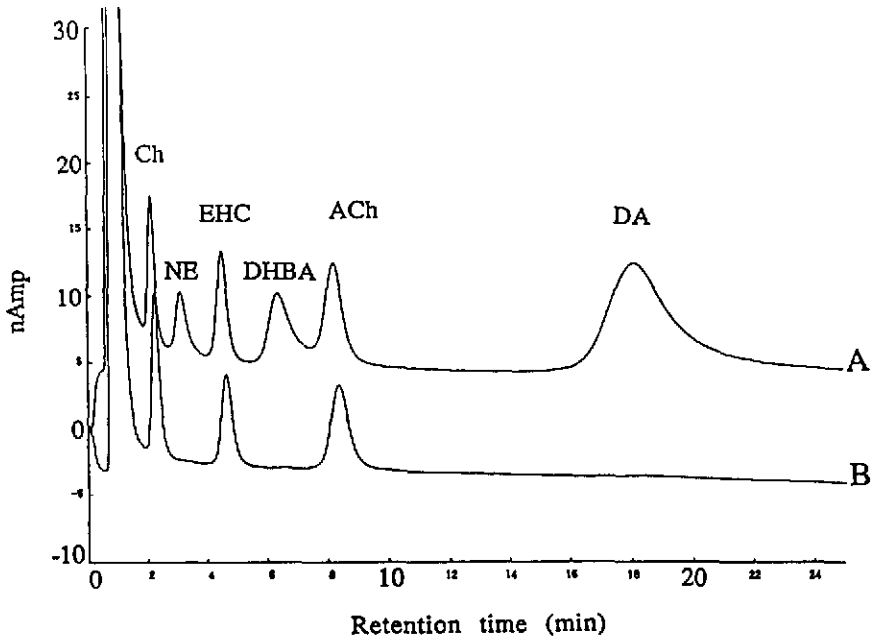

Fig. 3. Chromatograms obtained from injection of 10 μl of rat striatal homogenates with (B) and without (A) the use of the glassy carbon pre-column. The LC–ED system employed was that designed for the determination of ACh and Ch. The glassy carbon pre-column was 3 mm × 3 mm I.D.

TABLE III

CAPACITY OF GLASSY CARBON PRE-COLUMNS FOR ADSORPTION OF CATECHOLAMINES

Column size (length × I.D.) (mm)	Capacity (pmol)		
	NE	DHBA	DA
3 × 3	10	10	70
3.4 × 4	40	20	150
5.1 × 4	80	40	250
10 × 4	150	80	600

concentration. Using the largest pre-column, over 100 injections of typical brain tissue homogenates may be employed before appearance of the unknown peaks occurs. In addition to the greater adsorption capacity found for the pre-columns containing greater amounts of the glassy carbon packing material, we also observed a second and unexpected advantage of these units. As seen in Fig. 4 for hypothalamic tissue homogenates, the solvent front peak size was dramatically reduced, from approximately 500 to 10 nA, in going from the smallest to the largest pre-column. This reduction allows much more precise determination of the peak characteristics, in particular, for the early eluting Ch peak. More recent work in our laboratory (in progress) would indicate that the glassy carbon pre-columns are removing many of the non-retained, electrochemically active components which typically elute with the solvent front in the usual ACh/Ch analysis; thus, this removal is responsible for the reduction in the solvent peak observed. Similar pre-columns, incidentally, prepared with aluminum oxide and borate packing materials, yielded unsatisfactory results.

Glassy carbon, originally described by Yamada in 1968 [16], is a material categorized as a "polymeric carbon", containing carbon fibers and chars [17]. In general, the structure reveals a network of various arrangements of carbon microfibrils and ribbons. However, no previous report, to our knowledge, describes any selectivity of this material for the adsorption of catechol-

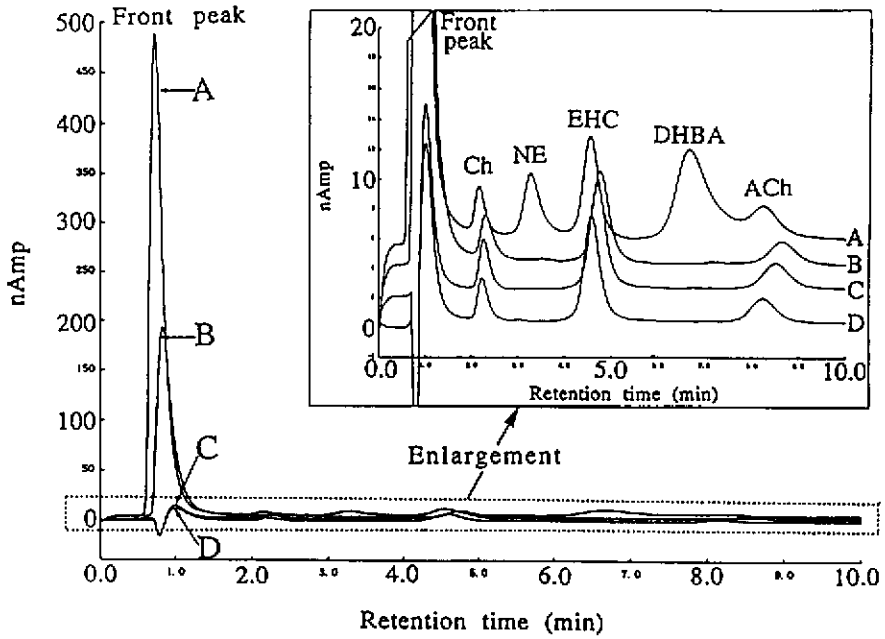

Fig. 4. Effect of the size of the glassy carbon pre-column on the solvent front peak size. Chromatograms were obtained from the injection of a 10-μl aliquot of rat hypothalamus homogenate into the LC–ED system designed for the determination of ACh and Ch. The dimensions of the glassy carbon pre-columns employed were none (A), 3 mm × 3 mm I.D. (B), 3.4 mm × 4 mm I.D. (C), and 10 mm × 4 mm I.D. (D).

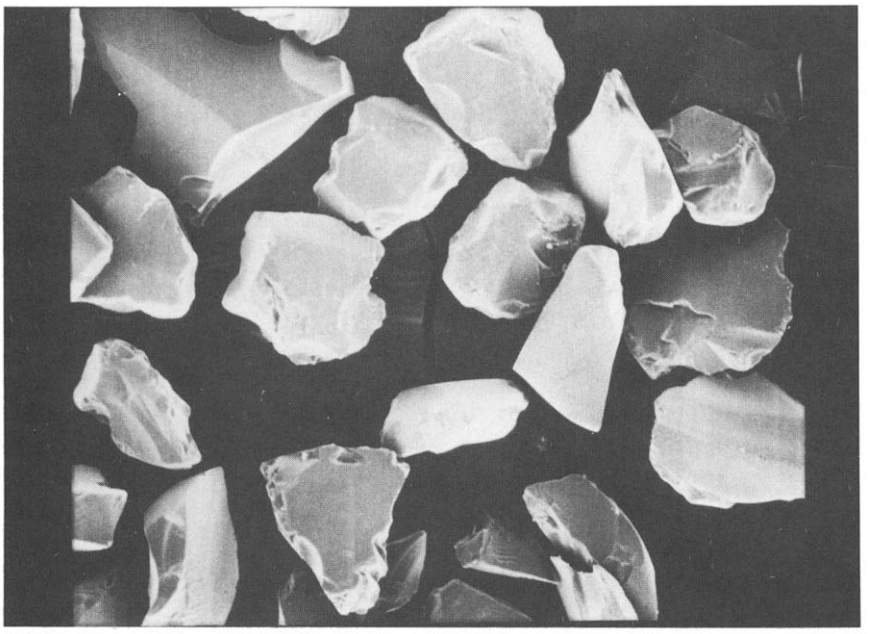

Fig. 5. Scanning electron micrograph of glassy carbon particles used to prepare glassy carbon pre-columns for the direct determination of ACh and Ch. The entire photograph shown is 280 μm in height and 490 μm in width; the individual particles have characteristic dimensions of 73 ± 24 μm (mean ± S.D.), with individual measurements ranging from 34 to 138 μm.

containing species over that for quaternary amine-related species. An electron micrograph of the glassy carbon particles used as pre-column packing materials in the current investigation, shown in Fig. 5, reveals an irregular shape with a typical dimension of 73 ± 24 μm (mean ± S.D., n = 39).

Glassy carbon pre-columns for ACh/Ch systems may thus be used for at least two purposes. First, they substantially decrease the required amount of sample preparation time by allowing the more direct injection of perchloric acid extracts of tissue samples. Secondly, they allow the same perchloric acid extract to be employed for both the ACh/Ch determination and, separately, for the determination of catecholamines, indoleamines and related compounds. The chromatographic time required per sample is decreased in the analysis of ACh and Ch when employing the glassy carbon pre-columns by approximately 50%, from 20 to 10 min. And, the decrease in the associated solvent front peak obtained with the glassy carbon pre-columns provides better precision in the determination of early-eluting components like Ch.

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REFERENCES

1 P. E. Potter, J. L. Meek and N. H. Neff, *J. Neurochem.*, 41 (1984) 188.
2 Y. Ikarashi, T. Sasahara and Y. Maruyama, *J. Chromatogr.*, 322 (1985) 191.
3 Y. Ikarashi, C. L. Blank, K. Itoh, H. Satoh, H. K. Inoue and Y. Maruyama, *Folia Pharmacol. Jpn.*, 97 (1991) 51.
4 N. Kaneda and T. Nagatsu, *J. Chromatogr.*, 341 (1985) 23.
5 P. Boer, B. H. C. Westerink and A. S. Horn, *Neurosci. Lett.*, 116 (1990) 357.

6 N. Bertrand, J. Bralet and A. Beley, *J. Neurochem.*, 55 (1990) 27.
7 M. Matsumoto, H. Togashi, M. Yoshioka, M. Hirokami, K. Morii and H. Saito, *J. Chromatogr.*, 526 (1990) 1.
8 Y. Maruyama, Y. Ikarashi and C. L. Blank, *Trends Pharmacol. Sci.*, 8 (1987) 84.
9 A. Beley, A. Zakhnini, S. Lartillot, D. Fage and J. Bralet, *J. Liq. Chromatogr.*, 10 (1987) 2977.
10 Y. Ikarashi, Y. Maruyama and W. B. Stavinoha, *Jpn. J. Pharmacol.*, 35 (1984) 371.
11 M. Asano, T. Miyauchi, T. Kato, K. Fujumori and K. Yamamoto, *J. Liq. Chromatogr.*, 9 (1986) 199.
12 Y. Ikarashi and Y. Maruyama, *Biogenic Amines*, 1 (1984) 341.
13 Y. Ikarashi and Y. Maruyama, *Biogenic Amines*, 2 (1985) 101.
14 J. Glowinski and L. L. Iversen, *J. Neurochem.*, 13 (1966) 655.
15 Y. Ikarashi, K. Itoh and Y. Maruyama, *Biol. Mass Spectrom.*, 20 (1991) 21.
16 S. Yamada, *A Review of Glass-like Carbons*, Defense Ceramic Center, Battelle Memorial Institute, Columbus, OH, 1968.
17 G. M. Jenkins and K. Kawamura, *Polymeric Carbons*, Cambridge University Press, London, 1976.