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Application of a novel, plastic formed carbon as a precolumn packing material for the liquid chromatographic determination of acetylcholine and choline in biological samples

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Abstract

A novel carbon material, plastic formed carbon (PFC), was prepared by mixing various amounts of pure graphite with an organic binder and pyrolysing the mixture to a "glassy carbon" at a modest final temperature of 1000–1400°C. This preparation procedure allows more convenient and precise control of the final graphite adsorption characteristics. Various PFC materials were constructed and tested both as bulk adsorbents and as precolumn packings for the direct determination of ACh and Ch in brain tissue homogenates. The PFC precolumns prepared from 12.5–50% graphite, by mass, were capable of selectively removing interfering species while not adsorbing any of the desired quaternary amine analytes. The usually large solvent front was also dramatically reduced with these precolumns. These PFC precolumns are useful for the direct determination of ACh and Ch in brain tissue homogenates and other biological samples.

1. Introduction

In the determination of acetylcholine (ACh) and choline (Ch) in brain tissue homogenates using systems based on reversed-phase ion-pair liquid chromatography with electrochemical detection (LC–ED), it is well known that catecholamines (CAs), in particular, interfere owing to the chromatographic overlap of these compounds with the targeted quaternary amines [1,2]. We recently reported that such interfering species could be effectively eliminated through the use of precolumns packed with glassy carbon particles prepared at a final curing temperature of 3000°C. The precolumn selectively removed CAs, indoleamines and related metabolites while not adsorbing any of the quaternary amines being analysed [3]. Further studies on the mechanism of such selective adsorption properties revealed that the adsorption sites of the glassy carbon were predominantly graphite-like domains, the number of which was enhanced by increasing the final curing temperature to 3000°C [4]. However, since the formation of the

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graphite-like domains is dependent on the heating process(es), it is difficult to prepare reproducibly glassy carbon particles containing constant levels of the critical adsorption sites.

Recently, a novel carbon material called plastic formed carbon (PFC) has been reported [5,6]. This material is prepared by mixing various amounts of pure graphite with an organic binder and then pyrolysing the mixture to form a "glassy carbon" at a relatively modest curing temperature of 1400°C. Formation of additional graphite-like adsorption sites is not observed as a result of heating at this relatively low temperature; any graphite-like domains in such a low-temperature process arise exclusively from the graphite added originally and not from the heating process. Thus, it is easier to control the final graphite adsorption site content of the product by simply controlling the fraction of the originally added pure graphite. The PFC materials were expected to be suitable replacements for the previously employed glassy carbon as precolumn adsorbents for, in particular, interfering CAs.

In this investigation, we initially examined the adsorption properties of the PFC particles towards both CAs and three quaternary amines related to ACh in a batch mode. We then constructed and examined precolumns packed with PFC particles prepared from various initial concentrations of graphite to assess the applicability of such precolumns in the direct LC–ED determination of ACh and Ch.

2. Experimental

2.1. Reagents

The following chemicals were purchased from Sigma (St. Louis, MO, USA): norepinephrine (NE) hydrochloride, dopamine (DA) hydrobromide, 3,4-dihydroxybenzylamine (DHBA) hydrobromide, acetylcholine (ACh) chloride and choline (Ch) chloride. Ethylhomocholine (EHC) was prepared as described previously [7]. Chemicals used in the LC–ED eluents were obtained at the highest available purity from various manufacturers.

2.2. PFC materials

Plastic formed carbon (PFC) materials initially containing 0–75% pure graphite, by mass, were prepared by mixing 0, 12.5, 25, 50 or 75% pure graphite with a vinyl chloride resin and pyrolysing the mixture to form a "glassy carbon" at a final annealing temperature of 1400°C [5,6]. These materials were transformed into smaller particles by an impact crusher. Particles in the 100–200-mesh range were collected by sieving and washed five separate times with a volume of acetone. A typical scanning electron micrograph for the 50% graphite PFC particles, which reveals irregular shapes with typical maximum dimensions of 74–149 μm, is shown in Fig. 1. For the investigations with the precolumns, approximately 100 mg of the PFC particles were

Fig. 1. Scanning electron micrograph of 50% graphite PFC particles. The individual particles are irregular in shape and exhibit characteristic dimensions in the range 74–149 μm.

suspended in acetone and packed in a stainless-steel column (10 mm × 4 mm I.D.) using conventional slurry packing procedures.

2.3. LC–ED system for determination of ACh and Ch

The LC system employed for the determination of ACh, Ch and EHC consisted of an LC100P pump, an LC100S injector and an LC100W/F work station for data processing from Yokogawa (Tokyo, Japan). Additional components, obtained from Bioanalytical Systems Japan (BAS Japan, Tokyo, Japan), included an LC-4A amperometric detector with a dual platinum working electrode (E_{app} = +0.500 V vs. Ag/AgCl), an LC-22A temperature controller (35 ± 1°C), an Acetylcholine Separation column (3 μm, 60 × 4 mm I.D., polymeric styrene-based packing material, Catalogue No. 51-5764) and an immobilized postcolumn enzyme reactor (5 × 4 mm I.D.) containing acetylcholinesterase and choline oxidase. The mobile phase was 0.050 *M* phosphate buffer (pH 8.40) containing 1.0 m*M* disodium ethylenediaminetetraacetate (EDTA) and 0.40 m*M* sodium 1-octanesulfonate; the flow-rate was 0.70 ml/min.

2.4. LC–ED system for determination of catecholamines

The liquid chromatographic system employed for the determination of NE, DA and DHBA primarily employed components from BAS Japan. These included a PM-60 pump, a CC-4 injector, a BioPhase ODS IV analytical column (3 μm, 110 × 4.6 mm I.D., Catalogue No. 51-6034), a dual glassy carbon electrode (E_{app} = +0.80 V vs. Ag/AgCl) and an LC-4B amperometric potentiostat. The temperature of the column was maintained at 35 ± 1°C by an LC-22 temperature controller. The mobile phase was a 0.050 *M* citrate buffer (pH 3.2), containing 0.80 m*M* sodium 1-octanesulfonate and 0.50 m*M* disodium EDTA. The flow-rate was typically 0.50 ml/min. Data collection and processing were accomplished with the aid of an LC100W/F work station (Yokogawa).

2.5. Batch studies of adsorption

Two separate sets of batch experiments were performed to examine the binding characteristics of the neurochemicals of concern. For the catecholamines, a 1.00-ml aliquot of 0.10 *M* phosphate buffer (pH 8.40), containing 5.0 nmol each of NE, DHBA and DA was added to 50 mg of the PFC particles. For the quaternary amines, a 1.00-ml aliquot of the same buffer containing 5.0 nmol each of ACh, Ch and EHC was added to 50 mg of the PFC particles. In both cases, the mixture was shaken on a vortex mixer for a few seconds. The samples were individually filtered through a 0.45-μm Millipore filter and a 4.0-μl aliquot of the filtrate was injected into the appropriate LC–ED system for quantification. The results for each substance were compared with those obtained from equivalent mixtures which had not been exposed to any PFC particles.

2.6. PFC precolumn investigations

When employed, the PFC precolumn was inserted into the LC–ED system, described above for the determination of ACh and Ch, between the injection port and the analytical column in the flow path of the eluting solvent. A 10-μl aliquot of a 1.0-ml solution of 0.10 *M* phosphate buffer (pH 8.40), containing 5.0 nmol each of ACh, Ch, EHC and NE, 10 nmol of DHBA and 20 nmol of DA was injected into the complete system. The results obtained with the PFC precolumn were compared with those obtained without the precolumn.

2.7. Use of the PFC precolumn for determination of rat striatal ACh and Ch

Male Wistar rats, 9 weeks of age and weighing 200–220 g at the time of use, were obtained from the Institute of Experimental Animal Research at Gunma University. The animals were killed by exposure, concentrated on the head region, 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) [8]. The brains were quickly removed from the skulls of the animals and subsequently dissected into seven brain regions. The striatal tissue was placed into 1.00 ml of a 0.050 *M* perchloric acid solution containing 1.00 nmol of EHC, the internal standard for the ACh and Ch determinations. Homogenization of the tissue was accomplished with a Model US-300T ultrasonic cell disrupter 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. Finally, the supernatant was purified by passage through a 0.45-μM Millipore filter. Aliquots, typically 10 μl, of the filtrate were injected into the ACh/Ch LC–ED system both with and without an added PFC precolumn.

3. Results and discussion

The results of the batch studies are shown in Fig. 2. As can be seen, no measurable amounts

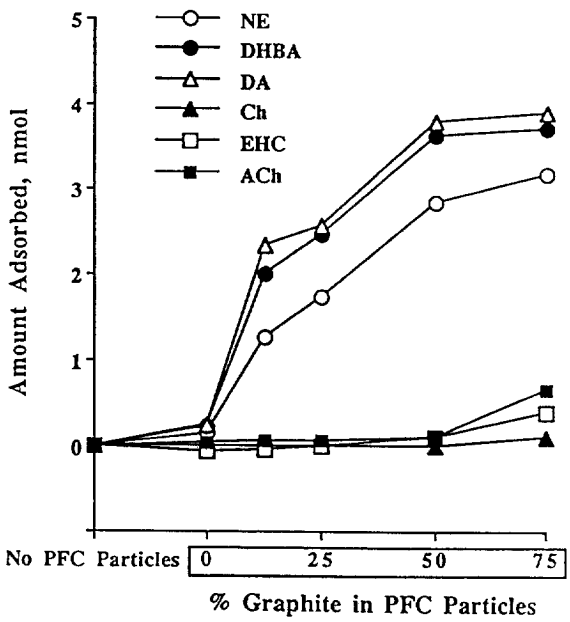

Fig. 2. Batch studies of adsorption of neurochemicals on PFC particles prepared with various amounts of graphite. Points represent the means of duplicate measurements. The total amount of neurochemical exposed to the particles was 5 nmol in each case; the total amount of particles used for each experiment was 50 mg.

of any of the compounds tested were adsorbed on the 0% graphite PFC particles prepared at the modest annealing temperature of 1400°C. This result is in complete agreement with our previous report showing no adsorption of these neurochemicals on glassy carbons prepared with a final annealing temperature of only 1000–1400°C [4]. Both of these results, combined, support the lack of formation, or at most very minimal formation, of graphite-like adsorption sites due to heating when the final annealing temperature is maintained at ≤1400°C. Exposure of the neurochemical-containing solutions to PFC particles prepared with increasing amounts of graphite, on the other hand, show the expected increase in adsorption for the catecholamines, with the maximum adsorption corresponding to the maximum graphite content of 75%. The quaternary amines (ACh, Ch, and EHC) revealed no adsorption on any of the PFC particles containing up to 50% graphite; however, in the 75% graphite PFC particles, significant adsorption of these three species was observed (13% of ACh, 4% of Ch and 15% of EHC). In general, these results suggested that a carefully selected graphite content in the PFC particles would, indeed, be applicable for use as a packing material in the precolumn for ACh and Ch determinations. The capacity of 50 mg of the 50% graphite PFC particles for catecholamines of concern, as seen in Fig. 2, is approximately 3–4 nmol. This capacity represents approximately 60- and 1000-fold larger amounts of DA and NE, respectively, than those found in a 10-μl injection sample of rat striatal homogenate [2].

We next constructed and incorporated a precolumn containing these PFC materials into the LC–ED system designed for the ACh/Ch analyses. A fixed amount of a six compound mixture was injected into the combined unit. The results are shown in Fig. 3. Typical chromatograms obtained from systems with PFC precolumns containing 0, 50 and 75% graphite particles are presented in Fig. 4. The catechols were adsorbed virtually completely on each of the 12.5, 25 and 50% graphite PFC precolumns whereas, simultaneously, no perceptible adsorption of any of

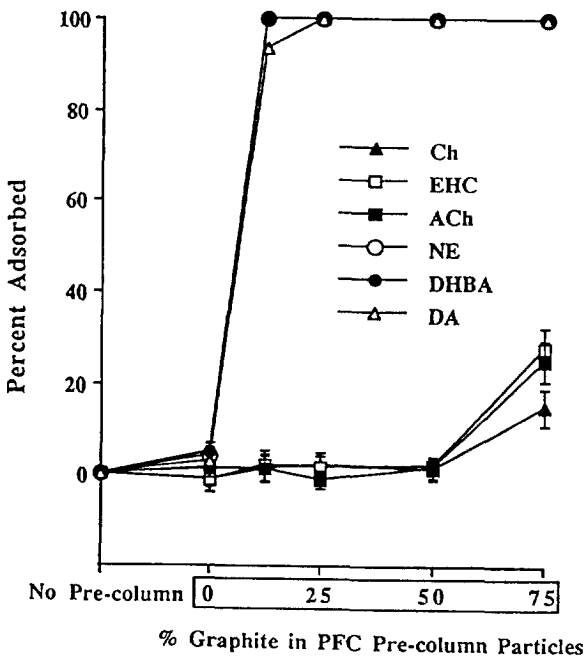

Fig. 3. Adsorption of neurochemicals by precolumns packed with PFC particles containing various amounts of graphite. Data points are means ± S.E. of five determinations.

the quaternary amines was observed in any of these cases. The 75% graphite PFC precolumn also absorbed all of the catechols; however, this precolumn also adsorbed noticeable amounts of the three targeted quaternary amines (25% of ACh, 15% of Ch and 28% of EHC). From these results, the 12.5, 25 and 50% graphite PFC precolumns were deemed applicable to the routine analysis of ACh and Ch, with the latter two being preferred. Precolumns containing graphite contents exceeding 50%, however, are not recommended for this purpose. Although the precolumns with 75% graphite clearly contain more adsorption sites, and thus have a greater capacity for adsorption of interferents, such precolumns also demonstrate the undesired adsorption of the targeted analytes.

The chromatograms shown in Fig. 5 were both obtained from the injection of 10-μl aliquots of rat striatal homogenates. The ACh/Ch LC–ED system employed either (A) did not or (B) did contain a 50% graphite PFC precolumn. The chromatograms obtained without the precolumn clearly show interferences afforded by the co-eluting CAs. NE partially overlaps the EHC peak, while DHBA partially overlaps the ACh peak. Notably, the presence of the DA peak ($t_R \approx 29$ min) effectively alters the single sample analysis time from 15 min to ca. 35 min. When an equal volume of the striatal sample was injected into the CA LC–ED system, the amounts of NE and DA in the 10-μl aliquots were determined to be 3.2 and 60.3 nmol,

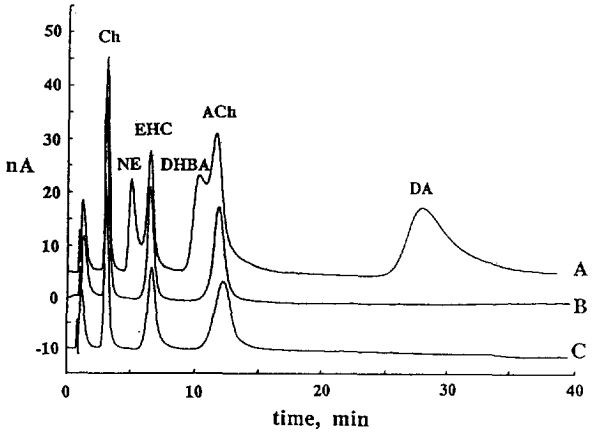

Fig. 4. Typical chromatograms obtained from ACh/Ch LC–ED systems having graphite PFC precolumns. The percentage of graphite used in the preparation of the PFC particles used to pack the pre-columns was (A) 0, (B) 50 and (C) 75%.

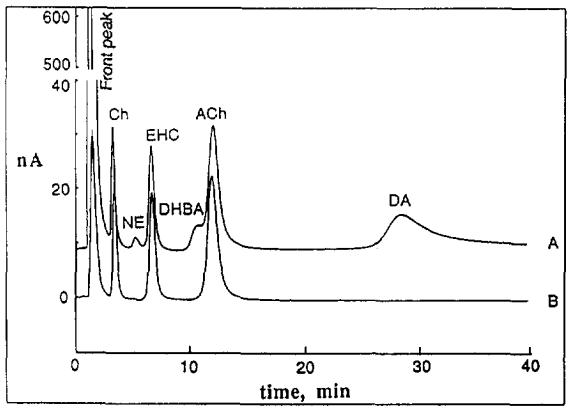

Fig. 5. Chromatograms obtained from a 10-μl injection of rat striatal homogenate, (A) without and (B) with the use of a 50% graphite PFC precolumn in the ACh/Ch LC–ED system.

respectively. We also investigated a more extensive mixture of components containing DA, NE, DOPAC, HVA, 5HT, 5HIAA, ACh, Ch and the internal standards DHBA, nMET and EHC, all at concentrations corresponding to those found in or appropriate to a rat striatal tissue homogenate; injection of 10 μl of such a mixture revealed a chromatogram indistinguishable from that seen in Fig. 5A, indicating that only the catecholamines (NE, DA, and DHBA) provided interference under normal circumstances. In all cases, insertion of the precolumn into the flow stream completely eliminated the three catecholamine peaks in the ACh/Ch LC–ED system. Further, the solvent front was dramatically reduced by the precolumn, from over 600 nA to ca. 30 nA. Previous studies with other carbon materials have demonstrated similar absorptive properties for many of these neurochemicals [3,4]. Hence, this solvent front reduction is presumably due to the adsorptive removal of many non-retained, electrochemically active compounds, which allows for much more precise assessment of the peak characteristics for the early-eluting compounds of concern, particularly Ch. These selective adsorption properties for the 50% graphite PFC precolumn described were found to be undiminished following more than 50 injections of striatal samples.

Generally, rat brain tissue homogenates must be subjected to precipitation or some other form of pretreatment prior to injection into an ACh/Ch LC–ED system. Precipitation with a Reinecke salt [1], precipitation with KI–I_2 [9] and solvent extraction [10] have all provided adequate results via such pretreatment. However, such sample purification is time consuming and may suffer from loss of the targeted species due to incomplete recoveries. Thus, direct injection of tissue homogenates and other biologically related samples is clearly desirable. The present investigation demonstrates that the use of 12.5–50% graphite PFC precolumns is entirely appropriate to the direct determination of brain ACh and Ch. These precolumns effectively adsorb interferents in the ACh/Ch LC–ED assay. Further, the PFC materials are easier to produce and more amenable to quality control than the previously reported glassy carbon materials [2–4].

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References

[1] P.E. Potter, J.L. Meek and N.H. Neff, J. Neurochem., 41 (1983) 188.
[2] Y. Ikarashi, H. Iwatsuki, C.L. Blank and Y. Maruyama, J. Chromatogr., 575 (1992) 29.
[3] Y. Ikarashi, C.L. Blank and Y. Maruyama, J. Chromatogr., 645 (1993) 219.
[4] Y. Ikarashi, C.L. Blank, T. Kawakubo, Y. Suda and Y. Maruyama, J. Liq. Chromatogr., 17 (1994) 287.
[5] T. Kawakubo, Y. Suda, H. Kaneko, A. Negishi and M. Yamada, Tanso, 152 (1992) 106.
[6] H. Kaneko, A. Negishi, Y. Suda and T. Kawakubo, Denki Kagaku, 61 (1993) 920.
[7] Y. Ikarashi, T. Sasahara and Y. Maruyama, Folia Pharamcol. Jpn., 84 (1984) 529.
[8] Y. Ikarashi, Y. Maruyama and W.B. Stavinoha, Jpn. J. Pharmacol., 35 (1984) 371.
[9] Y. Maruyama, M. Kusaka, J. Mori, A. Horikawa and Y. Hasegawa, J. Chromatogr., 164 (1979) 121.
[10] A. Beley, A. Zakhnini, S. Lartillot, D. Fage and J. Bralet, J. Liq. Chromatogr., 10 (1987) 2977.