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Letter to the Editor

Detection of choline and acetylcholine by high-performance liquid chromatography

Limitations, pitfalls, sample preparation

Sir,

The determination of choline esters [1] has received a new impulse from the introduction of a high-performance liquid chromatographic (HPLC) method with electrochemical detection, first described by Potter and co-workers [2,3]. The two enzymes acetylcholinesterase (AChE) and choline oxidase (ChO) used to generate electrochemically detectable hydrogen peroxide were later immobilized in a short post-column reactor [4–9]. The selectivity of this method is achieved in three ways: (1) by the number of theoretical plates of the cation-exchange separation column, (2) by the selectivity of the immobilized enzymes in the post-column reactor and (3) by the low oxidation potential of the electrochemical detector (+500 mV using a platinum electrode and an Ag/AgCl reference electrode) at which only few other substances are also taken up that could possibly interfere.

A major drawback of the originally described methods was the very short lifetime of the usually silica-based cation-exchange separation column under the assay conditions [silica is dissolved at alkaline pH (>7.2)] [7]. This paper compares the performance of commercially available cation-exchange columns and describes some experience in working with the HPLC method for the detection of choline and acetylcholine.

HPLC SYSTEM

The HPLC system consisted of a constant-flow pump (Model 600/200; Gynkotek, Munich, F.R.G.) and as an electrochemical detector either an ELDEC 102 (Chromatofield, Chateauneuf-les-Martiguez, France) or a model M 20 (Gynkotek). The platinum electrode, the detection chamber and the enzyme reactor with the immobilized enzymes were manufactured by Biometra (Göttingen, F.R.G.) [9]. The running buffer was 100 mmol/l phosphate buffer (pH 7.6) containing 7 mmol/l tetramethylammonium perchlorate (TMA). The flow-rate was varied between 0.5 and 1.5 ml/min.

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CATION SEPARATION COLUMNS

The cation-exchange separation columns tested are listed in Table I. In the first few days of use the Nucleosil columns provided the highest number of theoretical plates. Four Nucleosil columns were tested: three Nucleosil 5 SA (packed by Macherey-Nagel, Düren, F.R.G.) and one Nucleosil 5 SA (packed by Gynkotek). The best results were obtained with the column packed by Gynkotek. However, the performance of all the columns decreased after a few days, so that the assay became inadequate at a very early stage after starting the system (Fig. 1A). The lifetime never exceeded 10 days, the columns suddenly becoming completely blocked owing to the dissolution of the silica-based Nucleosil at pH 7.6 (see above). This pH of the running buffer was a compromise: a more acidic pH would have prevented dissolution of the silica, but would not have been tolerated by the enzymes in the enzyme reactor; on the other hand, an even more alkaline pH range (8.0–8.5) would have been optimum for the enzymatic reaction [10]. An acceptable retention time of < 10 min for acetylcholine could only be obtained with high flow-rates of 1.2–1.5 ml/min (Table I) at an extremely high back-pressure (> 150 bar). With such flow-rates, the lifetimes of the columns were even shorter.

Excellent results were obtained with Hamilton PRP-X 200 columns [a macroporous poly(styrene–divinylbenzene) copolymer; Hamilton, Reno, NV, U.S.A.]. Both of the columns tested could still be used after 4 weeks. During that time the performance of the columns did not decrease noticeably. The peaks were sharp and the retention times on the short column (No. 6, Table I) were 3.2 min for choline and 8.8 min for acetylcholine at a flow-rate of 0.7 ml/min (Fig. 1B). Under these conditions choline could still be distinguished from the solvent peak. With the long Hamilton PRP X-200 column (No. 5, Table I) the retention times were 3.1 min for choline and 11.3 min for acetylcholine at a flow-rate of 1.5 ml/min. Even under these conditions the back-pressure was very low (33 bar).

No separation of choline and acetylcholine could be achieved with the Bio-Gel TSK-SP-5PW column (No. 7, Table I) or the Microanalyzer MA7C cartridge (No. 8, Table I) (both supplied by Bio-Rad Labs., Munich, F.R.G.). On the Bio-Gel TSK-SP-5PW column only one peak with a retention time of 7.3 min at a flow-rate of 0.5 ml/min appeared for both substances. This peak could be shifted by varying the ionic strength of the running buffer between 10 and 100 mmol/l phosphate or by changing the flow-rate from 0.5 up to 1.5 ml/min. A separation of the two substances could never be achieved, and the peak height and peak area depended on the amount of choline and/or acetylcholine injected. This suggested that acetylcholine was cleaved on the column and detected as choline. With the Microanalyzer MA7C cartridge the choline esters appeared in the solvent peak, indicating that they were not retarded on the column.

DETECTION LIMIT

The detection limit (three times the signal-to-noise ratio) depended on the sample volume, on the cation separation column and on the age of the enzyme reactor. Increasing the injection volume fivefold (from 10 to 50 μl) resulted in only a 3.5-fold decrease in the relative detection limit owing to an increase in the peak width. The

TABLE I

COLUMN SPECIFICATIONS, RETENTION TIMES AND NUMBER OF THEORETICAL PLATES FOR THE CATION-EXCHANGE SEPARATION COLUMNS TESTED

No.	Column	Dimensions (length × I.D.) (mm)	Lifetime (days)	Flow-rate (ml/min)	Choline				Acetylcholine			
					Retention time (min)	Peak width (mm)	Theoretical plates		Retention time (min)	Peak width (mm)	Theoretical plates	
							Per column	Per m			Per column	Per m
1	Nucleosil 5 SA (Macherey, Nagel & Co.)	200 × 4.0	<10									
				After 4 days of use:								
				1.0	4.0	4.3	479	2397	15.7	7.1	2723	13614
2	Nucleosil 5 SA (Macherey, Nagel & Co.)	125 × 4.6	<10	1.0	8.3	5.5	1256	10045	17.0	8.2	2376	19004
3	Nucleosil 5 SA (Macherey, Nagel & Co.)	125 × 4.0	<10	1.2	5.4	3.6	1270	10158	10.2	5.7	1784	14276
4	Nucleosil 5 SA (Gynkotek)	125 × 4.6	<10	1.5	6.6	3.5	1958	15664	13.5	6.2	2627	21013
				8 days later:								
				1.5	5.7	6.2	468	3746	11.5	9.1	885	7078
5	PRP-X 200 (Hamilton)	250 × 4.1	>28	0.7	6.7	6.1	670	2683	25.2	24.0	611	2443
				1.5	3.2	3.7	404	1616	12.2	11.4	638	2550
6	PRP-X 200 (Hamilton)	150 × 4.1	>28	0.7	3.2	3.8	388	2586	8.8	6.0	1194	7963
7	Bio-Gel TSK-SP-5PW (Bio-Rad Labs.)	75 × 4.6	—[a]	0.5	7.3	18.0	263	3509	7.3	18.0	263	3509
					No separation between choline and acetylcholine							
8	Microanalyzer MA7C (Bio-Rad Labs.)	30 × 4.6	—[a]									
					No separation of sample peak from solvent peak							

[a] Lifetimes cannot be given for columns 7 and 8 as they were only used for 2–3 days to test the separation of choline and acetylcholine. By that time no decrease in performance or in the number of theoretical plates was noticed.

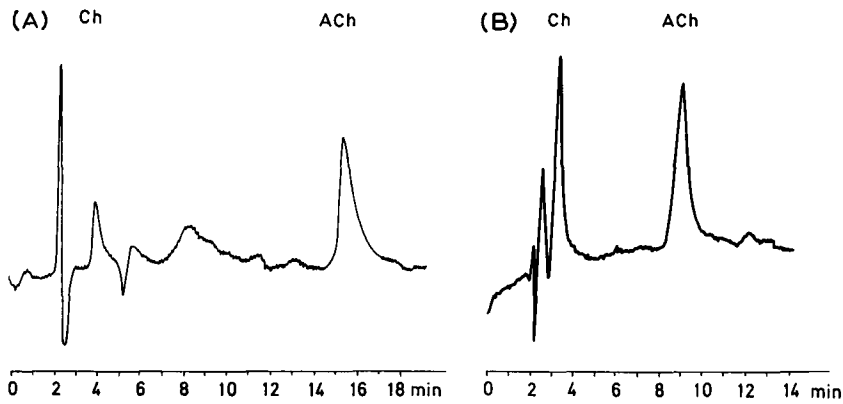

Fig. 1. Separation of choline and acetylcholine on various cation-exchange columns. (A) Nucleosil 5 SA column. 125 pmol of choline and 125 pmol of acetylcholine were injected in a sample volume of 10 μl onto a Nucleosil 5 SA column (No. 1, Table I). The column was used for 4 days continuously. Oxidation potential, +500 mV; detector sensitivity, 0.5 nA/V (full-scale); running buffer, 100 mmol phosphate buffer (pH 7.6) containing 7 mmol/l tetramethylammonium perchlorate; flow-rate, 1.0 ml/min. (B) Hamilton PRP-X 200 column. 266 pmol of choline and 414 pmol of acetylcholine were injected in a sample volume of 20 μl onto a Hamilton PRP-X 200 column (No. 7, Table I). The column was used for 6 days continuously. Oxidation potential, +500 mV; detector sensitivity, 0.02 nA per 10 mV (full-scale); running buffer, as in (A); flow-rate, 0.7 ml/min.

method was 4–8 times more sensitive for choline than for acetylcholine. With the Nucleosil 5 SA column the absolute detection limit was 9 pmol of choline and 39 pmol of acetylcholine in a 10-μl injection volume; with the Hamilton PRP-X 200 column it was 11 pmol of choline and 85 pmol of acetylcholine in a 20-μl injection volume. Shortly before the enzyme reactor broke down the detection limit increased.

SAMPLE PREPARATION

Deproteinization with perchloric acid is usually sufficient for sample preparation [11]. However, an extraction procedure may be necessary to increase the sample concentration of acetylcholine and/or to remove acetylcholine esterase inhibitors [12]. We modified the ion-pair extraction with dipicrylamine (DPA) as described by Eksborg and Persson [13,14] to obtain free acetylcholine for HPLC with electrochemical detection. The specimens were shaken twice for 8 min in a vertical shaker with 5 ml of $1.2 \cdot 10^{-3}$ mol/l DPA solution in dichloromethane followed by centrifugation for 10 min at 4°C. The aqueous phase was discarded, the combined organic phases were evaporated to dryness under a stream of nitrogen and the residue was resuspended in 1 ml of Bio-Rex-9 (Bio-Rad Labs.) in methanol. After sedimentation of the anion-exchange resin, the supernatant was transferred to a new tube, in which the methanol was evaporated at 37°C under a stream of nitrogen. The residue was resuspended in 25 μl of HPLC running buffer.

Choline and/or acetylcholine dissolved in Krebs–Ringer hydrogencarbonate solution (pH 7.4) used for perfusion of isolated guinea-pig hearts were thus treated. Acetylcholine could be concentrated by factor of 4; with an injection volume of 20 μl

the acetylcholine concentration of a 1.1 μmol/l solution could still be detected. Under the conditions described here, choline was extracted to only a very limited extent, resulting in its almost complete loss. The procedure is fast and simple to perform (ten samples within 2 h).

CONCLUSIONS

The HPLC method with electrochemical detection for the detection of choline esters is a very suitable quantitative method for routine analyses. It provides the highest selectivity of all currently available methods. The total amount of acetylcholine necessary for detection is in the range of that in other methods, *e.g.*, the radio-enzyme assay of Goldberg and McCaman [15] and McCaman and Stetzler [16] or the isotachophoretic method of Haen *et al.* [17], with the advantage of easier handling and possible automation. Not all cation-exchange separation columns are suitable. Silica-based columns yield optimum separations of choline esters within the first 3–4 days of use, but deteriorate under the assay conditions (pH 7.6), having a lifetime of less than 10 days. Reports in the literature recommend repacking these columns every week [10]. This situation is very unfortunate, as the sensitivity of the electrochemical detector and the stability of its baseline increase over 3–4 days. The detection limit depends on the sample volume, on the age of the enzyme reactor and on the cation separation column. The best results were obtained with Hamilton PRP-X 200 columns. With an HPLC injection volume of 20 μl, the absolute detection limit for acetylcholine is 85 pmol. The method is about 4–8 times more sensitive for choline than for acetylcholine. Enrichment of the acetylcholine concentration is possible by ion-pair extraction using dipicrylamine, with an enrichment by a factor of 4.

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Walther-Straub-Institute of Pharmacology and Toxicology, University of Munich, Nussbaumstr. 26, D-8000 Munich 2 (F.R.G.) E. HAEN*

Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen (F.R.G.) H. HAGENMAIER

Walther-Straub-Institute of Pharmacology and Toxicology, University of Munich, Nussbaumstr. 26, D-8000 Munich 2 (F.R.G.) J. REMIEN

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