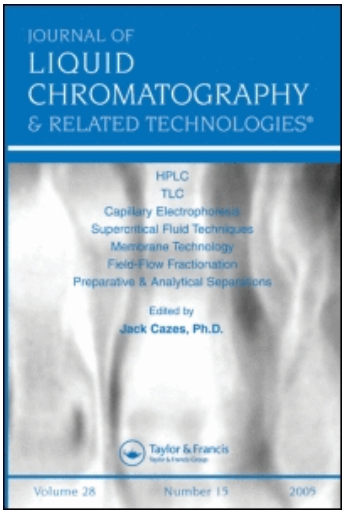

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PURIFICATION OF AN ENDOGENOUS MODULATOR FOR VOLTAGE-SENSITIVE CALCIUM CHANNELS USING THE ECCENTRIC MULTI-LAYER COIL PLANET CENTRIFUGE

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ABSTRACT

High-speed countercurrent chromatography using a two-phase solvent system was found to be the method of choice for purifying an endogenous Ca^{2+} channel modulator. The solvent system composed of n-butanol, acetic acid and water (4:1:5) was most suitable for resolving the active material from other unknown substances of low molecular weight. Countercurrent chromatography proved useful in validating the presence in rat brain of an endogenous ligand for dihydropyridine-sensitive Ca^{2+} channels. Moreover, the degree of purity of the active material obtained by countercurrent chromatography is suitable for consecutive analysis by mass spectrometry or nuclear magnetic resonance.

INTRODUCTION

Dihydropyridines regulate a membrane depolarization evoked entry of Ca^{2+} into smooth muscle, cardiac muscle and neuronal cells. In smooth and

cardiac muscle cells specific binding sites of dihydropyridines constitute a component of the Ca^{2+} channel complex (1-2). This was demonstrated by showing that cloning of the complementary DNA of the dihydropyridine receptor and injection of cDNA-derived messenger-RNA into xenopus oocytes led to the formation of functional dihydropyridine-sensitive Ca^{2+} channels (3).

The finding showing that dihydropyridines do not merely bind to a drug receptor sites on the surface of cell membranes but also to a physiological receptor site triggered our interest in searching for an endogenous ligand that interacts with dihydropyridine receptor sites and modulates Ca^{2+} channel activity. We have reported evidence for the presence in rat brain tissue of a substance that appears to act as an allosteric modulator of dihydropyridine binding sites (4-5) and an endogenous modulator of voltage-sensitive Ca^{2+} channels (6-7). Recently, Ebersole and coworkers (8) also described the isolation of an endogenous ligand for dihydropyridine binding sites in brain tissue. But it is not clear whether their endogenous inhibitor of dihydropyridine binding also interacts with the dihydropyridine-sensitive Ca^{2+} channels.

The elucidiation of the chemical structure of this endogenous Ca^{2+} channel modulator has not yet been accomplished because of difficulties in the separation technique. We previously reported that purification of the endogenous Ca^{2+} channel modulator by C_{18} reverse phase and partisil silica HPLC resulted in a single peak at absorbance of 256 nm, but analysis of the eluate by Californium 252 plasma desorption mass spectrometry indicated the

presence of three substances with different mass numbers suggesting that the active material had not been purified to homogeneity (7). Moreover, when HPLC columns with a silica matrix were used in the purification bleeding of silica polymers off the columns occurred. The presence of these silica polymers obscured the interpretation of subsequent mass spectrometric analysis.

In an attempt to improve purity of the substance we adopted countercurrent chromatography as a separation technique. One attractive feature of countercurrent chromatography that employs the planet centrifugal system recently developed by Ito and coworkers (9,12) is that impurities arising from conventional solid support used in high performance liquid chromatography are eliminated. Moreover, by using only liquid solvent systems, deactivation from solid adsorbance can be avoided. Several reports in the literature have shown that countercurrent chromatography may be used for preparative-scale purification of peptides and low molecular weight substances (9-11). The present report demonstrates that countercurrent chromatography provides a better separation of the endogenous modulator from contaminants coeluting from the partial column, increasing the yield of the purified endogenous ligand.

MATERIALS AND METHODS

Chemicals:

Methylene chloride, methanol and acetonitrile were obtained from Burdick Jackson Laboratories (Muskegon, MI) and n-butanol (chromatography

grade) from EM Science (Cherryhill, N.J.). Nifedipine was purchased from Sigma (St. Louis, MO) and ^{3}H-nitrendipine (specific activity: 177.4 ci/mmol) from New England Nuclear (Dupont, Wilmington, DE).

Isolation of Endogenous Ligand for ^{3}H-Nitrendipine Binding Sites from Rat Brain Tissue:

Specific binding of ^{3}H-nitrendipine to washed hippocampal membrane preparations was measured as described by Sanna and Hanbauer (4). Aliquots of membrane suspensions (150-200 μg protein) were incubated for 40 min. at 24°C in the presence of 50 mM Tris buffer (pH 7.5), 100 pM ^{3}H-nitrendipine, and aliquots of fractions to be tested for the presence of endogenous ligand. Nonspecific binding was measured in the presence of 2 μM nifedipine and was usually 15-25% of the total ^{3}H-nitrendipine bound. Inhibition of specific ^{3}H-nitrendipine binding was measured to determine the presence of endogenous ligand.

Partial purification of endogenous ligand was carried out as described by Hanbauer et al. (6). In brief, rat brains were homogenized in distilled water (10 v/w) and trichloroacetic acid was added (final concentration 3%) to precipitate bulk proteins. The supernatant fraction was washed four times with ether (1:4 v/v) and then freeze-dried. The dried residue was extracted into methanol and dried in a speed vac concentrator (Sargent). The residue was dissolved in distilled water, and loaded on C_{18} SEPPAK cartridges and the column washed with water. The effluent and water wash contained the total

^{3}H-nitrendipine binding-inhibitory activity and therefore were combined and freeze-dried. The residue was dissolved in a small volume of methanol, injected on partisil-10 silica HPLC column (4.6 mm x 250 mm; Whatman) that was equilibrated in 95% methylene chloride/5% methanol, and eluted with a linear gradient of 5% to 45% methanol in 40 min (flow rate: 1 ml/min; optical absorbance: 256 nm). The inhibitor of ^{3}H-nitrendipine binding was eluted as a single peak with 33% methanol/67% methylene chloride. After removal of the solvents by speed-vac centrifugation, the residue was further purified by countercurrent chromatography.

Countercurrent Chromatography:

Solvent Composition:

The solvent system that gave the optimal fractionation of the endogenous Ca^{2+} channel modulator was determined by measuring the partition coefficient (K) of the substance in various solvent systems.

Apparatus:

An eccentric multilayer coil planet centrifuge with a 10 cm revolution radius was used as described by Ito and Oka (12). In brief, four identical multilayer coils served as columns, and were symmetrically arranged around the coil holder shaft. Each column was prepared by winding PTFE tubing (1.6 mm ID) onto a hub to form six layers of coil with a capacity of about 50 ml. These columns were connected in series to hold a total volume of 200 ml.

Table 1

Meaurement of the Partition Coefficient in Various Solvent Systems for an Endogenous Ca^{2+} Channel Modulator

Solvent System (Ratio)	Endogenous Modulator Displacing Activity		Partition Coefficient ($K = C_U/C_L$)*
	UP	LP	
$CHCl_3 : CH_3OH : H_2O$ (5 : 5 : 3)	2.7	1.0	2.7
$EtOAc : H_2O$ (1 : 1)	0.92	3.5	0.26
$n\text{-}BuOH : H_2O$ (1 : 1)	0.98	1.8	0.54
$n\text{-}BuOH : CH_3COOH : H_2O$ (4 : 1 : 5)	1.3	1.7	0.76
$n\text{-}BuOH : TFA : H_2O$ (1 : 0.001 : 1)	1.0	2.1	0.47

*Solute concentration in the upper phase divided by that in the lower phase.

The lower aqueous phase was used as stationary phase. Partially purified endogenous ligand was dissolved in the aqueous phase and mixed with non-aqueous solvent until a two phase solvent system was obtained. The solution was injected into the column through the external end of the fourth column. The column was eluted by pumping the upper non-aqueous phase used as mobile phase into the external end of the fourth column. The fractionation was carried out at 800 rpm. The flow rate was 60 ml/hr using a Milton Roy

minipump. The optical absorbance of the eluate was monitored by an LKB Uvicord S at 254 nm and 3 ml/tube were collected with an LKB fraction collector.

RESULTS AND DISCUSSION

Ongoing research in our laboratories is aimed at purification of an endogenous Ca^{2+} channel modulator and analysis of its structure.

Measurements of the partition coefficients of partially-purified endogenous Ca^{2+} channel modulator in various solvent systems indicated that the best separation of the substance by countercurrent chromatography would be achieved by a two phase system formed from n-butanol/acetic acid/water (4:1:5, v/v/v) (Table 1).

Figure 1 shows that partially purified endogenous Ca^{2+} channel modulator, eluted from a Partisil 10 silica column can be further resolved from UV absorbing contaminants by countercurrent chromatography. The ^{3}H-nitrendipine binding-inhibiting activity was in fractions 90 to 110 and contributed the major absorbance peak at 256 nm.

Figure 2 shows progress of the purification by countercurrent chromatography as measured by chromatography on a Glyco PAK N HPLC column (Waters). The column was equilibrated in acetonitrile:H_2O (73:27) and eluted under isocratic conditions at a flow rate of 1 ml/min. at 256 nm. Fig. 2A shows that when material not purified by countercurrent chromatography was injected, the presence of contaminants prevented an efficient resolution of the peak (RT=13.8) containing the ^{3}H-nitrendipine

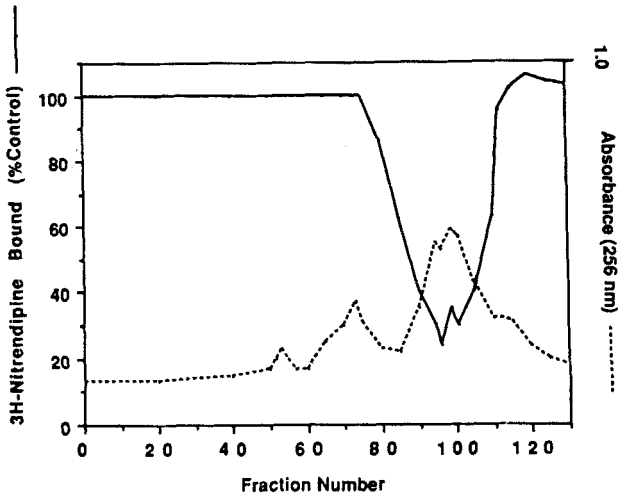

FIGURE 1. Countercurrent chromatography of an endogenous Ca^{2+} channel modulator isolated from rat brain tissue. Five mg. of partially purified sample was chromatographed with the upper, or non-aqueous phase on n-butanol/acetic acid/water (4:1:5) run as the mobile phase at 60 ml/hr. at a revolution of 800 rpm. The non-aqueous solvent front appeared in fraction 49. The retention of the stationary phase was 27%.

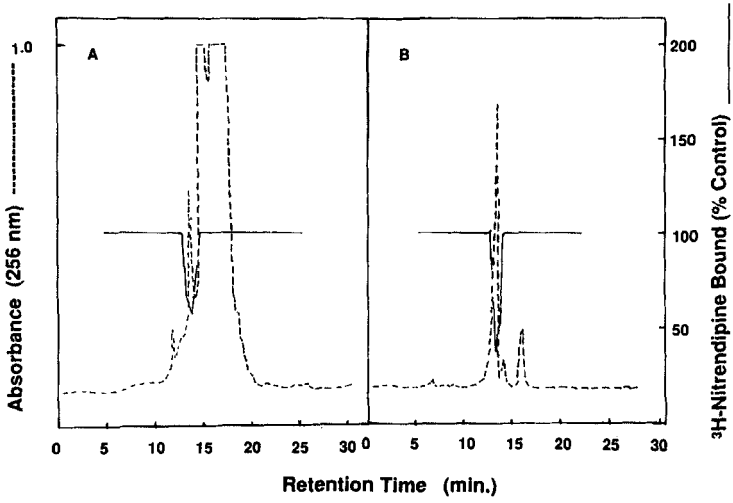

FIGURE 2. Separation of partially purified endogenous Ca^{2+} channel modulator by HPLC using a Glyco PAK-N column (4.6 mm x 25 cm; Waters Div., Millipore, Milford, MASS). The column was equilibrated in acetonitrile and water (73:27) and eluted with isocratic conditions. Flow rate: 1 ml/min.; UV: 256 nM, and a back pressure below 500 psi. **A**: Chromatography of sample before countercurrent chromatography, and, **B**: Sample purified by countercurrent chromatography.

binding-inhibitor from other material. Instead, when material previously purified by countercurrent chromatography was injected onto Glyco PAK N column (Fig. 1B) the peak (RT = 13.8) that contained the ^{3}H-nitrendipine-inhibiting activity was separated from other contaminants.

The present results clearly demonstrates that high-speed countercurrent chromatography is a valuable tool for separation of unknown biological materials. Preliminary studies that will be reported elsewhere of the purified material by mass spectrometric and nuclear magnetic resonance resulted in the identification of distinct functional groups (Drs. P. Driscoll and J.A. Omichinski, Laboratory of Chemical Physics, NIH, Bethesda, MD) that will be useful in elucidating the structure of the endogenous Ca^{2+} channel modulator.

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