Journal of Chromatography, 431 (1988) 192–196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4281

Note

Determination of *n*-butyl-β-carboline-3-carboxylate in aqueous brain extracts

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(First received February 18th, 1988; revised manuscript received April 29th, 1988)

After the demonstration by Braestrup et al. [1] that ethyl-β-carboline-3-carboxylate, a compound artificially formed during the extraction of urine or brain tissue with ethanol and strong acids, binds with high affinity to the benzodiazepine receptor, various derivatives of β-carboline-3-carboxylic acid have been shown to have similar binding properties [2–7]. Recently, we were able to isolate and identify *n*-butyl-β-carboline-3-carboxylate (β-CCB) as a natural component of brain, which displaces [^{3}H]flunitrazepam binding with a K_i of 3 n*M* [8]. However, the methods of extraction used required large amounts of tissue and a series of time-consuming purification steps.

Here we describe a rapid method for the extraction of β-carbolines from brain in which the prior use of Sep-Pak C_{18} reversed-phase cartridges permits the purification of the β-CCB by subsequent high-performance liquid chromatography (HPLC). An advantage of the method is that small amounts of tissue, corresponding to the cerebral cortices of four to eight rats, suffice for the assay of this natural ligand for the benzodiazepine receptor and for carrying out experimental studies.

0378-4347/88/$03.50

EXPERIMENTAL

Materials

Trifluoroacetic acid was obtained from Fluka (Buchs, Switzerland) and HPLC-grade acetonitrile from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All the synthetic β-carbolines were kindly provided by Dr. Leif H. Jensen (Ferrosan, Soeborg, Denmark). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). Vydac analytical columns, C_4 (214 TP 54) or C_{18} (218 TP 54), 250 mm × 4.6 mm I.D., with 5 μm particle diameter, were purchased from Separation Group (Hesperia, CA, U.S.A.). [^{3}H]Ethyl-β-carboline-3-carboxylate ([^{3}H]ethyl) (24 Ci/mmol) was obtained from Amersham (Amersham, U.K.) and [^{3}H]flunitrazepam ([^{3}H]methyl) (72.1 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]Butyl-β-carboline-3-carboxylate was tritiated at Amersham by catalytic reduction with tritium gas, using as starting material the *n*-butenyl ester of β-carboline-3-carboxylate synthesized at Ferrosan. The radiolabelled compound was purified in our laboratory using a C_{18} reversed-phase HPLC column, reaching a specific activity of 56 Ci/mmol.

Apparatus

The HPLC instrument consisted of two Constametric pumps, a solvent programmer and a UV detector, all from Laboratory Data Control (Riviera Beach, FL, U.S.A.). Samples were applied with a Rheodyne 7125 injector (Cotati, CA, U.S.A.), equipped with a 500-μl loop.

Sample preparation

Wistar rats of both sexes, weighing 200–250 g, were decapitated, the brains rapidly removed and put on ice and the cerebral cortices dissected. Tissue corresponding to four to eight brains was pooled, suspended in 7 volumes of ice-cold distilled water (pH 7.5) and homogenized for 30 s in a Polytron at setting 4. The suspension was stirred for 15 min in the cold and then centrifuged at 30 000 *g* for 30 min. The pellet was re-extracted into water and the pooled supernatants were heated at 90°C for 15 min, then centrifuged. We obtained similar results in experiments performed without heating. The residue was discarded and the supernatant was concentrated under vacuum at room temperature to a few millimeters. The concentrated supernatant was passed through a Sep-Pak C_{18} cartridge which had been previously activated and equilibrated with aqueous 0.1% trifluoroacetic acid (TFA). The cartridge was first eluted with 10 ml of 0.1% TFA, and then in successive steps with 3 ml of 10, 20 and 30% acetonitrile in 0.1% TFA, the washings being discarded. The active material was eluted with 6 ml of 40% acetonitrile in 0.1% TFA.

Chromatographic procedure

The active fraction was evaporated to 0.5 ml and applied to the C_4 column; chromatography was carried out with a linear gradient of acetonitrile in water. The gradient was made by mixing solvent A containing 15% acetonitrile and solvent B with 50% acetonitrile, from 10 to 45% B in 20 min, followed by isocratic

elution with 45% B. The flow-rate was 1 ml/min. On the C_{18} column the elution programme consisted of a 40-min gradient from 0 to 45% B, in which solvent A was 15% aqueous acetonitrile and solvent B was 80% aqueous acetonitrile. The active fraction was detected by measuring the displacement of [^{3}H]flunitrazepam or [^{3}H]ethyl-β-carboline-3-carboxylate binding to washed neural membranes of rat brain [8] from the various peaks of UV-absorbing material. For this purpose, aliquots of each fraction were evaporated almost to dryness and the residue was dissolved in saline for the assay., UV detection was performed at 280 nm.

RESULTS

Preliminary experiments had demonstrated that the Sep-Pak C_{18} was able to retain a sample of synthetic β-CCB and that this substance was completely eluted with 40% acetonitrile in 0.1% aqueous TFA but not with lower concentrations of acetonitrile. Analogously, the material from a brain extract retained by the Sep-Pak C_{18} cartridge could be eluted with increasing concentrations of acetonitrile in 0.1% aqueous TFA, but only the fraction corresponding to 40% acetonitrile contained a substance that displaced competitively and with high affinity the binding of [^{3}H]flunitrazepam to the central benzodiazepine receptor. Further purification of this substance was effected by HPLC. As shown in Fig. 1, in a C_4 column the active fraction was eluted between 35 and 40 min. This retention time agrees well with that shown by a sample of synthetic β-CCB which, using the same column and conditions, elutes at 37 min. Using samples of [^{3}H]β-CCB (28 000 cpm), we obtained a 94% recovery of radioactivity and the peak was localized exactly in the region of elution of the natural substance (not shown).

When the active material from the C_4 column (Fig. 1) is chromatographed in a C_{18} analytical column, the gradient of acetonitrile elutes the [^{3}H]flunitrazepam-

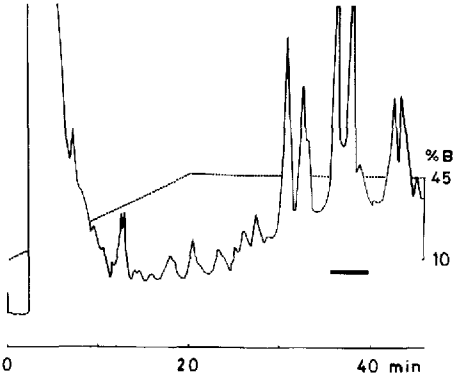

Fig. 1. HPLC on a reversed-phase C_4 column of the active fraction eluted from the Sep-Pak C_{18} cartridge loaded with the rat brain extract. HPLC was carried out with a 10–45% B linear gradient in 20 min followed by isocratic elution with 45% B. Solvent A was 15% and solvent B 50% aqueous acetonitrile. The eluate was monitored at 280 nm within a range of sensitivity such that full-scale deflection in the recorder corresponded to 0.02 absorbance units. The black bar indicates the fraction active in the displacement of [^{3}H]flunitrazepam binding.

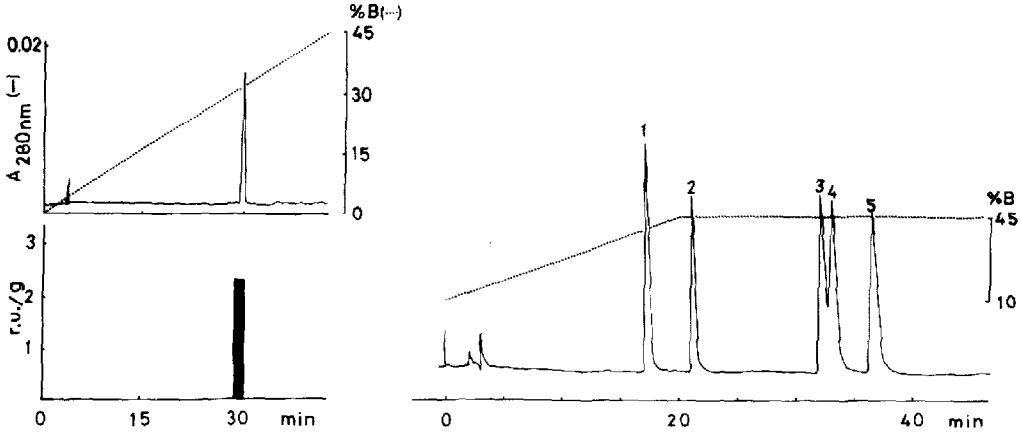

Fig. 2. Comparison between the HPLC retention time of the endogenous active material isolated from rat brain (see Fig. 1), rechromatographed on a C_{18} reversed-phase column (lower panel), and the same parameter obtained with a synthetic *n*-butyl ester of β-carboline-3-carboxylate (upper panel). Identical chromatographic conditions were applied in both instances, i.e., a 40-min gradient from 0 to 45% B, solvent A being 15% acetonitrile and solvent B 80% acetonitrile; the flow-rate was 1 ml/min. Displacement of [^{3}H]flunitrazepam binding to neural membranes, expressed in relative units per gram (r.u./g), was used to detect the natural compound in the eluate. The synthetic β-carboline derivative was detected at 280 nm, with the same sensitivity as in Fig. 1.

Fig. 3. HPLC profile of various esters of β-carboline-3-carboxylic acid. Peaks: 1 = methyl; 2 = ethyl; 3 = *tert.*-butyl; 4 = *sec.*-butyl; 5 = *n*-butyl. The HPLC conditions were as in Fig. 1 using 25 ng of each compound.

displacing substance at 30 min, exactly as occurs with a sample of synthetic β-CCB (Fig. 2).

As shown in Fig. 3, chromatography in the C_4 column allows the separation of various esters of β-carboline-3-carboxylic acid. It may be observed that as the hydrophobicity increases in the order methyl < ethyl < butyl ester, their retention times increase. The elution pattern also demonstrates that the *tert.*- and *sec.*-isomers of the *n*-butyl ester elute slightly ahead of the *n*-butyl derivative. This last derivative is the only substance whose retention time coincides with that of the active substance extracted from the brain.

The concentration of this active substance in the cerebral cortex of control rats, expressed in relative units (r.u.), i.e., the amount able to displace 50% of the [^{3}H]flunitrazepam binding at 0.5 n*M* to neural membranes, is 2.4 ± 0.3 r.u./g, equivalent to 1.85 ± 0.3 ng of β-CCB per gram of tissue [9]. This amount is near the limit of UV detection, but is clearly identifiable by the displacement of [^{3}H]flunitrazepam binding.

DISCUSSION

β-Carbolines have previously been isolated from various biological sources following long and cumbersome procedures. This is partly due to the fact that these

substances are present in very low concentrations, forcing the use of large amounts of starting material [1,8,10,11].

The method described here permits a simpler and faster analysis. The use of Sep-Pak C_{18} cartridges, eluted with a discontinuous acetonitrile gradient, as a first step in the purification eliminates large amounts of inactive materials and allows the use of analytical columns for further HPLC purification steps. It is therefore possible, in a few hours, to purify completely the *n*-butyl-β-carboline-3-carboxylate present in only a few grams of cerebral cortex of rats. The use of smaller amounts of tissue facilitates experiments performed with several groups of animals [9].

In previous work we were able to identify the endogenous active substance as β-CCB by mass spectrometry (M^+, *m/z* 268), ultraviolet and fluorescence spectrometry, coelution with β-CCB in several HPLC systems and by the correspondence of the K_i value with that of synthetic β-CCB in displacing [^{3}H]flunitrazepam binding [8]. Based on these results and the present findings, we can conclude that the new extraction and purification procedure truly isolates the *n*-butyl ester of the β-carboline-3-carboxylate from brain. In fact, we have observed here that the retention time of the active material, after HPLC in the C_4 column, corresponds to synthetic β-CCB and the same result is obtained after rechromatography on a C_{18} column (Fig. 2). The selectivity of the C_4 column chromatography is demonstrated further by the fact that it can discriminate between various esters of β-carboline-3-carboxylic acid and even between various isomers of the *n*-butyl ester (Fig. 3).

ACKNOWLEDGEMENTS

This work was supported by grants from CONICET, Argentina. We express our gratitude to Dr. Leif H. Jensen of Ferrosan, Soeborg, Denmark, for providing all of the synthetic β-carbolines used. The technical assistance of Miss M. Ramírez in the preparation of the manuscript is gratefully acknowledged.

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