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Note

Column liquid chromatography of calcium channel blockers

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Calcium channel blockers would be expected to inhibit the influx of calcium following pathological events and to prevent cellular impairment [1] When it is attempted to deliver the chemicals into the brain, their uptake by brain cells has to be monitored In this study we have developed a sensitive method for the determination of calcium antagonists of piperazine derivatives, and applied it to the monitoring of levels in plasma and uptake into a subcellular fraction of brains

Cinnarizine and related compounds have been determined by gas chromatography (GC) [2–4] and high-performance liquid chromatography (HPLC) [5] In most reported procedures [2–5], samples for chromatographic analysis were purified by solvent extraction under acidic conditions Although this seemed to be adequate for handling plasma or serum, in a preliminary experiment we noticed that the extraction method introduced considerable amounts of contaminants when tissue homogenates were used as the starting material We could overcome this problem simply by using an SP-Toyopearl column to clean up the samples As the calcium antagonists tested appeared to be partly degraded during GC analysis, according to our preliminary experiments, especially when very small amounts were analysed, HPLC was used in this study Employing a small column and a low wavelength enhanced the sensitivity about 10-fold compared with reported HPLC methods [5]

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EXPERIMENTAL

Preparation of samples

A mixture of flunarizine [II (Fig 1); Sigma St Louis, MO, U S A , 3 mg/kg body weight] and 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine [III (Fig 1), Kanebo, Osaka, Japan, 5 mg/kg] [6] was administered intravenously to 3-month-old female Fischer rats Cerebral tissues were homogenized with 0 32 *M* sucrose and centrifuged for 3 min at 1300 *g*. The subcellular particles in the supernatant were spun down for 10 min at 17 000 *g* to give the P_2 fraction [7] A sample of plasma (100 μl) was mixed with 10 μl of a methanol solution of cinnarizine [I (Fig 1) Eisai, Tokyo, Japan, 1 ng/μl] as an internal standard and a small amount of methanol, resulting in a ratio of methanol to water of 1 1 The sample solution was made alkaline with 200 μl of 2 *M* sodium hydroxide solution and shaken for 5 min at room temperature The solution was mixed with 750 μl of *n*-hexane (HPLC grade), vortex-mixed and centrifuged for 5 min at 1500 *g* The organic layer was removed and the aqueous layer was re-extracted with 750 μl of *n*-hexane

For the P_2 fraction, 400 μl of the sample (about 8 mg of protein) were taken and 20 μl of the internal standard solution were added The volumes of sodium hydroxide solution and *n*-hexane employed were four times greater than those used for plasma The combined extracts were concentrated in a stream of nitrogen and dissolved in 2 ml of methanol–1 *M* hydrochloric acid (9 1) The

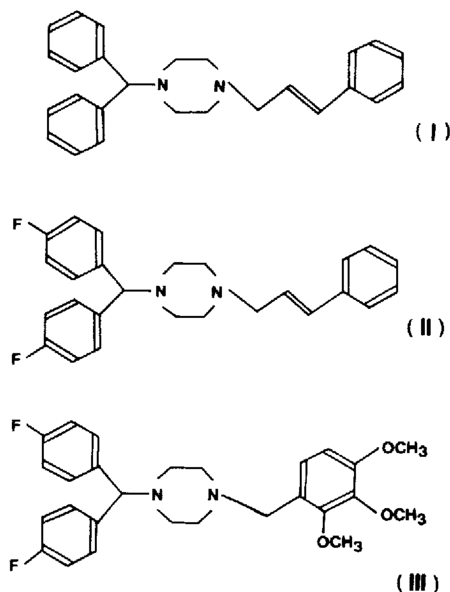

Fig 1 Structures of cinnarizine (I), flunarizine (II) and 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine (III) [6]

solution was applied to an SP-Toyopearl (particle size 44–88 μm) column (5 mm×5 mm I D) (Tosoh, Tokyo, Japan) After the column had been washed with 1 ml of methanol–water (9 1), the calcium antagonists were eluted with 1 ml of methanol–1 *M* ammonia solution (9 1) The effluent was evaporated to dryness in a stream of nitrogen and the residue dissolved in 50 μl of acetonitrile–20 m*M* potassium phosphate buffer (pH 5 5) (70 30) to provide as a sample for HPLC An aliquot (5–20 μl) was injected into a chromatograph

High-performance liquid chromatography

An LC-4A chromatograph equipped with a C-R4A integrator (Shimadzu Seisakusho, Kyoto, Japan) was used. An Inertsil C_8 column (20 cm×2 1 mm I.D , 5 μm particle size) was obtained from Gasukuro Kogyo (Tokyo, Japan) The mobile phase was acetonitrile–20 m*M* potassium phosphate buffer (pH 5 5) (69 31), which was degassed by bubbling helium, and the flow-rate was 0 2 ml/min The elution of calcium antagonists was monitored at 210 nm. The peak heights were used for the calculation of sample concentration

RESULTS AND DISCUSSION

Calcium channel blockers with a piperazine structure can be quantitatively extracted from biological fluids with organic solvents under mildly basic conditions (pH 8 5–10 8) [2–4] If the chemicals are incorporated into tissues or membranes, they may not easily be extracted under such mild conditions We used 1 *M* sodium hydroxide solution to degrade membranes and to release the chemicals therefrom Under this condition, the recovery into *n*-hexane of I, II and III from tissue homogenates was more than 94% Clean-up of the extracts has previously been carried out by re-extraction into an acidic aqueous layer and then back-extraction into an organic layer [2–4], and this procedure seemed to be appropriate for plasma samples However, tissue samples that were purified by back-extraction appeared still to contain large amounts of contaminants that interfered with the HPLC profiles We tested several clean-up methods and found that a small column of a strong cation-exchange resin, SP-Toyopearl, is excellent for removing most of the contaminants The recovery of drugs from this column was 97%, and the overall recovery in the present procedure was about 91%

The calcium antagonists employed could be separated with either a C_8 or a C_{18} column Under the present chromatographic conditions, good separations and symmetrical peak shapes were obtained using a C_8 column Compounds I and II are usually detected at 254 nm [5] As the molar absorptivity of these kinds of compounds are much higher at wavelengths lower than 254 nm, we employed 210 nm for detection, under which conditions as little as 100 pg of the samples could be determined The relative detector responses were 1 00 for I, 0 69 for II and 1 01 for III The coefficients of variation for these relative

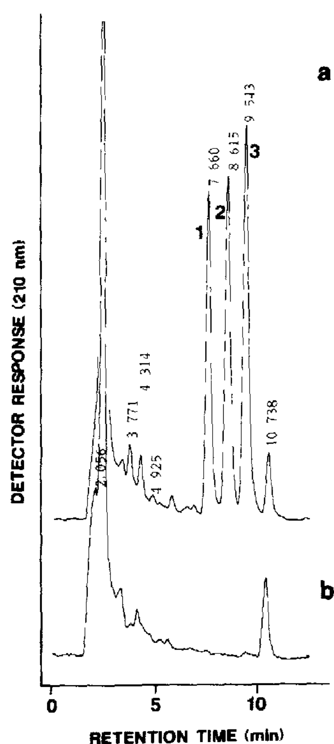

Fig 2 HPLC of calcium channel blockers (a) Rat plasma 7 h after intravenous injection of II and III, (b) control rat plasma Peaks 1=III (95 ng/ml), 2=I (100 ng/ml) as internal standard, 3=II (177 ng/ml)

TABLE I

TIME COURSE OF PLASMA CONCENTRATION OF II AND III, AND THEIR UPTAKE INTO CEREBRAL P_2 FRACTION

II (3 mg/kg body weight) and III (5 mg/kg) were injected intravenously into rats Blood was collected from the tail vein and brains were obtained 7 h post-injection Values are expressed as means ± S D

Time (h)	Number of animals	Plasma (ng/ml)		Cerebral P_2 fraction (ng/mg of protein)	
		II	III	II	III
0 5	3	423±33	318±56	-	-
1	4	377±21	230±28	-	-
2	3	333±22	199±14	-	-
4	4	263±26	86±25	-	-
7	3	162±21	81±14	3 14±0 23	1 16±0 26

responses during 2 months were 2 1% for II and 2 2 % for III when 5 ng of each compound were injected A typical HPLC profile of the calcium antagonists extracted from rat plasma is shown in Fig 2

This method was applied to the determination of calcium antagonists in plasma and a brain subcellular P_2 fraction. The latter preparation was free from any contribution of blood contamination This point seems to be very important in assessing the real uptake of chemicals into brain tissues through the blood–brain barrier Table I shows the time course of the plasma concentration of the chemicals and the uptake by brain tissue as expressed by incorporation into the P_2 fraction The results indicate that different turnover rates are observed for II and III in plasma and that appreciable amounts of both chemicals are actually incorporated into brain tissues This study would provide an accurate method for monitoring drug concentrations in tissues and in plasma when treating brain damage with calcium antagonists

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