Interference of Linoleic Acid Fraction in Some Receptor Binding Assays

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An extract of a suspension culture of *Tabernaemontana pandacaqui* Poir. was fractionated by centrifugal partition chromatography. Aliquots were tested in an adenosine A_1 receptor binding assay. This led to the isolation and identification of linoleic acid, which proved to be a noncompetitive inhibitor. This "false positive"effect also extended to some other binding assays.

Currently, pharmaceutical industries are becoming increasingly interested in screening natural products for new biologically active compounds because of the availability of high-throughput screening methods using receptor binding assays or enzyme assays. However, such screening can be hampered by the occurrence of ubiquitous compounds that have known bioactivities, or of compounds that cause nonspecific inhibition in the assay. Therefore, a prefractionation prior to screening might be useful.

Centrifugal partition chromatography (CPC) has been used as a tool for prefractionation of crude plant extracts in our laboratories.[1] CPC has particular advantages in natural product research. It combines higher sample-loading capacity with lower solvent consumption and a shorter run time compared to the conventional preparative LC techniques. Moreover, irreversible retention of a sample is eliminated since no solid support is used. CPC was first described by Murayama,[2] and the theoretical and the application aspects were extensively discussed by Foucault.[3]

In the present study, we combined CPC with radioligand binding studies as a rapid screening assay. We were particularly interested in the adenosine A_1 receptor since many natural products such as purines and flavonoids show affinity for this receptor subtype.

The affinities of the extracts from some plants and cell suspension cultures on the adenosine A_1 receptor were determined (Table 1). The ethanol extract from a *Tabernaemontana pandacaqui* Poir. cell suspension culture and the ethanol extract from a *Catharanthus roseus* (L.) G. Don cell suspension culture were two of the most active extracts, and were selected for further investigation.

The previously selected CPC system, heptane/ethyl acetate/methanol/water 6:1:6:1 (v/v/v/v)[1] was used as the prefractionation step of an ethanol extract of a *T. pandacaqui* cell culture. Adenosine A_1 receptor binding assay guided fractionation led to the isolation of the noncompetitive inhibitor, linoleic acid. This compound was identified by means of ^{1}H NMR, MS, and by comparison of the ^{1}H NMR spectrum and the TLC chromatogram with a reference compound. Its affinity for the adenosine A_1 receptor expressed as a half-maximal inhibiting concentration (IC_{50}) was 65 ± 14 μM (SD) (Figure 1). The maximum bindings observed from three separate saturation experiments carried out in the absence and in the presence of 56 μM linoleic acid were 603.4 ± 67.8 fmol/mg protein (SD) and 142.7 ± 56.1 fmol/mgprotein (SD) (Figure 2). This difference showed that linoleic acid acted as a noncompetitive inhibitor in the adenosine A_1 receptor binding assay. The activities of palmitic acid, stearic acid, oleic acid and arachidonic acid on the same assay were also tested. The unsaturated fatty acids showed a greater inhibitory effect than the two saturated fatty acids on this assay (Figure 3). Moreover, linoleic acid showed an effect in the opiate receptor binding assay and nucleoside transport protein binding assay (Table 2). In the opiate receptor binding assay, the amount of radioligand remaining in the assay increased in the presence of the high concentration of linoleic acid (10^{-3} M), but decreased in the presence of 10^{-4} M linoleic acid. On the other hand, in the nucleoside transport protein binding assay, only the high concentration of linoleic acid (10^{-3} M) affected the assay by decreasing the amount of radioligand remaining in the assay. It has been reported before that fatty acids cause noncompetitive or mixed-noncompetitive

Table 1. Percentage Specific Binding of [^{3}H]DPCPX Remaining on Adenosine A_1 Receptor after Incubation with 100 μL Aliquots of Some Plant and Plant Cell Culture Extracts (2.5 mg/mL)

		% specific binding[a] of [^{3}H] DPCPX		
		water extract.	ethanol extract.	toluene extract.
T. pandacaqui	cell suspension culture	75 ± 26	6 ± 1	14 ± 4
C. roseus	cell suspension culture	nd[b]	2 ± 1	0
R. tinctorum	cell suspension culture	72 ± 8	0	nd[b]
T. pachysiphon	dried leaves	nd[b]	80 ± 8	96 ± 6
A. xhenryi	roots	nd[b]	19 ± 7	nd[b]

[a] The results are the mean ± SD of 3 separate determinations in which duplicate samples were tested. [b] nd = not determined.

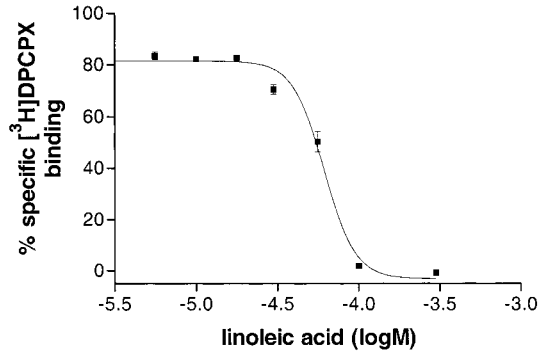

Figure 1. Affinity of linoleic acid on adenosine A_1 receptor binding assay. The specific binding of [^{3}H]DPCPX remaining was determined as described in the Experimental Section. Values are means of one typical experiment performed in duplicate.

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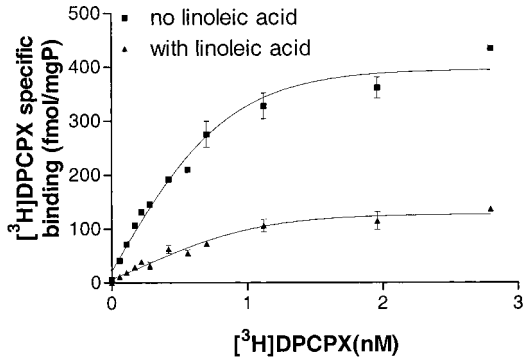

Figure 2. Saturation curve of [^{3}H]DPCPX binding to adenosine A_1 receptors in the absence and presence of 56 μM. linoleic acid The specific binding of [^{3}H]DPCPX remaining was determined as described in the Experimental Section. Values are means of one typical experiment performed in duplicate.

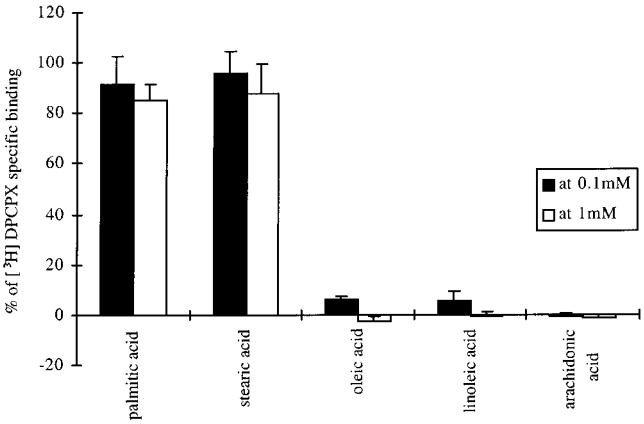

Figure 3. Percentage specific binding of [^{3}H]DPCPX remaining on adenosine A_1 receptor after incubation with 100 μL aliquots of 1 mM and 0.1 mM of some fatty acids.

Table 2. Effects of Linoleic Acid on Adenosine A_1 Receptor, Opiate Receptor, and Nucleoside Transport Protein Binding Assays

	% specific binding of radioligand remaining in the assays[a]		
	adenosine A_1 receptor binding assay	opiate receptor binding assay	nucleoside transport protein binding assay
10^{-3} M linoleic acid	0	>100	<0
10^{-4} M linoleic acid	3−8	42−58	100
10^{-5} M linoleic acid	85−94	100	100

[a] The results are the ranges of values from 3 separate determinations in which duplicate samples were tested.

inhibition on some receptors.[4−6] Vallette et al. suggested that binding of the synthetic glucocorticoid dexamethasone to the rat liver cytosolic glucocorticoid receptor was mixed-noncompetitively inhibited by physiological concentrations of fatty acids as a function of increasing dose, the degree of unsaturation and chain length of the unsaturated fatty acid.[4] Kato reported that arachidonic acid and other long-chain fatty acids showed a noncompetitive inhibitory effect on the specific binding of estrogen, progestin androgen, and glucocorticoid receptors from the central and peripheral tissues of rats.[5] Furthermore, Kang et al. found that eicosapentaenoic acid caused the inhibition of platelet-activating factor (PAF) binding due to a decrease in both affinity and number of PAF receptors.[6]

The mechanism by which unsaturated fatty acids affect adenosine A_1 receptor binding activity in the assay is not known. It is possible either that fatty acids bind to the receptor and change receptor conformation, decreasing the availability of binding sites; or that fatty acids alter the properties of membrane lipids by modifying membrane lipid-bilayer structure. The anomalous behavior of linoleic acid on the opiate receptor has no obvious explanation. As it is very reproducible, it might be a clue for further studies on the mechanism of the noncompetitive inhibitory effect.

Linoleic acid is a good example of a ubiquitous compound that can mislead a bioactive compound investigation by its nonspecific activity. The general prefractionation step could be useful to identify the fraction containing linoleic acid. This was in fact further proven by the fractionation of the *C. roseus* extract, another active crude extract, with the same CPC system. An active fraction was found at the same retention as that of the first CPC experiment with *T. pandacaqui*. The main compound of this fraction was identified as linoleic acid by TLC analyses. For this reason, a further investigation was not performed, thus saving us from devoting time and money to the fraction of the already-known compound.

In conclusion, CPC can be used as the prefractionation method prior to bioactivity screening. By determining the retention of known compounds, or of compounds giving noncompetitive effect on the bioassay used, the efficiency of screening programs can be improved.

Experimental Section

General Experimental Procedures. The CPC used was a modular Sanki (Kyoto, Japan) CPC type LLN. It consisted of a power supply (model SPL), a centrifuge (model NMF), and a triple-head constant flow pump (model LBP-V). A Panasonic pen recorder (model VP 67222A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV). Fractions were collected by means of a LKB 2211 Superrac fraction collector. In all experiments, six partition cartridges (total internal volume 125 mL) were used. The pressure was limited to 60 bar and the flow rate was set to 2 mL/min. The fraction size was 8 mL. GC−MS was performed on a Finnigan ITD 700 (Finnigan, San Jose, CA) in EI mode coupled with a Packard 438A gas chromatography. ^{1}H NMR (300 MHz) measurements were performed on a Bruker DPX-300 spectrometer in deuterated chloroform. TMS was used as an internal chemical shift reference.

Reagents. The reference fatty acids and dipyridamole were obtained from Sigma (St. Louis, MO). The radioligands were purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). N^6-Cyclopentyladenosine (CPA) were purchased from RBI (Natick, MA). Morphine was obtained from ACF Chemiefarma N. V. (Maarssen, The Netherlands).

Plant and Cell Suspension Cultures Material. The cell suspension cultures, *T. pandacaqui* (cell line 6OriB13)[7] (Apocynaceae), *C. roseus* (cell line A12A2)[8] (Apocynaceae), and *Rubia tinctorum* L.[9] (Rubiaceae) were grown in 2 L flasks. The cells were harvested and stored at −20 °C. *Tabernaemontana pachysiphon* Stapf (Apocynaceae) and roots of *Aconitum xhenryi* E. Pritz "Spark" (Ranunculaceae) were cultivated at the division of Pharmacognosy, Leiden University, Leiden, The Netherlands. The herbarium specimens are kept at Rijksherbarium, Leiden, The Netherlands.

Extraction and Isolation. The cell suspension cultures were thawed and extracted with 0.1 M phosphate buffer, pH 7, using a Turrax at high speed for 3 min. The buffer extract was filtered over filter paper and the filtrate was freeze-dried. Subsequently, the residual cells were extracted with ethanol and toluene (5 mL per gram fresh weight each). The ethanol filtrate as well as the toluene filtrate was evaporated until dryness under reduced pressure. Dried leaves of *T. pachysiphon* and roots of *A. xhenryi* were macerated with 5 mL ethanol per gram to prepare an ethanol extract and with 5 mL toluene per gram to make a toluene extract. After 1 week,

they were filtered. Then the ethanol filtrate and the toluene filtrate were evaporated until dryness under reduced pressure.

The first separation step of *T. pandacaqui* ethanol extract was done by means of CPC with the solvent system heptane/ethyl acetate/methanol/water, 6:1:6:1 (v/v/v/v). One gram of the dried ethanol extract from *T. pandacaqui* was dissolved in 4 mL of each of the two phases before injection into CPC. The nonsoluble part was separated by filtration over filter paper and named as fraction 1. The first 112 mL, excluding the void volume, was eluted in ascending mode. The mode of elution was then reversed to descending mode, where another 128 mL was eluted.

The eluate was grouped into fractions 2−7 according to the results of TLC analysis. Fraction 4 (33 mg) was subjected to the second CPC separation using solvent system heptane/acetonitrile/chloroform 5:5:1 (v/v/v). The first 160 mL, excluding the void volume, was eluted in ascending mode. The mode of elution was then reversed to descending mode where 104 mL was eluted. The eluate was grouped into fractions 4/1−4/6 according to the results of both TLC analysis and adenosine A_1 receptor binding assay. Fraction 4/6 was evaporated till dryness under reduced pressure and then applied to CPC, solvent system heptane/ethanol/water 6:5:1 (v/v/v). The first 192 mL, excluding the void volume was eluted in ascending mode. After mode reversion, 80 mL was eluted in descending mode. The eluate was grouped into 9 fractions (fractions 4/6/1−4/6/9) according to the results of TLC analysis. The main compound of fraction 4/6/4 was further purified by means of reversed-phase HPLC (Phenomenex Hypersil, 5 C18, 250 × 4.6 mm I.D., photodiode array detection, mobile phase acetonitrile/water 9:1).

The separation of *C. roseus* ethanol extract step was done by means of CPC with the solvent system heptane/ethyl acetate/methanol/water 6:1:6:1 (v/v/v/v). One gram of the dried ethanol extract of *C. roseus* was applied into the CPC. The CPC operation and the grouping of eluate were done in the same way as that in the first separation step of *T. pandacaqui*.

Radioligand Receptor Binding Assays. The adenosine A_1 receptor and the opiate receptor binding assays were carried out on cortical membranes from rat brains. Membranes were prepared according to the method of Lohse et al.[10] except that the membranes were incubated with 2 IU/mL adenosine deaminase (ADA) at 37 °C before storage, as described by Pirovano et al.[11] Protein concentrations were measured with the bicinchonic acid (BCA) method.[12]

The adenosine A_1 receptor binding assays were performed with 0.4 nM [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX) as the radioligand (K_d 0.39 nM). The assays were performed as originally described by Lohse et al. [13] Briefly, incubation mixtures consisted of 100 μL of [^{3}H]DPCPX, 100 μL of 10^{-5} M N^6-cyclopentyladenosine (CPA) as a displacer, 100 μL of 50 mM Tris/HCl buffer, pH 7.4, and 100 μL of rat brain homogenate containing 30 μg of brain tissue. After incubating at 25 °C for 60 min, the mixture was put on ice. The mixture was then filtered over glass-fiber filters (GF/B Whatman) under reduced pressure. The filters were washed 3 times with 2 mL of ice-cold 50 mM Tris/HCl buffer, pH 7.4. The activity of the washed filters was counted for 4 min by a Hewlett Packcard Tri-Carb 1500 liquid scintillation counter after adding 3.5 mL of scintillation liquid. Nonspecific binding was determined in the presence of 10^{-5} M CPA. Radioligand binding data were analyzed with the software package Prism (Graph Pad Inc, San Diego, CA).

The opiate receptor binding assays were performed as described by Cox[14] using 1.5 nM [^{3}H]naloxone as the radioligand (K_d 2.1 nM). Nonspecific binding was determined in the presence of 10^{-5} M morphine.

The nucleoside transport protein binding assay was carried out on membranes of calf lungs. Membrane preparation and assay were performed as described by IJzerman et al.[15] with 1.5 nM [^{3}H]nitrobenzylthioinosine (NBI) as the radioligand (K_d 0.65 nM). Nonspecific binding was determined in the presence of 3 μM dipyridamole.

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