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Rapid determination by high-performance liquid chromatography of free fatty acids released from rat platelets after derivatization with monodansylcadaverine

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ABSTRACT

The release of free fatty acids from rat platelets, triggered by thrombin stimulation, was monitored by high-performance liquid chromatography (HPLC) after precolumn derivatization with monodansylcadaverine (MDC). A rapid filtration procedure was devised for the precise determination of free fatty acids released from aggregated platelets, instead of the conventional method using a stop solution or enzyme reactions. The fatty acids thus collected were derivatized with MDC in the presence of diethyl phosphorocyanidate (DEPC). The simultaneous separation of MDC derivatives of fatty acids was achieved on a reversed-phase TSKgel ODS-80$_{TM}$ column within 60 min by linear gradient elution, using 0.2 *M* Tris–HCl buffer (pH 7.8)–methanol (50:50, v/v) and acetonitrile. The MDC derivatives were detected with excitation and emission wavelengths of 340 and 518 nm, respectively. The amounts of liberated fatty acids were in the range from 45.0 pmol for myristoleic acid ($C_{14:1}$) to 395.0 pmol for palmitic acid ($C_{16:0}$) per $1.9 \cdot 10^7$ platelets.

INTRODUCTION

Various labelling reagents have been developed for the trace determination of carboxylic materials. Fluorogenic derivatization reagents are especially effective in providing high sensitivities for the detection of biologically active carboxylic acids[1–4].

Recently, a new derivatization method was introduced for the sensitive determination of carboxylic acids with monodansylcadaverine (MDC) as a fluorophore[5] (Fig. 1). The method exceeds the conventional methods in rapidity of derivatization and in sensitivity and it has been successfully employed in the determination of fatty acids in rabbit blood plasma[6].

Blood platelets, stimulated by adenosine diphosphate (ADP), collagen, thrombin, etc., aggregate rapidly. This leads to a dynamic change in the fatty acid composition of membrane phospholipids, caused by activated phospholipases which liberate

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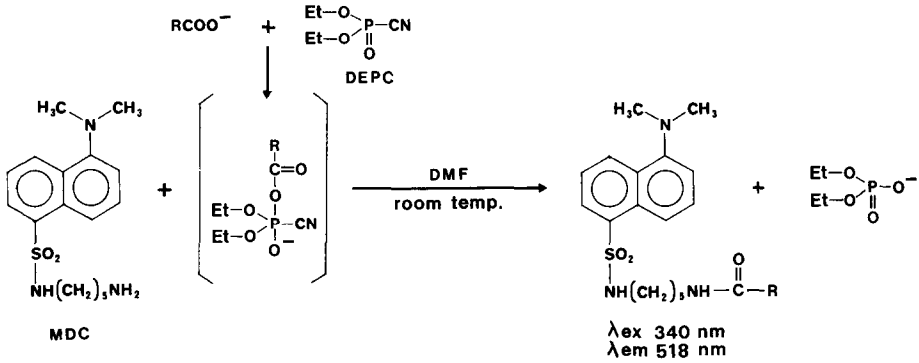

Fig. 1. Derivatization of carboxylic acids with monodansylcadaverine (MDC). DEPC = Diethyl phosphorocyanidate; DMF = N,N-dimethylformamide; Et = ethyl; λ_{ex} = excitation maximum; λ_{em} = emission maximum.

saturated and unsaturated fatty acids from the *sn*-1 and/or *sn*-2 sites in membrane phospholipids.

In this work, stimuli-induced free fatty acids in activated platelets were determined by high-performance liquid chromatography (HPLC) after derivatization with MDC.

EXPERIMENTAL

Chemicals

Saturated fatty acids ($C_{12:0}$–$C_{20:0}$) were obtained from Tokyo Kasei (Tokyo, Japan). Arachidonic acid ($C_{20:4}$) was purchased from Sigma (St. Louis, MO, U.S.A.) and other unsaturated fatty acids from Nacalai Tesque (Kyoto, Japan). MDC was purchased from Sigma and purified with absolute ethanol prior to use. Diethyl phosphorocyanidate (DEPC) was obtained from Wako (Osaka, Japan), and the fraction collected by distillation at 88–89°C under reduced pressure (*ca.* 8 mmHg) was used. All other reagents were of analytical-reagent grade.

Instrumentation

A Jasco (Tokyo, Japan) Trirotar-VI high-performance liquid chromatograph equipped with a reversed-phase TSKgel ODS-80$_{TM}$ column (250 mm × 4.6 mm I.D.) (Tosoh, Tokyo, Japan) was used. An FP-210 spectrofluorimeter (Jasco) equipped with a 15-μl micro-flow cell was used as a monitor.

Preparation of washed rat platelets

Wistar rats (male, *ca.* 250 g) were purchased from Saitama Zikken Doubutsu (Saitama, Japan). Volumes of 10 ml of blood were collected from the heart under diethyl ether anaesthesia by the use of a disposable polypropylene syringe (20 ml) containing 2 ml of ACD buffer (anticoagulant; 65 m*M* citric acid–85 m*M* sodium citrate–2.0 g per 100 ml glucose). To prevent the blood from coagulating, the syringe was shaken gently to mix the blood with the anticoagulant. After removing the sy-

ringe needle, the blood was transferred slowly to a Polyspits tube (10 × 1.4 cm I.D.) (Iwaki, Tokyo, Japan) with a PTFE cap and then centrifuged at 250 *g* for 10 min at room temperature. The upper phase, platelet-rich plasma, was collected and centrifuged at 800 *g* for 15 min. Precipitated platelets were washed with 3 ml of Tyrode–N-(2-hydroxyethyl)piperazine-N′-2-ethanesulphonic acid (HEPES) buffer (pH 7.2; 137 m*M* NaCl–2.7 m*M* KCl–10 m*M* HEPES–0.1% glucose) and centrifuged at 500 *g* for 10 min. Washing was repeated twice and then the number of platelets was adjusted to $1.9 \cdot 10^7$/ml by diluting with Tyrode–HEPES buffer.

Stimulation of washed rat platelets

A 1-ml volume of washed rat platelet suspension was transferred to a Polyspits tube with a PTFE cap. Platelets were stimulated in the presence of 2 m*M* calcium chloride by the addition of 2.5 units/ml of thrombin at 7°C.

Isolation of free fatty acids in platelets

After incubation at 37°C for 4 min, 100 pmol of margaric acid ($C_{17:0}$), dissolved in 50 μl of methanol, were added as an internal standard. The platelet pellets were removed quickly by passing the mixture through a membrane filter kit (pore size 0.45 μm, HA type) (Millipore, Milford, MA, U.S.A.), which was previously connected to a vacuum aspirator. The aqueous layer, collected in a glass test-tube, was acidified with 10 μl of 20% hydrochloric acid and then extracted with 2 ml of chloroform–methanol (1:1, v/v) followed by 1 ml of chloroform. After centrifugation at 800 *g* for 10 min, the lower organic layer was separated, combined and evaporated under a flow of nitrogen. The amounts of free fatty acids were determined by the following procedures.

Derivatization procedure

After the residue containing the fatty acids had been dissolved in 50 μl of N,N-dimethylformamide (DMF), 50 μl of 12 m*M* MDC in DMF and then 2 μl of DEPC were added. The reaction mixture was stirred for 10 s, allowed to stand at room temperature for 15 min, and then directly injected into the HPLC system.

HPLC conditions

The simultaneous separation of MDC derivatives of fatty acids was achieved by linear gradient elution using 0.2 *M* Tris–HCl buffer (pH 7.8)–methanol (50:50, v/v) (eluent A) and acetonitrile (eluent B). The initial condition was set at 50% each of eluents A and B and the proportion of B was increased to 90% in 60 min. The flow-rate was set at 1.0 ml/min. The column temperature was maintained at 40°C. The MDC derivatives of fatty acids were detected with an excitation wavelength of 340 nm and an emission wavelength of 518 nm.

RESULTS AND DISCUSSION

Although the complete mechanism of platelet activation is not yet known, the liberation of fatty acids from membrane phospholipids and the subsequent oxygenation of some fatty acids by cyclooxygenase or lipoxygenase are well known. Therefore the determination of liberated fatty acids is essential to the understanding of stimuli–response phenomena occurring in the cell membrane. With the procedure

described under Experimental, the extraction recoveries of fatty acids with chloroform–methanol (1:1, v/v) were more than 95% at the 100-pmol level. Further, the derivatization yield of arachidic acid ($C_{20:0}$) with MDC exceeded 95%. We reported previously that the MDC-labelled free fatty acids in rabbit plasma could be separated completely using the HPLC conditions described under Experimental[6]. By using this HPLC method, free fatty acids liberated from rat platelets were determined in this work at picomole levels. Fig. 2 shows typical HPLC patterns which illustrate stimuli-induced changes in intracellular free fatty acids of washed platelets with and without thrombin stimulation. As shown in Table I, the levels of all free fatty acids were increased after stimulation in comparison with intracellular levels. Saturated fatty acids generally show larger increases than unsaturated fatty acids; the net amounts of the increased levels were 169.3 pmol for myristic acid ($C_{14:0}$), 395.0 pmol for palmitic acid ($C_{16:0}$) and 106.6 pmol for stearic acid ($C_{18:0}$). The increased amounts of oleic acid ($C_{18:1}$) and linoleic acid (C18:2) were larger than those of the other unsaturated fatty acids. The large increase in saturated fatty acids supports the theory that the *sn*-1 site of phospholipids is mostly occupied by such saturated fatty acids, as they are liberated by means of hydrolysis by stimulated phospholipases. Especially palmitic acid ($C_{16:0}$) is known to occcupy *ca.* 62% of the total carboxylic acids in phosphatidylcholine (PC), which is the most abundant phospholipid in rat platelet membrane[7].

However, arachidonic acid ($C_{20:4}$) was not detected in the free form, although it is known to be liberated by phospholipase A_2 during platelet aggregation. Probably

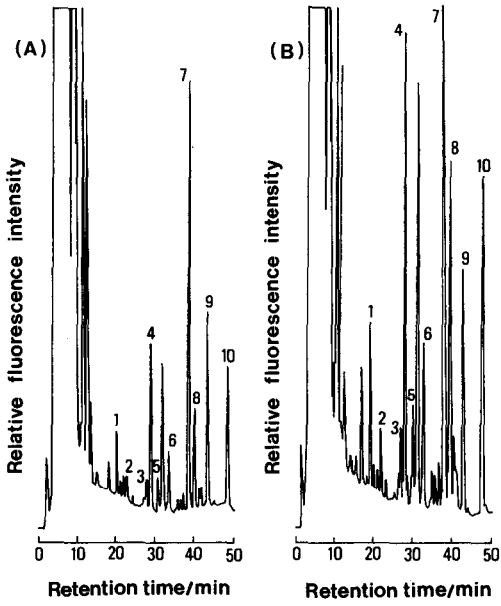

Fig. 2. HPLC profiles of MDC-labelled intracellular fatty acids in washed rat platelets (A) before and (B) after thrombin-induced aggregation. Peaks: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{16:1}$; 6 = $C_{18:2}$; 7 = $C_{16:0}$; 8 = $C_{18:1}$; 9 = $C_{17:0}$; (internal standard); 10 = $C_{18:0}$. For HPLC conditions, see Experimental.

TABLE I

CHANGES IN FREE FATTY ACID CONTENT OF WASHED RAT PLATELETS FOLLOWING THROMBIN-INDUCED AGGREGATION

Free fatty acid[a]	*Aggregated by thrombin (pmol fatty acids per 1.9 · 10^7 platelets)*		
	Before	*After*	*Increase*
12:0	111.1	165.4	54.3
14:1	80.3	125.3	45.0
18:3	68.9	120.9	52.0
14:0	177.7	347.0	169.3
16:1	55.0	126.4	71.4
18:2	70.2	161.3	91.1
16:0	381.8	776.8	395.0
18:1	133.5	336.0	202.5
18:0	146.3	252.9	106.6

[a] Fatty acids are designated by chain length:number of double bonds.

liberated arachidonic acid is converted quickly to its oxidized metabolites through the cyclooxygenase and/or lipoxygenase pathways. On the other hand, a peak which was eluted earlier than arachidonic acid appeared and tended to increase like free fatty acids after thrombin stimulation (Fig. 2). To identify the unknown peak, the incubation mixture (Fig. 2B) was mixed with the same volume of the MDC derivatives

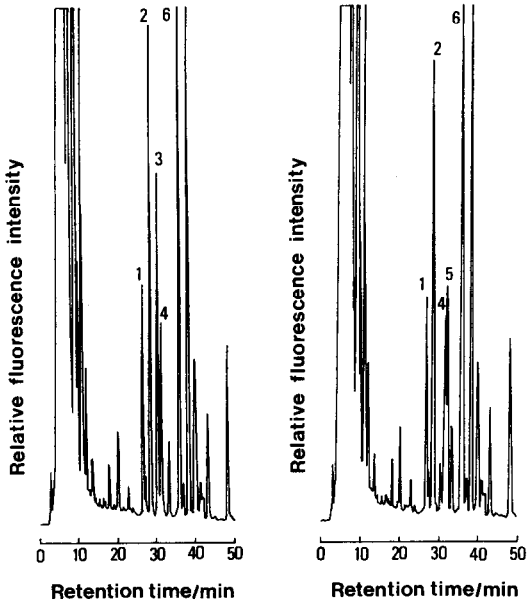

Fig. 3. Mixed chromatograms of MDC derivatives of fatty acids released from rat platelets with those of authentic compounds. The incubation mixture illustrated in Fig. 2B was spiked with a mixture of (A) $C_{20:5}$, $C_{14:0}$, $C_{22:6}$ and $C_{20:3}$ or (B) $C_{20:5}$, $C_{14:0}$, $C_{20:4}$ and $C_{20:3}$. Peaks: 1 = $C_{20:5}$; 2 = $C_{14:0}$; 3 = $C_{22:6}$; 4 = unknown; 5 = $C_{20:4}$; 6 = $C_{20:3}$. HPLC conditions as in Fig. 1.

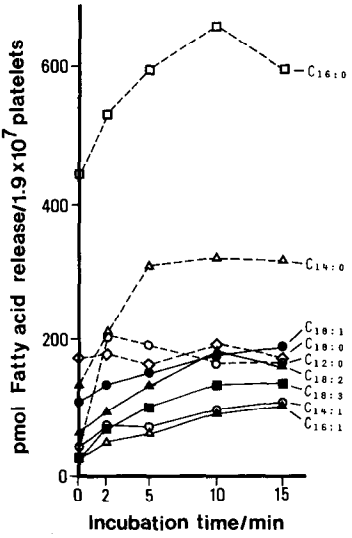

Fig. 4. Time courses of fatty acid release from washed rat platelets after thrombin stimulation.

of authentic fatty acids and injected into the HPLC system. The resulting mixed chromatograms are shown in Fig. 3. This unknown peak was eluted between docosahexaenoic acid ($C_{22:6}$) (Fig. 3A) and arachidonic acid (Fig. 3B). Therefore, it seems to be an oxidized intermediate of arachidonic acid associated with the oxygenase pathways. The identity of the unknown peak is under investigation.

The detection limit of this method was reported[5] to be 0.1 pmol at a signal-to-noise ratio of 3. In this study, application of this method with such high sensitivity was adequate for determining the free fatty acids at the intracellular level of 10^7 platelets.

Fig. 4 illustrates the changes in the liberated free fatty acids 2, 5, 10 and 15 min after the addition of thrombin at an incubation temperature of 25°C. A larger increase in palmitic acid ($C_{16:0}$) and stearic acid ($C_{14:0}$) was observed and reached maximum values 10 min after thrombin stimulation. The other fatty acids were observed to increase slightly, and arachidonic acid also was not observed.

As stated above, the present HPLC method, involving the use of MDC as a labelling fluorophore, was useful in the determination of the fatty acids liberated from platelets owing to its high sensitivity. Although Ikeda and Matsumoto[8] reported a precolumn derivatization method for the determination of released fatty acids from washed rat platelets with fluorescent 9-aminophenanthrene, their HPLC conditions and extraction procedure for fatty acids were inadequate for a precise determination. By using the membrane filter method developed here, the aggregated platelets were removed quickly from the reaction mixture. Hence the precise quantification achieved can be attributed to the reactivity of MDC with carboxylic acids and the rapid extraction system mentioned above. Therefore, this method will permit the precise determination of the total changes in released fatty acids originating from various biological reactions. However, the roles of the liberated fatty acids from activated platelets and their metabolites related to membrane flexibility remain to be elucidated in the future.

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