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Note

Separation of thromboxane B_2 by high-performance liquid chromatography

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Arachidonic acid is metabolized in platelets via two different pathways [1]. The cyclooxygenase enzyme produces thromboxane (TX) A_2 in addition to 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), prostaglandin (PG) D_2 and PGE_2 whereas 12-hydroperoxy-5,8,10,14,17-eicosapentaenoic acid (12-HPETE) is formed via the lipoxygenase pathway [2,3]. The importance of the cyclooxygenase pathway in platelet function is due to the formation of TXA_2, a potent inducer of aggregation [2]. The search for selective inhibitors of cyclooxygenase and thromboxane synthase is important in developing compounds with therapeutic potential to control pathological processes mediated by PGH_2 and TXA_2. A number of non-steroidal anti-inflammatory drugs (e.g. aspirin, indomethacin, fenamic acid) have been found to inhibit cyclooxygenase but do not affect thromboxane synthase. The availability of a selective thromboxane synthase inhibitor would be useful for studying the function of platelet aggregation, thrombosis formation and atherosis formation. To evaluate the thromboxane synthase activity, the accurate separation and determination of TXB_2 derived from TXA_2 will be needed.

In recent studies using human or animal platelets, purification by preparative thin-layer chromatography (TLC) after the incubation of arachidonic acid or PGH_2 with platelets is commonly used [4–10]. In the present study, we compared the TLC method with a high-performance liquid chromatographic (HPLC) technique.

EXPERIMENTAL

High-performance liquid chromatography

A Shimadzu Model LC-6A high-performance liquid chromatograph equipped with a variable-wavelength spectrophotometric detector (Shimadzu SPD-6AV,

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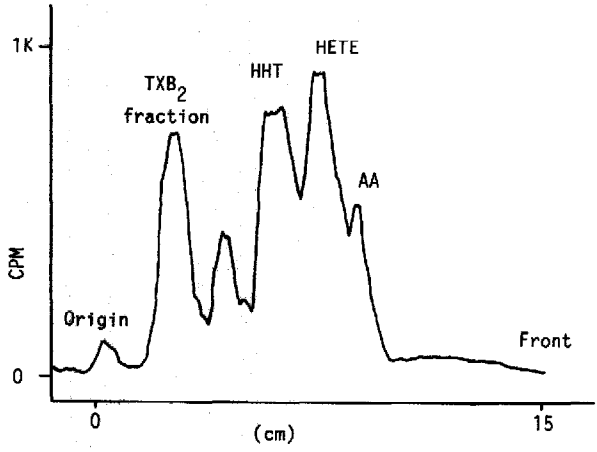

Fig. 1. Thin-layer radiochromatogram of the metabolites produced when rabbit platelets were incubated with [1-^{14}C]arachidonic acid. Solvent system: chloroform–ethyl acetate–methanol–acetic acid–water (70:30:8:1:0.5, v/v).

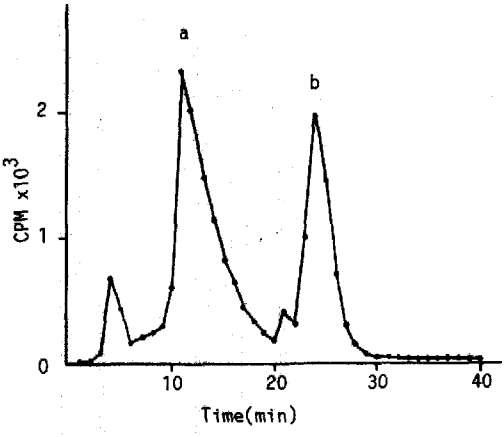

Fig. 2. Chromatogram of labelled TXB_2 fraction isolated from the incubation mixture of [1-^{14}C]arachidonic acid by TLC. Peaks: a = TXB_2; b = unknown compound.

Kyoto, Japan) was used. The column was packed with Hypersil ODS (5 μm, 250×4 mm I.D., Erma, Tokyo, Japan). The mobile phase was acetonitrile–water–sulphuric acid (35:65:0.03, v/v/v/). Other conditions were: column temperature, 25°C; flow-rate, 0.8 ml/min; detection wavelength, 196 nm; chart speed, 2.5 mm/min.

Reagents

[1-^{14}C]Arachidonic acid was purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabelled arachidonic acid, TXB_2 and prostaglandin were obtained from Funakoshi (Tokyo, Japan). Antimycin A was purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade.

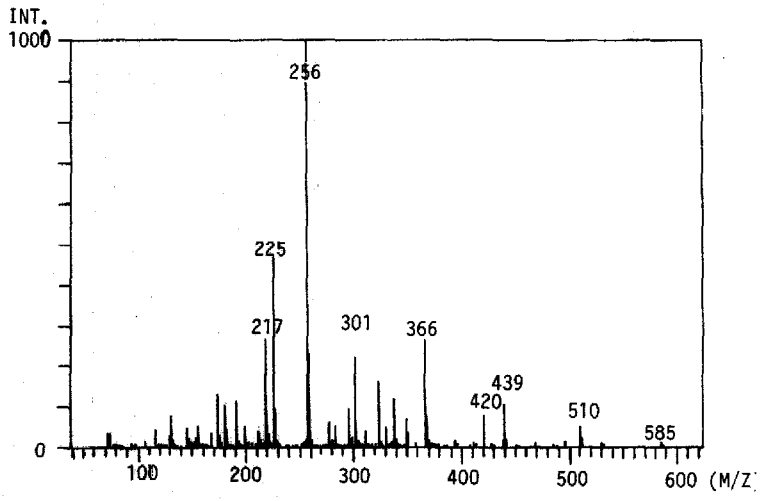

Fig. 3. Mass spectrum of TXB_2 (Me-TMS-TXB_2).

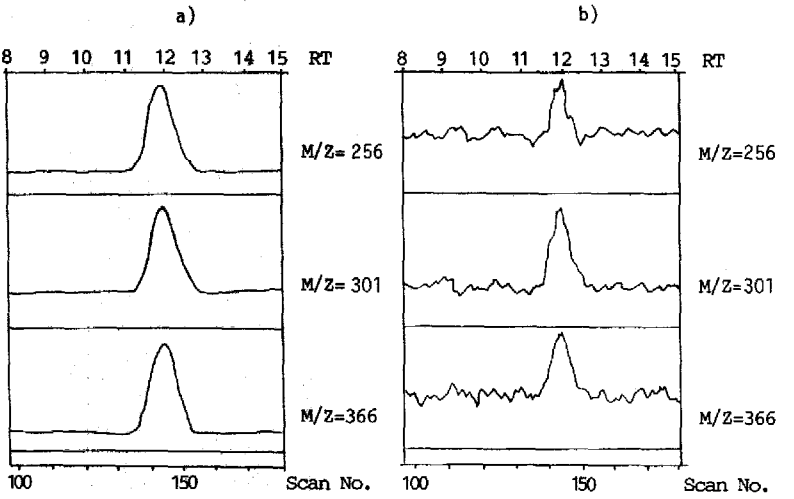

Fig. 4. Mass chromatogram of TXB_2 (Me-TMS-TXB_2). (a) Authentic TXB_2; (b) TXB_2 isolated from the incubation mixture of arachidonic acid. *m/z* 366, 301 and 256 are characteristic ions of Me-TMS-TXB_2.

Preparation of enzyme

Blood was obtained from normally fed New Zealand White rabbits (Charles River, Atsugi, Japan) weighing 2.5–3.0 kg under anaesthesia with somnopentyl (Pitman-Moore, Washington Crossing, NJ, U.S.A.). The blood, containing 3.8% trisodium citrate, was centrifuged at 200 *g* for 10 min. The platelet-rich plasma was centrifuged at 600 *g* for 15 min. The resulting platelet pellet was suspended in 25 m*M* Tris physiological saline buffer (pH 7.6) containing 1.8 μM antimycin A, 137 m*M* sodium chloride, 2.7 m*M* potassium chloride and 11 m*M* dextrose (TBS) and centrifuged again at 600 *g* for 15 min. This procedure was repeated twice. The washed platelet pellet was suspended in TBS and stored at $-80°C$.

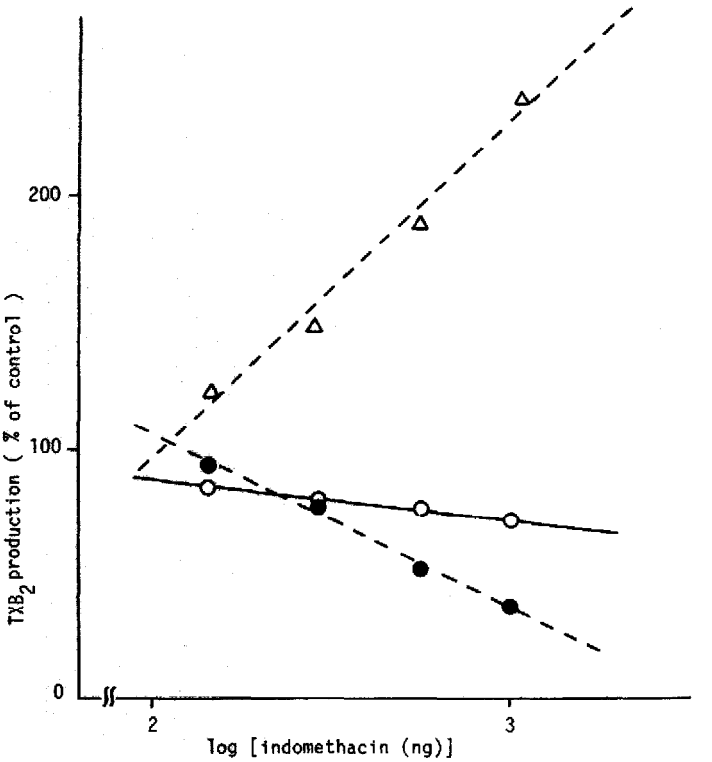

Fig. 5. Effect of indomethacin on the formation of TXB_2 purified by TLC and HPLC. ○, TXB_2 (TLC); ●, TXB_2 (HPLC); △, unknown compound (HPLC).

Before use, the pellet was thawed and sonicated. The protein content in this platelet fraction was 1.1 mg/ml by the Lowry method.

Incubation and chromatographic procedures

Platelet suspension, 2 ml (2.2 mg protein), was incubated for 15 min at 37°C with [1-^{14}C]arachidonic acid ($4 \cdot 10^5$ cpm). The reaction was stopped by the addition of 0.1 ml of 10% formic acid, and extracted with 10 ml of chloroform–methanol (2:1, v/v). The mixture was agitated with a vortex mixer and centrifuged. After the separation of the organic layer, authentic standards of TXB_2 and PGE_2 were added. The organic layer was evaporated in vacuo. The residue was redissolved in a small amount of methanol, applied to a TLC plate (Merck F_{254} glass plate) and developed with chloroform–ethyl acetate–methanol–acetic acid–water (70:30:8:1:0.5, v/v). Radioactive spots were detected with a radiochromatoscanner (Aloka, Tokyo, Japan). The TXB_2 fraction, which overlapped with the authentic unlabelled TXB_2, was scraped from the plate, eluted with methanol and further purified by HPLC on a reversed-phase column.

Preparation of the derivatives of TXB_2 for gas chromatography–mass spectrometry (GC–MS)

Diazomethane in diethyl ether was added to the samples dissolved in methanol, and the reaction mixture was allowed to stand for 60 min at room temperature.

The solvents were then evaporated under a stream of nitrogen. The methylated samples were treated with 50 μl of a mixed solution of hexamethyldisilazane and trimethylchlorosilane in an anhydrous pyridine (TMS-HT) (Tokyo Kasei, Tokyo, Japan) at room temperature for 120 min.

Gas chromatography–mass spectrometry

A JEOL DX-300 gas chromatograph–mass spectrometer interfaced with a JMA-3500 data system was used. A glass column 2 m × 4 mm I.D.) packed with 3% OV-1 on Chromosorb WAW DMSC (60–80 mesh) was used. The temperatures of the column, injection port and ionization chamber were maintained at 240, 260 and 190 °C, respectively. The flow-rate of helium was 40 ml/min. The electron-impact (EI) mass spectra were obtained at an electron energy of 30 eV, an emission current of 300 μA and an accelerating voltage of 3.0 kV.

RESULTS

Purification of TXB_2

The thin-layer radiochromatogram of the incubation products from [1-^{14}C]arachidonic acid is shown in Fig. 1. The sample contained one peak that corresponded to that of TXB_2 (R_F=0.16) in addition to the peaks of HHT (R_F=0.39), hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) (R_F=0.50) and arachidonic acid (R_F=0.61). The TXB_2 fraction, scraped from the thin-layer plate, was subjected to HPLC analysis, and the results are shown in Fig. 2. Two main peaks and a peak corresponding to TXB_2 were observed. Therefore, Me-TMS-TXB_2 was derived from the fractionated TXB_2 by HPLC and subjected to GC–MS analysis. Fig. 3 shows the mass spectrum of the authentic Me-TMS-TXB_2. Fig. 4 shows the mass chromatogram of Me-TMS-TXB_2. Specific ions at m/z 256, 301 and 366 for standard derivative were observed [11,12]. The specific ions of Me-TMS-TXB_2, purified by HPLC, corresponded well to those of the standard derivatives. From these results, the peak observed at 11 min was identified as TXB_2. The second peak, whose retention time was 24 min, could not be identified.

Comparative studies of both purification methods using TLC and HPLC

The radioactivity of the TXB_2 fraction obtained from the incubation mixture of [1-^{14}C]arachidonic acid by the purification method using HPLC after the preparative TLC was compared with that obtained by the purification method using TLC alone. The average formation rate of TXB_2 obtained by the purification method using HPLC was 7.4% (11 000 cpm) and that obtained by TLC only was 28.5% (42 600 cpm). The formation rate of the unknown compound whose retention time was 24 min was 3.8% (5700 cpm). Next, the inhibitory effects of indomethacin on the formation of TXB_2 were investigated. The results are summarized in Fig. 5. Indomethacin (0.3, 0.6 and 1.2 μg/ml) inhibited the TXB_2 formation in a dose-dependent manner when the HPLC method was applied. On the contrary, the formation of the unknown compound increased strongly after treatment with indomethacin. Indomethacin showed slight inhibition in the case of the purification using TLC alone.

DISCUSSION

Many investigators determined the radioactivity of the TXB_2 fraction scraped from the TLC plate for the screening of drugs [13–20]. Recently, purification of TXB_2 by HPLC has been reported in the case of the experiment using human platelets [21–25]. Their chromatograms show several peaks of the products from arachidonic acid, in which TXB_2 is the main peak. The same experiment using animal platelets has also been reported [26]. In the reports on rabbit platelets, HHT, HETE and TXB_2 were the main peak in both TLC and HPLC methods. Furthermore, the HPLC value for TXB_2 agreed well with TLC determination. In our experiment, the HPLC pattern was different from the reported results. The chromatogram obtained in our experiment showed two main peaks: the first peak was identified as TXB_2. The spots of PGE_2 and $PGF_{2\alpha}$, which had similar R_F values to those of TXB_2 in TLC, were not detected. The second peak was not identified with authentic samples, although it might be derived from arachidonic acid by the cyclooxygenase or lipoxygenase pathway in platelets. Furthermore, the radioactivity of the TXB_2 fraction obtained by the TLC method exceeded that obtained by the HPLC method. The IC_{50} value (50% inhibition constant) of indomethacin was 0.72 μg/ml in the HPLC method after purification by TLC. However, the IC_{50} value was not obtained by the TLC method in the range of doses used in this experiment, owing to weak inhibition. Conversely, the formation of the unknown compound was increased in a dose-dependent manner by treatment with indomethacin. Indomethacin (1.2 μg/ml) inhibited formation of TXB_2 by 62 and 26% in the HPLC and the TLC methods, respectively, and it increased the formation of the unknown compound by 145%. The reduced inhibitory effect of indomethacin in the TLC method could be due to the increase in the unknown compound in the TXB_2 fraction scraped from the TLC plate.

These results indicate that the HPLC method is more accurate than the TLC method in the determination of TXB_2 formation from the incubation mixture of arachidonic acid. We are currently investigating the structure of the remaining unknown compound, which increases during treatment with indomethacin.

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