107. A Radioimmunoassay for Thromboxane B_2 with Thromboxane B_2 - ^{125}I -Tyramide

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It has been reported that thromboxane A_2 ($\text{Tx}A_2$), which is derived from arachidonic acid within cell membrane, is more effective in inducing contraction of arterial smooth muscles and aggregation of platelets than are prostaglandin endoperoxides ($\text{PG} G_2$ and H_2).¹⁾⁻³⁾ Causal relationships between thromboxane A_2 and vascular injuries, in particular atherosclerotic vascular disease, have been given increasing attention.^{4),5)} However, a quantitative assay of $\text{Tx}A_2$ is impossible as the half life is less than 30 sec. Therefore, a simple and steady quantitative method for determination of thromboxane B_2 ($\text{Tx}B_2$), a stable metabolite of unstable $\text{Tx}A_2$, should enable clarification of the physiological role of $\text{Tx}A_2$. Currently, a gas chromatographic/mass spectroscopic method,⁶⁾ and radioimmunoassays⁷⁾⁻⁹⁾ have been reported.

For the radioimmunoassays, [^3H] $\text{Tx}B_2$, which is derived from [^3H]arachidonic acid enzymatically, was used as ligands. We developed a radioimmunoassay for $\text{Tx}B_2$ using $\text{Tx}B_2$ - ^{125}I -Tyramide (TA), in which the count is simplified and there is a greater sensitivity and selectivity. This method was utilized to study $\text{Tx}B_2$ formation during platelet aggregation, as induced by collagen.

Materials and methods. *Synthesis of $\text{Tx}B_2$ -TA.* Twenty mg of $\text{Tx}B_2$ (Fuji Pharmaceutical Co., Ltd.) and 8 mg of tyramine were allowed to react with 20 mg of N,N'-dicyclohexylcarbodiimide in 0.2 ml of N,N'-dimethylformamide. The reaction was carried out at 0 to 5°C for 1 hr and at a room temperature of 25°C for 6 hr. After removal of the precipitate by filtration, the mixture was evaporated to dryness. The evaporated residue was dissolved in a small amount of ethyl acetate and subjected to silica gel plate (0.2×20×20 cm, Merck). $\text{Tx}B_2$ -TA was purified by preparative Thin Layer Chromatography (TLC) using the solvent system ethyl acetate: acetic acid (99:1 v/v).

Iodination of $\text{Tx}B_2$ -TA. Iodination of $\text{Tx}B_2$ -TA was performed

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using the method of Hunter and Greenwood¹⁰⁾ with some modification. Five μg of TxB_2 -TA in 5 μl of ethanol was dissolved in 20 μl of 0.5 M phosphate buffer pH 7.4. Ten μl of $\text{Na } ^{125}\text{I}$ (Ca. 1.2 mCi) (CIS) was added and then 5 μg of Chloramine T in 5 μl of 0.05 M phosphate buffer pH 7.4 was added. The solution was allowed to react for 10 sec at a room temperature of 25°C and then 12.5 μg of sodium metabisulfite in 10 μl of 0.05 M phosphate buffer pH 7.4 was added. Thereafter, 20 μl of ethanol was added, and TxB_2 - ^{125}I -TA was purified by preparative TLC on silica gel plate in solvent system ethyl acetate : formic acid (80:1 v/v, $R_f=0.21$). Iodinated TxB_2 -TA had a specific activity of 216 $\mu\text{Ci}/\mu\text{g}$.

Preparation of immunogenic conjugate and immunization. Ten mg of TxB_2 was dissolved in 1.0 ml of tetrahydrofuran and cooled at 0°C. Twenty mg of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide HCL (Nakarai Chemicals Co., Ltd.) was added and the preparation was stirred for 20 min at 0°C. The mixture was dropwisely added to 40 mg of bovine serum albumin dissolved in 3 ml of distilled water, and the pH was adjusted to 5.5–6.0 with dilute hydrochloric acid, and stirred for 20 hr at a room temperature of 25°C. Then, the solution was dialyzed against tap water for 24 hr and lyophilized. This lyophilized TxB_2 -BSA conjugate was used for immunization of four New Zealand White rabbits. Immunization and collection of antiplasma were performed by the method of Granström *et al.*⁷⁾

Radioimmunoassay procedure. In the entire assay procedure, 0.02 M phosphate buffer pH 7.2 containing 5×10^{-3} M EDTA-2Na, 0.1% bovine serum albumin, 0.01% bovine r-globulin and 0.1% NaN_3 was used as assay buffer. Experiments to determine dilution of the rabbit antiplasma that bound 50% of the radioactive TxB_2 -TA were run as follows: 0.1 ml of TxB_2 - ^{125}I -TA (Ca. 8000 cpm) was added to 0.1 ml of assay buffer and 0.1 ml of diluted antiplasma in a small test tube. The tube was incubated for 20 hr at 4°C. 0.5 ml of the aqueous polyethylene glycol (PEG 6000) was added and the mixture was then centrifuged at 3,500 rpm, 5°C for 20 min. The supernatant solution was removed, and radioactivity of the precipitate was counted to determine Bo/T(%). For determination of the standard curve and cross reaction of various PGs (Fuji Pharmaceutical Co., Ltd.), 0.1 ml of assay buffer containing various concentrations PGs and 0.1 ml of antiplasma at a dilution (1:300) were added to each small test tube. 0.1 ml of TxB_2 - ^{125}I -TA (Ca. 8000 cpm) was then added to each tube. TxB_2 standard solutions were constituted so as to give concentrations ranging from 78 pg/ml to 100 ng/ml. The reaction was carried out for 20 hr at 4°C. Each tube was centrifuged after adding 0.5 ml of PEG 6000. Radioactivities

of the precipitate were counted to determine B/Bo(%).

Platelet aggregation studies. Platelet Rich Plasma (PRP) was prepared from a healthy male donor who had not taken any drugs for at least one week. Blood was collected from the cubital vein with 0.1 volume of 3.8% sodium citrate solution and centrifuged at 1000 rpm for 15 min at a room temperature of 25°C. The upper layer of the PRP was removed and kept at this same room temperature. Platelet counts of PRP were adjusted to about $30 \times 10^4/\text{cumm}$. Standard curve using PRP was prepared as follows: 0.1 ml of TxB₂-[¹²⁵I]-TA was added to 0.1 ml of PRP at dilution (1:10), 0.1 ml of standard solutions of TxB₂ and 0.1 ml of antiplasma at a dilution (1:300) in a small test tube. The preparations were then incubated for 20 hr at 4°C and centrifuged after adding 0.5 ml of PEG 6000. The precipitates were counted to determine B/Bo(%). In actual assay of platelet aggregation studies, collagen (Hormon-Chemie München), 3 µg/ml (final concentration), was added to each sample and aggregation was monitored by continuous recording of light transmission (Sienco dual sample aggregometer). Aliquots (10 µl) were removed from the cuvette at precise intervals, deposited in small test tubes containing 90 µl of assay buffer, pH 7.2, with 1×10^{-4} M indomethacin, and then, 0.1 ml of assay buffer and 0.1 ml of antiplasma at a dilution (1:300) were added. Thereafter, 0.1 ml of TxB₂-[¹²⁵I]-TA was added, the preparation, incubated, centrifuged, and counts done to determine B/Bo(%).

Results and discussion. TxB₂-TA was determined by TLC, in solvent system ethyl acetate : acetic acid (99:1 v/v, R_f=0.30) and by the spectrum of UV, NMR and IR. Purity of TxB₂-[¹²⁵I]-TA was more than 95% in TLC.

Two out of four rabbits, KI-1 and KI-4, responded to inoculation

Table I. Cross reaction of prostaglandins with thromboxane B₂ antiplasma

Compound	Nanograms required to displace 50% B/B ₀	Cross reaction (%)
Thromboxane B ₂	0.075	100
PG A ₁	>10,000	<10 ⁻⁵
PG B ₁	>10,000	<10 ⁻⁵
PG D ₂	4.6	1.6
PG F _{1α}	880	0.01
PG F _{2α}	420	0.02
PG F _{2β}	5,200	0.001
PG E ₁	3,400	0.002
PG E ₂	1,900	0.004
PG I ₂	>10,000	<10 ⁻⁵

Fig. 1. Accuracy of the radioimmunoassay for TxB_2 .

Fig. 2. Thromboxane B₂ formation during aggregation of platelet rich plasma (0.4 ml) with collagen (3 $\mu\text{g}/\text{ml}$). —○—: Thromboxane B₂.

with TxB₂-BSA conjugate after 3 to 5 months and showed 50% binding of TxB₂-[¹²⁵I]-TA at an antiplasma dilution of 1:300 to 1:600 (final antiplasma dilution, 1:1200 to 1:2400). KI-1 antiplasma was used in these experiments. No significant cross reaction was observed for any prostaglandin showing the highest affinity for TxB₂-[¹²⁵I]-TA (Table I). PGD₂ showed the greatest cross reaction, but in our assay system, the reaction was about 1.6% or less. In our radioimmunoassay system, the measurable ranges of TxB₂ level were from 5 to 500 pg/tube.

Furthermore, the recovery with this assay system was satisfactory as depicted in Fig. 1. We examined the TxB₂ level after addition of collagen to PRP by our radioimmunoassay system, and found that TxB₂ levels rose in a pattern similar to the aggregometer trace (Fig. 2). TxB₂ levels continued to rise for two min and such parallels the observations of Granström *et al.*⁷⁾ As lipids and fatty acids in PRP may have interfered with the absolute value of TxB₂ measured by our assay system, further studies concerning the absolute value of TxB₂ should be done. Relative comparisons between samples are however, feasible. The radioimmunoassay with TxB₂-[¹²⁵I]-TA provides a useful tool for detailed studies of the physiological role of TxA₂. On the other hand, in radioimmunoassays of TxB₂ so far reported, [³H]-TxB₂ was used as ligands and the radioactivities were measured by liquid scintillation counter. Our radioimmunoassay using TxB₂-[¹²⁵I]-TA enables simplification of the count compared with the radioimmunoassay using [³H]-TxB₂ as the radioactivity of TxB₂-[¹²⁵I]-TA could be easily measured by well type scintillation counter.

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