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Profiling of eicosanoids in inflamed gall bladder wall by gas chromatography with selected-ion monitoring

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ABSTRACT

The profiling of eicosanoids, including prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4), in dog and human gall bladders was carried out by a combination of an effective and convenient clean-up procedure and gas chromatography with selected-ion monitoring. The clean-up procedure was based on the stepwise elution of their methyl ester derivatives from a silica gel column with *n*-hexane–ethyl acetate and ethyl acetate–methanol in various ratios. The LTB_4 methyl ester was eluted with an *n*-hexane–ethyl acetate (2:1, v/v) fraction because LTB_4 is more lipophilic than the other eicosanoids. The present method permitted the quantitation of trace amounts of eicosanoids, including LTB_4, present in tissues in the order of pg/mg of protein, without interference from other endogenous substances. In experimental acalculous cholecystitis produced in dog, the levels of eicosanoids (except LTB_4) were significantly changed. Of these eicosanoids, the level of 6-keto-$PGF_{1\alpha}$ was significantly higher in the seromuscular layer and correlated with the observed severe morphological changes. In human chronic cholecystitis with gallstones, the level of 6-keto-$PGF_{1\alpha}$ in the mucosal layer was significantly higher than that in the seromuscular layer. These data suggest that prostaglandin I_2 may play an important pathophysiological role in the course of cholecystitis.

INTRODUCTION

The arachidonic acid (AA) cascade, which involves cyclooxygenase-derived prostaglandins (PG) and lipoxygenase-derived leukotrienes (LT), is thought to play an important role as a mediator of inflammation in the gall bladder [1–6]. PGE_2 causes vasodilation, increases vascular permeability, bile flow and net fluid secretion, decreases net fluid absorption, and exhibits a cytoprotective effect [7].

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$PGF_{2\alpha}$ causes muscle contraction and increases bile flow and net fluid secretion [8,9]. PGI_2 causes vasodilation, increases vascular permeability and mucosal mucin secretion, and inhibits platelet aggregation and leukocyte chemotaxis [10–12]. LTB_4 produces leukocyte chemotaxis, chemokinesis and degranulation, and increases vascular permeability mediated directly or indirectly by PGE_2 and PGI_2 [13].

Cholecystitis is one of the most important inflammatory processes in which the arachidonic cascade could play a role. The presence of PGE_2, $PGF_{2\alpha}$ and PGI_2 in gall bladder tissues in experimental cholecystitis and human chronic and acute cholecystitis has been reported [1–6].

The findings indicate that the eicosanoids play a role in the inflammatory process. Acute acalculous cholecystitis, which occurs against a background of sepsis, shock or other severe illness, has a very high mortality, and causes the severe histological gall bladder changes [14]. Acute acalculous cholecystitis can be induced experimentally by bolus injection of endotoxin [15].

Although the importance of eicosanoids is widely recognized, there has been no convenient method for their profile analysis in tissues by gas chromatography–mass spectrometry (GC–MS) in electron ionization mode because of the difficulty of purification. This paper describes a method for the profiling of eicosanoids in gall bladder tissue by combination of an effective and convenient clean-up procedure and GC–MS. Using this method, the profile of eicosanoids in the mucosal and seromuscular layers of the gall bladder in dogs with experimental acute acalculous cholecystitis and humans with chronic cholecystitis with gallstones were compared with histological findings.

EXPERIMENTAL

Reagents

PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, thromboxane B_2 (TXB_2) and LTB_4 were purchased from Funakoshi Yakuhin (Osaka, Japan). [3,3,4,4,$^2H_4$]-$PGF_{2\alpha}$, [3,3,4,4,-$^2H_4$]-6-keto-$PGF_{1\alpha}$ and ^{18}O-enriched water (97 atom % $H_2{}^{18}O$) were purchased from M.S.D. (Montreal, Canada). [3,3,4,4,-$^2H_4$]-TXB_2 was a gift from Ono Pharmaceutical (Osaka, Japan). [5,6,8,9,11,12,14,15,-$^2H_8$]-LTB_4 was kindly donated by Dr. T. Shimizu (Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo, Japan). Indomethacin was of medical grade as described in the Pharmacopoeia Japonica (11th ed.). AA-861, an inhibitor of leukotriene synthesis [16], was supplied from Takeda Chemical Industries (Osaka, Japan). Endotoxin (lipopolysaccharide from *Escherichia coli*) was purchased from Sigma (St. Louis, MO, USA). Dimethylisopropylsilyl (DMIPS) imidazole and methoxylamine hydrochloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Other reagents and solvent were of the highest purity commercially available.

Preparation of $^{18}O_2$-labelled 6-keto-$PGF_{1\alpha}$

$^{18}O_2$-labelled 6-keto-$PGF_{1\alpha}$ was prepared according to a modification of the procedure described by Strife and Murphy [17]. Ethereal diazomethane (2 ml) was added to a solution of 6-keto-$PGF_{1\alpha}$ (0.2 mg) in methanol (0.2 ml), and the resulting mixture was evaporated to dryness below 40°C under reduced pressure. The residue was dissolved in 0.2 *M* $Li^{18}OH$ (0.1 ml), and the resulting solution was sonicated for 2 h. The solution was acidified to pH 2 with 0.5 *M* hydrochioric acid and then extracted with ethyl acetate. The organic layer was washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate and evaporated. The above operation was repeated five times. The final product was dissolved in acetone (0.2 mg/ml) and stored at −20°C.

Animals

Mongrel dogs (10–30 kg) of either sex fasted for 6 h prior to experiments but allowed free access to water, were anaesthetized with pentobarbital (30 mg/kg) intravenously. Then, in the control group, six dogs underwent cholecystectomy. In the study group, five dogs were injected intravenously with endotoxin (0.8 mg/kg dissolved in 20 ml saline) and after 60 min underwent cholecystectomy. The gall bladder was immediately opened on the liver bed side and washed carefully with 100 ml of an ice-cold solution of 3 m*M* indomethacin dissolved in saline containing 30 μg of AA-861 to remove the bile. A small specimen of gall bladder tissue was placed in 10% formalin solution for one week, then placed on a glass slide and stained with hematoxylin-eosin for histological evaluation.

The remainder of the gall bladder was used for the analysis of eicosanoids. To separate the mucosal layer from the seromuscular layer, the specimen was placed on a glass slide and covered with another slide of the same size. One side of the slides was held by a double clip and the slides were immersed into *n*-hexane in a dry ice–acetone bath. The slides were separated from each other by gentle pressure [18]. The separated mucosal and seromuscular layers were dipped in 10 ml of ice-cold ethanol solution containing 5 ml of 3 m*M* indomethacin–ethanol and 5 ml of 3 m*M* AA-861–ethanol. The tissue in the ethanol solution was dissected with scissors and homogenized, and the homogenate was diluted to 20 ml with ethanol.

After the addition of a mixture of the internal standards (10 ng of $[^2H_4]$-PGE_2, 10 ng of $[^2H_4]$-$PGF_{2\alpha}$, 20 ng of $[^{18}O_2]$-6-keto-$PGF_{1\alpha}$, 10 ng of $[^2H_4]$-TXB_2 and 10 ng of $[^2H_8]$-LTB_4), 2 ml of the homogenate were used for quantitation of protein by the method of Lowry *et al.* [19].

Humans

Five patients, diagnosed as having chronic cholecystitis with gallstones, underwent cholecystectomy. The gall bladder specimens were removed and treated according to the procedure described above.

Extraction and purification

The homogenate (18 ml) was centrifuged at 7500 *g* for 15 min at 4°C. The upper layer was evaporated to complete dryness, and the residue was dissolved in 5 ml of 15% ethanol, and acidified to pH 3 with 0.1 *M* hydrochloric acid. After further centrifugation at 7500 *g* for 15 min at 4°C, the upper layer was poured over a Clin Elut 1005 cartridge (Analytichem International, Harbor City, CA, USA).

Prostaglandins and leukotrienes were eluted with 30 ml of ethyl acetate and evaporated to complete dryness under a nitrogen stream below 30°C. The residue was dissolved in 5 ml of 15% ethanol and acidified to pH 3, and the resulting solution was loaded on a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA) washed previously with ethanol and water.

The cartridge was washed with 10 ml of 15% aqueous ethanol solution and 10 ml of *n*-hexane, and the prostaglandins and leukotrienes were eluted with 10 ml of ethyl acetate. The extract was evaporated to dryness under reduced pressure and a nitrogen stream. The residue was dissolved in 0.5 ml of methanol.

Derivatization for GC–MS

Prostaglandins and leukotrienes were derivatized by the method described in the previous paper [20]. The methyl esters were obtained by treating the residue with 2 ml of freshly prepared diazomethane in methanol for 30 min at room temperature. Each methyl ester was evaporated to complete dryness under reduced pressure and a nitrogen stream, and dissolved in 3 ml of *n*-hexane–ethyl acetate (3:1, v/v). The methyl esters of prostaglandins and leukotrienes were purified and eluted stepwise from a silica gel column (50 mm × 8 mm I.D., 70–230 mesh, E. Merck, Darmstadt, Germany) using 20 ml of *n*-hexane–ethyl acetate solution with the ratios of 3:1 (v/v) and 2:1 (v/v), and 10 ml of the same mixture with a ratio of 1:1 (v/v), and then 20 ml of ethyl acetate–methanol (99:1, v/v).

The eicosanoid analysis was carried out using the *n*-hexane–ethyl acetate fraction with the ratio of 2:1 (v/v) for LTB_4, that of 1:1 (v/v) for TXB_2, and the ethyl acetate–methanol fraction with the ratio of 99:1 (v/v) for PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$. After evaporation to complete dryness, the residue was treated with 100 μl of methoxylamine hydrochloride (Tokyo Kasei Kogyo) in pyridine, and the resulting solution was kept at 60°C for 1 h. After methyloximation, the reaction mixture was evaporated to complete dryness under a nitrogen stream, 50 μl of DMIPS imidazole were added, and the mixture was allowed to stand at room temperature for 1 h. In order to remove excess silylating reagent, the resulting solution was applied to a Sephadex LH-20 column (30 mm × 8 mm I.D., Pharmacia, Uppsala, Sweden), and the final derivative was eluted with 2.5 ml of chloroform–*n*-hexane–methanol (10:10:1, v/v). After evaporation of the solvent, the residue was dissolved in 100 μl of *n*-hexane containing 1% pyridine (v/v). The LTB_4 methyl ester-DMIPS ether derivative was further purified by silica gel col-

umn chromatography using *n*-hexane–diethyl ether (8:2, v/v) as the eluting solvent. The samples were then analysed by GC–MS with selected-ion monitoring (SIM).

Gas chromatography–mass spectrometry

GC–MS was carried out using a Hitachi M-80B instrument with an M-0101 data-processing system. Chromatography was carried out on a 25 m × 0.32 mm I.D. fused-silica capillary column, cross-linked with methylsilicone (Ultral, Hewlett-Packard, Avondale, PA, USA), which was connected directly into the source of the mass spectrometer. Helium was used as a carrier gas at a flow-rate of 10 ml/min. The column temperature was 280°C for the analysis of PGE_2 and $PGF_{2\alpha}$, and was programmed from 200 to 280°C at 5°C/min and maintained at 280°C for the analysis of TXB_2, 6-keto-$PGF_{1\alpha}$ and LTB_4. SIM was carried out with an MS resolution ($M/\Delta M$) of 2000 for PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2, and of 5000 for LTB_4. The sample was injected by a moving needle-type solventless injector. The mass spectrometer was operated in the electron ionization mode. The ion source temperature was kept at 200°C, and the ionization potential and current were 70 eV and 200 μA, respectively.

Assay

Quantitation of eicosanoids was performed by SIM using the ions m/z 552.35 for PGE_2, m/z 556.38 for [$^2H_4$]-PGE_2, m/z 625.41 for $PGF_{2\alpha}$, m/z 629.44 for [$^2H_4$]-$PGF_{2\alpha}$, m/z 670.44 for 6-keto-$PGF_{1\alpha}$, m/z 674.44 for [$^{18}O_2$]-6-keto-$PGF_{1\alpha}$, m/z 670.44 for TXB_2, m/z 674.46 for [$^2H_4$]-TXB_2, 439.27 for LTB_4 and m/z 445.31 for [$^2H_8$]-LTB_4. The peak-area ratio of PGE_2 was calculated using the second eluting peak of its *syn/anti* isomers, and that of 6-keto-$PGF_{1\alpha}$ was carried out using the first peak of its *syn/anti* isomers.

Histological evaluation

Two full-thickness sections taken from each gall bladder strip were placed on a glass slide and stained with hematoxylin-eosin The slides were coded and evaluated in a blind manner by three pathologists, and the degree of inflammation was expressed using a scoring system. The degrees of muscle thickness, inflammatory cell infiltration, necrosis, edema, bleeding and ulceration were graded on a scale of 0 to 3, with 0 being normal, 1 being slight change, 2 being moderate change, and 3 being severe change. The final histological score for each gall bladder was taken as a sum and averaged.

RESULTS

Purification of LTB_4 and other eicosanoids in gall bladder tissue

Table I shows the absolute recovery of [^{3}H]-LTB_4 through clean-up procedure with a Clin Elut cartridge and with a Sep-Pak cartridge: the absolute recoveries

TABLE I

ABSOLUTE RECOVERY OF [^{3}H]-LTB$_4$

	Recovery (%)
Clin Elut	
Application	100.0
Ethyl acetate	86.9
Sep-Pak	
Application	100.0
Through	4.1
15% Ethanol	1.5
n-Hexane	1.4
Ethyl acetate	79.4
Silica gel chromatography by stepwise elution[a]	
Application	100.0
Through	0.6
n-Hexane–ethyl acetate	
5:1 (v/v), 10 ml	2.3
3:1 (v/v), 10 ml	7.4
2:1 (v/v), 10 ml	63.7
1:1 (v/v), 10 ml	13.8
Ethyl acetate–methanol	
99:1 (v/v), 20 ml	5.4

[a] Overall absolute recovery through the present purification was calculated to be 44.0%.

were 86.9 and 79.4%, respectively. The absolute recovery of the [^{3}H]-LTB$_4$ methyl ester through silica gel chromatography by stepwise elution is also shown in Table I. The major amount of LTB$_4$ was eluted with the *n*-hexane–ethyl acetate (2:1, v/v) fraction. The recovery of [^{3}H]-LTB$_4$ in 10 ml of *n*-hexane–ethyl acetate (2:1, v/v) was found to be 63.7%. In this assay, 20 ml of *n*-hexane–ethyl acetate (2:1, v/v) were used as the elution volume. The overall absolute recovery of [^{3}H]-LTB$_4$ was 44.0%, but there was no problem in quantitation by use of the internal standard. When there were sometimes interfering peaks in the analysis of LTB$_4$, further silica gel chromatography of the DMIPS derivative was found to be sufficient for their complete elimination, as described in the previous paper [21].

Fig. 1 shows the elution pattern on the SIM recordings of the methyl esters of LTB$_4$, TXB$_2$, 6-keto-PGF$_{1\alpha}$, PGE$_2$ and PGF$_{2\alpha}$.

Gas chromatography with SIM

Column temperature. GC separation of the methyl ester-methyloxime dimethylisopropylsilyl (Me-MO-DMIPS) ether derivatives of 6-keto-PGF$_{1\alpha}$ and its $^{18}O_2$-labelled analogue was remarkably improved by programming the temper-

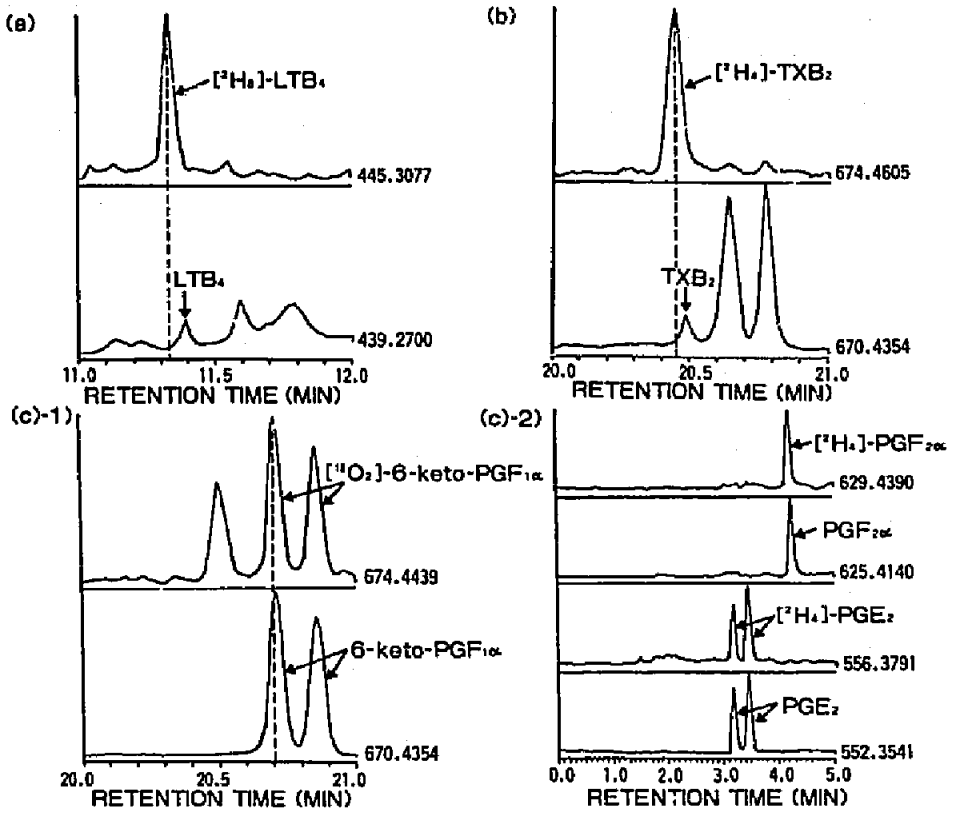

Fig. 1. Elution patterns on selected-ion recordings of the methyl esters of PGE_2, $PGF_{2\alpha}$, TXB_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 in gall bladder tissue. (a) *n*-Hexane–ethyl acetate (2:1, v/v) fraction: LTB_4. Selected-ion recordings of the Me-DMIPS derivatives of LTB_4 (*m/z* 439.27) and its $^2H_2$-labelled analogue (*m/z* 445.31). (b) *n*-Hexane–ethyl acetate (1:1, v/v) fraction: TXB_2. Selected-ion recordings of the Me-MO-DMIPS derivatives of TXB_2 (*m/z* 670.44) and its $^2H_4$-labelled analogue (*m/z* 674.46). (c) Ethyl acetate–methanol (99:1, v/v) fraction: 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$. (c-1) Selected-ion recordings of Me-MO-DMIPS derivatives of 6-keto-$PGF_{1\alpha}$ (*m/z* 670.44) and its $^{18}O_2$-labelled analogue (*m/z* 674.44). (c-2) Selected-ion recordings of Me-MO-DMIPS derivatives of PGE_2 (*m/z* 552.35) and its $^2H_4$-labelled analogue (*m/z* 556.38), Me-DMIPS derivatives of $PGF_{2\alpha}$ (*m/z* 625.41) and its $^2H_4$-labelled analogue (*m/z* 629.44).

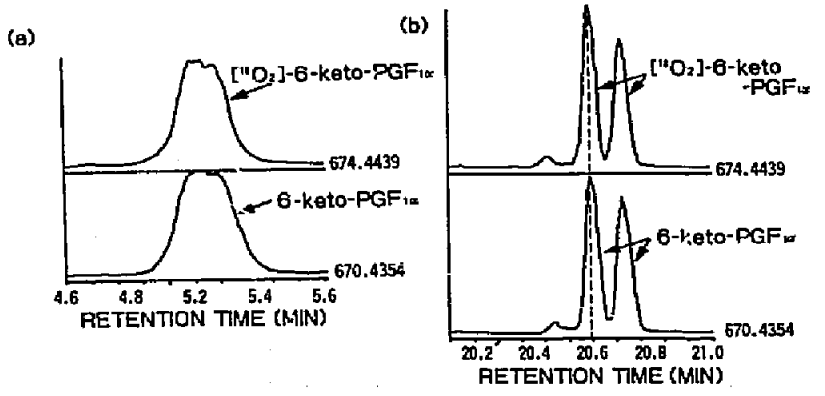

Fig. 2. Selected-ion recordings of the Me-MO-DMIPS derivatives of 6-keto-$PGF_{1\alpha}$ (*m/z* 670.44) and its $^{18}O_2$-labelled analogue (*m/z* 674.44), in the extract from gall bladder tissue, by monitoring the characteristic ion of $[M-43]^+$ (a) without temperature-programmed GC (maintained at 280°C) and (b) with temperature-programmed GC (programmed from 200 tot 280°C at 5°C/min and maintained at 280°C).

ature from 200 to 280°C at 5°C/min. Under this condition, baseline separation of the *syn* and *anti* isomers of 6-keto-$PGF_{1\alpha}$ was observed (Fig. 2).

Mass spectral resolution. Although SIM of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 was carried out after elevation of the mass spectral resolution from 2000 to 5000, there were no advantages in this procedure. In the case of LTB_4, however, the same increase prevented interference from endogenous substances.

Physicochemical isotope effect

On the stepwise elution of 6-keto-$PGF_{1\alpha}$ and its $^2H_4$-labelled analogue, a physicochemical isotope effect on the elution was observed in the *n*-hexane–ethyl acetate (1:1, v/v) and ethyl acetate–methanol (99:1, v/v) fractions: the peak-area ratios of the non-labelled form and its $^2H_4$-labelled analogue in the *n*-hexane–

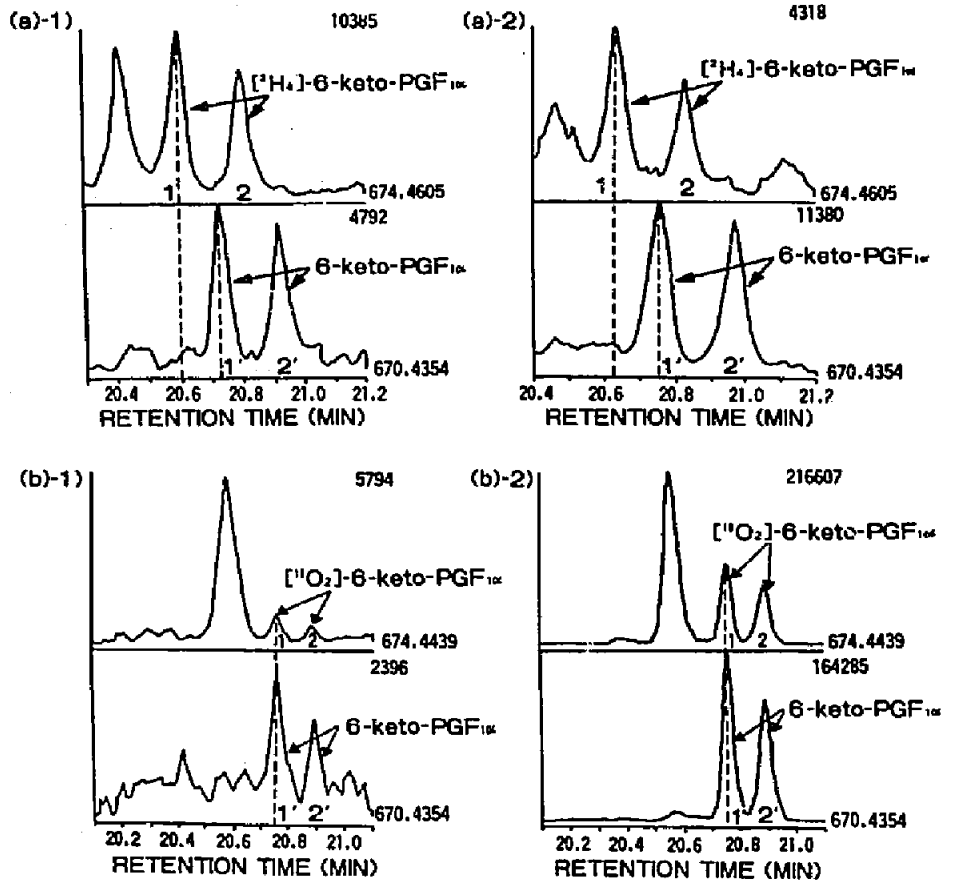

Fig. 3. (a) Selected-ion recordings of the Me-MO-DIMPS derivates of 6-keto-$PGF_{1\alpha}$ (*m/z* 670.44) and its $^2H_4$-labelled analogue (*m/z* 674.46) in the extract from gall bladder tissue. (a-1) *n*-Hexane–ethyl acetate (1:1, v/v) fraction; (a-2) ethyl acetate–methanol (99:1, v/v) fraction with temperature-programmed GC. (a-1) Area ratio = (peak 1')/(peak 1) = 0.45; (a-2) area ratio = (peak 1')/(peak 1) = 3.61. (b) Selected-ion recordings of the Me-MO-DMIPS derivatives of 6-keto-$PGF_{1\alpha}$ (*m/z* 670.44) and its $^{18}O_2$-labelled analogue (*m/z* 674.44) in the extract from gall bladder tissue. (b-1) *n*-Hexane–ethyl acetate (1:1, v/v) fraction; (b-2) ethyl acetate–methanol (99:1, v/v) fraction with temperature-programmed GC. (b-1) Area ratio = (peak 1')/(peak 1) = 1.77; (b-2) area ratio = (peak 1')/peak 1 = 1.67.

ethyl acetate and ethyl acetate–methanol fractions were found to be 0.45 and 3.61, respectively, as shown in Fig. 3a. This undesirable isotope effect was circumvented completely by use of the $^{18}O_2$-labelled analogue as an internal standard: the peak-area ratios of the non-labelled form and this analogue in the same fractions were found to be 1.77 and 1.67, respectively, as shown in Fig. 3b.

Accuracy of the eicosanoid analysis

In order to examine the accuracy of the present analysis, the recovery and reproducibility for PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2 and LTB_4 were examined statistically according to the orthogonal polynomial equation (Table II).

Clinical study

Normal dog gall bladder. Table III shows that the profiles of the eicosanoids are not significantly different in the mucosal and seromuscular layers of normal dog gall bladder. However, there was a tendency for the amount of 6-keto-$PGF_{1\alpha}$ in the seromuscular layer to be higher than that in the mucosal layer ($p<0.10$).

Dog gall bladder with cholecystitis. The profiles of the eicosanoids in the mucosal and seromuscular layers of dog gall bladder with cholecystitis are also shown in Table III. The levels of PGE_2, $PGF_{2\alpha}$, and TXB_2 were significantly higher in this circumstance than in normal dogs ($p<0.05$). The level of 6-keto-$PGF_{1\alpha}$ was also significantly higher in the mucosal layer ($p<0.05$), and especially in the seromuscular layer ($p<0.01$). However, the level of LTB_4 was not significantly higher in either layer. When the mucosal and seromuscular layers in experimental cholecystitis were compared, the level of PGE_2 in the mucosal layer showed a tendency to be higher than that in the seromuscular layer, and the level of LTB_4 showed the same tendency ($p<0.10$). The level of 6-keto-$PGF_{1\alpha}$ in the seromuscular layer was significantly higher than that in the mucosal layer ($p<0.01$). The level of TXB_2 in the mucosal layer was also significantly higher than that in the seromuscular layer ($p<0.05$). However, there was no significant difference in the levels of $PGF_{2\alpha}$ between the mucosal and seromuscular layers.

Human chronic cholecystitis with gallstones. Table IV shows the profile of eicosanoids in the mucosal and seromuscular layers of the gall bladder in human chronic cholecystitis with gallstones. The levels of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 in the mucosal layer were significantly higher than those in the seromuscular layer ($p<0.05$). There was no significant difference in the levels of LTB_4 between the mucosal and seromuscular layers. However, there was a tendency for the level of PGE_2 in the mucosal layer to be higher than that in the seromuscular layer.

Histological evaluation. Fig. 4 shows photomicrographs of representative histological sections of the mucosal and seromuscular layers in normal and inflamed dog gall bladders, where the inflammatory change was severe in the seromuscular layer and mainly edematous change. Table V indicates the histological scores for the gall bladder and morphological changes. There was a statistically high corre-

TABLE II

RECOVERIES OF EICOSANOIDS ADDED TO GALL BLADDER HOMOGENATE

The estimated amounts and their confidence limits were obtained according to the orthogonal polynomial equation.

Eicosanoid	Sample ($X_x + nx$)[a] ($n = 0, 1, 2, 3$)	Added (pg/mg protein)	Found (pg/mg protein)			Recovery (mean ± S.D.) (%)	Estimated amount ± 95% confidence limit
PGE_2	A (X_a)	0	150.00	152.47	153.72		156.72 ± 15.35
	B (X_a + a)	148.76	288.43	292.42	298.35	94.79 ± 1.77	
	C (X_a + 2a)	297.52	434.79	442.85	449.81	97.61 ± 1.55	
	D (X_a + 3a)	446.28	564.46	580.62	600.00	96.27 ± 2.92	
$PGF_{2\alpha}$	A (X_b)	0	26.12	24.67	26.86		26.02 ± 13.76
	B (X_b + b)	146.94	169.92	162.44	171.49	96.69 ± 2.08	
	C (X_b + 2b)	293.88	313.62	299.95	327.41	97.92 ± 3.51	
	D (X_b + 3b)	440.82	459.31	433.88	476.41	97.69 ± 3.76	
6-keto-$PGF_{1\alpha}$	A (X_c)	0	849.34	805.69	782.67		723.62 ± 137.14
	B (X_c + c)	386.85	1244.97	1184.88	1147.20	98.17 ± 3.28	
	C (X_c + 2c)	773.70	1665.36	1566.39	1504.00	99.01 ± 5.02	
	D (X_c + 3c)	1160.55	2177.94	2094.48	1985.12	109.71 ± 4.54	
TXB_2	A (X_d)	0	68.88	66.61	62.37		68.14 ± 15.07
	B (X_d + d)	13.26	82.67	78.94	75.21	97.94 ± 4.54	
	C (X_d + 2d)	26.52	96.52	90.71	86.89	95.84 ± 5.95	
	D (X_d + 3d)	39.78	109.94	102.92	100.90	97.13 ± 4.88	
LTB_4	A (X_e)	0	24.79	22.86	22.64		22.64 ± 13.23
	B (X_e + e)	82.64	110.41	101.76	94.91	95.51 ± 6.59	
	C (X_e + 2e)	165.28	187.66	168.50	195.21	96.69 ± 7.17	
	D (X_e + 3e)	247.92	264.63	251.02	286.63	98.42 ± 6.02	

[a] X_x = amount of eicosanoid present in gall bladder; nx = amount of eicosanoid added to gall bladder.

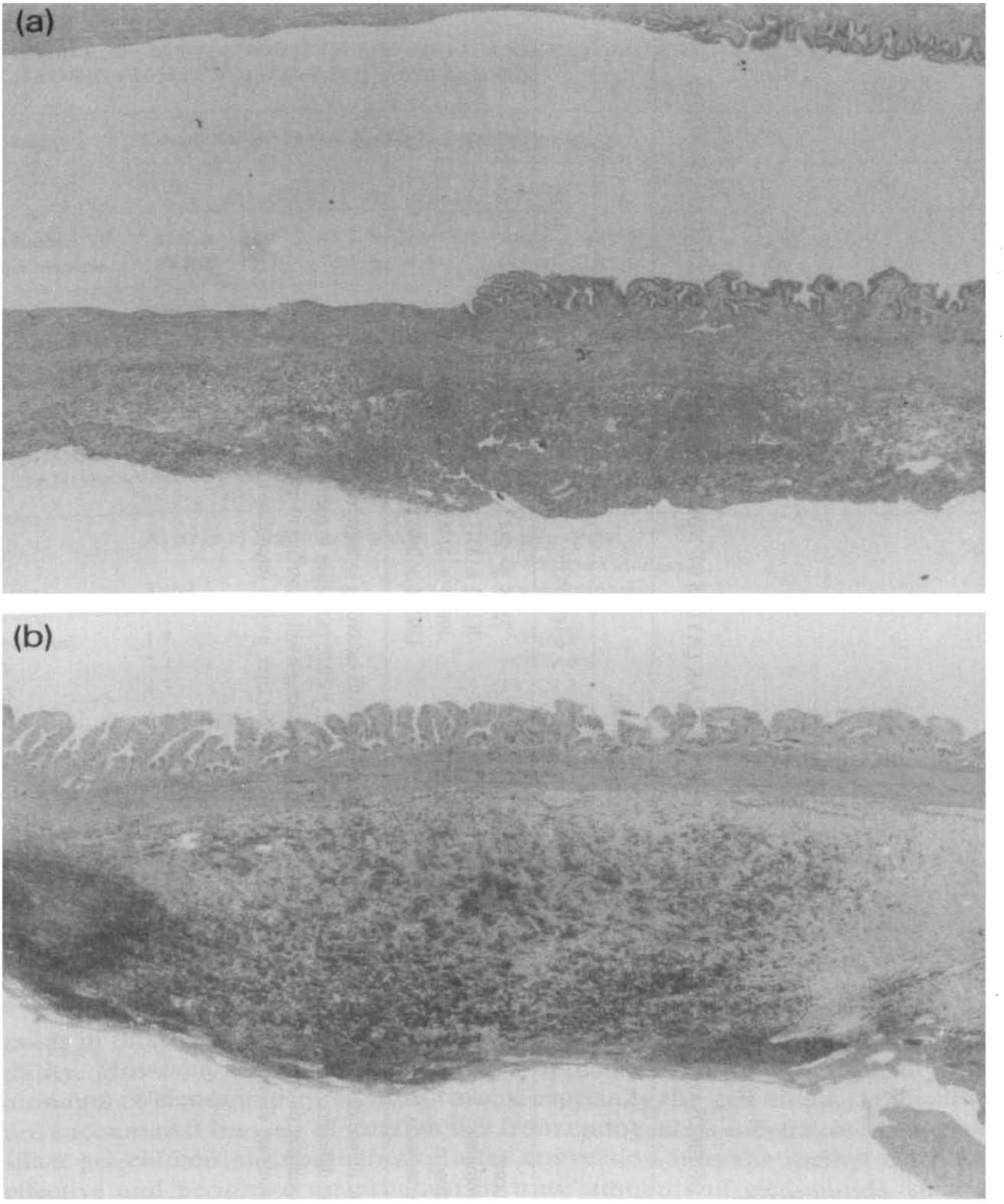

Fig. 4. Photomicrographs of representative histological sections of the mucosal and seromuscular layers in (a) normal dog and (b) inflamed gall bladder, stained with hematoxylin-eosin. (a) Initiation of mucosal removal on the left. (b) Initiation of inflammatory change involving marked edema and moderate bleeding with slight leukocyte aggregation in the seromuscular layer and minimal change in the mucosal layer.

TABLE III

PROFILE OF EICOSANOIDS IN MUCOSAL AND SEROMUSCULAR LAYERS OF NORMAL AND INFLAMED DOG GALL BLADDER

Sample	Concentration (mean ± S.D.) (pg/mg protein)				
	PGE_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TXB_2	LTB_4
Normal mucosa	151.4 ± 132.7	25.8 ± 10.6	728.5 ± 284.1	41.0 ± 23.8	20.8 ± 21.0
Normal seromuscle	123.5 ± 86.8	18.9 ± 9.1	3611.2 ± 3311.9	40.8 ± 15.5	7.6 ± 9.3
Inflamed mucosa	6840.3 ± 5355.2[a]	389.7 ± 267.1[a]	4965.3 ± 3174.0[a]	95.2 ± 43.5[a,b]	28.2 ± 12.9
Inflamed seromuscle	2470.5 ± 1678.1[a]	208.5 ± 101.6[a]	13803.6 ± 2606.8[c,d]	19.1 ± 12.3[a]	6.9 ± 4.0

[a] Significant difference between normal and inflamed gall bladder. $p<0.05$ (according to unpaired t-test).

[b] Significant difference between mucosal layer and seromuscular layer. $p<0.05$ (according to paired t-test).

[c] Highly significant difference between normal and inflamed gall bladder. $p<0.01$ (according to unpaired t-test).

[d] Highly significant difference between mucosal layer and seromuscular layer. $p<0.01$ (according to paired t-test).

TABLE IV

PROFILE OF EICOSANOIDS IN MUCOSAL AND SEROMUSCULAR LAYERS OF HUMAN CHRONIC CHOLECYSTITIS WITH GALLSTONES

Sample	Concentration (mean ± S.D.) (pg/mg protein)				
	PGE_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TXB_2	LTB_4
Mucosa	156.8 ± 152.2	43.3 ± 24.9[a]	306.7 ± 145.5[a]	34.1 ± 21.6[a]	19.7 ± 16.1
Seromuscle	30.5 ± 15.5	12.1 ± 6.7	110.4 ± 87.7	6.8 ± 4.1	2.8 ± 1.3

[a] Significant difference between mucosal layer and seromuscular layer: $p < 0.05$ (according to paired t-test).

TABLE V

HISTOLOGICAL SCORING OF INFLAMED GALL BLADDER AND MORPHOLOGICAL CHANGE; AVERAGE OF SCORING BY THREE PATHOLOGISTS

	Average of histological score		6-Keto-$PGF_{1\alpha}$ in seromuscular layer (pg/mg protein)
	Total	Edema	
Normal	1.7	0.7	5567.9
1	7.3	2.7	17 239.0
2	4.3	0.7	14 724.3
3	3.2	1.2	10 137.6
4	4.0	2.7	14 112.4
5	1.2	0.2	12 804.6

lation between the total score and the level of 6-keto-$PGF_{1\alpha}$ in the seromuscular layer ($r = 0.741$, $p < 0.01$), as shown in Fig. 5.

DISCUSSION

GC with SIM is the most suitable technique for the quantitation of eicosanoids in biological specimens because of its sensitivity, specificity and reproducibility. However, when this technique is applied to the quantitation of trace amounts of eicosanoids present in tissues, especially the gall bladder, difficulties are encountered because of interference from endogenous substances. The use of silica gel column chromatography after conversion into the methyl ester was effective and permitted quantitation of trace amounts of eicosanoids, such as PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2, and 11-dehydro-TXB_2, but not LTB_4, by GC with SIM [22–24]. Only a few papers have described the analysis of LTB_4 by GC with SIM in the electron ionization mode [25–27]. Recently, techniques using

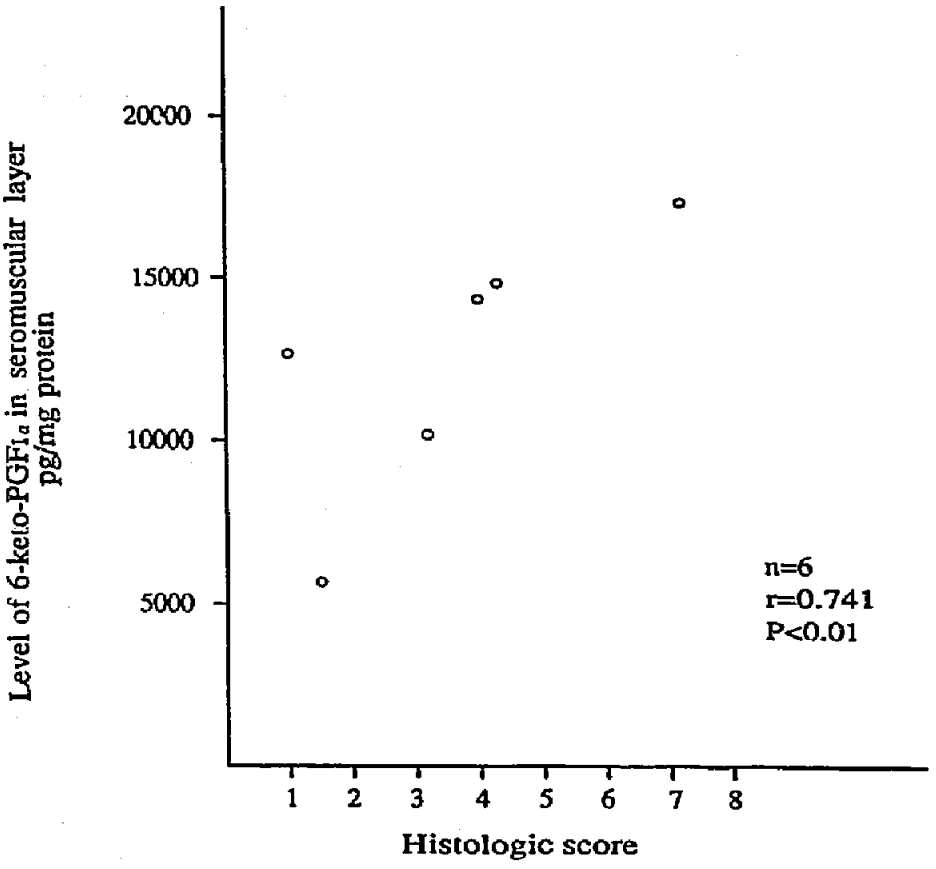

Fig. 5. Histological score plotted against the level of 6-keto-$PGF_{1\alpha}$ in the gall bladder seromuscular layer.

tandem mass spectrometry (MS–MS) and electron-capture negative-ion chemical ionization mass spectrometry (NICI-MS) have been applied to the analysis of LTB_4 in biological specimens [28–30] because of the difficulty of purification in combination with GC with SIM. In the present investigation, an effective and convenient clean-up procedure was developed. Using silica gel chromatography after conversion into the methyl ester, the elution pattern of LTB_4 was examined in detail by stepwise elution with mixtures of *n*-hexane and ethyl acetate in various ratios, and the LTB_4 methyl ester was found to be eluted with the *n*-hexane–ethyl acetate (2:1, v/v) fraction. This indicates that LTB_4 methyl ester is more lipophilic than the other eicosanoids. This method permitted quantitation of eicosanoids including LTB_4 in gall bladder tissue in combination with GC with SIM under the conventional electron ionization mode to be in the order of pg/mg of protein. Further clean-up of the LTB_4 methyl ester–DMIPS derivative by silica gel chromatography permits complete elimination of the interfering peaks.

On stepwise elution, TXB_2 was eluted with the *n*-hexane–ethyl acetate (1:1, v/v) fraction, and PGE_2 and $PGF_{2\alpha}$ were eluted with ethyl acetate–methanol (99:1, v/v). We found that the stepwise chromatographic behaviour of the methyl

esters of non-labelled and $^2H_4$-labelled 6-keto-$PGF_{1\alpha}$ was different. 6-Keto-$PGF_{1\alpha}$ was eluted with the *n*-hexane–ethyl acetate (1:1, v/v) and ethyl acetate–methanol (99:1, v/v) fractions, but mainly in the latter, whereas [$^2H_4$]-6-keto-$PGF_{1\alpha}$ was eluted mainly with the *n*-hexane–ethyl acetate (1:1, v/v) fraction. This was probably due to an isotope effect of the deuterated analogue on the elution, suggesting that the latter analogue was unsuitable as an internal standard. This isotope effect was successfully avoided by using the $^{18}O_2$-labelled analogue [17] as an internal standard: [$^{18}O_2$]-6-keto-$PGF_{1\alpha}$ as well as 6-keto-$PGF_{1\alpha}$ were eluted with the same fraction. In GC with SIM we found that a programming mode for the GC column temperature was very effective for separating the overlapping peaks of the syn and *anti* isomers of 6-keto-$PGF_{1\alpha}$ ME-MO-DMIPS ether. The use of an $^{18}O_2$-labelled internal standard and the improvement of GC conditions permitted the quantitation of 6-keto-$PGF_{1\alpha}$ in gall bladder tissue with high reliability: it was found to be much higher than that of TXB_2. The group separation of TXB_2 and 6-keto-$PGF_{1\alpha}$ by this stepwise elution is convenient for the quantitation of TXB_2 with high reliability.

The role of prostaglandins in cholecystitis has recently become a focus of interest [1–6]. Thornell *et al.* [3] demonstrated an increase of luminal PGE_2 in experimental cholecystitis, whereas Kaminski and coworkers [1,2,4,5] found an increase of PGE_2 in experimental cholecystitis and also of PGE_2 and PGI_2 in human cholecystitis. They suggested that prostaglandins might play an important pathophysiological role in the course of cholecystitis, and also speculated that other eicosanoids, such as leukotriene, might be involved. In the present study, we performed profile analysis of eicosanoids including LTB_4 in order to clarify their role in cholecystitis. We found no significant changes in the level of LTB_4, but did find significant changes in the levels of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 in inflamed gall bladder. Among these eicosanoids, the level of 6-keto-$PGF_{1\alpha}$ was found to be closely correlated with the morphological changes in both the mucosal and seromuscular layers.

In experimental cholecystitis induced by endotoxin, the levels of PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were significantly increased in both the mucosal and seromuscular layers, but not the level of LTB_4. The most pronounced increase was that of 6-keto-$PGF_{1\alpha}$ in the seromuscular layer. Becker *et al.* [15] reported that in experimental cholecystitis induced by endotoxin, the morphological change was observed mainly in the seromuscular layer, in agreement with the present investigation showing mainly marked edema, moderate bleeding and slight leukocyte aggregation in the seromuscular layer at this stage. Of five eicosanoids examined, 6-keto-$PGF_{1\alpha}$ was most closely related to the degree of inflammation, indicating that PGI_2 may play an important role in the occurrence of cholecystitis. TXB_2 was found to be decreased in the seromuscular layer in proportion to the increase of 6-keto-$PGF_{1\alpha}$, *ie.* thromboxane A_2 (TXA_2) has the opposite effect to PGI_2 in the mediation of the inflammatory process. PGE_2, PGI_2, and LTB_4 are thought to be possible mediators of the increase of vascular permeability and

plasma leakage [31,32]. The present findings indicate that edema in the seromuscular layer may be caused by PGI_2 via vasodilation and an increase of vascular permeability. Williams *et al.* [6] reported the increase of PGI_2 production in the gall bladder wall during endotoxin-induced septic shock, and indicated its cytoprotective effect on the mucosa. The increase of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the mucosal layer may thus exert mucosal cytoprotection to the endotoxin-induced inflammation. LTB_4 is known to mediate leukocyte aggregation [33]. However, only slight leukocyte aggregation was seen in both the mucosal and seromuscular layers, and there was no significant change in the levels of LTB_4. There have been several reports on the time course of proinflammatory eicosanoids, such as PGE_2, TXB_2, PGI_2 and LTB_4, during experimental inflammation [34–36]. The inflammation observed in the present study was thought to be an early stage of acute inflammation. Therefore, we speculate that LTB_4 is likely to increase and cause leukocyte aggregation in the later stage, and may play an important role in the course of cholecystitis. In the early stage of acute inflammation, the increase of PGI_2 seemed to be closely related to the degree of morphological change. Therefore, PGI_2 may be closely related to the induction of acute acalculous cholecystitis with endotoxemia.

In human chronic cholecystitis with gallstones, Kaminski and co-workers [4,5] noted an increase of PGE_2 in the mucosal layer and of PGI_2 in the seromuscular layer. In the present study, the level of PGE_2 in the mucosal layer tended to be higher than that in the seromuscular layer, but the difference was not significant. The levels of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ and TXB_2 in the mucosal layer were also found to be significantly higher than in the seromuscular layer. Samuelsson [13] found that PGI_2 exerted a proinflammatory effect and, in most systems, was more potent than PGE_2. In human chronic cholecystitis, the morphological change is more marked in the mucosal than in the seromuscular layer. We found that the level of PGI_2 seemed to be well correlated with the morphological changes observed in the gall bladder wall. LaMont *et al.* [10] reported that mucin secretion appeared to be regulated by endogenous PGI_2, and speculated that mucin secretion stimulated by prostaglandins favoured nucleation during gallstone formation [11]. It is not yet clear whether the increase of PGI_2 exerts a proinflammatory effect or an increase of mucin secretion that contributes to the cytoprotective mucosal diffusion barrier [37] and gallstone formation by promoting nucleation. Nevertheless, PGI_2 certainly plays an important role in the course of human chronic cholecystitis with gallstones.

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