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In-vitro Evaluation of 5-Lipoxygenase and Cyclo-oxygenase Inhibitors using Bovine Neutrophils and Platelets and HPLC

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Abstract—Inhibition of 5-lipoxygenase has been determined by monitoring the formation of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid in bovine polymorphonuclear leucocytes. For evaluating the inhibition of cyclo-oxygenase two different test systems are presented: the first uses 12-hydroxyheptadecatrienoic acid produced by bovine platelets as an indicator of the cyclo-oxygenase activity; the second test system monitors the prostaglandin E_2 formation by bovine platelets. All arachidonic acid metabolites are quantified by reverse-phase HPLC with UV-detection.

Cyclo-oxygenase and 5-lipoxygenase are key enzymes in the metabolism of arachidonic acid (AA). The metabolites formed possess a wide range of physiological and pathophysiological activities. The cyclo-oxygenase-derived prostaglandins participate in the genesis of inflammation, fever and pain and the 5-lipoxygenase-generated leukotrienes are postulated to play an important role in the pathophysiology of inflammatory diseases, asthma and psoriasis (Samuelsson 1983; Bailey 1985).

Published in-vitro test systems for the evaluation of 5-lipoxygenase inhibitors use radioimmuno-assays (Salmon et al 1982; Mita et al 1986; Ozaki et al 1986; Ikuta et al 1987) or HPLC (Hubbard et al 1979; Harvey & Osborne 1983; Iwakami et al 1986; Steinhilber et al 1986; Wagner & Fessler 1986; Buckle et al 1987; Musser et al 1987; Summers et al 1987; Sweeny et al 1987a,b; Mita et al 1988; Ninnemann 1988; Salmon et al 1989) to detect the 5-lipoxygenase products leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) generated in rat basophil leukaemia (RBL-1) cells or polymorphonuclear leucocytes (PMNL) of man, rat, swine or guinea-pig, respectively.

In-vitro cyclo-oxygenase assays mostly apply radioimmunoassays to determine the cyclo-oxygenase product prostaglandin E_2 (PGE_2) formed for example, by macrophages or RBL-1 cells (Brune et al 1981; Levine 1983). In other systems [^{14}C]AA is added to cyclo-oxygenase isolated from bovine or sheep semen, the produced prostaglandins are quantified by liquid scintillation after TLC separation (Terashita et al 1986; Wagner et al 1987). Although it is possible to measure prostaglandins after HPLC, using UV-detection (Desiderio et al 1981; Terragno et al 1981; van Rollings et al 1982; Rydzik et al 1984) this method has not been applied for the evaluation of cyclo-oxygenase inhibitors because the extinction coefficients and the maximum of absorption of the prostaglandins (192 nm) are low; the single cyclo-oxygenase product, which has been used to assess cyclo-oxygenase activity is 12-hydroxyheptadecatrienoic acid (12-HHT) (maximum absorption, 232 nm), formed in rat peritoneal cells (Sweeney et al 1987b).

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We report here effective, reliable and inexpensive test systems for the evaluation of inhibitors of 5-lipoxygenase and cyclo-oxygenase using bovine polymorphonuclear leucocytes and bovine platelets as enzyme sources and isocratic HPLC for the determination of the enzyme products LTB_4, 5-HETE, 12-HHT and PGE_2. These cells have not been used as an enzyme source for evaluating inhibitors of 5-lipoxygenase or cyclo-oxygenase.

Materials and Methods

Materials

All reagents used were of analytical grade and obtained as follows: salts for buffer solutions, solvents: Merck, Darmstadt (Germany); calcium ionophore A 23187 (free acid), nordihydroguaiaretic acid (NDGA), Histopaque 1077, indomethacin: Sigma, München (Germany); HPLC reference substances LTB_4, LTC_4, 5*S*,12*S*-DiHETE, 12-HHT, 5-HETE, 12-HETE, TXB_2 and PGE_2, HPLC internal stanards PGB_2 and 15-keto-PGE_2: Paesel, Frankfurt am Main (Germany); REV-5901 (Coutts et al 1985), E/Z-7-phenyl-7-(3-pyridyl)-6-heptenoic acid (CV-4151/CV-4193) (Kato et al 1985; Hecker et al 1986) and HPLC-internal standard, 3-(2,2-dimethyl-5,6-diphenyl-2,3-dihydro-1H-pyrrolizin-7-yl)-butyric acid (Dannhardt & Lehr 1990) were synthesized by the published procedures; bovine blood was obtained from the local abattoir.

Apparatus

Solid phase extraction was by use of a Baker-10 SPE system using octadecyl reverse-phase extraction columns (500 mg, 6 mL, Baker, Groß-Gerau, Germany).

HPLC was by use of a Kontron HPLC pump 420 equipped with a Rheodyne 7125 device (sample loop 2 mL), a Kontron Uvikon 735 LC UV-detector and a Merck Hitachi D-2000 Chromato-Integrator. The separation of the AA metabolites was performed on a Nucleosil 7 C_{18} column, 250 × 4 mm (Macherey & Nagel, Düren, Germany). A Sysmex Microcellcounter CC-130 was used for counting polymorphonuclear leucocytes and a Sysmex Plateletcounter PL-100 for platelets.

Cell preparations

Preparation of bovine polymorphonuclear leucocytes for the 5-lipoxygenase assay. Bovine polymorphonuclear leucocytes (PMNL) were isolated as described previously (Walstra et al 1984) with modification as follows. A solution of 0·077 M EDTA disodium in 0·2% saline (0·1 L per 1 L blood) was used to inhibit blood coagulation; the last centrifugation was carried out at 50 *g* for 15 min to remove most of the remaining platelets. The pellet was then resuspended in phosphate buffered saline (0·9% w/v) which had been stirred vigorously with a magnetic stirrer (5 min) to enrich the solution with oxygen. The final cell concentration was adjusted to 6×10^7 cells mL^{-1}. The yield was about 3×10^9 cells per 1 L blood.

Preparation of bovine platelets for the cyclo-oxygenase assays. One litre EDTA disodium solution (0·027 M) containing bovine blood was diluted with 0·5 L phosphate buffered saline and centrifuged at 200 *g* for 20 min in plastic tubes. The platelet-rich supernatant was carefully separated by aspiration. The leucocyte- and erythrocyte-containing lower layer was diluted with phosphate buffered saline to 1·5 L and centrifuged at 200 *g* for 20 min. The combined platelet-rich fractions were centrifuged at 1000 *g* for 15 min, the pellet was washed with phosphate buffered saline containing EDTA disodium (1 mg mL^{-1}). After centrifugation at 1000 *g* for 15 min the thrombocytes were resuspended in phosphate buffered saline which had already been saturated with oxygen as described above. The final cell concentration was adjusted to 5×10^8 cells mL^{-1} (1 mL of this suspension diluted with 9 mL phosphate buffered saline had an absorption of 0·25–0·30 at 800 nm). The yield was about 6×10^{10} cells per 1 L blood.

Incubation procedures

5-Lipoxygenase assay. A sample of dimethylsulphoxide (DMSO) solution of drug, 2·5 μL, and 0·8 mL of the bovine PMNL suspension were preincubated in ground glass borosilicate tubes for 10 min at 37°C, and after 5 min, 0·2 mL $CaCl_2$ solution (10 mM in 0·8% w/v saline) was added. The cells were stimulated by adding 2·5 μL of a DMSO solution of calcium ionophore A 23187 (4·2 mg mL^{-1} = final concentration of 20 μM) and the incubation was continued for 5 min (in the kinetic tests the incubation time varied) at 37°C. AA metabolism was terminated by the addition of 1 mL of a mixture of acetonitrile and methanol (1 : 1 v/v) containing 0·2 μg PGB_2 as internal standard and 6 μg NDGA as oxygen scavenger. The glass tubes were cooled for 20 min in an ice bath and then centrifuged at 4000 *g* for 15 min at 0°C. The supernatants were stored at −20°C.

Cyclo-oxygenase assay using 12-HHT as indicator of enzyme activity. The test was carried out under the conditions described for the 5-lipoxygenase assay (see above) except for the application of 0·8 mL bovine platelet suspension instead of PMNL suspension and the use of 3-(2,2-dimethyl-5,6-diphenyl-2,3-dihydro-1H-pyrrolizin-7-yl)-butyric acid (0·47 μg mL^{-1} acetonitrile-methanol solution) as internal standard instead of PGB_2 and incubation for 1 min instead of 5 min after calcium ionophore A 23187 stimulation. Using exogenous AA, 50 μL of a sodium arachidonate solution (0·14 mg mL^{-1} phosphate buffered saline = final concentration 20 μM) was added instead of calcium ionophore A 23187 solution.

Cyclo-oxygenase assay using PGE_2 as indicator of enzyme activity. A 7·5 μL sample of a DMSO solution of CV-4151/CV-4193 (0·28 μg mL^{-1} = final concentration 1 μM), 7·5 μL of a DMSO solution of drug and 2·4 mL of the bovine platelet suspension were preincubated in ground glass borosilicate tubes for 10 min at 37°C and after 5 min 0·6 mL $CaCl_2$ solution (10 mM in 0·8% w/v saline) was added. The cells were stimulated by adding 7·5 μL of a DMSO solution of calcium ionophore A 23187 (4·2 mg mL^{-1} = final concentration 20 μM) and the incubation was continued for 5 min (in the kinetic tests incubation time varied) at 37°C. By the addition of 3 mL of a mixture of acetonitrile and methanol (1 : 1 v/v) containing 0·8 μg 15-keto-PGE_2 as internal standard and 18 μg NDGA as oxygen scavenger the enzyme reaction was terminated. The glass tubes were cooled for 20 min in an ice bath and centrifuged at 4000 *g* for 15 min at 0°C. The supernatants were stored at −20°C.

Sample preparation

5-Lipoxygenase assay. The supernatants were diluted with 10 mL of water and the AA metabolites isolated with octadecyl reverse-phase extraction columns following the procedure of Verhagen et al (1986). The eluant (3 mL) was diluted with 3 mL of water and then subjected to HPLC. (Note: the octadecyl reverse-phase extraction columns were reused about 20 times.)

Cyclo-oxygenase assay using 12-HHT as indicator of enzyme activity. The sample preparation was carried out as described for the 5-lipoxygenase assay.

Cyclo-oxygenase assay using PGE_2 as indicator of enzyme activity. The sample preparation was carried out as described for the 5-lipoxygenase assay, except that the supernatants were diluted with 30 mL of water before application to the extraction column, the AA metabolites were eluted twice with 0·6 mL of methanol and the eluant was diluted with 4 mL of water before HPLC.

Reverse-phase HPLC analysis

Two mL samples were injected on a Nucleosil 7 C_{18} column (see apparatus). After each run the column was washed twice with 2 mL of methanol. The AA metabolites were quantified by peak area ratio. The absolute amount of the enzyme products was calculated using the molar absorption coefficients (Terragno et al 1981; Powell 1985). Further conditions were as follows.

5-Lipoxygenase assay. For the determination of LTB_4 a previously described mobile phase was used (Walstra et al 1984). Absorption was monitored at 270 nm. Where necessary, LTB_4 was separated from the test compound using acetonitrile-ethanol-water-acetic acid (30:30:40:0·1 v/v/v/v adjusted to pH 5·5 with conc. ammonium hydroxide) as the solvent system at a flow rate of 1·0 mL min^{-1}.

5-HETE was eluted with methanol-water-acetic acid (77:23:0·1, v/v/v, pH 5·5) at a flow rate of 1·0 mL min^{-1} and detection at 232 nm.

Cyclo-oxygenase assay using 12-HHT as indicator of enzyme activity. 12-HHT and 12-HETE were eluted with methanol/water/acetic acid (76:24:0·1 v/v/v, pH 5·5) at a flow rate of 1·0 mL min^{-1} and detection at 232 nm.

Cyclo-oxygenase assay using PGE_2 as indicator of enzyme activity. PGE_2 was eluted with acetonitrile/water/$NaH_2PO_4 \cdot 2H_2O$ (28:72:0·1 v/v/w) at a flow rate of 1·5 mL min^{-1} and detection at 192 nm. All solvents were degassed by sonication.

Solubility of the test compounds

To check the solubility of test compounds, 1·6 mL phosphate buffered saline and 0·4 mL of $CaCl_2$ solution (10 mM in 0·8% w/v saline) were added to 5 μL of a DMSO solution of the drug (usually 4 μmol $mL^{-1} = 10$ μM in the buffer solution). After centrifugation at 4000 *g* for 15 min the concentration of the test compound in the supernatant was measured by HPLC. To another 5 μL of DMSO stock solution of the drug, 2 mL of a mixture of methanol and water (1:1 v/v) was added and the drug concentration was determined. The ratio of both concentrations showed the solubility.

Statistics

For evaluation of enzyme inhibition the formation of the enzyme product (LTB_4, 5-HETE, 12-HHT, PGE_2 or 12-HETE) in the treatment group (n = 2 per incubation) was compared with the mean level in the control group (n = 4 per incubation). An average inhibition of 15% was significantly different from control as evaluated by Student's *t*-test ($P < 0.05$).

Results and Discussion

The formation of leukotrienes from AA by 5-lipoxygenase in bovine PMNL has been described (Walstra et al 1984). However, bovine PMNL has not been used as an enzyme source for evaluating inhibitors of 5-lipoxygenase.

The formation of 5-lipoxygenase products by bovine PMNL in our assay was initiated with calcium ionophore A 23187 in the presence of calcium. After 5 min the enzyme reactions were stopped by addition of a mixture of methanol-acetonitrile containing nordihydroguaiaretic acid (NDGA) as oxygen scavenger and prostaglandin B_2 (PGB_2) as internal standard. The centrifuged test solution was diluted with water and the 5-lipoxygenase products were isolated using octadecyl-reverse phase extraction columns (Verhagen et al 1986). In contrast, in our procedure the methanol eluate (3 mL) was not concentrated before HPLC analysis, but was diluted with water (2 mL). The solvent strength of the resulting mixture was weaker than that of the eluent allowing the injection of relatively large sample volumes (2 mL) (Powell 1985). This was an improvement compared with previously described procedures (Steinhilber et al 1986) because stressing of the sensitive samples by concentration was avoided and the time of analysis was reduced. Figs 1 and 2 show typical reverse-phase HPLC tracer under the test conditions used, without addition of 5-lipoxygenase inhibitors.

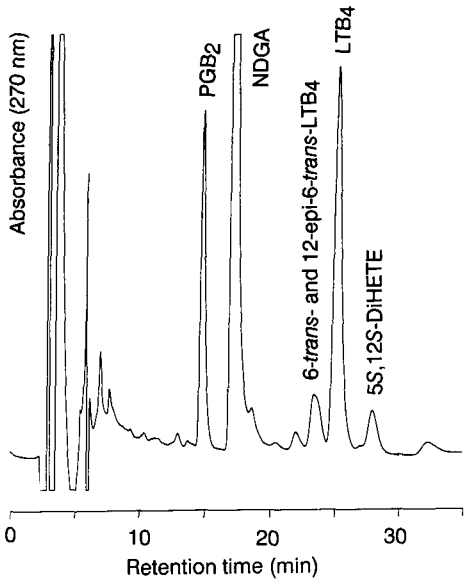

FIG. 1. Reverse-phase HPLC of arachidonic acid metabolites formed by bovine PMNL stimulated with calcium ionophore A23187; UV monitoring at 270 nm.

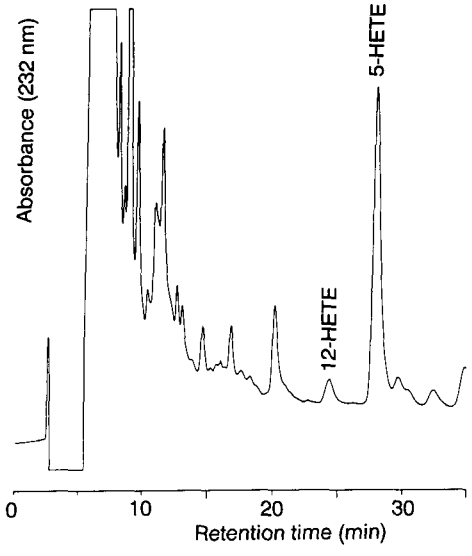

FIG. 2. Reverse-phase HPLC of arachidonic acid metabolites formed by bovine PMNL stimulated with calcium ionophore A23187; UV monitoring at 232 nm.

Besides small amounts of 5*S*,12*S*-Di-HETE and the two all-*trans* isomers of LTB_4, the main peak in the HPLC monitored at 270 nm was LTB_4 (Fig. 1). The presence of 5*S*,12*S*-Di-HETE, an interaction product of PMNL-5-lipoxygenase and platelet 12-lipoxygenase (Marcus et al 1982), showed that the PMNL preparation was slightly contaminated with platelets. Contrary to the studies of Walstra et al (1984) we could not detect appreciable amounts of LTC_4 due to the absence of eosinophiles in our PMNL preparations: eosinophile PMNL were found to produce almost exclusively LTC_4 whereas neutrophile PMNL produce only LTB_4 (Verhagen et al 1984). In agreement with Walstra et al (1984) metabolism of LTB_4 into 20-LTB_4 could

not be detected in bovine PMNL but could be detected in human PMNL.

5-HETE was the main component in the chromatogram recorded at 232 nm (Fig. 2). Corresponding to the contamination with platelets, the platelet 12-lipoxygenase product 12-HETE was detected.

To evaluate 5-lipoxygenase inhibitors the LTB_4 formation was first monitored. If a substance was found to be active, the inhibition of 5-HETE synthesis was also measured. This procedure gives information on whether the formation of LTA_4 by 5-lipoxygenase or only the conversion of LTA_4 to LTB_4 by LTA_4-hydrolase was affected. However, a simultaneous inhibition of the formation of LTB_4 and 5-HETE does not prove an inhibition of 5-lipoxygenase unambiguously; the inhibited release of AA, e.g. by inhibition of PLA_2 (see below), may also account for this effect.

Kinetic studies showed that LTB_4 increases linearly during the first 5 min after stimulation and therefore a 5 min incubation time was chosen for the 5-lipoxygenase assay.

The formation of cyclo-oxygenase metabolites by bovine platelets has not been previously reported. In contrast to the PMNL 5-lipoxygenase, which has to be activated selectively, e.g. by calcium ionophore, the platelet cyclo-oxygenase needs only free (exogenous or endogenous) AA to initiate the cascade.

The first cyclo-oxygenase test assay uses 12-HHT, which is produced in bovine platelets by cyclo-oxygenase and thromboxane synthase (TXS) besides thromboxane B_2 (TXB_2), as a marker for cyclo-oxygenase activity. 12-HHT is detected at 232 nm after HPLC-separation, platelet 12-lipoxygenase can also be measured by determining the simultaneously formed 12-HETE (Fig. 3).

A diminished production of 12-HHT does not necessarily reflect an inhibition of cyclo-oxygenase since the reduced release of AA, e.g. by inhibition of PLA_2 (see below), or the inhibition of the TXS may be responsible for this effect. Although the specific TXS inhibitor E/Z-7-phenyl-7-(3-pyridyl)-6-heptenoic acid (Kato et al 1985; Hecker et al 1986)

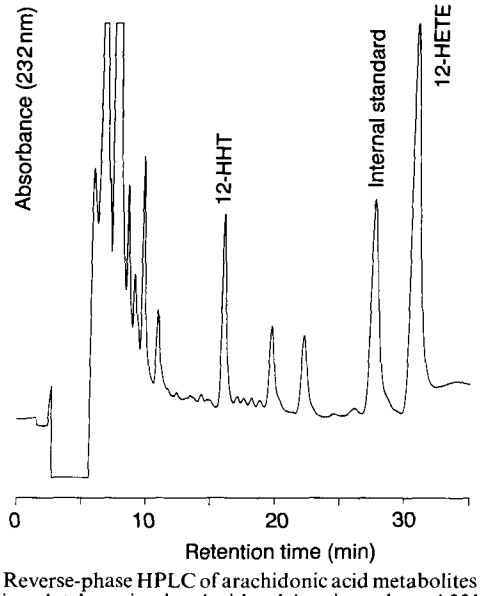

FIG. 3. Reverse-phase HPLC of arachidonic acid metabolites formed by bovine platelets stimulated with calcium ionophore A23187; UV monitoring at 232 nm.

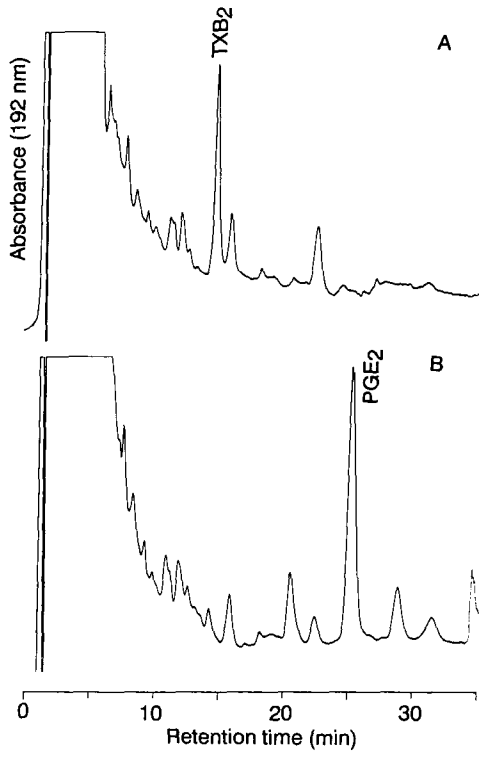

FIG. 4. Reverse-phase HPLC of arachidonic acid metabolites formed by bovine PMNL stimulated with calcium ionophore A23187. A, in the absence of the specific TXS inhibitor; B, in the presence of the specific TXS inhibitor E/Z-7-phenyl-7-(3-pyridyl)-6-heptenoic acid. UV monitoring at 192 nm.

inhibits the 12-HHT formation by about 50% at a concentration of 0·1 M, a total inhibition of the 12-HHT synthesis could not be achieved even at higher concentrations, because 12-HHT is not only formed from PGH_2 enzymatically by TXS but also nonenzymatically (Diczfaluzy et al 1977).

The second cyclo-oxygenase test system does not depend on the TXS activity. The formation of PGE_2 by the cyclo-oxygenase of bovine platelets after the addition of the specific TXS inhibitor E/Z-7-phenyl-7-(3-pyridyl)-6-heptenoic acid is measured. Under these conditions the PGE_2 concentration is high enough for UV detection (maximum at 192 nm), whereas the cyclo-oxygenase/TXS product TXB_2 cannot be detected (Fig. 4).

For the 12-HHT-assay, 3-(2,2-dimethyl-5,6-diphenyl-2,3-dihydro-1H-pyrrolizin-7-yl)-butyric acid (Dannhardt & Lehr 1990) and for the PGE_2-determination, 15-keto-PGE_2 were used as internal standards.

Kinetic studies on the formation of 12-HHT and PGE_2 demonstrated that 12-HHT increases only in the first minute and remains constant after 3 min whereas the PGE_2 concentration increases linearly over the first 5 min. The formation of 12-HETE shows nearly the same kinetic time-course as the formation of the 12-HHT. Similar results concerning the formation of 12-HHT and 12-HETE were obtained with horse platelets (Lapetina & Cuatrecasas 1979).

Reduced amounts of the AA metabolites LTB_4 and 5-HETE or 12-HHT and PGE_2 do not necessarily reflect an inhibition of 5-lipoxygenase or cyclo-oxygenase because the release of endogenous AA from the phospholipids by the phospholipase A_2 (PLA_2) could also be inhibited.

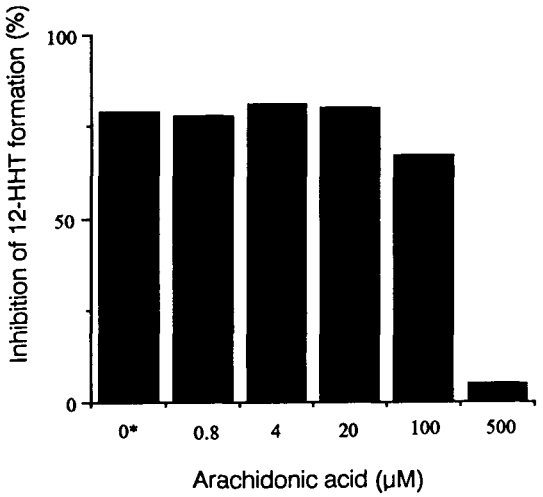

FIG. 5. Inhibition of 12-HHT formation (bovine platelets) by 6·6 nM indomethacin. * = stimulation with calcium ionophore A23187.

Table 1. IC50 values of lipoxygenase inhibitors and cyclo-oxygenase inhibitors evaluated with bovine PMNL and bovine platelets, respectively.

	IC50 (μM)			
	5-Lipoxygenase		Cyclo-oxygenase	
Inhibitors	Bovine PMNL	Other test systems	Bovine platelets	Other test systems
NDGA	0·8	0·43–1·57 (Salmon et al 1989)		
REV 5901	9·0	4·7 (Coutts et al 1985)		
Indomethacin			0·0025	0·0017–0·01 (Brune et al 1981)

An inhibition of AA release will be excluded if at least one of the three pathways (5-lipoxygenase-, cyclo-oxygenase- or 12-lipoxygenase-pathway) is not influenced by the test compound. If all three AA pathways are affected, an inhibition of AA release can be ruled out, e.g. by comparing the formation of 12-HHT from endogenous AA with the formation of this metabolite from exogenous AA. To obtain comparable results the inhibition of 12-HHT production from exogenous AA by selective cyclo-oxygenase inhibitor (indomethacin) at different concentrations of exogenous AA must be established (Fig. 5). At concentrations of 0·8, 4 and 20 μM the inhibition of 12-HHT formation from endogenous and exogenous AA by indomethacin was nearly the same; the highest amount of 12-HHT was found at a concentration of 20 μM AA. Higher AA concentrations (100 and 500 μM) decreased the inhibition. Therefore this assay was carried out with a concentration of 20 μM AA.

The data evaluated for NDGA and REV 5901 with indomethacin agree with previous recorded data for these compounds (Table 1) indicating that the test systems are valid for the evaluation of 5-lipoxygenase and cyclo-oxygenase inhibitors.

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