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Monitoring eicosanoid biosynthesis via lipoxygenase and cyclooxygenase pathways in human whole blood by single HPLC run

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Abstract

Eicosanoids play an important role as lipid mediators for physiological and pathological processes. Inhibitors of their biosynthesis have been developed as drugs for various diseases with major health political relevance. The search for more efficient inhibitors of eicosanoid formation requires simultaneous monitoring of various metabolic pathways. We developed an HPLC-based assay system, which quantifies lipoxygenase metabolites leukotriene B_4 (LTB_4), 5-hydroxyeicosatetraenoic acid (5-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE) and cyclooxygenase metabolite 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) in whole human blood. Eicosanoid formation in blood is initiated with calcium ionophore A23187, arachidonic acid and calcium and magnesium ions. After solid phase extraction the different eicosanoids were separated by isocratic RP-HPLC using prostaglandin B_1 as authentic standard. To verify the assay we determined the IC_{50} of known inhibitors of eicosanoid biosynthesis (zileuton, indomethacin, nordihydroguaiaretic acid). The test system is simple. It does not require extensive methodological experience and can be carried out in any biochemical laboratory. The analytical procedure can be robotized and thus, the assay appears suitable for medium-throughput testing of drugs.

Keywords: Human whole blood; Lipoxygenase; Cyclooxygenase; RP-HPLC

1. Introduction

In inflammation, the activation of proinflammatory signalling cascades induces the liberation of free arachidonic acid (AA) from membrane phospholipids and subsequently the synthesis of bioactive eicosanoids. Two cyclooxygenase (COX) and several lipoxygenase (LOX) isoforms have been identified as key enzymes of eicosanoid biosynthesis. Prostaglandins (PGs) such as PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxane A_2 (TxA_2) and leukotrienes (LT) are the major arachidonic acid metabolites [1]. Traditional non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and aspirin-like compounds are commonly used for the treatment of inflammatory symptoms [2]. They inhibit both COX isoforms and frequently cause gastrointestinal irritations or excessive bleeding. These negative side effects prompted the development of selective inhibitors of COX-2, but the advantages of these drugs have recently been challenged [3]. Furthermore, selective inhibition of COX pathway can switch in arachidonic acid metabolism to the LOX pathway. The formation of LTs as principle mediators of bronchial asthma can be induced, which is reported as aspirin-asthma [4,5]. The only 5-LOX inhibitor zileuton is usually prescribed for patients suffering from bronchial asthma [6]. An advanced concept of anti-inflammatory therapy constitutes the use of inhibitors of COX-1/2 and LOX pathways [7]. Licofelone, a dual COX/LOX inhibitor, is currently in phase III of clinical testing [8]. Other LOX isoforms, such as 12- and 15-LOXs have also been implicated in a series of pathophysiological processes [9,10]. The search for dual inhibitors and for inhibitors of 12- and 15-LOX as well requires the quantification of different key eicosanoids preferential in a single step. Human whole blood (HWB) appears as most suitable source for such an assay. Several systems have previously been reported in literature. Predominantly, eicosanoids are quantified in biological samples by immunological methods [11], while HPLC-based assays with UV detection [12], radioisotope detection [13], fluorescence detection [14] or electrochemical detection [15] are applied as well. Unfortunately, most of them suffer from disadvantages, which limit their

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application as routine screening system. Limitations of reported methods using human whole blood or human cell preparations are:

- quantification of mostly single eicosanoid [16] or separated record of different but at the most two metabolites in whole blood [17],
- cost-intensive materials and equipment for measuring radioactivity in radioimmunoassays (RIA) or for enzymimmunoassays (EIA) [17],
- time-intensive isolation of appropriate cells from human whole blood [18].

For the time being there is no HWB-based assay available, which can be used for concurrent quantification of the major COX- and LOX metabolites LTB_4, 5-hydroxyeicosatetraenoic acid (5-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT).

This lack of methodological knowledge prompted us to develop such an assay system, which is based on RP-HPLC quantification of the metabolites and only requires standard equipment. The method was verified using known inhibitors of the COX- and LOX pathways (indomethacin, zileuton, nordihydroguaiaretic acid).

2. Materials and methods

2.1. Reagents

All the reagents used were of analytical grade and obtained from the following sources: solvent for HPLC, methanol gradient grade: Roth, Germany; calcium ionophore A23187 (free acid): Serva, Germany; HPLC reference substances LTB_4, 5-HETE, 12-HETE, 15-HETE, 12-HHT and PGB_1 as internal standard for HPLC: Cayman Chemical, USA; zileuton: Abbott, USA; indomethacin: Synopharm GmbH, Germany; nordihydroguaiaretic acid (NDGA): Fluka Chemical, Germany; heparin: Roche, Switzerland.

2.2. Apparatus

Solid phase extraction was performed on an Adsorbex SPU system using Bakerbond SPE™ octadecyl C_{18} extraction columns (3 ml, 500 mg J.T. Baker, Philipsburg, USA).

For high-performance liquid chromatography a Merck Hitachi HPLC system was used, which consisted of a diode array detector L-4500, a pump module L-6000, an autosampler AS-2000A and an interface D-7000.

Arachidonic acid metabolites were separated on a LiChrospher® 100 RP-18 matrix (5 μm) packed in a LiChroCART® 250-4 reversed-phase column (Merck, Darmstadt, Germany).

2.3. Human whole blood

For the use of human blood we received the agreement by the ethical committee of our university. All vessels that got in contact with blood were made of polypropylene (PP). Human blood (≈60 ml) was drawn from the arm vein of healthy volunteers, who did not received NSAIDs for at least 7 days. Coagulation was prevented by the addition of 10 I.E. of heparin (Liquemin® N 10000) per ml blood. The blood was immediately aliquoted (3 ml) into a series of PP tubes.

To assay eicosanoid formation, blood samples were first incubated without preincubation in the absence (control) or presence of various concentrations of test compounds (6 μl of a drug solution in dimethylsulfoxide (DMSO) or only DMSO as vehicle) for 30 min at 37 °C in a water bath. Then $CaCl_2$ and $MgCl_2$ (final concentration 0.5 mM each) were added and eicosanoid formation was initiated by the addition of calcium ionophore A23187 (final concentration 30 μM) and AA (final concentration 10 μM). Incubation of the samples was continued for 40 min at a temperature of 37 °C. Next, 0.5 ml of ice-cold methanol containing the internal standard PGB_1 (final concentration 1.0 μM) was added to stop the reaction. The samples were immediately stored at −18 °C for at least 1 h. For validation of analytical procedure aliquots of human whole blood were mixed with appropriate amounts of eicosanoids (solved in ethanol) and taken through the same protocol.

2.4. Solid phase extraction

An octadecyl (C_{18}) reversed-phase extraction column was used to extract the eicosanoids from blood (see Section 2.2). The cartridges were pre-washed with 10 ml methanol and 10 ml water.

The blood samples were centrifuged at 2000 × *g* for 20 min after adding 4 ml water. The supernatants were placed on SPE columns. The columns were first washed with 2 ml of water and then with 2 ml of 10% methanol (in water). Eicosanoids were eluted from the columns with 100% methanol (two fractions 0.4 ml each). The methanol was removed under a stream of argon at 37 °C, the residue was reconstituted in 120 μl of ethanol and aliquots were subjected to HPLC. The SPE columns were used four times without losing their extraction efficiency.

2.5. Reversed-phase HPLC analysis

For validation of HPLC method eicosanoids were solved in ethanol at specified concentrations. Aliquots of 20 μl were injected. Isocratic elution was performed at room temperature with the solvent system methanol/water/acetic acid (78:22:0.01, v/v/v) at a flow rate of 1.0 ml/min. The AA metabolites were identified comparing their retention times with those of authentic standards and quantified by the peak area. Extraction efficiency was quantified with respect to recovery of the authentic standard. The UV spectra were monitored in the wavelength range between 200 and 320 nm. For quantification, following peaks were analyzed: retention time = 6.3 min, λ_{max} = 280 nm: PGB_1 (internal standard); retention time = 9.3 min, λ_{max} = 270 nm: LTB_4; retention time = 11.9 min, λ_{max} = 230 nm: 12-HHT; retention time = 21.5 min, λ_{max} = 235 nm: 15-HETE; retention time = 26.3 min, λ_{max} = 235 nm: 12-HETE; retention time = 31.7 min, λ_{max} = 235 nm: 5-HETE.

Table 1
Stability of internal standard PGB_1 at −18 °C for 28 day and of eicosanoids LTB_4 and 15-HETE at −18 °C for 7 day and at room temperature (RT) for 7 days

PGB_1	LTB_4		15-HETE	
−18 °C, 28 days Concentration: *t*-value	−18 °C, 7 days Concentration: *t*-value	RT, 7 days *t*-value	−18 °C, 7 days Concentration: *t*-value	RT, 7 days *t*-value
4 μg/ml: 1.94	0.4 μg/ml: 1.32	0.83	0.5 μg/ml: 1.56	1.90
8 μg/ml: 1.01	0.9 μg/ml: 1.74	1.07	1.0 μg/ml: 1.39	0.05
12 μg/ml: 2.03	1.4 μg/ml: 0.44	2.04	1.5 μg/ml: 0.55	0.03

$P = 0.05$; $n = 6$; $t_{tab} = 2.57$.

2.6. IC_{50} determination

IC_{50} values were determined by the non-linear regression module of Microcal Origin® 6.0 software package. Four to six different inhibitor concentrations were assayed on 3 consecutive days and blood of various donors was used. Statistical significance was determined by *t*-test analysis referred to the 95% confidence limits ($P < 0.05$).

3. Results

3.1. Validation of HPLC method

The HPLC method was validated according to ICH Guidelines (1995). The validation consisted in studying the linearity of the chromatographic response, the precision, the accuracy, the limit of detection (LOD) and the limit of quantification (LOQ) of the HPLC method as well as the stability of eicosanoid solutions.

Stability of internal standard PGB_1 was studied on standard solutions, which was stored for at least 28 days at −18 and +6 °C. Using *t*-test ($P = 0.05$; $n = 6$) it could be demonstrated, that no significant degradation of the substances occurred storing at −18 °C (see Table 1). Probes, which were kept at +6 °C for the reported time were found to decompose.

During the test procedure solutions of extracted eicosanoids were stored for at least 1 day and for repeated determination for at least 7 days. Therefore, the stability was controlled at −18 °C and at room temperature for 7 days exemplary for LTB_4 and 15-HETE to minimize the costs of validation. The performed *t*-test established that investigated eicosanoids possess a sufficient stability in solution at −18 °C likewise at room temperature during analytical run time (Table 1).

Further validation characteristics are presented in Tables 2 and 3. All parameters were computed using the ratio of the peak areas of different eicosanoids and internal standard PGB_1. The calibration curves were linear in the ranges given in Table 2, the least square method was used to calculate the regression equations. The limit of detection and limit of quantification are expressed as the concentration at a 3-to-1 and 10-to-1 signal-to-noise ratio as well, which were determined according to the European Pharmacopoeia [19].

Precision is performed as repeatability and intermediate precision and is expressed as the percent relative standard deviation (R.S.D.). Repeatability was determined from six determinations per concentration covering the specified range of the procedure (three concentrations). The measurements of intermediate precision were carried out on 3 different days. The accuracy of the HPLC method was assessed by comparing the analyte amount spiked at three different concentration levels with six replicates for each concentration level investigated. The data are reported as the percent recovery of the known, added amount with intervals of confidence (IC).

3.2. Formation of eicosanoids in human whole blood

The extent of eicosanoid formation in whole human blood depends on the assay conditions, such as ionic composition of the assay mixture, and on the chemical nature of the stimuli. The impact of Ca^{2+} and Mg^{2+} ions on eicosanoid formation by human whole blood is shown in Fig. 1. In the absence of exogenous cations we observed an almost linear increase in LTB_4 biosynthesis depending on ionophore concentration up to 40 μM (panel A). However, an increase in ionophore concentration up to 50 μM did not lead to augmented LTB_4 formation. Addition of Ca^{2+} or Mg^{2+} at a final concentration of 1 mM decreased LTB_4 biosynthesis at 15 μM but was without any effect at 30 μM. There was no difference between the two cations at either concentration. Maximal LTB_4 formation was observed when both cations were present at 0.5 mM concentrations and when an ionophore concentration between 30 and 45 μM was used. Similar observations were made when the formation of 12-HETE was profiled. However, for this metabolite we did not observe major differences when its biosynthesis was quantified in the absence or in the presence of 0.5 mM Ca^{2+} and 0.5 mM

Table 2
Calibration curve data as well as limits of detection (LOD) and quantification (LOQ)

Eicosanoid	Linearity ($n = 9$; $k = 10$)	Range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
LTB_4	$y = 0.21724x - 0.01582$, $r^2 = 0.9990$	0.3–1.7	0.1	0.3
5-HETE	$y = 0.06768x - 0.01074$, $r^2 = 0.9994$	0.5–3.0	0.2	0.5
12-HETE	$y = 0.09314x - 0.00004$, $r^2 = 0.9910$	2.5–30.0	0.1	0.5
15-HETE	$y = 0.15239x - 0.04465$, $r^2 = 0.9992$	0.3–2.0	0.1	0.3
12-HHT	$y = 0.2894x - 0.0476$, $r^2 = 0.9973$	0.3–2.5	0.1	0.3

Table 3
Precision and accuracy of HPLC method[a]

Eicosanoid	Concentration (μg/ml)	Repeatability, R.S.D. (%)	Intermediate precision, R.S.D. (%)	Accuracy, recovery ± IC (%)
LTB_4	0.70	5.4	4.5	92.7 ± 5.2
	1.00	4.7	2.8	91.6 ± 4.6
	1.50	2.8	1.7	93.8 ± 2.7
5-HETE	0.50	2.2	3.6	95.3 ± 2.2
	1.25	3.9	2.9	93.5 ± 3.8
	2.00	2.4	4.3	97.4 ± 2.5
12-HETE	1.50	6.5	6.8	103.8 ± 9.9
	2.50	2.2	6.9	100.4 ± 2.7
	7.50	1.8	4.1	103.2 ± 3.4
15-HETE	0.50	3.3	9.2	91.7 ± 3.2
	0.80	3.8	3.3	93.0 ± 3.7
	1.50	2.4	4.3	96.6 ± 2.1
12-HHT	0.90	5.8	3.6	91.3 ± 5.6
	1.25	1.9	2.6	95.4 ± 1.9
	1.60	2.8	1.5	99.6 ± 3.0

[a] Precision as repeatability and intermediate precision are expressed as the percent relative standard deviation (R.S.D.), accuracy is expressed as percent recovery with intervals of confidence (IC); $P = 0.05$ and $n = 6$.

Mg^{2+} (Fig. 1, panel B). Addition of exogenous AA significantly stimulated the formation of LTB_4, 5-HETE and 12-HETE. In contrast, biosynthesis of 15-HETE and 12-HHT did not alter (Fig. 2).

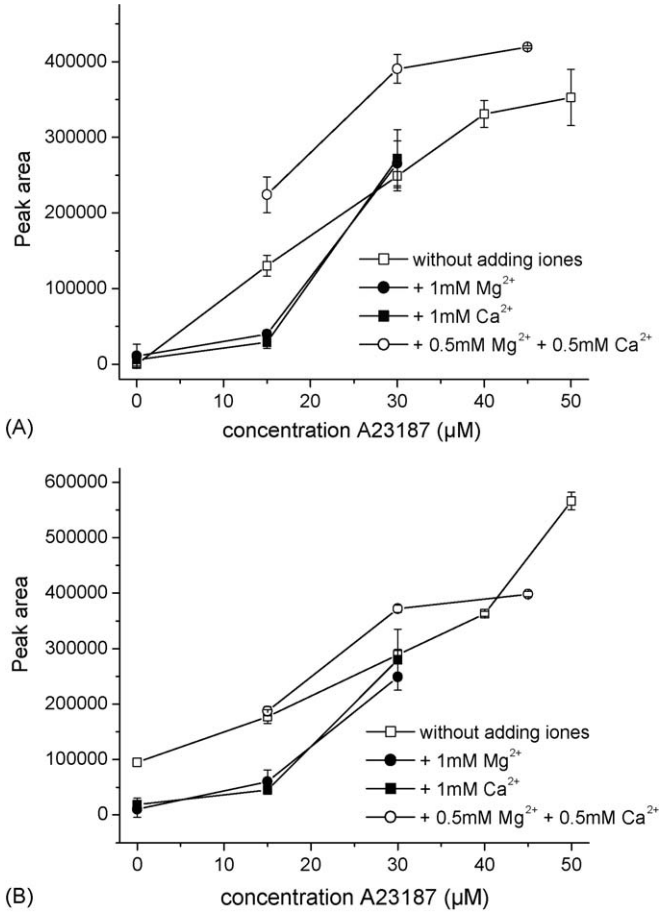

Fig. 1. Dependence of biosynthesis (A) of LTB_4 and (B) of 12-HETE on the concentration of cations and A23187 in human whole blood ($n = 4$).

To investigate the time course of eicosanoid formation human blood samples were incubated for various time periods with calcium ionophore A23187, arachidonic acid as well as Ca^{2+}/Mg^{2+} ions and the metabolites were quantified by HPLC. After 45 min of incubation we observed maximal generation of LTB_4 and 15-HETE. Biosynthesis of 5-HETE and 12-HHT was maximal after 60 min (Fig. 3A and C) whereas the highest concentration of 12-HETE was reached after 90 min (Fig. 3B). For our standard assay system an incubation period of 40 min was selected. Worth mentioning, a preincubation time before adding inhibitors rapidly decreases the ability of whole blood to generate AA metabolites under A23187 stimulation [20].

The solvent for test compounds and reagents should be inert to enzyme reactions. Water represents the ideal medium for test procedure but most of the substances are insoluble in water. Therefore, we analyzed the influence of dimethylsulfoxide (DMSO) and methanol on the eicosanoid formation.

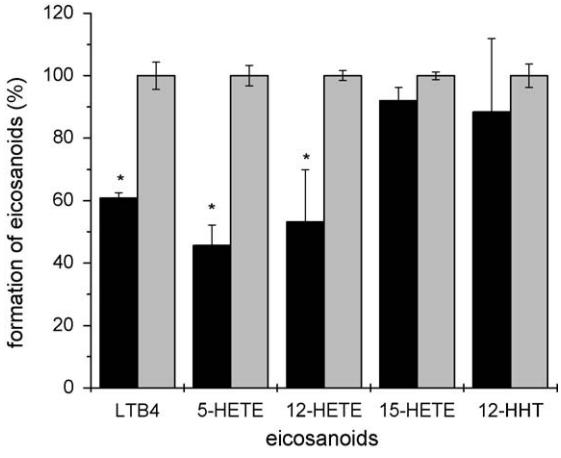

Fig. 2. Influence of AA addition to the stimulation mix on eicosanoid formation, black column: without AA, grey column: with addition of 10 μM AA (=100 %); $n = 4$. *Significance to value obtained by addition of AA, $P < 0.05$.

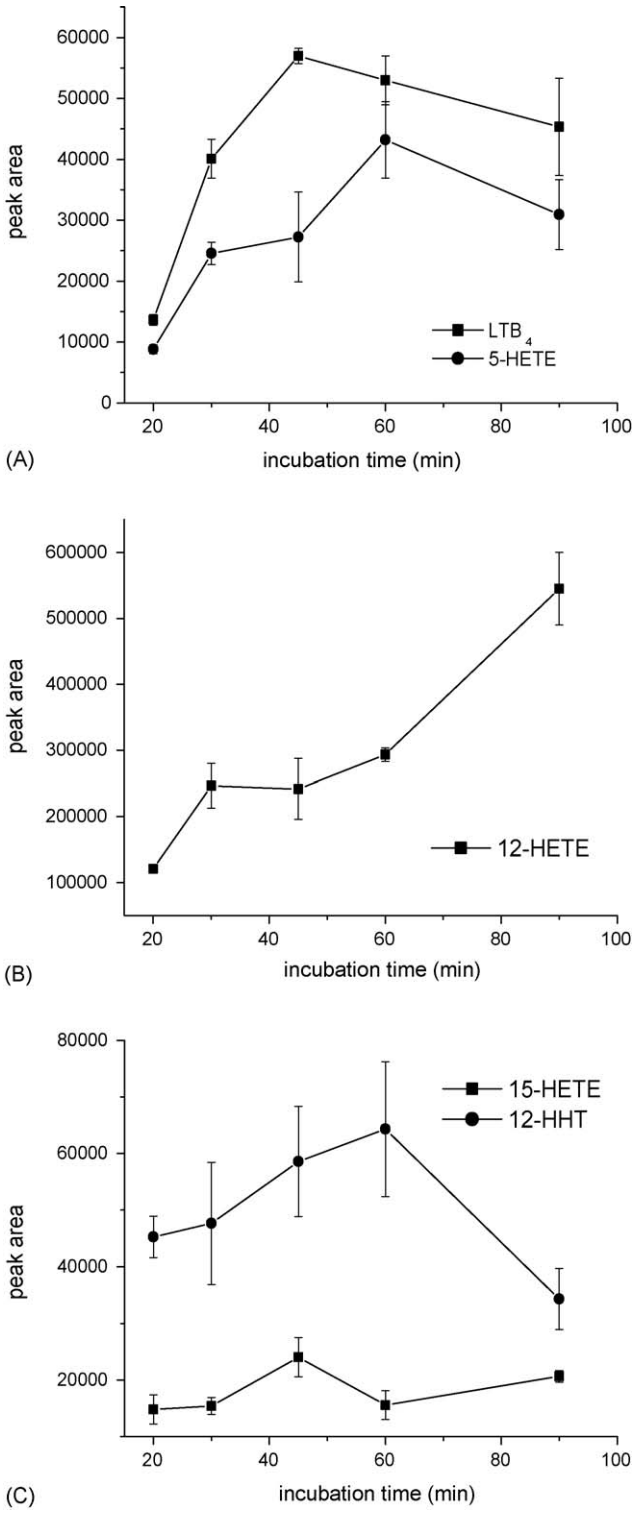

Fig. 3. Time course of formation (A) of LTB_4 and 5-HETE, (B) of 12-HETE and (C) of 12-HHT and 15-HETE in human whole blood after stimulation with A23187, cations and arachidonic acid ($n = 3$).

In comparison to water, DMSO has a more stimulating and methanol a more inhibiting effect (Fig. 4). Since DMSO indicates to be more qualified for solving the inhibitors, the stimulating effect was accepted in course of the same effect in control samples. Thus, DMSO was used as solvent for A23187 and

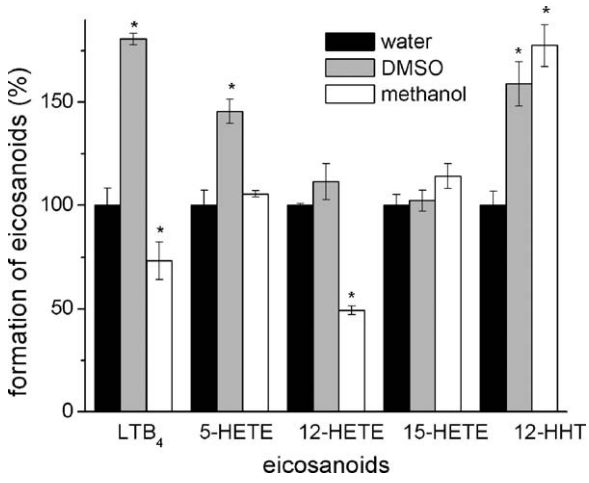

Fig. 4. Formation of eicosanoids (%) in dependence on solvent for inhibitors; black column: probes with 6 μl water (100%), $n = 3$; grey column: probes with 6 μl DMSO, $n = 6$; white column: probes with 6 μl methanol, $n = 3$.

for inhibitors as well, which results in 0.4% DMSO in whole blood.

Recovery of eicosanoids was determined by comparing chromatographic peak areas of eicosanoids in whole blood samples with those obtained by direct injection of corresponding amount of eicosanoid (0.75, 1.5 and 3.0 μg/ml solved in ethanol). For this purpose, samples of 1 ml human whole blood were spiked with 90, 180 and 360 ng eicosanoid and then the samples were taken through our analytic protocol. The results, which are shown in Table 4 indicate that the recovery varied between 45 and 75% depending on the chemistry of the eicosanoids.

Furthermore, the concentration of eicosanoids in stimulated whole blood can be obtained by extrapolating the peak areas of HPLC chromatogram from calibration curves. Each run for IC_{50} determination involves three samples for control. Using this data pool of uninhibited control samples, the concentration of eicosanoids in stimulated blood can be concluded. For evaluation of inhibition and for determining IC_{50} values it is advantageous using blood samples of different subjects, which minimizes the impact of interindividual variations. From Table 4 it can be seen that except for 15-HETE the variation within the same blood sample (intraindividual variation) is relatively small. On the other hand, the interindividual variations of eicosanoid generation between different subjects were much more pronounced.

3.3. Impact of known LOX/COX inhibitors

To validate our assay, eicosanoid biosynthesis was quantified in the presence of known inhibitor of different pathways of arachidonic acid cascade. For this purpose, we used the 5-LOX inhibitor zileuton, the non-steroidal anti-inflammatory drug indomethacin (COX inhibitor) and the unspecific LOX inhibitor NDGA. Typical chromatograms of a stimulated human whole blood sample and of a sample, which was incubated in the presence of the COX inhibitor indomethacin, are shown in Fig. 5.

The internal standard PGB_1 and the 5-LOX metabolite LTB_4 were early eluted. The COX metabolite 12-HHT eluted with

Table 4
Absolute recovery rates ± standard deviation (S.D.) of eicosanoids using the optimized procedure ($n = 3$) as well as concentration of eicosanoids in blood samples in the same and in different subjects

Eicosanoid	Recovery rates (%, ±S.D.)	Concentration (ng/ml blood) (R.S.D.)	
		Same subject ($n = 3$)	Different subjects ($n = 6$)
LTB_4	74.2 ± 1.7	105.2 (18.1)	91.4 (50.5)
5-HETE	45.0 ± 3.8	160.0 (9.2)	170.8 (33.1)
12-HETE	65.1 ± 1.2	990.6 (15.7)	1700.3 (41.2)
15-HETE	69.5 ± 5.8	41.1 (34.6)	45.8 (40.0)
12-HHT	75.6 ± 0.2	49.6 (18.8)	84.1 (50.0)

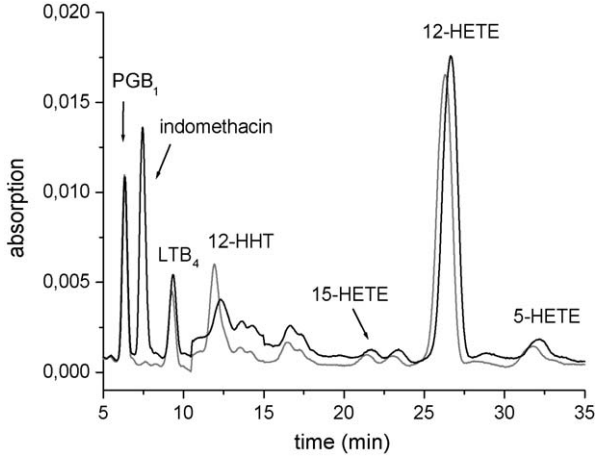

Fig. 5. Chromatograms of stimulated human whole blood (grey) and stimulated whole blood after inhibition by 2 μM indomethacin (black), UV-detection: 230 nm: 12-HHT; 235 nm: 15-HETE, 12-HETE, 5-HETE; 270 nm: LTB_4; 280 nm: PGB_1.

a retention time of about 12 min in the middle part of the chromatogram. The LOX metabolites 15-HETE, 12-HETE and 5-HETE appear in the later part of the chromatogram between 22 and 32 min.

Indomethacin (2 μM) inhibited the formation of the COX product 12-HHT whereas the LOX products LTB_4, 15-HETE, 12-HETE and 5-HETE were hardly impacted. The unspecific LOX inhibitor NDGA inhibited the formation of all LOX products (LTB_4, 5-HETE, 12-HETE, 15-HETE) in a dose-dependent manner (Fig. 6). Surprisingly, 12-HHT originating from the COX pathway was also inhibited dose-dependently.

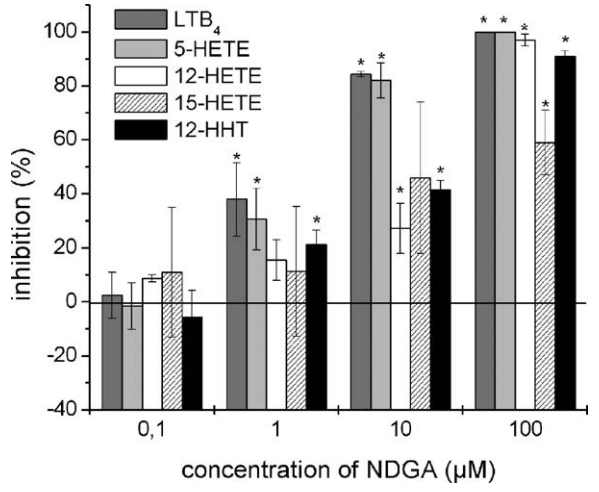

Fig. 6. Profile of inhibition of eicosanoid formation in human whole blood assay by NDGA. * Significance to control, $P < 0.05$.

Table 5
Inhibitory effects of zileuton, indomethacin and NDGA on enzymes of arachidonic cascade related to eicosanoid biosynthesis using developed HWB assay

Inhibitor	Enzyme	IC_{50} ± IC (μM)[a] (related to eicosanoid)
Zileuton	5-LOX	0.60 ± 0.09 (LTB_4), 1.6 ± 0.11 (5-HETE)
Indomethacin	COX-1	0.34 ± 0.02 (12-HHT)
NDGA	5-LOX	1.8 ± 0.19 (LTB_4), 2.4 ± 0.26 (5-HETE)
	12-LOX	16.9 ± 5.6 (12-HETE)
	15-LOX	54.4 ± 15.4 (15-HETE)
	COX-1	13.1 ± 4.5 (12-HHT)

[a] The data show IC_{50} value ± 95% interval of confidence (IC).

Based on a minimum of four concentrations of test compound we determined IC_{50} values for the inhibition of eicosanoid biosynthesis in blood samples of three to five different volunteers. The specific 5-LOX inhibitor zileuton reduced the formation of LTB_4 and 5-HETE with a similar efficiency indicating the 5-LOX origin of the two metabolites. As shown in Fig. 5 the COX inhibitor indomethacin prevents the formation of 12-HHT with a fairly low IC_{50} (Table 5) but was ineffective with respect to the biosynthesis of the LOX metabolites. The reference NDGA inhibited the formation of the 5-LOX metabolites LTB_4 and 5-HETE with IC_{50} in the lower μM range. The formation of 12- and 15-HETE as well as 12-HHT was found to be reduced at higher concentration of NDGA.

4. Discussion

Human whole blood constitutes a suitable ex vivo system to investigate the activity of different COX and LOX isoforms since various cell types interact to synthesize eicosanoids endogenously and transcellularly, simulating in vivo conditions [20]. In the present study, we introduced a simple analytical method to quantify LOX and COX products in HWB. As shown previously [16,21] stimulation of blood ex vivo and the evaluation of metabolites from AA represent a valuable model for the assessment of the in vivo efficiency of drugs acting on AA metabolism.

Calcium ionophore A23187 is known to stimulate eicosanoid biosynthesis in whole blood in concentrations up to 50 μM

[16,22]. We found that under our experimental conditions 30 μM of A23187 is sufficient to stimulate eicosanoid production (Fig. 1). Addition of AA to the incubation mix is predominantly used in assays with isolated enzymes, where AA acts as substrate for enzyme reaction but it has a stimulating effect on eicosanoid biosynthesis as well [23]. In our assay (see Fig. 2), we assume that AA fulfils both roles, namely as a stimulant that supplies the blood additionally with enough enzyme substrate during prolonged incubation times. The consequence of addition of both, Ca^{2+} and Mg^{2+} ions as well to human whole blood mixture, comparing to the addition of one ion has not yet been investigated in literature. Separate addition of Ca^{2+} or Mg^{2+} ions to the blood samples does not lead to increased higher eicosanoid biosynthesis (Fig. 1). In contrast, a mixture of the two ions considerably augmented production of LTB_4 at all calcium ionophore concentrations tested. The formation of 12-HETE was slightly enhanced at single calcium ionophore concentration of 30 μM. The amount of solvent for inhibitor is applied in the usual range until 1.0% DMSO in whole blood assays [24,25].

Using calibration curves for the different eicosanoids the following average concentrations in stimulated human whole blood were determined (six different human subjects): LTB_4 91 ng/ml, 5-HETE 171 ng/ml, 12-HETE 1.7 μg/ ml, 15-HETE 46 ng/ml and for 12-HHT 84 ng/ml blood (Table 4). These data are consistent with previously reported results obtained an assay (LTB_4 66 ng/ml) [26], 12-HETE 1.3 μg/ml [27]). It should, however, been stressed that we observed large interindividual differences between different human subjects. In contrast, the intraindividual differences turned out to be quiet low suggesting the reliability of the method. Although relatively large interindividual differences hamper comparison of the absolute eicosanoid concentrations of different subjects. This disadvantage does not impair the suitability of the assay for determination of IC_{50} values of potential inhibitors of eicosanoid formation. For this purpose, relative eicosanoid concentrations (related to the uninhibited reaction) must be determined.

The HWB assay described here is based on the quantification of final products of different eicosanoid synthesizing pathways. It allows separate quantification of various LOX pathways (5-LOX, 12-LOX, 15-LOX) but it does not differentiate between the two COX isoforms. Appropriate methods for this intention have been described in literature [16,28]. Moreover, the assay is not capable of differentiating between different 15-LOX isoforms (15-LOX-1 and 15-LOX-2) since the final products of the two pathways are identical. However, so far no major expression of 15-LOX-2 has been reported in blood cells and thus, this disadvantage may not be of major importance.

The results of IC_{50} value determination of standard inhibitors of eicosanoid biosynthesis (Table 5) are consistent with literature data obtained in comparable test systems [17,29–31]. However, when other cellular systems or even enriched enzyme preparations were used as test medium quite different results were obtained. Amagata et al. [32] investigated the inhibitory potency of the standard LOX inhibitor NDGA for isolated human 15- and 12-LOXs. They observed lower IC_{50} values (0.11 μM for 15-LOX, 5.1 μM for 12-LOX) for both enzymes and calculated a selectivity ratio (15-LOX versus 12-LOX) of about 46. Therefore, this compound was called as selective 15-LOX inhibitor. However, in a preparation of human leukocytes/platelets suspension NDGA was reported to inhibit 12- and 15-LOXs with equal potency (IC_{50} = 30 μM) and the 5-LOX pathway was also effectively inhibited with an IC_{50} of 0.2 μM [33]. Furthermore, in human endothelial cells the IC_{50} values for the 12-LOX pathway varied between 0.1 μM [34] and 5 μM [35]. All these data reflect the extremely difficult evaluation of inhibitory potency and selectivity using isolated enzymes or cell preparations. There is no apparent correlation between different assays. Despite the variations of activity of NDGA, no data were available from human whole blood. The results obtained with the described whole blood assay (Table 2) indicate that NDGA has a predominantly greater potency for 5-LOX but a distinct selectivity towards one enzyme of the AA cascade could not been detected.

In summary, the method we have described herein is an efficient whole blood assay to quantify the concentrations of various eicosanoids. Studies of a selective activity in particular of LOX inhibitors can be managed under near physiological conditions. For the time being most information about selectivity of drugs for 12- and 15-LOX were commonly obtained using isolated enzymes or cell preparations [32,33]. The developed assay performs the simultaneous quantification of activity not only of 5-, 12- and 15-LOX but also of COX-1 and seems to be suited for an evaluation of potential dual inhibitors. The handling of whole blood is simple, convenient and time saving because there are no steps necessary for cell isolation. In addition, the methodology can be used in conjunction with other detection methods for the measurement of eicosanoids like prostaglandins and thromboxane which were not detectable by UV as well as for the determination of 5-/12-HETE ratio as a marker of inflammation especially in untreated asthmatic patients [27].

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