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Note

High-performance liquid chromatographic quantitation of cyclooxygenase and lipoxygenase metabolites of arachidonic acid from rat polymorphonuclear leukocytes

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Arachidonic acid (AA) metabolites synthesized by cells or tissues can be detected by various methods. Only few of these techniques allow rapid and precise analysis of every major metabolite in a single assay. Reversed-phase high-performance liquid chromatography (RP-HPLC) has been described as one of the more reliable techniques for the separation and characterization of AA metabolites. Most of the techniques published have described specific and accurate methods to analyse lipoxygenase metabolites [1–3], and some have reported sequential methods to analyse major AA metabolites in a single assay [4–7]. Considering these techniques, we have developed a simple and rapid RP-HPLC method to investigate, *in vitro*, drug interactions with AA metabolism and especially with cyclooxygenase and lipoxygenase enzymes. As an example, 1-^{14}C-labelled AA metabolites released *in vitro* by polymorphonuclear leukocytes (PMNLs) stimulated with the ionophore A23187 were analysed in a single run. This biological model was used because several potent metabolites, such as prostaglandins (PGs), thromboxane (TX), leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) were synthesised in a very short time [8].

EXPERIMENTAL

Chemicals

1-^{14}C-Labelled AA (2.16 GBq/mmol, Amersham France, Les Ulis, France) was evaporated under a stream of nitrogen, and the residue was dissolved in ethanol at a concentration of 400 μg/ml; tritium-labelled standards of PGE_2, TXB_2, LTC_4 and LTB_4, 5HETE, 12HETE and 15HETE were purchased from Du Pont de Nemours (New England Nuclear Products, Paris, France). Calcium ionophore A23187 was obtained from Calbiochem (La Jolla, CA, U.S.A.). Calcium- and magnesium-free phosphate-buffered saline (PBS) was from Flow Labs.

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(Puteaux, France). All other chemicals were reagent grade, and solvents (Farmitalia Carlo Erba, Paris, France) were HPLC grade. Indomethacin and nordihydroguaiaretic acid (NDGA) (Sigma France, La Verpillière, France) were dissolved, respectively, in 0.1 *M* NaOH–H_2O and dimethyl sulphoxide (DMSO) and added to the cells; the final solvent concentration was lower than 2% (v/v). Scintillation cocktail (Zinsser 303) was obtained from Berthold (Wildbad, F.R.G.).

Materials

The automated RP-HPLC system consisted of a dual-pump programmable solvent module 126 (Beckman Instruments France, Gagny, France) equipped with a NEC 8400 controller, a Nucleosil C_{18} column (3 μm particle size, 150 mm × 4.6 mm I.D.) (Interchim, Paris, France), an automated sampling system with a thermostatted tray and a liquid scintillation flow-through detector LB506C (Berthold) computerized to an IBM AT for data recording and analysis. The scintillation cocktail was delivered to a mixing tee by a pump LB5034 (Berthold).

Sample preparation

Male Lewis rats (Charles River France, Cleon, France) weighing 160–180 g were injected intraperitoneally with 6 ml of a 2% glycogen solution in 0.9% sterile saline. After 4 h, the animals were killed with diethyl ether and the PMNLs were obtained by washing the peritoneal cavity twice with 5 ml of PBS containing 10% of 0.109 *M* sodium citrate; the peritoneal fluid was filtered through a nylon sift (Nybolt PA-60/33, Nybolt, Zürich, Switzerland), centrifuged at 900 *g* for 10 min at 4°C, and washed twice in PBS. When more cells were needed, several rats were injected with the glycogen solution, and cell suspensions were pooled just before cell counting. The cell suspension was adjusted to $7.5 \cdot 10^6$ cells per ml and left for 30 min at 37°C before use. Under these conditions, the viability of the cells, as determined by the blue trypan technique, was greater than 90%.

Aliquots of the cell suspension (3 ml) were preincubated with test drug or vehicle for 5 min at 37°C. The suspension was then completed with $CaCl_2$ and $MgCl_2$ (2 and 1 m*M*, respectively), and the cells were stimulated with ionophore A23187 (4 μM) in the presence of [1-^{14}C]AA (14 MBq per 3 ml, 6.5 μM). Incubation was done for 3 min under constant gentle stirring at 37°C. The reaction was stopped by the addition of 0.3 ml of 1 *M* hydrochloric acid and 15 ml of ethyl acetate. The suspension was stirred in a vortex for 30 s, cooled in an ice-bath and centrifuged at 1500 *g* for 10 min. An aliquot (10 ml) of the organic layer was evaporated under a stream of nitrogen. The evaporated extract was resuspended in 30 μl of methanol, and 10 μl of a mixture of the eight tritiated standards in methanol were added (0.74 kBq of each).

The integrity of the cells after preincubation with drugs was checked by dosing the supernatant lactate dehydrogenase (LDH) content (kit, Roche Diagnostica Neuilly, France).

Separation of arachidonic acid metabolites by RP-HPLC

The extract containing [1-^{14}C]AA metabolites synthesized by the PMNLs and tritiated standards was applied to the RP-HPLC column. Elution was carried out using a combination of two solvents in a single run of 70 min with three major steps (flow-rate 1.0 ml/min).

During the first step solvent A (0.05 *M* NaH_2PO_4, pH 5 0–acetonitrile, 62.5:37.5, v/v) was used for 20 min. Then a gradient with solvent B (0.05 *M* NaH_2PO_4, pH 5–methanol–acetonitrile, 30:35:35, v/v) was carried out in the second step as follows: a 10-min linear gradient from 0% B to 60% B, a 10-min isocratic step (40% A and 60% B), then a 5-min linear gradient to 20% A and 80% B. The third step was isocratic: 25 min at 20% A and 80% B. Elution of residual [1-^{14}C]AA was achieved by solvent C 100% methanol at a flow-rate of 1.5 ml/min.

Metabolites of [1-^{14}C]AA were identified by comparing their retention times with those of tritiated standards and quantitated by measuring the areas under the respective peaks. Each peak count was adjusted relative to the total radioactivity of the corresponding sample. For each drug assay, the amount of every metabolite was compared with the control assay (vehicle). This calculation method attenuates the variability due to the loss of material during ethyl acetate extraction and allows a rapid and comparative quantitation of drug interactions with the AA metabolism *in vitro*. Each assay was done in duplicate.

RESULTS

The biotransformation of exogenous [1-^{14}C]AA by control-stimulated PMNLs was 63.4 ± 2.6% (confidence interval for $\alpha = 0.05$) from six assays. The extraction of AA metabolites with a direct liquid–liquid method provided sufficient recovery (more than 50% for each metabolite) for accurate radioactivity measurements and a clean sample, which could be directly applied to the column after evaporation. AA metabolites were identified by reference to the authentic tritiated standards. Fig. 1 shows a typical pattern of AA metabolite separation; the major products formed were lipoxygenase derivates: 5HETE, 12HETE and LTB_4. A non-negligible amount (more than 30%) of cyclooxygenase compounds — PGE_2, TXB_2 and 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) — was also produced.

Cyclooxygenase metabolites were eluted in the first 20-min isocratic step by solvent A except for HHT, which was eluted in the third step. The second step allowed an accurate identification of LTB_4. HHT and HETEs were separated by the isocratic third step; residual [1-^{14}C]AA was eluted by a 10-min flow of 100% methanol. The total time of the analysis was *ca.* 70 min for the separation of cyclooxygenase and lipoxygenase derivatives, with a further 15 min for AA elution. As shown in Table I, the variability of the retention times [as determined by the coefficient of variation (C.V.)], dependent on the column quality, was low in these conditions.

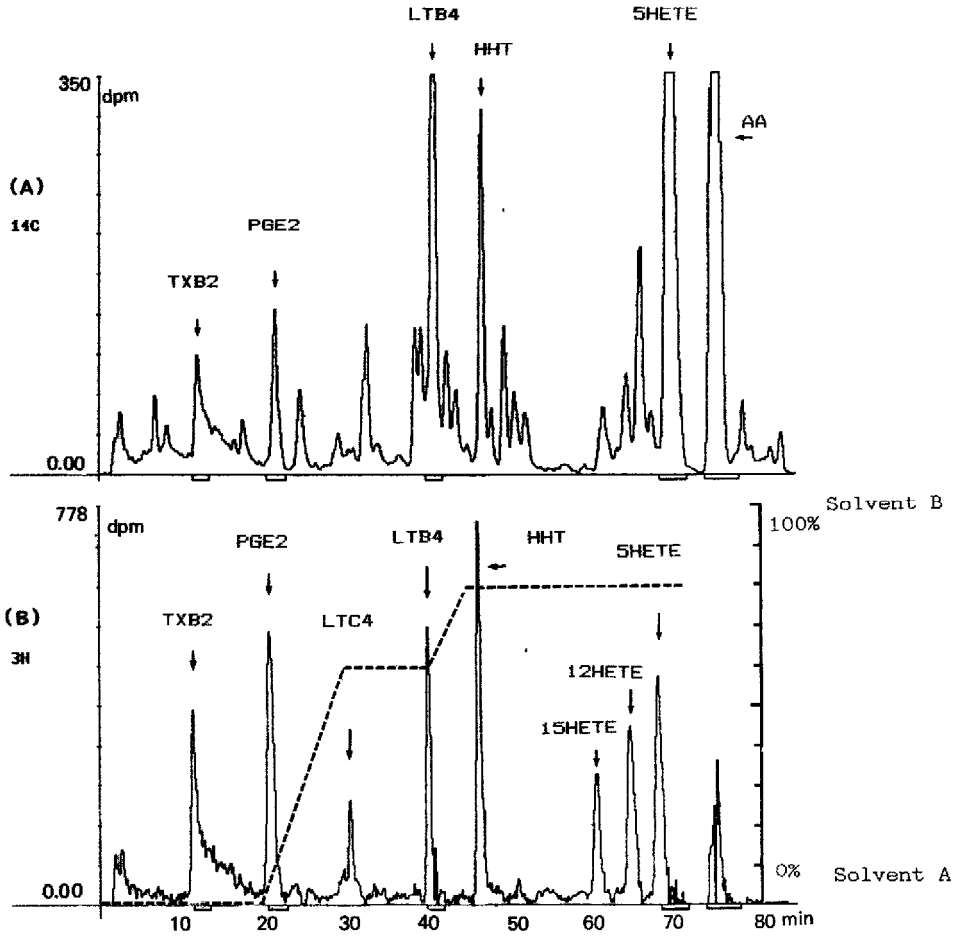

Fig. 1. RP-HPLC analysis of AA metabolites from rat PMNLs stimulated with ionophore A23187 in presence of [1-^{14}C]AA (A) with on-line dual-channel radioactive monitoring. Elution was carried out with comigration of tritiated standards (B) The solvent system is described in Experimental, and the gradient curve is shown in B After 70 min, unchanged AA was eluted with 100% methanol (10 min).

This chromatographic system was applied to the rapid analysis of AA metabolites contained in an extract of *in vitro* stimulated rat PMNLs preincubated with a potent cyclooxygenase inhibitor (indomethacin) or a lipoxygenase inhibitor (NDGA) used at the concentrations of 10^{-6} and $5 \cdot 10^{-7}$ *M*. Elution profiles of [1-^{14}C]AA metabolites are shown in Fig. 2; TXB_2, PGE_2, LTB_4, 5HETE and residual AA were quantitated for each sample.

At the concentrations used, indomethacin inhibited selectively the biosynthesis of cyclooxygenase derivates ($\geqslant 58\%$), and NDGA inhibited the production of LTs and monoHETEs ($\geqslant 73\%$) without any action on the prostaglandin syn-

TABLE I

COMPARISON OF THE RETENTION TIMES OF THE MAJOR METABOLITES OF [1-^{14}C]AA PRODUCED BY RAT PMNLs

Assay	n	Retention time (mean ± S.E.M.) (min)			
		TXB_2	PGE_2	LTB_4	5HETE
1	2	11.04 ± 0.04	19.40 ± 0.13	38 74 ± 0 14	64.13 ± 0.40
2	2	12.03 ± 0.10	21.17 ± 0.30	39 43 ± 0 10	65.00 ± 0.40
3	2	12 70 ± 0.23	21.67 ± 0.40	39 34 ± 0 14	63 27 ± 0.00
4	2	11.94 ± 0.14	20.33 ± 0.20	39.03 ± 0.10	64 54 ± 0 74
5	2	12 44 ± 0.44	21.20 ± 0.87	39 70 ± 0 17	64.54 ± 0.27
6	2	10 80 ± 0 13	19.37 ± 0.24	39.30 ± 0 23	66 34 ± 0 34
C.V. (%) (inter-assay)		6.40	4.78	0.84	1 58

thesis (Table II). This inhibition was not due to cytotoxicity of the drugs since no increase of the LDH level was observed at the concentration of 10^{-6} M (results not shown).

DISCUSSION

In this study, we have described a simple and rapid automated HPLC system for characterization and quantitation of the major AA metabolites in a single run. This system was developed using synthetic metabolites: Fig. 3 shows an elution pattern of eighteen AA derivatives separated in a single run of 65 min with multiple-wavelength UV detection. The lowest amount of PGE_2, LTB_4 or 5HETE detected was *ca.* 10 ng injected.

TABLE II

COMPARATIVE ACTIVITIES OF INDOMETHACIN AND NDGA ON [1-^{14}C]AA METABOLISM BY RAT PMNLs STIMULATED WITH IONOPHORE A23187

Results are expressed as mean percentage change from control obtained in two assays

Compound	Concentration (M)	Activity (%)			
		TXB_2	PGE_2	LTB_4	5HETE
Indomethacin	10^{-6}	−66	−76	+16	+19
	$5 \cdot 10^{-7}$	−58	−66	+29	+20
NDGA	10^{-6}	+23	+17	−89	−86
	$5 \cdot 10^{-7}$	+ 7	+10	−72	−74

As a trial the system was applied to the analysis of AA metabolites released *in vitro* by rat PMNLs stimulated with ionophore A23187, but their concentrations in the cell medium were below the UV detection level. The sensitivity of the detection method was increased by using [1-^{14}C]AA as a marker of the endogenous AA metabolism. Detection of AA metabolites was achieved with a dual-channel continuous radioactive detector. This detection mode, which avoids tedious collection of fractions and determination of each fraction count, allows a

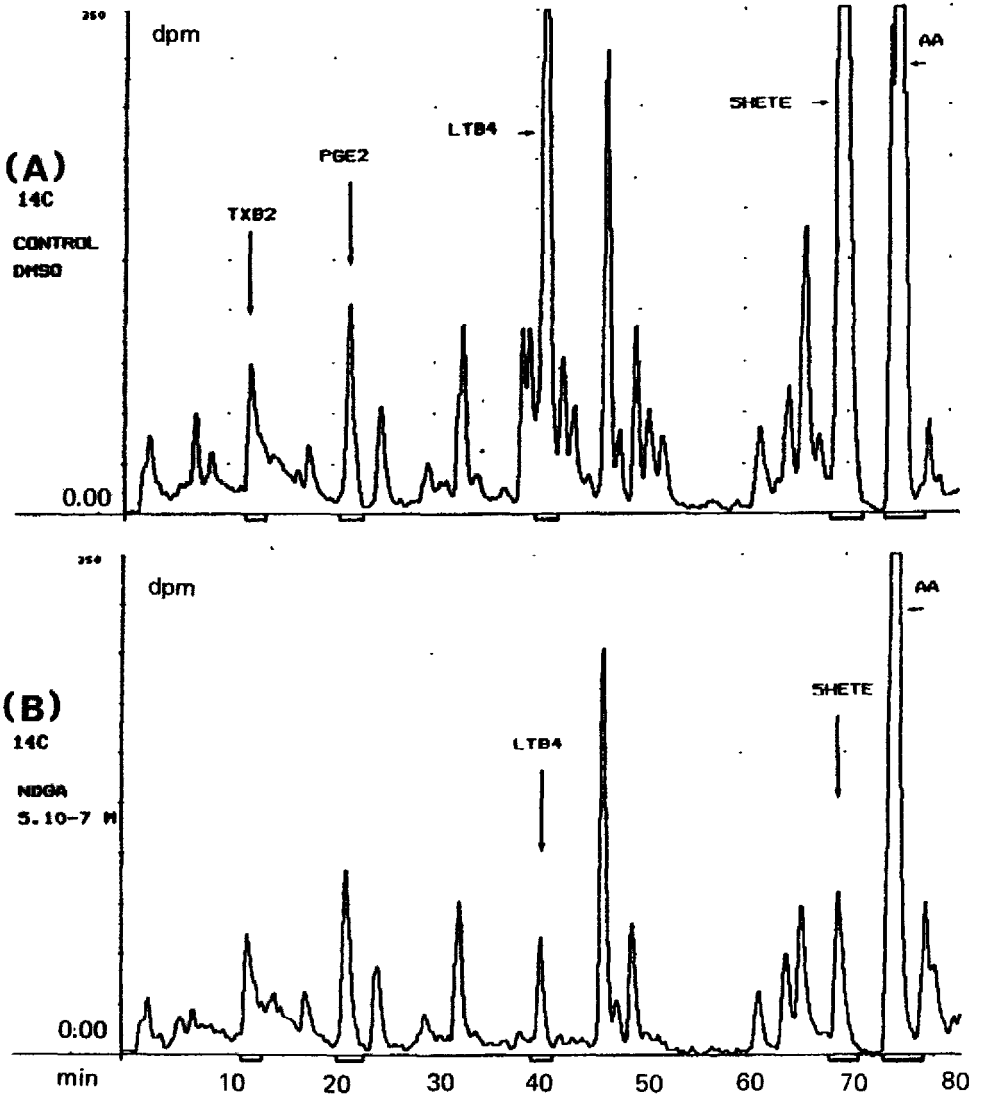

Fig. 2

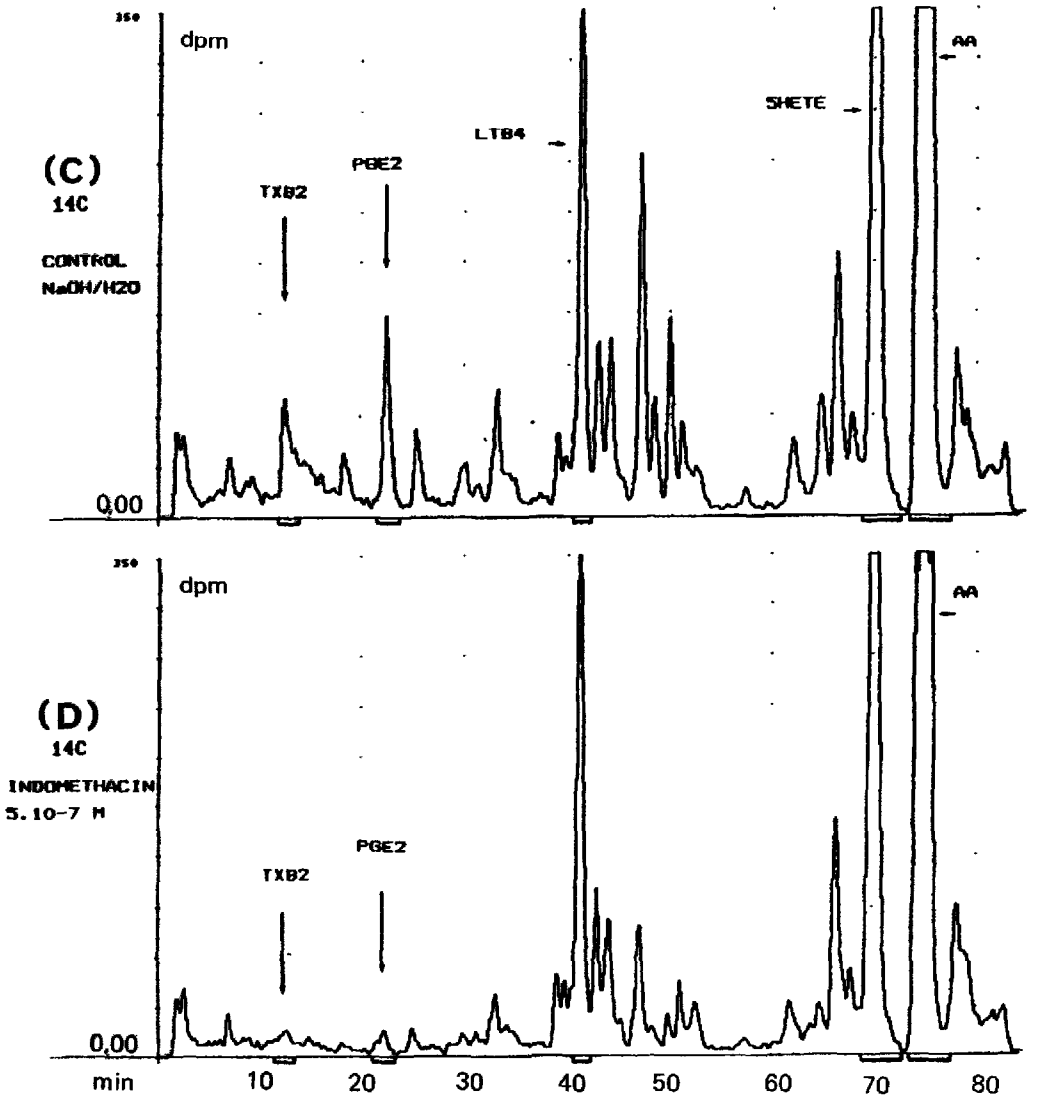

Fig. 2. Selective inhibition by indomethacin and NDGA of AA cyclooxygenase and lipoxygenase metabolites from rat PMNLs stimulated with Ca ionophore A23187. Cells were preincubated for 5 min with $5 \cdot 10^{-7}$ *M* NDGA (B) or a vehicle (DMSO) (A), $5 \cdot 10^{-7}$ *M* indomethacin (D) or a vehicle (0.1 *M* NaOH–H_2O) (C)

better resolution with sharper peaks, and therefore a reliable identification and a more accurate quantitation of each metabolite were obtained (Fig. 1). In addition, with respect to the separation of LTB_4 from HHT, a higher resolution was obtained by introducing an isocratic step during the second gradient step. This automated RP-HPLC system may be applied to the analysis of a great number of

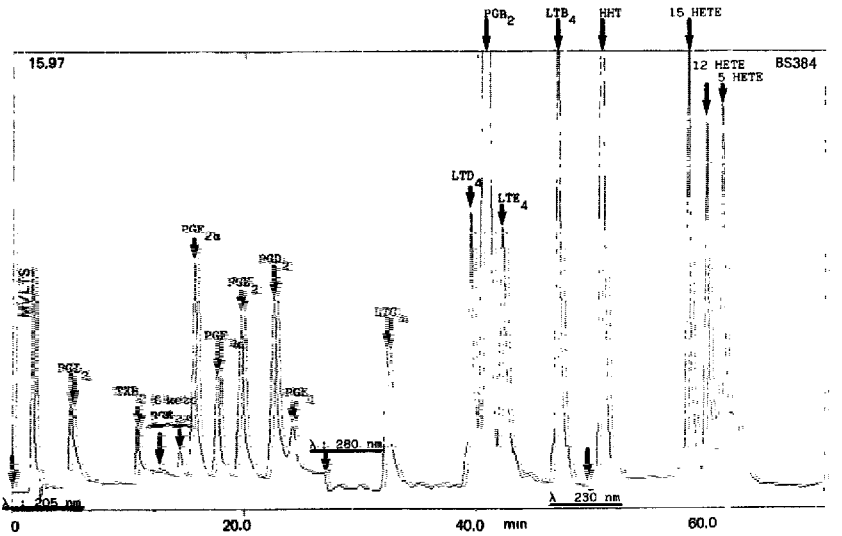

Fig. 3. Elution pattern in a single run of cyclooxygenase and lipoxygenase products (synthetic standards) by a gradient of two solvents in three steps with UV detection Solvent A was 0.05 *M* NaH_2PO_4 (pH 5)–acetonitrile (72.5·27.5, v/v), and solvent B was 0.05 *M* NaH_2PO_4 (pH 5)–methanol–acetonitrile (30:35:35, v/v). First step, 100% A for 20 min (λ = 205 nm); second step, linear gradient 100% A to 100% B in 25 min (λ = 280 nm); third step, 100% B for 20 min (λ = 230 nm). The flow-rate was 1 0 ml/min

samples (fifteen samples per 24 h) with high resolution and may be reproduced easily from one sample to another with low variability of the retention time (Table II); comigration with authentic standards allows a reliable identification of each AA metabolite. Variations from one experiment to another, due to the column quality and cell response to the stimulation by ionophore, were attenuated by the comparative calculation method used.

Introduction of internal standards or use of another extraction process for these metabolites, such as a Sep-Pak cartridge [9], may be carried out to increase the precision of analysis; however, this would increase the analysis time. Results obtained under our conditions after preincubation of the cells in the presence of a selective inhibitor of cyclooxygenase (indomethacin) or of lipoxygenase (NDGA) have shown that this system permits a comparative and quantitative determination of drug interactions with AA metabolism *in vitro* (Table II).

In conclusion, this HPLC system offers a simple model for the rapid and precise analysis of the cyclooxygenase and lipoxygenase metabolites produced by cells or tissues using a detection mode (UV or continuous-flow radioactivity) adapted to their concentrations in the assay medium. This system constitutes a good model for the first pharmacological screening of drugs acting on the AA metabolism and for the rapid investigation of their specificity for the cyclooxygenase or the lipoxygenase pathway.

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