

Material and Methods

Reagents

Lymphoprep was bought from Medinor (Stockholm, Sweden). Microbeads CD16 and MACS column was bought from Göteborgs Termometerfabrik (Göteborg, Sweden). Dextran (T500) was bought from GE Healthcare (Uppsala, Sweden). NH₄Cl was bought from Merck Solna (Sweden). Tris-HCl was bought from Scharlau (Spain) Arachidonic acid was bought from Nu-Check-prep (Elysian, MN, USA). Lysin-aspirin was bought from Apoteket (Huddinge, Sweden). A23187 was bought from Sigma (Stockholm, Sweden). Enzyme Immunoassays from Cayman, 15-HETE and EXC₄, was bought from AH Diagnostics (Stockholm, Sweden) and Cayman Chemicals (Ann Arbor, Michigan, USA) respectively. Enzyme immunoassay LTC₄ was bought from GE Healthcare (Uppsala, Sweden). Synthetic Prostaglandin E₂ (PGE₂), D₂ (PGD₂), F₂α (PGF_{2α}), 15-Ketoprostaglandin E₂ (15-Keto-PGE₂), 6-Keto prostaglandin F_{1α} (6-Keto-PGF_{1α}), Thromboxane B₂ (TxB₂), Eoxin C₄ (EXC₄), Leukotriene C₄ (LTC₄) and Lipoxin A₄ (LXA₄) were bought from the SMS-gruppen (Denmark).

Identification of eicosanoids by LC-MS/MS

The comprehensive identification by LC-MS/MS was performed on a Surveyor MS coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corporation, Sweden). Reverse phase LC was performed using a Gemini-NX 3μm C18 column 2.0 x 50 mm (Phenomenex) with flow rate constantly held at 400 μl/min. Mobile phase A consisted of 2% acetonitril in water and 0.1% acetic acid, and mobile phase B consisted of 80% acetonitrile in water and 0.1% acetic acid. All water used was of MilliQ grade and organic solvents of analysis grade. Three different analyses methods were performed on prostaglandins, EXC₄/LTC₄ and LXA₄. Incubations were stopped by adding acetonitrile containing internal standard for quantitative analyses of prostaglandins and LXA₄ by LC-MS/MS.

For prostaglandins the chromatography started isocratically for 1 min at 90 % A was followed by a 7 min linear gradient reaching 70% of B. After direct switching to 100 % B the system

was washed for 2 min and subsequently equilibrated at 90% A for 2 min. The mass spectrometer was operated using electrospray atmospheric pressure ionization source in negative mode. The spray voltage was set to 3300 V, capillary temperature was 350°C and sheath and auxiliary gas was optimal at 50 and 6 respectively (arbitrary units). Skimmer offset was at 10-12 V and tube lens 128 V. The transition utilized in the SRM scan was for PGE₂ and PGD₂ 351.2 → 271.0 m/z, PGF_{2α} 353.2 → 309.0 m/z, 15-Keto-PGE₂ 349.1 → 287.1 m/z, 6-Keto-PGF_{1α} 369.2 → 245.2 m/z, TxB₂ 369.2 → 169.0 m/z at collision energies between of 20-26 eV.

For EXC₄/LTC₄ the chromatography started isocratically for 1.5 min at 90 % A was followed by a 4.5 min linear gradient reaching 100% of B. The system was washed at 100 % B for 2 min and subsequently equilibrated at 90% A for 2 min. The mass spectrometer was operated using electrospray atmospheric pressure ionization source in positive mode. The spray voltage was set to 4500 V, capillary temperature was 350°C and sheath, ion sweep gas and auxiliary gas pressure was optimal at 60, 5 and 5 respectively (arbitrary units). Skimmer offset was at 10 V and tube lens 122 V. The transition utilized in the SRM scan for LTC₄ was 626.2 → 189.0 m/z and for EXC₄ 626.2 → 205.1 m/z at a collision energy of 19 and 21 eV, respectively. The EXC₄ and LTC₄ were also separated chromatographically by approximately 0.4 min.

For LXA₄ an Sunfire 3.5 μm C18 column 2.1 x 30 mm (Waters, Sweden) was used and the chromatography started isocratically for 1 min at 90 % A was followed by a 5 min linear gradient reaching 70% of B. After direct switching to 100 % B the system was washed for 2 min and subsequently equilibrated at 90% A for 2 min. The mass spectrometer was operated using electrospray atmospheric pressure ionization source in negative mode. The spray voltage was set to 3000 V, capillary temperature was 350°C and sheath ion, sweep gas and auxiliary gas was optimal at 60, 5 and 5 respectively (arbitrary units). Skimmer offset was at 10 V and tube lens 96 V. The transition utilized in the MRM scans was for LXA₄ 351.2 → 289.2 and 217.1 m/z at collision energy of 15 and 21 eV, respectively. Standard calibration curve at ten concentration levels was prepared in PBS (0.5 ml) in the following interval; 1.2-600 pmol/ml for all prostaglandins, 0.6-320 pmol/ml for EXC₄ and LTC₄ and 0.9-473 pmol/ml. Three qualitative controls at 3 different concentration levels were used for each analyte. Warfarin was used as internal standard at 16 nM added to the acetonitril solution used to stop the incubations. The transition utilized for warfarin in the SRM scan was in negative mode 307.0 → 161.0 m/z and in positive mode 309.0 → 163.1 m/z. The standard samples were

treated in the same manner as the eosinophil incubation samples. One standard series was analysed in the beginning and one at the end of the analysis list. Limit of quantification (S/N 5) was for PGE₂ 1.2 pmol/ml, PGD₂ 1.2 pmol/ml, PGF_{2α} 4.7 pmol/ml, 15-Keto-PGE₂ 4.7 pmol/ml, 6-Keto-PGF_{1α} 18.8 pmol/ml, TxB₂ 1.2 pmol/ml, EXC₄ 1.3 pmol/ml, LTC₄ 1.3 pmol/ml and LXA₄ 7.4 pmol/ml.