Rapid Simultaneous Analysis of Prostaglandin E_2, 12-Hydroxyeicosatetraenoic Acid and Arachidonic Acid Using High Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry

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Arachidonic acid (AA) can be metabolized to a variety of lipid mediators including prostaglandins (PGE), and hydroxyeicosatetraenoic acids (HETE) by cyclooxygenase, lipoxygenase and cytochrome P450-dependent monooxygenase enzymatic pathways. Traditional experimental procedures to quantify these lipid mediators require purification, often by high performance liquid chromatography (HPLC), prior to derivatization for gas chromatography/mass spectrometry (GC/MS) analysis. This paper describes a rapid and simple technique for the simultaneous quantitative analysis of PGE_2, 12-HETE, and AA by HPLC/electrospray ionization mass spectrometry on cultured human dermal fibroblast supernatants. Extension of the method to analyse 5-HETE and 15-HETE was investigated. The advantages of this method include minimal sample preparation and elimination of the problem associated with thermal stability for GC/MS analysis. A detection limit of 20pg on column for PGE_2 and 5pg on column for 12-HETE and AA was determined. **© 1997 by John Wiley & Sons, Ltd.**

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Arachidonic acid (AA) can be metabolized to a variety of molecules by cyclooxygenase, lipoxygenase and cytochrome P450-dependent monooxygenase enxymatic pathways.[1–3] Products of these pathways include prostaglandin E_2,a nd 12-hydroxyeicosatetraenoic acid (12-HETE) which are important mediators in inflammatory skin disorders.[4] Established methods for the analysis of PGE_2, 12-HETE and AA are often based on gas chromatography/mass spectrometry (GC/MS).[5] First, lipid mediators are extracted either by organic extraction[6] or by using a reverse phase solid phase extraction (SPE) column.[2,7,8] Further purification by high performance liquid chromatography (HPLC) is frequently required, particularly for HETE analysis[7,8] before purified extracts can be derivatized for quantitative analysis by GC/MS. Although successful, these methods are cumbersome when handling a large throughput of samples. Thermal stability under GC/MS conditions is also a problem for HETE analysis.[5,9] As the hydroxyl substitution position in the lipid chain becomes closer to the carboxylic acid moiety, increased evidence of degradation is seen. The analysis of 5-HETE can be particularly problematic.

Directly linking HPLC to electrosparay ionization mass spectrometry (ESI-MS) removed the need for off-line HPLC purification and the requirement for derivatizing these lipid mediators. The problem associated with the thermal stability of these compounds under GC/MS analysis was removed. Cultured human dermal fibroblast supernatants were examined as a source of lipid mediators because of our interest in inflammatory skin disorders. PGE_2, 12-HETE and AA were purified using reverse phase SPE columns and quantified by HPLC/ESI-MS using a stable iostope dilution technique. The possible presence of 5 and 15-HETE in the samples was also monitored. This paper describes a rapid simple multi-component technique for quantitative analysis of these lipid mediators from cultured dermal fibroblasts.

EXPERIMENTAL

Chemicals

Dulbecco's modified Eagles medium (DMEM), heat inactivated fœtal bovine serum, 2 mM L-glutamine, 100 U/L penicillin, 0.2 mg/L streptomycin supplement and 0.25% trypsin solution were purchased from Sigma (Poole, UK) > Recombinant human interleukin-1α (rhIL-1α) was obtained from R&D Systems (Abingdon, UK). AA, AA-d_8, 5(±)-HETE, 12(±)-HETE, 12(*S*)-HETE-d_8, 15(±)-HETE, PGE_2 and PGE_2-d_4 (Cayman Chemicals, USA) solutions were prepared in methanol at 1 ng/μL. Acetonitrile, methanol and glacial acetic acid (analytical grade) were purchased from Fischer Scientific (Loughborough, UK).

Cell culture preparation

Human dermal fibroblasts were isolated from skin circumcisions and maintained as described by Sly and Grubb.[10] Cultures were grown in DMEM supplemented with 10% heat inactivated fœtal bovine serum plus 2 mM L-glutamine, 100 U/L penicillin, 0.2 mg/L streptomycin and passaged using trypsin. For experiments, cells from the third passage were seeded in 12 well culture plates with 1mL culture medium and grown to 90% confluence. Cells were stimulated by replacing the growth media with 1ml of serum-free

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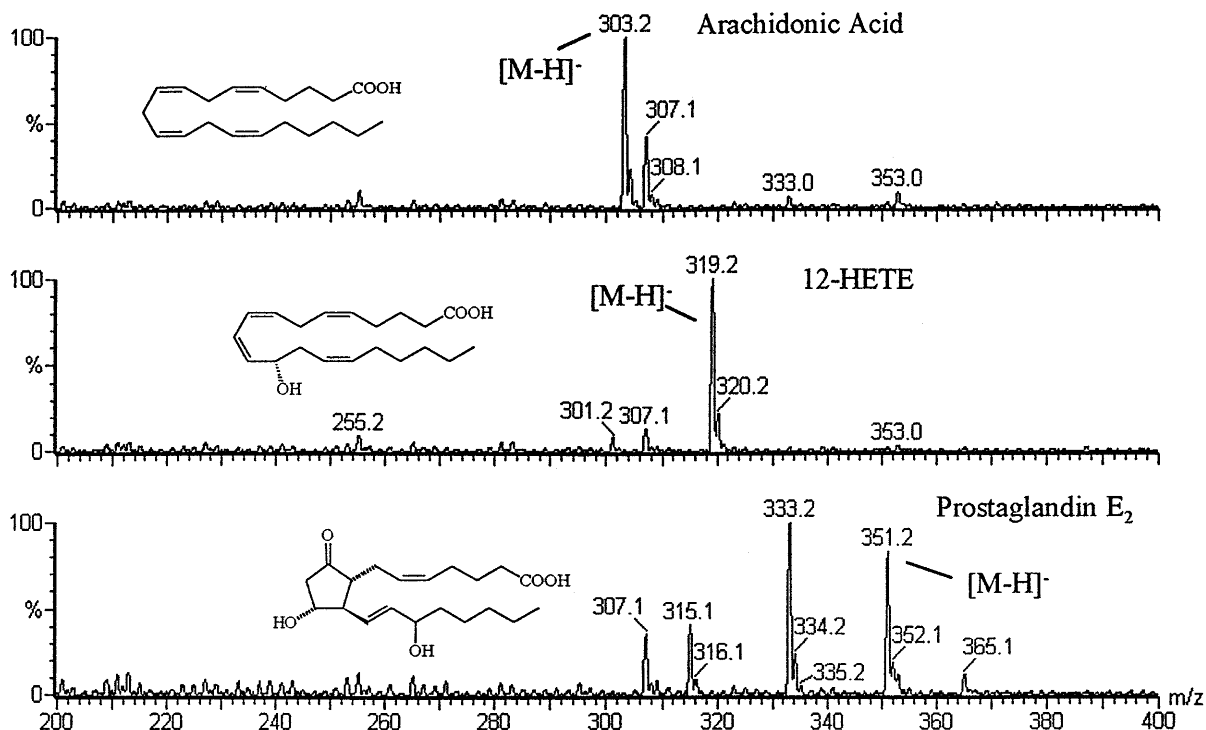

Figure 1. Negative ion electrospray mass spectra of 100 pg/μL arachidonic acid, 12-HETE and prostaglandin E_2. Capillary = 3.6 kV, Cone = 20V.

DMEM containing 100 pg/mL rhIL-1α. IL-1α treatment was omitted for the control. Cell culture supernatants were removed at appropriate times within the experiment and used for lipid analysis.

Sample preparation

The 1mL culture supernatants were centrifuged (100 g/5 min.) and the liquid decanted in order to remove particulates and cell debris. 10 μL of AA-d_8 solution, 10 μL of 12(*S*)-HETE-d_8 solution and 10 μL or PGE_2-d_4 solution were added to the samples as internal standards. Disposable octadecyl SPE columns (Isolute C_{18} endcapped 100 mg sorbent (Jones Chromatography, Hengoed, UK), were equilibrated successively with 2 × 1mL of acetonitrile and 3 × 1mL of de-ionized water. Samples were acidified by the addition of 20 μL glacial acetic acid and applied to the columns. After washing with 2 × 1mL of de-ionized water, lipids were eluted by addition of 2 × 0.5 mL 98% acetonitrile. The eluted fractions were then evaporated to dryness in a Savant vacuum centrifuge (Life Sciences International, Basingstoke, UK) at room temperature and the residue reconstituted in 100 μL of 50% acetonitrile/de-ionized water for analysis.

Table 1. Linearity of calibration curves for lipid mediators using HPLC/ESI/MS. (10 μL on-column)

Conc^n (pg/μL)	*Ratio lipid/internal standard* PGE$_2$	12-HETE	AA
10	0.149	0.156	0.190
50	0.651	0.719	0.935
100	1.200	1.353	1.925
200	2.567	2.8279	4.088
400	5.152	5.3324	7.508
Slope=	0.0129	0.0133	0.0189
Intercept=	−0.009	0.054	0.054
R^2=	1.000	0.999	0.998

Recovery (spiked) samples

To 1 mL of serum-free culture medium, 10 μL of PGE_2-d_4, 12(*S*)-HETE-d_8 and AA-d_8 internal standard solutions were added. The solution was then spiked with 10 μL of PGE_2, 5(±)-HETE, 12(±)HETE, 15(±)HETE and AA stock solutions and acidified with 20 μL glacial acetic acid. This produced a spiked sample containing 10 ng of each lipid mediator. Spiked serum-free culture media were than applied to disposable C_{18} SPE columns and treated as described for the sample preparation.

Standard calibration curve preparation

100 μL of PGE_2-d_4 solution, 100 μL of 12(*S*)-HETE-d_8 and 100 μL of AA-d_8 solution were added to a series of 2 mL sample containers. 10, 50, 100, 200 or 400 μL of PGE_2, 5(±)-HETE, 12(±)-HETE, 15 (±)-HETE and AA solutions were subsequently added in order to construct a standard curve. The standard solutions were evaporated to dryness (rotary vacuum, room termperature) and the residue reconstituted in 1mL of 50% acetonitrile/de-ionized water for analysis. Subsequent dilutions of a prepared 10 pg/μL standard solution with 50% acetonitrile/de-ionized water were used to assess the on-column limits of detection.

Instrumentation

Applied Biosystems 140B modular high pressure binary syringe pump, Applied Biosystems 112A modular oven/injector (Perkin Elmer, Warrington, Cheshire, UK) and Micromass Platform I (Wythenshawe, Manchester, UK) mass spectrometer with electrospray ionization source.

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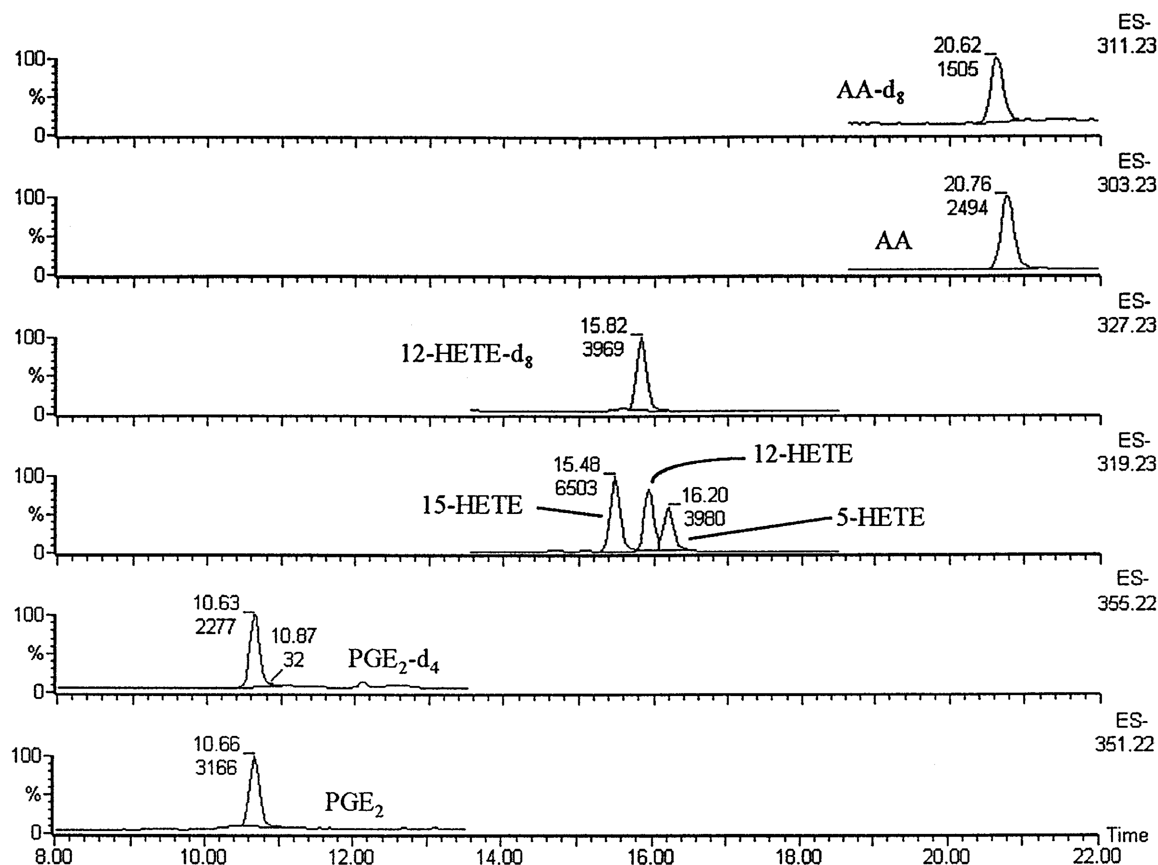

Figure 2. Chromatogram obtained from spiked culture media at 10ng lipid per mL (1ng of lipid on-column for a 10µL injection). Each trace is a SIR for the $[M-H]^-$ anion corresponding to the mediators or the deuterated internal standards.

Chromatography

Chromatographic separation of PGE_2, 5/12/15-HETE's and AA was achieved using a Microbore™ 15 cm × 1 mm i.d. column packed with Spherisorb 3µm ODS-2 (Phase Separations, Clywd, UK). The column was maintained at 40 °C. Phase A was 20% acetonitrile 72% (0.02% aqueous formic acid), phase B was 90% acetonitrile, 10% (0.02% aqueous formic acid). Mobile phases were delivered at 60 µL/min. using 100% A between 0 min. and 1 min. followed by a linear gradient to 100% B at 17 min. then isocratic to 22 min. with 100% B. 10 µL of the prepared solutions were applied to the column at 0 min. following suitable equilibration.

Mass spectrometry

MS was configured to detect the *m/z* for the $[M-H]^-$ anion of each acid (Fig. 1) or internal standard as elution from the HPLC column occurred. The source temperature was maintained at 90 °C, capillary 3.6 kV, cone 20 V. The drying gas maintained at *ca.* 300 l/hr and the nebulizing gas at *ca.* 20 L/hr. Selected ion recording (SIR) 8 to 13.5 min. for PGE_2/ PGE_2-d_4 *m/z* = 351.2 and 355.2, 13.5 to 18.5 min. for HETE/HETE-d_8, *m/z* = 319.2 and 327.2, 18.5 to 22 min. for AA/AA-d_8, *m/z* = 303.2 and 311.2. Dwell times of 1s were used to monitor each ion. Data was collected and analyzed using Mass Lynz V 2.22.

Table 2. Recovery of 10 ng/ml (1ng on-column) lipid mediators from spiked cell culture medium using HPLC/ESI/MS. Data is the mean of five determinations (*n*=5) ± standard deviation

Compound	*Recovery %*
Prostaglandin E2	117 ± 13.3
12-Hydroxyeicosanoic acid	105 ± 12.3
Arachidonic acid	102 ± 10.4

RESULTS

Figure 1 shows the negative ion electrospray (ESI) spectra of AA, 12-HETE and PGE_2 collected using the same parameters as described in the experimental mass spectometry section. Solutions were at concentrations of 100 pg/µL in 45% acetonitrile, 45% aqueous formic acid (0.22%) and 10% methanol. An intense $[M-H]^-$ anion was readily detected for all the chosen lipid mediators that could be monitored by SIR for HPLC detection. We observed no reduction in the signal intensities when formic acid was present in the mobile phase compared to when it was excluded. The use of formic acid was included to permit reproducible HPLC. Under the described HPLC/ESI-MS conditions, limits of detection where the signal was greater than three times the noise, were determined to be 20 pg on column for PGE_2 and 5 pg on column for 12-HETE and AA.

The response from the HPLC/ESI-MS method was found to be linear for PGE_2, 12-HETE and AA. Table 1 demonstrates linearity for ratios of analyte to internal standard signal for up to 4 ng of lipid on-column. Cell culture media examined without the presence of lipids

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or internal standards showed no interfering peaks. Figure 2 shows the chromatogram obtained from 1ng on-column spiked culture medium. Good recoveries from culture media, within expected handling errors, were obtained for PGE_2, 12-HETE and AA as demonstrated in Table 2. The recovery of 1ng on-column for 5 and 15-HETE was deterined with respect to the 12-HETE-d_8 internal standard. The recovery of 15-HETE was calculated to be 133.7 ± 14.2% (mean ± standard deviation) and 5-HETE to be 61.0 ± 7.1% for five observations ($n = 5$). The differing responses could be taken into account when quantifying HETE's found in 'real' samples removing the need for three separate HETE internal standards. The responses were determined to be linear, with low pg on-column sensitivity and the sample preparation gave good recovery with specificity.

Analysis of human dermal fibroblasts was undertaken on account of our interest with lipid mediators in inflammatory skin disorders of which PGE_2[11] and HETE's[12] have particular relevance. IL-1α can play a primary role in the initiation of inflammatory skin conditions.[13] Figure 3 shows the results of a time course analysis for the stimulation of cultured dermal fibroblasts with rhIL-1α. Data is represented as the mean of three determinations ($n = 3$) ± standard deviation. IL-1α can be seen to stimulate the production of PGE_2 but does not increase the production of 12-HETE within fibroblast culture supernatants. Traces of 15-HETE were encountered with no increase upon IL-1α stimulation and no 5-HETE was detected. The redistribution of AA was judged to be unaffected by the IL-1α stimulus. The distribution of data obtained was considered within experimental error for cell culture procedures.

DISCUSSION

We have demonstrated that HPLC/ESI-MS gave a rapid and reproducible method for the analysis of PGE_2, 12-HETE and AA in fibroblast cell culture supernatants. The method has the potential for expansion to include 5 and 15-HETE where requried. This procedure gave good recovery, reproducibility and overcame several disadvantages of previously described methods.[5,7,8] Sample preparation was quick and simple requiring only purification through a C_{18} SPE column, removing the need for HPLC fraction collection and derivatization prior to analysis by GC/MS. The problem of thermal stability for HETE analysis under GC/MS conditions was avoided and the method permitted multi-component analysis. Under

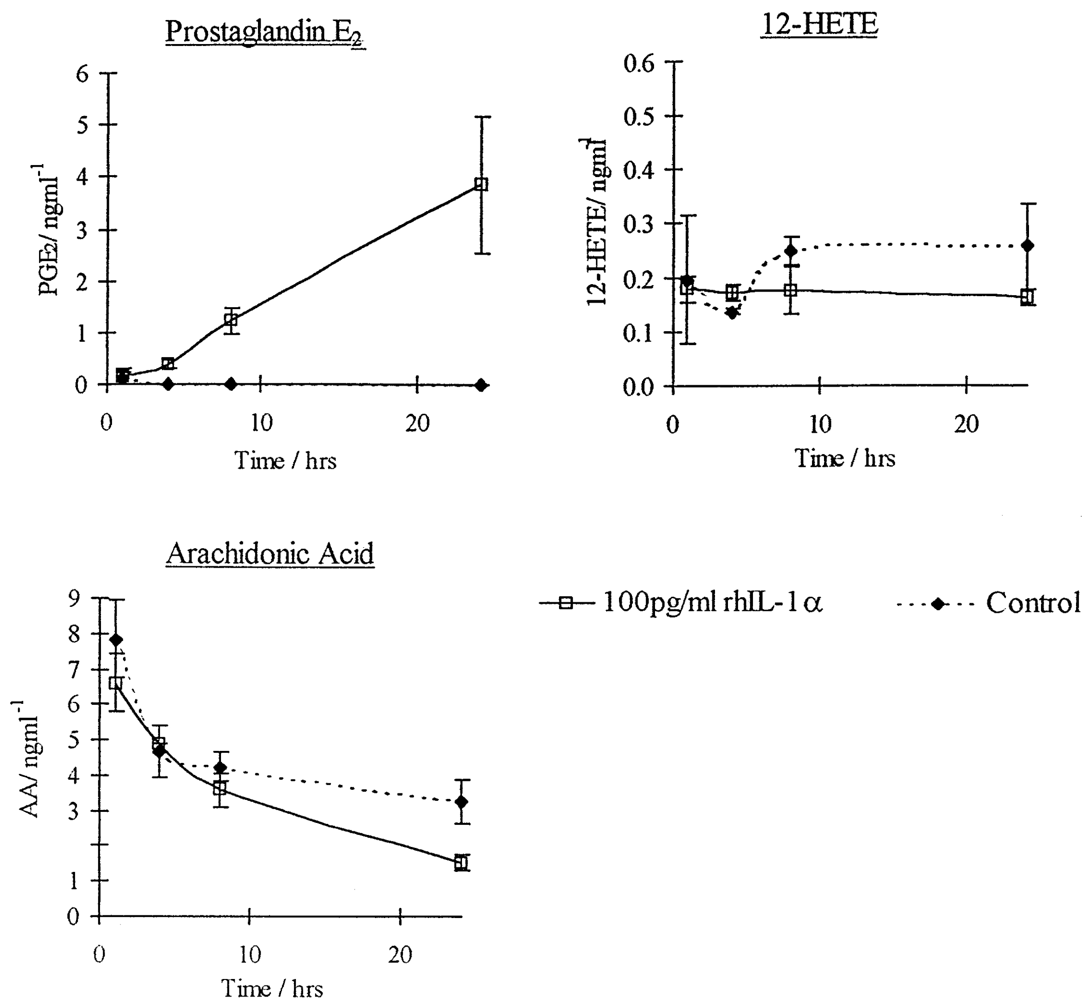

Figure 3. HPLC/ESI-MS analysis of cultured dermal fibroblasts stimulated by IL-1α against control (no stimulus) for lipid mediators. 24 hour time course for PGE_2, 12-HETE and AA. Each data point is the mean of three determinations ($n = 3$) ± standard deviation. IL-1α stimulation results in increasing PGE_2 production but not 12-HETE production.

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the described conditions, 20pg of PGE_2 and 5pg of 12-HETE and AA on-column were detected. The HPLC mobile phase included formic acid to permit reproducible chromatography. Although this leads to 'Wrong way round' electrospray ionisation,[14,15] we observed no reduction in the signal obtained for the lipid mediators with or without the presence of the formic acid. This is in agreement with other reported observations.[15] The procedure can be applied to other important lipid mediators of oxidised products of polyunsaturated fatty acids such as LBT_4 and may be applicable to other cell culture experiments.

CONCLUSION

HPLC/ESI-MS proved to be a powerful tool for the quantitative analysis of lipid inflammatory mediators in cultured dermal fibroblasts. The technique has several advantages over previously described GC/MS producedures, including reduced sample preparation, multicomponent analysis and the removal of thermal stability problems.

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