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THERMOSPRAY–MASS SPECTROMETRIC ANALYSIS OF UNDERIVATIZED MONOHYDROXY FATTY ACIDS: APPLICATION TO STIMULATED PLATELETS

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SUMMARY

Monohydroxylated fatty acids prepared from polyunsaturated fatty acids of nutritional value were analysed by thermospray–mass spectrometry without prior chemical derivatization. Positive and negative ionization modes were compared. The highest sensitivity was observed with the negative ionization mode with detection limits of 10 pmol based on the 12-hydroxy derivative of eicosatrienoic acid (12-OH-8,10,14–20:3). This is comparable to that obtained by high-performance liquid chromatography with UV detection at 234 nm. Selected ion monitoring based on the fragment $[M-H]^-$ allowed a variety of standard monohydroxy fatty acids to be detected. This approach makes possible the analysis of various derivatives generated by thrombin-stimulated platelets (10^9 cells) pre-enriched with minor polyunsaturated fatty acids, even when these derivatives co-elute from the column (e.g., 12-HETE and 14-OH-22:6).

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are metabolized by lipoxygenases to the hydroperoxides [1]. Subsequent enzymic or non-enzymic events lead to the formation of either further oxygenated products or to the corresponding monohydroxy derivatives via a peroxidase pathway [2]. Cyclooxygenase may also produce monohydroxy derivatives from prostanoid precursors [3]. These different oxygenated products appear to be capable of modulating platelet functions [4–8].

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The various hydroxy derivatives differ according to their chain length, the number and position of double bonds and the position of the hydroxy group. They can be all detected by high-performance liquid chromatography (HPLC) with UV detection (235 nm) because of the *trans-cis* conjugated double bond in the α-position to the hydroxy group. Gas chromatography–mass spectrometry (GC–MS) has also been used to measure a wide range of monohydroxy fatty acids with high specificity and sensitivity [9–15]. However, this technique requires derivatization of the molecules and hydrogenation of the double bonds because of a lack of stability, particularly with compounds with a high degree of unsaturation [16]. These procedures are time consuming and involve delicate multi-step processes.

Thermospray–liquid chromatography–mass spectrometry (LC–MS) has great potential for the analysis of labile polar biological samples as the spectrum of the intact molecule can be readily obtained without prior derivatization [17–19]. This technique allows on-line LC–MS operation at conventional flow-rates.

In this study, we used this approach for the analysis of a wide range of monohydroxy fatty acids obtained from the action of soybean lipoxygenase and platelet oxygenases. We also monitored the different compounds produced in thrombin-stimulated platelets pre-enriched with various PUFAs.

EXPERIMENTAL

Reagents

Fatty acids and soybean lipoxygenase were obtained from Sigma (St. Louis, MO, U.S.A.) and [1-^{14}C]arachidonic acid (60 Ci/mol) was supplied by the Radiochemical Centre (Amersham, U.K.). Organic solvents (analytical-reagent grade) were provided by SDS (Peypin, France). Silica gel G plates were purchased from Merck (Darmstadt, F.R.G.). Nucleosil C_{18} (3 μm) was purchased from Macherey, Nagel & Co. (Düren, F.R.G.).

High-performance liquid chromatography

A Waters Model 6000 A pump was connected to a Hewlett-Packard Model 1040 A diode-array detector. A 15 cm $\times$ 6.35 mm O.D. $\times$ 3.2 mm I.D. column was filled with Nucleosil C_{18} (3 μm) according to a published method for Partisil 5 [20]. A Rheodyne Model 7125 injection valve equipped with a 100-μl loop was used. The column was eluted with methanol–water (pH 3, adjusted with acetic acid) (74:26, v/v) at a flow-rate of 0.5 ml/min. The hydroxy fatty acids were detected at 234 nm and their UV spectra measured between 200 and 400 nm. Assays of sensitivity were performed under the same conditions with a Kontron Model 735 LC UV detector.

Thermospray–MS

All experiments were carried out on a Nermag R10-10C quadrupole mass spectrometer equipped with a digital data system and fitted with a Vestec thermospray (Vestec, Houston, TX, U.S.A.). The thermospray source modified by Nermag included a repeller. The optimal temperatures for the vaporizer and

downstream jet for this monohydroxy fatty acid analysis were found to be 145 and 195°C, respectively. Most of the liquid coming into the source was removed by a mechanical pump, connected opposite to the thermospray vaporizer, through a liquid nitrogen cold-trap. The analyser pressure was maintained at approximately $2.6 \cdot 10^{-3}$ Pa with a total mobile phase flow-rate of 1.2 ml/min, including the buffer (0.1 *M* ammonium acetate), which was added post-column at a flow-rate of 0.7 ml/min to allow ionization of the analytes.

Preparation of standard hydroxy fatty acids

n−8 hydroxy fatty acids. Human platelets ($3 \cdot 10^8$/ml) were obtained as described previously [21] and incubated with 10^{-4} *M* 20:5*n*−3, 20:3*n*−6, 20:3*n*−9, 20:4*n*−6 or 22:6*n*−3 for 30 min at 37°C. After acidification to pH 3 with 3 *M* hydrochloric acid the hydroxy fatty acids synthesized were extracted twice with 10 volumes of diethyl ether. They were separated from other lipids on silica gel G plates with hexane–diethyl ether–acetic acid (60:40:1, v/v).

n−5 hydroxy fatty acids. Hydroperoxides from 18:2*n*−6, 20:3*n*−6, 20:4*n*−6, 20:5*n*−3 and 22:4*n*−6 were prepared as reported previously [22] using soybean lipoxygenase. They were then reduced to the corresponding hydroxy derivatives treatment with sodium borohydride and purified by thin-layer chromatography as described above.

Biological assay

Human platelets were obtained from normal volunteers who had not taken any drug for at least 10 days prior to venous puncture. Platelets were isolated from plasma as described previously [21] and resuspended in Tyrode HEPES buffer (pH 7.4) (THB) containing 3.5 g/l fatty acid-free human albumin pre-coated with a mixture of PUFAs (20:3*n*−6, 20:3*n*−9, 20:5*n*−3, 22:4*n*−6 and 22:6*n*−3) as described [23], except that the molar ratio of PUFA to albumin was 0.5. After incubation for 2 h at 37°C with gentle shaking, the platelet suspension ($3 \cdot 10^8$/ml) was acidified to pH 6.4 with citric acid and centrifuged for 10 min at 700 *g*. The pellets were then resuspended in THB. One half of the platelet suspension was stimulated by 1 U/ml human thrombin and the other half was incubated with the vehicle.

Extraction and separation of lipids

The reaction was terminated after 4 min and the platelet suspension was acidified to pH 3 with 3 *M* hydrochloric acid. Lipids were immediately extracted with ten volumes of diethyl ether. The organic phase was evaporated and monohydroxy derivatives were separated from other lipids by thin-layer chromatography. The monohydroxy fatty acid spot (R_F 0.30) was scraped from the plate and extracted twice with 1 ml of diethyl ether.

RESULTS AND DISCUSSION

Modes of ionization

The ionization modes available with the thermospray technique (positive and negative chemical ionization) were used with the 12-hydroxy derivative of eicosa-

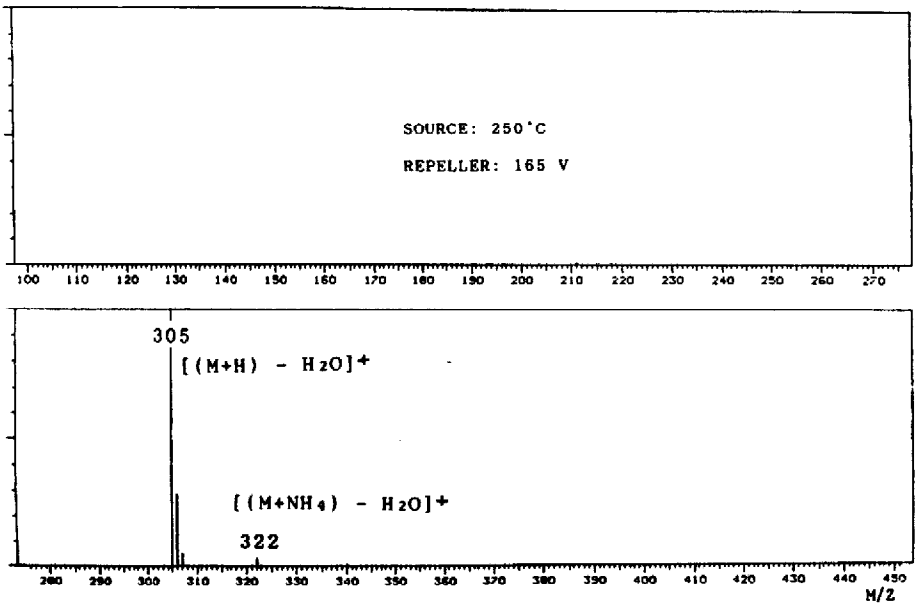

Fig. 1. Positive-ion mass spectrum of 12-OH-8,10,14-20:3 obtained on a Nermag R10-10C instrument with direct injection using a 50-μl loop (without a chromatographic column). The carrier liquid was methanol–water (pH 3, adjusted with acetic acid) (74:26) at a flow-rate of 0.5 ml/min, and 0.7 ml/min of 0.1 *M* ammonium acetate was added just before the thermospray.

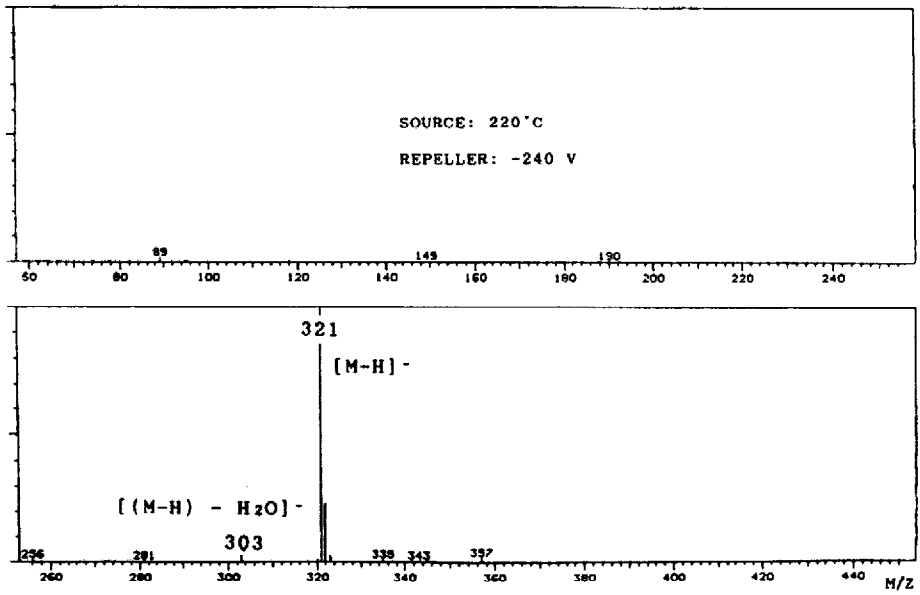

Fig. 2. Negative-ion mass spectrum of 12-OH-8,10,14-20:3. Conditions as in Fig. 1.

trienoic acid (12-OH-8,10,14–20:3). With the positive mode, two fragments were observed. That at m/z 305, corresponding to $[(M+H)-H_2O]^+$, is the base peak and that at m/z 322 could be attributed to $[(M+NH_4)-H_2O]^+$ as has been

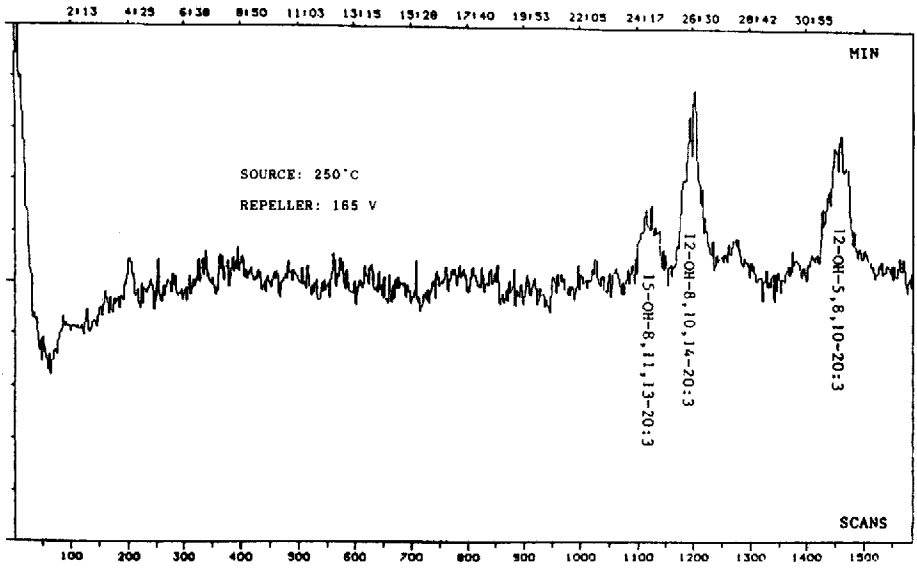

Fig. 3. Selected ion monitoring trace obtained with ca. 25 pmol of each monohydroxy derivative from 20:3 isomers. The positive chemical ionization mode was used and the fragment of m/z 305 was detected. Each peak was observed with 300 ms per scan. Separation of derivatives was obtained with a Nucleosil C_{18} (3 μm) column and methanol–water (pH 3, adjusted with acetic acid) (74:26) as the mobile phase. The flow-rate was 0.5 ml/min and 0.7 ml/min of 0.1 *M* ammonium acetate was added post-column just before the thermospray. Relative intensity (%) is displayed on the ordinate.

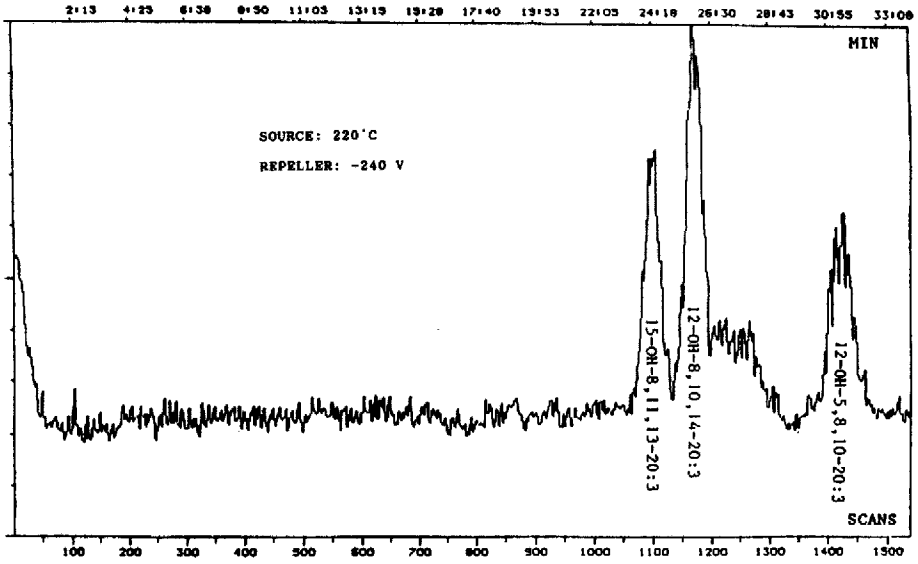

Fig. 4. Selected ion monitoring trace obtained as described in Fig. 3 except that the negative chemical ionization mode was used and the fragment of m/z 321 was measured.

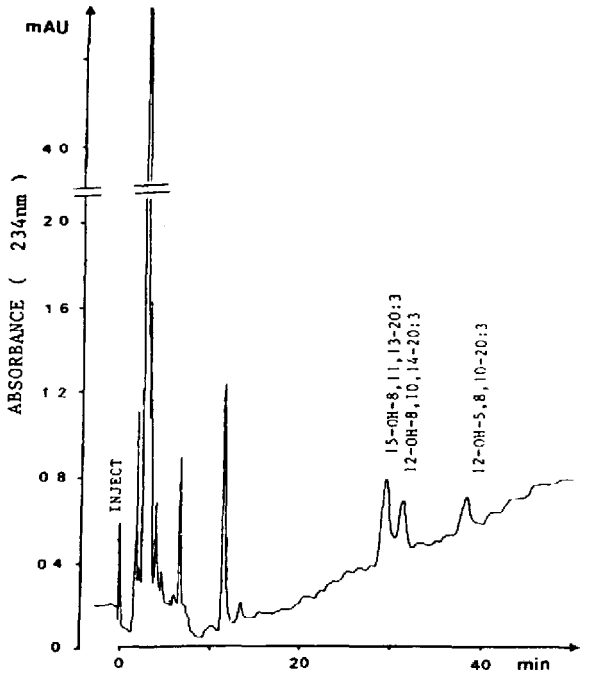

Fig. 5. Reversed-phase HPLC of ca. 25 pmol of the three monohydroxy fatty acids measured by LC-MS (Figs. 3 and 4) using a Nucleosil C_{18} (3 μm) column and methanol–water (pH 3, adjusted with acetic acid) (74:26) as the mobile phase. The absorbance at 234 nm is displayed on the ordinate and time on the abcissa.

reported with carbohydrates [24]. The intensity of the second fragment was very low (Fig. 1). In the negative ionization mode (Fig. 2), we observed a base peak at m/z 321 which represents the molecular ion $[M-H]^-$, and another ion of low intensity at m/z 303, corresponding to $[(M-H)-H_2O]^-$.

Neither mode is able to elucidate the structure of the monohydroxy fatty acid. In fact, the same spectra were obtained from 15-OH-8,11,13–20:3 and from 12-OH-5,8,10–20:3 (results not shown). Despite this disadvantage, this approach may be considered promising because of the expected high sensitivity owing to the small number of fragments produced.

Optimatization and sensitivity

The temperature of the ion source and the voltage of the repeller were optimized to obtain the highest sensitivity. With the positive ionization mode, the best conditions were reached at 250°C and 165 V. Under these conditions, around 25 pmol of each 15-OH-8,11,13–20:3, 12-OH-8,10,14–20:3 and 12-OH-5,8,10–20:3 could be detected with a signal-to-noise ratio of 5 (Fig. 3). With the negative ionization mode, the highest sensitivity was obtained with the source at 220°C and the repeller set at −240 V (maximal value allowed with the apparatus). Under these conditions, the signal-to-noise ratio was around 15 for the same amount of monohydroxy derivatives (25 pmol) (Fig. 4). This indicates that the

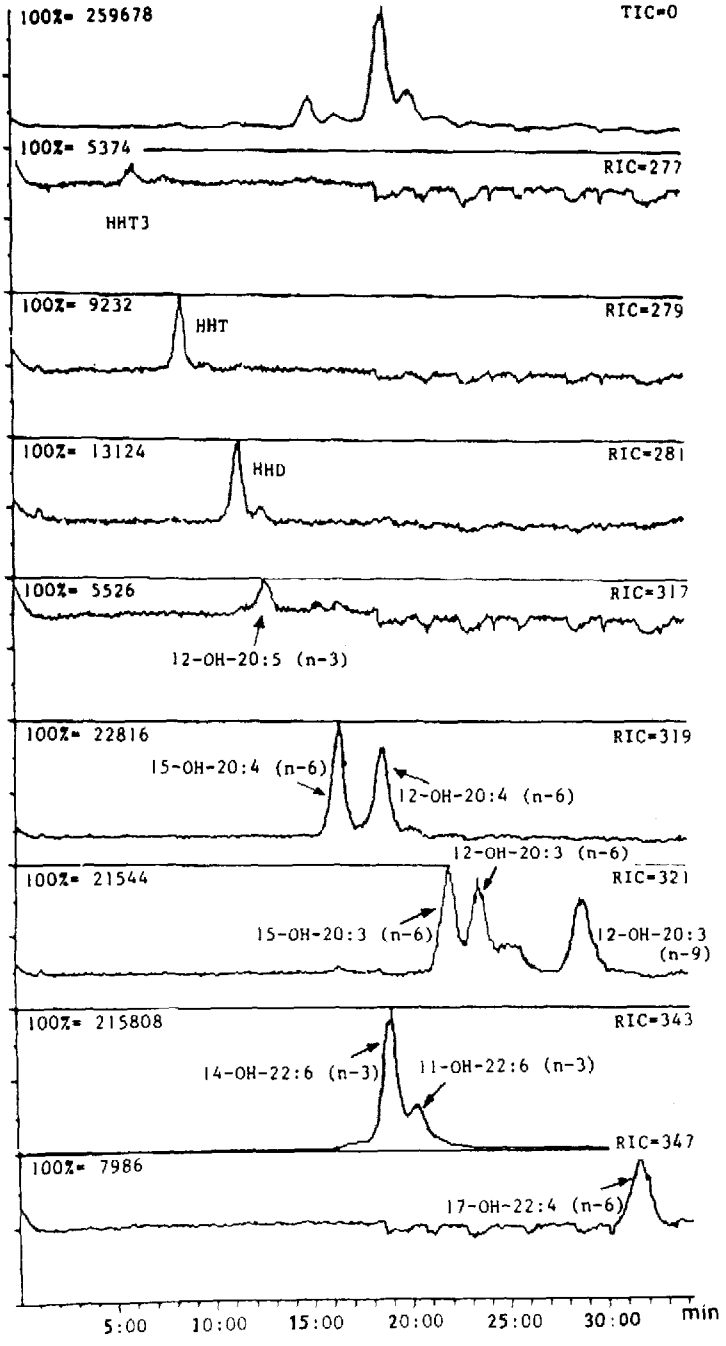

Fig. 6. Selected ion monitoring of different standard monohydroxy fatty acids. Each peak was observed with 300 ms per scan. Conditions as in Fig. 4.

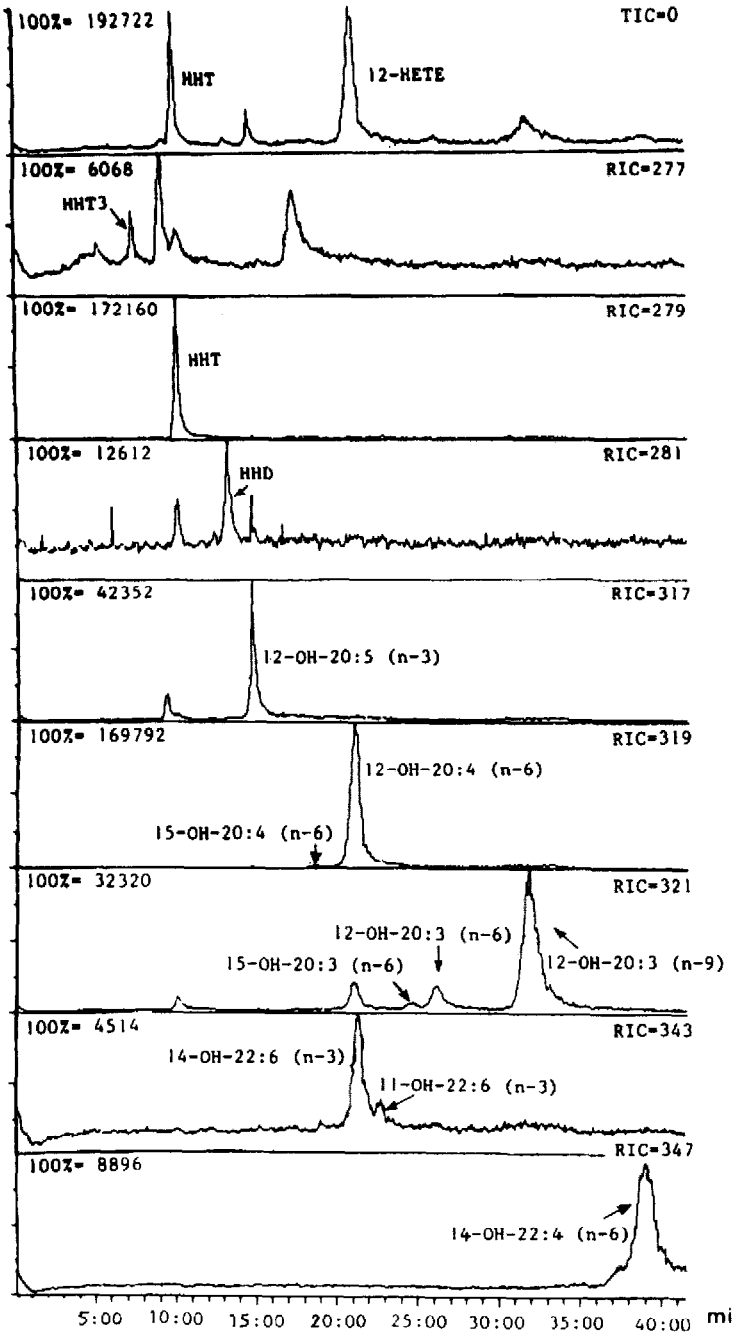

Fig. 7. Selected ion monitoring of monohydroxy fatty acids obtained from platelets stimulated by 1 U/ml thrombin. 10^9 platelets were pre-loaded with a mixture of different fatty acids (20:5$n-3$, 20:3$n-6$, 20:3$n-9$, 22:4$n-6$ and 22:6$n-3$). Conditions as in Fig. 4.

negative ionization mode is around three times more sensitive than the positive mode and would allow a detection limit between 5 and 10 pmol. Such a sensitivity is similar to that expected with UV detection. As an example, Fig. 5 shows UV detection at 234 nm of the same amount of the three monohydroxy derivatives. Fragmentometry is much more selective, however, and discriminates between monohydroxy derivatives co-eluted from the column. A good example is given with 12-HETE (12-OH-5,8,10,14–20:4) and 14-OH-4,7,10,12,16,19–22:6, which have the same retention time in reversed-phase HPLC (RP-LC). Their corresponding ions at m/z 319 and 343 can be measured separately (Fig. 6).

Biological application

A variety of standard monohydroxy fatty acids, prepared as described under Experimental, were detected according to their molecular ion $[M-H]^-$. A high resolution and a short analysis time were obtained using RP-LC with a column packed with 3-μm particles (Fig. 6).

Monohydroxy derivatives obtained from platelets (10^9) pre-enriched with various polyunsaturated fatty acids and stimulated by 1 U/ml thrombin were also analysed. As shown in Fig. 7, the relative peak intensities can be compared on a qualitative basis (100% values). As expected, 12-HETE and HHT (12-OH-5,8,10-heptadecatrienoic acid) were the main hydroxy derivatives. We may also note the efficient formation of 12-monohydroxylated fatty acids from 20:3$n-9$. Lipoxygenase products from 20:3$n-6$ were much less synthesized, also in agreement with our previous results [23]. The data also confirm that 22:6$n-3$ is poorly liberated from platelet stores, resulting in a weak conversion into oxygenated derivatives [25,26]. In comparison, 22:4$n-6$ appears to be substantially liberated and/or lipoxygenated during the platelet activation process.

We conclude that the use of thermospray–mass spectrometry for underivatized monohydroxy fatty acids offers several advantages over conventional techniques. With a sensitivity similar to that of UV detection, it is more selective and allows discrimination between co-eluting derivatives. We succeeded in detecting a variety of monohydroxy derivatives from polyunsaturated fatty acids of nutritional value in thrombin-stimulated platelets pre-enriched with these acids.

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