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QUALITATIVE AND QUANTITATIVE MEASUREMENT OF HYDROXY FATTY ACIDS, THROMBOXANES AND PROSTAGLANDINS USING STABLE ISOTOPE DILUTIONS AND DETECTION BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY*

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SUMMARY

Methods for measurement of the metabolites of arachidonic acid (AA), namely prostaglandins (PGs), thromboxanes (TXs) and hydroxy fatty acids, using stable isotope dilution gas chromatography—mass spectrometry are described. With a few exceptions, labelled species of the various AA metabolites are not commercially available and were therefore synthesized in our laboratory. [$^{2}H_{8}$]AA, produced by deuteration of eicosatetraynoic acid, was used for comparing the metabolism of exogenously added and endogenously present AA in fibroblast cultures. After derivatization and catalytic hydrogenation, structure elucidation and quantification of the different hydroxy fatty acids was carried out by determination of the

*This paper is dedicated to Prof. Dr. H.G. Klingenberg on his 65th anniversary.

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fragment ions resulting form α-cleavage at the site of the hydroxy function. During catalytic hydrogenation a significant hydrogen—deuterium exchange was observed. To eliminate this problem, ^{18}O-labelled standards were prepared by exchanging the oxygen of the carboxylic acid group. The preparation and the use of hydroxy fatty acids, PGs and TXs labelled with ^{18}O is described.

INTRODUCTION

Stable isotope dilution in combination with gas chromatography—mass spectrometry (GC—MS) provides a powerful tool for metabolic investigations because of its high accuracy, sensitivity and specificity, as will be demonstrated for arachidonic acid (AA). The products of the AA cascade, namely hydroxy fatty acids, prostaglandins (PGs) and thromboxanes (TXs) are potent tissue hormones. Each cell type produces its own pattern of AA metabolites with different biological action. For example, human skin fibroblasts (HSFs) produce in addition to the different PGs, 12-hydroxyheptadecatrienoic acid (HHT), 11-hydroxyeicosatetraenoic acid (11-HETE) and 15-HETE [1]. HHT and 11-HETE are cyclooxygenase products like PGs, whereas 15-HETE is formed via the lipoxygenase pathway.

We are interested in a method which allows the quantitative measurement of a pattern of AA metabolites and has the ability to monitor the entire AA metabolism including the influence of drugs. GC—MS, using selected-ion monitoring (SIM) and substances labelled with stable isotopes as tracers, is well suited for this purpose.

PRINCIPLES AND PROBLEMS OF THE METHOD

Substances labelled with stable isotopes like ^{2}H, ^{13}C or ^{18}O have nearly identical chemical and physical properties to those of the unlabelled, naturally occurring molecules, but can be recorded by GC—MS simultaneously with the non-labelled analogues due to differences in molecular weight or fragment-ion mass. Stable isotopes are harmless to humans and animals, and non-toxic compounds can therefore be handled without special care and even be used for in-vivo experiments. For quantification of a metabolite, the labelled analogue is added in a known amount to the biological sample at the beginning of the work-up procedure and acts as a carrier during preparation and as an internal standard for the measurement of the biological analogue. The final step is the comparison of the peak areas obtained by MS for the natural substance and for the labelled standard. All losses during the preparation are assumed to be identical for labelled and unlabelled material, barring isotope effects, and are therefore compensated for in the best possible way. Another experimental design is to add AA or an intermediate product in the labelled form to the incubation medium and to follow its metabolism alongside that of the endogenous presently unlabelled substance.

We have to consider the natural abundance of stable isotopes like ^{29}Si (4.7%), ^{30}Si (3.1%) and ^{13}C (1.1%), causing intense isotope peaks. In order to avoid interference between isotope peaks, and the labelled standard on the one

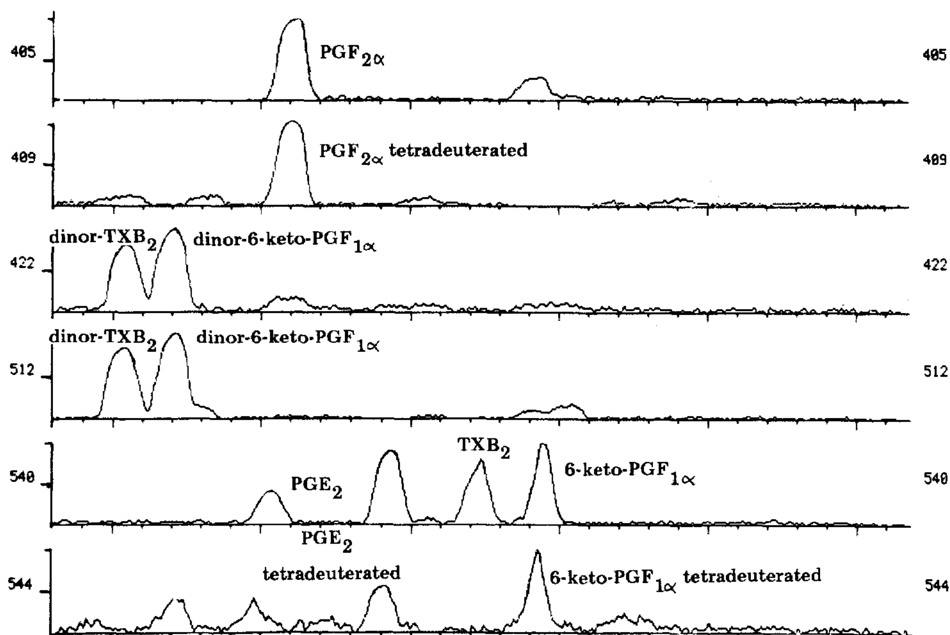

Fig. 1. Mass chromatogram of $PGF_{2\alpha}$ (m/z 405), tetradeuterated $PGF_{2\alpha}$ (m/z 409), dinor-TXB_2 (m/z 512 and 422), dinor-6-keto-$PGF_{1\alpha}$ (m/z 512 and 422), PGE_2 (double peak with m/z 540), tetradeuterated PGE_2 (double peak with m/z 544), TXB_2 (m/z 540), 6-keto-$PGF_{1\alpha}$ (m/z 540), and tetradeuterated 6-keto-$PGF_{1\alpha}$ (m/z 544) obtained from the methyl ester—silyl ether—methoxime derivatives using PICI with ammonia as CI gas and SIM.

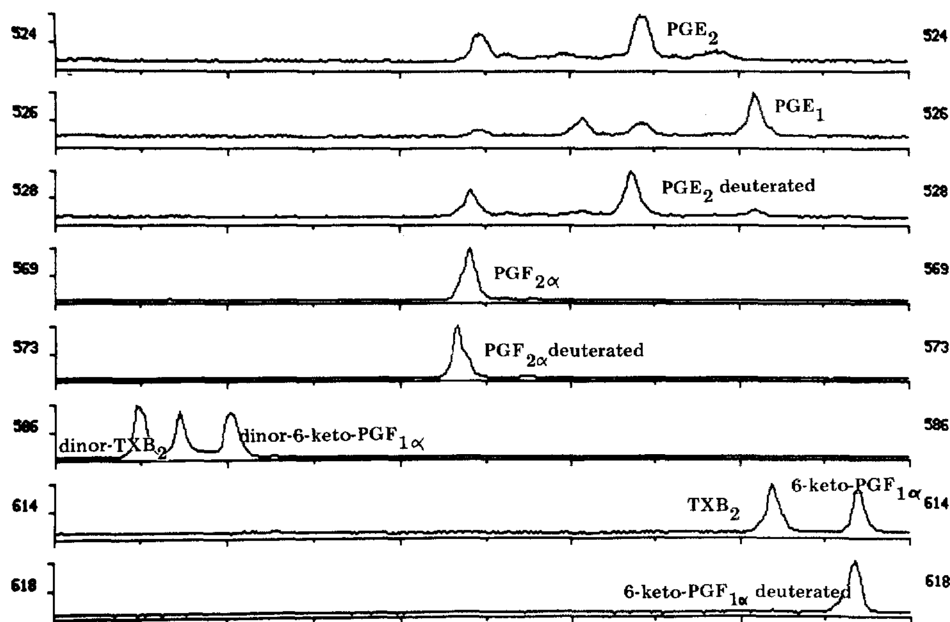

Fig. 2. Mass chromatogram of PGE_2 (double peak with m/z 524), PGE_1 (double peak with m/z 526) tetradeuterated PGE_2 (double peak with m/z 528), $PGF_{2\alpha}$ (m/z 569), tetradeuterated $PGF_{2\alpha}$ (m/z 573), dinor-TXB_2 (m/z 586), dinor-6-keto-$PGF_{1\alpha}$ (double peak with m/z 586), TXB_2 (m/z 614), 6-keto-$PGF_{1\alpha}$ (m/z 614) and tetradeuterated 6-keto-$PGF_{1\alpha}$ (m/z 618) obtained from the pentafluorobenzyl ester—silyl ether—methoxime derivatives using NICI with methane as CI gas and SIM.

hand, and a greater difference in GC retention time on the other, a difference of 3—6 mass units between the labelled and unlabelled substance is recommended. From the point of view of the stability to exchange reactions, labelling with ^{13}C would be preferable but requires synthesis of the whole molecule. In this laboratory labelling is carried out with ^{2}H and with ^{18}O. Substances labelled with ^{2}H are relatively stable in biological media but unstable to hydrogenation. On the contrary, ^{18}O-labelling compounds are stable to hydrogenation but unstable to esterases present in biological media. [$^{2}H_{8}$]AA is applied for metabolic studies in biological media, whereas ^{18}O-labelled HETE is preferred as an internal standard. A further advantage of ^{18}O-labelling is the minor kinetic isotope effect observed for ^{18}O in comparison with ^{2}H. This kinetic isotope effect causes differences in retention time between labelled and unlabelled material. However, [$^{2}H_{4}$]PGs are used as an internal standard, when commercially available, as the work-up procedure for the different PGs includes no hydrogenation step and the differences in retention time are negligible (Figs. 1 and 2).

EXPERIMENTAL

Instrumental conditions

A Finnigan 9610 gas chromatograph coupled to a Finnigan 4000 mass spectrometer with positive-ion electron impact (EI) and pulsed positive—negative-ion chemical ionization (CI) and an Incos data system were used. The chromatograph was equipped with a 30 m × 0.25 mm fused-silica column, coated with chemically bonded DB-5 (J&W Scientific, Rancho Cordova, CA, U.S.A.). The injector and transfer line were kept at 260°C, the column at 100°C for 1 min, then programmed to 320°C at 40°C/min. The ion source of the mass spectrometer was operated at 260°C. EI was carried out with an electron energy of 70 eV. Positive-ion chemical ionization (PICI) was achieved with ammonia as CI gas and negative-ion chemical ionization (NICI) with methane as CI gas, the pressure in the ionizer being ca. 50 Pa, the electron energy 120 eV and the emission current 0.1 A. The scan time for a mass range of m/z 100—700 was chosen to be 1 sec; in SIM mode a total acquisition time of 250-350 msec was used. In this way each peak consists of at least eight acquisitions.

Preparation of HETEs and ^{18}O-labelling of AA metabolites

For quantification of the different AA metabolites, calibration curves have to be established with known amounts of unlabelled substance and against a constant amount of the labelled analogue, which is also used as an internal standard for measurement of the samples. Only a part of the reference material could be purchased or obtained as a gift, and all the other AA metabolites had to be prepared. When preparing unlabelled standards starting with AA, a very small quantity of radioactive AA was added for calculation of the exact amount obtained after reaction and purification. Another possibility for quantification is measurement of the UV absorption. Contrary to the unlabelled reference material, the absolute amount of the labelled internal standard has not to be known, it is only essential to add them always in exactly the same quantity. The preparation of HETEs starting from AA can be done

enzymatically or chemically. 15-HETE was synthesized with soy bean lipoxygenase as described by Hamberg et al. [2].

An example of the formation of a 5-HETE standard by a chemical reaction using the method [3] yielding 5-HETE from AA will be described briefly. For calculation of the exact amounts obtained after reaction and purification, ^{14}C-labelled AA was added to 500 mg AA dissolved in 17.5 ml tetrahydrofuran followed by shaking under nitrogen at 0°C for 8 h with a solution of 0.69 g sodium bicarbonate in water + 2.2 g potassium iodide + 6.3 g iodine. Sodium thiosulphate pentahydrate (7 g) was then added and the 5-hydroxy-6-iodo-8,11,14-*cis*-eicosatrienoic acid δ-lactone extracted with hexane. The hexane was evaporated and the residue dissolved in 15 ml benzene. 1,5-Diazabicyclo-[5.4.0]undec-5-ene (400 μl) was slowly added under nitrogen followed by shaking for 6 h. Water (13 ml) and hexane (13 ml) were then added and the mixture acidified with acetic acid. The crude 5-HETE δ-lactone was extracted three times with hexane, the solvent evaporated and the residue dissolved in 1.5 ml methanol and 0.54 ml triethylamine. After setting aside for 30 min, 2 ml acetic acid were added and the methyl ester was extracted with hexane (tested by GC—MS, Fig. 3). The product has now been purified, the radioactivity measured and the total amount of 5-HETE calculated and used as a standard, or labelled and used as an internal standard for quantification.

As described above, labelled substances with high isotopic purity and

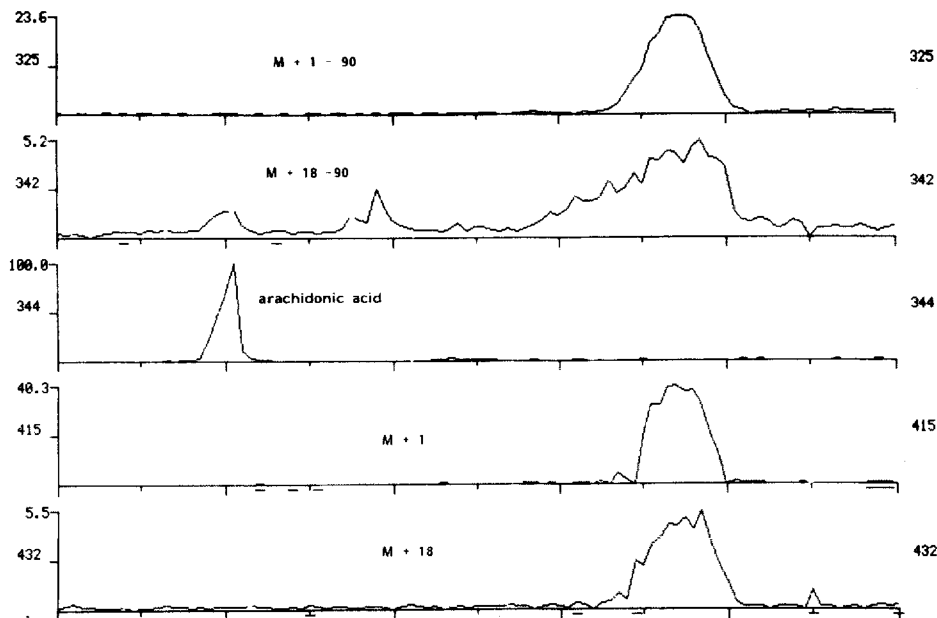

Fig. 3. Mass chromatogram obtained from 5-HETE derived from AA. The crude reaction product isolated from the reaction mixture as described was methylated, silylated and analysed by GC—MS using PICI with ammonia as CI gas. For AA we looked at *m/z* 344 as the major ion, whereas the presence of 5-HETE was indicated by the peaks with *m/z* 415, 432 = M + 18 corresponding to M + ammonia, 325= M + 1 — 90 corresponding to the loss of trimethylsilanol, 342 = M + 18 — 90 corresponding to the loss of trimethylsilanol and addition of ammonia. The number at the upper end of the scales indicates the relative intensity of the ions.

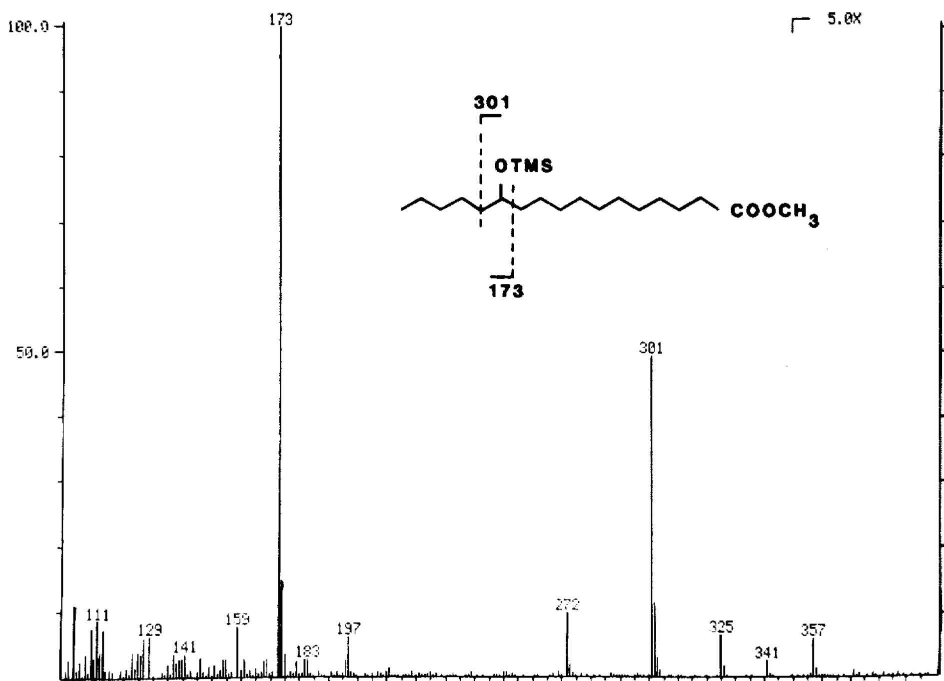

Fig. 4. Mass spectrum of hydrogenated HHT as the methyl ester—silyl ether derivative obtained by EI. The ions *m/z* 301 and 173, resulting from α-cleavage, are the most prominent fragments.

stability to hydrogenation are required for quantification of HETEs. The AA metabolites contain a carboxylic acid group; the exchange of their ^{16}O atoms by ^{18}O atoms, according to the studies of Strife and Murphy [4], is useful as the products obtained are stable to catalytical hydrogenation and a high isotopic purity can be reached by repeated cycling of the procedure.

HETEs are difficult to separate by GC as they have the same molecular weight and the only difference is in the position of the hydroxy group. Methylation, hydrogenation and silylation are necessary to detect and measure different HETEs in the EI mode by use of the two fragment ions resulting from α-cleavage, as shown in Fig. 4 for HHT (*m/z* 173 and 301). The specific fragment ions of the HETEs are: *m/z* 173 and 343 for 15-HETE, *m/z* 215 and 301 for 12-HETE, *m/z* 229 and 287 for 11-HETE and *m/z* 203 and 313 for 5-HETE. After labelling with ^{18}O the mass of the fragment ion containing the $COOCH_3$ moiety is increased by 4 mass units, equivalent to the exchange of two ^{16}O atoms by two ^{18}O atoms. Therefore the following ions were used for quantification: *m/z* 343 and 347 for 15-HETE, *m/z* 301 and 305 for 12-HETE, *m/z* 287 and 291 for 11-HETE and *m/z* 203 and 207 for 5-HETE. The mass of the other fragment ion remains unaltered by the labelling and is only used for identification purposes.

The labelling procedure was the same for all AA metabolites, only the number of cycles necessary to obtain a product with the desired isotopical purity differing from metabolite to metabolite. We used the base-catalyzed exchange of ^{18}O. Briefly, $Li^{18}OH$ was prepared by adding lithium metal to $H_2{}^{18}O$.

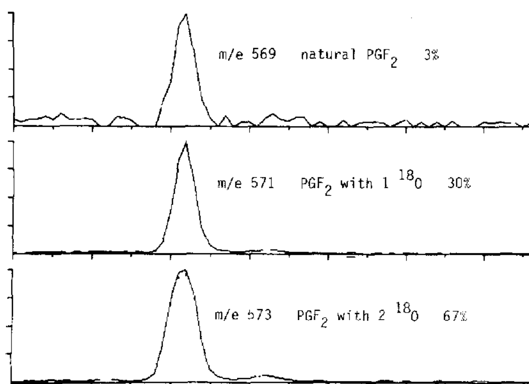

Fig. 5. Mass chromatogram of $PGF_{2\alpha}$ obtained by NICI with methane as CI gas after three cycles with $H_2{}^{18}O$ and derivatization with PFB-Br and BSTFA. The traces show that 67% of the product has two ^{18}O atoms incorporated (*m/z* 573), 30% with one ^{18}O atom (*m/z* 571) and only 3% remained unlabelled (*m/z* 569).

A 100-μl volume of this solution was added to less than 1 mg methylated substance. After shaking for 1 h the solution was acidified with 0.01 *M* formic acid and extracted with ethyl acetate. After removal of the solvent the residue was remethylated with diazomethane in methanol—diethyl ether (1:1, v/v) and the procedure was repeated. For $PGF_{1\alpha}$, three cycles yielded a substance containing mainly (67%) the $^{18}O_2$ product, 30% ^{18}O, ^{16}O and 3.0% $^{16}O_2$, as shown in Fig. 5. For TXB_2, four cycles resulted in a substance containing less than 0.5% of the ^{16}O analogue and similar results were obtained for 5-HETE. A poor reaction of the other HETEs forced us to label them by the enzymatic procedure.

Use of [$^2H_8$] AA for incubation experiments on human skin fibroblast cultures

Another important use of substances labelled with stable isotopes is in metabolic studies. The use of ^{18}O-labelled precursors for investigations in biological media is not recommended because of the $^{18}O/^{16}O$ exchange induced by esterases. For such purposes, deuteration is preferable, as the deuterated substances are more stable to enzymatic exchange reactions.

[$^2H_8$] AA was prepared according to Hamberg et al. [5] by catalytic deuteration of eicosatetraynoic acid (ETYA) with deuterium gas. For studying the AA metabolism in HSF [1, 6], we measured the quantity of AA bound to the cellular lipids using [$^2H_8$] AA as an internal standard. Dependent on the cell line, a relatively high amount of ca. 3.5 μg AA per 10^6 cells was estimated. We were also interested as to whether the endogenous AA is metabolized in the same way as exogenously added AA. The endogenous AA is released from the phospholipids by the action of the phospholipase A_2; the activity of this enzyme can be stimulated by the addition of the calcium ionophore A 23187 or bradykinin to the incubation medium. For the present investigation we added 150 μg [$^2H_8$] AA to ca. $2 \cdot 10^7$ cells and analyzed the metabolites formed. The products derived from the endogenous unlabelled AA were compared with those obtained from [$^2H_8$] AA using CI-MS. PICI spectra of the fatty acids and related substances using ammonia as CI gas show representative ions for M+1

and for M+18, the latter resulting form the addition of ammonia to the molecule. The octadeuterated analogues have increased in molecular weight by 8 mass units, corresponding to the exchange of eight H atoms by eight deuterium atoms. Therefore we monitored in SIM mode the ions M+1 and M+18 for the natural product and M+8+1 and M+8+18 for the octadeuterated analogue; e.g., the ions *m/z* 407 and 425 were recorded for HETE and *m/z* 415 and 433 for octadeuterated HETE, or *m/z* 367 and 385 for HHT and *m/z* 375 and 393 for octadeuterated HHT. Under the conditions chosen for this experiment, a ^{1}H:^{2}H ratio of 1:30 was estimated for all AA products investigated. This experiment suggests, barring isotope effects, that exogenously added and endogenously present AA are metabolized in the same way.

Method for the measurement of PG and TX

This method was elaborated according to that described by Blair et al. [7] and Robinson et al. [8], and allows the accurate measurement of all AA metabolites, providing the labelled analogues can be purchased or prepared. At present we are able to quantify PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ with tetradeuterated analogues (PGE_2 and $PGF_{2\alpha}$ purchased from Merck Sharp & Dohme/Isotopes; 6-keto-$PGF_{1\alpha}$ was a gift from J. Pike, Upjohn Company). PGE_1 is calculated using tetradeuterated PGE_2 as a standard, whereas TXB_2 and the different HETEs are measured using ^{18}O-labelled compounds as reference materials. ^{18}O-Labelled analogues of 2,3-dinor-TXB_2 and of 2,3-dinor-6-keto-$PGF_{1\alpha}$ are still being sought. The labelling with ^{18}O was carried out as described under *Preparation of HETEs and ^{18}O-labelling of AA metabolites.* When measuring positive ions, 50 ng of each labelled compound were added to the sample and transformed into the methyl esters. When utilizing negative ions, the sensitivity is increased, and therefore only 5 ng labelled standard were used and converted into pentafluorobenzyl (PFB) esters [9]. Except for these variations, both methods have identical preparative procedures.

The labelled standards were added to the sample, mixed well and adjusted to pH 3.2 with hydrochloric acid. The mixture was placed on a 6-ml C_{18} Bond-Elut reversed-phase column (Analytichem International, Harbor City, CA, U.S.A.) which was washed with 6 ml water and then eluted with 4 ml ethyl acetate. The solvent in the effluent was removed under nitrogen and the residue dissolved in 150 μl methanol. This solution was placed on a thin-layer plate (Merck Kieselgel 60) and developed with the organic layer of ethyl acetate–acetic acid–isooctane–water (11:2:5:10). The zones containing the PGs, TXs or hydroxy fatty acids were detected using reference materials co-chromatographed on the same plate, cut off and visualized by spraying with phosphomolybdate. The zones of interest were scraped off and extracted three times with 2% acetic acid in methanol. The extracts were evaporated to dryness under reduced pressure, and the residue dissolved in 3 ml phosphate–citrate buffer pH 3.8. This solution was applied to a 3-ml C_{18} Bond-Elut column, which was washed three times with phosphate–citrate buffer and three times with water and eluted with 3 ml ethyl acetate. The effluent was evaporated to dryness under nitrogen. A 50-μl volume of 2% methyloxime in pyridine (only for substances with keto groups) was added and allowed to react for 2 h

at 60°C. The pyridine was evaporated. To the residue, (a) for PICI, diazomethane in methanol—diethyl ether (1:1) was added and allowed to react for 30 min at room temperature; (b) for NICI, 40 μl of 7% pentafluorobenzyl bromide (PFB-Br) in acetonitrile and 10 μl diisopropylamine were added and allowed to react for 10 min at room temperature. To the dry products obtained from (a) and (b) were added 50 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA)—pyridine (1:1) and allowed to react for 30 min at 60°C. The solution was evaporated under nitrogen and the residue dissolved in 50 μl hexane. Aliquots of 1—10 μl were injected splitless on the GC column [measured in the multiple-ion detection (MID) mode].

RESULTS

The methods described above enabled us to investigate the structure of the unsaturated hydroxy fatty acids formed by HSFs [1, 6] and to show that they are partly derived from AA by a cyclooxygenase activity (HHT and 11-HETE), as their formation could be inhibited by indomethacin. The other part (15-HETE) is formed via a lipoxygenase activity; its formation can be prevented only by ETYA, not by indomethacin. Through the use of [$^{2}H_{8}$] AA we were able to demonstrate that similar results were obtained when AA was liberated from the cellular lipids by a stimulation of the phospholipase A2 activity, or when AA was added exogenously to the culture media.

The labelling with ^{18}O and the method described for the quantification of AA metabolites enable the quantitative measurement of HHT, of different HETEs, of PGs and TXs from biological media by recording positive or negative ions in the CI mode, or in the EI mode as is sometimes useful for the separate quantification of different HETEs. The sensitivity, however, depends on the mode of detection. Depending on the substance, we found that the detection limit is ca. 1 ng in the EI mode, 500 pg in the PICI mode and < 1 pg in the NICI mode. This is easy to understand as the abundance of the M $-$ 181 fragment, recorded in NICI, is striking. This fragment is obtained by the elimination of the PFB moiety as a very stable tropylium cation, allowing the carboxylate anion to be detected in the NICI mode. Mass chromatograms of some PG and TX standards are given in Fig. 1 under PICI and in Fig. 2 under NICI conditions. Comparing these figures, it is observed that much lower amounts were injected for detection in the NICI mode than for detection in the PICI mode.

The high sensitivity obtained in the NICI mode renders it possible to measure AA metabolites not only from incubation media or urine samples, but also from blood samples as well as from saliva. The measurement of AA metabolites from saliva is possibly of importance, as the collection of saliva is non-invasive and can be carried out even in newborn and preterm babies and the results are probably very closely related to the blood level. A further advantage is that blood sampling irritates the endothelial cells of the vessel wall and thus alters the level of AA metabolites under investigation; such a problem is avoided by measuring in saliva. Our first results, obtained from saliva, revealed the presence of $PGF_{2\alpha}$ (up to 10 ng per ml saliva) which rapidly decreased upon intake of aspirin to less than 1 ng per ml saliva, 3 h after oral ingestion of one tablet of aspirin.

The high number of chromatographic steps combined with the mass-specific detection renders the method highly specific. The accuracy and reliability are ensured by the compensation for losses by use of the labelled internal standard. A correlation coefficient of 0.998 is obtained, the fourth digit depending on the substance measured. For pure substances the standard deviation is 2.5% and for biological samples the standard deviation is less than 5%.

DISCUSSION

Our work clearly shows the usefulness of stable isotope dilution and GC—MS measurement for metabolic studies. Substances labelled with stable isotopes are used as standards and allow an exact calculation of all losses. They are added to the biological sample immediately after collection and are detected simultaneously with the natural analogue. The addition of an excess of the marked standard diminishes losses and increases sensitivity by contributing to irreversible adsorption and similar processes. The usefulness of GC separation and mass-specific detection can even be seen in the mass chromatograms obtained from pure substances; chromatographic separation alone would not be sufficient. A calculation of the losses without the use of a labelled internal standard would be incorrect, especially as the losses during preparation, which vary from substance to substance, are caused by the large number of steps, and by a different fragmentation in the ion source. The temperature in the ion source, like all the other parameters, contributes to the fragmentation, which depends very closely on the structure. The labelled analogue, however, behaves in a very similar way to the natural substance, and acts therefore as an ideal internal standard. An exact quantification can only be achieved if labelled and unlabelled substances are available. The method described allows the quantitative detection of AA metabolites from biological samples under routine conditions with a standard deviation of less than 5%. With a more sophisticated technique, including an additional column chromatographic purification, a standard deviation of even less than 1% can be reached, thus proving that this method is well suited as a reference method. The high sensitivity enables the mesurement of AA metabolites in very small amounts of biological samples, like 0.1 ml saliva, and is therefore well suited for measurements on premature babies. Finally, the possibility of preparing deuterated or ^{18}O-labelled analogues extends the applicability of this method. The deuterated metabolites are more stable in biological media to enzymatic exchange of the label, the ^{18}O-labelled derivatives are better suited to the analytical procedures.

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