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Isolation and quantification of fluoroacetate in rat tissues, following dosing of Z-Phe-Ala-CH_2-F, a peptidyl fluoromethyl ketone protease inhibitor

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Abstract

Peptidyl fluoromethyl ketones (PFMK) are irreversible inhibitors of cathepsin B, a cysteine proteinase thought to be involved in the degradation of cartilage. It has been speculated that PFMK inhibitors may metabolize in rodents to form fluoroacetate (FAC), an extremely toxic poison. A highly selective and sensitive separation and detection scheme was developed to measure trace levels of FAC in rat tissues following PFMK dosing. The procedure consisted of extracting FAC from tissue and spiking the extract with $[^{18}O]_2$-fluoroacetate (^{18}O-FAC) as an internal standard. FAC and ^{18}O-FAC were further isolated from matrix components using ion-exchange, solid-phase extraction. The pentafluorobenzyl esters of FAC and ^{18}O-FAC were formed to facilitate the chromatographic separation. Two-dimensional gas chromatography coupled with selected-ion-monitoring detection provided the final measurement. The assay had a limit of detection of 2 ng FAC per g tissue, and was capable of accurately quantitating as little as 10 ng FAC per g tissue with a S/N ratio of 40:1. Linearity was established over two orders of magnitude, from 2–500 ng ml^{-1}, with 5 μl injected on-column. The method was used to demonstrate that FAC was formed in rats following dosing with Z-Phe-Ala-CH_2-F, a PFMK cathepsin enzyme inhibitor. © 1997 Elsevier Science B.V.

Keywords: Fluoromethyl ketones; Fluroacetate; Proteinase inhibitors; Two-dimensional gas chromatography; Mass spectrometry

1. Introduction

Osteoarthritis is a disease characterized by the destruction of the articular joint cartilage due to breakdown of the collagen and proteoglycan matrix. Cathepsin proteases, members of the cysteine protease family, are known to play a major role in the destruction of cartilage tissues [1,2]. Peptidyl fluoromethyl ketones (PFMK) have been shown to be irreversible inhibitors of some members of the cathepsin enzyme family and may offer a potential therapy for the treatment of osteoarthritis [3–5]. A concern existed, however, that PFMK inhibitors might be metabolized to

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form fluoroacetate (FAC), Compound 1080, an extremely potent rodent poison ($LD_{50} = 1.1$ mg kg^{-1}). The utility of PFMK analogs as a potential therapeutic treatment for osteoarthritis could be negated by the formation of even low levels of such a toxic metabolite. To evaluate the potential metabolic formation of FAC, a PFMK inhibitor, Z-Phe-Ala-CH_2-F (Fig. 1), was administered to rats. Tissues were then subsequently acquired for FAC analysis.

In order to analyze tissues for even trace FAC levels, a sensitive analytical method was required. However, a highly selective method was also required to avoid potential false positives. The requirements posed by the requisite assay sensitivity and selectivity presented a unique analytical challenge. Although a number of sensitive GC methods have been reported for FAC analysis, and portions were adopted in this work, none exactly fit the particular needs of sensitivity and selectivity [6–12]. An analytical approach that utilized two-dimensional gas chromatography (2D-GC) coupled with programmed temperature vaporization (PTV) injection and selected-ion-monitoring (SIM) detection was developed. Briefly, FAC and ^{18}O-FAC were isolated from tissue extracts using solid phase extraction (SPE). A GC separation followed that utilized two capillary columns of differing polarity. Detection was performed by monitoring a single ion each for FAC and ^{18}O-FAC following electron ionization (70 eV). The methodology was used to determine FAC levels in rat tissue following dosing with Z-Phe-Ala-CH_2-F.

2. Materials and methods

2.1. Two-dimensional chromatography

Two dimensional chromatography was chosen as the separation technique because the authors thought it would best assure a positive identification of the analyte. This technique which is described in detail by W. Dale Snyder [13], and developed by Deans [14], is typically called a 'Deans switch'. Our particular instrument consisted of a Hewlett Packard GC which had been modified by Analytical Controls Inc. (Bensalem, PA) to perform the necessary cryogenic trapping as well as the pneumatic column switching. The trap was externally isolated from the oven compartment and was situated just above it. The trap, connecting the first and second columns, consisted of a stainless steel coil through which a section of fused silica was threaded. The trap was insulated from the atmosphere and cooled with liquid carbon dioxide to trap components as they eluted from the first column (heartcut). An electrical current could be passed through the coil to provide resistance heating, thus rapidly heating the trap and automatically releasing the trapped components onto a second fused silica column, at the prescribed time.

2.2. Reagents and materials

Z-Phe-Ala-CH_2-F was purchased from Enzyme Systems Products (Dublin, CA). Sodium monofluoroacetate was purchased from Sigma Chemical Co. (St. Louis, MO). 2-[^{14}C]-Sodium monofluoroacetate (^{14}C-FAC), containing 1.88 mCi $mmol^{-1}$, was obtained from ICN Biomedicals, Inc. (Costa Messa, CA). [^{18}O]Water (95%) was obtained from Sigma Aldrich Companies (Milwaukee, WI). Pentafluorobenzylbromide (PFBBr) was obtained from Pierce Chemical Co. (Rockford, IL). Acetone, potassium hydroxide, potassium phosphate, were all reagent grade, from J.T. Baker (Phillipsburg, NJ). Liquified CO_2 cylinders equipped with dip tubes were purchased from Liquid Carbonic Corp. (Chicago, IL).

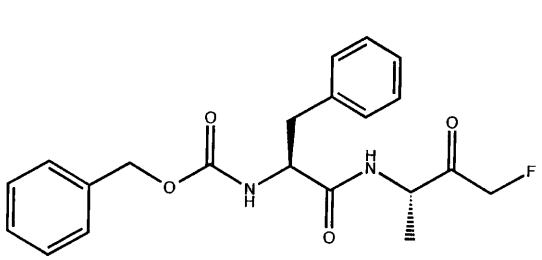

Fig. 1. Structure of Z-Phe-Ala-CH_2-F.

2.3. Description of animal studies

Six male Sprague–Dawley rats received a per-oral (p.o.) dose (100 mg kg^{-1}) of Z-Phe-Ala-CH_2-F and two rats each were sacrificed at 2, 4 and 8 h post-dose. Two additional rats received only vehicle (5% povidone and 0.25% Tween 80) and were sacrificed at 8 h post-dose. Liver, kidney, and heart tissues were removed from each animal after sacrifice. In a second study, two rats received an intravenous (i.v.) injection (10 mg kg^{-1}) of Z-Phe-Ala-CH_2-F in the same vehicle as above. These rats were sacrificed at 1 h post-dose and the livers were removed for FAC analysis. Dosing solutions were freshly made prior to each experiment. All tissues were stored at -80°C until analyzed.

2.4. Preparation of the stable isotope-labeled internal standard (SIL-IS)

The ^{18}O-FAC was prepared in our laboratory by incorporation of [^{18}O] into the carboxylic acid moiety using an acid-catalyzed exchange reaction initially described by Murphy and Clay [15]. Briefly, [^{18}O]water was saturated with anhydrous HCl gas and 0.2 ml was added to 1 mg of FAC. Acetonitrile was slowly added ($\sim$0.3 ml) until the FAC completely dissolved. The solution was heated at 50°C for 24 h and then diluted to 10 ml with 0.01 M phosphate buffer (pH 6.2). This stock solution of ^{18}O-FAC was stored at 4°C until further dilution was performed to yield the working solutions. Working solutions of ^{18}O-FAC were prepared by transferring 0.1 ml of the stock ^{18}O-FAC solution into a 4-dram screw cap vial, taking it to dryness and redissolving the residue in 10 ml of acetone. The solution was transferred to a clean vial and sealed with a Teflon-lined cap. Assuming a 100% isotope incorporation, this procedure would yield a 1 μg ml^{-1} working solution of ^{18}O-FAC.

2.5. Extraction of FAC from rat tissues

Tissue samples were thawed at room temperature and either the entire organ or a portion of the organ was taken for analysis. Generally, the entire heart and kidney were used, while only a 1 gram portion of the liver was analyzed by excising with stainless steel scissors. The tissue was placed in a glass Tenbroeck tissue grinder (Wheaton 200 series), together with 2.0 ml of acetone/0.01 M potassium phosphate, pH 6.2 (80:20; v/v). Following grinding, all homogenized tissue and suspended solids were added to the culture tube. Solids remaining in the grinding apparatus were reground two additional times by subsequent additions of 1.0 ml of extraction solvent. Following each additional grinding, all homogenized tissue and suspended solids were transferred into the 16×100 mm disposable culture tube containing the 50 ng of ^{18}O-FAC. Following the third grinding, any remaining solid material was also transferred to the culture tube containing the 50 ng ^{18}O-FAC. The tube was then vortexed for 1–3 s and centrifuged for 10 min to clarify the sample. The supernatant was removed and placed in a clean culture tube. The residue was washed two additional times with 2 ml of the extraction solvent, vortexed thoroughly for 12–15 s and centrifuged as before. The supernatants from the two washing steps were combined with the original supernatant and the tissue solids discarded. The combined supernatant sample was further processed by SPE.

2.6. Absolute recovery of ^{14}C-FAC from spiked tissue samples

The recovery of FAC from the extraction procedure was monitored by spiking rat tissue samples with ^{14}C-FAC. The ^{14}C-FAC was directly injected into several locations of a thawed tissue sample using a small-volume syringe. Three samples of each tissue were spiked with 2 μg ^{14}C-FAC per g tissue and extracted. The radioactivity in the extracts was determined by liquid scintillation counting and the recovery was determined relative to the known amount of activity added to the samples.

2.7. Solid phase extraction procedure

Quaternary amine ion-exchange SPE cartridges (3 ml Bakerbond spe, J. T. Baker) were condi-

tioned with 3.0 ml of 0.01 M potassium phosphate buffer, pH 6.2. The entire tissue extract was then loaded onto the SPE cartridge at a flow rate not exceeding 1 ml min^{-1}. The cartridge was subsequently washed with 3.0 ml of 0.01 M potassium phosphate buffer, pH 6.2. Analytes were then eluted with 5 ml of a 0.1 M KCl solution buffered with 0.01 M potassium phosphate buffer, pH 6.2. The pH of the SPE effluent was adjusted to between 7–8 with 400 μl of 1 N NaOH and the solvent removed at 37°C under a stream of nitrogen using a Turbo-Vap™ (Zymark, Hopkington, MA) evaporator. The recovery of FAC from the SPE procedure was determined by adding ^{14}C-FAC to blank tissue extracts and determining the radioactivity in the SPE eluent by liquid scintillation counting before and after solvent removal. Recoveries of ^{14}C-FAC were expressed relative to the known amount of activity added to the sample extracts.

2.8. Derivatization procedure

The dried SPE residues were derivatized with a mixture of 0.9 ml of acetone:water (95:5; v/v) and 0.1 ml of 10% PFBBr in acetone. The samples were placed on a heating block (60°C) for 1 h. The samples were removed at 15 min intervals from the heating block, vortexed for 30 sec and then returned to the heating block during this 1-h interval. The samples were removed and allowed to cool. A small portion of each sample (approximately 10%) was transferred to autosampler vials for chromatographic analysis.

2.9. Preparation of FAC spiked tissues for accuracy and precision

A portion of normal rat liver (0.8–1.5 g) was removed from the parent organ with stainless steel scissors. The tissue was ground according to the described procedure. Before the first grinding, either 10, 50 or 200 ng of FAC was added to the grinding apparatus, and the FAC was thoroughly mixed with the tissue homogenate by additional grinding. The homogenate was transferred to a culture tube and the remainder of the extraction procedure was performed.

2.10. Preparation of calibration standards

An aliquot of FAC, in the sodium salt form, dissolved in acetone, containing 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng was added to individual disposable screw cap culture tubes, each containing 50 ng of ^{18}O-FAC. The solvent was removed under a nitrogen stream, and the residue was derivatized using 1.0 ml of derivatization reagent according to the previously described procedure.

2.11. Instrument conditions

The GC instrumentation included a Hewlett Packard (HP, Avondale, PA) 5890 gas chromatograph, equipped with an Analytical Controls (ACI, Bensalem, PA) Deans column switching system, an ACI PTV injector, and an HP model 7673A autoinjector with sample tray. The mass selective detector (MSD) was a HP model 5970 equipped with a direct interface.

A 5-μl sample volume was injected into the PTV injector that was initially maintained at 70°C. One min after the injection, the PTV injector was rapidly ramped to 180°C. An Rtx-1 (15 m × 0.53 mm I.D., 3.0 μm dimethylpolysiloxane film) from Restek (Bellefonte, PA) served as the precolumn. The precolumn was maintained at a constant helium flow rate of 9.5 cc min^{-1}. An initial oven temperature of 70°C was maintained for 4 min, followed by a 3°C min^{-1} ramp to 120°C. From 19.6 to 20.6 min the flow from the precolumn was diverted from waste by the Deans switching system into a small section of fused silica (0.5 m × 0.18 mm I.D., 0.2 μm film of 95% dimethyl/5% diphenylpolysiloxane) which served as the trap. The two analytes were cryogenically trapped in the fused silica which was maintained at −30°C with liquid CO_2. The analytes were held on the trap while the oven temperature was cooled to 65°C, the initial temperature of the analytical separation. Following equilibration, the trap flow was diverted to the analytical column consisting of a Stabilwax column (30 m × 0.25 mm I.D., 0.1 μm film) from Restek which was maintained at a helium head pressure of 16 psi. The trap was then rapidly heated to release the analytes. After 2 min at 65°C, the oven was ramped at 12°C min^{-1} to a final temperature of 160°C.

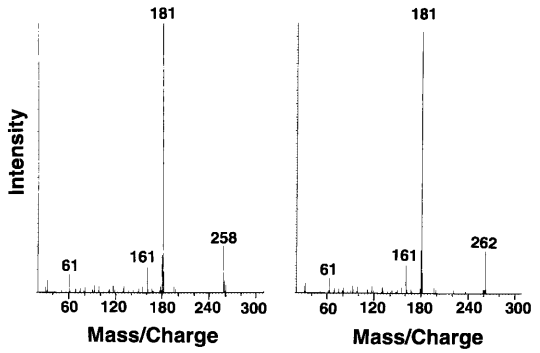

Fig. 2. Electron ionization (70 eV) mass spectra of pentafluorobenzyl esters of: (A) FAC, and (B) ^{18}O-FAC.

The MSD conditions used to monitor the effluent from the analytical column involved electron ionization (70 eV) using SIM at m/z 258 (FAC) and 262 (^{18}O-FAC) with a dwell time of 125 ms for each ion and a transfer line temperature of 300°C.

2.12. Quantitative determination of FAC in rat tissues

Measurement of FAC and ^{18}O-FAC was performed by measuring the abundance of ions m/z 258 and 262 for FAC and ^{18}O-FAC, respectively. Electronically integrated peak heights were produced, and the ratio of the mass (FAC/^{18}O-FAC) was plotted vs. the height ratio (FAC/^{18}O-FAC). Calibration was performed by analyzing known quantities of FAC covering a range from 2–500 ng ml^{-1}, each containing 50 ng ml^{-1} of ^{18}O-FAC. A regression line was fitted to the data using a linear least squares regression analysis. The mass of FAC contained in unknown and control samples was determined by computing the height ratio for the sample or control and extrapolating the mass ratio from the calibration line.

3. Results and discussion

3.1. Mass spectra of FAC and ^{18}O-FAC

The mass spectra for FAC and ^{18}O-FAC derivatized with PFBBr are shown in Fig. 2. The molecular ion for FAC was observed at m/z 258, while that for the ^{18}O-FAC occurred at m/z 262 due to the incorporation of two [^{18}O] atoms. The level of unlabelled FAC to that of the [^{18}O]$_2$-FAC was less than 0.9% as determined by SIM analysis. For quantitative analysis, the molecular ions were monitored for FAC and ^{18}O-FAC, since these ions represented the only ion pair where an isotopic difference, due to the [^{18}O]-label, was retained.

3.2. Calibration curve

The PTV injection allowed the introduction of a relatively large sample volume, 5 μl, in a fully automated mode. The ability to inject this sample volume increased the sensitivity of the FAC analysis. As is typical for GC/MS based methods, the LOD varied somewhat depending upon the condition of the instrumentation, in particular the cleanliness of the source and the injection port liner. A typical SIM chromatographic profile for a 2 ng ml^{-1} standard (10 pg on column) is shown in Fig. 3. A signal to noise ratio of 3 was obtained

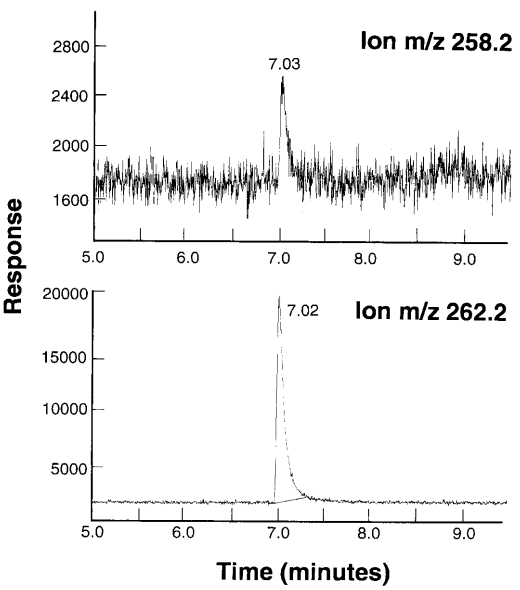

Fig. 3. Typical SIM chromatogram obtained for a 2 ng ml^{-1} FAC calibration standard containing 50 ng ml^{-1} ^{18}O-FAC. Equivalent to 10 pg FAC on-column.

for this standard, approximating the LOD for this particular day. The instrument portion of the method proved to be very rugged, despite its complexity. The retention time (t_R) of the analyte, as it eluted from the pre-column, held constant and never needed to be altered over a several month period. Linear calibration curves were obtained from 2 to 500 ng ml^{-1} (5–2500 pg on column), with coefficients of determination greater than 0.999 ($n = 5$). Replicate injections ($n = 5$) of the 5 and 50 ng ml^{-1} standards resulted in R.S.D. values of 4.2 and 1.7%, respectively.

3.3. FAC recovery from tissue

The isolation of FAC from the various tissues was optimized using ^{14}C-FAC. Due to its high volatility, FAC had be kept in the ionized state prior to derivatization to avoid losses. The tissue grinding solvent, containing 80% acetone and 20% 0.01 M potassium phosphate buffer (pH 6.2), was chosen because it provided good extraction recoveries and kept FAC in the ionized form. Mean recoveries of ^{14}C-FAC spiked into kidney, liver and heart tissue, at the 2 μg g^{-1} level, were 95% ($\pm$ 1.6, $n = 4$), 89% ($\pm$ 4.3, $n = 3$) and 100% ($\pm$ 1.4, $n = 3$), respectively.

3.4. Absolute FAC recovery from SPE isolation

Once extracted from tissue, additional sample preparation was necessary to isolate FAC prior to derivatization. Ion-exchange SPE was a natural choice, since FAC could be maintained in the ionic state. The absolute recovery of ^{14}C-FAC from the SPE extraction was 98% ($\pm$ 3.5%, $n = 14$). A critical step in the SPE process was the addition of a small amount of 1 N NaOH to the SPE eluent before solvent removal. The addition of the base insured that FAC remained in the ionized state during solvent removal. Failure to add the base, prior to solvent removal, resulted in a significant decrease in the absolute recovery of ^{14}C-FAC, ca. 50%.

3.5. Derivatization efficiency

The derivatization of FAC from the dried SPE effluent proved to be the most problematic step in the sample preparation procedure. It was necessary to find an appropriate solvent, or solvent mixture, that would allow analyte solubility, thus permitting derivatization to proceed, while at the same time keeping the salts insoluble. Since it has been reported that the ester formation proceeds best when performed under anhydrous conditions [9,11], we would have preferred to eliminate the use of water in the derivatization reaction. Several attempts to dissolve the ^{14}C-FAC from the solid SPE salts using acetone or methanol, in combination with repeated grinding and sonication, were unsuccessful. Also, an attempt to perform an extractive derivitization procedure on dried SPE residue containing ^{14}C-FAC using an acetone/PFBBr mixture was unsuccessful. However, when dried SPE sample residue containing ^{14}C-FAC was reacted for 1 h in the presence of the acetone/water/PFBBr derivitization mixture, 72% of the activity was contained in the reaction solvent. Reversed-phase HPLC with radiochemical detection was used to demonstrate that $>95\%$ of the isolated radioactivity existed as the pentafluorobenzyl ester (data not shown). Thus, FAC was successfully isolated and derivatized in a single step, allowing the direct injection of the reaction solvent without further need to isolate the pentafluorobenzyl ester of FAC.

3.6. Assay selectivity

The use of 2D-GC, in conjunction with SIM analysis, resulted in a highly selective assay for FAC. No endogenous interferences were detected in the chromatographic trace of normal tissue extracts, but rather, only baseline electronic noise was observed (Fig. 4A). The excellent degree of selectivity, in combination with ultratrace detection, allowed a high degree of confidence in the identification of trace levels of FAC from tissue extracts.

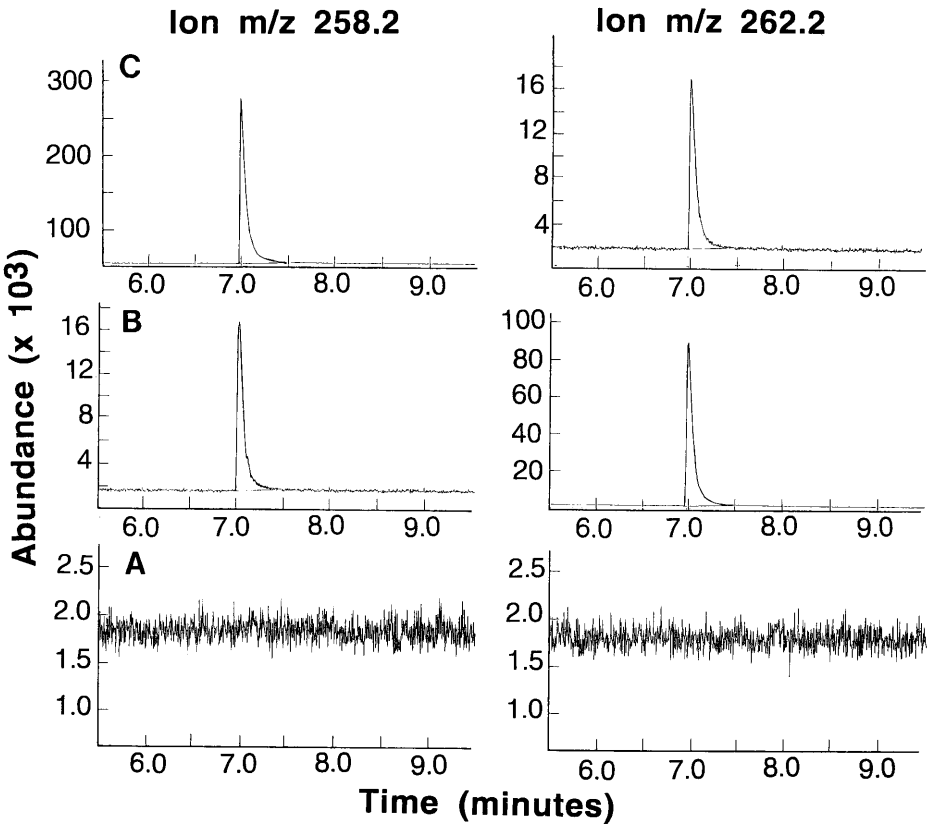

Fig. 4. Typical SIM chromatograms obtained for: (A) a blank rat liver, (B) a blank rat liver spiked with 10 ng FAC and 50 ng of ^{18}O-FAC per g of tissue, and (C) a 2-h post-dose rat liver extract obtained following a p.o. dose (100 mg kg^{-1}) of Z-Phe-Ala-CH_2-F.

3.7. Assay accuracy and precision

Liver was used as a model tissue to evaluate the accuracy and precision of the assay. Portions of liver were spiked with known amounts of FAC and were prepared for analysis as described previously. The mass of FAC found in liver tissues spiked at the 10, 50, and 200 ng levels were 9.45 ng ($\pm$ 2.2%, $n = 2$), 51.8 ng ($\pm$ 5.9%, $n = 5$), and 201.9 ng ($\pm$ 5.2%, $n = 5$), respectively. The lowest mass we attempted to quantitate from a spiked tissue sample was 10 ng FAC g^{-1} tissue. For the 10 ng spiked samples, the final derivatized solutions were concentrated prior to analysis by placing them under a stream of nitrogen. Since derivatized FAC may be volatile, the samples were not taken to complete dryness, rather the volume was reduced to 0.1 mL. FAC was easily quantitated from the 10 ng g^{-1} spike, giving a S/N ratio of greater than 40:1 (Fig. 4B). Clearly, based on the S/N ratio obtained for the 10 ng g^{-1} spiked sample, FAC could be quantitated at much lower levels using this methodology. Although a similar detection limit was achieved by others [6,9–11], the two dimensional approach, coupled with the use of a stable-isotope internal standard offers a much higher degree of selectivity and a higher degree of certainty in the quantitative results.

Additionally, the potential for back exchange of the [^{18}O]-label was examined by spiking blank tissue samples with ^{18}O-FAC. The spiked tissue samples were then extracted and the extracts derivitized and analyzed for FAC as previously

Table 1
FAC levels in rat tissues following PFMK dosing

Treatment	Route	Time (h)	Tissue	FAC (ng g^{-1} tissue)
Study 1				
Z-Phe-Ala-CH_2F	p.o.	2	Heart	600
	p.o.	2	Liver	840
	p.o.	2	Liver	280
	p.o.	2	Kidney	860
	p.o.	4	Heart	760
	p.o.	4	Liver	560
	p.o.	4	Liver	510
	p.o.	4	Kidney	540
	p.o.	4	Kidney	510
	p.o.	8	Heart	690
	p.o.	8	Liver	430
	p.o.	8	Liver	280
	p.o.	8	Kidney	410
Vehicle	p.o.	8	Liver	0.0
	p.o.	8	Kidney	0.0
	p.o.	8	Kidney	0.0
Study 2				
Z-Phe-Ala-CH2F	i.v.	1	Liver	2.4
	i.v.	1	Liver	80

described. No detectable levels of FAC were observed in the extracts indicating that no back exchange of the [^{18}O]-label occured (data not shown).

3.8. FAC levels in tissues following PFMK dosing

The levels of FAC observed in rat tissues, following p.o. and i.v. dosing with Z-Phe-Ala-CH_2-F are shown in Table 1. Surprisingly, a p.o. dose of Z-Phe-Ala-CH_2-F resulted in high levels, >250 ng g^{-1}, of FAC in all tissues for up to 8 h post-dose. A typical SIM chromatogram for a liver extract from a dosed animal is shown in Fig. 4C. All of the samples collected from the p.o. study were not analyzed since it became evident that FAC had been produced and distributed into all the vital organs. However, at least one sample for each type of tissue was examined, with the exception of the control group where no heart sample was analyzed. Blank liver extracts spiked with Z-Phe-Ala-CH_2-F were analyzed to insure that FAC formation was not an artifact of the sample preparation procedure. No measurable levels of FAC were detected in these spiked samples, confirming a metabolic route of FAC formation.

FAC was also found following i.v. dosing with Z-Phe-Ala-CH_2-F, suggesting that digestive enzymes of the gastrointestinal tract were not necessary for the metabolic generation of FAC. Two additional proprietary PFMK analogs were also studied and found to generate FAC by the subcutaneous and i.v. routes of administration (data not shown). These results suggest that the metabolic pathway leading to the formation of FAC may be general for molecules of the PFMK class.

The metabolic pathway involved in the conversion of PFMK analogs to FAC is not known. A similar metabolic pathway has been described in the literature for the metabolism of progesterone using microsomes from pig testes [16]. Progesterone is metabolized by the pig microsomes to yield 17-*O*-acetyltestosterone through the enzymatic equivalent of a Bayer–Villiger rearrangement. Further work will be required to define the exact mechanism of FAC formation from PFMK analogs.

4. Conclusions

A highly selective and sensitive analytical approach utilizing 2D-GC was developed for the analysis of FAC in rodent tissues. The methodology was employed to demonstrate that a PFMK analog, Phe-Ala-CH_2-F, was metabolized by rats to yield a highly toxic metabolite, FAC. The formation of FAC was shown to be independent of the route of administration and was also demonstrated to occur with other analogs of the PFMK class. At the present time the metabolic route involved in the formation of FAC from the PFMK analogs is not known.

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