# Rapid measurement of deuterium-labeled long-chain fatty acids in plasma by HPLC-ESI-MS

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**Abstract Imbalanced fatty acid metabolism contributes significantly to the increased incidence of metabolic disorders. Isotope-labeled fatty acids ($^{2}$H, $^{13}$C) provide efficient means to trace fatty acid metabolism in vivo. This study reports a new and rapid method for the quantification of deuterium-labeled fatty acids in plasma by HPLC-MS. The sample preparation protocol developed required only hydrolysis, neutralization, and quenching steps followed by high-performance liquid chromatography-electrospray ionization-mass spectrometry analysis in negative ion mode using single ion monitoring. Deuterium-labeled stearic acid (d7-C18:0) was synthesized to reduce matrix interference observed with d5 analog, which improved the limit of detection (LOD) significantly, depending on the products analyzed. Linearity $>$ 0.999 between the LOD (100 nM) and 30 μM, accuracy $>$ 90%, precision $>$ 88%, and adequate recovery in the dynamic range were obtained for d7-C18:0 and d7-oleic acid (C18:1). Upon oral dosing of d7-C18:0 in rats, the parent compound and its desaturation and β-oxidation products, d7-C18:1 and d7-C16:0, were circulating with a maximal concentration ranging from 0.6 to 2.2 μM, with significant levels of d7-fatty acids detected for up to 72 h.**—Gagné, S., S. Crane, Z. Huang, C. S. Li, K. P. Bateman, and J-F. Lévesque. **Rapid measurement of deuterium-labeled long-chain fatty acids in plasma by HPLC-ESI-MS.** ***J. Lipid Res.*** **2007.** 48: **252–259.**

**Supplementary key words** deuterium-labeled stearic acid • deuterium-labeled oleic acid • deuterium-labeled palmitic acid • high-performance liquid chromatography-electrospray ionization-mass spectrometry • electrospray ionization negative mode • quantification of plasma levels

Fatty acid biosynthesis and degradation are of increasing interest in a context in which obesity affects a growing proportion of the population of the United States and is associated with higher risks of medical problems (1, 2). Analysis of natural fatty acids such as palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), and oleic (C18:1) acids in biological samples is commonly performed by gas chromatography coupled to flame ionization detection or electron impact/chemical ionization mass spectrometry (3). However, the presence of an important in vivo endogenous pool disables short-term in vivo studies of fatty acid biosynthesis and metabolism. An alternative approach commonly used consists of dosing isotopically labeled fatty acids ($^{2}$H or $^{13}$C) to generate a mass shift that allows selective detection of the tracer using either GC-MS or GC coupled to isotope ratio mass spectrometry, with minimal interference attributable to natural fatty acids (4–8). Tracers such as [$^{13}$C]C18:0 and deuterium-labeled (d$_4$)-linoleic acid were dosed orally, and the disappearance of parent compound and the formation of labeled metabolites were reported in rat, cat, and human over a short time period (4–6, 8).

The use of GC for fatty acid analysis is generally associated with time-consuming sample preparation resulting from the liquid-liquid extraction and chemical derivatization steps (4–6, 8, 9). HPLC and HPLC-MS were previously considered as alternative approaches (8, 10–20), but all methodologies reported still involved long derivation and/or labor-intensive liquid-liquid extraction steps. There are increasing needs in the pharmaceutical industry to develop a fast and simple approach for the analysis of fatty acids. The objective of this work was to find a novel and rapid analytical method using high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) for the analysis of natural and deuterium-labeled fatty acids in plasma involving a simple sample preparation procedure that would significantly increase the sample preparation throughput without compromising repeatability, accuracy, and robustness.

## MATERIALS AND METHODS

### Chemicals

C16:0, C16:1, C18:0, C18:1, and tricosanoic acid were obtained from Sigma-Aldrich (Milwaukee, WI) and were used without any

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Abbreviations: C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; HPLC-ESI-MS, high-performance liquid chromatography-electrospray ionization-mass spectrometry; LOD, limit of detection; MRM, multiple reaction monitoring; SIM, single ion monitoring.

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further purification (99% purity). d5-C18:0 was purchased from C/D/N Isotopes (Pointe-Claire, Canada), whereas d7-C18:0 and d7-C18:1 were synthesized in-house (S. N. Crane, K. Bateman, S. Gagne, and J-F. Levesque, unpublished data). Methanol and acetone (optima grade) were obtained from Fisher Scientific, and $NH_4OH$ was from American Chemical, Ltd. (Montreal, Canada). Water was deionized using Milli-Q Plus from Millipore. NaOH (10 N) was obtained from J. T. Baker (Phillipsburg), and formic acid (99%) was purchased from Acros Organics.

### Instruments

The HPLC-ESI-MS/MS system consisted of a Waters 2790 Alliance HT chromatograph coupled to a Quattro Ultima triple quadrupole mass spectrometer (Manchester, England) equipped with an electrospray source. The analytical column used was the X-Terra® MS C8 2.5 μm (2.1 mm × 50 mm) from Waters (Santry, Ireland), which is stable at high pH. For exact mass measurement of the endogenous and deuterated standards, a Micromass Premiere quadrupole-time of flight apparatus from Waters coupled to a Waters Acquity ultra-performance liquid chromatograph was used. All exact masses were acquired by direct infusion using a Pump 11 from Harvard Apparatus.

### Analytical conditions

The mobile phase was composed of methanol plus 0.1% $NH_4OH$ (eluent A) and 0.1% aqueous $NH_4OH$ (eluent B) to maximize the negative ionization of the acid function of fatty acids. HPLC separation was achieved using a gradient from 60% to 95% eluent A in 10 min. The eluent A composition was then held constant for 2 min, followed by a 5 min equilibration period at 60% eluent A. The flow rate was 0.25 ml/min, and the column was kept at room temperature. The flow was diverted from the mass spectrometer during the 0–1.5 and 12–17 min time periods to the waste containers. The injection volume was 5 μl. The Quattro was operated in ESI negative mode, the capillary voltage was set at −3.5 kV, the cone at −30 V, the extractor at −1 V, the source temperature at 120°C, the desolvation temperature at 350°C, the desolvation flow at 458 l/h, the nebulizer at 21 l/h, and data were acquired in single ion monitoring (SIM) mode (**Table 1**). The MS/MS spectrum of the C18:0 was acquired using optimal collision energy of 25 and a full scan on Q3 of *m/z* 50–300. For the exact mass measurement with a lock spray, the quadrupole-time of flight system was operated in ESI negative mode, the capillary voltage was set at 2.0 kV, the cone at 40 V, the extractor at 4 V, the ion guide at 1 V, the collision energy at 10, the source temperature at 120°C, the desolvation temperature at 350°C, the desolvation flow at 500 l/h, the nebulizer at 20 l/h, and data were acquired using a scan time of 1 s and a *m/z* range of 100–1,200. Leucine-Enkephalin $(M\text{-}H)^-$ (*m/z* 554.2615) was the lock mass of the lock spray.

### Rat dosage and plasma collection

Male Sprague-Dawley rats and C57BL mice were supplied by Charles River Canada (St. Constant, Canada), squirrel monkeys by PAHO and Osage Research Primates LLC, and beagle dogs by Marshall Farms. Blood samples were collected from healthy animals in heparinized tubes, and plasma was prepared by centrifugation at 11,000 *g* for 3 min at 4°C.

For the rat pharmacokinetic study of d7-C18:0, Sprague-Dawley rats were fasted overnight, and 0 h blood sample was obtained by tail bleeding. d7-C18:0 was then administered orally at 10 or 30 mg/kg in 1% Methocel at 5 ml/kg, and plasma samples were collected at 1, 2, 4, 6, 24, 48, and 72 h after dose. Plasma samples were frozen right after collection. The rats were allowed to receive food 4 h after dosing, and water was provided at all times during the study.

### Sample and standard curve preparation

Plasma samples were kept at −78°C and were thawed on ice at 4°C before analysis. Fifty microliters of plasma was transferred in 1.5 ml Eppendorf tubes, and 100 μl of 2 N NaOH (containing 5 μM of the internal standard tricosanoic acid) was added. The sample was vortexed and heated at 65°C for 1.5 h. Then, the solution was cooled at room temperature for 5 min, and addition of 15 μl of formic acid and 150 μl of acetone were done successively in combination with proper sample mixing at each step. The final solution was centrifuged at 14,000 rpm for 10 min, and 150 μl of the supernatant was transferred in a 300 μl preinserted vial and analyzed by HPLC-MS.

The standard curve samples were prepared using the same protocol except that 2 μl of a stock solution of d7-C18:0 and d7-C18:1 was added to the initial 50 μl of plasma for each desired concentration between the limit of detection (LOD) and 30 μM.

### Analytical performance evaluation

The SIM descriptor used for each fatty acid was optimized by loop injection for each standard available. The SIM descriptors for unavailable compounds were determined empirically based on the theoretical mass of a deuterium atom replacing a proton on the endogenous standard. The sensitivity of the method was evaluated by spiking a known amount of d7-C18:0 and d7-C18:1 in blank rat plasma, which was subsequently treated as described above. The sensitivity is reported based on a signal-to-noise ratio of 3:1 for each deuterium-labeled standard available. The dynamic range, the repeatability, the precision, and the recovery were

TABLE 1. Chemical structures of fatty acids analyzed and SIM ions used

| Name | Abbreviation | Structure | SIM | Exact Mass |
|---|---|---|---|---|
| Palmitic acid | C16:0 | $CH_3(CH_2)_{14}COO^-$ | 253.5 | 255.2316 |
| d5-palmitic acid | d5-C16:0 | $CD_3CD_2(CH_2)_{13}COO^-$ | 258.5 (expected) | Not available |
| d7-palmitic acid | d7-C16:0 | $CD_3CD_2CD_2(CH_2)_{12}COO^-$ | 260.5 (expected) | Not available |
| Palmitoleic acid | C16:1 | $CH_3(CH_2)_5CH = CH(CH_2)_7COO^-$ | 255.5 | 253.2171 |
| d5-palmitoleic acid | d5-C16:1 | $CD_3CD_2(CH_2)_4CH = CH(CH_2)_7COO^-$ | 260.5 (expected) | Not available |
| d7-palmitoleic acid | d7-C16:1 | $CD_3CD_2CD_2(CH_2)_3CH = CH(CH_2)_7COO^-$ | 262.5 (expected) | Not available |
| Stearic acid | C18:0 | $CH_3(CH_2)_{16}COO^-$ | 283.5 | 283.2631 |
| d5-stearic acid | d5-C18:0 | $CD_3CD_2(CH_2)_{15}COO^-$ | 288.5 | Not acquired |
| d7-stearic acid | d7-C18:0 | $CD_3CD_2CD_2(CH_2)_{14}COO^-$ | 290.5 | 290.3075 |
| Oleic acid | C18:1 | $CH_3(CH_2)_7CH = CH(CH_2)_7COO^-$ | 281.5 | 281.2504 |
| d5-oleic acid | d5-C18:1 | $CD_3CD_2(CH_2)_6CH = CH(CH_2)_7COO^-$ | 286.5 (expected) | Not available |
| d7-oleic acid | d7-C18:1 | $CD_3CD_2CD_2(CH_2)_5CH = CH(CH_2)_7COO^-$ | 288.5 | 288.2940 |
| Tricosanoic acid | C23:0 | $CH_3(CH_2)_{21}COO^-$ | 353.6 | Not acquired |

SIM, single ion monitoring.

also determined using the same procedure. The repeatability was calculated from six measurements at five different concentrations in the desired dynamic range on a single day. The precision was calculated from five different concentrations spread over the entire dynamic range and repeated five times for each measurement by the same person, on the same instrument, but on 3 different days. The recovery was established by spiking a known concentration in rat plasma and quantified using a standard curve. The recovery was done at five different concentrations and repeated five times for each measurement.

## RESULTS AND DISCUSSION

### Mass spectrometric approach

Typical methods reported in the literature for fatty acid analysis necessitate long sample preparation, including multiple liquid-liquid extractions, hydrolysis, and chemical derivatization steps, followed by GC-MS analysis (4–6). This paper describes a rapid and new method developed for the measurement of natural and deuterium-labeled fatty acids in plasma by HPLC-ESI-MS. The proposed approach consists of the hydrolysis of fatty acids incorporated into the triglyceride and other fat pools, followed by the acidification of the hydrolyzate (4–6), and a rapid and simple protein precipitation step using organic solvent. Then, samples are centrifuged and supernatants are analyzed by HPLC-ESI-MS. This protocol significantly reduces the sample preparation time required to analyze total fatty acids by avoiding numerous evaporation steps involved in derivation and liquid-liquid extraction procedures (4–6).

As a first attempt, fatty acids were analyzed using multiple reaction monitoring (MRM) as an MS/MS approach to minimize interference from the plasma matrix, using C18:0 as a model compound for the optimization (21). As shown in **Fig. 1**, good ionization of C18:0 was obtained in negative electrospray ion mode, but its fragmentation did not allow an optimal sensitivity compared with SIM detection. The maximal fragment represented 15% of the parent ion, as already reported (19) with a $[\mathrm{M\text{-}18}\mu]^-$ ion observed, which can be correlated with a loss of water from the carboxylic acid group. This poor fragmentation efficiency does not allow good sensitivity. The SIM mode was found to give better sensitivity compared with the MRM mode, despite the fact that more analytical interference may be expected in SIM mode than in MRM mode.

The simple sample preparation procedure combined with the HPLC-ESI-MS method in SIM mode allowed good detection of the natural fatty acids C14:0, C14:1, C16:0,

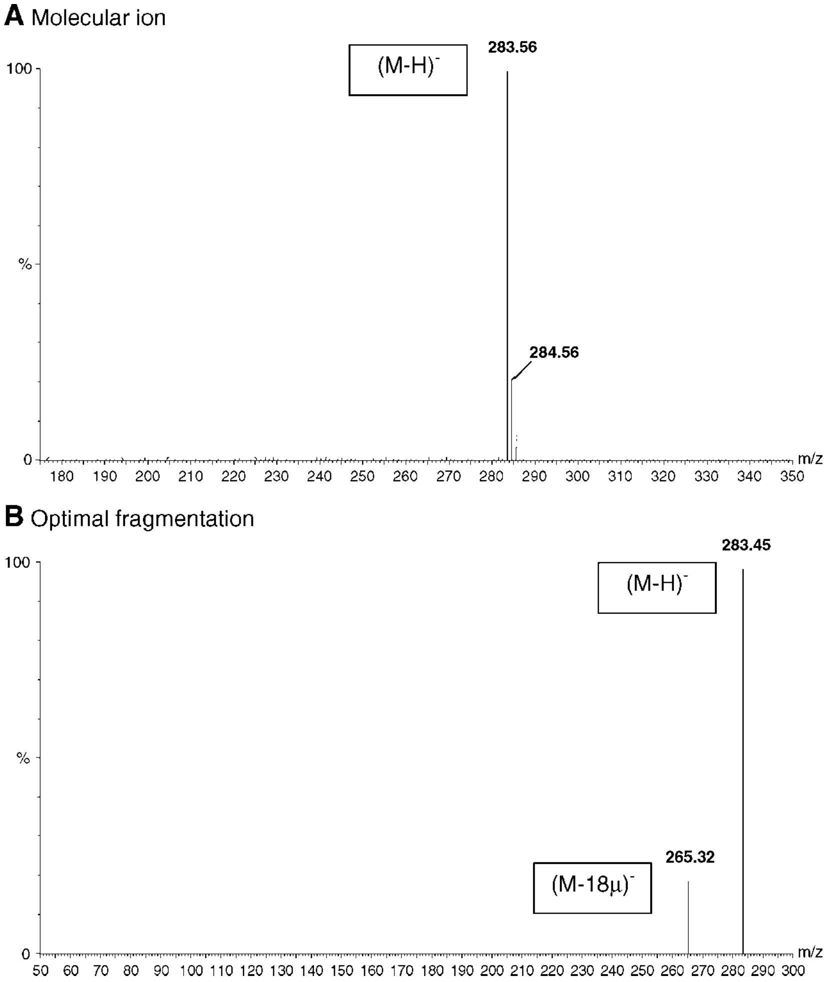

**Fig. 1.** Ionization (A) and fragmentation (B) of stearic acid (C18:0).

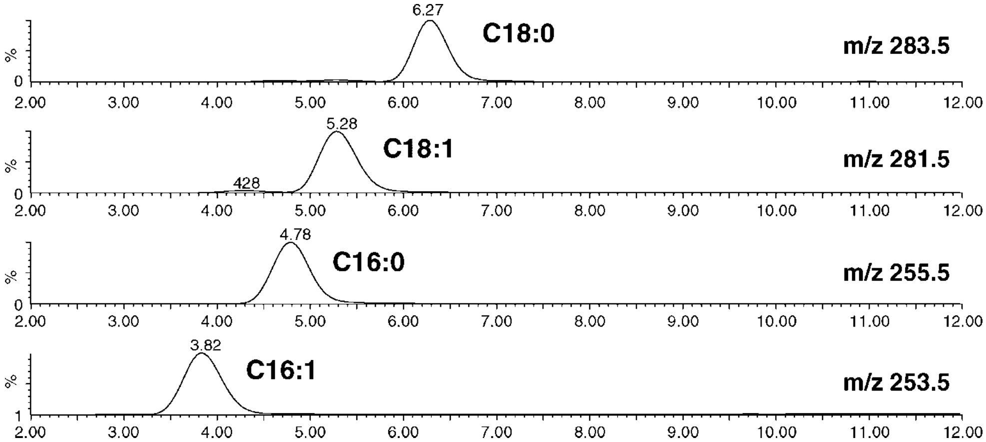

**Fig. 2.** Total endogenous fatty acids detected in rat plasma by high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS).

C16:1, C18:0, and C18:1, as shown in **Fig. 2**. As expected, plasma concentrations of these endogenous fatty acids were estimated to be in the high micromolar or low millimolar range in rat, mouse, dog, and monkey plasma (data not shown) (6, 22). Such high levels of natural fatty acids justify the need to use isotopically labeled fatty acids to monitor in vivo biosynthesis and metabolism of fatty acids over a short period with minimal interference from endogenous material. $^2$H and $^{13}$C tracers have been widely used for such studies, in which $^2$H- and $^{13}$C-labeled essential fatty acid biotransformations were followed in rat, cat, and human (4–6, 8). C18:0 was selected as a prototypical fatty acid to evaluate the potential use of this new analytical methodology for the detection of isotopically labeled tracers.

Commercially available sources for labeled C18:0 materials are d2, d3, d4, d5, d35, $^{13}C_1$, and $^{13}C_{18}$. In theory, the

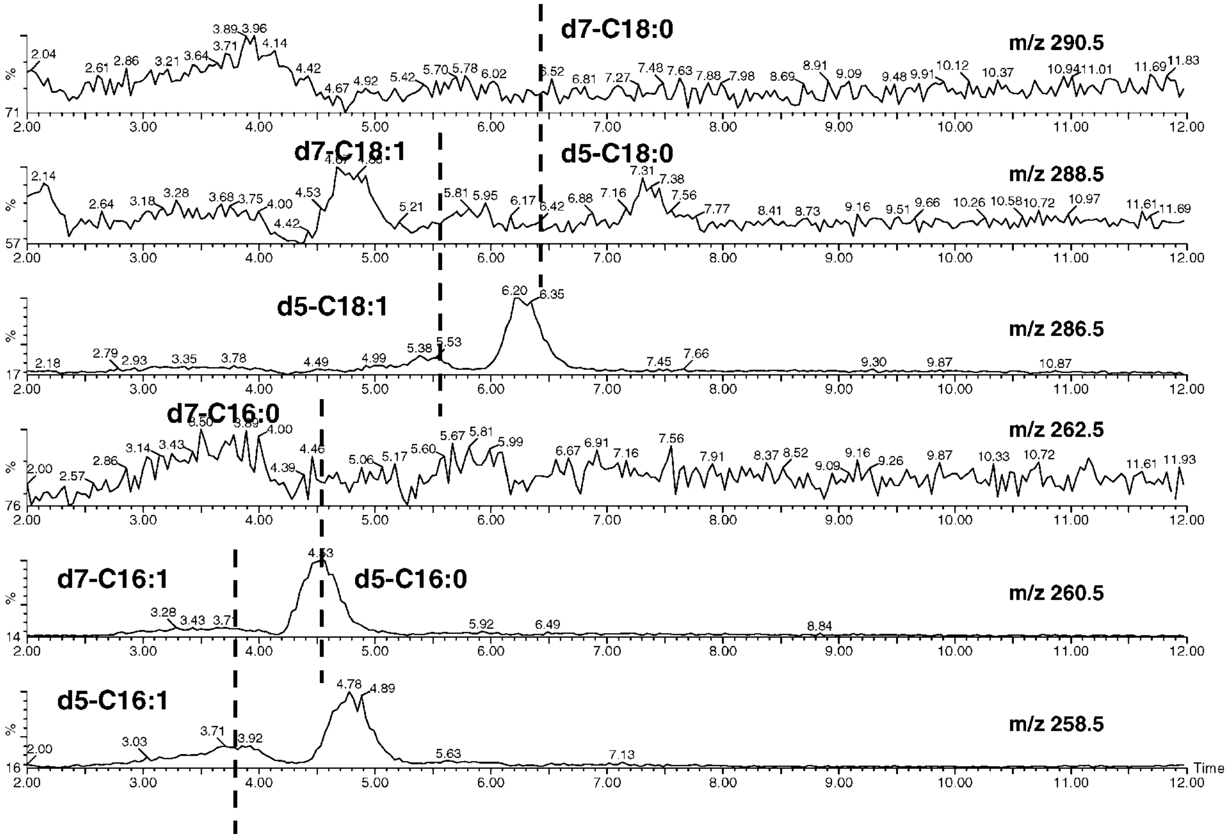

**Fig. 3.** Deuterium-labeled palmitic acid (d5- and d7-C16:0), palmitoleic acid (d5- and d7-C16:1), d5- and d7-C18:0, and oleic acid (d5 and d7-C18:1) single ion monitoring (SIM) traces from blank plasma extraction of rat, mouse, squirrel monkey, and beagle dog. The expected elution windows are indicated by the dotted lines.

presence of a $^{2}H$ or $^{13}C$ label on fatty acids should minimize the contamination of the SIM trace by endogenous fatty acid. However, the mass contribution from the added isotope must be superior to the natural isotopic effect of the endogenous form, because natural and isotopically labeled materials coelute by chromatography (4–6). In addition, the presence of isotopes on C18:0 were minimized to avoid potential isotopic effects on the activity of enzymes involved in fatty acid metabolism (6, 23). Because at least five deuterium atoms were needed to minimize signal contamination attributable to the isotopic composition of endogenous fatty acids, the commercially available d5-C18:0 was first considered as a potential tracer for in vivo studies in rats. To evaluate the presence of interference from the plasma matrix, blank plasma from Sprague-Dawley rat, mouse, squirrel monkey, and beagle dog was extracted and analyzed with SIM descriptors corresponding to putative C16:0, C16:1, C18:0, and C18:1 labeled with five deuterium atoms.

SIM traces obtained in blank rat plasma are presented in **Fig. 3**. These traces are representative of results obtained across species. In summary, interference was detected in the SIM trace of a d5-C18:1 and more importantly for a d5-C16:0, whereas clean traces were observed for d5-C16:1 and d5-C18:0 at the chromatographic retention times expected based on the endogenous form. This interference issue can be addressed by the use of SIM traces calculated for d7-C16:0, d7-C16:1, d7-C18:0, and d7-C18:1. For these traces, the chromatogram is exempt from interference at the expected chromatographic retention times of all compounds in both rodent and nonrodent species. The use of d7-labeled fatty acids would allow easy detection of the fatty acids of interest, which would be difficult using a commercially available d5-labeled fatty acid because of background noise. In dog plasma, for example, the detection of d5-C18:1 was compromised as a result of high levels of interference, whereas the d7-C18:1 trace was cleaner (**Fig. 4**), proving once more the advantage of d7-labeled fatty acids as tracer.

For the purposes of this study, d7-C18:0 and d7-C18:1 were synthesized in-house and fully characterized by NMR (S. N. Crane, K. Bateman, S. Gagne, and J-F. Levesque, unpublished data) and by mass accuracy using an ultra-performance liquid chromatography-quadrupole-time of flight apparatus. All exact masses measured were in good agreement with the theoretical expected mass within 10 ppm.

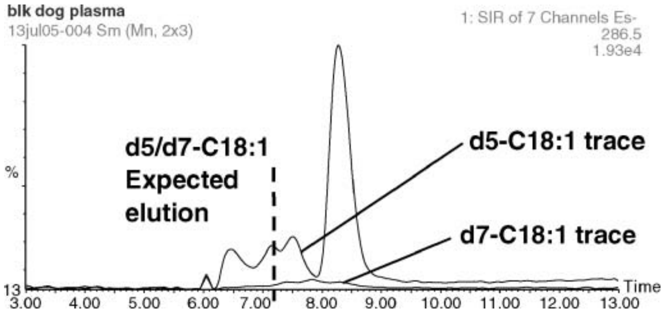

**Fig. 4.** SIM traces from beagle dog plasma extraction at *m/z* 286.5 (d5-C18:1) and *m/z* 288.5 (d7-C18:1).

TABLE 2. Analytical performances for d7-C18:0 and d7-C18:1 by HPLC-ESI-MS

| Variable | d7-C18:0 | d7-C18:1 |
|---|---|---|
| LOD (nM) | 100 | 100 |
| Linearity ($R^2$) | LOD to 30 μM (0.999631) | LOD to 30 μM (0.998580) |
| Selectivity (SIM *m/z*) | 290.5 | 288.5 |
| Repeatability (%) | >92 | >90 |
| Precision (%) | >90 | >88 |

HPLC-ESI-MS, high-performance liquid chromatography-electrospray ionization-mass spectrometry; LOD, limit of detection.

## Analytical performance

The analytical methodology presented above was adapted for d7-C18:0, d7-C18:1, d7-C16:0, and d7-C16:1 in rat plasma. The ester forms of each d7-fatty acid being unavailable, the analytical performance discussion cannot take into account the hydrolysis efficiency. Nevertheless, the hydrolysis efficiency was evaluated previously (data not shown) in the optimization of the sample preparation procedure for endogenous fatty acids in plasma. Parameters such as hydrolysis time and hydrolysis medium were optimized. Similar conditions were also reported in the literature (4–6).

**Table 2** summarizes all of the analytical parameters evaluated. The LOD for both d7-C18:0 and d7-C18:1 was 100 nM, and minimal interfering background was detected, compared with the d5-fatty acid analogs (**Figs. 3–5**). The linearity was evaluated between the LOD and a maximal concentration fixed at 30 μM, and for both standards, $R^2 \geqslant 0.999$ was obtained. Repeatability $> 90\%$ and precision $> 88\%$ were obtained for both compounds, confirming the stability of their mass spectrometric ionization in the conditions optimized.

In addition, the recovery was measured for d7-C18:0 and d7-C18:1, and the results obtained are presented in **Table 3**. Over the entire dynamic range reported, the recovery allowed an accurate measurement for each d7-fatty acid ± 15% at concentrations of $\geqslant 1$ μM and within an

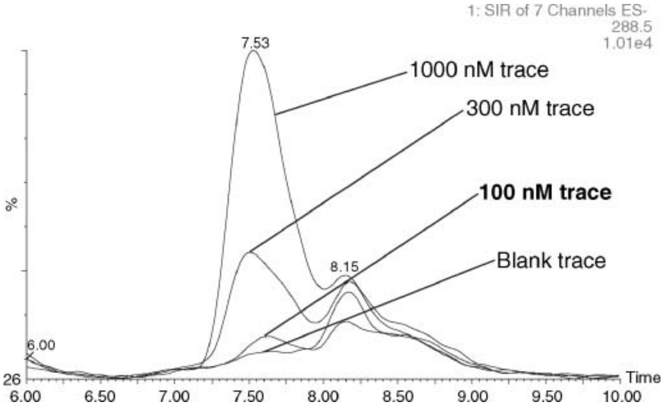

**Fig. 5.** SIM trace of beagle dog plasma spiked with various concentrations of d7-C18:1.

TABLE 3. Recovery evaluation for d7-C18:0 and d7-C18:1 by HPLC-ESI-MS

| Concentration Spiked | Percentage Recovery, d7-C18:0 | Percentage Recovery, d7-C18:1 |
|---|---|---|
| 0.3 μM | 69.9 ± 27.4 | 156.7 ± 10.6 |
| 1 μM | 96.0 ± 15.8 | 109.9 ± 4.4 |
| 3 μM | 110.6 ± 12.0 | 111.7 ± 6.1 |
| 10 μM | 101.4 ± 6.8 | 92.3 ± 9.6 |
| 30 μM | 99.7 ± 2.6 | 101.0 ± 9.0 |

acceptable range at 0.3 μM for d7-C18:0 and d7-C18:1. Similar analytical performances were presented for fatty acid analysis by LC-MS in other matrices than plasma (18, 19). This validated analytical methodology for the extraction and analysis of d7-fatty acids was then used to analyze plasma samples from an in vivo experiment.

### In vivo evaluation in rat

To evaluate the potential of this new d7 tracer to study fatty acid metabolism, d7-C18:0 was administered orally at two doses and the plasma samples collected were treated using the optimized analytical method. Typical transformations expected (24) for d7-C18:0 should include CoA formation, unsaturation, and β-oxidation of its alkyl chain as well as esterification to triglycerides or incorporation into fat pools. However, the plasma hydrolysis step in the sample preparation disables the detection of d7-fatty acid esters.

Rat plasma samples collected were analyzed, and a typical HPLC-ESI-MS chromatogram is shown in **Fig. 6**. Excellent signal-to-noise ratios were observed for d7-C18:0 and d7-C18:1 at all time points, whereas d7-C16:0 was detected only up to 24 h. For both dosages, only the d7-C16:1 stayed under the LOD at all time points. **Figure 7** depicts the phar-

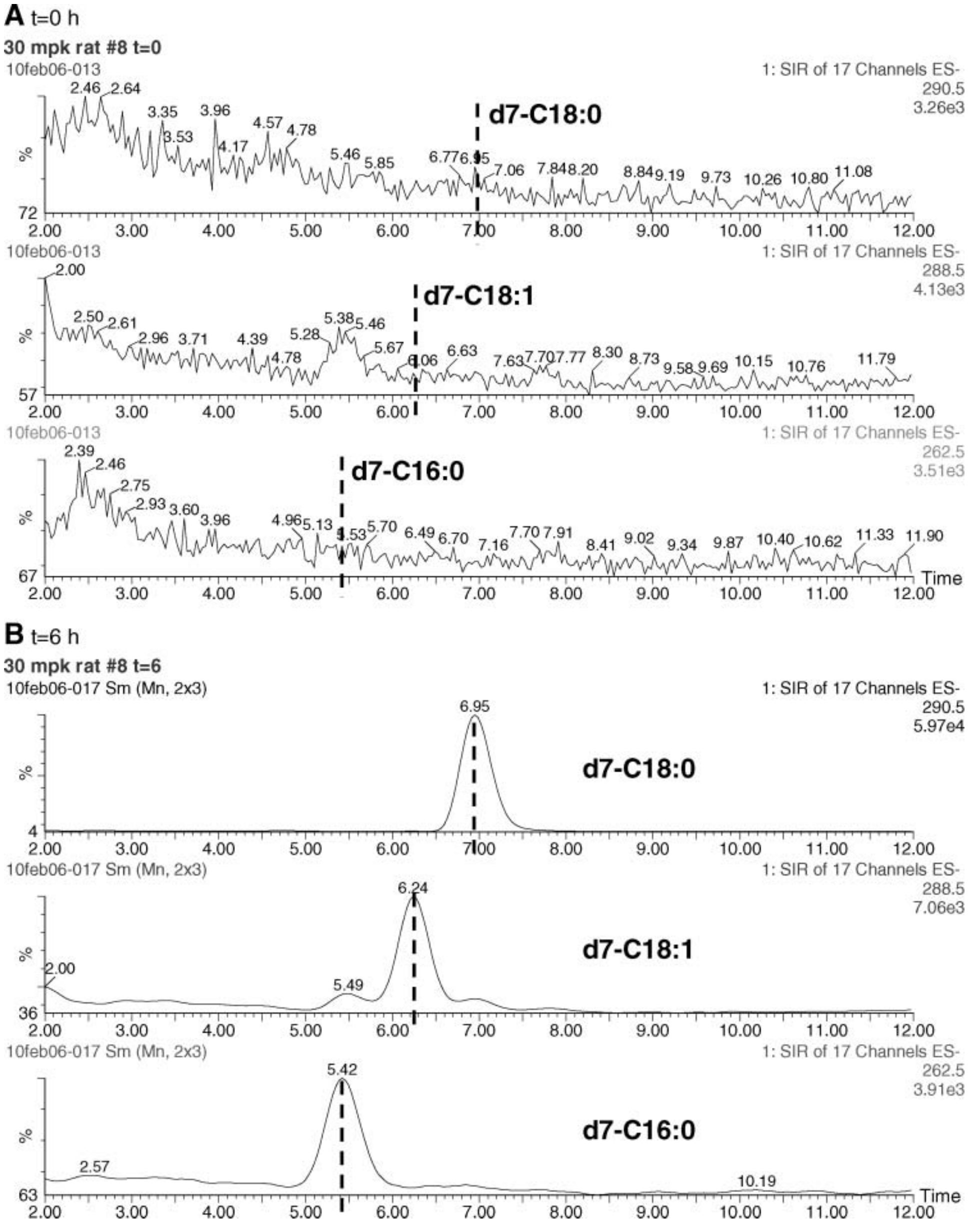

**Fig. 6.** Plasma samples of rat dosed orally with 30 mg/kg d7-C18:0 at 0 (A) and 6 h (B).

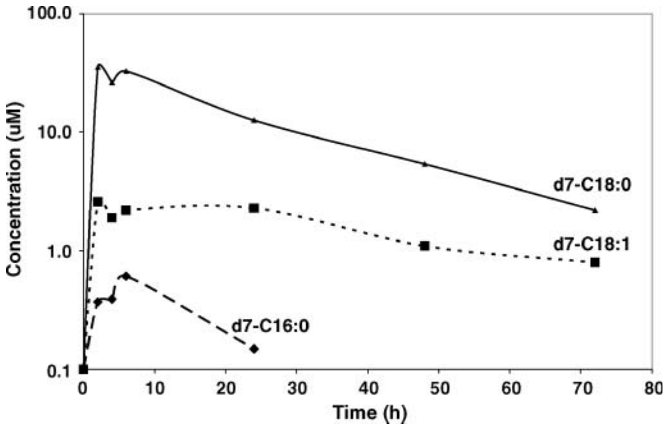

**Fig. 7.** Plasma profile of rat dosed orally with 30 mg/kg d7-C18:0.

macokinetic profile of d7-fatty acids upon a 30 mg/kg dose of d7-C18:0. Data obtained for the 10 mg/kg dose were similar, except that lower concentrations were observed for each d7-fatty acid (data not shown). As expected, slow rates of elimination were observed for the d7-fatty acids analyzed. After 72 h, important levels of d7-C18:0 and d7-C18:1 was still detected, and elimination half-lives were estimated to be 17 and 45 h, respectively. These results clearly demonstrate that unsaturation and β-oxidation are the main biotransformations involved in d7-C18:0 metabolism in vivo in rats, as reported in the literature for the de novo fatty acid biosynthesis (24, 25). Interestingly, d7-C16:0 has a shorter elimination half-life than d7-C18:0 and d7-C18:1 (7 h), as shown previously in rat (26).

In summary, an improved methodology was developed for the analysis of natural fatty acids (C14:0, C16:0, and C18:0 and their monounsaturated versions) and deuterium-labeled analogs in plasma samples, involving only hydrolysis, neutralization, and quenching steps followed by HPLC-ESI-MS analysis in negative ion mode using SIM. This method is rapid and robust and is useful for the detection of fatty acids from 100 nM to the high micromolar range. A new d7-fatty acid tracer, d7-C18:0, was synthesized to reduce matrix interference observed with its d5 analog, which significantly improved the LOD depending on the products analyzed. Upon the oral dosing of d7-C18:0 in rats, the parent compound and its desaturation and β-oxidation products, d7-C18:1 and d7-C16:0, were circulating with a maximal concentration ranging from 0.6 to 2.2 μM, with significant levels of d7-fatty acids detected for up to 72 h. This methodology could be extended to other d7-fatty acids, as needed, based on results obtained with endogenous fatty acids. In the course of these studies, only d7-C18:0 and d7-C18:1 were synthesized, because monodesaturation was expected to be the main biotransformation involved in d7-C18:0 metabolism, and the synthesis of labeled material can be time- and resource-consuming.

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