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Identification in Rat Plasma and Urine by Linear Trap Quadrupole−Orbitrap Mass Spectrometry of the Metabolites of Maslinic Acid, a Triterpene from Olives

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ABSTRACT: Maslinic acid is a natural pentacyclic triterpenoid widely distributed in edible and medicinal plants with health-promoting activities. The identification and quantification of its metabolites is a requirement for a better understanding of the biological effects of this triterpene. Therefore, maslinic acid was orally administered to Sprague−Dawley rats at a dose of 50 mg/kg of body weight. Blood and urine were withdrawn at 45 min. Samples were extracted with ethyl acetate prior to liquid chromatography−atmospheric pressure chemical ionization−linear trap quadrupole−Orbitrap (LC−APCI−LTQ−Orbitrap) analysis. Screening of plasma yielded four monohydroxylated derivatives (M1−M4), one monohydroxylated and dehydrogenated metabolite (M5), and two dihydroxylated and dehydrogenated compounds (M6 and M7). In urine, M1, M4, M5, and M6 were detected. Quantification by LC−APCI−mass spectrometry (MS) revealed maslinic acid as the prevalent compound in both plasma (81.8%) and urine (73.9%), which indicates that metabolism is low and mainly attributable to phase I reactions.

KEYWORDS: *maslinic acid, metabolism, rat plasma, pentacyclic triterpenes, LC−MS*

INTRODUCTION

Metabolite identification and quantitation play an important role in the investigation of bioactive food components. Metabolism affects not only their bioavailability, by influencing the distribution and rate or route of excretion, but also the evaluation of their actions, given that metabolites could either contribute to the biological activities or have toxicological effects independent of the parent compound.[1] In addition, the identification of metabolites is important for their potential application as biomarkers of intake in dietary intervention studies.[2] One of the natural molecules being assessed as a future nutraceutical is maslinic acid [(2α,3β)-2,3-dihydroxyolean-12-en-28-oic acid], a biologically active pentacyclic triterpene that has been isolated from different vegetal species, including edible and medicinal plants.[3,4] This compound is especially prevalent in the leaves and fruits of *Olea europaea* L. and has recently been found in different fresh vegetables,[5] legumes,[6] and fruits.[7] Maslinic acid has attracted much interest because of its proven toxicological safety[8] and its multiple biological activities, such as antitumoral, antidiabetic, cardioprotective, and neuroprotective.[9] Recently, the pharmacokinetics of this compound has been assessed through a population approach that indicated that maslinic acid had a fairly rapid absorption with a maximal concentration of 4.03 μM at 30 min after the oral administration of 50 mg/kg of body weight and bioavailability of 5.13%,[10] but the information about its metabolism remains unknown.

Therefore, the present study aims to investigate the metabolites of maslinic acid in rat plasma and urine by linear trap quadrupole (LTQ)−Orbitrap mass spectrometry (MS), because the robust and accurate mass data that this high-resolution mass spectrometer provides allows for its unambiguous identification. Furthermore, maslinic acid and its metabolites were quantified by liquid chromatography−atmospheric pressure chemical ionization (LC−APCI)−MS to obtain a thorough understanding of the biotransformation underwent by this bioactive compound. The knowledge of the circulating derivatives of maslinic acid is relevant for not only providing information about the metabolic fate of this pentacyclic triterpene applicable to its future use as a nutraceutical but also putative biomarkers of table olives and olive oil consumption.

MATERIALS AND METHODS

Chemicals and Reagents. Maslinic acid was derived from olive pomace by the patented method by García-Granados.[11] Extrasynthèse (Genay, France) supplied betulinic acid [3β-3-hydroxy-lup-20(29)-en-28-oic acid], which was used as the internal standard (IS). Sigma-Aldrich (St. Louis, MO) provided (2-hydroxypropyl)-β-cyclodextrin and sodium carboxymethylcellulose. Acetonitrile was purchased from Scharlau Chemie S.A. (Barcelona, Spain), and ethyl acetate and methanol were from J.T. Baker (Deventer, Netherlands). All of them were high-performance liquid chromatography (HPLC)-grade solvents. Other chemicals and solvents were analytical-grade reagents supplied by Sigma-Aldrich. Ultrapure water (18 mΩ, Milli-Q water, Millipore, Milan, Italy) was used in all of the experiments.

Animals. Adult male Sprague−Dawley rats of 250−300 g were maintained in cages (n = 2/cage) at the Animal House Facility of the

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Facultat de Farmàcia of the Universitat de Barcelona. Animals were kept in an environmentally controlled room with stable humidity (50 ± 10%) and temperature (22 ± 2 °C) and a 12 h light/dark cycle. A commercial diet (2014 Teklad Global 14%, Harlan, Barcelona, Spain) and water were supplied *ad libitum*. Rats were fasted overnight and anesthetized by intraperitoneal injection of 90 mg/kg of ketamine (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain) and 10 mg/kg of xylacine (Rompun 2%, Química Farmacéutica Bayer S.A., Barcelona, Spain). All animal manipulations accomplished the requirements set by the European Community guidelines for the care and management of laboratory animals. In addition, the experiments were conducted in the morning to lessen the effects of circadian rhythms. The research protocol (reference 164/10) was approved by the Ethic Committee of Animal Experimentation of the Universitat de Barcelona.

Dose Administration and Sample Collection. Maslinic acid was administered to overnight fasted rats at a dose of 50 mg/kg of body weight by single oral gavage. Pentacyclic triterpene was dissolved in an aqueous solution of (2-hydroxypropyl)-ß-cyclodextrin (40%, w/v) and sodium carboxymethylcellulose (0.5%) as previously described.[12] After oral administration, blood was withdrawn from the saphenous vein[13] at 45 min and placed in tubes containing ethylenediaminetetraacetic acid (EDTA)−K_2 (Microvette CB300, Sarstedt, Granollers, Spain) to avoid coagulation. Samples were maintained on ice until centrifugation at 1500*g* (Centrifuge Megafuge 1.0R, Heraeus, Boadilla, Spain) for 15 min at 4 °C. Plasma was subsequently separated and stored at −20 °C until analysis. At 45 min post-administration, urine samples were directly collected from the urinary bladder of previously anesthetized animals and frozen at −20 °C until processing.

Preparation of Rat Plasma and Urine Samples. Plasma and urine were thawed before analysis, and urine was centrifuged at 25000*g* for 30 min at 2 °C (Centrifuge 5417R, Eppendorf Ibérica S.L.) to obtain a clear supernatant. A total of 200 μL of either plasma or urine was spiked with 10 μL of 50 μM betulinic acid [IS, dissolved in 80:20 (v/v) methanol/water] before being extracted with 2 mL of ethyl acetate. Each sample was strongly stirred in a vortex mixer for 3 min and then centrifuged at 1500*g* for 15 min at 4 °C (Centrifuge Megafuge 1.0R, Heraeus). The ethyl acetate layer was carefully removed and placed in a clean conical tube, and a second extraction of the residue was performed. The organic supernatants were gathered together and evaporated to dryness in a vacuum concentrator (Concentrator 5301, Eppendorf Ibérica S.L., San Sebastián de los Reyes, Spain) at 45 °C. The dry residue was dissolved by adding 100 μL of methanol/water (80:20, v/v), followed by vigorous shaking in a vortex mixer for 5 min. Samples were immediately sonicated for 1 min and centrifuged at 25000*g* for 15 min at 4 °C (Centrifuge 5417R, Eppendorf Ibérica S.L.). Finally, an amber vial was employed to place the clean supernatant before the analysis.

Metabolite Identification by LC−APCI−LTQ−Orbitrap. Samples were analyzed using an Accela liquid chromatograph (Thermo Scientific, Hemel, Hempstead, U.K.) equipped with a thermostated autosampler and a quaternary pump and coupled to a LTQ−Orbitrap Velos mass spectrometer (Thermo Scientific). The equipment was available at the Centres Científics i Tecnològics of the Universitat de Barcelona. Chromatographic separations was carried out on a Luna C_{18} column (150 × 2.0 mm, 5 μm) kept at 40 °C and protected with a security guard cartridge of the same packing material (Phenomenex, Micron Analítica S.A., Madrid, Spain). Gradient elution was performed with water (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.8 mL/min. The volume of injection was 10 μL. Separation was carried out within 19 min under the following conditions: 0 min, 95% A and 5% B; 4 min, 50% A and 50% B; 8 min, 40% A and 60% B; 12 min, 25% A and 75% B; 15 min, 0% A and 100% B; and 19 min, 0% A and 100% B. An additional period of 8 min was needed for re-equilibration of the column before the ensuing injection.

Mass spectrometric analysis was conducted using an APCI source in the negative-ion mode. Mass calibration was performed daily according to the guidelines of the manufacturer. The LTQ−Orbitrap was tuned to the optimal conditions for maslinic acid. Therefore, a 100 μM standard solution was delivered by the LTQ syringe pump at a constant flow rate of 10 mL/min in the presence of LC mobile phase under isocratic conditions of 25% A and 75% B. Operation parameters were set as follows: vaporizer temperature, 450 °C; sheath gas, 50 arbitrary units (au); auxiliary gas flow, 5 au; sweep gas, 0 au; discharge current, 5 μA; S-lens radio frequency (RF) level, 70%; and capillary gas, 300 °C. The data were acquired using Fourier transform full scan with a resolving power of either 60 000 or 100 000 (at m/z 400) and the microscan count fixed to unity. The automatic gain control (AGC) was set at 1×10^6. The mass spectrometric data were collected in a range of m/z from 100 to 1000.

MS Data Processing. Mass chromatograms and spectral data were acquired with the Xcalibur software 2.0 (Thermo Scientific). Maslinic acid metabolites were searched both manually with Xcalibur Qual Browser and also employing MetWorks software (version 1.3, Thermo Scientific). Qual Browser was employed for MS data processing and calculation of elementary compositions. The elemental composition was selected according to the accurate masses and the isotopic pattern. Mass accuracy was determined by the deviation of measured and theoretical masses relative to theoretical values and was expressed in millidaltons. MetWorks software allowed for the identification of metabolites by comparing the samples to controls, of either blank plasma or urine. The most commonly observed biotransformations in phase I and phase II metabolism were searched to find putative metabolites of maslinic acid with a mass deviation of less than 5 ppm from the predicted values.

Quantification of Maslinic Acid and Its Metabolites by LC−APCI−MS in Rat Plasma and Urine. After the identification of maslinic acid metabolites by LC−APCI−LTQ−Orbitrap, the compounds were quantified by LC−APCI−MS. The liquid chromatograph (PerkinElmer series 200, PerkinElmer, Norwalk, CT) included a quaternary pump and a refrigerated autosampler plate and was coupled to a single quadrupole mass spectrometer (PE Sciex API 150 EX, AB SCIEX, Spain) with an APCI source at 500 °C in negative-ion mode. The LC−MS system was controlled by the 1.4.2 Analyst software supplied by Applied Biosystems (Foster City, CA). The chromatographic conditions were the conditions described for the LC−APCI−LTQ−Orbitrap system. For the mass spectrometer, nitrogen was used as both curtain gas (12 au) and nebulizer gas (10 au) with a nebulizing flow of −3 au. The optimum LC−APCI−MS pre-collision cell voltages were −30 V for declustering potential, −100 V for focusing potential, and −5 V for entrance potential. The selected ion monitoring (SIM) mode was used for quantification of maslinic acid at m/z 471.3 and betulinic acid (IS) at m/z 455.3. The metabolites identified by LC−APCI−LTQ−Orbitrap were quantified at m/z 487.3 (M1−M4), 485.3 (M5), and 501.3 (M6 and M7). The dwell time was set at 165 ms. Concentrations of maslinic acid in plasma and urine samples were calculated from the ratio of the peak area of triterpene to the IS interpolated on an external calibration curve. Because of the absence of synthetic standards, it was assumed that LC−APCI−MS response to derivatives is similar to that of maslinic acid; thus, both plasma and urine metabolites were quantified using the standard curve of the parent compound. Results were expressed as micromoles per liter (μM).

The determination of maslinic acid in plasma and urine was validated for recovery, matrix effect, precision, accuracy, linearity, and sensitivity, according to the European Medicines Agency (EMA) Guideline on Bioanalytical Method Validation.[14] Calibration standards were freshly prepared by spiking 190 μL of rat blank plasma or urine with 10 μL of standard solutions of maslinic acid at different concentrations and were treated as previously indicated for the specimens collected from dosed rats. The post-extraction spiking method proposed by Matuszewski et al.[15] was used to establish the recovery and matrix effect. Briefly, recovery was assessed by comparing the peak areas of calibration standards to the peak areas obtained from blank plasma or urine spiked with maslinic acid after the extraction. The matrix effect was evaluated by comparing the peak areas obtained from blank plasma or urine spiked with pentacyclic triterpene after the extraction to those of a solution of 80% methanol spiked with the expected concentration. Recovery and matrix effect were determined

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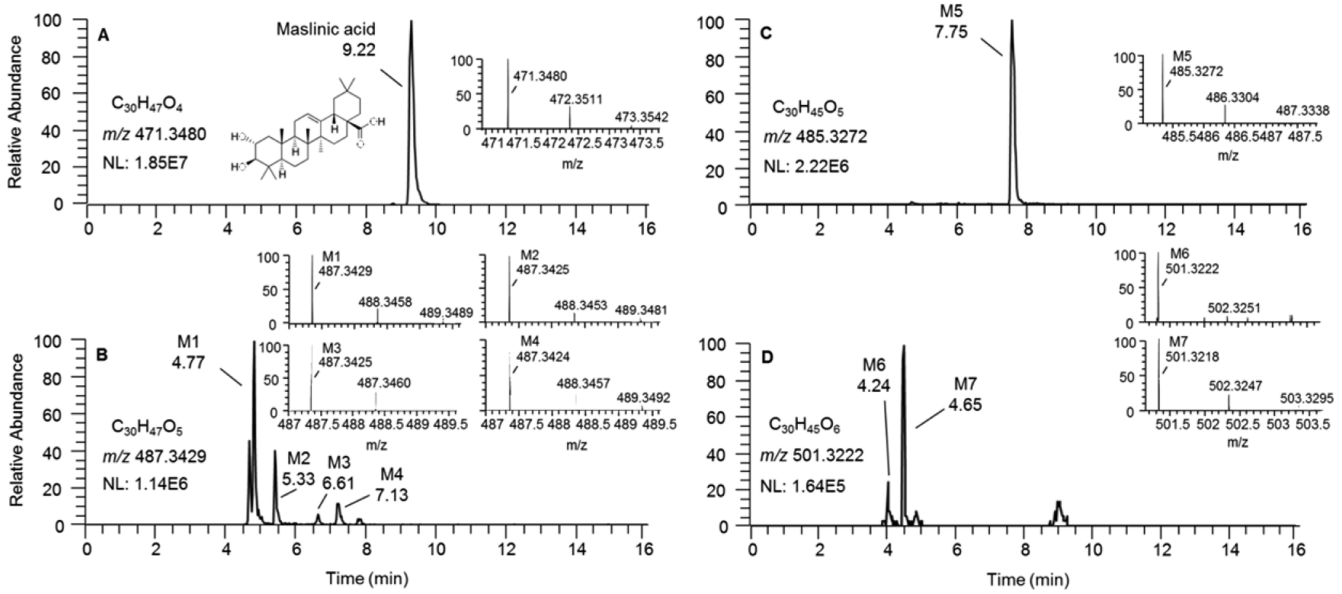

Figure 1. Representative extracted ion chromatograms (EICs) and MS spectra of maslinic acid and its phase I metabolites of the rat plasma sample obtained 45 min after the oral administration of 50 mg/kg of body weight of triterpene. A full-scan analysis was performed by LC−APCI−LTQ−Orbitrap using a scan range from *m/z* 100.00 to 1000.00. EICs: (A) maslinic acid (*m/z* 471.32), (B) monohydroxylated metabolites (*m/z* 487.34), (C) monohydroxylated and dehydrogenated metabolites (*m/z* 485.32), and (D) dihydroxylated and dehydrogenated metabolites (*m/z* 501.32). MS spectra: (A1) maslinic acid, (B1−B4) monohydroxylated metabolites, (C1) monohydroxylated and dehydrogenated metabolite, and (D1 and D2) dihydroxylated and dehydrogenated metabolites. NL = normalized level.

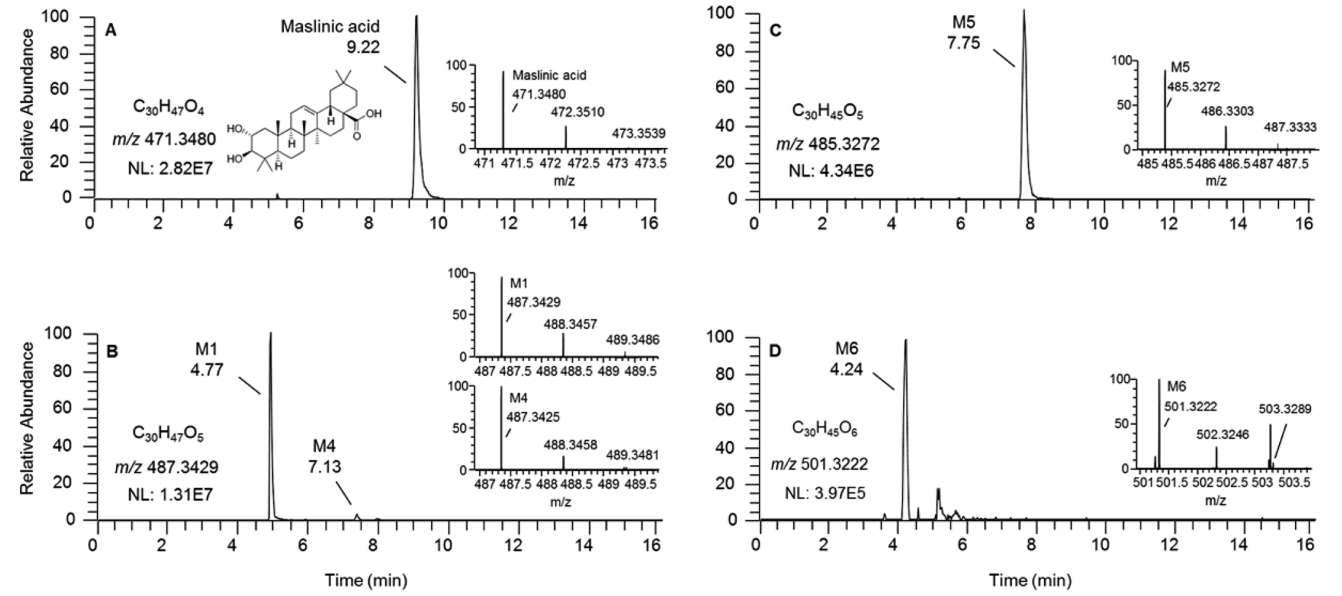

Figure 2. Representative EICs and MS spectra of maslinic acid and its phase I metabolites of the rat urine sample withdrawn 45 min after the oral administration of 50 mg/kg of body weight of triterpene. A full-scan analysis was performed by LC−APCI−LTQ−Orbitrap using a scan range from *m/z* 100.00 to 1000.00. EIC chromatograms: (A) maslinic acid (*m/z* 471.32), (B) monohydroxylated metabolites (*m/z* 487.34), (C) monohydroxylated and dehydrogenated metabolites (*m/z* 485.32), and (D) dihydroxylated and dehydrogenated metabolites (*m/z* 501.32). MS spectra: (A1) maslinic acid, (B1 and B2) monohydroxylated metabolites, (C1) monohydroxylated and dehydrogenated metabolite, and (D1) dihydroxylated and dehydrogenated metabolite. NL = normalized level.

at four concentration levels (0.5, 1, 5, and 10 μM), while precision and accuracy were analyzed at 1, 5, and 10 μM. Linearity was assayed in the range of 0.005−10 μM, which included eight concentration levels that covered the expected amounts in the samples. The limit of detection (LOD) was considered as the minimum concentration of maslinic acid that could be recognized by the detector and produced a signal-to-noise ratio of 3:1. The lowest limit of quantification (LLOQ) was defined as the lowest concentration of the standard curve that could be accurately quantified and with a signal at least 10 times above the noise level.

RESULTS

LC−APCI−LTQ−Orbitrap Analysis of Maslinic Acid and Its Metabolites in Rat Plasma and Urine. Maslinic acid ($C_{30}H_{48}O_4$) was unambiguously identified in rat plasma and urine by comparing its retention time (9.22 min) and MS

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Table 1. Maslinic Acid and Its Metabolites Identified by LC−APCI−LTQ−Orbitrap in Rat Plasma and Urine Samples Obtained after the Oral Administration of 50 mg/kg of Body Weight of Pentacyclic Triterpene

					error		metabolite appearance	
	retention time (min)	molecular formula	theoretical mass (m/z)	measured mass (m/z)	mDa	ppm	plasma	urine
M0	9.22	$C_{30}H_{48}O_4$	471.3480	471.3479	0.04	−0.22	+	+
			Monohydroxylated Metabolites					
M1	4.77	$C_{30}H_{48}O_5$	487.3429	487.3426	−0.05	−0.30	+	+
M2	5.33	$C_{30}H_{48}O_5$	487.3429	487.3425	0.13	−0.35	+	ND[a]
M3	6.61	$C_{30}H_{48}O_5$	487.3429	487.3425	0.25	−0.80	+	ND[a]
M4	7.13	$C_{30}H_{48}O_5$	487.3429	487.3424	0.9	−0.50	+	+
			Monohydroxylated and Dehydrogenated Metabolites					
M5	7.75	$C_{30}H_{46}O_5$	485.3272	485.3271	0.23	0.03	+	+
			Dihydroxylated and Dehydrogenated Metabolites					
M6	4.24	$C_{30}H_{46}O_6$	501.3222	501.3216	−0.37	0.18	+	+
M7	4.65	$C_{30}H_{46}O_6$	501.3222	501.3218	−0.05	−1.13	+	ND[a]

[a]ND = not detected.

data to the standard reference. In addition, the injection into the high-resolution system LTQ−Orbitrap gave its accurate mass (m/z 471.3480) with an error of 0.4 mDa. The spectra generated for pentacyclic triterpene in plasma and urine showed the deprotonated molecule $[M - H]^-$ without the presence of any fragment (Figures 1A and 2A, respectively).

Possible derivatives of maslinic acid were first predicted on the basis of knowledge of metabolism and then searched in full-scan chromatograms of rat plasma and urine samples. Afterward, the formulas of potential metabolites were compared to that of the parent compound to refer metabolic pathways. The high mass accuracy of the LTQ−Orbitrap analyzer enabled an extraction of individual chromatograms in a narrow interval of the m/z scale (± 5 mDa), which is useful for discarding other compounds with similar masses.[16] The initial screening of plasma and urine samples obtained after the oral administration of maslinic acid revealed the formation of derivatives with mass shifts of +16, +14, and +30 Da in comparison to the parent compound. Mass shifts of +32 Da, which corresponded to a double hydroxylation, were searched but were not found in the analyzed plasma and urine samples. On the other hand, phase II metabolites of maslinic acid were not detected after screening with LC−APCI−LTQ−Orbitrap and MetWorks software, indicating that this compound undergoes mainly phase I metabolism.

Table 1 shows the list of metabolites identified through LTQ−Orbitrap analysis, along with retention times, accurate masses, molecular formulas, and mDa and ppm of error between the mass found and the accurate mass of each derivative. The observed and calculated masses agree to within less than 1 mDa, which demonstrates the high accuracy of the measured masses. Neither maslinic acid nor its metabolites were detected in plasma and urine of the control group at the corresponding retention times.

M1−M4 (m/z 487.3). Four polar metabolites, namely, M1−M4, were detected in plasma as deprotonated molecules at m/z 487.3 and a molecular formula of $C_{30}H_{48}O_5$, according to the Formula Predictor software (Table 1). The mass of these compounds was 15.9949 Da higher than that of maslinic acid, thus indicating that the biotransformation might be the addition of a hydroxyl group. All of the metabolites eluted earlier than the parent compound, showing retention times of 4.77, 5.33, 6.61, and 7.13 min for M1, M2, M3, and M4, respectively. As shown in Figure 1B, the peak of M1 was the most abundant, followed by M2 and M4 peaks, while that of M3 was less intense. The accurate mass of each of these metabolites differed by less than 1 mDa from the theoretical mass, and the respective spectra showed an isotopic distribution that was in accordance with the theoretical spectra of m/z 487.3, indicating a correct identification (panels B1−B4 of Figure 1).

On the other hand, in urine samples, only metabolites M1 and M4 were detected (Figure 2B). The compound M1 was the most abundant, as was observed in plasma. However, M2 and M3 were not present in any of the samples analyzed, and M4 appeared in urine with low intensities. The corresponding spectra are shown in panels B1 and B2 of Figure 2.

M5 (m/z 485.3). A single peak corresponding to a deprotonated molecule at m/z 485.3 was identified as M5. This metabolite had a retention time of 7.75 min and was found in both plasma (Figure 1C) and urine (Figure 2C) samples. The mass increment of 13.9792 Da with respect to maslinic acid (Table 1), together with the molecular formula ($C_{30}H_{46}O_5$) calculated by the Formula Predictor software, suggested that this biotransformation consists of the incorporation of a hydroxyl group and the loss of two hydrogen atoms. M5 was identified with an error of 0.23 mDa (Table 1) and a correct isotopic distribution pattern (Figures 1C1 and 2C1).

M6 and M7 (m/z 501.3). Two additional compounds (M6 and M7) eluting earlier than the parent compound yielded a deprotonated molecule at m/z 501.3. The retention times were 4.24 and 4.65 min for M6 and M7, respectively. Because the molecular formula of both metabolites was $C_{30}H_{46}O_6$, as calculated by the Formula Predictor software (Table 1) and the mass shift was +29.9442 Da, this biotransformation was attributed to dihydroxylation and dehydrogenation of maslinic acid. In plasma samples, M7 showed a higher relative abundance than M6 (Figure 1D), whereas in urine, only M6 was detected and with very low intensities, indicating small concentrations (Figure 2D).

Quantification of Maslinic Acid Metabolites by LC−APCI−MS in Rat Plasma. The mass chromatograms from LC−APCI−LTQ−Orbitrap were consistent with the LC−APCI−MS chromatograms and allowed for the identification of all of the metabolites, taking into account the relative retention times. The method was adequate for the quantification of maslinic acid in plasma samples, as indicated by the results obtained in the validation. The mean recovery and matrix effect

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Table 2. Matrix Effect and Recovery of the Analytical Methods To Determine Maslinic Acid in Rat Plasma and Urine by LC–APCI–MS

theoretical concentration (μM)	recovery (%)[a]		matrix effect (%)[a]	
	plasma	urine	plasma	urine
0.5	98.8 ± 1.6 (n = 9)	95.6 ± 2.7 (n = 3)	108.8 ± 3.1 (n = 3)	110.6 ± 1.8 (n = 3)
1	96.8 ± 2.1 (n = 9)	96.7 ± 2.2 (n = 8)	124.7 ± 1.1 (n = 3)	112.0 ± 4.7 (n = 6)
5	100.2 ± 4.2 (n = 8)	98.7 ± 1.8 (n = 8)	116.1 ± 2.2 (n = 3)	111.5 ± 3.0 (n = 10)
10	100.4 ± 5.2 (n = 6)	99.4 ± 2.0 (n = 6)	126.7 ± 2.3 (n = 3)	109.7 ± 2.3 (n = 3)

[a]Values are expressed as means ± standard error of the mean (SEM).

Table 3. Precision and Accuracy of the Analytical Methods To Determine Maslinic Acid in Rat Plasma and Urine by LC–APCI–MS

theoretical concentration (μM)	intraday precision (%)[a]		interday precision (%)[a]		accuracy (%)[a]	
	plasma	urine	plasma	urine	plasma	urine
1	1.30 (n = 3)	5.62 (n = 3)	5.46 (n = 8)	7.59 (n = 12)	0.54 (n = 5)	−4.36 (n = 8)
5	2.64 (n = 3)	6.90 (n = 3)	5.93 (n = 9)	6.91 (n = 10)	2.34 (n = 3)	0.85 (n = 10)
10	3.30 (n = 3)	3.54 (n = 3)	6.42 (n = 9)	5.10 (n = 6)	0.94 (n = 4)	14.5 (n = 3)

[a]Values are expressed as means ± SEM.

at the four concentration levels assayed were 99.1 and 119.1%, respectively (Table 2). The intra- and interday precision and accuracy are summarized in Table 3, showing that, at all concentrations, the results were well below the 15% limit established by the EMA Guideline on Bioanalytical Method Validation.[14] Calibration curves were linear within the concentration range from 0.005 to 10 μM, and a representative curve is $y = 2.39x + 0.25$. Correlation coefficients were above 0.99. The sensitivity was suitable, because the LOD and the LLOQ were 2 and 5 nM, respectively.

Maslinic acid was the main compound in plasma samples withdrawn at 45 min after the oral administration of 50 mg/kg of body weight with a concentration of 4.83 ± 0.85 μM (Figure 3A). The identified metabolites were found in plasma at much lower concentrations than the parent compound. The most abundant derivative was the monohydroxylated and dehydrogenated metabolite M5 (m/z 485.3) at 0.77 ± 0.18 μM, followed by the monohydroxylated metabolite M1 (m/z 487.3) that reached the amount of 0.15 ± 0.03 μM. The other monohydroxylated metabolites were found at concentrations of 57.0 ± 12.7, 22.6 ± 3.8, and 34.2 ± 10.9 nM for M2, M3, and M4, respectively (Figure 3). Similarly, the dihydroxylated and dehydrogenated compounds were present in plasma in minor amounts, of 25.1 ± 4.1 nM for M7 and 10.3 ± 2.3 nM for M6 (Figure 3).

Quantification of Maslinic Acid Metabolites by LC–APCI–MS in Rat Urine. The analytical method for the determination of maslinic acid in urine was also validated to ensure a reliable quantification of both the parent compound and its metabolites. Recovery and matrix effect were analyzed at concentrations of 0.5, 1, 5, and 10 μM (Table 2). The extraction method was appropriate for the analysis of maslinic acid in urine, because it yielded recoveries between 95.6 and 99.4% (Table 2) and matrix effect values from 109.7 to 112%. Table 3 displays the intra- and interday precision and accuracy, which were assayed by spiking blank urine at three concentrations (1, 5, and 10 μM). The method was precise, as evidenced by relative standard deviations (RSDs) of intra- and interday assays lower than 6.90 and 7.59%, respectively, which fall below the 15% limit stipulated by the EMA Guideline on Bioanalytical Method Validation.[14] The accuracy of the

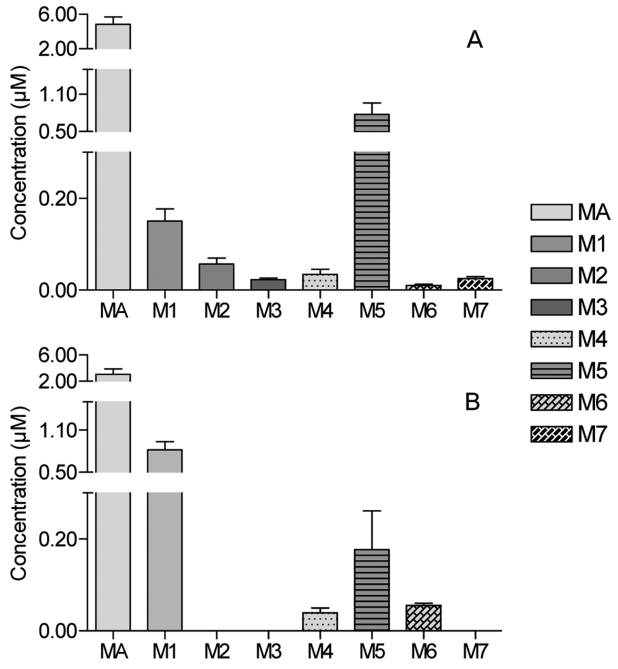

Figure 3. Concentrations of maslinic acid and its metabolites in (A) plasma and (B) urine collected at 45 min after the oral administration of 50 mg/kg of body weight of triterpene. Values are represented as means + SEM (n = 4–5).

method met the same criterion. Linearity was observed over the concentration range assayed (0.005–10 μM), with a representative calibration curve of $y = 0.58x + 0.05$ and correlation coefficients that surpassed 0.99. The LOD was 2 nM, and the LLOQ was 5 nM, meaning appropriate sensitivity.

Maslinic acid and its metabolites were quantified by LC–APCI–MS in urine samples obtained 45 min after the administration of triterpene (Figure 3B). As previously observed in plasma, maslinic acid was the major compound in urine (73.9%). However, the metabolite profile in this biological fluid differed from that found in plasma. The most abundant compound was the monohydroxylated derivative M1, representing 19.9%, whereas the monohydroxylated and dehydrogenated metabolite (M5) accounted for only 4.30%.

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The other monohydroxylated metabolite (M4) and the dihydroxylated and dehydrogenated compound M6 represented only 0.71 and 1.23%, respectively.

DISCUSSION

A large variety of drugs and bioactive compounds from the diet, such as maslinic acid, are considered as xenobiotics by the organism. Consequently, a detoxification process is activated, consisting of the increase of its hydrophilicity via metabolism to facilitate their excretion.[17] The biotransformation of xenobiotics can take place through phase I reactions on the basis of oxidation, reduction, and hydrolysis processes and phase II reactions, which comprise glucuronidation, sulfation, methylation, acetylation, and glutathione conjugation.[1,18] To devise a comprehensive strategy for the detection in plasma and urine of maslinic acid and its metabolites, the derivatives arising from both processes were considered, but only phase I metabolites were found.

The analysis by LTQ−Orbitrap allowed the identification of a monohydroxylated metabolite with four isomers in plasma samples (M1−M4) and two in urine (M1 and M4). The metabolism of pentacyclic triterpenes has been scarcely assessed with only an *in vivo* study for boswellic acids.[19] In agreement with our study, Krüger et al.[19] found in plasma two monohydroxylated derivatives after the oral administration of 12.5 mg/kg of boswellic acids to female albino Wistar rats. The fact that hydroxylation is the principal metabolic route of pentacyclic triterpenes has been confirmed by *in vitro* assays consisting of incubations of oleanolic acid[20] or boswellic acids[19,21] with rat and human liver microsomes. With regard to maslinic acid, the biotransformation of this pentacyclic triterpene has only been investigated *in vitro* with the use of fungi, such as *Cunninghamella*[22] and *Rhizomucor*.[23] These species express cytochrome P450 (CYP) enzymes that enable equivalent phase I reactions to mammalian metabolism, thus being widely used in the assessment of the biotransformation of drugs and xenobiotics.[24] The results obtained with these microbial models of mammalian drug metabolism are in concordance with our findings. Feng et al.[22] described that maslinic acid was transformed by *Cunninghamella blakesleana* into three different monohydroxylated derivatives (7β-hydroxy, 15α-hydroxy, and 13β-hydroxy with double bond migration) and one dihydroxylated metabolite (7β,15α-dihydro derivative). Moreover, the bioconversion of maslinic acid by *Rhizomucor miehei* also yielded a monohydroxylated metabolite, but in this case, the addition of the hydroxyl group took place at the angular methyl group on position 30.[23] Taking into account these published data and considering that, in our study, four isomers of the monohydroxylated maslinic acid were detected in plasma, it could be suggested that the hydroxyl groups were added to the positions 7, 13, 15, and 30.

Maslinic acid underwent a biotransformation that yielded M5, which was detected in both plasma and urine. The mass shift of +14 Da exhibited by this metabolite was compatible with monohydroxylation, followed by dehydrogenation, which could be due to the oxidation of the corresponding secondary alcohol to a ketone. The pentacyclic ring skeleton of maslinic acid has two alcohols at the 2 and 3 positions, respectively, which are likely to be converted to a ketone. Up to now, this modification of maslinic acid has not been reported. However, this reaction has been described *in vitro* for boswellic acids after incubation with rat liver microsomes[19] and also for betulinic acid after incubations with different microbial models of drug metabolism.[25,26] Additionally, metabolites M6 and M7 showed a mass shift of +30 Da, which corresponded to the addition of two hydroxyl groups plus the conversion of a secondary alcohol to a ketone. This result is consistent with the biotransformation observed for boswellic acids[19] as well as ursolic acid after the incubation with the fungus *Pestalotiopsis microspora*.[27] Under our experimental conditions, no metabolites resulting solely from oxidation (−2 Da) or dihydroxylation (+32 Da) reactions were found in either plasma or urine.

Once the metabolites of maslinic acid were identified by LTQ−Orbitrap, their plasmatic concentrations were quantified to know the degree of the biotransformation of this compound by LC−APCI−MS. After the administration of 50 mg/kg of body weight of maslinic acid, this bioactive molecule was the prevalent compound in plasma and urine, where it accounted for 81.8 and 73.9%, respectively. Therefore, although the results indicate that maslinic acid undergoes phase I metabolism, yielding seven derivatives in plasma and four in urine, their contribution to the elimination of this pentacyclic triterpene is not preeminent, because the parent compound is excreted in urine mainly unaltered. These findings are consistent with our previous assessment of the pharmacokinetics of maslinic acid that indicated a low clearance that was attributed to minor hepatic and renal metabolism along with unchanged renal excretion.[10] Moreover, the oral bioavailability of 5.13% observed cannot be mainly attributed to metabolism. At present, experiments on the intestinal absorption of this compound are being conducted in our laboratory, and the results suggest poor absorption from the gastrointestinal tract (data not shown).

In summary, the present study thoroughly characterizes for the first time the metabolite profile of maslinic acid in rat plasma and urine. Although the oral bioavailability of similar pentacyclic triterpenes has been reported and the derivatives of some of them have been identified after incubation with different microorganisms[19,25−27] or rat and human microsomes,[19,20] none of these studies quantified the metabolites in biological fluids. Our results reveal that maslinic acid undergoes mainly phase I metabolism through hydroxylation and oxidation reactions but is not subjected to phase II biotransformations. A total of seven metabolites were identified in plasma, four monohydroxylated derivatives (M1−M4), one monohydroxylated and dehydrogenated metabolite (M5), and two dihydroxylated and dehydrogenated derivatives (M6 and M7). Only M1, M4, M5, and M6 were detected in urine. This study provides comprehensive insight into the metabolite profile of maslinic acid, thus increasing our knowledge about the bioavailability of this bioactive food component.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

LC−MS, liquid chromatography−mass spectrometry; LOD, limit of detection; LLOQ, lowest limit of quantification; RSD, relative standard deviation

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