# Assessment of a Maslinic Acid Derivative and its Metabolite in Rat Blood by Liquid Chromatography Coupled with Mass Spectrometry

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*A new rapid high-throughput liquid chromatography coupled with tandem mass spectrometry method (LC-MS/MS) was developed for the blood assessment of a maslinic acid derivative (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide) and its metabolite. Separation was performed using a Zorbax SB-C18 column with a mobile phase composition of methanol and 0.2% formic acid in water (78:22, v/v) in isocratic flow, with a flow rate of 0.6 mL/min. Detection was performed by multiple reaction monitoring (MRM) mode, monitoring the transitions 338.3; 526.6; 544.6; 527.5; 545.5 m/z derived from 646.6 m/z for the maslinic acid derivative and 409; 338.3; 203; 205; 526.5 m/z derived from 562.6 m/z for its metabolite, using atmospheric pressure chemical positive ionisation source. Single-step protein precipitation with methanol was used for preparation of rat blood samples. The method was validated with respect to selectivity, linearity (r > 0.9937), within-run and between-run precision (CV < 12.0%) and accuracy (bias < 7.9%) over the ranges of 20.5 - 656 ng/mL blood for maslinic acid derivative, and 20 - 640 ng/mL blood for maslinic acid derivative metabolite. The analytical method is simple, fast, selective and is suitable for bioanalysis of maslinic acid derivative and its metabolite from rat blood.*

*Keywords: LC/MS analysis, maslinic acid derivative, metabolite, rat blood*

Maslinic acid is a natural compound belonging to the class of pentacyclic triterpenes [1, 2]. It possesses various pharmacological activities: antitumor, anti-inflammatory, antioxidant, antiparasitic, antiviral and antidiabetic [1]. Lozano-Mena *et al.* reported maslinic acid's anticancer activity on various tumor cell lines: HT-29 and Caco-2 (human colorectal adenocarcinoma), HepG2 (Human hepatocellular carcinoma), MCF-7 (Human breast adenocarcinoma) etc [1].

Several maslinic acid derivatives were obtained from olives (*Olea europaea* L.) and tested for their antitumor activity [3-5]. Siewert *et al.* established that some of them exhibited high antitumor activity against various tumor cell lines [3]. Among them, a benzylamide derivative of maslinic acid (*EM2*) (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide) elicited high antitumor activity, in the same time beeing less toxic to primary human fibroblasts, making it suitable for further studies [3].

Our previous studies revealed that the maslinic acid derivative (*EM2*) showed antimicrobial activity against *Streptococcus pyogenes* and *Staphylococcus aureus* and cytotoxic properties on two melanoma cell lines (B164A5 murine melanoma and A375 human melanoma cell line) [6].

Sanchez-Gonzalez *et al.* performed a pharmacokinetic study in order to assess the bioavailability of maslinic acid after i.v. and oral administration (1 and 50 mg/kg) in Sprague-Dawley rats; interestingly, they reported a rapid absorption for maslinic acid (peak concentration at 0.51 h) after oral administration [7].

Determination of maslinic acid has been previously reported by 2 groups that used high-performance liquid chromatography (HPLC) [8] and, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) [9, 10], respectively.

The present study was aimed, to assess the blood level of a benzylamide derivative of maslinic acid (fig.1) and its metabolite (fig.2) by high-throughput liquid chromatography coupled with tandem mass spectrometry method (LC-MS/MS) after oral and intraperitoneal administration, respectively.

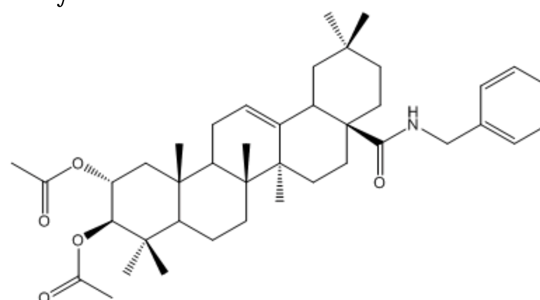


Fig. 1. Chemical structure of maslinic acid derivative

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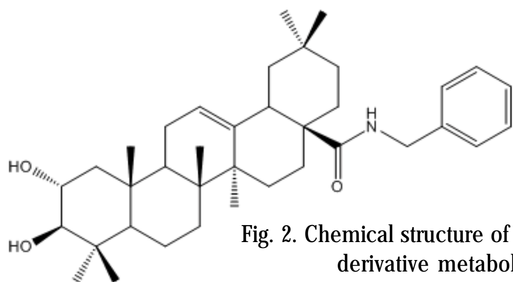


Fig. 2. Chemical structure of maslinic acid derivative metabolite.

## Experimental part

### *Materials and methods*

Chemicals and reagents

The maslinic acid derivative with a cytostatic activity comparable to maslinic acid (being more toxic to cancer cells than to primary human fibroblasts) was a kind gift from Prof. Rene Csuk [3].

Reference standards for maslinic acid derivative and its metabolite (laboratory synthesis) [3] were used for standard solution preparation. High purity methanol of gradient grade for liquid chromatography and analysis reagent formic acid used were manufactured by Merck KGaA (Darmstadt, Germany). Bidistilled deionized water was also used for mobile phase preparation.

Equipment

An Agilent 1100 Series (Agilent, USA) chromatography system (binary pump, online degasser, autosampler, thermostat) coupled with Agilent Ion Trap detector was used. Other equipment used: Sigma (Osterode am Harz, Germany) 204 series centrifuge; Mettler-Toledo (Greifensee, Switzerland) Analytical Plus and Precision Standard series analytical balances; Scientific Industries (Bohemia NY, USA) Vortex Genie 2 vortex mixer; Biohit (Helsinki, Finland) Proline series automatic pipettes.

Liquid chromatography tandem mass spectrometry conditions

Analytical column used for chromatographic separation was an Agilent Zorbax SB-C18 100 mm x 3.0 mm I.D kept at 45°C. Mobile phase composition was 78:22 mixture of methanol and 0.2% (v/v) formic acid in water in isocratic flow with a flow rate of 0.6 mL/min. Injection volume was set at 1 μL. Detection was performed by multiple reaction monitoring (MRM) mode, monitoring the transitions 338.3; 526.6; 544.6; 527.5; 545.5 m/z derived from 646.6 m/z for the maslinic acid derivative and 409; 338.3; 203; 205; 526.5 m/z derived from 562.6 m/z for its metabolite, using atmospheric pressure chemical positive ionisation source. The total analysis time was of 4.5 min for each sample.

Standard solutions

Stock solutions of 1025 μg/mL concentration of maslinic acid derivative in methanol and 800 μg/mL concentration of the metabolite in methanol were prepared. The stock solutions were further diluted with methanol to obtain working solutions with a concentration of 2050 ng/mL for maslinic acid derivative and 2000 ng/mL for its metabolite, respectively. The working solutions were then used to prepare calibration standards with concentrations of 20.5 (lower limit of quantification), 41, 82, 164, 246, 328, 492 and 656 ng/mL, and quality control (QC) samples with concentrations of 82 ng/mL (lower), 246 ng/mL (medium) and 492 ng/mL (higher) for maslinic acid derivative and calibration standards with concentrations of 20 ng/mL (lower limit of quantification), 40, 80, 160, 240, 320, 480 and 640 ng/mL, and quality control (QC) samples with concentrations of 80 ng/mL (lower), 240 ng/mL (medium) and 480 ng/mL (higher) for maslinic acid derivative metabolite.

Sample preparation

Blood samples (100 μL) were deproteinized with methanol (300μL), mixed (10 seconds) and centrifuged (for 5 min at 12000 rpm). 100μL of supernatant were transferred to chromatographic vials and inserted into the auto-sampler. Volumes of 1 αL of sample were injected into the LC/MS.

Method validation

The analytical method was fully validated with regards to selectivity, linearity, within-run and between-run precision and accuracy for both maslinic acid derivative and its metabolite respectively. The evaluation of specificity of the method was carried out by analyzing six sources of blank matrix for interference against a sample spiked at the lower limit of quantification. Absence of interfering components was be accepted as the response in blank samples were less than 20% of the lower limit of quantification for the analyte.

Concentrations of samples were calculated by the instrument data system using external standard method. Calibration curves were linear and constructed from single calibration standards, using the equation $y=ax+b$, the weighting factor was $1/y^2$, where **y** is the area of the analyte, **x** is the analyte concentration, **a** is the slope of the curve, **b** is the intercept. Calibration curves were considered acceptable if the coefficient of determination was higher than 0.98 and accuracy for backcalculated concentrations did not exceed ±20% for the lower limit of quantification and ±15% for the other calibration standards. At least 6 out of 8 calibration standards needed to fulfil this criterion for every calibration curve, including the upper and lower limit of quantification.

Five replicate samples (n=5) of each quality control sample (lower, medium and higher) were injected and analysed for both maslinic acid derivative and its metabolite in the same run to determine within run accuracy and precision. Five samples of each quality control sample (lower, medium and higher) were injected and analysed for both maslinic acid derivative and its metabolite in different runs to establish between run accuracy and precision. Precision was expressed as coefficient of variation, CV %, while accuracy was expressed as relative difference between obtained and theoretical concentration, bias %. Accuracy and precision are acceptable if their values do not exceed ±15%.

The lower limit of quantification is the lowest point on the calibration curve where accuracy and precision have acceptable values. Both within and between run accuracy and precision were studied and calculated at the lower limit of quantification. Accuracy and precision are considered acceptable if their values do not exceed ±20%.

Method application

The maslinic acid derivative (50 mg/kg of body weight) was given via the oral and intraperitoneal route [3, 7]. Blood was automatically collected using the Culex System [BASi Corporate, Indiana, USA]. Maslinic acid derivative was dissolved in a mixture of ethanol and saline solution in a 3:1 ratio for oral administration and in dimethylsulfoxide (DMSO) for intraperitoneal administration.

After oral administration, blood samples were collected at 15 min, 30 min, 1h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h, 48 h and 72 h post-administration, respectively, after intraperitoneal administration, blood samples were

collected at 15, 45 min, 1 h, 1,5 h, 2 h, 3 h, 5 h, 7 h, 9 h, 12 h, 18 h, 24 h, 48 and 72 h post-administration.

Animals were fed *ad libitum* and kept under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). All experimental procedures were approved by the Institutional Animal Ethical Committee of the Iuliu Hatieganu University of Medicine and Pharmacy Cluj-Napoca.

## Results and discussions

*The analytical method*

The analytical method which was developed for the simultaneous analysis of the maslinic acid derivative and the maslinic acid derivative metabolite showed good specificity due to the tandem mass spectrometry (MS/MS) detection method. The analysis of blank samples against samples spiked at the lower limit of quantification showed good selectivity as no interfering endogenous peaks in blank rat blood at the retention times of maslinic acid derivative (3.8 min) (fig. 3) and maslinic acid derivative metabolite (1.45 min) (fig. 4).

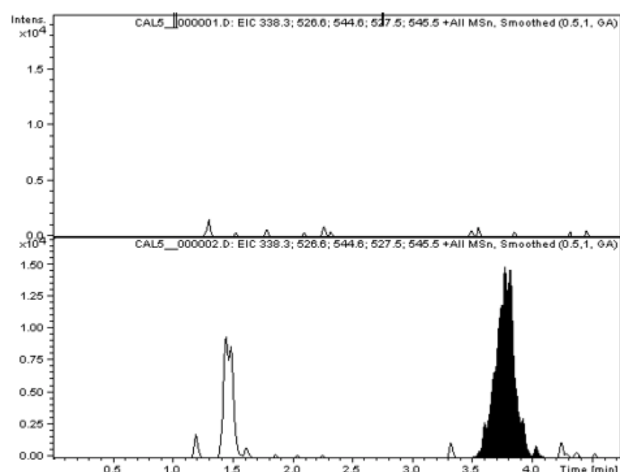


Fig. 3. Blank sample and LLOQ calibration standard sample of maslinic acid derivative

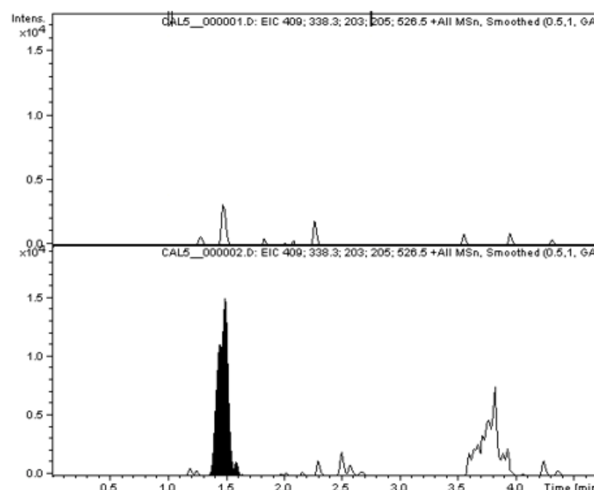


Fig. 4. Blank sample and LLOQ calibration standard sample of maslinic acid derivative metabolite

Calibration curves were linear having correlation coefficients higher than 0.99 throughout the whole range of concentrations studied for both analytes (table 1 and table 2). Accuracy of backcalculated concentrations for calibration standards were calculated for both maslinic acid derivative (table 1) and maslinic acid derivative metabolite (table 2) and were within the acceptance criteria. Backcalculated concentrations show that the

**Table 1**
ACCURACY OF BACKCALCULATED CONCENTRATIONS FOR CALIBRATION STANDARDS OF MASLINIC ACID DERIVATIVE

| | Accuracy % | | | | |
|---|---|---|---|---|---|
| Nominal concentration (ng/ml) | Batch 1 | Batch 2 | Batch 3 | Batch 4 | Batch 5 |
| 20.50 | 2.3 | 1.9 | 10.4 | 12.6 | 5.8 |
| 41.00 | -4.8 | -3.0 | -13.8 | -13.2 | -10.4 |
| 82.00 | 1.4 | -1.8 | 1.3 | -3.5 | 4.2 |
| 164.00 | -3.0 | -2.6 | -2.8 | -8.7 | -1.9 |
| 246.00 | 1.5 | -0.9 | 3.8 | 6.2 | 0.1 |
| 328.00 | 0.6 | -2.3 | -2.0 | 3.9 | -1.7 |
| 492.00 | 2.5 | 4.1 | 4.5 | 4.9 | 6.4 |
| 656.00 | 0.6 | 6.2 | 7.3 | 9.9 | 2.3 |
| Parameters of calibration curve | | | | | |
| Gradient | 6951.00 | 6844.00 | 6862.00 | 8428.00 | 7399.00 |
| Intercept | -14524.00 | -23973.00 | -6524.00 | 942.50 | -15131.00 |
| R | 0.9997 | 0.9973 | 0.9964 | 0.9937 | 0.9983 |

| Nominal Concentration (ng/ml) | Accuracy % | | | | |
|---|---|---|---|---|---|
| | Batch 1 | Batch 2 | Batch 3 | Batch 4 | Batch 5 |
| 20.00 | -1.5 | 2.2 | 2.2 | 14.5 | 15.1 |
| 40.00 | 13.4 | -7.4 | -0.9 | -14.5 | 3.0 |
| 80.00 | -10.2 | 10.0 | -1.3 | -6.8 | -1.2 |
| 160.00 | -4.2 | -0.4 | -11.4 | 0.5 | 1.0 |
| 240.00 | 3.5 | 7.2 | 9.5 | 7.2 | -0.8 |
| 320.00 | -3.7 | -7.5 | -2.5 | 1.9 | -7.3 |
| 480.00 | 4.4 | 2.4 | 3.2 | 10.8 | 7.0 |
| 640.00 | 6.2 | -0.8 | 7.1 | -0.9 | -2.1 |
| Parameters of calibration curve | | | | | |
| Gradient | 4818.00 | 4946.00 | 4094.00 | 5827.00 | 5201.00 |
| Intercept | -19634.00 | -16136.00 | 12165.00 | -3550.00 | -20621.00 |
| R | 0.9970 | 0.9984 | 0.9958 | 0.9958 | 0.9973 |

**Table 2**
ACCURACY OF BACK CALCULATED CONCENTRATIONS FOR CALIBRATION STANDARDS OF MASLINIC ACID DERIVATIVE METABOLITE

| Nominal concentration ng/ml | Measured concentration ng/ml (± SD) | | Precision % | Accuracy % |
|---|---|---|---|---|
| 20.50 | 22.12 | 2.65 | 12.0 | 7.9 |
| 82.00 | 83.82 | 6.45 | 7.7 | 2.2 |
| 246.00 | 252.58 | 1.20 | 0.5 | 2.7 |
| 492.00 | 498.55 | 24.75 | 5.0 | 1.3 |

**Table 3**
WITHIN RUN ACCURACY AND PRECISION DATA FOR MASLINIC ACID DERIVATIVE

| Nominal concentration ng/ml | Measured concentration ng/ml (± SD) | | Precision % | Accuracy % |
|---|---|---|---|---|
| 20.50 | 21.04 | 1.67 | 7.9 | 2.7 |
| 82.00 | 84.60 | 5.46 | 6.4 | 3.2 |
| 246.00 | 250.47 | 7.35 | 2.9 | 1.8 |
| 492.00 | 508.82 | 20.76 | 4.1 | 3.4 |

**Table 4**
BETWEEN RUN ACCURACY AND PRECISION DATA FOR MASLINIC ACID DERIVATIVE

residuals do not have a specific tendency depending on concentration for none of the analytes.

Within run and between run precision and accuracy during method validation was acceptable for both analytes at all concentrations studied, all results obtained were within the acceptance criteria. Results are shown in table 3-6.

Blood samples collected from rats were analyzed using the validated analytical method and maslinic acid derivative and maslinic acid derivative metabolite blood

**Table 5**
WITHIN RUN ACCURACY AND PRECISION DATA FOR MASLINIC ACID DERIVATIVE METABOLITE

| Nominal concentration ng/ml | Measured concentration ng/ml (± SD) | | Precision % | Accuracy % |
|---|---|---|---|---|
| 20.00 | 21.49 | 1.57 | 7.3 | 7.4 |
| 80.00 | 80.09 | 2.40 | 3.0 | 0.1 |
| 240.00 | 242.00 | 5.42 | 2.2 | 0.8 |
| 480.00 | 504.80 | 17.16 | 3.4 | 5.2 |

**Table 6**
BETWEEN RUN ACCURACY AND PRECISION DATA FOR MASLINIC ACID DERIVATIVE METABOLITE

| Nominal concentration ng/ml | Measured concentration ng/ml (± SD) | | Precision % | Accuracy % |
|---|---|---|---|---|
| 20.00 | 20.96 | 1.52 | 7.3 | 4.8 |
| 80.00 | 78.92 | 5.41 | 6.9 | -1.4 |
| 240.00 | 242.92 | 16.91 | 7.0 | 1.2 |
| 480.00 | 491.35 | 21.39 | 4.4 | 2.4 |

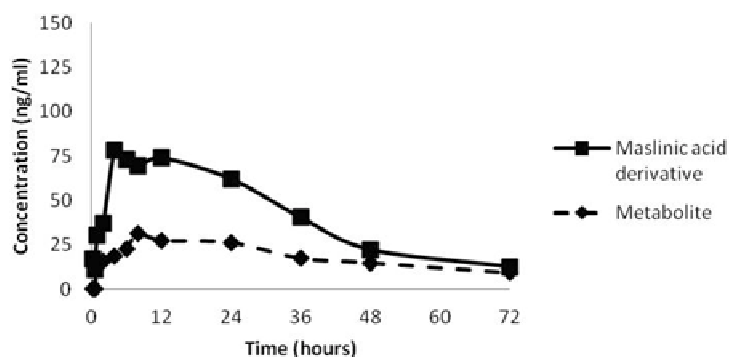


Fig. 5. Typical blood profiles of maslinic acid derivative and its metabolite after oral administration in rat

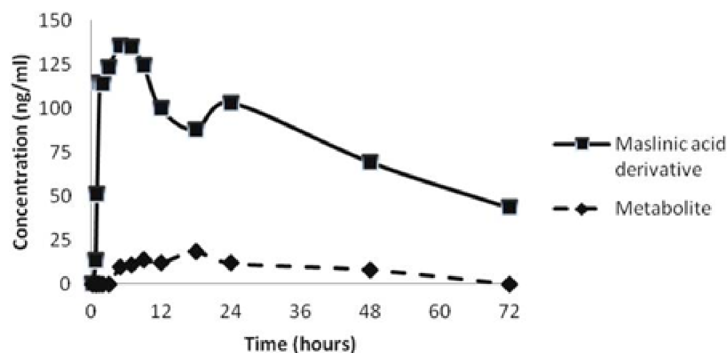


Fig. 6. Typical blood profiles of maslinic acid derivative and its metabolite after intraperitoneal administration in rat

levels were determined. Tipical blood levels of maslinic acid derivative and its metabolite after oral and intraperitoneal administration in rats are shown in figure 5 and figure 6, respectively.

Our results showed that maslinic acid derivative had a maximal concentration at 4 h after oral administration, and at 5 h after i.p. administration, respectively. We also obtained higher concentration in rat blood after i.p. administration than after oral administration. In rat blood, the analyzed derivative has a superior bioavailability as compared to maslinic acid [7-10].

The group of Joana Planas performed a pharmacokinetic study and reported that maslinic acid has a rapid absorption

(maximal concentration at 0.51 h) after oral administration (50 mg/kg) and is widely distributed into tissues after i.v. administration (1 mg/kg) [7]. When given orally in the high dose (50 mg/kg), maslinic acid was detected in plasma by HPLC 10 minutes after its administration, and was still found 60 min later [8]. Sanchez-Gonzales *et al.* identified maslinic acid in rats plasma by using the LC-APCI-MS method [9]. Different doses of the compound were given orally (10, 25 and 50 mg/kg). Maslinic acid at all three doses was identified in rats plasma, 24 h after administration. The paper does not indicate when the compound reaches the maximal concentration in rat plasma [9].

## Conclusions

We presented here an analytical method allowing the analysis of maslinic acid derivative and its metabolite in blood after oral and intraperitoneal administration in rats. The LC-MS/MS method developed and validated in this paper has a short runtime, a simple and inexpensive sample preparation technique, and is robust, all important features for high-throughput methods used in routine bioavailability studies. The compound showed a lower bioavailability after oral *vs.* intraperitoneal administration.

Further studies are required to assess the pharmacokinetics and pharmacodynamics of this compound in the setting of experimental models of chronic, non-communicable diseases, including cancer.

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