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Development and validation of a GC-MS method for determination of metformin in normal brain and in glioblastoma tissues

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ABSTRACT

Metformin hydrochloride (MH) has recently been repurposed as an anticancer agent, showing antiproliferative activity in vitro and in vivo. In particular, experimental evidence has suggested its potential clinical efficacy in glioblastoma (GBM), a very aggressive tumor frequently characterized by gloomy prognosis. Unfortunately, the published literature concerning experimental applications of MH in glioblastoma animal models report no data on metformin levels reached in the brain, which, considering the high hydrophilicity of the drug, are likely very low. Therefore, new sensitive analytical methods to be applied on biological tissues are necessary to improve our knowledge of MH in vivo biodistribution and biological effects on tumors. In this research work, a GC-MS method for MH quantification in brain tissues is proposed. MH has been derivatized using *N*-methyl-bis(trifluoroacetamide), as already described in the literature, but the derivatization conditions have been optimized; moreover, deuterated MH has been selected as the best internal standard, after a comparative evaluation including other internal standards employed in published methods. After ascertaining method linearity, its accuracy, precision, specificity, repeatability, LOD and LOQ (0.373 μM and 1.242 μM, respectively, corresponding to 0.887 and 2.958 pmol/mg of wet tissue) have been evaluated on mouse brain tissue samples, obtained through a straightforward preparation procedure involving methanolic extraction from lyophilized brain homogenates and solid phase purification. The method has been validated on brain samples obtained from mice, either healthy or xenografted with GBM cells, receiving metformin dissolved in the drinking water. This analytical method can be usefully applied in preclinical studies aiming at clarifying MH mechanism of action in brain tumors.

1. Introduction

Metformin hydrochloride (*N,N*-dimethylimidodicarbonimidic diamide hydrochloride, MH), a biguanide derivative used as a first-line drug in type-2 diabetes, has recently been investigated as a possible antitumor drug in different types of cancer [1–4], showing a time-dependent antiproliferative activity in cancer stem cells, which is independent of the hypoglycemic effect of the drug [5]. Several possible anticancer mechanisms of MH have been proposed, involving the activation of an AMPK/LKB1 pathway causing a stress response affecting tumor cells survival [6,7], the inhibition of the pro-survival kinase Akt [8], and the blockade of CLIC1, a chloride channel whose activity is related to the tumorigenicity of cancer stem cells [9].

Glioblastoma (GBM) is the most aggressive brain tumor [10], characterized by a very poor prognosis even after radical therapeutic approaches. The current gold standard treatment for GBM patients includes maximal tumor surgical resection followed by radiotherapy with concomitant treatment with the alkylating drug temozolomide, and then six cycles of temozolomide alone [11]. In spite of this aggressive protocol, patients' survival extension does not reach two years, as

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median value; therefore, there is urgent need for more effective therapies.

MH has proved to interfere with GBM stem cell survival, proliferation and invasiveness in several preclinical models [12,13] and has been proposed in combination with other drugs for the treatment of GBM. For example, a synergy with temozolomide on AMPK activation has been hypothesized [14], and in vitro [15], in vivo [16] and clinical studies [17] are ongoing. Unfortunately, MH presents a poor pharmacokinetic profile and, being highly polar, is subjected to complex distribution in the target tissues; therefore, new analytical methods to quantify it in the brain tissue are needed.

Although HPLC has been used for MH quantification in biological tissues [18,19], low sensitivity is often a limiting factor of this approach; hence, several more sensitive GC-MS methods have been proposed. Uçaktürk [20] developed and validated a GC-MS method for MH detection in human plasma, selecting diphenylamine as internal standard (IS), and derivatizing MH with *N*-methyl-bis(trifluoroacetamide) (MBTFA): interestingly, the author investigated the kinetics of the derivatization reaction, and the study yielded optimal reaction conditions of 80 °C and 60 min. In another work [21], a GC-MS method was applied to determine MH content in surface water: in this study, buformin hydrochloride was chosen as IS because, being structurally analogous to metformin, it has quite the same chemical behavior and, like MH, can be derivatized using MBTFA. Following this work, Tao et al. [22] carried out an optimization study to improve MH derivatization reaction and its biotransformation product guanylurea with MBTFA, with the aim of detecting both analytes in aqueous samples. In a recent paper, Arbouche et al. [23] described a GC-MS method for MH quantification in human hair specimens: the authors used deuterated MH ([$^2H_6$]-metformin hydrochloride, $^2H_6$-MH) as IS, and the derivatization reaction was performed using MBTFA under the same conditions set as optimal by Uçaktürk [20].

The aim of the present work was to develop a new GC-MS procedure for MH quantification in mouse brain tissue, also affected by GBM, streamlining the sample preparation set up and studying different chromatographic conditions to obtain an adequately specific, sensitive, and robust method.

2. Materials and methods

2.1. Chemicals

Metformin hydrochloride was purchased from Metapharmaceutical (Barcelona, Spain); [$^2H_6$]-metformin hydrochloride (100%) was purchased from Alsachim (Illkirch-Graffenstaden, France); *N*-methyl-bis (trifluoroacetamide) (MBTFA for GC derivatization, ≥99%) was supplied by Sigma-Aldrich (St. Louis, MO, US); ammonium acetate was purchased from VWR Chemicals (Radnor, Pennsylvania, US); formic acid 98–100% was purchased from Merck (Burlington, Massachusetts, US). Gradient grade methanol (VWR Chemicals, Radnor, Pennsylvania, US) was used for the preparation of all standard and working solutions, and for the extraction procedures.

2.2. Preparation of standard solutions

A MH stock standard solution (0.48 mM) was prepared by dissolving an accurately weighed amount of MH in methanol. Working standard solutions were obtained by diluting the stock standard solution with methanol to different concentrations for preparing the calibration curve or for spiking the brain tissue for recovery studies. For the IS stock standard solution, 2.0 mg of $^2H_6$-MH were dissolved in 5.0 mL of methanol; the IS working standard solution was prepared by diluting the IS standard solution to 5.83 μM.

2.3. Instrumental conditions

GC-MS analyses were performed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a Hewlett Packard 5971 A mass selective detector. A 25 m × 0.2 mm × 0.3 μm cross-linked methyl silicone gum column (Part number 19091Z-102, Agilent Technologies, US) was used, with helium as the carrier gas; the column was equipped with a 1000 × 0.25 mm deactivated retention gap (Part number 160–2255–1, Agilent Technologies, US). The analysis was performed by thermal gradient: the initial oven temperature of 120 °C was increased to 160 °C at a rate of 10 °C/min, ramped to 175 °C at 15 °C/min, and finally to 220 °C at a rate of 20 °C/min; the solvent delay was set at 4 min. The analyses were performed in selected ion monitoring (SIM) mode using a cool on-column injector at constant flow (0.5 mL/min) and with oven tracking; the inlet initial pressure was 0.823 bar, while the inlet initial temperature was 120 °C. The transfer line and detector temperature were 280 °C and 185 °C, respectively. The $m/z = 303$ (derivatized MH, dMH) and $m/z = 309$ (derivatized IS, dIS) ions were monitored; moreover, $m/z = 288$ and $m/z = 125$ were used as qualifying ions for dMH, and $m/z = 291$ and $m/z = 128$ were used as qualifying ions for dIS. The formation of MH and IS monoacetyl derivatives was evaluated by acquisition of $m/z = 207$ and $m/z = 213$ ions, respectively. To maximize sensitivity, the SIM acquisition was carried out by setting two m/z windows: the first one, starting from 4.0 min (after the solvent delay) and ending at 5.1 min, monitored ions with $m/z = 303$ and 309 plus the qualifying ions; the second one, starting at 5.1 min, monitored ions with $m/z = 207$ and 213. The injected volume was about 1 μL, and the samples were stored at − 20 °C until injection time.

2.4. Animals and brain tissue sampling

All experiments respected ARRIVE guidelines and the EU Council Directive 2010/63/EU, and were approved by the Italian Ministry of Health (authorization #981/2020-PR). Adult (age > postnatal day 60) wild type C57BL/6 J mice were bred in the animal facility of the National Research Council (CNR) (Pisa, Italy) and housed in a 12-hour light/dark cycle, with food and water available ad libitum. The murine glioma GL261 cells were grown as previously described in [24]. To induce the formation of glioma, mice under a cocktail of ketamine and xylazine (100:10 mg/kg i.p.) received a stereotaxically-guided injection of 40000 GL261 syngeneic GBM cells (20,000 cells/μL PBS solution) into the motor cortex (coordinates from bregma: 1.75 mm lateral to the midline, 0.5 anterior) with a Hamilton syringe guided by an automatized pump (Kd Scientific, US) at a depth of 0.9 mm from the pial surface. MH (10 mg/mL, 1.5 mg/kg/die) was administered in the drinking water starting from day 8 and until day 15 after tumor injection. Concentration and volume were set on the basis of previous pharmacological studies [25] where an average of 4–7 mL/die of water consumption by single animal was estimated. At the end of each day of treatment, the water of both treated and control groups was measured to confirm the administration of the drug and verify that the amount of water assumed by the two groups was similar. At the end of the treatment, all animals were sacrificed with cervical dislocation to avoid administration of other drugs, and brain tissues were rapidly collected and frozen.

2.5. Sample preparation

Frozen mouse brain samples (ranging from 140 to 220 mg) were homogenized in ultrapure deionized water using a glass homogenizer fitted with a Teflon pestle, and then lyophilized using a lyophilizer (Labconco, US) at − 35 °C and residual pressure lower than 20×10^{-3} mbar for 48 h, followed by a secondary drying phase at 20 °C for 1 h. The lyophilized brain tissue was extracted with 2 mL of methanol. The methanolic suspension was vortexed for 60 s and sonicated for 10 min, and then centrifuged for 30 min at 900 rpm at 20 °C. The extraction procedure was repeated, and the collected supernatants were mixed.

Blank samples, to evaluate interference or method specificity, were obtained from healthy mice that did not receive MH; in the treatment of these samples, IS addition was omitted.

The extractive sample solutions, as well as accurately measured volumes of MH working standard solutions for calibration curve, were dried under nitrogen flow at 70 °C. The residues were solubilized in 0.25 mL of aqueous ammonium acetate buffer (pH 7.5; 100 mM) and underwent solid phase extraction (SPE) purification. A weak cation exchanger (Strata-X-CW, 1 mL, 30 mg sorbent mass, Phenomenex, Aschaffenburg, Germany) was washed with 0.5 mL of methanol and conditioned with 2 × 1 mL of ammonium acetate buffer. After sample loading, the cartridges were washed with 0.5 mL of ammonium acetate buffer and 2 × 1 mL of methanol, then the analytes were eluted directly in silanized derivatization conical vials (Supelco, Bellefonte, PA, US), using 3 × 1 mL of methanol-formic acid (95:5, v/v) mixture. IS working standard solution (20 μL) was added to the eluate, then the mixture was slowly dried at 70 °C under nitrogen flow, and the dried residue was derivatized in the vials, capped with a Mininert® valve (Supelco, Bellefonte, PA, US), using 15 μL of MBTFA and 45 μL of acetonitrile and heating at 70 °C for 30 min. After derivatization, the solutions were stored at − 20 °C for at least 20 min. Before the first injection, 165 μL of acetonitrile were added to each vial and the mixture was vortexed. After each injection, the derivatized solutions were stored at 4 °C.

2.6. Method validation

2.6.1. Linearity study and calibration curve

For the study of MH response linearity, different concentrations of MH ranging from 0.24 to 4.12 μM in acetate buffer were used. A standard curve was obtained by plotting the ratio of peak areas corresponding to $m/z = 303$ (dMH) and $m/z = 309$ (dIS) ions against the concentration of MH used. Ordinary least squares regression was performed. The confidence interval for ordinary least squares regression was calculated applying the equation reported in [26].

2.6.2. Specificity

To prove method specificity, blank samples were processed following the sample preparation procedure described above omitting IS addition. The obtained derivatized mixtures were analyzed through SIM procedure to detect the presence of any peak with $m/z = 303$ or 309 at the typical retention times of dMH and dIS under these chromatographic conditions.

2.6.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined as the MH levels whose SIM chromatographic dMH peaks reach at least 3 or 10 times, respectively, the signal noise of the baseline. LOD and LOQ were obtained by adding increasing amounts of MH to blank samples from healthy mice and identifying the first detectable and quantifiable concentrations.

2.6.4. Accuracy and precision

Accuracy and precision were evaluated in accordance with ICH guideline M10 on bioanalytical method validation and study sample analysis as adopted by EMA (EMA/CHMP/ICH/172948/2019). Quality control (QC) samples were prepared from separate stock solutions at a minimum of 4 concentration levels within the calibration curve range: the LLOQ (about 0.24 μM), low QC sample (about 1.04 μM), medium QC (about 2.52 μM) and high QC (about 4.12 μM). At least 5 replicates at each QC concentration level were analyzed. The acceptance criteria for accuracy at each concentration level was set within ± 15% of the nominal concentration, except at the LLOQ, where it was set within ± 20%. The precision (CV%) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

2.6.5. Recovery, intra-day and inter-day repeatability

The recovery study was planned in accordance with 3 × 3 randomized Latin Square at three levels in untreated healthy mice brain samples of about 126.5 ± 12.7 mg, which were spiked with 0.30, 0.60 or 0.91 nmol of MH; Table 1 shows the Latin Square where the rows report the three days of analysis (day 1, 10 and 30) and the columns report the daily run order of analyzed samples. Latin Square design assumes that the effects of the factors considered for recovery robustness evaluation are additive and do not interact.

2.6.6. Matrix effect

The matrix effect evaluation was carried out at two different MH concentrations (1.20 and 3.64 μM), by spiking 3 eluates from healthy mice blank brain samples for each concentration level, with 0.30 and 0.91 nmol of MH respectively. The resulting solutions, after addition of the IS, were derivatized and analyzed, and the peak area ratios were compared to the ones obtained from the analysis of methanol/formic acid solutions (95:5 v/v) containing the same amounts of MH and IS and equally derivatized.

3. Results and discussion

The GC-MS approach for identification and quantification of trace amounts of small molecule drugs in biological tissues with good selectivity and high sensitivity represents an important tool, especially in studies involving small animals and low drug levels in tissues [27]. The repositioning of MH as a possible antitumor drug for the treatment of GBM, and the necessity of detecting and dosing MH in the brain of mice orally treated with MH, prompted us to the development of a GC-MS SIM method.

First, a GC-MS method for quantifying low amounts of analytes in biological samples requires an appropriate choice of the internal standard, and in previously published works regarding MH either diphenylamine [20] or buformin hydrochloride [21] have been used. In the first stages of method development, we investigated the possibility of using these molecules as internal standards; however, diphenylamine is not the best choice because of the dissimilarities in chemical structure from MH, determining differences in the chemical and chromatographic behavior, for example excessively different retention time with respect to the main analyte. The use of buformin hydrochloride as internal standard also failed, since the product of buformin derivatization was thermally labile. Stable isotopically labeled molecules, such as deuterated derivatives, are considered the ideal internal standards, since they exhibit physical and chemical features similar to the target analyte, leading to similar extraction recovery, ion fragmentation pattern during EI-MS, and chromatographic retention time [28,29]. Therefore, since dMH and dIS co-eluted with almost superimposable peaks due to optimal chemical and chromatographic characteristics, $^{2}H_{6}$-MH was selected as IS.

The methanolic extractive solutions from lyophilized brain and calibration samples were purified through SPE, using weak cation exchange cartridges, already identified as the most suitable for metformin [30]. The dried residue of the extractive solutions was resuspended in the smallest volume of buffer, in order to solubilize all the MH and load a concentrated solution on the cartridge.

The minimum volume of aqueous ammonium acetate buffer to

Table 1
Scheme of the 3 × 3 randomized Latin Square used for the evaluation of the recovery and intra-day and inter-day repeatability.

	INTRADAY		
INTERDAY	**I**	**II**	**III**
1	0.30	0.91	0.60
10	0.60	0.30	0.91
30	0.91	0.60	0.30

solubilize MH was determined in a preliminary study, by comparing the dMH/dIS peak area ratios obtained by analyzing 3 blank brain extracted residues spiked with accurately measured 0.06 nmol of MH, dried and solubilized in 250 μL of buffer with the ones obtained by directly dissolving the same amount of MH in the same buffer volume, and no significant difference was found ($p > 0.05$). The use of higher buffer volume did not change the result.

For the SPE elution, different volumes of methanol-formic acid were tested (2, 3, 4 and 5 mL): 3 mL resulted to be the minimum volume able to elute the total amount of analyte.

The decision to add the IS after SPE purification was based on reproducibility issues of the response when IS was added to the methanolic extracts, possibly because of MH and IS different affinities for the stationary phase. The study on the different interaction of MH and IS with the stationary phase has been performed and reported in the Supplementary Materials (Table S1).

For GC-MS analysis, both MH and $^{2}H_{6}$-MH, the selected IS, have to be transformed into volatile and thermally stable compounds. Several derivatization procedures are available, and a possible versatile derivatization reaction involves silylation, obtained by using reagents like *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), MSTFA/imidazole and *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA); however, these derivatizing agents present some issues, as reported by Uçaktürk [20]: indeed, it has been observed that multiple derivatives formed from the reaction of MH with MSTFA and MSTFA/imidazole, even under different reaction conditions. MTBSTFA, widely used to obtain tert-butyldimethylsilyl (TBDMS) derivatives, is known to generate more stable and less moisture-sensitive compounds; however, from the reaction with MH, the bis-TBDMS derivative formed, and it was reported that the excess of MTBSTFA interfered with analyte dosing [20].

Acylation is another commonly used strategy to derivatize analytes, more often using trifluoroacetyl derivatives as acylating agents, requiring rigorously anhydrous conditions. Among the different acylating derivatizing agents, MBTFA was selected since, as reported in the literature [31], it reacts rapidly under soft conditions with primary and secondary amines. Besides preventing the presence of water in the reaction mixture, the absence of residual methanol is also required, since the protic solvent interferes with the derivatization, therefore the reaction was carried out in dried acetonitrile.

As depicted in Fig. 1, in the derivatization reaction two equivalents of MBTFA reacted with one equivalent of MH or IS ($^{2}H_{6}$-MH) yielding the two diacetyl-derivatives, whose GC-MS retention time is approx. 4.75 min and which can be monitored by selecting the ion fragments with m/z equal to 303 and 309, respectively.

The derivatization conditions reported by Uçaktürk [20] were initially adopted, so the reaction was carried out at 80 °C for 60 min, but the presence of a further peak eluting at about 5.2 min, identified as the monoacetyl-derivative of MH and IS, with $m/z = 207$ and 213 respectively, was evidenced (Fig. 2). This can result from an incomplete reaction, or from the partial hydrolysis of the diacetyl-derivative, but the first hypothesis was excluded by performing the reaction in the presence of a higher molar ratio between derivatizing agent and substrate. The increase of reaction time and temperature led to augmented production of the monoacetyl-derivatives; therefore, the reaction conditions were modified to 70 °C [32] and 30 min, thus obtaining the complete transformation of MH and IS in diacetyl-derivatives, avoiding the by-product formation.

In order to maximize the sensitivity of the MH and IS response, two time windows were set for MS detection of the selected analytical and qualifying ions. A preliminary study confirming the absence of peaks whose mass spectrum included $m/z = 207$ and 213 at the retention time of dMH and dIS was performed and is reported in the Supplementary Materials (Fig. S1).

The minimum amount of derivatizing agent to be employed in the reaction was also evaluated, considering that an excess of MBTFA can damage the stationary phase of the chromatographic column [21]. Using incremental volumes of MBTFA (from 5 to 20 μL), 15 μL was identified as the optimal amount of derivatizing agent. Moreover, to preserve GC column integrity, a deactivated retention gap was used, and the mixture was diluted with dried acetonitrile before injection.

After the reaction time, the mixture was quenched at − 20 °C for at least 20 min before injection. This procedure assured the repeatability of the derivatization, reducing the loss of derivatized molecules by volatilization, possibly occurring during the opening of the valve cap to drain the sample volume for injection. As shown in Fig. 3, dMH and dIS almost co-eluted at 4.75 ± 0.1 min and 4.72 ± 0.1 min, respectively: actually, the peak shown in Fig. 3a is slightly asymmetrical, since it results from the overlapping of the peaks of the two analytes. It has been reported in the literature that deuterium-labeled IS might exhibit the isotope effect [33] due to the different lipophilicity caused by the difference of deuterium and hydrogen bonds, leading to a partial resolution of the two peaks; this phenomenon is particularly evident in the case of deuterated-metformin, having six deuterium atoms [28,34,35]. Moreover, the different ionic density of MH and IS can explain their different affinity for SPE, which led us to add IS after SPE purification.

The analytical method is selective, as demonstrated by the data obtained from the blank samples, which did not show any interfering peaks affecting the quantification of MH or IS, both in SIM (Fig. 4) and TIC chromatograms. As for specificity, the analyses of zero samples (i.e., samples of brain tissue from untreated mice, added only with IS) confirm the absence of MH: indeed, MH is not metabolized, and the animals did not receive any other drug, possibly interfering with the analyte.

The sensitivity and robustness of the method are also related to the cool on-column injection mode: indeed, the cool on-column inlet ensures high accuracy and reproducibility, since the direct sample injection in the GC column allows quantitative sample transfer without thermal degradation of the analytes. However, the direct introduction of the sample, possibly containing contaminants, into the column requires the use of a retention gap to preserve capillary column integrity.

Calibration samples containing accurately measured concentrations of MH ranging from 0.24 to 4.12 μM were analyzed. The relationship

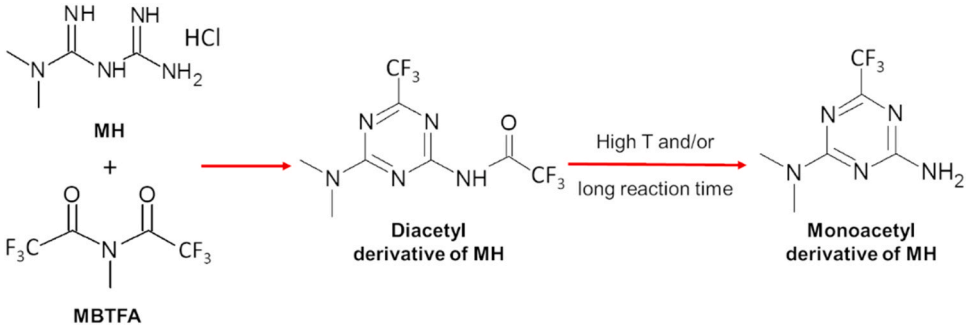

Fig. 1. Possible steps of the reaction between MH and MBTFA.

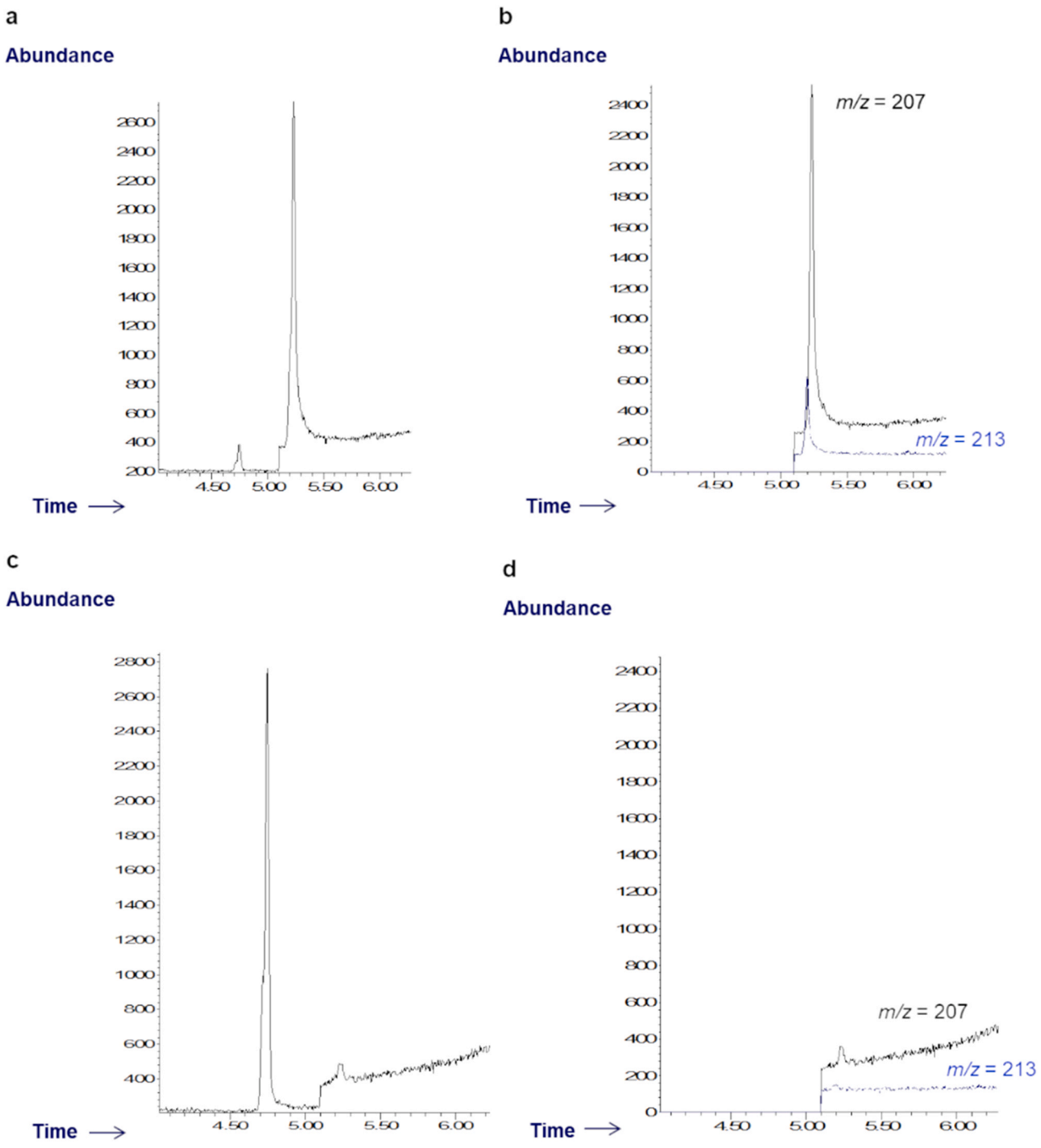

Fig. 2. a) SIM chromatogram obtained from the derivatization of MH and IS, carrying out the reaction at 80 °C for 60 min; b) Ion extraction of the chromatogram in a), evidencing that the peak eluting at 5.2 min is associated with fragments with $m/z = 207$ (black) and 213 (blue dashed), typical of the monoacetyl-derivatives of MH and IS, respectively; c) SIM chromatogram obtained from the derivatization of MH and IS, carrying out the reaction at 70 °C for 30 min; d) Ion extraction of the chromatogram in c), evidencing the negligible presence of fragments with $m/z = 207$ (black) and 213 (blue dashed), typical of the monoacetyl-derivatives of MH and IS, respectively.

obtained by plotting the SIM peak area ratio (dMH/dIS) against the concentration of MH ($y = 3.673x - 0.041$, $S_{Yx} = 0.7323$) was linear ($R^2 = 0.975$), and the intercept value was not significantly different from zero ($p = 0.815$).

Moreover, the developed method featured low LOD (0.373 μM, corresponding to 0.887 pmol/mg of wet brain tissue) and LOQ (1.242 μM, corresponding to 2.958 pmol/mg of wet brain tissue) values, determined on the basis of the signal-to-noise-ratio of 3 and 10 for the least intense signal, respectively. The LOD and LOQ values obtained are higher than the values declared by other authors [21,32]; however, it is important to consider that methods reporting very low LOD and LOQ values are related to MH quantification in wastewater, where the analyte can be isolated from large volumes of sample, while in our case the sample (i.e. mouse brain, surely a rare matrix) is very small and its amount cannot be increased by pooling brain samples. Indeed, our sensitivity is comparable to the one declared by Arbouche et al. [23],

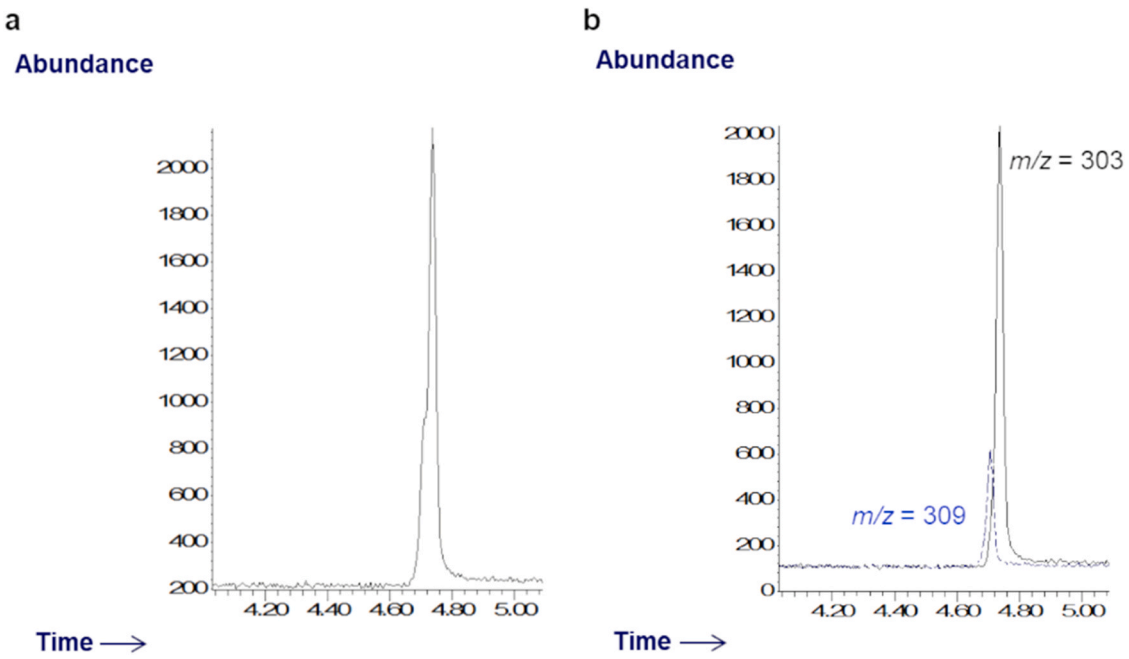

Fig. 3. a) SIM chromatogram obtained from the reaction mixture of a MH working standard solution; b) Superimposition of the extracted-ion chromatograms corresponding to $m/z = 303$ (black) and $m/z = 309$ (blue dashed) ions.

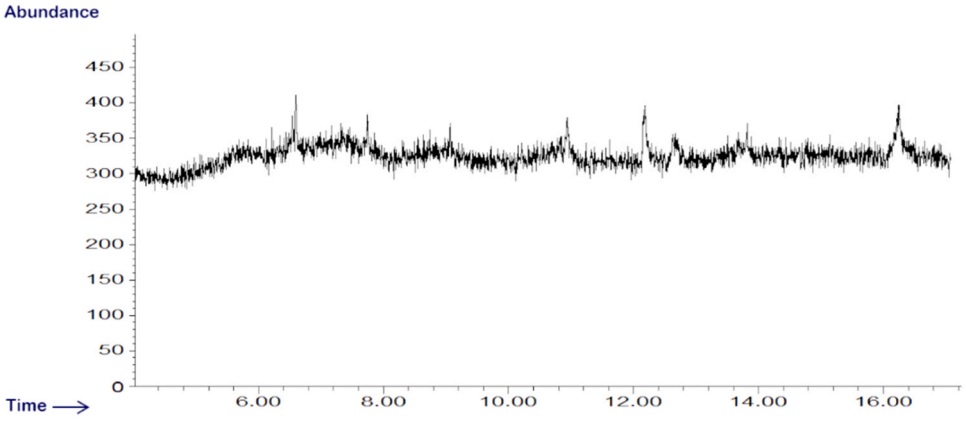

Fig. 4. SIM chromatogram of a blank sample, obtained by monitoring the $m/z = 303$ and $m/z = 309$ ions, showing the absence of any interfering substance eluting at the dMH and dIS typical retention times (4.75 ± 0.1 min and 4.72 ± 0.1 min, respectively).

which dosed MH from human hair.

For LLOQ, accuracy was − 16.3% and precision was 2.2%. For the other concentration levels, the accuracy ranged from − 3.2–4.6% and precision was lower than 5.4%.

The recovery was validated through a Latin Square experimental design, which produces a substantial reduction in error over the randomized blocks design. The recovery study was conducted at three levels, spiking brain samples deriving from healthy, untreated mice with three different amounts of MH (0.30, 0.60 and 0.91 nmol); a randomized 3 × 3 Latin Square design (Table 1) was carried out, where the rows are the days of the assay (day 1, 10 and 30) and the columns are the daily order of analysis (first, second, third assay). The ANOVA of the mean percent recoveries, reported in Table 2, evidenced that the recovery, which was always higher than 77%, did not depend on the day of the assay ($p = 0.501$), on the daily order of analysis ($p = 0.202$), and on the amount of MH added ($p = 0.972$) (Fig. 5).

The matrix effect at the two concentration levels resulted 98 ± 5% and 96 ± 6%, respectively (Table S2), compliant with ICH M10 Guideline on bioanalytical method validation and indicating no noteworthy effect of the mice brain tissue.

Table 2
Percent recoveries expressed as mean ± IC 95% (n = 3).

	INTRADAY		
INTERDAY	**I**	**II**	**III**
1	86.9 ± 6.4	84.6 ± 6.5	85.4 ± 6.5
10	88.6 ± 6.3	87.0 ± 6.1	85.2 ± 6.2
30	88.5 ± 6.2	83.7 ± 6.4	83.5 ± 6.5

The assay was tested in a murine experimental model, administering MH both to healthy animals and to mice undergone GBM cell orthotopic xenografting. Considering that MH intestinal absorption and distribution to the central nervous system are limited by the hydrophilic nature of the molecule, whose crossing of lipid membranes occurs only via specific transporters, we hypothesized that, in a potential MH antitumoral application, a more continuous exposition to the drug may result in higher activity compared to administration *in bolo*, in which the

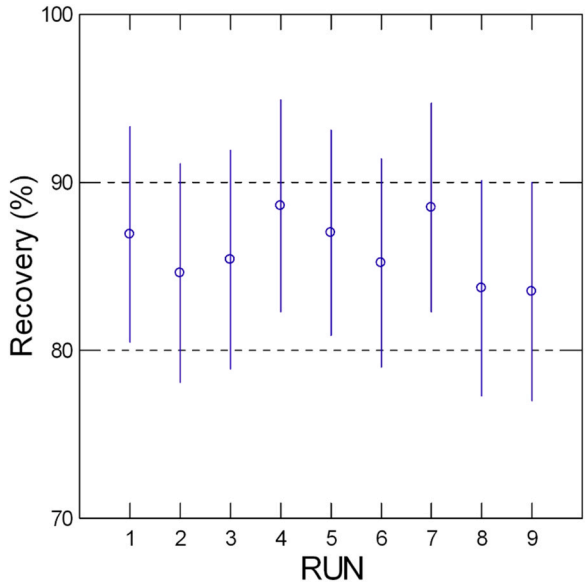

Fig. 5. Percent recovery of the 9 experiments (mean value ± IC 95%).

expected higher concentrations are hampered by the transporters' saturation. Thus, we administered MH in the drinking water, allowing a continuous absorption of the drug, which might determine a lower peak brain concentration but, on the other hand, might allow continuous activity of MH on the tumor cells. Moreover, in this way a steady state of the drug concentration in the brain may be reached, thus this type of treatment is not affected by sampling time issues, unlike any acute administration. To this aim, animals previously injected with GL261 cells in the motor cortex were treated with MH in the drinking water (1.5 mg/kg/die, corresponding to 9.06 μmol/kg/die) for seven days starting from day 8 after tumor infection, while control mice received pure water. The drug administration was evaluated by measuring the water consumed by each animal every day.

Samples of brain tissue deriving from four healthy MH-treated mice were analyzed, revealing a MH content in the range of 3.47–8.15 pmol/mg of wet tissue. Four samples of brain tissue from MH-treated mice bearing experimental GBM revealed a MH content in the range between 3.21 and 7.85 pmol/mg of wet tissue (Fig. 6). The results are summarized in Table 3.

4. Conclusions

Repurposing MH as a therapeutic tool for treating different types of cancer is the goal of several ongoing preclinical and clinical studies. In particular, the possibility to take advantage of the selectivity of this drug to target cancer stem cells supported the idea of using it as add-on therapy to conventional cytotoxic drugs. This possibility is particularly relevant for those tumours for which only scarcely effective therapies are available and patients' prognosis is still very dismal; specifically, MH application has been hypothesized for the treatment of GBM and preclinical and clinical studies are currently in progress. However, due to the low penetration of MH in the central nervous system due to its hydrophilic nature, the detection of its presence in brain tissue and the determination of the modalities of administration achieving therapeutically effective concentrations are still open issues. We have developed a sensitive and specific analytical method to measure MH content in the mice brain. The straightforward work-up procedure on lyophilized brain samples involving SPE purification allows to extract MH from the tissue without interference from such a complex matrix; the following derivatization procedure, rapid and not expensive, has been optimized to achieve full conversion of the analyte; accuracy and precision of the method comply with ICH limits; the recovery study performed by

Table 3
Results obtained from the analysis of brain samples of healthy (H) and tumor-affected (T) mice receiving MH, expressed as picomoles of MH/mg of wet brain tissue.

	Mean ± IC 95% (pmol/mg)		Mean ± IC 95% (pmol/mg)
H1	6.8 ± 0.1	**T1**	6.9 ± 0.1
H2	8.2 ± 0.2	**T2**	3.2 ± 0.1
H3	3.5 ± 0.1	**T3**	7.9 ± 0.1
H4	5.9 ± 0.1	**T4**	5.1 ± 0.1

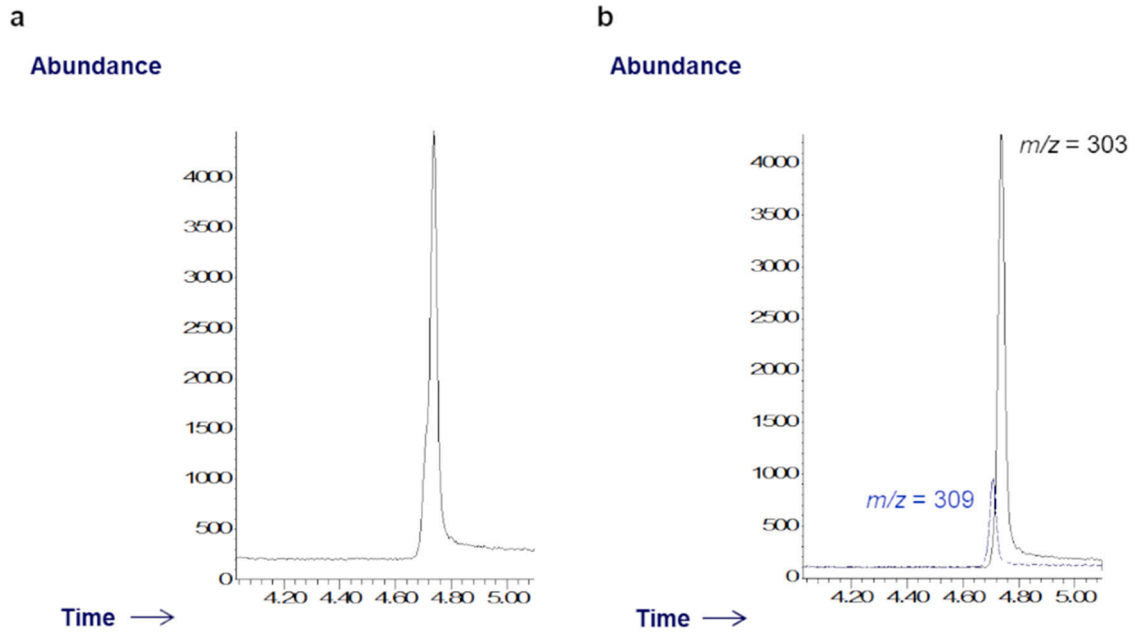

Fig. 6. a) SIM chromatogram obtained from a brain of a tumor-affected mouse receiving MH; b) Superimposition of the extracted-ion chromatograms corresponding to $m/z = 303$ (black) and $m/z = 309$ (blue dashed) ions.

spiking brain tissues with different amounts of MH has demonstrated that the method allows almost quantitative extraction of this small, highly hydrophilic analyte from the tissue and has ruled out any intra/interday effect; moreover, no significant matrix effect has been observed. The method has been successfully applied to MH quantification in brains of mice, both healthy and affected by GBM.

These preliminary results appear promising and entice to proceed to full validation of this method, which might be useful to possibly identify a direct correlation between the undeniable beneficial effects of MH emerged from preclinical studies and its tissue concentration, one missing aspect in the literature published on this topic so far; this method might represent a relevant tool to assess the performance of this drug ahead of clinical studies.

CRediT authorship contribution statement

Giorgia Ailuno: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Sara Baldassari:** Conceptualization, Writing – original draft. **Alice Balboni:** Formal analysis, Investigation, Writing – original draft. **Giuliana Drava:** Methodology. **Cristina Spalletti:** Formal analysis, Investigation; **Elena Tantillo:** Formal analysis, Investigation. **Michele Mazzanti:** Conceptualization, Methodology. **Federica Barbieri:** Methodology. **Stefano Thellung:** Methodology. **Tullio Florio:** Conceptualization, Methodology, Supervision. **Gabriele Caviglioli:** Conceptualization, Methodology, Supervision. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michele Mazzanti reports financial support was provided by AIRC Italian Foundation for Cancer Research.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115503.

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