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Validation of a routine gas chromatography mass spectrometry method for 2-hydroxyglutarate quantification in human serum as a screening tool for detection of *idh* mutations

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ABSTRACT

High circulating levels of 2-hydroxyglutarate (2HG) have been reported in patients with determinate isocitrate dehydrogenase (IDH) mutated tumors. Recent studies indicate that in malignancies such as acute myeloid leukemia (AML), measurements of 2HG in serum provide useful diagnostic and prognostic information and improve patient selection and monitoring of IDH-targeted treatments. In the current study, we validated a sensitive and specific gas chromatography mass spectrometry (GC–MS) method specifically intended to quantify serum levels of 2HG in routine clinical laboratories. Extraction was liquid-liquid with ethyl acetate, and derivatization was reduced to 3 min of microwave irradiation. The analytical method was linear over a wide dynamic range, presenting acceptable intraday and day-to-day precision and accuracy. The limit of quantification was 10 ng/mL, process efficiency ranged between 38% and 49%, and recovery of added 2HG was 99–105%. 2HG was found to be stable in serum for up to 48 h at both 4 °C and at ambient temperature, and after three freeze-thaw cycles. Microwave derivatizated extracts in the autosampler were found to be stable for up to 120 h. In summary, the present method is useful for quantification of 2HG serum levels in patients with IDH mutated malignancies in clinical laboratories.

1. Introduction

Isocitrate dehydrogenase (IDH) mutations have been reported in a wide range of human tumors, including hematopoietic malignancies and solid tumors [1]. IDH enzymes (IDH1 and IDH2) normally catalyze the conversion of isocitrate to alpha-ketoglutarate. However, mutated IDH enzymes produce 2-hydroxyglutarate (2HG) from alpha-ketoglutarate [2,3]. Accordingly, high intratumoral and circulating levels of 2HG have been reported in patients with IDH-mutated malignancies such as acute myeloid leukemia (AML), intrahepatic cholangiocarcinoma or breast adenocarcinoma among others [1,4–6].

Recent studies have evaluated the clinical value of 2HG levels in serum of patients with AML. Consistent associations between IDH mutations and serum levels of 2HG have been found in AML patients. Furthermore, according to recent studies, serum measurements of 2HG in AML patients provide useful diagnostic and prognostic information, can improve patient selection for IDH-targeted therapies and can be useful to monitor response to therapy [4,7–9]. More recently, serum 2HG has also been proposed as a useful biomarker in patients with IDH-mutant intrahepatic cholangiocarcinoma. In these patients, 2HG serum levels predict IDH mutational status and correlate with tumor burden as with other clinical variables [5].

Thus, determination of 2HG in serum has a recognized clinical value in AML and its clinical usefulness is likely to extend to other clinical oncological scenarios linked to IDH mutations. Methods used in clinical studies include liquid chromatography-tandem mass spectrometry [2,10], gas chromatography mass spectrometry [11,12] and gas chromatograph–time-of-flight mass spectrometry [7]. However, very few methods specifically validated for the analysis of 2HG in serum in clinical laboratories have been reported [12,13]. In the current study we present a gas chromatography mass spectrometry (GC–MS) method specifically devised and validated [14] for the determination of serum

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levels of 2HG in routine clinical laboratories. The matrix effect was exhaustively evaluated to see the adequacy of the method to the analysis of serum samples and a microwave assisted derivatization procedure was used. In fact, one of the main drawbacks in the routine application of methods based on GC–MS is the need for derivatization. The search for more efficient derivatization protocols is focused not only in obtaining better yields and stabilities, but also in the reduction of the incubation time. Among the strategies that have been employed to enhance this process, few have the potential of microwave-accelerated derivatization (MAD). Several publications have already demonstrated the potential of MAD for the derivatization of different types of analytes [15]. In steroids, we have previously observed the efficacy of this procedure even in the sylilation of hindered hydroxyl groups [16].

The intended use of the proposed method is measurement of serum 2HG levels in AML patients but it may also be useful in patients with other IDH-mutated tumors in which 2HG determination possesses clinical significance.

2. Material and methods

2.1. Chemicals reagents

D-α-hydroxyglutaric acid disodium salt, D-α-hydroxyglutaric acid-$^{13}C_5$ disodium salt, ethyl acetate, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane (BSTFA:TMCS; 99:1), methoxyamine hydrochloride and *N*-trimethylsilylimidazole were obtained from Sigma (Steinheim, Germany). Organic acid quality control material (ORG-01) was obtained from MCA Laboratory (Winterswijk, The Netherlands). Hydrochloric acid (HCl, 37%) and methanol were purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained using a Millipore Milli-Q purification system.

2.2. Instrumentation

GC–MS analyses were performed on a Shimadzu GCMS QP2010 Ultra instrument (Kyoto, Japan). Final extracts were injected in splitless mode (valve opened at 1 min) into the gas chromatograph interfaced with a mass selective detector. Chromatographic separation was achieved on a Sapines-5MS+ capillary column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness) from Teknokroma (Barcelona, Spain) with helium as a carrier gas at a constant velocity of 50 cm/s. The temperature program was set to begin at 100 °C, maintained at this temperature for 3 min, elevated at 20 °C min^{-1} to 240 °C, then increased at 30 °C min^{-1} until 300 °C and finally maintained for 6 min at 300 °C. The total run time was 21 min, which included 2 min of temperature stabilization and four pre and post-injection washes (with cyclohexane). Electron multiplier voltage was 1.0 kV. The ion source and transfer line temperatures were set to 250 °C and 280 °C, respectively. Mass detector was operated in selected ion monitoring (SIM) mode following a 7 min solvent delay. 2HG (*m/z* 349, 247, 203) and 2HG$^{13}C_5$ (*m/z* 354, 252 and 208) were acquired using a dwell time of 150 ms. Quantitation ions were *m/z* 247 for 2HG and *m/z* 252 for 2HG$^{13}C_5$. Identification of the analytes in the sample extracts was achieved by GC retention time comparison with reference standards.

2.3. Preparation of stock solutions, working solutions, calibrators and quality controls samples

2HG is an endogenous compound present in blank human serum. Therefore, calibration curves were prepared in water or in methanolic solutions as 2HG free surrogate matrices. The analytical response differences between serum and free surrogate matrices were evaluated by a recovery assessment. Stock solutions of 2HG and 2HG$^{13}C_5$ were prepared at concentration of 1 g/L in methanol and stored at −20 °C. Working solutions were prepared by diluting the stock solutions in methanol to final concentrations of 100 μg/mL for 2HG and of 200 ng/mL for 2HG$^{13}C_5$. Seven-point calibration curves (10, 100, 500, 1000, 5000, 10,000 and 50,000 ng/mL) were prepared for the calibration of 2HG by diluting 2HG working solutions in water or methanol. For quality controls (QC), three concentrations were prepared (250 ng/mL, 2500 ng/mL and 25,000 ng/mL) by serially adding 2HG working solutions in water, methanol or human serum.

2.4. Sample preparation

For determination of 2HG in calibrators, QC and serum samples, 133 ng of 2HG$^{13}C_5$ were added as internal standard and samples (300 μL) were acidified with 90 μL of 5 M HCl. Then 4 mL of ethyl acetate were added and after centrifugation (2000 g for 5 min), the organic phase was transferred and evaporated to dryness under nitrogen at 37 °C. Trimethylsilyl ether derivatives were formed by derivatization (80 μL BSTFA:TMCS 99:1, microwave irradiation for 3 min at 800 W). After derivatization, 1 μL was injected in the chromatographic system.

2.5. Microwave assisted derivatization

Derivatization yields obtained when performing the silylation under thermal or microwave assisted energy transfer were compared. When conducting the classical heating approach, trimethylsilyl ethers were formed by adding 80 μL of BSTFA:TMCS 99:1 to the extracts and heating for 60 min at 60 °C. For microwave assisted derivatization (MAD), 80 μL of BSTFA:TMCS 99:1 were added to the extracts and the silylation was performed in a domestic microwave at 800 W (SpeedyGrill, Taurus). Time for MAD was optimized by testing three derivatization times (1, 2 and 4 min). The relative response factors (RRFs) for 2HG in methanolic standards and serum samples were calculated by comparing the area ratios obtained by MAD and thermal derivatization.

2.6. Method validation

2.6.1. Linearity of calibration curves

Linearity was evaluated over a range between 10 and 50,000 ng/mL using seven concentrations (10, 100, 500, 1000, 5000, 10,000 and 50,000 ng/mL). Complete calibration curves were analyzed on 5 separate days. A 1/x weighted linear regression was used to plot the peak area ratio (2HG to 2HG$^{13}C_5$) versus the corresponding 2HG concentration. Slope, y-intercept and correlation coefficient were calculated for each standard curve. A minimum value of $r^2 = 0.99$ was required to pass this validation step. Back-calculated concentrations of the standards were acceptable when within ± 15% of the nominal values except for the limit of quantification for which a ± 20% was considered [14].

The analytical responses of 2HG in serum were assessed to ensure that the calibration curve built in water or in methanolic standards could be used to quantify clinical serum samples. The slope coefficient (α) of 3-point QC curves spiked in serum from 4 different healthy sources was compared with their respective curves spiked in water or methanolic solutions. Response factors (RF) were calculated as $\alpha_{spiked\ serum}/\alpha_{water}$ or $\alpha_{spiked\ serum}/\alpha_{methanol}$. The back-calculated concentrations of QC samples in serum with and without RF correction were used to calculate the sum of the absolute values of relative residuals to evaluate the three methodologies (relative residual = $100 \times (C_{spiked\ serum} - C_{nominal}) / C_{nominal}$).

2.6.2. Accuracy and precision

To evaluate the accuracy and precision of the analysis method, back-calculated results of multiple analysis of the three QC, the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) were used. The LLOQ and ULOQ were set at the lowest (10 ng/

mL) and highest (50,000 ng/mL) calibration standards values, respectively. In the case of intra-day accuracy and precision, five replicates were performed for each concentration on the same day. Inter-day accuracy and precision were calculated on five different days. To pass the accuracy test, the mean values should be within 100 ± 15% of the theoretical value. Accuracy was determined as the difference between calculated concentrations of 2HG with theoretical concentrations expressed in percent. Precision at each concentration level was expressed as relative standard deviation (%RSD) for each QC and should not exceed 15%. In the case of the LLOQ, accuracy should stand within 100 ± 20% and precision below 20%.

2.6.3. Process efficiency and recovery of added 2HG

The process efficiency in serum samples was determined by comparing the area (A) of 2HG in 8 individual sources of healthy blank serum and the same serums spiked with 2HG (250, 2500 and 25,000 ng/mL). Due to the presence of endogenous concentrations in the blank serums, the individual areas of the blank serum samples were subtracted from 2HG added sample values. Process efficiency (%) was calculated according to the following formula: $100 \times (A_{spiked} - A_{blank}) / A_{neat}$. In addition, process efficiency was also determined by comparing the area of $2HG^{13}C_5$ in extracted serum samples and nonextracted standards according to the formula: $100 \times (A_{serum} / A_{standard})$. On the other hand, recovery of added 2HG was determined by comparing the 2HG concentrations in 8 individual sources of healthy blank serum and the same serums spiked with 2HG (250 and 2500 ng/mL).

2.6.4. Selectivity and carry-over

The selectivity was investigated by analyzing 15 different serum samples from 15 healthy subjects and was indicated by the absence of any endogenous interference at retention times of 2HG. Ion ratios between the three acquired ions of 2HG (*m/z* 349, 247, 203) were calculated for standards and compared with serum samples. The carry-over was evaluated by injecting 1 μL of cyclohexane immediately after the injection of a standard with ULOQ concentration in three separate occasions.

2.6.5. Stability

The stability in serum was evaluated at low and high QC levels by three replicates stored under different conditions and durations: three freeze-thaw cycles, ambient temperature for 48 h and 4 °C for 48 h. Bench-top stability at ambient temperature before ethyl acetate extraction was also evaluated for 4 h and 24 h, respectively. The stability of MAD extracts on the autosampler was evaluated by reanalyzing serum QC samples stored inside the autosampler at ambient temperature for up to 120 h.

2.6.6. Method applicability

Serum samples of 15 subjects without AML and of 4 patients with AML were analyzed. Samples from patients with AML included 3 samples from patients with AML not associated to *IDH1–2* mutations and one sample from a patient with AML and *IDH2* mutation.

2.7. Statistics

Statistical calculations were performed with the GraphPad Prism 6 (GraphPad Prism Software Inc. San Diego, California, USA).

3. Results and discussion

3.1. Method optimization

The aim of the present work was to develop a simple, sensitive and reproducible method to allow the accurate quantification of 2HG in human serum which may be useful in the diagnosis, prognosis and follow-up of patients with IDH-mutated AML. Liquid-liquid extraction was conducted for its simplicity. An acceptable extraction efficiency (45%) and high cleanliness was achieved using ethyl acetate. The initial tests revealed the need of a previous acidification of serum samples to achieve 2HG stability. The amount of 90 μL HCl 5 M per 300 μL of serum sample presented an optimum performance. The signal-to-noise ratio of 2HG was not improved when additional volumes of water were added to serum before ethyl acetate extraction.

Table 1
Derivatization yields of 2HG. Results are expressed as the normalized relative areas (RRFs, mean ± standard deviation) of microwave irradiation versus 60 min 60 °C thermal block incubation. n = 3 replicates.

Sample	Time (min)	Derivatization yield (RRFs)
Standard (methanol)	1	0.321 ± 0.06
Standard (methanol)	2	0.816 ± 0.04
Standard (methanol)	4	1.027 ± 0.08
Serum samples	2	0.947 ± 0.19
Serum samples	4	1.083 ± 0.25

RRFs: relative response factors.

To improve both the selectivity of the analysis and the efficiency of the chromatography, as well as to enhance the detectability of GC–MS, 2HG should be derivatizated. Derivatization with the mixture of methoxyamine hydrochloride with *N*-trimethylsilylimidazole or with the mixture of BSTFA with 1% TMCS were tested. Both methods achieved derivatization in a similar effective manner. Derivatization with BSTFA with 1% TMCS was chosen for its readiness.

In the current study MAD derivatization of 2HG was initially tested with methanolic solutions of 2HG (100 ng). A successful silylation was achieved after 1 min of microwave irradiation of the BSTFA:TMCS 99:1. As shown in Table 1, peak areas after 2 or 4 min of microwave irradiation were similar to those obtained after incubation at 60 °C for 60 min. To further investigate the applicability of MAD of 2HG in serum extracts, this alternative heating approach was compared with the classical block heating method also in serum samples (n = 5). Two aliquots of each sample were analyzed by both methods and derivatization yields for 2HG were compared. Table 1 shows the average RRFs values of MAD normalized to classical derivatization (60 min, 60 °C). Both derivatization methods produced similar absolute yields. RRFs ranged from 0.95 to 1.08. Thus, by using the described MAD procedure, the derivatization step can be reduced to only very few minutes of microwave irradiation in the same injection vial. Although only 2HG derivatization yields were evaluated, results suggest the possibility that this MAD procedure may also be useful for the analysis of other organic acids or for methods aiming at the comprehensive acids organics profiling determinations.

After extraction and derivatization, the chromatographic conditions led to the profile shown in Fig. 1. 2HG was eluted after 8.1 min. Full scan spectra for 2HG tri-TMS is also shown in Fig. 1. Selected ion monitoring (SIM) was used to achieve low detection limits. Three acquisition ions for each analyte were selected in SIM analysis to maximize specificity.

3.2. Method validation

Calibration curves were prepared in water to avoid the potential bias resulting from endogenous 2HG at different concentrations in human serums. The method was linear for 2HG concentrations ranging from 10 to 50,000 ng/mL, obtaining r^2 values of 0.997 (mean) ± 0.003 (S.D.). A wide range of standard concentrations was selected since very high levels of 2HG in serum (> 25,000 ng/mL) have been observed in some patients with IDH mutated tumors whereas healthy subjects and patients without IDH mutations typically present values between 20 and 300 ng/mL [4,7,8,17]. In spite of the wide range, calibration samples showed an accuracy above 85% and a precision below 15%, as

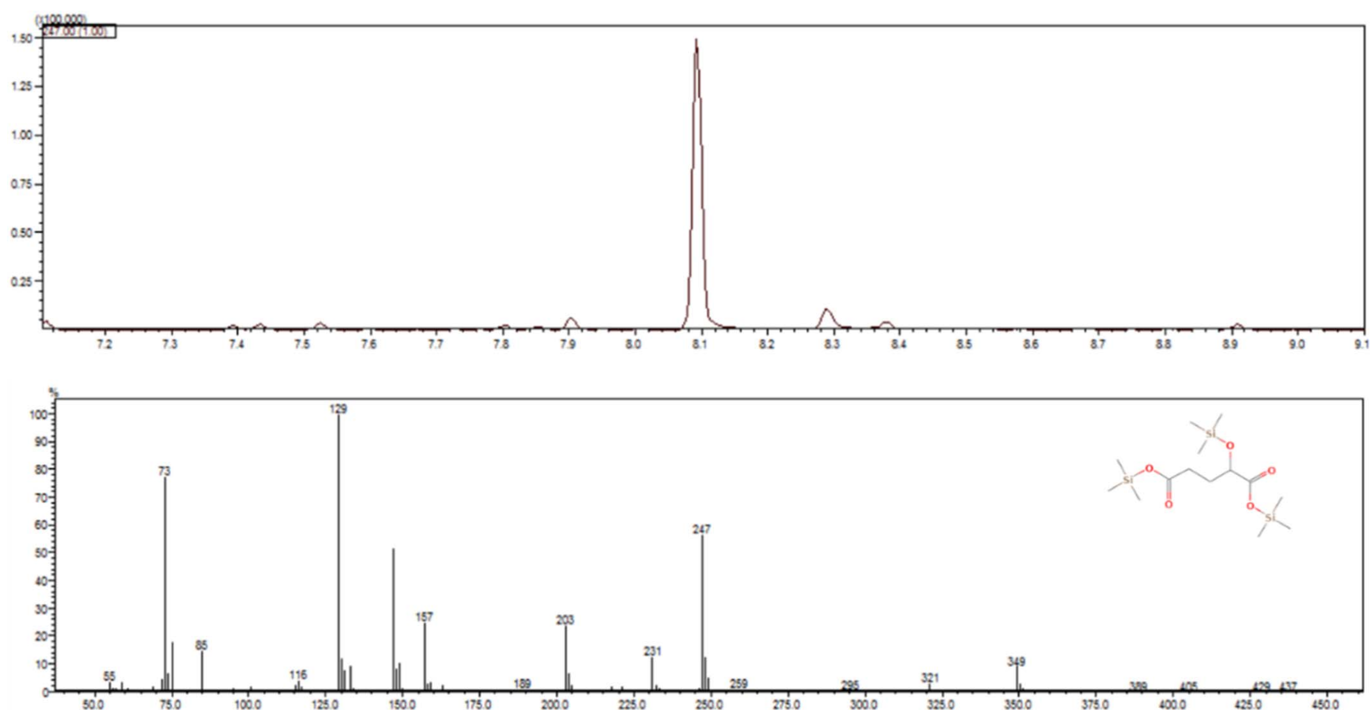
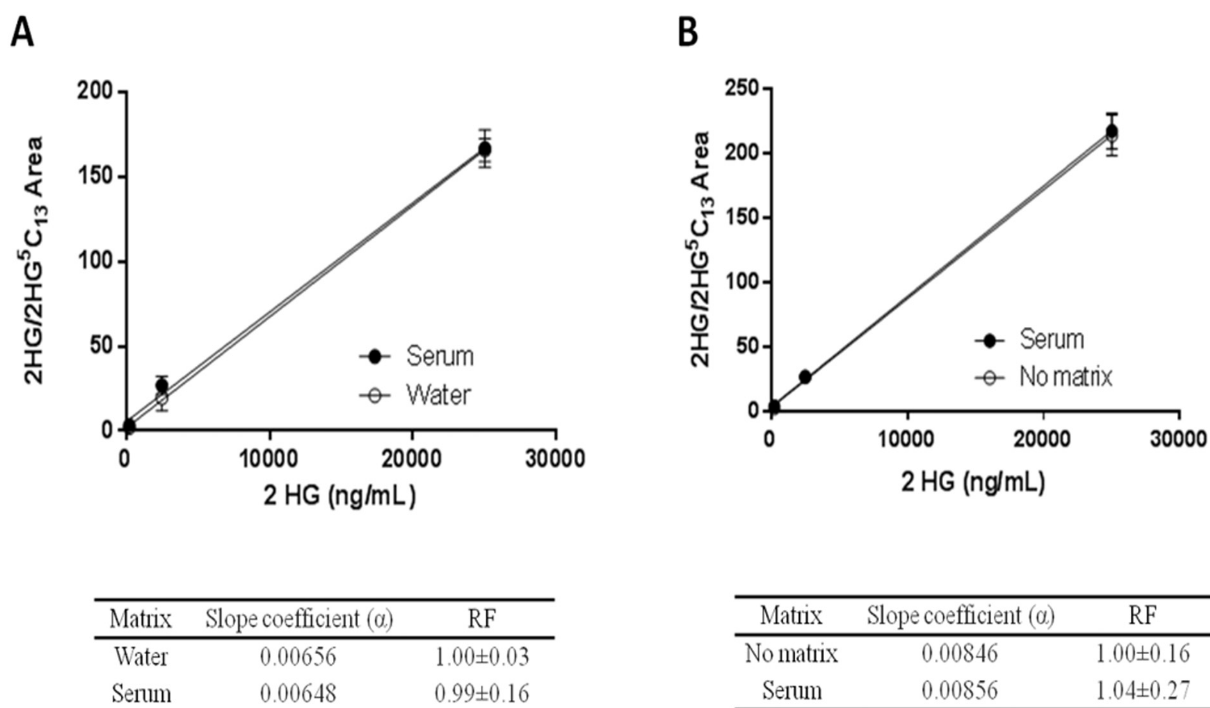


Fig. 1. Chromatogram of a serum sample (*m/z* 247) and mass spectrum of 2HG tri-TMS.

specified in the current guidelines for analysis [14]. The calibrator of 10 ng/mL concentration was chosen for the low limit of quantification (LLOQ). The accuracy of the LLOQ was above 80%, whereas precision was below 20%.

Although $2HG^{13}C_5$ was used as internal standard to compensate for any variations during sample processing, additional validation procedures were necessary to evaluate the appropriateness of preparation of calibrations in water. Experiments were performed to evaluate the differences on recoveries between serum and water and also between serum and no matrix (methanolic standard solutions). As shown in Fig. 2A, no significant differences were observed in slope coefficient (α) or RF of 3-point QC 2HG-spiked curves in serum or water. Back calculated results after applying both RF ranged 0.96–1.02 for QC1 (250 ng/mL), 0.97–1.03 for QC2 (2500 ng/mL) and 0.97–1.03 for QC3 (25,000 ng/mL). The implementation of RF for QC samples spiked in serum did not result in better accuracy or precision. Thus, a RF was not necessary despite using different matrix for calibration curves and QC samples compared to clinical samples. This supports the parsimonious



Matrix	Slope coefficient (α)	RF
Water	0.00656	1.00±0.03
Serum	0.00648	0.99±0.16

Matrix	Slope coefficient (α)	RF
No matrix	0.00846	1.00±0.16
Serum	0.00856	1.04±0.27

Fig. 2. (A) 3-point QC curves spiked in human serum compared with the respective curves spiked in water (n = 4). (B) 3-point QC curves spiked in human serum compared with the respective curves spiked in no matrix (n = 4). RF: response factor.

Table 2
Precision expressed as relative standard deviation and accuracy values of 2HG measurement.

Quality control	2HG (ng/mL)	Precision (%)		Accuracy (%)	
		Intra-day (n = 5)	Inter-day (n = 5)	Intra-day (n = 5)	Inter-day (n = 5)
LLOQ	10	7.2	19.5	108.2	108.4
QC-1	250	2.9	4.6	94.0	107.8
QC-2	2500	2.3	7.1	102.7	107.6
QC-3	25,000	6.7	5.2	98.0	99.3
ULOQ	50,000	2.1	2.4	102.1	99.5

LLOQ: Low Limit of Quantification, QC: Quality Control, ULOQ: Upper Limit of Quantification.

Table 3
Process efficiency in serum samples. Efficiency 1 was determined by comparing the area of 2HG in healthy blank serums and the same serums spiked with 2HG. Efficiency 2 was determined by comparing the area of $2HG^{13}C_5$ in extracted serum samples and non-extracted standards.

Added 2HG (ng/mL)	Efficiency 1 (n = 6)	Efficiency 2 (n = 8)
0	–	47 ± 16%
250	49 ± 11%	46 ± 13%
2500	45 ± 22%	38 ± 9%
25,000	40 ± 14%	41 ± 16%

Table 4
Recovery of added 2HG in serum samples.

Added (ng/mL)	Expected (ng/mL)	Mean detected (ng/mL)	Mean recovery (%)
0	NA	139	NA
250	389	388	99.5
2500	2639	2781	105.5

NA. Not applicable.

Table 5
Stability of 2HG in spiked human serum (% of degradation).

	2HG (ng/mL)	25 °C			4 °C	F&T (3 cycles)
	–	4 h	24 h	48 h	48 h	–
Serum QC-1	250	−2.4	1.0	−2.9	−8.6	11.1
Serum QC-3	25,000	−12.6	−10.4	−7.8	−2.7	−9.1

F&T. Freeze and thaw.

approach of not compensating for the different matrices. Similar results were obtained when comparing serum and methanolic solutions (Fig. 2B).

Values of intra- and inter-assay precisions and accuracies of the LLOQ, the three quality control levels and ULOQ are summarized in Table 2. In addition to a good precision and accuracy, it is convenient to use quality control materials that externally evaluate over time the standards and controls prepared in house. We used the organic acid control material (ORG-01, ERNDIMQA) diluted 1/100 obtaining mean values of 165 ng/mL (CV = 8%, n = 4).

Values for process efficiency were determined by comparing the peak areas of serum QC samples with and without spiked 2HG. As shown in Table 3, process efficiency was between 40 and 49% (efficiency 1). Similar values of process efficiency (between 38 and 47%) were obtained when comparing $2HG^{13}C_5$ areas of extracted serums with nonextracted methanolic solutions (efficiency 2). Excellent recoveries of added 2HG were obtained when analyzing replicates of 8 spiked serums with two different 2HG amounts (Table 4).

No interfering signals were observed in 2HG or $2HG^{13}C_5$ when analyzing 15 different human serum samples (including 4 patients diagnosed with AML). Ion ratios for standard samples were *m/z* 247 (100), *m/z* 203 (47) and *m/z* 349 (17). The same ion ratios were observed for evaluated serum samples. Also, there were no carry-over effects for 2HG or $2HG^{13}C_5$.

Stability of 2HG in serum stored at 4 °C, ambient temperature and after three freeze and thaw cycle was evaluated. The accuracy of low and high serum QC levels was above 85% (Table 5). These results are in agreement with those reported by Poinsignon et al. [13]. Finally, 2HG extracts in the autosampler at ambient temperature were highly stable for at least 120 h (Table 6). This result further supports the efficacy of MAD derivatization of 2HG.

Serum 2HG analysis by mass spectrometry can accurately identify patients with AML with and without IDH mutations and is a diagnostic, prognostic, and therapeutic monitoring tool in AML [4,7–9]. However, very few methods specifically validated for the analysis of 2HG in serum in clinical laboratories have been reported. Recently, He et al. developed and validate a GC–MS method for the simultaneous determination of tricarboxylic acid cycle acids and 2-Hydroxyglutarate in serum from patients with nasopharyngeal carcinoma [12]. Also recently, Poinsignon et al. developed and validated a liquid chromatography tandem mass spectrometry method suitable for clinical and preclinical applications including determination in blood samples allowing quantification of both L and D enantiomers of 2HG [13]. Samples were extracted by solid phase extraction and a derivatization step with (+)-o,o′-diacetyl-L-tartaric anhydride was used to separate the two isomers. Retention time was 5 min and run time was 8 min. In comparison, our method is based on GC–MS and a liquid-liquid extraction and does not separate 2HG isomers but quantifies them together. Although retention time was 8 min, a total run time of 21 min was used for a better protection of the column between injections. However, based on column temperatures a shorter run time of 15 min should probably achieve also the same aim. Table 7 summarizes performance characteristics among validated methods for 2HG quantification in serum.

Serum levels of 2HG in healthy subjects ranged 21.5–158.8 ng/mL. Fig. 3 shows the results of determination by this method of 3 samples from patients with AML not associated to *IDH1–2* mutations (2HG concentration range 21.0–43.1 ng/mL) and one patient with AML and *IDH2* mutation (2HG concentration 545.1 ng/mL). Table 8 compares previously published serum 2HG levels in AML patients with and without *IDH1–2* mutations. We obtained lower median values in comparison with previous clinical studies. This is probably related to the

Table 6
Stability in the autosampler at ambient temperature expressed as accuracy values of 2HG measurements in serum after 24, 48, 96 and 120 h.

	2HG (ng/mL)	Accuracy (%)			
		24 h	48 h	96 h	120 h
Unspiked	150	100.4 ± 2.3	109.3 ± 0.7	101.7 ± 3.3	113.9 ± 1.8
Serum QC-1	250	101.1 ± 0.5	100.0 ± 0.0	100.8 ± 0.7	102.9 ± 0.3
Serum QC-2	2500	97.5 ± 3.1	100.1 ± 0.0	97.0 ± 2.2	99.7 ± 0.0
Serum QC-3	25,000	100.2 ± 0.8	99.9 ± 1.1	98.5 ± 0.6	99.5 ± 2.6

Table 7
Characteristics of 2HG validated methods for serum 2HG quantification.

	Poinsignon et al. [13]	He et al. [12]	This method
Method	LC-MS	GC–MS	GC–MS
Extraction	Solid phase extraction	Liquid:liquid (methanol)	Liquid:liquid (ethyl acetate)
Derivatization	DATAN (80 min, 40 °C)	BSTFA + TMCS (60 min, 60 °C)	BSTFA + TMCS (3 min, MAD)
Retention time (min)	5	11	8
Run time (min)	8	–	21
Total vs enantiomers	D and L enantiomers	Total 2HG	Total 2HG
Sample volume (μL)	250	100	300
Linear range (ng/mL)	50–20,000	36–780	10–50,000
LOQ (ng/mL)	30	36	10
Interday accuracy	< 5.3%	–	< 7.8%
Interday precision	< 3.1%	< 9.0%	< 7.1%

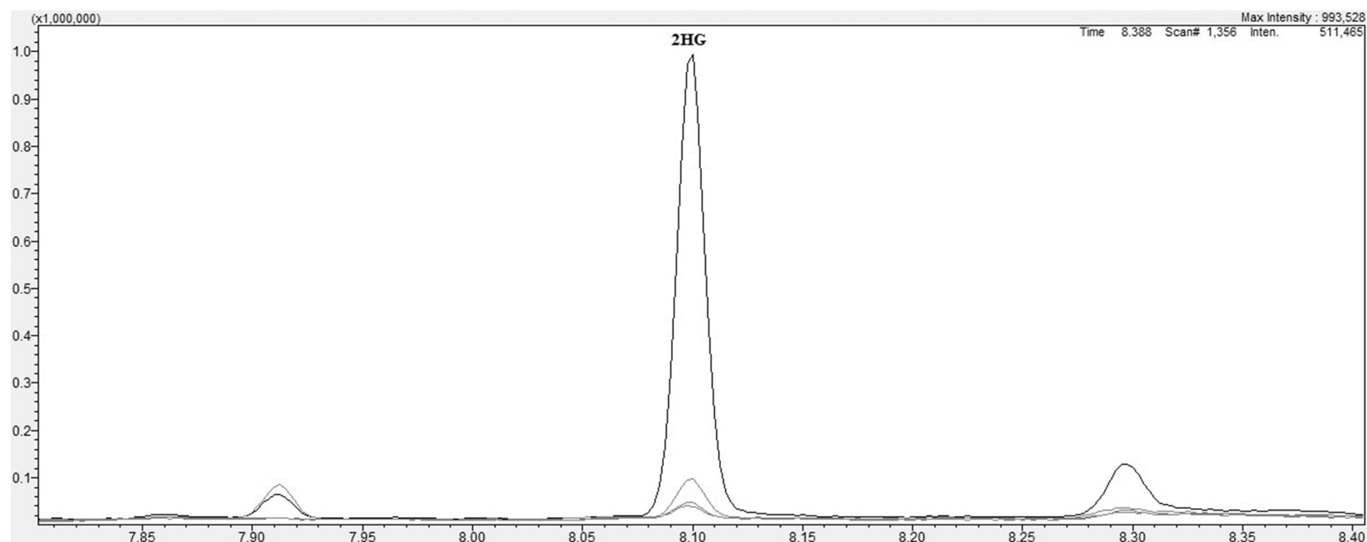


Fig. 3. Superimposed chromatograms (*m/z* 247) of one serum sample of a patient with acute myeloid leukemia (AML) and *IDH2* mutation (black) and three additional patients with AML lacking *IDH1–2* mutations (grey).

Table 8
Median serum levels of 2HG in AML patients without or with IDH1/2 mutation.

2HG levels (non IDH1/2 mutation)	2HG levels (IDH1/2 mutation)	Method	Reference
61 ng/mL	3004 ng/mL	LC-MS	[8]
178 ng/mL	3170 ng/mL	GC–MS/MS	[17]
87 ng/mL	1863 ng/mL	LC-MS	[9]
28 ng/mL	545 ng/mL	GC–MS	This study

low number of patients included in our evaluation. However, as can be observed in Fig. 3 and Table 8, the patient with *IDH2* mutation presented > 10-fold higher serum levels of 2HG than patients without *IDH1–2* mutations.

4. Conclusions

A sensitive GC–MS method was developed and validated for the quantitative measurement of 2HG in human serum. A simple liquid-liquid extraction method was used and derivatization was reduced to a 3 min microwave irradiation. The intra- and inter-assay precision and accuracy of this method are well within the EMEA guideline limits. 2HG in serum was found to be stable under different storage conditions. The technique was assessed and found to be free of matrix effect as determined by experiments involving different serum sources. This method is useful for quantification of 2HG serum levels in patients with IDH mutated malignancies in clinical laboratories.

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