Journal of Chromatography B, 876 (2008) 123–128

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Quantitative analysis of 5′-deoxy-5′-methylthioadenosine in melanoma cells by liquid chromatography-stable isotope ratio tandem mass spectrometry

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ARTICLE INFO

*Article history:*
Received 17 September 2008
Accepted 23 October 2008
Available online 30 October 2008

*Keywords:*
5′-Deoxy-5′-methylthioadenosine
Methylthioadenosine phosphorylase
LC–MS/MS
Stable isotope dilution
Melanoma

ABSTRACT

The frequent deletion of the human chromosomal region 9p21, including the methylthioadenosine phosphorylase (*MTAP*) gene, is hypothesized to lead to the intra- and/or extracellular accumulation of 5′-deoxy-5′-methylthioadenosine (MTA) in cancer cells and the subsequent promotion of tumor progression. The lack of sensitive methodology for the direct measurement of MTA in tumor cells has hampered the testing of this hypothesis to date. A liquid chromatography electrospray ionization tandem mass spectrometry method (LC–MS/MS) was developed for the absolute quantitative determination of MTA in cell culture media and cell extracts using stable isotope labeled MTA as an internal standard. Limit of detection (LOD) and lower limit of quantification (LLOQ) were 62.5 pM and 2 nM, respectively, and allowed the direct measurement of MTA in biological samples without prior enrichment. Average imprecision of MTA extraction from cells and cell media, as well as LC–MS/MS analysis were 9.7, 3.8 and 1.9%, respectively. The method enabled the demonstration of the accumulation of MTA in melanoma cell culture media reaching a steady-state level within 24 h. Only a slight difference in extracellular MTA concentrations was observed between cells with and without MTAP expression. However, there was a fourfold increase in intracellular MTA concentration in melanoma cells lacking MTAP, thus confirming the hypothesized accumulation of MTA in human cancer cells harboring a chromosome 9p21 deletion.



## 1. Introduction

5′-Deoxy-5′-methylthioadenosine (MTA) is formed from S-(5′-deoxy-5′-adenosyl)-L-methionine, a condensation product of adenosine and L-methionine, in the course of spermidine synthesis, by loss of the alanine moiety [1]. The first step of MTA catabolism is the cleavage of MTA to adenine and methylthioribose-1-phosphate, an enzymatic reaction catalyzed by MTA phosphorylase (*MTAP*, MIM 156540) [2]. MTA is ubiquitously distributed in rat tissues at concentrations of 2–7 nmol/g fresh tissue weight as determined by HPLC–UV after sample pre-concentration [3]. Cancer cells commonly lack MTAP and, consequently, MTA is presumably not metabolized but accumulates intracellularly and/or is excreted, thereby increasing the invasive potential of the cancer cells due to molecular mechanisms yet to be elucidated in detail [4–8]. It was furthermore observed that tumor cells lacking MTAP show a worse response to interferon therapy [9].

A major impediment to the study of MTA and its role in cancer has been the lack of a sensitive and specific analytical method for its direct quantification in cell lysates and cell culture media. Mass spectrometry has been applied to the analysis of urinary MTA. Liquid chromatography coupled to an ion trap MS and MALDI-TOF-MS yielded lower detection limits (LODs) in the lower pmol range [10] and 100 fmol [11], respectively. However, data on absolute quantification, reproducibility and accuracy were reported for neither method. Further, there have been reports on the HPLC separation of MTA and its detection by means of UV absorbance [12–14]. Although LODs as low as 1 nmol were obtained using boronate affinity chromatography for pre-concentration of MTA, LC/UV has proven too insensitive and insufficiently selective for the direct analysis of MTA in tumor cells.

Here, we report the development of a sensitive and selective analytical method for the quantitative determination of MTA by means of stable isotope ratio liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) and its successful application to the *in vitro* measurement of the extra- and intracellular accumulation of MTA in various melanoma cancer cell lines.

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doi:10.1016/j.jchromb.2008.10.038

## 2. Experimental

### 2.1. Chemicals

All solvents for sample preparation and LC–MS were HPLC grade and purchased from Fisher-Scientific (Schwerte, Germany). MTA was obtained from Sigma–Aldrich (Taufenkirchen, Germany) and labeled adenosine from Omicron Biochemicals (South Bend, IN, USA). The water used was purified by means of a PURELAB Plus system (ELGA LabWater, Celle, Germany). All chemicals for synthesizing labeled MTA (thionylchloride, pyrimidine, ammonia and sodium methanethiolate) were purchased from Fluka (Taufenkirchen, Germany).

### 2.2. Internal standard preparation

A 2.24 mM stock solution of stable isotope labeled [1′,2′,3′,4′,5′-$^{13}C_5$]MTA was synthesized (purity 96.1%) in house according to Robins et al. [15]. The stock was diluted with water to obtain a working solution of 224 nM [1′,2′,3′,4′,5′-$^{13}C_5$]MTA, which was spiked into the samples and calibration standards. The final concentration of the internal standard in samples and standards was 22.4 nM.

Stock solutions of unlabeled MTA were prepared in water and serially diluted over a concentration range of 12.5 pM to 1 mM. Spiking with internal standard was done immediately before calibration. For calibration, 10 μL of internal standard were transferred into a 0.2-mL micro-insert (VWR, Darmstadt, Germany) in a 1.5-mL glass vial (Fisher-Scientific), dried by evaporation using an infrared vortex-vacuum evaporator (CombiDancer, Hettich AG, Bäch, Switzerland), and then reconstituted with 100 μL of the respective aqueous MTA standard.

### 2.3. Cell culture

The melanoma cell line Mel Im has been described in detail previously [16]. Further, two clones of Mel Im that either lack (Mock D) or express (Clone 5) *MTAP* were used [5]. Cells were grown at 37 °C/5% $CO_2$ in Dulbecco's modified Eagle medium (DMEM; PAN Biotech GmbH, Aidenbach, Germany) supplemented with penicillin (400 U/mL), streptomycin (50 μg/mL), L-glutamine (300 μg/mL) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany).

Melanoma cells, 200,000 each, were seeded in Falcon™ six-well-plates (Becton Dickinson GmbH, Heidelberg, Germany) and cultured in 2 mL DMEM as described above, for different periods of time. If not stated otherwise, cells were cultured for 24 h and were then harvested with trypsin (PAN Biotech GmbH).

### 2.4. Extraction of MTA from cell culture media

Methanol (600 μL) was added to 200 μL of cell culture medium, followed by the addition of 10 μL internal standard (224 nM). The sample was vortexed and centrifuged at 9000 × *g* for 5 min at 4 °C. The supernatant was transferred to a glass vial. The protein pellet was washed twice with 200 μL methanol and all supernatants were combined. The solvent was evaporated and the residues were reconstituted in 100 μL water.

A matrix spike and a standard addition experiment were carried out in cell culture media to check the efficiency of the extraction procedure and to evaluate ion suppression. To generate a representative matrix sample, 500-μL aliquots from 13 different cell culture media were combined. For the matrix spike experiment, aliquots of 200 μL of the matrix sample were spiked with MTA at three molar levels (resulting in final concentrations of 25, 50 and 75 nM of MTA after reconstitution) and extracted as described above. For the standard addition, 200-μL aliquots of the matrix sample were extracted as described above. The obtained extract was then spiked with MTA at three levels (resulting in final concentrations of 25, 50 and 75 nM after reconstitution). All samples were fortified with stable isotope labeled internal standard prior to extraction.

### 2.5. Cell harvesting and extraction of MTA from cell pellets

The adherently growing cells used in this study were harvested by incubation in 200 μL of either a solution containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA or 5 mM EDTA only. Trypsination was stopped after 5 min with 700 μL of cell culture medium. Following centrifugation at 100 × *g* (5 min, room temperature), the supernatant was removed and the cell pellets were stored at −80 °C until extraction. Alternatively, cells were scraped directly into pure methanol. To that end, 500 μL methanol spiked with 2.48 nmol/L stable isotope labeled MTA were added to the well after the cell culture medium had been removed and the cells were washed with PBS buffer. Cells were scraped, centrifuged (100 × *g*, 5 min, room temperature) and the supernatant was collected. The cell pellet was washed twice with 200 μL methanol, centrifuged and all supernatants were combined. After drying and reconstitution in 100 μL of water the concentration of the internal standard was 22.4 nM in case of 100% recovery.

For the extraction of MTA from cell pellets, different solvent combinations (MeOH, MeOH/$H_2O$ 50:50 (v/v), acetonitrile, and acetonitrile/$H_2O$ 50:50 (v/v)) were tested. Briefly, 600 μL of the different solvents were added to the frozen pellets and internal standard was spiked as described above. Then, the sample was slowly thawed on ice. To complete the cell lysis the cells were again shock-frozen in liquid nitrogen and thawed on ice. The freeze/thaw cycle was performed three times and the sample was vortexed in between each cycle. The sample was centrifuged at 9000 × *g* for 5 min at 4 °C and the supernatant was transferred to a 1.5-mL glass vial. The pellet was washed twice with 200 μL of methanol and all supernatants were combined. The extract was further treated as described above.

For cell pellets, a standard addition experiment was carried out to evaluate ion suppression. The cell pellets were prepared as described above and a representative set of cell extracts was pooled. The experiment was carried out with cells removed by trypsin and EDTA, respectively. Ten microliters of MTA standard solution with concentrations of 2.5, 5.0 and 7.5 μM were dried and then reconstituted with 100 μL of the pooled cell extracts. All concentrations were prepared in triplicate.

### 2.6. Instrumentation

LC–ESI-MS/MS was performed using an Agilent 1200 SL HPLC system (Böblingen, Germany) and a PE Sciex API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany), which was equipped with a turbo ion spray source (completely controlled by Analyst version 1.4.2). The column oven was kept at 25 °C. An Atlantis T3 3 μm (1.0 mm i.d. × 150 mm) reversed phase column (Waters, Eschborn, Germany) was used. LC separation was carried out using a mobile phase consisting of 0.1% acetic acid in water (Solvent A) and 0.1% acetic acid in acetonitrile (Solvent B). The gradient employed was as follows: 0–10 min linear increase from 0 to 100% solvent B, hold at 100% solvent B for 5 min. The flow-rate was set to 125 μL $min^{-1}$. Sample volumes of 10 μL were injected.

The API 4000 QTrap mass spectrometer was operated in positive mode using turbo ion spray with the following parameters: gas 1 as 50, gas 2 as 30 and the curtain gas as 10 (all arbitrary units). The turbo ion spray source was heated to 250 °C. The declustering potential was set to 60.0 V and the entrance potential to 10.0 V.

Quantitative determination was performed in the multiple reaction monitoring (MRM) mode using the following ion transitions: $m/z$ 298.2 $(M+H)^+$ to $m/z$ 136.1 (product ion) for MTA and $m/z$ 303.2 $(M+H)^+$ to $m/z$ 136.1 (product ion) for the internal standard ($^{13}C_5$-labeled MTA). Collision-induced dissociation was performed with nitrogen as collision gas. The collision energy and the collision exit potential were set at 23 eV and 9 V, respectively. The electron multiplier was set to 2100 V. All MS parameters were optimized by direct infusion and the source parameters by flow injection. Data analysis was performed using Analyst version 1.4.2.

### 2.7. Data analysis

Standard calibration curves were plotted as the chromatographic peak area ratio (MTA/IS) versus the corresponding nominal concentration ratio (MTA/IS). A $1/x^2$ weighted regression analysis was used to determine the slope, intercept and coefficient of determination ($r^2$). A $t$-test was used to determine whether the means of the intra- and extracellular amounts of MTA measured in Mel Im clones either lacking or showing expression of MTAP are distinct.

## 3. Results and discussion

### 3.1. Method validation

Fig. 1 shows a product ion spectrum of MTA after ionization in positive mode. At $m/z$ 298.1, the quasi molecular ion of MTA occurs and the main product ion is the protonated adenine at $m/z$ 136.1, which was used to set up the MRM transition for quantitative analysis. Representative MRM-chromatograms of an MTA standard, a cell culture medium sample and a cell pellet sample are shown in Fig. 2. As can be seen, MTA elutes as a symmetric peak without any interferences even in the sample extracts.

A calibration was carried out using standards in the range of 12.5 pM to 1 mM. During analysis, it was observed that the internal standard contains minute amounts of unlabeled MTA. Therefore, a calibration without internal standard was performed to determine the instrumental detection limit. This resulted in a LOD of 62.5 pM at a signal to noise ratio ($S/N$) of 3. The lower limit of quantification (LLOQ) was defined as five times the background MTA level. Due to the contamination of the internal standard with unlabeled MTA the LLOQ was 2 nM and could be determined with an accuracy of 100.15% and an imprecision of 2.96%. This concords well with the FDA guidelines for bioanalytical method validation that require an analyte response at the LLOQ of at least five times the response compared to a blank and an accuracy and imprecision of 80–120 and <20%, respectively [17]. The calibration curve was linear from the LLOQ to 1 μM (intercept: 0.0212; slope: 0.949; $r^2$: 0.999). The relative standard deviation (R.S.D.) of triplicate injections was 1.77%.

A matrix spike and a standard addition experiment of MTA in cell culture medium samples were carried out. Fig. 3A shows the results in comparison to the calibration curve over this concentration range. R.S.D.s for triplicate samples in the matrix spike and standard addition ranged from 1.3 to 7.0 and 1.6–5.9%, respectively. It can be seen that the internal standard corrects very well for potential ion suppression or incomplete extraction because the slope of the three curves is similar. To further evaluate the extraction efficiency, the absolute areas of the MTA without internal standard correction were plotted versus the concentration (Fig. 3B). Interestingly, the R.S.D. values were still in the range of 0.9–3.9 and 1.9–5.2% for the matrix spike and standard addition, respectively. Fig. 3B shows a nearly identical slope for the matrix spike and the standard addition experiment. This demonstrates that extraction of MTA from the sample is absolute. Using the values of the standard addition experiment as reference, recovery rates were calculated for the matrix spike samples and found to range from 94.6 to 112.4%. However, the slope of matrix spike and standard addition experiments is only 60% of the slope of the calibration curve, indicating substantial ion suppression. This observation is identical to the recovery of the internal standard from cell culture media, namely about 60%.

In addition to MTA excretion into the cell culture media, the intracellular levels were determined. For cell pellet extraction (pellets of 1,000,000 cells), different solvent combinations (MeOH, MeOH/$H_2O$ 50:50 (v/v), acetonitrile and acetonitrile/$H_2O$ 50:50 (v/v)) were tested. The highest amount of extracted MTA and the

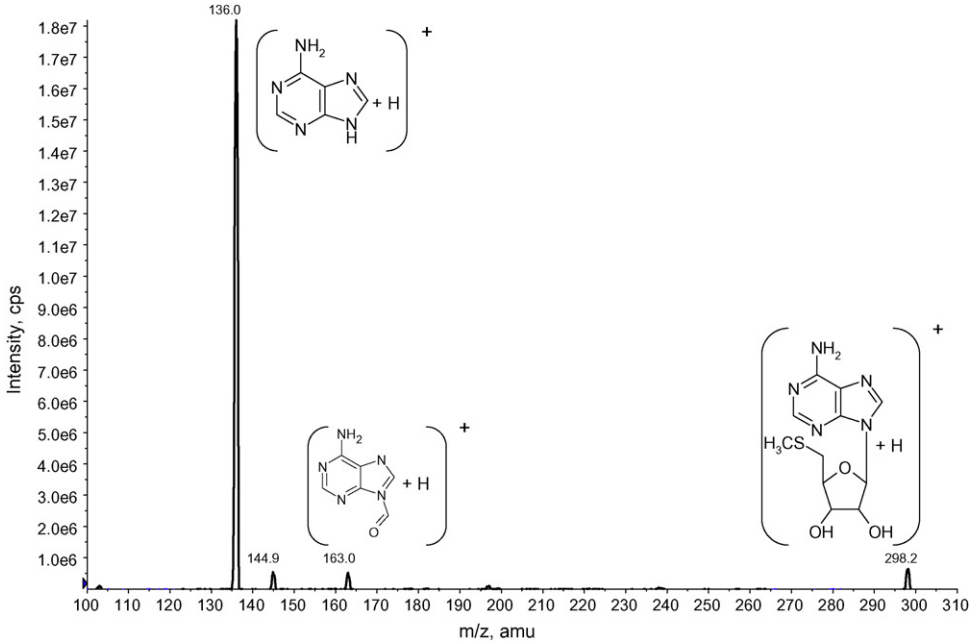


**Fig. 1.** Product ion spectrum of MTA obtained on a PE Sciex API 4000 QTrap mass spectrometer. Source temperature: 250 °C; declustering potential: 60 V; collision energy: 23 eV.

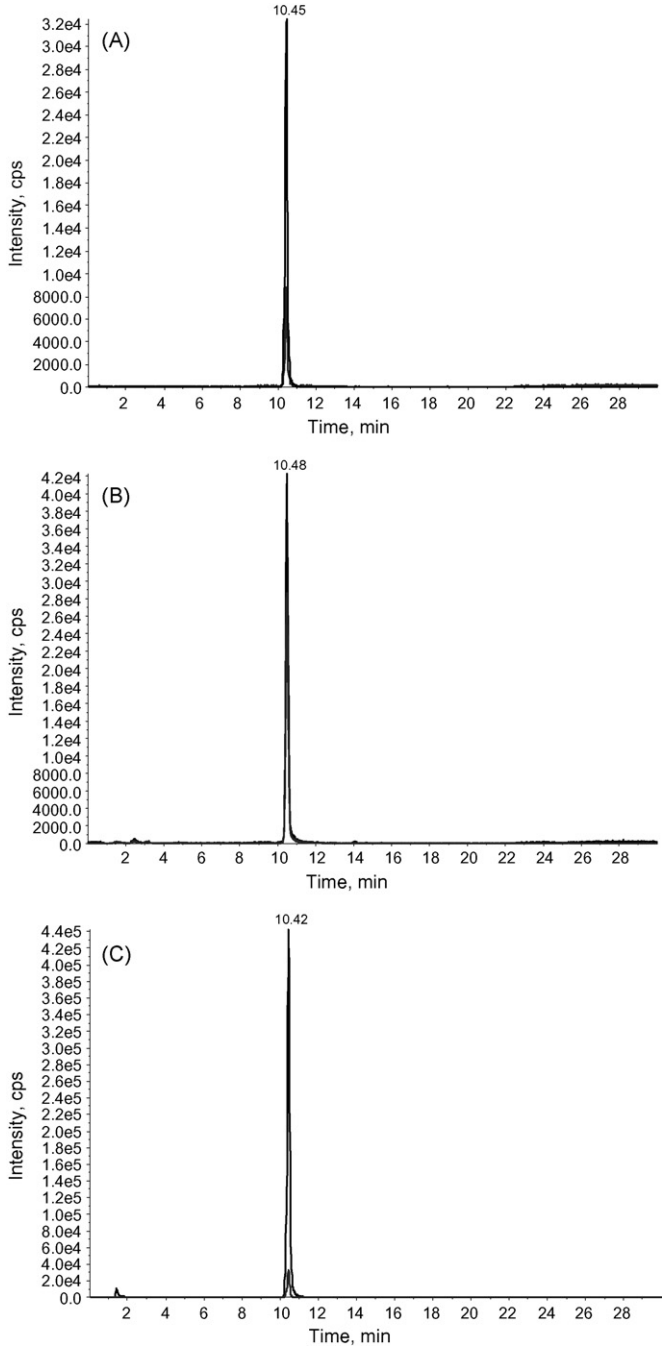


**Fig. 2.** MRM-chromatograms of co-eluting unlabeled and stable-isotope labeled MTA (minor peak). (A) MTA standards, and methanol extracts of (B) cell culture medium and (C) Mel Im cell pellet. Column: Waters Atlantis T3 3 μm 1.0 mm × 150 mm; mobile phase: 0.1% acetic acid; gradient: 0–100% acetonitrile in 10 min; flow-rate: 125 μL min$^{-1}$; column temperature: 25 °C.

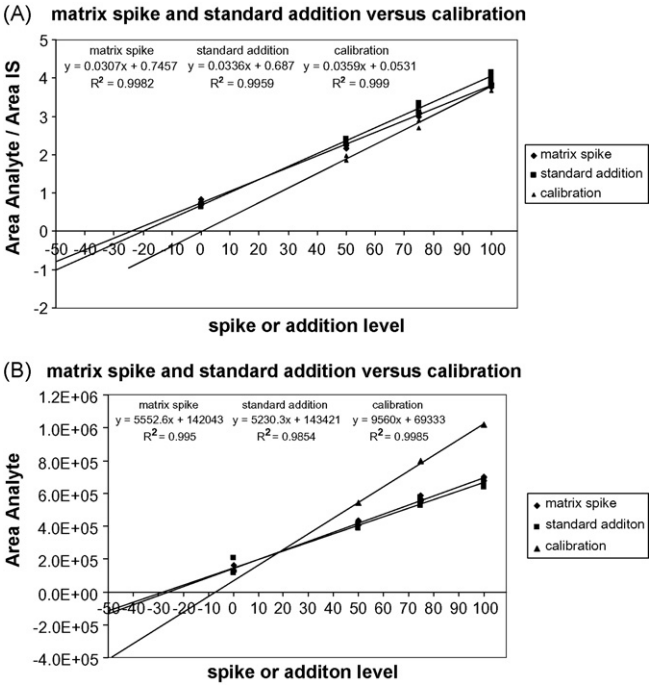


**Fig. 3.** Matrix spike and standard addition experiment for cell media versus calibration curve with internal standard correction (A) and matrix spike and standard addition experiment for cell media versus calibration curve without internal standard correction (B).

best recovery of the internal standard were achieved with pure methanol (see Fig. 4).

Leakage of the adherently growing cells during harvesting can present a serious problem, if intracellular metabolite concentrations need to be determined. The conventional method to release cells is trypsination. In addition, we tested a reportedly less aggressive method, namely a 5-mM EDTA solution. We observed very similar amounts of extracted MTA in both instances. Hence, cell leakage must be similar (Fig. 5). We also tested cell harvesting by scraping the cells directly into methanol spiked with IS, so that all

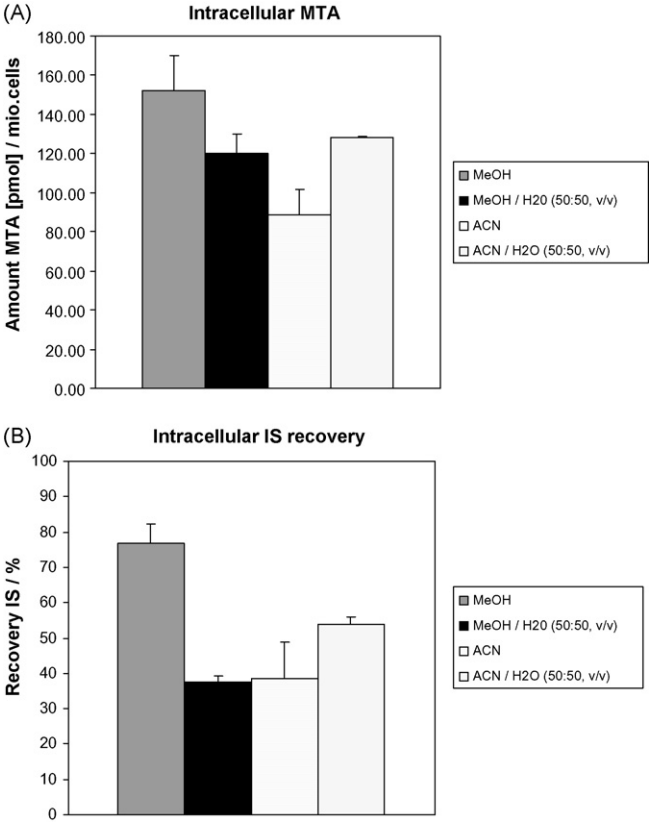


**Fig. 4.** Detected absolute amount of MTA (A) and recovery of internal standard (B) in pellets of 1,000,000 cells each using different extraction protocols.

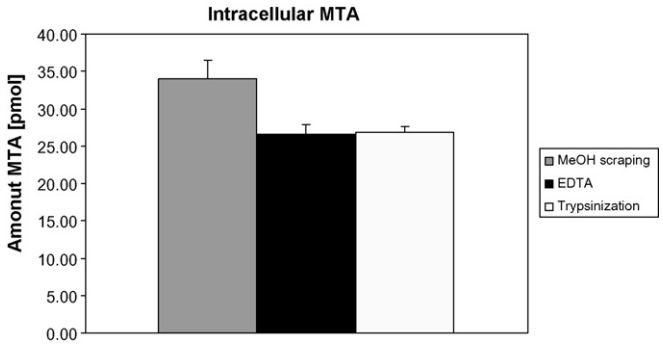


**Fig. 5.** Arithmetic means and standard deviations of intracellular amount of MTA for three different cell harvesting procedures after incubation of 200,000 seeded Mel Im cells for 24 h.

of the intracellular MTA would be extracted. With this extraction protocol, the amount of extracted MTA is about 40% higher than for the other two methods (Fig. 5). However, direct scraping does not allow the determination of cell count, a parameter often used for normalization in cell culture experiments. Overall, trypsination allowed the extraction of MTA and recovery of internal standard with the lowest imprecision, namely 2.82 and 2.33%, respectively. The respective values for EDTA were 5.32 and 4.52%, while those for scraping in pure methanol were 7.41 and 15.28%.

For cell pellets a standard addition experiment was carried out. Fig. 6 shows the results of the standard addition experiment (A) and the recovery of the internal standard (B). Spike recoveries ranged from 70.44 to 81.87% and from 66.13 to 68.65% for EDTA and trypsination, respectively. Interestingly, the recovery of the internal standard was between 95 and 97% for cells removed with EDTA, but only 75% for trypsinated cells. No significant difference in extracted MTA amount could be observed. The R.S.D.s of the absolute MTA peak areas ranged from 0.6 to 3.7 and 0.2 to 0.9% for cells treated with EDTA and trypsin/EDTA, respectively.

Based on these results, trypsination and pure methanol were used in all subsequent experiments for cell detachment and the extraction of MTA from cell pellets, respectively.

During sample analysis, quality controls (QCs) were measured after every 10–15 biological samples. The QC was 25 nM of MTA spiked with stable isotope labeled MTA. The calibration check samples yielded accuracy values of approximately 96%, and the recovery of the internal standard was about 97% in all QCs. The measured blanks (pure water) did not show MTA or internal standard.

### 3.2. MTAP activity in FCS and stability of MTA

In 1984, Riscoe et al. [18] observed MTAP activity in both native and heat-inactivated human and fetal calf sera. To investigate potential degradation of MTA during cell culture experiments, cell culture media with and without FCS were spiked with an MTA standard (with a final concentration of 300 nM) and incubated for 24 h at 37 °C, to check for MTAP activity in FCS. To determine a potentially time dependent decrease of the MTA concentration, samples were taken in triplicate at 0, 8 and 24 h. Fig. 7 shows the concentration of MTA in both experiments. No significant change ($P = 0.96$) in the MTA concentration was observed in the presence or absence of FCS, thus demonstrating that there is no significant MTAP activity in FCS, and that MTA is stable over 24 h at 37 °C.

### 3.3. MTA and melanoma cell lines

To measure the extracellular accumulation of MTA, 200,000 cells each of the parental Mel Im cell line were seeded in triplicate and cultured in 2 mL DMEM for 0, 2, 4, 6, 8, 16, 24, 30, 48, 72 and 96 h, before MTA was extracted from the cell medium and analyzed by LC–MS/MS. Extracellular concentrations of MTA reached a steady-state concentration of approximately 20 nM in the first 24 h of incubation (data not shown).

As a first examination of the hypothesis that cells lacking MTAP do not metabolize MTA, two Mel Im cell clones that either lack (Mock D) or show expression of MTAP (Clone 5) [5] were analyzed. The clones showed a slight, albeit significant ($P = 0.0086$), difference in extracellular MTA concentrations measured in quadruplicate after 24 h of incubation. The respective values for Mock D and Clone 5 were $11.6 \pm 0.2$ and $12.7 \pm 0.4$ nM. Intracellular amounts of MTA, in comparison, differed by a factor of 4 with $44.3 \pm 3.5$ pmol in Mock D versus $11.5 \pm 5.0$ pmol in Clone 5 ($P = 0.0012$). These values correlate inversely with MTAP expression.

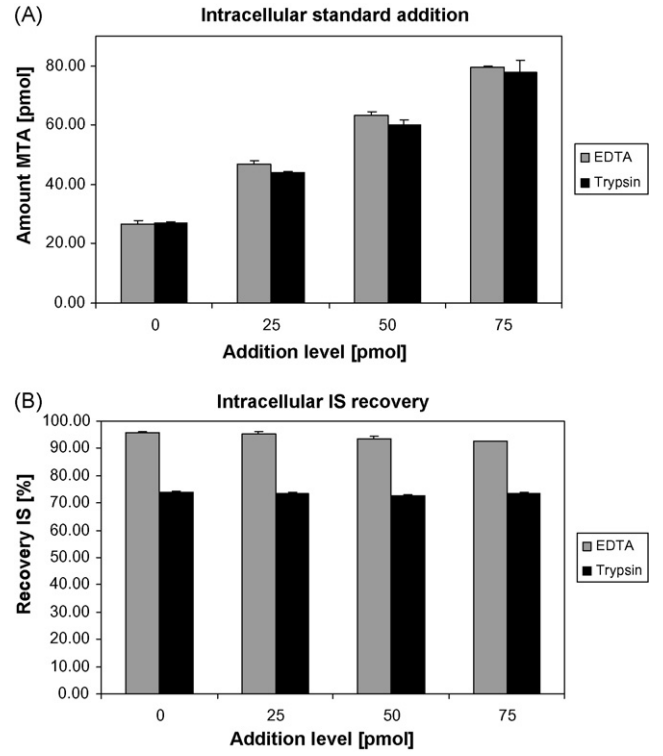


**Fig. 6.** Arithmetic means and standard deviations of intracellular amount of MTA (A) and internal standard recovery (B) in standard addition experiments employing pellets of 200,000 Mel Im cells each.

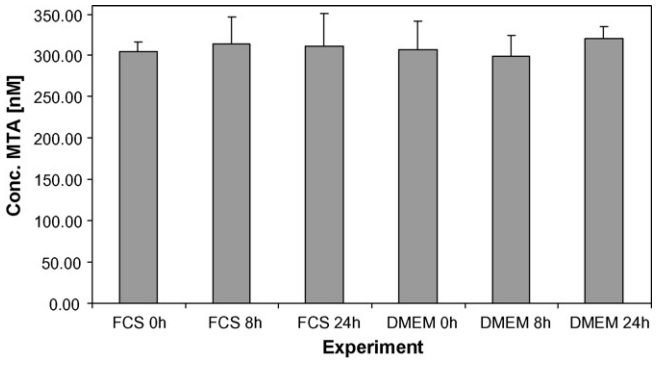


**Fig. 7.** Stability of MTA in cell culture medium (DMEM) with and without FCS over 24 h at 37 °C. Arithmetic means and standard deviations of MTA are shown at 0, 8 and 24 h (DMEM–DMEM without FCS; FCS–DMEM with FCS).

## 4. Conclusions

An LC–ESI-MS/MS method was developed for the quantitative determination of MTA in cell culture media and cell pellets. The method is sensitive, specific, precise and accurate in the concentration range of 2 nM to 3 μM. The LOD was determined at 62.5 pM and the LLOQ at 2 nM. The method is reproducible with an RSD of triplicate injections of 1–7% in cell media and 0.2–3.7% in cell extracts. All quality controls showed an accuracy of 95–97% with an internal standard recovery of 97%.

The method developed will prove essential in the elucidation of the molecular mechanism, by which MTA promotes tumor progression, and the utility of MTA as a diagnostic and/or prognostic tumor marker.

## Acknowledgements

This study was supported by BayGene and the intramural ReForM C program. A.P.S. would like to thank Martin Link for help in the synthesis of the internal standard.

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