Metabolomics of Opiate-Induced Changes in Murine Brain by GC/Q-TOF

Application Note

Metabolomics

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Abstract

A study was performed to elucidate opiate-induced metabolic changes in murine brain. The EI MS, EI MS/MS, and PCI capabilities of the Agilent 7200 Series GC/Q-TOF MS, in combination with Agilent MassHunter Software tools, enabled a very flexible and comprehensive workflow for identifying metabolomic differences. The workflow was used to distinguish between morphine-sensitive and resistant murine strains, determine differences in their response to morphine administration, and identify compounds using various techniques.

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Introduction

Metabolomics is a powerful approach for investigating the biochemistry of morphine addiction, as it provides the most direct information about a system's physiological state. There is currently very little information available regarding metabolomic changes that mediate neurobehavioral responses. It is likely that clinically important opiate responses could be mediated by opiate-induced metabolomic changes.

This application note describes a study that employed an untargeted metabolomics approach using gas chromatography/mass spectrometry (GC/MS) to characterize opiate-induced metabolic changes in murine strains that display drastically different levels of sensitivity to morphine. The Agilent 7200 GC/Q-TOF was used in order to take advantage of the accurate mass information, full spectrum sensitivity, MS/MS capabilities, and both EI and CI modes. This approach was combined with the full featured capabilities of Agilent MassHunter and Agilent Mass Profiler Professional (MPP) software to enable identification and confirmation of metabolites whose levels were changing in these murine strains. It was of particular interest to identify inter-strain differences in metabolite levels that happened in response to morphine administration, since these changes help in understanding the mechanism of morphine addiction. This metabolomics study revealed a significant change in adenosine levels between control and morphine administration in a morphine-sensitive murine strain. We also observed changes in the levels between the two strains for a few other metabolites, including adenosine 5'-monophosphate, glyceric acid, cholesterol, and the neurotransmitter N-acetylaspartylglutamic acid.

Experimental

Materials

C57BL/6 and 129Sv1 murine strains (males, 7–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Morphine was obtained from Sigma Chemicals (St. Louis, MO).

Instruments

This study was performed on an Agilent 7890B GC system, coupled to an Agilent 7200 GC/Q-TOF system. The instrument conditions are listed in Table 1.

Table 1. GC and Mass Spectrometer Conditions

GC run conditions	
Column	Agilent DB-5 MS Ultra Inert, 30 m × 0.25 mm, 0.25 µm film (p/n 122-5532UI)
Injection volume	1 µL
Split mode ratio	Split 10:1 (EI) and Splitless (PCI and MS/MS)
Inlet temperature	250 °C
Oven temperature program	60 °C for 1 minute, 10 °C/min to 325 °C, 3.5 minutes hold
Carrier gas	Helium at 1 mL/min constant flow
Transfer line temperature	290 °C
MS conditions	
Ionization mode	EI, positive CI (20% methane flow)
Source temperature	230 °C
Quadrupole temperature	150 °C
Mass range	40 to 600 *m/z*
Spectra acquisition rate	5 Hz, collecting both in centroid and profile modes

Sample preparation

Mice were administered either with morphine for four consecutive days, or with saline control over the same period of time. Brainstem tissue was collected from eight week old male C57BL/6 (morphine-sensitive) and 129Sv1 (morphine-resistant) mice in seven to eight biological replicates. Metabolites were extracted by the Folch method [1]. The aqueous fraction was collected, dried under vacuum, and subsequently derivatized by methoximation using a saturated solution of hydroxylamine HCl in pyridine followed by silylation with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and 1% trimethylchlorosilane (TMCS).

Data processing and statistical analysis

The data were processed by chromatographic peak deconvolution using the Unknowns Analysis tool from MassHunter Quantitative Analysis Software package, followed by compound identification by comparison to the Agilent Fiehn GC/MS Metabolomics Retention Time Locked (RTL) Library. Molecular Structure Correlator (MSC) software was used to further validate the structures of tentatively identified compounds.

Statistical analysis was performed by Mass Profiler Professional (MPP), a multivariate statistical analysis package. It was used to visualize data clustering as well as to determine significant differences in compound abundance levels between pairs-wise conditions.

Results and Discussion

Chromatographic peak deconvolution and library search

Chromatographic peak deconvolution using the Unknowns Analysis tool was able to find approximately 700 components in each sample (Figure 1). The corresponding hits were assigned using the Agilent-Fiehn metabolomics MS library with a Match Factor score > 50 for approximately 70–100 components in each sample. After all the components that did not display significant changes were filtered out in MPP, the unidentified components, as well as those identified by the Agilent-Fiehn library were searched against the NIST MS library for confirmation and identification of the components not present in the Agilent-Fiehn library. Some of the abundant components that did not have a good match in either library but did display significant changes, were taken through additional analysis steps such as elucidation of the empirical formula using PCI and EI MS/MS, as well as proposal of a structure using accurate mass information and the Molecular Structure Correlator (MSC) software tool, as will be described later.

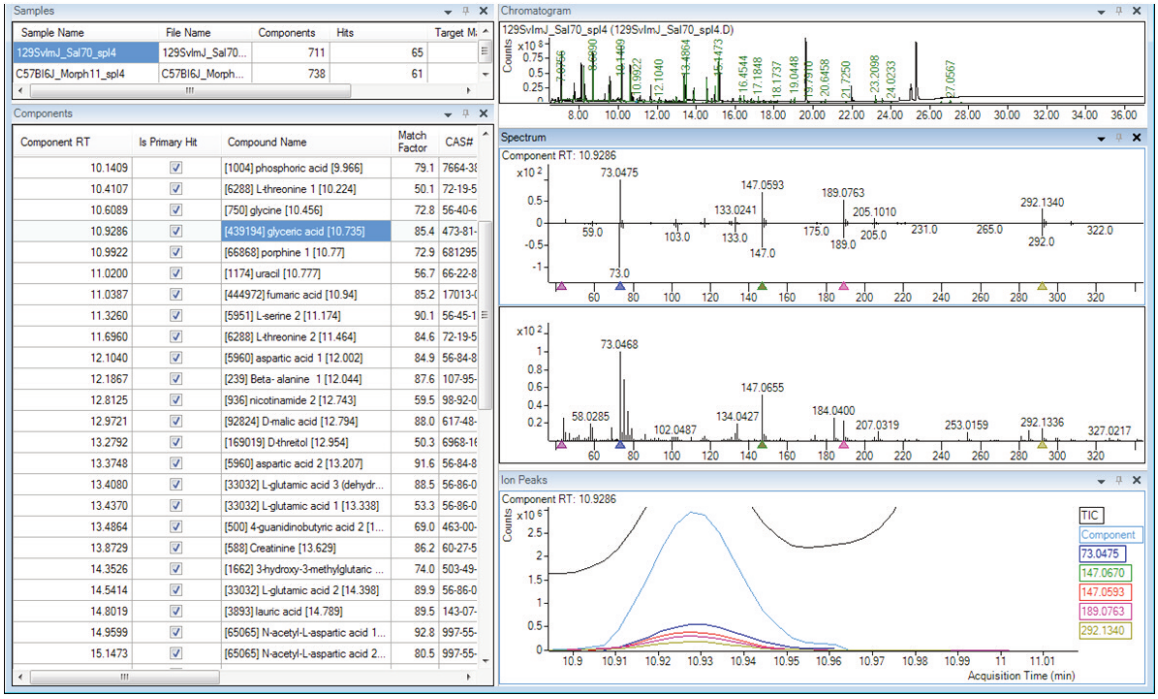

Figure 1. The Unknowns Analysis tool was used to perform deconvolution and the initial library search. The lower right-hand panel shows deconvoluted ions of the component that have the same peak shapes, thus confirming that they all belong to the same component.

Approximately 60 metabolites previously identified in each sample using the Agilent-Fiehn unit mass metabolomics library were confirmed using accurate mass information. This allowed us to rule out any false positives. The Fragment Formula Annotation (FFA) tool in MassHunter Qualitative Analysis Software was combined with library search results to enable easy confirmation of an identified compound (Figure 2). A fragment is annotated and colored green when the formula of the fragment is a subset of the molecular formula identified by a library search. This is useful for rapid confirmation of the compound identity (Figure 2). The majority of identified metabolites represented amino acids, organic acids, and carbohydrates (Figure 3).

Statistical analysis workflow

Analysis of metabolomics data is often a tedious and time-consuming process. MPP software is ideal for filtering, interpretation, sample model creation, and prediction; this is required to efficiently evaluate complex and noisy data. MPP provides an easy-to-follow workflow that helps the user decide how best to evaluate their data. For more sophisticated users, MPP also provides access to different statistical operations, and users can treat their data in many different ways in order to optimize their data analysis (see the Mass Profiler Professional brochure 5990-4164EN for further details).

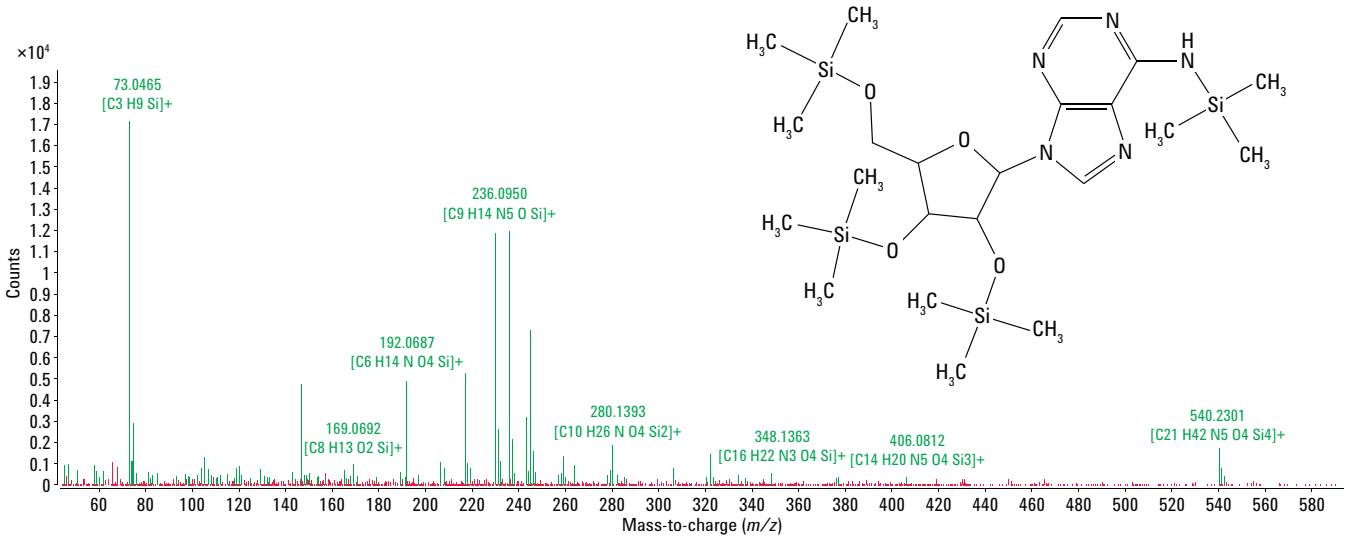

Figure 2. Annotated spectrum of one of the compounds (adenosine) using FFA in Agilent MassHunter Qualitative Analysis. When the library hit was found, FFA automatically recognized the molecular ion (MI), and, based on the empirical formula of the hit and accurate mass spectral data, it assigns fragment formulas. These annotated ions are colored in green. Whenever a good match for an ion fragment cannot be found, the ions from the spectrum have the original color (red).

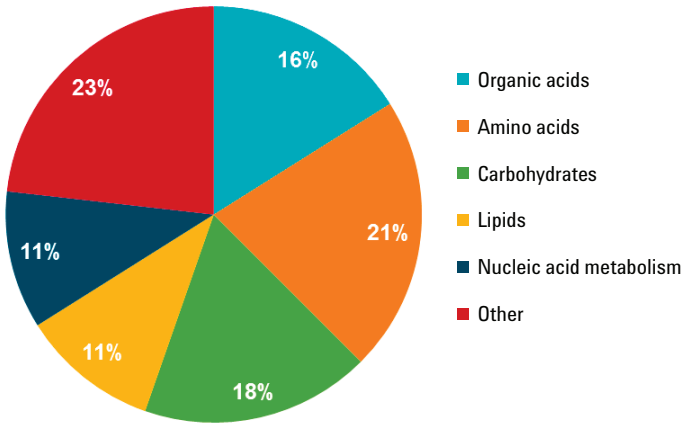

Figure 3. Distribution by metabolite class of the 60 compounds identified using the Agilent-Fiehn library.

Once deconvolution was completed in Unknowns Analysis, compound information was saved in an xml-based, compound exchange file format (.CEF). These files were imported into MPP to determine which compounds had significant changes in their abundance levels between pairs of conditions or murine strains. An important part of the MPP workflow is a correct setup of the compound filters to ensure that a significant proportion of the noise in the data is filtered out. This is so that it does not interfere with statistical analysis. The inherent degree of variance in the data was evaluated using Principal Component Analysis (PCA) (Figures 4 and 5), followed by significance and fold-change analysis visualized by Volcano Plots (Figures 6 and 7).

Evaluation of data using Principal Component Analysis

PCA is a frequently employed unsupervised multivariate statistical analysis technique for data dimensionality reduction. It is performed through the transformation of measured variables into uncorrelated principal components, each being a linear combination of the original variables. Figures 4 and 5 show representative examples of PCA analyses. The PCA plots reveal clear separation between control and morphine-administered groups, as well as separation between the two murine strains.

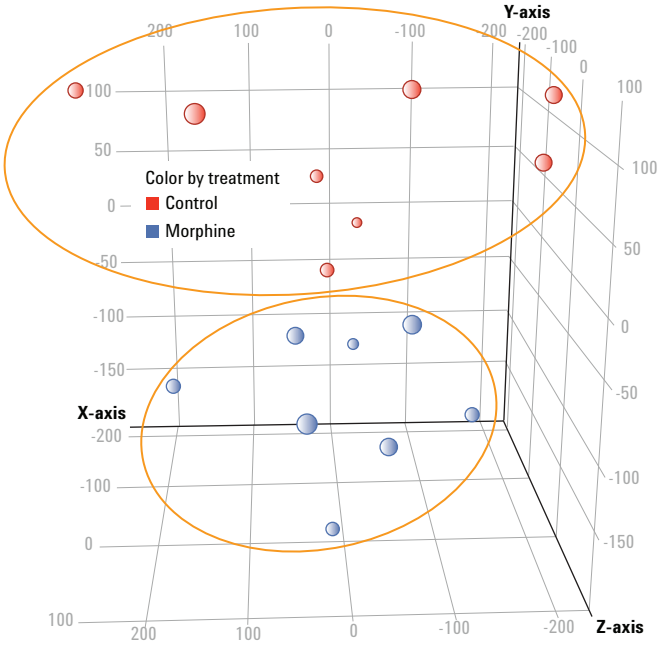

Figure 4. PCA of control versus morphine-treated mice in the 129Sv1 strain confirms the existence of two distinct clusters. Eight biological replicates (mice) were analyzed for each condition (control, red; morphine, blue) in the clustering analysis. A similar plot was obtained for the C57BL/6 strain.

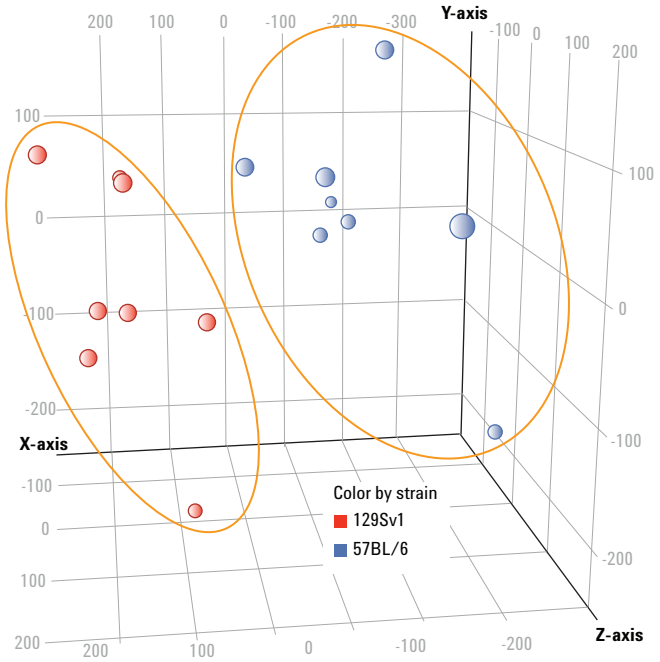

Figure 5. PCA of an inter-strain comparison without morphine administration, showing two distinct clusters. Eight biological replicates (mice) were analyzed for mouse strain (129Sv1, red; 57BL/6, blue) in the clustering analysis. A similar plot was obtained comparing the same strains after morphine administration.

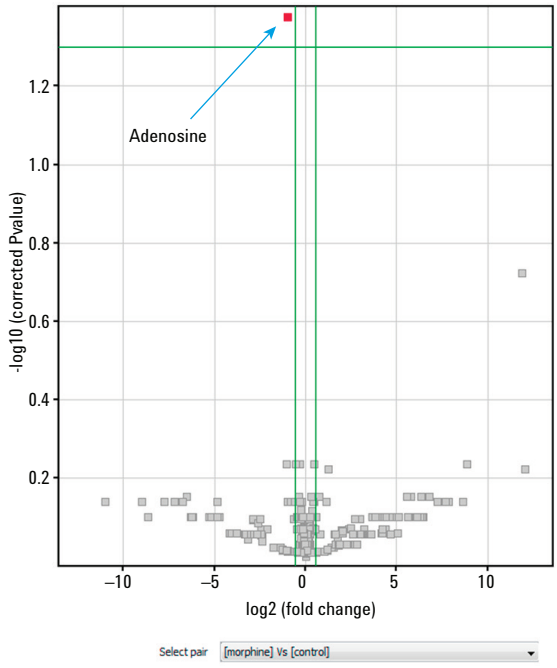

Figure 6. Volcano plot of fold change in concentration versus probability value for morphine-treated versus control morphine-sensitive mice (C57BL/6). The level of adenosine is decreased in morphine-administered mice as compared to control in the C57BL/6 strain ($p < 0.05$), and it is the only compound whose level changes significantly between the two conditions. The green lines indicate cutoff values of $p = 0.05$ (vertical axis) and a fold change of 1.5 (x-axis).

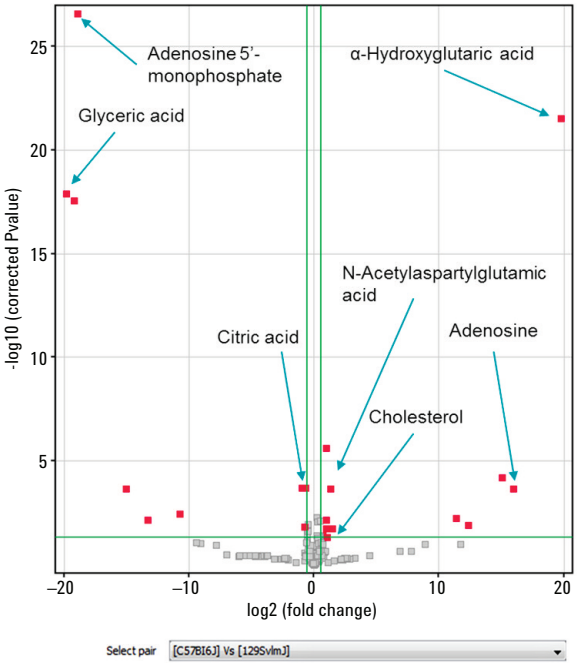

Figure 7. Volcano plot of fold change in concentration versus probability value for morphine-sensitive (C57BL/6) versus morphine-resistant mice (129Sv1). The levels of 21 compounds significantly differ between the two strains ($p < 0.05$). The green lines indicate cutoff values of $p = 0.05$ (vertical axis) and a fold change of 1.5 (x-axis).

Metabolite Fold Change Analysis

Following PCA, Fold Change (FC) analysis was performed on morphine-treated and control samples for both morphine-sensitive and resistant mice (C57BL/6 and129Sv1, respectively). Initially, the magnitude of a biologically significant fold change in the concentration of any given compound between conditions and murine strains was determined. This analysis resulted in the identification of compounds (entities) with substantial abundance differences between the selected classes.

Next, in order to determine if the differences found between the evaluated pairs of conditions or strains by fold change were statistically significant, a series of t-tests was performed. The results of fold change analysis and statistical significance are displayed in a Volcano Plot. In a comparison between morphine-treated and control samples for C57BL/6 (morphine-sensitive) mice, we noticed that adenosine was the only compound that showed a statistically significant change: a reduction in concentration for morphine-administered mice as compared to control (p-value < 0.05; Figure 6). In contrast, a comparison of C57BL/6 and 129Sv1 mice in the absence of morphine treatment revealed a number of compounds (including adenosine, adenosine 5'-monophosphate, glyceric acid, cholesterol, neurotransmitter and N-acetylaspartylglutamic acid) with significant fold changes at a p-value < 0.05 (Figure 7), indicating significant metabolic differences between the two murine strains.

Empirical formula determination of an unknown using EI MS/MS and PCI

The combination of accurate mass/high resolution information with Molecular Formula Generation (MFG) using the FFA tool in MassHunter Qualitative Analysis helped identify possible formulas for the fragments. However, the molecular ion is often uncertain in EI, making it difficult to confirm the correct annotation and narrow down the class of the compound. One of the advantages offered by the use of the accurate mass 7200 GC/Q-TOF system is its ability to obtain additional information through the use of MS and MS/MS, with PCI and EI ionization techniques. One example in this study was an unknown compound eluting at 10.34 minutes that accumulated after morphine administration in the morphine-resistant 129Sv1 murine strain relative to the control, but did not show significant changes in its levels in the morphine-sensitive strain (Figure 8).

An MS/MS experiment can be performed on the ions generated in the EI scan in order to identify product ions of abundant precursor ions in the EI spectrum and, therefore, distinguish possible interferences. In this way, one can easily filter out interfering ions with relatively high *m/z*. Figure 9 illustrates that ions at *m/z* 129.1022 and *m/z* 72.0808 are not product ions of *m/z* 228.0665, that was used as a precursor ion. In fact, abundances for the 129.1022 and 72.0808 *m/z* ions differed between the two murine strains, but the abundances of the ion at *m/z* 228.0665 and its product ions did not, suggesting that the *m/z* 228.0665 ion might belong to a different compound that coelutes with the unknown. Thus, MS/MS helped distinguish interferences in the spectrum.

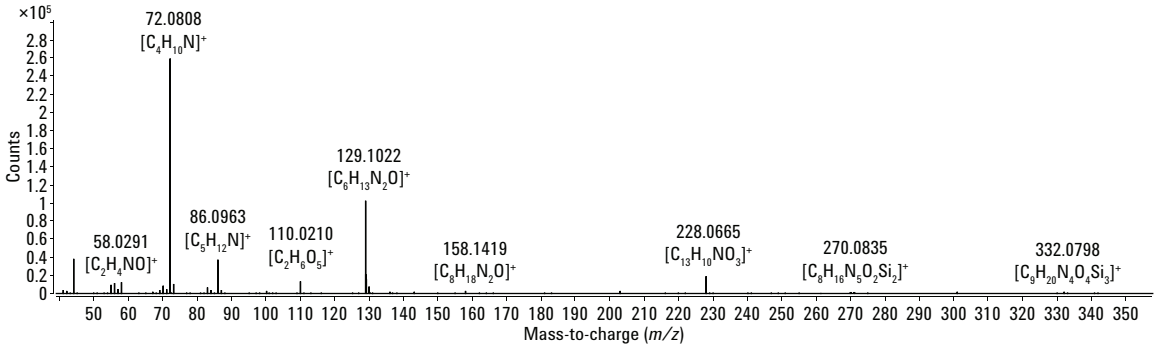

Figure 8. EI spectrum of the unknown peak eluting at 10.34 minutes, which accumulates in the morphine-treated 129Sv1 mice. The spectrum is annotated using the MFG tool of Agilent MassHunter Qualitative Analysis Software.

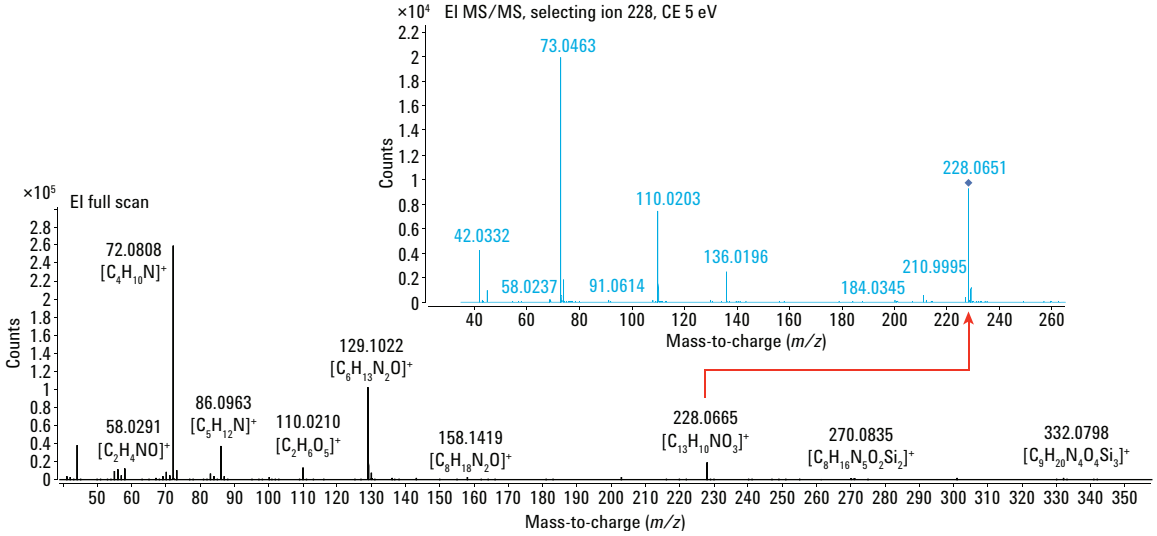

Figure 9. MS/MS performed on the Agilent 7200 GC/Q-TOF system can be used to find contaminating compounds in a peak of interest. In this case, the two ions in the spectra having the highest abundance (m/z 72.0808 and m/z 129.1022) are clearly not derived from ion 228.0665 m/z, possibly making it a contaminant. This hypothesis was confirmed by tracking the changes in the abundance of these ions when comparing morphine-treated versus control conditions.

PCI in MS mode, using methane as a reagent gas, was then used to identify the molecular ion as the *m/z* 158.1419 ion, for which there were methane adducts that help confirm the molecular formula ($C_8H_{19}N_2O$; Figure 10). The high isotope abundance score and isotope spacing score increased confidence in the identification. Having an accurate empirical formula was one of the first steps in compound identification workflow, and it also helped determine if the compound was of interest, or outside the focus of the study, as it was in this case.

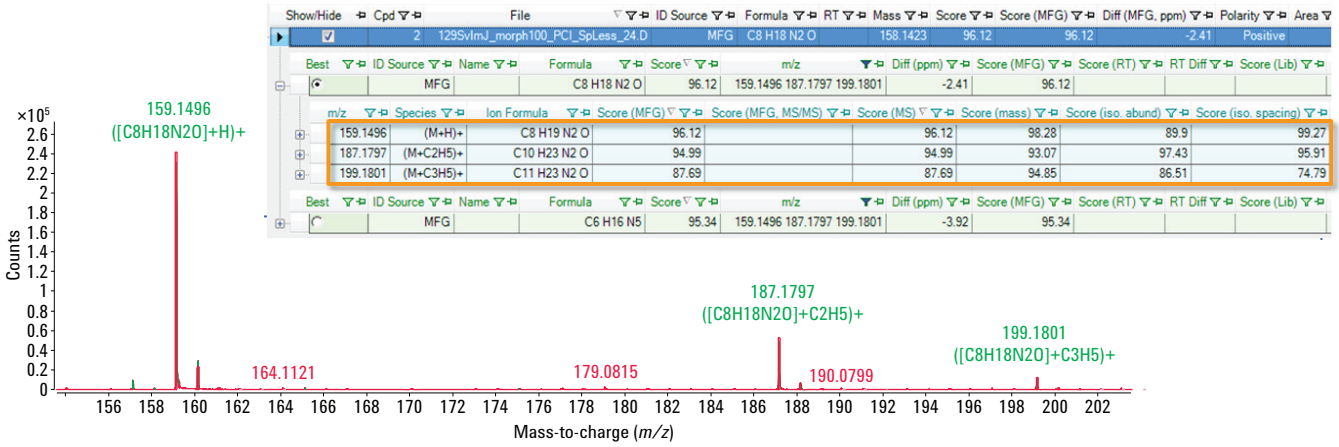

Figure 10. Methane PCI spectral data on the unknown compound eluting at 10.34 minutes confirms that the molecular ion is m/z 158.1419, and the high mass accuracy, isotope abundance, and isotope spacing scores increase confidence in the identification. After performing MFG with FFA, the PCI spectrum shows typical PCI methane adducts of m/z 158.1419: 159.1496 (M+H)+, 187.1797 (M+C_2H_5)+, and 199.1801 (M+C_3H_5)+. The theoretical isotopic spacing abundance is overlaid in red. The tabular results of the FFA workflow are shown above the spectrum.

Validating the structure of a tentatively identified compound using the Molecular Structure Correlator tool

Since α-hydroxyglutaric acid, accumulated in morphine-sensitive strain C57BL/6, was tentatively identified using the NIST library but not the Agilent-Fiehn RTL library, we took additional steps to confirm the identity of this compound. First, using accurate mass information of the EI spectrum and the MFG tool of MassHunter Qualitative Analysis, we verified the consistency between the empirical formula of α-hydroxyglutaric acid and the annotated fragment ions (Figure 11). MSC software was used to further validate the structure of the tentatively identified compound (Figure 12). First, the spectrum was imported into MSC as a CEF file, and MSC predicted fragment formulas using accurate mass information. Then the ChemSpider database was searched to find all possible structural isomers. α-Hydroxyglutaric acid as well as two other structures had the highest compatibility score (92.88). In addition to high compatibility score, α-hydroxyglutaric acid had a higher number of literature references than the other two structures with an equal score. Although this type of confirmation is not completely unambiguous, it provides additional validation for a tentatively identified compound.

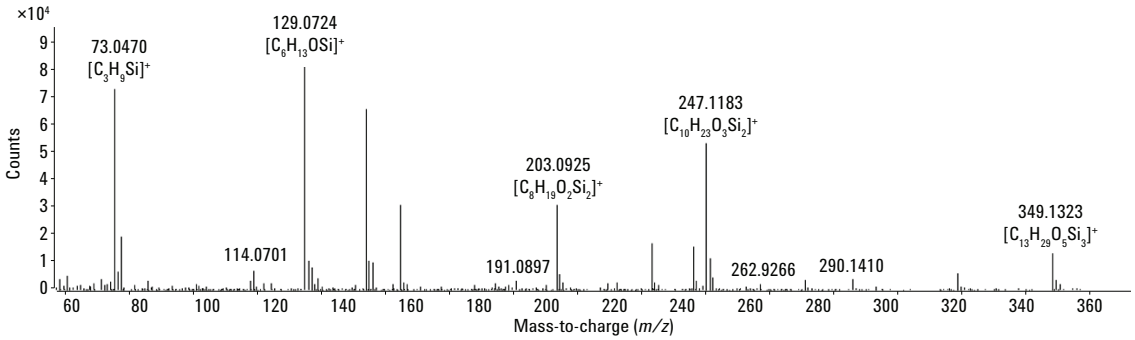

Figure 11. Accurate mass information and MFG were used to identify possible fragment formulas, and thus confirm the identity of the compound accumulated in the morphine-sensitive strain C57BL/6 as α-hydroxyglutaric acid.

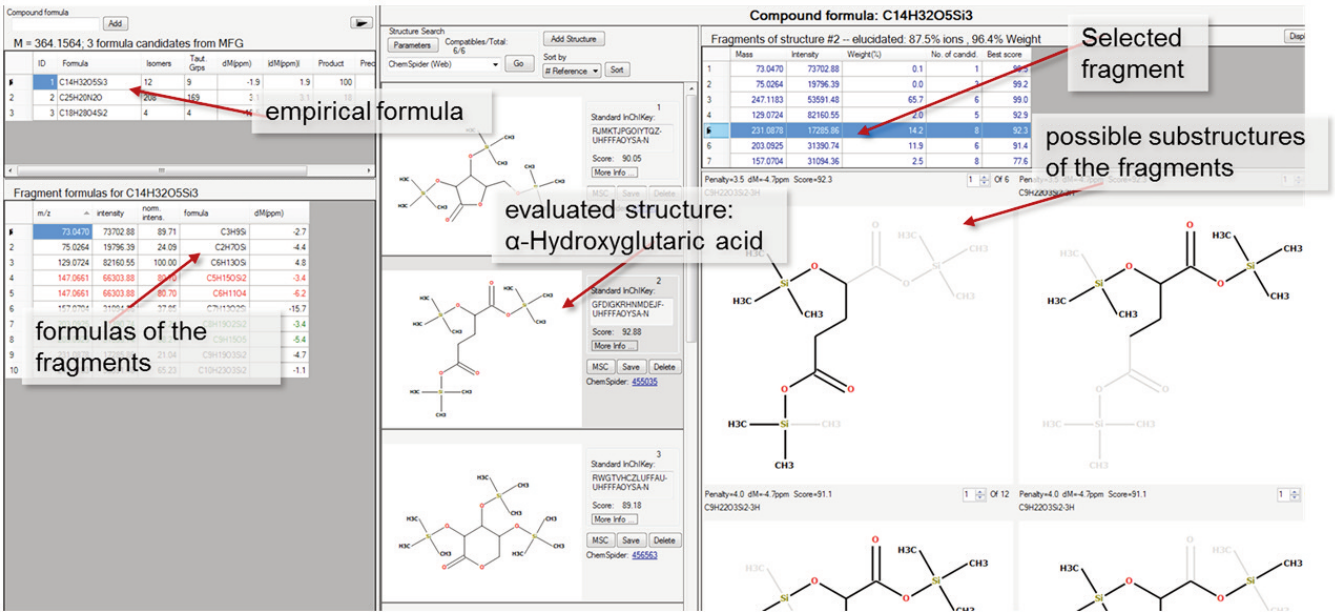

Figure 12. Structure validation results for the compound tentatively identified as α-hydroxyglutaric acid using Molecular Structure Correlator. Each individual fragment ion is ranked based on mass error corresponding to the proposed formula, along with a penalty based on how many bonds needed to be broken to generate that proposed formula.

Conclusions

Metabolomics studies can benefit from multiple features of the Agilent 7200 GC/Q-TOF such as accurate mass information, high sensitivity in full spectrum mode, MS/MS capabilities, and the ability to easily switch between EI and CI modes. The use of numerous Agilent MassHunter and Agilent Mass Profiler Professional Software capabilities such as deconvolution, statistical analysis, automatic fragment formula annotation, and structure elucidation enabled the identification and confirmation of metabolites whose levels were changing in morphine-sensitive and resistant murine strains. Both GC/MS and LC/MS approaches have been applied to provide a more complete picture of the metabolome, and the results presented in this application note are consistent with previous LC/MS findings [2].

References

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2. D. Liang, G. Liao, J. Wang, J. Usuka, Y. Y. Guo, G. Peltz, J. D. Clark "A genetic analysis of opioid-induced hyperalgesia in mice", *Anesthesiology* 104, 1054–1062 (2006a).

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