Software-assisted, high-throughput identification of main metabolites of pharmaceutical drugs

Rapid data acquisition by Agilent 1290 Infinity LC, TOF and Q-TOF instrumentation, and subsequent identification of metabolites by Agilent MassHunter Metabolite Identification software

Application Note

Metabolite identification in drug discovery and drug development

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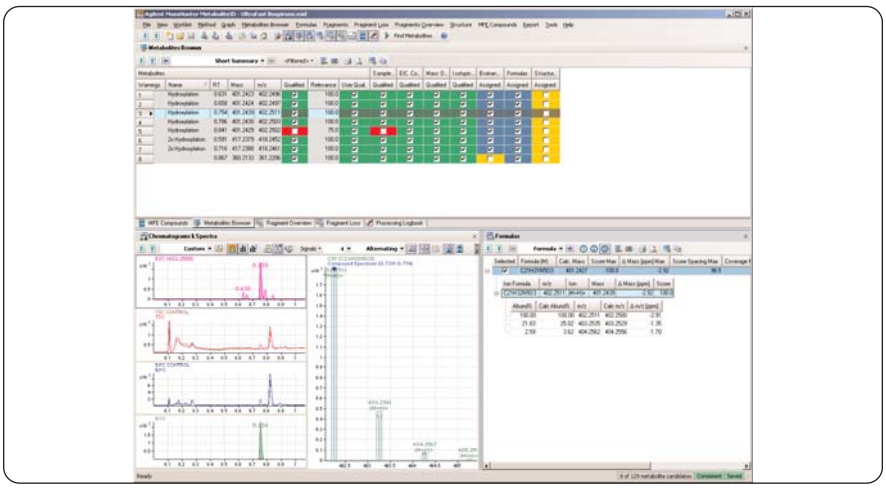

Abstract

This Application Note describes:

- Rapid separation of metabolites generated from in-vitro experiments using the Agilent 1290 Infinity LC, system
- Fast acquisition of TOF mass spectra using Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS systems
- Fast, software-assisted identification of main metabolites from in-vitro experiments using Agilent MassHunter Metabolite Identification software
- Generation of reports for the identified metabolites using Agilent MassHunter software

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Introduction

In modern pharmaceutical drug development it is of crucial importance to analyze the adsorption, distribution, metabolism and excretion (ADME) properties of possible new drug candidates as quickly as possible in order to make decisions about further investments in the development of a special compound. To find compounds with the correct properties it is essential to screen a large number of compounds for their ADME properties, which requires to work in an high-throughput environment. This Application Note describes the application of the Agilent 1290 Infinity LC system, the Agilent 6530 Q-TOF MS system and the MassHunter Metabolite Identification software for fast, high-throughput identification of main metabolites of new pharmaceutical drug candidate compounds.

Experimental

Equipment

- Agilent 1290 Infinity LC system consisting of 1290 Infinity Binary Pump with integrated degasser, 1290 High Performance Autosampler with thermostat, and 1290 Infinity Thermostatted Column compartment
- Agilent 6530 Accurate-Mass Q-TOF LC/MS system
- Agilent MassHunter Metabolite Identification (MetID) software
- Column: ZORBAX SB-C18, 2.1 x 50 mm, 1.8 µm

Sample preparation

The following stock solutions were used:

- 20 mg/mL microsomal S9 preparation
- 0.1 mg/mL buspirone in water
- 1.6 mg NADP in 1.6 mL 0.1 M phosphate buffer, pH 7.4
- 50 mM isocitrate/$MgCl_2$ (203 mg $MgCl_2.6H_2O$ + 258.1 mg isocitrate in 20 mL H_2O)
- Isocitrate dehydrogenase 0.33 unit/µL

NADPH regeneration system: 1.6 mL NADP solution + 1.6 mL Isocitrate solution + 100 µL IDH solution.

Incubation mixture: 3.85 µL substrate + 200 µL NADPH regeneration system + 746.15 µL phosphate buffer + 50 µL S9.

Incubation was carried out at 37 °C for 60 minutes. A 100 µL aliquot was taken at the beginning (t=0) and at t=60 min. The reaction was stopped by adding 6 µL perchloric acid and 100 µL acetonitrile followed by centrifugation for 15 min at 14,000 rpm. The supernatant was evaporated to dryness using a SpeedVac concentrator and reconstituted with water containing 0.1 % formic acid for LC/MS analysis. The incubation sample stopped at 0 min was used as control.

LC method

Solvent A:	Water + 0.1 % formic acid
Solvent B:	ACN + 0.1 % formic acid
Flow:	0.8 mL/min
Gradient	0 min, 5 %B; 0.10 min, 5 %B; 1.10 min, 75 %B;
Stop time:	1.1.0 min
Post time:	1 min.
Injection:	Volume 5 µL, sample cooler at 4 °C, needle wash in 50 % methanol for 5 s, injection loop to bypass at 0.1 min with flush out factor 16
Column:	Temperature 60 °C

TOF MS method

Source:	ESI positive
Capillary:	3500 V
Dry gas:	12 L/min
Nebulizer:	55 psi
Gas temp.:	350 °C
Skimmer:	65 V
Fragmentor:	200 V
Mass range:	100-1000 m/z
Acquisition rate:	5 spectra/s
Reference masses:	121.0508 and 922.0080

Data analysis method in the MetID software

The first step in the analysis comprised a comparison between the data file that contained the metabolite compounds (metabolite sample) and the data file that contained only the parent drug (control sample). All detectable mass signals were extracted from the MS level data using the Molecular Feature Extraction (MFE) algorithm. Related compound isotope masses and adduct masses were grouped together into discrete molecular features, and chemical noise was removed. The compounds lists of the metabolized sample and the control were then compared.

All new compounds or those that increased twofold in the metabolized sample were considered potential metabolites and were subjected to further analysis by different algorithms. The algorithms can identify and qualify new metabolites, or just qualify metabolites found by another algorithm. In this high-throughput experiment all algorithms' results were weighted equally and combined into a final identification relevance score. Metabolites were qualified when their final score was above the stringently defined relevance threshold. The results from all algorithms were collated in a results table, which could be inspected at-a-glance and reported[1].

Results and discussion

To achieve fast separation of the metabolites on a 50 mm, 1.8 µm particle size column, a 1 minute gradient was applied by the Agilent 1290 Infinity LC system. The metabolites were generated from the pharmaceutical test compound buspirone in an in-vitro assay. For adequate detection with the time-of-flight mass spectrometer the instrument was operated at a data rate of 5 Hz.

After generation the data was loaded into the MetID software and analyzed using a common method. The result was displayed by the MetID software in an at-a-glance table, in which the result for each metabolite could be examined in more detail (figure 1). From the results table a summary report was generated, which showed the available information for each metabolite (figure 2). The more extensive report contained the detailed results for each metabolite. As example the result for a mono-hydroxyl metabolite (figures 3 to 5) and a dihydroxy metabolite (figures 6 to 8) of buspirone are discussed here.

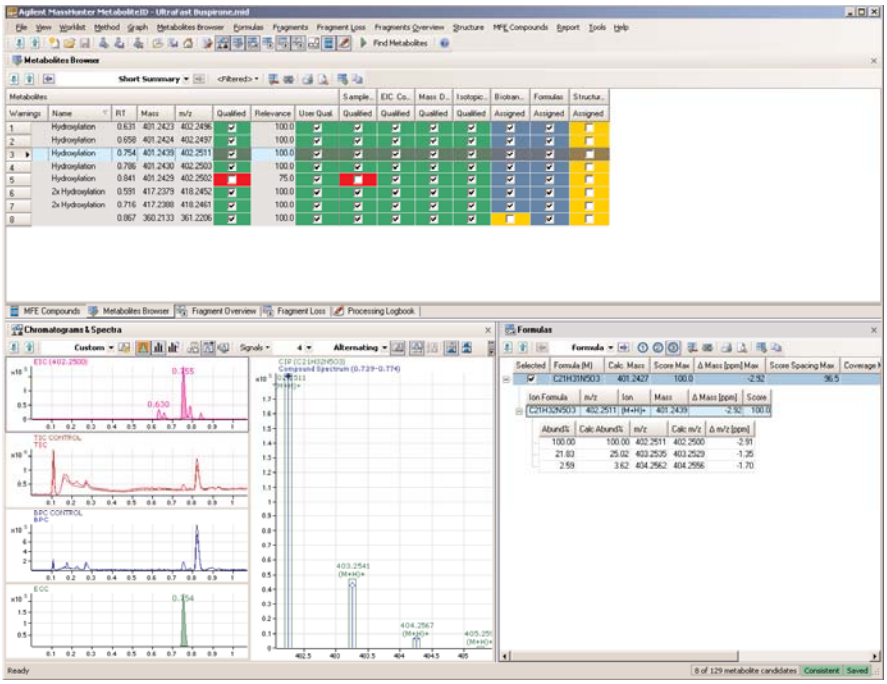

Figure 1
Result table showing an at-a-glance summary of buspirone metabolite analysis with overall identified metabolites, extracted ion chromatograms (EIC), extracted compound chromatograms (ECC), isotopic pattern analysis and calculated formulas.

Name	Mass	RT	Rel.	Qual.	User	SC	IPM	EIC	MDF	Form.	BioXF
2x Hydroxylation	417.2379	0.59	100.00	✓	✓	✓	✓	✓	✓	✓	✓
Hydroxylation	401.2423	0.63	100.00	✓	✓	✓	✓	✓	✓	✓	✓
Hydroxylation	401.2424	0.66	100.00	✓	✓	✓	✓	✓	✓	✓	✓
2x Hydroxylation	417.2388	0.72	100.00	✓	✓	✓	✓	✓	✓	✓	✓
Hydroxylation	401.2439	0.75	100.00	✓	✓	✓	✓	✓	✓	✓	✓
Hydroxylation	401.2430	0.79	100.00	✓	✓	✓	✓	✓	✓	✓	✓
Buspirone	385.2478	0.82	_		_	_	✓	✓	✓	✓	_
Hydroxylation	401.2429	0.84	75.00	×	✓	×	✓	✓	✓	✓	✓

Figure 2
Summary result report, including qualified metabolites sorted by their retention times (RT), with their metabolite names and relative score, molecular mass and the passed flag for individual algorithm results. SC=Sample-control comparison, IPM = Isotopic Pattern Matching, EIC = Extracted Ion Chromatogram, MDF = Mass Defect Filter, Form. = Calculated Formula, BioXF = Assigned Biotransformation, Qual. = Qualified by Score, User = Qualified by User.

The extensive report for the mono-hydroxyl metabolite, which eluted after 0.75 minutes at m/z 402.2511, showed the detailed information about the metabolite itself such as measured accurate mass, calculated formula, assigned biotransformation and ion species. Further, the report showed more detailed information about the result of each individual algorithm, for example, Molecular Feature Extraction (MFE), Extracted Ion Chromatogram (EIC) compound search and Mass Defect Filter Result (figure 3). For the hydroxyl metabolite the possible formula was calculated based not only on a defined mass error window but also on the measured isotopic pattern, which increased the quality of the calculated formula and limited the possible number of hits significantly. These results were also displayed in the detailed metabolite result report for the formula (figure 4).

Metabolite Information

Name	Hydroxylation	**BioXF Name**	Hydroxylation
Formula	C21H31N5O3	**Mass**	401.2439
m/z	402.2511	**Species**	(M+H)+
RT	0.754	**Sample Type**	MetaboliteSample

MFE Compound Search

Mass	m/z	Species	RT	Start Time	End Time	Volume	Height
401.2439	402.2511	(M+H)+	0.754	0.739	0.774	192448	187344

EIC Compound Search

Mass	m/z	Species	RT	Start Time	End Time	Area	Area %
401.2427	402.2500	(M+H)+	0.755	0.739	0.774	149323	100.00

Sample Comparison Results

Qualified	Changed	Resp. Ratio	Corr. RT	Normalized Height
☑	New			

Isotopic Pattern Matching Results

Qualified	Score	Delta m/z
☑	95.91	0.00

Mass Defect Filter Results

Qualified	Delta Mass [mDa]
☑	-3.91

Formula Results

Assigned	Neutral Formula	Calc. Mass	Delta Mass [mDa]	Delta Mass [ppm]	Calculation Base
☑	C21H31N5O3	401.2427	-1.17	-2.92	MfeCompoundMsSpectrum

Biotransformation Results

Assigned	Name	Phase	Offset Formula	Delta Mass [mDA]	Delta Mass [ppm]	Calc. Mass
☑	Hydroxylation	I	+O	1.17	2.92	401.2427

Figure 3
Detailed metabolite report for the buspirone hydroxy metabolite at retention time 0.75 min. This part of the report gives detailed information about the identified metabolite and the identifying algorithms. Other detailed information about formula (figure 4), chromatograms and isotopic pattern (figure 5) are also available.

Metabolite Information

Name	Hydroxylation	**BioXF Name**	Hydroxylation
Formula	C21H31N5O3	**Mass**	401.2439
m/z	402.2511	**RT**	0.754

Formula Summary

Selected	Score	Formula	Ion Formula	Mass	Calc. Mass	⊿ Mass [ppm]
TRUE	100.0	C21H31N5O3	C21H32N5O3	401.2439	401.2427	-2.92

Formula Details

Formula (M)	Selected
C21H31N5O3	TRUE
Species	**m/z**
(M+H)+	402.2511

Formula Results

Ion Formula	Score	Mass	⊿ Mass [mDa]	⊿ Mass [ppm]	DBE
C21H32N5O3	100.0	401.2439	-1.17	-2.92	9

Isotopic Peak Information

Abund %	Calc Abund%	m/z	Calc m/z	⊿ m/z [ppm]
100.00	100.00	402.2511	402.2500	-2.91
21.83	25.02	403.2535	403.2529	-1.35
2.59	3.62	404.2562	404.2556	-1.70

Figure 4
Detailed metabolite report about the formula including isotopic pattern, calculated for the buspirone hydroxy metabolite at retention time 0.75 min.

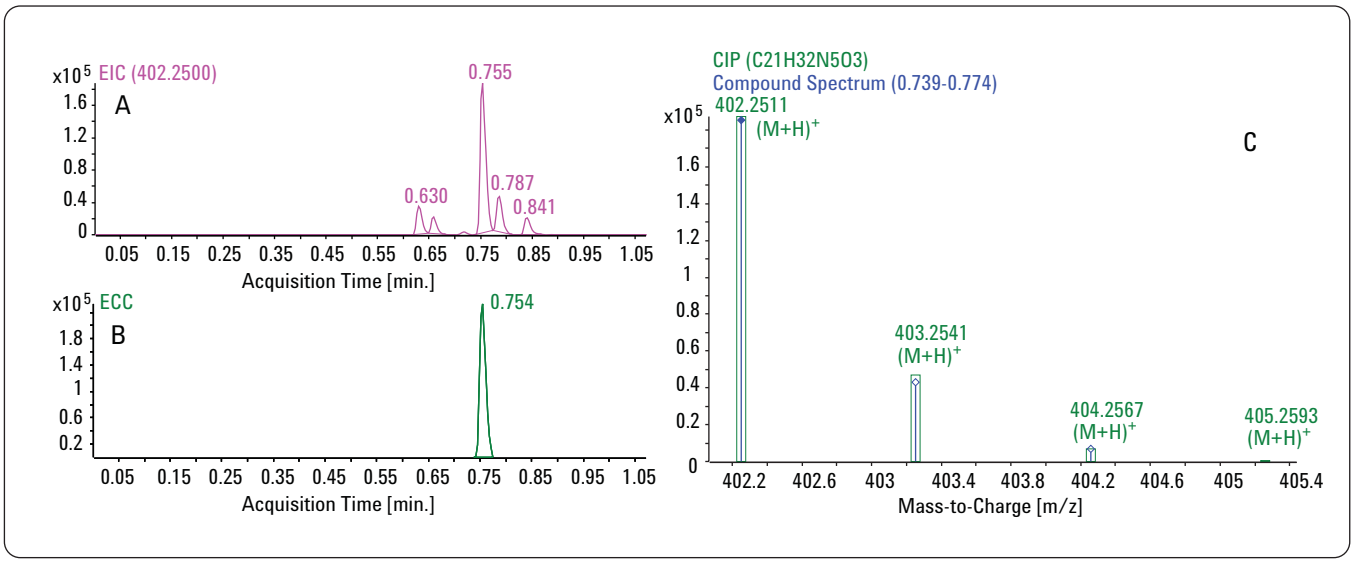

Figure 5
Detailed metabolite report for buspirone hydroxy metabolite at retention time 0.75 min:
A) Extracted Ion Chromatograms (EIC) of compounds with mass 402.25
B) Extracted Compound Chromatogram (ECC) of buspirone hydroxy metabolite at retention time 0.75 min
C) Measured isotopic pattern of buspirone hydroxy metabolite at retention time 0.75 min (blue lines) and caclulated isotopic pattern (CIP, green box).

Finally, the EIC, ECC and isotopic pattern were displayed (figure 5). The EIC of m/z 402.25 showed 5 peaks for possible hydroxyl metabolites of buspirone with the selected one at retention time 0.75 minutes (figure 5A). The ECC showed the extracted MFE compound for the molecular mass of 401.2439 at retention time 0.75 minutes identical to the EIC (figure 5B). The measured isotopic pattern of this compound showed an excellent fit to the calculated isotopic pattern as a basis for the formula calculation (figure 5C).

Within the same data analysis the dihydroxy metabolites at a level of two orders of magnitude below the monohydroxy metabolites were also identified. The extensive report showed detailed information about the dihydroxy metabolite, which elutes after 0.71 minutes at m/z 418.2461 and the detailed information about each algorithm (figure 6).

Metabolite Information

Name	2x Hydroxylation	BioXF Name	2x Hydroxylation
Formula	C21H31N5O4	Mass	417.2388
m/z	418.2461	Species	(M+H)+
RT	0.716	Sample Type	MetaboliteSample

MFE Compound Search

Mass	m/z	Species	RT	Start Time	End Time	Volume	Height
417.2388	418.2461	(M+H)+	0.716	0.700	0.726	3865	3889

EIC Compound Search

Mass	m/z	Species	RT	Start Time	End Time	Area	Area %
417.2376	418.2449	(M+H)+	0.713	0.703	0.739	3483	100.00

Sample Comparison Results

Qualified	Changed	Resp. Ratio	Corr. RT	Normalized Height
☑	New			

Isotopic Pattern Matching Results

Qualified	Score	Delta m/z
☑	91.50	0.00

Mass Defect Filter Results

Qualified	Delta Mass [mDa]
☑	-8.97

Formula Results

Assigned	Neutral Formula	Calc. Mass	Delta Mass [mDa]	Delta Mass [ppm]	Calculation Base
☑	C21H31N5O4	417.2376	-1.20	-2.87	MfeCompoundMsSpectrum

Biotransformation Results

Assigned	Name	Phase	Offset Formula	Delta Mass [mDA]	Delta Mass [ppm]	Calc. Mass
☑	2x Hydroxylation	I	+O2	1.20	2.87	417.2376

Figure 6
Detailed metabolite report for dihydroxy metabolite of buspirone at retention time 0.71 min. This part of the report gives detailed information about the identified metabolite and the identifying algorithms. Other detailed information about formula (see figure 7), chromatograms and isotopic pattern (see figure 8) are also available.

The calculation of the formula was outlined in the detailed formula report (figure 7).

The EIC of m/z 418.24 showed about five significant peaks for possible dihydroxylated metabolites of buspirone with the selected peak at 0.71 minutes (figure 7A). The ECC showed the extracted MFE compound for the molecular mass of 417.2388 at retention time 0.71 identical to the EIC (figure 7B). The measured and calculated isotopic pattern of this compound is shown in figure 7C.

Metabolite Information

Name	2x Hydroxylation	BioXF Name	2x Hydroxylation
Formula	C21H31N5O4	Mass	417.2388
m/z	418.2461	RT	0.716

Formula Summary

Selected	Score	Formula	Ion Formula	Mass	Calc. Mass	Δ Mass [ppm]
TRUE	100.0	C21H31N5O4	C21H32N5O4	417.2388	417.2376	-2.87

Formula Details

Formula (M)	Selected
C21H31N5O4	TRUE
Species	**m/z**
(M+H)+	418.2461

Formula Results

Ion Formula	Score	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE
C21H32N5O4	100.0	417.2388	-1.20	-2.87	9

Isotopic Peak Information

Abund %	Calc Abund%	m/z	Calc m/z	Δ m/z [ppm]
100.00	100.00	418.2461	418.2449	-2.86
23.90	25.06	419.2488	419.2478	-2.35

Figure 7
Detailed metabolite report about the formula, including isotopic pattern, calculated for dihydroxy metabolite of buspirone at retention time 0.71 min.

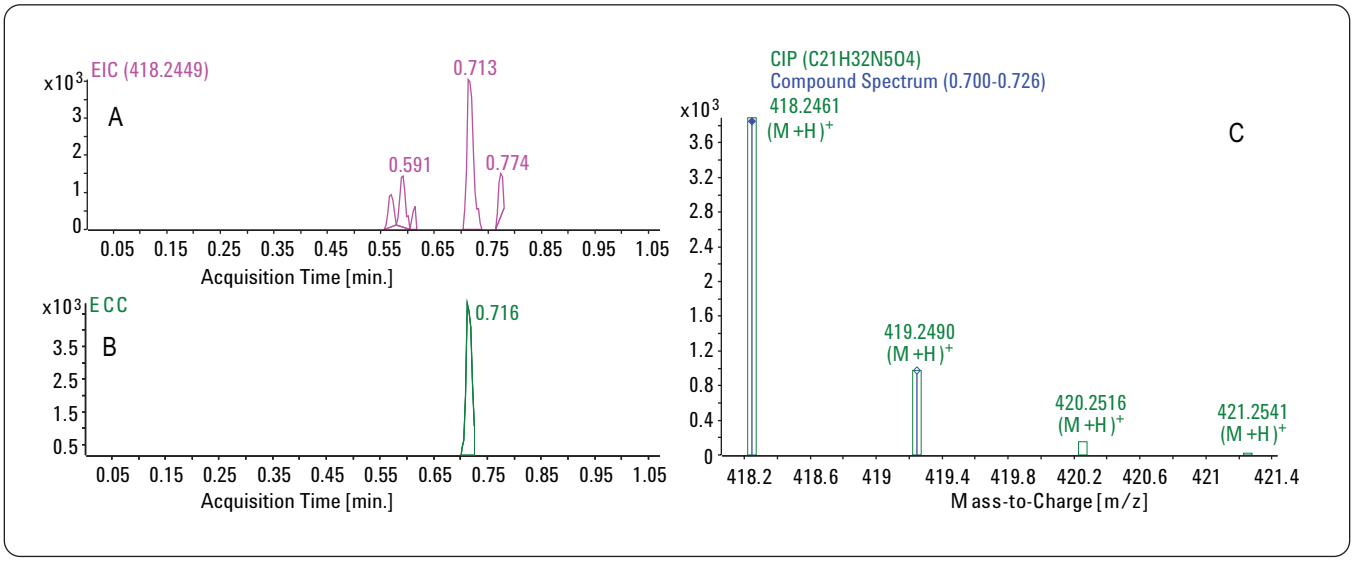

Figure 8
Detailed metabolite report for dihydroxy metabolite buspirone at retention time 0.71 min:
A) Extracted Ion Chromatograms (EIC) of compounds with mass 418.24
B) Extracted Compound Chromatogram (ECC) of dihydroxy metabolite of buspirone at retention time 0.71 min
C) Measured and calculated isotopic pattern of dihydroxy buspirone metabolite at retention time 0.71 min.

Conclusion

This Application Note demonstrated the use of the Agilent 1290 Infinity LC system with an Agilent Q-TOF LC/MS system for fast separation and accurate mass measurement of compounds in an in-vitro metabolite sample under high-throughput conditions. The metabolite compounds were separated in a run time below one minute and the width of the peaks extracted by the Metabolite ID software were below one second (FWHH). The major metabolites were identified quickly by means of the Agilent Metabolite Identification software.
A summary report as well as detailed reports for each metabolite were generated.

References

1.
E. Naegele, F. Wolf, U. Nassal, R. Jäger, H. Lehmann, F. Kuhlmann, K. Subramanian, "An interwoven, multi-algorithm approach for computerassisted identification of drug metabolites", *Agilent Technologies Application Note, publication number 5989-7375EN,* **2007.**

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Published May 1, 2009
Publication Number 5989-9924EN

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