

1 High Resolution and High Precision Analysis of Barbiturates and
2 Metabolite in Human Body Fluids Using a Monolithic Spin Tip and UPLC-
3 Q-ToF-MS
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17 Running title: High resolution and high precision analysis of barbiturates
18

1 **Abstract**

2

3 A high-throughput method was developed for determination of five
4 barbiturates (phenobarbital, cyclobarbital, amobarbital, secobarbital, and
5 thiopental) and a metabolite in human body samples using a new
6 Monolithic C₁₈ gel-packed Spin Tip and ultra-performance liquid
7 chromatography (UPLC)-quadrupole-time of flight (Q-ToF) mass
8 spectrometry (MS). Plasma (20 µl) or urine (100 µl) spiked with the five
9 barbiturates and 5-(4-methylphenyl)-5-phenylhydantoin (internal standard,
10 IS) were mixed with distilled water. The mixture was extracted with
11 Monolithic C₁₈ Spin Tip. The analytes retained on the C₁₈ phase were then
12 eluted with methanol. The eluate was injected directly into analytical
13 column (Waters Acquity BEH C₁₈, 50 mm × 2.1 mm i.d., particle size 1.7
14 µm). Quantification was performed by multiple reaction monitoring with
15 negative-ion electrospray ionization (ESI). Good separation and clear peak
16 shapes of the five drugs were achieved within an analysis time of 6 min,
17 including the extraction time. All drugs spiked in plasma showed
18 recoveries of 86–98%. The regression equations for the five drugs showed
19 excellent linearities in the range of 5–500 ng/20 µl of plasma, and the limits
20 of detection and i-Fit was 1 ng/20 µl. The method was successfully applied
21 to determine the level of amobarbital and its metabolite in human plasma

1 and urine, respectively, after oral administration to a volunteer. This new
2 method can lead to a wide range of applications in quantitative
3 determination of drugs and metabolites in the clinical and forensic fields.

4

5

6 **Keywords** Barbiturate drugs, Monolithic C₁₈ Spin Tip, UPLC-Q-ToF-MS

1 **Introduction**

2

3 Barbiturates are a class of drugs derived from barbituric acid that act as
4 sedative-hypnotics to the central nervous system [1, 2]. These drugs are
5 frequently encountered in emergency toxicology screening, drug abuse
6 testing, and forensic medical examinations [3, 4]. Methods for determining
7 the concentration of barbiturates in human samples are required for
8 diagnosis and effective treatment of intoxication and for forensic purposes.

9 Several methods have been reported for the determination of the levels
10 of barbiturates in various matrices using gas chromatography [5], gas
11 chromatography-mass spectrometry [6, 7], high-performance liquid
12 chromatography (HPLC) [8, 9], and HPLC-mass spectrometry (MS) or
13 HPLC-tandem MS (MS-MS) [10, 11]. Most of these techniques employ
14 extraction methods such as liquid-liquid extraction (LLE) [6, 8-10], solid-
15 phase extraction (SPE) [11], or solid-phase microextraction [7] to remove
16 impurities contained in human body fluid samples. Although LLE and SPE
17 can successfully extract drugs from biological fluids, these procedures are
18 generally performed in the off-line mode and are often labor-intensive and
19 time-consuming.

20 In this study, we present and discuss new and unique monolithic silica
21 SPE techniques that use spin tips. An advantage of Monolithic C₁₈ gel-

1 packed SPE Spin Tip for sample preparation is that extraction can be
2 carried out more easily and rapidly than with the conventional SPE
3 cartridges (Fig 1). The small bed volume and sorbent mass within the
4 monolithic spin tip allow for the use of a reduced solvent volume, smaller
5 elution volume, reduced time for the extraction step, and higher throughput.

6 Currently, HPLC-MS/MS mainly based on triple quadrupole instruments,
7 have been widely applied for the analysis of biological samples [10-17].
8 When using HPLC-MS/MS operating in multiple reaction monitoring
9 (MRM) mode, although quantitative information can be obtained, the
10 qualitative information needed to support the structural elucidation of
11 analytes is poor. Recently, HPLC/quadrupole time-of-flight (Q-TOF)-MS
12 has been used for the unequivocal confirmation of the compounds from
13 biological and environment samples by accurate mass measurement of
14 protonated, and deprotonated molecules [18-21]. Accurate mass
15 measurements of MS/MS product ions have also become particularly
16 important in the structural elucidation of unknowns. Q-ToF-MS is unique
17 in its ability to give accurate mass measurements of product ions, assuring
18 the correct identification of target compounds and unknowns.

19 In the present study, we established a recommendable procedure for
20 analyzing five barbiturates (phenobarbital, cyclobarbital, amobarbital,
21 secobarbital, thiopental) from human plasma samples and the metabolite of

1 amobarbital from human urine using the Monolithic C₁₈ Spin Tip and
2 UPLC-Q-ToF-MS analysis. This is the first report on use Monolithic C₁₈
3 Spin Tip for the extraction of barbituric acid drugs from human body fluids.

4 5 6 **Materials and methods**

7 8 *Materials*

9 Amobarbital was provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan).
10 Cyclobarbital was purchased from Tokyo Kasei Industry Co. Ltd. (Tokyo,
11 Japan). Phenobarbital, secobarbital, thiopental, and 5-(4-methylphenyl)-5-
12 phenylhydantoin (internal standard, IS) was purchased from Sigma-Aldrich
13 (St. Louis, MO). HPLC-MS grade acetonitrile was obtained from Wako
14 Pure Chemical Industries Ltd. (Osaka, Japan). Other common chemicals
15 used were of the highest purity commercially available. Ultra-pure water
16 from the Milli-Q ultra-pure system (Komatsu Electronics Co., Ltd.,
17 Ishikawa, Japan) was used in all experiments. Monolithic C₁₈ Spin Tips
18 (C₁₈-bonded monolithic silica gel with a diameter of 2.8 mm, thickness of 1
19 mm, weight of 2.5 mg, mesopore size of 10 nm, through pore size of 5 μm,
20 and surface area of 350 m²/g) were purchased from GL Sciences.

1 *Preparation of plasma samples*

2 Drug-free whole blood samples from healthy volunteers, who were
3 recruited among laboratory personnel, were obtained intravenously in the
4 presence of heparin sodium as an anticoagulant. To prepare drug-free
5 plasma samples, heparinized whole blood was centrifuged at $1700 \times g$ for
6 10 min at 4°C , and the plasma was decanted into a clean centrifuge tube.
7 The drug-free plasma samples were stored at -80°C until use.

8

9 *Preparation of standard solutions and quality control samples*

10 Individual stock standard solutions (1 mg/ml) of the five barbiturates and
11 the IS were prepared separately by dissolving an accurately weighed
12 quantity of each drug in methanol. The solutions were then stored at 4°C .
13 Working standard solutions of these drugs were prepared by appropriate
14 dilution of the stock standard solutions using the HPLC mobile phase (10
15 mM ammonium acetate in 50% acetonitrile). All working standard
16 solutions were freshly prepared every week and stored at 4°C . Calibration
17 standards were prepared by mixing appropriate amounts of the working
18 standard solutions and drug-free plasma to achieve seven different
19 concentrations ranging from 5 to 500 ng/20 μl (5, 10, 25, 50, 100, 250, and
20 500 ng/20 μl) for five barbiturates, and 100 ng/20 μl for the IS. Quality

1 control (QC) samples (10–500 ng/20 μ l) for all test drugs were also
2 prepared using the same procedure.

3 4 *Extraction procedure using Monolithic C₁₈ Spin Tip*

5 Extraction of five barbiturates and IS from human plasma and urine
6 were achieved using a Monolithic C₁₈ Spin Tip. Monolithic C₁₈ Spin Tips
7 were conditioned with 100 μ l methanol and centrifuged at 1000 \times g for 15 s,
8 followed by 100 μ l of ultra-pure water at 1000 \times g for 15 s. For new tips,
9 this procedure was repeated to reduce the background noise. To 20 μ l of a
10 human plasma sample containing 10 μ l of drug mixture (five barbiturates
11 and IS), 10 μ l of 1 N HCl solution and 160 μ l of ultra-pure water were
12 added. For human urine samples of 100 μ l of urine containing 10 μ l of drug
13 mixture (five barbiturates and IS), 10 μ l of 1 N HCl solution and 80 μ l of
14 ultra-pure water were added. The sample solution was applied to the
15 conditioned Monolithic C₁₈ Spin Tip. The spin tip was centrifuged at 1000
16 \times g for 15 s. The spin tip was then washed with 100 μ l of ultra-pure water
17 at 1000 \times g for 15 s. After washing, the analytes were eluted from the spin
18 tip with 50 μ l of methanol at 1000 \times g for 10 s. A 5- μ l aliquot of the eluate
19 was directly analyzed by UPLC-Q-ToF-MS.

20 21 *UPLC-Q-ToF-MS conditions*

1 A UPLC-Q-ToF-MS system consisting of an Acquity UPLC liquid
2 chromatograph (Waters, Milford, MA, USA) and a Xevo G2 Q-TOF mass
3 spectrometer (Waters, Milford, MA, USA) was used for all measurements.

4 The Waters Acquity UPLC system was equipped with a binary solvent
5 manager, sample manager, and column oven. The chromatographic
6 separation of the five barbiturates and IS was achieved on a Waters
7 Acquity UPLC BEH C₁₈ column (50 mm × 2.1 mm., particle size 17- μ m)
8 with a linear gradient elution system composed of 10 mM ammonium
9 acetate (pH 6.8) and methanol at a flow rate of 0.4 ml/min. Solvent A was
10 ultra-pure water containing 10 mM ammonium acetate, and solvent B was
11 methanol. Gradient runs were programmed to change from 90% solvent
12 A/10% solvent B to 50% solvent A/50% solvent B within 2.5 min, and then
13 to 1% solvent A/99% solvent B within 2.5 min. The column was
14 subsequently maintained with 1% solvent A/99% solvent B for 1.0 min and
15 then re-equilibrated with 90% solvent A/10% solvent B for 2.0 min before
16 the next injection. The total chromatographic run time was 8 min.

17 For confirmation, mass spectrometric measurements were performed
18 using a Xevo G2 Q-TOF mass spectrometer. The analyses were carried out
19 using the ESI setting in the negative mode (ESI⁻). The mass spectrometer
20 was operated in negative ionization mode, with a capillary voltage of 2.8
21 kV and a cone voltage of 30 V. The source and desolvation temperatures

1 were 150°C and 500°C, respectively. The desolvation gas flow was 1000
2 l/hr and the cone gas flow 50 l/hr (both N₂). The mass range considered
3 was 100–1000 Da. Data were collected in centroid mode, and the
4 sensitivity analyzer mode was selected. The accuracy and reproducibility of
5 all the analyses were guaranteed by the use of a LockSpray. Leucine-
6 enkephalin was used as the lock mass at a concentration of 1 ng/ml in 50%
7 of ultra-pure water-acetonitrile with 0.1% formic acid and a flow rate of 5
8 µl/min. MassLynx version 4.1 (Waters, Milford, MA, USA) was used to
9 analyze the samples. The parameter settings were as follows: the analysis
10 time was 0–6 min; the spectrum was above the relative intensity of 2%; the
11 maximum tolerance of mass error was set as 5 ppm. The prediction rules of
12 elemental composition (EC) were defined as follows: atom numbers of
13 carbon, hydrogen, oxygen, nitrogen, and sulfur were set to ranges of 0–100,
14 0–200, 0–20, 0–20, and 0–6, respectively. The molecular formula
15 assignments were obtained with the MassLynx i-Fit algorithm. For
16 barbiturates and IS, the search was restricted to molecules containing
17 CHONS only and the best Fit was obtained on both mass accuracy and
18 isotope intensity pattern (i-Fit). Blank human plasma or urine samples were
19 used as controls for comparison with the analytic samples, and all were
20 processed under the same conditions.

21

1 *Methods validation*

2 The method was validated for linearity, selectivity, precision, accuracy,
3 and recovery according to the US Food and Drug Administration
4 guidelines for bioanalytical method validation [22]. Regression equations
5 of the barbiturates were obtained by plotting the peak-area ratio of
6 analytes/IS (y-axis) against the analyte concentration (x-axis). The slope
7 and y-intercept of the regression line were estimated in duplicate for each
8 of ten different calibrations and on six consecutive days. The acceptance
9 criterion for the correlation coefficient was > 0.998 . The limit of detection
10 (LOD) was defined as the lowest concentration of analyte spiked in plasma
11 that could be detected with a signal-to-noise ratio of at least 3. The lower
12 limit of quantification (LLOQ) was defined as the lowest concentration on
13 the calibration curve that could be measured with a signal-to-noise ratio of
14 at least 10.

15 The selectivity of the method was estimated by analyzing blank human
16 plasma matrix samples. The responses of the interfering substances or
17 background noises at the retention time of barbiturates and IS were
18 acceptable if they were less than 5% of the mean response of the LLOQ.
19 Intra-day precision and accuracy were carried out by analyzing QC samples
20 spiked with the five barbiturates at three different concentrations (10, 100,
21 and 500 ng/20 μ l) in six replicate samples on the same day. The

1 concentration of analytes in the QC samples was calculated using the
2 calibration curves. The precision was determined by calculating the
3 coefficient of variation (CV), while the accuracy was expressed as a
4 percentage of the mean of measured concentration against the nominal
5 concentration. The evaluations of precision were based on previously
6 published criteria [22]. The acceptance criterion for precision (percentage
7 CV) was $\leq 15\%$. Recovery was calculated by comparing the
8 chromatographic peak areas of the analyte in QC samples with those
9 obtained by direct injection of analyte standards dissolved in 10 mM
10 ammonium acetate, and determined at different concentration levels (10,
11 100, and 500 ng/20 μ l).

12

13 *Administration of amobarbital to healthy volunteer*

14 The present method was applied to real samples of human plasma to
15 confirm its utility. A therapeutic dose of amobarbital (300 mg) was
16 administered orally to a 40-year-old male volunteer (body weight, 73 kg).
17 Informed consent was obtained from the subject. Whole blood samples
18 were collected pre-dose (0 h) and 1.5 h after drug administration, and
19 transferred to centrifuge tubes containing heparin sodium. The heparinized
20 blood samples were centrifuged at $1700 \times g$ for 10 min at 4 °C and plasma

1 samples were stored at $-80\text{ }^{\circ}\text{C}$ until analysis. This study was approved by
2 the Ethics Committee of Showa University School of Medicine (No 860).

3 4 5 **Results**

6
7 The extracted ion chromatogram (XIC) chromatograms and mass
8 spectral data for the five barbiturates and IS obtained by UPLC-Q-ToF-MS
9 using an ESI are shown in Fig. 2 and Table 1. Phenobarbital, cyclobarbital,
10 amobarbital, secobarbital, thiopental, and the IS detected in negative-ion
11 mode gave the deprotonated molecule $[\text{M}-\text{H}]^{-}$ at m/z 231.0765, 235.1076,
12 225.1231, 237.1232, 241.1006, and 265.0970, respectively, in the full-scan
13 mode. These deprotonated molecules were identified as $\text{C}_{12}\text{H}_{11}\text{N}_2\text{O}_3$ (-2.2
14 ppm mass error, 0.020 i-Fit) for phenobarbital, $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_3$ (-3.0 ppm
15 mass error, 0.015 i-Fit) for cyclobarbital, $\text{C}_{11}\text{H}_{17}\text{N}_2\text{O}_3$ (-3.6 ppm mass error,
16 0.093 i-Fit) for amobarbital, $\text{C}_{12}\text{H}_{17}\text{N}_2\text{O}_3$ (-3.0 ppm mass error, 0.010 i-Fit)
17 for secobarbital, $\text{C}_{11}\text{H}_{17}\text{N}_2\text{O}_2\text{S}$ (-2.1 ppm mass error, 0.461 i-Fit) for
18 thiopental, and $\text{C}_{16}\text{H}_{13}\text{N}_2\text{O}_2$ (-1.6 ppm mass error, 0.017 i-Fit) for IS,
19 respectively.

20 The regression equations of the five barbiturates showed good linearity
21 from 5 to 500 ng/20 μl with correlation coefficients of at least 0.9984

1 (Table 2). The LOD and LLOQ of the barbiturates under optimal
2 conditions were 1 ng/20 μ l and 5 ng/20 μ l for plasma, respectively (Table
3 2). Intra-day precisions and accuracies were evaluated by assessing QC
4 samples prepared from human plasma (Table 3). The intra-day CVs were
5 no greater than 7.8%, and the accuracies ranged from 86.3% to 97.9% for
6 all concentrations. The recoveries of the five barbiturates from human
7 plasma samples determined at three different concentrations ranged within
8 89.6–97.1% (Table 4).

9 In addition to analysis of spiked human plasma, the present method was
10 applied to human plasma and urine samples collected following oral
11 administration of amobarbital (300 mg) to a male volunteer. The amount of
12 the IS was 100 ng/20 μ l for plasma and 100 ng/100 μ l for urine,
13 respectively. Figure 3 shows UPLC-Q-ToF-MS XICs chromatograms and
14 typical spectra of EC of human plasma 1.5 h after oral administration. The
15 drug concentration in plasma calculated by internal calibration was 44.1
16 ng/20 μ l 1.5 h after administration of amobarbital. The amobarbital and its
17 hydroxide metabolite were identified using the present method from human
18 urine 8 h after oral administration (Fig. 4). The deprotonated molecule was
19 assigned as $C_{11}H_{17}N_2O_4$ (−2.5 ppm mass error, 0.354 i-Fit) for 3-
20 hydroxyamobarbital (Fig. 4).

21

1

2 **Discussion**

3

4 *Optimization of extraction conditions for Monolithic C₁₈ Spin Tip*

5 The entire Monolithic C₁₈ Spin Tips extraction process, including
6 conditioning, sample loading, washing, and elution required approximately
7 2 min. In contrast, the time required to manually perform conventional
8 cartridge SPE was reported to be >20 min [23–26]. The eluate from the
9 Monolithic C₁₈ Spin Tips was directly injected into the UPLC-Q-ToF-MS
10 without evaporation and reconstitution steps, which is particularly
11 important for rapid and simple analysis. Therefore, the use of Monolithic
12 C₁₈ Spin Tips is recommended for rapid extraction of barbiturates and its
13 metabolites from human body fluids. The total solvent volume used for
14 each step of the extraction process was 500 µl, which is lower than
15 volumes required by conventional SPE cartridges (around 5.6–65 ml)
16 [23–26]. Furthermore, the required plasma or urine sample volume was
17 reduced to 20 or 100 µl, respectively, which corresponds to 4.5–50 times
18 less than volumes previously reported for barbiturates analysis in plasma
19 and urine samples [23–26]. Thus, the small volumes of solvent and samples
20 needed with the method used in this study represent a significant advance
21 in sample preparation miniaturization and the overall procedure can be

1 considered to be “green” because it requires little solvent and produces
2 little waste.

3

4 *Method performance*

5 Figure 2 shows TIC and XIC chromatograms obtained by UPLC-Q-ToF-
6 MS for barbiturate drugs from human plasma containing the test
7 compounds (50 ng per 20 μ l). Distinct peaks appeared for each drug and
8 the IS on the chromatograms within 4 min. Blank chromatograms gave
9 small impurity peaks and no interfering peaks appeared around the
10 retention times of the test compounds (data not shown). The spectra
11 obtained showed the accurate corresponding masses for the deprotonated
12 molecular ions. The criteria of the potential candidate ions used for EC
13 analysis included mass error (mDa and ppm), double bond equivalent
14 (DBE, total number of rings and double bonds in a molecule), empirical
15 formula, and i-Fit value (the likelihood that the isotopic pattern of the EC
16 matches a cluster of peaks in the spectrum). The potential calculated
17 masses, mass accuracy, DBE, i-Fit value, and ECs associated with the
18 measured mass of the marker ion were generated and analyzed with the
19 ChemSpider and SciFinder libraries. The lower i-Fit value and the better
20 the Fit Conf for barbiturates and IS were obtained (Table 1).

1 The present method showed good linearity for the known components
2 tested, with LOD and LLOQ for human plasma under optimal conditions
3 corresponding to 1 ng/20 μ l and 5 ng/20 μ l, respectively. Furthermore,
4 lower i-Fit value was obtained under LLOQ concentration (Table 2). With
5 therapeutic levels in plasma or serum of barbiturates reported to be
6 10000–40000 ng/ml for phenobarbital [27], 2000–6000 ng/ml for
7 cyclobarbital [28], 2500–4000 ng/ml for amobarbital [29], 1000–2200
8 ng/ml for secobarbital [30], and 25000–50000 ng/ml for thiopental [31], the
9 present method is sensitive enough to analyze these drugs at therapeutic
10 levels.

11 The intra-day CVs at three concentrations examined were below 7.8%,
12 and the accuracies ranged from 86.3% to 97.9% for all concentrations
13 (Table 3), leading us to consider this variability acceptable for method
14 validation based on current criteria [32, 33]. The recoveries of the five
15 barbiturates from human plasma samples determined at three different
16 concentrations ranged within 89.6–97.1% (Table 4). The reduced recovery
17 (below 100%) is probably due to a loss of analyte during overall sample
18 preparation steps. However, the reduction was not problematic, because
19 satisfactory quantification was achieved using the method described, as
20 shown in Table 2 and 3.

21

1 *Actual measurements of amobarbital in human plasma after oral*
2 *administration*

3 In addition to analysis of spiked human plasma, the present method was
4 applied to human plasma samples collected following oral administration
5 of amobarbital to a male volunteer. Typical XIC chromatograms from this
6 analysis are shown in Fig. 3. The drug concentration in plasma calculated
7 by internal calibration was 44.1 ng/20 μ l 1.5 h after administration of
8 amobarbital. This concentration was within therapeutic levels and UPLC-
9 Q-ToF-MS analysis [29].

10
11
12 **Conclusions**

13
14 We have established a detailed procedure for the quantitative
15 determination and identification of barbiturates and a metabolite in human
16 body samples via a novel Monolithic C₁₈ Spin Tip and UPLC-Q-ToF-MS
17 analysis. The use of Monolithic C₁₈ Spin Tips represents an ideal sample
18 preparation technique because of the simple extraction, and minimal
19 sample and solvent requirements. The recoveries of the five drugs in
20 plasma were 86–98% and the LLOQ was 5 ng/20 μ l. The intra-day CVs for
21 all the drugs in plasma were less than 7.8%. This method will be useful for

1 high-throughput determination of barbiturates in clinical and toxicological
2 analyses. We are currently developing this technique for the detection of
3 other classes of drugs in human body fluid samples.

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5
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12
13 **Conflict of interest disclosure**

14 The authors declare no conflict of interest associated with this
15 manuscript.

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Figure captions

1

2

3 **Fig. 1.** Appearance of a Monolithic C₁₈ gel-packed Spin Tip and electron
4 micrograph of the monolithic silica gel.

5

6 **Fig. 2.** TIC and XIC chromatograms of UPLC-Q-TOF-MS for barbiturate
7 drugs from human plasma in negative ESI mode. The mixture of drugs (50
8 ng each) and 5-(4-methylphenyl)-5-phenylhydantoin used as IS (100 ng)
9 were spiked into 20 µl of plasma. The MS spectra of XIC chromatograms
10 are consistent with those reported in Table 1.

11

12 **Fig. 3.** UPLC-Q-Tof-MS XICs chromatograms and spectra of elemental
13 composition from human plasma 1.5 h after oral administration of
14 amobarbital (300 mg). The amount 5-(4-methylphenyl)-5-phenylhydantoin
15 used as IS was 100 ng/20 µl of plasma.

16

17 **Fig. 4.** UPLC-Q-Tof-MS XICs chromatograms and spectra of elemental
18 composition from human urine 8 h after oral administration of amobarbital
19 (300 mg). The amount 5-(4-methylphenyl)-5-phenylhydantoin used as IS
20 was 100 ng/100 µl of urine.

Monolithic C₁₈ Spin Tip

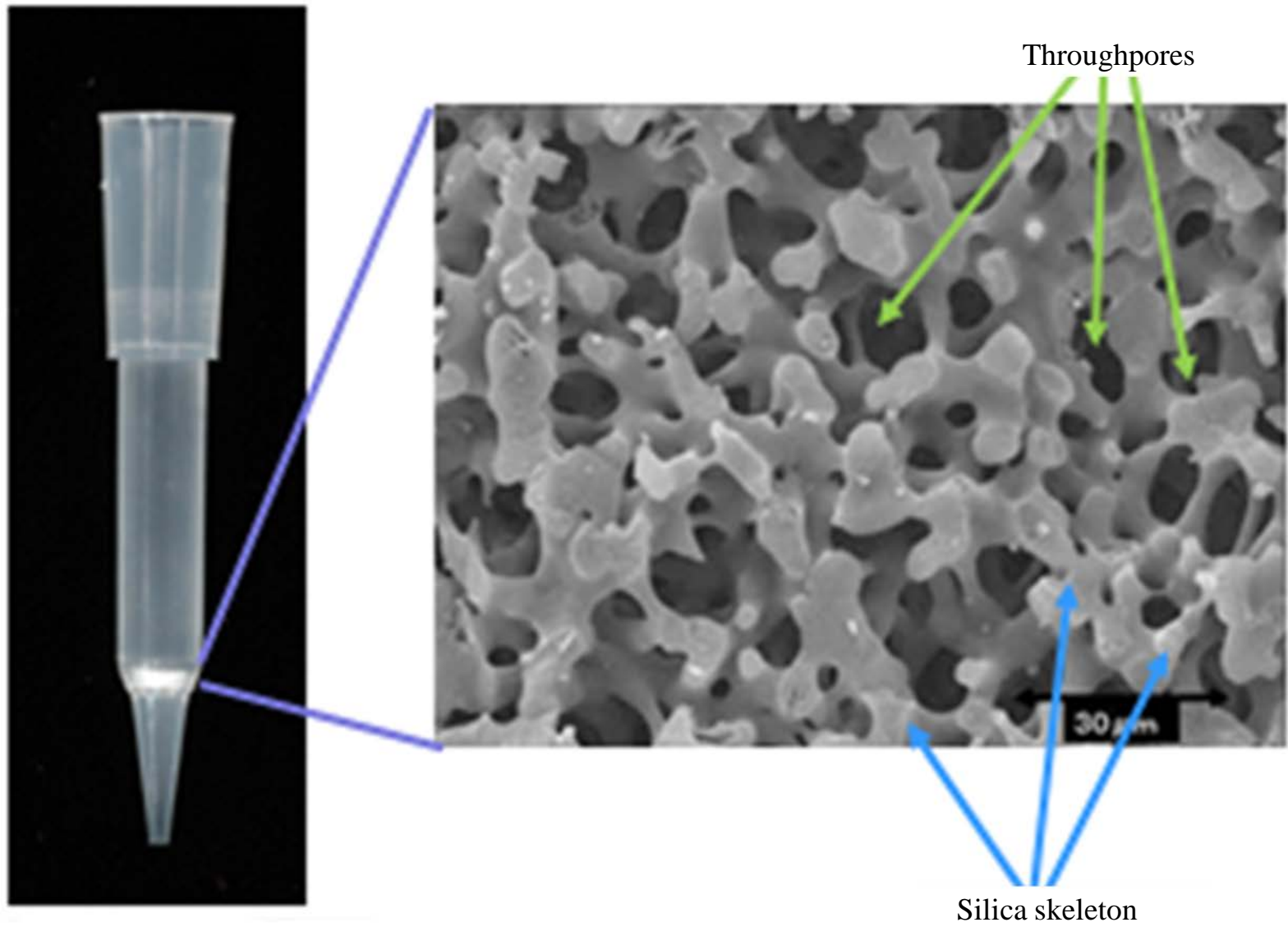


Fig. 1 Shouji et al

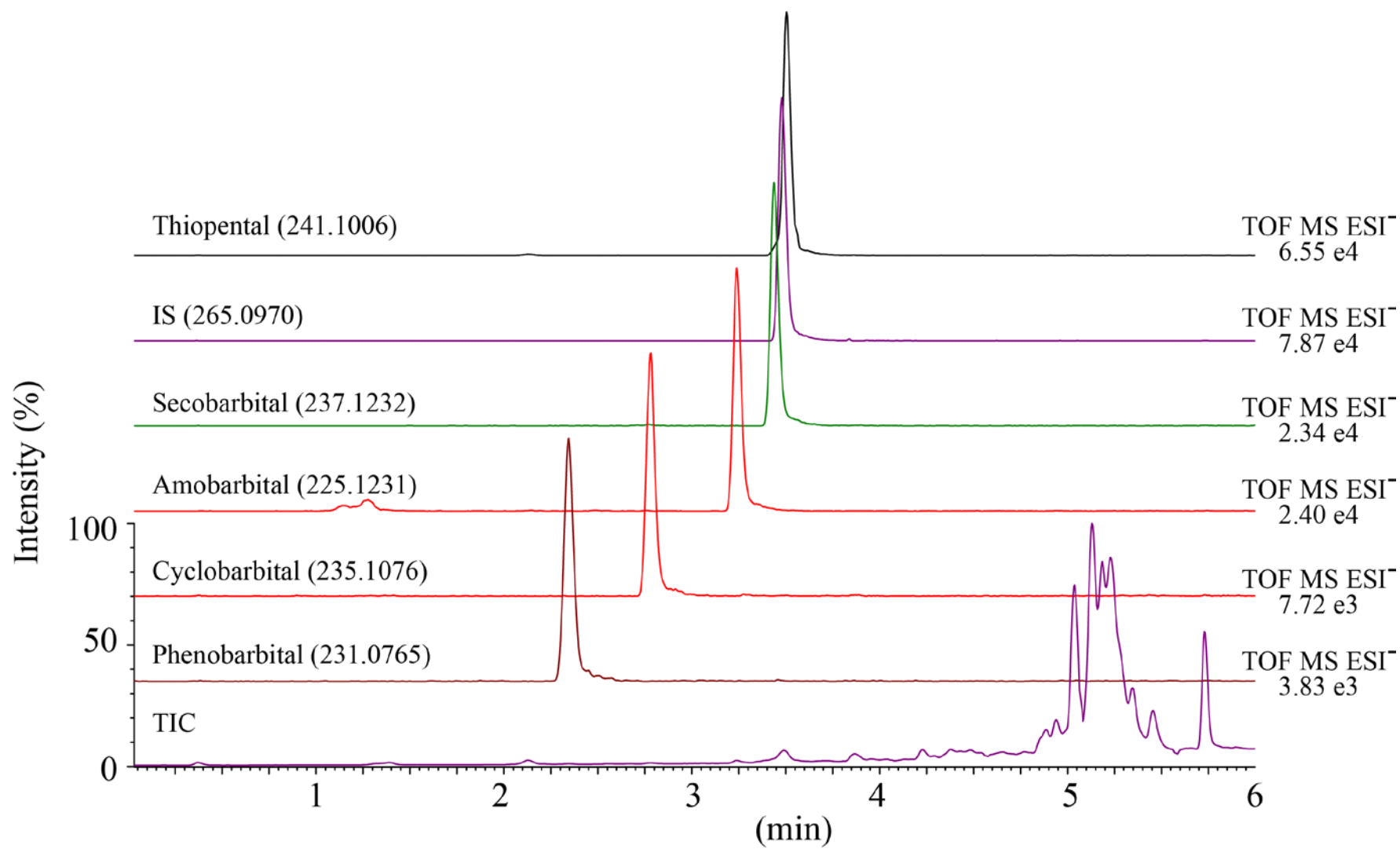


Fig. 2 Shouji et al

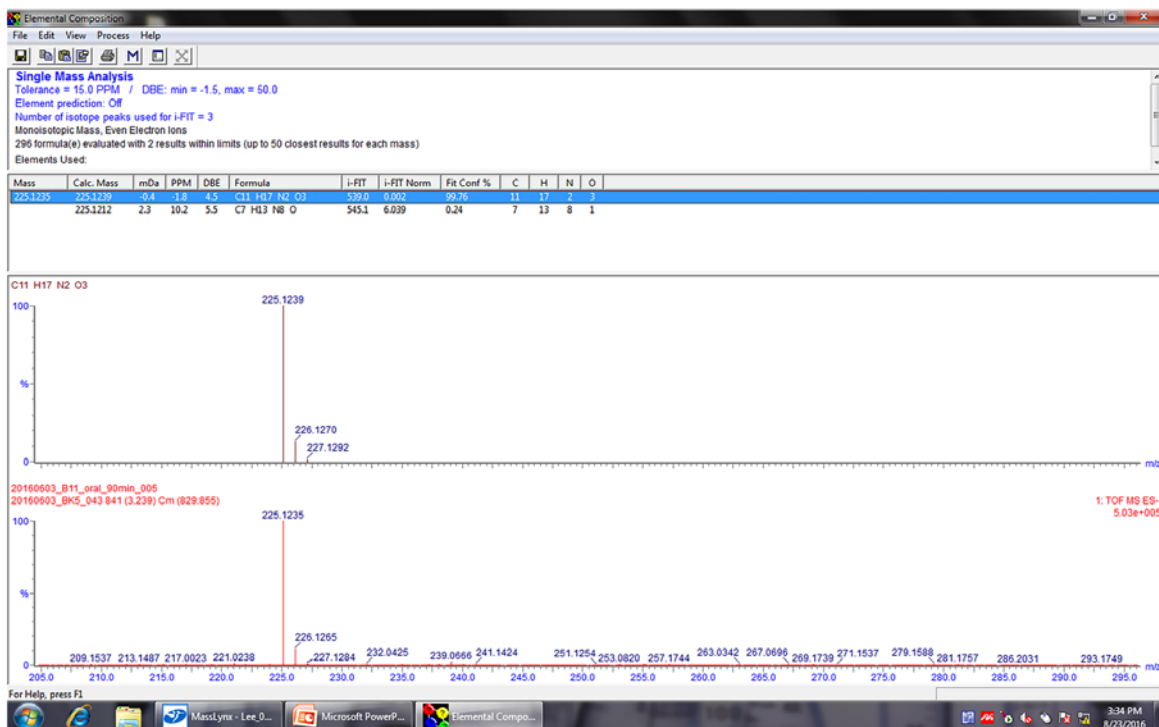
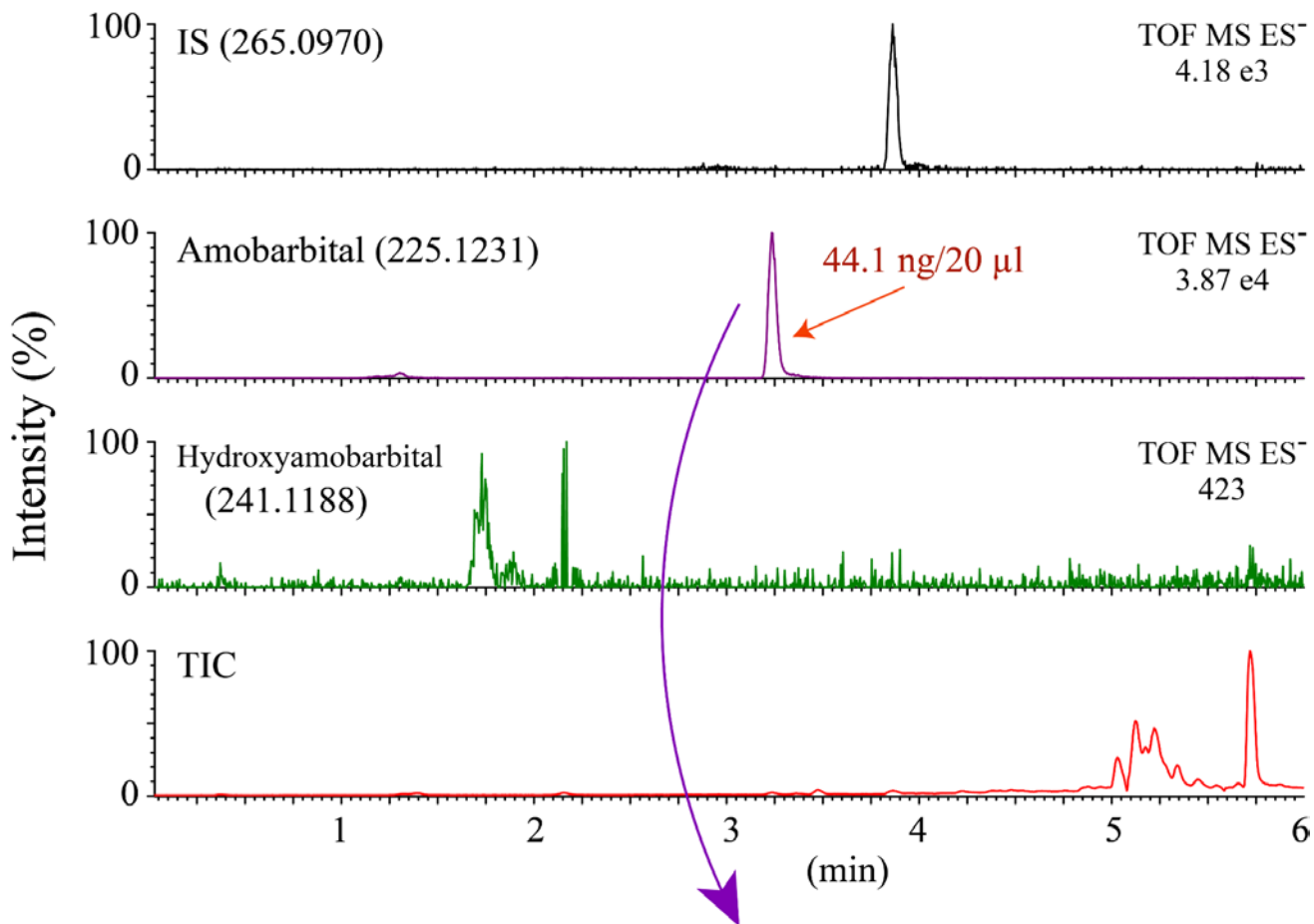
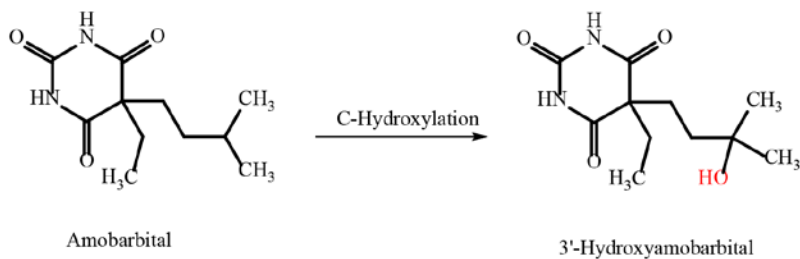
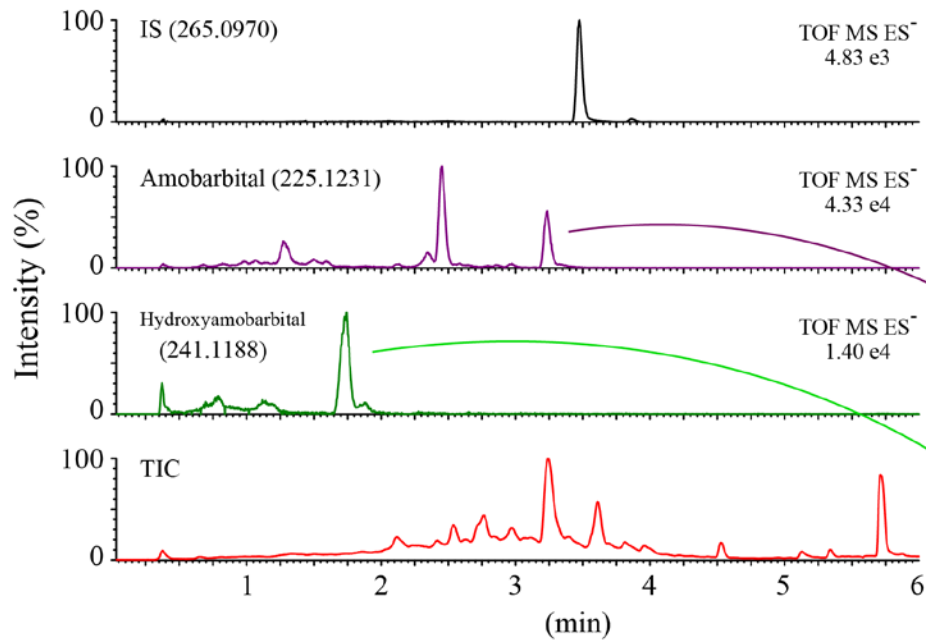


Fig. 3 Shouji et al



Metabolic pathway of amobarbital in human



Fig. 4 Shouji et al