# Supplementary Material

## S1. Materials and Methods

**Table S1.** Summary of group information.

| Groups | Drug (Dose) | Mice | | | | | Aim |
| | | Number | Age | Sex | Treatments | Sample Type | |
|---|---|---|---|---|---|---|---|
| EFI & Control (four NDRDs (CD, MA, DR and HS)) | EFI: 500 mg/100g (6 × LD50) <br><br> NDRDs: non-drug related deaths | 10, respectively | 7–8 weeks | Female: Male= 1:1 | EFI: Death from drug poisoning <br> The DR, MA and HS groups were given cervical dislocation at the time of almost dying. | Plasma, Brainstem | Differential metabolites screening & Classification model construction |
| EFI & New Control (four NDRDs (CD, MA, DR and HS)) | EFI: 500 mg/100g (6 × LD50) | 10, respectively | 7–8 weeks | Female: Male = 1:1 | ibid | Plasma | Testing of classification model predictive power in validation set |
| EFI & Control (three NDRDs (CD, MA, DR)) | EFI: 500 mg/100g (6 × LD50) | EFI: 8; (CD, DR, MA, n = 6, respectively) | 7–8 weeks | Female: Male = 1:1 | ibid | Plasma | Stability analysis of Classification Model within 20 days. |
| EFI & Four other sedative-hypnotics fatal intoxication | EFI: 500 mg/100g (6 × LD50) <br> diazepam: 280 mg/100g (5 × LD50) <br> nitrazepam: 275 mg/100g (5 × LD50) <br> zaleplon: 280 mg/100g (Same as diazepam) <br> sodium pentobarbital: 20 mg/100 g (≈ 1.6 × LD50) | 10, respectively | 7–8 weeks | Female: Male = 1:1 | Death from drug poisoning | Plasma, Brainstem | Specificity evaluation of the Classification Model |
| EFI & Four other toxicants fatal intoxication | EFI: 500 mg/100g (6 × LD50) <br> Heroin: 21.8 mg/100g <br> DL-METH: 43.2 mg/100 g <br> CO/CO2: Replace the anesthesia box volume at a rate of 10-30% per minute into the box, ≈2 min | 10, respectively | 7–8 weeks | Female: Male = 1:1 | Death from drug poisoning <br><br> Death from toxicant poisoning | Plasma, Brainstem | Specificity evaluation of the Classification Model |
| EFI & Three other different doses of Estazolam intoxication | EFI: 500 mg/100g (6 × LD50) <br> EFI: 250 mg/100g (3 × LD50) <br> 50 x Estazolam therapeutic: 2.59 mg/100 g <br> 100 x Estazolam therapeutic: 5.17 mg/100 g | 10, respectively | 7–8 weeks | Female: Male = 1:1 | Death from drug poisoning <br><br> Administered for 24 h, euthanized by cervical dislocation | Plasma, Brainstem | Sensitivity evaluation of the Classification Model |
| EFI & EIND | EFI/EIND: 500 mg/100g (6 × LD50) | 10, respectively | 7–8 weeks | Female: Male = 1:1 | EFI: Death from drug poisoning | Brainstem | Toxicological mechanism analysis and validation (QEA & qPCR & TEM) |
| EFI & EIND & Control (CD) | EFI/EIND: 500 mg/100g (6 × LD50) | 10, respectively | 7–8 weeks | Female: Male = 1:1 | EIND:After administration not dead for more than 8 h and treated with cervical dislocation | Brainstem | Toxicological mechanism validation (Metabolite Quantification) |

### S1.1. Experimental Design

Plasma concentration is not an uncontested criterion for identifying fatal estazolam intoxication as the cause of death. Estazolam fatal intoxication and intoxication non-death plasma concentration data demonstrated this result (quantified by UPLC (ExionLCTM AC, SCIEX, Framingham, MA, USA) in conjunction with a 5500 Q-Trap system (SCIEX) in multiple reaction monitoring modes). Therefore, a non-targeted metabolomics approach was introduced to construct a classification model for the fatal intoxication caused by this drug to determine the death cause and explore its new toxicological mechanism. There are four sets of animal models of estazolam fatal intoxication and intoxication at different doses. Four sets of non-drug intoxication death groups associated with hypoxia (control) and four sets of other fatal intoxication poisons. The other drugs and toxicants fatal intoxication mice models were prepared by intragastric administration except for gas intoxication. After a series of treatments, plasma samples from the abdominal aorta and brainstem tissue samples from each mouse group were analyzed by liquid chromatography-high resolution tandem mass spectrometry (LC-HR MS/MS) in full scan mode. The instrument utilized in this step is a UPLC (Ultimate 3000) combination of a Q Exactive mass spectrometer. The raw data from the LC-HR MS/MS were further processed with Compound Discoverer 3.1. Those metabolites with MS2 information were

subjected to multivariate analysis with MetaboAnalyst 5.0 to complete screening of potential differential metabolite and the classification model construction between estazolam fatal intoxication group and four sets of non-drug intoxication death groups for plasma samples and brain stem tissue samples. The specificity of the classification model was verified in the lethal group of four other poisons and four sedative-hypnotic drugs, and the sensitivity of the classification model was verified in the different dose groups of estazolam. The classification model's stability was also verified in the blood samples. Finally, metabolic pathway enrichment analysis was conducted in the groups of estazolam fatal intoxication and not death, and the most disturbed differential metabolic pathway was obtained. This pathway was further verified to explore the new toxicological mechanism of estazolam.

*S1.2. Chemicals and Reagents*

Heroin and DL-METH were provided by Beijing Municipal Public Security Bureau (Beijing, China). Carbon monoxide (liquefaction) and carbon dioxide (liquefaction) were purchased from Shijiazhuang Xisanjiao Practical Gas Co. LTD (Shijiazhuang, China).

*S1.3. Animals Diets and Grouping*

Subsequently, to assess the specificity of the classification model, four other toxicant fatal intoxication models ($n = 10$, respectively, F: M = 1:1) were introduced; namely, Heroin (21.8 mg/100g), DL-METH (43.2 mg/100g), Acute carbon monoxide fatal intoxication model and Acute carbon dioxide fatal intoxication model, CO and CO2 models were made according to the euthanasia treatment of mice.

*S1.4. Determination of Plasma Concentration*

In total, 200 μL whole blood samples after thawing at 4 °C were added to tubes with 790 μL ice-cold extractant ($V_{acetonitrile}$: $V_{Milli-Q\ water}$ = 3:1), and 10 μL SKF525A (100 ug/mL) was used as an internal standard. Another blank plasma was mixed with different volumes of 1 mg/mL of estazolam stock solution (dissolved in methanol) to configure 1 mL of 250, 500, 1000, and 2000 μg/mL of estazolam plasma, then 200 μL of which was taken into 790 mL of ice-cold extractant and 10 μL of internal standard working solution as described above to configure a series of the calibration curve (50, 100, 200, 400 μg/mL of estazolam) for quantification Each sample was then vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 20 min at −20 °C to allow protein precipitation. The mixtures were centrifuged at 12,000 g for 10 min at 4 °C. The resulting supernatants diluted 100 times with methanol were transferred to HPLC vials for LC-MS/MS analysis.

*S1.5. Multiple Reaction Monitoring (MRM) Analysis by LC-MS/MS*

The LC-MS/MS system was an ExionLC™ AD (AB SCIEX, Framingham, MA, USA) coupled with a SCIEX QTRAP 5500 triple quadrupole mass spectrometry (AB SCIEX) with an ESI source used for separation and detection the plasma concentration of estazolam. The column used for this study was a Kinetex C18 column (2.1 × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 40 °C. The elution gradient with a gradient elution of mobile phase A (1 mmol/L ammonium acetate with 0.01% formic acid in Milli-Q water) and mobile phase B (acetonitrile) at a flow rate of 0.4 mL/min for 12 min. and an injection volume of 5 μL. The elution gradient was set as follows: 0 min, 97% A, 0.5 min, 97% A; 9 min, 5% A; 10 min, 5% A; 10.1 min, 97% A;12 min, 97% A. The Mass spectrometry was performed in positive electrospray ionization utilizing multiple reaction monitoring (MRM) modes. The source-dependent parameters were as follows: ion spray voltage 5500 V, curtain gas 30 psi, vaporizer temperature 550 °C, nebulizing gas (GS1) 60 psi, and drying gas (GS2) 70 psi. Data acquisition and processing were performed using Analyst software version 1.6 (SCIEX).

*S1.6. Saccharopine and Lysine Measurement*

To measure saccharopine and lysine levels in mice brainstems, 50 mg of brain stem tissue ($n_{EFI}$ = $n_{EIND}$ = 10, F:M = 1:1) was thawed at 4 °C mixed with 500 μL of ice-cold methanol. An appropriate amount of grinding beads was added and ground at −4 °C for 90 s to form a homogenized slurry. Then, it vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 20 min at −20 °C to allow protein precipitation. The mixtures were then centrifuged at 12,000 g for 10 min at 4 °C, and the supernatant was collected and evaporated to dryness under nitrogen and redissolved with 50 μL of methanol containing SKF525A (10 ng/mL). Since a plasma sample devoid of these two standards is unavailable, methanol calibration curves were prepared. Briefly, 1 mg/mL of lysine and saccharin stock solution was serially diluted to generate the calibration curve. A 495 μL aliquot of the appropriate dilution was transferred into a chromatographic tube and mixed with 5 μL internal standard-SKF525A (1 μg/mL) for each point. All samples were stored at 4 °C for LC-MS/MS analysis. In the LC-MS/MS system as above, the column used for this part was a Luna PFP (2) column (4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA). The column temperature and mobile phase are consistent with the previous. The flow rate was 0.3 mL/min, and an injection volume of 2 μL. The elution gradient was set as follows: 0 min, 98% A; 1 min, 98% A; 5 min, 2% A; 8 min, 2% A; 8.1 min, 98% A; and 10 min, 98% A. The Mass spectrometry was performed in positive electrospray ionization utilizing multiple reaction monitoring (MRM) modes. The source-dependent parameters were as follows: ion spray voltage 5500 V, curtain gas 30 psi, vaporizer temperature 550 °C, nebulizing gas (GS1) 55 psi, and drying gas (GS2) 55 psi. Data acquisition and processing were performed using Analyst software version 1.6 (SCIEX).

*S1.7. Quantitative Real-time PCR*

RNA was isolated from brainstem tissues ($n_{EFI}$ = $n_{EIND}$ = 10, F:M = 1:1) after low-temperature grinding using a Trizol. PrimeScript RT reagent Kit (Takara) was used to reverse-transcribe RNA in a 20-μl reaction mixture. Quantifying SDH and LKR mRNA expression was performed using the TB Green PremixEx TaqTM II kit (Takara) by a real-time PCR system (QuantStudio 7 Flex, Applied Biosystems, Waltham, MA, USA) in quadruplicate. Gapdh was amplified as the internal control. The data were analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$). The gene sequences of the primers are as follows:

AASS-LKR forward primer: 5'-AGGGTCTCGGATAGTGGCTTTCG-3',
AASS-LKR reverse primer: 5'-GGCTGCTGTTCCTGTAGTTGTGAG-3',
AASS-SDH forward primer: 5'-CCACCACAGGATGAAGCACATAAGG-3',
AASS-SDH reverse primer: 5'-GTTGGCAAGCAAGAGGCAAAGC-3'.

*S1.8. TEM Analysis*

After the mice in the EFI group died, and the mice in the EIND and control groups were euthanized by cervical dislocation, the brain stem tissue was taken immediately within 1–3 min. A sharp blade was used to cut and harvest fresh tissue blocks（no more than 1 mm³) to minimize mechanical damage. The small tissue pieces were then immediately transferred to Petri dishes on ice in ice-cold fresh TEM fixative to trim the size and then transferred into EP tubes with ice-cold fresh fixative for further fixation for 24 h. Then the tissues were washed thrice with 0.1 M PB (pH 7.4) for 15 min each. Then the samples were washed with phosphate buffer thrice (5 min for each time). Tissues avoid light post-fixed with 1% $OsO_4$ in 0.1 M PB (pH 7.4) for 2 h at room temperature. After removing OsO4, the tissues are rinsed in 0.1 M PB (pH 7.4) thrice for 15 min each. Dehydration at room temperature was performed by a series of graded ethanol solutions (30, 50, 70, 80, and 95% ethanol solutions) for 20 min for each step, and the 100% ethanol step was performed twice (20 min for each time), and a 100% acetone step twice more (15 min each). Then samples were infiltrated and embedded by acetone and embedding media 812 with different proportions. After a series of resin embedding procedures at 37 °C for approximately 36 h, it transferred to 65 °C to polymerize for more than 48 h. Sections were cut by ultra-microtome into 60–80 nm and stained with uranyl acetate and

lead citrate to capture images with the transmission electron microscope (HT7800, Hitachi, Tokyo, Japan).

**S2. Results**

**Table S2.** Metabolite classes and proportions in plasma and brainstem tissue samples

| Class (HMDB) | Metabolites | | | |
| | Plasma | | Brainstem | |
| | Number | Percentage | Number | Percentage |
| --- | --- | --- | --- | --- |
| Organic acids and derivatives | 106 | 30.11% | 78 | 35.78% |
| Lipids and lipid-like molecules | 101 | 28.69% | 47 | 21.56% |
| Organoheterocyclic compounds | 49 | 13.92% | 37 | 16.97% |
| Benzenoids | 40 | 11.36% | 21 | 9.63% |
| Organic oxygen compounds | 22 | 6.25% | 10 | 4.59% |
| Nucleosides, nucleotides, and analogues | 12 | 3.41% | 10 | 4.59% |
| Alkaloids and derivatives | 7 | 1.99% | 0 | 0 |
| Organic nitrogen compounds | 7 | 1.99% | 0 | 0 |
| Others | 8 | 2.27% | 15 | 6.88% |
| | | 89.80% | | 89.34% |
| No-class | 40 | 10.20% | 26 | 10.66% |
| Total | 392 | | 244 | |

*S2.1. A Brief Procedure for Screening the Constituent Components of the EFI Classification Models*

During the screening we selected the combinations of candidate differential metabolites in Table 1 that had relatively large effects on the EFI group, using the values of *P* value, Combined Log FC, et al. as criteria for selection. In addition, metabolites that were stably present in several pre-experiments were only included. In order to construct a classification model with better discriminatory power, we controlled the inclusion criteria for the modeling components more strictly than the common differential metabolites screening.

The ROC curve analysis (in the training set) based on SVM algorithm was first performed with five compounds combinations, and next it was found that the combination of four compounds could also achieve an AUC value of 1. When candidate differential metabolites were gradually included in order of importance, the AUC value of combination 7 decreased, then "Valine" was deleted. Three of the remaining six candidate differential metabolites were randomly selected for recombination, and then ROC curve analysis was done. Four combinations all had an AUC value of 1. The ROC test was further continued in a new set of samples, and combination 9 had an AUC value of 1 under all algorithms, with higher predictive power than the remaining three combinations. The three candidate differential metabolites of combination 9 (creatine, phenylacetylglycine, and indole-3-lactic acid) were finally identified as the constituent components of the classification model. For details, please refer to the results in Table S3 below. The construction of the classification model in brainstem tissues had the experience of screening in plasma samples. We directly used three candidate differential metabolites to constitute different combinations. Then the AUC curve analysis was performed, the process that was more concise than in plasma samples. As shown in the results of Table S8 (Prostaglandin D2 and DL-Tryptophan were not included because they could not be identified in each batch of brainstem samples) in the Supplementary Material and will not be repeated here.

**Table S3.** AUC values of different candidate differential metabolite combinations in plasma samples

| Candidate differential metabolites | Combinations of different candidate differential metabolites | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Combined Log FC > 1, or < -1, $P < 0.05$, mzCloud Best Match > 90 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Phenylacetylglycine | √ | | | √ | | | | √ | √ | √ | √ |
| Creatine | √ | √ | | √ | √ | | | √ | √ | √ | √ |
| Methionine | √ | √ | √ | √ | √ | √ | | √ | | | |
| Xanthurenic acid | √ | √ | √ | √ | √ | √ | √ | | | √ | |
| Acetyl-L-carnitine | √ | √ | √ | | √ | √ | √ | | | | √ |
| Indole-3-lactic acid | | √ | √ | | | √ | √ | | √ | | |
| Valine | | √ | | | | | √ | | | | |
| **AUC-value** | 1 | 1 | 1 | 1 | 1 | 1 | 0.999 | 1 | 1 | 1 | 1 |
| **AUC-value (new samples)** | | | | | | | | 0.999 | 1 | 0.996 | 0.998 |
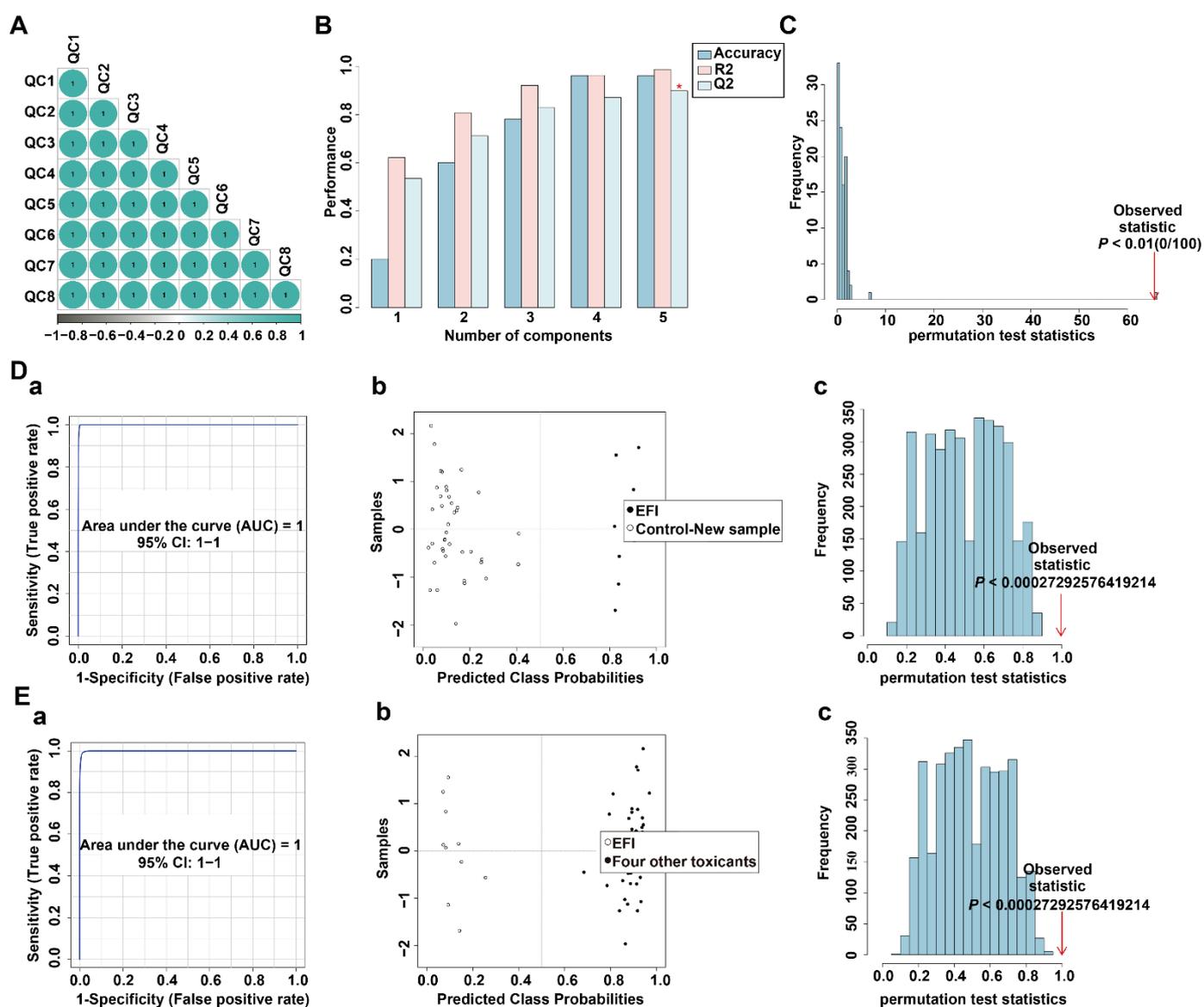


**Figure S1.** Evaluation of PCA and PLS-DA models for plasma samples and the results of EFI classification model validation. A: Correlation analysis plot of QC samples. B: PLS-DA model cross-validation plot by 10-fold CV algorithm, the five-component model was the best (marked ∗), R2 =

0.98675 and Q2 = 0.89801. C: PLS-DA model with 100 permutation-test plots, $p < 0.01$. D: Discriminatory ability evaluation in the test set ($n_{EFI, CD, DR, MA, HS}$ = 10, respectively). (a) ROC plot: AUC = 1 (95% CI: 1−1) (b) Confusion matrix plot showing samples without misclassification. (c) Classification model with 100 permutation-test plots, $p < 0.01$. E: Predictive performance of classification models in the EFI group relative to the four toxicants fatal intoxication models (Heroin, DL-METH, CO, CO2, $n_{animal}$ = 10 respectively) in plasma samples. (a) The ROC plot shows that the AUROC value is 1 (95% CI: 1−1) (b) Confusion matrix plot showing samples without misclassification. (c) Classification model with 100 times permutation test plot, $p < 0.01$.

To further verify the Specificity of this classification model, four more toxicant lethal models (Heroin, DL-METH, CO, $CO_2$) in plasma samples were introduced for the above ROC testing. The results of the ROC plot (AUC = 1) and the distribution states of the confusion matrix plot showed that the classification model could well distinguish the EFI group from the other four toxic poisoning lethal groups, and the result of the 100 times permutation test ($P < 0.01$) showed that the model had good predictive power. The model has good predictive power and strong specificity. (Figures S1Ea–c)

**Table S4.** Prediction results of new plasma samples (Validation set).

| Sample No. | Probability | Class |
|:---:|:---:|:---:|
| 1 | 0.94691 | EFI |
| 2 | 0.94093 | EFI |
| 3 | 0.99081 | Control |
| 4 | 0.97394 | Control |
| 5 | 0.99239 | Control |
| 6 | 0.96297 | Control |
| 7 | 0.99167 | Control |
| 8 | 0.97816 | Control |
| 9 | 0.93148 | Control |
| 10 | 0.93038 | Control |

**Table S5.** Relative blood drug concentration of estazolam in different dose groups (plasma samples).

| No. | 6 × LD50 | 3 × LD50 | 100 × | 50 × |
|:---:|:---:|:---:|:---:|:---:|
| 1 | $8.84 \times 10^{10}$ | $6.70 \times 10^{10}$ | $1.66 \times 10^{7}$ | $2.45 \times 10^{7}$ |
| 2 | $9.12 \times 10^{10}$ | $7.91 \times 10^{10}$ | $1.63 \times 10^{7}$ | $7.58 \times 10^{6}$ |
| 3 | $7.75 \times 10^{10}$ | $7.56 \times 10^{10}$ | $2.46 \times 10^{7}$ | $4.80 \times 10^{7}$ |
| 4 | $8.30 \times 10^{10}$ | $8.31 \times 10^{10}$ | $1.99 \times 10^{7}$ | $1.62 \times 10^{7}$ |
| 5 | $8.51 \times 10^{10}$ | $7.19 \times 10^{10}$ | $1.80 \times 10^{7}$ | $1.98 \times 10^{7}$ |
| 6 | $8.02 \times 10^{10}$ | $7.15 \times 10^{10}$ | $1.16 \times 10^{7}$ | $9.47 \times 10^{6}$ |
| 7 | $9.08 \times 10^{10}$ | $5.94 \times 10^{10}$ | $5.28 \times 10^{7}$ | $1.32 \times 10^{7}$ |
| 8 | $7.38 \times 10^{10}$ | $6.27 \times 10^{10}$ | $7.38 \times 10^{6}$ | $1.66 \times 10^{7}$ |
| 9 | $9.14 \times 10^{10}$ | $6.62 \times 10^{10}$ | $1.41 \times 10^{7}$ | $1.00 \times 10^{7}$ |
| 10 | $1.00 \times 10^{11}$ | $6.86 \times 10^{10}$ | $2.16 \times 10^{7}$ | $1.49 \times 10^{7}$ |

**Table S6.** Sensitivity validation results of classification models in estazolam different dose groups (plasma samples).

|  | 6 × LD50 | 3 × LD50 | 100 × | 50 × |
|---|---|---|---|---|
| TP | 10 | 10 | 8 | 8 |
| TN | 40 | 40 | 32 | 35 |
| FP | 0 | 0 | 8 | 5 |
| FN | 0 | 0 | 2 | 2 |
| **Precision (PPV)** | 1 | 1 | 0.8 | 0.8649 |
| **NPV** | 1 | 1 | 0.8 | 0.8140 |
| **Recall** | 1 | 1 | 0.8 | 0.8000 |
| **F1-score** | 1 | 1 | 0.8 | 0.8386 |

**Table S7.** Stability validation results of classification models in different storage time groups (plasma samples).

|  | 0day | 1day | 5day | 10day | 15day | 20day |
|---|---|---|---|---|---|---|
| **AUC-value** | 1 | 1 | 0.998 | 0.991 | 0.994 | 0.971 |
| TP | 8 | 8 | 8 | 8 | 8 | 7 |
| TN | 18 | 18 | 17 | 17 | 17 | 16 |
| FP | 0 | 0 | 1 | 1 | 1 | 2 |
| FN | 0 | 0 | 0 | 0 | 0 | 1 |
| **Precision (PPV)** | 1.0000 | 1.0000 | 0.9474 | 0.9474 | 0.9474 | 0.8873 |
| **NPV** | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9412 |
| **Recall** | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.8750 |
| **F1-score** | 1.0000 | 1.0000 | 0.9730 | 0.9730 | 0.9730 | 0.8811 |
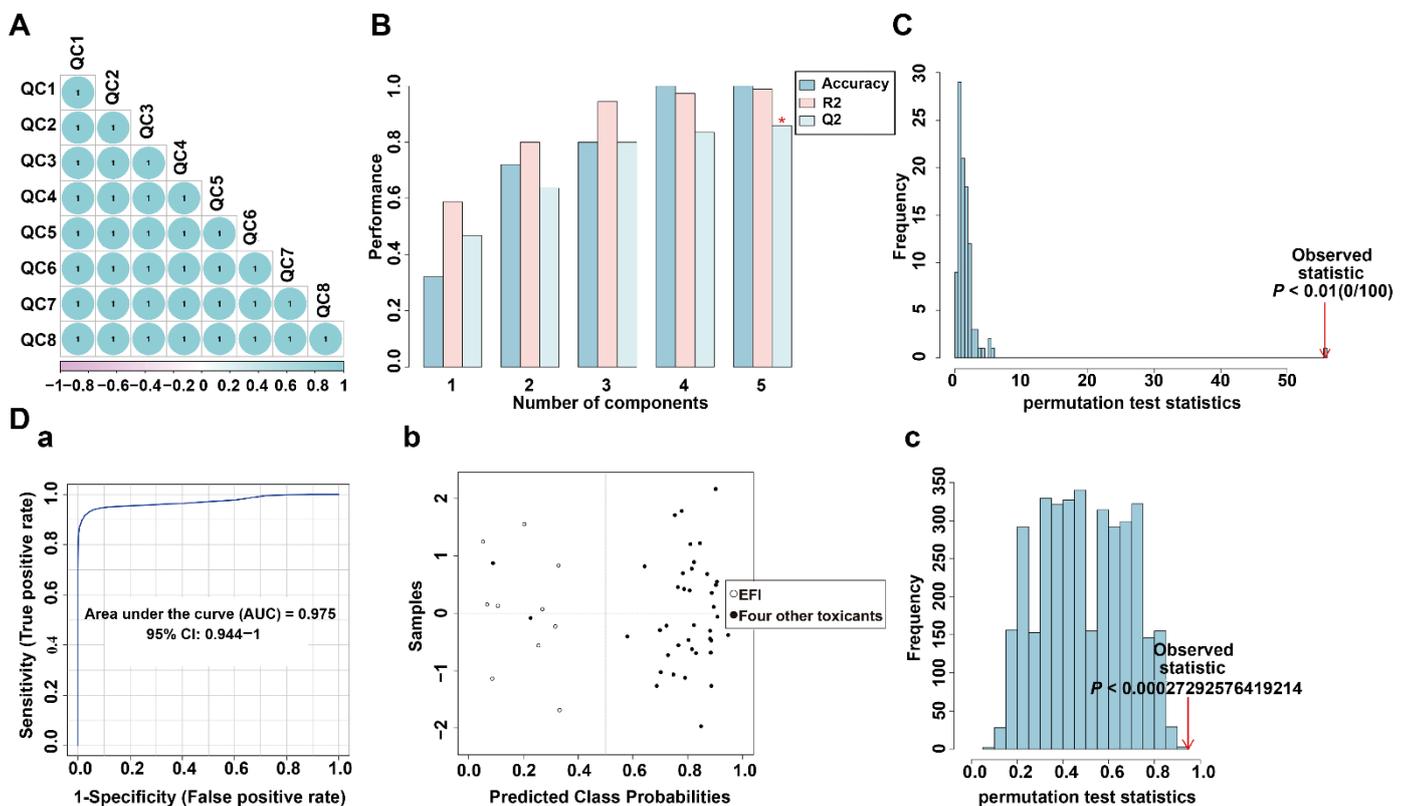


**Figure S2.** Evaluation of PCA and PLS-DA models for brainstem samples and the results of EFI classification model validation. A: Correlation analysis plot of QC samples. B: PLS-DA model

cross-validation plot by 10-fold CV algorithm, the five-component model was the best (marked ∗), R2 = 0.98959 and a Q2 = 0.85946. C: PLS-DA model with 100 permutation-test plots, $p < 0.01$. D: **B**: Predictive performance of classification models in the EFI group relative to the four other toxicants fatal intoxication models (Heroin, DL-METH, CO, CO2, $n_{animal}$ = 10 respectively) in brainstem samples. (**a**) The ROC plot shows that the AUROC value is 0.975 (95% CI: 0.944−1) (**b**) The confusion matrix shows that two cases in the control group were wrongly diagnosed as EFI. (**c**) Classification model with 100 times permutation test plot, $p < 0.01$.

To further verify the specificity of this classification model in brainstem samples, four more toxicant lethal models (Heroin, DL-METH, CO, CO2) were also introduced for the above ROC testing. The results of the ROC plot (AUC = 0.975) and the distribution states of the confusion matrix plot showed that the classification model could well distinguish the EFI group from the other four toxic poisoning lethal groups, and the result of the permutation test ($P < 0.01$) shows that the model was not overfitted (Figures S2Da–c). The classification model exhibits high specificity in brainstem samples.

**Table S8.** AUC values of different candidate differential metabolite combinations in brainstem tissue samples.

| Candidate differential metabolites | Combinations of different candidate differential metabolites | | | |
|---|---|---|---|---|
| Combined Log FC > 1, or < -1, $P < 0.05$ | 1 | 2 | 3 | 4 |
| Palmitic acid | √ | | √ | √ |
| Indole-3-lactic acid | √ | √ | | √ |
| Creatine | √ | √ | √ | |
| Indole-3-acrylic acid | | √ | √ | √ |
| AUC-value | 0.999 | 0.994 | 0.998 | 0.991 |

**Table S9.** Prediction results of new brainstem samples (Validation set).

| Sample No. | Probability | Class |
|---|---|---|
| 1 | 0.98832 | EFI |
| 2 | 0.97902 | EFI |
| 3 | 0.99817 | Control |
| 4 | 0.99585 | Control |
| 5 | 0.95477 | Control |
| 6 | 0.93889 | Control |
| 7 | 0.99797 | Control |
| 8 | 0.99834 | Control |
| 9 | 0.91156 | Control |
| 10 | 0.90506 | Control |

**Table S10.** Sensitivity validation results of classification models in estazolam different dose groups (brainstem samples).

| | 6 x LD$_{50}$ | 3 x LD$_{50}$ | 100 x | 50 x |
|---|---|---|---|---|
| **AUC-value** | 0.999 | 1 | 0.908 | 0.872 |
| TP | 10 | 10 | 8 | 8 |
| TN | 39 | 40 | 33 | 34 |
| FP | 1 | 0 | 7 | 6 |
| FN | 0 | 0 | 2 | 2 |
| **Precision (PPV)** | 0.9756 | 1 | 0.8372 | 0.8421 |
| **NPV** | 1 | 1 | 0.8919 | 0.8095 |
| **Recall** | 1 | 1 | 0.8000 | 0.8000 |
| **F1-score** | 0.9877 | 1 | 0.8182 | 0.8205 |
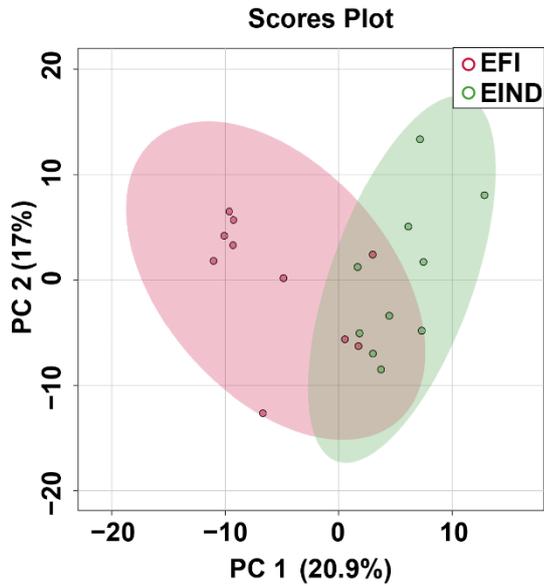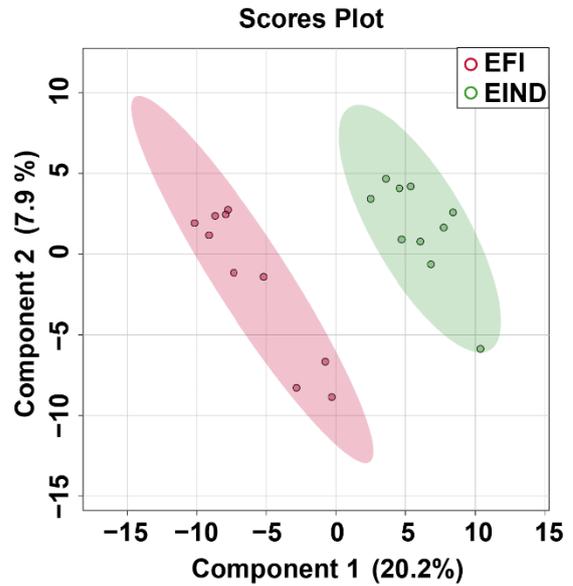
**Figure S3.** Overview of metabolic profiles of the EFI and EIND ($n_{animal} = 10$, respectively) groups. A: PCA score plot. B: PLS-DA score plot.

**Table S11.** Endogenous differential metabolites in the EFI group vs the EIND group.

| FC > 1.5 or FC < 0.67, $P$ < 0.05 | FC | $P$ | VIP | Trend |
|---|---|---|---|---|
| N-Arachidonylglycine | 2.6661 | 0.00010073 | 1.79766 | up |
| NADH | 2.6048 | 0.00034098 | 1.70344 | up |
| Adenosine diphosphate ribose | 2.3382 | 0.0013691 | 1.57474 | up |
| Stearoyl ethanolamide | 2.0853 | 0.037478 | 1.20522 | up |
| N-Acetylvanilalanine | 1.8335 | 0.0006948 | 1.64066 | up |
| Acetylcarnitine | 1.7892 | 0.0077646 | 1.3639 | up |
| (R)-3-hydroxybutyrylcarnitine | 1.591 | 0.00045466 | 1.71114 | up |
| Adenine | 1.5507 | 0.0017718 | 1.56552 | up |
| Uric Acid | 1.5027 | 0.00050132 | 1.6744 | up |
| 15,16-DiHODE | 0.66153 | 0.016455 | 1.30794 | down |
| 3-Hydroxydecanoic acid | 0.61526 | 0.0021894 | 1.53144 | down |
| D-lysopine | 0.60846 | 0.00023753 | 1.73162 | down |
| Kynurenine | 0.58367 | 0.038273 | 1.11644 | down |
| 2-Hydroxycaproic acid | 0.56784 | 0.0076504 | 1.385 | down |
| D-Gluconic acid | 0.53105 | 0.00057824 | 1.65746 | down |
| 3-Oxotetradecanoic acid | 0.51422 | $4.4 \times 10^{-5}$ | 1.8536 | down |
| alpha-Aminoadipic acid | 0.49592 | $8.47 \times 10^{-6}$ | 1.99162 | down |
| Indole-3-lactic acid | 0.49278 | 0.0050607 | 1.44342 | down |
| Leu-Val | 0.47859 | 0.00040455 | 1.7118 | down |
| 3-Hydroxysebacic acid | 0.45214 | 0.0022586 | 1.5244 | down |
| Arg-pro | 0.43829 | 0.0004477 | 1.6837 | down |
| Isovalerylglutamic acid | 0.42847 | 0.0097986 | 1.34168 | down |
| Homovanillic acid sulfate | 0.286 | 0.0031413 | 1.4989 | down |
| Hippuric acid | 0.28531 | 0.00048126 | 1.6788 | down |

**Table S12.** Results from Quantitative Enrichment Analysis of EFI group vs EIND group.

| Metabolite Set | Total | Hits | Statistic | Expected | $P$ | Holm $P$ | FDR |
|---|---|---|---|---|---|---|---|
| Lysine degradation | 25 | 2 | 67.684 | 5.2632 | $8.47 \times 10^{-6}$ | 5.08E-05 | $5.08 \times 10^{-5}$ |
| Purine metabolism | 65 | 3 | 45.623 | 5.2632 | $3.12 \times 10^{-5}$ | 1.56E-04 | $9.35 \times 10^{-5}$ |
| Phenylalanine metabolism | 10 | 1 | 50.091 | 5.2632 | $4.81 \times 10^{-4}$ | 0.001925 | $8.67 \times 10^{-4}$ |
| Pentose phosphate pathway | 22 | 1 | 49.109 | 5.2632 | $5.78 \times 10^{-4}$ | 0.001925 | $8.67 \times 10^{-4}$ |
| Pyrimidine metabolism | 39 | 1 | 24.023 | 5.2632 | 0.028244 | 0.056487 | 0.033892 |
| Tryptophan metabolism | 41 | 1 | 21.735 | 5.2632 | 0.038273 | 0.056487 | 0.038273 |

**Table S13.** Quantification of metabolites in brainstem tissue samples(ng/mg).

| | Control | EIND | EFI |
|---|---|---|---|
| **Lysine** | 0.528432 | 0.717235 | 1.092008 |
| | 0.625512 | 0.720427 | 1.340978 |
| | 0.583638 | 0.755141 | 0.937166 |
| | 0.687387 | 0.746447 | 1.166628 |
| | 0.524275 | 0.797177 | 0.841333 |
| | 0.4395 | 0.740366 | 1.007781 |
| | 0.570175 | 0.837613 | 1.153898 |
| | 0.700907 | 0.660676 | 0.909991 |
| | 0.698202 | 0.685484 | 1.14234 |
| | 0.555585 | 0.754073 | 1.298486 |
| **Saccharopine** | 0.755505 | 1.010357 | 1.107189 |
| | 0.750406 | 0.742726 | 1.085818 |
| | 0.801162 | 0.881176 | 1.089089 |
| | 0.44937 | 0.734578 | 1.518584 |
| | 0.765673 | 1.030368 | 1.073638 |
| | 0.617363 | 0.898919 | 1.108023 |
| | 0.616964 | 1.458254 | 1.443831 |
| | 0.671605 | 1.09252 | 1.243551 |
| | 0.488346 | 0.800901 | 1.048675 |
| | 0.761186 | 0.883453 | 1.807271 |