Identification of absorbed constituents and evaluation of the pharmacokinetics of main compounds after oral administration of yindanxinnaotong by UPLC-Q-TOF-MS and UPLC-QqQ-MS†

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Yindanxinnaotong capsule (YDXNT), a traditional Chinese formula, has been used to treat cardiovascular diseases for several decades. Previous research has focused on evaluating the pharmacological properties and main compounds of YDXNT in vitro and in vivo. However, the multiple bioactive compounds in vivo remain poorly understood. In the present research, an integrative strategy using UPLC-Q-TOF-MS combined with UPLC-QqQ-MS was employed to detect the absorbed constituents and investigate the pharmacokinetics of main compounds in the plasma after oral administration of YDXNT. UPLC-Q-TOF-MS was developed to detect the absorbed constituents and their metabolites in the plasma after oral administration in rats. A total of 52 constituents, including 44 prototype compounds and 8 metabolites, were identified or tentatively characterized. Then, nine main compounds (quercetin, isorhamnetin, kaempferol, ginkgolide A, ginkgolide B, ginkgolide C, bilobalide, tanshinone IIA, and salvianolic acid B) were chosen to further investigate the pharmacokinetic behavior of YDXNT using UPLC-QqQ-MS. The concentration of nine main constituents were in the range of 27.85–76.54 ng mL⁻¹. This research provides a systematic approach for rapid qualitative analysis of absorbed constituents and for evaluating the pharmacokinetics of the main ingredients of YDXNT following its oral administration. More importantly, this work provides key information on the identification of bioactive compounds and the clarification of their action mechanisms, as well as on the pharmacological actions of YDXNT.

1. Introduction

Traditional Chinese medicine (TCM), one of the oldest phyto-medicine systems in health care, has been used in Asia, such as in China, Korea, and Japan, for thousands of years.¹–⁴ The herbs used in TCM usually have complex formulas and function mainly via their multiple constituents, targets and modes of action (3M) in a network-based and holistic manner.⁵ Thus, the therapeutic effects of TCM formulae rely on the joint effect of multiple ingredients. Most TCM formulae are taken orally, and only ingredients absorbed into the blood can exert their bioactivities.⁶ Therefore, it is necessary to trace the compounds in a TCM prescription in vivo and evaluate their pharmacokinetics to provide more in-depth insight into the main active components and therapeutic mechanisms of TCM formulae. In recent years, an integrative strategy using ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) combined with ultra-high-performance liquid chromatography with triple quadrupole mass spectrometry (UPLC-QqQ-MS) has been widely used to identify the absorbed constituents and determine main compounds in complex matrices.⁷–¹ⁱ Hence, they are valuable analytical techniques for identifying key compounds and evaluating the pharmacokinetics of TCM formulae in vivo.

Yindanxinnaotong capsule (YDXNT), a classic TCM formula, comprises eight herbs: *Ginkgo biloba* leaf (yinxingye, YXY), *Salvia miltiorrhiza* (danshen, DS1), *Gynostemma gynostemmatis*...
in vivo and salvianolic acid B (SAB) in rats, with baicalein used as the addition, UPLC-QqQ-MS was used for simultaneous quantitation of the main components quercetin (QCT), kaempferol (KMF),isorhamnetin (ISR), ginkgolide A (GA), ginkgolide B (GB),ginkgolide C (GC), bilobalide (BB), tanshinone IIA (TSIIA), and salvianolic acid B (SAB) in rats, with baicalein used as the internal standard (IS). These compounds exert anti-atherosclerotic and endothelium-independent vasodilator effects, prevent inflammatory damage and have a protective effect on nitric oxide, antioxidant action, and anti-vascular inflammation. The pharmacokinetics of QCT, KMF, ISR, GA, GB, GC, BB, TSIIA, and SAB were investigated in rats after oral administration of YDXNT, and the pharmacokinetics properties of this formula were further speculated. This research represents the first detailed investigation into the absorbed constituents, metabolites, and pharmacokinetics of YDXNT. It may provide valuable information for better understanding the pharmacological mechanisms and clinical applications of YDXNT.

2. Experiments

2.1 Chemicals and reagents

YDXNT soft capsules were provided by Guizhou Bailing Group Pharmaceutical Co., Ltd. Standards of rutin and cryptotanshinone were purchased from Chengdu Chroma-Biotechnology Co., Ltd. (Sichuan, China). Kaempferol, quercetin, tanshinone IIA, salvianolic acid B, ginkgolide A, ginkgolide B, bilobalide, and baicalein were purchased from Chengdu Herb Purify Co., Ltd. (Sichuan, China). Isorhamnetin, ginkgolide C, ginsenoside Rg1, ginsenoside Re, ginsenoside Rd, and notoginsenoside R1 were purchased from the National Institutes for Food and Drug Control (Beijing, China), Ltd., and HPLC-grade acetonitrile and methanol were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). Formic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and the water used in the experiments was purified with a Milli-Q system (Sartorius Arium® Pro, Germany).

2.2 Preparation of YDXNT sample

The samples were first processed by completely removing the outer capsule layer. Then, a 48.0 g sample was precisely weighed and soaked with 100 mL de-ionized water. The sample was ultrasonically dissolved for 30 min and then stored at −4 °C until it was orally administered to rats.

2.3 Animal experiments

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Center for Laboratory Animal Care, China Academy of Chinese Medical Science and approved by the Animal Ethics Committee of Institute of Chinese Material Medica, China Academy of Chinese Medical Science.

Twenty-four male Sprague-Dawley rats (250 ± 30 g) were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and divided into four groups (six rats each). The rats were housed and fed at a temperature of 25 ± 2 °C and a relative humidity of 50% for three days in the breeding room. Standard chow and deionized water were provided prior to the oral treatment. All rats were fasted for 12 h with free access to water before the experiment. YDXNT was administered orally to rats at a dose of 4.8 g kg⁻¹ d⁻¹, and blood was collected from the abdominal aorta after 20% urethane anesthesia and then centrifuged at 3000 rpm for 10 min at 4 °C to obtain the plasma. All samples were stored at −20 °C until analysis. Blank plasma samples were prepared in the same manner.
2.4 Serum sample preparation

Six milliliters of 75% methanol was added to two milliliters of prepared plasma and then vortexed for 60 s. The mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was transferred to another EP tube and dried using a pressure blowing concentrator. The residue was redissolved with 200 μL of 75% methanol, vortexed for 60 s, and then centrifuged at 13 000 rpm for 10 min. To an aliquot of 200 μL plasma, 120 μL of 0.2 mol L⁻¹ acetic acid–sodium acetate buffer solution (pH = 4.6), 100 μL of the IS baicalein and 80 μL of 980 U mL⁻¹ β-glucuronidase and sulfatase was added and then vortexed for 1 min. The mixture was hydrolyzed in a water bath at 37 °C for 30 min, and 600 μL acetone was added to stop the hydrolysis. The sample was cooled, followed by vortexing for 2 min and then centrifugation at 13 000 rpm for 10 min. The supernatant fluid was evaporated to dryness under a flow of nitrogen gas.

2.5 Preparation of standard, calibration, and quality control solutions

Stock solutions of QCT, ISR, KMF, GA, GB, GC, BB, TSIIA, SAB and IS were separately prepared by dissolving the compounds in methanol at a concentration of 200 ng mL⁻¹; all solutions were stored at 4 °C. Working solutions for calibration and QC samples were prepared by adding the diluted stock solutions to blank rat plasma. Calibration standards of plasma-derived working solutions with QCT, ISR, KMF, GA, GB, GC, BB, and TSIIA were all prepared with final concentrations ranging from 1–100 ng mL⁻¹. The SAB solution was prepared with final concentrations ranging...
from 5–500 ng mL\textsuperscript{−1}. The IS solution was prepared at a final concentration of 100 ng mL\textsuperscript{−1} in methanol. For method validation, QC plasma samples of QCT, ISR, KMF, GA, GB, GC, BB, and TSIIA were all prepared separately at three concentrations (1, 10 and 100 ng mL\textsuperscript{−1}). The QC plasma sample of SAB was prepared at three concentrations (5, 50, and 500 ng mL\textsuperscript{−1}).

### 2.6 UPLC-Q-TOF-MS for qualitative analysis

UPLC data were produced using the Waters Synapt G2-S UPLC system (Waters, Milford, USA) equipped with a quaternary pump system, online degasser, autosampler, thermostatically controlled column compartment, and diode array detector. The system was controlled with Masslynx V4.1 software. The chromatographic column ACQUITY BEH C18 (100 × 2.1 mm, 1.7 μm) equipped with VanGuard™ BEHC18 1.7 μm was used. The column was eluted with a gradient of A (0.1% formic acid in deionized water) and B (CAN) at a flow rate of 0.3 mL min\textsuperscript{−1} and a temperature of 30 °C: 0–2.5 min, 10–16% B; 2.5–6.5 min, 16–16% B; 6.5–9.5 min, 16–25% B; 9.5–11.0 min, 25–25% B; 11.0–12.0 min, 25–45% B; 12.0–15.0 min, 45–55% B; 15.0–18.0, 55–

#### Table 1

| Analyte | MRM transitions | Fragmentor (V) | Collision energy (V) | Dwell time (ms) |
|---------|-----------------|----------------|----------------------|-----------------|
| QCT     | 301.1 → 151.0   | 350            | 26                   | 20              |
| ISR     | 315.0 → 300.1   | 350            | 22                   | 20              |
| KMF     | 285.0 → 142.9   | 350            | 35                   | 20              |
| GA      | 453.1 → 351.1   | 350            | 16                   | 20              |
| GB      | 423.1 → 367.1   | 350            | 15                   | 20              |
| GC      | 439.1 → 383.1   | 350            | 12                   | 20              |
| BB      | 325.1 → 163.1   | 350            | 16                   | 20              |
| TSIIA   | 295.0 → 249.0   | 350            | 26                   | 20              |
| SAB     | 717.1 → 519.0   | 350            | 38                   | 20              |
| IS      | 269.0 → 195.0   | 350            | 28                   | 20              |

Fig. 2 The UPLC-Q-TOF-MS results for drug-containing plasma. (A) negative ionisation mode-analysis of drug-containing plasma. (B) positive ionisation-mode analysis of drug-containing plasma.
Table 2  (1) Chromatographic and MS data of absorbed constituents analysed by UPLC-Q-TOF-MS in negative mode. (2) Chromatographic and MS data of absorbed constituents analysed by UPLC-Q-TOF-MS in positive mode

| Peak no. | RT (min) | [M – H]− | Tolerance ppm | MS² | Molecular formula | Tentative identity | Source |
|----------|----------|------------|---------------|-----|------------------|-------------------|--------|
| 1        | 1.01     | 217.0338   | 2.284         | 135.0470 [M – H-C6H4O]− | C7H10O3 | Unknown | 16 |
| 2        | 2.03     | 197.0449   | 2.691         | 109.0326 [M – H-CO2]− | C7H8O4 | Tanshinol | 16 |
| 3        | 2.37     | 153.0188   | 0.950         | 108.0453 | C7H8O3 | Protocatechuic acid | 16 |
| 4        | 3.18     | 137.0233   | 0.150         | 108.4153 | C7H8O3 | Protocatechualdehyde | 16 |
| 5        | 3.62     | 179.0349   | 5.686         | 178.9783, 134.963 | C7H9O4 | Caffeic acid | 16 |
| 6        | 4.99     | 755.2035   | 6.4           | 753.1502 [M – H-C6H4O]− | C7H11O5 | Quercetin 3-O-[2,6-di-O-bis(2-0-C-glucosyl)-β-d-glucoside] | 16 |
| 7        | 5.82     | 193.0501   | 0.0           | 183.0453, 139.037 | C7H9O4 | Kaempferol 3-O-[2,6-di-O-bis(α-L-rhamnosyl)-β-d-glucoside] (a) | 16 |
| 8        | 6.00     | 739.2123   | 5.810         | 737.1650 [M – H-C6H4O]− | C7H10O4 | Ferulic acid | 16 |
| 9        | 6.19     | 769.2180   | 0.741         | 767.1707, 315.074, 193.047 | C7H12O8 | Bilobalide | 16 |
| 10       | 6.73     | 609.1464   | 2.280         | 607.1097, 351.065, 239.037 | C7H10O4 | Ginkgolide C | 16 |
| 11       | 6.83     | 325.0914   | 0.9           | 323.0454, 163.1108 | C7H9O3 | Apigenin 7-O-rutinoside | 16 |
| 12       | 7.31     | 439.1245   | 2.305         | 437.0877, 241.050, 123.0236 | C10H10O4 | 16 |
| 13       | 9.20     | 609.1459   | 2.280         | 607.1097, 351.065, 239.037 | C7H10O4 | Kaempferol 3-O-[2,6-di-O-bis(α-L-rhamnosyl)-β-d-glucoside] | 16 |
| 14       | 9.34     | 593.1501   | 0.006         | 591.1033, 335.065, 241.037 | C7H10O4 | 16 |
| 15       | 9.76     | 623.1625   | 2.951         | 621.1154, 351.065, 239.037 | C7H10O4 | 16 |
| 16       | 9.96     | 577.1567   | 2.630         | 575.1097, 329.065, 239.037 | C7H10O4 | 16 |
| 17       | 10.52    | 359.0775   | 3.777         | 357.1033, 209.065, 145.037 | C7H8O3 | 16 |
| 18       | 10.57    | 593.1501   | 0.006         | 591.1033, 335.065, 241.037 | C7H10O4 | 16 |
| 19       | 11.30    | 755.1843   | 3.323         | 753.1376, 321.065, 153.037 | C7H10O4 | 16 |
| 20       | 11.39    | 977.3546   | 3.099         | 975.4078, 391.071, 279.044 | C7H10O4 | 16 |
| 21       | 11.67    | 717.1407   | −0.6011       | 715.1335, 363.096, 181.060 | C7H10O4 | 16 |
| 22       | 11.91    | 407.1346   | 2.312         | 405.1078, 259.071, 153.037 | C7H9O3 | 16 |
| 23       | 11.93    | 423.1321   | 8.335         | 421.0854, 271.048, 145.021 | C7H9O3 | 16 |
| 24       | 11.98    | 845.4844   | −5.810        | 843.4376, 457.070, 279.044 | C7H10O4 | 16 |
| 25       | 12.01    | 991.5478   | 0.584         | 989.4910, 541.048, 279.044 | C7H10O4 | 16 |
| 26       | 12.29    | 739.1883   | 2.332         | 737.1415, 481.076, 279.044 | C7H10O4 | 16 |
| 27       | 13.10    | 301.0343   | 0.07          | 300.0275, 150.013, 75.007 | C7H9O3 | 16 |
| Peak no. | RT (min) | [M – H]⁻ | Tolerance ppm | MS² | Molecular formula | Tentatively identity | Source |
|---------|----------|-----------|---------------|-----|-------------------|---------------------|--------|
| 28      | 13.28    | 637.1726  | 3.939         |      |                   | C₂₉H₅₄O₁₆         | Unknown | 16    |
| 29      | 14.47    | 269.0482  | –5.138        |      |                   | C₂₃H₄₀O₆          | Apigenin | 16    |
| 30      | 14.66    | 285.0453  | –4.546        |      |                   | C₂₅H₄₂O₁₂         | Kaempferol | 16    |
| 31      | 14.83    | 1153.5948 | –4.546        |      |                   | C₆₃H₉₄O₂₅         | Ginsenoside Rb₁ | 16 |
| 32      | 14.88    | 315.0522  | 7.208         |      |                   | C₁₆H₁₂O₇           | Isorhamnetin | 16 |
| 33      | 14.97    | 829.4974  | 3.619         |      |                   | C₄₁H₇₁O₁₃         | Ginsenoside F₂/20(s)-ginsenoside Rg₃ | 16 |
| 34      | 15.05    | 683.4370  | 0.748         |      |                   | C₁₆H₂₃O₉           | Ginsenoside F₁/ginsenoside Rh₁ | 16 |
| 35      | 15.22    | 683.4365  | 0.016         |      |                   | C₁₆H₂₃O₉           | Ginsenoside F₁/ginsenoside Rh₁ | 16 |
| 36      | 15.54    | 991.5486  | 1.391         |      |                   | C₁₉H₂₄O₁₈         | Ginsenoside Rd  | 16 |
| 37      | 18.44    | 313.1482  | 4.3           |      |                   | C₁₇H₁₄O₆           | Salvianolic acid F | 16 |

| Peak no. | RT (min) | [M + H]⁺ | Tolerance ppm | MS² | Molecular formula | Tentatively identity | Source |
|----------|----------|-----------|---------------|-----|-------------------|---------------------|--------|
| 38       | 14.72    | 311.1254  | –9.3          | 293.0783 [M + H⁻H₂O]⁻  | C₁₉H₁₄O₄         | Tanshinone II B | 16 |
| 39       | 15.10    | 311.1256  | –7.3          | 293.1178 [M + H⁻H₂O]⁻  | C₁₉H₁₄O₄         | 3α-Hydroxytanshinone II A | 16 |
| 40       | 15.33    | 309.1118  | –1.085        | 293.1057 [M + H⁻2H₂O]⁻ | C₁₉H₁₂O₄         | Tanshinaldehyde | 16 |
| 41       | 17.07    | 311.1254  | –9.3          | 293.1173 [M + H⁻H₂O]⁻  | C₁₉H₁₂O₄         | Przewaquinone A | 16 |
| 42       | 17.91    | 279.1017  | 1.179         | 261.0872 [M + H⁻H₂O]⁻  | C₁₉H₁₂O₃         | Dihydrotanshinone I | 16 |
| 43       | 20.77    | 297.1495  | 3.295         | 279.1382 [M + H⁻H₂O]⁻  | C₁₉H₁₂O₃         | Cryptotanshinone | 16 |
| 44       | 21.80    | 295.1323  | –1.935        | 277.1293 [M + H⁻H₂O]⁻  | C₁₉H₁₂O₃         | Tanshinone IIA | 16 |
### Table 3

| Peak no. | RT (min) | Molecular formula | Tentatively identified | Source |
|----------|----------|-------------------|------------------------|--------|
| M1       | 11.87     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M2       | 14.59     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M3       | 17.46     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M4       | 20.38     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M5       | 23.29     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M6       | 26.20     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M7       | 29.11     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |
| M8       | 32.02     | C_{9}H_{13}O_{4}  | 1,2; 1,3; 2,3-dehydrotanshinone IIA | 35,46 |

#### 2.7 UPLC-QqQ-MS for quantitative analysis

Chromatographic analysis was performed using an Agilent 1290 Series UPLC from Agilent Technologies (Palo Alto, CA, USA), equipped with a quaternary pump an online degasser, autosampler and column oven. Chromatographic separation was performed on the Agilent ZORBAX SB-Aq C18 column (100 mm × 2.1 mm, 1.8 μm, Agilent, USA). The analytical column temperature was maintained at 30 °C and eluted with a mobile phase comprising 0.1% formic acid in water (A) and acetonitrile (B) using the following gradient program: 25–90% B from 0 to 1 min, 90% B from 1 to 3.5 min, and 90–25% B from 3.5 to 5 min at a flow rate of 0.3 mL min⁻¹. The equilibration time was 5 min, and the injection volume of sample and reference constituents was 5 μL.

Mass spectrometry was performed on the Agilent 6490 triple-quadrupole mass spectrometer (Palo Alto, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface. The mass spectrometer was set in negative ionization mode, with the capillary voltage set at 3500 V. The other parameters in the source were set as follows: desolvation gas temperature, 350 °C; nebulizer gas (N2) pressure, 50 psi; flow, 12 L min⁻¹. The mass spectrometer scanned in multiple reaction monitoring (MRM) mode. The UPLC and QqQ-MS methods have been optimised (ESI 1†).

#### 2.8 Method validation

Method validation was performed according to the guidelines for bioanalytical method validation published by the US FDA. The analytical method used in this research was validated to demonstrate its specificity, selectivity, linearity, lower limit of quantification (LLOQ), precision, accuracy, stability, extraction recovery rates and matrix effects of the samples (ESI 3†).

#### 2.9 Pharmacokinetics study

Six male rats were used in the pharmacokinetics study. All the rats were orally administered 2.4 g kg⁻¹ YXCNT suspended in an aqueous solution of 0.5% carboxymethyl cellulose sodium (QCT: 4.118 mg; KMF: 5.067 mg; ISR: 0.965 mg; GA: 1.416 mg; 98% B; 18.0–21.0 min, 98–98% B; 21.0–25.0 min, 98–10% B. An injection volume of 10 μL was used for analysis.
GB: 0.680 mg; GC: 0.796 mg; BB: 0.326 mg; TSIIA: 0.624 mg; SAB: 3.84 mg). After dosing for 0 min, 5 min, 15 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, and 24 h, blood samples (500 μL) were drawn from the retro-orbital plexus of each mouse and collected in heparinized tubes according to the specific schedule. All the collected blood samples were immediately centrifuged at 13 000 rpm for 10 min. The plasma layer was transferred to clean tubes and stored at −20 °C until analysis. The plasma concentrations of QCT, ISR, KMF, GA, GB, GC, BB, TSIIA, and SAB were expressed as the mean ± standard deviation (SD), and the mean concentration–time curve was plotted. Non-compartmental pharmacokinetic parameters were computed using DAS 2.1.1 software.

3. Results and discussion

3.1 Identification of prototype constituents and metabolites in drug-containing plasma

The optimized LC-MS protocol was used for the separation and identification of compounds in drug-containing plasma. Exogenous constituents of YDXNT in the plasma were divided into prototype components and metabolites. Ultimately, a total of 52 compounds, including 44 prototype constituents and 8
metabolites, were detected by comparison to the blank plasma sample (Fig. 2, Tables 2 and 3).

3.2 Analysis of prototype constituents of YDXNT in rat plasma

Owing to the high sensitivity of UPLC-Q-TOF-MS and extracted ion chromatograms, 44 prototype constituents were tentatively identified by comparison with standards, mass data, fragment information, and our previous compound research. Of the 44 peaks, 36 were detected in negative-ionization mode, and 7 peaks were detected in positive-ionization mode. The peaks included 15 flavonoids, 3 ginkgolide, 8 phenolic, 8 ginsenosides, and 7 diterpenoid tanshinones. Among them, 14 peaks were further confirmed with standards by comparing retention time and MS data, including peaks 10, 11, 12, 20, 21, 22, 23, 24, 26, 27, 30, 32, 36, 43, and 44.

3.3 Analysis of the metabolites of YDXNT in rat plasma

The full-scan mass spectrum was achieved from the plasma sample after YDXNT was orally administered to rats and compared to the blank plasma sample to discover possible metabolites in rat plasma. Eight metabolites, including 1 monophenol, 4 ginkgolides, 2 flavonoids, and 1 diterpenoid tanshinone, were tentatively identified from the plasma by
comparing their retention times, deprotonated molecules, and characteristic fragments to those reported in the literature.\textsuperscript{31-47} The precursors of these metabolites were deduced to be protocatechuic aldehyde, ginkgolide A, ginkgolide B, ginkgolide C, apigenin, kaempferol, and tanshinone II A. Characterization of the structures of these metabolites is described below.

In our research, one monophenolic-related metabolite (M1) was detected. M1 (t<sub>r</sub> = 2.59 min) showed a deprotonated molecular ion at m/z 313.0555, and product ions of m/z 137.0233 were obtained. The neutral loss of 176 Da (C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>) indicated that M1 was the glucuronide conjugate\textsuperscript{32,33} (Fig. 3). Based on retention time, accurate quasi-molecular ion, and MS/MS data, as well as the related literature,\textsuperscript{33-34} M1 was tentatively identified as the characteristic ion of protocatechuic aldehyde glucuronide.

Ginkgolide-related metabolites were the main possible xenobiotic metabolites in plasma.\textsuperscript{35,36} In the present study, 3 ginkgolides and 4 ginkgolide-related metabolites were detected. The 3 ginkgolides (ginkgolide A, ginkgolide C and ginkgolide B) were identified by comparison to standards. The characteristic ions at m/z 351, m/z 383 and m/z 367 were observed in ginkgolide A, ginkgolide C, and ginkgolide B, respectively. The common fragmentation pattern of ginkgolide was the successive loss of 2CO.\textsuperscript{37} M2, M3 and M4 have [M – H]\textsuperscript{−} ions at m/z

### Table 4

| Biosamples | Analyte | Linear range (ng mL\textsuperscript{-1}) | Linear equation | R\textsuperscript{2} | LLOQ (ng mL\textsuperscript{-1}) |
|------------|---------|------------------------------------------|----------------|----------------|-------------------------------|
| Plasma     | QCT     | 1–100                                    | \(Y = 0.0443X + 0.0621\) | 0.9993 | 1               |
|            | ISR     | 1–100                                    | \(Y = 1.8431X - 16.561\) | 0.9982 | 1               |
|            | KMF     | 1–100                                    | \(Y = 0.0031X + 0.0222\) | 0.9971 | 1               |
|            | GA      | 1–100                                    | \(Y = 0.00741X + 0.3012\) | 0.9987 | 1               |
|            | GB      | 1–100                                    | \(Y = 0.1353X + 1.3265\) | 0.9987 | 1               |
|            | GC      | 1–100                                    | \(Y = 0.0541X + 0.4030\) | 0.9995 | 1               |
|            | BB      | 1–100                                    | \(Y = 0.204X + 0.1212\) | 0.9978 | 1               |
|            | TSIIA   | 1–100                                    | \(Y = 0.3066X + 0.3920\) | 0.9992 | 1               |
|            | SAB     | 5–500                                    | \(Y = 0.0063X + 0.0542\) | 0.9985 | 5               |

### Table 5

| Biosamples | Analytes | Analyte concentration (ng mL\textsuperscript{-1}) | Inter-day | Intra-day |
|------------|----------|--------------------------------------------------|-----------|-----------|
|            |          |                                                  | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) |
| Plasma     | QCT      | 1                                                | 94.16 ± 2.36 | 2.51 | 94.56 ± 4.05 | 4.28 |
|            |          | 10                                               | 107.52 ± 4.82 | 4.48 | 101.12 ± 5.32 | 5.26 |
|            |          | 100                                              | 98.73 ± 6.29 | 6.37 | 96.18 ± 7.43 | 7.73 |
|            | ISR      | 1                                                | 97.75 ± 7.39 | 7.56 | 98.33 ± 6.71 | 6.82 |
|            |          | 10                                               | 93.12 ± 5.48 | 5.88 | 95.26 ± 7.93 | 8.32 |
|            |          | 100                                              | 102.48 ± 3.32 | 3.24 | 100.11 ± 5.19 | 5.18 |
|            | KMF      | 1                                                | 96.49 ± 5.19 | 6.71 | 94.20 ± 6.90 | 7.32 |
|            |          | 10                                               | 103.28 ± 8.48 | 8.21 | 99.89 ± 10.15 | 10.16 |
|            |          | 100                                              | 92.27 ± 4.31 | 4.67 | 96.37 ± 6.54 | 6.79 |
|            | GA       | 1                                                | 100.35 ± 6.40 | 6.38 | 101.18 ± 9.64 | 9.53 |
|            |          | 10                                               | 97.57 ± 2.45 | 2.51 | 95.47 ± 3.19 | 3.34 |
|            |          | 100                                              | 103.63 ± 8.81 | 8.5 | 101.54 ± 9.30 | 9.16 |
|            | GB       | 1                                                | 95.53 ± 4.17 | 4.37 | 94.15 ± 6.99 | 7.42 |
|            |          | 10                                               | 101.13 ± 3.23 | 3.19 | 98.24 ± 5.59 | 5.69 |
|            |          | 100                                              | 99.28 ± 7.45 | 9.87 | 97.11 ± 8.97 | 9.24 |
|            | GC       | 1                                                | 94.25 ± 8.31 | 7.5 | 95.35 ± 7.80 | 8.18 |
|            |          | 10                                               | 101.37 ± 9.22 | 9.1 | 99.40 ± 10.20 | 10.26 |
|            |          | 100                                              | 97.46 ± 5.34 | 5.48 | 95.27 ± 6.24 | 6.55 |
|            | BB       | 1                                                | 105.38 ± 7.24 | 6.87 | 101.33 ± 7.20 | 7.11 |
|            |          | 10                                               | 94.21 ± 4.22 | 4.48 | 93.62 ± 5.27 | 5.63 |
|            |          | 100                                              | 96.48 ± 5.29 | 5.48 | 94.71 ± 6.03 | 6.37 |
|            | TSIIA    | 1                                                | 97.36 ± 6.53 | 6.71 | 94.41 ± 4.23 | 3.85 |
|            |          | 10                                               | 102.42 ± 4.73 | 4.62 | 98.56 ± 6.73 | 2.73 |
|            |          | 100                                              | 106.26 ± 10.49 | 9.87 | 99.18 ± 5.59 | 8.93 |
|            | SAB      | 5                                                | 92.65 ± 6.78 | 7.32 | 103.11 ± 6.94 | 4.97 |
|            |          | 50                                               | 94.57 ± 4.69 | 4.96 | 95.44 ± 7.26 | 6.20 |
|            |          | 500                                              | 101.77 ± 7.82 | 7.68 | 98.84 ± 5.38 | 9.44 |
M5 and M7 were tentatively identified as apigenin-3-O-glucuronide (Fig. 5) and kaempferol-3-O-glucuronide by comparing retention times, accurate quasi-molecular ions, and MS/MS fragmentation data as well as by referring to the related literature.40–44

One tanshinone-related metabolite was tentatively identified from rat plasma on the basis of accurate deprotonated molecular data, MS/MS fragmentation data, and previous reports.45–47 M7 gives rise to a protonated molecule \([M + H]^+\) at \(m/z\) 293.0816. This molecule was 2 Da smaller than TSIIA. The neutral loss of \(m/z\) 15, \(m/z\) 18, and \(m/z\) 28 indicated the loss of \(\mathrm{CH}_2\), \(\mathrm{H}_2\mathrm{O}\), and \(\mathrm{CO}\), respectively (Fig. 6). Corresponding to the deprotonated molecular ion and its fragments, M7 was tentatively identified as dehydrogenated TSIIA, which has been reported in the literature.44,45–47

### 3.4 Assay validation

#### 3.4.1 Specificity. Under the UPLC-MS/MS conditions, a specific voltage at the optimal value was set to obtain the best sensitivity and specificity for each analyte. According to the results, there was no obvious interference from endogenous components, indicating good method selectivity. The chromatographic peaks were at retention times of 2.435, 2.580, 2.557, 2.499, 2.494, 2.324, 2.454, 3.384, 2.285 and 2.612 min for QCT, ISR, KMF, GA, GB, GC, BB, TSIIA, SAB and IS, respectively.

#### 3.4.2 Linearity. The calibration curves showed good linearity \((R^2 > 0.9931)\) over the concentration ranges of the nine

| Biosamples | Analytes | Analyte concentration (ng mL\(^{-1}\)) | Matrix effect | Extraction recovery | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) |
|------------|----------|----------------------------------------|---------------|---------------------|--------------|---------|--------------|---------|
| Plasma     | QCT 1    | 1                                      | 100.69 ± 9.02 | 8.96                | 84.42 ± 8.51 | 10.08   |
|            | 10       | 97.51 ± 3.91                          | 4.51          | 79.87 ± 5.69        | 9.26        |
|            | 100      | 96.51 ± 5.94                          | 6.16          | 93.89 ± 3.40        | 3.62        |
| ISR 1      | 97.56 ± 4.78                          | 4.9           | 83.03 ± 4.57        | 5.5        |
|            | 10       | 96.33 ± 8.08                          | 8.38          | 79.84 ± 2.27        | 2.85        |
|            | 100      | 102.33 ± 10.89                         | 10.64         | 90.31 ± 6.74        | 7.46        |
| KMF 1      | 96.13 ± 5.84                          | 6.08          | 79.73 ± 5.88        | 7.37        |
|            | 10       | 100.04 ± 8.39                         | 8.38          | 89.14 ± 3.13        | 3.52        |
|            | 100      | 98.81 ± 3.84                          | 5.91          | 95.77 ± 5.63        | 5.88        |
| GA 1       | 103.25 ± 7.22                          | 6.99          | 83.17 ± 3.24        | 3.89        |
|            | 10       | 102.75 ± 8.69                          | 8.45          | 87.72 ± 3.75        | 4.27        |
|            | 100      | 98.48 ± 6.99                          | 7.09          | 93.89 ± 3.40        | 3.62        |
| GB 1       | 99.29 ± 5.99                           | 6.04          | 87.26 ± 3.27        | 5.26        |
|            | 10       | 100.39 ± 5.82                          | 5.8           | 85.72 ± 3.28        | 3.83        |
|            | 100      | 94.71 ± 3.26                          | 3.44          | 91.12 ± 5.14        | 5.64        |
| GC 1       | 101.18 ± 9.92                          | 9.13          | 87.72 ± 3.75        | 4.27        |
|            | 10       | 98.50 ± 4.62                           | 4.69          | 83.47 ± 6.25        | 7.49        |
|            | 100      | 91.12 ± 5.14                           | 5.64          | 87.72 ± 3.75        | 4.27        |
| BB 1       | 94.25 ± 7.36                           | 7.8           | 76.86 ± 5.23        | 6.8         |
|            | 10       | 97.57 ± 5.82                           | 5.97          | 81.33 ± 4.89        | 4.27        |
|            | 100      | 94.56 ± 3.50                           | 3.7           | 91.22 ± 3.18        | 6.01        |
| TSIIA 1    | 94.63 ± 3.91                           | 4.13          | 87.72 ± 3.75        | 4.27        |
|            | 10       | 93.28 ± 5.21                           | 5.59          | 93.92 ± 8.18        | 8.71        |
|            | 100      | 99.11 ± 12.27                         | 12.38         | 72.43 ± 6.26        | 8.64        |
| SAB 5      | 96.92 ± 5.83                           | 6.02          | 77.65 ± 5.72        | 7.37        |
|            | 50       | 103.64 ± 11.20                         | 10.80         | 83.97 ± 7.24        | 8.62        |
|            | 500      |                                          |               |                      |             |
constituents in rat plasma. The correlation coefficients of the standard curves and the linear ranges of the plasma are listed in Table 4.

3.4.3 Precision and accuracy. The precision and accuracy data for six replicates of the QC samples at three concentrations are shown in Table 5. The results demonstrated that the precision (RSD%) of the method was 10.26%.

3.4.4 Extraction recovery rates and matrix effects. The extraction recovery and matrix effects of each QC concentration are listed in Table 6. The extraction recoveries ranged from 76.86% to 95.77% at different concentrations in plasma. All ratios for matrix effects were in the range of 94.56–103.25% in plasma samples. Thus, no significant matrix effects were observed for the analytes.

3.4.5 Stability. The data for freeze–thaw stability, short-term temperature stability, long-term stability, and autosampler stability under different storage conditions are summarized in Table 7. The results were well within the acceptable limits, therefore validating the established method for sample extraction, storage and intermittent analysis and indicating its suitability for pharmacokinetic study.

3.5 Pharmacokinetics studies
The developed and validated UPLC-QqQ-MS/MS method was successfully applied in the pharmacokinetics study of QCT, ISR, KMF, GA, GB, GC, BB, TSIIA, and SAB in rat plasma after an oral dose of 4.8 g kg⁻¹ d⁻¹ YDXNT. Fig. 7 shows the mean plasma concentration–time profiles of nine constituents in rat plasma. Table 8 shows the main pharmacokinetic parameters of QCT, ISR, KMF, GA, GB, GC, BB, TSIIA, and SAB in rat plasma. GA and GB have similar pharmacokinetic parameters and may be connected to similar polar constituents, similar to the absorption and elimination processes. The Cₘₐₓ of GC was the lowest among the four ginkgolides, indicating that GC may be partially converted to hydrolyzed ginkgolide C, as we detected. The time required to reach the maximum plasma concentration (Tₘₐₓ) was 0.75 h for GA, 1.00 h for GB, 1.50 h for GC, and 0.75 h for BB. The T₁/₂ of four ginkgolides in YDXNT were 7.52 h, 7.84 h, 5.45 h, and 5.55 h. Absorption of the four ginkgolides was slower than that of the flavonols, TSIIA and SAB, while their distribution occurred relatively faster than that of the flavonols, TSIIA and SAB. Additionally, the flavonols showed double peaks in the mean plasma concentration curves. This phenomenon has been reported previously, indicating that these components might have enterohepatic recirculation. The double peaks suggested that enterohepatic recirculation, as well as intertransformation among the compounds, might have occurred. One possible explanation for this phenomenon is that some of the other compounds with similar structures might have transformed into these compounds. Since drug absorption is
a complex process, more detailed adsorption studies are needed to ascertain the mechanism of the double-peak phenomenon. The $C_{\text{max}}$ of TSIIA (38.34 ng mL$^{-1}$) is higher than that of SAB (32.00 ng mL$^{-1}$), and the $T_{\text{max}}$ and $T_{1/2}$ of TSIIA (0.25 h, 7.04 h) are lower than those of SAB (0.75 h, 12.17 h), indicating the absorption and distribution rates of TSIIA are faster than those of SAB in rats.

Fig. 7 Plasma concentration–time profiles of the analytes following oral administration of the YDXNT to rats (mean ± SD, $n = 6$).

| Analytes | $C_{\text{max}}$(ng mL$^{-1}$) | $T_{\text{max}}$ (h) | AUC$_{0-\text{t}}$ (ng mL$^{-1}$ h$^{-1}$) | AUC$_{0-\text{INF}}$ (ng mL$^{-1}$ h$^{-1}$) | $T_{1/2}$ (h) | MRT (h) |
|----------|-------------------------------|----------------------|------------------------------------------|------------------------------------------|----------------|--------|
| QCT      | 45.02 ± 11.28                 | 0.33 ± 0.11          | 410.34 ± 73.15                           | 412.46 ± 82.67                           | 2.69 ± 0.65    | 8.44 ± 1.24 |
| ISR      | 27.85 ± 8.38                  | 0.33 ± 0.14          | 339.17 ± 49.27                           | 384.49 ± 55.13                           | 8.19 ± 2.42    | 13.91 ± 3.71 |
| KMF      | 49.90 ± 13.82                 | 0.50 ± 0.23          | 401.33 ± 84.37                           | 539.04 ± 82.29                           | 8.19 ± 2.42    | 17.11 ± 4.42 |
| GA       | 76.31 ± 18.19                 | 0.75 ± 0.29          | 490.92 ± 72.63                           | 545.10 ± 78.91                           | 7.52 ± 2.08    | 9.42 ± 1.77  |
| GB       | 76.54 ± 15.43                 | 1.00 ± 0.35          | 610.18 ± 91.45                           | 691.00 ± 96.83                           | 7.84 ± 3.53    | 10.58 ± 2.63 |
| GC       | 35.35 ± 10.28                 | 1.50 ± 0.23          | 281.80 ± 41.33                           | 297.49 ± 46.59                           | 5.45 ± 1.48    | 7.77 ± 2.62  |
| BB       | 48.70 ± 12.34                 | 0.75 ± 0.50          | 365.97 ± 48.37                           | 383.98 ± 51.49                           | 5.55 ± 2.67    | 9.62 ± 3.18  |
| TSIIA    | 38.34 ± 17.35                 | 0.25 ± 0.23          | 167.20 ± 69.49                           | 182.83 ± 77.41                           | 7.04 ± 2.18    | 9.89 ± 1.50  |
| SAB      | 32.00 ± 15.43                 | 0.75 ± 0.18          | 118.17 ± 58.46                           | 135.72 ± 64.24                           | 12.17 ± 3.33   | 9.46 ± 2.37  |
4. Conclusion

In this research, we developed the UPLC-Q-TOF-MS method for analyzing and identifying the main absorbed constituents of YDXNT and their possible metabolites in rat plasma. A total of 52 constituents, including 44 prototype compounds and 8 metabolites, were identified or tentatively characterized. Among the prototype constituents, 15 flavonoids, 4 ginkgolides, 8 phenolic, 8 ginsenoside, and 7 diterpenoid tanshinones were identified. The metabolic routes of 8 metabolites in rat plasma after oral administration were hypothesized. The result indicated that the metabolites of YDXNT undergo the phase I metabolite pathway of hydrolysis (M2, M3, M4, and M6), dehydrogenation (M8) and the phase II metabolic route of glucuronide (M1, M5 and M7). Furthermore, a sensitive and reliable method based on UPLC-QqQ-MS was established for simultaneous quantification of nine abundant main compounds in rat plasma. The developed assay was successfully applied to assess the pharmacokinetics of ginkgetin aglycone, tanshinone IIA, salvianolic acid B, and tanshinone from YDXNT applied to assess the pharmacokinetics of ginkgetin aglycone, 15738

This work provides more in-depth insight into the main active constituents of YDXNT in vivo as well as helpful information for clinical application. More importantly, our findings are helpful for better understanding the material foundation and action targets underlying the efficacy of YDXNT.

Author contributions

All the authors have approved the manuscript and agree with submission to your esteemed journal.

Conflicts of interest

There are no conflicts of interest to declare.

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