Comparative pharmacokinetics of baicalin and geniposide in juvenile and adult rats after oral administration of Qingkailing Granules

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Objective: To explore the effect of age on Qingkailing Granules disposition by comparing the pharmacokinetics of geniposide and baicalin in juvenile and adult rats.

Methods: A simple and rapid LC-MS/MS method was developed and validated to simultaneously determine geniposide and baicalin in rat plasma after a simple protein precipitation. The analytes were separated on an Agilent ZORBAX Extend-C18 column. The mobile phase consisted of acetonitrile and water with 0.1% (volume percent) formic acid at a flow rate of 0.6 mL/min. The ionization was conducted using an ESI source in negative ion mode. Multiple reaction monitoring was used for quantification at transitions of m/z 445.0 → m/z 268.9 for baicalin, m/z 433.2 → m/z 225.0 for geniposide, m/z 431.0 → m/z 341.0 for vitexin (IS). Juvenile and adult rats were administrated Qingkailing Granules (3 g/kg) orally. Plasma concentrations of baicalin and geniposide were determined by LC-MS/MS.

Results: The linear ranges of the analytes were 1–1000 ng/mL for baicalin and 2–2000 ng/mL for geniposide. The method was successfully applied to compare the pharmacokinetics of the analytes between juvenile and adult rats after oral administration of Qingkailing Granules. AUC was bigger in adult rats, while t1/2 was longer in juvenile rats.

Conclusion: These results suggested that the absorption and elimination of baicalin and geniposide in juvenile rats was lower than that in adult rats. Additional attention should be paid to the pharmacokinetic difference when Qingkailing Granules were used in children.

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1. Introduction

Qingkailing (QKL) Granules preparation is developed from Angong Niuhuang Pills, a traditional Chinese medicine (TCM) formula that has been used for thousand years in China. Now, QKL is widely used for the treatment of fever and colds, infection of the upper respiratory tract, pneumonia, and hepatitis, especially for the treatment of cold and flu in children (Niao, Zhou, Li, Liao, & Zheng, 2015). Recently, injection preparation of QKL was warned to be used in children due to severe cases of adverse drug reactions (ADR), including sudden death (Li, Deng, Yue, Zhang, & Sun, 2015). Oral dosage forms of QKL by contrast, have higher safety and are more suitable for children (Yang, Wang, Bai, Li, & Ai, 2009). However, the pharmacokinetics of oral formulation has not been fully understood, which makes it still risky when used in children.

According to Chinese Pharmacopoeia, QKL is prepared by eight herb medicines that exhibit excellent antiviral and anti-inflammatory activities. The major bioactive components of QKL consisted of baicalin, geniposide, chlorogenic acid, and cholic acid, which could synergistically contribute to its therapeutic effects (Ma et al., 2006). Among them, the content ranges of cholic acid, geniposide and baicalin are required to meet the standard of Chinese Pharmacopoeia (2015). Recently, the pharmacokinetics of QKL injection in rats has been reported (Peng et al., 2014). Furthermore, age, disease, and combined drugs directly affect the pharmacokinetics (Batchelor & Marriott, 2015; Huo et al., 2017; Zhao et al., 2017); However, little is known about their pharmacokinetics in paediatric populations. Considering the huge physiological difference between paediatric and adults, it is necessary to make a deep study on the pharmacokinetic profiles of the main chemical components in QKL Granules in paediatric populations. Baicalin and geniposide were then taken as the analytes for pharmacokinetic study except cholic acid for inevitable interference by endogenous
bile acids. In the present study, a rapid, sensitive and accurate LC-MS/MS analytical method was developed to simultaneously quantify baicalin, and geniposide in rat plasma after oral administration of QKLGranules. Using the method, the pharmacokinetic difference of QKL Granules in juvenile and adult rats after oral administration was studied. The results of present study would provide useful information for the safe and rational clinical application of QKL Granules in children.

2. Materials and methods

2.1. Materials

Baicalin (B20570), geniposide (B21661), and vitexin (B33310, IS) with purity more than 98% were purchased from the Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). QKL Granules were bought from Guangzhou Baiyunshan Pharmaceutical Holdings Co., Ltd. (Lot No. 450308, Guangzhou, China). Methanol and formic acid were obtained from Tedia (OH, USA). Deionized water was prepared in our laboratory (Millipore, MA, USA).

2.2. Equipment and LC–MS/MS condition

LC-MS/MS system was consisted of an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) and an API 3200 triple quadrupole instrument (Applied Biosystems, Foster City, CA, USA). Liquid chromatography condition was as follows. An Agilent ZORBAX Extend-C18 column (100 mm × 4.6 mm, 5 µm) was used for the separation of analytes at 30 °C with a flow rate of 0.6 mL/min. The mobile phase was in gradient manner which was consisted of acetonitrile (A) and 10 mmol/L ammonium acetate (B) and the gradient elution was set as: 0–1.0 min, 5% A; 1.0–5.0 min, 5% A → 52% A; 5.0–5.5 min, 52% A → 5% A; 5.0–8.0 min, 5% A.

The analytes and IS at 1 µg/mL in methanol were used to optimize mass spectrometric parameters. Mass spectrometric determination were conducted in the negative mode with an electrospray ionization source (ESI) and performed on multiple reaction monitoring (MRM) mode. Ion spray voltage was –4.5 kV. Turbo temperature was 500 °C. Curtain gas pressure was 20 psi. Gas source 1 and gas source 2 were set at 40 and 60 psi, respectively. EP and CXP were set at –10 eV and –13 eV for all analytes. The transitions used for determination and optimized DP and CE were listed in Table 1. Injection volume was 10 µL.

2.3. Preparation of standard and quality control samples

A mixed stock standard solution of baicalin and geniposide was prepared in methanol at final concentrations of 0.4 and 1.0 ng/mL, respectively. The working solution at series concentrations was obtained by further diluting the stock solution with methanol. The internal solution was prepared at a final concentration of 100 ng/mL. All solutions were kept at 4 °C until use.

To prepare the calibration samples, 100 µL standard working solutions were evaporated to dryness using nitrogen gas at 37 °C in a water bath. Then, blank rat plasma (100 µL) acidified with 10% formic acid (2 µL) (Xing, Chen, & Zhong, 2005) was added to redissolve the analytes. The concentration ranges of calibration samples for baicalin, and geniposide were 1.0–1000 ng/mL and 2.0–2000 ng/mL, respectively.

Quality control (QC) samples were prepared at three levels in the same way mentioned above as follows: 2.0, 40.0 and 800 ng/mL for baicalin, 4.0, 80.0 and 1600 ng/mL for geniposide.

2.4. Sample preparation

Acidified plasma samples (100 µL) were spiked with 20 µL of IS working solution and 200 µL of methanol for protein precipitation. After vortex and centrifugation at 20 000 rpm for 20 min, an aliquot of 250 µL supernatant was transferred to a new tube then evaporated to dryness at 37 °C using nitrogen. The residue was dissolved in 100 µL mobile phase and an aliquot of 10 µL was used for LC-MS/MS analysis.

2.5. Method validation

Validation of the proposed method was performed with respect to specificity, linearity, matrix effect, accuracy and precision according to FDA guidelines for bioanalytical method validation.

2.5.1. Selectivity, linearity and lower limit of quantification

Selectivity was tested by comparison of blank plasma from six individual rats with corresponding spiked plasma samples at lower limit of quantification (LLOQ). Calibration curve samples containing a zero sample processed without analyte but with IS and six non-zero samples were analyzed in six replicates. Peak area ratios of analytes to the IS were plotted versus the corresponding concentrations to obtain the calibration curve using a 1/x² weighted linear least-squares regression model. The linearity of each calibration curve was defined by a correlation coefficient (r) of at least 0.995. LLOQ was defined as the lowest concentration of each analyte determined with acceptable precision and accuracy (six replicates with relative standard deviation below 20% and relative error within ±20%). Moreover, LLOQ should producing a signal-to-noise (S/N) ratio larger than 10.

2.5.2. Accuracy and precision

LLOQ and QC samples at three concentration levels in six replicates were analyzed within day (intra-day) and over six consecutive days (inter-day) to assess the accuracy and precision. Accuracy were calculated as [(Cobs–Cspiked)/Cspiked] × 100%. Precision were evaluated by relative standard deviation (RSD, = [standard deviation (SD)/Cobs] × 100%). Accuracy and precision values varied within ±15%, except for the LLOQ, which varied by up to ±20%.

2.5.3. Matrix effect and recovery

Extraction recoveries were determined by comparing the peak area obtained from plasma samples at the three QC levels spiked before extraction with those from plasma samples spiked after extraction. The matrix effect was evaluated by comparing the peak area obtained from plasma samples at the three QC levels spiked after extraction with those from the analytes in neat solvent. The same procedure was performed for IS.

2.5.4. Stability

The stabilities of analytes were evaluated by analyzing QC samples in six replicates after storage at ambient temperature for 2 h, in auto-sampler for 24 h at 4 °C after protein precipitation, at −20 °C for 30 d and after three freeze-thaw cycles from −20 °C to room temperature. These results were compared with those obtained for freshly prepared QC sample. Stability (%) = (Cobs/ Cspiked) × 100.

2.6. Pharmacokinetic study

Male Sprague-Dawley rats weighing from 48 to 56 g (juvenile, 3 weeks, n = 72) and 180–220 g (adult, 6–8 weeks, n = 6) were used for PK study. The study protocol was approved by the Animal
the recoveries were stable and reproducible (Table 4). The of two analytes and IS ranged from 90.2% to 97.3%, indicating good precision and accuracy. The extraction recoveries of plasma of two analytes were all within ±15% at all three QC levels, and inter-day precisions ranged from 4.14% to 8.02% and 4.56% to 9.58% for two analytes, respectively. The corresponding accuracy ranged from 101.6% to 108.9% and 98.8% to 104.0% for two analytes (Fig. 2).

3.2. Method validation

According to the representative chromatograms of blank plasma samples, no interfering peak was observed in blank plasma under the established chromatographic condition (Fig. 2), indicating a good selectivity. Regression equations for calibration curves for three analytes were list in Table 2 and the r values were all higher than 0.9983, indicating excellent linearity over the concentration range of 1.0–1000 ng/mL for baicalin and 2.0–2000 ng/mL for geniposide in the plasma (Table 2). Moreover, the LLOQs for baicalin and geniposide were validated as 1.0 ng/mL and 2.0 ng/mL, respectively (Table 2). The intra- and inter-day precisions ranged from 4.14% to 8.02% and 4.56% to 9.58% for two analytes, respectively. The corresponding accuracy ranged from 101.6% to 108.9% and 98.8% to 104.0% for two analytes, respectively (Table 3). The data derived from QC samples of two analytes were all within ±15% at all three QC levels, indicating good precision and accuracy. The extraction recoveries of two analytes and IS ranged from 90.2% to 97.3%, indicating the recoveries were stable and reproducible (Table 4).

3.3. Pharmacokinetic study

Using the established method, the plasma concentration of baicalin and geniposide was determined after oral administration of QKL Granules in juvenile and adult rats. The difference in pharmacokinetics of QKL Granules between juvenile and adult rats was obviously found. In adult rats, baicalin and geniposide in plasma reached their peak concentrations at 0.75 h and 2 h, respectively (Fig. 3). When given to juvenile rats,\( T_{\text{max}} \) of three components was earlier (Fig. 3). Moreover,\( C_{\text{max}} \) of baicalin and geniposide in adult rats were significantly higher than these in juvenile rats (Fig. 3). A non-compartmental analysis was performed to obtain the pharmacokinetic parameters of baicalin and geniposide (Table 6). AUCs of baicalin and geniposide in adult rats increased by 23.0% and 42.8% compared these in juvenile rats, respectively (Table 6). In addition,\( T_{\text{1/2}} \) values of baicalin and geniposide in juvenile rats was 3.8-fold and 1.27-fold of these in adult rats, respectively (Table 6). These results suggested that the absorption and elimination of baicalin and geniposide in juvenile rats was lower than that in adult rats. Physiological changes during childhood have an impact on the absorption, distribution, metabolism, and elimination of a compound, which contributes to altered pharmacokinetic profile. It has reported that intestinal transit time was shorter in young children which may reduce the amount of drug absorbed, particularly for poorly soluble drugs or sustained release products (Grand, Watkins, & Torti, 1976; Pedersen & Steffensen, 1987). Meanwhile, the elimination of a substance mediated by either renal excretion or liver metabolism or bile secretion is generally lower in newborns owing to the immaturity of renal and liver function (Anderson & Holford, 2013; Barter et al., 2007; Perez de la Cruz Moreno et al., 2006). The higher levels of drugs in adult rats than that in juvenile rats were also observed in others' research (Boulamery, Marsot, Bruguerolle, & Simon, 2014). Therefore, the lower levels of AUC and\( C_{\text{max}} \) of baicalin and geniposide may attribute to the special physiological characteristics of juvenile rats. Currently, QKL is widely used for the treatment of cold and flu in children. As special populations, information regarding safe and rational use of drugs for children is limited. There is even no record about Pediatric Use in drug labels. In present study, we found that juvenile rats exhibited lower absorption and slower elimination of baicalin and geniposide after treated with QKL Granules at the same dosage compared with adult rats. The findings suggested that higher oral dose and longer delivery intervals might be necessary to reach effective therapeutic concentration and to avoid excessive accumulation when QKL Granules were used in children. Besides, the plasma concentrations of chlorogenic acid were also determined and lower than LLOQ (1 ng/mL) after 1 h and the data was too little to calculate the pharmacokinetic parameters (data not shown). Yang et al developed a LC-MS/MS method for the quantitative analysis of chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid and geniposide in rat plasma using LC-electrolyte switch in a contiguous time segments (Yang et al., 2019), which might improve the LLOQ of chlorogenic acid in this study. Further study was needed to give suggestion about dose adjustments.
**Fig. 1.** Product ion mass spectra of baicalin (A), geniposide (B) and IS (C) in negative mode.

**Fig. 2.** Representative chromatograms for baicalin (A), geniposide (B) and IS (C) in rat plasma samples. Left panel, MRM chromatograms of blank plasma sample; Middle panel, MRM chromatograms of blank plasma spiked with baicalin (2.0 ng/mL), geniposide (4.0 ng/mL) and IS (100 ng/mL); Right panel, MRM chromatogram of plasma sample at 30 min following oral administration of QKL Granules (3 g/kg body weight).

| No. | Compounds   | Monitoring ion | Precursor ion (m/z) | Product ion (m/z) | DP  | CE  |
|-----|-------------|----------------|---------------------|-------------------|-----|-----|
| 1   | Baicalin    | [M–H]^-        | 445.0               | 268.9             | -80 | -29 |
| 2   | Geniposide  | [M + HCOO]-    | 433.2               | 225.0             | -90 | -25 |
| 3   | Vitexin (IS)| [M–H]^-        | 431.0               | 341.0             | -110| -30 |
Table 2
Regression equations, linear ranges and LLOQs of analytes.

| No. | Compounds | Linear range/(ng mL\(^{-1}\)) | Regression equations | r      | LLOQ/(ng mL\(^{-1}\)) | Retention time/min |
|-----|-----------|-------------------------------|----------------------|--------|-----------------------|-------------------|
| 1   | Baicalin  | 1.0–1000                      | \(Y = 0.341X-0.162\) | 0.9990 | 1.0                   | 4.74              |
| 2   | Geniposide | 2.0–2000                      | \(Y = 0.575X-0.467\) | 0.9983 | 2.0                   | 4.25              |

Table 3
Precision and accuracy of analytes.

| No. | Compounds | Concentrations/(ng mL\(^{-1}\)) | Intra-day (n = 6) | Inter-day (n = 3) |
|-----|-----------|--------------------------------|-------------------|-------------------|
|     |           |                                | Precision/RSD %   | Accuracy/%        | Precision/RSD % | Accuracy/% |
|     |           |                                |                    |                   |                  |            |
| 1   | Baicalin  | 2                              | 8.02               | 108.9             | 9.58             | 100.5      |
|     |           | 80                             | 4.14               | 107.1             | 4.56             | 102.8      |
| 2   | Geniposide | 4                             | 6.93               | 106.1             | 7.57             | 104.0      |
|     |           | 160                            | 5.49               | 106.3             | 5.47             | 102.4      |
|     |           | 1600                           | 4.84               | 102.8             | 5.20             | 98.5       |

Table 4
Matrix effect and recovery of analytes.

| No. | Compounds | Spiked/(ng mL\(^{-1}\)) | Matrix effect/% | Recovery/% |
|-----|-----------|--------------------------|-----------------|------------|
|     |           |                          |                  |            |
| 1   | Baicalin  | 2                        | 97.6             | 90.2       |
|     |           | 80                       | 99.8             | 97.3       |
| 2   | Geniposide | 4                        | 92.5             | 92.5       |
|     |           | 160                      | 96.0             | 95.5       |
|     |           | 1600                     | 94.9             | 92.5       |
| 3   | IS        | 100                      | 90.8             | 95.2       |

Table 5
Stability of analytes.

| No. | Compounds | Added Con./(ng mL\(^{-1}\)) | Benth-top stability \(^{a}\) | Autosampler stability \(^{a}\) | Freeze/thaw cycles \(^{c}\) | Long-term stability \(^{d}\) |
|-----|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|     |           |                             | RSD/% | RE/% | RSD/% | RE/% | RSD/% | RE/% | RSD/% | RE/% |
| 1   | Baicalin  | 2                            | 9.1   | 8.0  | 12.4  | 9.2  | 4.2   | –9.5 | 6.0   | 7.1  |
|     |           | 80                           | 7.2   | 1.6  | 7.1   | –3.0 | 3.9   | –3.9 | 2.7   | –10.7|
| 2   | Geniposide | 4                           | 0.9   | –5.0 | 4.1   | –5.6 | 1.1   | 5.0  | 1.4   | –2.8 |
|     |           | 160                          | 1.6   | 5.7  | 5.4   | 9.2  | 5.7   | 5.8  | 2.5   | –7.8 |
|     |           | 1600                         | 1.9   | –1.0 | 8.0   | 1.5  | 3.9   | 2.3  | 2.0   | –3.3 |

\(^{a}\) Exposed at ambient temperature (25 °C) for 2 h.
\(^{b}\) Kept at autosampler temperature (4 °C) for 24 h.
\(^{c}\) After three freeze/thaw cycles.
\(^{d}\) Stored at –20 °C for 30 d.

Fig. 3. Plasma concentration-time profiles of baicalin (A) and geniposide (B) in juvenile and adult rats after orally administration of QKL Granules (3 g/kg) (mean ± SD, n = 6). *, \(P < 0.05\) vs adult rats.
Table 6
Pharmacokinetic parameters of baicalin and geniposide.

| Parameters | Units             | Baicalin | Geniposide |
|------------|-------------------|----------|------------|
|            |                   | Juvenile | Adult      | Juvenile | Adult      |
| AUC(0-t)   | μg mL⁻¹ min⁻¹     | 156 ± 46 | 198 ± 58   | 2.22 ± 0.63  | 3.16 ± 0.13 |
| MRT(0-t)   | min               | 537 ± 101| 420 ± 129  | 508 ± 202  | 425 ± 171  |
| t₁/2a      | min               | 588 ± 118*| 154 ± 59   | 826 ± 381  | 652 ± 210  |
| tmax       | min               | 57.7 ± 18.1| 69.2 ± 14.6| 102 ± 42  | 120 ± 40   |
| Cmax       | ng mL⁻¹           | 448 ± 110| 599 ± 242  | 23.6 ± 7.2 | 30.2 ± 0.5 |

* P < 0.05 vs adult rats.

4. Conclusions

A rapid and sensitive LC-MS/MS method was developed and validated for the simultaneous determination of baicalin and geniposide in rat plasma. The main active constituents of QKL Granules were found to exhibit different pharmacokinetic characterization between juvenile rats and adult rats for the first time. Juvenile rats exhibited lower absorption and slower elimination of baicalin and geniposide.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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