EFFECT OF A CHINESE HERBAL FORMULATION ON THE PHARMACOKINETICS OF PARACETAMOL IN RATS.


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ABSTRACT
The effect of a Traditional Chinese herbal formulation, K-601, on the pharmacokinetics of paracetamol (acetaminophen) was investigated. A simple high performance liquid chromatography-single quadrupole mass spectrometric (LC/MS) analytical method was developed, validated according to ICH guidelines and applied for the pharmacokinetics determination of paracetamol in the plasma of rats. The results of the pharmacokinetics revealed significant differences in all the parameters compared. It was observed that co-administering paracetamol with K-601, the half-life ($t_{1/2}$), the time of maximum concentration ($T_{\text{max}}$), the area under concentration-time curve (AUC), the mean residence time (MRT) and the volume of distribution ($V_d$) were lower than those from the paracetamol-alone treated group. The maximum plasma drug concentration ($C_{\text{max}}$) and clearance (CL) however were higher than those from the paracetamol-alone treated group. In conclusion, K-601 was found to significantly affect the metabolism of paracetamol in the liver.

KEYWORDS: Paracetamol (acetaminophen), pharmacokinetics, high performance liquid chromatography-single quadrupole mass spectrometry.

INTRODUCTION
Paracetamol (acetaminophen) is a very common drug usually prescribed for its analgesic and antipyretic effects. It is a readily available over-the-counter medication. The use of herbal medicines has been on the increase particularly in the developing countries of Africa and
Asia. In China for instance, herbal medicines have been integrated into the healthcare system, and their manufacture and use is regulated. Due to this integration, there is the common occurrence of co-administration of western medications (Orthodox Medicines) with Traditional Chinese Medicines (TCM) some of which could have serious interactions and possibly dire consequences. There is therefore the need to continuously monitor the potential orthodox Medicines - TCM interactions especially those in common use.

In this study we investigated the effect of a TCM liquid formulation, K-601 on the pharmacokinetics of paracetamol (acetaminophen). We also investigated if the influence of the K-601 on the pharmacokinetics of paracetamol was significant. K-601 is a multicomponent herbal formulation comprising five herbs namely; *Lonicera japonica* Thunb., *Isatis indigotica* Fort., *Rheum palmatum* L., *Phellodendron chinense* Schneid., and *Scutellaria baicalensis* Georgi. It is indicated for use in the treatment of common cold and commonly used in hospitals in Nanjing, China.

An analytical method was developed, validated and used for the pharmacokinetic determination of paracetamol in the plasma of rats.

**MATERIALS AND METHODS**

**Fig.1 Chemical structures of Paracetamol and Caffeic acid (internal standard)**

**Chemicals and Reagents**

HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany), distilled water was purified by Milli-Q system (Millipore, USA), HPLC grade methanol was purchased from Jiangsu Hanbon Sci. & Tech. Co. Ltd, Jiangsu, China. Analytical grade formic acid was purchased from Nanjing Chemical Factory (Nanjing, China). Paracetamol reference standard was purchased from Aladdin (purity of 99.0%), K-601 was gotten from Nanjing Children...
Hospital. Caffeic acid was isolated from its plant source in our lab. Its structure was identified by spectroscopic methods of U.V, IR, MS, $^1$HNMR, and $^{13}$CNMR and its purity determined to be over 98% by HPLC.

**Equipment and LC/MS condition**

Chromatographic analysis was performed on an Agilent 1290 Series (Agilent Corp., Santa Clara, CA, USA,) HPLC system equipped with a binary pump, micro degasser, an auto sampler and a thermostatically controlled column compartment coupled with single quadrupole mass spectrometer (Agilent technologies 6110 quadrupole LC/MS). Chromatographic separation was carried out at 25°C on a Sapphire- C18 column(4.6 x 250mm, 5µm). The mobile phase consisted of 0.1% formic acid-water solution (A) and ACN (B) using a gradient elution of 0-5% B at 0–2 min, 5-15% B at 2-7 min, 15 - 35% B at 7 - 10 min, 35-65% B at 10 - 12 min and 65 - 100% B at 12 - 15 min. The operating parameters were as follows: method of spray chamber,API-ES; active signals, channel 1; scan data storage, full; mode, SIM; polarity, positive; drying gas flow (L/min), 10.0; nebulizer pressure (psig),30; drying gas temperature, 350°C; capillary voltage (V),3500; collision energy,30; fragmentor voltage,70.

**Preparation of standard solutions, calibration samples and quality control samples**

The stock solution of paracetamol was prepared in methanol to a concentration of 100µg/mL. The internal standard, caffeic acid (CA) was prepared in the stock solution to a final concentration of 2µg/mL with methanol. Six (6) calibration standard solutions at 0.04, 0.2, 1, 2, 4, 10 µg/mL were prepared by spiking blank plasma with appropriate amounts of standards. Quality control (QC) plasma samples at 0.03, 1 and 5µg/mL were prepared in the same way as the calibration standards. Blank plasma samples without analyte and internal standard were also prepared.

**Biosample Preparation**

100µL of plasma, 100µL of internal standard (CA) and 1000µL of ethyl acetate/ methanol (v: v, 9:1) were combined and vortexed for 1min and finally centrifuged at 13000rpm for 10min at 4°C. The supernatant was transferred into a sample tube and evaporated to complete dryness with nitrogen gas. The residue was dissolved in 100µL of mobile phase with vortex-mixing for 1min and centrifuged again at 13000rpm for 10min. 10µL of the supernatant was injected into the LC-MS system for analysis.
Method Validation

Linearity, LOD and LOQ
Linearity was evaluated by using plasma calibration graph. The linear plasma calibration curves were generated by plotting the concentration against the corresponding peak area ratios of the analyte (paracetamol) and the internal standard (CA). The limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions were determined at a signal-to-noise ratios (S/N) of about 3 and 10 respectively.

Accuracy and Precision
Intra-day precision and accuracy were determined by analyzing five replicate injections of each concentration of QC samples in the same day. Inter-day accuracy and precision were determined by analyzing five replicate injections of QC samples on six consecutive days. Accuracy was calculated as the relative error (RE) of the observed concentration from the spiked concentration.

%RE = [(mean observed-theoretical value)/theoretical value] x 100

Precision was expressed as the coefficient of variation (CV):

%CV= (standard deviation/mean) x 100.

Recovery and Matrix Effect
Recoveries of the analyte (paracetamol) at the three QC concentrations were determined by comparing the responses of the analyte from QC samples with the responses of analyte spiked with post-extracted matrix at equivalent concentrations.

Blank plasma samples from five untreated rats (blood taken from rats at time 0min) were spiked paracetamol and CA prior to the extraction process (set 1). Another set of blank plasma samples from five untreated rats were spiked with paracetamol and CA post-sample extraction (set 2). In a third set, paracetamol and CA were prepared in the chromatographic mobile phase solution. Matrix effects in the ionization of the analyte was examined by comparing the set 2 with set 3. The efficiency of extraction (EE) and matrix effects (ME) are expressed as follows.

%EE = (peak area ratio of paracetamol + CA in set 1/peak area ratio of paracetamol + CA in set 2) x 100.
%ME = 100 - [(peak area ratio of paracetamol + CA in set 2 / peak area ratio of paracetamol + CA in set 3)] x 100.

**Freeze-thaw stability**
This was done by analyzing the three concentrations of the QC samples stored at -80°C. Two freeze-thaw cycles were determined at 4 and 24 hours.

**Animal Experiment**
Male Wistar albino rats from Yangzhou University Cooperative Medicine Center weighing 180-220g were used for the study. They were housed in acryl fibre cages at 24 ± 2°C, humidity 50± 1.0% and were kept on a 12h light/dark cycle. They were fed with standard pellet diet from Jiangsu Provinical Synergistic Engineering Co. Ltd and water ad libitum and made to acclimatize with the new environment for 7 days before experimentation. The rats were fasted 12h before the test. The animal care and use complied with the Provisions and General Recommendations of the Chinese Experimental Animals Administration Legislation. The experiments were performed with the approval from the Animal Ethics Committee of China Pharmaceutical University.

After acclimatization, the rats were randomly divided into 2 groups: Group 1 (paracetamol treated group, n = 5), Group 2 (paracetamol and K-601 treated group, n = 5). Paracetamol, 50 mg/kg body weight was given to the animals in both groups. K-601, 4mL was given to each animal in group 2.

Under light ether anaesthesia, blood samples (0.5mL) were collected from the retro-orbital plexus of the rats into heparinized tubes at time points of 0, 30, 45, 60, 90, 150, 240min after dosing. Plasma was obtained by centrifuging the blood samples at 4500 rpm for 10min at 4°C, as the supernatant. These samples were stored at -80°C until further analysis.

The following pharmacokinetic parameters were determined: maximum plasma concentration ($C_{\text{max}}$), time to maximum plasma concentration ($T_{\text{max}}$), mean residence time (MRT), the volume of distribution (Vd), half-life ($t_{1/2}$), area under the concentration-time curve (AUC) and plasma clearance (CL). These data were obtained via a non-compartmental method with PKSolver software (an add-in program for pharmacokinetics and pharmacodynamics analyses in Microsoft Excel).
Statistical Analysis
The results were expressed as mean ± S.E.M (standard error of mean). Statistical differences between control and treatment group were analyzed using a two-tailed t-test with GraphPad Prism 5.0 software. p-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Fig. 2 Chromatogram showing analyte (paracetamol) and internal standard (cafeic acid)

Method validation
Specificity
The method was found to be selective for the analyte and internal standard. This was done by comparing the chromatograms of six different blank plasma with the corresponding plasma. By using the selective ion mode in the LC/MS set-up, the detections of analyte and internal standard were specific, and based on their m/z values. Hence by using the optimized LC/MS conditions, the retention times of paracetamol and cafeic acid were respectively 9.12min and 13.22min. No significant interference from the endogenous component was observed, further revealing the specificity of the method.

Linearity, LOD and LOQ
The equation of the line for the paracetamol in the plasma of the rats was, y = 0.4125x + 0.7048, r² = 0.9968. This was linear in the range of 0.04-10µg/mL. A series of concentrations were prepared from the plasma spiked sample of concentration 0.04µg/mL and analyzed to obtain data corresponding to concentrations that gave the noise-to-signal ratios of 3 and 10
respectively as LOD and LOQ. The values for LOD and LOQ were respectively 0.00015µg/mL and 0.00036µg/mL.

Fig.3 A calibration curve for paracetamol using LC/MS

Accuracy and Precision
The data for the intra-day and inter-day precision are presented below as well as that for accuracy. Precision is presented as percentage coefficient of variation (%CV) and accuracy as percentage bias (%RE). The values from these tests were within acceptable limits.

Table 1 Intra-day Precision and Accuracy for Paracetamol in Rat Plasma

<table>
<thead>
<tr>
<th>Theoretical value(µg/mL)</th>
<th>Observed value(µg/mL)</th>
<th>%CV</th>
<th>%RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.033 ± 0.0012</td>
<td>3.64</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>1.09 ± 0.08</td>
<td>7.34</td>
<td>9</td>
</tr>
<tr>
<td>15</td>
<td>15.61 ± 2.1</td>
<td>13.45</td>
<td>4.07</td>
</tr>
</tbody>
</table>

Table 2 Inter-day Precision and Accuracy of Paracetamol in Rat Plasma

<table>
<thead>
<tr>
<th>Theoretical value(µg/mL)</th>
<th>Observed value(µg/mL)</th>
<th>%CV</th>
<th>%RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>0.028 ± 0.0019</td>
<td>6.79</td>
<td>-6.67</td>
</tr>
<tr>
<td>1</td>
<td>0.8 ± 0.12</td>
<td>15</td>
<td>-20</td>
</tr>
<tr>
<td>15</td>
<td>17.01 ± 1.62</td>
<td>9.52</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Recovery and Matrix Effect
Table 3 below shows the extraction efficiencies as well as the matrix effects for paracetamol. At all three concentrations of the analyte, the extraction recoveries were all within acceptable range. The results for the matrix effect revealed that for the various concentrations of the QC samples, its effect was moderate (averagely 19.36).
Table 3 Extraction Efficiency and Matrix Effect for Paracetamol in Rat Plasma

<table>
<thead>
<tr>
<th>Amount added (µg/mL)</th>
<th>% Extraction Efficiency (n = 5)</th>
<th>% Matrix Effect (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>72.25 ± 15.14</td>
<td>20.33 ± 4.22</td>
</tr>
<tr>
<td>1</td>
<td>88.64 ± 10.91</td>
<td>21.09 ± 2.10</td>
</tr>
<tr>
<td>5</td>
<td>96.47 ± 6.98</td>
<td>16.67 ± 1.21</td>
</tr>
</tbody>
</table>

**Freeze-thaw stability**

Using the QC samples the stability of paracetamol was tested over two freeze-thaw cycles. After 4h the relative errors of paracetamol during freeze-thaw cycles were lower than 7% and also lower than 10.4% at 24h as presented in table 4.

Table 4 Freeze-thaw Stability of Paracetamol in Rat Plasma

<table>
<thead>
<tr>
<th>Theoretical value (µg/mL)</th>
<th>% Relative Error(4h)</th>
<th>% Relative Error (24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>-4.55</td>
<td>-10.31</td>
</tr>
<tr>
<td>1</td>
<td>-6.98</td>
<td>-8.86</td>
</tr>
<tr>
<td>5</td>
<td>-4.2</td>
<td>-9.7</td>
</tr>
</tbody>
</table>

**Pharmacokinetics Study**

*Fig. 4 Pharmacokinetic profile of paracetamol alone and in the presence of K-601*

*p values < 0.05 indicating statistical significance upon comparison. Each value represents the mean ± S.E of 5 rats. t_{1/2}: half-life, T_{max}: time reached for maximum plasma concentration to be attained, C_{max}: maximum plasma concentration, AUC: the area under the concentration-time curve, MRT: the mean residence time, Vd: volume of distribution, CL : clearance*
Table 5 Results of pharmacokinetic parameters from

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Free Paracetamol</th>
<th>Paracetamol + K-601</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$</td>
<td>min</td>
<td>1412.54 ± 280.65*</td>
<td>188.56 ± 36.38*</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>min</td>
<td>90 ± 17.32*</td>
<td>45 ± 3.67*</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>μg/mL</td>
<td>1.26 ± 0.12*</td>
<td>3.93 ± 0.38*</td>
</tr>
<tr>
<td>AUC</td>
<td>μg/mL*min</td>
<td>2660.06 ± 0.31.57*</td>
<td>1146.66 ± 0.08*</td>
</tr>
<tr>
<td>MRT</td>
<td>min</td>
<td>2061.62 ± 407.07*</td>
<td>297.66 ± 58.45*</td>
</tr>
<tr>
<td>Vd</td>
<td>mL/kg</td>
<td>38304.98 ± 3621.97*</td>
<td>11862.18 ± 894.65*</td>
</tr>
<tr>
<td>CL</td>
<td>mL/min</td>
<td>18.79 ± 29.14*</td>
<td>43.6 ± 9.36*</td>
</tr>
</tbody>
</table>

DISCUSSION

The method developed for the determination of paracetamol in the plasma of rat and applied for its pharmacokinetic determination was validated according to the ICH guidelines. The method proved to be accurate, specific and precise for the determination of paracetamol in plasma. The method was also validated for LOD, LOQ, freeze-thaw stability and matrix effects and good results obtained as elaborated above.

The aim of this study was to investigate the effect of K-601, a multicomponent herbal oral liquid formulation, on the pharmacokinetics of paracetamol. This is because patients using K-601 for the treatment of common cold are usually prescribed paracetamol, a common antipyretic agent, to be co-administered. The potential influence of herbal formulation on western drugs is one area which receives very little attention. Part of the reasons for this neglect lies in the fact that, the herbal formulations in question are usually multicomponent in nature and very much difficult to characterize. Seasonal variations in the raw materials of these herbal formulations coupled with the difficulty of producing consistent batches of the same formulation also account for the difficulties encountered in this area of research interest.

It is well known that cytochrome P450 enzymes (CYP) are responsible for the bioactivation of paracetamol in the liver\[3,8\]. Paracetamol is mainly excreted in its glucuronide or sulphate form in urine. CYP-dependent oxidation of paracetamol results in the formation of toxic N-acetyl-p-benzoquinoneimine, causing hepatocellular damage in humans and experimental animals. It has also been demonstrated from previous reports that CYP3A4, CYP1A2, CYP2D6 and CYP2E1 are involved in the metabolism of paracetamol\[1,2,4,5,6\].

From the results of the study as presented in Table 5, the all pharmacokinetic parameters between the two groups of rats were significant ($p < 0.05$) upon conducting a two-tailed t-test. It can be observed from the results that upon co-administering paracetamol with K-601, the $t_{1/2}$, $T_{\text{max}}$, AUC, MRT and Vd were lower than those from the paracetamol-alone treated
group. The $C_{\text{max}}$ and CL, however were higher than those from the paracetamol-alone treated group. It can be said that K-601 significantly affects the pharmacokinetics of paracetamol. The metabolism of paracetamol increased leading to higher CL rate, lower AUC values, lower MRT, lower Vd and lower $t_{1/2}$. The time required to reach maximum concentration, $T_{\text{max}}$, was also reduced by 50% (45min) in comparison with the paracetamol-alone treated group. The result of this study provides a foundation for further research. Our next aim would be to determine which of the cytochrome P450 enzymes is/are affected and to which extent. There is also ongoing research specifically on K-601 in our laboratory.

CONCLUSION
The method developed proved to be accurate, specific and precise for the determination of paracetamol in rat plasma. When validated for LOD, LOQ, freeze-thaw stability and matrix effects and good results were obtained. The TCM, K-601 had a significant effect on the pharmacokinetics of paracetamol.

CONFLICT OF INTEREST
The authors hereby declare that there are no competing interests regarding this study.

REFERENCES
