Investigation on pharmacokinetics, tissue distribution and excretion of 1-triacontanol in rats by gas chromatography-tandem mass spectrometry (GC-MS/MS)

Chunfeng Wang¹, Ali Fan¹, Shuhua Deng¹, Wenchao Gao¹, Wei Zhang¹, Wei Yang¹, Xiaojie Zhu¹, Yang Lu², and Xijing Chen¹

¹Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, People’s Republic of China and ²Division of Pharmacotherapy and Experimental Therapeutics, Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract
1. 1-Triacontanol (TA) recently shows promising anti-tumor activity. The present study was aimed to develop a sensitive gas chromatography-tandem mass spectrometry method to explore the pharmacokinetic profiles, distribution and excretion of TA in Sprague-Dawley rats after oral administration of TA. Chromatography separation was performed on a HP-5MS column. 1-Octacosanal was used as the internal standard (IS). Quantification of TA and IS was carried out at m/z 495.6 → 97.0 and m/z 467.5 → 97.0, respectively, in positive electron ionization and multiple reaction monitoring mode. The pharmacokinetic parameters were calculated by non-compartmental analysis.

2. The area under concentration-time curve AUC₀–₆ h and AUC₀–∞ for TA at 60 mg/kg were 87.737±13.574 and 93.617±17.62, respectively. The mean residence time was 3.25 ± 0.17 h. In addition, the elimination half-lives (t½) were (2.37±1.23, 1.27±0.49, 2.07±0.93) h after single oral administration of 30, 60 and 120 mg/kg of TA. After oral administration, TA was extensively distributed in stomach and intestine. The majority of TA excreted via feces, and its accumulative excretion ratio during the period of 72 h was 26.68 ± 7.14%, but only 0.0023 ± 0.0015% and 0.0027 ± 0.0006% for urines and bile, respectively. The absolute bioavailability (F, %) of TA was about 2.0%.

Keywords
1-triacontanol, GC-MS/MS, pharmacokinetics

Introduction
1-Triacontanol (TA, Figure 1) is a long chain fatty alcohol with 30 carbon atoms. It is a component of policosanol purified from rice bran, wheat germ and beeswax (Dullens et al., 2008; Más, 2000). According to previous reports, policosanol could lower total cholesterol level in many species, including rats, rabbits, dogs and monkeys (Arruzazabala et al., 1994; Hernández et al., 1992; Menendez et al., 1996; Mesa et al., 1994; Noa et al., 1995; Pons et al., 1994; Rodriguez-Echenique et al., 1994). Similar cholesterol level lowering effect was also found in healthy volunteers and patients with type II hypercholesterolemia after policosanol administration (Castaño et al., 2000a,b, 2001, 2003; MÁS et al., 1998, 2001). Recently, TA exhibits promoting anti-tumor activity and was used in the treatment of cancer. Its indications include liver, colon and lung cancer (Zhang et al., 2008). Hence, TA is regarded as a promising anti-cancer compound with less or no toxicity. Due to low bioavailability and undesirable half-life, many promising compounds failed to become new drugs. It is of great significance to establish a reliable method to quantify TA and study the pharmacokinetic profiles of TA.

Till now, the methods for the quantitative determination of TA are rarely reported. Although Haim et al. developed a GC/MS method for the determination of TA in rat plasma (Haim et al., 2009), the application of that method is limited due to non-specificity or inadequate sensitivity for pharmacokinetic studies. For the pharmacokinetic study of TA, an ideal sensitivity of 2.0 ng/mL is required according to our preliminary study. However, in the report of Haim et al., the sensitivity is only 8.4 ng/mL. In this report, we established a reliable gas chromatography-tandem mass spectrometry (GC-MS/MS) method with high sensitivity for the quantitative determination of TA concentration in rat plasma. More importantly, we fully assessed the pharmacokinetic profile of 1-TA, including pharmacokinetics, tissue distribution and excretion in rats using GC-MS/MS.
Flow. The oven temperature program was used: 100 °C in 2.5 min, then increased to 200 °C in 2 min. The trap was set at 200 °C. The collision energy was 12 EV for TA and 10 EV for IS. The detection of the ions was performed in the multiple reaction monitoring (MRM) mode monitoring the ion transitions at m/z 495.6 → 97.0 for TA and m/z 467.5 → 97.0 for IS (Figure 2), respectively. The interface was heated at 300 °C. The mass spectrometer operating conditions were ion source temperature at 250 °C, electron energy of 70 EV. Solvent delay was set at 3 min.

Standard and sample preparation

Preparation of stock and working solutions, calibration samples and quality controls

Stock solutions of TA and IS were prepared by dissolving accurate amounts of reference standards in heptane at the concentration of 0.1 mg/mL and stored at 4 °C. TA (0.004, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4 and 1 µg/mL) working solutions were prepared by serial dilution of the stock solution with heptane. The working solutions for quality control (QC) samples were prepared in the same way. The working solution of IS (100 ng/mL) was used for sample pretreatment. All working solutions were stored at −20 °C until analysis.

Preparation of calibration standards and QC samples

Calibration standards for TA were prepared by spiking the appropriate amounts of the standard solutions into 100 µL of blank plasma to yield final concentrations of 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL. QC samples for the low, midle and high levels were prepared similarly at concentrations of 5, 50 and 400 ng/mL, respectively. All samples were stored at −20 °C until analysis.

Sample preparation

Biological samples were stored at −20 °C, and taken out and thawed at room temperature before use. For pharmacokinetics study, an aliquot of 100 µL plasma was spiked with 100 µL of IS (100 ng/mL) and was added 1 mL of ethanolic NaOH solution (1 mol/L NaOH dissolved in ethanol distilled water (80/20, v/v)). Then the sample was mixed by vortex for 1 min, saponified at 80 °C for 1 h and acidified with 300 µL HCl (5 mol/L). Each sample bathed at 70 °C for 10 min before extraction by adding 2 mL of heptane and mixed by vortex 2 min. The supernatant was transferred to a new tube and washed with 2 mL ultrapure water. The steps including extraction and wash were conducted for three times for each sample. After that, all the supernatant was transferred to a new tube to evaporate to dryness by a centrifugal thickener (Centrivap console, Labconco Co., Kansas City, MO). After dryness, the sample was derivatized with 300 µL of BSTFA at 80 °C for 20 min. After drying the sample again, the residue was reconstituted in 50 µL heptane and 2 µL of the solution was injected into the GC-MS/MS for analysis.

To study tissue distribution of TA, each weighed tissue sample was homogenized in ice-cold physiological saline solution (1:4, w/v). Then a 50 µL of tissue homogenate was taken out and processed further like the plasma samples. Tissue homogenates which concentrations were greater than the upper limit of the calibration curve were reanalyzed by appropriate dilution with blank tissue homogenate.

Experimental

Reagents and chemicals

1-TA (purity ≥99%) and 1-octacosanol (internal standard (IS), purity ≥99%) reference substance (used for standard curve preparation) were provided by Sigma-Aldrich Chemical Co. (St. Louis, MO). 1-TA (purity ≥96%) (used for animal study) was provided by Kunming Longjin Inc. (Kunming, Yunnan, China). N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA, purity ≥99%) (used for silylation) was purchased from Tedia Co. (Fairfield, OH). All other reagents were of analytical grade.

Animals

Male and female Sprague-Dawley rats weighing 180–200 g were provided by Shanghai SIPPR/BK Experimental Animal Co. (Shanghai, China). Animals were housed under standard conditions of temperature, humidity and light. Food and water were supplied for three successive days prior to study. The rats were fasted 12 h before the administration of TA but with unrestricted access to water. The pharmacokinetic study was carried out according to the guidance of the Animals Experimental Ethics Committee in China Pharmaceutical University (Nanjing, China).

Instrument and analytical conditions

Separation of TA and IS was carried out using an Agilent 6890A gas chromatograph on a HP-5MS column (30 m × 250 µm I.D., 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA). Helium was used as the carrier gas at 1 mL/min flow. The oven temperature program was used: 100 °C, then to 200 °C in 2.5 min, kept for 7.5 min, increased to 300 °C in 5.0 min and lasted for 5.0 min. Argon (Ar) was used as a collision gas at a flow rate of 1 mL/min. Splitless injection was performed at 300 °C. The total run time for sample analysis was 16 min.

MS/MS spectrometry (Micromass Quattro Micro, Waters, Milford, MA) was equipped with an electron ionization (EI) source operating in the positive ionization mode. The Masslynx software version 4.1 package was used for data acquisition and analysis (Applied Waters, Milford, MA). Mass parameters, including repeller, extraction lens, focus lens 1 and focus lens 3 were optimized at 10, 23, 250 and 27 V. The trap was set at 200 µA. The collision energy was 12 EV for TA and 10 EV for IS. The detection of the ions was performed in the multiple reaction monitoring (MRM) mode monitoring the ion transitions at m/z 495.6 → 97.0 for TA and m/z 467.5 → 97.0 for IS (Figure 2), respectively. The interface was heated at 300 °C. The mass spectrometer operating conditions were ion source temperature at 250 °C, electron energy of 70 EV. Solvent delay was set at 3 min.
For excretion study, the feces samples were pulverized with a mortar and pestle, weighed 10 mg and homogenized in 2 ml of ethanolic NaOH solution. An aliquot of 50 μL bile or urine was spiked with 100 μL of IS (100 ng/mL). Then the procedure left were in a similar manner as the plasma samples.

**Method validation**

This method was fully validated according to US FDA guidelines for bioanalytical method validation. Selectivity was evaluated by analyzing blank plasma samples collected from six different rats with a plasma sample spiked with TA and IS to investigate the potential interferences at the peak region. Calibration curve \( (Y = AX + B) \) was constructed using weighted \((1/X^2)\) least squares linear regression. LLOQ was defined as the minimum concentration of plasma samples that could be quantified by this method (relative standard deviation, RSD < 20%), and signal-to-noise ratio (S/N) was at least 10 (Figure 3). The matrix effects were evaluated by comparing the peak areas of the analytes in post-extracted blank plasma samples spiked with TA at three QC levels, the pure standard solutions with same concentration were dried directly and reconstituted with the heptane. The precision and accuracy were assessed by analyzing QC samples (5, 50 and 400 ng/mL) on three consecutive days. The intra- and inter-day precision and accuracy were evaluated by the RSD, respectively. The relative error and RSD were all within ±15%. Recovery was determined by comparing the peak areas of processed QC samples with those of the pure standards without extraction. The stability of the stock solution prepared in heptane was studied after storing at 4 °C for nearly one month. The stability of TA in plasma was evaluated under various conditions using three levels of QC samples after short-term (pre-processed samples stored at room temperature for 4 h) and long-term storage conditions (stored at −20 °C for one month). The stability of QC samples after three freeze (−20 °C)-thaw (room temperature) cycles and post-preparative stability (post-processed samples stored at 4 °C for 24 h) was also analyzed.

**Pharmacokinetic study**

Rats were fasted for 12 h before dosing with free access to water and were randomized into four groups, each group consisting of six rats. The dosages of the three groups were 30, 60 and 120 mg/kg for oral administration, respectively, the other was 3 mg/kg for intravenous injection. Blood samples
were collected in heparinized tubes prior to and at 0.5, 0.75, 1, 1.5, 2, 3, 4 and 6 h after the oral administration and 2, 5, 10, 15, 30, 60, 120, 240 and 360 min for intravenous injection. Plasma (100 μL) was centrifuged at 12,000 rpm for 5 min after harvesting and the supernatant was stored at −20°C until analyzed by GC-MS/MS.

Tissue distribution study

Four groups of SD rats (six rats per group) were i.g. administrated at a single dose of 60 mg/kg TA. After oral administration, the tested animals were sacrificed by bleeding the femoral artery at 0.5, 1.5 and 3 h (six animals per time point, three male and three female), and the normal tissues,
including heart, liver, spleen, lung, kidney, brain, stomach, intestine, muscle, body fat, testis/ovary and uterus were immediately taken, weighed and collected with animal carcasses remained. The blood samples were collected at the same time. All the tissues were washed in normal saline (4°C), blotted on filter paper, accurately weighed, and stored at −20°C until analysis.

Excretion study

For biliary excretion study, six rats (three male and three female) were anesthetized and a cannula was implanted into the bile duct to collect bile. TA at a single dose of 60 mg/kg was i.g. administrated. Bile samples were collected at 0–0.5, 0.5–0.75, 0.75–1, 1–2, 2–4, 4–6, 6–8 h and 8–12 h post-dosing and stored at −20°C after the volume of each collection was recorded.

For urinary and fecal excretion study, six rats (three male and three female) were i.g. administrated at a single dose of 60 mg/kg TA. Then the rats were individually placed in stainless-steel metabolic cages which allowed the separate collection of urine and feces. The urine and feces were collected at 0–2, 2–4, 4–8, 8–12, 12–24, 24–36 and 36–72 h. The specimens were stored at −20°C till quantitative determination of TA by GC-MS/MS after the volume of urine and the dry weight of feces for each collection period were measured.

Calculation

All the pharmacokinetic parameters were calculated by non-compartmental analysis using Drug and Statistics software (DAS 2.1.1 version, Mathematical Pharmacology Professional Committee of China), including area under the plasma concentration-time curve (AUC), the mean residence time (MRT), half-time (T_{1/2}), oral clearance (CL/F) and clearance (CL). All data were expressed as mean ± standard deviation (SD).

Oral bioavailability (F) was calculated according to the following equation:

\[
F(\%) = \left( \frac{\text{AUC}_{ig}}{\text{AUC}_{iv}} \right) \times \left( \frac{\text{Dose}_{iv}}{\text{Dose}_{ig}} \right) \times 100\%.
\]

Results

Assay validation

Under the described conditions, no endogenous interferences were observed at the retention times of TA (T_R = 14.25 min) and IS (T_R = 12.13 min), respectively. Representative chromatograms of blank rat plasma, LLOQ (2 ng/mL) plasma sample and test plasma sample obtained at 1 h after the single dose administration of TA (30 mg/kg) were demonstrated in Figure 3.

The linearity was evaluated by the calibration curves determined in five separated days. The calibration curve was obtained by plotting the peak-area ratio of TA to the IS versus the TA concentration, showing a good linearity over the concentration range of 2–500 ng/mL with a typical equation of \( Y = 0.0218X + 0.101 \) \( (r^2 = 0.9957) \). This method was sensitive enough to investigate the pharmacokinetic study of TA with a good signal-to-noise ratio \( \text{(S/N} > 20) \) at LLOQ.

The RSD values of intra-day and inter-day precision were all less than 8.0%. The accuracy results were 94.8–107.2% for intra-day and 96.0–102.7% for inter-day, respectively (Table 1). The results indicated that this analytical method was quite reliable and reproducible.

As shown in Table 2, the mean values of matrix effects for TA at low, middle and high QC concentrations were 94.6%, 100.7% and 93.9%, respectively. The mean recovery was all more than 90.0% at three QC levels for TA. The mean matrix effects for IS was 92.2%. The results suggested that processing procedure could provide high extraction efficiency. No ion suppression or enhancement was detected under the current conditions.

The data of stability test for TA in rat plasma under the described conditions are summarized in Table 3. The results indicated that TA could maintain its stability during the whole analytical process.

Pharmacokinetic study

The method was successfully applied to the pharmacokinetic study after a single dose of 30, 60 and 120 mg/kg for oral administration and at 3 mg/kg for intravenous injection to rats. Mean plasma concentration-time profiles of intravenous injection dose and different oral administration doses of TA are illustrated in Figure 4. The non-compartmental pharmacokinetic parameters for both i.g. and i.v. administration are summarized in Table 4.

The \( t_{1/2} \) were 2.37±1.23, 1.27±0.49, 2.07±0.93 h after single oral administration of 30, 60 and 120 mg/kg of TA. The \( t_{1/2} \) was 1.63±0.36 h after intravenous administration of 3 mg/kg of TA. After oral administration of TA to rats, the plasma drug concentration reached the maximum point at about 1.5 h. Then the plasma concentration decreased gradually with \( t_{1/2} \) between 2 and 3 h. The mean \( t_{1/2} \) was 1.56 h,
showing that TA was eliminated rapidly in rats. C\text{max} and AUC were linearly related to the doses, indicating that the pharmacokinetic process of TA was consistent with dose-proportional pharmacokinetics. Based on the calculation of AUC\text{0–6} obtained from i.g. and i.v. administration, the absolute bioavailability (F, %) of TA was calculated to be 2.0% in rats.

**Tissue distribution**

As shown in Figure 5, TA in most tissues reached peak level at 1.5 h after oral administration. The highest concentrations of TA were detected in intestine and stomach, followed by lung and spleen, concentrations in body adipose and ovary were minimal among all the tissues. One and half an hour after TA treatment, the mean TA concentration in the intestine (2.62 ± 0.27 \mu g/g tissue) was more than three times higher than that in the lung (0.74 ± 0.12 \mu g/g tissue). The tissue distribution profile showed that the lowest TA concentration was found in the brain (0.19 ± 0.01 \mu g/g tissue at 1.5 h) and testis (0.15 ± 0.00 \mu g/g tissue at 1.5 h).

**Excretion**

The accumulative excretion of TA by urine and feces from 0 to 72 h, by bile from 0 to 10 h, after oral administration at 60 mg/kg, is showed in Figure 6. It is clear that the major route of excretion of TA was via feces, 26.68% ± 7.14% of TA being excreted within 72 h. Fecal excretion reached the maximum nearly 12 h after the treatment of TA (23.37% ± 6.51% of dose/12-h period). The contribution of urine and bile made for the elimination of TA was insignificant, which only dealt with 0.0023% ± 0.0015% and 0.0027% ± 0.0006% amount of drug tested. The results were in agreement with the previous studies about the main excretion path of other common saturated fatty alcohols drugs, which fecal excretion is responsible for the major of elimination. As the half-life was 1.56 h, more than 12 h is needed to achieve >90% excretion.
Discussion

It is necessary to use an IS to get high accuracy and precision when a mass spectrometer is equipped with GC as a detector (Haim et al., 2009). An ideal IS should be a structurally similar analog compound. 1-Octacosanal was chosen as the IS for the quantification of TA due to its similar extraction recovery, ionization response in EI mass spectrometry, and a similar chromatographic retention time. Based on the full scan of the mass spectrum of TA and IS and their product ion fragments under daughter ion scanning (DAU) mode, the daughter ions of TA after derivatization were +71, 83, 97, 111, 125 and 137 and that of 1-octacosanal were +75, 83, 97, 111, 125 and 153. By observing the maximum response of the product ions in the MRM model, we chose 97 as the qualitative daughter ion. Finally, we selected MRM of precursor-product ion transitions with m/z 495.6 → 97.0 for TA and m/z 467.5 → 97.0 for IS, respectively. Product ion spectra of TA and IS were shown in Figure 2.

In this work, the sensitivity of GC-MS/MS method (LLOQ, 2 ng/mL) was higher than the reported GC-MS method (LLOQ, 8.4 ng/mL) (Haim et al., 2009), which were suitable for the present pharmacokinetic study of TA. Take the group treated with TA at 120 mg/kg body weight, for example, a peak plasma concentration for total TA of 98.74 ng/mL was reached immediately and declined sharply to 12.27 ± 2.97 ng/mL within 6 h, which was almost consistent with the previous studies that the plasma concentration of TA reached maximum point (120 ng/mL) in 1 h, after oral administration of 100 mg/kg of TA (Haim et al., 2009). Moreover, C_max reached 21.07 ng/mL, 41.41 ng/mL and 98.74 ng/mL in 1.5 h after single oral administration of 30, 60 and 120 mg/kg, respectively; indicating that the pharmacokinetic process of TA was consistent with dose-proportional pharmacokinetics. With the calculation of the pharmacokinetic parameters, the F (%) of TA was only about 2.0% in rats.

The tissue distribution study demonstrated that the majority of TA was in intestine and stomach, followed by lung, spleen and liver (Figure 5). This is similar to the earlier studies that TA may become a broad spectrum anti-cancer drug, especially with a best treatment for liver cancer, colon cancer and lung cancer applications (Fan et al., 2011; Zhang et al., 2008). Moreover, it is surprising and interesting that TA concentration in brain (0.19 ± 0.01 μg/g tissue at 1.5 h) and testis (0.15 ± 0.00 μg/g tissue at 1.5 h) was the lowest among the tested tissues. In addition, concentration of TA in plasma was quite low and decreased along with the time, being in agreement with the results obtained from plasma pharmacokinetics.

It is widely acceptable that the main elimination path of saturated fatty alcohols-based anti-tumor drugs is via feces, with very little TA recovered in urine and bile. The fecal excretion of TA was 26.68% ± 7.14%, while the contribution of urine and bile made for the elimination of TA was 0.0023% ± 0.0015% and 0.0027% ± 0.0006%. Therefore, fecal excretion of TA is higher than that of urine and bile. There is one possible reason that TA is highly lipophilic and hardly dissolved in urine and bile accounting for their differences in excretion.

Conclusion

A validated GC-MS/MS method for the determination of TA in rat biological matrices was developed and applied to
investigate the pharmacokinetics, tissue distribution and excretion of TA in rats. The $t_{1/2}$ of TA in plasma after i.g. and i.v. administration, which were $1.56 \pm 0.89$ h and $1.63 \pm 0.36$ h, respectively, showed that TA can be rapidly absorbed and quickly eliminated in plasma. The $F$ (%) was estimated as 2.0%, indicating the poor oral absorption of TA. Tissue distribution studies showed that TA could be rapidly and widely distributed into tissues like intestine and stomach, but showed the lowest TA concentration in the brain ($0.19 \pm 0.01 \mu g/g$ tissue at 1.5 h) and testis ($0.15 \pm 0.00 \mu g/g$ tissue at 1.5 h). The excretion study indicated the major route of excretion of TA was via feces (26.68% ± 7.14%) while the contribution of urine ($0.0023\% \pm 0.0015\%$) and bile ($0.0027\% \pm 0.0006\%$) made for the elimination of TA was insignificant. To the best of our knowledge, this is the first report for the thorough studies of the pharmacokinetics, tissue distribution and excretion profiles of TA in rats and would be useful for new drug developments and applications.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

**References**