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Application of a sensitive liquid chromatographic/tandem mass spectrometric method to a pharmacokinetic study of allylestrenol in healthy Chinese female volunteers

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ABSTRACT

A sensitive, specific and rapid liquid chromatographic/tandem mass spectrometric (LC/MS/MS) assay for the determination of allylestrenol in human plasma was established. Plasma samples were extracted by *tert*-butyl ether and separated by LC/MS/MS using a Phenomenex Curosil-PFP column (250 mm × 4.6 mm ID, dp 5 μm) with a mobile phase of methanol–water (95:5, v/v). The analytes were monitored with atmospheric pressure chemical ionization (APCI) by selected reaction monitoring (SRM) mode. The linear calibration curves covered a concentration range of 0.04–20.0 ng/mL with lower limit of quantification (LLOQ) at 0.04 ng/mL. The mean extraction recovery of allylestrenol was greater than 81.8%. The intra- and inter-day precisions were less than 1.3% and 3.1% respectively, determined from quality control (QC) samples of three representative concentrations. The method has been successfully applied to determining the plasma concentration of allylestrenol and a clinical pharmacokinetics study in healthy Chinese female volunteers.

1. Introduction

Allylestrenol, 17α-allylestr-4-en-17β-ol (Fig. 1A), is a synthetic steroid which bears progestational activity. In clinic, it was initially used in case of progesterone deficiency, for treating threatened and recurrent miscarriage, and to prevent premature labour [1]. Later, it was used as antiandrogen therapy for treating prostate cancer or hyperplasia as well [2,3]. Recently, problems of the adverse effects in clinic usage, e.g. sexual disfunction [4] have been considered to prescribe allylestrenol. Therefore, it is important, especially for clinic usage, to know the pharmacokinetic parameters of allylestrenol in human being. However, the pharmacokinetic study of allylestrenol has rarely been reported although decades of its prescription in clinic.

So far as we know, only two paper up to now reported their traditional HPLC and gas chromatography assays, which is not suitable for pharmacokinetic study owing to the detection limitation, for the determination of allylestrenol from its marketed formulation [5,6].

In this study, we developed a sensitive, specific, and rapid LC/MS/MS assay for the determination of allylestrenol in human plasma based on optimizing the sample extraction and the mass spectrometric condition, with an LLOQ of 0.04 ng/mL and a lineal detection range of 0.04–20 ng/mL. Then, we successfully applied this method to a pharmacokinetic study involving 20 healthy Chinese female volunteers after a single oral dose of 20 mg allylestrenol.

2. Experimental

2.1. Chemical and reagents

Allylestrenol was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and Mifepristone (internal standard, IS, Fig. 1B) provided by Zizhu Pharmaceutical Factory Co. Ltd. (Beijing, China). The test drug of allylestrenol tablets (5 mg/tablet) was kindly provided by the Fourth Pharmaceutical Co. Ltd. (Changzhou, China). Methanol and *tert*-butyl ether of HPLC grade were purchased from Merck (Darmstadt, Germany). Water used to prepare all aqueous solutions throughout the study was deionized and purified using a Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals and reagents are of analytical grade. Heparinized blank human plasma was obtained from Xijing hospital (Xi'an, China).

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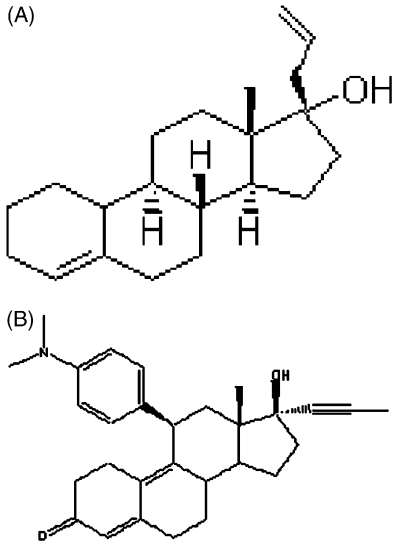

Fig. 1. Chemical structure of allylestrenol (A) and mifepristone (B).

2.2. Instrumentation and analytical conditions

The LC/MS/MS system consisted of a Surveyor LC pump, a Surveyor auto-sample, a TSQ Quantum Ultra AM triple–quadrupole tandem mass spectrometer with an ion max source, and the Xcalibur 1.1 software for data acquisition and analysis (Thermo Finnigan, San Jose, CA). Separation of the analytes from plasma was done at 25 °C on a Curosil-PFP column (250 mm × 4.6 mm ID, dp 5 μm; Phenomenex, Torrance, CA, USA). A mobile phase of methanol–water (95:5, v/v) at a flow rate of 1.0 mL/min was employed.

The MS/MS was detected by atmospheric pressure chemical ionization (APCI) source in positive mode. The injection volume was 50 μL. Separation was conducted under isocratic conditions and the total running time was within 6 min. The TSQ quantum parameters were optimized and set as following: charging current of 16.0 μA, capillary temperature of 200 °C, sheath gas pressure of 20 psi, auxiliary gas pressure of 10 psi, heater gas temperature at 420 °C and argon collision gas pressure of 1.3 mTorr. Typical product ion-scan spectra for the allylestrenol and the internal standard are shown in Fig. 2. Quantification was carried out using selected reaction monitoring (SRM) with ion transitions of *m*/*z* 241–91 with collision energy (CE) of 38 eV for allylestrenol, and *m*/*z* 430–372 with CE of 35 eV for mifepristone (IS).

2.3. Preparation of standard and quality control (QC) samples

Two stock solutions of allylestrenol were prepared by dissolving the accurately weighed reference substance in methanol. One solution was then serially diluted with methanol to give working solutions at the following concentrations: 0.40, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 160.0 and 200.0 ng/mL. The other stock solution was independently diluted in a similar way to achieve QC solutions at concentrations of 2.0, 40.0 and 160.0 ng/mL. Internal standard working solution (15 ng/mL) was prepared by diluting the 200 μg/mL stock solution of mifepristone with methanol. All the solutions were kept at 4 °C and were brought to room temperature before use.

Both the calibration standard samples and the quality control samples, which were used in the pre-study validation and during the pharmacokinetic study were prepared by spiking 0.5 mL blank plasma with 50 μL working solutions correspondingly.

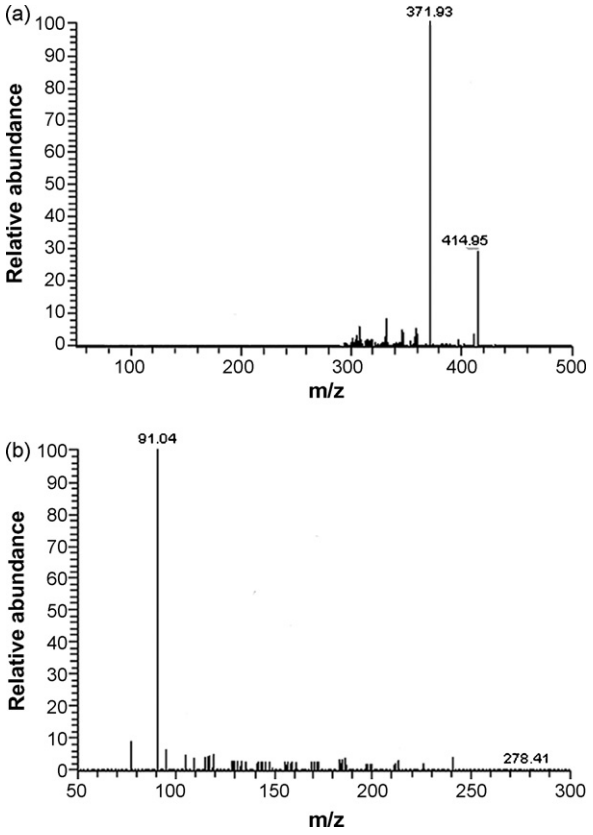

Fig. 2. Product ion mass spectra of $[M+H]^+$ of (A) miferpristone and (B) allylestrenol (internal standard).

2.4. Sample preparation

The frozen human plasma samples were thawed at ambient temperature, then a 0.5 mL aliquot plasma sample was mixed with 50 μL IS (1.5 ng/mL) solution and 100 μL of 0.1 M hydrochloric acid solution. The mixture was extracted with 5 mL of *tert*-butyl ether, vortex-mixed for 4 min, and then centrifuged at 1700 × *g* for 5 min. The 4 mL of organic layer was removed and evaporated under a stream of nitrogen gas in the thermostatically controlled water-bath maintained at 40 °C until completely dry. The residue was dissolved in 150 μL of mobile phase, vortex-mixed for 1 min, centrifuged at 6000 × *g* for 5 min, and a 50 μL of the supernatant was then injected into the LC/MS/MS for analysis.

2.5. Method validation

2.5.1. Selectivity

To investigate the selectivity of the method, human blank plasma samples from six different donors were pretreated and analyzed at LLOQ. LLOQ was defined as the lowest concentration of analyte within the acceptable precision and accuracy (five replicates with relative standard deviation (R.S.D.) below 20% and relative error (R.E.) within ±20%). Moreover, the analyte's response at this concentration level should be >5 times of the baseline noise.

2.5.2. Linearity of calibration curves and lower limits of quantification

Linearity was evaluated by assaying calibration curves in human plasma in four separate runs. Each validation run consisted of a set of the spiked calibration standards at 10 concentration levels of 0.040, 0.10, 0.20, 0.40, 1.0, 2.0, 4.0, 10.0, 16.0 and 20.0 ng/mL

($n = 4$ at each concentration). Calibration curves ($Y = aC + b$) were represented by plotting the peak area ratios (Y) of allylestrenol to IS versus the concentrations (C) of the calibration standards. Calibration curves were obtained from weighted ($1/C^2$) least-squares linear regression analysis of the data.

The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20%, and it was established based on five samples independent of standards.

2.5.3. Precision and accuracy

To evaluate the precision and accuracy of the method, QC sample at three concentration levels (0.20, 4.00 and 16.0 ng/mL) were analyzed in six replicates on three validation days. The assay precision was calculated by using the relative standard deviation and a one-way analysis of variance (ANOVA). It separates out the sources of variance due to within- and between-run factors. The assay accuracy was expressed as

$$\text{relative error (R.E.\%)} = \frac{\text{observed concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100$$

The accuracy was required to be within ±15%, and the intra- and inter-day precisions not to exceed 15%.

2.5.4. Extraction recovery

Before extraction procedure, we tested three commonly used extraction solutions, diethyl ether, ethyl acetate and the mixture of acetonitrile and diethyl ether. The recoveries of allyestrenol from human plasma were all lower than 70% in these three extraction solutions. The *tert*-butyl ether was therefore chosen as the extraction solvent for its higher extraction efficiency to the two target compounds. In addition, the pH of the plasma samples was lowered via adding 100 μL of 0.1 M hydrochloric acid solution to 0.5 mL plasma sample before extraction to promote recovery. The extraction recoveries of allylestrenol were determined at low, medium and high concentrations (0.20, 4.00 and 16.0 ng/mL) by comparing the allylestrenol extracted from plasma samples with standard solutions without extraction. The extraction recovery was calculated by the formula:

$$\text{recovery}(\%) = \frac{\text{detector response of extracted analyte}}{\text{detector response of non-extracted analyte}} \times 100$$

where detector response is the area of the chromatographic peak for extracted or non-extracted analyte divided by the area of the chromatographic peak for the IS added.

2.5.5. Stability

The stability experiments aimed at testing all possible conditions that the samples might be exposed to during shipping and handling. The stock solutions were investigated by storing under refrigeration at −20 °C. To evaluate allylestrenol stability in human plasma, blank plasma samples were spiked at 4.0 ng/mL ($n = 3$ per test and each concentration). The short-term temperature stability was assessed after samples were kept at ambient temperature (25 °C) for 6 h. Freeze–thaw stability (−20 °C in plasma) was checked through three freeze–thaw cycles within 30 days. The long-term stability was performed at −70 °C in plasma for 30 days. The stability of extracted and dried residues of plasma samples containing allylestrenol was also evaluated.

2.6. Pharmacokinetic study design

The validated method was applied to a pharmacokinetic study of oral administration of 20 mg allylestrenol, four tablets of 5 mg formation. Twenty female Chinese healthy volunteers (aged 31.8 ± 3.6, body weight 56.7 ± 3.9 kg) were involved in the study after a thorough medical, biomedical and physical examinations. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved to the Human Investigation Ethical Committee at Xijing Hospital of the

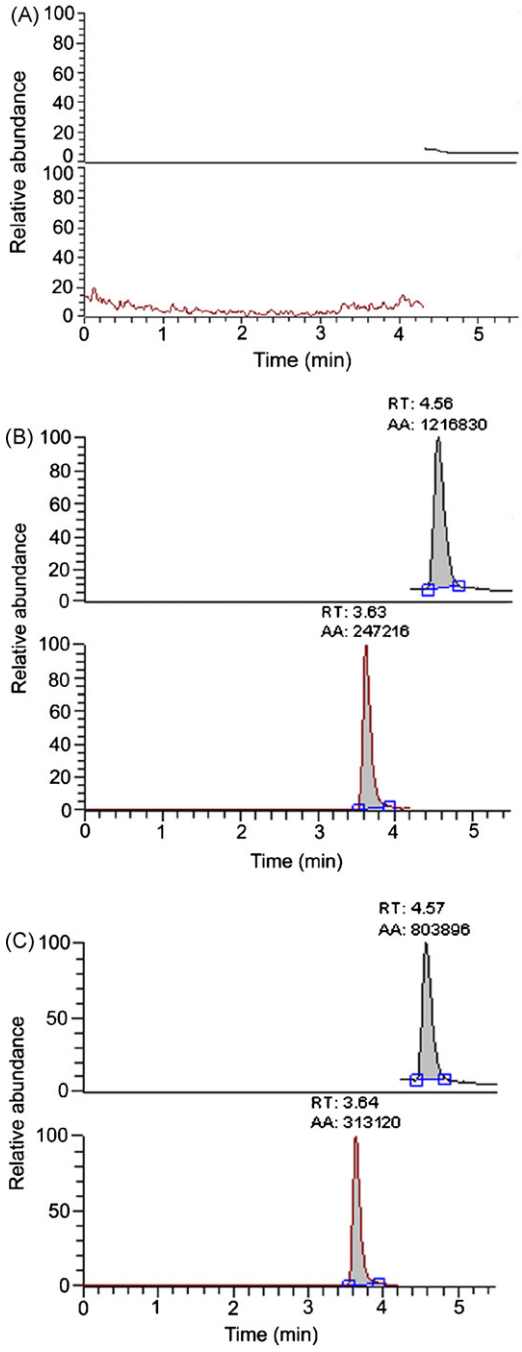

Fig. 3. Representative SRM chromatograms for allylestrenol and IS (mifepristone) in human plasma sample: (A) blank plasma sample; (B) a blank plasma sample spiked with allylestrenol (16.0 ng/mL, $t_R = 4.56$ min) and IS (1.5 ng/mL, $t_R = 3.63$ min); and (C) a plasma sample from a volunteer 1.5 h after an oral administration of 20 mg allylestrenol.

Table 1
Validation from QC samples of human plasma extracts ($n = 5$)

Added C (ng/mL)	Detected C (ng/mL)					Mean	±S.D.	R.S.D. (%)
0.20	0.21	0.21	0.22	0.21	0.21	0.20	0.01	5.1
	0.19	0.20	0.19	0.20	0.18			
4.00	4.09	4.44	4.01	4.07	3.82	4.2	0.22	5.3
	4.18	4.51	4.40	4.41	4.12			
16.0	17.4	17.9	16.5	18.2	17.8	17.4	0.7	4.3
	18.1	17.6	15.9	17.2	17.1			

Fourth Military Medical University. The study was performed according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of good clinical practice (GCP). Each volunteer was fasted and administered a single dose of 20 mg allylestrenol with 250 mL water. Venous blood samples about 4 mL were collected in heparin containing tubes before drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, 36 h after dosing. The plasma samples were centrifuged at $1600 \times g$ for 10 min and were frozen at $-20\,^{\circ}C$ until required for analysis.

The plasma concentrations of allylestrenol versus time profiles were acquired for each subject. Then major PK parameters of allylestrenol were calculated by non-compartment model. The maximum allylestrenol plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were determined by inspection of the individual plasma concentration–time profiles of the drug. The elimination rate constant (k) was calculated as the slope of the linear regression fit of the logarithm scale plasma concentrations versus time data for the last four measurable points. Apparent elimination half-time ($t_{1/2}$) was obtained as $0.693/k$. The area under the plasma concentration–time curve (AUC) was calculated according to the linear trapezoidal rule to the last measurable point (AUC_{0-t}) or to $_{\infty}$ ($AUC_{0-\infty}$) by $AUC_{0-t} + C_t)/k$, where C_t is the last measurable drug concentration. The CL/F were calculated based on the equations: $CL/F = kV_c$, where V_c is the apparent volume of distribution calculated as dose/C_0.

3. Results and discussion

3.1. Method development

Sample preparation is critical in many occasions for the determination of pharmaceuticals in biological samples with low interfering matrix components and high selectivity and sensitivity. Comparing with protein precipitation and solid–liquid extraction, liquid–liquid extraction (LLE) was advantageous in that it can purify and concentrate the sample simultaneously. In the present work, we chose *tert*-butyl ether for extraction after comparing the extraction efficiencies of three commonly adopted extraction solvents, and it produced a clean chromatogram for a blank plasma sample with extraction recoveries more than 81.8%. A Phenomenex Curosil-PFP column (250 mm × 4.6 mm ID, dp 5 μm) was used for the chromatographic separation. In our study, we tested multiple chromatographic conditions. As far as the mobile phase was concerned, acetonitrile results in higher background noise than methanol does. A mixture of methanol–water (95:5, v/v) was found to be optimal for the present study, which provided symmetric peak shapes of the analytes and the internal standard as well as a short run time. For the selection of the internal standard, the similar extracted recovery and the retention time was of significant important. Several compounds were tried and mifepristone was finally adopted as the internal standard in this work.

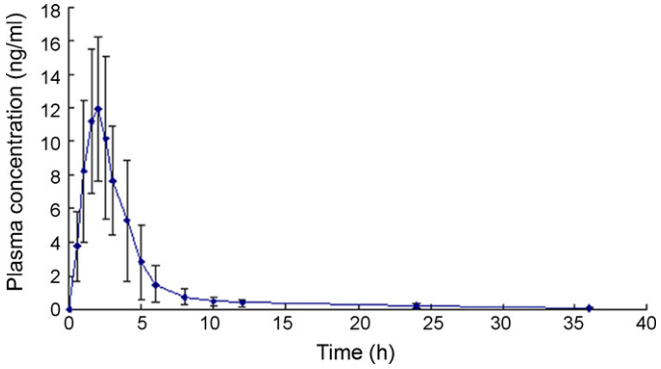

Fig. 4. Mean plasma concentration–time profile of allylestrenol in 20 healthy volunteers after an oral dose of allylestrenol tablets (20 mg). Each point represents mean ± S.D. ($n = 20$).

3.2. Method validation

3.2.1. Assay selectivity

Fig. 3 shows the typical MRM chromatograms of blank plasma, a plasma sample from a healthy volunteer 1.5 h after an oral administration of 20 mg of allyestrenol, and a spiked plasma sample with allyestrenol (16 ng/mL) and mifepristone (1.5 ng/mL). As shown, the retention times of allylestrenol and mifepristone in our system were 4.56 ± 0.05 min and 3.63 ± 0.05 min, respectively. No interference was observed at the retention times of either allylestrenol or internal standard in all plasma samples used for analysis. The results showed that the method exhibited good specificity and selectivity and was applicable to clinical use.

3.2.2. Calibration curve and sensitivity

Five calibration analyses were performed on five consecutive days and the back-calculated values for each level were recorded. The regression equation for calibration curves in the range of 0.04–20.0 ng/mL was $y = 0.2770C + 0.04703$. The correlation coeffi-

Table 2
Accuracy and precision for the analysis of allylestrenol in human plasma

Added concentration (ng/mL)	Mean measured concentration (ng/mL)	R.E. (%)	Intra-assay R.S.D. (%)	Inter-assay R.S.D. (%)
0.20	0.18	−12.0	1.3	1.9
4.00	4.30	7.4	0.6	3.1
16.0	17.4	8.9	0.4	0.2

Table 3
Main pharmacokinetic parameters of allylestrenol in female Chinese volunteers after an oral dose of 20 mg allylestrenol ($n = 20$, mean ± S.D.)

Parameters	Mean ± S.D.
C_{max} (ng/mL)	13.63 ± 4.05
T_{max} (h)	1.9 ± 0.5
$t_{1/2}$ (h)	9.46 ± 2.88
V_d (L)	6363 ± 4123
CL/F (h^{-1})	481 ± 192
AUC_{0-36} (ng h/mL)	46.88 ± 17.48
$AUC_{0-\infty}$ (ng h/mL)	48.30 ± 17.72

cient was 0.9997, indicating a good linearity. Standard curves were prepared daily and checked by QC samples (Table 1).

The LLOQ was established at 0.04 ng/mL, which was sensitive enough to investigate the pharmacokinetics of an oral dose (20 mg) of allylestrenol as demonstrated by this paper. The precision and accuracy at this concentration level were acceptable, with R.S.D. = 8.8% ($n = 10$), indicating a good sensitivity of our method.

3.2.3. Assay precision and accuracy

The precision was calculated by using one-way ANOVA. The intra- and inter-run precision and accuracy of the assay were studied by analyzing QC samples. All the values are summarized in Table 2. The data in the table showed that intra- and inter-day variability values were less than 1.3% and 3.1% respectively, and the relative error was within ±12%, all within the acceptable range, suggesting that the method is accurate and precise.

3.2.4. Extraction recovery

The extraction recoveries of allylestrenol from the human plasma were 81.8 ± 5.8%, 84.7 ± 11.1% and 88.7 ± 6.2% at concentration levels of 0.20, 4.00 and 16.0 ng/mL, and the R.S.D. were 7.1%, 13.1% and 6.9% ($n = 5$), respectively, showing a good extraction recovery.

3.2.5. Stability

The stability of allylestrenol was studied under a variety of storage and handling conditions. The results were obtained by comparing the freshly prepared solutions with those handled as described above. For the freeze–thaw stability, the plasma samples were found to be stable for at least three freeze–thaw cycles with the R.S.D. below 4.15%. In the short-term stability study, the plasma samples (4.0 ng/mL) were found to be stable for 6 h at ambient temperature (25 °C). In the long-term stability study, our results showed that no significant degradation occurred when stored at −70 °C for at least 30 days with the R.S.D. below 2.25%. Extracted and dried residues of plasma samples containing allylestrenol were stable at −70 ± 5 °C. All the deviations were within ±5%.

3.3. Pharmacokinetic study

No pharmacokinetic study of allylestrenol has been reported yet as far as we know, so we applied our present method to a pharmacokinetic study in which plasma concentration of allylestrenol in 20 healthy Chinese female volunteers were determined up to 36 h after an oral administration of 20 mg allylestrenol tablets. The mean plasma concentration–time curves were shown in Fig. 4. The non-compartmental analysis pharmacokinetic parameters were listed in Table 3. The main pharmacokinetic parameters of allylestrenol were as follows: C_{max} 13.63 ± 4.05 ng/mL, T_{max} 1.9 ± 0.5 h, $t_{1/2}$ 9.46 ± 2.88 h, V_d 6363 ± 4123 L, CL/F 481 ± 192 L, AUC_{0-36} 46.88 ± 17.48 ng h/mL, $AUC_{0-\infty}$ 48.30 ± 17.72 ng h/mL.

4. Conclusion

A sensitive and efficient LC/MS/MS method with high selectivity was developed and validated for the determination of allyestrenol in human plasma. The sample was prepared by *tert*-butyl ether extraction and followed by the analysis of a total running time of 6 min per sample with the LLOQ of 0.04 ng/mL. The method was successfully applied to characterize the pharmacokinetics of allyestrenol in Chinese healthy female volunteers after a single oral administration of 20 mg.

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