

Determination of Phenazopyridine in Human Plasma by GC–MS and its Pharmacokinetics

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Abstract

A sensitive, selective, and simple gas chromatography–mass spectrometry method is developed for quantitation of phenazopyridine (PAP) in human plasma using internal standard (diazepam). PAP and IS are extracted from plasma by liquid–liquid extraction and analyzed on a DB-5MS column with mass selective detector. Excellent linearity is found between 5–500 ng/mL ($r = 0.9992$, $n = 7$) for PAP in human plasma. The limit of detection is 0.3 ng/mL. Intra- and Inter-day precisions expressed as the relative standard deviation for the method are 1.37–6.69% and 1.24–6.01%, respectively. Extraction efficiency is more than 90%, and recoveries are in the range of 92.65–96.21%. This method is successfully applied for the pharmacokinetics and bioequivalence of 2 formulations of PAP in 18 healthy male volunteers who received a single 200 mg dose of each formulation.

Introduction

Phenazopyridine hydrochloride exerts an analgesic effect on the mucosa of the urinary tract and is used to provide symptomatic relief of pain in conditions such as cystitis and urethritis (1). It is given in conjunction with an antibacterial agent for the treatment of urinary-tract infections (2,3).

Phenazopyridine (PAP) [2,6-diamino-3-(phenylazo)pyridine] (Figure 1A) was adopted by USP in 1928 (4). Many methods have been developed for the measurement of PAP such as a polarographic and voltammetric study of copper-phenazopyridine monohydrochloride system in phosphate buffer medium electroanalysis (5), adsorptive stripping voltammetric assay of phenazopyridine hydrochloride in biological fluids and pharmaceutical preparations (6) and determination of phenazopyridine in tablets by high-performance liquid chromatography (HPLC) (7,8), have been reported. However, these methods all have disadvantages for determination of biological matrices. LC and LC–mass spectrometry (MS) methods for determination of PAP in human plasma have been reported (9,10). The HPLC–UV method was developed to determine phenazopyridine concentrations in plasma at 405 nm, but the limit of quantitation (LOQ) was as high as 50 ng/mL. Some treatment procedures given showed that the extraction recoveries were lower than 80% and

a great deal of plasma sample (1 mL) and extract (5 mL) were necessary (10).

This paper reports that a simple, sensitive, and specific gas chromatography (GC)–MS method was investigated for the quantitation of PAP in human plasma samples with liquid–liquid extraction as the sample preparation method for further detection based on the GC–MS method for rats (11). We have applied this method for bioequivalence study of two oral dosage forms of PAP (test and reference). The open randomized, cross over study performed on a group of 18 healthy, Chinese male volunteers confirmed the bioequivalence of both formulations. It has proven that the method can be widely applied in phenazopyridine clinical practice, such as bioequivalence and bioavailability study.

Experimental

Chemicals and Reagents

Phenazopyridine hydrochloride (purity > 99.0%) reference and reference tablets were supplied by Jia-lun Pharmaceutical Company of Hong Kong. Test tablets were supplied by Institute of Pharmaceutical Research of Gui-zhou. Diazepam (purity > 98.8%) was bought by National Institute for the Pharmaceutical and Biological Products of China. HPLC grade methanol, ethyl acetate, and diethyl ether were obtained from Fisher Scientific (Pittsburg, PA). Helium (purity, 99.999%) was from Xi'an Analytical Instrument Factory (Xi'an, China). Other reagents used in the experiment were analytical grade and from commercial sources.

GC–MS conditions and instrumentation

A capillary GC/MS-QP2010 (Shimadzu, Kyoto, Japan) with a

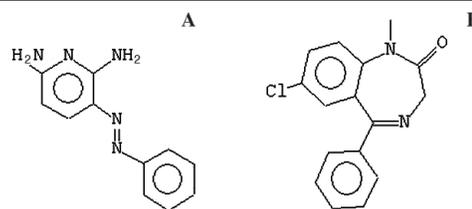


Figure 1. The structures of phenazopyridine and diazepam.

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DB-5MS capillary column (30 m × 0.32 mm i.d, 0.25 μm film thickness, Agilent Technologies, Palo Alto, CA) was used. The inlet temperature was maintained at 280°C. The oven temperature was initially held at 140°C for 2 min, and then programmed to 280°C at 10°C/min where it was held constant for 4 min. Helium was used as carrier gas at a constant flow rate of 2.0 mL/min. The biological samples were analyzed by GC–MS with the pulsed splitless injection mode. 1 μL was injected for each assay.

The source and electrodes of the quadrupole mass filter were both set to 200°C. Ionization was carried out in electron impact ionization (EI) mode at 70 eV. Detection was operated under selected ion monitoring (SIM) mode. The ions for base peak (m/z 213 for PAP and m/z 283 for IS) were selected for quantitative assay under investigation.

Preparation of analytical standard solutions, PAP quantitation, method validation

The stock solution of PHP and IS were prepared by dissolving PAP and I.S in methanol to make a 1.0 mg/mL solution, respectively. The regression line of peak-area ratios versus standard concentrations calculated with respect to IS was used in quantitation of PAP. As well as 5.3 μg/mL working solution of IS, the working standard solution for each concentration point on

the standard curve was prepared. Calibration curves were obtained from plasma samples spiked with various amounts of PAP (5, 10, 20, 50, 100, 200, and 500 ng/mL) and 50 μL of working IS solution was added for all quantitated samples to evaluate the linearity, precision (%CV, $n = 5$), and accuracy (the bias between theoretical and actual concentrations).

The intra-day and inter-day precision and accuracy were determined by analyzing spiked plasma samples containing 5, 20, 100, and 500 ng/mL of PAP from 5 analyses per day for 5 consecutive days, respectively. Recoveries of PAP and IS were calculated by comparing the peak areas of 5 extracted samples from plasma to that of standards injected directly at the concentrations of 5, 20, 100, and 500 ng/mL with the mean peak area of standards and 5.3 μg/mL of IS.

Sample preparation

A liquid–liquid extraction method was used for the extraction of PAP and IS in bio-samples. To every plasma sample, 50 μL of IS (μg/mL) was added to 0.5 mL plasma in glass centrifuge tube. Then, every solution was subjected to addition of 0.1 mL of 1M NaOH at room temperature. Next, 2.5 mL ethyl acetate–diethyl ether (1:1) was added to every one, and the mixture was vortexed for 5 min. After centrifugation for 10 min at 4000 rpm, the upper organic layer was transferred to a clean tube. The organic solution was evaporated under a stream of nitrogen at 40°C. 0.1 milliliters of ethyl acetate was added to the residue and centrifugation procedure mentioned above was repeated. An aliquot (1 μL) of the supernatant was injected into the GC–MS systems for analysis.

Results and Discussion

Pharmacokinetic studies

PAP was given to healthy male volunteers after having obtained their informed consents before enrolment to participate to the study. The clinical protocol was reviewed and approved by the Ethics Committee of Xi'an Jiaotong University Hospital, China. Eighteen healthy male volunteers aged from 21 to 28 and with body weight between 58 and 70 kg were included in a randomized, single-dose, 2-period, 2-sequence, cross-over study with a 1-week washout period. The volunteers were non-alcoholic, free from cardiac, hepatic, renal, gastrointestinal, and hematological diseases, and also assessed by physical examination and following laboratory tests, had

complete blood count, hepatic function, and renal function tests. During each period, the volunteers were admitted to the Clinical Pharmacokinetic Laboratory, Xi'an Jiaotong University Hospital at 18:00 h and had an evening meal before 20:00 h. After an overnight fasting, they received a single 200 mg PAP dose of each of the formulations of test and reference drug at 07:00 h along with 240 mL of water. They were then in the seated position for at least 1 h and then fasted for 2 h. A standard lunch and an evening meal were provided at 4 and 10 h after dosing. Blood samples (5 mL) were

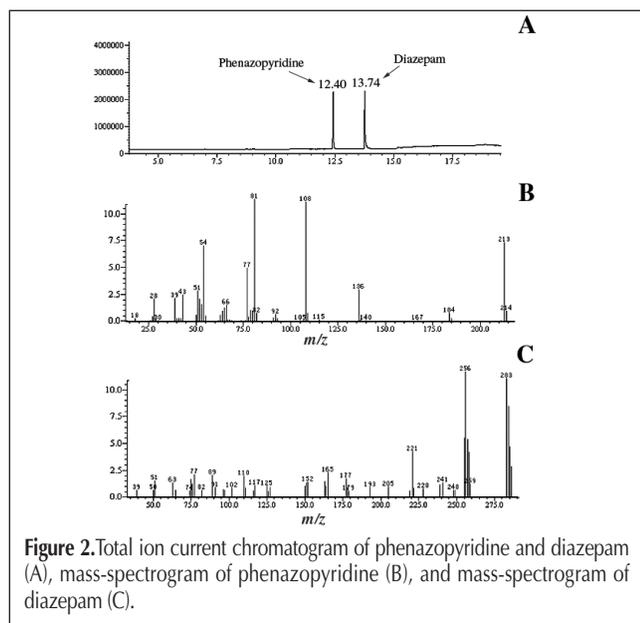


Figure 2. Total ion current chromatogram of phenazopyridine and diazepam (A), mass-spectrogram of phenazopyridine (B), and mass-spectrogram of diazepam (C).

Table I. Precision and Accuracy of the GC–MS Method for Determining PAP Concentration in Human Plasma Samples

Concentration added (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	CF* (mean ± SD, ng/mL)	Precision (%)	Accuracy (%)	CF (mean ± SD, ng/mL)	Precision (%)	Accuracy (%)
5	5.160 ± 0.345	6.69	103.2	5.090 ± 0.279	5.48	101.8
20	20.48 ± 0.444	2.17	102.4	19.90 ± 1.196	6.01	99.5
100	94.50 ± 3.374	3.57	94.5	93.20 ± 2.488	2.67	93.2
500	499.0 ± 6.836	1.37	99.8	483.5 ± 5.995	1.24	96.7

* CF = Concentration found.

withdrawn from antecubital vein at 0, 0.25, 0.50, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after and then transferred to vacutainer tubes and centrifuged at 4000 rpm for 15 min (4°C); the plasma samples obtained were stored at -20°C until analysis. The concentrations of total plasma of PAP were determined as the mean of duplicate samples. The maximal concentration (C_{\max}) and the time for maximal concentration (T_{\max}) were determined by visual inspection from each volunteer's plasma concentration-time plots for PAP. The area under the plasma concentration-time curve [$AUC_{(0-t)}$] was calculated by the linear trapezoidal method from 0 to 24 h. The AUC extrapolated to infinity [$AUC_{(0-\infty)}$] was calculated as $AUC_{(0-t)} + C_t/K_e$, where C_t is the last measurable concentration and the elimination rate constant (K_e) was obtained from the least square fitted terminal log-linear portion of the plasma-concentration-time profile (The Pharmacological Society of China, 1997). Plasma elimination half-life ($T_{1/2}$) was calculated as $0.693/K_e$.

Separation

A typical gas chromatography-total ion current (GC-TIC) mass spectrogram chromatogram obtained for PAP standard and IS is shown in Figure 2 as analyzed by GC-MS in the electron impact (EI) mode. The retention time of PAP was 12.5 min and IS was 13.8 min. It is obvious that the GC-TIC chromatogram and mass spectrogram can provide the elective ion and the time program: From 3–13.2 min the m/z was 213 (PAP) and 13.2–20 min the m/z was 283 (I.S).

Table II. The Extraction Recovery of Measurement of PAP and IS in Human Plasma

Samples	Sample concentration (ng/mL)	Extraction recovery (mean \pm SD, $n = 5$)
Phenazopyridine	5	95.34 \pm 6.59
	20	93.34 \pm 3.49
	100	92.65 \pm 4.21
	500	96.21 \pm 3.57
Diazepam	106	93.21 \pm 2.48

Table III. Stability of PAP in Human Plasma

	Nominal concentration (ng/mL) ($n = 5$)	Mean found concentration (mean \pm SD, ng/mL)	Precision (%)	Accuracy (%)
Bench top stability*	5	5.195 \pm 0.379	7.3	103.9
	20	20.48 \pm 1.024	5.0	102.4
	100	99.90 \pm 2.697	2.7	99.9
	500	493.0 \pm 5.916	1.2	98.6
Freeze and thaw stability†	5	5.125 \pm 0.384	7.5	102.5
	20	20.26 \pm 1.256	6.2	101.3
	100	99.60 \pm 2.789	2.8	99.6
4-week storage stability‡	5	5.120 \pm 0.353	6.9	102.4
	20	20.04 \pm 1.042	5.2	100.2
	100	101.3 \pm 2.431	2.4	101.3
	500	486.0 \pm 5.832	1.2	97.2

* Exposed at room temperature (25°C) for 15 h.

† After three freeze-thaw cycles.

‡ Stored at -20°C.

The GC-SIM chromatograms obtained for blank samples, PAP, and IS are shown in Figure 3 as analyzed by GC-MS. The presence of specific fragmentations, such as m/z of 213 and 283 are shown in Figure 2. It is obvious that the GC-SIM method simplifies the chromatogram very efficiently and provides a single peak for identification.

Method validation and linearity of calibration curve

The peak area of PAP in human plasma was linear with respect to the analyte concentration over the range of (5–500 ng/mL). The mean linear regression equation of calibration for PAP was $y = (0.03 \pm 0.011) + (0.021 \pm 0.001)x$, $r = 0.9992$, $n = 7$, where y was the peak area of the analyte and x was the concentration of the analyte. LOQ was established as (0.3 ng/mL) for PAP with an accuracy of 104% and precision less than 12%. The information of precision and accuracy are shown in Table I. For an intra-day run ($n = 5$), the CVs of PAP at 5, 20, 100, and 500 ng/mL were 6.69%, 2.17%, 3.57%, and 1.37%, respectively. The accuracy which mean the bias between theoretical and actual concentrations at the 5, 20, 100, and 500 ng/mL were 93.2–103.2%. The extraction recoveries of PAP and IS from spiked human plasma were determined at the concentrations of 5, 20, 100, and 500 ng/mL and 106 ng/mL in five replicates as shown in Table II. The recoveries of PAP and IS were 91.89% and 93.21%, respectively (12).

Stability

Table III lists data for bench top, freeze/thaw, and storage stability. Bench top stability was investigated to ensure that PAP was

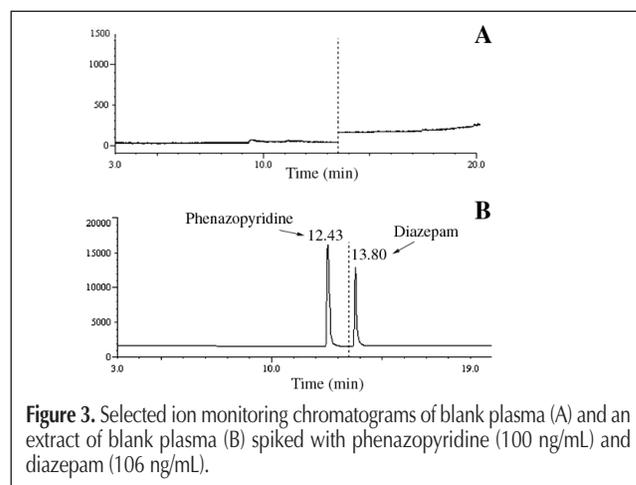


Figure 3. Selected ion monitoring chromatograms of blank plasma (A) and an extract of blank plasma (B) spiked with phenazopyridine (100 ng/mL) and diazepam (106 ng/mL).

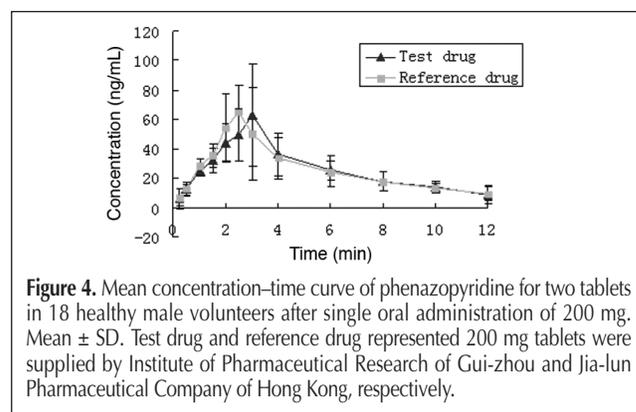


Figure 4. Mean concentration-time curve of phenazopyridine for two tablets in 18 healthy male volunteers after single oral administration of 200 mg. Mean \pm SD. Test drug and reference drug represented 200 mg tablets were supplied by Institute of Pharmaceutical Research of Gui-zhou and Jia-lun Pharmaceutical Company of Hong Kong, respectively.

Table IV. Mean Pharmacokinetic Parameters Obtained from 18 Human Volunteers After Oral Administration of 200 mg of PAP Tablets

Pharmacokinetic parameters	Test drug Mean \pm S.D	Reference drug Mean \pm S.D
AUC _(0–12 h) (ng.h/mL)	354.15 \pm 92.04	345.67 \pm 81.52
AUC _(0–∞) (ng.h/mL)	421.61 \pm 77.29	431.77 \pm 87.82
C _{max} (ng/mL)	62.76 \pm 30.68	65.00 \pm 29.23
T _{max} (h)	2.65 \pm 0.38	2.48 \pm 0.50
T _{1/2} (h)	9.61 \pm 1.97	9.41 \pm 2.04
K _e (h ⁻¹)	0.076 \pm 0.018	0.076 \pm 0.019

not degraded in plasma samples at room temperature for a time period to cover the sample preparation. Four sets of plasma samples at concentrations of 5, 20, 100, and 500 ng/mL were left at room temperature for 12 h. The samples were then processed and analyzed. The results indicated that PAP was stable during the exposure period.

Freeze-thaw stability was evaluated for PAP using QC samples. The QCs were exposed to three freeze-thaw cycles; each cycle consisted of removing the QCs from the freezer, thawing them unassisted to room temperature, kept at room temperature for 4 h and re-freezing at -20°C . The samples were processed along with a standard curve and concentrations were determined. This result indicated that PAP had an acceptable stability after three freeze-thaw cycles in human plasma. The storage stability at -20°C was also tested using QC samples.

The stability was closely monitored during validation and sample analysis periods, and no degradation of the compound was observed. The 4-week stability data are listed in Table III. The result indicated that PAP was stable in plasma for at least 6 weeks.

Clinical application in healthy volunteers

This method has been successfully used to the pharmacokinetic study of PAP after a single oral dosing of two PAP formulations (200 mg) for 18 healthy male volunteers by Latin-square cross-over design. Figure 4 shows the mean plasma concentration profiles of two formulations of PAP in 18 volunteers. Pharmacokinetic parameters are shown in Table IV. These parameters of 2 formulations such as AUC, C_{max}, T_{max}, and T_{1/2} are comparable to the corresponding parameters obtained by single oral dose of 200 mg PAP in the results of Zhou Y.D. et al. (13). In the bioequivalence test, the maximum concentration (C_{max}) and the areas under the curve [AUC_(0–12 h)] were compared Zhou Y.D. et al. The geometric mean and 90% confidence interval (CI) of C_{max} and AUC_(0–12 h) were 100.5% (90% CI = 92.42–117.99) and 100.9% (90% CI = 90.57–110.13), respectively. Because both 90% CI for C_{max} and AUC_(0–12 h) were included in the 80–125% interval proposed by the China Food and Drug Administration, it could be concluded that two types of 200 mg tablets were considered to be bioequivalent according to pharmacokinetic results of PAP obtained from time–concentration profiles of the two formulations. Diazepam, as the internal standard, is commonly used in clinical practice, so it may interfere with the assay results of phenazopyridine. Therefore, a full range mass scan was performed in volunteers' blank plasma, and no drug of benzodiazepines was found.

Conclusions

A simple, sensitive and specific GC–MS method was investigated for the quantification of PAP in plasma samples with liquid–liquid extraction as sample pretreatment. Diazepam was selected as internal standard (IS, Figure 1B). The limit of detection of PAP was up to 0.1 ng/mL (S/N > 3) in biological samples. The whole procedure is sensitive, accurate, and reproducible, which should be suitable to support this study. The applicability of the method is demonstrated in the bioequivalence test of two different PAP formulations in 18 healthy male volunteers.

Acknowledgments

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