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Determination of penehyclidine by gas chromatographic–mass spectrometry and its application to pharmacokinetics in humans, rabbits and mice

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Abstract

A sensitive and specific gas chromatographic–mass spectrometry with the extracted ion chromatograms (GC–MS/EIC) method has been developed and validated for the identification and quantification of penehyclidine (PH) in human and animal blood. The chromatography was on HP-5 capillary column (12 m × 0.2 mm × 0.3 μm). PH-d_5 was selected as the internal standard (IS). Simultaneous MS detection of PH and IS was performed at *m/z* 315 (PH) and *m/z* 320 (PH-d_5), and the EIC of the two compounds was at 175 and 180. PH and PH-d_5 eluted at approximately 8.2 min and no endogenous materials interfered with their measurement. Linearity was obtained over the concentration range of 0.1–100 ng/ml in the blood. The lower limit of quantification (LLOQ) was reproducible at 50 pg/ml in both human and animal blood. The within-day and between-day precisions were no more than 9.1%. This method was successfully applied to quantification and pharmacokinetic studies of PH in the subjects. The concentration–time profiles of PH in humans, rabbits and mice were all fitted to first order absorption two-compartment open model after i.m. a single dose. The differences in absorption, distribution and elimination of PH among the species were found. The results provided the important information for developing a novel anti-cholinergic drug and for obtaining a more effectual remedy in clinical practice.

Keywords: Penehyclidine; Anticholinergic drug; Quantification; Pharmacokinetics; GC–MS/SIM

1. Introduction

Penehyclidine [3-(2-hydroxy-2-cyclopentyl-2-phenoxyl) quincycloethane, PH] is a novel anti-cholinergic drug developed by the Beijing Institute of Pharmacology and Toxicology of China. Pharmacological evaluation has proved that PH has anti-muscarinic and anti-nicotinic activities, while retaining potent central and peripheral anti-cholinergic activities [1–7]. The clinical results have demonstrated that PH has a positively preventive and curative effect for organic phosphorus pesticide poisoning and can be used before general anesthesia [8–10].

Determination of the pharmacokinetic profile of PH is very important for gaining a better understanding of the mechanism of action and for ensuring more efficient therapeutic application. Because of the low therapeutic dose of PH (1–4 mg/day for the intramuscular dose in human), a sensitive analytical method is needed for the quantification of PH in the biologic fluids after intramuscular administration. In this paper, the quantification and the pharmacokinetics of PH in humans, rabbits and mice have been firstly investigated by the stable isotope tracer method in conjunction with gas chromatography–mass spectrometric (GC–MS) technique. The method is sensitive, reliable and suitable for quantification and the pharmacokinetic studies of PH in the subjects. The results could provide important information for developing a novel anti-cholinergic drug and for obtaining more effectual remedy in clinical practice.

2. Experimental

2.1. Chemicals and reagents

PH and the internal standard (IS, penehyclidine-d_5, PH-d_5) were prepared and provided from the Beijing Institute of Phar-

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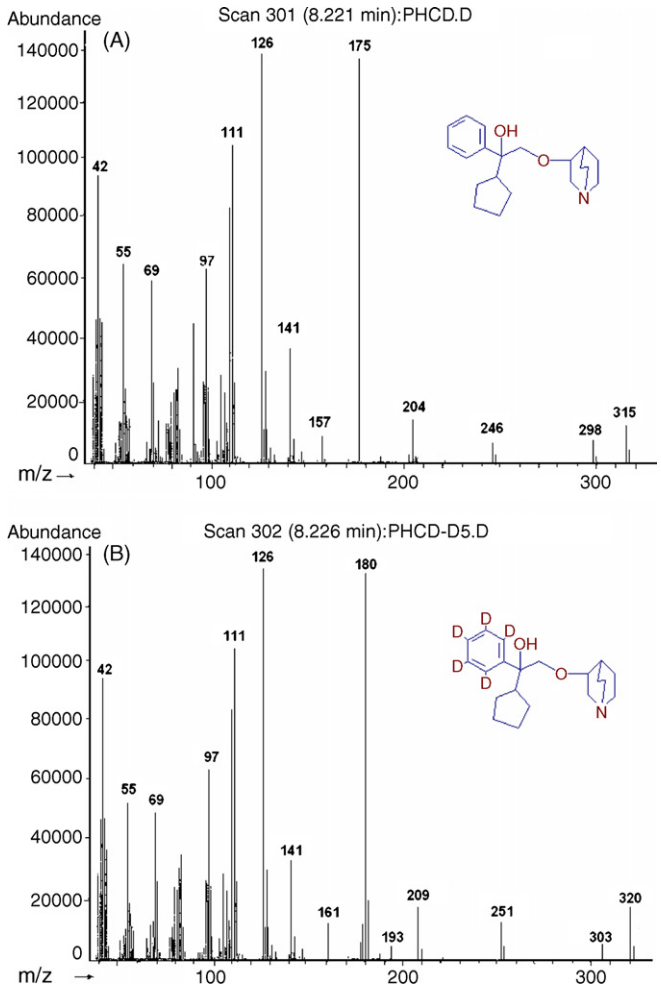

Fig. 1. Mass spectra (EI) for: (A) penehyclidine and (B) the internal standard, penehyclidine-d_5. (A) EI-MS of *m/z* 315 → 175 for penehyclidine. (B) EI-MS of *m/z* 320 → 180 for penehyclidine-d_5.

macology and Toxicology of China, which the structure was identified as pure compound from the melting point, UV, IR, MS and NMR [11–14]. The purity of PH or PH-d_5 was measured by the HPLC method and the purities were all more than 99%. Their chemical structures are shown in Fig. 1. Standard solutions of PH and IS were prepared in methanol and kept at 4 °C for 2 months. Methanol used was of LC grade and was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). All other reagents and chemicals were of analytical grade and obtained from Fisher Chemical (Fair Lawn, NJ, USA) and Dikma Reagent Company (Beijing, China).

2.2. Instrumentation and operating conditions

GC–MS experiments were performed with a HP 5971A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) equipped with HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). A HP-5 capillary column (length 12 m, 0.2 mm I.D., film thickness 0.33 μm) was employed with helium as carrier gas (flow 1.0 ml/min). A 1 μl volume of sample was split off injected into the GC. The column temperature was programmed as follows: first from 100 °C to 220 °C at 20 °C/min held for 6 min, then to 250 °C at a rate of 25 °C/min held for 10 min. The MS-EI ion source temperature was 200 °C, and the temperature of injection and the interface between the chromatograph and the spectrometer was set at 280 °C. The MS was operated in an electron impact (EI) ionization mode. The electron collision energy was 70 eV. The full scan range of the sample for the qualitative analysis was from *m/z* 40 to *m/z* 500 and then the quantification was the extracted ion chromatograms (EIC) from the full scans and for quantification the extracted ion traces *m/z* 175 (PH) and *m/z* 180 (IS) were used.

The mass spectrum peaks of *m/z* 175 and *m/z* 180 during the retention time of PH were detected with the EIC in GS-MS. The peak area of PH and IS was measured using HP ChemStation data acquisition system with the integration of peak area. A HP ChemStation data system was used to control the GC–MS system and to collect and quantify the data. The chromatographic data were processed using the IS method of plotting peak area ratios of analyte/IS versus the relative concentration followed by least square regression of these data.

2.3. Collection and preparation of samples

Eight healthy male volunteers, aged 22–38 years, weighing 59.4 ± 4.5 kg, were chosen as the subjects for this study after clinical and physical examinations and review of their medical histories. The volunteers were required to abstain from any drug, cigarette and alcohol for at least 2 weeks prior to the study. The protocol was approved by the Ethics Committee of the Hospital in Beijing (Academy of Medical Sciences, Beijing, China) and conducted in accordance with good clinical practice. Each volunteer was given a written consent to participate the experiment after receiving written and verbal information describing the study.

Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory animals as adopted and promulgated by the National Health Ministry of China. Six healthy male rabbits, weighing 3.0 ± 0.2 kg, and healthy male Kunming mice, weighing 20 ± 0.1 g, were all purchased from Animal Center of the Academy of Medical Sciences of China.

Each subject (human, rabbit and mouse) was given intramuscularly an injection containing a single dose (0.1 mg/kg) of PH after an overnight fast. Serial blood samples of the subjects were collected via an intravenous catheter at pre-dose ($t = 0$ h) and at 0.0167, 0.033, 0.083, 0.167, 0.33, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 24.0 h post-dose into heparinzed tubes after administration. Pooled drug-free blood was obtained from the subjects, after aliquoting, all the blood controls were stored at −20 °C and then thawed at room temperature for use in calibration curves and quality control (QC).

Stock solutions were prepared by dissolving 10 mg of PH and 10 mg of the IS in 10 ml of LC-grade methanol. A working standard solution was prepared by the serial dilution of the stock solution-using methanol. QC samples were also prepared in the same way, using a separately weighed stock solution. All solutions were stored at 4 °C prior to use.

2.4. Method validation

Calibration samples in the blood were prepared by mixing solutions of PH and IS with the blank blood of the subjects at a volume ratio to form a concentration series of 0.1, 1, 5, 25, 50, 100 ng/ml PH and 50 ng/ml IS. The precision and accuracy of the assay were determined by performing replicate analyses of QC samples against calibration standards. The precision and accuracy of the method were calculated as the relative standard deviation (R.S.D.) and the percentage deviation of observed concentration from theoretical concentration, respectively. The extraction recovery was determined by calculating the ratio of the amount of extracted compound from drug-free blood spiked with known amounts of PH to the amount of compound added at the same concentrations to methanol. The stability of the sample was also investigated by measuring QC samples: (1) allowed to stand at ambient temperature for at least 24 h before extraction; and (2) subjected to three freeze–thaw cycles for at least 5 days. The lower limit of detection (LLOD) was considered as three times the signal/noise ratio (S/N) and the LLOQ was 10 times the S/N ratio. The specificity of the method was characterized by assessing to accurate quantification of PH in the presence of endogenous compound and the metabolites of PH, confirmed by the analysis of blank and spiked QC samples.

2.5. Extraction procedure

Blood samples (0.5 ml) were spiked with 50 μl each of PH working solutions and 50 μl of IS stock solution. Then 0.1 ml of 0.2 mol/l NaOH and 2 ml of the mixed solvent (ethyl ether–dichloromethane 2:1, v/v) were added to the tubes of the blood samples. The combined samples were adjusted to pH 10, vortex-mixed for 1 min, and centrifuged at $3000 \times g$ for 5 min to get the upper organic portion, and the upper layer was transferred to another tube. The combined blood samples were extracted twice and the upper organic portions were combined. Then 1 ml of 0.1 mol/l HCl was added to the combined organic potions and the pH was adjusted to 2. The mixed system was vortex-mixed and centrifuged as above, and the upper organic layer was discarded. Then 1 ml of 0.2 mol/l NaOH and 2 ml of the mixed solvent were added to the lower aqueous phase and the pH was adjusted to 10, which was vortex-mixed, centrifuged and extracted twice. And the upper organic potions were combined and evaporated to dryness under nitrogen at 40 °C, the residue was dissolved in 50 μl of methanol, and an aliquot (1 μl) of was injected into the GC–MS system.

2.6. Pharmacokinetic studies

The GC–MS method was successfully applied to the pharmacokinetic studies of PH in humans, rabbits and mice. The subjects were fasted for 12 h before the test, with water available ad libitum. Blood was collected from the subject's vein before and after receiving a single i.m. dose of PH (0.1 mg/kg). All blood samples were sealed and stored at −20 °C until analysis. The blood samples were then extracted and separated and quantified as in Section 2.5.

The pharmacokinetic model and the parameters were calculated by the practical pharmacokinetic program-version 87 (3P87), edited by the Committee of the Mathematic Pharmacology, the Chinese society of Pharmacology. The compartment model was established by the methods of the survival square sum (SUM), the Akaike's information criterion (AIC) and the fitted degree (r^2). Data were analyzed statistically by a *t*-test, with the level of significance set at $p < 0.05$.

3. Results and discussion

3.1. Method development

Sample preparation plays an important role for determination of drugs in biological samples. After several trials, the method of the ionization with base and acid and liquid–liquid extraction was found to be suitable for the determination of PH in the blood. An HP-5 capillary column was used for the chromatographic separation, while chromatographic conditions were optimized through several trails to obtain high resolution and relative symmetric peak shapes, as well as a short run time. PH-d_5, the analog of PH, was found to be optimal for using the IS. Some parameters related to gas chromatographic and mass spectrometric detection were investigated and optimized.

The mass spectra of PH and PH-d_5 in EI-MS from different matrices were all similar when the electron collision energy was 70 eV. The corresponding values of PH were all identical when the same concentration of these compounds was in different matrices and the interferences were removed by the method of ionization with base and acid and liquid–liquid extraction. Simultaneous PH and the IS of the qualitative analysis were performed from *m/z* 40 to *m/z* 315 (PH) and from *m/z* 40 to *m/z* 320 (IS), and the EIC was used for the quantitative detection of both PH and the IS at *m/z* 175 and *m/z* 180.

3.2. Selectivity

The results for selectivity are shown in Fig. 2. The GC–MS/EIC method described was selective and specific and no endogenous interfering peaks were visible in the blank blood of the animal matrices. And the peak of PH is a little tailing at some concentration in human blood in which there could be some endogenous interfering. The retention times of PH and PH-d_5 were both 8.20 min.

3.3. Linearity

The quantitation was achieved by the chromatographic peak area of PH to the calibration standards at the concentration levels in the range of 0.1–100 ng/ml. For a standard curve the ratio of the chromatographic peaks area (PH/IS) as ordinate variables were plotted versus the concentration of PH as abscissa. The standard curve was linear over the range of 0.1–100 ng/ml

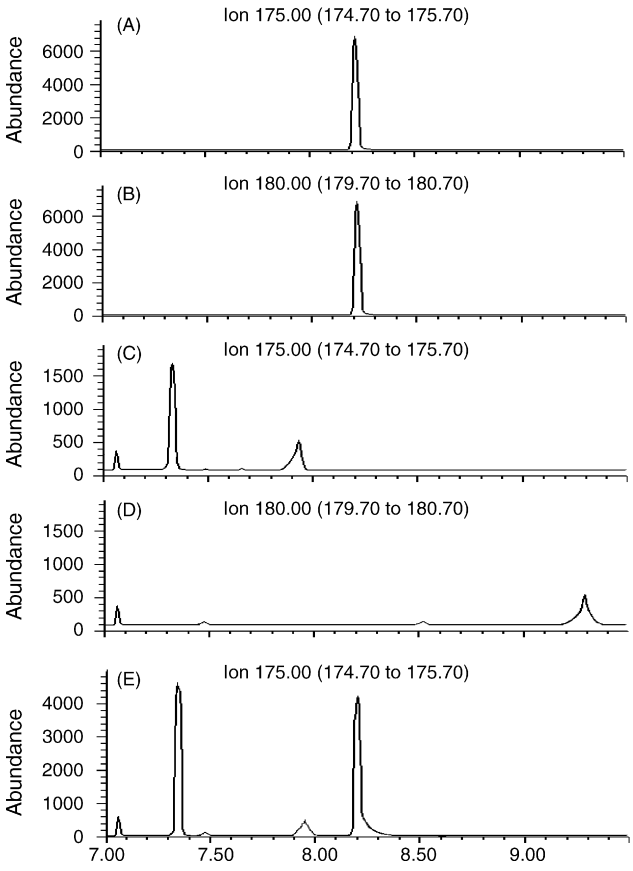

Fig. 2. Extracted ion chromatograms of penehyclidine and penehyclidine-d_5: (A) penehyclidine; (B) penehyclidine-d_5; (C and D) blank blood; (E) a blood sample from a human containing 12 ng/ml for penehyclidine.

in blood of the subjects, and the mean values of regression equation were $Y = 0.0642 + 0.214X$ ($\gamma = 0.998$, humans), $Y = 0.0839 + 0.311X$ ($\gamma = 0.997$, rabbits), $Y = 0.0358 + 0.206X$ ($\gamma = 0.995$, mice), respectively.

3.4. Precision and accuracy

The precision and accuracy of the method were assessed in the blood by performing replicate analyses of the spiked samples against calibration standards. The procedure was repeated on the same day and for different days on the same spiked standard series. The within-day and between-day precision and accuracy of the method are presented in Table 1. The data indicate that the precision and accuracy of the method are acceptable.

Sensitivity was evaluated by determining the LLOQ, which is defined as the lowest concentration that can be reliably and reproducibly measured at least five replicates. To determine the LLOQ, pooled blood samples were spiked to contain 50 pg/ml PH and were analyzed on 5 different days. The PH peak had to be distinct from noise peaks and for verification of LLOQ; the peak area in chromatograms for the spiked blood samples containing 50 pg/ml PH was compared with the noise signal. The LLOQ had to have precision of $\leq$12% and a signal/noise ratio $\geq$10.

3.5. Recovery

The extraction recovery was determined for five replicates of the subject blood spiked with low, medium and high concentrations (1, 5 and 50 ng/ml) of PH. The results are summarized in Table 2. The data indicate that the extraction recovery of PH from the blood was concentration-independent in the concentration range evaluated and was acceptable.

3.6. Stability

The stability of the solution kept at 20 °C and frozen (−20 °C) blood samples, as well as frozen blood extracts, was checked. Blood QC samples were: (1) allowed to stand at ambient temperature for at least 24 h before extraction; (2) subjected to three freeze–thaw cycles for at least 5 days. Analysis of these samples consistently afforded values that were nearly identical to those of freshly prepared QC samples, thus confirming the overall stability of PH in blood under frozen storage, assay processing and freeze–thaw conditions.

3.7. Pharmacokinetics of penehyclidine in humans, rabbits and mice

The GC–MS method was successfully used for the pharmacokinetic study of PH following an intramuscular administration (0.1 mg/kg) in humans, rabbits and mice. The pharmacokinetic parameters were evaluated by the compartmental analysis. After the data of the concentration–time of PH were treated by program 3P87, the blood drug concentration–time curves of PH in humans, rabbits and mice were all best fitted to first order absorption two-compartment open model after administrating a single dose PH. The mean blood concentration–time profiles for PH are shown in Fig. 3 and the values of the pharmacokinetic parameters in the subjects are shown in Table 3.

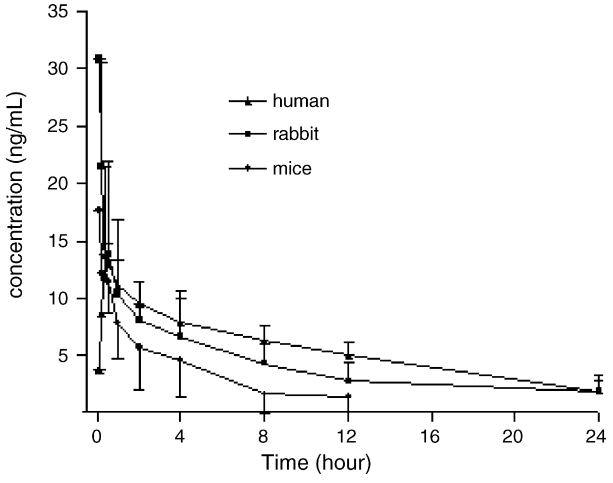

Fig. 3. Pharmacokinetic profile of penehyclidine in the blood after administrating a single intramuscular dose (0.1 mg/kg) to humans, rabbits and mice.

Table 1
Precision and accuracy of the GC–MS analysis of penehyclidine

Matrix	Theoretical concentration (ng/ml)	*n*	Experimental concentration (ng/ml)	Precision (R.S.D.%)	Accuracy percent error (%)
Humans	Within-day				
	1.0	5	0.917 ± 0.079	8.62	8.32
	5.0	5	4.89 ± 0.112	2.29	2.24
	50.0	5	47.5 ± 1.85	3.90	5.02
	Between-day				
	1.0	5	1.08 ± 0.096	8.89	8.11
	5.0	5	5.13 ± 0.250	4.87	2.68
	50.0	5	46.6 ± 1.87	4.01	6.87
Rabbits	Within-day				
	1.0	4	0.891 ± 0.068	7.63	10.9
	5.0	4	4.69 ± 0.256	5.46	6.22
	50.0	4	51.1 ± 2.82	5.52	2.18
	Between-day				
	1.0	4	0.926 ± 0.079	8.53	7.41
	5.0	4	5.35 ± 0.278	5.20	6.96
	50.0	4	45.6 ± 3.11	6.83	8.85
Mice	Within-day				
	1.0	4	0.934 ± 0.082	8.78	6.63
	5.0	4	4.82 ± 0.356	7.39	3.67
	50.0	4	48.1 ± 1.46	3.03	3.76
	Between-day				
	1.0	4	0.893 ± 0.081	9.07	9.02
	5.0	4	4.72 ± 0.422	8.94	5.64
	50.0	4	48.3 ± 1.61	3.32	3.45

Table 2
Recovery of penehyclidine

Matrix	Added (ng/ml)	*n*	Determined (ng/ml)	Recovery ± S.D. (%)	R.S.D. (%)
Humans	1.0	5	0.815 ± 0.069	81.52 ± 4.76	5.84
	5.0	5	4.17 ± 0.242	83.45 ± 3.35	4.01
	50.0	5	44.6 ± 0.972	89.16 ± 1.24	1.39
Rabbits	1.0	4	0.793 ± 0.082	79.33 ± 6.25	7.88
	5.0	4	4.07 ± 0.256	81.31 ± 3.49	4.29
	50.0	4	43.3 ± 1.12	86.52 ± 3.37	3.89
Mice	1.0	4	0.806 ± 0.082	80.58 ± 5.17	6.42
	5.0	4	4.13 ± 0.356	82.65 ± 2.98	3.61
	50.0	4	43.7 ± 1.46	87.24 ± 2.86	3.28

Table 3
The main pharmacokinetic parameters describing disposition of penehyclidine in humans, rabbits and mice after im a single dose (0.1 mg/kg), respectively

Parameter/unit	Humans ($n=8$)	Rabbits ($n=6$)	Mice ($n=5$)
$T_{1/2\alpha}$ (h)	0.41 ± 0.34	0.12 ± 0.097	0.13 ± 0.17
$T_{1/2\beta}$ (h)	10.4 ± 1.22	8.42 ± 2.79	$3.28 \pm 0.58^{\Delta\Delta,\#\#}$
$T_{1/2Ka}$ (h)	0.16 ± 0.065	$0.024 \pm 0.017^{**}$	$0.013 \pm 0.0036^{\Delta\Delta}$
AUC (ng h/ml)	133.2 ± 14.8	$106.5 \pm 26.3^{*}$	$50.4 \pm 6.72^{\Delta\Delta,\#\#}$
$Cl_{(S)}$ (ml/h kg)	6. 29 ± 0.68	$0.00097 \pm 0.00032^{**}$	$0.0021 \pm 0.00032^{\Delta\Delta,\#\#}$
T_{max} (h)	0.56 ± 0.17	$0.068 \pm 0.032^{**}$	$0.061 \pm 0.024^{\Delta\Delta}$
C_{max} (ng/ml)	13.2 ± 2.11	$30.2 \pm 6.09^{**}$	$18.8 \pm 2.29^{\Delta\Delta,\#\#}$

$^{*}p<0.05$ vs. human group; $^{**}p<0.01$ vs. human group; $^{\Delta\Delta}p<0.01$ vs. human group; $^{\#\#}p<0.01$ vs. rabbit group.

3.8. Comparison of the main pharmacokinetic parameters of the species

The comparison of the main pharmacokinetic parameters of PH in humans, rabbits and mice by the *t*-test are as follows:

$T_{1/2Ka}$: There was no statistically significant variation in the absorption between rabbits and mice but there was a statistical difference between humans and rabbits/mice ($p < 0.01$). $T_{1/2\beta}$: There was no statistically significant difference in the elimination between human and rabbit, but there was a statistically significant difference between human/rabbit and mice ($p < 0.01$). T_{max}: There was a statistically significant difference between humans and rabbits/mice ($p < 0.01$), but there was no statistical difference between rabbits and mice. C_{max}: The sequence of the peak concentration of the PH: $C_{rabbit} > C_{mice} > C_{human}$. AUC: $AUC_{human} > AUC_{rabbit} > AUC_{mice}$. The above analysis shows that there were some differences in the absorption and elimination for the PH in humans, rabbits and mice.

The results indicate that PH was absorbed and distributed and eliminated slowly in the species compared to the other anticholinergic drug such as atropine, and that the effects were long-lasting. The pharmacological potency was essentially dependent upon the metabolism and elimination of PH and the affinities to cholinergic receptors [15–17]. The studies provided important information for developing a novel anti-cholinergic drug and for obtaining a more effectual remedy for organic phosphorus pesticide poisoning of and for safe use before general anesthesia.

4. Conclusion

A GC–MS/EIC method has been developed for the determination of PH in the blood from humans, rabbits and mice. The performance criteria for specificity, precision, accuracy, recovery, sensitivity, linearity and stability have been assessed and were within the SFDA recommended guidelines. The results indicated that the method could be used for determination of PH in the blood of humans and animals. The pharmacokinetic profile showed that there were some differences in the disposition of PH among humans, rabbits or mice.

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