

8. Myal, Y., Robinson, D. B., Iwaszow, B., Tsuyuki, D., Wong, P., and Shiu, R. P. (1991) The prolactin-inducible protein (PIP/GCDFP-15) gene: cloning, structure and regulation. *Mol. Cell Endocrinol.* **80**, 165–715.
9. Sidransky, D., Tokino, T., Helzlsouer, K., Zehnbauser, B., Rausch, G., Shelton, B., Prestigiacomo, L., Vogelstein, B., and Davidson, N. (1992) Inherited p53 gene mutations in breast cancer. *Cancer Res.* **52**, 2984–2986.
10. Brennan, J. A., Mao, L., Hruban, R. H., Boyle, J. O., Eby, Y. J., Koch, W. M., Goodman S. N., and Sidransky, D. (1995) Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.* **332**, 429–435.

Pharmacokinetic Study and Determination of Imperialine, the Major Bioactive Component in Antitussive *Fritillaria cirrhosa*, in Rat by High-Performance Liquid Chromatography Coupled with Evaporative Light-Scattering Detector

Shun-Wan Chan,* Song-Lin Li,*† Ge Lin,*¹ and Ping Li†

*Department of Pharmacology, Faculty of Medicine, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, SAR, China; and †Department of Pharmacognosy, China Pharmaceutical University, Nanjing, People's Republic of China

Received April 6, 2000

Imperialine (Fig. 1) is the major biologically active isosteroidal alkaloid present in the most commonly used antitussive traditional Chinese medicinal herb, *Bulbus Fritillaria*. It has been identified from several species in genus *Fritillaria*, including *Fritillaria cirrhosa*, the primary plant source for this herbal medicine (1–5). The antitussive effect of imperialine and the crude *fritillaria* alkaloid extracts of various *Fritillaria* spp. has been extensively studied in both *in vitro* and *in vivo* models, and imperialine has been demonstrated to be the most potent *fritillaria* alkaloids (6). However, to date there are no reports on the pharmacokinetic data of imperialine and other *fritillaria* alkaloids. Imposed by the low sensitivity of the chromophore in *fritillaria* alkaloid, a direct HPLC analysis with ultraviolet or fluorescence detection of this type alkaloid is very difficult (7). Consequently, the reported HPLC–UV assay with precolumn derivatization requires extensive sample preparations, and restricted and well-controlled chemical reactions (8). Therefore, the

present study attempts to develop a direct HPLC analytical method for the analysis of imperialine in blood samples by using evaporative light scattering detector (ELSD),² a universal mass detector that responds to all eluates regardless of their structure and/or chromophore (9, 10). Moreover, the pharmacokinetic profiles of imperialine in rats via intravenous and oral administrative routes were investigated by using the developed HPLC–ELSD analytic method.

Materials and methods. Imperialine was isolated from *F. cirrhosa*. The purity and identity were determined by TLC, HPLC, NMR, and MS analyses. Solanidine was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade solvents were obtained from Labscan Asia Co. (Bangkok, Thailand). HPLC analysis was performed on an HP1100 (Hewlett–Packard) system equipped with an Alltech 500 ELSD (Alltech Associates Inc., Deerfield, IL). A Supelco reversed-phase C₈ analytical column (150 × 4.6 mm, i.d., 3 μ) coupled with a C₈ guard column (20 × 4.0 mm, 5 μ) was utilized with a gradient elution at flow rate of 1 ml/min and column temperature of 28°C. The mobile phase consisted of distilled water (A), acetonitrile (B), and methanol containing 0.6% triethylamine (C) was eluted as follows: 0–6 min A:B:C = 7:35:58; 6–7 min linear increase to A:B:C = 0:42:58 and maintained for 25 min, 25–30 min for returning to the initial conditions. The nitrogen gas flow of 2.22 standard liters per minute and drift tube temperature of 72°C were set for ELSD.

Male Sprague–Dawley rats (180–220 g) supplied by the Laboratory Animal Services Centre at the Chinese University of Hong Kong were fed on a standard laboratory diet with free access to water under the controlled temperature at 20–22°C and relative humidity of 50% with 12-h light/dark cycles prior to the study. Rats were surgically cannulated with polyethylene catheters on the right jugular veins under anesthesia with diethyl ether vapor. The animals recovered in individual metabolic cages and fasted but were allowed to have free access to water overnight. Two groups of conscious cannulated rats with at least five in each group were dosed with the HCl salt of imperialine intravenously (20 mg/kg) and orally (100 mg/kg), respectively. Serial venous blood samples (0.25 ml) were collected from the right jugular vein via the cannulated catheter into heparinized tubes at suitable time intervals up to 360 min after dose. At each blood sampling, an equivalent volume of heparinized normal saline (25% v/v, 0.25 ml) was injected into the animals to maintain a constant blood volume.

¹ To whom correspondence should be addressed. Fax: 852-2603-5139. E-mail: linge@cuhk.edu.hk.

² Abbreviation used: ELSD, evaporative light scattering detector.

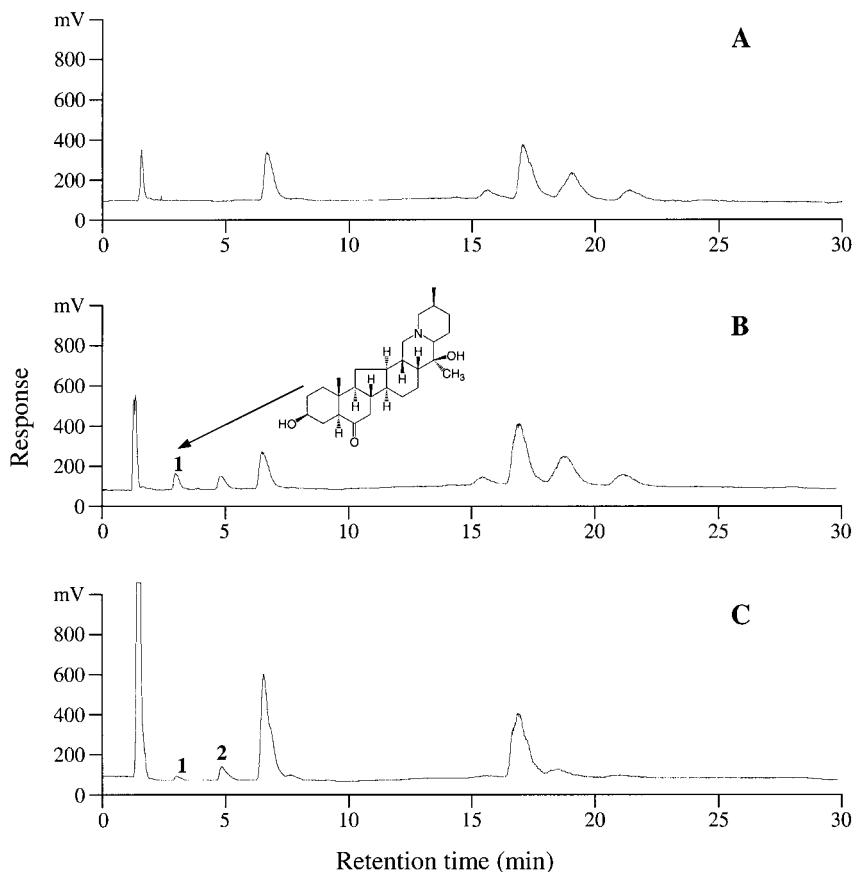


FIG. 1. Representative HPLC chromatograms of (A) blank plasma, (B) blank plasma spiked with imperialine and solanidine (the internal standard), and (C) plasma sample obtained from rat dosed with imperialine (20 mg/kg, iv). Peak 1, imperialine; Peak 2, the internal standard.

The blood samples (0.25 ml) were centrifuged at 9000g to harvest the plasma. To the aliquots of plasma 150 μ l solanidine was added, an internal standard (30 μ l, 0.279 mg/ml), alkalized by ammonium hydroxide (20 μ l), and then shaken with diethyl ether (2×1 ml) for 30 min followed by centrifugation at 1780g for 10 min. The supernatants were combined and dried by a vacuum concentrator. The residues were reconstituted into methanol (200 μ l) and filtrated through a syringe filter (0.45 μ m). Aliquots (50 μ l) of the extracts were subjected to HPLC–ELSD analysis. Blank blood samples spiked with imperialine and solanidine with an appropriate range of concentrations (5–50 μ g/ml) were prepared for the construction of calibration curve. The samples were then extracted and analyzed identically as for the test blood samples. Calibration curve was constructed by plotting the blood concentration of imperialine as a function of peak area ratio of imperialine to the internal standard.

Pharmacokinetic parameters, including area under the plasma concentration–time profile from time zero to time infinity ($AUC_{0-\infty}$), apparent plasma clearance (CL), apparent volume of distribution (V_d),

distribution half-life ($t_{1/2\alpha}$), plasma elimination half-life ($t_{1/2\beta}$), and maximum plasma concentration and time (C_{max} and T_{max}), were calculated by a PK Solutions 2.0 software (Summit Research Services, Ashland, U.S.A.) with a model-independent approach. Bioavailability (F) was calculated as the ratio of the dose-normalized $AUC_{0-\infty}$ after oral administration (100 mg/kg) to that after bolus iv injection (20 mg/kg) of imperialine.

Results and discussion. The representative HPLC chromatograms of the extracts of plasma samples are shown in Fig. 1. For both administrative routes only the intact imperialine was found in the plasma of the dosed rats. Under the developed condition, both imperialine and the internal standard were separated well from the endogenous compounds present in plasma. The calibration curve derived for imperialine was nonlinear and followed a binomial regression ($y = ax^2 + bx + c$, $r^2 = 0.993$) (Fig. 2), which is a commonly reported feature of ELSD (11, 12). It is well recognized that imperialine like other *fritillaria* alkaloids cannot be detected by conventional ultra-

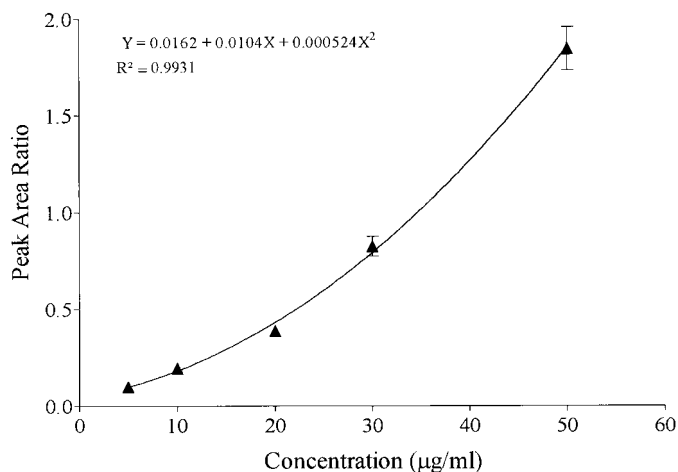


FIG. 2. Calibration curve for imperialine. Each concentration was analyzed in triplicate.

violet and fluorescence detectors since it is nonchromophoric (7, 8). The present on-line HPLC–ELSD analysis was, for the first time, able to determine imperialine in biological samples owing to this unique detector responsible to any elute that does not evaporate before passing through the laser beam (13, 14). As summarized in Table 1, the analytical method developed was fully validated and demonstrated that the quantification of imperialine in blood samples provided an excellent reproducibility with overall intraday and interday variations of less than 10.5%, and a good overall accuracy of greater than 86%, respectively. In addition, an acceptable detection limit of 50 ng/ml in blood sample was obtained.

Imperialine found in plasma was quantified by the developed HPLC–ELSD method. The plasma concentration–time profiles of imperialine with two different administrations are shown in Fig. 3. The pharmacokinetic parameters summarized in Table 2 suggested a linear one-compartment model for the

TABLE 1

Intra- and Interday Variability for the HPLC–ELSD Assay on Plasma Imperialine Concentration

	Imperialine added (µg/ml)	Imperialine detected (µg/ml, n = 3)	RSD ^a (%)	Accuracy ^b (%)
Intraday	8	9.06 ± 0.53	5.85	86.7
	25	25.92 ± 0.93	3.59	96.3
Interday	5	5.39 ± 0.57	10.50	92.2
	30	31.17 ± 2.25	7.22	96.1

^a RDS (%) (relative standard deviation) = (SD/mean) × 100%.

^b Accuracy (%) = [(1 – (mean concentration measured – concentration spiked)/concentration spiked)] × 100%.

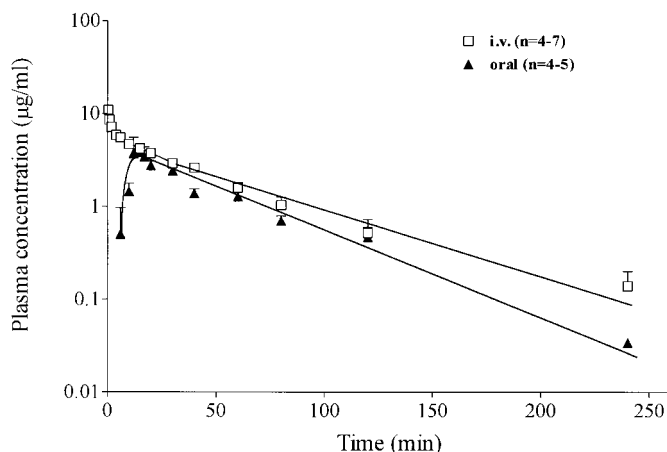


FIG. 3. Pharmacokinetic profile of imperialine in rats after a single intravenous (20 mg/kg) and oral (100 mg/kg) administration of imperialine.

oral administration, a two-compartment model for the bolus iv injection, and the first-order kinetics for all processes. After the iv administration, the concentration of imperialine was about 11 µg/ml at the first sampling point (0.5 min), and the blood level declined biexponentially with very rapid distribution ($t_{1/2\alpha}$ of 11 min) and quick elimination ($t_{1/2\beta}$ 39 min). In the case of oral route, the results demonstrated that imperialine was absorbed rapidly from the gastrointestinal tract. The blood concentration reached its maximum (C_{\max} 5.0 µg/ml) at T_{\max} of 15 min, and then quickly decreased monoexponentially with an elimination half-life of 33 min. Furthermore, a large volume of distribution (V_d) was observed in both routes (3.7 liter/kg for iv and 3.4 liter/kg for oral), indicating that imperialine, a lipophilic alkaloid, was distributed extensively into the extravascular tissues in rats. The compatible mean plasma clearance (68 ml/min/kg for iv and 70 ml/min/kg for oral) and V_d values obtained for the two different routes ex-

TABLE 2

Pharmacokinetic Parameters Following a Single Intravenous (20 mg/kg) and Oral (100 mg/kg) Administration of Imperialine to Rats

Parameters	iv (n = 4)	Oral (n = 5)
$T_{1/2\alpha}$ (min)	10.7 ± 0.2	—
$T_{1/2\beta}$ (min)	38.7 ± 7.3	32.6 ± 3.2
AUC _{0-∞} (µg/ml · min)	308 ± 78	189 ± 68
V_d (L/kg)	3.70 ± 0.64	3.37 ± 1.44 ^a
CL (ml/min/kg)	67.7 ± 14.8	70.2 ± 20.2 ^a
C_{\max} (µg/ml)	—	5.00 ± 1.58
T_{\max} (min)	—	14.8 ± 1.79

^a Normalized for bioavailability. Values are expressed as means ± SD.

hibited a linear kinetic process in both dosage regimens. Consequently, the oral bioavailability of 12% was determined by comparison of the dose-normalized $AUC_{0-\infty}$ ($189 \mu\text{g/ml} \cdot \text{min}$, 100 mg/kg) of oral dose to the $AUC_{0-\infty}$ ($308 \mu\text{g/ml} \cdot \text{min}$, 20 mg/kg) of iv injection of imperialine. This very low bioavailability suggested an extensive first-pass metabolism in oral administration of imperialine in rats.

In summary, the developed novel HPLC–ELSD analytical method allowed us to successfully determine imperialine in the blood samples obtained from the dosed rats. This assay is simple and accurate and provides good reproducibility. The results of pharmacokinetic study demonstrated that imperialine is very rapidly absorbed orally and has a quick onset (15 min) and a short duration ($t_{1/2}$ of $\sim 30 \text{ min}$) of action in rats. However, the low oral bioavailability may indicate a relative low therapeutic efficacy in oral ingestion of imperialine-containing herbal remedies. Further investigations into the correlation between antitussive activity and pharmacokinetic fate of imperialine and other active *fritillaria* alkaloids are currently under progress in our research team.

Acknowledgment. This investigation was supported by the Research Grant Council (RGC) of Hong Kong for the Competitive Earmarked Grant (CUHK 4240/97M).

REFERENCES

1. Ministry of Public Health of the People's Republic of China (1995) Pharmacopoeia of the People's Republic of China, Beijing.
2. Li, S. L., Li P., Lin, G. Chan, S. W., and Ho, Y. P. (2000) *J. Chromatogr. A* **873**, 221–228.
3. Xu, D. M., and Xu, Y. J. (1991) *Chin. Traditional Herbal Drugs* **8**, 132–139.
4. Huang, K. C. (1999) *The Pharmacology of Chinese Herbs*, CRC Press, Boca Raton, FL.
5. Mir, G. N., and Ray Ghatak, B. J. (1965) *Indian J. Exp. Biol.* **3**, 249–252.
6. Chan, S. W., Kwan, Y. W., Lin, G., Ho, Y. P., and Li, P. (1998) *PharmSci* **1**, S-369.
7. Li, S. L., Chan, S. W., Li, P., Lin, G., Zhou, G. H., Ren, Y. J., and Chiu, F. C. K. (1999) *J. Chromatogr. A* **859**, 183–192.
8. Ding, K., Lin, G., Ho, Y. P., Cheng, T. Y., and Li, P. (1996) *J. Pharm. Sci.* **85**, 1174–1179.
9. Gunnarsson, T., Karlsson, A., Hansson, P., Johnson, G., Alling, C., and Odham, G. (1998) *J. Chromatogr. B* **705**, 243–249.
10. Niemi, R., Taipale, H., Ahlmark, M., Vepsäläinen, J., and Järvinen, T. (1997) *J. Chromatogr. B* **701**, 97–102.
11. Christie, W. W. (1985) *J. Lipid Res.* **26**, 507–512.
12. Christie, W. W. (1986) *J. Chromatogr.* **361**, 396–399.
13. Christie, W. W. (1995) in *Advances in Lipid Methodology* (Christie, W. W., Ed.), Chap. 7, Vol. 1, pp. 239–271, Oily Press, Ayr.
14. Hopia, A. I., and Ollilainen, J. (1993) *J. Liq. Chromatogr.* **16**, 2469–2469.

A Double-Injection DNA Electroporation Protocol to Enhance *in Vivo* Gene Delivery in Skeletal Muscle

Frank Hoover¹ and John Magne Kalhovde

Department of Physiology, Institute of Basic Medical Sciences, University of Oslo, Norway

Received June 2, 2000

Key Words: soleus; extensor digitorum longus; regeneration; gene transfer.

Skeletal muscle represents an easily accessible tissue that is amenable to transfection *in vivo* (1). Among the nonviral transfection methods, intramuscular injection of plasmid or “naked” DNA into muscle has received much attention since the first report by Wolff and colleagues (2). One advantage of this technique is that it provides stable epi-chromosomal expression over long periods of time (3, 4). Therapeutic application of this strategy already has shown promise in the preparation of DNA vaccines for use in livestock and humans (5, 6). However, despite optimization and modification, this method is hindered by a low level of transfection efficiency (about 1–2% of muscle fibers).

Recently, direct intramuscular DNA injection in combination with electrical stimulation (*in vivo* electroporation) has resulted in increased numbers of transfected fibers and reporter gene activity (7–9). This method also directs long-term and stable transgene expression. One consequence of *in vivo* muscle electroporation is that regions of muscle become damaged and necrotic. This condition is temporary, since skeletal muscle has the ability to regenerate (10). Interestingly, intramuscular injection of plasmid DNA into myotoxin-induced regenerating rat skeletal muscle produces significantly higher levels of reporter gene expression compared to normal muscle (11). Here we report that a second dose of intramuscularly injected DNA into electroporated muscle will result in further increases in the number of muscle fibers transfected *in vivo*. This technique will provide a useful protocol to enhance transfection efficiency for gene expression studies and promoter analysis *in vivo*.

¹ To whom correspondence should be addressed at University of Bergen, Center for Research in Virology, Bergen High Technology Center, P.O. Box 7800, 5020 Bergen, Norway. Fax: (47) 55 58 45 12. E-mail: frank.hoover@vir.uib.no.