Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1083–1087
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous determination of Gastrodin and Ligustrazine hydrochloride in dog plasma by gradient high-performance liquid chromatography

L.L. Li, Z.R. Zhang, T. Gong*, L.L. He, L. Deng

Renmin Nan Road, West China School of Pharmacy, Sichuan University, 610041, PR China

Received 15 November 2005; received in revised form 14 February 2006; accepted 14 February 2006
Available online 29 March 2006

Abstract

A novel reversed-phase HPLC method was developed for the simultaneous determination of Gastrodin (Gas) and Ligustrazine hydrochloride (LZH) in dog plasma after oral administration of the preparation 'Tianxiong Capsule'. The assay involves deproteinization, extraction and subsequent detection with a gradient solvent system at two different wavelengths. Retention times were 10.6 and 18.9 min for Gas and LZH, respectively. Linear responses were observed over a wide range (0.40–200.0 μg/ml for Gas and 0.0999–39.96 μg/ml for LZH) in plasma. The mean intra- and inter-assay variation coefficients were 2.7 and 3.4% for Gas and 3.4 and 4.2% for LZH, respectively. The average extract recoveries were 76.77% for Gas and 75.8% for LZH. This assay has been successfully used to provide pharmacokinetic data for Gastrodin with oral administration of Tianxiong capsules.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Gastrodin; Ligustrazine hydrochloride

1. Introduction

Traditional Chinese medicine (TCM) is the natural therapeutic agent used under the guidance of the theory of traditional Chinese medical sciences and has been used to treat human diseases in China for centuries. People are becoming increasingly interested in traditional Chinese medicines because of their low toxicity and good therapeutic performance.

The preparation known as 'Dachuanxiong Pill' is one of the ancient TCMs that can be dated back to the Chinese Jin Dynasty. It has been widely used in both China and Japan to treat migraine, and has been proved to be effective and of low toxicity through thousands of years' use [1–3]. Dachuanxiong pills are composed of Tianma (*Gastrodia elata* Bl.) and Chuanxiong (*Ligusticum chuanxiong* Hort.) in certain proportions, and the two components were both recorded in Chinese Pharmacopoeia (2005 Edition). Gastrodin (Gas) and Ligustrazine (structures shown in Fig. 1A and B) are the major constituents and bioactive components in *G. elata* Bl. and *L. chuanxiong* Hort., respectively [4,5]. Gastrodin has been reported to have sedative and anticonvulsant actions, a neuroprotective effect facilitating memory consolidation and retrieval, and antioxidant and free radical scavenging activities [6–10]. While Ligustrazine has been reported to be able to inhibit platelet aggregation in vitro and lower blood pressure by vasodilation, and this has been used to treat a variety of cardiovascular disorders, e.g., myocardial and cerebral infarction [11–14].

Under the guidance of the 'Dachuanxiong Pill' prescription and TCM theory, a new preparation named 'Tianxiong Capsule', which is formed from Gastrodin and Ligustrazine hydrochloride (which has the same pharmacologic and pharmacodynamic properties as Ligustrazine but nicer physicochemical properties, structure shown in Fig. 1C) in certain proportions with other excipients, was prepared in our lab.

It is important to quantify Gastrodin and Ligustrazine hydrochloride (LZH) in dog plasma and investigate their pharmacokinetics for evaluating the clinical applications of Tianxiong capsules. There are some reported quantitative methods and pharmacokinetic studies on Gas or LZH in biological samples [15–18]. But simultaneous determination of Gastrodin and Ligustrazine hydrochloride in dog plasma and their pharmacokinetic study was not previously reported. The present study describes a sensitive and rapid RP-HPLC method to simultaneously determine Gastrodin and Ligustrazine hydrochloride in

* Corresponding author. Tel.: +86 28 85501615; fax: +86 28 85501615.
E-mail address: lily913@sohu.com (T. Gong).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.02.023

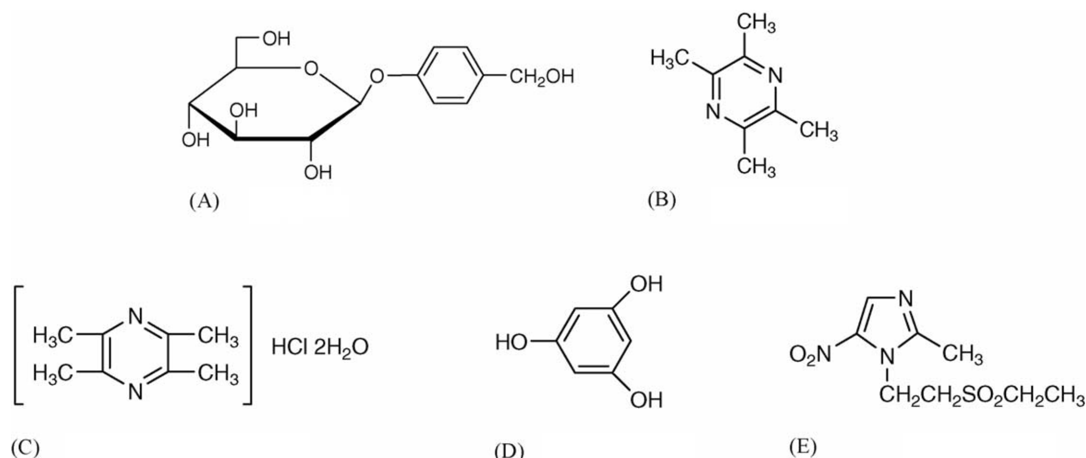

Fig. 1. Chemical structure of analysts. (A) Gastrodin; (B) Ligustrazine; (C) Ligustrazine hydrochloride; (D) Phloroglucinol (internal standard); (E) Tinidazole (internal standard).

dog plasma after oral administration of Tianxiong capsules, so as to take a limited view of their pharmacokinetic profiles.

2. Experimental

2.1. Chemicals and reagents

Gastrodin (Gas) was purchased from Chengyi Pharmaceutical Co. Ltd. (Zhejiang, China). Ligustrazine hydrochloride (LZH) was purchased from Weifang Fine Chemical Co. Ltd. (Shanghai, China). Tianxiong capsule was prepared in our laboratory. Internal standard Phloroglucinol (Fig. 1D) was obtained from Taishan Chemical Factory (Guangdong, China), while another internal standard, Tinidazole (Fig. 1E) was kindly supplied by Kelun Pharmaceutical Co. Ltd. (Sichuan, China). Methanol and acetonitrile (HPLC grade) were purchased from Sanli Chemical Factory (Zhejiang, China). Water was prepared in a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents used were of analytical grade.

2.2. Preparation of standard solutions

Concentrated stock solutions of Gas and LZH were prepared separately at concentrations of 500.0 and 199.8 μg/ml in methanol and was further diluted into 1.0–500.0 and 0.4995–199.8 μg/ml for the preparation of plasma calibration standards. All solutions were stored at −20 °C in dark glass. Pure standards prepared for the recovery calculations were diluted with methanol to appropriate concentrations.

2.3. Preparation of calibration standards

Plasma calibration curves were constructed with 10 different plasma standards covering the expected concentration ranges (0.40–200.0 μg/ml for Gas and 0.0999–39.96 μg/ml for LZH).

Different standard solutions (Gas 200 μl each and LZH 100 μl each) were transferred into test tubes, which contained 500 μl of drug-free dog plasma, 20 μl of Phloroglucinol solution, 50 μl of Tinidazole solution, and 300 μl of 0.01 mol/l NaOH methanol solution. The mixture was deproteinized with 1 ml methanol, added with 2 ml dichloromethane, vortexed for 5 min for fully deproteinization and extraction, and then centrifuged for 10 min at 4000 r/min. Both layers of organic phase were transferred into another test tube and added with 400 μl of 0.05 mol/l HCl methanol solution. After being vortexed for 1 min, the organic phase was removed under a nitrogen stream in a water bath at 50 °C. Then the residue was reconstituted in 0.2 ml of water, vortexed for 3 min and centrifuged for 10 min at 12,000 r/min. A 20-μl volume of supernatant fluid was subjected to HPLC analysis. Calibration curves were constructed using peak area ratios of analyte to internal standard.

2.4. Apparatus and chromatographic conditions

The analysis was performed using Shimadzu instruments (Chiyoda-Ku, Kyoto, Japan) consisting of a 20-μl injector loop, a CTO-10A column thermostat, two LC-10AT pumps and an SPD-10A UV detector. The system was controlled through an SCL-10A system controller and a personal computer.

Separations were carried out using a Shimadzu Shim-Pack C reverse phase column (200 mm × 6 mm i.d.) with 5-μm pore size column (Chiyoda-Ku, Kyoto, Japan) protected by a Shimadzu Shim-Pack G guard column (4 mm × 1 mm i.d.) (Chiyoda-Ku, Kyoto, Japan).

A gradient solvent system of acetonitrile in water was used as the mobile phase. The content of acetonitrile in the mobile phase was 2.5% (v/v) for 11 min followed by a linear increase to 50% in 4 min which was maintained for another 6 min. The column effluent was detected at 221 nm at first for 14 min, and then at 14.01 min changed to 295 nm and maintained for another 6 min.

The mobile phase was filtered, degassed by sonication and pumped through the system at a flow rate at 1.0 ml/min at 35 °C. The normal operating pressure was 90–120 MPa and the analytical time was ∼20 min.

2.5. Animal experiment

Three male and three female beagle dogs (weighing 10–12 kg) were fasted for 15 h with a free supply water before drug administration. The formulations were swallowed with 200 ml water. The dogs were provided a standardized meal 6 h after drug intake. Blood samples were collected into heparinised polystyrene tubes at time 0, 0.167, 0.333, 0.5, 0.667, 0.833, 1.5, 4.5, 9, 14, 24 and 28 h. Plasma samples were obtained from the supernatant of centrifuged blood and they were immediately frozen and kept at −20 °C until assay.

3. Results and discussion

3.1. Sample preparation procedure

Good chromatographic results can be obtained for Gastrodin with the extracts of samples being deproteinized merely by either methanol, ethanol or acetonitrile, but for Ligustrazine hydrochloride the extraction recovery was low all the time by these means, which is probably because LZH was absorbed by protein and then co-precipitated. Thus, in the preparation procedure, the sample was added with both methanol for deproteinization and dichloromethane to extract LZH. Ligustrazine hydrochloride was converted to Ligustrazine (tetramethylpyrazine) by over amountive NaOH for more efficient extraction, and then was converted back to Ligustrazine hydrochloride by HCl after being separated with the precipitates. Fig. 2 shows the effect of the volume of HCl on the extraction recovery of both Gas and LZH. The profiles demonstrated that stable recoveries (>70%) were obtained with 400 μl or more HCl solution for both Gas and LZH of either low, medium or high concentration.

Both the two layers of organic phase were transferred and removed altogether. Reconstitution of the residue either in methanol, ethanol or acetonitrile led to low reproducibility. Since both Gas and LZH are highly water soluble, good chromatographic results were obtained when the residue was reconstituted in water.

In addition, because the sample has been deproteinized during the preparation procedure, no striking change was observed even after more than 100 plasma samples (500 μl each) had been analyzed by the pre-column.

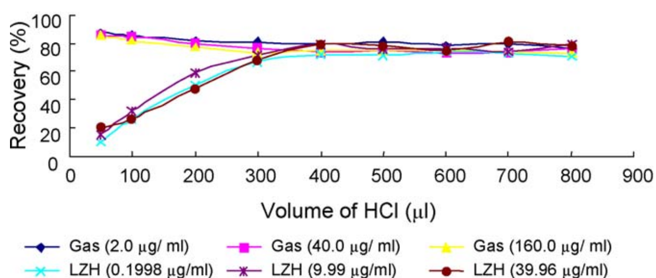

Fig. 2. Effect of the volume of HCl on the extraction recovery of Gas and LZH.

Table 1
Extract recoveries of Gas and Ligustrazine hydrochloride from plasma

Compound	Concentration (μg/ml)	% Recovery (mean ± S.D., $n = 3$)
Gas	2.0	82.1 ± 4.2
	40.0	74.5 ± 2.8
	160.0	73.7 ± 3.6
LZH	0.1998	71.8 ± 5.1
	9.99	80.2 ± 2.3
	39.96	75.4 ± 4.3

3.2. Chromatography

Although a complete separation of Gas, LZH and their internal standards can also be achieved by an analytical mobile phase of methanol–water, the peak shape of Gas was not very good at high concentration, and moreover, the analytical time was a little too long for ∼35 min. Acetonitrile–water system as the analytical mobile phase improved the peak shape quite well, and shortened the analytical time to ∼20 min.

Since Gas and Ligustrazine hydrochloride have their maximum UV absorptions (λ_{max}) at different wavelengths, in order to obtain the highest sensitivity to analyze each analyte, two different wavelengths at 221 and 295 nm, respectively were chosen.

Thus, both Gas and LZH and their own internal standards were eluted with baseline separation under the optimized chromatographic condition. Typical chromatograms obtained from blank plasma, plasma sample and drug-spiked plasma are shown in Fig. 3. Under the chromatographic conditions described previously, Gas and LZH have retention times of 10.6 and 18.9 min, respectively, with complete baseline resolution between peaks of interest. The selectivity of the assay was investigated by injecting the blank plasma. No chromatographic interference derived from endogenous substance or system peaks was observed.

3.3. Recovery

Extraction recoveries of the components were assessed at three concentration levels by calculating the peak area of the analytes in extracts and comparing them with that in standard solutions. Concentration levels of 2.0, 40.0 and 160.0 μg/ml for Gas and 0.1998, 9.99 and 39.96 μg/ml for LZH were applied to the analysis. All analyses were performed in triplicate. Table 1 shows the mean recoveries of Gas and LZH at the concentrations tested. The mean recoveries were 72.8% and 76.1% for Phloroglucinol and Tinidazole, respectively.

3.4. Linearity, detection limit and LOQ

Calibration curves for Gas and LZH were generated by linear regression of peak area ratios against their respective concentrations. The calibration curves showed good linearity between peak-area ratios against concentrations over the calibration ranges in plasma (Gas, $y = 0.0209x + 0.0499$, $r^2 = 0.996$; LZH, $y = 0.033x + 0.0304$, $r^2 = 0.997$). The detection limit for

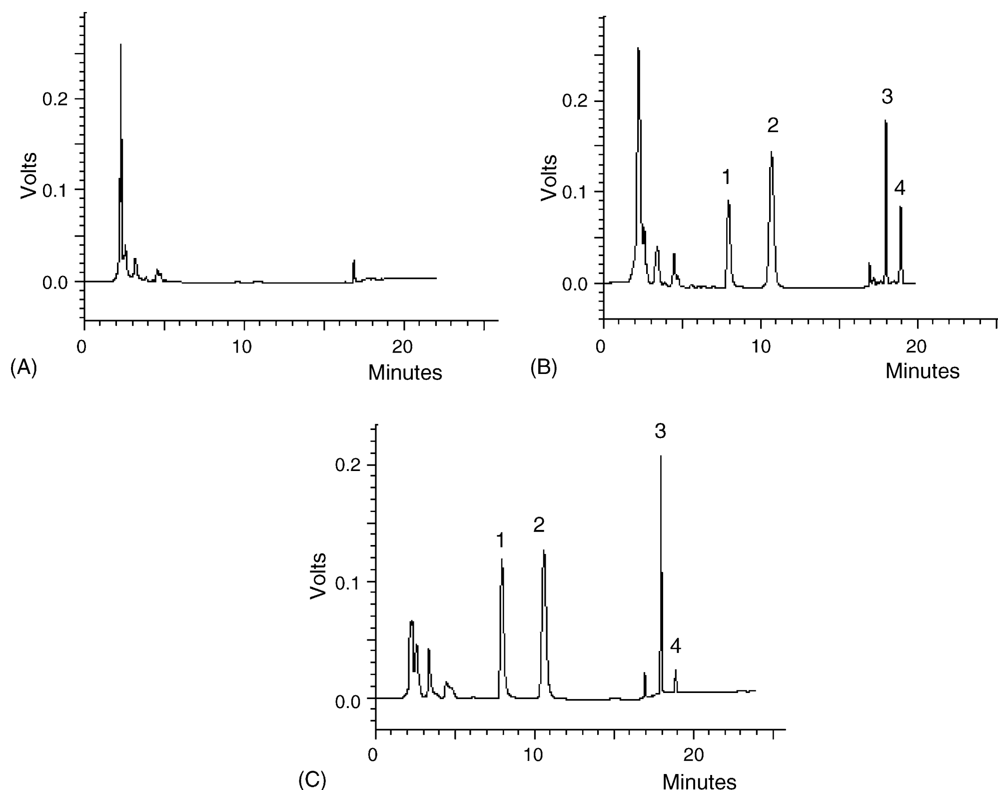

Fig. 3. Representative chromatograms of: (A) blank plasma; (B) plasma spiked with Gas, LZH and their internal standards; (C) dog plasma sample at 4.5 h after oral administration of Tianxiong capsules. Chromatographic peaks: (1) Phloroglucinol (internal standard), (2) Gas, and (3) Tinidazole (internal standard) and LZH.

the methods, defined as a signal-to-noise ratio of 3:1, was 0.08 μg/ml for Gas and 0.03 μg/ml for LZH. The limit of quantification (LOQ) was defined as the lowest drug concentration, which can be determined with a within-day relative standard deviation R.S.D. ≤ 20%. The LOQ was estimated as 0.15 and 0.05 μg/ml for Gas and LZH, respectively.

3.5. Method precision and standard stability

The reproducibility of the assay was determined by analyzing the plasma standards prepared at 2.0, 40.0 and 160.0 μg/ml for Gas and 0.1998, 9.99 and 39.96 μg/ml for LZH. The results of intra-assay and inter-assay precision are expressed as the coefficient of variation (C.V.). The assay was reproducible with an average intra-day coefficient of variation less than 5% and an average inter-day coefficient of variation less than 10% (Table 2).

The stability of Gas and LZH were investigated after storing spiked plasma samples (40.0 and 9.99 μg/ml for Gas and LZH, respectively) at −20 °C for 1 month. During the storing period, three freeze-thaw cycles were carried out. The mean concentration following this storage period was 99.5% (R.S.D. = 2.3%, n = 3) and 99.3% (R.S.D. = 3.6%, n = 3) of the normal value for Gas and LZH, respectively, indicating that plasma samples containing Gas and LZH were stable at −20 °C for at least 1 month.

3.6. Assay application

The mean plasma profile of Tianxiong capsule after oral uptake by dogs is shown in Fig. 4. The pharmacokinetic parameters were estimated by the DAS ver1.0 (Drug and Statistics for Windows) program. A two-compartment model was applied to

Table 2
Intra- and inter-assay precision during assay for Gas and LZH

Compound	Intra-assay (n = 3) concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Inter-assay (n = 3) concentration (mean ± S.D.) (μg/ml)	C.V. (%)
Gas	1.95 ± 0.08	4.1	2.06 ± 0.11	5.4
	39.3 ± 1.14	2.9	39.1 ± 1.41	3.6
	161.4 ± 1.94	1.2	161.1 ± 1.93	1.2
LZH	0.194 ± 0.007	3.6	0.224 ± 0.006	2.7
	10.3 ± 0.43	4.2	9.88 ± 0.80	8.1
	37.9 ± 0.87	2.3	39.6 ± 0.75	1.9

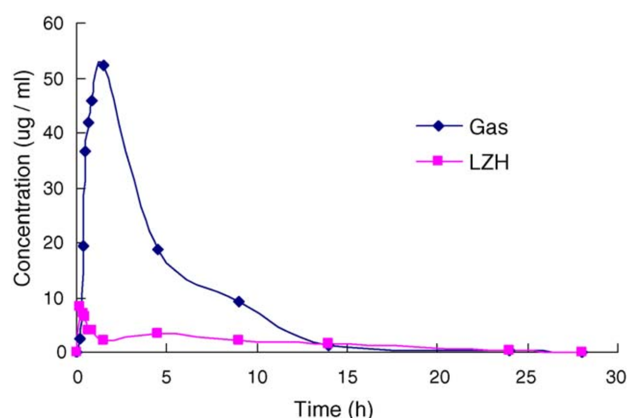

Fig. 4. Plasma concentration–time profiles of Gas and LZH after oral administration of Tianxiong capsules.

Table 3
Mean pharmacokinetic parameters of Gas and LZH in plasma after oral administration of Tianxiong capsules ($n=6$)

Parameters	Gas	LZH
α (h^{-1})	0.28	0.15
β (h^{-1})	0.11	0.14
$t1/2\alpha$ (h)	2.49	1.60
$t1/2\beta$ (h)	6.43	4.69
k_{10} (h^{-1})	0.26	0.15
k_{12} (h^{-1})	0.01	0.01
k_{21} (h^{-1})	0.11	0.14
$AUC_{0–24}$ (mg/l h)	674.9	198.6
$AUC_{0–\infty}$ (mg/l h)	682.1	201.2
T_{max} (h)	1.50	0.17
C_{max} (mg/l)	51.0	8.86

estimate the parameters of Gastrodin and a one-compartment model for Ligustrazine hydrochloride. Statistical moment was applied to calculate area under curve (AUC). The main pharmacokinetic parameters are listed in Table 3.

4. Conclusion

The analytical method described above is a useful, simple HPLC assay for simultaneous quantification of Gastrodin and Ligustrazine hydrochloride in dog plasma after oral administration of Tianxiong capsules, and is useful for its pharmacokinetic studies. The method shows good overall recovery, accuracy, precision, and low detection limits of two compounds. Gas and LZH were assayed with appropriate retention times under the same chromatographic conditions, although there is a great difference in lipophilicity between the two compounds, indicating that it is valid to apply the gradient solvent system described above. The method was validated to meet the requirements of the pharmacokinetic investigation of the two compounds. The pharmacokinetic results are useful to provide a basis for evaluating the clinical efficacy of Tianxiong capsules.

References

[1] Z.L. Huang, J. Mod. Dev. Tradit. Media 5 (1985) 251–257.
[2] J. Ju, J. Du, J. Tradit. Chin. Med. 15 (1995) 183–184.
[3] K.J. Chen, K. Chen, Chin. Med. J. (Engl.) 105 (1992) 870–873.
[4] H.C. Feng, Acta Chem. Sin. 37 (1979) 175–179.
[5] M.C. Sutter, Y.X. Wang, Cardiovasc. Res. 27 (1993) 1891–1901.
[6] H.J. Kim, S.R. Lee, K.D. Moon, Phytother. Res. 17 (2003) 909–912.
[7] Y.H. Cao, X. Zhang, Y.Z. Fang, J.N. Ye, Analyst 126 (2001) 1524–1528.
[8] H.J. Kim, K.D. Moon, S.Y. Oh, S.P. Kim, S.R. Lee, Neurosci. Lett. 314 (2001) 65–68.
[9] M.T. Hsieh, C.R. Wu, C.F. Chen, J. Ethnopharmacol. 56 (1997) 45–54.
[10] J. Liu, A. Mori, Neuropharmacology 31 (1992) 1287–1298.
[11] X.G. Mao, J. Contin. Educ. Phys. 8 (1989) 31–36.
[12] J.L. Zhang, Z.X. Shi, Y.T. Shi, Chung-kuo Chung His I Chieh Ho Tsa Chih 14 (1994) 478–481.
[13] L.N. Lin, W.T. Wang, Z.J. Xu, Chungkuo Chung His I Chieh Ho Tsa Chih 17 (1997) 261–266.
[14] X.Z. Dai, R.J. Bache, J. Cardiovasc. Pharmacol. 7 (1985) 841–849.
[15] H.B. Li, F. Chen, J. Chromatogr. A 1052 (2004) 229–232.
[16] Y.K. Zhao, Q.E. Cao, Y.Q. Xiang, Z.D. Hu, J. Chromatogr. A 849 (1999) 277–292.
[17] H.B. Li, F. Chen, J. Chromatogr. A 1047 (2004) 249–253.
[18] X.G. Chen, et al., J. Chromatogr. A 1040 (2004) 169–178.