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Ion-pair reverse-phase high performance liquid chromatography method for determination of Huperzine-A in beagle dog serum

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Abstract

Huperzine-A (Hup-A), a biologically potent, reversible acetylcholinesterase inhibitor for the treatment of Alzheimer disease (AD) in China, has very low blood concentration. In order to study the pharmacokinetics of newly developed Hup-A transdermal patches in animal, a rapid and sensitive ion-pair reverse-phase high performance liquid chromatography (RP-HPLC) method for the determination of Hup-A in beagle dog serum using mebendazole as internal standard has been developed and validated. The analyte and internal standard were extracted from serum using chloroform–isopropanol (95:5, v/v), analyzed on a C (18) column (5 μm, 150 mm × 4.6 mm i.d.) with a mobile phase consisting of methanol–water–glacial acetic acid (50:48.5:1.5, v/v/v), using sodium dodecylsulfonate as an ion-pair reagent, and detected with UV detector at 313 nm. The chromatographic run time was within 15 min. The assay was linear over the concentration range of 1–12 ng/ml and intra- and inter-day precision over this range was not more than 12.8%. The limit of quantification in serum was 1 ng/ml. The method was successfully applied to characterize the Hup-A concentration–time profiles and study the single and multiple doses phamacokinetics of Hup-A transdermal patches in beagle dogs. The pharmacokinetic study results showed that Hup-A patches has the characteristic of sustained or controlled drug release in vivo.

Keywords: Ion-pair RP-HPLC; Huperzine-A; Dog serum

1. Introduction

Huperzine-A (Hup-A, Fig. 1), a natural alkaloid isolated from Chinese herb *Huperzia serrata*, is a potent, reversible acetylcholinesterase inhibitor, which crosses the blood–brain barrier smoothly, and shows high specificity for acetylcholinesterase. It has been approved as a drug for the treatment of Alzheimer disease (AD) in China, and marketed in USA as a dietary supplement [1]. Results of clinical trials [2,3] showed that Hup-A is efficient and safe in treatment of patients with mild to moderate AD if used properly and also enhance the memory and learning performance of adolescent students. Currently, the marketed medicines of Hup-A are orally administered immediate-release products, including tablets and capsules, and have to be administered for 2–3 times per day. It was reported [4] that rat liver was affected during the acute exposure to Hup-A, especially for overdose of Hup-A. Patients with AD have decreased memory, and this could easily lead to overdosing to get adverse effect or underdosing not to reach therapeutical level when the drug was administered frequently with bad compliance. Recently, our institute developed Hup-A transdermal patches, which is designed to deliver Hup-A continuously and constantly over 3–4-day interval, after application to intact skin, to overcome the disadvantage of orally administered products. In order to characterize the Hup-A concentration–time profiles, study phamacokinetics of Hup-A transdermal patches in beagle dogs and to evaluate its sustained or controlled drug release characteristic in vivo, a simple and rapid method for deter-

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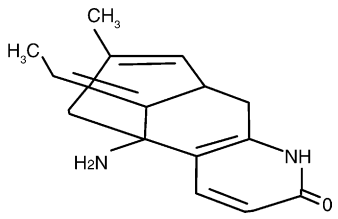

Fig. 1. Chemical structure of Huperzine-A.

mination of Hup-A in blood should be established. Hup-A is a biologically potent molecule (maximum recommended human daily dose: 450 μg). Its blood concentration is very low and difficult to detect by routine HPLC method. For this reason, there is a little information available on the pharmacokinetic of Hup-A. Recently, Liquid chromatographic–tandem mass spectrometric method [5,6] for the quantitation of Hup-A in dog plasma and in rat blood has been established. Even though LC–MS or LC–MS–MS is considered to be the most promising technique in pharmacokinetics studies because of its sensitivity, specificity and versatility, the high cost and the feasibility of such instrument still do not allow the routine use for most of analytical laboratories. In this paper, a rapid and sensitive ion-pair RP-HPLC method we developed for determination of Hup-A in dog serum was described and validated.

2. Experimental

2.1. Chemicals and reagents

Hup-A was provided by Weng Ling Pharmaceutical Co. (Zhejiang, China). Its purity was 99.6%, which was verified by Zhejiang Provincial Institute for Drug Control. Hup-A patches (4 mg/20 cm^2) was developed by Institute of Materia Medica, Zhejiang Academy of Medical Sciences (lot 030208). The internal standard (I.S.), mebendazole, was bought from National Institute for the Control of Pharmaceutical and Biological Product of China. Sodium dodecylsulfonate was purchased from Shanghai Shengzhong Fine Chemical Co. Ltd (Shanghai, China). Borax, sodium carbonate, Chloroform, glacial acetic acid and all other chemicals or solvents were analytical reagent or chromatographic grade and purchased from commercial sources. Blood samples were collected from beagle dogs by puncture of the femoral vein, and then processed for serum by centrifugation at 3000 × *g* for 10 min. Serum samples were frozen and maintained at −20 °C until analysis. Purebred beagle dogs were obtained from Yu Hang animal center (Zhejinang, China).

2.2. Equipment and chromatographic condition

The HPLC equipment consisted of two LC-10A pumps, one SPD-10A UV detector, a manual injection with a 50 μl fixed loop, and a C-R6A data processor (Shimadzu, Japan). The analytical column was a Shimpack CLC-ODS (150 mm × 4.6 mm i.d., 5 μm). The mobile phase was a mixture of methanol–water–glacial acetic acid (50:48.5:1.5, v/v/v), which contains 0.0015 mol/L dodecylsulfonate as ion-pair reagent, with a flow rate of 1.0 ml/min. The wavelength of UV detector was set at 313 nm and the temperature of column oven was set at 35 °C.

2.3. Preparation of stock and standard solution

Hup-A and mebendazole were accurately weighted, transferred to volumetric flasks and dissolved in methanol to make individual stock solutions of 100 μg/ml. The solutions were stored at 4 °C and stable for at least 5 months. The stock solution was diluted with methanol or water to certain concentration before use according to the analytical requirement.

2.4. Extraction and assay procedure

The pH of 2.5 ml serum was adjusted to 9.5 by adding 1 ml of Borax-sodium carbonate buffer (pH 10.8–11.2). Thirty-five microliters of mebendazole (0.5 μg/ml in water) was added as the I.S. Hup-A and I.S. were extracted with 3.0 ml chloroform–isopropanol (95:5, v/v) by vortex-mixing for 2 min. The organic layer was separated by centrifugation at 3000 × *g* for 10 min, transferred to a clean spiky bottom tube and evaporated to dryness under a gentle stream of nitrogen at 50 °C. The extraction procedure was performed twice. The residue was dissolved with 100 μl of mobile phase and then centrifugated at 2000 × *g* for 2 min to precipitate solid impurity of the residue. An aliquot of 50 μl of the resulting solution was injected into the HPLC system.

2.5. Application of the assay

The present assay has been used to quantify the dog serum concentrations of Hup-A in pharmacokinetic studies of newly developed Hup-A transdermal patches. The Hup-A patches 20 cm^2 (containing 4 mg of Hup-A) was given and applied to an area of clean and dry skin on the back of the dogs immediately after removal the protective liner. The site of skin was molted 12 h before application. In the single dose study, the patch was worn 84 h (i.e.3.5 days). Blood samples were collected from each dog by puncture of the femoral vein at 0 (pre-dose), 4, 6, 8, 10, 12, 24, 32, 48, 56, 72, 84(↓), 96 and 104 h after transdermal wearing. The patches were removed at 84 h. 6 ml of blood was processed for serum by centrifugation at 3000 × *g* for 10 min. Serum samples were analyzed as described under extraction and assay procedure.

3. Results

3.1. Assay specificity

The specificity of the assay was evaluated with three independent sources of serum. The results showed in Fig. 2(A)

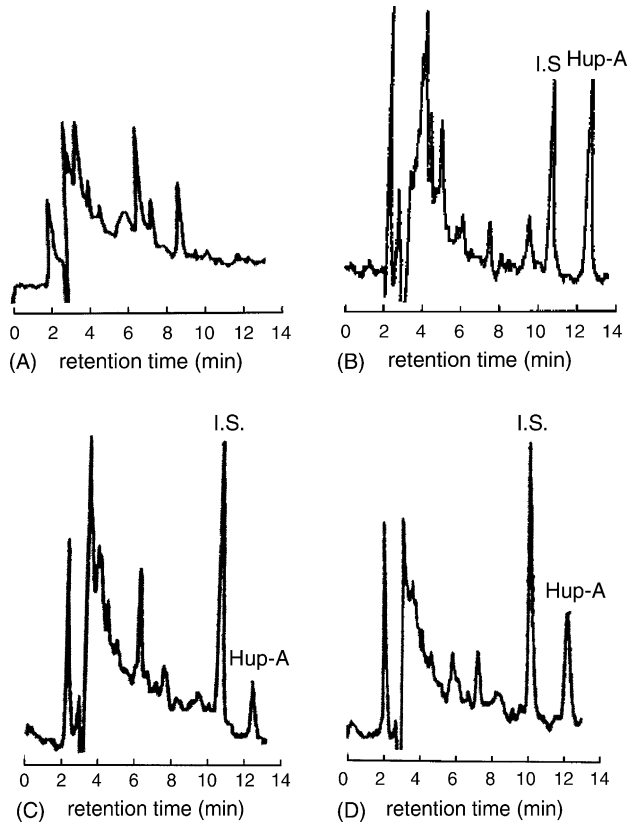

Fig. 2. chromatograms of (A) blank serum; (B) blank serum spiked with Hup-A and I.S.; (C) LOQ ($C_{\text{Hup-A}}$: 1 ng/ml) and (D) dog serum sample.

and (B) indicated that the method was specific for determining Hup-A under the chromatographic conditions employed. The peak locations of I.S. and Hup-A were not interfered by the compounds in serum. I.S. and Hup-A were resolved from each other completely with retention time of approximately 10.8 and 12.8 min, respectively.

3.2. Calibration curves

The calibration curve for the assay was constructed by analyzing a series of blank serum spiked with Hup-A (0.5 μg/ml in water) in the concentration range from 0.5 to 12 ng/ml. The calibration curve of Hup-A was linear over the concentration ranges from 1 to 12 ng/ml by performing a regression linear analysis of the peak-area ratios (R) of Hup-A to I.S. versus the Hup-A concentrations (C). The regression equation was $R = 0.1388C + 0.0247$ and coefficient of correlation (r) was 0.9998. Experimental peak-area ratios were interpolated on the calibration curve and the back-calculated concentrations were made. The mean back-calculated concentrations approach to the spiked concentration with R.S.D. < 15%. In addition, three calibration curves of independent sources of blank serum from three different dogs were analyzed for 3 consecutive days, respectively. The results showed that the calibration curve has good reproducibility.

3.3. Limit of quantification (LOQ)

The limit of detection (LOD) was 0.5 ng/ml in serum for this assay, which was measured based on signal/noise (S/N) ≥ 3. The LOQ of the method was defined as the lowest concentration of Hup-A measured in five replicate with acceptable precision (±20% R.S.D.) and accuracy (±20%). R.S.D stands for the relative standard deviation. LOQ determined was set at 1 ng/ml of Hup-A in serum and at this level the R.S.D. was 13.2%. The chromatogram of LOQ was showed in Fig. 2(c).

3.4. Recovery

A series of blank serum, spiked with various amounts of Hup-A were processed as described under extraction and assay procedure. The method recovery was obtained by comparing the peak-area ratios of Hup-A to I.S. of extracted samples with which calculated from the calibration curve of Hup-A. While extraction recovery were determined by comparing the peak-area ratios of Hup-A to I.S. of extracted samples with the peak-area ratios obtained from direct injections of a standard solution, containing the same concentration of Hup-A and I.S. The average method and extract recovery of assay was 99.6–109.4% and 72.1–76.0%, respectively (Table 1, $n = 5$).

3.5. Precision

The drug-free serum, spiked with Hup-A at different concentrations of 1, 5, 10 ng/ml were used for precision studies. Intra- and inter-day assay variability were determined by analyzing five parallel samples and samples on five consecutive days, respectively. The precision was evaluated by intra-day and inter-day R.S.D. R.S.D was ⩽12.8% for intra-day assay and ⩽10.8% for inter-day assay (Table 1, $n = 5$).

3.6. Stability

The stability of Hup-A in serum was evaluated by comparing fresh dog blank serum spiked with 2 and 6 ng/ml of Hup-A at indicated time. The samples were stored at room

Table 1
Recovery and precision for assay of Hup-A in blank serum ($\bar{x} \pm s$, %, $n = 5$)

Concentration spiked (ng/ml)	Recovery (%)		Average relative standard deviation (%)	
	Method recovery ($X \pm s$, %)	Extract recovery ($X \pm s$, %)	Intra-day	Inter-day
1	109.4 ± 14.4	76.0 ± 10.2	12.8	10.8
5	99.6 ± 3.1	72.1 ± 2.3	3.0	5.9
10	102.8 ± 5.5	74.0 ± 4.0	5.4	5.8

Table 2
Stability of Hup-A in dog serum in different storing condition ($\bar{x} \pm s$, %, $n = 3$)

Concentrations spiked (ng/ml)	Storing condition of serum samples	Mean back-calculated concentrations
2	Room temperature for 0 h	2.2 ± 0.25
2	Room temperature for 24 h	2.1 ± 0.26
2	−20 °C for 32 days	2.2 ± 0.23
6	Room temperature for 0 h	6.2 ± 0.40
6	Room temperature for 24 h	5.9 ± 0.40
6	−20 °C for 32 days	5.6 ± 0.32

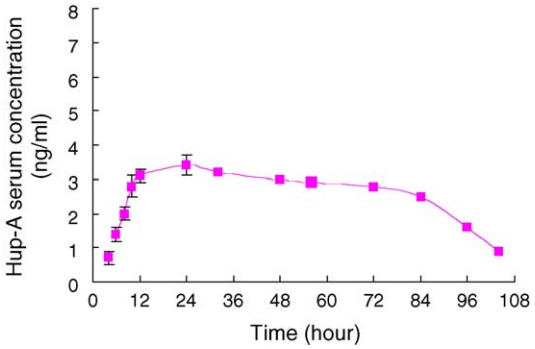

Fig. 3. Profile of mean serum concentration versus time after single dose transdermal administration in six dogs.

temperature (about 25–34 °C) for 0 h, 24 h and at −20 °C for 32 days, then processed and analyzed as described under extraction and assay procedure. The concentrations calculated from calibration curve showed that no degradations were observed (Table 2, $n = 3$).

3.7. Application to pharmacokinetic study

The present assay has been used to quantify the concentration of Hup-A in dog serum in pharmacokinetic study of Hup-A transdermal patches. Fig. 2(D) shows the typical chromatogram of serum sample collected from Hup-A transdermal patches treated beagle dog. The pharmacokinetic study results showed that Hup-A patches has the characteristic of sustained or controlled- drug release in vivo. Following application of the first patch (4 mg/20 cm^2), Hup-A serum concentrations increased for approximately 12–24 h, reaching average maximum concentration of 3.4 ng/ml. Thereafter, blood concentrations were maintained up to 84 h (i.e. 3.5 days) during wearing period. The results indicate that the Hup-A transdermal patches is suitable for twice-weekly application. Fig. 3 shows mean Hup-A serum concentration–time profile after single dose transdermal administration of Hup-A patches (4 mg/20 cm^2) in six beagle dogs.

4. Discussion

A simple, rapid and sensitive analytical method is highly required because of very low Hup-A concentration in blood. We once developed a reverse-phase HPLC method for determining Hup-A in serum but the limit of quantification of the method was 4 ng/ml, which still is not sensitive enough to meet the pharmacokinetics study.

As a naturally weak alkaloid, the serum was adjusted to alkalescence (pH 9.5) for extraction of Hup-A by adding alkaline buffer, which make sure that the Hup-A binding to blood constituents, if any, can be dissociated and increase the extraction recovery.

Ion-pair reagent and its concentration were optimized. To choose the proper reagent, alkyl chain lengths should be taken into consideration. The chain lengths enable selective separation of the analyte. The longer the chain, the more hydrophobic the counterion, and therefore, greater the retention. Sodium dodecylsulfate and sodium dodecylsulfonate was evaluated by determining their effects on retention time, sensitivity in detection and interference under the same chromatographic condition. The result showed that Hup-A and I.S. were difficult to separate from each other and sensitivity in detection of Hup-A was relatively lower when using sodium dodecylsulfate as ion-pair reagent. While sodium dodecylsulfonate is idea in terms of increasing sensitivity in detection for Hup-A, suitable retention time and complete resolution among Hup-A, I.S. and impurities in serum. In addition, the retention time of both Hup-A and I.S. is prolonged as the pH increases.

Hup-A is a moderate fat-soluble compound, based on the consideration to achieve high extract recovery and to avoid to interfering compounds from serum, Some organic solvent were evaluated for their extraction efficiency (Table 3). The extraction recovery of Hup-A and I.S., and assay interference were taken as evaluation criteria. The results showed that extraction recovery of Hup-A and I.S. is very low when using cyclohexane, hexane and chloroform as extraction solvents. After isopropanol was used as the second solvent, the extraction recovery of analytes increased, but if the content of isopropanol surpassed 5%, the organic layer is difficult to evaporate to dryness. 2-Butanol is ideal in term of Hup-A extraction recovery, unfortunately, the peak location of I.S., was interfered with impurities in serum. So, mixture of chloroform–isopropanol (95:5, v/v) was finally used as the extraction solvent.

Table 3
Extraction recovery of Hup-A and I.S. using different organic solvents (pH 9.5, $C_{Hup\text{-}A}$: 4 ng/ml, $C_{I.S.}$: 7 ng/ml, $n = 3$)

Organic solvents	Extraction recovery (%)		Assay interference
	Hup-A	I.S.	
Cyclohexane	49.7	40.5	–
Cyclohexane–isopropanol (95:5, v/v)	70.8	65.4	–
Hexane	50.1	41.9	–
2-Butanol	78.8	Interfered	+
Chloroform	59.5	50.4	–
Chloroform–isopropanol (95:5, v/v)	73.5	70.5	–
Chloroform–isopropanol (90:10, v/v)	76.3	77.9	–

5. Conclusion

Ion-pair RP-HPLC method for the determination of Hup-A in beagle dog serum using mebendazole as internal standard has been developed and validated. The HPLC method described here was accurate, precise, rapid and sensitive, and has been successfully applied to characterize the Hup-A concentration–time profiles and phamacokinetics of newly developed Hup-A transdermal patches in beagle dogs.

Acknowledgements

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References

[1] H. Jiang, X. Luo, D. Bai, Curr. Med. Chem. 10 (2003) 2231.
[2] Z. Zhang, X. Wang, Q. Chen, L. Shu, J. Wang, G. Shan, Zhonghua Yi Xue Za Zhi 82 (2002) 941.
[3] Q.Q. Sun, S.S. Xu, J.L. Pan, H.M. Guo, W.Q. Cao, Zhongguo Yao Li Xue Bao 20 (1999) 601.
[4] X.C. Ma, J. Xin, H.X. Wang, T. Zhang, Z.H. Tu, Acta Pharmacol. Sin. 24 (2003) 247.
[5] Y. Wang, D. Chu, J. Gu, J.P. Fawcett, Y. Wu, W. Liu, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 803 (2004) 375.
[6] C. Li, F. Du, C. Yu, X. Xu, J. Zheng, F. Xu, D. Zhu, Rapid Commun. Mass Spectrom. 18 (2004) 651.