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Pharmacokinetic study with N-Ile[1]-Thr[2]-63-desulfato-r-hirudin in rabbits by means of bioassay*

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Abstract: Aim: To study the pharmacokinetic (PK) properties in rabbits treated with N-Ile[1]-Thr[2]-63-desulfato-r-hirudin (rH) newly developed in China by means of bioassay in order to provide preclinical experiment basis for its development as a novel anticoagulant agent. Methods: rH plasma concentration was determined using bioassay based on ex vivo antithrombin activity of rH. Normal rabbits received iv rH 4.0, 2.0 and 1.0 mg/kg or sc rH 2.0 mg/kg, respectively. The rabbits with acute severe renal failure were given iv rH 2.0 mg/kg. Results: The bioassay described in this paper met requirements for study of PK in rabbits. The major PK parameters after iv dosing were as follows: $t_{1/2\beta}$ 58.4~59 min. V_d 0.09~0.12 L/kg, *CL* 0.0035~0.0040 L/(kg·min); *AUC* were proportional to the doses, $t_{1/2}$ and *CL* did not change significantly with the doses. The sc bioavailability reached 94%. The rabbits suffering from acute severe renal failure presented 11-fold longer $t_{1/2\beta}$ and 13-fold greater *AUC* than normal healthy rabbits. Conclusion: rH exhibited rapid elimination, distribution was only limited to extracellular space and good absorption from sc site. The excretion of rH by kidneys played a very important role in the elimination of rH. The PK of rH could be described by the two- and one-compartment model after iv and sc dosing, respectively, and followed linear kinetics.

Key words: r-hirudin, Pharmacokinetics, Bioassay, Thrombin time, Rabbit
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INTRODUCTION

Hirudin, a naturally occurring polypeptide from salivary glands of medicinal leech (*Hirudo Medicinalis*) and an important effective component of traditional Chinese medicine Shui Zhi, has proven to be a highly selective direct thrombin inhibitor (Markwardt, 1994). Currently, in China recombinant-hirudin has been developed by DNA recombination technique and is undergoing extensive preclinical and clinical evaluation.

Over the past decade, much effort in our lab was devoted to pharmacodynamic (PD) and pharmacokinetic (PK) studies of N-Ile[1]-Thr[2]-63-desulfato-r-hirudin (rH), a novel r-hirudin newly developed in China (Lu *et al*., 2002a; 2002b; Jiang *et al*., 2003; Zhou *et al*., 2005). In a previous paper we described PK profiles of rH in rats by means of ELISA method (Jiang *et al*., 2003). In the present paper we report the PK properties of rH in rabbits investigated by a bioassay called thrombin time (TT) method, so as to provide preclinical experiment data for its development as a novel anticoagulant and antithrombotic drug.

MATERIALS AND METHODS

Drugs and reagents

rH being examined was provided by Dalian Guoxin Biopharmaceutical Co. Ltd., Lot No. 991101 (purity>99.0%, anticoagulant potency>16000 ATU/mg) and identified as N-Ile[1]-Thr[2]-63-desulfato-r-hirudin composed of 65 amino acids with MW 7000.

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It was available as a white, sterile lyophilized powder for dissolution in saline before injection. Thrombin was purchased from Shanghai Biological Institute, Lot No. 200011.

Animal and instruments

Male Big Ear White rabbits weighing (2.0±0.2) kg were supplied by the Animal Center of Dalian Medical University (Liaoning Province Experiment animal ratification No. 22); PlSC 20000-4 Coagulometer was the product of Pulisheng Co. Ltd.

Determination of rH concentration in plasma of test rabbits

rH concentration in rabbit's plasma was determined by a bioassay called the thrombin-time (TT) method developed in our lab (Ren *et al*., 2005), which was based on ex vivo antithrombin activity of rH. Briefly, 50 μl of plasma, 0.1 mol/L pH 7.4 Tris-HCl buffer and thrombin solution (5 U/ml) were each successively pipetted into a cuvette kept at 37 °C in a coagulometer, and then tested as instructed by User's Guide. The time from addition of thrombin to clot formation was recorded as plasma thrombin clotting time, briefly called thrombin-time (TT). The concentration of rH in plasma was read from the calibration curve the relating log*TT* prolongation rate to plasma concentration of rH. The validation of methodology using quality control (QC) samples at high, medium and low concentrations showed that log*TT* prolongation rate was linearly correlated to the concentration of rH in plasma (r=0.999) and ranged from 0.12 to 0.60 μg/ml with limit of quantitation of 0.12 μg/ml. The intra- and inter-day precisions (RSD) were 5.6%~9.1% and 9.2%~11.5%, respectively. The method's recovery was 97.5%~99.2% and dilution recovery was 92.4%~94.8% at 2~100 fold dilution made for high concentration samples. The plasma samples were stable for at least 1 weeks during storage at −20 °C.

PK study of rH in rabbits

Twenty-five rabbits were randomly divided into 5 groups, 5 animals each. Groups 1, 2 and 3 received iv administration of rH 4.0, 2.0 and 1.0 mg/kg, respectively; Group 4 was given sc rH 2.0 mg/kg. Group 5 was subjected to ligation of bilateral renal arteries causing the animals to suffer from acute severe failure of renal functions which then received iv administration of 2.0 mg/kg rH.

Blood samples were taken from ear marginal vein of rabbits at pre-dosing and 5, 10, 20, 30, 40, 60, 90, 120, 150, 180, 240 min post-dosing for Groups 1, 2, 3 and 5; and 15, 30, 60, 90, 120, 150, 180, 240 min post-dosing for Group 4, respectively. The rH concentration in plasma of rabbits was measured as stated above. The PK parameters of rH were calculated by 3P87 Program developed by China Mathematical Pharmacological Society.

Statistical analysis

All data were presented as mean±standard deviation (SD). The statistical analysis was performed using t-test and ANOVA.

RESULTS

PK of iv rH in normal rabbits

Fig.1 shows that after iv administration of 1.0, 2.0 and 4.0 mg/kg rH yields a biphasic plasma concentration-time profile that could be simulated in a two-compartment model, with first order kinetics. The PK parameters are presented in Table 1 showing rapid elimination of rH from blood of rabbits with $t_{1/2\beta}$ being 58.4~59.0 min with a distribution limited to extra-cellular fluid with V_d of 0.090~0.122 L/kg.

sc bioavailability of rH in rabbits

After sc administration, rH pharmaco-kinetically behaved as the one-compartment model of extravascular administration, as indicated in Fig.1. The main parameters were as follows: $t_{1/2}k_e$ (48±7) min; $t_{1/2}k_a$ (31±4) min; AUC (522.3±81.0) (mg·min)/L; t_{max} (56.0±7.0) min; C_{max} (3.43±0.70) μg/ml; F 0.94.

PK of rH in acute severe renal failure rabbits

As shown by Fig.1, the rH plasma concentrations in the acute severe renal failure rabbits were significantly elevated in comparison with that in the normal rabbits, and maintained at constant and high level after 1 h postdose, had a markedly prolonged $t_{1/2\beta}$ (697 min) and greatly increased AUC (7084.4 (mg·min)/L). The above calculated data indicated that acute severe renal failure resulted in 11-fold longer $t_{1/2\beta}$ and 13-fold greater AUC than normal renal functions.

Table 1 PK parameters of rH in normal rabbits receiving iv rH 1.0, 2.0 and 4.0 mg/kg

Parameter	Dose (mg/kg)		
	1.0	2.0	4.0
$t_{1/2\alpha}$ (min)	9.0±1.5	10.8±1.8	8.3±2.9
$t_{1/2\beta}$ (min)	58.6±5.0	59.0±4.0	58.4±6.3
k_{21} (min^{-1})	0.034±0.015	0.025±0.009	0.043±0.008
k_{10} (min^{-1})	0.034±0.010	0.032±0.007	0.043±0.008
k_{12} (min^{-1})	0.039±0.020	0.020±0.008	0.026±0.007
V_d (L/kg)	0.090±0.007	0.122±0.023	0.105±0.019
CL (L/(kg·min))	0.0035±0.0006	0.0040±0.0015	0.0038±0.0004
AUC ((mg·min)/L)	297.8±55.9	553.6±203.8	1050.0±109.5

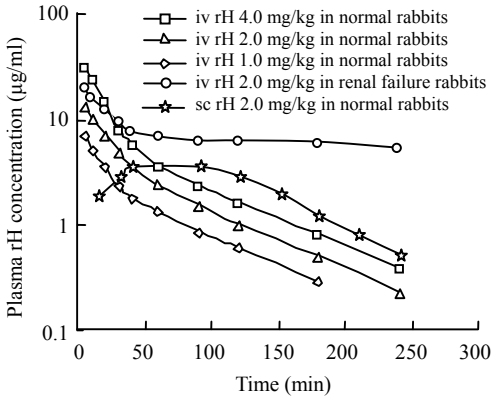

Fig.1 Concentration-time curve of rH in plasma of rabbits

DISCUSSION

It is widely accepted that for biotechnology drugs 3 kinds of assays, namely, immunoassay, radioassay and bioassay, should be used to fully elucidate their PK properties from different aspects (Tang *et al*., 1996). Unlike the other 2 assays, bioassay-based concentration represents the active concentration. The immunoassay and radioassay-based concentration only represent the so-called antigen concentration and radio concentration, respectively. The determined concentration using the bioassay reported by us includes both rH itself and its anticoagulantly active degraded products, which are undoubtedly, very useful for gaining insight into the PK profiles of rH, although the sensitivity obtained by bioassay is not as high as that of ELISA and radioassay.

The bioassay developed by us showed a good precision and high recovery at dilution of 2~100-fold dilution and therefore, completely meet the requirements for PK study of rH.

The PK behaviors of rH in rabbits after iv and sc administration could be described by two-compartment model and one-compartment model, respectively, followed the first-order kinetics. The obtained PK parameters indicated that rH was eliminated from the body rapidly and was distributed only in extra-cellular space. AUCs were found to be proportional to rH doses, with $t_{1/2}$, CL not changing significantly with doses, implying that the PK of rH in rabbits in the range of doses utilized obeyed linear kinetics. The findings that bioavailability of rH after sc administration reached 94%, with t_{max} being less than 1 h and $t_{1/2}k_a$ being about 0.5 h showed sc dosing resulted in a relatively complete and fast absorption. The above results all accorded with those obtained by radioassay and immunoassay as reported in (Markwardt, 1994; Han, 2003). The very rapid elimination of rH from plasma of rabbits suffering from acute severe renal failure indicated that kidneys play an extremely important role in excretion of rH.

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