Excretion of r-Hirudin in urine and bile of rabbits and its pharmacokinetics investigated by bioassay

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Abstract  Aim To develop a bioassay for determination of r-hirudin (rH) in urine and bile of rabbits and thereby study urinary and biliary excretion and pharmacokinetics (pK) of rH in rabbits. Methods rH urine and bile concentrations were measured by thrombin time (TT) method based on ex vivo antithrombin activity of rH. rH plasma concentration was determined according to the bioassay reported before by us. Creatinine concentration in urine and plasma were assayed with reference to Jaffe’s picrate method and used to calculated CLCR and thereby to estimate GFR. Sigma-minus method was used for urinary pharmacokinetic analysis. Results LogTT prolongation rate was found to be linearly related to rH concentration in urine and bile over the range of 0.49-2.50µg·mL⁻¹ (r>0.995). The validation of methodology showed high precision and recovery. It was found that rH was undetectable in bile samples. The 6-h cumulative excretion of rH in urine accounted for about 60% of dose. The main pharmacokinetic parameters were t₁/₂ 58.08-63.97 min, MRT 66.59-75.37 min, CLR 1.67-1.78 ml·min⁻¹·kg⁻¹ (urinary pK study) and t₁/₂ 59.76-62.71 min, MRT 70.41-71.39 min, CLR 2.80-2.98 ml·min⁻¹·kg⁻¹ (plasma pK study), as well as CLCR 3.18-4.52 ml·min⁻¹·kg⁻¹. Conclusion The urinary excretion is the main elimination route of rH in rabbits with no rH biliary excretion being found. The t₁/₂ and MRT determined using urinary date are in good agreement with those using plasma data, and all indicate a rapid elimination of rH in rabbits. rH follows linear pharmacokinetics. It is estimated that approximately 40% of rH dosed is cleared through non-renal and non-biliary routes. Since CLR is smaller than CLCR, it is reasoned that rH excretes into urine by both renal glomerular filtration and tubular reabsorption.

Key words r-hirudin, urine, bile, plasma, excretion, pharmacokinetics, rabbits

Introduction

Recombined-Hirudin (rH), as a gene-engineering product of natural hirudin contained in salivary glands of bloodsucking leech Has been proven to be an highly selective directly-acting thrombin inhibitor with powerful anticoagulant and antithrombotic effects[1], and currently, is undergoing extensive preclinical studies and clinical trials in China. Over the past years our lab has addressed itself to pharmacodynamic (PK) and pharmacokinetic studies of N-Ile¹-Thr²-63-desulfatohirudin, a newly developed r-hirudin in China[2-7]. In previous papers we reported plasma PK profiles of rH in rats by ELISA method and in rabbits by bioassay[3,4]. So far, there is no report available regarding rH urinary and biliary pharmacokinetics. In view of the fact, the purpose of this paper is to develop a bioassay for determination of rH in urine and bile of rabbits and thereby study the urinary and biliary excretion and its pharmacokinetics in rabbits, and further to investigate the disposition of rH by kidneys through the comparison between rH CLR and CLCR, and also to find out whether urinary PK results are in agreement with plasma PK results so as to provide experimental data for its development and clinical application.

Materials and Methods

Drugs and Chemicals
Recombined-Hirudin to be examined (purity >99.0%, anticoagulant potency >16000 ATU·mg⁻¹)
was provided by Dalian Guoxin Biopharmaceutical Co., Ltd. This drug was produced using Hansun yeast cell expression system and identified as N-Ile1-Thr2-63-desulfatohirudin which was comprised of 65 amino acids with MW of 7 KD. It was available as a white, sterile lyophilized powder for injection and stored at 0-4°C before use and dissolved in saline prior to use. Thrombin was purchased from Biopharmaceutical Co., Ltd Lot No. 20060101).

**Animals and Instruments**

Male Big Ear White rabbits weighing 2.5±0.2 kg were supplied by the Animal Center of Dalian Medical University (Liaoning Province experiment animal ratification No.22); PISC 2000-4 Coagulometer and LD5-2A centrifuge were products of Pulishing Co., Ltd and Beijing Co., Ltd, respectively.

**Determination of thrombin time (TT)**

10 µl of urine or bile being examined, 40 µl each of citrated blank rabbit plasma and 0.1mol/L pH7.4 Tris-HCL buffer were mixed in cuvette and incubated at 37°C in a coagulometer. After addition of 50 µl thrombin solution (5 AT U·mL⁻¹) the clotting time was automatically recorded and was designated by the name of thrombin clotting time, briefly called thrombin time (TT).

**Preparation of calibration curve and concentration determination of rH in urine and bile samples**

1 mg·mL⁻¹ rH standard stock solution was serially diluted with blank urine or bile of rabbit to produce rH urine or bile standard solution of 2.50, 1.67, 1.11, 0.74 and 0.49 µg·mL⁻¹. 10 µl of the blank and resulting standard solutions were used to determine TT, respectively, as described under section determination of TT. The TT prolongation rate (%) was calculated using the formula: TT prolongation rate (%) = (TTₙ₉ - TT₀) / TT₀ × 100, where TTₙ₉ and TT₀ were the determined TT values of rH-containing and rH-free urine or bile solutions, respectively. The plot of log TT prolongation rate vs rH concentration in urine and bile yielded respective calibration curve.

The rH concentrations in urine and bile samples collected from rabbits receiving iv administration of rH were read from the aforementioned calibration curve. In order to enable the assayed concentration to be in linear range of the calibration curve, the proper dilution of samples whose rH concentration was higher than linear range with blank samples became compelling. The determination of each set of samples was accompanied by calibration curve.

**Validation of methodology and observation of stability**

The validation of developed method including linearity, precision and recovery was carried out by routine procedures (n=5). Method recovery was obtained by found concentration divided by added concentration. Dilution recovery (%) was calculated by the formula: determined concentration (Cₙ) of diluted samples × diluted ratio / added concentration × 100.

In order to inspect the stability of samples, the urine and bile standard solutions were prepared at high (2.50 µg·mL⁻¹) and low (0.49 µg·mL⁻¹) concentrations and stored at room temperature and -20°C and examined at different time with respect to their concentrations.

**Measurement of rH plasma concentration and endogenous creatinine level in plasma and urine**

rH plasma concentration was measured according to the ex vivo antithrombin activity-based bioassay reported before by us[5]. The creatinine levels in urine and plasma were spectrophotometrically assayed by Jaffe’s picrate method[8].

**Animal Experiment**

Fifteen male rabbits, which were randomly divided into high, middle and low dose groups, were anesthetized with iv urethane (25%-4mL·kg⁻¹ body weight) and then were subjected to the surgical opening of abdominal cavity and cannulation of bilateral ureters and bile duct common with plastic tube for collection of samples. Physiological saline was infused into ear marginal vein at constant rate of 0.5mL·min⁻¹ to maintain stable urinary flow. rH (1 mg·mL⁻¹) was iv given to 3
groups of rabbits via ear marginal vein at doses of 2.1 and 0.5 mg·kg⁻¹, respectively. Samples of urine and bile were collected from respective catheter prior to dosing and different time (10, 20, 30, 40, 60, 90, 120, 180, 240, 360min) postdosing. Meanwhile, the citrated blood was taken from ear marginal vein prior to dosing and 5, 15, 25, 35, 50, 75, 105, 150, 210, 300min (middle point time of urine collection intervals) after dosing, and then centrifuged at 3000r/min for 15 min to yield plasma samples.

**PK analysis**

The program 3p97 was used to judge the compartment model corresponding to C-T curve and to calculate PK parameters such as t½, AUC, CLt, CLR, CLNR, AUMC, MRT. The sigma-minus method was chosen for urinary data processing[9]. The following equations were used: CLt = dose iv / AUC, AUMC = ∫₀^∞ tcdt, MRTplasma = AUMC / AUC, MRTurine = ∫₀^∞ (Xu - X₀)dt / X₀, CLR = CLt × fe, CLNR = CLt - CLR, where CLt, CLR and CLNR were total body clearance, renal clearance and non-renal clearance of rH, respectively. MRTplasma and MRTurine were mean residence time derived from plasma and urine data, respectively. The glomerular filtration rate (GFR) was estimated by measuring the creatinine clearance (CLCR) which was calculated using the equation: CLCR = XuCR / Cpmid, where XuCR was the amount of creatinine excreted over urine collection intervals, Cpmid was creatinine plasma concentration at middle point time of urine collection intervals.

The PK parameters were compared between urine data and plasma data to judge the consistency of results derived from 2 kinds of datum sources. Both plasma and urinary PK parameters at 3 different doses were used to investigate if rH followed linear PK. The comparison between CLR and CLCR was used to probe the mechanism by which rH was excreted by kidneys.

**Statistical analysis**

All data were expressed as the mean ± standard deviation. The significance was determined by the t test between two means for unpaired data.

**Results**

**Validation of methodology**

A good linear relationship was obtained between the log TT prolongation rate(Y, %) and rH concentrations(X, µg·mL⁻¹) ranged from 0.49 to 2.50 µg/ml in both urine and bile, with the liner equation: $Y = 0.7621 + 1.0096$ (r=0.995) for urine and $Y = 0.6637X + 0.9871$ (r=0.997) for bile. The limit of quantitation was 0.49µg·mL⁻¹.

The intra- and inter-day precisions (RSD) at each concentration level (2.50, 1.11 and 0.49 µg·mL⁻¹) of 3 QC samples were <10% for both urine and bile.

The method recovery approximated 100%; dilution recovery for 5–200 –fold diluted samples was >95% for both urine and bile.

The urine and bile samples were found to be stable for at least 15 days when stored at −20°C and for at least 8 hours when store at room temperature.

**Excretion of rH in urine and bile**

As seen in Fig 1, the cumulative amount of rH excreted in urine reached plateau at 6h post dosing with 2.23±0.56mg ±0.13±0.05mg and 0.63±0.03mg for doses of 2mg/kg, 1mg/kg and 0.5mg/kg, respectively, accounting for 60.70±15.25% and 57.83±6.30% and 59.37±5.36 % of the dose. In contrast to urine samples, rH was undetectable in bile samples over all sampling period of 3 doses.

Fig 2 gave excretion rate at different urine collection intervals showing that rH was detectable in the first collection intervals (0–10min) and reached the maximum during 0–10min and afterwards declined gradually.

**Urinary PK of rH**

As seen in Fig 3, a plot of logarithm of rH amount remaining to be excreted [log (Xu – X₀)] against time t, depicting that log (Xu – X₀) = ct curve was a biexponential curve characterized by initial rapid decay and secondary relative slow decline. Consequently, based on the urinary data the kinetic behavior of rH could be depicted by the two-compartment model with the following equation:
\[ X_{u}\infty - X_u = A e^{-\alpha t} + B e^{-\beta t} \]

The urinary PK parameters could be obtained by method of residuals (Table 1).

The plasma PK parameters of rH were presented in Table 2. Consistent with urinary PK, the plasma \( t_{1/2} \beta \) was about 1h, MRT was about 70min, indicating that rH eliminated rapidly from body. AUC was proportional to the doses; The \( t_{1/2} \), CL MRT did not change significantly with the doses, exhibiting that rH followed linear PK in the range of doses used.

![Fig 1. The cumulative excretion amount of rH in urine](image1.png)

**Fig 1.** The cumulative excretion amount of rH in urine

![Fig 2. A bar graph of urinary excretion rate of rH at different collection intervals](image2.png)

**Fig 2.** A bar graph of urinary excretion rate of rH at different collection intervals

Clearance results and renal excretion mechanism of rH

As shown in Table 3, CL\( R \) accounted for about 60% of CL\( t \), indicating that rH eliminated from body mainly by kidneys. In view of no excretion in bile, it could be considered that 40% of rH was
eliminated via non-renal and non-biliary routes. The findings that CLR was less than CLCR, namely, GFR and Ra was greater than 47.20% suggested that there existed not only renal glomerular filtration but also tubular reabsorption of rH during its renal excretion.

Table 1. Urinary PK parameters of rH (n=5)

<table>
<thead>
<tr>
<th>dose [mg·kg⁻¹]</th>
<th>Xu₀-6h [mg]</th>
<th>fₚ %dose</th>
<th>t₁/₂β [min]</th>
<th>MRT [min]</th>
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</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2.23±0.56</td>
<td>60.70±15.25</td>
<td>58.08±0.57</td>
<td>66.59±0.38</td>
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<tr>
<td>1.0</td>
<td>1.13±2.72</td>
<td>57.83±6.30</td>
<td>59.27±4.34</td>
<td>66.99±3.10</td>
</tr>
<tr>
<td>0.5</td>
<td>0.63±0.03</td>
<td>59.37±5.36</td>
<td>63.97±0.34</td>
<td>75.37±0.20</td>
</tr>
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</table>

Table 2. Plasma PK parameters of rH (n=5)

<table>
<thead>
<tr>
<th>dose [mg·kg⁻¹]</th>
<th>t₁/₂β [min]</th>
<th>AUC [µg·min·ml⁻¹]</th>
<th>MRT [min]</th>
<th>CLt [ml·min⁻¹·kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>62.27±1.40</td>
<td>682.73±15.89</td>
<td>71.39±0.94</td>
<td>2.93±0.07</td>
</tr>
<tr>
<td>1.0</td>
<td>62.71±2.86</td>
<td>335.58±3.66</td>
<td>70.88±2.04</td>
<td>2.98±0.03</td>
</tr>
<tr>
<td>0.5</td>
<td>59.76±4.09</td>
<td>179.51±13.81</td>
<td>70.41±2.44</td>
<td>2.80±0.21</td>
</tr>
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</table>

Table 3. Results of rH clearances and reabsorption

<table>
<thead>
<tr>
<th>dose [mg·kg⁻¹]</th>
<th>CLt [ml·min⁻¹·kg⁻¹]</th>
<th>CLR [ml·min⁻¹·kg⁻¹]</th>
<th>CLNR [ml·min⁻¹·kg⁻¹]</th>
<th>CLCR (ml·min⁻¹·kg⁻¹)</th>
<th>Ra (%)</th>
</tr>
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<tbody>
<tr>
<td>2.0</td>
<td>2.93±0.07</td>
<td>1.78±0.44</td>
<td>1.15±0.46</td>
<td>4.26±0.76</td>
<td>58.61±2.98</td>
</tr>
<tr>
<td>1.0</td>
<td>2.98±0.03</td>
<td>1.72±0.18</td>
<td>1.26±0.20</td>
<td>4.52±0.75*</td>
<td>42.15±6.41</td>
</tr>
<tr>
<td>0.5</td>
<td>2.80±0.21</td>
<td>1.67±0.25</td>
<td>1.13±0.11</td>
<td>3.18±0.64*</td>
<td>41.61±5.47</td>
</tr>
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</table>

*P<0.05 vs CLR
Discussion

In this paper we described a bioassay for determination of rH in urine and bile of rabbits by means of rH ex vivo antithrombin activity. Validation of methodology showed that the TT method developed by us had good linearity, and high precision and recovery. Actual application demonstrated that this method completely meets requirements for PK study. The method developed in the present paper has not been seen in literature to date.

For construction of calibration curve, logarithmic transformation of data is inevitable. The comparison between 3 different transformation patterns exhibited that log TT prolongation rate – C plot is much superior than TT prolongation rate – \( \log C \) plot and double log plot \( r=0.995, 0.984 \) and \( 0.853 \), respectively (urine) and \( r=0.997, 0.847 \) and \( 0.947 \), respectively ( bile). This kind of logarithmic transformation is similar to heparin bioassay described in China Pharmacopoeia \(^{10}\), and clinical therapeutic drug monitoring of heparin reported in literature \(^{11}\).

Unlike TT method for determination of rH in plasma that was reported before by us \(^4\), determination of rH in urine and bile, which are plasma-free, should be carried out by addition of blank rabbit plasma to urine and bile to provide blood clotting matrix. In this way, TT method can be also used to measure rH concentration in urine and bile.

Although studied before in our lab, rH plasma PK was also investigated in this paper so that the comparison of results between 2 kinds of different biological samples (urine and plasma) derived from the same individuals could be done and clearance data could be obtained. The results of 2 classes of data all showed that rH pharmacologically behaved as two-compartment model and obeyed the linear kinetics and eliminated from body rapidly, as evidenced by the short half-life and MRT.

The urinary data exhibited that the cumulative amount of rH excreted in urine accounted for 60% of dose; Moreover, no rH could be found in bile. This indicated that about 40% of rH dosed is eliminated through non-renal and non-biliary route. In addition, the finding that rH CL\(_R\) is smaller than CL\(_{CR}\) suggests the involvement of not only renal glomerular filtration but also tubular reabsorption in the disposition of rH by kidneys, with the reabsorption rate amounting to 40.61-58.61 %. The exact mechanism of the reabsorption needs to be studied further.

References


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