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根据受体三维结构搜寻配体活性构象的方法及在凝血酶抑制剂中的应用[1]

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关键词 分子构象；膦酰肽；凝血酶受体；配体；结构-活性关系；分子力学

目的：发展一种根据受体三维结构搜寻配体活性构象的方法. 方法：结合系统构象搜寻方法和配体—受体分子对接(Dock)方法，我们发展了一种根据受体三维结构搜寻配体小分子活性构象的方法. 结果：应用这一方法，我们搜寻出了膦酰肽类凝血酶抑制剂的活性构象，由搜寻结果，我们又用分子力学方法，计算了膦酰肽类抑制剂和凝血酶的结合能. 结论：计算结果表明，这些凝血酶抑制剂和凝血酶的结合能与其抑制活性之间有很好的相关性，这说明我们计算方法的可靠性. 同时，计算结果能很好地解释膦酰肽类凝血酶抑制剂的作用机理.

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Pharmacokinetics of recombinant human granulocyte colony-stimulating factor in rabbits and mice

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KEY WORDS granulocyte colony-stimulating factor; gel chromatography; pharmacokinetics

AIM: To study the pharmacokinetics of recombinant human granulocyte colony-stimulating factor (rhGCSF) in rabbits and mice. **METHODS**: ^{125}I-rhGCSF was prepared by iodogen method and determined by size exclusive HPLC (SEHPLC). **RESULTS**: Concentration-time curves after iv ^{125}I-rhGCSF in rabbits were best fitted with 2-compartment open model. The α and terminal elimination $T_{\frac{1}{2}}$ were 0.25 - 0.33 and 3.2 - 4.6 h, respectively. AUC increased with doses, and Cl_s and K_{10} were similar. T_{peak} was 0.59 ± 0.25 h after sc, and elimination $T_{\frac{1}{2}}$ was similar to that after iv. The bioavailability after sc was 1.0. In mice the highest level was found in renal system, the next was bile-enteric system. Levels in lymph nodes, bone marrow, and spleen were approximately equal to or slightly lower than that in plasma, while the levels in brain, fat, and muscles were the lowest. About 68 %-86 % were recovered in urine and feces. **CONCLUSION**: Pharamcokinetics of ^{125}I-rhGCSF in rabbits and mice provided a useful index for clinical trial.

Granulocyte colony-stimulating factor (GCSF) is a 18.8 kDa protein that stimulates the proliferation of bone marrow precursor cells and their differentiation into granulocyte colonies. The bacterially synthesized recombinant human GCSF (rhGCSF) supports the formation of granulocyte colonies from precursor cells. In this paper the pharmacokinetic profile of rhGCSF[1] was studied.

MATERIALS AND METHODS

Iodogen, synthesized by Prof LI De-Yu of the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences. $Na^{125}I$, 0.74 TBq · L^{-1} (Amersham, radiochemical purity 99.4 %, specific activity 577.2 TBq/g of iodine). rhGCSF was produced by Associate Professor CHEN Hui-Peng (batch number 9403, purity >98 %, 0.83 g · L^{-1} in 0.60 mL) in our Institute of Radiation Medicine. Sephacryl S-200 HR (Pharmacia); Protein-Pak 125 column, Waters, packed by Dalian Elite Scientific Instruments Co. ID

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10 mm × 30 cm. Other reagents were of AR (Beijing Chemical Industry Factory). γ counter (Clinigamma, Pharmacia).

Iodogen 20 μg in 100 μL chloroform was blown to dryness with N_2. After adding rhGCSF 498 μg and $Na^{125}I$ 185 MBq in 50 μL, shaking at 20 ℃ for 30 min, the reaction mixture was applied on S-200 column. Gel filtration condition: column 10 mm × 92 cm, eluent: Tris-HCl buffer 10 mmol·L^{-1}, pH 8.0, containing edetic acid 2 mmol·L^{-1}, flow rate 7.5 mL·h^{-1}. Eluted fractions were detected by γ counter. The eluted fraction with chromatographic behavior as standard rhGCSF, was identified by size exclusive high performance liquid chromatography (SEHPLC). The result of S-200 gel filtration indicated that ^{125}I-rhGCSF and $Na^{125}I$ were eluted at 167 - 198 mL and 300 - 598 mL, respectively. The recovery of radioactivity was 93.5 %. The SEHPLC behavior of ^{125}I-rhGCSF appeared as a single radioactive peak. The retention volume was the same as standard unlabeled rhGCSF (including Batch No 9403 and standard from Amgen-Kirin). Radioactive purity was 96.3 ± 0.8 % (n = 5). The specific activity of prepared ^{125}I-rhGCSF was 3.6 TBq · mnl^{-1}. The most purified fractions were selected for pharmacokinetic study.

LACA mice bred in Animal Center of Academy of Military Medical Sciences, weighted 22 ± s 2 g. Each group consisted of 3 ♂ and 2 ♀. Each mouse was injected iv ^{125}I-rhGCSF 2.5 μg (48.7 kBq, about 125 μg·kg^{-1}). At 0.167, 0.5, 1.5, 4.0, 8.0, and 24 h after iv injection. Samples from blood, urine, and 22 organs or tissues were taken. The plasma and urine were analyzed by SEHPLC. The % of radioactivities of ^{125}I-rhGCSF main peak in SEHPLC profiles in the samples was converted to ^{125}I-rhGCSF in plasma. The analysis effectively discriminated rhGCSF from ovalbumin, rhIL-2, EGF, small molecular amino acids and I^-. For detection of very low activity sample, the detection time was set at 5 min. The detection limit for ^{125}I-rhGCSF was 10 μg·L^{-1} in plasma. Urine was absorbed on filter paper. At 8, 24, 32, and 48 h urine and feces were collected for γ counting.

Rabbits (n = 16) produced by Animal Center of Academy of Military Medical Sciences, aged 70 - 90 d, weighing 2.5 ± 0.2 kg, were divided randomly, with 2 ♂ and 2 ♀ in each group. Each rabbit was injected 1.6 mL containing ^{125}I-rhGCSF + unlabeled rhGCSF 15, 30, and 60 μg·kg^{-1} for iv group and 30 μg·kg^{-1} for sc group (118.7 kBq/rabbit). Rabbit plasma obtained at 5, 15, 30 min, 1, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, and 24 h was determined for ^{125}I-rhGCSF. The rabbit urine and feces were collected for assay of γ radioactivity. The ^{125}I-rhGCSF concentration-time data were fitted with computer program 3p87.

RESULTS

Biodegradation The SEHPLC profiles of plasma taken at 0.167, 0.5, 1.5, 4, and 8 h after iv ^{125}I-rhGCSF showed that the ^{125}I-rhGCSF peak area decreased with time, and smaller molecular fractions relatively increased. The radioactivity of plasma taken after 8 h was too low for reliable SEHPLC assay (Fig 1).

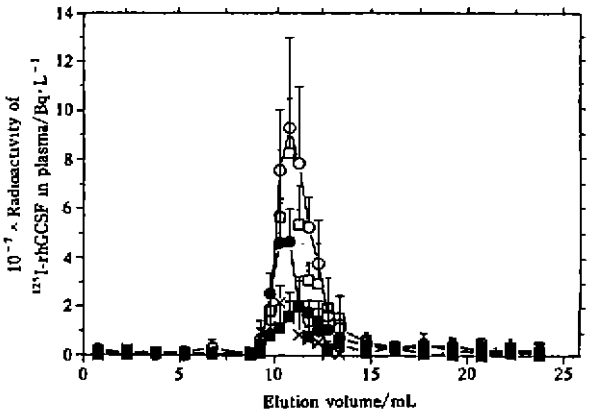

Fig 1. Protein-Pak 125 SEHPLC profiles of plasma taken at 0.167 h (○), 0.5 h (□), 1.5 h (●), 4 h (×), 8 h (■) after iv ^{125}I-rhGCSF 2.5 μg/mouse (n = 5, $\bar{x}$ ± s).

Pharmacokinetics The ^{125}I-rhGCSF concentration-time curve assayed by SEHPLC in mice was best described by 2-compartment model with α and terminal half-lives of 0.49 ± 0.17 h and 3.9 ± 2.6 h, respectively (Tab 1).

Distribution of radioactivity in tissues The radioactivity in urine, urinary bladder, kidney, bile, and gallbladder were higher than that in plasma at 10 min following administration. The concentration gradients were ovaries > blood > adrenal glands > skeletal muscles > heart > thymus > intestinal content > lungs > mesentery lymph nodes > spleen > liver > jejunum > fat > feces in colon > testes > brain. The radioactivity in thyroid was only 0.2 ± 0.1 % of the injected radioactivity. The radioactivity was the highest at 10 min after iv, but those in except spleen, thymus, intestinal content and feces in colon reached their peak at 30 min after injection (Tab 1).

SEHPLC behavior of urine collected at 1.5 and 4.0 h found that 94 ± 3 % (n = 2) and 90 ± 3 % (n = 4) appeared as ^{125}I-rhGCSF, respectively. The metabolites increased with time. The accumulation of ^{125}I in thyroid increased markedly

Tab 1. Radioactivity distribution in tissues (Bq/g tissue) after iv ^{125}I-rhGCSF (125 μg·kg^{-1}, 48.7 kBq/mouse). n = 5 (3♂, 2♀). $^{b}P<0.05$ and $^{c}P<0.01$ *vs* plasma.

Tissue	10 min	30 min	1.5 h	4 h	8 h	24 h	AUC Bq·h·g^{-1}
Bone marrow (Bq/femur)	109 ± 33	66 ± 31	47 ± 45	26 ± 9	9 ± 11	6.5 ± 6.7	-
Brain	9.5 ± 3.6^{c}	11 ± 10^{c}	8 ± 12^{c}	4.7 ± 2.2^{c}	0.7 ± 0.9^{c}	1.2 ± 0.6^{b}	58
Muscles	139 ± 186^{a}	42 ± 18^{c}	19 ± 7^{c}	13 ± 10^{c}	10 ± 9^{c}	1.2 ± 1.0^{b}	278
Heart	103 ± 45^{b}	54 ± 14^{c}	31 ± 12^{c}	15 ± 6^{c}	10.1 ± 1.7^{c}	3.0 ± 1.1^{a}	293
Fat	61 ± 47^{c}	54 ± 52^{c}	20 ± 14^{c}	16 ± 7^{c}	9.8 ± 2.5^{c}	2.5 ± 1.2^{a}	296
Jejunum	62 ± 22^{c}	54 ± 18^{c}	29 ± 10^{c}	18 ± 9^{c}	15 ± 12^{c}	3.5 ± 2.6^{a}	340
Liver	88 ± 31^{c}	65 ± 11^{c}	35 ± 9^{c}	18 ± 6^{c}	17 ± 11^{c}	4.4 ± 1.9^{a}	393
Mesenteric lymph nodes	90 ± 37^{b}	77 ± 25^{c}	41 ± 16^{c}	24 ± 7^{c}	16 ± 4^{c}	3.3 ± 1.8^{a}	417
Testes	36 ± 2^{c}	65 ± 39^{c}	46 ± 7^{b}	23 ± 7^{a}	18 ± 3^{c}	3.2 ± 2.7^{a}	420
Ovaries	154 ± 108^{a}	84	37 ± 12^{b}	32 ± 17^{a}	16 ± 9^{c}	3.9 ± 3.7^{a}	458
Lungs	130 ± 63^{a}	97 ± 26^{c}	52 ± 10^{c}	29 ± 7^{b}	22 ± 3^{c}	4.1 ± 1.6	533
Adrenal gland	139 ± 75^{a}	108 ± 56^{a}	42 ± 69^{a}	68 ± 43^{a}	8 ± 6^{c}	9 ± 10^{a}	557
Spleen	88 ± 30^{c}	114 ± 54^{a}	65 ± 22^{a}	35 ± 8^{a}	21.0 ± 2.4^{c}	4.1 ± 2.9^{a}	573
Kidneys	407 ± 222^{a}	117 ± 28^{b}	58 ± 15^{b}	32 ± 15^{a}	21 ± 8^{c}	4.5 ± 3.2	647
Blood	147 ± 57^{a}	109 ± 14^{c}	68 ± 22^{a}	36 ± 14^{a}	27 ± 5^{c}	4.9 ± 3.6^{a}	660
Plasma	212 ± 76	175 ± 34	97 ± 25	50 ± 18	40 ± 7	8 ± 5	977
Intestinal content	132 ± 65^{a}	186 ± 58^{a}	89 ± 14^{a}	50 ± 18^{a}	46 ± 16^{a}	11 ± 10^{a}	1 033
Thymus	136 ± 65^{a}	156 ± 124^{a}	69 ± 17^{a}	46 ± 25^{a}	70 ± 66^{a}	5.5 ± 4.3^{a}	1 160
Feces in colon	56 ± 26^{c}	129 ± 54^{a}	72 ± 18^{a}	112 ± 50^{b}	108 ± 59^{b}	29 ± 19^{b}	2 238
Gallbladder & bile	451 ± 227^{a}	433 ± 304^{a}	285 ± 141^{b}	200 ± 88^{c}	93 ± 69^{a}	12 ± 16^{a}	2 577
Bladder	1 199 ± 1 423^{a}	955 ± 1 796^{a}	151 ± 88^{a}	206 ± 310^{a}	159 ± 69^{a}	7 ± 16	3 553
Urine	6 776 ± 7 301^{a}	7 195 ± 7 872^{a}	1 080 ± 274^{c}	744 ± 447^{c}	1 134 ± 1 812^{a}	153 ± 240^{a}	23 715
Thyroid	555 ± 446^{a}	2 963 ± 2 214^{b}	7 063 ± 3 610^{b}	14 771 ± 10 422^{b}	7 112 ± 3 907^{c}	20 466 ± 10 664^{c}	131 150

with time and reached its highest level 8 ± 6 % at 24 h. This phenomenon revealed the biodegradation of ^{125}I-rhGCSF again.

Excretion At 24 h after iv the radioactivities recovered from urine and feces were 70 ± 19 % and 2.60 ± 0.20 % of the injected dose, respectively.

Pharmacokinetics after iv and sc injections of ^{125}I-rhGCSF in rabbits Plasma ^{125}I-rhGCSF concentrations after iv and sc of ^{125}I-rhGCSF were shown in Fig 2. The curves after iv were best fitted with two-compartment model as in mice.

The areas under curves (AUC) were almost linearly increased with dose (r = 0.936, n = 3, P > 0.05). Systemic clearance (Cl_s) and K_{10} were similar in different dosages. The deposition of ^{125}I-rhGCSF could be considered nearly linear pharmacokinetics in the dosage range studied. The time to maximal concentration was 0.59 ± 0.25 h after sc of ^{125}I-rhGCSF, and elimination rate constant was approximated to those after iv. Bioavailability was 1.0 (Tab 2).

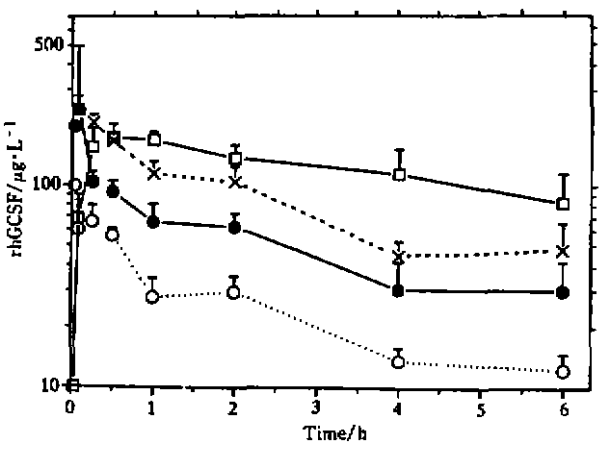

Fig 2. rhGCSF in plasma after *iv* 15 μg·kg^{-1} (○), 30 μg·kg^{-1} (●), 60 μg·kg^{-1} (×), or sc 30 μg·kg^{-1} (□) of ^{125}I-rhGCSF.

Tab 2. Pharmacokinetic parameters of ^{125}I-rhGCSF in rabbits. $n = 4$, $\bar{x} \pm s$.

Parameters	iv 15 μg·kg^{-1}	iv 30 μg·kg^{-1}	iv 60 μg·kg^{-1}	sc 30 μg·kg^{-1}
$T_{\frac{1}{2}\alpha}$/h	0.25 ± 0.16	0.29 ± 0.13	0.34 ± 0.14	–
$T_{\frac{1}{2}\beta}$/h	4.2 ± 1.7	4.6 ± 2.3	3.2 ± 0.7	3.7 ± 2.1
V_c/L·kg^{-1}	0.21 ± 0.09	0.20 ± 0.07	0.23 ± 0.04	0.29 ± 0.04
AUC/mg·h·L^{-1}	215 ± 42	519 ± 241	683 ± 159	526 ± 164
Cl_s/L·h^{-1}·kg^{-1}	0.072 ± 0.014	0.067 ± 0.027	0.091 ± 0.018	0.063 ± 0.023
k_{21}/h^{-1}	3.4 ± 4.4	1.3 ± 0.4	1.3 ± 0.7	–
k_{12}/h^{-1}	3 ± 5	1.3 ± 1.1	0.9 ± 0.4	–
k_{10}/h^{-1}	0.41 ± 0.25	0.37 ± 0.18	0.40 ± 0.09	–
C_{max} # /mg·L^{-1}	–	–	–	95 ± 11
T_{peak} # /h	–	–	–	0.6 ± 0.2
F_s	–	–	–	1.0
Lag Time/h	–	–	–	0.02 ± 0.03

The recoveries of radioactivity in urine and feces were listed in Tab 3. About 66 % – 84 % of the injected radioactivity was excreted in urine, only 0.4 % – 0.9 % in feces.

DISCUSSION

One important challenge in the research of pharmacokinetics of peptides or proteins is about the method for assay. Bioassays and immunoassays are methods most often used, but are often nonspecific, inaccurate and imprecise. Specifically, these assays *do not necessarily detect the structurally intact* molecule[2-4]. One disadvantage of radiolabelling method is the labeled amino acids liberated through catabolism that limits the use of labels in studying the disposition of peptides and proteins in animals and human[2-4]. Harris *et al* successfully used size exclusion HPLC combining with ^{125}I-labeling method to investigate the catabolism of recombinant tissue plasmonogen activator in biological samples[10].

The pharmacokinetics of rhGCSF had been extensively studied by using bioassay[7], sandwich enzyme-linked immunosorbent assay[6], and solid-phase radioimmunoassay[5]. In this paper we used ^{125}I-rhGCSF and size exclusion HPLC combined method to assay the plasma ^{125}I-rhGCSF concentration in rabbits and mice. The results showed that there was obvious biodegradation of ^{125}I-rhGCSF in the body, indicating that it is necessary to incorporate a HPLC method to enhance the resolution of the general radiolabeling method. The AUC, Cl_s, and terminal half-life were very close to that reported by other methods[5,6].

The excretion of ^{125}I-rhGCSF in mice was very similar to the data of ^{125}I-rh-IL2 reported by our Lab, and similar to low molecular peptides or proteins. The major route of excretion is renal-urinary system, the excretion through feces is less than 1 %. The influence of renal and hepatic failure on the pharmacokinetics of rhGCSF[8] is consistent with this conclusion. Tissue distribution and excretion of rhGCSF have not been published in the literature. The distribution profile was

Tab 3. Recoveries of radioactivity in urine and feces after iv ^{125}I-rhGCSF. $\bar{x} \pm s$.

Route	Dosage mg·kg^{-1}	Rabbits	Radioactivity (% of injected) Urine	Feces	Urine + Feces	Thyroid	Total
iv	15	3	83.6 ± 6.8	0.9 ± 0.9	86.1 ± 4.3	16.1 ± 2.9	100 ± 26
iv	30	4	81.8 ± 5.9	0.9 ± 0.7	82.7 ± 6.2	12.8 ± 11.1	85 ± 7
iv	60	4	67.8 ± 14.6	0.4 ± 0.3	68.2 ± 14.4	14.8 ± 4.0	83 ± 16
sc	30	4	65.9 ± 15.1	0.9 ± 0.8	66.7 ± 15.0	11.2 ± 7.4	78 ± 19

somewhat different from ^{125}I-rh-IL2. The levels in liver and lungs were relatively low. We do not know why the radioactivity concentrations in target organ, bone marrow and spleen were only approximately equal to or slightly lower than that in plasma.

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基因重组人粒细胞集落刺激因子在兔和小鼠中的药物动力学

R977-6

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关键词 粒细胞集落刺激因子；凝胶色谱；药物动力学

目的：研究家兔和小鼠中重组人粒细胞集落刺激因子的药物动力学。**方法**：用 Iodogen 法制备^{125}I-rhGCSF。用分子筛 HPLC 测定浓度。**结果**：iv 后浓度时间曲线符合二房室模型。初期和末端 $T_{1/2}$分别为 0.25 - 0.33 h 和 3.2 - 4.6 h。AUC 和剂量接近正比。sc 达峰时间 0.59 ± 0.25 h，生物利用度为 1.0。药物主要以原型从尿排泄。给药后泌尿系统放射性最高，胆胃肠道次之，骨髓和脾脏略低或接近血浆。**结论**：家兔和小鼠 rhGCSF 药物动力学数据为临床试验提供有用参考。