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**Notes**

# Dose-dependent pharmacokinetics of human granulocyte/macrophage colony-stimulating factor in rabbits

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## Summary

The pharmacokinetic parameters of HGM-CSF, such as $t_{1/2}$ (11.3, 10.4, 39.2, 39.2 vs 51.6 min), MRT (11.6, 10.9, 22.9, 20.3 vs 18.3 min), CL (25.0, 21.1, 6.98, 7.94 vs 6.51 ml $min^{-1}$ $kg^{-1}$), $CL_{NR}$ (24.6, 19.9, 6.87, 6.92 vs 4.24 ml $min^{-1}$ $kg^{-1}$) and $V_{ss}$ (292, 211, 162, 162 vs 120 ml $kg^{-1}$) were dose-dependent after intravenous administration of the factor to rabbits at the doses of 0.05, 0.1, 0.5, 1.0 and 2.5 mg $kg^{-1}$, respectively: The dose-dependent pharmacokinetics of HGM-CSF in rabbits appeared to represent saturable metabolism of the factor at the dose ranges studied. HGM-CSF was highly concentrated in the kidney and liver, and less concentrated in other tissues or organs studied at 2 h after subcutaneous administration of the factor, 0.5 mg $kg^{-1}$ to rabbits.

Human granulocyte/macrophage colony-stimulating factor (HGM-CSF), a hematopoietic growth factor, can increase the granulocyte counts, and reduce the period and severity of neutropenia after myelosuppressive therapy (Morstyn et al., 1988). The native form of HGM-CSF, a variably glycosylated protein, has a molecular mass of 15–31 kDa (Clark and Kamen, 1987). Following the cloning of the HGM-CSF gene, biosynthetic HGM-CSF has been produced and purified from yeast (Moonen et al., 1987), mammalian cells (Metcalf et al., 1986), and *E. coli* (Burgess et al., 1987). Recently, Lucky Ltd (Seoul, South Korea) produced yeast-derived recombinant HGM-CSF having a molecular mass of approx. 14.5–15.5 kDa.

If the metabolism of HGM-CSF is capacity limited, it is conceivable that nonlinearity of HGM-CSF kinetics might be observed if the factor is given in a high enough dose to saturate the metabolic pathway. Although the pharmacokinetics (Cebon et al., 1988; Morstyn et al., 1989), and Phase I (Bronchud et al., 1987; Cebon et al., 1988) and Phase II (Bronchud et al., 1987) studies on HGM-CSF in patients have been reported, the dose-dependent pharmacokinetics of HGM-CSF appear not to have been thoroughly investigated especially over wide ranges of dose.

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The purpose of this paper is to report the dose-dependent pharmacokinetics of HGM-CSF after intravenous (i.v.) administration of the factor, 0.05, 0.1, 0.5, 1.0 and 2.5 mg $kg^{-1}$, respectively, to rabbits. The plasma level-time profiles and tissue distribution of HGM-CSF after subcutaneous (s.c.) administration of the factor, 0.5 mg $kg^{-1}$ to rabbits are also reported.

49 male New Zealand White rabbits (1.5–2.2 kg, Korea Laboratory Animal Development, Seoul, South Korea) were anesthetized with 30–50 mg of i.v. ketamine (kindly supplied by the Yu-han Research Center, Kunpo, South Korea) via the ear vein. The left jugular vein (i.v. study only) and carotid artery were catheterized with silastic tubing (Dow Corning Inc., Midland, MI) for administration of the factor and blood sampling, respectively. The animals were allowed to recover for 4–5 h from anesthesia before the study and fasted during the experiment.

HGM-CSF (1 mg $ml^{-1}$ per vial; kindly supplied by Lucky Ltd) was freshly diluted with injectable distilled water before use. The factor, 0.05, 0.1, 0.5, 1.0 and 2.5 mg $kg^{-1}$ was infused (injection volume approx. 2–5 ml) in 1 min through the cannula placed in the jugular vein to rabbits, 1–7, 8–15, 16–23, 24–32 and 33–40, respectively. Blood samples (0.5–2.5 ml, depending on blood sampling times and doses) were collected from the carotid artery, and centrifuged immediately to minimize the potential 'blood storage effect' (Lee et al., 1984; Shin et al., 1992) in the plasma concentrations of HGM-CSF. Urine samples were also collected for up to 24 h after the dose via a pediatric Foley catheter (Sewoon Medical Co., Seoul, South Korea) which was introduced into the urinary bladder (Yoon et al., 1991).

HGM-CSF, 0.5 mg $kg^{-1}$ was injected (injection volume approx. 2 ml) s.c. to the dorsal side of the neck of rabbits 41–46, and blood and urine samples were appropriately collected. HGM-CSF, 0.5 mg $kg^{-1}$ was also similarly injected s.c. to rabbits 47–49. At 2 h after the injection, as much whole blood as possible was collected through the carotid artery, and the liver, spleen, kidney, heart, lung, stomach, large intestine and muscle were excised from rabbits 47–49. The liver was perfused with cold normal saline solution which was also employed to wash the other organs or tissues in order to remove blood remaining in each organ or tissue. After blotting dry with paper tissue, exactly 1 g of each organ or tissue was weighed. Each organ or tissue was minced into small pieces with scissors and then homogenized with 3 volumes of phosphate buffer of pH 7.4 using a tissuemizer (Ultra-Turrax T25, Janke & Kunkel, IKA-Labortechnik, Germany). Plasma was also mixed with 3 volumes of the buffer.

The concentrations or amounts of HGM-CSF in the above biological samples were determined by the modifications of the reported ELISA method (Cebon et al., 1988; Konito, 1991) as follows:

Production of murine monoclonal antibodies to HGM-CSF: Balb/C female mice (6–8 weeks of age, Lucky Ltd) were first immunized s.c. with 50 $\mu$g of HGM-CSF in Freund's complete adjuvant (Gibco, Grand Island, NY), and further immunized with the same amounts of HGM-CSF in Freund's incomplete adjuvant (Gibco) at intervals of 2 weeks more than three times. 3 days after the last boost, spleens from the immunized mice were removed, then the cells were fused with SP 2/0 mouse myeloma cell line (ATTC, Rockville, MD) and hybrids were propagated. The reactivity of hybrid supernatants with HGM-CSF was assayed by a direct ELISA method using HRP-conjugated anti-mouse IgG (Cappel, West Chester, PA). Hybrids that produced antibodies reacting with HGM-CSF were selected and cloned by repeated limiting dilution. Cloned hybrids were injected intraperitoneally into pristane-primed Balb/C mice, and ascitic fluid was collected 14–28 days later. Monoclonal antibody was purified by ammonium sulfate precipitation followed by protein G-agarose affinity chromatography (Pierce, Rockfort, IL). The subclass of each monoclonal antibody was determined by enzyme immunoassay using a Mouse Typer Sub-isotyping kit (Bio-Rad, Richmond, CA) according to the manufacturer's instruction. The monoclonal antibody, GMCC8E6 (Lucky Ltd) was selected, since it gave the highest sensitivity and lowest background when HGM-CSF levels were measured in whole serum.

Conjugation of HRP to monoclonal antibodies: 10 mg of HRP (Boehringer-Mannheim, Mennheim, Germany) was oxidized with 0.1 M $NaIO_4$ for 20 min at room temperature and then purified by using a G-25 column to eliminate less oxidized HRP. 0.6 mg of purified HRP was mixed with 1 mg of monoclonal antibody in 10 mM sodium carbonate buffer of pH 9.6 and incubated overnight at 4°C. 0.11 M $NaBH_4$ was added and the mixture was further incubated for 2 h at 0°C. Conjugated antibody was precipitated by 45% ammonium sulfate and dialyzed against phosphate-buffered saline (PBS).

Production of guinea pig polyclonal antibody to HGM-CSF: Guinea pigs were immunized with 100 $\mu$g HGM-CSF in Freund's complete adjuvant (Gibco) followed by boosts at monthly intervals in Freund's incomplete adjuvant. Antibody titer was assayed by direct ELISA using HRP-conjugated anti-guinea pig IgG (Cappel, West Chester, PA). Polyclonal antibody was purified by ammonium sulfate precipitation followed by protein A-agarose affinity chromatography (Pierce).

HGM-CSF EIA: Immulon I microtiter plate wells (Dynatech, Chantilly, VA) were coated at room temperature (20–25°C) for 18–24 h with the optimized concentration of the polyclonal antibody in coating buffer (10 mM sodium carbonate, pH 9.6). The plates were washed with distilled water and nonspecific binding sites were blocked with 60 $\mu$l of ELISA buffer (PBS with 0.05% Tween 20 and 30% FBS) for 2 h at room temperature. Plates were washed with distilled water, and test samples or controls (25 $\mu$l) were added to the wells simultaneously with HRP-conjugated monoclonal antibody (25 $\mu$l) in ELISA buffer. Sample-conjugate mixtures were incubated for 2 h at 37°C. Then the wells were washed three times with PBS containing 0.05% Tween 20 and exposed to substrate solution (2 mg per ml OPD, Sigma Chemical Co., St. Louis, MO, and 0.03% $H_2O_2$ in 50 mM citrate-acetate buffer of pH 5.0) for 30 min at room temperature under conditions of darkness. Color development was stopped by adding 100 $\mu$l of 2 N $H_2SO_4$, and the resulting absorbance was measured at a wavelength of 492 nm using a microtiter plate reader (Beckman, Palo Alto, CA).

The pharmacokinetic parameters, such as the area under the plasma concentration-time curve from time zero to infinity (AUC), first moment of AUC (AUMC), apparent volume of distribution at steady state ($V_{ss}$), time-averaged total body (CL), renal ($CL_R$) and nonrenal ($CL_{NR}$) clearances, and mean residence time (MRT) were estimated by the standard methods (Gibaldi and Perrier, 1982; Yoon et al., 1991). Harmonic mean was employed for the calculation of the mean values of $t_{1/2}$, each clearance and $V_{ss}$ (Chiou, 1979).

The mean arterial plasma concentration-time profiles of HGM-CSF after i.v. infusion of the factor, 0.05, 0.1, 0.5, 1.0 and 2.5 mg $kg^{-1}$ to rabbits 1–7, 8–15, 16–23, 24–32 and 33–40, respectively, are shown in Fig. 1, and the relevant pharmacokinetic parameters are listed in Table 1.

TABLE 1

*Mean (±SD) pharmacokinetic parameters of HGM-CSF after intravenous infusion, 0.05, 0.1, 0.5, 1.0, and 2.5 mg $kg^{-1}$ and subcutaneous injection, 0.5 mg $kg^{-1}$ of the factor to rabbits 1–7, 8–15, 16–23, 24–32, 33–40 and 41–46, respectively*

| Dose | 0.05 mg $kg^{-1}$ (i.v.) | 0.1 mg $kg^{-1}$ (i.v.) | 0.5 mg $kg^{-1}$ (i.v.) | 1.0 mg $kg^{-1}$ (i.v.) | 2.5 mg $kg^{-1}$ (i.v.) | 0.5 mg $kg^{-1}$ (s.c.) |
|---|---|---|---|---|---|---|
| $t_{1/2}$ (min) | 11.3 ± 2.33 | 10.4 ± 3.21 | 39.2 ± 20.5 | 39.2 ± 7.07 | 51.6 ± 6.93 | 134 ± 53.3 |
| AUC (ng min $ml^{-1}$) | 2000 ± 562 | 4970 ± 1820 | 68100 ± 26100 | 126000 ± 39400 | 384000 ± 198000 | 57100 ± 26700 |
| MRT (min) | 11.6 ± 2.10 | 10.9 ± 4.49 | 22.9 ± 7.83 | 20.3 ± 5.18 | 18.3 ± 3.07 | |
| CL (ml $min^{-1}$ $kg^{-1}$) | 25.0 ± 8.38 | 21.1 ± 6.90 | 6.98 ± 2.11 | 7.94 ± 3.17 | 6.51 ± 3.61 | |
| $CL_R$ (ml $min^{-1}$ $kg^{-1}$) | 0.312 ± 0.453 | 0.223 ± 0.205 | 0.149 ± 0.170 | 0.434 ± 0.754 | 1.05 ± 1.06 | 0.132 ± 0.208 |
| $CL_{NR}$ (ml $min^{-1}$ $kg^{-1}$) | 24.6 ± 8.22 | 19.9 ± 6.70 | 6.87 ± 2.01 | 6.92 ± 3.31 | 4.24 ± 3.00 | |
| $V_{ss}$ (ml $kg^{-1}$) | 292 ± 64.5 | 211 ± 61.8 | 162 ± 34.8 | 162 ± 34.8 | 120 ± 66.1 | |
| $X_U$/dose (%) | 1.78 ± 1.49 | 1.40 ± 0.477 | 3.08 ± 1.36 | 10.9 ± 9.23 | 23.4 ± 13.3 | 2.22 ± 1.93 |

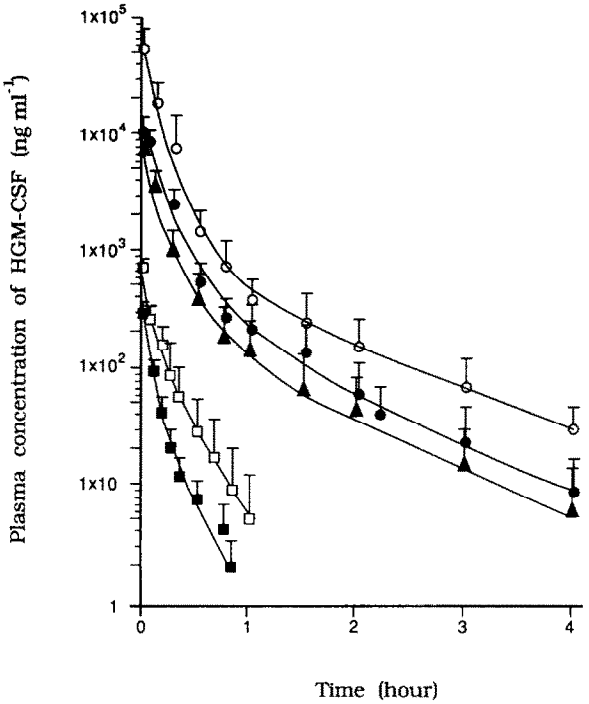


Fig. 1. Mean arterial plasma concentration-time profiles of HGM-CSF after intravenous administration of the factor, 0.05 mg $kg^{-1}$ (■), 0.1 mg $kg^{-1}$ (□), 0.5 mg $kg^{-1}$ (▲), 1.0 mg $kg^{-1}$ (●), and 2.5 mg $kg^{-1}$ (○) to rabbits 1–7, 8–15, 16–23, 24–32 and 33–40, respectively. Bars represent standard deviation.

The plasma levels of HGM-CSF declined polyexponentially in all the rabbits studied and fell more slowly with increasing doses. It should be noted that some of the pharmacokinetic parameters of HGM-CSF were dose-dependent over the dose ranges studied. A 50-fold increase in i.v. dose of HGM-CSF (0.05–2.5 mg $kg^{-1}$) produced a 74.0 and 82.8% reduction in the mean values of CL and $CL_{NR}$, respectively. The marked reduction of the CL with increasing doses was due to the reduced mean values of $CL_{NR}$ with increasing doses, since the mean $CL_R$ remained relatively constant over the dose ranges studied and the mean contributions of $CL_{NR}$ to CL were 98.4, 94.3, 98.4, 87.2 and 65.1% for the doses of 0.05, 0.1, 0.5, 1.0 and 2.5 mg $kg^{-1}$, respectively. Therefore, the dose-dependent CL and $CL_{NR}$ of HGM-CSF could be due to saturable metabolism in rabbits. The higher values of the contribution of $CL_{NR}$ to CL suggested that most of the i.v. administered HGM-CSF could be metabolized in rabbits. Although the exact metabolizing organ(s) for HGM-CSF has not yet been demonstrated, the kidney might be the main organ for metabolizing HGM-CSF as demonstrated for interferon (Bino et al., 1982a,b), and human growth hormone (Owens et al., 1973; Wallace and Stacy, 1975; Retegui-Sardou et al., 1977). The role of the kidney in metabolizing low-molecular-mass proteins has been reviewed (Maack et al., 1979).

The mean values of the terminal half-lives were also dose-dependent; the values were 11.3, 10.4, 39.2, 39.2 and 51.6 min for the doses of 0.05, 0.1, 0.5 1.0 and 2.5 mg $kg^{-1}$, respectively. The longer terminal half-lives of HGM-CSF, 150 min (Cebon et al., 1988) and 80–150 min (Morstyn et al., 1988), were reported when HGM-CSF, 0.3–1 $\mu$g $kg^{-1}$ and 3 $\mu$g $kg^{-1}$, respectively, were administered i.v. to patients. The differences in terminal half-lives could be due to species differences and/or differences in assay sensitivity and blood sampling schedules as reported for bumetanide (Choi et al., 1991). Dose-dependent half-lives of another hematopoietic growth factor, G-CSF, were also reported; the values were $1.4 \pm 0.3$ and $3.7 \pm 1.1$ h when G-CSF, 0.5–1.5 $\mu$g $kg^{-1}$ and 5–30 $\mu$g $kg^{-1}$, respectively, were administered to patients (Morstyn et al., 1989).

The mean values of $V_{ss}$ tended to decrease with increasing doses of HGM-CSF, and might be due to saturable binding of the factor to tissues; the mean values decreased by 58.9% for a 50-fold increase in the dose of the factor. The mean values of MRT tended to increase with higher doses of the factor. The % of dose excreted in 24 h urine as unchanged HGM-CSF ($X_U$/dose) appeared to increase with increasing doses; the mean values were 1.78, 1.40, 3.08, 10.9 and 23.4% at the dose of the factor, 0.05, 0.1, 0.5, 1.0 and 2.5 mg $kg^{-1}$, respectively. This may also support the saturable metabolism of HGM-CSF in rabbits.

The mean (solid line) and individual arterial plasma concentration-time profiles after s.c. administration of the factor, 0.5 mg $kg^{-1}$ to rabbits 41–46 are shown in Fig. 2, and the relevant mean pharmacokinetic parameters are also listed in Table 1. After s.c. administration, the peak concentration was reached at 2–5 h after injection

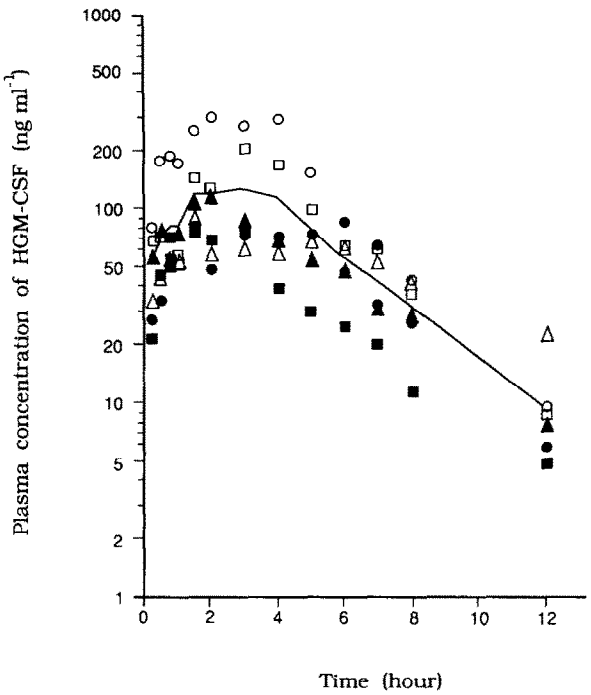


Fig. 2. Mean (solid line) and individual arterial plasma concentration-time profiles of HGM-CSF after subcutaneous administration of the factor, 0.5 mg kg$^{-1}$ to rabbits 41 (○), 42 (□), 43 (△), 44 (●), 45 (■) and 46 (▲).

and a second peak was evident in the plasma concentrations of HGM-CSF. Similar results were also reported when HGM-CSF, 3 $\mu$g kg$^{-1}$ was administered s.c. to patients (Morstyn et al., 1989). After reaching the second peak, the plasma concentrations declined monoexponentially with a mean terminal half-life of 134 min.

The amounts (ng per g tissue or organ) of HGM-CSF remaining in each tissue or organ at 2 h after s.c. injection are listed in Table 2. HGM-CSF was highly concentrated in the kidney and liver, and less concentrated in the other tissues or organs studied; the mean values of the tissue to plasma ratio (T/P) were 6.06, 1.14, 0.711, 0.694, 0.866, 0.891, 0.691, 0.753, 0.714, 0.674, 0.641, and 0.697 for the kidney, liver, small intestine, stomach, heart, lung, spleen, large intestine, muscle, brain, fat, and mesentery, respectively. This suggested that HGM-CSF does not appear to have a strong affinity for the tissues or organs studied except the kidney and liver. This was also supported in the present i.v. studies that the $V_{ss}$ of HGM-CSF was relatively small as listed in Table 1. Human growth hormone (Lee et al., 1993) and interferon-$\alpha$A (Jang et al., 1992) were also found to be highly concentrated in the kidney.

TABLE 2

*Amounts (ng per tissue) of HGM-CSF remaining in each tissue at 2 h after subcutaneous administration of the factor, 0.5 mg kg$^{-1}$ to rabbits 47–49*

| Rabbits | Kidney | Liver | S.I. [a] | Stomach | Heart | Lung | Spleen | L.I. [b] | Muscle | Brain | Fat | Mesentery | Plasma |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 47 | 69.8 | 33.5 | 19.1 | 18.5 | 25.4 | 25.0 | 18.2 | 21.0 | 18.7 | 19.3 | 16.5 | 18.3 | 23.8 |
| | (2.93) [c] | (1.41) | (0.803) | (0.777) | (1.07) | (1.05) | (0.765) | (0.882) | (0.786) | (0.811) | (0.693) | (0.769) | (1.00) |
| 48 | 239 | 26.4 | 18.2 | 18.1 | 22.3 | 22.9 | 18.1 | 19.0 | 18.5 | 16.9 | 16.6 | 18.1 | 18.1 |
| | (13.2) | (1.46) | (1.01) | (1.00) | (1.23) | (1.27) | (1.00) | (1.05) | (1.02) | (0.934) | (0.917) | (1.00) | (1.00) |
| 49 | 121 | 32.5 | 19.0 | 18.0 | 21.2 | 21.0 | 18.2 | 19.2 | 20.0 | 16.5 | 18.6 | 19.1 | 59.3 |
| | (2.04) | (0.548) | (0.320) | (0.304) | (0.357) | (0.354) | (0.307) | (0.328) | (0.337) | (0.278) | (0.314) | (0.322) | (1.00) |
| Mean | 143 | 30.8 | 18.8 | 18.2 | 23.0 | 23.0 | 18.2 | 19.7 | 19.1 | 17.6 | 17.2 | 18.5 | 33.7 |
| | (6.06) | (1.14) | (0.711) | (0.694) | (0.866) | (0.891) | (0.691) | (0.753) | (0.714) | (0.674) | (0.641) | (0.697) | (1.00) |
| S.D. [d] | 86.8 | 3.84 | 0.493 | 0.265 | 2.18 | 2.00 | 0.0577 | 1.10 | 0.814 | 1.51 | 1.18 | 0.529 | 22.3 |
| | (6.20) | (0.513) | (0.354) | (0.355) | (0.465) | (0.478) | (0.352) | (0.378) | (0.347) | (0.349) | (0.305) | (0.345) | (0.00) |

[a] Small intestine.
[b] Large intestine.
[c] Tissue to plasma ratio (T/P).
[d] Standard deviation.

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