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Simultaneous determination of glycyl–L-histidyl–L-lysine and its metabolite, L-histidyl–L-lysine, in rat plasma by high-performance liquid chromatography with post-column derivatization

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Abstract

A selective and sensitive high-performance liquid chromatographic (HPLC) method was developed for the determination of glycyl-L-histidyl-L-lysine (GHK), a liver-cell growth factor isolated from human plasma, and its metabolite, L-histidyl-L-lysine (HK), in rat plasma. Both high selectivity and sensitivity were achieved by the use of solid-phase extraction with a Bond-Elut Certify cartridge, ion-pair chromatography with 1-pentanesulfonate on a 5-μm Capcell Pak C_{18} UG120 column (250×4.6 mm I.D.) with a guard column, and by post-column derivatization with *o*-phthalaldehyde (OPA). GHK and HK were extracted from 0.1 ml of rat plasma after addition of *o*-phenanthroline to protect against degradation. The limit of detection for GHK and HK were 50 and 15 ng/ml, respectively, and the calibration curves were linear in the range 0.1–5.0 μg/ml. The developed method was applied to the pharmacokinetic study of GHK after a single dose was administered intravenously to rats. GHK was rapidly degraded to HK, which was eliminated rapidly.

Keywords: Derivatization, LC; Glycy-L-histidyl-L-lysine; L-Histidyl-L-lysine; Peptides

1. Introduction

Glycyl-L-histidyl-L-lysine (GHK) is a tripeptide isolated from human plasma [1] and seems to be involved in the transport of ionic copper(II) [2] and as a growth factor for a variety of cultured cells [3]. In addition, the ability of GHK to form complexes with copper(II) ions suggests that it has a physiological role in the processes of wound healing and tissue repair [4].

To evaluate it possible use in clinical applications, its pharmacokinetic profile should be established. For this purpose, an analytical method for the determination of GHK in plasma becomes necessary. However, to date, no method has been available for the highly sensitive determination of GHK due to the difficulty of selective extraction, its low UV absorbance and its instability in plasma.

Due to its high hydrophilicity, GHK and its metabolite, L-histidyl-L-lysine (HK), are difficult to extract selectively from plasma and to separate from other endogenous plasma components on a C_{18} column. Therefore, we sought to establish a method for the accurate determination of GHK and HK in plasma.

We report a highly selective and sensitive method for the simultaneous determination of GHK and HK in rat plasma by extraction with a Bond-Elut Certify

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cartridge, as the first step, followed by high-performance liquid chromatography (HPLC) and post-column derivatization with *o*-phthalaldehyde (OPA). The developed method was applied to the determination of GHK and HK after intravenous administration of a single dose to rats.

2. Experimental

2.1. Chemicals and reagents

Glycyl–L-histidyl–L-lysine (GHK) acetate and L-histidyl–L-lysine (HK) HBr were purchased from Peptide Institute (Osaka, Japan) and Bachem Feinchemikalien (Bubendorf, Switzerland), respectively. *o*-Phthalaldehyde (OPA) was of fluorogenic detection grade (Nacalai Tesque, Kyoto, Japan). 1-Pentanesulfonate was of ion-pair reagent grade (Wako, Osaka, Japan) and 2-mercaptoethanol was of biochemical grade (Wako). Methanol and acetonitrile were of HPLC grade (Wako). All other reagents were of analytical grade. Water was purified with a Milli-Q system (Nihon Millipore, Tokyo, Japan) after deionization and distillation.

The OPA reagent solution was prepared as follows: 4 ml of a freshly prepared methanolic solution of OPA (0.2 g/ml), 1 ml of 2-mercaptoethanol and 6 ml of 10% (w/v) Brij 35 aqueous solution were added to 1 l of 0.4 *M* potassium hydroxide aqueous solution containing 0.4 *M* boric acid. The obtained solution was sonicated for approximately 10 min.

2.2. Chromatographic system and conditions

The HPLC system (Fig. 1) consisted of two PU-980 pumps, an AS-950 autosampler, an LCSS-900 system controller, a CO-965 column oven, a DG-980-50 degasser, an FP-920 fluorescence detector (all from JASCO, Tokyo, Japan) and a C-R5A integrator (Shimadzu, Kyoto, Japan). The Capcell Pak C_{18} UG120 column (250×4.6 mm I.D., 5 μm particles) and the Capcell Pak UG120 guard column (10×4.0 mm I.D.) were obtained from Shiseido (Tokyo, Japan). The preheating coil (3 m×0.1 mm) and the reaction coil (2 m×0.5 mm) were made from stainless steel tubing for HPLC (GL Sciences, Tokyo, Japan). The temperature of the columns, the preheating coil and the reaction coil was maintained at 55°C using the column oven. Excitation and emission wavelengths were 340 and 450 nm, respectively. The mobile phase was 0.05% (v/v) *o*-phosphoric acid containing 7% (v/v) acetonitrile and 22.5 m*M* 1-pentanesulfonate. The flow-rate was maintained at 0.9 ml/min. The OPA reagent was pumped at a flow-rate of 0.3 ml/min.

2.3. Sample preparation

Plasma samples (0.1 ml) were diluted with 2.5 ml of 0.1 *M* potassium phosphate buffer (pH 3) con-

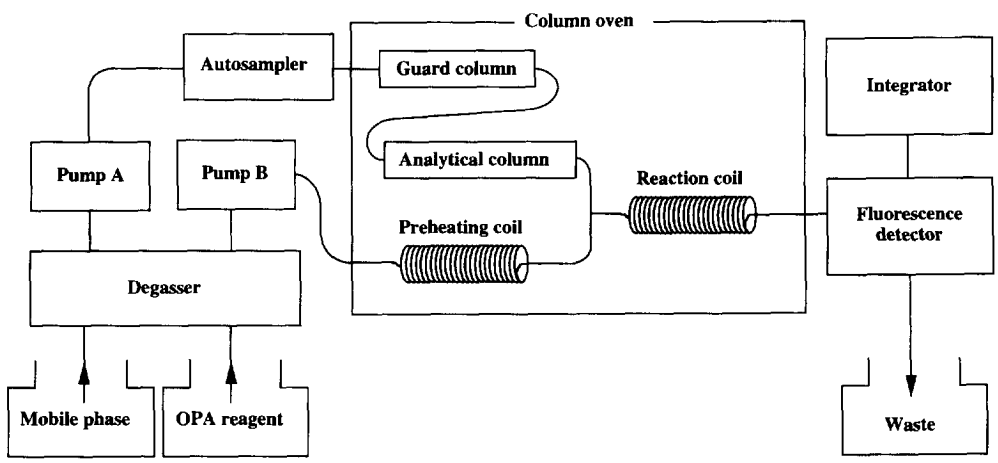

Fig. 1. Scheme of the chromatographic system.

taining 0.1% (w/v) disodium ethylene diamine tetraacetic acid (EDTA). The mixtures were applied onto the Bond-Elut Certify cartridges (130 mg) obtained from Varian Associates (Harbor City, CA, USA), which were preconditioned with 2.5 ml of methanol and 2.5 ml of 0.1 *M* potassium phosphate buffer (pH 3). The cartridges were then sequentially washed with 2.5 ml of 0.01 *M* acetic acid, 2.5 ml of methanol and 2.5 ml of methanol–25% ammonium hydroxide (100:0.04, v/v). Finally, GHK and HK were eluted with 2.5 ml of methanol–25% ammonium hydroxide (5:1, v/v). The samples were evaporated under a stream of nitrogen at room temperature and the residues were redissolved in 200 μl of the mobile phase. The samples were centrifuged in a Microfuge E (Beckman Instruments, Palo Alto, CA, USA) for 1 min and 50 μl of the supernatants were injected onto the HPLC column.

2.4. Standard solution and calibration curve

Both stock solutions of GHK and HK were prepared at 100 μg/ml in 0.1 *M* potassium phosphate solution containing 0.1% (w/v) disodium EDTA and were stored at 4°C. Working solutions containing identical amounts of GHK and HK were prepared at 1, 2, 10 and 50 μg/ml by appropriate dilution of the stock solutions with the phosphate solution.

To 0.1 ml volumes of blank rat plasma, 10 μl of a 50% (w/v) aqueous solution of *o*-phenanthroline were added and mixed well. The plasma was spiked with 10 μl of working solution containing the same amounts of GHK and HK to provide final concentrations of 100, 200, 1000 and 5000 ng/ml. These plasma samples were analyzed as described above. Each standard calibration curve for GHK and HK was obtained by plotting the peak height against concentration.

2.5. Validation of the method

The absolute recoveries of GHK and HK were assessed at both a low (100 ng/ml) and a high (5000 ng/ml) concentration by comparing the peak height after extraction with the peak height obtained from direct injection of the same amount of GHK and HK standard. The analytical method was further evaluated to assess intra-day and inter-day variation. The intra-day repeatability of the method was determined by multiple analysis of individual samples on the same day. Inter-day reproducibility was assessed on three different days.

3. Results and discussion

3.1. Sample preparation, chromatography and derivatization

Both selective extraction and high recovery were achieved by solid-phase extraction with Bond-Elut Certify cartridges. The washing procedure of the cartridge with 2.5 ml of methanol–25% ammonium hydroxide (100:0.04, v/v) was very effective for elution of the majority of interfering endogenous substances and it did not result in a significant loss of GHK and HK. Other cartridges for solid-phase extraction such as C_{18}, C_8, CN and CH did not permit selective extraction.

GHK and HK were separated from the remaining endogenous components by ion-pair chromatography using 1-pentanesulfonate. Retention of GHK and HK on the C_{18} column was selectively enhanced by the addition of 1-pentanesulfonate as the ion-pairing agent.

As GHK and HK do not have sufficient UV-absorbing power for the required sensitive determination, these compounds have to be derivatized. OPA has been used for the detection of amino acids and various peptides as fluorescent derivatives [5,6]. The OPA derivatives can be generated rapidly and simply by mixing amino acids/peptides with the OPA-reagent. However, the OPA derivatives are labile and, for this reason, pre-column derivatization with OPA is not suitable for routine analysis. So, GHK and HK were reacted with OPA by means of "on-line" post-column derivatization. In order to achieve optimum derivatization of GHK and HK, several conditions were studied, such as reaction temperature, reaction time, reagent flow-rate and the concentration of OPA and of 2-mercaptoethanol. The optimized conditions are given in the Section 2. The OPA derivatives of GHK and HK were rapidly generated at 55°C within 30 s.

3.2. Stability of GHK and HK in rat plasma

GHK is reported to be unstable in human plasma [7], as it is rapidly degraded by aminopeptidases [8]. For this reason, the stability of GHK and HK in rat plasma was examined at 37°C. Chelating agents such as *o*-phenanthroline and EDTA are well known as inhibitors of metalloenzymes [9], and some aminopeptidases are also inhibited by these agents [10,11]. Thus, the effect of *o*-phenanthroline on the stability of GHK and HK in rat plasma was evaluated (Fig. 2). GHK and HK were unstable in fresh rat plasma at 37°C and more than 90% of GHK and HK was degraded within 15 min. Conversely, in rat plasma containing *o*-phenanthroline at a final concentration of 5 mg/ml, both GHK and HK were stable at 37°C for at least 60 min. As a consequence, *o*-phenanthroline was added to plasma or blood samples that were collected during the pharmacokinetic study.

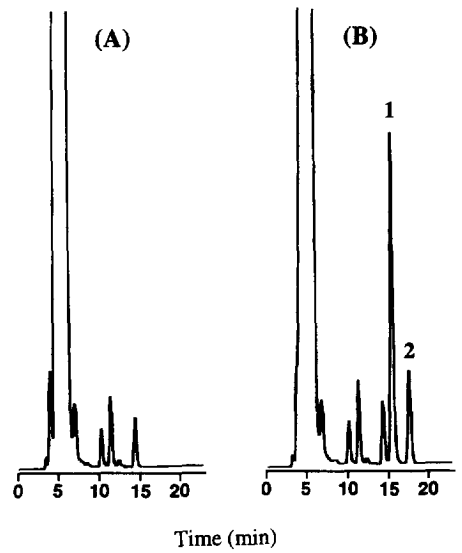

Fig. 3. HPLC chromatograms of blank plasma (A) and plasma spiked with 5 μg/ml GHK and HK (B). Peaks: 1=HK; 2=GHK.

3.3. Validation of the method

Fig. 3 shows chromatograms of the blank plasma (A) and blank plasma cantaining GHK and HK (B). The retention times of HK and GHK were 15.9 and 18.1 min, respectively. The chromatogram of blank plasma showed some additional peaks that were attributable to endogenous components, but these signals did not have any influence on either chro-

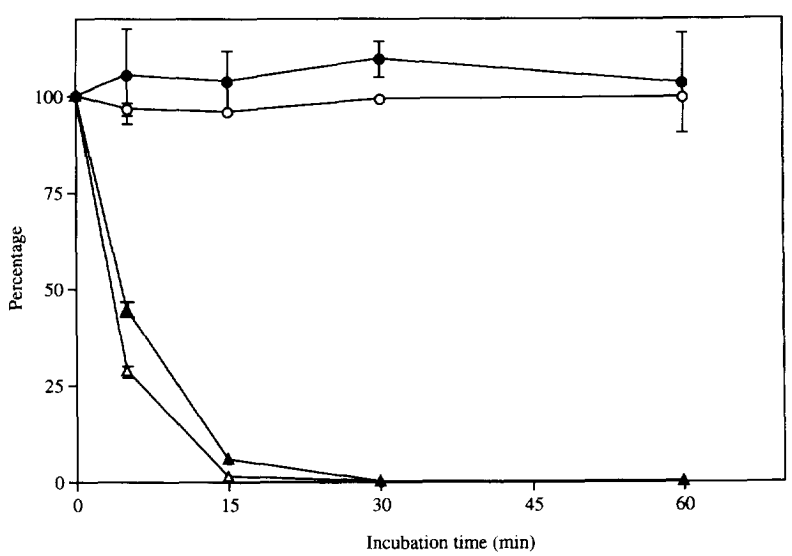

Fig. 2. Stability of GHK (●, ▲) and HK (○, △) in rat plasma with or without 5 mg/ml of *o*-phenanthroline. (●, ○) Samples were incubated in rat plasma with 5 mg/ml of *o*-phenanthroline. (▲, △) Samples were incubated in rat plasma without *o*-phenanthroline. Each point represents the mean±S.D. of results obtained from three samples.

Table 1
Precision and accuracy

Compound	Added concentration (ng/ml)	Intra-day ($n=5$)			Inter-day (three days, $n=5$)		
		Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)	Accuracy (%)	Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
GHK	100	94.4±6.4	6.8	−5.6	91.8±5.0	5.4	−8.2
	5000	5435.2±318.3	5.9	8.7	4996.7±408.0	8.2	−0.1
HK	100	95.9±5.98	6.2	−4.1	88.9±7.48	8.4	−11.1
	5000	5031.1±354.3	7.0	0.6	4931.5±148.2	3.0	−1.4

matographic separation or quantitation. The calibration curves for GHK and HK were linear in the range of 100 to 5000 ng/ml and the limits of detection (signal-to-noise ratio of three) were 50 and 15 ng/ml, respectively. The correlation coefficients (r^2) were greater than 0.999. The extraction recoveries for GHK and HK were greater than 88.3 and 90.8%, respectively. The intra- and inter-day assay precision and accuracy for low and high concentrations of GHK and HK are summarized in Table 1. The intra-day coefficient of variation (C.V.) and the accuracy for GHK or HK were 7.0% or less and 8.7% or less, respectively. The inter-day C.V. and accuracy for GHK or HK were less than 8.4 and 11.1% or less, respectively.

3.4. Application to a pharmacokinetic study

The established method was applied to the pharmacokinetic study of GHK in male rats. A GHK saline solution (1%, w/v) was injected into the tail vein of rats at a dose of 10 mg/kg. Blood samples (0.3 ml) were collected prior to and at 0.5, 1, 2, 3, 5, 10, 15, 20, 30 and 60 min after administration. Blood samples were transferred immediately to heparinized microfuge tubes, each containing 3 μl of 50% (w/v)

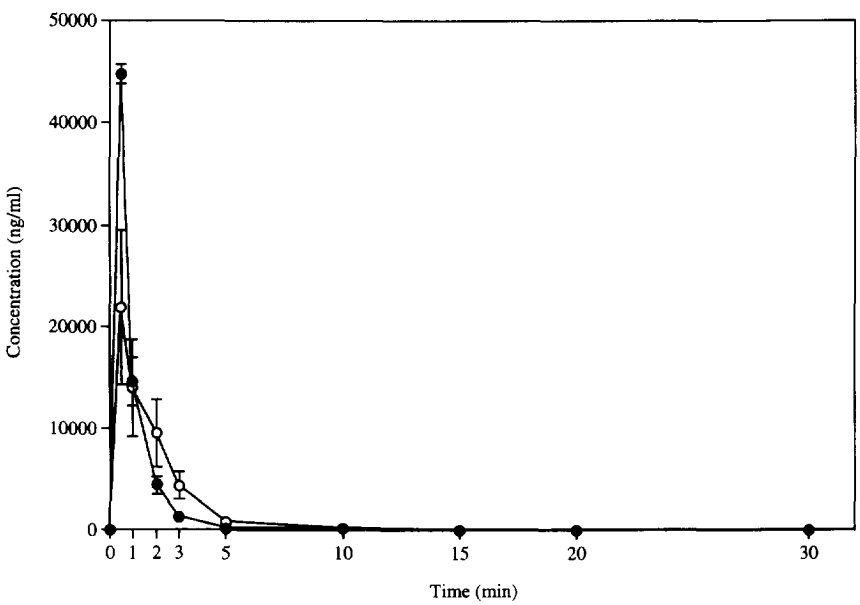

Fig. 4. Plasma concentration of GHK (●) and its metabolite HK (○) after a single intravenous administration of GHK at a dose of 10 mg/kg to male rats. Each point represents the mean±S.D. of results obtained from three rats.

o-phenanthroline, and were centrifuged for 1 min. The resulting plasma (0.1 ml) was analyzed by the method described above. Samples with a concentration of greater than 5 μg/ml were reanalyzed after appropriate dilution with blank plasma.

The plasma concentration–time profiles of GHK and HK after intravenous administration to male rats are shown in Fig. 4. GHK and HK were not detected in pre-dose plasma samples. After the injection, GHK was rapidly degraded to HK, which was also rapidly eliminated from circulating blood.

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