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RESEARCH ARTICLE

Determination and Pharmacokinetics of Recombinant Human Growth Hormone in Rabbit Plasma by Spectrofluorometry Method [1]

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Summary

In this study, a new spectrofluorometry method was developed for the determination of human growth hormone (somatotropin) in rabbit plasma. The validation was assessed under a variety of conditions (FDA, Bioanalytical Method Validation Guidline). Calibration curves were linear between the concentration range of 0.125-8.0 µg/mL. Intra- and inter-day precision values for somatotropin in plasma were less than 13.5, and accuracy (relative error) was better than 12.0%. The recoveries for all samples were >84.0%. The limits of detection (LOD) and quantification (LOQ) of somatotropin were 0.075 and 0.10 µg/mL, respectively. The described spectrofluorometry method has adequate sensitivity and specificity to study pharmacokinetics of somatotropin in rabbits, and could be adapted also to clinical pharmacokinetic study.

Keywords: *Human growth hormone, Spectrofluorometry, Liquid-liquid extraction, Rabbit, Pharmacokinetic study*

Spektroflorometri Yöntemi Kullanarak Tavşan Plazmasında Rekombinant İnsan Büyüme Hormonunun Tayini ve Farmakokinetiği

Özet

Bu çalışmada, insan büyüme hormonunun (somatotropin) tavşan plazmasında tayini için yeni bir spektroflorometri yöntemi geliştirildi. Geçerlilik FDA'nın "Bioanalitik Yöntem Validasyonu" rehberinde belirtildiği koşullarda yapıldı. Kalibrasyon eğrileri 0.125-8.0 µg/mL derişim aralığında doğrusaldır. Plazmada somatotropin için gün içi ve günler arası kesinlik değerleri %13.5'den küçüktü ve doğruluk (bağıl hata) %12.0'dan daha iyiydi. Bütün örnekler için geri kazanım >%84.0 idi. Somatotropinin belirlenebilen (LOD) ve ölçülebilen (LOQ) en düşük değeri sırasıyla 0.075 and 0.10 µg/mL'dir. Tanımlanan spektroflorometri yöntemi tavşanlarda somatotropinin farmakokinetiğini incelemek için yeterli duyarlılığa ve özgüllüğe sahiptir ve klinik farmakokinetik çalışmalara da uyarlanabilir.

Anahtar sözcükler: *İnsan büyüme hormonu, Spektroflorometri, Sıvı-sıvı ekstraksiyon, Tavşan, Farmakokinetik çalışma*

INTRODUCTION

Synthetic human growth hormone (somatotropin) is manufactured by recombinant DNA technology. It is a 191 amino acid polypeptide (MW 22 kDa) with an amino acid sequence and two internal disulphide bridges identical to that of the major component of human pituitary growth hormone. Therapeutically, it is used in children to treat growth retardation, for example short stature due to insufficient growth hormone secretion, Turner's syndrome or chronic renal insufficiency. In adults, it is used as a treatment for growth hormone deficiency and for

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management of HIV-related wasting and cachexia [1].

Several analytical methods for the quantitative and qualitative estimation of somatotropin have been described, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) frequently combined with immunoblotting [2-4], capillary zone electrophoresis [5], size-exclusion [6], hydrophobic-interaction [7,8], reversed phase [9,10] high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) [11] and enzyme-linked immunosorbent assay (ELISA) [12,13].

The pharmacokinetics and the quantitation of somatotropin has been described in sheep [1], rabbit [13] human plasma [14] and dog serum [15] by chemiluminescent immunometric assay, ELISA, RIA, immunoradiometric assay, respectively. But, pharmacokinetics of somatotropin has not been described in rabbits by spectrofluorometry in the literature.

As we known, non-immune method for somatotropin detection had insufficient sensitivity compared with immune method because of the interferences from the sample matrix. However, the fluorescence spectroscopy [16] has excellent sensitivity in determining many fluorescence compounds (pharmaceutical, biomedical, etc.), including aromatic aminoacides that exhibit particularly intense fluorescence.

Therefore, the aim of this work was to develop a method using a sensitive and specific spectrofluorometry method, and to validate the whole analytical method, according to international guidelines [17] in order to obtain an efficient tool for further pharmacokinetic studies in rabbit plasma.

MATERIAL and METHODS

Spectrofluorometry System

All fluorescence measurements were done on a SHIMADZU RF-5301 PC spectrofluorometer equipped with a 150 W Xenon lamp. Experimental parameters were: slit width; 5.0 nm, l_{exc}=275 nm, l_{em}=330 nm.

Chemicals and Reagents

Somatotropin was provided by Eli Lilly & Co. (Indianapolis, USA). Humatrope (1.33 mg somatropin) was obtained from Department of Endocrinology and Metabolism, Faculty of Medicine, Ataturk University. Water was Milli-Q grade and all other chemicals and solvents used were of analytical grade. Buffer solution (potassium dihydrogen phosphate, KH_2PO_4) was prepared with deionised water.

Preparation of Plasma Standards and Controls

A standard stock solution containing somatotropin was prepared in 25 mM phosphate buffer solution. Phosphate buffer was prepared in double distilled filtered water and its pH was adjusted to 7.4±0.02 with sodium hydroxide solution. Final concentration of somatotropin is 100 µg/mL. Standard calibration solutions were prepared by spiking drug-free rabbit plasma with stock standard solutions, which were then further diluted to achieve final concentrations of between 0.125-8.0 µg/mL (0.125, 0.25, 2, 4, 6, 8 µg/mL) of somatotropin. Somatotropin plasma control samples were prepared from a separate stock solution at concentrations of 2, 5, 7 µg/mL.

Extraction Procedure

Rabbit plasma (0.25 mL) was placed in a 12 mL capacity glass tube. Standard solution (1 mL) was added into the plasma and the solution was thoroughly vortexed. Then, 2.5 mL acetic acid was added into the plasma and the solution was vortexed for 2 min. The sample was centrifuged for 5 min at 6.000 rpm. The supernatant was transferred into another glass tube and evaporated to dryness at 60°C under a stream of nitrogen. The dried residue was reconstituted in 4 mL of 25 mM buffer solution. All samples were filtered through a Phenomenex membrane of 0.45-µm pore size (25 mm filter).

Animal Material

The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University (2006/1). The rabbits are female which is 2.2-2.5 kg weight. Five rabbits were housed with free access to food and water, except for the final 2 h before experiment. After placing the animals in a restraining box, cannulations of the auricular artery and vein in the opposite ears were done. This validated method was then applied to determination of somatotropin concentrations in five rabbits after intravenous administration of 0.1 mg/kg of Humatrope. 1 mL volume of blood was obtained from the artery at time zero and at 5, 10, 15, 20, 30, 45 and 60 min after 0.1 mg/kg intravenous administration of somatotropin to rabbits. The blood was collected into EDTA tubes. The blood was immediately centrifuged 6000 × *g* for 10 min at ambient temperature. The plasma was separated and analyzed for somatotropin concentrations as described above.

RESULTS

Linearity

Fluorescence emission intensity was measured immediately at 330 nm exciting at 275 nm. The linearity of calibration graphs was demonstrated by the good determination coefficients (r^2) obtained for the regression line *(Fig. 1)*.

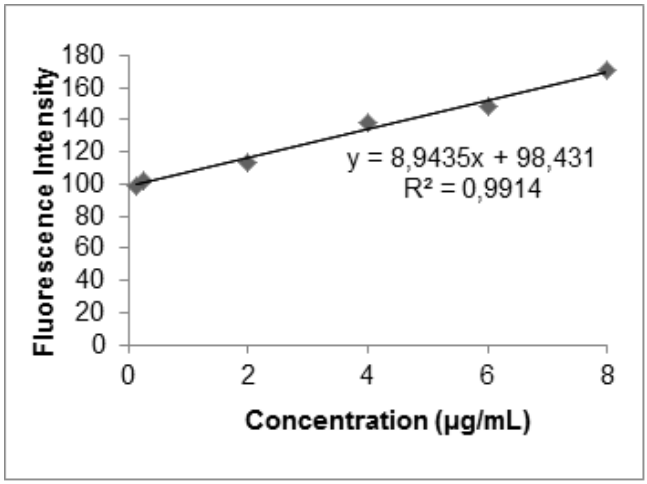

Fig 1. Calibration curve for plasma somatotropin standards
Şekil 1. Plazma somatotropin standartları kalibrasyon eğrisi

The mean regression equation is y=8.9435x+98.431 (0.9914). The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope (S_b) and intercept (S_a) on the ordinate. S_a, standard deviation of intercept of regression line is 14.780 and S_b, standard deviation of slope of regression line is 1.738.

Precision and Accuracy

Precision and accuracy were determined on spiked rabbit plasma samples at six concentrations with respect to a calibration graph prepared every day. The precision of the method was evaluated as the intra- and inter-day RSD of the measured peak intensity by assaying spiked plasma samples at three different concentrations. All samples for these purposes were freshly prepared including preparing the standard solution from the same stock solution (100 µg/mL). Three replicates from each pool were assayed on each of 2 days so that both intra- and inter-day precision and accuracy could be determined. The results for somatotropin in rabbit plasma are shown in *Table 1*.

Precision and accuracy studies in plasma showed an acceptable the RSD values and the relative errors were <13.5% and high accuracy for both intra- and inter-day (n = 3) studies (≤12%).

Limits of Quantitation and Detection

The limit of quantitation (LOQ) is defined as the lowest plasma concentration in the calibration curve that could be measured routinely with acceptable precision and accuracy (RSD<20%). The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOQ and LOD values for somatotropin in rabbit plasma were determined 0.10 µg/mL and 0.075 µg/mL, respectively.

Recovery

The extraction recovery of somatotropin in rabbit plasma was determined by comparing with precision and accuracy studies. The one-step extraction procedure was fairly rapid. Acetic acid solvent was selected for our liquid-liquid extraction method. The solvent acetic acid gave good recovery and the absolute recoveries of somatotropin from plasma were between 84 and 112 % as shown in *Table 2*.

Table 1. *Intra-day and inter-day precision and accuracy of somatotropin in plasma (n=6)*
Tablo 1. *Plazmada somatotropinin gün-içi ve günler-arası kesinlik ve doğruluğu (n=6)*

Added (µg/mL)	Intra-day			Inter-day		
	Found (Mean±SD[a])	Precision %RSD	Accuracy[b]	Found (Mean±SD[a])	Precision %RSD	Accuracy[b]
Plasma[c]						
2	2.03±0.049	2.41	1.50	2.24±0.238	10.63	12.00
5	5.23±0.176	3.37	4.60	5.51±0.646	11.72	10.20
7	7.16±0.633	8.84	2.29	7.64±1.029	13.47	9.14

SD[a]: *Standard deviation of six replicate determinations,* ***RSD:*** *Relative standard deviation,* ***Accuracy[b]:*** *(% relative error) (found-added)/addedx100,* ***[c]:*** *Plasma volume (0.25 mL)*

Table 2. *Recovery of somatotropin in plasma (n=3)*
Tablo 2. *Plazmada somatotropinin geri kazanımı (n=3)*

Added (µg/mL)	Found±SD	Recovery Ratio[a] (%)	Accuracy Relative Error (%)	%RSD
2	2.24±0.238	112.0	12.00	10.63
6	5.05±0.796	84.17	-15.83	15.76
8	8.60±1.391	107.5	7.50	16.17

[a] *Mean values*

Stability

For the determination of the stability of somatotropin in plasma at room temperature, +4°C and -20°C refrigeration temperature, low (2.0 µg/mL) and high (6.0 µg/mL) somatotropin concentrations were kept for 24 h and 3 days. Then the stability measurements were carried out. Plasma sample were found to be stable after 24 h with no significant change in concentration when stored at +4 and -20°C. The results of these stability studies are given in *Table 3*.

Application of the Method

The new method had been successfully applied to the analysis of samples from a pharmacokinetic study consisting of five rabbits *(Fig. 2)*.

In particular, the data obtained from rabbits were satisfactorily fitted to a linear one-compartment open model. *Fig. 3* represents the mean plasma concentration-time profile of five rabbits.

Table 3. *Stability of somatotropin in plasma (n=3)*
Tablo 3. *Plazmada somatotropinin stabilitesi (n=3)*

Concentration (µg/mL)	Room Temperature 24 h	Room Temperature 72 h	Refrigaratory +4°C, 24 h	Refrigaratory +4°C, 72 h	Frozen -20°C, 24 h	Frozen -20°C, 72 h
2	109.6±9.28	122.1±13.65	108.4±8.22	117.7±10.54	106.5±5.97	116.3±11.32
6	108.7±6.32	117.6±9.53	107.5±7.53	114.8±9.21	104.9±6.53	112.8±8.76

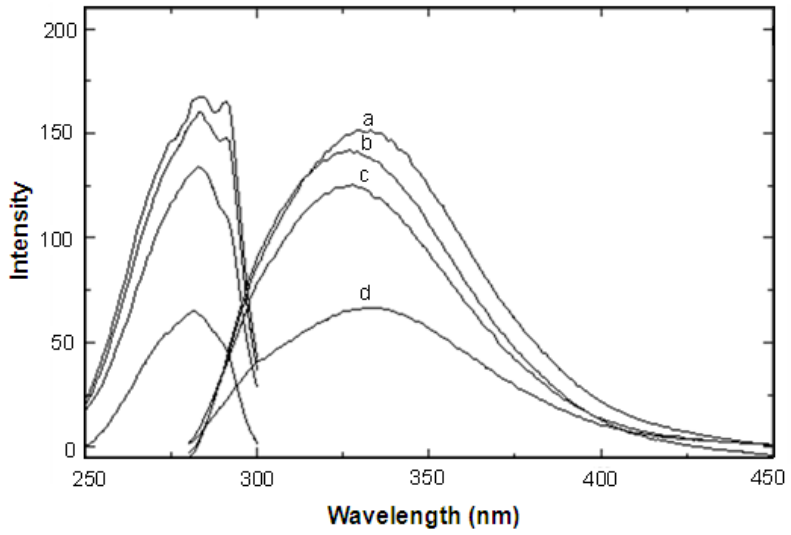

Fig 2. Excitation and emission spectra of a plasma sample collected from rabbit after intravenous administration of 0.1 mg/kg of Humatrope (d) rabbit sample prior to infusion, (a) rabbit sample at 5 min postinfusion, (b) rabbit sample at 15 min postinfusion, (c) rabbit sample at 30 min postinfusion

Şekil 2. Humatrope'un 0.1 mg/kg intravenöz verilmesinden sonra tavşandan alınan plazma örneğinin uyarma ve yayılma spektrumları (d) infüzyondan önceki tavşan örneği, (a) infüzyondan 5 dakika sonraki tavşan örneği, (b) infüzyondan 15 dakika sonraki tavşan örneği, (d) infüzyondan 30 dakika sonraki tavşan örneği

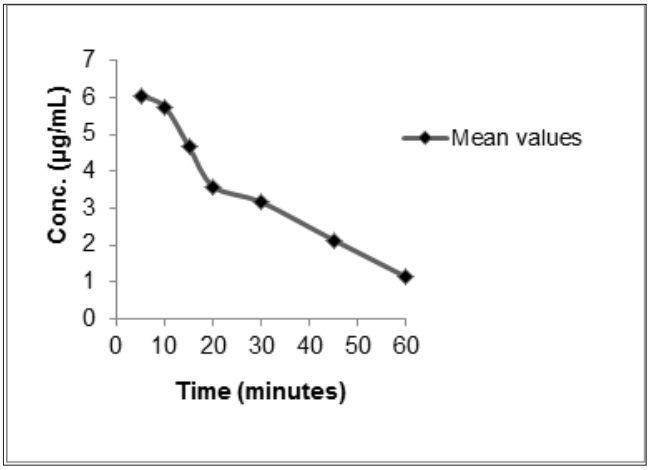

Fig 3. Mean plasma somatotropin concentration-time profile for five rabbits after a intravenous injection of 0.1 mg/kg somatotropin

Şekil 3. Beş tavşan için 0.1 mg/kg somatropinin tek intravenöz verilmesinden sonra ortalama plazma somatotropin derişim-zaman profili

In the developed method, C_{max} for somatotropin was 8.94 µg/mL. Somatotropin was well tolerated after a single intravenous infusion. Plasma somatotropin concentration was declined rapidly after 30 minutes somatotropin infusion. Somatotropin began to disappear from plasma after 1 h. The $AUC_{0-1\,h}$ and $t_{1/2}$ were 3886.57 µg h/L and 0.3416 h, as different from that of previous report [13]. The major pharmacokinetic parameters are reported in *Table 4*.

DISCUSSION

For spectrofluorometry method, various solvent systems (water, methanol and phosphate buffer) were investigated. The final decision for using phosphate buffer as the solvent was based on sensitivity, suitability for drug content determination and stability studies.

It has good precision and accuracy that this sensitive

Table 4. *Mean pharmacokinetic parameters of somatotropin for five rabbits after intravenous injection of 0.1 mg/kg somatotropin*

Tablo 4. *Beş tavşan için 0.1 mg/kg somatotropin intravenöz verilmesinden sonra somatotropinin ortalama farmakokinetik parametreleri*

Parameter	Mean	SD	% RSD
C_{max} (µg/mL)	8.94	2.743	30.68
$AUC_{0\text{-}1\ h}$ (µg h/L)	3886.57	898.66	23.12
$t_{1/2}$ (h)	0.3416	0.156	45.67
Cl (L/h)	89.80	3.810	4.24
k_{el} (h^{-1})	2.45	1.181	48.20
V_d (L)	0.025	0.011	44.00

C_{max}: *Maximum concentration,* ***$AUC_{0\text{-}1h}$:*** *Area under the plasma concentration-time curve,* ***$t_{1/2}$:*** *Half-life,* ***Cl:*** *Clearance,* ***k_{el}:*** *Elimination constant,* ***V_d:*** *Volume of distribution*

method has been studied in plasma. Plasma samples were extracted with acetic acid in a one-step liquid-liquid extraction. And only one-step extraction procedure was fairly rapid and advantage. In the developed method, 0.25 mL of plasma was used in the sample preparation.

Pharmacokinetic parameters such as half-life ($t_{1/2}$), apparent distribution volume (V_d), clearance (Cl) and area under the plasma concentration-time graph (AUC) were calculated by standard formulas [18]. Somatotropin elimination after a single intravenous injection was regarded a first-order reaction kinetic following the equation $C = C_0e^{-kt}$, where C represents somatotropin concentration in any time points, C_0 is the concentration when time (t) equals zero and elimination rate constant (k_{el}) is the first-order rate constant expressed in units of concentration per hour. *Kel* and $t_{1/2}$ were calculated from the slope of the linear regression line in the elimination phase of the semi-logarithmic plot of plasma concentration versus time as $\log C = \log C_0 - k_{el}\, t/2.3$. Results were expressed as V_d, Cl, k_{el}, $t_{1/2}$ and AUC as they apply to one-compartment open linear model. $t_{1/2}$ was calculated as $0.693/k_{el}$. The area under the plasma concentration-time curve $AUC_{0-1\ h}$ was calculated on the experimental values (trapezoidal rule) with extrapolation to infinity, obtained by the elimination rate constant. V_d and Cl were calculated as D/C_0 and V_dk_{el}, respectively.

In previous reports, chemiluminescent immunometric assay [1], ELISA [13], RIA [14] and immunoradiometric assay [15] methods were developed for the pharmacokinetics of somatotropin in sheep, rabbit, human plasma and dog serum, respectively. Pharmacokinetic application with intravenous somatotropin in dogs revealed that somatotropin followed a two-compartment open model with one distribution phase and one elimination phase. Elimination half-life in sheeps was 0.574 h [15].

In conclusion, we have developed a rapid, sensitive, precise and accurate spectrofluorometry method for determination of somatotropin in rabbits. To our knowledge, this is the first description of somatotropin pharmacokinetics in rabbit plasma by spectrofluorometry method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of somatotropin for clinical use.

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