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Quantitative Analysis of Thymosin α1 in Human Serum by LC-MS/MS

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ABSTRACT A high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was developed to measure the thymosin alpha 1 (Tα1) concentration in human serum. Tα1 in human serum was determined by solid phase extraction and reverse phase LC-MS/MS. The high-performance liquid chromatography (HPLC) system interfaced with the MS/MS system with a Turbo Ion spray interface. Positive ion detection and multiple reaction monitoring (MRM) mode were used for this human serum quantitation. Eight different concentration standards were used to establish the detection range. Six quality control (QC) and 2 matrix blanks were checked by calibration curves performed on the same day. The lower quantitation limit was 0.5 ng/mL T α1 in human serum. Calibration curves were established between 0.5 to 100 ng/mL by weighted linear regression. The correlation coefficients for different days were 0.9955 or greater. Quantitation of Tα1 by the LC-MS/MS method is fast, accurate, and precise.

KEYWORDS: Thymosin α1, LC-MS/MS, solid phase extractiuon, peptide analysis.

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INTRODUCTION

Thymosin alpha 1 (Tα1) is a peptide that has been evaluated for its immunomodulatory activities and therapeutic potential in several diseases, including chronic hepatitis B [1-3] and C [4-6], acquired immunodeficiency syndrome (AIDS) [7,8], depressed response to vaccination [9-11], and cancer [12,13]. The basis for Tα1 effectiveness in these conditions may be through modulation of immunological responsiveness, as Tα1 has been shown to have effects on immune modulators and to increase T-cell differentiation and maturation. Tα1 has been shown to increase production of IFNγ[interferon gamma? Per AMA the abbreviation for interferon is INF.], interleukin 2 (IL-2), IL-3, and expression of IL-2 receptor following activation by mitogens or antigens [1,14,15]. This pattern of enhanced cytokine production (ie, IFNγ and IL-2) suggests that Tα1 favors progression to a type 1 T helper cells (Th1) type of immune response. Tα1 has also been shown to increase natural killer (NK)-cell activity [16,17]and increase antibody response to T-cell-dependent antigens [18,19]. Recently, Tα1 was shown to antagonize dexamethasone-induced apoptosis in thymocytes in vitro in a dose-dependent fashion [20]. Tα1 stimulates thymopoiesis in a human coculture system by increasing the number of thymocytes and expanding CD44+25+3- and CD3+CD4+ T cells [21].

Tα1 is a 28– amino acid peptide with an acetylated N-terminus. It was first isolated from a preparation of bovine thymus, called thymosin fraction 5, by CM- and DEAE-cellulose chromatography and 3-column gel filtration steps [22,23]. Partial

sequencing of Tα1 isolated from human-derived thymosin fraction 5 did not reveal any structural differences from the bovine peptide [24], suggesting the conservation of the sequence of this peptide in mammals. Tα1 has also been synthesized by solid-phase peptide synthesis; this is the material that has been used in recent clinical trials.

Endogenous Tα1 can be detected in serum, where levels measured in more than 600 healthy adults by enzyme-linked immunosorbent assays (ELISA) were found to be in the 0.1 to 1 ng/ml range [24-27]. The circulating concentration of Tα1 tends to be lower in diseased individuals and higher during pregnancy [28-30]. The source and mechanisms of release and regulation of circulating Tα1 are unknown. Tα1 is contained in the sequence of prothymosin, a 126– amino acid peptide that is reported to be found in the cell nucleus and has been examined in terms of its potential effects on cell proliferation. Tα1 is found in highest concentrations in the thymus but has also been found in spleen, lung, kidney, brain, blood, and a number of other tissues.

Although circulating concentrations of Tα1 have been measured by ELISA and radioimmunoassay (RIA), these methods have prolonged sample preparation time or have not been very sensitive or specific.

The study presented here describes a fast and reproducible LC-MS/MS method to determine concentrations of Tα1 in human serum between 0.5 ng/mL and 100 ng/mL, the concentrations seen in both healthy subjects and in patients after pharmacologic administration. The method employs a simple reverse-phase SPE extraction combined with LC-MS/MS multiple reaction monitor (MRM) detection.This method is accurate and precise, as well as sensitive, with only 30 µ L required for injection. The run time is only 8 minutes, so the method can be used in both clinical trial samples as well as for pharmacokinetic studies [31].

MATERIALS AND METHODS

Materials

SciClone Pharmaceuticals, Inc. (San Mateo, CA) kindly provided Tα1. Internal standard, β -Endorphin Ac (1-26) human 99%, was purchased from the American Peptide Company, Inc. (Sunnyvale, CA). Both compounds were used without purity correction. All solvents were high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific Company (Pittsburgh, PA). Acetic acid 99.8%, ammonium acetate 99.99%, ammonium hydroxide A.C.S. reagent, and formic acid 96% were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Human serum was purchased from Tennessee Blood Source Corporation (Memphis, TN).

Sample Preparation

Standard Preparation: An accurately weighed amount of Tα1 was dissolved in water to produce 100 µ g/mL stock solution. (Tα 1 is stable in water.) Further dilutions were made to produce a series of Tα1 working stock solutions. A 100 µ g/mL stock internal standard solution was also made in water. A 600 ng/mL internal standard working stock solution was further diluted from this stock solution. New dilutions were made when needed. All solutions were stored at –20° C. All working stock solutions were thawed and vortexed at room temperature before use.

Serum Extraction: Human serum samples were spiked with 50 µ L of different concentrations of Tα1 working stock solutions and 50 µ L of 600 ng/mL internal standard working stock solution to achieve different drug serum concentrations. Human serum control blank samples were spiked with 50 µ L water and the same amount of internal standard working stock solution. Tα1 calibration curve standards (0.5, 1, 5, 20, 30, 50, 90, and 100 ng/mL) were prepared by spiking appropriate Tα1 working stock solutions (10, 20, 100, 200, 400, 1000, 1800, and 2000 ng/mL) and internal standard working stock solution into 1000 µ L of human serum. Quality control (QC) samples (1, 2, 5,

20, 70, 90 ng/mL) were prepared by using another set of 40, 100, 200, 400, 1400, and 1800 ng/mL Tα1 working stock solutions. Acetic acid solution was added to the sample and shaken violently. Ethanol, water, and acetic acid solutions were used to pretreat Supelclean™ LC-18 SPE Tubes (6 mL) (Supelco, Bellefonte, PA). The samples were loaded on to the SPE cartridges and washed with 3-mL acetic acid solution and 10 mL water. The analyte and internal standard were eluted with 6 mL 4% acetic acid in 86% ethanol. The eluant was collected and dried under N_2 in a 37° C water bath. Samples were reconstituted with 1000 µ L 5 mM NH_4OAc solution (pH=7.2 by NH_4OH). They were then centrifuged and transferred to HPLC vials for analysis.

HPLC Conditions

The HPLC system consisted of 2 Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) LC-10ATvp binary pumps, an SIL-10ADvp auto injector, and an SCL-10Avp system controller. This system is capable of delivering a 2-solvent gradient. A UV detector (Shimadzu SPD-10AVvp) was also online but was not used. The mobile phases were (1) 5 mM NH_4OAc with 0.1% formic acid (vol/vol) and (2) 5 mM NH_4OAc acetonitrile/H_2O (90/10, vol/vol) solution with 0.1% formic acid (vol/vol). A 30-µ L sample was injected by the Shimadzu auto injector. The analytical column used was a Phenomenex (Torrance, CA) Columbus 2 × 50-mm C18 column. The particle size was 5 µ m, and the flow rate was 0.3 mL per minute. The total chromatography time was 8 minutes. Retention times were determined by injecting a standard solution into the HPLC system for analyte and internal standard respectively.

MS Conditions

The mass spectrometer was a PE Sciex API-365 LC/MS/MS system. The Turbo ionspray interface was used. Turbo ionspray gas was set at 8 L per minute at 25 psi. The instrument was calibrated before sample analysis. Preliminary Q1 scan and product ion scan of analyte, Tα1, and internal standard were carried out. The specific precursor ions and product ions were chosen based on these results. Instrument optimization was performed to achieve more sensitive detection based on these ion pairs. Serum samples were analyzed and Tα1 was quantitated by the MRM mode. An example of detailed instrumental parameters is listed in **Table 1**. This method employs highly specific LC-MS/MS for the detection mode, coupled with the characteristic retention time observed for the analyte on the appropriate HPLC column. Therefore no further confirmation is required.

Table 1: Example of LC-MS/MS System Parameters

LC/MS/MS Parameter List (V)	
(IS) Ion Source:	4000.00
(TEM) Temperature (°C):	400.00
(OR) Orifice Plate:	40.00
(RNG) Focusing Ring:	220.00
(Q0) Quad 0 Rod Offset:	-9.00
(IQ1) Inter Quad 1 Lens:	-11.00
(ST) Stubbies (Prefilter):	-16.00
(R01) Quad 1 Rod Offset:	-11.00
(IQ2) Inter Quad 2 Lens:	-56.00
(R02) Quad 2 Rod Offset:	-61.00
(IQ3) Inter Quad 3 Lens:	-76.00
(R03) Quad 3 Rod Offset:	-66.00
(DF) Deflector:	-300.00
(CEM) Channel Electron Multiplier:	2800.00
(NEB) Nebulizer Gas (N2):	8 L/min
(CUR) Curtain Gas (N2):	7.00
(CAD) Collisionally Activated Dissociation Gas (N2):	9.00

RESULTS AND DISCUSSION

Q1 AND PRODUCT ION SCAN RESULTS

The molecular weight of Tα1 is 3108 Da. Its single charged molecular ion could not be observed by the LC-MS/MS instrument because it is above the instrument's mass detection range. A preliminary Q1 scan study of Tα1 demonstrated that under experimental conditions Tα1 forms a triple-charged species $(M+3H)^{3+}$ at Q1, whose m/z ratio is 1037 (**Figure 1**). The double-charged molecular ion (m/z=1555, $(M+2H)^{2+}$) and quadruple-charged molecular ion (m/z=778, $(M+4H)^{4+}$) were also observed in other experiments with much lower intensity. Other experiments showed that a lower pH value, eg, having more formic acid in the mobile phase, favors higher charged species.

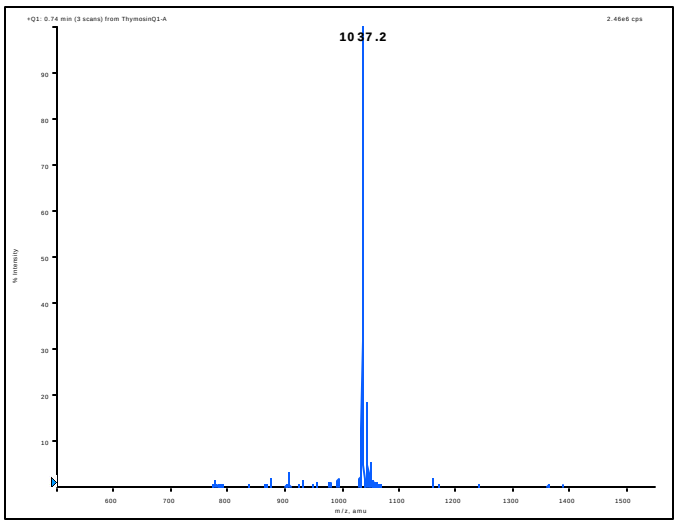

Figure 1: Q1 Mass Spectrum of Thymosin α1

Using the triple-charged ion (m/z = 1037) as the precursor ion in the product ion scan mode produced m/z = 316 and 387 as major fragments, along with a few other fragments (**Figure 2**). Ion 316 was assigned to Ala-Glu-Asn from the Tα1 C terminus and ion 387 was assigned to Tα1's acetylated N-terminus (Ac-Ser-Asp-Ala-Ala). Some other fragments were also assigned: the 244 ion was assigned to Glu-Asn and the 227 ion was assigned to (Ac-Ser-Asp)- H_2O.

The precursor molecular ion with m/z 1037 and the product ion with m/z 316 were chosen to represent Tα1 in the MRM experiment. Internal standard endorphin Ac (1-26) was also tested under Q1 and MS/MS mode. Molecular ion and product ion pair for the internal standard (968.2→ 320) MRM mode detection were also chosen based on the Q1 and MS/MS experimental results. The Q1 scan of endorphin Ac (1-26) showed a triple-charged ion $[M+3H]^{+3}$ with m/z 968.2. The product ion scan of the m/z 968.2 precursor gave a dominant product ion with m/z 320, which could be assigned to (Ac-Tyr-Gly-Gly), a fragment from the endorphin N terminus.

HPLC Retention

Tα1 and the internal standard endorphin Ac (1-26) solvent standard solutions were injected separately to obtain their retention times. MRM detection was used in the experiments. A matrix standard, which contained

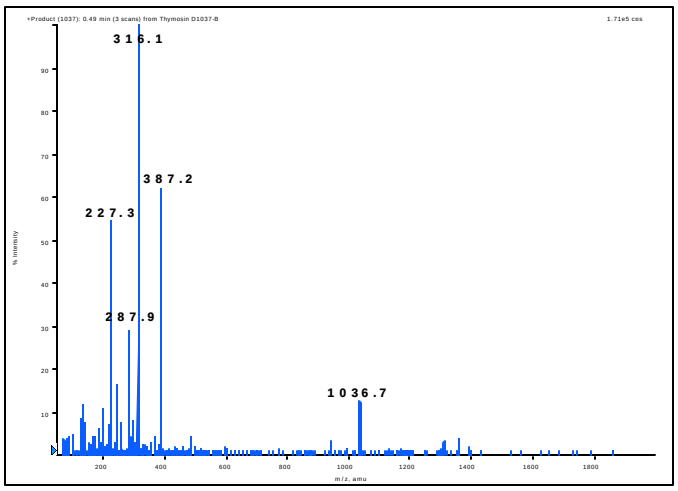

Figure 2: MS/MS Spectrum of Thymosin α 1 ion m/z=1037

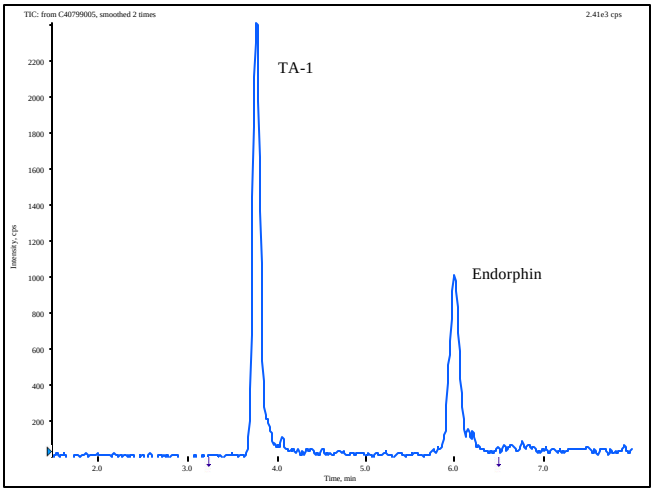

Figure 3: Tα1 and Internal Standard (Endorphin) Human Serum Chromatogram. The drug concentrations in human serum were 20 ng/mL for Tα1 and 30 ng/mL for internal standard.

both Tα1 and internal standard endorphin, was tested under the same conditions to reveal matrix effects and possible interferences. A typical matrix standard chromatogram is shown in **Figure 3.** No interference was observed in the analyte or internal standard retention windows. Their retention times were 3.8 minutes and 5.9 minutes, respectively.

Linearity, Limit of Detection (LOD), and Limit of Quantitation (LOQ)

Three sets of 8 human serum standard curve samples, 6 levels of QC samples using different working stock

solutions, and 2 serum blank samples were prepared as described above. The calibration standards ranged from 0.5 ng/mL to 100 ng/mL (0.5, 1, 5, 20, 30, 50, 90, and 100 ng/mL). Each standard was injected at least once in the sample set. All data points acquired on the same day were collected to construct the calibration curve. Linearity was obtained by fitting all data points with weighted linear regression (1/concentration) using analyte and internal standard peak areas. All 3 sets were analyzed on 3 consecutive days. Results are summarized in **Table 2**. It is clear that the analytical results are reproducible and accurate (relative error [RE%] from –9.9% to 6.2%) and precise (correlation of variation [CV%] from 2.2% to 10.6%) in the entire concentration range (N=9). Correlation coefficients for each day were 0.9955 or greater. A typical calibration curve is shown in **Figure 4.** The LOD is 30 µL of 0.5 ng/mL drug serum concentration (15 pg injected on column), with a good signal to noise ratio of 6. The low LLOQ, therefore, was 0.5 ng/mL in human serum. This LC-MS/MS method demonstrates relatively good linearity over a wide linearity range. Typical chromatograms of 1 ng/mL Tα 1 and 30 ng/mL internal standard endorphin in human serum are shown in **Figures 5** and **6.** For comparison, "A" shows chromatograms for blank serum and "B" for Tα 1 and internal standard, respectively. No interference is observed at the Tα1 or internal standard retention windows (retention time [RT] = 3.8 and 5.9 minutes**)**.

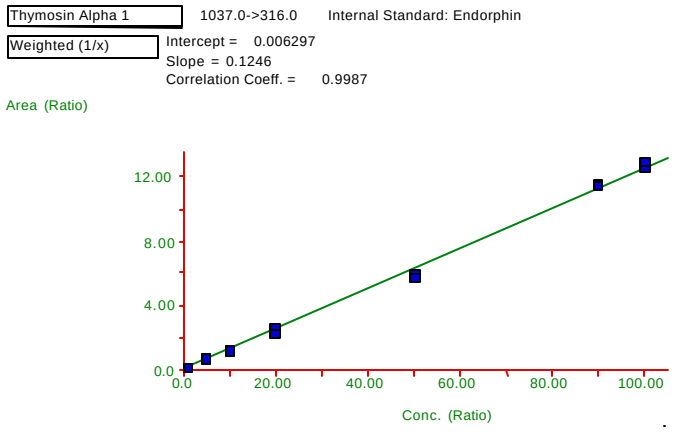

Figure 4: Calibration Curve for Thymosin α 1

Table 2: Calibration Standards Results Summary (N=9)

Calibration Standards Statistical Results

	Drug Serum Concentration (ng/mL)							
	0.5	1	5	10	20	50	90	100
1	0.483	1.144	5.125	10.480	22.418	46.606	92.616	102.629
2	0.512	0.981	5.098	8.415	18.484	40.953	88.443	111.445
3	0.504	1.083	5.220	10.789	22.897	46.080	93.791	103.277
4	0.493	1.104	5.100	9.983	21.412	47.865	90.358	100.254
5	0.550	0.993	4.869	8.352	18.904	41.546	88.445	108.412
6	0.501	1.058	5.021	10.523	22.754	48.254	94.234	102.455
7	0.486	1.102	5.201	10.235	22.235	46.873	91.864	98.562
8	0.536	0.980	4.986	8.561	18.874	41.235	88.165	107.235
9	0.505	1.112	5.134	10.754	22.145	46.237	93.654	99.687
Average	0.5078	1.0619	5.0838	9.7880	21.1248	45.0721	91.2856	103.7729
SD	0.0223	0.0623	0.1099	1.0395	1.8301	2.9584	2.4858	4.3573
CV%	4.3879	5.8707	2.1617	10.6204	8.6634	6.5637	2.7231	4.1989
RE%	1.5556	6.1889	1.6756	-2.1200	5.6239	-9.8558	1.4284	3.7729

SD, standard deviation; CV%, correlation of variation; RE%, relative error.

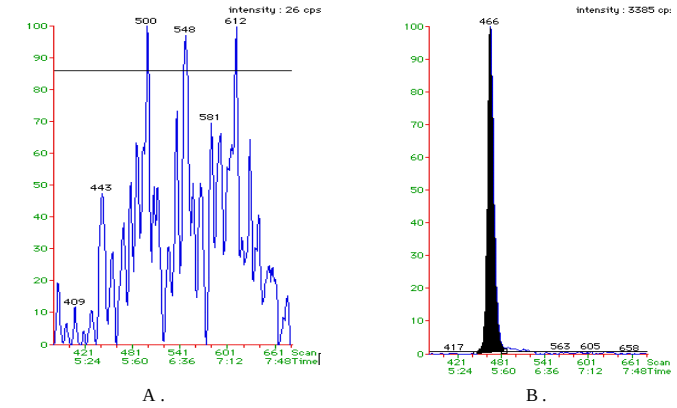

Figure 5: Typical Human Serum Blank and 1 ng/mL Calibration Standard Chromatogram

A. Human serum blank chromatogram, 100% scale is 14 cps

B. 1 ng/mL Tα1 calibration standard chromatogram, 100% scale is 118 cps

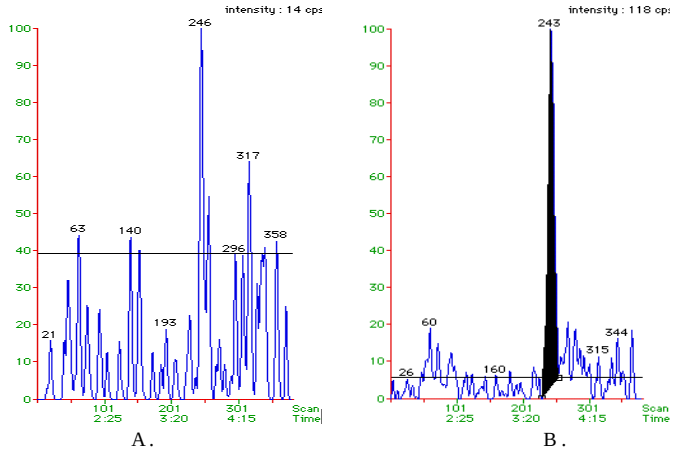

Figure 6: Typical Human Serum Blank and 30 ng/mL Internal Standard Chromatogram

A. Human serum blank chromatogram, 100% scale is 26 cps

B. 30 ng/mL Internal standard chromatogram, 100% scale is 3385 cps

Table 3. Statistical Results of all QC Sample Data

QC Sample Statistical Results

	Drug Serum Concentration (ng/mL)					
	1	2	5	20	70	90
1	0.942	1.842	5.831	23.397	56.518	81.287
2	0.981	1.823	5.512	22.933	65.232	92.234
3	1.145	2.062	5.045	19.356	71.245	95.687
4	0.954	1.805	6.018	23.457	56.232	85.944
5	0.998	1.865	5.621	22.879	66.356	92.125
6	1.125	2.012	5.187	19.365	71.684	95.147
7	0.951	1.862	5.947	23.125	56.627	83.214
8	0.987	1.830	5.574	22.743	65.458	92.687
9	1.102	2.035	5.061	19.208	71.498	95.888
Average	1.0206	1.9040	5.5329	21.8292	64.5389	90.4681
SD	0.0804	0.1017	0.3684	1.9040	6.5671	5.5529
CV%	7.8745	5.3418	6.6589	8.7223	10.1755	6.1379
RE%	2.0556	-4.8000	10.6578	9.1461	-7.8016	0.5201

Accuracy, Precision, Stability, and Specificity

Six levels of QC samples were analyzed in each sample set, as well as a mobile phase blank and 2 human serum controls. No detectable levels of Tα1 were observed in the mobile phase blank or control serum blanks.

All QC samples were also analyzed on 3 consecutive days. The results are summarized in **Table 3**. CV% and RE% statistical calculations show that they are all < 15%. Thus, this method is accurate and precise in the calibration range.

Stability tests were also performed. Bench top (12 hours) and – 20° C freezer storage samples (3 months) as well as 3 cycles of freeze/thaw at –20° C were tested. No degradation was observed for Tα1 or internal standard in serum. The analytes were stable under the above analytical conditions.

A carryover problem was observed for the higher concentration sample of Tα1 on the auto injector. Appropriate methods should be used for eliminating the carryover problem in order to detect a low level of Tα1. In our case, 500 µ L of rinse solution on the Shimadzu SIL-10ADvp Auto injector was used. After each injection, this pretreatment with a large volume rinse involved several rinse steps, and eliminated the carryover problem.

CONCLUSIONS

This LC-MS/MS method demonstrates high accuracy and precision for analysis of Tα1 in a human serum matrix. Tα1 was extracted from serum by reverse phase SPE extraction and analyzed on a reverse phase LC-MS/MS instrument. In these experiments, the sample preparation time for 1 person to process all 46 samples (3 sets of 8 standards, 5 LLOQ samples, 2 serum controls, and 3 levels of QC samples in 5 replicates) was 6 hours. The HPLC and MS/MS acquisition time was 8 minutes. This analytical method is thus quick, reliable, and accurate for determination of Tα1 in serum matrix. This could prove to be useful for analysis of clinical trial samples and for pharmacokinetic studies.

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