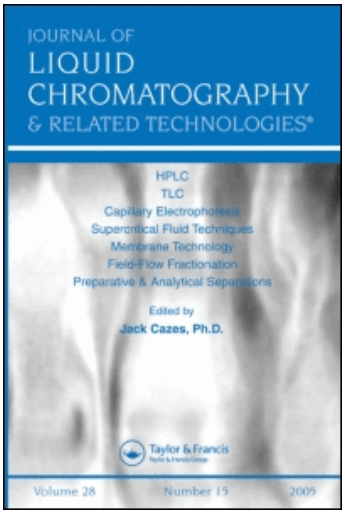

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UTILIZATION OF HPLC-ELISA TO ASSESS SERUM LEVELS OF THYMOSIN α_1 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO HUMAN SUBJECTS

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ABSTRACT

Thymosin α_1 ($T\alpha_1$) is a 28 aa peptide which is prepared synthetically for clinical use in settings where an immune modulating peptide is predicted to have efficacy. With the increase in clinical studies utilizing $T\alpha_1$ as a single agent or in combination with other therapeutic agents the need to accurately assess the pharmacokinetics of serum levels of the authentic $T\alpha_1$ in serum has become clear. Utilizing radioimmunoassays, previous studies have demonstrated elevated levels of thymosin like material in serum for 3-6 hours following administration. This study reports the development of an extraction-analysis procedure which combines HPLC specificity with ELISA sensitivity to measure the levels of authentic $T\alpha_1$ following subcutaneous administration. The precision, specificity and sensitivity data for the procedure is presented as well as data confirming the ability of the procedure to assess levels in human subjects following subcutaneous administration.

INTRODUCTION

Thymosin α_1 ($T\alpha_1$) is a 28 amino acid peptide currently undergoing evaluation in clinical trials where modulation of immune response may be a

critical factor in the control or clearance of disease (1-6). $T\alpha_1$ is administered subcutaneously, generally as two or three injections over the period of a week. The in vivo events which result in positive effects such as the clearance of the hepatitis B virus, an enhancement of response to vaccination or improvement in the survival time of cancer patients receiving various therapies are believed to center around $T\alpha_1$'s capacity to enhance immune responses. The specific mechanism(s) of action, however, has not been defined. In vitro studies suggest that an important mechanism may be the induction of cytokine production or an increase in cytokine receptors (7,8). Initially isolated from the thymus, $T\alpha_1$ has also been identified in high levels in spleen tissue, is secreted by lymphocytes and is present in serum (9-14). As with many cytokines and protein hormones, lower but detectable levels are also found in essentially all tissues and cells (13-15).

Using a radioimmunoassay based on a rabbit antibody which is specific for $T\alpha_1$ and has been shown to recognize epitopes contained in the N-terminal and C-terminal regions, elevated serum levels of thymosin like material (TLM) have been detected after subcutaneous (sc) administration (16). The subjects in the published study were part of a Phase II clinical trial of thymosin as a follow-up to irradiation in lung cancer patients. In all subjects, pharmacological levels of TLM were measured between 1 and 6 hrs. Similar results have been obtained in individuals who were HIV positive and in elderly individuals. (unpublished results of Naylor et al).

The studies presented here, describe the development of an HPLC-ELISA method to determine the levels of authentic 28 aa $T\alpha_1$ in serum following sc administration. The ELISA is required for detection since even following injection of 1.6 mg of $T\alpha_1$, serum levels are not high enough to be measured with the uv detector of the HPLC unit. The specificity, sensitivity, linearity, precision and accuracy of the method is defined. The utility of the method is demonstrated using serum from an individual receiving a sc injection of $T\alpha_1$.

MATERIALS AND METHODS

Sep-Pak Extraction Procedure

Prior to HPLC analysis, the $T\alpha_1$ in samples was extracted using a Waters C18 Sep-Pak. The sep-pak contains C18 ubondapak reverse phase resin and was prepared by sequentially washing with Methanol, HPLC grade water and the buffer of choice (Buffer A = 20 mM Potassium Phosphate, PH 6.0). The sample (typically 2 mls of serum) was loaded onto the column and vacuum applied using a Waters Sep-Pak Manifold. The effluent was collected and in the initial studies the removal of thymosin α_1 verified by ELISA. After two washes with buffer A (2 ml ea), 2 mls of Buffer B(50% acetonitrile,50 % Buffer A) was drawn through the sep-pak and collected. A final wash using 50% acetonitrile was also collected in the initial studies. All collected samples were evaporated using a Savant Speed-Vac and resuspended into 500 ul of HPLC quality water.

HPLC procedure

The HPLC procedure utilized a Perken-Elmer Series 4 Liquid Chromatograph with an ISS-100 autoinjector, LC235 uv Detector and a programmable ISCO fraction collector. The flow rate was 1 ml/minute, the gradient was 10-50% Buffer B in Buffer A (see above), and the uv detector set for 214 nm. Injection volumes were typically 100 ul.

ELISA procedure

The ELISA procedure was a modification of that previously published by Weller et al (11, 14). In brief, rabbit antibody specific for $T\alpha_1$ (1/2000 dilution in PBS-Tween-Azide buffer(PTA)) was incubated overnight at 4oC with equal volumes of standard or sample. $T\alpha_1$ coated ELISA plates were prepared by incubating $T\alpha_1$ in PBS overnight at 4oC in the wells of Immunolon 4 96 well plates. After washing the ELISA plates, an aliquot (50 ul) of the overnight incubation mixture was added and the plates incubated for 40 minutes at 37oC. The

free antibody (ie that which was left over following the overnight establishment of the equilibrium) was captured by the $T\alpha_1$ on the plate. The antibody bound to the $T\alpha_1$ on the plate was detected by sequential additions, (following washes with PTA) of biotinylated-rabbit antibody(Vector Antibodies Inc), avidin-biotinylated alkaline phosphatase (Vector Antibodies Inc) and p-nitrophenyl-phosphate substrate.(Sigma). The optical density at 405 nm was determined and the amount of $T\alpha_1$ in the sample determined by comparison to a curve generated using know standard concentrations of $T\alpha_1$. The curves were typically log-linear with correlation coefficients of at least 0.95 and slopes of at least -.5. Standard deviations for the standards of less that 2-10% were typically obtained.Sample variations were 10-20%. Trimming of the means of serial dilutions yielded the 5-10% errors in the reported data.

RESULTS

The HPLC was used as the criterion for evaluating the extraction procedure. The retention time of $T\alpha_1$ in the HPLC was highly reproducible (0.2% error). The system was also linear with respect to the size of the peak area as compared to varying concentrations of known $T\alpha_1$ (R value = 0.999) and recoveries(99.4 =/- 9.1%. (n= 9)(Table 1)). The reproducibility of the system with respect to injection of the same sample was 5.7% for the Peak Area and 3.4% for the peak height. (n=6)

The extraction procedure resulted in recoveries of $T\alpha_1$ from serum samples spiked with known concentrations of $T\alpha_1$ of 95% based on HPLC Area and 91% when based on HPLC peak height (n= 6). The standard deviation between samples was 6.6 % based on peak area and 9.9 % based on peak height. There was no detectable $T\alpha_1$ in the non-retained sample, or the washes. The serum from a normal individual was subjected to the procedure in parallel to the sample which was spiked with synthetic Tal. Due to the lack of sensitivity of the UV detector, no $T\alpha_1$ was

TABLE I
Accuracy of HPLC Method by Percent Recovery

Concentration $T\alpha_1$ in ng/ml	Peak Area	Calculated Peak Area*	%Recovery**
0.0310	2013313	64945580	87.7
0.0310	2006401	64722612	87.4
0.0310	2099191	67715838	91.4
0.0625	4759637	76154192	102.8
0.0625	4704228	75267648	101.6
0.0625	4637822	74205152	100.2
0.1250	10085500	80684000	108.9
0.1250	10210900	81687200	110.3
0.1250	10137690	81101520	109.5

Average Recovery: 99.4 +/- 9.1

* Peak area/concentration
** % Recovery = Calculated Peak Area/Average Peak Area per mg(74053749)

detected in normal serum, thus the HPLC could be used to define recoveries in the spiked samples. Figure 1 shows that the $T\alpha_1$ in normal serum could be assessed by ELISA and that consistent with the HPLC results,spiked serum had higher levels than normal serum. There were no additional peaks as assessed by HPLC in the spiked serum and when TLM was assessed by ELISA there were no additional peaks in either the normal or spiked sample.

The specificity of the HPLC retention time for $T\alpha_1$ was assessed by comparing retention time of $T\alpha_1$ with the 14 aa N-terminal and 14 aa C-terminal peptide (Figure 2). The retention time for $T\alpha_1$ was 14.0, for N-14 $T\alpha_1$ it was 11.4 and for C-14 $T\alpha_1$ was 9.1. The procedure clearly differentiates between the three peptides.

Serum from an individual injected with 1.6 mg of $T\alpha_1$ was obtained prior to injection and at 0.5,1,2,3,4,6, and 24 hrs. TLM at levels significantly above baseline was present between 0.5 and 4 hrs (Figure 2). A decline was clearly evident at 6 hrs and levels were at baseline by 24 hrs. Samples were then subjected to the Sep-Pak,HPLC, ELISA procedure to determine whether the TLM was present as the authentic 28 aa peptide. As indicated in Figure 3, the only detectible immunoreactivity had a retention time identical to $T\alpha_1$.

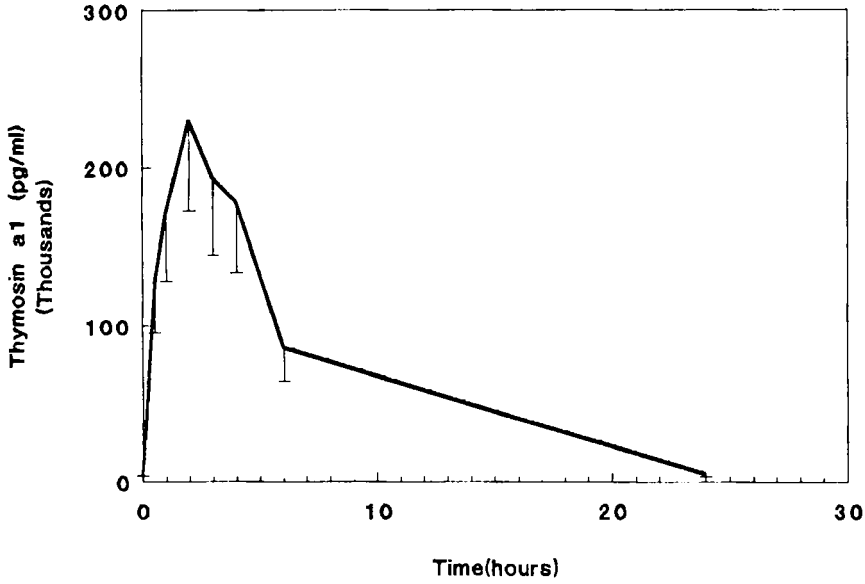

FIGURE 1

Serum samples with and without added Tal were subjected to the Sep-Pak-HPLC procedure and the presence of $T\alpha_1$ assessed by ELISA. Identical single immunoreactive peaks were identified in both samples. The values are expressed as pg/ml of resuspended extracted samples to demonstrate the lack of background in the HPLC-ELISA system.

DISCUSSION

Since $T\alpha_1$ has only recently entered Phase 3 trials, there is little published data regarding the circulating levels following administration to man. These studies are unique since for the first time the levels of the authentic 28 aa peptide have been measured. This is in contrast to the only previous study where an RIA was used to measure TLM in the serum (16). The HPLC-ELISA system thus confirms that following sc injection, the authentic 28 amino-acid $T\alpha_1$ is

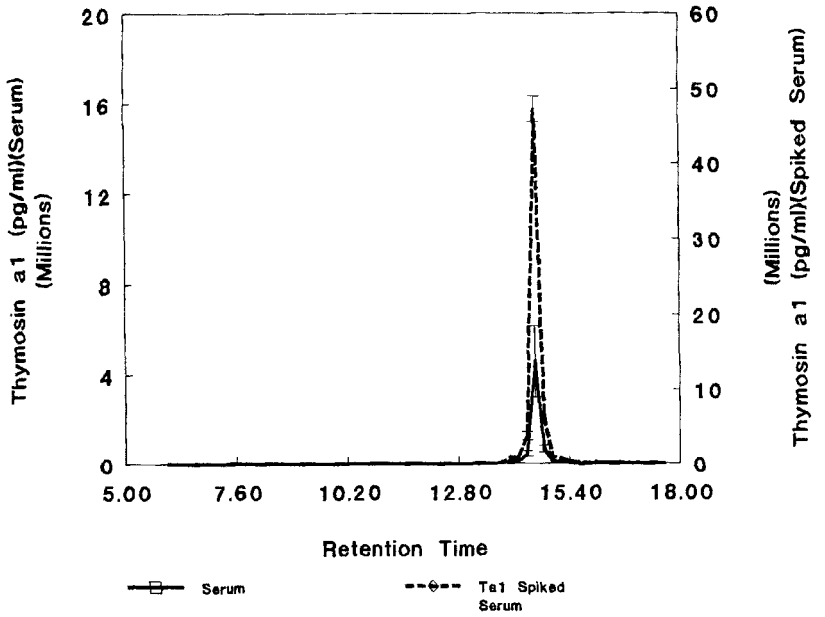

FIGURE 2

Serum was drawn at the times indicated after a 1.6 mg subcutaneous injection of $T\alpha_1$ to an individual with autoimmune hepatitis. The levels of immunoreactive $T\alpha_1$ were assessed by ELISA. The error bars represent the standard error of the mean of 8-10 values.

present in serum as defined by the retention time of the immunoreactive material. Recoveries for the procedure are at least 90%, resulting in a high degree of confidence in the observation. The maximum achievable levels approached 200 ng/ml compared to basal levels of 6 ng/ml. These results are similar to those reported using the RIA to detect TLM in serum from lung cancer patients undergoing $T\alpha_1$ treatment. In these studies, peak levels 10-50 times greater than baseline were reported. As here, the peak levels of $T\alpha_1$ (presumably now validated as authentic by this report) were 3-6 hrs post sc injection. The kinetics are similar to those reported for other proteins, such as interferon-α,

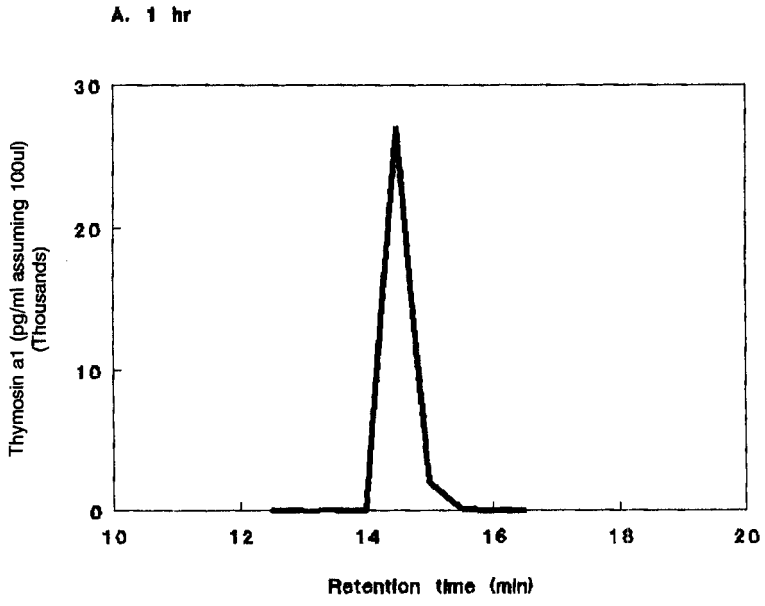

FIGURE 3

Serum at various times post injection of $T\alpha_1$ (1.6 mg,sc as in figure 2) was subjected to the Sep-Pak-HPLC-ELISA procedure. Data from two representative time points where pharmacological levels were detected (at 1 and 3 hrs post injection) are shown. In all samples only a single immunoreactive peak at the retention time of authentic $T\alpha_1$ was detected. The results are expressed as pg/ml of resuspended sample to demonstrate the relationship between sample and non-specific background.

where clearance is generally via filtration through the renal glomeruli followed by proximal tubular reabsorption with subsequent proteolytic degradation by lysosomal enzymes (17). The liver typically has little role unless the proteins are highly glycosylated. The presence of only the authentic peptide at all time points is consistent with this route of clearance.

Thus this study reports a methodology for assessing the levels of authentic $T\alpha_1$ in serum following administration to humans. Although levels approaching 0.2

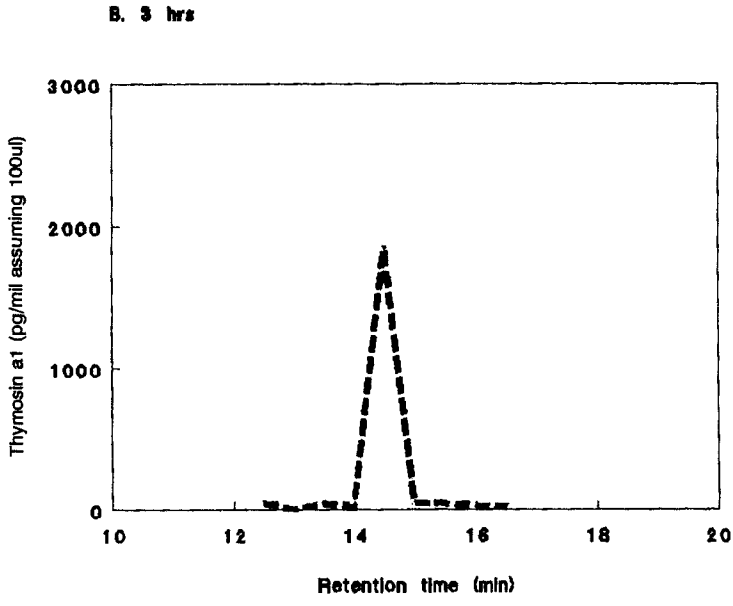

FIGURE 3 (Continued)

ug/ml were achieved, these levels are not adequate for measurement by the uv where minimal detectable levels of thymosin a1 were 1 ug/ml. The sensitivity of the HPLC-ELISA method also allows for the determination of the kinetics in normal individuals since sub bioactive doses (10-100 fold lower) could be used and detected. An additional utility of this methodology is that it detects authentic $T\alpha_1$ in serum from normal individuals. Consistent with our previous study where a sizing HPLC column was used, only a single peak with retention time identical to that of $T\alpha_1$ was detected (13). This methodology is predicted to be especially important in subsequent studies where Ta1 levels in individuals following long term $T\alpha_1$ administration are evaluated. Based on the previous study in lung cancer patients, it is predicted that long term treatment will result in an increase in baseline $T\alpha_1$ levels. This HPLC-ELISA method will allow the determination of whether these levels are due to authentic $T\alpha_1$ or a TLM

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