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Simple, Rapid and Sensitive Method for Determination of Tacrolimus in Human Blood by using Liquid Chromatography / Tandem Mass Spectrometry

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Abstract

A simple, rapid method using an isocratic liquid chromatography/ Tandem mass spectrometry was developed and validated for the assay of Tacrolimus in the Human Blood. Linearity was observed between the Tacrolimus concentration and the peak area ratio from 0.52 ng/mL to 61.00 ng/mL. Blood samples containing Tacrolimus were extracted with Zinc sulphate and methanol to precipitate proteins followed by SPE. The observed recovery of Tacrolimus was 66.7 %. The intra-day and inter-day accuracies ranged from 91.3 – 110.5% and from 93.9 – 100.6% respectively, at three different concentrations. The method will be used in the determination of the pharmacokinetic parameters of Tacrolimus after oral administration of Tacrolimus formulation.

Keywords: Tacrolimus, LCMS/MS, Human Blood

Introduction

Tacrolimus is a macrolide immunosuppressant and has unpredictable pharmacokinetics, therefore regular monitoring is required in patients receiving Tacrolimus. It is used in organ transplantation to prevent graft rejection. However, its use is not devoid of side effects, making it important to maintain blood concentrations within therapeutic ranges. Several analytical methods are currently available for routine drug monitoring. We have developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for measuring Tacrolimus concentrations in whole blood.

Materials and Methods

Materials: Tacrolimus (Biocon LTD), Sirolimus (Biocon LTD), HPLC grade Acetonitrile (JT Baker), HPLC grade methanol (JT Baker), Ammonium acetate (GR Grade Merck), Sodium hydroxide (GR Grade Merck), Zinc sulphate (GR Grade Merck),Water was deionized and triple distilled.

Preparation of Tacrolimus standard solutions: Standard solution of Tacrolimus (1.0 mg/ml) was prepared by dissolving an accurately weighed amount of 10.27 mg of Tacrolimus in 5 ml of methanol, sonicated for 5 minutes and the volume was adjusted to 10 ml with methanol. The standard solution was stored subsequently at 2-8 ^{0}C. The appropriate concentrations of standard solution were prepared by

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diluting the stock solution with 80: 20 methanol : water.

Preparation of Sirolimus Internal standard solutions: Standard solution of Sirolimus (1.0 mg/ml) was prepared by dissolving an accurately weighed amount of 10.22 mg of Sirolimus in 5 ml of methanol, sonicated for 5 minutes and the volume was adjusted to 10 ml with methanol. The standard solution was stored subsequently at 2-8 ^{0}C. The appropriate concentrations of internal standard solution were prepared by diluting the stock solution with 80: 20 methanol : water.

Treatment of serum:

1. Blood samples of Healthy Human Volunteers (supplied by the Prathama Blood Bank Ahmedabad) were taken and allow them to thaw at room temperature for 30 minutes.

2. A series of blood samples (0.5 ml) in polypropylene tubes were prepared by mixing 0.495 ml of the blood with 5 μL of Spiking solution containing varying amounts of Tacrolimus, ranging from 0.05 to 6.21 μg/mL (corresponding to 0.52 – 62.14 ng/mL).

3. Followed by addition of 50 μL of ISTD (Sirolimus 2.5μg/mL), then basified it with 50 μL of 0.2N Sodium hydroxide solution vortexed and then deproteinized with 1ml of zinc sulphate.

4. Followed by addition of 2ml of methanol which also dissolved Tacrolimus that was bound to those proteins and vortex the samples for one minute.

5. The blood was then Centrifuge at 4500 rpm for 10 minutes at 5°C.

6. Supernatant is loaded on the equilibrated HLB 3CC cartridge, after the washing of the cartridge with water and 20 % methanol.

7. Tacrolimus is eluted with 1 ml of acetonitrile/water solution (0.75/0.25) and vortexed for 30sec.

8. Finally, the resulting solution was injected into the analytical column.

Instrumentation and conditions: The HPLC and MS system consisted of a pump and auto injector connected to a personal computer (MS of Applied biosystems and HPLC of Agilent). The column used was a reverse phase C18, 5 μm (Hypersil HyPurity).

The mobile phase consisted of a mixture of methanol and 5 Mm Ammonium Acetate at a ratio of 90:10 respectively. The mobile phase was degassed by passing through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) prior to use. The mobile phase was pumped isocratically at a flow rate of 0.5 ml/min. The injector was filled with an injector loop of 10 μl. Tandem mass spectrometric detection and quantification was performed using multiple reaction monitoring (MRM).

Results and Discussion

Optimization of HPLC conditions for rapid extraction of Tacrolimus from Human Blood: The extraction procedure developed for Tacrolimus from Human Blood allowed samples to be available for HPLC analysis in approximately 30 minutes. Conditions for simple and rapid HPLC separation with MS detection were developed using an isocratic elution with a mobile phase composed of methanol and 5 Mm Ammonium acetate at a ratio of 90:10. These conditions gave a well defined, sharp peak of Tacrolimus and Sirolimus (ISTD) with a retention time of approximately 1.78 minutes for both. Under these conditions an amount of Tacrolimus as low as 10pg/mL could be detected. With these retention times, analysis could be completed in about 2.5 minutes.

Method validation

Linearity: The quantification of the chromatogram was performed using the peak area ratio of Tacrolimus and Sirolimus (ISTD). Nine standard solutions were prepared (0.52 ng/mL, 1.03 ng/mL, 2.20 ng/mL, 6.29 ng/mL, 12.27 ng/mL, 25.14 ng/mL, 36.97 ng/mL, 49.30ng/mL and 61.62 ng/mL) and subjected analyses by HPLC MS/MS. Five linearities were injected. The peak area ratio was determined and plotted versus the concentration of Tacrolimus. Statistical analysis using least square regression analysis indicated excellent linearity for Tacrolimus with the concentration range studied as shown in Table 1. In constructing the standard curve, samples of Tacrolimus in Human Blood identical to those in the standard solutions were prepared and the Tacrolimus response ratios were plotted against the concentrations of Tacrolimus in ng/mL as shown in figure 1. The linearity of the concentration and response relation was established over the range of 0.52 – 61.62 ng/mL (R^2 = 0.9898). Figure 2 shows the LC MS/MS chromatograms of pure drug (Tacrolimus), drug-free Human Blood and standard Blood sample containing the drug at a concentration of 0.52 ng/mL.

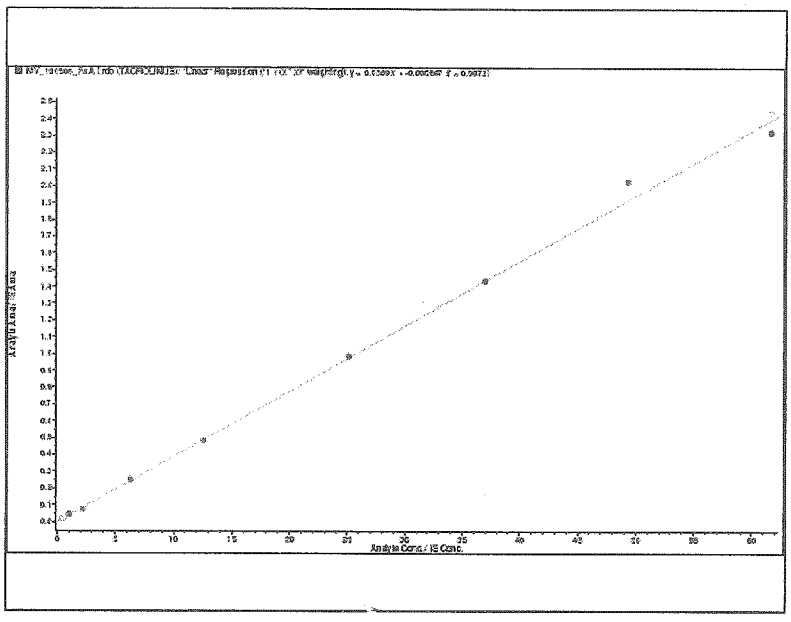

Figure 1. Linearity of standard samples of Tacrolimus in Human Blood.

Table 1. Statistical analysis of liner regression of Tacrolimus

CC ID	STD 9-1	STD 9-2	STD 8	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1-1	STD 1-2	Slope	Intercept	r2
Nominal Conc. ng/mL	61.62	61.62	49.30	36.97	25.14	12.27	6.29	2.20	1.03	0.52	0.52			
CC1	62.69a	59.75	52.17	36.94	25.33	12.42	6.43	1.92	1.14	0.50a	0.51	0.0389	0.00085	0.9946
CC2	64.54a	58.96	49.89	38.35	24.8	12.77	6.37	1.94	1.18	0.5	0.47a	0.038	-0.00107	0.9934
CC3	65.45a	59.29	49.31	37.18	25.7	11.99	6.52	1.80a	1.07	0.49a	0.51	0.0407	5.75E-05	0.9986
CC4	61.67	64.09 a	51.89	37.98	26.49	13.44	5.78	2.03	0.9	0.57a	0.56	0.0406	-0.00292	0.9924
CC5	60.34	65.68 a	52.50	39.56	26.01	13.73	5.62	1.99	0.90	0.57	0.61a	0.0392	-0.00178	0.9898

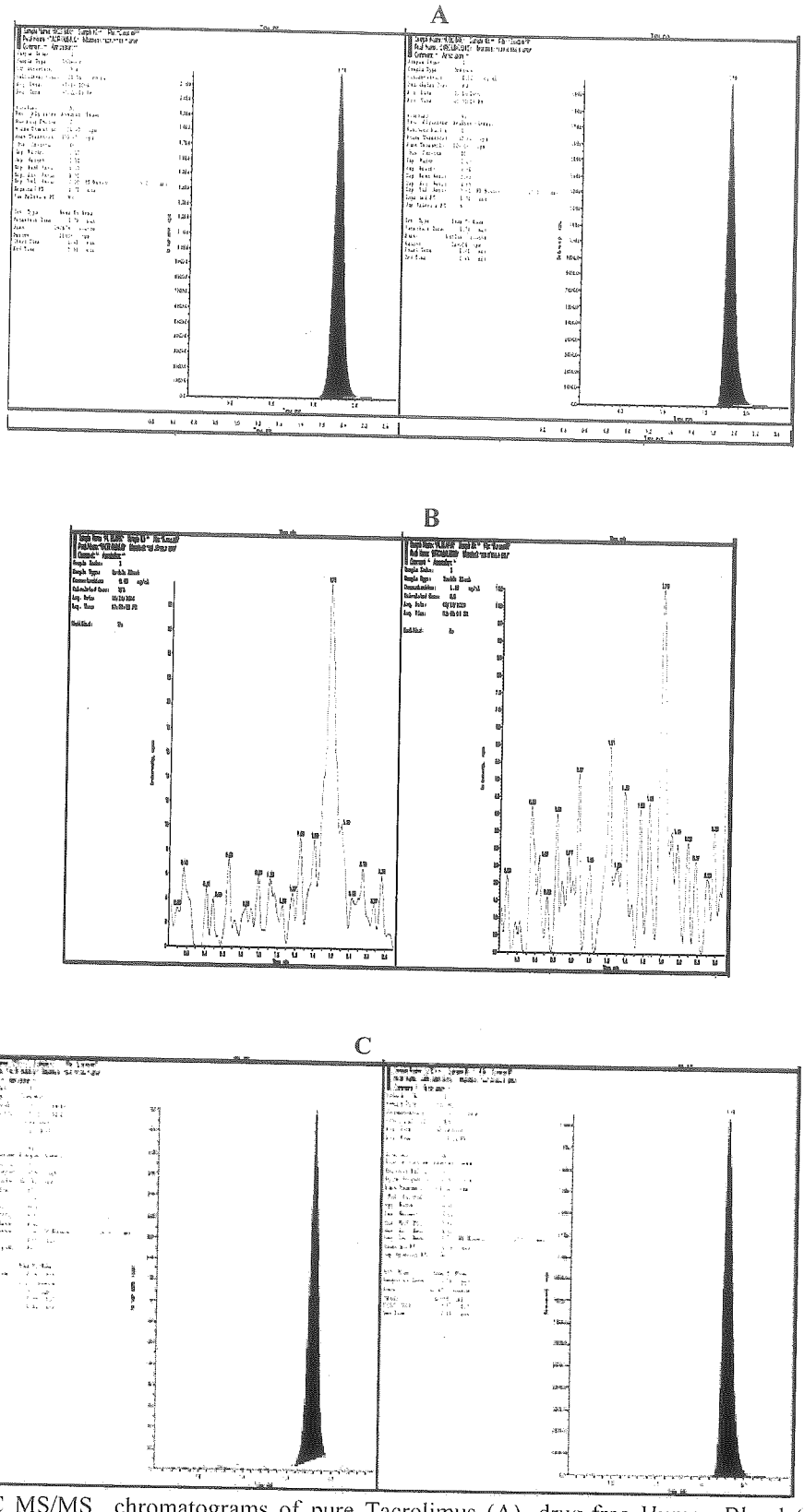

Figure 2. LC MS/MS chromatograms of pure Tacrolimus (A), drug-free Human Blood (B) and of standard Blood sample containing the Tacrolimus and Sirolimus at a concentration of 0.52ng/mL (C).

Accuracy and precision: The intra-day accuracy and precision of the assay was evaluated by analyzing six replicates of the Blood containing Tacrolimus at three different concentrations. The intra-day precision of the analyzed samples as determined by R.S.D. (%) range from 4.1 to 9.2%, while the intra-day accuracy ranged from 93.9 – 100.6%. The inter-day precision of the assay was measured by analyzing six replicates of Tacrolimus Blood samples for three consecutive days. The inter-day precision of the analyzed samples as determined by R.S.D. (%) range from 2.1 to 7.8%, while the inter-day accuracy ranged from 91.3 – 110.5%.

Recovery: The absolute recovery was calculated by comparing the peak areas of Tacrolimus and Sirolimus standards to those assessed by extraction of Tacrolimus and Sirolimus at the three different concentrations. Results of absolute recovery of Tacrolimus and Sirolimus ranged from 60.00 to 83.42% and 60.66 to 82.25 respectively as shown in Table 2.

Table 2. Absolute recovery of Tacrolimus and Sirolimus from Human Blood.

P & A II	Aqueous Area		Extracted Area		% of Recovery	
	DRUG	ISTD	DRUG	ISTD	DRUG	ISTD
HQC	359090	179652	225225	114988	62.72	64.01
	377018	185343	255412	126002	67.75	67.98
	375243	179506	229214	116857	61.08	65.10
	385545	193115	236324	117147	61.30	60.66
	388181	186593	245142	129472	63.15	69.39
	379754	178516	227845	118487	60.00	66.37
Mean	377471.8	183787.5	236527.0	120492.2	62.666	65.585
S.D +	10277.51	5665.95	11726.00	5826.09	2.7399	3.0919
% CV	2.7	3.1	5.0	4.8	4.4	4.7
MQC	183952	176351	116234	114586	63.19	64.98
	181457	183281	126254	133800	69.58	73.00
	182312	174709	118228	123396	64.85	70.63
	182915	176788	118762	123932	64.93	70.10
	183602	172512	127299	131104	69.33	76.00
	184387	175780	120010	127384	65.09	72.47
Mean	183104.2	176570.2	121131.2	125700.3	66.160	71.196
S.D +	1094.42	3624.58	4551.18	6776.49	2.6452	3.6915
% CV	0.6	2.1	3.8	5.4	4.0	5.2
LQC	9282	150689	7492	123942	80.72	82.25
	10784	183280	7248	120451	67.21	65.72
	8841	148850	7375	118163	83.42	79.38
	10991	176282	7654	121640	69.64	69.00
	10823	177376	6847	121728	63.26	68.63
	11065	173699	7013	112746	63.38	64.91
Mean	10297.7	168362.7	7271.5	119778.3	71.271	71.649
S.D +	973.19	14751.69	301.07	3927.30	8.7440	7.3340
% CV	9.5	8.8	4.1	3.3	12.3	10.2
			Global Mean		66.7	69.5
			Global S.D +		6.32	5.54
			Global %C.V.		9.5	8.0
			Global recovery%		66.7	69.5

Summary

This method was validated for its specificity, sensitivity, accuracy, linearity, precision (repeatability & reproducibility), % recovery, stability of samples (Freeze thaw, Bench top and Auto sampler stability, short-term and long term stability of stock solution and Internal Standard), dilution integrity and for ruggedness.

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