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"ANALYTICAL TECHNOLOGY: LC-MS/MS FOR THERAPEUTIC IMMUNOSUPPRESSANTS DRUGS MONITORING IN WHOLE BLOOD."

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Abstract: This article describes the simultaneous determination of immunosuppressants drugs in whole blood by tandem mass spectrometry. Here, a convenient ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method was develop and validate for the simultaneously determination of four compounds in whole blood. The whole blood samples, 50µL for each, extracted using a simple protein precipitation extraction method. The chromatographic separation was achieved on an Ultisil XB-CN column (50 mm × 2.1 mm, 3µm) at 40°C by a gradient elution within 2.8min. The mobile phase was a mixture of methanol (phase A) and 5mM ammonium acetate with 0.1% glacial acetic acid (phase B) with a total flow rate of 0.4mL/min. The positive-ionization mode with multiple reaction monitoring was used for detection. The sensitivity was good with no carry-over detected, and the lower limit of quantification range. All standard curves showed favorable linearities with $r^2 > 0.99$. The method has been successfully verified using authentic case samples that had previously been quantified using different methods. The assay is suitable for clinical utilization and management of patients on these medications.

KEYWORDS: Immunosuppressants; Therapeutic drug monitoring; UPLC-MS/MS; human whole blood .

Introduction:

Immunosuppressants are an important class of compounds which are commonly used by transplant recipients to avoid organ rejection. Immunosuppressants drugs are currently used to suppress the immune system and prevent cytokine-associated tissue damage. Tacrolimus, Sirolimus, Everolimus and Cyclosporine A are successfully applied in kidney, heart, lung, pancreas, intestinal tract, skin, and liver Transplantations (Ferrara JL, Deeg HJ 1991). In clinical routine, ligand binding assays of different designs and mass spectrometry setups with a predominance of lab developed tests are dominating technologies (Uwe, et al., 2015). In addition, the therapeutic ranges of the different immunosuppressants are even dependent on the transplanted organ, the age of the patient, the comedication, and the period after transplantation (Staatz CE, Tett SE et al., 2005) Constant patient therapeutic drug monitoring (TDM) is therefore mandatory. At the moment, several pharmacokinetic markers are used, including (limited sampling) area under the curve monitoring, C2 (2 hours after administration), and through blood levels (Kahan et al., 2002). In addition, they are used for the treatment of immune-mediated diseases or disorders of the immune system and non-autoimmune inflammatory reactions such as heavy allergic asthma. The therapeutic concentration range of these compounds, typically narrow, requires careful monitoring from whole blood to ensure the correct patient dosage.

Mass spectrometry instruments over the last decade have lead to increased utilization of high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as a means of providing assays with increased specificity and sensitivity, with the aim of improving the quality of patient care (Sallustio BC et al., 2010). The use of LC coupled with electrospray tandem mass spectrometry has become the very popular technique in bioavailability studies due to the fast, sensitive, and reliable results generated by its use (Silva *et al*., 2006). UPLC has been evaluated as a faster and more efficient analytical tool compared to current HPLC (Villiers *et al*., 2006). There are several methods for measuring TAC in whole blood, including LC-MS/MS and various immunoassays. Several analytical methods mainly based on immunoassay method (Barau C *et al*., 2009, Moes DJ *et al*., 2010, Laha TJ *et al*., 2012), fluorescent

polarization immunoassay for whole blood (Salm P *et al*., 2006), flow chromatography combined with tandem mass spectrometry (Ceglarek U *et al*., 2004), dried blood spots as a minimally invasive method (McDade *et al*., 2007), DBS specimen combined with high-sensitivity detection systems has the potential to significantly increase the feasibility of pharmacokinetic studies in children and radically improve therapeutic outcomes (Pandya *et al., 2011*), liquid chromatography-electrospray tandem mass spectrometry in DBS spots (Qin Li *et al., 2012),* liquid chromatography-electrospray tandem mass spectrometry in whole blood (Thomas *et al., 2013),* simultaneous liquid chromatography tandem mass spectrometry (Judith *et al., 2020),* Several LC-MS/MS methods have been described so far, yet most of them require online extraction procedures (Wallemacq *et al., 2003,* Korecka *et al., 2006,* Keevil *et al., 2002).* The online extraction procedures also require an additional pump, switch valve, and trapping column, making the method more complicated and vulnerable to instrument problems. In other cases, different LC-MS/MS configurations are required to analyze all immunosuppressants (Keevil *et al., 2002,* Salm *et al., 2005).*

The described and validated method for the selective determination of immunosuppressants contains a combination of solid-phase extraction, higher run time, reversed-phase LC and tandem mass detection. In this article a simple, rugged and reproducible method is described for the analysis of as low a concentration in whole blood by protein precipitation extraction and LC-MS/MS detection. The run time is only 2.8 min. Furthermore, all analytes are directly measurable without the need for any additional derivatization step, and positive ion modes were used to achieve the best sensitivity and specificity. This method consolidates all four analytes thus allowing for the diagnosis of disorders in related pathways.

Experimental

Chemicals and reagents.

The Calibrator & Controls standard set lyophilised of Immunosuppressants drugs (Figure 1) Tacrolimus, Sirolimus, Everolimus and Cyclosporine A were procured from Recipe, Munich, Germany. High purity water used for the LC-MS/MS was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). HPLC methanol and acetonitrile were purchased from. J.T.Baker (USA). All other regents and solvents were obtained from general commercial suppliers.

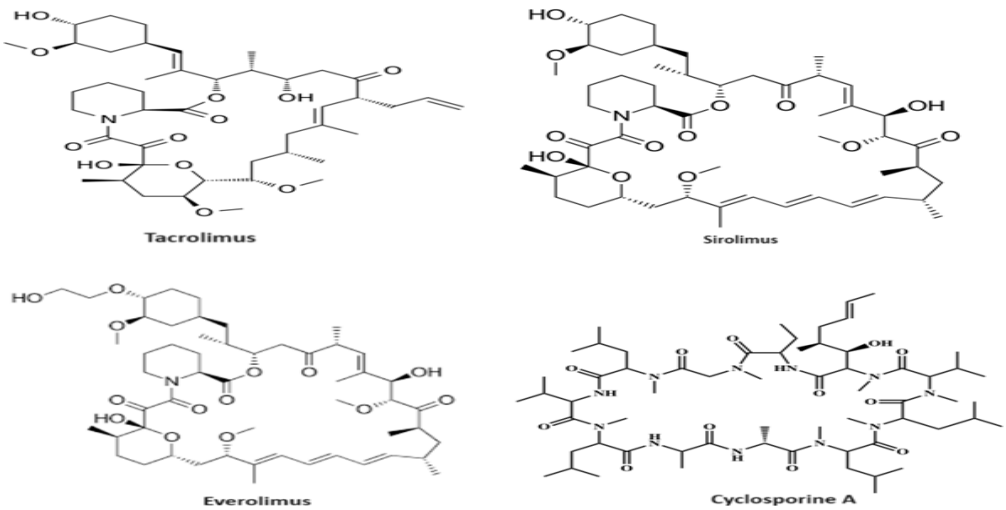

Figure 1. Structure of Immunosuppressants drugs.

Calibration curves:

The Calibration curve was established using the recipe clinical calibrator set lyophilised for Tacrolimus, Sirolimus, Everolimus and Cyclosporine A (Recipe, Munich, Germany). Three levels of calibration curve (CC) and three levels of quality controls (QC) were reconstituted as per company recommendation.

Sample Preparation

Sample preparation was achieved by simple protein precipitation. Fifty microliters of human whole blood was mixed with 0.2 mL of 100mM zinc sulfate. To this 0.3 mL of methanol was added after vortex mixing

for 60 s, Centrifuge the sample for 5 minutes at 10000 RPM at 5± 5°C. The organic supernatant layer was transferred to an auto sampler vial for LCMS/MS system.

Chromatographic conditions

Chromatography separation was performed on Nexera UPLC system (Shimadzu, Japan) with cooling auto-sampler and column oven enabling temperature control of the analytical column. The chromatography separations were performed on a Ultisil XB-CN column (50 mm × 2.1 mm, 3µm) and temperature maintained at 40˚C. The mobile phase was a gradient of 100% methanol (solvent A) and 5mM ammonium acetate with 0.1% glacial acetic acid (solvent B), with a constant flow rate of 0.4 mL/min (Table 1). The total running time was 2.80 min for each injection. Mobile phase was used as weak wash and strong wash solvent to avoid any carry over from previous injection. The auto-sampler was maintained at 10˚C and the injection volume was 10µL.

Table 1. Chromatographic conditions (gradient).

Time (min)	Flow (ml/min)	Conc. A (%)	Conc. B (%)
0.00	0.400	30.0	70.0
0.20	0.400	30.0	70.0
0.70	0.400	90.0	10.0
2.50	0.400	90.0	10.0
2.60	0.400	30.0	70.0
2.80	0.400	30.0	70.0

Result and Discussion

The objective of the present work is to develop a simple and rapid LC–MS/MS method for the simultaneous determination of Immunosuppressants drugs. Chromatographic separation was performed in gradient mode. The separation of analytes could be achieved by changing the composition of methanol and acetonitrile in the mobile phase. The use of volatile buffer namely ammonium acetate and ammonium formate and acidic buffer like formic acid and acetic acid for the separation of analytes had been evaluated also. A gradient mobile phase composed of methanol and 5mM ammonium acetate with 0.1% glacial acetic acid as gave symmetric peak shape, better separation and best sensitivity for the analytes. Among the various chromatographic columns tested for their suitability Ultisil XB-CN column (50 mm × 2.1 mm, 3µm) column gave good peak shape and response even at lowest concentration level for all analytes. The mobile phase flow rate was set at 0.4 mL/min and all peaks are evaluated in 2.4min but run time set of 2.8 min for the column to run longer.

Mass Spectrometry

The mass spectrometer was tuned in both positive and negative ionization modes to check for optimum response of Immunosuppressants drugs. The Immunosuppressants drugs molecule gave fragment ions at low and high collision energy (CE). The setting of the MS method for the detection of levetiracetam (especially) in MRM mode was done with the aim of achieving the best specificity with respect to any other available matrix ion. The first quadrupole (Q1) of the MS system was given a filter for scanning only this ion with unit resolution and keeping the third quadrupole (Q3) filter to scan the product molecular ion with low resolution. The tandem mass spectrometer was operated in electro spray with multiple reactions monitoring acquisition parameters shown in Table 2. The Desolvation and Heat Block Temperature were set at 220°C and 400°C respectively. Nitrogen was used as nebulizing and drying gas, flow was set at 3.0 and 15.0 L/min respectively.

Table 2. Optimization of MRM parameters, Ionization mode Q1 pre bias, Collision energy, Q3 pre bias and retention time (RT) of Immunosuppressants drugs.

Drugs	Precursor ion (m/z)	Product ion (m/z)	Ionization mode	Q1 pre bias (v)	Collision energy (v)	Q3 pre bias (v)	Dwell time(msec)	RT (minutes)
Tacrolimus	821.50	768.45	+	-10.0	-20.0	-28.0	50	1.99
Sirolimus	931.55	864.40	+	-35.0	-15.0	-11.0	50	2.08
Everolimus	975.50	908.40	+	-11.0	-20.0	-38.0	50	2.08
Cyclosporine A	1219.80	1202.70	+	-10.0	-20.0	-15.0	50	2.10

Specificity and Selectivity

The specificity and selectivity of this method was evaluated by analyzing 15 different sources of matrix in comparison with lower limit of quantification (LLOQ) samples. As shown in Figure 2(A) & 2(B) no significant direct interference in the blank serum traces were observed from endogenous substances in drug-free human serum at the retention time of the Immunosuppressants drugs respectively.

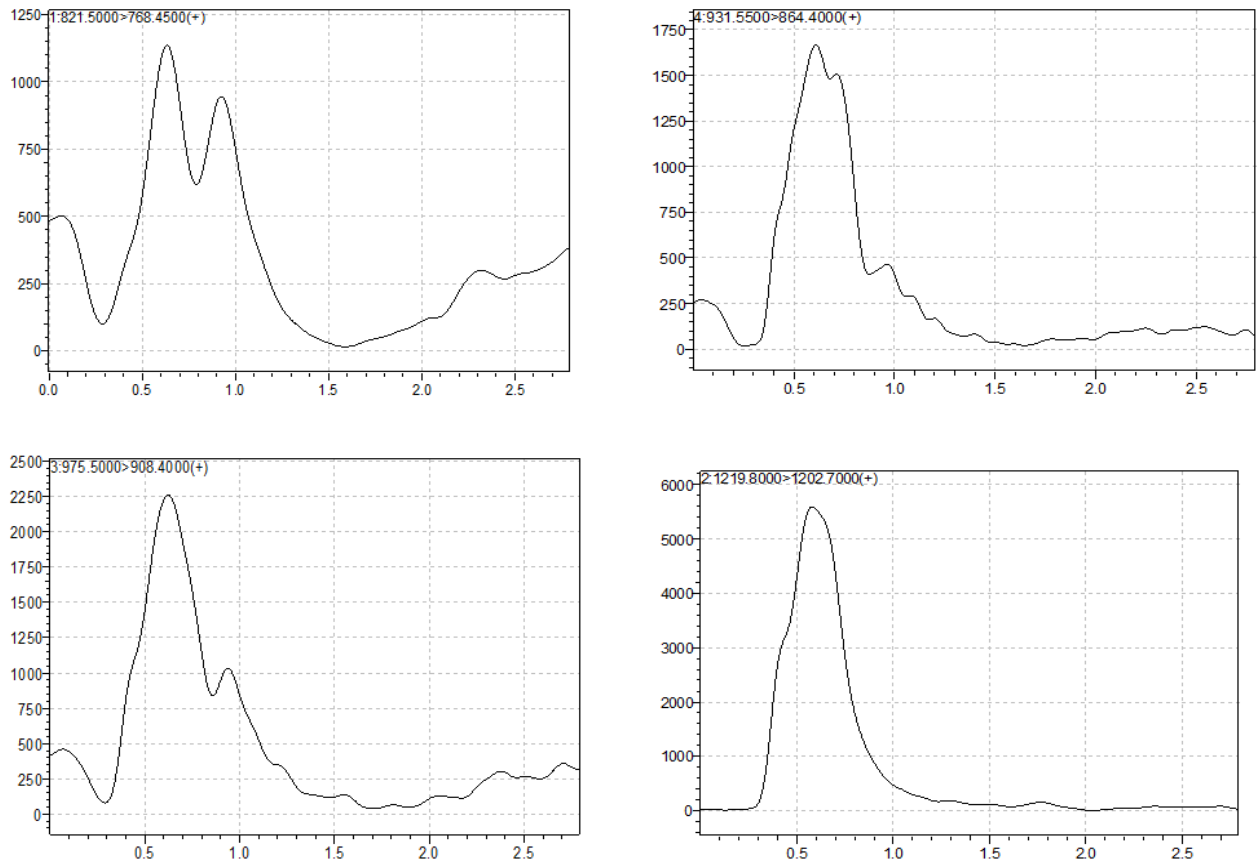

Figure 2(A): Representative chromatograms of Extracted blank plasma sample.

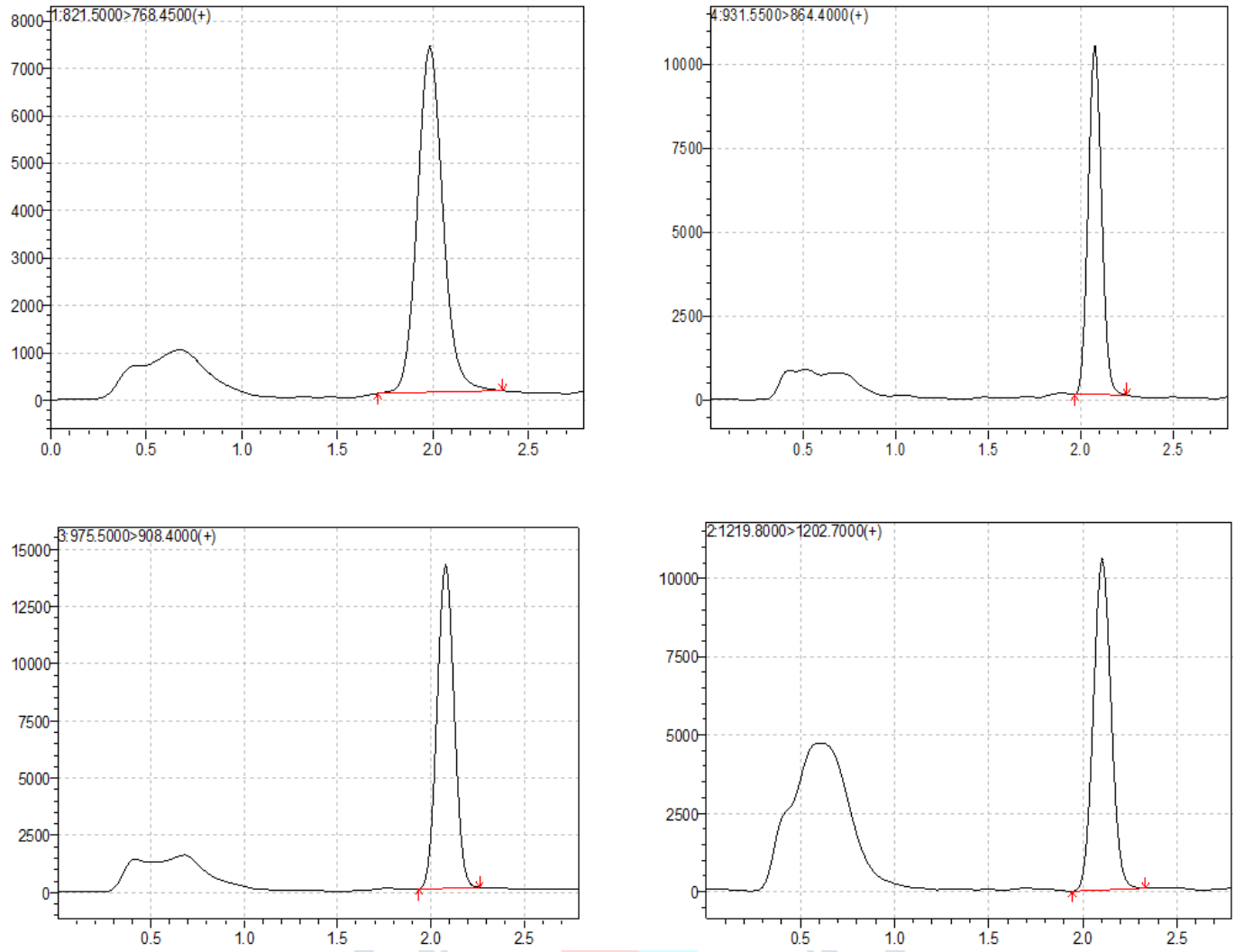

Figure 2(B): Representative chromatograms of Extracted lower limit of quantification plasma sample.

Linearity and lower limit of detection (LOD)

The linearity of Immunosuppressants drugs were determined by weighted least square regression analysis of standard plot that consisted of 3 point standard curve. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drugs concentration were found to produce the best fit for the chromatographic response versus concentrations for both the analytes in whole blood. The correlation coefficient was constantly greater than 0.99 during the entire course of validation. LOD was determined by repeated analyses of spiked samples at decreasing concentrations. Five different sources of matrix samples were spiked at decreasing concentrations and were processed and analyzed by proposed extraction procedure. Table 3 summarizes the calibration range, lower limit of quantification and concentration of LOD.

Table 3. Calibration range, LLOQ and LOD for Immunosuppressants drugs in whole blood.

Drugs	Calibration range (ng/mL)	Quality Control (ng/mL)			LLOQ (ng/mL)	LOD (ng/mL)
		Level-1	Level-2	Level-3		
Tacrolimus	1.240-20.200	3.490	7.110	14.400	1.240	0.420
Sirolimus	1.340-19.600	3.340	10.500	17.900	1.340	0.450
Everolimus	1.200-18.700	3.200	10.400	17.300	1.200	0.400
Cyclosporine A	23.200-468.000	51.000	105.000	204.00	23.20	7.740

Precision and accuracy

Intra–day precision and accuracy results were calculate using two different batches analyzed on a single day, whereas inter–day results were calculated using five different batches analyzed on a three successive day. The acceptable intra–day and inter day precision and accuracy results of Immunosuppressants drugs are presented in Table 4.

Table 4. Precision an accuracy of the method for determining Immunosuppressants drugs concentration in whole blood samples.

Analytes	Concentration added (ng/mL)		**Intra-day precision(n=5)**		**Inter-day precision (n=5)**	
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Tacrolimus	Level-1	3.490	6.9	102.8	7.7	98.9
	Level-2	7.110	6.8	95.0	8.6	96.6
	Level-3	14.400	3.4	99.6	5.8	98.2
Sirolimus	Level-1	3.340	3.8	105.3	5.5	107.0
	Level-2	10.500	3.2	98.1	2.9	103.6
	Level-3	17.900	6.2	104.2	3.2	96.4
Everolimus	Level-1	3.200	4.2	106.0	3.4	107.6
	Level-2	10.400	3.2	105.7	3.9	102.1
	Level-3	17.300	6.3	105.2	4.4	102.5
Cyclosporine A	Level-1	51.000	4.4	101.7	3.8	100.2
	Level-2	105.000	4.8	102.9	4.2	102.4
	Level-3	204.000	3.3	99.7	2.3	99.8

System suitability And Carryover effect

LC–MS/MS system performance was evaluated through system suitability test. Six consecutive injections of a Calibrator sample were injected in to the LC–MS/MS system every day before start of the analysis. The precision (% CV) for system suitability test was found to be less than 1% for retention time and 3.0% for area ratio of Immunosuppressants drugs.

Carryover effects must be evaluated during assay validation intended for confirmation and/or quantitation. Carryover effect was assessed by injecting the processed blank sample just after the highest calibrator of CC in triplicate. Carryover in the blank samples following the highest calibration standard should not be greater than 20% of the analyte response at the LLOQ.

Stability

All the stability tests for Immunosuppressants drugs were studied at Low and High levels. Stability experiments were performed exhaustively to evaluate the stability of Immunosuppressants drugs in whole blood samples under different conditions, simulating the same conditions which occurred during study sample analysis: auto sampler stability and bench top stability for whole blood. The stability results summarized in Table 5 showed that Immunosuppressants drugs spiked into whole blood were stable for at least 6 h in bench top, for at least 52 h in the mobile phase at 10°C under autosampler storage condition.

Table 5. Stability samples result for Immunosuppressants drugs concentration in whole blood samples.

Analytes	Concentration added (µg/mL)		Mean calculated comparison sample concentration for BT	Mean calculated stability sample concentration for BT	Mean percentage change for BT	Mean calculated comparison sample concentration for ASS	Mean calculated stability sample concentration for ASS	Mean percentage change for ASS
Tacrolimus	Level-1	3.490	3.429	3.327	-3.0	3.287	3.109	-5.4
	Level-3	14.400	14.405	14.218	-1.3	14.192	13.676	-3.6
Sirolimus	Level-1	3.340	3.575	3.729	4.3	3.369	3.570	6.0
	Level-3	17.900	17.888	17.251	-3.6	17.178	17.805	3.6
Everolimus	Level-1	3.200	3.312	3.363	1.5	3.010	2.943	-2.2
	Level-3	17.300	17.627	18.199	3.2	17.093	17.937	4.9
Cyclosporine A	Level-1	51.000	51.393	50.085	-2.5	44.977	47.262	5.1
	Level-3	204.000	202.701	204.345	0.8	202.617	206.600	2.0

Conclusion

In Conclusion, A high-efficiency LC-MS/MS method for the determination of Immunosuppressants drugs in whole blood was developed and validated. This method was proven to be sensitive, rapid, and convenient due to its simple sample preparation and other experimental conditions. The extraction method gave consistent and reproducible for Immunosuppressants drugs from whole blood, with minimum interference, ion suppression and a short chromatographic run time is only 2.8 min. This all provides better and faster patient care at lower costs. We provide a platform that can be easily adapted to a number of clinical settings, particularly those which have complex and challenging patient populations with relatively low specimen numbers. Practically, this would facilitate economical, single day turnaround time for immunosuppressants requiring maintenance of a single assay.

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