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Development And Validation of Bioanalytical Method for The Determination of Imatinib In Human Plasma by LC-MS/MS

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ABSTRACT

A sensitive method has been developed for the determination of imatinib in human plasma by using liquid chromatography coupled with tandem mass spectrometer (LC-MS/MS) with deuterated imatinib (imatinib D8) as internal standard having the sample volume of 150 µl. Sample preparation was performed using precipitation technique and chromatographic separation was achieved using the mobile phase consisting the mixture of 0.1% formic acid and methanol in the ratio of 65:35 with the flow rate of 0.6 ml/min. The retention times of imatinib and imatinib D8 were found to be 1.5 minutes and the method was found to be linear over the calibration curve range of 10 to 4000 ng/ml. The method was validated for accuracy, precision, linearity, recovery and stability evaluations in compliance to international regulatory guidelines for bioanalytical method validation.

Key words: Imatinib, Plasma, Protein Precipitation, LC-MS/MS and Validation.

INTRODUCTION

Imatinib mesylate (imatinib, Fig. 1) is widely used for the treatment of different types of cancer, such as chronic myelogenous leukemia and gastrointestinal stromal tumors. It is a tyrosine kinase inhibitor which binds to the active side of the enzyme and prevents the binding of ATP (1). Imatinib has demonstrated efficacy in cancer treatment by virtue of its ability to potently and selectively inhibit the BCR-ABL tyrosine kinase as well as the platelet-derived growth factor receptors a-PDGFR and ß-PDGFR and KIT receptor tyrosine kinases (2) and is considered as the first line of non stem cell transplantation treatment for patients diagnosed with Chronic Myeloid Leukemia (3) and it has received fast track approval by the US Food and Drug Administration (FDA) for treatment of chronic myeloid leukemia (4).

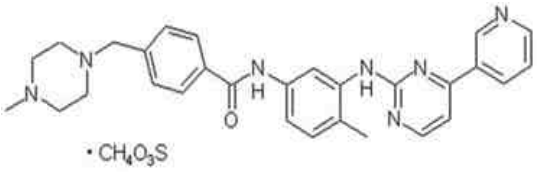

Figure 1. Imatinib mesylate

Bioequivalence and/or pharmacokinetic studies become an integral part of generic drug applications and a simple, sensitive, reproducible validated bioanalytical method should be used for the quantification of intended analyte, i.e. imatinib from the biological matrix. The following literature for the quantification of imatinib from dosage forms and biological matrix was reported by using various instrumentation techniques. Widmer et al.and Schleyer et al. reported an HPLC method for the determination of imatinib from plasma with a solid phase extraction and online extraction technique respectively (5-6). Bakhtiar et al and Parise et al reported an LCMSMS and LCMS method for imatinib and its metabolite using protein precipitation technique the later were reported the sensitivity of 30 ng/ml (4,7). Jelena et al reported the LCMSMS method for imatinib in whole human blood and cell culture using trazadone as internal stanadard (8).

Guetens et al reported the quantification of imatinib in plasma and erythrocytes (9) and patient tumor tissues (10) using LCMSMS and sediment technology. They also reported an HPLC UV method for the simultaneous determination of imatinib along with another molecule in cultured tumor cells with the limit of quantification of 50 ng/ml (11). Oostendorp et al reported an ion pairing HPLC method for the determination of imatinib in plasma and murine specimens (12). Velpandian et al reported an HPLC UV method for the estimation of imatinib from biological samples of patients with chronic myeloid leukemia with the sensitivity of 50ng/ml (13). Maria et al reported an HPLC method for the quantification of imatinib mesylate from pharmaceutical dosage forms using UV detection at 237nm (14). Satyanarayana et al reported an HPLC method using UV detection at 240nm for the estimation of imatinib from bulk and pharmaceutical dosage forms (15). Zafer et al reported an LC-MS method for the quantification of imatinib from plasma and bone marrow samples with the ion trap method using 250 µl of sample volume (16). Teoh et al reported an HPLC method for the determination of imatinib from mice plasma and tissue after multiple dose administration with the sensitivity of 100 ng/ml (17).

Validation of the bioanalytical method is the primary requirement for a method which is part of the pharmacokinetic study and/or bioequivalence study. Guidelines for the bioanalytical method validation have been available since a decade by USFDA (18) and recently the EMA has also released the guidelines for the same (EMA BMV) indicating the requirement of bioanalytical method validation (19). The aim of the present work is to develop and validate sensitive and simple bioanalytical method with the low sample volume for the quantification of imatinib from plasma using deuterated internal standard with the desired calibration range applicable

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for bioequivalence studies. The method can be readily applicable for usage during the bioequivalence evaluation of various generic formulations for submission as part of abbreviated new drug applications.

MATERIALS AND METHODS

Chemicals

Imatinib standard was procured as a gift sample from a Pharma company and HPLC grade methanol, acetonitrile were commercially procured and all other chemicals were of analytical grade.

Preparation of solutions

0.1% formic acid solution was prepared by diluting 0.1 ml of formic acid to 100 ml in a volumetric flask with milli Q water and the pH of solution was measured to conform the pH range of 2.6±0.2. 80% acetonitrile was prepared by mixing 1600 ml of acetonitrile and 400 ml of water. 25% methanol in 0.1% formic acid was prepared by mixing 200 ml of methanol and 800 ml of 0.1% formic acid in a reagent bottle. Dilute hydrochloric acid solution-1 was prepared by diluting 8.5 ml of HCl to 100 ml with milli Q water and dilute hydrochloric acid solution-2 was prepared by mixing 2 ml of 1N HCl with 98 ml milli Q water. Mobile phase consists of 0.1% formic acid and methanol in the ratio of 65:35 respectively.

Preparation of standards

Imatinib and Imatinib D8 stock solutions were prepared at a concentration of 1 mg/ml by dissolving in dilute hydrochloric acid solution-2 and the stock solutions were stored in the refrigerator. Spiking solutions of imatinib for the preparation of calibration standards and quality control samples were prepared in 80% acetonitrile and spiked in to the plasma at the ratio of 1:50. The calibration curve from 10.0 to 4000.1 ng/ml was generated using eight calibration standards at the concentrations of 10.0 ng/ml (STD 1), 20.2 ng/ml (STD 2), 100.5 ng/ml (STD 3), 250.2 ng/ml (STD 4), 750.1 ng/ml (STD 5), 2000.1 ng/ml (STD 6), 3250.8 ng/ml (STD 7) and 4000.1 ng/ml (STD8). The Quality Control samples were prepared at the concentrations of 10.2 ng/ml (LLOQQC), 29.9 ng/ml (LQC), 1612.5 ng/ml (MQC) and 3550.2 ng/ml (HQC). The bulk spiked calibration standards and quality control samples were stored in the freezer. Internal standard dilution was prepared at a concentration of 400 ng/ml using 80% acetonitrile.

Sample preparation and extraction

Imatinib from the plasma was extracted using protein precipitation technique. Plasma aliquot of 0.15 ml (150µl)was added to the tube containing 50µl of internal standard dilution and vortexed the tubes. 1 ml of methanol was added to the above tube and vortexed on vibramax for 5 minutes and then the tubes are centrifuged at 12000 rpm for 10 minutes and after centrifugation, 250 µl of supernatant was added to the tube containing 750µl of 0.1% formic acid and vortexed to mix the contents and the same was transferred to the auto sampler vials.

Chromatographic conditions

HPLC coupled with Mass Spectrometer (LC-MS/MS) with the C18 column (3 x 50mm, 3.5µ) was used and the m/z of 494.3/394.2 and 502.3/394.2 were used in Multiple Reaction Monitoring (MRM) mode with turbo ion spray in positive mode for the quantification of imatinib and internal standard respectively. The other mass spectrometric conditions are optimized for reproducible response. The mobile phase used was 0.1% formic acid and methanol in the ratio of 65:35.

Validation

The method performance was evaluated for selectivity, accuracy, precision, linearity, and robustness, stability during various stress conditions including bench top stability, freeze thaw stability, auto sampler stability, stability of stock solutions etc, dilution integrity and recovery.

Selectivity

Selectivity was evaluated by extracting different blank plasma samples. The absence of interfering peaks at the retention time of analyte or internal standard was considered as evidence for selectivity.

Linearity

Calibration curves were constructed after evaluating the linear regression for the best fit using weighing of none, 1/x and 1/x2 for the calibration curve range of 10.0 to 4000.1 ng/ml.

Recovery

Recovery of analyte was evaluated by comparing the imatinib and internal standard response in extracted samples versus equivalent aqueous samples. Recovery was evaluated at three levels of quality control samples (LQC, MQC and HQC levels). The mean recovery of analyte and internal standard was evaluated.

Precision and accuracy

For precision and accuracy studies, samples were prepared at four concentration levels, limit of quantification (LOQQC), low (LQC), medium (MQC) and high (HQC) quality controls. Corresponding to 10.2, 29.9, 1612.5 and 3550.2 ng/ml respectively with six replicates each. Precision and accuracy was evaluated at inter and intra day.

Dilution Integrity

Dilution integrity was evaluated by diluting the sample having the concentration of approx. 7000 ng/ml (approx. two times of HQC) with 1/5 and 1/10 dilutions and quantified against the calibration curve to evaluate the ability to dilute the pharmacokinetic samples.

Stability studies

The stability of the imatinib in solutions and plasma samples was also evaluated during method validation. Imatinib stability was evaluated using two concentration levels (low and high quality control, corresponding to 29.9 and 3550.2 ng/ml respectively). The stability of imatinib was also evaluated in post-extracted samples kept in the auto sampler at 10 °C as well as in plasma samples kept at freezer and after being stressed to freeze-thawing cycles (24 hours each cycle). All samples described above were quantified using fresh calibration curve and compared to freshly prepared quality control samples at the same concentration level.

RESULTS AND DISCUSSION

Chromatographic optimization

Liquid chromatography coupled with the mass spectrometer (LC-MSMS) has now become a universally acceptable technique for the estimation of drugs from the biological fluids as part of bioequivalence evaluations. Imatinib and internal standard were scanned in the positive mode for the parent ion and reproducible daughter ion and the m/z ratio of 494.3/394.2 and 502.3/394.2 respectively were selected for imatinib and internal standard. The quantification was performed in multiple reaction monitoring (MRM) mode in analyst software. The compound specific mass spectrometric parameters are optimized to produce the reproducible responses for the analyte and internal standard. Chromatographic conditions are optimized to achieve good resolution and symmetric peak shape for the analyte at the lower level of quantification. The chromatographic conditions like flow rate (0.6 ml/min) and column (C18 column) conditions were also optimized with the runtime of 5 minutes. The analyte and internal standard were quantified at 1.5 minutes. Other conditions are optimized for the reproducible quantification method.

Sample preparation

Protein precipitation technique was chosen for the simple and cost effective

extraction procedure and the conditions are optimized to yield cleaner extract of the sample to avoid the quantification issues with the LCMSMS. Sample volume of 150µl was optimized to have the sensitivity and quantifiable and acceptable peak shape at the lower limit of quantification of 10 ng/ml.

The Quality control samples were prepared at the concentrations specified in the bioanalytical method validation guidelines. The LOQQC was prepared at approximately same concentration of lowest calibration standard. The LQC was prepared at the concentration less than three times of lowest

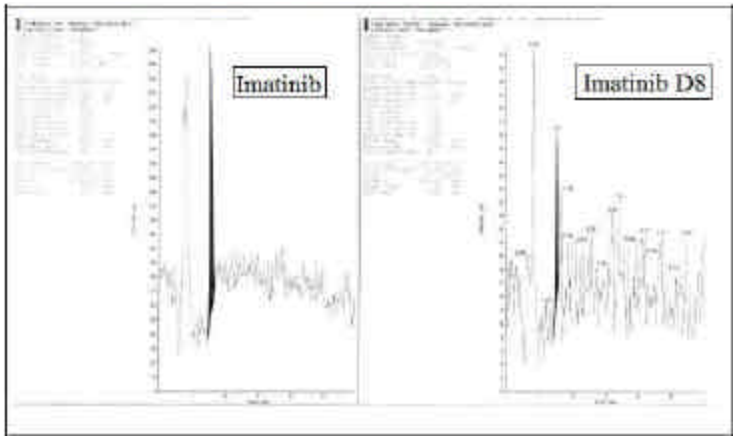

Fig. 2. Representative chromatogram of blank plasma sample

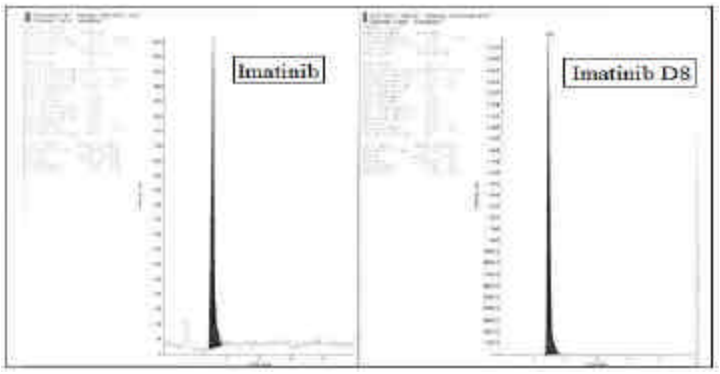

Fig. 3. Representative chromatogram of lowest calibration standard

calibration standard. MQC concentration was prepared at approximately 40% of the highest calibration standard. HQC concentration was prepared at the concentration of approximately 85% of the highest calibration standard.

Selectivity

The LCMSMS method was selective for the intended analyte since the quantification is based on the mass to charge ratio of parent as well as product ion in MRM transition mode which are selective and specific. The selectivity was also established for the blank plasma lots with the acceptance criteria of analyte response and internal standard response observed in the blank plasma samples shall be less than 20% and 5% of the analyte and internal standard response of LLOQ sample. No interference was observed for the blank plasma lots at the analyte and internal standard retention times. **Figures 2,3 and 4** represent the chromatogram of blank plasma sample and lowest calibration standard and highest calibration standards respectively.

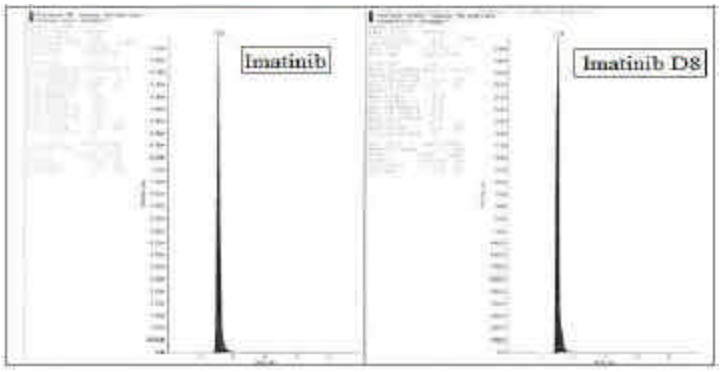

Fig. 4. Representative chromatogram of highest calibration standard

Linearity

Linearity was demonstrated from 10.0-4000.1 ng/ml. Table 1 shows data from calibration curves analysed for the evaluation of precision and accuracy during different days. The calibration curve includes 8 calibration standards which are distributed throughout the calibration range. Correlation coefficient was considered for the evaluation of goodness fit. The average correlation coefficient was found to be 0.9988 with goodness of fit. The details of calibration curves are represented in Table 1.

Precision and accuracy

Precision and accuracy was evaluated by analysing 3 precision and accuracy batches. Each precision and accuracy batch consists of calibration curve and six replicates of LOQQC, LQC, MQC and HQC. Precision and accuracy was evaluated both inter and intra batches. The intraday and inter day precision and accuracy of the method for each imatinib concentration level (10.2, 29.9, 1612.5 and 3550.2 ng/ml) are presented in Table 2. The mean accuracy for each concentration level ranged from 101.0 to 103.2 and the mean precision for each concentration level ranged from 2.4 to 7.6.

Recovery

The recovery was evaluated by comparing response of extracted and unextracted samples. Extracted samples include six replicates of extracted LQC, MQC and HQC samples. Unextracted samples included the aqueous solutions equivalent to extracted samples. Internal standard recovery was evaluated in the same manner at MQC level. The average recovery for imatinib in plasma was ranged from 86.2 to 92.7% for the low, medium and high quality control samples respectively with an average of 89.3%. The average recovery of the internal standard was 91.5%.

Stability studies

Stability studies were performed to evaluate the stability of imatinib both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include stock solution stability of imatinib and Imatinib D8 in stock solution, stock dilution stability of imatinib in dilutions, bench top stability in plasma, freeze thaw stability in plasma,

Table -1: Precision and accuracy of Calibration standards

Batch	STD 1	STD2	STD3	STD 4	STD 5	STD 6	STD 7	STD 8	Regression
	Concentration (ng/ml)								Coefficient (r value)
	10	20.2	100.5	250.2	750.1	2000.1	3250.8	4000.1	
P&A-1	10.2	19.3	96.6	244.6	785.3	2050.2	3240	4086.4	0.9989
P&A-2	10.4	18.5	98.7	243.4	805.5	2124.5	3100.5	4058.3	0.9978
P&A-3	10.1	19.8	98.7	242	801.7	2053.4	3238.6	4071.4	0.9997
Average	10.23	19.20	98.00	243.33	797.50	2076.03	3193.03	4072.03	0.9988
Standard Deviation	0.153	0.656	1.212	1.301	10.735	42.004	80.139	14.061	
%CV	1.5	3.4	1.2	0.5	1.3	2.0	2.5	0.3	

long term storage stability in plasma, and auto sampler stability of processed samples. All stability evaluations were performed as per international regulatory guidelines.

Table 2: Between and within batch precision and accuracy of QC samples

Nominal Concentration	LOQQC 10.2	LQC 29.9	MQC 1612.5	HQC 3550.2
Precision and	11.4	31.2	1691.0	3602.6
Accuracy Batch-1	10.8	28.8	1684.8	3736.0
	10.6	30.3	1662.4	3650.4
	11.3	31.2	1618.3	3726.8
	11.4	27.7	1601.8	3567.0
	10.6	30.3	1596.0	3554.0
Average	11.02	29.92	1642.38	3639.47
Standard Deviation	0.392	1.396	42.289	78.715
%CV	3.6	4.7	2.6	2.2
% Nominal	108.0	100.1	101.9	102.5
Precision and	9.1	32.8	1603.3	3730.5
Accuracy Batch-2	10.4	32.9	1561.0	3749.6
	10.6	32.1	1586.7	3631.8
	9.4	29.9	1624.1	3571.2
	9.1	30.8	1595.8	3559.5
	9.6	30.3	1650.3	3507.7
Average	9.70	31.47	1603.53	3625.05
Standard Deviation	0.651	1.303	30.862	97.617
%CV	6.7	4.1	1.9	2.7
% Nominal	95.1	105.2	99.4	102.1
Precision and	11.3	30.4	1654.7	3498.2
Accuracy Batch-3	10.0	30.9	1682.1	3513.3
	9.3	30.0	1674.5	3565.4
	10.3	32.4	1641.1	3499.5
	10.1	30.9	1680.4	3507.2
	10.1	32.4	1638.2	3512.3
Average	10.18	31.17	1661.83	3515.98
Standard Deviation	0.646	1.013	19.775	25.011
%CV	6.3	3.3	1.2	0.7
% Nominal	99.8	104.2	103.1	99.0
Global Precision and Accuracy				
Average	10.30	30.85	1635.92	3593.50
Standard Deviation	0.778	1.361	39.282	89.593
%CV	7.6	4.4	2.4	2.5
% Nominal	101.0	103.2	101.5	101.2

Imatinib and Imatinib D8 stock solutions (1 mg/ml) remained stable when stored at refrigerator conditions for 5 days including the storage at room temperature for 8 hours. Imatinib was stable in plasma samples when stored at room temperature for 20 hours. Imatinib was found to be stable for 3 freeze and thaw cycles. Imatinib was stable and did not show any degradation when stored in the freezer for 106 days. Imatinib in the processed samples was stable for 40 hours when stored in the auto sampler at 10°C. The method characteristics are represented in table 3.

Table 3: Method Characteristics

Analyte	Imatinib
Internal Standard	Imatinib D8
Method Description	Protein Precipitation with LCMSMS detection
Regression Model	Linear regression with $1/Conc^2$ weighing
Analysis method	Peak area ratios
Limit of Quantification	10.0 ng/ml
Selectivity	No interference from the endogenous matrix components
Recovery of analyte	89.3 %
Recovery of internal standard	91.5 %
Linearity Range	10.0 to 4000.1 ng/ml
Quality Controls concentrations	LLOQQC: 10.2 ng/ml LQC: 29.9 ng/ml MQC: 1612.5 ng/ml HQC: 3550.2 ng/ml
QC interday accuracy range	101.0 to 103.2 %
QC interday precision range	2.4 to 7.6 %
Bench Top stability	20 hours at room temperature
Freeze-Thaw stability	3 Cycles
Auto injector stability	40 hours
Long term stability	106 days
Stock solution stability	5 days
Stock dilution stability	29 hours
Dilution integrity	1/5 and 1/10
Re-injection Reproducibility	One time

CONCLUSIONS

We described here the development of a new, selective, precise and accurate method for the quantification of imatinib in human plasma using Liquid Chromatography Mass Spectrometric method with the simple protein precipitation technique using the less volume of plasma and is suitable for application to a pharmacokinetic, bioequivalence and drug interaction studies for the estimation of imatinib from plasma. The limit of quantification of the method was set to 10 ng/ml considering the dosage of imatinib administered and it is determined not only by detection technique but also by the effective clean-up of sample and thus improving the signal to noise ratio. The method reported here uses a simple and cost effective extraction technique with good and reproducible recovery.

REFERENCES

1. Marek D, Heidemarie B. (2011). Imatinib quantification in human serum for clinical purposes using high-performance liquid chromatography with a diode array detector. Acta Chim. Slov., 58: 347–350.
2. Hans PG, Ulrike P, Felix W, Markus Z, Claudia S, Peter Z, Michael H, Rolf P, Michael S, Monique BA, Bin P, Gerhard G. (2005). Metabolism and disposition of imatinib mesylate in healthy volunteers. DMD, 33:1503–1512.
3. Tripathi AK, Ashutosh K, Anant R. (2011). Total leukocyte counts and the requirement of dose reduction due to cytopenias as prognostic indicators affecting response to imatinib in chronic myeloid leukemia. Indian J Hematol Blood Transfus., 27(1):7–13.
4. Bakhtiar R, Lohne J, Ramos L, Khemani L, Hayes M, Tse F. (2002). High-throughput quantification of the anti-leukemia drug STI571 (Gleevec) and its main metabolite (CGP 74588) in human plasma using liquid chromatography-tandem mass spectrometry. J. Chromatogr. B., 768:325–340.
5. Widmer N, Beguin A, Rochat B, Buclin T, Kovacsovics T, Duchosal MA, Leyvraz S, Rosselet A, Biollaz J, Decosterd LA. (2004). Determination of imatinib (Gleevec®) in human plasma by solid-phase extraction–liquid chromatography–ultraviolet absorbance detection, J. Chromatogr. B., 803:285–292.
6. Schleyer E, Pursche S, Kohne CH, Schuler U, Renner U, Gschaidmeier H, Freiberg-Richter J, Leopold T, Jenke A, Bonin M, Bergemann T, Le CP, Gruner M, Bornhauser M, Ottmann OG, Ehninger G. (2004). Liquid chromatographic method for detection and quantitation of STI-571 and its main metabolite N-desmethyl-STI in plasma, urine, cerebrospinal fluid, culture medium and cell preparations. J. Chromatogr. B., 799: 23–36.
7. Parise RA, Ramanathan RK, Hayes MJ, Egorin MJ. (2003). Liquid chromatographic-mass spectrometric assay for quantitation of imatinib and its main metabolite (CGP 74588) in plasma, J.Chromatogr. B., 791: 39–44.
8. Jelena K, Yan LZ, Jost K, Nora A, Natalie JS, and Uwe C. (2009). Development and validation of a sensitive assay for the quantification of imatinib using LC/LC-MS/MS in human whole blood and cell culture. Biomed Chromatogr., 23(12): 1251–1258.
9. Guetens G, De GB, Highley M, Dumez H, Van OAT, Bruijn EAD. (2003). Quantification of the anticancer agent STI-571 in erythrocytes and plasma by measurement of sediment technology and liquid chromatography–tandem mass spectrometry. J. Chromatogr. A., 1020: 27–34.
10. Guetens G, Prenen H, Boeck GD, Highley M, Wever ID, Oosterom ATV, Bruijn EAD. (2006). Imatinib (Gleevec®, Glivec®) tumour tissue analysis by measurement of sediment and by liquid chromatography tandem mass spectrometry. J. Sep. Sci., 29: 453–459.
11. Guetens G, Prenen H, Boeck GDB, Oosterom AV, Schoffski P,

Highley M, Bruijn EAD. (2007). Simultaneous determination of AMN107 and Imatinib (Gleevec®, Glivec®, STI571) in cultured tumour cells using an isocratic high-performance liquid chromatography procedure with UV detection. J. Chromatogr. B., 846: 341–345.

12. Oostendorp RL, Beijnen JH, Schellens JHM, Olaf VT.(2007). Determination of imatinib mesylate and its main metabolite (CGP74588) in human plasma and murine specimens by ion-pairing reversed-phase high-performance liquid chromatography. Biomed. Chromatogr., 21:747–754.
13. Velpandian T, Mathur R, Agarwal NK, Arora B, Kumar L, Gupta SK. (2004). Development and validation of a simple liquid chromatographic method with ultraviolet detection for the determination of imatinib in biological samples. J. Chromatogr. B., 804:431–434.
14. Maria AR, Maria AM, Maria TP, Adriana IS. (2005). Validation of an HPLC Method for the Determination of Imatinib Mesylate in Pharmaceutical Dosage. J Liq. Chrom. & Rel. Tech., 28(20): 3283-3292.
15. Satyanarayana G, Ramesh E, Jitendra KP, Hanumantha RK, Sridhar B, Nagaraju P. (2010). Development and validation of new reversed phase high performance liquid chromatography method for the estimation of imatinib in bulk and pharmaceutical dosage forms. Int. Jour. Res. Pharm. & Biomed. Sci., 1(1):6-9.
16. Zafar I, Moira E, David GW, Tessa H And Heather J. (2011). Analysis of imatinib in bone marrow and plasma samples of chronic myeloid leukaemia patients using solid phase extraction LC-ESI-MS. Pak. J. Pharm. Sci., 24(3):285-291.
17. Teoh M, Narayanan P, Moo KS, Radhakrisma. (2010). HPLC determination of imatinib in plasma and tissues after multiple oral dose administration to mice. Pak. J. Pharm. Sci., 23(1):35-41.
18. Guidance for industry, (2001). Bionalytical method validation, Food and Drug Administration. Center for Drug Evaluation and Research, May 2001.
19. European Medicines Agency, (2011). Guideline on bioanalytical method validation, 21 July 2011, EMEA/CHMP/EWP/192217/ 2009.

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