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LC-MS/MS Bioanalytical Approach for the Quantitative Analysis of Dabigatran in Biological Fluids

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A novel bioanalytical methodology has been developed to establish a rapid and sensitive procedure for the detection of dabigatran in biological fluid, specifically human plasma. In this study, dabigatran $^{13}C_6$ was utilized as an internal standard (IS). The solid phase extraction method was employed to extract dabigatran and internal standard (IS) from a plasma sample. The separated components are determined by utilizing a eluting mixture composed of 2 mM ammonium formate:methanol:acetonitrile (20:40:40 v/v/v). The procedure utilized a GL Sciences Ace C18 stationary phase with dimensions of 150 mm × 4.6 mm; 5 µm. The identification of dabigatran and IS was carried out using the MRM mode, which was chosen to minimize interference from the surrounding matrix. The measured *m/z* of the protonated precursor ion of dabigatran was 472.20, while the corresponding product ion has a *m/z* value of 289.10. The precursor ion with a *m/z* of 478.20 and the product ion with a *m/z* of 295.20 were generated by the internal standard (IS). Linearity was observed within the range of 1.016-304.025 ng/mL; r^2 of 0.9956 and a sample size (n) of 5. The stability of the drug in plasma was evaluated through a series of tests. These tests included a 17 h period on a laboratory bench, 24 h in an injector, six cycles of freeze-thaw and a storage period of 7 days and 22 h at -20 ± 5 ºC. The results of these stability studies indicated that the drug remained stable in plasma. The method was subjected to validation and was determined to be qualified. The proposed analytical approach demonstrates potential for the routine analysis and pharmacokinetic study of dabigatran in biological fluids.

Keywords: Dabigatran, LC-MS/MS, Sample size, Biological fluid.

INTRODUCTION

Dabigatran is an aromatic amide compound which is synthesized *via* the formal condensation reaction. Dabigatran etexilate, a type of prodrug, undergoes a process of metabolic activation in order to generate an active metabolite that serves as an anti-coagulant. The utilization of this pharmacological agent is intended to prevent embolism and stroke. The substance exhibits multiple functions, such as serving as an anticoagulant, inhibiting the enzyme thrombin (EC 3.4.21.5) and inhibiting the enzyme ribosyl dihydro nicotinamide dehydrogenase (quinone) (EC 1.10.99.2). The compound under consideration is categorized as an aromatic amide, specifically belonging to benzimidazole group. The compound can also be classified as a carboxamidine, which is a derivative of pyridines and β-alanine [1]. Dabigatran is also referred to as Pradaxa, demonstrates reversible binding to the active site of the thrombin molecule. As a result, it hinders the activation of coagulation components that are mediated by thrombin. There is a possibility that it demonstrates a diminished antagonistic effect on thrombin-induced platelet aggregation. Additionally, dabigatran possesses the capability to inactivate thrombin, even when fibrin is present. This action reduces the inhibitory effect of thrombin on fibrinolysis, potentially enhancing the process of fibrinolysis [2]. This medication is indicated to avoid embolism and stroke in individuals suffering from nonvalvular atrial fibrillation post replacement procedures [3].

A comprehensive literature review has identified numerous methods for the estimation of dabigatran in its pure form, prodrug form, both alone and in combination, as well as in the presence of metabolites and impurities. In their study, Gouveia *et al.* [4] devised a liquid chromatographic method to analyze direct oral anticoagulant drugs in biological samples. Korostelev *et al.* [5] determined rivaroxaban and dabigatran levels in human plasma by LC-MS/MS, whereas the detection of DBT in human plasma was performed using UPLC MS/MS as reported by Delavenne *et al.* [6]. Li *et al.* [7] developed method for the analysis of a prodrug of dabigatran and its associated metabolites using LC-MS/MS. Hu *et al.* [8] employed the triple quadrupole technique to identify metabolites of dabigatran. Furthermore, the prodrug and active metabolites in human plasma were assayed using LC-MS/MS [9], whereas Nagadeep *et al.* [10] performed the analysis of a prodrug in the presence of impurities employing HPLC. In 2015, Amrani *et al.* [11] identified a metabolic pathway for a prodrug using LC-MS/MS. Similarly, the quantification of total dabigatran in human plasma was performed by Abd Allah *et al.* [12] using LC-MS/MS technique. Using advanced technique, Wang *et al.* [13] performed fluorescence chemometric comparison between dabigatran and a prodrug. The present work elucidates a novel rapid approach that possesses, cost-effectiveness and sensitivity for dabigatran, while considering its significance as a pharmaceutical agent.

EXPERIMENTAL

The pure form of dabigatran and dabigatran $^{13}C_6$ (IS) were provided by Clearsynth Limited, India, Hyderabad, India. Methanol and acetonitrile (ACN) (JT Baker), HCl, citric acid, ammonium formate (Merck), formic acid (AVRA), Water of Rankem; Strata-X 33 µm, polymeric sorbent of Pheno-menex make were procured and finally the human plasma sample was collected from the local blood bank.

HPLC and MS operating conditions: The separation process involved the utilization of the API 3000 LC-MS/MS instrument in positive ionization mode, employing atmospheric pressure ionization. A stationary phase of Ace 3 (150 mm × 4.6 mm, 5 µm) was used. The elution mixture consisted of a combination of 2 mM ammonium formate with 0.1% formic acid HPLC grade methanol and acetonitrile in a ratio of 20:40:40 v/v/v was passed by isocratic mode at 1.0 mL/min at ambient conditions. The instrument conditions were maintained at curtain gas (CUR) 12 psi; Temperature (TEM ºC) 500 ºC; collision gas (CAD) 10 psi; dwell time 200 ms. The observation of transition pairs, specifically the precursor to product ion, was conducted within the *m/z* range of 472.20 to 289.10.

Solutions: 1 mg/mL concentration of dabigatran was prepared by transferring 2 mg of drug into 2 mL volumetric flask. Mobile phase was used as diluent for this purpose. Suitable and caluculated concentrations of linearity and QC samples were prepared as per the standard procedures and stored at 2 to 8 ºC.

Sample preparation: After thawing, samples were vortexed and a volume of 200 µL of plasma sample was transferred to 5 mL RIA tubes to which 20 µL of an IS was added. After thorough mixing, 500 µL of 0.1% formic acid solution was added and mixed using a vortexer. Using Strata-X 33 µm cartridges, extraction was performed. The eluted samples were carefully transferred into for further analysis.

Method validation: The procedures were validated using industrial standards for bioanalytical technique validation [14].

Selectivity and system suitability: The selectivity of the approach was evaluated through the analysis of human plasma samples obtained from six separate batches. The purpose of this assessment was to investigate interferences to dabigatran and IS. The concentration of 54.370 ng/mL for dabigatran and 40.788 ng/mL for dabigatran-$^{13}C_6$ were employed. Aqueous mixture was utilized for the injection of the system suitability test solution. Aqueous samples were prepared in accordance with a recovery basis. To prepare the system suitability sample, a total of 20 µL of analyte with a concentration of 2718.475 ng/mL and 40 µL of the working concentration of the internal standard at 1019.700 ng/mL were combined with 940 µL of the mobile phase.

System performance: In order to evaluate the system's performance, a technique validation study was conducted. As part of this study, a single LLOQ sample was prepared, which included an internal standard. This sample was then injected at the beginning of each analytical batch.

Specificity: The novel approach's validity was evaluated by examining an unmodified control sample, known as the standard blank, without introducing any modifications of dabigatran. The analysis involved the use of seven standard plasmas that were preserved using K_2EDTA anticoagulant. Additionally, one lipidemic plasma and one haemolyzed plasma, both preserved with K_2EDTA anticoagulant, were also included in the analysis. Furthermore, the study incorporated a single heparin plasma sample.

Linearity: Studies were performed in the range of 1.016 ng/mL to 304.025 ng/mL. The quality control samples were prepared at 1.048 ng/mL (LLOQ); 3.083 ng/mL (LQC); 41.107 ng/mL (MQC1); 137.023 ng/mL (MQC2); 232.243 ng/mL (HQC). The evaluation of the calibration curves involved performing tests using two types of samples: a blank sample without an internal standard (IS) and a zero sample with an internal standard.

Precision and accuracy: To evaluate the intra-assay precision and accuracy, six replicates of human plasma samples containing dabigatran at LLOQ were analyzed and three levels of quality control on different days for each of the four levels.

Recovery and matrix effect: The evaluation of the extraction efficiency of dabigatran and IS from human plasma consisted of comparing the analytes extracted from triplicate quality control (QC) samples at low, medium and high concentrations with those obtained from the post-extracted plasma reference sample at similar quantities. By conducting a comparative analysis, the influence of plasma constituents on the ionization of analytes and the internal standard (IS) was assessed. This analysis involved post-extracted plasma standard quality control (QC) samples, which had a sample size of four. The response of analytes from these plasma samples to the response of analytes from aqueous samples with equivalent concentrations were also compared. The matrix effect calculation was conducted using analyte concentrations that were the same as those used in the recovery investigation.

Dilution integrity: A total of 12 sets of dilution integrity samples were meticulously prepared. Each set involved the addition of 1.55 times the highest standard concentration (470.106

ng/mL) to ensure accurate and reliable results. A total of six sets of dilution integrity samples were subjected to a two-fold dilution process, while an additional six sets underwent a four-fold dilution process. The accuracy and precision (PA-2) were determined by analyzing quality control samples alongside undiluted calibration curve standards covering the same concentration range. The concentrations of the quality control samples were determined by taking into consideration the appropriate dilution factor. The results obtained from the dilution of dabigatran were considered acceptable for both the 2- and 4-fold dilutions. Each of the six replicates must have a precision level of 15% and an accuracy level of 100%.

Ruggedness: The system's robustness was assessed through the utilization of three distinct sets of measurements, each of which was distinguished by its precision and accuracy. Each batch was subjected to testing using a unique combination of equipment and staff. This combination included one column, one analyst and three pieces of equipment.

Stability experiments: In order to evaluate the stability of dabigatran and IS in the injection solvent, the processed samples were reintroduced into the auto-sampler within a specified time period. To evaluate the stability of the analytes over time, a comparison was performed between their peak areas and those of the internal standard (IS) acquired in the initial cycle. The stability of the analyte in plasma at room temperature (bench top) was evaluated by performing six replicate tests on three distinct concentrations. The assessment of freezer stability for the analytes in plasma involved the analysis of quality control samples. These samples were stored at a temperature of -20 ºC for a minimum duration of 7 days. The stability of analytes in plasma was evaluated by exposing quality control samples to four freezing and thawing cycles, performed at -20 ºC and -70 ºC. During the course of this procedure, the samples underwent spiking with analytes. The aforementioned procedures were executed on the samples. The stability of assay values was assessed by evaluating their conformity to the acceptable ranges for accuracy (defined as a standard deviation of 15%) and precision (defined as a relative standard deviation of 15%).

RESULTS AND DISCUSSION

Method optimization: The liquid-liquid extraction method has been tested with ethyl acetate, dichloromethane, diethyl ether and *n*-hexane solvents. An alternate method for estimating dabigatran concentrations in human plasma samples was also investigated. This approach precipitates with methanol and acetonitrile. A methanol solvent system with 2 mM ammonium acetate in an 80:20 volume-to-volume ratio separated DBT. The separation was done using a 150 × 4.6 mm Ace 3 C18 column with 5 μm particles. The mobile phase flow rate was 0.8 mL/min. Dabigatran $^{13}C_6$ was chosen as the internal standard because of its excellent analyte correlation. Plasma analyte was extracted by liquid-liquid extraction (LLE). Methanol and ethyl acetate were extraction buffers and solvents. Optimal chromatograms indicate minimal matrix interference. In just 3 min, the process is complete. Dabigatran and the in-house standard dabigatran-$^{13}C_6$ retained 1.55 min.

The protonated precursor ions $[M+H]^+$ for dabigatran were found at *m/z* 472.20 to 289.10 and for IS from 478.20 to 295.20. The Q1 full-scan mass spectra of these ions showed the most abundance. The precursor ion was $(M+H^+)$ and thus the positive ionization of dabigatran and the internal standard was achieved.

Validation: Fig. 1 shows the matrix selectivity in extracted blank plasma chromatograms from plasma screened batches. The mass transitions of dabigatran and the internal standard were not affected by endogenous components. When injecting dabigatran-$^{13}C_6$ at an upper limit of quantification (ULOQ) concentration, analyte selectivity analysis showed no interference during the retention time. The working concentration of dabigatran-$^{13}C_6$ did not impact with dabigatran retention time. The coefficient of variation (CV) for dabigatran and IS retention time was 1.23% to 2.16%. Additionally, area ratio CV ranged from 0.89% to 2.81%. The quantifiable lower limit of dabigatran concentration in human plasma was found to be 1.016 ng/mL. The precision and accuracy of dabigatran at this concentration were 4.70% and 106.89%. The blank samples from each batch were free of endogenous interferences. When testing dabigatran's internal standard retention time, batches showed no substantial interferences. The batches above were used to prepare CC and QC samples. A regression equation with a weight of $1/(\text{concentration ratio})^2$ of drug to IS concentration best fit the concentration-detector response correlate for dabigatran in human plasma. Dabigatran extracted samples were compared to non-extracted samples. The internal standard's response in extracted samples of LQC, MQC2 and HQC was compared to the whole set of eighteen quality control samples. The average dabigatran recovery rate was 60.89% with variations across 1.65% to 3.77%; for IS it was 69.07%, with 2.53% to 2.70% precision.

No significant matrix effect was found in any of the eight samples. The IS normalized matrix factor had 2.73% and 1.40% precision at LQC and HQC levels. Additionally, the IS normalized factor was 0.976 for LQC and 1.009 for HQC. By adding 1.55 times the highest standard concentration (470.106 ng/mL), 12 dilution integrity samples were developed. Dilution integrity samples were diluted to two-fold and six sets four-fold. The samples were evaluated with undiluted processed calibration curve standards. These standards have a concentration range similar to batch-2's precision and accuracy. The right dilution factor determined the quality control sample concentrations. The research conducted into dabigatran revealed that both two-fold and four-fold dilutions possessed adequate dilution integrity. With a dilution factor of 2, dabigatran had 0.91% precision and 109.38% accuracy. At a dilution factor of 4, dabigatran had 0.83% precision and 107.47% accuracy. Studies performed using different made columns and solutions on the same instrument. Precision values are tabulated in Table-1.

Stability studies: Stability tests required experimental changes to temperature, time and other parameters. Analyte accuracy and % recoveries remain unchanged after stability tests under varied settings. These conditions include stock solution stability in an ice bath for 7 h, refrigerator stock stability for 7 days and 22 h, auto-injector stability for 49 h and 51 min, bench top stability for 17 h and 44 min in ice water bath.

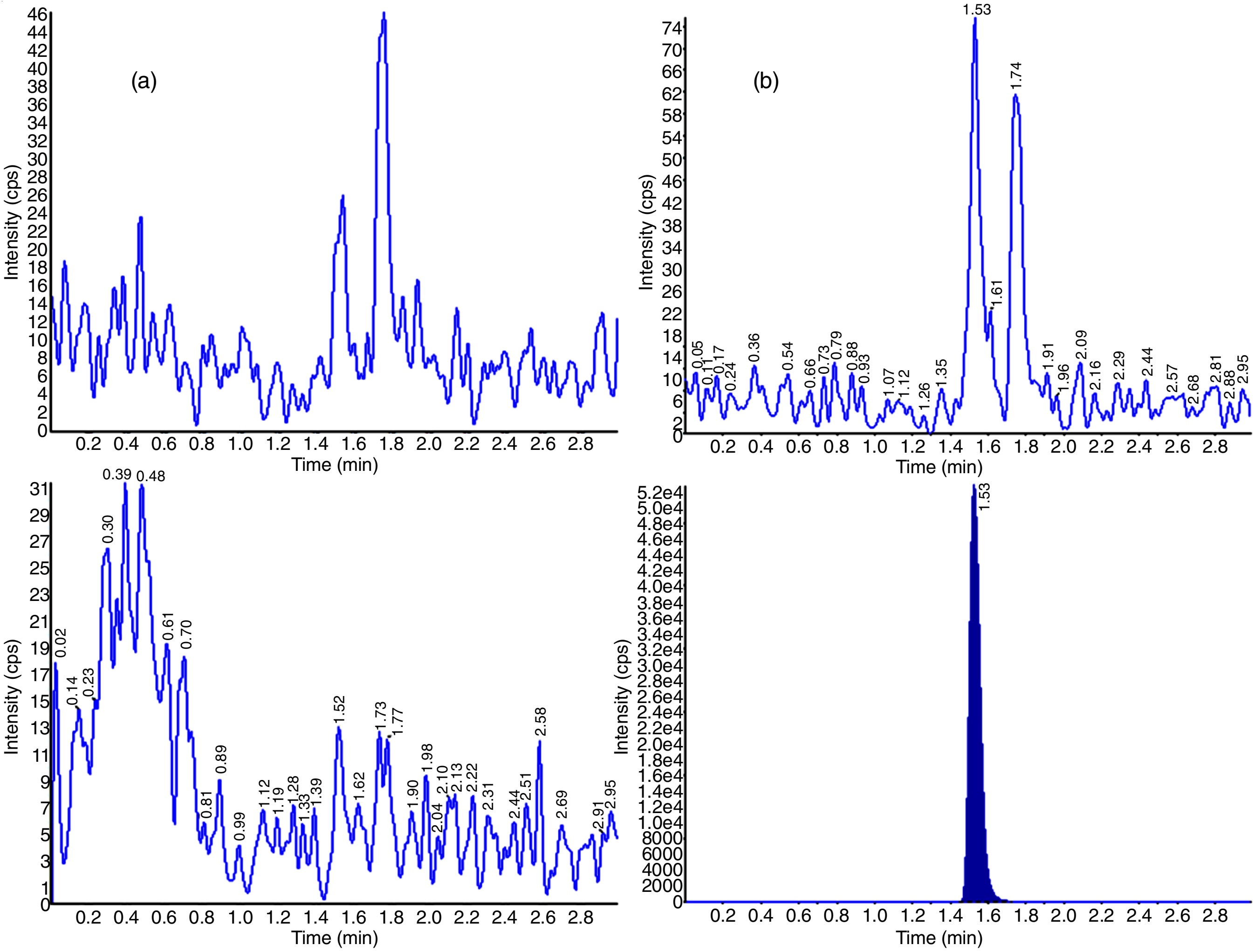

Fig. 1. Representation of plasma sample of (a) dabigatran and (b) with IS of dabigatran

TABLE-1
CONCENTRATION-RESPONSE LINEARITY DATA FOR DABIGATRAN

DAB	Nominal concentration (ng/mL)									
	STD-A	STD-B	STD-C	STD-D	STD-E	STD-F	STD-G	STD-H	STD-I	STD-J
CC	1.016	2.032	5.080	15.163	30.327	60.653	121.306	182.415	243.220	304.025
Accuracy (%)	101.07	101.38	92.59	94.67	99.24	102.67	106.89	98.90	102.12	100.48
Mean	1.0428	1.9562	4.9202	14.8132	29.8754	61.3040	126.1264	180.0324	251.8442	306.2952
S.D.	0.03545	0.13917	0.19970	0.37311	0.29362	0.97977	2.12674	1.29928	4.03531	3.07168
C.V. (%)	3.40	7.11	4.06	2.52	0.98	1.60	1.69	0.72	1.60	1.00
Nominal (%)	102.64	96.27	96.85	97.69	98.51	101.07	103.97	98.69	103.55	100.75
N	5	5	5	5	5	5	5	5	5	5

Dabigatran showed stability after six freeze-thaw cycles. The stability of re-injection (24 h and 52 min) and whole blood (3 h and 18 min) were also examined.

Concomitant drug effect: Pantoprazole, dicyclomine, paracetamol, ibuprofen, nicotine, caffeine and diphenhydramine were added to screened plasma at their maximum concentrations (5063.896 ng/mL for pantoprazole, 10039.000 for paracetamol, 26020.733 for ibuprofen, 103.934 for diphenhydramine, 15579.905 for caffeine, 52.309 for dicyclomine and 50.620 for nicotine). There were 9.49%, 3.22% and 0.47% values in between batches and HQC, whereas the LQC, LQC and HQC samples had within-batch accuracy of 113.20%, 91.00% and 93.50%, respectively. The summary of validated parameters using LC-MS/MS technique are shown in Table-2.

Conclusion

An efficient method has been devised to detect dabigatran levels in plasma accurately. This method uses LLE and HPLC-API/MS/MS, which is rapid, sensitive method and ideal for the quantitative in the biological samples. Following a series of chromatography optimization tests, methanol and acetonitrile were selected as the preferred organic solvents.

TABLE-2
SUMMARY OF VALIDATION PARAMETERS

Validation parameters	Dabigatran	
	Nominal/Stability (%)	Precision
Matrix effect at LQC	0.976	2.73
Matrix effect at HQC	1.009	1.40
Sensitivity	106.89%	4.70%
Coefficient of correlation (r^2)	0.9964-0.9994	0.9964-0.9994
Within batch precision and accuracy	90.20%-102.71%	30.27%-11.61
Intra-day precision and accuracy	93.28%-102.81%	0.74-4.44%
Between batch/inter-day precision and accuracy	92.23%-103.71%(LQC)	2.09-8.84%
Re injection stability (24 h 52 min)	99.63%-106.81%	1.17%-11.44%
Stock solution stability in ice water bath (7 h)	99.85%	1.01%-1.57%
Spiking solution stability in ice water bath (7 h)	105.12%	1.01%-1.34%
Refrigerated stock solution stability (7 days)	100.62%	0.74%-1.42%
Auto sampler stability (49 h 51 min)	99.35%-106.76%	1.03%-1.66%
Wet extract stability (44 h 7 min)	98.91%-106.48%	2.91%-4.27%
Freeze thaw stability (VI Cycles)	94.18%-104.21%	1.43%-2.89%
Bench top stability in ice water bath (17 h 44 min)	95.49%-103.30%	2.25%-2.52%
Short term-20 °C stability (6 days 19 h)	99.45%-99.69%	1.25%-1.54%
Recovery	60.89%	1.65%-3.77%
Dilution integrity: Two times dilution	109.38%	0.91%
Dilution integrity: Four times dilution	107.47%	0.83%
Ruggedness	91.97%-103.21%	1.07%-9.59%
Drug interactions	91.00%-113.20%	0.47%-9.49%
Stability in whole blood (3 h 18 min) at LQC	103.07%	2.41%-3.18%
Stability in whole blood (3 h 18 min) at HQC	100.01%	0.90%-1.07%

This can be attributed to the elevated levels of dabigatran and IS sensitivity and resolution. The mobile phase in this study was 2 mM ammonium formate mixed with methanol and acetonitrile. The experimental conditions in this study maximized dabigatran and IS sensitivity. Bioanalytical research focuses on co-elution, the occurrence of naturally occurring chemicals eluting with the analytes. The factor above influences analyte ionization efficiency, reducing repeatability and accuracy and preventing the predetermined limits from being met. The analyte extraction solvent was selected and modified with careful consideration of this issue. According to matrix studies, the presence of low values indicates a high degree of efficiency in the extraction of analyte, resulting in minimal generation of byproducts.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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