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Evaluation of Aspirin and Dipyridamole Using Low Concentration Potassium Fluoride as a Stabilizer in Human Plasma by LC-MS/MS Mode

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The objective of this work is to develop a stable, specific, accurate and precise bioanalytical method for the determination of aspirin and dipyridamole in human plasma using LC-ESI-MS/MS. Sample extraction is carried out using solid phase extraction technique. Methanol: 0.10 % formic acid in water (90:10 v/v) is used as a mobile phase and separation was achieved in 3 min on a Chromolith® high resolution, 100 × 4.6 mm, 5 µ analytical column. Detection is performed using MRM transitions 178.9/136.8 with positive polarity for aspirin and 525.7/505.6 with negative polarity for dipyridamole on a API 4000 mass spectrometer. This method is specific and calibration range is found linear over the range 5.073 to 802.820 ng/mL for aspirin and 50.051 to 4004.069 ng/mL for dipyridamole. Method is validated as per EMA and USFDA guidance on bioanalytical method. Method is successfully applied to conduct bioequivalence studies.

Keywords: LC-MS/MS method, Aspirin, Dipyridamole, Human plasma, Stability, Selectivity, Matrix effect.

INTRODUCTION

Aspirin in combination with dipyridamole is an antiplatelet agent intended for oral dosage. 200 mg dipyridamole in an extended-release form and 25 mg aspirin as an immediate-release formulations are available in the market. Dipyridamole molecular formula is $C_{24}H_{40}N_8O_4$ with molecular weight of 504.63 g/mol; whereas aspirin (acetylsalicylic acid) molecular formula is $C_9H_8O_4$ (with molecular weight of 180.16 g/mol) [1].

Multiple methods are available in public domain for the determination of aspirin and dipyridamole in plasma [2-7] individually and various methods are available for the simultaneous determination of aspirin and dipyridamole in dosage forms [8-15]. Literature survey reveals limited methods are available for the determination of aspirin and dipyridamole simultaneously. Further, the literature survey reveals no reports on the aspirin stability issues in plasma and/or whole blood. Bae *et al.* [16] reported the determination of acetylsalicylic acid and its major metabolite, salicylic acid, in human plasma using liquid chromatography-tandem mass spectrometry. Na *et al.* [17] investigated a simultaneousness determination of aspirin and dipyridamole.

To best of our knowledge, it is very intricate to develop a simultaneous determination method of the combination in plasma due to complex nature of the combination. Aspirin is unstable either in whole blood or plasma where as dipyridamole is very sensitive to haemolysis effect. Aspirin is polar analyte and is not stable at neutral pH either in aqueous or in matrix like whole blood or plasma.

With this background, it is necessary for comprehensive investigation to overcome the stability issues. This conundrum can be overcome by adding a stabilizer, to maintain the sample integrity and at the same time stabilizer impact on dipyridamole is not to be neglected. Also, active metabolite of aspirin will tend to convert into parent in electron source ionization (ESI) source if temperature is not optimized properly. More importantly that the whole blood samples should not be kept in ice cold water bath conditions after addition of stabilizer as this will severely impact the dipyridamole analysis particularly at low concentrations due to hemolysis.

During our earlier simultaneous acquisition method development, we found that with polarity switching results in poor resolution between aspirin and dipyridamole due to acidified mobile phase, which is strongly required to eliminate matrix effects at low source temperature especially for aspirin. Hence, we intend to develop individual acquisition methods that would be applied successfully developed for single extraction method, which will be cost effective.

Our earlier investigation revealed the unstable nature of aspirin in plasma and whole blood. For this reason, we intend

to carefully optimize the percentage of stabilizer to be added in whole blood for aspirin to prevent degradation and at the same time no haemolysis will occur which affects the dipyridamole reproducibility.

In the current study, we report a novel selective, accurate, precise and stable liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of aspirin and dipyridamole in human plasma. Stability problems will be overcome by maintaining the low pH for aqueous samples and low pH for blood samples using potassium fluoride. Metabolite conversion into parent in electron source ionization source for aspirin will be prevented by using low source temperature. Hemolysis effect on dipyridamole will be eliminated completely by maintaining the blood samples at room temperature up to separation. The novel method will be tested using bioequivalence study samples including incurred sample reanalysis. We herein report a successful individual acquisition determination method that will have no constraints and will be very suitable for unknown sample analysis.

EXPERIMENTAL

Pure certified working reference standards of aspirin and dipyridamole and aspirin D4 & dipyridamole D20 from third party vendors, methanol and acetonitrile of HPLC grade of JT Baker were procured.

Preparation of stock and working solutions: 1 mg/mL Stock solution for each aspirin, dipyridamole, aspirin D4 and dipyridamole D20 were prepared by weighting respective working standard accurately using micro balance. Aspirin and aspirin D4 were dissolved in 1 % formic acid in water, whereas dipyridamole and dipyridamole D20 were dissolved in 100 % methanol. These stocks solutions were stored in a refrigerator maintained between 2-8 °C. Working spiking solutions of aspirin and dipyridamole were prepared in 50 % methanol in water for further usage.

Preparation of calibration curve standards and quality control samples: Eight calibration curve standards were prepared in the bulk over the calibration curve range by spiking 5 % of the respective working solution in a screened K_2EDTA blank stabilized plasma (20 % stabilizer added to whole blood). Four levels of quality controls were also prepared from their respective working solutions along with calibration curve standards. All spiked samples were aliquoted and were stored in a deep freezer maintained at -70 °C ± 10 °C.

Sample extraction method: To an aliquot of 0.3 mL plasma sample in a RIA vial, 50 µL of internal standard working solution was added and vortexed for proper mixing and then samples were subjected to solid phase extraction using Strata X, HLB 1cc/30 mg cartridges. Cartridges were conditioned with 1 mL methanol followed by equilibration with 1 mL 0.1 % formic acid in water. Prepared samples were then loaded onto equilibrated cartridges and passed slowly at low positive pressure. Cartridges were washed with 1 mL 100 % water then eluted with mobile phase (0.500 mL). Eluent was directly taken for acquisition.

Instrumentation: (i) The samples were acquired on the API 4000 ESI-MS/MS Coupled with Shimazdu HPLC while the separation was achieved on a Chromolith® high resolution, 100 × 4.6 mm, 5 µ column using mobile phase consisting of methanol: 0.10 % formic acid in water (90:10 v/v) and eluted with isocratic flow of 1.0 mL/min. Injection volume for aspirin is 10 µL whereas it is 5 µL for dipyridamole. (ii) The mass spectrometer was operated in positive ion mode with unit resolution for dipyridamole and negative mode with unit resolution for aspirin. Source parameter IS set at 5500 kv. Temperature set at 450 °C for dipyridamole and 200 °C for aspirin.

RESULTS AND DISCUSSION

Method validation was carried out for aspirin and dipyridamole as per EMA & USFDA guidance on bioanalytical method validation [18,19]. The data acquired and quantified on LC-MS/MS system with analyst version 1.6.2 for the determination of aspirin and dipyridamole.

Specificity: The specificity of this method was tested by screening eight individual lots of blank human plasma containing K_2EDTA as an anticoagulant including a hemolytic and a lipemic plasma lot. Each blank plasma lot was tested for interferences at retention times of analyte and internal standard against lower limit of quantitation sample which is prepared in the respective blank. All lots were showed below 20 % interference at analyte retention time and below 5 % interference at internal standard retention time. Sample blank and standard 1 chromatograms were presented at Figs. 1-4.

Linearity, precision and accuracy: Four precision and accuracy batches (including one ruggedness for column/analyst change) were analyzed for linearity, precision and accuracy. Eight standards for calibration curve and six replicates of four levels of quality controls (lower limit of quantitation, lower, middle and higher) were processed in every individual batch and acquired on LC-MS/MS for both aspirin and dipyridamole. The criteria applied for the acceptability of accuracy is within ± 15 % deviation from the nominal values except lower limit of quantitation where the accuracy was within ± 20 % and a precision was within ± 15 % relative standard deviation except lower limit of quantitation where it's precision within ± 20 %.

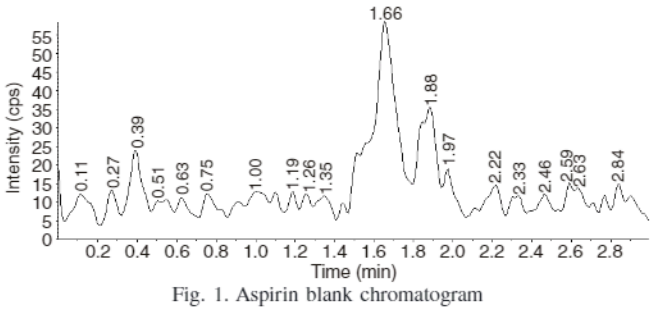

Fig. 1. Aspirin blank chromatogram

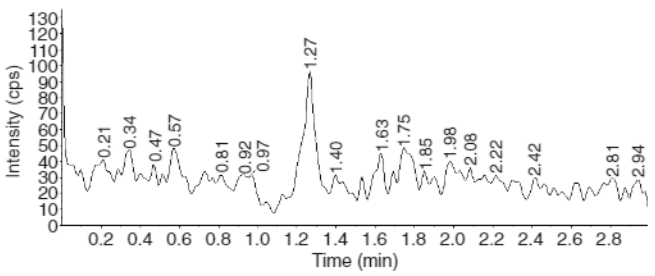

Fig. 2. Dipyridamole blank chromatogram

TABLE-1
RESULTS OF STABILITY EXPERIMENTS

Stability	Temperature	Duration	
		Aspirin	Dipyridamole
Long term stock solution stability of analyte	2 to 8 °C	9 days	9 days
Long term stock solution stability of ISTD	2 to 8 °C	9 days	9 days
Long term working solution stability of analyte and ISTD	2 to 8 °C	9 days	9 days
Auto sampler stability	5 °C	47 h	47 h
Wet extract bench top stability	Ambient	07 h	07 h
Wet extract stability	2 to 8 °C	74 h	74 h
Bench top stability matrix	Ambient	08 h	08 h
Freeze thaw stability	-70 ± 10 °C	5 cycles	5 cycles
Long term stability in matrix	-70 ± 10 °C	80 days	80 days
Whole blood stability	Room temperature	2 h	2 h

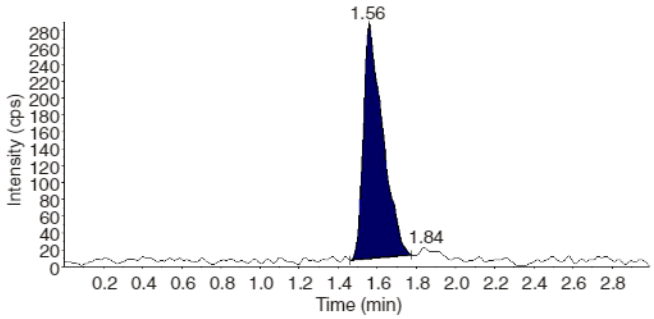

Fig. 3. Aspirin LLOQ (STD 1) chromatogram

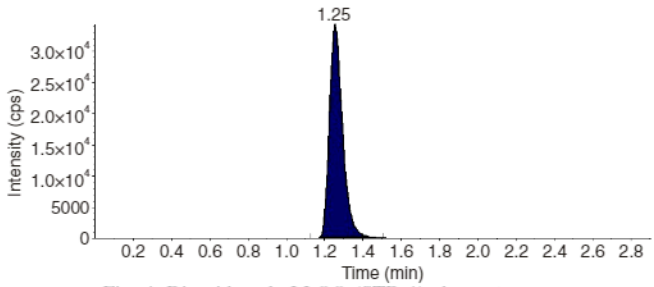

Fig. 4. Dipyridamole LLOQ (STD 1) chromatogram

Aspirin: Regression was found always greater than 0.9968, precision was found in the range 0.89 to 4.43 %, whereas accuracy found in the range 95.12 to 100.03 %.

Dipyridamole: Regression was found always greater than 0.9978, precision was found in the range 0.47 to 1.96 % and accuracy found in the range 98.97 to 106.83 %.

Extraction recovery: The recoveries of analytes and internal standards were determined by comparing the peak area obtained for quality control samples (3 levels, lower, middle and higher) that were subjected to the sample extraction procedure with those obtained from pure aqueous (authentic) samples prepared equivalent to the extracted samples. Global recovery for aspirin was found 95.10 % whereas it was 71.37 % for dipyridamole. Coefficient of variation found within 15 %.

Matrix effect: Matrix effect was performed using 6 plasma lots including 4 normal, one hemolytic and one lipemic plasma lot. Lower and higher quality control samples were prepared in post extracted blank samples and equivalent aqueous samples; and then acquired together. Coefficient of variation (%) of internal standard normalized matrix factor was calculated, acceptance criteria for the same is less than or equals to 15 %. % Coefficient of variation of internal standard normalized matrix factor was found 1.80 to 2.40 % for aspirin, whereas it was 1.94 to 3.80 % for dipyridamole.

Selectivity in presence of metabolites: Mobile phase and column conditions are optimized in such a way that the metabolites will neither elute at the retention time of aspirin and dipyridamole nor convert into parent in the electron source ionization source. Method selectivity was evaluated in presence of salicylic acid, aspirin glucuronide and dipyridamole glucuronide using 6 blank samples. Interferences at retention times were evaluated for acceptance. Interferences found within acceptance limits for all.

Stabilities: Different stability conditions like bench top stability, autosampler stability, evaporation stability, wet extract stability, wet extract bench top stability, freeze-thaw stability, whole blood stability, long term stability, short term & long term stock solution stability were evaluated. Dipyridamole found stable during all stability experiments, whereas, aspirin is found not stable in whole blood and plasma without any stabilizer. Hence formic acid is added as stabilizer to stock solutions and potassium fluoride as stabilizer is added to whole blood immediately after sample collection. At the same time method development is revealed that the aspirin is light sensitive. Summary of stability experiments were presented in Table-1.

Other method validation experiments: Apart from the experiments mentioned above, dilution integrity, reinjection reproducibility, limit of quantitation, haemolysis effect, lipemic effect were conducted and found acceptable.

Conclusion

Stable, specific, accurate and precise method has been developed for the determination of aspirin and dipyridamole in human plasma using liquid chromatography tandem mass spectrometry. Aspirin and dipyridamole stability in aqueous, whole blood and plasma have been addressed using suitable stabilizer. Bioequivalence studies were conducted in accordance with the IEC approved protocol successfully using this method.

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