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DETERMINATION OF BETAINE, A NATURAL POTENTIAL ANTI-INFLAMMATORY COMPOUND, USING SILDENAFIL AS AN INTERNAL STANDARD IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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Abstract: Betaine is a naturally occurring cationic functional group which shows a wide range of therapeutic activities. The present research work involves rapid; robust and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method developed and validated for s analysis of betaine in human plasma. This method involved a solid phase extraction and a subsequent analysis performed on an LC-MS system which contained the turbo spray ionization interface. Chromatographic separation was performed using a Gemini C18 column. Good linearity was observed over the concentration range of 10.00 ng/mL to 1203.26 ng/mL with a correlation coefficient ($r^2 \geq 0.9984$). Sildenafil was used as the internal standard in the present method. The method involves simple isocratic chromatography conditions and mass spectrometric detection in the positive ionization mode. The intra-day and inter-day precision and accuracy of the quality control samples were within the acceptance criteria of ±15%. The total elution time was about 5 min which allows higher throughput. This validated method can be applied for analysis of real samples from a bioequivalence study involving administration of formulations containing betaine as their active therapeutic components.

Key words: Betaine; Sildenafil; LC-MS/MS; Solid phase extraction

INTRODUCTION

Achyranthes aspera commonly known as prickly chauff flower or apamarga is a species of plant found in amaranthaceae family. It is distributed throughout the tropical world. It can be found in many places growing as an introduced species and a common weed. Betaine is the therapeutically active component found in *Achyranthes aspera*. The plant is reported to have several medicinal properties and used as purgative, diuretic, antimalarial, antihyperlipidemic, anti-inflammatory, antispasmodic, antibacterial, and antiviral agents in traditional systems of medicine [1, 12] In addition, Traditional healers claim that addition of *A. aspera* would enhance the efficacy of any drug of plant origin and is therefore used in numerous commercially available herbal formulations and dietary supplements. [2]

Achyranthes aspera is used as a diuretic, carminative, digestive, expectorant and an anti-inflammatory agent. These medicinal properties are attributed to the major alkaloid Betaine extracted from the plant. [3] The effect of aqueous extract of *Achyranthes aspera* was studied on animal models for inflammation. The plant extract exhibit promising anti-inflammatory activity attributed to flavonoids, alkaloids, saponins and triterpenoids phytoconstituents.[1,18] Betaine is in a class of medications called nutrients. It works by decreasing the amount of homocysteine in the blood. [4] Studies in rats suggest that betaine may help protect against harmful fatty deposits in the liver. These deposits can be caused by alcohol abuse, obesity, diabetes, and other causes.

Preliminary studies in people have shown same results. Studies show that this compound can profoundly benefit individuals suffering from digestive upsets such as hypochlorhydria, a deficiency of stomach acid production. [5] Considering the wide therapeutic effects of *Achyranthes aspera*, traditional healers claim that addition of the extracts of plant would enhance the efficacy of any drug of plant origin.[2] Additionally, aqueous methanolic extract of the whole plant have been shown to possess hypoglycaemic activity [6] Therefore, to support preclinical and clinical development, determination of Betaine from biological matrix such as human plasma is essential for evaluation of pharmacokinetic/pharmacodynamic parameters.

Literature survey revealed that very few methods have been reported for determination of Betaine from biological fluids. HPLC method reported for Betaine has a very long run time of 15 minutes making it time consuming. Further the method quantitates the amount of Betaine in-vitro and not from the biological fluid. [7] Further, a HPLC/MS/MS method has been reported for Betaine which used isotope dilution method that is not cost effective and involves laborious extraction.[8]

In the present research work, a sensitive, selective, rapid and reproducible HPLC/ESI-MS/MS method has been developed and validated for quantification of Betaine using sildenafil as an internal standard from the human plasma. The detection of both Betaine and IS was performed in same ionization (positive) mode without the use of any adduct ion formation.

MATERIALS AND METHODS

Chemicals: HPLC grade methanol and acetonitrile were procured from JT Baker, (USA). Formic acid (reagent grade) and ammonia solution (AR grade) were purchased from Sigma-Aldrich. Reference standards of Betaine (purity >98%) and Sildenafil (purity >8%) were purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany). Ammonium formate (AR grade), was procured from Merck Specialities Pvt. Ltd. (Worli, Mumbai, India).

Preparation of solutions: The stock solution of Betaine was prepared for bulk spiking of calibration curve

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and quality control samples for the method validation exercise. For bulk spiking, screened blank plasma from six different lots with least interference at the retention time of the analytes and the internal standards was pooled together and used.

The stock solutions of Betaine and the internal standard Sildenafil were prepared in 50% methanol (methanol: water 50:50v/v) solution at concentration of 1000 µg/mL. Aliquots of these stock solutions were kept stored under refrigeration at 2–4°C for determination of stock solution stability.

Primary dilutions were prepared from stock solutions by dilution with water: methanol (40:60, v/v). The secondary dilutions and subsequent working solutions were prepared as and when required using the same diluent as those for the primary dilutions. These working standard solutions thus prepared were used to prepare the calibration curve and quality control samples.

An eight-point standard curve was prepared by spiking the previously screened blank plasma with appropriate amount of Betaine. The calibration curve ranged from 10.00 to 1203.26 ng/mL.

Quality control samples were prepared at three concentration levels of 27.36, 600.92 and 994.90ng/mL in a manner similar to the preparation of calibration curve samples from the stock solutions.

Instruments and Equipments: The experiment was performed on Shimadzu prominence UFLC system coupled with mass spectrometer API- 4000. Chromatographic separation was performed on Gemini C 18 (150 x 4.6mm, 5µ) column with Acetonitrile, buffer (5mM ammonium formate) and formic acid (60:40:1 v/v) as mobile phase. The flow rate was maintained at 1.000 mL/min and the auto sampler temperature at 6°C. The HPLC system was interfaced with an API 4000 mass spectrometer (AB-MDS Sciex, Toronto, Canada) equipped with an ESI source by Analyst 1.5.1 data acquisition and analysis software (AB Sciex, USA). Solid phase extraction (SPE) was performed on an Ezypress 48 positive-pressure SPE instrument (Orochem, Lombard, IL, USA).

Mass Spectrometric conditions: MS detection was carried out in the positive ion mode with full-mass scan spectra recorded over the mass range 50–700. The optimization of ESI and MS parameters was performed by direct infusion of Betaine and IS (500ng/mL) at a flow rate of 10µL/min using Intellistart optimization process. Selective ion monitoring (SIM) mode was used for the quantification of the analytes monitoring precursor ion of m/z 118 for Betaine (Fig.1) and m/z 475 (Fig.2) for Sildenafil. The various MS parameters set for Betaine and Sildenafil are summarized in Table 1.

Table 1: MS parameters

Compound parameters						
Compound	**Q1 Mass (amu)**	**Dwell (msec)**	**DP(V)**	**EP (V)**	**CE(V)**	**CXP(V)**
Betaine	118	200.00	50.00	10.00	19.00	10.00
Sildenafil	475	200.00	46.00	7.00	18.00	6.00
Source parameters						
CAD (V)	**CUR (V)**	**GS 1 (V)**	**GS 2 (V)**	**IS (V)**	**TEM (°C)**	ihe
12.00	30.00	70.00	70.00	3500.00	650.00	On

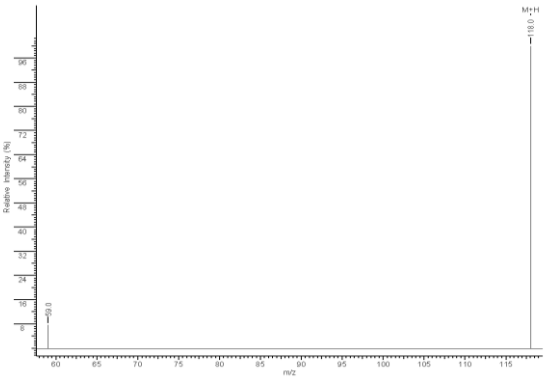

Fig.1: ESI mass spectrum of Betaine in full scan positive ionization mode

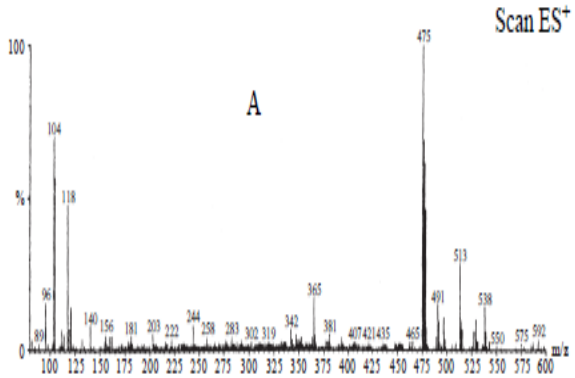

Fig.2: ESI mass spectrum of Sildenafil in full scan positive ionization mode

Sample extraction: All frozen plasma samples (System suitability, blank plasma, zero standard, calibration curve standards and quality control samples) were thawed at room temperature and vortex mixed at 70 rpm for 1 minute to ensure complete mixing of the contents. 50µL of mixture of sildenafil (approximately 100.00ng/ml) was taken in prelabelled polypropylene tube as an internal standard except in blank sample wherein 50µL of diluent solution (50% methanol in water) was added. An aliquot of 250 µL of the thawed plasma samples were added into above tubes and vortexed for 1 minute at 70 rpm. To each tube was then added 500µL of 2% formic acid solution and samples were vortexed. Then these plasma samples were processed on Waters Oasis MCX cartridges (30 mg/ml). Condition the

cartridges using 1 ml methanol followed by equilibration with 1 ml of 2% formic acid in water. The prepared plasma samples were loaded on to the cartridges and allow them to elute out at constant flow of nitrogen stream at 2 psi. Washing was performed with 1 mL of methanol, twice. Final elution was carried with 1 mL of elution solution (5% Ammonia in methanol) and 10 µL was injected into the LC/MS system.

Method validation: The method has been validated for selectivity, linearity, precision, accuracy, recovery and stability following the USFDA guidelines [17]. Selectivity was performed by analyzing the blank plasma samples from 10 different sources (or donors) to test for interference at the retention time of Betaine and the internal standard Sildenafil. These 10 sources comprised of six normal controlled plasma lots and two controlled plasma lots each of haemolysed and lipemic plasma containing heparin as the anticoagulant.

The intrarun and interrun accuracy were determined by replicate analysis of the three quality control levels along with the LLOQ (lower limit of quantitation) level of each analyte in human plasma. In each of the precision and accuracy batches, six replicates (n = 6) at each quality control level inclusive of the LLOQ level were analysed.

Accuracy is defined as the percent relative error (%RE) and was calculated using the formula % RE = $(E-T)$ $(100/T)$ where E is the experimentally determined concentration and T is theoretical concentration. Assay precision was calculated by using the formula % R.S.D. = (S.D. /M) (100) where M is the mean of the experimentally determined concentrations and S.D. is the standard deviation of M.

The extraction efficiencies of Betaine and Sildenafil were determined by analysis of six replicates at low, medium and high quality control concentrations for Betaine and at one concentration for the internal standard, Sildenafil. The percent recovery was evaluated by comparing the peak areas of extracted analytes to the peak areas of non-extracted standards.

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) constitutes an important and integral part of validation for quantitative LC–MS–MS method for supporting pharmacokinetics studies. It was performed by processing six lots of different normal controlled plasma samples in quadruplet (n = 4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The R.S.D. for six values at each level was calculated by taking the mean value obtained by injecting the post extracted samples prepared in duplicate from each plasma lot. The R.S.D. of the area ratios of post spiked recovery samples at LQC and HQC levels were within 6.25% and 2.69% respectively for Betaine. The internal standard Sildenafil the R.S.D. of the area ratios over both LQC and HQC levels was less than 3%. These results found were well within the acceptable limit set i.e. the R.S.D. of area ratio to be within ±15% at each level tested for the two analytes and within ±20% over both the levels tested for the internal standard.

As a part of the method validation, stability was evaluated. Analytes were considered stable if the recovery of the mean test responses were within 15% of appropriate controls. The stability of spiked human plasma kept at room temperature of about 25°C (bench-top stability) was evaluated for 24 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after keeping in the auto sampler at 10°C for 79 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed five times, with freshly spiked quality control samples. Six aliquots of each low and high concentration were used for the freeze-thaw stability evaluation.

RESULTS AND DISCUSSION

Method development: The goal of this work was to develop and validate a simple, selective, rapid and sensitive assay method for the extraction and quantification of Betaine, suitable for determining the pharmacokinetics of these compounds in clinical studies. To achieve the goal, during method development different options were evaluated to optimize sample extraction, detection parameters and chromatography. The Oasis MCX cartridges were selected for the current assay as they were found to be the most reproducible and gave less batch to batch variation when compared with other cartridges of the same make. Electro spray ionization (ESI) was evaluated to get better response of analytes. It was found that the best signal was achieved with ESI positive ion mode. Further optimization in chromatographic conditions increased signal of analytes. A mobile phase containing 5mM ammonium formate buffer in combination with acetonitrile resulted in improved signal and further addition of formic acid to the mobile phase helped improve the ionization. The total runtime for the analysis was 5 min thus enabling higher throughput.

Selectivity: Representative chromatogram obtained from blank plasma and plasma spiked with LLOQ (lower limit of quantification) standard for Betaine is presented in Fig.3. Similarly a representative chromatogram obtained from blank plasma and at its nominal concentration for Sildenafil (Internal Standard) is shown in Fig. 4. No interfering peak of endogenous compounds was observed at the retention time of analyte or the internal standard in blank human plasma containing heparin as anti-coagulant from six different lots of normal controlled plasma and two each of lipemic and heamolysed plasma.

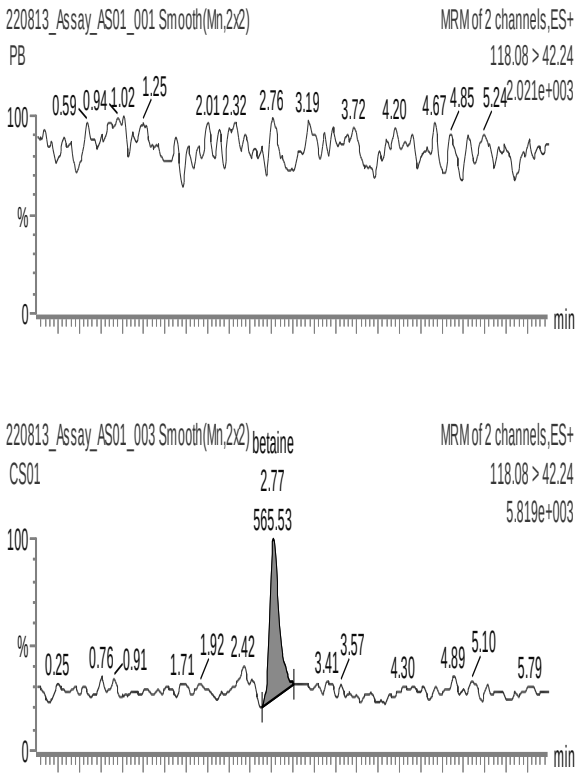

Fig.3: Representative chromatogram of plasma blank and plasma spiked with betaine at the lower limit of quantification (10ng/mL).

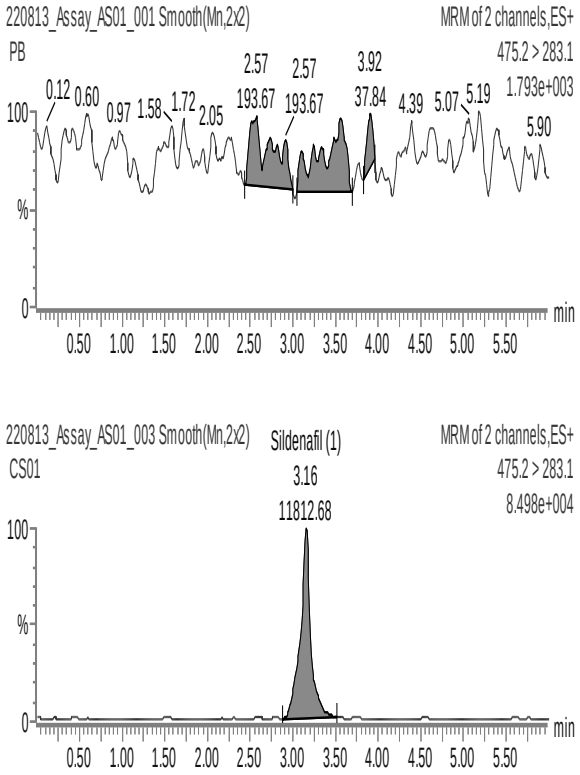

Fig.4: Representative chromatogram of plasma blank and plasma spiked with sildenafil at the lower limit of quantification (about 100 ng/mL)

Linearity: The peak area ratios of calibration standards were proportional to the concentration of analyte in each assay over the nominal concentration range of 10.00 – 1203.26 ng/mL for Betaine. The calibration curves appeared linear and were well described by least squares lines. A weighing factor of 1/concentration^2 was chosen to achieve homogeneity of variance. The correlation coefficients were ≥0.998 for Betaine. Across the eight points taken as calibration standards, the R.S.D. obtained over three batches was ≤2.0.

Precision and accuracy: The intrarun precision was ≤4.90% for Betaine whereas at the LLOQ levels it was ≤6.70%. The % R.S.D. calculated at each level are mentioned in the table below (Table 2). The interrun precision and accuracy were determined by pooling all individual assay results of replicate (n = 18) quality control samples over the three separate batch runs. The interrun precision was ≤9.00% for Betaine whereas at the LLOQ levels it was ≤8.39%. The % R.S.D. calculated at each level are mentioned in the table below (Table 3).

Table 2: Intrarun precision and accuracy (n = 6) of Betaine in human plasma

Analyte	Spiked Conc. (ng/mL)	Mean Calculated Conc. (ng/mL)	SD (±)	% RSD
Betaine	10.05	10.613	0.7112	6.70
	27.360	27.621	1.346	4.87
	600.920	616.624	29.141	4.73
	994.900	1016.018	41.259	4.06

Table 3: Interrun precision and accuracy (n = 18) of Betaine in human plasma

Analyte	Spiked Conc. (ng/mL)	Mean Calculated Conc. (ng/mL)	SD (±)	% RSD
Betaine	10.05	10.323	0.8658	8.39
	27.360	27.982	2.485	8.88
	600.920	602.854	45.572	7.56
	994.900	1003.509	54.504	5.43

Recovery: Six replicates at low, medium and high quality control concentration for Betaine were prepared for recovery determination. The mean recovery for Betaine was 92.13% with R.S.D. value of 1.27%. The mean recovery for Sildenafil was 85.53% with an R.S.D. of 1.39%.

Stabilities: Bench top and process stabilities for Betaine were investigated at LQC and HQC levels. The results revealed that Betaine was stable in plasma for at least 24 h at room temperature of about 25°C and 79 h in the auto sampler at 10°C. It was confirmed that repeated freeze and thawing (five cycles) of plasma samples spiked with betaine at LQC and HQC level did not affect the stability of Betaine. In addition to these stability exercises, the stability of the main stock solutions of Betaine and Sildenafil were also proved by comparing their stored aliquots at refrigerated temperature of 4°C versus freshly prepared stocks. This was done by comparing the areas obtained from aqueous samples prepared at the MQC level from both the stability and the freshly prepared stock solutions. It was found that the stock solutions of betaine and sildenafil were stable for at least 24 h at room temperature (about 25°C) and under refrigeration at 4°C for up to 7 days.

Application and scope of the method: The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine. Determination of pharmacokinetic and pharmacodynamic parameters for active components of plant extracts and herbal formulations is an important aspect for their wide acceptability in world market. The present research work is an attempt to provide standardized protocol that can be used as a quality control tool for standardization of herbal medicines containing the major phytoconstituent Betaine. The proposed method has been validated for a wide range of concentration of Betaine in human plasma. The method can be applied to pharmacokinetic and bioavailability studies in humans of plant extracts (e.g. *A. aspera, Asparagus officinalis*) or herbal formulations containing Betaine as their active component.

CONCLUSION

A precise, simple, specific, rapid and sensitive analytical method for the determination of Betaine using sildenafil as an internal standard in human plasma has been developed and validated. Further, the feasibility of the method can be studied by application to therapeutic monitoring of Betaine in *in vivo* studies in human volunteers.

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