Effect of deprotenizing agent and quantification of donepezil hydrochloride in human plasma

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Abstract: The effect of deprotenizing agents on recovery of donepezil hydrochloride in the development of a simple, rapid, selective and sensitive high performance liquid chromatography method for quantification of donepezil hydrochloride in human plasma was described. The deprotenizing agents were comprised of, perchloric acid, methanol, acetonitrile, chloroform and their mixtures. The chromatographic separation was carried out using reversed phase C18 column (Agilent Eclipse Plus C18) with UV detection at 268 nm. The mobile phase was comprised of 0.01 M potassium dihydrogen phosphate buffer, methanol and acetronitrile (50:30:20, v/v) adjusted to pH 2.7 with phosphoric acid (80%). A combination of perchloric acid and methanol gave a cleaner sample with a good recovery of donepezil hydrochloride of above 96%. The method showed intraday precision and accuracy in the range of 6.82% to 1.5% and 3.13% to 1.12% respectively, while interday precision and accuracy ranged between 1.06% to 4.71% and 13.01% to 6.43% respectively. The standard calibration curve was linear from 30ng/mL to 4000ng/mL, with a correlation coefficient of 0.9965±0.0034. The retention time of donepezil was 5.9 min with a run time of 7.0 min. The method can be applied to analyze large batch plasma samples in pharmacokinetic studies.

Keywords: Donepezil hydrochloride; HPLC; human plasma; deproteinization.

INTRODUCTION

Donepezil hydrochloride is a reversible inhibitor of acetylcholinesterase. Acetylcholinesterase inhibitors are commonly prescribed for mild to moderate stage of Alzheimer's disease (Reddy *et al*., 2004). Deficiency of cholinergic neurotransmission was proposed as the pathogenesis in Alzheimer's disease (Kafkala *et al*., 2008). The proposed mechanism of donepezil hydrochloride is to increase acetylcholine concentration by blocking its hydrolysis by acetyl cholinesterase and enhancing cholinergic function (Pappa *et al*., 2002). The molecular structure of donepezil hydrochloride is shown in fig. 1. Donepezil has shown has shown its therapeutic efficacy in improving cognitive function and maintaining daily activity levels in patients with mild to moderate Alzheimer disease (Kim *et al*., 2011). There was a non-randomized-controlled trial to test the effectiveness of donepezil hydrochloride in treating Down syndrome and the result was promising (Lott *et al*., 2002).

Therefore, in view of the clinical significance of this drug, the quantification of donepezil hydrochloride in biological fluids has its importance and value. Tiseo *et al.* (1998) reported a pharmacokinetic study of donepezil in healthy human volunteers but the extraction method was not described in detail and the method required 1mL of plasma. Furukori *et al.* (2002) reported a HPLCUV method, which required a large blood sample of 1mL and a complicated sample treatment method involving 4 solvents and a few extraction steps. Radwan *et al.* (2006) reported a HPLC method with limit of quantification of 50ng/mL to determine donepezil hydrochloride concentration in rat plasma. Shetti *et al.* (2011) used LCMS to quantify donepezil in human plasma, employing 4mL of extraction solvent. Analytical methods using LC-MS/MS which were more selective and sensitive have been reported (Kim *et al.,* 2011; Matsui *et al.,* 1995; Matsui *et al.,* 1999; Patel *et al.,* 2008; Apostolou *et al.,* 2007; Park *et al.,* 2008). Nevertheless, these methods involve expensive instrumentation.

The objective of the study is to develop and validate a simple, rapid, sensitive and affordable method to quantify donepezil hydrochloride in human plasma.

METHODS

Materials

Donepezil hydrochloride was obtained from Ind-Swift Laboratory Limited (Chandigarh, India). Perchloric acid, potassium dihydrogen phosphate, phosphoric acid, HPLC-grade acetronitrile and chloroform were obtained from Merck (New Jersey, USA). HPLC-grade methanol was obtained from J.T. Baker (Philipsburg, USA).

Instrumentation

The HPLC system was comprised of a Shimadzu (VP series, Kyoto, Japan) pump (LC-10AT vp/FCV-10AL-vp) with solvent cabinet, an auto-injector (SIL-10AD vp), UV/VIS detector (SPD-20AD vp), and a computer software (VP-CLASS).

Chromatography condition

The separation was carried out using a reversed phase C-18 Agilent Eclipse Plus column (Agilent, USA) fitted

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with analytical guard column (Zorbax Eclipse Plus) packed with replaceable C-18 cartridge (12.5 X 4.6 mm ID, 5 µm). The mobile phase was comprised of potassium phosphate buffer (0.01M)-methanol-acetronitrile (50:30:20, v/v) adjusted to pH 2.7 with 80% phosphoric acid. The sample was run at a flow rate of 1ml/min at a detection wavelength of 268 nm. Sample of 80µl was injected onto the column.

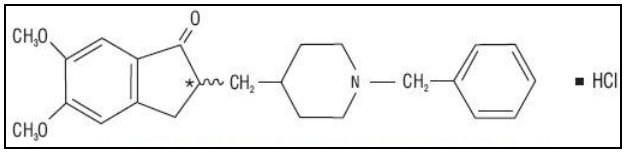

Fig. 1: Molecular Structure of Donepezil Hydrochloride

Preparation of stock standard solution

10mg of donepezil hydrochloride working standard was weighed. The powder was then dissolved in 100mL volumetric flask. Mobile phase was added into volumetric flask and sonicated using ultrasonic vibrator for 5 min. The stock standard solution had concentration of 100 µg/mL of donepezil hydrochloride.

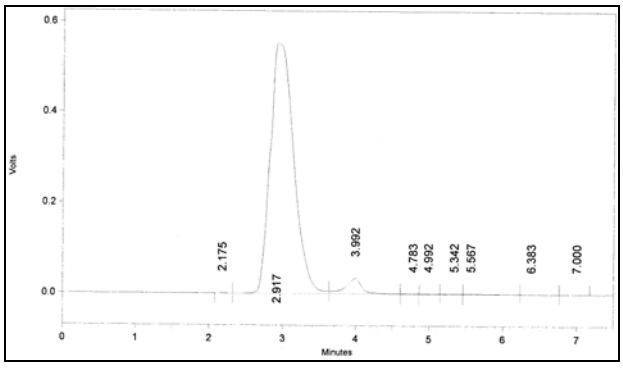

Fig. 2: Chromatogram of blank plasma. Experimental condition: C18 (250x 4.6 mm ID, 5um) Agilent eclipse plus column: Mobile phase: 0.01mM potassium dihydrogen phosphate: Methanol: Acetonitrile with ratio 50:30:20. Mobile phase was adjusted pH to 2.7 with phosphoric acid. Flow rate: 1ml/min: Detection wavelength: 268 nm: Injection Volume: 80ul

Preparation of working standard solution

5mL and 0.5mL of stock standard solution were pipetted accurately into two separate 50mL volumetric flasks. The solutions were diluted with mobile phase to volume and mixed well. The working standard solutions had concentration of 10µg/mL and 1µg/mL respectively.

Protein precipitation

An aliquot of 250µl of plasma was measured accurately into 2.0 ml Eppendorf® tubes, followed by the addition of deprotenizing agents to precipitate the plasma protein. The mixture was vortexed using a vortex mixer for 1 min and centrifuged at 12000 rpm for 10 min. 200µL supernatant was transferred into autosampler vials and 80 µl supernatant was injected into the HPLC.

The deprotenizing agents evaluated were, 20µl of perchloric acid (70%), 500µl of methanol, 500µl of acetonitrile, a mixture of 10µl of perchloric acid (70%) and 100µl of acetonitrile, 10µl of perchloric acid (70%), 100µl of methanol as well as 250µl of methanol and 250µl of chloroform. Deproteinizing agent that gave the highest recovery was used in subsequent method validation.

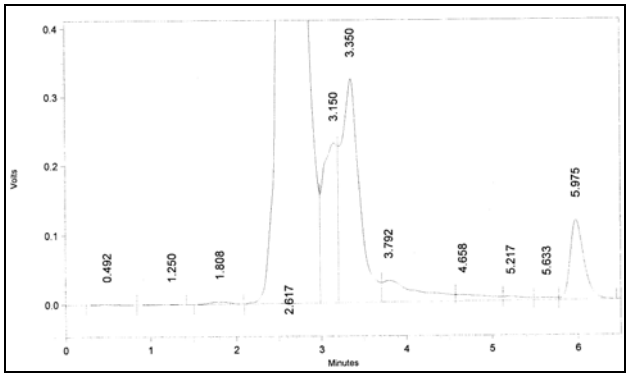

Fig. 3: Chromatogram of plasma sample containing 15ug/ml donepezil hydrochloride. Experimental condition: C18 (250x 4.6 mm ID, 5um) Agilent eclipse plus column: Mobile phase: 0.01mM potassium dihydrogen phosphate buffer: Methanol: acetonitrile with ratio 50:30:20. Mobile phase was adjusted pH to 2.7 with phosphoric acid. Flow rate: 1ml/min: Detection wavelength: 268 nm: Injection Volume: 80ul

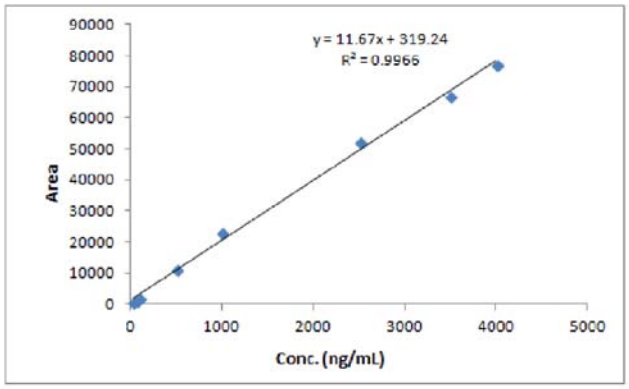

Fig. 4: Standard calibration curve of donepezil hydrochloride in plasma samples. The linearity is in the range of 30-4000ng/ml. Mean ± SD, n=6.

Preparation of quality control and LOQ plasma samples

Quality control (QC) plasma samples at concentrations of 125ng/ml (low), 1500ng/ml (medium) and 3500ng/ml (high), and LOQ sample (30ng/ml) were prepared. 87.5µl and 37.5µl of 10µg/mL working standard solution were pipetted into Eppendorf® tube and make up the volume to 250µl with human blank plasma. These plasma samples had concentrations of 3500ng/ml and 1500 ng/ml of donepezil hydrochloride. 31.25µl and 7.5µl of 1µg/mL working standard solution were pipetted into Eppendorf®

tube and make up the volume to 250µl with blank plasma. These plasma samples had concentrations of 125ng/ml and 30 ng/ml of donepezil hydrochloride.

Method validation
The Quality control (QC) plasma samples at concentrations of 125ng/ml (low), 1500ng/ml (medium) and 3500ng/ml (high), and LOQ sample (30ng/ml) were used for method validation.

Absolute recovery
The absolute recovery was defined as the ratio of response of donepezil hydrochloride in the supernatant of extracted samples to response of freshly prepared standard drug solutions of similar concentration.

Selectivity
The selectivity of donepezil hydrochloride from plasma interferents or endogenous compounds was investigated by using 6 sources of plasma obtained from Blood Transfusion Centre of General Hospital Penang.

Precision and accuracy
Inter- and intra-day precision and accuracy of the assay method were evaluated for donepezil hydrochloride. For inter-day precision and accuracy, six replicates of each analyte concentrations were injected over six consecutive days while for intra-day precision and accuracy, six replicates of each analyte concentrations were injected on the same day. According to FDA guideline, the precision should not exceed the limit of 15% of the coefficient of variation (CV) and LOQ should not exceed 20% of the CV. As for accuracy, the mean value should not exceed 15% of the actual value and LOQ should not exceed 20% of the mean true values (FDA, 2001).

Linearity
A standard calibration curve of 30-4000 ng/ml was constructed for donepezil hydrochloride. Six standard curves were constructed to calculate the system linearity response.

Stability
Five stability studies, namely freeze and thaw stability, short term stability, long term stability, stock solution stability and post-preparative stability, were carried out. QC plasma samples of 125ng/ml (low) and 3500 ng/ml (high) were used for the study.

Freeze and thaw stability
Freeze and thaw stability was determined through three freeze and thaw cycles. Three replicates of low and high concentrations were frozen in freezer at -20°C for 24 hours and thawed at ambient room temperature (26°C). The thawed samples were again frozen in the freezer. The freeze and thaw cycle was repeated twice and analyzed on third cycle.

Short- term stability
Short term stability was determined by keeping three replicates of high and low concentration at room temperature (26°C). The samples at 6 hour were analyzed and the instrumental responses were compared with that of freshly prepared samples at zero hour.

Stock solution stability
Stock solution stability was determined by keeping stock solutions of donepezil hydrochloride at concentration of 100 µg/ml for 6 hours. The solution was analyzed and the instrumental responses were compared with that of freshly prepared solutions.

Long term stability
Long term stability was measured by storing three aliquots of each high and low concentrations at -20°C for 1 month.

Post-preparative stability
USFDA has defined post-preparative stability as the stability of processed samples, including the resident time in the autosampler for at least the time for one batch run. In this study, three replicates of high and low concentration of samples were kept in the autosampler at room temperature (26°C) and analyzed after 24 hours.

Limit of quantification (LOQ)
The limit of quantification was determined as the lowest concentration of donepezil hydrochloride in the standard calibration curve.

RESULTS

The recover results are presented in table 1. Fig. 2 and 3 show chromatograms of blank plasma and plasma spiked with 3500ng/ml of donepezil hydrochloride. The results of interday and intraday precision and accuracy for donepezil are shown in table 2. The method showed intraday precision and accuracy in the range from 6.82% to 1.5% and 3.13% to 1.12%. The interday precision and accuracy showed values in the range from 1.06% to 4.71% and 13.01% to 6.43%. The precision and accuracy conform to the requirement of USFDA which stated the mean value should be within ±15% deviation except at LOQ where ±20% of deviation is allowed.

The results of linearity are presented in table 3. This method showed linearity in the concentration of 30ng/ml to 4000ng/ml. The linear curve is presented in fig. 4. The regression line could be described by $y = 11.67x + 319.24$ with a coefficient correlation of greater than 0.99. The LOQ was determined to be 30ng/ml. The method showed excellent linearity. The stability data are presented in table 4.

Table 3: Linearity obtained for regression analysis of the method for determining donepezil hydrochloride in human plasma.

No.	Slope	Intercept	r^2
1	7.2481	236.1700	0.9989
2	14.2810	542.9600	0.9998
3	14.1600	-588.2900	0.9980
4	10.0630	1293.8000	0.9916
5	10.1240	1043.5000	0.9928
6	14.1290	-612.6800	0.9979
Mean	11.6675	319.2433	0.9965
Stdev	2.9526	802.9143	0.0034

DISCUSSION

Protein sample cannot be directly injected into reverse phase C-18 HPLC column. The huge protein molecule easily clots the column. As a result, a sample preparation method which extracts drug molecule from plasma is needed. Extraction and protein precipitation are most common methods used for sample preparation involving plasma samples. However, extraction normally involves large volume of organic solvent, heating, drying of samples and reconstitution. It is very tedious and laborious. In contrast, protein precipitation is a fast and simple method by adding in protein precipitation solvent before injecting into HPLC system (Chamberlain, 1995). The different protein precipitation techniques (organic solvent, acid, salt and metal ion) involve different modes of protein precipitation. Perchloric acid and acetonitrile combination produced a clear supernatant but turned into gel on standing at room temperature after 4 hours. Methanol or acetonitrile produced a supernatant, which was clear initially but turned cloudy after standing at room temperature for 6 hours. Other deproteinizing agents produced samples with clear supernatants. This method was then validated and was proved reproducible, accurate and precise.

A high recovery value was obtained when mixture of perchloric acid and methanol was used as protein precipitating agent, with an average value of above 96%. Although methanol, acetonitrile, mixture of perchloric acid and acetonitrile, also provided high recovery, they were not used as a result of the problem encountered as described earlier. Perchloric acid and mixture of methanol and chloroform yielded low drug recovery of below 40%. As a result, mixture of perchloric acid and methanol was thus selected as protein precipitating agent.

The donepezil peak was well resolved and there was no interference from the endogenous compounds in the plasma. The retention time of donepezil hydrochloride was 5.9 minute. The method showed high selectivity.

The freeze and thaw stability study showed that the drug remained stable after undergoing harsh exposure. Short-term stability revealed that the plasma samples were stable when kept under room temperature for 6 hours. For long-term stability, the results suggested that the samples were stable even after storage for 1month at -20˚C. Post-preparative stability indicated that the processed samples were stable when kept under room temperature for 24 hours.

CONCLUSION

In summary, a simple, rapid, sensitive and affordable HPLC-UV method for the determination of donepezil hydrochloride in human plasma samples was successfully developed and validated. The short sample run time and simple sample preparation method made the assay method applicable for pharmacokinetic and bioequivalence studies.

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Table 4: Stability data for donepezil hydrochloride. Mean ± SD, N=3.

Types of study	Percentage remaining (%)		
	1500 (ng/ml)	3500(ng/ml)	100(ug/ml)
Freeze and thaw stability	98.67±3.14	101.97±2.92	-
Short term stability	102.41±9.01	99.65±3.29	-
Long term stability	101.23±3.36	101.43±1.21	-
Post-preparative stability	95.19±1.56	100.15±3.57	-
Stock solution stability	-	-	101.92±0.66

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