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Original research paper

Determination of donepezil hydrochloride in human plasma and pharmaceutical formulations by HPLC with fluorescence detection

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A sensitive, isocratic reversed-phase high performance liquid chromatographic method involving fluorescence detection was developed for the determination of donepezil hydrochloride in tablets and in human plasma. Pindolol was used as an internal standard. Good chromatographic separation was achieved by using an analytical column C18. The system operated at room temperature using a mobile phase consisting of methanol, phosphate buffer (0.02 mol L^{-1}) and triethyl amine (pH 3.5) (55 : 45 : 0.5, *V*/*V*/*V*) at a flow rate 0.9 mL^{-1} min. The analyte and internal standard were extracted from human plasma *via* liquid-liquid extraction. The proposed method was validated for sensitivity, selectivity, linearity, accuracy and precision. The calibration curve was linear over the range of 5–2000 ng mL^{-1} of donepezil with detection limit of 1.5 ng mL^{-1}. Intra- and inter-day relative standard deviations were less than 2.5 %. The method was found to be suitable for quality control of donepezil hydrochloride in bulk drug as well as in human plasma.

Keywords: donepezil hydrochloride, RP-HPLC, fluorescence detection, dosage form, plasma

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Donepezil hydrochloride, 2-[((1-benzylpiperidin-4-yl)methyl)]-5,6-dimethoxy-2,3-dihydoinden-1-one monohydrochloride (DP) (Fig. 1) is a centrally and selectively acting acetylcholinesterase inhibitor. Donepezil has been reported to be effective in the treatment of cognitive impairment and memory loss in patients with Alzheimer's disease. It is well tolerated when 5 mg of the drug is prescribed daily (1).

In clinical trials, significant correlations were found between the plasma concentration of donepezil and percentage of acetylcholinesterase inhibition. A 50 % inhibition of acetylcholinesterase activity was obtained at a plasma drug concentration of 15.6 ng mL^{-1} and inhibition plateaus at the plasma concentration of donepezil higher than 50 ng mL^{-1} (2). Therefore, plasma drug concentration can be a useful tool to predict the clinical outcome of donepezil in the treatment of Alzheimer's disease.

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Previous studies have reported quantification of donepezil in pharmaceutical preparations (3–5) and in plasma (6, 7) by HPLC with UV detection. However, most of the above mentioned methods have a common limitation of low sensitivity and long chromatographic run time. Further, LC-MS (8, 9) and capillary electrophoresis (CE) (10) were presented for the determination of DP. Although LC-MS is selective and sensitive and has been successfully applied to analysis of DP, both LC-MS and CE required expensive instrumentation.

To the best of our knowledge, only one HPLC method with fluorescence detection was developed (11). Fluorescence detection was employed because it can provide excellent selectivity and sensitivity. The reported HPLC-FL method for the assay of DP in plasma and microdialysate samples was developed using micellar liquid chromatography with a short C30 column (11). Micellar liquid chromatographic technique (MLC) is used mainly to enhance retention and selectivity of various solutes, which would otherwise be inseparable or poorly resolved. One of the main drawbacks of this technique is the reduced efficiency of separation caused by the micelles (12, 13).

The main purpose of this study was to develop a sensitive, simple, and reliable method to quantitate donepezil hydrochloride in a relatively short time with high linearity. Therefore, this study was focused on the development of a simple and rapid isocratic RP-HPLC-FL method that can be employed for the routine analysis of donepezil hydrochloride in bulk drug formulations and in human plasma.

EXPERIMENTAL

Reagents and materials

Donepezil and pindolol were obtained from Sigma Chemical Co. (USA). HPLC-grade methanol, analytical grade triethylamine and phosphoric acid were purchased from BDH Chemicals (UK). Bidistilled water was purified using a Milli-Q plus cartridge purification system (Millipore, Waters, USA) to get ultra pure water of 18 μΩ. Aricept® tablets, 5 and 10 mg, products of Eisai Co., Ltd (Japan) were obtained from the local market. Human blood was obtained from male adult healthy volunteers and whole blood was received from the blood blank unit of the King Khalid University Hospital (Riyadh, KSA). It was kept frozen until use. This work was approved by the Deanship of Scientific Research at the King Saud University.

Instrumentation and chromatographic conditions

The LC analysis was carried out on a Water HPLC system (USA) equipped with a 1500 series HPLC pump, operated in isocratic mode to deliver the mobile at a flow rate of 0.9 mL min^{-1}. A dual wavelength fluorescence detector (2475) and an autosampler (717 plus) were used. The data was collected with an Empower pro Chromatography Manager Data collection system. Chromatographic separations were performed on an analytical column Phenyl Hypersil C18 (125 mm × 4.6 mm i.d. × 3 μm particle diameter) manufactured by Phenomenex (USA). All solutions were degassed by ultrasonication (Technal, Brazil) and filtered through a 0.45-μm Millex filter (Millipore).

The mobile phase consisted of methanol, 0.02 mol L^{-1} (pH 3.5) buffer phosphate and triethylamine (55: 45: 0.5, *V/V/V*). It was filtered and degassed. Buffer phosphate was prepared from monobasic sodium phosphate, triethylamine and phosphoric acid. The samples (15 μL each) were injected with the aid of an auto-sampler. The fluorescence detector was set at 290 nm as the excitation wavelength and 315 nm as the emission wavelength.

Preparation of standard solutions

Stock solutions of both donepezil and pindolol as internal standard (IS) were prepared by dissolving the appropriate amount of each compound in methanol to yield a concentration of 1 mg mL^{-1}. Stock solutions were stable for at least two months when stored in refrigerator, and no evidence of degradation of the analyte was observed on the chromatograms during that period. Working solutions of DP (10.0, 1.0 and 0.1 μg mL^{-1}) and 100 μg mL^{-1} of IS were obtained by suitable dilutions of stock solutions with methanol.

Determination of donepezil in the pharmaceutical dosage forms

Commercially available formulations (Aricept® tablets) labeled to contain either 5 or 10 mg donepezil hydrochloride were analyzed. Ten tablets of each formulation were weighed and then powdered. Powder samples, equivalent to 5 or 10 mg of DP, were placed in a 100-mL volumetric flask with the aid of methanol. The content of the flask was vortexed for 3 min and sonicated for 15 min. The content in the flask was made up to the volume with methanol. Aliquot was filtered and further diluted with the mobile phase to obtained the final sample solution of 5 μg mL^{-1} of DP. Donepezil was determined by using external standard working solutions from pure reference DP run simultaneously.

Extraction of plasma sample and preparation of plasma quality control samples

Hundred microliters of human plasma were spiked with 15, 500 and 1000 ng of DP in 2.0-mL disposable polypropylene microcentrifuge tubes. Each was vortexed for 30 s. The solution was mixed with 600 μL of acetonitrile, vortexed at high speed for 1 min and centrifuged at 10,000 rpm for 30 min. The supernatant was transferred to a 2.0-mL disposable microcentrifuge tube and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 100 μL of mobile phase and 15 μL was injected into the HPLC system for DP determination. Blank human plasma samples were processed in the same manner using methanol instead of DP.

The quality control (QC) samples at three concentration levels (15, 500 and 1000 ng mL^{-1} were prepared by spiking drug-free plasma with appropriate volumes of DP and IS. Before spiking, the drug-free plasma was tested to make sure that there were no endogenous interferences at the retention time of DP and IS.

Validation

Validation was preformed according to the criteria set in references 14–17.

Linearity range. – Calibration plots for the DP were prepared by diluting stock solutions to yield seven concentration levels (5, 20, 100, 200, 500, 1000 and 2000 ng mL^{-1}).

Calibration curves were constructed using the observed analyte peak area ratio *versus* the nominal concentration of analyte.

Precision and accuracy. – Intra-day precision was expressed through the relative standard deviation of five replicate assays of samples at three concentration levels. Inter-day precision was determined by analyzing the same set of samples on five different days.

Accuracy was determined (using the data from precision assessment) as the closeness of spiked samples to the nominal value. The recovery was evaluated by comparing the peak area of spiked analyte samples to pure analyte from the stock solution that was injected directly into the HPLC system.

Accuracy and precision of the method were determined for DP according to the FDA guidance for bioanalytical method validation (14).

Selectivity. – The selectivity of the assay was checked by analyzing six independent blank plasma samples. Chromatograms obtained by analyzing human plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analyte. Further, the selectivity of the assay was checked by analyzing 10 Ariecpt® Tablets samples. Chromatograms were compared with the chromatograms obtained by analyzing the standard solution containing the drug.

Limit of detection and limit of quantification. – The limit of detection (*LOD*) and the limit of quantification (*LOQ*) (14) were determined at 3.3 and 10 times the base-line noise, respectively.

RESULTS AND DISCUSSION

Method development

Determination of DP in human plasma using HPLC with fluorescence detection using micellar liquid chromatography has been reported (11). The main drawback of this technique is the reduced efficiency of separation caused by the micelles (12, 13). The present work represents an HPLC method for the determination of DP in human plasma at nano level. Table I. summarizes the general characteristics of the proposed HPLC method.

Chromatographic conditions were aimed to achieve efficient separation and resolution. Also, the response should be adequate with a sharp peak shape and a short run time per analysis for both the analyte and IS. This includes selection of the mobile-phase,

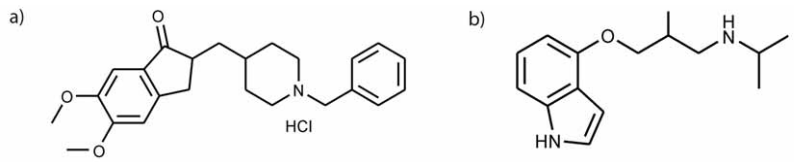

Fig. 1. Chemical structure of: a) donepezil hydrochloride and b) pindolol.

flow rate, column type and injection volume. Different ratios of methanol/water and acetonitrile/water combinations were tried as the mobile phase, along with phosphate buffer, on μ-bonded silica C18. Use of Phenyl Hypersil C18 (150 × 3.9 mm × 4 μm) chromatographic column helped fast separation and elution of the drug and IS. An optimum mobile phase was very critical for their separation as they had similar retention mechanisms and retention times. It was observed that a mobile phase consisting of methanol/ 0.02 mol L^{-1} phosphate buffer (pH = 3.5) /triethylamine (55:45:0.5) was most appropriate for good resolution, elution, and peak shape. Pindolol as an internal standard was easily separated and eluted along with the analyte. Total chromatographic run time was 15 min using 0.9 mL min^{-1} flow rate. There was no effect of IS on analyte recovery and sensitivity of detection. Typical chromatograms of DP spiked in human plasma are shown in Fig. 2. Retention times were 11.4 and 8.1 min, for DP and IS, respectively.

Peak characteristics

In order to determine adequate resolution and repeatability of the proposed method, suitability parameters including the retention factor, selectivity, and resolution and peak asymmetry were investigated and the results are abridged in Table I. The peak retention factor (k') for both the drug and IS was calculated from $k' = (t_R - t_o)/t_o$, where t_R and t_o are retention times of the peak of interest and the solvent front, respectively. The separation factor (α) was estimated from $\alpha = k'_2/k'_1$ where k'_1 and k'_2 are retention factors of the drug and IS, respectively. Useful and practical measurement of peak shape, the peak asymmetry factor, A_s, was calculated at 10 % of peak height. The resolution factor (R_s) was calculated by $R_s = 1/4\ (\alpha - 1)\ (N^{1/2})\ [(k'/(1 + k')]$, where N is the column plate num-

Table I. Analytical parameters for the determination of donepezil using the proposed method[a]

Parameter	
Calibration line	
Slope ± SD	0.004 ± 0.0003
Intercept ± SD	0.0179 ± 0.0024
R	0.998
Linearity range (ng mL^{-1})	5.0–2000.0
LOD (ng mL^{-1})[a]	5.0
LOQ (ng mL^{-1})[a]	1.5
Retention time for DP (min)	11.4
Retention time for IS (min)	8.1
Capacity factor (k')	5.01
Separation factor (α)	1.5
Resolution factor (R_s)	3.02
Peak asymmetry factor at 10 % peak height	1.05

[a] Values are the mean of six measurements.

ber and k' is the average retention factor for the two bands. The column plate number was determined using the formula, $N = 5.54\ (t_R/w_h)^2$, where w_h is the bandwidth at 50 % of peak height.

Validation of the method

The method was extensively validated as per the United States Food and Drug Administration (FDA) guidelines.

Linearity, LOD and LOQ. – Excellent linear relationship was demonstrated between the peak area ratio of DP and IS *vs.* plasma DP concentration over a range of 5.0–2000.0 ng mL^{-1}. The mean linear regression equation of the peak ratio *(y) versus* drug concentration (ng mL^{-1}) in spiked plasma samples (γ) showed the correlation coefficient $R > 0.998$ ($y = 0.004\gamma + 0.0179$). Good linearity of the calibration graphs and negligible scatter of experimental points are evident from the values of the correlation coefficient and standard deviation of the obtained data (18). Table I shows the statistical analysis of experimental data obtained by the least-squares treatment of the results. The *LOD* was 1.5 ng mL^{-1}. The LOQ of the calibration graph was 5.0 ng mL^{-1}(18).

Selectivity. – Analytical figures of merit for this method are shown in Fig. 2. Donepezil and IS (pindolol), were well separated under the HPLC conditions applied. Retention times were 11.4 ± 0.1 and 8.1 ± 0.1 min for donepezil and IS, respectively. This has indicated appropriate selectivity of the elaborated procedure. Excipients commonly co-formulated with DP such as magnesium stearate, cellulose, starch, calcium hydrogenphosphate, colloidal silicon dioxide and coloring agents did not interfere with donepezil determination, indicating high selectivity of the method. Also, no interfering peaks were co-eluted with the compounds of interest (Figs. 3a and b), which could originate from endogenous substances in human plasma.

Precision and accuracy. – The precision of the method was evaluated in terms of repeatability (intra-day) and intermediate precision (inter-day) (15–17). Three different concentrations of QC samples were analyzed in six independent series during the same day and in different days; each sample was injected in triplicate. The RSD values of intra- and inter-day studies for DP showed that the precision of the method was satisfactory (Table II) with intra- and inter day RSDs less than 1.5–1.8 %. The results obtained for relative error (e_r) (15–17) at three concentrations are shown in Table II and were (± 0.2–2.4 %).

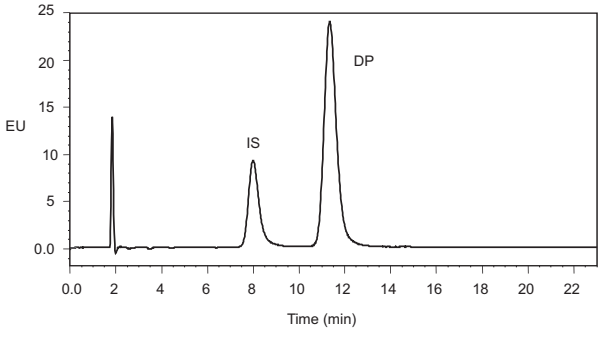

Fig. 2. Chromatogram of 500 ng mL^{-1} donepezil (DP) from the formulation with 15 μg mL^1 of pindolol (IS). First peak is solvent fronting.

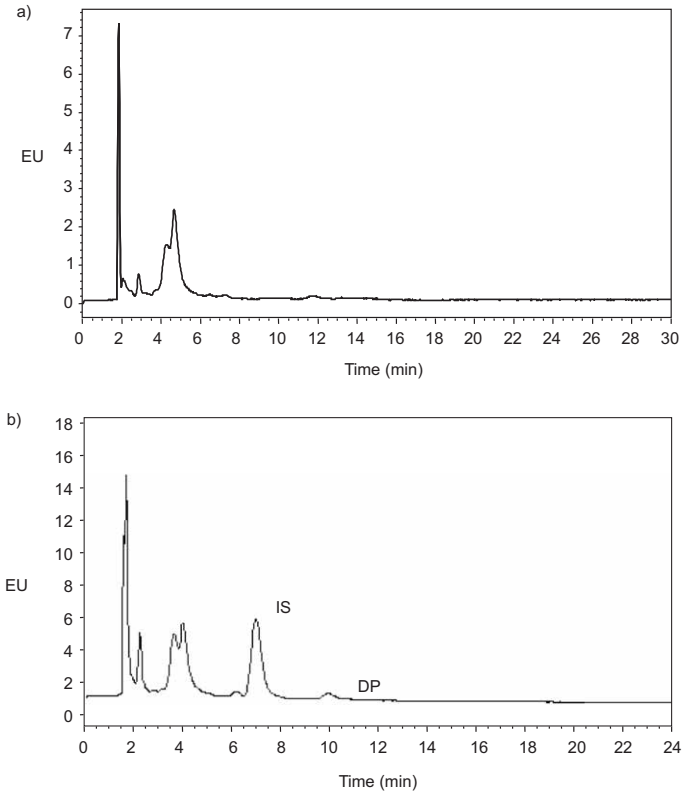

Fig. 3. Chromatogram of: a) blank human plasma (drug-free plasma), and (b) reference spiked with 5 ng mL^{-1} donepezil and 15 mg mL^{-1} pindolol (IS). First three peaks in Fig. 3b is the plasma background.

Ruggedness. – Ruggedness of the HPLC method was evaluated by carrying out the analysis using two different analysts and different instruments on different days. RSD values of less than 2 % were obtained for repetitive measurements and operators. The results indicated that the method is rugged enough.

Application of the method to pharmaceutical formulations and plasma

Reliability of the proposed method for donepezil quantification was assessed first for its determination in water. Determination of donepezil in solutions (five replicates) by direct HPLC or standard addition method gave average recovery values of 97.6–99.8 % with relative standard deviation of 1.5–1.8 (Table II). This indicates high model accuracy of the proposed method.

Results obtained for the analysis of DP in each formulation by the proposed and the HPLC USP methods (19) are given in Table III.

Table II. Accuracy and precision data for donepezil in standard solution

Actual concentration (ng mL^{-1})	Found concentration (ng mL^{-1})[a]	RSD (%)	e_r (%)
5.00	$4.88 \pm 0.00_1$	1.8	2.4
10.00	9.80 ± 0.00	1.8	2.0
20.00	19.60 ± 0.00	1.8	2.0
100.00	$99.60 \pm 0.00_2$	1.7	0.4
500.00	499.00 ± 0.01	1.6	0.2
1000.00	998.00 ± 0.02	1.5	0.2
2000.00	1996.00 ± 0.03	1.5	0.2

[a] Mean ± SD, $n = 6$.

Table III. Determination of DP means in pharmaceutical dosage forms

Dosage form		Proposed method found (mg)[a]	HPLC metod (19) found (mg)[a]	$\|t\|_2$	*F*-test[b]
Aricept®	5 mg	4.88 ± 0.02	4.90 ± 0.01	1.70	3.1
	10 mg	9.80 ± 0.02	9.79 ± 0.02	1.60	2.8

[a] Mean ± SD, $n = 6$.
[b] The tabulated value of t is 3.36 and of F is 6.38.

Comparison the experimental means for the two methods was carried out using the null hypothesis of $|t|_2$ at $p = 0.05$ and $n = 5$. It was found that $|t|_2$ was 1.7 and 1.5, which was less than the tabulated value ($|t|_2 = 3.36$) (18). This indicate comparable accuracy of the proposed method to that of as USP method (19). Comparison of the precision of the proposed method with that of the USP method was also carried out using the two-tailed

Table IV. Accuracy and precision data for donepezil in spiked human plasma

DP	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1})	Recovery (%)[a]	RSD (%)[a]
Within-a-day	15.0	13.8	92.2	6.5
	500.0	472.5	94.5	5.0
	1000.0	950.0	95.0	5.0
Between-days	15.0	13.7	91.4	6.5
	500.0	470.0	94.0	6.0
	1000.0	945.0	94.5	5.0

$n = 6$

F-test (18). It is clear that experimental $F_{4,4}$ values were 3.1 and 2.8, which are obviously lower than the tabulated value of $F_{4,4}$ for $p = 0.05$ and $n = 5$ (6.38) (18). This proves comparable precision of both methods.

Recovery of donepezil was also determined by analysis of spiked plasma. As shown in Table IV recovery ranged from 92.3 to 94.5 % in the linearity range of 5.0–2000.0 ng mL^{-1} of DP. In human plasma samples with DP concentration 15–1000 ng mL^{-1} RSD was less than 7 %.

Table V summarizes the characteristics of the reported HPLC methods with the developed one.

Table V. A summary of the characteristics of the earlier reported HPLC methods and the improved method

Stationary phase	Analytical range	*LOD*	Precision (RSD, %)	Correlation coefficient (*R*)	Application	Reference
C18 (Microsorb-MV)	10–60 μg mL^{-1}	10 μg mL^{-1}	0.5	0.9995	Pharmaceutical formulations	4
C18 Uptispher ODB (250×4.6 mm, 5 μm)	0.35–0.64 mg mL^{-1}	0.06 μg mL^{-1}	< 0.31	0.9980	Pharmaceutical formulations and impurities	3
C18 STR ODS-II (250×4.6 mm, 5 μm)	3–90 ng mL^{-1}	3 ng mL^{-1}	7.3–7.6	0.9987	Human plasma	6
Chiralcel OD (chiral column)	0.05–2 μg mL^{-1}	20 ng mL^{-1}	≤ 10	0.994	Pharmaceutical formulations and human plasma	7
C30 Develosil Combi-RP-5 (250×4.6 mm, 5 μm)	5–500 nmol L^{-1} 10–500 nmol L^{-1} 1–50 nmol L^{-1}	2.5 nmol L^{-1} 5.0 nmol L^{-1} 0.5 nmol L^{-1}	≤ 9.3	0.999 1.0 0.999	Rat plasma microdialysate human plasma	11
C18 Phenyl Hypersil (125×4.6 mm, 3 μm)	5–2000 ng mL^{-1}	2.0 ng mL^{-1}	≤ 6.5	0.998	Pharmaceutical formulations and human plasma	This paper

CONCLUSIONS

In a conclusion, a simple, sensitive and reliable RP-HPLC-FL method for measuring DP in human plasma and pharmaceutical formulations has been developed and validated. The low volume of plasma needed, the simplicity of separation procedure, and the short run time make this method suitable for quick and routine analyses. Hence, it can be recommended for routine quality control and for pharmacokinetic studies.

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S A Ž E T A K

Određivanje donepezil hidroklorida u humanoj plazmi i ljekovitim oblicima pomoću HPLC s detekcijom fluorescencije

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Ovaj rad opisuje HPLC metodu određivanja donepezil hidroklorida (DP) u tabletama i u ljudskoj plazmi u nano području. Postavljena je osjetljiva metoda izokratične HPLC s fluorescencijskom detekcijom. Kao unutarnji standard upotrebljen je pindolol. Dobro kromatografsko odjeljivanje postignuto je primjenom analitičke kolone C18. Radna temperatura bila je sobna, a kao mobilna faza upotrebljena je smjesa metanola, fosfatnog pufera (0,02 mol L^{-1}) i trietilamina (pH 3,5) (55 : 45 : 0.5, *V/V/V*). Analit i unutarnji standard su ekstrahirani iz ljudske plazme ekstrakcijom tekuće-tekuće. Predložena metoda je validirana s obzirom na selektivnost, područje linearnosti, ispravnost i preciznost. Kalibracijska funkcija bila je linearna u području od 5-2000 ng mL^{-1} donepezila, a granica detekcije iznosila je 2 ng mL^{-1}. Relativna standardna devijacija za repetabilnost i intermedijarnu preciznost bila je manja od 2,5 %. Metoda je primjenljiva u kontroli kvalitete ljekovitih formulacija s DP-om i u praćenju DP-a u ljudskoj plazmi.

Ključne riječi: donepezil, RP-HPLC, dozirani oblik, plazma

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