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Determination of dapoxetine, an investigational agent with the potential for treating depression, and its mono- and di-desmethyl metabolites in human plasma using column-switching high-performance liquid chromatography

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ABSTRACT

A column-switching high-performance liquid chromatographic (HPLC) method is described for the determination of dapoxetine and its mono- and di-desmethyl metabolites in human plasma. The analytes, including an internal standard, were extracted from plasma at basic pH with hexane–ethyl acetate. The organic extract was evaporated to dryness and the residue reconstituted with acetonitrile. The analytes were separated from late-eluting endogenous substances on a Zorbax RX-C_8 pre-column. The front-cut fraction containing the analytes was further separated on a second RX-C_8 column. The analytes were detected by their native fluorescence, using excitation and emission wavelengths of 230 and 330 nm, respectively. The limit of quantitation was determined to be 20 ng/ml, and the response was linear from 20 to 200 ng/ml. The method has been successfully applied to human plasma samples in a Phase I study.

INTRODUCTION

Dapoxetine (LY210448 hydrochloride), or (+)-N,N-dimethyl-α-[2-(1-naphthalenyloxy)ethyl]benzenemethanamine hydrochloride (Fig. 1), is a specific neuronal inhibitor of serotonin re-uptake [1]. Selective serotonin re-uptake inhibitors have been shown to have clinical antidepressant activity [2,3], and dapoxetine is currently undergoing clinical trials for treatment of depression. Microsomal metabolism studies from mice, rats, dogs, monkeys, and humans showed the formation of both mono- and di-desmethyl metabo-

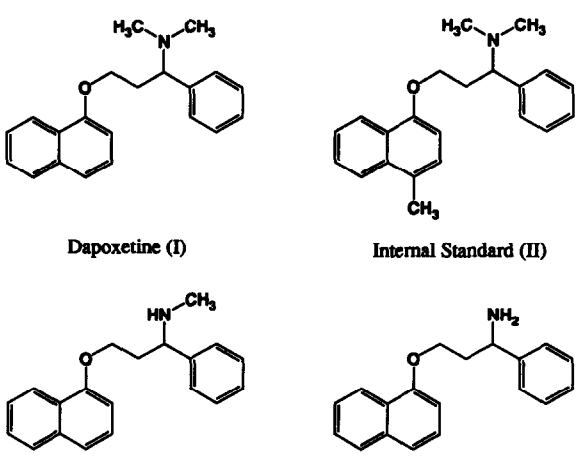

Fig. 1. Structures of dapoxetine (I), the internal standard (II), the mono-desmethyl metabolite (III), and the di-desmethyl metabolite (IV).

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lites of dapoxetine (Fig. 1). These metabolites were subsequently observed in the plasma of the animals following oral dosing with dapoxetine [4,5].

In order to facilitate pharmacokinetic and pharmacodynamic studies of dapoxetine, a sensitive and selective method for the determination of dapoxetine and its two N-demethylated metabolites in plasma was required. The development of such a method proved to be particularly challenging. Late-eluting interferences disrupted the chromatography when liquid–liquid extractions were used to effect sample clean-up. The use of solid-phase extraction protocols eliminated these late-eluting interferences, but the recovery of the analytes from reversed-phase and ion-exchange extraction cartridges was variable from sample to sample.

Column switching as a means of sample clean-up has received much attention in the literature [6]. Applying a front-cut strategy, column switching was used to eliminate the late-eluting interferences remaining following liquid–liquid extractions of dapoxetine and the N-demethylated metabolites. This report describes this sensitive and selective method for the determination of dapoxetine and the potential mono- and di-desmethyl metabolites in human plasma.

EXPERIMENTAL

Chemicals and reagents

Dapoxetine hydrochloride, the di- and mono-desmethyl metabolites of dapoxetine, and the internal standard, (±)-N,N-dimethyl-α-[2-(4-methyl-1-naphthalenyloxy)ethyl]benzene-methanamine ethanedioic acid (Fig. 1), were obtained from Lilly Research Labs. (Eli Lilly and Company, Indianapolis, IN, USA). HPLC-grade methanol, hexane, ethyl acetate, and acetonitrile were purchased from Burdick & Jackson (Division of Baxter Healthcare, Muskegon, MI, USA). Purified water (Milli-Q system, Millipore) was used in all aqueous solutions. All other chemicals were of analytical-reagent grade. Control (blank) human plasma and serum were obtained from healthy volunteers.

Liquid chromatography

The HPLC system consisted of two pumps (Model 600E gradient pump and Model M45 isocratic pump, Waters Chromatography, Division of Millipore, Milford, MA, USA), one of which was capable of controlling a six-port automated switching valve (Waters Chromatography, Division of Millipore Corporation), a variable-wavelength fluorescence detector (LS-4, Perkin-Elmer, Norwalk, CT, USA), and an autosampler (WISP Model 712, Waters Chromatography, Division of Millipore). The pre-column, used for further purification of the plasma samples, was a Zorbax RX-C_8 column (1.25 cm × 4 mm I.D., 5 μm, Du Pont, Wilmington, DE, USA); the analytical column was also a Zorbax RX-C_8 column (15 cm × 4.6 mm I.D., 5 μm, Du Pont). An in-line filter (2 μm, Upchurch Scientific, Oak Harbor, WA, USA) was positioned directly in front of the analytical column. A Hewlett-Packard Model 1000 computer was used for on-line data acquisition and subsequent calculations. The equipment configuration is illustrated in Fig. 2.

The mobile phase for the analytical column was 100 m*M* sodium acetate (pH 6)–acetonitrile (45:55, v/v). The wash solvent for the pre-column was 100 m*M* sodium acetate (pH 6)–acetonitrile (10:90, v/v). The flow-rate through the analytical column was 1.0 ml/min, and the flow-rate through the pre-column during the wash period was 1.5 ml/min. The column temperatures were ambient. A total chromatography time of 25 min was required per sample. The analytes were detected using an excitation wavelength of 230 nm and an emission wavelength of 330 nm.

Preparation of standard solutions

A standard stock solution containing dapoxetine and its mono- and di-desmethyl metabolites was prepared in 50 m*M* sodium acetate (pH 3), at a concentration of 5 μg/ml (free base). The internal standard solution was prepared by sonicating 0.5 mg of the compound in 2 ml of methanol plus ~40 ml of water for 5 min and then diluting to 50 ml with water, resulting in a concentration of 10 μg/ml.

Plasma standards were prepared at analyte

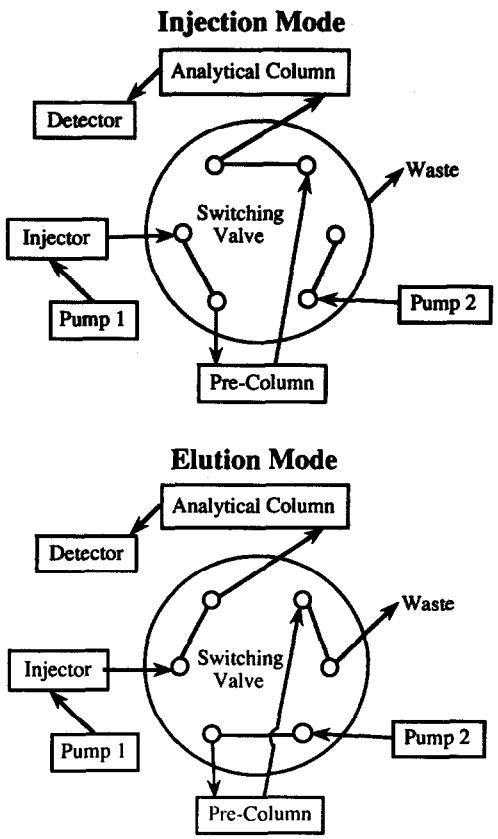

Fig. 2. Schematic representation of column-switching HPLC operation. The valve is switched from the injection mode to the elution mode at 1.2 min and switched back from the elution mode to the injection mode at 20 min.

concentrations of 20, 65, 100, 150, and 200 ng/ml by diluting appropriate aliquots of the stock solution with blank plasma.

Sample preparation procedures

Aliquots of plasma samples or standards (1 ml for analyte concentrations of 20–200 ng/ml or 0.1 ml for analyte concentrations of 201–800 ng/ml) were dispensed into 15-ml disposable glass tubes with PTFE-lined screw caps. When a 0.1-ml aliquot size was used, 0.9 ml of blank plasma was added to the tube to bring the total plasma volume to 1 ml. Following the addition of internal standard solution (150 μl), samples were vortex-mixed and then made basic by the addition of 1 ml of 1 *M* sodium carbonate buffer, pH 10. After mixing, hexane–ethyl acetate (90:10, v/v) (5 ml) was added to each sample. The samples were mixed on a rotary mixer at approximately 6 rpm for 20 min and then centrifuged at approximately 2500 × *g* for 10 min at 5°C. The lower aqueous layer was immediately frozen by immersing the tubes in a dry ice–acetone bath; the upper organic layer was decanted into silanized glass culture tubes. The supernatant was dried at 40°C under a stream of nitrogen. The samples were reconstituted with 1 ml of acetonitrile, vortex-mixed, and transferred to glass HPLC autosampler vials. This procedure can also be applied to human serum.

Calculations

A least-squares calibration curve was obtained by plotting the concentrations of the plasma standards *versus* the peak-height ratios. The peak-height ratios were calculated by dividing the peak heights of the analytes by the peak heights of the internal standard. The concentration of each compound was determined from the peak-height ratio relative to the calibration curve.

Determination of recovery, precision, and accuracy

The extraction efficiency (recovery) of the sample preparation procedure was tested by comparing the peak heights obtained from the chromatography of aqueous standards (not extracted) to those of extracted plasma samples which had been spiked with the analytes. Recovery was determined at three concentrations (30, 100, and 180 ng/ml) over the standard curve range.

The precision and accuracy of the method were determined by performing replicate analyses of six pools of plasma spiked with known concentrations of the compounds. The pool concentrations were selected to cover the range of the standard curve. These concentrations included the limit of quantitation as well as a pool with a concentration approximately four times the high calibration standard. The latter sample was diluted ten-fold with blank plasma at the time of analysis to bring the concentration within the standard

curve range. Five replicates of each pool were analyzed on three different days by the same analyst on the same instrument. All samples were analyzed in random order. Two standard curves were included each day, one at the end and one at the beginning of the run.

Determination of stability

The stability of dapoxetine in plasma was determined by preparing pooled plasma with known amounts of dapoxetine (40 and 180 ng/ml). The pools were aliquoted (1.3 ml) into polypropylene plastic tubes, capped, and stored at −70°C, −20°C, 4°C, and room temperature. Five replicates of each pool were assayed from each storage condition at set time points following initial storage.

Freeze–thaw stability of dapoxetine was determined through three freeze–thaw cycles. Pooled plasma spiked with dapoxetine at 40 and 180 ng/ml was frozen at −70°C. Five replicates of each concentration were evaluated after each cycle.

Processed sample stability of dapoxetine was evaluated by reinjecting a set of 38 samples starting 24 and 48 h after the run was first initiated.

Analysis of samples from a clinical study

Plasma samples from volunteers in a clinical fed–fasted study were frozen and maintained at −20°C prior to analysis. On thawing, aliquots of each sample were dispensed into 15-ml disposable glass screw-capped tubes and processed as stated above under *Sample preparation procedures*. Quality control samples (high, medium, and low concentrations), prepared from a separate weighing of standards, were included in duplicate in each run.

RESULTS AND DISCUSSION

Method development and chromatography

The assay conditions described in this report evolved from the evaluation and optimization of the sample preparation, chromatography, and detection of dapoxetine, its N-demethylated metabolites, and internal standard. The required sensitivity of the assay (less than 5 ng/ml) led to a decision to utilize an HPLC separation with fluorescence detection, which capitalizes on the intense native fluorescence of the analytes.

Both liquid–liquid and solid-phase extractions were explored when developing a sample preparation scheme. A variety of different reversed-phase solid-phase extraction cartridges were evaluated, including CH, C_2, C_8, C_{18}, CN, phenyl, and NH_2 (Analytichem Bond Elut) phases. Cationic cartridges such as SCX and Certify I (Analytichem Bond Elut), which exploit the amine functional group of the analytes, were screened as well. Even though some extraction cartridges yielded reasonably clean extracts, in general, recoveries were low and reproducibility was poor.

The use of a liquid–liquid extraction sample preparation protocol met with greater success. Several different solvents were evaluated including hexane–butanol (96:4, v/v), hexane–ethyl acetate (90:10, v/v), pentane, hexane, heptane, *n*-butyl chloride, and ethyl acetate. Hexane–ethyl acetate proved to be the optimal extraction solvent in that it maximized recovery of the analytes while minimizing interferences from plasma components. Back extractions with 0.2 *M* hydrochloric acid further eliminated interferences, but decreased recovery by approximately 50%.

In evaluating the chromatographic separation, several columns were screened for optimal peak shape and selectivity as well as ruggedness. The columns included Jones CN, Zorbax ODS, C_8, CN, phenyl, and silex, and the base-deactivated columns SynChrom SynChropak SCD100 and Zorbax RX-C_8. The RX-C_8 column yielded the best combination of peak shape, resolution of analytes from endogenous interferences, and ruggedness. The amine group present on the analytes caused some tailing even with base-deactivated chromatographic phases. Many buffers were screened in order to minimize this tailing. The addition of the modifier triethylamine to eliminate residual tailing dramatically reduced peak heights.

This assay is sensitive to plasticizer interferences. For example, substantial interferences were observed with the use of plastic autosampler vials, which were apparently components leached

from the plastic vials by the acetonitrile. This sensitivity to plasticizers necessitated reconstituting the samples in acetonitrile rather than mobile phase. When the samples were reconstituted in mobile phase, particulates were observed that could not be readily removed without introducing interferences from the filtration devices.

The use of column switching with this method was adopted to eliminate late-eluting interferences that caused significant problems with the chromatography (attempts to eliminate these interferences by changing the sample preparation procedure were unsuccessful). Using a front-cut strategy, the samples were injected onto a pre-column equilibrated with mobile phase (Fig. 2). The analytes eluted onto the analytical column within 1.2 min. At this point, the valve was switched, allowing a stronger solvent to wash the interferences off of the pre-column while the mobile phase was diverted directly to the analytical column. The analytes were resolved from each other and any remaining interferences on the analytical column. Wash compositions of at least 90% acetonitrile were required to completely eradicate the interference problem. The valve was switched back at 20 min to allow reequilibration of the pre-column with mobile phase prior to the next injection. The pre-column had to be changed with each new run (~50 samples).

Representative chromatograms of extracted samples are shown in Fig. 3. Good resolution was obtained between the four analytes with no interference from endogenous substances. The chromatogram obtained from the pre-dose samples indicates the absence of interferences at the retention time of the analytes (Fig. 3). Chromatograms of plasma and serum extracts were essentially identical.

Sample recovery

The recovery of dapoxetine and the di-desmethyl metabolite was consistent over the three concentrations (Table I), with the recovery of dapoxetine essentially 100%. The mono-desmethyl metabolite recovery was concentration-dependent, with greater recoveries at higher concentrations.

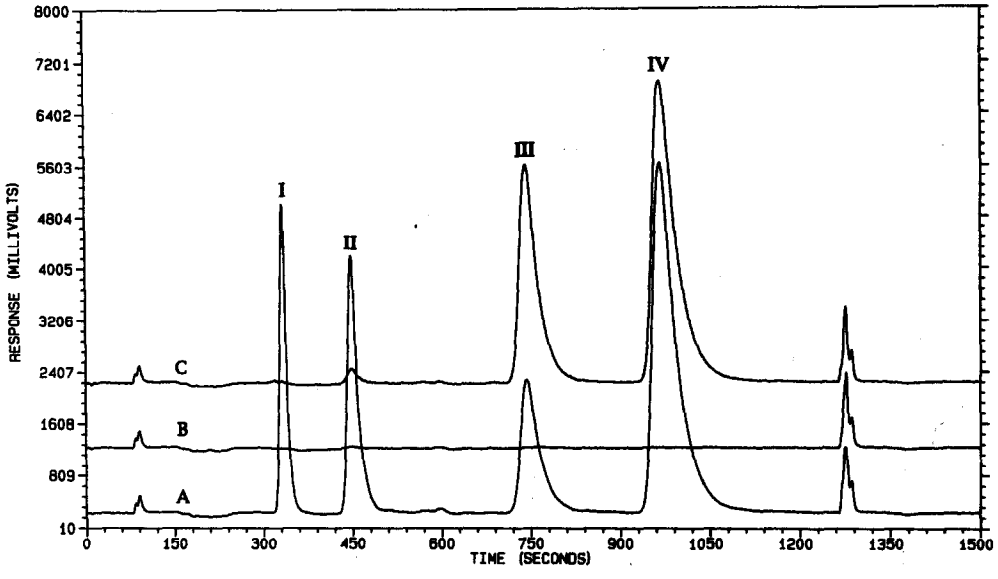

Fig. 3. Representative chromatograms from a patient administered a single oral dose of 40 mg of dapoxetine. (A) Human plasma standard (100 ng/ml); (B) patient sample at 0 min; (C) patient sample at 140 min. Peaks: I = dapoxetine; II = internal standard: III = mono-desmethyl metabolite; IV = di-desmethyl metabolite.

TABLE I

RECOVERY DATA (n = 8)

Values in parentheses are relative standard deviations (%).

~Analyte concentration (ng/ml)	Recovery (%)		
	Dapoxetine	Mono-desmethyl metabolite	Di-desmethyl metabolite
30	93.9 (3.9)	63.6 (22.9)	57.4 (9.1)
100	91.6 (8.7)	80.3 (20.7)	58.5 (10.3)
180	93.5 (8.1)	92.0 (8.0)	60.7 (8.2)

Precision and accuracy

The determination of dapoxetine and its two N-demethylated metabolites was evaluated for precision and accuracy by replicate analyses of plasma pools spiked with the analytes at various concentrations. Replicates of the same pools were evaluated on three different days so that both within-day and between-day precision and accuracy could be determined. Overall, the precision and accuracy of the method were excellent; relative standard deviations (R.S.D.) and accuracies were within 5–10% (Tables II–IV). The determination of dapoxetine in human serum yielded within-day precision and accuracy values consistent with those obtained with plasma (Table V). Between-day precision and accuracy for serum were not determined.

Sensitivity

The limit of quantitation of the assay was 20 ng/ml. This limit could be lowered to 2 ng/ml by reconstituting the samples in 200 μl of acetonitrile and using a standard curve range of 2–25 ng/ml. The limit of quantitation was defined to

TABLE II

PRECISION AND ACCURACY DATA FOR DAPOXETINE IN HUMAN PLASMA

Day		Theoretical concentration (ng/ml)				
		21.2 ng/ml	74.3 ng/ml	127 ng/ml	174 ng/ml	849 ng/ml
1	Mean (n = 5)	22.2	73.7	128	171	835
	R.S.D. (%)	2.7	1.4	2.2	1.8	2.9
	% of theory	105	99.2	101	98.3	98.4
2	Mean (n = 5)	20.3	71.9	126	174	820
	R.S.D. (%)	1.1	1.3	1.8	0.5	2.4
	% of theory	95.8	96.8	99.2	100	96.6
3	Mean (n = 5)	20.8	72.7	125	175	830
	R.S.D. (%)	2.5	5.2	5.0	1.1	2.4
	% of theory	98.1	97.8	98.4	101	97.8
Overall	Mean (n = 15)	21.1	72.8	126	173	828
	R.S.D. (%)	5.1	3.2	3.3	1.7	2.6
	% of theory	99.5	98.0	99.2	99.4	97.5

TABLE III

PRECISION AND ACCURACY DATA FOR THE MONO-DESMETHYL METABOLITE IN HUMAN PLASMA

Day		Theoretical concentration (ng/ml)				
		19.8 ng/ml	69.2 ng/ml	119 ng/ml	162 ng/ml	790 ng/ml
1	Mean (n = 5)	21.5	61.6	115	159	797
	R.S.D. (%)	3.7	8.9	5.5	5.1	2.0
	% of theory	109	89.0	96.6	98.1	101
2	Mean (n = 5)	20.8	68.0	121	165	778
	R.S.D. (%)	2.8	1.0	1.5	0.7	2.0
	% of theory	105	98.3	102	102	98.5
3	Mean (n = 5)	19.6	72.5	129	174	825
	R.S.D. (%)	2.3	4.4	4.9	4.5	4.2
	% of theory	99.0	105	108	107	104
Overall	Mean (n = 15)	20.5	66.7	118	164	790
	R.S.D. (%)	6.8	7.1	4.6	3.9	2.3
	% of theory	104	96.4	99.2	101	100

be equivalent to the lowest validation pool which, under the given conditions, yielded a value of $\leq$ 15% R.S.D. and $\leq$ 15% relative error both within and between day.

Linearity

The linearity of the responses with 1-ml sample volumes was established over the concentration range 20–200 ng/ml. Typical correlation coefficients were greater than 0.99. Samples with analyte concentrations between 200 and 800 ng/ml can be analyzed by ten-fold dilution with blank plasma. Dilution of samples greater than ~800 ng/ml was not examined.

Stability

Dapoxetine is stable in plasma (<10% degradation) for at least 48 h at room temperature, for

TABLE IV

PRECISION AND ACCURACY DATA FOR THE DI-DESMETHYL METABOLITE IN HUMAN PLASMA

Day		Theoretical concentration (ng/ml)				
		20.2 ng/ml	70.7 ng/ml	121 ng/ml	166 ng/ml	808 ng/ml
1	Mean (n = 5)	19.7	62.6	122	163	815
	R.S.D. (%)	3.4	12.9	3.8	3.2	3.5
	% of theory	97.5	88.5	101	98.2	101
2	Mean (n = 5)	20.1	69.8	122	173	808
	R.S.D. (%)	3.6	1.4	4.4	1.1	2.8
	% of theory	99.5	98.7	101	104	100
3	Mean (n = 5)	19.6	72.5	129	174	825
	R.S.D. (%)	2.3	4.4	4.9	4.5	4.2
	% of theory	97.0	103	107	105	102
Overall	Mean (n = 15)	19.8	68.3	124	170	816
	R.S.D. (%)	3.2	10.0	5.3	4.6	3.5
	% of theory	98.0	96.6	102	102	101

TABLE V

PRECISION AND ACCURACY DATA FOR DAPOXETINE IN HUMAN SERUM

	Theoretical concentration (ng/ml)				
	20.1 ng/ml	70.3 ng/ml	120 ng/ml	166 ng/ml	803 ng/ml
Mean (n = 5)	19.9	71.9	120	165	827
R.S.D. (%)	5.7	3.4	4.7	2.8	2.4
% of theory	99.0	102	100	99.4	103

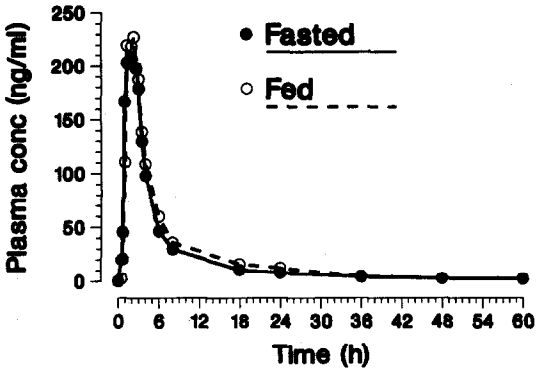

Fig. 4. Representative fed/fasted profiles obtained for dapoxetine plasma concentrations *versus* time following a single oral dose of 40 mg of dapoxetine hydrochloride.

at least two weeks at 4°C, and for at least nine months at −20°C and −70°C. The data for the last time point at each temperature is shown in Table VI. Plasma samples spiked with dapoxetine are stable through at least three freeze–thaw cycles. Processed samples containing dapoxetine are stable for greater than 48 h. Standard stock solutions of dapoxetine are stable for at least three months at 4°C. The stability of the metabolites has not been examined.

Application of the method in pharmacokinetic studies

The validated procedure was used to provide pharmacokinetic data for dapoxetine in man fol-

TABLE VI

STABILITY DATA FOR DAPOXETINE IN HUMAN PLASMA

Theoretical concentration (ng/ml)	Storage time	Mean (n = 5) assay result	R.S.D. (%)	% of theory	Storage temperature[a]
40.9	Initial	42.1	4.0	103	N.A.
184	Initial	185	4.7	101	N.A.
40.9	48 h	42.5	12.3	104	R.T.
184	48 h	186	5.4	101	R.T.
40.9	2 weeks	42.7	5.0	104	4°C
184	2 weeks	196	3.6	107	4°C
40.9	9 months	39.6	5.3	96.7	−20°C
184	9 months	178	7.5	96.6	−20°C
40.9	9 months	38.3	5.0	93.6	−70°C
184	9 months	171	9.1	92.7	−70°C

[a] N.A. = not applicable; R.T. = room temperature.

lowing the administration of single oral doses in a fed–fasted study. Plasma samples were obtained at defined time intervals post-administration, extracted, and analyzed by HPLC. Analysis of plasma samples collected prior to dapoxetine administration demonstrated that interferences from endogenous plasma components were not compromising the quality of the results. Representative chromatograms are shown in Fig. 3. The mono- and di-desmethyl metabolite levels were below the limit of quantitation (20 ng/ml). Representative plasma profiles of dapoxetine as a function of time comparing the fed and fasted samples of one volunteer are given in Fig. 4.

CONCLUSIONS

Dapoxetine and its mono- and di-desmethyl metabolites may be accurately determined in plasma by the described procedure. The use of column switching has eliminated late-eluting interferences and greatly enhanced assay ruggedness. This method was used to analyze Phase I samples and will be used to facilitate pharmacokinetic studies and therapeutic monitoring of dapoxetine in Phase Ib/II/III studies.

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