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Determination of terfenadine and terfenadine acid metabolite in plasma using solid-phase extraction and high-performance liquid chromatography with fluorescence detection

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ABSTRACT

This work describes the methodology for the analysis of terfenadine and the acid metabolite of terfenadine in plasma using high-performance liquid chromatography. The use of solid-phase extraction allows the use of robotic or manual sample preparation for the efficient clean-up of terfenadine and terfenadine acid metabolite from plasma. Additional selectivity is obtained through the use of fluorescence detection. For terfenadine, the validated quantitation range of this method is 10.0–84.2 ng/ml with coefficients of variation of 5.7–30%. For terfenadine acid metabolite, the validated quantitation range of this method is 8.2–500 ng/ml with coefficients of variation of 4.1–24%.

INTRODUCTION

Terfenadine (I, Fig. 1a) has been shown to be efficacious for the symptomatic relief of allergic rhinitis and a variety of skin allergies [1] without the sedation and anticholinergic effects of conventional antihistamines.

Terfenadine undergoes extensive first-pass metabolism with more than 99% of the absorbed dose eventually metabolized [2,3]. A major metabolite resulting from the oxidation of the *tert.*-butyl group to give the carboxylic acid metabolite (II, Fig. 1b) appears to exert most of the pharmacological actions associated with administration of the parent drug. After the administration of either single or twice daily doses of a 60-mg tablet, plasma levels of terfenadine are typically below 10 ng/ml while plasma levels of the acid metabolite are readily detectable [4]. For this reason, acid metabolite rather than parent terfenadine levels have been used for pharmacokinetic and pharmacodynamic assessments [5]. Terfenadine levels are monitored to allow for unique clinical situations although in normal subjects terfenadine levels are below the limit of quantitation for this method.

This paper details the analytical methodology used for the quantification of the terfenadine acid metabolite and terfenadine in such studies. The validation of this methodology with regards to accuracy, precision, sensitivity, and specificity is presented.

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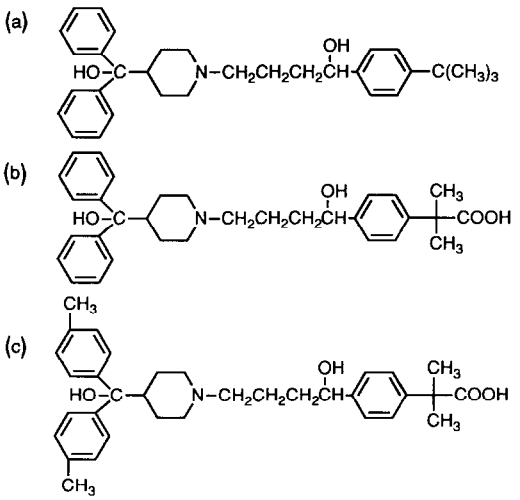


Fig. 1. Chemical structures of (a) terfenadine (I), (b) terfenadine acid metabolite (II), and (c) the internal standard (III).

EXPERIMENTAL

Materials

If not specified, reagent-grade chemicals were used throughout this study. The ammonium acetate used for mobile phase buffer was high-performance liquid chromatography (HPLC) grade (J. T. Baker, Phillipsburg, NJ, USA). Water was distilled and deionized. All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI, USA). The terfenadine acid metabolite (II, MDL 16,455) reference material, the terfenadine reference material, and the internal standard reference material (III, MDL 26,042, Fig. 1c) were obtained from the Marion Merrell Dow Research Institute (Cincinnati, OH, USA). Drug-free EDTA human plasma was supplied by Carolina Biological Supply (Burlington, NC, USA).

Standards

Standards of I and II were prepared in drug-free EDTA plasma. An initial solution of 0.5 mg/ml in methanol was used to dissolve II and a solution of 0.5 mg/ml in ethanol to dissolve I. For II the 0.5 mg/ml solution was diluted with water to give a stock solution of 25 μg/ml. For I the 0.5 mg/ml solution was diluted with methanol to give a stock solution of 25 μg/ml. Various volumes of these stock solutions were added to plasma to give plasma standards of 0, 10, 20, 50, 100, 200, and 500 ng/ml of both I and II. These solutions were divided into 3-ml aliquots and kept frozen until use. The internal standard (III) was also initially dissolved in methanol to give a solution of 0.5 mg/ml. This solution was then diluted with water to give a solution of 5 μg/ml and then diluted with acetate buffer (0.2 *M*, pH 4.0) to give a working solution of 75 ng/ml III.

Validation study

To test the accuracy and precision of the analytical method, 46 randomly coded unknowns were analyzed over a six-day period. To determine the within-day precision of the method, six aliquots of six different concentrations were analyzed on one day. Ten blank plasma samples were assayed to check for endogenous interferences. In addition, a variety of different compounds such as pseudoephedrine, which might be present in patients, were tested for possible interferences. The concentrations of II were chosen to be representative of the concentration ranges previously observed in clinical samples.

Sample preparation

Samples and standards were prepared by solid-phase extraction (SPE) on C_{18} minicolumns (Analytichem SPE C_{18}, 3 ml, 500 mg packing). Clean SPE columns were preconditioned with two 2.0-ml volumes of methanol followed by one 2.0-ml volume of water and one 1.5-ml volume of acetate buffer (0.2 *M*, pH 4.0) routed to waste. The internal standard working solution (1 × 1.0 ml) was added to the column but not forced through. A 1.0-ml aliquot of plasma (sample or standard) was then added to the solution in the SPE column and both solutions were blown through the column with nitrogen. The SPE column was rinsed with 1 × 2.0 ml of water, 2 × 1.0 ml of methanol–water (50:50), and 1 × 1.0 ml of methanol. The SPE column was then dried by blowing nitrogen through the column for approximately 5 min at 8 ml/min. The sample was eluted from the column into a clean test tube with 2 × 0.5 ml of 0.05 *M* triethylamine (TEA) in methanol. A heating block at 55°C and a nitrogen stream were then used to evaporate the sample to dryness. The sample was reconstituted in 200 μl of mobile phase, and 150 μl injected were into the HPLC system. This sample preparation can be done manually or by using a robotics system. The data in this study were obtained by using a robotic system (Zymark, Hopkinton, MA, USA) configured for SPE extraction and injection into the HPLC system which has been previously described [6].

High-performance liquid chromatography

The HPLC pumping system used in this study was a Waters Model 6000A pump (Millipore, Waters Chromatography Division, Milford, MA, USA). The mobile phase was acetonitrile–methanol–0.012 *M* ammonium acetate buffer (19:29:52, v/v). The 0.012 *M* ammonium acetate buffer was prepared by dissolving 0.5 g of ammonium acetate and 5 ml of acetic acid in 520 ml of water. The flow-rate was 1.8 ml/min. The HPLC column (250 mm × 4.6 mm I.D.) was a Spherisorb S5CN, 5-μm cyano column (Phase Separation, Norwalk, CT, USA) and used with a 2-μm guard filter (Rheodyne, Cotati, CA, USA). Column temperature was maintained at 35°C with a Model TC-50 (FIAtron, Milwaukee, WI, USA) column heater. An electrically actuated Model 7010 injector (Rheodyne), fitted with a 200-μl loop, was partially backfilled to a injection volume of 150 μl by a syringe controlled by the robot system.

A Model 1046A fluorometer (Hewlett-Packard, Avondale, PA, USA) was used to monitor the HPLC effluent at an excitation wavelength of 230 nm and an emission wavelength of 280 nm with an excitation slit of 2 nm and emission slits of 4 nm. A Beckman CALS laboratory data system (Beckman Instruments, Allendale, NJ, USA) was used to acquire, integrate, and analyze the chromatographic data.

Calculations

The peak heights of I, II, and the internal standard were determined using the laboratory data system described above. The general form of the calibration used was $y^n = ax + b$ where y is the peak-height ratio found by dividing either I or II by the internal standard, x is the concentration of the compound, a is the slope of the fitted line, and b is the intercept. Assuming $b = 0$, the natural logarithm of both sides was determined and the equation rearranged to $\ln y = \ln A/n + \ln x/n$. A linear regression on $\ln x$ *versus* $\ln y$ for each standard in the standard curve permitted calculation of n and a from the slope and intercept. For typical standard curves of I and II the resulting line fit is essentially linear with correlation coefficients greater than 0.999.

RESULTS AND DISCUSSION

HPLC and SPE

The development of the SPE method for I and II was based on several considerations. The primary consideration was to isolate, purify, and concentrate both I and II in order to produce the highest analytical sensitivity and selectivity possible. A second factor included compatibility with either manual or robotic sample preparation of the same methodology. Another area of consideration during the method development was to include the flexibility to allow for the consecutive if not simultaneous assay of additional components in the sample. The development of formulations containing not only terfenadine but also pseudoephedrine made it advantageous to consider the ability to analyze at least two components from the same plasma sample solution. These were the primary factors in choosing SPE and the specific wash/extraction steps used for sample preparation. The HPLC column and conditions were chosen to complete resolution of the compounds of interest within the cycle time constraints of the robotic system. In a typical chromatogram, the retention times of II, internal standard, and terfenadine were 7.7, 9.3, and 13.5 min, respectively. A chromatogram for an extracted plasma standard at the 200 ng/ml level is shown in Fig. 2.

Validation study

The absolute extraction recovery of II and I from plasma samples was determined by comparing the peak heights of the respective peaks from extracted standards in human plasma to the peak heights of unextracted standards. The

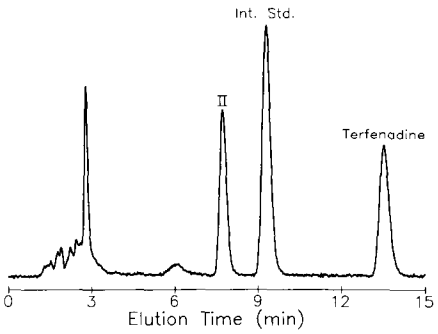


Fig. 2. Typical chromatogram of a plasma standard containing 200 ng/ml terfenadine and terfenadine acid metabolite (II).

extraction efficiency was found to be 77.9–87.6% for II, 59.6–66.5% for I, and 73,9% for the internal standard (III). These results are presented in Table I.

The accuracy and precision of the method were determined between days by assaying 46 randomly coded unknowns on six different days. The within-day accuracy and precision was determined by analyzing six aliquots of six different concentrations on the same day. Additionally, in order to better define the limit of quantitation, these unknowns contained concentrations below the lowest point on the standard curve. To determine the between-day precision for II, the range of concentrations was 8.2–409 ng/ml. These data are shown in Table II. For levels of II above 8.2 ng/ml the accuracy ranged from 99.2 to 103.5% with coefficients of variation (C.V.) ranging from 4.1 to 9.8%. For the samples containing 8.2

TABLE I

RECOVERY OF TERFENADINE ACID METABOLITE (II), TERFENADINE, AND INTERNAL STANDARD EXTRACTED FROM 1 ml PLASMA ON C_{18} SOLID-PHASE EXTRACTION COLUMNS

Recoveries were determined by dividing the peak heights found after injection of unextracted solutions to peak heights of extracted plasma samples. Unextracted solutions were prepared to be equivalent to the concentrations expected for 100% recovery.

Concentration (ng/ml)	Recovery (%)		
	II	Terfenadine	Internal standard
500	85.5	61.1	
200	87.6	66.5	
100	83.0	61.5	73.9
50	80.9	60.5	
20	77.9	59.6	

TABLE II

TERFENADINE ACID METABOLITE (II) PLASMA CONCENTRATIONS FOUND IN 46 UNKNOWNS RUN OVER SIX DAYS: BETWEEN-DAY PRECISION

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	*n*	C.V. (%)	Accuracy (%)
409.00	415.06 ± 18.44	5	4.40	101.50
294.00	300.87 ± 12.23	6	4.10	102.30
196.00	202.85 ± 8.34	6	4.10	103.50
81.80	84.73 ± 4.13	6	4.80	103.50
49.10	48.73 ± 3.40	6	7.00	99.20
32.70	33.55 ± 3.25	6	9.80	102.60
8.20	9.30 ± 2.25	6	24.00	113.40
0	0 ± 0	6		

ng/ml II the accuracy was 113.4% with a C.V. of 24%. The within-day accuracy and precision samples ranged in concentration from 8.2 to 294 ng/ml II and these results are shown in Table III. The accuracy for within-day samples above 8.2 ng/ml was from 96.6 to 100.7%, with C.V.s from 1.0 to 6.0%. The samples containing 8.2 ng/ml II had a accuracy of 95.2% with a C.V. of 26%. As expected the C.V. increases as the level of II decreases. The limit of quantitation (at 8.2 ng/ml) is strongly influenced by the performance of the HPLC system and requires careful monitoring of the detector performance and column efficiency. For II, the valid concentration range for quantitation by this method is 8.2–500 ng/ml.

Similar studies were conducted for the determination of terfenadine. Between-day accuracy and precision results are shown in Table IV for concentrations of I

TABLE III

TERFENADINE ACID METABOLITE (II) PLASMA CONCENTRATIONS FOUND IN 36 UNKNOWNS RUN ON 1 DAY: WITHIN-DAY PRECISION

Concentration added (ng/ml)	Concentration found (mean ± S.D., $n = 6$) (ng/ml)	C.V. (%)	Accuracy (%)
294.00	296.10 ± 9.04	3.00	100.70
196.00	195.25 ± 2.05	1.00	99.60
81.80	79.15 ± 3.84	5.00	96.80
49.10	47.42 ± 2.83	6.00	96.60
32.70	32.10 ± 1.43	4.00	98.20
8.20	7.81 ± 2.00	26.00	95.20

TABLE IV

TERFENADINE PLASMA CONCENTRATIONS FOUND IN 46 UNKNOWNS RUN OVER 6 DAYS: BETWEEN-DAY PRECISION

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	*n*	C.V. (%)	Accuracy (%)
84.30	90.84 ± 6.58	5	7.30	107.80
60.60	68.00 ± 3.85	6	5.70	112.20
40.40	45.12 ± 2.60	6	5.80	111.60
16.80	17.88 ± 4.04	6	22.30	106.50
10.10	11.88 ± 3.53	6	30.00	117.60
6.70	3.23 ± 5.10	6	158.00	47.80
1.7	0.0 ± 0.0	6		
0	0 ± 0	6		

from 1.7 to 84.2 ng/ml. At 10.1 ng/ml and above the accuracy was 106.5–117% with C.V.s ranging from 5.7 to 30%. Within-day results are shown in Table V for concentrations of I from 1.7 to 60.6 ng/ml. The accuracy varied from 108.2 to 115.7% with C.V.s from 2.0 to 23%. For both the between-day and within-day studies, a peak was detected at a concentration of 6.7 ng/nl in only two out of six samples and at 1.7 ng/ml no peaks were detected. As in the case of II, the performance of the HPLC column has a great effect on the precision, accuracy, and detectability of terfenadine at low concentrations. Because the retention time of I is greater than that of II, the resulting increase in peak width contributes to the higher C.V.s determined at the lower levels of 10.1 and 16.8 ng/ml. For I, the valid quantitation range of this method is 10.1–84.2 ng/ml.

During sample preparation, plasma samples may remain at room temperature for varying amounts of time before analysis. The stability of II and I in human plasma at room temperature was checked by preparing a pool sample containing both compounds and dividing it into several aliquots. Some aliquots were frozen

TABLE V

TERFENADINE PLASMA CONCENTRATIONS FOUND IN 36 UNKNOWNS RUN ON ONE DAY: WITHIN-DAY PRECISION

Concentration added (ng/ml)	Concentration found (mean ± S.D., $n = 6$) (ng/ml)	C.V. (%)	Accuracy (%)
60.60	65.57 ± 1.59	2.00	108.20
40.40	45.77 ± 3.79	8.00	113.30
16.80	18-73 ± 2.41	13.00	111.50
10.10	11.69 ± 2.73	23.00	115.70
6.70	3.25 ± 5.04	155.00	48.50
7.7	0.0 ± 0.0	0.0	

TABLE VI

STABILITY OF TERFENADINE ACID METABOLITE (II) IN PLASMA FOR NINE DAYS AT ROOM TEMPERATURE

Sample age (days)	500 ng/ml (n = 6)		50 ng/ml (n = 6)	
	Mean found (ng/ml)	Percentage found	Mean found (ng/ml)	Percentage found
1	502.6	100.5	47.8	95.6
3	500.0	100.0	48.0	96.0
8	491.9[a]	98.4[a]	49.2[a]	98.4
9	522.5[a]	104.0	51.8	103.6

[a] n = 5.

TABLE VII

STABILITY OF TERFENADINE IN PLASMA FOR NINE DAYS AT ROOM TEMPERATURE

Sample age (days)	500 ng/ml (n = 6)		50 ng/ml (n = 6)	
	Mean found (ng/ml)	Percentage found	Mean found (ng/ml)	Percentage found
1	505.1	101.2	49.2	98.4
3	489.3	97.9	49.1	98.2
8	475.8[a]	95.2	49.3[a]	98.6
9	536.6[a]	107.3	48.8	97.6

[a] n = 5.

and others were left at room temperature for various lengths of time. No statistically significant degradation of II or I in human plasma was observed over the nine-day period of the experiment. These results are presented in Tables VI and VII. This concentration range of II and I stored frozen in EDTA plasma at −20°C have been shown to be stable for up to sixteen months.

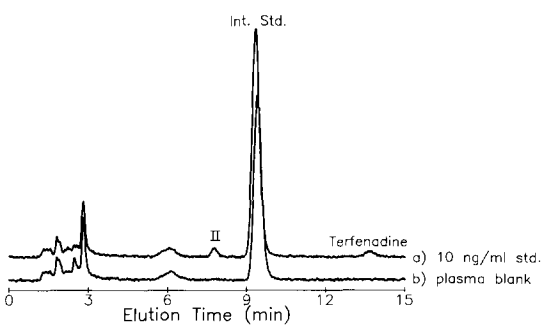


Fig. 3. Chromatograms of (a) a plasma standard at 10 ng/ml terfenadine and terfenadine acid metabolite (II) along with (b) a plasma blank for comparison.

TABLE VIII

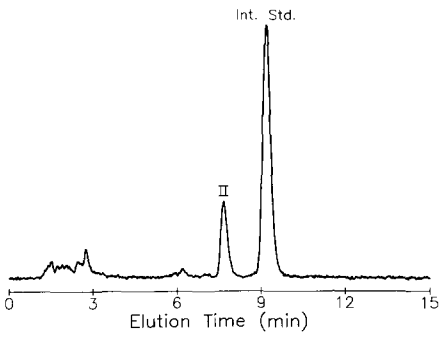
COMPOUNDS USED FOR SPECIFICITY TEST

MDL specification	Common name	Relative retention time
MDL 4829	Terfenadine metabolite	0.69
MDL 17,523	Terfenadine alcohol metabolite	0.95
MDL 16,455	Terfenadine acid metabolite (II)	0.84
	Terfenadine (I)	1.36
MDL 26,042	Internal standard (III)	1.00
	Ephedrine	0.65
	Norpseudoephedrine	0.50
	Pseudoephedrine	0.53
	Ibuprofen	0.32
	Ibufenac	0.28
	Acetaminophen	0.32

No interfering peaks were present in eight different blank plasma samples analyzed. Fig. 3 shows the chromatograms of a blank plasma standard along with a 10 ng/ml plasma standard for comparison. A variety of different compounds which might be present in samples were tested for possible interference at the level of 8.0 μg injected and are listed in Table VIII. None of these compounds had relative retention times that would interfere with the measurement of II, I, or the internal standard.

Application of the method

This procedure has been applied successfully to the analysis of samples from a number of pharmacokinetic and bioavailability studies. The chromatogram shown in Fig. 4 is from a subject who was dosed with 30 mg of I. This time point



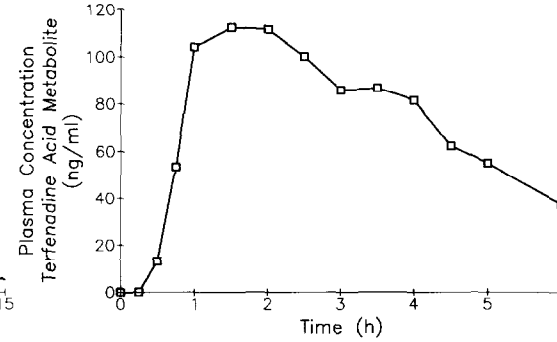


Fig. 4. Chromatogram of a plasma sample from a subject dosed with 30 mg terfenadine. Time point is 3½ h post-dose. Calculated terfenadine acid metabolie (II) level is 86 ng/ml.

Fig. 5. Concentration of terfenadine acid metaboltie *versus* time for a subject dosed with 30 mg terfenadine. Plasma concentration *versus* time profile is for 0–6 h post-dose.

was 3½ h post-dosing on the start of day 1 and represents a calculated level of 86 ng/ml of II. Fig. 5 shows the plasma concentration of II versus time for this subject following the dose of 30 mg terfenadine from 0 to 6 h post-dose.

CONCLUSIONS

This method provides for the accurate and precise determination of terfenadine acid metabolite and terfenadine in human plasma. The use of SPE allows rapid and efficient sample clean-up and is applicable to either manual or automated (robotic) sample preparation.

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