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Analysis of hydroxylated and *N*-dealkylated metabolites of terfenadine in microsomal incubates by liquid chromatography–mass spectrometry

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Abstract

This report describes an assay for the H_1-receptor antagonist, terfenadine, and its two primary metabolites, terfenadine alcohol (TOH) and azacyclonol (AZ), using positive-ion, electrospray ionization–liquid chromatography–mass spectrometry. The assay was developed in support of kinetic studies of terfenadine oxidative metabolism in human liver and intestinal microsomes, which required quantification of incubate metabolites at low nanomolar concentrations. Terfenadine metabolites were extracted from basified microsomal incubates into methylene chloride. Reconstituted extracts were subject to liquid chromatographic separation on a cyano-reverse phase column. The $[M+H]^+$ ions of terfenadine, terfenadine metabolites, and internal standard were monitored in the effluent by quadrupole mass spectrometry. The assay demonstrated linearity over an incubate concentration range of 5–250 and 12.5–1250 ng/ml for the metabolites and the parent drug, respectively. The respective limits of detection and quantitation for all three analytes were 1.5 and 5 ng/ml of microsomal incubate. Replicate analysis of quality control samples exhibited intra-day coefficients of variation ranging from 3.3% to 7.8% for the three analytes. The corresponding inter-day coefficients of variation ranged from 4.2% to 8.6%. The reproducibility and sensitivity of the assay, combined with the selectivity of mass spectrometric detection, should allow an accurate kinetic characterization of terfenadine oxidation mediated by the high affinity CYP3A enzymes in human liver and intestinal microsomes.

Keywords: Terfenadine; Terfenadine alcohol; Azycyclonal

1. Introduction

Terfenadine (Seldane™) was the first non-sedating antihistamine introduced for the treatment of seasonal allergic rhinitis. Terfenadine was widely prescribed until recently when it was withdrawn from the US market because of safety concerns over potentially fatal cardiac arrhythmia. The primary risk factor has been recognized to be metabolic drug interactions in patients who receive concurrent azole antifungals, macrolide antibiotics, and other drugs capable of inhibiting the cytochrome P450 enzyme, CYP3A4. In healthy, un-medicated individuals extensive first-pass metabolism of terfenadine (~99%) results in very low concentrations (<1 ng/ml) of the

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parent drug in the systemic circulation [1]. In patients who developed cardiac arrhythmia (Torsade de Pointes) following an adverse drug interaction with terfenadine, plasma terfenadine concentrations exceeding 5–10 ng/ml have been observed [2]. Since terfenadine is known to be a potent blocker of slow potassium channels in the myocardium, an elevated level of terfenadine in circulation is recognized to be the cause of Torsade de Pointes [3].

Terfenadine undergoes two parallel oxidative metabolism pathways: hydroxylation of the *tert.*-butyl side chain of terfenadine to yield the alcohol metabolite (TOH), and *N*-dealkylation to form azacyclonol (AZ) (Fig. 1). Terfenadine alcohol undergoes sequential oxidation metabolism to the acid metabolite (TCOOH), which is the active antihistaminic species. The alcohol metabolite also undergoes *N*-dealkylation to AZ, but to a much lesser extent than the acid metabolite [4,5]. Yun et al. [4] were the first to show that CYP3A4 is the exclusive enzyme responsible for the two primary oxidative pathways of terfenadine. Later, Ling et al. [5] examined the sequential C-oxidation steps to the acid metabolite using TOH as substrate, and concluded that CYP3A4 is also the main enzyme catalyzing these secondary reactions. Their data indicated that C-oxidation accounts for 75% of the primary oxidation pathways.

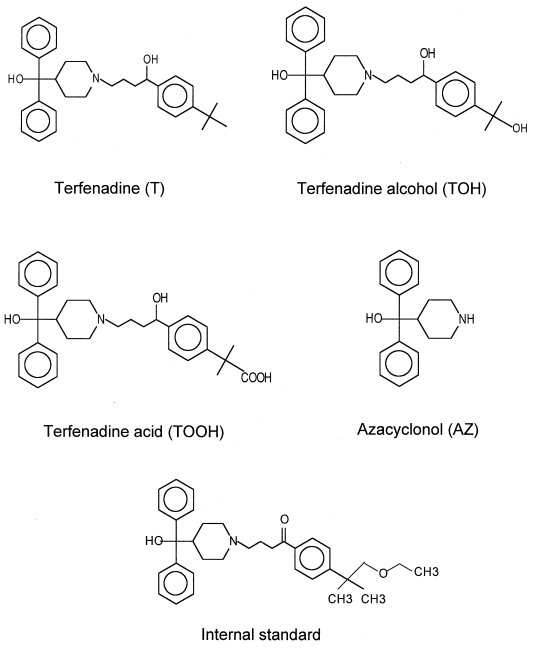

Fig. 1. Chemical structures of terfenadine, its oxidative metabolites, and the internal standard.

Aside from toxicological interest, terfenadine metabolism has attracted considerable attention because of its potential utility as a high-turnover catalytic probe for in vitro investigation of CYP3A4 activity in microsomes prepared from the gut mucosa and the liver. Accurate kinetic characterization of CYP3A4-mediated oxidation of terfenadine to TOH and AZ at below-K_M substrate concentrations (<5 μM) requires an assay capable of quantifying around 10 ng/ml or 20 nM of the primary metabolites in 0.5–1 ml volume of microsomal incubate.

A number of chromatographic methods for intact terfenadine and its acid metabolite (TCOOH) in plasma and urine have been reported [6–12]. Even though some of the more recently reported assays for terfenadine are highly sensitive [11,12], none of them can readily be adapted to the measurement of TOH and AZ in microsomal incubates. Other assays have been reported for the determination of terfenadine metabolites in urine [13] or AZ in serum [14] with modest sensitivity. High-performance liquid chromatography (HPLC) methods using fluorescence or UV detection have been described for simultaneous determination of the three oxidative metabolites of terfenadine in human liver microsomes [4,5,15]. All have inadequate sensitivity with limits of quantitation at or over 50 ng/ml of incubate. Recently, Rodrigues et al. [16] described a sensitive radio-HPLC assay for quantifying [^{3}H]-terfenadine metabolites in microsomal incubates. However, radiolabeled terfenadine is not commercially available. Hence, there is need to develop a sufficiently sensitive, non-radiolabel assay of terfenadine metabolites for microsomal studies.

This paper describes a liquid chromatographic–mass spectrometric (LC–MS) procedure that allows quantitative determination of TOH and AZ, along with the parent drug, in human liver and intestinal microsomes with sufficient sensitivity to fully characterize the high affinity (low K_M) Michaelis–Menten kinetics of terfenadine oxidation.

2. Experimental

2.1. Chemicals and materials

The following reagents were purchased from J.T. Baker (Phillipsburg, NJ, USA): potassium phosphate monobasic, ammonium acetate (HPLC grade), triethylamine (HPLC grade), and glacial acetate acid. HPLC-grade acetonitrile and methylene chloride were purchased from Burdick and Jackson (Muskegon, MI, USA). Methanol was purchased from Fisher Scientific Co. (Santa Clara, CA, USA). Distilled and deionized water were obtained from a Nanopure™ filtration system.

Terfenadine (MDL 9918), terfenadine alcohol (MDL 17523), azacyclonol (MDL 4829), terfenadine acid (MDL 16455), and the internal standard (MDL 16232), a keto- and *O*-ethylated derivative of terfenadine, were obtained from Hoechst Marion Roussel, Inc. (Kansas City, MS, USA). Fig. 1 shows the chemical structures of terfenadine, its metabolites as well as the internal standard.

2.2. Instrumentation

LC–MS analysis of the extracts was performed on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) fitted with a Shimadzu LD-10AD solvent delivery system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) and an Alcott 738R autoinjector (Alcott Chromatography, Norcross, GA, USA). The LC conditions followed those described by Ling et al. [5] with some modification. Chromatographic separation was accomplished with a 5-μm Spherosorb CN analytical column (4.6 mm I.D.×25 cm; Phase Separation Inc., Norwalk, CT, USA) under isocratic condition. The mobile phase consisted of 40% acetonitrile and 60% 0.1 *M* ammonium acetate buffer (pH=4.7) containing 0.1% triethylamine (TEA). The flow-rate of mobile phase was set at 1.2 ml/min. A flow of 60 μl/min was split from the column effluent and subjected to positive ion electrospray (ES) ionization mass spectral analysis.

The mass spectrometer was operated at a source temperature of 100°C and an ES probe voltage of 3.8 kV. Nitrogen served as the nebulizing and bath gas. Signal responses for AZ, T, TOH, and the internal standard were optimized as a function of cone voltage; the final settings were 25 V for AZ and 30 V for the other three compounds. The selection of ion windows (±0.25 Da) for selected-ion monitoring (SIM), data acquisition, and tuning of the quadrupole were accomplished using the PC-based Micromass MassLynx™ 2.1 software (Micromass, Ltd., Manchester, UK). The monitored ions were m/z 268.2, 472.3, 488.3 and 528.3, which corresponded to the $[M+H]^+$ of AZ, terfenadine, TOH and the keto- and ethylester derivative of terfenadine (i.e. internal standard), respectively. Dwell times were set at 100 ms/ion.

2.3. Standards and quality control

Stock solutions at a concentration of 1 mg/ml in methanol were prepared separately for terfenadine and each metabolite, and stored in tightly capped, amber glass vials at −20°C. Working solutions of standards were prepared as 'cocktails' of TOH, AZ and T in 50:50 (v:v) mixture of 0.1 *M* monobasic potassium phosphate (pH=7.4) solution and acetonitrile (ACN). Working solutions were stored in a glass container at 4°C for up to 6 months.

Stock solution of the internal standard (keto- and *O*-ethylated derivative of terfenadine) was prepared at a concentration of 4 mg/ml in methanol. The working solution was prepared by diluting the stock solution to a concentration of 80 μg/ml with a 50:50 (v:v) mixture of 0.1 *M* ammonium acetate and acetonitrile. The working solution was stored in a glass container at 4°C and remained stable for 6 months.

A quality control working solution was also prepared. Five microliters of the stock solutions (1 mg/ml) of TOH and AZ and 25 μl of terfenadine stock solutions were diluted to final concentrations of 50 ng/ml of TOH and AZ, and 250 ng/ml in 50:50 (v:v) mixture of 0.1 *M* KH_2PO_4:acetonitrile.

Calibration standards and quality controls were prepared in human liver microsomal suspensions at protein concentrations of 0.075 and 0.1 mg/ml, which were the protein concentrations used in the kinetic experiments for liver and intestinal micro-

somes, respectively. Calibration curves for AZ and TOH consisted of six standards: 5, 10, 20, 50, 100, and 250 ng/ml; terfenadine calibration curves consisted of seven standards: 12.5, 25, 50, 100, 250, 500, and 1250 ng/ml. The low and high quality control samples were prepared to contain respectively 10 and 50 ng/ml of each metabolite (TOH and AZ), and 25 and 250 ng/ml of terfenadine.

2.4. Extraction procedure

Twenty microliters of internal standard (8 mg/100 ml) and 200 μl of 1 *M* Na_2 CO_3 (pH=10.3) were added to 1 ml of microsomal samples, standards or quality control in disposable 15 ml screw-capped, glass culture tubes (16×125 mm) with Teflon-lined caps. Five milliliters of methylene chloride were added to each tube, followed by 5 min of vigorous shaking. After centrifugation at 2000×*g* for 10 min at room temperature, the bottom organic layer was transferred into a clean set of glass tubes, and evaporated to dryness under a stream of nitrogen gas in a 40°C water bath. The residue was reconstituted in 300 μl of mobile phase; a 100 μl aliquot was injected onto the column.

2.5. Microsomal incubation

Microsomal reactions were conducted in 15 ml screw-capped, disposable glass culture tubes in duplicates. Each incubate (1 ml) was prepared by suspending 0.075 mg protein/ml of human liver microsomes or 0.1 mg protein/ml of human intestinal microsomes in 0.1 *M*, pH 7.4, monobasic potassium phosphate buffer. An aliquot of the terfenadine stock solution was added to the microsomal suspension to achieve a pre-designated concentration of the substrate. The incubates were placed in a 37°C shaking water bath. After 5 min of pre-warming, the reaction was started with 100 μl of NADPH in phosphate buffer to yield a final cofactor concentration of 1 m*M*. Following 6 min of incubation, the reaction was stopped by the addition of 5 ml methylene chloride. The microsomal samples were processed as described earlier.

3. Results and discussion

3.1. Sample preparations

Initial efforts were made to minimize sample preparation by precipitating microsomal protein with acetonitrile and subjecting the supernatant to LC–MS analysis directly. In principle, this approach has the advantage of detecting all forms of terfenadine metabolites, i.e. acidic, neutral and basic metabolites. Unfortunately, background interference was observed in some human liver and intestinal microsomes. Nevertheless, we were able to perform several preliminary experiments to show that, under the chosen incubation condition, formation of terfenadine acid metabolite (TCOOH) was not detected over the range of substrate concentrations studied (0.1–8 μ*M*). Secondary oxidation did not appear to be a complication. Moreover, formation of TOH and AZ accounted for the amount of terfenadine consumed (i.e. mass balance). Hence, there was no need to account for TCOOH in our incubates. The direct protein precipitation method also resulted in a twofold dilution of the sample with acetonitrile, which hindered low level quantitation of metabolites (<20 ng/ml). To overcome the background and sensitivity problems, we adopted a solvent extraction method that is a modification of a procedure described earlier by Raeissi et al. [15]. The original procedure used an excess amount of NaCl (salt) to promote extraction of terfenadine metabolites into the organic solvent at basic pH, most likely because of the high protein concentration (>1 mg/ml) used in Raeissi's microsomal incubation. At the relatively low protein concentrations used in our protocol (<0.1 mg/ml), extraction recovery was excellent without resorting to salting of the aqueous phase. Also, elimination of salting avoided the extraction of basic and neutral endogenous substances, which cause build up of contaminants on the column and the LC–MS interface.

The recovery from extraction by methylene chloride was calculated by comparing peak heights of extracted standards with those of direct injections of reference solutions containing analytes at amounts that are expected for complete recovery. The recovery was estimated to be 92–100% for AZ, 78–

100% for TOH, and 88–100% for T over the range of calibration standard concentrations ($n=6$). The internal standard recovery was assessed at 90–100%.

3.2. Chromatography and mass spectrometry

Fig. 2 shows the mass spectra obtained after positive electrospray ionization of internal standard

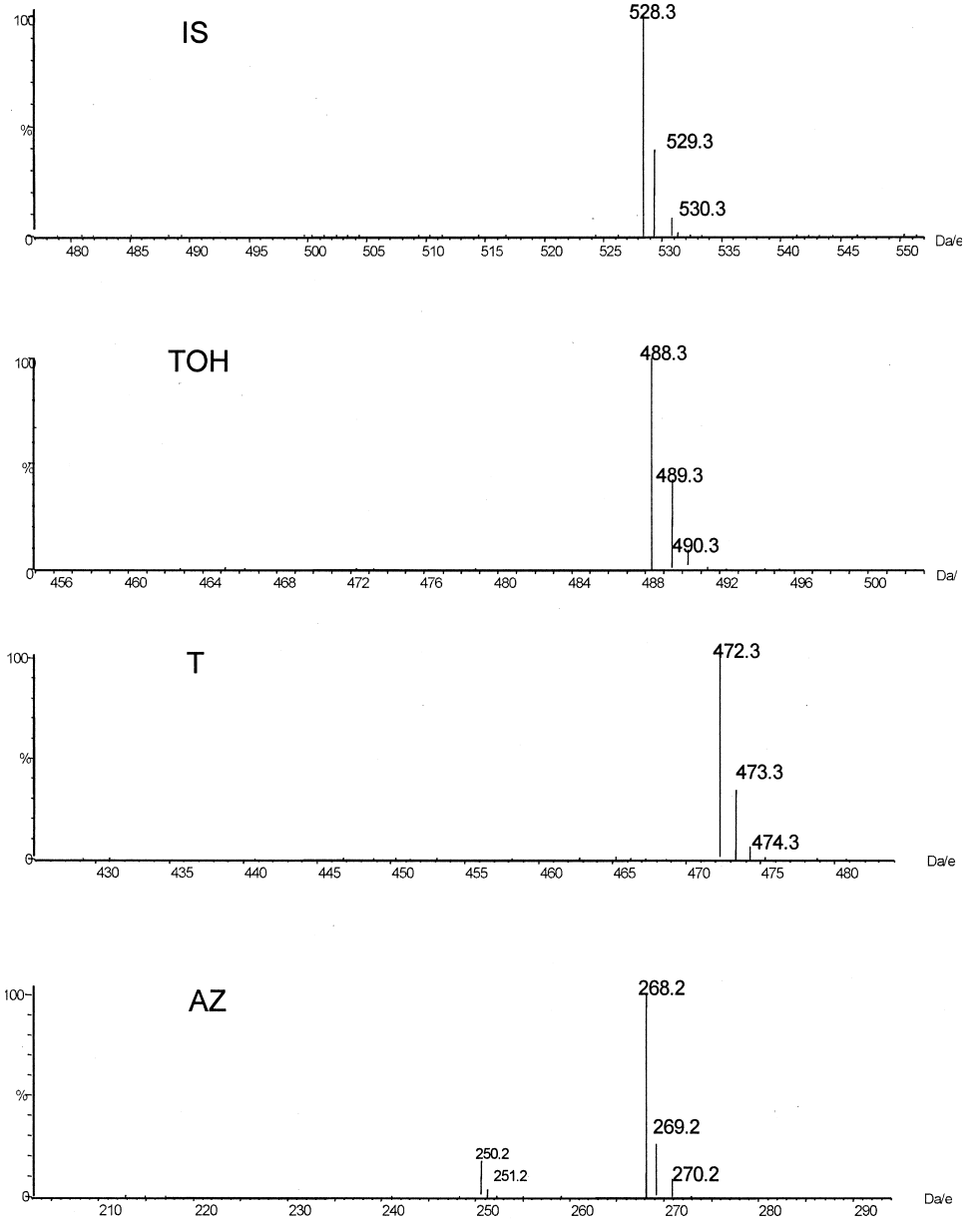

Fig. 2. Mass spectra of internal standard and authentic TOH, T and AZ obtained by positive electrospray ionization at a probe voltage of +3.8 kV.

and the analytes, TOH, T, and AZ. The cone voltage was optimized to achieve the highest signal sensitivity without fragmentation of $[M+H]^+$, which occurred by the loss of water molecules. The $[M+H]^+$ of internal standard, TOH and T did not fragment until the cone voltage was raised beyond 30 V. However, AZ was more readily fragmented, losing water molecules at 30 V cone voltage. Hence, a lower voltage of 25 V was set for AZ, which provided sufficient sensitivity.

Fig. 3 shows representative SIM chromatograms of extracts for 5 ng/ml standards of the metabolites, 25 ng/ml standard of terfenadine, and 1.6 μg/ml of internal standard. Retention times were 4.56, 5.95, 8.25 and 8.96 min for AZ, TOH, T, and internal standard, respectively. Figs. 4 and 5 show examples of selected-ion chromatograms for extracts of blank (drug metabolite-free) human liver microsomes and microsomal incubates at 3 μ*M* of terfenadine, respectively. As can be seen in Fig. 4, no ion interference was observed at retention times corresponding to those of the analytes in either incubated or unincubated human liver and intestinal microsomes. Incubations with terfenadine often yielded a significant background peak in the *m*/*z* 488 channel, which appeared at a retention time of 7.4 min, well resolved from the TOH ion peak occurring at 6.0 min. A small background peak was also found to precede AZ in some incubates of human liver microsomes; this peak became interfering only below the limit of quantitation (<5 ng/ml).

3.3. Assay performance

The limit of detection was 1–2 ng/ml of incubate for both the metabolites and terfenadine. The limit of quantitation for all three analytes was around 3–5 ng/ml. The calibration curves consisting of six standard points were constructed by plotting the peak-height ratios of terfenadine or metabolites over the internal standard against concentration. The resulting calibration curves were linear over the range of 5–250 ng/ml for the metabolites (AZ and TOH), and 12.5–1250 ng/ml for terfenadine per milliliter of sample. The correlation coefficients were consistently greater than 0.998. Signal-to-noise ratio at the limit of quantitation varied from 3 to 5 depending on the condition of the mass spectrometer. For the initial validation of assay performance, ten replicate quality control samples at low and high

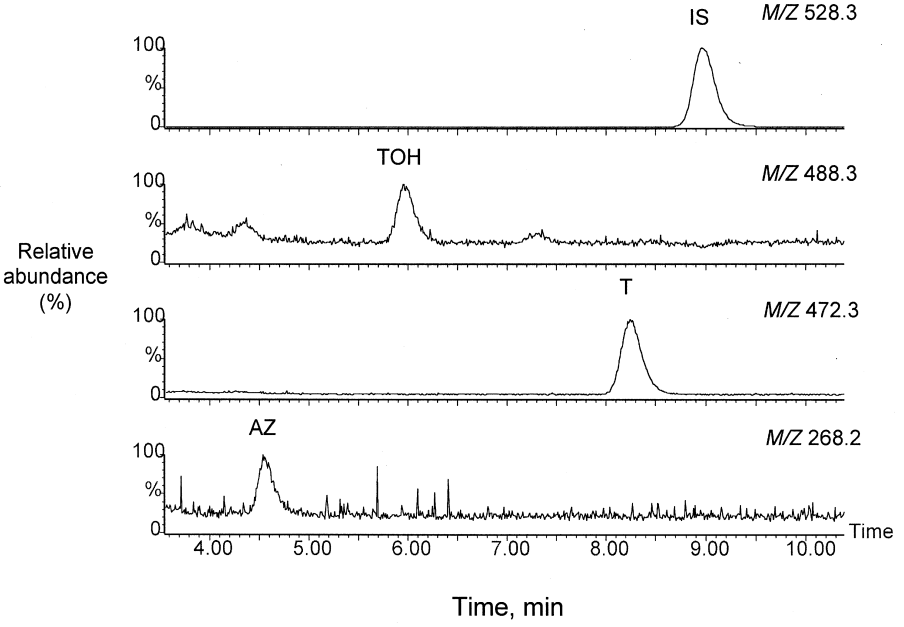

Fig. 3. Examples of selected-ion chromatograms of extracts of calibration standards, which contained TOH (5 ng), T (12.5 ng) and AZ (5 ng), along with internal standard (1.6 μg).

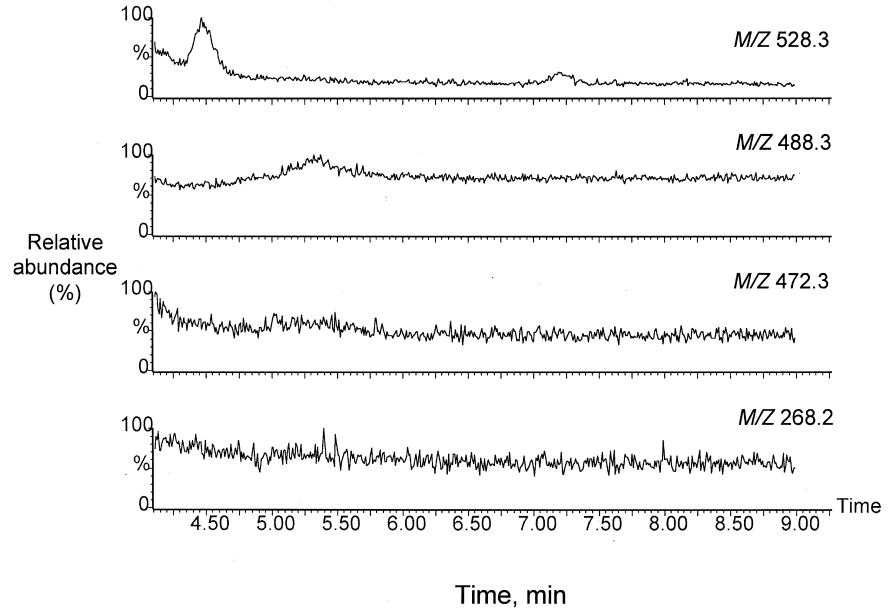

Fig. 4. Representative selected-ion chromatograms of an extract of unincubated, blank (drug metabolite free) human liver microsomes. Incubated microsomes showed similar background chromatograms.

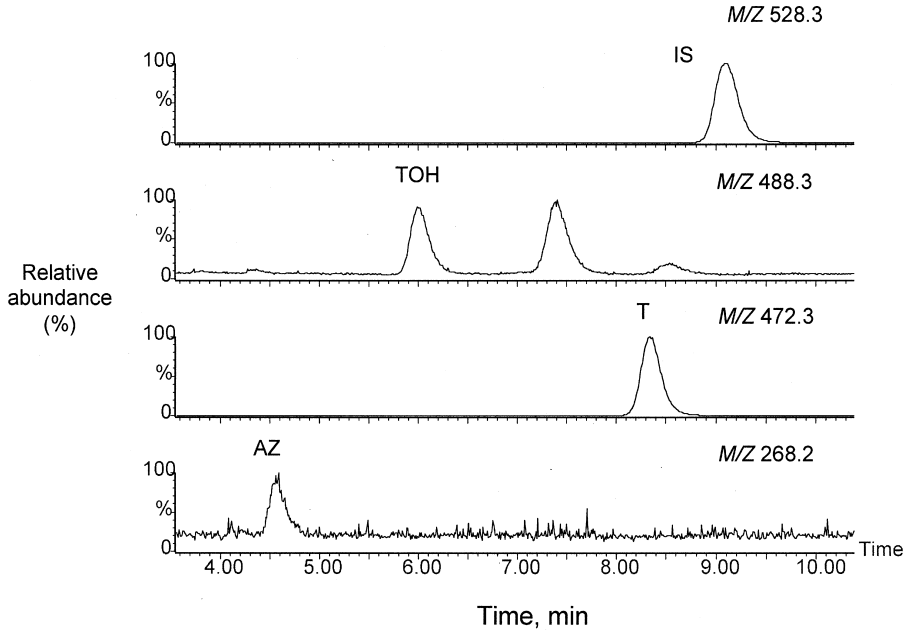

Fig. 5. Selected-ion chromatograms of a typical extract of a human liver microsomal incubate (HL-150) at a terfenadine concentration of 3 μM.

analyte levels were assayed within a single run. The bi-level set of quality control samples were included with incubate samples during each routine assay run. Assay precision and accuracy as indicated by the quality control data are summarized in Table 1. Comparable coefficients of variation were obtained for the intra- and inter-day analyses that ranged from 3 to 9%. Also, averages of the quality control data were well within ±10% of the nominal values.

3.4. Application

Fig. 6 shows initial rate versus substrate concentration profiles for the formation of TOH and AZ from terfenadine in microsomes prepared from a representative human liver (HL-148) and intestinal mucosa (HI-21). Kinetic modeling of TOH and AZ data yielded respective K_M estimates of 0.4 and 0.7 μ*M* for HL-148. The corresponding values for HI-21 were 0.2 and 0.9 μ*M*. Previous K_M literature reports for TOH ranged from 1.8 μ*M* [15] to 60 μ*M* [17]. Our recent work revealed that the apparently high K_M values obtained by the earlier investigators can be attributed to inappropriate incubation conditions (i.e. insufficiently low substrate concentrations, substrate depletion, and secondary metabolism); which were dictated by the limited sensitivity of their terfenadine metabolite assays. These examples illustrate the need to perform kinetic studies at terfenadine concentrations less than 3 μ*M*, which had not been feasible using previously reported assay procedures.

Table 1
Precision and accuracy for quality control samples

	AZ	TOH	T
	50 ng	50 ng	250 ng
Intra-day variability (n=10)			
Mean (ng)	61.6	52.2	240.4
SD[a]	3.9	2.3	8.0
C.V. (%)[b]	6.3	4.4	3.3
Inter-day variability (n=20)			
Mean (ng)	62.1	53.2	250.1
SD	3.8	2.2	10.4
C.V. (%)	6.4	4.2	4.2
	10 ng	10 ng	50 ng
Inter-day variability (n=16)			
Mean (ng)	9.5	10.0	49.4
SD	0.8	0.6	3.3
C.V. (%)	8.6	6.2	6.6
	5 ng	5 ng	25 ng
Inter-day variability (n=16)			
Mean (ng)	5.0	4.7	22.6
SD	0.4	0.6	1.8
C.V. (%)	8.8	13.4	8.0

[a] SD, standard of deviation.
[b] CV, coefficient of variation.

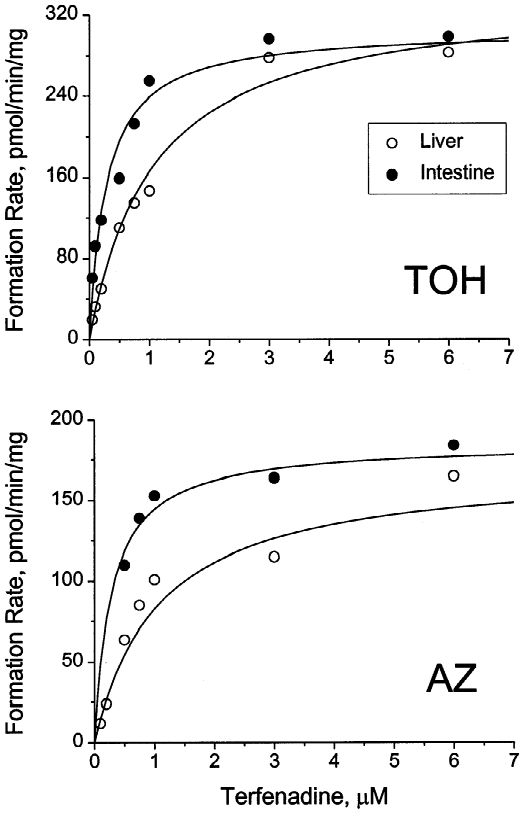

Fig. 6. A plot of the formation rates of TOH (upper panel) and AZ (lower panel) versus terfenadine concentrations in representative human liver (HL-148) and intestinal (HI-21) microsomes.

4. Conclusions

The present method for the analysis of terfenadine metabolites in microsomal incubates involves a rapid, single step solvent extraction procedure, followed by liquid chromatography coupled to positive-ion, electrospray ionization–mass spectrometry. The

assay was shown to have the requisite sensitivity for characterizing the high affinity or low K_M kinetics of CYP3A4-mediated terfenadine oxidation in human liver and intestinal microsomes. More than 400 microsomal samples have been analyzed with this LC–MS assay with proven reproducibility and reliability.

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