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Journal of Chromatography B, 693 (1997) 345–351

JOURNAL OF CHROMATOGRAPHY B

# Analysis of terfenadine in human plasma using microbore high-performance liquid chromatography–electrospray ionisation mass spectrometry

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Received 30 September 1996; revised 7 January 1997; accepted 21 January 1997

**Abstract**

An assay based on combined microbore high-performance liquid chromatography–positive ion electrospray ionisation mass spectrometry with selected ion recording has been developed for the measurement of the antihistamine drug terfenadine in human plasma. A deuterated analogue of terfenadine was synthesised for use as an internal standard and extraction of terfenadine was carried out on $C_{18}$ solid phase extraction columns. The limit of detection of terfenadine in plasma is 0.1 ng/ml and the intra-assay coefficient of variation at 1 ng/ml is 10.1%. Plasma concentrations of terfenadine measured in six normal subjects following a 120 mg oral dose are reported.

*Keywords:* Terfenadine

## 1. Introduction

Terfenadine is a selective $H_1$-receptor antagonist administered orally for the symptomatic relief of allergic rhinitis and various skin allergies. Many antihistamine drugs cause sedation and have anticholinergic effects [1,2] but terfenadine has the advantage of being essentially free of these undesirable side effects [3]. The compound undergoes extensive pre-systemic metabolism by cytochrome P450-3A4 (CYP3A4) in the gut wall and liver to give initially azacyclonol and primary alcohol metabolites. The alcohol is then oxidised to a carboxylic acid metabolite which appears to exert most of the pharmacological actions associated with administration of the parent drug [4]. As more than 99% of terfenadine undergoes pre-systemic biotransformation, peak plasma levels of the drug following therapeutic doses of 60 mg once or twice daily are typically below 5 ng/ml [5].

Plasma concentrations of terfenadine are of clinical interest because the parent drug is cardiotoxic and has been associated with ventricular arrhythmias such as *torsades de pointes*. Incidents of cardiotoxicity have been described in patients suffering from overdosage [6], or where metabolism of terfenadine is inhibited either by liver disease [7,8] or by the concommitant administration of other drugs that inhibit CYP3A4 activity [9,10]. As part of our studies into compounds occurring in the human diet that may be potential inhibitors of CYP3A4, a sensitive assay for terfenadine in plasma was re-

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*PII* S0378-4347(97)00083-2

quired. A radioimmunoassay for the drug in plasma with a detection limit of 0.25 ng/ml has been reported [11] but the antiserum used is not commercially available. An assay based on high-performance liquid chromatography (HPLC) with fluorescence detection has also been published [12] but this assay has a limit of detection of 10 ng/ml, which is not sensitive enough for terfenadine measurement after therapeutic doses of the drug. We have therefore developed a more sensitive assay for terfenadine in human plasma based on microbore HPLC positive ion electrospray ionisation (ESI) mass spectrometry (MS). A deuterated analogue of terfenadine was synthesised for use as an internal standard in the assay.

## 2. Experimental

### 2.1. Materials

Terfenadine was purchased from Sigma (Poole, UK) while [$^2H_3$]terfenadine was synthesised as described below. All chemicals and deuterated reagents used in the synthesis were obtained from Aldrich (Gillingham, UK). Stock solutions of terfenadine and [$^2H_3$]terfenadine in methanol (100 and 400 ng/ml, respectively) were prepared and stored at −20°C until required. Bond Elut$^R$ $C_{18}$ disposable solid phase extraction columns (3 ml/200 mg size, Varian) were supplied by Anachem (Luton, UK). Methanol used in the extraction procedure was of Analar$^R$ grade (BDH-Merck, Lutterworth, UK). Deionised water used in the extraction procedure and for the preparation of the HPLC mobile phase was generated by a Milli-Q water purification system (Millipore, Watford, UK) and had a resistivity of 18.2 MΩ.cm. Acetonitrile, methanol and ammonium acetate required for the HPLC mobile phase were obtained from Fisher Scientific (Loughborough, UK) and were of HPLC grade.

### 2.2. Synthesis of [$^2H_3$]terfenadine

A mixture of terfenadine (250 mg), pyridinium dichromate (200 mg) and acetone (50 ml) was stirred for 31 h at room temperature, after which more pyridinium dichromate (100 mg) and acetone (25 ml) were added and the reaction mixture stirred for a further 17 h. Acetone was then removed by rotary evaporation and 0.7 *M* sodium carbonate (150 ml) was added to the residue. The aqueous mixture was extracted with chloroform (3×100 ml) and the organic extracts combined, washed with water and then dried over anhydrous sodium sulphate. After filtration and removal of the solvent, oxidised terfenadine (1-(4-*tert.*-butylphenyl)-4-[4-(α-hydroxybenzhydryl)piperidino]butan-1-one) was obtained as a yellow–orange oil. Oxidised terfenadine (10 mg) was dissolved in a mixture of hexadeuteroacetone (15 ml) and deuterium oxide (3 ml). Sodium deuteroxide in deuterium oxide (30% solution, 14 μl) was added and the mixture allowed to stand at room temperature. Deuterium incorporation to produce 1-(4-*tert.*-butylphenyl)-2,2-($^2H_2$)-4-[4-(α-hydroxybenzhydryl)piperidino]butan-1-one was complete after 24 h. The reaction mixture was taken to dryness by rotary evaporation followed by lyophilisation. Half of the residue (5 mg) was redissolved in tetradeuteromethanol (2 ml) and treated with sodium borodeuteride (4 mg) in deuterium oxide (1.4 ml). Reduction to [$^2H_3$]terfenadine{(*RS*)-1-(4-*tert.*-butylphenyl)-1,2,2-($^2H_3$)-4-[4-(α-hydroxybenzhydryl)piperidino]butan-1-ol} was complete after 2 h. Water (10 ml) was added to the reaction mixture and the product extracted with chloroform (3×20 ml). The organic extracts were combined, washed with water and then dried over anhydrous sodium sulphate. After filtration and removal of the solvent, the reaction product (1.5 mg) was redissolved in chloroform (3 ml) and stored at −20°C. The identities of synthetic intermediates and [$^2H_3$]terfenadine were confirmed by positive ion fast atom bombardment (FAB) MS (Fig. 1) using a Finnigan 4500 quadrupole mass spectrometer with glycerol as sample matrix and xenon (10 kV) as the primary ionising species.

### 2.3. Extraction procedure

[$^2H_3$]Terfenadine (20 ng in 50 μl methanol) was added to every plasma sample (2 ml) which was thoroughly mixed by manual inversion. The samples were then applied to Bond Elut $C_{18}$ solid phase

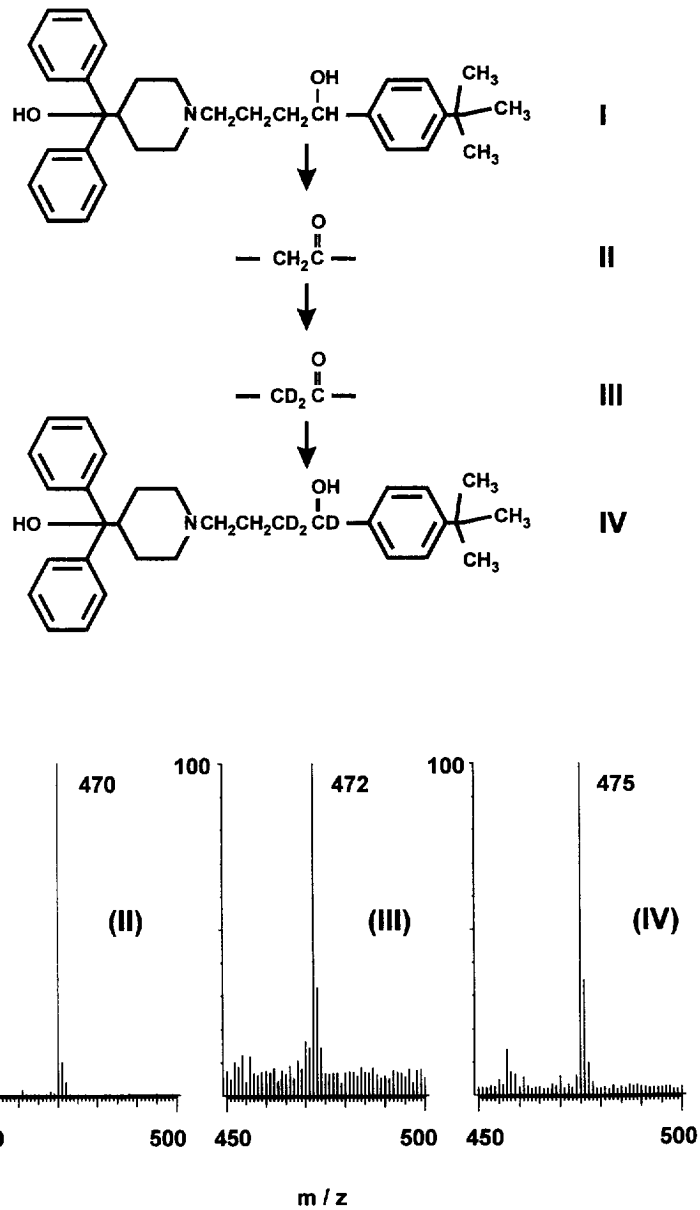


Fig. 1. Synthesis of [$^2H_3$]terfenadine (IV) from terfenadine (I). Deuterium atoms ($^2H$) are shown as D. Fast atom bombardment mass spectrometry was used to characterise the intermediates II ($MH^+$ at $m/z$ 470) and III ($MH^+$ at $m/z$ 472) and the final product IV ($MH^+$ at $m/z$ 475).

extraction columns which had been preconditioned sequentially with methanol (2×2 ml) and deionised water (2×2 ml). The $C_{18}$ columns were washed with deionised water (2×2 ml) and methanol–water (50:50, v/v, 2 ml) and these washings discarded. Terfenadine and deuterated terfenadine were then eluted from the columns with methanol (2×2.5 ml). The eluates were collected in glass vials (10 ml capacity) and evaporated to dryness under nitrogen. The residue in each vial was transferred to a smaller glass vial (1.8 ml capacity) with methanol (2×0.75 ml) and these extracts stored at −20°C. Immediately

prior to analysis, the extracts were brought to room temperature, methanol removed by evaporation under nitrogen and the residues reconstituted in 15 μl acetonitrile–methanol–100 m*M* ammonium acetate buffer pH 4 (20:20:60, v/v/v). A 10 μl aliquot of each sample was injected onto the HPLC column.

### 2.4. Microbore HPLC–electrospray ionisation mass spectrometry

An HPLC system consisting of a Waters 616 liquid chromatography pump and 600S flow controller (Waters, Watford, UK), and a Rheodyne Model 8125 injector (Rheodyne, Cotati, CA, USA) fitted with a 20 μl sample loop was used. Chromatographic separation was carried out on a Spherisorb S5CN column, 25 cm×1 mm I.D., (Hichrom, Theale, UK) fitted with a Phenomenex (Macclesfield, UK) cyano guard cartridge (3 cm×1 mm I.D.) under isocratic conditions, using a mobile phase of acetonitrile–methanol–100 m*M* ammonium acetate buffer pH 4 (30:30:40, v/v/v). At a flow-rate of 50 μl/min, the retention times of terfenadine and [$^2H_3$]terfenadine were 7 min. The HPLC column was connected directly to the ESI interface of a VG Quattro II triple quadrupole mass spectrometer (VG Biotech, Altrincham, UK). This instrument, supplied with nitrogen as both drying gas and nebulising gas, was operated in the positive ion ESI mode with a source temperature of 70°C, a cone voltage of 35–40 V and a capillary voltage of 3.2 kV. For selected ion recording (SIR) work, the mass spectrometer was set, with Q1 acting as a mass filter at unit resolution, to monitor ions *m/z* 472.3±0.05 and *m/z* 475.3±0.05, the protonated molecular ions of terfenadine and [$^2H_3$]terfenadine, respectively (Fig. 2). The dwell time per ion was 0.5 s with a 0.02 s interchannel delay and peak area ratios were determined. Selected reaction monitoring (SRM) of the parent-product ion pairs *m/z* 472→57, 475→57, 472→436 and 475→438 was undertaken with a span of ±0.15 u and a dwell times of 0.08 s for each ion. Argon was used as collision gas at a pressure of $9\times10^{-4}$ mbar and collision energy was set at 70 eV (*m/z* 472→57 and 475→57) and 40 eV (*m/z* 472→436 and 475→438). Peak area ratios were determined. Data acquisition and processing were performed with Masslynx software.

### 2.5. Standard curve

Nine standards in half-dram glass vials were prepared from stock solutions of terfenadine (100 ng/ml methanol) and [$^2H_3$]terfenadine (400 ng/ml methanol). The standards all contained [$^2H_3$]terfenadine (20 ng) as well as terfenadine (0, 0.2, 0.5, 1, 2, 5, 10, 20 and 30 ng). After evaporation to dryness under nitrogen, standards were reconsti-

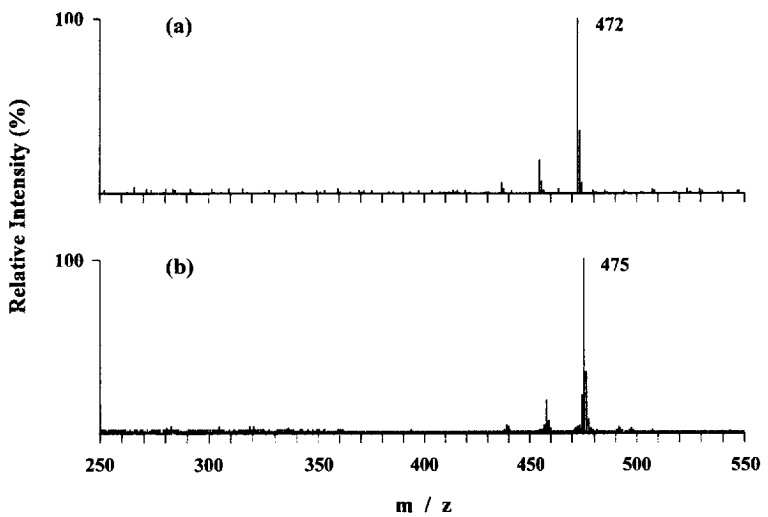


Fig. 2. Positive ion electrospray ionisation mass spectra of (a) terfenadine and (b) [$^2H_3$]terfenadine.

tuted in 30 μl acetonitrile–methanol–100 m*M* ammonium acetate buffer pH 4 (20:20:60, v/v/v). Ten microliter aliquots of standards were injected onto the HPLC column.

### 2.6. Plasma samples

Six healthy male volunteers, having fasted from the previous evening, received 120 mg terfenadine (taken orally as two 60 mg tablets). Blood samples (10 ml) were drawn via an indwelling venous catheter into lithium heparin tubes prior to dosing and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5.5, 7 and 24 h post-drug. Plasma was separated from blood cells by centrifugation (1000*g* at 4°C) and stored for analysis at −20°C.

## 3. Results and discussion

Mass spectrometry, particularly gas chromatography–mass spectrometry (GC–MS), has been widely used for the quantitative analysis of drugs and their metabolites in biological fluids. Despite the initial high cost of instrumentation, analytical methodology using mass spectrometric detection has the advantages of high specificity and sensitivity. In initial studies, we found that terfenadine was not amenable to GC–MS analysis because of the poor yield in converting it to a suitable volatile derivative. Whilst the secondary hydroxyl group could be satisfactorily converted to, for example, a trimethylsilyl or bis-trifluoromethylbenzoyl derivative, the tertiary hydroxyl group was much less reactive, with decomposition occurring under forcing conditions. Partial derivatisation of terfenadine gave a product with poor chromatographic behaviour which was unsuitable for use in a quantitative assay (data not shown).

The ESI and atmospheric pressure chemical ionisation interfaces used in conjunction with an atmospheric pressure ion source for combined HPLC–MS have provided an important alternative technology for compounds not amenable to analysis by GC–MS. These soft ionisation techniques can give high sensitivity and often produce molecular ions that undergo little fragmentation, an ideal situation for quantitative analysis by SIR. Using a cyano microbore HPLC column (1 mm I.D.) and a solvent flow-rate of 50 μl/min, terfenadine eluted with a retention time of 7 min and generated a simple positive ion ESI mass spectrum (Fig. 2). The base peak at *m/z* 472 [$MH^+$] was accompanied by two less intense fragment ions at *m/z* 454 and 436, corresponding to the loss of one and two molecules of water, respectively. Sensitivity for the detection of terfenadine by microbore HPLC–ESI-MS was high. When ions of *m/z* 472 were monitored by SIR, terfenadine (20 pg) could be detected with a signal-to-noise ratio (*S/N*) of 5.

A normal requirement for a reliable and precise assay is the availability of a stable isotope labelled analogue of the analyte for use as an internal standard. A trideuterated analogue of terfenadine ([$^2H_3$]terfenadine) was therefore synthesised for this purpose from terfenadine in three steps (Fig. 1). The secondary alcohol in terfenadine was oxidised to a carbonyl group with pyridinium dichromate in acetone. Two deuterium atoms were then incorporated into the oxidised terfenadine by keto–enol tautomerism of the carbonyl group under alkaline conditions. Finally, reduction of deuterated oxidised terfenadine with sodium borodeuteride gave a terfenadine analogue containing three deuterium atoms. The identities of the intermediates in and final product of the synthesis were confirmed by FAB-MS (Fig. 1). [$^2H_3$]Terfenadine coeluted with terfenadine on HPLC and generated an analogous positive ion ESI mass spectrum, with the protonated molecular ion shifted by 3 u to *m/z* 475 (Fig. 2). Standard mixtures containing [$^2H_3$]terfenadine (20 ng) and terfenadine (0–30 ng) were prepared and analysed by HPLC–ESI-MS with SIR of ions *m/z* 472 and 475. The unextracted standard curves obtained ($n=3$) were linear with a gradient of $0.0411 \pm 0.0004$, an intercept of $0.008 \pm 0.002$ (mean ± S.E.M.) and a correlation coefficient of $>0.999$. The low intercept of the standard curves is a result of the $<1\%$ of terfenadine in the deuterated internal standard.

A solid phase extraction procedure was developed for the isolation of terfenadine from plasma. Plasma to which [$^2H_3$]terfenadine had been added was passed through a $C_{18}$ column, which was then washed with water and methanol–water (50:50, v/v). Terfenadine and its deuterated analogue were then eluted from the column with methanol and the eluate

subjected to HPLC–ESI-MS with SIR. Absolute recovery through the extraction, assessed by comparison of internal standard peak areas in unextracted and extracted standards, was between 50–70% depending on the source of the plasma, which illustrates the requirement of this assay for a chemically similar internal standard added to each sample. An extracted standard curve was generated from blank plasma samples to which known amounts of terfenadine (0–30 ng/2 ml) had been added. A representative HPLC–ESI-MS trace for plasma containing terfenadine at a concentration of 0.1 ng/ml is shown in Fig. 3. Optimal peak shape is obtained with an injection volume of 10 μl, although up to 20 μl can be injected on the column without appreciable peak broadening. The extracted standard curve obtained ($y=0.0437x+0.005$, $r^2>0.999$) had a similar slope and intercept to the unextracted standard curve. In all blank plasma samples examined by us there was no direct interference in either the $m/z$ 472 or 475 ion channels and so unextracted standard curves could be used for the analysis of these samples. The intra-assay coefficient of variation was determined as 10.1% ($n=7$) at 1 ng terfenadine per ml plasma. While the limit of detection of terfenadine by SIR was 20 pg (0.02 ng) on column ($S/N$ of 5), the operational limit of detection for terfenadine in a 2 ml plasma sample was set at 0.2 ng, a level that could be clearly distinguished from the plasma blank (Fig. 3).

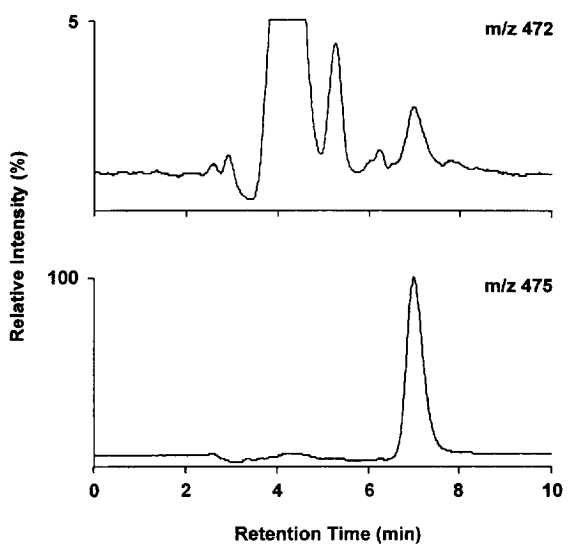


Fig. 3. Selected ion recording trace for the analysis of terfenadine in plasma (0.1 ng/ml).

The use of a triple quadrupole mass spectrometer for this work permitted the detection of product ions of terfenadine and [$^2H_3$]terfenadine produced by collision induced dissociation (CID). With argon as collision gas, terfenadine fragments to give ions $m/z$ 454 ($MH^+-H_2O$), 436 ($MH^+-2H_2O$) and 57 ($C_4H_9^+$) while [$^2H_3$]terfenadine generates analogous ions $m/z$ 457 ($MH^+-H_2O$), 438 ($MH^+-H_2O-{}^2HOH$) and 57 ($C_4H_9^+$). The optimal collision energies for production of ions of $m/z$ 57 and 436/438 were found to be 70 eV and 40 eV, respectively and, when the mass spectrometer was operated in the SRM mode for either of these ion transitions, terfenadine in plasma extracts could be measured. However, the benefit of reduction in background noise obtained with SRM was offset by an even greater reduction in signal strength of the ions monitored so that the sensitivity of the assay was limited to 0.5 ng/ml. As chromatographic interference has not proved a problem when using SIR for the measurement of terfenadine in plasma, the use of SRM did not offer us any advantages. There have been two recent, detailed reports of HPLC–MS assays for terfenadine, each of which uses SRM [13,14]. The limits of detection quoted by these authors are similar to those reported here for our SIR based method, and five to ten fold better than we obtained with SRM, suggesting that there may be differences in the MS–MS capability of different instruments.

Using the assay described above, plasma concentrations of terfenadine were measured in six subjects following a 120 mg oral dose of the drug (Fig. 4). Although peak levels were below 4.5 ng/ml (and hence less than the limits of detection of HPLC-fluorescence assays), they were well above the minimum level of detection of this HPLC–MS assay and the drug could readily be monitored up to 24 h after administration. This assay should therefore prove suitable for use in future clinical studies on the pharmacokinetics of terfenadine metabolism at therapeutic doses of the drug.

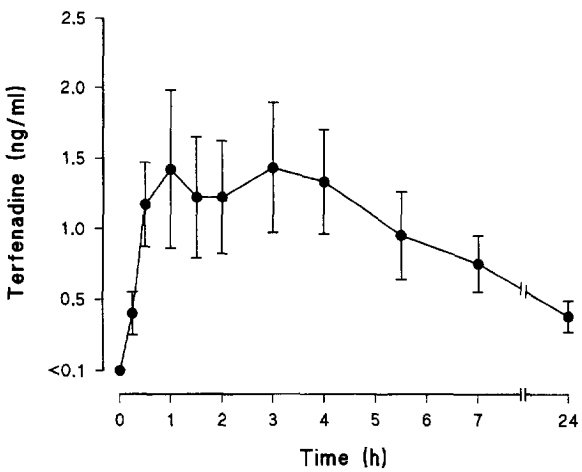


Fig. 4. Plasma concentrations of terfenadine in six subjects following a 120 mg oral dose (mean±S.E.M.).

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