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High-performance liquid chromatographic-tandem mass spectrometric method for the determination of clemastine in human plasma

Viola Horváth[a,*], Antal Tolokán[b], Andrea Egresi[c], Tímea Pap[c], George Horvai[c], Katalin Balogh-Nemes[d], Imre Klebovich[e]

[a] *Research Group of Technical Analytical Chemistry, Hungarian Academy of Sciences—Budapest University of Technology and Economics, Gellért tér 4., Budapest H-1111, Hungary*
[b] *Dr. E. Wessling Chemical Laboratory Ltd., Fóti út 56., Budapest H-1047, Hungary*
[c] *Department of Chemical Information Technology, Budapest University of Technology and Economics, Gellért tér 4., Budapest H-1111, Hungary*
[d] *Department of Pharmacokinetics, EGIS Pharmaceuticals Ltd., Keresztúri út 30.–38., Budapest H-1106, Hungary*
[e] *Department of Pharmaceutics, Semmelweis University, Hőgyes Endre utca 7., Budapest H-1092, Hungary*

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Abstract

A highly sensitive high-performance liquid chromatographic-tandem mass spectrometric method (HPLC–MS–MS) has been developed to quantitate clemastine in human plasma for the purpose of pharmacokinetic studies. Sample preparation was carried out by liquid–liquid extraction using deuterated clemastine as an internal standard. Chromatographic separation used a C18 reversed phase polymer column giving an extremely fast total run time of 2 min. The method was validated and used for the bioequivalence study of clemastine tablets in healthy male volunteers ($n = 28$). The lower limit of detection proved to be 0.01 ng/ml for clemastine.
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1. Introduction

Clemastine hydrogen fumarate (Fig. 1a) is an antihistaminic drug used to relieve the symptoms of allergic conditions such as urticaria and allergic rhinitis.

Clemastine competes with histamine for the H_1 receptor binding sites. Being an ethanolamine derivative it also possesses anticholinergic activity, resulting in a sedative effect. It is administered orally in small doses (2×1 mg up to 3×2 mg per day) which in turn leads to very low blood levels (C_{max} is around 0.6 ng/ml/1 mg dose) [1]. The lack of sufficiently sensitive analytical methods therefore hindered the extensive study of its pharmacokinetic properties for a long time.

The first clinical studies used radiolabeled drug. An early gas-chromatographic method oxidized clemastine to chlorobenzophenone prior to analysis of plasma samples [2]. The lowest concentration of clemastine that could be measured was 1 ng/ml. The selectivity of the method is questionable since metabolites of the parent compound are oxidized to the same compound. A GC–MS method was developed later with a similar quantitation limit for the determination of clemastine in human plasma after dermatological application of the drug [3]. A recent gas chromatographic method with nitrogen–phosphorous detection was validated having a lower quantitation limit of 0.1 ng/ml [4]. Radioimmunoassay has proven to be selective and sensitive enough to measure pharmacokinetics and bioavailability of clemastine in several studies with 0.1 ng/ml minimum measurable concentration [1].

Recently an HPLC–MS–MS method was presented that quantified clemastine from blood plasma along with 17 other antihistamine drugs [5]. The LOQ value of this method was 0.5 ng/ml for clemastine.

* Corresponding author. Tel.: +36 1 4631415.
E-mail address: vhorvath@mail.bme.hu (V. Horváth).

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The present paper for the first time describes a new HPLC–MS–MS method and its validation that can quantify clemastine between the 0.01 and 10 ng/ml concentration range. This concentration range corresponds to therapeutic levels and makes it possible to follow its elimination. Since clemastine is rapidly hydrolized at the ether bond in the presence of acid [6], a basic liquid–liquid extraction was applied to extract the drug from plasma. A deuterated internal standard was used throughout the measurements.

The method was used to measure the pharmacokinetics of the drug in a bioequivalence study.

2. Experimental

2.1. Reagents and chemicals

Ethylacetate, acetonitrile and methanol were super purity solvents purchased from Romil (Cambridge, England). Acetic acid and sodium hydroxide were supplied by Fluka (Buchs, Switzerland). *n*-Heptane (Merck, Darmstadt, Germany) was analytical grade.

Clemastine ((+)-2-{2-[1-(4-chlorophenyl)-1-phenylethoxy]-ethyl}-1-methylpyrrolidine hydrogen fumarate) was produced by EGIS Pharmaceuticals Ltd. (Budapest, Hungary), clemastine-d_3 hydrogen fumarate (Fig. 1b) was synthetized by the Institute of Chemistry, Chemical Research Centre, Hungarian Academy of Sciences (Budapest, Hungary).

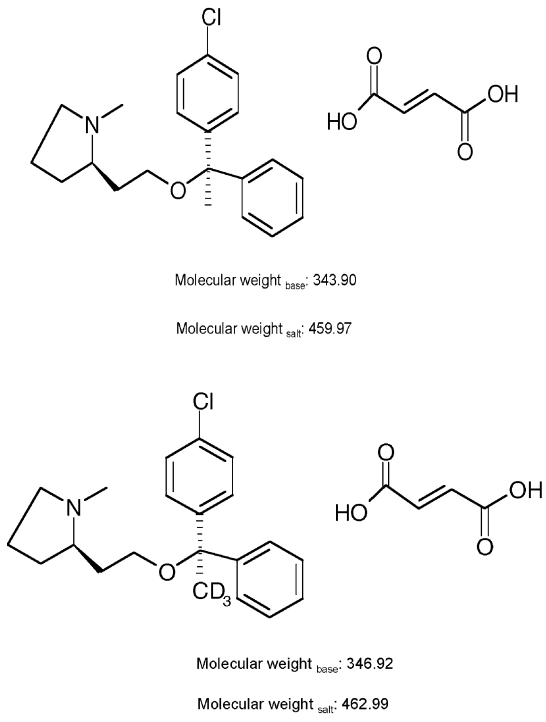

Fig. 1. Structure of clemastine hydrogen fumarate (a) and clemastine-d_3 hydrogen fumarate (b).

2.2. Instrumental

2.2.1. Chromatography

Separation was carried out on a Perkin Elmer Series 200 HPLC system (Perkin Elmer Instruments, Norwalk, CT, USA). The chromatographic column used to separate the components of pre-treated plasma samples was an Asahipak ODP 50 polymer phase column (125 mm × 4 mm i.d., particle size 5 μm) (Hewlett Packard, Palo Alto, CA, USA) connected to a Purospher RP-18e pre-column (4 mm × 4 mm i.d., particle size 5 μm) (Merck, Darmstadt, Germany). The mobile phase contained 50% acetonitrile and 50% water each component having 0.5% acetic acid in it. The eluent was run through the system with a flow rate of 1 ml/min. A 50-μl aliquot of extracted plasma samples was injected into the chromatograph.

2.2.2. Mass spectrometry

MS–MS measurements were run on a Perkin Elmer/MDS Sciex API 365 instrument (MDS Sciex, Toronto, ON, Canada). Mass spectra of clemastine and clemastine-d_3 are shown in Fig. 2a and b. It is evident from Fig. 2b that the deuterated internal standard contains minor amounts of clemastine-d_1 and clemastine-d_2 impurities too. The product ion spectra of clemastine and the deuterated internal standard can be seen in Fig. 3. Clemastine hydrogen fumarate and its deuterated derivative, clemastine-d_3 hydrogen fumarate were detected in the multiple reaction monitoring mode scanning the following transitions with 200 ms dwell time and 5 ms pause time with unit mass resolution: m/z 344.1 → 215.2 amu for clemastine and m/z 349.0 → 220.2 amu for the internal standard. For the deuterated internal standard, the M + 3 peak (m/z 349.0) was chosen as the precursor ion since at M + 1 (m/z 347.0), clemastine exhibits a relatively large peak due to the contribution of naturally occurring ^{37}Cl and ^{13}C isotopes (see insert in Fig. 2a). At M + 3 (m/z 349.0), clemastine-d_3 has a peak about one-third the intensity of the M + 1 peak due to the contribution of the naturally occurring ^{37}Cl isotope, which is still intensive enough to use it as an internal standard (see insert in Fig. 2b). TurboIonSpray (electrospray) ion source was used in the positive ionization mode, heated to 450 °C, its voltage set to 4500 V. The flow rate of the N_2 nebulizing gas was 12 units; that of the N_2 heater gas was 7 l/min; and 10 and 3 units for the N_2 curtain gas and collision gas, respectively.

2.3. Preparation and dilution of stock solutions

Separate stock solutions and diluted solutions were prepared for calibration and quality control samples in the following way. Methanolic stock solutions of clemastine hydrogen fumarate (1 mg/ml) were diluted with methanol to yield 1 μg/ml solutions. One of the 1 μg/ml solutions was further diluted with methanol to 0.2, 1, 2, 10, 20 and 100 ng/ml to give spiking standard solutions for calibration plasma samples. The other was diluted in a similar way to 0.2, 1, 10

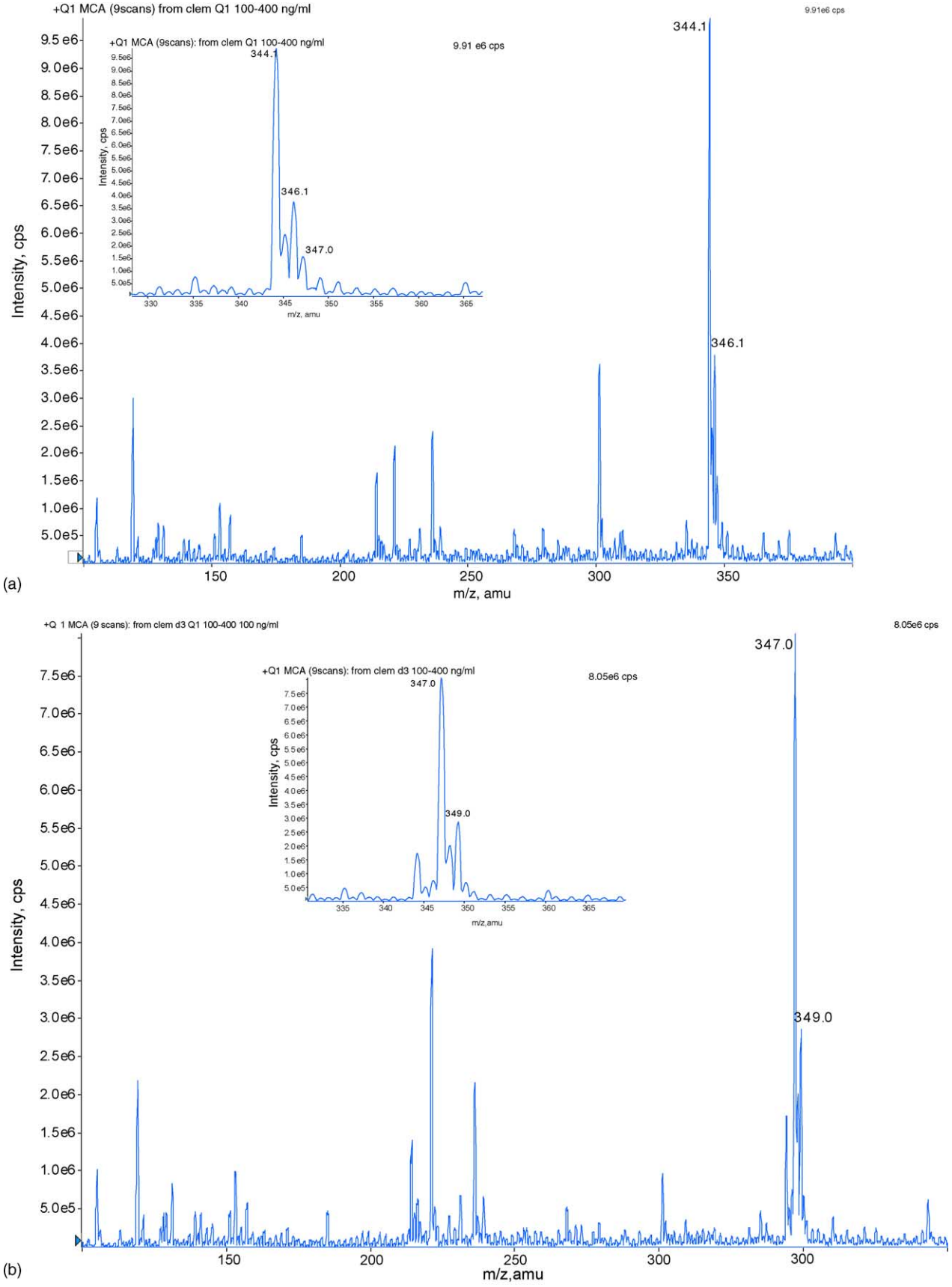

Fig. 2. Mass spectra of clemastine (a) and clemastine-d_3 (b). (Experimental conditions: concentration of infused analyte, 100 ng/ml; infusion speed, 10 μl/min; mass range, 100–400 amu; step size, 0.1 amu; dwell time, 10 ms; pause time, 2 ms; scan speed, 30 s; number of scans, 9.) A zoom on the protonated molecule ion is inserted into the mass spectrum in order to have a better view of the isotopic distribution.

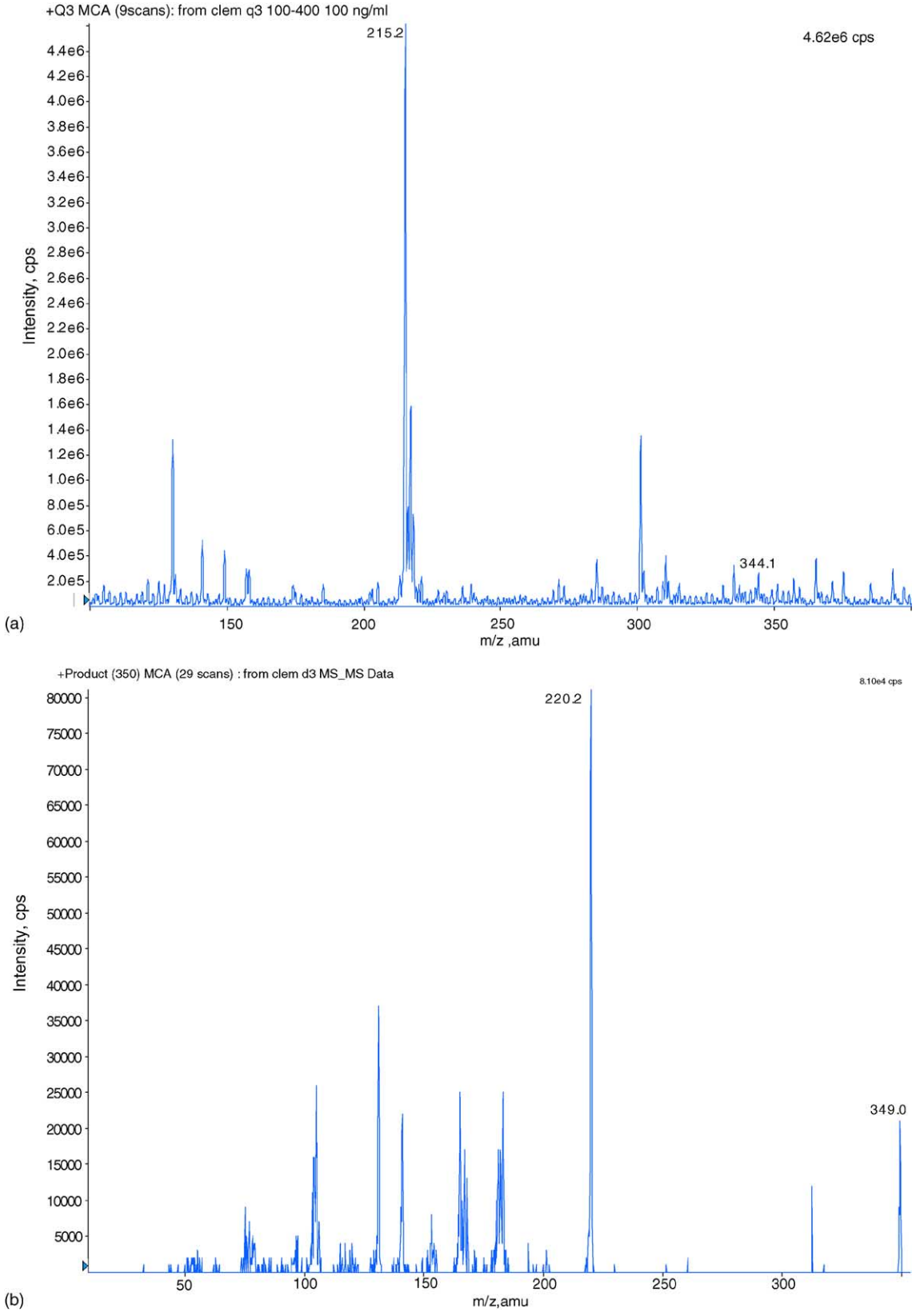

Fig. 3. Product ion spectra of clemastine (a, precursor ion: *m*/*z* 344.1) and clemastine-d_3 (b, precursor ion: *m*/*z* 349.0). (Experimental conditions: concentration of infused analyte, 100 ng/ml; infusion speed, 10 μl/min; mass range, 100–400 amu for clemastine, 10–354 amu for clemastine-d_3; step size, 0.1 amu for clemastine, 0.2 amu for clemastine-d_3; dwell time, 10 ms for clemastine, 1 ms for clemastine-d_3; pause time, 2 ms; scan speed, 30 s for clemastine, 1.72 s for clemastine-d_3; number of scans, 9 for clemastine, 29 for clemastine-d_3.)

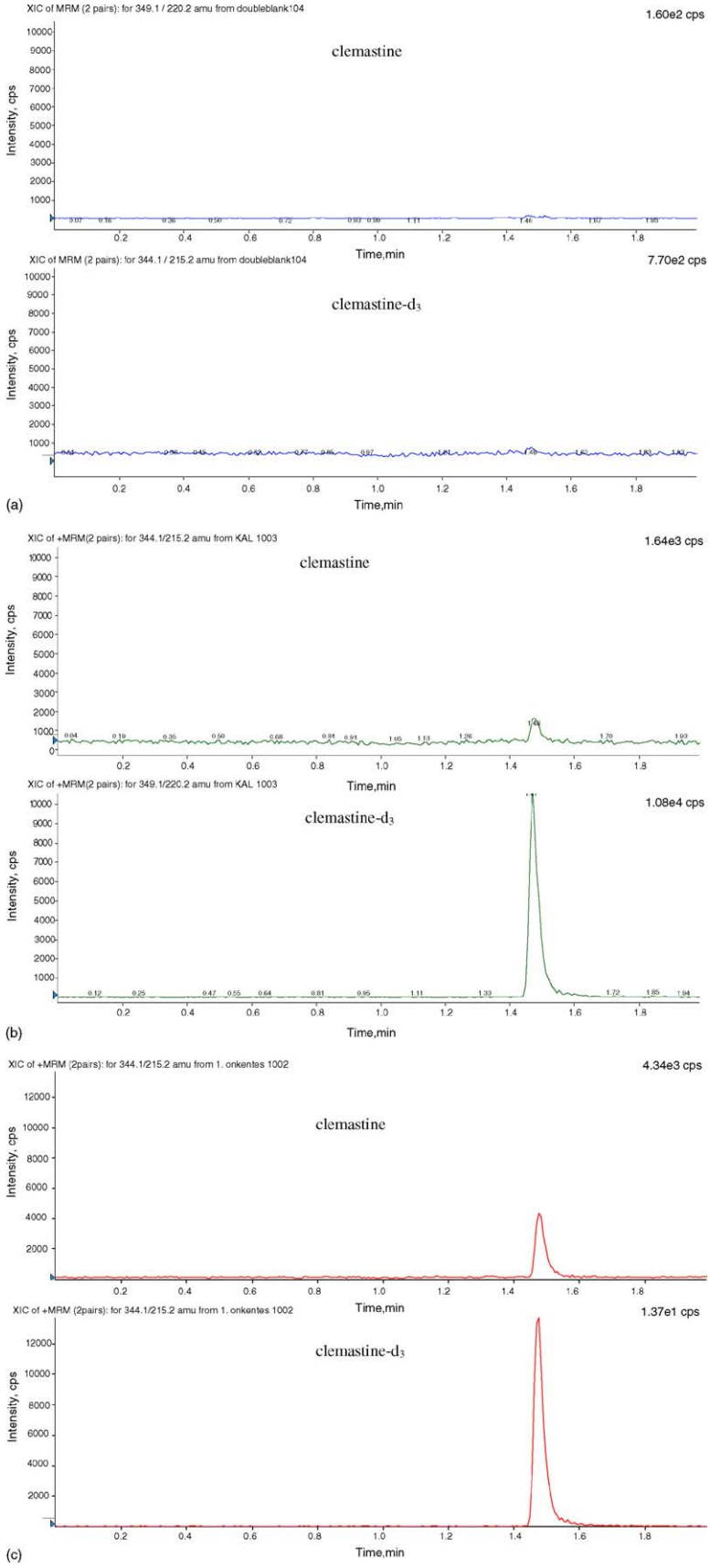

Fig. 4. (a) Chromatograms of blank plasma. (b) Chromatograms of a plasma standard containing 0.01 ng/ml clemastine and 0.2 ng/ml internal standard. (c) Chromatograms of a healthy volunteer's plasma sample 0.5 h after the oral administration of 3 × 1 mg clemastine containing tablet.

and 100 ng/ml to yield spiking standards for quality control samples.

A 1 mg/ml stock solution of clemastine-d_3 hydrogen fumarate was consecutively diluted to 4 ng/ml with methanol. This solution was used to spike plasma samples with internal standard at a concentration level of 0.2 ng/ml.

All solutions were stored at 4 °C.

2.4. Preparation of calibration and quality control samples

Calibration plasma samples were freshly prepared before each measurement by spiking 950 μl blank human plasma with 50 μl of the spiking standards to 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 ng/ml concentration.

Quality control samples (0.01, 0.05, 0.5 and 5 ng/ml) were prepared before the validation measurements in large batches and distributed in 1.1 ml aliquots into polypropylene tubes and stored at −20 °C.

2.5. Sample pre-treatment

A 1-ml aliquot of plasma was spiked with 50 μl 4 ng/ml internal standard solution and basified with 200 μl 1 M NaOH. The mixture was extracted with 3 ml 90% *n*-heptane:10% ethylacetate extraction solvent mixture for 30 min on an orbital shaker (Heidolph UNIMAX 2010, Heidolph Elektro GmbH, Keilheim, Germany). The organic and aqueous layer was separated by centrifuging at 3000 × *g* for 10 min at room temperature (Sigma 204, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). A 2.50-ml aliquot of the organic layer was pipetted into clean centrifuge tubes and evaporated to dryness at 50 °C in a TurboVap LV evaporator (Zymark Corporation, Hopkinton, USA) under N_2. The residue was dissolved in 40 μl acetonitrile by vortexing for 30 s. Afterwards 160 μl 0.5% acetic acid solution was added and vortexed for 15 s. A 50-μl aliquot of the sample solution was injected into the HPLC–MS–MS.

2.6. Evaluation of the results

Data acquisition and manipulation was done by the Masschrom Ver. 1.1 software (Perkin Elmer Sciex Instruments, Foster City, CA, USA) running on a Power Macintosh G3 computer. Calibration curves were constructed by plotting the peak area ratio of the analyte and internal standard versus their concentration ratio. Standard curves were fitted by the least squares method using $1/y^2$ weighting. Concentration of the samples (calibration, quality control and real samples) was back calculated from the daily calibration curve. Precision of a result is given as the R.S.D. of replicate measurements, accuracy is calculated as the percentage deviation of the result from the nominal value.

3. Results and discussion

3.1. Selectivity, linearity and lower limit of quantification (LLOQ)

Chromatograms of a blank plasma, a calibration standard and a healthy volunteer's plasma sample (obtained 0.5 h after the oral administration of three 1 mg clemastine containing tablets), respectively, are shown in Fig. 4a–c.

Calibration curves were prepared using seven standards in the 0.01–10 ng/ml range. The slope and intercept values of six replicate calibration curves taken on different days were averaged, giving the following equation: $y = 2.3373\ (\pm 0.0474)\ x + 0.1773\ (\pm 0.0296)$. The average regression coefficient was $r = 0.9990 \pm 0.0011$. The calibration could be extended up to 20 ng/ml by diluting the samples with blank plasma.

The LLOQ for clemastine was the lowest standard on the calibration curve, i.e. 0.01 ng/ml.

3.2. Intra-day repeatability

Precision and accuracy of five replicate quality control samples of 0.05, 0.5 and 5 ng/ml concentration are shown in Table 1. Intra-day precision values ranged from 2.68 to 7.54%, while accuracies were between −0.7 and 5.6%.

3.3. Inter-day reproducibility

Precision and accuracy values were determined on six different days by measuring three replicates of quality control samples at four concentration levels. The results are listed in Table 2. Even at the LLOQ the precision and accuracy values did not exceed 15%.

3.4. Recovery

Recovery of clemastine from plasma samples was studied at three concentration levels with five replicates comparing the detector response obtained from the analyte added to and extracted from the plasma to the detector response obtained

Table 1
Intra-day ($n = 5$) precision and accuracy of clemastine measured in human plasma by HPLC–MS–MS

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Precision (%)	Accuracy (%)	*n*
0.05	0.053	7.0	5.6	5
0.5	0.514	2.7	2.9	5
5	4.966	7.5	−0.7	5

Table 2
Inter-day ($n = 6$) precision and accuracy of clemastine measured in human plasma by HPLC–MS–MS

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Precision (%)	Accuracy (%)	n
0.01	0.010	14.1	0.0	10*
0.05	0.054	7.6	8.4	15*
0.5	0.556	3.9	11.2	18
5	5.245	3.9	4.9	18

* Outlier values were excluded.

for the pure standard. The mean recovery was found to be $62.6 \pm 7.4\%$.

3.5. Stability

Stability of clemastine containing samples were studied at three concentration levels (0.05, 0.5 and 5 ng/ml) measuring three replicates at each level. The samples were kept under different conditions, assayed and their concentration calculated from the daily calibration curve was compared to that of freshly spiked samples.

3.6. Extracted samples

Extracted and redissolved samples were stable for at least 8 h at room temperature. Extracted and dried samples were stable for 24 h at 4 °C.

3.7. Stability during repeated freeze–thaw cycles

Plasma samples were subjected to five freeze–thaw cycles. Even after this procedure no degradation of the clemastine was observed.

3.8. Long term stability

No significant degradation of clemastine has been observed after storing the samples at −20 °C for 98 days.

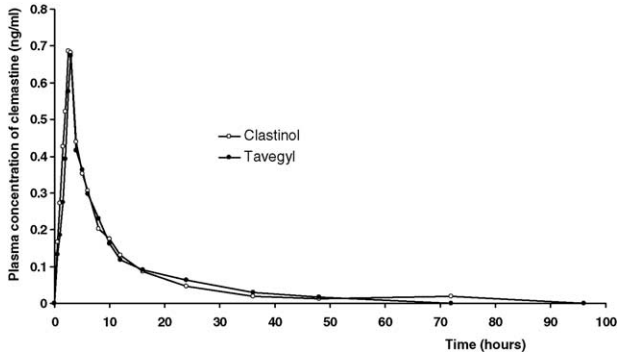

Fig. 5. Pharmacokinetic profiles of a volunteer after the administration of 3×1 mg clemastine (Tavegyl® or Clastinol® tablets).

3.9. Pharmacokinetic study

A bioequivalence study of clemastine has been carried out on male non-smoking volunteers. The pharmacokinetic profiles of one volunteer are shown in Fig. 5 after the administration of 3×1 mg clemastine (Clastinol® or Tavegyl® tablets). The results of the study will be reported elsewhere.

4. Conclusion

We have successfully developed and validated an HPLC–MS–MS method for the quantitation of clemastine from human plasma that has, to the best of our knowledge, a lower quantitation limit than any other former method described in the literature. The method uses liquid–liquid extraction of the drug from basified human plasma and electrospray ionization tandem mass spectrometry after reversed phase chromatographic separation. The use of a deuterated internal standard considerably improves the precision and LLOQ of the analysis. The elaborated method has been successfully applied in a bioequivalence study of the drug.

Acknowledgement

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