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Development and validation of high-throughput liquid chromatography–tandem mass spectrometric method for simultaneous quantification of loratadine and desloratadine in human plasma

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Abstract

As a continuation of effort to improve our high flow on-line bioanalytical approach for high-throughput quantification of drugs and metabolites in plasma by high-throughput liquid chromatography tandem mass spectrometry (HTLC–MS/MS), we have developed a simple, sensitive and reliable method for simultaneous quantification of loratadine and desloratadine in human plasma. We have performed on-line coupling of extraction with Cyclone P 50 mm × 0.5 mm 50 μm HTLC column and chromatographic separation is performed with Zorbax XDB C18 50 mm × 2.1 mm 5 μm, followed by quantification with mass detector. The method is validated and showed good performances in terms of linearity, sensitivity, precision, accuracy and stability. A marked improvement in sample throughput efficiency is realized with this method and the proposed method will be useful for pharmacokinetic and/or bioequivalence studies.

Keywords: Loratadine; Desloratadine; High-throughput liquid chromatography; Mass spectrometric detection; Human plasma; Validation

1. Introduction

Loratadine [1] is an orally effective, nonsedating, long-acting H1 receptor antagonist, with no autonomic anticholinergic effects in humans [2–4]. Metabolic studies in humans [5–7] have established that loratadine is rapidly absorbed and undergoes extensive first-pass metabolism to descarboethoxy loratadine (desloratadine, DL). DL is also pharmacologically active and is present in the plasma at low concentrations due to metabolism to several hydroxylated metabolites including the active metabolite 3-hydroxydesloratadine (3-OH-DL), which are excreted as conjugates [8] identified CYP2D6 and CYP3A4 as the primary human liver enzymes responsible for the metabolism of LOR to DL with a K_m range for LOR of 7–35 μM. Loratadine is 97–99% plasma protein bound with an apparent oral clearance of 142.0 ± 56.5 mL/(min kg) for a 40-mg dose [9]. The clinical plasma C_{max} for a 10-mg dose is 4.7 ± 2.7 ng of LOR/mL [6]. For DL and 3-OH-DL, the clinical plasma C_{max} for a 10-mg dose are 4.0 and 2.8 ng/mL, respectively [6,10,11]. Loratadine is used generally in treatment of allergic bronchial asthma [12] and urticaria [13].

Analytical methods so far reported for determination of loratadine, employing HPLC-coupled ultraviolet detection [14,15], fluorescence detection [16], mass spectrometric detection [17], gas–liquid chromatographic method [20], tandem mass spectrometric detection [19], UPLC method [21] and one HPLC method was reported for simultaneous determination of loratadine and its metabolite [18]. In literature some of the

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automated method were reported using solid-phase extraction technique [32,33].

The objective of the present work is to develop and validate the first automated, high-throughput liquid chromatography tandem mass spectrometric method for the simultaneous determination of loratadine and desloratadine in human plasma using citrizine as an internal standard. Robotic liquid handling systems are employed to all liquid transfer steps including the sample preparation procedure as well as to the addition/removal of the organic solvent. The current method includes a simple and rapid sample preparation as a result of robotic systems utilization that enabled parallel processing as well as significantly shorter analysis run time compared to previously published methods.

2. Experimental

2.1. Chemicals and reagents

Loratadine and desloratadine are supplied by Vasudha Laboratories, Hyderabad, India and citrizine is supplied by Praveen Laboratories, Bombay, India. Ammonium acetate, ammonium formate, formic acid, isopropyl alcohol and acetone are obtained from Merck Chemicals, and acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from JT Baker. Healthy human plasma was obtained from CR Bio Clinical Research (I) Laboratory, Hyderabad. Double distilled water is obtained from Sartorius apparatus.

2.2. Data processing

Chromatograms are acquired using the Thermo LCQuan 2.5 software version 1.4 supplied by the manufacturers. Robotic liquid handling system is operated using the software package supplied from the cohesive technologies Aria^{TM}. The calibration curve is constructed by weighted $1/x^2$ least-square linear regression analysis of the peak area ratio (drug/ISTD) vs. the concentration of drug and (metabolite/ISTD) vs. the concentration of metabolite.

2.3. Standard solutions preparation

2.3.1. Stock solution preparation

Approximately 5 mg of loratadine (A)/desloratadine (B)/citrizine (IS) working standard is weighed and transferred to 10.0 mL volumetric flask, to this 5.0 mL of methanol is added and sonicated to aid dissolution and the final volume is made up with methanol.

2.3.2. Drug intermediate stock solution (A + B)

Aliquot requires volume of drug stock solution A and drug stock solution B into 10 mL volumetric flask to get the concentration approximately 10 μg/mL for each. (If required separate intermediate stocks can be prepared.) Volume is made up with methanol and sonicated to aid dissolution.

2.3.3. Preparation of internal standard dilution

The citrizine internal standard (ISTD) dilution of about 10 ng/mL from the ISTD stock solution (IS stock) using methanol as the diluent is prepared.

2.3.4. Preparation of calibration curve (CC) standards and quality control (QC) samples

CC concentration range from 20 ng/mL to 1000 ng/mL and QC concentration range from 30 ng/mL to 800 ng/mL from the drug intermediate stock using methanol as the diluent are prepared.

2.4. Solutions used for robotic on-line sample extraction system

Pure acetonitrile is used in pump A, 10 mM ammonium formate buffer is used in pump B (eluting pump), 0.5% formic acid is used in pump C and washing solution in the ratio of 60:25:15 (acetonitrile:IPA:acetone) is employed in pump D.

2.5. Sample preparation

Retrieve the frozen CC, QC samples and subject samples from the deep freezer and thaw in water bath maintained at room temperature, vortex to mix. Remove the caps from the polypropylene tubes. Aliquot 0.5 mL of CC, QC and subject samples into pre-labelled Eppendorf vials. Add 50.0 μL of ISTD dilution (about 10 ng/mL) into Eppendorf vials and vortex to mix. Centrifuge the Eppendorf vials at 14,000 rpm and at 10 °C for 20 min, transfer the 0.400 mL of supernatant to pre-labelled injection vials. Add 0.400 mL of 10 mm ammonium formate and vortex to mix and subsequently transfer the injection vials to auto sampler.

3. Results and discussion

3.1. Chromatographic and mass spectrometric conditions

The LC/MS/MS system consisted of four pumps for gradient solvent delivery, and a divert valve to direct LC effluent to the mass spectrometer in the analyte elution window.

The analytical column effluent is directed through the divert valve to a thermo electron TSQ quantum discovery mass spectrometer. Source/gas parameters such as spray voltage is operated at 3000, sheath gas pressure, auxiliary gas and capillary temperature settings are maintained at 40 psi, 10 cm^3 min^{-1} and 350 °C, respectively. Compound specific parameters such as col-

Table 1
Compound specific mass spectrometric parameters

Parameters	MS/MS (SRM[a])		
	Loratadine	Desloratadine	Citrizine (IS)
Collision energy (CE)	22	13	26
Tube lens offset	113	97	90
Skimmer offset	−10	−10	−10

[a] Scan type.

Table 2
Steps involved in on-line robotic method

Step	Start	Sec.	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	60	2.00	Step	0.0	100.0	0.0	0.0	–	Out	0.60	Step	50.0	50.0
2	1.00	60	0.20	Step	50.0	0.0	50.0	0.0	T	In	0.40	Step	50.0	50.0
3	2.00	60	2.00	Step	0.0	0.0	0.0	100.0	–	In	0.60	Ramp	50.0	50.0
4	3.00	30	2.00	Step	0.0	0.0	0.0	100.0	–	Out	0.60	Step	50.0	50.0
5	3.50	30	2.00	Step	0.0	0.0	0.0	100.0	–	In	0.60	Step	50.0	50.0
6	4.00	30	2.00	Step	100.0	0.0	0.0	0.0	–	In	0.60	Step	50.0	50.0
7	4.50	90	2.00	Step	0.0	100.0	0.0	0.0	–	Out	0.60	Step	50.0	50.0

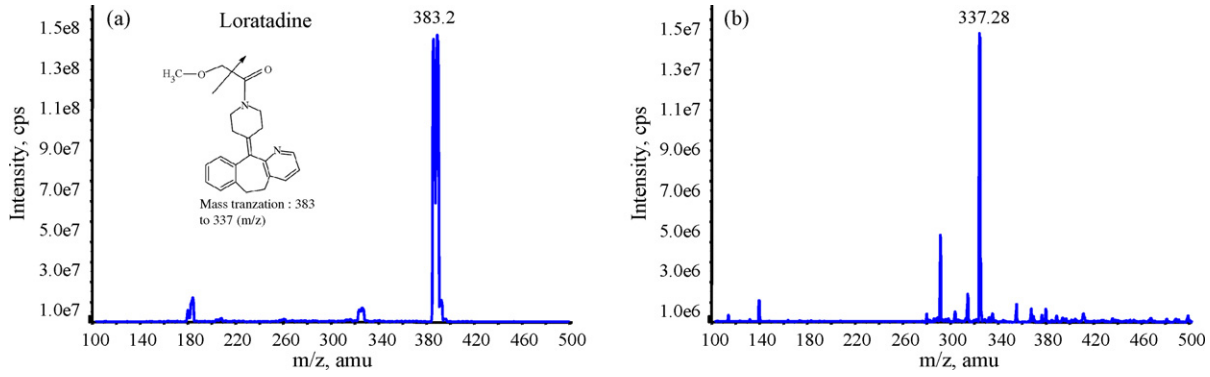

Fig. 1. Mass spectra of the (a) loratadine precursor and (b) major loratadine fragment.

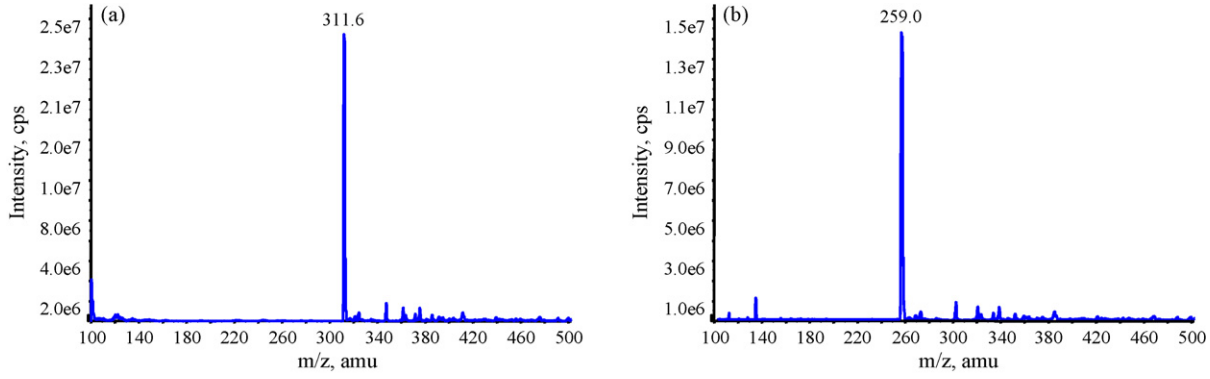

Fig. 2. Mass spectra of the (a) desloratadine precursor and (b) major desloratadine fragment.

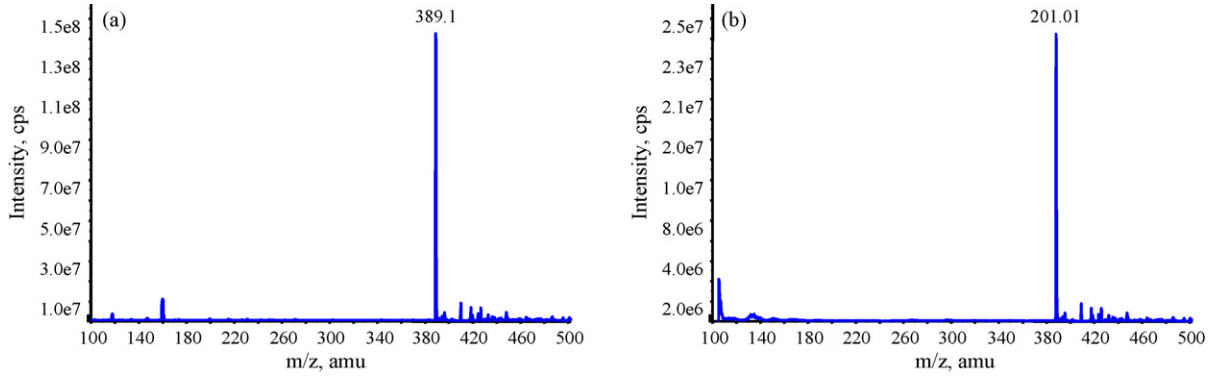

Fig. 3. Mass spectra of the (a) citrizine precursor and (b) major citrizine fragment.

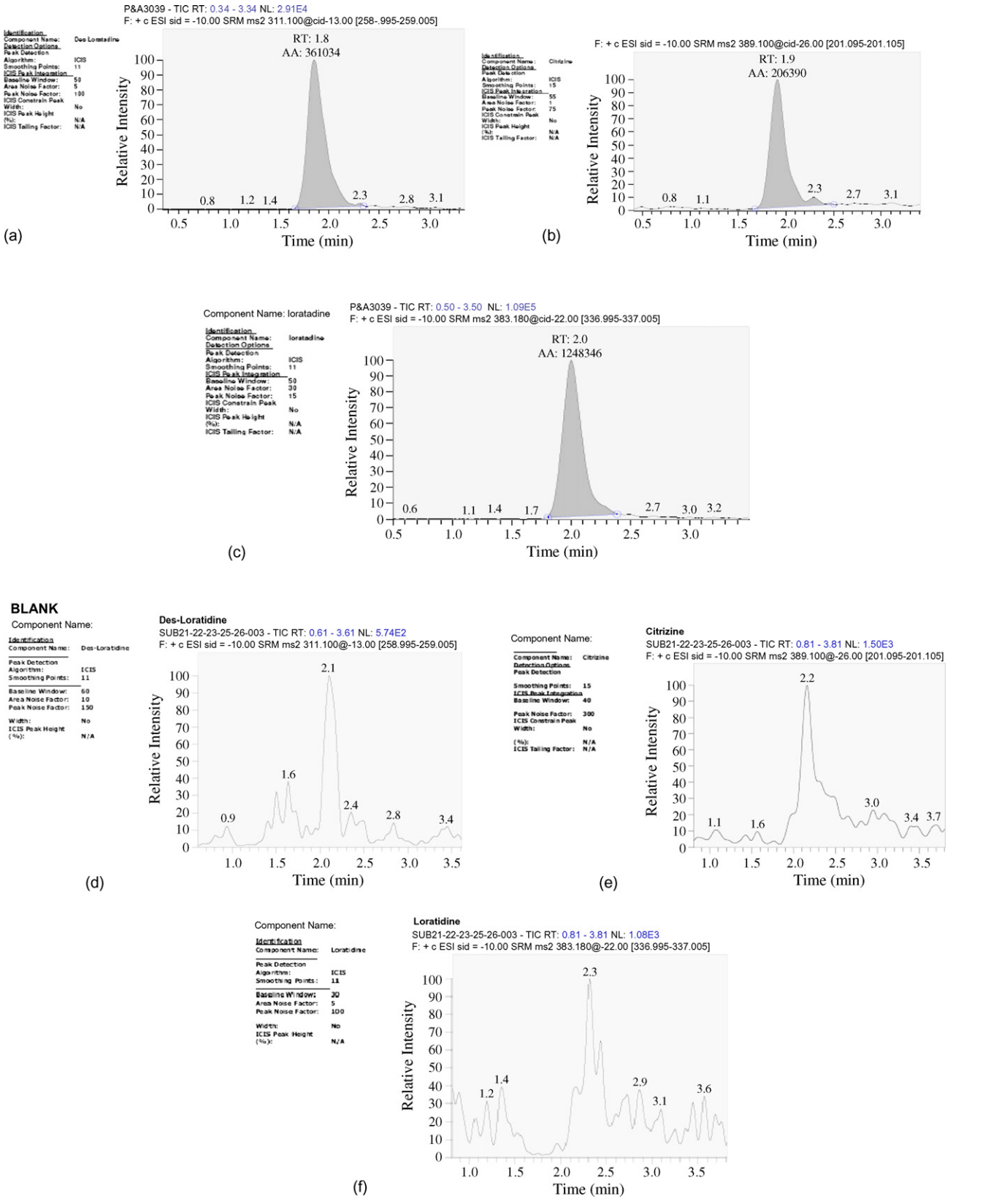

Fig. 4. Representative chromatogram of (a) desloratadine at high QC level, (b) citrizine at standard concentration, (c) loratadine at high QC level and (d–f) at blank concentration.

lision energy, tube lens offset and skimmer offset are presented in Table 1. The instrument is operated in the positive ion mode. The precursor $[M{\cdot}H]^+$ ions at *m/z* 383.20, 311.16 and 389.10 for loratadine, desloratadine and citrizine, respectively are selected by the first quadrupole (Q1). After collision-induced fragmentation in Q2, the product ions at *m/z* 337.20, 259.00 and 201.101 for loratadine, desloratadine and citrizine, respectively, are monitored in Q3. A resolution of one unit (at half peak height) is used for both Q1 and Q3. The full scan and product ion spectra are shown in Figs. 1–3.

3.1.1. Steps involved in on-line robotic method development

A typical two-column setup featuring two six-port switching valves as described by Herman [29] is employed for method development. The procedure consisted of four steps:

(1) The eluent loop is filled with 50% acetonitrile in 10 mM ammonium formate.
(2) The sample is loaded onto the Cyclone 50 mm × 0.5 mm HTLC (50 μm) column at a flow rate of 2 mL/min during 60 s.
(3) The eluent loop is discharged at 0.4 mL/min for 60 s to transfer the analytes from HTLC column onto the Zorbax XDB C18 50 mm × 2.1 mm i.d., 5 μm column and 0.5% aqueous formic acid at 0.2 mL/min in added post column.
(4) LC–MS/MS is performed using ballistic gradient at 2.0 mL/min (10–90% acetonitrile in 0.5% formic acid).

3.2. On-line sample extraction

The gradient program accomplished a Cyclone HTLC column for sample extraction, elution with four pumps as reported in Table 2. TLX turbo flow on-line technique is employed for separation of analyte from sample molecules. The mechanism involved in sample preparation may be affinity. The small drug molecules bind to the HTLC column, and molecules that have lower binding affinity quickly diffuse into the column particles and large sample molecules are flushed to waste, then the mobile phase elutes the analyte molecules that are binded at HTLC column to analytical column, from this analytical column analytes are entered to mass detector. To achieve required chromatograms with consistency we have performed different combinations of the solvents and gradient system. Finally we succeeded with the solution combinations as mentioned in Table 2.

3.3. Ion suppression

One important factor that can affect the quantitative performance of a mass detector is ion suppression. Sample matrix, coeluting compounds and cross-talk can contribute to this effect. Ionization suppression typically observed in sample extracts from biological samples is not likely to be caused by reactions occurring in the gas phase. It is most likely that ionization suppression is the result of high concentrations of nonvolatile materials present in the spray with the analyte. King et al. [30] suggested that the effect is more generally applicable to any nonvolatile solute, including analyte. The exact mechanism by which the nonvolatile materials inhibit release of analyte into the gas phase has not been clearly demonstrated, although a likely list of effects relating to the attractive force holding the drop together and keeping smaller droplets from forming should account for a large portion of the ionization suppression observed with ESI. Once nonvolatile materials has been removed from sample preparation, there is no guarantee that suppression of ionization will no longer be a problem, other mechanisms such as impairing agents (e.g. trifluoro acetic acid) may play a role in ionization suppression. Bonfiglio et al. [31] reported the effects of sample preparation methods on the variability of ESI response. According to their results precipitation method showed the greatest amount of ESI response suppression followed by solid-phase extraction while liquid–liquid extracts demonstrated the least. In our study robotic liquid handling system was employed for sample extraction from plasma and 0.5% formic acid was employed as mobile phase additive to minimize ion suppression.

3.4. Assay validation

3.4.1. Specificity and selectivity

No significant interferences are found at the retention time of required analytes and internal standard. The representative chromatograms are shown in Fig. 4c–f. Six different sources of drug-free human plasma are screened and no endogenous interferences are observed at the retention of loratadine, desloratadine and internal standard (Fig. 4a–c).

3.4.2. Linearity

Linearity means that the assay provides test results that are proportional to the concentration of the analyte in the sample with directly or via a mathematical transformation [23]. The relationship between the experimental response value and known concentrations of the analyte is referred to as calibration curve. In our study calibration curve is constructed by weighted $1/x^2$ of the peak area ratio (drug/IS) vs. the concentration of drug and (metabolite/IS) vs. the concentration of metabolite with the above calibration standards to generate a calibration curve. The linearity range obtained in the present study is 0.409–20.310 ng/mL ($r^2 = 0.99$) for loratadine, 0.403–17.751 ng/mL ($r^2 = 0.99$) indicates the method is within the acceptance criteria.

3.4.3. Recovery

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the nominal concentrations of the pure authentic standard [22]. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations with unextracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible. In our method we got 73.2%, 57.1% and 61.3% recovery for loratadine, desloratadine and

Table 3
Stability in terms of % accuracy[a]

Stability parameter	Loratadine	Desloratadine
Short-term stock solution	95.9	104.4
Long-term stock solution	98.5	102.2
Freeze–thaw		
LQC	92.1	87.5
HQC	96.1	99.7
Bench top		
LQC	87.4	111.8
HQC	99.3	96.4
Wet extraction		
LQC	85.6	87.0
HQC (auto sampler)	99.1	93.1

[a] Mean of six determinations.

citrizine, respectively, which are within the acceptance criteria [28].

3.4.4. Precision and accuracy

The global means within batch and between batch precision obtained is 4.4–11.1 for low, middle and high quality control samples and 15.2 for lower limit of quality control sample, respectively. The global accuracy obtained for within batch and between batches is 96.8, 107–109.1 for low, middle and high quality control samples and 97.4 for lower limit of quantification control sample, respectively; the results obtained are within the acceptance criteria.

3.4.5. Stability

Stability is prominently an important preanalytic variable for the determination of analytes in biological matrices. It is extremely important to perform the stability study of the analyte and internal standard in biological fluids as soon as possible in the lifetime of the project in order to obtain information concerning the conditions and times of samples storage so that sample integrity before assay is assumed. Inaccuracies resulting from losses of analytes during sample storage and processing might occur before any instrumental methods of analysis. Although LC/ESI-MS/MS methods have demonstrated the capability of reducing the needs of sample clean up procedures because of its inherent selectivity and sensitivity as compared to conventional HPLC methods, the duration of time required for sample processing is not short enough to complete instability issues. Therefore analyte stability during sample transport, storage and preparation is a concern for the interpretation of the concentrations of therapeutic agents, their metabolites, or degradation products in drug metabolism, pharmacokinetics [24], toxicological [25], clinical [26] and bioanalytical [27] studies. According to FDA guidelines for industry [28] effect of freeze–thaw, bench top, short-term, long-term, stock solution and post preparative stability assessments are evaluated as a part of bioanalytical method validation. In our study quality control plasma samples are used subject to bench top (6 h), in injector (10–98 h), freeze–thaw (−20 to +20 °C) cycles, short term (24 h) at room temperature and long term (30 days) at deep freezer (at −20 °C) tests are performed. The values obtained for present stability studies are tabulated (Table 3), which are within the acceptance criteria [28].

4. Conclusion

On-line coupling requires some modifications to the offline extraction techniques. The coupling is most commonly performed with the help of multiport valves and one or more pumps for the dynamic extraction or transfer of the extract to the chromatographic system, the extraction can be performed in either static or dynamic mode or as a combination of these so long as the extraction system allows the on-line transfer of the extract to the chromatographic system. In on-line systems, the whole extract is transferred to the chromatographic column, in contrast to traditional off-line techniques where only a small part is injected. This means that the sensitivity of the on-line method is much better. However, the high sensitivity easily leads to overloading of the analytical column. Miniaturisation of the extraction system is often required to avoid this. In our method miniaturisation is achieved with small extraction in extraction vessels and the total analysis means sample extraction, chromatographic separation and mass spectrometric detection has been completed within 8 min for one sample quantitation.

References

[1] N. Levine, C.C. Levine, Dermatology Therapy, Springer, Berlin, 2006, p. 360.
[2] R.L. Batenhorst, A.S. Batenhorst, D.A. Graves, T.S. Foster, M. Kung, R.P. Gural, H.J. Amkraut, Eur. J. Clin. Pharmacol. 31 (1986) 247.
[3] P.M. Bedard, J.D. Carpio, A. Gutkowski, Ann. Allergy 55 (1985) 233 (Abstract 29).
[4] F.J. Villani, C. VMagatti, D.B. Vashi, J. Wong, T.L. Popper, Arzneimittel-Forschung 36 (1986) 1311.
[5] B. Katchen, J. Cramer, M. Chung, R. Gura, J. Hilbert, V. Luc, V. Mortizen, D.R. Sousa, S. Symchowicz, N. Zampaglione, Ann. Allergy 55 (1985) 393.
[6] J. Hilbert, E. Radwanski, R. Weglein, V. Luc, G. Perentesis, S. Symchowicz, N. Zampaglione, J. Clin. Pharmacol. 27 (1987) 694.
[7] F.E.R. Simons, K.J. Simons, Clin. Pharmacokinet. 36 (1999) 329.
[8] N. Yumibe, K. Huie, K. Chen, R.P. Clement, M.N. Cayen, Int. Arch. Allergy Immunol. 107 (1995) 420.
[9] L.N. Jensen, P. Damkier, T.B. Bidstrup, R.S. Pedersen, F. Nielsen, K. Brosen, Eur. J. Clin. Pharmacol. 62 (2006) 342.
[10] J. Villacampa, F. Alcántar, J.M. Rodriguez, J.M. Morales, J. Herrera, R. Rosete, Clin. Ther. 22 (2003) 25.
[11] M. Luis, J. Salmun, M. Herron, B. Christopher, D. Padhi, R. Lorber, M.B. Affrime, Clin. Ther. 22 (2000) 61.
[12] J.-L. Menardo, F. Horak, M.R. Danzig, W. Czarlewski, Clin. Ther. 19 (1997) 1278.
[13] E.W. Monroe, Clin. Ther. 19 (1997) 232.
[14] M. Qi, P. Wang, Y. Geng, J. Pharm. Biomed. Anal. 38 (2005) 355.
[15] P.K. Kunicki, J. Chromatogr. B: Biomed. Sci. Appl. 755 (2001) 331.
[16] H. Amini, A. Ahmadiani, J. Chromatogr. B 809 (2004) 227.
[17] I.I. Salem, J. Idrees, J.I. Al Tamimi, J. Pharm. Biomed. Anal. 34 (2004) 141.
[18] O.Q.P. Yin, X. Shi, M.S.S. Chow, J. Chromatogr. B 796 (2003) 165.
[19] W. Naidong, T. Addison, T. Schneider, X. Jiang, T.D.J. Halls, J. Pharm. Biomed. Anal. 32 (2003) 609.
[20] R. Johnson, J. Christensen, C.-C. Lin, J. Chromatogr. B: Biomed. Sci. Appl. 657 (1994) 125.
[21] J.X. Shen, H. Wang, S. Tadros, R.N. Hayes, J. Pharm. Biomed. Anal. 40 (2006) 689.

[22] S. Braggio, R.J. Barenaby, P. Grossi, M.J. Cugula, Pharm. Biomed. Anal. 14 (1996) 375.
[23] G. Srinubabu, B. Sudharani, B. Sridhar, J.V.L.N. Seshagiri Rao, Chem. Pharm. Bull. 54 (2006) 819.
[24] C. Dufresnce, P. Favetta, C. Paradis, Ther. Drug. Monit. 23 (2001) 550.
[25] R. Ventura, C. Jimenez, N. Closas, J. Chromatogr. B 795 (2003) 167.
[26] B.Y. Chan, K.A. Buckley, B.H. Durham, Clin. Chem. 49 (2003) 2083.
[27] A.L. Sailaja, K.K. Kumar, D.V.R. Ravi Kumar, C.M. Kumar, N.M. Yugandhar, G. Srinubabu, Chromatographia 65 (2007) 359.
[28] http://www.fda.gov/cder/guidance/4252fnl.pdf.
[29] J.L. Herman, Rapid Commun. Mass Spectrom. 16 (2002) 421.
[30] R.C. King, R. Bonfiglio, M.C. Fernandez, S.C. Miller, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[31] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[32] J. Zhang, W. Zeng, C. Kitchen, A.Q. Wang, D.G. Musson, J. Chromatogr. B 806 (2004) 167–175.
[33] N. Brignol, L.M. McMahon, S.L. Francis, L.S. Tse, Rapid Commun. Mass Spectrom. 15 (2001) 898–907.