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Simultaneous determination of ebastine and its three metabolites in plasma using liquid chromatography-tandem mass spectrometry

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Abstract

We developed a method for determining ebastine, a new generation of antihistamines, and its three metabolites (hydroxyebastine, carebastine and desalkylebastine) in plasma simultaneously using LC/MS/MS. Four compounds and terfenadine, an internal standard, were extracted from plasma using a mixture of diethylether and dichloromethane in the presence of 1 M HCl. After drying the organic layer, the residue was reconstituted in mobile phase (acetonitrile:5 mM ammonium acetate, 50:50, v/v) and injected onto a reversed-phase C_{18} column. The isocratic mobile phase was eluted at 0.2 ml/min. The ion transitions monitored in multiple reaction-monitoring mode were *m*/*z* 470.7 → 167.1, 486.7 → 167.1, 500.6 → 167.1, 268.4 → 167.1 and 472.7 → 436.0 for ebastine, hydroxyebastine, carebastine, desalkylebastine and terfenadine, respectively. The coefficient of variation of the assay precision was less than 12.5%, and the accuracy exceeded 88%. The limit of detection was 0.5 ng/ml for desalkylebastine; 0.2 ng/ml for ebastine, hydroxyebastine and carebastine, respectively. This method was used to measure the plasma concentration of ebastine and its three metabolites from healthy subjects after a single 20 mg oral dose of ebastine. This analytic method is a very simple, sensitive, and accurate to determine the pharmacokinetic profiles of ebastine including its metabolites.
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Keywords: Ebastine; Desalkylebastine; Hydroxyebastine; Carebastine; LC/MS/MS

1. Introduction

Ebastine is a new generation of antihistamines which has potent and selective histamine H_1-receptor antagonistic effect, but negligible anticholinergic and antiserotonergic properties [1,2]. The drug is further characterized by a lack of electrocardiologic side effects such as QT interval prolongation which has been reported in the use of terfenadine or other antihistamines [3–5]. Ebastine is effective for the treatment of chronic idiopathic urticaria and allergic diseases with once daily regimen, and the antihistaminic action is mainly induced by the active metabolite (carebastine) rapidly generated in the small intestine and in the liver [6]. Although the other two metabolites, desalkylebastine and hydroxyebastine are pharmacologically inactive, they should be still kept an eye on in pharmacokinetic point of view, because different cytochrome P450 enzymes, CYP3A4 and CYP2J2 are involved in dealkylation and in hydroxylation of ebastine to generate desalkylebastine and hydroxyebastine, respectively [7,8].

To date, a couple of chromatographic methods have been reported to quantify ebastine and its metabolites in physiological samples, no publication, however, has appeared to simultaneously measure the four compounds in human plasma due to the limitation of detection [9]. Rohatagi et al. recently improved the assay methodology using a tandem mass spectrometry, but they only measured ebastine and carebastine in human plasma [10].

Therefore, we develop a sensitive and accurate method for determining ebastine and its three metabolites at the same time in human plasma using liquid chromatography with a

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tandem mass spectrometry. This method was successfully applied to characterize the pharmacokinetics of ebastine and its metabolites in humans.

2. Experimental

2.1. Reagents and materials

Ebastine, desalkylebastine, hydroxyebastine, and carebastine were kindly donated by Almirall Prodesfarma, SA (Barcelona, Spain). Terfenadine (internal standard, IS) and HPLC-grade organic solvents (dichloromethane and diethylether) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany), respectively. All the other chemicals and solvents were of the highest analytical grade available.

2.2. Preparation of standards and quality controls

Ebastine, its three metabolites and IS were dissolved in methanol at 1 mg/ml, respectively. The standard solutions were serially diluted with methanol and added at drug-free plasma to obtain the concentrations of 0.5, 2, 5, 10, 20, 50 and 100 ng/ml for carebastine and desalkylebastine, respectively, and 0.5, 1, 2, 5 and 10 ng/ml for ebastine and hydroxyebastine, respectively. Calibration graphs in plasma were derived from the peak area ratio of ebastine and three metabolites to IS with a linear regression, respectively.

Quality controls were prepared daily in 1 ml of blank human plasma by adding 100 μl of standard solution, respectively. They were prepared for 0.5, 5, 20 and 100 ng/ml for carebastine and desalkylebastine, respectively, and 0.5, 2, 5 and 10 ng/ml for ebastine and hydroxyebastine, respectively, to evaluate the inter- and intra-day precision and accuracy of this assay method.

2.3. Characterization of the product ions using tandem mass spectrometry

One micromolar ebastine, desalkylebastine, hydroxyebastine, carebastine and IS solutions were infused into the mass spectrometer separately at a flow rate of 10 μl/min to characterize the product ions of each compound. The precursor ions $[M+\mathrm{H}]^+$, and the pattern of fragmentation were monitored using positive ion mode. The major peaks observed in the MS/MS scan were used to quantify ebastine, its three metabolites and IS.

2.4. Analytical system

The concentrations of ebastine and its three metabolites in human plasma were quantified using liquid chromatography-mass spectrometry with a PE SCIEX API 3000 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface used to generate positive ions $[M+\mathrm{H}]^+$. The compounds were separated on a reversed-phase column (Luna C_{18}, 2 mm × 50 mm internal diameter, 3 μm particle size; Phenomenex, Torrance, CA, USA) with an isocratic mobile phase consisting of acetonitrile and 5 mM ammonium acetate buffer (50:50%, v/v). The mobile phase was eluted using an HP 1100 series pump (Agilent, Wilmington, DE, USA) at 0.2 ml/min.

The turboion spray interface was operated in the positive ion mode at 5500 V and 350 °C. The operating conditions were optimized by flow injection of a mixture of all anlalytes and were determined as follows: nebulizing gas flow, 1.04 l/min; auxiliary gas flow, 4.0 l/min; curtain gas flow, 1.44 l/min; orifice voltage, 80 V; ring voltage 400 V; collision gas (nitrogen) pressure, 3.58×10^{-5} Torr. Quantitation was performed by multiple reaction monitoring (MRM) of the protonated precursor ion and the related product ion for ebastine and its three metabolites using the internal standard method with peak area ratios and a weighting factor of $1/x$. The mass transition used for ebastine, hydroxyebastine, carebastine and terfenadine were m/z 470.7 → 167.1, 486.7 → 167.1, 500.6 → 167.1 and 472.7 → 436.0, respectively (collision energy 40 eV, dwell time 200 ms); that for desalkylebastine was m/z 268.4 → 167.1 (collision energy 15 eV, dwell time 200 ms). Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.2, Applied Biosystems, Foster City, CA, USA).

2.5. Sample preparation

Fifty microliters of internal standard (2.5 ng/ml) and 1 ml of 1 M HCl were added to 1 ml of plasma, followed by 1 min liquid–liquid extraction with 5 ml of a mixture of dichloromethane and diethylether (1:1). The organic layer was separated and evaporated to dryness at ambient temperature in a Speed-Vac (Savant, Holbrook, NY, USA). The residue was reconstituted into 100 μl of mobile phase by vortex mixing for 15 s; 5 μl of this solution was injected onto the column.

2.6. Validation procedure

The validation parameters were selectivity, extraction recovery, precision, and accuracy. Five batches of blank heparinized human plasma were screened to determine the specificity. The results from drug-spiked plasma extracts were compared to blank plasma extracts with drug added after extraction to determine the extraction recovery. The precision and accuracy of the intra- and inter-day assay validation were estimated using the inverse prediction of the concentration of the quality controls from the calibration curves. For the stability study in plasma, control drug-free plasma samples were spiked with 5 and 20 ng/ml for carebastine

and desalkylebastine, respectively, and with 2 and 5 ng/ml for ebastine and hydroxyebastine, respectively. Short-term stability was assessed after 12 h of storage at room temperature; long-term stability was assessed after 2 weeks of storage in a freezer at −80 °C. The stability of ebastine and three metabolites in plasma samples was tested after three freeze–thaw cycles (−80 °C to room temperature). The stability in extracts was also examined after 10 h of storage at room temperature.

2.7. Clinical application

Ten healthy subjects who gave written informed consent took part in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were exclusion criteria. This study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, South Korea). After an overnight fast, all the subjects were given a single 20 mg oral dose of ebastine. Blood samples (6 ml) were taken before and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48 and 72 h after drug administration and stored at −80 °C until analysis.

3. Results and discussion

3.1. Mass spectra

Precursor ions for ebastine, its three metabolites and terfenadine, and their corresponding product ions were determined from spectra obtained during the infusion of standard solutions into a mass spectrometer using an electrospray ionization source, which operated in positive ionization mode with collision nitrogen gas in Q2 of MS/MS system. Ebastine, desalkylebastine, hydroxyebastine, carebastine and terfenadine mainly produced protonated molecules at *m/z* 470.7, 268.4, 486.7, 500.6 and 472.7, respectively. Their product ions were scanned in Q3 after collision with nitrogen in Q2 at *m/z* 167.1 for ebastine, desalkylebastine, hydroxyebastine, carebastine, respectively, and at *m/z* 436.0 for terfenadine. These are the most sensitive product ions for quantification (Fig. 1). Forty electron voltage of the collision energy was optimal to produce the fragment ion of *m/z* 167.1 (benzhydryl ion) from ebastine, carebastine and hydroxyebastine, respectively, and of *m/z* 436.0 dissociating two molecules of H_2O from terfenadine [11], while 15 eV of collision energy should

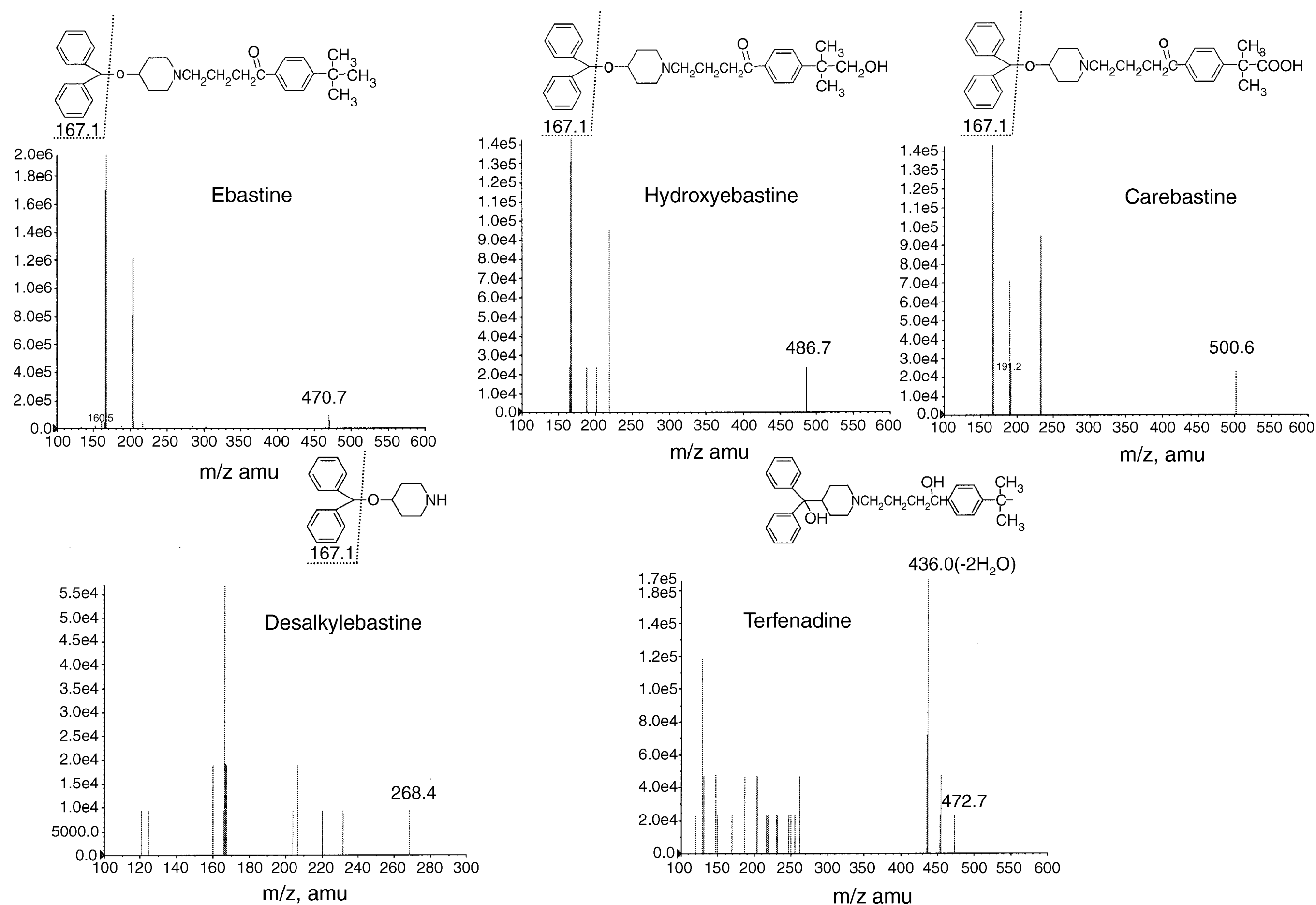

Fig. 1. Mass–mass spectra of ebastine, hydroxyebastine, carebastine, desalkylebastine and terfenadine obtained using electrospray ionization mode.

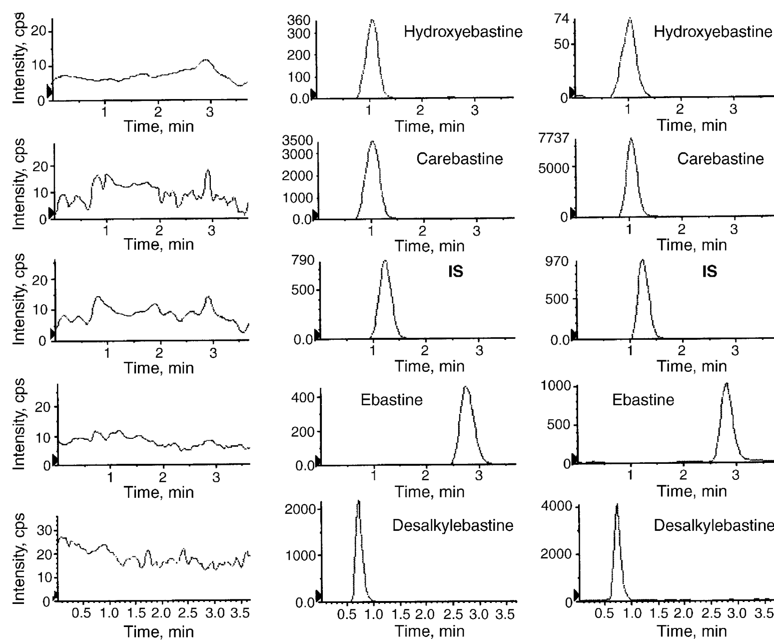

Fig. 2. Chromatograms of ebastine, its three metabolites and terfenadine. Left column: blank plasma; middle column: plasma spiked with 5 ng/ml of ebastine and hydroxyebastine, respectively, and 50 ng/ml of carebastine and desalkylebastine, respectively; right column: plasma sample equivalent to 0.9, 92, 12 and 87 ng/ml for hydroxyebastine, carebastine, ebastine and desalkylebastine, respectively, from a volunteer 2 h after the oral dose of 20 mg ebastine.

be needed to detect desalkylebastine and its fragment, benzhydryl ion in the multiple reaction mode.

3.2. Determination of ebastine and its three metabolites

There were no interfering peaks at the elution times for either analytes (desalkylebastine, 0.8 min; hydroxyebastine, 1.1 min; carebastine, 1.1 min; ebastine, 2.7 min) or IS (terfenadine, 1.3 min). Fig. 2 represents the typical chromatograms for blank plasma (left column) and plasma spiked with 5 ng/ml for ebastine and hydroxyebastine, respectively, and 50 ng/ml for carebastine and desalkylebastine, respectively, and 2.5 ng/ml for IS (middle column). The plasma sample from a volunteer is shown in the right column of Fig. 2.

In the previous study, to purify ebastine and metabolites, plasma sample was deproteinized with organic mixture, and was diluted with acidic solution followed by solid phase extraction [9]. The eluent was further evaporated, and the residue was reconstituted with mobile phase before loading onto the column. We found that a liquid–liquid extraction in acidic condition could simultaneously remove ebastine and its three metabolites from plasma, and that was much simpler than the pretreatment procedure used previously. Such a liquid–liquid extraction method was also used by Rohatagi et al. [10]. They used isopropyl ether as an extraction solvent in neutral condition, but the acidic condition was much better to recover all analytes from plasma.

Furthermore, the present method was sensitive and accurate to measure ebastine, desalkylebastine and hydroxyebastine. Previously, carebastine was only detected in plasma sample after an oral administration of ebastine because of the limitation of UV detection [9]. However, we quantified not only ebastine and its active metabolite, carebastine, but also desalkylebastine and hydroxyebastine which were not measured in the study using LC/MS/MS [10].

3.3. Linearity and detection limit

The calibration curves in plasma provided a reliable response from 0.5 to 100 ng/ml for carebastine and desalkylebastine, and from 0.5 to 10 ng/ml for ebastine and hydroxyebastine. The mean equations of the regression lines in plasma were $y = (0.101 \pm 0.005)x + (0.004 \pm 0.001)$ for ebastine ($r^2 > 0.998$, $n = 5$), $y = (0.089 \pm 0.011)x + (0.003 \pm 0.002)$

Table 1
The precision and accuracy of the intra-day assay ($n = 5$)

Concentration (ng/ml)	Ebastine	Hydroxyebastine	Concentration (ng/ml)	Carebastine	Desalkylebastine
0.5	88 ± 11[a] (12.5)[b]	91 ± 9 (9.9)	0.5	93 ± 9 (9.7)	92 ± 5 (5.4)
2	96 ± 5 (5.2)	93 ± 4 (4.3)	5	99 ± 5 (5.1)	98 ± 10 (10.2)
5	92 ± 10 (10.9)	101 ± 5 (5.0)	20	97 ± 6 (6.2)	92 ± 7 (7.6)
10	95 ± 7 (7.4)	98 ± 6 (6.1)	100	95 ± 8 (8.4)	97 ± 6 (6.2)

[a] Accuracy (mean% ± S.D.).
[b] CV, coefficient of variance (%).

Table 2
The precision and accuracy of the inter-day assay ($n = 5$)

Concentration (ng/ml)	Ebastine	Hydroxyebastine	Concentration (ng/ml)	Carebastine	Desalkylebastine
0.5	90 ± 8[a] (8.9)[b]	93 ± 10 (10.8)	0.5	92 ± 9 (9.8)	91 ± 8 (8.8)
2	94 ± 9 (9.6)	95 ± 8 (8.4)	5	99 ± 8 (8.1)	98 ± 7 (7.1)
5	102 ± 8 (7.8)	99 ± 6 (6.1)	20	94 ± 7 (7.4)	95 ± 6 (6.3)
10	95 ± 8 (8.4)	102 ± 9 (8.8)	100	96 ± 9 (9.4)	99 ± 8 (8.1)

[a] Accuracy (mean% ± S.D.).
[b] CV, coefficient of variance (%).

Table 3
Stability of ebastine and its three metabolites after storage under indicated conditions

Compounds	Concentration (ng/ml)	10 h, ambient in extracts	12 h ambient	2 weeks −80 °C	Three freeze–thaw cycles
Ebastine	2	1.9 ± 0.3[a]	1.8 ± 0.4	1.9 ± 0.4	2.2 ± 0.3
	5	5.1 ± 0.2	5.0 ± 0.3	4.8 ± 0.5	5.3 ± 0.5
Hydroxyebastine	2	2.0 ± 0.3	1.9 ± 0.3	1.9 ± 0.4	2.1 ± 0.4
	5	4.8 ± 0.3	5.1 ± 0.4	4.7 ± 0.5	5.1 ± 0.5
Desalkylebastine	5	5.1 ± 0.2	5.2 ± 0.4	4.8 ± 0.5	5.0 ± 0.4
	20	19.8 ± 0.5	20.2 ± 0.6	19.5 ± 0.8	20.5 ± 0.6
Carebastine	5	4.7 ± 0.4	4.9 ± 0.2	5.1 ± 0.5	4.9 ± 0.4
	20	19.5 ± 0.3	20.5 ± 0.7	19.8 ± 0.6	20.1 ± 0.5

[a] Mean ± S.D. ($n = 3$).

for carebastine ($r^2 > 0.999$, $n = 5$), $y = (0.091 \pm 0.012)x + (0.008 \pm 0.003)$ for hydroxyebastine ($r^2 > 0.998$, $n = 5$), $y = (0.051 \pm 0.008)x + (0.002 \pm 0.001)$ for desalkylebastine ($r^2 > 0.998$, $n = 5$). The limit of detection was 0.5 ng/ml for desalkylebastine; 0.2 ng/ml for ebastine, hydroxyebastine and carebastine, respectively, at a signal-to-noise (S/N) ratio of 3.

3.4. Precision, accuracy, recovery and stability

The intra- and inter-day precision and accuracy of our method were listed in Tables 1 and 2, respectively. The coefficients of variation of the precision of the intra- and inter-day validation were less than 12.5 and 10.8%, respectively. The accuracy of the intra- and inter-day validation were 88–101 and 90–102%, respectively. The extraction recoveries were ranged 88–105% ($n = 3$).

Ebastine and its three metabolites were stable in plasma at room temperature for up to at least 12 h; they also remained intact at −80 °C for up to 2 weeks. With respect to the run-time stability of processed samples, no significant loss of the four compounds was observed at room temperature, and no degradation was observed after three cycles of freezing

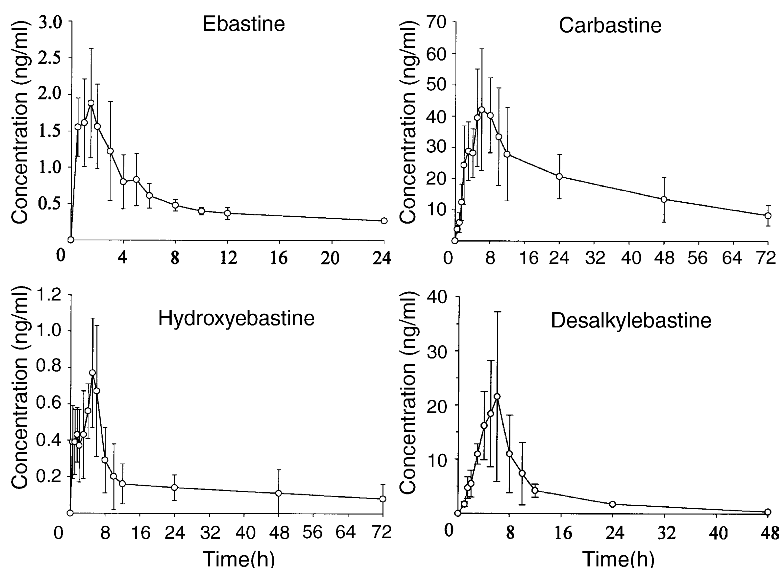

Fig. 3. Time course of the plasma concentrations of ebastine and its three metabolites in healthy subjects after a single 20 mg oral dose of ebastine. Each point represents the mean ± S.D. ($n = 10$).

and thawing (Table 3). This information provides the confidence to perform repeated analyses of clinical samples within 12 h.

3.5. Clinical application

Time profiles of plasma ebastine-, hydroxyebastine-, carebastine-, desalkylebastine-concentration after an oral administration of 20 mg ebastine were illustrated in Fig. 3. Since CYP2J2 and CYP3A4 have been known to be involved in the metabolism of ebastine, the time profiles of each metabolite in plasma could inform us the enzyme activities of CYP3A4 for desalkylebastine, and of CYP2J2 for hydroxyebastine as well as carebastine. Further study in subjects with a genetic polymorphism of those enzymes would be interesting to evaluate the effect of genetic variation on the metabolic profiles of ebastine.

In conclusion, our LC/MS/MS method is a very simple, sensitive, and accurate way to simultaneously determine ebastine and its three metabolites (carebastine, desalkylebastine, hydroxyebastine) in plasma, and is suitable for in vitro and in vivo pharmacokinetic studies of ebastine.

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