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Journal of Chromatography B, 757 (2001) 173–179

JOURNAL OF CHROMATOGRAPHY B

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Short communication

Simultaneous determination of the histamine H_1-receptor antagonist ebastine and its two metabolites, carebastine and hydroxyebastine, in human plasma using high-performance liquid chromatography

Michiaki Matsuda*, Yasuyuki Mizuki, Yoshiaki Terauchi

Department of Pharmacokinetics, Developmental Research Laboratories, Dainippon Pharmaceutical Co. Ltd., 33-94 Enoki-cho, Suita, Osaka 564-0053, Japan

Received 21 March 2000; received in revised form 10 August 2000; accepted 30 August 2000

Abstract

Ebastine (CAS 90729-43-4) is an antiallergic agent which selectively and potently blocks histamine H_1-receptors in vivo. A simple and sensitive high-performance liquid chromatography (HPLC) method is described for the simultaneous determination of ebastine and its two oxidized metabolites, carebastine (CAS 90729-42-3) and hydroxyebastine (M–OH), in human plasma. After a pretreatment of plasma sample by solid-phase extraction, ebastine and its metabolites were analyzed on an HPLC system with ultraviolet detection at 254 nm. Chromatography was performed on a cyano column (250×4.0 mm I.D.) at 40 °C with the mobile phase of acetonitrile–methanol–0.012 *M* ammonium acetate buffer (20:30:48, v/v/v) at a flow rate of 1.2 ml/min. Accurate determinations were possible over the concentration range of 3–1000 ng/ml for the three compounds using 1 ml plasma samples. The intra- and inter-day assay accuracy of this method were within 100±15% of nominal values and the precision did not exceed 12.4% of relative standard deviation. The lower limits of quantitation were 3 ng/ml for ebastine and its metabolites in human plasma. This method was satisfactorily applied to the determination of ebastine and its two oxidized metabolites in human plasma after oral administration of ebastine. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ebastine; Carebastine; Hydroxyebastine

1. Introduction

Ebastine (4′-*tert*.-butyl-4-[4-(diphenylmethoxy)-piperidino]butyrophenone, CAS 90729-43-4, Fig. 1) is a potent and selective histamine H_1-receptor antagonist in vivo, which has little anticholinergic and antiserotoninergic properties [1–3]. This agent also has no effects on cardiovascular and psychomotor functions [3–7], which occured during treatment with classical antihistamine agents such as chlorpheniramine and diphenhydramine.

It has been often reported that ebastine undergoes virtually complete first-pass biotransformation to the pharmacologically active carboxylic acid metabolite, carebastine (4-[4-[4-(diphenylmethoxy)-1-piperidinyl]-1-oxobutyl]-α,α-dimethylbenzeneacetic acid, CAS 90729-42-3, Fig. 1) in both experimental animal and human subjects [8–12]. However, some assay methods in those reports were only for the determination of carebastine, and some of them lack

*Corresponding author. Fax: +81-6-6338-7656.

E-mail address: michiaki-matsuda@dainippon-pharm.co.jp (M. Matsuda).

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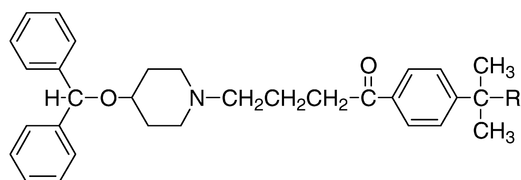
PII: S0378-4347(00)00494-1



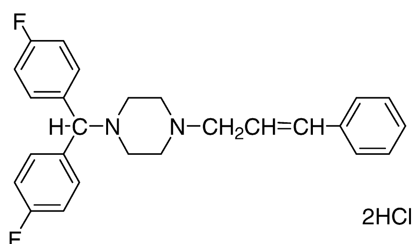


Fig. 1. Chemical structures of ebastine, its metabolites and internal standard (flunarizine).

the sensitivity to determine both ebastine and carebastine. In the in vitro studies for ebastine metabolism by human liver microsomes, hydroxyebastine (hydroxy metabolite of ebastine; M–OH, Fig. 1) is formed [13]. The metabolite is considered an intermediate to be further oxidized to carebastine. But it is not clear whether M–OH can be detected in human plasma after dosing with ebastine.

This paper describes a simple and sensitive high-performance liquid chromatography (HPLC) method for the simultaneous determination of ebastine and its two oxidized metabolites (carebastine and M–OH) in human plasma.

2. Experimental

2.1. Materials

Ebastine and its two oxidized metabolites, carebastine and M–OH, were supplied by Laboratorios Almirall (Barcelona, Spain) [14] (Fig. 1). Flunarizine dihydrochloride (CAS 30484-77-6), which was purchased from Sigma Chemical Co. (St. Louis, MO, USA), was used as an internal standard substance for HPLC.

Unless otherwise specified, guaranteed reagent-grade chemicals (Wako Pure Chemical Industries, Osaka, Japan) were used throughout this study. Acetic acid used for 0.2 *M* acetate buffer and mobile phase buffer was of specially prepared reagent-grade for spectrum analysis (Nacalai Tesque, Kyoto, Japan). Methanol used for sample preparation and dissolution of ebastine, its two metabolites and internal standard was of residual PCB grade (Katayama Kagaku, Osaka, Japan). All other solvents were of HPLC grade (Nacalai Tesque).

The concentration of each metabolite was expressed as the equivalent of unchanged drug, ebastine.

2.2. Chromatography

HPLC was carried out using a Model 800 series high-performance liquid chromatography (Japan Spectroscopic, Tokyo, Japan), consisting of a Model

801-SC system controller, a Model 880-PU pump, a Model 850-AS autosampler and a Model 870-UV ultraviolet detector (254 nm). The data recording, analysis and reporting were performed using Millennium[32] chromatography manager software (version 3.01, Waters, Milford, MA, USA) operating on a Pentium processor personal computer (Digital PC 5100, DEC, Maynard, MA, USA).

A stainless steel column packed with Spherisorb S5CN (5-μm, 250×4.0 mm I.D., Waters) was used as the analytical column. Column temperature was maintained at 40°C with a Model CC-P2 column heater (ADVANTEC TOYO, Tokyo, Japan).

The mobile phase consisted of acetonitrile–methanol–0.012 *M* ammonium acetate buffer (20:30:48, v/v/v). The 0.012 *M* ammonium acetate buffer was prepared by dissolving 0.5 g of ammonium acetate and 5 ml of acetic acid in 520 ml of distilled water. The mobile phase was filtered through a 0.45-μm filter (Zetapor® Membrane, CUNO Inc., Meridene, CT, USA) and degassed under reduced pressure. The flow-rate was 1.2 ml/min.

2.3. Standard solutions

Stock solutions were prepared by dissolving ebastine and its two metabolites in methanol (50 μg/ml) and then stored at 4°C. These stock solutions were mixed and further diluted with methanol to prepare working standard solutions at the following seven concentrations: 30, 50, 200, 500, 2000, 5000 and 10 000 ng/ml.

The internal standard was also dissolved in methanol (50 μg/ml) and the solution stored at 4°C (stock solution). The stock solution of internal standard was then diluted with methanol to the final concentration of 5 μg/ml (working solution).

2.4. Sample preparation

Human plasma sample was processed by solid-phase extraction (SPE) using a C_{18} column (Bond Elut C_{18} cartridge, 3cc/200 mg, Varian Sample Preparation Products, Harbor City, CA, USA). The SPE column was preconditioned twice with 3 ml of methanol followed twice by 3 ml of distilled water prior to sample loading. To 1 ml of plasma sample in a disposable glass culture tube (16×125 mm, Iwaki Glass, Tokyo, Japan) was added 2 ml of methanol–acetonitrile (1:1, v/v). After vortex-mixing for 10 s, 2 ml of distilled water and 1 ml of 0.2 *M* acetate buffer (pH 4.0) were added. The mixture was vortex-mixed, followed by centrifugation at 1300 *g* for 10 min. The supernatant was loaded to the preconditioned SPE column. The column was washed twice with 3 ml of distilled water and then once with 2 ml of methanol. Ebastine and its metabolites were eluted with 2 ml of methanol–50 m*M* phosphate buffer (pH 2.5) (9:1, v/v). To the eluate was added 0.1 ml of internal standard working solution (5 μg/ml in methanol) for correcting errors, if any, at the following steps. The mixture was then evaporated to dryness in vacuo at 55°C. The residue was dissolved in 0.1 ml of the mobile phase and a 40-μl aliquot of the solution was injected into the HPLC. Preconditioning and washing of the SPE column were performed under reduced pressure, but sample loading and eluting were not forced through.

2.5. Validation study

Standard curves were obtained at the following seven concentrations: 3, 5, 20, 50, 200, 500 and 1000 ng/ml. Quality control samples were prepared at the following concentrations: 3, 5, 200 and 1000 ng/ml. To produce the range of concentrations described above, 0.1 ml of each working standard solution (30–10 000 ng/ml) in a disposable glass culture tube was evaporated, and the residue was dissolved in 1 ml of blank human plasma obtained from healthy volunteers.

Human plasma samples spiked with known amounts of ebastine and its metabolites were analyzed according to the procedure described in the previous section. Each standard curve was obtained by plotting the peak-area ratios of ebastine or its metabolites to the internal standard (y) against the plasma concentrations of each compound added (x), using a weighted ($1/x^2$) least-square regression. To test the reproducibility of this analytical method, the quality control samples were analyzed for intra-day ($n=4$) and inter-day ($n=2$ on 3 separate days) accuracy and precision. To check the endogenous

interference, six blank human plasma samples were also assayed.

2.6. Analysis of plasma samples from a healthy volunteer and a patient

Plasma samples were obtained from a healthy volunteer and a patient receiving ebastine during clinical trials performed in Japan, and immediately frozen to −20°C until analysis.

In human plasma at −20°C, ebastine is stable over 5 months, and carebastine over 16 months [15]. M–OH retained 98.6% of the initial value in human plasma (36.5 ng/ml) after long-term storage at −20°C for 14 months.

3. Results and discussion

3.1. Assay specificity

Six blank human plasma samples were assayed to check the endogenous interference. Typical chromatograms of human plasma with or without ebastine, its two oxidized metabolites and the internal standard are shown in Fig. 2. With the assay method,

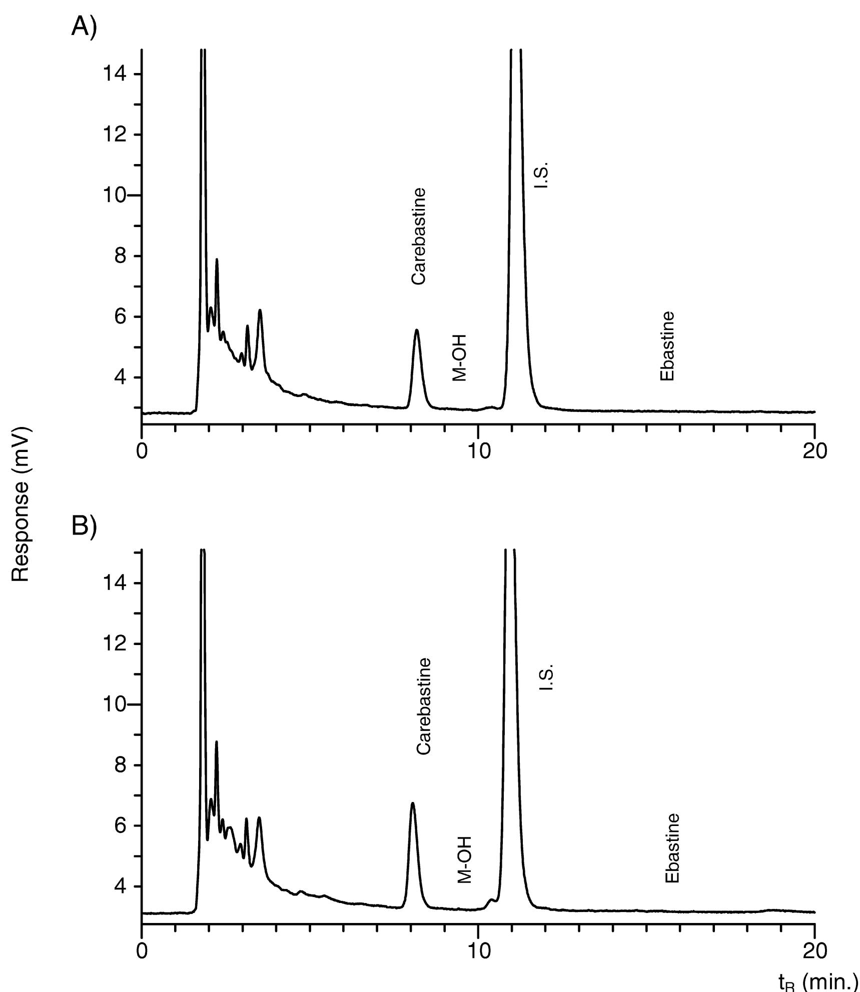


Fig. 2. Typical chromatograms of human plasma. (A) Blank human plasma. (B) Human plasma spiked with the test compounds (Carebastine, M-OH, Ebastine 20 ng/ml, I.S. 500 ng/ml).

Table 1
Intra-day assay accuracy and precision for the determination of ebastine and its metabolites in human plasma

Compound	Nominal conc. (ng/ml)	Observed conc. (Mean±SD, n=4) (ng/ml)	Precision (RSD) (%)	Accuracy (%)
Ebastine	3.13	3.23±0.02	0.6	103.2
	5.22	5.32±0.22	4.1	101.9
	209	208±9	4.3	99.5
	1040	1049±8	0.8	100.9
Carebastine	3.06	3.11±0.30	9.6	101.6
	5.10	5.43±0.06	1.1	106.5
	204	202±8	4.0	99.0
	1020	1006±11	1.1	98.6
M–OH	2.44	2.18±0.27	12.4	89.3
	4.06	3.75±0.18	4.8	92.4
	162	161±8	5.0	99.4
	812	822±8	1.0	101.2

peaks of ebastine, its metabolites and the internal standard were well separated from each other and from peaks derived from the endogenous compounds.

3.2. Linearity

Linearity tests were conducted using 1 ml plasma samples spiked with a wide range of concentrations (final concentrations 3–1000 ng/ml). A good linearity was obtained between the peak-area ratios to the internal standard (y) and the corresponding plasma concentrations (x) on the standard curve of ebastine or its metabolites. The equations for the regression line were $y=(0.00152\pm0.00014)x-(0.00120\pm0.00187)$ for ebastine, $y=(0.00148\pm0.00017)x+(0.00125\pm0.00133)$ for carebastine and $y=(0.00161\pm0.00020)x+(0.00051\pm0.00128)$ for M–OH (mean±SD, $n=6$). The relative standard deviation (RSD) of the standard curve slopes was 8.9% for ebastine, 11.6% for carebastine and 12.4% for M–OH. The correlation coefficients (r) were

Table 2
Inter-day assay accuracy and precision for the determination of ebastine and its metabolites in human plasma[a]

Compound	Nominal conc. (ng/ml)	Observed conc. (Mean±SD, n=6) (ng/ml)	Precision (RSD) (%)	Accuracy (%)
Ebastine	3.13	3.35±0.37	11.0	107.0
	5.22	5.10±0.47	9.2	97.7
	209	193±8	4.1	92.3
	1040	895±61	6.8	86.1
Carebastine	3.06	2.67±0.25	9.4	87.3
	5.10	4.59±0.47	10.2	90.0
	204	180±22	12.2	88.2
	1020	873±106	12.1	85.6
M–OH	2.44	2.44±0.20	8.2	100.0
	4.06	3.94±0.37	9.4	97.0
	162	145±15	10.3	89.5
	812	710±83	11.7	87.4

[a] Results were obtained from the experiments for 3 days.

between 0.9955 and 0.9986 for ebastine, 0.9932 and 0.9990 for carebastine and 0.9938 and 0.9990 for M–OH.

3.3. Reproducibility

The intra-day accuracy and precision of this assay method are shown in Table 1. The accuracy at the concentrations examined ranged between 99.5 and 103.2% of the nominal values with relative standard deviations (RSDs) ranging between 0.6 and 4.3% for ebastine, 98.6–106.5% with RSDs of 1.1–9.6% for carebastine and 89.3–101.2% with RSDs of 1.0–12.4% for M–OH, respectively.

Table 2 shows the inter-day accuracy and precision for the three compounds. Their accuracy varied from 85.6 to 107.0% with RSDs of 4.1–12.2%.

The reproducibility of this method was considered to be satisfactory for the determination of ebastine and its two metabolites in human plasma.

With this method, the lower limits of quantitation for ebastine, carebastine and M–OH were 3 ng/ml.

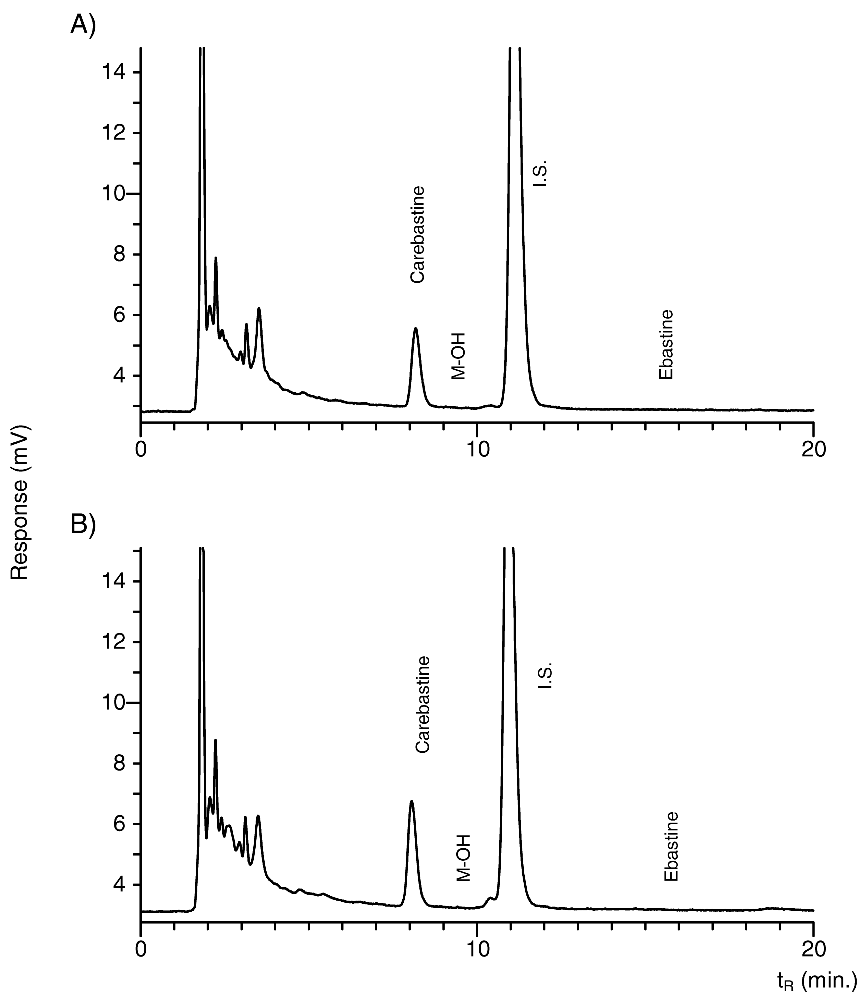


Fig. 3. Analysis of plasma samples from a healthy volunteer and a patient receiving ebastine. (A) Chromatogram of a plasma sample from a healthy volunteer following single oral administration at a dose of 10 mg of ebastine. Time point was 6 hours after dosing. Ebastine and M-OH were not detected. The level of carebastine was 87 ng/ml. (B) Chromatogram of a plasma sample from a patient following multiple oral administration of ebastine (10 mg once daily for 14 days). Time point was 15 hours after the last dosing. Ebastine and M-OH were not detected. The level of carebastine was 127 ng/ml.

This method has at least 3-fold higher sensitivity than the previous methods for ebastine and carebastine [9,10,12].

3.4. Analysis of plasma samples from a healthy volunteer and a patient

This method has been applied successfully to the analysis of samples from clinical trials. The chromatograms shown in Fig. 3 are from a healthy volunteer and a patient following single (10 mg, at 6 h) and multiple (10 mg, once daily for 14 days, at 15 h) oral administration of ebastine, respectively. In their plasma, ebastine and M–OH were not detected (under 3 ng/ml). As reported in previous papers [10,11], the unchanged form of ebastine was virtually absent from plasma.

Although the volunteer was concomitantly administered ebastine and doxepin hydrochloride and the patient received ebastine, betametazone, *d*-chlorpheniramine maleate and tranexamic acid, little interference by the other medications was observed.

4. Conclusion

A high-performance liquid chromatography method has been developed for the simultaneous determination of ebastine and its metabolites in human plasma. Using this assay method, ebastine, carebastine and M–OH in human plasma can be quantified sensitively and simultaneously. Therefore, the developed method is considered to be applicable to pharmacokinetic and pharmacodynamic studies on ebastine in human subjects.

Acknowledgements

We would like to thank Professor Kazuhiko Yanai (Department of Pharmacology, Tohoku University School of Medicine, Miyagi, Japan) and Dr. Terumichi Fujikura (Department of Otorhinolaryngology, Yatsu Hoken Hospital, Chiba, Japan) for providing plasma samples from a healthy volunteer and a patient receiving ebastine. We are also grateful to Dr. Antoni Martínez-Tobed (Department of Pharmacokinetics and Drug Metabolism, Laboratorios Almirall, Barcelona, Spain) for his helpful advice.

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