BIOLOGICAL MASS SPECTROMETRY, VOL. 23, 385–390 (1994)

Identification of Rat Faecal Metabolites of Ebastine by *B/E* Linked Scanning Liquid Secondary Ion Mass Spectrometry

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The identification of rat faecal metabolites of a new antihistaminic agent, ebastine, 4′-*tert*-butyl-4-[4-(diphenylmethoxy)piperidino]butyrophenone, is presented. After oral administration of (^{14}C)ebastine (20 mg kg^{-1}) to rats, 84% of the radioactive dose was excreted in the 24 h faeces. Unchanged drug and five metabolites were isolated from the faeces by thin-layer chromatography and solid-phase extraction, and their structures were identified by liquid secondary ion mass spectrometry using the *B/E* linked scanning technique. The main metabolic pathways were oxidation of a terminal methyl group to give the hydroxymethyl and carboxyl derivatives, and hydroxylation of a phenyl ring in the diphenylmethoxy moiety. In addition to the oxidative mechanism, metabolism of ebastine involved sulphate conjugation. It is noteworthy that M-4, having both phenolic and alcoholic hydroxyl groups, was sulphated selectively in the latter position.

INTRODUCTION

Ebastine, 4′-*tert*-butyl-4-[4-(diphenylmethoxy)piperidino]butyrophenone (**1**), is a novel antihistaminic agent. A pharmacokinetic study in man has demonstrated[1] that during the first pass ebastine (**1**) is extensively metabolized to carebastine, 4-[4-[4-(diphenylmethoxy)-piperidino]butyryl]-α-methylhydratropic acid (**2**), which has been shown to be the actual active principle possessing a potent antihistamine activity.[2] However, little has appeared concerning the additional metabolic pathways of ebastine (**1**).

This paper describes the identification and characterization of rat faecal metabolities of orally administrated (^{14}C)ebastine by liquid secondary ion (LSI) mass spectrometry using the constant *B/E* linked scanning technique.[3]

EXPERIMENTAL

Materials

(^{14}C)Ebastine labelled uniformly in the benzene ring of the diphenylmethoxy moiety with a specific radioactivity of 1.96 MBq (53.1 μCi)/mg was synthesized in our laboratories according to the procedure described earlier.[4] Non-labelled ebastine (**1**) and carebastine (**2**) were a gift from Laboratories Almirall (Barcelona, Spain). 4′-*tert*-Butyl-4-[4-[(4-hydroxyphenyl)phenylmethoxy]piperidino]butyrophenone (**3**) was obtained by hydrolysis of its corresponding acetate (**4**) prepared by reaction of *p*-acetoxybenzhydryl bromide[5] with 4′-*tert*-butyl-4-[4-hydroxypiperidino]butyrophenone.[4] An *O*-methyl ether of **3** (**5**) was prepared by methylation of **3** with diazomethane.

Animal studies

(^{14}C)Ebastine was administrated orally as a suspension in 0.5% tragacanth gum solution to three male Wistar rats weighing about 220 g in a dose of 20 mg kg^{-1}. Faeces were collected over 24 h in metabolism cages.

Separation and purification

Faeces was homogenized in 5 vol. (v/w) of water. The homogenate was mixed with 4 vol. of methanol and centrifuged; this procedure was repeated four times. The combined supernatant solution was evaporated to dryness under reduced pressure, and the residue was subjected to thin-layer chromatography (TLC) on silica gel 60 F_{254} of 0.25 mm thickness (E. Merck, Darmstadt, Germany) in solvent A, $CHCl_3$/n-hexane/acetic acid (1:1:2; v/v/v). The thin-layer radiochromatogram gave five peaks. Each peak component, designated as **M-1** to **M-5** in descending order of the R_f value, was scraped off and extracted with methanol. Further purification on these crude metabolites was carried out as follows.

The crude **M-1** was immersed in water and extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated to dryness to give **M-1**.

The crude **M-2** and **M-3** were purified by solid-phase extraction using a Bond Elut C_{18} cartridge (Analytichem, Harbor city, California, USA). After successive washing with water and methanol, each cartridge was eluted with methanol/acetic acid (20:1, v/v).

The crude **M-4** was purified by repeated preparative TLC in solvent A, and then by solid-phase extraction as described.

The crude **M-5** was purified by repeated preparative TLC in solvent B, n-butanol/acetic acid/water (4:1:1; v/v/v), and in solvent C, $CHCl_3$/methanol/aq. ammonia (20:20:1; v/v/v). On the latter TLC, **M-5** gave two radioactive zones: **M-5a** and **M-5b**, having R_f value of

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CCC 1052-9306/94/070385-06

Received 20 December 1993
Revised 28 February 1994

0.56 and 0.46, respectively. Each zone was extracted with methanol and concentrated to dryness. The residue was dissolved in water and applied to solid-phase extraction using a bond Elut C_{18} cartridge. After washing with water, each cartridge was eluted with methanol.

Enzymatic hydrolysis

Incubations of purified **M-5a** and **M-5b** with sulphatase (from abalone entrails, type VIII, Sigma, St Louis, Missouri, USA) were carried out in 0.2 M acetate buffer (pH 5.0) at 37 °C for 24 h. After addition of 4 vol. ice-cooled ethanol and centrifugation, the supernatant was evaporated. The residue was dissolved in methanol and subjected to TLC.

Radioactivity measurement

Faecal homogenate was solubilized in Soluene 350 (Packard Ins., Co., Meriden, Connecticut, USA) and mixed with Dimilume 30 scintillation cocktail (Packard Ins., Co.). Aquasol-2 (NEN Research Products, Boston, Massachusetts, USA) was used as a scintillator for measurement of the extracted radioactivity in the organic layer. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer model 2200 CA using a quenching correction by gamma standardization.

The developed TLC plates were scanned for radioactivity using an automatic linear analyser (Laboratoirium Berthold, Wildbad, Germany).

Mass spectrometry

Electron impact (EI) and isobutane chemical ionization (CI) mass spectra were recorded on a JMS-D300 double-focusing mass spectrometer (JEOL, Tokyo, Japan) using 70 and 200 eV electrons, respectively. LSI mass spectra and linked-scan spectra at constant *B/E* (*B/E* spectra) were recorded on a Hitachi M-80B double-focusing mass spectrometer (Hitachi Ltd, Tokyo, Japan) using Xe^+ of 8 keV as primary ions, and either glycerol or *m*-nitrobenzyl alcohol (NBA) as the liquid matrix. No collision gas was used for obtaining the *B/E* spectra. Samples were introduced into mass spectrometers using the direct inlet probe.

^{1}H Nuclear magnetic resonance

The ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectrum of **M-3** was measured in $CDCl_3$ solution on a Varian XL-300 NMR spectrometer (Varian, Palo Alto, California, USA) with tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

Radiochromatogram

Twenty-four hours after administration of (^{14}C)ebastine to rats, faeces contained 84% of dosed radioactivity. The methanol extract of faeces contained more than 90% of the faecal radioactivity and gave five peaks on the thin-layer radiochromatogram (**M-1** to **M-5**, Fig. 1). Of the five peak components, the least polar two, **M-1** and **M-2**, had R_f values corresponding to those of compounds **1** and **2**, respectively.

B/E spectra of ebastine and related compounds

For a successful characterization of a metabolite by linked-scan mass spectrometry, it is essential to select an appropriate ionization technique which provides an abundant molecular ion (M^+) or protonated molecule (MH^+) for the parent compound. Ebastine (**1**) as well as synthetic compounds **2–5** were examined by EI, isobutane CI and LSI mass spectrometry. The latter was found to yield abundant MH^+ ions for compounds **1–5**, whereas EI hardly yielded an M^+ ion and isobutane CI failed to give an MH^+ ion of compound **2**. These findings, coupled with the TLC data indicating that some of the metabolites to be identified are highly polar, suggested that LSI ionization is appropriate for the present study.

For obtaining a good-quality LSI spectrum, it is important to use an optimum matrix compound. We selected glycerol and NBA as candidates for the present study, since both are versatile matrices[6,7] being widely used in LSI mass spectrometry. The LSI spectrum of 1 μg of compound **1** in 2 μl glycerol exhibited an MH^+ ion at *m/z* 470, but the spectrum was dominated by peaks due to the matrix. Whereas the corresponding spectrum using NBA displayed a distinct MH^+ ion, despite its absolute intensity it was comparable to that obtained using glycerol. A low 'chemical noise' spectrum would facilitate the assignment of an MH^+ ion of a metabolite, which is also relevant for the present study. We therefore selected NBA as the matrix for the analysis of metabolites other than **M-1a** and **M-1b**. The NBA was replaced by glycerol for the two polar metabolites, considering that their solubilities in NBA would be low compared to those in glycerol.

The *B/E* spectrum of compound **1** exhibited product ions at *m/z* 161, 167 and 203, as shown in Table 1. The *m/z* 161 ion (type A ion) and *m/z* 167 ion (type B ion) corresponded to *tert*-butylbenzoyl and phenyltropylium

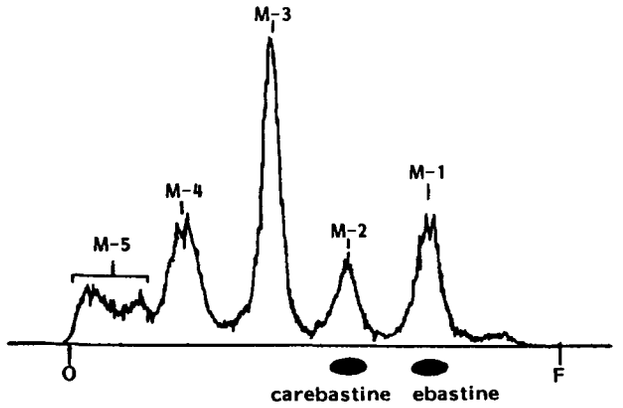
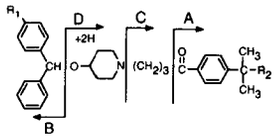


Figure 1. Radiochromatogram of a methanol extract of 24 h pooled faeces after oral administration of (^{14}C)ebastine (20 mg kg^{-1}) in rats. Solvent system: $CHCl_3$/n-hexane/acetic acid (1 : 1 : 2; v/v/v).

Table 1. Summary of *B/E* spectra of compounds 1–5 (matrix: *m*-nitrobenzyl alcohol)



1: $R_1 = H$; $R_2 = CH_3$ (ebastine)
2: $R_1 = H$; $R_2 = COOH$ (carebastine)
3: $R_1 = OH$; $R_2 = CH_3$
4: $R_1 = OCOCH_3$; $R_2 = CH_3$
5: $R_1 = OCH_3$; $R_2 = CH_3$

Cpd	Mol. wt	Product ion (relative intensity %) A	B	C	D	Others[a]
1	469	*m/z* 161(10)	*m/z* 167(100)	*m/z* 203(38)		
2	499	191(13)	167(100)	233(59)		
3	485	161(12)	183(74)	203(64)	*m/z* 304(100)	
4	527	161(11)	225(55)	203(49)		*m/z* 485(6), 183(100)
5	499	161(7)	197(100)	203(31)		

[a] Ions of less than 5% relative intensity were omitted.

ions, respectively. The *m/z* 203 ion (type C ion) resulted from cleavage of the C—N bond between the piperidine ring and the butyrophenone group with charge retention on the latter. Since ions corresponding to the type A, B and C ions of compound **1** were commonly prominent in the *B/E* spectra of compounds **2–5** (Table 1), they could be used as diagnostic ions indicating the moiety in which the metabolic modification occurred. It was noteworthy that the *B/E* spectrum of compound **3** exhibited another significant ion at *m/z* 304 (type D ion) corresponding to protonated 4-'*tert*-butyl-4-[4-hydroxypiperidino]butyrophenone. This ion was actually absent in the *B/E* spectra of compounds **1**, **4** and **5**, suggesting the type D ion to be indicative of a hydroxy group in the diphenylmethoxy moiety of a metabolite.

Identification of metabolites

Although purified as described above, **M-1** gave many peaks in the mass spectrum (Fig. 2a). Among them, the peak at *m/z* 470 attributable to an MH^+ ion of the unchanged drug was observed, whereas the diagnostic ions, type A–C ions, were obscure. On the other hand, type A–C ions were clearly visible in the *B/E* spectrum (Fig. 2b) at the same masses as those of compound **1**, thus confirming that **M-1** is unchanged ebastine (**1**).

M-2 was attributed to carebastine (**2**) by its mass spectrum exhibiting an MH^+ ion at *m/z* 500 (Fig. 3a). This assignment was confirmed by comparison of the *B/E* spectrum (Fig. 3b) with that of compound **2**.

The peak at *m/z* 349 in Fig. 3(b) could be an extraneous peak derived from a faecal contaminant; extraneous ions are frequently observed in *B/E* linked scans, owing to the poor precursor ion resolution.

The mass spectrum of **M-3** exhibited an MH^+ ion at *m/z* 516 (Fig. 4a), which is 16 u higher than that of **M-2**. The increase of 16 u strongly indicated that **M-3** is a mono-oxygenated **M-2**. It was readily apparent that the oxygenation had to be hydroxylation in the diphenylmethoxy moiety because **M-3** gave a type D ion (*m/z* 334) and a type B ion at *m/z* 183 (+16 u) in the *B/E* spectrum (Fig. 4b). In addition, type A and C ions were observed in the spectrum at the same *m/z* values as those of **M-2** (*m/z* 191 and 233, respectively), indicating no change had occurred in the α-methylhydratropic acid moiety. Furthermore, the ^{1}H-NMR spectrum of **M-3** exhibited A_2B_2 type signals at $\delta = 6.80$ and 7.17 characteristic of a *p*-hydroxyphenyl group (Fig. 5). Based on the mass and NMR spectral evidence, **M-3** was finally assigned as a mono-hydroxylated **M-2**, 4-[4-[4-(4-hydroxyphenyl)phenylmethoxy)piperidino]-butyryl]-α-methylhydratropic acid.

The mass spectrum of **M-4** exhibited an MH^+ ion at *m/z* 502 (Fig. 6a). The MH^+ ion at *m/z* 502 represented an increase in mass of 32 u over that of **M-1**, indicating a di-oxygenated **M-1**. The *B/E* spectrum of *m/z* 502 (Fig. 6b) exhibited type A, B, C and D ions at *m/z* 177, 183, 219 and 320, respectively, all of which except the type D ion are 16 u higher than those of **M-1**. The increase in mass of 16 u of the type B ion along with the presence of the type D ion in the spectrum indicated a hydroxyl group in the diphenylmethoxy moiety, and the

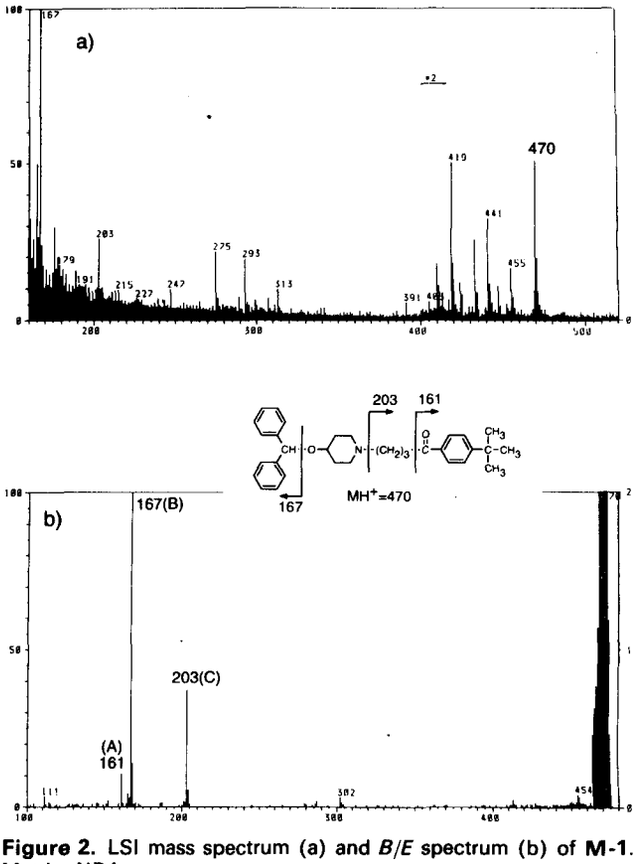


Figure 2. LSI mass spectrum (a) and *B/E* spectrum (b) of **M-1**. Matrix: NBA.

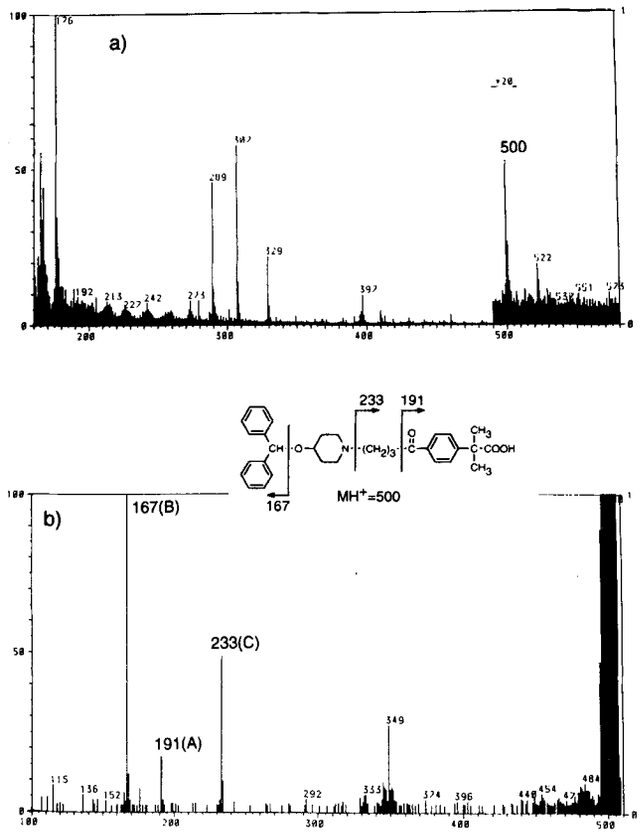


Figure 3. LSI mass spectrum (a) and *B/E* spectrum (b) of **M-2**. Matrix: NBA.

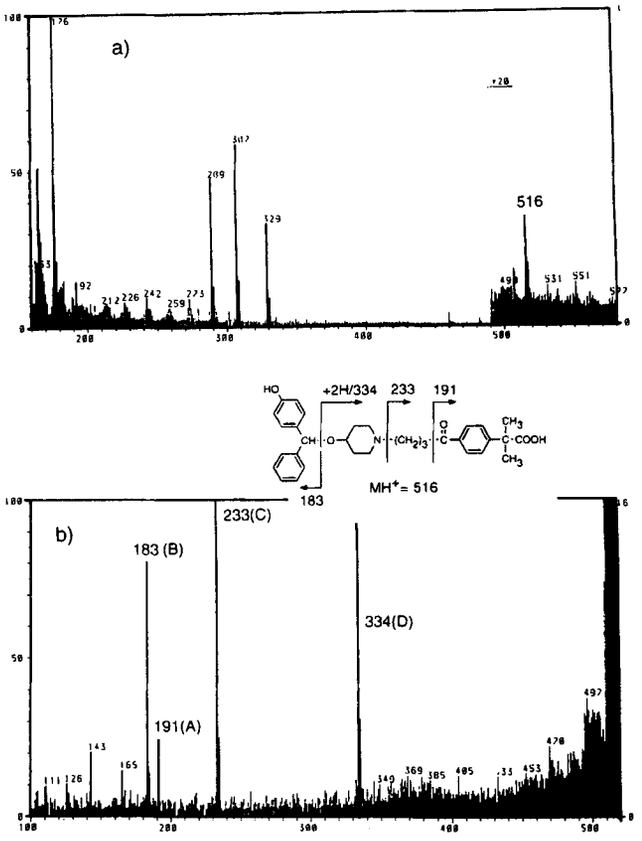


Figure 4. LSI mass spectrum (a) and *B/E* spectrum (b) of **M-3**. Matrix: NBA.

increase of 16 u of both type A and C ions indicated oxygenation in the *tert*-butylbenzoyl moiety of the molecule. This oxygenation was finally defined as hydroxylation in the terminal methyl group since **M-4**, when treated with diazomethane, afforded a mono-methylated **M-4** (MH^+ ion at *m/z* 516), whose *B/E* spectrum showed an increase in mass of 14 u (corresponding to methylation) only in the type B ion and did not show any change in masses of either type A or C ions. Therefore, **M-4** was assigned as a di-hydroxylated **M-1** possessing one hydroxyl group in the diphenylmethoxy moiety and the other in the terminal hydroxymethyl group.

M-4 could be considered as an intermediate metabolite yielding **M-3**, and hence the phenolic hydroxyl group of **M-4** would locate at the *para* position.

M-5a gave prominent ions at *m/z* 566, 588 and 610 in the mass spectrum (Fig. 7a). These ions appeared at 22 u intervals and were attributed to MH^+, $M + Na^+$ and $[M - H + 2Na]^+$ of the metabolite, respectively. The MH^+ of mass 566, which was verified by the negative LSI mass spectrum showing a molecular anion ($[M - H]^-$) at *m/z* 564, is 96 u higher than that of **M-1**,

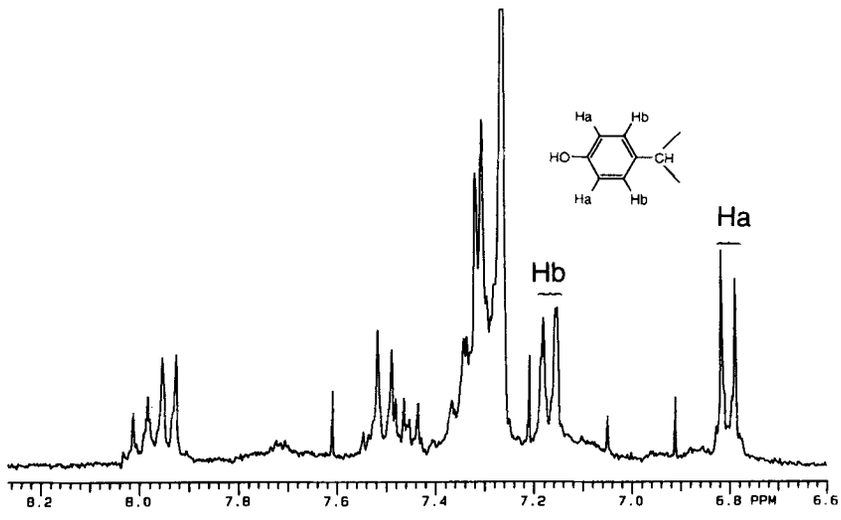


Figure 5. Partial NMR spectrum of **M-3**. Solvent: $CDCl_3$.

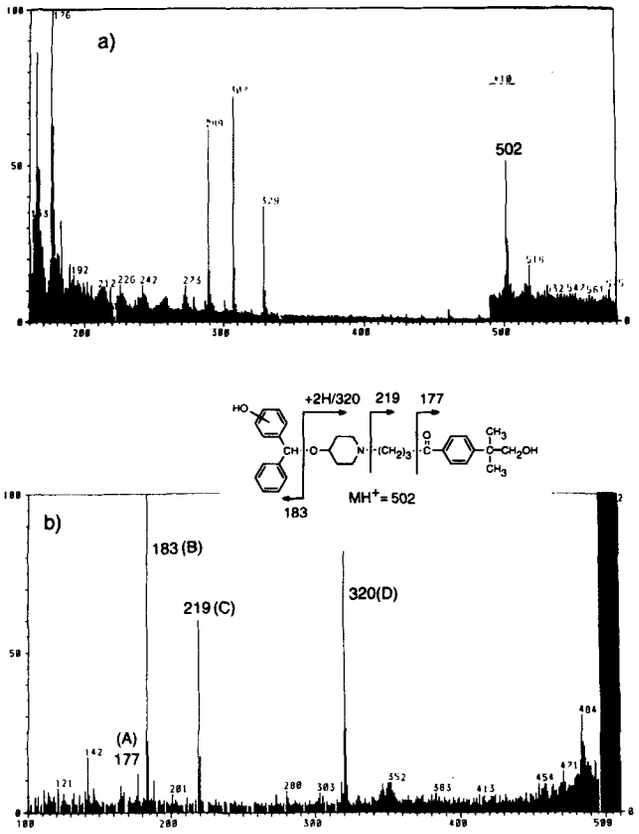


Figure 6. LSI mass spectrum (a) and *B/E* spectrum (b) of **M-4**. Matrix: NBA.

suggesting that hydroxylation (+16 u) and subsequent sulphate conjugation (+80 u) had occurred in the drug. The *B/E* spectrum of the MH^+ ion at *m/z* 566 (Fig. 7b) exhibited product ions at *m/z* 167 (type B ion), 299 (type C ion) and 486 ($MH^+ - SO_3$). The *m/z* 486 ion characterized the sulphate structure for **M-5a**, since loss of SO_3 from MH^+ is considered to be a common fragmentation in the LSI mass spectra of sulphates.[8] The type B ion representing the diphenylmethoxy moiety of the molecule remained unchanged to that of **M-1**, while the type C ion showed a shift by 96 u to *m/z* 299, indicating that the *O*-sulphate group is located in the *tert*-butylbenzoyl moiety of the molecule. Furthermore, **M-5a** was not hydrolysed by arylsulphatase, corroborating that the metabolite is not a phenolic sulphate but an alcoholic sulphate. **M-5a** was therefore assigned as a sulphate conjugate of the hydroxymethyl derivative of **M-1**, 2-methyl-2-[4-[4-[4-(diphenylmethoxy)piperidino]-butyryl]phenyl]propyl hydrogensulphate.

The mass spectrum of **M-5b** (Fig. 8a) exhibited an MH^+ ion at *m/z* 582 and corresponding sodium adducts at *m/z* 604 and 626. The MH^+ ion at *m/z* 582 represented an increase of 16 u over that of **M-5a**, indicating a monopoxygenated **M-5a**. This oxygenation was attributed to hydroxylation in the diphenylmetoxy moiety on the basis of fragmentation shown in the *B/E* spectrum: the presence of a type D ion (*m/z* 400) and a type B ion at *m/z* 183 (+16 u). Similarly to **M-5a**, the alcoholic sulphate structure for **M-5b** was indicated by the presence of ions of masses of 299 (type C ion) and 502 ($MH^+ - SO_3$) in the *B/E* spectrum, and corroborated by an enzymatic test. Therefore, **M-5b** was assign-

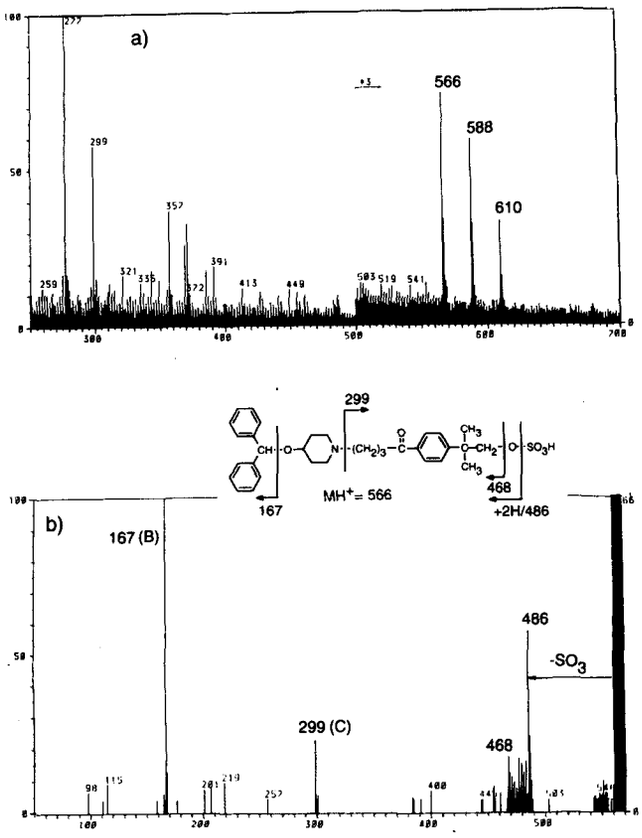


Figure 7. LSI mass spectrum (a) and *B/E* spectrum (b) of **M-5a**. Matrix: glycerol.

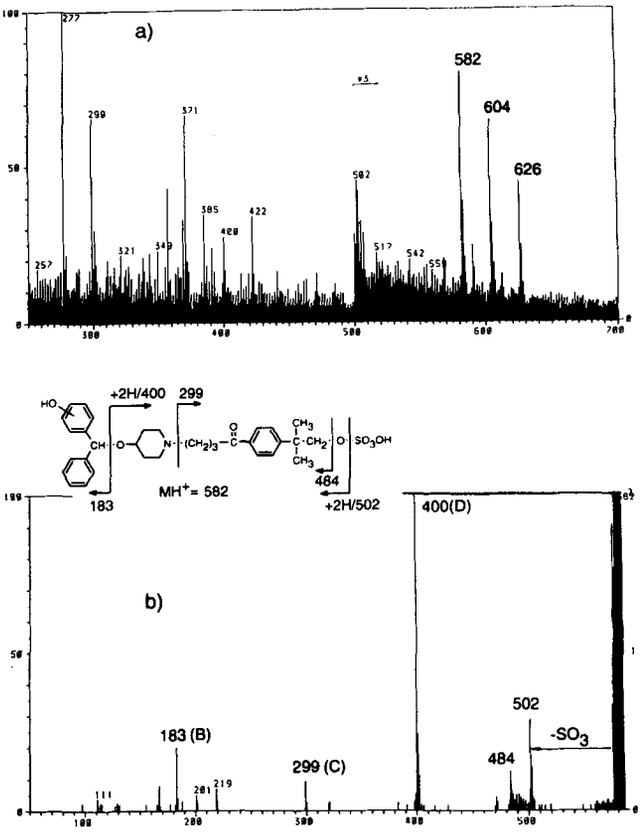
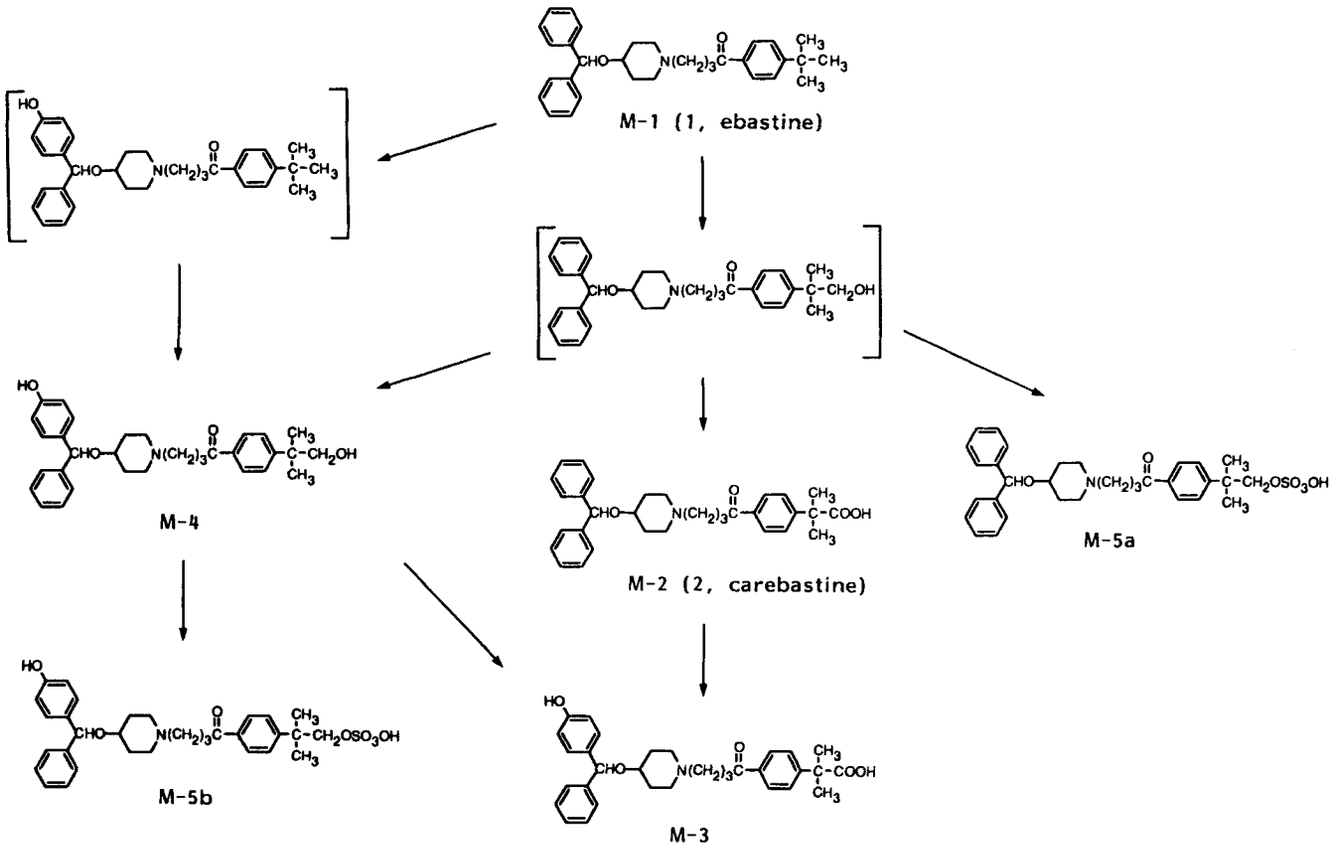


Figure 8. LSI mass spectrum (a) and *B/E* spectrum (b) of **M-5b**. Matrix: glycerol.



Scheme 1. Possible metabolic pathway of ebastine in rats. Brackets indicate a presumed intermediate.

ed to be hydroxylated **M-5a** or an alcoholic sulphate conjugate of **M-4**.

Possible metabolic pathways

The present study demonstrated that the major metabolic pathways of ebastine in rats are oxidation in the terminal methyl group to give hydroxylmethyl and carboxyl derivatives, and hydroxylation in a phenyl ring of the diphenylmethoxy moiety. The hydroxymethyl derivative, although not detected in the present study, could be an intermediate in the formation of **M-2** (carebastine) and **M-4**. Likewise, a hydroxyebastine with a phenol group in the diphenylmethoxy moiety is a possible precursor of **M-4**. In addition to the oxidative pathways, sulphate conjugation giving **M-5a** and **M-5b** was also observed. Therefore, the metabolic pathway of ebastine (**1**) in rats can be summarized as in Scheme 1. It is worth noting that **M-4** having both phenolic and alcoholic hydroxyl groups is sulphated selectively in the latter, although phenolic sulphates appear to be more common metabolites in rodents.

Acknowledgements

The authors wish to thank Dr J. Matsumoto, director of our laboratory, for his support to this work, and Mrs C. Sasaki for technical assistance.

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