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Identification of metabolites of halofantrine, a new candidate anti-malarial drug, by gas chromatography–mass spectrometry

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ABSTRACT

Two previously unknown metabolites of halofantrine, a candidate anti-malarial drug, have been isolated by thin-layer chromatography from the plasma of dogs administered a single oral dose of 60 mg/kg. Their identities were investigated after trimethylsilylation by gas chromatography–mass spectrometry under electron-impact and negative-ion chemical ionization conditions. The structural assignment was further confirmed by using a combination of elemental composition analysis of all the isotope peaks at low mass resolution and isotope pattern matching. These two metabolites were formed by modification of the dibutylaminopropyl side-chain of the parent compound involving deamination and oxidation or reduction.

INTRODUCTION

Halofantrine (Fig 1) is a phenanthrene derivative which has been shown to have *in vitro* and *in vivo* activity against both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* [1] and is being developed as a new anti-malarial drug. As part of this development programme, investigations on metabolism of the drug have been performed in the dog using ^{14}C-labelled halofantrine.

Little is known about the metabolism of halofantrine in animal species although it has previously been found that its monodebutylated derivative is one of the major metabolites in the dog [2] and in man [3,4]. In the present study, two other unknown metabolites have been detected in plasma samples by thin-layer chromatography (TLC) followed by autoradiography. The identities of these two metabolites were examined as their trimethylsilyl derivatives by capillary gas chromatography–mass spectrometry (GC–MS).

Accurate mass measurements were also carried out in the GC–MS mode to characterise the ion structures of these metabolites. Although accurate mass measurement is a long established technique for elemental composition analysis [5,6], its

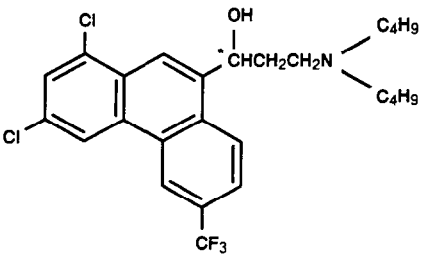

Fig. 1. Structure of halofantrine. The asterisk denotes the position of carbon-14.

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application is often restricted to measurements carried out at high resolution with extensive use of mass references. It is often difficult to apply this technique to GC–MS applications especially if capillary GC is employed. We report here how elemental composition can be determined from less precise data by considering all the isotope peaks within an ion cluster in addition to matching observed isotope patterns to theoretical calculations.

EXPERIMENTAL

Materials

[Carbinol-^{14}C]halofantrine (specific activity 14 Ci/mol) was supplied by Research Triangle Institute (Research Triangle Park, NC, USA). Unlabelled halofantrine and its monodebutylated derivative were supplied by the Walter Reed Army Institute of Research (Washington, DC, USA). N,O-Bis(trimethylsilyl)trifluoroacetamide containing 10% trimethylchlorosilane was purchased from Chrompack UK (London, UK). Pre-layered glass-backed Kieselgel F_{254} plates of layer thickness 0.25 mm were obtained from E. Merck (Darmstadt, Germany).

Sample collection and processing

Six male beagle dogs with body weights in the range of 11–15 kg were given an oral dose (60 mg/kg) of [^{14}C]halofantrine (specific activity 0.194 μCi/mg). Blood samples were collected at pre-determined time intervals (between 2 and 8 h post-dose) and the plasma was separated immediately by centrifugation. Acetonitrile extracts of a pooled plasma sample were examined by radiochromatographic analysis on Kieselgel F_{254} plates using chloroform–methanol–35% aqueous ammonia (70:30:1, v/v) as the solvent system under chamber saturation condition. Radiochromatograms of the plates were obtained and quantified using a Berthold automatic TLC linear analyser (Model No. 2842, Berthold UK, St. Albans, UK) linked to an IBM AT computer. For MS analysis, samples of metabolites were isolated from extracts of pooled plasma by preparative TLC to obtain about 10 μg each of metabolite A (R_F 0.21) and metabolite B (R_F 0.29).

Derivatisation

The isolated metabolites (approximately 5 μg) were derivatised with N,O-bis(trimethylsilyl)trifluoroacetamide containing 10% trimethylchlorosilane (15 μl) and silylation-grade pyridine (10 μl) in capped vials. The vials were heated at 100°C for 10 min. The reaction mixtures were analysed directly by GC–MS.

Gas chromatography–mass spectrometry

Analysis of the trimethylsilyl (TMS)-derivatized samples were performed on a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Wokingham, UK) fitted with a HP Ultra 1 (methyl silicone) 25 m × 0.25 mm I.D., 0.1 μm film thickness, fused-silica column coupled to a VG TS-250 mass spectrometer (VG Analytical, Manchester, UK). The samples (1 μl) were injected splitless at 250°C with the split valve opened 0.5 min after injection and the column temperature programmed from 100 to 300°C at 20°C/min. Helium was used as the carrier gas at an injector pressure of 103 kPa giving a linear velocity of 34 cm/s. Electron-impact (EI) mass spectra were acquired at 70 eV over the mass range 50–600 a.m.u. at a rate of 1 scan/s. In the chemical ionization (CI) mode isobutane was used as the reagent gas at an indicated source pressure of 0.03 Pa and a source temperature of 150°C. Negative-ion CI (NICI) spectra were acquired over the mass range 100–600 a.m.u. at a rate of 1 scan/s. The mass resolution in both cases was 800 (10% valley).

All data processing including determination of possible elemental compositions for various unknown fragment ions were computed using VG software on an 11-250J data system.

RESULTS AND DISCUSSION

In the analysis of halogen-containing compounds, CI is often the preferred ionization method for detecting negative ions because of the enhanced sensitivity and reduced background interferences over EI ionization. This technique is particularly useful in analysing crude extracts from biological matrices.

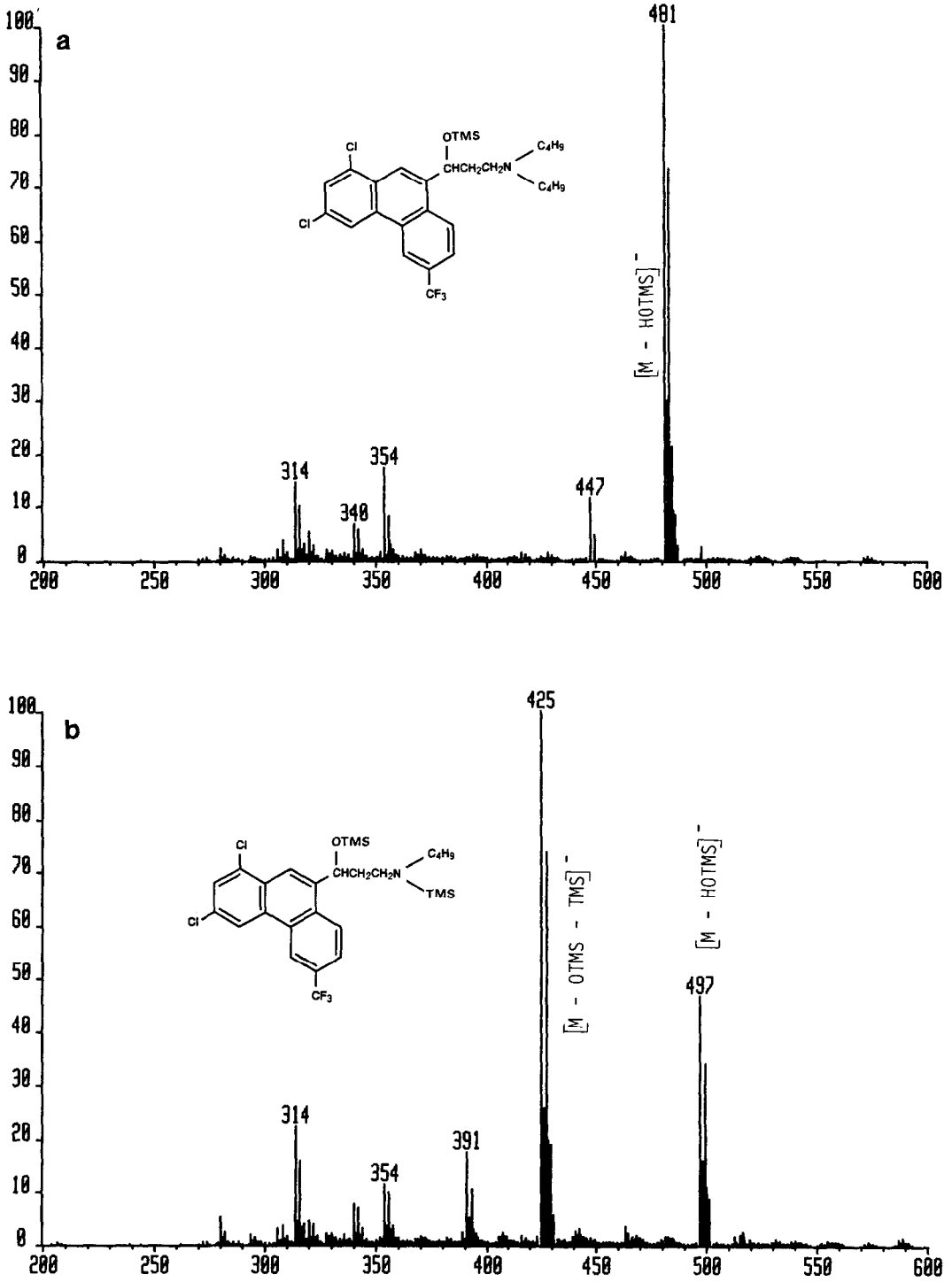

Fig. 2. NICI mass spectra of the TMS derivatives of (a) halofantrine and (b) N-desbutylhalofantrine.

The NICI spectrum of the trimethylsilylated derivative of halofantrine is given in Fig. 2a showing a weak (< 1%) molecular ion at m/z 571 and a base peak at m/z 481 representing loss of the neutral HO-Si$(CH_3)_3$ (HOTMS) fragment. Analysis of the diTMS derivative of the monobutyl analogue of halofantrine also gave a weak molecular ion (< 1%) at m/z 587 and prominent ions at m/z 497 and 425 corresponding to $[M - HOTMS]^-$ and $[M - OTMS - TMS]^-$, respectively (Fig. 2b).

Analysis of the TMS derivative of metabolite A in the EI mode gave a complex total-ion chromatogram (TIC) as expected from a crude extract. Although the metabolite contained two chlorine atoms which could be used as a marker, it was difficult to locate the metabolite peak in the chromatogram due to the level of impurities present in the sample. Analysis of the sample in the NICI mode gave an equally complex TIC but a reconstructed partial TIC of 400–600 a.m.u. gave only one major peak at 10.4 min (Fig. 3). The NICI mass spectrum of this GC peak is shown in Fig. 4. Only two major fragment ions (m/z 456 and 340) were observed in the mass spectrum. As these ions corresponded to the losses of TMS functional moieties, no structural information about the metabolite was apparent.

One special feature of the TS-250 sector mass spectrometer is the use of an air-core magnet which eliminates non-linearity and hysteresis effects [7]. Mass measurements carried out on these

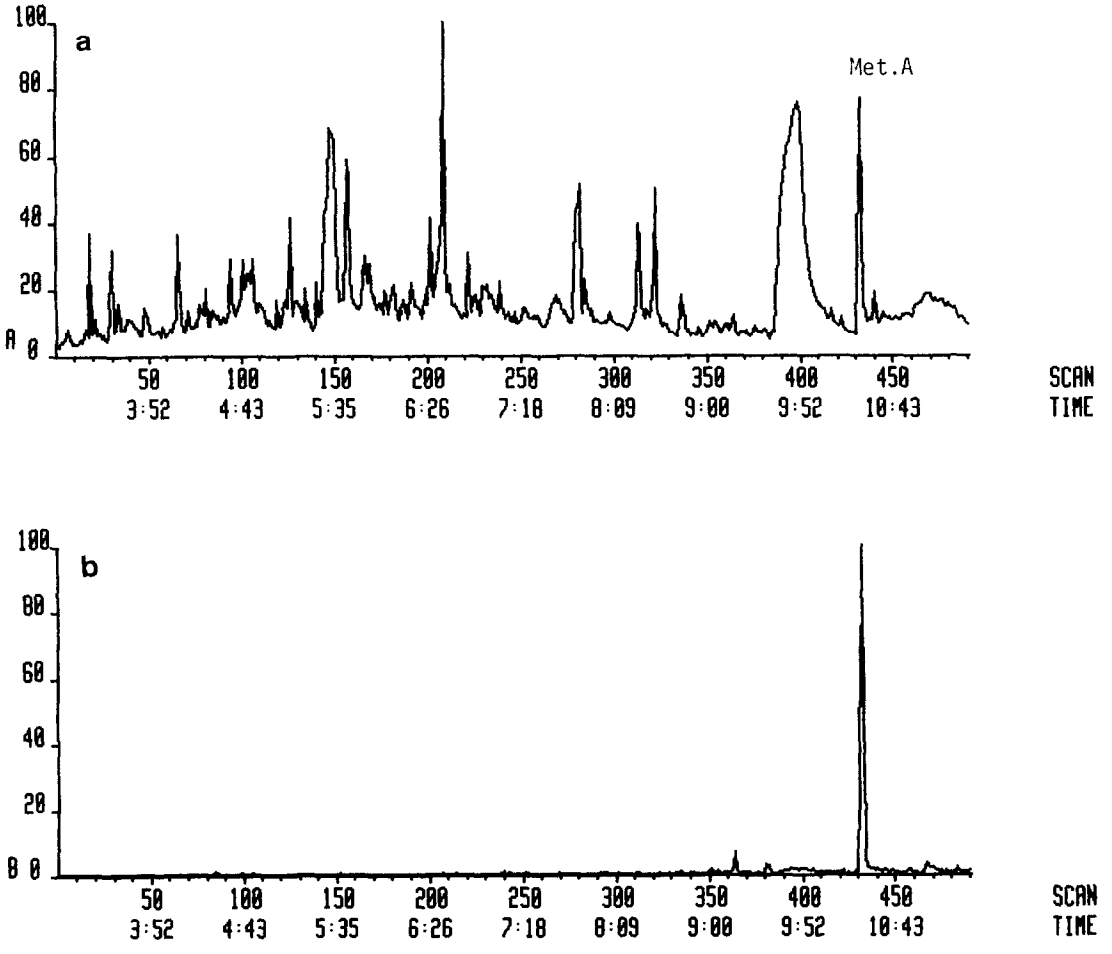

Fig. 3. (a) TIC and (b) partial TIC of m/z 400 to 600 obtained from NICI analysis of metabolite A after trimethylsilylation.

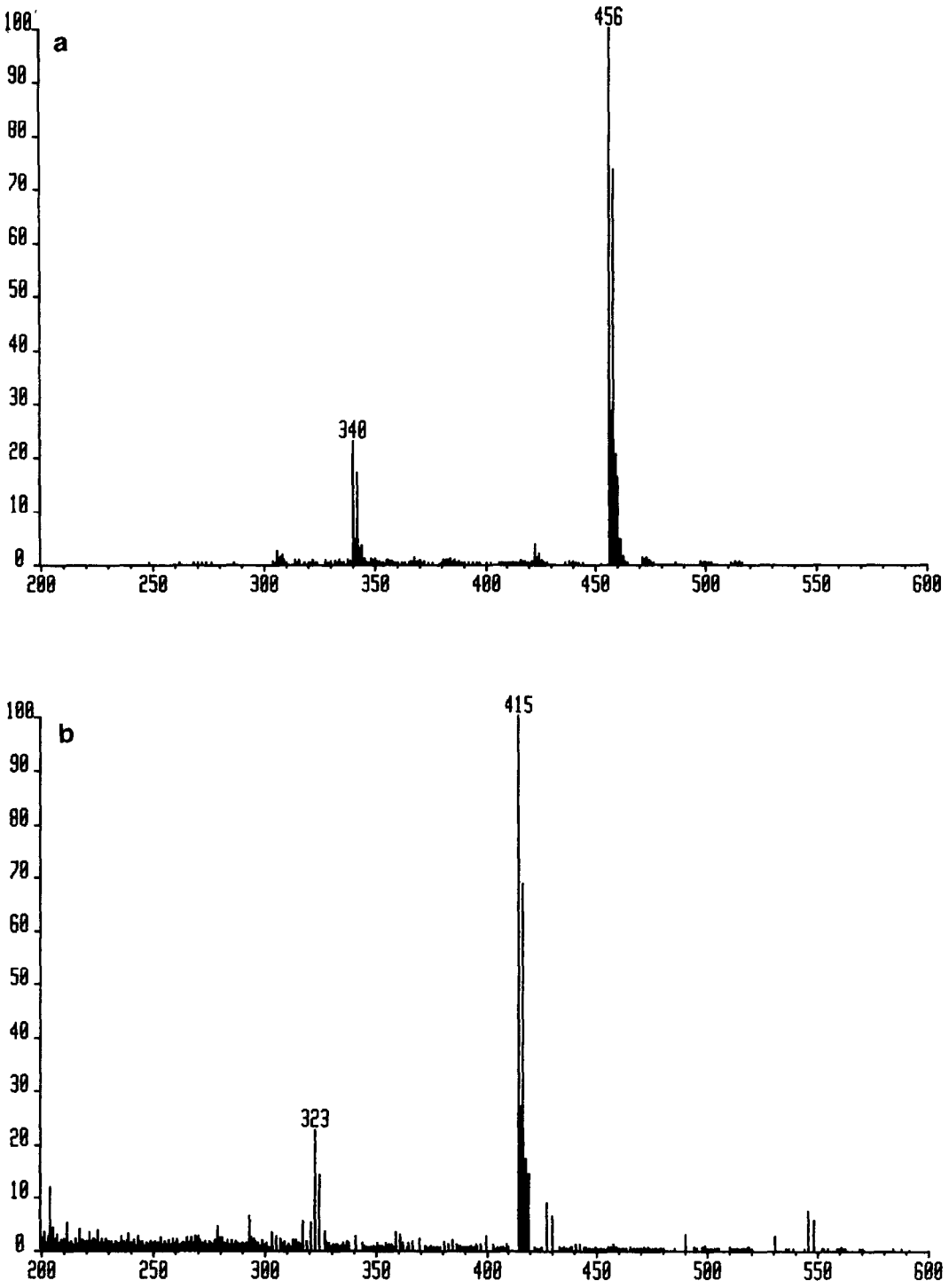

Fig. 4. (a) NICI and (b) positive-ion EI mass spectra of the TMS derivative of metabolite A.

instruments can be calculated directly from the magnet coil current without complicated calibration even at relatively high scan speeds used in GC–MS applications. However, results obtained under these conditions (often at low mass resolution) do not have sufficient precision for determining elemental compositions. In our experience, the errors are often up to 30–40 ppm.

Table I shows the elemental composition report of the ions at *m/z* 456–458 from the spectrum of metabolite A. In order to reduce the number of possible elemental formulae for each ion mass, we included a number of restrictive conditions for each isotope peak in the ion cluster. In the example given here, *m/z* 456 is expected to contain no ^{13}C, two ^{35}Cl and no ^{37}Cl, *m/z* 457 is expected to contain one ^{13}C, two ^{35}Cl and no ^{37}Cl, while *m/z* 458 would contain no ^{13}C, one ^{35}Cl and one ^{37}Cl. With the inclusion of such "isotope filters" as underlined in Table I, the only elemental formula which is common to all three isotope peaks is $C_{21}H_{17}O_2F_3Cl_2Si$.

With reference to the mass spectra of halofantrine and its analogues (see Fig. 2), this fragment ion corresponded to the loss of HOTMS from the molecular ion. Hence the molecular formula of the derivatised metabolite was $C_{24}H_{27}O_3F_3Cl_2Si_2$. Based on this information, it could be deduced that the metabolite contained a side-chain of $-C_3H_5O_3$ and the proposed structure is shown in Fig. 6. Further confirmation of the fragment ion structure (*m/z* 456) was obtained by the good agreement between the observed and the theoretical isotope pattern calculated using a computer algorithm as shown in Table II. Although the halofantrine used in the dose contained carbon-14, the percentage of carbon-14 present was insignificant (<0.2%).

The presence of a carboxylic functional group in the metabolite was supported by the fragment ion at *m/z* 340 (see Fig. 4a) which corresponded to the sequential loss of –OTMS and $-CO_2TMS$ fragments from the molecular ion. Finally the EI spectrum of this trimethylsilylated metabolite is

TABLE I

ELEMENTAL COMPOSITION REPORT OF THE IONS AT *m/z* 456 TO 458 FROM THE SPECTRUM OF METABOLITE A

Many of these elemental formulae can be eliminated by only accepting those with the expected number of $^{12}C/^{13}C$ and $^{35}Cl/^{37}Cl$ isotopes as underlined.

Observed mass	^{12}C	^{13}C	H	N	O	F	^{35}Cl	^{37}Cl	Si	MMU[a]	DBE[a]
458.0455	22	0	19	0	1	3	0	2	1	2.0	12.0
	21	1	18	0	1	3	0	2	1	−2.4	12.5
	23	0	18	0	1	3	1	0	2	8.2	15.0
	21	1	20	0	1	3	1	1	1	16.2	11.5
	20	0	19	0	1	3	2	0	2	−15.1	11.0
	19	1	18	0	1	3	2	0	2	−19.6	11.5
	22	1	18	0	2	3	0	2	0	15.5	12.5
	21	0	17	0	2	3	1	1	1	−15.8	12.0
	21	0	19	0	2	3	2	0	1	2.8	11.0
	20	1	18	0	2	3	2	0	1	−1.6	11.5
	21	0	17	1	1	3	0	2	1	−10.5	12.5
	20	1	16	1	1	3	0	2	1	−15.0	13.0
	21	0	19	1	1	3	1	1	1	8.1	11.5
	20	1	18	1	1	3	1	1	1	3.6	12.0
	22	0	17	1	2	3	0	2	0	7.4	12.5
	21	1	16	1	2	3	0	2	0	3.0	13.0
	20	0	17	1	2	3	2	0	1	−9.7	11.5
	19	1	16	1	2	3	2	0	1	−14.2	12.0

TABLE I (*continued*)

Observed mass	^{12}C	^{13}C	H	N	O	F	^{35}Cl	^{37}Cl	Si	MMU[a]	DBE[a]
457.0496	22	0	18	0	1	3	0	2	1	−10.0	12.5
	21	1	17	0	1	3	0	2	1	−14.4	13.0
	22	0	20	0	1	3	1	1	1	8.6	11.5
	21	1	19	0	1	3	1	1	1	4.2	12.0
	23	0	18	0	2	3	0	2	0	8.0	12.5
	22	1	17	0	2	3	0	2	0	3.6	13.0
	21	0	18	0	2	3	2	0	1	−9.1	11.5
	20	1	17	0	2	3	2	0	1	−13.6	12.0
	21	0	18	1	1	3	1	1	1	−3.9	12.0
	20	1	17	1	1	3	1	1	1	−8.4	12.5
	21	0	20	1	1	3	2	0	1	14.7	11.0
	20	1	19	1	1	3	2	0	1	10.2	11.5
	19	1	20	1	2	3	0	1	2	18.1	11.5
	22	0	16	1	2	3	0	2	0	−4.5	13.0
	21	1	15	1	2	3	0	2	0	−9.0	13.5
	22	0	18	1	2	3	1	1	0	14.1	12.0
	21	1	17	1	2	3	1	1	0	9.6	12.5
456.0345	22	0	17	0	1	3	0	2	1	−2.7	13.0
	21	1	16	0	1	3	0	2	1	−7.1	13.5
	22	0	19	0	1	3	1	1	1	15.9	12.0
	21	1	18	0	1	3	1	1	1	11.5	12.5
	20	0	17	0	1	3	2	0	2	−19.8	12.0
	23	0	17	0	2	3	0	2	0	15.3	13.0
	22	1	16	0	2	3	0	2	0	10.8	13.5
	21	0	17	0	2	3	2	0	1	−1.9	12.0
	20	1	16	0	2	3	2	0	1	−6.3	12.5
	21	0	15	1	1	3	0	2	1	−15.3	13.5
	20	1	14	1	1	3	0	2	1	−19.7	14.0
	21	0	17	1	1	3	1	1	1	3.3	12.5
	20	1	16	1	1	3	1	1	1	−1.1	13.0
	20	1	18	1	1	3	2	0	1	17.5	12.0
	22	0	15	1	2	3	0	2	0	2.7	13.5
	21	1	14	1	2	3	0	2	0	−1.7	14.0
	21	1	16	1	2	3	1	1	0	16.9	13.0
	20	0	15	1	2	3	2	0	1	−14.4	12.5
	19	1	14	1	2	3	2	0	1	−18.9	13.0

[a] MMU and DBE refer to errors in milli-mass units and number of double bond equivalent, respectively.

given in Fig. 4b showing the molecular ion at m/z 546 and the base peak at m/z 415 representing $[M - CH_3 - CO_2TMS + H]^+$.

The analysis of unknown metabolite B under the conditions as described gave a GC peak at 10.6 min. Its NICI spectrum is given in Fig. 5 showing a base peak at m/z 458 and the sequential loss of another TMS group to m/z 385 which could suggest the presence of a keto-alcohol structure. A tentative structure proposed for this metabolite is shown in Fig. 6.

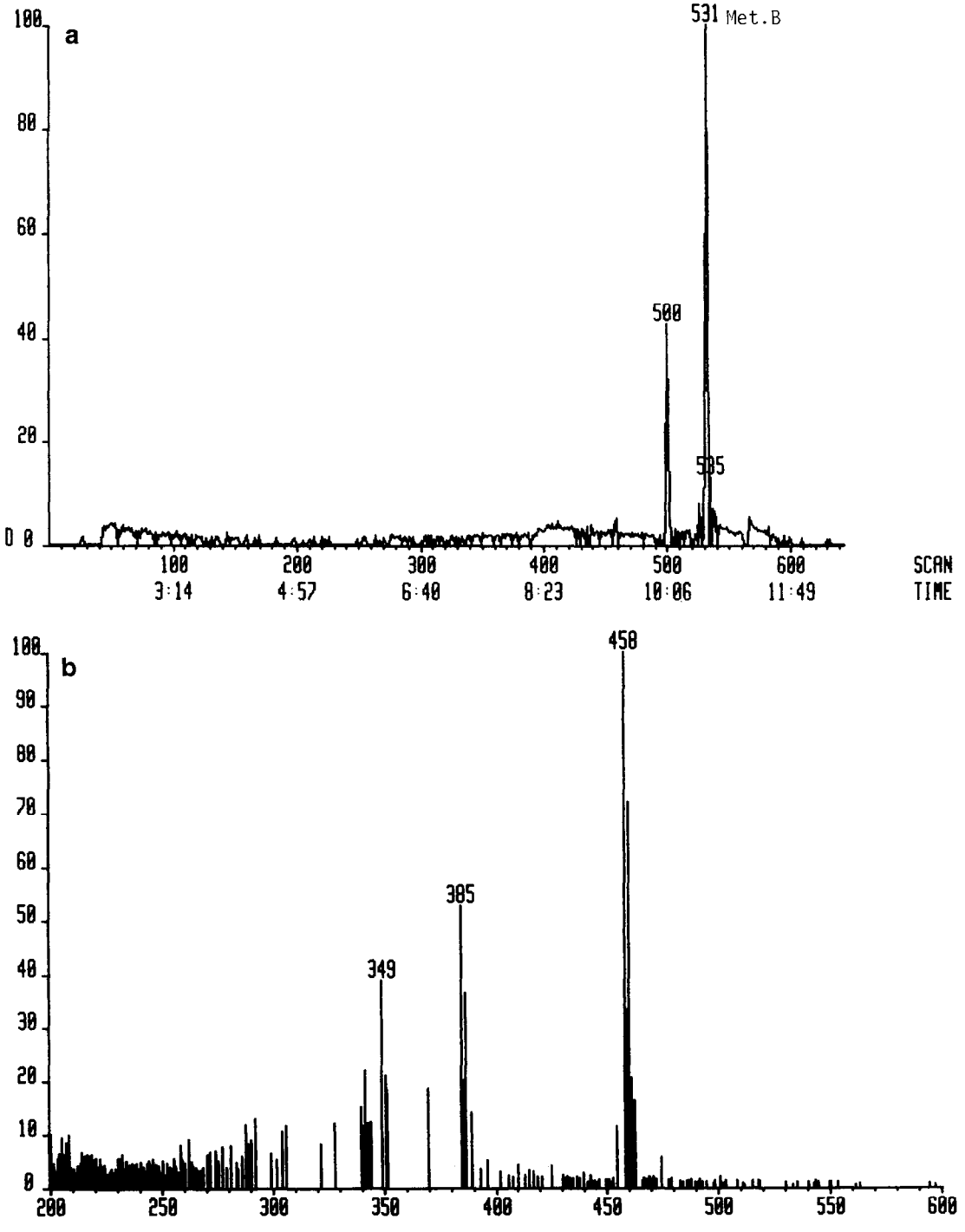

Fig. 5. (a) Summed ion chromatogram of m/z 458 and 460 of metabolite B after trimethylsilylation and (b) NICI mass spectrum of the GC peak at 10.6 min.

TABLE II

THEORETICAL *VERSUS* OBSERVED ISOTOPE PATTERN OF THE PROPOSED FRAGMENT ION STRUCTURE FOR METABOLITE A

m/z	Relative intensity (%)	
	Theoretical	Observed
456	100.0	100.0
457	29.1	28.7
458	71.7	73.9
459	19.8	20.4
460	15.3	16.2
461	3.8	4.6
462	0.9	1.6

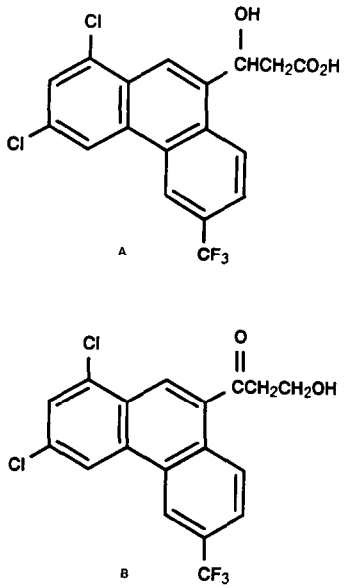

Fig. 6. Proposed structures for metabolites A (upper) and B (lower).

These two previously unknown metabolites of halofantrine were formed by modification of the dibutylaminopropyl side-chain involving deamination followed by oxidation or reduction. A full report on the metabolism of halofantrine in the dog will be published elsewhere.

CONCLUSIONS

Two previously unknown dog metabolites of halofantrine have been identified in plasma by GC–MS. The use of NICI gave improved sensitivity but structural elucidation was difficult because very few fragment ions were observed in the spectrum. The incorporation of an air-core magnet design in a sector mass spectrometer is unique for performing accurate mass measurements in GC–MS applications. We have demonstrated here that less precise mass measurements can still be used for determining elemental composition by examining other isotope peaks within the cluster. If the results give more than one possible elemental formula, the observed isotope pattern may be used to compare with the theoretical pattern in order to reduce the number of possibilities.

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