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Detection of Quinine and Its Metabolites in Horse Urine by Gas Chromatography–Mass Spectrometry

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After oral administration of quinine sulfate to a thoroughbred mare, seven urine samples were obtained over a 45.5 h period. Using gas chromatography–electron impact ionization and positive-ion chemical ionization mass spectrometry, quinine and five putative metabolites were detected and tentatively identified in enzyme-hydrolysed post-administration urine; all metabolites involved some form of oxidation. The parent drug could be detected for about 16 h and some phase I biotransformation products for up to 40 h post-administration.

Keywords: *Quinine; equine metabolites; horse urine; gas chromatography–mass spectrometry*

Introduction

The cinchona alkaloid quinine (**I**, Fig. 1) is widely used in the treatment of malaria in humans.[1] It is also used in veterinary medicine because of its potent analgesic and antipyretic properties.[2] However, its use is prohibited in race horses before racing because of its stimulant property and is restricted to medical purposes. Detection of its metabolites is important in equine forensic science as this compound can be used as an illegal stimulant. Quinine metabolism and breakdown have been studied in humans, rats and dogs,[3] but have not been described in horses. Therefore, a detailed knowledge of the biotransformation and excretion of quinine in thoroughbred horses was of particular interest for the development of screening and confirmatory methods to detect its use in horse racing.

Several different methods have been used to detect quinine and its breakdown products, including HPLC,[4,5] GC–MS[6] and NMR spectrometry.[7] Different extraction and derivatization procedures and ionization techniques have been used for the detection of metabolites in urine samples.

GC–MS has played a crucial role in the investigation of quinine metabolites in horse urine. It provides precise information on their structures and also can provide molecular mass information. In this work, we used GC–MS for the detection of quinine and its metabolites. Proposed structures were obtained by interpretation of mass spectrometric ions. Different ionization techniques yield different structural information, so a sensible approach is to combine electron impact (EI) ionization and positive chemical ionization (PCI) methods. The former technique provides largely fragment ions, with less information about molecular ion clusters, whereas the latter method is one of soft ionization, giving molecular mass information.

Samples of urine obtained from controlled feeding of a thoroughbred racehorse with quinine were analysed. In a preliminary report,[8] we described the detection of two metabolites of quinine. In this work, we extended the study to six metabolites, followed by using a time profile after administration of quinine, and a procedure was developed for the detection of the quinine and its major metabolites in horse urine based on solid-phase extraction (SPE), the formation of trimethylsilyl (TMS) derivatives and analysis by combined capillary column GC–MS in the EI and PCI modes.

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Experimental

Materials

All reagents were of analytical-reagent grade. Methanol was obtained from Rathburn (Walkerburn, UK), ammonia solution (32%) and glacial acetic acid from Merck (Poole, UK), anhydrous quinine from Fluka (Gillingham, Dorset, UK), *Helix pomatia* juice (mixture of β-glucuronidase and aryl sulfatase enzymes) from Uniscience (London, UK) and ethyl acetate, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma (Poole, UK). Bond-Elut Certify (10 ml) solid-phase extraction columns were obtained from Analytichem International (Harbor City, CA, USA) and supplied by Jones Chromatography (Hengoed, UK).

Animal Administration

Quinine sulfate (2.0 g; 1.55 g of quinine as free base) was administered orally with food to a thoroughbred mare (body mass 432 kg) and naturally voided urine samples were collected for up to 2 days after administration. The collection times of samples after administration are given in Tables 1 and 2. The samples are referred to as U_1–U_7. The urine samples were stored in polyethylene bottles at −20 °C until required for analysis.

Enzymic Hydrolysis of Conjugated Metabolites

Acetate buffer (0.2 mol l^{-1}, pH 4.8, 2.0 ml) was added to aliquots of blank and post-administration urines (5.0 ml) and the pH was adjusted to 4.8. The urine samples were then enzymically hydrolysed with *Helix pomatia* juice (25 μl) by incubation overnight at 37 °C.

Solid-phase Extraction

The extraction adapted in this study is a slightly modified form of the standard method for basic drugs described previously.[9–11] Hydrolysed urine samples were adjusted to pH 6.0 and phosphate buffer (0.1 mol l^{-1}, pH 6.0, 2.0 ml) was added. The SPE column was preconditioned under a vacuum manifold by washing with methanol (2.0 ml) and phosphate buffer (2.0 ml).

During this activation step, air must not reach the stationary phase. The samples were passed slowly through the activated column. The column was rinsed with acetic acid (1.0 mol l^{-1}, 1.0 ml), dried under full vacuum for at least 5 min, washed with

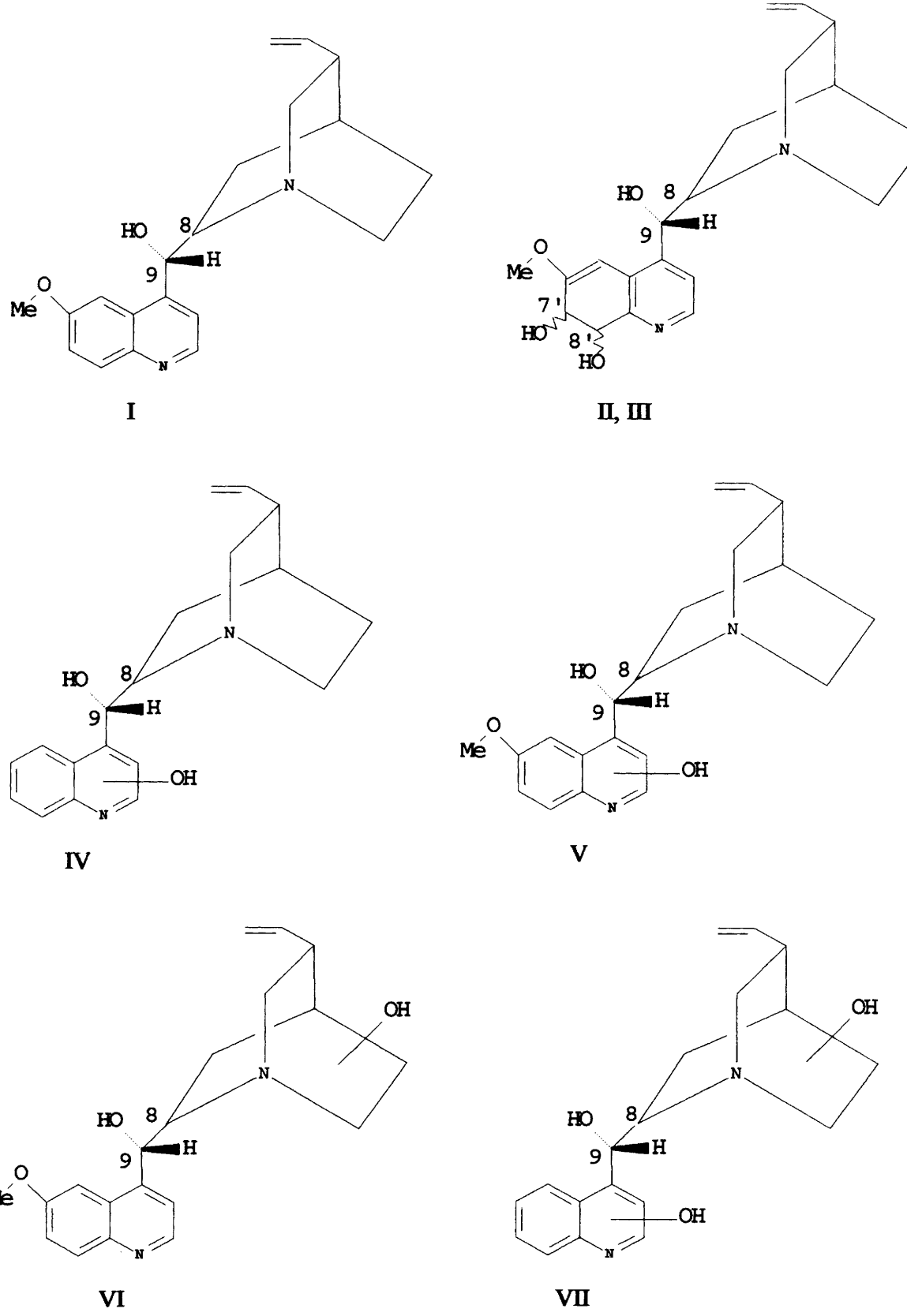

Fig. 1 Structure of quinine (**I**) and proposed structures of the phase I metabolites of quinine (**II**–**VII**) identified in this study. **II** and **III** are two regioisomeric compounds or vicinal *cis*–*trans*-diol stereoisomers at carbons 7′ and 8′.

methanol (6.0 ml) and re-dried under vacuum for a further 2 min. Finally, the analytes were recovered with ethyl acetate (3.0 ml) containing 3% v/v of concentrated (32%) ammonia solution. The extracts were transferred into screw-capped vials and evaporated to dryness under nitrogen at 40 °C.

Derivatization

TMS derivatives were prepared by adding BSTFA (50 μl) to the dry residue. After vortex mixing, the sample was heated at 80 °C for 1 h. The extracts were cooled to room temperature and then the reagents were removed under nitrogen (40 °C). The derivatized extract was dissolved in toluene–MSTFA (99 + 1 v/v, 50 μl) and 1 μl of sample was injected into the GC–MS system.

GC–MS Conditions

GC–MS was carried out on a Finnigan MAT (San Jose, CA, USA) TSQ-700 mass spectrometer interfaced to a Varian 3400 gas chromatograph with a septum-equipped temperature-programmable injector (SPI). EI mass spectra were recorded at 70 eV. A Chrompack (Middleburg, The Netherlands) capillary column (CPSIL 5-CB; 50 m × 0.32 mm ID; 0.12 μm film thickness) was used with helium as carrier gas at 68.95 kPa (10 psi). The oven temperature was programmed as follows: initial temperature, 100 °C; initial hold, 4.0 min; temperature programming rate, 8 °C min^{-1}; final temperature, 300 °C; final hold, 10 min. The transfer line temperature was 300 °C. The injector was programmed from 90 to 300 °C at 200 °C min^{-1}. Full-scan EI mass spectra were acquired by scanning the 40–650 u range. The GC conditions used in this work are generally applicable to a variety of metabolites and are not specific for quinine. When analysing real samples, the nature of the metabolites will not be known in advance.

Under PCI conditions, ammonia was used as a reagent gas at a pressure of 50 kPa.

Results and Discussion

Mass Spectrum of Quinine and Selection of Ions for Monitoring

The EI and PCI mass spectra of the TMS derivative of pure quinine are given in Figs. 2(*a*) and (*b*), respectively. The EI spectrum shows a molecular ion ($M^{+\cdot}$) at *m/z* 396 and fragment ions at *m/z* 381 ($M^{+\cdot}$ − 15), and 261 ($M^{+\cdot}$ − 135) and the base peak at *m/z* 136 corresponding to the quinuclidine moiety. The corresponding PCI spectrum [Fig. 2(*b*)] shows the $[M + H]^{+\cdot}$ ion at *m/z* 397, a fragment ion at *m/z* 307 ($M^{+\cdot}$ − 90) and the base peak at *m/z* 136 . The parent drug was identified in hydrolysed urine as peak A (Tables 1 and 2). Many metabolites arise from oxidation of quinine. The addition of hydroxyl involves an increase in molecular mass of 88 in derivatized samples (OTMS), so the ion at *m/z* 224 indicates an oxidized quinuclidine moiety (136 + 88). For this reason, peaks at *m/z* 136 and 224 were used for the detection of quinine breakdown products in the EI mode. Additionally, the ion at *m/z* 397 was also used in the PCI mode for the detection of quinine in urine samples.

Time Profiles of the Metabolites in Urine Samples

The total ion chromatograms (TIC) and mass chromatograms for various diagnostic ions generated from the GC–MS analysis of the derivatized isolate of a representative post-administration urine sample (U_3) are illustrated in Fig. 3(*a*) (EI mode) and (*b*)

Table 1 GC–EI-MS results (scan numbers) of analysis of post-administration samples (scan numbers approximately 1 scan s^{-1})

		Sample						
Peak	Parent compound	U_1 (1.50 h)	U_2 (3.50 h)	U_3 (16.50 h)	U_4 (20.00 h)	U_5 (26.00 h)	U_6 (40.00 h)	U_7 (45.50 h)
A	**I**	nd*	1011	1011	nd	nd	nd	nd
B	**II**	1043	1027	1027	1033	1036	1031	1027
C	**III**	1053	1040	1039	1043	1045	1045	1040
D	**IV**	1069	1056	1055	1058	nd	nd	nd
E	**V**	nd	1097	1098	1100	nd	nd	nd
F	**VI**	1160	1145	1145	1149	1151	nd	nd
G	**VII**	1188	1173	1173	1176	1178	1172	nd

* nd = not detected.

Table 2 GC–PCI-MS results (scan numbers) of analysis of post-administration samples (scan numbers approximately 1 scan s^{-1})

		Sample						
Peak	Parent compound	U_1 (1.50 h)	U_2 (3.50 h)	U_3 (16.50 h)	U_4 (20.00 h)	U_5 (26.00 h)	U_6 (40.00 h)	U_7 (45.50 h)
A	**I**	nd*	1019	1027	nd	nd	nd	nd
B	**II**	1080	1034	1044	1051	1041	1021	nd
C	**III**	1055	1057	1055	1061	nd	nd	nd
D	**IV**	1097	1065	1071	1077	nd	nd	nd
E	**V**	nd	1116	1115	nd	nd	nd	nd
F	**VI**	1189	1154	1162	1169	1160	nd	nd
G	**VII**	1217	1180	1190	1196	1183	1184	nd

* nd = not detected.

(PCI mode). The GC–EI-MS analysis profile of the derivatized isolate of a pre-administration (blank) urine is illustrated in Fig. 3(*c*). It shows the absence of metabolites in the blank sample. The peaks suspected of being derived from quinine, detected by the ions at *m/z* 136 and 224 (EI mode) and *m/z* 136, 224 and 397 (PCI mode) are labelled A–G [Figs. 3(*a*) and (*b*)]. The results of their detection in other post-administration urine samples (U_1–U_7) by GC–MS are given in Tables 1 (EI mode) and 2 (PCI mode). The slight difference in data points in the two tables for the metabolites is due to the variability of the GC conditions.

The excretion periods of different compounds in urine can be compared across the seven samples. As can be seen from Tables 1 and 2 some of the metabolites can be detected in urine up to 40 h after administration. After this time, the mass spectra

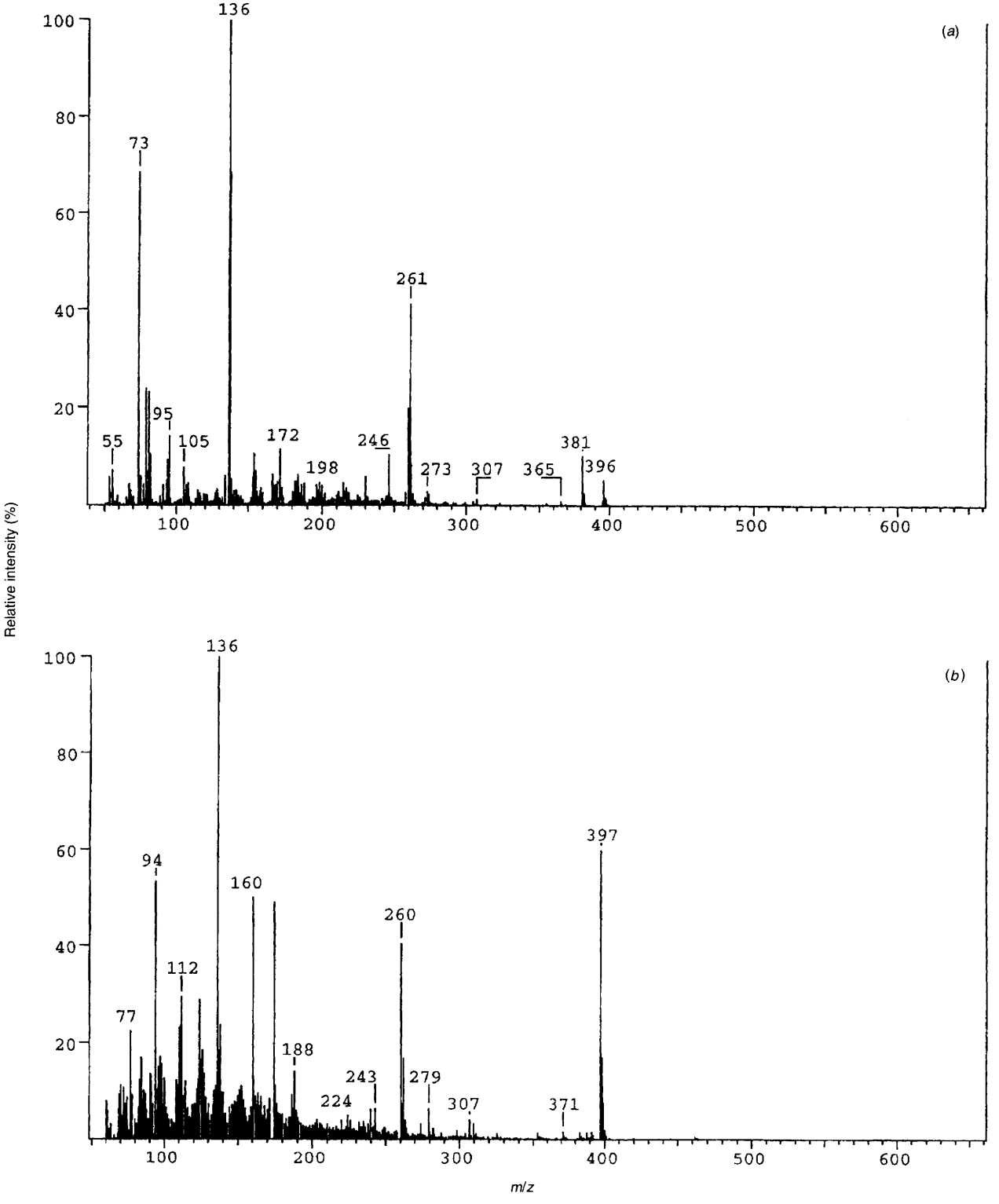

Fig. 2 (*a*) EI and (*b*) PCI mass spectra of pure quinine.

contain only low-abundance ions and are not acceptable. Therefore, for the calculation of the detection limits of quinine and its metabolites in urine the use of some other analytical method is required. Further experiments, using a quantitative method, should also be carried out to define more accurately the detection times of drug and metabolites in urine.

By comparison of the TIC and ion chromatograms obtained from GC–MS analysis of post-administration urine samples

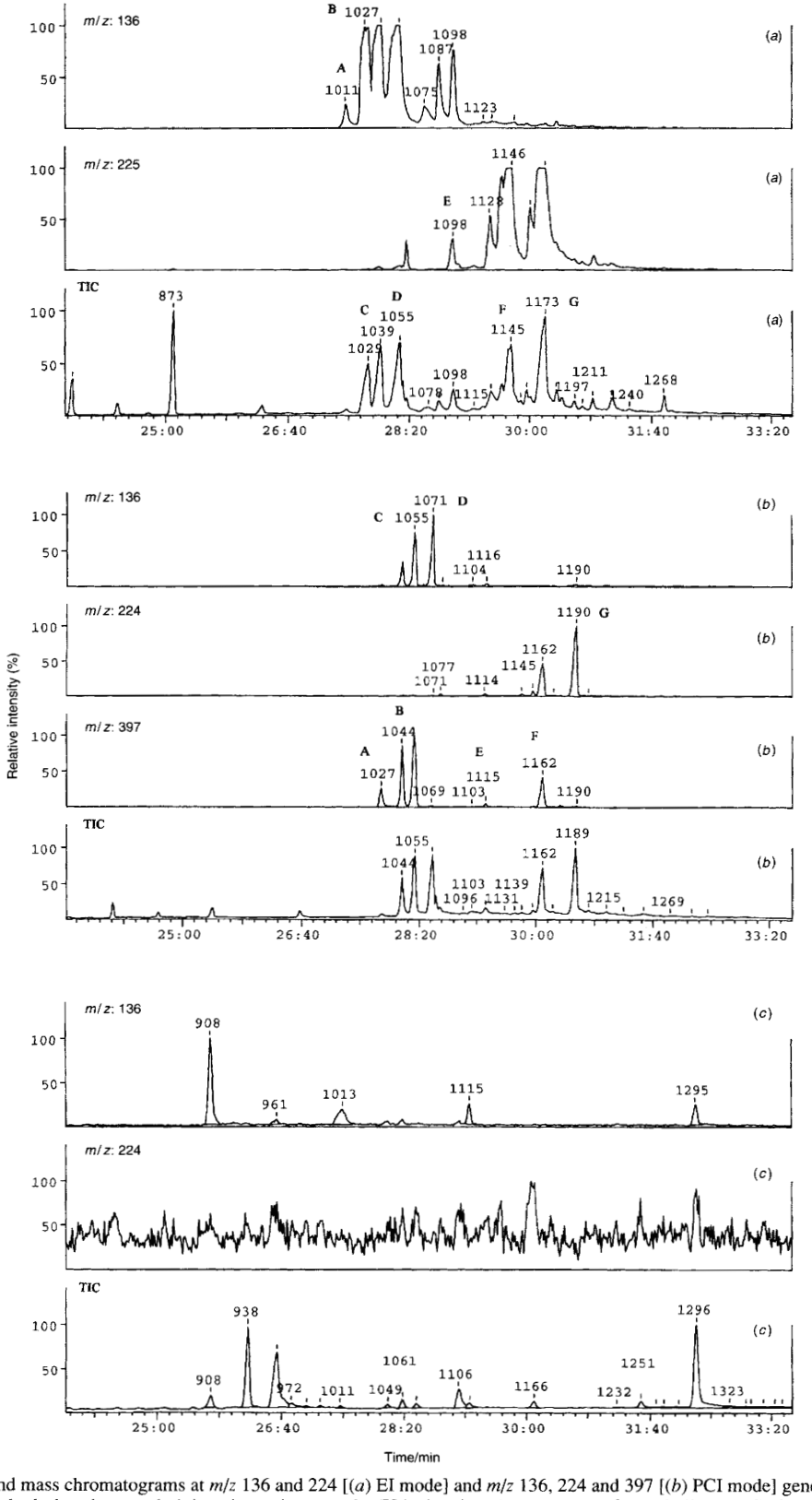

Fig. 3 TIC (bottom) and mass chromatograms at *m/z* 136 and 224 [(*a*) EI mode] and *m/z* 136, 224 and 397 [(*b*) PCI mode] generated from the TMS ether-derivatized isolate of a hydrolysed post-administration urine sample (U_3) showing the presence of metabolites A–G. 3(*c*) TIC (EI mode) and mass chromatograms at *m/z* 136 and 224 generated from the TMS ether-derivatized isolate of hydrolysed blank urine showing the absence of metabolites.

with those of blank urine, it was confirmed that the compounds listed in Tables 1 and 2 are derived from quinine administration.

Identification of the Metabolites in Urine Samples

In man, quinine is metabolized by oxidation of the aromatic part (quinoline) of the molecule to produce hydroxy derivatives.[12] In

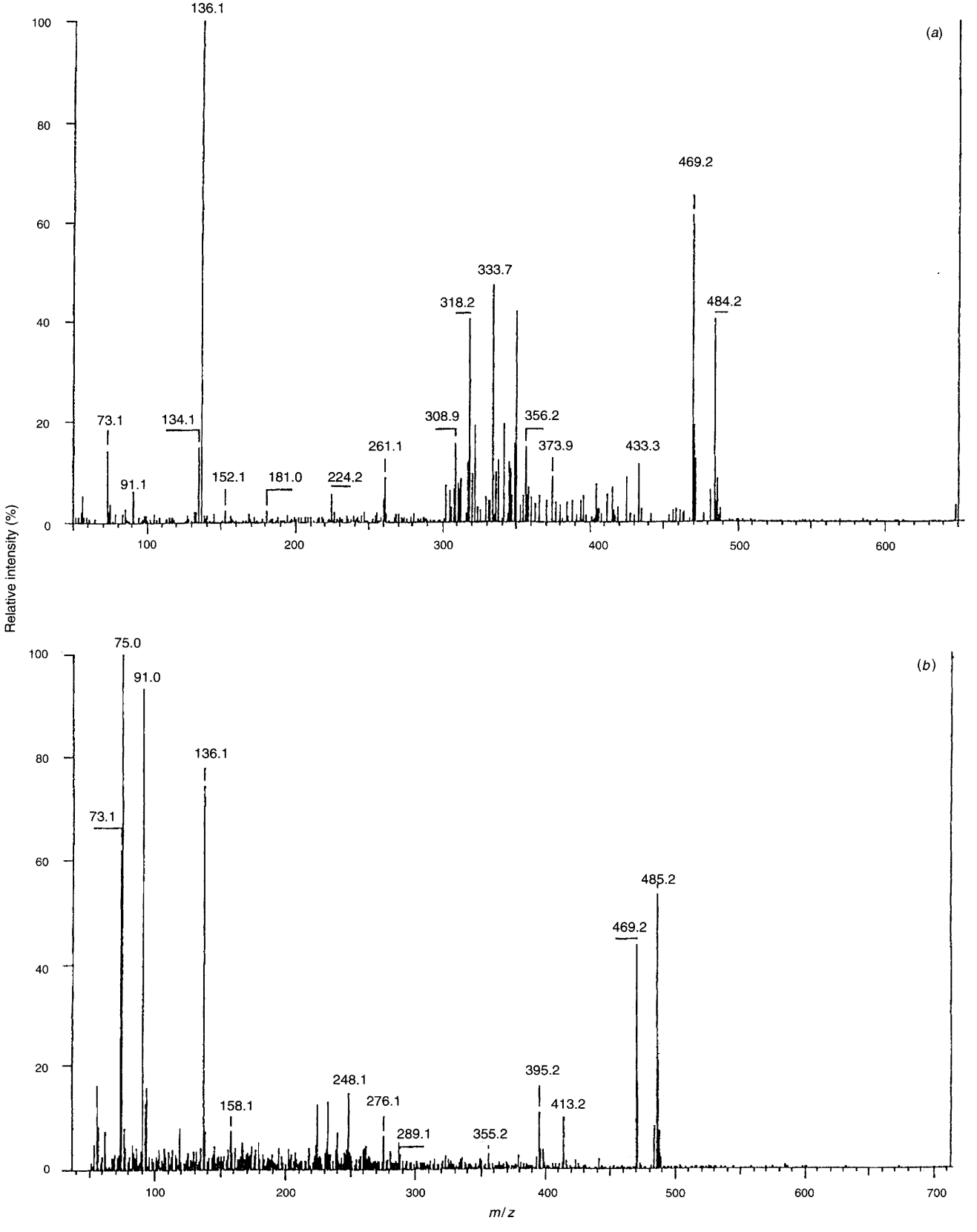

Fig. 4 (*a*) EI and (*b*) PCI mass spectra of the TMS ether derivative of **V** (peak E, Fig. 3) isolated from hydrolysed post-administration urine.

the present study, hydroxylation was obtained. Hydroxylation is observed in the quinoline ring and non-aromatic (quinuclidine) moieties. The presence of hydroxy group(s) detected in the structures of the phase I metabolites of quinine excreted in horse urine is based on the interpretation of the EI and PCI mass spectral fragmentation data. In the horse, as in humans,[13,14] at least six hydroxylated metabolites of quinine can be detected.

The EI and PCI mass spectra of the TMS derivatives of the six compounds **II–VII** (Tables 1 and 2) are given in Figs. 4(*a*)–9(*a*) and 4(*b*)–9(*b*), respectively. The silylated derivatives of structure **I** and the proposed structures **II–VII** (Fig. 1) were detected and tentatively identified in the samples corresponding to peaks A–G in Tables 1 and 2.

In addition, six putative metabolites of quinine excreted in horse urine were found in different samples. The EI and PCI mass spectra of the bis-TMS ether derivatives of two isomeric

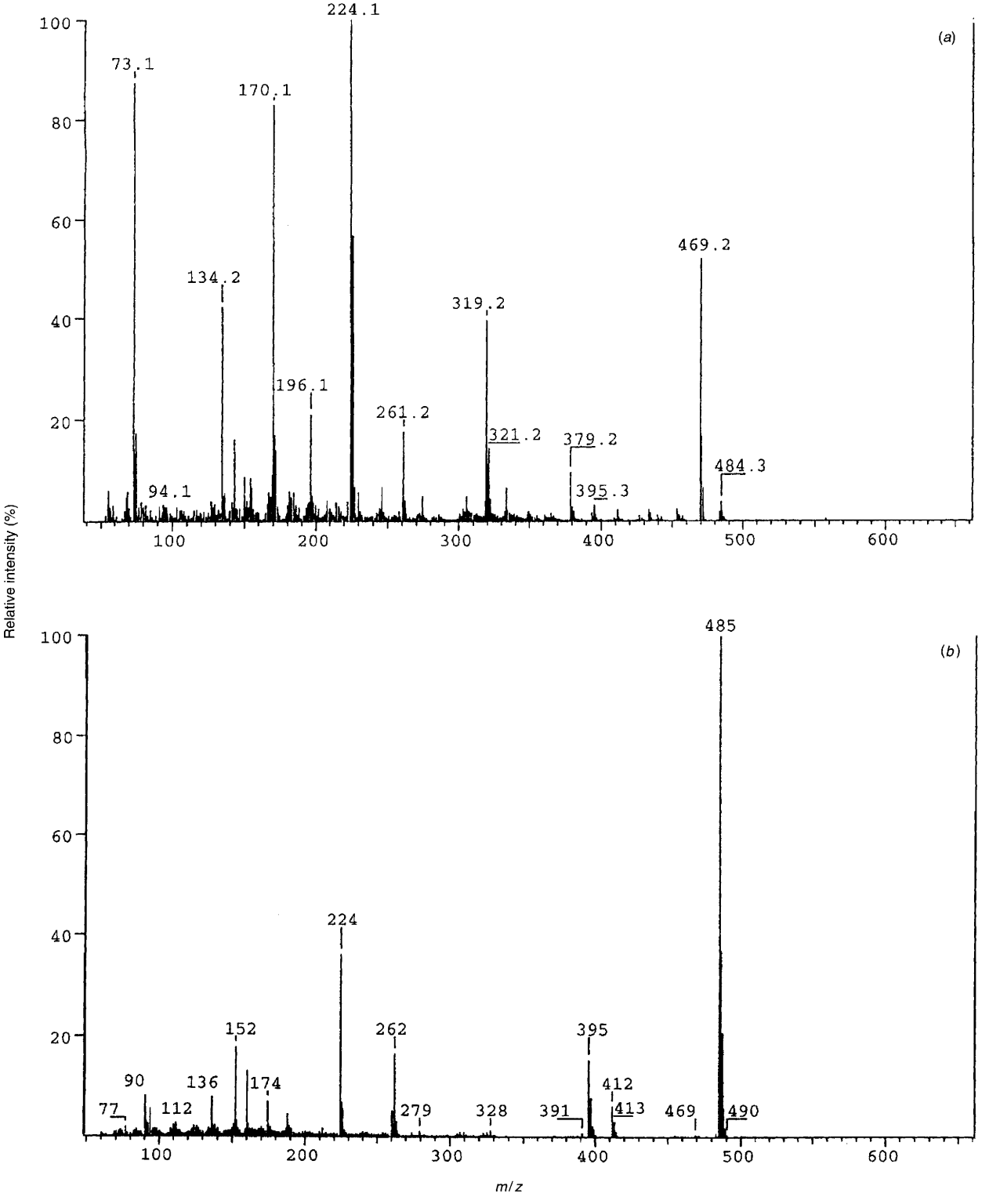

Fig. 5 As Fig. 4 for **VI** (peak F, Fig. 3).

monohydroxylated metabolites of quinine (**V** and **VI**, Fig. 1) are shown in Figs. 4(*a*) and (*b*) and 5(*a*) and (*b*), respectively. The PCI mass spectra of these compounds [Figs. 4(*a*) and 5(*b*)] show the protonated molecular ion [$(M + H)^{+\cdot}$] at *m/z* 485 compared with that of the derivatized parent drug quinine at *m/z* 397. This indicates the addition of one OTMS group to the TMS derivative of the quinine molecule. The presence of the ion at *m/z* 136 seen in the spectra of compounds **V** [Figs. 4(*a*) and (*b*)] indicate that hydroxylation occurs in the quinoline (aromatic) part of the molecule. The spectra of the isomeric compound **VI**, however, show the corresponding ion at *m/z* 224 [Fig. 5(*a*) and (*b*)]. This is due to the increase of 88 u caused by the addition of one OTMS group to the quinuclidine fragment of the molecule. Hence these two compounds are identified as regioisomeric monohydroxylated metabolites of the parent quinine. The exact site of attachment (oxidation) and the

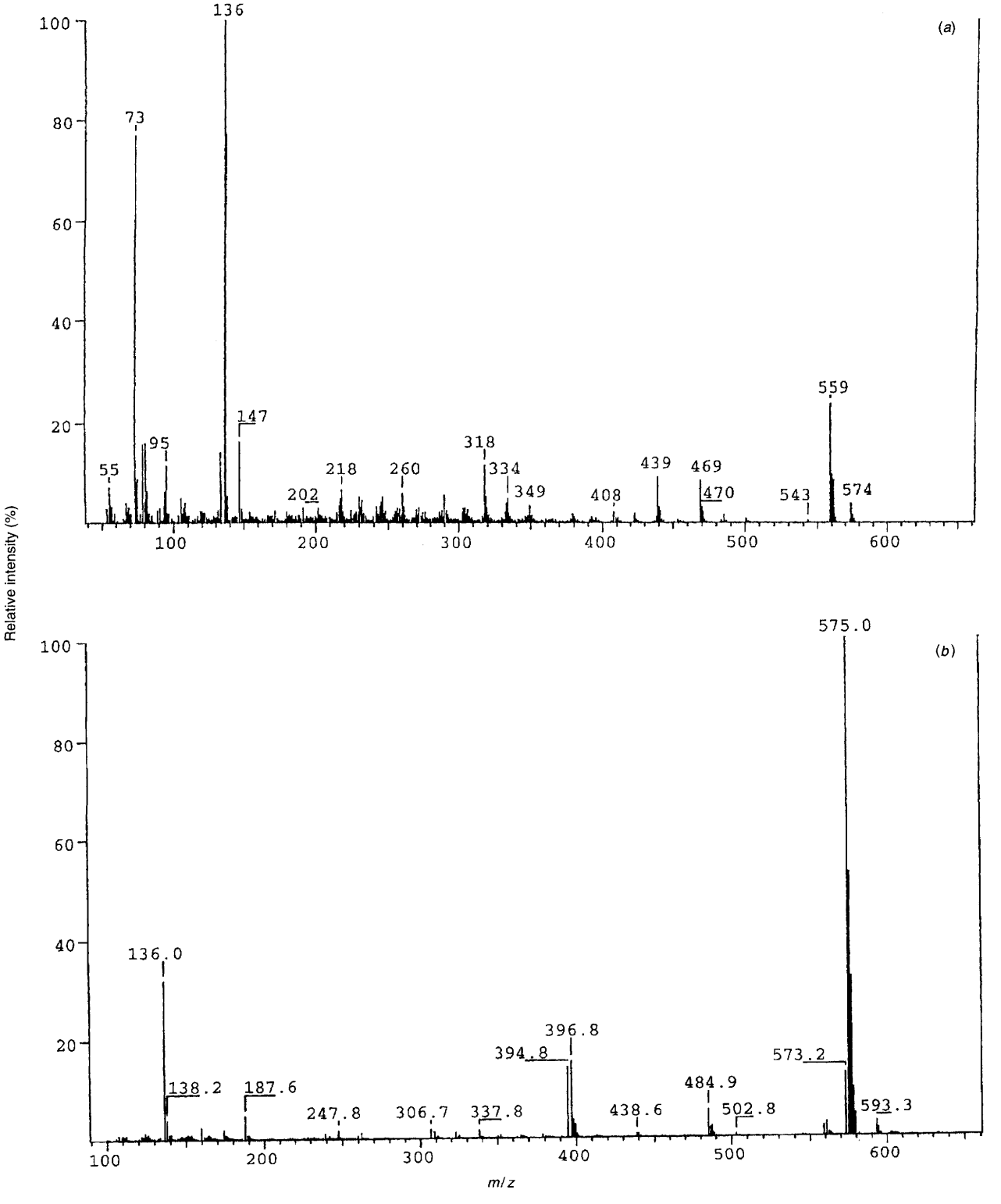

Fig. 6 As Fig. 4 for **II** (peak B, Fig. 3).

stereochemistry of the hydroxy group cannot be established from mass spectral data.

The EI mass spectra [Figs. 6(*a*) and 7(*a*)] of the tris-TMS ether derivatives of two isomers of a metabolite of quinine (**II** and **III**, Fig. 1) show a weak molecular ion ($M^{+\cdot}$) at *m/z* 574 and fragment ions at *m/z* 559 ($M^{+\cdot} - 15$), 469 [$M^{+} - (90 + 15)$], 439 ($M^{+\cdot} - 135$), and 349 [$M^{+\cdot} - (90 + 90 + 15)$] and the base peak at *m/z* 136 corresponding to the quinuclidine moiety. The

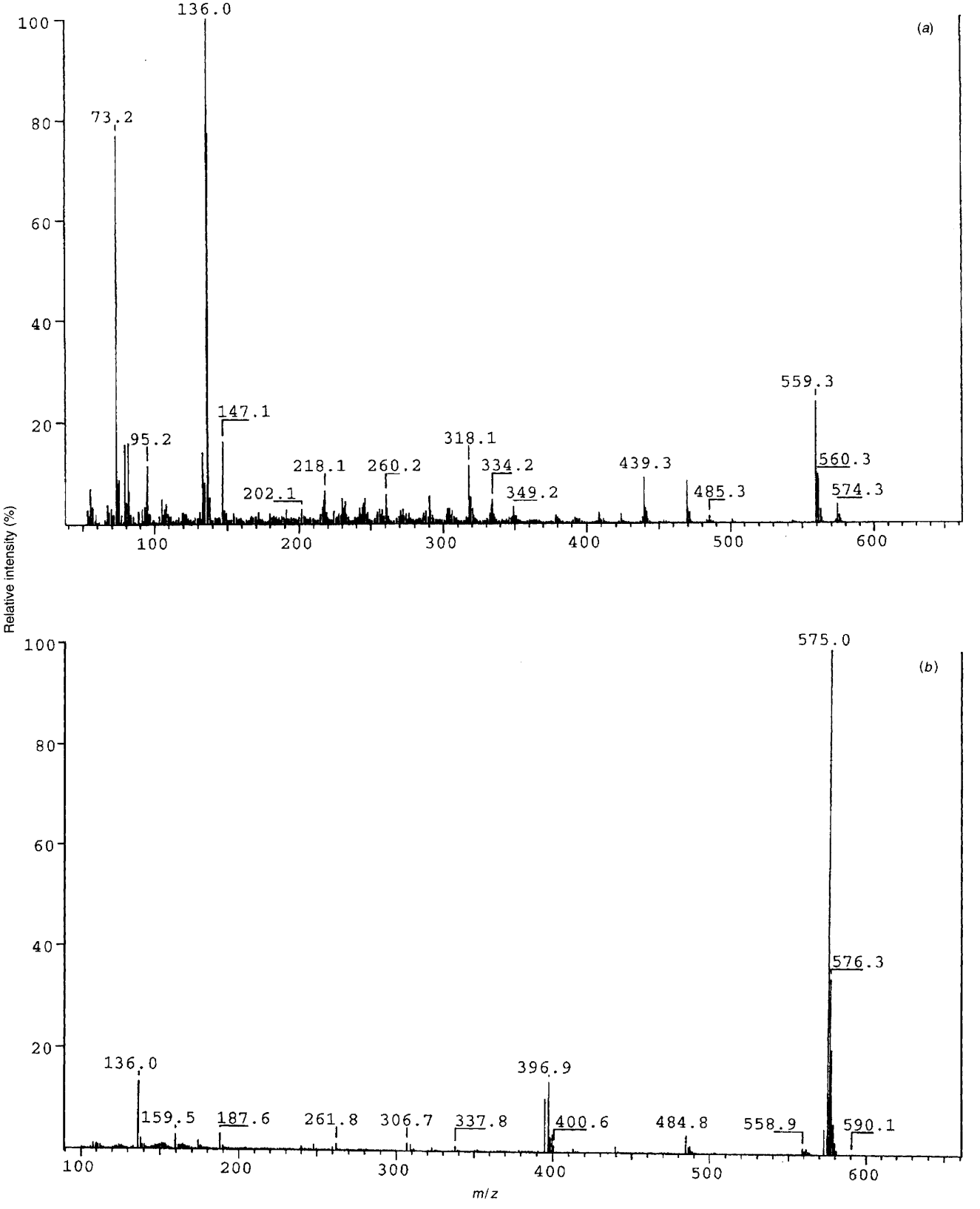

Fig. 7 As Fig. 4 for **III** (peak C, Fig. 3). **III** is the isomer of **II**.

protonated molecular ion $[M + H]^{+\bullet}$ at *m/z* 575 in the PCI spectra [Figs. 6(*b*) and 7(*b*)] confirms the addition of two vicinal OTMS groups following reduction of a double bond in the quinoline moiety and the resultant loss of aromaticity. From the above data, it is concluded that these two compounds are isomeric dihydrodiol metabolites of parent quinine. Whether they are two (positional) regioisomer compounds or vicinal *cis–trans*-diol stereoisomers separated on the GC column cannot be established from the mass spectral data. However, the *in vivo* formation, urinary excretion and GC–MS identification of a dihydrodiol metabolite of the β-antagonist drug propranolol in the horse have been reported previously.[15]

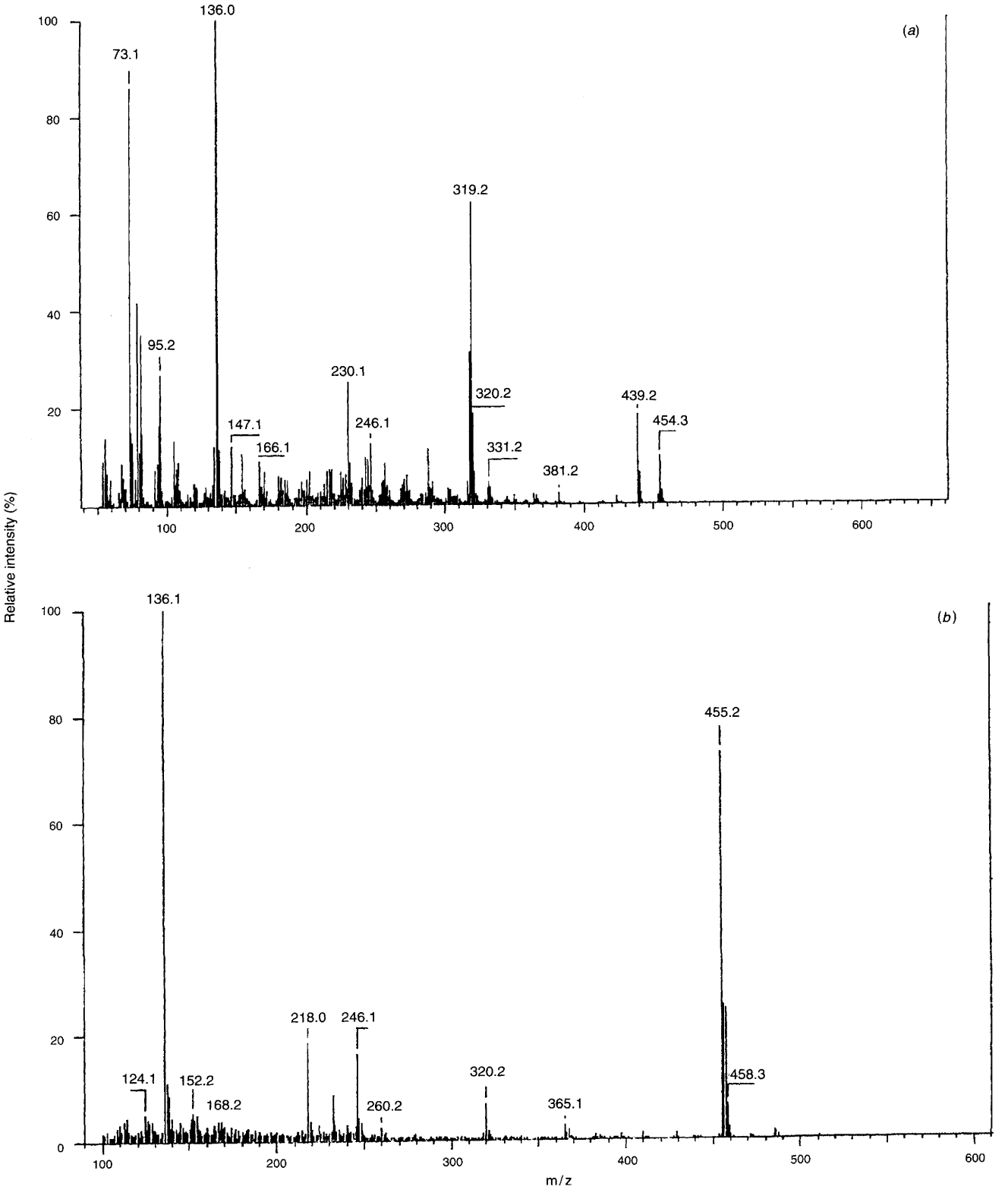

Fig. 8 As Fig. 4 for **IV** (peak D, Fig. 3).

A second type of *in vivo* biotransformation pathway for quinine involves demethoxylation in the quinoline moiety.[16] This pathway was also observed in the present study. The EI mass spectrum [Fig. 8(*a*)] of the TMS ether derivative of a putative metabolite of quinine shows a molecular ion $M^{+\cdot}$ at *m/z* 454 with fragment ions at *m/z* 439 ($M^{+\cdot} - 15$), the base peak at *m/z* 136 (quinuclidine moiety) and an ion at *m/z* 319 ($M^{+\cdot} - 135$). The corresponding PCI spectrum [Fig. 8(*b*)] shows the $[M + H]^{+\cdot}$ ion at *m/z* 455 and fragment ions at *m/z* 365 ($M^{+\cdot} - 90$), the base peak at *m/z* 136 and the corresponding radical loss at *m/z* 320 [$(M + H)^{+\cdot} - 135$]. From these data, it is concluded that the compound is a demethoxy monohydroxy metabolite of

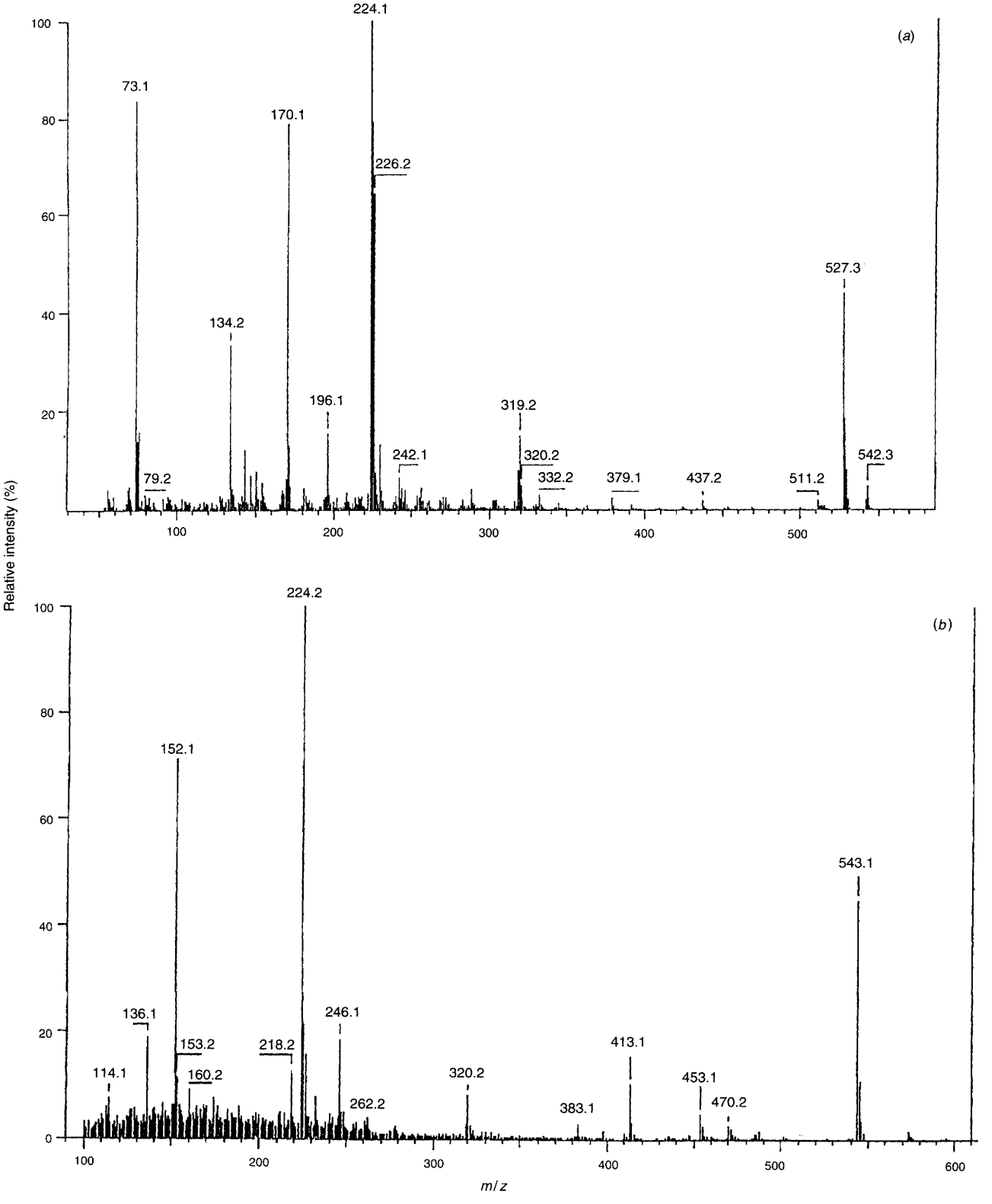

Fig. 9 As Fig. 4 for **VII** (peak G, Fig.3).

quinine (**IV** Fig. 1) with hydroxylation present in the quinoline part of the molecule identified in post-administration urine as its bis-TMS ether derivative.

The EI mass spectrum [Fig. 9(*a*)] of the TMS ether derivative of another putative *in vivo* metabolite of quinine shows a weak molecular ion at *m/z* 542 with fragment ions at *m/z* 527 ($M^{+\cdot}$ − 15), 437 [($M^{+\cdot}$ − (90 + 15)], the base peak at *m/z* 224 (α-cleavage ion corresponding to hydroxyquinuclidine-TMS ether fragment) and ions at *m/z* 134 (loss of TMSOH group from *m/z* 224) and 319 ($M^{+\cdot}$ − 223). The PCI spectrum of this compound [Fig. 9(*b*)] shows the $[M + H]^{+\cdot}$ ion at *m/z* 543 and a fragment ion at *m/z* 453 ($M^{+\cdot}$ − 90), the base peak at *m/z* 224 and the corresponding radical loss at *m/z* 320 ($M^{+\cdot}$ − 223). From these data, it is concluded that the compound is a demethoxydihydroxy metabolite of quinine (**VII**, Fig. 1) with hydroxylation in both the quinoline and quinuclidine moieties of the molecule identified in post-administration urine samples as its tris-TMS ether derivative. Whether demethoxylation is the primary pathway or occurs following initial oxidative transformations cannot be ascertained.

Conclusion

A method for the detection of quinine and its *in vivo* phase I metabolites in hydrolysed horse urine has been developed. All compounds were identified from their EI and PCI mass spectra after trimethylsilylation of the isolates and GC separation. The compounds **I**, **II**, **III**, **IV** and **V** showed base peaks at *m/z* 136 and the others (**VI** and **VII**) at *m/z* 224. These peaks could also be observed in the PCI mode, although their intensity was lower. Peaks at *m/z* representing $[M + H]^{+\cdot}$ in the PCI mode are useful for estimating molecular masses of compounds. The six metabolites in horse urine were detected by GC–MS. However, other metabolites may be present that are not detectable using this technique.

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