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Rapid characterization of artemether and its in vitro metabolites on incubation with bovine hemoglobin, rat blood and dog blood by capillary gas chromatography–chemical ionization mass spectrometry

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Abstract

A fast and sensitive analytical method was developed to characterize artemether and its metabolites in small amounts in body fluids. The extracts were derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, separated on an optimized capillary gas chromatographic system and identified by chemical ionization mass spectrometry by using ammonia as reagent gas. The analytical assay is demonstrated on samples extracted from bovine hemoglobin, rat blood and dog blood. Full mass spectra of artemether and three metabolites were obtained at a level of $1 \cdot 10^{-6}$ g/ml. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Artemether is one of the new, promising semi-synthetic anti-malarial drugs (sesquiterpene lactone endoperoxides) derived from the natural product artimisinin, extracted from the plant *Artemisia annua*. It is used for treatment against the erythrocytic stages of chloroquine-resistant *Plasmodium falciparum* and for cerebral malaria [1].

A number of methods have been reported for the quantitative and qualitative analysis of artemether, its ethoxy analogue (arteether), the respective metabolites and related sesquiterpene lactones. These analytical assays include thin-layer chromatography (TLC) [2–7], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [8–11], HPLC with electrochemical detection (ED) [12–15], HPLC in combination with mass spectrometry (LC–MS) [16–20], supercritical fluid chromatography [21], gas chromatography (GC) [22,23] and GC–mass spectrometry (GC–MS) [24–26]. However, in practice, all these assays suffer from several inherent limitations.

In many cases the peak capacity of TLC and HPLC is insufficient for the separation of all the isomeric and epimeric metabolites and matrix compounds in pharmacological applications. Moreover, the HPLC peaks of some metabolites drastically broaden (e.g., dihydroartemisinin), thereby reducing the separation power and the detection limit (e.g.,

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Ref. [16]). Secondly, owing to the lack of suitable chromophores in sesquiterpene lactone endoperoxides UV detection, commonly used in HPLC, is usually not applicable and of restricted use only. Reductive ED showed good, specific response for peroxy compounds, but failed for deoxygenated metabolites [18]. At the moment, MS with thermospray (TSP) (e.g., Ref. [16]) or electrospray ionization (ESI) [20] is the only universal and sensitive HPLC detection method to analyze sesquiterpene lactones in samples of pharmacological interest.

However, ESI mass spectra of artemether and related compounds show extensive, concentration-dependent multimer formation. In practice, this behavior has impeded quantification in pharmacokinetic studies [20].

In those cases where capillary GC has been exploited as a more powerful alternative separation technique, pyrolysates or degradation products were analyzed instead of the actual sesquiterpene lactone endoperoxides [21,22]. So far, GC–MS has not been used as a tool for characterizing the structures of intact sesquiterpene lactone metabolites in pharmacokinetic studies.

In this work we want to show, that GC–MS can be successfully used for the characterization of artemether and its metabolites, provided measures are taken to chromatograph the thermally labile peroxides below their degradation temperatures. For this purpose, the samples were injected at room temperature (cold on-column injection) on a short, tailor-made, microbore, thin-film capillary column. Hydrogen was used as a carrier gas. It is demonstrated that under these experimental conditions all examined peroxides could be separated without any degradation.

2. Experimental

2.1. Chemicals and supplies

The reference compounds were supplied by the Department of Pharmaceutical Research and Development, Novartis Pharma, Basel, Switzerland and Professor Charles D. Hufford, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, (University, MS, USA). The chromatographic elution order of the single compounds is shown in Fig. 1.

N-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Fluka (Buchs, Switzerland).

Artemether (CGP 56696, batch 81094, supplied by Pharmaceutical Development, Novartis Pharma AG) was used for the ex vivo study. A stock solution in ethanol–water (1:1, w/w) at a concentration of 10 mg/g solvent was prepared for the incubation of blood and the control samples.

2.2. Studies with hemolysates and hemoglobin

2.2.1. Artemether stock solution

Two stock solutions of artemether (CGP 56696) were freshly prepared in ethanol–water (1:1, w/w) prior to the start of the incubation experiments. Artemether (CGP 56696) was first dissolved in ethanol and then slowly diluted with water (see Table 1).

2.2.2. Animals

Rat blood was collected from two male albino rats [Tif:RAIf (SPF)]. The animals were first anaesthetized with CO_2–O_2 (65:35) and blood was obtained by heart cut. Two drops of Liquemin (containing 5000 U/ml; Roche, Basle, Switzerland) were added into the thorax to avoid coagulation of blood. The blood of all animals was pooled in heparinized vials (Cryovial, Type T310-4A), Simport Plastics, Quebec Canada). Blood of a single male dog (Beagle) was obtained by injection of the cephalic vein using heparinized tubes (containing 15 U/ml blood; Sarstedt, Nümbrecht, Germany). Plasma was prepared from blood by centrifugation at 2800 *g* for 10 min (LC 1-K, Sarstedt, Nümbrecht, Germany).

2.2.3. Incubation conditions

The incubations were carried out in conical glass tubes containing either saline (control incubation), hemoglobin or blood in the presence or absence of artemether (blank incubations). The incubation experiments were performed in two series on different days with different artemether stock solutions. The incubation volume was 11.3–12.8 g for incubations in saline, hemoglobin, rat blood and dog blood. The incubation volume of blank incubations was 2.69–

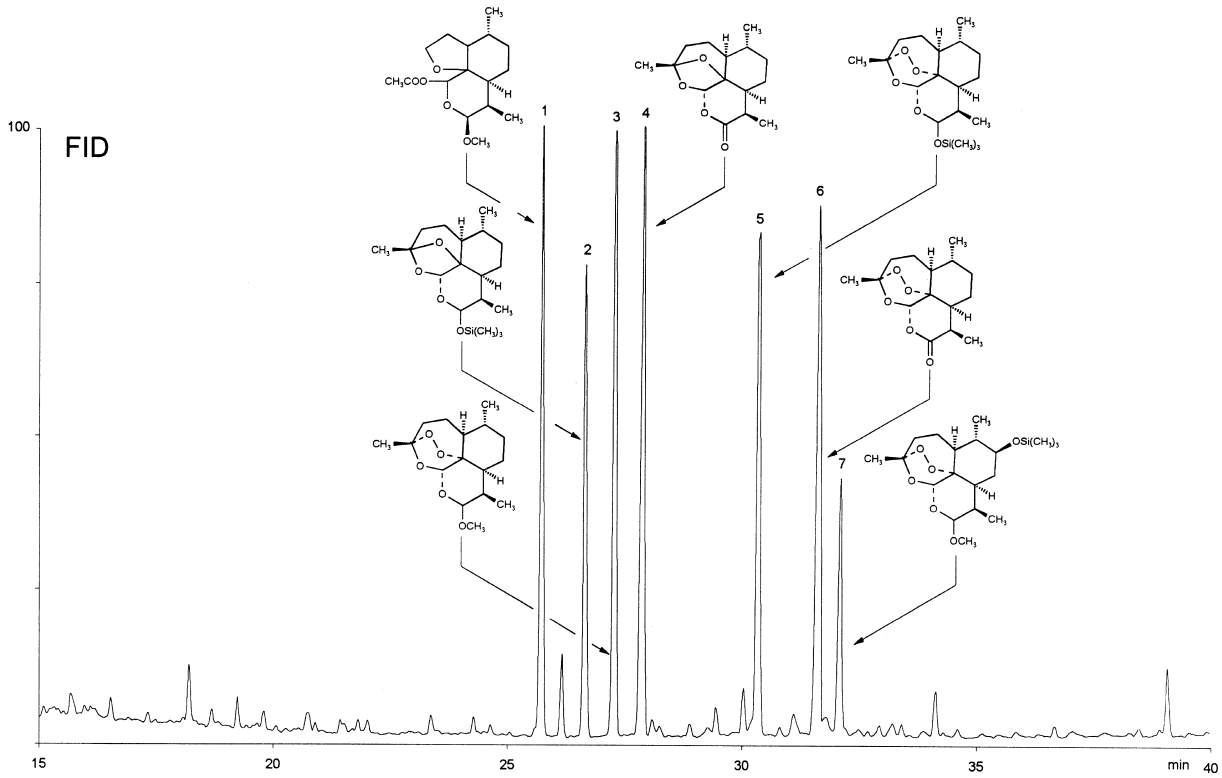


Fig. 1. FID chromatogram of an artificial mixture of endoperoxides related to artemether. Column: 8 m×0.2 mm glass capillary coated with PS-086 [OH-terminated poly(diphenyl/dimethyl)siloxane copolymer] [28]. Temperature: 40°C to 250°C with 4°C/min. Carrier gas: hydrogen.

3.22 g. The incubations in bovine hemoglobin were carried out at final concentration of 5 g/l and 150 g/l. All incubations were heated for 15 min to 37°C in a shaking water bath (Infors HT, Bottmingen, Switzerland) before 100 μl of the artemether stock solution (containing 1.08 mg artemether corresponding a final concentration in the incubation of 0.09 mg/g) was added. To the blank incubations 25 μl solvent (ethanol–water; 1:1, w/w) was added. All incubations were kept at 37°C during shaking.

Samples (2 ml) were taken from the incubation mixture at zero time and at 30, 60 and 120 min, respectively. The reaction in saline was stopped by addition of 3 ml ethyl acetate and rapid mixing with a vortex mixer. The hemoglobin samples were first diluted with 3 ml water and the reaction was stopped by addition of 6 ml ethyl acetate.

From all blood samples the hematocrit was determined by the packed cell volume method and the degree of hemolysis was visually investigated. Data

Table 1
Artemether stock solutions

	CGP 56696 (mg)	Ethanol (g)	Ethanol–water (1:1, w/w) (g)	Concentration of CGP 56696 (mg/g)
Stock solution I	25.2	1.01	2.11	11.79
Stock solution II	29.3	1.22	2.45	11.80

not shown. Blood samples were cooled in an ice bath and then immediately centrifuged for preparation of plasma.

2.3. Extraction and derivatization

The blank samples (saline), the plasma samples obtained from incubations (rat blood and dog blood) and the hemoglobin samples were extracted twice with 3 ml ethyl acetate. The organic phase was separated from the aqueous phase by freezing the mixture in dry ice. The combined organic phases were evaporated under reduced pressure to a volume of 200 μl. All samples were stored at −20°C until GC–MS analysis.

A 20-μl aliquot of this stock solution was taken up with 20 μl acetonitrile and 20 μl MSTFA. After a reaction time of 10 min, 1 μl of the solution was used for the GC–MS analysis.

2.4. Instrumental

2.4.1. Gas chromatography

GC separation was performed by using a 4160 gas chromatograph (Carlo Erba) equipped with a constant flow/constant pressure regulator (CP/CF 516, Carlo Erba) and a 8 m×0.2 mm glass capillary column coated with PS-086 [OH-terminated poly(diphenyl/dimethyl)siloxane copolymer] as described previously [28]. All samples were introduced by cold on-column injection.

The analytical column was protected from nonvolatile by-products in the samples by a deactivated, but uncoated 5 m×0.3 mm glass capillary (retention gap), installed in a separate GC oven. Both capillaries were linked by a heated transfer line. The retention gap was heated separately and programmed from 70°C to 120°C at a rate of 10°C/min. The temperature program of the analytical column was started, when the temperature of the retention gap exceeded 120°C [29]. The analytical column was temperature-programmed from 40°C to 250°C with 4°C/min. Carrier gas was hydrogen.

2.4.2. Gas chromatography–mass spectrometry

The separation equipment described above was linked by a temperature-programmable glass interface [30] to a 4600 quadrupole mass spectrometer (Finnigan MAT, San Jose CA, USA). The ionization technique employed was chemical ionization (CI) with ammonia as reagent gas. The ion source temperature was 150°C. The transfer line was programmed from 100 to 200°C with 4°C/min. The scan range was m/z 100–700.

3. Results and discussion

3.1. GC–MS analysis

The gas chromatographic working conditions were optimized with a silylated, artificial mixture consisting of 3,3α,4,5,6,6α,7,8-octahydro-8-methoxy-4,7-dimethyl-8-oxo-2H,10H-furo[3,2-*i*]benzopyran-10-yl acetate, deoxydehydroartemisinin, artemether, deoxyartemisinin, dehydroartemisinin, artemisinin and 6-hydroxyartemether. Besides the column characteristics, some instrumental prerequisites are important for a successful analysis of thermally labile compounds.

First, the sample should be injected at room temperature directly into the column, in order to avoid early degradation in the (over) heated injection port. Secondly, hydrogen should be used as carrier gas. The optimum gas velocity is higher for hydrogen compared to other common carrier gases, helium or nitrogen. At optimal flow conditions, hydrogen driven gas chromatography is 1.5-times faster than with helium, while the plate height remains same. Moreover, using hydrogen, the carrier gas velocity can be increased above the optimum, with only comparable little loss in separation efficiency [31]. Finally, it should be ensured, that not only the temperature of the chromatograph but also the temperature of the entire GC–MS system is kept below the degradation temperature of the most thermally labile compound, in this case dihydroartemisinin. For this reason, the GC–MS interface line is temperature programmed likewise. An interface temperature above the degradation temperature of the substrates influences the quality of the mass spectra.

Under the above-mentioned conditions all test endoperoxides could be separated as single, undegraded peaks in the temperature range between 40–170°C (Fig. 1). The test compounds were stable over several weeks in a solution of acetonitrile–MSTFA.

Artemether and related compounds are known to produce significant fragment ions even under soft ionization conditions [16,32,33]. The CI/NH_4 mass spectrum of artemether shown in Fig. 2 is representative for this class of compounds.

The main fragmentation pathway is the loss of methanol in position 12 and the loss of ethanol by a rearrangement in the seven-ring from the respective Lewis acid adducts $[M+NH_4]^+$ or Broensted acid adducts $[M+H]^+$. Therefore, the ion current is mainly concentrated on fragments at m/z 284, $[M+NH_4-CH_3OH]^+$, at m/z 267 $[MH-CH_3OH]^+$, at m/z 253, $[MH-C_2H_5OH]^+$, at m/z 221 $[MH-CH_3OH-C_2H_5OH]^+$, respectively (see Fig. 3). The most dominant and characteristic ions at m/z 284 and m/z 267 can be monitored for quantitative measurements. In single ion monitoring mode the limit of detection was 10^{-8} g/ml.

The CI/NH_4 mass spectrum of the isomeric furano acetate (3,3α,4,5,6,6α,7,8-octahydro-8-methoxy-4,7-dimethyl-8-oxo-2H,10H-furo[3,2-*i*]benzopyran-10-yl acetate, metabolite III) shows mainly elimination of acetic acid from the respective Lewis acid adduct $[M+NH_4-CH_3COOH]^+$, at m/z 256 and from the Broensted acid adduct $[MH-CH_3COOH]^+$, at m/z 239, and subsequently elimination of methanol $[MH-CH3COOH-CH_3OH]^+$ at m/z 224, or carbon monoxide $[MH-CH_3COOH-CO]^+$, at m/z 211, respectively (see Fig. 4).

The mass spectra of the trimethylsilylated deoxydihydroartemisinin metabolites show simpler fragmentation behavior. Both spectra are dominated by $[MH-OH\text{-}Si(CH_3)_3]^+$ fragment ions at m/z 251 (TMS-deoxydihydroartemisinin, metabolite IV) and at m/z 339 (TMS-3-hydroxy-deoxydehydroartemisinin, metabolite V), respectively (see Fig. 5).

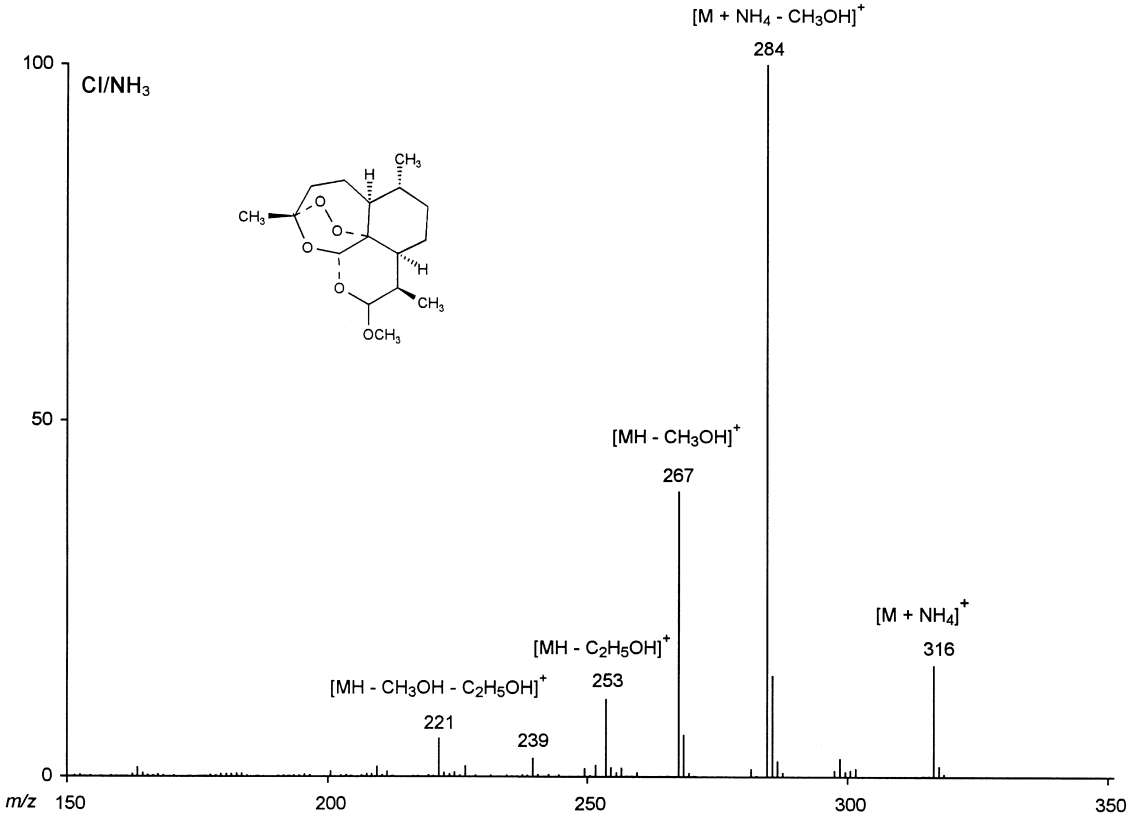


Fig. 2. Partial CI/NH_4 mass spectrum (m/z 150–350) of artemether.

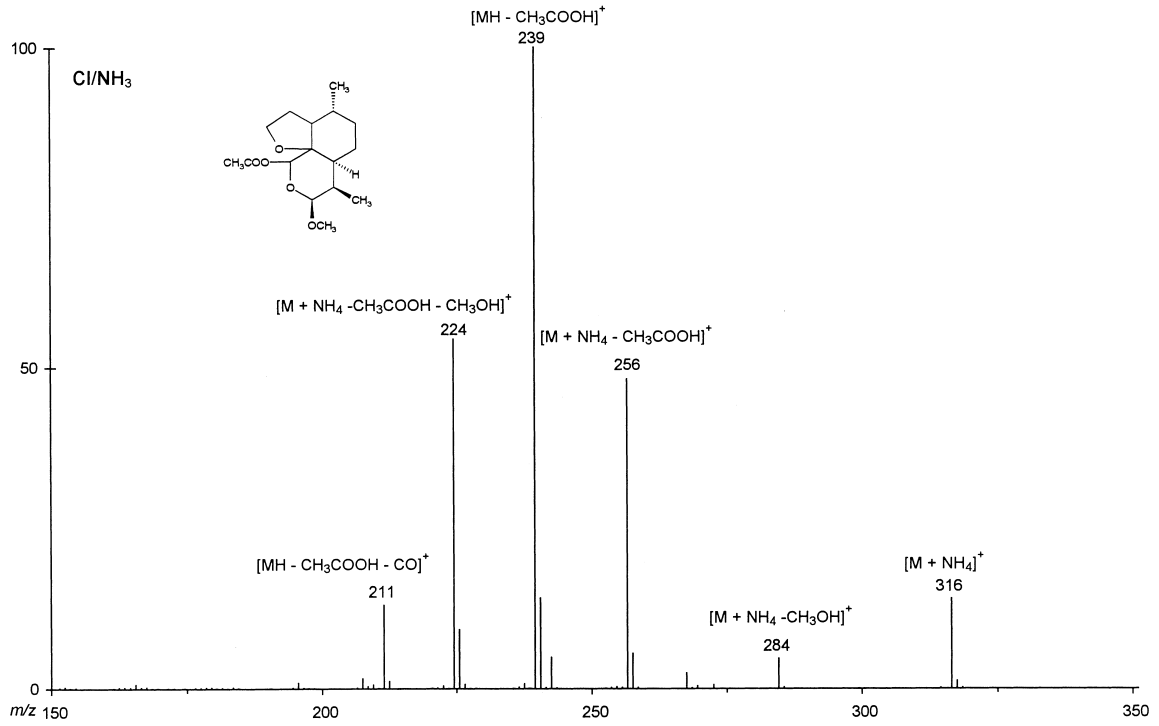


Fig. 3. Partial CI/NH_4 mass spectrum (m/z 150–500) of artemether furano acetate [metabolite III 3,3α,4,5,6,6α,7,8-octahydro-8-methoxy-4,7-dimethyl-8-oxo-2H,10H-furo[3,2-*i*]benzopyran-10-yl acetate)].

3.2. Incubation of artemether with hemoglobin or blood

Incubation of artemether (I), with bovine hemoglobin or blood from rats or dogs resulted, time-dependent, in the formation of at most three metabolites (metabolites III–V, see Figs. 1,6–8). With saline, used as control incubation in order to control possible degradation of artemether at 37°C in solution, 2 h after incubation only traces of metabolite III and IV could be observed (chromatogram not shown). Similar results were obtained after incubation with bovine hemoglobin at low concentration (chromatogram not shown). With higher amounts of bovine hemoglobin (150 mg/l, see Fig. 6), a degradation of artemether to two main products (metabolites III and IV) and one minor product (metabolite V) was observed. With rat blood and dog blood only metabolites III and IV could be detected (see Figs. 7 and 8). As expected, the formation of metabolites increased with the duration of incubation time.

In rat blood and in bovine hemoglobin at high concentrations, the metabolites III and IV were formed rapidly (Figs. 6 and 7). In dog blood, the formation of degradation products was very slow. Two hours after incubation only low amounts of compounds III and IV were observed (Fig. 8).

3.3. Iron-catalyzed decomposition of artemether

The mode of action of artemisinin and related compounds towards resistant *Plasmodium falciparum* is the subject of current investigations [34–39]. Studies with radiolabeled artemisinin and dihydroartemisinin showed a rapid uptake of both compounds into *Plasmodium falciparum* infected erythrocytes reaching concentrations more than 100-fold higher compared to the non-infected stage [40–42].

A markedly reduced recovery of radiolabeled artemether in hemin, hematin, hemoglobin and methemoglobin [27] and of artemether during storage in whole blood, to at least two main degradation products, was reported in various studies [19,36,39].

In studies with artemisinin, the so far identified

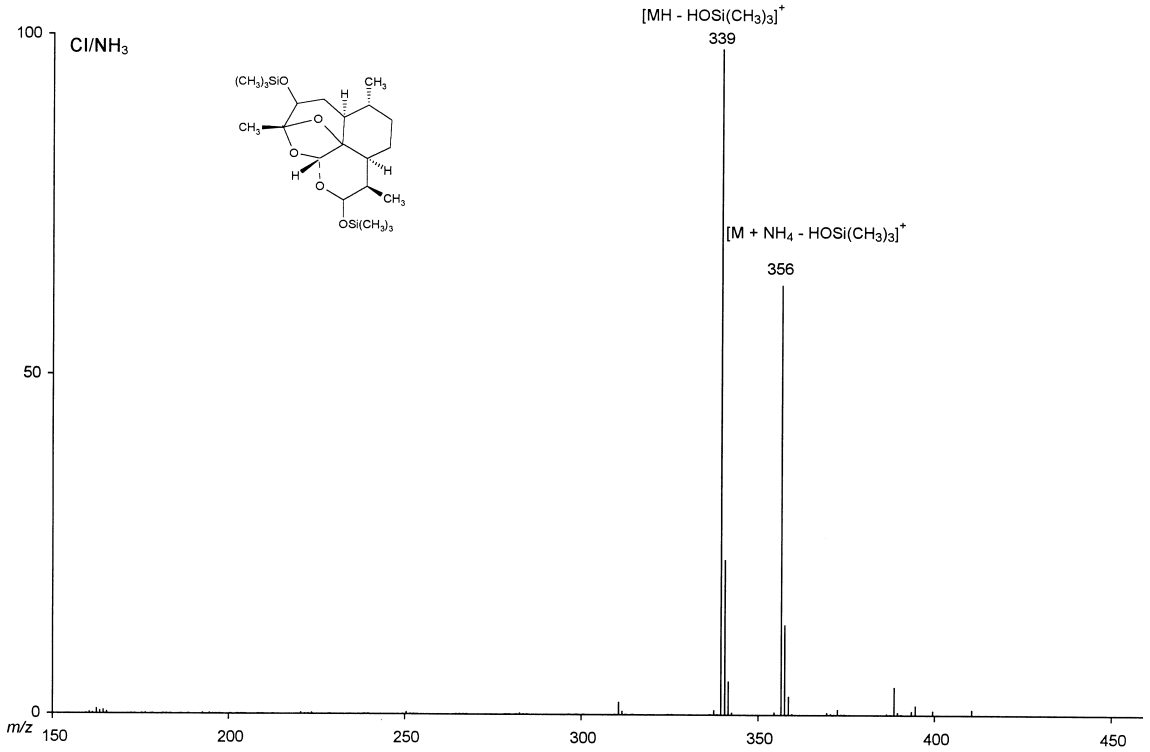


Fig. 4. Partial CI/NH_4 mass spectrum (m/z 150–500) of trimethylsilylated 3-hydroxydeoxydihydro-artemisinin.

reaction products were: (i) furano acetate isomer, (ii) hydroxydeoxy-artemisinin and (iii) deoxygenated artemisinin [39]. In this relationship, two main reaction pathways were suggested. Once the formation of a carbon-centered radical followed, via an oxy radical, by an intramolecular 1,5-hydrogen shift [36] or in a more recent study two separate unrelated reactions [39]. However, both suggested reaction mechanisms have the iron-catalyzed homolytic cleavage of the peroxide and the formation of a C-centered radical in common.

The mechanism of the formation of deoxydehydroartemisinin (metabolite V) is hitherto unknown. The degradation of artemether with ferriprotoporhyrin is – due to its additional demethylation reaction – apparently different from the degradation with $FeCl_2$. One possible explanation could be the presence of Fe(III) formed during the incubation which could cleave the acetal to form a hemiacetal in a similar way as published previously with the polyether nigericin [43]. Since neither dihydroartemisinin nor hydroxydeoxyartemether could be detected in the present study, the demethylation could take place concomitantly during the iron-catalyzed rearrangement of artemether.

The total ion current gas chromatograms of the bovine hemoglobin, rat blood and dog blood samples at 30 and 120 min after incubation of artemether, are shown in Figs. 6–8. Besides the parent compound (artemether), two to three peaks arise (species dependent) with increasing incubation time. The relevant mass spectra and the derived structures are given in Figs. 3–5.

Although the mechanism of the protoporphyrin-catalyzed degradation of artemether is not completely explained so far, the findings of this study agree to a large extent with those reported previously (e.g., Refs. [35,36,39]). The main reaction products in all investigated samples were the respective artemether furano acetate (metabolite III, see Scheme 1a) and 3-hydroxydeoxydihydro-artemisinin (metabolite IV, see Scheme 1b). However, the degradation of ar-

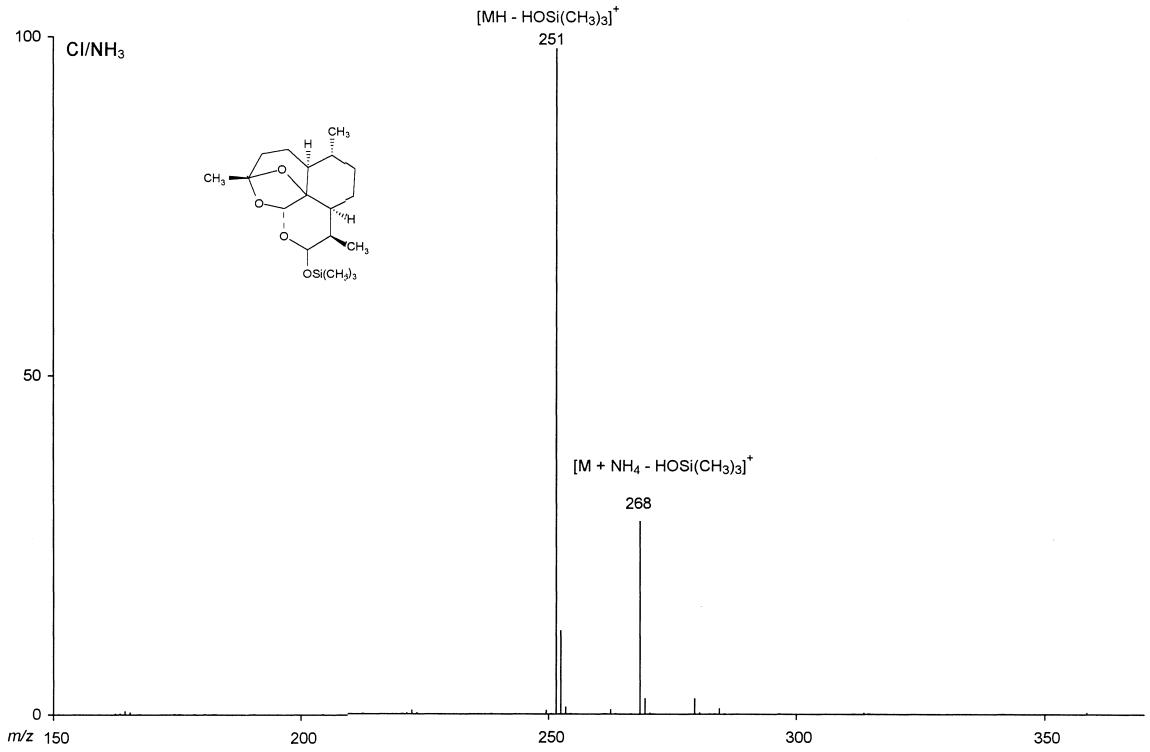


Fig. 5. Partial CI/NH_4 mass spectrum (m/z 150–500) of trimethylsilylated deoxydihydro-artemisinin.

temether differs from the decomposition of artemisinin, in respect to the additional demethylation step at the acetal moiety. Since in this examination neither dihydroartemisinin nor hydroxydeoxyartemether could be detected as intermediates, the demethylation possibly takes place concomitantly during the iron-catalyzed rearrangement of artemether.

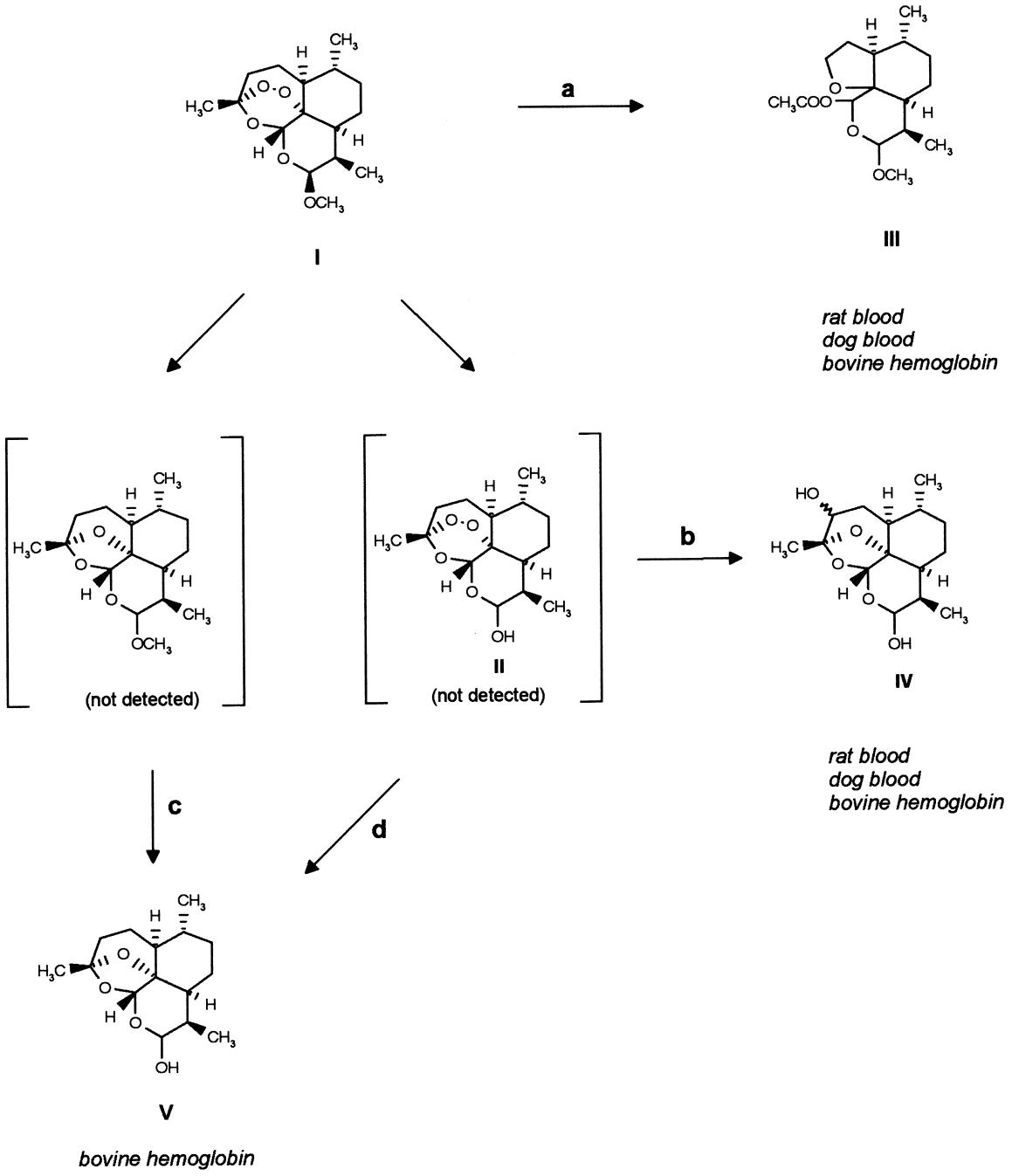
The reason for different amounts and ratios of metabolites III and IV in the experiments with bovine hemoglobin (Fig. 6), rat blood (Fig. 7) and dog blood (Fig. 8) is not known. A possible explanation could be that the coordination of heme-iron to the endoperoxide varies with the hemoglobin of different species.

The deoxygenation without rearrangement of artemether to deoxydihydroartemisinin (metabolite V, see Scheme 1c,d) was only observed with high amounts of bovine hemoglobin (Fig. 6) which reflected roughly the concentration of hemoglobin in human blood.

4. Conclusion

The results of the present study confirm that the recovery of artemether after storage in hemoglobin, rat blood and dog blood is reduced. The formation of at least two metabolites, depending on the incubation time, is observed in all examined ex vivo samples. This suggests that in vivo a similar metabolic pathway can not be excluded.

For the first time GC–MS has been successfully used as a sensitive method for the mixture analysis of artemether and related compounds in pharmacological studies. Further-reaching examinations reveal that the method can also be used for the analyses of iron-catalyzed reaction products in other body fluids.



Scheme 1. Proposed pathway for the ferriprotoporphyrin catalyzed degradation of artemether (the intermediates in parentheses are hypothetical).

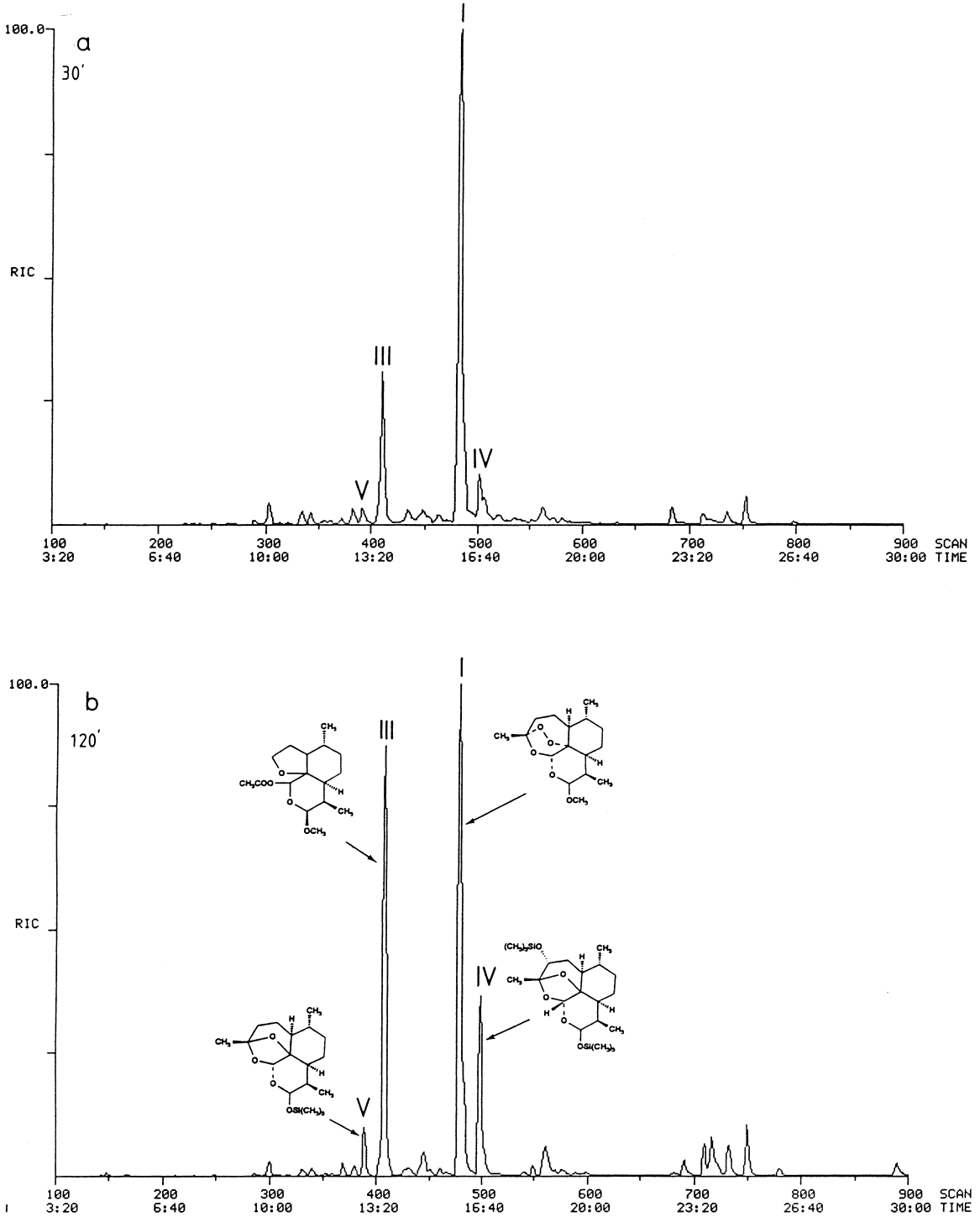


Fig. 6. CI/NH_4 TIC chromatograms of derivatised bovine hemoglobine extracts: (a) 30 min incubation time, (b) 120 min incubation time. Column: 8 m×0.2 mm glass capillary coated with PS-086 [OH-terminated poly(diphenyl/dimethyl)siloxane copolymer] [28]. Temperature: 40°C to 250°C with 4°C/min. Carrier gas: hydrogen.

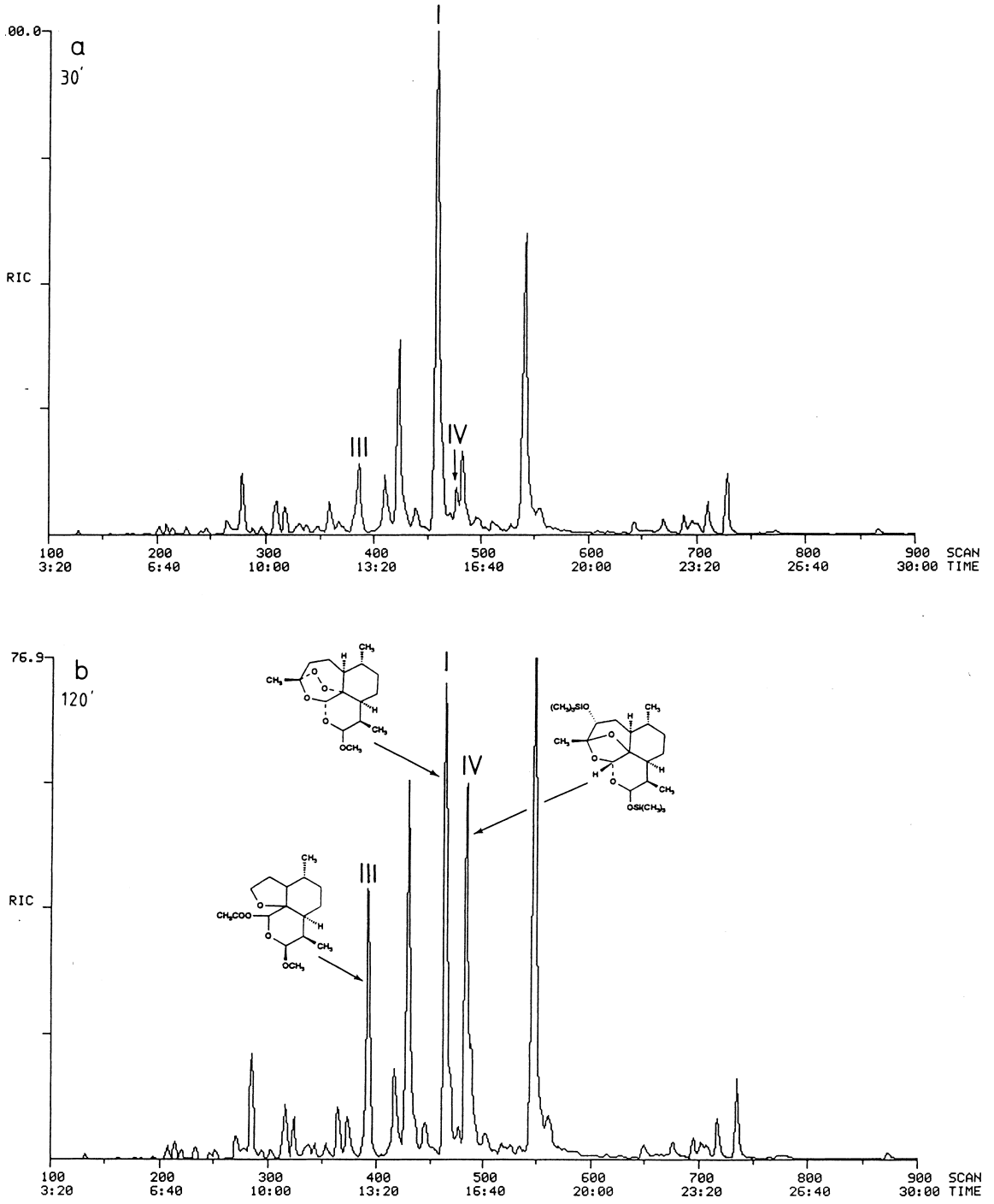


Fig. 7. CI/NH_4 TIC chromatograms of derivatised rat blood extracts: (a) 30 min incubation time, (b) 120 min incubation time. Column: 8 m×0.2 mm glass capillary coated with PS-086 [OH-terminated poly(diphenyl/dimethyl)siloxane copolymer] [28]. Temperature: 40°C to 250°C with 4°C/min. Carrier gas: hydrogen.

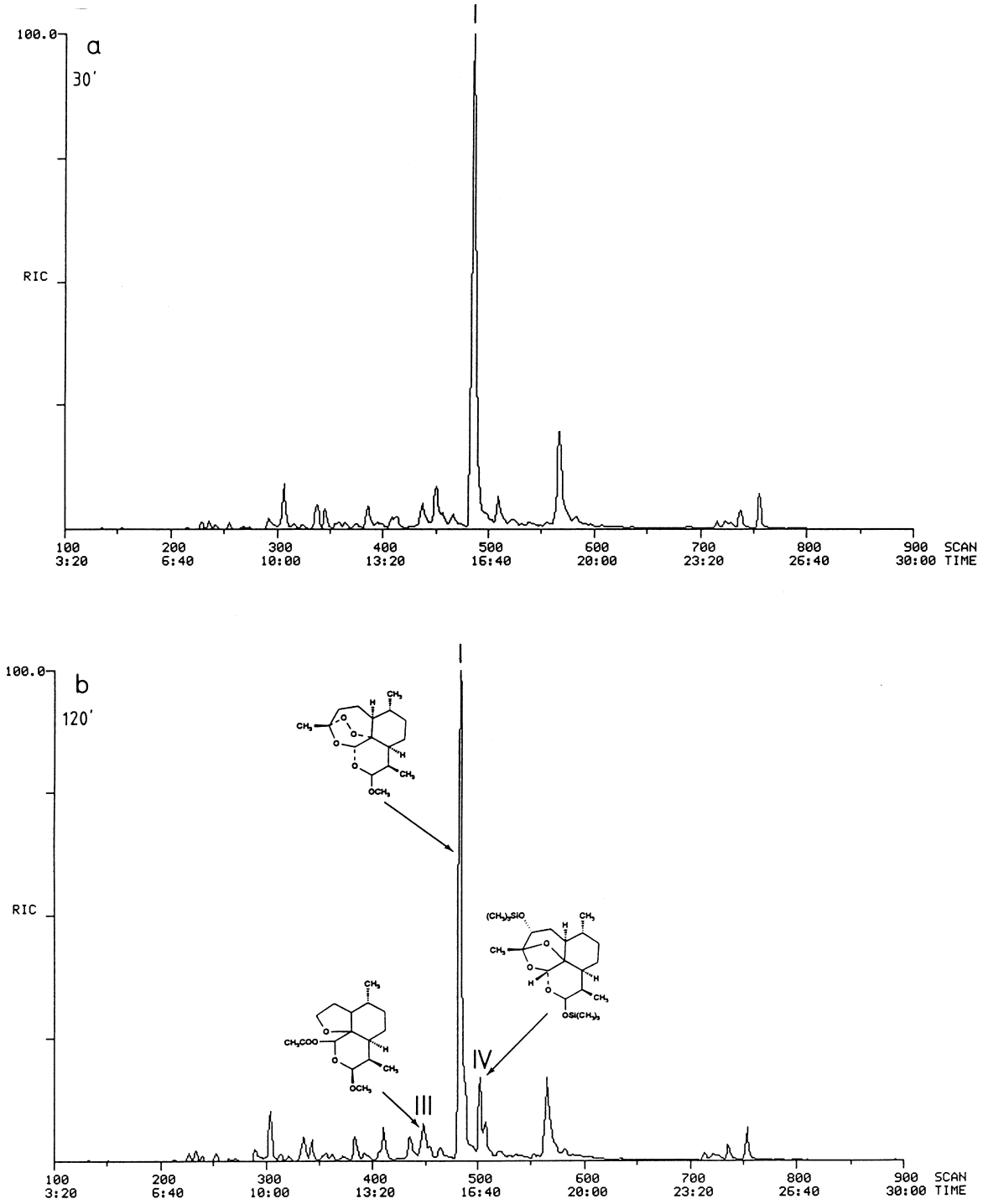


Fig. 8. CI/NH_4 TIC chromatograms of derivatised dog blood extracts: (a) 30 min incubation time, (b) 120 min incubation time. Column: 8 m×0.2 mm glass capillary coated with PS-086 [OH-terminated poly(diphenyl/dimethyl)siloxane copolymer] [28]. Temperature: 40°C to 250°C with 4°C/min. Carrier gas: hydrogen.

However, compared to previously reported alternative analytical assays, the application range, chromatographic resolution and detection limits could be significantly improved.

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