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Studies on the metabolism and toxicological detection of the *Eschscholtzia californica* alkaloids californine and protopine in urine using gas chromatography–mass spectrometry☆

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Abstract

Eschscholtzia californica preparations are in use as phytopharmaceuticals and as herbal drugs. Studies are described on the metabolism and the toxicological analysis of the *Eschscholtzia californica* alkaloids californine and protopine in rat urine using gas chromatography–mass spectrometry. The identified metabolites indicated that californine is extensively metabolized by *N*-demethylation and/or single or double demethylenation with consecutive catechol-*O*-methylation of one of the hydroxy groups. Protopine, however, only undergoes extensive demethylenation of the 2,3-methylenedioxy group followed by catechol-*O*-methylation. All phenolic hydroxy metabolites were found to be partly conjugated. The authors' systematic toxicological analysis procedure using full-scan gas chromatography–mass spectrometry after acid hydrolysis, liquid–liquid extraction and microwave-assisted acetylation allowed the detection of the main metabolites of californine and protopine in rat urine after a dose which should correspond to that of drug users. Therefore, use of *Eschscholtzia californica* preparations should also be detectable in human urine by the authors' systematic toxicological analysis procedure.

Keywords: *Eschscholtzia californica*; Alkaloids; Californine; Protopine

1. Introduction

Eschscholtzia californica, the state flower of California, is a native plant of this state and grows wild in pacific regions of North America from Oregon to Mexico. It was introduced into Europe in the early 19th century as a decorative garden plant [2]. The herbaceous genus belongs to the papaveraceae and was named by Adelbert von Chamisso in honor of his friend Johann Friedrich Eschscholtz, a physician and naturalist who participated in the Russian Kotzebue expedition visiting California. Common names of the genus are California poppy or Yellow poppy [3,4].

The whole plant contains a complex mixture of tertiary and quaternary isoquinoline alkaloids with a higher content in flowering plants. The main alkaloids can be subdivided into several constitutional types, the pavine alkaloids californidine and californine (Eschscholtzine), the protopine alkaloids protopine and allocryptopine, the aporphine lauro-

☆Part of these results were reported at the 40th International TIAFT Meeting, Paris, August 26–30, 2002 [1].

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scholtzine (*N*-methyllaurotetanine), and to a minor extent the benzophenanthridines sanguinarine and chelerythrine [5–14]. The distribution of the various alkaloids in the organs of the plant differs considerably.

Eschscholtzia californica is a traditional medicinal plant of the American Indians and early settlers of California. It gained a reputation of having hypnotic and analgesic properties [2,15]. Until today, it has been in use in America and Europe as a mild sedative. In recent years, the biological effects of *Eschscholtzia californica* extracts have been investigated in various animal models and in vitro studies. From these studies, various effects were concluded, e.g. sleeping time prolongation or spasmolytic, sedative and anxiolytic activities [15–19]. However, little is known about pharmacology and toxicology in humans.

Nevertheless, the herb has been described as a substitute drug for marijuana as it should evoke a mild euphoria after ingestion or smoking lasting 20–30 min [20–23]. It has been proposed to stepwise increase the dose until the desired effect occurs [3]. In the 1960s, Cheney reported that regular and prolonged use would not be habit-forming [2].

So far, the metabolism of the *Eschscholtzia californica* alkaloids has not been studied. However, the knowledge about metabolic steps is a prerequisite for developing toxicological screening procedures and for toxicological risk assessment, as in both cases the metabolites may play a major role. So far, procedures for determination of or screening for the main *Eschscholtzia* alkaloids and/or their metabolites have not yet been published, although this might be important in the context of monitoring herbal drug use.

Therefore, the aim of this study was first to identify the metabolites of main *Eschscholtzia californica* alkaloids in rat urine using gas chromatography–mass spectrometry (GC–MS) in the electron impact (EI) and positive-ion chemical ionization (PICI) mode. The study was focussed on californine and protopine because the most abundant alkaloid californidine should not be bioavailable because of its quaternary amine structure. Rat urine samples after defined drug dosage were used, since authentic human urine samples were not available. Second, the detectability of californine and protopine and/or their metabolites within the authors' systematic toxicological analysis (STA) procedure in urine by GC–MS in the EI mode was studied [24].

2. Experimental

2.1. Chemicals and reagents

All chemicals used were obtained from E. Merck, Darmstadt (Germany) and were of analytical grade. Californine and protopine were kindly provided by Boehringer Ingelheim, Ingelheim (Germany).

2.2. Urine samples

The investigations were performed using urine of male rats (Wistar, Ch. River, Sulzfleck, Germany) which were administered a single 25 mg/kg body mass dose (for metabolism studies) or a 1 mg/kg body mass dose (for development of the screening procedure) of californine or protopine in aqueous suspension by gastric intubation. Urine was collected separately from the faeces over a 24 h period. All samples were directly analyzed and then stored at −20 °C until further analysis. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

2.3. Sample preparation for metabolism studies

A 5 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 *M*) and incubated at 37 °C for 12 h with 100 μl of a mixture (100 000 Fishman units per ml) of glucuronidase (EC No. 3.2.1.31) and arylsulfatase (EC No. 3.1.6.1), then adjusted to pH 8–9 and extracted with 5 ml of a dichloromethane–isopropanol–ethylacetate mixture (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred into pear-shaped flasks and evaporated to dryness and the residue was derivatized.

Acetylation was conducted with 100 μl of an acetic anhydride–pyridine mixture (3:2, v/v) for 5 min under microwave irradiation at about 440 W

[25,26]. After evaporation, the residue was dissolved in 100 μl of methanol and 2 μl of this solution were injected into the GC–MS. The same procedure with the exception of enzymatic hydrolysis was used to study which metabolites are excreted as glucuronide and/or sulfate.

A second urine sample was worked up as described above, with the exception of the extraction which was carried out at pH of 4–5. The corresponding extract was analyzed after methylation followed by acetylation. Methylation was conducted after reconstitution of the extraction residue in 50 μl of methanol with 50 μl of a solution of diazomethane in diethyl ether, synthesized according to the procedure of McKay et al. [27]. The reaction vials were sealed and left at room temperature for 15 min. Thereafter, the mixture was once again gently evaporated to dryness under a stream of nitrogen and acetylated as described above.

2.4. Sample preparation for toxicological analysis

The urine samples (5 ml) were divided into two aliquots. One aliquot was refluxed with 1 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, the sample was mixed with 2 ml of 2.3 mol/l aqueous ammonium sulfate and 1.5 ml of 10 mol/l aqueous sodium hydroxide to obtain a pH value of 8–9. Before extraction, the other aliquot of native urine was added. This mixture was extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v/v). After phase separation by centrifugation, the organic layer was transferred into a pear-shaped flask and evaporated to dryness. The residue was derivatized by acetylation with 100 μl of an acetic anhydride–pyridine mixture (3:2, v/v) for 5 min under microwave irradiation at about 440 W [24]. After evaporation of the derivatization mixture, the residue was dissolved in 100 μl of methanol and 2 μl were injected into the gas chromatograph.

2.5. Gas chromatography–mass spectrometry

The alkaloids and their metabolites were separated and identified in acetylated urine extracts using a Hewlett-Packard (Agilent, Waldbronn, Germany) 5890 Series II GC combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m×0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100 to 310 °C at 30 °/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode, m/z 50–550 u; EI ionization mode: ionization energy, 70 eV; chemical ionization using methane, positive mode (PICI): ionization energy, 230 eV; ion source temperature, 220 °C; capillary direct interface heated at 260 °C.

For toxicological detection of the alkaloids and their metabolites, mass chromatography was used with the selected ions m/z 174, 188, 190 and 232 (for californine) and m/z 136, 148, 165 and 190 (for protopine). Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros [28] (the macros can be obtained from the authors: e-mail: hans.maurer@uniklinik-saarland.de). The identity of the peaks in the mass chromatograms was confirmed by computerized comparison [29] of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study (Fig. 1).

3. Results and discussion

3.1. Sample preparation

Cleavage of conjugates was necessary before extraction and GC–MS analysis of the suspected metabolites in order not to overlook conjugated metabolites. For studies on the metabolism, gentle enzymatic hydrolysis was preferred, whereas for studies on the toxicological detection, rapid acid hydrolysis was performed. Acid hydrolysis has proved to be very efficient and fast for cleavage of conjugates [24,26,30–33]. However, some compounds covered by this STA are altered or destroyed during acid hydrolysis [24,34]. Therefore, one part of

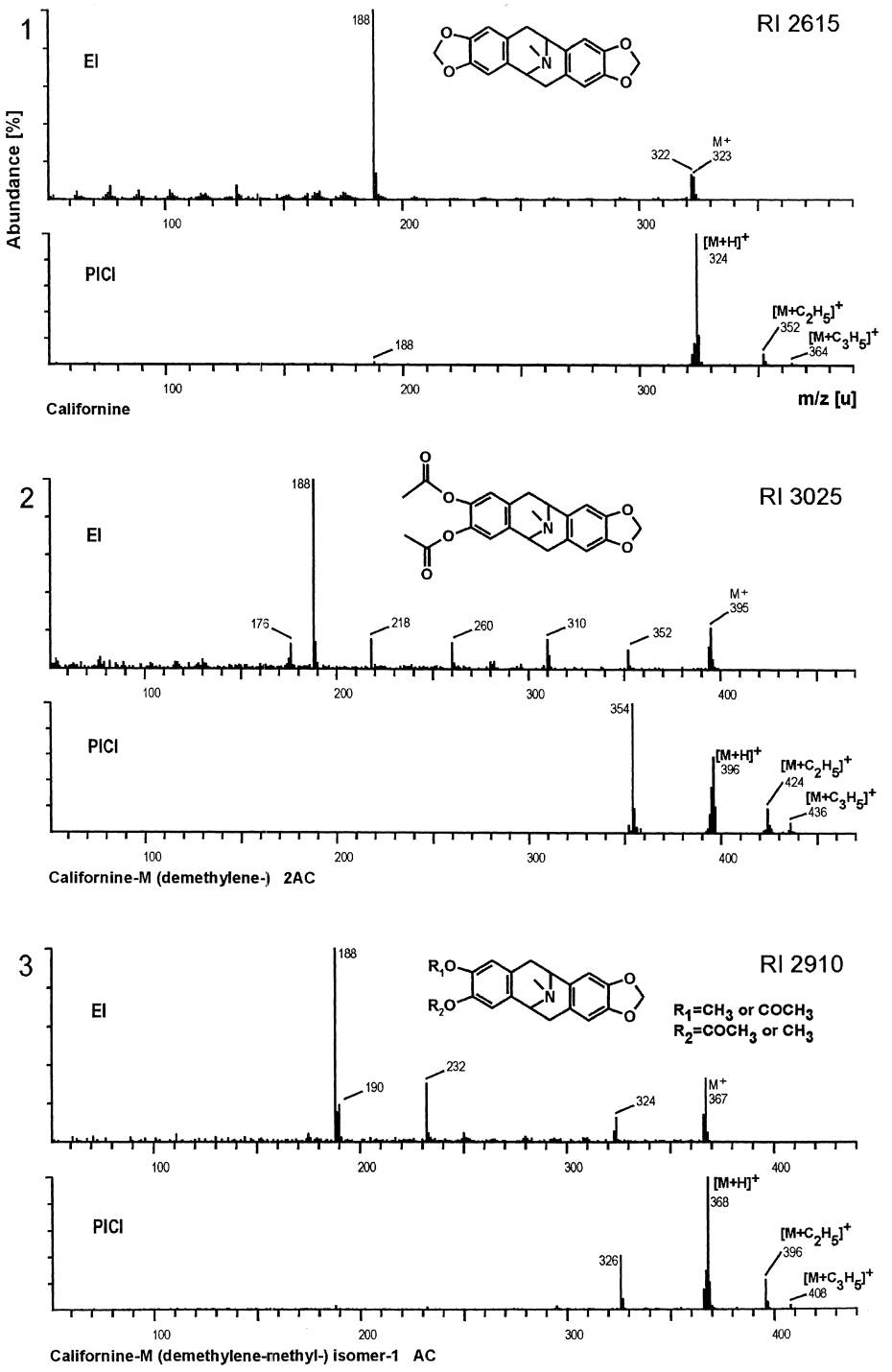
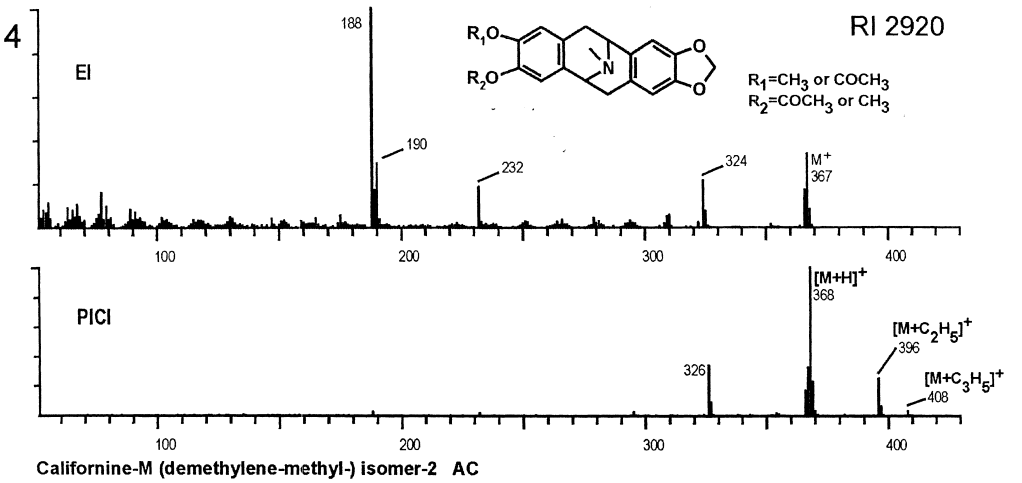
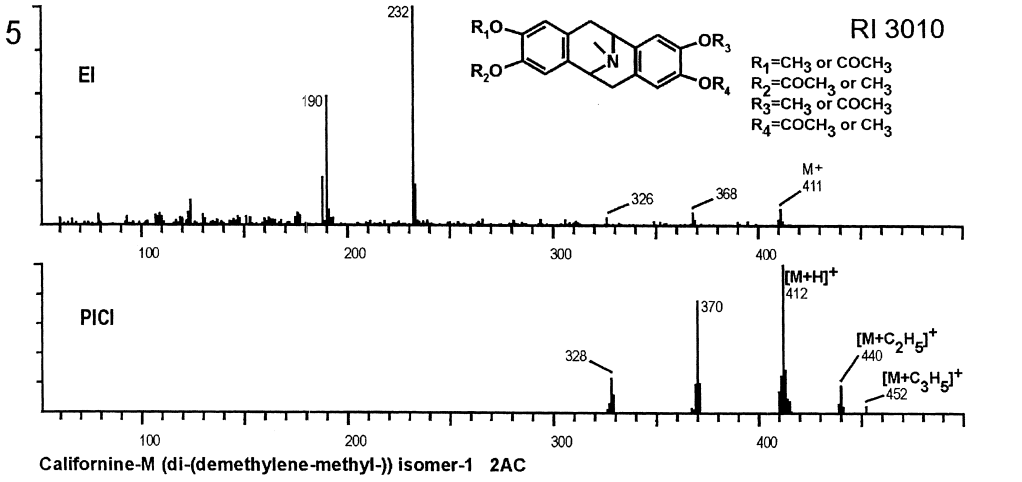
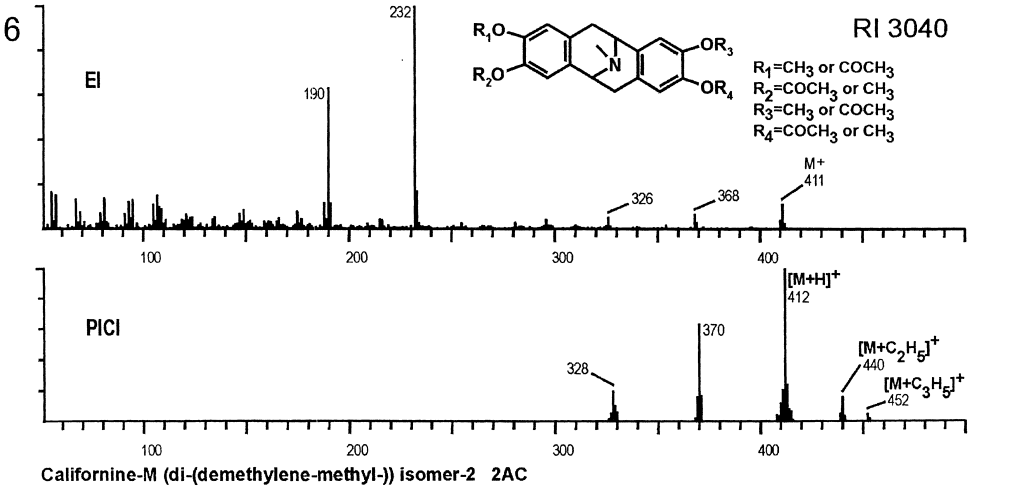


Fig. 1. EI and PICI mass spectra, gas chromatographic retention indices (RI) and structures of californine and protopine and their metabolites after acetylation. The numbers of the spectra correspond to those in Figs. 2–5. The axes are only labelled for spectrum 1.







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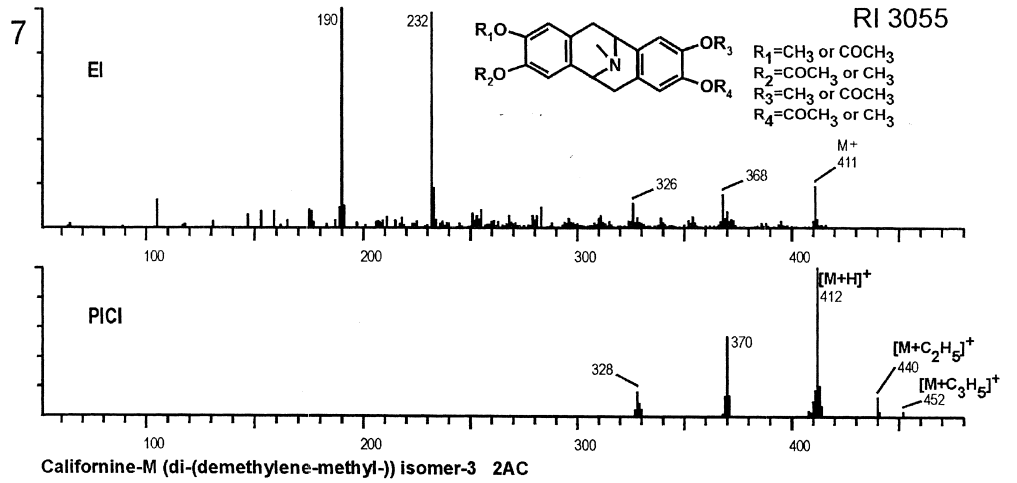

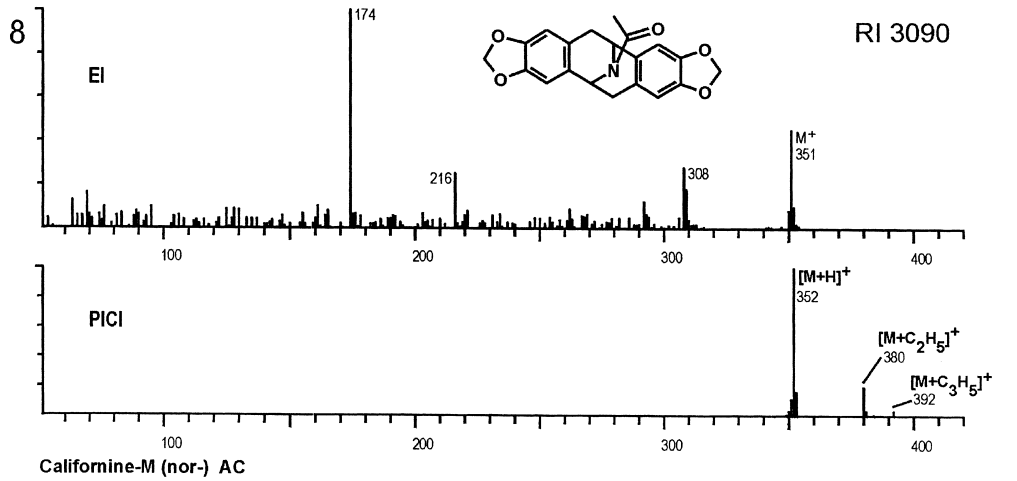

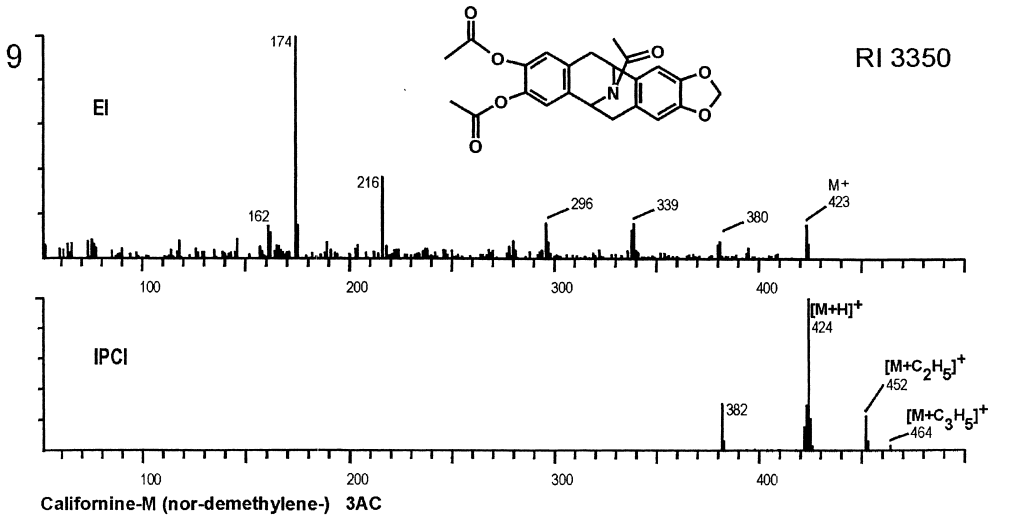

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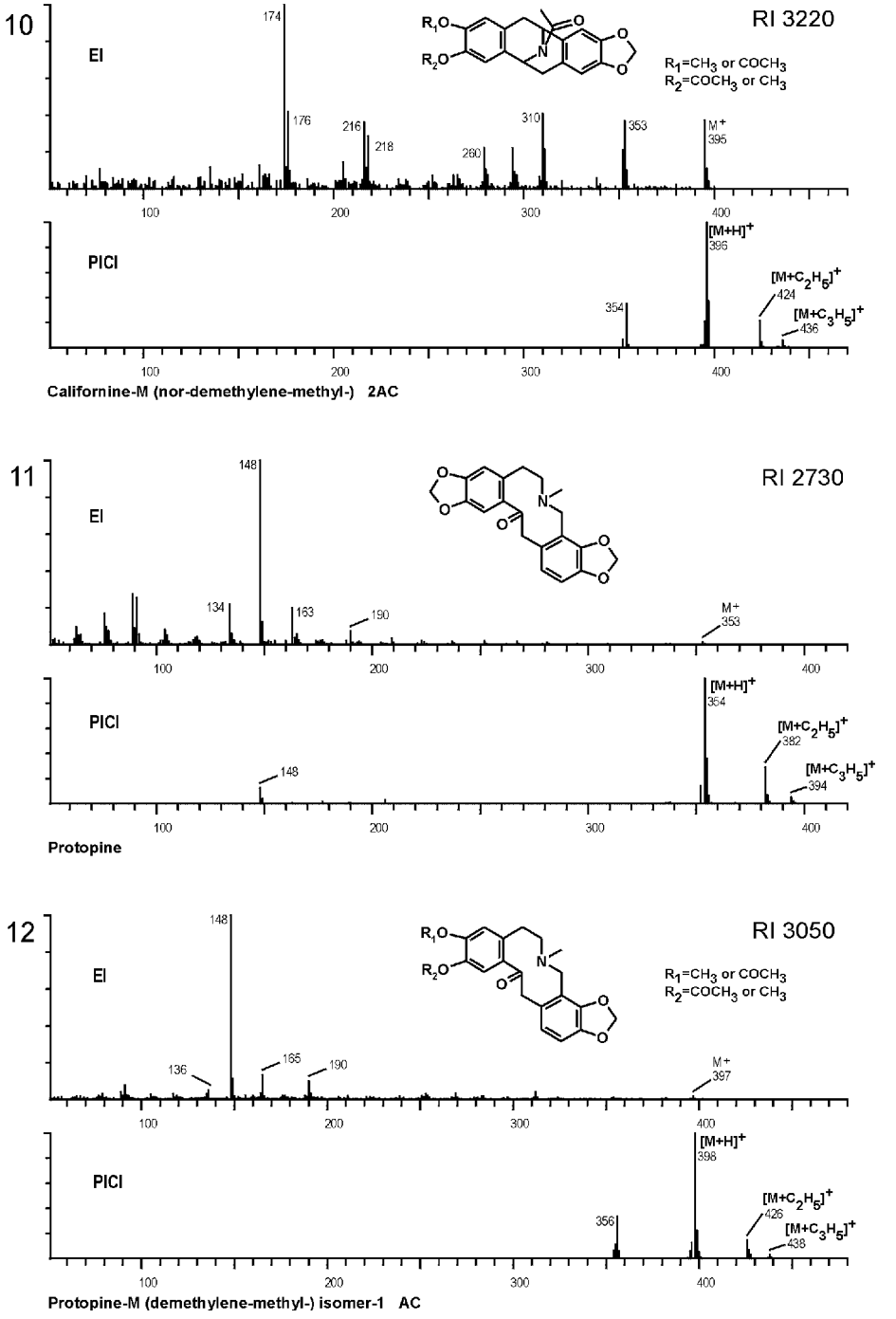

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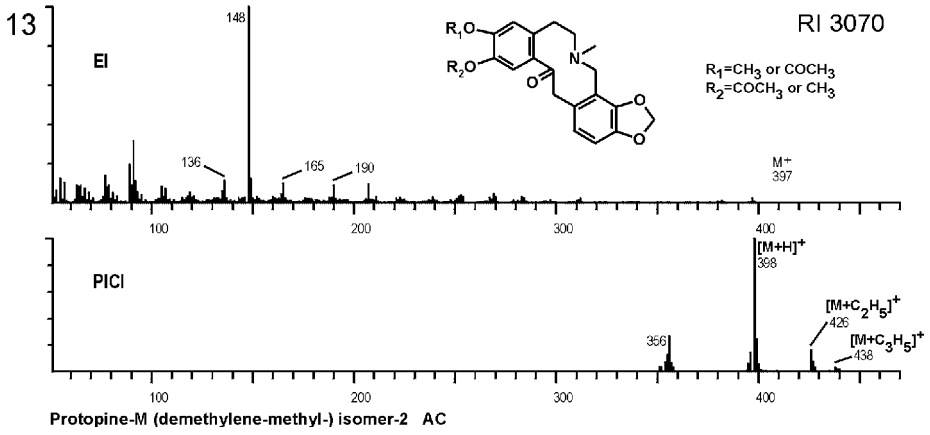

Fig. 1. (*continued*)

unhydrolyzed urine was added before extraction. This modified sample preparation was a compromise between the necessity of a quick cleavage of conjugates and the detectability of compounds and metabolites destroyed during acid hydrolysis. Although the modification of the STA procedure led to lower extract concentrations of compounds excreted in conjugated form, this modified procedure was suffi-

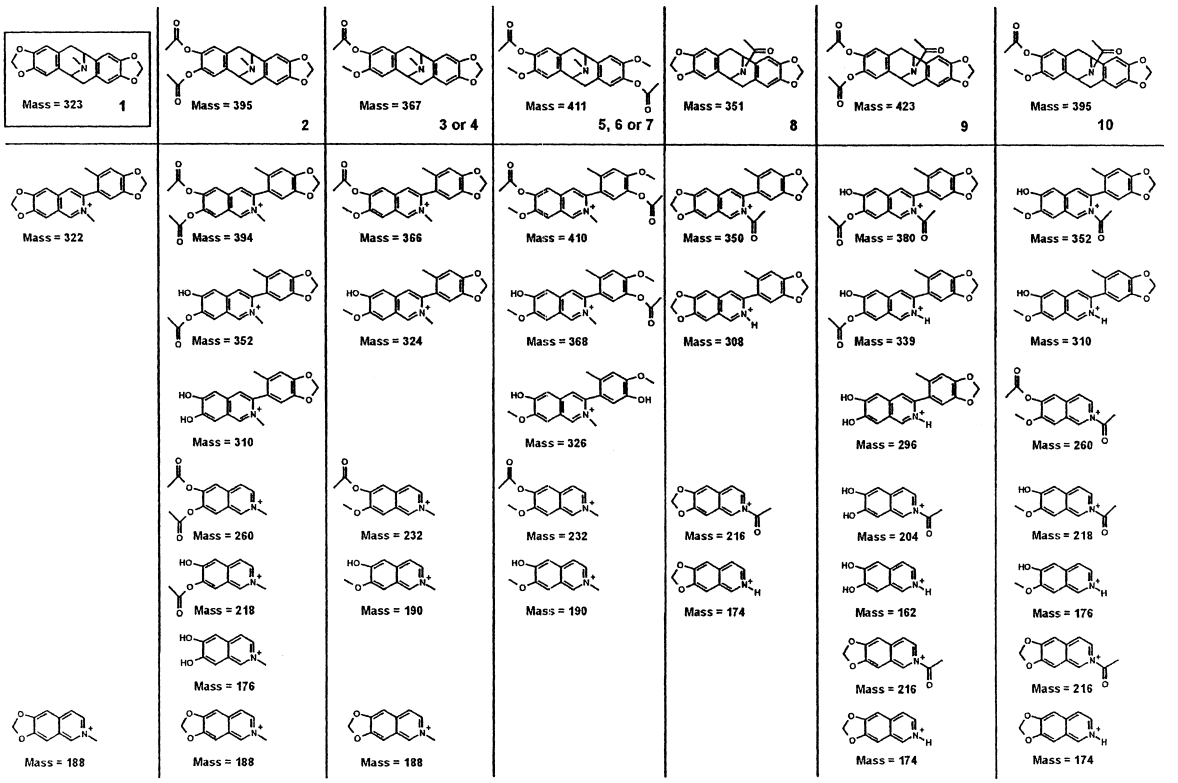

Fig. 2. Predominant fragments of the EI mass spectra of californine and its metabolites after acetylation. For compounds 3–4, 5–7, 10 and 14, the structures of only one isomer each are shown.

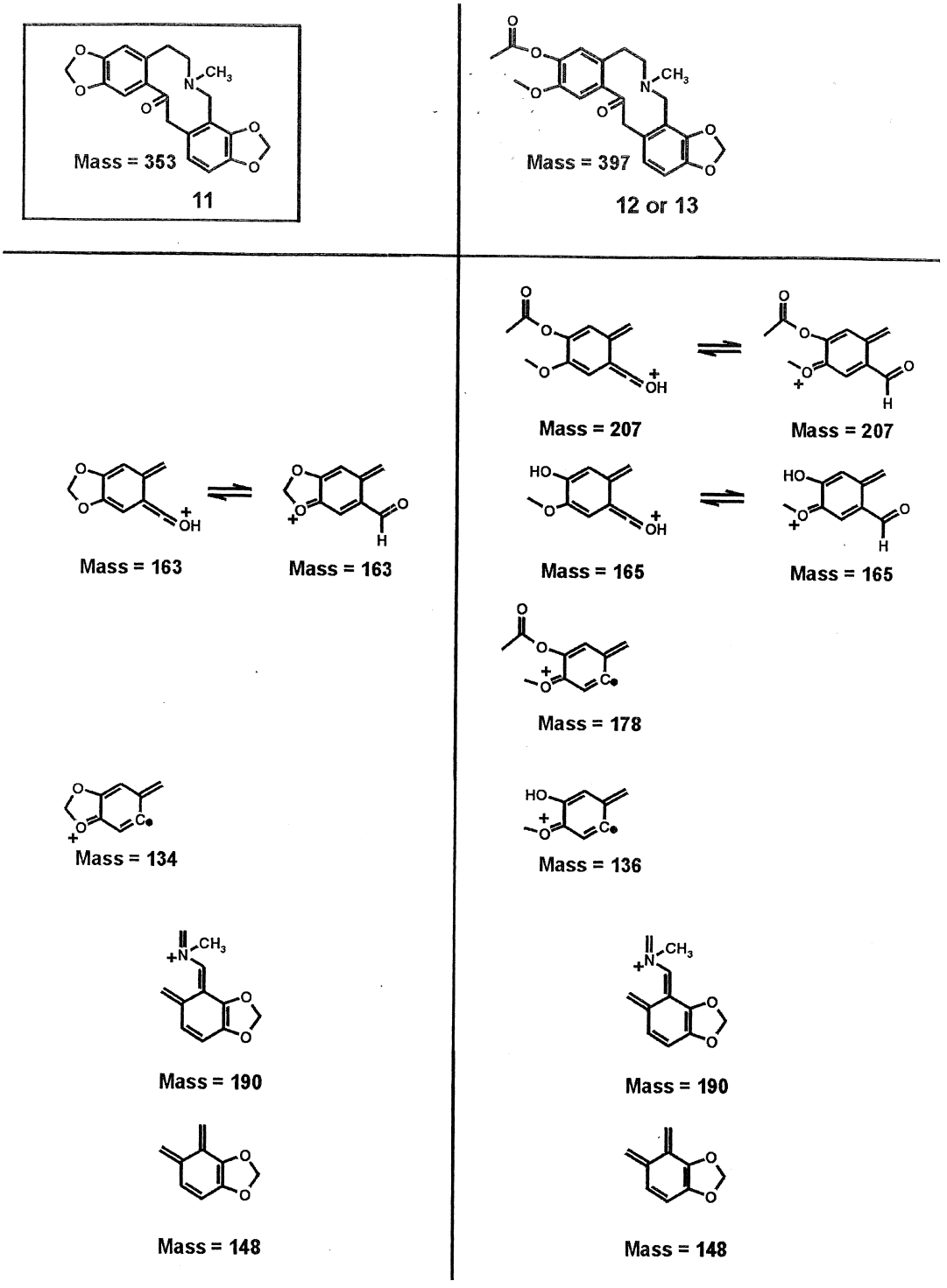

Fig. 3. Predominant fragments of the EI mass spectra of protopine and its metabolites after acetylation. For compounds 12 and 13, the structures of only one isomer are shown.

cient, because of the high sensitivity of modern GC–MS apparatus [24,34].

The samples were extracted at pH 8–9 to achieve the best extraction conditions for the metabolites with aromatic hydroxy groups (phenolbases) which are ionized at higher pH values. However, those metabolites are often excreted for a longer time than the parent compounds [30,35–39]. Derivatization of the extracts was indispensable for sensitive detection.

The extraction efficiencies were studied using spiked urine samples and were found to be 93±2% for californine and 96±3% for protopine ($n=5$). However, as both alkaloids are almost completely metabolized, the determination of the extraction efficiencies is of little use.

3.2. Identification of metabolites

The urinary metabolites of californine and protopine were separated by GC and identified by EI and PICI MS after enzymatic hydrolysis, extraction and acetylation. The EI and PICI mass spectra, gas chromatographic retention indices (RI) and structures of californine and protopine and their metabolites after acetylation are shown in Fig. 1 and will be included in the forthcoming update of the authors' handbook and library [29,40]. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode which were interpreted in correlation to those of the parent compound according to the rules described by McLafferty and Turecek [41]. In addition, studies on mass spectral fragmentation of pavine and protopine alkaloids have also been taken into consideration [42–46]. The predominant fragments of the EI spectra of californine, protopine and their metabolites after acetylation are shown in Figs. 2 and 3 (only one isomer each is shown). In order to verify the molecular mass of the postulated metabolites, PICI mass spectra were recorded, because they contain strong molecular peaks (M+H) with adduct ions typical for PICI using methane as reagent gas.

Californine and protopine (mass spectra nos. 1 and 11 in Fig. 1) were extensively metabolized and could only be detected in very small amounts after the administration of 25 mg/kg body mass. The follow-

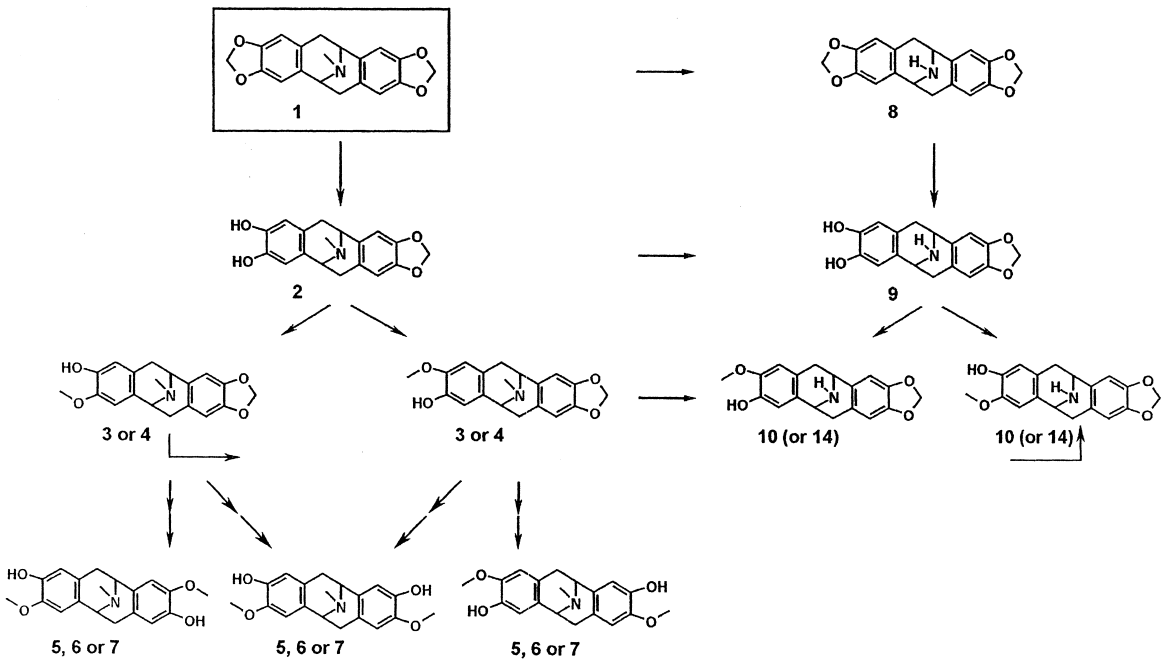

Fig. 4. Proposed scheme for the metabolism of californine in rats. The phenolic metabolites were also excreted as glucuronides and/or sulfates.

ing metabolites could be identified in rat urine: for californine, mono-demethylene californine (mass spectrum no. 2), two isomers of mono-demethylenemethyl californine (mass spectra nos. 3 and 4), three isomers of di-demethylenemethyl californine (mass spectra nos. 5–7), *N*-demethyl californine (mass spectrum no. 8), *N*-demethyl-mono-demethylene californine (mass spectrum no. 9), *N*-demethyl-mono-demethylenemethyl californine (mass spectrum no. 10). A second isomer of the latter metabolite (no. 14 in Fig. 4) should be formed, but it could not be detected due to very low concentration. For the same reason, the expected isomers of *N*-demethyl-di-demethylenemethyl californine could also not be detected. For protopine, two isomers of the mono-demethylenemethyl metabolite (mass spectra nos. 12 and 13) could be found. The intermediate demethylene metabolite could not be detected.

Based on the identified metabolites, the following partially overlapping metabolic pathways could be postulated for californine (Fig. 4): *N*-demethylation and/or single or double demethylenation with consecutive catechol-*O*-methylation of one of the hydroxy groups. For protopine, the following metabolic pathway could be postulated (Fig. 5): demethylenation only of the 2,3-methylenedioxy group (confirmed by fragmentation pattern) followed by catechol-*O*-methylation.

All the phenolic compounds are partially excreted as conjugates, since the peak areas were greater after cleavage of conjugates. As a mixture of glucuronidase and arylsulfatase has been used for conjungate cleavage, a differentiation whether the metabolites are excreted as glucuronides or sulfates was not possible.

In order to check for acidic metabolites, the urine samples had also been extracted after cleavage of conjugates at acidic pH and the corresponding extracts had been analyzed after methylation plus acetylation. However, no acidic metabolites were found.

3.3. Detection of californine and protopine metabolites by GC–MS within the STA

Californine, protopine and their metabolites were separated by GC and identified by EI MS after acid hydrolysis, extraction and acetylation within the authors' standard STA. Mass chromatography with the following ions *m*/*z* 174, 188, 190 and 232 was used to indicate the presence of californine and its metabolites and *m*/*z* 136, 148, 165 and 190 to indicate the presence of protopine and its metabolites. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros. Fig. 6 shows reconstructed mass chromatograms indicating the presence of californine metabolites in an acetylated extract of a rat urine sample collected over 24 h after ingestion of 1 mg/kg body mass of californine. Fig. 7 shows reconstructed mass chromatograms indicating the presence of protopine and its metabolites in an acetylated extract of a rat urine sample collected over 24 h after ingestion of

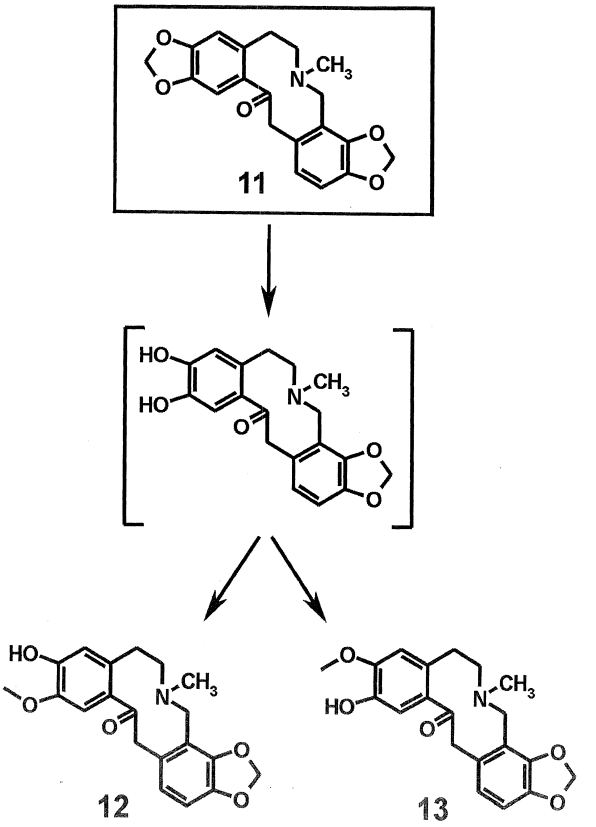

Fig. 5. Proposed scheme for the metabolism of protopine in rats. The phenolic metabolites were also excreted as glucuronides and/or sulfates.

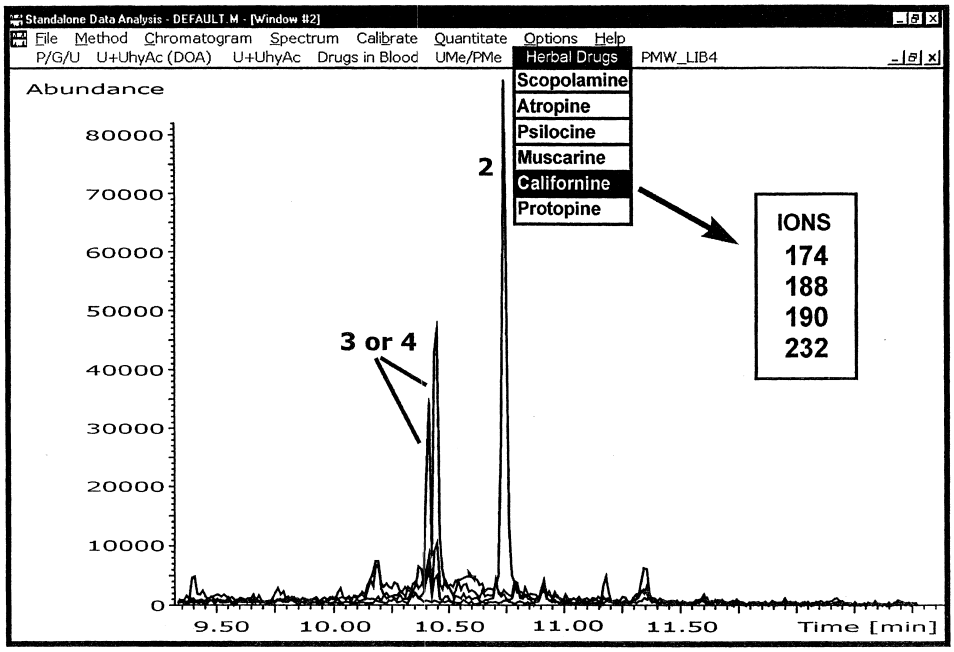

Fig. 6. Typical mass chromatograms with the ions m/z 174, 188, 190 and 232. They indicate the presence of californine metabolites in an acetylated extract of a rat urine sample collected over 24 h after ingestion of 1 mg/kg body mass of californine. The numbers of the peaks correspond to those in Figs. 1, 2 and 4. The merged chromatograms can be differentiated by their colors on a color screen.

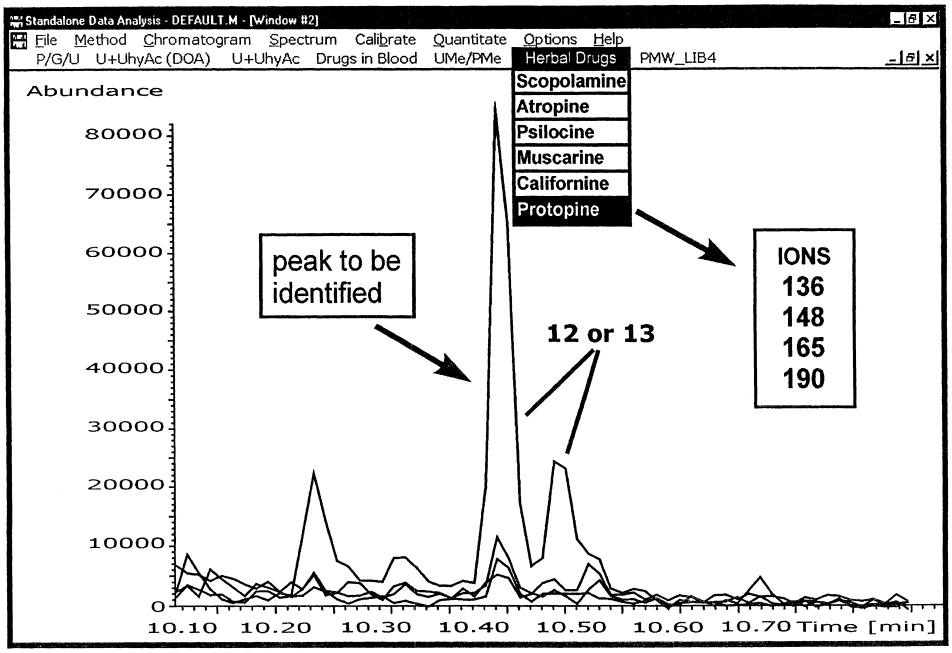

Fig. 7. Typical mass chromatograms with the ions m/z 136, 148, 165 and 190. They indicate the presence of protopine metabolites in an acetylated extract of a rat urine sample collected over 24 h after ingestion of 1 mg/kg body mass of protopine. The numbers of the peaks correspond to those in Figs. 1, 3 and 5.

1 mg/kg body mass of protopine. The identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra (Fig. 1) recorded during this study [29]. Fig. 8 shows the mass spectrum underlying the marked peak in Fig. 7, the reference spectrum, the structure, and the hit list found by computer library search. The gas chromatographic RIs given in Fig. 1 allowed differentiation of isomers. They were recorded during the GC–MS procedure (Section 2.5) and calculated in correlation with the Kovats' indices [47] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [48,49]. The reproducibility of retention indices measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats.

Although the limit of detection of californine in urine was as low as 0.5 ng/ml and that of protopine 2 ng/ml (S/N 3) under routine MS conditions, no more parent compound could be detected, which means that both alkaloids are excreted almost completely in metabolized form. As already mentioned in Section 3.2, the alkaloids themselves could only be detected after administration of the higher dose.

The authors' STA procedure allowed the detection of the metabolites of the *Eschscholtzia californica* alkaloids californine and protopine in rat urine after administration of a dose which should correspond to a common drug user's dose extrapolated from a dose commonly applied in *Eschscholtzia californica* phytopharmaceuticals. Assuming similar metabolism in man, the analytical targets in urine in case of a poisoning with *Eschscholtzia* should be the main metabolites demethylene and demethylenemethyl californine and demethylenemethyl protopine.

As far as known, californine is a unique alkaloid of *Eschscholtzia californica*, while protopine is a widespread alkaloid in papaveraceae or fumariaceae. Therefore, californine and/or its metabolites should be the analytical markers for use of *Eschscholtzia*

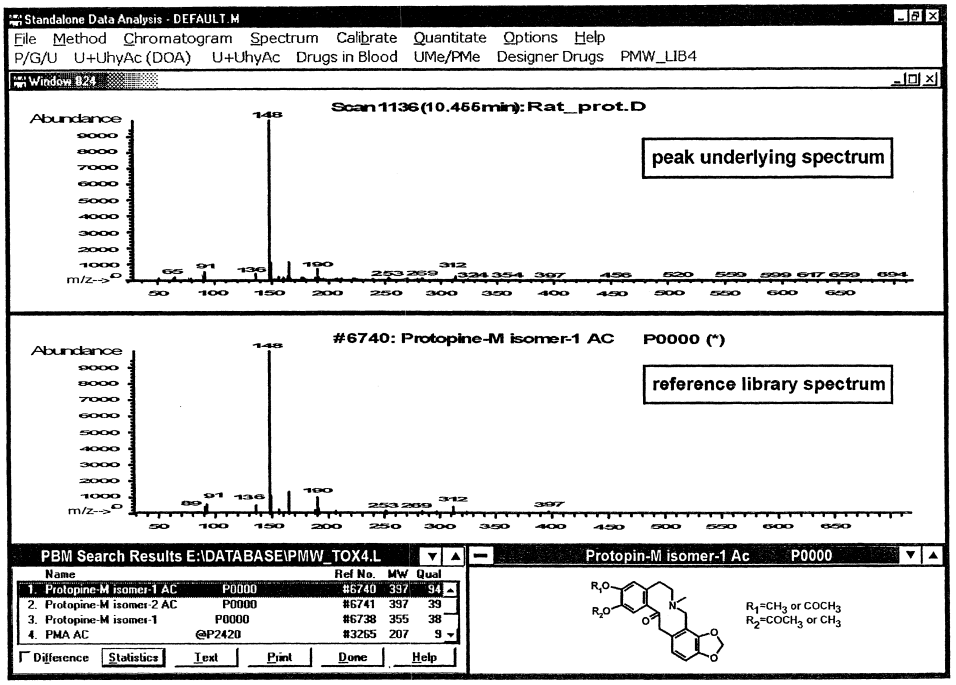

Fig. 8. Mass spectrum underlying the marked peak in Fig. 7, the reference spectrum, the structure, and the hit list found by computer library search.

californica preparations. Due to full-scan mass spectral detection, interferences with other drugs or biomolecules are improbable [40,50].

4. Conclusions

The presented studies showed that californine and protopine are extensively metabolized. A clinical or forensic analysis in body samples, especially in urine, should be focussed on the main metabolites each. According to the authors' experience in metabolism and analytical studies in rats and humans, it should be possible to detect the metabolites found in rat urine also in human urine samples. This study is an example for the necessity of metabolism studies as a prerequisite for developing of a toxicological screening procedure.

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