Biotransformation of Urapidil: Isolation and Identification of Metabolites in Mouse, Rat, Dog and Man

Ernst Sturm and Karl Zech
Research Laboratories of Byk Gulden Lomberg Chemische Fabrik GmbH, Box 6500, D–7750 Konstanz, FRG

The identification of biotransformation products of the new antihypertensive drug urapidil in mouse, rat, dog and man has been performed by means of high-performance liquid chromatographic and mass spectrometric techniques. In urine, three metabolites were found in addition to the unchanged drug. The para-hydroxylated product (1) (6-{3-[4-(*o*-methoxy-*p*-hydroxyphenyl)piperazinyl]-propylamino}-1,3-dimethyl-uracil), the *O*-demethylated compound (2) (6-{3-[4-(*o*-hydroxyphenyl)piperazinyl]-propylamino}-1,3-dimethyluracil) and the uracil-*N*-dealkylated compound (3) (6-{3-[4-(*o*-methoxyphenyl)piperazinyl]-propylamino}-1-methyl-uracil). In urine of dog, the metabolite with the *N*-oxide structure (5) was also identified, but only in trace amounts (6-{3-[4-(*o*-methoxyphenyl)piperazinyl-*N*-oxide]-propylamino}-1,3-dimethyluracil).

INTRODUCTION

Urapidil,[1] 6-{3-[4-(*o*-methoxyphenyl)piperazinyl]-propylamino}-1,3-dimethyluracil (Ebrantil®, Byk Gulden), is a new antihypertensive drug, the efficacy of which has been proven in animals[2] as well as in man.[3] Biotransformation of this compound was studied in urine and serum samples[4] of mouse, rat, dog and man. Comparison of metabolic patterns of the investigated species revealed that all species break urapidil down to the same metabolites, but to a different degree. Serum concentration, urinary excretion and biological activity of the metabolites in comparison with urapidil in *in vitro* and *in vivo* systems are desribed elsewhere.[5] This paper deals with the isolation and identification of these metabolites in urine of animals and man following administration of the unlabeled drug. After isolation of metabolites from urine of the species with the highest concentration of the respective metabolite by means of semipreparative high-performance liquid chromatography (HPLC), structural elucidation was realised using electron impact (EI) and chemical ionization (CI) mass spectrometry. After synthesis of the metabolites, co-chromatography of the synthetic compound with the isolated metabolites and comparison of the mass spectra of synthetic and isolated metabolites was performed additionally.

EXPERIMENTAL

Collection of samples

Five groups of NMRI mice (four animals in each group) and Sprague–Dawley SIV 50 rats (two animals in each group) for each sex and four male and two female Beagle dogs were used. The animals, weighing on average 26 g (mice), 230 g (rats) and 13 kg (dogs), were fasted for 12 h before administration of 30 mg kg^{-1} urapidil solution to mice and rats (5 and 30 mg ml^{-1}, respectively) and of 10 mg kg^{-1} urapidil as tablets to dogs. The animals of these three groups were held in metabolic cages to ensure a clean separation of urine and faeces. The urine of animals and of man (after administration of one sustained release 30 mg capsule) was collected in two fractions, 0–8 and 8–24 h, and immediately frozen until analysis.

Separation of metabolites

Urapidil and metabolites were fractionated by HPLC[6] (Fig. 1) after direct injection of centrifuged urine. Omission of sample extraction before analysis was necessary because of metabolite instability and loss of polar metabolites which was quantitative for urapidil-*N*-oxide. Using a semipreparative separation column (8 × 250 mm) with a flow of the mobile phase adjusted to 4 ml min^{-1}, approximately 100–150 μg of pure substance per component was obtained. Repeated injection and collection of timed fractions[7] resulted in enough material for different modes of mass spectrometry and for checking the fraction by analytical HPLC.

Preparation of samples for mass spectral analysis

High-performance liquid chromatographic fractions containing the metabolites were neutralized, acetonitrile was partly removed from the fractions on a rotary evaporator and the remaining aqueous layer lyophilized. The residue was dissolved in dichloromethane, Na_2SO_4 was added and centrifuged. After concentration of the organic solution, the sample was applied to the solid insertion probe of the mass spectrometer from a microliter syringe.

CCC–0306–042X/84/0011–0211 $03.00

© Wiley Heyden Ltd, 1984

Mass spectral analysis

Low resolution electron impact and chemical ionization spectra were recorded on a VG MM 7070F double-focusing mass spectrometer interfaced to a VG 2035 data system. Determination of elemental composition involved peak matching at a resolution of $M/\Delta M = 5000$. The sample was warmed up by induction from the ion chamber. The mass spectrometer conditions for EI were: electron energy, 70 eV; trap current, 100 μA; source temperature, 200 °C; scan rate, 3 s/decade. The mass spectrometer conditions for isobutane CI were: electron energy, 50 eV; emission current, 500 μA; source temperature, 190 °C; source housing pressure, 2×10^{-5} Torr.

RESULTS AND DISCUSSION

Mass spectral identification of metabolites

The structures of metabolites **1**, **2**, **3** and **5** were elucidated by mass spectral analysis of high-performance liquid chromatographic fractions using electron impact and isobutane chemical ionization. The EI mass spectra of the metabolites isolated from urine of man, rat or dog were compared to those obtained with urapidil and synthesized metabolites (Figs 2 and 3). HPLC co-chromatography of biological and reference samples provided an additional control. The metabolic pathway is demonstrated in Fig. 5.

Internal α-cleavage of the piperazine moiety[8,9] is the predominant fragmentation process of urapidil and its metabolites following electron impact ionization. The charge resides with either the uracil- or the aryl-ring containing fragments. Figure 4 shows the principal fragmentation pattern proposed for urapidil, which was substantiated by the mass spectra of structurally related compounds, especially by the mass spectrum of a deuterium labeled analogue of urapidil containing a quadruple deuterated ethylene group in the piperazine ring. Consequently, the presence of the base ion m/z 225 can be used to characterize the unchanged uracil nucleus, whilst mass shifts of the ions *d*–*m* can be used to monitor the change in the substituent(s) in the phenyl ring. Surprisingly, cleavage of the propylene bridge forming ion *f* is of minor importance. The abundances of the $[M-2]^+$ and $[M-15]^+$ ions are related to the concentration of sample in the ion source and to the source temperature. As the loss of both $CH_3^{\cdot}$ and $C^2H_3^{\cdot}$ is observed in the mass spectrum of the deuterium-labeled analogue, formation of the $[M-15]^+$ ion involves fragmentation and rearrangement of the piperazine ring.

The elemental composition of the molecular ions of **1**, **2** and **3** obtained by peak matching, together with the base ions observed in the CI spectra, are given in Table 1. The CI spectra of **1–3**, exhibiting dominant protonated molecular ions, were useful for confirmation of the molecular weight but did not give more structural information. In **5**, neither $[M]^{+\cdot}$ nor $[MH]^+$ could be detected in EI and CI modes, respectively.

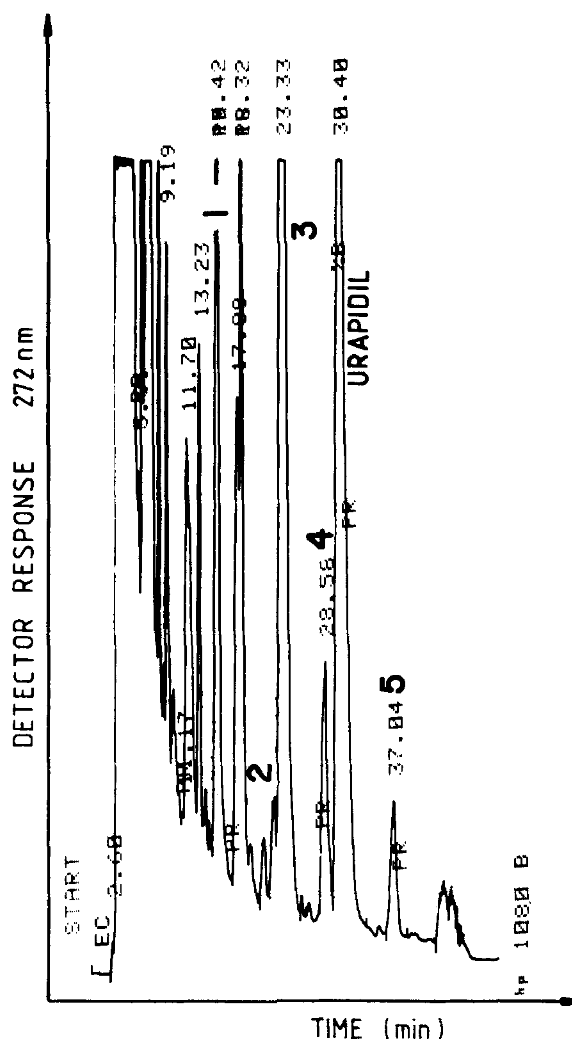

Figure 1. Typical high-performance liquid chromatogram of *semi-preparative* separation of sample from 0–8 h dog urine used for isolation of metabolites **3**, **4**, and **5**. Injection volume: 50 μl; column: Lichrosorb®, RP8-7 μm, 8×250 mm; mobile phase: water (pH 2.0 with sulphuric acid) with 10% acetonitrile, gradient elution with 0.5% min^{-1}; flow 4 ml min^{-1}; detection at 272 nm. **1–5** denote metabolites. Most of the other peaks correspond to background as known from comparison with blank urine.[6]

Metabolite 1

The elemental composition of the molecular ion of **1** (Table 1) shows that compared with urapidil, **1** contains an additional oxygen atom. The presence of the base

Table 1. Elemental composition of urapidil and its metabolites, accurate mass data and base ions observed in CI mode

	Elemental composition	Exact mass of $[M]^{+\cdot}$ Observed	Calculated	Base ion in CI mode
Urapidil	$C_{20}H_{29}N_5O_3$	387.2275	387.2270	388 $[MH]^+$
1	$C_{20}H_{29}N_5O_4$	403.2229	403.2219	404 $[MH]^+$
2	$C_{19}H_{27}N_5O_3$	373.2119	373.2114	374 $[MH]^+$
3	$C_{19}H_{27}N_5O_3$	373.2120	373.2114	374 $[MH]^+$
5	$C_{20}H_{29}N_5O_4$	—	403.2219	388 $[MH-O]^+$

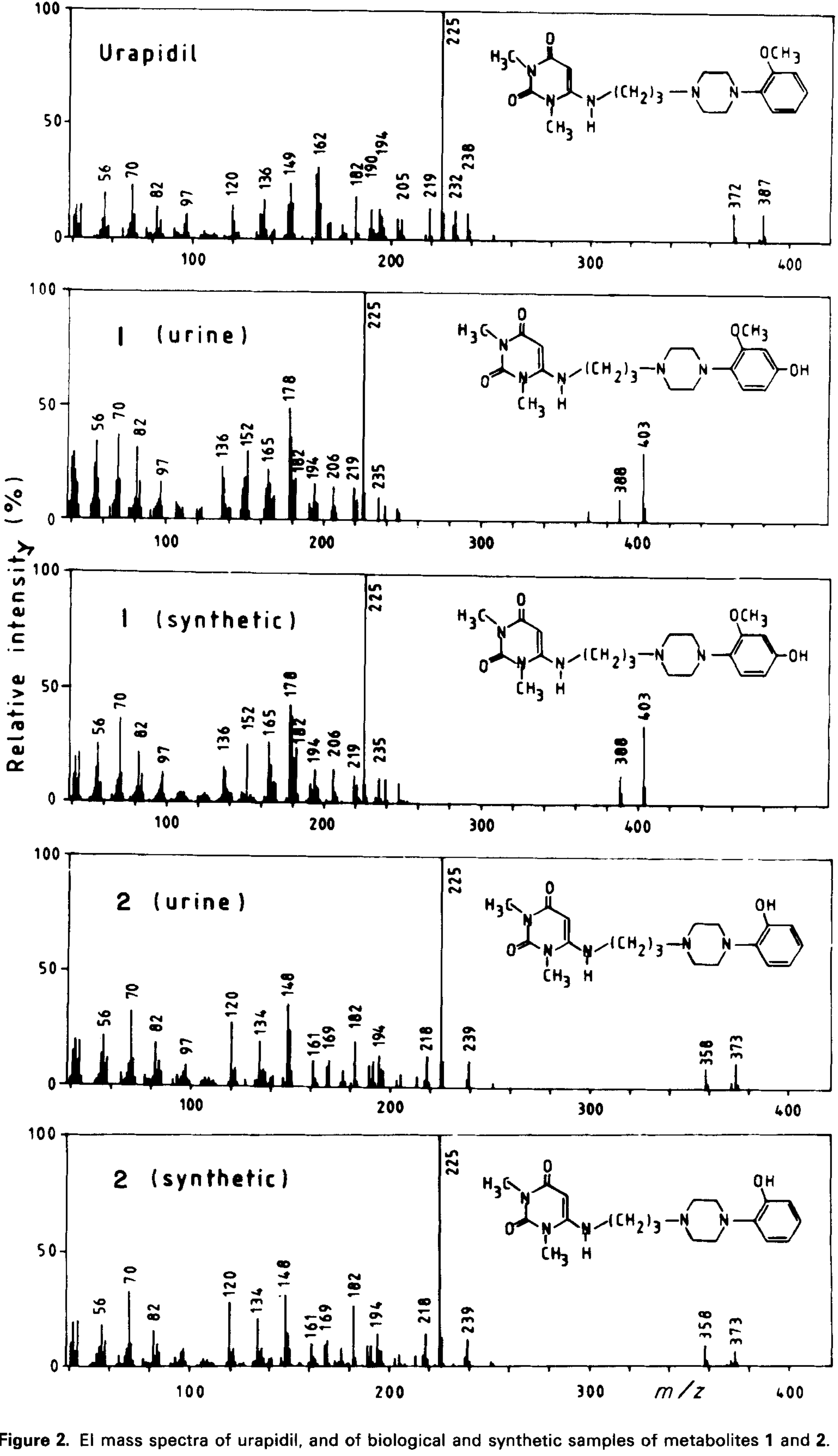

Figure 2. EI mass spectra of urapidil, and of biological and synthetic samples of metabolites **1** and **2**.

ion m/z 225, together with the shift of the ions d–m by 16 u to higher mass numbers, indicate that **1** is formed by aromatic ring hydroxylation, probably by introduction of an oxygen atom in p-position. In solution, **1** decomposes slowly to 6-[3-(1-piperazinyl)propylaminol]-1,3-dimethyluracil. Formation of this product was evidenced by the presence of its protonated molecular ion $[MH]^+$ (m/z 282) in CI mass spectra of samples containing **1** after prolonged storage, and by comparison of high-performance liquid chromatography

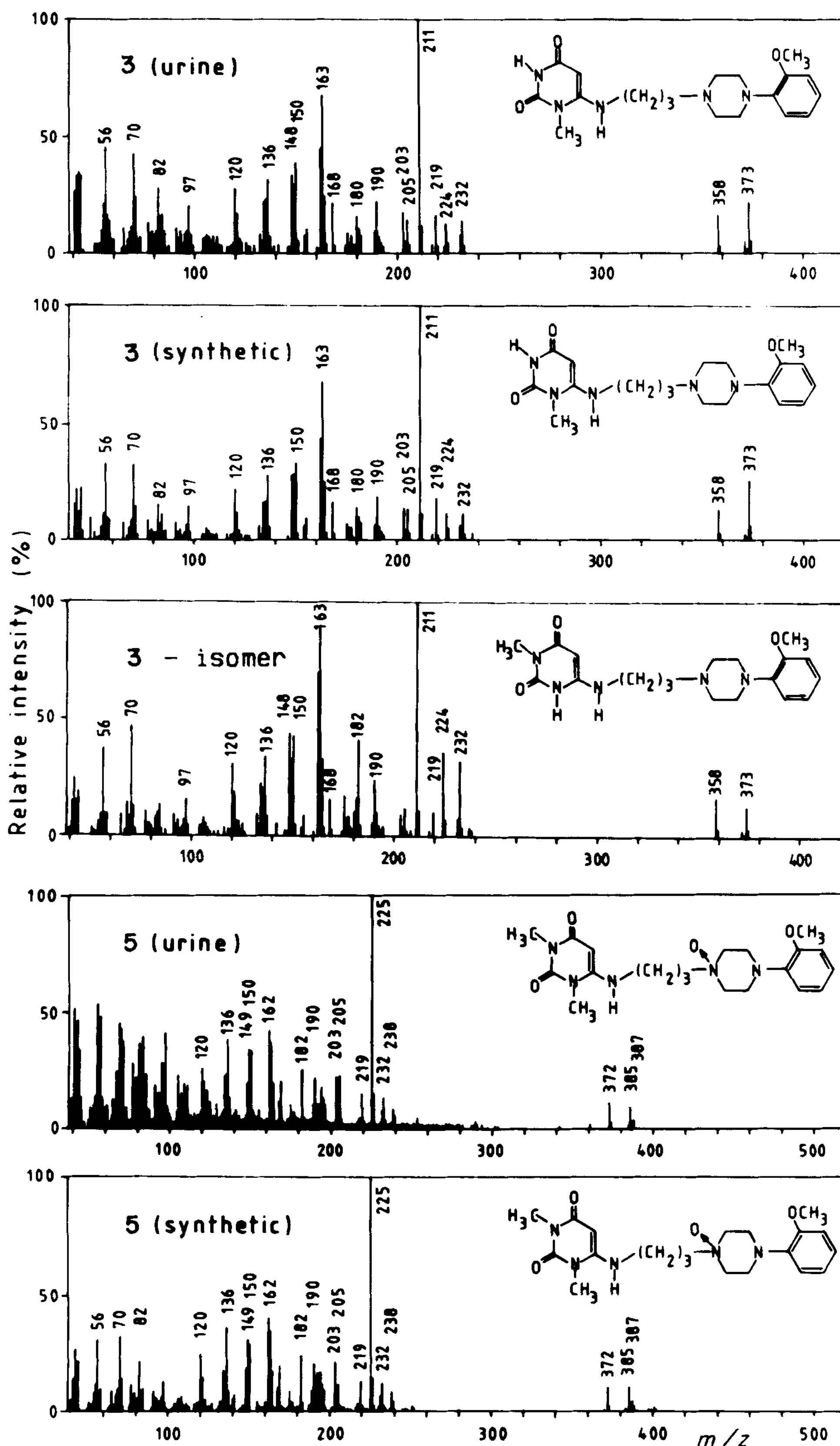

Figure 3. EI mass spectra of biological samples of metabolites **3** and **5** and of respective reference compounds.

retention times using an authentic sample. Degradation of **1** in solution appears plausible, since the presence of the OH-group in **1** offers the possibility of oxidation to a quinoid system with subsequent hydrolysis.

Metabolite 2

The EI mass spectrum of **2** shows the molecular ion at m/z 373, whose elemental composition (Table 1) for-

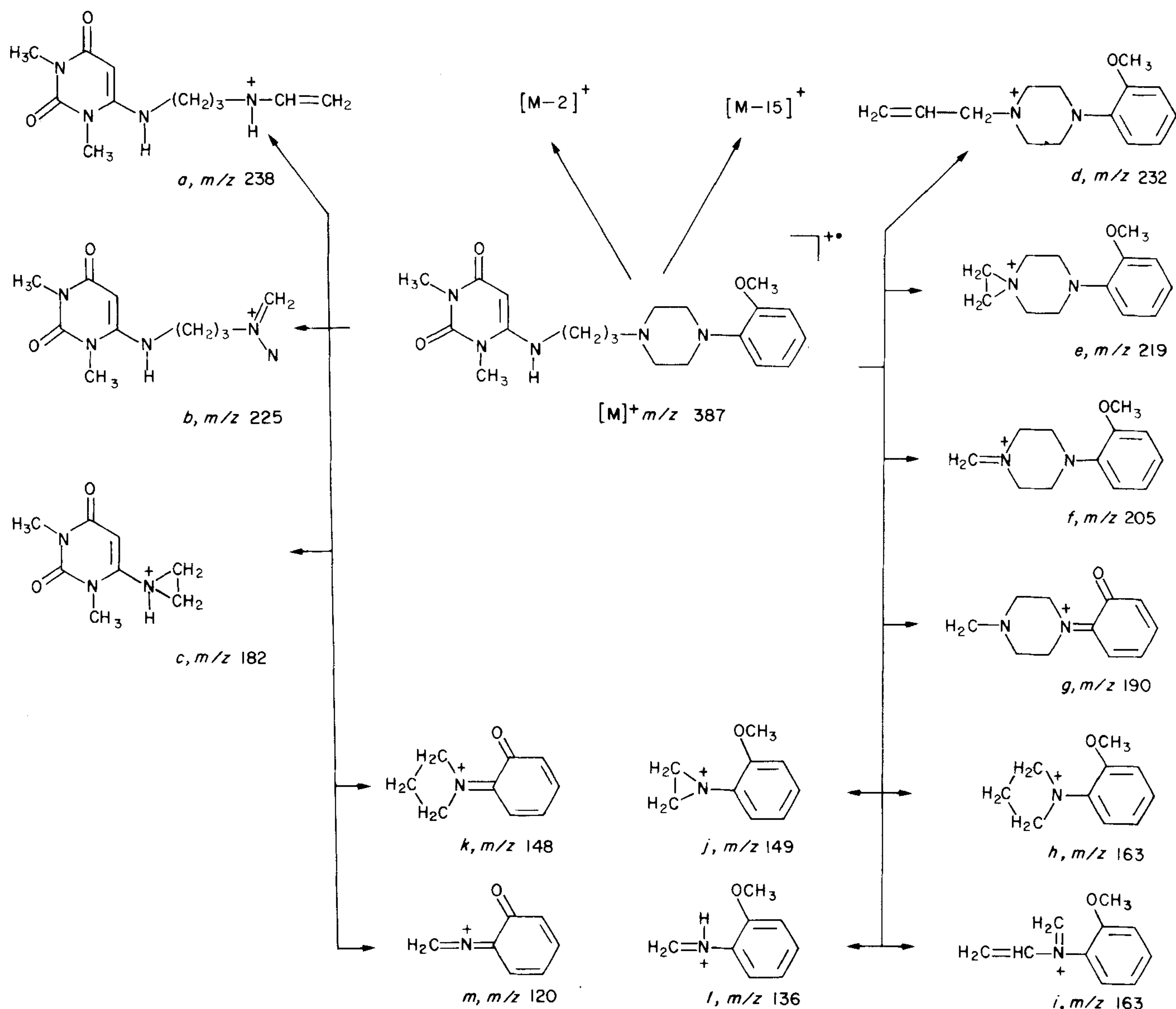

Figure 4. Fragmentation pattern proposed for urapidil.

mally suggests formation of **2** from urapidil by loss of a CH_2-group, hence by demethylation. As for urapidil and **1**, the spectrum shows the base ion typical for the unchanged uracil ring. The fragments *d–j*, *l* in the spectrum of urapidil are shifted in **2** by 14 u to lower mass numbers while maintaining their relative intensities. Therefore, **2** is formed by *O*-demethylation from urapidil, as confirmed by the mass spectrum of the synthetic product.

Metabolite 3

The elemental composition of **3** (Table 1) is identical to that of **2**; **2** and **3** being isomers. Comparison of the mass spectra of urapidil and **3** shows that the fragment m/z 225 typical for the uracil nucleus is shifted in **3** by 14 u to m/z 211, while all fragments containing the aryl ring of urapidil and **3** are identical. Thus, formation of **3** occurs via demethylation of the uracil nucleus resulting in one of two possible isomers. Similar fragment ions were observed in the spectra for both synthetic isomers, but the significant difference in relative intensities of m/z 182 allowed for positive identification of **3**. This result was confirmed by the high-performance liquid chromatographic retention properties of the two synthetic isomers. **3** is formed by specific demethylation of urapidil in position 3 of the uracil ring.

Metabolite 5

Except for background ions in the low mass range, the mass spectrum of **5** is almost identical to that of urapidil. There is, however, a significant difference in the relative intensities of the peaks m/z 387 and 385, which could be explained by the assumption, that **5** is a *N*-oxide of urapidil allowing formation of m/z 387, 386, 285 by loss of O, OH and H_2O, respectively. The additional oxygen atom can reside on either piperazine nitrogen atom. For compounds with several N-atoms, like urapidil, that *N*-oxide will be formed as a rule whose oxygen atom is localized at the most basic *N*-atom.[10]

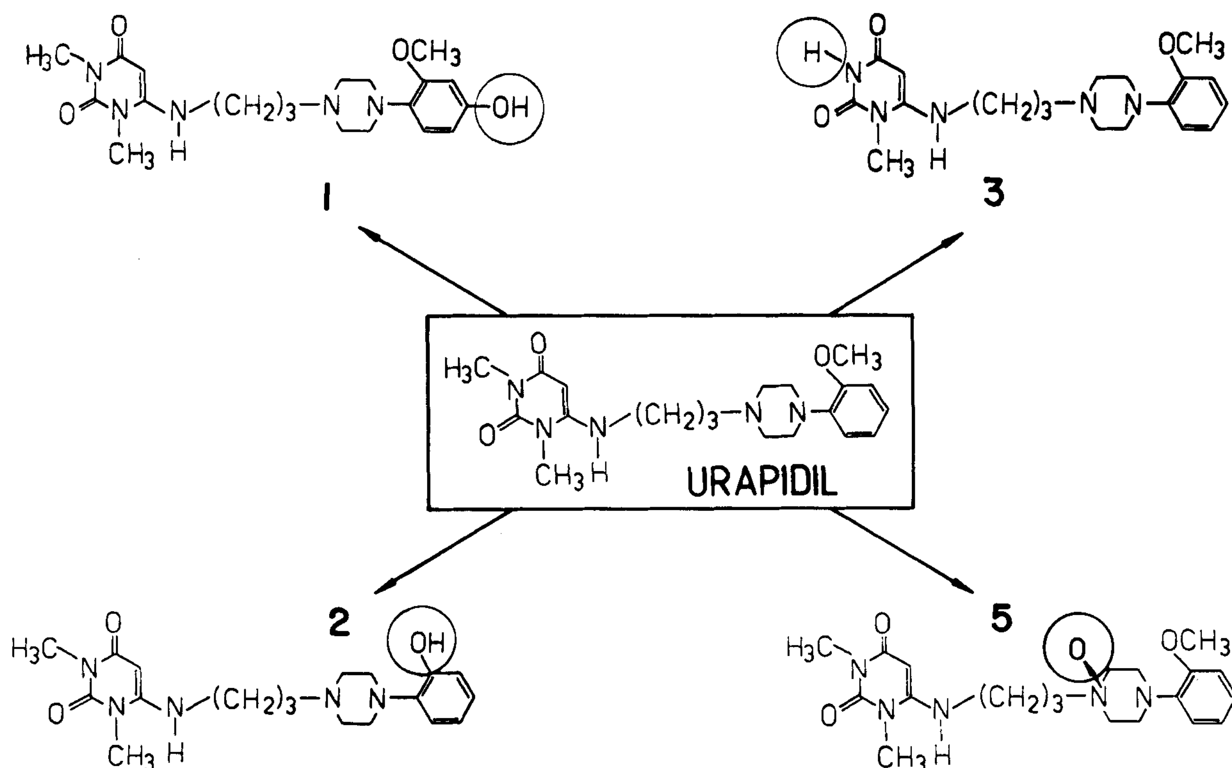

Figure 5. Metabolic pathways of urapidil.

Therefore it appeared appropriate to compare **5** with the synthetic *N*-oxide the oxygen of which is localized at the more basic piperazine-N-centre of urapidil. This reference product shows the same major fragments in the EI spectrum as **5**. Also, in CI mode, **5** and the synthetic *N*-oxide give identical behaviour: m/z 388 is observed as base ion in both cases, an ion that corresponds to protonated urapidil probably formed by thermal degradation of the *N*-oxide. The identity of **5** and the synthetic *N*-oxide was further confirmed by HPLC co-chromatography and by reduction of both compounds to the parent compound urapidil. This reaction was performed in dichloromethane using PCl_3, an agent commonly employed for deoxygenation of aromatic *N*-oxides.[11] Quantitative conversion of **5** to urapidil was then confirmed by HPLC.

Metabolite **4**, the structure of which could not be elucidated by mass spectrometry, shows after reduction with PCl_3 the same chromatographic behaviour as urapidil. Although the reference compound was not synthesized, metabolite **4** is supposed to be the di-*N*-oxide.

Acknowledgements

The authors wish to thank Dr W. Prüsse for the preparation of synthetic reference substances. We are also grateful to P. Arnold for his valuable technical assistance.

REFERENCES

1. K. Klemm, W. Prüsse and U. Krüger, *Arzneim.-Forsch./Drug Res.* **27**, 1859 (1977).
2. W. Schoetensack, P. Bischler, E. Ch. Dittman and V. Steinijans, *Arzneim.-Forsch./Drug Res.* **27**, 1908 (1977).
3. A. Scriabine (Ed.), *New Drugs Annual: Cardiovascular Drugs*, W. Schoetensack, E. G. Bruckschen and K. Zech, p. 19. Raven Press, New York (1983).
4. G. Ludwig, H. Vergin and K. Zech, *Arzneim.-Forsch./Drug Res.* **27**, 2079 (1977).
5. K. Zech, M. Eltze, U. Kilian, K. Sanders and N. Kolassa, in preparation.
6. K. Zech, P. Arnold, H. Liechti and G. Ludwig, *Chromatographia* **12**, 354 (1979).
7. K.-P. Hupe, H. H. Lauer and K. Zech, *Chromatographia* **13**, 413 (1980).
8. K. Biemann, *Mass Spectrometry* pp. 184–186. McGraw-Hill, New York (1962).
9. D. N. B. Mallen and J. M. Smith, *Biomed. Mass Spectrom.* **6**, 472 (1979).
10. G. Bouse and M. Metzler, *Biotransformation organischer Fremdsubstanzen* p. 29. Thieme, Stuttgart (1978).
11. E. Ochiai, *Aromatic N-Oxides*, Elsevier, Amsterdam, London, New York (1967).

Received 30 May 1983; accepted (revised) 14 August 1983