ELSEVIER

Journal of Chromatography A, 970 (2002) 117–130

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Verapamil: new insight into the molecular mechanism of drug oxidation in the human heart

Markus Walles, Thomas Thum, Karsten Levsen, Jürgen Borlak*

Fraunhofer Institute of Toxicology and Aerosol Research, Center of Drug Research and Medical Biotechnology, Nicolai-Fuchs-Str. 1, D-30659 Hannover, Germany

Abstract

Verapamil is a commonly prescribed cardiovascular drug, but surprisingly its metabolism in the target tissue of pharmacotherapy is basically unknown. We therefore investigated its biotransformation in human heart tissue and correlate the production of metabolites with the gene expression of major drug metabolising enzymes. Using electrospray LC–MS–MS and LC–MS^3 experiments, a total of nine metabolites were observed in incubation experiments with verapamil and microsomes isolated from the human heart tissue, and this included a carbinolamine-, *N*-formyl-, ahemiacetale-, and formate-intermediate of *N*-demethyl- and *O*-demethylverapamil. We also observed a hydroxylation product at the benzylic position of atom C-7 (M9). Metabolites M5–M9 are novel and were not observed in previous studies with liver or other human tissues. A fine example of the considerable metabolic competence of human heart is the formation of M1–M4, e.g. dealkylverapamil, norverapamil and isomers of *O*-demethylverapamil, which were believed to be exclusively produced by the liver.

Keywords: Drug oxidation; Heart; Verapamil; Cytochrome P450

1. Introduction

The cytochrome P450 enzymes play an important role in the detoxification of foreign compounds, but surprisingly, little is known about the metabolic competence of the human heart. World-wide a substantial number of drugs are prescribed for the treatment of hypertension, acute and chronic heart failure, and/or other forms of chronic heart disease and information on cytochrome P450 enzyme expression in human heart might explain drug inefficacy and/or the cardiomyopathy observed with certain cardiovascular drugs [1] and with tricyclic antidepressives, as recently reported by Reilly et al. [2].

The calcium antagonist verapamil is commonly prescribed for antianginal therapy and myocardial ischemia, but suffers from extensive first pass metabolism [3]. This results in low drug bioavailability and considerable variability of therapeutic plasma levels [4–6]. Metabolism of verapamil leads to pharmacological inactivation and thus, patients require frequent dosage of this particular drug. Although the therapeutic benefit of verapamil is well documented [7,8], the tissue specific metabolism may impact its mode of action in diseased heart tissue.

Biotransformation of calcium antagonists in heart tissue is potentially linked to adverse drug reactions. It is tempting to speculate that the tissue-specific metabolic breakdown in diseased human heart tissue

*Corresponding author. Tel.: +49-511-535-0559; fax: +49-511-535-0155.

E-mail address: borlak@ita.fhg.de (J. Borlak).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00641-6

is linked to therapeutic inefficacy and more generally, to the adverse drug reactions seen with these drugs.

As no information is available on the metabolism of verapamil in human heart tissue, we wanted to investigate the metabolic pathway of this particular calcium antagonist in the target tissue of drug treatment. We report the identification of novel and tissue specific biotransformation products and link the metabolism to the gene expression of major drug metabolising enzymes in human heart tissue.

2. Experimental

2.1. Materials and chemicals

AcCN (Malinckrodt–Baker, Deventer, Holland), MeOH (Malinckrodt–Baker, Deventer, Holland), NH_4Ac (Merck, Darmstadt, Germany), acetic acid (Fluka, Buchs, Switzerland), Norverapamil (5-*N*(3,4-dimethoxyphenethyl)amino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) (Research Biochemicals International, Natick, MA, USA), Verapamil (5-*N*(3,4-dimethoxyphenethyl)methylamino-2-(3′,4′-dimethoxyphenyl)-2-isopropyl-valeronitrile) (Lot56H-0925/Sigma–Aldrich, Steinheim, Germany). The metabolite M2 (PR 23) and the ^{13}C- and deuterium-labelled verapamil shown in Fig. 4 were the kind gift from Professor Nelson, University of Seattle (USA).

2.2. Human heart tissue and preparation of microsomes

Ethical approval for the use of biopsy material was obtained from the Medical School of Hannover (J. Borlak). The age of the patients ranged from 13 to 58 years and patients were diagnosed as dilatative cardiomyopathy ($n=3$). After explantation, biopsy material was obtained immediately and shock-frozen in liquid nitrogen and stored at −80 °C until further use.

Tissue pieces from human heart were cut into small pieces and homogenised with an ultraturrax (IKA, Germany) in KCl-buffer (0.15 *M*, pH 7.4). After centrifugation for 30 min with 11 000 *g* at 4 °C the supernatant was centrifuged at 170 000 *g* and 4 °C for 60 min. The pellet was resuspended in KCl-buffer (0.15 *M*, pH 7.4) and recentrifuged for 40 min at 200 000 *g* and 4 °C and the microsomal fraction was transferred into Tris–sucrose buffer (0.25 *M* sucrose, 20 m*M* Tris-buffer, 5 m*M* EDTA, pH 7.4). Microsomal solutions were shock frozen in liquid nitrogen and stored at −80 °C until further use.

2.3. Incubation assays with cultured cardiomyocytes

The isolation was done as described previously [9,10]. In brief, the thorax was opened by surgical procedures and the aorta ascendens was anatomically prepared. The heart was perfused in situ with a washing solution for 1 min. After the initial wash, the heart was swiftly removed and mounted onto the perfusion apparatus. Then, different enzyme digests were pumped through the rat heart to obtain cardiomyocytes [10]. The cell yield was determined with the Neubauer's counting chamber (Hecht, Germany) and cardiomyocytes were cultured at 37 °C at 5% CO_2 for 1 day before starting the experiment. The purity was >95% and further improved by preplating cell suspension on plastic flasks for 2 h as described previously [11].

2.4. Metabolism studies with ^{13}C- and deuterium-labelled verapamil

In addition to the studies described above, cultured rat cardiomyocytes were used for incubation assays with ^{13}C- and deuterium-labelled verapamil and unlabelled verapamil as control: cultured cardiomyocytes of adult rats were incubated with 2 μ*M* of ^{13}C- or deuterium-labelled verapamil for 24 h. At the end of the incubation, cardiomyocytes were lysed using an ultrasonic water bath, centrifuged at 1000 rpm for 10 min and the supernatant was collected and frozen at −20 °C until further analysis.

2.5. Measurement of protein concentration

Microsomal protein concentrations were determined as described earlier [12]. Protein content was adjusted to approximately 5 mg protein/ml.

2.6. Microsomal incubation assays

Microsomes (200 μg) were incubated with 2 μ*M* verapamil, 21.4 μ*M* glucose-6-phosphate (Sigma, Deisenhofen, Germany), 4.6 μ*M* nicotinamide-adenine-dinucleotide-phosphate (Sigma, Deisenhofen, Germany) and 5 U of glucose-6-phosphate-dehydrogenase (Sigma, Deisenhofen, Germany) in a total volume of 2 ml Tris buffer (20 m*M*, Sigma, Deisenhofen, Germany) for 4 h at 37 °C in a shaking water bath and were stored at −80 °C until further analysis.

2.7. Sample preparation

Proteins were precipitated with 6 ml of methanol and removed by centrifugation at 200 rpm for 5 min. The supernatant was dried under a gentle stream of nitrogen.

2.8. Measurements of verapamil and its metabolites

Verapamil and its basic metabolites were analysed by solid-phase extraction (SPE) followed by high-performance liquid chromatography–mass spectrometry (HPLC–MS).

For SPE, a lipophilic cartridge (RP18, Merck, Germany) was used which was conditioned with methanol followed by equilibration with water. The sample was loaded onto the cartridge without any organic solvent and washed with 3% methanol to separate any sample matrix. Verapamil and its basic metabolites eluted with 65% methanol containing 3% acetic acid. Eluants were evaporated to dryness and reconstituted in 200 μl of acetonitrile/ammonium acetate (0.01 *M*, pH 6.0, 50/50, v/v). Aliquots of 20 μl were injected onto the HPLC–MS system.

2.9. Recovery

Recovery experiments were done in quadruplicate. For this purpose, ventricular microsomal suspensions were spiked with 50 ng/ml of verapamil and norverapamil. Solid-phase extraction was done as described above and the resultant eluent was reduced in volume to 200 μl. Measurement of these extracts was done as described above and recoveries of 85±4 and 81±5% for verapamil and norverapamil, respectively were determined.

2.10. HPLC-mass spectrometry analysis (HPLC–MS)

HPLC–MS analyses were done on a Waters HPLC instrument (pumps 590) coupled to an ion trap mass spectrometer (Esquire; Bruker Daltonik, Germany) operated under positive ion electrospray (ESI) conditions in the full scan, MS^2 and in some instances in the MS^3 mode. The nebulizer pressure was set to 40 p.s.i. and the dry gas temperature to 300 °C, while +3 kV were applied to the nebulizing capillary. Full mass spectra were acquired by scanning the mass range of m/z 100–500. CID spectra were obtained from the protonated molecules $(M+H)^+$. HPLC analysis was carried out with an isocratic elution of 1:1 ammonium acetate buffer (0.01 *M*, pH 6.0)/acetonitrile. The total run time was 60 min and the flow-rate 0.2 ml/min. Separation of verapamil and its metabolites was achieved on a 250×2 mm RP select B column with a particle size of 4 μm (Merck, Germany).

2.11. Gene expression studies using reverse transcriptase polymerase chain reaction (RT-PCR)

Tissue pieces were pooled and total RNA was isolated and translated to cDNA as described earlier [13]. The cDNA was amplified using oligonucleotides for cytochrome P450 isoforms (CYP1A1, CYP1A2, CYP2A6/7, CYP2B6/7, CYP2C8-19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP3A4, CYP3A5, CYP3A7, CYP4B1), epoxide hydrolase, flavin-containing monooxygenases 2 and 5 and the 18*S* rRNA as a housekeeping gene.

3. Results

3.1. Gene expression studies

As shown in Fig. 1 gene copies of CYP1A1, CYP2B6/7, CYP2C8-19, CYP2D6, CYP2E1, CYP2J2, CYP4B1, epoxide hydrolase, the flavin-containing monooxygenases 2 and 5 and 18*S* rRNA could be detected in human heart muscle tissue.

Fig. 1. RT-PCR of several drug-metabolising enzymes expressed in the human heart (EH, epoxide hydrolase; FMO, flavin-containing-monooxygenase).

3.2. Metabolite identification

Human heart tissue was isolated as described in the Experimental section.

Identification of metabolites from human heart tissue was done by collision-induced dissociation (CID) experiments in multiple LC–MS–MS and LC–MS^3 experiments. MS–MS spectra of metabolites are shown in Fig. 2 while the few MS^3 experiments are only mentioned in the text. Metabolism of verapamil proceeded predominantly with the production of norverapamil (M2) with microsomes isolated from the right ventricle of the heart. In strong contrast, no detectable production of oxidation products was observed in left ventricular microsomal incubation experiments [13].

The MS spectrum of verapamil shows a protonated molecule (MH^+) of $m/z=455$ (Fig. 2j). Fragmentation in the MS^2-mode (Table 1) leads to abundant ions of m/z 165 (N–C-cleavage at the lower substituted side and charge transfer) and $m/z=303$ (cleavage of the C–C-bond in alpha-position to the nitrogen accompanied by hydrogen and charge transfer). Further fragment ions are absorbed at $m/z=150$ (cleavage of the alpha-C–C-bond and charge transfer). These fragmentation routes are in part also observed with the metabolites discussed below and thus can be used for their identification. In addition, a typical ion chromatogram of verapamil and its metabolites is shown in Fig. 6.

3.2.1. Metabolite M1 (D 617)

The MS spectrum shows a protonated molecule (MH^+) of $m/z=291$ and points to oxidative dealkylation of the lower substituted phenylalkylamine moiety (M=164 Da less than verapamil). Fragmentation in the MS^2-mode (Table 1 and Fig. 2a) gives rise to two abundant ions of $m/z=248$ (loss of the isopropyl group) and $m/z=260$ (loss of methylamine by N–C cleavage on the higher substituted side accompanied by proton transfer), which supports the structure M1 given in the metabolic pathway of Fig. 3.

3.2.2. Metabolite M2 (norverapamil)

The full scan MS spectrum displays a protonated molecule (MH^+) of $m/z=441$ and this points to an oxidative dealkylation of a methyl group. Fragmentation in the MS^2-mode (Table 1 and Fig. 2b) produces an abundant ion of $m/z=165$ and further, less abundant ions at $m/z=398$, 289 and 151, which are indicative for oxidative *N*-demethylation. The prominent ion of $m/z=165$ can be explained by *N*-alkyl-cleavage with charge transfer and of $m/z=289$ by C–C-cleavage in alpha-position of the nitrogen with subsequent proton transfer. The fragment of $m/z=398$ is formed by loss of isopropyl, $m/z=151$ by α-cleavage with charge transfer. The structure of metabolite 2 was confirmed by comparing the LC and MS properties with those of a synthetic reference compound and with incubation assays of ^{13}C-labelled verapamil (see C-24) as shown in Table 2 (biotransformation of verapamil to norverapamil leads to loss of the labelled methyl group and thus the m/z values for all ions are identical to those of the unlabeled compound).

3.2.3. Metabolite M3 (PR 23)

The full scan MS spectrum shows a protonated molecule (MH^+) of $m/z=441$. This again is highly suggestive for oxidative dealkylation of a methyl group. Fragmentation in the MS^2-mode (Table 1 and Fig. 2c) corresponds largely to that observed for verapamil and leads to abundant ions of $m/z=289$,

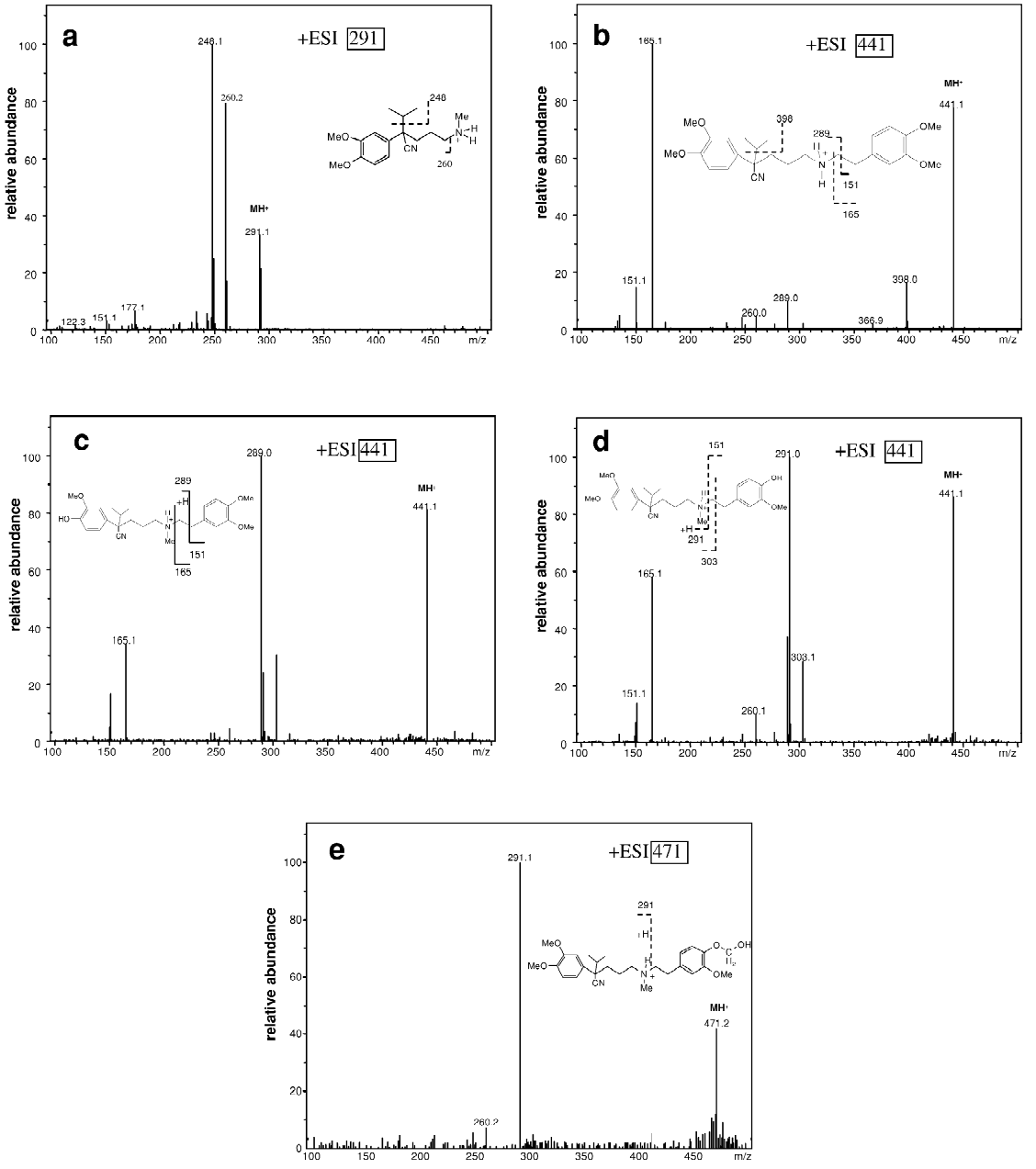


Fig. 2. Collision-induced dissociation mass spectra (MS–MS) of verapamil metabolites (a = M1, b = M2, c = M3, d = M4, e = M5, f = M6, g = M7, h = M8, i = M9) and verapamil (j).

165 and 151 which suggests oxidative demethylation in position C-31 or C-33 (R3 and R4 in the scheme in Fig. 1). The prominent ions of $m/z = 289$ and 151 point to C–C-cleavage in alpha-position to the nitrogen (the former accompanied by proton transfer). The ion of $m/z = 246$ is formed by N–C-cleavage at the higher substituted moiety and thus supports the view of oxidative demethylation at position

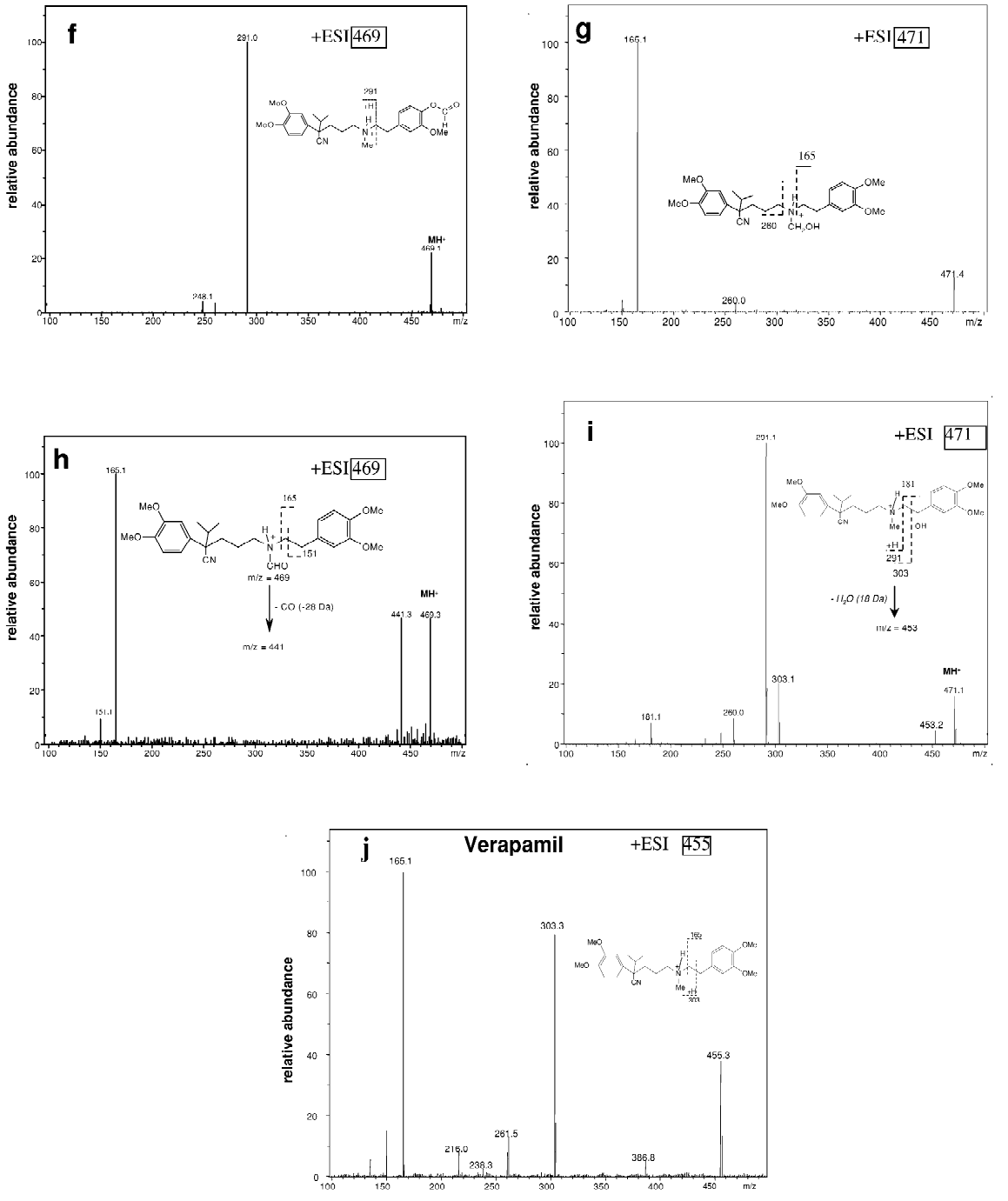
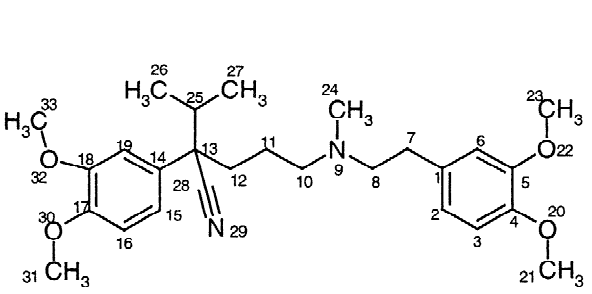
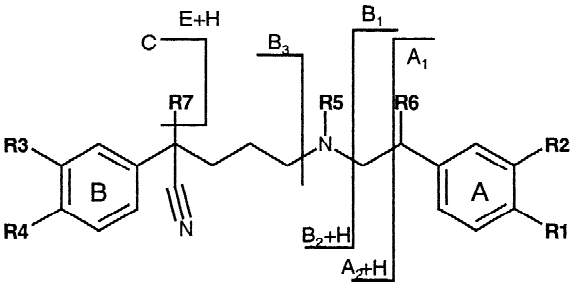


Fig. 2. (*continued*)

Table 1
Fragmentation scheme and main fragment ions of nine metabolites of verapamil





Number									α-cleavage		N–C-cleavage				
	R1	R2	R3	R4	R5	R6	R7	M+H	A_1	A_2+H	B_1	B_2+H	B_3	C	Others
Verapamil	OCH_3	OCH_3	OCH_3	OCH_3	CH_3	H	C_3H_7	455	150	**303**	**165**	–	–	–	–
M1(D617)	–	–	OCH_3	OCH_3	CH_3	–	C_3H_7	291	–	–	–	–	**260**	**248**	–
M2 (Norverapamil)	OCH_3	OCH_3	OCH_3	OCH_3	H	H	C_3H_7	441	151	289	**165**	–	–	398	–
M3 (PR22[a])	OCH_3	OCH_3	OCH_3	OH	CH_3	H	C_3H_7	441	151	**289**	**165**	–	246	–	–
M4 (D-702)	OH	OCH_3	OCH_3	OCH_3	CH_3	H	C_3H_7	441	–	303	151	**291**	–	260	248-
M5	OCH_2OH	OCH_3	OCH_3	OCH_3	CH_3	H	C_3H_7	471	–	–	–	**291**	260	–	248
M6	OCH_2O	OCH_3	OCH_3	OCH_3	CH_3	H	C_3H_7	469	–	–	–	**291**	260	248	441(–CO)
M7	OCH_3	OCH_3	OCH_3	OCH_3	CH_2OH	H	C_3H_7	471	151	–	**165**	–	260	–	453 ($-H_2O$)
M8	OCH_3	OCH_3	OCH_3	OCH_3	CH_2O	H	C_3H_7	469	151	–	**165**	–	–	–	441 (–CO)
M9	OCH_3	OCH_3	OCH_3	OCH_3	CH_3	OH	C_3H_7	471	–	303	181	**291**	260	–	248, 453 ($-H_2O$)

The most abundant fragments are shown bold m/z values.
[a] Also known as D-703.

31 or 33 (Fig. 3). It was shown previously that *O*-demethylation occurs predominantly in position 31 [14]. The peak at $m/z=303$ stems from metabolite 4 as both isomers have similar retention times in the chromatogram and thus, their mass spectra partially overlap. The structure of metabolite 3 was confirmed by comparing the LC and MS properties with those of a synthetic reference compound.

3.2.4. Metabolite M4 (PR 24)

The full scan MS spectrum shows a protonated molecule (MH^+) of $m/z=441$ and this indicates again oxidative dealkylation of a methyl group. Fragmentation in the MS^2-mode (Table 1 and Fig. 2d) produces an abundant ion of $m/z=291$. This is highly suggestive for demethylation at C-atoms 21 or 23 (R3 and R4 in the scheme in Fig. 1). The prominent ion at $m/z=291$ stems from a *N*-alkyl-cleavage with proton transfer. The other fragmentation ions of $m/z=303$ (C–C-cleavage in alpha-position to the nitrogen accompanied by proton transfer), $m/z=248$ and 260 (loss of the isopropyl or a methylamine group from m/z 291) confirm an *O*-demethylation in position 21 or 23. It was shown previously that *O*-demethylation occurs predominantly in position 21 [14]. Because of insufficient separation, the mass spectra of M3 and M4 overlap. Thus, the fragment of $m/z=165$ stems from metabolite 3.

3.2.5. Metabolite M5

The full scan MS spectrum shows a protonated molecule (MH^+) of $m/z=471$ and this is highly suggestive for direct hydroxylation of verapamil itself. Fragmentation in the MS^2-mode gives rise to an abundant ion of $m/z=291$ and further ions of $m/z=248$ and 260. The prominent peak at $m/z=291$ can be explained by *N*-alkyl-cleavage with proton transfer and this points to hydroxylation at the phenylalkylamine-moiety. Corroborative evidence is the loss of the isopropyl-group ($m/z=248$) and the N–C-cleavage on the higher substituted side of the nitrogen ($m/z=260$) from m/z 291 shown in Fig. 2e. Consequently, the hydroxylation reaction could

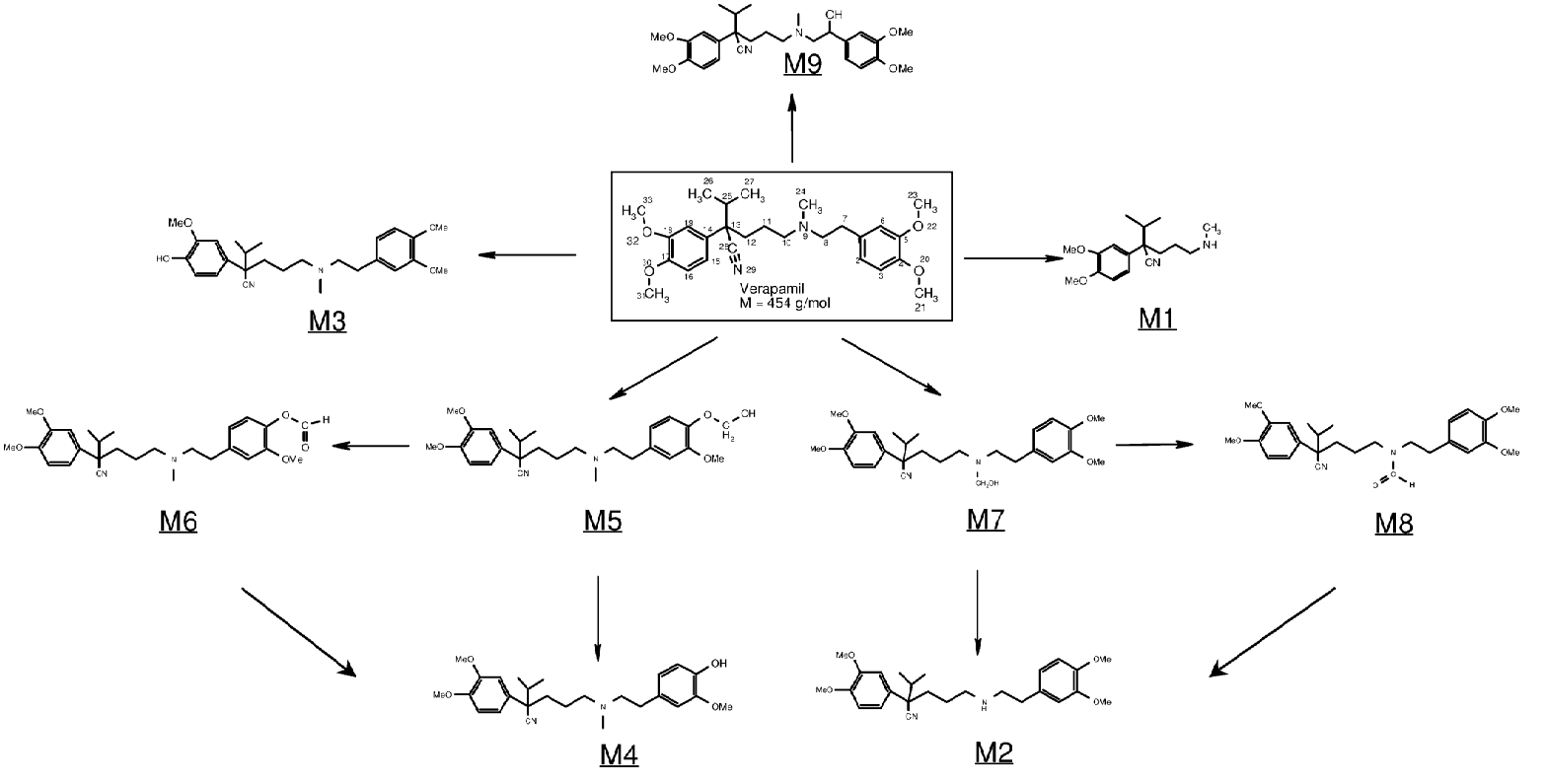


Fig. 3. Metabolic pathway of verapamil in human heart tissue.

Table 2
Metabolite fragments of unlabelled and ^{13}C-labelled Verapamil (VP)

	Unlabelled	^{13}C-labelled
VP	165, 303, 455	165, 304, 456
M1	248,260,291	248, 260, 291
M2	165, 289,398, 441	165, 289, 398, 441
M3	165, 289,303,441	165, 304, 442
M4	151, 260, 291,303,441	151, 260, 292,442
M7	165, 360, 453, 471	165, 260, 454, 472
M8	165, 441,469	165, 441, 470
M9	181, 260, 291, 303, 453, 471	181,260,291,454,472

occur at C-atoms 2, 3, 6, 7, 8 or at one of the methoxy groups. When comparing the fragmentation pattern of this hydroxylated metabolite with that of the *O*-demethyl-verapamil (M4) and the isomeric metabolite M9 (discussed below) no loss of water is observed from the $[MH]^+$ of both metabolites. In contrast, collision-induced dissociation of the hydroxyverapamil-M9 metabolite (hydroxyl group in the benzylic position) shows loss of water, which suggests (but does not prove unambiguously) M5 to be formed as the oxidative precursor of *O*-demethylverapamil (M4). This conclusion is corroborated by more recent studies on the metabolism of verapamil in hepatocytes [15].

3.2.6. Metabolite M6

The full scan MS spectrum shows a protonated molecule (MH^+) of $m/z=469$. This is highly suggestive for direct oxidation of verapamil. Fragmentation in the MS^2-mode (Fig. 2f and Table 1) leads to an abundant ion of $m/z=291$ and further subsequent fragments at $m/z=248$ and 260. The prominent peak at $m/z=291$ results from a *N*-alkyl-cleavage with proton transfer demonstrating that oxidation occurred in the phenylalkylamine-moiety. The fragment ions of $m/z=248$ and $m/z=260$ originate from $m/z=291$ and indicate loss of the isopropyl and methylamine (by N–C-cleavage on the higher substituted side of the nitrogen), respectively. With the exception of the molecular ion, only small differences in the fragmentation pattern of M6 and *O*-demethylverapamil are observed upon collision-induced decomposition (CID). The MS-data are consistent with (but do not prove unambiguously) the aldehyde structure shown in Fig. 3 which is corroborated by a weak loss of CO (not shown in Fig. 2f). Thus the metabolite M6 is a precursor of M4.

3.2.7. Metabolite M7

The full scan MS exhibits a protonated molecule (MH^+) of $m/z=471$. This indicates oxidative hydroxylation. Fragmentation in the MS^2-mode (Fig. 2g and Table 1) leads to an abundant ion at $m/z=165$ and further ions at $m/z=260$, 150 and 151. The prominent ion of $m/z=165$ and $m/z=151$ is consistent with N–C-cleavage with charge migration and α-cleavage on the lower substituted moiety and therefore oxidation of the phenylalkylamine moiety can be ruled out. Furthermore, we observe an ion of $m/z=260$, which originates from N–C-cleavage at the higher substituted side and thus excludes hydroxylation of the higher substituted moiety. Therefore hydroxylation of the methyl group bound to nitrogen or the nitrogen itself (*N*-hydroxylamine stemming from the *N*-oxide) is likely. Previous studies have shown that the *N*-oxide of verapamil is not stable in solution [26]. Decomposition via cope elimination occurs in solution and two cleavage products are formed, a methyl hydroxyl amine and dimethoxystyrene. Both decomposition products were not detected by us and the fragmentation pattern of the synthesised reference compound shows different fragment ions [16], which exclude M7 to be the *N*-oxide (the mass spectral data of the *N*-oxide recorded with liquid secondary ion mass spectrometry forms also an ion of $MH^+=471$, but shows a fragment ion of $m/z=455$, which corresponds to verapamil. As we do not observe this ion but $m/z=165$ and 260 and 453, we exclude the formation of a *N*-oxide. Only slight differences are obvious, when the spectral data of M7 are compared with norverapamil. We propose hydroxylation of a *N*-bound methyl group to yield a carbinolamine. A weak loss of water from the protonated molecule observed in some experiments supports this conclusion, In general, carbinolamines are unstable in solution, but there are exceptions, which will be discussed further below.

3.2.8. Metabolite M8

The full scan MS spectrum shows a protonated molecule (MH^+) of $m/z=469$. Fragmentation in the MS^2-mode (Fig. 2h and Table 1) produces an

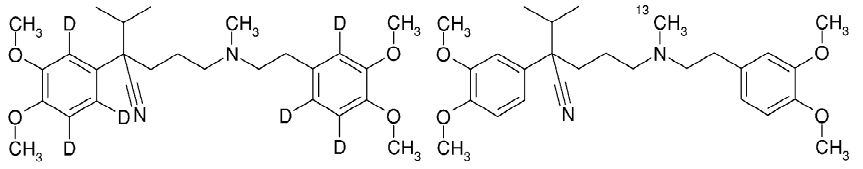


Fig. 4. Structure of ^{13}C- and deuterium-labelled verapamil.

abundant ion of $m/z=165$ and further ions at $m/z=441$ and 151. The ion of $m/z=441$ can be explained by loss of CO and provides strong evidence for the presence of a carboxyl group. The prominent ion of $m/z=165$ results from a N–C-cleavage. This indicates oxidation of the higher substituted moiety or the methyl group bound to nitrogen. Further fragmentation of the ion of $m/z=441$ (as shown by MS^3 experiments) leads to no other fragments apart from $m/z=165$. When the MS data of M8 are compared with that of norverapamil, only slight differences are obvious. We propose oxidation of the methyl group bound to the nitrogen atom. This structure is corroborated in incubation assays with verapamil ^{13}C-labelled at C-24, as shown in Table 2; $m/z=441$ is not shifted to $m/z=442$ demonstrating that C-24 is incorporated in the CO lost from M8. M8 is an intermediate of M2 (norverapamil) and an oxidation product of M7.

3.2.9. Metabolite M9

The full scan MS spectrum shows a protonated molecule (MH^+) of $m/z=471$ indicating an hydroxylation of verapamil. Fragmentation in the MS^2-mode produced an abundant ion of $m/z=291$ and further peaks at $m/z=453$, 303, 260, 248 and 181 (Table 1 and Fig. 2i). Loss of water yields an ion of $m/z=453$. The prominent ion of $m/z=291$ can be explained by N–C cleavage (with proton transfer) at the lower substituted side and is highly suggestive for hydroxylation of the phenylalkylamine moiety. This is confirmed by the presence of further fragment ions of $m/z=248$ and $m/z=260$ formed by loss of isopropyl and methylamine from $m/z=291$. The peak at $m/z=303$ is formed by C–C-cleavage (with proton transfer) in the alpha-position to the nitrogen. The ion of $m/z=181$ is formed by *N*-alkyl-cleavage with charge transfer. Consequently, the hydroxylation may have occurred at the benzylic position (C-7) or the aromatic ring (C-2, -3, or -6), but aromatic hydroxylation is less likely, as comparison of the MS data of the hydroxylated metabolite (loss of water) with that of *O*-demethylverapamil (no loss of water), is highly suggestive for the hydroxylation at the benzylic position. This conclusion is corroborated by more recent studies on the metabolism of verapamil in hepatocytes [15].

3.3. Metabolism studies with ^{13}C- and deuterium-labelled verapamil (Fig. 4)

In addition to the studies with microsomes isolated from human heart tissue, experiments with rat cardiomyocytes and ^{13}C- and deuterium-labelled verapamil were carried out to confirm the above proposed structural assignments. The results are summarised in Table 2 and, in part, have been discussed above.

With the use of the stable ^{13}C-isotope, we confirm the proposed structures of M2 and M8. The deuterium-labelled verapamil did not provide conclusive information on the metabolic pathways leading to M5, M6 and M7 as the ^{2}H label is not subject to metabolic attack and did not provide additional MS-fragmentation data.

4. Discussion

Our study demonstrates the utility and effectiveness of electrospray ionization and collision-induced dissociation mass spectrometry in the identification and structural elucidation of novel metabolites of verapamil. Mass spectrometry is thus the first choice in the search for new metabolites as the concentrations of biotransformation products in human heart

tissue are too low to obtain informative data. The collision-induced dissociation of the protonated molecules of all metabolites can be described by a general fragmentation scheme shown in Fig. 1. The fragmentation leads to structure-specific ions formed by bond cleavages in the aliphatic moiety of the molecule (with and without hydrogen transfer). This fragmentation predominantly leads to thermochemically stable products, i.e. even-electron ions and neutral molecules (see also Table 1), with the exception of the loss of the isopropyl group observed with several metabolites, which leads to an odd electron fragment ion and a neutral radical. This surprising loss of the isopropyl radical may be influenced by the cyano group at the same C-atom. Note, however, that the competing (thermodynamically more favourable) loss of an isopropene molecule is often observed. The neutral losses are particularly valuable for the identification of structural features. Thus, loss of water is characteristic of a hydroxy group in the aliphatic moiety of the molecule, loss of methylamine from the protonated molecule or a primary fragment proves the presence (or collision-induced formation) of a secondary amine and loss of CO shows the presence of a carbonyl group. The relative abundance of the fragment ions of the parent compound and the nine metabolites depends on the substitution pattern. For instance, oxidation of the phenylalkylamine moiety (M4, M5, M6 and M9) leads to N–C-cleavage with charge retention on the nitrogen, oxidation in the remaining part of the molecule to a corresponding cleavage with charge migration (M2, M3, M7, M8) to the phenylalkyl moiety.

Further, the extensive metabolism of verapamil is well documented, both in vitro and in vivo, but its biotransformation in target tissue of drug treatment is basically unknown. To the best of our knowledge, this is the first report on the tissue-specific metabolism of verapamil in the human heart and we report the identification of a total of nine metabolites, of which M5 to M9 are novel and have not been observed in other human tissues. We present experimental evidence that these are key intermediates in the *N*-demethylation and *O*-demethylation pathway and provide new information on drug oxidation in human heart.

Whether P450 monooxygenase catalyse oxidation of amines (which would lead to the formation of metabolite M7) remains controversial, but Karki and Dinnozenz demonstrated oxidation of substituted *N*,*N*-dimethylanilines via an initial hydrogen abstraction to yield an alpha-amino-radical [17]. Subsequent hydroxylation gives rise to carbinolamines, which are unstable and usually collapse to the corresponding secondary amine and formaldehyde.

Some investigators report the isolation of stable carbinolamines and, as shown with *N*-methylcarbazole, metabolism leads to the production of the stable carbinolamine 3-OH-NHMC [18]. Similar, metabolism of medazepam also proceeds via the production of a stable carbinolamine [19]. Additionally, Hollenberg demonstrated *N*-demethylation of tertiary amines to proceed via a carbinolamine and/or an *N*-oxide [20] and more recently, Upthagrove and Nelson reported the identification of stable carbinolamines, imines, and oxazolidines, all resulting from metabolism of fluorinated propranolol analogs [21]. We thus propose a carbinolamine as an intermediate in the *N*-demethylation of verapamil, even though *N*-formyl derivatives are usually chemically more stable. It has been demonstrated that some carbinolamines can be dehydrogenated (instead of hydrolysed) yielding stable *N*-formyl-derivatives [22] and *N*-formyl derivatives of prenylamine [23] and *N*-formyl derivative for bepridil (a class III antiarrhythmic drug) are good examples [24].

We suggest *N*-demethylation of verapamil proceeds via a carbinolamine (M7) and an *N*-formyl intermediate (M8), which are sufficiently stable in microsomal extracts of heart tissue to be identified by mass spectrometry (Fig. 5). As shown in Fig. 3 we suggest that verapamil is oxidised via a hemiacetale (M5) and formate (M6) intermediate to yield *O*-demethylverapamil (M4 = D702) and similarly, production of norverapamil proceeds via a carbinolamine (M7) and *N*-formyl-intermediate (M8) to yield the *N*-demethyl product (M2). M9 is a further novel metabolite and hydroxylation occurred, most likely, at the benzylic position of C-7 (Fig. 3), though aromatic ring hydroxylation at atom C-2, -3 or -6 cannot be ruled out.

Noteworthy, oxidative intermediates of both *N*- and *O*-demethylation products have not been observed previously, and therefore our data provide valuable experimental evidence for CYP catalysed

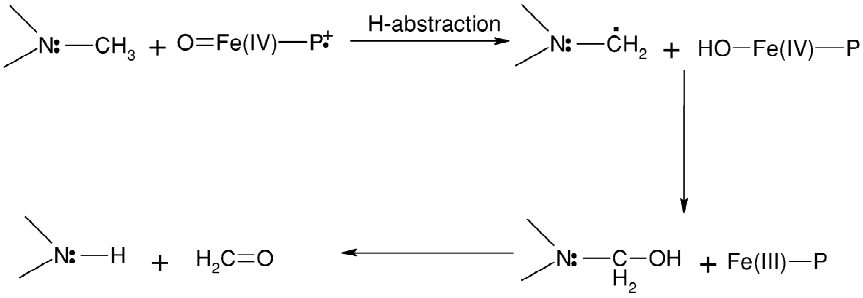


Fig. 5. *N*-Demethylation of verapamil via production of a carbinolamine.

oxidations of verapamil in human heart. The metabolism of (*R*/*S*)-verapamil in man was studied after oral administration in healthy human volunteers [14] and approximately 67–71% of the administered dose was excreted into urine within 5 days. The early investigations of Eichelbaum et al. [25] show extensive first pass metabolism with only 3–4% of the dose being excreted into urine as unchanged drug. In this study, extensive *O*- and *N*-dealkylation reactions were observed leading to norverapamil and *O*-demethylverapamil products.

The enzymes involved in the metabolism in verapamil have been characterised using the microsomal fraction of 21 human livers, which had been previously been characterised for their individual expressions of various P450 enzymes [26]. The maximum rate of formation of M1 (D-617), e.g. dealkylverapamil, and of norverapamil was linked to CYP3A and CYP1A2 expression and further studies

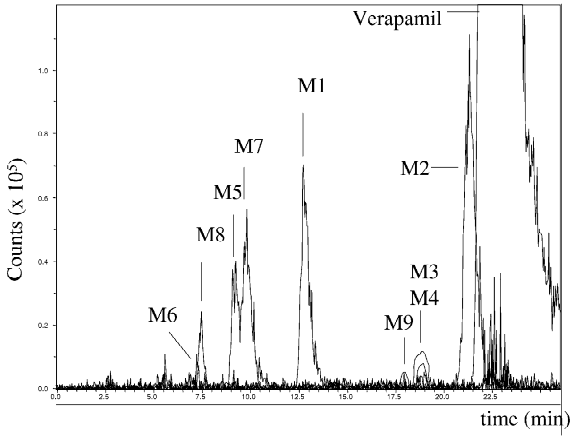


Fig. 6. Ion chromatogram of verapamil and metabolites in microsomal suspensions of the human heart.

show cytochromes of the P4502C subfamily to be major contributors to the *O*-demethylation of verapamil and particular of M4 (D-702) and M3 (D-703) [27]. Interestingly, both M4 (D-702) and M3 (D-703) were produced by recombinant CYP2C9 and CYP2C18 protein, whereas CYP2C8 selectively yielded M3 (D-703).

In a recent publication, the cytochrome P450 isoforms involved in the metabolism of the enantiomers of verapamil and norverapamil were investigated [28]. The results confirm earlier findings of Kroemer et al. [29] and Busse et al. [27] and show CYP3A4, CYP3A5 and CYP2C8 to play a key role in verapamil metabolism with CYP2C8 expressing stereoselective preference for the (*S*)-enantiomer of norverapamil, but it should be noted that verapamil is prescribed as a racemic mixture but not as a single enantiomer.

Eichelbaum et al. discussed as early as 1979 the *N*-dealkylation of verapamil via oxidation in the alpha-position [25] and their GC–MS-spectra indicate the presence of weak formylamines. This was supported by Nelson and Olsen [14,26] who were able to trap two aldehydes as their *O*-methyloximes. Later these aldehydes were identified as artefacts and thus earlier findings [25,26] need to be viewed with caution.

Although the molecular mechanism in verapamil oxidation remains uncertain, the identification of novel and key intermediates reported here point to a mechanism based on hydrogen abstraction and hydroxy transfer. We provide experimental evidence for *N*-demethylation of verapamil to proceed via a carbinolamine and a *N*-formyl-intermediate.

The mechanism of action of verapamil in the various therapeutic indications is not fully under-

stood, but includes inhibition of the inward flow of calcium ions through permeable calcium ion channels on the cell surface membrane [30]. In the case of arrhythmia, electrical activity through the AV-node depends upon calcium influx through the slow calcium channel. By decreasing the influx of calcium, verapamil prolongs the effective refractive period and thus slows AV-conduction in a rate-related manner [31,32]. Although the molecular mechanisms leading to the acclaimed progressive risk of myocardial infarction upon drug treatment with verapamil are unknown [33], we suggest pharmacogenetic risk factors and idiosyncratic metabolism in heart tissue to be linked to adverse drug reactions. Indeed, verapamil is metabolised by various cytochrome P450 isoforms with known single nucleotide polymorphisms [27–29,34–37]. It is quite likely that the risk of adverse drug reactions can arise from the distorted pharmacology of a drug which fails to be metabolised and builds up to abnormally high concentrations in human heart tissue, as recently suggested by Idle [38]. Alternatively, idiosyncratic metabolism of verapamil may also be responsible for the malignant diseases in breast and colon tissue, as reported by several clinical research groups [39–41].

Initially, verapamil was prescribed for the treatment of angina pectoris and myocardial ischemia [42], but the results of long-term studies cast doubt over its therapeutic benefit in particular indications [33,43–47]. Moreover, the results of long-term studies provide evidence for an association between the use of calcium channel blockers and an increased risk for malignant diseases, and particularly breast and colon cancer [39–41]. There may also be a dose-dependent increase in coronary heart disease upon drug treatment with calcium antagonists [33]. In some patients, clinical studies also suggest extrapyramidal (Parkinson-like) symptoms associated with calcium channel blockers [48].

This study clearly shows that the human heart is a metabolic tissue with the capacity to produce novel and tissue specific metabolites and it is tempting to speculate that metabolic breakdown of verapamil in diseased human heart tissue is linked to therapeutic inefficacy and adverse drug reactions. Whether any of the herein identified biotransformation products modulate ion channels and electric conductivity of heart muscle cells requires further investigation.

Acknowledgements

We would like to thank Professor Nelson (University of Washington, Seattle) for providing the ^{13}C- and deuterium-labelled verapamil. The loan of an Esquire ion trap mass spectrometer by Bruker Daltonik, Bremen, Germany, is kindly acknowledged.

References

[1] J.G. Killian, K. Kerr, C. Lawrence, D.S. Celermajer, Lancet 354 (1999) 1841.
[2] J.G. Reilly, S.A. Ayis, I.N. Ferrier, S.J. Jones, S.H. Thomas, Lancet 355 (2000) 1048.
[3] R.G. McAllister Jr., G.L. Schloemer, S.R. Hamann, Am. J. Cardiol. 57 (1986) 16D.
[4] R.L. Woosley, D.M. Roden, Am. J. Cardiol. 52 (1983) 3C.
[5] D. Piovan, R. Padrini, G. Svalato Moreolo, G. Magnolfi, O. Milanesi, R. Zordan, P.A. Pellegrino, M. Ferrari, Ther. Drug Monit. 17 (1995) 60.
[6] R. Padrini, E. Barbieri, D. Piovan, M. Toffoli, A. Motta, G.P. Trevi, M. Ferrari, Eur. J. Clin. Pharmacol. 28 (1985) 653.
[7] W.D. Weaver, Circulation 74 (1986) IV94.
[8] D.R. Rosing, K.M. Kent, B.J. Maron, J. Condit, S.E. Epstein, Chest 78 (1980) 239.
[9] T. Thum, J. Borlak, Br. J. Pharmacol. 130 (2000) 1745.
[10] T. Thum, J. Borlak, Xenobiotica 30 (2000) 1063.
[11] T. Chajek, O. Stein, Y. Stein, Biochim. Biophys. Acta 488 (1977) 140.
[12] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[13] T. Thum, J. Borlak, Lancet 355 (2000) 979.
[14] W.L. Nelson, L.D. Olsen, Drug Metab. Dispos. 16 (1988) 834.
[15] M. Walles, T. Thum, K. Levsen, J. Borlak, Drug Metab. Dispos. 29 (2001) 761.
[16] J.R. Cashman, Mol. Pharmacol. 36 (1989) 497.
[17] S.B. Karki, J.P. Dinnocenzo, Xenobiotica 25 (1995) 711.
[18] T. Ebner, C.O. Meese, M. Eichelbaum, Xenobiotica 21 (1991) 847.
[19] M.A. Schwartz, S.J. Kolis, J. Pharmacol. Exp. Ther. 180 (1972) 180.
[20] P.F. Hollenberg, FASEB J. 6 (1992) 686.
[21] A.L. Upthagrove, W.L. Nelson, Drug Metab. Dispos. 372 (2001) 1114.
[22] B. Testa, P. Jenner, Drug Metab. Rev. 7 (1978) 325.
[23] G. Remberg, M. Eichelbaum, G. Spiteller, H.J. Dengler, Biomed. Mass Spectrom. 4 (1977) 297.
[24] W.N. Wu, J.F. Hills, S.Y. Chang, K.T. Ng, Drug Metab. Dispos. 16 (1988) 69.
[25] M. Eichelbaum, M. Ende, G. Remberg, M. Schomerus, H.J. Dengler, Drug Metab. Dispos. 7 (1979) 145.

[26] W.L. Nelson, L.D. Olsen, D.B. Beitner, R.J. Pallow Jr., Drug Metab. Dispos. 16 (1988) 184.
[27] D. Busse, J. Cosme, P. Beaune, H.K. Kromer, M. Eichelbaum, Naunyn Schmiedebergs Arch. Pharmacol. 353 (1995) 116.
[28] T.S. Tracy, K.R. Korzekwa, F.J. Gonzalez, I.W. Wainer, Br. J. Clin. Pharmacol. 47 (1999) 545.
[29] H.K. Kroemer, J.C. Gautier, P. Beaune, C. Henderson, C.R. Wolf, M. Eichelbaum, Naunyn Schmiedebergs Arch. Pharmacol. 348 (1993) 332.
[30] L.H. Opie, W.H. Frishman, U. Thadani, Drugs Heart (1995) 50.
[31] D.P. Zipes, J.C. Fischer, Circ. Res. 34 (1974) 184.
[32] L.B. Mitchell, J.S. Schroeder, J.W. Mason, Am. J. Cardiol. 49 (1982) 629.
[33] B.M. Psaty, S.R. Heckbert, T.D. Koepsell, D.S. Siscovick, T.E. Rahunathan, N.S. Weiss, F.R. Rosendaal, R.N. Lemaitre, N.L. Smith, P.W. Wahl, J. Am. Med. Assoc. 274 (1995) 620.
[34] K. Iwahashi, E. Okuyma, A. Furukawa, K. Nakamura, R. Miyatake, Y. Matsuo, Y. Ichkawa, Clin. Chim. Acta 252 (1996) 197.
[35] C.R. Bhasker, J.O. Miners, S. Coulter, D.J. Birkett, Pharmacogenetics 7 (1997) 51.
[36] C.A. Felix, A.H. Walker, B.J. Lange, T.M. Williams, N.J. Winick, N.K. Cheung, B.D. Lovett, P.C. Nowell, I.A. Blair, T.R. Rebbeck, Proc. Natl. Acad. Sci. USA 95 (1998) 13176.
[37] C. Sachse, J. Brockmoller, S. Bauer, I. Roots, Br. J. Clin. Pharmacol. 47 (1999) 445.
[38] J.R. Idle, Lancet 355 (2000) 1824.
[39] A.L. Fitzpatrick, J.R. Daling, C.D. Furberg, R.A. Kronmal, L. Weissfeld, Cancer 80 (1997) 1438.
[40] M. Pahor, J.M. Guralnik, L. Ferrucci, M.C. Carti, M.E. Salive, J.R. Cerhan, R.B. Wallace, R.J. Havlik, Lancet 348 (1996) 493.
[41] L. Hardell, O. Axelson, M. Fredrikson, Lancet 348 (1996) 542.
[42] D.A. Weiner, M.D. Klein, S.S. Cutler, Am. J. Cardiol. 59 (1987) 215.
[43] C.D. Furberg, B.M. Psaty, Herz 20 (1995) 365.
[44] C.D. Furberg, B.M. Psaty, J.V. Meyer, Circulation 92 (1995) 13326.
[45] M.H. Aldermann, H. Cohen, R. Roque, S. Madhavan, Lancet 349 (1997) 594.
[46] D. Waters, Can. J. Cardiol. 13 (1997) 757.
[47] C.M. Jespersen, Cardiovasc. Drugs Ther. 13 (1999) 301.
[48] J.R. Daniel, V.F. Mauro, Ann. Pharmacother. 29 (1995) 73.