ELSEVIER

Journal of Chromatography A, 729 (1996) 341–354

JOURNAL OF CHROMATOGRAPHY A

Location of the hydroxyl functions in hydroxylated metabolites of nebivolol in different animal species and human subjects as determined by on-line high-performance liquid chromatography–diode-array detection

J. Hendrickx*, M. Bockx, C. Zwijsen, C. Borgmans, G. Mannens, W. Meuldermans, J. Heykants

Department of Drug Metabolism and Pharmacokinetics, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium

Abstract

Nebivolol hydrochloride (R067555), is a new antihypertensive drug. Aromatic and alicyclic hydroxylation at the benzopyran ring systems of nebivolol are important metabolic pathways.

Generally, NMR is used to unambiguously assign the sites of hydroxylation. Because of the low dose rates and the extensive metabolism of nebivolol in the different species, NMR identification is not always possible, and therefore another spectroscopic technique was searched for to address this problem.

UV-chromophore absorption is affected by the kind and arrangement of adjacent atoms and groups (auxochromes). The effect of these auxochromes (e.g. $-NH_2$, $-NR_2$, –SH, –OH, –OR and halogens) can be strongly influenced by the pH.

This paper proves that HPLC at high pH combined with on-line diode-array detection is an excellent technique for the location of the hydroxyl functions in hydroxylated metabolites of nebivolol. With this technique it is also possible to differentiate between glucuronidation at the aromatic and aliphatic or alicyclic hydroxyl functions.

Keywords: Nebivolol

1. Introduction

Nebivolol hydrochloride (R067555), (±) - [2*R**[*R**[*R**(*S**)]]] - α,α′ - [iminobis(methylene)]bis-[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol] hydrochloride, a new antihypertensive drug, is a racemate of two enantiomers with four chiral centres (Fig. 1). The *SRRR*-enantiomer (R067138; *d*-nebivolol) is a potent and cardioselective β1-adrenergic blocker. The *RSSS*-enantiomer (R067145; *l*-nebivolol) has a favourable hemodynamic profile, in that normal energy supply during exercise is not affected [1–3].

UV-chromophore absorption is affected by the kind and arrangement of adjacent atoms and groups. A significant effect is exerted by the free-valence groups that contain atoms with free electron pairs and which can take part in the conjugation. Groups of this kind are generally known as auxochromes. The auxochrome is a functional group, which does

*Corresponding author. Address for correspondence: Department of Non-clinical Pharmacokinetics, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium.

0021-9673/96/$15.00
SSDI 0021-9673(95)01114-5

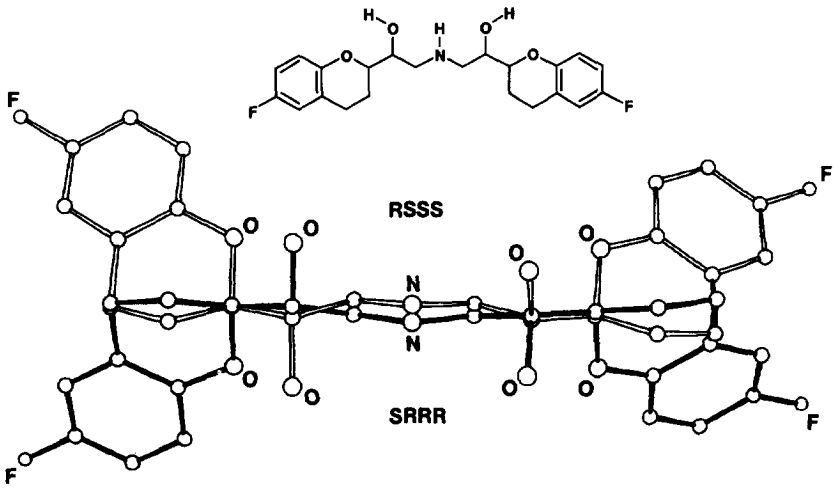

Fig. 1. Structure of nebivolol and its isomers.

not absorb radiation in the UV–Vis range but, if attached to a chromophore, increases the absorption intensity and shifts the absorption to a longer wavelength (red or bathochromic shift). Common auxochromes are typical electron donor groups, (e.g. $-NH_2$, $-NR_2$, $-SH$, $-OH$, $-OR$ and halogens). The effect of these auxochromes can be strongly influenced by the pH. The strongest pH-induced changes in UV–Vis absorption spectra are observed in compounds that undergo dissociation, such as phenols, and a more distinct effect is also noticed in protolytic solvents (H_2O, MeOH), where the dissociation of these compounds is more complete. Substitution on the benzene ring of auxochromic groups shifts the E- and B-bands to longer wavelengths, frequently with intensification of the B-band and loss of its fine structure. Conversion of a phenol to the corresponding phenolate results in a red shift of the E2 (ethylenic) band and the B (benzenoid) band and an increase in the E_{max} because of the availability of the non-bonding electrons in the anion for interaction with the *p*-electrons of the ring [4]. Because the ionization of a phenol is blocked by its glucuronidation, the UV spectra of the glucuronidated metabolites before and after enzymatic hydrolysis will give an indication whether the glucuronidation took place on an aromatic, aliphatic or alicyclic hydroxyl function of the molecule.

Thus at a pH>10 at which the phenolic group is ionized, marked differences in the UV spectra of the different aromatic hydroxy metabolites of nebivolol are expected (Fig. 2). An HPLC solvent system of pH 11.6 was chosen because at this pH there was also a good separation between the aromatic hydroxy and alicyclic hydroxy reference compounds and between the different metabolites. The use of methanol and water as elution solvents intensified the auxochromic effect.

The UV spectra of the various metabolites were compared with those of reference compounds and of identified metabolites.

2. Experimental

2.1. Sample preparation

Analytical samples

Aliquots of urine up to 2 ml, without or after enzymatic hydrolysis (*β*-glucuronidase/arylsulphatase from *Helix pomatia*), were injected after centrifugation onto a C_{18} column (Hypersil ODS, 5 μm).

Purification of the metabolites

Urinary metabolites of the different species (dog, rat, rabbit and man) were concentrated on XAD-2 adsorbent and isolated by semi-preparative HPLC on a reversed-phase column (Hypersil ODS 5 μm) and further purified on a Polygosyl-$N(CH_3)_2$ normal-phase column (Macherey-Nagel 5 μm). To check

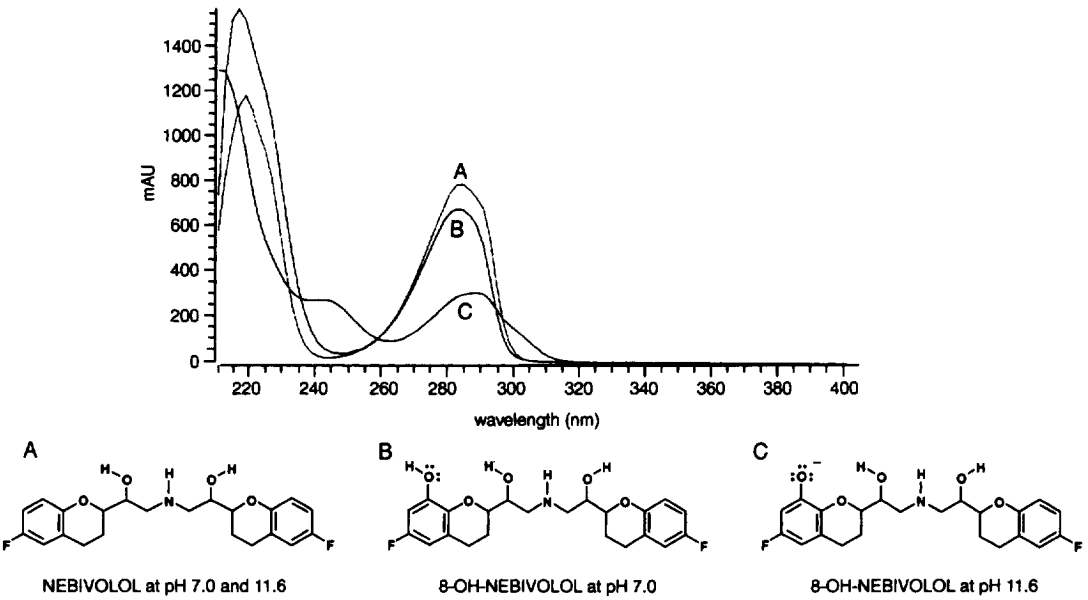

Fig. 2. Spectra of nebivolol and 8-hydroxynebivolol at different pH values.

their stability and purity, the purified metabolites were re-injected on the reversed-phase column and eluted with the same HPLC method as for the analytical samples. Aliquots of the purified metabolites were treated with β-glucuronidase/arylsulphatase for enzymatic hydrolysis of the conjugates and analyzed with the same analytical HPLC method. During the re-injection of the purified metabolites, on-line DAD spectra were extracted from the metabolites and their aglycons.

2.2. High-performance liquid chromatography

The HPLC apparatus consisted of a Waters 600-MS pump system, equipped with a 715-Ultra-WISP (Waters) automatic injector and a Rheodyne 2-ml loop injector for manual injections. UV diode-array spectra of the purified metabolites were extracted at the occasion of their reinjection on the C_{18} column, with a Hewlett-Packard 1040A photodiode-array detector (DAD), coupled on-line to the HPLC system. The detector was interfaced with a 79994A Analytical Workstation (Hewlett-Packard).

Stainless-steel columns (30 cm×4.6 mm I.D.) were packed with Hypersil C_{18} (5 μm, Shandon) or Polygosyl-$N(CH_3)_2$ (Macherey-Nagel, 5 μm), by a balanced density slurry procedure (Haskel DSTV 122-C pump, 7×10^7 Pa).

A gradient system with linear steps was applied to the C_{18} column. Elution started at 1.0 ml/min with a first very short gradient from 100% of 0.2% diethylamine, adjusted to pH 11.6 with acetic acid (solvent system A) to 70% of solvent system A and 30% of 2% diethylamine adjusted to pH 11.6 with acetic acid–distilled water–methanol (10:10:80) (solvent system B) over 1 min. Then a gradient to 95% of solvent system B was applied over 60 min. The latter solvent composition was held for 5 min and a short gradient to 100% solvent system B was applied over a 1-min time interval. The latter solvent composition was held for another 5 min before returning to the starting conditions. UV detection was performed at 290 nm by the Hewlett-Packard 1040A photodiode-array detector, and on-line radioactivity detection was carried out with a Berthold Radioactivity Monitor LB 507 A system, equipped with an 1.0-ml flow-through cell. The eluates were mixed with Picofluor 30 (Packard) as a scintillation cocktail, delivered by a FMI LB 5031 pump at a flow-rate of 4 ml/min. Detector outputs were connected to the Nelson 3000 chromatography data system (CDS) via Nelson A/D interfaces.

The $-N(CH_3)_2$ column was eluted with a gradient elution of diisopropyl ether containing 0.2% formic acid (solvent system C) and increasing concentrations of a mixture of 2% formic acid in water–methanol (15:85, solvent system D). The column was eluted with 100% methanol before returning to the starting conditions.

3. Results and discussion

3.1. UV diode-array spectroscopy of reference compounds

Fig. 3A and B shows UV spectra of examples of the different hydroxylated compounds. There is no difference in UV spectrum between the same positional isomers whether they are hydroxylated on the *R*S** or on the *S*S** side of the molecule.

The 4-keto, 4-hydroxy, 8-hydroxy, 7-hydroxy and 5-hydroxy derivatives were synthesized in our laboratories.

The UV spectrum of the parent compound nebivolol comprises the sum of the two benzopyran moieties with a maximum at 283 nm. The spectrum of the 4-keto derivative shows two additional maxima (250 nm, 330 nm) due to the extra C=O chromophore in one of the benzopyran moieties. The maximum at 283 nm refers to the unchanged benzopyran ring. The spectrum of the 4-hydroxy derivative is identical to that of nebivolol, because hydroxylation at the alicyclic part of the benzopyran ring has no influence on the chromophores. The other spectra, viz. of 8-hydroxynebivolol, 5-hydroxynebivolol and 7-hydroxynebivolol, are all of aromatic hydroxy derivatives and they show a mixture of both the unchanged benzopyran maximum and the red-shift maximum by the ionized hydroxyl function of the hydroxylated benzopyran ring. There is a clear difference between the spectra of the different positional hydroxy isomers.

The structure of dog metabolite A1 (Fig. 3B, 5F-6OH-nebivolol) was elucidated with MS and NMR, as 5-fluoro-6-hydroxy metabolite of nebivolol, formed by a NIH-shift (i.e. aromatic carbon hydroxylation via arene oxide and proton-catalyzed epoxide opening with simultaneous migration of a ring substituent, and resulting in 6-hydroxy-5-fluoro metabolites) [5] of the fluorine atom from the 6 to the 5 position. Metabolite A2 (Fig. 3B; 7F-6OH-nebivolol) was identified with MS as an aromatic hydroxy metabolite of nebivolol. The UV spectrum, however, was different from those of the 5-hydroxy, 8-hydroxy, and 7-hydroxy reference compounds and also from the 5-fluoro-6-hydroxy metabolite. The only possibility left is a 7-fluoro-6-hydroxynebivolol metabolite, thus also resulting from a NIH shift.

3.2. Separation of the metabolites

Like other β-blockers [6], nebivolol was extensively metabolised in all species (see Section 3.4). Because of the extreme complexity of the biotransformation of nebivolol it was attempted to obtain a separation of similar metabolites or groups of metabolites, rather than of single metabolites. Fig. 4 and Fig. 5 show radio-HPLC chromatograms, before and after enzymatic hydrolysis, of urine samples of different species. Single metabolites are indicated with characters and/or arabic numerals. Metabolic groups are indicated with roman numerals. These groups were classified as N-dealkylated metabolites (I), combined dihydroxy metabolites (II) (i.e. hydroxylated at an alicyclic and an aromatic part of the benzopyran moieties), aromatic monohydroxy metabolites (III), alicyclic dihydroxy metabolites (IV), alicyclic hydroxy-keto metabolites (V), alicyclic mono-oxidized metabolites (VI) (hydroxy- or keto-), glucuronides of nebivolol (gluc UD) and finally unchanged nebivolol (UD)). Within these groups it was attempted to separate the different metabolites.

Via pH and solvent (tetrahydrofuran, methanol, acetonitrile) screening methods on synthesized reference compounds and on urine and faeces samples of studies with radiolabelled nebivolol, the HPLC separation method described above was selected.

With this method the two goals, i.e. ionisation of the aromatic hydroxylated metabolites and separation of the different groups of metabolites, were achieved. The stability and lifetime of the column appeared to be affected to a less extent by the high pH of the elution solvents than by the matrix of the samples (direct injection of urine and bile, and of crude faeces extracts).

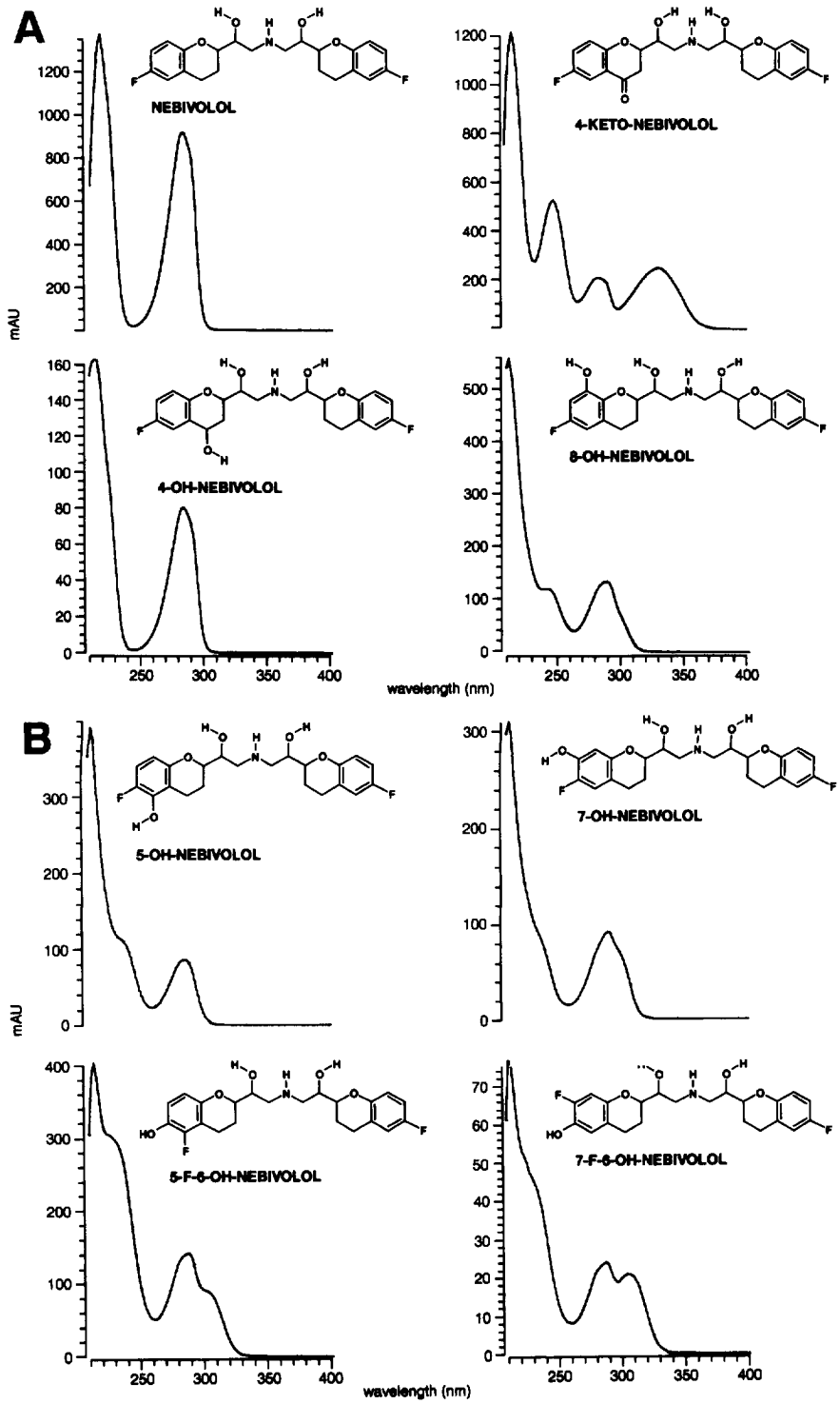

Fig. 3. Spectra of reference compounds.

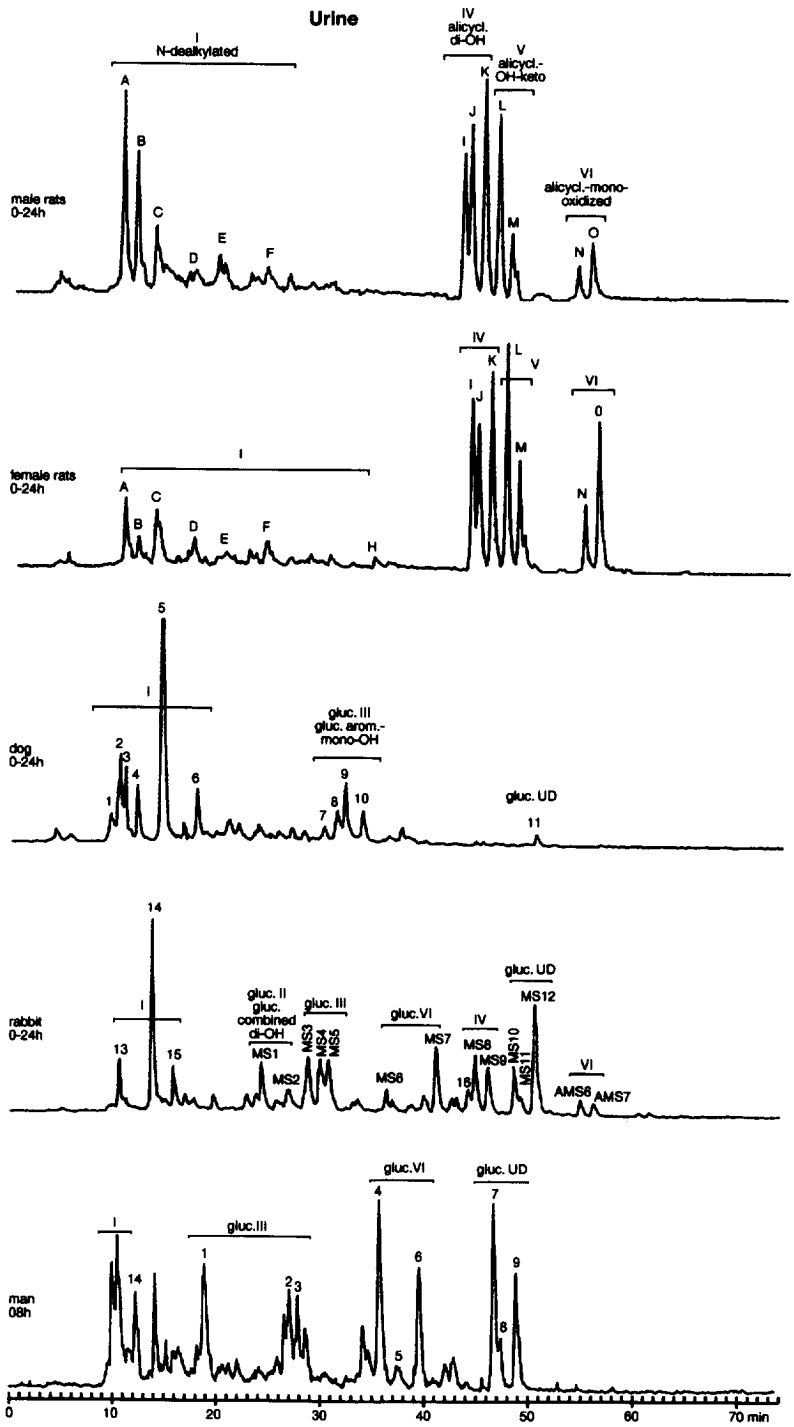

Fig. 4. Radio-HPLC chromatograms obtained for urine samples of animals and man. The different groups of metabolites are given by the roman numerals (see text).

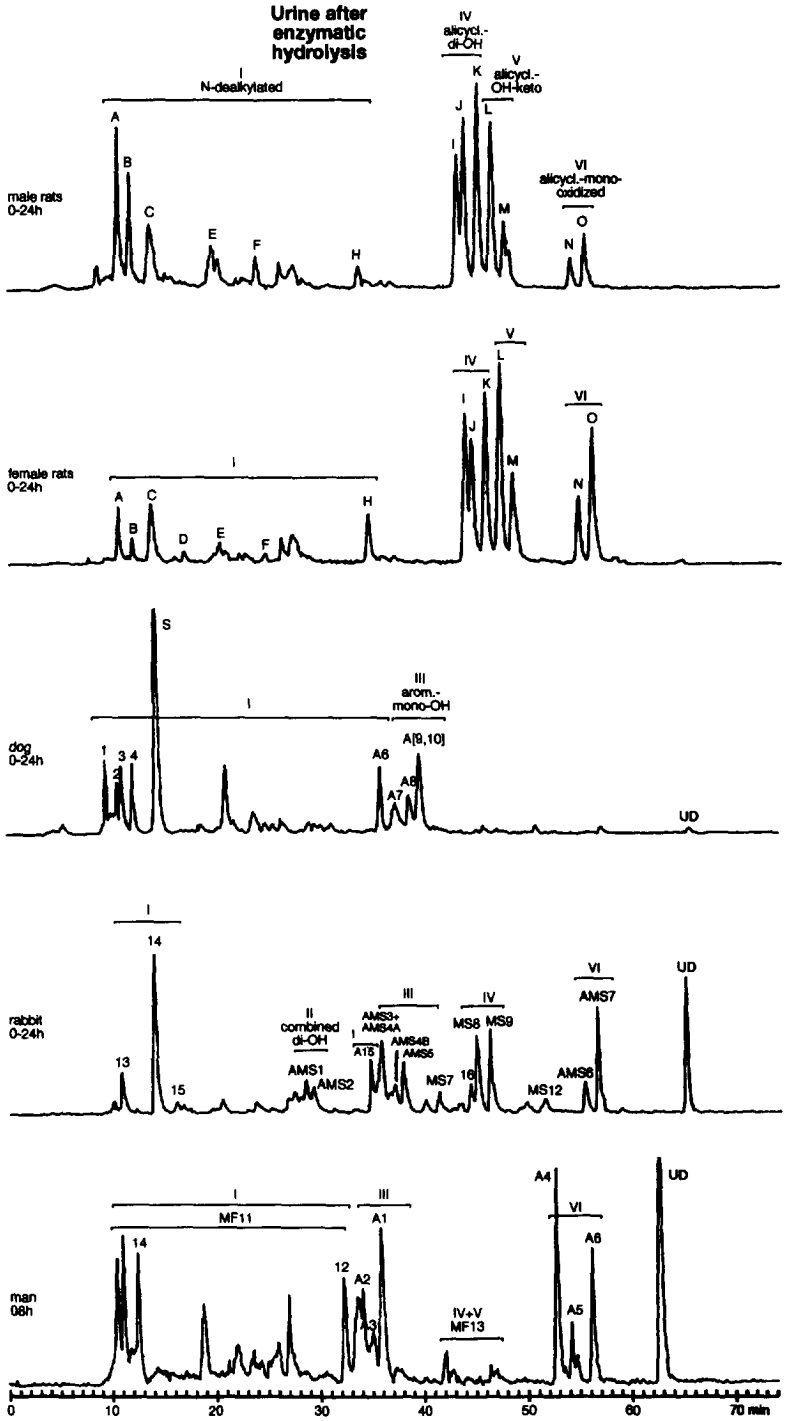

Fig. 5. Radio-HPLC chromatograms obtained for enzymatically hydrolysed urines of animals and man. The different groups of metabolites are given by the roman numerals (see text).

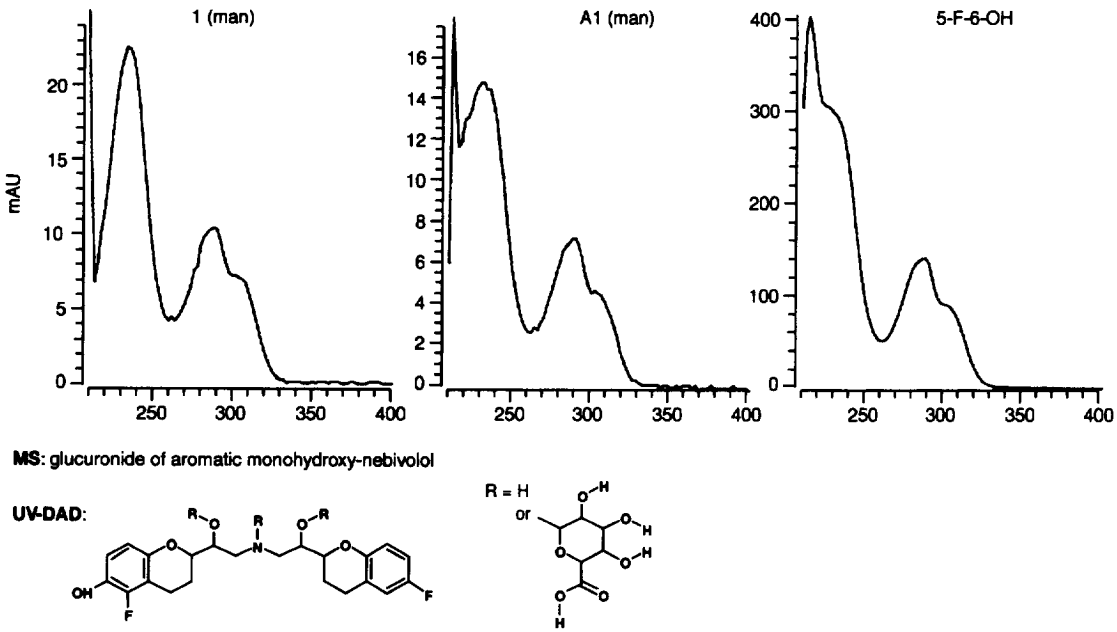

Fig. 6. On-line UV-DAD spectra of the purified metabolite 1 (man) before and after enzymatic hydrolysis in comparison with the UV spectrum of the metabolite A1 of the dog, identified with MS and NMR as 5-fluoro-6-hydroxynebivolol.

3.3. UV diode-array spectroscopy of some isolated metabolites

The on-line UV-DAD spectra of the purified metabolite 1 (from man) before and after enzymatic hydrolysis are shown in Fig. 6 in comparison with the UV spectrum of the dog metabolite A1. Metabolite 1 was identified with MS as a glucuronide of an aromatic hydroxy metabolite of nebivolol. The resemblance of the UV spectrum of the aglycon of metabolite 1 (A1) with the UV spectrum of dog metabolite A1 confirmed that the metabolic hydroxyl function was aromatic, because the spectrum is the sum of a red-shifted benzopyran spectrum caused by introduction of an ionized hydroxyl function and an unchanged benzopyran spectrum. Because the red-shift in metabolite 1 is of the same intensity as in the dog metabolite A1, the position of the fluorine atom and the hydroxyl function is the same in both metabolites (5-fluoro-6-hydroxy). There was no difference in the UV spectra before and after enzymatic hydrolysis indicating that the glucuronidation took place on an aliphatic hydroxyl function of the molecule.

The on-line UV-DAD spectra of the purified metabolite 2 (from man) before and after enzymatic hydrolysis are shown in Fig. 7 in comparison with the UV spectrum of the metabolite A1 of the dog. Metabolite 2 was identified with MS as a glucuronide of an aromatic hydroxy metabolite of nebivolol.

The resemblance of the UV spectrum of the aglycon of metabolite 2 (A2) with the UV spectrum of metabolite A1 confirms that the metabolic hydroxyl function is aromatic. Because the red-shift in metabolite 2 is of the same intensity as in the metabolite A1, the position of the fluorine atom and of the hydroxyl function is the same in both metabolites (5-fluoro-6-hydroxy).

The difference between the UV spectrum before and after enzymatic hydrolysis indicates that in this case the glucuronidation took place on the aromatic hydroxyl function of the molecule.

The on-line UV-DAD spectra of the purified metabolite 3 (from man) before and after enzymatic hydrolysis are shown in Fig. 8 in comparison with the UV spectrum of the metabolite A2 of the dog. Metabolite 3 (man) was identified with MS as a glucuronide of an aromatic hydroxy metabolite of

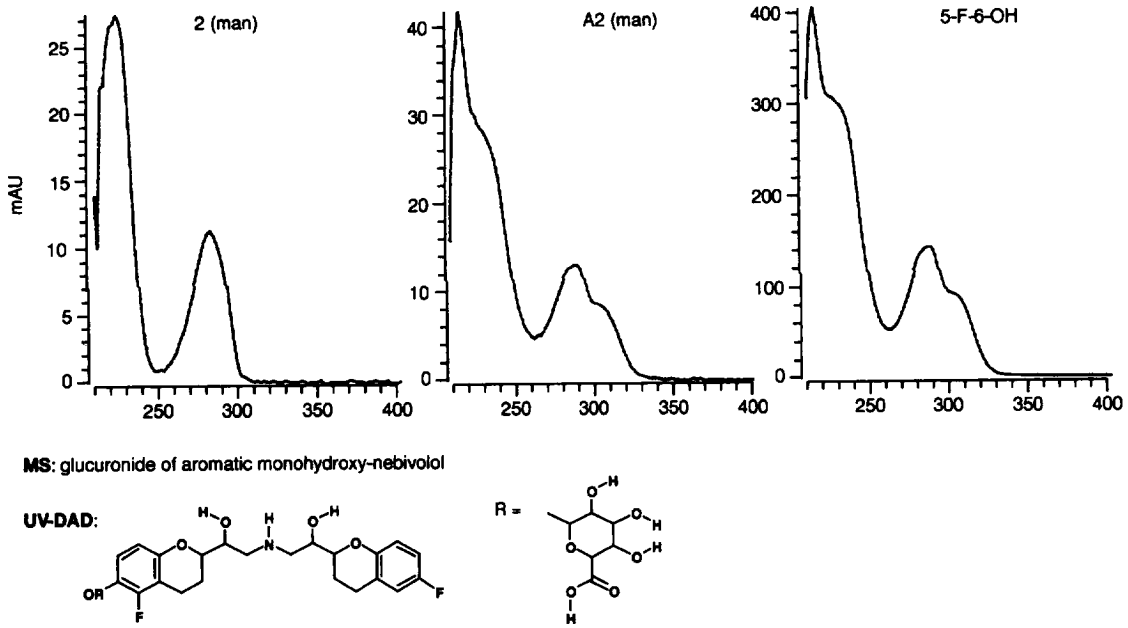

Fig. 7. On-line UV-DAD spectra of the purified metabolite 2 (man) before and after enzymatic hydrolysis in comparison with the UV spectrum of the metabolite A1 of the dog, identified with MS and NMR as 5-fluoro-6-hydroxynebivolol.

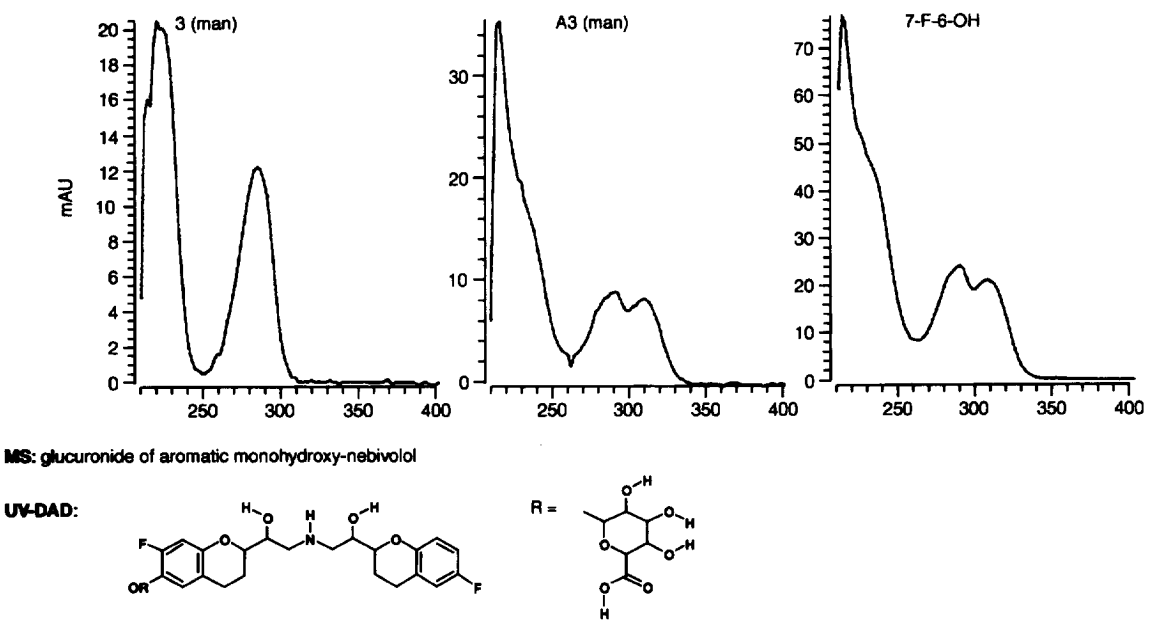

Fig. 8. On-line UV-DAD spectra of the purified metabolite 3 (man) before and after enzymatic hydrolysis in comparison with the UV spectrum of the metabolite A2 of the dog, identified with MS and NMR as 7-fluoro-6-hydroxynebivolol.

nebivolol. The resemblance of the UV spectrum of metabolite 3 (A3) after deconjugation with the UV spectrum of A2 confirms that the metabolic hydroxyl function is aromatic. Because the red-shift in the metabolite 3 is of the same intensity as in the metabolite A2, the position of the fluorine atom and hydroxyl function is the same as in this metabolite (7-fluoro-6-hydroxy).

The difference between the UV spectra before and after enzymatic hydrolysis again indicates that the glucuronidation took place on the phenolic hydroxyl function of the molecule.

The UV spectrum of metabolite 4 (man) (Fig. 9) and of its aglycon is similar to the UV spectrum of 4-hydroxynebivolol. Its retention time however increased with more then 10 min after enzymatic hydrolysis, indicating that this metabolite was a conjugate. MS identified metabolite 4 as a glucuronic acid conjugate of an alicyclic hydroxy derivative of nebivolol. The UV spectrum confirms this identification. Neither alicyclic hydroxylation nor glucuronidation of the alcohol function affects the chromophores. Metabolite 4 did not co-elute with any of the synthesized 4-hydroxy reference compounds, and therefore probably was a non-synthesized 4-hydroxy or a 3-hydroxy metabolite.

The UV spectrum of the purified metabolite 6 (man) (Fig. 10) corresponds with the UV spectrum of 4-ketonebivolol. MS identified this metabolite as a glucuronic acid conjugate of an alicyclic keto metabolite of nebivolol. The UV spectrum together with the retention times confirm these identifications. The carbonyl group is responsible for the two maxima at 250 nm and 330 nm. Glucuronidation on an aliphatic alcohol function does not influence the chromophore. The maximum at 283 nm refers to the unchanged benzopyran ring.

The on-line UV-DAD spectra of the purified metabolite MS1 (rabbit) before and after enzymatic hydrolysis are shown in Fig. 11 in comparison with the UV spectrum of the dog metabolite A2. MS1 was identified with MS as a glucuronic acid derivative of a dihydroxy metabolite of nebivolol with one hydroxyl group on the aromatic part of one benzopyran group and the other hydroxyl group on the alicyclic part of the other benzopyran group. The resemblance of the UV spectrum of the aglycon of MS1 (AMS1) with that of metabolite A2 (dog) confirms that only

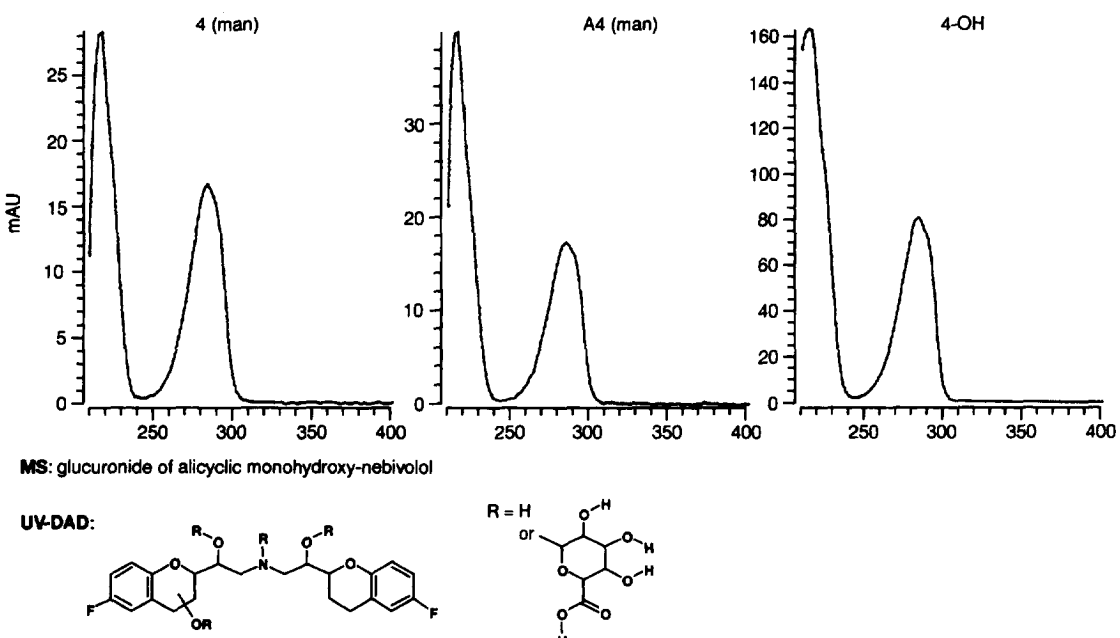

Fig. 9. On-line UV-DAD spectra of the purified metabolite 4 (man) before and after enzymatic hydrolysis in comparison with the UV spectrum of a synthesized 4-hydroxy derivative of nebivolol.

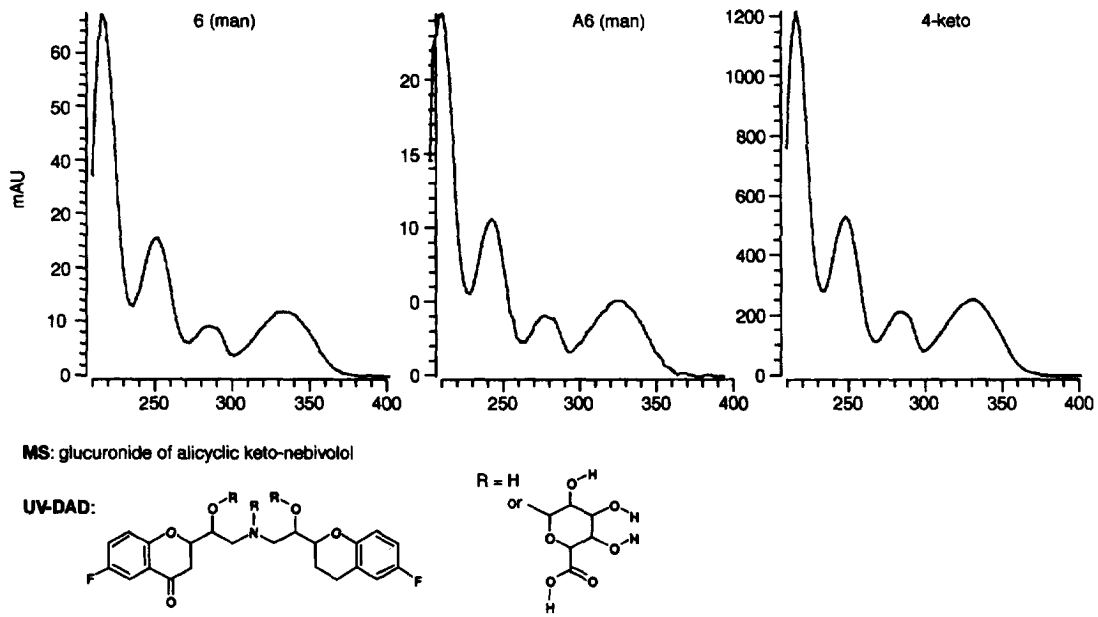

Fig. 10. On-line UV-DAD spectra of the purified metabolite 6 (man) before and after enzymatic hydrolysis in comparison with the UV spectrum of a synthesized 4-keto derivative of nebivolol.

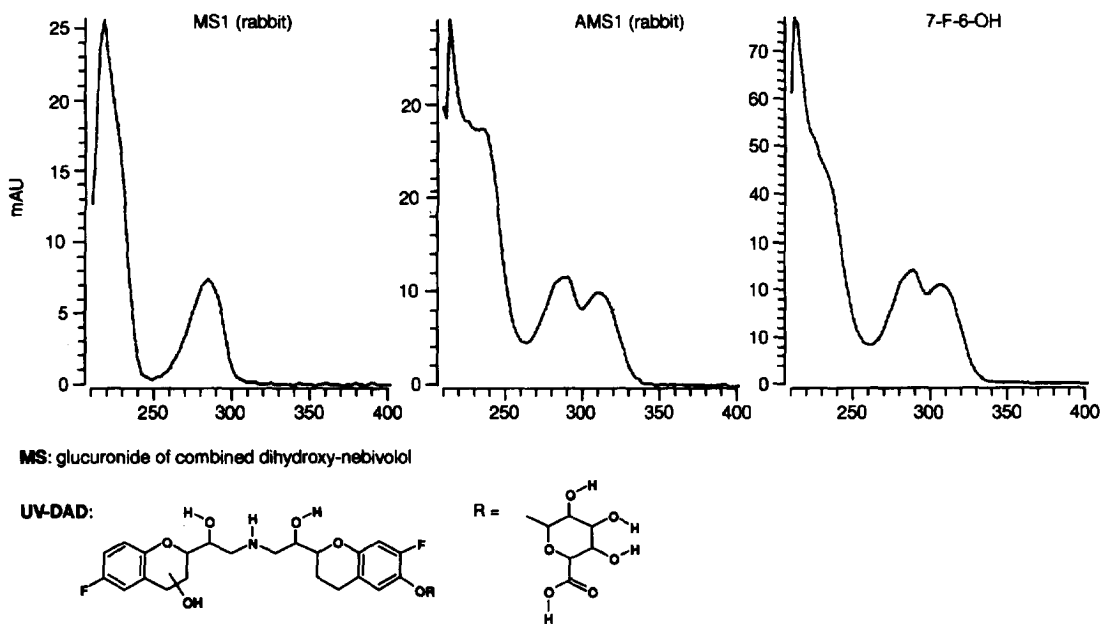

Fig. 11. On-line UV-DAD spectra of the purified metabolite MS1 (rabbit) before and after enzymatic hydrolysis in comparison with the UV spectrum of the dog metabolite A2 (7-fluoro-6-hydroxynebivolol).

one metabolic hydroxyl group is aromatic, because the spectrum is the sum of a red-shifted benzopyran spectrum caused by the introduction of an ionized hydroxyl group, and an unchanged benzopyran spectrum. Introduction of an hydroxyl group on the alicyclic part of the molecule does not change the UV spectrum.

Because the red-shift in the metabolite MS1 metabolite is of the same intensity as in the metabolite A2, the positions of the fluorine atom and hydroxyl group must be identical in the two metabolites (7-fluoro-6-hydroxy).

The difference between the UV spectra before and after enzymatic hydrolysis indicates that the glucuronidation took place on the aromatic hydroxyl function of the molecule. Glucuronidation blocks the ionization of the phenol and also its auxochromic effect.

The UV and MS spectra of metabolite MS2 (rabbit) (Fig. 12) and its aglycon (AMS2) are identical to those of MS1. Consequently MS2 has the same chemical structure as MS1. However because these two metabolites are completely separated on HPLC, the only explanation is that one metabolite is hydroxylated on the aromatic part of the benzopyran at the R^*S^*-side of the molecule and hydroxylated on the alicyclic part of the benzopyran at the S^*S^*-side and vice versa for the other metabolite.

3.4. Overal metabolism in the different species

The major metabolic pathways of nebivolol in different species are shown in Fig. 13. The species are ranked according to decreasing importance of the different metabolic pathways. When bile is mentioned it means that that particular pathway was also detected in the bile. A species name within parentheses means that the metabolic pathway is detected in that species, but that it accounts for a very small fraction of the dose.

4. Conclusion

This paper proves that HPLC at high pH combined with on-line diode array detection is an excellent technique for the location of the hydroxyl functions in hydroxylated metabolites of nebivolol. With this technique it is also possible to differentiate between

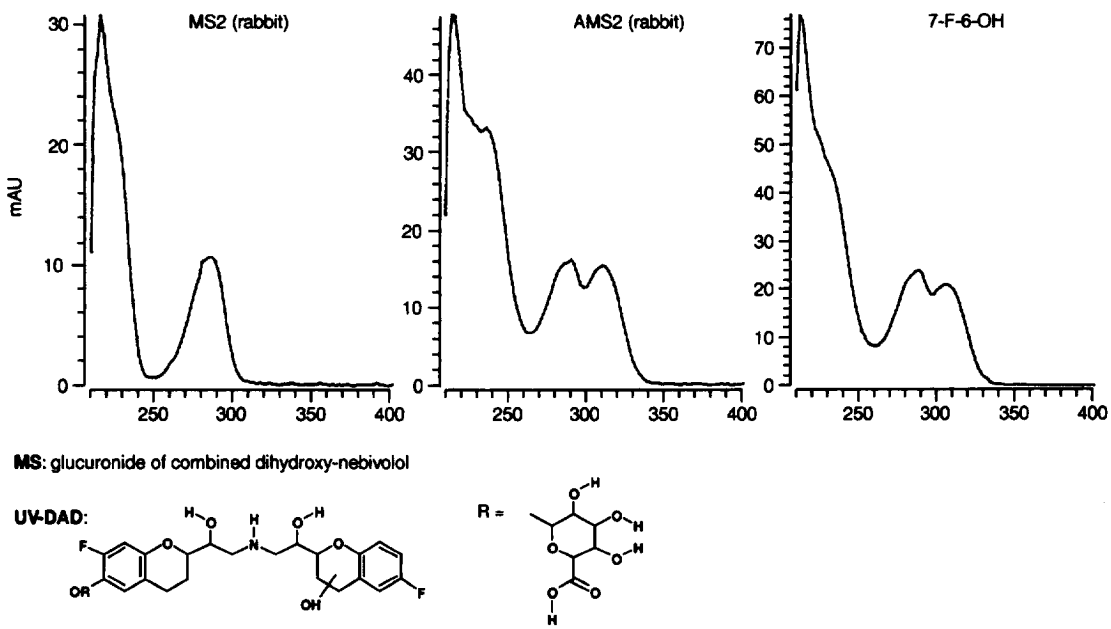

Fig. 12. On-line UV-DAD spectra of the purified metabolite MS2 (rabbit) before and after enzymatic hydrolysis in comparison with the UV spectrum of the dog metabolite A2 (7-fluoro-6-hydroxynebivolol).

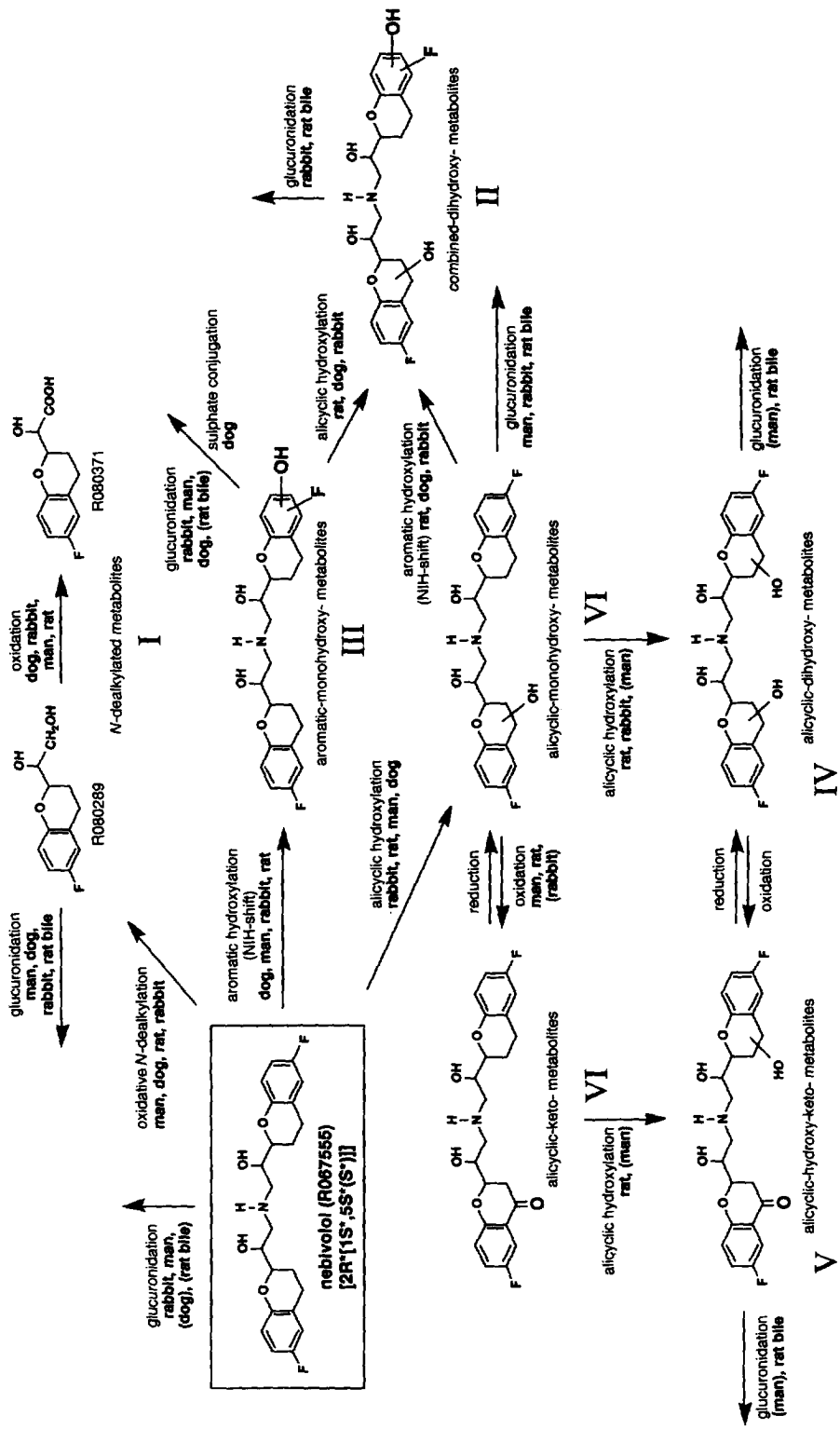

Fig. 13. Major metabolic pathways of nebivolol in animals and man.

glucuronides of aromatic and aliphatic or alicyclic hydroxy metabolites.

Acknowledgements

The authors are grateful to H. Lenoir and C. Janssen for the synthesis of ^{14}C-nebivolol and of the various reference compounds. They wish to thank D. Deleersnijder for his expert technical assistance and S. De Cauwer and L. Leijssen for the preparation of the manuscript.

References

[1] A. Van de Water, R. Xhonneux, R. Reneman and P. Janssen, Eur. J. Pharmacol., 156 (1988) 95–103.

[2] P. Pauwels, W. Gommeren, G. Van Lommen, P. Janssen and J. Leysen, Mol. Pharmacol., 34 (1988) 843–851.

[3] R. Xhonneux, L. Wouters, R. Reneman and P. Janssen, Eur. J. Pharmacol. 181 (1990) 261–265.

[4] T. Nowicka-Jankowska, K. Gorczynska, A. Michalik and E. Wieteska, Analytical Visible and Ultraviolet Spectrometry –Comprehensive Analytical Chemistry, Vol. XIX, Elsevier, Amsterdam, 1986.

[5] B. Testa and P. Jenner, in J. Swarbrick (Editor), Drug Metabolism: Chemical and Biochemical Aspects, Part 1 (Drugs and the Pharmaceutical Sciences, Vol. 4), Marcel Dekker, New York, 1976, pp. 49–52.

[6] R.L. Smith, Eur. J. Clin. Pharmacol., 25 [Suppl] (1985) 77–84.