Journal of Chromatography, 259 (1983) 301–310
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,584

MULTIPLE INVERSE ISOTOPE DILUTION ASSAY FOR OXPRENOLOL AND NINE METABOLITES IN BIOLOGICAL FLUIDS

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(Received November 29th, 1982)

SUMMARY

An isotope dilution assay for the specific determination of ^{14}C-labelled oxprenolol and nine of its metabolites in the same biological sample is described. After addition of unlabelled carriers to the sample, oxprenolol and the metabolites were isolated by base- and acid-specific extraction and separated by normal-phase high-performance liquid chromatography using two different mobile phases. Quantitation of the various peaks was performed by on-line ultraviolet detection at 275 nm and off-line radiometry by liquid scintillation counting. Endogenous compounds and unknown metabolites did not interfere in the assay. The analysis of rat and dog blood, plasma and urine samples spiked with [^{14}C]oxprenolol hydrochloride, showed mean recoveries between 98.7 and 99.8%. The assay was used to investigate the metabolic fate of [^{14}C]oxprenolol in the dog. Analyses of blood and urine demonstrated the quantitative significance of the various metabolites in the biotransformation of oxprenolol.

INTRODUCTION

Oxprenolol hydrochloride, 1-[2-(allyloxy)phenoxy]-3-isopropylamino-2-propanol hydrochloride, is an important cardioprotective β-adrenergic blocking agent[1]. In rat, dog and man the drug is extensively metabolized[2,3]. Various metabolites have been qualitatively identified[4–7]. The metabolic pathways include oxidation at the propanolamine and allyl side chains, aromatic hydroxylation and glucuronidation or sulphatation. In addition, N-methylation[8,9] and N-acetylation[10] of the primary amine metabolite was found in dogs and rats.

Several gas chromatographic[11–16] and high-performance liquid chromatographic (HPLC)[17–19] methods have been reported for the quantification of oxprenolol in biological fluids. Recently, a combination of HPLC separation and mass spectrometry was used for the determination of two phenolic metabolites[20].

The present paper describes a multiple inverse isotope dilution assay (IDA) for the specific and sensitive determination of unchanged drug and nine of its metabolites

0021-9673/83/0000–0000/$03.00

in biological fluids after administration of ^{14}C-labelled oxprenolol hydrochloride. The application of the method is demonstrated in a dog study.

EXPERIMENTAL

Chemicals

Oxprenolol hydrochloride (I) and two of its ^{14}C-labelled forms were synthesized by Ciba-Geigy (Basle, Switzerland). One form was uniformly labelled in the phenyl ring (Batch No. G 443.3 D; specific radioactivity 3.04 μCi/mg, radiochemical purity *ca.* 99%) and the second at the C-2 of the isopropylamino group (Batch No. W 44.2 A-3; specific activity 3.54 μCi/mg; radiochemical purity *ca.* 99%). The following unlabelled carrier substances were prepared by Ciba-Geigy: 1-(2-allyloxy-4-hydroxyphenoxy)-1-isopropylamino-2-propanol semioxalate (II); 1-[2-(allyloxy) phenoxy]-3-amino-2-propanol hydrochloride (III); 1-[2-(allyloxy)phenoxy]-3-methylamino-2-propanol hydrochloride (IV); 1-(2-hydroxyphenoxy)-3-isopropylamino-2-propanol hydrochloride (V); 3-[2-(allyloxy)phenoxy]-2-hydroxypropionic acid (VI); 2-[2-(allyloxy)phenoxy]acetic acid (VII); (2-hydroxyphenoxy)acetic acid (VIII); 3-[2-(allyloxy)phenoxy]-1,2-propanediol (IX); N-{3-[2-(allyloxy)phenoxy]-2-hydroxypropyl}acetamide (X) (for structural formulae of the free compounds see Figs.1 and 2).

1,2-Dichloroethane (Chemische Werke Hüls, Marl, G.F.R.) was distilled before use. 2-(4′-*tert.*-Butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD), Irgasolv® and Irgascint® A 300 were obtained from Ciba-Geigy. β-Glucuronidase/arylsulphatase (*Helix pomatia*), B-grade, was purchased from Calbiochem (La Jolla, CA, U.S.A.). All other solvents and chemicals were of analytical or spectroscopic grade and were obtained from E. Merck (Darmstadt, G.F.R.).

Chromatographic conditions

Separation of oxprenolol (I) and compounds II–X was achieved by normal-phase HPLC on a 250 × 10 mm I.D. stainless-steel column (Altex; Beckman, Berkeley, CA, U.S.A.) packed with 10-μm LiChrosorb® Si 60 (E. Merck) using two different solvent systems. The separation columns were protected by precolumns (50 × 4.6 mm I.D.) packed with identical material. For injection, a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) was used. Oxprenolol (I) and compounds II–V were chromatographed with a basic mobile phase (1,2-dichloroethane–2-propanol–1-butanol–conc. aqueous ammonia, 50:50:10:2), whereas compounds VI–X were separated with an acidic mobile phase (1,2-dichloroethane–tetrahydrofuran–dioxan–formic acid–water, 80:10:10:4:1). The solvent systems were pumped through the column at a flow-rate of 4.0 ml/min at ambient temperature with a constant flow pump (Altex, Model 110 A). The compounds were detected with a Cecil CE 212 UV detector (Cecil, Cambridge, Great Britain) at 275 nm and range 2 and quantified on-line with a W + W Model 1100 integrating recorder (Kontron, Zürich, Switzerland).

Inverse isotope dilution analysis (IDA)

To a 0.2–2.0 ml sample of urine, plasma or haemolysed blood a solution containing the non-labelled carrier substances I (0.7 mg), II (2.0 mg), III (0.4 mg), IV (1.0 mg), V (1.0 mg), VI (1.0 mg), VII (0.5 mg), VIII (0.7 mg), IX (1.0 mg) and X (1.5 mg) in 1.0 ml of water was added. The mixture was vortexed for 1 min and allowed to stand for 15 min.

Extraction of compounds VI–X. 5.0 ml of glycine–hydrochloric acid buffer, pH 1.0 (Titrisol®, Merck) were added to the biological sample and the acidic and neutral compounds were extracted twice with 1,2-dichloroethane (8 ml each time) by vortexing for 1 min and centrifuging at 550 *g* for 10 min. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness (rotary evaporator). The residue was dissolved in 0.1 ml of the acidic solvent system and chromatographed as described above.

Extraction of compounds I–V. The remaining biological aqueous phase was adjusted to pH 8–9 with 1 *M* sodium hydroxide solution (about 0.8 ml). Then 2.0 ml of concentrated borate buffer pH 10.0 (Titrisol, Merck; undiluted) were added. The basic compounds were extracted twice with 1,2-dichloroethane (8 ml each time). The extract was processed as described above. The residue was dissolved in 0.1 ml of the basic solvent system and chromatographed (see *Chromatographic conditions*).

After the chromatographic separations, the eluate fractions corresponding to the peak sections, which were integrated for each of the ten compounds, were collected directly in counting vials. Approximately 15 ml of scintillation cocktail were added to each sample and the radioactivity was measured. The quantity of each compound in a sample can be determined from the spectrophotometric and radiometric results.

Deconjugation of metabolites

For the determination of conjugated parent compound and metabolites in blood, plasma and urine, enzymatic hydrolysis was carried out prior to the IDA. After dilution with the individual carrier substances, 1–2 ml of 1.8 *M* sodium acetate buffer pH 5.12 (Elemente Analytik, Ciba-Geigy) and 1 ml of β-glucuronidase/arylsulphatase, diluted 1:10 in the same buffer, were added to the biological specimen. The mixture was placed in a water-bath at 37°C for 16 h. Following the incubation, the sample was subjected to IDA.

Calibration of the HPLC method

Replicate samples containing various amounts of one of the ten compounds were injected into the HPLC system. The range of each compound was selected to cover the expected concentrations of samples containing the carrier substances. Each chromatographic peak was integrated automatically by the integrating recorder. Calibration curves were fitted by means of a computer using a linear regression program. The resulting coefficients were used for the calculations in the IDA.

Radiometry

Radioactivity in biological samples and chromatographic eluate fractions was measured in a TriCarb® Model 460 C liquid scintillation counter (Packard, Downers Grove, IL, U.S.A.) with external standardization equipment. The scintillation cocktail used was 0.6% Butyl-PBD in toluene–methanol (3:1) for eluates and urine, and Irgascint for blood after dissolution in Irgasolv.

Recovery of ^{14}C-labelled oxprenolol hydrochloride from spiked biological samples

Samples of 1 ml rat blood, rat plasma, rat urine, dog blood, dog plasma and dog urine were spiked with 2.9–18.4 μmol/l of [^{14}C]oxprenolol hydrochloride (spe-

cific radioactivity 3.54 μCi/mg). The spiked samples were carried through the analytical procedure. The results obtained were compared to the amount added to each respective sample to determine the recoveries of [^{14}C]oxprenolol hydrochloride.

Pattern of urinary metabolites

To check the two HPLC separations of the various compounds for possible interference from unknown metabolites, enzymatically hydrolysed urine samples from a dog treated with [^{14}C]oxprenolol hydrochloride were subjected to IDA. Unlike the procedure described above, however, the total effluent from the chromatographic separation was collected in small fractions of 0.8 ml, which were then counted for radioactivity. The UV-absorption profile was compared with the radioactivity pattern.

Animal experiment

One male beagle dog (Tif: CAB^{21}), weighing 12.5 kg, was obtained from the Tierfarm Sisseln, Switzerland. The animal was deprived of food for 15 h before and 4 h after administration of the compound; water was available *ad libitum*. [^{14}C]Oxprenolol hydrochloride (3.04 μCi/mg) was orally administered in a dose of

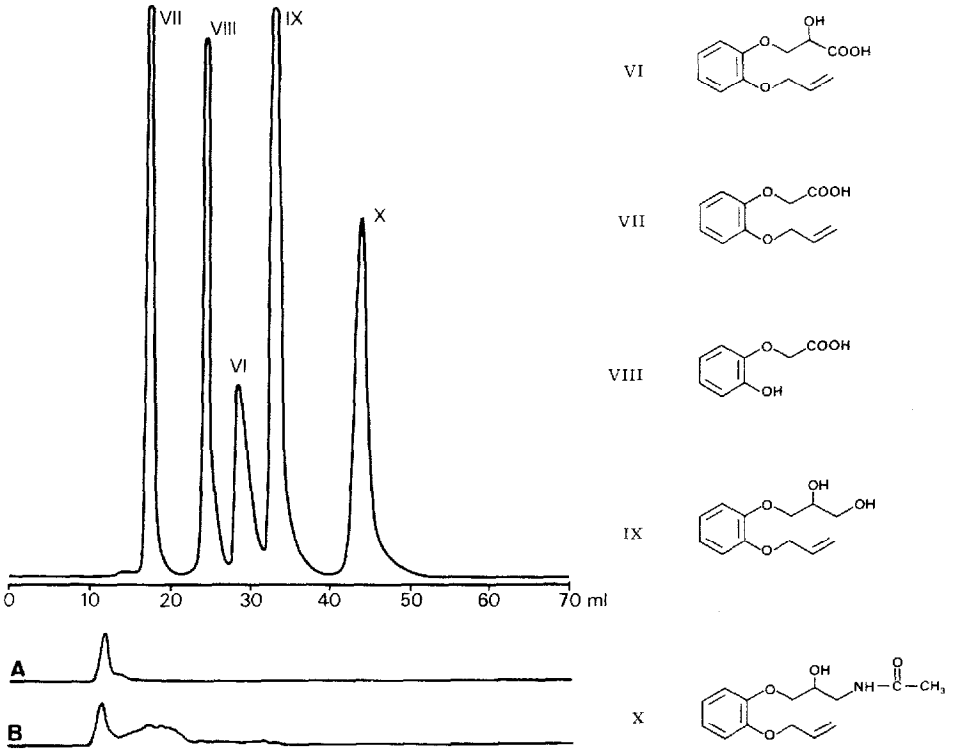

Fig. 1. HPLC separation of oxprenolol metabolites VI–X, and chromatograms from blank dog blood (A) and urine (B), as obtained following the IDA procedure. HPLC conditions: column, 10-μm LiChrosorb Si 60 (25 × 1.0 cm I.D.); eluent, 1,2-dichloroethane–tetrahydrofuran–dioxan–formic acid–water (80:10:10:4:1); flow-rate, 4 ml/min; UV-detection at 275 nm.

3 mg/kg. Blood was withdrawn from the foreleg vein 15 and 30 min and 1, 2, 3, 4, 6, 8, 12 and 24 h after dosing. Urine was sampled for 24 h. Heparinized blood samples and urine were stored at −20°C before analysis.

RESULTS AND DISCUSSION

Specificity of the HPLC separation

The HPLC separation of the acidic extract containing compounds VI–X is illustrated in Fig. 1. The acidic mobile phase employed was found to provide a good separation of the carboxylic acids VI–VIII, the glycol IX and the acetamido compound X. Analysis of blank samples of blood and plasma from rat and dog demonstrated that endogenous compounds do not interfere with the UV-detection (see Fig. 1). In rat and dog urine there is some interference with compound VII, which, however, can be overcome by reducing the sample volume.

Fig. 2 depicts the corresponding chromatograms of the basic extract (compounds I–V) and of blank samples. Using the described HPLC system with a basic mobile phase, oxprenolol (I), 4-hydroxy-oxprenolol (II), the desisopropyl compound (III), the methylamino compound (IV) and the desallyl compound (V) were com-

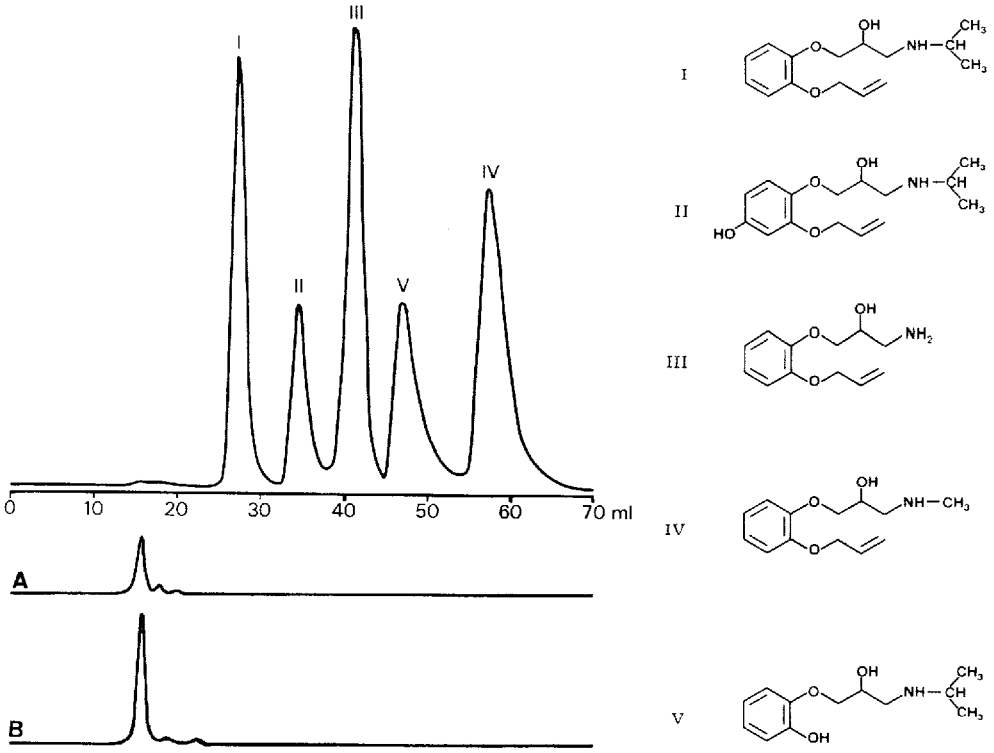

Fig. 2. HPLC separation of oxprenolol (I) and metabolites II–V, and chromatograms from blank dog blood (A) and urine (B), as obtained following the IDA procedure. HPLC conditions: column, 10-μm LiChrosorb Si 60 (25 × 1.0 cm I.D.); eluent, 1,2-dichloroethane–2-propanol–1-butanol–conc. aqueous ammonia (50:50:10:2); flow-rate, 4 ml/min; UV-detection at 275 nm.

pletely separated from each other. Blood, plasma or urine compounds showed no interference.

It is essential to perform the acidic extraction step first. The glycol (IX) and the acetamido compound (X) can be extracted under both acidic and basic conditions. However, separation of these two neutral compounds can be achieved only in

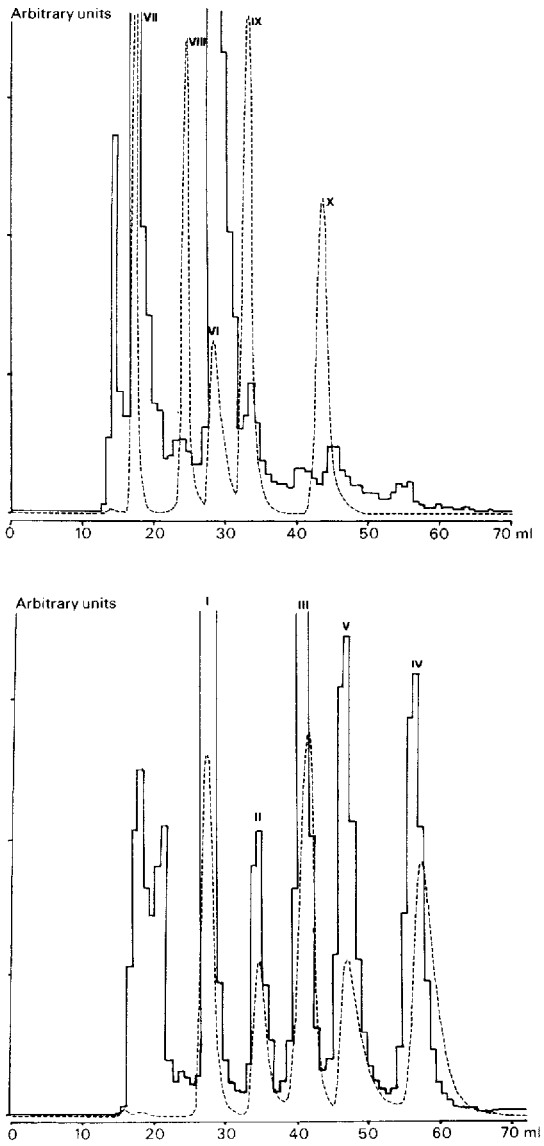

Fig. 3. HPLC separation of oxprenolol metabolites VI–X (upper part), and oxprenolol (I) and metabolites II–V (lower part), in IDA from urine of one dog dosed with [^{14}C]oxprenolol hydrochloride, monitored by radioactivity (———) and UV-detection (– – –). Urine was analysed after enzymatic hydrolysis with β-glucuronidase/arylsulphatase. HPLC conditions as in Figs. 1 and 2.

the acidic solvent system. Under the conditions of the acidic extraction, none of the basic compounds I–V is extracted.

Good coincidence was observed in both HPLC separations between the radioactivity pattern and the UV-absorption profile, when urine of dogs dosed with [^{14}C]oxprenolol hydrochloride was analysed according to the IDA procedure (Fig. 3). This indicates that unknown metabolites of oxprenolol do not interfere with the chromatographic separation of compounds I–X.

Calibration and reproducibility of the spectrophotometric determination

Excellent linearity ($r > 0.994$) was observed for the calibration curves of all compounds. The coefficients of the curves are displayed in Table I.

TABLE I

CALIBRATION AND REPRODUCIBILITY OF UV-DETECTOR RESPONSE TO OXPRENOLOL (I) AND COMPOUNDS II–X

FOR HPLC CONDITIONS SEE FIGS. 1 AND 2

Compound No.	*Quantities injected (μg)*	*Coefficients of the calculated calibration curves* $y = a + bx$*			*Coefficient of variation*** C.V. (%)*	*No. of samples*
		a	*b*	*r***		
I	150– 700	7.380	0.260	0.9940	± 2.7	17
II	200– 600	11.918	0.306	0.9994	± 1.5	11
III	500–1900	5.862	0.127	0.9963	± 3.8	17
IV	250– 700	28.336	0.328	0.9998	± 0.7	6
V	250–1050	9.030	0.307	0.9998	± 1.6	9
VI	350– 900	24.257	0.404	0.9963	± 3.3	6
VII	80– 300	9.699	0.328	0.9976	± 2.9	15
VIII	130– 700	16.688	0.390	0.9991	± 2.5	13
IX	80– 400	12.115	0.324	0.9979	± 4.3	15
X	200–1000	6.986	0.308	0.9979	± 2.5	18

* Calibration curves were calculated by linear regression analysis; y = integration units, x = amount injected.

** r = coefficient of correlation.

*** For the ratio of x (calculated) to x (given).

The reproducibility of the method expressed as the coefficient of variation (C.V.) was highest for compound IV (± 0.7%) and lowest for the glycol IX (± 4.3%). The C.V. values of all compounds I–X are summarized in Table I.

Accuracy of the IDA method

Biological samples were spiked with 2.9–18.4 μmol/l of ^{14}C-labelled oxprenolol hydrochloride (specific radioactivity 3.54 μCi/mg) and analysed by the assay. The calculated recoveries for oxprenolol hydrochloride had means ± C.V. of 99.8 ± 2.3% ($n = 4$) in rat blood, 98.8 ± 1.8% ($n = 5$) in dog blood, 99.5 ± 2.7% ($n = 4$) in rat plasma, 98.7 ± 2.5% ($n = 6$) in dog plasma, 99.1 ± 3.6% ($n = 4$) in rat urine and 99.4 ± 2.0% ($n = 6$) in dog urine.

The sensitivity of the method would be about 0.013 μmol/l (4ng/ml) using a 1-ml sample and a specific radioactivity of 3.5 μCi/mg.

Application of the IDA

The analytical procedure was applied to the determination of unchanged oxprenolol (I) and metabolites II–X in enzymatically hydrolysed blood and urine as well as in native urine of a dog orally dosed with 3 mg/kg of [^{14}C]oxprenolol hydrochloride. Fig. 4 shows the blood concentration curves of the various compounds together with the curve of total radioactive substances. The results of the analysis of the 0–24 h urine expressed in per cent of total ^{14}C in urine, are depicted in Fig. 5.

[^{14}C]Oxprenolol was extensively metabolized in the dog. The carboxylic acids VI and VII and the glycol IX were the predominant metabolites in blood. Compounds VIII and X were not detectable (Fig. 4). The results from the analysis in urine

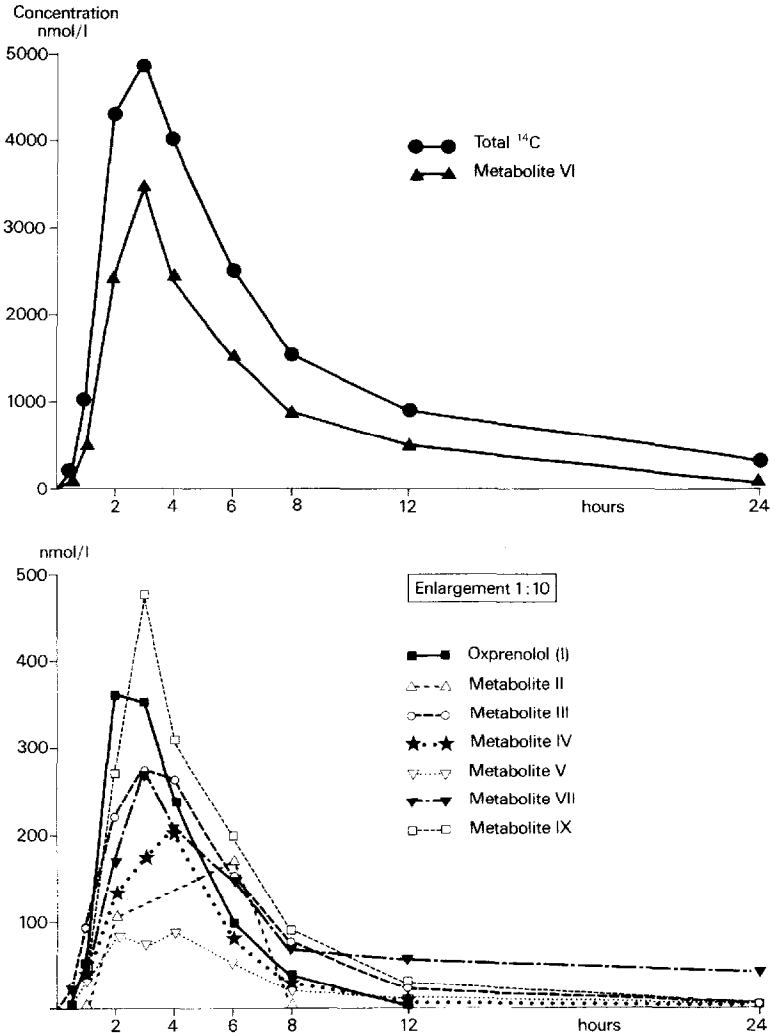

Fig. 4. Blood concentration profiles, obtained after enzymatic hydrolysis, of total ^{14}C-labelled substances, unchanged oxprenolol (I) and metabolites II–VII and IX in one dog after oral dosage of 3 mg/kg [^{14}C]oxprenolol hydrochloride.

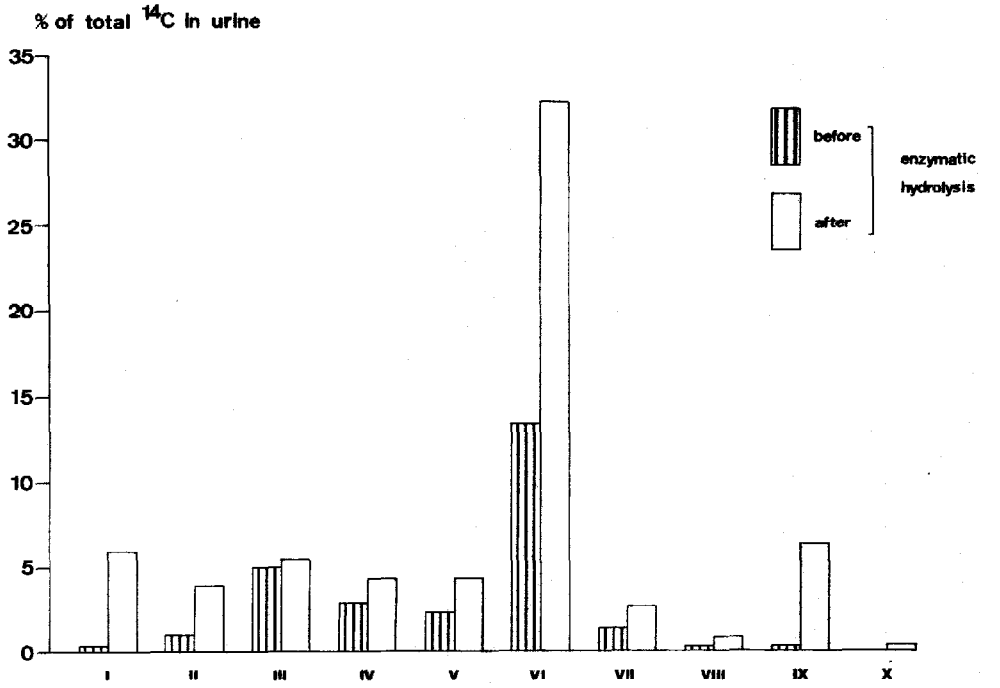

Fig. 5. Excretion of unchanged oxprenolol (I) and metabolites II–X in urine of one dog after oral dosage of 3 mg/kg [^{14}C]oxprenolol hydrochloride. Urine was analysed in native form and after enzymatic hydrolysis with β-glucuronidase/arylsulphatase.

indicate that oxprenolol and its various metabolites existed, at least partly, as conjugates, which can be liberated by β-glucuronidase/arylsulphatase. Only the primary amine metabolite III was excreted preferentially in unconjugated form (Fig. 5). Similar to the findings in blood, the major urinary products were the carboxylic acid VI and the glycol IX. The sum of compounds I–X in free and conjugated form constituted 65.5% of urinary radioactivity.

CONCLUSION

In isotope dilution analysis the carrier acts as ideal internal standard. In the multiple mode this is valid for all the simultaneously determined compounds. The entire analytical procedure is therefore perfectly controlled and provides high specificity, accuracy and reproducibility.

The present method allows one to determine the disposition and metabolism of oxprenolol in a quantitative manner. This is not possible with any of the previously published methods.

ACKNOWLEDGEMENTS

We thank Drs. W. Küng, F. Ostermayer and K. A. Jaeggi of Ciba-Geigy for the supply of labelled oxprenolol and of the synthetic metabolites, and Miss G. David for excellent technical assistance.

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