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MULTIPLE INVERSE ISOTOPE DILUTION ASSAY FOR THE STEREOSPECIFIC QUANTITATIVE DETERMINATION OF *R*(−) AND *S*(+)-OXAPROTILINE IN BIOLOGICAL FLUIDS

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SUMMARY

An isotope dilution assay for the determination of both oxaprotiline enantiomers in biological samples after administration of the racemic mixture has been developed. The enantiomers were reacted with synthetically prepared, optically pure N-trifluoroacetyl-*S*(−)-prolyl chloride, followed by high-performance liquid chromatographic separation of the diastereoisomers formed. Quantitation was performed by on-line UV detection at 260 nm and off-line radiometry by liquid scintillation counting. Endogenous compounds and metabolites do not interfere in the assay. Analysis of water and the blood and urine of rats spiked with [^{14}C]oxaprotiline · HCl showed recoveries for *S*(+)-oxaprotiline · HCl (mean ± coefficient of variation, n = 4–6) of 98.0 ± 1.0% (water), 100.5 ± 0.6% (blood) and 101.5 ± 2.0% (urine), and for *R*(−)-oxaprotiline · HCl of 101.3 ± 2.0% (water), 102.2 ± 2.1% (blood) and 103.2 ± 0.2% (urine).

A pilot study to determine blood levels of the two enantiomers in two rats dosed with racemic [^{14}C]oxaprotiline · HCl (10 mg/kg i.v.) was carried out to test the method. The results indicated stereoselective disposition of oxaprotiline enantiomers in the rat. The ratio of the areas under the blood concentration curves for *R*(−)- to *S*(+)-oxaprotiline · HCl was 1.14.

INTRODUCTION

Oxaprotiline · HCl, a highly selective and potent inhibitor of noradrenaline uptake, possesses a chiral centre in the aliphatic side-chain and contains two optically active enantiomers. Only the *S*(+)-form showed strong noradrenaline uptake inhibition in rats; the *R*(−)-form proved inactive in this test (for formulae see Fig. 1). Both enantiomers exhibit anti-aggressive and anti-histaminic properties[1].

Stereoselective disposition of the two oxaprotiline enantiomers can result in different pharmacokinetic profiles, owing to selective biotransformation reactions, tissue uptake, protein binding and elimination[2]. To investigate this, an inverse isotope dilution analysis (IDA) was elaborated which allows one to measure simultaneously the individual enantiomers after administration of the racemic mixture.

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The methodology involves derivatization of both isomers from biological samples by an optically pure derivatizing agent, separation of the resulting diastereoisomers by high-performance liquid chromatography (HPLC) and their quantitation by on-line UV detection and off-line radiometry.

Experiments on the application of this type of analysis to stereospecific kinetics of oxaprotiline · HCl in the rat are described.

EXPERIMENTAL

Chemicals

R(−)-Oxaprotiline · HCl (melting point 231–232°C, $[\alpha]_D^{20} = -9°$ in methanol), *S*(+)-oxaprotiline · HCl (melting point 231–232°C, $[\alpha]_D^{20} = +9°$ in methanol) and racemic oxaprotiline · HCl (melting point 234–235°C) were prepared by Ciba-Geigy (Basle, Switzerland). [^{14}C]Oxaprotiline · HCl, labelled at the C-1 position of the 1-methylamino-2-hydroxypropyl side-chain (Batch No. Z 511.3 B; specific radioactivity 6.78 μCi/mg; radiochemical purity *ca.* 99%), was synthesized by Ciba-Geigy. *S*(−)-Proline (puriss. grade) ($[\alpha]_D^{20} = -85.3°$ in water), trifluoroacetic anhydride (puriss. biochem. grade) and thionyl chloride were obtained from Fluka (Buchs, Switzerland). 1,2-Dichloroethane (Chemische Werke Hüls, Marl, G.F.R.) was distilled before use. Ethanol, methylene chloride and *n*-heptane were of analytical-reagent grade and were obtained from E. Merck (Darmstadt, G.F.R.). Butyl-PBD [0.8% (w/v) in toluene], Irgasolv® and Irgascint® A 300 were obtained from Ciba-Geigy.

Synthesis of N-trifluoroacetyl-S(−)-prolyl chloride (TPC)

The resolving agent TPC was synthesized using a modification of a previously reported technique[3]. *S*(−)-Proline (1.0 g, dried at 100°C for 15 h under high vacuum) was dissolved in trifluoroacetic anhydride (10 ml) by magnetic stirring with cooling in an ice-bath. The bath was removed and the excess of anhydride was evaporated at ambient temperature under a stream of dry nitrogen. To the fairly yellow oily residue freshly distilled thionyl chloride (10 ml) was added. After being allowed to stand for 15 min at ambient temperature, the excess of thionyl chloride was evaporated under dry nitrogen (ice-bath). The residue was dissolved in 10 ml of methylene chloride and evaporated under nitrogen. This step was repeated twice. Finally, the clear and colourless residue was dissolved in 50 ml of methylene chloride and stored at −20°C in glass vials with septum stoppers sealed under helium. Under these conditions the TPC solution is stable for at least 4 months.

General procedure for derivatization of oxaprotiline enantiomers by TPC (see Fig. 1)

R(−)-Oxaprotiline · HCl, *S*(+)-oxaprotiline · HCl or racemic oxaprotiline · HCl (30 mg) was dissolved in water (2 ml). Phosphate buffer (pH 10) (10 ml) was added and the free amines were extracted with 1,2-dichloroethane (2 × 3 ml) by vortexing for 1 min and centrifuging at 550 *g* for 10 min. The organic phase was evaporated to dryness (rotary evaporater) and the residue redissolved in 1,2-dichloroethane (4 ml). TPC reagent (0.6 ml, see above) was added and the mixture was alowed to stand for 15–20 min. After adding 0.1 *M* sodium hydroxide solution (5 ml), vortexing for 2 min and centrifuging at 550 *g* for 10 min, the organic phase was washed

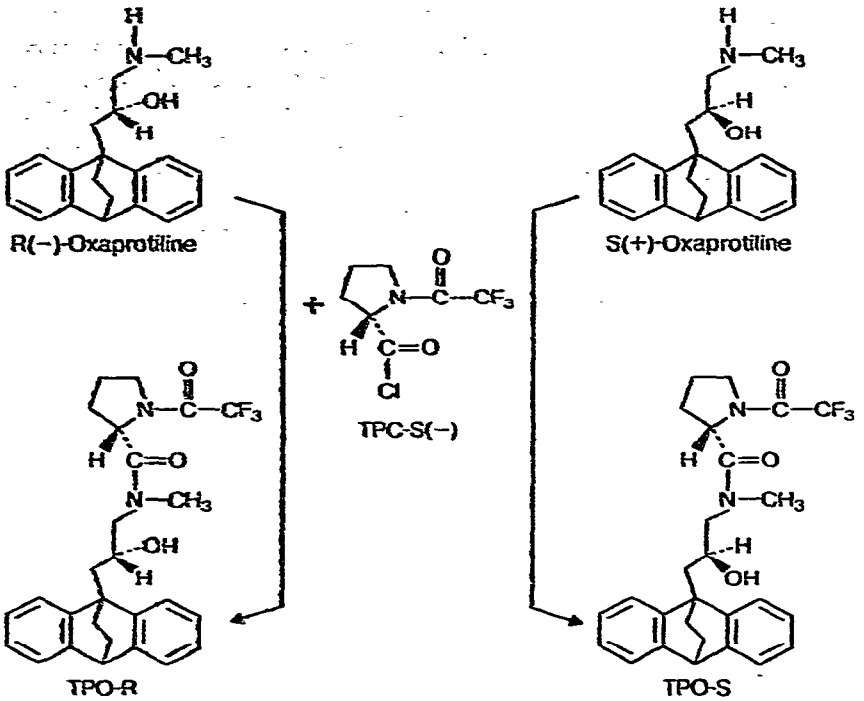

Fig. 1. Reaction of *R*(−)oxaprotiline and *S*(+)-oxaprotiline with N-trifluoroacetyl-*S*(−)-prolyl chloride (TPC) to form the corresponding N-oxaprotiline derivatives TPO-*R* and TPO-*S*, respectively.

with water (3 × 5 ml) and evaporated to dryness. Aliquots of the residue were dissolved in the chromatographic solvent system for chromatography.

For calibration purposes, the N-trifluoroacetyl-*S*(−)-prolyl-N-derivatives of *R*(−)-oxaprotiline (TPO-*R*) and *S*(+)-oxaprotiline (TPO-*S*) were prepared on a preparative scale (500 mg each of the enantiomers) and purified by preparative HPLC using the chromatographic conditions described below. As attempts to crystallize the pure material failed, the oily residue was evaporated under high vacuum, whereby a white foam was obtained.

The elemental analyses were as follows: TPO-*S* ($C_{27}H_{29}N_2O_3F_3$) calculated, C 66.65, H 6.01, N 5.76, F 11.71%; found, C 66.75, H 6.10, N 5.79, F 11.25%; TPO-*R* ($C_{27}H_{29}N_2O_3F_3$) calculated, C 66.65, H 6.01, N 5.76, F 11.71%; found, C 66.57, H 6.13, N 5.96, F 11.87%.

Chromatographic conditions

A solution of TPO-*S* and TPO-*R* in the chromatographic solvent system (1,2-dichloroethane–*n*-heptane–ethanol, 82:15:3) was separated by HPLC on a 250 × 10 mm I.D. stainless-steel column (Altex) packed with LiChrosorb® Si 60, 10 μm (E. Merck). For injection, a U6K injector (Waters Assoc., Milford, MA, U.S.A.) was used. The solvent system was pumped through the column at a flow-rate of 4.0 ml/min at ambient temperature using an Altex Model 110 A constant-flow pump. The compounds were detected and quantified on-line with a UV detector at 260 nm and range 2 (Cecil CE212) connected to an integrating recorder (W + W Model 1100).

Inverse isotope dilution analysis (IDA)

To 0.2–2.0 ml of the biological sample (urine, blood) or water, a solution of *ca.* 3000 μg of non-labelled oxaprotiline · HCl in 2.0 ml of water was added. The mixture was vortexed for 1 min and allowed to stand for 15 min. After extraction of the free amines and derivatization with TPC as described above, the mixture of the diastereoisomeric oxaprotiline derivatives was chromatographed. The eluate fractions corresponding to the peak fractions, which were integrated for each of the two diastereoisomers, were collected directly in counting vials. Approximately 15 ml of scintillation cocktail were added to each sample and the radioactivity was measured. The amount of each substance in a sample can be determined from the spectrophotometric and radiometric results.

Calibration of the HPLC method

Replicate samples containing various amounts of synthetic TPO-*S* and TPO-*R* were injected into the HPLC system. The range of concentrations for both compounds was selected to cover the expected concentrations of samples mixed with the carrier compounds. Each sample was integrated automatically by the recorder. The ratios of the amount of *S*(+)-oxaprotiline · HCl and *R*(−)-oxaprotiline · HCl to integration units (factor *f*) were determined for each sample.

Radiometry

The radioactivity in biological samples and chromatographic eluate fractions was measured in a liquid scintillation counter (Packard Model 3375) 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 having dissolved it in Irgasolv.

Recovery of ^{14}C-labelled R(−)-oxaprotiline · HCl and S(+)-oxaprotiline · HCl from samples spiked with [^{14}C]oxaprotiline · HCl

Samples of 2 ml water, rat urine and rat blood were spiked with racemic [^{14}C]oxaprotiline · HCl (48.21 and 14.49 μmol/l) and the spiked samples were carried through the analytical procedure. The results obtained for ^{14}C-labelled *R*(−)-oxaprotiline · HCl and *S*(+)-oxaprotiline · HCl were compared to the amount of racemic [^{14}C]oxaprotiline · HCl added to each sample to determine the recovery. For the evaluation of the results, it is assumed that synthetic [^{14}C]oxaprotiline · HCl is a 1:1 mixture of ^{14}C-labelled *R*(−)-oxaprotiline · HCl and *S*(+)-oxaprotiline · HCl.

Animal experiment

Two male albino rats [Tif: RAIf (SPF)], weighing 300 g, were obtained from the Tierfarm Sisseln, Switzerland. The animals were deprived of food 15 h before and during the experiment; water was available ad libitum. For intravenous administration of 10 mg/kg into the caudal vein, [^{14}C]oxaprotiline · HCl (6.78 μCi/mg) was dissolved in water (3.23 mg/ml). Blood was withdrawn from the orbital sinus under carbon dioxide anaesthesia 5, 10, 15 and 30 min and 1, 2, 3, 4 and 6 h after dosing. Heparinized blood samples were stored at −20°C before analysis.

RESULTS AND DISCUSSION

Structure of the diastereoisomeric oxaprotiline derivatives

The reaction of the two oxaprotiline enantiomers with TPC led to the proposed structures, *viz.*, the diastereoisomeric N-trifluoroacetyl-*S*(−)-prolyl-N-derivatives of *S*(+)- and *R*(−)-oxaprotiline (see Fig. 1), as shown by elemental analysis (see Experimental) and by mass and NMR spectroscopy. The mass spectra of both products were identical. The observed molecular ion of *m/e* 486 and the fragmentation pattern are consistent with a monotrifluoroacetylprolyloxaprotiline derivative. Conclusive evidence for the reaction of TPC with the secondary amino group of oxaprotiline was deduced from NMR spectra. Formation of an amide results in a chemical shift of the NCH_3 signal from 2.5 ppm to 3.05 and 3.31 ppm (in $CDCl_3$). As the N-derivative is a tertiary amide, it exists as a mixture of the two rotamers, and thus leads to the observed splitting of the NCH_3 signal.

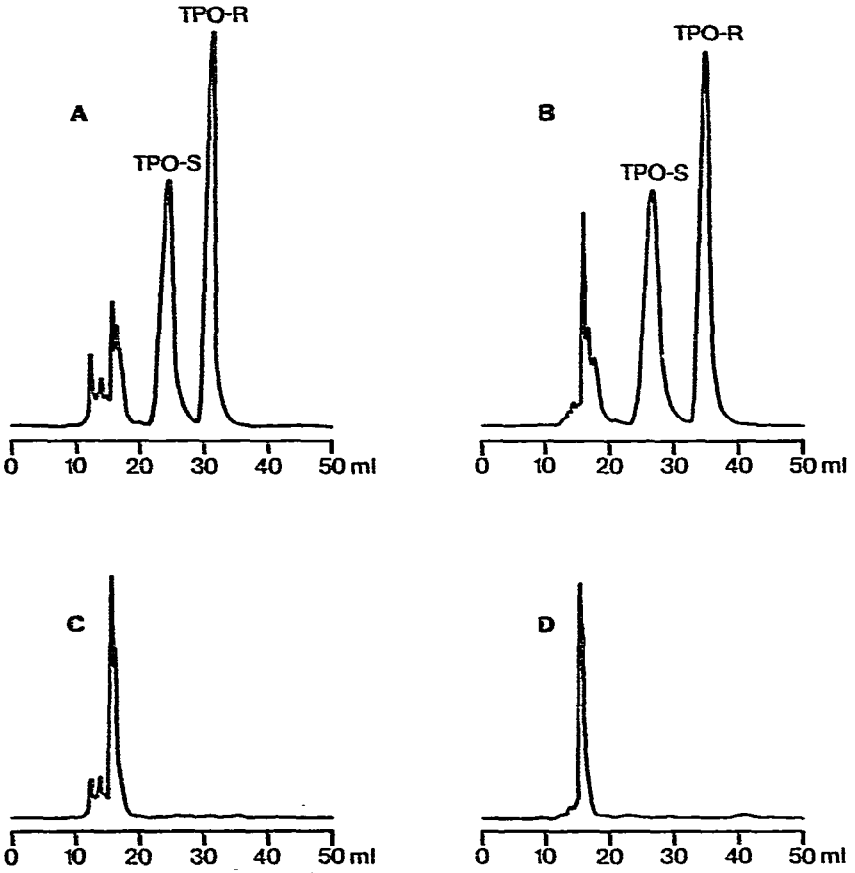

Fig. 2. HPLC separation of TPO-*S* and TPO-*R* in IDA from (A) blood and (B) urine of rats spiked with racemic oxaprotiline, and chromatograms from blank rat (C) blood and (D) urine, as obtained following the IDA procedure. HPLC: LiChrosorb Si 60, 10 μm (25 × 1.0 cm I.D. column); 1,2-dichloroethane–*n*-heptane–ethanol (82:15:3); flow-rate, 4 ml/min; UV detection at 260 nm.

Specificity of the HPLC separation

Using the described HPLC system, the two diastereoisomeric oxaprotiline-TPC derivatives were completely (baseline) separated from each other, as shown in Fig. 2. Blood or urine components did not interfere (Fig. 2). Also, there was no indication that metabolites of oxaprotiline interfere in the assay. As shown in Fig. 3, two symmetric radioactive peaks appeared at the positions of TPO-*S* and TPO-*R* when the effluent from the analysis of urine of rats dosed with [^{14}C]oxaprotiline · HCl was monitored for radioactivity.

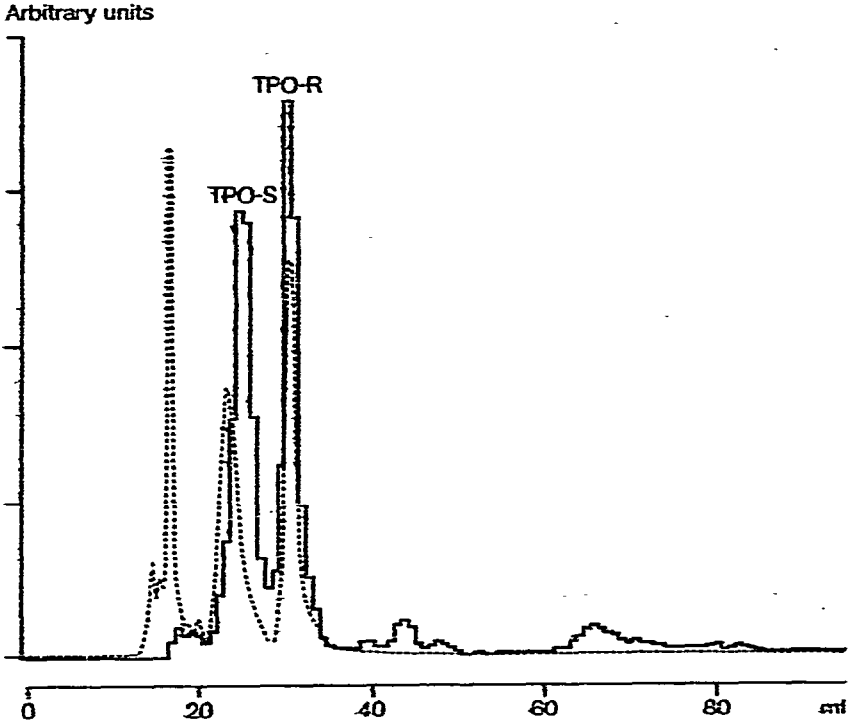

Fig. 3. HPLC separation of TPO-*S* and TPO-*R* in IDA from urine of rats dosed with racemic [^{14}C]oxaprotiline · HCl, monitored by radioactivity (——) and UV detection (——). HPLC conditions as in Fig. 2.

Calibration and reproducibility of the spectrophotometric determination

A calibration graph for the quantitative spectrophotometric determination of the derivatives of the two oxaprotiline enantiomers is shown in Fig. 4. With amounts ranging from 350 to 1500 μg of each of the compounds, a linear response was obtained ($r = 0.9989$).

The calibration graphs for the two diastereoisomers coincide completely. The reproducibility or precision of the method, expressed as the coefficient of variation (CV) was ±3.2% ($n = 17$) for TPO-*R* and TPO-*S*.

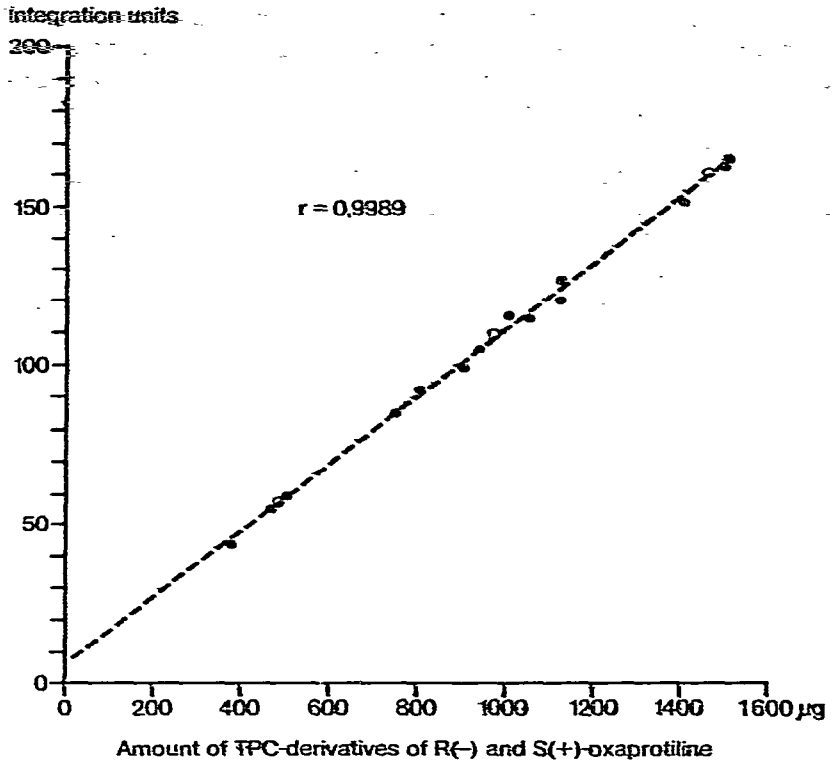

Fig. 4. Calibration graph for the spectrophotometric determination of the N-trifluoroacetyl-*S*(−)-prolylderivatives of *R*(−)-oxaprotiline (●) and *S*(+)-oxaprotiline (○).

Accuracy of the IDA method

The recoveries for *S*(+)-oxaprotiline · HCl from water and urine and blood samples from rats, spiked with 48.21 and 14.49 µmol/l (water only) of racemic [^{14}C]oxaprotiline · HCl (specific radioactivity 6.78 µCi/mg) had means ± CV of 98.0 ± 1.0% (96.7–99.0%, $n = 6$), 101.5 ± 2.0% (98.9–103.7%, $n = 4$) and 100.5 ± 0.6% (100.1–101.3%, $n = 4$), respectively. The corresponding values for *R*(−)-oxaprotiline · HCl from water, urine and blood were 101.3 ± 2.0% (99.5–104.5%, $n = 6$), 103.2 ± 0.2% (102.7–103.3%, $n = 4$) and 102.2 ± 2.1% (99.8–104.7%, $n = 4$), respectively. The data were calculated with the assumption that synthetic [^{14}C]oxaprotiline is a 1:1 mixture of ^{14}C-labelled *R*(−)-oxaprotiline · HCl and *S*(+)-oxaprotiline · HCl. In fact, the sum of the individual values of *S*(+)-oxaprotiline · HCl and *R*(−)-oxaprotiline · HCl resulted in means ± CV for the recovery of racemic oxaprotiline · HCl of 99.7 ± 1.4% (water), 102.2 ± 1.0% (urine) and 101.4 ± 1.1% (blood).

The sensitivity would be about 0.006 µmol/l (2 ng/ml) using a 1-ml sample and a specific radioactivity of 6.8 µCi/mg.

Concentration of R(−)- and S(+)-oxaprotiline · HCl in the blood of rats dosed with [^{14}C]oxaprotiline · HCl

A pilot study on two rats was conducted to demonstrate the feasibility of the

method. Each of the rats was given a 10 mg/kg intravenous dose of [^{14}C]oxaprotiline · HCl. Blood samples were collected up to 6 h from each individual animal and analysed for total radioactive substances and the two oxaprotiline enantiomers. The blood concentration curves are depicted in Fig. 5.

From the results, it is evident that the disposition of intravenously administered [^{14}C]oxaprotiline · HCl is stereoselective in rats. Up to 15 min after dosing, the levels for *R*(−)- and *S*(+)-oxaprotiline · HCl were identical, but after that time the concentration of the *S*(+)-enantiomer always exceeded that of the *R*(−)-enantiomer. The ratios of the areas under the blood concentration–time curves between 5 min and 6 h for *R*(−) and *S*(+)-oxaprotiline · HCl were 1.15 and 1.13 in the two rats.

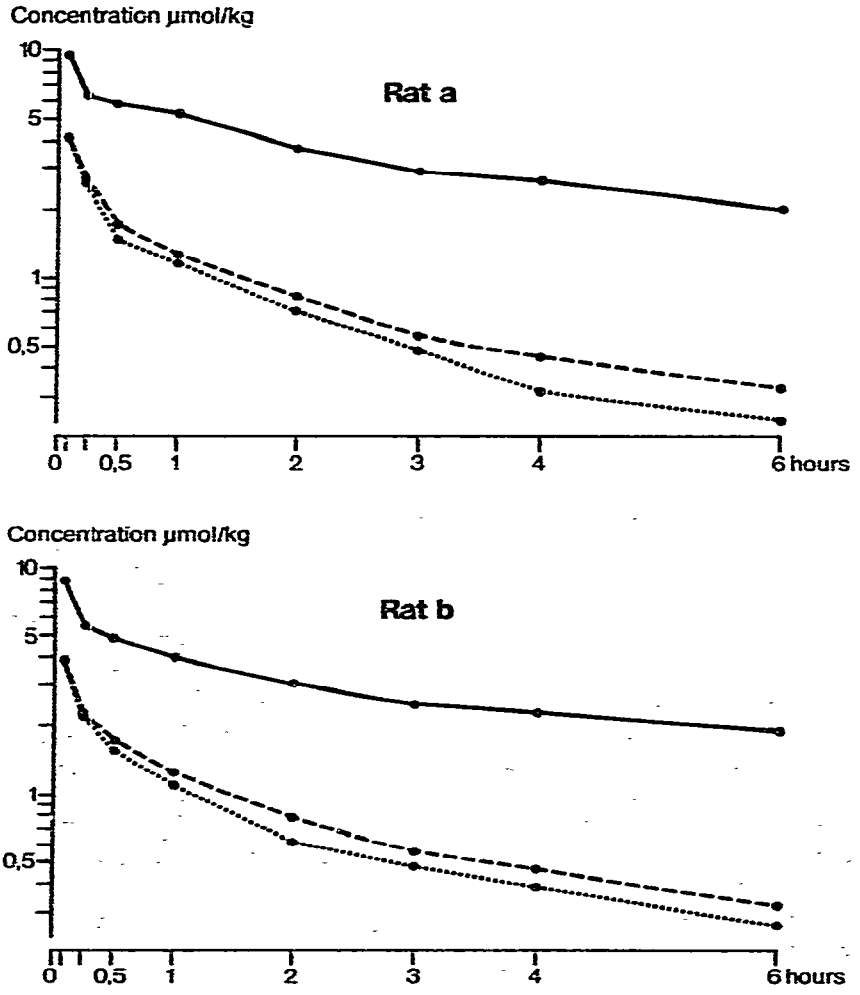

Fig. 5. Blood concentration profiles of total ^{14}C-labelled substances, *S*(+)-oxaprotiline · HCl and *R*(−)-oxaprotiline · HCl in two individual rats after intravenous dosing with racemic [^{14}C]oxaprotiline · HCl (10 mg/kg). ●——●, Total ^{14}C; ●——●, *S*(+)-oxaprotiline · HCl; ●·····●, *R*(−)-oxaprotiline · HCl.

CONCLUSION

In recent years, advances have been made in the simultaneous determination of enantiomers by chromatographic techniques. They include separation by thin-layer chromatography[4,5], gas chromatography[3] and HPLC[6–9].

For the resolution of optical isomers two different approaches have been developed: first, the use of chiral solvents or sorbents, and second, derivatization with suitable chiral reagents followed by separation on an achiral stationary phase[7,9]. The second approach was chosen for the described multiple isotope dilution assay using TPC as the derivatization reagent[10,11] and HPLC as the separation technique. To our knowledge, this is the first example in which the principle of inverse isotope dilution[12] has been employed for the quantitative simultaneous determination of enantiomers as diastereoisomeric derivatives.

Although this technique is restricted to radioactively labelled compounds, its great advantage lies in the use of an ideal internal standard which allows one to control perfectly the analytical procedure, including especially extraction, derivatization and chromatography. In fact, the described method for the stereospecific quantitative determination of *R*(−)- and *S*(+)-oxaprotiline in biological fluids proved to be of high specificity, accuracy and reproducibility.

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