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

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SUMMARY

An isotope dilution assay has been developed for the determination of both oxprenolol enantiomers in biological samples after administration of the racemic ^{14}C-labelled mixture. The enantiomers were reacted with optically pure *S*(−)-1-phenylethyl isocyanate and the diastereoisomeric urea derivatives formed were separated by normal-phase high-performance liquid chromatography. Quantitation was performed by on-line ultraviolet detection at 275 nm and off-line radiometry. Endogenous compounds and oxprenolol metabolites did not interfere with the assay. Analysis of water and blood, plasma and urine samples of rats and dogs spiked with [^{14}C]oxprenolol hydrochloride showed mean recoveries for *R*(+)-oxprenolol hydrochloride of 99.2% (water), 99.3% (blood), 99.1% (plasma) and 97.9% (urine), and for *S*(−)-oxprenolol hydrochloride of 99.7% (water), 98.1% (blood), 98.6% (plasma) and 96.9% (urine). In a pilot study, the presented method was used to investigate the metabolic fate of the enantiomers in two dogs dosed orally with racemic [^{14}C]oxprenolol hydrochloride (3 mg/kg). The results show that conjugation of *R*(+)-oxprenolol exceeded that of *S*(−)-oxprenolol.

INTRODUCTION

Oxprenolol hydrochloride, 1-[2-(allyloxy)phenoxy]-3-isopropylamino-2-propanol hydrochloride, is an important cardioprotective β-adrenergic agent. It is a racemic mixture of which the *S*(−)-enantiomer is 10–35-fold more active than the *R*(+)-enantiomer[1]. However, no method is available to investigate the stereoselective disposition of the two optical isomers.

High-performance liquid chromatographic (HPLC) techniques have been successfully used in the simultaneous determination of enantiomers[2–5]. Several methods have been published which describe the determination of the isomers of adrenergic β-receptor blocking drugs (propranolol, metoprolol, alprenolol) by HPLC of diastereoisomeric derivatives[6–10]. In a recent study on the separation of enantiomeric

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amines by ion-pair chromatography, the optical antipodes of oxprenolol were only incompletely resolved[11].

Previously[12] we developed an isotope dilution technique for the quantification of *R*(−)- and *S*(+)-oxaprotiline. It included reaction of the secondary amino group of the molecule with N-trifluoroacetyl-*S*(−)-prolyl chloride and separation of the diastereoisomers by HPLC. Applying this technique to oxprenolol, baseline separation of the enantiomers was not possible. We therefore used *S*(−)-1-phenylethyl isocyanate as the optically active reagent. Chiral isocyanates have been employed for the resolution of racemic mixtures of alcohols[13], and, more recently, for the resolution of racemic propranolol[14].

This paper describes the development of an isotope dilution assay which allows one to measure simultaneously the *R*(+)- and *S*(−)-isomers in biological fluids after administration of racemic [^{14}C]oxprenolol. Experiments on the application of this type of analysis to the stereospecific disposition of [^{14}C]oxprenolol hydrochloride in the dog are also presented.

EXPERIMENTAL

Chemicals

R(+)-Oxprenolol hydrochloride (m.p. 72–74°C, $[\alpha]_D^{20} = +19°$ in ethanol), *S*(−)-oxprenolol hydrochloride (m.p. 73–75°C, $[\alpha]_D^{20} = -19°$ in ethanol) and racemic oxprenolol hydrochloride (m.p. 107–109°C) were prepared by Ciba-Geigy (Basle, Switzerland). [^{14}C]Oxprenolol hydrochloride, labelled uniformly in the phenyl ring (Batch No. G 443.3 D; specific radioactivity 3.04 μCi/mg; radiochemical purity *ca.* 99%) or at the C-2 of the isopropylamino group (Batch No. W 44.2 A-2; specific radioactivity 11.67 μCi/mg; radiochemical purity *ca.* 99%) were synthesized by Ciba-Geigy. *S*(−)-1-Phenylethyl isocyanate (purum, $[\alpha]_{546}^{20} = -1.7°$ in benzene) was obtained from Fluka (Buchs, Switzerland). 1,2-Dichloroethane (Chemische Werke Hüls, Marl, G.F.R.) was distilled before use. 2-(4′-*tert.* -Butylphenyl)-5-(4″-bipenylyl)-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 chemicals and solvents were of analytical or spectroscopic grade and were obtained from E. Merck (Darmstadt, G.F.R.).

General procedure for derivatization of oxprenolol enantiomers by S(−)-1-phenylethyl isocyanate

Racemic oxprenolol, hydrochloride *R*(+)-oxprenolol hydrochloride or *S*(−)-oxprenolol hydrochloride (302 mg, 1 mmol) was dissolved in water (5 ml). Borate buffer pH 10.0 (3 ml) was added and the free amines were extracted with toluene (2 × 8 ml) 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 redissolved in toluene (5 ml) and reacted with *S*(−)-1-phenylethyl isocyanate (147 mg, 1 mmol; dissolved in 15 ml of toluene) for 5–10 min at room temperature. After washing with water, the organic phase was evaporated to dryness. The residue was dissolved in an appropriate volume of the chromatographic solvent system and chromatographed (see below).

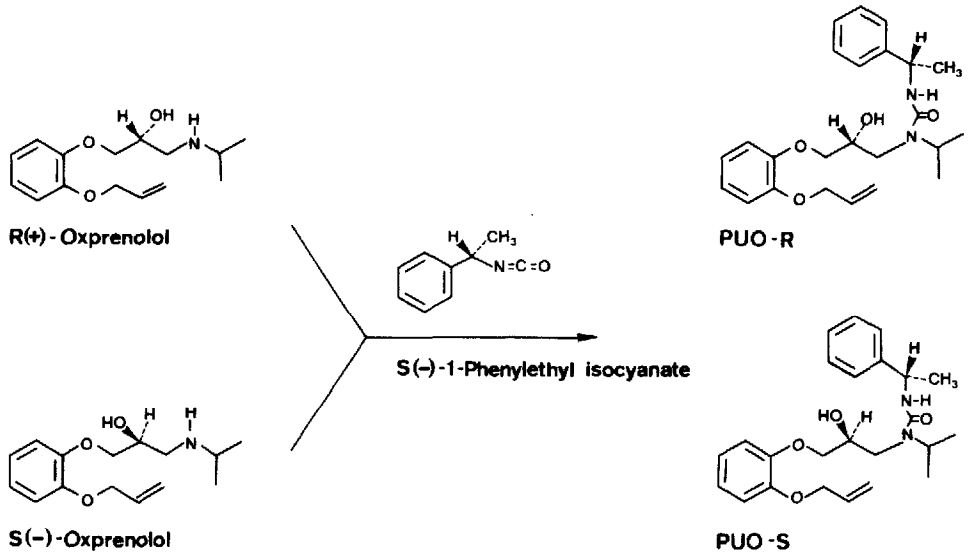

Fig. 1. Reaction of *R*(+)-oxprenolol and *S*(−)-oxprenolol with *S*(−)-1-phenylethyl isocyanate to form the corresponding urea derivatives PUO-R and PUO-S, respectively.

For calibration purposes, the *S*(−)-1-phenylethyl urea derivatives of *R*(+)-oxprenolol (PUO-R) and *S*(−)-oxprenolol (PUO-S) (see Fig. 1) were purified by preparative HPLC using the chromatographic conditions described below. The urea derivative of *R*(+)-oxprenolol was crystallized (m.p. 95–99°C) from ethanol–*n*-heptane (2:1). The derivative of *S*(−)-oxprenolol was only obtained as an oily residue. Elemental analyses: PUO-R ($C_{24}H_{32}N_2O_4$) calculated, C 69.88, H 7.82, N 6.79%; found, C 70.00, H 7.80, N 6.66%; PUO-S ($C_{24}H_{32}N_2O_4$) calculated, C 69.88, H 7.82, N 6.79%; found, C 70.15, H 7.84, N 6.84%.

Chromatographic conditions

The diastereoisomeric urea derivatives of *R*(+)- and *S*(−)-oxprenolol were separated by 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). For injection, a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) was used. The mobile phase (1,2-dichloroethane–*n*-heptane–methanol–2-propanol, 95:60:2:1) was pumped through the column at a flow-rate of 4.0 ml/min at room temperature using an Altex Model 110 pump. Peaks 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 the biological fluid (blood, plasma, urine) or water, a solution of *ca.* 2.5 mg of non-labelled racemic oxprenolol hydrochloride 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 amine and derivatization with *S*(−)-1-phenylethyl isocyanate as described above, the diastereoisomeric urea derivatives of oxprenolol were dissolved in 0.1 ml of the solvent system and chromatographed. The eluate

fractions corresponding to the integrated peak fractions of each of the two diastereoisomers were collected directly in counting vials. About 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.

Calibration of the HPLC method

Replicate samples containing various amounts of synthetic PUO-R were analysed with the HPLC system. A few determinations were also carried out with PUO-S to confirm the calibration curve obtained with the *R*(+)-derivative. The range of calibration was selected to bracket the expected concentrations of samples containing the carrier substances. Each chromatographic peak was integrated by the integrating recorder.

Recovery of enantiomers from samples spiked with racemic [^{14}C]oxprenolol hydrochloride

Samples of 1 ml water, rat blood, plasma and urine, and dog blood and plasma, were spiked with racemic [^{14}C]oxprenolol hydrochloride (18.9 and 20.1 μmol/l). The spiked samples were analysed by IDA. The results obtained were compared to the amount of racemic [^{14}C]oxprenolol hydrochloride added to each sample to determine the recovery. It was assumed that synthetic [^{14}C]oxprenolol hydrochloride is a 1:1 mixture of ^{14}C-labelled *R*(+)-oxprenolol hydrochloride and *S*(−)-oxprenolol hydrochloride.

Animal experiment

Two male beagle dogs (Tif: CAB[16]), weighing 12.5 and 13.6 kg, were obtained from the Tierfarm Sisseln, Switzerland. The animals were deprived of food for 15 h before and for 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 3 mg/kg. Blood was withdrawn from the foreleg vein 15 and 30 min and 1, 2, 3, 4, 6, 8 and 12 h after dosing. Urine was sampled for 24 h. Heparinized blood samples and urine were stored at −20°C before analysis.

Other methods used

Radiometry was done by liquid scintillation counting. The pattern of urinary metabolites was determined by HPLC. Conjugates of oxprenolol were hydrolysed enzymatically. Details of these methods are described elsewhere[15].

RESULTS AND DISCUSSION

Structure of the diastereoisomeric oxprenolol derivatives

The reaction of the two oxprenolol enantiomers with *S*(−)-1-phenylethyl isocyanate led to the diastereoisomeric urea derivatives PUO-R and PUO-S (see Fig. 1), which was confirmed by elemental analysis (see Experimental) and by mass and nuclear magnetic resonance (NMR) spectroscopy . The mass spectra are consistent with the proposed structure, *m/e* (%): 412 (< 1), M; 394 (1), M − H_2O; 263 (11), M − $O(C_6H_4)OCH_2CHCH_2$; 221 (8), 263 − C_3H_6; 150 (6), $HO(C_6H_4)OCH_2$

$CHCH_2$; 147 (15), $(C_6H_5)CH(CH_3)NCO$; 132 (35), 147 − CH_3; 105 (11), $(C_6H_5)CHCH_3$; 72(100), $CH_2=N^+HCH(CH_3)_2$. As deduced from NMR spectra, acylation of the amino group results in a chemical shift of the NCH_2 signal from 2.8 to 3.36 ppm (in deuterochloroform) for both derivatives. The position of the signal of the C-2 proton remains unchanged. The spectra of the derivatized isomers show three methyl doublets, indicating non-equivalence of the two methyl groups in the isopropylamine. All these findings confirm the formation of N-substituted ureas and not of carbamates which would result from the reaction of the isocyanate with the C-2 carbinol.

Specificity of the HPLC separation

Using the described HPLC system, the two diastereoisomers PUO-R and PUO-S were completely separated, as illustrated in Fig. 2. The analysis of blank samples of blood, plasma and urine from rat and dog under the conditions of the IDA showed that endogenous components do not interfere with the UV detection (see Fig. 2). Also, good coincidence was observed between the radioactivity pattern and the UV-absorption profile, when urine of dogs dosed with [^{14}C]oxprenolol hy-

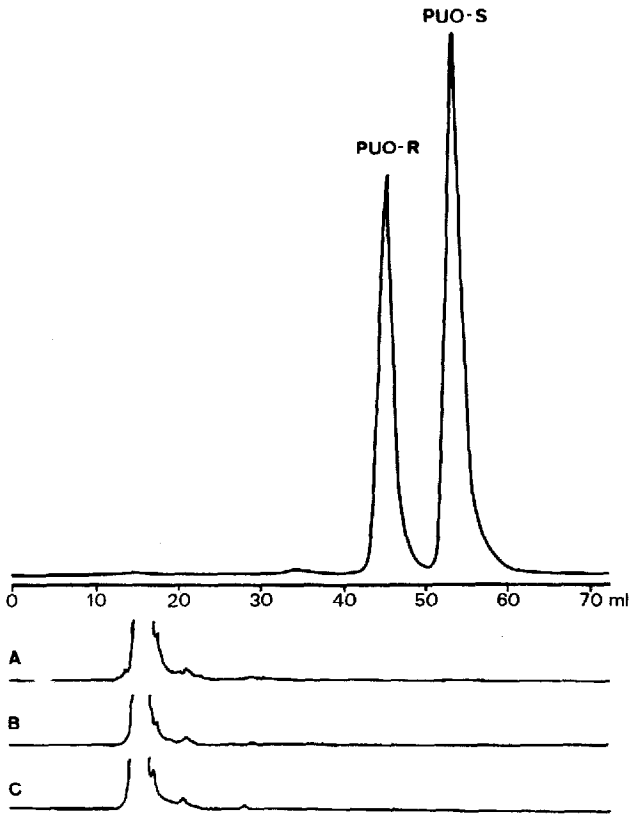

Fig. 2. HPLC separation of the diastereoisomeric urea derivatives of oxprenolol, PUO-R and PUO-S, and chromatograms from blank dog blood (A), plasma (B) and urine (C), as obtained following the IDA procedure. HPLC conditions: column, 10-μm LiChrosorb Si 60 (25 × 1.0 cm I.D.); eluent 1,2-dichloroethane–*n*-heptane–methanol–2-propanol (95:60:2:1); flow-rate, 4 ml/min; UV-detection at 275 nm.

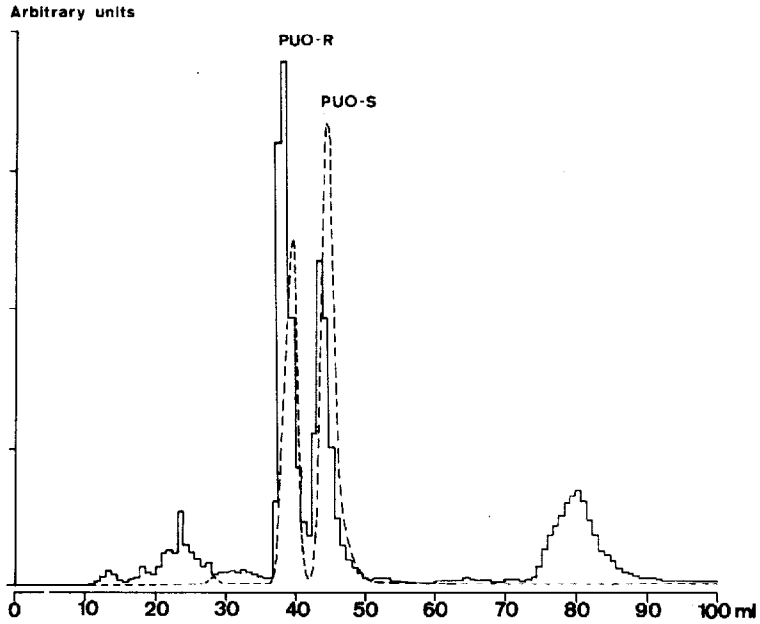

Fig. 3. HPLC separation of the diastereoisomeric urea derivatives of oxprenolol, PUO-R and PUO-S, in IDA from urine of dogs, dosed with racemic [^{14}C]oxprenolol hydrochloride, monitored by radioactivity (————) and UV-detection (– – – –). HPLC conditions as in Fig. 2.

drochloride was enzymatically hydrolysed and subjected to IDA (see Fig. 3). This indicates that metabolites of oxprenolol do not interfere with the HPLC separation.

Calibration and reproducibility of the spectrophotometric determination

A linear calibration curve was obtained for PUO-R in the range 600–1300 μg. Some additional calibration points with PUO-S demonstrated that both diastereoisomers possess the same spectrophotometric response. The reproducibility of the method, expressed as the coefficient of variation (C.V.), was ± 2.8% (n = 17).

Accuracy of the IDA method

Water and biological samples from rat and dog were spiked with 18.9 and 20.1 μmol/l of racemic [^{14}C]oxprenolol hydrochloride (specific radioactivity 3.04 and 11.67 μCi/mg) and analysed by the IDA assay. The recoveries for R(+)-oxprenolol hydrochloride had means ± C.V. of 99.2 ± 2.5% (n = 6) in water, 99.3 ± 2.3% (n = 7) in blood, 99.1 ± 1.9% (n = 7) in plasma and 97.9 ± 1.8% (n = 5) in urine. The corresponding values for S(−)-oxprenolol hydrochloride in water, blood, plasma and urine were 99.7 ± 3.9%, 98.1 ± 2.4%, 98.6 ± 2.5% and 96.9 ± 1.2%, respectively. The data were calculated with the assumption that synthetic [^{14}C]oxprenolol hydrochloride is a 1:1 mixture of the two enantiomers. In fact, the sum of the individual values of R(+)-oxprenolol hydrochloride and S(−)-oxprenolol hydrochloride resulted in means ± C.V. for the recovery of racemic oxprenolol hydrochloride of 99.5 ± 3.1% (water), 98.7 ± 2.1% (blood), 98.9 ± 2.1% (plasma) and 97.5 ± 1.4% (urine).

The sensitivity of the method would be about 0.015 μmol/l (5 ng/ml) or 0.003 μmol/l (1 ng/ml) using a specific radioactivity of 3 or 11.7 μCi/mg, respectively, and a 1-ml biological sample.

Concentration of $R(+)$- and $S(-)$-oxprenolol hydrochloride in blood and their excretion in urine of dogs dosed with [^{14}C]oxprenolol hydrochloride

A pilot study on two dogs was carried out to demonstrate the applicability of the method. The dogs received an oral dose of 3 mg/kg of racemic [^{14}C]oxprenolol hydrochloride. Blood and urine samples from each individual animal were analysed before and after hydrolysis with β-glucuronidase/arylsulphatase. In blood the concentrations of the free optical isomers reached values close to the limit of detection. Therefore, only the blood level profiles obtained after enzymatic hydrolysis are depicted in Fig. 4. The amount of the free and conjugated oxprenolol enantiomers excreted in 0–24 h urine are shown in Table I.

The results show that the amount of conjugated $R(+)$-oxprenolol exceeded that of $S)-)$-oxprenolol by a factor of about 1.5 This is evident from both the blood level profiles and the urinary excretion. On the other hand, the free enantiomers were found in equal amounts in urine.

Determination of $R(+)$- and $S(-)$-oxprenolol together with various metabolites

In the preceding paper[15] a multiple inverse isotope dilution assay for oxprenolol and nine of its metabolites was reported. When this method is combined with the IDA described in the present study, it is possible to measure simultaneously the oxprenolol enantiomers and the individual metabolites in the same biological sample.

After the HPLC separation in the IDA previously reported[15], the unresolved peak of the enantiomers of oxprenolol is collected. The enantiomers are reacted with $S(-)$-1-phenylethyl isocyanate and the diastereoisomeric urea derivatives formed are submitted to the further steps of the assay described herein.

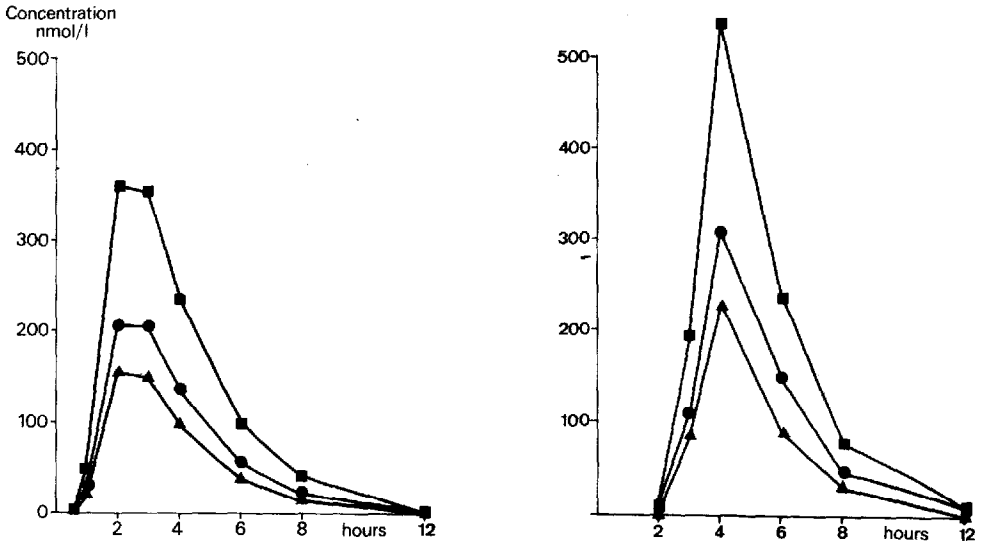

Fig. 4. Blood concentration profiles, obtained after enzymatic hydrolysis, of $R(+)$-(●) and $S(-)$-oxprenolol hydrochloride (▲) and the sum of both (total oxprenolol hydrochloride, ■) in two dogs after oral dosage of 3 mg/kg [^{14}C]oxprenolol hydrochloride.

TABLE I

EXCRETION OF *R*(+)- AND *S*(−)-OXPRENOLOL IN URINE OF TWO DOGS 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.

Compound	*% of total ^{14}C-labelled substances excreted in urine*			
	Before enzymatic hydrolysis		*After enzymatic hydrolysis*	
	Dog a	*Dog b*	*Dog a*	*Dog b*
R (+)-Oxprenolol hydrochloride	0.2	0.6	3.4	5.6
S (−)-Oxprenolol hydrochloride	0.2	0.7	2.5	3.8
Racemic oxprenolol hydrochloride	0.4	1.3	5.9	9.4
Σ[*R*(+) plus *S* (−)]				

CONCLUSION

The inverse isotope dilution assay described allows one to measure the enantiomers of oxprenolol in biological fluids with high specificity, accuracy and reproducibility. The present assay can be combined with the multiple isotope dilution technique for oxprenolol and nine of its metabolites[15].

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