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Percentages of the Deuterium Retained After *para*-Hydroxylation of (*R*) (+) 4-^{2}H-Phenytoin and (*S*) (−) 4-^{2}H-Phenytoin in Rat

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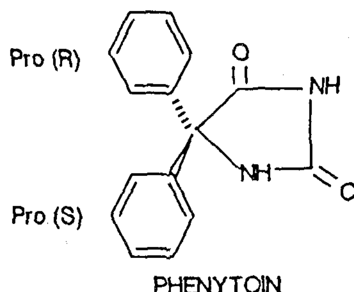
Abstract☐ (*R*) (+) and (*S*) (−) 4-^{2}H-phenytoin have been used as substrates for the determination of the percentage of deuterium retention (NIH shift) after *para*-hydroxylation of the substrates in rat. By using GC-MS analyses, the percentages of deuterium retention were found to be 69% and 70% for the (*R*) and (*S*) phenyl rings, respectively. *The results add additional evidence for the involvement of arene oxide in the oxidation* of the *pro* (*R*) and *pro* (*S*) phenyls of phenytoin. The oxidation process of each ring could be mediated by independent enzyme systems, a rapid oxidative enzyme for the *pro* (*S*) phenyl and a slow oxidative enzyme for the *pro* (*R*) phenyl.

Keywords☐ Phenytoin, metabolism, NIH shift, GC-MS analysis.

Racemic monodeuterated phenytoin, (*R,S*) 5 (4-^{2}H-phenyl)-5-phenylhydantoin, has been used to determine the importance of arene oxide-NIH shift during the metabolic conversion of phenytoin (5,5-diphenylhydantoin) to its major metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-HPPH) in rat and man[1-4]. However, values of deuterium retention reported (68-72%)[1] are global for what is occurring in both the *pro* (*R*) and *pro* (*S*) phenyl rings of phenytoin.



PHENYTOIN

The stereoselectivity studies on the *para*-hydroxylation of phenytoin revealed that the enantiomeric composition of 4-HPPH isolated from urine of rats pretreated with phenobarbitone composed of 73:27 (*S/R*) ratio of enantiomers[5], while 4-HPPH isolated from rats not similarly treated was 89:11 (*S/R*)[6]. The preferential formation of the (*S*) isomer of 4-HPPH, (*S*) 4-HPPH, over the (*R*) isomer, (*R*) 4-HPPH, has been discussed elsewhere[5], but no experimental clarification has been reported. Because the stereoselective formation of (*S*) 4-HPPH could probably be due to difference in the mechanism of hydroxylation of the *pro* (*S*) and *pro* (*R*) phenyls, thus, and with the objective of giving an explanation for the preferential hydroxylation of the *pro* (*S*) over the *pro* (*R*) phenyl ring, it was planned to determine the importance of the arene oxide-NIH shift pathway, separately, on each of the two phenyls of phenytoin during 4-HPPH formation. This has been achieved by the use of the (*S*) and (*R*) enantiomers of 4-^{2}H-phenytoin as substrates in rat for the estimation of the percentage of deuterium retained after *para*-hydroxylation of these substrates.

EXPERIMENTAL METHODS

Materials

(*R*) (+) 4-^{2}H-phenytoin, (*R*) (+) 5-(4-^{2}H-phenyl)-5-phenylhydantoin (98% ^{2}H content) and (*S*) (−) 4-^{2}H-phenytoin, (*S*) (−) 5-(4-^{2}H-phenyl)-5-phenylhydantoin (96% ^{2}H content) were prepared by the se-

paration of the two enantiomers of 4-HPPH by the use of brucine and by application of the method cited in lit[7], followed by formation of the phenyltetrazole ethers of (*R*) (+) 4-HPPH and (*S*) (−) 4-HPPH, which were then subjeeted to deuterolysis as described in lit[8], to afford the (*R*) (+) and (*S*) (−) 4-^{2}H-phenytoin, respectively. Authentic 4-HPPH was synthesized from 4-methoxybenzophenone by the method of Melton and Henze[9]. 5-(4-Hydroxyphenyl)-5-(1,2,3,4,5-pentadeuterio-phenyl)hydantoin ($^2H_5$-4′-HPPH, 90% ^{2}H content) was prepared by the method cited in lit[8]. β-Glucuronidase was obtained from Sigma Chemical Co., St. Louis, USA.

Instrumentatoin

Gas chromatography-mass spectrometer (GC-MS), LKB 9000S, equipped with 8 ft. 3% OV_1-column operated at 210℃, and a flow rate 25 ml/min. The on-column methylation technique was applied for the analysis of the *para*-hydroxy metabolites using trimethylanilinium hydroxide (TMAH) as methylating agent. The mass spectra were recorded at 70 eV

Metabolic studies

Two groups of albino rats (each of 5 rats weighing 150-200g) were used. Group A was used for the metabolic study of (*R*) (+) 4-^{2}H-phenytoin and group B was used for the metabolic study of the (*S*) enantiomer. Each animal was injected with a single intraperitoneal dose of 50 mg/kg of the deuterated substrate as a solution in DMSO/propylene glycol (1:4). Urine was then collected and pooled together, separately, of each rat during 24 hours (10-15 m*l*). To the urine sample of each rat, 50 μg/m*l* of the internal standard $^2H_5$-4′-HPPH was added. After adjustment with acetate buffer to pH 5, each urine sample was subjected to enzymatic deconjugation with β-glucuronidase (1000 units/m*l*) at 37℃ for 40 hours. Urine was then centrifuged and extracted twice with ethyl acetate (20 m*l* each). The organic extract was then concentrated under reduced pressure to 2 m*l*. The *para*-hydroxy metaboiite of the deuterated substrate together with the internal standard were separated on preparative silica gel thin layer chromatographic plates using $CHCl_3$:CH_3OH:CH_3COOH (90:10:1, v/v) as a developing system. The R_f value of 4-HPPH and the internal standard, $^2H_5$-4′-HPPH, was 0.8. The separated *para*-hydroxy metabolites together with the internal standard were extracted from silica gel using methanol, and the extract was divided into two aliquot portions, one for the GC-MS analysis of (*R*) or (*S*) 4-HPPH before acid treatment and the other for (*R*) or (*S*) 4-HPPH after acid treatment (Schemes 1 & 2).

Analysis of (R) or (S) 4-HPPH before acid treatment [(R) or (S) 4-HPPH]

TLC extract was evaporated to dryness and equal volumes of TMAH and methanol (20 μ*l*) were added to the residue and 1 μ*l* of the solution was injected into the GC-MS unit 7 times. The peak height ratio of the molecular ions of trimethyl (*R*) or (*S*) 4-HPPH (*m/z* 310) and trimethyl-$^2H_5$-4′-HPPH (*m/z* 315) were recorded.

Analysis of (R) or (S) 4-HPPH after acid treatment [(R) or (S) 4-HPPH]H⁺

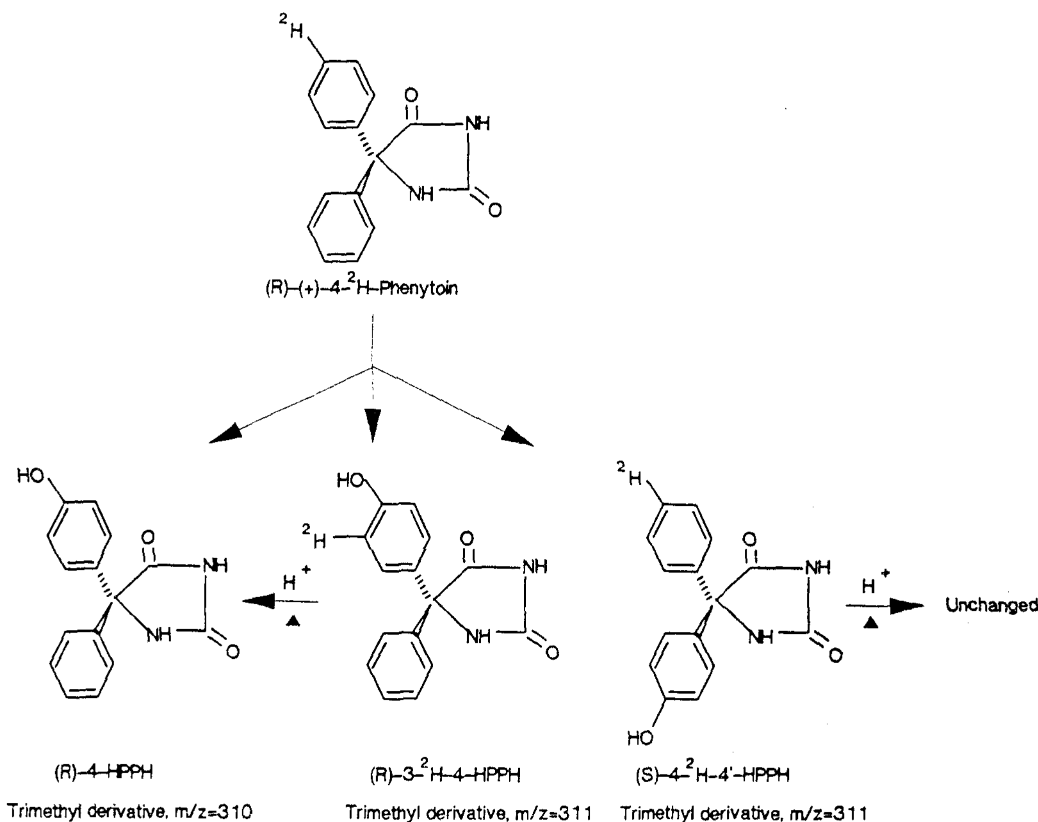
Hydrochloric acid (2 m*l*) was added to the second aliquot portion and the solution was heated under reflux for 3 hours. The solution was evaporated under reduced pressure and the residue was treated with TMAH and methanol (30 μ*l*) and then subjected to GC-MS analysis as mentioned before. The peak height ratio of (*m/z* 310) and (*m/z* 315) were determined.

GC-MS calibration curve for 4-HPPH against $^2H_5$-4′-HPPH

A calibration curve relating the peak height ratio of trimethyl-4-HPPH (*m/z* 310) and trimethyl-$^2H_5$-4′-HPPH (*m/z* 315) was produced. 10 μ*l* of $^2H_5$-4′-HPPH (stock solution of 80 mg/100 m*l* methanol) was added to 6, 12, 20, 40, 80 and 100 μ*l* of 4-HPPH (stock solution of 40 mg/100 m*l* methanol). The peak height ratio of *m/z* 310/ *m/z* 315 was determined by GC-MS analysis for each concentration and a calibration curve was produced. Parameters and standard deviations of the regression line were ($y = bx + a$), $b = 0.091 \pm 0.003$ and $a = 0.002 \pm 0.031$.

RESULTS

In order to determine the deuterium retention value (NHI shift) which occurs during *para*-hydroxylation of the *pro* (*R*) and *pro* (*S*) phenyls of phenytoin, (*R*) (+) and (*S*) (−) 4-^{2}H-phenytoin were used as substrates. As described in Scheme 1 and 2, it is clear that in each case, there are three possible *para*-hy-



Scheme 1. Arene oxide-NIH shift pathway for the *para*-hydroxylation of (*R*) (+) 4-^{2}H-Phenytoin.

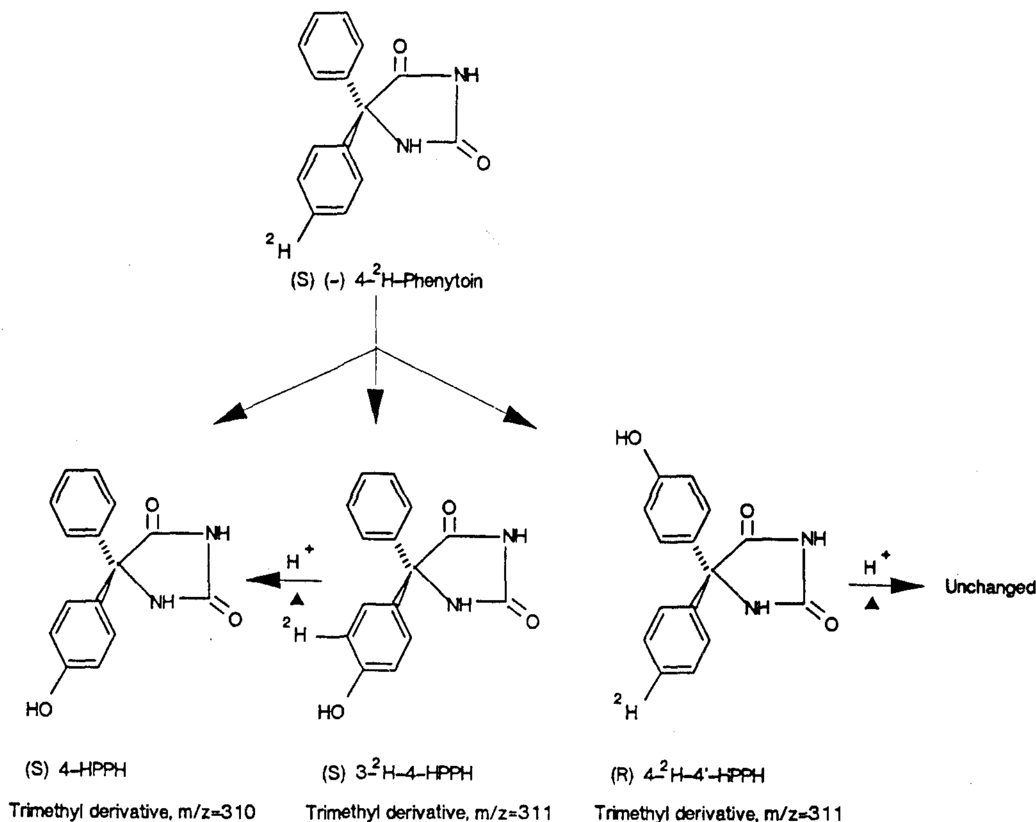
droxy metabolites as a result of *para*-hydroxylation of the substrates; assuming that the oxidation process proceeds predominantly *via* an intermediate arene oxide. In Scheme 1 and 2, the metabolite (*R*) or (*S*) 3-^{2}H-4-HPPH is that one on which deuterium is retained as the result of the NIH shift. Consequently, the percentage of deuterium retained can theoretically be presented *e.g.* in the case of the (*R*) isomer, as follows:

$$\% \text{ of } {}^2\text{H retention} = \frac{(R)\text{-3-}{}^2\text{H-4-HPPH}}{(R)\text{-3-}{}^2\text{H-4-HPPH} + (R)\text{-4-HPPH}} \times 100 \quad (1)$$

It is experimentally proved[1] that deuterium is liable to exchange with proton when it occupies an activated position as in case of (*R*) or (*S*) 3-^{2}H-4-HPPH while it is highly stable when not activated as in (*R*) or (*S*) 4-^{2}H-4'-HPPH (Scheme 1 and 2) Based on these facts, the percentages of deuterium retention can practically be determined by GC-MS analyses of the chromatographically separated metabolites before and after acid treatment as discussed in the experimental part. The peak height of the molecular ion of the trimethyl (*R*) or (*S*)-4 HPPH (*m/z* 310) as in Scheme 1 or 2, respectively, is measured before and after acid treatment relative to the peak height of the molecular ion of $^2H_5$-4'-HPPH (trimethyl derivative, *m/z* 315) as an internal standard, Thus, equation (1) could be rewritten as follows:

$$\% \text{ of } {}^2\text{H retention} = \frac{[(R)\text{ 4-HPPH}]\text{H}^{+} \text{- } [(R)\text{ 4-HPPH}]\text{O}}{[(R)\text{ 4-HPPH}]\text{H}^{+}} \times 100 \quad (2)$$

From the peak height ratio of *m/z* 310/ *m/z* 315 measured from the GC-MS analyses before and after acid treatment, equation (2) could be replaced by equation (3):



Scheme 2. Arene oxide-NIH shift pathway for the *para*-hydroxylation of (*S*) (−) 4-^{2}H-Phenytoin.

Table I. Percentage of deuterium retention after *para*-hydroxylation of (*R*) (+) and (*S*) (−) 4-^{2}H-phenytoin in rat

Subject No.	(*R*) (+) 4-^{2}H-Phenytoin*	(*S*) (−) 4-^{2}H Phenytoin*
1	66 ±0.2	69±0.2
2	70 ±0.1	72±0.1
3	72 ±0.1	71±0.2
4	68 ±0.2	68±0.1
5	70 ±0.2	70±0.2
Mean± SEM	69.2±1.0	70±0.3

*Values are expressed as a mean of seven measurements± S.E.

% of ^{2}H retention=

$$\frac{[m/z\ 310/\ m/z\ 315]\mathrm{H}^+ - [m/z\ 310/\ m/z\ 315]\mathrm{O}}{[m/z\ 310/\ m/z\ 315]\mathrm{H}^+} \times 100 \qquad (3)$$

It should be noted that a correction factor for the isotopic purities of the substrates should be applied for the peak height of *m/z* 310 in GC-MS analysis. This factor is applied only on the denominator of equation (3) and is obtained from X/(X+2Y), where, X=% isotopic purity of the substrate and Y=100−X. The correction factor is 0.96 and 0.92 for (*R*) and (*S*) enantiomers of deuterated phenytoin, respectively.

The percentage of deuteriun retention calculated after *para*-hydroxylation of (*R*) (+) and (*S*) (−) 4-^{2}H-phenytoin in each group of rats are presented in Table I.

DISCUSSION

The arene oxide is the presumptive intermediate in the oxidation of phenytoin as reflected by dihydrodiol formation[5] and indirectly by the occurrence of NIH shift[1,8] The magnitude of NIH shift represents a minimal estimate for the role of arene oxide. The values of deuterium retention, 69% on the (*R*) and 70% on the (*S*) enantiomer, obtained by the GC-MS analyses of the metabolites of the (*R*) and

(*S*) isomers of *para*-deuterated phenytoin indicate clearly that both prochiral phenyl rings are subjected to the same oxidative mechanism. Moreover, the values of deuterium retention obtained in the present investigation are very close to those obtained with racemic phenytoin (68-72%)[1].

On the basis of the obtained results, it is safe to assume that the preferential formation of (*S*) 4-HPPH is not due to difference in the oxidative mechanism between the *pro* (*R*) and *pro* (*S*) phenyls and both rings are subjected to the same oxidative mechanism. In addition, the results add more evidence for the role of arene oxide as a sole pathway for phenytoin hydroxylation. Maguire *et al.*[5] proposed that the minor formation of (*R*) 4-HPPH could be due to different fate of the arene oxide formed on the *pro* (*R*) phenyl; it could be involved with covalent binding to macromolecules, conjugation with glutathione, etc., rather than isomerization to (*R*) 4-HPPH, or alternatively, it could be that (*R*) 4-HPPH is formed largely by a direct hydroxylation mechanism. A recent report of Moustafa *et al.*[10] and other previous results[11] had excluded the involvement of a direct hydroxylation process in the oxidation of phenytoin.

Differential metabolism of drug enantiomers containing chiral centers such as mephenytoin[12], methadone[13], hexobarbital[14] and warfarin[15,16] had been reported. The difference in the metabolic behaviour of the (*R*) and (*S*) enantiomers of warfarin has been explained on the basis of probable interaction of the two enantiomers with two different forms of cytochrome P-450[16].

On the light of the above demonstration and the results obtained in this investigation, we could conclude that both *pro* (*R*) and *pro* (*S*) phenyls of phenytoin are hydroxylated *via* the same oxidative mechanism. However, the hydroxylation process might be mediated by two different enzyme systems, a rapid oxidative enzyme for the *pro* (*S*) phenyl ring and a slow oxidative enzyme for the *pro* (*R*) phenyl nucleus. We could also assume that the slow oxidative enzyme is more sensitive to induction because pretreatment of rats with phenobarbitone alters the enantiomeric composition of 4-HPPH from 89:11[6] to 73:23[5] (*S*/*R*); that is in the favour of the (*R*) 4-HPPH formation.

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