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DIRECT ENANTIOMERIC RESOLUTION OF MEPHENYTOIN AND ITS N-DEMETHYLATED METABOLITE IN PLASMA AND BLOOD USING CHIRAL CAPILLARY GAS CHROMATOGRAPHY

P.J. WEDLUND, B.J. SWEETMAN, C.B. McALLISTER, R.A. BRANCH and G.R. WILKINSON*

Department of Pharmacology, Vanderbilt University, Nashville, TN 37232 (U.S.A.)

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SUMMARY

A gas chromatographic method was developed for the determination of the *R*- and *S*-enantiomers of the anticonvulsant, mephenytoin, and its N-demethylated metabolite, 5-phenyl-5-ethylhydantoin (PEH), in plasma and blood. Direct enantiomeric separation of mephenytoin and its internal standard was obtained using a chiral capillary column (Chirasil-Val®) followed by nitrogen specific detection. However, resolution of the enantiomers of PEH and its internal standard required propylation at the 3-position of the hydantoin ring prior to analysis. Similar linear and reproducible standard curves were obtained from both plasma and blood over the concentration range 50 ng/ml to 5 μg/ml, and above 100 ng/ml the reproducibility was less than 8% (coefficient of variation).

Pronounced stereoselective differences in the plasma concentration—time curves for both mephenytoin and PEH were observed in a normal subject who received a single oral dose of 300 mg racemic mephenytoin. The peak plasma level of *S*-mephenytoin was only one-fifth that of the *R*-enantiomer and its elimination half-life was less than 3 h compared to over 70 h for *R*-mephenytoin. Similarly, *S*-PEH levels were barely detectable whereas concentrations of *R*-metabolite steadily increased over 4—6 days before slowly declining.

INTRODUCTION

Molecular interactions in biological systems frequently exhibit stereoselectivity of which drug action is a particular example. Numerous examples exist where optical enantiomers exhibit qualitatively and quantitatively different pharmacological activities as a result of differences in receptor interactions and/or drug disposition within the body. For example, the metabolism of the anticonvulsant, mephenytoin, has been shown to be stereoselective in both the dog [1] and in the majority of humans [2]. In the latter case, the hydroxyla-

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tion of the S-(+) enantiomer appears to be almost stereospecific [2]. The degree of stereoselectivity in drug disposition processes may be assessed by separately studying each enantiomer in vivo; however, this cumbersome approach neglects the possibility that one of the isomers may have an effect on the other. The latter is an important consideration since the majority of chiral drugs are clinically evaluated and marketed as racemic mixtures. Accordingly, stereoselective drug disposition is best and most directly studied by an analytical technique which resolves the two enantiomers following administration of the racemate.

The use of a pseudoracemate consisting of differentially labelled enantiomers based on either radioactive [2] or heavy elements [3] has been successfully used, but this elegant approach often has significant limitations and is not too well suited to the usual therapeutic situation, where unlabelled drug is administered. For example, the earlier studies with mephenytoin [2] were based on urinary excretion profiles using radiolabelled enantiomers, the specific activities of which were too low to permit measurements of the circulating plasma concentrations of the compounds of interest. Gas—liquid chromatography has been successfully applied to the enantiomeric resolution problem using two approaches. The first is dependent on derivatization with a pure enantiomer of an optically active reagent and separation of the resulting diastereoisomers on a non-chiral stationary phase [4—8]. Alternatively, direct separation of the enantiomers on a chiral column is possible [9—14]. The second approach is obviously more desirable because of its analytical simplicity, and the recently developed chiral phases based on polysiloxanes with covalently bound amino acid or peptide groups [15—17] potentially offer several advantages over more established methods [9—14]. Accordingly, the suitability of this type of phase, specifically Chirasil-Val®, to separate the enantiomers of mephenytoin and its demethylated metabolite was investigated, and an analytical procedure was developed to routinely determine these compounds in biological fluids, including blood and plasma.

EXPERIMENTAL

Chemicals

The purified enantiomers of mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin) and 5-phenyl-5-ethylhydantoin (PEH) were kindly supplied by Dr. Adrian Küpfer (University of Bern, Bern, Switzerland). Racemic mephenytoin was a gift from Sandoz Pharmaceuticals (Hanover, NJ, U.S.A.). Propiophenone, butyrophenone, isobutyrophenone, 5-phenyl-5-methylhydantoin and hydantoin were obtained from Aldrich (Milwaukee, WI, U.S.A.). Iodomethane, iodoethane, 1-iodopropane, 2-iodopropane, 1-iodobutane and 2-iodo-2-methylbutane were purchased from Eastman Kodak (Rochester, NY, U.S.A.). Glass-distilled methanol and dichloroethane were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All chemicals and reagents were used without further purification.

Synthesis

The syntheses of racemic PEH, 5-phenyl-5-propylhydantoin and 5-phenyl-5-

isopropylhydantoin were carried out according to the methods of Henze and Isbell [18] utilizing propiophenone, butyrophenone and isobutyrophenone, respectively, as starting material. Alkylation of the hydantoins at the 3-position was achieved by dissolving the hydantoin in 0.1 *M* sodium hydroxide in methanol—iodoalkane (4:1) and stirring the mixture at 50°C for 24 h, except for the reaction with iodomethane which was carried out at room temperature. Alkylation was stopped by adding 4 parts 0.1 *M* hydrochloric acid and extracting the final product into 12 parts dichloroethane. The organic layer was then washed twice with an equal volume of aqueous 0.1 *M* sodium hydroxide to remove any unreacted hydantoin. The dichloroethane layer was then evaporated to dryness at room temperature under nitrogen and the resulting residue was utilized without further purification.

Possible internal standard for mephenytoin and its N-demethylated metabolite, PEH, were examined by synthesizing the methyl through butyl series of alkylated derivatives of 5-phenyl-5-methyl-, 5-phenyl-5-ethyl-, 5-phenyl-5-propyl-, and 5-phenyl-5-isopropylhydantoins, respectively. Solutions of the selected compounds, 3-methyl-5-phenyl-5-isopropylhydantoin (3MIPPH) and 5-phenyl-5-propylhydantoin (PPH), were prepared in ethyl acetate to provide concentrations of 8 and 17 μg/ml, respectively.

Analytical procedure

Internal standard solutions (0.1 ml each) were added to 1 ml of plasma or blood which was then acidified with 1 ml of 0.01 *M* acetic acid and extracted with 6 ml of dichloromethane by reciprocal shaking. Following centrifugation, the supernatant was removed by aspiration and the organic phase transferred to a 5-ml Reacti-Vial™ (Pierce Chemical, Rockford, IL, U.S.A.) and evaporated to dryness under nitrogen at room temperature. The residue was dissolved in 1 ml of 4:1 solution of 0.1 *M* sodium hydroxide in methanol—iodopropane and then incubated in a water bath at 50°C for 18 h. Derivatization was stopped by addition of 1 ml of 0.1 *M* hydrochloric acid and 3 ml of dichloroethane were added to each vial. Following extraction as described previously, the organic phase was transferred to a 1-ml Reacti-Vial with a PTFE-lined septum, evaporated to dryness under nitrogen at room temperature, and the residue dissolved in 10 μl of ethyl acetate. A 0.5—1.0 μl aliquot of the resulting solution was chromatographed on a Varian 2100 gas chromatograph modified for capillary use and with a thermionic nitrogen—phosphorus specific detector. The column was a 25 m × 0.25 mm I.D. Chirasil-Val glass capillary (Applied Science, Deerfield, IL, U.S.A.) with the following conditions: injector temperature, 225°C; oven temperature 165°C, detector temperature, 300°C; helium flow-rate, 1.2 ml/min with a 20:1 split ratio; make-up, 2.5 ml/min; hydrogen flow-rate, 3.5 ml/min; and air flow-rate, 175 ml/min.

RESULTS AND DISCUSSION

Preliminary studies showed that separation of hydantoin enantiomers on the Chirasil-Val column was only possible if there was a 3-alkyl group located on the hydantoin ring; in the absence of such a group or when the 1,3-dialkyl

derivatives were chromatographed, enantiomeric resolution was lost. Accordingly, a mild and selective procedure was developed for converting the N-demethylated metabolite, PEH, and its internal standard, PPH, to their 3-propyl derivatives. Since mephenytoin was unaffected by this procedure, it was considered important to use a separate internal standard with a similar characteristic. Of the various hydantoin derivatives which were synthesized and examined, most were either poorly resolved or had retention times that overlapped with the other compounds of interest. Only 3MIPPH was found to have suitable chromatographic properties.

Under the described conditions, excellent chromatographic separations were obtained between the different compounds of interest and their enantiomers (Fig. 1) and no interfering peaks were observed from either plasma or blood. The *S*-enantiomers eluted earlier than the corresponding *R*-enantiomers with almost baseline separation between the two, and the respective retention times (*S*/*R*) were: mephenytoin, 19.2/19.9 min; 3MIPPH, 23.5/25.6 min; PEH, 28.8/30.1; and PPH, 40.3/42.7 min. Standard curves for the mephenytoin and PEH exhibited good linearity between peak height ratio of the enantiomer to the appropriate internal standard and plasma concentrations over the range 50 ng/ml to 5 μg/ml (Fig. 2). The curves were reproducible in that the coefficient of variation of the slopes was less than 5% for the mephenytoin enantiomers and 13% for those of PEH (n = 8), and the lowest measurable concentration was about 50 ng/ml. Analysis of blood gave identical results to those with plasma; however, repetitive analyses quickly contaminated the injection port leading to a decrease in peak resolution. Intraday variability in the assay was less than 4.5% for mephenytoin (n = 6—8) over the concentration range 50 ng/ml to 1.0 μg/ml and below 8% for PEH. However, poorer reproducibility (< 19%) was observed with PEH at concentrations below 100 ng/ml. A similar situation was apparent with the interday reproducibility; the

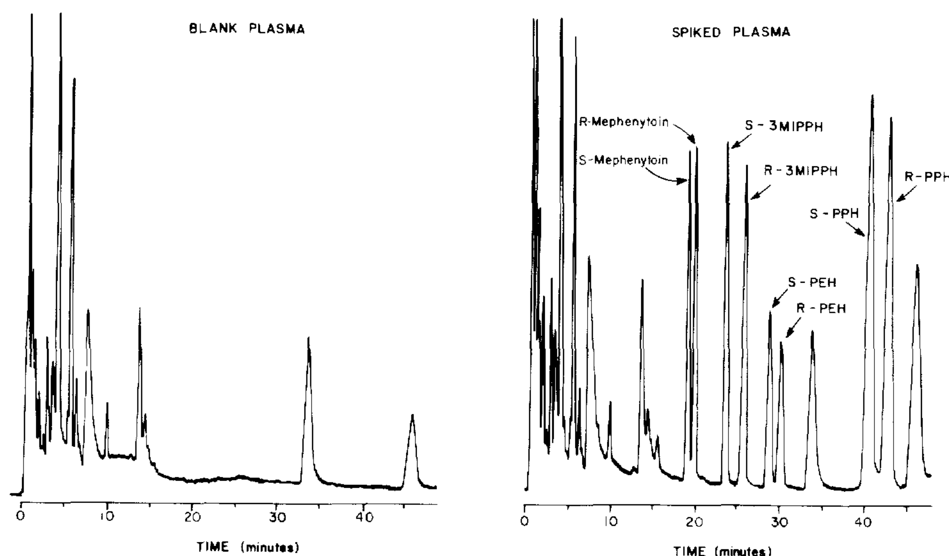

Fig. 1. Separation of the *R*- and *S*-enantiomers of mephenytoin, PEH and their internal standards following extraction from plasma.

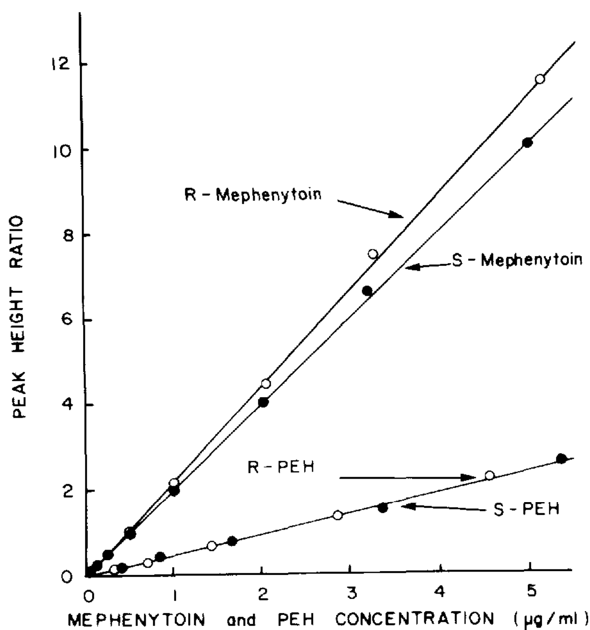

Fig. 2. Standard curves for the *R*- and *S*-enantiomers of mephenytoin and PEH extracted from plasma.

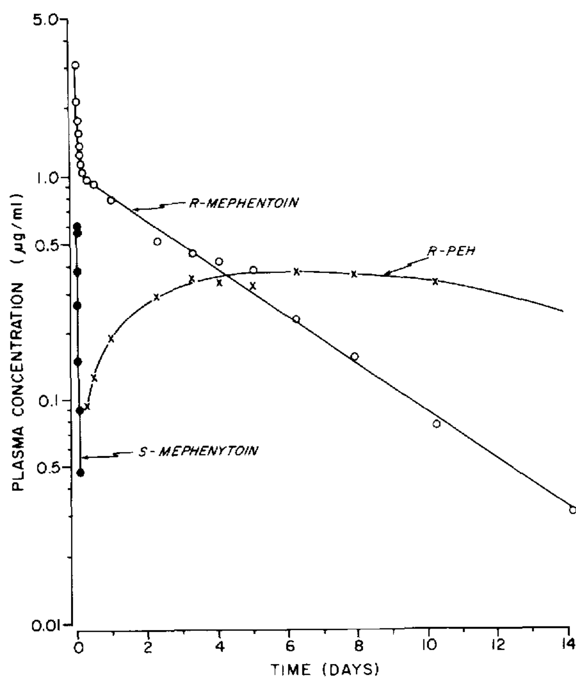

Fig. 3. Plasma concentration—time curves of the *R*- and *S*-enantiomers of mephenytoin and its N-demethylated metabolite (PEH) in a normal subject after receiving a 300-mg oral dose of racemic mephenytoin.

coefficient of variation (n = 8) being less than 8% for both mephenytoin and PEH over the above range except for the lowest concentrations of PEH where only 20% reproducibility was obtainable. The interday reproducibility studies were performed over a 6—8 month period, but no trends were apparent to suggest that either mephenytoin or PEH were unstable in frozen plasma during this time.

In order to examine the clinical applicability of the developed procedure to determine the circulating plasma concentrations of mephenytoin and PEH enantiomers, a normal subject was given a 300-mg oral dose of racemic mephenytoin. This was followed by serial blood sampling over the next two weeks, and the plasma concentrations of the enantiomers were determined (Fig. 3). Pronounced stereoselective differences were apparent in the plasma concentration—time curves for both mephenytoin and PEH. For example, the peak concentration of *R*-mephenytoin was five-fold greater than that of the *S*-enantiomer and the rates of elimination were widely disparate; the half-life of *S*-mephenytoin being less than 3 h compared to over 70 h for the *R*-isomer. Similarly, large enantiomeric differences were present with the N-demethylated metabolite; *S*-PEH concentrations were barely measurable and rapidly fell below the detection limit of the assay whereas *R*-PEH levels steadily increased over 4—6 days to exceed these of *R*-mephenytoin and then declined very slowly. These findings are consistent with earlier studies based on urinary excretion profiles [2] and support the conclusion that the metabolism of *S*-mephenytoin is very rapid and involves 4-hydroxylation followed by conjugation. In contrast, the *R*-enantiomer is mainly N-demethylated at a much slower rate to the pharmacologically active metabolite, *R*-PEH. Since this metabolite is not further metabolized and its renal clearance is small, *R*-PEH accumulates in the plasma and is only slowly removed from the body. Such differences in drug disposition and their clinical consequences clearly demonstrate the need for analytical methodology capable of separating and quantifying the two stereoisomers and their metabolites. The described direct separation procedure using a chiral gas chromatographic column will, therefore, allow investigation of the determinants of mephenytoin's stereoselective metabolism and clinical effectiveness.

Chirasil-Val is synthesized by coupling L-valine, *tert.*-butylamide to a copolymer of dimethylsiloxane and carboxyalkylmethylsiloxane units [15]. It was originally developed for analyzing the optical purity of amino acids derived from synthetic peptides [15, 16]. However, preliminary studies [17] have shown that it is also applicable to the enantiomeric resolution of certain sympathomimetic amines and catecholamines, glycols and α-hydroxycarboxylic acids [17]. The mechanism of separation of enantiomers in the gas phase probably involves formation of hydrogen-bonded diastereomeric association complexes with the stationary phase. The degree of enantiomeric separation appears to be dependent on the sequence and spatial relationship between hydrogen-donating amino groups and hydrogen-accepting carbonyl groups around the chiral center [17]. The resolution of the enantiomers of mephenytoin on this phase was excellent, but as indicated with PEH, the degree of alkylation of the molecule was critical. The ease and simplicity of this direct analytical approach to the important area of stereoselective drug disposi-

tion would suggest that further studies should be pursued to characterize the necessary structural requirements for enantiomeric resolution of other types of compounds using Chirasil-Val or other chiral stationary phases.

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REFERENCES

1 A. Küpfer and J. Bircher, J. Pharmacol. Exp. Ther., 209 (1979) 190.
2 A. Küpfer, R.K. Roberts, S. Schenker and R.A. Branch, J. Pharmacol. Exp. Ther., 218 (1981) 193.
3 T.A. Baillie, Pharmacol. Rev., 33 (1981) 81.
4 B. Halpern and J.W. Westley, Biochem. Biophys. Res. Commun., 19 (1965) 361.
5 E. Gil-Av, R. Charles-Sigler, G. Fischer and D. Nurok, J. Gas Chromatogr., 4 (1966) 51.
6 B.L. Karger, R.L. Stern, W. Keane, B. Halpern and J.W. Westley, Anal. Chem., 39 (1967) 228.
7 C.J.W. Brooks, M.T. Gilbert and J.D. Gilbert, Anal. Chem., 45 (1973) 896.
8 W.A. König, W. Rahn and J. Eyem, J. Chromatogr., 133 (1977) 141.
9 B. Feibush and E. Gil-Av, J. Gas Chromatogr., 5 (1967) 257.
10 B. Feibush and E. Gil-Av, Tetrahedron, 26 (1970) 1361.
11 W. Koenig, W. Parr, H. Lichtenstein, E. Bayer and J. Oró, J. Chromatogr. Sci., 8 (1970) 183.
12 W. Parr, C. Yang, E. Bayer and E. Gil-Av, J. Chromatogr. Sci., 8 (1970) 591.
13 W. Parr, J. Pleterski, C. Yang and E. Bayer, J. Chromatogr. Sci., 9 (1971) 141.
14 J.A. Corbin, J.E. Rhoad and L.B. Rogers, Anal. Chem., 43 (1971) 327.
15 H. Frank, G.J. Nicholson and E. Bayer, Angew. Chem. Int. Ed. Engl., 17 (1978) 363.
16 H. Frank, G.J. Nicholson and E. Bayer, J. Chromatogr., 146 (1978) 197.
17 I. Abe, K. Izumi, S. Kuramoto and S. Musha, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 549.
18 H.R. Henze and A.F. Isbell, J. Amer. Chem. Soc., 76 (1954) 4152.