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ANALYSIS OF HYDROXYLATED AND DEMETHYLATED METABOLITES OF MEPHENYTOIN IN MAN AND LABORATORY ANIMALS USING GAS—LIQUID CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Separation of urinary mephenytoin metabolites was evaluated under various gas—liquid chromatographic (GLC) and high-performance liquid chromatographic (HPLC) conditions. A simple and rapid alkylation procedure is described for GLC using a nitrogen sensitive thermionic detector. The in situ formation of sodium methinylsulfinylmethide is used as base for the perpropylation of hydantoins and their metabolites. Normal-and reversed-phase HPLC of the underivatized compounds was performed using four different types of stationary phases. None of the GLC systems separated all the six hydantoin compounds tested, whereas, normal- and reversed-phase HPLC were able to obtain a complete separation of these compounds.

The major metabolites of mephenytoin were 5-phenyl-5-ethylhydantoin, 3-methyl-5-(4-hydroxyphenyl)-5-ethylhydantoin and 5-(4-hydroxyphenyl)-5-ethylhydantoin in man, rat, mouse, rabbit, and guinea pig. 3-Methyl-5-phenyl-5-(2-hydroxyethyl)-hydantoin and 3-methyl-5-(3-hydroxyphenyl)-5-ethylhydantoin are major metabolites in the dog.

INTRODUCTION

The antiepileptic drug, mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin), is methylated at position 3 of the hydantoin ring. The 5-phenyl-5-ethyl substitution makes it a close congener of diphenylhydantoin (DPH). Oxidative demethylation of mephenytoin yields the pharmacologically active metabolite, 5-phenyl-5-ethylhydantoin (PEH, nirvanol) [1, 2]. In man, this metabolite accumulates in the body and contributes to the pharmacological response [3, 4]. In addition, mephenytoin can be hydroxylated to a variety of products [3, 5—8] the predominant one being 3-methyl-5-(4-hydroxyphenyl)-5-ethyl-

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hydantoin (4-OH-M). There is stereoselective formation of this metabolite in man [9] with preferential formation of S-4-OH-M. The hydroxylated metabolites are rapidly excreted largely as glucuronides. Rapid renal excretion of hydroxylated products prevents their accumulation in plasma under normal circumstances [6, 9].

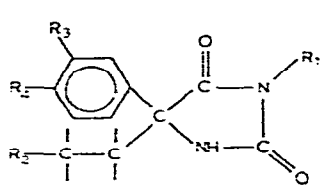
Quantitative assays for the determination of concentrations of mephenytoin, PEH and 4-OH-M by gas—liquid chromatography (GLC) [10—16] and high-performance liquid chromatography (HPLC) [6] have been published in several papers. However, a detailed description of the separation characteristics of major metabolites of mephenytoin using a variety of GLC and HPLC systems has not previously been presented. The objective of this paper is to present the results of our experience obtained while developing analytical methods for this group of compounds.

The thermionic nitrogen-sensitive detector provides a high sensitivity towards N-alkylated drugs and their metabolites [15]. As a consequence, we concentrated on alkylation of compounds to obtain suitable derivatives for GLC separation. In contrast, HPLC did not require derivatization and normal-phase chromatography was compared with reversed-phase chromatography. The assay methods developed have been used to investigate species differences in mephenytoin metabolism. The information presented in this paper should facilitate the selection of the appropriate GLC or HPLC procedure in the future investigation of mephenytoin dispostion in laboratory animals and man.

EXPERIMENTAL

Materials

The syntheses for mephenytoin [9], 4-OH-M [17], PEH, 5-phenyl-5-propylhydantoin (PPH) [17, 18], 5-(4-hydroxyphenyl)-5-ethylhydantoin (4-OH-PEH) [8] and 5-(4-bromphenyl)-5-ethylhydantoin (4-Br-PEH) [9] were carried out according to the references given in square brackets. The 3-OH- and 2-OH-isomers of 4-OH-M were kindly donated by Professor U.P. Schlunegger from the Department of Organic Chemistry at the University of Berne (Berne, Switzerland). 3-Methyl-5-(2-hydroxyethyl)-5-phenylhydantoin (OH-ethyl-M) was obtained from Professor T.M. Harris of the Department of Organic Chemistry at Vanderbilt University (Nashville, TN, U.S.A.) (Fig. 1). Details of synthesis nuclear magnetic resonance (NMR) and gas chromatographic—mass spectrometric (GC—MS) data of OH-ethyl-M will be reported in detail elsewhere. Trimethylanilinium hydroxide 0.1 *N* in methanol (TMA) was prepared as earlier reported [19].



R_1	R_2	R_3	R_4	
CH_3	H	H	H	Mephenytoin
CH_3	H	H	OH	4-OH-M
CH_3	H	OH	H	3-OH-M
CH_3	OH	H	H	OH-ethyl-M
H	H	H	OH	4-OH-PEH
H	H	H	H	PEH, Nirvanol

Fig. 1. Mephenytoin and its major metabolites in various laboratory animals and in man.

1-Iodoethane and 1-iodopropane were obtained from Eastman-Kodak (Rochester, NY, U.S.A.) for ethylation and propylation, respectively. The alkyl halides were redistilled to remove traces of the rapidly alkylating methyl iodide from the derivatization reaction. Dimethylsulfoxide (DMSO, methylsulfoxide) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) and dried over powdered calcium hydride (Fisher Scientific). Sodium hydride (NaH), obtained from Aldrich (Milwaukee, WI, U.S.A.) in a 50% suspension in mineral oil was washed with heptane prior to its use.

Methods

Racemic mephenytoin was administered to rats (60 mg/kg), mice (80 mg/kg), dogs (40 mg/kg), rabbits 80 mg/kg, guinea pigs (80 mg/kg) and man (5 mg/kg) and urine was collected for 24 h with the exception of the dog studies (8 h).

To one ml of urine either 2 μmol of PPH for GLC assay or 2 μmol of PPH, DPH, or 4-Br-PEH for HPLC were added as internal standards. Acid hydrolysis to liberate the aglycone was performed with hydrochloric acid (6 *N* final concentration) at 100°C for 2 h [20]. The hydrolyzed urines were extracted with 5 ml of ethyl acetate and the ethyl acetate was transferred to a dry test tube. The organic solvent was evaporated at 60°C under a stream of nitrogen. The residue was either dissolved in 200 μl of methanol and injected for HPLC or derivatized for GLC.

GLC separation

Compounds were injected in the GLC system underivatized or derivatized by peralkylation by methylation, ethylation, and propylation. On-column methylation, using trimethylanilinium hydroxide (0.1 *N*) in methanol (TMA), was performed as previously reported [19]. Ethylation and propylation were performed at room temperature using a modified Hakamori procedure [21—23], where the DMSO anion is formed in situ by direct addition of NaH to the final reaction mixture containing approximately 50 μg of the dry standard drug, 200 μl of dry DMSO, 50 μl of the iodoalkane and 50 mg of washed sodium hydride suspended in 1 ml of heptane. After 30 min of constant vortexing, the reaction was stopped by addition of 4 ml of water and the derivatives were extracted into 6 ml of diethyl ether. The diethyl ether was evaporated to dryness and after addition of 200 μl of methanol, an aliquot of 4 μl was injected into a perkin-Elmer Model 3920 B gas chromatograph equipped with temperature programmer and a thermionic nitrogen—phosphorous-sensitive detector (NPD, Perkin-Elmer). Stationary phases and temperature conditons are given in Table I. Carrier gas flow-rates and detector gas supply (air and hydrogen) were adjusted according to the manufacturers recommendations. Peak detection and retention times were obtained by a Varian CDS 211 C electronic integrator as well as by a standard pen recorder.

HPLC separation

A Waters Model 6000A solvent delivery system (Waters Assoc. Milford, MA, U.S.A.) equipped with a Waters Model 450 UV/VIS spectrophotometric variable-wavelength detector was used at a flow-rate of 1 ml/min under iso-

TABLE I

RETENTION TIMES (min) OF MEPHENYTOIN AND ITS METABOLITES UNDER VARIOUS DERIVATIZING AND GLC CONDITIONS

Column length and materials used: 1.8 m OV-17 5% on Chromosorb W HP 80—100 mesh (Applied Science Lab., State College, PA, U.S.A.); 1.8 m SP-2100 3% on Supelcoport 100—120 mesh (Supelco, Bellefonte, PA, U.S.A.).

Column	Derivatization	Temperature	MEPH	PEH	PPH	2-OH-M	3-OH-M	OH-ethyl-M	4-OH-M	4-OH-PEH
OV-17	None	220°C isothermal	3.4	6.5	—	—	—	9.2	11.0	—
OV-17	Methylation	200°C isothermal	5.6	5.6	6.6	11.3/13.0	12.9	11.0	16.0	16.0
OV-17	Propylation	230°C isothermal	2.2	3.5	4.1	—	6.6	6.0	8.8	12.3
SP-2100	Ethylation	8 min 180°C, 8°C/min to 220°C	3.6	4.0	—	8.4	9.6	11.6	10.8	11.6
SP-2100	Propylation	8 min 180°C, 8°C/min to 220°C	4.0	6.5	8.0	10.6	11.7	11.2	13.3	15.1

TABLE II

RETENTION TIMES OF UNDERIVATIZED MEPHENYTOIN AND ITS METABOLITES IN NORMAL- AND REVERSED-PHASE HPLC USING VARYING SOLVENT COMPOSITIONS

Column	Mobile phase			Retention time (min)										
				MEPH	PEH	2-OH-M	3-OH-M	OH-Ethyl-M	4-OH-M	4-OH-PEH	PPH	Phenobarbital	DPH	4-BrPEH
Normal-phase HPLC														
μPorasil	3% 2-propanol in heptane			7.5	12.0	9.6	15.3	85.2	28.2	39.9	8.7	8.1	9.3	11.0
Zorbax CN	11% 2-propanol in heptane			6.3	6.5	7.8	10.8	12.6	15.3	15.9	6.0	6.9	6.9	4.8
Reversed-phase HPLC														
	Water	Methanol	Acetonitrile											
Zorbax CN	400	—	100	18.3	10.5	10.5	10.3	7.5	9.0	6.6	—	—	—	—
Zorbax C_8	500	—	100	41.0	16.3	16.2	13.1	10.1	11.2	6.0	41.0	22.0	—	72.0
μBondapak C_{18}	140	100	—	12.0	8.3	—	7.5	7.0	6.5	4.7	12.8	—	—	21.0
μBondapak C_{18}	280	100	—	46.0	23.0	—	19.0	17.0	15.0	10.0	—	—	—	—
μBondapak C_{18}	400	100	—	—	—	—	34.0	30.0	26.4	13.2	—	—	—	—

cratic conditions. The columns used were (a) Waters μPorasil 10 μm particle size 30 cm × 3.9 mm and a Waters μBondapak C_{18} column (30 cm × 3.9 mm). The nitrilo column CN (cyanopropyl—silica) and the C_8 column were both of the Zorbax series (6 μm particle size, 25 cm × 4.6 mm) as commercially supplied by DuPont (Wilmington, DE, U.S.A.). Solvents were glass-distilled as obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Underivatized samples were analyzed after acid hydrolysis. Peak detection was performed at 211 nm according to a previously established UV scan of mephenytoin and PEH in methanol—water (1:1, v/v). Retention times were reported uncorrected according to the peak time obtained by measuring peak location on the strip chart recorder.

RESULTS AND DISCUSSION

Table I summarizes the retention times for mephenytoin, its various metabolites and the internal standard 5-phenyl-5-propylhydantoin (PPH) under various GLC conditions. In their underivatized form, the compounds separated well on the OV-17 column and we used this approach successfully for the structural analysis of urinary metabolites in conjunction with MS [6, 17]. This method had the advantages inherent with the injection of smaller, underivatized molecules with respect to GC—MS identification. However, there was relatively low sensitivity in comparison to alkylated derivatives. Furthermore, a pronounced tailing phenomenon made this procedure inadequate for quantitative metabolite determinations.

On-column methylation (flash-heater methylation) is a simple derivatizing procedure recommended for flame ionization detectors only. However, the TMA reagent is of limited use in connection with the thermionic nitrogen sensitive detector because the excess of TMA present (and its breakdown products) result in a high and prolonged background signal. The utility of this approach is also limited because the permethylated products of mephenytoin and PEH, as well as 4-OH-M and 4-OH-PEH are the same. A separate analysis of these compounds, however, can be achieved by the use of deuterated TMA in combination with MS, where the native and the added methyl groups are distinguishable by their isotopic composition [24].

An additional problem was encountered with the flash-heater methylation of 2-OH-M. This *ortho*-substituted phenolic compound consistently produced two peaks (Table I) of similar peak height ratios. This problem has not been pursued but it seems probable that steric hindrance of the *ortho*-OH-group prevents quantitative methylation, at least under the conditions of flash-heater methylation.

Ethylation derivatization was considered to be unsatisfactory in the separation of compounds as the separation between mephenytoin and PEH was small, and OH-Ethyl-M had the same retention time as 4-OH-M (Table I). In contrast, propylation provided baseline separation for mephenytoin, PEH, 3-OH-M, 4-OH-M, 4-OH-PEH, and internal standard on both OV-17 and SP-2100 columns; however, OH-ethyl-M separation from 3-OH-M was incomplete. Attempts to achieve the total separation of OH-ethyl-M from 3-OH-M was not

undertaken because these two metabolites could only be identified as major metabolites in the dog (see Table III). Thus, derivatization by propylation followed by column separation on either OV-17 and SP-2100 columns provided adequate methods for the development of quantitative assays for studies in other species.

Table II contains the retention times of the underivatized hydantoins in normal- and reversed-phase HPLC with varying qualitative and quantitative solvent compositions. Table II also contains the retention times for phenobarbital and DPH as potential internal standards since PPH was not an appropriate internal standard under all the various conditions presented. In addition, we evaluated 4-Br-PEH because it was used in the synthesis of 3-methyl-5-[^{3}H-phenyl]-5-ethylhydantoin by catalytic tritiation in some of our experiments [9].

For practical purposes, mephenytoin can be omitted in the urinary analysis of samples since less than 2% of the administered dose is excreted in urine within 24 h and 2-OH-M was not identified within the limits of GLC or HPLC detection in any of the species studied (Table III).

TABLE III

MAJOR PRODUCTS OF MEPHENYTOIN METABOLISM IN VARIOUS LABORATORY ANIMALS AND MAN

Species	Oxidative demethylation of mephenytoin	Aromatic hydroxylation to *meta*- or *para*-phenols	Aliphatic hydroxylation of mephenytoin	2-Hydroxy-mephenytoin (2-OH-M)
Man	+ mainly during chronic mephenytoin therapy	*para*	—	—
Dog	+	*meta* and *para*	+	—
Rat	+	*para*	—	—
Mouse	+	*para*	—	—
Guinea pig	+	*para*	—	—
White New Zealand rabbit	+	*para*	—	—

Complete separation of all samples of interest was obtained with normal-phase HPLC using a μPorasil column under isocratic conditions (Table II). However, long collection times were required for 4-OH-PEH and OH-Ethyl-M. The cyanopropyl column (Zorbax CN) in normal-phase chromatography did not fully separate 4-OH-M and 4-OH-PEH but had good resolution for the remaining compounds.

For reversed-phase chromatography, optimal resolution between compounds was obtained with the Zorbax C_8 column (Table II). Mephenytoin, PEH, and metabolites separated within a 17-min retention time. The reversed-phase cyanopropyl (CN) column provided baseline separation of all metabolites except for 3-OH-M and PEH. It was important to use acetonitrile in both C_8 and CN systems, as substitution with methanol yielded substantially lower resolution. Reversed-phase chromatography with the commonly used C_{18} column did not give baseline separation for 3-OH-M, 4-OH-M and OH-ethyl-M.

Resolution was not improved either by increasing the polarity of the solvents because corresponding peak width increased at at higher retention times, or by using acetonitrile instead of methanol. Thus, both normal-phase chromatography with a μPorasil column or reversed-phase chromatography with a C_8 column were adequate for quantitative assay development for all species. The latter system had the advantages of an easy solvent system do use, and a relatively short retention time for all compounds of interest except mephenytoin itself.

A qualitative assessment of urinary metabolites found in a number of species is presented in Table III. Two aspects deserve special attention. Firstly, the dog has a distinctive pattern of aromatic as well as of aliphatic hydroxylation of mephenytoin. In addition to 4-OH-M, the isomeric phenolic metabolite, 3-OH-M, as well as OH-ethyl-M represent major metabolic end products in this species. One of these routes of metabolism is analogous to DPH, where it has been shown, that the *meta*-isomer of the phenolic DPH metabolite is a major urinary constituent in addition to *para*-substituted phenol [25-27]. Secondly, in man, the demethylated metabolite PEH is a minor urinary metabolite in single mephenytoin does studies, but accounts for approximately 30% of the total metabolites present during chronic drug administration [4].

Thus, the requirements of the separation procedure are dependent on the range of metabolic products to be expected and this in turn is dependent on the species under investigation. If the species to be studied is known, then the information provided in this paper can be used to select an appropriate analytical separation procedure to develop a quantitative assay to study the fate of mephenytoin under various experimental conditions.

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REFERENCES

1 T.C. Butler, J. Pharmacol. Exp. Ther., 104 (1952) 299.
2 A.S. Troupin, P. Friel, M.P. Lovely and A.J. Wilensky, Ann. Neurol., 6 (1979) 410.
3 A.S. Troupin, L. Moretti-Ojemann and C.B. Dodrill, Epilepsia, 17 (1976) 403.
4 A. Küpfer, R.K. Roberts, J.F. Gerkens, S. Schenker and R.A. Branch in: R. Preisig and J. Bircher (Editors), The Liver, Quantitative Aspects of Structure and Function, Editio Cantor, Aulendorf, G.F.R., 1979.
5 R.K. Lynn, J.E. Bauer, W.P. Gordon, R.G. Smith, D. Griffin, R.M. Thompson, R. Jenkins and N. Gerber, Drug Metab. Dispos., 7 (1979) 138.
6 A. Küpfer, G.M. Brilis, J.T. Watson and T.M. Harris, Drug Metab. Dispos., 8 (1980) 1.
7 N. Gerber, R.M. Thompson, R.G. Smith and R.K. Lynn, Epilepsia, 20 (1979) 287.
8 T.C. Butler, J. Pharmacol. Exp. Ther., 117 (1956) 160.
9 A. Küpfer, R.K. Roberts, S. Schenker and R.A. Branch, J. Pharmacol. Exp. Ther., 218 (1981) 193.
10 P. Friel and A.S. Troupin, Clin. Chem., 21 (1975) 751.
11 V.A. Raisys, A.M. Zebelman and S.F. MacMillan, Clin. Chem., 25 (1979) 172.
12 H.J. Kupferberg and W. Yonekawa, Drug Metab. Dispos., 3 (1975) 26.
13 W. Yonekawa and H.J. Kupferberg, J. Chromatogr., 163 (1979) 161.
14 A. Küpfer and J. Bircher, J. Pharmacol. Exp. Ther., 209 (1979) 190.

15 B. Kolb and J. Bischoff, J. Chromatogr. Sci., 12 (1974) 625.
16 R. de Sagher, J. Pocius and J.P. Thenot, J. Chromatogr., 156 (1978) 43.
17 G. Karlaganis, A. Küpfer, J. Bircher, U.P. Schlunegger, H. Gfeller and P. Bigler, Drug Metab. Dispos., 8 (1980) 173.
18 A. Küpfer, J. Bircher and R. Preisig, J. Pharmacol. Exp. Ther., 203 (1977) 493.
19 E. Brochmann-Hanssen and T. Olawuyi-Oke, J. Pharm. Sci., 58 (1969) 370.
20 T. Chang and A.J. Glazko, J. Lab. Clin. Med., 75 (1970) 145.
21 H. Björndal, C.G. Hellerquist, B. Lindberg and S. Svensson, Angew. Chem., Int. Ed. Engl., 9 (1970) 610.
22 J.S. Brimacomb, B.D. Jones, M. Stacey and J.J. Willard, Carbohyd. Res., 2 (1966) 167.
23 P.A. Leclerq and D.M. Desiderio, Jr., Anal. Lett., 4 (1971) 305.
24 L.K. Wong, C.E. Costello and K. Biemann, J. Chromatogr., 116 (1976) 321.
25 A.J. Atkinson, Jr., J. MacGee, J. Strong, D. Garteiz and T.E. Gaffney, Biochem. Pharmacol., 19 (1970) 2483.
26 T.C. Butler, K.M. Dudley, P. Johnson and S.B. Roberts, J. Pharmacol. Exp. Ther., 199 (1976) 82.
27 J.H. Maguire, T.C. Butler and D.H. Dudley, J. Med. Chem., 21 (1974) 1294.