foxides VIII, X, XVII, and XXIII, the latter affording M^+ ions having <15% relative abundance. The variations in the abundance of this ion are not surprising because plausible processes leading to its formation (including charge transfer to M, loss of H· from MH^+, and direct ionization by electron impact) are sensitive to slight variations in temperature, pressure, and chemical entities in and near the source.

Fragmentation processes involving the trimethylene bridge appeared to be common to the spectra of II–V, VII–XV, and XVIII–XXIX (phenothiazine–$CH_2CH_2CH_2$–base) and to XVI and XVII, producing weak ions that corresponded in mass to the (substituted) phenothiazin-10-yl nucleus (Ion A) and to its methylene homolog (Ion B). Generally more abundant ions, corresponding in mass to the terminal methylene unit plus the attached aliphatic nitrogen atom and its substituents (Ion C, CH_2–base or CH_3CH–base) and to the ethylene homolog (Ion D, C_3H_6–base), were observed at *m/e* 113 and 141 (XVIII–XXIII) or 143 and 171 (XXIV–XXIX), respectively. Although the corresponding ions in the spectra of III–XVIII had *m/e* values <100 and were, therefore, not measured in this study, the chemical ionization (methane) mass spectra of XII and two other hydroxylated homologs of XII were found (6) to exhibit prominent ions at *m/e* 58 and 86, corresponding to $[CH_2N(CH_3)_2]^+$ and $[C_3H_6N(CH_3)_2]^+$, respectively, in accord with the idea that these form by processes general for compounds of this type.

Molecules in this study having polar substituents (halogen or hydroxyl groups) also generally appeared to undergo loss of a hydrogen atom plus the substituent from the MH^+ species. Fragments resulting from such processes were relatively abundant in the chemical ionization (methane) spectra but minor or not observed in the corresponding chemical ionization (isobutane) spectra. The derivatives of 2-chlorophenothiazine (I–XII, XVIII–XXI, and XXIV–XXVI) also exhibited an $MH^+ - 34$ ion that did not have a satellite ion heavier by two mass numbers; this resulted from contamination by the respective halogen-free homologs.

In summary, derivatives of phenothiazine generally form very stable MH^+ and M^+ ions under conditions of chemical ionization. Although no simple correlation is obvious to account for the variations in relative intensity of fragment ions formed by different compounds in this study, decomposition appears to be limited mainly to cleavage of exocyclic carbon–carbon single bonds, plus loss of water or hydrogen halide in appropriately substituted derivatives. The prominence of these stable proton-capture ions in the chemical ionization spectra affords a measurable property that is not only characteristic of a given drug or metabolite but also exquisitely sensitive for its detection. These two conditions suggest that selective ion monitoring techniques (12) in conjunction with chemical ionization are potentially useful for bioanalytical studies of phenothiazines.

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Possible Antineoplastic Agents II

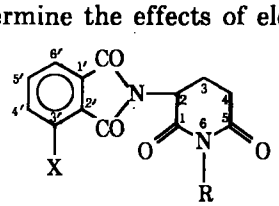
A. U. DE ˣ and D. PAL

Abstract □ Various glutarimide derivatives were synthesized, and some showed significant activity against Ehrlich ascites carcinoma in Swiss albino mice.

Keyphrases □ Glutarimides, substituted—various derivatives synthesized, antineoplastic activity evaluated, mice □ Antineoplastic activity, potential—substituted glutarimides synthesized and screened, mice □ Structure–activity relationships—various substituted glutarimides synthesized and screened for antineoplastic activity in mice

Synthesis of a few glutarimide analogs of the types 2-phthalimidoglutarimide (thalidomide) (I) and 3-phenylglutarimide (II) and their biological evaluation in Ehrlich ascites carcinoma were reported previously (1). Meanwhile, more derivatives of I and II were synthesized and evaluated biologically according to the same procedures (1) (Table I).

In the previous article (1), the rationale behind the projected synthesis of these glutarimides (I and II) and others with some structural variations (II*a*–II*l*, III, and IV) was discussed. These structural changes were designed to determine the effects of electron-repelling or electron-



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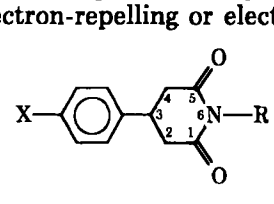


II

attracting groups on carcinolytic activity. The inclusion of a sulfonamide moiety in either the cyclic or noncyclic parts of the glutarimide analogs was utilized to evaluate antineoplastic effects. Synthesis and biological evaluation of 6-substituted 3-*p*-methoxyphenylglutarimides (IIc–II*l*), 2-benzenesulfinocarboximidoglutarimide (III) and 2-*p*-substituted benzenesulfonamidoglutarimides (IV) have been accomplished. The *p*-acetamido derivative (IV*c*) was selected with the hope that it might be hydrolyzed *in vivo* to the therapeutically active sulfanilamido derivative.

DISCUSSION

Chemistry—Treatment of 3-*p*-methoxyphenylglutaric anhydride (2) with appropriate amines furnished the described imides (IIc–II*l*) (3). Possible formation of the diamides was ruled out by elemental analyses and IR spectra.

Reaction between saccharin sodium and 2-bromoglutarimide (4) in the presence of dimethylformamide resulted in the desired imide (III), as confirmed by elemental analysis and IR spectra.

Interaction of potassium benzenesulfonamides and 2-bromoglutarimide in dimethylformamide or water did not give the desired imides (IV*a*–IV*c*) because the anionic parts of the benzenesulfonamides, acting as strong bases, brought about the β-elimination-type reaction. Hence, 2-benzenesulfonamidoglutaric acids (5) were either converted to the corresponding dichlorides with thionyl chloride or to the diethyl esters to avoid any acid hydrolysis of the *p*-acetamido group by thionyl chloride. Interaction of these dichlorides and diesters with ammonia led to the diamides. These compounds were heated in the presence of refluxing nitrobenzene, with liberation of ammonia, to provide the desired imides (IV*a*–IV*c*).

Biological Activity (6)—Antineoplastic potency was evaluated against Ehrlich ascites carcinoma in Swiss albino mice according to the procedure described previously (1, 7) (Table I). In the present case also, both test and control groups contained five animals. The compounds were administered at a dose level of 50 mg/kg ip in buffered saline (pH 7.2); both the cell count and weight of ascitic fluid were regarded as activity parameters. Mitomycin at 1 mg/kg ip in buffered saline was used as a standard. Compounds I*a*, II*f*, and IV*a* showed significant activity.

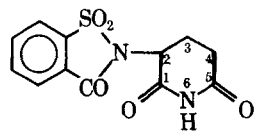
Structure–Activity Relationships—Although a systematic interpretation has not been attempted, some logical deductions can be made after examination of the observed antineoplastic potency of the compounds listed in this article as well as in a previous one (1). In the 2-phthalimidoglutarimide series (I*a*–I*d*), an alkyl or aryl substituent at the 6-position and an electronegative substituent like a nitro group in the benzene ring separately increased the activity. But an aralkyl or cycloalkyl group at the 6-position or the simultaneous combination of a nitro group at the 3-position and an alkyl group such as hexyl at the 6-position had the reverse effect.

In the 3-phenylglutarimides (II*a*–II*l*), introduction of an alkyl substituent at the 6-position and an electron-donating group such as methoxyl at the *para*-position of the benzene ring increased the activity. Aliphatic substituents such as ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, and *n*-hexyl at the 6-position increased the activity, which reached a maximum when the substituent was *n*-propyl. Cycloalkyl, aralkyl, and aryl groups had the opposite effect.

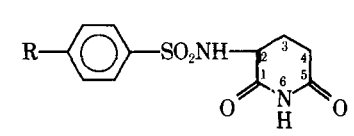
In the sulfonamidoglutarimides (III and IV*a*–IV*c*), incorporation of the sulfonamide group in a ring structure tended to decrease the activity more than when it was in a straight chain; significant activity was observed in the latter case. Introduction of an electron-donating group such as methyl at the *para*-position of the benzene ring did not improve the activity, while an acetamido group decreased the activity considerably. However, the most significant feature is that all the glutarimide derivatives had higher activity than the glutarimide itself.

EXPERIMENTAL[1]

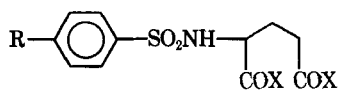
6-Substituted 3-*p*-Methoxyphenylglutarimides (IIc–II*l*)—A mixture of 3-*p*-methoxyphenylglutaric anhydride (1 mole) and the appropriate amine (1 mole) was heated in a sealed tube on an oil bath at 180° for 8 hr. The reaction mass was washed with sodium bicarbonate solution and crystallized from dilute ethanol after charcoal treatment (Table I).



III



IV



V*a*: R = H, X = Cl
V*b*: R = CH_3, X = Cl
V*c*: R = $NHCOCH_3$, X = OC_2H_5
V*d*: R = H, X = NH_2
V*e*: R = CH_3, X = NH_2
V*f*: R = $NHCOCH_3$, X = NH_2

6-Methyl-3-*p*-methoxyphenylglutarimide (II*d*) showed the characteristic carbonyl stretching frequency at 1675 cm^{-1} and no amino stretching frequency, the absence of which ruled out the possibility of diamide structure formation.

2-Benzenesulfinocarboximidoglutarimide (III)—A mixture of 2-bromoglutarimide (2 g), saccharin sodium (2.2 g), and dimethylformamide (20 ml) was stirred at room temperature for 10 hr until a negative Beilstein test was observed. Dimethylformamide was removed as completely as possible by distillation under reduced pressure in an oil bath, and the residual colored mass was washed with a little water to remove the excess saccharin sodium. The desired imide (III) was obtained by crystallization from dilute ethanol with charcoal treatment. Its physical characteristics are recorded in Table I.

Compound III showed characteristic sulfonyl stretching frequencies at 1165 and 1330 cm^{-1}, a carbonyl stretching frequency at 1705 cm^{-1}, and an amino stretching frequency at 3120 cm^{-1}.

2-*p*-Substituted Benzenesulfonamidoglutaryl Dichlorides (V*a* and V*b*)—A mixture of the diacids (10 mmoles) and thionyl chloride (15 ml) was refluxed on a steam bath until hydrogen chloride evolution ceased. Excess thionyl chloride was exhaustively removed, first by distillation under reduced pressure and then by adding 3 × 10 ml of benzene to the mass with subsequent removal by distillation. The acid chlorides solidified on cooling and were sufficiently pure for the next step. Compound V*a* had a melting point of 95–97°; the melting point of V*b* was 58–60°.

Diethyl 2-*p*-Acetamidobenzenesulfonamidoglutarate (V*c*)—A mixture of 2-*p*-acetamidobenzenesulfonamidoglutaric acid (6 g), absolute ethanol (25 ml), and sulfuric acid (specific gravity 1.84; 0.36 ml) was refluxed for 8 hr and worked up in the usual way. Compound V*c* had a melting point of 104–106°.

2-*p*-Substituted Benzenesulfonamidoglutaricdiamides (V*d*–V*f*)—The acid dichlorides (V*a* and V*b*, 5 g) were slowly basified with liquor ammonia (20 ml) under cooling in an ice bath to give the crude diamides (V*d* and V*e*). Compounds V*d* and V*e* were then crystallized from dilute ethanol after charcoal treatment to yield the pure product. The diethyl ester (V*c*, 2 g) and liquor ammonia (10 ml) were heated in a sealed tube in a steam bath for 8 hr to yield the crude diamide (V*f*), which was crystallized from dilute ethanol with charcoal treatment. Compound V*d* had a melting point of 214–216°, V*e* had a melting point of 219–221°, and V*f* had a melting point of 231–233°.

2-*p*-Substituted Benzenesulfonamidoglutarimides (IV*a*–IV*c*)—Mixtures of the diamides (V*d*–V*f*, 2 g) and nitrobenzene (10 ml) were refluxed on an oil bath until ammonia evolution ceased. The reaction mass then was cooled and diluted with benzene or petroleum ether (bp 60–80°) to yield the crude imides. These compounds were purified by crystallization from dilute ethanol after charcoal treatment as glistening needles. Their physical characteristics are recorded in Table I.

2-Benzenesulfonamidoglutarimide (IV*a*) showed sulfonyl stretching frequencies at 1165 and 1340 cm^{-1}, a carbonyl stretching frequency at 1650 cm^{-1}, and an amino stretching frequency at 3500 cm^{-1}. 2-*p*-Toluenesulfonamidoglutarimide (IV*b*) showed sulfonyl stretching frequencies at 1165 and 1346 cm^{-1}, a carbonyl stretching frequency at 1655 cm^{-1}, and an amino stretching frequency at 3505 cm^{-1}. 2-*p*-Acetamidobenzenesulfonamidoglutarimide (IV*c*) showed sulfonyl

[1] Melting points were recorded with a Gallenkamp instrument and are uncorrected. IR spectra were recorded in potassium bromide pellets using a Perkin-Elmer spectrophotometer, Infracord model 137.

Table I—Physical Characteristics and Antineoplastic Activity of Some Glutarimides

Compound	R	X	Yield, %	Melting Point	Formula	Analysis, %		Antineoplastic Activity[a]	
						Calc.	Found	Inhibition of Ascitic Cells, %	Inhibition of Ascitic Fluid, %
I*a*	Phenyl	H	60	158–160°	$C_{19}H_{14}N_2O_4$	C 68.26 H 4.19 N 8.38	67.91 4.47 8.49	87.31	98.40
I*b*	Benzyl	H	62	176–178°	$C_{20}H_{16}N_2O_4$	C 68.97 H 4.60 N 8.05	69.27 4.84 8.42	100.00[b]	84.00
I*c*	Cyclohexyl	H	64	108–110°	$C_{19}H_{20}N_2O_4$	C 67.06 H 5.88 N 8.24	67.37 6.24 7.95	47.60	43.99
I*d*	*n*-Hexyl	3′-NO_2	55	182–184°	$C_{19}H_{21}N_3O_6$	C 58.91 H 5.43 N 10.85	58.72 5.03 11.22	15.72	64.07
II*a*	Benzyl	H	73	94–96°	$C_{18}H_{17}NO_2$	C 77.42 H 6.09 N 5.02	77.37 5.84 5.29	100.00[b]	80.64
II*b*	Cyclohexyl	H	60	204–206°	$C_{17}H_{21}NO_2$	C 75.28 H 7.75 N 5.17	74.89 8.02 5.18	85.55	80.64
II*c*	H	OCH_3	70	169–171°	$C_{12}H_{13}NO_3$	C 65.75 H 5.94 N 6.39	65.79 6.29 6.44	33.39	30.00
II*d*	Methyl	OCH_3	69	115–117°	$C_{13}H_{15}NO_3$	C 66.95 H 6.44 N 6.01	67.13 6.70 6.19	44.96	60.00
II*e*	Ethyl	OCH_3	64	113–115°	$C_{14}H_{17}NO_3$	C 68.02 H 6.88 N 5.67	67.87 6.94 5.32	63.03	97.33
II*f*	*n*-Propyl	OCH_3	62	75–77°	$C_{15}H_{19}NO_3$	C 68.97 H 7.28 N 5.36	69.11 7.02 4.98	100.00[c]	100.00[c]
II*g*	*n*-Butyl	OCH_3	60	81–83°	$C_{16}H_{21}NO_3$	C 69.82 H 7.64 N 5.09	69.89 8.01 5.33	28.50	92.31
II*h*	*n*-Pentyl	OCH_3	58	52–54°	$C_{17}H_{23}NO_3$	C 70.59 H 7.96 N 4.84	70.32 7.57 5.17	76.48	92.31
II*i*	*n*-Hexyl	OCH_3	65	96–98°	$C_{18}H_{25}NO_3$	C 71.29 H 8.25 N 4.62	71.38 8.16 4.41	62.94	91.77
II*j*	Phenyl	OCH_3	75	158–160°	$C_{18}H_{17}NO_3$	C 73.22 H 5.76 N 4.75	73.61 6.07 4.66	76.08	84.00
II*k*	Benzyl	OCH_3	73	80–82°	$C_{19}H_{19}NO_3$	C 73.79 H 6.15 N 4.53	73.69 6.27 4.72	66.41	88.89
II*l*	Cyclohexyl	OCH_3	55	218–220°	$C_{18}H_{23}NO_3$	C 71.76 H 7.64 N 4.65	71.95 7.37 5.00	26.87	58.82
III	—	—	51	254–256°	$C_{12}H_{10}N_2O_5S$	C 48.98 H 3.40 N 9.52	48.62 3.36 9.89	5.61	34.12
IV*a*	H	—	55	190–192°	$C_{11}H_{12}N_2O_4S$	C 49.25 H 4.48 N 10.45	49.29 4.53 10.19	100.00[c]	100.00[c]
IV*b*	Methyl	—	53	192–194°	$C_{12}H_{14}N_2O_4S$	C 51.06 H 4.97 N 9.93	50.73 5.12 10.17	84.45	88.39
IV*c*	Acetamido	—	51	238–240° dec.	$C_{13}H_{15}N_3O_5S$	C 48.00 H 4.62 N 12.92	48.25 5.02 12.67	57.34	48.58

[a] Mitomycin at a dose level of 1 mg/kg was used as standard showing 100% inhibition of ascitic cell and ascitic fluid. [b] Ascitic fluid could not be drawn by a syringe. [c] Ascitic fluid did not form.

stretching frequencies at 1160 and 1340 cm^{-1}, a carbonyl stretching frequency at 1680 cm^{-1}, and an amino stretching frequency at 3405 cm^{-1}.

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Radioimmunoassay of the Anticonvulsant Agent Clonazepam

W. R. DIXON [x], R. L. YOUNG, R. NING, and A. LIEBMAN

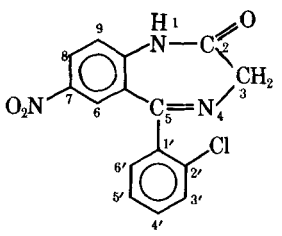
Abstract □ A simple and specific radioimmunoassay was developed for the determination of the anticonvulsant agent clonazepam directly in plasma without extraction. Antibodies to clonazepam were produced in rabbits after immunization with an immunogen prepared by covalently linking the 3-hemisuccinyloxy derivative of clonazepam to bovine serum albumin. When employing ^{3}H-clonazepam as the tracer, the radioimmunoassay has a limit of sensitivity of 5 ng/ml using a 0.1-ml sample of plasma. The antibodies exhibited a high degree of specificity for clonazepam; no cross-reactivity was observed with its 7-amino and 7-acetylamino metabolites nor with a number of other widely prescribed anticonvulsant agents that might be administered in conjunction with clonazepam. Satisfactory agreement was obtained for the plasma levels of clonazepam in humans when samples were assayed by the radioimmunoassay and an established electron-capture GC technique. By virtue of its simplicity, the radioimmunoassay offers a distinct advantage to the clinician for monitoring plasma clonazepam levels and the compliance of patients undergoing anticonvulsant therapy with the drug.

Keyphrases □ Clonazepam—radioimmunoassay, human plasma □ Radioimmunoassay—clonazepam, human plasma □ Anticonvulsants—clonazepam, radioimmunoassay, human plasma

Clonazepam (I) is a member of the 1,4-benzodiazepine class of compounds which has recently been found to be clinically effective in controlling minor motor seizures (petit mal) in humans (1–4). Studies on the pharmacokinetics and metabolism of the drug also have been reported (5–7).

At the present time, only electron-capture GC methods have been reported for the determination of the nanogram levels of clonazepam that exist in the plasma of subjects undergoing anticonvulsant therapy (8–10). These electron-capture–GC methods have a limit of sensitivity in the order of 1 ng of clonazepam/ml of plasma using a 2-ml sample. However, apart from requiring skilled technical operation, these techniques are time consuming and thereby restrictive for the routine analysis of the numerous samples obtained during clinical studies.

The present report concerns the development of a specific and rapid radioimmunoassay for clonazepam in plasma. This assay allows the clinician to relate therapeutic response to plasma levels of the drug in individual patients.



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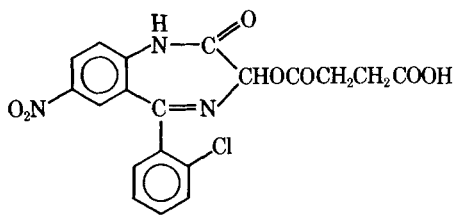

II

EXPERIMENTAL

Tritium-Labeled Clonazepam—^{3}H-Clonazepam was prepared by tritium exchange using dimethylformamide–tritiated water (specific activity 100 Ci/g). The product was purified by silica gel column chromatography, yielding material with a specific activity of 4.23 mCi/mg. This method of introducing tritium probably provided exchange mainly at the C-3 position.

Preparation of Immunogen—The 3-hemisuccinyloxy derivative of clonazepam (II) was covalently coupled to bovine serum albumin using the mixed anhydride procedure of Erlanger *et al.* (11). After successive dialysis against dioxane–water (1:1), 0.05 *M* borate buffer (pH 9), and water, the immunogen was isolated by lyophilization.

On the basis of its absorbance at 365 nm in 0.1 *N* NaOH against a standard solution of II mixed with albumin, it was estimated that the immunogen consisted of 35 moles of II covalently coupled to 1 mole of albumin.

Antibody Production—Two New Zealand White female rabbits were immunized intradermally at multiple sites with 1 mg of the immunogen as an emulsion in Freund's complete adjuvant as previously described (12). The animals were then boosted intravenously with 50 μg of immunogen at monthly intervals, and serum was harvested 10–14 days after each administration. Both rabbits produced satisfactory titers of antibodies to clonazepam within 3 months following the initial immunization, and their serum was pooled. At a 1:2000 dilution, 1 ml of diluted serum bound approximately 60% of the ^{3}H-clonazepam used for the assay.