analogue was compared with the slope of the estradiol response to determine parallelism in this assay system. The relative uterotropic activity of each compound was expressed as a percentage of estradiol activity according to the method of Bliss (11).

RESULTS

The dose–response curve produced by each of the selenium analogues is presented in Fig. 1. The potency of each compound relative to estradiol is summarized in Table I.

Among the selenosteroids that were examined in this study, III produced the greatest uterotropic activity while I produced the least activity. The selenoanalogues of ethynylestradiol (III and IV) were more active than those of estradiol or estrone. This may be due to the differences in the activity of the parent compounds (12). None of the test compounds produced a significant degree of antiestrogenic activity in this study.

DISCUSSION

In Table I the relative binding affinity of the test compounds was compared with the uterotropic activity. The potency estimates were in good agreement with the relative binding affinities with few exceptions. To correlate *in vitro* and *in vivo* results, metabolism has to be taken into consideration. Even though VI demonstrated a low *in vitro* binding affinity, its *in vivo* potency is almost equal to that of V. This could be as a result of demethylation *in vivo*. Sulfur-containing organic compounds are metabolized to sulfoxides (13); analogously, selenides are expected to oxidize to selenoxides *in vivo*. Reports indicate that aromatic selenoxides are less stable than the aliphatic derivatives (14–16). Therefore, we assume that the greater activity of III as compared with IV, despite a lower affinity for estrogen receptors, is due to the degradation of III to the more potent ethynylestradiol.

It is reasonable to assume that VII has a greater *in vivo* uterotropic activity than V because estradiol (the parent compound of analogue VII) is more potent than estrone (the parent compound of analogue V) (12).

This study indicates that increasing the size of the substituent on C-17 decreases the *in vitro* binding and the *in vivo* potency. These data confirm the importance of the β-hydroxyl group on C-17 to retain the estrogenic activity (17). Since these selenosteroids retain a high degree of estrogenic activity (especially III and IV) *in vivo*, as shown in the present study, and display potent receptor binding *in vitro* (8), it is possible that one of these derivatives might be a suitable imaging agent for estrogen-dependent tumors and metastatic foci. Selenium-75 labeling of II has been accomplished utilizing newly developed methodology (18). Biodistribution studies of this ^{75}Se-labeled compound in tumor-bearing animals are underway.

REFERENCES

(1) W. L. McGuire, K. L. Horwitz, D. T. Zava, R. E. Garola, and G. C. Chamness, *Metabolism*, **27**, 487 (1978).

(2) C. K. Osborne and W. L. McGuire, *Bull. Cancer (Paris)*, **66**, 203 (1979).

(3) J. A. Katzenellenbogen, K. D. McElvany, S. G. Senderoff, K. E. Carlson, S. W. Landvatter, M. J. Welch, and the Los Alamos Medical Radioisotope Group, *J. Nucl. Med.*, **23**, 411 (1982).

(4) R. N. Hanson, D. E. Seitz, and J. C. Botarro, *J. Nucl. Med.*, **23**, 431 (1982).

(5) G. P. Basmadjian, R. R. Hetzel, and R. D. Ice, *Int. J. Appl. Radiat. Isotopes*, **26**, 695 (1975).

(6) F. F. Knapp, in "Radiopharmaceuticals: Structure Activity Relationships," R. P. Spencer, Ed., Grune & Stratton, New York, N.Y., 1981, p. 345.

(7) S. A. Sadek, S. M. Shaw, W. V. Kessler, and G. C. Wolf, *J. Org. Chem.*, **46**, 3259 (1981).

(8) S. A. Sadek, W. V. Kessler, S. M. Shaw, J. N. Anderson, and G. C. Wolf, *J. Med. Chem.*, **25**, 1488 (1982).

(9) J. T. Pento, R. A. Magarian, R. T. Wright, M. M. King, and E. T. Benjamin, *J. Pharm. Sci.*, **70**, 399 (1981).

(10) B. L. Rubin, A. S. Dorfman, L. Black, and R. I. Dorfman, *Endocrinology*, **49**, 429 (1951).

(11) C. I. Bliss, "The Statistics of Bioassay," Academic, New York, N.Y., 1952.

(12) J. P. Raynaud, M. M. Bouton, D. Gallet-Bourguin, D. Philibert, C. Tournemine, and G. Azadian-Barlanger, *Mol. Pharmacol.*, **9**, 520 (1973).

(13) N. P. Salzman and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **118**, 46 (1956).

(14) R. Walter and J. Roy, *J. Org. Chem.*, **36**, 2561 (1971).

(15) K. B. Sharpless, M. W. Young, and R. F. Lauer, *Tetrahedron Lett.*, **1973**, 1979.

(16) G. Ayrey, D. Barnard, and D. T. Woodbridge, *J. Chem. Soc.*, **1962**, 2089.

(17) S. G. Korenman, *Steroids*, **13**, 163 (1969).

(18) S. A. Sadek, G. P. Basmadjian, and R. D. Ice, *Nucl. Med. Commun.*, **3**, 247 (1982).

Diaspirins of Methylenecitric Acid

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Received October 25, 1982, from the *Department of Chemistry, Northwestern University, Evanston, IL 60201.* Accepted for publication January 19, 1983.

Abstract □ A series of bissalicylic esters of methylenecitric acid have been prepared and, as a probe of their potential as antisickling agents, tested for their ability to modify hemoglobin. Substantial acylation of hemoglobin was obtained with these dicarboxylate esters at 1–5 mM concentrations.

Keyphrases □ Methylenecitric acid—bissalicylic esters, synthesis, hemoglobin-modifying potential □ Antisickling agents—potential, bissalicylic esters of methylenecitric acid, synthesis, hemoglobin-modifying ability

Diaspirins have been found (1) to modify hemoglobin S and to change its solubility markedly. Consequently, this class of compounds may provide promising antisickling agents. Just as aspirin is an acetate of salicylic acid, so are diaspirins the alkanedioates of salicylic acid. Alkanedioic acids of 4–5 carbon chain length seem to be optimal in modification of hemoglobins, probably because they can readily span the β-cleft of the hemoglobin molecule and form a covalent bridge between the two Lys 82 residues. Such a bridge evidently locks the protein into a conformation out of register for the aggregation that occurs in sickling.

The modified pentanedioic acid, 4-oxo-1,3-dioxolane-5,5′-diacetic acid, also known as methylenecitric acid (A), is a five-carbon span dicarboxylic acid whose monoesters have been examined previously for pharmacological ac-

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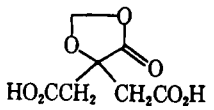
Table I—Modification of Hemoglobin by Bissalicylic Esters of Methylenecitric Acid

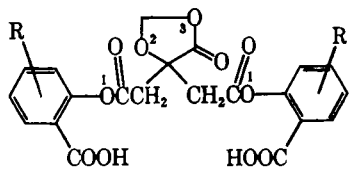
Compound	Melting Point, °C	Yield, %	Formula[a]	Conc., mM	Modification of Oxyhemoglobin A, %[b]
I	155–157	72	$C_{21}H_{16}O_{11}$	5	34
				1	20
II	153–155	54	$C_{21}H_{14}F_2O_{11}$	5	46
				1	24
III	161–163	51	$C_{21}H_{14}Cl_2O_{11}$[d]	5	70
				1	35
IV	168–169	45	$C_{21}H_{14}Br_2O_{11}$[e]	5	74
				1	38
V	167–168	53	$C_{21}H_{14}I_2O_{11}$	5	83
				1	42
VI	165–166	45	$C_{21}H_{14}Cl_2O_{11}$[f]	5	69
				1	35
VII[c]	155–157	69	$C_{21}H_{12}Cl_4O_{11}$[g]	1	27
VIII[c]	183–185	53	$C_{21}H_{12}Br_4O_{11}$[h]	1	32
IX[c]	146–148	76	$C_{21}H_{12}I_4O_{11}$[i]	1	35
X	[Bis(4,6-dibromo-*z*-carboxyphenol)fumarate]			1	87
				1[j]	88

[a] Compounds I–IX underwent elemental analyses for C, H, and the appropriate halogen. Unless otherwise noted, all analyses were within ±0.4% of the theoretical values. [b] Hemoglobin concentration, 1 mM. [c] Unstable compared with the monosubstituted compounds; they hydrolyzed slowly on standing. Repeated recrystallization did not improve the analysis. [d] Calc. for Cl, 13.82; found, 13.21. [e] Calc. for Br, 26.54; found, 26.04. [f] Calc. for Cl, 13.82; found, 12.98. [g] Calc. for H, 2.08; Cl, 24.36. Found for H, 2.72; Cl, 19.75. [h] Calc. for Br, 42.06; found, 39.06. [i] Calc. for I, 53.55; found, 48.62. [j] In aqueous solution containing 2.6% ethanol to aid in solubilizing the compound.

tivity (2, 3). It seemed interesting, therefore, to prepare salicylic derivatives (B) of this diacid and to examine their effects on hemoglobin. The significant parts of B for its chemical and biological activity are the three C—O—C moieties designated O(1), O(2), and O(3). Classification of the functionality of the C—O—C at O(1) is acid anhydride, at O(2) acetal, and at O(3) acylal, and their relative reactivities toward amino groups are O(1) > O(3) > O(2).



A



B′

I: R = H
II: R = 5-F
III: R = 5-Cl
IV: R = 5-Br
V: R = 5-I
VI: R = 4-Cl
VII: R = 3,5-Cl_2
VIII: R = 3,5-Br_2
IX: R = 3,5-I_2

EXPERIMENTAL[2]

To a stirred mixture of salicylic acid (2.00 mmol) or its halogen derivatives and dimethylaniline (4.13 mmol) in 100 mL of dry benzene was added in a dropwise manner 4-oxo-1,3-dioxolane-5,5-bis(acetylchloride) (methylene-citroyl dichloride, 1.02 mmol) dissolved in 20 mL of benzene. After 0.5 h at room temperature, the mixture was further warmed on a water bath (at 60–70°C) for ~10 min and then stirred overnight at room temperature. The solvent was removed *in vacuo*; the residue was suspended in 100 mL of ethyl acetate and then extracted twice with ice-cold 1 M aqueous H_2SO_4 (50 mL) and once with water (50 mL). After the organic layer had been dried ($MgSO_4$), filtered, and evaporated (*in vacuo*), the residue was triturated with several milliliters of petroleum ether to give solid material. Recrystallization either from benzene or a mixture of chloroform–petroleum ether (30–60°C) provided purified bis(substituted 2-carboxyphenyl)4-oxo-1,3-dioxolane-5,5′-diacetates, commonly known as bis(substituted salicyl)anhydromethylene citrates.

The compounds were identified by elemental analysis (Table I), IR, and ^{1}H-NMR spectrometry (1). The C=O stretching vibration of esters occurs in the range 1690–1770 cm^{-1}. NMR data were consistent with the proposed structures. The NMR spectra (DMSO-d_6 tetramethylsilane as internal standard) showed the following common absorption peaks: δ 3.3–3.5 (s, —C—CH_2—CO), 5.4–5.52 (s, O—CH_2—O), and 7.2–8.2 ppm (ArH).

Isoelectric focusing (6, 7) was used to assess the extent of modification of oxyhemoglobin. The acylated protein was separated from the parent protein in a flat gel by electrophoresis[3]. Minor alterations (8) were made in the procedures described by the manufacturer. Before application to the gel surface (on small pieces of filter paper) all hemoglobin samples were saturated with carbon monoxide and made to 0.01 M in NaCN. After focusing had been achieved, the gel was fixed (8) and dried overnight, and a densitometric scan was made with a soft laser scanning densitometer[4] and integrator. From this pattern the extent of modification was calculated.

RESULTS AND DISCUSSION

The extent of modification of oxyhemoglobin by these diesters is shown in Table I. The prototype of this series, the diester with unsubstituted salicylate (I), shows significant modification of the protein even at concentrations as low as 1 mM. The effectiveness increases progressively as one proceeds from the fluoro to the chloro, bromo, and iodo monosubstituted salicylates (II–V). With the monochlorosalicylate, a comparison was also made of the 4-substituted (VI) with the 5-substituted derivative (III), but the difference in behavior is insignificant. Derivatives with disubstituted halosalicylates (VII–IX) are not more effective as acylating agents for hemoglobin than are the monosubstituted derivatives.

Using [^{14}C]acetyl-labeled salicylate, we established previously (8) that acylation of hemoglobin occurs on lysine side chains. Subsequently, with the "double-headed" aspirins, chemical work supplemented by X-ray diffraction (9, 10), showed that the β-subunits of hemoglobin were cross-linked by —C(=O)—CH_2—CH_2—C(=O)— or —C(=O)—CH=CH—C(=O)— bridges connecting β_1 Lys 82 to β_2 Lys 82. Similar cross linking has been observed with five-atom bridges (11). Thus, the methylene citric acid series became a natural candidate for the extension of these structure-function investigations.

At first glance one would have expected (1) the dihalosalicylate diesters to be more effective than the monohalo ones since the additional halogen substituents should increase binding to hemoglobin. However, one must recognize that the extra electron-withdrawing substituents also activate the ester bond and make the compound more sensitive to hydrolysis. Such effects have already been observed (11, 12) with derivatives containing polar substituents on a four-carbon bridge; oxygen moieties adjacent to the carbonyl groups decrease the stability of the bis(dibromo)salicylate

[1] Numbering of substituents in structure B is in terms of salicylate ring.

[2] Elemental analyses were performed by Micro-Tech Laboratories of Skokie, Ill. IR spectra (in KBr pellets) were obtained with a Perkin-Elmer 283 spectrometer. ^{1}H-NMR spectra were obtained with Varian EM-360 (60 MHz) and JEOL FX-270 spectrometers. Melting points were taken in open capillary tubes and are uncorrected. Salicylic acids used herein were purchased from the Aldrich Chemical Co. Methylenecitric acid was prepared from citric acid and paraformaldehyde, and its subsequent conversion into dichloride was by treatment with PCl_5. The physical properties in all cases were in close agreement with those cited in the literature (2, 4, 5).

[3] LKB 2117 Multiphor with a Savant power source.

[4] Zeineh.

esters. Corresponding results have also been evident when electron-withdrawing groups were placed on the salicylate ring (13). The doubly halogenated salicylate derivatives of methylenecitric acid have activating groups both on the aromatic rings and on the middle carbon of the five-carbon bridge so that hydrolytic instabilities are magnified. Even in the solid state, traces of moisture lead to slow cleavage of these esters. Thus, as has been emphasized previously (11, 12) a careful balance between stability and reactivity is essential for optimal activity of the double-headed aspirins.

REFERENCES

(1) I. M. Klotz, D. N. Haney, and L. C. King, *Science*, **213**, 724 (1981).

(2) A. Dulin and J. W. Martin, Jr., *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 851 (1958).

(3) R. J. Warren, R. W. Gardier, A. B. Richards, and W. M. Alexander, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 853 (1958).

(4) C. A. Nau, E. B. Brown, and J. R. Bailey, *J. Am. Chem. Soc.*, **47**, 2596 (1925).

(5) Ger. pat. 185,800, 1906; *Chem. Abstr.*, **2**, 351 (1908); Ger. pat. 186,659, 1906; *Chem. Abstr.*, **2**, 354 (1908).

(6) H. F. Bunn, *Ann. N.Y. Acad. Sci.*, **209**, 345 (1973).

(7) P. G. Righetti and J. W. Drysdale, in "Laboratory Techniques in Biochemistry and Molecular Biology," Vol. 5, Part 2, T. S. Work and E. Work, Eds., Elsevier, Amsterdam, 1976.

(8) M. Shamsuddin, R. G. Mason, J. M. Ritchey, G. R. Honig, and I. M. Klotz, *Proc. Natl. Acad. Sci. USA*, **71**, 4693 (1974).

(9) J. A. Walder, R. Y. Walder, A. Arnone, and I. M. Klotz, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **38**, 461 (1979).

(10) J. A. Walder, R. Y. Walder, and A. Arnone, *J. Mol. Biol.*, **141**, 195 (1980).

(11) L. E. Wood, D. N. Haney, J. R. Patel, S. E. Clare, G.-Y. Shi, L. C. King, and I. M. Klotz, *J. Biol. Chem.*, **256**, 7046 (1981).

(12) E. J. Delaney, S. E. Massil, G.-Y. Shi, and I. M. Klotz, *Arch. Biochem. Biophys.*, in press.

(13) R. H. Zaugg, J. A. Walder, R. Y. Walder, J. M. Steele, and I. M. Klotz, *J. Biol. Chem.*, **255**, 2816 (1980).

ACKNOWLEDGMENTS

This work was supported in part by Grant No. HL22719 from the National Heart, Lung, and Blood Institute of the National Institutes of Health. We are also very grateful to Professor C. D. Hurd for his advice on proper nomenclature of the compounds studied and his helpful comments on this manuscript.

COMMUNICATIONS

Computer-Interfaced Capacitive Sensor for Monitoring the Granulation Process

Keyphrases □ Granulation—computer-interfaced capacitive sensor, high-intensity mixer, moisture measurement, instrumentation, process control

To the Editor:

Wet granulation of powders is a fundamental process in pharmaceutical technology. The phenomenon of granulation, however, is not well understood because of the many variables involved in the process. The need for a better understanding and control of wet granulation has increased in recent years because greater utilization of high-intensity mixers has dramatically reduced the total time required to reach or exceed a proper granulation end point. Several workers have reported results from studies of the granulation process using instrumented mixers (1–8). These studies have shown that measurements of power consumption by the mixer, torque on the mixer shaft, change in rotation-rate of the mixer shaft, or the force with which granules deflect a strain-gauged beam, provided insight into the changes that occur as a powder is massed.

We wish to report a new approach to study the dynamics of wet granulation, which uses a computer-interfaced capacitive sensor that has been designed in our laboratories. The sensor is constructed from a polytetrafluoroethylene cylinder 1.5 cm in diameter, to which two insulated stainless steel electrodes are attached. The sensor is threaded to fit the existing thermocouple port in the wall of a high-intensity mixer[1]. The sensor extends into the mixer a distance of 5 cm and is 12 cm below the mixer center, with the distal 3 cm of each electrode exposed.

The two electrical leads from the sensor are connected to a moisture analyzer[2] which acts as the interface to the computer. The electronics of the moisture analyzer have been modified to filter signal noise caused by the rotation of the mixer plowshares and to allow for initial baseline adjustments. The moisture analyzer with sensor operates on the principle of power loss from a tuned radio-frequency (R/F) circuit as the dielectric of the medium surrounding the sensor changes. The analog DC voltage output of the moisture analyzer is converted to a digital value by an A/D converter and is fed to a 16-bit microprocessor[3] which is used for data processing and system control. System software is stored in a nonvolatile form in an onboard, programmable, read-only memory and allows for operator control of data sampling rate and text information for hard-copy output. Each data point, taken at 4-s intervals, is the average of 5000 A/D conversions.

Granulations were prepared by charging the mixer with 20 kg of lactose powder and using only the plowshare mixing action. Figure 1 shows the resulting voltage *versus* mixing time profile as incremental additions of water were made to the lactose[4]. The decreases in voltage are the result of rapid moisture distribution throughout the powder after each addition. The plateau reached between each addition is an equilibrium voltage value that is a function of the amount of water in the moist powder. The granules formed with this simple lactose water system, however, were not of good quality.

Figure 2 shows the voltage response when 2.5 kg of a 4% w/w methylcellulose 15 cps[5] aqueous solution was added to 20 kg of lactose at time zero. A similar voltage response

[1] Model FM-50, Littleford-Lödige, Florence, Ky.

[2] Moisture Register Model G8R, Moisture Register Co., Berwind Instruments Group, North Hollywood, Calif.

[3] iSBC 88/25, Intel Inc., Santa Clara, Calif.

[4] Lactose Hydrous USP, No. 80M, Sheffield Products, Memphis, Tenn.

[5] Methocel A 15 Premium, The Dow Chemical Co., Midland, Mich.

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