(25). Gaur and Palrecha (26) also determined the stability constants polarographically with a sodium perchlorate supporting electrolyte (no buffer given) and found that 1:1, 1:2, and 1:3 species were formed, having stability constants of 80, 540, and 1610, respectively. Williams *et al.* (10) attempted to determine a stability constant potentiometrically but were unable to do so due to the rapid hydrolysis of acetylsalicylate. It is possible that at the concentrations employed and in the absence of excess acetylsalicylate ion, little complexed species exists. In this case, hydrolysis rates should not be different for solutions made from the copper complex than for those of aspirin.

In summary, the pH-dependent hydrolysis of aspirin was found to occur at the same rate in the free form as in a dilute solution of the tetrakis-μ-acetylsalicylato-dicopper(II) complex. Preparation of this complex in aqueous solution, especially a basic solution, yielded a compound contaminated with significant amounts of salicylic acid.

REFERENCES

(1) W. Hangarter and A. Lubke, *Dtsch. Med. Wochenschr.*, **77**, 870 (1952).

(2) W. Hangarter, *Z. Rheum.*, **25**, 289 (1966).

(3) J. Schubert, *Chimia*, **11**, 113 (1957).

(4) J. R. J. Sorenson, *J. Med. Chem.*, **19**, 135 (1976).

(5) J. R. J. Sorenson, *Inflammation*, **1**, 317 (1976).

(6) J. R. J. Sorenson, *J. Pharm. Pharmacol.*, **29**, 450 (1977).

(7) K. D. Rainsford and M. W. Whitehouse, *ibid.*, **28**, 83 (1976).

(8) K. D. Rainsford and M. W. Whitehouse, *Specialia*, **32**, 1172 (1976).

(9) K. D. Rainsford and M. W. Whitehouse, *Life Sci.*, **21**, 371 (1977).

(10) D. A. Williams, D. T. Walz, and W. O. Foye, *J. Pharm. Sci.*, **65**, 126 (1976).

(11) E. Boyle, P. C. Freeman, A. C. Goudie, F. R. Mangan, and M. Thomson, *J. Pharm. Pharmacol.*, **28**, 865 (1976).

(12) M. W. Whitehouse and W. R. Walker, *Agents Actions*, **8**, 85 (1978).

(13) V. Weser, L. Richter, A. Wendel, and M. Younes, *Bioinorg. Chem.*, **8**, 201 (1978).

(14) A. J. Lewis, *Agents Actions*, **8**, 244 (1978).

(15) A. J. Lewis, *Br. J. Pharmacol.*, **63**, 413p (1978).

(16) J. R. J. Sorenson and W. Hangarter, *Inflammation*, **2**, 217 (1977).

(17) J. R. J. Sorenson. *Prog. Med. Chem.*, **15**, 211 (1978).

(18) D. A. Williams, MsC thesis, Massachusetts College of Pharmacy, Boston, Mass., 1962.

(19) L. Manojlovic-Muir, *J. Chem. Soc., Chem. Commun.*, **1967**, 1057.

(20) G. W. Stevenson, *Anal. Chem.*, **32**, 1522 (1960).

(21) K. S. Bose and C. C. Patel, *Ind. J. Chem.*, **8**, 840 (1970).

(22) A. L. Edwards, "Statistical Methods for the Behavioral Sciences," Rinehart, New York, N.Y., 1955.

(23) W. R. Walker and S. J. Beveridge, *Inorg. Perspect. Biol. Med.*, **2**, 93 (1979).

(24) M. Kato, H. B. Jonassen, and J. C. Fanning, *Chem. Rev.*, **64**, 99 (1964).

(25) P. A. Pella and W. C. Purdy, *J. Electroanal. Chem.*, **10**, 51 (1965).

(26) J. N. Gaur and M. M. Palrecha, *J. Inst. Chem. (India)*, **41**, 25 (1969).

ACKNOWLEDGMENTS

Supported by the Miller-Dwan Medical Center Foundation and the Faculty Development Fund of the College of St. Scholastica.

NOTES

Quantitation of the Antineoplastic Agent Indicine-*N*-oxide in Human Plasma by Differential Pulse Polarography

MADELINE McCOMISH, ITAMAR BODEK, and ALAN R. BRANFMAN ˣ

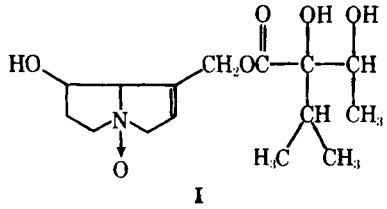
Received September 27, 1979, from *Arthur D. Little, Inc., Acorn Park, Cambridge, MA 02140.* Accepted for publication January 14, 1980.

Abstract □ A sensitive and reproducible differential pulse polarographic method of analysis was developed for indicine-*N*-oxide (NSC 132319) in human plasma. Lyophilized plasma is extracted with methanol, and the extract is chromatographed over partially deactivated aluminum oxide and reversed-phase silica gel columns. Indicine-*N*-oxide is eluted from the silica gel column with 25% aqueous methanol and quantitated by differential pulse polarography by measurement of the peak current at -0.72 ± 0.03 v (*versus* the saturated calomel electrode). Recovery of indicine-*N*-oxide from plasma was $88 \pm 7\%$ (*SD*) in the 1–20-μg/ml range. The method was linear over the range of 0.5–10 μg/ml of pH 4.6 buffer.

Keyphrases □ Indicine-*N*-oxide—quantitation by differential pulse polarography, human plasma □ Antineoplastics—indicine-*N*-oxide, quantitation by differential pulse polarography, human plasma □ Polarography, differential pulse—indicine-*N*-oxide, quantitation in human plasma

Indicine-*N*-oxide (I) (NSC 132319) is an antineoplastic agent (1) that recently entered clinical trials. The purpose of this investigation was to develop a polarographic method of analysis for use in clinical studies on the physiological disposition of the drug. Assays based on GLC (2) and GLC–mass spectrometry (3–5) have been reported. These assays required derivatization of either indicine-*N*-oxide or its reduced form to a volatile species prior to analysis. The method reported here for the analysis of the drug in biological samples does not require derivatization and is not subject to interference from the reduced form.

With recent advances in instrumentation (6), differential pulse polarography has become a rapid and inexpensive technique. A quantitative polarographic assay was developed in these laboratories for indicine-*N*-oxide in human plasma that combines sufficient sensitivity, reproducibility, and ease of operation for the potential routine analysis of suitable clinical samples (7).



I

0022-3549/80/0600-0727$01.00/0

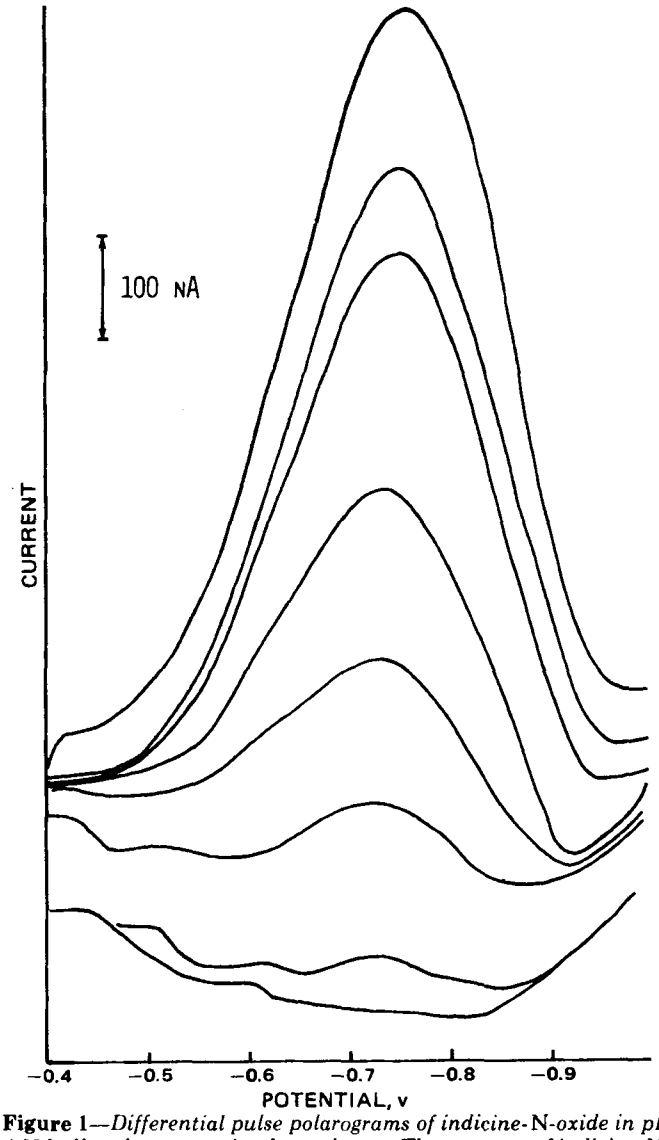


Figure 1—*Differential pulse polarograms of indicine-N-oxide in pH 4.63 buffer after extraction from plasma. The amounts of indicine-N-oxide added to 1 ml of plasma were 0 (plasma blank), 1, 3, 6, 9, 13, 15, 17, and 20 μg. A scale change to 2 μamp was necessary to record the 20-μg samples.*

EXPERIMENTAL

Conditions for Polarographic Analysis—Differential pulse polarograms were run on a polarographic analyzer[1] equipped with a drop timer and used in conjunction with a three-electrode polarographic cell comprised of a dropping mercury electrode, a saturated calomel electrode, and a platinum wire as the auxiliary electrode. The mercury drop time was 0.5 sec using a mercury column 70 cm in height. The current range typically was 1–2 μamp for a peak response of full-scale deflection, and the scan rate was 5 mv/sec.

Argon, cleansed of oxygen by passage through an acidic chromium(II) chloride solution, was used to deoxygenate the sample solutions. While polarograms were recorded, argon was passed continually over the surface of the solution. Samples were scanned from −0.4 to −1.3 v *versus* the saturated calomel electrode, and the differential pulse polarograms were recorded on an *x*–*y* recorder[2].

Reagents and Other Equipment—Indicine-*N*-oxide[3] and indicine[3] were used without further purification. The human plasma used contained the anticoagulant citrate dextrose. All other reagents were analytical grade and were used without further purification. Glass-distilled methanol[4] and deionized water[5] were used in the extraction. Standard buffer solutions[6] (pH 4.63, 5, 6, 7, and 8) were made with distilled water.

Table I—Effect of pH on the Position and Intensity of the Differential Pulse Polarographic Maxima of Indicine-*N*-oxide

pH	E_p[a], v	Intensity at E_p, μamp/μg/ml
1.65[b]	−0.70	0.133
4.63[c]	−0.735	0.141
5.00[d]	−0.775	0.141
5.60[d]	−0.830	0.097
6.00[d]	−0.860	0.091
7.00[e]	−1.08	0.050
8.00[e]	—[f]	~0

[a] Reduction peak potential. [b] 0.05 *M* HCl. [c] 0.05 *M* Sodium acetate–acetic acid. [d] 0.05 *M* Potassium biphthalate. [e] 0.05 *M* Monobasic potassium phosphate–dibasic potassium phosphate. [f] No wave detected.

Standard Solutions—Stock solutions for the preliminary work and standard curves were prepared containing indicine-*N*-oxide in water at 15.8 (5×10^{-2} *M*) and 0.15 (4.8×10^{-4} *M*) mg/ml. Aliquots of the stock solutions (10–200 μl) were added to 15 ml of the appropriate buffers. Most quantitative work was done in the pH 4.63 buffer and with a 2-ml cell. Biological samples were prepared by the addition of suitable aliquots of drug from an aqueous stock solution containing 0.5 mg/ml. Standard curves were prepared by plotting the peak current, corrected for the blank, at −0.72 ± 0.03 v (saturated calomel electrode) *versus* concentration.

Procedure for Analysis in Human Plasma—A known concentration of indicine-*N*-oxide was added to 1 ml of human plasma contained in a 15-ml glass-stoppered centrifuge tube, and the mixture was vortexed for 30 sec. The glass stopper was removed, the tube was covered with a thin film of silanized glass wool, and the mixture was lyophilized[7] overnight. After lyophilization, 3 ml of methanol was passed through the glass wool cover. The glass wool cover then was discarded, the glass stopper was replaced, and the mixture was vortexed for 30 sec. After centrifugation at 700×*g* for 10 min, the supernate was drawn off and set aside.

Methanol (3 ml) was added to the residue, and the mixture again was vortexed and centrifuged as described. The resulting supernate was combined with that from the previous extraction and evaporated under a nitrogen stream to ~1 ml. This 1-ml volume was loaded onto a 3.5-cm column of partially deactivated aluminum oxide[8] (94.5% aluminum oxide–5.5% water, w/w) packed in methanol in a disposable Pasteur pipet. The column was eluted with an additional 10 ml of methanol, and the eluate was evaporated to dryness under a nitrogen stream. This residue was dissolved in 1 ml of water, and the container was washed with an additional 1 ml of water.

Both samples were loaded onto a C_{18} bonded silica gel column[9]. The water effluent was discarded, and 10 ml of 25% aqueous methanol was used to elute the indicine-*N*-oxide from this column. The aqueous methanol eluate was collected and concentrated to 3 ml under a nitrogen stream, and the concentrate was lyophilized overnight. The residue from lyophilization was dissolved in 2 ml of pH 4.6 buffer containing 0.01 *M* KCl. The sample was deoxygenated for 5–10 min and analyzed by differential pulse polarography using the previously described conditions. The final concentrations of indicine-*N*-oxide in the plasma samples used to determine drug recovery were 0, 1, 3, 6, 9, 13, 15, 17, and 20 μg/ml of plasma.

pKa Determinations—The pKa of indicine-*N*-oxide was determined (in duplicate) by titration with 1 *N* HCl at 23°. The *N*-oxide (120 mg) was dissolved in 10 ml of 0.05 *M* KCl (freed of carbon dioxide by bubbling argon through the reagent for 15 min). Hydrochloric acid (1 *N*) was added in 25-μl increments, and the pH was recorded with each increment. The pKa was determined at the point where half-neutralization occurred. The method was checked by determining the pKa of tris(hydroxymethyl)-aminomethane, which has a value of 8.07 at 25° (8) (found 8.04 at 25°).

TLC—TLC was carried out on precoated silica gel plates[10] (20 cm × 20 cm × 0.25 mm) with chloroform–methanol–ammonium hydroxide

[1] PAR model 174A, Princeton Applied Research Corp., Princeton, N.J.
[2] Hewlett-Packard model 7044A.
[3] Obtained from Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (indicine-*N*-oxide was lot 76-08-12-01).
[4] Burdick & Jackson, Muskegon, Mich.
[5] Milli-RO4 purification system, Millipore Corp., Bedford, Mass.
[6] Fisher Scientific Co., Medford, Mass.
[7] Model 10-100, The Virtis Co., Gardiner, N.Y.
[8] W200, neutral, supergrade 1, ICN Pharmaceuticals, Cleveland, Ohio.
[9] C_{18} Sep Pak, Waters Associates, Milford, Mass.
[10] EM Laboratories, Elmsford, N.Y.

Table II—Recovery of Indicine-*N*-oxide from Plasma Samples

Amount Added, μg	Amount Found[a], μg	Recovery, %
1	0.78 ± 0.04	78
3	3.07 ± 0.04	102
6	5.33 ± 0.07	89
9	7.99 ± 0.04	89
13	11.7 ± 0.1	90
15	12.9 ± 0.1	86
17	15.2 ± 0.1	90
20	16.1	81
Average		88 ± 7

[a] Average of duplicate samples.

(10:9:1) as the developing solvent. Components were visualized with Dragendorff's spray reagent (9).

RESULTS AND DISCUSSION

Solutions of indicine-*N*-oxide in pH 4.6 buffer exhibited a differential pulse polarographic maximum at −0.72 ± 0.03 v (saturated calomel electrode) (Fig. 1). The potential for this maximum was pH dependent, becoming more negative at higher pH values (Table I). Since similar reduction peak potentials and pH dependencies have been observed for other *N*-oxides (10–12), these data are consistent with reduction of an *N*-oxide moiety. In support of this reduction, no wave was detected for indicine (NSC 136052), the reduced form of indicine-*N*-oxide, under similar polarographic conditions. The pKa of indicine-*N*-oxide was 4.25 ± 0.02 at 23°. This value is consistent with values of other *N*-oxides, which are typically in the 4.3–5.4 range (10–12). The average purity of the drug was 95.9 ± 3% based on the number of millimoles of hydrogen ion added at the equivalence point to the millimoles of *N*-oxide added (based on the weight).

A pH of 4.63 was chosen for the analysis medium because the effect of pH on the peak potential in this region was minimal and the intensity of response was the greatest (Table I). A linear polarographic response was observed over a range of 0.5–10 μg/ml of pH 4.6 buffer. The standard curve parameters determined by a least-squares fit of the data indicated a response of 0.122 ± 0.002 μamp/μg/ml and a correlation coefficient of 0.9997.

Extraction of indicine-*N*-oxide from plasma samples was possible only after lyophilization. Both normal aluminum oxide and reversed-phase (C_{18} bonded silica gel) chromatographic columns were needed to remove other chemical species that gave polarographic responses in the same region as the drug. Qualitative confirmation of the drug in the methanolic extract of the lyophilized residue, the methanolic eluate from the aluminum oxide column, and the methanol–water eluate from the reversed-phase silica gel column was determined by TLC (R_f 0.41). Indicine-*N*-oxide was not retained to any significant extent by either column, as indicated by the recovery data.

Analysis of plasma spiked with known concentrations of indicine-*N*-oxide gave an overall recovery of 88 ± 7% (*SD*) (Table II) over a concentration range of 1–20 μg/ml of plasma. The plasma assay presented here is a reliable, sensitive method that is applicable for clinical use. The method is suitable for the analysis of the parent *N*-oxide in the presence of the known metabolic reduction product, indicine (2).

REFERENCES

(1) M. Kugelman, W. C. Liu, M. Axelrod, T. J. McBride, and K. V. Rao, *Lloydia*, **39**, 125 (1976).

(2) M. M. Ames and G. Powis, *J. Chromatogr.*, **166**, 519 (1978).

(3) J. V. Evans, A. Peng, and C. J. Nielsen, "26th Annual Conference on Mass Spectrometry and Allied Topics," American Society for Mass Spectrometry, 1978, abstr. MC3.

(4) J. V. Evans, S. K. Daley, and C. J. Nielsen, "27th Annual Conference on Mass Spectrometry and Allied Topics," American Society for Mass Spectrometry, 1979, abstr. WAMOA4.

(5) J. V. Evans, A. Peng, and C. J. Nielsen, *Biomed. Mass Spectrom.*, **6**, 38 (1979).

(6) J. H. Christie, J. Osteryoung, and R. A. Osteryoung, *Anal. Chem.*, **45**, 210 (1973).

(7) A. R. Branfman, I. Bodek, and M. McComish, "177th National Meeting of the American Chemical Society," Abstr. Anal. 022 (1979).

(8) "Handbook of Analytical Chemistry," L. Meites, Ed., McGraw-Hill, New York, N.Y., 1963, pp. 1–27.

(9) "Handbook of Chromatography," vol. II, CRC Press, Cleveland, Ohio, 1972, p. 113.

(10) A. H. Beckett, E. E. Essien, and W. F. Smyth, *J. Pharm. Pharmacol.*, **26**, 399 (1974).

(11) H. Hoffmann, *Arch. Pharm.*, **304**, 741 (1971).

(12) P. Jenner, *Xenobiotica*, **1**, 399 (1971).

ACKNOWLEDGMENTS

Supported by Contract NO1-CM-87163 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.

The authors thank Dr. C. J. Nielsen of the National Cancer Institute Frederick Cancer Research Center for helpful advice concerning the extraction of indicine-*N*-oxide from biological fluids and Dr. E. Modest of the Sidney Farber Cancer Institute for supplying the human plasma. Discussions with J. Valentine and T. Gilbert are gratefully acknowledged.

Synthesis of Phenylurethans of 1,2-Dialkyl-4-pyrazolidinols as Anticonvulsant Agents

MILTON J. KORNET

Received December 10, 1979, from the *College of Pharmacy, University of Kentucky, Lexington, KY 40506.* Accepted for publication January 18, 1980.

Abstract □ Twelve phenylurethans of 1,2-dialkyl-4-pyrazolidinols were synthesized and tested for anticonvulsant activity. Several compounds exhibited activity.

Keyphrases □ Phenylurethans—synthesized from 1,2-dialkyl-4-pyrazolidinols, evaluation as anticonvulsant agents □ Anticonvulsant activity—phenylurethans of 1,2-dialkyl-4-pyrazolidinols, synthesis and evaluation for activity □ 1,2-Dialkyl-4-pyrazolidinols—phenylurethan derivatives, synthesis and testing for anticonvulsant activity

4-Pyrazolidinyl benzoates, ester derivatives of 1,2-dialkyl-4-pyrazolidinols (I), reportedly possess local anesthetic activity (1). Since lidocaine (2) and many urethans (3) show anticonvulsant properties, synthesis and testing of a series of phenylurethans (III*a*–III*l*) of 1,2-dialkyl-4-pyrazolidinols as potential anticonvulsant agents were desired.

DISCUSSION

The necessary 1,2-dialkyl-4-pyrazolidinols (I) were obtained from the reaction of epichlorohydrin and 1,2-dialkylhydrazines according to a reported procedure (4). Hydrazinoalcohols possessing methyl (I*a*), ethyl (I*b*), and *n*-propyl (I*c*) substituents were prepared. The 1,2-dialkyl-4-

0022-3549/80/0600-0729$01.00/0