Englewood Cliffs, N.J., 1962.
(5) S. Prakongpan, W. I. Higuchi, K. H. Kwan, and A. M. Molokhia, *J. Pharm. Sci.*, **65**, 685 (1976).
(6) C. V. King and S. S. Brodie, *J. Chem. Soc.*, **59**, 1375 (1937).
(7) D. J. Merchant, R. H. Kant, and W. H. Murphy, Jr., "Handbook of Cell and Organ Culture," Burgess, Minneapolis, Minn., 1969.
(8) J. C. Drack and J. M. Novack, *Anal. Biochem.*, **52**, 633 (1973).
(9) J. H. Wang, *J. Chem. Soc.*, **74**, 1182 (1952).
(10) D. Dworkin and K. Keller, *J. Biol. Chem.*, **252**, 864 (1977).
(11) P. J. Niebergall and E. T. Sugita, *J. Pharm. Sci.*, **57**, 1805 (1968).
(12) S. Prakongpan, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1974, p. 89.
(13) H. Nogami, T. Nagai, and A. Suzuki, *Chem. Pharm. Bull.*, **14**, 329 (1966).
(14) A. Riddiford, in "Advances in Electrochemistry and Electrochemical Engineering," vol. 4, P. Delahay, Ed., Interscience, New York, N.Y., 1966.
(15) I. Sarkany and P. M. Gaylarde, *Trans. St. John's Hosp. Dermatol. Soc.*, **59**, 241 (1973).
(16) J. Cory and R. Suhadolnik, *Biochemistry*, **4**, 1733 (1965).
(17) O. D. Laerum, *J. Invest. Dermatol.*, **52**, 204 (1969).

GLC Assay of Verapamil in Plasma: Identification of Fluorescent Metabolites after Oral Drug Administration

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Abstract □ The fluorometric assay for verapamil in plasma is not useful after oral drug administration because of interference by inactive, but fluorescent, metabolites extracted along with the parent drug. A GLC method using a flame-ionization detector after silylation allows the separation of unchanged active verapamil from the metabolites and quantitation to a sensitivity of 0.025 μg/ml. After a single oral dose of drug in dogs, up to 80% of "fluorescent verapamil" represented inactive metabolites.

Keyphrases □ Verapamil—GLC analysis, plasma fluorometric analysis, metabolite interference □ GLC—analysis, verapamil in plasma □ Vasodilators—verapamil, GLC analysis in plasma

Verapamil, 5-[(3,4-dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (I), is an experimental antiarrhythmic and antianginal agent (1–5) and is currently undergoing clinical evaluation in the United States. Its pharmacological activity is related to suppression of transmembrane calcium fluxes in cardiac and other tissues (6, 7). These "slow" calcium currents may be involved in the genesis of fatal arrhythmias during myocardial ischemia and associated with sudden cardiac death (8).

BACKGROUND

A fluorometric assay for verapamil in plasma (9) has been used to determine its elimination kinetics in dogs after intravenous injection (10) and to demonstrate a linear correlation between plasma drug concentrations and both electrophysiologic and hemodynamic effects in dogs following systemic dosing (11). However, after oral administration of verapamil to dogs, no relationship between plasma level and effect was found (12), raising the possibility that metabolites generated during the first passage through the liver interfere with the fluorometric assay.

Verapamil is known to be metabolized extensively in rats and dogs, primarily to *O*- and *N*-dealkylated derivatives through loss of the 3,4-dimethoxyphenylethyl moiety (13). Schomerus *et al.* (14) used mass fragmentography to confirm the extensive metabolism of the drug in three normal human subjects after a single oral dose but did not identify the specific metabolites produced.

Since verapamil may become an important addition to the drugs available for treatment of cardiovascular disorders, the assay validity must be defined before further kinetic and pharmacological studies can be carried out. Two major metabolites of verapamil, 5-amino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (D620) (II) and 5-methylamino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (D617) (III)[1], were studied to determine possible interference with the fluorometric assay for parent verapamil. Furthermore, a GLC procedure specific for unchanged verapamil in the presence of II or III was developed and is described.

EXPERIMENTAL

Fluorometric Assay—The fluorometric assay for verapamil in plasma (9) involves extraction of drug from alkaline plasma with heptane and back-extraction into acid. Fresh plasma from normal drug-free human subjects was pooled. To one group of aliquots, verapamil was added to achieve concentrations of 0.1, 0.5, and 1.0 μg/ml; to two other aliquots, II or III were added similarly. All samples were prepared in triplicate.

The fluorometric assay was performed on each sample by an experienced technician under blind conditions, and the resulting fluorescence at an excitation/emission spectra of 275/310 nm was recorded[2]. The calculated recovery from these standards ranged from 85 to 94%.

To additional aliquots of plasma from the same pool, combinations of verapamil and II and III were added, each in a concentration of 0.5 μg/ml. The assay was carried out as previously described, and total fluorescence was determined[2].

GLC Assay—All reagents and solvents were of analytical grade, and glassware was cleaned by superheating after a distilled water rinse (15). Fresh plasma was collected from normal human volunteers as well as from drug-free mongrel dogs; no differences were found between the two for the work described here, and the term "plasma" used here may apply equally to either species.

Known amounts of verapamil and/or II and III were added to 2-ml plasma samples. After addition of 1.0 ml of 1 *N* NaOH and 5.0 ml of heptane, the mixture was shaken for 15 min and centrifuged at 1800 rpm for 10 min. Four milliliters of the heptane layer was transferred to a 15-ml centrifuge tube containing 2.0 ml of 0.1 *N* HCl; each tube was shaken and centrifuged as before. The heptane layer was aspirated and discarded, and the aqueous phase was washed twice with 2.0 ml of heptane. One milliliter of 1.0 *N* NaOH was added, and the acid layer was extracted with 4.0 ml of heptane, with agitation and centrifugation as before.

Three milliliters of the heptane layer was then transferred to another tube and evaporated under a nitrogen stream during heating to 60° on

[1] Kindly supplied by Prof. K. Hahn, Knoll AG, Ludwigshafen, West Germany.

[2] Aminco-Bowman spectrophotofluorometer with ratio photometer unit, American Instrument Co.

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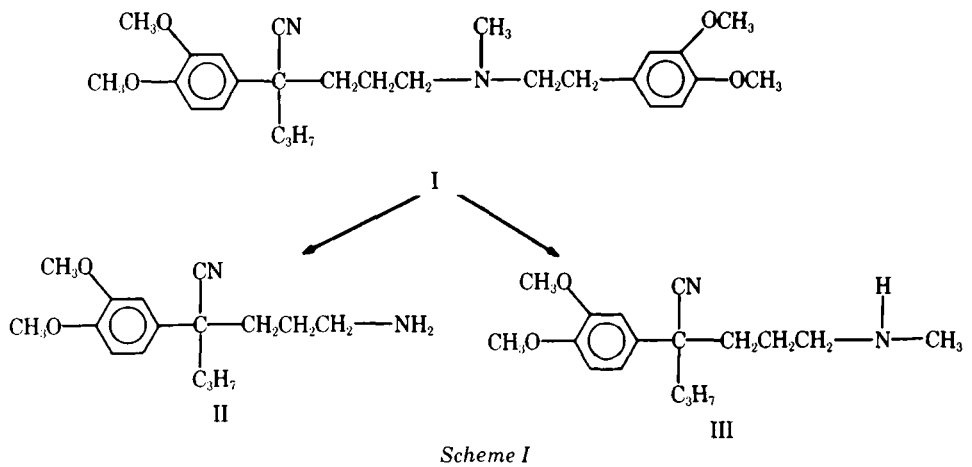

Scheme I

a waterbath. To the residue was added 100 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (IV)[3], with heating for 1 hr at 65–70°. After cooling, evaporation was again effected under a nitrogen stream; the sample was then reconstituted with 25 μl of methanol, and 5-μl amounts were injected into the gas chromatograph.

The gas chromatograph[4] was equipped with a glass column (1.8 m × 2 mm i.d.) packed with 3% Dexsil-300[3] on 100–200-mesh WHP[3]. The column was silanized and conditioned at 325° for 72 hr prior to use. The conditions were as follows: carrier gas (nitrogen) flow rate, 30 ml/min; inlet temperature, 310°; column temperature, 270°; and detector temperature, 350°. The flame-ionization unit was supplied with hydrogen at 30 ml/min and with air at 300 ml/min. Detector responses were recorded[5], and peak areas were integrated electronically[6].

Animal Study—Verapamil (3 mg/kg in 30 ml of water) was administered by nasogastric tube to a healthy 20-kg mongrel dog which had been fasted overnight. Serial blood samples were collected over the following 4 hr. Plasma was analyzed for verapamil by the fluorometric method (9) and the GLC technique described here.

RESULTS AND DISCUSSION

Fluorometric Assay—The fluorescence of verapamil, II, and III is shown in Fig. 1. Both metabolites fluoresced maximally at the same excitation/emission wavelength combination as the parent drug, and both were extracted with high recoveries (80–90%) in the plasma–heptane–acid procedure used for verapamil. The potential for interference by these metabolites with the fluorometric verapamil assay is illustrated in Fig. 2; various combinations of the compounds were extracted from plasma, and total fluorescence was determined. Both II and III showed a concentration–fluorescence intensity relationship similar to that for unchanged verapamil; addition of one or both metabolites to plasma containing the parent drug resulted in a proportionate increase in total fluorescence.

The pharmacological activity of these fluorescent metabolites has not

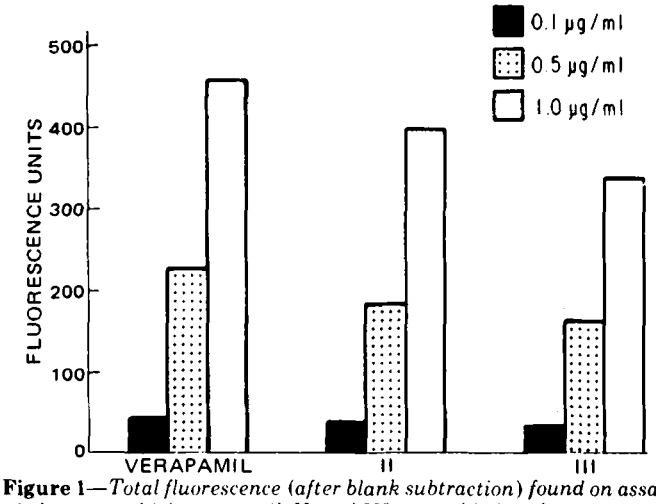

Figure 1—*Total fluorescence (after blank subtraction) found on assay of plasma to which verapamil, II, and III were added in the concentrations shown.*

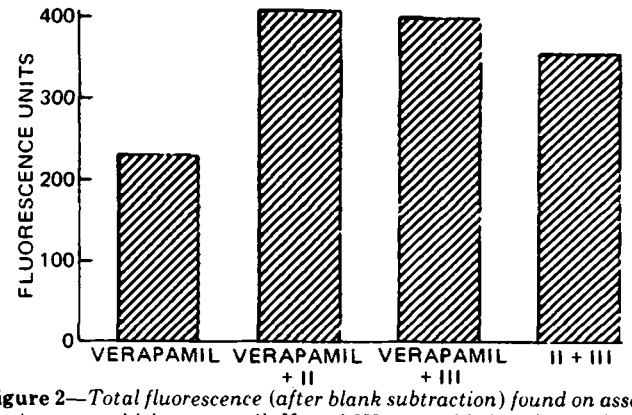

Figure 2—*Total fluorescence (after blank subtraction) found on assay of plasma to which verapamil, II, and III were added in the combinations indicated; 0.500 μg of each compound/ml was present.*

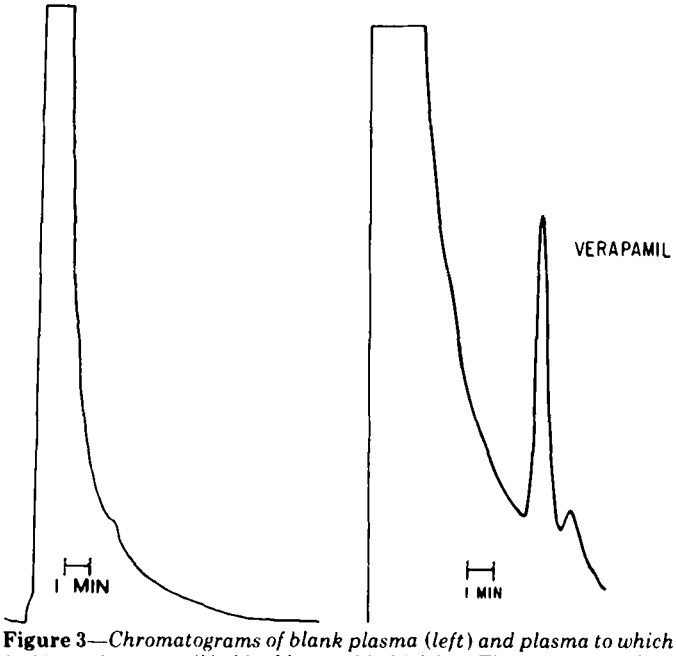

Figure 3—*Chromatograms of blank plasma (left) and plasma to which 0.100 μg of verapamil/ml had been added (right). The assay procedure is described in the text.*

[3] Pierce Chemical Co., Rockford, Ill.
[4] Model 3700 dual-column gas chromatograph, Varian Co., Walnut Creek, Calif.
[5] Model 9176 recorder, Varian Co., Walnut Creek, Calif.
[6] CDS 111 integrator, Varian Co., Walnut Creek, Calif.

Table I—Linearity and Precision of Plasma Verapamil Concentrations Measured by GLC

Plasma Concentration, μg/ml	Peak Area[a], mean ± *SD* × 10^4	*CV*, %
0.025	0.514 ± 0.056	10.8
0.050	1.013 ± 0.106	10.4
0.100	2.030 ± 0.168	8.3
0.250	6.531 ± 0.521	7.9
0.500	12.123 ± 0.885	7.3
1.000	24.674 ± 1.517	6.1

[a] $n = 6$ at each plasma concentration.

Figure 4—*Chromatograms of plasma to which II or III was added, using a column temperature of 220°. Both compounds were added in a concentration of 0.100 μg/ml.*

been specifically determined. However, since the concentration of plasma verapamil determined fluorometrically is linearly related to the drug's effects after intravenous administration (10, 11) but not after oral administration (12), these (and possibly other) fluorescent metabolites probably are generated during the first passage through the liver and are either inactive or have little pharmacological effect. In any event, the fluorometric assay for verapamil in plasma is not a valid reflection of the concentration of active drug in the presence of drug metabolites after oral dosing.

GLC Assay—Under the conditions described, verapamil could be extracted from plasma with good recoveries (range of 89–96%) in a concentration range of 0.025–1.000 μg/ml. Figure 3 shows chromatograms of blank plasma and of plasma containing 0.100 μg of added verapamil/ml. The retention time for verapamil was 7.3 min. Table I gives data on the reproducibility and sensitivity of the GLC procedure. The assay was linear over the concentration range studied ($r = 0.9995$; $p < 0.001$), which reflects the plasma drug concentrations likely to be of clinical interest (10, 11).

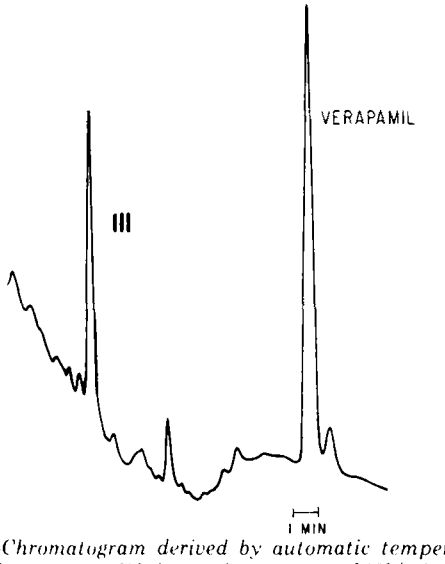

Figure 5—*Chromatogram derived by automatic temperature programming, from 190 to 270°, increasing at a rate of 10°/min. Compound III eluted at a column temperature of approximately 220°, while verapamil emerged at 270°.*

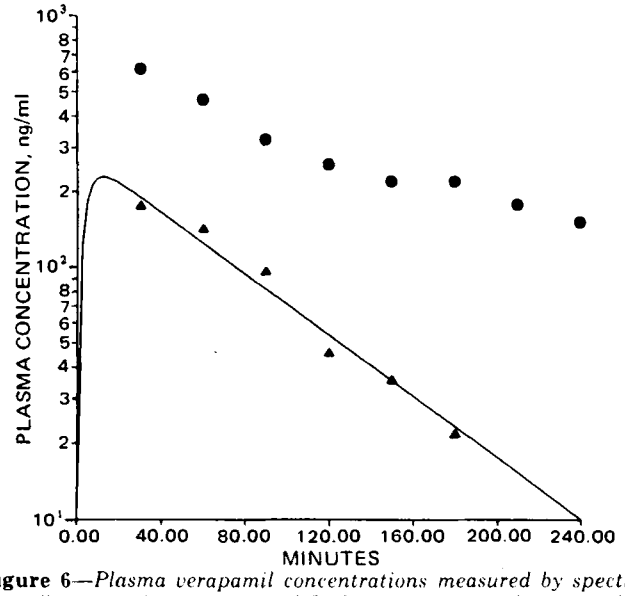

Figure 6—*Plasma verapamil concentrations measured by spectrophotofluorometric assay (●) and GLC (▲); serial samples were taken from a 20-kg mongrel dog given 3 mg of verapamil/kg at time zero.*

Of the two metabolites studied, II did not appear to form a silyl derivative and could not be detected under the GLC conditions used. Compound III, however, was easily and quantitatively detected at a column temperature of 220° (Fig. 4). Automatic temperature programming, beginning at a column temperature of 190° and increasing at 10°/min, could be used to detect both III and parent verapamil in the same plasma sample (Fig. 5). At the column temperature of 270° used to measure parent verapamil concentrations, III emerged with the solvent front.

Since this assay is specific for unchanged verapamil, it will be particularly useful in pharmacokinetic and pharmacodynamic studies of the drug after oral dosing. For example, when both spectrophotofluorometric and GLC techniques were used to measure serial drug plasma levels in a dog given a single oral dose of verapamil, fluorometric analysis gave results four- to fivefold higher than those measured by GLC, indicating the presence of metabolites interfering with the fluorometric procedure (Fig. 6).

On the basis of these and prior studies (10–12), it appears that the fluorometric assay for plasma verapamil is a valid reflection of the concentration of unchanged, active drug only after intravenous verapamil administration. After oral doses, a more specific assay, such as the described GLC procedure, must be used to quantitate the parent compound.

REFERENCES

(1) B. N. Singh and E. M. Vaughan Williams, *Cardiovasc. Res.*, **6**, 109 (1972).

(2) F. Hagemeijer, *Circulation*, **57**, 751 (1978).

(3) L. Schamroth, D. M. Krikler, and C. Garrett, *Br. Med. J.*, **1**, 660 (1972).

(4) W. G. Nayler, A. Grau, and A. Slade, *Cardiovasc. Res.*, **10**, 650 (1976).

(5) O. Parodi, I. Simonetti, and A. Maseri, *Circulation (Suppl. III)*, **55–56**, 224 (1977).

(6) M. R. Rosen, J. P. Ilvento, H. Gelband, and C. Merker, *J. Pharmacol. Exp. Ther.*, **189**, 414 (1974).

(7) A. L. Wit and P. R. Cranefield, *Circulation (Suppl. III)*, **49–50**, 146 (1974).

(8) B. Surawicz, *Am. J. Cardiol.*, **33**, 689 (1974).

(9) R. G. McAllister and S. M. Howell, *J. Pharm. Sci.*, **65**, 431 (1976).

(10) R. G. McAllister, D. W. A. Bourne, and L. W. Dittert, *J. Pharmacol. Exp. Ther.*, **202**, 38 (1977).
(11) L. M. Mangiardi, R. Hariman, R. G. McAllister, V. Bhargava, B. Surawicz, and R. Shabetai, *Circulation*, **57**, 366 (1978).
(12) A. L. Cornish, H. G. Hanley, T. G. Tan, T. A. Patrick, and R. G. McAllister, *Clin. Res.*, **26**, 288 (1978).
(13) H. M. McIlhenny, *J. Med. Chem.*, **14**, 1178 (1971).
(14) M. Schomerus, B. Spiegelhalder, B. Stieren, and M. Eichelbaum, *Cardiovasc. Res.*, **10**, 605 (1976).
(15) S. M. Howell and R. G. McAllister, *Lab. Med.*, **7**, 34 (1976).

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Stability-Indicating High-Performance Liquid Chromatographic Determination of Chlorpropamide, Tolbutamide, and Their Respective Sulfonamide Degradates

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Abstract □ A quantitative high-performance liquid chromatographic method for the determination of chlorpropamide, tolbutamide, and their respective hydrolysis products, *p*-chlorobenzenesulfonamide and *p*-toluenesulfonamide, in solid dosage forms was developed. The method is stability indicating and can be used to determine the sulfonamide hydrolysis product and the intact drug in the presence of minor degradates. Method reproducibility, demonstrated by repeated injections of a calibration standard, was 1.21%. The lower limit of quantitation of the hydrolysis products, *p*-chlorobenzenesulfonamide and *p*-toluenesulfonamide, was 0.2 μg/5-μl injection. The accuracy of the method for intact drugs was determined by comparison of the HPLC results to those obtained by the appropriate USP or BP assays. The mean of the results obtained by the two methods differed by 0.7% for chlorpropamide and 0.3% for tolbutamide. Pure drug samples were spiked with amounts of the hydrolysis products ranging from 20 to 120% of the intact drug content. The mean percent recovery for *p*-chlorobenzenesulfonamide was 98.6%; for *p*-toluenesulfonamide, it was 100.6%. A qualitative TLC procedure for the detection of chlorpropamide, *p*-chlorobenzenesulfonamide, dipropylurea, propylurea, *n*-propylamine, tolbutamide, *p*-toluenesulfonamide, dibutylurea, butylurea, and *n*-butylamine is also described.

Keyphrases □ High-performance liquid chromatography—analysis of chlorpropamide, tolbutamide, *p*-chlorobenzenesulfonamide, *p*-toluenesulfonamide, stability in solid pharmaceutical dosage forms □ Chlorpropamide—high-performance liquid chromatographic analysis of stability in solid pharmaceutical dosage forms, sulfonamide degradates □ Tolbutamide—high-performance liquid chromatographic analysis of stability in solid pharmaceutical dosage forms, sulfonamide degradates □ Sulfonamides—degradation products of chlorpropamide and tolbutamide, high-performance liquid chromatographic analysis □ Antidiabetic agents—chlorpropamide, tolbutamide, high-performance liquid chromatographic analysis of stability and sulfonamide degradation products

The antidiabetic agents chlorpropamide and tolbutamide decompose under various experimental conditions. The major degradation route is hydrolysis (1, 2), mainly to *p*-chlorobenzenesulfonamide or *p*-toluenesulfonamide, but thermal dissociation in various solvents has been reported also (3, 4).

The USP XIX (5) spectrophotometric assay for tablets containing these drugs lacks specificity since the sulfonamide degradates retain the UV chromophore of the intact drug. The BP 1973 (6) titrimetric procedures are selective because the sulfonamide degradates are weak acids that are not neutralized by the aqueous solution of sodium hydroxide. However, the method cannot be used to assay highly discolored samples due to interference in the visual detection of the end-point.

Colorimetric procedures have been reported (2, 7, 8), but these methods require prior separation of the degradates before the intact drug can be quantitated. A high-performance liquid chromatographic (HPLC) method for the quantitation of sulfonylureas in pharmaceuticals has been reported (9), but separation and quantitation of the hydrolysis products were not demonstrated. An HPLC procedure for the determination of chlorpropamide in tablet formulations was described (10) but was not suitable for resolution of the sulfonamide from the main drug.

This paper describes a TLC technique for the detection of the major degradates of chlorpropamide and tolbutamide and an HPLC assay of intact chlorpropamide, tolbutamide, and their sulfonamide impurities, *p*-chlorobenzenesulfonamide and *p*-toluenesulfonamide, in solid dosage formulations.

EXPERIMENTAL

Materials—Chlorpropamide[1] and tolbutamide[2] were recrystallized from acetone and found to meet USP XIX (5) specifications. *p*-Chlorobenzenesulfonamide[3], *p*-toluenesulfonamide[4], *n*-propylurea[3], *n*-butylurea[4], *n*-dipropylurea[5], *n*-dibutylurea[6], *n*-propylamine[7], *n*-butylamine[3], micronized prednisone[8], acetic acid[9], hydrochloric acid[9], chlo-

[1] F. W. Horner Ltd., Montreal, Quebec, Canada.
[2] Paul Maney Laboratories, Toronto, Ontario, Canada.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Eastman Organic Chemicals, Rochester, N.Y.
[5] Authentic samples, B.P. Commission.
[6] K & K Laboratories, Plainsview, N.Y.
[7] J. T. Baker Chemical Co., Phillipsburg, N.J.
[8] Roussel (Canada) Ltd., Montreal, Quebec, Canada.
[9] Analytical reagent grade.

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