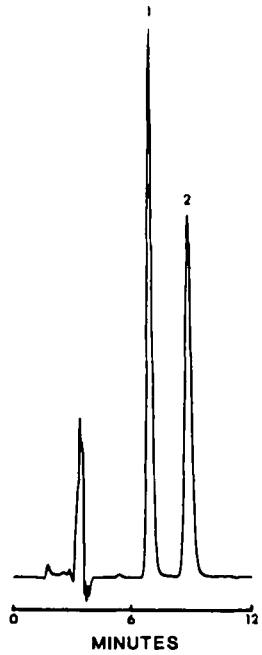

Figure 2—*Chromatogram of sodium levothyroxine and sodium liothyronine; detector at 229 nm and 0.02 AUFS. Key: (1) sodium liothyronine; (2) sodium levothyroxine, each at a level of ~4 μg/mL.*

The chromatographic system parameters were adequate to separate sodium levothyroxine from sodium liothyronine for testing the bulk drug substances for impurities; Fig. 2 shows a chromatogram of this separation. Liothyronine was found at levels of 0.04–0.96% in five sodium levothyroxine bulk drug samples analyzed by HPLC.

Sample Analysis—Table I lists the results obtained from survey by the HPLC procedure. A comparison of results for composite samples, obtained with the HPLC and the USP XX methods, shows that the latter gave a higher result in practically every case. This is not surprising since the USP XX assay is nonspecific for levothyroxine and measures total iodine content. However, the difference in assay values could not be totally accounted for by a total area summation of HPLC peaks. The major impurities and degradation products probably are not eluted from the column with this mobile phase.

Low assay values were a problem experienced by most manufacturers; this problem would not be recognized if the assays were based only on the results from the USP method. Some tablet composite samples gave suitable assay values for total iodine by the USP method, but gave extremely low assays for sodium levothyroxine by HPLC. All samples which gave low assay values by HPLC gave suitable assays by the USP method. This fact indicates that the problems of low assays of marketed sodium levothyroxine are, in all probability, attributable to sodium levothyroxine instability.

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Determination of Isosorbide 5-Mononitrate in Human Plasma by Capillary Column Gas Chromatography

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Received February 22, 1983, from the *University of Bern, Department of Medicine, Inselspital, CH-3010 Bern, Switzerland.* Accepted for publication August 25, 1983.

Abstract □ An electron-capture gas chromatographic method for the determination of isosorbide 5-mononitrate in human plasma using a capillary column is described. Isosorbide 5-mononitrate and the internal standard (isosorbide dinitrate) are extracted from the alkalinized plasma with ether. The lower limit of detection for isosorbide 5-mononitrate is 1 ng/mL of plasma.

Keyphrases □ Gas chromatography—isosorbide 5-mononitrate, human plasma □ Isosorbide 5-mononitrate—GC, human plasma

Isosorbide 5-mononitrate [1,4:3,6-dianhydro-D-glucitol 5-nitrate (I)] is the primary metabolite of isosorbide dinitrate [1,4:3,6-dianhydro-D-glucitol dinitrate (II)] which has been used for many years in the treatment of angina pectoris and congestive heart failure. Recent studies of the hemodynamic effect of the mononitrate (1) indicate that, after acute administration, cardiac work load decreases at rest and during exercise. Pharmacokinetic studies (2–4) showed that the mononitrate is rapidly and completely absorbed from the GI tract without undergoing first-pass elimination. The maximum concentrations were reached within 1 h after oral administration, and the substance was eliminated with a half-life of ~4 h. Thus, the mononitrate has a half-life which is at least four times as long as the half-life of the dinitrate (5). Published gas chromatography (GC) assays (2, 6, 7), using older column

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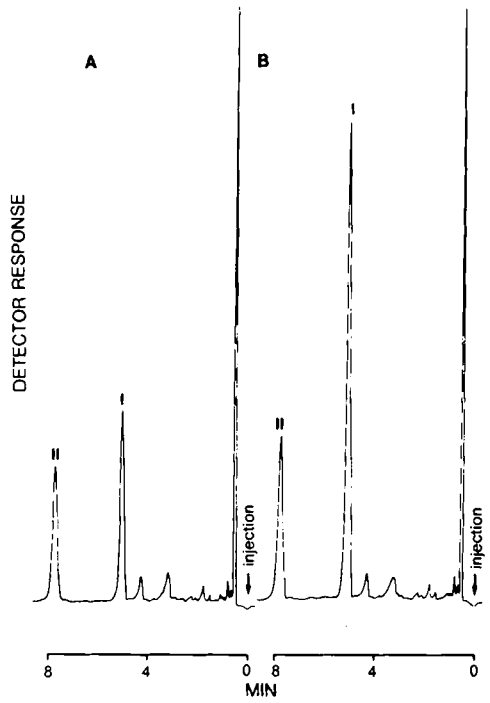

Figure 1—*Chromatograms of 1-mL human plasma extracts. Key: (A) plasma spiked with 25 ng of isosorbide 5-mononitrate (I) and 20 ng of isosorbide dinitrate (II); (B) plasma spiked with 50 ng of I and 20 ng of II.*

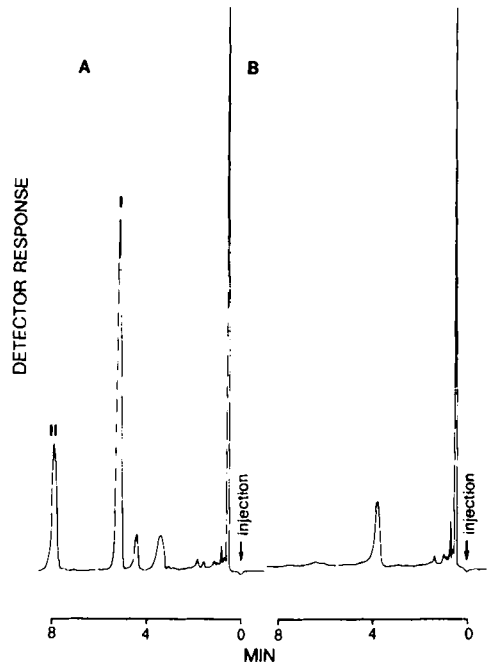

Figure 2—*Chromatograms of 1-mL plasma sample extracts obtained from a subject before (B) and 40 min after (A) an infusion of 5 mg of isosorbide 5-mononitrate; 20 ng of isosorbide dinitrate was added as the internal standard (A).*

coating or packing (OV-17, QF_1) and complicated extraction procedures, lack sufficient sensitivity and fail to give reproducible results.

EXPERIMENTAL SECTION

Apparatus—The analysis was performed on a GC[1] with a Grob-type, nonvaporizing, specially cooled on-column injector and equipped with a nickel-63 electron-capture detector.

Column—A wall-coated open tubular (WCOT) capillary column (16 m × 0.33 mm i.d.), with immobilized stationary phase SE-54[2], was used. Acidic leaching, silylation, and static coating, including immobilization, was carried out according to the method of Grob and Grob (8, 9). The film thickness was ~0.8 μm.

Reagents—The ethyl acetate[3] was pesticide grade, while the ether[3] and potassium carbonate[4] were analytical grade. Compounds I and II were used as received[5].

Instrumental Conditions—The carrier gas was helium with a flow rate of ~4 mL/min. The detector was operated with argon gas and 5% methane (v/v) at a flow rate of ~20 mL/min. The column was 160°C, the injector port was 200°C, and the electron-capture detector was 225°C. Direct on-column injection was used.

Procedure—Isosorbide dinitrate [the internal standard (20 ng in 50 μL of 0.9% NaCl)], 1 mL of patient plasma, 2 mL of saturated solution of potassium carbonate (pH 12.2), and 2 mL of ether were shaken in a 10-mL glass tube, with a polytef-lined screw cap, for 40 min at low speed (~25 rpm). After centrifugation (10 min at 4000 rpm) the organic phase was removed by aspiration in a conical tube. The solvent was evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was redissolved in 10–50 μL of ethyl acetate to give the desired concentration, and 0.4 μL of the solution was injected. All glassware was cleaned in an ultrasonic bath but not silanized; no plastic material was used.

Calibration—For calibration, human plasma (1 mL) was spiked with increasing concentrations of I (5–400 ng in 50 μL of 0.9% NaCl) and the internal standard, II (20 ng in 50 μL of 0.9% NaCl). Two milliliters of a saturated solution of potassium carbonate and 2 mL of ether were added to each solution. Extraction was performed as described above. The ratios of the peak heights of the mononitrate to the internal standard were plotted *versus* the concentrations of the mononitrate. Values of unknown plasma drug concentrations were determined from this calibration curve. Calibration chromatograms are shown in Fig. 1.

RESULTS AND DISCUSSION

This GC assay provides a method for determining I in plasma following a single extraction. A linear calibration curve, determined daily, was obtained for concentrations between 5 and 200 ng/mL of plasma. The mean slope was 0.058 ± 0.001 (peak height ratio/ng·mL^{-1} ± *SD*) and the mean intercept was 0.077 ± 0.09 (peak height ratio ± *SD*). The reproducibility from day-to-day analyses gave a coefficient of variation of 3.03% for 10 control plasma samples of 78 ng/mL of the mononitrate (78.71 ± 3.9), which were assayed on different days over 2 months. Within-day precision was assessed by analyses of five replicate plasma samples to which 10 and 50 ng/mL of I were added. The coefficients of variation were 1.6% for 10 ng (10.30 ± 0.165) and 1.49% for 50 ng (49.61 ± 0.793).

The use of a chemically similar drug as the internal standard, the quality of our column, and the on-column injection technique are probably the main reasons for the good reproducibility of the results. The attempted use of glyceryl trinitrate as internal standard was a failure because of its instability.

The recoveries for I and II were similar, varying between 70 and 75%. The retention times were 5.2 min for the mononitrate (mp 89°C) and 7.8 min for the dinitrate (mp 71°C). The lower limit of detection was ~1 ng/mL of plasma for the mononitrate and 0.5 ng/mL for the dinitrate. In some cases, small amounts of the mononitrate (<5 ng) were difficult to determine due to an unknown interfering peak. The use of peak height ratios was sufficient because the calibration curve passed through the origin and was linear up to 200 ng/mL. The column was washed every month with dichloromethane[3] and pentane[3], analytical grade.

Two chromatograms of human plasma extracts are shown in Fig. 2: a blank plasma extract and a plasma extract from the same subject 40 min after an infusion of 5 mg of the mononitrate. The dinitrate was added as an internal standard before analysis. Apart from an unknown peak with the retention time of 4.3 min (which always occurred), there is a second peak with about the same retention time as isosorbide 2-mononitrate (3.2 min). This peak, due to a contaminant in the internal standard, was eliminated later by recrystallization of isosorbide dinitrate.

Further investigations showed that this method could easily be adapted to determine the concentrations of the dinitrate and isosorbide 2-mononitrate by using the chemically similar substances isomannide dinitrate and isoidide dinitrate as internal standards, respectively. The use of pure solvents for ex-

[1] Fractovap 4160 series; Carlo Erba, Milano, Italy.
[2] Supelco, Bellefonte, Pa.
[3] Merck, Darmstadt, Federal Republic of Germany.
[4] Fluka, Buchs SG, Switzerland.
[5] A gift of the Schweizerische Sprengstoffabrik Dottikon, Switzerland.

traction and chromatography is essential for good results. The capillary column with an immobilized stationary phase and the possibility of solvent washing was also a big advantage in this assay.

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Binding of Sulfadimethoxine to Isolated Human Blood Protein Fractions

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Abstract □ The binding of sulfadimethoxine to selected human blood protein fractions and to fresh serum has been examined by means of a new equilibrium dialysis technique which minimizes experimental error and permits the evaluation of low-level binding. Certain α-globulin fractions, containing mixtures of proteins, were found to bind the drug. Scatchard analysis of the binding of sulfadimethoxine to fresh serum, calculated as though all of the binding is due to albumin, gives a different result from that obtained with isolated albumin. This may be a reflection of the contribution of the α-globulins to the overall binding of sulfadimethoxine in fresh serum. Although sulfadimethoxine is amphoteric, it did not bind to the α_1-acid glycoprotein. The drug behaves as an acidic compound when binding to the blood proteins.

Keyphrases □ Sulfadimethoxine—blood fraction binding, equilibrium dialysis, humans □ Blood fraction binding—humans, sulfadimethoxine, equilibrium dialysis

Sulfadimethoxine is a long-acting sulfanilamide antibacterial drug which is extensively bound to the proteins of the blood (1, 2). It has been shown that the antibacterial activity and rate of metabolic *N*-acetylation of sulfanilamide drugs depend on the concentration of free, unbound drug in the plasma (3, 4). Nonlinearity in the pharmacokinetics of sulfadimethoxine has been associated with dose-dependent changes in the percentage of the drug bound to plasma proteins (5). Furthermore, the volume of distribution of sulfadimethoxine is altered significantly by small changes in the concentration of plasma albumin (6).

Albumin is the most important of the blood proteins in the binding of sulfanilamide drugs (2, 6–11), although the involvement of other plasma proteins has been implied (12). Sulfadimethoxine is an amphoteric compound (13) with an acidic center at the sulfonamide (pK_a 6.7) and a basic center at the aniline-like primary amino group (pK_b 11.98). The isoelectric point is at pH 4.36. Albumin is effective in binding acidic drugs such as warfarin (14–16), fenoprofen (17, 18) and phenylbutazone (19). Unlike these compounds, basic drugs such as imipramine (20), alprenolol (21), propranolol, (22) and lidocaine (23) bind significantly to α_1-acid glycoprotein and lipoproteins in addition to albumin. The individual contributions of different proteins to the binding of drugs becomes significant in disease states which lead to changes in the concentration of one or more of the blood proteins (24–27). The present study was undertaken to determine whether proteins other than albumin are involved in the overall binding of the amphoteric drug sulfadimethoxine.

The binding of sulfadimethoxine to isolated human plasma protein fractions was measured by a newly developed equilibrium dialysis technique (28). This method employs calibration and control procedures that minimize artifactual errors and permit statistical evaluation of control and test data. This stringent control technique also permits correction to be made for concentration-dependent binding to the dialysis membrane or other equipment; thus, it is possible to demonstrate statistically significant binding at low levels (<10%).

EXPERIMENTAL SECTION

Equilibrium dialysis was performed using 20-cm strips of dialysis tubing[1], 1-cm diameter, 4.8-nm pore diameter, with a molecular weight cut-off of 12,000. These membranes were immersed in boiling water and then stirred for 2 h as they cooled. The tubing was then stirred with 70% methanol for 30 min, stored in 50% methanol overnight, rinsed with distilled water, and soaked in phosphate buffer (pH 7.4) for 2–3 h prior to use. The membranes were used immediately after preparation. The tubing was tied with a double knot at one end, and then filled with 2 mL of protein solution in phosphate buffer (pH 7.4) containing sulfadimethoxine. The protein solutions were as follows: Cohn Fraction I (fibrinogen)[2] 0.34% (w/v); Cohn Fraction II (α-globulin)[3], 0.74% (w/v); Cohn Fraction IV-1 (α-globulin)[3], 0.81% (w/v); Cohn Fraction IV-4 (α-globulin)[3], 0.81% (w/v); Cohn Fraction V (albumin)[3], 4% (w/v); Cohn Fraction VI (α_1-acid glycoprotein)[3], 0.1% (w/v); lyophilized serum[3], 7.3%; and fresh serum[4], reduced to half volume by ultrafiltration. Except for the fresh serum, the protein solutions were prepared initially at double strength in buffer and then diluted with an equal volume of buffer containing sulfadimethoxine. The fresh serum, concentrated to half volume, was diluted with

[1] Fisher Scientific Co., Toronto, Ontario, Canada.
[2] ICN Pharmaceuticals Inc., Cleveland, Ohio.
[3] United States Biochemical Corp., Cleveland, Ohio.
[4] Collected from a healthy young-adult male volunteer.

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