Table VI—Timed-Release Dosage Forms Analyzed

Active Ingredient	Label, mg	Assay, mg
Capsule A[a]		
Pseudoephedrine hydrochloride	120.0	118.8
Chlorpheniramine maleate	8.0	7.8
Timed-Release Tablet B[b]		
Phenylpropanolamine hydrochloride	40.0	44.0
Phenylephrine hydrochloride	10.0	10.7
Chlorpheniramine maleate	5.0	4.8
Phenyltoloxamine citrate[c]	15.0	—
Capsule C[d]		
Chlorpheniramine maleate	8.0	7.43
Phenylephrine hydrochloride	15.0	20.4
Methscopolamine[c]	2.5	—

[a] Deconamine capsules, Cooper Laboratories. [b] Naldecon timed-release tablets, Bristol. [c] Could not be analyzed due to lack of reference standard. [d] Cosea-D capsules, Center.

other. By changing the counterion from heptanesulfonic acid to pentanesulfonic acid, the naphazoline retention was reduced without affecting the propylparaben retention, and a complete resolution of these compounds was possible.

To ensure the specificity of the proposed method, all drugs were artificially degraded. All drugs were well separated from their degradation products. Twelve pharmaceutical preparations were analyzed by this method. The versatility of the method was demonstrated by the analysis of various combination preparations (Tables IV–VI). The reproducibility of the method was excellent. Five replicate analyses of Liquid Preparation I (Table IV) had a coefficient of variation of 0.9%. The variation coefficients of five replicate analyses of two solid dosage forms (Preparation I, Table V; and Preparation I, Table VI) were 1.4 and 1.9%, respectively.

The proposed method is useful for assaying of cough and cold drugs. The method is simple, requires no elaborate sample preparation techniques, and is specific, accurate, and reproducible. Many separations can be achieved by proper manipulation of the mobile phase.

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Specific and Sensitive High-Performance Liquid Chromatographic Determination of Glyburide

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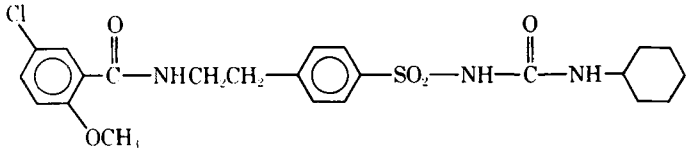
Received December 18, 1978, from the *Pharmaceutical Research and Development and Control Research and Development Laboratories, The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication March 21, 1979.

Abstract □ A specific and sensitive high-performance liquid chromatographic method has been developed for the rapid determination of intact glyburide in dog serum. With butylparaben as an internal standard, 1 ml of acid-buffered serum was extracted with toluene and an aliquot of the toluene was evaporated to dryness. The redissolved residue was chromatographed on a microparticulate reversed-phase column, and quantitation was achieved by monitoring the UV absorbance of the eluate at 228 nm. The response was linear, and the lower detection limit was ~20 ng/ml. Assay precision, as estimated by analyzing replicate samples of a laboratory standard, was better than 6% (*CV*). The utility of the analytical methodology for the determination of this highly potent sulfonylurea in pharmacokinetic studies in the dog was demonstrated.

Keyphrases □ Glyburide—analysis, high-performance liquid chromatography, dog serum □ Antidiabetic agents—glyburide, high-performance liquid chromatographic analysis, dog serum □ High-performance liquid chromatography—analysis, glyburide in dog serum

Glyburide[1] (I), 1-[[4-[2-(5-chloro-2-methoxybenzamido)ethyl]phenyl]sulfonyl]-3-cyclohexylurea, is a potent, orally active sulfonylurea for the management of maturity-onset diabetes mellitus (1). A number of analytical methods for the determination of glyburide in serum have been reported. These methods include procedures based on UV spectrophotometry, colorimetry following reaction with 2,4-dinitrofluorobenzene, fluorometry (2), and competitive protein binding (3, 4). However, only a recently developed radioimmunoassay (4) is sufficiently sensitive for monitoring blood levels following administration of



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therapeutic doses (2.5–10 mg). Although this radioimmunoassay is highly sensitive, with the lower limit of detection being ~4 ng/ml of serum, the method lacks specificity because of cross-reactivity of the hydroxy metabolites of glyburide. In addition, it is necessary to prepare standard curves using pretreatment serum from each subject because the assay response is subject dependent (4).

GLC has been used extensively for the analysis of sulfonylureas in biological fluids (5–14). However, this technique requires a time-consuming derivatization step to form volatile, thermally stable derivatives unless on-column derivatization is employed with concomitant loss in reproducibility[2] (12). Furthermore, the technique frequently lacks specificity since the intact compound and its metabolites may form identical derivatives (14, 15). Finally, the high molecular weight and inherently low volatility of glyburide or its derivatives make GLC analysis difficult if not impossible.

[1] Micronase, The Upjohn Co., Kalamazoo, MI 49001.

[2] D. G. Kaiser, The Upjohn Co., Kalamazoo, MI 49001, personal communication.

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The utility of high-performance liquid chromatography (HPLC) for the determination of sulfonylureas in plasma and serum was demonstrated recently[3] (15–19), with quantitation of concentrations as low as 200 ng/ml (19). HPLC with UV detection represents a particularly sensitive technique for the determination of glyburide because of its high molar extinction coefficient at 228 nm (2). The present report describes a rapid, specific, and sensitive HPLC method for the determination of glyburide in dog serum.

EXPERIMENTAL

Reagents—Acetonitrile[4], toluene[4], monobasic ammonium phosphate[5], monobasic sodium phosphate[6], and butylparaben[7] were used as received. The inorganic reagents were prepared in distilled, deionized water. Glyburide[8] and synthetic samples of the major metabolites of glyburide in the dog (2), 3-*cis*-[1-[[4-[2-(2-methoxy-5-chlorobenzamido)ethyl]-phenyl]sulfonyl]ureido]cyclohexanol, 1-[[4-carboxyphenyl]sulfonyl]3-cyclohexylurea[9], and 2-methoxy-5-chlorobenzamide[8], were used without further purification.

Standard Solutions—A stock solution containing 10 μg of glyburide/ml was prepared by dissolving an accurately weighed sample of the reference standard material in 40 ml of acetonitrile containing 0.2 ml of blank dog serum and diluting to 100 ml with toluene. Appropriate dilutions were made with toluene to obtain calibration curve standards containing 1500, 1200, 900, 600, 300, 150, 75, and 37.5 ng of glyburide/ml. These solutions were stable for at least 2 months when stored at −15°.

A stock solution containing 300 μg of butylparaben/ml was prepared by dissolving an appropriate quantity of the compound in toluene. Two milliliters of the stock solution was diluted to 1 liter with toluene to provide a working internal standard solution containing 600 ng of butylparaben/ml.

Calibration Curves—Calibration curves were prepared each day samples were analyzed to establish the linearity and reproducibility of the assay. The calibration curve standards were prepared by adding 1-ml aliquots of the appropriate glyburide standards to 16 × 125-mm screw-capped culture tubes fitted with aluminum-lined caps[10] and containing 1 ml of blank serum. These standards were analyzed along with the samples.

Sample Analysis—One-milliliter aliquots of serum were added to 16 × 125-mm culture tubes fitted with aluminum-lined caps along with 1 ml of toluene. After 1 ml of working internal standard was added to all samples, including the calibration curve standards, 1 ml of 2.55 *M* NaH_2PO_4 and 8 ml of toluene were added. The samples were then extracted at 280 cpm on a two-speed reciprocating shaker[11] for 30 min. Following separation of the phases by centrifugation for 15 min at 1800 rpm, 8-ml aliquots of the organic phase were transferred to clean 16 × 125-mm culture tubes and evaporated to dryness at 40° using a gentle dry nitrogen stream[12].

The residues were redissolved in 300–600 μl of chromatographic mobile phase and thoroughly mixed on a high-speed vortex mixer[13], and 250-μl aliquots were chromatographed.

Chromatographic Analysis—The samples were chromatographed on a commercially prepared reversed-phase column[14] (25 cm × 4.6 mm) packed with 5-μm LiChrosorb RP-8[15] using a constant flow liquid chromatograph[16], a loop injection valve[17], and a variable-wavelength UV detector[18]. Separation of glyburide and the internal standard from coextracted endogenous components in the serum was accomplished using a mobile phase composed of 0.05 *M* $NH_4H_2PO_4$–acetonitrile (1:1 v/v) at a 1.2-ml/min flow rate (~1000 psig). The UV absorbance of the column eluate was monitored at 228 nm.

Under these chromatographic conditions, the internal standard and glyburide retention times were ~7.6 and 9.8 min, respectively. Slight changes in retention times were produced by small variations in the solvent composition.

Quantitation—Peak height ratios were calculated by dividing the glyburide peak height by the internal standard peak height. Calibration curves were constructed by plotting the peak height ratios of calibration curve standards *versus* their concentrations, expressed in nanograms per milliliter of serum. The glyburide concentration was calculated from the peak height ratio using the slope and intercept obtained by linear regression analysis of the calibration curve data.

***In Vivo* Studies**—Six beagle dogs, 12.2–16.3 kg, were fasted for 16 hr prior to and 4 hr after oral treatment with a 2.5- or a 10.0-mg compressed glyburide tablet. Blood was collected using 10-ml vacutainers[19] at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. The blood was allowed to clot (~30 min) and centrifuged at 1800 rpm for 20 min, and the collected serum was stored frozen (−15°) until analysis.

Table I—Glyburide Assay Precision

Trial	Glyburide, ng/ml		
	Within Day		Between Day
1	1036.0	999.0	924.0
2	1065.0	1001.0	985.0
3	1009.0	966.0	906.0
4			870.0
5			1002.0
Average	1037.0	989.0	937.0
CV, %	2.7	2.0	5.9

RESULTS AND DISCUSSION

The high molecular weight, low volatility, and large molar extinction coefficient of glyburide make HPLC the technique of choice for the analyses of the bulk drug, compressed tablets (20), and biological fluids. The simplicity of the cited methodology for glyburide determination in dog serum, coupled with its high specificity and sensitivity, provides a particularly suitable analytical method for pharmacokinetic studies and monitoring the bioavailability/bioequivalence of pharmaceutical formulations.

Chromatographic Analysis—During assay development, commercially prepared reversed-phase columns from a number of manufacturers and a variety of mobile phases were investigated before adoption of the

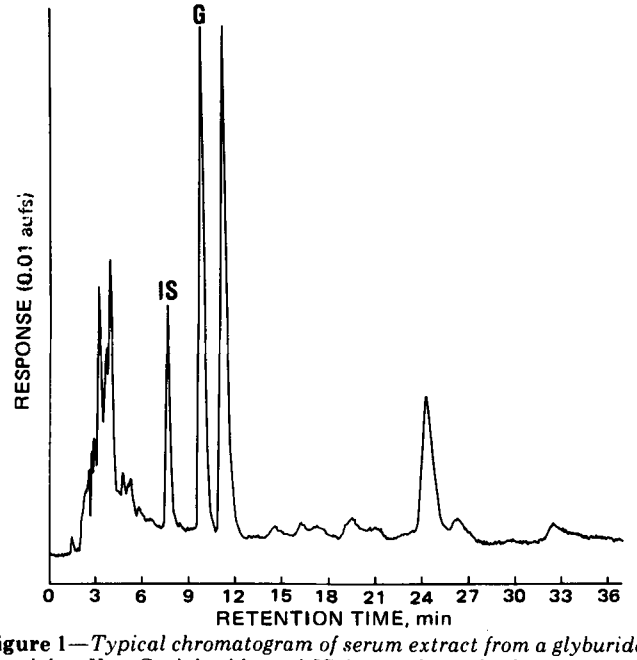

Figure 1—*Typical chromatogram of serum extract from a glyburide-dosed dog. Key: G, glyburide; and IS, internal standard.*

[3] Unpublished data.
[4] Burdick & Jackson Laboratories, Muskegon, Mich.
[5] Analytical reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Analytical reagent, Mallinckrodt, St. Louis, Mo.
[7] Eastman Kodak Co., Rochester, N.Y.
[8] Farbwerke Hoechst AG, Frankfurt, West Germany.
[9] The Upjohn Co., Kalamazoo, MI 49001.
[10] Brockway Glass Co., Parkersburg, W. Va.
[11] Eberbach and Sons, Ann Arbor, Mich.
[12] Organomation Associates, Shrewsburg, Mass.
[13] Lab-line Instruments, Melrose Park, Ill.
[14] Brownlee Labs RP-5A, Rheodyne, Inc., Berkeley, Calif.
[15] E. Merck, Darmstadt, West Germany.
[16] Model 100, Altex Scientific Inc., Berkeley, Calif.
[17] Model 70-10, Rheodyne, Inc., Berkeley, Calif.
[18] Altex/Hitachi model 155-40, Altex Scientific Inc., Berkeley, Calif.
[19] Fisher Scientific Co., Pittsburgh, Pa.

Table II—Selected Pharmacokinetic Parameters: Glyburide in Dogs

Dose, mg	Average Peak Serum Concentration, μg/ml	Average Half-Life, hr	*AUC*, (μg hr)[a]/ml
2.5	0.291	2.3	0.156
10.0	1.386	2.1	0.632

[a] Average area under the serum concentration–time curve, 0–24 hr.

described chromatographic conditions. Optimum chromatography was achieved using a reversed-phase column packed with 5-μm LiChrosorb RP-8 and a mobile phase of 0.05 *M* $NH_4H_2PO_4$–CH_3CN (1:1 v/v). A typical chromatogram of a serum extract is shown in Fig. 1. Baseline resolution of glyburide from the coextracted interference eluting at ~11.2 min was ordinarily achieved for all concentrations and animals studied. On occasion, a small interference having a retention time similar to that of glyburide (~10.5 min) was observed. Resolution of these peaks was nearly complete for low glyburide concentrations. Utilization of a precolumn[20] to protect the analytical column is recommended when large numbers of samples are analyzed.

Separation of glyburide from the major interference at ~11.2 min was sensitive to both the pH and percentage of acetonitrile in the mobile phase. Increasing the pH and/or the percentage of acetonitrile resulted in poorer resolution, or even coalescence, of these peaks. Above pH 6, the elution order was reversed.

Major glyburide metabolites in the dog (2), 3-*cis*-[1-[[4-[2-(2-methoxy-5- chlorobenzamido]ethyl]phenyl]sulfonyl]ureido]cyclohexanol, 1-[[4-carboxyphenyl]sulfonyl]-3-cyclohexylurea, and 2-methoxy-5-chlorobenzamide, elute prior to the internal standard and do not interfere. An additional proposed metabolite of glyburide in the dog is 1-[[4-[2-(2-methoxy-5-chlorobenzamido)ethylenyl]phenyl]sulfonyl] -3- cyclohexylurea (2). The structure of this metabolite has not yet been confirmed, and a synthetic sample was not available for chromatographic analysis. Evidence of its presence in dog serum following glyburide administration could not be found in chromatograms of serum extracts. The major metabolites of glyburide in humans (2) do not interfere.

Assay Recovery and Sensitivity—The absolute recovery of glyburide from serum containing 100, 400, 800, 1200, or 1600 ng/ml was determined by comparing the peak heights obtained when 250-μl aliquots of the redissolved serum residues and standards were chromatographed. No statistically significant differences in the recovery were found ($p = 0.38$), with the average recovery being 96 ± 5% (*SD*, $n = 19$).

Glyburide extraction from the acidified serum was essentially insensitive to the monobasic sodium phosphate buffer concentration; however, high ionic strength minimized the coextraction of endogenous components, which could potentially interfere. A small amount of serum was added to the stock standard glyburide solution because previous researchers (4) reported binding of glyburide to glass in the absence of serum. Preparation of glyburide in organic solvents, *e.g.*, acetonitrile, obviates the need for serum to prevent binding to glass. Comparable analytical results can be obtained by preparing glyburide standards in acetonitrile and evaporating the solvent under dry nitrogen prior to addition of control serum.

The lower glyburide detection limit was ~20 ng/ml of serum extracted. This concentration gave rise to a response equivalent to approximately three times the peak-to-peak noise level, or 2% of full-scale response at 0.01 absorbance unit full scale (aufs).

Assay Precision—Linear regression analyses of calibration curve data indicated no significant deviations from linearity for concentrations up to 1500 ng of glyburide/ml of serum. Coefficients of variation were better than 4.8% for all curves, with the average coefficient of variation being 2.8 ± 1.0% (*SD*) for standard curves run on 6 successive days.

The precision of the methodology was assessed further by analyzing replicate samples of a laboratory standard prepared by pooling serum collected from glyburide-dosed dogs. Triplicate samples were analyzed on 2 different days to establish within-day precision, and single samples were analyzed on 5 different days to establish between-day precision (Table I). Within-day coefficients of variation were comparable to the coefficients of variation of the standard curves. Not unexpectedly, the between-day coefficient of variation, 5.9%, was approximately equal to the standard deviation in the recovery of glyburide from serum, ±5%.

[20] LiChrosorb RP-8, 10 μm, 4.2 cm × 3.2 mm i.d., Altex Scientific Inc., Berkeley, Calif.

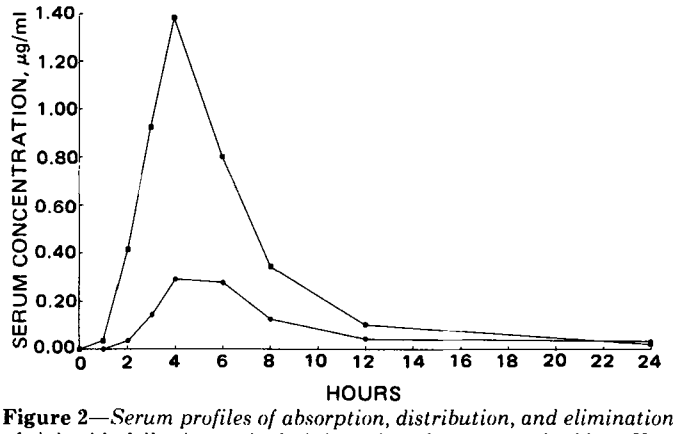

Figure 2—*Serum profiles of absorption, distribution, and elimination of glyburide following oral administration of compressed tablets. Key:* ●, *2.5 mg of glyburide; and* □, *10.0 mg of glyburide.*

Methodology Applicability—The utility of the analytical method for the determination of glyburide in pharmacokinetic studies was demonstrated by monitoring serum profiles in the dog in a pilot study. Average serum profiles for three beagle dogs, each dosed with a 2.5- or a 10-mg compressed tablet, are shown in Fig. 2. The average peak serum concentrations and areas under the average serum concentration–time curves were proportional to the administered dose (Table II). Peak serum concentrations occurred 4–5 hr after oral administration of the compressed tablets, essentially identical to the mean time of 4.3 ±1.4 hr (*SD*) found in a recent human study (21). Serum glyburide concentrations then decreased by apparent first-order kinetics, with average half-lives of 2.3 and 2.1 hr for the 2.5- and 10.0-mg compressed tablets, respectively.

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