Table II—Hemolysis of Erythrocytes in Isosmotic Solutions

Substances	Isosmotic Concentration, % w/v	Hemolysis, %	Approximate pH
Carbazochrome salicylate	2.57	82[a]	9.4
Cefazolin sodium	8.77	0	4.6
Cephapirin sodium	7.80	3[b]	6.7[c]
Dopamine hydrochloride	3.11	6	4.6
Etidocaine hydrochloride	5.08	100	4.3
Isoetharine hydrochloride	4.27	8	4.1
Ketamine hydrochloride	4.29	41	3.3
Potassium thiocyanate	1.52	0	6.3
Sodium nitroprusside	3.30	50[d]	9.1
Sodium thiocyanate	1.27	Trace	5.0
Terbutaline sulfate	6.75	11	4.1
Ticarcillin disodium	4.62	Trace	5.2

[a] Solution turned orange. [b] Solution turned yellow. [c] The pH was determined after addition of erythrocytes. [d] Solution turned red brown.

The percent of hemolysis found for the 12 isosmotic solutions studied, the isosmotic concentration used for each, and the solution's approximate pH before the addition of erythrocytes are listed in Table II. Any noticeable change in appearance of the erythrocytes or the solution was indicated. Of the 12 compounds studied, only four isosmotic solutions prevented hemolysis of human erythrocytes.

A compilation of the 305 substances whose isosmotic solutions were studied using the present hemolytic method shows that 144 failed to prevent hemolysis while 161 prevented hemolysis. Care must be taken not to equate isotonicity and isosmoticity without knowledge of the corresponding data whenever a biological membrane is utilized. This aspect was discussed previously (2, 4, 6–9).

Earlier studies (3–5) showed apparent aggregation of some substances in aqueous solution. In the present study, the cryoscopic graphs of cefamandole nafate and cefoxitin sodium (whose aqueous solutions foamed considerably) showed a slight discontinuity at 1%, suggesting that some aggregation may have taken place above that concentration; however, this phenomenon was not as distinct as with the previously reported drugs. Likewise, solutions of piperacillin sodium, propantheline bromide, and propranolol foamed considerably, suggesting that their surface tensions also were lowered; however, a plot of their cryoscopic graphs did not indicate aggregation. A sufficient number of compounds in the total study did tend to aggregate in solution, which suggests that this interfacial phenomenon may play a role in drug action by affecting biological activity. Associations taking place in the complex biological medium undoubtedly affect the thermodynamic activity of a given drug at the molecular level and, therefore, warrant increased study.

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Improved Liquid Chromatographic Assay for Serum Fluorouracil Concentrations in the Presence of Ftorafur

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Received January 5, 1981, from the * *Department of Laboratory Services and the* ‡ *Department of Pharmacy, Veterans Administration Medical Center, San Diego, CA 92161*, and the § *Division of Radiation Oncology, University of California Medical Center, San Diego, CA 92103.* Accepted for publication March 23, 1981.

Abstract □ An improved liquid chromatographic assay for serum ftorafur and fluorouracil is shown to be routine, sensitive, and reproducible using 200 μl of serum. Dilute ammonium acetate buffer at pH 10.2 is used for solubilization of the evaporated ethyl acetate extract for injection into the liquid chromatograph. A stability study indicated little or no *in vitro* formation of fluorouracil from ftorafur under the conditions described. Low serum fluorouracil levels were found after administration of therapeutic doses of ftorafur.

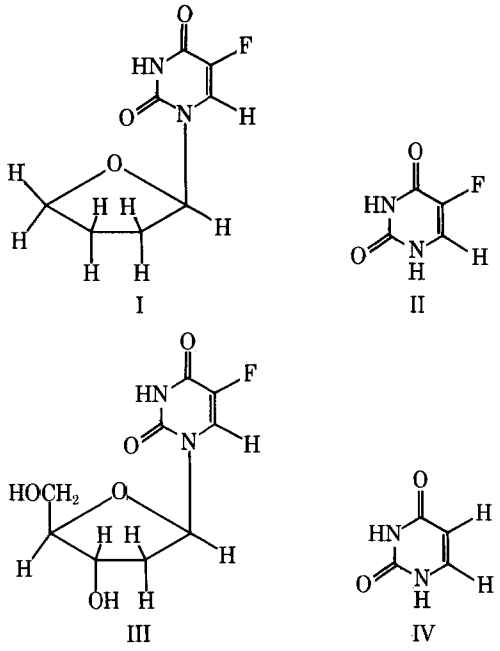
Keyphrases □ Liquid chromatographic assay—improved results for fluorouracil in presence of ftorafur □ Ftorafur—stability study, no *in vitro* formation of fluorouracil □ Anticancer agent—ftorafur potential for treatment of GI cancers

The recent development of an oral dosage form of the antineoplastic ftorafur [R_1S-*1*-(tetrahydro-2-furanyl)-5-fluorouracil] (I) has added new potential for the treatment of GI cancers on an outpatient basis. Ftorafur is generally considered to be a prodrug for fluorouracil (II), which, in turn, derives its cytotoxicity from its structural resemblance to uracil (IV). Inspection of the structure of I, II, and IV reveals the structural similarities between these molecules, and comparison of I and III indicates the unusual tetrahydrofuranyl ring of ftorafur.

BACKGROUND

During initial human studies with bolus ftorafur, investigators reported circulating levels of fluorouracil greater than those found after continuous intravenous infusion of fluorouracil (1, 2). In addition, there is some clinical similarity between bolus ftorafur and infusional fluorouracil in terms of patient toxicity (3). These findings suggested that the cytotoxic mechanism of ftorafur was probably due to the slow release of fluorouracil into the systemic circulation.

However, in a more recent study with bolus ftorafur, very low circulating levels of fluorouracil were reported and fluorouracil was not detected in the serum 8 hr after ftorafur administration (4). This report suggested that the high fluorouracil levels reported previously were the result of *in vitro* breakdown of ftorafur or perhaps its more labile dehydro metabolite (5). This metabolite is thought to differ from ftorafur by only a double bond located in an undetermined position in the furanyl ring



(5, 6). In view of these divergent results and the importance of determining whether serum fluorouracil levels resulting from ftorafur administration could be related to patient efficacy and toxicity as indicated with infusional fluorouracil (7), levels of circulating fluorouracil were determined in patients on bolus ftorafur protocols (6).

In initial attempts to adopt existing liquid chromatographic assays for ftorafur and fluorouracil in this laboratory, great difficulty was experienced in obtaining results either routinely or reproducibly for fluorouracil. These problems were caused mainly by interfering peaks of varying sizes from serum to serum and partly by peak broadening with the use of methanol as the injection solvent. To overcome these difficulties, an assay originally developed by other researchers (8) was modified. The modified assay is very sensitive and reproducible and is readily adaptable to routine therapeutic drug monitoring of fluorouracil in the presence of ftorafur and its labile metabolite, dehydroftorafur. A stability study revealed little or no formation of *in vitro* fluorouracil during assay or storage, and patient serum data confirm low levels of circulating fluorouracil with ftorafur doses as high as 5 g/m^2.

EXPERIMENTAL

Subjects and Clinical Protocols—Blood samples were taken from five patients with various GI carcinomas during 15 treatment cycles of a Phase I clinical trial of combined ftorafur and radiation therapy. Each cycle consisted of total daily ftorafur doses of 1.5–2.0 g/m^2 for 5 days and concurrent daily radiation doses of 250 rads starting on the 2nd day for 4 days. Each cycle was followed by 9 days of rest before the next treatment cycle. Ftorafur was administered orally three times daily with meals, and the serum samples were drawn 1–4 hr after the midday dose. One patient was given a single intravenous 2-hr infusion of 5.0 g of ftorafur/m^2. Blood samples were taken periodically during and for up to 24 hr after the infusion. All serum samples were frozen at −20° until analysis.

Reagents and Apparatus—Analytical standards of ftorafur[1], fluorouracil[2], and thymidine[2] were used as supplied. All solvents were nanograde quality.

The analysis of serum fluorouracil, ftorafur, and dehydroftorafur was performed on a microprocessor-controlled high-performance liquid chromatograph[3] equipped with a variable-wavelength detector, automatic sampling system, and a 4-mm × 30-cm reversed-phase column[4]. The column was run at ambient temperatures at a flow rate of 2 ml/min. The elution solvent varied from 3 to 6% methanol in 0.01 *M* aqueous sodium acetate (pH 4.0), yielding retention times of 2.9, 23, and 27 min for fluorouracil, ftorafur, and dehydroftorafur, respectively. The variable UV

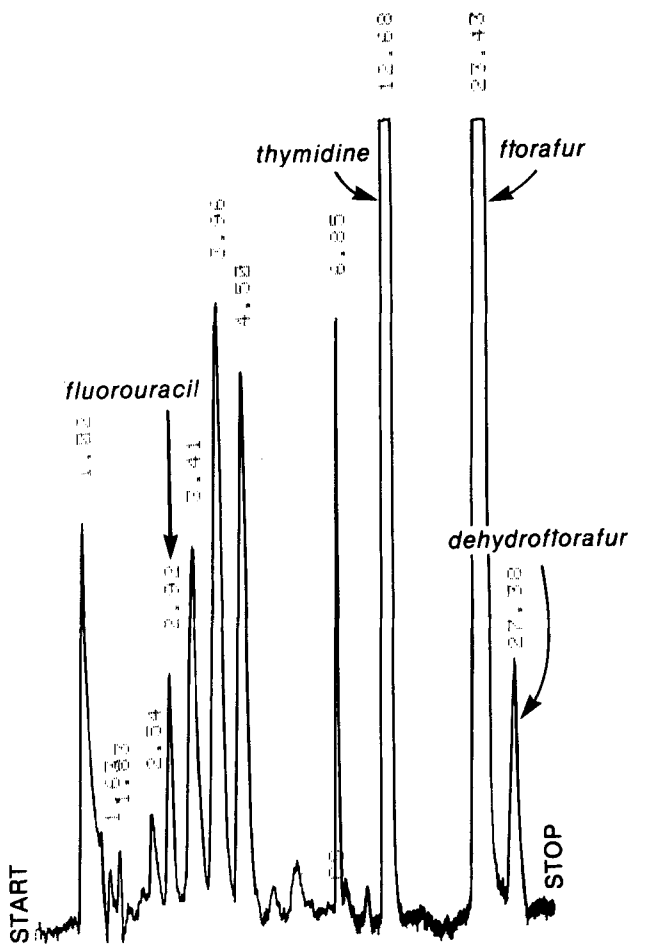


Figure 1—*Liquid chromatographic profile of a patient serum using 100 µl of 0.01* M *ammonium acetate (pH 10.2) as the injection solvent for the evaporated ethyl acetate extract of 200 µl of serum. Chart speed was changed from 0.8 to 0.15 cm/min at 6.5 min.*

detector was set at a sample wavelength of 270 nm and a reference wavelength of 330 nm with full-scale absorbance set at 0.003 unit.

Extraction Procedure—To a 10-ml screw-capped disposable glass tube were added 200 µl of serum; either 100 µl of water, fluorouracil, or ftorafur solution; 40 µl of thymidine internal standard (100 µg/ml serum); 40 µl of 0.5 *M* sodium phosphate buffer; and 6 ml of ethyl acetate. The tubes were shaken vigorously for 3 min and centrifuged at 2000 rpm for 15 min (1000×*g*). The organic phase was transferred to a second 10-ml tube and evaporated to dryness under nitrogen at 50°. Then 100 µl of 0.01 *M* ammonium acetate (pH 10.2) was added to the dried extract, and the tube was sonicated for 30 min. The extract was transferred to a microvial and capped, and 25 µl was injected into the liquid chromatograph.

Along with patient samples and the control, each assay included two duplicate reference standards for fluorouracil at concentrations of either 50 or 100 ng/ml and two ftorafur reference standards at concentrations of 50 and 100 µg/ml for quantitation of both ftorafur and dehydroftorafur. When filtration was used as a substitute for extraction and concentration, serum was passed through centriflow-membrane filters[5] before injection into the liquid chromatograph.

RESULTS AND DISCUSSION

The most important modification to the original assay (8) is the use of 0.01 *M* ammonium acetate at pH 10.2 instead of methanol to take up the dried ethyl acetate extract. This approach leads to other modifications by taking advantage of the anionic form of the fluoropyrimidines occurring at alkaline pH. Many materials extracted by ethyl acetate are not soluble in the aqueous pH 10.2 solvent. With the modified procedure, the

[1] Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.
[2] Sigma Chemical Co., St. Louis, MO 63178.
[3] Model 1084B, Hewlett-Packard, Avondale, PA 19311.
[4] µBondapak C_{18}, Waters Associates, Milford, MA 01757.
[5] Type CF50A, Amicon Corp., Lexington, MA 02173.

extraction ratio of ethyl acetate to serum can be increased from 8:1 to 16:1; this change increases the recovery of fluorouracil from ~50 to 70% and still eliminates interferences with the fluorouracil peak.

Figure 1 is a typical serum profile. When methanol is used as the injection solvent, mixing occurs more slowly, which effectively broadens peaks not well retained by the column. These faster eluting peaks are much sharper with the use of the aqueous solvent. This increased peak definition, combined with cleaner spectra and greater fluorouracil recovery, results in a lower and constant serum requirement of 200 μl from which concentrations as low as 10 ng/ml of fluorouracil can be reliably and routinely detected. Many blank serum samples as well as patient serum samples have been tested with this procedure, and none produced interfering peaks.

The procedure offers two other benefits that are not clearly demonstrated in Fig. 1. Thymidine in microgram amounts was proven to be a reliable internal standard for the fluoropyrimidines after numerous runs showed that very few peaks of any size other than ftorafur and dehydroftorafur eluted after ~7–8 min from injection. Naturally occurring thymidine appears in low nanogram amounts in serum and contributes <1% to the much higher amounts of thymidine spiked into serum. Another advantage is a decrease in solubilization of less polar and more strongly retained compounds extracted by ethyl acetate. These materials are soluble in methanol and often become interferences in subsequent injections into the liquid chromatograph. This decrease in slowly eluting peaks allows a far more reliable use of automated injection capabilities. Ten to 20 samples can be processed for injection in 2–3 hr, and the liquid chromatograph can be programmed to inject automatically day or night.

To determine truly representative systemic fluorouracil levels in the presence of ftorafur, it is important to demonstrate that the fluorouracil concentration measured is not a result of the breakdown of ftorafur or the more labile dehydroftorafur during storage or assay. Briefly, ftorafur and dehydroftorafur did not contribute *in vitro* to fluorouracil concentrations in serum when stored at −20° for up to 6 weeks or when stored for up to 10 hr in 0.01 *M* ammonium acetate (pH 10.2) at room temperature. No fluorouracil could be detected after extraction and chromatography of purified ftorafur spiked into blank serum. A negligible fluorouracil concentration was detected after extraction and chromatography of dehydroftorafur spiked into blank serum; however, the dehydroftorafur had been concentrated by lyophilization during which conversion of dehydroftorafur to fluorouracil previously was demonstrated.

Storage and reproducibility were conveniently checked by monitoring the control samples included in daily assays. These samples were from a ftorafur patient serum, which had been divided into 200-μl aliquots and frozen at −20° until assayed. Day-to-day variations in the fluorouracil levels of 10 of these aliquots were small and random, indicating that no measurable fluorouracil originated from ftorafur and dehydroftorafur for up to 6 weeks of storage at −20°. The coefficient of variation for the control samples was 4.8% for fluorouracil at a mean concentration of 44 ng/ml. The mean and coefficient of variation for ftorafur were 64 μg/ml and 5.8%, respectively, while the corresponding values for dehydroftorafur were 1.1 μg/ml and 9.4%.

The contribution to fluorouracil from ftorafur at a concentration of ~100 μg/ml was negligible during extraction and chromatography. This result was determined by injecting a very concentrated aqueous solution of ftorafur into the liquid chromatograph to separate it from any contaminating fluorouracil. The ftorafur peak was collected, and an aliquot of the unconcentrated and fluorouracil-free ftorafur solution was spiked into a blank serum to give a concentration of ~100 μg/ml. The spiked serum was extracted and chromatographed as usual; no measurable fluorouracil peak was found in the elution profile.

Since a purified and concentrated form of dehydroftorafur was not available, it was not possible to repeat an identical experiment with the metabolite. Therefore, dehydroftorafur had to be isolated from fluorouracil and ftorafur by liquid chromatography of patient serum. For isolation of dehydroftorafur, the column elution buffer was changed from sodium acetate to ammonium acetate; the collected dehydroftorafur fraction was immediately titrated to pH ~10 for concentration by lyophilization. The reconstituted dehydroftorafur was spiked into a blank serum to give an approximate concentration of 1 μg/ml. Extraction and chromatography of the spiked serum resulted in a small 5-fluorouracil concentration of 15 ng/ml. However, it previously had been shown that similar lyophilization of ftorafur resulted in measurable fluorouracil formation. Since dehydroftorafur is even more labile, this would indicate that the formation of fluorouracil was a result of the lyophilization step, which is not part of the routine assay. In addition, serum dehydroftorafur concentrations have been typically <5% of ftorafur serum concentrations.

Table I—Mean and Standard Deviation of the Serum Concentrations for the Three Fluoropyrimidines Monitored in Five Patients on Oral Ftorafur Chemotherapy [a]

Patient	Total Daily Dose, g/m²	Ftorafur, μg/ml	Dehydroftorafur, μg/ml	Fluorouracil, ng/ml	n [b]
BH	1.5	76 ± 14	0.87 ± 0.35	70 ± 15	6
RJ	1.5	50 ± 14	0.76 ± 0.31	63 ± 17	8
RW	1.5	96 ± 35	—	45 ± 36	9
FT	2.0	79 ± 15	—	64 ± 22	7
NR	2.0	73 ± 23	1.6 ± 0.45	76 ± 30	10

[a] Ftorafur range = 34–147 μg/ml. Fluorouracil range = 9–133 ng/ml. [b] Number of serum samples monitored.

If the instrument is used unattended in the automatic injection mode, the last sample to be injected will sit in the pH 10.2 solution for up to 10 hr more than the first sample injected. To test this situation, multiple extractions were performed on another patient serum having a ftorafur concentration of 124 μg/ml and a dehydroftorafur concentration of 1.5 μg/ml. The dried extracts were brought up in the pH 10.2 buffer, and the samples were injected so that 10 hr elapsed from the first to last injection. The initial concentration of fluorouracil measured in this sample was 53 ng/ml, and only small random changes (coefficient of variation of 4.3%) were seen over the 10-hr period in the three fluoropyrimidine concentrations. These results were routinely confirmed several times by placing the first sample injected behind the last sample for automatic reinjection.

Table I shows the average serum levels for five patients treated with several cycles of concurrent radiation and oral ftorafur chemotherapy. On a molar basis, the dosages are approximately equivalent to the 5-day dosage of fluorouracil given during direct intravenous infusion of 25 mg/kg/24 hr. This dosage of fluorouracil generates serum fluorouracil levels starting near 100 ng/ml to >500 ng/ml (9). The highest serum fluorouracil concentrations achieved in the ftorafur patients were comparatively low, and only three of the 40 values exceeded 100 ng/ml. In a different protocol, Patient BH was given a single intravenous bolus dose of 5.0 g/m² of ftorafur and serum levels of the three fluoropyrimidines were monitored for 24 hr after the dose. This dose was more than three times this patient's daily 1.5 g/m² of oral ftorafur given during the regular 5-day cycle. The ftorafur level at 2 hr was 250 μg/ml with a fluorouracil level of 150 ng/ml. At 24 hr, the ftorafur concentration had dropped to 90 μg/ml with a fluorouracil level of only 85 ng/ml.

Serum fluorouracil concentrations in the 1–2-μg/ml range were reported for up to 24 hr after a ftorafur intravenous bolus dose of 5 g/m² (2). However, the latter data were acquired using a somewhat different method. The serum had been filtered through a 50,000 molecular weight cutoff filter and injected directly into the liquid chromatograph with water as the column eluant. As a check on methodological differences, the 2- and 24-hr serum samples from Patient BH were repeated using the filtration method with 0.1 *M* sodium acetate, 5% methanol (pH 4.0), or water as the column eluant. Both 200- and 1000-ng/ml fluorouracil standards were run concurrently with these samples, which demonstrated fluorouracil peaks smaller than the 200-ng/ml standard with both column eluants.

The assay is sensitive and reproducible, and there appears to be little or no *in vitro* formation of fluorouracil during storage or assay under the conditions described. With just minor variations, the assay is now routinely used to monitor fluorouracil serum and cerebrospinal fluid concentrations in fluorouracil infusion patients instead of the more complex GLC–mass spectrometric assay used previously (7, 10). Ftorafur patient data indicate that ftorafur does not release fluorouracil into the serum in concentrations comparable to levels seen during fluorouracil infusion regimens. Furthermore, the data indicate that fluorouracil does not accumulate in the serum to infusional fluorouracil levels during repeated 5-day oral administration of therapeutic doses of ftorafur.

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High-Performance Liquid Chromatographic Assay of Tolbutamide and Carboxytolbutamide in Human Plasma

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Received October, 10, 1980, from the *Division of Biopharmaceutics and Pharmacokinetics, Department of Pharmaceutics, College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.* Accepted for publication March 4, 1981.

Abstract □ A high-performance liquid chromatographic method was developed for the simultaneous measurement of tolbutamide and its major metabolite, carboxytolbutamide, in plasma. The assay involves the ether extraction of 1 ml of plasma, using chlorpropamide as an internal standard. The extract is dried, the residue is taken up in acetonitrile, and 5 μl is injected into a reversed-phase column. The mobile phase consisted of 35% acetonitrile and 65% 0.05 *M* phosphoric acid buffer (pH 3.9). A fixed-wavelength detector was set at 254 nm. The sensitivity limits for the tolbutamide and carboxytolbutamide assay were 2 and 0.1 μg/ml, respectively. The ratio of carboxytolbutamide to tolbutamide in plasma obtained from a subject given a 500-mg tolbutamide tablet was ~1:20.

Keyphrases □ Tolbutamide—simultaneous high-performance liquid chromatographic assay with carboxytolbutamide, human plasma □ Carboxytolbutamide—simultaneous high-performance liquid chromatographic assay with tolbutamide, human plasma □ High-performance liquid chromatography—simultaneous assay of tolbutamide and carboxytolbutamide

Tolbutamide is an oral hypoglycemic agent used for the chronic treatment of diabetes mellitus of the maturity-onset type. After oral administration to humans, ~85% of the dose is excreted in the urine as the carboxy metabolite (1-butyl-3-*p*-carboxyphenylsulfonylurea) and the hydroxymethyl metabolite (1-butyl-3-*p*-hydroxymethylphenylsulfonylurea) (1), both inactive.

BACKGROUND

The pharmacokinetics of tolbutamide in patients is highly variable, with apparent half-lives ranging from ~2 to 25 hr (2, 3). Because of this variability, it was suggested that plasma tolbutamide concentrations should be determined in patients receiving this drug (2, 4). The bioavailability of tolbutamide after oral administration was shown to be influenced by the salt form (5), the surface area (6), and the excipients present in the formulation (7). These observations are particularly significant since changes in the serum glucose concentrations show a good relationship to the time course of tolbutamide in plasma (5, 7). Therefore, an assay method for tolbutamide and its metabolites would be useful in pharmacogenetic studies, therapeutic drug monitoring, and bioavailability studies.

Colorimetric (8–12) and UV (13) methods lack sensitivity and specificity. Methods involving GLC (14–17) require derivatization of the drug. More recently, high-performance liquid chromatographic (HPLC) methods were developed (18, 19). One method (18) is suitable for the determination of tolbutamide and its major metabolite, carboxytolbutamide, but does not use an internal standard and is not sensitive enough to quantitate the low metabolite levels reported previously (20). Another method (19) uses chlorpropamide as an internal standard but does not measure carboxytolbutamide simultaneously.

EXPERIMENTAL

Reagents and Chemicals—Tolbutamide[1], carboxytolbutamide[1], chlorpropamide[2], ether[3], acetonitrile[3], hydrochloric acid[4], and phosphoric acid[5] were used.

HPLC—Deionized water (1 ml) and 1 ml of chlorpropamide internal standard (30 μg/ml) were added to 1 ml of plasma in a 20-ml centrifuge tube with a polytef-lined screw cap. After mixing, 0.1 ml of 1.3 *N* HCl was added; the mixture was extracted with 10 ml of ether for 15 min by gentle shaking on a platform shaker. After centrifugation at −10° for 15 min at 3000 rpm, the ether layer was transferred to a 15-ml conical tube and evaporated under nitrogen at 40°. The residue was taken up in 100 μl of acetonitrile and vortexed for 30 sec, and 5 μl was injected into the HPLC system.

The HPLC system consisted of a sample injector[6], a mobile phase pump[7] operated at 2.5 ml/min (2000 psi), a reversed-phase column[8], a fixed-wavelength UV absorbance detector[9] at 254 nm (aufs 0.01), and a recorder[10] operated at 0.5 cm/min. The mobile phase consisted of 35% acetonitrile and 65% 0.05 *M* phosphoric acid buffer (pH 3.9). Column temperature was maintained at 28°. After each use, the column was washed with water followed by methanol to avoid damage by the buffer in the mobile phase.

Preparation of Standard Curves—A standard solution was prepared to contain 100 μg of tolbutamide/ml and 5 μg of carboxytolbutamide/ml in 0.1 *N* NaOH. Aliquots of this solution were diluted with deionized water to yield standards containing 2, 5, 10, 20, 40, 60, and 80 μg of tolbutamide/ml and 0.1, 0.25, 0.5, 1, 2, 3, and 4 μg of carboxytolbutamide/ml. The internal standard solution contained 30 μg of chlorpropamide/ml in 0.0005 *N* NaOH. One milliliter of the tolbutamide–carboxytolbutamide solution and 1 ml of the chlorpropamide solution were added to 1 ml of pooled human plasma, and the mixture was assayed. Standard curves were prepared using duplicate samples at each concentration by plotting peak height ratio (tolbutamide to internal standard or carboxytolbutamide to internal standard) *versus* drug or metabolite concentration.

Stability Studies—Three 20-ml samples of pooled plasma were spiked with 20 ml of water, 20 ml of an aqueous standard containing 10 μg of tolbutamide/ml and 0.5 μg of carboxytolbutamide/ml, or 20 ml of an aqueous standard containing 80 μg of tolbutamide/ml and 4 μg of carboxytolbutamide/ml. Each portion of spiked plasma was separated into individual 8-ml portions and stored frozen at −10° for subsequent assay after 1, 2, and 4 weeks. A sample of each also was assayed immediately after preparation.

[1] Provided by The Upjohn Co., Kalamazoo, Mich.
[2] Provided by Pfizer, Brooklyn, N.Y.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] Mallinckrodt, Paris, Ky.
[6] Model U6K, Waters Associates, Milford Mass.
[7] Model M6000, Waters Associates, Milford, Mass.
[8] μBondapak C_{18}, Waters Associates, Milford, Mass.
[9] Model 440, Waters Associates, Milford, Mass.
[10] Recordall Series 5000, Fisher Scientific Co., St. Louis, Mo.

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