Assay of Flucytosine and Furosemide by High-Pressure Liquid Chromatography

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Abstract □ A high-pressure liquid chromatographic method for the analysis of flucytosine and furosemide concentrations in biological fluid is described. The separations were carried out on a pellicular cation-exchange resin eluted with an ammonium phosphate buffer. Detection of elution peaks was by UV absorption at 280 nm and fluorescence monitoring. Advantages of the method are specificity, minimal preanalysis sample workup, and small sample size.

Keyphrases □ Flucytosine and furosemide—high-pressure liquid chromatographic analysis in plasma and urine □ Furosemide and flucytosine—high-pressure liquid chromatographic analysis in plasma and urine □ High-pressure liquid chromatography—analysis, flucytosine and furosemide in plasma and urine

In recent years, high-pressure liquid chromatography (HPLC) has gained increasing use in the analysis for drugs in biological fluids, *e.g.*, barbiturates (1), phenytoin (diphenylhydantoin) (1), and phenacetin (2). The fact that many drugs are highly soluble in water or polar organic solvents but are nonvolatile (troublesome problems for GC analysis and many extraction procedures) is advantageous in HPLC, especially when using aqueous elution from ion-exchange resins. HPLC also has the advantages of specificity, small sample size, and minimal preanalysis sample workup.

These advantages led to the development of an HPLC analysis system for two drugs: flucytosine and furosemide. Flucytosine was recently recognized as a useful antifungal agent in the treatment of systemic *Candida albicans, Cryptococcus neoformans* (3–6), and *Cladosporium trichoides* (7). While studying the pharmacokinetics of both flucytosine and furosemide in normal and uremic volunteers, a rapid and sensitive assay procedure applicable to serum and urine samples was needed to enable the administration of a low drug dosage as an extra margin of safety to the patients. Since 80–95% of flucytosine is excreted unchanged in the urine (8) and dose-related hepatotoxicity has been reported (3), a rapid analysis for plasma drug concentration in clinical samples, particularly in patients with renal insufficiency, was desirable. An HPLC method for the determination of flucytosine concentrations in both serum and urine is reported. With minor modifications of the conditions, an analysis for furosemide for use in pharmacokinetic studies can also be conducted. This method replaces the fluorometric technique reported earlier (9). Although not generally useful for furosemide for clinical purposes, the HPLC method for the pharmacokinetic study of both drugs has shown decided advantages over existing methods (9–15) for the determination of furosemide and flucytosine concentrations in both serum and urine.

EXPERIMENTAL

Flucytosine[1] (5-fluorocytosine) (for assay development), furosemide[2] (4-chloro-*N*-furfuryl-5-sulfamoylanthranilic acid), 4-chloro-5-sulfamoylanthranilic acid[2] (I), ^{35}S-furosemide[2] (for standard and clinical studies), and probenecid sodium[3] were used. All other chemicals were analytical reagent grade.

The high-pressure liquid chromatograph[4] was equipped with an absorbance monitor[5]. The absorbance monitor was equipped with an optical unit[6], a 280–310-nm filter, and high-pressure microflow cells[7], and the output was recorded[8]. For the analysis of furosemide, the eluant from the absorbance monitor was passed through a fluoromonitor[9] (excitation at 320–400 nm; emission at 400–700 nm) and the signal output was recorded on a dual-pen recorder[10]. A pellicular cation-exchange resin, prepared in 304.8 × 0.1-cm i.d. stainless steel tubing[11] with a precolumn[12] containing the same packing material, was used for both serum and urine.

Five-microliter aliquots of urine or serum were injected directly onto the column resin bed (stop-flow technique) with a 10-μl syringe[13] equipped with a 7-cm needle.

For the analysis of flucytosine, the column was eluted with a monobasic ammonium phosphate buffer solution (16 m*M*) with the pH adjusted to 2.00 with phosphoric acid. The column pressure was 2000 psi, the eluent flow rate was 0.24 ml/min, and the column temperature was 78°. Recordings were made at a range of 0.01 absorbance unit and a recorder span of 1 v full scale.

Standard flucytosine samples were prepared in concentrations ranging from 0 to 10 mg/liter in water, serum, and urine. Aqueous samples were stable for at least 1 month at ambient temperature.

Furosemide working standards were prepared fresh each day from injectable furosemide (10 mg/ml) from the same lot number used in each study and was, in turn, standardized against a solution of the pure compound. Compound I standards were prepared from chemically pure compound dissolved in water. All standards, urines, and serums containing furosemide were protected from light.

The column was eluted with the monobasic ammonium phosphate buffer solution (50 m*M*) with the pH adjusted to 2.50 with phosphoric acid. The column conditions were: pressure, 1300 psi; eluent flow rate, 0.33 ml/min; and temperature, 74°. Recordings were made at a range of 0.02 absorbance unit with a recorder span of 10 mv.

Drug concentrations were determined by measuring the net peak height above the baseline. Baselines were determined by a trough-to-trough tangent method. Drug concentrations were calculated by a computer-assisted, unweighted, least-squares fit of the standard concentrations and peak heights to a straight line. When serum samples were analyzed, a preadministration sample was also analyzed to determine if extraneous compounds, which might interfere, were already present in the serum. A preadministration urine sample, suitably diluted in proportion to the increased urine

[1] Hoffmann-La Roche, Inc., Nutley, NJ 07110
[2] Hoescht Pharmaceutical Co., Cincinnati, OH 45229
[3] Merck Sharp and Dohme Research Laboratories, West Point, Pa.
[4] Varian model LCS-1000, Varian Aerograph, Walnut Creek, CA 94598
[5] ISCO model UA-5, Instrument Specialties Co., Lincoln, NE 68505
[6] Type 6.
[7] Series 0080-014.
[8] Houston Omniscribe recorder model 51112, Houston Instrument, Bellaire, TX 77401
[9] LDC model 1209, Laboratory Data Control, Inc., Riviera, FL 33404
[10] Varian model A-25, Varian Aerograph, Walnut Creek, CA 94598
[11] Varian No. 07-000036-00, Varian Aerograph, Walnut Creek, CA 94598
[12] Varian No. 07-000038-00, Varian Aerograph, Walnut Creek, CA 94598
[13] No. 801 SN, Hamilton Co., Whittier, CA 90608

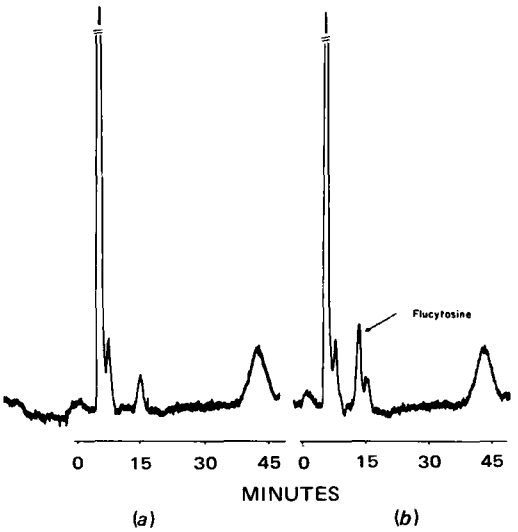

Figure 1—*HPLC elution pattern (280 nm) for serum without (a) and with (b) flucytosine (2.1 mg/liter), depicting the separation of flucytosine from other materials in serum which absorb at 280 nm.*

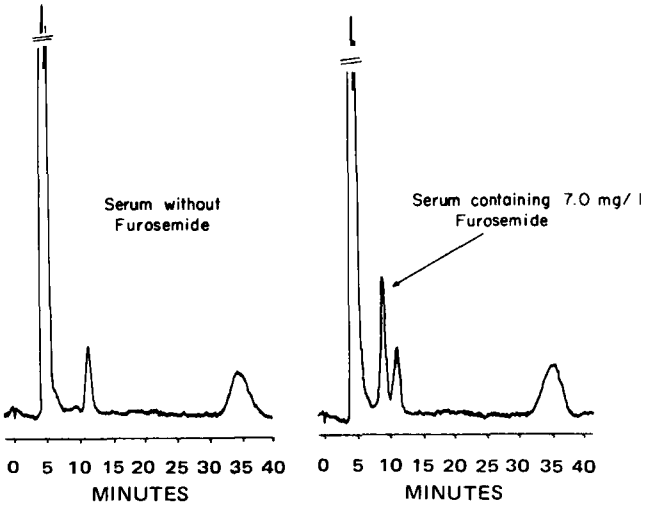

Figure 3—*HPLC elution patterns (280 nm) for serum without and with furosemide, depicting the separation of furosemide (elution time of 9 min) from other serum constituents that absorb at 280 nm.*

flow rate in the case of furosemide (9), was analyzed as a check for interfering substances.

Liquid scintillation counting and quench correction of samples containing ^{35}S-furosemide were carried out as reported earlier (9). Drug concentrations were calculated from the disintegrations per minute resulting from a dilution of the prepared injection solution of known specific activity, which was counted simultaneously.

Due to the high concentration of flucytosine in the urine samples following administration of the drug, both pre- and postadministration urines were diluted by a factor of either 20 or 100, depending on the actual flucytosine concentration, to obtain concentrations within the range of the standard curve.

RESULTS AND DISCUSSION

Following the described chromatographic methods, pure flucytosine or pure furosemide, when added to samples of serum or urine, was particularly well resolved from the other constituents in serum and urine (Figs. 1–4).

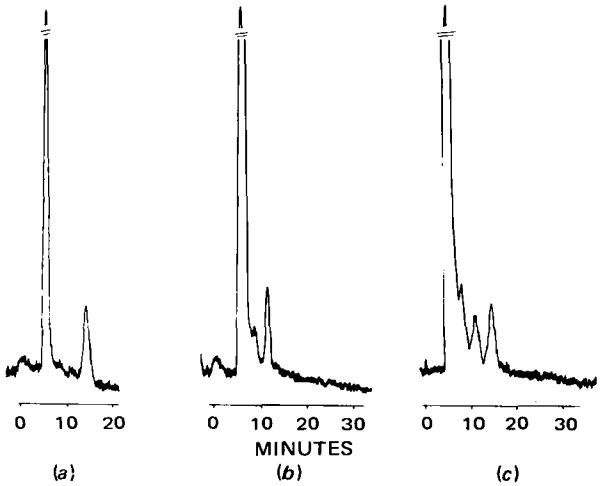

Figure 2—*HPLC elution pattern (280 nm) for patient urine with and without flucytosine. Different dilutions show effect on chromatogram peak height. Peak at 11 min in 1:20 dilution is a urine constituent that is almost diluted out in the 1:100 dilution. Flucytosine concentrations are those calculated after dilution. Key: (a), urine diluted 1:100 containing flucytosine (2 mg/liter); (b), urine diluted 1:20 without flucytosine; and (c), urine diluted 1:20 containing flucytosine (1.5 mg/liter).*

Flucytosine—With the reported conditions, flucytosine elutes from the column 13.5 min after sample injection (Fig. 1). Altering the buffer molarity to a higher concentration reduces the elution time but also tends to cause shifts of elution time, resulting in some overlap of the flucytosine peak with the small serum constituent peak, which normally elutes at approximately 15 min. Increasing the pH to 2.80 has little effect on the elution of flucytosine; however, with pH's higher than 2.00, resolution of flucytosine and the 15-min serum peak disappears.

The time involved to analyze a single serum sample (*i.e.*, for all peaks to elute) is 55–60 min; however, the flucytosine concentration may be calculated within 15 min after injection, making this technique relatively rapid for monitoring patient therapy. Since a large flat area in the elution pattern exists between the 15- and the

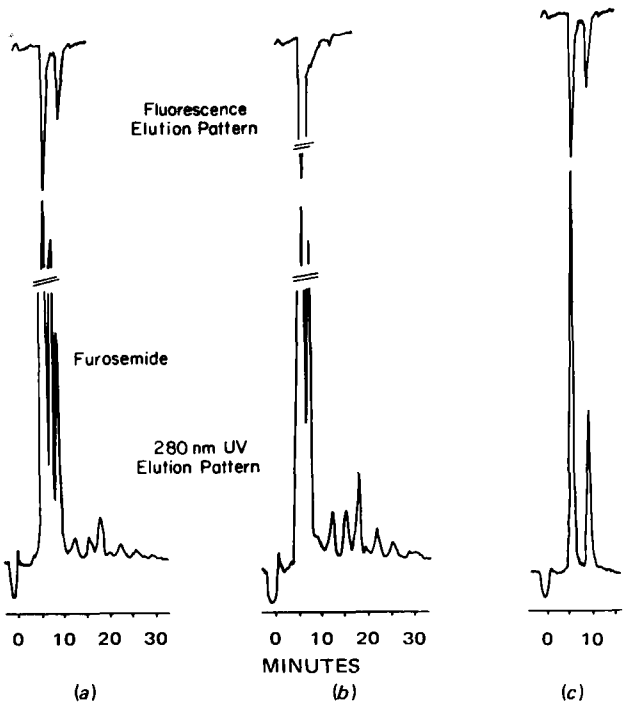

Figure 4—*HPLC elution patterns (280 nm) for urine with and without furosemide. Fluorescence pattern (top) shows clear separation from other fluorescent material in urine. Key: (a), urine diluted 1:20 containing furosemide (15 mg/liter); (b), urine diluted 1:10; and (c), urine from normal volunteer receiving furosemide.*

Table I—Comparison of HPLC Method with a Microbiological Method for Flucytosine in Serum (B) and Urine (U)

Sample	Flucytosine Concentration, mg/liter	
	HPLC Method	Microbiological Method
B_0	0	0
B_4[a]	0.8	0
B_5	6.7	6.8
B_6[a]	3.2	3.2
B_7	6.48	4.5
B_9	5.5	6.1, 5.6[b]
B_{11}	5.8	9.5
B_{12}	5.3	5.4
B_{15}	2.3	3.2
B_{17}[a]	1.5	1.8
B_{19}[a]	0.6	0
U_1	659	651
U_1[a]	108	164
U_2[a]	392	651
U_3	126	41

[a] Samples from a second study. [b] Duplicate sample to check reproducibility of the microbiological method.

55-min serum peaks, a second sample can be injected 25–30 min after the first injection, thereby allowing the analysis of at least two samples/hr. The urine samples from normal volunteers treated with flucytosine and diluted 1:20 showed no significant peaks (Fig. 2) due to urine constituents following the elution of flucytosine, thereby allowing a second injection 15 min after the first. Thus, four urine samples may be analyzed per hour.

The differences between peak heights for each set of standards prepared from pure flucytosine added to water, serum, and urine were not outside the relative standard deviation for repetitive injections of the same standard aqueous solution. Therefore, aqueous standards were used for calculation of serum and urine flucytosine concentrations in the clinical studies. The HPLC method was utilized for 3 months for the analysis of flucytosine in serum and urine samples. A standard curve for aqueous standards is shown in Fig. 5. The day-to-day relative standard deviation in the slope of the standard curve was 8%, and daily analysis of the same aqueous standard showed a relative standard deviation of 5.5% at 10 mg/liter. The within-day relative standard deviation for repetitive injections of the same aqueous standard solution was 2% at a concentration of 10 mg/liter.

The results from the HPLC method were also checked against a microbiological method performed by an independent laboratory[14] (Table I). The serum samples correlated reasonably well with a few exceptions, whereas the correlation between methods for urine samples was only fair at best.

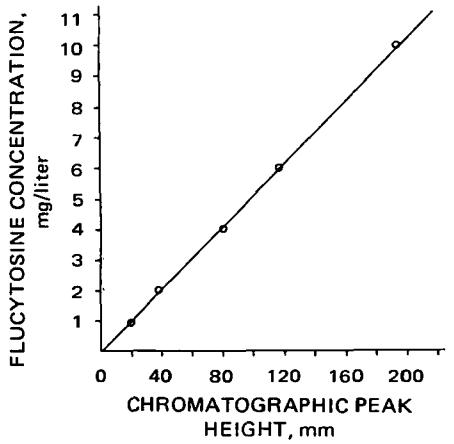

Figure 5—*Standard curve for aqueous flucytosine standards ranging from 1 to 10 mg/liter (slope = 0.052, SE = 0.061, and Y intercept = 0.042).*

[14] Bio-Science Laboratories, Van Nuys, CA 91405

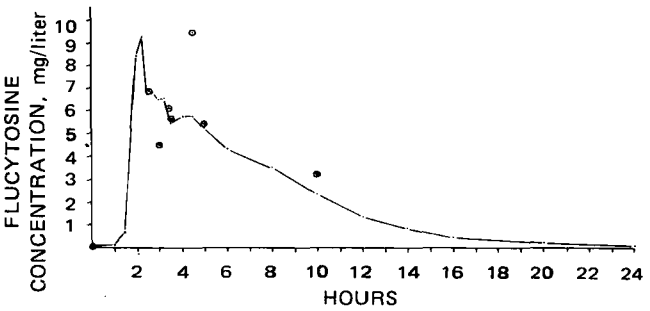

Figure 6—*Pharmacokinetic curve for a normal volunteer receiving 500 mg of flucytosine orally after a meal, comparing duplicate samples assayed by the HPLC method (•—•) and by a microbiological method (⊙).*

When the microbiological assay values are plotted on the same graph as those by HPLC from a complete patient study (Fig. 6), the plot depicts the greater precision of the HPLC method; this increased precision leads to more accurate values of the fitted parameters for the pharmacokinetic curve. When measured by the microbiological method, the quantity of recovered flucytosine for Sample U_2 (Table I) alone was 114% of the ingested dose compared to a total of 135% recovered overall; this example depicts the magnitude of the gross error in the microbiological method. In contrast, 80.5% of the ingested dose was recovered overall when the

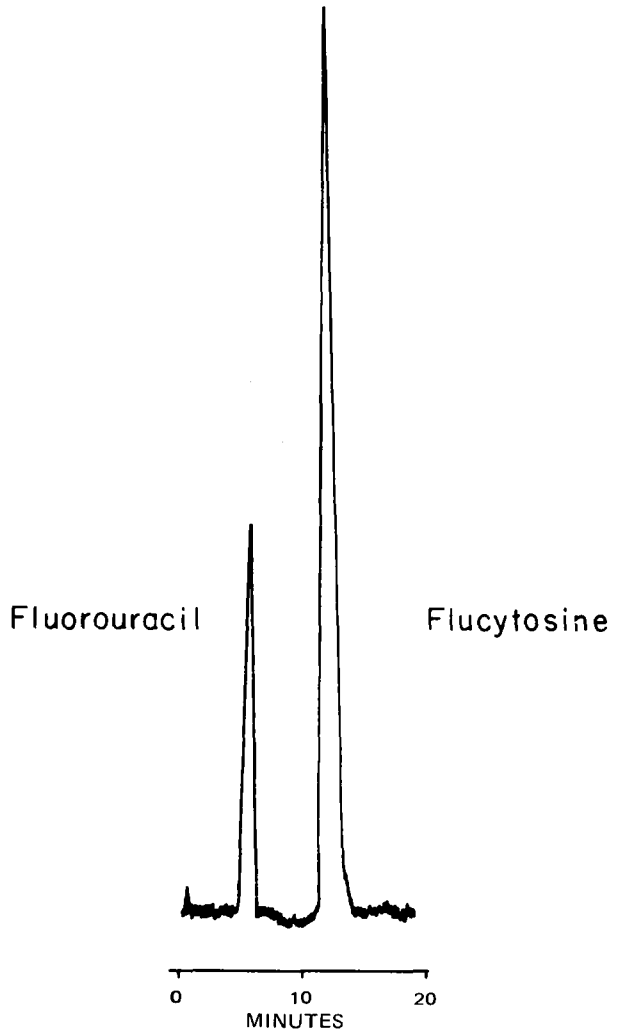

Figure 7—*HPLC elution pattern for an aqueous solution of flucytosine (10 mg/liter) and fluorouracil (10 mg/liter), depicting the clear separation of these two compounds.*

Table II—Comparison of Furosemide Concentrations (Milligrams per Liter) in Urine (U) and Plasma (P) as Determined by the HPLC and Radiochemical Techniques after an Oral Dose of 242 mg of Furosemide (Specific Activity 13.186 μCi/μmole)

Sample	Minutes	HPLC	Radiochemical
U_0	0	0	0
U_1	35	2.3	1.5
U_2	52	5.9	5.4
U_3	64	7.3	7.4
U_4	75	7.5	7.3
U_5	89	8.6	8.5
U_6	97	9.3	8.3
U_7	105	9.5	10.5
U_8	124	10.4	10.9
U_9	137	11.6	11.9
U_{10}	152	10.9	10.0
U_{11}	166	10.1	9.3
U_{12}	176	7.5	7.3
U_{13}	188	5.8	6.0
U_{14}	207	4.9	5.0
U_{15}	219	5.3	5.8
U_{16}	233	3.6	4.1
U_{17}	250	3.0	3.0
U_{18}	260	2.6	2.6
U_{19}	273	2.2	2.3
U_{20}	282	2.1	2.3
U_{21}	294	1.6	1.9
U_{22}	308	1.4	1.7
U_{23}	323	1.3	1.7
U_{24}	333	1.3	1.5
U_{25}	348	1.3	1.5
P_0	0	0	0
P_1	11	0	0
P_2	23	0	0
P_3	33	0	0.6
P_4	42	1.6	1.2
P_5	50	2.0	1.4
P_6	60	2.0	1.4
P_7	70	2.4	2.0
P_8	80	2.7	2.6
P_9	100	3.2	3.4
P_{10}	120	3.4	3.8
P_{11}	150	3.9	3.2
P_{12}	180	2.4	2.0
P_{13}	210	1.7	1.2
P_{14}	240	0.5	0.6

same urine samples were assayed by the HPLC method.

The HPLC method for the analysis of flucytosine has certain advantages over existing reported methods (13–15). This method requires no lengthy workup of serum or urine samples. Serum is applied directly to the column, and urine is simply diluted before application to the column. The sample size is small (5 μl), which facilitates analysis of samples derived from uremic patients, who normally have low hematocrits, and from infants. Pharmacokinetic and toxicity studies in small animals would also be facilitated by this method. The method is simple and fast.

Although flucytosine was reported not to be metabolized to fluorouracil in mammalian tissue (4, 16), it was of interest to show that fluorouracil does not interfere with the analysis of flucytosine, for instance, in concomitant therapy with these two drugs. In an aqueous solution under the reported conditions, fluorouracil elutes from the column 6 min postinjection, well resolved from the flucytosine peak (Fig. 7).

Furosemide—Using the chromatographic conditions described for furosemide, this drug was well resolved from other serum and urine constituents (Figs. 3 and 4). Furosemide was also completely resolved from its major breakdown product (I) (Fig. 8), both in biological samples and in aqueous solution. A separation of the two compounds in aqueous solution was shown in Fig. 8. Injectable furosemide is normally alkaline (pH 10.00) to prevent acid-catalyzed degradation to I. Urine, however, may be acidic, and furosemide will be quickly degraded unless alkalinized. Since the reaction is also light catalyzed, urine samples are also protected from light. As a further precaution, the samples are usually analyzed immediately. At the pH of analysis (pH 2.50), furosemide is highly fluorescent, whereas I is only weakly so (9, 10). This attribute allows

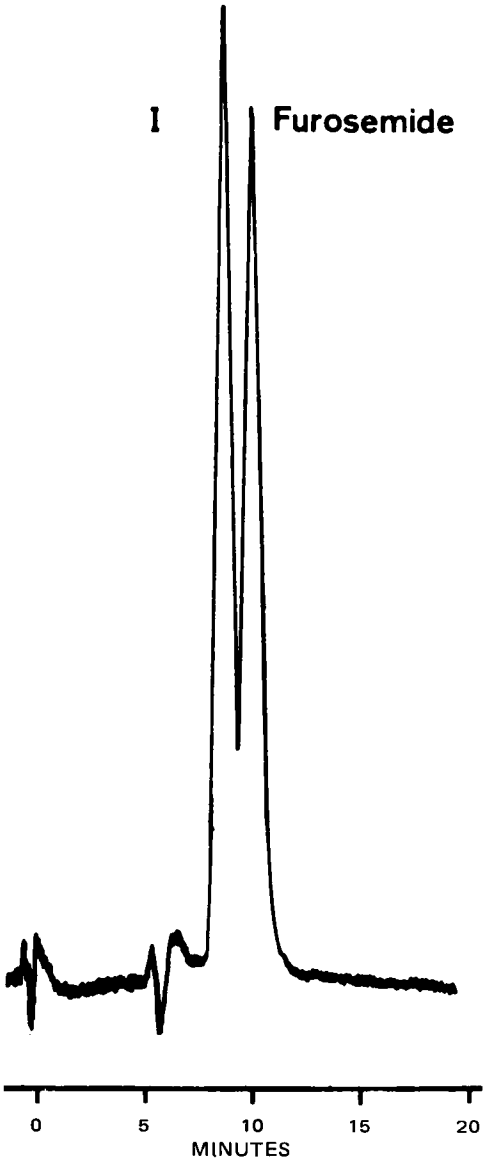

Figure 8—*HPLC elution pattern for an aqueous solution of furosemide and its major breakdown product, 4-chloro-5-sulfamoylanthranilic acid (I), showing that I does not interfere with the determination of the furosemide peak height.*

furosemide to be distinguished from other constituents in urine and plasma. Normally, only two fluorescent peaks are seen in elution patterns, one due to the breakthrough peak and the other due to furosemide. There is no peak in nonfurosemide-containing serum or urine, which exhibits both absorbance and fluorescence, and also elutes at the position corresponding to furosemide shown in Fig. 4.

The urine of patients who respond normally to furosemide shows only one urine peak at the solvent front due to the diluting effect of the drug. In the more concentrated urines from patients who do not respond to furosemide with a diuresis or from the normal concentrated urine from an untreated individual, there are a number of small unidentified peaks (Fig. 4). This figure shows, however, that the furosemide peak is, in all cases, uniquely identified and well resolved from any other fluorescent or absorbing material in the urine.

In these cases, the pH of the monobasic ammonium phosphate buffer is very critical. Changes of the monobasic ammonium phosphate buffer pH affect the retention time of furosemide only minimally over several pH units. However, changes on the order of 0.05 pH unit drastically alter the retention time of the other urine constituents. As a result, analyses during pharmacokinetic studies of

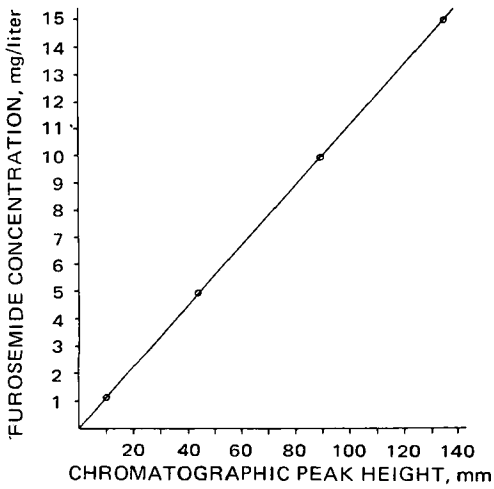

Figure 9—*Standard curve for aqueous furosemide standards ranging from 1 to 15 mg/liter (slope = 0.112, SE = 0.043, and Y intercept = 0.038).*

patients resistant to the diuretic action of furosemide require more careful monitoring of the buffer pH to maintain the separation of furosemide from other urine peaks. This phenomenon is also important in the analysis of urine samples in species such as mice, which do not exhibit a diuretic response to furosemide, even when extremely high doses of up to 400 mg/kg body weight are used (17).

Increasing the buffer molar concentration causes a shortening of the retention time of furosemide. The retention times of many compounds contained in the urine are also greatly affected by changes in buffer molarity, since some compounds undergo ion-exchange processes while others are being ion excluded. These other urine components have not been identified. Thus, while the effects on the elution profile of varying the buffer molarity are more subtle, they turn out to be less predictable than the effects of changing only the eluent pH.

The HPLC method was used for more than 9 months to measure furosemide levels in clinical studies. A standard curve for aqueous standards is shown in Fig. 9. The day-to-day relative standard deviation in the slope of the standard curve was 2.1%, and the day-to-day variation in the analysis of the same aqueous standard showed a relative standard deviation of 1.5%. The relative standard deviation of the concentration of repetitive injections of the same aqueous standard solution (within day variation) was 1.2% at a concentration of 15 mg/liter. Results from repetitive analysis of serum and urine samples containing 10 mg/liter of furosemide on the same day were 3 and 2%, respectively.

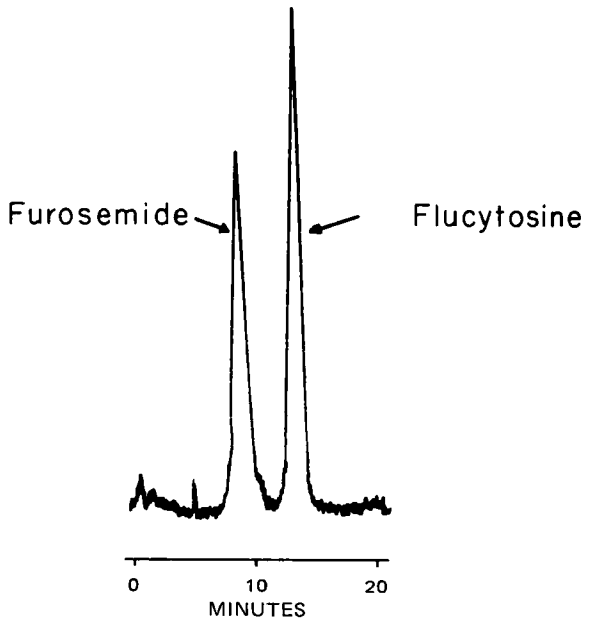

Figure 10—*HPLC elution pattern for an aqueous solution of flucytosine (5 mg/liter) and furosemide (5 mg/liter), using the conditions for separation of flucytosine from serum and urine.*

Table III—Comparison of Furosemide Concentrations (Milligrams per Liter) in Urine (U) and Plasma (P) as Determined by the HPLC and Radiochemical Techniques after an Intravenous Injection of 232 mg of Furosemide (Specific Activity 11.588 μCi/μmole)

Sample	Minutes	HPLC	Radiochemical
U_0	0	0	0
U_1	14.5	93.8	92.2
U_2	25.2	70.0	64.8
U_3	37.5	41.5	44.0
U_4	47.4	28.0	30.6
U_5	57.5	22.0	24.3
U_6	68.0	17.3	18.9
U_7	82.8	15.3	16.5
U_8	103.0	12.0	12.4
U_9	116.6	10.0	10.8
U_{10}	135.0	8.5	9.1
U_{11}	152.0	7.2	8.0
U_{12}	168.3	6.7	7.3
U_{13}	201.0	7.0	7.6
U_{14}	245.1	8.5	9.7
P_0	0	0	0
P_1	11.9	29.5	32.3
P_2	20.2	20.4	23.6
P_3	30.5	13.6	18.4
P_4	40.0	10.4	13.3
P_5	50.0	9.8	18.4
P_6	60.0	7.3	9.1
P_7	70.0	5.2	7.1
P_8	80.5	4.9	6.6
P_9	100.6	3.5	4.7
P_{10}	121.0	4.2	3.8
P_{11}	150.0	1.1	3.0
P_{12}	181.8	1.1	2.1

To estimate the accuracy of the HPLC method, it was compared to the measurement of ^{35}S-furosemide concentration determined radiochemically by means of the ^{35}S-label after administration to a normal subject by both oral and intravenous routes. In a normal subject, the drug virtually disappeared from the serum by 4 hr. The results (Tables II and III) demonstrate good agreement between the HPLC measurement and that made radiochemically, indicating that little metabolism of furosemide took place.

As with any chromatographic assay method, care must be taken to ensure that other drugs do not interfere with the determination of the drug to be analyzed. In initial studies using a combination of probenecid and furosemide, these two drugs were found to have the same elution time under the conditions described. However, by lowering the column temperature to 60°, furosemide was resolved from the probenecid peak. This lowering of the temperature results in a longer elution time and a 20% reduction in the furosemide peak height, which makes the assay somewhat less sensitive at low drug concentrations.

The size of the sample required for analysis (5 μl) makes this assay particularly attractive in clinical research studies for measuring serum concentrations of furosemide in infants and anemic patients and for pharmacokinetic and metabolism studies in small animals. In the analysis of rat serum and urine, no interferences during measurement of furosemide levels were found. By using the conditions for the separation of flucytosine from serum or urine, furosemide, when it is present, is well resolved from the flucytosine peak. Furosemide elutes at 9 min and flucytosine elutes at 13.5 min (Fig. 10). Thus, the analysis of flucytosine would not be interfered with by concomitant furosemide therapy.

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High-Pressure Liquid Chromatographic Analysis of Triflubazam and Its Metabolites in Human and Animal Blood and Urine

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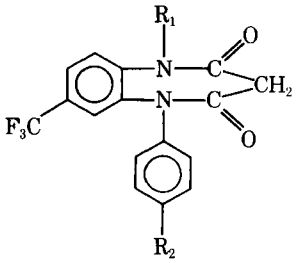
Abstract □ A high-pressure liquid chromatographic method is described for analyzing triflubazam [1-methyl-5-phenyl-7-trifluoromethyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione] and its primary metabolites in blood and urine. Adsorption chromatography, using pellicular silica gel as the stationary phase and dioxane–isooctane as the mobile phase, permitted rapid sample analysis. After extraction of blood and urine samples with toluene, quantitation is achieved using liquid chromatography with an internal standard. The method is sensitive above 50 ng/ml of triflubazam and its known metabolites. Recoveries for all compounds from blood or urine averaged above 95%. The specificity of the method was established by collecting samples separated by liquid chromatography and characterizing them by mass spectrometry. Human and animal data are presented to illustrate the utility of the method.

Keyphrases □ Triflubazam (a 1,5-benzodiazepine) and its metabolites—high-pressure liquid chromatographic analysis in human and animal blood and urine □ 1,5-Benzodiazepines—high-pressure liquid chromatographic analysis of triflubazam and its metabolites in human and animal blood and urine □ High-pressure liquid chromatography—analysis, triflubazam (a 1,5-benzodiazepine) and its metabolites in human and animal blood and urine

The quantitative analysis of drugs and their metabolites in physiological fluids is important for correlating pharmacological activity. Various methods requiring preliminary purification and/or lengthy analysis time to obtain the sensitivity and specificity needed to generate an accurate profile of the drug's metabolism have been described. Polarography (1), colorimetry (2), UV spectrophotometry (3), GLC (4), TLC (5), and liquid chromatography (6) have been employed to analyze the benzodiazepine class of compounds in blood and urine. A comprehensive review (7) of all methods for the analysis of benzodiazepines was published recently.



	R_1	R_2
I:	CH_3	H
II:	H	H
III:	CH_3	OH
IV:	H	OH

The methodology presented in this paper for the analysis of triflubazam [1-methyl-5-phenyl-7-trifluoromethyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione], Compound I[1], and its metabolites (II–IV) in blood and urine by high-pressure liquid chromatography (HPLC) exemplifies the advantages of this technique to this area of research.

EXPERIMENTAL

Reagents—All solvents were analytical reagent quality and were used without further purification.

[1] Synthesized by Boehringer-Ingelheim G.m.b.H., Ingelheim, Germany; referred to as ORF-8063.