An Improved HPLC Method for the Determination of Furosemide in Plasma and Urine

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AN IMPROVED HPLC METHOD FOR THE DETERMINATION OF FUROSEMIDE IN PLASMA AND URINE

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ABSTRACT

A sensitive HPLC method with minimal sample preparation and good reproducibility for the determination of furosemide in plasma and urine is described. Acidified plasma samples were extracted using CH_2Cl_2 containing desmethylnaproxen as internal standard (IS). Fresh urine samples were incubated with β-glucuronidase for 15 minutes and then treated with CH_3CN containing IS.

Chromatography was performed on a C18 column with 10 mcl sample injection. Mobile phases were: a) for plasma: 0.01 M NaH_2PO_4, pH 3.5 - CH_3OH (65:35), and b) for urine: acetic acid, pH 3.5 - CH_3OH (60:40) at 3 ml/min and fluorescence detection at Ex 235/Em 389 nm. The plasma standard curve was linear from 0.01 to 15.0 mcg/ml and the urine from 0.5 to 200.0 mcg/ml. The within run CV's were 3.2% at 0.74 mcg/ml plasma and 2.0% at 10.7 mcg/ml urine. Recovery from plasma was 69.9% at 2.0 mcg/ml and 98.6% from urine at 5.0 mcg/ml. The stability of furosemide and its glucuronide were studied. Both methods have been applied to the analysis of plasma and urine samples obtained from human volunteers.

INTRODUCTION

Furosemide is a fast acting diuretic which affects the
ascending loop of Henle. The drug is useful when treating renal
disease, liver cirrhoses, edema, and hypertension. The dosing
regimen for a patient may vary between 40-200 mg furosemide per
day depending on the severity of the condition which is being
treated. Furosemide levels can be determined in patients' plasma
or urine samples. Urine samples contain both the drug and its
glucuronide conjugate.

Early analytical methods for monitoring furosemide levels in
plasma or urine were spectrophotometric (1-3) and gas chromato-
graphic (4). In order to improve sensitivity and specificity,
high performance liquid chromatographic methods (5-10) have been
developed. These methods use either protein precipitation or
solvent extraction for sample preparation, and either UV or
fluorescence detection.

The stability of furosemide and its glucuronide metabolite
have been questioned by several researchers (4,6,8,11).Decom-
position has been attributed to photolytic degradation as well as
to chemical reactions (11). Stability problems of this type have
put into question the quantitation of furosemide in biological
fluids. The present study was undertaken to evaluate certain of
these stability factors and to develop methods for both plasma
and urine analysis which are specific and sensitive, and which
require minimal sample preparation. Several factors affecting
the hydrolysis of furosemide glucuronide were studied in order to
optimize the method.

MATERIALS AND METHODS

Instrumentation:

Chromatography was performed with a Hewlett Packard model
1084B liquid chromatograph equipped with an autoinjector, and an
octadecylsilyl column, 4.6 mm diameter x 15 cm long, and packed
with 5 micron spherical material (Zorbax ODS, Dupont). A
Schoeffel Fluorescence Detector Model FS970 was used to detect
the compounds and the response was recorded on a Hewlett Packard
97850B LC terminal, The effluent was monitored at excitation
wavelength 235 nm with a type 389 nm emission cut off filter.
The degassed mobile phases were pumped through the column at 3.0
ml/min and the column compartment was maintained at 45$^{\mathrm{o}}$C.

Reagents:

Acetonitrile (HPLC grade) was obtained from MCB. Methanol,
methylene chloride, sodium acetate (HPLC grade), and sodium

phosphate monobasic (certified ACS) were obtained from Fisher
Scientific. Tetrahydrofuran (distilled in glass) was obtained
from Burdick and Jackson Laboratories, Inc. Acetic acid (HPLC
grade) was obtained from Baker Chemical Company. Furosemide (USP
Lot #RC1412) was obtained from Hoechst-Roussel, Inc. The β-gluc-
uronidase type VII Lot 22F-6824 was obtained from the Sigma
Chemical Company and the 6-desmethylnaproxen was obtained from
Syntex.

Drug Solutions:

       Plasma Assay - A stock solution of furosemide was prepared
at 500 mcg/ml CH_3OH. A working dilution of 5 mcg/ml CH_3OH was
prepared from the stock solution.

       Urine Assay - Working solutions of furosemide were prepared
at 500 mcg/ml CH_3CN and at 50 mcg/ml CH_3CN.

Internal Standard (IS) Solutions:

       Plasma Assay - A stock solution of 6-desmethylnaproxen was
prepared at 500 mcg/ml CH_3OH. A dilution of 5 mcg/ml CH_3OH was
prepared from the stock solution. The extraction solution con-
sisted of a 4 ml aliquot of the diluted IS solution per liter of
methylene chloride (20 ng/ml CH_2CL_2).

       Urine Assay - A stock solution of 6-desmethylnaproxen was
prepared at 500 mcg/ml CH_3CN. A working dilution of 1.5 mcg/ml
CH_3CN was prepared from the stock solution and used to precipi-
tate proteins from urine samples.

Other Solutions:

       Plasma Assay - Acetic Acid, 8.5 M, was prepared by the
addition of 1 ml glacial acetic acid to 1 ml H_2O.

       Urine Assay - Sodium phosphate buffer: 0.075 M NaH_2PO_4 was
prepared, and the pH was then adjusted to 6.8 with NaOH.

       Urine Assay - β-glucuronidase solution: The lyophilized
contents of a vial of β-glucuronidase was reconstituted with
sodium phosphate buffer (0.075 M, pH 6.8) to give a final concen-
tration of 2000 U/ml buffer.

Mobile Phases:

       Plasma Assay - 0.01 M NaH_2PO_4 was prepared in distilled
deionized water and the pH was adjusted to 3.5 with phosphoric
acid. The mobile phase consisted of 65% buffer and 35% CH_3OH.

Urine Assay - Glacial acetic acid was diluted to obtain a pH of 3.5. The mobile phase consisted of 60% diluted acid and 40% CH_3OH.

Preparation of Plasma Standards:

To 500 mcl of heparinized plasma, in a screw capped centrifuge tube, were added an aliquot of the drug working solution, 50 mcl of 8.5 M acetic acid and 5 ml extraction solution. The mixture was vortexed for 10 seconds and centrifuged for 5 minutes at 1000 x g. The organic layer was then transferred to an evaporating tube (Concentratube[R], Laboratory Research Co., Los Angeles, CA). The sample was evaporated to dryness under a gentle stream of dry N_2 at 30°C, reconstituted in 100 mcl of CH_3OH and transferred to a polypropylene microvial (P. Weidman & Co., Romanshorn, Switzerland). A 10 mcl aliquot was injected onto the column.

Preparation of Urine Standards:

To a known volume of urine an aliquot of a working drug solution was added. A 100 mcl aliquot of the urine was then placed in a 10x75 mm glass tube, and 50 mcl of β-glucuronidase solution was added. After mixing, the tube was incubated for 15 minutes at room temperature. The proteins were then precipitated with 800 mcl of CH_3CN containing the internal standard. The tubes were vortexed for 10 seconds and centrifuged for 10 minutes at 1500 x g. An aliquot of the supernatant was transferred to a polypropylene microvial before injection of 15 mcl into the chromatograph.

Quantitation:

Standard curves were constructed for plasma and urine utilizing four replicates for plasma samples and five replicates for urine samples at several concentrations. The peak heights were measured and the ratios of furosemide to internal standard were plotted against concentration (mcg drug/ml plasma or urine).

Recovery:

Plasma Assay - Samples containing 2.0 mcg furosemide/ml plasma were carried through the analysis and the peak heights of drug and IS were measured. An equivalent amount of furosemide was added to 5 ml of extraction solution, vortexed, evaporated to dryness, and reconstituted in CH_3OH for injection into the HPLC. The peak heights of drug and IS were measured for comparison with the extracted samples to estimate percent recovery.

Urine Assay - Samples containing 50 mcg furosemide/ml of urine or water were carried through the analysis and the peak

heights of drug and IS were measured to estimate percent
recovery.

Patient Samples:

Plasma or urine samples from patients receiving furosemide
were analyzed using the same procedures. The amount of drug in
patient samples was calculated by comparison to a standard curve
prepared daily.

Quality Control (QC) Samples:

Plasma and urine were spiked with known concentrations of
furosemide solutions prepared in CH_3OH and CH_3CN, respectively.
The samples were stored frozen and used to determine within-run
and day-to-day precision. After the samples were brought to room
temperature, the samples were carried through the plasma or urine
assays. The amount of drug in the QC samples was calculated by
comparison to a standard curve prepared daily.

Stability of Furosemide:

Solutions of furosemide in H_2O, CH_3OH, CH_3CN, CH_2Cl_2, and
THF were prepared and used to assess the stability of furosemide
under room temperature conditions, with and without exposure to
room light. The H_2O, CH_3OH, CH_3CN, and THF solutions (containing
5 mcg drug/ml) were injected directly into the HPLC. A 5 ml
aliquot of the CH_2Cl_2 solution (containing 0.2 mcg drug/ml) was
evaporated to dryness under N_2, and reconstituted in CH_3OH for
injection into the HPLC. The various solutions were tested at
timed intervals.

Enzyme Hydrolysis of Furosemide Glucuronide (FG):

The conditions necessary for complete enzymatic hydrolysis of
FG in urine were studied using freshly collected urine from volun-
teers taking furosemide. β-glucuronidase was added in varying
amounts (0, 500, 1000, 2000, or 4000 units/ml urine). The tubes
were incubated at room temperature and at 37°C. At timed inter-
vals the hydrolysis reaction was stopped by addition of CH_3CN .
containing IS, and the samples were analyzed.

Stability of Furosemide Glucuronide (FG):

The stability of FG in urine and organic solvents was
studied using freshly collected urine from volunteers taking
furosemide. The urine was left untreated or was mixed with CH_3OH,
CH_3CN, or THF. The samples were analyzed by HPLC at timed
intervals.

In order to differentiate between the decomposition of FG in aqueous media and its β-glucuronidase hydrolysis, the effect of water, sodium acetate buffer (0.04 M, pH 7), and sodium phosphate buffer (0.075 M, pH 6.8) without enzyme were tested.

RESULTS AND DISCUSSION

In reviewing the analytical literature for furosemide, the following factors were considered: 1) the need to analyze both plasma and urine samples, 2) the presence of furosemide glucuronide in urine samples, 3) the sensitivity levels and ranges of linearity required, 4) the stability of furosemide and its glucuronide during the analytical procedures, 5) the choice of chromatographic conditions and detection, 6) the choice of extraction and protein precipitation solvents, 7) the choice of an internal standard, 8) the conditions necessary for β-glucuronidase hydrolysis, and 9) the convenience of the overall sample processing. The methods for analysis of furosemide in plasma and urine which are described in this paper are sensitive and have minimized or eliminated several potential degradation problems.

Chromatographic Conditions:

The plasma and urine methods described in this paper employ the same ODS column and both use fluorescence detection, but different mobile phases are utilized. The urine determination method was developed first using acetic acid (pH 3.5) and methanol to elute the drugs (Figure 1). An endogenous plasma constituent coeluted with furosemide using the acetic acid/ methanol mobile phase. Therefore that mobile phase could not be applied to the plasma assay. Changing the organic modifier to acetonitrile changed the elution order of peaks, but did not improve the separation of furosemide from an interfering plasma peak and did reduce the fluorescence intensity by approximately half. The mobile phase buffer was changed to sodium dihydrogen phosphate (0.01 M, pH 3.5) and resulted in good separation of all the peaks (Figure 2).

The use of a Schoeffel fluorescence detector set for excitation wavelength 235 nm and using a type 389 nm emission cut off filter gave excellent sensitivity for detection of furosemide and the internal standard. The detectability limit for furosemide by fluorescence was 0.1 ng "on column", which was approximately ten times the sensitivity for UV at 275 nm (λ max). The use of fluorescence detection has the additional advantage of minimizing interference from other drugs since few drugs would be expected to co-elute with furosemide and give a fluorescent signal.
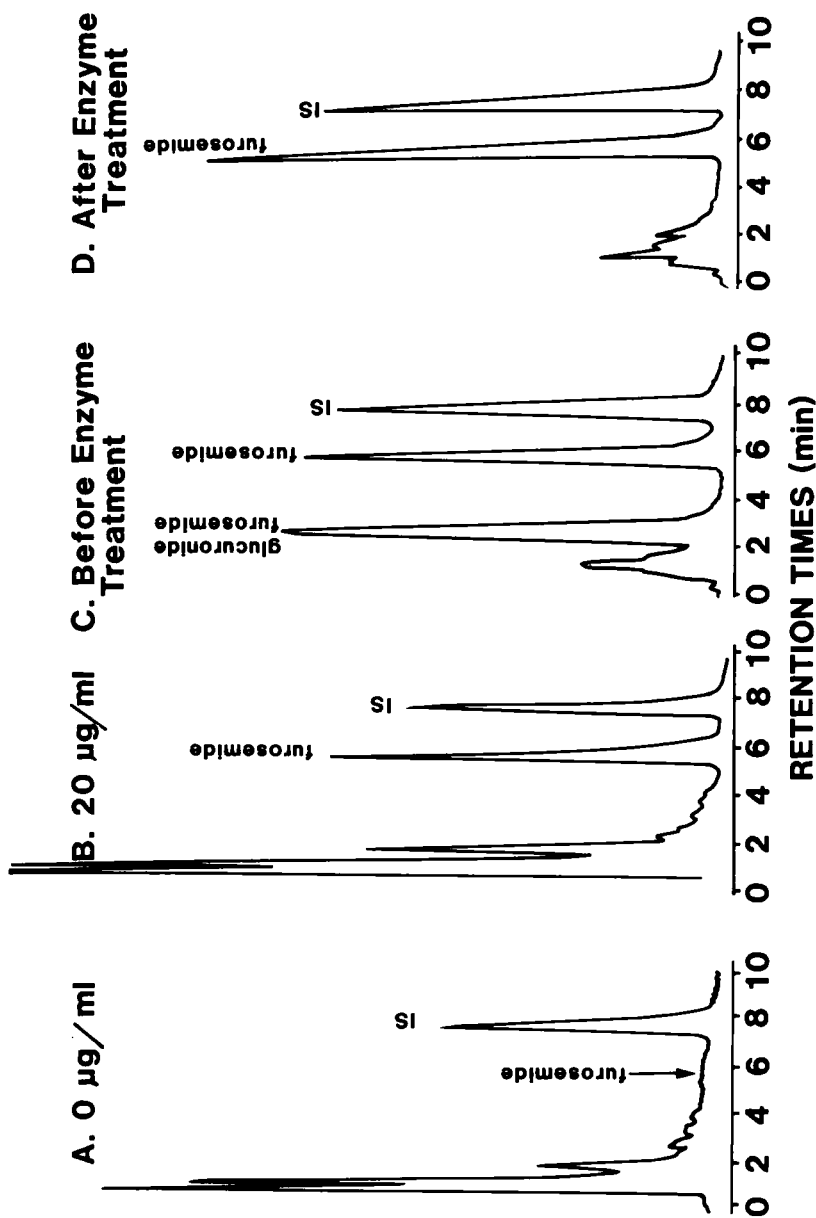
FIGURE 1.   HPLC Chromatograms of urine precipitations.
Mobile Phase: Acetic acid pH 3.55-CH_3OH (72:28)

A: 0 µg/ml   **B: 0.5 µg/ml**   **C: patient**

RETENTION TIME (MIN)
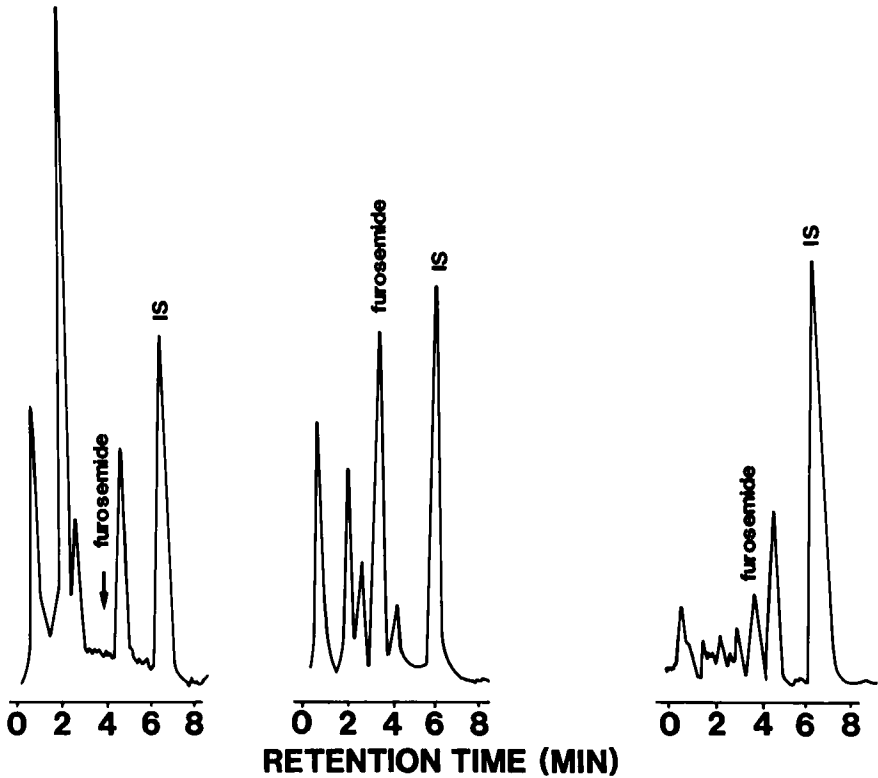
FIGURE 2.   HPLC Chromatograms of plasma extracts.
Mobile Phase: Na N_2PO_4 0.01M, pH 3.5-Ch_3OH (65:35)

Plasma Analysis:

The plasma assay presented here combines portions of several methods found in the literature. The result is a procedure which is sensitive and convenient, and minimizes furosemide decomposition. The stability of furosemide under the conditions of the analysis is documented.

The plasma method of Kerremans, et al. (8) formed the basis for the procedure described in this paper. Segments taken from that method include acidification with acetic acid, use of 6-

desmethylnaproxen as internal standard, and chromatography under
similar conditions. A major difference in the methods is our
substitution of methylene chloride for their ether-hexane
extraction solvent. They chose not to use methylene chloride,
citing extraction of excessive endogenous fluorescent materials.
We did not find this to be a problem, possibly due to use of a
different type of fluorescence detector and the use of different
wavelengths. Kerremans, et al. (8) also went to great lengths to
protect their plasma samples from light during the extraction
procedure. We did not find this to be necessary.

Furosemide decomposition has been noted in the presence of
hydrochloric acid, phosphate buffers and sulfate ions, and is
catalyzed with light (8,11). The use of acetic acid for plasma
extraction minimizes hydrolysis (8). In our laboratory we tested
furosemide stability in each of the solvents that are used for the
plasma assay. The drug was stable in methanol for at least four
days and in methylene chloride for at least 24 hours while exposed
to normal fluorescent room lighting. Desmethylnaproxen solutions
also showed excellent stability in CH_3OH and CH_2Cl_2. These
results indicate that special treatment to protect the samples
from light during processing is unnecessary. The stability of
frozen plasma samples has also been confirmed by the present
study for a period of at least 4 months. This data is in good
agreement with the literature (8).

The assay for furosemide in plasma demonstrates good within-
run and day-to-day reproducibility (Table 1). The standard curve
shows good precision and is linear from 10.0 ng/ml to 15.0 mcg/ml
plasma (Table 2). The method is 2 to 10 times more sensitive than
recently published methods (4-10), and the range of linearity is
wider than that reported in the other papers. The recovery of
furosemide from plasma was 69.9% at 2.0 mcg/ml of plasma.

Urine Analysis:

The analysis of urine for furosemide involves several
special considerations. Both furosemide and its glucuronide
conjugate are present in patient samples, and both compounds may
exhibit instability under certain conditions. To quantitate the
drug and metabolite accurately, one must use processing conditions
which minimize or avoid decomposition. The methods found in the
literature all have certain disadvantages which the present
procedure seeks to avoid. These problems include long or compli-
cated sample processing, decomposition of the analytes, hydrolysis
of the furosemide glucuronide using harsh conditions which
probably cause simultaneous decomposition reactions, low sensi-
tivity, and narrow range of assay linearity (2,6,8-10).

An improved analysis for urine furosemide (F) with a simpli-
fied hydrolysis of furosemide glucuronide (FG) is presented here.
The method takes into account the stability of F and FG in

TABLE 1

REPRODUCIBILITY OF THE FUROSEMIDE PLASMA ASSAY

WITHIN-RUN

	$\overline{X}$(MCG/ML)	SD	CV(%)	N
High	1.47	0.032	2.2	3
Low	0.74	0.024	3.2	3

DAY-TO-DAY

	$\overline{X}$(MCG/ML)	SD	CV(%)	N
High	1.47	0.098	6.7	12
Low	0.66	0.056	8.5	12

various organic solvents and aqueous solutions and utilizes mild
conditions for the hydrolysis of FG by β-glucuronidase. The
relative fluorescence of F and FG are discussed, and data is
presented to show the quantitative conversion of FG to F by enzyme
hydrolysis. Each of these topics is discussed in the following
sections.

The method described in this paper for analysis for total
furosemide in patient urine samples involves hydrolysis of FG to F
with β-glucuronidase, followed by precipitation of proteins with
acetonitrile. The samples are then centrifuged and the super-
natant injected into the HPLC.

Furosemide Glucuronide Stability and Hydrolysis:

Chromatography of urine samples obtained from healthy male
volunteers who took furosemide produced two peaks not found in
drug-free urine (Figure 1). One peak corresponded to furosemide
and the second peak which eluted earlier, appeared to be somewhat
unstable, and could be readily hydrolyzed to furosemide by the
addition of β-glucuronidase. It was therefore assumed to be FG.
The chromatographic elution of the F and FG peaks is similar to
that reported by Carr, et al. (6), with the same mobile phase.

TABLE 2

FUROSEMIDE PLASMA STANDARD CURVE

mcg/ml Added	mcg/ml Found*	CV%
0.010	0.013 + 0.001	7.7
0.020	0.024 + 0.002	8.3
0.030	0.033 + 0.003	9.1
0.050	0.055 + 0.005	9.1
0.100	0.099 + 0.010	10.1
0.300	0.314 + 0.016	5.1
0.500	0.535 + 0.026	4.9
1.000	1.077 + 0.022	2.0
2.000	2.002 + 0.103	5.1
4.000	3.813 + 0.170	4.5
6.000	5.890 + 0.159	2.7
8.000	8.084 + 0.300	3.7
10.000	9.881 + 0.133	1.3
12.500	12.238 + 0.453	3.7
15.000	14.341 + 0.484	3.4

Total N = 60

Correlation Coefficient (R) = 0.9991

*mean + standard deviation

Furosemide glucuronide appears to be a relatively unstable compound both in untreated urine and in urine to which water, buffers, or organic solvents have been added. We did not have pure FG available to use for preparing standard solutions, so instead we used urine collected from volunteers who took the drug, precipitated the proteins, and measured relative changes in the HPLC peak size of FG compared to F and the IS. Urine stored at room temperature for periods up to 48 hours showed a substantial decrease in the relative size of the FG peak. However, the furosemide concentration did not show a simultaneous increase, indicating that either furosemide glucuronide was decomposing to something other than F, or that the F itself was also decomposing. No additional fluorescence peaks were detected.

A protein precipitation method of preparing urine samples for analysis was chosen to allow for rapid processing of a large number of samples. Three solvents were compared as possible precipitating agents - CH_3OH, CH_3CN, and THF. The stability of furosemide and furosemide glucuronide in each was tested at ambient temperature with exposure to normal fluorescent room light (Table 3). Furosemide showed no decomposition in either CH_3OH or CH_3CN, but was not stable in THF. Furosemide glucuronide concentrations decreased in all 3 solvents with time. Acetonitrile was chosen over methanol for use in the assay, since it gives a more complete precipitation of proteins.

The use of β-glucuronidase to hydrolyze FG to F has been reported in the literature (6,8), incubating the urine with the enzyme for 21 to 24 hours at $37^{O}C$. Such long incubation periods pose two problems - potential decomposition of the compounds and excessive processing time. To minimize these problems, we undertook a short study to determine the effect of incubation of urine with water, buffers, and enzyme at several time intervals and at two temperature levels (ambient and $37^{O}C$). We found that a moderate enzyme concentration (2000 U/ml urine) completely hydrolyzed the glucuronide to furosemide in 10 seconds as evidenced by a complete disappearance of FG and a simultaneous increase in the furosemide peak. The incubation of FG urine with water, sodium acetate buffer, or sodium phosphate buffer each showed a decrease in the FG peak size after only 30 minutes at room temperature. At $37^{O}C$, the FG completely decomposed in the aqueous and acetate buffered solutions within 3 hours, while the FG in the phosphate solution disappeared within 20 hours (Table 4). There was not a significant increase or decrease in F in any of these non-enzyme media, indicating that FG must be decomposing to something other than F under those conditions. The present study shows that the urine sample hydrolysis may be simplified greatly from what has been previously reported in the literature. In the present work, we routinely hydrolyzed 100 mcl aliquots of patient urine samples with 50 mcl of enzyme solution (100 U) for 15 minutes at room temperature, thus eliminating the need for

TABLE 3

PERCENTAGE OF FUROSEMIDE (F) AND FUROSEMIDE GLUCURONIDE (FG)
REMAINING IN SOLUTION AFTER PRECIPITATION OF URINE SAMPLES
WITH VARIOUS SOLVENTS AT ROOM TEMPERATURE

Time	CH_3CN		CH_3OH		THF	
	%F	%FG	%F	%FG	%F	%FG
10 sec	100	100	100	100	100	100
24 hr	99.5	62	101.2	37.7	86	92

TABLE 4

PERCENTAGE OF FUROSEMIDE (F) AND FUROSEMIDE GLUCURONIDE (FG)
MEASURED CHROMATOGRAPHICALLY AFTER INCUBATION AT DIFFERENT CONDITIONS
IN THE PRESENCE AND ABSENCE OF β-GLUCURONIDASE
MOBILE PHASE-ACETIC ACID pH 3.55-CH_3OH (72:28)

Incubation Conditions	H_2O		Sodium Acetate 0.04 M pH 7		Sodium Phosphate 0.075M pH 6.8		Enzyme in Phosphate Buffer 2000 u/ml	
	%F	%FG	%F	%FG	%F	%FG	%F	%FG
10 sec, 22°C	100	100	100	100	100	100	100	100
30 min, 22°C	102	95	104	94	105	95	115	0
5 min, 37°C	102	93	98	86	97	90	112	0
30 min, 37°C	99	56	93	55	96	81	110	0
3 hr., 37°C	104	0	100	0	103	60	118	0
20 hr., 37°C	106	0	102	0	105	0	115	0

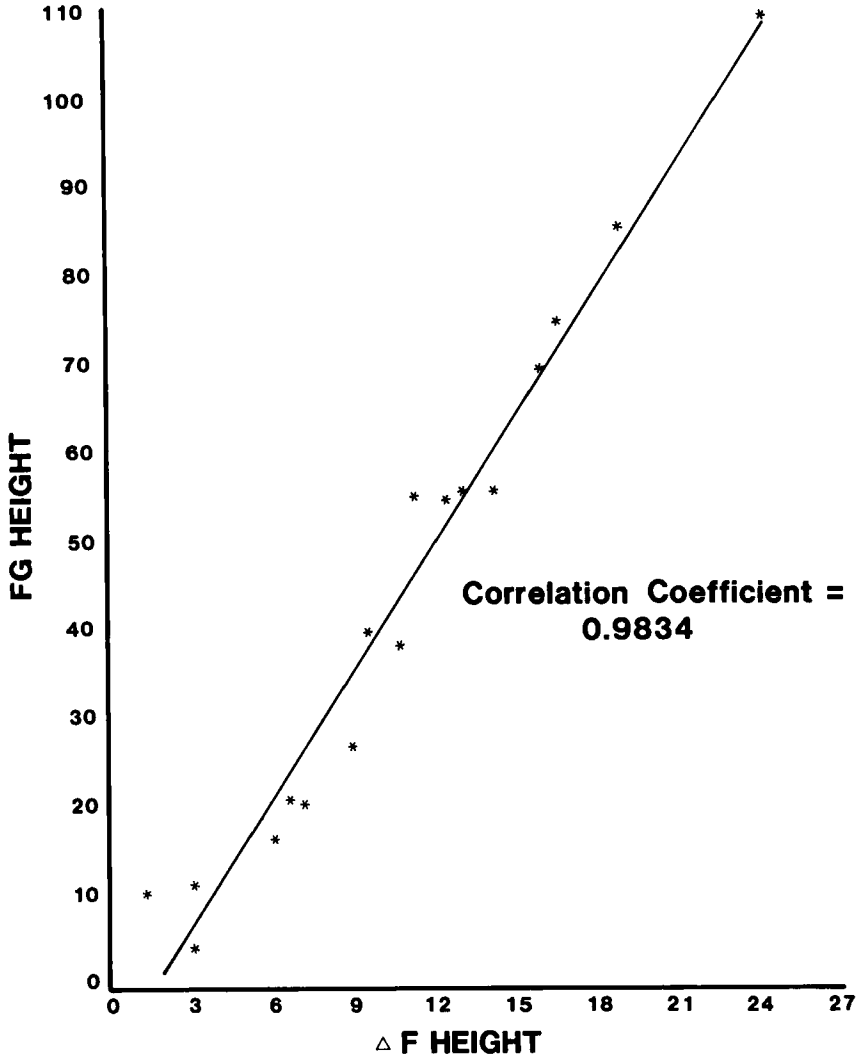**FIGURE 3.** FG Height versus increase in furosemide after enzyme incubation.

TABLE 5

REPRODUCIBILITY OF THE FUROSEMIDE URINE ASSAY

WITHIN-RUN

	$\bar{X}$(MCG/ML)	SD	CV(%)	N
High	27.1	0.519	1.9	4
Medium	10.3	0.201	2.0	4
Low	5.4	0.141	2.6	4

DAY-TO-DAY

	$\bar{X}$(MCG/ML)	SD	CV(%)	N
High	29.2	1.059	3.6	12
Medium	10.4	0.574	5.5	12
Low	5.2	0.419	8.1	12

constant temperature (37°C) incubation and greatly reducing
processing time.

The correlation between relative FG peak height and the
change in F concentration after enzyme incubation was studied.
Urine from two volunteers taking furosemide orally was collected
at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 12, and 24 hours. The samples
were analyzed immediately upon collection after treatment with
and without β-glucuronidase. The results are plotted in Figure 3
and show a linear relationship demonstrating conversion of FG to F
over a wide concentration range. The correlation coefficients for
the plots from the individual volunteers were 0.9657 and 0.9943
with a combined correlation of 0.9834.

The response of the fluorescence detector signal to FG seems
to be several orders of magnitude greater than the signal response
for furosemide under the present assay conditions. This is
evidenced by urine enzyme hydrolysis experiments where the
disappearance of a large FG peak resulted in only a small
increase in F peak size (Figure 1). No other chromatographic
peaks were noted while this phenomenon occurred, so we assume
quantitative enzyme conversion of FG to F. The large difference

TABLE 6

FUROSEMIDE URINE STANDARD CURVE

mcg/ml Added	mcg/ml Found*	CV%
0.5	0.52 ± 0.03	5.8
1.5	1.34 ± 0.007	0.5
2.5	2.31 ± 0.009	0.4
5.0	4.91 ± 0.032	0.7
10.0	10.18 ± 0.020	0.2
20.0	19.98 ± 0.034	0.2
40.0	38.16 ± 2.53	6.6
80.0	80.82 ± 3.25	4.0
120.0	120.75 ± 1.32	1.1
160.0	153.82 ± 2.95	1.9
200.0	205.23 ± 3.86	1.9

Total N = 55

Correlation Coefficient (R) = 0.9990

*mean ± standard deviation

in fluorescence detector response is probably due to the structures of the compounds. The ratio of FG to F peak size also varied slightly from day to day in our laboratory, possibly due to variations in mobile phase composition and retention time changes. Attempts to synthesize or recover FG from urine were unsuccessful so that further study and quantitation of FG could not be included in the present work.

The furosemide urine assay demonstrates good reproducibility for both within-run and day-to-day quantitation (Table 5). The standard curve had good precision and was linear from 0.5 to

200 mcg/ml urine (Table 6). The recovery of furosemide from urine was 98.6% at 50 mcg/ml. The sensitivity of the method is higher than or equivalent to that from extraction methods presently available (6,8,9).

CONCLUSIONS

The methods for plasma and urine furosemide analysis presented in this paper have several advantages over other procedures in the literature. The plasma assay provides higher sensitivity and has good linearity and reproducibility. Furosemide degradation is minimized by appropriate choices of solvents and extraction conditions. Photolytic degradation is not observed using the method.

The assay for urine furosemide uses a simple CH_3CN protein precipitation step instead of extraction, and yet has equivalent or higher sensitivity than other procedures. The method is linear and reproducible. The enzyme hydrolysis of FG to F was studied, and the incubation period is reduced to 15 minutes at room temperature, a substantial savings in processing compared to the 21 and 24 hour incubations at $37^{O}C$ which were described in the literature. The immediate treatment of fresh urine samples with β-glucuronidase insures that the FG is converted quantitatively to furosemide without decomposition to other by-products.

The sensitivity, simplicity, reproducibility, and wide ranges of linearity for both the plasma and urine assays make them applicable for monitoring furosemide levels in patients and for use in bioequivalency studies. These methods have been used to analyze several hundred plasma and urine samples in our laboratory.

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