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Comparison of extraction and precipitation methods for the HPLC determination of furosemide in plasma and urine

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Abstract: Comparisons have been made between an ether extraction method and an acetonitrile precipitation method for the HPLC determination of furosemide (frusemide) in human plasma and urine. Recoveries of furosemide were 81–89% (ether extraction method for plasma), 73–103% (acetonitrile precipitation method for plasma) and 62–89% (ether extraction method for urine) for the concentration ranges studied. Values of correlation coefficients were 0.9998, 0.9991 and 0.9997 for standard curves for the three methods, respectively. Accuracy and precision (RSD) were: 92.4–114% ± 3.57–20% for ether-extracted plasma; 98.1–103% ± 3.47–19.9% for acetonitrile-precipitated plasma; and 103–107% ± 4.03–13.2% for ether-extracted urine. Because of carryover of endogenous urine components, the acetonitrile-precipitation assay was unacceptable for urine. Furosemide was stable in frozen plasma for at least 113 days and in frozen urine for at least 204 days. No artifactual appearance of the hydrolysis product of furosemide, 4-chloro-5-sulfamoylanthranilic acid (CSA), was detected by the ether-extraction method under normal assay conditions.

Keywords: *Furosemide; reversed phase HPLC; ether extraction; acetonitrile precipitation.*

Introduction

Furosemide (frusemide) is 4-chloro-*N*-furfuryl-5-sulfamoylanthranilic acid (Fig. 1); it is widely used as a potent diuretic. A variety of analytical methods exist for the determination of furosemide in biological fluids (for example, [1–5]); most approaches are based on separation of furosemide by high-performance liquid chromatography and measurement by fluorimetric detection. Some laboratories [1–3] use an ether-extraction procedure before chromatography. Typically, samples containing furosemide are acidified and extracted from the biological fluid matrix with several volumes of diethyl ether. After evaporation of the organic phase, the residue is dissolved in a suitable solvent and subjected to chromatography. Other laboratories [4–5] have employed an acetonitrile precipitation step in place of ether extraction. Addition of two volumes of

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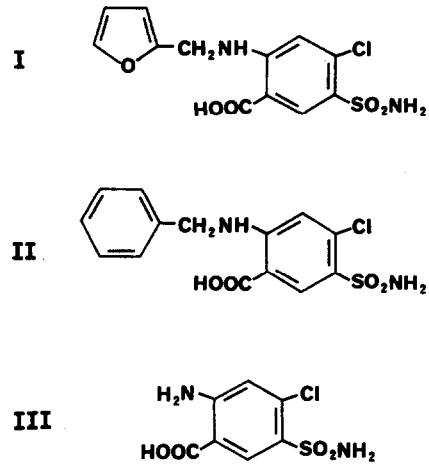

Figure 1
Structures of furosemide (I), internal standard (II) and CSA (III).

acetonitrile to a sample of plasma or urine containing furosemide results in precipitation principally of proteinaceous materials, while furosemide remains in the solution phase. A portion of the solution phase is subsequently concentrated and subjected to analysis by HPLC. Notably, Smith *et al.* [4] have employed this method to support the hypothesis that the appearance in chromatograms of 4-chloro-5-sulfamoylanthranilic acid (CSA; III in Fig. 1) from biological samples containing furosemide is an analytical artifact, rather than indicating the presence of an authentic metabolite of furosemide.

Samples of plasma and urine from pharmacokinetic studies and subsequently analysed in the authors' laboratory led to an interest in the relative merits of the ether-extraction and acetonitrile-precipitation procedures. In the present study, the accuracy, precision, sensitivity and required analysis time for each method is appraised. Extended stability data for furosemide in frozen plasma and urine samples are also reported.

Materials and Methods

Materials

Furosemide and CSA were provided by Hoechst-Roussel Pharmaceuticals Inc. (Somerville, NJ, USA) and *N*-benzyl-4-chloro-5-sulfamoylanthranilic acid (internal standard II) by Hoechst AG (Frankfurt, FRG). These standard compounds were used as received after determining homogeneity by HPLC (see below). Chromatographic solvents were of HPLC grade. All other chemicals were reagent grade. Stock solutions of furosemide (approximately 2 mg/ml) and internal standard II (approximately 1 mg/ml) were prepared in either methanol or acetonitrile and were stable for at least 3 weeks when stored protected from light at 4°C. Control plasma was obtained from the local plasma centre and was pooled from an unknown number of donors. Control urine was obtained from one of the authors (M.B.). Several different batches of this urine showed similar chromatographic characteristics in the ether-extraction method.

Ether extraction of plasma samples

To 1 ml of plasma was added 10 μl of a four-fold diluted solution of internal standard II stock solution (final concentration approximately 2.5 μg/ml) and, for standard curve samples, 100 μl of furosemide stock solution appropriately diluted to yield final

concentrations of furosemide up to 5.2 μg/ml. The sample was acidified with 100 μl of 6 M HCl and immediately extracted with 5 ml of anhydrous diethyl ether by manual shaking for 2 min. After centrifugation (1875*g* for 10 min), 4 ml of the ether phase was transferred to another tube, evaporated under a gentle stream of nitrogen, and reconstituted in 0.25 ml of 0.02 M glycine buffer, pH 11. A 100-μl portion of the glycine buffer phase was subjected to chromatography using an IBM HPLC system comprising a 150 × 4.6 mm i.d. column packed with 5-μm ODS (Altex) with fluorescence detection (Gilson Spectra/Glo filter fluorimeter, excitation filter 330–400 nm, emission filter 460–600 nm). The mobile phase was methanol–water–acetic acid (40:57:3, v/v/v); the flow rate was 1.45 ml/min. Figure 2 shows a chromatogram obtained under the above conditions. Furosemide was typically eluted with a k' of 6.3 while the internal standard II was typically eluted with a k' of 17.

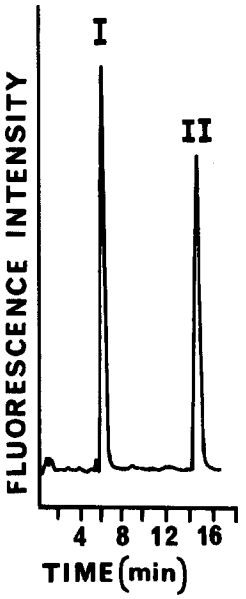

Figure 2
Chromatogram of furosemide (I) and internal standard (II) from a sample of ether extracted spiked plasma. Concentrations of furosemide and internal standard were 2.60 and 2.80 μg/ml, respectively.

Ether extraction of urine samples

The method for urine extraction and chromatography was similar to that for plasma, with the exception that 4 ml of the ether phase obtained from the extraction (inversion mixer, 2 min) of the acidified sample was transferred to another tube and back extracted with 1 ml of 0.02 M glycine buffer, pH 11. A 100-μl portion of the glycine buffer phase was then subjected to chromatography as described for plasma. A chromatogram obtained under the above conditions is shown in Fig. 3.

Acetonitrile precipitation of plasma and urine samples

To 1 ml of plasma or urine was added sufficient volume of the stock solution of internal standard II to give a final concentration of 5.50 μg/ml. For standard curve samples, 100 μl of furosemide stock solution (in acetonitrile), appropriately diluted, was added to give final concentrations of 0.26 to 5.20 μg/ml; 2 ml of acetonitrile was then added and the samples were shaken manually for 2 min. After centrifugation (1875*g* for 10 min) to sediment the precipitated material, a 2-ml portion of the supernatant solution was

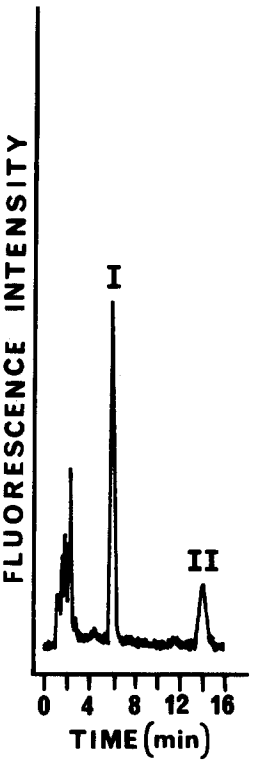

Figure 3
Chromatogram of furosemide (I) and internal standard (II) from a sample of ether extracted spiked urine. Concentrations of furosemide and internal standard were 10.2 and 2.55 μg/ml, respectively.

transferred to a clean tube, evaporated under a gentle stream of nitrogen at 40°C, and reconstituted in 1 ml of CH_3CN–0.015 M H_3PO_4 (35:65, v/v [4]). HPLC separation was accomplished with the CH_3CN–H_3PO_4 mixture as the mobile phase; the other column conditions were as described for the ether extraction method. Figure 4 shows a chromatogram obtained for a plasma sample. Furosemide was typically eluted with a k' of 4.0, while internal standard II was typically eluted with a k' of 7.2. When the acetonitrile-precipitation assay was used, 1-ml urine samples yielded a large amount of endogenous material which was carried through the separation process. Chromatograms indicated inadequate chromatographic resolution of furosemide from the endogenous substances.

Results

In the development, modification and comparison of analytical methods, absolute recovery, sensitivity, standard curve characteristics, accuracy and precision must be considered. Table 1 shows values of absolute recoveries of furosemide from spiked plasma samples for each of the two analytical procedures compared in this study, an ether extraction procedure and an acetonitrile precipitation procedure. As indicated in this table, percentage recoveries and standard deviations are good for the concentration range examined, although the sensitivity was five times less (approximately 0.26 μg/ml) for the acetonitrile precipitation method than that for the ether extraction method (approximately 0.05 μg/ml). Although Smith *et al.* [4] have reported a sensitivity limit of approximately 8 ng/ml by use of fluorescence detection in a sample of one-fifth the

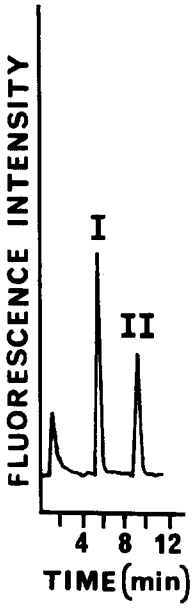

Figure 4
Chromatogram of furosemide (I) and internal standard (II) from a sample of acetonitrile precipitated spiked plasma. Concentrations of furosemide and internal standard were 10.4 and 5.50 μg/ml, respectively.

Table 1

Method	Concentration range (μg/ml)		% Recovery*			
	Furosemide	Internal standard II	Furosemide		Internal standard II	
Ether extraction						
Plasma	0.05–5.0	0.25–27	98.9 ± 9.73	(*n*=21)	74.4 ± 8.82	(*n*=20)
Urine	0.1–50	0.25–28	104 ± 7.40	(*n*=25)	79.3 ± 8.43	(*n*=24)
Acetonitriole precipitation						
Plasma	0.25–5.0	0.25–25	98.3 ± 10.6	(*n*=19)	89.0 ± 11.5	(*n*=20)

*Percentage recovery ± relative standard deviation.

volume used in the present study, this result could not be repeated under the conditions described above.

Absolute recoveries of furosemide from drug-spiked urine samples (ether extraction method only) are also shown in Table 1. Recoveries and relative standard deviations are acceptable at or above 0.5 μg/ml, which was the practical lower sensitivity limit for the urine assay using the ether extraction method. When the acetonitrile precipitation assay was used with urine samples, large amounts of endogenous material were carried through the separation process. In the chromatograms furosemide was not adequately resolved from endogenous urine constituents; the acetonitrile precipitation method was therefore unacceptable for urine samples of 1-ml volume.

The potential loss of furosemide by acid-catalysed hydrolysis during extraction of HCl-acidified plasma was examined. Table 2 presents the results of stability experiments of

Table 2
Loss of furosemide* as a function of time in acidified PBS and acidified plasma

Sample extracted	Time (min)	Mean % furosemide remaining† Set 1	Set 2
PBS	0	(100)	(100)
	5	98.4 ± 8.09	96.8 ± 6.41
	10	98.9 ± 6.57	96.4 ± 6.65‡
	30	95.6 ± 8.14	93.0 ± 8.96
	60	89.5 ± 5.60	95.3 ± 18.1
Plasma	0	(100)	(100)
	5	101 ± 7.90§	101 ± 3.53
	10	102 ± 9.14	100 ± 5.56
	30	97.9 ± 5.95§	97.4 ± 3.38
	60	100 ± 5.85	94.3 ± 6.16§

*Phosphate-buffered saline (PBS; 137 mM Nacl, 8.31 mM Na_2HPO_4, 1.66 mM KH_2PO_4, pH 7.4) or plasma (1 ml) spiked with furosemide and acidified with 6 M HCl (100 μl) as described in the text for the ether extraction assays of plasma. Determinations were normalized to 100% recovery at zero time. Values shown are mean relative standard deviation for four determinations (PBS) or six determinations (plasma), unless otherwise indicated in the table.

†Set 1: furosemide concentrations 0.252–0.262 μg/ml and 0.248–0.262 μg/ml (plasma). Set 2: furosemide concentrations 2.02–2.10 μg/ml (PBS) and 1.98–2.10 μg/ml (plasma).

‡n = 3 determinations.

§n = 5 determinations.

samples of furosemide in acidified phosphate-buffered saline (PBS) and plasma. Essentially quantitative recoveries of furosemide were obtained when samples were acidified and extracted within 2 min, which is readily achievable under normal assay conditions. The data in Table 2 indicate that this is well within the time prior to significant acid-catalysed loss of furosemide. Figures 5A and 5B show chromatograms of zero hour PBS (A) and plasma (B) samples representative of the data shown in Table 2. The arrow indicates the retention time of authentic CSA subjected to chromatography under identical conditions. Figure 5C is a chromatogram of blank plasma. Peaks in the retention time range of 2–3 min were always present in the blank samples and did not increase in area as a function of time; it was therefore concluded that these materials were not CSA.

Regression characteristics for the standard curve data were acceptable for the concentration ranges 0.052–5.20 μg/ml (ether extraction method for plasma), 0.260–5.20 μg/ml (acetonitrile precipitation method for plasma) and 0.260–26.0 μg/ml (ether extraction method for urine). Correlation coefficients (n=3) were 0.9991, 0.9998 and 0.9997 for these three methods, respectively. Actual lower and upper limits for daily standard curves would be expected to be based on values expected for clinical samples for a given experimental design. As noted above, carryover of endogenous material made chromatographic resolution, and thus standard curve construction, impossible for urine samples subjected to the acetonitrile-precipitation method.

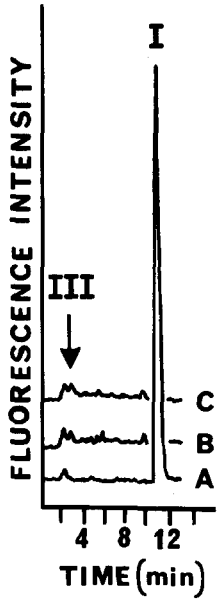

Figure 5
Chromatograms of zero hour PBS (A) and plasma (B) samples spiked with furosemide (I) and subjected to the ether extraction method described in the text. The arrow indicates the retention time of authentic CSA (III) subjected to chromatography under the same conditions. The chromatogram shown in (C) represents blank plasma assayed under identical conditions. The flow rate was 0.8 ml/min.

To test the accuracy and precision of each assay, samples of plasma and urine spiked with a variety of concentrations of furosemide were subjected to both ether extraction and acetonitrile precipitation methods. As indicated in Table 3, accuracy and precision were acceptable for both methods with respect to plasma samples of drug, and were of a higher order at or above concentrations of 0.26 μg/ml (ether extraction) and 1.1 μg/ml (acetonitrile precipitation). Ether extracted urine samples exhibit a decrease in precision below approximately 1 μg/ml, with an unacceptable level of precision below 0.5 μg/ml. Normally, the lower limit of sensitivity for the urine ether extraction assay was 0.5 μg/ml.

Experiments on the stability of furosemide in frozen biological matrices indicated that the drug was stable for at least 113 days in frozen plasma; and at least 204 days in frozen urine.

Discussion

The results indicated that an ether extraction method was effective in the analysis of biological samples containing furosemide. In the plasma assay, the sensitivity was approximately five times greater than that attained with an acetonitrile precipitation method. The reasons for the differences in sensitivity of the ether extraction method between the present data and those of Smith *et al.* [4] are not clear, although one possibility rests in potential differences in the measurement of fluorescence emission. In the present experiments, a filter fluorimeter with a bandpass emission filter of 460–600 nm was used; this allowed measurement of fluorescence intensity over most of the emission wavelengths of furosemide. Smith *et al.* [4] employed a fluorescence spectrophotometer equipped with dual monochromators and a lower sensitivity limit of 8.2 ng/ml of furosemide was claimed for 0.2-ml plasma samples. Sensitivity in the present experiments, however, may have been partially compromised by significant levels of photodetector noise over the wide emission wavelength range employed.

Table 3
Comparison of accuracy and precision of furosemide determinations*

Assay	Concentration range (μg/ml)	Mean recovery† (%)	RSD‡ (%)	*n*
Plasma				
Ether extraction	0.05–0.25	114	20.0	5
	0.26–1.0	100	6.61	8
	1.1–2.5	94.8	3.45	4
	2.6–5.0	92.4	3.57	5
Acetonitrile precipitation	0.25–1.0	98.1	19.9	9
	1.1–2.5	104	10.4	7
	2.6–5.0	103	3.47	5
Urine				
Ether extraction	0.50–1.0	105	13.2	12
	1.1–2.5	103	8.72	4
	2.6–5.0	103	3.64	4
	5.1–10.0	106	9.93	3
	11.0–25.0	105	4.18	5
	26.0–50.0	107	4.03	4

*Lower sensitivity limits for ether extraction and acetonitrile precipitation plasma assays were approximately 0.05 and 0.25 μg/ml, respectively; that for the urine ether extraction assay was approximately 0.5 λg/ml.
†Mean recovery is expressed as a percentage of the spiked concentration.
‡RSD = relative standard deviation.

Use of the acetonitrile-precipitation assay for furosemide-containing urine samples was unacceptable owing to the large amounts of endogenous urine components that were carried through during the precipitation step (urine sample volume of 1 ml). Pharmacokinetic studies often require measurement of low concentrations of drug, thus making the larger initial sample size (1.ml compared with 0.2 ml used by Smith *et al.*) attractive if not mandatory.

Whereas the time required per sample for extraction or precipitation and chromatographic separation was identical for each type of assay, the acetonitrile precipitation protocol suffers from the amount of time required to evaporate a relatively large volume (2 ml) of acetonitrile, a solvent considerably less volatile than diethyl ether. This extra manipulation, which is necessary to concentrate the sample sufficiently for HPLC analysis, increased the assay time per sample by approximately 30–45 min. The time required per sample would be extended further if sample composition and pH were adjusted, as suggested by Rapaka *et al.* [5].

The interpretation of the appearance of presumptive CSA in chromatograms of furosemide-containing samples has been controversial. Aside from the issue of whether or not CSA is an authentic furosemide metabolite present in biologically derived samples, the problem of potential loss of furosemide by hydrolysis to CSA as an assay artifact must be addressed. Cruz *et al.* [6] reported substantial hydrolysis of furosemide under acidic conditions and calculated a loss of furosemide of approximately 20% for a sample at pH 1 for 1 h at 37°C. Similarly, under conditions in the present work where a sample spiked with furosemide was acidified (pH less than or equal to 1) prior to extraction (ether extraction method), approximately 5–10% of furosemide was lost over 1 h at ambient temperature. Loss of furosemide due to photolytic decomposition in

acidic media has also been reported [3], thereby necessitating protection of furosemide-containing samples from light [7]. It is not known whether or not Smith *et al.* [4] took this precaution, but under conditions in the present work in which the ether extraction assay was employed (extraction with ether immediately following acidification) and with adequate protection of stock solutions and samples from light, there was no evidence of the production of other substances, in particular, CSA.

Finally, since the pharmacological activity of furosemide seems to correlate best with urinary furosemide content [8–10], a method that reliably measures levels of furosemide in human urine as well as plasma is highly desirable. It was found that the acetonitrile precipitation method was unsuitable for the measurement of furosemide in urine owing to large amounts of endogenous urine components that were carried through the procedure, thereby reducing the selectivity of the assay.

In summary, an ether extraction method is recommended for the analysis of biological fluids containing furosemide. Compared with the acetonitrile precipitation method using a common HPLC system, the ether extraction assay exhibited greater sensitivity and, for urine samples, greater selectivity. Processing time required for each sample was less for the ether extraction protocol and no artifactual production of furosemide degradation products was encountered.

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