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Simplified method to quantify furosemide in urine by high-performance liquid chromatography and ultraviolet detection

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Abstract

Simplified reversed-phase high-performance liquid chromatographic method with ultraviolet detection at 280 nm without extraction procedure is described to quantify furosemide in rabbit and human urine. An internal standard was not used. The lower limit of quantitation was 0.750 μg/ml using 50 μl urine samples (100 μl of total injection volume), and linear response was tested from 0.750 to 250 μg/ml in both humans and rabbits. Within and between-day accuracy and precision were always below 10% at all analyzed concentrations. Validation data showed that this method is linear, sensitive, selective, specific, accurate and reproducible.

Keywords: Furosemide

1. Introduction

Furosemide is a potent diuretic drug currently used in clinical practice. Despite its introduction a long time ago, a recent interest in the pharmacokinetic/pharmacodynamic relationship emerged not only for furosemide but also for other diuretics acting at the loop of Henle [1–4].

Different methods using high-performance liquid chromatography (HPLC) to measure furosemide have been described [5–9], some of them requiring fluorometric or radioimmunoassay detection, while most require an extraction procedure.

The purpose of this study was to examine an HPLC method to quantify furosemide in urine samples by direct injection and ultraviolet detection.

In relation to the HPLC method, our starting point was three methods previously proposed elsewhere to quantify furosemide in urine [8,10,11].

2. Experimental

2.1. Materials

Standard of furosemide was either kindly donated by Hoescht Marion Roussel S.A. de C.V. (Mexico City, Mexico) or obtained from Sigma–Aldrich Química S.A. de C.V. (Mexico City, Mexico). The methanol (HPLC grade) was obtained from Sigma–

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Aldrich Química, whereas glacial acetic acid 100% reagent grade was obtained from Merck Mexico S.A. (Mexico City, Mexico). An internal standard was not used. The pure deionized water was obtained by means of a Milli-Q plus water system (Millipore Corporation, Bedford, MA, USA). Filter papers No. 42, ashless circles were obtained from Whatman Limited, England, UK. Furosemide used for the pharmacokinetic analysis in a human volunteer was an oral formulation containing 20 mg (Lasix, Hoescht Marion Roussel), while an intravenous formulation also containing 20 mg (Lasix, Hoescht Marion Roussel) was used for the rabbit.

Urine was obtained from New Zealand male rabbits, lightly anesthetized with one dose of ketamine i.m. (6–10 mg/kg), xylazine chlorhydrate i.v. (2 mg/kg), and diazepam i.v. (1 mg/6 h), by inserting a 10 Fr-Foley catheter into the bladder through the urethra. Human urine was obtained from healthy volunteers by spontaneously voiding the bladder.

2.2. Sample preparation

The furosemide standard solution (1 mg/ml) was prepared daily, firstly by dissolving furosemide in an 800-μl volume of methanol and then adding the mobile phase.

The standard urine solution of furosemide (1 mg/ml), prepared for either rabbits or humans, was prepared daily, firstly by dissolving furosemide in an 800-μl volume of methanol and adding urine. The urine was filtered, acidified to pH 3.0 by adding acetic acid, and then diluted to 50:50 (v/v) with methanol. This urine was used for construction of the standard curves.

The standard solution and standard urine solution were stored at −80°C. For use, a sample was placed in a glass vial and 100 μl was injected into the HPLC system.

2.3. Chromatography

A Beckman HPLC system was equipped with a Model 126 solvent delivery system, a Model 507 injector (the autosampler was automatically operated and used chromatographic nitrogen), and a Model 168 UV–VIS variable-wavelength detector. The analytical column was a C18 Spherisorb column (150× 4.6 mm I.D., 5 μm particle size) (Phase Separations Inc., Norwalk CT, USA). The whole system was automatically operated by a Compaq Prolinea 4/50 computer, using the System Gold v. 8.10 (Beckman Instruments, Inc., Fullerton, CA, USA).

The chromatographic separation was carried out at room temperature, and the solvent flow-rate was 0.750 ml/min. The mobile phase was prepared by mixing methanol and water (acetic acid 3%) in a ratio of 40:60, v/v. The detection wavelength was 280 nm, band width was 8 nm and 0.005 AUFS. Neither filtration nor extraction was performed to the urine samples.

Urine was collected, centrifuged at 4000 *g*, pooled, filtered through filter paper, acidified to pH 3.0, and stored at 4°C. Calibration curves were constructed from furosemide, which was placed in a 10 ml-flat-bottom flask, diluted with 800 μl of methanol, had the urine added to it, and then the mobile phase (50:50, v/v) was used to make the volume up to 10 ml. Serial dilutions were prepared and concentrations of 0.750, 5.0, 25.0, 50.0, and 250.0 μg/ml of furosemide were obtained. The final solution was then vortexed at median speed for 1 min, and then transferred to the autosampler vials. Sets of quality control (QC) samples were prepared in batchs in the same manner at same concentrations, including one at 1000 μg/ml. The QC sample containing the highest concentration was used to investigate the effect of sample dilution.

2.4. Validation

Method validation was performed according to the guidelines recorded in the conference report on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [12], with minor modifications as described previously [13]. All validations were run on four consecutive days and included calibration curves processed in triplicate and a set of QC samples in quintuplicate analyzed with one cycle of freezing and thawing.

2.5. Pharmacokinetic study

The application of the method for pharmacokinetic studies was evaluated according with the Ethical and

Research Committee of the Hospital de Especialidades, Centro Médico Nacional ''Siglo XXI'', IMSS, Mexico City, Mexico. Urine samples were obtained at selected times over a period of 8 h from one rabbit receiving 3 mg/kg i.v. of furosemide. Whereas, the urine samples from a volunteer, who had had one 20 mg tablet, was collected for a period of 4 h by spontaneously voiding the bladder. All urine samples were collected in glass tubes without a preservative, centrifuged at 4000 *g* for 5 min, transferred into 1.5 ml microcentrifuge tubes of polypropylene, and stored in the dark at −80°C. Prior to injection into the HPLC system, they were allowed stand at room temperature for 10–15 min, and centrifuged at 4000 *g* for 5 min. Furosemide concentration was determined by the procedure described above.

3. Results

3.1. Specificity

Fig. 1 displays typical chromatograms resulting from HPLC analysis of direct injection of urine samples. The upper (A) and lower (B) left panel represent a direct injection of rabbit and human urine samples, respectively. Whereas, a urine sample from rabbit and human that had been spiked to contain 25 μg/ml of furosemide were displayed on the upper (C) and lower (D) right panel, respectively.

Certain drugs that are commonly used clinically and potentially could be co-administered with furosemide were selected to check for potential interference in the assay. These included amikacin, ampicillin, calcium, cephalothin, ciprofloxacin, clin-

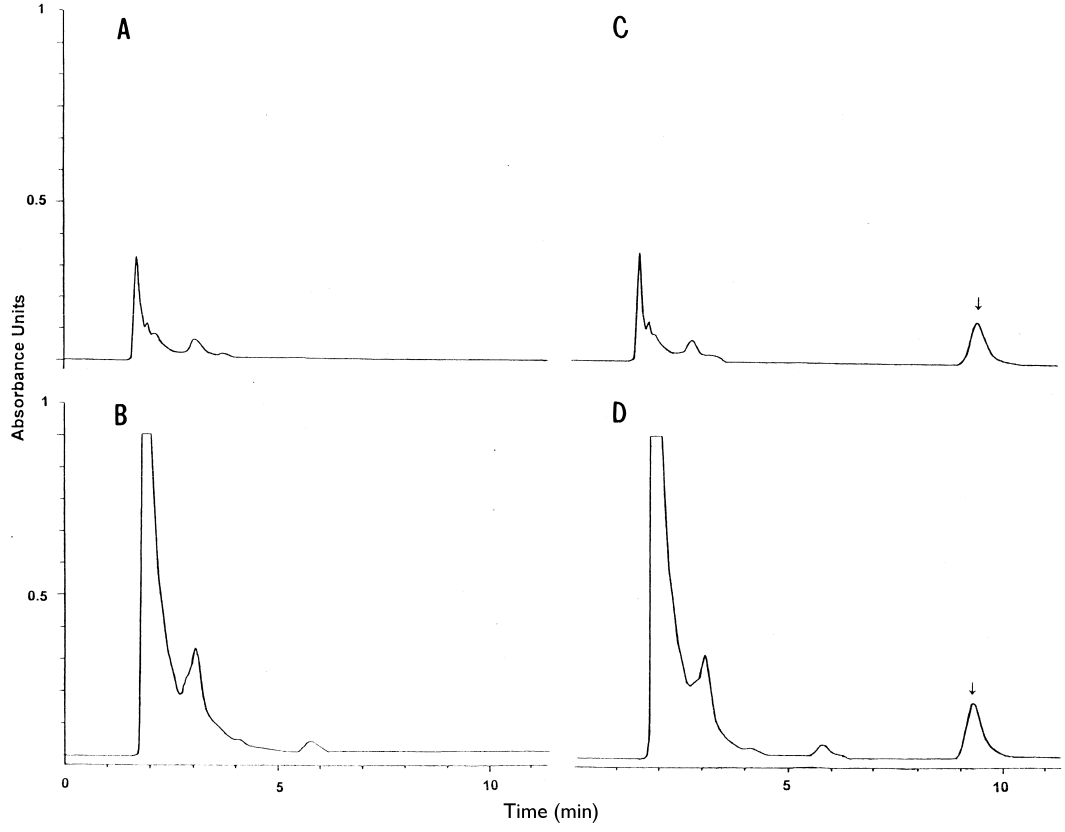

Fig. 1. Typical chromatogram of furosemide-free rabbit (A) and human (B) urine samples (upper and lower panel at left side, respectively). The same rabbit and human urine samples spiked with furosemide (C and D, respectively) are shown at the right side of the figure.

damycin, conjugated estrogens, dexamethasone, dipyrone, estriol, imipenem, medroxypregesterone, metoprolol, metronidazole, nalbuphine, octreotide, pentoxifylline, phenazopyridine, ranitidine, trimethoprim/sulfamethoxazole, spironalactone, and vitamin C. Under the analytical conditions, none interfered with the analysis of furosemide.

3.2. Validation characteristics

Typical chromatograms of direct injection of urine samples are shown in Fig. 1. The retention times of furosemide were of 9.89±0.16 min in the mobile phase and of 10.9±0.35 min in the urine samples. The relationship between the peak area and furosemide concentration in the mobile phase and rabbit urine was evaluated over the range 0.750–250.0 μg/ml and was found to be linear: $y=3.02x+2.41$ ($r=0.9999$, $n=6$), $y=3.0657x+1.0096$ ($r=0.9999$, $n=6$), respectively. In relation to human urine the relationship between the peak area and furosemide concentration was found to be linear: $y=3.20x+2.20$ ($r=0.9999$, $n=6$). As previously suggested [14], the upper and lower limit of quantification are the highest and lowest calibration standards of the method, respectively. In addition, relationships between calibration curves performed on the mobile phase and rabbit urine ($y=1.0137x-1.6$, $r=0.9999$) and for human urine ($y=1.0590x-0.71$ ($r=0.9999$) were also found to be linear in the same range of 0.750–250.0 μg/ml.

The detection limits, defined as the furosemide concentration producing a signal-to-noise ratio of 3, were 0.050 μg/ml and 0.200 μg/ml in the mobile phase and urine, respectively.

The intra-assay coefficient of variation of the method for furosemide in urine was always less than 10%, i.e. 1.63% (24.74±0.41 μg/ml, $n=6$) and 4.43% (249.63±11.09 μg/ml, $n=6$) in rabbits (Table 1) and 1.66% (249.70±4.15 μg/ml, $n=6$) and 4.49%(24.86±1.15 μg/ml) in humans (Table 2).

The stability of furosemide in urine samples was studied at room temperature and without any light protection. The calibration curves were obtained in the range 0.750–250 μg/ml, and concentration of furosemide decreased 4.36% (from 5.02±0.03 to 4.80±0.005 μg/ml) in 24 h. The stability at −80°C was also established up to three months without any significant decrease in furosemide concentrations.

The method was thereafter applied to the pharmacokinetic studies described above. The observed cumulative urinary concentrations vs time curve for the human healthy volunteer and rabbit are shown in Fig. 2.

4. Discussion

In the present paper we describe a method to quantify furosemide in human and rabbit urine by direct injection of urine samples. In fact, the samples were only centrifuged and diluted before injection. Filtration did not improve either the signal or any analytical parameter and the use of an internal standard was not required.

In addition, by adding furosemide (diluted in the

Table 1
Accuracy and inter-assay precision of the high-performance liquid chromatographic assay used to quantified furosemide in rabbit urine samples by direct injection

Theoretical concentration (μg/ml)	Measured concentrations (μg/ml; mean±S.D.) ($n=6$)	Accuracy (%)	C.V. (%)
0.750	0.70±0.17	94.63	1.60
5.00	4.45±0.17	89.00	3.49
25.00	24.73±0.41	98.94	1.63
50.00	52.06±1.65	104.14	3.14
250.00	249.63±11.09	99.85	4.43

S.D.=Standard deviation.
C.V.=Inter-assay coefficient of variation.

Table 2
Accuracy and inter-assay precision of the high-performance liquid chromatographic assay used to quantified furosemide in human urine samples by direct injection

Theoretical concentration (μg/ml)	Measured concentrations (μg/ml; mean±S.D.) (n=6)	Accuracy (%)	C.V. (%)
0.750	0.73±0.09	97.33	3.20
5.00	4.58±0.16	91.60	2.92
25.00	24.86±1.15	99.40	4.49
50.00	51.66±2.01	103.32	3.85
250.00	249.70±4.15	99.88	1.66

S.D.=Standard deviation.
C.V.=Inter-assay coefficient of variation.

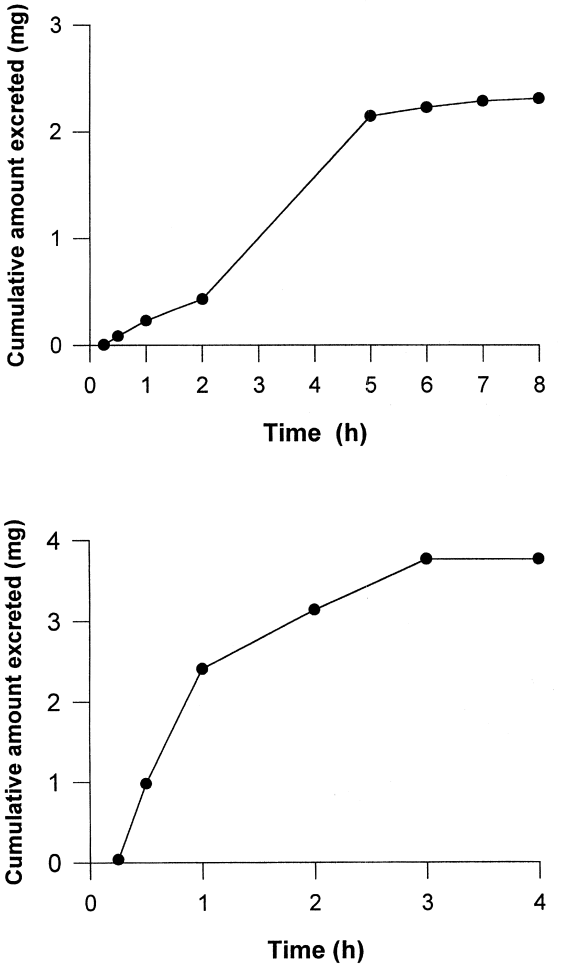

Fig. 2. An observed cumulative urinary concentrations against time curve for human and rabbit (upper and lower panel, respectively) by using the method describe in the paper.

mobile phase) to the urine samples, 1:1, to a final concentration of 1.0 μg/ml, increased the simplicity of the method without affecting any analytic parameter and allowing us to easily quantify amounts under 0.750 μg/ml.

Previously, some methods quantifying furosemide in plasma or urine by employing gas chromatography with electron capture detection or high-performance liquid chromatography with fluorometric detection have been described. These methods have required some time-consuming and costly steps and only two methods used a direct injection of urine samples in humans [10,11]. These two methods used acetonitrile as a component of the mobile phase. In the current method, we used methanol, which possesses a very similar polarization constant to acetonitrile, is less toxic, and has a lower cost, e.g. 1 l of methanol costs US$ 20.60, while 1 l of acetonitrile costs US$ 38.10 (data obtained from the 1997 Sigma catalogue, Sigma–Aldrich Química). Furosemide was eluted in a short enough time to be quantified without any interfering signal and calibration curves were constructed in a range similar to a previously described method using direct injection of urine [10] and with a wider range than another previously described method [11].

5. Conclusions

In conclusion, the method described in the current paper fulfills the precision, linearity, sensitivity, and specificity requirements to quantify furosemide in urine samples. Drugs probably co-administered with

furosemide do not interfere with the assay. Furthermore, the procedure appears to be cheaper than those previously described. Altogether these factors make this method a useful tool to be used in pharmacokinetic studies in healthy subjects and patients, and also probably for routine monitoring of furosemide levels in small volumes of urine samples.

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