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Short Communication

Automated high-performance liquid chromatographic method for determination of furosemide in dog plasma

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ABSTRACT

An automated high-performance liquid chromatographic method for the determination of the diuretic drug furosemide has been established. Dog plasma was injected directly into a two-column system with a BSA–ODS (ODS column coated with bovine serum albumin) precolumn and a C_{18} analytical column for the separation of furosemide. The two columns were automatically switched. Furosemide remained trapped on the precolumn while proteins were eluted to waste. After column switching, furosemide was washed onto the analytical column and analysed without interference. The greatest advantage of the method is its easy performance without manual sample preparation; it requires no extraction or deproteinization. The method allows determination of 0.1–10 μg/ml of furosemide with accuracy and precision comparable with previously reported values. The coefficients of variation obtained from replicate measurements of 1 μg/ml and 5 μg/ml samples were 1.65% and 2.40%, respectively. This method was used to measure the plasma levels of furosemide in beagle dogs to whom the drugs was administered, as a reference, in a toxicological study.

INTRODUCTION

The toxicity of a new drug has been evaluated by administering to animals and monitoring its plasma level in a toxicokinetic study. In the study, a higher dose of drug than used in clinical therapy has been administered for a long period. An assay method with high sensitivity is not necessarily required, but it is advantageous to enable analysis with simple sample treatment using less plasma. Furosemide was chosen as a reference drug for toxicity testing in the development of a new diuretic drug. However, previously reported high-performance liquid chromatographic (HPLC) methods, although they have higher sensitivity for plasma (human and animal), require pretreatment before the HPLC analysis. Typically, extraction with organic solvent [1–3], solid phase extraction [4,5] or deproteinization with acetonitrile [6–9] are needed. In order to decrease sample preparation time, we used an on-line column-switching technique. We have previously reported the use of a similar technique for the determination of an antibiotic human plasma [10]. The two-column system introduces plasma to the precolumn for sample clean-up and then, after the proteins have been washed to waste (drain), the flow lines are changed automatically by a column-switching operation. This changes the mo-

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bile phase and elutes the analyte onto the analytical column for separation. A protein-coated BSA–ODS (bovine serum albumin) precolumn and a reversed-phase C_{18} analytical column were used.

In this study, our automated system was used to assay furosemide in dog plasma. We were able to establish an automated HPLC method that allowed direct injection of plasma with on-line clean-up. Only 50 μl of plasma were needed, which is less than in the other reported methods. We assayed furosemide in beagle dog plasma collected after its administration at the dose of 10 mg/kg. This amount was given daily over a 91-day period, and the levels in the animals were measured at the 86-day administration.

EXPERIMENTAL

Materials and reagents

Furosemide that conformed to the Japanese Pharmacopoeia was used for animals. Standard furosemide was purified by recrystallization of the commercial chemical (for biochemistry) from Wako (Osaka, Japan). A solution of 0.5 mg/ml in 50% acetonitrile and water was prepared and used after dilution as a standard solution. Acetonitrile, methanol and water were of HPLC grade from Wako. All other reagents were of Japan Industrial Standard special grade.

Sampling from animals

In a repeated-dose toxicity study, the drug and its reference material, furosemide, were administered orally to 7-month-old beagle dogs. Furosemide was given for 91 days at 10 mg/kg per day to each of five male and five female dogs. Blood collections were made on the first day and on the 86th day, near the end of the test. The collection times were before (0) and after administration at 1, 3, 6 and 24 h. Blood was transferred to a heparinized polyethylene tube and centrifuged. Separated plasma was stored at −20°C.

Apparatus

The HPLC system was the same as that used previously [10]. The detection wavelength was set at 254 nm. A centrifuge Model XX42 CF06T (Japan Millipore) was used.

Mobile phase

The pretreatment mobile phase (S_1) was 2.5 m*M* ammonium phosphate (pH 5)–methanol (50:1). The washing mobile phase (S_2) for the precolumn was acetonitrile–water (1:1). The mobile phase for analysis (S_3) was 2.5 m*M* ammonium phosphate (pH 2.5)–acetonitrile (69:31).

Column-switching procedure

Solvents S_1, S_2, and S_3 were passed through the two valves 1 and 2 (Fig. 1). Each value turns to two directions called "1" and "2".

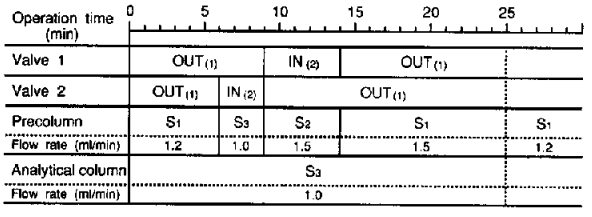

Fig. 1. Flow diagram of the column-switching system. S_1 = pretreatment mobile phase; S_2 = washing mobile phase; S_3 = mobile phase for analysis.

Conditioning of the columns. The two valves were positioned at "1". S_1 and S_3 flowed through the precolumn and the analytical column, respectively, along the dotted lines in valve 2 [10] at the flow-rates of 1.2 and 1.0 ml/min, respectively. Both solvents were finally flushed into the drain.

Injection of the sample. A 50-μl volume of the sample was injected at 0 min. S_1 flowed for 6 min at a flow-rate of 1.2 ml/min. Furosemide was retained near the head of the precolumn.

Transfer of the analyte and washing of the precolumn. At 6 min after sample injection, valve 2 was positioned at "2". The solvent delivery into the precolumn was changed from S_1 to S_3. The analyte in the precolumn was back-flushed (flow-rate, 1.0 ml/min) along the solid lines in valve 2 [10] to the analytical column. S_1 was flushed into the drain.

Separation of the analyte and washing of the precolumn. At 9 min after sample injection, valves

1 and 2 were switched to positions "2" and "1", respectively. Through the continuous flow of S_3 at the same rate (1.0 ml/min), further separation of the analyte and coeluted substances from plasma occurred, with elution at 19.7 min. The precolumn was then washed with S_2 (flow-rate 1.5 ml/min) to remove any remaining substances.

Conditioning of the precolumn. At 14 min after sample injection, valve 1 was turned back to the original position ("1"). The precolumn was re-equilibrated by elution with S_1 at a flow-rate of 1.5 ml/min until the next sample injection. The injection interval for this procedure was 25 min.

The switching times for the two valves and the flow-rates of the three solvents in the chromatographic run (time programme) were programmed and stored in the system controller.

Assay procedure

The collected samples were thawed, and 200 μl each were transferred to a cylindrical cup (Ultrafree (C3-GV), Japan Millipore). After centrifugation at 2000 *g* for 1 min, 50 μl of the supernatant were injected into the column. Solvent delivery proceeded automatically according to the time programme. The peak area at 19.7 min was measured.

Calibration graph

The stock solution was diluted with 50% aqueous acetonitrile to make three standard solutions (1.0, 2.5 and 5 μg/ml). The plot of the peak area of each standard solution obtained by injection against the concentration gave a linear line as a calibration graph.

RESULTS AND DISCUSSION

The chromatographic system established previously [10] was applied to the furosemide analysis. Combination of the two columns with the delivery of three solvents enabled both the pretreatment and the analysis to be performed on-line. Solvent S_1 for clean-up of plasma sample and solvent S_2 for washing of the precolumn consisted of similar constituents to those used in the previous method. The mobile phase S_3 was changed for a pH 2.5 buffer (2.5 m*M* ammonium phosphate)–acetonitrile solution to retain furosemide on the ODS column. The pK_a value of furosemide was 4.61 (50% methanol). Delivery of these solvents under the same time programme as described in the previous method (flow-rate and switching time) separated the analyte without interference from plasma components. A typical chromatogram obtained by the direct injection of dog plasma sample is shown in Fig. 2.

Analysis of spiked samples

Beagle dog plasma was spiked with furosemide in the range 0.5–10 μg/ml, and 50 μl were assayed. When the spiked plasma was assayed using this calibration graph, the correlation between the added and the measured values (Table I) gave a good linear relationship with a slope of *ca.* 1.0, indicating little or no effect of plasma components on the assay. The linearity was reproducible with a standard deviation of 0.102 μg/ml, proving the accuracy of the method.

Precision and reproducibility

Spiked samples of 1.00 and 4.98 μg/ml were

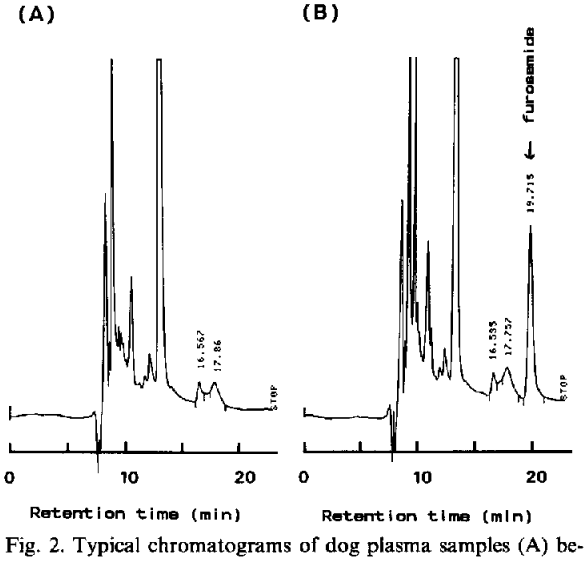

Fig. 2. Typical chromatograms of dog plasma samples (A) before administration and (B) from the same dog 1 h after oral administration of 10 mg/kg of furosemide. Plasma concentration of furosemide, 2.51 μg/ml.

TABLE I

REGRESSION ANALYSIS DATA FOR THE ASSAY OF FUROSEMIDE IN DOG PLASMA (0.5–10 μg/ml)

Run	Regression equation	s^a	r
1	$y = 0.976x + 0.039$	0.070	0.9998
2	$y = 0.998x - 0.061$	0.128	0.9995
Total[b]	$y = 0.987x - 0.011$	0.102	0.9996

[a] $n = 6$.
[b] Covariance analysis.

TABLE II

PRECISION AND RECOVERY LEVEL OF THE METHOD

Run	Concentration (μg/ml)	
	1.00 μg/ml	4.98 μg/ml
1	0.98	4.88
2	0.95	5.20
3	0.98	4.96
4	0.95	5.01
5	0.98	4.92
Mean ± S.D.	0.97 ± 0.016	4.99 ± 0.12
C.V. (%)	1.65	2.40

analysed repeatedly. As shown in Table II, five replicate measurements showed the same level of precision with a coefficient of variation (C.V.) of 1.65–2.40%. The day-to-day measurements reproduced the assay values within the variation limits.

Limit of determination

The lower limit of the determination was 0.1 μg/ml plasma. The peak area at this concentration was on the extrapolated calibration graph. Replicate analyses of the 0.1 μg/ml samples reproduced the spiked amount with a C.V. of 20%.

Stability of furosemide

Furosemide was stable in aqueous acetonitrile solution for at least 5 h. No lowering phenomenon was observed. Samples were stable for 90 days at −20°C.

Plasma level of beagle dogs

The plasma concentration–time profiles (Fig. 3) showed similar patterns after each administration between the first and the last days, and little or no differences was observed between male and female dogs. The C_{max} value was 2–3 μg/ml, at *ca.* 1–2 h. On near the final day, as on the first day, the drug disappeared after 24 h. The results indicate little accumulation of the drug after multiple administration to dogs.

This HPLC method involves less samples handling than previous methods, and was successfully applied to dog plasma specimens in toxicity testing. No deterioration of the BSA–ODS column was observed after analyses of several hundred plasma samples.

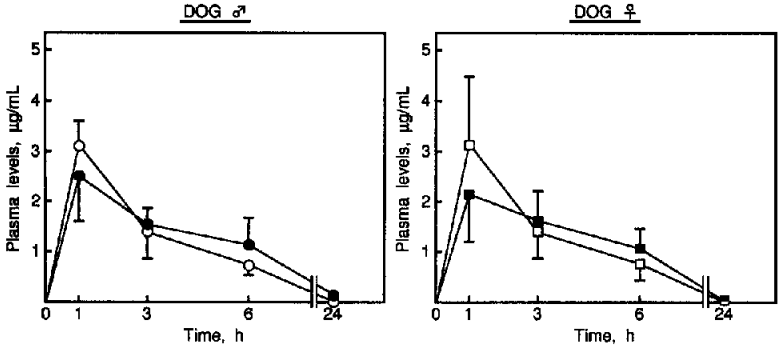

Fig. 3. Plasma levels of furosemide during 3-month repeated dose test: (○) and (□) after the first administration of 10 mg/kg per day; (●) and (■) after the 86th administration.

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