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Short communication

Determination of furosemide in plasma and urine using monolithic silica rod liquid chromatography

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Abstract

In the present study we developed a fast and reliable HPLC assay for the determination of the loop diuretic furosemide in plasma and urine, using a Chromolith® RP 18e (100 mm × 4.6 mm) monolithic silica rod HPLC column. After liquid–liquid extraction with diethylether, plasma or urine samples were separated with a gradient consisting of solvent A (20% acetonitrile) and solvent B (80% acetonitrile), both in 0.25% acetic acid. The flow rate was 3.5 ml/min and the effluent was monitored by fluorescence with excitation at 230 nm and emission at 410 nm. The retention times for the internal standard (naproxen) and for furosemide were 2.1 and 3.7 min, respectively, and total run time was 8 min. The calibration curves were linear between 7.8 and 1000 ng/ml, and within-assay and between-assay coefficients of variation were <6.5% and <10%, respectively. The proposed assay for furosemide in plasma and urine using monolithic silica rod chromatography is fast, sensitive, and reliable, and, thus, well suited for pharmacokinetic studies.

Keywords: Furosemide; Monolithic silica rod liquid chromatography; HPLC; Plasma; Urine

1. Introduction

Furosemide is a potent and widely used loop diuretic in the treatment of edematous states and/or associated with chronic renal failure, hypertension, congestive heart failure, or cirrhosis of the liver [1–7]. It can also be used for forced diuresis in cases of poisoning or overdosage of drugs [8].

Furosemide is administered both orally and intravenously. The pharmacokinetics of furosemide is well documented in healthy subjects. Absorption is rapid and peak levels occur after 60–90 min post-dose. It has a high plasma protein binding (97–98%) and is eliminated by hepatic and renal glucuronidation and by renal secretion and filtration [9]. The elimination half-life is relatively fast ($t_{0.5}$ 0.5–2 h). However, biphasic elimination kinetics with a slow second half-life of 20–30 h due to enterohepatic cycling of the furosemide acyl glucuronide has been described [10]. The interindividual variability of the pharmacokinetic behaviour of furosemide is large and is influenced by the underlying disease process [11].

Determination of furosemide in plasma and urine is warranted in pharmacokinetic studies in patients, for instance treated with multiple drug combinations (e.g. drugs which use the same renal secretory pathways) and in bioequivalence studies. Several HPLC, HPLC/MS and CE methods have been developed for the determination of furosemide in biological fluids [12–19]. However, employing classical HPLC columns can be rather time consuming, especially in larger pharmacokinetic studies. Recently, highly porous monolithic silica rod columns have been introduced, which have a bimodal pore structure with a large surface area [20–23]. Due to this feature, higher flow rates can be applied while the column back pressure is still low. These new columns enable flow rates up to 10 ml/min with good column performance, resulting not only in very short run times, but also in very rapid column equilibration, allowing a fast method change over.

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On this basis, the aim of the present study was to develop and optimise a fast and reliable HPLC method using monolithic silica rod column technology for the routine determination of furosemide in plasma and urine samples from patients.

2. Experimental

2.1. Chemicals

Furosemide and naproxen (internal standard) were from Sigma–Aldrich Corp., St. Louis, MO, USA. All other chemicals were of analytical grade and were purchased from Merck, Darmstadt, Germany. Double distilled water was used for all solutions.

2.2. Instrumentation and liquid chromatography

HPLC analyses have been performed on a LaChrome® system (VWR International, Darmstadt, Germany) which consisted of a model 7100 quarternary pump with on-line degassers, model 7200 autosampler, model 7485 fluorescence detector operating at an excitation wavelength of 230 nm and an emission wavelength of 410 nm, and a model 7360 column oven. The LaChrom® Elite software was used for controlling the HPLC system and for data acquisition and processing. Furosemide and internal standard (naproxen) were separated on a RP 18e (100 mm × 4.6 mm) Chromolith™ Performance monolithic silica rod column (VWR International, Darmstadt, Germany), including the corresponding 5 mm × 4.6 mm guard column, maintained at 32 °C. The mobile phase consisted of binary gradient consisting of solvent A (20% acetonitrile) and solvent B (80% acetonitrile), both in 0.025% acetic acid. The gradient started at 91% A for 1 min, decreased to 36% A within 2.4 min, followed by 100% B for 1.3 min, and finally returned to the starting conditions within 0.5 min. The flow rate was 3.5 ml/min, leading to a backpressure of 87 bar, and total run time was set to 8 min.

2.3. Sample preparation and standards

The extraction procedure for plasma and urine samples was similar as described before [16] with some modifications. To 0.5 ml of plasma or diluted urine, 50 μl of internal standard solution (20 μg/ml in water) and 0.15 ml of 2.5 M hydrochloric acid were added and extracted with 5 ml diethylether by vortexing at maximal speed for 75 s. Urine samples were prediluted 1:10 (v/v) with drug free urine prior to extraction. After centrifugation at 1500 × *g* for 3 min, the two phases were separated by freezing out of the water phase, and the organic layer was transferred in a second tube and evaporated to dryness in a turbovap® evaporator (Zymark Corporation, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. The residues were dissolved in 0.25 ml of mobile phase, and after mixing and sonication for 10 s, 25 μl of the supernatant were injected onto the column for HPLC analysis. Standards containing 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 ng/ml furosemide were prepared by dilution of a stock solution of furosemide (0.1 mg/ml) in methanol by appropriate volumes of drug free plasma or urine and stored frozen in aliquots at −20 °C.

2.4. Assay validation

Calibration curves were prepared in concentration ranges of 31.2–1000 ng/ml. For quality control studies, the calibration curves were extended down to 7.8 ng/ml. Precision and accuracy were determined by running quality controls at low and high concentrations covering the calibration range on the same (intra-day) and on different days (inter-day variability). Extraction recovery was calculated by comparing the peak areas of extracted spiked plasma or urine samples with the unextracted spiking solution peak areas. The limit of detection (LOD) was calculated at signal-to-noise ratio of 3 and the approximate limit of quantitation (LOQ) was defined for the lowest concentration measured with a signal-to-noise ratio of about 10. Specificity of the method was tested by injecting drugs which are often co-administered with furosemide at concentrations above the normal therapeutic range. In addition, furosemide acyl glucuronide in urine samples was de-conjugated by the method of Vree et al. [13], and the samples were analysed before and after de-conjugation.

2.5. Method application

After having given written informed consent, a healthy volunteer received on two separate study days a single dose of 20 mg furosemide by intravenous or oral route, respectively. Blood samples were withdrawn into heparinised glass tubes before, and after 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 6, and 8 h after drug intake. Urine samples were taken before, and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 24 h after drug intake. Plasma samples and, after recording of the volume, aliquots of the urine samples were stored frozen at −70° until analysis. All samples were light protected during the whole study, e.g. amber vials were used during analysis.

3. Results and discussion

With a flow rate of 3.5 ml/min and a backpressure of 89 bar, furosemide and the internal standard could be separated with retention times of 2.1 and 3.7 min, respectively, resulting in a total run time of 8 min. A mobile phase gradient was found of advantage to shorten the analysis time and to wash down late eluting endogenous products. Examples of chromatograms of a plasma and urine sample from healthy volunteers are shown in Fig. 1. Because column backpressure is still low despite a flow rate of 3.5 ml/min there, there would be room for a further reduction of the total run time by employing even higher flow rates. For example, after adaptation of the gradient pattern the flow rate can be raised to 5.2 ml/min leading to a total run time of 5.5 min with a corresponding back pressure of 142 bar without loosing peak resolution. In comparison, total run times of published HPLC methods are in the order between 10 min [21] and 42 min [13].

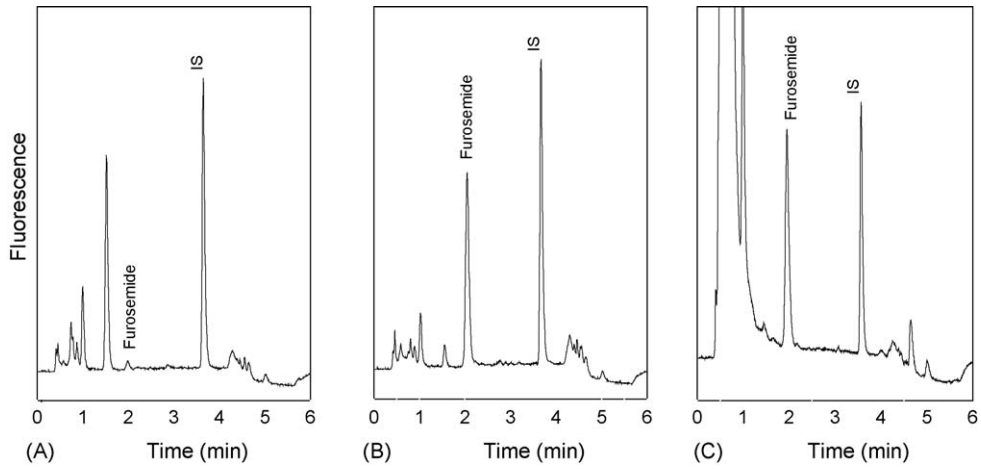

Fig. 1. Chromatograms of furosemide and internal standard (IS) extracted from plasma (A) and (B), or urine (C). The concentrations of furosemide in plasma were 12 ng/ml (A) and 218 ng/ml (B), and in urine 254 ng/ml (C), respectively.

Table 1
Intra-day and inter-day precision and accuracy for furosemide in plasma ($n = 6$)

Intra-day				Inter-day			
Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)	Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)
45.2	42.7	2.4	94.6	31.3	34.0	6.3	109
250	258	6.4	103	286	275	7.0	96.2

Standard curves showed a good linearity between 7.8 and 1000 ng/ml. The correlation coefficients (R^2) of the linear calibration curves were between 0.9980 and 0.9999 for both matrixes. Extraction recoveries for furosemide and internal standard from plasma were $76.5 \pm 2.51\%$ and $84.7 \pm 3.25\%$, respectively ($n = 5$). The corresponding recoveries from urine were $90.8 \pm 3.8\%$ and $95{,}9 \pm 3.0\%$, respectively ($n = 5$). Intra- and inter-day precision and accuracy at low and high furosemide concentrations in plasma and urine are summarized in Tables 1 and 2. As a result of the short retention times, high and narrow peaks were achieved, leading to good assay sensitivity with lower limit of quantitation (LLOQ) of 12 ng/ml and lower limit of detection (LLOD) of 3 ng/ml. At 12 ng/ml (LLOQ) the intra-day coefficient to variation (C.V.) was 5.4%, and the accuracy 101% ($n = 12$). To determine the specificity of the method, drugs which are often used in combination with furosemide (spironolacton, propranolol, metoprolol, enalapril, captopril, digoxine, and losartan) were injected under assay condition. As a result, no interfering peaks were observed for all these compounds. In addition, urine samples with high furosemide concentrations taken 2 h after drug intake were analysed before and after enzymatic de-glucuronidation to verify that the furosemide acyl glucuronide peak is not interfering with furosemide. Urine samples with high furosemide concentrations were also measured after extraction as described and by direct injection of the samples. No significant difference between the results was observed, indicating, that there is no degradation of the samples, e.g. hydrolysis of the glucuronide, during sample preparation.

The concentration time data in human plasma after an oral or intravenous dose of 20 mg of furosemide are shown in Fig. 2. The corresponding urinary recoveries of furosemide after the two different applications in the same volunteer are shown in Fig. 3. The long-term stability of the monolithic silica rod column was excellent. At present more than 2000 plasma and urine samples have been investigated with the same column without any substantial loss of column performance. This is in agreement with observation by others [23,24].

The use of the silica rod technology for the fast and effective separation of drugs and other compounds is a very promising concept. Unfortunately, the available selection of stationary phases is quite limited so far. In addition, at present, the num-

Table 2
Intra-day and inter-day precision and accuracy for furosemide in urine ($n = 6$)

Intra-day				Inter-day			
Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)	Theoretical concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)
45.2	43.6	4.7	96.5	99.6	96.9	10.3	96.9
747	728	2.9	97.5	747	714	3.0	95.6

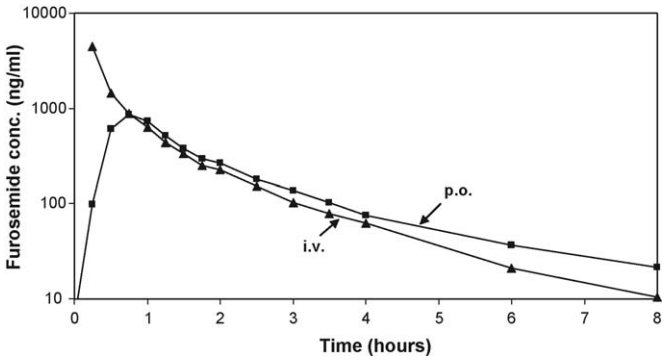

Fig. 2. Concentration–time curves in plasma of a subject receiving 20 mg furosemide p.o. (■) or i.v. (▲).

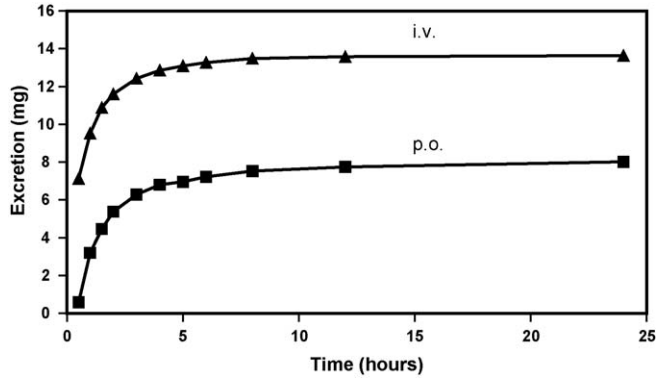

Fig. 3. Cumulative urinary excretion of furosemide over 24 h of a subject receiving 20 mg furosemide p.o. (■) or i.v. (▲).

ber of published applications for the use of silica rod HPLC for pharmacokinetic studies and therapeutic drug monitoring is rather scarce. This might change, if additional stationary phases are introduced in the near future. The use of very fast HPLC columns with a good performance is also of great advantage in the field of therapeutic drug monitoring (TDM), because the TDM laboratory is often faced with the situation that different HPLC methods have to be applied for only a few patient samples, and the results have to be forwarded to the clinicians on the same day. Thus, the application of high flow rates offers not only the advantage of short run times but also of short equilibration times after column exchange.

In conclusion, a rapid, sensitive, and reliable and robust HPLC assay has been developed for the determination of furosemide in plasma and urine using a monolithic silica rod column at a high flow rate. The application of fast monolithic columns proves to be a useful approach for pharmacokinetic studies with a large amount of plasma and urine samples. Another advantage using this technique is the possibility for a rapid method change over because of short column equilibrium times.

References

[1] L.Z. Benet, J. Pharmacokinet. Biopharm. 7 (1979) 1–27.
[2] M.M. Hammarlund, L.K. Paalzow, B. Odlind, Eur. J. Clin. Pharmacol. 26 (1984) 197–207.
[3] M. Hammarlund-Udenaes, L.Z. Benet, J. Pharmakin. Biopharm. 17 (1989) 1–46.
[4] L.L. Ponto, R.D. Schoenwald, Clin. Pharmacokinet. 18 (1990), 381–408, 460–471.
[5] F. Fiaccadori, G.C. Pasetti, G. Pedretti, P. Pizzaferri, G.F. Elia, Cardiology 84 (1994) 80–86.
[6] G. Romano, G. Favret, E. Federico, E. Bartoli, Pharmacol. Res. 37 (1998) 409–419.
[7] F. Cavaliere, S. Masieri, Curr. Drug Targets 3 (2002) 197–201.
[8] J. Prandota, Am. J. Ther. 9 (2002) 317–328.
[9] T.B. Vree, A.J. van der Ven, J. Pharm. Pharmacol. 51 (1999) 239–248.
[10] D.C. Brater, R. Seiwell, S. Anderson, A. Burdette, G.J. Dehmer, P. Chennavasin, Kidney Int. 22 (1982) 171–176.
[11] D. Farthing, T. Karnes, T.W. Gehr, C. March, I. Fakhry, D.A. Sica, J. Pharm. Sci. 81 (1992) 569–571.
[12] J.S. Sidhu, B.G. Charles, J. Chromatogr. 612 (1993) 161–165.
[13] T.B. Vree, M. van den Biggelaar-Martea, C.P. Verwey-van Wissen, J. Chromatogr. 22 (1994) 53–62.
[14] T. Okuda, K. Yamashita, M. Motohashi, J. Chromatogr. B: Biomed. Appl. 682 (1996) 343–348.
[15] C.D. Mills, C. Whitworth, L.P. Rybak, C.M. Henley, J. Chromatogr. B: Biomed. Sci. Appl. 701 (1997) 65–70.
[16] H.S. Abou-Auda, M.J. Al-Yamani, A.M. Morad, S.A. Bawazir, S.Z. Khan, K.I. Al-Khamis, J. Chromatogr. 12 (1998) 121–128.
[17] D. Teshima, T. Taniyama, R. Oishi, J. Clin. Pharm. Ther. 26 (2001) 387–390.
[18] V. Sanz-Nebot, I. Toro, R. Berges, R. Ventura, J. Segura, J. Barbosa, J. Mass Spectrom. 36 (2001) 652–657.
[19] J. Caslavska, W. Thormann, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 770 (2002) 207–216.
[20] N. Tanak, H. Kobayashi, N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Hosoya, T. Ikegami, J. Chromatogr. A 965 (2002) 35–49.
[21] Y.S. el-Saharty, J. Pharm. Biomed. Anal. 33 (2003) 699–709.
[22] F.C. Leinweber, D. Lubda, K. Cabrera, U. Tallarek, Anal. Chem. 74 (2002) 2470–2477.
[23] J.H. Smith, H.M. McNair, J. Chromatogr. Sci. 41 (2003) 209–214.
[24] A.M. van Nederkassel, A. Aerts, A. Dierick, D.L. Massart, Y. Vander Heyden, J. Pharm. Biomed. Anal. 32 (2003) 233–249.