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High-performance liquid chromatography using on-line solid-phase extraction: determination of furosemide in human serum

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Abstract

An on-line solid-phase extraction technique based on column switching (heart-cutting) was developed for direct injection analysis of furosemide in human serum. In order to minimize the influence of deterioration in pre-treatment column efficiency, which was caused by protein precipitation with repeated injections of serum, furosemide was completely enriched at the top of the analytical column by ion-pair formation with tetra-*n*-butylammonium ion during heart-cutting. The robustness of the established on-line solid-phase extraction system was confirmed under routine conditions. As a result, almost comparable chromatograms could be obtained even though 50 repeated injections of a 100-μl volume of serum were carried out using one pre-treatment column. The linearity of the calibration curves was demonstrated by the correlation coefficient which was greater than 0.99999 (5–1000 ng/ml). The relative errors and C.V. of quality control samples were within 4.00 and 5.88%, respectively (furosemide concentration: 5, 100 and 1000 ng/ml).

Keywords: Furosemide

1. Introduction

In order to elucidate the mechanism of drug metabolism in humans, determination of the metabolites as well as of the parent drug in biological fluid is frequently required in clinical pharmacokinetic studies. Administered drug is generally oxidized to the hydrophilic form in the body, in which the carboxylic acid of the parent drug is often recognized. Since there are many hydrophilic endogenous organic acids, laborious sample pre-treatment procedures have to be carried out for the sensitive and selective high-performance liquid chromatographic (HPLC) determination of acidic metabolites.

HPLC determination based on direct injection of serum has been increasingly used, due to the more widespread use of the column-switching technique. Generally, two methods are used for direct injection analysis. One is a method in which a restricted access column is used as a pre-treatment column [1–8] and the other is on-line solid-phase extraction [9–14]. The former enables one to analyze many samples by only one pre-treatment column, without the loss of column efficiency caused by protein precipitation, since serum protein cannot permeate into the hydrophobic region of the column [1–8]. Some problems, however, have been pointed out, e.g., restriction to small sample volumes, low per-

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formance and the high cost involved [1,2,6,7]. In the latter case, peak compression is usually attempted by back-flushing mode in order to minimize peak broadening. Thus, good separation of a drug from endogenous substances and high sensitivity could be obtained [9–14]. However, it is difficult to analyze many samples because of deterioration of the efficiency of the pre-treatment column by adsorption of serum protein [9–11].

For a simple and sensitive routine analysis of methotrexate (MTX) in monkey plasma, we reported on an on-line solid-phase extraction method [15]. In the method, enrichment of the MTX peak at the top of the analytical column was accomplished by using ion-pair chromatography in order to minimize the influence of deterioration in the pre-treatment column efficiency, thus, the problems with respect to on-line solid-phase extraction discussed above have been conquered. Since MTX is a hydrophilic carboxylic compound, the principal used in the MTX determination could be expanded to the determination of acidic metabolites. Therefore, we decided to confirm the applicability of our proposed principal with another carboxylic compound. Furosemide (FS, 5-(aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)-amino]benzoic acid) can be considered as a model compound for acidic metabolites, because FS is one of the carboxylic drugs. In the present study, the applicability of the proposed direct injection system under routine analytical conditions was confirmed by establishing a direct injection method for FS in human serum.

2. Experimental

2.1. Materials

FS was of biochemical grade and acetonitrile was of HPLC grade. These reagents were purchased from Wako (Osaka, Japan). Tetra-*n*-butylammonium bromide (TBAB, ion-pair chromatographic grade) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Deionized water was further purified using a Milli-Q laboratory water purification system (Nihon Millipore, Yonezawa, Japan). All other chemicals were of reagent grade and used without further purification. Drug-free serum was obtained from healthy volunteers.

2.2. Instrumentation and HPLC conditions

The HPLC system consisted of three LC-6A pumps, an SPD-10A ultraviolet (UV) detector, an FCV-2AH six-port switching valve and an SIL-6B autosampler, all of which were controlled by an SCL-6B controller (all from Shimadzu, Kyoto, Japan). UV detection was carried out at 271 nm. The peak height of FS was measured by a Waters 805 data station (Nihon Waters, Osaka, Japan). Samples injected onto the HPLC system were kept at 4°C using a WIG 7000A cooling system (Ishido, Chiba, Japan) just before analysis.

The pre-treatment column (C1) was Guard-pak μBondapak C_{18} (10 μm particle size; Cat No. 88070; column size, not opened; Waters, Milford, MA, USA). The analytical column (C2) was YMC Pack ODS column (A-type, 5 μm particle size, 150×4.6 mm I.D.; Yamamura Chemicals, Kyoto, Japan). The mobile phases (MP1, MP2 and MP3) were as follows: MP1, 0.02 *M* phosphate buffer (pH 7); MP2, acetonitrile; MP3, 0.02 *M* phosphate buffer (pH 7)–acetonitrile (65:35, v/v) containing 15 m*M* TBAB. The temperature was ambient for C1 and 40°C for C2. The flow-rates of MP1 and MP2 are described in Table 1 and that of MP3 was 1 ml/min.

Table 1
Time program for the column-switching system

Time (min)	MP1+MP2 (ml/min)[a]	MP1 concentration (%)	MP2 concentration (%)	Valve position
0	1	100	0	1
1	1			
1.01	2			
4.60				2
6.00		82	18	
7.00		82	18	1
7.01		40	60	
11.00		40	60	
11.01		100	0	
16.00	2			
16.01	1			
17.00	1	100	0	1

[a] Flow-rate of a mixture of MP1 and MP2.

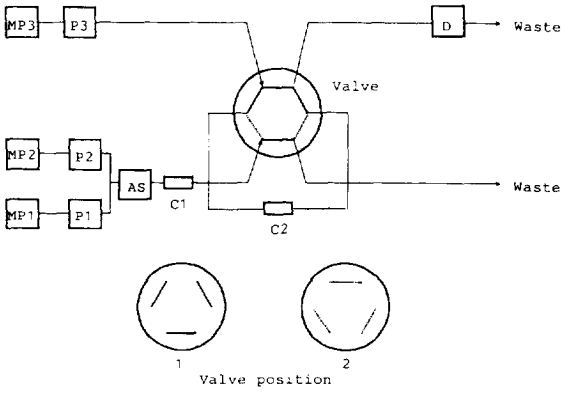

Fig. 1. HPLC system used: MP1–MP3, mobile phases 1–3; P1–P3, pumps 1–3; AS, autosampler; C1–C2, columns 1–2; D, detector.

2.3. Analytical system and procedure

A schematic diagram of the HPLC system is shown in Fig. 1. The time program of the system is described in Table 1. Following serum injection onto C1, gradient elution with MP1 and MP2 was carried out. After serum protein and endogenous substances were washed off to waste (from 0 to 4.6 min), FS was transferred to C2 using the heart-cutting technique between 4.6 and 7.0 min. FS introduced to C2 was eluted by MP3 and was monitored at 271 nm. C1 was washed with a mixture of MP1 and MP2 (40:60, v/v) for about 4 min and then equilibrated with MP1 for 5 min at a flow-rate of 2 ml/min. The analysis of sample was completed within 17 min.

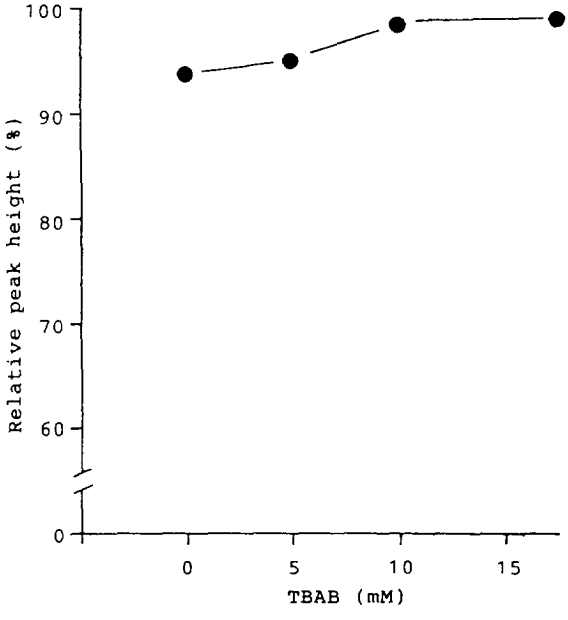

Fig. 2. Effect of the concentration of TBAB on the relative height of the FS peak. The peak height was taken as 100% when FS was directly injected onto C2. MP3 composition: 0.02 *M* phosphate buffer (pH 7)–acetonitrile (80:20, v/v) or (65:35, v/v) containing TBAB (5, 10 and 15 m*M*).

2.4. Preparation of quality control (QC) samples

FS (3 mg) was dissolved in 3 ml of methanol to obtain a 1 mg/ml stock solution which was stored at −20°C. Working solutions of FS (300, 30 and 1.5 μg/ml) for QC samples were prepared by diluting the stock solution with methanol. Then, 30-μl volumes of the working solutions were added to 8970-μl volumes of drug-free serum to obtain QC samples at concentrations of 1000, 100 and 5 ng/ml, respectively. The QC samples were stored at −20°C before use.

2.5. Preparation of calibration standards

Working solutions of FS (100, 20, 10, 2 and 0. 5 μg/ml) for calibration standards were prepared by diluting the stock solution with methanol. Then, 10 μl of the working solutions were added to 990 μl of drug-free serum to obtain spiked serums to be used as calibration standards at the concentrations of 1000, 200, 100, 20 and 5 ng/ml, respectively. The spiked serums were prepared just before analysis.

2.6. Drug analysis

A 170-μl volume of serum sample (calibration standard or QC sample) was filtered through a membrane filter (UFC30HV00 membrane filter; pore size, 0.45 μm; Nihon Millipore, Tokyo, Japan) and a 100-μl aliquot was injected onto the HPLC system.

External standards were used for calculating the FS concentration. It is difficult to use internal standards because the heart-cutting technique is applied to the analytical system. A calibration curve was obtained as a weighted linear regression curve ($1/c$), which was calculated by using FS peak heights of the calibration standards.

3. Results and discussion

3.1. Effect of TBAB on enrichment of the FS peak in the analytical column

In the direct injection analysis, deterioration in efficiency of a pre-treatment column (C1), which is caused by adsorption of serum protein, is unavoidable [15]. Under this condition, on-line solid-phase extraction is rarely applied to routine analysis since the peak of analyte in C1 gradually broadens. If the analyte could be enriched at the top of an analytical column (C2) during introduction of the analyte from C1 to C2 by heart-cutting, the influence of peak broadening in C1 should become negligible in C2. It has been reported that MTX was enriched at the top of C2 by using ion-pair chromatography with TBAB in C2, even though deterioration in efficiency of C1 occurred [15]. Since MTX is a dicarboxylic acid, the effect of the ion-pair formation with tetra-*n*-butylammonium ion would become greater than that found in the case of a monocarboxylic acid. Therefore, the effect of TBAB concentration in MP3 on the enrichment of FS, one of the monocarboxylic acids, was investigated in C2. Fig. 2 shows the ratios of FS peak heights obtained from column-switching to direct injection onto C2. Comparable peak heights were obtained with and without column-switching at a TBAB concentration of more than 10 m*M*. This is explained by the enrichment of the FS peak at the top of C2. Under this condition, the influence of the deterioration in efficiency of C1 could be minimized.

In Fig. 2, the acetonitrile concentration in MP3 with TBAB (35%) was much higher than both that without TBAB (20%) and during heart-cutting (14.4–18%). To exclude the possibility that the obtained peak compression could be attributed to a gradient effect caused by differences in the con-

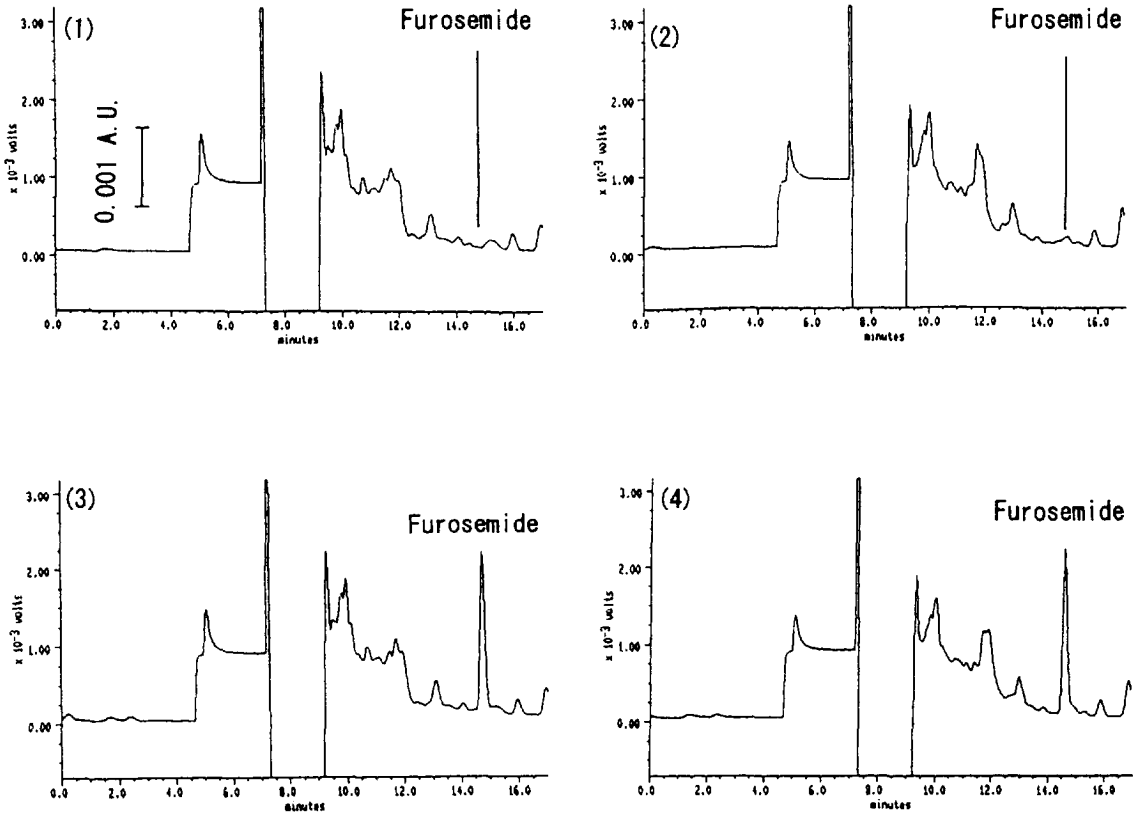

Fig. 3. Chromatograms of drug-free serum and QC sample (100 ng/ml): drug free serum before (1) and after (2) 50 repeated injection of the QC sample and the QC sample for the 1st (3) and the 50th (4) injection.

centration of acetonitrile, the acetonitrile concentration in MP3 was adjusted, as the retention time of FS in C2 was almost the same with and without TBAB in MP3. If the peak compression with TBAB was caused by the gradient effect, a similar peak compression phenomenon should also be obtained without TBAB. Such a phenomenon was observed only by using TBAB. Therefore, the obtained peak compression was attributed to the enrichment of the FS peak at the top of C2.

3.2. Establishment of a direct injection system for FS

A direct injection system for the determination of FS in human serum was established by using the enrichment technique discussed in Section 3.1. In order to confirm the applicability of the system to routine analysis, the strength, linearity, accuracy, precision and reproducibility were investigated.

When 50 repeated injections of a 100-μl volume of serum were carried out using only one pre-treatment column (Fig. 3), almost comparable peak heights of FS could be obtained. Chromatograms of the drug-free serum obtained before and after the 50 repeated injections were also comparable, and no interference peak was observed at the retention time of FS. It could be concluded that the direct injection system was stable for at least 50 pre-treated injections of serum samples onto a single pre-treatment column.

The linearity, accuracy, precision and reproducibility were satisfactory from 5 to 1000 ng/ml (Table 2 and Table 3). The linearity of the calibration curve was demonstrated by the correlation coefficient which was greater than 0.99999. The relative errors and C.V. values of QC samples were within 4.00 and 5.88%, respectively. The lower quantitation limit of FS was 5 ng/ml, which was defined as the lowest concentration at which the relative errors and C.V. were not more than 20% (Table 3) [16].

Thus, the proposed direct injection system for the determination of FS in human serum was sufficiently stable for the conditions used in routine analysis. This technique could be applied to the determination of various acidic compounds in serum.

Table 2
Regression parameters for the validation of FS in human serum[a]

Day	Slope	Intercept	Correlation coefficient
1	21	−16	>0.99999
2	20	− 1	>0.99999
3	22	− 1	>0.99999

[a] Weighted linear regression: $1/c$.
Concentration: 1000, 200, 100, 20 and 5 ng/ml.

Table 3
Accuracy, precision and reproducibility of the determination method for FS in human serum

Day	Parameter[a]	5 ng/ml	100 ng/ml	1000 ng/ml
Intra-day assay (n=5)				
1	R.E. (%)	2.00	−3.60	2.73
	C.V. (%)	5.88	0.97	1.65
2	R.E. (%)	4.00	−1.60	−1.08
	C.V. (%)	5.77	0.61	1.59
3	R.E. (%)	0.00	0.30	−0.08
	C.V. (%)	2.00	0.80	1.17
Inter-day assay (n=3)				
	R.E. (%)	2.00	0.77	0.52
	C.V. (%)	1.96	2.61	1.97

[a] R.E.=relative error; C.V.=coefficient of variation.

4. Conclusion

The direct injection technique for serum was proposed. Applicability of the technique was confirmed by establishing the determination method of FS. This technique was applicable to the conditions used in routine analysis.

References

[1] C.M. Dawson, H.J.C.R. Belcher, S.J. Rainbow and T.R. Tickner, J. Chromatogr., 534 (1990) 267.
[2] S.H.Y. Wong, L.A. Butts and A.C. Larsen, J. Liq. Chromatogr., 11 (1988) 2039.
[3] Y. Oda, N. Asakawa, S. Abe, Y. Yoshida and T. Sato, J. Chromatogr., 572 (1991) 133.
[4] D.J. Gisch, B.T. Hunter and Feibush, J. Chromatogr., 433 (1988) 264.
[5] H. Riva, R. Merati and L. Cavenaghi, J. Chromatogr., 533 (1991) 35.
[6] M. Yagi, A. Shibukawa and T. Nakagawa, Chem. Pharm. Bull., 38 (1990) 2513.

[7] T.C. Pinkerton, T.D. Miller and L.J. Janis, Anal. Chem., 61 (1989) 1171.
[8] J. Haginaka and J. Wakai, Anal. Chem., 62 (1990) 997.
[9] K. Zech and R. Huber, J. Chromatogr., 353 (1986) 351.
[10] R. Huber, W. Müller, M.C. Banks, S.J. Rogers, P.C. Norwood and E. Doyle, J. Chromatogr., 529 (1990) 389.
[11] R. Wyss and F. Bucheli, J. Chromatogr., 593 (1992) 55.
[12] A. Matsuura, T. Nagayama and T. Kitagawa, J. Chromatogr., 617 (1993) 339.
[13] H. Bloemhof, K.N. van Dijk, S.S.N. de Graaf, D.E.M.M. Vendrig and D.R.A. Uges, J. Chromatogr., 572 (1991) 171.
[14] P. Altmayer, U. Büch, H.P. Büch and R. Larsen, J. Chromatogr., 612 (1993) 326.
[15] T. Okuda, M. Motohashi, I. Aoki and T. Yashiki, J. Chromatogr. B, 662 (1994) 79.
[16] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 81 (1992) 309.