Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 792 (2003) 49–54
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Direct analysis of drugs in plasma by column-switching liquid chromatography–mass spectrometry using a methylcellulose-immobilized reversed-phase pretreatment column

Shin-ichi Kawano[a], Hiroyuki Murakita[a,*], Eiichi Yamamoto[b], Naoki Asakawa[b]

[a]*Analytical Applications Department, Shimadzu Corporation, 380-1 Horiyamashita, Hadano, Kanagawa 259-1304, Japan*
[b]*Analytical Research Laboratories, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan*

Received 8 November 2002; received in revised form 10 March 2003; accepted 24 March 2003

Abstract

A novel methylcellulose-immobilized reversed-phase pretreatment column (MC-ODS) for column switching liquid chromatography–mass spectrometry (LC–MS) was investigated to improve recovery and durability. Pretreatment and analytical conditions were optimized so that high throughput and high selectivity was ensured during mass spectrometric analysis. Analytical runs, including deproteinization and gradient LC analysis, were conducted in a 6-min cycle. As a consequence, recoveries for test drugs (metoprolol, propranolol, lidocaine, dibucaine, bupivacaine) were greater than 90% and more than 300 plasma samples spiked with target compounds were directly injected and measured without compromising MS detection or system performance.

Keywords: Column switching; Methylcellulose

1. Introduction

Ion suppression in mass spectrometry can have a profound effect on the quantitative measurement of drugs in plasma samples. To minimize non-specific interferences, plasma proteins are typically removed using manual pretreatment procedures, for example, organic solvents (such as acetonitrile), liquid–liquid extraction or solid-phase extraction. Although such methods have been applied to a considerable range of applications, the principle limitation is that the methods are manual and as a consequence time consuming. To automate sample pretreatment, on-line column switching procedures have been successfully developed to increase sample throughput [1–7]. Previously, we reported a newly developed methylcellulose-immobilized reversed-phase pretreatment column (MC-ODS) and its evaluation using a column-switching with UV detection [8]. High recovery, stability and reproducibility were shown after the repetitive injections of plasma sample totally up to 40 ml, including direct injection of a 1-ml plasma sample. Given the inherent sensitivity and specificity of MS detection it is highly desirable to consider such on-line approaches using API-MS. The key factors that affect on-line column switching include the stability of the bonded phase and mobile phase composition used in elution and de-salting. Bonded phase instability may result in an increase in

*Corresponding author.
E-mail address: murakita@shimadzu.co.jp (H. Murakita).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00309-X

background ion signal or may even physically block the inlet to the MS [9]. Mobile phase composition optimized for MS detection must take into account the possibility of ion suppression but should also consider time for method development, a rapid analysis time and use a fast gradient as described by Romanyshyn et al. [10]. Analysis of Pranlukast and its polar metabolites succeeded by step gradient liquid chromatography–tandem mass spectrometry (LC–MS–MS) with on-line solid-phase extraction [11]. To reduce the time for column equilibration and accelerate sample throughput a 10-mm long analytical column was used. Heinig et al. investigated a short (10×2.1 mm) analytical column for analysis of oxazepam and lorazepam [12]. MC-ODS has the advantage that it is highly stable and reproducible regardless of the organic solvent composition. In this paper we describe an on-line extraction method using a column switching technique initially developed for isocratic separations [13–17] now modified for fast linear gradient analysis using LC–MS.

2. Experimental

2.1. Materials

Metoprolol, propranolol, lidocaine, dibucaine, bupivacaine, ammonium acetate, dimethylsulfoxide and HPLC grade acetonitrile (ACN) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Rat plasma was obtained from Japan SLC (Shizuoka, Japan). Water used for sample dilution, extraction mobile phase and mobile phase was purified using a Millipore (Tokyo, Japan) Milli-Q Gradient system.

2.2. Sample preparation

Standard stock solutions (1000 μg/ml) of propranolol dissolved in water and other test compounds dissolved in dimethyl sulfoxide were prepared. Analytical standards were prepared by mixing stock solutions and diluting using water. Rat plasma samples spiked with target compounds were prepared by adding mixed standard solutions to aliquots of plasma. Concentrations of test compounds ranged from 0.01 to 1 μg/ml.

2.3. Chromatographic conditions

The liquid chromatograph was a Shimadzu (Kyoto, Japan) LC-VP system with SIL-HT autosampler, high pressure gradient pump system (0.5-ml mixer), vacuum degasser, column oven and UV–Vis detector. A six-port flow changeover valve and an auxiliary pump were added for sample pretreatment. A bypass line was used for an eightfold dilution of the sample solution. A MC-ODS (10×4.6 mm) was held at 45 °C with an eluent composition of 10 m*M* ammonium acetate and 5% acetonitrile at a flow-rate of 3 ml/min for pretreatment. A VICI-Jour in-line filter (Onsala, Sweden) preceded the pretreatment column. After extraction (1 min for 5- or 10-μl injection, or 2 min for 50-μl injection), the changeover valve was switched and mobile phase for analysis was introduced at a flow-rate of 0.8 ml/min. Trapped and enriched target compounds were then eluted from the MC-ODS pretreatment column and delivered to a MercuryMS (LUNA 5 μ C_{18}(2), 10×4.0 mm) analytical column purchased from Phenomenex (Torrance, CA, USA) using the following gradient conditions: 5% ACN (0–0.5 min)–90% ACN (3–4 min)–5% ACN (4.01–5 min) for 5- or 10-μl injection, or 5% ACN (0–1.5 min)–90% ACN (4–5 min)–5% ACN (5–6 min) for a 50-μl injection. The flow changeover valve was set at the initial position at 4 min (5- or 10-μl injection) or 5 min (50-μl injection). A tee positioned downstream of the UV–Vis detector was used to split the mobile phase into the mass spectrometer (25%) and to waste (75%). The system flow diagram is shown in Fig. 1.

2.4. Mass spectrometry

Analyses were performed on a Shimadzu LCMS-2010 single stage quadrupole mass spectrometer (Kyoto, Japan) equipped with an electrospray ionization interface. Selected ion monitoring (SIM) was used for detection of protonated molecules of metoprolol (*m*/*z* 268), propranolol (*m*/*z* 260), lidocaine (*m*/*z* 235), dibucaine (*m*/*z* 344), bupivacaine (*m*/*z* 289). The probe voltage was set at +4.5 kV. The nebulizer gas flow was set at 4.5 l/min. Data

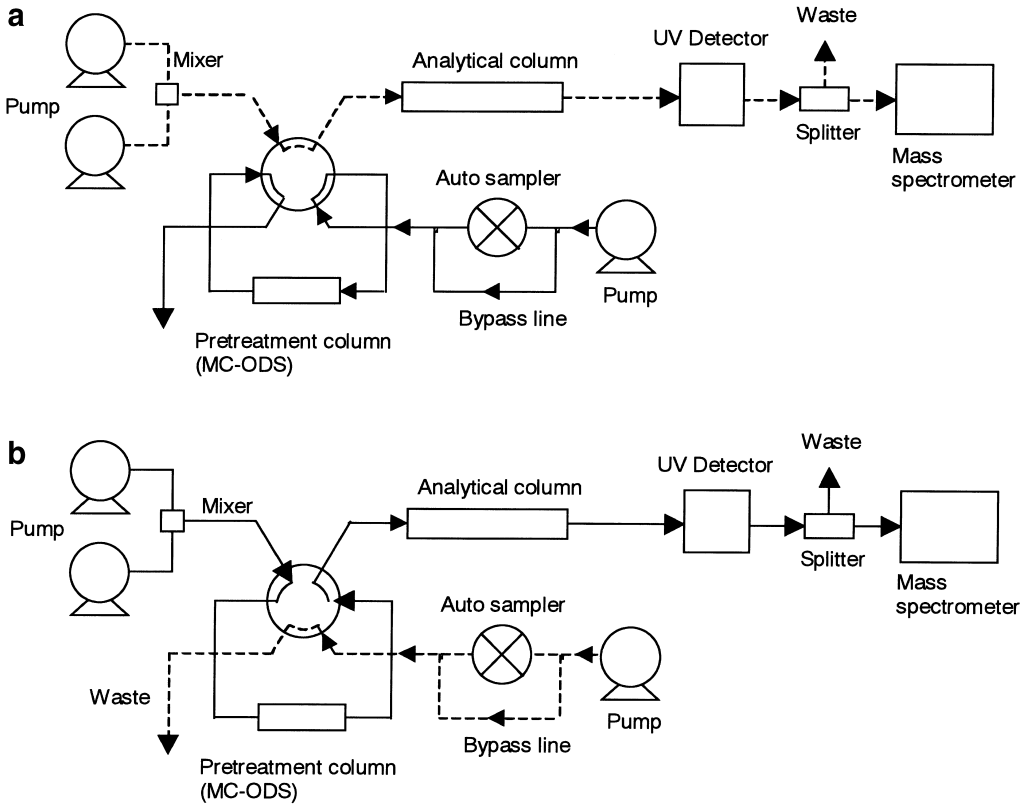

Fig. 1. Flow diagram of the system (a) pretreatment, (b) analysis.

processing were carried out using LCMS Solution software.

3. Results and discussion

3.1. Repeatability and recovery

Fig. 2 shows SIM chromatograms obtained for the target compounds in standard aqueous solution and plasma. Elution profiles were similar to each other and no matrix interference was observed. As shown in Tables 1, the repeatability was between 1.0 and 2.6%, even when plasma samples spiked with target compounds were injected. The recovery of each compound was greater than 90%. Repeatability and recovery of propranolol were comparable with those previously reported using UV detection [8].

3.2. Calibration curve and limit of quantitation

Calibration curves for each compound spiked in plasma (0.01–1 μg/ml) are shown in Fig. 3. The correlation factor (r) was greater than 0.999. The limit of quantitation (LOQ, $S/N=10$) of each compound is as follows: metoprolol, 2.6 ng/ml; propranolol, 5.5 ng/ml; lidocaine, 2.4 ng/ml; dibucaine, 2.7 ng/ml; bupivacaine, 2.5 ng/ml. No peaks corresponding to any of the target compounds were observed in the chromatograms obtained during the analysis of blank plasma.

3.3. Effect of acetonitrile in extraction mobile phase

Addition of low percentage (<10%) of organic solvent improved recovery. Fig. 4 shows a com-

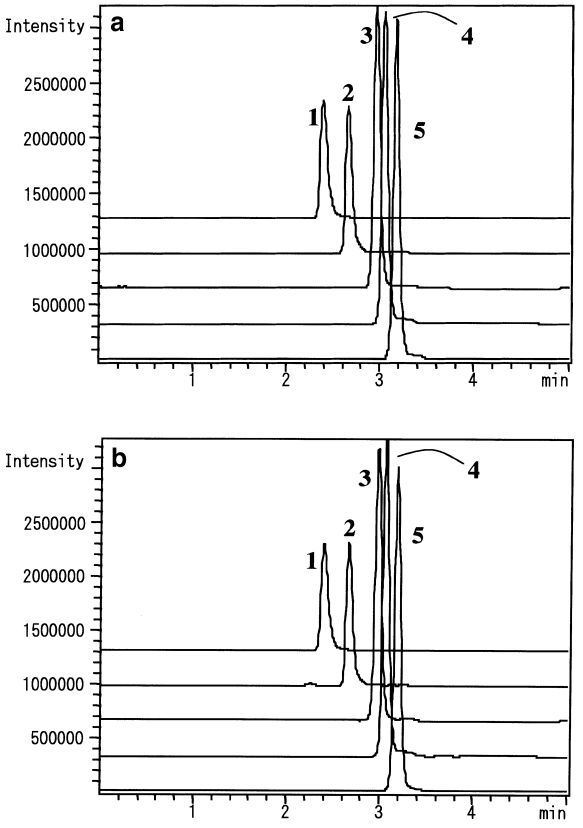

Fig. 2. SIM chromatograms of test compounds [(a) standard, (b) plasma spiked]. 1, metoprolol; 2, propranolol; 3, lidocaine; 4, dibucaine; 5, bupivacaine. Concentration of each compound was 1 μg/ml. Injection volume was 5 μl. Baselines were shifted intentionally.

parison of the recovery between different extraction mobile phases. As Nakagawa et al. described [18], acetonitrile would affect drug–protein binding.

Table 1
Repeatability, recovery of each test compound (1 μg/ml, 5 μl)

Compound	RSD, % (n=5)		Recovery, %
	Standard	Plasma spiked	
Metoprolol	1.2	1.0	95.0
Propranolol	1.0	1.4	97.4
	0.581[a]	0.861[a]	95.6–104.2[a]
Lidocaine	0.9	2.6	94.0
Dibucaine	1.6	2.5	107.7
Bupivacaine	0.7	1.5	97.1

[a] RSD (%) or recovery (%) of propranolol obtained using UV detector [8].

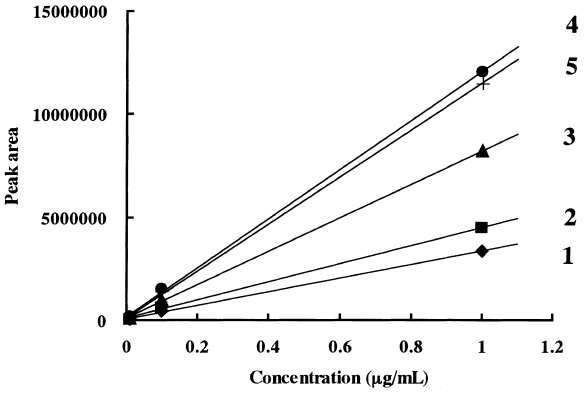

Fig. 3. Calibration curves of test compounds. Rat plasma spiked with drugs were injected (10 μl). Concentration ranged from 0.01 to 1 μg/ml. At each concentration, samples are injected repeatedly (n=5). Correlation factor (r) of each compound was as follows: 1, metoprolol 0.99954; 2, propranolol 0.99958; 3, lidocaine 0.99981; 4, dibucaine 0.99978; 5, bupivacaine 0.99986.

3.4. Effect of bypass line

A bypass line is advantageous when injecting large amounts of sample dissolved in organic solvent. Fig. 5 shows a comparison of peak response of metoprolol between with and without a bypass line. The sample solvent was water, water/acetonitrile (1:1) or acetonitrile. Peak response using a bypass line showed good correlation with injection volume regardless of the sample solvent used. Without the bypass line, the addition of acetonitrile in the sample solution caused poor retention, and as a result poor linearity between injection volume and peak response was observed as seen in typical reversed-phase liquid chromatography. A bypass line may be useful when a large volume of a stability test sample is analyzed after manual pretreatment, for example, using acetonitrile to stop an enzymatic reaction.

3.5. Reproducibility

Representative SIM chromatograms obtained by the repeated injections of the same sample (n=300) are shown in Fig. 6. Variation of ion intensity for each target compound was minimal over a 30-h analysis (a total of 15 ml plasma injected). Pressures of the pretreatment column and analysis column at injection were approximately 3 MPa. The pressure

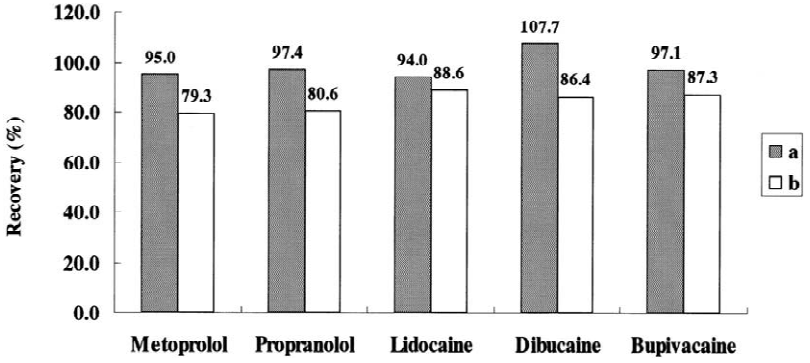

Fig. 4. Effect of acetonitrile in extraction mobile phase on recovery. (a) Extraction mobile phase: 10 m*M* ammonium acetate/acetonitrile= 95:5; (b) extraction mobile phase: 10 m*M* ammonium acetate. Test mixture (1 μg/ml each, 5 μl) was injected.

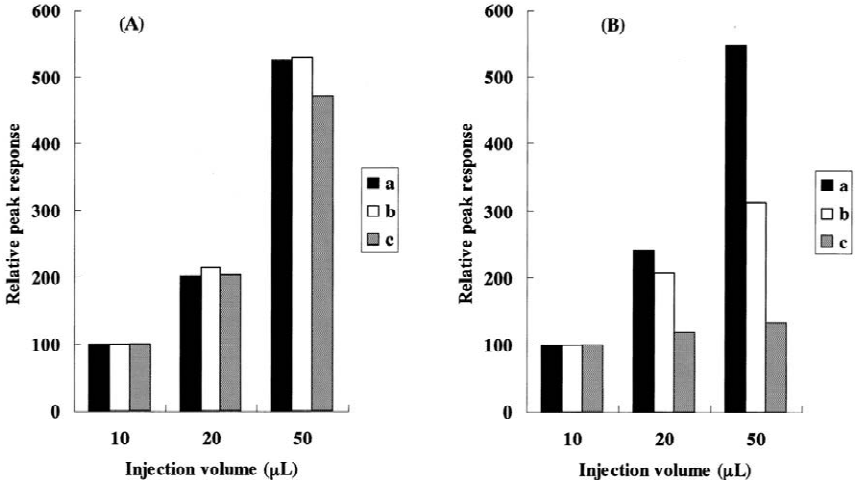

Fig. 5. Effect of bypass line. (A) With bypass line; (B) without bypass line. Sample solvents were as follows: (a) water; b, water/acetonitrile=1:1; (c) acetonitrile. Metoprolol (0.1 μg/ml) was injected.

Table 2
Comparison of RSD between internal and external standard methods

Compound	RSD, % (n=16)[a]	
	Internal standard[b]	External standard
Metoprolol	7.2	16.9
Propranolol	5.0	16.6
Lidocaine	4.5	16.4
Dibucaine	3.4	16.4
Bupivacaine	–	14.1

[a] 1st, 21st, 41st, . . . , 281st and 301st injection.
[b] Bupivacaine as internal standard.

across the MC-ODS pretreatment column gradually increased but was below 3.5 MPa with minimal variation. The relative standard deviation was calculated using 16 points evenly distributed throughout the 300 injection experiment. Table 2 shows the RSD(%) calculated for each compound by internal standard method or external standard method.

4. Conclusion

The performance of column-switching LC–MS using MC-ODS was investigated. MC-ODS resulted in a highly efficient on-line extraction of the target

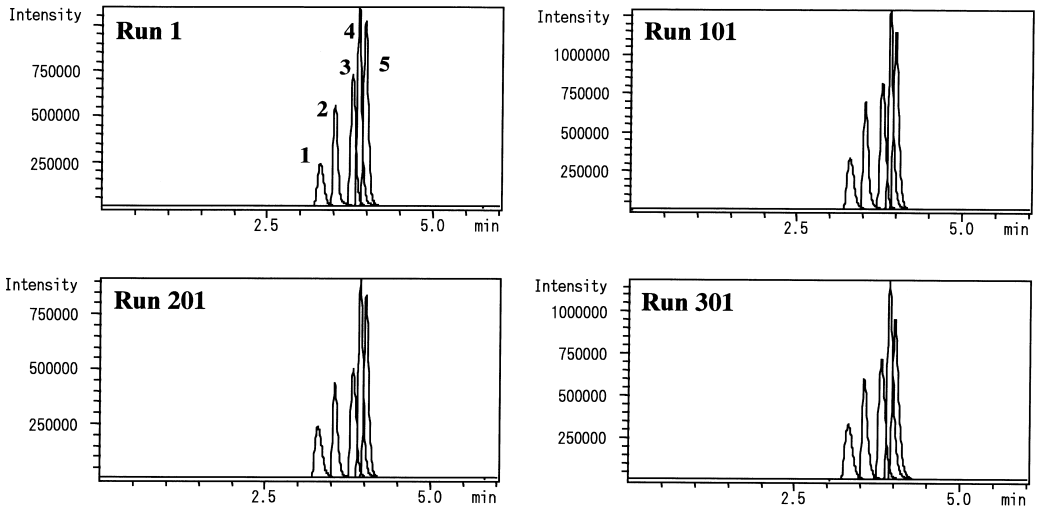

Fig. 6. SIM chromatograms of repetitive 300 analysis. 1, metoprolol; 2, propranolol; 3, lidocaine; 4, dibucaine; 5, bupivacaine. Injection volume was 50 μl.

compounds whilst at the same time minimizing non-specific matrix effects. MS performance was maintained throughout the rapid analysis without compromising either the reliability or reproducibility of the assay. Although automated pretreatment LC using column-switching with other packing materials have been demonstrated with a triple quadrupole mass spectrometer [13–15,17] or an ion trap mass spectrometer [16], we have demonstrated the use of a single stage quadrupole mass spectrometer and obtained reliable results of test compounds. It is a technique that can be considered for a broader range of target molecules in biological samples using selectivity in the bonded phase of pretreatment columns and single stage quadrupole MS systems.

References

[1] H. Yoshida, I. Morita, T. Masujima, H. Imai, Chem. Pharm. Bull. 30 (1982) 2287.
[2] I.H. Hagestam, T.C. Pinkerton, Anal. Chem. 57 (1985) 1757.
[3] J. Haginaka, N. Yasuda, J. Wakai, H. Matsunaga, H. Yasuda, Y. Kimura, Anal. Chem. 61 (1989) 2445.
[4] K. Kimata, R. Tsuboi, K. Hosoya, N. Tanaka, T. Araki, J. Chromatogr. 515 (1990) 73.
[5] Y. Oda, N. Asakawa, S. Abe, Y. Yoshida, T. Sato, J. Chromatogr. 572 (1991) 133.
[6] J. Hermansson, A. Grahn, J. Chromatogr. A 660 (1994) 119.
[7] N. Nimura, H. Itoh, T. Kinoshita, J. Chromatogr. A 689 (1995) 203.
[8] E. Yamamoto, K. Murata, Y. Ishihama, N. Asakawa, Anal. Sci. 17 (2001) 1155.
[9] T. Arinobu, H. Hattori, M. Iwai, A. Ishii, T. Kumazawa, O. Suzuki et al., J. Chromatogr. B 776 (2002) 107.
[10] L. Romanyshyn, P.R. Tiller, R. Alvaro, A. Pereira, C.E.C.A. Hop, Rapid Commun. Mass Spectrom. 15 (2001) 313.
[11] A. Marchese, C. McHugh, J. Kehler, H. Bi, J. Mass Spectrom. 33 (1998) 1071.
[12] K. Heinig, F. Bucheli, J. Chromatogr. B 769 (2002) 9.
[13] V.K. Boppana, C. Miller-Stein, W.H. Schaefer, J. Chromatogr. B 678 (1996) 227.
[14] R.A.M. van der Hoeven, A.J.P. Hofte, M. Frenay, H. Irth, U.R. Tjaden, J. van der Greef et al., J. Chromatogr. A 762 (1997) 193.
[15] F. Beaudry, J.C. Yves Le Blanc, M. Coutu, N.K. Brown, Rapid Commun. Mass Spectrom. 12 (1998) 1216.
[16] S.R. Needham, M.J. Cole, H.G. Fouda, J. Chromatogr. B 718 (1998) 87.
[17] H. Keski-Hynnilä, K. Raanaa, M. Forsberg, P. Männistö, J. Taskinen, R. Kostiainen, J. Chromatogr. B 759 (2001) 227.
[18] T. Nakagawa, A. Shibukawa, N. Shimono, T. Kawashima, H. Tanaka, J. Haginaka, J. Chromatogr. 420 (1987) 297.