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Column-switching techniques for high-performance liquid chromatography of ibuprofen and mefenamic acid in human serum with short-wavelength ultraviolet detection

KENJI YAMASHITA*, MICHIO MOTOHASHI and TAKATSUKA YASHIKI

Analytical Laboratories, Takeda Chemical Industries, Ltd., Juso-Honmachi, Yodogawa-ku, Osaka 532 (Japan)

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ABSTRACT

Column-switching techniques for high-performance liquid chromatography of two acidic drugs, ibuprofen and mefenamic acid, in human serum with short-wavelength ultraviolet detection are described. The method involved extraction of the analyte from acidified serum followed by the chromatographic analysis using column switching. Three ODS columns were used each with different mobile phase, utilizing the difference of ion-pair formation or of ionization caused by pH change. The method offered high sensitivity and selectivity, with short-wavelength ultraviolet detection at 221 nm for ibuprofen and at 219 nm for mefenamic acid. The detection limits were 0.5 ng/ml (2.4 pmol/ml) for ibuprofen and 0.1 ng/ml (0.4 pmol/ml) for mefenamic acid using 1 ml of serum, both at a signal-to-noise ratio of 3. With some modifications, the principle of the method would be applicable to other acidic compounds in biological fluids.

INTRODUCTION

The need for the development of the sensitive method for the determination of drugs in biological fluids is increasing, and the sensitivity required has also become higher and higher. High-performance liquid chromatography (HPLC) is now the most widely used method for this purpose. However, we often encounter the difficulty that the required sensitivity cannot be obtained because of background peaks due to endogenous substances in the chromatogram. A selective detection method will reduce the background peaks and offer high selectivity and sensitivity, but this is rarely available. Ultraviolet (UV) detection, especially at wavelengths longer than *ca.* 240 nm, is now most widely used. However, many compounds show strong UV absorption only in the short-wavelength region, which is poorly selective and is not usually suitable for their sensitive determination in biological fluids. To obtain high selectivity and sensitivity for basic drugs in biological fluids with this poorly selective detection method, we successfully developed a column-switching technique combined with ion-pair chromatography [1–4].

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This paper describes the development of a similar sensitive method for acidic drugs, using ibuprofen (IB) and mefenamic acid (MA) as model compounds. Since IB and MA are both simple carboxylic acids, they were considered to be suitable as model compounds to predict the applicability of the method to other acidic compounds. Further, both IB and MA show strong UV absorbance only in the short-wavelength region, the molar absorptivity being 8200 at 221 nm for IB and 32 000 at 219 nm for MA.

EXPERIMENTAL

Reagents and materials

IB and MA were obtained from Sigma (St. Louis, MO, USA). Tetrabutylammonium bromide (TBAB) and *n*-octylamine were of ion-pair reagent grade (Tokyo Kasei, Tokyo, Japan). Acetonitrile, *n*-hexane, 2-propanol and acetone were of HPLC grade (Wako, Osaka, Japan). All other reagents were of analytical-reagent grade and were used without further purification.

Extraction from serum

The extraction procedure for IB from serum was as follows. To 1.0 ml of human serum were added 50 μl of 1 *M* hydrochloric acid. The mixture was extracted twice with 4 ml of *n*-hexane–2-propanol (85:15, v/v) by vortex-mixing for 30 s and centrifuging for 5 min at 2000 *g*. The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 300 μl of the mobile phase for column 1 (MP1), which will be described later. An aliquot of 200 μl was injected into the HPLC system. The extraction procedure for MA from serum was the same, except that the organic solvent was replaced by *n*-hexane–acetone (85:15, v/v).

Intrument and conditions

The same HPLC system was used for IB and MA, which consisted of three LC-6A pumps, an SPD-6A UV detector, a CTO-6A column oven, two FCV-2AH six-port switching valves and an SIL-6A autosampler, all of which were controlled by an SCL-6A controller (all from Shimadzu, Kyoto, Japan). A C-R3A integrator (Shimadzu) was used for the quantitation. Three ODS columns were used: YMC ODS (A type, 5 μm particle size, 70 mm × 4.6 mm I.D.; Yamamura Chemical, Kyoto, Japan) for both columns 1 and 2 (C1 and C2), and TSK gel ODS 80 TM (5 μm particle size, 150 mm × 4.6 mm I.D.; Tosoh, Tokyo, Japan) for column 3 (C3). The mobile phases for C1, C2 and C3 (MP1, MP2 and MP3) are described in Table I. The temperature and the flow-rate for all columns were 40°C and 1.0 ml/min, respectively. Detection was carried out at 221 nm for IB and 219 nm for MA.

TABLE I

MOBILE PHASES FOR C1, C2 AND C3

Compound	Mobile phase[a]
IB	MP1, 20 m*M* pH 3.5 PB–CH_3CN (60:40, v/v) containing 5 m*M* TBAB MP2, 20 m*M* pH 7 PB CH_3CN (70:30, v/v) containing 5 m*M* TBAB MP3, 20 m*M* pH 7 PB–CH_3CN (70:30, v/v)
MA	MP1, 20 m*M* pH 5 PB–CH_3CN (65:35, v/v) MP2, 20 m*M* pH 3.5 PB–CH_3CN (55:45, v/v) MP3, 20 m*M* pH 6 PB–CH_3CN (55:45, v/v)

[a] PB = phosphate buffer, CH_3CN = acetonitrile.

Analytical system and procedure

A schematic diagram of the HPLC system is shown in Fig. 1. The injected sample was first analysed on C1 with MP1 (valve 1 position: A). The eluate fraction containing the analyte was transferred from C1 to C2 by switching the valve 1 position from A to B. Immediately after the elution of the analyte from C1 to C2, the valve 1 position was switched back to A and the heart-cut fraction was further separated on C2 with MP2. The eluate fraction containing the analyte was

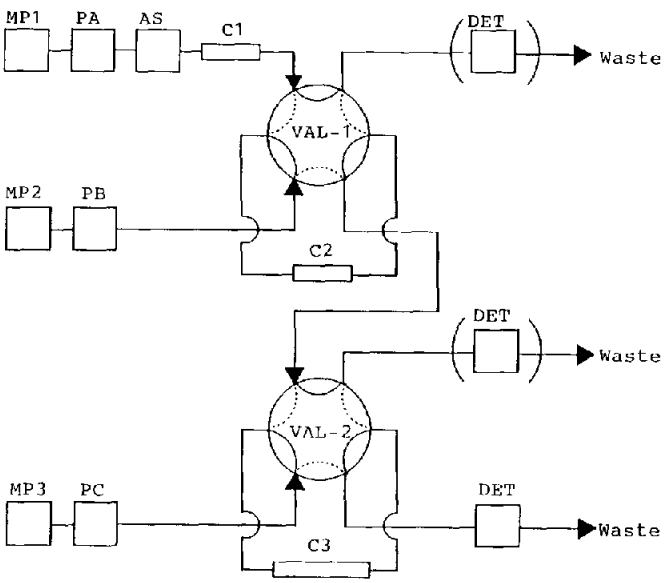

Fig. 1. Schematic diagram of the HPLC system. PA, PB and PC = pumps A, B and C; AS = autosampler; VAL-1 and VAL-2 = six-port valves 1 and 2; C1, C2 and C3 = columns 1, 2 and 3; DET = UV detector; MP1, MP2 and MP3 = mobile phases 1, 2 and 3. The solid and dotted lines in the six-port valves indicate valve positions A and B, respectively.

then similarly transferred from C2 to C3 by the valve 2 operation. The heart-cut fraction was finally analysed on C3 with MP3, and the UV absorbance was monitored at the appropriate wavelength. The valve operations were carried out automatically by the SCL-6A controller according to a predetermined time programme.

RESULTS AND DISCUSSION

The detection limits for analytes in biological samples depend on the sensitivity and the selectivity of the detector, the amount of sample injected and the background in the chromatogram. If the background is decisive for the detection limit, the use of a larger sample does not improve matters because it leaves the ratio of the peaks of interest and the background peaks unchanged. One must therefore improve either the selectivity of the detection or the selectivity of the

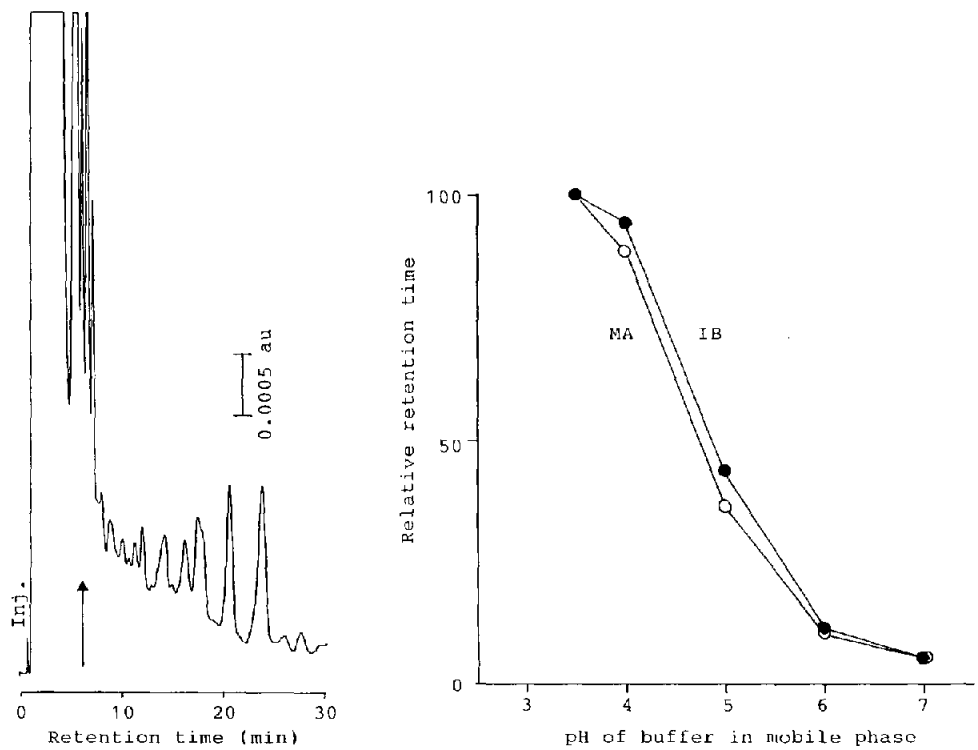

Fig. 2. Typical chromatogram of drug-free serum obtained with C3 alone. The arrow indicates the retention time of IB.

Fig. 3. Effect of the buffer pH in the mobile phase on the retention times of IB and MA. Mobile phases: 20 m*M* phosphate buffer (pH 3.5–7)–acetonitrile (70:30, v/v) for IB, and 20 m*M* phosphate buffer (pH 3.5–7)–acetonitrile (65:35, v/v) for MA. Column: C1. The retention time is expressed as a percentage of that obtained with pH 3.5 mobile phase.

separation itself [5]. Gradient elution is an effective method of separating the analytes from interferences and to obtain sharp peaks, but it cannot generally be used with short-wavelength UV detection owing to a large baseline drift. The heart-cutting technique, with an appropriate combination of different separation modes, is one of the most promising approaches to compensate for the poor selectivity of this detection method and, as a result, it allows the injection of a larger volume of biological sample. In previous papers [1–4], we reported the successful development of the column-switching technique utilizing ion-pair formation for this purpose, which was applied to various basic drugs in biological fluids. The difference of the separation mode and of the elution ability derived from the combination of ion-pair and non-ion-pair chromatography using only ODS columns, offered high selectivity and sensitivity even with poorly selective short-wavelength UV detection.

In the present study, we first attempted to apply this technique to an acidic compound, IB. It was clearly impossible to obtain high sensitivity with a single ODS column (C3 with MP3) owing to the background peaks at the retention time

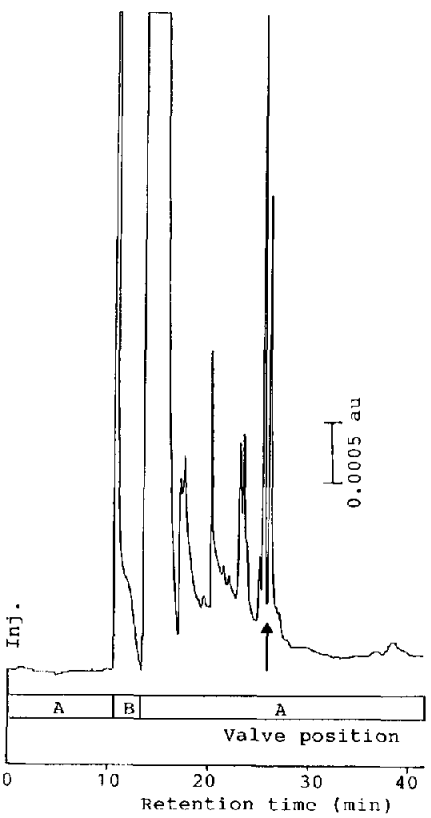

Fig. 4. Typical chromatogram of drug-free serum obtained with C2 plus C3. The arrow indicates the retention time of IB.

of IB (Fig. 2). IB, which has a carboxylic acid functional group, shows typical retention behaviour as a simple acid (Fig. 3), and its elution was predicted to be delayed by ion-pair formation with TBAB at *ca.* pH 7. Therefore, initially, the similar two-column system described previously for basic compounds [1 4] utilizing ion-pair formation was tried to eliminate interferences. The injected sample was first separated by ion-pair chromatography (C2 with MP2). The heart-cut fraction was then analysed by reversed-phase chromatography (C3 with MP3). However, as shown in Fig. 4, some interferences were still observed at the retention time of IB. There are many more acidic than basic endogenous compounds in biological fluids, and this may be one of the reasons why this method could not separate the analyte from interferences. The replacement of TBAB by *n*-octylamine as the ion-pair reagent did not improve matters (data not shown). Therefore, prior to ion-pair chromatography (C2 with MP2), another column with a different pH (C1 with MP1) was incorporated into the system using additional six-port switching valve (Fig. 1). The resulting combination of three mobile phases (Table I) produced chromatograms free from interferences for IB (Fig. 5). The

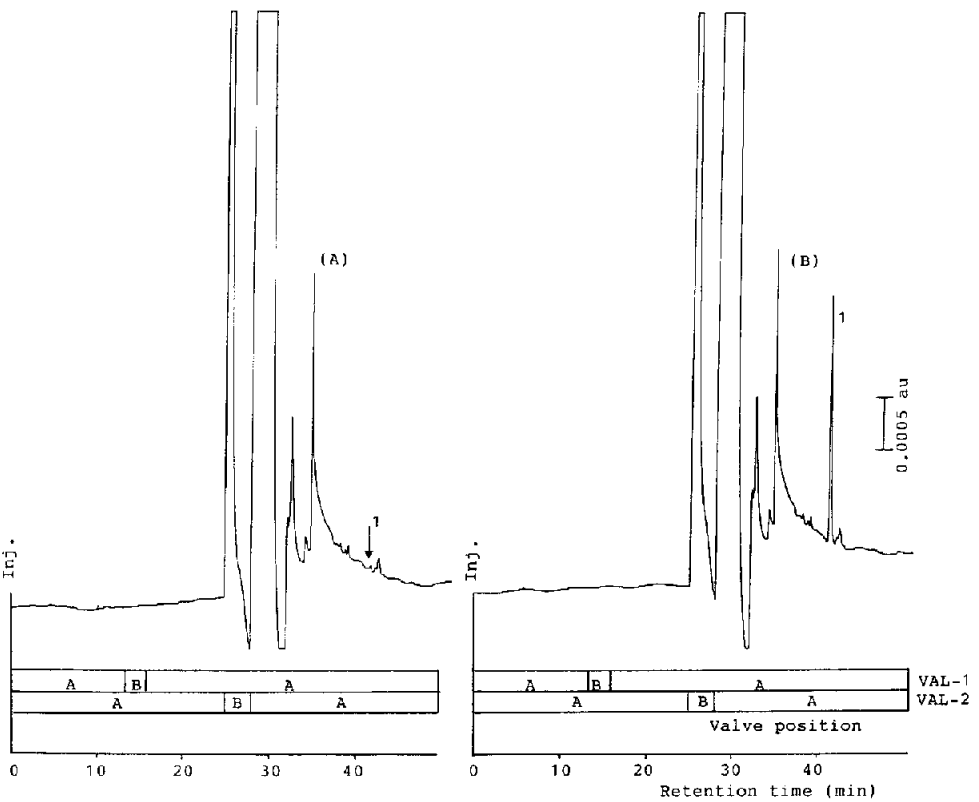

Fig. 5. Typical chromatograms of (A) drug-free serum and (B) serum spiked with IB (20 ng/ml) obtained with C1, C2 plus C3. Peak 1 = IB.

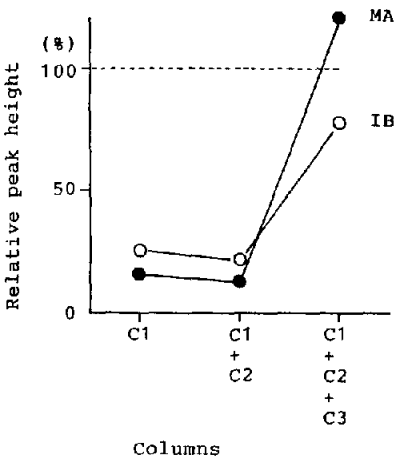

Fig. 6. Effect of column-switching on the peak height of IB and MA. Peak height is expressed as a percentage of that obtained with C3 alone.

peak height obtained by C1, C2 plus C3 was almost the same as that obtained by C3 alone (Fig. 6), indicating no peak broadening after column-switching. Satisfactory compression of the IB peak after column switching shows the sufficient difference between the elution abilities of MP2 and MP3, derived from the presence and absence of ion-pair reagent. The addition of TBAB to MP1 (pH 3.5) did not affect the elution of IB, because IB is not ionized and does not form an ion-pair. However, the introduction of MP1 without TBAB into C2 by column-switching resulted in abnormally fast elution of IB on C2 after column-switching (data not shown), probably owing to the prevention of the ion-pair formation in MP2. Therefore, TBAB was also added to MP1.

Another mode of column switching, a combination of three mobile phases with different pH values without an ion-pair reagent, was also developed, which was applied to MA in human serum. It was clearly impossible to obtain high sensitivity for MA with a single ODS column (C3 with MP3) owing to interferences (Fig. 7). MA also shows typical retention behaviour as a simple acid similar to IB (Fig. 3). The elution of the analyte was greatly affected by the pH of the mobile phase owing to the change of ionization, which seemed to be usable for column switching to eliminate interferences. From an investigation of various combinations of mobile phases with different pH values (data not shown), the conditions described in Table I were finally selected. The proposed combination of three different pH values eliminated interferences (Fig. 8) and also offered satisfactory peak compression of MA after column-switching (Fig. 6) because of the sufficient difference between the elution abilities of MP2 and MP3 derived from the difference of ionization.

The peak height finally obtained after column switching (C1, C2 plus C3) was almost the same as that obtained with C3 alone (Fig. 6). The chromatograms

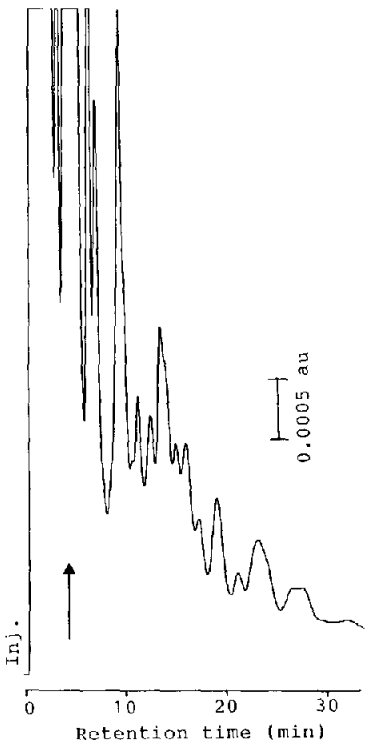

Fig. 7. Typical chromatogram of drug-free serum obtained with C3 alone. The arrow indicates the retention time of MA.

were free from interferences (Figs. 5 and 8), and quite different from those obtained with C3 alone (Figs. 2 and 7), which clearly demonstrates the satisfactory clean-up efficiency of the proposed column-switching technique for both IB and MA in serum, even with short-wavelength UV detection.

The calibration graph was obtained by analysing spiked serum samples over the concentration ranges 3–1000 ng/ml for IB and 0.5–1000 ng/ml for MA, respectively. The least-squares regression fit showed good linearity, passing through the origin for both IB and MA (correlation coefficient = 0.9999). The recovery of IB from spiked serum samples was 85.1%, with a coefficient of variation (C.V.) of 0.8% (20 ng/ml, $n = 6$), and that of MA was 83.8%, with a C.V. of 4.8% (5 ng/ml, $n = 6$). The detection limits in serum were 0.5 ng/ml (2.4 pmol/ml) for IB and 0.1 ng/ml (0.4 pmol/ml) for MA, both at a signal-to-noise ratio of 3.

To obtain high sensitivity by the heart-cutting technique, satisfactory peak compression, *i.e.* no peak-broadening after column-switching, is essential. This can be achieved if the elution abilities of the two mobile phases are sufficiently different. For this purpose, the content of organic modifier has usually been varied, with the combination of an ODS analytical column and the same ODS or

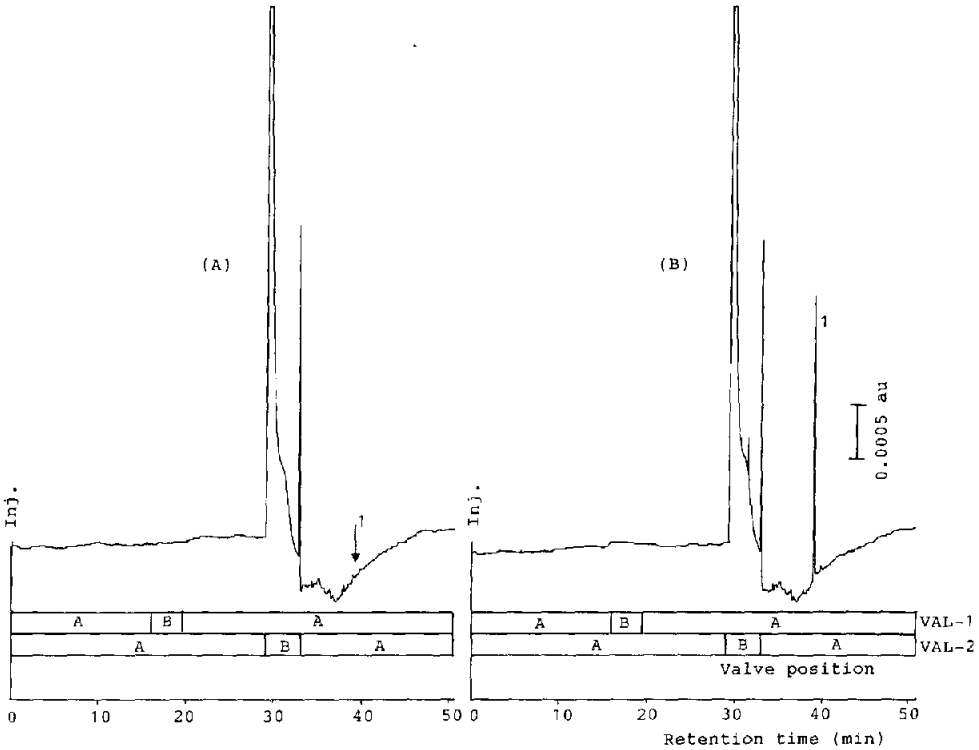

Fig. 8. Typical chromatograms of (A) drug-free serum and (B) serum spiked with MA (5 ng/ml) obtained with C1, C2 plus C3. Peak 1 = MA.

a less hydrophobic preseparation column (C_8, CN and gel chromatography) [6–12]. However, in the proposed method utilizing the difference of ion-pair formation or of ionization, the proportion of the organic modifier was the same in MP2 and MP3. Therefore, the baseline disturbance after column switching would be smaller in the present method than in the systems reported previously [6–12]. The smaller baseline disturbance allows faster elution of the analyte after column switching, resulting in higher sensitivity. Further, the proposed method uses only ODS columns, which are now most widely used, because they usually show higher and more reproducible column efficiency, and have longer column lives than others commercially available. Therefore, the present method using only ODS columns would allow more sensitive, constant and reproducible analysis than those using other columns.

With some modifications, the principle of the present column-switching technique would be applicable to other acidic compounds in biological fluids.

REFERENCES

1 K. Yamashita, M. Motohashi and T. Yashiki, *J. Chromatogr.*, 487 (1989) 357.
2 T. Miyabayashi, K. Yamashita, I. Aoki, M. Motohashi, T. Yashiki and K. Yatani, *J. Chromatogr.*, 494 (1989) 209.
3 K. Yamashita, M. Motohashi and T. Yashiki, *J. Chromatogr.*, 527 (1990) 103.
4 K. Yamashita, M. Motohashi and T. Yashiki, *J. Chromatogr.*, 527 (1990) 196.
5 R. Huber and K. Zech, in R. W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in High-Performance Liquid Chromatography, Part A*, Elsevier, Amsterdam, 1988, Ch. 2, p. 124.
6 H. T. Smith and W. T. Robinson, *J. Chromatogr.*, 305 (1984) 353.
7 H. Takahagi, K. Inoue and M. Horiguchi, *J. Chromatogr.*, 352 (1986) 369.
8 L. Weidolf, *J. Chromatogr.*, 343 (1985) 85.
9 H. Humbert, J. Denouel, J. P. Chervet, D. Lavene and J. R. Kiechel, *J. Chromatogr.*, 417 (1987) 319.
10 L. Karlsson, *J. Chromatogr.*, 417 (1987) 309.
11 M. Johansson and C. Svensson, *J. Pharm. Biomed. Anal.*, 6 (1988) 211.
12 K. Hikida, Y. Inoue, T. Miyazaki, N. Kojima and Y. Ohkura, *J. Chromatogr.*, 495 (1989) 227.