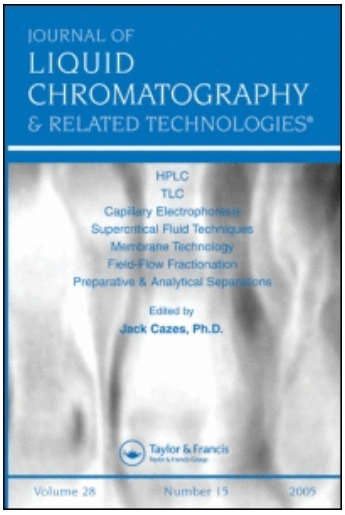

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Resolution of 1-Benzyl-4-[(5, 6-dimethoxy-1-indanon)-2-yl] Methylpiperidine Hydrochloride Enantiomers in Plasma by High-Performance Liquid Chromatography with Direct Injection Into Avidin-Conjugated Column

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RESOLUTION OF 1-BENZYL-4-[(5,6-DIMETHOXY--1-INDANON)-2-YL] METHYLPIPERIDINE HYDROCHLORIDE ENANTIOMERS IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIRECT INJECTION INTO AVIDIN-CONJUGATED COLUMN

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ABSTRACT

A high-performance liquid chromatographic method for the chiral separation of 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl] methylpiperidine hydrochloride (E2020) and its plausible metabolites in plasma has been developed using an avidin column. The hydrophobicity (alkyl chain length) of a spacer, combining avidin to the silica support, affected significantly the retention and separation of E2020 enantiomers. A new column-switching technique using avidin columns for pretreatment and chiral separation enabled the direct injection analysis of plasma samples and the enrichment of E2020, which gave high-sensitivity. This occurs because plasma proteins are excluded from the avidin column, while E2020 enantiomers are retained on the column. This column-switching technique and liquid chromatography-mass spectrometry were used to separate E2020 enantiomers from its plausible metabolites in plasma. This system enables to determine the level of E2020 enantiomers in plasma with high-selectivity and high-sensitivity without any pretreatments.

INTRODUCTION

Possible differences in pharmacological effects of drug enantiomers necessitate the development of analytical methods for the differentiation of these compounds (1). Although high-performance liquid chromatography (HPLC) is an efficient method for the separation of enantiomers, its application to biological samples is not straightforward. Because most chemically synthesized chiral stationary phases for direct separation are based on normal-phase chromatography (2), the analytes must be transferred from an aqueous to an organic phase prior to injection (3). On the other hand, some protein-bonded columns, which can be used with an aqueous mobile phase, have wide applicability for the separation of various drug enantiomers (4-6). However, since these columns have a low peak capacity (2), it is very difficult to optimize the chromatographic conditions which avoid interferences from endogenous compounds in plasma. Consequently the tedious and time-consuming procedures are required for the selective sample pretreatments, which sometimes causes racemization (2). Some direct injection analysis of drug enantiomers in plasma by HPLC have been reported (8-10). These methods allow for the accurate and fast determination of drug enantiomers with easy operation and no manual pretreatment of the plasma. However, these reports have not been focused on the separation of parent drug from its metabolites in plasma sample. Maybe the application to biological sample is not straightforward. By the way, during the last few years liquid chromatography (LC) - mass spectromerty (MS) has been extensively used for drug analysis in biological fluids (11-13). Owing to the high selectivity of MS, sample clean-up can be kept to a minimum.

In this study, we used 1 - benzyl - 4 - [(5, 6 - dimethoxy - 1 - indanon) - 2 - yl] methylpiperidine hydrochloride (E2020) as a model compound. E2020,newly synthesized in our laboratories, is a potent centrally acting acetylcholinesterase inhibitor which is expected to serve as a drug for Alzheimer's disease (14). This drug easily racemizes due to the presence of a carbonyl group at the α-position of an asymmetrical carbon (Fig. 1). The aim of this paper is ; (a) to investigate the effect of a spacer, which combines avidin to silica support, on the retention and separation of E2020 enantiomers; (b) to develop a new column-switching method to preconcentrate the low levels of drug enantiomers following direct injection of plasma sample ; and (c) to demonstrate high-selectivity of E2020 analysis in the presence of plausible metabolites and plasma with combining avidin column to MS directly.

MATERIALS AND METHODS

Reagents and materials

Racemic E2020 and its plausible metabolites (15) were synthesized in our laboratories and their structures are shown in Fig. 1. HPLC grade acetonitrile and water were obtained from Wako Pure Chemical Industries (Osaka, Japan). Disuccinimidyl carbonate (DSC) was purchased from Aldrich (Milwaukee, WIS, USA). Ammonium acetate, acetic acid and glycerol of analytical reagent grade were from Kanto Chemical Co. (Tokyo, Japan). Human plasma was collected from our own blood and was used after being filtered through 0.45 μm membranes.

HPLC equipment

A schematic diagram of the chromatographic system is given in Fig. 2. The system consists of two pumps (Shimadzu LC-9A,

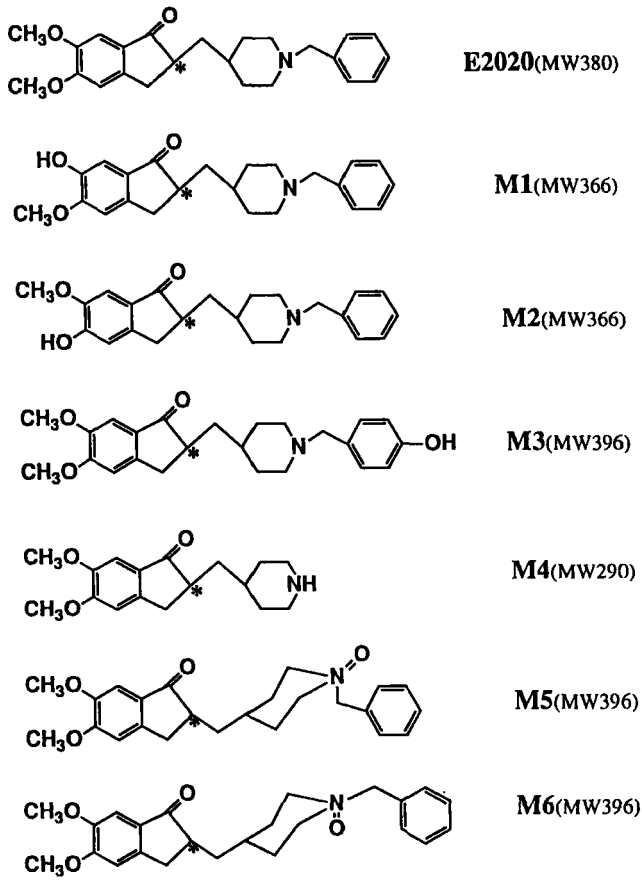


Fig. 1, Structure of E2020 and its metabolites.

Shimadzu Inc., Kyoto, Japan)(pump 1, pump 2), a variable-wavelength UV detector (Shimadzu SPD-6A)(DET), a six-port switching valve (Rheodyne Inc., Cotati, CA, USA)(V) and a model 7125 injector (Rheodyne)(INJ) equipped with a 1-ml loop. The avidin column (250 mm X 4.6 mm I.D.) was made in our laboratories as previously described (10). The avidin DSC column was prepared by the same method as the avidin column, except using DSC instead

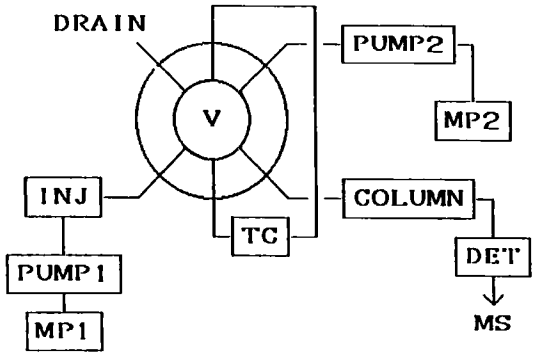


Fig. 2, Coupled-column liquid chromatographic system.

of disuccinimidyl suberate (DSS). The trapping column (TC) (10 mm X 4.0 mm I.D.) was packed with the same packing materials as the avidin column. The eluents used were as follows: the mobile phase 1 (MP1) for trapping E2020 was 0.2 M ammonium acetate buffer (pH 7.5) and delivered by pump 1 at a flow rate of 1.0 ml/min, and the mobile phase 2 (MP2) for separating E2020 consisted of 0.1 M ammonium acetate buffer (pH 5)/acetonitrile = 95/5 (v/v) and delivered by pump 2 at a flow rate of 1.0 ml/min. All procedures were performed at ambient temperature. E2020 was detected at 271 nm.

LC/MS equipment

Positive-ion fast atom bombardment (FAB) mass spectra were obtained using a Jeol (Tokyo) JMS-SX102 with a JMA-DA6000 data system connected to an HPLC system by a Jeol frit-FAB interface. The ionization chamber temperature was 50 °C, and the xenon fast atoms at 4 KeV were used for FAB ionization. The accelerating voltage was 8 KeV, the emission current was 10 mA and the

resolving power was 1000. The range m/z 100 - 1000 was scanned over approximately 3 s. For compatibility with the vacuum system of the mass spectrometer, the HPLC eluate was split so that approximately 5 µl/min entered the HPLC-frit-FAB-MS interface. At a column flow-rate of 1.0 ml/min, this resulted in a split ratio of 1:199.

Sample preparations

A known amount of racemic E2020 was dissolved in saline and the solution was diluted with the saline (saline sample), or plasma (plasma standard sample), to the desired concentration.

RESULTS AND DISCUSSION

Effect of eluent pH, acetonitrile content and buffer concentration on avidin column.

Saline samples were injected into the avidin column directly (without TC) to examine the effects of MP2 on retention and enantioselectivity. Table I shows the effect of acetonitrile content on the retention of E2020. Both the avidin column and the avidin DSC column retain E2020 in a reversed-phase mode. The retention on the avidin column is stronger than that on the avidin DSC column, because the avidin column has a hydrophobic DSS phase. Table I also gives the effects of acetonitrile content on the enantioselectivity, and Fig. 3 shows the representative chromatograms of E2020 enantiomers. The enantioselectivity of the avidin column is high compared with the avidin DSC column. This indicates that the hydrophobicity (alkyl chain length) of a spacer may be effective for the resolution of E2020 enantiomers. It is suggested that E2020 enantiomers in the avidin column may

TABLE I Effect of acetonitrile content on the enantioselectivity

content(%)	1.0%	2.5%	3.6%	5.0%	6.2%	7.5%	10.0%	12.5%
Avidin column								
k_1	-	10.85	-	5.60	-	3.57	2.57	1.93
k_2	-	21.12	-	9.80	-	5.65	3.82	2.72
α	-	1.95	-	1.75	-	1.58	1.49	1.41
Rs	-	4.20	-	4.20	-	3.42	2.43	1.60
Avidin DSC column								
k_1	0.92	0.82	0.47	0.45	0.36	0.30	-	-
k_2	1.25	1.13	0.68	0.45	0.36	0.30	-	-
α	1.36	1.39	1.40	1	1	1	-	-
Rs	0.94	1.14	1.50	0	0	0	-	-

k_1 and k_2 mean the capacity factor of R-E2020 and S-E2020, respectively.

α and Rs mean the separation factor and the resolution, respectively

Mobile phase: 0.1 M ammonium acetate buffer (pH 5.0)

distribute themselves between the mobile phase and the avidin phase, and between the mobile phase and the hydrophobic spacer (DSS phase). Therefore, the chiral selectivity of avidin phase may be enhanced as a result of the shift of the partition equilibrium by increasing the hydrophobicity in the spacer. Due to this two-step equilibrium, protein bonded columns may have generally a low efficiency. Table II shows the effects of the eluent pH on the retention and enantioselectivity, respectively. The isoelectric point of the native avidin is pH 9-10, although

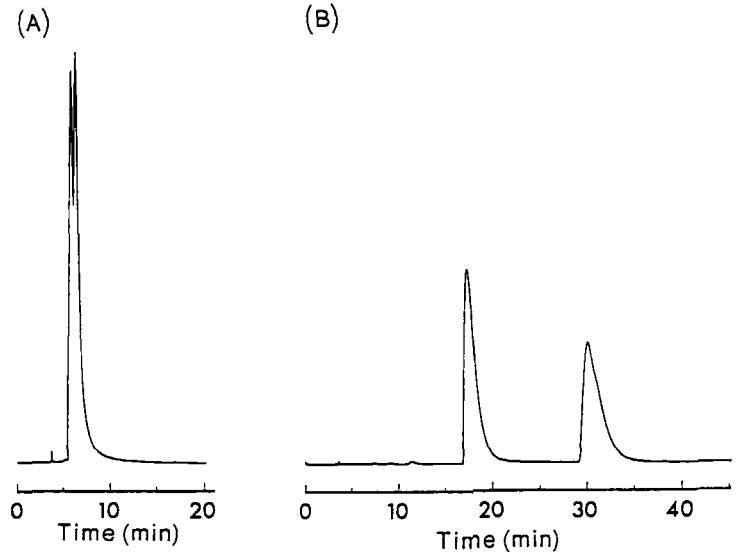


Fig. 3, Chromatograms of E2020 enantiomers (injection amounts: 5 μg) (A) on avidin DSC column, (B) on avidin column. Eluent: 0.1 M ammonium acetate buffer (pH 5), (A) 5 % acetonitrile, (B) 1 % acetonitrile
Other conditions, see text.

TABLE II Effect of eluent pH on enantioselectivity

PH	6.9	6.6	6.4	6.1	5.9	5.7	5.4	5.0	4.6
Avidin column									
k_1'	15.31	-	11.17	-	9.08	-	7.08	5.59	4.18
k_2'	24.40	-	18.47	-	15.53	-	12.40	9.77	6.96
α	1.59	-	1.65	-	1.71	-	1.75	1.75	1.67
Rs	2.19	-	2.72	-	3.16	-	3.88	4.20	3.82
Avidin DSC column									
k_1'	-	0.67	-	0.58	-	0.52	0.49	0.46	0.38
k_2'	-	0.79	-	0.70	-	0.60	0.49	0.46	0.38
α	-	1.18	-	1.20	-	1.17	1	1	1

Mobile phase: 0.1 M ammonium acetate buffer/acetonitrile = 95/5 (v/v)

TABLE III Effect of buffer concentration on enantioselectivity

Concentration	10 mM	50 mM	100 mM	200 mM	300 mM
Avidin column					
k_1	1.72	4.03	5.60	6.65	7.32
k_2	3.08	7.16	9.80	11.11	11.86
α	1.79	1.78	1.75	1.67	1.62
Rs	2.73	4.17	4.20	3.97	3.60
Avidin DSC column					
k_1	0	0.16	0.49	0.76	0.97
k_2	0	0.16	0.49	0.76	0.97
α	1	1	1	1	1

Mobile phase: ammonium acetate buffer(pH 5.0)/acetonitrile = 95/5(v/v)

that of the avidin column may be changed by conjugating DSS. On the other hand, the pKa of E2020 is around pH 9. These factors influence the retention of E2020 on the avidin column. The highest enantioresolution was obtained with the eluent of pH 5.0. The protein may take suitable conformation for chiral recognition at this pH. Severe peak tailing was observed with the eluent pH above 5, because of the dissociation of carboxyl groups, which are the degradation residues of DSS, and/or of the dissociation of the silanol groups. Table III shows the effects of buffer concentration. The retention of E2020 on the avidin column was enhanced by increasing the buffer concentration. It appears that a salting out occurs on the avidin. The enantioselectivity was affected little by changing the buffer concentration, although

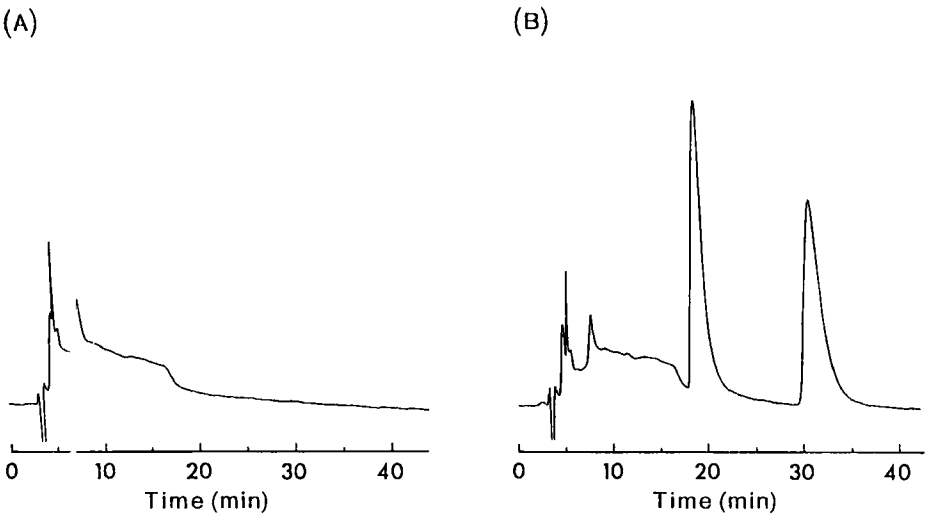


Fig. 4, Chromatograms of (A) plasma blank and (B) plasma sample (E2020: 1 μg/ml) after column-switching procedure.
Injection volume: 0.5 mL
Other conditions, see text.

the presence of buffer is needed. Thus, the optimum mobile phase (MP2) described in the experimental section and the avidin column were selected.

Direct injection of plasma samples by column-switching method.

In previous papers, we reported that the avidin column could be applied to the measurement of drugs in plasma without a clean-up procedure (10). However the recovery of plasma proteins from the avidin column was low at around pH 4.5 of the mobile phase (10 , while the separation of E2020 enantiomers is best at pH 5 on the mobile phase (Table II). Therefore, the direct injection analysis of plasma can not be performed under the optimum mobile phase for the separation of E2020 enantiomers. To overcome this

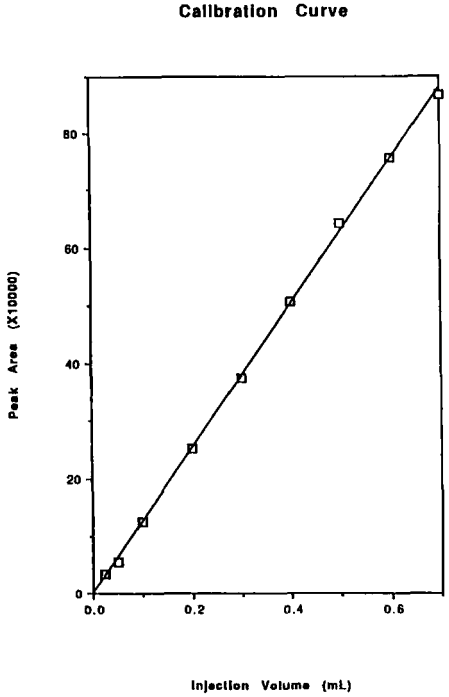
TABLE IV Reproducibility and recovery for E2020 with column-switching

	t_1	t_2	recovery
mean	16.90	28.55	98.5 %
C.V. (%)	0.49	0.74	1.33

t_1 and t_2 mean the retention time of R- and S- E2020 enantiomers, respectively.

C.V. means the coefficient value (n = 7).

problem, a manual column-switching technique was introduced for the analysis of E2020 enantiomers. The plasma samples were directly injected into the TC from the INJ without any pretreatments. Proteins and other hydrophilic constituents were washed out with 3.5 ml of MP1. Then, the valve was switched and the analytes were transferred to the avidin column by MP2. Fig. 4 shows the chromatograms of control plasma (0.5 mL) and a plasma containing 1 μg/ml E2020, obtained by the column-switching procedures. Plasma proteins were washed out from the TC, and E2020 retained on the TC was optically resolved on the avidin column without interfering peaks. The reproducibility and the percent recovery for E2020 enantiomers (injection volume: 0.1 μg/mL, 0.25mL) studied are given in Table IV. These data indicate that this method can measure the concentration of E2020 enantiomers in plasma with good reproducibility. Fig. 5 shows the calibration line of E2020 obtained by using direct injection in the range from 0.025 mL to 0.7 mL of plasma samples (1 μg/ml). The linearity was good (r = 0.997). This result means that the TC



F.g. 5, Calibration line of E2020 in plasma.
Total peak area was plotted vs. injection volume of plasma samples (E2020: 1 μg/ml)
Other conditions. see text.

can enrich E2020 in plasma to obtain a high sensitivity. Thus, this column-switching method allows not only direct injection of plasma samples, but also high sensitivity analysis.

Liquid chromatography-mass spectrometry (LC/MS)

HPLC is currently the most useful technique for the separation of drug enantiomers. Most of the applications, however, have been only described to relatively uncomplicated samples. These have been little reports to separate drug

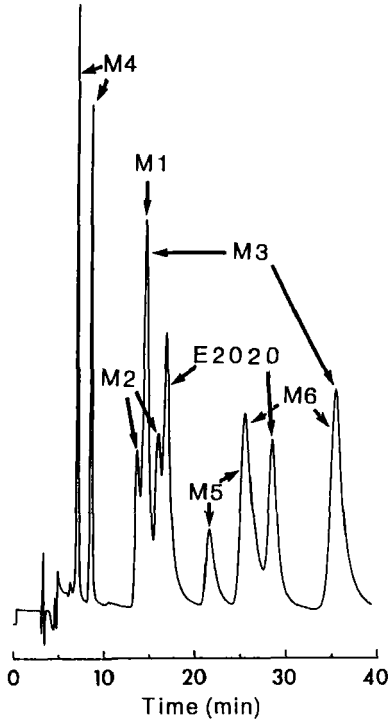


Fig. 6, Chromatogram of E2020 and its six metabolites in plasma after column-switching procedure. Conditions, see text.

enantiomers simultaneously from each other, its metabolites enantiomers and plasma components without any pretreatments. Fig. 6 shows the chromatogram of E2020 and its plausible six metabolites in plasma standard sample obtained by the column-switching method. E2020 and the metabolites (except M1) were optically resolved, but some overlapping between enantiomers and within each enantiomer class were observed. It seems to be difficult to find the proper conditions to separate E2020 enantiomers completely from all its plausible metabolites. Owing

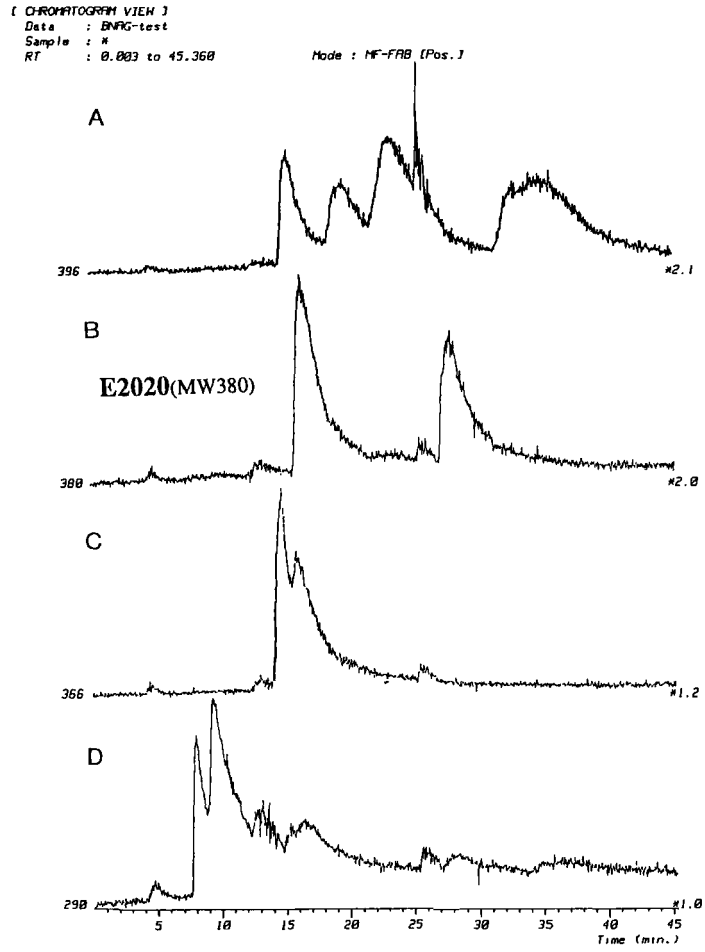


Fig. 7, Mass chromatogram after column-switching procedure. Molecular weight (MW), A: 396, B: 380, C: 366, D:290. Conditions are the same as Fig. 9.

to the high selectivity of LC-MS, the target analytes can be identified even if they are not separated completely by HPLC. To introduce the mobile phase from LC to MS while on-line, 0.8 % glycerol was added in MP2 as the FAB matrix (16). Fig. 7 shows good performance with respect to the separation of E2020

enantiomers from its plausible metabolites enantiomers in plasma standard sample. Under the present conditions, the peaks of E2020 molecule (m/z = 380)(Fig. 7 C), M3, M5 and M6 molecules (m/z = 396) (Fig.7 A), M1 and M2 molecules (m/z = 360) (Fig. 7 B), and M4 molecule (m/z = 290) (Fig.7 D) are observed in scan mode, although small amounts of glycerol in MP2 have caused the slight increments of the peak widths of samples on the avidin column (17) as diffusion might occur at the interface. This method can easily distinguish E2020 enantiomers in the presence of its plausible metabolites in plasma. If isotopically labeled internal standards were used in this system, the determination of E2020 enantiomers in plasma is possible with the direct injection method. In previous our paper, the use of a micro bore ovomucoid column led to high sensitivity (18), so a micro bore avidin column will be required not only to decrease the split ratio at the interface, but also to obtain a high mass sensitivity. To achieve maximum sensitivity, the mass spectrometer should be operated in the selected-ion monitoring mode. When this LC/MS system is used, the sample work-up can be minimized. It is expected that the application of this technique will be useful in clinical study.

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