Journal of Chromatography, 572 (1991) 133–141
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6089

Avidin protein-conjugated column for direct injection analysis of drug enantiomers in plasma by high-performance liquid chromatography

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(First received June 3rd, 1991; revised manuscript received July 30th, 1991)

ABSTRACT

A new concept in high-performance liquid chromatography supports is proposed for the direct injection analysis of drug enantiomers in plasma. The new supports are designed with disuccinimidyl suberate as a hydrophobic internal region, and avidin protein as a hydrophilic and bulky surface region. Plasma proteins are excluded by the avidin phase and are eluted immediately from the column, whereas low-molecular-mass analytes can penetrate the surface region and interact with disuccinimidyl suberate. Enantiomers interact differentially with avidin, and are thereby separated. This column was used in reversed-phase high-performance liquid chromatographic analysis to determine ketoprofen enantiomers in plasma by direct injection. The recovery of racemic drug from plasma was almost 100%.

INTRODUCTION

High-performance liquid chromatography (HPLC), especially reversed-phase chromatography, is very widely used for drug analysis. Some drugs are administered as racemic mixtures, but generally the separation of optical isomers is very important in biochemistry and in the pharmaceutical industry, where one isomer of a drug may be therapeutically active and the other may be inactive, inhibitory or even toxic [1]. The number of HPLC columns that are available to separate enantiomers has grown considerably since Pirkle *et al.* [2] first introduced a chiral stationary phase [3,4]. Most such columns are used under normal-phase chromatographic conditions, except for cyclodextrin-bonded silica [5] and protein-conjugated phases [6–8]. It is preferable to carry out drug analysis by HPLC in the reversed-phase mode owing to the water contained in plasma samples. But there are some significant problems facing the analysis of drugs in plasma by HPLC. The direct injection of plasma samples onto reversed-phase columns such as C_{18} HPLC columns causes column clogging or the loss of efficiency because of adsorption and accumulation of proteins on support particulates [9]. Accordingly, time-consuming pretreatment procedures such as deproteinization or extraction

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are necessary prior to sample injection. Various attempts have been made to achieve direct injection analysis by HPLC for drugs or drug metabolites in plasma in order to speed up and simplify sample handling [10]. Yoshida *et al.* [11] modified a C_{18} reversed-phase coating by using human plasma proteins. Hagestam and Pinkerton [12] prepared an internal surface reversed-phase (ISRP) column by using a hydrophobic peptide in internal pores and a hydrophilic diol phase as an external phase. Haginaka *et al.* [13] and Kimata *et al.* [14] modified the ISRP column by changing the hydrophobic regions in the internal phase to strengthen the retention of the compounds of interest. Gisch *et al.* [15] reported a novel type of phase which excluded proteins while interacting with small molecules. Although these columns can be used for direct injection of drug analysis in plasma, enantiomeric resolution cannot be performed. Recently, Haginaka and Wakai [16] reported cyclodextrin-bonded silica for direct injection analysis of drug enantiomers in serum by HPLC. In this paper, we will describe an avidin column based on a new concept, which is different from the column developed by Haginaka and Wakai, for the direct injection analysis of drug enantiomers in plasma using ketoprofen as a model compound.

EXPERIMENTAL

Reagents and materials

Aminopropyl silica gel (particle diameter, 5 μm) was obtained from Macherey-Nagel (Düren, Germany). Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, IL, USA). Avidin protein, ovomucoid protein and ovalbumin were isolated from eggs in our laboratories [17]. Ketoprofen and Coomassie brilliant blue (CBB) were from Sigma (St. Louis, MO, USA). Ethanol, acetonitrile and water of HPLC grade were obtained from Wako (Osaka, Japan). Other reagents were of analytical grade. Human plasma was collected from our own blood samples and was used after being filtered through 0.45-μm membranes (Toyo Roshi Kaisha, Tokyo, Japan).

Preparation of protein-conjugated column

Fig. 1. illustrates the synthetic scheme for the preparation of the new column. Aminopropyl silica gel was packed into 250 mm × 4.6 mm I.D. stainless-steel columns by conventional high-pressure slurry-packing procedures. Then this phase was activated by recycling DSS (300 mg) in 0.1 *M* sodium hydrogencarbonate buffer–acetonitrile (1:2, 30 ml) for 4 h followed by washing with water–acetonitrile (1:2, 20 ml). Then the mobile phase was replaced with 0.1 *M* sodium hydrogencarbonate buffer, and the protein (500 mg) dissolved in the same buffer was recycled in the column for 4 h.

Chromatographic apparatus

The analyses were performed with a Shimadzu LC-9A pump, SPD-6A detec-

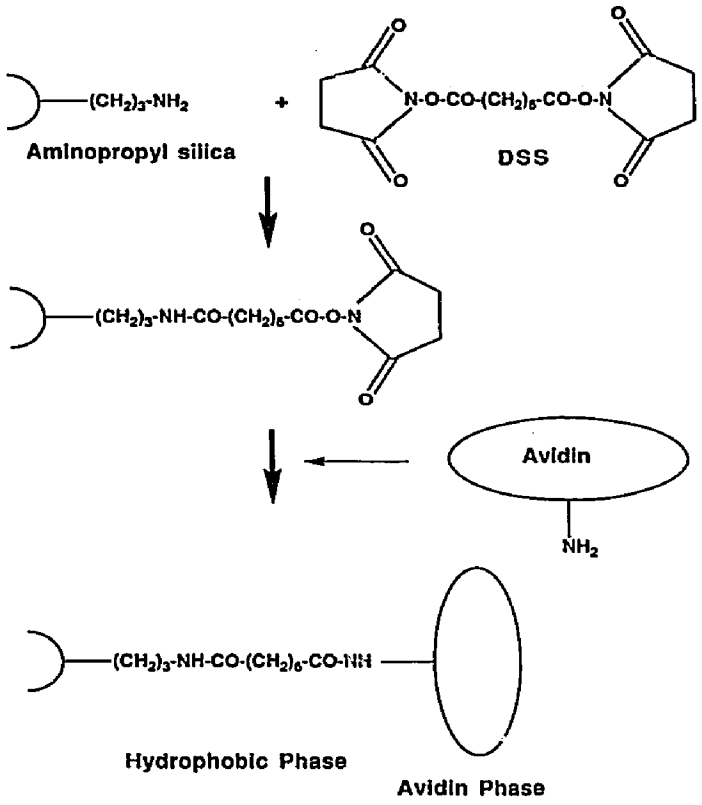

Fig. 1. Scheme for the preparation of the avidin column.

tor, SIL-6B autoinjector, SCL-6B system controller and C-R4AX integrator (Shimadzu, Kyoto, Japan). The mobile phase was delivered at a flow-rate of 1 ml/min. All procedures were carried out at room temperature.

Recovery of plasma protein from the new support column

CBB reagent was prepared by dissolving 100 mg of CBB in 50 ml of ethanol, adding 100 ml of phosphoric acid, and diluting the mixture to 1 l with water [18]. The recovery of human plasma protein from the new support was examined as follows [14]. A 20-μl portion of human plasma was injected onto the new support and the eluate was collected for 10 min. A 0.5-ml portion of the eluate was mixed with the CBB reagent, and after 2 min the absorbance of the mixture was measured at 595 nm. The recovery was calculated from the absorbance ratio with and without the column.

RESULTS AND DISCUSSION

Production of avidin column

The new avidin column was produced from a commercially available aminopropyl silica support. Aminopropyl silicas remain popular as bonded normal

stationary phases [19] but are little used in the reversed-phase mode because they have C_3 chains, which are very weakly hydrophobic compared with C_{18} chains. For this reason, longer carbon chains needed to be introduced onto the aminopropyl support to increase the hydrophobicity. N-Hydroxysuccinimide (NHS) esters have been introduced as cross-linking reagents [20]. The most significant properties of NHS esters are their reactivity at physiological pH and their long half-lives [21]. DSS, which is a kind of NHS, is a non-cleavable, amine-reactive homobifunctional cross-linker [22]. This cross-linker has been widely used for conjugating a radiolabelled ligand to a cell surface receptor [23]. DSS also has C_6 chains, and should be able to play a hydrophobic role in reversed-phase chromatography. DSS (300 mg) was dissolved in acetonitrile (20 ml) at first, and sodium hydrogencarbonate buffer (10 ml) was mixed with this DSS solution for the reaction with the primary amine function on the aminopropyl phase. Then, after washing out the organic solution to avoid denaturation of the protein, amine moieties of avidin were cross-linked to DSS in sodium hydrogencarbonate buffer.

Recovery of plasma from avidin column

The avidin column consists of carbon chains of DSS as hydrophobic internal regions, and avidin as hydrophilic and bulky surface regions, which prevent interactions between the internal hydrophobic carbon chains and large water-soluble molecules such as plasma proteins. The plasma proteins showed essentially no retention on the avidin column in the mobile phase at pH 2 and 7 (Table I). At pH 4.5, plasma proteins were retained on the avidin column and were not eluted within 10 min. The reason for this is presumably that the isoelectric point of many plasma proteins is around pH 4–5, so those proteins increase in hydrophobicity at this pH and are retained on the avidin column. On the other hand, the avidin may also change its character at this pH. Ovomucoid and ovalbumin, which were conjugated by exactly the same method as the avidin column, were also examined for use as the bulky and hydrophilic matrix to exclude the plasma proteins from hydrophobic regions, but the recoveries of plasma proteins from those two columns were poor, as shown in Table I. This may be related to the character of

TABLE I

RECOVERY OF PLASMA PROTEIN FROM PROTEIN-CONJUGATED COLUMNS

pH[a]	Recovery (%)			
	Avidin	Ovomucoid	Ovalbumin	Avidin (DSS-C_{10})
2	97	30	87	28
4.5	24	6	3	4
7	92	38	72	13

[a] Mobile phase: 0.1 *M* phosphate buffer–acetonitrile (90:10), 1 ml/min.

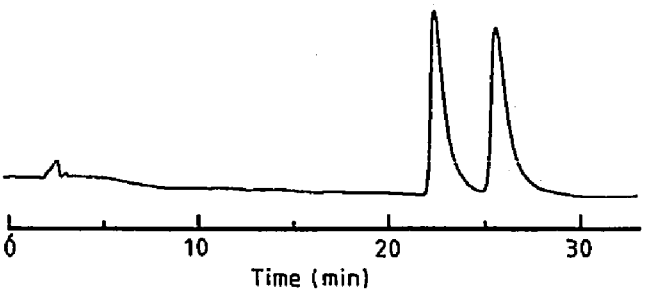

Fig. 2. Chromatogram of ketoprofen enantiomers on the avidin column. Mobile phase, 0.1 *M* potassium phosphate buffer (pH 2)–acetonitrile (95:5); detection, UV 260 nm; flow-rate, 1.0 ml/min; injection volume, 20 μl.

these proteins such as molecular size. When DSS reacts with the aminopropyl supports, one or both ends of DSS may conjugate to the primary amines. Thus, not all DSS moieties have a free end available to couple with an amino group of a protein molecule. Since ovomucoid (MW = 28 000) and ovalbumin (MW = 45 000) are small compared with avidin (MW = 70 000), they may not completely block access to the internal hydrophobic carbon chains, taking account of the fact that some DSS moieties with both ends bound to the support would not be directly shielded. We examined the effect of extending the carbon chains of DSS to C_{10} (DSS-C_{10}) from C_6 in our laboratories to increase the hydrophobic inter-

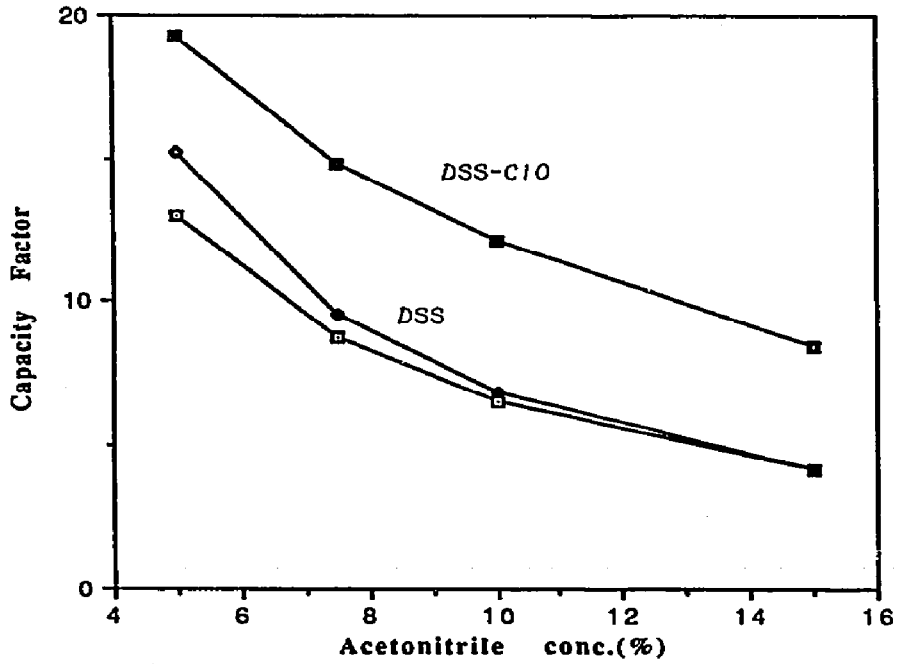

Fig. 3. Effect of acetonitrile on the avidin column and the avidin C_{10} column. (□) Ketoprofen racemate; (◆) and (⊡) ketoprofen enantiomer. Other conditions were the same as those in Fig. 2.

actions between the compounds and the stationary phase, but unfortunately the avidin column made from DSS-C_{10} (avidin C_{10} column) retained plasma proteins (Table I). This may be because both ends of DSS-C_{10} are more likely to react with the aminopropyl silica compared with DSS, so avidin protein may not be sufficiently conjugated to the DSS-C_{10}, and/or the degree of freedom of carbon chains (DSS-C_{10}) may be so large that access to them is not adequately blocked by avidin.

Retention behavior of ketoprofen on the avidin column

Fig. 2. shows a typical clution pattern of ketoprofen from an avidin column; the ketoprofen enantiomers were retained and resolved completely. Fig. 3 shows

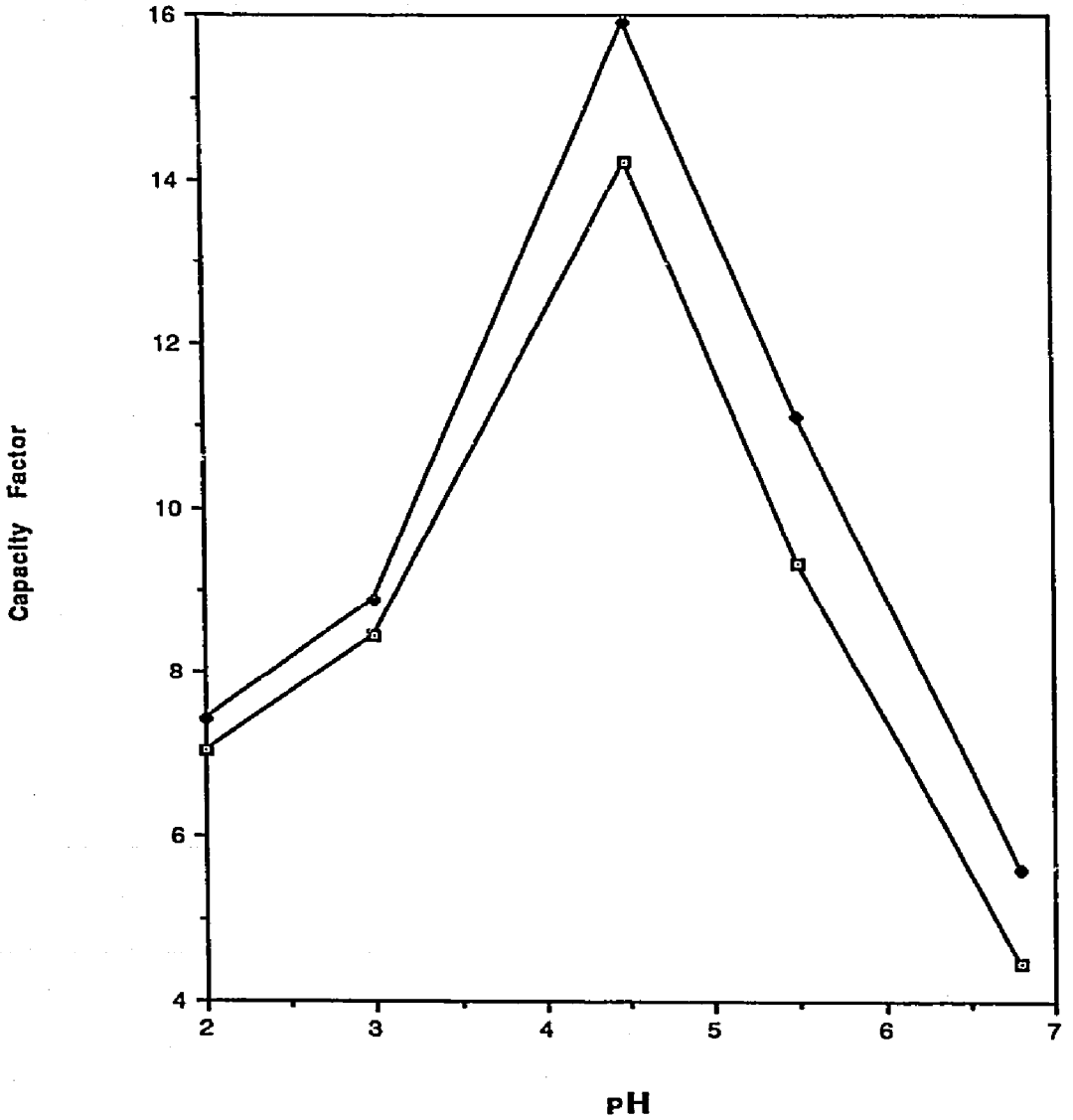

Fig. 4. Effect of pH of the mobile phase. Other conditions were the same as those in Fig. 2.

the effects of acetonitrile on the avidin column and the avidin C_{10} column. (The avidin C_{10} column was made by exactly the same method as the avidin column.) The retention of ketoprofen decreased with increasing acetonitrile concentration in each column. This indicates that both columns have reversed-phase characteristics. The ketoprofen enantiomers were not resolved with the avidin C_{10} column, although the retention time of ketoprofen with the avidin C_{10} column was longer than that with the avidin column. The reason for this may be that ketoprofen enantiomers, which are retained on the carbon chains of DSS-C_{10}, do not interact enough with avidin, because the avidin protein molecules do not cover the hydrophobic regions sufficiently. Fig. 4 shows the pH profile of the retention of ketoprofen enantiomers on the avidin column. The elution of ketoprofen enantiomers was latest in the mobile phase at pH 4.5. It should be noted that the isoelectric point of avidin (pH 10–11) may be changed by conjugation of DSS to the primary amine of avidin protein. Enantiomeric resolution of ketoprofen was best at pH 7. This may also be related to the isoelectric point of avidin conjugated to DSS or/and the dissociation of ketoprofen (pK_a = *ca.* 4). The ionized form may be more favorable for the differential enantiomeric interactions with the avidin column. The effect of salt concentration is shown in Fig. 5. The retention and the enantiomeric resolution of ketoprofen were affected only slightly by varying the salt concentration of the mobile phase.

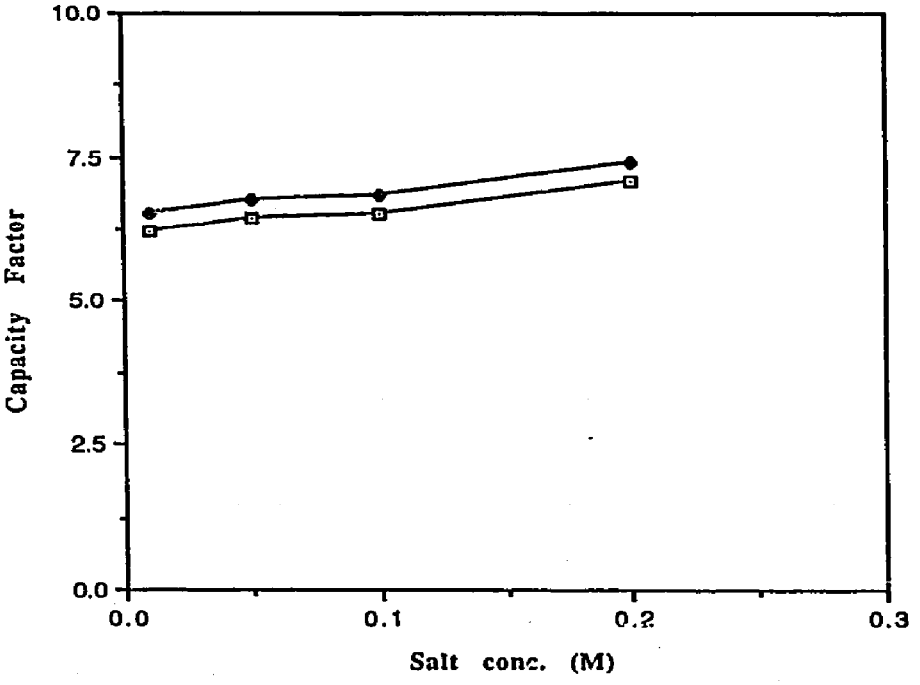

Fig. 5. Effect of salt concentration of the mobile phase. Other conditions were the same as those in Fig. 2.

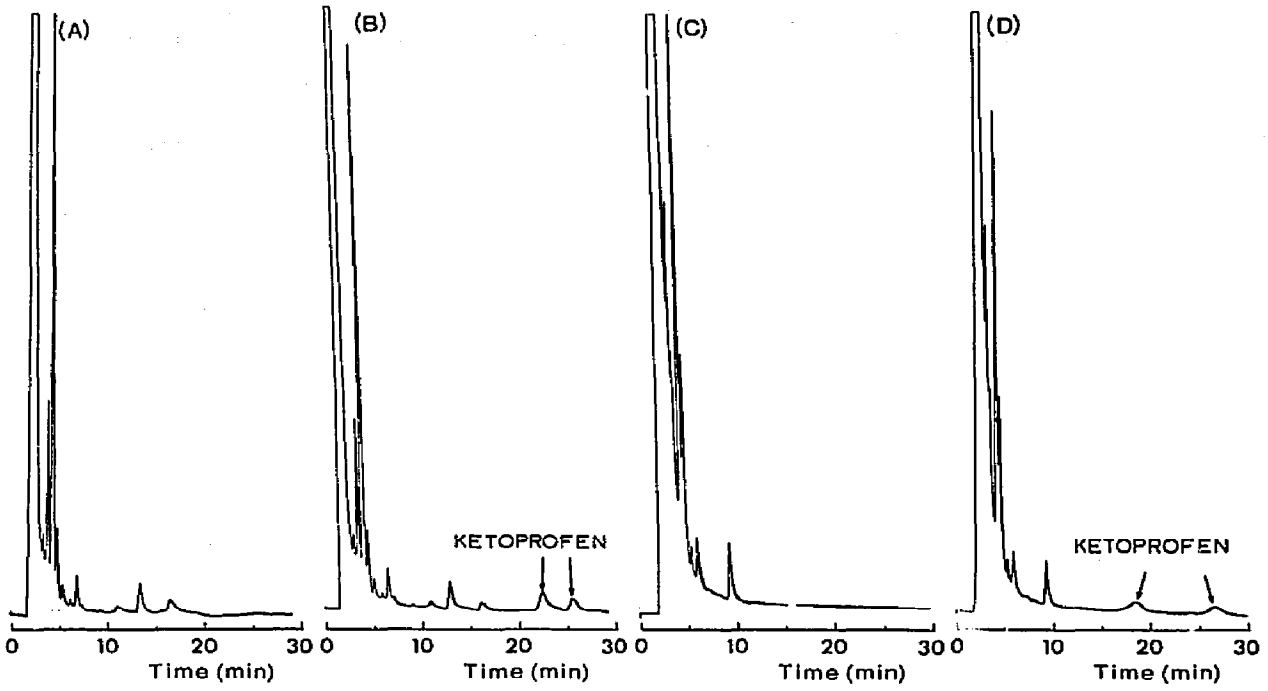

Fig. 6. Chromatograms of (A) and (C) plasma blank and (B) and (D) plasma spiked with ketoprofen enantiomers (2.5 μg/ml). Mobile phase: (A) and (B) pH 2 and (C) and (D) pH 7. Other conditions were the same as those in Fig. 2.

Direct injection analysis of ketoprofen enantiomers in plasma

Miwa *et al.* [17] reported previously that an avidin-conjugated column exhibited chiral recognition of ketoprofen. But the retention of this column was very weak, so ketoprofen was not separated from plasma components. Fig. 6 shows chromatograms obtained by injection of 20-μl samples of a plasma blank and plasma spiked with ketoprofen enantiomers (2.5 μg/ml). The plasma proteins were immediately eluted from the avidin column without retention in the mobile phase at pH 2 and 7. Retention of a low-molecular-mass compound of interest can be adjusted by modifying the mobile phase conditions. The ketoprofen enantiomers were well separated from each other and from plasma components with an acetonitrile–0.1 *M* phosphate buffer (5:95, v/v) mobile phase (pH 2 and 7), as shown in Fig. 6. The recovery of ketoprofen from the human plasma samples were quantitative (100.6% with a pH 2 mobile phase and 98.7% with a pH 7 mobile phase). In these conditions the avidin column was stable with no change of back-pressure and separation after more than 40 injections, although it had no guard columns. This indicates that the assay with the avidin column also allows the determination of the total drug concentration in plasma.

CONCLUSION

Plasma proteins are excluded from the avidin column by the hydrophilic and bulky avidin surface phase, being eluted without retention, whereas drugs are retained by internal hydrophobic regions composed mainly of DSS carbon chains and are separated from the plasma components. The drug enantiomers interact differentially with the avidin phase during penetration into and exit from the hydrophobic regions, and so are resolved. The avidin column should thus prove to be a powerful tool for direct injection analysis of drug enantiomers in plasma.

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