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Direct injection assay of drug enantiomers in serum on ovomucoid-bonded silica materials by liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the determination of drug enantiomers in serum was developed. The method involves direct injection of serum samples on to an ovomucoid-bonded column, which is prepared by bonding of ovomucoid proteins to an aminopropyl-silica gel by the N,N'-disuccinimidyl carbonate activation method and separation of drug enantiomers on the column using a mixture of phosphate buffer and an organic solvent. High recoveries of serum proteins were obtained using eluent pH values of 3, 4, 6 and 7 at phosphate buffer concentrations above 50 m*M*, whereas the recovery was *ca.* 70% at an eluent pH of 5. The recovery of each enantiomer of basic and acidic drugs from serum was almost 100%.

INTRODUCTION

Since the introduction of internal-surface reversed-phase silica materials [1] for direct serum injection assays of drugs, various materials have been developed for assays of achiral drugs by this method [2–4], including shielded hydrophobic phase [5], dual-zone [6], semi-permeable surface [7] and mixed functional phase (MFP) [8] materials. With such materials, large molecules such as serum proteins are eluted in the void volume without destructive accumulation, but small molecules such as drugs can reach the hydrophobic sites and be separated. Haginaka and Wakai [9] extended the MFP concept to the determination of drug enantiomers in serum by direct injection by preparing MFP materials having β-cyclodextrin (CD) and diol phases. However, a limited number of drug enantiomers in serum could be separated on the β-CD and diol phases, because β-CD resolved only a small range of drug enantiomers.

Many protein-bonded stationary phases based on albumins such as bovine serum albumin [10] and human serum albumin [11] and glycoproteins such as α_1-acid glycoprotein [12], ovomucoid [13], avidin [14] and cellulase [15] have been developed for the resolution of enantiomers and for the first five protein-bonded columns are now commercially available. The advantages of these phases include the possible resolution of a wide range of drug enantiomers and the possible use of an aqueous eluent, inluding a buffer and organic modifier, as in the reversed-phase mode.

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Generally, a protein has a tendency for its outer and inner surface to consist of hydrophilic and hydrophobic amino acids, respectively. Also, a glycoprotein might have much more hydrophilic outer surfaces than the deglycosylated protein. These facts suggest that protein-bonded materials have both hydrophilic and hydrophobic phases, or in other words, mixed functionality, in the ligand, and could be used for direct serum injection assays of drug enantiomers. Recently, Oda *et al.* [16] reported an avidin-bonded column for direct serum injection assays of ketoprofen enantiomers. Avidin is a glycoprotein in egg white. The avidin was bound to aminopropylsilica gels activated by N,N′-disuccinimidyl suberate (DSS), where C_6 carbon chains remain after crosslinking of the aminopropylsilica and protein. The disadvantages of the use of DSS as a crosslinker are that the superfluously achiral and non-specific interactions of enantiomers with the hydrophobic chains might diminish the observed enantioselectivity, and that the interaction of protein molecules could result in a low recovery of proteins from the silica materials. Oda *et al.* [16] reported that the recovery of serum proteins was *ca.* 5–40% from the ovomucoid-bonded materials by the DSS activation method.

This paper deals with direct serum injection assays of drug enantiomers on an ovomucoid-bonded column, using N,N′-disuccinimidyl carbonate (DSC) as a crosslinker. The use of DSC could overcome the disadvantages described above. The recovery of serum proteins from the ovomucoid-bonded materials was high except when an eluent of pH 5 was used. The materials were successfully applied to direct serum injection assays of drug enantiomers.

EXPERIMENTAL

Materials

Chlorpheniramine maleate and benzoin were purchased from Sigma (St. Louis, MO, USA). Ketoprofen and oxazepam were kindly donated by Kaken Pharmaceutical (Tokyo, Japan) and Banyu Pharmaceutical (Tokyo, Japan), respectively. Ovomucoid proteins were purchased from Eisai (Tokyo, Japan). DSC and control human serum (control serum I) were obtained from Wako (Osaka, Japan). Other reagents and solvents were of analytical-reagent or HPLC grade. Water purified with a Nano-pure II system (Barnstead, Boston, MA, USA) was used for the preparation of sample solutions and eluents.

Coomasie Brilliant Blue G-250 (CBB) reagent was prepared by dissolving 10 mg of CBB in 5 ml of ethanol and diluting to 100 ml with distilled water after addition of 10 ml of 85% phosphoric acid.

Preparation of ovomucoid-bonded silica materials

Aminopropylsilica gel (Ultron-NH_2, 5 μm, 12-nm pore size; Shinwa Chemical Industries, Kyoto, Japan) was activated by DSC as reported by Miwa *et al.* [13]. A 2-g amount of DSC-activated aminopropylsilica gel was slurried in 20 ml of 20 m*M* phosphate buffer (pH 6.6). To the slurry, 2 g of ovomucoid dissolved in 10 ml of 20 m*M* phosphate buffer (pH 6.6) were gradually added and the mixture was gently rotated at 30°C for 20 h. The mixture was filtered and washed with water and methanol. The isolated silica gel was dried *in vacuo* of P_2O_5 at 40°C overnight. The ovomucoid-bonded silica materials were packed into a 100 × 4.6 mm I.D. stainless-steel column by conventional high-pressure slurry-packing procedures.

Chromatography

The HPLC system was composed of an LC-9A pump, an SPD-6A spectrophotometer, a SIL-6B autoinjector, a C-R4A integrator and an SCL-6B system controller (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.8 ml/min. All separations were carried out at 25°C using a CO-1093C column oven (Uniflows, Tokyo, Japan). Chromatographic parameters such as capacity factor (k'), enantioseparation factor (α) and resolution (R_s) were calculated from data obtained. The eluents used are specified in the figures and tables.

Preparation of human serum sample

Ketoprofen and chlorpheniramine was dis-

solved in human serum at known concentrations and an appropriate volume of serum sample was applied to the ovomucoid-bonded silica materials after filtration through a 0.22-μm membrane filter (Nippon Millipore, Tokyo, Japan).

Recovery of serum proteins from the ovomucoid-bonded silica materials

The recovery of human serum proteins from the ovomucoid-bonded silica materials was examined according to the procedures described previously [17]. Briefly, a 20-μl portion of a control serum sample was loaded on to the ovomucoid-bonded silica material using 50 m*M* phosphate buffer–ethanol (85:15, v/v) as the eluent at a flow-rate of 0.8 ml/min, and the eluate was collected for 10 min. The eluate obtained without the column was used as a blank. A 0.5-ml portion of the eluate was mixed with 5 ml of CBB reagent and after 2 min the absorbance of the mixture was measured at 595 nm using the eluent treated with CBB reagent as the reference solution. The recovery was calculated from the absorbance ratio with and without the column.

RESULTS AND DISCUSSION

Recovery of serum proteins from ovomucoid-bonded silica

Table I shows the recovery of serum proteins from ovomucoid-bonded silica using 50 m*M* phosphate buffer–ethanol (85:15, v/v) as the eluent. At an eluent pH of 5, the recovery of serum proteins was about 70%, whereas at pH 3, 4, 6 and 7 the serum protein recoveries were high (93–97%). Oda *et al.* [16] reported that the recovery of serum proteins from an avidin-bonded column was 97, 24 and 92% at eluent pH values of 2, 4.5 and 7, respectively. The low recovery of serum proteins from the ovomucoid- and avidin-bonded columns at an eluent pH of 4.5–5.0 might be due to serum albumin. It has been reported that the isoelectric points of human serum albumin and ovomucoid proteins are 4.7–5.2 [18] and 4.1 [19], respectively. Also, assuming that the change in the isoelectric point of an ovomucoid protein might be small before and after immobilization, both serum albumin and ovomucoid were negatively charged above an eluent pH of 6, whereas below an eluent pH of 3 both proteins were positively charged, so the recovery of serum proteins was high. On the other hand, at an eluent pH of 5, both the protein of ovomucoid and serum albumin have an isoelectric point, *i.e.* both proteins are positively and negatively charged. Also, as the isoelectric point of avidin is 10.0 [19], the avidin-bonded column should be positively charged over an eluent pH range of 2–7. Therefore, around the isolectric point of serum albumin a low recovery of serum proteins was observed on the avidin-bonded column.

TABLE I

RECOVERY OF SERUM PROTEINS FROM THE OVOMUCOID-BONDED COLUMN AT VARIOUS ELUENT pH VALUES

HPLC conditions: column, ovomucoid-bonded column (100 × 4.6 mm I.D.); eluent, 50 m*M* phosphate buffer–ethanol (85:15, v/v); flow-rate, 0.8 ml/min.

Eluent pH[a]	Mean recovery (%)[b]	R.S.D. (%)
3.2	97.4	4.03
4.0	96.3	4.30
5.1	71.3	7.08
6.0	93.8	1.80
6.9	97.5	2.44

[a] Phosphate buffer pH.
[b] $n = 5$.

These results suggest that direct serum injection assays of drug enantiomers could be achieved on ovomucoid-bonded silica materials except at an eluent pH around 5. However, a gradual decrease in the performance of the ovomucoid-bonded coloumn was observed when 20 m*M* phosphate buffer (pH 6.8)–ethanol (90:10, v/v) was used as the eluent. When 50 m*M* phosphate buffer–ethanol (90:10, v/v) was used, the column performance was maintained as described below.

Repetitive injections of serum samples

Prior to serum injections, the stability of the ovomucoid-bonded column towards washing

TABLE II

CHANGES IN CAPACITY FACTOR (k'_1), ENANTIOSELECTIVITY (α) AND RESOLUTION (R_s) OF RACEMIC SOLUTES WITH REPETITIVE INJECTIONS OF SERUM SAMPLES

The capacity factor (k'_1) of the first-eluted enantiomer, enantioselectivity (α) and resolution (R_s) of racemic solutes were measured before and after 200 injections of a 20-μl portion of serum sample and after washing with 500 ml of 75% acetonitrile followed by 200 injections of a 20-μl portion of serum sample. The HPLC conditions used were as follows: column, ovomucoid-bonded column (100 × 4.6 mm I.D.); eluent, 20 m*M* phosphate buffer (pH 5.1)–ethanol (90:10, v/v) for chlorpheniramine and ketoprofen, and 20 m*M* phosphate buffer (pH 6.9)–ethanol (90:10, v/v) for benzoin and oxazepam; flow-rate, 0.8 ml/min; column temperature, 25°C.

Solute	Parameter	Before injection	After 200 injections[a]	After washing with 75% CH_3CN
Chlorpheniramine	k'_1	1.97	2.33	2.67
	α	1.79	1.59	1.70
	R_s	3.07	2.00	2.68
Ketoprofen	k'_1	11.4	8.07	10.9
	α	1.11	1.11	1.13
	R_s	1.29	1.15	1.31
Benzoin	k'_1	4.74	3.58	4.78
	α	2.11	2.07	2.14
	R_s	7.10	6.23	7.05
Oxazepam	k'_1	7.57	6.09	8.05
	α	2.24	2.18	2.28
	R_s	7.72	7.02	7.77

[a] The HPLC conditions for repetitive serum injections were the same as those described at the top of the table except that 50 m*M* phosphate buffer (pH 6.9)–ethanol (90:10, v/v) was used as the eluent.

with organic solvents was checked. Pure ethanol, acetonitrile and 2-propanol were pumped at a volume of about 300 ml each with no decrease in column performance. Also, no change in the capacity factor of chlorpheniramine occurred. Hence the ovomucoid-bonded column is stable towards treatment with such organic solvents.

Volumes of 20 μl of serum samples were repetitively injected on to an ovomucoid-bonded column using 50 m*M* phosphate buffer (pH 6.9)–ethanol (90:10, v/v) as the eluent. Table II illustrates changes in the column performance before and after 200 injections of 20-μl serum samples. For basic chlorpheniramine, an increase in the capacity factor of the first-eluted enantiomer (k'_1) was observed after the repetitive injections, whereas a decrease in enantioselectivity (α) and resolution (R_s) occurred. For acidic ketoprofen, a decrease in k'_1 and R_s occurred, whereas the α value remained unchanged. For uncharged benzoin and oxazepam, a slight decreasse in k'_1, α and R_s was observed. Next, the column was washed with about 500 ml of 75% acetonitrile and the column performance was checked again. The k'_1, α and R_s values of all solutes except chlorpheniramine recovered to the original values before the injections of serum samples. For chlorpheniramine, k'_1 was slightly increased whereas α and R_s were slightly decreased compared with the values before the repetitive injections. It is unclear why an increase in the capacity factor of chlorpheniramine occurs after the repetitive injections and further after washing with 75% acetonitrile. Taking into account the recovery of serum proteins with repetitive injections, the achiral and chiral recognition site(s) of the ovomucoid-bonded column might be blocked with a hydrophobic component(s) in human serum. However, the component(s) could be removed from the column by washing with 75% acetonitrile.

Tabel III shows the peak areas of chlorpheni-

TABLE III

CHANGES IN PEAK AREAS OF CHLORPHENIRAMINE AND KETOPROFEN ENANTIOMERS AFTER REPETITIVE INJECTIONS OF SERUM SAMPLES

HPLC conditions as in Figs. 1 and 2.

Solute	Enantiomers	Peak area (μV s $\times$ 10^{-5})		
		Before injection	After 200 injections	After washing with 75% CH_3CN
Chlorpheniramine	First-eluted	1.76	1.72	1.73
	Second-eluted	1.74	1.70	1.72
Ketoprofen	First-eluted	1.04	1.00	1.08
	Second-eluted	1.03	0.97	1.06

ramine and ketoprofen before and after 200 injections of serum samples and after washing with 75% acetonitrile. These results reveal that constant drug recoveries are obtained throughout the 200 repetitive injections, and the method should be applicable to the determination of drug enantiomers. Also, no increase in the back-pressure of the column was observed before and after the injections. These results indicate that direct serum injection assays of drug enantiomers on the ovomucoid-bonded column could be attainable.

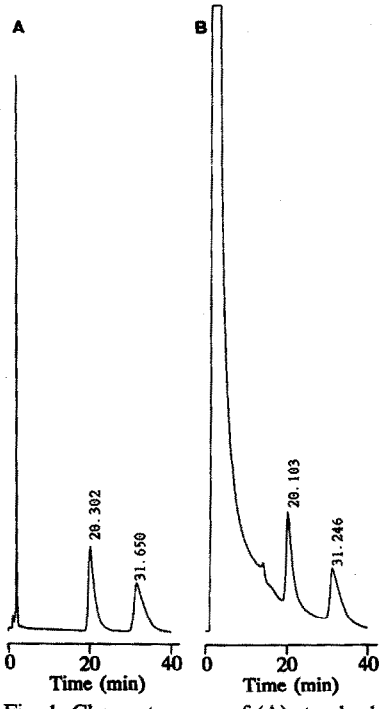

Fig. 1. Chromatograms of (A) standard chlorpheniramine and (B) control serum spiked with chlorpheniramine by direct serum injection on to the ovomucoid-bonded column. Concentration of racemic chlorpheniramine = 50 μg/ml. Chromatographic conditions: eluent, 50 m*M* phosphate buffer (pH 6.0)–acetonitrile (92:8, v/v); flow-rate, 0.8 ml/min; detection wavelength, 254 nm; injection volume, 20 μl.

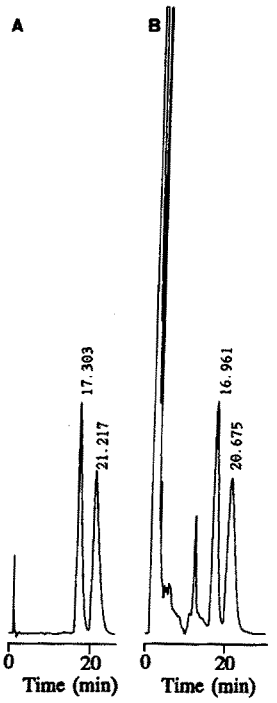

Fig. 2. Chromatograms of (A) standard ketoprofen and (B) control serum spiked with ketoprofen by direct serum injection on to the ovomucoid-bonded column. Concentration of racemic ketoprofen = 25 μg/ml. Chromatographic conditions: eluent, 50 m*M* phosphate buffer (pH 3.2)–ethanol (94:6, v/v); flow-rate, 0.8 ml/min; detection wavelength, 254 nm; injection volume, 10 μl.

TABLE IV

REPRODUCIBILITY AND RECOVERY OF EANTIOMERS OF CHLORPHENIRAMINE AND KETOPROFEN FROM HUMAN SERUM

HPLC conditions as in Figs. 1 and 2.

Solute	Enantiomer	R.S.D. (%)[a]	Recovery (%)
Chlorpheniramine	First-eluted	2.67	99.9
	Second-eluted	1.49	99.0
Ketoprofen	First-eluted	0.81	99.0
	Second-eluted	1.22	101

[a] $n = 5$.

Direct serum injection assay of drugs in serum

Figs. 1 and 2 show chromatograms for direct injection assays of chlorpheniramine and ketoprofen enantiomers, respectively, in human serum. Eluent pH values of 3.2 and 6.9 were used for the separations of chlorpheniramine and ketoprofen enantiomers from the background components. The serum proteins were eluted in the void volume with almost 100% recovery, followed by the elution of the enantiomers. Table IV gives the relative standard deviation (R.S.D.s) of the asays of chlorpheniramine and ketoprofen enantiomers in serum and their recoveries from the serum samples. Each enantiomer of chlorpheniramine and ketoprofen was almost completely recovered from serum with good reproducibility.

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