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Direct injection analysis of carbamazepine and its active 10,11-epoxide metabolite in plasma by use of a semipermeable surface (SPS) silica column in LC

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Abstract: A semipermeable surface (SPS) silica column was applied for the simultaneous determination of carbamazepine (CBZ) and its active 10,11-epoxide metabolite (EPO) in plasma following direct injection in LC. The SPS packing material consists of an ODS ligand as the hydrophobic inner phase and a polyoxyethylene network as the hydrophilic outer phase. When a 5-μl portion of intact plasma was injected onto the column using a mobile phase of phosphate buffer (pH 7.1, ionic strength 0.1)–acetonitrile (4:1, v/v), the plasma proteins were size-excluded, whereas the drug and its metabolite were retained and separated both from each other and from other commonly co-administered drugs such as phenobarbital (PB) and phenytoin (DPH). The calibration graphs (peak area vs concentration) of CBZ, EPO and PB were linear over the therapeutic range of plasma concentration ($r > 0.998$) with good relative standard deviations (RSD < 3.98%, $n = 5$). The recoveries from plasma were almost complete (>96.6%). The analysis time was 17 min. The method as developed was applied in studies on the time course of plasma concentrations of unchanged CBZ and EPO after i.v. administration of CBZ to the rat.

Keywords: *Carbamazepine; carbamazepine-10,11-epoxide; direct sample injection; semipermeable surface silica; restricted access column; reversed-phase liquid chromatography.*

Introduction

HPLC analysis following direct injection of biological samples has been recently explored for pharmacokinetic and toxicologic studies and for therapeutic drug monitoring (TDM). The advantages of direct sample analysis would include enhanced efficiency, precision, possible automation and potential higher efficiency performance [1, 2]. The recent development of so-called 'restricted access' HPLC packing materials is very significant for direct sample analysis. So far, several 'restricted access' type HPLC packing materials such as ISRP, SHP, SPS, MFP and DZM [3, 4] have been developed for this purpose. These new materials have the common function that large molecules such as plasma proteins are size-excluded, whereas small molecules such as drugs and metabolites are retained in the pores, which are designed to permit hydrophobic interaction. Consequently, the new materials allow repeated injection of intact plasma samples with little loss of column efficiency.

A semipermeable surface (SPS) silica, one of the restricted access type packing materials [5], has a hydrophilic outer phase consisting of a polyoxyethylene network and a hydrophobic inner phase of common reversed-phase ligands such as ODS. The outer phase prevents plasma proteins from reaching the inner phase, while small molecules can penetrate through the outer phase and are consequently retained by the hydrophobic inner phase.

Carbamazepine (CBZ) is a widely used anticonvulsant. The therapeutic plasma level of CBZ lies in a relatively narrow range (4–12 μg ml^{-1}) [6], and its major metabolite, carbamazepine-10,11-epoxide (EPO), also exhibits anticonvulsant activity in rats [7]. Therefore, the simultaneous determination of CBZ and EPO in plasma is important for therapeutic drug monitoring and pharmacokinetic studies.

The analysis of CBZ and its principal metabolite EPO in plasma sample has been mainly based on liquid extraction pretreatment [8–10]. Hartley *et al.* reported a simple HPLC analysis for CBZ and EPO as well as for another metabolite, CBZ-diol, based on manual solid extraction pretreatment [11]. It has also been reported that a column-switching

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system can be used for the analysis of anticonvulsants, including CBZ and its metabolites [12, 13].

This paper deals with the application of a SPS silica column to the simultaneous determination of CBZ and EPO in plasma following direct sample injection, without the need for manual pretreatment or for column-switching technique, thus permitting the fully automatic analysis of CBZ and its metabolites in serum.

Experimental

Reagents and materials

Carbamazepine (CBZ), phenobarbital (PB) and phenytoin (DPH) of guaranteed reagent grade were purchased from Wako Pure Chemicals (Osaka, Japan). Carbamazepine-10,11-epoxide (EPO) was obtained from Yoshitomi Pharmaceutical Industries (Osaka, Japan). Human plasma was prepared from fresh human blood in the usual manner.

Apparatus

An HPLC system equipped with a LC-6A pump (Shimadzu, Japan), a UV detector, SPD-2AM (Shimadzu, Japan) and a data analyser, Chromatopac C-R3A (Shimadzu, Japan) was employed. A Rheodyne 7125 injector with a 20-μl loop was used for sample injection.

HPLC conditions

A SPS-5PM-5S-100-C18 column (15 cm × 4.6 mm i.d.; Regis Chemical Co., IL, USA) was used at a temperature of 30°C, controlled by a water bath. The mobile phase was a mixture of sodium phosphate buffer (pH 7.1, ionic strength 0.1)–acetonitrile (4:1, v/v). The flow rate was 1.0 ml min^{-1} (0–17 min) and 1.5 ml min^{-1} (after 17 min).

Standard plasma samples

Stock solutions of CBZ (240 μg ml^{-1}), EPO (80 μg ml^{-1}) and DPH (400 μg ml^{-1}) were made up in acetonitrile, while a solution of PB (700 μg ml^{-1}) was made up in sodium phosphate buffer (pH 7.4, ionic strength 0.17). Standard plasma solutions were prepared by spiking human plasma with these stock solutions. The concentration ranges of the spiked plasma are shown in Table 1. Each 5-μl portion of plasma solution was injected directly onto the HPLC system without any pretreatment.

Results and Discussion

The mobile phase conditions for direct injection analysis need be selected carefully to avoid clogging of the column with plasma proteins. Pinkerton *et al.* recommended that the content of organic modifiers in the mobile phase should be <25% for acetonitrile, <20% for isopropanol, and <10% for tetrahydrofuran [14]. It has been suggested that the mobile phase used in combination with the SPS column should ensure that organic modifier concentration is lower than 25%, pH is between 5.5 and 7.5 and that buffer concentration is in the range of 0.05–0.1 M. The separation of CBZ and EPO from plasma proteins were optimized taking these limitations into account. Also, the simultaneous separation of PB and DPH, antiepileptic agents often co-administered with CBZ, were also investigated. As shown in Fig. 1(a), the capacity factors of CBZ and EPO were almost unaffected by the change in pH of mobile phase, since they are neutral over the pH range tested, whereas those of PB and DPH decreased with increasing pH due to the proton dissociation. Figure 1(b) shows the effect of the phosphate buffer concentration. The capacity factors did not change very much for ionic strengths between 0.08 and 0.15. Figure 1(c) shows the effect of organic modifier. A decrease in acetonitrile content from 20 to 15% caused an increase in the capacity

Table 1
Calibration data and recoveries of plasma samples

	Slope*	Intercept*	r†	Conc. (μg ml^{-1})	Recovery (%)‡
CBZ	68.1390	20.1247	>0.9984	1.44–24.0	96.6
EPO	68.7481	−6.8440	>0.9998	0.48–8.00	99.0
PB	22.2334	−5.7033	>0.9999	4.20–70.0	102.8
DPH	13.2759	−9.5096	>0.9991	2.40–40.0	106.6

*Area = slope × concentration + intercept.
†Correlation coefficient.
‡Ratio of the slopes of the calibration line: (plasma sample/standard solution).

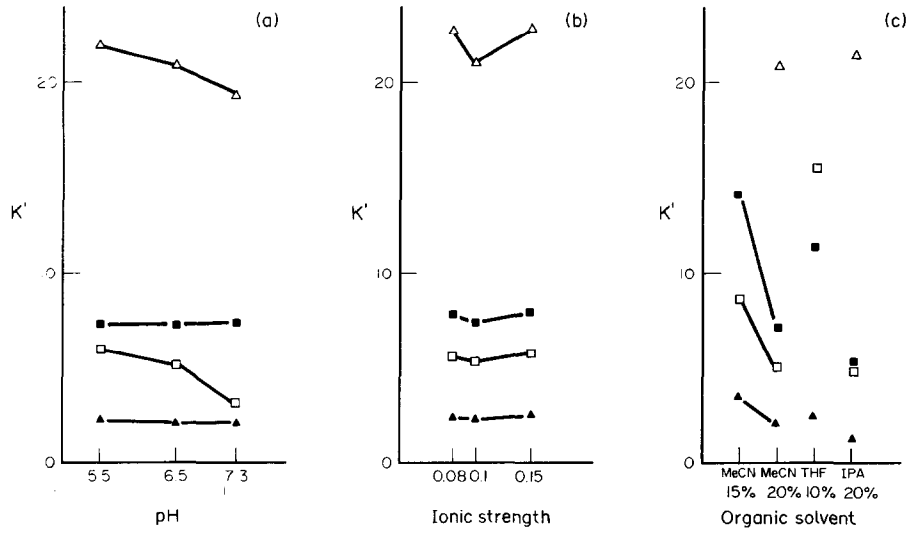

Figure 1
Effect of (a) pH, (b) ionic strength and (c) organic solvent on capacity factors (k') of CBZ (■), EPO (▲), PB (□) and DPH (△). HPLC conditions: column, SPS C18 (15 cm × 4.6 mm, i.d.); flow rate, 1.0 ml min^{-1}; detection, UV 214 nm; injection volume, 5 μl; column temperature, 30°C; mobile phase, phosphate buffer (I = 0.1)–MeCN (4:1, v/v) for (a); phosphate buffer (pH 6.5)–MeCN (4:1, v/v) for (b); and phosphate buffer (pH 6.5, I = 0.1) containing organic modifier for (c), where I denotes ionic strength, MeCN acetonitrile, THF tetrahydrofuran and IPA isopropanol.

factors of CBZ, EPO and PB. The use of a mobile phase containing 20% acetonitrile gave better separation of CBZ and PB than 20% isopropanol. When the mobile phase contained 15% acetonitrile or 10% tetrahydrofuran, the retention time for DPH was longer than 60 min. From these results, the mobile phase selected was: phosphate buffer (pH 7.1, ionic strength 0.1) containing 20% acetonitrile.

Sample volume is a critical factor for direct injection analysis. A small injection volume is preferable for the determination of total drug concentration, because the bound drug is quickly and completely released from plasma proteins by dilution with the mobile phase and is then eluted together with unbound drug as a single peak. Consequently, the total concentration can be determined from the peak area. On the other hand, a large injection volume tends to suffer from the effect of drug–protein binding; because the release of bound drug is suppressed, band-broadening appears, sometimes accompanied by splitting of the drug peak [15, 16]. It is worth noting that if the injection volume and the mobile phase conditions are selected carefully, the unbound drug concentrations in the protein-binding equilibrium can be determined by use of a restricted access type HPLC column [16–20]. In this paper, the injection volume was 5 μl. This volume is large enough for suitable detection of the therapeutic levels of these drugs by UV absorption and, as mentioned later, is small enough to avoid the effect of protein binding.

Figure 2(a) and (b) show the chromatograms of plasma samples and buffer solution of these drugs, respectively. The flow rate was increased from 1.0 to 1.5 ml min^{-1} 17 min after injection in order to expedite the elution of DPH. The peak of each drug was not interfered with by plasma proteins or other endogenous components.

The calibration graphs were prepared by plotting the peak areas vs concentration, over the therapeutic range. As shown in Table 1, the calibration data indicated good linearity ($r > 0.998$), and the recoveries were almost complete, which means that the bound drug was readily released from proteins to enable the total concentration to be determined. As shown in Table 2, the RSD values of the peak areas were lower than 3.98% (n = 5) for CBZ, EPO and PB, indicating good reproducibility for these three solutes. One analysis required about 17 min. However, the method as developed does not seem to be suitable for the assay of DPH, because the reproducibility of DPH was rather worse than for the other three drugs and the elution time was very long.

The column stability parameters are shown in Table 3, indicating no significant deterior-

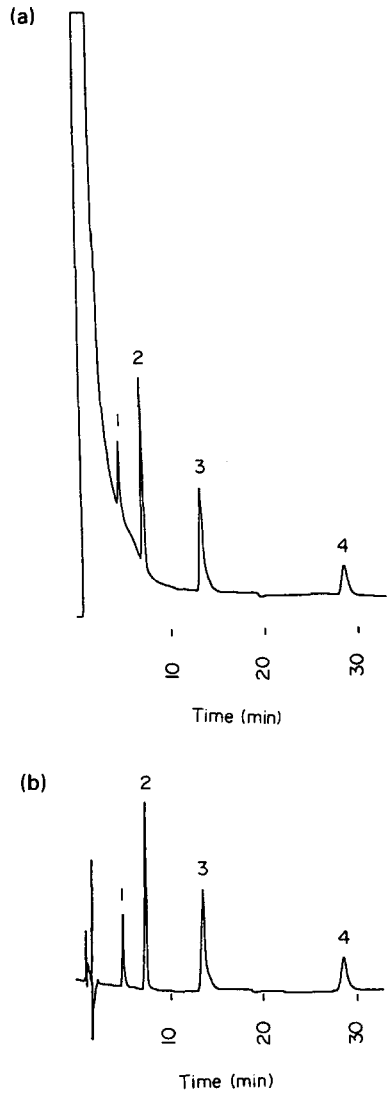

Figure 2
Chromatogram of (a) spiked plasma and (b) standard solution samples: 1, EPO; 2, PB; 3, CBZ; and 4, DPH. HPLC conditions: column, SPS C18 (15 cm × 4.6 mm i.d.); mobile phase, phosphate buffer (pH 7.1, ionic strength 0.1)–acetonitrile (4:1, v/v); flow rate, 1.0 ml min^{-1} for 0–17 min, 1.5 ml min^{-1} after 17 min; detection, UV 214 nm; injection volume, 5 μl; column temperature, 30°C.

ation of column efficiency after injections repeated 100 times.

The HPLC method thus developed was applied to the simultaneous determination of CBZ and EPO in rat plasma after intravenous administration of 10 mg kg^{-1} CBZ. Figure 3 shows the chromatogram of rat plasma obtained 15 min after i.v. administration. The peak eluted at 15 min is due to the pentobarbital used to anaesthetize the rat. The plasma concentration–time course is shown in Fig. 4. These results agreed with those reported by Remmel *et al.* [21], where the CBZ and EPO concentrations were determined by HPLC after a manual extraction procedure.

Table 2
Reproducibility of peak area of the drugs in human plasma sample

	Conc. (μg ml^{-1})	Within-run RSD (n = 5)	Day-to-day RSD (n = 5)
CBZ	12.0	1.40	0.77
EPO	4.0	1.28	3.98
PB	35.0	0.83	1.23
DPH	20.0	5.30	5.84

HPLC conditions: column, SPS C18 (15 cm × 4.6 mm, i.d.); mobile phase, phosphate buffer (pH 7.1, ionic strength 0.1)–acetonitrile (4:1, v/v); flow rate, 1.0 ml min^{-1}; detection, UV 214 nm; injection volume, 5 μl; column temperature, 30°C.

Conclusions

The SPS silica column is applicable to the simultaneous determination of CBZ and EPO in plasma following direct sample injection. PB

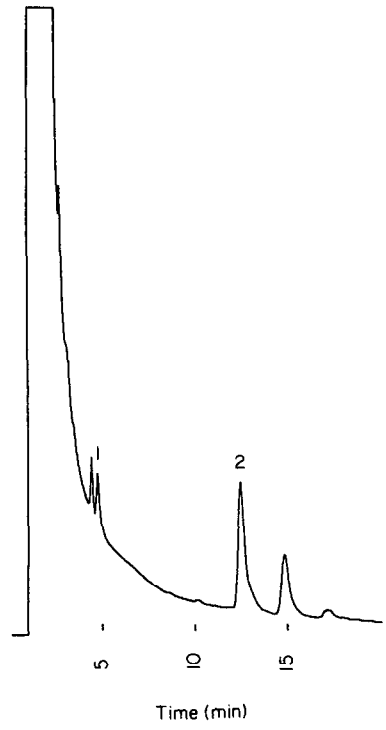

Figure 3
Chromatogram of rat plasma obtained 15 min after i.v. administration of 10 mg kg^{-1} CBZ samples: 1, EPO; 2, CBZ. HPLC conditions: column, SPS C18 (15 cm × 4.6 mm i.d.); mobile phase, phosphate buffer (pH 7.1, ionic strength 0.1)–acetonitrile (4:1, v/v); flow rate, 1.0 ml min^{-1}; detection, UV 214 nm; injection volume, 5 μl; column temperature, 30°C.

Table 3
Stability after repeated use of a SPS column

Utilization period: 3 months	Retention time (min)	
Compound	Initial	After 3 months
CBZ	13.0	13.5
EPO	4.8	4.9
PB	7.4	7.4
DPH	27.9	28.7

Number of injections: 44 times for standard solution + 56 times for plasma samples = 100 times in total.

Total volume of injections: 220 μl for standard solutions + 280 μl for plasma samples = 500 μl in total.

Total volume of mobile phase used: 1 l.

	Pressure	
Flow rate	Initial	After 3 months
1.0 ml min^{-1}	<80 kgf cm^{-2}	<90 kgf cm^{-2}
1.5 ml min^{-1}	<120 kgf cm^{-2}	<130 kgf cm^{-2}

HPLC conditions: column, SPS C18 (15 cm × 4.6 mm, i.d.); mobile phase, phosphate buffer (pH 7.1, ionic strength 0.1)–acetonitrile (4:1, v/v); flow rate, 1.0 ml min^{-1} for 0–17 min, 1.5 ml min^{-1} after 17 min; detection, UV 214 nm; injection volume, 5 μl; column temperature, 30°C.

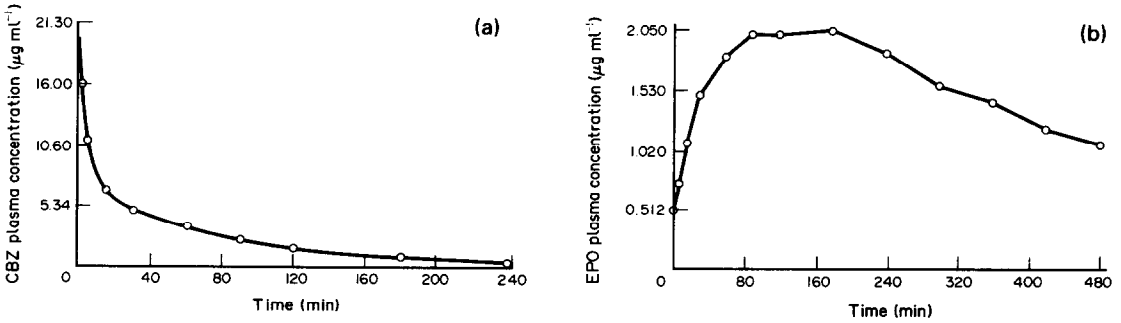

Figure 4
The plasma concentration–time course of (a) CBZ and (b) EPO after i.v. administration of 10 mg kg^{-1} CBZ to a rat.

can also be determined under the same HPLC conditions. The proposed HPLC assay is easy to perform and requires 17 min for one analysis. The total concentrations of drug and metabolite are determined simultaneously with good reproducibility using a 5 μl portion of plasma without any pretreatment.

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