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SOLID-PHASE EXTRACTION OF CHLORAMPHENICOL WITH GRAPHITIZED CARBON BLACK

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SUMMARY

The sorption and desorption properties of graphitized carbon black (GCB) were evaluated for enrichment of highly polarizable chloramphenicol (CP) from biological fluids. The distribution of CP between GCB and water lay strongly toward GCB. Among the organic solvents examined, methanol gave the lowest adsorption coefficient. The optimum packing amount of GCB, and the optimum volume and flow-rate of the eluent were deduced from the breakthrough curve and methanol elution curve, respectively. The inner diameter of GCB extraction columns was chosen from the sorption efficiency curve obtained by plotting CP recovery against column inner diameter. The solid-phase extraction method using GCB was applied to the assay of CP in serum samples in the concentration range of 5 to 50 μg/ml.

INTRODUCTION

Chloramphenicol (CP) is an effective broad spectrum antibiotic with activity against a host of aerobic and anaerobic Gram-positive and Gram-negative bacterias. CP is the drug of choice in infants and children for treating meningitis since it is effective against ampicillin-resistant *Haemophilus influenzae*[1].

Because of a marked interpatient variation in pharmacokinetics of CP, quick and accurate determination of serum concentrations for each individual is necessary to avoid concentration-dependent toxicity during therapy. CP is also widely used for the treatment of mastitis in cattle. It is gradually eliminated in milk after intramammary administration and the control of its residues in milk is thus a continuing problem[2].

The high-performance liquid chromatographic (HPLC) methods have been well developed for the assay of CP in biological fluids[3–10]. These HPLC methods were reported to be superior to other conventional methods in speed, precision and ease of performance. They are ideally suitable for CP quantitation in pediatric patients due to the very small sample required.

Prior to HPLC assay, either solvent extraction[2–5] or deproteinization[6–10] pro-

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cedures have been applied for the trace enrichment or purification of CP from complex biological samples. Precipitation of plasma proteins followed by direct injection of the supernatant will affect column life adversely due to the build-up of solids on the HPLC column. Besides this, CP, which is about 50% bound to plasma proteins, is likely to precipitate along with proteins when using a very efficient precipitant[8], or entrapped by the precipitated protein disk.

In recent years, liquid–liquid partitioning extraction generally has been replaced by liquid–solid adsorbing extraction, namely solid-phase extraction, in routine analyses of drugs and their metabolites in biomedical fields.

Among the commonly used hydrophobic solid sorbents, graphitized carbon black (GCB) possesses characteristics of an ideal sorbent for the trace enrichment of highly polarizable aromatic compounds[11]. GCB is a chemically and thermally stable inorganic sorbent with a homogeneous and non-polar surface consisting of graphite basal hexagonal planes resembling condensed aromatic rings. Aromatic compounds are thus more strongly adsorbed than aliphatic compounds[12]. Retention on the GCB surface is, however, solely through dispersion forces because of its non-polar nature. Consequently, retentions are not a function of dipole moments of compounds, but are dependent more on the molecular size, molar volume and polarizability[13].

GCB has gained widespread acceptance for the extraction of trace amounts of organic pollutants such as polycyclic aromatic hydrocarbons[14–16], chlorinated pesticides[11,17–19] and phenolic compounds[13,20,21]. It has also been used for extracting some metabolites or drugs from biological samples[22–25]. However, this effective sorbent has not been extensively used in the biomedical fields, even though most of biologically active drugs are polar aromatic compounds containing polarizable groups.

CP is unique among natural compounds in that it contains very polarizable nitro, phenyl and dichloro groups. The presence of these highly polarizable groups will enhance the ability of CP to associate with the GCB surface, leading to the strong retention by GCB.

We investigated the sorption and desorption properties of GCB for CP as a sorbent to optimize the solid-phase extraction procedure, suitable for handling microvolumes of blood samples from pediatric patients.

EXPERIMENTAL

Materials and reagents

Carbopak B (60–80 mesh) served as the GCB was purchased from Supelco (Bellefonte, PA, U.S.A.). GCB was sequentially cleaned with methanol, acetone, dichloromethane and petroleum ether, followed by drying at 200°C under vacuum for 3 h before use. CP and ethenzamide (EA) were obtained from Kookje Pharmaceutical (Seoul, Korea) and Ilyang Pharmaceutical (Seoul, Korea), respectively. Methanol, dichloromethane and acetonitrile were of HPLC grade purchased from Kokusan (Japan). Water was deionized and double distilled before use. All other reagents were of reagent grade.

Standard solutions and serum samples

CP stock solution wass prepared by dissolving CP in water at a concentration

of 1 mg/ml. EA was dissolved in methanol at a concentration of 1 mg/ml to be served as the internal standard solution. Spiked serum samples containing CP in the range of 5.0 to 50 μg/ml were prepared by adding aliquots of CP stock solution to serum.

HPLC analysis

A Waters Assoc. Model ALC 200 HPLC apparatus was equipped with a Model 440 absorbance detector, a Model 6000A solvent delivery system, a Model U6K universal injector and a Model 730 data module (Milford, MA, U.S.A.). A μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D.) was used. Methanol–water (40:60, v/v) was used as the mobile phase at a flow-rate of 1.0 ml/min. The sample size was 10 μl and the detection was made at 280 nm. All quantitative calculations were made by measuring the peak heights of CP relative to those of EA and comparing them with those of the standard curve.

Determination of static adsorption coefficients in GCB–liquid systems

We selected as the liquid systems water, acetonitrile, methanol and methanol modified with dichloromethane. Constant amounts of CP (250 μg) and GCB (50 mg) were added to a constant volume of each liquid (5 ml). Each suspension was shaken for 8 h at 25 ± 1°C to reach the adsorption equilibrium according to the procedure of Laganà and co-workers[13,15]. Each liquid was then filtered through a 0.45-μm Millipore sample clarification filter (Bedford, MA, U.S.A.). An aliquot of internal standard solution (10 μl) was added into each filtrate (1 ml) and analyzed by HPLC for the CP quantitation. Static adsorption coefficients, K_D, were calculated as follows:

$$K_D = \frac{\mu g \text{ of CP adsorbed by GCB per g of GCB}}{\mu g \text{ of CP in liquid per ml of liquid}}$$

Breakthrough studies

To perform breakthrough studies of CP in on-line mode, we prepared a GCB column by packing a Valco guard column (5 cm × 4.6 mm I.D.) with 400 mg of GCB. The HPLC column was replaced by this GCB column between injector and detector in the HPLC system.

To evaluate the influence of methanol flow-rate on CP recovery, aliquots of either 5, 10, or 15 μl of CP stock solutioon were injected into the GCB column. Following each injection, methanol was pumped through at a flow-rate of 0.5, 1.0, or 3.0 ml/min, and each effluent was directly monitored for CP.

Aliquots of either 5, 10, or 25 μl of CP stock solution were injected into the GCB column, and 150 ml of water as the mobile phase was then pumped through at a flow-rate of 5 ml/min prior to elution with methanol at 0.5 ml/min. The effluents were continuously monitored for CP breakthrough. The plate numbers of the eluted peaks before and after the water passage were determined.

Test for the optimal inner diameter of GCB extraction column

GCB extraction columns were constructed with Pyrex glass tubing with the inner diameters of 2, 5 or 7 mm. A constant amount of GCB (30 mg) was packed into each of the extraction columns and both ends secured with small plugs of silanized glass wool. Each was then washed with methanol and equilibrated with water

before use. A constant volume of water (5 ml) containing 50 μg of CP was passed through each of the extraction columns at a flow-rate of 2 ml/min and each filtrate was collected. After the traces of water were removed by vacuum (water pump) CP was eluted with methanol by applying positive nitrogen pressure to keep the elution rate at about 0.5 ml/min. The internal standard solution (10 μl) was added to the first 1 ml of the methanol eluate. Both filtrate and eluate were analyzed by HPLC for CP measurement.

Test for the efficiency of GCB extraction procedure

GCB extraction columns were prepared by packing columns of 2 mm I.D. with an amount of 50 mg of GCB. They were then washed with methanol, followed by equilibration with water. An aliquot of 100 μl of each serum sample spiked with CP in the range of 5.0 to 50 μg/ml was loaded onto the GCB extraction column. The column was washed with 3 ml of water to remove water soluble components. After removing the traces of water, it was washed with 3 ml of petroleum ether to remove fat-soluble interfering compounds. CP was then eluted with 600 μl of methanol. The internal standard solution (10 μl) was added to the methanol eluate (500 μl), followed by the HPLC analysis for CP quantitation.

RESULTS AND DISCUSSION

The adsorption coefficients, K_D of CP between GCB and water, and between GCB and organic solvents were measured in static sorption mode (Table I). The distribution of CP in water lay strongly toward GCB at 50 μg/ml which is higher than the normal toxic level in blood. This high K_D in water demonstrates that the graphite-like surface of GCB has a high affinity for CP molecules which are aromatic compounds carrying polar functional groups. Due to its reversed-phase property, for GCB, solvents such as dichloromethane, alkanes and benzene are stronger than methanol[12]. However, methanol gave the lowest adsorption coefficient, indicating CP completely favored methanol because of its enhanced solubility in methanol.

The static capacity of GCB for CP is estimated to be higher than 5 μg of CP per mg of GCB. The dynamic capacity of GCB might be, however, lower than this value. This is because sorption efficiency is apparently impaired during the dynamic sorption of CP from the migrating aqueous samples. Therefore, the amount of GCB was increased when performing the breakthrough studies.

TABLE I

STATIC ADSORPTION COEFFICIENTS IN GCB–LIQUID SYSTEMS

Conditions: GCB, 50 mg; liquid, 5 ml; CP, 250 μg. Shaken for 8 h at room temperature; $n = 2$.

Liquid system	*Adsorption coefficient*
Methanol	$\leqslant 0.001$
Acetonitrile	0.021
Methanol–dichloromethane (90:10, v/v)	0.029
Methanol–dichloromethane (80:20, v/v)	0.004
Methanol–dichloromethane (70:30, v/v)	0.075
Water	$\geqslant 1000$

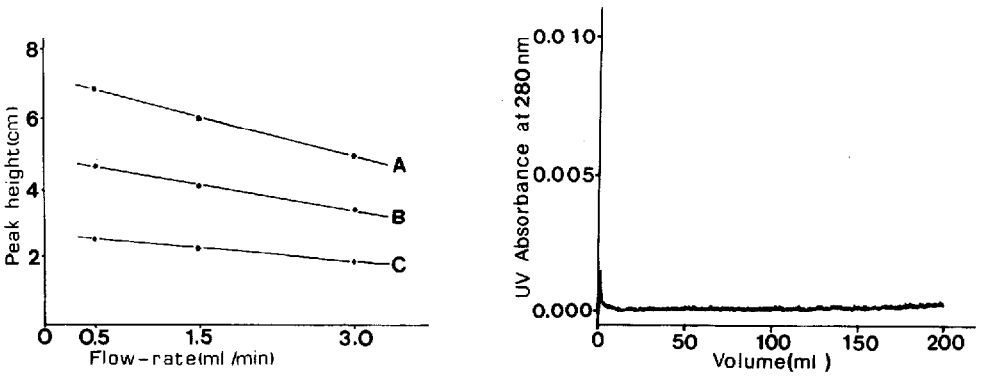

Fig. 1. Effect of methanol flow-rate on recovery of CP. (A) 15 μg, (B) 10 μg and (C) 5 μg.

Fig. 2. Breakthrough plot, 25 μg of CP on 400 mg of GCB.

As seen in Fig. 1, the recovery of CP from GCB exhibited a high dependence on the methanol flow-rate. This suggests that the mass transfer becomes slower and incomplete as the flow-rate is increased. A longer residence time is thus required for methanol during the elution step to ensure quantitative recovery of CP.

No breakthrough was observed even after 150 ml of water passed through after loading 25 μg of CP onto the GCB column (Fig. 2) This verifies that CP will be quantitatively retained on GCB from a volume of water that is sufficient to wash off water-soluble interfering components in serum. Breakthrough volume of 25 μg of CP seemed to be higher than 150 ml for 400 mg of GCB.

After the breakthrough plots, methanol was pumped through the GCB column to elute the adsorbed CP. The eluted peaks were of gaussian shape with little tailing (Fig. 3). They exhibited about 80% of the total plate numbers of the CP peaks of the direct methanol elution. This indicates that CP was strongly retained on the top

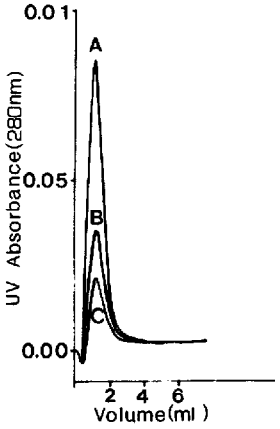

Fig. 3. Methanol elution curves of CP. (A) 25 μg, (B) 10 μg and (C) 5 μg.

TABLE II

EFFECT OF COLUMN INNER DIAMETER ON THE DYNAMIC ADSORPTIVITY OF GCB

Conditions: sample, 5 ml of water containing 50 μg of CP; GCB, 30 mg; $n = 5$.

Column I.D. (mm)	*Amount found (μg)*			*Recovery (%)*
	Filtrate	*Methanol eluate (mean ± S.D.)*	*R.S.D. (%)*	
2	–*	46.6 ± 0.6	1.29	93.2
5	3.5	42.1 ± 1.5	3.56	84.2
7	8.9	38.5 ± 1.4	3.64	77.0

* Not detectable.

of the GCB column as a narrow band and band broadening due to diffusion during the water passage was negligible. Methanol (4 ml) seems to be sufficient for the quantitative elution of CP from 400 mg of GCB. As the column I.D. increases for a fixed amount of sorbent, the packing density tends to be less uniform, thus multiple path effect and wall effect are likely to be enhanced during the sorption and desorption steps.

The influence of the geometry of extraction columns on the sorption of CP was evaluated by varying the column I.D. As expected, the sorption efficiency and overall precision had very high I.D. dependence (Table II).

Fig. 4 shows a typical chromatogram obtained from human serum spiked with CP at 40 μg/ml. The very clean background shows that the present solid-phase extraction using GCB is very effective in removing interfering components from serum.

A series of six serum samples spiked with CP in the range of 5.0–50.0 μg/ml was assayed. Recovery of CP was quantitative with a mean value of 90.8 ± 4.2% over the concentration range studied (Table III). The calibration plot of peak height ratios against concentrations of CP in serum samples was linear with a correlation coefficient of 0.9966.

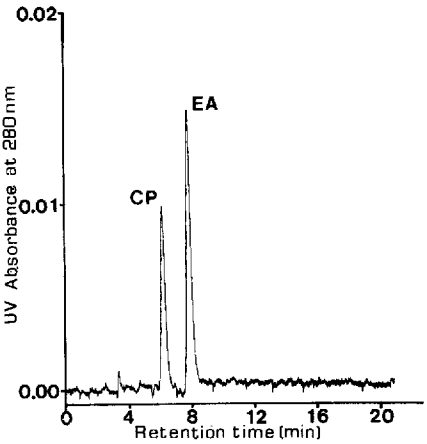

Fig. 4. Typical chromatogram of serum spiked with CP (40 μg/ml).

TABLE III

RECOVERIES OF KNOWN AMOUNTS OF CHLORAMPHENICOL ADDED TO HUMAN SERUM

Conditions: serum, 100 μl; GCB, 50 mg packed into 2-mm I.D. column; methanol eluate, 500 μl.

Amount added (μg/ml)	*Amount found (μg/ml)*		*Recovery (%)*
	Mean ± S.D.	*R.S.D. (%)*	
5.0	4.6 ± 0.2	4.3	92.4
10.0	9.3 ± 0.5	5.4	93.3
20.0	17.8 ± 0.9	5.0	89.2
30.0	27.3 ± 0.7	2.6	90.9
40.0	36.0 ± 1.8	5.0	89.9
50.0	44.7 ± 2.4	5.4	89.4

In conclusion, the present work proved that GCB is an ideal sorbent for the solid-phase extraction of CP from microvolumes of biological samples without laborious sample manipulations. The usefulness of the present method can be further extended by incorporating a GCB precolumn into the HPLC system for on-line sample clean-up and enrichment.

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