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Preparation and evaluation of octadecyl- or phenylpropyl-treated porous glass for the high-performance liquid chromatographic analysis of bufadienolides in *Bufonis venenum*

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ABSTRACT

The retention and selectivity of bufadienolides (cinobufagin, resibufogenin, bufalin) in *Bufonis venenum* were studied using high-performance liquid chromatography on octadecyl- or phenylpropyl-treated porous glass. From elemental analysis data for carbon, the maximum number of bonded octadecyl or phenylpropyl surface groups per 100 Å^2 of glass (mean pore diameter 157 Å, specific surface area 213 m^2/g) in octadecyl or phenylpropyl gel was calculated to be 2.15 and 1.97, respectively. Using acetonitrile–water mixtures as the eluent, bufadienolides were separated on octadecyl or phenylpropyl gels, but with different degrees of resolution.

INTRODUCTION

At present, chemically bonded stationary phases are used most widely as column-packing materials for reversed-phase high-performance liquid chromatography (HPLC). These materials consist of organic functional groups, such as octadecyl, octyl, ethyl and phenyl groups, bonded to silicas. In previous papers [1–4], I have suggested that the features of silicas, which are important in determining the number of accessible alkylamino or phenyl groups per 100 Å^2 are the pore diameter and the specific surface area. The identification of drugs and determination of their concentration, especially in oriental formulations and for forensic science purposes, require several types of column gels for HPLC and several types of column gels for gas chromatography. This paper therefore considers how the chromatographic properties of octadecyl- or phenylpropyl-modified glass gels make them, like chemically modified silica gels, important HPLC or micro-HPLC columns gels. However, there have been few reports of HPLC analyses on octadecyl- or phenylpropyl-treated glass columns in physical and chemical research [5–9]. Therefore, the analysis of bufadienolides in *Bufonis venenum* (toad cake) using HPLC was compared with previous high-performance thin-layer chromatographic (HPTLC) analysis [10].

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EXPERIMENTAL

Reagents

Octadecyldimethylchlorosilane (ODS) and phenylpropyldimethylchlorosilane (PHP) were obtained from Petrach Systems (Bristol, PA, U.S.A.). Uracil, benzene, naphthalene, diphenyl and anthracene were obtained from Wako (Osaka, Japan). Porous glass and porous silicas differing in their mean particle size, mean pore diameter, specific surface area and pore volume (Table I) were purchased from Fuji-Davison (Nagoya, Aichi, Japan). Cinobufagin, resibufogenin and bufalin were provided by Kampo Labs., Kanebo Co. (Osaka, Japan). The other reagents and organic solvents were of analytical reagent grade.

Apparatus

The HPLC measurements were carried out on a Twincle instrument (Jasco, Tokyo, Japan), equipped with a Uvidec-100IV variable-wavelength detector (Jasco, Tokyo, Japan) and a column of 150 × 4.6 mm I.D., packed with ODS- or PHP-treated glass (or silica).

Stationary phase and elemental analysis

As described previously [1–4], 7-g samples of dried glass were added to 70 ml of a 3.4% solution of ODS (or PHP) in dry toluene containing 3 ml of triethylamine. The glass or silica suspension was refluxed for 5 h, filtered through a glass filter (1 μm), washed several times with toluene, chloroform, methanol and acetone, and then dried *in vacuo* at 70°C for 2 days. The final products are listed in Table II as glass-

TABLE I

CHARACTERISTICS OF ORIGINAL GLASSES AND SILICAS

Sample[a]	Means particle size (μm)	Mean pore diameter (Å)	Specific surface area (m^2/g)	Pore volume (ml/g)
Glasses	5.2	157	213	1.48
Silicas	5.5	131	330	1.08

[a] These names were assigned for convenience and are not commercial names.

TABLE II

CHARACTERISTICS OF TREATED GLASSES AND SILICAS

Treated gel	Specific surface area (m^2/g)	Carbon content (%)	Average pore diameter (Å)	Pore volume (ml/g)	Number of surface groups per gram ($\times 10^{21}$)	Number of surface groups per 100 $Å^2$
Glass-ODS	133	13.8	152	0.97	0.457	2.15
Glass-PHP	164	8.2	155	1.16	0.419	1.97
Silica-ODS	228	19.7	122	0.70	0.456	2.00
Silica-PHP	244	12.5	120	0.73	0.505	2.07

ODS, glass-PHP, silica-ODS and silica-PHP. The characteristics of these materials are also given in Table II. The carbon contents of the treated glass or silicas were determined by elemental analysis using an MT-3 CHN elemental analyser (Yanagimoto, Kyoto, Japan). The specific surface areas, mean pore diameters and pore volumes of the column glass and column silicas were determined with an MOD-220 porosimeter (Carlo Erba, Milan, Italy) and SA-1000 surface-area, pore-volume analyser (Shibata, Tokyo, Japan), and the data are shown in Table II.

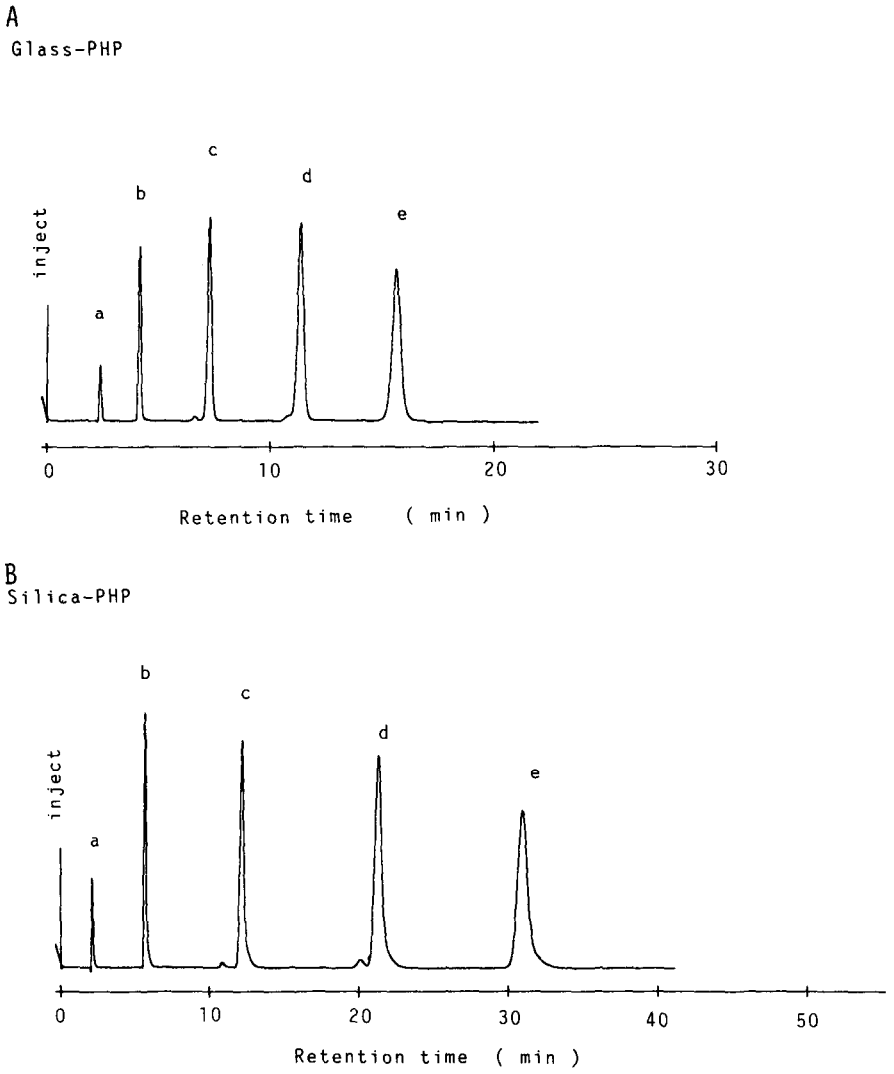


Fig. 1. Typical liquid chromatograms obtained with some aromatic hydrocarbons on glass-PHP or silica-PHP. (A) Glass-PHP: phenylpropyl-treated porous glass. (B) Silica-PHP: phenylpropyl-treated porous silica. Mobile phase: methanol–distilled water (60:40). Flow-rate: 1 ml/min. Detection: 254 nm UV. Peaks: a = uracil; b = benzene; c = naphthalene, d = diphenyl, e = anthracene.

Column preparation

The column glass or column silicas were packed into the stainless-steel column (150 × 4.6 I.D.) using the slurry technique.

Sample preparation

As previously described [10], 100 mg of *Bufonis venenum* were extracted four times with 10 ml of acetonitrile–distilled water (1:1). The mixture was warmed in a hot water bath at 50°C for 15 min, and was mixed ultrasonically (45 W, 38 kHz, 10 min). After centrifugation (1500 *g*, 5 min), the upper phase was filtered through a membrane filter (0.22 μm). The upper phase was made up to volume with acetonitrile–distilled water (1:1) in a 50-ml volumetric flask, then an aliquot of the upper phase was injected onto the HPLC system.

RESULTS AND DISCUSSION

From the elemental analysis of the treated glass, the number of accessible ODS or PHP surface groups per 100 Å^2 of glass can be calculated as previously described [1–4]. The results are given in Table II. Fig. 1 shows typical liquid chromatograms obtained with some aromatic hydrocarbons on glass-PHP or silica-PHP.

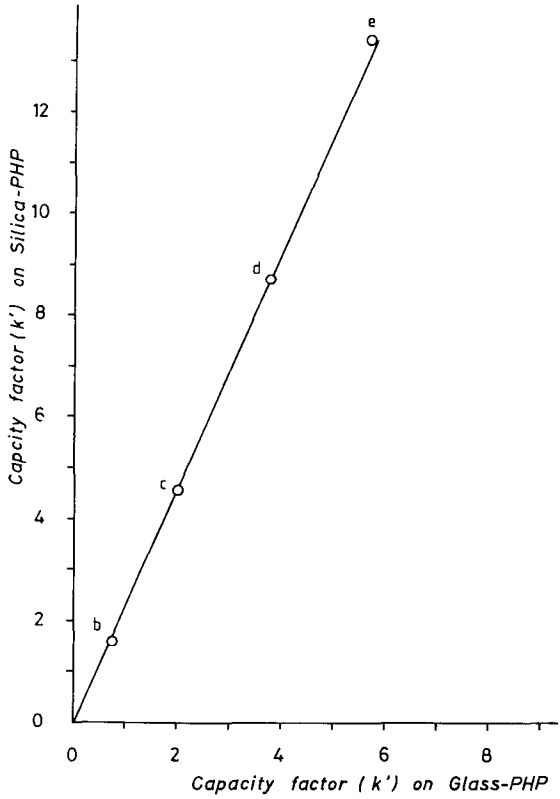


Fig. 2. Correlations between the capacity factors (k') of some aromatic hydrocarbons on glass-PHP *versus* silica-PHP. Chromatographic conditions as in Fig. 1. Points: b = benzene; c = naphthalene; d = diphenyl, e = anthracene.

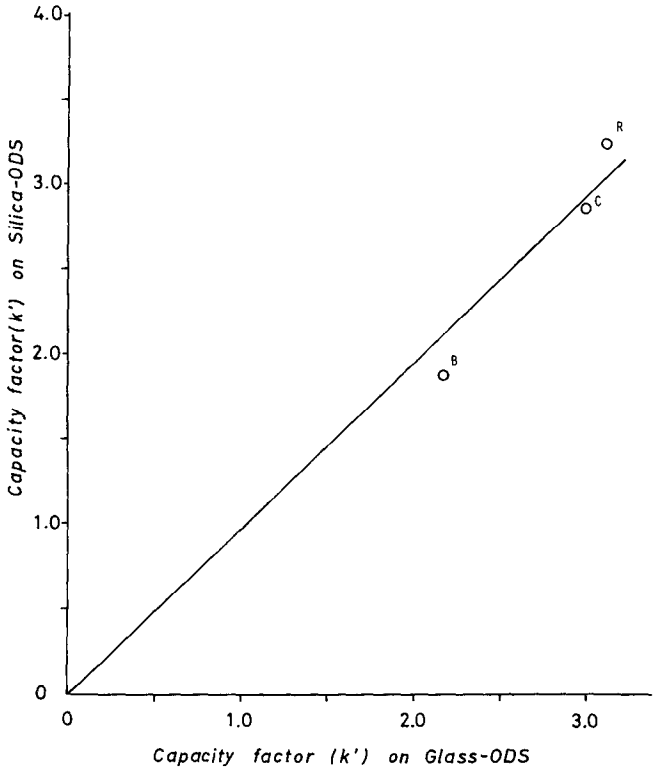


Fig. 3. Correlations between the capacity factors (k') of bufadienolides on glass-ODS *versus* silica-ODS. Mobile phase: acetonitrile–distilled water (50:50). Detection: 300 nm UV. Flow-rate: 1 ml/min. Points: B = bufalin; C = cinobufagin; R = resibufogenin.

Fig. 2. shows the correlations between the capacity factors (k') of some aromatic hydrocarbons on glass-PHP *versus* silica-PHP.

Figs. 3–5 show the correlations between the capacity factors (k') of bufadienolides of glass-ODS *versus* silica-ODS, glass-ODS *versus* glass-PHP and glass-PHP *versus* silica-PHP. The curves of capacity factors (k') obtained with the glass-ODS, glass-PHP series provide similar resolution and retention in Figs. 3–5.

Typical calibration graphs of peak area *versus* amount of bufalin, resibufogenin and cinobufagin are linear in the range 0.10–0.60 μg, 0.10–1.10 μg and 0.14–1.40 μg respectively. The extraction of bufalin, resibufogenin and cinobafagin from commercial *Bufonis venenum* was substantially complete within the 5–30, 5–55 and 7–70 μg/ml ranges, and the reproducibility of the assays was good down to a concentration of 5 μg/ml bufalin, 5 μg/ml resibufogenin and 7 μg/ml cinobufagin. The mean recovery from five samples containing 5–30 μg of bufalin, 5–55 μg of resibufogenin and 7–70 μg of cinobufagin per ml were 96.4, 97.4 and 98.3% [coefficients of variation (C.V.) 3.37, 3.57 and 1.87%], respectively.

Figs. 6 and 7 show typical liquid chromatograms obtained with bufadienolides from commercial *Bufonis venenum* on glass-ODS or silica-ODS. The resolution value

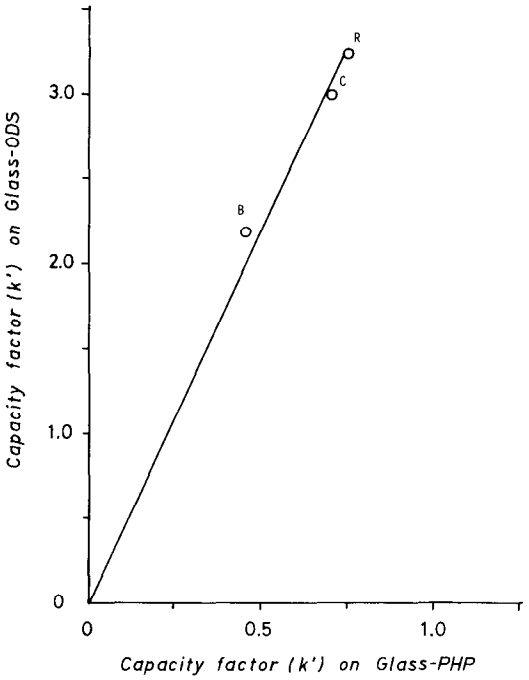


Fig. 4. Correlations between the capacity factors (k') of bufadienolides on glass-ODS *versus* glass-PHP. Conditions as in Fig. 3.

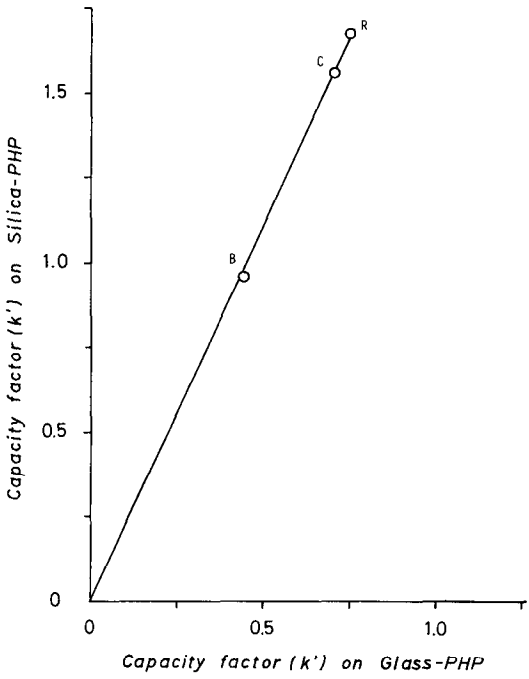
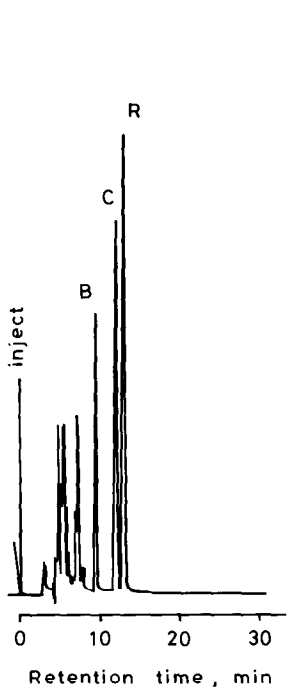


Fig. 5. Correlations between the capacity factors (k') of bufadienolides on glass-PHP *versus* silica-PHP. Conditions as in Fig. 3.



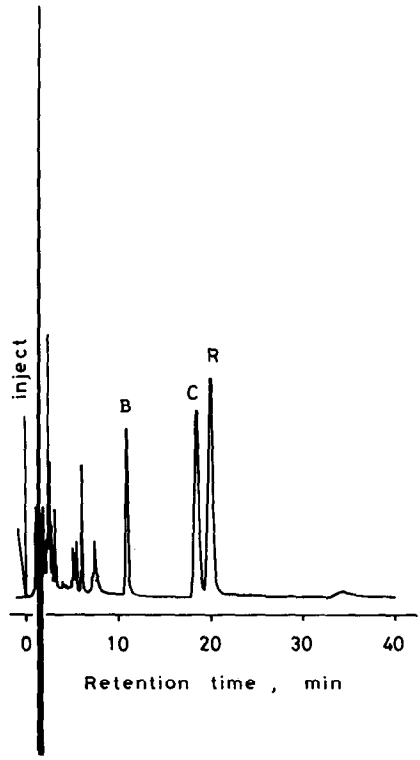


Fig. 6. Typical liquid chromatogram obtained with bufadienolides in commercial *Bufonis venenum* on glass-ODS. Column: 400 × 4.6 mm I.D. Peaks: B = bufalin; C = cinobufagin; R = resibufogenin. Other chromatographic conditions as in Fig. 3.

Fig. 7. Typical liquid chromatogram obtained with bufadienolides in commercial *Bufonis venenum* on silica-ODS. Column: 150 × 4.6 mm I.D. Other chromatographic conditions and peaks as in Fig. 6.

(R_s) of resibufogenin *versus* cinobufagin on glass-ODS and silica-ODS was 1.38 and 1.33 with the HPLC conditions in Figs. 6 and 7. Table III shows the bufalin, resibufogenin and cinobufagin contents in commercial *Bufonis venenum*.

TABLE III

BUFADIENOLIDES CONTENTS IN COMMERCIAL *BUFONIS VENENUM*
$n = 7$.

Sample	Bufadienolides (%)			Recovery (%)	C.V. (%)
	Bufalin	Cinobufagin	Resibufogenin		
Sample 1	2.08	5.20	6.52	96.1–98.3	2.05
Sample 2	1.84	4.51	3.02	94.7–98.7	2.33
Sample 3	1.06	1.83	2.85	93.5–97.3	2.34
Sample 4	2.30	5.80	2.78	95.3–98.6	2.16
Sample 5	1.51	2.80	4.80	96.5–98.8	2.07

These results also show that bufadienolides and some aromatic hydrocarbons can be separated on column glass treated with ODS or PHP groups using either an acetonitrile–distilled water or a methanol–distilled water mixture as the eluent.

It is concluded from the present investigation that it is not sufficient to evaluate column gels solely on the basis of the carbon content of chemically bonded reversed-phase materials. The pore size distribution of the glass supports, the bulkiness of the ligands bonded to the glass and the molecular size of the solute must also be considered.

On the other hand, in the analysis of bufadienolides from *Bufonis venenum*, the sensitivity of this HPLC method was three times that of previous HPTLC method [10].

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