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Void volume markers in reversed-phase and biomimetic liquid chromatography

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ABSTRACT

A comparative investigation of 15 void volume marker candidates was carried out on two reversed-phase (RP), two immobilized artificial membrane (IAM) and two immobilized protein columns, namely Human Serum Albumin (HSA) and α_1-acid-glycoprotein (AGP), using different mobile phases in respect to pH and buffer composition. Pycnometric analysis of the 6 chromatographic columns was also employed for reasons of comparison. The results revealed the possible overestimation of stationary phase void volume if a general void volume marker is used under different chromatographic conditions. In particular, overestimation of the investigated columns' void volumes was a common phenomenon when using acidic eluents or High Purity Water (HPW) as mobile phase. For this purpose, a classification of the recommended chemicals according to the column type or the pH of the mobile phase is suggested for the accurate determination of retention factors.

1. Introduction

Void volume in liquid chromatography is recognized as the total volume of the mobile phase in the column [1]. This parameter is crucial for an accurate determination of the retention factor (k) of a chemical, according to the formula:

$$k = \frac{t_r - t_0}{t_0} \quad (1)$$

where t_r and t_0 are the retention times for the investigated and an unretained compound, respectively. Retention factors have been widely used for system suitability issues, for theoretical descriptions of both small and large molecules and determination of thermodynamic parameters associated with chromatographic retention as well as the estimation of partitioning processes associated with drug design and fate of chemicals [2,3]. In particular, reversed-phase liquid chromatography has been widely used to reproduce octanol–water partition coefficients as a measure of lipophilicity [4–6]. The most popular practice in determining the void volume of a column is the use of unretained chemicals, such as inorganic salts and non-ionizable small molecules [2,3]. However, there is no truly chromatographic unretained chemical in practice [2,3]. Any physical adsorption or repulsion of the probe marker will cause an overestimation or underestimation in the accessible void volume [3].

As void volume markers in reversed-phase chromatography, small organic molecules, such as acetone, uracil, urea and thiourea have been suggested [2,3]. However, most of the small molecules proved to have elution times that increased linearly with decreasing mobile phase strength. This indicates that retention is occurring and the corresponding volume yielded is greater than the "true" void volume [2]. Many authors consider salts to be the best choice as void volume markers because of the simplicity of use and ease of detection [2]. Among them, inorganic salts, especially $NaNO_3$ and KBr, have been the most popular [2,3]. However, one of the greatest limitations in using such salts is the possibility of the exclusion of charged species from the pores of the stationary phase due to the presence of residual silanol groups (Donnan effect) [2]. The problem may be partially faced when using stationary phases with reduced silanol sites, such as Discovery end-capped columns or polar embedded RP-Amide stationary phases, such as the ABZ$^+$ column with improved behaviour towards acids, bases and zwitterions.

Biomimetic stationary phases are currently available. Immobilized artificial membrane (IAM) columns are consisted on phospholipids chemically bonded on HPLC grade silica skeleton. Such columns mimic the lipid environment of a fluid membrane and they are widely employed for the assessment of drug-membrane interactions [4] and/ or the estimation of permeability

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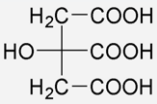
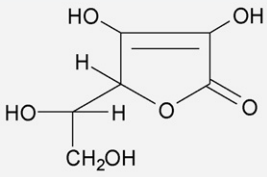
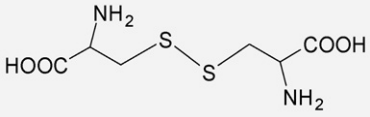
Table 1
Chemical structures and pK_a values of formic, acetic, oxalic, citric and ascorbic acid and L-Cystine.

Analyte	Structure	pK_a values	Reference
Formic acid	HCOOH	pK_{a1} = 3.75	[22]
Acetic acid	CH_3COOH	pK_{a1} = 4.75	[23]
Oxalic acid	HOOC–COOH	pK_{a1} = 1.27 pK_{a2} = 4.82	[24]
Citric acid		pK_{a1} = 3.13 pK_{a2} = 4.76 pK_{a3} = 6.40	[25]
Ascorbic acid		pK_{a1} = 4.04 pK_{a2} = 11.34	[26]
L-Cystine		pK_{a1} = 1.51 pK_{a2} = 2.79 pK_{a3} = 8.25 pK_{a4} = 8.97	[27]

through cell membranes [7,8]. For this type of stationary phases, citric acid is recommended by the manufacturer as a void volume marker [9]. A recent publication [3] stated substantial overestimation of an IAM.DD2 column dead volume in acidic conditions using small organic acids and inorganic salts as void volume markers.

Another type of biomimetic liquid chromatography uses stationary phases containing plasma proteins, such as human serum albumin (HSA) and α-acid glycoprotein (AGP), immobilized by attachment to silica support materials [4]. Retention factors on HSA and AGP columns may be suitable for the estimation of the binding affinity and the percentage of a drug bound to serum proteins in human blood [4]. In recent studies water and $NaNO_3$ have been used as void volume markers for both HSA [10,11] and AGP [11,12] columns.

In the present work, a comprehensive comparative study of 15 void volume marker candidates, namely High Purity Water (HPW), HCOOH, CH_3COONa, KBr, KI, KIO_3, $LiClO_4$, $NaNO_3$, $(COONa)_2$, $(COONH_4)_2$, $KMnO_4$, $K_2Cr_2O_7$, sodium citrate, ascorbic acid and L-Cystine was carried out using two RP (Discovery and ABZ) and two IAM (DD2 and MG) columns, as well as a HSA and a AGP stationary phase under different chromatographic conditions. The effect of both buffer composition and pH of the mobile phase to the consistency of the 15 void volume marker candidates was studied. As reference values, pycnometrically determined void volumes of the 6 columns were considered. The results presented and discussed provide some practical guidelines for the use of void volume markers especially in biomimetic chromatography.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. For the preparation of buffers, KCl (≥99.5%, Riedel-de Haen), KH_2PO_4 (≥99.5%, Merck), Na_2HPO_4 (≥99.0%, Sigma–Aldrich), NaCl (≥99.5%, Sigma–Aldrich), morpholine-propanesulfonic acid (MOPS) (≥98%, Merck) and CH_3COONH_4 (≥97%, Panreak) were used. As void volume markers, $CH_3COONa{\cdot}3H_2O$ (≥99%, Panreak), $NaNO_3$ (≥99.5%, Carlo-Erba), sodium citrate·$2H_2O$ (≥99.0%, Sigma–Aldrich), KIO_3 (≥99.9%, Carlo-Erba), $(COONH_4)_2{\cdot}H_2O$ (≥99.5%, Riedel-de Haen), $(COONa)_2$ (≥99.5%, Riedel-de Haen), KBr (Riedel-de Haen), KI (≥99.0%, Sigma–Aldrich), L-Cystine (≥99%, Fluka Biochemika), HCOOH (85%, Carlo-Erba), $KMnO_4$ (≥99%, Merck) were used. High Purity Water (HPW) was obtained by means of an EASYpure II (Barnstead International, USA) water purification system. Acetonitrile (Merck), used for pycnometric determination of columns void volume was of HPLC grade.

The chemical formulae of formic acid, acetic acid, oxalic acid, citric acid, ascorbic acid and L-Cystine along with their pK_a values are presented in Table 1.

2.2. Chromatographic conditions

The liquid chromatographic system consisted of a Knauer K-1001 HPLC gradient pump with a K-1500 degasser and solvent organizer (Model Wellchrom) and a Knauer K-2501 UV–Vis detector (180–800 nm). The flow rate was adjusted at 1 ml min^{-1} and an injection loop of 50 μl was used. A 20 μl loop was also employed for reasons of comparison, in particular for the short columns (IAM.PC.DD2, HSA and AGP). No differences in the elution volumes were obtained. All void volume markers were detected at 205 nm. Chromatographic data were processed with the Eurochrom 2000 Version 2.05 software. As stationary phases, the following columns were used:

(A) Discovery end-capped BIO Wide Pore RP-18 (Supelco, USA, 150 mm × 4.6 mm I.D., 3 μm particle size).
(B) Supelcosil ABZ+ plus (Supelco, USA, 150 mm × 4.6 mm I.D., 5 μm particle size).
(C) Rexchrom IAM.PC.DD2 Drug-Discovery (Regis Technologies, Inc., USA, 30 mm × 4.6 mm, 12 μm particle size).
(D) Rexchrom IAM.PC.MG Drug-Discovery (Regis Technologies, Inc., USA, 150 mm × 4.6 mm, 10 μm particle size).
(E) ChromTech Chiral HSA (Supelco, USA, 50 mm × 4.0 mm, 5 μm particle size).
(F) ChromTech Chiral-AGP (Supelco, USA, 50 mm × 4.0 mm, 5 μm particle size).

The mobile phases tested on each stationary phase are presented in Table 2. All experiments were carried out under constant temperature (25 °C).