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Separation of anionic and cationic compounds of biomedical interest by high-performance liquid chromatography on porous graphitic carbon

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ABSTRACT

The separation of small, ionizable compounds of biomedical interest on porous graphitic carbon is described. The retention of anionic compounds is dominated by electronic interaction between the solute and the delocalized electron clouds on the graphitized carbon, while cationic compounds are mainly retained by reversed-phase interaction with the hydrophobic carbon surface. Anionic and cationic compounds can be separated simultaneously with a mobile phase containing an electronic modifier (*e.g.*, trifluoroacetic acid) and an organic modifier (*e.g.*, acetonitrile) for elution. Examples of applications include the measurement of oxalic acid in urine, the determination of creatine and creatinine in urine and in serum, the separation of basic drugs (remoxipride and FLA 981) and the simultaneous analysis of pertechnetate anion and the cationic technetium–amine complexes.

INTRODUCTION

Porous graphitic carbon (PGC) is a new column packing available recently for high-performance liquid chromatography (HPLC)[1–3]. It was considered a "pure" reversed-phase material because it contains no unreacted silanols but only hydrophobic carbon surfaces. It is stable to strong acids and alkalis and therefore eluents at any pH can be used. In addition to the extremely hydrophobic nature, PGC is also unique in possessing a conduction band of delocalized electrons. Thus, retention on PGC is usually a mixture of hydrophobic and electronic interactions. The study by Bassler *et al.*[4] on the retention behaviour of 24 substituted aromatic compounds under non-polar solvent conditions has concluded that direct electronic interactions can arise from both the mixing of the HOMO and LUMO orbitals of the solute with the electronic distribution of PGC or from charge transfer between the solute and PGC. Using the separation of TcO_4^- and ReO_4^- as an example, Lim[5] has demonstrated that electronic-interaction chromatography (EIC), based totally on electronic interaction, is also possible.

The aim of the present study is to exploit further the extreme hydrophobicity and

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the electronic properties of PGC for the separation of small anionic and cationic compounds of biomedical interest, particularly compounds which are difficult or impossible to retain on ODS silicas.

EXPERIMENTAL

Materials and reagents

Creatine, creatinine, ethylenediamine, stannous tartrate and oxalic acid were from Sigma (Poole, U.K.). Trifluoroacetic acid (TFA), hydrochloric acid and sodium chloride were AnalaR grade from BDH (Poole, U.K.). 1,5,8,12-Tetraazadodecane was from Aldrich (Gillingham, U.K.). Remoxipride and FLA 981 were gifts (to Dr. N. Veall) from Astra Läkemedel (Södertälje, Sweden). Acetonitrile was of HPLC grade from Rathburn (Walker, U.K.).

Preparation of technetium–amine complexes

The method of Emery and Lim[6] was used. Sodium pertechnetate was eluted from an Ultratechnekow FM generator (Malinckrodt Diagnostia, The Netherlands). The eluate was diluted with 0.9% sodium chloride to give a working solution of 0.2–1 mCi/ml. A saturated solution of stannous tartrate (as the reductant) was made by adding the solid to nitrogen-purged 0.01 *M* hydrochloric acid. The solution was filtered through a Millex GS 0.22-μm filter (Millipore, Watford, U.K.) into a nitrogenfilled vial and constantly purged with nitrogen. Ethylenediamine and 1,5,8,12-tetraazadodecane solutions (20–50 m*M*) were made up in water. The reaction was carried out by mixing 1 ml of ligand solution, 1 ml of sodium pertechnetate working solution and 1 ml of filtered stannous tartrate solution in a capped vial. The mixture was shaken and allowed to stand at room tempertature for 20 min.

Preparation of urine sample for oxalic acid measurement

A Varian UK (Walton-on-Thames, U.K.) AASP Vac-Elut system was used with C_8 AASP extraction cartridges for sample preparation. Urine (500 μl) was vortex-mixed with 200 μl of 10% TFA. The mixture was loaded rapidly under positive nitrogen pressure onto the C_8 cartridge, which had previously been washed successively with 0.5 ml of methanol and 1 ml of water. The eluate was collected. The cartridge was then washed with 300 μl of 10% TFA and the eluate was again collected. A 10–20-μl volume of the pooled eluate was injected into the PGC column for oxalic acid separation and quantitation.

Preparation of urine sample for determination of creatine and creatinine

Urine (100 μl) was mixed with 0.1% TFA (100 μl) and loaded onto a C_8 AASP extraction cartridge, which had been pre-conditioned by washing with 0.5 ml of methanol, followed by 1 ml of 0.1% TFA. The eluate was collected. The cartridge was then washed with 1 ml of 0.1% TFA and the eluate was again collected. A 10–20-μl volume of the pooled eluate was used for HPLC analysis.

Preparation of serum sample for creatine and creatinine analysis

Serum (200 μl) was vortex-mixed with 500 μl of 10% TFA and centrifuged at 3000 *g* for 5 min. The supernatant was loaded onto a C_8 AASP extraction cartridge and

the eluate was collected. The cartridge was washed with 1 ml of 0.1% TFA and the eluate was again collected. A 100-μl volume of the pooled eluate was injected into the HPLC column for analysis.

HPLC

A Varian Model 5000 liquid chromatograph was used with a Varian UV-100 variable-wavelength detector and/or a laboratory-built radiometric detector for the detection of technetium complexes[6]. The separations were carried out on a 10 cm $\times$ 4.6 mm I.D. Hypercarb column (Shandon Scientific, Runcorn, Cheshire, U.K.) With 0.1–1% TFA with or without acetonitrile as mobile phases. The flow-rate was 1 ml/min for all separations. The particle diameter of PGC was 7 μm with a surface area of 150 m^2/g,a mean pore diameter of 300 Å and a particle porosity of 70%.

RESULTS AND DISCUSSION

Separation of oxalic acid by EIC

In an earlier study on the separation of the inorganic anions pertechnetate (TcO_4^-) and perrhenate (ReO_4^-), it was demonstrated conclusively that retention was exclusively a result of electronic intereaction between solutes and the delocalized electrons on PGC[5]. In order to find out whether EIC can also be extended to the separation of organic molecules, the separation of oxalic acid was investigated. Oxalic acid was chosen, because it is clinically important and is difficult to retain on ODS silicas. It also possesses lone pairs of electrons on the carboxyl groups, ideal for studying electronic interaction.

Like the oxo-anions of Tc and Re, oxalic acid could not be eluted with water as mobile phase and addition of an organic modifier (acetonitrile) had no effect. Since oxalic acid ionizes completely in water, it cannot be retained on a reversed-phase column without ion pairing or ion suppression. Also, there are no ion-exchange sites on PGC. Thus total retention of oxalic acid with water as eluent can only be due to electronic interaction. This was confirmed by addition of TFA, an electronic modifier[5], to the mobile phase, when oxalic acid was eluted as a sharp peak. It is now clear that to separate anionic compounds on PGC, an electronic modifier (competitor) rather than an organic modifier is of prime importance. The retention of oxalic acid decreases with increasing electronic modifier concentration. This provides further evidence in support of an electronic-interaction mechanism.

Separation of oxalic acid in urine

Oxalic acid in urine can be separated from endogenous impurities with 0.08% TFA as mobile phase (Fig. 1). Sample preparation, particularly removal of hydrophobic compounds which will contaminate the column, is essential for the analysis of biological fluids on PGC. For urinary oxalic acid, this can simply be carried out by loading acidified urine onto a silica-based reversed-phase extraction cartridge, which will remove most hydrophobic components but allow the hydrophilic oxalic acid to pass straight through. Urine samples must be acidified to pH 0.5 or below with 10% TFA or concentrated hydrochloric acid before loading onto the cartridge. This prevents the precipitation of oxalic acid (mainly as the Ca^{2+} and Mg^{2+} salts) and will also dissolve any salts that have already formed in the urine sample. The recovery was

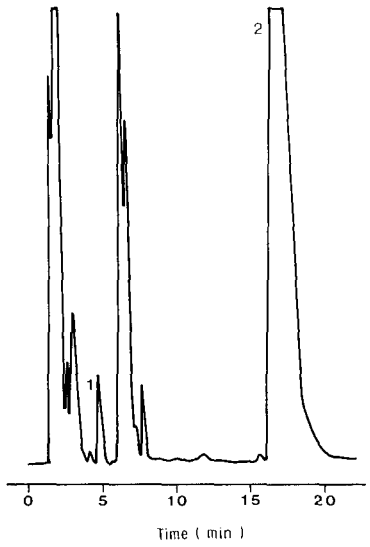


Fig. 1. Chromatography of oxalic acidin human urine. Eluent, 0.08% TFA; flow-rate, 1 ml/min; detector, 210 nm, 0.1 a.u.f.s. Peaks: (1) oxalic acid; (2) creatinine.

virtually 100%. The detection limit at 210 nm and 0.1 a.u.f.s. was 0.3 μg injected (signal-to-noise ratio of 3), which is adequate for the determination of oxalic acid in normal and in pathological urine.

Separation of creatine and creatinine in body fluids

Although many HPLC methods are described for the separation of creatinine in body fluids, very few are suitable for the simultaneous determination of creatine and creatinine[7]. Creatine is difficult to retain on ODS columns and it is often eluted together with other early-eluted interfering compounds. However, creatine and creatinine are expected to be retained much more strongly on the highly hydrophobic PGC column.

The separation of creatine and creatinine with 3% (v/v) acetonitrile in 0.1% TFA as the mobile phase is shown in Fig. 2. The study on the relationship between TFA concentration and capacity ratios (k') of creatine and creatinine (Fig. 3) indicates that retention was based mainly on reversed-phase ion-pair chromatography. The k'value increased with increasing TFA (ion-pairing agent) concentration. There was probably also a slight electronic interaction involved in the retention of these compounds, as at low TFA concentrations (below 0.01%) increasing the TFA concentration caused a small drop in k' (Fig. 3). At higher TFA concentration (above 0.05%), however, ion-pairing predominated. With 3% (v/v) acetonitrile in water as eluent, resolution was poor. The percentage acetonitrile content of the mobile phase can also be used to control the retention and resolution of creatine and creatinine, as shown in Fig. 4. The effect is that, as expected for reversed-phase chromatography, k' decreases with increasing organic modifier content.

From the above retention behaviour studies, it was concluded that 3% (v/v) acetonitrile in 0.1% TFA is the optimal system for the separation of creatine and

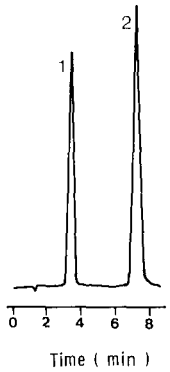


Fig. 2. Separation of creatine (1) and creatinine (2) on porous graphitic carbon. Eluent, 3% (v/v) acetonitrile in 0.1% TFA; flow-rate, 1 ml/min; detector, 210 nm.

creatinine in urine (Fig. 5a and b) and in serum (Fig. 5c). Removal of hydrophobic contaminants was again effected by a C_8 extraction cartridge, in which creatine and creatinine were not retained (see Experimental). The recoveries were virtualy 100%. The detection limits for creatine and creatinine were 20 and 10 ng injected, respectively.

Separation of remoxipride and FLA 981

Remoxipride and FLA 981 (Fig. 6) are potential neuroleptic agents[8]. These basic drugs were well known to behave badly with severe peak tailing and broadening on silica-based reversed-phase columns[9] because of partial ionization and interaction with residual silanol groups, despite the inclusion of a silanol masking agent in the mobile phase. An example is shown in Fig. 7.

The major advantages of PGC column over silica-based materials are that it contains no silanol groups and is stable at high pH. It is therefore commonly believed that PGC is an ideal reversed-phase packing for the chromatography of basic

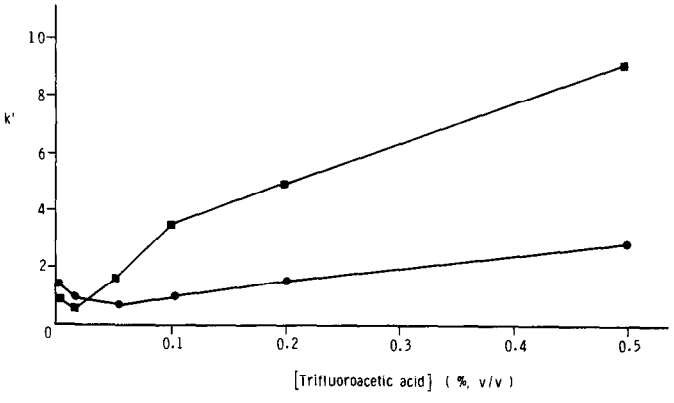


Fig. 3. Relationship between TFA concentration and capacity ratios of creatine (●) and creatinine (■).

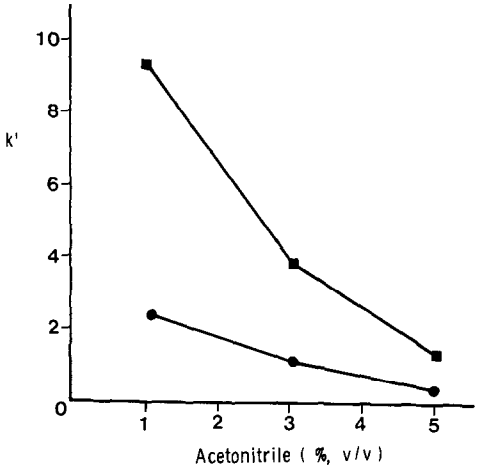


Fig. 4. Relation between percentage acetonitrile content of the mobile phase and retention of creatine (●) and creatinine (■).

compounds by ion-suppression at high pH. However, suppression of ionization greatly increases the hydrophobicity of the solute, and this can lead to strong interaction with the highly hydrophobic carbon surface, resulting in excessive retention.

A better approach to the separation of basic compounds on PGC is the use of a mobile phase containing TFA as eluent. TFA can function as an ion-pairing agent for bases, but, unlike long-chain ion-pairing agents it does not confer strong

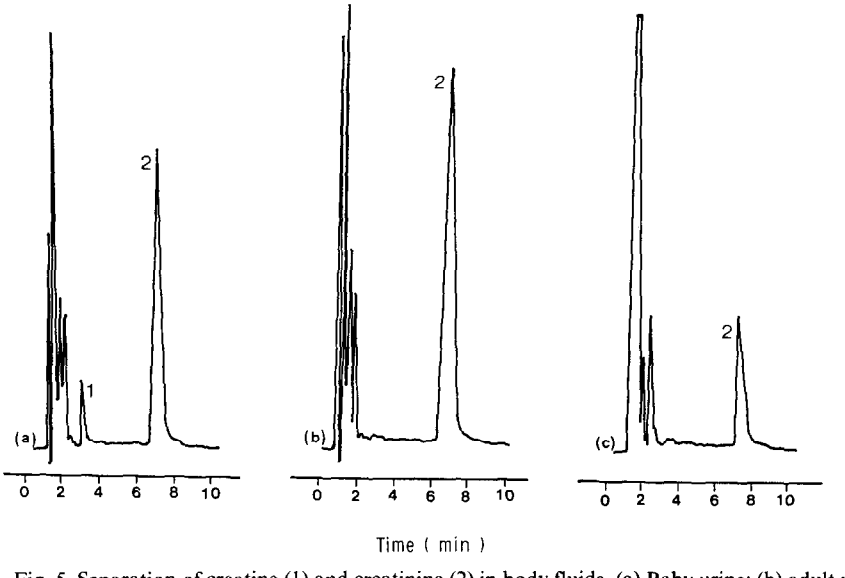
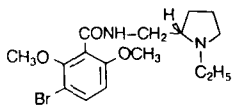
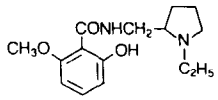


Fig. 5. Separation of creatine (1) and creatinine (2) in body fluids. (a) Baby urine; (b) adult urine; (c) adul serum. Eluent, 3% (v/v) acetonitrile in 0.1% TFA; flow-rate, 1 ml/min; detector, 210 nm, 0.1 a.u.f.s.



Remoxipride



FLA 981

Fig. 6. Structures of remoxipride and FLA 981.

hydrophobicity on the solute. The separations of remoxipride and FLA 981 by ion suppression at pH 10 (50% acetonitrile in 0.1 *M* ammonium hydroxide) and by ion pairing with TFA (50% acetonitrile in 0.1% TFA) are shown in Fig. 8a and b, respectively. The superiority of the TFA mobile phase system is clearly demonstrated, remoxipride and FLA 981 being eluted within convenient retention times. With ion suppression, excessive retention was observed for both compounds and FLA 981 could not be eluted at 50% acetonitrile concentration.

Simultaneous separation of the oxo-anion of technetium and cationic ^{99m}technetium–amine complexes

Pertechnetate (TcO_4^-), the oxo-anion of technetium, is the starting material for the preparation of many radiopharmaceuticals[10], and cationic ^{99m}technetium–amine complexes are typical examples[11]. To monitor the radiochemical purity of these potential imaging agents and to study their stability and metabolic fate, a chromato-

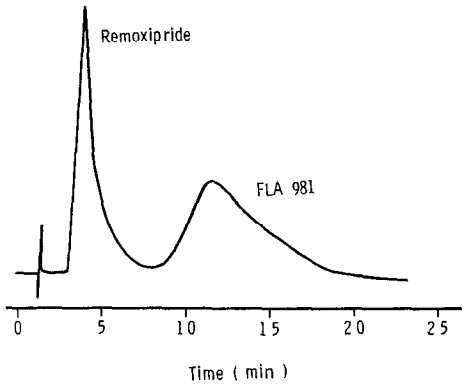


Fig. 7. Separation of remoxipride and FLA 981 on 5-μm Hypersil ODS (10 cm × 5 mm). Eluent, 50% (v/v) acetonitrile in water, containing 1% triethylamine; flow-rate, 1 ml/min; detector, 254 nm.

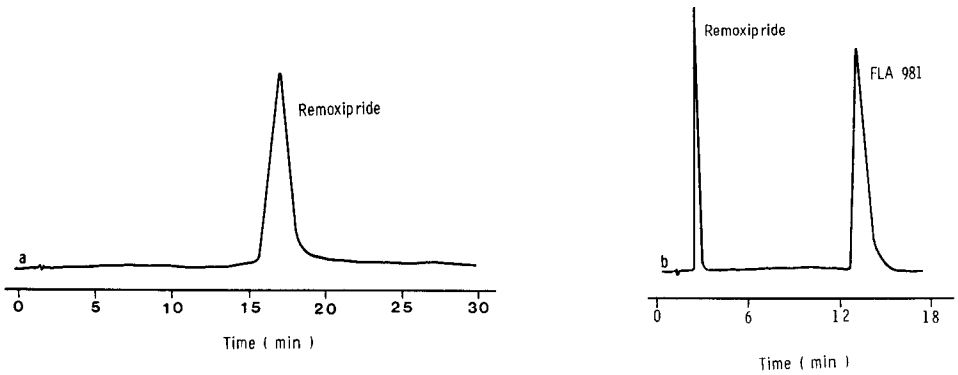


Fig. 8. Separation of remoxipride and FLA 981 on porous graphitic carbon. (a) With 50% (v/v) acetonitrile in 0.1 *M* ammonium hydroxide (pH 10) as eluent; (b) with 50% (v/v) acetonitrile in 0.1% TFA as mobile phase; flow-rate, 1 ml/min; detector, 254 nm.

graphic system capable of simultaneously separating anionic and cationic compounds is required. Previous studies[6] have shown that this is possible on a PGC column with TFA and acetonitrile as mobile phase.

The separation of TcO_4^- and the cationic complexes dioxo(bisethylenediaminato)technetium and dioxo(1,5,8,12-tetraazadodecane)technetium (Fig. 9) is shown Fig. 10a and b. TcO_4^- was retained exclusively by electronic interaction while the cationic complexes were retained by reversed-phase interaction. It is therefore possible to control the separation precisely according to the nature of application by altering the TFA concentration or acetonitrile content in the mobile phase. TFA functions as an electronic modifier and has a significant effect on the retention of TcO_4^- (Fig.10a and b). The acetonitrile content in the mobile phase, on the other hand, affected the retention of the cationic complexes but not the anionic TcO_4^-.

The electronic modifier strengths of acetates have been studied and the order TFA > sodium acetate > acetic acid was observed. Acetic acid is a weak electronic modifier and requires a concentration of above 5% (v/v) for elution of TcO_4^-. Sodium acetate ionizes better than acetic acid and is also a stronger electronic modifier, because the lone-pair electrons on the carboxyl group are more available for electronic interaction. TFA is a strong carboxylic acid, which ionizes easily to provide lone-pair electrons for interaction. Its electronic-modifier strength is thus the strongest among the acetates.

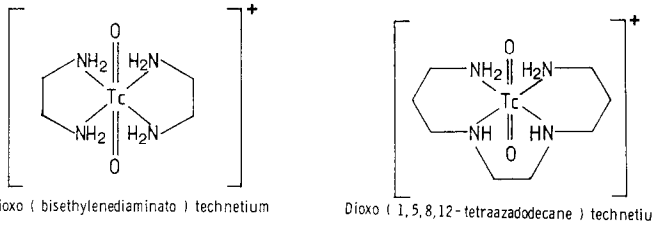


Fig. 9. Structures of dioxo(bisethylenediaminato)technetium and dioxo(1,5,8,12-tetraazadodecane)technetium cations.

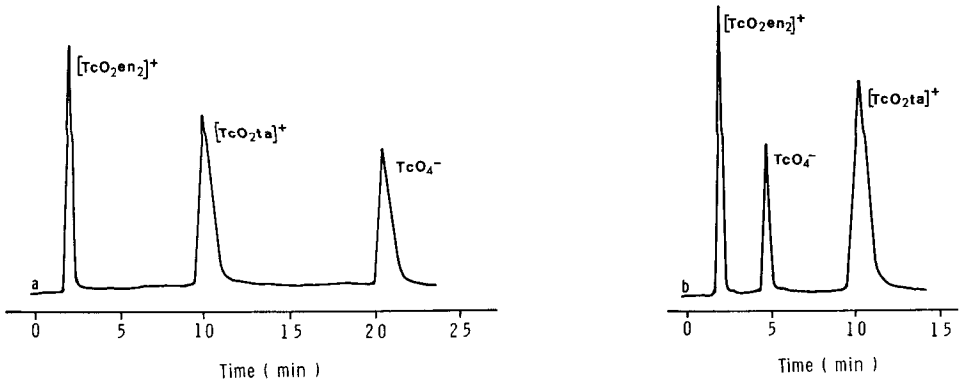


Fig. 10. Separation of pertechnetate (TcO_4^-), dioxo(bisethylenediaminato)technetium, $[TcO_2en_2]^+$, and dioxo(1,5,8,12-tetraazadodecane)technetium, $[TcO_2ta]^+$, on porous graphitic carbon. (a) With 2% (v/v) acetonitrile in 0.1% TFA as eluent; (b) with 2% acetonitrile in 1% TFA as eluent; flow-rate, 1 ml/min; detector, radiometric.

CONCLUSIONS

Retention on PGC is usually a mixture of reversed-phase and electronic interactions, although pure EIC is also possible. Retention of anionic compounds is dominated by electronic interaction and the mobile phase must contain an electronic modifier with or without an organic modifier. For the separation of cationic compounds, ion suppression with basic eluents at high pH increases the hydrophobicity of the solute, resulting in excessive retention on the highly hydrophobic carbon surface. Hydrophobic ion-pairing agents also lead to strong retention of cationic compounds for the same reason.

TFA is a universal mobile phase additive for chromatography of ionic compounds on PGC. It is an excellent electronic modifier, which can also function as an ion-pairing agent that does not confer excessive hydrophobicity on the solute. Sticking to one mobile phase additive will improve the reproducibility of separation on PGC.

To avoid excessive contamination, sample preparation is essential when biomedical samples are analysed on PGC. It is recommended that, after a separation, the column be washed with a gradient of TFA in acetonitrile or methanol in order to remove both ionic and hydrophobic contaminants. The column is then stored in methanol or aqueous methanol.

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