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HIGH-PERFORMANCE LIQUID CHARGE-TRANSFER CHROMATOGRAPHY

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SUMMARY

Acriflavin has been covalently attached to microparticulate silica (10 μm) and used for the resolution of low-molecular-weight biochemical substances by high-performance charge-transfer chromatography. The acriflavin–silica adsorbent is able to resolve mixtures of purines and pyrimidines, nucleosides and nucleotides, pyridine nucleotide coenzymes, flavins and aromatic amino acids. The simultaneous operation of electrostatic and hydrophobic interactions, in addition to the charge-transfer effects, permits considerable flexibility in the mode of operation of this new type of adsorbent. Furthermore, these separations may be achieved in a fraction of the time required for similar separations by low-pressure liquid chromatography.

INTRODUCTION

The formation of neutral molecular complexes by electron displacement or transfer from one component, the donor (D), to a second component, the acceptor (A) has been recognised for some time[1]:

$$D + A \leftrightarrow (D,A) \leftrightarrow (D^+:A^-)$$

Charge-transfer complexes of this type most commonly involve π–π interactions between conjugated systems, although n–π complexes between the lone-pair electrons of oxygen or sulphur and aromatic rings and σ–π complexes between polyhydroxylic adsorbents and π-electron-rich solutes may also be implicated[2,3]. Desirable properties in the donor and acceptor are influenced by the type and number of aromatic rings constituting the conjugated system and by the presence or absence of electron-donating or electron-withdrawing substituents.

If one of the components of the interacting system is anchored to a neutral insoluble matrix, the latter will be converted into an adsorbent for the complementary

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binding partner of the charge-transfer complex. However, early attempts to exploit such charge-transfer processes for the chromatography of biochemically interesting substances proved somewhat elusive[4-6], although recent investigations with adsorbents comprising several aromatic moieties coupled to Sephadex have proven more promising for the resolution of aromatic amino acids and nucleotides[7,8]. Acriflavin was selected for detailed investigation into the utility of charge-transfer chromatography since it displays excellent electron-acceptor properties towards nucleotides and produced the most efficient adsorbent for a number of tested substances[8,9]. The choice of acriflavin as acceptor is justified by the formation of charge-transfer complexes between purines, aromatic amino acids and flavins[8] and by its proven utility in the resolution of DNA fragments[10], oligonucleotides[11] and single-stranded from double-stranded nucleic acids[11]. To date, however, no attempt has been made to unite the techniques of high-performance liquid chromatography (HPLC) and charge-transfer chromatography in order to capitalise on the biological specificity of the charge-transfer process and the inherent speed and resolving power of HPLC. The combination of HPLC and affinity chromatography by exploiting the interactions between silica-immobilised coenzymes[12], immunoligands[12], boronates[13] and triazine dyes[14,15] and their complementary enzymes and proteins is now well established. The present report demonstrates the ability of microparticulate silica-bonded acriflavin to resolve, rapidly and selectively complex mixtures of biological substances by high-performance liquid charge-transfer chromatography.

EXPERIMENTAL

Chemicals

Microparticulate porous silica (LiChrosorb Si 60, 10 μm) was obtained from E. Merck (Darmstadt, G.F.R.). L-Tyrosine, L-tryptophan, L-phenylalanine, adenine, guanine, cytosine, xanthine, hypoxanthine, thymine, uracil, acriflavin, riboflavin, flavin–adenine dinucleotide (FAD), flavin mononucleotide (FMN), adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate, uridine, cytidine, guanosine, adenosine and N-ethylmorpholine were purchased from Sigma (London) (Poole, Great Britain). The pyridine nucleotide coenzymes, NAD^+ and $NADP^+$, were obtained from Boehringer Mannheim (G.F.R.). The organofunctional silane, γ-glycidoxypropyltrimethoxysilane was from Silor Labs. (Scotia, NY, U.S.A.).

Synthesis of acriflavin-substituted silica

LiChrosorb Si 60 (10 μm) was epoxysilylated with the organofunctional silane, γ-glycidoxypropyltrimethoxysilane, as described previously[14,15]. Epoxy-substituted silica (5 g dry weight) was added to 0.1 *M* $NaHCO_3$–Na_2CO_3 buffer, pH 8.5 (25 ml) containing acriflavin (0.26 g, 1 mmol), and the slurry was sonicated for 10 min under reduced pressure and incubated overnight at 30°C with gentle agitation. The resulting acriflavin–silica was exhaustively washed with water, methanol and 1 *M* potassium chloride solution alternately until the washings were clear of desorbed acriflavin. The silica matrix was sucked moist on a sintered funnel and added to 10 m*M* hydrochloric acid (50 ml). The slurry was heated to 75°C for 30 min to hydrolyse excess oxirane groups and subsequently washed with water (1 l), 50% (v/v) aqueous methanol (500

ml), 100% methanol (200 ml) and finally diethyl ether (100 ml) prior to drying in air to a yellow powder. The concentration of immobilised acriflavin was determined by spectrophotometric analysis of alkaline hydrolysates at 450 nm as described previously[14]. The immobilised ligand concentration, typically 18–20 μmol/g dry weight silica, was calculated using a molar absorption coefficient of 3300 l mol^{-1} cm^{-1} for acriflavin at 450 nm[16].

Chromatographic procedures

The acriflavin–silica conjugate (1.5 g) was packed in a stainless steel column (100 × 4.5 mm I.D., total volume *ca.* 1.5 ml) with the upward slurry packing technique[14] in methanol at 20.7 MPa (3000 p.s.i.). All chromatographic procedures were performed at ambient temperature (20–22°C) using Waters Assoc. (Hartford, Northwich, Great Britain) HPLC equipment comprising two Model 6000 solvent metering pumps, a Model 450 variable-wavelength detector (190–800 nm), a Model U6K sample injector and a Model 660 solvent programmer.

RESULTS AND DISCUSSION

Preparation of acriflavin–silica

Modification of the surface silanol groups of microparticulate silica with the organofunctional silane, γ-glycidoxypropyltrimethoxysilane, not only yields an epoxy-activated support capable of reacting with amino-containing ligands such as acriflavin, but also produces, on subsequent hydrolysis of excess epoxy groups, a neutral hydrophilic surface which minimises non-specific interactions with sensitive biological substances. The structure of the conjugate produced on coupling acriflavin directly to epoxysilylated LiChrosorb Si 60 is shown in Fig. 1. In general, acriflavin–silica prepared by this procedure had a ligand substitution level of 18–20 μmol/g dry weight silica although higher or lower degrees of substitution could be readily achieved by suitable modification of the coupling procedure. Reference columns of epoxy- or glycol-silylated silica showed some retardation of the biological substances investigated in this report. Table I gives the reduced elution volumes for several substances on HPLC on glycol-silylated silica. With the exception of riboflavin, adenine and adenosine very little retardation was observed on the control adsorbent. Furthermore, the recovery of the substances was nearly quantitative when applied to the reference columns.

Resolution of bases, nucleosides and nucleotides

It is well established that acriflavin interacts strongly with purines such as adenine and guanine and that this interaction is related to the energies of the de-

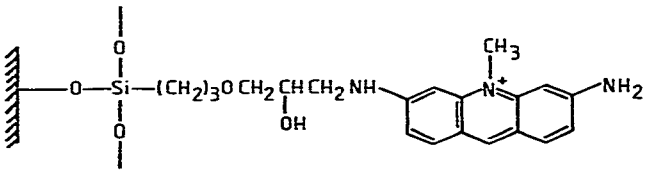


Fig. 1. The structure of the acriflavin–silica charge-transfer adsorbent.

TABLE I

REDUCED ELUTION VOLUMES OF BIOLOGICAL SUBSTANCES ON ACRIFLAVIN–SILICA

Chromatographic conditions were as follows: column buffer, 0.1 *M* N-ethylmorpholine–acetate (pH 7.0); temperature, 20–22°C; flow-rate, 1 ml/min; pressure, 3.5 MPa (500 p.s.i.); V_e = elution volume; V_o = void volume (1 ml).

Biological substance	*Reduced elution volume* (V_e/V_o)	
	Acriflavin–silica	*Control glycol-silylated silica*
FMN	5.2	1.2
FAD	5.0	1.2
Riboflavin	4.4	2.2
Adenine	2.75	2.6
Guanine	2.2	–
$NADP^+$	2.0	–
cAMP	1.9	–
Adenosine	1.9	1.8
L-Tryptophan	1.9	–
Xanthine	1.85	–
Hypoxanthine	1.85	–
Nicotinamide	1.85	–
p-Aminobenzoic acid	1.85	–
Ascorbic acid	1.8	–
NAD^+	1.75	–
Thymine	1.7	–
AMP	1.7	1.0
ATP	1.7	1.0
Thiamine	1.7	–
ADP	1.65	1.0
L-Phenylalanine	1.6	–
L-Tyrosine	1.6	–
Cytidine	1.6	–
Guanosine	1.6	–
Pyridoxamine	1.6	–
Cytosine	1.55	–
Uridine	1.5	–
Uracil	1.5	–

localised π-electrons in the highest filled molecular orbitals[11]. To a first approximation a strong charge-transfer complex is formed when the energy difference between the highest occupied level of the donor is close to the lowest unoccupied level of the acceptor. However, pure charge-transfer phenomena rarely exist since the effect is largely hidden by solvent effects, hydrophobic interactions with the aromatic moieties involved and by the fact that most π-electron-rich substances can act as both π-donors and π-acceptors in the charge-transfer complex. Nevertheless, these considerations suggest that the purines, adenine and guanine should act as better electron donors than thymine, uracil and cytosine and thus permit resolution of purine and pyrimidine bases by high-performance charge-transfer chromatography. Fig. 2a demonstrates the group resolution of purines and pyrimidines on acriflavin–silica in 0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0). As anticipated, adenine was the most

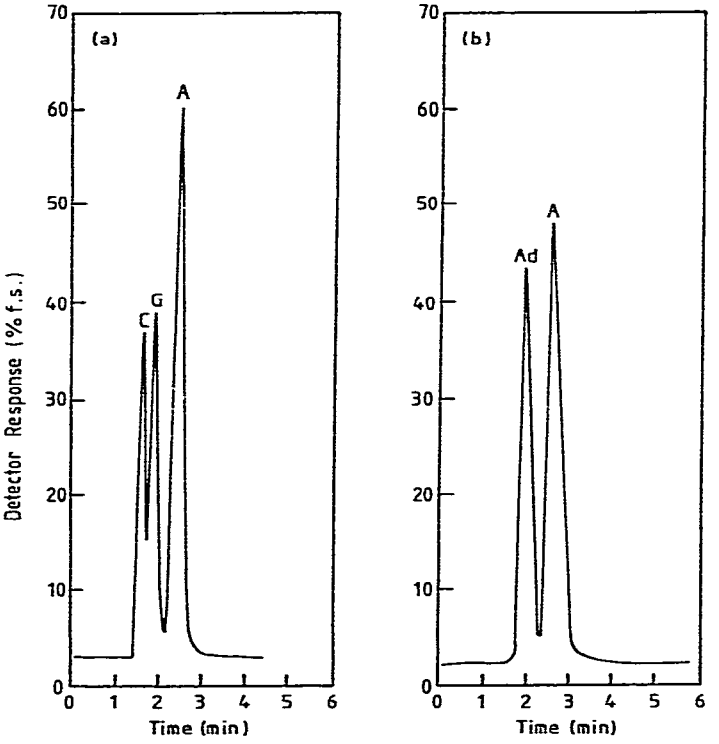


Fig. 2. Resolution of purines and pyrimidines on acriflavin–silica. (a) Adenine (1.8 μg) (A), guanine (2.0 μg) (G) and cytosine (C) (2.0 μg) in 0.1 *M* N-ethylmorpholine–acetate (pH 7.0) in a total volume of 40 μl applied to column at time zero. (b) Adenine (2.0 μg) (A) and adenosine (Ad) (2.0 μg) in 20 μl of 0.1 *M* N-ethylmorpholine–acetate (pH 7.0) applied at time zero. Column buffer, 0.1 *M* N-ethylmorpholine–acetate (pH 7.0); flow-rate, 1 ml/min; pressure, 3.5 MPa (500 p.s.i.); detector, 260 nm, 1.0 a.u.f.s.

retarded heterocyclic base, followed by guanine and cytosine. Furthermore, as illustrated in Fig. 2b, adenine was more retarded than its riboside, adenosine, presumably because the sugar moiety obstructs the charge-transfer overlap recognised between the adenine and the indole ring of acriflavin[17].

The positive charge on the nitrogen at position 9 (N^9) imparts a cationic character to the acriflavin and is able to provide an additional electrostatic effect for the resolution of nucleotides. Thus, at low ionic strength, solutes were eluted or retarded in accordance with their charge transfer or electrostatic interactions with the immobilised acriflavin, whilst at high ionic strength, charge-transfer effects predominate. As Table I illustrates, in 0.1 *M* N-ethylmorpholine–acetate buffer pH 7.0, AMP, ADP and ATP are retarded almost equally and are therefore unresolvable from each other, although clearly separable from adenosine and adenine. In contrast, when 0.01 *M* N-ethylmorpholine–acetate (pH 7.0) is used as isocratic column irrigating buffer, AMP is significantly more retarded than adenine with a reduced elution volume, V_e/V_0, where V_e is the elution volume of the sample and V_0 is the void volume of the column, of 5.6 compared with 2.5 for adenine and 1.9 for adenosine. Fig. 3 shows that increasing the number of anionic phosphate groups in the series AMP, ADP and ATP increased the interaction with silica-immobilised acriflavin and

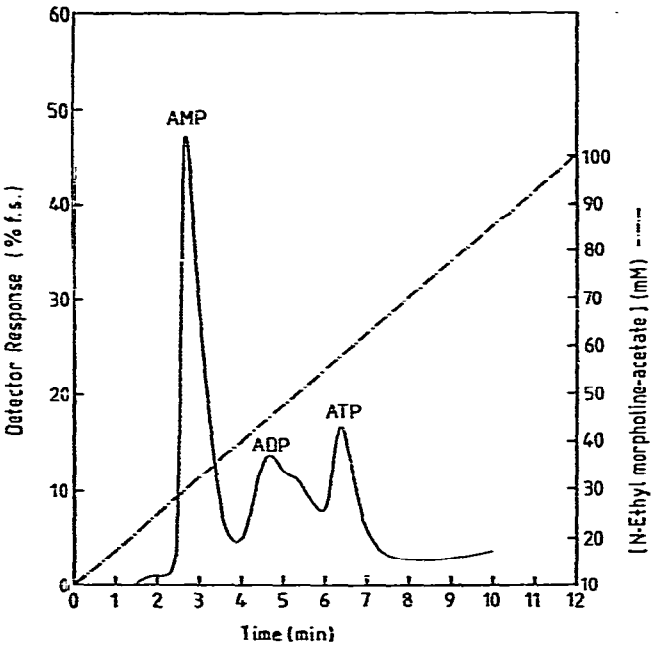


Fig. 3. Resolution of adenosine phosphates by high-performance charge-transfer chromatography on acriflavin–silica. AMP (10 μg), ADP (10 μg) and ATP (10 μg) in a total sample volume of 20 μl were applied to a column equilibrated with 0.01 *M* N-ethylmorpholine–acetate (pH 7.0); flow-rate, 1 ml/min; detector, 260 nm, 20 a.u.f.s.; pressure, 3.5 MPa (500 p.s.i.). Elution was effected at time zero with a linear gradient 0.01–0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0).

permitted the resolution of these adenosine phosphates by linear gradient elution with 0.01–0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0). Similar results were obtained when adenosine and its nucleotides were co-chromatographed on acriflavin–Sephadex G-25 in 0.1 *M* buffer[9], although the separation was achieved in hours rather than minutes as in the present report.

The resolution of the pyridine nucleotide coenzymes, NAD^+ and $NADP^+$, may also be achieved by suitable adjustments to the ionic strength of the irrigating buffers. Table I demonstrates that in 0.1 *M* N-ethylmorpholine–acetate buffer pH 7.0, $NADP^+$ is slightly more retarded (V_e/V_0 2.0) than NAD^+ (V_e/V_0 1.75). On the other hand, at low ionic strength, in 0.01 *M* N-ethylmorpholine–acetate buffer (pH 7.0), NAD^+ is eluted isocratically (V_e/V_0 2.2) whilst $NADP^+$ is strongly adsorbed and requires a pulse (500 μl) of 0.1 *M* N-ethylmorpholine–acetate (pH 7.0) for subsequent desorption (Fig. 4). Quantitative recovery of the coenzymes was achieved under conditions where individual coenzymes were eluted when applied separately to the adsorbent. It seems likely that the resolution of NAD^+ and $NADP^+$ on acriflavin–silica may be attributed to an enhanced electrostatic interaction between the more anionic $NADP^+$ and the cationic acridine derivative. It is also conceivable, however, that the significantly greater interaction with $NADP^+$ may be due to the 2′-phosphate inducing a more favourable configuration of the nucleotide molecule and thus favouring the formation of the charge-transfer complex. Similar considerations may account for the greater retention of cyclic-AMP (cAMP, V_e/V_0 1.9) than AMP (V_e/V_0 1.7) on

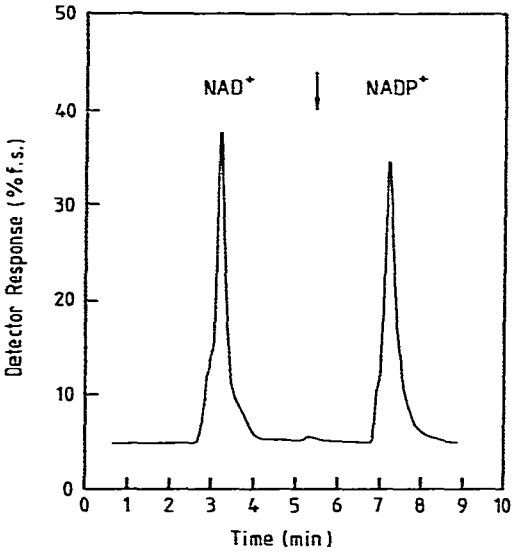
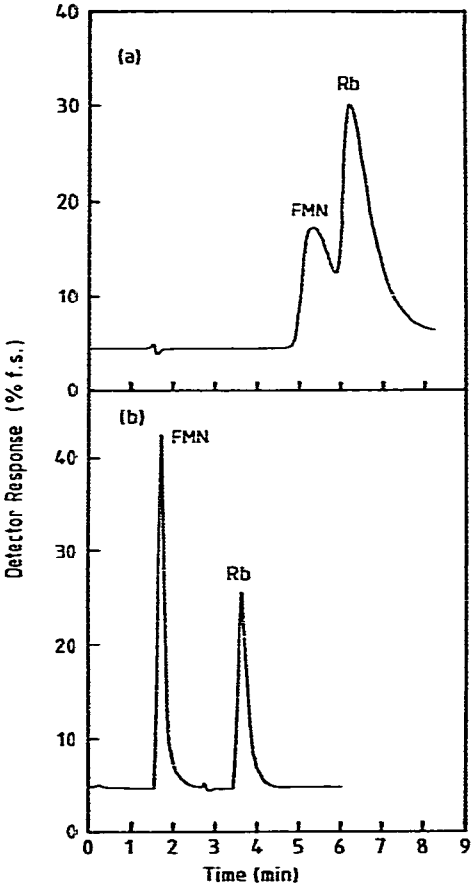


Fig. 4. Separation of NAD^+ and $NADP^+$ on acriflavin–silica. NAD^+ (10 μg) and $NADP^+$ (10 μg) in a total sample volume of 20 μl were applied to the column at time zero. Column buffer, 0.01 *M* N-ethylmorpholine–acetate (pH 7.0); pressure, 3.5 MPa (500 p.s.i.); flow-rate, 1 ml/min; detector, 260 nm, 2.0 a.u.f.s.; eluent added at arrow, 0.1 *M* N-ethylmorpholine–acetate (pH 7.0) (500 μl).

acriflavin–silica (Table I). It has been suggested that the 5′-phosphate group of AMP does not play a direct role in the formation of the charge-transfer complex but that at high ionic strength, the phosphomonoester group can partially shield the aromatic ring of adenosine, thereby decreasing the nucleotide-acriflavin interaction[9]. Furthermore, the anionic phosphates influence the resolution of riboflavin (V_e/V_0 4.4), FMN (V_e/V_0 5.2) and FAD (V_e/V_0 5.0) on the charge-transfer adsorbent in 0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0) (Table I). Fig. 5a shows that a mixture of riboflavin (10 μg) and FMN (10 μg) in a sample volume of 40 μl are incompletely resolved when chromatographed on acriflavin–silica at 3.5 MPa (500 p.s.i.) in 0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0) containing 10% (v/v) methanol. In this case, it seems likely that the major interaction is the charge-transfer interaction between the closely aligned isoalloxazine rings of riboflavin and FMN and the indole ring of acriflavin, with the contribution due to the interaction between the phosphate group of FMN and the cationic N^9 of acriflavin being minimal. The reduced retention of FAD (Table I) compared with FMN supports this view and suggests that the geometry of the dinucleotide is less conducive to the formation of a strong charge transfer complex. Excellent resolution of FMN and riboflavin is obtained on chromatography in 50% (v/v) aqueous methanol (Fig. 5b).

Resolution of aromatic amino acids

The chromatography of aromatic amino acids on acriflavin–Sephadex G-25 provides convincing evidence that the adsorption is due primarily to the π-electron-donor capacity of the ligands[7,8]. Fig. 6 shows that a mixture of two aromatic amino acids, L-tyrosine and L-tryptophan may be resolved by high-performance charge-transfer chromatography on acriflavin–silica at 20–22°C in 0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0).



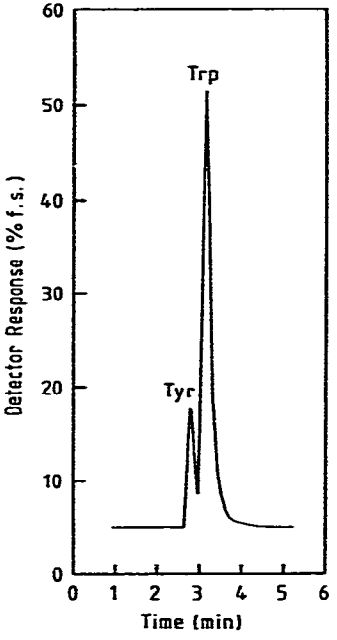


Fig. 5. Resolution of flavins by high-performance charge-transfer chromatography on acriflavin–silica. Flavin mononucleotide (FMN, 10 μg) and riboflavin (Rb, 10 μg) in appropriate solution (40 μl) were added at time zero. Column buffer: (a) 0.1 *M* N-ethylmorpholine–acetate (pH 7.0) containing 10% (v/v) methanol; (b) 50% (v/v) aqueous methanol. Pressure, 3.5 MPa (500 p.s.i.); flow-rate, 2 ml/min; detector, 266 nm, 0.4 a.u.f.s.

Fig. 6. Separation of L-tyrosine and L-tryptophan by charge-transfer chromatography. L-Tyrosine (10 μg) and L-tryptophan (10 μl) in 0.1 *M* N-ethylmorpholine–acetate (pH 7.0; 20 μl) were applied to the column at time zero. Column buffer, 0.1 *M* N-ethylmorpholine–acetate (pH 7.0); pressure, 3.5 MPa (500 p.s.i.); flow-rate, 1 ml/min; detector, 260 nm, 1.0 a.u.f.s.

Charge-transfer chromatography of other substances

Table I demonstrates that a number of other low-molecular-weight biological materials are retarded by passage through columns of the charge-transfer adsorbent, acriflavin–silica, in 0.1 *M* N-ethylmorpholine–acetate buffer (pH 7.0). The vitamins, nicotinamide, *p*-aminobenzoic acid and ascorbic acid are more retarded (V_e/V_0 > 1.8) than thiamine (V_e/V_0 1.7) or pyridoxamine (V_e/V_0 1.6). Furthermore, some retardation of the antihistamine drugs trimeprazine tartrate, dimethothiazine mesylate and mepyramine maleate may be achieved by high-performance charge-transfer chromatography in 50% (v/v) methanol–0.1 *M* N-ethylmorpholine–acetate (pH 7.0).

CONCLUSIONS

Acriflavin–silica is a versatile new high-performance charge-transfer chromatography support matrix that should find wide application in the resolution of biological substances such as bases, nucleosides, nucleotides, oligo- and polynucleotides, coenzymes and coenzyme analogues, vitamins, drugs and a variety of other aromatic compounds. High-performance chromatography on acriflavin–silica drastically reduces the separation time without loss of resolution when compared with conventional low-pressure charge-transfer chromatography on acriflavin–Sephadex G-25. Furthermore, the implication of co-operative adsorption effects such as aromatic and ionic interactions in addition to the charge-transfer effect permits greater flexibility in the practical operation of the technique than is the case with other high-performance systems based on ion-exchange, reversed-phase or boronate adsorbents[13,18].

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