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Dynamically Modified Silica and its Applications in Drug Control and Drug Metabolism Studies

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Abstract—An alternative to the well known reversed-phase separations on chemically-bonded phases has been developed. The approach is based on a dynamic modification of bare silica with long chain quaternary ammonium ions. The influence of the concentration and type of quaternary ammonium ion, the pH value and the ionic strength of the eluent on the selectivity towards test solutes has been investigated. The large number of parameters that can be varied in the system offers numerous possibilities by which a desired selectivity can be attained. Once established, a high degree of reproducibility of the selectivity between solutes is obtained even when using different brands of silica; this is in contrast to the situation when using chemically-bonded phases, such as, for example, different brands of octadecylsilyl-bonded silica materials. Examples of the use of the system in pharmaceutical analysis and drug metabolism studies are given.

Separation methods based on reversed-phase high-performance liquid chromatography (HPLC) on column packing materials with chemically-bonded phases have been used with ever increasing success during the last decade. This increased use has been particularly pronounced in the field of analysis of samples of biological origin; this success will doubtlessly continue in the years to come. Despite all their indisputable advantages, these packing materials still suffer from one serious drawback, namely the lack of reproducibility as regards the selectivity obtained when using packing materials of theoretically the same nature from different sources. These problems have been discussed in the literature[1~9]. Using the dynamically modified silica approach the reproducibility of the selectivity of packing materials from different manufacturers is considerably improved, as discussed below.

Experimental

Apparatus—Testing of the individual chromatographic systems was performed on a Waters (Milford, MA, USA) liquid chromatograph consisting of a model 6000 A pump, a model 710 A WISP autoinjector, a model 440

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ultraviolet absorbance detector (operated at 254 nm), a model 730 data module and a model 720 system controller; alternatively a liquid chromatograph was assembled, consisting of a Kontron model 410 LC pump, a Pye-Unicam (Cambridge, UK) LC-UV detector (operated at 254 nm) and a Rheodyne model 7125 injection valve. Chromatograms were recorded on a Kipp and Zonen Model BD-8 recorder. Retention data were collected on a Waters model 730 data module or on a Hewlett-Packard Model 3353 A laboratory system.

Procedures—Determination of the amounts of cetyltrimethylammonium (CTMA) bromide adsorbed on the column material by the breakthrough method or by the elution method was performed as previously described.[10~11]

Chromatography—All experiments were performed on 120×4.6 mm i.d. columns from Knauer (Berlin, FRG), packed by the dilute slurry technique, unless otherwise indicated, with 5-μm Lichrosorb Si-60 (E. Merck, Darmstadt, FRG). The eluent was methanol-water-phosphate buffer (50:45:5, v/v/v) with the addition of various types and concentrations of quaternary ammonium compounds. All pH values stated are those measured in the buffers before dilution in the final eluent. The buffers were prepared from potassium dihydrogen phosphate or orthophosphoric acid by titration to the required pH with 5 M potassium hydroxide, followed by dilution to the final concentration of 0.2 M. During chromatography the column was protected by a silica saturation column situated between the pump and the injection device to saturate the eluent. The chromatographic system was equilibrated by elution overnight. Following each adsorption experiment the column was brought to its initial status by eluting with methanol-0.05 M nitric acid (1:1, v/v) and finally with methanol.

Chemicals—Stearyltrimethylammonium (STMA) bromide was prepared as described previously[12]. Dodecyltrimethylammonium (DTMA) bromide and tetradecyltrimethylammonium (TTMA) bromide were obtained from Sigma (St Louis, MO, USA). Cetyltrimethylammonium (CTMA) bromide, tetrabutylammonium (TBA) bromide, tetrapentylammonium (TPA) bromide and all other reagents were of analytical grade from E. Merck. All chemicals were used as received from the manufacturers.

Results and Discussion

The use of bare silica as the packing material and aqueous mobile phases might be considered contradictory to the old perception still found among many chromatographers that silica is an adsorbent used in straight-phase adsorption chromatography with relatively non-polar eluents. However, in recent years an increasing number of papers dealing with chromatographic systems based on the combination of bare silica and aqueous mobile phases have appeared[12~14]. The use of bare silica in reversed-phase chromatography may be performed by impregnating the silica with a non-polar liquid immiscible with the eluent thus creating a liquid-liquid partition system. Such a system requires, however, a lot of skill to operate, and it is in practice very difficult to maintain a stable system. In the dynamically modified silica approach the surface of the bare silica is covered with a layer of quaternary ammonium ions as shown schematically in Fig. 1. The amount attached (primarily by electrostatic forces) to the silica surface is dependent on the composition of the eluent[15]. It forms a dynamic coating in which the quaternary ammonium ions on the silica surface are in equilibrium with the quaternary ammonium ions that are continuously present in the eluent. The lipophilic quaternary ammonium ions exhibit a high

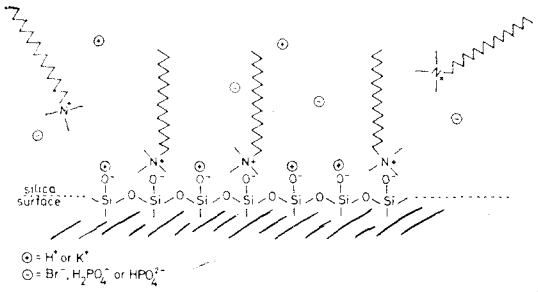

Fig. 1. Bare silica dynamically modified with long chain quaternary ammonium ions. A typical system would be: solid phase, 5-μm Lichrosorb Si-60, 120×4.6 mm i.d.; mobile phase, methanol-water-0.2 M potassium phosphate (pH 7.5) (50:45:5, v/v/v) with 2.5 mM of cetyltrimethylammonium bromide added.

affinity towards the ionized silanol groups. The observed pK_a-value of the silica is about 7[16], and below a pH value of about 5 only very small amounts of quaternary ammonium ions

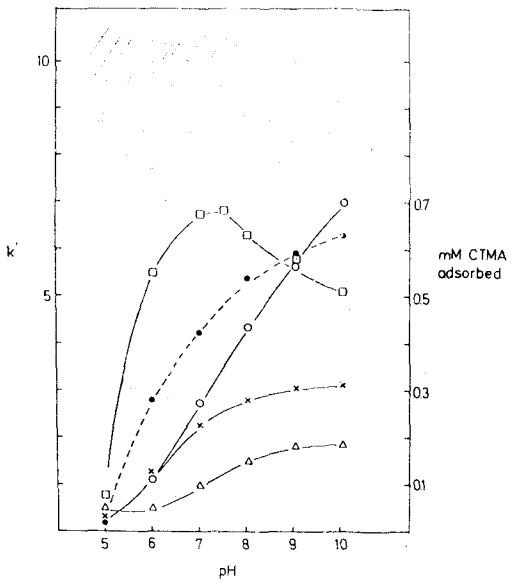

Fig. 2. Relationship of the pH of the buffer used in the eluent with:

(a) the amount of cetyltrimethylammonium (CTMA) bromide adsorbed; and (b) retention(k') of various test solutes.

Eluent: methanol-water-0.2 M phosphate buffer (50:45:5, v/v/v with 2.5 mM CTMA added. Symbols; •, CTMA; △, phenethylamine; ×, benzene; ○, phenol; □, benzoic acid.

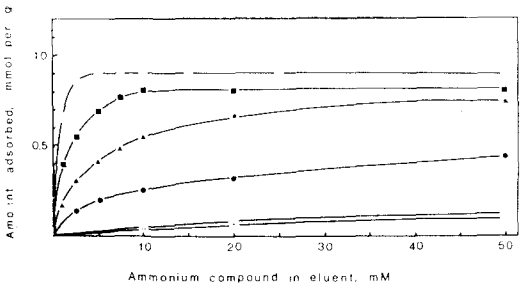

Fig. 3. Amount of quaternary ammonium salt adsorbed per gram of silica vs concentration in eluent.

Key: ○, tetrabutylammonium bromide; △, tetrapentylammonium bromide; •, dodecyltrimethylammonium bromide; ▲, tetradecyltrimethylammonium bromide; ■, cetyltrimethylammonium bromide; □, stearyltrimethylammonium bromide. Eluent: methanol-water-0.2 M potassium phosphate (pH 7.5) (50:45:5, v/v/v) with the appropriate concentration of quaternary ammonium compound added.

are adsorbed on to the silica. At pH values above 8 the solubility of the silica increases rapidly. To avoid any dissolution of the silica in the analytical column it is essential to saturate the eluent with silica even at lower pH values. This saturation is most conveniently performed by the use of a saturation column, packed with e.g. Lichroprep Si-60, 15~25 μm, and situated between the HPLC-pump and the injection device.

When the chromatographic system has reached equilibrium it behaves very much like a reversed-phase system using chemically bonded phases[15]. However, the dynamically modified system offers more parameters to vary in order to obtain a given selectivity, e.g. the amount of stationary phase (and thereby the retention) may be varied by changing the concentration and nature of long chain quaternary ammonium ions, the pH, or the nature of the organic modifier in the eluent[15,17~18]. The eluting strength of the mobile phase may be increased by increasing the concentration of organic

modifier and/or the ionic strength. Also gradient elution may be used in the dynamically modified silica mode[19] either by increasing the ionic strength (thereby causing a selective gradient elution of anionic solutes), by increasing the concentraiton of the long chain quaternary ammonium ions from an initial concentration above the critical micellar concentration (micellar gradient elution) or by increasing the concentration of organic modifier. The latter method can, however, only be used under very strictly controlled conditions. (Fig. 4, 5) It has been found that under the conditions investigated and with a minimum of 5% of organic modifier in the eluent only a monomolecular layer of the long chain quaternary ammonium compound is formed on the silica surface[10,14]. A coverage in the order of 1~

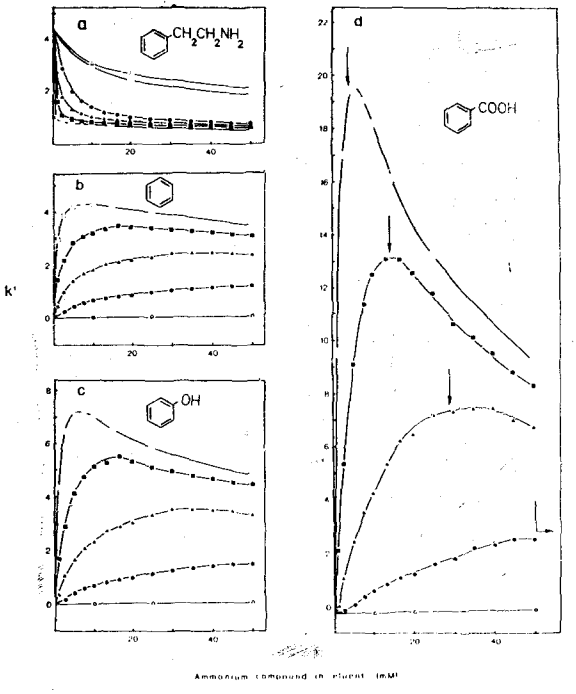

Fig. 4. Phase capacity ratio(k') values for four test solutes vs the concentration of quaternary ammonium salt in the eluent: a, phenethylamine; b, benzene; c, phenol; d, benzoic acid. Symbols and eluent composition as in Fig. 3. The arrows in d indicate the estimated critical micellar concentrations.

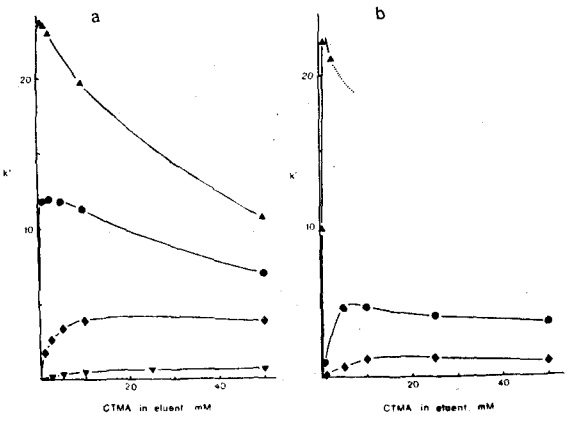

Fig. 5. Relationship between the concentration of CTMA in the eluent and the retention of benzene using various concentrations of methanol (a) or acetonitrile (b) Symbols: ▲, 10%; ●, 30%; ◆, 50%; ▼, 75%.

2 μmol ammonium compound per m^2 surface of the silica is obtained. This is to be compared to about 3 μmol/m^2 in the most heavily loaded monomolecular layers of chemically bonded phases. Using eluents without organic modifiers a multilayer may be formed[20]. Three main retention mechanisms may be observed in chromatography on dynamically modified silica. All solutes will be subject to a general reversed phase retention mechanism. Anionic solutes will form ion-pairs with the long chain quaternary ammonium ions in the eluent and as such be retained by the reversed phase mechanism. Due to the formation of the very lipophilic ion-pairs anions are most often relatively strongly retained. Cations are mainly retained by a reversed phase mechanism and only very lipophilic cations are in fact able to compete with the quaternary ammonium ions for the ionic sites on the silica.

Biomedical and pharmaceutical application—The dynamically modified silica approach has been successfully used in the analysis of drugs and other organic substances in biological matrices. In the following sections applications covering determination of drugs and their

metabolites in biological samples, analysis of natural products, determination of endogenic metabolites and the metabolites of an occupational chemical are discussed. These applications will also exemplify some of the other features of dynamically modified silica.

1. Assay of 5-aminosalicylate and its acetylated metabolite

5-aminosalicylic (5-ASA) acid is active in the treatment of inflammatory bowel disease. The drug is readily oxidized and it is difficult to extract from aqueous solution due to its

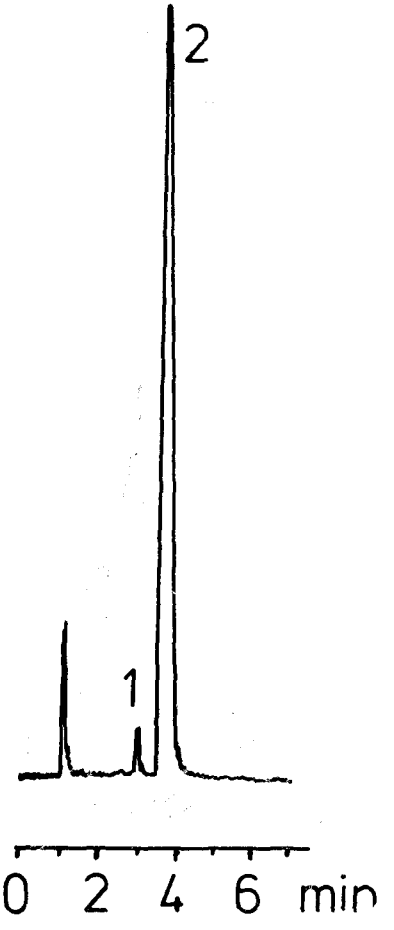

Fig. 6. Separation of 5-aminosalicylate (5-ASA) (30 ng/ml) and acetyl-5-aminosalicylate (Ac-5-ASA) (1.02 μg/ml) in plasma. Column: Lichrosorb Si-60 (120×4.6 mm). Eluent: methanol-water-0.2 M potassium phosphate buffer (pH 7.5) (50:45:5) with the addition of 2.5 mM CTMA. Flow rate: 1.5 ml/min. Detection: Fluorescence ex. 315 nm, em. 470 nm. Temperature: 30°. Peaks: 1, 5-ASA; 2, Ac-5-ASA.

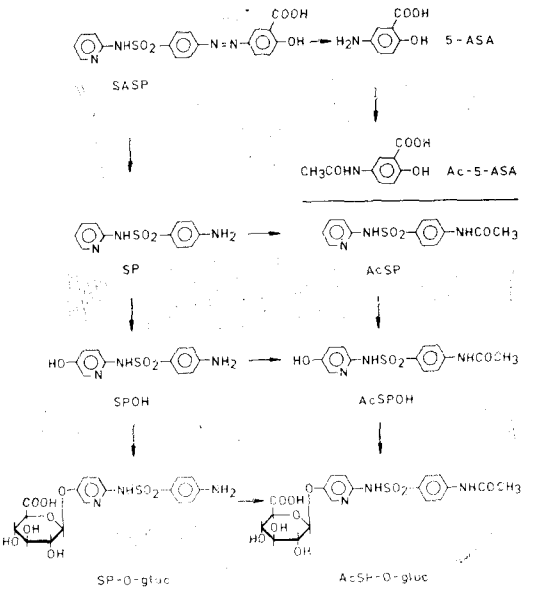

Fig. 7. The metabolism of salicylazosulphapyridine (SASP).

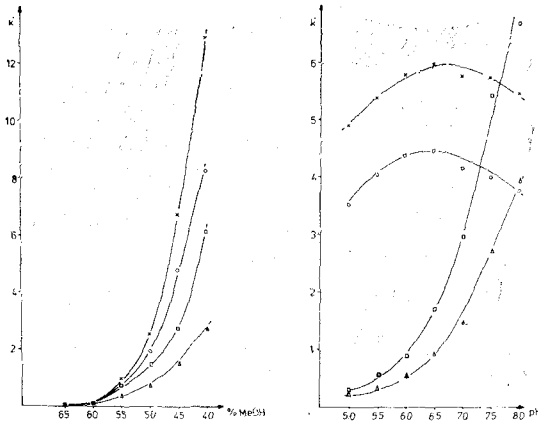

Fig. 8. The influence of pH and modifier concentration in the eluent on the retention of the metabolites of SASP. Column: Lichrosorb Si-60(120×4.6)mm. Eluent: A: methanol-water-0.2 M potassium phosphate buffer (pH from 5 to 8) (45:50:5) with the addition of 2.5 mM of CTMA; B: As in A with buffer pH 7.0 and with various amounts of methanol. Symbols: ○, 5-ASA; ×, Ac-5-ASA; △, sulphapyridine; □, acetylsuphapyridine.

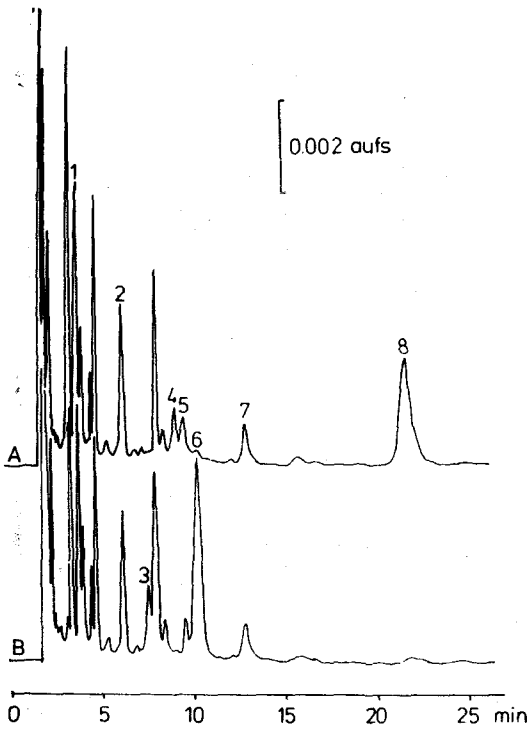

Fig. 9. Chromatogram obtained with a urine sample from a patient treated with SASP. A, Before and, B, After treatment with β-glucuronidase. Detection: UV, 254 nm. Other chromatographic conditions as in Fig. 8 A with buffer pH 6.8. Peaks: 1, sulphapyridine; 2, acetylsulphapyridine; 3, OH-sulphapyridine; 4, sulphapyridine-O-glucuronide; 5, 5-ASA; 6, OH-acetylsulphapyridine; 7, Ac-5-ASA; 8, acetylsulphapyridine-O-glucuronide.

high polarity. Accordingly, the drug is only weakly retained in reversed-phase systems. A method was developed including sample preparation by mixing the plasma with 4 parts of methanol[21]. Since the eluent contains 50 per cent of methanol (Fig. 6) no problems were encountered in injecting samples containing 80 per cent of methanol. The CTMA ions form lipophilic ion-pairs with the salicylates thereby causing a suitable retention. This method has been extended to a separation of all major metabolites of salicylazosulphapyridine (SASP) (Fig. 7), a drug used for similar clinical purposes. The influence of the pH and methanol

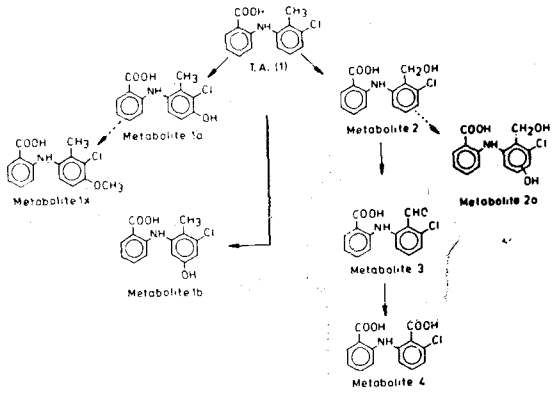

Fig. 10. Metabolism of tolfenamic acid (T.A.).

concentration on the retention of some of the metabolites is seen in Fig. 8[22]. The hydroxylated metabolites of sulphapyridine exhibit a higher retention than the corresponding non-hydroxylated metabolites. This is a common phenomenon in the dynamically modified silica approach and it is due to the high affinity of CTMA to groups capable of exchanging a proton. A chromatogram of a urine sample from a patient treated with SASP is seen in Fig. 9.

2. Determination of tolfenamic acid and its metabolites

Tolfenamic acid is a non-steroid anti-inflammatory drug having at least seven metabolites (Fig. 10). A chromatographic system based on dynamically modified silica was developed for the separations of this mixture[23]. In Table I. is shown investigations concerning the modifier used and the surface area of the column as parameters for optimizing the system, including the time of analysis. The optimized separations is seen in Fig. 11.

3. Phenols in urine

In a study on the occupational exposure to toluene one of the objects was to investigate whether a prolonged, daily exposure (more than 10 years) to toluene could alter the kinetics of the metabolism and excretion of the inhaled

Table I. Retention a tolfenamic acid and some of its metabolites when changing the organic modifier in the eluent containing 0.01M potassium phosphate buffer (pH 7.5) and 1.25 mM CTMA. Flow rate: 1.5 ml/min.

Organic modifier	% v/v in eluent	Column packing material	k'			
			Metabolite la	Metabolite 3	Metabolite 2	Tolfenamic acid
Methanol	60	Lichrosorb Si-60	4.8	5.0	6.5	14.4
Acetonitrile	40		5.9	8.7	9.0	19.8
Acetonitrile + methanol	25 / 20		7.0	8.0	9.9	23.0
Acetonitrile + methanol	25 / 20	Polygosil 60	3.4	4.1	5.0	11.0

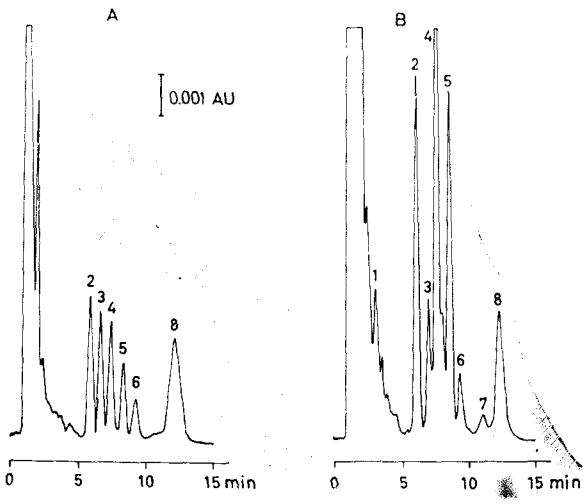

Fig. 11. Chromatograms of a plasma sample spiked with tolfenamic acid and its metabolites (10 μg of each per ml plasma) (A), and of a urine sample from volunteer no. 1 on day no. 5 at 12 h in a multiple dose study (B). Chromatographic conditions as stated in Table 8 for the Polygosil 60 column. Peaks: 1, metabolite 2a; 2, metabolite la; 3, metabolite 3; 4, metabolite 2; 5, metabolite 1b; 6, metabolite 4; 7, metabolite 4; 8, tolfenamic acid.

solvent. The major metabolite of toluene is benzoic acid, which is excreted in urine as the glycine conjugate, hippuric acid. Unfortunately, this substance is a common endogen metabolite normally present in concentrations ranging between 0.2 and 1.0 mg/ml in urine. o-Cresol is a minor metabolite since only ab. 0.1 per cent of the toluene is excreted in this form. It is excreted as conjugates with glucuronic acid and sulphuric acid. o-Cresol is present only in very low amounts in urine from normal, non-

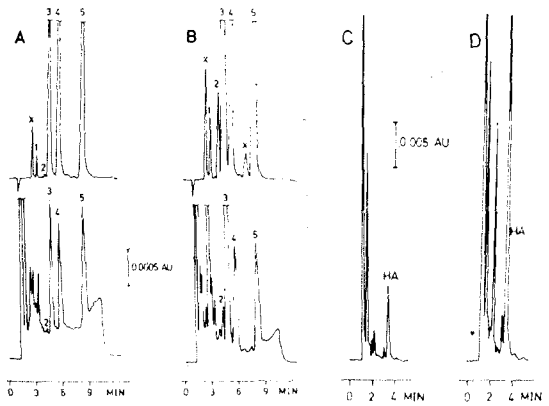

Fig. 12. Determination of urinary o-cresol. A: Normal urine (0.03 μg o-cresol per ml). B: Urine from a person exposed to toluene (0.9 μg o-cresol per ml). Upper traces: Fluorescence detection (ex. 273 nm, em. 298 nm) Lower traces: UV detection at 254 nm. Column: Lichrosorb Si-60 (120×4.6 nm). Eluent: acetonitrile-water-0.2 M potassium phosphate buffer (pH 7.5) (30:65:5) with the addition of 1.25 mM CTMA. Peaks: 1, phenol; 2, o-cresol; 3, *m*-and *p*-cresol; 4, 2,5-dimethylphenol: X, unknowns. Determination of urinary hippuric acid (HA). C: Normal urine (0.29 mg HA per ml). O: Urine from a person exposed to toluene (3.35 mg HA per ml).

smoking humans[24].

The method developed, based on dynamically modified silica, made it possible to use the same HPLC system for the determination of both hippuric acid and o-cresol. In Fig. 12 typical chromatograms are shown. o-Cresol elutes just prior to *m*- and *p*-cresol. This critical selectivity was kept constant during a period of more than three years, in which period of time the method was set up two to three times a year and using several columns (the columns were also used for other purposes).

4. Separation of catecholamines

Catecholamines in biological samples as well as in pharmaceutical preparations have been determined using the dynamically modified silica approach[25] The polar, biogene amines are retained even when using 80 per cent of methanol in the eluent (Fig. 13). It is therefore possible to use a very limited sample preparation before introducing the sample into HPLC-system. The urine is simply diluted with

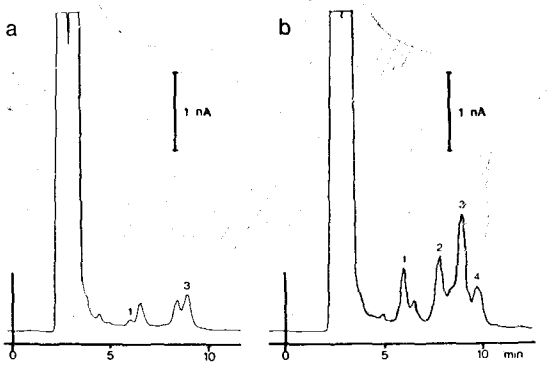

Fig. 13. Chromatograms of urine samples with (b) and without (a) the addition of catecholamines and internal standard (3.5 μg injected of each). Column: Lichrospher Si-100 (250×4.6 mm). Eluent: methanol-water-0.2 M potassium phosphate buffer (pH 7.0) (80:15:5) with the addition of 2.5 mM CTMA. Electrochemical detection: polarization voltage: 400 mV. Flow rate: 1 ml/min. Peaks: 1, noradrenaline; 2, 3,4-dihydroxybenzylamine; 3, dopamine; 4, adrenaline.

an equal amount of methanol. Of importance in simplifying the analysis is also the fact that all non-ionic and anionic compounds, which are retained by reversed-phase partition only, are eluted at short retention times, due to the high modifier concentration and the low amount of CTMA adsorbed (ab. 0.1mmole/g of silica). Thus in this case the most important effect of adding CTMA to the eluent is that it improves the peak shape of catecholamines and at the same time prevents the retention of cationic compounds by an ion-exchange mechanism.

5. Assay of opiates in opium

Determination of opiates in opium and in pharmaceutical preparations has always been a challenge to the chromatographer due to a considerable difference in polarity between the five major alkaloids. A study of the effect of changing the pH and the amount of methanol in the eluent is seen in Fig. 14.[26]. The relatively apolar alkaloids papaverine and noscapine seem to be retained by reversed-phase partition only, while the retention mechanism for the more polar alkaloids, which exhibits a stronger cationic character, may be explained as being composed of reversed-phase partition and cation exchange. At lower pH, where the alkaloids are highly ionized and the loading on CTMA on the silica surface is low, the retention is primarily due to cation exchange, However, as the pH is increased, the protonation of the alkaloids decreases and the CTMA-loading on the silica surface increases with the result that the reversed-phase mechanism is dominant. Thus codeine is eluted before thebaine. The reason for the still increasing retention of morphine with increasing pH is an ion-pair formation between the phenolate group in morphine and CTMA. Combining these findings with those of the study on the effect of the modifier concentration makes it possible to devise an HPLC-system with a sufficient selec-

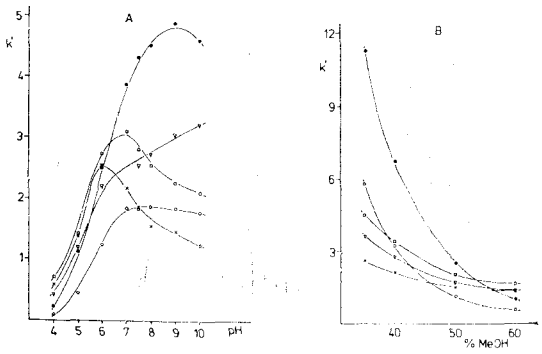

Fig. 14. A: Retention of five opiates versus the pH in the buffer used in the eluent. Column: Lichrosorb Si-60 (120×4.6 mm). Eluent: methanol-water-0.2 M potassium phosphate buffer (50:45:5) with the addition of 2.5 mM CTMA.

B: Retention of five opiates versus the concentration of methanol in the eluent. Buffer pH 7.0, other chromatographic conditions as in A. Symbols: ▽, morphine; ×, codeine; □, thebaine; ○, papaverine; ●, noscapine.

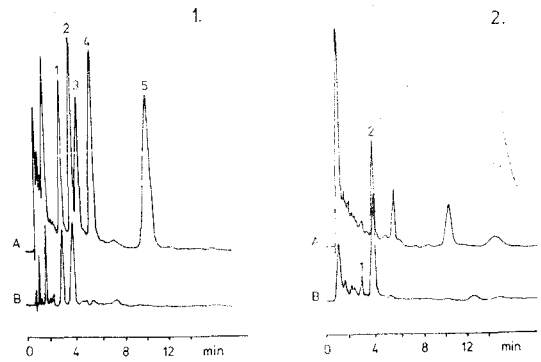

Fig. 15. Determination of opiates in opium (1) and in a cough mixture (2). Column: As in Fig. 14. Eluent: As in Fig. 14 with 35% of methanol and buffer pH 7.0. Detection: A: UV 254 nm. B: Fluorescence, ex. 285 nm, em. 325 nm. Peaks: 1, codeine; 2, morphine; 3, thebaine; 4, papaverine; 5, noscapine.

tivity and a reasonable retention of the five solutes. It should be noticed that codeine elutes

Table II. Separation factors between propranolol and three of its possible impurities, measured on eight different ODS-silica columns

Column packing material	Pore size(nm)	Separation factor		
		II	III	IV
Silica				
Lichrosorb Si 60	6	0.26	1.86	2.22
Nucleosil 50-5	5	0.26	1.76	2.13
Zorbax SIL	7	0.24	1.76	2.08
Partisil 5	7~8	0.25	1.66	1.98
Spherisorb S 5 W	8	0.28	1.74	2.01
Lichrosorb Si 100	12	0.31	1.39	1.60
Nucleosil 100-5	10	0.26	1.43	1.66
Hypersil	10	0.26	1.40	1.66
ODS-silica				
Lichrosorb RP-18		0.40	2.43	3.81
Nucleosil-5 C_{18}		0.61†	2.40	4.69
Hypersil ODS		0.38	2.62	3.34
Zorbax ODS		0.38	2.97	4.38
Partisil 10 ODS		1.19†	2.05	4.48
Partisil 10 ODS 2		0.61	3.21	6.53
Partisil 10 ODS 3		0.56	2.42	3.71
Spherisorb S5 ODS		0.60	1.84	2.68

*Separation factor is defined as relative time with respect to propranolol.

Substances	R=1-naphthyl
I (Propranolol)	R−CH_2−CHOH−CH_2−NH−$CH(CH_3)_2$
II	R−CH_2−CHOH−CH_2OH
III	R−CH_2−CHOH−CH_2−N(−$CH(CH_3)_2$)−CH_2− CHOH−CH_2−O−R
IV	R−CH_2−CHOH−CH_2−O−R
V	R−CH_2−CH(O)−CH_2
VI	R−CH_2−CHOH−CH_2Cl

†The propranolol peak overlaps the peak in the chromatogram

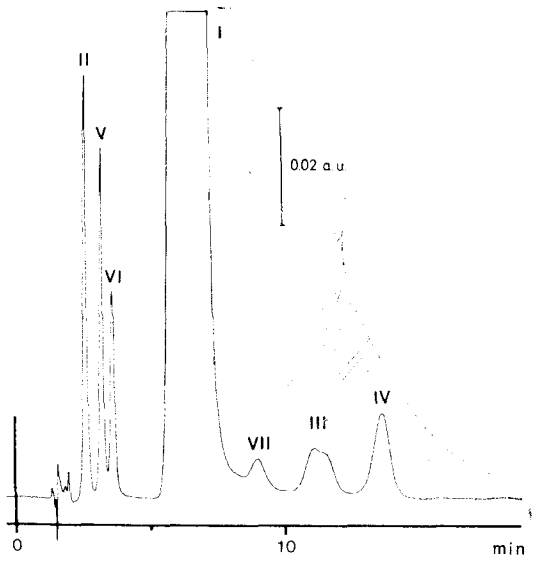

Fig. 16. Chromatogram of propanolol spiked with 0.3% m/m of each possible impurity. Column, Zorbax SIL (120×4.6 mm, i.d.); eluent, methanol-water-0.2 M potassium phosphate buffer (pH 8.0) (70:25:5, v/v/v), containing 2.5 mM CTMA; detection wavelength, 292 nm; flow rate, 1 ml/min. Peak identification: as in Table II, except for VII, which is an unknown impurity.

before morphine at pH values above 6.5. This is due to the ion-pairing between CTMA and morphine as mentioned above. The first eluted solute (codeine) has a k' value of about 3 and is thus well separated from the solvent front. Accordingly, the method is suited to the analysis of raw opium and other complex matrices as seen in Fig. 15.

6. Reproducibility of the selectivity

One of the most obvious advantages of the proposed approach is the high reproducibility of selectivity from batch and even between brands of bare silica from various manufacturers[10]. An example of this reproducible selectivity is given by the determination of impurities in propranolol[27]. The relative retention values of propranolol and some of its impurities on different brands of chemically bonded ODS-materials, and also on different brands of bare silica dynamically modified with CTMA, are given in Table Ⅱ. A chromatogram of the separationin in the latter system is shown in Fig. 16.

Conclusion

Reversed-phase HPLC based on dynamic modification of bare silica with quaternary ammonium ions has been developed. The main advantage of the approach is high reproducibility of the selectivity. This is especially important, when the same analysis has to be performed in different laboratories. The approach has been used in studies on drug metabolism. The determination of the toluene metabolite in urine, for example, emphasizes the importance of the reproducibility of selectivity, since many peaks due to endogenous phenols are present in the

chromatogram. Over a period of eight years using several new columns for this assay, no problems in reproducing the selectivity have appeared. Thus, chromatographic systems based on dynamically modified silica are very stable and may be used for long periods of time without loss in efficiency and without changes in selectivity. The solubility of the silica is a disadvantage that can be readily overcomed by installing a saturation column between the pump and the injection device.

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