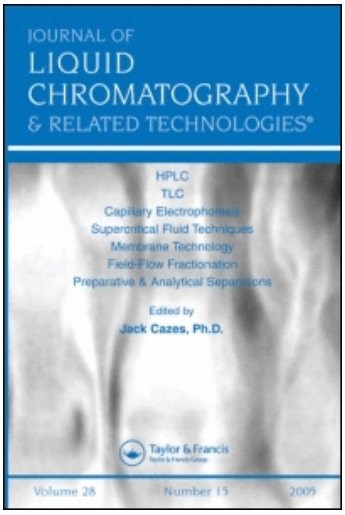

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Influence of the Structure of Chemically Bonded C_{18} Phases on HPLC Separation of Naproxen Glucuronide Diastereoisomers

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INFLUENCE OF THE STRUCTURE OF CHEMICALLY BONDED C_{18} PHASES ON HPLC SEPARATION OF NAPROXEN GLUCURONIDE DIASTEREOISOMERS §

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ABSTRACT

On the basis of Nucleosil-100 a series of materials with varying structure and different coverage density of chemically bonded C_{18} phases (CBP) were prepared. The physico-chemical characteristics of these packings e.g. porosity and carbon content were studied by the BET method and CHN analysis. The structure of the C_{18} CBP was determined by solid state CP/MAS NMR. The prepared packings and columns have been applied for HPLC separation of naproxen and its two diastereoisomeric conjugates with glucuronic acid. Material with monomeric C_{18} CBP structure and with high coverage density has given good and reproducible separation results.

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INTRODUCTION

Chemically bonded phases (CBP) are widely used in HPLC and it is estimated that about 80 % of chromatographic analyses are carried out with these phases. However, widely variable capacity factors (k') and selectivity (α_{ij}) are observed for the same solutes using various commercially available reversed-phase (RP) silica packings of the same type.

Several factors influence the properties of RP-silica packings [1,2]. One of these factors is the functionality of the silane used as a silica surface modificator. Monofunctional silanes are known to form well defined products whereas di- and trifunctional silanes can produce oligomer or polymer layers on the silica surface [3]. Although monomeric phases are usually considered to have the most reproducible properties, manufacturers use mono-, di- and trifunctional silanes to produce CBP [1,2].

In a previous study, we showed that the packing density of CBP had an important influence on the separation mechanism in RP HPLC [4]. The packing materials with varying structures of C_{18} CBP and different coverage density could be considered as an interesting application in analysis of some natural products and/or drugs.

Naproxen, 6-methoxy-α-methyl-2 naphtalene acetic acid, one of the nonsteroidal antiinflammotary drugs, and its two diastereoisomeric glucuronide conjugates were selected as testing substances. Naproxen contains a chiral carbon atom (Fig. 1.) and when conjugated with glucuronic acid forms two diastereoisomeric S- and R-naproxen glucuronides.

The separation of the diastereoisomeric glucuronides has been a goal of multiple studies [5,6]. Because of the impact of the stereoselective

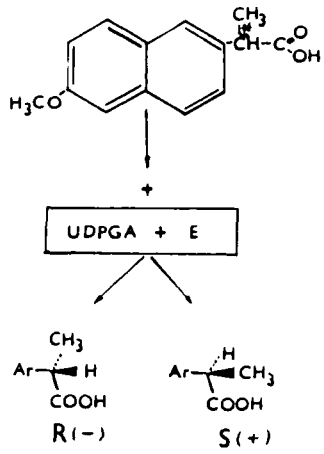

Fig.1. Stereochemical representations of naproxen and its two glucuronic acid conjugates R and S; where: UDPGA: uridine 5'-diphosphoglucuronic acid, E: UDP - glucuronyltransferase.

glucuronidation of drugs containing a chiral carbon atom on the pharmacokinetic and pharmacodynamic parameters, the isolation and quantitation of these conjugates were of upmost importance in clinical medical practice [7]. The selection of definitive separation system is dependent on the characteristics of the packing and the mobile phase composition in a way to be suitable for biological sample analysis.

EXPERIMENTAL

Materials and reagents

Nucleosil-100 with 7μm particles (Machery,Nagel & Co, Düren, FRG) was used as the support for the CBP.

Chemical modification was carried out using monochlorodimethyloctadecylsilane (ODMCS), dichloromethyloctadecylsilane (ODDCS) and trichlorooctadecyl-silane (ODTCS)(Wacker Chemie GmbH, München, FRG). Moreover, the following reagents were used: methanol, toluene, benzene, morpholine and hexane (E. Merck, Darmstadt, FRG). All reagents were of analytical grade.

The phases were packed into 125 x 4.6 mm I.D. stainless-steel tubes purchased from the Bischoff GmbH. (Leonberg, FRG).

The mobile phases were 70-30 % v/v MeOH-H_2O when testing all columns [4] and 0.05 M ammonium acetate with acetonitrile (80-20 % v/v) adjusted to pH = 6.0 with glacial acetic acid for separation of naproxen and its two diastereoisomeric glucuronides [5].

As test substances, diphenyl (Merck) and racemic naproxen (Syntex Corp., Palo Alto, CA, USA) were used.

Formation of glucuronic acid conjugates and sample preparation.

Diastereoisomeric naproxen glucuronides were synthetized enzymatically by incubating rat liver microsomes with 2 mM racemic naproxen at 37°C in the presence of 5 mM UDPGA and 2 mM $MgCl_2$ [5]. The incubation was carried out at pH = 6.5 to minimize the possibility of rearrangement of the formed glucuronides. The reaction was stopped by protein precipitation with 0.4 M HCl followed by centrifugation. The supernatant was then analyzed by HPLC.

Synthesis of CBP.

After placing the individual portion of the bare silica gels in glass reactors having the valves closed to prevent contact of the reagents with the environment

they were heated at 200 °C under vacuum (10^{-3} Pa) for 8 h. The reaction was started in the flask by injecting the agent mixture composed of silane and morpholine as activator in exact molar ratios [8]. Then, the reactor was heated for 10 h at 120 ± 5 °C. These controlled conditions of synthesis allowed the formation of packings with high and low surface concentration of alkyl ligands. The physico-chemical characteristics of the prepared packings were listed in Table 1.

Column packings and HPLC evaluation.

The columns were packed under a pressure of 50 MPa, using the Shandon pump (Shandon, Frankfurt, FRG). As a dispersing medium iso-propanol was used, but methanol as a packing mobile phase [9].

Evaluation of the prepared columns was made by determining the characteristic basic parameters such as number of theoretical plates (n_T)(per 125 mm column length), capacity factor ($k' = t_R - t_o/t_o$) and asymmetry factor (f_{As})[10]. In this instance, diphenyl was used as a test substance in the RP-HPLC systems and the flow-rate was 1 ml/min. Dead time (t_o) was determined on the basis of the solvent peaks [11].

The resolution of the diastereoisomeric conjugates was characterized by calculation of the separation selectivity ($\alpha_{RS} = k'_R/k'_S$) and resolution factor $R_S = 2(t_{RR} - t_{RS})/(w_S + w_R)$, where: t_{RR} is retention time of the peak R, t_{RS} of the peak S, w_R and w_S are the widths of R and S naproxen glucuronide peaks, respectively.

Apparatus.

Chromatographic measurements were made using a liquid chromatograph system consisting of a pump Model M-6000A (Waters Assoc., Milford, CA,

USA), an injection valve Model U6K (Waters Assoc.), and Waters Model 481 variable-wavelength spectrophotometer for measuring the UV absorbance at 285 nm and CI 4100 computing integrator (Milton Roy, Staffodshire, U.K.).

The porosity parameters characterizing the starting material and chemically modified packings were determined by the low-temperature nitrogen adsorption-desorption method using a Model 1800 Sorptomatic apparatus (Carlo Erba, Milan, Italy).

The degree of coverage of the surface by alkylsilyl ligands, α_{RP}, was calculated on the basis of the carbon percentage [12] determined with a Model 240 CHN analyzer (Perkin Elmer, Norwalk, USA).

Solid state NMR measurements were performed on a Bruker MSL 200 spectrometer with samples of 200 - 300 mg in double bearing rotors of ZrO_2. Magic Angle Spinning was carried out at a spinning rate of 4 KHz. Spectra of ^{29}Si CP/MAS NMR were recorded with a pulse length of 5 μs together with a contact time of 5 ms and a pulse repetition time of 2 s. All NMR spectra were externally referenced to liquid tetramethylsilane (TMS) and the chemical shifts were given in parts per million (ppm).

RESULTS AND DISCUSSION

Characterization of surface silanized packings.

Table 1 lists the physico-chemical characteristics for bare silica gel and after chemical modification by three various octadecylsilane.

Comparison of the percentage carbon contents of the prepared materials with dense coverage showed that a high coverage was achieved with monofunctional

Table 1. Physico-chemical characteristics of the packings, where: S_{BET} - specific surface area (m^2/g); V_p - pore volume (cm^3/g); D - mean pore diameter (nm); α_{RP} - concentration of C_{18} groups ($\mu mol/m^2$); ODMCS - monochlorodimethyloctadecylsilane; ODDCS - dichloromethyloctadecylsilane; ODTCS - trichlorooctadecylsilane.

Packing Number	Type of the packing	Column No.	Porosity S_{BET}	V_p	D	Coverage %C	α_{RP}
1	Nucleosil 100	-	283	1.092	15.45	-	-
2	Nucleosil ODMCS	I	181	0.652	14.34	18.22	3.51
3	Nucleosil ODMCS	II	215	0.855	14.86	7.86	1.29
4	Nucleosil ODDCS	III	192	0.683	14.25	15.25	2.94
5	Nucleosil ODDCS	IV	211	0.767	14.55	8.05	1.39
6	Nucleosil ODTCS	V	170	0.624	14.13	17.02	3.62
7	Nucleosil ODTCS	VI	188	0.723	14.42	13.35	2.66

modifier (packing No. 2; α_{RP}=3.51 $\mu mol/m^2$). However, the same high α_{RP} value was obtained with trifunctional silane (packing No. 6; α_{RP}=3.62 $\mu mol/m^2$). On the other hand, the difunctional silane revealed relatively larger difference in α_{RP} value, but this was consistent with blocking mechanism of the surface by modifier molecules [1,2]. Analysis of data from Table 1 for packings with low coverage density showed that the α_{RP} values could be arranged according to their silane functionality ODTCS > ODDCS > ODMCS.

The data obtained from elemental analysis could not be used as the only origin of information about the structure of CBP which wereformed after chemical modification. Hence, the surface characterization by solid state NMR spectroscopy of the prepared packings was necessary. Moreover, the structure of CBP

and porosity of the support of CBP had an important influence on the change of the mass transfer in the chromatographic process [2,10,12].

In Figures 2 - 4, the ^{29}Si CP/MAS NMR spectra of the starting silica gel (a) are shown together with spectra (b & c) resulting from different surface loads after mono-, di- or trifunctional modification. The changes in surface species concentration of the silica gel caused by chemical modification reaction are easily recognized in all sets of NMR spectra [12-15].

The ^{29}Si CP/MSA NMR spectrum of the bare silica gel exhibits three signals at - 91 ppm, - 100 ppm and -109 ppm, which can be assigned to geminal silanol groups Q_2,silanol groups Q_3 and siloxane groups Q_4, respectively (Fig. 2a, 3a & 4a). Because of the monofunctional modification an additional signal at + 13 ppm can be observed (Fig. 2b), the intensity of which is increasing with increasing carbon load (Fig. 2c). Correspondingly, the signal intensity of geminal silanol groups Q_2 and of silanol groups Q_3 is reduced, which is indicated the absence of a Q_2 resonance in the spectrum of the compound with highest carbon load.

In case of difunctional modification, overlapping broad signals of D_1, D_3, $D_4 + D_4'$ units can be seen. The increasing signal intensity of D_1 - D_3 units shows that under the used reaction conditions formation of D_4 cross-links is negligible. In contrast to difunctional modification, treatment with a trifunctional silane results in an increasing number of cross-linked T_4 and T_4' units with increasing surface load (Fig. 4b & c). Whereas in case of the phase with carbon load of 2.66 μmole/m^2 besides bidentates T_2 and polydentates T_4 and T_4' monodentates T_1 are formed, this type of species is totally concerted into crosslinks T_4 and T_4' in the phase with highest carbon load (Fig. 4c). Simultaneously with increasing number of cross-links, a clear decrease in number of silanol groups Q_3 is attained (Fig. 2c).

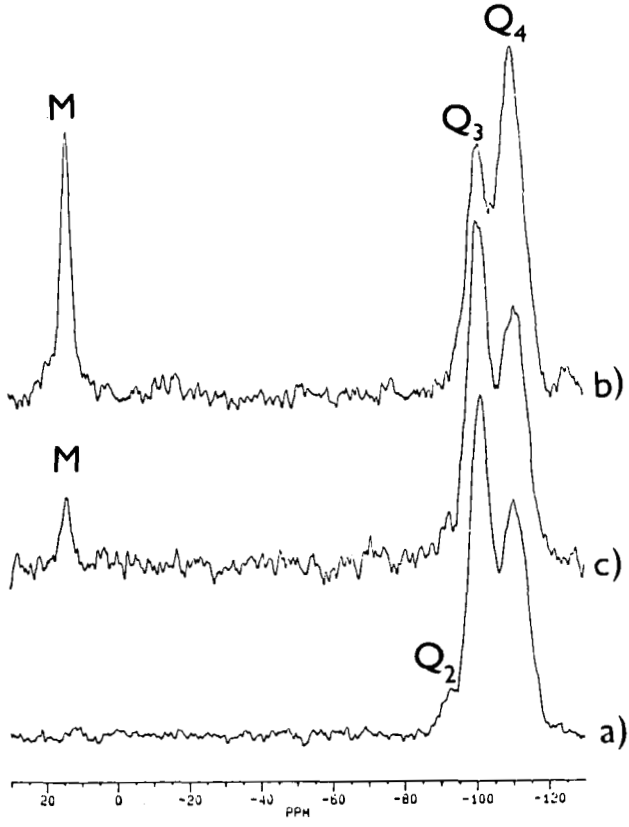

Fig.2. ^{29}Si CP/MAS NMR spectra of silica gel modified by monochlorosilane;

a) packing No.1. - unmodified silica gel,

b) packing No.2. - α_{RP} = 3.51 µmol/m²,

c) packing No.3. - α_{RP} = 1.29 µmol/m².

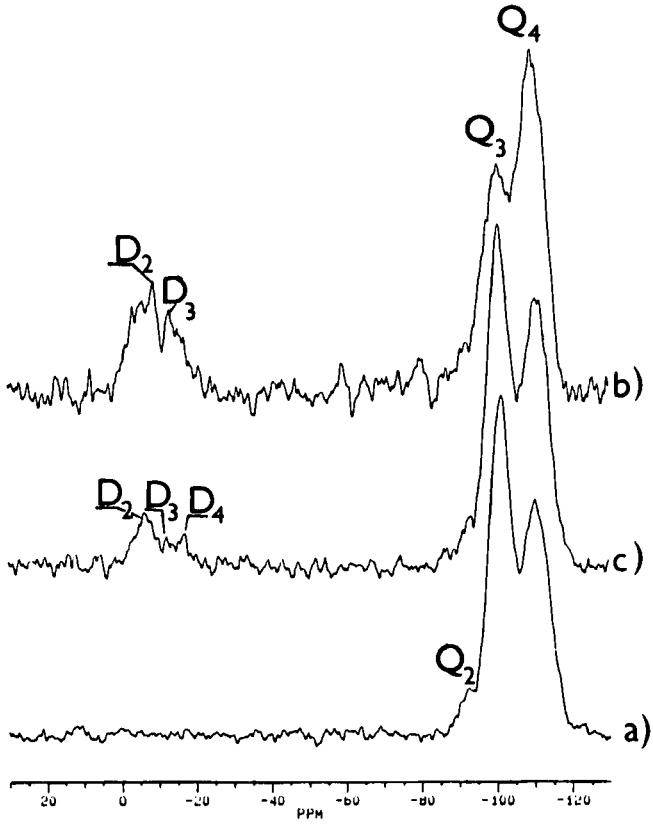

Fig.3. ^{29}Si CP/MAS NMR spectra of silica gel modified by difunctionally silane;
a) packing No.1. - unmodified silica gel,
b) packing No.4. - α_{RP} = 2.49 μmol/m²,
c) packing No.5. - α_{RP} = 1.39 μmol/m².

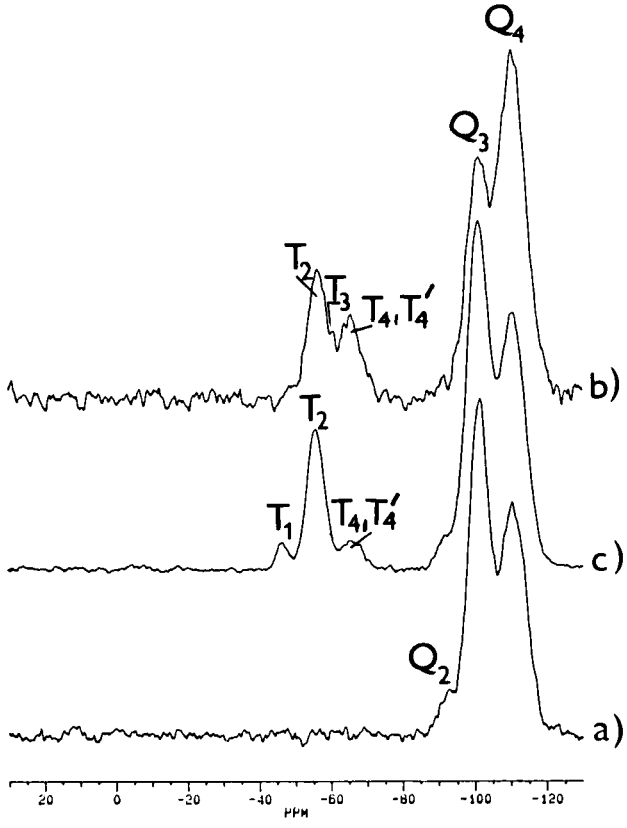

Fig.4. ^{29}Si CP/MAS NMR spectra of silica gel modified by trifunctionally silane;
a) packing No.1. - unmodified silica gel,
b) packing No.6. - α_{RP} = 3.62 μmol/m²,
c) packing No.7. - α_{RP} = 2.66 μmol/m².

The correlation of NMR spectra and CHN data with porosimetrical data (Table 1) showed that the surface area for material with high coverage density (packings No. 2, 4 & 6) was decreased by about 45 - 55 % when compared to bare silica. However, with low coverage density (packings No. 3, 5 & 7) the reduction was only 24 - 35%. Also, moderate decrease in the mean pore diameter and pore volume were observed. This is consistent with previously published data [4,12].

Columns evaluation.

Parameters characterizing the quality of the prepared columns are listed in Table 2.

Table 2. Chromatographic properties of RP columns (125 x 4.6 mm I.D.), mobile phase - 70 -30 % v/v MeOH - H_2O, where: k'- capacity factor; n_T - number of theoretical plates; h - reduced height equivalent to a theoretical plate; f_{As} - asymmetry factor; Δp - pressure at flow rate 1 ml/min.

Column number	Diphenyl as solute				Δp
	k'	n_T	h	f_{As}	
I	6.67	6230	2.82	1.08	55
II	2.11	5155	3.50	1.19	60
III	6.14	5735	3.11	1.19	45
IV	4.58	5400	3.33	1.28	51
V	6.48	5660	3.15	1.17	50
VI	2.61	5130	3.48	1.32	59

The columns investigated were characterized by similar high numbers of theoretical plates (n_T) which were counted per 125 mm column length. The reduced height equivalent to a theoretical plate (h) was within the typical range of 1.5 - 5.0 for "good" columns [10]. Differences in performance between the individual types of columns were related to different arrangement of packing particles in beds. The arrangement of the packing particles was affected by the structure and coverage density of CBP in surface [8].

The capacity ratio (k') shown in Table 2 correlated well with the coverage density. The influence of free silanol groups on the k' values was clearly expressed. The values of h and k' were inversely proportional with coverage density.

Similar finding appeared with comparison of the asymmetry factors (f_{As}). The columns with high coverage density packings (columns No. I, III & V; Table 2) showed lesser tendency to tailing of peaks (f_{As}=1.08 - 1.19) than columns with small coverage density (columns No. II, IV & VI; Table 2) (f_{As}=1.19-1.32). For example the column No. VI (Table 2) in spite of its intermediate coverage density (α_{RP}=2.66 μmol/m² - trifunctional silane) also gave nonsymmetrical peaks (f_{As}=1.32). Here, evidently appears the influence of the all residual silanol groups which have an equal participation in adsorption. This effect was described in detail in Ref. [16].

The working pressure was relatively low, but here were also differences between low and high coverage density resulting from large surface energy (mobile phase - stationary phase interactions) by packings with thinly coverage.

In general, these columns could be considered as good or even very good according to Bristow and Knox nomenclature [10].

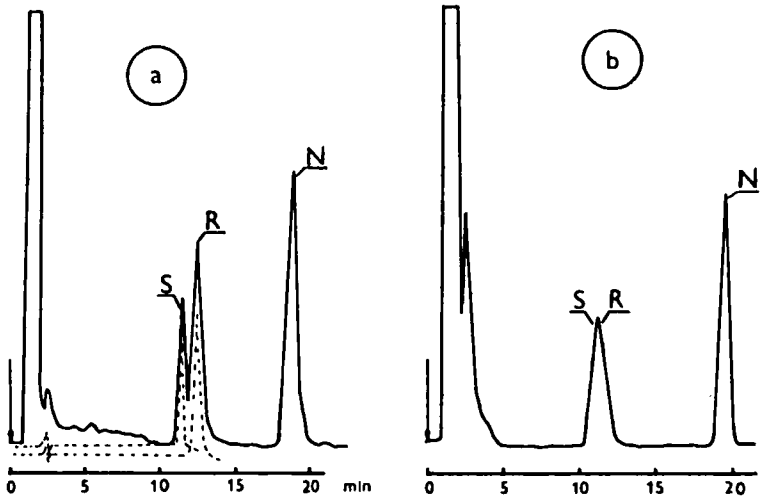

Fig.5. Separation of naproxen (N) and its two glucuronic acid conjugates R & S on the columns packed with monofunctionally C_{18} CBP;
a) high coverage density (column No.I.),
b) low coverage density (column No.II.).
Chromatographic conditions were as described in experimental part.

HPLC separation of the naproxen glucuronide diastereoisomers.

Chromatograms of naproxen and its two conjugated diastereoisomers obtained with the different columns are illustrated in Fig 5 - 7. Comparison of the separations on Fig. 5a - 7a obtained on columns with high coverage density materials (columns No. I, III & V, Tabele 1 & 3) and with different structure of CBP revealed that the values of selectivity (α_{RP}) and resolution (R_S) for these columns could be arranged according to silanes functionality i.e. mono- > di- > trifunctional. The capacity ratio (k'), α_{RS} and R_S values for both diastereoisomeric glucuronides R and S were presented in Table 3.

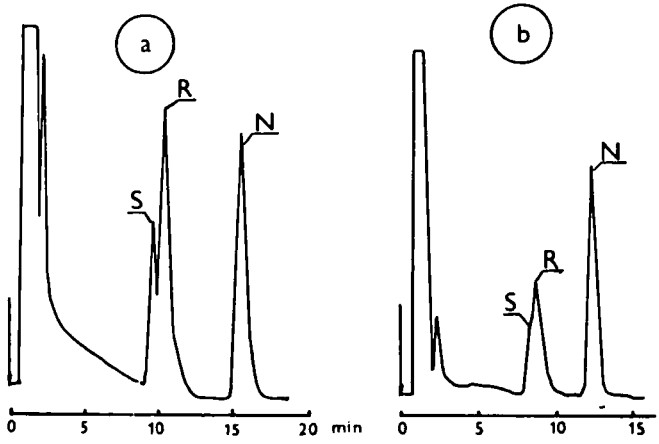

Fig.6 . Separation of naproxen (N) and its two glucuronic acid conjugates R & S on the columns packed with difunctionally C_{18} CBP;
a) high coverage density (column No.III.),
b) low coverage density (column No.IV.).
Chromatographic conditions were described in experimental part.

Table 3. Retention and resolution data for both diastereoisomeric naproxen glucuronides.

Column number	Capacity ratio k'_S	Capacity ratio k'_R	α_{RS}	R_S
I	3.63	4.25	1.176	1.18
II	3.54	3.64	1.00	-
III	3.58	3.92	1.096	0.94
IV	2.48	2.61	1.053	0.57
V	3.36	3.66	1.091	0.80
VI	2.95	3.20	1.085	0.76

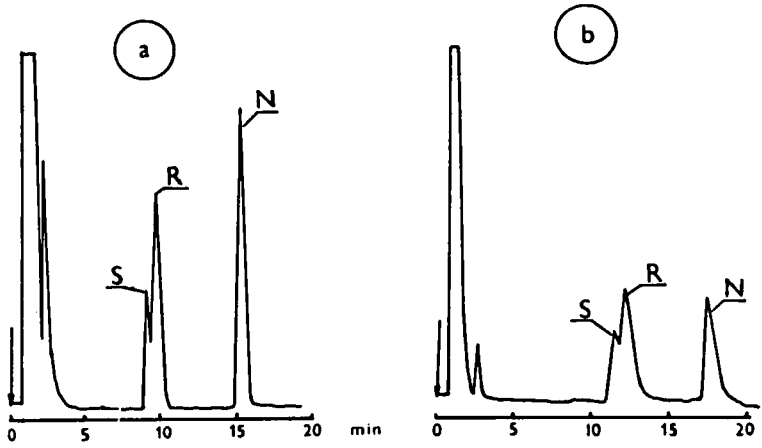

Fig.7 . Separatin of naproxen (N) and its two glucuronic acid conjugates R & S on the columns packed with trifunctionally C_{18}CBP;
a) high coverage density (column No.V.),
b) low coverage density (column No.VI.).
Chromatographic conditions as in experimental part.

The better resolution was obtained with column No. I which characterized by monomeric structure and high coverage density of CBP (α_{RP}= 3.55 μmol/m^2). This resolution was resulted from lower specific interaction between the separated solute molecules and the residual silanol groups (strong chain - chain interaction). Moreover, the change of mass transfer was also better, because of the monomeric structure of CBP material characterized by easier penetration of separated molecules [17-19].

The formation of the polymer structure of CBP on the support surface lowered the k' values and consequently, the α_{RS} and R_S values were also decreased. It appers that the participation of residual silanol groups developed interactions

between: solute - mobile phase - packing during the separation process (Table 3). Moreover, the change of the mass transfer was more difficult [17,18].

The reverse situation was observed with materials of low coverage density (columns No. II, IV & VI, Table 1 & 3). Analysis of the chromatograms (Fig. 5b - 7b) and data from Table 3 revealed that α_{RS} and R_S values were proportional with silane functionality. Consequently, change of coverage density altered the specific interaction between: solutes - mobile phase - packing. For example in the chromatogram presented in Fig. 7b (column No. VI) appeared two peaks corresponding to S and R naproxen glucuronides.

This effect was better illustrated by the relationship between R_S and α_{RP} (concentration of alkylsilyl ligands) presented in Fig 8.

This relationship indicated that:

(i) higher α_{RP} values were not always accompanied with better resolution,

(ii) material with cross-linked structure of C_{18} of CBP reduced the chances of mass transfer, probably due to reduced chain - chain interaction,

(iii) monofunctional packings with high coverage density permitted reproducible results in routine analysis of biological samples.

These suggestions were confirmed in Table 4 where the concentration of individual compounds of the mixture and their ratio are shown. The S/R ratio of diastereoisomers obtained from packings with high coverage density were relatively higher (column No. I) than those with low coverage density which were not possible to determine (Fig.5b & Fig.8; Table 3 & 4). Similary, a recovery ratio of diastereoisomeric naproxen glucuronides obtained from packing with high coverage density was near entity (Table 4).

In other instances the values were deviated from the unity possibly because of the irreversible sorption of one of the compounds on the packing surface (strong specific interaction).

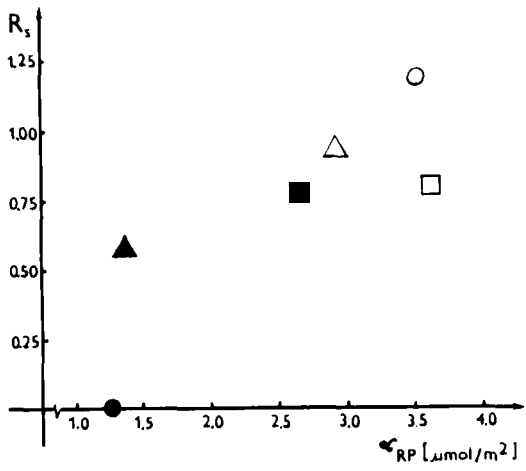

Fig. 8. Relationship between the resolution (R_S) of the two glucuronic acid conjugates R and S and the coverage density of C_{18} ligands (α_{RP}).

Table 4. Quantitative separation of standard diastereoisomeric glucuronides mixture.

Column number	Concentration in μg S	R	Ratio of S/R	Ratio of recovery
I	37.5	65.5	0.57	0.93
II	-	-	-	-
III	39.9	80.2	0.50	0.81
IV	-	-	-	-
V	34.3	80.9	0.42	0.69
VI	27.8	73.6	0.38	0.61

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