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INFLUENCE OF SOLUTE POLARITY IN COLUMN-SWITCHING CHROMATOGRAPHY FOR THE ASSAY OF DRUGS IN PLASMA AND URINE

JEAN-BAPTISTE LECAILLON*, NICOLE FEBVRE and CLAIRE SOUPPART
Ciba-Geigy, Biopharmaceutical Research Centre, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

SUMMARY

The polarity of a drug is one of the most important parameters for the elaboration of switching systems. If the polarity of the drug is low or medium, "reversed-phase" chromatography is well adapted. The plasma or urine sample is diluted with water, centrifuged and injected first into a column of medium polarity (C_2, CN or diol bonded phases). The compounds of interest are stopped on the top of the column and rinsed with water, then eluted and chromatographed on a C_8 or C_{18} analytical column. A third column of still lower polarity can be added to improve the specificity of the system. In each successive step, the polarities of the mobile phases and columns should be decreased to reconcentrate the sample and reduce the band broadening that occurred in the previous step.

Compounds of high polarity show almost no retention on reversed-phase columns, and normal-phase chromatography should be used. Aqueous solutions cannot be injected into polar bonded-phase columns as they lead to excessive band broadening. This problem can be solved by diluting plasma or urine with a large volume of a water-miscible organic solvent and injecting the clear supernatant. The compounds to be assayed are first reconcentrated on a polar column (NH_2 or $N(CH_3)_2$ bonded phase) and then eluted. The selected "heart cut" of the eluate is chromatographed on another, more polar column.

The influence of the polarity of drugs on the choice of switching systems is exemplified by assay methods for drugs of low, medium and high polarity.

INTRODUCTION

Column-switching systems previously applied in liquid chromatography[1–4] are of particular interest when one or several minor components are to be quantitated in a complex matrix[3–6], as in the determination of drugs in biological fluids, where the drug and possible metabolites are present at low concentrations in a complex medium.

Methods of liquid chromatography without column switching can be classified in two categories:

(a) Single-step methods, in which a small volume of the biological medium, diluted, if necessary, is directly injected into the analytical column after centrifuga-

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tion[7]. Such methods can be applied only if the concentration of the drug is high and the response of the detector is selective and high enough.

(b) Multiple-step methods, in which several purification steps are carried out before chromatography (extraction, back-extraction, cartridge purification, etc.[8]).

Switching systems should permit most of the multiple-step methods to be transformed into single-step procedures by on-line chromatographic purification. These systems should allow first the injection of a large volume of sample to achieve adequate sensitivity and second good reproducibility of the chromatographic steps.

When large volume are injected, good reproducibility may be obtained if the columns are not overloaded, *i.e.*, if the linear capacity of the columns is not exceeded[9]. This means that the first column should be filled with some high-load-capacity packing material (totally porous material, stable and highly bonded material, average particle diameter 10–40 μm). Overloading can also be partly overcome by diluting the sample before injection.

Separation and sensitivity may be improved if sample dilution on the columns (band broadening) is minimized throughout the analysis. This involves reconcentrating selected parts of the sample at the end or during each step, which may be achieved by using columns with successively stronger retention, and mobile phases with increasing elution strength.

Biological samples are very polar mixtures, as their major component is water. Reversed-phase chromatography is well adapted to the injection of aqueous samples and was used as the basis of previously described switching methods[8,10–19].

Nevertheless, for drugs of high polarity, the retention on reversed phases is poor and makes reconcentration of the sample difficult. In this instance, other chromatographic modes should be used. The chromatographic choices[20–23] are thus mainly governed by the polarity of the drug: solvophobic reversed-phase chromatography for non-ionizable and ionizable compounds of low or medium polarity[22–27], ion-suppression chromatography for weakly acidic or basic compounds[20,22], ion-pair chromatography for ionized compounds[22,28–29], use of silanophilic interaction for ionized basic compounds[29–31] and normal-phase chromatography with polar mobile phases for polar hydrophilic compounds[20,32]. The selectivity may be reinforced by making successive steps different from each other (different types of packing material, different manufacture[33–36], different chromatographic mode). The structures of commercially available bonded materials have recently been studied[33] and provide useful information for the choice of bonded materials.

This paper presents three examples of column-switching systems, applied to the quantitative assay of drugs with low, medium and high polarity, and demonstrates that systems are available, irrespective of the polarity of the drug.

EXPERIMENTAL

Equipment

The chromatographic systems were constructed from the following components: HPLC pumps: Model 302 and 303 (Gilson, Villiers-le-Bel, France), Constametric Model III (LDC, Riviera Beach, FL, U.S.A.) or Model SP 8700 (Spectra-Physics, Santa Clara, CA, U.S.A.); an automatic sample injector, composed of a Model SC6 (Gilson) or Model IV sampler (Technicon, Tarrytown, NY, U.S.A.), a

Minipuls Model II (Gilson) or Model III (Technicon) peristaltic pump and a Model AH-CV-UHPa-N60 air-actuated sample injection valve (Valco, Houston, TX, U.S.A.), equipped with a 1- or 2-ml sample loop. The system was controlled by a Model 4270 computing integrator (Spectra-Physics), which actuated the valves and the sampler, recorded the chromatogram and displayed the areas of the chromatographic peaks. It also controlled the SP 8700 pump for elution gradient or flow-rate modifications. Detection was performed with a Model SF 970 spectrofluorimetric detector (Schoeffel, Westwood, NJ, U.S.A.) for switching system I, with a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.) equipped with a 405-nm filter for switching system II and with a Model Spectroflow 773 UV spectrophotometric detector (Kratos, Ramsey, NJ, U.S.A.) for switching system III.

Columns

Laboratory-prepared columns were used in this study. Their characteristics are given in Table I.

TABLE I

CHARACTERISTICS OF PACKINGS AND COLUMNS

Packing	*Manufacturer*	*Nominal particle diameter (μm)*	*Column* length (cm)*
Nucleosil C_8	Macherey, Nagel & Co. (Düren, F.R.G.)	5	10
Nucleosil C_{18}		3,5	10, 15
LiChrosorb RP-2	Merck (Darmstadt, F.R.G.)	5, 10, 25–40	10, 25
LiChrosorb RP-8		5	10, 25
LiChrosorb RP-18		5	10, 25
Hypersil C_8	Shandon Southern Products (Cheshire, U.K.)	3	10
Hypersil C_{18}		3	10
Co:Pell ODS	Whatman (Clifton, NJ, U.S.A.)	30–38	5
LiChrosorb diol	Merck	5	10
LiChrosorb CN		10	10
Nucleosil CN	Macherey, Nagel & Co.	5	10
Nucleosil $N(CH_3)_2$		10	10
Nucleosil NH_2		5	10, 25
Polygosil NH_2		5	10
LiChrosorb NH_2	Merck	5,10	10
Vydac PBP	Separations Group (Hesperia, CA, U.S.A.)	10	10
Spherisorb SW (not bonded)	Phase Separations (Queensferry, U.K.)	5	10
Chromegabond diamine	E. S. Industries (Marlton, NJ, U.S.A.)	5	10

* I.D. 4.7 mm.

Chemicals and reagents

Metoprolol, alprenolol, oxiracetam and 4-nitrodiphenylamine derivatives, containing N-methylpiperazinyl or N-methyl-N-oxide-piperazinyl moieties were supplied by Ciba-Geigy (Basle, Switzerland). All solvents and reagents were of analytical-reagent grade; acetonitrile was of spectroscopy quality (Merck, Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Switching system I (low-polarity drug)

Metoprolol is a secondary amine, with a pK_a in water of 8.9. Its *n*-octanol–aqueous buffer (pH 7.4) partition coefficient is 0.6; this is greatly increased when the pH is increased and is reduced at acidic pH. On reversed-phase material its retention time was increased at a basic pH of the mobile phase, corresponding to ion suppression of metoprolol, and considerably decreased when the drug was ionized under acidic conditions. An increase in the ionic concentration strongly decreased the retention at acidic pH owing to the increased ionization of metoprolol (Table II).

The influence of the acetonitrile content of the acidic mobile phase on the metoprolol retention shown in Fig. 1 can be explained by solvophobic and silanophilic interactions. With a low acetonitrile content, the retention increased, corre-

TABLE II

INFLUENCE OF IONIC CONCENTRATION ON THE CAPACITY FACTOR OF METOPROLOL

Column: LiChrosorb RP-18, 5 μm, 10 cm long. Mobile phase: 20% acetonitrile in 0.02 *M* acetic acid–sodium acetate.

CH_3COONa concentration (M)	*k'*
0.002	> 50
0.005	47
0.01	28
0.02	19

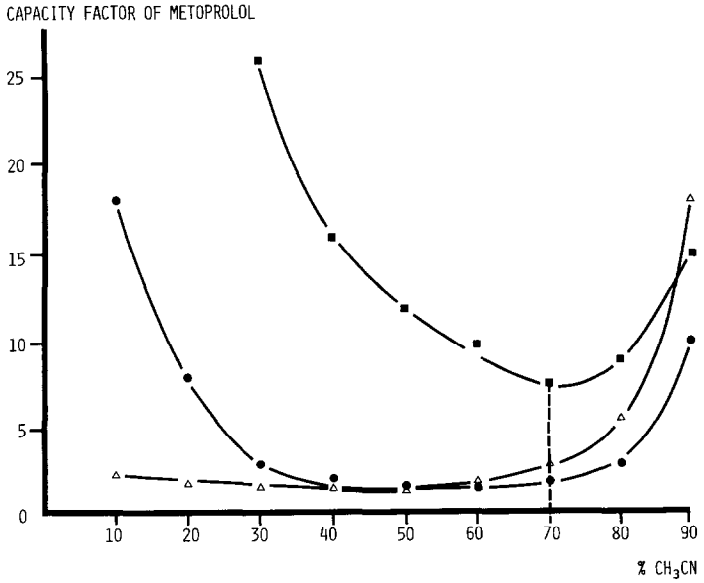

Fig. 1. Influence of the acetonitrile content in the mobile phase on the retention of metoprolol. Columns, LiChrosorb CN, 10 μm (△), LiChrosorb RP-2, 10 μm (●) and RP-8, 5 μm (■) 10 cm long; mobile phase, acetonitrile in 0.004 *M* CH_3COONa–0.04 *M* CH_3COOH.

sponding to solvophobic chromatography[23–26]. With an intermediate content of the organic solvent, a silanophilic interaction occurred[29–31]. At high organic solvent concentrations, the retention was considerably increased by silanophilic interactions, which became predominant. This previously described phenomenon[30,31] can increase the selectivity of chromatography.

As shown in Fig. 1, on more polar bonded materials (C_2 and CN), the retention was less marked in the solvophobic mode. When the silanophilic interaction appeared, the retention was more strongly modified on the CN packing. With 80% or more of organic solvent, the system behaved as in normal-phase chromatography.

The chromatographic system finally elaborated is shown in Fig. 2 and a sample chromatogram is given in Fig. 3. It involves both solvophobic and silanophilic interactions. The first, second and third steps, *viz.*, injection of 2 ml of diluted sample

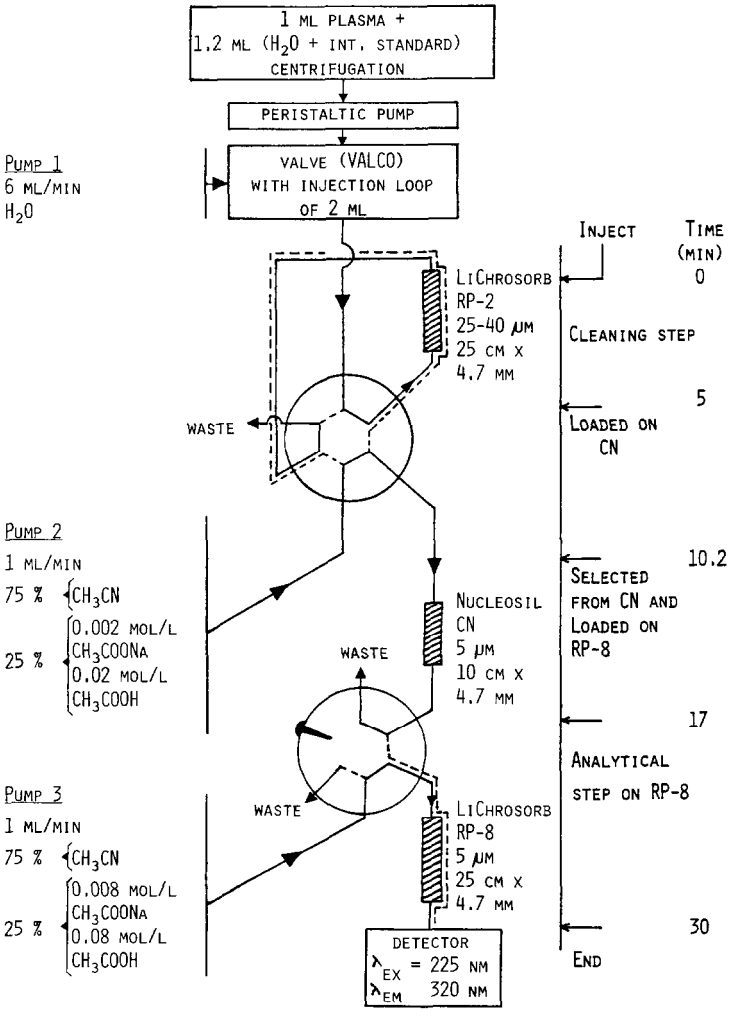

Fig. 2. Diagram of the column switching system for the determination of metoprolol.

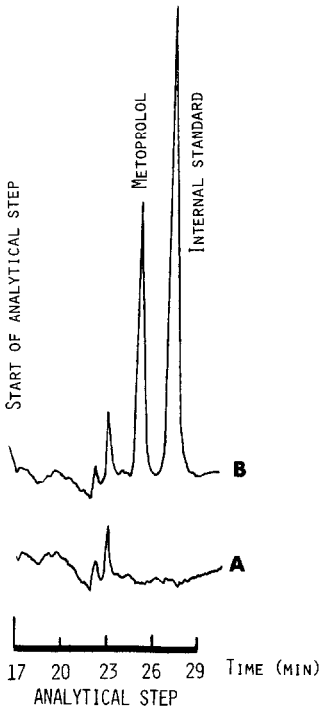

Fig. 3. Chromatograms of (A) a plasma blank and (B) the same plasma spiked with metoprolol and the internal standard. Chromatographic conditions as in Fig. 2.

(plasma–water, 1:1.2), reconcentration of the compounds of interest at the column head and washing the C_2 column with water and elution, involved the solvophobic mode. The fourth and fifth steps, heart cutting on the CN column and analytical chromatography on the C_8 column, involved both solvophobic and silanophilic interactions.

It should be noted that the procedure, in agreement with the two requirements defined above, involves no overloading of the column, thanks to the injection of a diluted sample into a long column, filled with totally porous material[9], and reconcentration of the sample at the end of each step by the use of mobile phases of increasingly greater elution strengths on columns with increasing retention characteristics. The reproducibility of the method is adequate and its sensitivity is comparable to that of a manual method[37].

Switching system II (medium-polarity drug)

The two 4-nitrodiphenylamine derivatives studied contain an N-methylpiperazinyl moiety (derivative 1) and an N-methyl-N-oxide-piperazinyl moiety (derivative 2). Both have amine functions and a pK_a value, measured in methyl Cellosolve solution, of 5.7. The partition coefficient of derivative 1 in *n*-octanol–aqueous buffer is 15 at pH 5.2, 430 at pH 7.4 and 640 at pH 9.

As shown in Fig. 4, the retention times of this compound on C_2, C_{18} and diol packings indicated solvophobic interactions when the acetonitrile or methanol con-

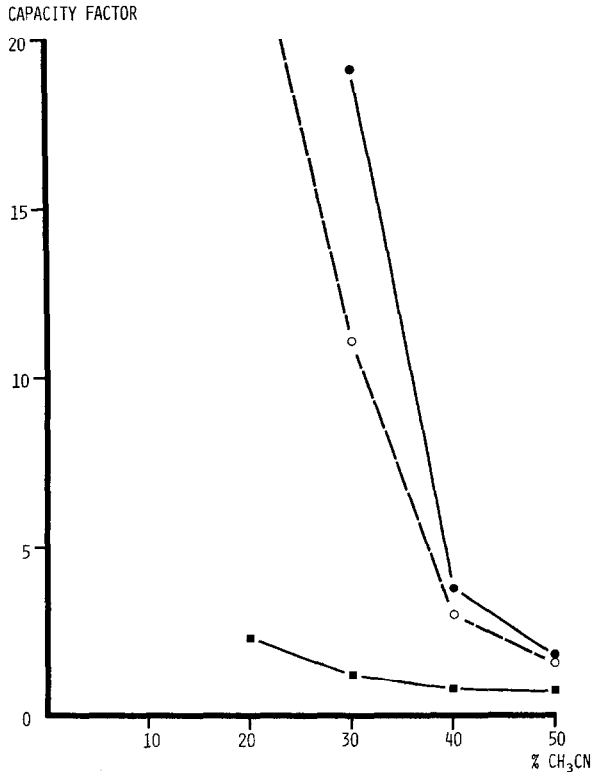

Fig. 4. Influence of acetonitrile content in the mobile phase on the retention of a nitrodiphenylamine derivative (Derivative 1). Columns, 10 cm long, LiChrosorb diol, 5 μm (■), LiChrosorb RP-2, 5 μm (○) and Hypersil C_{18}, 3 μm (●); mobile phases, acetonitrile in 0.0665 *M* phosphate buffer (pH 8.2).

tent of the mobile phase was less than 50%. The retention of the two derivatives on different packing materials in reversed-phase chromatography with the same mobile phase is shown in Table III. Large differences in capacity factors (k') (up to a factor

TABLE III

INFLUENCE OF PACKING MATERIAL ON THE RETENTION OF NITRODIPHENYLAMINE DERIVATIVES

Column as in Table I. Mobile phase: 40% acetonitrile in 0.0665 *M* phosphate buffer (pH 8.2).

Packing	k'	
	Derivative 1	*Derivative 2*
LiChrosorb NH_2	0.74	0.76
LiChrosorb diol	0.79	0.83
LiChrosorb RP-2	3.03	1.23
Hypersil C_8	3.18	0.96
Hypersil C_{18}	3.54	0.94
LiChrosorb RP-8	5.56	1.65
Nucleosil C_{18}	8.53	2.15
Nucleosil C_8	16.7	6.51

of 7) were observed between different C_8 or C_{18} packings. The stronger retention on Nucleosil C_8 than on Nucleosil C_{18} might be due to the end-capping of the C_{18} material and may indicate silanophilic interaction. The influence of the acetonitrile-to-methanol ratio on the retention at an constant organic solvent content of the mobile phase is shown in Fig. 5. The selectivity was apparently modified by merely changing from acetonitrile to methanol.

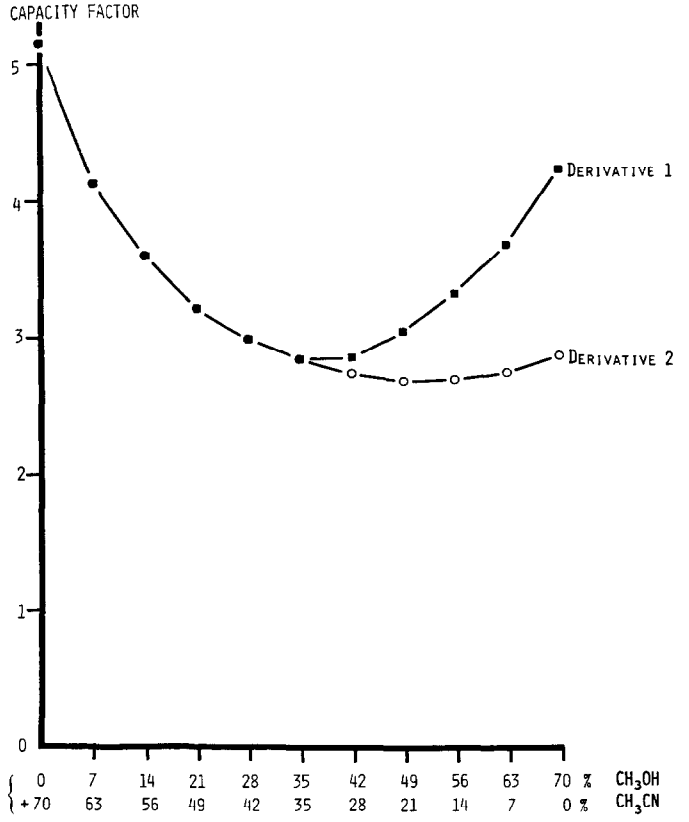

Fig. 5. Influence of the ratio of organic solvents on the selectivity of the nitrodiphenylamine derivatives. Column, Nucleosil C_8, 5 μm, 15 cm long; mobile phase, modifier 70% (acetonitrile + methanol) in 0.0665 *M* phosphate buffer (pH 8.2).

The switching system elaborated for the quantitation of the two 4-nitrodiphenylamino derivatives is presented in Fig. 6. A 2-ml volume of diluted sample (plasma–water, 1:4) was injected and the compounds of interest were concentrated at the top of a C_2 column. After washing and back-elution, the compounds were separated on a C_8 column. A sample chromatogram corresponding to the analytical step is shown in Fig. 7. Such a simple system appeared convenient for quantitation because of the selectivity of the detection of the two compounds at 405 nm, where few endogenic compounds absorb.

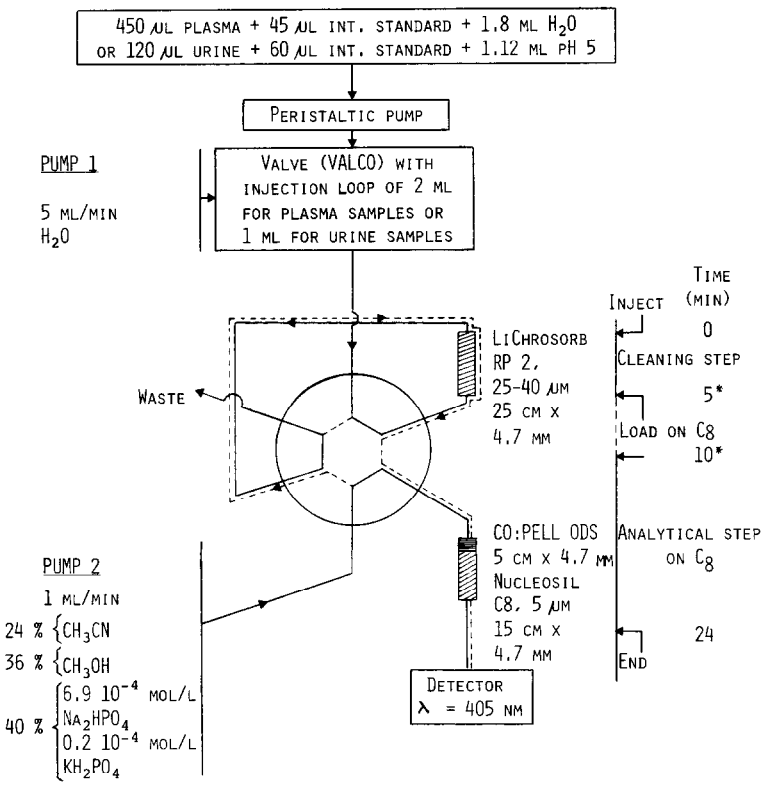

Fig. 6. Diagram of the column switching system for the determination of nitrodiphenylamine derivatives. Broken lines show the path between 5 and 10 min after injection.

Switching system III (high-polarity drug)

Oxiracetam has two amide functions and is very polar. Conditions for its quantitation were not favourable: its aqueous solubility is 0.3 g/ml in water, its *n*-octanol–aqueous buffer (pH 7.4) partition coefficient is 0.005 and that for *n*-octanol–0.1 *M* HCl is 0.006. Further, its maximum absorption is at about 200 nm, a wavelength at which many endogenic plasma components and organic solvents absorb. Initial results with C_{18} and C_8 columns and aqueous acidic or basic mobile phases without organic solvents indicated that oxiracetam was almost unretained, and that reversed-phase systems were inconvenient for switching chromatography. Normal-phase chromatography with totally organic mobile phases was not feasible, as attempts at extraction by organic solvents had been unsuccessful. Moreover, the injection of aqueous solutions into organic mobile phases led to chromatographic disturbances, extensive band broadening, elution and partial loss of the sample. Oxiracetam was slightly retained on polar columns when a polar organic mobile phase, containing a small amount of water (acetonitrile–water, 95:5) was used. Fig. 8 shows that the retention was greatly increased by adding dichloromethane or sulphuric acid to the mobile phase. This can be explained by the polarity decrease of the mobile phase due to dichloromethane and by the increased interaction of the solute with the polar sites of the stationary phase with acidic mobile phases[20,32]. The nature of the

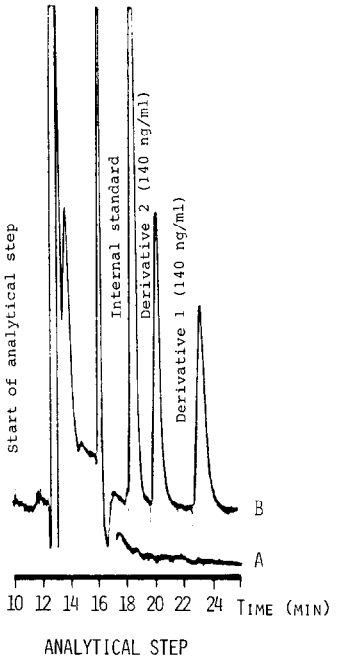

Fig. 7. Chromatograms of (A) a plasma blank and (B) the same plasma spiked with derivatives 1 and 2 and the internal standard. Chromatographic conditions as in Fig. 6.

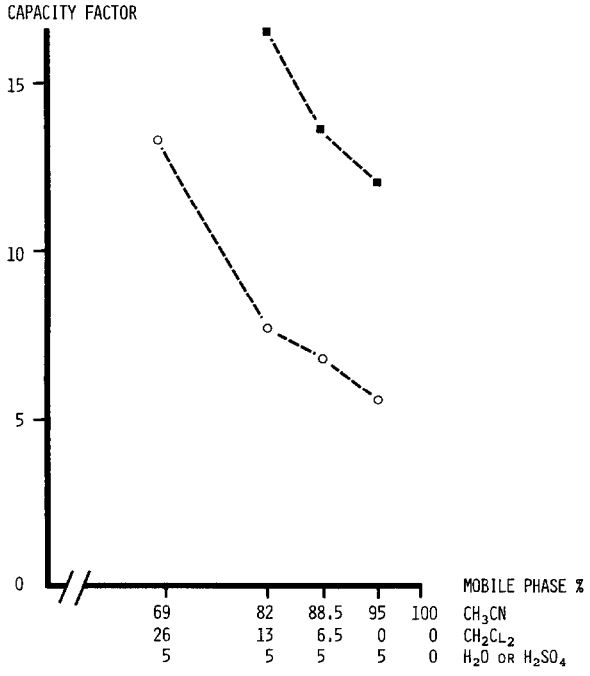

Fig. 8. Influence of dichloromethane and acid contents in the mobile phase on the retention of oxiracetam (normal-phase chromatography). Column, LiChrosorb NH_2, 5 μm, 10 cm long; mobile phase, acetonitrile–dichloromethane–water (○) or 0.14 *M* sulphuric acid (■).

polar bonded material and the origin of the packing material (manufacturer) also demonstrated strong influence on the retention (Table IV).

A satisfactory switching system was developed by injecting, after centifugation, 1 ml of an acidified plasma–acetonitrile–dichloromethane mixture, as shown in Fig.

TABLE IV

INFLUENCE OF PACKING MATERIAL ON THE RETENTION OF OXIRACETAM (NORMAL-PHASE CHROMATOGRAPHY)

Column as in Table I. Mobile phase: acetonitrile–water (95:5).

Packing	*k'*
Vydac PBP	0.65
Nucleosil $N(CH_3)_2$	1.50
Chromegabond diamine	2.89
Spherisorb SW	3.05
LiChrosorb NH_2	3.07
Polygosil NH_2	3.78
Nucleosil NH_2	6.62

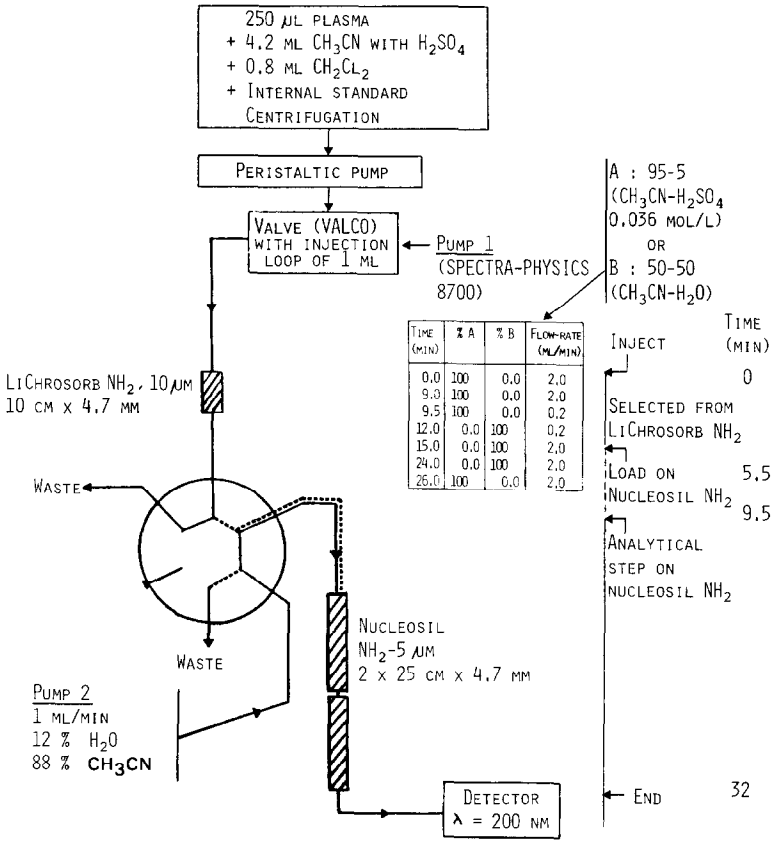

Fig. 9. Diagram of the column-switching system for the determination of oxiracetam. Broken lines show the path between 5.5 and 9.5 min after injection.

9. The sample was concentrated on the LiChrosorb NH_2 column and eluted. The selected heart cut was reconcentrated on the second column, having a stronger retention [Nucleosil NH_2 (Table IV)] and then eluted. A sample chromatogram is shown in Fig. 10. During the analytical step on the Nucleosil NH_2 column, the first column (LiChrosorb NH_2) was flushed with a more polar solvent to purge the strongly retained plasma components that interfered with the next oxiracetam analysis. The limit of quantitation was 0.2 μg/ml of oxiracetam in plasma (signal-to-noise ratio = 20), whereas a reported assay method involving direct plasma injection into a reversed-phase column for piracetam, an analogue of oxiracetam having a similar absorption, gave a sensitivity limit of about 30 μg/ml (ref. 38).

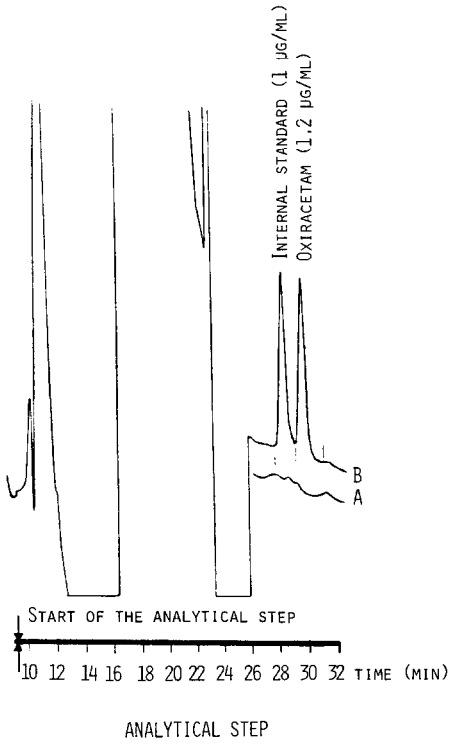

Fig. 10. Chromatograms of (A) a plasma blank and (B) the same plasma spiked with oxiracetam and the internal standard. Chromatographic conditions as in Fig. 9.

Elaboration of switching systems

The three methods reported above for compounds with large polarity differences indicate that a variety of components can be quantitated provided that their retention characteristics are different on two columns. Arrangement of bonded packings in order of decreasing polarity[20,32] (diamino > amino > cyano > dimethylamino > nitro > diol > C_2 > C_8 > C_{18}) gives an indication of the order in which the columns are to be used. In reversed-phase chromatography, the first column is usually packed with C_2 material and the subsequent columns with C_8 or C_{18} mate-

rials. For compounds of very low polarity, the first column of the system can be a CN column. The order of packings may be subject to variation, as shown in Tables III and IV, mainly owing to the functional groups of the sample compounds, the type of chromatography used, the characteristics of the silica, residual silanols and end-capping[33]. For instance, we have found that retention on Nucleosil C_{18} is always greater than on analogous LiChrosorb RP-18 or μBondapak C_{18} materials. Classification of analogous bonded materials has been proposed previously[24,28,29,34–39] and is important for the choice of columns.

When reversed-phase chromatography cannot be used, for instance for compounds of high polarity, polar columns with mobile phases of intermediate polarity, containing mainly organic solvents, can give convenient retention for switching systems. The first column would be a CN or an amino column and subsequent columns would have greater polarity. Selectivity and retention can also be increased by using bonded materials with a bonded moiety similar to the functional groups of the compound to be assayed[40].

CONCLUSION

The polarity of the solute is one of the most important factors for the elaboration of switching systems. Various possible choices have been described and exemplified for compounds with large polarity differences. This study has shown that the chromatography of polar hydrophilic drugs, which is a difficult problem, can also be solved with these systems.

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