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STRAIGHT-PHASE ION-PAIR CHROMATOGRAPHY OF ZIMELIDINE AND SIMILAR DIVALENT AMINES

II. THE CHROMATOGRAPHIC SYSTEM

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SUMMARY

The properties of a straight-phase ion-pair liquid chromatographic system are described. The system is based on perchlorate as the anion component in a strongly acidic stationary phase with methylene chloride–*n*-butanol as the mobile phase, and has been used for the separation of zimelidine, a divalent hydrophobic amine, and related compounds.

Batch distribution data for some of the amines as bases and as 1 + 1 and 1 + 2 ion pairs with perchlorate are presented and used to calculate capacity ratios, which were found to be in good agreement with the experimental chromatographic data. It is concluded that the retention mechanism is based mainly on liquid–liquid distribution and that selectivity factors can be calculated from batch extraction constants. The ion-pair equilibria were found to include an association of a 1 + 2 ion pair in the aqueous phase and also dissociation of the 1 + 1 ion pair in the organic phase.

The relationship between chemical structure and selectivity is discussed, and it is emphasized that it is complicated because of the possible existence of two kinds of ion pairs with the divalent amines. The baseline separation of four compounds that are both geometric and bromo-positional isomers demonstrate the excellent selectivity of the system in practice.

The capacity ratios increase both with increasing flow-rates and at very low flow-rates, but with maintained selectivities, and possible reasons are discussed.

The effects of the injection of large sample volumes (up to 500 μl) on chromatographic efficiencies and resolutions are demonstrated, and linear relationships between the standard deviation (σ) of the dispersion and the injected volume were obtained.

INTRODUCTION

Ion-pair extraction is a well established technique in analytical chemistry, and its fundamental properties regarding theory and applications in batch extractions and

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in modern liquid chromatography have recently been treated in a book[1] and several reviews[2–5].

This paper describes some chromatographic properties of a straight-phase ion-pair system based on a strongly acidic perchlorate solution as the stationary phase on irregular silica gel microparticles (5 μm) with a mixture of methylene chloride and *n*-butanol as the mobile phase. The compounds studied were a divalent amine, zimelidine, and some chemically related compounds.

The application of this system to the analysis of the compounds in biological material has been described earlier[6].

EXPERIMENTAL

Apparatus

The equipment used for chromatography, photometric measurements, pH measurements and ultrasonic homogenizations has been described elsewhere[6]. Two-phase titrimetric experiments and potentiometric titrations were performed with equipment obtained from Radiometer (Copenhagen, Denmark), namely a pH meter (PHM 26) with glass (9202 B) and calomel (K 401) electrodes or, in the determinations of extraction constants at ionic strength 1, an Orion Model 90-02 double-junction electrode, with 1 *M* sodium chloride solution as the salt bridge. The titrant was added with a 0.5-ml Agla micrometer syringe (Wellcome Reagents, London, Great Britain) or a Dosimat E 535 (Metrohm, Herisau, Switzerland) with an E 552-1B micro-exchange unit.

Chemicals

Most of the chemicals, including the chromatographic support (Partisil 5), have been described previously[6]. Zimelidine, norzimelidine, zimelidine N-oxide, zimelidine *E*-isomer, norzimelidine *E*-isomer, compounds I, II, III, V, VI, XI, XII and XIII were obtained from the Department of Organic Chemistry, Astra Läkemedel AB (Södertälje, Sweden), and compounds IV, VII, VIII, IX and X from AB Hässle, Gothenburg, Sweden (see Table IX). They were in the form of bases or salts with chloride, oxalate or maleate and were used as received.

Sodium chloride, sodium sulphate, sodium perchlorate and perchloric acid were of analytical (pro analysi) quality from Merck (Darmstadt, G.F.R.); sulphuric acid (concentrated) was Chemtam® (P-H Tamm, Gothenburg, Sweden).

Column packing and coating

The procedure has been described in detail elsewhere[6]. With methylene chloride–*n*-butanol (89:11) as the mobile phase the volume of the stationary phase (V_s) on the column (150 × 4 mm) was 0.79 ml, as determined by eluting the column with anhydrous methanol and measuring the water content by a Karl Fischer titration. The interstitial volume (V_m) was determined to be 1.02 ml by the injection of an unretained sample (toluene).

The contents of perchlorate in the stationary and mobile phases were determined by a quantitative extraction with dimethylprotriptylin (MPT) into methylene chloride and photometric measurements, according to the principles described by Borg[7].

Determination of distribution data

The batch extractions were carried out in centrifuge tubes either at room temperature or in a thermostated bath at 23.0°C. The approximate shaking time for the determination of extraction constants was 30 min or more, and for the determination of distribution constants for bases 60 min or more. The tubes were then centrifuged at 1600–1900 *g* for 5–10 min. The concentrations were determined by photometry in both the aqueous and the organic phase. Molar absorptivities of the compounds are given in Table I.

TABLE I

MOLAR ABSORPTIVITIES

Aqueous phase: 0.5–1 *M* $HClO_4$. Organic phase: methylene chloride–*n*-butanol (89:11), saturated with aqueous phase.

Compound	*Aqueous phase*		*Organic phase*	
	Wave-length (nm)	*log ε*	*Wave-length (nm)*	*log ε*
Zimelidine	250	4.278	250	4.342
Zimelidine *E*-isomer	220	4.281	239	4.230
Norzimelidine	250	4.296	250	4.294
Norzimelidine *E*-isomer	222	4.303	241	4.281
Chlorpheniramine	265	3.922	266	3.896

In the two-phase titrations the pH set was calibrated before and after every titration with two commercially available buffers (pH 4.01 ± 0.02 and 7.01 ± 0.02) (Radiometer). The titrations were performed in a closed vessel to prevent disturbance by carbon dioxide from the air. The vessel was kept in a thermostated water-bath (23.0 ± 0.2°C). The two phases were saturated with each other prior to the titration in order to avoid volume changes.

A 10^{-2} *M* solution of the amine in 30 ml of the organic phase (methylene chloride–*n*-butanol, 89:11) was prepared by extraction from a small volume of an alkalinized aqueous solution of the amine salt. After centrifugation, 25.0 ml of the organic phase were transferred into the titration vessel and 25.0 ml of aqueous phase were added. The two-phase system was then titrated with 1 *M* perchloric acid for ion-pair extraction constants and 0.5 *M* sulphuric acid for base distributions.

The titrant was delivered in equal portions with vigorous stirring. The pH was measured, with stirring, about 60 sec after the addition. Blank titrations were performed in both instances and calculations were made according to Johansson and Gustavii[8,9].

RESULTS AND DISCUSSION

Distribution data

The compounds studied are divalent amines and in the chromatographic system used, which contains a strongly acidic stationary aqueous phase, they can be distributed both as 1 + 1 (HAX_{org}) and 1 + 2 (H_2AX_{2org}) ion pairs. Constants for the distribution of the compounds as bases and as ion pairs to the mobile phase, methylene chloride–*n*-butanol (89:11), are given in Tables II–IV.

TABLE II

CONSTANTS FOR DISTRIBUTION AS BASES

Determined by two-phase titrations. Organic phase: methylene chloride–*n*-butanol (89:11) equilibrated with the aqueous phase. Number of experimental points: 9–12. Aqueous phase: 0.033 *M* Na_2SO_4; titrant, 0.5 *M* H_2SO_4 (I = 0.1).

Amine	$C_A^\circ \cdot 10^3$	$C'_A \cdot 10^3$	*pH*	$-\log k_d \cdot K'_{HA}$	pK'_{H_2A}
Zimelidine	5.01	3.04–3.26	4.52–3.45	3.97	3.76*
Norzimelidine	5.05	3.76–3.94	5.13–3.83	5.36	3.83**
Norzimelidine *E*-isomer	5.03	2.87–3.86	5.38–3.51	5.55	4.29
Zimelidine *E*-isomer	5.02	2.93–3.07	4.55–3.58	4.07	4.25
Chlorpheniramine	5.02	3.13–3.51	5.10–3.33	4.92	3.81

* 3.78 ± 0.03 (n = 11) by potentiometric titration.
** 3.92 ± 0.01 (n = 11) by potentiometric titration.

The distribution of the compounds as bases is negligible at pH < 2 (equal phase volumes), as is the case with increasing ionic strength, as it can be expected that the base distribution will then decrease, in a similar manner to the results for the 1 + 1 ion pairs (Table III) where the difference is about two-fold. Acid dissociation constants of zimelidine and norzimelidine at an ionic strength of 1 were found to be 4.08 ± 0.01 and 4.24 ± 0.01 (n = 11), respectively, as determined by potentiometric titrations, *i.e.*, the acidities are about halved on increasing the ionic strength 10-fold.

The ion-pair extraction constants at an ionic strength of 1.0 were determined according to the principles outlined by Modin and Schill[10]; 1 + 1 ion-pairs could be determined without any influence of 1 + 2 ion pairs at pH 2.88 and 4.15 for zimelidine and norzimelidine, respectively. In the determinations of 1 + 2 ion pairs (Table IV) the co-extraction of the 1 + 1 ion pairs was compensated for by using the determined constants (Table IIIB). At high ionic strength the 1 + 1 ion pairs were found to dissociate in the organic phase (Table IIIB); the determinations of constants at low ionic strength (Table IIIA), however, were performed with much higher concentrations, that is, under conditions where dissociation of an ion pair in the organic phase does not occur.

With variation of the perchlorate concentration under acidic conditions (1 + 2 ion pair, Table IV) the conditional extraction constants for zimelidine and norzime-

TABLE III

EXTRACTION CONSTANTS FOR 1 + 1 PERCHLORATE ION PAIRS

(A) Ionic strength = 0.1

Determination technique: two-phase titrations. Aqueous phase: 0.1 *M* $NaClO_4$; titrant, 1 *M* $HClO_4$.

Compound	$C_A^\circ \cdot 10^3$	$C'_A \cdot 10^5$	*pH*	$\log K_{ex(HAX)}$
Zimelidine	10.06	3.48–6.27	6.19–5.63	3.06
Norzimelidine	10.05	5.77–12.24	7.41–6.79	2.74
Zimelidine *E*-isomer	9.97	5.90–12.52	6.11–5.47	2.73
Norzimelidine *E*-isomer	9.99	19.84–32.44	6.96–6.46	2.33
Chlorpheniramine	10.10	1.73–3.88	7.54–6.80	3.27

(B) Ionic strength = 1

Determination technique: batch extraction and UV photometric measurements. Aqueous phase: 0.2 *M* $HClO_4$ + $NaClO_4$ and NaCl to give $I = 1.0$.

Compound	$C_A^\circ \cdot 10^5$	$C'_A \cdot 10^5$	*pH*	$\log K_{ex(HAX)}$	$-\log k_{diss}$
Zimelidine	2.25–28.36	1.049–15.60	2.88	2.82	5.23*
Norzimelidine	2.21–22.60	0.479–5.67	4.15	2.48	4.75**

* $r = 0.9986$.
** $r = 0.9888$.

TABLE IV

EXTRACTION CONSTANTS FOR 1 + 2 PERCHLORATE ION PAIRS

Determination technique: batch extractions and UV photometric measurements. Organic phase: methylene chloride–*n*-butanol (89:11). Aqueous phase: 0.2 *M* $HClO_4$ + $NaClO_4$ and NaCl to give $I = 1.0$.

Compound	$C'_{Aorg} \cdot 10^5$	$C'_A \cdot 10^2$	C'_{ClO_4}	$K_{ex(HAX_2)}$	$k_{a(H_2AX_2)}$	*n*
Zimelidine	6.68–8.20	1.20–1.42	0.4–0.8	7.5	14.5	14
Norzimelidine	1.92–5.42	1.72–2.05	0.2–1.0	3.1	13.1	8

lidine decreased with increasing concentration of perchlorate in the aqueous phase. This may be due to dissociation of an ion pair in the organic phase or association reactions in the aqueous phase. The assumption of an association between the amine in divalent form and two molecules of perchlorate to form an ion pair (H_2AX_2) in the aqueous phase gave the best fit to the data as treated by slope analysis. Other possibilities tested were extraction of a 1 + 1 ion pair only and association of amine and perchlorate to form the ion pair HAX in the aqueous phase; extraction of a 1 + 1 ion pair and dissociation of this ion pair in the organic phase; and extraction of a 1 + 2 ion pair and dissociation in the organic phase to form either H_2AX_{org} and X_{org} or

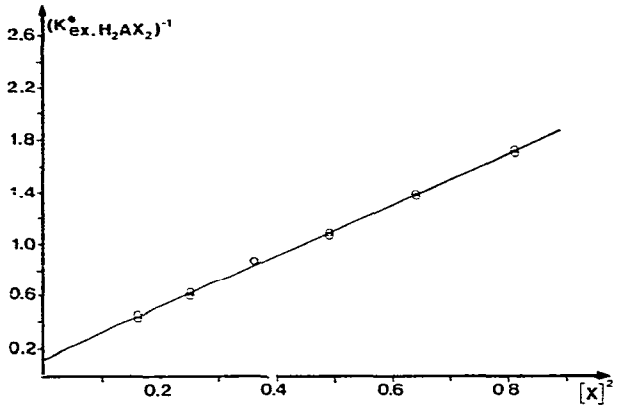


Fig. 1. Ion-pair association in aqueous phase. Organic phase: methylene chloride–*n*-butanol (89:11). Aqueous phase: 0.2 *M* $HClO_4$ + $NaClO_4$ and NaCl to give I = 1.0. Calculation of data from Table IV according to eqn. 1 (r = 0.9991).

H_2A_{org} and 2 X_{org}. Computations according to these principles gave, however, low correlation coefficients and in many instances improbable values of the constants. As an example, the association of zimelidine with two perchlorate molecules in the aqueous phase is illustrated in Fig. 1 according to the equation

$$(K^{\circ}_{ex(H_2AX_2)})^{-1} = (K_{ex(H_2AX_2)})^{-1} + k_a [X]^2 (K_{ex(H_2AX_2)})^{-1}$$

The extraction and association constants obtained from the slope and intercept for the two compounds are reported in Table IV.

The magnitude of the association constants means that the ion-pair formation in the aqueous phase is significant at perchlorate concentrations above $7 \cdot 10^{-4}$ *M*. The dissociation of the 1 + 1 ion pairs in the organic phase for zimelidine and norzimelidine is of importance at organic phase concentrations $\leqslant 10^{-5}$ *M*.

The determinations of constants for the 1 + 2 ion pairs were carried out under conditions where the concentrations in the organic phase were low, but as perchloric acid is extracted (see below) the dissociation of the amine–perchlorate ion pairs in the organic phase may be suppressed by perchlorate ions from the acid[11] and remain undetected.

The distribution ratios for actual amines were also determined by batch extractions under conditions identical with those used in the chromatographic system (Table V). As remarked earlier, in this system both 1 + 1 and 1 + 2 ion pairs will distribute simultaneously and complicate discussions on selectivity, as discussed in depth later (see *Chemical structure and selectivity*).

Data on extraction selectivity, expressed as the difference between the logarithmic values for the constants, are given in Table VI. In base distribution high selectivity is obtained between tertiary and secondary amines, which obviously depends on a combination of differences in both distribution constants and acid dissociation constants. High selectivity for geometrical isomers is obtained by 1 + 1 ion-pair extractions, where the selectivity between the tertiary and secondary amines is also

TABLE V

DISTRIBUTION OF ION PAIRS BETWEEN PHASES USED IN THE CHROMATOGRAPHIC SYSTEM

Determination technique: batch extraction and UV photometric measurements. Organic phase: methylene chloride–*n*-butanol (89:11). Aqueous phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$.

Compound	$C'_{Aorg} \cdot 10^5$	$C'_A \cdot 10^5$	*Distribution ratio*	*s*	*n*
Chlorphenir-amine	1.768–9.005	1.938–10.123	0.8706	0.0135	8
Zimelidine	0.614–3.062	1.138–5.796	0.5329	0.0067	10
Zimelidine *E*-isomer	0.4710–2.420	1.241–6.430	0.3709	0.0066	4
Norzimelidine	0.4417–2.198	1.183–5.987	0.3745	0.0045	5
Norzimelidine *E*-isomer	0.3766–1.899	1.244–6.00	0.3258	0.0168	5

good. For 1 + 2 ion pairs data only for zimelidine and norzimelidine are available and for this pair of compounds the selectivity is similar to the 1 + 1 ion pairs. In phases used in the chromatographic system D (Table VI), however, where both 1 + 1 and 1 + 2 ion pairs are distributed, the selectivities are lower; this system was, however, preferred for the chromatography mainly because of higher stability and buffer capacity[12].

Capacity ratios

In a chromatographic system based on the distribution of ion pairs, the capacity ratios can be calculated according to

$$k'_c = V_s (V_m D_{A(X)})^{-1} \quad (2)$$

The determined capacity ratios are 1.5–2 times higher than the calculated values, as demonstrated in Table VII. The difference may be due to the influence of the support on the properties of stationary phase or on the sample. Larger differences have been found earlier in similar systems for amines[13] and steroidal conjugates[14], and a close correspondence was obtained for some phenylacetic acid derivatives[15].

A quantitative determination of perchlorate in the stationary phase showed that its concentration increased during the course of the equilibration from 1 to 1.23 *M*, which is analogous to the results found for quaternary ammonium compounds in similar systems[14,15]. The chromatographic results indicated in those instances that the additional amount of the counter ion did not take part in the ion-pair distribution. This also seems to be the case in the present system. A significant amount of perchlorate is also extracted into the mobile phase, which was found to have a concentration of $2.01 \cdot 10^{-3}$ *M*, probably mainly consisting of perchloric acid, as this species has a much higher distribution than sodium perchlorate[12].

TABLE VI

EXTRACTION SELECTIVITY

Calculated from values in Tables II–V.

Compounds	*A, base distribution:* $\Delta \log k_d \cdot K'_{HA}$ *(I = 0.1)*	*B, 1 + 1 ion-pair distribution:* $\Delta \log K_{ex(HAX)}$		*C, 1 + 2 ion-pair distribution:* $\Delta \log K_{ex(H_2AH_2)}$ *(I = 1)*	*D, mixed ion-pair distribution:* $\Delta \log D_A$ *(I = 1)*
		I = 0.1	*I = 1*		
Zimelidine–norzimelidine	1.39	0.32	0.34	0.34	0.15
Zimelidine *E*–norzimelidine *E*	1.47	0.39			0.06
Zimelidine–zimelidine *E*	0.11	0.34			0.16
Norzimelidine–norzimelidine *E*	0.19	0.41			0.06
Zimelidine–chlorpheniramine	0.96	0.24			0.21

TABLE VII

DETERMINED AND CALCULATED CAPACITY RATIOS

Support: Partisil 5. Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: methylene chloride–*n*-butanol (89:11). V_s/V_m = 0.77. k'_f and k'_c = determined and calculated (Table V) capacity ratios, respectively.

Compound	k'_f	k'_f/k'_c
Chlorpheniramine	1.78	2.02
Zimelidine	2.36	1.63
Norzimelidine	3.10	1.50
Zimelidine *E*-isomer	3.25	1.56
Norzimelidine *E*-isomer	3.78	1.60

The good agreement between determined and calculated capacity ratios indicates that the retention is due mainly to liquid–liquid distribution. This conclusion is supported by the fact that selectivity factors can be calculated from extraction constants obtained by batch extractions, as demonstrated in Table VIII. It is noteworthy that about the same selectivity is obtained between *Z*- and *E*-isomers as between tertiary and secondary amines.

TABLE VIII

SELECTIVITIES FROM EXTRACTION CONSTANTS AND CHROMATOGRAPHIC DATA

The selectivity factors (α) were calculated from Tables V and VII.

Compounds	α *calculated from*	
	k'_f	$D_{A(V)}$
Zimelidine/zimelidine *E*-isomer	1.38	1.44
Zimelidine/chlorpheniramine	1.33	1.63
Zimelidine/norzimelidine	1.31	1.42
Zimelidine *E*-isomer/ norzimelidine *E*-isomer	1.16	1.14
Zimelidine/norzimelidine *E*-isomer	1.05	1.01
Norzimelidine/norzimelidine *E*-isomer	1.22	1.15

Chemical structure and selectivity

The discussion of factors that determine the selectivity in this chromatographic system is complicated by the possible existence of two kinds of ion pairs in the organic phase, H_2AX_2 and HAX, when the compounds concerned are divalent amines. The ratios of the concentrations of these ion pairs are obtained by the equation

$$R = \frac{\text{total concentration of 1 + 2 ion pair in organic phase}}{\text{total concentration of 1 + 1 ion pair in organic phase}}$$

$$= K^x_{ex(H_2AX_2)}\, a_{H^+}\, [X]\, [K^x_{ex(HAX)}\, K'_{H_2A}]^{-1} \qquad (3)$$

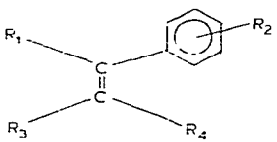
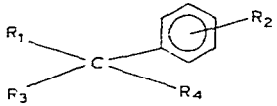
As the pH and the counter ion concentration are kept constant in the chromatographic system, the ratio, R, depends on the quotient of the extraction constants and the acid dissociation constant of the pyridine-nitrogen. R values for zimelidine and norzimelidine, using data from Tables II, IIIB and IVA, are 1.94 and 2.53, corresponding to 66 and 72% of the 1 + 2 ion pair in the chromatographic mobile phase, respectively. The R values are calculated assuming that the dissociation of the 1 + 1 ion pair is negligible as the co-extraction of significant amounts of perchloric acid (see above) will probably suppress this reaction. It can be estimated that for the compounds mentioned in Tables II–V between 60 and 99% of the species present in the organic phase will correspond to the 1 + 2 ion pair.

Capacity ratios for zimelidine and related compounds are summarized in Table IX, and some selectivity factors calculated from these figures are given in Table X.

TABLE IX

CAPACITY RATIOS

Chromatographic conditions: see Table VI.

Compound	R_1	R_2	R_3	R_4	*log* k'_f*
					
Zimelidine	3-Pyridyl	*p*-Br	$-CH_2N(CH_3)_2$	H	0.373
Norzimelidine	3-Pyridyl	*p*-Br	$-CH_2NHCH_3$	H	0.491
I	3-Pyridyl	*p*-Br	$-CH_2NH_2$	H	0.821
II	3-Pyridyl	*p*-Br	$-CH_2NHCOCH_3$	H	≤ -0.52
Zimelidine N-oxide	3-Pyridyl	*p*-Br	$-CH_2\overset{\downarrow}{N}(CH_3)_2$ O	H	0.137
III	3-Pyridyl	*p*-Br	$-COOH$	H	-0.523
IV	3-Pyridyl	H	$-CH_2N(CH_3)_2$	H	0.807
Zimelidine *E*-isomer	3-Pyridyl	*p*-Br	H	$-CH_2N(CH_3)_2$	0.512
Norzimelidine *E*-isomer	3-Pyridyl	*p*-Br	H	$-CH_2NHCH_3$	0.577
V	3-Pyridyl	*o*-Br	$-CH_2N(CH_3)_2$	H	0.316
VI	3-Pyridyl	*o*-Br	H	$-CH_2N(CH_3)_2$	0.599
VII	2-Pyridyl	H	$-CH_2N(CH_3)_2$	H	0.398
VIII	2-Pyridyl	*p*-F	$-CH_2N(CH_3)_2$	H	0.433
IX	2-Pyridyl	*o*-OCH_3	$-CH_2N(CH_3)_2$	H	0.097
X	2-Pyridyl	*p*-CH_3	$-CH_2N(CH_3)_2$	H	0.000
					
XI	3-Pyridyl	*p*-Br	$-OH$	$-CH_2CH_2N(CH_3)_2$	0.508
XII	3-Pyridyl	*p*-Br	$-OH$	$-CH_2CH_2NHCH_3$	0.665
XIII	3-Pyridyl	*p*-Br	$=O$	–	-0.984
Chlorpheniramine	2-Pyridyl	*p*-Cl	H	$-CH_2CH_2N(CH_3)_2$	0.250
Brompheniramine	2-Pyridyl	*p*-Br	H	$-CH_2CH_2N(CH_3)_2$	0.146

* k'_f values are means of two or three experiments.

TABLE X

CHEMICAL STRUCTURES AND SELECTIVITIES

Structural difference	*Compounds*	α
Secondary/tertiary amine	Norzimelidine/zimelidine	1.31
	Norzimelidine/zimelidine *E*-isomers	1.16
Primary/secondary amine	I/norzimelidine	2.14
Geometric isomerism	Zimelidine *E*/zimelidine	1.38
	Norzimelidine *E*/norzimelidine	1.22
Tertiary amine/amine oxide	Zimelidine/zimelidine N-oxide	1.72
Primary amine/acetylated primary amine	I/II	>21.9
Tertiary amine/carboxylic acid	Zimelidine/III	7.87
>C(OH)—CH_2—/>CH=CH< (*Z*-isomers)	XI/zimelidine	1.49
(*E*-isomers)	XI/zimelidine *E*-isomer	1.22
3-Pyridyl/2-pyridyl	IV/VII	2.56
H/*p*-Br	IV/zimelidine	2.72
p-F/H	VIII/VII	1.08
H/*o*-CH_3O	VII/IX	2.00
H/*p*-CH_3	VII/X	2.50
p-Cl/*p*-Br	Chlorpheniramine/brompheniramine	1.27

Owing to the complicated nature of the chromatographic system presented above, the following discussion on selectivities obtained is mainly of a qualitative nature and difficult to compare with other extraction systems.

Amines of different degrees of substitution are easy to separate; the selectivity, however, is strongly dependent on the content of alcohol in the mobile phase, as demonstrated in Fig. 2. The order of elution is reversed with 30 % of *n*-butanol in the mobile phase, the secondary amine being strongly solvated by the alcohol. The selectivity factors decrease continuously (1.34, 1.16, 1.00 and 0.95, respectively) with increasing concentration of *n*-butanol in the mobile phase, as does the number of theoretical plates (from about 7000 to 6000, 4500 and 3000, respectively). The mobile phase volume, V_m (toluene), was constant, indicating that the same amount of stationary phase is adsorbed to the support in all instances.

The excellent selectivity between two pairs of geometrical isomers is illustrated in Fig. 3. A and D are geometric isomers as well as B and C; A and B are furthermore bromo-positional isomers as well as C and D. Selectivity factors for positional isomerism are 1.14 (B/A) and 1.22 (D/C), respectively, while the geometric isomerism gives $\alpha = 1.92$ (D/A) and $\alpha = 1.38$ (C/B) in this instance.

Zimelidine N-oxide ($k' = 1.37$) elutes before zimelidine ($k' = 2.36$) in this chromatographic system, which is probably mainly a consequence of protolytic properties (the formation of an N-oxide often decreases the basic character of the compound by 4–5 pK_s units[16]), which increases the possibilities for the extraction of a 1 + 1 ion pair.

Acetylation of a primary amine usually decreases the polarity of a compound, but it also eliminates the protolytic properties. Compound II (Table IX) is consequently extracted as a 1 + 1 ion pair only, which accounts for the high selectivity

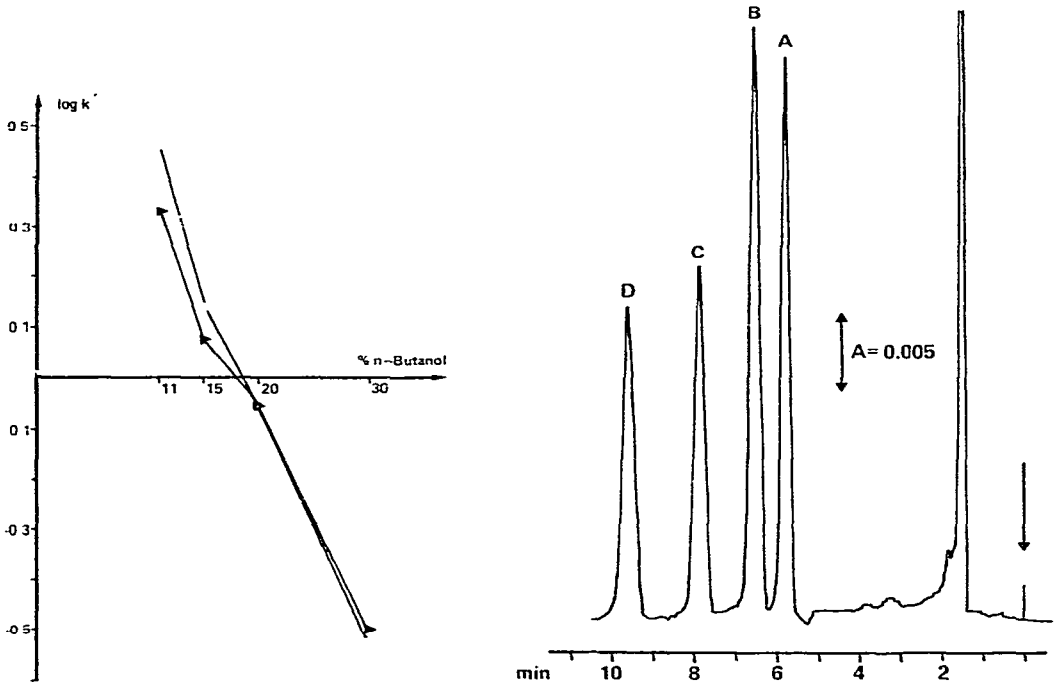


Fig. 2. Retention with different contents of *n*-butanol in the mobile phase. Support: Partisil 5. Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: methylene chloride–*n*-butanol. Sample: 600 pmol of each substance in 40 µl of mobile phase. Each point is the mean of three or four determinations, and the chromatographic system was allowed to equilibrate for not less than 20 h before the experiments. ▼, Zimelidine; ○, norzimelidine.

Fig. 3. Separation of geometric and positional isomerism. Support: Partisil 5. Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: methylene chloride–*n*-butanol (89:11) saturated with stationary phase. A, No. V in Table IX; B, zimelidine; C, zimelidine *E*-isomer; D, No. VI in Table IX.

against the primary amine (compound I) in this instance.

A further illustration of the higher extraction of 1 + 1 ion pairs is given by the carboxylic acid (III), which has a very low retention. The introduction of the polar carboxylic group should normally have increased the capacity ratio.

The exchange of $-CH=CH-$ for $-CH(OH)CH_2-$ in the propyl chain (zimelidine → XI and norzimelidine → XII) increases the affinity to the aqueous phase, as expected. The increase in the capacity ratios (0.13–0.17 log units) is, however, considerably smaller than on introduction of an alcohol group in an alkyl chain of a 1 + 1 ion pair. Extraction constants for choline picrate into 1-pentanol and methylene chloride are, for example, 0.6 and 1.0 log units lower, respectively, than the corresponding constants for the trimethylammonium ion pair[17].

The high selectivity between 2- and 3-pyridyl-substituted compounds (IV and VII) is remarkable and is probably an effect of differences in hydrophobicity and/or affinities to the support, as pK'_{H_2A} values for the two kinds of structures seem to be similar, as indicated, for example, by the pK'_{H_2A} of the 3-pyridyl derivative zimelidine and the 2-pyridyl derivative brompheniramine of 3.84 and 3.93, respectively[9].

Effects of variation in flow-rate

The dependence of H on the flow-rate for norzimelidine and the primary amine (I) is demonstrated in Table XI. H is constant at flow-rates between 0.4 and 1.8 mm/sec but increases at higher velocities. The reason behind this performance is not, however, a simple consequence of a velocity increase because the retention is also affected; the capacity ratios for both compounds are minimal at a flow-rate of about 0.8 mm/sec, as illustrated in Fig. 4. The selectivity is, however, only slightly affected, suggesting that the basic retention mechanism is maintained at all velocities. The retention is temperature dependent, as demonstrated in Table XII, which shows capacity ratios and selectivity factors obtained at 23.0 and 25.7°C. At the higher temperature the capacity ratios increase by 25–30% (0.027 unit per 0.1°C) but the

TABLE XI

INFLUENCE OF FLOW-RATE ON CHROMATOGRAPHIC PERFORMANCE

Amounts injected: 140 ng of norzimelidine and 600 ng of I. Volume injected: 20 µl. Chromatographic conditions: see Fig. 2.

Flow-rate (mm/sec)	*Compound*	*HETP (µm)*	*As**
0.41	Norzimelidine	23	1.38
0.41	I	23	1.26
0.81	Norzimelidine	21	1.22
0.81	I	22	1.21
1.81	Norzimelidine	23	1.14
1.81	I	23	1.14
2.50	Norzimelidine	34	1.13
2.50	I	34	1.25
3.28	Norzimelidine	41	1.23
3.28	I	39	1.20

* Asymmetry factor = back/front of peak at the baseline level.

TABLE XII

TEMPERATURE DEPENDENCE OF CAPACITY AND SELECTIVITY FACTORS

Compound	*23.0°C*		*25.7°C*	
	log k'_f	*α*	*log k'_f*	*α*
Chlorpheniramine	0.250		0.348	
		1.33		1.39
Zimelidine	0.373		0.491	
		1.31		1.31
Norzimelidine	0.491		0.607	
		1.22		1.17
Norzimelidine *E*-isomer	0.577		0.676	

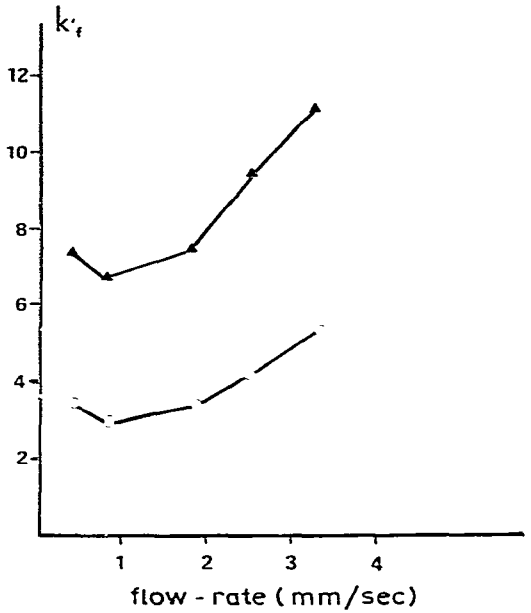


Fig. 4. Dependence of capacity ratios on flow-rate. Chromatographic conditions: see Fig. 3. Amounts injected: 140 ng of norzimelidine and 600 ng of I (primary amine). ○, Norzimelidine; ▲, compound I (Table IX).

selectivity is unchanged. It is known that heat due to friction develops within a column with large pressure drops[18], but such temperature effects can be responsible for only part of the observed difference in retention times in the present instance.

In an ion-pair retention mechanism the capacity ratios are directly proportional to V_s, the volume of the stationary phase (eqn. 2). Data obtained for V_m (the volume available for an unretained compound), however, increased from 1.026 to 1.066 with increasing flow-rate from 0.83 to 3.26 mm/sec, indicating that V_s decreases by about 5% with this flow-rate change. As the capacity ratios increase with increasing flow-rate another retention mechanism must be responsible, possibly an increased availability for adsorption of the compounds by the support with decreasing V_s. The increase in capacity ratios at the lower flow-rates may, however, partly depend on the increasing stationary phase volume.

Injection of large sample volumes

A perfect injection results in a plug with no mixing of the sample and mobile phase occurring but simply a displacement of mobile phase at the top of the column. However, depending on the flow pattern in the injection device, a more or less asymmetric injection profile is obtained in practice.

The dispersion starts as soon as the sample hits the column and results in band broadening at the top of the column as the delivery of the sample takes a certain amount of time. The magnitude of the dispersion also depends on the capacity ratio, as a more retained compound occupies a smaller volume at the top of the column, provided that rapid equilibrium between mobile and stationary phase is established.

The effective volume (V_e) occupied by the sample under these conditions is described by $V_e = V_i (1 + k')^{-1}$, where V_i is the volume injected.

The total dispersion, σ_t^2, is the sum of band-broadening effects at the injection, σ_i^2, and on the column, σ_c^2, provided that other contributions are negligible. It has been demonstrated in some chromatographic systems[19–21] that under certain conditions σ_i^2 is directly proportional to the volume injected by computing the total dispersion against V_i^2 according to

$$\sigma_t^2 = V_i^2/K^2 + \sigma_c^2 \tag{4}$$

where K is a constant representing the effects of the injection valve design and the capacity factor on the dispersion. Plots of eqn. 4, for three compounds with different k' values, X, norzimelidine and I with injection volumes ranging from 10 to 500 μl gave no straight lines, however.

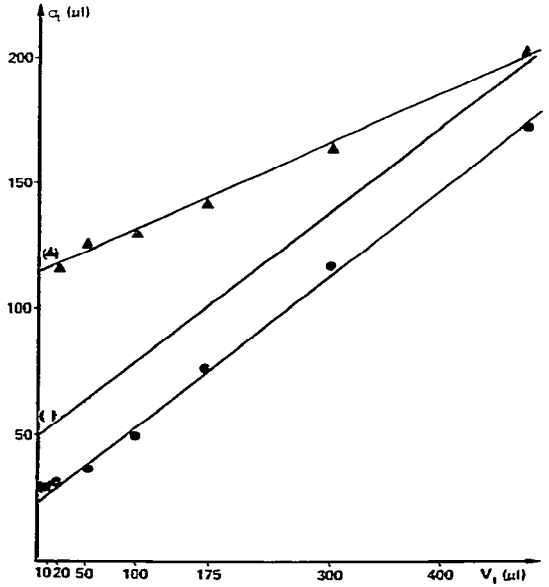


Fig. 5. Dispersion by injection of large sample volumes. Chromatographic conditions: see Fig. 3. Volumes injected: 10–500 μl. ●, Compound X (Table IX); ○, norzimelidine; ▲, compound I (Table IX).

Straight-line relationships were obtained by computing V_i against σ_t (Fig. 5), illustrating that the peak widths increase in proportion to the injected volumes according to the following empirical equations:

Compound X ($k'_f = 1.023 \pm 0.032$):

$$\sigma_t = 0.3040\ V_i + 22.15\ (r = 0.9993) \tag{5}$$

Norzimelidine ($k'_f = 3.57 \pm 0.046$):

$$\sigma_t = 0.3019\ V_i + 49.08\ (r = 0.9986) \tag{6}$$

Compound I ($k'_f = 8.49 \pm 0.053$):

$$\sigma_t = 0.1748\ V_i + 113.58\ (r = 0.9981) \quad (7)$$

Data for $V_i = 10\ \mu l$ are not included in the computation of the equations as they seem to deviate from a straight line, possibly because of excessive dispersion effects by the injection valve on such small volumes.

Replacing σ_t with the more frequently used parameter H by utilizing the relationship $\sigma_t = t_0 (1 + k') H^{1/2} L^{-1/2}$ and plotting against V_e (Fig. 6) gives a common straight line for the two compounds with low capacity ratios (< 3.6) while the third compound ($k' = 8.49$) seems to deviate at large V_e values.

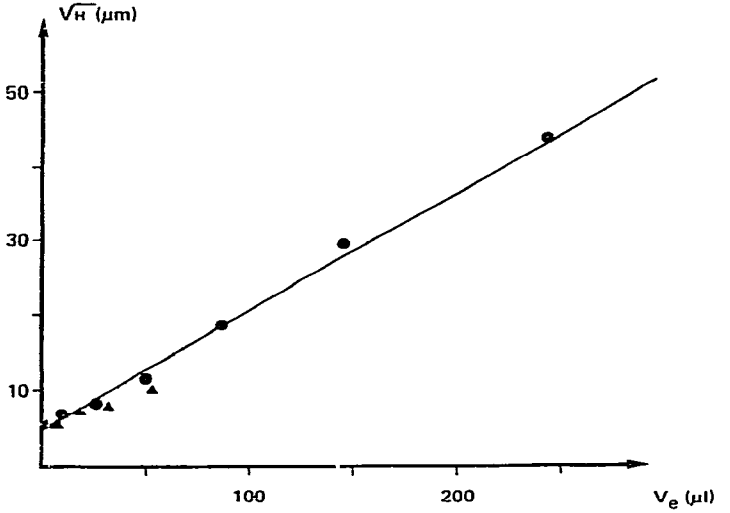


Fig. 6. Dispersion by injection of large volumes, effective volume injected against efficiency. Conditions: see Fig. 5. $V_e = V_i (1 + k')^{-1}$.

Similar results have been obtained for penicillins in a reversed-phase system[22].

The influence of increasing injection volume on the asymmetry factor, illustrated in Fig. 7, initially shows an increasing tailing tendency that stabilizes at a characteristic level for each compound when $50\ \mu l \leqslant V_i \leqslant 200\ \mu l$. With a further increase in V_i the asymmetry decreases for moderately and highly retained compounds, whereas it increases rapidly for the least retained compound. This effect is probably due to the asymmetric profile of the volume injected; for norzimelidine and compound I the asymmetric profile is smoothed out when the compounds are retained at the top of the column.

The influence of the sample volume on the resolution between the geometrical isomers of norzimelidine, which represents a very difficult separation[6], is illustrated in Fig. 8. The resolution decreases rapidly if $V_i > 20\ \mu l$ and is only about 50% of the maximal when 200 μl are injected. The efficiency and selectivity of the chromatographic system are so great, however, that a baseline separation ($R_s = 1.5$) is still achieved at $V_i = 190\ \mu l$.

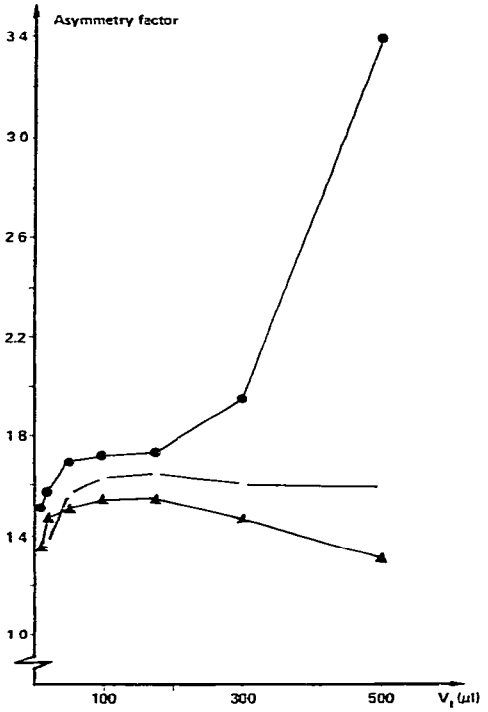


Fig. 7. Dependence of asymmetry factors on volumes injected. Conditions: see Fig. 5.

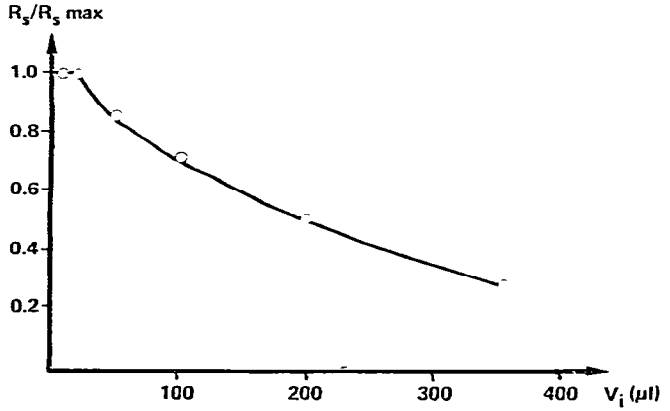


Fig. 8. Dependence of resolution on volumes injected. Chromatographic conditions: see Fig. 2. Injected compounds: norzimelidine and its geometric isomer. $R_s = R_{smax}$ when $V_i \leq 10$ µl.

Application to studies of purity

Although the chromatographic system was developed mainly for use in bioanalytical work, it was utilized in some instances for control of the purity of some batches of zimelidine and norzimelidine hydrochloride. Fig. 9 shows an example of a chromatogram obtained from a batch of norzimelidine hydrochloride, which con-

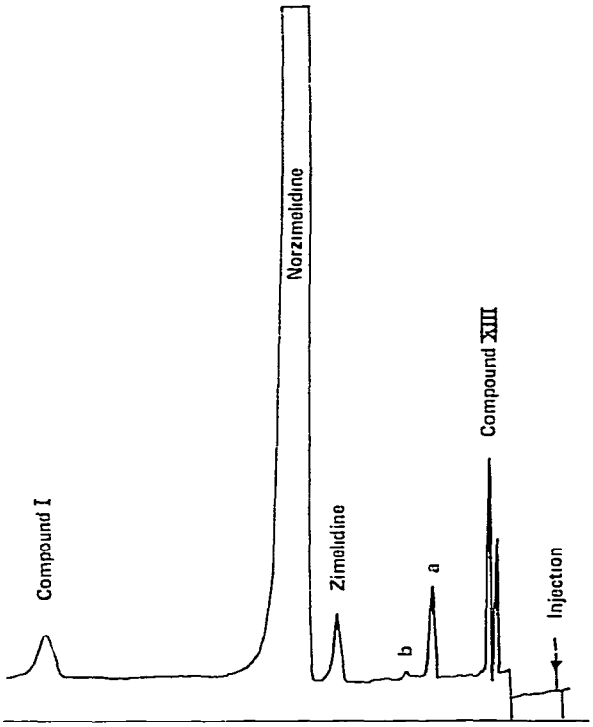


Fig. 9. Chromatographic purity of a batch of norzimelidine hydrochloride. Sample: 3 μg of salt in 100 μl of mobile phase. Chromatographic conditions: see Fig. 3. Estimated degree of impurity: zimelidine 0.2%, compound I 0.5%.

tained zimelidine, the primary amine (compound I), the ketone (compound XIII) and some unknown compounds (a, b) as impurities. The amount of the ketone (XIII) is difficult to interpret reliably as it has a very low capacity ratio (*ca.* 0.1) and is frequently interfered with by front disturbances that occur at $k' \leq 0.5$.

The high selectivity between geometrical isomers can be utilized for studies on isomeric purity, as illustrated in Fig. 10. In an injected amount of 2.4 μg of zimelidine, 0.01% of its *E*-isomer can be detected. However, it is an advantage if the impurity elutes before the main peak, because otherwise it may be masked by the tail of the large peak, so the detection limit of zimelidine as an impurity in the *E*-isomer (see Fig. 10B) is still lower.

SYMBOLS

ε = molar absorptivity;

C_A^c, C_X^o = initial concentrations of amine and perchlorate, respectively;

C'_{Aorg}, C'_{Xorg} = equilibrium concentrations of amine and perchlorate, respectively, in organic phase;

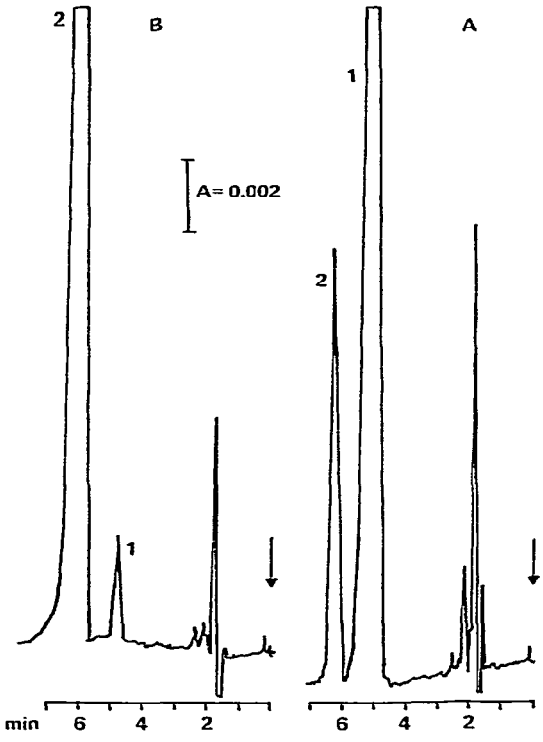


Fig. 10. Studies on purity from geometric isomers. A, Zimelidine (1) containing 2% of *E*-isomer (2); B, zimelidine *E*-isomer (2) containing 0.3% of zimelidine (1). Chromatographic conditions: see Fig. 3. Amounts injected: 2.4 μg in 80 μl of mobile phase.

C'_A, C'_X = equilibrium concentrations of amine and perchlorate, respectively, in aqueous phase;

$[H_2A]$, $[HA]$, $[X]$, $[H_2AX_2]$, $[HAX]_{org}$, $[H_2AX_2]_{org}$ = concentration of respective species in the appropriate phase;

$$k_d = \frac{[A]_{org}}{[A]} = \text{base distribution coefficient;}$$

$D_{A(X)}$ = distribution ratio of A as ion pair with X;

$$K'_{H_2A} = \frac{a_{H^+}[HA]}{[H_2A]} = \text{first apparent acid dissociation constant;}$$

$$K'_{HA} = \frac{a_{H^+}[A]}{[HA]} = \text{second apparent acid dissociation constant;}$$

$$K_{ex(HAX)} = \frac{[HAX]_{org}}{[HA][X]} = \text{extraction constant (1 + 1);}$$

$K^{x}_{ex(HAX)} = \dfrac{C'_{Aorg}}{C'_A\ C'_X}$ = conditional extraction constant (1 + 1);

$K_{ex(H_2AX_2)} = \dfrac{[H_2AX_2]_{org}}{[H_2A][X]^2}$ = extraction constant (1 + 2);

$K^{x}_{ex(H_2AX_2)} = \dfrac{C'_{Aorg}}{C'_A\ (C'_X)^2}$ = conditional extraction constant (1 + 2);

$k_{a(H_2AX_2)} = \dfrac{[H_2AX_2]}{[H_2A][X]^2}$ = ion-pair formation constant;

k'_f = determined capacity ratio;

k'_c = calculated capacity ratio.

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REFERENCES

1 G. Schill, *Separation Methods*, Apotekarsocieteten, Stockholm, 1978.
2 G. Schill, in J. A. Marinsky and Y. Marcus (Editors), *Ion Exchange and Solvent Extraction*, Vol. VI, Marcel Dekker, New York, 1974, p. 1.
3 G. Schill, K. O. Borg, R. Modin and B.-A. Persson, in E. R. Garrett and J. L. Hirtz (Editors), *Drug Fate and Metabolism —Methods and Techniques*, Marcel Dekker, New York, 1977, p. 135.
4 G. Schill, in E. Reid (Editor), *Assay of Drugs and Other Trace Compounds in Biological Fluids*, Longmans, London, 1975, p. 195.
5 G. Schill, K. O. Borg, R. Modin and B.-A. Persson, in E. Wänninen (Editor), *Essays in Memory of Anders Ringbom*, Pergamon Press, Oxford, New York, 1977, p. 379.
6 D. Westerlund, L. B. Nilsson and Y. Jaksch, *J. Liquid Chromatogr.*, 2 (1979) 373.
7 K. O. Borg, *Acta Pharm. Suecica*, 6 (1969) 425.
8 P.-A. Johansson and K. Gustavii, *Acta Pharm. Suecica*, 13 (1976) 407.
9 P.-A. Johansson, *Acta Pharm. Suecica*, 14 (1977) 363.
10 R. Modin and G. Schill, *Acta Pharm. Suecica*, 7 (1970) 585.
11 D. Westerlund, *Acta Pharm. Suecica*, 11 (1974) 581.
12 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
13 P.-O. Lagerström, I. Carlsson and B.-A. Persson, *Acta Pharm. Suecica*, 13 (1976) 157.
14 B. Fransson, K.-G. Wahlund, I. M. Johansson and G. Schill, *J. Chromatogr.*, 125 (1976) 327.
15 P.-O. Lagerström, *Acta Pharm. Suecica*, 13 (1976) 213.
16 M. Bieganowska, *Chromatographia*, 9 (1976) 168.
17 R. Modin and S. Bäck, *Acta Pharm. Suecica*, 8 (1970) 585.
18 I. Halász, R. Endele and J. Asshauer, *J. Chromatogr.*, 112 (1975) 37.
19 J. F. K. Huber, J. A. R. J. Hulsman and C. A. M. Meijers, *J. Chromatogr.*, 62 (1971) 79.
20 C. A. M. Meijers, J. A. R. J. Hulsman and J. F. K. Huber, *Z. Anal. Chem.*, 261 (1972) 347.
21 B. L. Karger, M. Martin and G. Guiochon, *Anal. Chem.*, 46 (1974) 1640.
22 D. Westerlund, J. Carlqvist and A. Theodorsen, *Acta Pharm. Suecica*, 16 (1979) 187.