Journal of Chromatography, 367 (1986) 171–180
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 820

Note

Dependence of the separation of some biological substances on the carbon content of C_{18} chemically bonded phases*

B. BUSZEWSKI**,*
Polymer Institute, CCR, Slovak Academy of Sciences, 842 36 Bratislava (Czechoslovakia)
K. ŠEBEKOVÁ
Medical Bionics Research Institute, 833 08 Bratislava (Czechoslovakia)
P. BOŽEK
State Hospital, 812 72 Bratislava (Czechoslovakia)
and
D. BEREK
Polymer Institute, CCR, Slovak Academy of Sciences, 842 36 Bratislava (Czechoslovakia)
(First received November 15th, 1985; revised manuscript received May 21st, 1986)

From the point of view of sensitivity and selectivity of separation and sample pretreatment, high-performance liquid chromatography (HPLC) is the method of choice for quantification of various compounds from biological materials.

It is often difficult and time consuming to find suitable conditions for separation, *e.g.*, the column packing (physical and chemical properties of sorbents), particle and column size, temperature, volume flow-rate and the eluent composition (type and concentration of components and pH)[1–6]. Compared to the mobile phase composition and its influence on the retention data of the separated substances[2,7–9], less attention is usually paid to the influences of the column packing material, particularly because commercially available columns are routinely used. Therefore we tried to investigate how the coverage of the surface of the sorbent by C_{18} chains influences the retention data.

We were interested in two groups of biological substances:

(i) hydroxyindoles: 5-hydroxytryptamine (serotonin) and its main metabolite, 5-hydroxyindolacetic acid (5-HIAA), determination of which is important in the investigation of the pathophysiological mechanisms of hypertension, mental disorders and platelet disorders[10–13]

(ii) purines: theophylline, theobromine, caffeine, oxyphylline [7-(2-hydroxyethyl)theophylline] and 8-methoxymethyltheophylline. Their quantification is of great importance in monitoring the levels of theophylline in long-term treatment of bronchial asthma[14].

---

* Presented in part at the *Conference on High Performance Chromatographic and Electrophoretic Techniques in Biochemistry, Siófok, September 1985.*

** On leave from the Institute of Chemistry, M. Curie-Skłodowska University, 200 31 Lublin, Poland.

0021-9673/86/$03.50

A number of papers have dealt with the determination of these compounds by means of HPLC[15–20], but the influence of the physical and chemical properties of sorbents on the separation was not investigated.

In this paper we present the results of our investigations on the HPLC separation of serotonin and 5-HIAA contained in urine and on the separation of theophyline and its derivatives contained in sera, as a function of density of coverage of the chemically bonded C_{18} phase.

EXPERIMENTAL

Apparatus

An liquid chromatograph (LKB, Bromma, Sweden) with an electrochemical detector 656 (Metrohm, Herisau, Switzerland) was employed for the determination of hydroxyindoles. Potentials of 0.55 and 0.7 V were used for 5-HIAA and serotonin analysis, respectively. Purines were detected with variable wavelength detector operated at 273 nm (LKB).

The samples (20 μl) were injected into a 7125 sampling valve (Rheodyne, Berkely, CA, U.S.A.).

Columns and reagents

Monomeric chemically bonded C_{18} stationary phases based on spherical, 8-μm silica gel SG-7/G[21,22], were prepared by the method of Buszewski[23]. They were characterized by various densities of coverage. Their physical and chemical properties are listed in Table I.

The specific surface area, S_{BET}, pore volume, V, and pore diameter, D were determined by nitrogen adsorption on a Sorptomatic Model 1800 (Carlo Erba, Milan, Italy). The extent of surface coverage of the packing, α_{RP}, was calculated by means of Berendsen's equation[7] on the basis of carbon loading, determinated by C, H, N analysis on a Model 240 instrument (Perkin-Elmer, Norwalk, CA, U.S.A.). The concentration of surface silanol groups, α_{SiOH}, was determined by the method described by Nondek and Vyskočil[24].

The columns (100 mm × 4 mm I.D.) were packed according to the ascending technique as described by Buszewski *et al.*[25] After packing, the columns were tested according to Bristow and Knox[26].

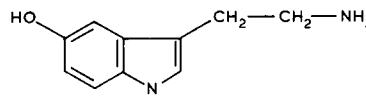
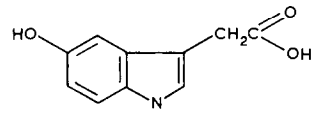
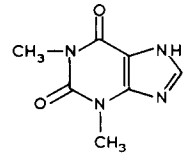
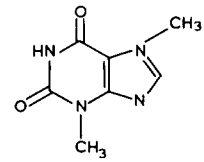
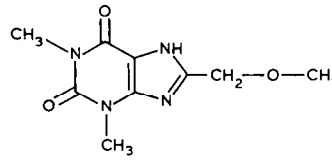
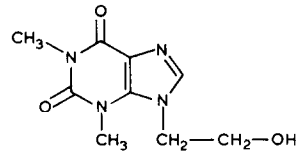
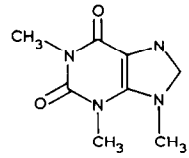
The eluent for the analysis of biological substances was selected by changing the type and the concentration of the amphiphilic ion, the pH, ionic strength and percentage of organic modifier, using a column of LiChrosorb RP-18 (5 μm, 150 mm × 4 mm I.D.; Merck, Darmstadt, F.R.G.).

The dead volume was calculated on the basis of solvent peaks, by considering the first positive peak eluted[23].

The following analytical grade reagents were used: methanol, acetonitrile and acetic acid (Merck). Other reagents were supplied by Lachema (Brno, Czechoslovakia). Doubly distilled water was used. The commercial standards used were: serotonin–creatinine sulphate (Merck), 5-hydroxyindolacetic acid (Koch-Light Laboratories, Colnbrook U.K.), caffeine, theophylline, theobromine and 2-7-hydroxyethyltheophylline (Léčiva, Prague, Czechoslovakia). 8-Methoxymethyltheophylline was synthesized by Dr. L. Štibrányi.

TABLE II

CHARACTERISTICS OF THE SEPARATED SUBSTANCES

No.	*Substance*	*Abbrevation*	*Formula*	*pK_a**	*Source of substance*
1	Serotonin	5HT		9.8–10	Urine
2	5-Hydroxyindol acetic acid	5-HIAA		4.7	Urine
3	Theophylline	TH		8.7	Sera
4	Theobromine	THB		9.9	Sera
5	8-Methoxymethyl theophylline	THOx		–	Sera
6	7-(2-Hydroxyethyl)-theophylline	HTTh		–	Sera
7	Caffeine	CAF		12.3	Sera

* According to ref. 32.

Preparation of standard mixtures and biological samples

Urine samples were pretreated off-line on Separcol[27] precolumns. The preseparation of serotonin was performed by the method of Koch and Kissinger[20]. For the isolation of 5-HIAA, standard Separcol SI C-18 precolumns were employed[28].

Stock solutions of serotonin and 5-HIAA were prepared in doubly distilled water. To obtain working standards, these solutions were diluted in artificial urine prepared according to Beck *et al.*[29], simulating the natural composition and ionic strength of human urine. The concentrations of serotonin and 5-HIAA were calculated by the external standard method and were plotted against peak height.

Theophylline and its derivatives were isolated from sera on microcrystalline cellulose[30]. Standards were made up in doubly distilled water. 8-Methoxymethyltheophylline was used as an internal standard.

RESULTS AND DISCUSSION

Table II lists the characteristics of the substances studied. Fig 1a presents a chromatogram of untreated human urine injected directly on the column, whereas Fig. 1b presents a chromatogram of the same urine sample but which had been previously purified on a precolumn.

From earlier investigations[23], it was known that the best support for preparation of the chemically bonded phase is that which has the greater diameter and volume of pores. It is characterized by the following parameters: average pore diameter, D = 20 nm; pore volume, V_p = 2.1 $cm^3\ g^{-1}$; surface area, S_{BET} = 360 $m^2\ g^{-1}$ and number of hydroxyl groups, α_{SiOH} = 5.3 $\mu mol\ m^{-2}$.

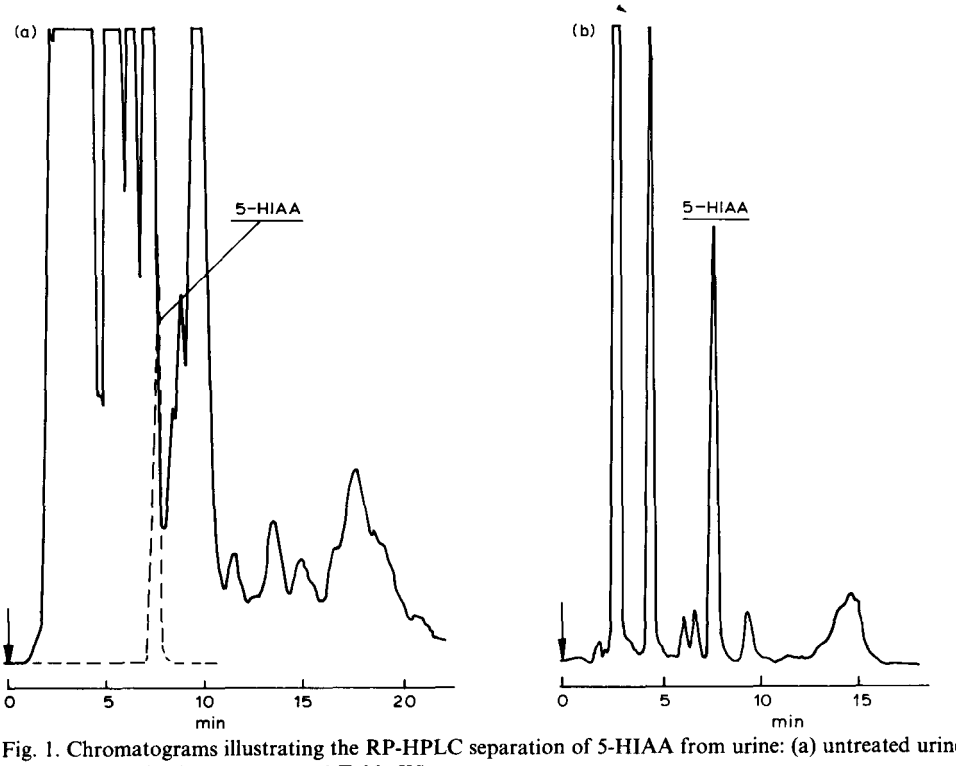


Fig. 1. Chromatograms illustrating the RP-HPLC separation of 5-HIAA from urine: (a) untreated urine; (b) preseparated urine (see text and Table IV).

The chemical modification was carried out to obtain a monomeric chemically bonded alkyl phase with various densities of surface coverage (Table I)[23]. From the model chemically bonded C_{18} phase proposed by Berendsen[7] and other authors[2,8,9,23], it was determined that monochloroactadecylsilane leads to a pure monomeric coverage of the silica gel.

Table I contains data characterizing the sorbents prepared. As the surface coverage by the alkyl phase increases, the number of silanol groups diminishes and the parameters characterizing the physical structure of the packing material, *i.e.*, pore volume and diameter and the surface area, become smaller. The coverage varied between 0.72 and 4.2 μmol m^{-2} for non-endcapped sorbents and from 1.18 to 4.28 μmol m^{-2} for endcapped sorbents. From a stereochemical point of view, this coverage is possible, as long as the maximum alkyl chain concentration is 2.8 alkyls per nm^2 of the surface. The smallest difference in carbon concentration before and after the endcapping was observed for packing 5 (ref. 23).

The chromatographic columns were characterized by Knox and Bristow parameters (Table III). Naphthalene was selected as a test substance. Upon increasing the degree of coverage, the amount of methanol added to the mobile phase must also be increased, illustrating the more hydrophobic character of the packing material. Such mobile phase composition (Table III) yields k' values of 4–5 for naphthalene at different degrees of surface coverage.

The separation efficiency of the columns is considered to be good. The number of theoretical plates varied from 2500 to 3500 per 10 cm for spherical 8-μm particles, which corresponds to a reduced plate height, h = 3.9–6.6. The flow resistance parameter, φ, of the colums was about 500–1500, mainly because of relatively low working pressures, which did not exceed 6 MPa. The endcapping reduced the tailing of peaks, as shown by the asymmetry factors, f_{As}. The columns were used to separate biological substances, by employing ion-suppression reversed-phase HPLC.

TABLE III

COLUMN PROPERTIES

$\bar{d}_p$ = Particle diameter, v = reduced velocity.

Column No.	*Coverage of OH groups (%)*	*Mobile phase (methanol–water) composition (%, v/v)*	$\bar{d}_p$ *(μm)*	*Naphthalene*				
				k'	h	v	φ	f_{As}
1	13.3	50:50	8	5.1	6.6	6.1	782	1.42
6	21.8			4.8	5.7	6.0	823	1.23
2	34.4	60:40	8	4.7	5.7	6.2	778	1.39
7	41.0			4.55	5.15	6.05	794	1.20
3	53.2	70:30	8	4.5	5.20	6.1	742	1.42
8	67.5			4.4	4.90	5.9	892	1.21
4	67.6	75:25	8	4.3	4.95	5.9	717	1.47
9	83.7			4.15	4.60	5.8	870	1.19
5	89.8	80:20	8	4.05	4.09	6.2	695	1.25
10	94.8			3.92	3.90	6.02	698	1.17

TABLE IV

MOBILE PHASE COMPOSITIONS

Substance	*Mobile phase composition (%, v/v)*	*Optimum pH*	*Concentration of modifier (mol l^{-1})*
Serotonin	Methanol–water (20:80)	4.5	0.02 Ammonium acetate
5-Hydroxyindolacetic acid	Methanol–water (15:85)	4.5	0.08 Sodium acetate
Theophylline Theobromine 7-(2-Hydroxyethyl)theophylline 8-Methoxymethyltheophylline Caffeine	Acetonitrile–water (10:90)	4.05	0.1 Acetic acid

Mobile phase compositions suggested for use with LiChrosorb RP-18 covered by a polymeric alkyl phase characterized by $\alpha_{RP} = 3.82$ μmol m^{-2} according to Bidlingmeyer[6] are listed in Table IV. The composition was slightly different from that used for separation of 5-HIAA because serotonin contains two nitrogen atoms. Purines were separated under different chromatographic conditions (Table IV). Acetonitrile was selected because of its lower viscosity and better UV transparency as compared with methanol.

Fig. 2 shows the dependence of k' for the separated substances on the surface coverage of silica gel by monomeric chemically bonded C_{18} phase. The biological substances with different pK_a values (dissociation constants) had the highest k' values on the sorbents densely covered with monomeric C_{18} phase, *i.e.*, $\alpha_{RP} \geqslant 2.8$ μmol m^{-2}, mainly $\alpha_{RP} = 4$ μmol m^{-2}.

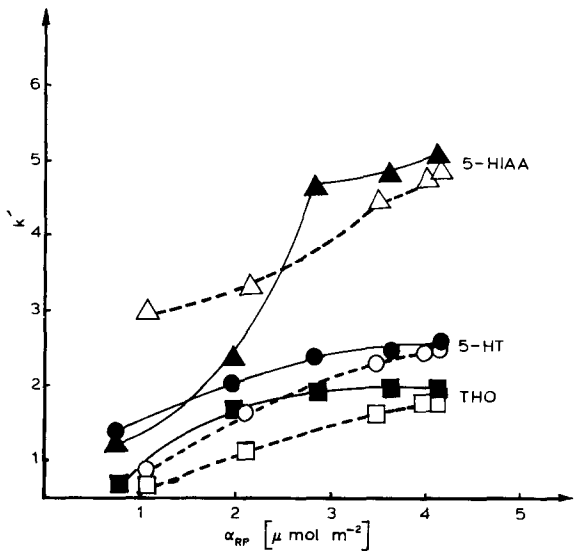


Fig. 2. Influence of the concentration of the chemically, bonded C_{18} phase on the capacity factor, k'. —, Non-endcapped sorbents; – – –, endcapped sorbents.

The endcapping markedly influences the carbon content and α_{RP} in sorbents with the lower densities of coverage by the chemically C_{18} bonded phase, up to 2.5 μmol m^{-2}.

The influence of silanol groups on the separation and interaction is evident for non-endcapped sorbents, especially in the case of 5-HIAA. Free electron pairs on nitrogen as well as carboxyl groups preferentially interact with unblocked silanols of sorbents with lower C_{18} coverages.

Fig. 3 shows the chromatograms of serotonin and 5-HIAA on the commercial LiChrosorb RP-18 column and our column with a coverage of 3.66 μmol m^{-2}. We were able to separate 5-hydroxytryptamine in a shorter time, and in both cases the hydrodynamic resistance was lower in our sorbent. Both factors are of great importance from the point of view of routine analysis.

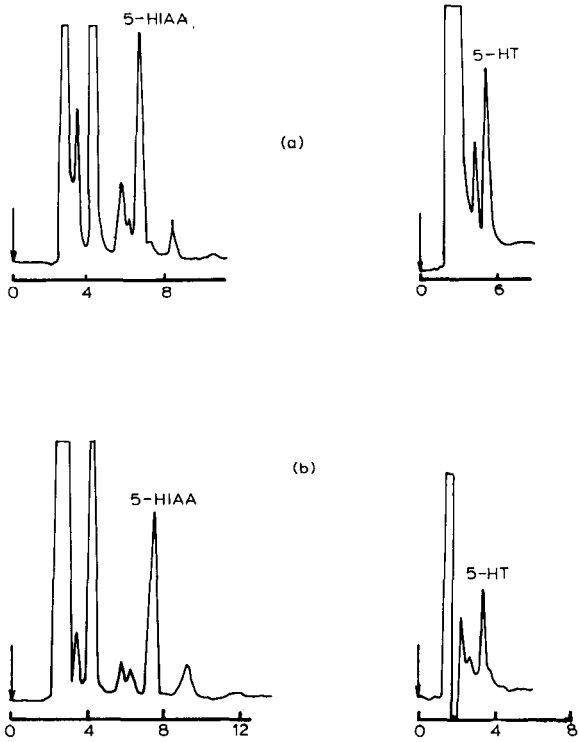


Fig. 3. Comparison of the separation of 5-HIAA and of 5-HT on (a) LiChrosorb RP-18 and (b) column 4 with α_{RP} = 3.66 μmol m^{-2} (see text and Table IV).

In the separation of theophylline and its derivatives (Fig. 4) the best resolution and higher selectivity for oxyphylline and caffeine was achieved on our column with a surface carbon coverage, α_{RP} = 4.20 μmol m^{-2}. This seems to be due to the different structure of the support (compared with LiChrosorb RP-18) and the different composition of the C_{18} chemically bonded phase, which explains the small differences in k', especially in the case of peaks 4 and 5 in Fg. 4. Employment of a sorbent with greater pore volume and size makes the column capacity 50% higher in comparison

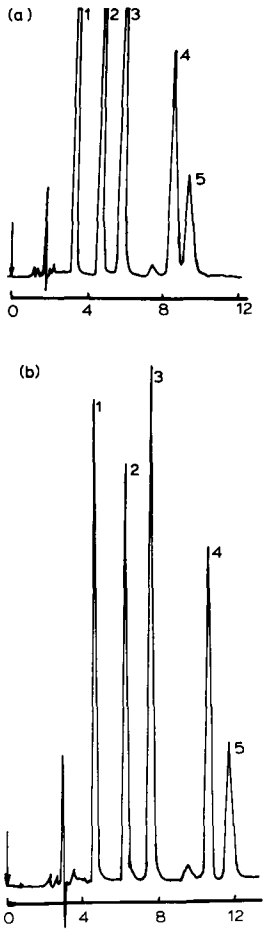


Fig. 4. Comparison of the separation of purines on (a) LiChrosorb RP 18 and (b) column 5 with α_{RP} = 4.2 μmol m^{-2} (see text and Table IV). Peaks: 1 = THB; 2 = THO; 3 = HTTh; 4 = THOx; 5 = CAF.

to commercial material (LiChrosorb RP-18)[23,31], enabling the injection volume to be increased two-fold. The effect is that the detection limit is decreased ten-fold to about $5 \cdot 10^{-5}$ g l^{-1}.

As the last two figures show, the separation efficiency on one of the most widely used sorbents — LiChrosorb for purine derivatives and 5-HIAA — is relatively good. For the separation of serotonin on polymeric LiChrosorb, it is necessary to use a mobile phase which yields a relatively high capacity factor and therefore the retention time increases. The new types of sorbents offer the possibility to obtain the same or even better results in a shorter time and with lower hydrodynamic resistance.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. L. Nondek for measurement of silanol groups and Dr. L. Štibrányi for synthesizing 8-methoxymethyltheophylline.

REFERENCES

1 G. D'Agostino, L. Castagnetta, F. Mitchell and M. J. O'Hare, *J. Chromatogr.*, 338 (1985) 1.
2 J. L. M. van de Venne, *Ph. D. Thesis*, Technische Hogeschool Eindhoven, 1979.
3 H. A. H. Billiet, A. C. J. H. Drouen and L. de Galan, *J. Chromatogr.*, 316 (1984) 231.
4 A. P. Goldberg, E. Nowakowska, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 316 (1984) 241.
5 Z. Suprynowicz, B. Buszewski, J. Rogalski and E. Malarczyk, *J. Chromatogr.*, 286 (1984) 253.
6 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525.
7 G. E. Berendsen and L. de Galan, *J. Liq. Chromatogr.*, 1 (1978) 561.
8 K. Szabo, N. L. Ha, Ph. Schneider, P. Zelter and K. sz. Kováts, *Helv. Chim. Acta*, 67 (1984) 2128.
9 L. C. Sander and S. A. Wise, *J. Chromatogr.*, 316 (1984) 163.
10 P. M. Vanhoutte, in F. de Clerck and P. M. Vanhoutte (Editors), *5-Hydroxytryptamine in Peripheral Reactions*, Raven Press, New York, 1982.
11 B. Shopsin and N. F. Feiner, *Adv. Biol. Psychiat.*, 14 (1984) 1.
12 S. Cortellazo, P. Viero and G. de Gastano, in F. de Clerck and P. M. Vanhoutte (Editors), *5-Hydroxytryptamine in Peripheral Reactions*, Raven Press, New York, 1982.
13 F. de Clerck and P. M. Vanhoutte, in F. de Clerck and P. M. Vanhoutte (Editors), *5-Hydroxytryptamine in Peripheral Reactions*, Raven Press, New York, 1982.
14 M. Bukowski, K. Nakatsu and P. W. Munt, *Ann. Intern. Med.*, 101 (1984) 63.
15 B. Petruccelli, G. Bakris, T. Miller, E. R. Korpis and M. Linnoila, *Acta Pharmacol. Toxicol.*, 51 (1982) 421.
16 J. De Jong, U. R. Tjaden, W. van 't Hof and C. F. M. van Valkenburg, *J. Chromatogr.*, 282 (1983) 443.
17 E. Kwarts, J. Kwarts and H. Rutgers, *Ann. Clin. Biochem.*, 21 (1984) 425.
18 D. Fretthold and J. Sunshhire, *Clin. Chem.*, 26 (1980) 1054.
19 N. Kucharczyk and F. H. Segelman, *J. Chromatogr.*, 340 (1985) 243.
20 D. D. Koch and P. T. Kissinger, *J. Chromatogr.*, 164 (1979) 441.
21 D. Berek and I. Novák, *U.S. Pat.*, 4, 255, 286 (1981).
22 I. Novák and D. Berek, *U.S. Pat.*, 4, 382, 070 (1985).
23 B. Buszewski, *Ph. D. Thesis*, Technical University, Bratislava, 1986.
24 L. Nondek and V. Vyskočil, *J. Chromatogr.*, 206 (1981) 581.
25 B. Buszewski, Z. Suprynowicz and T. Pastuszak, *Chem. Anal. (Warsaw)*, 28 (1983) 527.
26 P. A. Bristow and J. H. Knox, *Chromatographia*, 10 (1977) 279.
27 L. Šoltés, I. Novák and D. Berek, *Czech. Pat.*, 234, 100 (1983).
28 P. Božek, K. Šebeková and G. Brimmichová, *Biochim. Clin. Bohemoslov*, in press.
29 O. Beck, G. Palmskog and E. Hullman, *Clin. Chim. Acta*, 79 (1977) 149.
30 P. Božek, O. Uličná, J. Zemek and L. Štibrányi, *Biochim. Clin. Bohemoslov.*, 15 (1986) 175.
31 B. Buszewski, D. Berek, I. Novák and J. Garaj, *Budapest Symposium on Chromatography, Budapest, June 1985.*
32 D. D. Perrin, *Dissociation Constants of Organic Bases in Aqueous solutions*, Butterworths, London, 1965.