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Note

Effect of chemically bonded alkyl chain length on the recovery of serotonin and its metabolite from urine by a solid extraction clean-up procedure

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Serotonin is a neurotransmitter in cerebral centres and its perturbations can produce humour and behavioural disorders. The investigation of tryptophan (TRP) and serotonin (5-HT) metabolism is important in neurochemistry also for the early detection and supervision of treatment of carcinoid tumours.

Different procedures have been devised for the determination of the tryptophan, serotonin and the major metabolite of serotonin, *i.e.*, 5-hydroxyindole-3-acetic acid (5-HIAA). Ultraviolet absoprtion, spectrophotometric and fluorimetric determination have been thoroughly reviewed[1]. Radioenzyme methods mass spectrometry and gas chromatography–mass spectrometry have been also used[2,3]. Because of its high separating power, the application of high-performance liquid chromatography (HPLC) is still increasing in importance. Many investigators employ fluorescence detection[4–6] but it generally requires a derivatization step for high sensitivity. LC methods using UV detection suffer from a lack of sensitivity[7,8]. Most simple and sensitive for neurochemical applications seems to be the use of HPLC with electrochemical detection (ED)[9–11]. ED and UV methods have been compared and the superiority of the former has been demonstrated[12]. The sensitivity of ED is higher for 5-hydroxytryptophan (5-HTP) and 5-HIAA whereas the opposite holds for the fluorimetric detection of serotonin and tryptophan[6,13–15].

Tryptophan metabolites have been determined in brain tissue[14–22], cerebrospinal fluid[23,24], urine[23], serum and plasma[17,23] and biological materials[25,26]. There are different methods for the isolation of material of biological origin[23], *e.g.*, TRP and 5-HTP using a strong cation-exchange resin, *e.g.*, Dowex AG-50, 5-HT on a weak cation exchange resin, *e.g.*, Amberlite GC-50, and 5-HIAA on Sephadex G-10 gel. "Off-linc" isolation pre-columns are usually used. Attempts to use "on-line" preconcentration columns resulted in a *ca.* 100-fold increase in detection sensitivity[17,20].

Dense layered bonded stationary phases have been used successfully for the isolation of 5-HIAA from urine[27] and in HPLC separations[28]. Some attempts to isolate tryptophan and its metabolites have been made[29,20].

The properties of sorbents with alkyl bonded stationary phases are strongly dependent on many factors, such as coverage density[31], porous structure and chemical nature of the solid silica support[32,33], amount of remaining silanol groups (endcapping) and bonded layer structure (functionality of silane modifier). Monochloro-

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dimethylalkylsilanes seem to be the most promising modifiers because their use allows precisely defined and dense alkyl bonded layers to be obtained[31,34,35]. Inconvenient interaction of remaining silanol groups is usually eliminated by secondary silanization (end-capping) and its effectiveness may differ depending on how dense a layer is obtained in primary silanization[36,37].

EXPERIMENTAL

The chromatographic system consisted of an HPP-4001 pump (Laboratorní Přístroje, Prague, Czechoslovakia), a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 50-μl sample loop and 100 × 4.6 mm I.D. stainless-steel column (Chemical Reagents Factory, ZOCh, Lublin, Poland) packed with LiChrosorb RP-18 (5-μm) (Merck, Darmstadt, F.R.G.). An ELDEC 102 electrochemical detector (Chromatofield, Chateauneuf-Les Martiques, France) with a glass carbon electrode was employed at a potential of +0.6 V (5-HT, 5-HIAA) *vs.* a silver–silver chloride reference electrode.

The specific surface areas (S_{BET}) of the sorbents for the pre-columns were determined by the BET method from nitrogen adsorption data using a Sorptomatic Model 1800 instrument (Carlo Erba, Milan, Italy). The degree of surface coverage of the sorbents (α_{RP}) was calculated by means of the Berendsen equation[38] on the basis of the carbon loading (% C) determined by a Model 185 CHN analyser (Hewlett-Packard, Palo Alto, CA, U.S.A.).

LiChrosorb Si-60 was chemically modified by C_6, C_8 and C_{18} monochlorodimethylsilanes (Petrarch Systems, Levittown, PA, U.S.A.) in the absence and presence of an activator according to the method proposed by Buszewski *et al.*[35,39]. C_1 and secondary silanization (end-capping) were performed using hexamethyldisilazane (HMDS) (POCh, Gliwice, Poland).

Analytical-reagent grade chemicals used for off-line clean-up isolation and for chromatography were obtained from POCh. The mobile phase consisted of analytical-reagent grade chemicals dissolved in water [0.15 *M* sodium phosphate buffer (pH 4.2) containing 13% (v/v) of methanol]. Doubly distilled, deionized water was used.

The standards used were tryptophan (Reanal, Budapest, Hungary), serotonin (Merck), 5-hydroxyindole-3-acetic acid (Koch-Light Labs., Colnbrook, U.K.). Standard solution consisted of TRP (10 μl/ml, 5-HT (1 μg/ml) and 5-HIAA (2 μl/ml) dissolved in water, prepared freshly every month and kept frozen until used.

The off-line clean-up pre-columns consisted of 2-ml plastic extraction tubes packed with various types of synthesized stationary phases to yield a 1.8-cm high bed. The simultaneous isolation of 5-HT and 5-HIAA from the standard solutions and urine was carried out according to the procedure described in ref. 29.

RESULTS AND DISCUSSION

From the viewpoints of detectability, a constant potential of the working electrode (+0.6 V) and the most interesting information in neurochemistry, we investigated the recoveries of 5-HT and 5-HIAA using the pre-column clean-up procedure (Table I). The recovery of TRP was also tested at a detector potential of +0.9 V and

it was close to the values for 5-HIAA. The physico-chemical characteristics of off-line clean-up pre-columns with various sorbents are given in Table I.

The results may be discussed in terms of the chemically bonded surface hydrophobicity and availability of the remaining silanol groups for solute molecules. Surface hydrophobicity is directly proportional to the number of bonded main alkyl groups[31,34,35] or, more precisely, inversely proportional to the relaxation time (alkyl chain mobility) in CP-MAS NMR measerements[36,37]. Our results support the conclusions of Bayer *et al.*[36] that a high hydrophobicity (low relaxation times) may be obtained not only by dense coverage with main alkyl groups but also by exhaustive secondary end-capping of the partially covered silica surface. This is in agreement with our results for shorter bonded alkyl chains (C_6 and C_8) (sorbents 5, 6, 9 and 10 in Table I). A similar situation exists for the sorbents with a long C_{18} bonded alkyl chain (sorbents 13 and 14 in Table I), but there are evident hindrances to narrow pore penetration in bare silica gel[39].

Comparison of C_1 sorbents cannot be taken into account because primary and secondary silanization were effected with the use of the same modifier, *i.e.*, HMDS, which introduces the same trimethylsilyl radicals on the silica surface. The presence of an activator does not influence the surface reaction (lower surface coverage of sorbent 2 than 1; Table I) and, moreover, the recoveries of 5-HT and 5-HIAA are low and non-reproducible. Of course, the highest coverage density of the surface with main alkyl chains and hence the highest silica gel surface hydrophobicity may be obtained in the presence of morpholine activator[34,35].

Considering the influence of bonded alkyl chain length on most hydrophobic surfaces (EC and A + EC), it can be seen that the best 5-HT and 5-HIAA recoveries are given by C_8 pre-columns (sorbents 9 and 10, Table I). Similar results were obtained for C_{18} packings (sorbents 13 and 14, Table I). Bonding of shorter alkyl chain leads to lower recoveries of 5-HT for C_1 and C_6 (except sorbent 5, Table I) and a high 5-HIAA recovery (except sorbent 6, Table I). The coefficients of variation of the results obtained with the above sorbents, calculated for the average values from six independent measurements of recovery, are relatively low (Table I).

The above discussion of recoveries is valid for the results obtained with a standard two-component solute mixture. Test measurements with human urine showed that of the two available groups of alkyl-bonded sorbents of highest hydrophobicity (EC and A + EC), the latter gives a better performance (Fig. 1). Sorbents obtained without an activator gave a poor recovery of 5-HT from urine samples, in contrast to those obtained for standard solutions (Table I).

The 5-HT and 5-HIAA recoveries shown in Table I were obtained using a standard clean-up procedure[29] in which the investigated compounds were sorbed on "off-line" pre-columns from 2 ml of standard solution and the same volume of displacing agent [0.1 *M* ammonia solution containing 25% (v/v) of methanol] was used. Samples of 10 μl were taken from the eluate injected into the chromatograph.

This method guarantees the use of less than half of the clean-up pre-column capacity, because the breakthrough volumes correspond to *ca.* 5 ml of a standard solution or urine. Hence it is possible to obtain an almost 2-fold urine sample enrichment if 4 ml of urine and only 2 ml of displacing agent are used. Fig. 1 shows chromatograms that illustrate the 5-HT and 5-HIAA recoveries obtained for double urine samples on clean-up pre-columns with different sorbents. Because of the low

TABLE I

CHARACTERISTICS OF SORBENTS FOR OFF-LINE CLEAN-UP PRE-COLUMNS AND COMPARISON OF RECOVERIES OF 5-HT AND 5-HIAA FROM STANDARD SOLUTION (n = 6)

No.	*Type of sorbent for pre-column**	*Coverage density*		*Recovery of 5-HT ± S.D. (%)*	*Coefficient of variation*	*Recovery of 5-HIAA ± S.D. (%)*	*Coefficient of variation*
		C (%)	*α_{RP} (μmole/m²)*				
1	SG-Si-60 + C_1	6.45	5.81	4.2 ± 1.5	35.7	91.8 ± 3.0	3.3
2	+ C_1 + A	4.49	3.90	8.5 ± 2.7	31.8	Irreproducible.	
3	+ C_6	6.26	2.07	2.0 ± 0.7	35.0	84.6 ± 2.5	3.0
4	+ C_6 + A	10.59	3.89	5.6 ± 1.9	33.9	90.0 ± 1.3	1.4
5	+ C_6 + EC	10.43	5.87	48.4 ± 2.3	4.8	74.6 ± 1.9	2.5
6	+ C_6 + A + EC	13.95	5.60	23.4 ± 1.3	5.6	44.1 ± 2.3	5.2
7	+ C_8	7.89	2.12	2.6 ± 0.5	19.2	82.8 ± 3.1	3.7
8	+ C_8 + A	13.01	3.08	3.5 ± 1.0	28.6	85.7 ± 1.2	1.4
9	+ C_8 + EC	11.98	5.66	64.9 ± 2.2	3.4	86.3 ± 2.7	3.1
10	+ C_8 +A + EC	15.10	5.54	31.9 ± 2.4	7.5	60.0 ± 2.9	4.8
11	+ C_{18}	10.16	1.39	4.0 ± 0.9	22.5	79.0 ± 3.4	4.3
12	+ C_{18} + A	17.26	2.65	1.7 ± 0.4	23.5	79.5 ± 2.3	2.9
13	+ C_{18} + EC	13.49	4.22	46.5 ± 1.8	3.8	80.5 ± 3.2	3.9
14	+ C_{18} + A + EC	18.66	3.80	56.1 ± 1.9	3.4	62.7 ± 2.2	3.5

* SG-Si-60 = bare silica gel. S_{BET} = 348 m²/g, mean pore diameter (D) = 8.6 nm, pore volume (V_p) = 0.884 cm³/g, particle size (d_p) = 40–60 μm. A = activator. EC = end-capped (HMDS). C_1–C_{18} = length of bonded alkyl chain. Modifiers: C_6–C_{18} monochlorodimethylalkylsilanes.

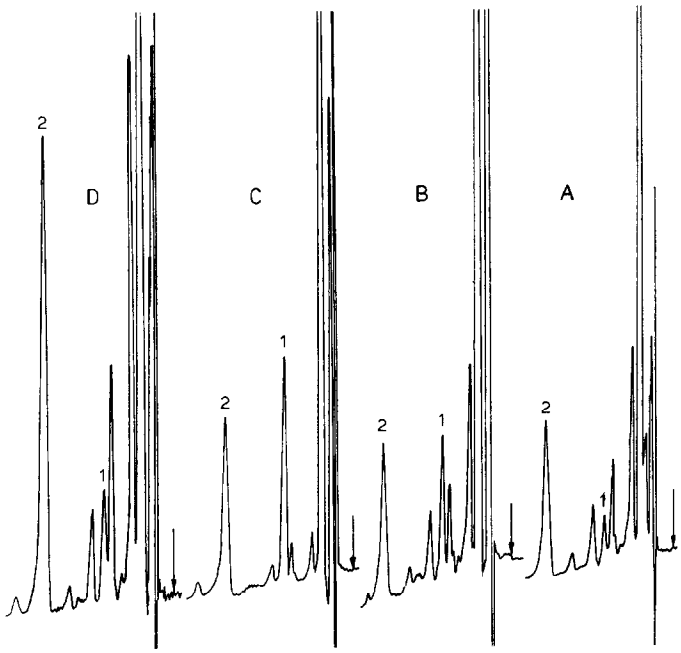

Fig. 1 Chromatograms of 5-HT and 5-HIAA obtained for double human urine samples using clean-up pre-columns with different sorbents. (A) Untreated urine; (B) urine with addition of 0.5 μl/g of 5-HT standard; after pre-column osilation and preconcentration, (C) C_{8+A+EC} and (D) C_{8+A+EC}. Chromatographic conditions: C_{18} column (250 × 4 mm I.D.) packed with 7-μm spherical particles (ZOCh); electrochemical detector (+0.6 V); mobile phase, 0.15 *M* sodium phosphate buffer (pH 4.2) containing 12.5% (v/v) of methanol; flow-rate, 0.9 cm^3/min; inlet pressure, 80 bar; sample volume, 10 μl. Peaks: 1 = 5-HT; 2 = 5-HIAA.

serotonin concentration (*ca.* 0.1 μg/ml), standard 5-HT solution (0.5 μl/ml) was added to common urine in order to show the real differences in the investigated pre-columns.

On a C_{8+A+EC} pre-column (C, Fig. 1) real enrichment of both solutes takes place. The $C_{18+A+EC}$ pre-column (D, Fig. 1) gives a better pre-concentration of 5-HIAA but poorer that might be expected for 5-HT, which may be the result either of a lower coverage density (sorbents 13 and 14, Table I) or of a slightly different sorption mechanism (poor pre-cleaning). This problem may be partially solved by the use of larger pore silicas[29,30], but even this procedure does not permit the same mechanism and the sample capacity in relation to that of C_{8+A+EC} localized on the surface of Si-60 silica gel.

A clear explanation of the phenomena discussed is difficult on the basis of the data in Table I. Taking into account literature data[29–31] and our latest results[40], we suggest that the most active compound towards silanol groups, 5-HT (amine), needs the best screening of the silica surface with a chemically bonded film to avoid undesirable interactions. This is probably achieved on $C_{18+A+EC}$ sorbent bonded on silica gel Si-60, where the narrow pores are completly closed with alkyl groups, in contrast to the effect of the shorter C_8 alkyl chain.

The disadvantage of the $C_{18+A+EC}$ material in practical pre-cleaning applications is its low selectivity, and from this point of view C_{8+A+EC} sorbent has a better performance (see the chromatograms in Fig. 1). It is noteworthy that the end-capping process is important, but it cannot solve the problem of elimination of surface activity because of steric hindrance (a trimethylsilyl group has *ca.* twice the cross-sectional diameter of a hydroxyl group) and allows the elimination of only half the silanol groups.

The different chemical natures of the investigated solutes influences their recoveries strongly and are similiar shapes of the molecules are less important. The low recovery of 5-HT is connected with irreversible sorption during the pre-cleaning procedure rather than its degradation and the latter effect is obviated through the standardization procedure during analysis.

We consider that the combination of clean-up samplers with different chemically bonded materials and a suitable choise of solvents for sorption–desorption processes will allow real practical applications to urine analysis. The limits of detection were established as 1 ng of 5-HIAA and 0.5 ng of 5-HT. If larger samples (up to 50 μl) are injected on to the HPLC column and a simple enrichment procedure is used, the limit of detection may be lowered by *ca.* one order of magnitude.

CONCLUSIONS

The hydrophobicity of the bonded alkyl layer and the availability of the remaining silanol groups on the silica gel surfaces to solute molecules are the main factors influencing the recoveries of 5-HT and 5-HIAA. Exhaustive silanization of the silica gel surface, including end-capping, gives usable sorbents for the isolation of 5-HT and 5-HIAA. C_8 sorbents synthesized on the basis of SI-60 silica gel in the presence of an activator show sufficient selectivity and recovery of 5-HT and 5-HIAA from urine samples and a sample capacity that allows simple enrichment before analysis.

REFERENCES

1 R. P. Maickel, *Methods Neurochem.*, 2 (1972) 101.
2 J. M. Saavedra, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 36 (1977) 2134.
3 J. M. Artigas and E. Gelpi, *Anal. Biochem.*, 92 (1979) 233.
4 G. M. Anderson and W. C. Purdy, *Anal. Chem.*, 51 (1979) 283.
5 T. P. Davis, C. W. Gehrke, C. W. Gehrke, Jr., T. D. Cunningham, K. C. Kuo, K. O. Gerhardt, H. D. Johnson and C. H. Williams, *Clin. Chem.*, 24 (1979) 1317.
6 A. M. Krstulovic and C. Matzura, *J. Chromatogr.*, 163 (1978) 72.
7 C. M. Riley, E. Tomlinson, T. M. Jefferies and P. H. Redfern, *J. Chromatogr.*, 162 (1979) 153.
8 N. Fornstedt, *Anal. Chem.*, 50 (1978) 1342.
9 P. T. Kissinger, C. S. Bruntlett and R. E. Shoup, *Life Sci.*, 28 (1981) 455.
10 A. M. Krstulović and P. R. Brown, *Reversed Phase High-Performance Liquid Chromatography*, Wiley, New York, 1982, pp. 134-181.
11 V. Pacaková and K. Štulik, *Nahrung*, 29 (1985) 293.
12 D. A. Richards, *J. Chromatogr.*, 175 (1979) 293.
13 G. M. Anderson and W. C. Purdy, *Anal. Chem.*, 51 (1979) 283.
14 B. A. Shaywitz, *J. Chromatogr.*, 233 (1981) 315.
15 B. H. C. Westerink, *J. Chromatogr.*, 233 (1982) 69.
16 J. M. Tusell, C. Sund, F. Artigas, E. Martinez and E. Gelpi, *Chromatographia*, 16 (1982) 112.

17 S. Sasa, C. L. Blank, D. C. Wenke and C. A. Szczupak, *Clin. Chem.*, 24 (1978) 1509.
18 W. H. Lyness, N. M. Friedle and K. E. Moore, *Life Sci.*, 26 (1980) 1109.
19 J. N. Mafford and J. D. Barchas, *J. Chromatogr.*, 181 (1980) 187.
20 D. D. Koch and P. T. Kissinger, *Life Sci.*, 26 (1980) 1099.
21 K. Oomagari, H. Whimury, T. Matsinoto, H. Matsuo, M. Saito, M. Hirano, C. Kin and M. Shimamura, *Neurosci. Kolbe Jpn.*, 8 (1982) 64.
22 A. Shum, M. J. Sole and G. R. van Loon, *J. Chromatogr.*, 228 (1982) 123.
23 D. D. Koch and P. T. Kissinger, *J. Chromatogr.*, 164 (1979) 441.
24 G. M. Anderson, J. G. Young and D. J. Cohen, *J. Chromatogr.*, 164 (1979) 501.
25 T. M. Kenyherez and P.T. Kissinger, *J. Food Sci.*, 43 (1978) 1354.
26 C. Hansson and E. Rosengren, *Anal. Lett.*, B 11 (1978) 901.
27 B. Buszewski, K. Šebeková, P. Božek, L. Štibranyi, J. Jendrichovský and L. Novák, *Chromatographia*, 22 (1986) 299.
28 B. Buszewski, K. Šebeková, P. Božek and D. Berek, *J.Chromatogr.*, 367 (1986) 171.
29 D. Sieńko, B. Buszewski and Z. Suprynowicz, *Anal. Chim. Acta*, in press.
30 B. Buszewski, D. Sieńko and Z. Suprynowicz, *J. Chromatogr.*, in press.
31 B. Buszewski and Z. Suprynowicz, *Chromatographia*, 24 (1987) 573.
32 H. Engelhard, B. Drayer and A. Schmidt, *Chromatographia*, 14 (1982) 11.
33 K. K. Unger, *Porous silica*, Elsevier, Amsterdam, 1979.
34 B. Buszewski and Z. Suprynowicz, *Anal. Chim. Acta*, 208 (1988) 263.
35 B. Buszewski, A. Jurášek, J. Garaj, L. Nondek, I. Novák and D. Berek, *J. Liq. Chromatogr.*, 10 (1987) 2325.
36 E. Bayer, A. Paulus, B. Peters, G. Laupp, J. Rainers and K. Albert, *J. Chromatogr.*, 364 (1986) 25.
37 A. Paulus, *Ph.D. Dissertation*, Tübingen, 1986.
38 G. Berendsen and L. de Galan, *J. Liq. Chromatogr.*, 1 (1978) 561.
39 B. Buszewski, *Ph.D. Dissertation*, Slovak Technical University, Bratislava, 1986.
40 D. Buszewski, D.Sieńko and Z. Suprynowicz, *J. Chromatogr.*, submitted for publication.