Journal of Chromatography, 91 (1974) 425–431

CHROM. 7256

PRELIMINARY STUDIES IN THE ANALYSIS OF BIOLOGICAL AMINES BY MEANS OF GLASS CAPILLARY COLUMNS

I. STUDIES WITH MODEL COMPOUNDS

J. J. FRANKEN and M. M. F. TRIJBELS*

Laboratory of Instrumental Analysis, Eindhoven University of Technology, Eindhoven (The Netherlands)

SUMMARY

Combination of the high resolving power of capillary columns and the extreme sensitivity of electron capture detection gives a powerful tool for the analysis of neurotransmittors. The preparation of glass capillary columns suitable for analyses at the sub-nanogram level is discussed. Several methods of derivative formation are compared. Model compounds, each containing one functional group, were used to evaluate procedures and some results obtained with biologically active compounds are presented.

INTRODUCTION

Neurotransmittors, their precursors and their metabolites occur in cerebrospinal fluid and various tissues, sometimes together with non-biogenic amines of therapeutic origin. Current research on these compounds is mainly carried out using analytical methods which detect individual compounds or groups of compounds. Gas chromatographic (GC) multi-component analysis, however, is very advantageous.

For gas–liquid chromatography these polar compounds, often containing both acidic and basic functional groups, must be converted quantitatively to more volatile and less polar derivatives. Even then, for successful quantitation the GC system should be very inert with respect to catalytic and adsorptive activity. This implies the use of an all-glass surface-deactivated injection system and of well deactivated glass columns. The extremely low concentrations experienced, about 1 to 100 ppb, necessitate the use of highly sensitive and selective detectors. Though most of the work was done on standard compounds using a flame ionization detector (FID), some authors have reported gas chromatographic–mass spectrometric (GC–MS) techniques (mass fragmentography)[1,2] and electron capture (EC) detection[2–5].

Separations are invariably carried out using packed columns. Yet the combination of the high resolving power of capillary columns with the extreme sensitivity of

* Present address: Psychiatric University Clinic, Division of Biological Psychiatry, Oostersingel 59, Groningen, The Netherlands.

an electron capture detector (ECD) could be a powerful tool for these analyses. The objective of this study is to investigate and select procedures for derivative formation and column preparation applicable to the quantitative analysis of neurotransmittors and related compounds at the sub-nanogram level.

STUDIES WITH MODEL COMPOUNDS

Method of approach

To compare the behaviour of the various functional groups towards different derivatizing reagents and different columns, model compounds, each containing only one functional group, were individually studied, *viz.* catechol, *p*-cresol, 1-phenylethanol, 2-phenylethanol, N-benzylmethylamine, phenethylamine and indole. It was assumed that any procedure for derivative formation and column preparation that was not completely satisfactory for all of the model compounds would not be suitable for the analysis of the biological compounds.

Gas chromatography

Apparatus and methods. A Model 5750 Hewlett-Packard gas chromatograph (Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with a FID and a ^{63}Ni ECD was used with special adaptions for the connection of capillary columns to the ECD[6]. Injections were performed using a slightly modified solids injection system[7]. When splitting was necessary a simple all-glass splitter was inserted into the injection block. Connections to the injector and detector adaptors were made by shrinkable PTFE tubes. To the small effluent stream of the capillary column additional purge gas was added at the detector junction to optimize the sensitivity of both the FID and the ECD[6].

The structure of the derivatives was studied using an AEI MS-12 mass spectrometer (AEI, Elmsford, N.Y., U.S.A.) coupled directly to the capillary column[8].

All columns were drawn from rather thick-walled capillary tubes of such dimensions that the resulting columns were of 1.2 mm O.D. and 0.4–0.6 mm I.D. and lengths of 15–25 m. Prior to coating, the columns were pretreated by silanization or surface-active agents to deactivate the glass wall. As column material we compared two kinds of glass commonly available, *viz.* Pyrex and soda lime glass.

Silanization was carried out in the gas phase according to Novotný *et al.*[9]. This method has been extensively studied in our laboratory[10] and is known to yield excellent columns for steroid analysis. Deactivation with surface-active agents was described by Metcalfe and Martin[11], who used Gas-Quat L (trioctadecylmethylammonium bromide), and by Malec[12], who used benzyltriphenylphosphonium chloride (BTPPC). Recently, our laboratory introduced the use of the gravimetric reagent for potassium, Kalignost (sodiumtetraphenyl borate) (KGn)[10,13], having a structure somewhat "antagonistic" to that of BTPPC. In this study we used the latter two agents. Experimental details were described by Rutten and Luyten[10].

After each step of the pretreatment, an intermediate test was performed so as to decide whether the glass wall was sufficiently deactivated. The idea for this test was introduced by Grob and Grob[14] and recently modified and studied by Rutten and Luyten[10]. The test consists of injection on to the uncovered column of a series of compounds of different adsorptive properties and of different acidity, *viz. n*-

undecane, dibutyl ketone, 2-propylcyclohexanol and 2,6-dimethylaniline. Columns exhibiting an asymmetrical elution peak for any of the test compounds are insufficiently deactivated. The pretreatment is then repeated and so is the intermediate test. Only columns that passed the test were coated.

Coating of capillary columns can be performed dynamically or statically. The static procedure of Bouche and Verzele[15] requires some experience and skill but the resulting columns are superior to those coated dynamically. The phase ratio can be predicted, which is not the case for dynamic methods. Hence, throughout this work we used a slightly modified version of this static method. SE-30 or OV-101 was used as the stationary phase with a film thickness of about 0.7–0.8 μm.

After the columns had been coated they were tested by a series of injections of the model compounds and of their derivatives (HFB-derivatives, *cf. derivative formation*). For comparative purposes some non-deactivated columns were included in the experiments.

Results and discussion. Non-deactivated Pyrex columns, being acidic in character, showed severe tailing and reduced peak areas for amines and alcohols. Only *p*-cresol was eluted with a reasonable peak shape. Non-deactivated soda lime glass columns were weakly basic and on them the secondary amine and alcohol were eluted with only slight tailing and *p*-cresol tailed considerably but the peak area was quite normal.

Surprisingly, silanization did not improve the peak shape nor the area of the non-derivatized compounds. As silanization reagents attack surface silanol groups only (Brönsted acid sites), it appears that at least some of the active sites are of a different nature. An explanation might be found in the presence of Lewis acid sites, associated with network-modifying cations (Ca^{2+}, Mg^{2+}, Ba^{2+}) or network-forming cations (Al^{3+}, B^{3+}). The latter possibility is the more likely one, as it is known that the incorporation of aluminium oxide or boron oxide in the vitreous silica framework yields a surface with high catalytic activity, due to the formation of both Lewis and Brönsted acid sites. Note that silanized columns nevertheless passed the intermediate test and were fully satisfactory for, *e.g.*, steroid analysis.

BTPPC and KGn are supposed to adhere to the glass wall rather than to react with it. Provided that shielding of the surface is complete, this might be a versatile method of deactivating both silanol groups and Lewis acid sites. Using these agents, it turned out to be much easier to deactivate soda lime glass than Pyrex. Yet, as a rule, the pretreatment had to be carried out at least twice. In Fig. 1 an example is given of the effect of repeated deactivation on the peak shape of the test compounds.

From comparisons of the coated columns it followed that soda lime glass columns were generally less active than Pyrex columns. The best results were obtained with KGn-treated soda lime glass columns. With these columns as little as 1 ng of indole could be chromatographed as such without notable adsorption or breakdown. Because all other model compounds, except catechol, were also eluted reasonably, it must be concluded that these columns are quite neutral and well deactivated.

After derivative formation all model compounds are eluted symmetrically from all types of column. Even non-deactivated columns yield quite reasonable peaks. These results, however, were obtained using a FID, the injected amounts being of the order of 1 to 10 ng. As previously reported[13] it is necessary to differentiate between

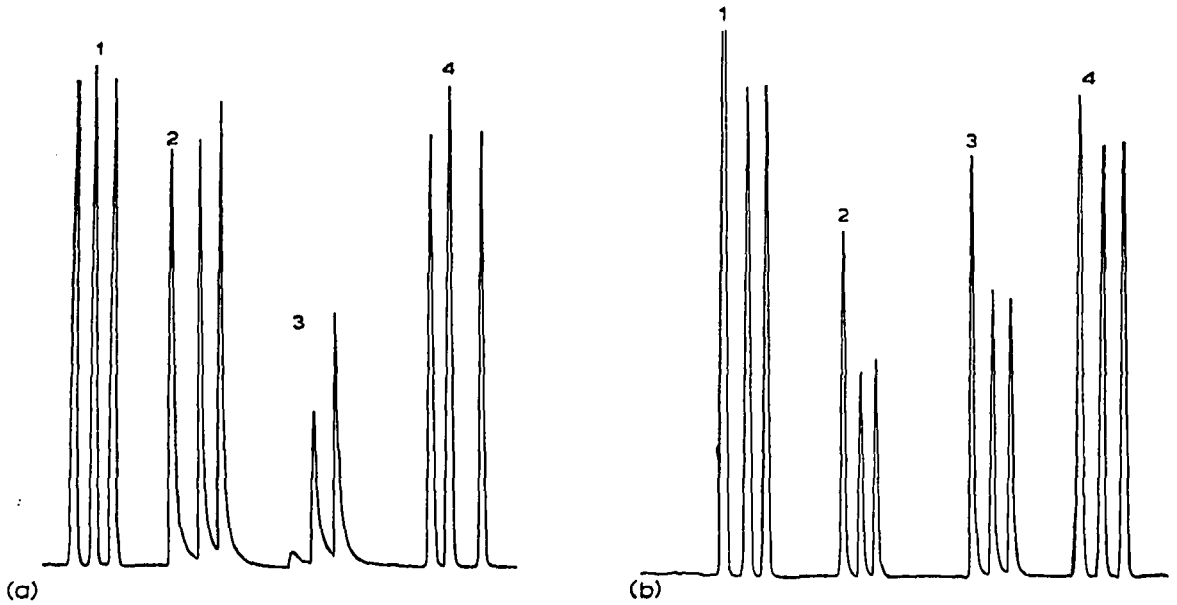

Fig. 1. Influence of repeated pretreatment on the peak shape of the test compounds. Column, 15 m × 0.55 mm I.D. soda lime glass; deactivation, KGn; temperature, 190°; linear velocity, 2 cm/sec. 1 = n-$C_{11}H_{24}$; 2 = dibutyl ketone; 3 = 2-propylcyclohexanol; 4 = 2,6-dimethylaniline. Each compound was injected three times, at 1.5 μg per injection. (a) Result after one rinsing. (b) Result after repeated rinsing.

results obtained at the ng level and results obtained at the pg level. Thus, the experiments were repeated using an ECD, the injected amount being about 10 pg of each derivative.

A marked difference was now observed between the columns. Generally, results with Pyrex columns were disappointing: tailing occurred and peak areas were irreproducible. With soda lime glass columns the results were much better and even non-deactivated columns showed rather symmetrical peaks. With respect to peak area, only the KGn-treated columns were fully satisfactory, provided that they had passed the intermediate test.

Thus, for the analysis of neurotransmittors at the sub-nanogram level, KGn-deactivated soda lime glass columns are the best choice.

Derivative formation

From the results obtained with the non-derivatized compounds, it follows that all functional groups, except perhaps the indole group, must be derivatized.

Using strong silanization reagents all the functional groups can be immediately silylated[16], yielding products with good GC properties but poor EC properties. With chlorinated silanization reagents the EC ability is improved but the attainable sensitivity is not high enough for analyses at the pg level.

A number of methods have been described which centre on N,O-acylation. The need for good EC properties suggests the use of halogeno-acyl derivatives, of which the heptafluorobutyryl (HFB) and pentafluoropropionyl (PFP) amides and

esters yield excellent sensitivity and are sufficiently stable[17]. Using a solids injection system, the somewhat less volatile HFB derivatives are to be preferred.

Alternatives, such as 2,4-dinitrophenyl, pentafluorobenzyl and several polyfluorobenzene derivatives, are reported not to be formed quantitatively[18,19] or are less universally applicable[20].

HFB-imidazole (HFBI)[21], purchased from Serva (Heidelberg, G.F.R.), and HFB anhydride (HFBA)[22], from Pierce (Rockford, Ill., U.S.A.) were used as reagents.

Using an ECD, the injection of a large excess of HFB reagent should be avoided. However, the complete removal of the HFB reagent proved not to be possible or desirable, because of the relative instability of some of the compounds of interest. So the reaction conditions were studied in order to adopt conditions as mild as possible with respect to the concentration of reagents. The influence of reaction time and temperature, the nature of the solvent and the addition of a catalyst were also studied.

Results and discussion. The conversion of aliphatic amines and alcohols proceeds smoothly under widely different reaction conditions. Excellent results were obtained with 3% v/v of either HFBA or HFBI in ethyl acetate as solvent. Conversion is completed in less than 1 min at room temperature without the addition of a catalyst.

Using HFBI, it was essential that the reaction was carried out in the dark. If light is admitted to the reaction mixture, a greenish yellow colour becomes apparent and the conversion is not reproducible. As the non-derivatized compounds chromatograph well on the columns described above, the reaction could easily be followed using a FID. As the model compound gradually disappeared, one single derivative peak was formed. After 1 min the model compound could not be detected any more. From the established limit of detection the minimal conversions of the model compounds were calculated to be 95, 99.5, 95 and 99.5% for the primary and secondary alcohols and the primary and secondary amines, respectively.

As solvents *n*-hexane and benzene were also tried, but the conversion was neither complete nor reproducible.

Under the conditions described above, aromatic hydroxyl groups were less reactive and behaved differently towards HFBI and HFBA. With HFBA the conversion of *p*-cresol needed 3 h. Catechol was not converted completely even after 75 h and in the presence of triethylamine (TEA) as catalyst. With HFBI in the dark *p*-cresol reacted quantitatively within 15 min and catechol within 30 min without catalyst. The minimal conversion of *p*-cresol, calculated as above, was 99%. As catechol does not elute as such the minimal conversion of this compound could not be calculated.

To study the concentration dependency of the reaction rate and yield, solutions of these six model compounds, containing approximately 5 mg/ml and 5 μg/ml, were derivatized using HFBI. The 5 mg/ml samples were diluted 1000 times and immediately analysed using EC detection. As internal standard *p*-chlorobromobenzene was used. The peak areas were compared to those obtained from the 5 μg/ml samples. As no significant differences could be found, it was concluded that the reaction rate and yield are independent of the concentration of the compound to be derivatized.

The nature of the indole nitrogen differs markedly from that in the aliphatic amines. Indole proved to be completely stable towards HFBI. Even after 75 h in the presence of TEA the peak area of indole was not reduced at all. With HFBA

indole disappeared slowly from the reaction mixture but without the formation of the desired product.

Ehrsson[23] reported the trifluoroacylation of the indole group using TFA anhydride and concluded that an acid-catalysed dimerization may occur. To inhibit this unwanted reaction, a tertiary amine was added. Yet, in the presence of TEA and in ethyl acetate as solvent the conversion was slow and incomplete. In *n*-hexane or benzene the reaction was reported to proceed smoothly. As stated above, however, these solvents cannot be used for the derivative formation of amines and alcohols. Although all functional groups can be derivatized under more drastic conditions[21], the indole group can be chromatographed as such without notable adsorption or breakdown (*cf. Gas chromatography*). Thus the formation of an indole derivative is not absolutely necessary. To prevent the dimerization of the free indole ring 1% by volume of TEA was added to the solvent as a routine precaution. This addition was found not to influence the formation of HFB derivatives using HFBI.

BIOLOGICALLY ACTIVE COMPOUNDS

Procedures for derivative formation of biologically active compounds can be deduced from the results obtained with the model compounds. The compounds under study can be derivatized in ethyl acetate as solvent containing 3% by volume of HFBI and 1 % of TEA. The reaction is completed at room temperature after 30 min. Exposure to light should be avoided.

Hydroxyl and amine groups are converted, while the less reactive indole group remains unchanged. The derivatives are separated using soda lime glass capillary columns deactivated by rinsing with a solution of KGn in acetone.

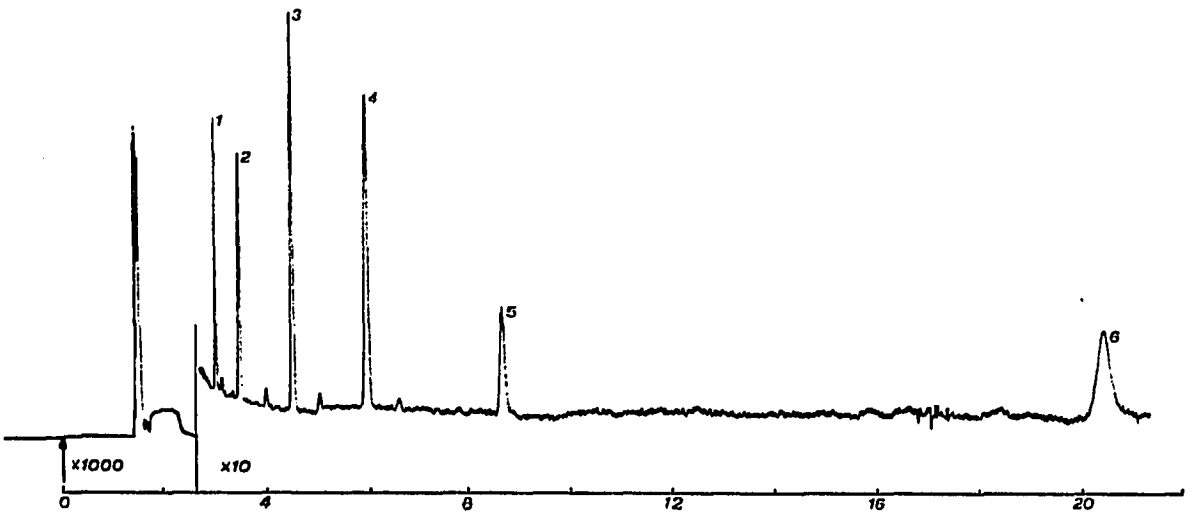

Fig. 2. Isothermal separation of HFB derivatives of reference compounds using a capillary column deactivated as in Fig. 1. Column, 22.5 m × 0.45 mm I.D. soda lime glass; stationary phase, SE-30; temperature, 175°. 1 = Ephedrine; 2 = 2-(3-methoxy-4-hydroxy)phenylethanol; 3 = 4-chloromethylamphetamine; 4 = tryptophol; 5 = indoleacetic acid ethyl ester; 6 = 5-methoxytryptamine.

The expected results were obtained in some preliminary experiments with ephedrine, 2-(3-methoxy-4-hydroxy)phenylethanol, 4-chloromethylamphetamine, tryptophol, indoleacetic acid ethyl ester and 5-methoxytryptamine. In Fig. 2 a chromatogram is reproduced.

REFERENCES

1 L. Bertilsson, A. J. Atkinson, Jr., J. A. Althaus, Å. Härfast, J. E. Lindgren and B. Holmstedt, *Anal. Chem.*, 44 (1972) 1434.
2 B. Sjöquist and E. Änggård, *Anal. Chem.*, 44 (1972) 2297.
3 K. Imai, M. Sugiura and Z. Tamura, *Chem. Pharm. Bull.*, 19 (1971) 409.
4 K. Imai, N. Arizumi, M.-T. Wang, S. Yoshine and Z. Tamura, *Chem. Pharm. Bull.*, 20 (1972) 2436.
5 E. Arnold and R. Ford, *Anal. Chem.*, 45 (1973) 85.
6 J. Franken and H. Vader, *Chromatographia*, 6 (1973) 22.
7 P. v. d. Berg and T. Cox, *Chromatographia*, 5 (1972) 301.
8 J. G. Leferink and P. A. Leclercq, *J. Chromatogr.*, 91 (1974) 385.
9 M. Novotný, K. Bartle and L. Blomberg, *J. Chromatogr. Sci.*, 8 (1970) 390.
10 G. A. F. M. Rutten and J. A. Luyten, *J. Chromatogr.*, 74 (1972) 177.
11 L. Metcalfe and R. Martin, *Anal. Chem.*, 39 (1967) 1204.
12 E. Malec, *J. Chromatogr. Sci.*, 9 (1971) 1318.
13 J. Franken and G. Rutten, in S. G. Perry (Editor), *Gas Chromatography 1972*, Applied Science Publishers, London, 1973, pp. 75–88.
14 K. Grob and G. Grob, *Chromatographia*, 4 (1971) 422.
15 J. Bouche and M. Verzele, *J. Gas Chromatogr.*, 6 (1968) 501.
16 P. W. Albro and L. Fishbein, *J. Chromatogr.*, 55 (1971) 297.
17 N. K. McCallum and R. J. Armstrong, *J. Chromatogr.*, 78 (1973) 303.
18 I. C. Cohen, J. Norcup, J. H. A. Ruzicka and B. B. Wheals, *J. Chromatogr.*, 44 (1969) 251.
19 F. Kawahara, *Anal. Chem.*, 40 (1968) 1009.
20 A. C. Moffat, E. C. Horning, S. B. Matin and M. Rowland, *J. Chromatogr.*, 66 (1972) 255.
21 J. Vessman, A. Moss, M. Horning and E. Horning, *Anal. Lett.*, 2 (1969) 81.
22 S. Clark and H. Wotiz, *Steroids*, 2 (1963) 535.
23 H. Ehrsson, *Acta Pharm. Suecica*, 9 (1972) 419.