CHROM. 13,915

GAS CHROMATOGRAPHIC DETERMINATION OF SOME BIOGENIC AMINES AS THEIR PENTAFLUOROBENZOYL DERIVATIVES IN THE PICOGRAM RANGE AND ITS APPLICABILITY TO BIOLOGICAL MATERIALS

URSULA E. G. BOCK[*,*] and PETER G. WASER

Institute of Pharmacology, University of Zürich, Gloriastr. 32a, CH 8006 Zürich (Switzerland)

(First received March 27th, 1980; revised manuscript received April 22nd, 1981)

SUMMARY

The catecholamines dopamine and noradrenaline were converted into the stable pentafluorobenzoyl (PFB) derivatives for their specific and quantitative gas chromatographic (GC) assay. This allowed their detection in the picogram range using an electron-capture detector. Acylation was performed with pentafluorobenzoyl chloride in the presence of pyridine and with acetonitrile as solvent. The structures of the PFB-catecholamines were confirmed by GC–mass spectrometry. A good separation was obtained on 5% OV-17 at 265°C. The high adsorption activity of the PFB-catecholamines could be overcome by optimizing the reaction conditions and applying special GC precautions. Linearity of the method was demonstrated for 50–500 ng of the catecholamines with detection at the picogram level. The application of the method to biological materials is demonstrated.

INTRODUCTION

The discovery of catecholamines (CAs) in the central nervous system by bioassays[1,2] has stimulated research with the development of analytical methods for their more specific and more sensitive determination in order to study their physiological importance. Not surprisingly, advances in the understanding of the biochemical bases of brain function are closely linked with all of these technological and methodological developments. Not all of the existing assays, such as spectrofluorimetric methods[3,4], radioenzymatic assays[5,6], gas chromatographic[7–20] and high-performance liquid chromatographic[21,22] methods combine sensitivity, specificity and reproducibility, the three requirements for their advantageous applicability. The method described here is specific, by employing the high separation capability of the gas chromatographic (GC) system, sensitive, with detection in the picogram range by using the high electron-capture detector (ECD)-specific pentafluorobenzoyl (PFB)

---

* Present address: Hommel AG, Postfach, CH 8134 Adliswil/ZH, Switzerland.

derivative together with the ECD, and reproducible, by optimizing the acylation and applying special GC precautions.

The polar nature of the CAs requires their conversion into suitable derivatives prior to gas-phase analysis. Various types of derivatives of the phenolic and alcoholic hydroxyl groups as well as of the amino group have been applied, and the kind of derivative and the kind of detector used determine above all the sensitivity of detection. Many derivatization procedures have been employed: N- and O-trimethylsilylation[7,8], O-trimethylsilylation together with N-ylidene formation[9,10], N-(2,4-dinitrophenyl) derivative formation[11], N-isothiocyanate formation[12] or N-acylation[13], cyclic boronate formation (only qualitative)[14] and, most important, N,O-acylation, especially trifluoroacetylation[15,16], pentafluoropropionylation[17–19] and heptafluorobutyrylation[20].

We chose the previously undescribed PFB derivative for the CAs because the N,O-acylation requires only a one-step reaction and, most important, the fluorinated aromatic system promised thermal stability and the highest ECD response. The latter was presumed as a consequence of the following known facts: (1) fluorine atoms in aromatic systems confer more electron-capturing properties to a molecule than those in aliphatic groups[23,24]; (2) the perfluoroaromatic ring is able to enhance the polarity of an adjacent carbonyl group[25], providing a coplanar, highly electron-delocalized system which probably accounts for the observed excellent electron-capture characteristics of the PFB derivatives; (3) O-perfluoroacylation is more important for sensitive electron-capture detection than N-perfluoroacylation[23,26], which favours the sensitive determination of the CAs; and (4) the pentafluorobenzamides of aliphatic amines, such as the CAs, exhibit much better sensitivity in the ECD than the pentafluorobenzamides of aromatic amines or any other non-aromatic fluoro-substituted aliphatic or aromatic amines[23,27]. In spite of these facts, the ECD sensitivity of the PFB-CAs could not be predicted with certainty. All aspects of the structural differences present within the whole molecule affect the efficiency of electron capture. In addition, it had to be established whether nanogram amounts of the polyfunctional CAs could be reproducibly pentafluorobenzoylated and whether the products, the PFB-CAs, could be analysed by GC in spite of their high molecular weight [945.43 for PFB-noradrenaline (PFB-NA)]. Associated with this, it had to be determined whether the GC conditions would be able to separate the PFB-CAs within reasonable retention times and, above all, whether this method would be applicable to biological materials.

EXPERIMENTAL

Reagents and chemicals

The CAs were obtained from Fluka (Buchs, Switzerland) and were of the purest grade available. Pentafluorobenzoyl chloride (PFBCl) and N-pentafluorobenzoylimidazole (PFBI) were Pierce products, purchased from Kontron (Zürich, Switzerland). All reagents and solvents were of analytical-reagent grade, supplied by Fluka, Merck (Darmstadt, G.F.R.) or Mallinckrodt (purchased from Oryx Pharmazeutika, Zürich, Switzerland). The purity of the solvents was checked by GC.

Equipment and operating conditions

GC was carried out with a Carlo Erba Fractovap GI gas chromatograph fitted with either a flame-ionization detector (FID), operated at 295°C with a hydrogen flow-rate of 25–30 ml/min and an air flow-rate of 250 ml/min, or a ^{63}Ni (10 mCi) ECD, combined with a Model 200 non-linearized exciter. The ECD was used in the pulse mode, amplitude 30 V, pulse width 10 μsec, pulse period 300 μsec, at a cell temperature of 300°C and with nitrogen as the scavenger gas at a flow-rate of 25 ml/min.

Silanized (according to Walle and Ehrsson[28]) glass columns (1 m × 2.5 mm I.D.) were used, packed with 5 % OV-17 on Chromosorb W, acid-washed and dimethylchlorosilane-treated, 80–100 mesh, sealed with silanized glass-wool. Before connection to the detector, the packed columns were conditioned overnight at 300°C, then silanized at 200°C with Silyl 8 (Pierce, Rockford, IL, U.S.A.) and additionally, prior to use, specifically conditioned at 265°C with an acetonitrile solution of the PFB-CAs. GC was performed isothermally at 265°C. The flash heater was maintained at 295°C. Nitrogen was used as the carrier gas with a flow-rate, measured at 265°C, as indicated in the figures.

Injection of the PFB-CAs on the picogram scale was carried out with ephedrine as priming substance to avoid memory effects. This so-called ephedrine injection was performed as follows: 1 μl of a solution of ephedrine in acetonitrile (1 μg/μl), prepared fresh daily) was first drawn up with a Hamilton 701N syringe (10 μl) and then separated by an air volume of 0.4 μl, 1.0 μl of the PFB-CA solution was drawn up.

All quantitative measurements were based on manual peak area measurements and expressed as area units (A.U.), corresponding to the injection of 1.0 μl: peak height (mm) multiplied by peak width at peak half-height (mm) multiplied by the electrometer sensitivity settings divided by the injection volume (μl).

Mass spectrometry (MS) was performed at 70 eV with an LKB Model 9000 GC–MS system. The GC conditions were as described above, except that the column used had an inner diameter of 3 mm.

Preparation of PFB derivatives

Derivatization in the milligram range. A 1-ml volume of acetonitrile was added to 1–2 mg of authentic CA (1 mg of DA = 6.5 μmol), followed by 0.2 ml of pyridine (2.48 mmol) and 0.1 ml of PFBCl [0.43 mmol, d = 1 (assumed)]. The reaction vial was closed and left at room temperature for 15 min. The solvents were removed by a flow of nitrogen at room temperature. The residue was dried for 60 min under high vacuum (0.1 Torr) and finally dissolved in 5 ml of acetonitrile. This gave a solution (200 ng of amine/μl, acylating 1 mg of amine) suitable for detection with the FID.

Derivatization in the nanogram range. These reactions were carried out in conical glass tubes, about 6 cm long and of I.D. *ca.* 7 mm with a volume of *ca.* 1 ml. Silanization of the glass tubes was unnecessary.

Samples containing nanogram amounts of the CA were prepared from stock solutions in 0.1 N hydrochloric acid. These solutions were lyophilized prior to acylation. A 50-μl volume of acetonitrile was added to the lyophilized residue (500 ng of DA = 3.26 nmol), followed by 2 μl of pyridine (24.8 μmol) and 2 μl of PFBCl (8.7 μmol), with a Hamilton syringe. The tube was tightly sealed with a polyethylene cap and the contents were thoroughly mixed at room temperature for 30 min. Before

opening, the tube was centrifuged for 5 min at 6000 g (0°C). Excess of derivatization agents were removed first by blowing off with nitrogen at room temperature and then by drying the residue under high vacuum (0.1 Torr) for 30 min. The PFB-CAs were dissolved in 0.5–1.0 ml of acetonitrile and were ready for GC analysis with electron-capture detection.

Preparation of tissue extracts

A male albino rat was killed by decapitation, the brain was removed as quickly as possible and a frontal slice, containing the corpus striatum, was dissected manually and immediately frozen with dry-ice. Homogenization and all further extraction steps were carried out at 0–4°C. The extraction procedure was performed according to Schlumpf *et al.*[4] with only a few variations. A 5-μl volume of standard solution (100 ng/μl of α-methyldopamine in 0.1 N hydrochloric acid) was added to the tissue sample and sufficient hydrochloric acid–n-butanol (0.85 ml of 37% hydrochloric acid in 1 l of n-butanol) to give a tissue to liquid ratio of 1 mg:10 μl. The sample was homogenized for 1 min in a glass homogenizer and the homogenate centrifuged for 10 min at 6000 g. A 400-μl volume of the supernatant was shaken with 1 ml of n-heptane and 50 μl of 0.1 N hydrochloric acid for 30 sec on a Vortex mixer and then briefly centrifuged to separate the layers. The upper layer was discarded and 30 μl of the lower hydrochloric acid phase was transferred to a reaction vial, as described for the acylation in the nanogram range, and lyophilized. The residue was pentafluoroben-zoylated as described above for nanogram amounts.

RESULTS AND DISCUSSION

Pentafluorobenzoylation

The pentafluorobenzoylation of the CAs on the milligram scale was easily performed and the electron-capture detection of the appropriate diluted PFB-CAs was reproducible even in the lower picogram range. However, first attempts to transfer these evaluated and optimized reaction conditions to the nanogram scale failed. There resulted a high adsorption capacity of the GC system for the PFB-CAs and therefore unreproducible and insensitive electron-capture detection. This was caused by the use of too large amounts and different molarities of reactants in comparison with acylation on the milligram scale. This required many additional optimization trials to evaluate the lowest concentration of reagents necessary or the use of other reactants that could guarantee complete acylation together with GC reproducibility. All of the trials indicated that reproducibility of this method could be achieved only by the use of the reactants PFBCl and pyridine in a very small concentration range.

These results are in agreement with the observations of numerous other workers who were unsuccessful or had difficulties in introducing the pentafluorophenyl group into a molecule at the nanogram level, regardless of whether they performed acylation with PFBCl[23,29–31], acylation or alkylation with pentafluorobenzyl bromide[32–34], oxime formation with pentafluorobenzylhydroxylamine[35], carbamate formation with pentafluorobenzyl chloroformate[36], N-ylidene formation with penta-fluorobenzaldehyde[24], etc. These difficulties did not occur when aliphatic per-fluoroacyl groups were introduced into a molecule, but were exclusively connected with the perfluoro-aromatic system. Many workers who claimed high ECD sensitivity

with these derivatives obtained their results after having derivatized milligram or microgram amounts[23,32,37]; nevertheless, some of them concluded that the method would be applicable to nanogram amounts[32]. In their studies the minimal derivatizable amount did not correlate with the minimal detectable amount. Some of these workers indicated that the application of their method to the nanogram scale or to biological materials was in progress[29,30,36] and some that they[38,39] and also others had succeeded[10,40]. Some workers[29,34,35] in work similar to ours, described the application of different reaction conditions, depending on the amount being derivatized, in order to introduce the pentafluorophenyl group. A further difficulty with the CAs was that all the above-mentioned workers had to modify only one chemical function, whereas CAs are polyfunctional molecules. For example, Wilkinson[23] could only obtain the N-PFB derivative of ephedrine, its alcoholic hydroxyl group remaining unacylated.

The applicability of different reactants is described below.

Reagents. PFBI, an acylating agent which does not require a basic catalyst, was tested as an alternative to PFBCl and pyridine. However, even the derivatization of milligram amounts of CAs in a 3 % or 4 % PFBI–acetonitrile solution was unsuccessful. Neither heating, addition of more PFBI nor longer reaction times were successful. We could not establish whether this was caused by a lower acylation efficiency of PFBI compared with PFBCl.

Catalysts. As pyridine particularly enhanced the degree of adsorption of PFB-CAs, other possible basic catalysts were tested for their usefulness in catalysing the acylation of CAs with PFBCl. The acylation conditions and a summary of the results for the different ranges and amines are given in Table I.

Some workers[32,41] utilized the catalytic power of potassium carbonate in acetone for alkylation and acylation with pentafluorobenzyl bromide. However, they did not modify amino groups. In our studies potassium carbonate (Fluka) was effec-

TABLE I

RESULTS OF THE PENTAFLUOROBENZOYLATION OF INDIVIDUAL AMINES IN THE MILLIGRAM, MICROGRAM AND NANOGRAM RANGES WITH PENTAFLUOROBENZOYL CHLORIDE IN ACETONITRILE IN THE PRESENCE OF DIFFERENT BASIC CATALYSTS

Acylation in the milligram range: 1–2 mg of amine, 1 ml of acetonitrile, 200 μl of pyridine (100 mg of potassium carbonate), 100 μl of PFBCl. Acylation in the microgram range: 40–50 μg of amine, 100 μl of acetonitrile, 1 mg of potassium carbonate (5 μl of TEA), 10 μl of PFBCl. Acylation in the nanogram range: 50–500 ng of amine, 50 μl of acetonitrile, 2 μl of pyridine (1–2 μl of TEA), 2 μl of PFBCl (TEA: 1 μl of PFBCl). Reaction time with pyridine: 15 min (milligram range), 30 min (nanogram range); potassium carbonate, 60 min; TEA, 30 min. DA = dopamine; NA = noradrenaline; AD = adrenaline; 5-HT = serotonin; PFBCl = pentafluorobenzoyl chloride; TEA = triethylamine. + = 100 % yield; (−) = yield < 100 %; − = several products; 0 = not tested.

Compound	Pyridine			K_2CO_3			TEA		
	mg	μg	ng	mg	μg	ng	mg	μg	ng
DA	+	0	+	+	+	0	0	+	−
NA	+	0	+	0	(−)	0	0	−	0
AD	−	0	−	0	−	0	0	−	0
5-HT	+	0	+	0	0	0	0	−	0

tive for the tested DA pentafluorobenzoylation in the milligram range, using 100 mg of freshly dried potassium carbonate, acetonitrile as the solvent and a reaction time of 60 min at room temperature (Fig. 1). After acylation the potassium carbonate was removed by centrifugation. As can be seen from Fig. 2, the two kinds of reactions gave the same yield, but the PFBCl–potassium carbonate acylation gave better GC reproducibility. A disadvantage of the PFB-CAs catalysed by potassium carbonate was their poorer stability. On acylating microgram amounts (for method, see Table I), the analysis of DA could only be performed immediately after derivatization, NA gave a poor yield (also with longer reaction times) and showed higher adsorbability and AD yielded a mixture of products. Even less reproducible results were obtained using acetone as the solvent. Possibly the reason was the formation of a Schiff base, as has been described for primary amines in the presence of potassium carbonate and acetone[9]. Replacing potassium carbonate with anhydrous sodium carbonate (Fluka), as used by Hartvig and Vessman[36], in the microgram range gave only one quarter of the yield for PFB-DA and even less for PFB-NA compared with potassium carbonate.
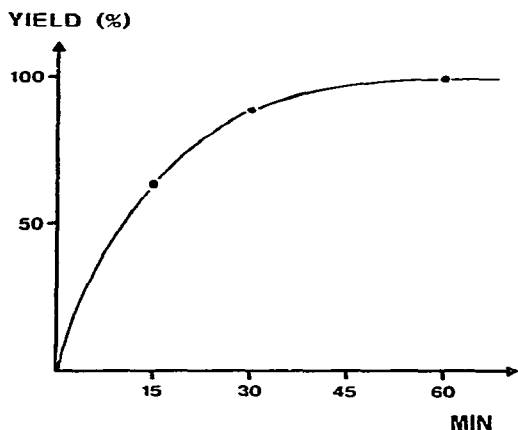

Fig. 1. Time dependence of the pentafluorobenzoylation of dopamine; reaction with pentafluorobenzoyl chloride and potassium carbonate in the milligram range.

Tertiary amines such as trimethylamine (TMA)[28,33], triethylamine (TEA)[29,34,40] and tripropylamine[42] have been widely used for the catalysis of the acylation and alkylation of different substances, including alcohols, phenols and amines. The advantages using TEA compared with potassium carbonate[34] and TMA compared with pyridine[28] have been described, but not the corresponding poorer catalytic capacities of the various tertiary amines. TEA (Fluka) was tested for the catalysis of the pentafluorobenzoylation of CAs at the microgram level (for method, see Table I). During the reaction TEA was converted into insoluble triethylammonium chloride, which was easily removed by centrifugation. The following results were obtained in the microgram range. DA could be acylated reproducibly, the derivative was stable for several weeks and the GC determination was reproducible. NA, adrenaline (AD) and serotonin (5-HT) each yielded a mixture of products (3–5 peaks
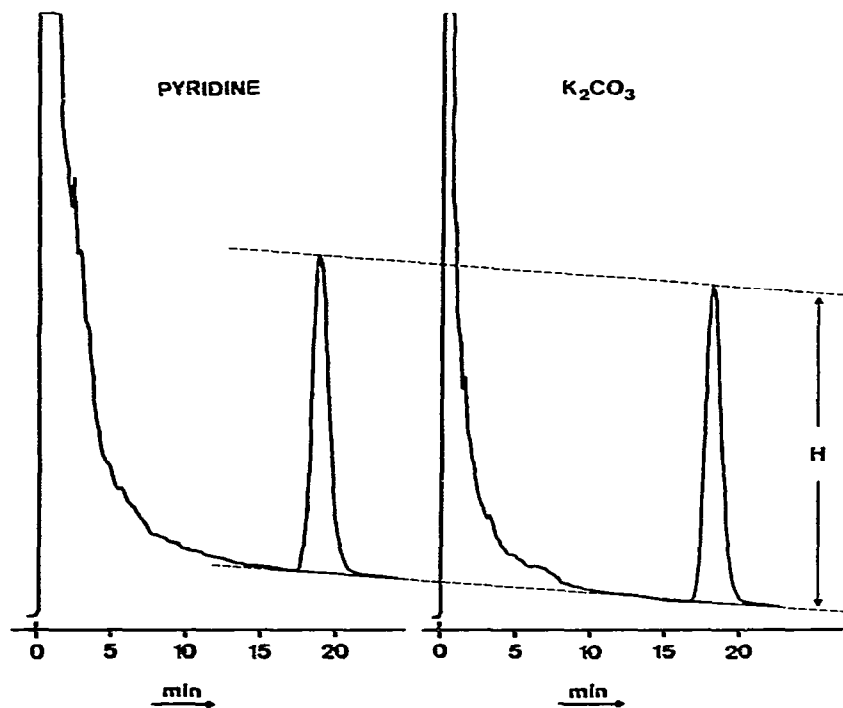
Fig. 2. Comparison of the gas chromatograms of PFB-DA (H = peak height) produced by the PFBCl–pyridine- and by the PFBCl–potassium carbonate reaction at the milligram level (1.6 mg of DA). FID (320 ng of DA per μl of acetonitrile).

each, depending on the amine). DA was also tried at the nanogram level, but in this range the TEA concentration was critical. Using 0.14 M TEA (1.0 μl per 53 μl), a second PFB-DA peak appeared with an area of about 10 % of that of the main peak, and 0.28 M TEA (2 μl per 54 μl) even increased the relative area of this second peak to about 50 %, although only one reproducible PFB-DA peak had been obtained in the microgram range using 0.31 M TEA (5 μl per 110 μl). Probably this second peak resulted from a tetrapentafluorobenzoylated DA with a diacylated amino group. because its retention time corresponded with that of the PFB-NA peak (four PFB groups) and because it has been reported that increasing concentrations of TMA[43] and TEA[34] favour the disubstitution of a functional group. On the other hand, the tertiary amine must be used in excess over the amine so that it can act as an effective catalyst[28,33]. In addition, the concentration itself differentiates not only the mono- or diacylation of a functional group[34,43] but also the kind of functional group that can be acylated[29]. The critical concentration of TEA was the reason for its uselessness in our trials.

The use of pyridine together with fluorine-containing agents causes a high ECD background, severe disturbances in the chromatograms and ready contamination of the detector[28,44]. Moreover, incompatibility of pyridine with fluorine-containing compounds has been mentioned[24]. Likewise in our trials, pyridine readily

caused undesirable secondary effects such as the formation of polymerization products, which led to poor GC reproducibility. The problems mentioned with pyridine could be overcome to give better GC reproducibility, resolution and sensitivity even at the nanogram level by application of special precautions and conditions, *e.g.*, the use of a minimal amount of pyridine and gentle reaction conditions, a clean-up procedure and special GC conditions, silanization of the system and ephedrine injection. The results of reducing the amount (concentration) of pyridine and PFBCl to the minimum in a given volume of acetonitrile (50 μl) are given in Table II. A concentration ratio of pyridine to PFBCl of greater than 1 is necessary for complete pentafluorobenzoylation of DA and NA. Each of these amines required different minimal concentrations: for DA, 2 % of pyridine and PFBCl in 50 μl of acetonitrile were sufficient, whereas NA required 4 % of each, which also resulted in reproducible acylation of DA. Not only the amounts of pyridine and PFBCl were important, but also their concentrations; the use of a different volume of acetonitrile would have required a new optimization of the reactants to be added. In this way NA, DA, 5-HT and also α-methyl-CA, α-methyl-DA and α-methyl-NA could be pentafluorobenzoylated reproducibly in the nanogram range.

TABLE II

INFLUENCE OF THE PYRIDINE AND PENTAFLUOROBENZOYL CHLORIDE CONCENTRATIONS (AMOUNTS) ON THE ACYLATION RATE OF DOPAMINE (DA) AND NORADRENALINE (NA) AT THE NANOGRAM LEVEL

Pentafluorobenzoylation of 444 ng of DA and 480 ng of NA in 50 μl of acetonitrile, electron-capture detection as 444 pg/μl (DA) and 480 pg/μl (NA). n = Number of acylations; PFBCl = pentafluorobenzoyl chloride.

Pyridine (μl)	PFBCl (μl)	Acylation rate ± S.D. (%)	
		DA acylation	NA acylation
0.5	0.5	41 (n = 1)	
0.5	1.0	21 (n = 1)	
1.0	1.0	96 ± 5.9 (n = 2)	32 ± 8.8 (n = 2)
1.0	2.0		12 (n = 1)
2.0	1.0	97 ± 8.7 (n = 2)	29 (n = 1)
2.0	2.0	107 (n = 1)	102 ± 2.1 (n = 2)
3.0	3.0		95 (n = 1)

Reaction medium and reaction conditions. The lipophilic solvents benzene and chloroform were not suitable for the PFBCl–pyridine pentafluorobenzoylation, a more polar solvent being required. On comparing acetonitrile, ethyl acetate and tetrahydrofuran, the reaction in acetonitrile was found to be the quickest and required the mildest reaction conditions: standing for 15–30 min at room temperature were sufficient for quantitative acylation. Longer reaction times or heating of the reaction mixture resulted in reduced yields of the PFB-CAs and erratic results.

Clean-up procedure. After the acylation process, the removal of excess of reagents and by-products was essential. The use of a high vacuum was sufficient for this purpose and no loss of the PFB-CAs occurred. At the nanogram level the PFB-CAs

had to be dissolved in an end-volume of 0.5–1.0 ml of acetonitrile. As the sensitivity of this method can be increased if the final volume can be proportionally reduced, a selective solvent extraction procedure was carried out for the complete removal of all interfering substances. Neither 0.1 N sodium hydroxide solution (pH 13), 5% aqueous ammonia (pH 10), phosphate buffer (pH 6) nor 0.1 N hydrochloric acid (pH 1) could be used after the PFB-CAs had been dissolved in benzene, as an amine- and pH-dependent decomposition of the PFB-CAs occurred. For example, PFB-NA was unstable and PFB-α-methyl-DA was stable in all solutions tested. PFB-DA and PFB-α-methyl-NA decomposed only at pH 1. These results were unexpected because of the excellent stability of the PFB-CAs in acetonitrile.

Stability of the PFB-amines

The stability of PFB-DA, PFB-NA and PFB-5-HT was examined over a period of 4 weeks. The amines had been acylated in the milligram range, dissolved in acetonitrile (nanograms per microlitre concentrations) and stored at room temperature. No decomposition could be observed with any of the compounds, the peak areas remaining identical. Even the PFB-CA solutions obtained by the acylation of nanogram samples from biological materials were stable for several months. This showed the great advantage of the PFB-CAs over the aliphatic perfluoroacyl derivatives. The latter had to be chromatographed immediately after acylation and stored in the cold in special solvents, sometimes with addition of a defined concentration of acylating agent (beware of overloading the ECD) for the prevention of hydrolysis[15,17–20,26]. This capacity of the pentafluorophenyl group to impart stability to the whole molecule being modified has been mentioned in connection with various derivatives containing this group[29–33,35,36,38,39].

Mass spectrometric studies

The products corresponding to the peaks attributed to the single amines in the chromatograms had been identified as the appropriate pentafluorobenzoyl derivatives by GC–MS and also by direct inlet mass spectrometry: PFB-DA three-fold (two phenolic hydroxyl, one amino group), molecular weight 735.37 (Fig. 3), PFB-NA four-fold (two phenolic hydroxyl, one alcoholic hydroxyl, one amino group), molecular weight 945.43 (Fig. 4) and PFB-5-HT doubly pentafluorobenzoylated (one hydroxyl, one amino group), molecular weight 564.34. Only when applied by direct inlet did PFB-AD yield a mass spectrum of a four-fold pentafluorobenzoylated derivative (two phenolic hydroxyl, one alcoholic hydroxyl, one *sec.*-amino group), molecular weight 959.45. Because PFB-AD always yielded three peaks in the gas chromatogram, it probably decomposed in the GC system. The structures of the PFB-CAs were in accordance with those of the aliphatic perfluoroacylated CAs[16,18]. 5-HT showed a difference, as its indole-NH was not pentafluorobenzoylated, although the three-fold acylation (one hydroxyl, one indole-NH and one amino group) of this amine has been described[16,18]. The PFB-amines would also be suitable for quantitative analysis by mass fragmentography because each produced one or more specific, characteristic intense ions that could be monitored by multiple ion detection (*m/e* values: PFB-DA, 524; PFB-NA, 733; PFB-AD, 238, 552, 747; PFB-5-HT, 340, 353).
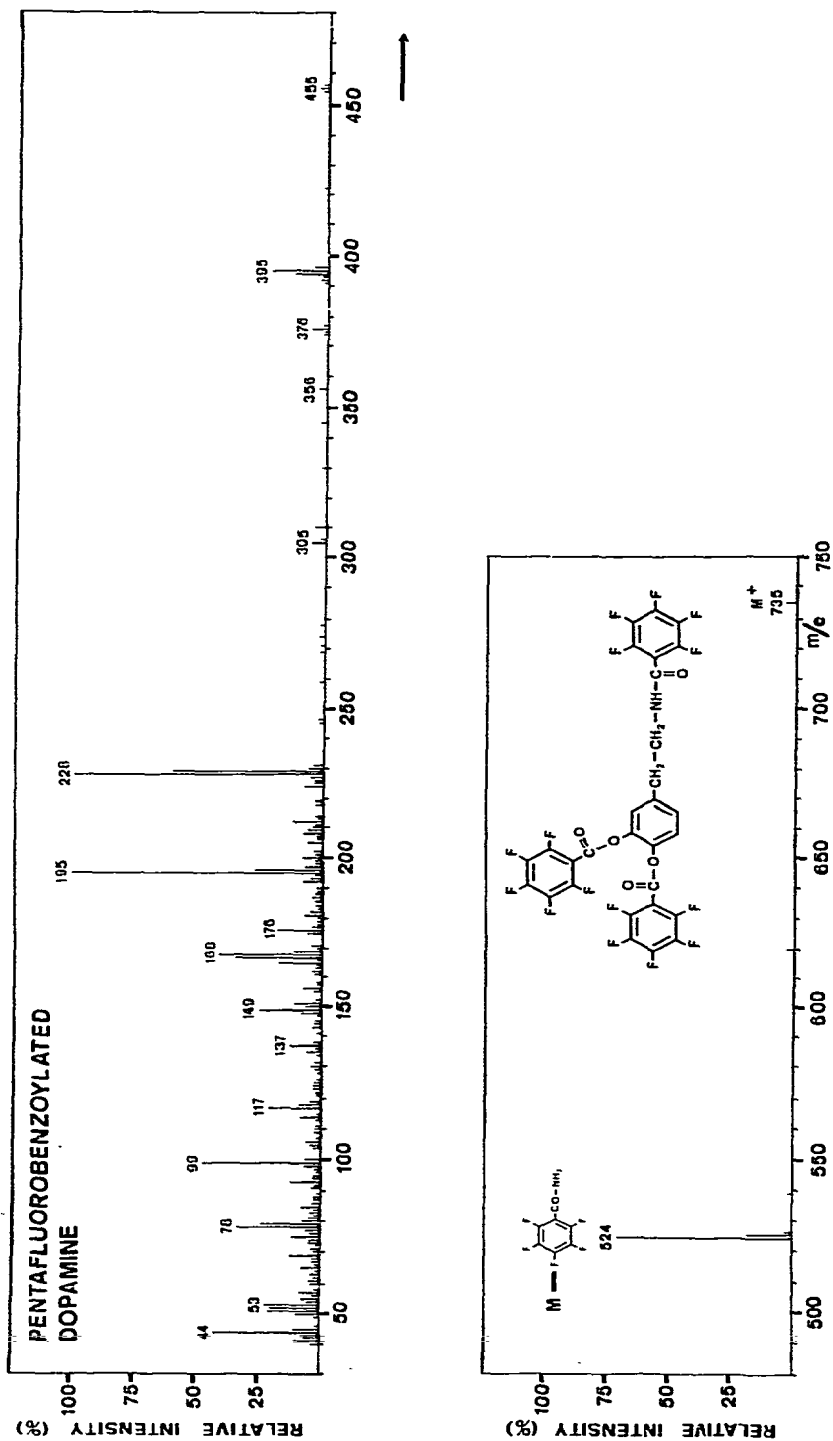
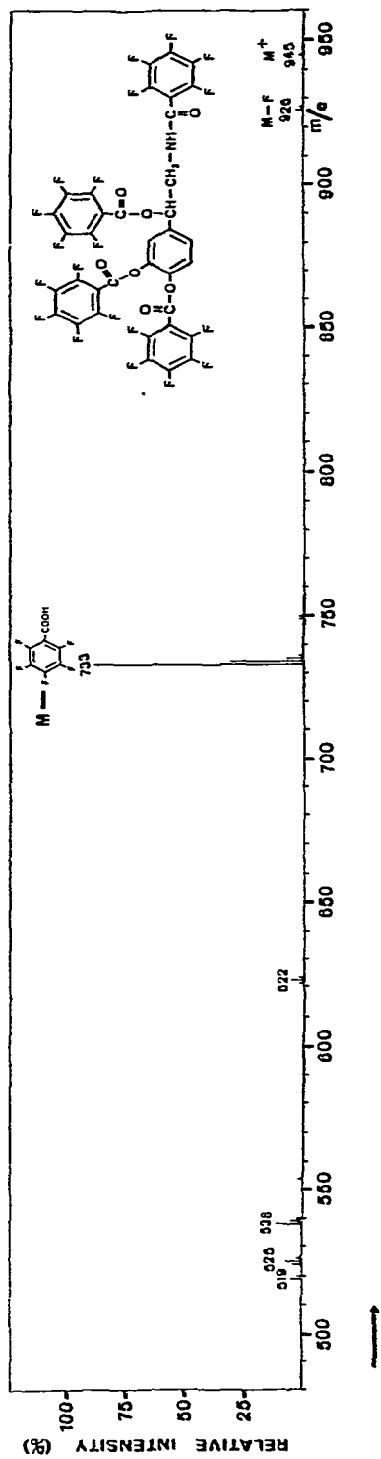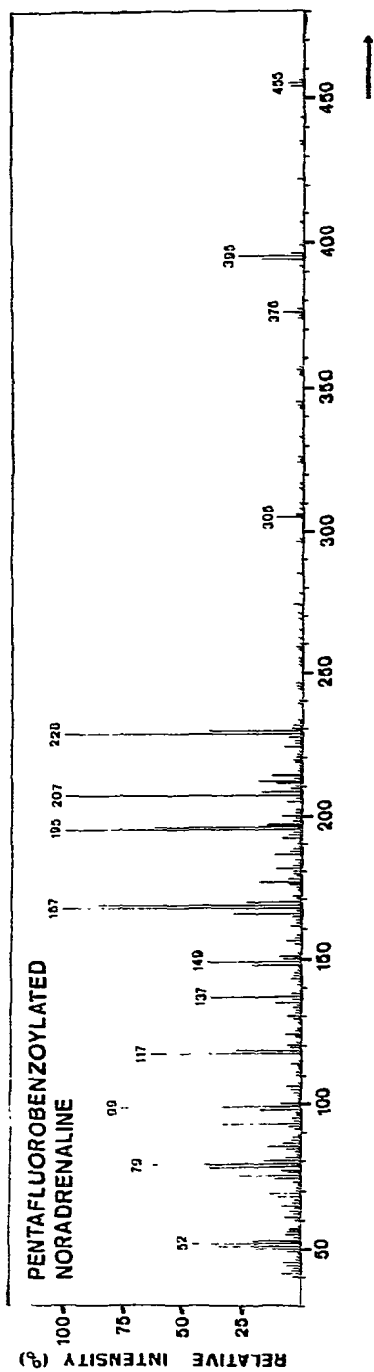Fig. 3. Mass spectrum (GC–MS) of pentafluorobenzoylated dopamine.

Fig. 4. Mass spectrum (GC–MS) of pentafluorobenzoylated noradrenaline.

Gas chromatographic analysis of the PFB-amines

Separation. The large increase in molecular weight that occurs on introducing PFB-groups into the amines was partly overcome by the degree of volatility imparted to the molecule by these groups. In spite of the moderate volatility of the PFB-amines, their similar structures and their high adsorbability, a good GC separation with reasonable retention times and symmetrical, quantitatively measurable peaks at the lower picogram level could nevertheless be achieved. Short, silanized glass columns, silanized supports and silanized glass-wool and OV-17 as the stationary phase and ephedrine injection had to be used (Fig. 6). For detection at the nanogram level, silanization and ephedrine injection were not required (Fig. 5).
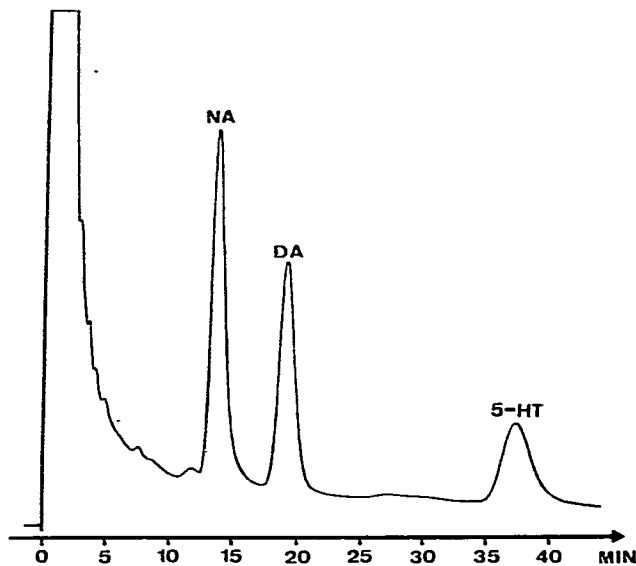

Fig. 5. Separation of noradrenaline (NA, 540 ng), dopamine (DA, 339 ng) and serotonin (5-HT, 398 ng) as their pentafluorobenzoyl derivatives. PFBCl–pyridine acylation at the milligram level. FID. Conditions: I.D., 3 mm; nitrogen flow-rate, 36 ml/min; electrometer sensitivity settings, 10/16.

The sequence of the elution of the PFB-amines was unexpected: PFB-α-methyl-NA, PFB-NA, PFB-α-methyl-DA, PFB-DA, PFB-5-HT. The retention times of the derivatives were inversely proportional to their molecular weights and also to the number of PFB groups present. These results corresponded with those obtained by other workers with aliphatic perfluoroacyl-CA derivatives on silicone stationary phases[16]; only once has another sequence, on GE-XF-1105, been described[20]. The very poor volatility of the indoleamine derivatives has been confirmed[16,18].

Gas chromatographic reproducibility. The occasional high adsorption selectivity for special structures or functional groups[45], the ready adsorbability of perfluoroacylated amines containing the catechol group[46] and disturbances and irreproducibilities in the GC analysis of amine derivatives[28,47] are all well known.

To avoid the adsorbability of the PFB-CAs (PFB-NA being more active than PFB-DA) and to achieve reproducible results, the following preventive measures, as described under Experimental, were required:

(a) Special reagents and reaction conditions and a reagent removal procedure.

(b) Silanization of all parts of the GC system (column, support and glass-wool). Silanized glass-wool was particularly necessary for the reproducible determination of PFB-NA.

(c) Specific preconditioning of the column with the PFB-CAs to saturate the adsorption-active sites.

(d) From time to time conditioning of the column with Silyl 8.

(e) Replacement of the column filling at the injection site when it has turned brown, probably owing to a deposit of adsorption-active secondary products.

(f) "Ephedrine injection" to avoid memory effects (Fig. 6). With the sensitive electron-capture detection, small "ghost peaks" were caused by acetonitrile injection subsequent to injection of the PFB-CAs. These ghost peaks had the same retention times as those of the PFB-CAs in the preceding injection. Therefore, ghosting must be due to adsorption of the PFB-CAs at the injection site of the GC system. For effective CA analysis, complete removal of the ghost peaks was required. This could be done by time-consuming rewashing of the column three to five times with acetonitrile until all traces of the preceding PFB-CA injection had been eluted or, more elegantly, by the "ephedrine injection" technique. This use of a priming substance, occupying all active sites in the GC system responsible for memory effects, was described earlier by Brötell et al.[39], who employed desipramine to suppress pentazocine ghosting. In our trials only the described ephedrine injection was successful. The use of other priming
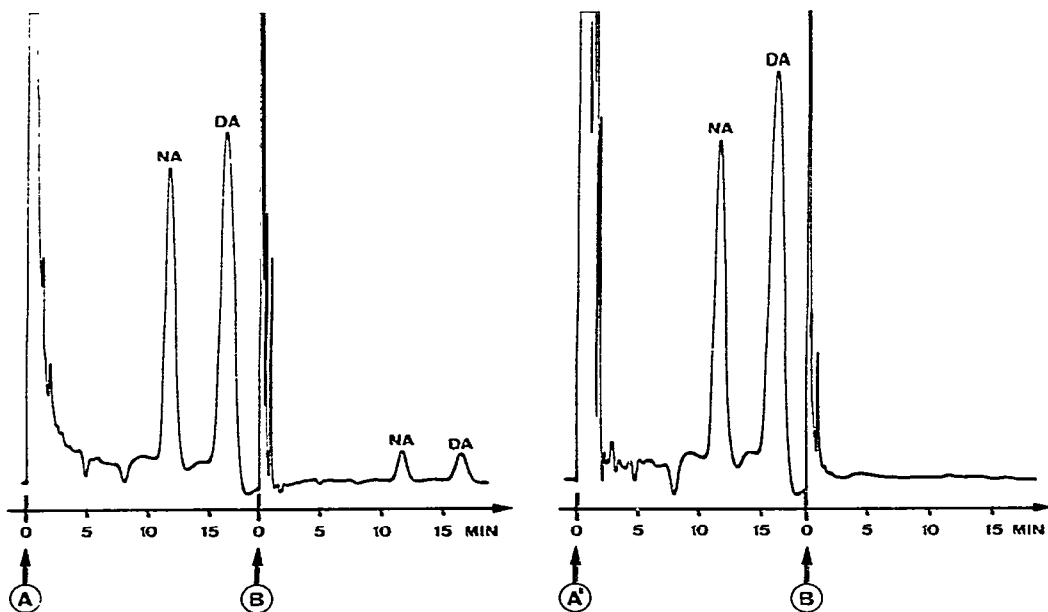

Fig. 6. Application of "ephedrine injection" to avoid the memory effect and ghost peaks. (A) Injection of 1.0 μl of PFB-CA solution (400 pg of NA and DA per μl of acetonitrile); (A') injection of 1.0 μl of the same PFB-CA solution together with 1.0 μl of ephedrine solution (1 μg/μl) (= "ephedrine injection"); (B) injection of 1.0 μl of acetonitrile. Conditions: nitrogen flow-rate, 48 ml/min; electrometer sensitivity settings, 100/16 (ECD).

substances or solvents, the separate injection of ephedrine immediately before the PFB-CA injection and the storage of the PFB-CAs in an ephedrine solution did not suppress the memory effects.

, There was a clear relationship between heat and the ease of PFB-CA adsorbability: adsorption of the PFB-CAs occurred only on heating the reaction mixture or in a hot GC system. No adsorption of the PFB-CAs to the glass surface of the reaction vials could be observed after they had been acylated at room temperature. Therefore, it was of no importance if the reaction vials were silanized or not (although this kind of adsorption has been described for derivatives containing the pentafluorophenyl group[39,40]), but in contrast it was essential to silanize the hot GC system.

ECD response. The variable parameters of the ECD, such as pulse mode setting, scavenger flow-rate and temperature, were optimized for the most sensitive PFB-CA detection. The settings in each instance are given under Experimental part. Use of a scavenger flow-rate of 18 ml/min or greater resulted in a 10% smaller peak width. Further increases gave no further improvement in the resolution. Therefore, a scavenger flow-rate of about 25 ml/min was always used. Only a small temperature range could be used: only cell temperatures of 275°C (= column temperature as the lower limit) and 300°C (= maximum operating temperature for the radioactive ^{63}Ni source) could be compared. Using PFB-DA (500 pg/μl of DA) a poorer resolution (5% larger peak width) and a poorer sensitivity (only 80% of the peak area) were obtained at 275°C compared with 300°C. Therefore, the ECD was operated at its temperature limit. These results indicate that the capture process of the PFB-CAs must be linked with a dissociation in which bond breaking is favoured by high temperatures.

To demonstrate the high ECD specifity of the PFB derivatives, the ECD to FID detection ratios calculated for PFB-DA and PFB-NA, using peak-area units per nanogram (FID) or per picogram (ECD), were 10,748:1 and 11,210:1, respectively. These values are more of theoretical interest, however, because the ECD had to be operated with a 10–20 times greater attenuation than the FID on account of its greater background noise.

The detection limit for the PFB-CAs was 20–30 pg per injection, which is a sensitivity comparable to that of fragmentographic assays[17,48].

As can be seen from Fig. 7, PFB-NA was less sensitive than PFB-DA to the ECD, although it contains one more PFB group, which was unexpected. On the other hand, it also exhibited a poorer FID response, possibly owing to its greater adsorbability, so that a slightly higher ECD to FID detection ratio resulted.

Linearity and accuracy. The results of all evaluations and optimizations are summarized in Fig. 7, which shows the calibration graphs for the PFB-CAs. The values for these plots were obtained by pentafluorobenzoylating increasing amounts of the CAs (50–500 ng) and detecting these nanogram amounts as picograms per microlitre by dissolving the PFB-CAs in 1 ml. Each point on the plot represents a mean value obtained by repeated acylation of the individual amounts and also by repeated injection of the PFB-CA solutions. Linear relationships were obtained for all of the amines tested. The linear regression equation was calculated for each CA plot (n = number of points):

DA   : $y = 36.124 + 3.592x$ $(n = 6)$
NA   : $y = 116.989 + 2.030x$ $(n = 6)$
α-methyl-DA: $y = 76.665 + 3.111x$ $(n = 3)$

The reproducibility of the acylation and of the GC analysis was calculated for each amine. The standard deviation of the whole method varied between 3.55 ± 2.03% and 5.99 ± 1.16%. The reproducibility was excellent, although no internal standard had been used and the peak areas were measured manually.
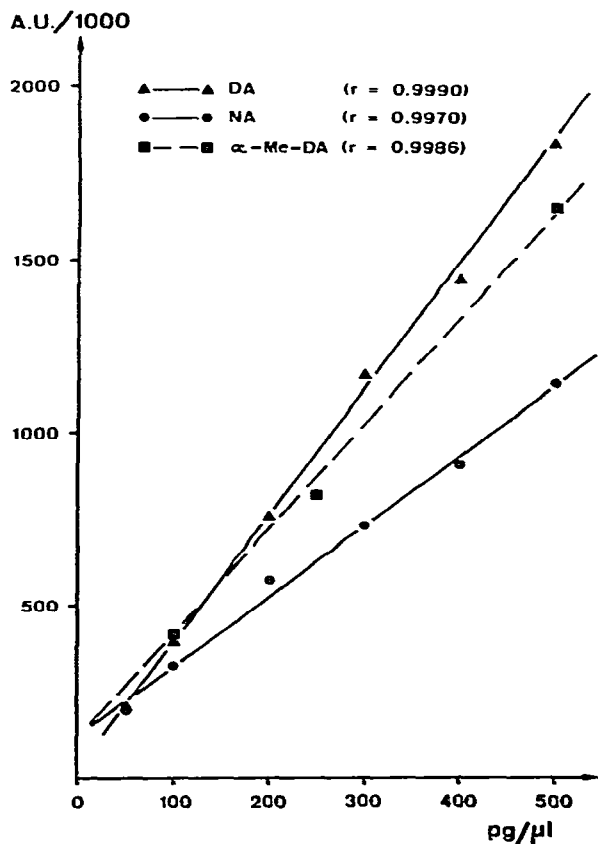

Fig. 7. Calibration graphs for PFB-DA (▲), PFB-NA (●) and PFB-α-methyl-DA (■).

Biological applications
To demonstrate the applicability of the method to biological materials, the DA content of the corpus striatum of the rat was analysed. The extraction was relatively simple but proved successful, so no more trials were necessary. No other acid-soluble compounds, present in the brain extract and hence subjected to the derivatization process, were found to interfere in the GC analysis of PFB-CAs. The DA content of the corpus striatum of the rat was 8.7 μg/g, which is in accordance with the values cited in the literature[17,49].

REFERENCES

1 U. S. von Euler, *Acta Physiol. Scand.*, 12 (1946) 73.
2 P. Holtz, *Acta Physiol. Scand.*, 20 (1950) 354.
3 R. Laverty and D. F. Sharman, *Br. J. Pharmacol.*, 24 (1965) 538.
4 M. Schlumpf, W. Lichtensteiger, H. Langemann, P. G. Waser and F. Hefti, *Biochem. Pharmacol.*, 23 (1974) 2437.
5 J. K. Saelens, M. S. Schoen and G. B. Kovacsics, *Biochem. Pharmacol.*, 16 (1967) 1043.
6 J. M. Saavedra, R. Kvetnansky and I. J. Kopin, *Brain Res.*, 160 (1979) 271.
7 N. P. Sen and P. L. McGeer, *Biochem. Biophys. Res. Commun.*, 13 (1963) 390.
8 F. P. Abramson, M. W. McCaman and R. E. McCaman, *Anal. Biochem.*, 57 (1974) 482.
9 P. Capella and E. C. Horning, *Anal. Chem.*, 38 (1966) 316.
10 J. C. Lhuguenot, B. F. Maume and P. Padieu, *J. Chromatogr. Sci.*, 12 (1974) 411.
11 T. Walle, *Acta Pharm. Suecica*, 5 (1968) 367.
12 N. Narasimhachari and P. Vouros, *Anal. Biochem.*, 45 (1972) 154.
13 M. G. Horning, A. M. Moss, E. A. Boucher and E. C. Horning, *Anal. Lett.*, 1 (1968) 311.
14 G. M. Anthony, C. J. W. Brooks and B. S. Middleditch, *J. Pharm. Pharmacol.*, 22 (1970) 205.
15 D. D. Clarke, S. Wilk, S. E. Gitlow and M. J. Franklin, *J. Gas Chromatogr.*, 5 (1967) 307.
16 I. L. Martin and G. B. Ansell, *Biochem. Pharmacol.*, 22 (1973) 521.
17 S. H. Koslow, F. Cattabeni and E. Costa, *Science*, 176 (1972) 177.
18 E. Gelpi, E. Peralta and J. Segura, *J. Chromatogr. Sci.*, 12 (1974) 701.
19 T. Kawano, M. Niwa, Y. Fujita, M. Ozaki and K. Mori, *Jap. J. Pharmacol.*, 28 (1978) 168.
20 M. G. Bigdelli and M. A. Collins, *Biochem. Med.*, 12 (1975) 55.
21 J. Merzhäuser, E. Röder and Ch. Hesse, *Klin. Wochenschr.*, 51 (1973) 883.
22 M. J. Cooper, R. F. O'Dea and B. L. Mirkin, *J. Chromatogr.*, 162 (1979) 601.
23 G. R. Wilkinson, *Anal. Lett.*, 3 (1970) 289.
24 A. C. Moffat and E. C. Horning, *Anal. Lett.*, 3 (1970) 205.
25 A. C. Moffat, E. C. Horning, S. B. Matin and M. Rowland, *J. Chromatogr.*, 66 (1972) 255.
26 E. Anggard and G. Sedvall, *Anal. Chem.*, 41 (1969) 1250.
27 L. M. Cummins, in I. I. Domsky and J. A. Perry (Editors), *Recent Advances in Gas Chromatography*, Marcel Dekker, New York, 1971, p. 313.
28 T. Walle and H. Ehrsson, *Acta Pharm. Suecica*, 7 (1970) 389.
29 A. Zmigrod, S. Ladany and H. R. Lindner, *Steroids*, 15 (1970) 635.
30 H. Ehrsson and B. Mellström, *Acta Pharm. Suecica*, 9 (1972) 107.
31 H. A. Schwertner, T. M. Ludden and J. E. Wallace, *Anal. Chem.*, 48 (1976) 1875.
32 F. K. Kawahara, *Anal. Chem.*, 40 (1968) 1009.
33 O. Gyllenhaal and H. Ehrsson, *J. Chromatogr.*, 107 (1975) 327.
34 T. Walle, *J. Chromatogr.*, 114 (1975) 345.
35 K. T. Koshy, D. G. Kaiser and A. L. VanDerSlik, *J. Chromatogr. Sci.*, 13 (1975) 97.
36 P. Hartvig and J. Vessman, *Anal. Lett.*, 7 (1974) 223.
37 A. Zlatkis and B. C. Pettitt, *Chromatographia*, 2 (1969) 484.
38 P. Hartvig, W. Handl, J. Vessman and C. M. Svahn, *Anal. Chem.*, 48 (1976) 390.
39 H. Brötell, H. Ehrsson and O. Gyllenhaal, *J. Chromatogr.*, 78 (1973) 293.
40 E. R. Garrett and C. A. Hunt, *J. Pharm. Sci.*, 62 (1973) 1211.
41 L. G. Johnson, *J. Ass. Offic. Anal. Chem.*, 56 (1973) 1503.
42 S. Wilk and B. Zimmerberg, *Biochem. Pharmacol.*, 22 (1973) 623.
43 H. Ehrsson and H. Brötell, *Acta Pharm. Suecica*, 8 (1971) 591.
44 R. A. Sams and L. Malspeis, *J. Chromatogr.*, 125 (1976) 409.
45 K. Grob, *Chromatographia*, 7 (1974) 94.
46 B. L. Goodwin, C. R. J. Ruthven, M. Sandler and B. Hine, *Clin. Chim. Acta*, 44 (1973) 271.
47 G. S. King and M. Sandler, *Clin. Chim. Acta*, 49 (1973) 295.
48 C. L. Galli, F. Cattabeni, T. Eros, P. F. Spano, S. Algeri, A. Di Giulio and A. Groppetti, *J. Neurochem.*, 27 (1976) 795.
49 A. S. Horn, A. C. Cuello and R. J. Miller, *J. Neurochem.*, 22 (1974) 265.