CHROM. 4607

Comparison of formaldehyde gas and Procházka reagents for the detection of biogenic monoamines by thin-layer chromatography

Formaldehyde gas was recently introduced as a sensitive and specific chromatographic detection reagent for a variety of biogenic phenylethylamines and indolylethylamines[1-6]. Previously, various modifications of the Procházka reagent[7-9] served the same purpose. STAHL AND KALDEWEY[8] recommended a mixture of formalin and hydrochloric acid (the classical Procházka reagent) for the visualization of indolamines on a silica gel thin layer; SEILER AND WIECHMANN[9] used a mixture of formalin and ammonia followed by the classical Procházka reagent for the detection of some catecholamine derivatives. From preliminary observations made in this laboratory, it was clear that the colors and fluorescence intensities of amine fluorophores, induced by exposure to formaldehyde gas, differed considerably from those of the fluorophores obtained from the same compounds with the various Procházka reagents. Theoretical possibilities exist for a variety of condensation reactions between amines and aldehydes[10]. One possible reaction is the well-known Pictet–Spengler reaction in which catecholamines and indolamines are condensed to highly fluorescent isoquinoline and β-carboline derivatives, respectively[11,12]. This reaction forms the basis for the histochemical method of FALCK AND HILLARP (see refs. 12 and 13), and it has been suggested that the formaldehyde condensation of catecholamines and indolamines on silica gel thin layers proceeds along similar lines[3,5,6]. In this paper the sensitivity of gaseous and aqueous formaldehyde as chromatographic reagents for the detection of certain biogenic aromatic amines and amino acids has been studied. Furthermore, the spectral characteristics of the various formaldehyde-induced amine fluorophores have been registered by means of microspectrofluorometry. It was found that the various types of formaldehyde reagents supplement each other by reacting characteristically with the phenylethylamine and indolylethylamine derivatives studied. Thus, the various reagents could be used in conjunction, thereby providing means for the chromatographic identification of individual tissue amines.

Material and methods

Thin layers. Silica gel thin layers were prepared by coating glass plates (standard histological cover slips, 24 × 32 mm) with approximately 100 μ of Kieselgel H (Merck, Darmstadt). The layer was applied as a slurry consisting of 20 g of silica gel suspended in 50 ml of 0.01 *M* phosphate buffer (pH 7.0) enriched with 10^{-3} *M* EDTA. The plates were dried at room temperature before use. Aqueous solutions of catecholamines, indolamines and some related amino acids in various concentrations were applied to the thin layers in a volume of 0.5 μl.

Detection reagents. The thin layers were treated according to seven different procedures. (1) Formaldehyde gas. Formaldehyde condensation of the various compounds on silica gel thin layers was induced by exposure to formaldehyde gas at 80–100° for 30 min. The reaction took place in a closed glass jar (1000 cc) containing about 5 g of paraformaldehyde previously equilibrated in air at about 50% relative humidity. (2) Formaldehyde gas + HCl. Treatment with formaldehyde gas was as described under (1) except that 0.2 ml of concentrated HCl was contained in the reaction

vessel. (3) Formaldehyde gas + NH_3. Treatment with formaldehyde gas was as described under (1) except that 0.2 ml of conc. ammonia was contained in the reaction vessel. (4) Formaldehyde gas + NH_3 + subsequent HCl treatment. The procedure was as described under (3) but followed by exposure to HCl vapor (0.2 ml. conc. HCl in a closed vessel at + 80° for 1/2 h). (5) Procházka I. The silica gel thin layers were sprayed with a mixture of formalin, 6 *N* hydrochloric acid, and ethanol (1:2:1) and dried in an oven at 100° for 30 min. (6) Procházka II. The thin layers were sprayed with a mixture of formalin and conc. ammonia (1:1) and then dried. (7) Procházka II + Procházka I. The thin layers were sprayed first with Procházka II, dried in the oven, sprayed with Procházka I, and then again dried. In addition, thin layers treated according to (1) were subsequently sprayed with phosphate buffer (pH 8.0), boric acid–KCl–NaOH buffer (pH 9.0 and pH 10.0) and then dried at 80°.

The sensitivity of the various detection reagents was evaluated by establishing the lowest concentration visible in the spot when examined under UV light (Sterisol UV-lamp, Original Hanau, applied with a UG 1 filter).

Microspectrofluorometric analysis. Analysis of the various fluorophores was performed with a modified Leitz microspectrograph[14]. For recording the excitation spectra, the optical system of the exciting light consisted of quartz components and the glass cover slips were mounted upside-down with the thin layer facing the quartz bright-field dry condenser. The thin layer outside the amine spot was used to obtain blank spectra. All spectra were corrected for instrumental errors as previously described[14].

All values given for excitation and emission maxima are the means of 4–8 separate recordings.

Results

Sensitivity of the detection reagents. Using the different procedures, the fluorescence intensity obtained from one and the same compound varied markedly. The various gaseous formaldehyde reagents proved to have a sensitivity similar to that of the corresponding aqueous formalin reagents (Table I). The Procházka II and the Procházka II + I spray reagents were somewhat better in visualizing the catecholamines and the 5-methoxyindoles. The Procházka I reagent and formaldehyde gas + HCl (type 2 treatment) gave strong fluorescence only with tryptamine and tryptophan, weak or no fluorescence with the 3-methoxylated phenylethylamines, the 5-hydroxyindoles, 5-methoxyindoles, and catecholamines. With the 3-methoxylated phenylethylamines, the Procházka II + I spray was found to induce more intense fluorescence than any of the other treatments, in agreement with previous observations[9]. Tryptamine could be detected with approximately the same degree of sensitivity with either of the reagents. The Procházka I and II + I spray reagents and the formaldehyde gas + HCl treatment were favorable in visualizing tryptophan.

The fluorescence induced from the various phenylethylamines by the formaldehyde gas treatment (type I) was found to vary markedly in intensity from one experiment to another. The reason for this is unclear. When the formaldehyde gas treatment was followed by spraying the thin layer with buffers of various pH, a pH dependence was revealed for the fluorescence intensities of the catecholamine fluorophores (Table II, *cf.* ref. 15); maximum fluorescence intensities for these compounds were regularly observed after spraying with buffer of pH 8.0. The fluorescence

J. Chromatog., 47 (1970) 530–536

TABLE I

MINIMUM DETECTABLE AMOUNT (μg) OF FORMALDEHYDE-TREATED PHENYLETHYLAMINES AND INDOLYLETHYLAMINES ON SILICA GEL

Compounds	*Gaseous formaldehyde reagents*				*Aqueous formaldehyde reagents*		
	Formaldehyde alone (type 1)	*Formaldehyde + HCl (type 2)*	*Formaldehyde + NH_3 (type 3)*	*Formaldehyde + NH_3 + HCl (type 4)*	*Procházka I (HCl) (type 5)*	*Procházka II (NH_3) (type 6)*	*Procházka II + I (NH_3 + HCl) (type 7)*
Tryptamine	0.01–0.03	0.01	0.01	0.003	0.01	0.01	0.003
Tryptophan	0.1	0.01	0.03	0.03	0.003–0.01	0.1–0.3	0.003–0.01
5-Hydroxytryptamine	0.03	1	0.01	0.03	—	0.03	0.03
5-Hydroxytryptophan	0.03	—	0.03	0.03	—	0.03	0.01
5-Methoxytryptamine	0.03	—	0.01	0.03	—	0.003	0.003
5-Methoxytryptophan	0.1	—	0.1	0.03	—	0.01–0.03	0.01
Dopamine	0.03	—	0.03	0.03	—	0.01–0.03	0.01
DOPA	0.1	—	0.03	0.1	—	0.03	0.03–0.1
Adrenaline	0.1	—	0.03	0.1	—	0.01–0.03	0.03
Noradrenaline	0.03	—	0.1	0.1	—	0.03	0.1
Metanephrine	—	—	—	—	0.1–0.3	—	0.03–0.1
Normetanephrine	0.3–1	—	—	0.3	0.1–0.3	1	0.01–0.03
3-Methoxytyramine	0.03	—	0.3	0.03	0.1–0.3	0.1–0.3	0.03

TABLE II

EFFECT OF pH ON FLUORESCENCE INTENSITIES OF CATECHOLAMINE FLUOROPHORES[a]; MINIMUM DETECTABLE AMOUNT (μg)

Compounds	*Formaldehyde gas alone*	*Formaldehyde gas + buffer pH 8*	*Formaldehyde gas + buffer pH 9*	*Formaldehyde gas + buffer pH 10*
Dopamine	0.03	0.01	0.03–0.1	0.1
Noradrenaline	0.03	0.01	0.03–0.1	0.1
Adrenaline	0.1	0.03	0.1–0.3	0.1–0.3

[a] Formaldehyde condensation was performed on silica gel thin layers, prepared with phosphate buffer (pH 7). Following this treatment the thin layers were sprayed with buffers of the pH values indicated.

observed after this combined formaldehyde–buffer treatment appeared to have a more consistent intensity.

The results were similar when treatment with the various aqueous Procházka reagents were replaced by treatments with gaseous formaldehyde combined with HCl or NH_3 vapor (treatments 2–4, *cf. Material and methods*; see Table I). When HCl vapor was included in the formaldehyde condensation procedure, the sensitivity for tryptamine and tryptophan was increased or undiminished while the sensitivity for the other compounds was reduced; as a consequence this technique was more selective for tryptamine and tryptophan than treatment with formaldehyde gas alone and similar to the Procházka I spray. Although in general gaseous formaldehyde and aqueous formalin appeared to have similar sensitivity as detection reagents, it should be noted that the sensitivity of the gaseous formaldehyde reagents was markedly affected by environmental factors, which became evident after having applied the methods in different local laboratories. This contrasted with the aqueous formalin reagents which were found to give more consistent fluorescence intensities. The difficulty in obtaining reproducible results with gaseous formaldehyde was most notable with the easily oxidized catecholamines (*cf.* refs. 6, 16 and 17).

Spectral characteristics of the fluorophores. The various detection reagents resulted in the formation of amine fluorophores with different spectral characteristics. The maxima of the excitation and the emission spectra of the fluorophores formed with formaldehyde gas and with the various Procházka reagents are given in Table III.

Discussion

Recent chromatographic and microspectrofluorometric studies[1–9,18] suggest that formaldehyde condensation has a considerable potential as a method for the chromatographic detection of biogenic phenylethylamines and indolylethylamines and some related amino acids. By varying the reaction conditions and the treatments applied after completion of the condensation, the properties of the formaldehyde-induced amine fluorophores (*i.e.* their fluorescence intensity and spectral characteristics) can be made to vary characteristically, thereby providing additional possibilities for the chromatographic identification of tissue amines (see also refs. 6, 12, 13, 17, 18).

Variations in pH have been found to induce marked changes in the properties of the formaldehyde-induced fluorescence of the above-mentioned compounds on silica gel, as well as in histochemical models and in tissues[12,13,17,18]. The pH can affect the final fluorescence intensity in two ways: (1) by affecting the rates and equilibria of the various reactions involved; (2) by affecting the ionization of the resulting fluorophores. If the pH-dependent effects are due mainly to the second alternative, the results would be similar irrespective of whether the change in pH is made before or after the formaldehyde condensation. This appeared to be the case with combined formaldehyde–ammonia treatments. Thus, spraying with ammonia after the formaldehyde condensation gave principally the same results as the Procházka II spray reagent (unpublished results, see also ref. 18). The Procházka I spray reagent resulted in a strong fluorescence only with tryptamine and tryptophan. In this case, the characteristic results could not be reproduced if HCl treatment was applied after condensation with gaseous formaldehyde (unpublished results and ref. 18); the formaldehyde-induced fluorescence of the compounds tested was largely unaffected by the subsequent HCl exposure. It is thus likely that in this case the pH-dependent

TABLE III

SPECTRAL PROPERTIES OF FORMALDEHYDE-INDUCED MONOAMINE FLUOROPHORES ON SILICA GEL

Spectra were recorded from spots containing 1–3 $\mu g/cm^2$ of the compound.

Compound	*Formaldehyde gas*	*Procházka I*	*Procházka II*	*Procházka II + I*
Tryptamine	370 (370)/510 (500–520)	365 (360–370)/560 (550–570)	365 (360–380)/500 (500)	370[c] (365–380)/500 (500)
Tryptophan	375 (370–380)/440 (430–450)	365 (360–370)/550 (545–560)	375 (370–380)/420 (410–425)	380–430[c]/485 (470–500)
5-Hydroxytryptamine	420 (415–420)/530 (520–550)	—	400 (380–410)/530 (530)	410 (400–420)/560 (550–580)
5-Hydroxytryptophan	410 (400–420)/530 (520–540)	—	380 (380)/520 (510–530)	410 (400–420)/560 (535–590)
5-Methoxytryptamine	410 (390–420)/570 (560–580)	—	370 (360–370)/525 (515–535)	435 (425–440), 360[a]/525 (520–530)
5-Methoxytryptophan	410 (400–420)/505 (500–510)	—	405 (400–410)/470 (460–480)	435 (430–440), 360[a]/515 (505–520)
Dopamine	400 (380–420); 320[a]/500 (490–520)	—	405 (400–410)/500 (485–505)	370 (360–380)/475 (440–490)
DOPA	415 (410–420); 320[a]/500 (490–520)	—	[b]/485 (470–500)	380 (370–390)/465 (440–490)
Noradrenaline	415 (410–420); 320[a]/500 (490–520)	—	395 (390–400)/500 (480–500)	420 (420–430)/470 (450–500)
Adrenaline	420 (420); 320[a]/450 (430–460)	—	365 (360–370)/465 (450–485)	415 (410–420)/475 (460–490)
3-Methoxytyramine	370 (360–380); 320[a]/480 (470–490)	< 340, 370[a]/480 (480)	[b] < 320, 415[a]/515 (500–525)	390, 330[a]/485 (480–490)
Normetanephrine	—	345 (335–350)/ < 410[a]; 490 (480–495)	[b] < 320, 390[a]/475 (440–500)	approx. 330/500 (500)
Metanephrine	—	335 (330–340)/ < 410[a]; 510 (505–515)	[b] < 320/470 (440–490)	< 320; 390[a]/495 (480–510)

[a] Second peak.
[b] Weak fluorescence.
[c] Broad peak.

difference in the results is due to changes in the reaction conditions, which can yield other condensation products or affect the extent of oxidative degradation of the amine reactants and of their formaldehyde condensation products.

In an earlier investigation[6] it was found that the most intense fluorescence was obtained from the catecholamines on silica gel thin layers when the formaldehyde gas treatment was followed by gentle reduction with sodium borohydride. However, sodium borohydride in higher concentrations completely abolished the fluorescence. Thus, difficulties were encountered in standardizing this technique to give reproducible results. At present, the most favorable method for the chromatographic detection of catecholamines in routine work is the condensation with gaseous formaldehyde on EDTA-enriched thin layers, followed by spraying with alkaline buffer (pH 8).

The results obtained with the use of the aqueous formaldehyde reagents could be reproduced fairly closely by formaldehyde gas treatment in combination with HCl or NH_3 (Table I). When the conditions of the gaseous treatments are properly standardized to give reproducible results, they should be preferred in chromatographic work because of minimum diffusion of the amine spots and because the use of gaseous formaldehyde allows the direct microspectrofluorometric comparison with the fluorophores obtained in fluorescence histochemistry[5,18]. The gaseous formaldehyde treatment has also been applied successfully in the detection of amines on paper (unpublished results and refs. 1 and 19), giving fluorophores with spectral properties and fluorescence intensities essentially similar to those obtained on silica gel thin layers.

Conclusions

Certain biogenic phenylethylamines and indolylethylamines react readily with formaldehyde in forming highly fluorescent condensation products. The formation of such fluorophores can be induced by aqueous formaldehyde (*i.e.*, Procházka reagents) as well as by formaldehyde gas. The spectral characteristics and the fluorescence intensities of the fluorophores formed with the various Procházka reagents, which involve the use of highly acid or highly alkaline formalin, were compared with those of the fluorophores obtained with formaldehyde gas. It was found that the fluorescence properties of the formaldehyde-indured fluorophores formed from a number of biogenic aromatic amines and amino acids differed markedly with the various types of formaldehyde detection reagents used. It was also found that the fluorescence properties obtained with different Procházka reagents could largely be reproduced by using gaseous formaldehyde in combination with HCl or NH_3 vapour. It is suggested that the proper selection of reaction conditions for the formaldehyde condensation in combination with microspectrofluorometric analysis will provide a valuable tool for the chromatographic identification of tissue amines.

This study was supported by grants from the Magnus Bergwall Foundation and from the United States Public Health Service (project No. 06701-04) and was carried out within a research organization sponsored by the Swedish Medical Research Council (projects No. B70-14X-712-05 and B70-14X-56-06).

Departments of Histology and Pharmacology,
University of Lund,
Lund (Sweden)

A. BJÖRKLUND
B. FALCK
R. HÅKANSON

1 C. E BELL AND A. R. SOMERVILLE, *Biochem. J.*, 98 (1966) 1C.
2 R. HÅKANSON, M. N. LOMBARD DES GOUTTES AND CH. OWMAN, *Life Sci.*, 6 (1967) 2577.
3 D. AURES, R. FLEMING AND R. HÅKANSON, *J. Chromatog.*, 33 (1968) 480.
4 E. J. COWLES, G. M. CHRISTENSEN AND A. C. HILDING, *J. Chromatog.*, 35 (1968) 389.
5 D. AURES, A. BJÖRKLUND AND R. HÅKANSON, *Z. Anal. Chem.*, 243 (1968) 564.
6 A. BJÖRKLUND, B. FALCK AND R. HÅKANSON, *J. Chromatog.*, 40 (1969) 186.
7 I. M. HAIS AND K. MACEK, *Handbuch der Papierchromatographie*, Bd. I., Fischer Verlag, Jena, 1958.
8 E. STAHL AND H. KALDEWEY, *Z. Physiol. Chem.*, 323 (1961) 182.
9 N. SEILER AND H. WIECHMANN, *Z. Physiol. Chem.*, 337 (1964) 229.
10 M. M. SPRUNG, *Chem. Rev.*, 26 (1940) 297.
11 W. M. WHALEY AND T. R. GOVINDACHARI, in R. ADAMS (Editor), *Organic Reactions Coll.*, Vol. 6, John Wiley and Sons, New York, 1951, p. 151.
12 H. CORRODI AND G. JONSSON, *J. Histochem. Cytochem.*, 15 (1967) 65.
13 B. FALCK AND CH. OWMAN, *Acta Univ. Lund. II*, 7 (1965) 1.
14 A. BJÖRKLUND, B. EHINGER AND B. FALCK, *J. Histochem. Cytochem.*, 16 (1968) 263.
15 H. CORRODI, G. JONSSON AND T. MALMFORS, *Acta Histochem.*, 25 (1966) 367.
16 G. B. WEST, *J. Pharm. Pharmacol.*, 4 (1952) 560.
17 A. BJÖRKLUND, B. FALCK AND R. HÅKANSON, *Acta Physiol. Scand.*, (1968) Suppl. 318.
18 A. BJÖRKLUND, B. FALCK AND R. HÅKANSON, *Anal. Biochem.*, (1970) in press.
19 L. CEGRELL, B. FALCK AND A.-M. ROSENGREN, *Acta Physiol. Scand.*, 77 (1969) 23.

Received December 23rd, 1969

CHROM 4586

Isolation of cyclic 3′,5′-adenosine monophosphate on a neutral silicic acid–glass microfiber matrix

The isolation of cyclic 3′,5′-adenosine monophosphate (cyclic-AMP) has concerned an increasing number of investigators. The multitude of published methods attests to the difficulties encountered with such an isolation[1–10]. This nucleotide is formed from adenosine 5′-triphosphate (ATP) by the enzyme, adenyl cyclase, and apparently functions as an intracellular mediator for a large array of hormones and neurotransmitters[11].

The isolation of cyclic-AMP on a silicic acid–glass microfiber matrix, as outlined in this communication, suggests a combination of advantages over other published procedures: (1) rapidity of development (approximately 50 min); (2) no significant interference by trichloroacetic acid (TCA), salts or Tris buffer; (3) ease of spot removal and dispersal for liquid scintillation counting; (4) separation from inosine, hypoxanthine, adenine, adenosine, as well as other nucleotide phosphates; (5) good recovery (95 %) from 40 nmoles to less than 0.01 pmole of cyclic-AMP when eluted following chromatography. Our chromatographic techniques have been applied to the assay of adenyl cyclase activity in tissue (manuscript in preparation) and are suited also to quantitative isolation of endogenous cyclic-AMP.

Materials and methods

Chemicals. Non-radioactive cyclic 3′,5′-adenosine monophosphoric acid was obtained from PL Biochemicals, Inc. N^6-2′-O-Dibutyryl cyclic 3′,5′-adenosine monophosphate was purchased from Schwarz Bioresearch, Inc. Cyclic 3′,5′-guanosine monophosphate was purchased from Boehringer Mannheim Corporation. All other non-radioactive purine bases, nucleosides and nucleoside mono-, di-, and triphosphates