ISOLATION AND TENTATIVE CHARACTERIZATION OF A TRACE FLUORESCENT LIPID FROM BOVINE, RAT AND RABBIT BRAIN

D.W. VIDRINE, C.H. MARTIN and H.J. NICHOLAS

Institute of Medical Education and Research, and *Department of Biochemistry, St. Louis University School of Medicine, 1605 South Fourteenth Street St. Louis, Mo. 63104, USA*

Received 11 April 1973

1. Introduction

During the course of examining bovine brain lipid concentrates for still unidentified hydrocarbons [1–3] some trace non-polar fluorescent lipids were observed in the concentrates. Our attention was subsequently directed towards at least four other recent manuscripts [4–7] which have indicated the presence of highly fluorescent trace lipids in brain tissue. Our preliminary studies have lead us to suggest that one of the compounds, which may be identical to that described by Geiger and Bampton [4], has the structure 9-seco-cholesta-5(10)-6,8-triene.

2. Materials and methods

2.1. Chemicals

Solvents were distilled in all-glass apparatus, and special precautions were taken throughout the operations to exclude contaminants, as in previous work [2]. The petroleum ether (b.p. 30–60°C) was allowed to stand over concentrated H_2SO_4 before distillation. Ethyl ether was freshly distilled over $LiAlH_4$. Gas-liquid chromatography (GLC) packings on 100/120 mesh Gas Chrom Q were obtained from Applied Science Laboratories (State College, Pa.).

2.2. Thin-layer chromatography (TLC)

TLC was performed on 20 × 20 cm glass plates coated with Silica Gel H, 0.75 mm in thickness applied as a water slurry and subsequently activated at 110°C overnight. Before use the plates were "washed" by ascending chromatography with benzene and ethyl acetate respectively, then reactivated at 70°C for 1 hr before use. Ascending chromatography for purification of the fluorescent compound in question was performed in two stages: first, using the solvent system I (hexane–ethyl ether, 100:5, v/v). In this step the fluorescent band (R_f 0.70) was visualized under long wave ultraviolet light (~ 280 nm) as a blue fluorescence. This band was scraped off the plate, eluted from the silicic acid with ethyl acetate, and rechromatographed in the same manner in solvent system II (hexane–ethyl acetate, 100:5, v/v). The fluorescent band (R_f 0.70; lanosteryl palmitate standard R_f 0.75) was subsequently eluted from the scraped area (visualized under U.V. light as above) with ethyl acetate, which was evaporated at room temp. under N_2 for further examination of the residue.

2.3. Physical analytical methods

Fluorescent spectra were measured on an Aminco–Bowman spectrofluorimeter, using 1 mm slit widths and a 1 cm square quartz cuvette containing the sample in n-heptane.

Gas-liquid chromatograph (GLC) was accomplished with a Varian 1740-10 chromatograph, using on-column injection onto 1% SE-30 or 3% SE-30 dichlorodimethylsilane-treated columns, 2 m × 2 mm diameter. Carrier gas flow was 30 ml/min STP argon. Column temperature (200–230°C) was adjusted to give 7–10 min elution times for 5α-cholestane with each column. Columns containing OV-17, OV-210, or OV-225 decomposed the fluorescent compound under examination completely in the amounts injected.

Mass-spectrometry (MS) was performed on an LKB-9000 mass spectrometer, using either direct injection or introduction via gas chromatography, with 40 ml/min STP helium flow. Ionizing voltage was 70 eV. GLC-MS was accomplished using 1% SE-30 on Gas Chrom Q, in a 2 m × 2 mm diameter dichlorodimethylsilane-treated glass column. Column temperature was 180°C, and molecular separator temperature was 250°C.

2.4. Isolation and partial purification of one of the fluorescent lipids (FC-1) from bovine brain

Preliminary examination of lipid concentrates of small quantities (50–500 g) of rat, rabbit and bovine brain indicated that the fluorescent compound described by Geiger and Bampton [4] was present in the brains of all three species. Since other minor trace fluorescent lipids were detected in bovine brain (see below) we will refer to Geiger and Bampton's compound as Fluorescent Compound 1 (FC-1). Since this compound seemed to be present in all of the brain tissues examined in very minute amount, a large quantity (20 kg) of the more readily available bovine brain tissue was processed for isolation and attempted purification of FC-1. The brains, freshly obtained from a local slaughter house, were freed of blood and meninges, homogenized in a Waring Blender in methanol, and chloroform was added to make the solvent concentration approx. 2:1 chloroform–methanol. The resulting mixture was boiled briefly on a steam bath, filtered through glass wool, and freed of solvent by distillation. An acetone concentrate of the residue was prepared essentially as described by Nicholas et al. [1]. This was subjected to chromatography on alumina as described by Ramsey et al. [2]. Following elution of the column with petroleum ether, FC-1 was detected by TLC in the first fractions eluted with benzene and was purified as indicated under sect. *2.3.*

3. Results

3.1. Purification and properties of FC-1

Repetition of Geiger and Bampton's procedure [4] using 50 g rabbit brain gave a fluorescent fraction weighing 30 mg and containing a fluorescent component (FC-1) which eluted at R_f 0.95 in their alumina TLC system in good agreement with their results. In our hands this proved to be 98–100% cholesterol as assayed by GLC or TLC. Removal of the cholesterol by crystallization followed by TLC assay of the filtrates gave the highly fluorescent compound (R_f 0.70 in system II).

When the large quantities of crude benzene fraction from Al_2O_3 chromatography are examined at least 3 other fluorescent compounds can be visualized under certain conditions (R_f's 0.52, 0.23 and 0.17, respectively). These will be described in a more detailed manuscript to be published later.

FC-1 showed a single major peak at a relative retention time of 0.95 on 1% SE-30, corresponding to a weight concentration of 1–2 ng per gram brain wet weight. Spectrofluorimetry gave the fluorescence spectrum shown in fig. 1. This spectrum is compatible with the structure proposed in fig. 3. Not enough material was available for a precise UV spectrum, but an absorption band between 280–310 nm was noted. This absorption, and the activation peak of the fluorescence spectrum, are both similar to the UV absorption spectra of known conjugated steroid trienols [8]. FC-1 is nonsaponifiable, and $LiALH_4$ reduction failed to change its chromatographic or fluorescent properties. The fluorescence is destroyed by H_2-Pt reduction at room temp. (H_2 : 40 psi).

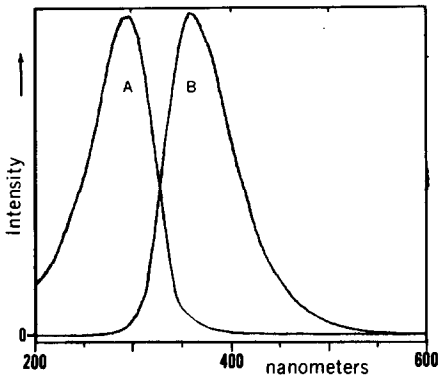

Fig. 1. Fluorescence spectrum of FC-1. A) Activation spectrum for 360 nm emission. B) Emission spectrum for 295 nm activation.

3.2. Mass spectrometry (MS)

Direct probe MS of the TLC purified FC-1 gave the spectrum shown in fig. 2. GLC-MS gave a spectrum consisting mostly of column background, especially at lower *m/e* (mass/charge) values. Only 5 peaks did not correspond to background peaks: *m/e* 213, 247, 260, 353, and 368. The fact that these are identical to the only major peaks in the direct probe spectrum which would not be expected to be obscured by column background, supports the conclusion that the GLC peak represents the same compound seen in the direct probe spectrum.

The major peaks in fig. 2 were interpreted to have resulted from the fragmentations shown in fig. 3. Metastable peaks support the fragmentation of *m/e* 368 into *m/e* 353, 260 and 247; and *m/e* 353 into *m/e* 255 and 213.

3.3. Structure

We propose the structure of FC-1 to be 9-seco-cholesta-5(10),6,8-triene (fig. 3). Our conclusions are based on the following evidence:

1) It is nonpolar on TLC and column chromatography, having the mobility expected of a steroid hydrocarbon.
2) It is resistant to $LiALH_4$ reduction (and saponification), signifying lack of any carboxyl oxygen.
3) It is readily reduced by H_2-Pt, the fluorescence disappearing on such reduction.
4) Its retention time on GLC suggests a molecular weight of 300–400.

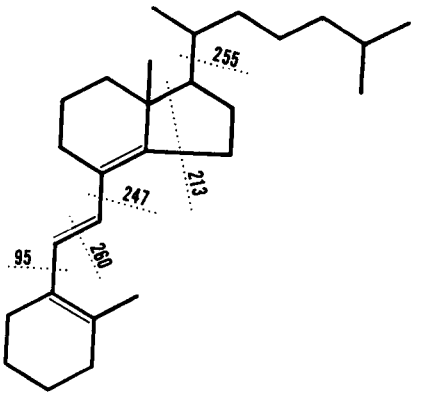

Fig. 3. Proposed structure of FC-1 with major fragmentation noted.

5) The fluorescence spectral data are consistent with the suggested structure.
6) A molecule of the proposed structure would be expected to undergo fragmentations like those observed in the direct probe mass spectrum.

Not enough is known about the fragmentation of this type of compound to rule out positional isomers of the proposed double bond system.

4. Discussion

It seems possible that one of the causes of discrepancies in the literature on trace fluorescent components of brain may be due to the extremely low concentrations of the fluorescent components and the difficulty

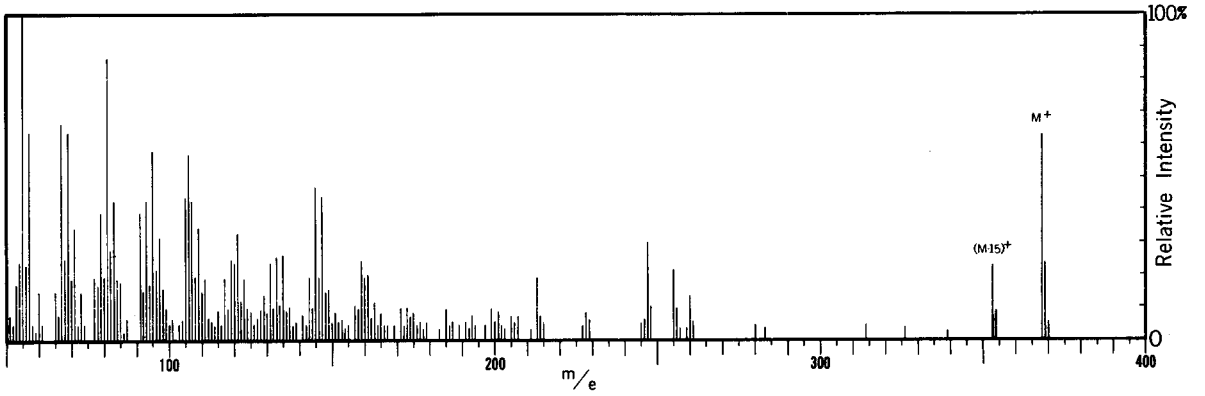

Fig. 2. Direct probe mass spectrum of FC-1.

in separation from the large amounts of other lipids, principally cholesterol.

It would be premature at this time to speculate on the origin of FC-1. Lunt and Rowe [5] and Khan and Hess [7] have suggested that some of the highly fluorescent trace lipids in brain may have a sterol structure. However, the compounds they studied are considerably more polar than FC-1, and no direct comparison with our work can therefore be made. The fluorescence spectra published by Khan and Hess [7] indicate that the chromophoric groups responsible for fluorescence in the components they studied are not the same as those of FC-1. Some preliminary synthetic preparations involving removal of the hydroxyl group from cholecalciferol have tended to verify our suggested structure for FC-1. At the moment FC-1 appears closely related to cholecalciferol and 7-dehydrocholesterol, and may be a metabolite thereof. Other tissues must be examined for the presence of the compound to assist in establishing its origin, since it may not be unique to brain tissue.

Acknowledgements

This work was supported by NIH Grant No. NS 06011-07 and NIH Biochem. Training Grant No. 2442492. We are grateful to Drs. Wm. H. Elliott and T.J. Curphey, St. Louis University School of Medicine, for assistance in interpreting the mass spectral data.

References

[1] H.J. Nicholas, R.C. Hiltibran and C.L. Wadkins, Arch. Biochem. Biophys. 59 (1955) 246.
[2] R.B. Ramsey, R.T. Aexel and H.J. Nicholas, J. Neurochem. 18 (1971) 2245.
[3] R.B. Ramsey and H.J. Nicholas, in: Advances in lipid research, Vol. 10, eds. R. Paoletti and D. Kritchevsky (Academic Press, New York, 1972) p. 143.
[4] W.B. Geiger and A.E. Bampton, Nature 212 (1966) 510.
[5] G.G. Lunt and C.E. Rowe, Life Sciences 8 (1969) 885.
[6] R. Richter and H. Dannenberg, Z. Physiol. Chem. 350 (1969) 1213.
[7] A.A. Khan and H.H. Hess, Lipids 6 (1971) 670.
[8] L.F. Fieser and M. Fieser, Steroids (Reinhold Publishing Corp., 1959) p. 90.