CHROMATOGRAPHIC ISOLATION AND DETERMINATION OF LONG-CHAIN N-ACYLETHANOLAMINES

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INTRODUCTION

The isolation of N-(2-hydroxyethyl)-palmitamide from soya lecithin, hexane-extracted peanut meal, and hen's egg-yolk was reported by KUEHL *et al.*[1]. Their procedures were not quantitative, and included a saponification step which facilitated the removal of esterified fatty acids but left doubt as to whether this compound actually occurred, as such, in the starting materials. Moreover, it was possible that other N-acylethanolamines ($R \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot OH$) were present but undetected. We have therefore devised quantitative methods for isolating and determining long-chain N-acylethanolamines that are equally applicable to non-saponifiable fractions and to intact lipids from plant and animal sources.

EXPERIMENTAL

Isolation by column chromatography

Silicic acid (Mallinckrodt, A.R.) was activated overnight *in vacuo* at 110° after removal of the largest particles by passage through a 100-mesh sieve and the smallest by sedimentation in methanol (after HIRSCH AND AHRENS[2]). A.R. chloroform was passed through a column of Linde 5A molecular sieve and sampled in a 1 mm infrared cell to verify the absence of hydroxy compounds; 1.00 % (v/v) methanol was added promptly.

A column (d. 19 mm) was prepared from a slurry of 10 g of silicic acid with 40 ml of 1 % methanol in chloroform, and washed until the "veil"[3] was lowered 1 cm. Lipids ($\lesssim$ 250 mg) were then applied, and washing was continued with 250 ml of 1 % methanol. Then 2 % methanol ($\lesssim$ 100 ml) was used to elute N-acylethanolamines, which were recovered by evaporation at $< 40°$.

(The "veil" recedes during development, as the adsorbent withdraws methanol from the eluent, until an equilibrium is established[4]; then the whole column is almost transparent. It is not necessary to reach this stage before lipids are applied. At equilibrium with 1 % methanol in chloroform, Mallinckrodt silicic acid adsorbs about 0.1 ml of methanol per g.)

Thin-layer chromatography

Non-activated layers of Silica Gel G, 0.25 mm in thickness, were used with either (A) benzene–pyridine–acetic acid (40:10:1, v/v/v)[5] or (B) chloroform–acetic

acid–water (180:20:1, v/v/v) as developing solvent. Developed plates were dried at room temperature, lightly sprayed with aqueous NaOCl (13 %, w/w, available Cl), left in a covered dish for 30 min, and then sprayed with ethanol and left exposed for 90 min. Finally, they were sprayed with a mixture (freshly made) of saturated *o*-tolidine in 2 % aqueous acetic acid and an equal volume of 0.05 *N* aqueous KI. N-Acylethanolamines gave blue zones on colourless backgrounds. In solvent A their R_F was about 0.4, and in solvent B it varied between 0.4 and 0.6.

Conversion to N-(2,4-dinitrophenyl) (DNP)-ethanolamine

A solution containing N-acylethanolamines (0.1 μg to 1 mg) was evaporated in a Pyrex test tube (16 × 150 mm) and 1 ml of 6 *N* aqueous HCl was added. The tube was sealed near the top and left overnight in a vertical position in an oven at 108°. When cool it was opened and 1 ml of ether was added. After careful shaking the ether layer was removed by pipette; this washing was repeated twice. Solid $NaHCO_3$ was then added in small excess, followed by 10 mg of 1-fluoro-2,4-dinitrobenzene dissolved in 2.5 ml of ethanol. The tube was closed with a cotton-wool plug and left in a dark cupboard overnight. All subsequent manipulations were carried out with minimum exposure to light. Most of the ethanol was removed in a stream of nitrogen. With 2 ml of water added, the residual mixture was transferred to a separating funnel (PTFE stopcock) and extracted four times with 3 ml portions of chloroform. The combined extracts were evaporated at room temperature, affording an orange residue.

Isolation and measurement of DNP-ethanolamine

The orange residue was chromatographed in ether on a column containing 5 g of activated silicic acid. The first 40 ml of effluent were rejected. The next 40 ml were collected, partly evaporated if necessary, and made up to a suitable volume, and the extinction was measured at 340 mμ. Calculated for N-(2-hydroxyethyl)-palmitamide, $E^{1\%}_{1cm}$ was 450 ± 30.

Confirmation of ethanolamine

HCl hydrolysates were evaporated with warm air and tested for ethanolamine (R_F 0.63) by paper chromatography in *n*-propanol–acetone–0.25 *N* aqueous ammonia (4:1:1, v/v/v)[6].

DNP-ethanolamine (R_F 0.2) was confirmed by thin-layer chromatography in solvent A as described above; samples that had been exposed to light for long periods gave several unidentified yellow zones.

DISCUSSION

Long-chain N-acylethanolamines were found to be eluted from silicic acid columns by 1 % methanol in A.R. chloroform, but retained in A.R. chloroform alone. There was some uncertainty about retention, however, depending upon how much ethanol the manufacturer had added as stabilizer to the chloroform, and so it was preferable to replace the ethanol with methanol at a known concentration. The critical content for elution then became 2 %. (On Florisil the same effect was observed.) Since triglycerides, free sterols, fatty acids, *etc.* were rapidly eluted by 1 % methanol, and most phospholipids and glycolipids by > 2 % methanol, a quantitative and

fairly specific procedure was thus available for isolating N-acylethanolamines occurring in any proportion in any lipid mixture.

The identity of eluted N-acylethanolamines was readily confirmed by infrared analysis (see Fig. 1) and by acidic hydrolysis to fatty acids and ethanolamine. Thin-layer chromatography, employing basic and acidic solvent systems, was useful for

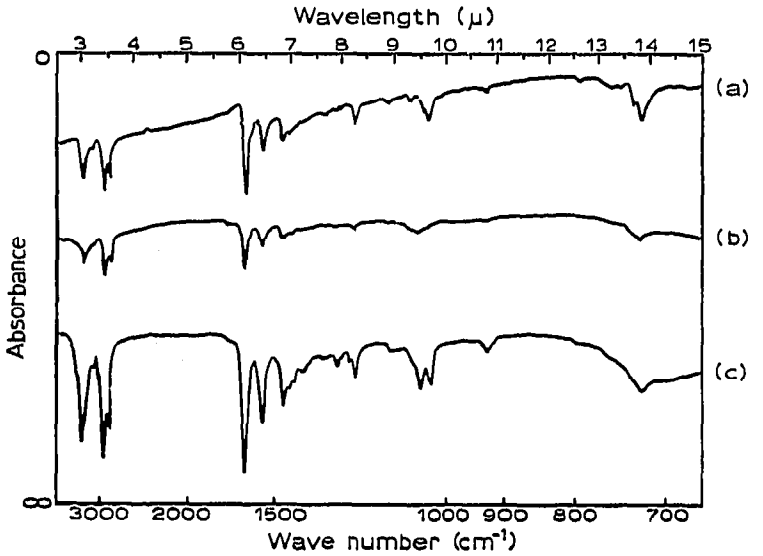

Fig. 1. Infrared spectra of films, cast from chloroform solution, on NaCl plates. (a) Synthetic N-(2-hydroxyethyl)-palmitamide. (b) N-Acylethanolamines obtained after saponification of egg cephalins. (c) N-Acylethanolamines obtained after saponification of corn oil in the presence of ethanolamine.

identification and for detection of N-acylethanolamines in impure eluates. Zones were best revealed by a specific chlorination test for CO·NH, which was adapted from those described by REINDEL AND HOPPE[7] and PAN AND DUTCHER[8]. The limit of detection for N-(2-hydroxyethyl)-palmitamide was 2.5 μg.

For micro-determination, eluted N-acylethanolamines were converted to N-(2,4-dinitrophenyl)-ethanolamine, using essentially the method of AXELROD *et al.*[9]. This derivative was then isolated by column chromatography, in diethyl ether on silicic acid, and measured by spectrophotometry. Excellent separation occurred, provided that the column was not overloaded. In a control experiment, which was designed to test the behaviour of likely impurities, 1-fluoro-2,4-dinitrobenzene (colourless, $\lambda_{max}^{Et_2O} < 228$ mμ), 2,4-dinitrophenol (colourless, $\lambda_{max}^{Et_2O}$ 256 and 330 mμ, $\lambda_{infl}^{Et_2O}$ 281 mμ), and 2,4-dinitraniline (yellow, $\lambda_{max}^{Et_2O}$ 329 mμ) appeared, in order, in the first (rejected) portion of effluent; DNP-ethanolamine (yellow, $\lambda_{max}^{Et_2O}$ 340 mμ) was then eluted in a fairly compact zone. The column separation made this determination considerably more specific than others (for lipid-bound ethanolamine) that are described in the literature[10]. The complete determination was calibrated with synthetic[11] N-(2-hydroxyethyl)-palmitamide.

Milligram quantities of N-acylethanolamines were often determined by direct weighing with, if necessary, correction for any minor impurities revealed by infrared analysis. When major impurities were present the method described for micro-determination was applied.

These methods have facilitated a study of egg-yolk lipids[12] and are now being used in studies of lipids from other sources. Recently UDENFRIEND and co-workers[13,14] used a silicic acid method, which was apparently less specific than that described here, to isolate radioactive N-acylethanolamines, and confirmed their identity by gas–liquid chromatography.

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SUMMARY

Elution from silicic acid with 2 % methanol in purified chloroform is the basis of a fairly specific procedure described for the isolation of long-chain N-acylethanolamines from lipid extracts and fractions. Identification and determination procedures are also described, including conversion to DNP-ethanolamine and determination of this derivative by a new method.

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