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The use of radioisotopically labelled methyl esters in the determination of the 15-epimer content of prostaglandins

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The quantitative determination of the 15-epimer content of prostaglandins by formation of their isotopically-labelled methyl esters is described. Either [^{3}H]dimethyl sulphate or [^{14}C]diazomethane are used as esterification reagents. The reaction products are separated by thin layer chromatography and the epimer ratio determined by scintillation counting of the labelled ester zones. The accuracy of the technique is illustrated by the determination of 15-epimer in ICI 74 205, a close analogue of $PGF_{2\alpha}$. Evidence for the uncatalysed reaction of diazomethane with the alcoholic hydroxyl groups of prostaglandin molecules, which could be significant in other prostaglandin analysis techniques (e.g. g.l.c.) which depend on quantitative esterification with this reagent, is also presented.

The presence of up to 5 asymetric centres in the naturally occurring prostaglandins (PG) has led to many problems in the resolution of the optical isomer mixture often produced in synthetic schemes, e.g. Corey, Albonico & others (1971).

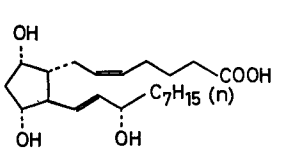
Since large differences in biological activity have been noted, particularly between 15-epimeric isomers, analytical control methods for the determination of this epimer ratio assume an importance in the development of prostaglandins and prostaglandin analogues as pharmaceutical preparations. So far, we have not found it possible to separate the 15-epimers by gas-liquid chromatography, due possibly to disturbance of the spatial inter-relations of the hydroxyl groups by the derivatization procedure.

Weinshenker & Longwell (1972) have used high pressure liquid chromatography in the determination of small amounts of 15-epi-$PGF_{2\alpha}$ in $PGF_{2\alpha}$. We now describe a quantitative technique for this determination utilizing thin-layer chromatography, which is rapid and precise and may be adapted readily to the study of many different epimeric pairs of prostaglandin-related compounds.

MATERIALS AND METHODS

The compound used primarily for this study was ICI 74205 (I), a compound closely related to $PGF_{2\alpha}$.

[^{3}H]Dimethyl sulphate supplied by the Radiochemical Centre; [^{14}C]diazomethane generated from [^{14}C]Diazald; (*N*-[^{14}C]methyl-*N*-nitroso-*p*-toluene sulphonamide)



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supplied by the Radiochemical Centre. For [^{3}H]dimethyl sulphate esterifications, chromatography was accomplished on plastic sheets precoated with kieselgel (Camlab Polygram). In the [^{14}C]diazomethane esterifications, 0·25 mm layers of kieselgel GF_{254} on glass plates were used.

Samples were counted in an Intertechnique SL 30 scintillation spectrometer at 4°.

Esterification using [^{3}H]dimethyl sulphate

Procedure. Labelled methyl esters were prepared using [^{3}H]dimethyl sulphate in an adaptation of the procedure of Stodola (1962). The strongly hindered base, *NN'*-dicyclohexylethylamine (DICE) was used as a proton acceptor rendering the reaction of [^{3}H]dimethyl sulphate with the carboxyl group rapid and specific, with minimal production of methyl ethers. The epimeric esters were separated by t.l.c. and the epimer ratio determined by liquid scintillation counting. No spots corresponding to hydroxyl methylation productions were visualized with iodine vapour. These ethers have been found to have R_F values slightly higher than 15-epi ICI 74205 methyl ester.

Method

350μg ICI 74205 ($\hat{=}$ 1μ mol) is dissolved in methanol (150 μl Analar) and is then treated with 4 μl DICE and 20 μl ($\hat{=}$ 13μ mol) of a solution of [^{3}H]dimethyl sulphate (1 mmol : specific activity = 100 μCi mmol^{-1}) in benzene (25 ml). A blank consisting of 150 μl Analar methanol is treated in the same way. The solutions are heated in a closed glass vial for 30 min at 60°.

The solutions are acidified with 0·5% hydrochloric acid to pH 3 and shaken with n-butyl acetate (150 μl Analar). When phase separation is complete, 20 μl aliquots of the n-butyl acetate extracts are applied to a precoated t.l.c. plate. The plate is developed in toluene–dioxan–acetic acid (20 : 20 : 1).

The chromatogram is visualized with iodine vapour and after the zones in the sample run corresponding to ICI 74205-Me ester and 15-epi ICI 74205-Me ester have been marked, the iodine is removed by warming the plate with an air blower. To remove excess [^{3}H]dimethyl sulphate from the chromatogram, the plate is sprayed with 0·880 ammonia solution and allowed to stand for 5 min before drying it with an air blower. The ammonia treatment is repeated and the plate redried.

The sections of the chromatogram containing the two methyl esters (approximate R_F values:—ICI 74205-Me = 0·4, 15-epi ICI 74205-Me = 0·5) are cut from the plate and placed in scintillation counting vials containing 10 ml of dioxan–naphthalene–Butyl-PBD–Aerosil scintillation fluor and shaken thoroughly. The samples are then counted for 20 min each.

Sections of plate from the chromatography of the blank solution of the same size and R_F value as the methyl ester sections, are cut and counted in the same way. The counting efficiency of each sample is determined by the internal standardization technique (using [^{3}H]hexadecane) and the d min^{-1} calculated.

Then:

x = d min^{-1} of 15-epi ICI 74205-Me ester spot — equivalent blank d min^{-1}
y = d min^{-1} of ICI 74205-Me ester spot — equivalent blank d min^{-1}

$$\therefore \%\ 15\text{-epimer} = \frac{x}{x+y} \times 100$$

Typical results are shown in Table 1.

Table 1. *Results of 15-epimer determinations of typical samples, using dimethyl sulphate.*

Sample	% 15-Epimer
Batch 1251/88: Crude synthetic product	6·7; 6·7
Batch 1251/88: after preliminary purification Mean Value (6 exp.)	4·4 ± 0·65
Batch 1251/88: final purification	0·72; 0·54

Esterification with diazomethane

Preparation of reagent. [^{14}C]Diazomethane was generated by the slow addition of sodium hydroxide solution (8% w/v in methanol–water; 9:1) to a gently boiling solution of *N*-[^{14}C]methyl-*N*-nitroso-*p*-toluene sulphonamide (50 mg, specific activity 2·6 μCi mmol^{-1}) in diethyl ether (8 ml). The distillate was collected in a tube containing diethyl ether (2 ml), cooled in ice. Distillation was continued until all the ether had been collected; the diazomethane solution so produced was used to esterify three 200μg samples of ICI 74205.

Method

The solid samples are dissolved in 2 ml of diethyl ether containing 10% methanol which enhances the solubility of the prostaglandin, and increases the rate of esterification (Schlenk & Gellerman, 1960). Roughly equal portions of the [^{14}C]diazomethane solution are added to these solutions, and to 2 ml of the ether–methanol solvent (solvent blank). The mixtures are allowed to stand, typically for 20 min at room temperature (20°). Excess [^{14}C]diazomethane is removed together with solvents, by evaporation under a stream of nitrogen at 30°.

The residues from each evaporation are dissolved in 100μl of Analar methanol, and 40μl aliquots of the solutions are chromatographed on a kieselgel GF_{254} layer developed in toluene–dioxan–acetic acid (20:20:1).

The labelled esters are visualized, removed from the layer by scraping, and counted in the same way as those produced by [^{3}H]dimethyl sulphate esterification. The blank samples are scraped from the solvent blank chromatogram and are of the same size and R_F value as the methyl ester sections.

The % 15-epimer content is calculated as previously described, and some typical results are shown in Table 2.

DISCUSSION

Whichever method of esterification is employed, it is of course necessary to ensure that impurities other than the 15-epimer are absent from the samples, by preliminary

Table 2. *Results of 15-epimer determinations of typical samples, using diazomethane.*

Sample	% 15-Epimer
Batch 2177/115: final purification	0·72; 0·66
Batch 2307/115; first purification	1·60; 1·33
Batch 2307/115: second purification	1·06; 1·15; 1·17
Batch 2307/115: third purification	<0·1; <0·1

t.l.c. examination. If other impurities are detected, allowance may be made in the calculation for the presence of their [^{14}C]methyl esters (and of their 15-epimeric esters, if applicable) when the relevant R_F values have been determined, and the zones counted.

In all samples of ICI 74205 treated with diazomethane so far encountered, chromatography of the [^{14}C]methyl esters has revealed two further zones made visible by iodine vapour, other than the two expected zones. (Approximate R_F values: 0·55: and 0·60). Reaction times of down to 1 min, with the reaction mixture stored in ice, still produced easily observed signs of these by-products. Under these conditions the supposedly fast -COOH esterification is still incomplete. "Titration" of a prostaglandin reaction solution with diazomethane solution to the first appearance of the first yellow colour of unreacted diazomethane, in order to cut down the excess of reagent present, still produced by the by-products. Substitution of unlabelled diazomethane for the ^{14}C-labelled material gave the same result.

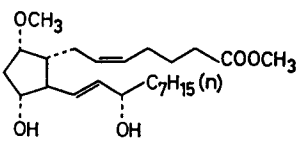
Quantitative measurement of the two by-products by scraping their chromatogram zones and those of ICI 74205 and its epimer from the t.l.c. plate and counting led to a combined figure of 6·1 % of the total reaction product, after 10 min reaction time at 0°.

Esterification of 30 mg ICI 74205 led to the isolation by preparative t.l.c. of sufficient of the by-products for spectroscopic examination.

Fourrier Transform nmr showed the double bond system to be undisturbed and that each impurity contained a -OCH_3 function as well as the expected -$COOCH_3$.

The impurities possessed almost identical mass spectra, with $M^+ = 410$, corresponding to a dimethylated product. The similarity of the spectra implied a close relationship between the compounds. Investigation of the trimethyl silyl ethers confirmed the replacement of -OH by -OCH_3.

It seems likely, therefore, that the by-products produced in the diazomethane esterification of ICI 74205 are the closely related ethers formed by methylation of the ring -OH groups, i.e.



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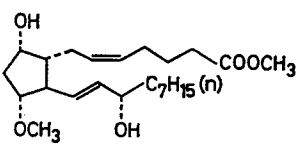

III

The possibility of the methylation of the 15-hydroxyl group is not however completely excluded.

The reaction of diazomethane with alcoholic hydroxyl groups catalysed by Lewis acids (Meerwein & Hinz, 1930), or boron compounds (Bawn & Ledwith, 1958) or when carboxyl groups are adjacent in the molecule (Schmidt & Zeiser, 1934) is well known. It has also been reported that primary alcoholic groups can be attacked in the absence of these conditions (Holloway & Deas, 1971). I feel, however, that the possibility of diazomethane attack on secondary hydroxyl groups as found in this case should be borne in mind in investigations of the quantitative esterification of substituted carboxylic acids with this reagent.

Conclusions

Either of the techniques described allowed a fairly rapid determination of the 15-epimer content of $PGF_{2\alpha}$-like compounds. Although it was expected that diazomethane would be the preferred reagent, the ether by-products which could be formed at different rates by the two epimers limit the reliability of this method as described. However, its rapidity and convenience outweigh this disadvantage in normal circumstances where amounts of 15-epimer are small. Although it is illustrated by its use in connection with ICI 74205, the method is applicable to $PGF_{2\alpha}$ itself with no modification, and to other similar compounds whose 15-epimers are resolved by t.l.c. For example the R_F values of $PGF_{2\alpha}$–Me and 15-epi–$PGF_{2\alpha}$–Me in the t.l.c. system described are 0·39 and 0·47 respectively. The use of [^{14}C]Diazald or [^{3}H]dimethyl sulphate of higher specific activity would enable even greater precision, and higher sensitivities to be obtained.

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