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Note

Gas-phase analytical methods in the characterization of ^{2}H- and ^{3}H-labeled 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid

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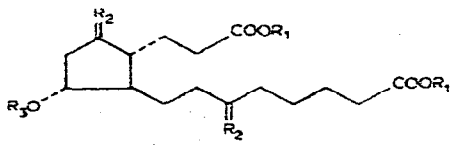
Gas-phase analytical methods [1] [i.e., gas—liquid chromatography (GLC)-Based separations combined with "normal" detectors, radioactivity monitors, and mass spectrometers] are employed extensively in drug metabolism and related studies. The use of radioisotopes in combination with gas—liquid radiochromatography (GLRC) in such research is well established [2, 3]. Stable-isotope labeling provides internal standards for GLC—mass spectrometry (MS)-based assays [4, 5] and characteristic isotope clusters for MS recognition of compounds as being drug or substrate-related [6, 7]. We now wish to report our use of these methods to characterize ^{2}H (deuterium)- and ^{3}H (tritium)-labeled 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (I)



I $R_1, R_3 = H$; $R_2 = O$
II $R_1 = CH_3$; $R_2 = NOC^2H_3$; $R_3 = H$
III $R_1 = CH_3$; $R_2 = NOCH_3$; $R_3 = Si(CH_3)_3$

Hamberg [8] and Seyberth et al. [9] have employed the bis-($^{2}H_3$-methyloxime),bis-methyl ester of 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (II)* as the internal standard in a GLC—MS assay to demonstrate the effect of certain drugs upon human urinary excretion of I, the major human urinary metabolite of prostaglandins E_1 and E_2. In using II as the internal standard it is introduced after endogenous I has been partially purified and derivatized to its bis-methyloxime,bis-methyl ester. We have sought to obtain labeled I in which the ^{2}H and ^{3}H are both present in the prostaglandin metabolite per se, so that the internal standard can be added to the urine sample at

*The underivatized compound, labeled with ^{3}H in the 2-position, is prepared biosynthetically from tritiated 15-keto-prostaglandin E_2 (15-keto-PGE$_2$) [8, 9].

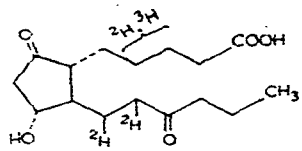
the beginning of the assay, thus compensating for yield losses incurred in the entire analysis. The present paper describes the use of several gas-phase analytical methods in characterizing $^2H,^3H$-labeled I prepared by two different means.

EXPERIMENTAL

GLRC was carried out using a Barber-Colman Model 5000 instrument in which the column effluent is split between a flame ionization detector and a combustion tube (CuO—steel-wool at 800°) connected to a proportional counter. GLC—MS data were obtained using an LKB Model 9000 instrument with the following settings: source temperature, 270°; electron energy, 70 eV; accelerating voltage, 3.5 kV; trap current, 60 μA. Derivatization was carried out as previously reported [9].

RESULTS AND DISCUSSION

The first approach to producing the ^2H, ^3H-labeled internal standard involving its biosynthesis by rabbits from ^2H, ^3H-labeled 15-keto-PGE_0* (see below) employed the dosing and isolation procedures reported by Seyberth et al. [9]. However, the second reversed-phase partition chromatographic step used by these authors was omitted, as it is carried out on the esterified (diazomethane) isolate and we wished to obtain the free acid.



By the very nature of its method of isolation $^2H,^3H$-labeled I must be less pure than if it had been esterified and carried through the complete isolation procedure [9]. As radioactivity is used as the measure of the quantity of internal standard added to an aliquot of urine, the mass added can only be a maximum. Further, as a result of the side-chain degradation the specific activity of the biosynthesized metabolite must be assumed to be one-half that of the tritiated precursor, and this approximation also results in the introduction of an additional inaccuracy. Finally, because of the extent and nature of the ^2H labeling, the internal standard (in its derivatized form) exhibits a signal at *m/e* 365 (see below), the ion monitored for quantitation of the endogenous metabolite in human urine.

The partial mass spectrum of $^2H,^3H$-labeled 15-keto-PGE_0 as its bis-methyloxime,bis-methyl ester trimethylsilyl (TSM) ether is presented in Fig. 1. The most intense signals of the isotopic clusters associated with the M−31 (loss of OCH_3) and M−(31 + 90) (loss of OCH_3 + TMSOH) ions, *m/e* 470 and 380, respectively, indicate that the compound contains three ^2H per mole, as the corresponding signals for the unlabeled compound are found at *m/e* 467 and 377.

*Prepared by Rosegay [10].

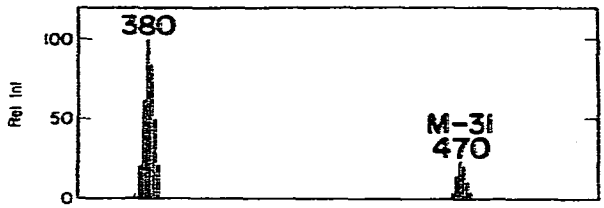


Fig. 1. Partial mass spectrum of ^2H, ^3H-labeled 15-keto-PGE_0 as its bis-methyloxime,bis-methyl ester, TMS ether.

The isolate from the rabbit urine was processed through the assay procedure [9] and the final thin-layer chromatographic (TLC) zone containing the bis-methyloxime,bis-methyl ester of the metabolite eluted and trimethylsilylated. Analysis of this derivatized radioactive material by GLRC gave the results shown in Fig. 2b and c. A radioactive component was observed which possessed the same retention time as the C_{24} fatty acid methyl ester (Fig. 2a) on a non-polar dimethyl polysiloxane stationary phase; this is the GLC behavior reported by Hamberg and Samuelson [11] for derivatized I from human urine. GLC—MS of this radioactive component from rabbit urine demonstrated that its mass spectrum was identical, except for the isotope clusters, to that published [11] for the human metabolite. The partial mass spectrum of this radioactive compound is given in Fig. 3. Whereas the M–31 and base peak M–(31 + 90) ions for derivatized unlabeled metabolite are found at *m/e* 455 and 365, respectively [11], the analogous ions for the radioactive rabbit metabolite are found at *m/e* 457 and 367. The isotope cluster patterns further characterize the compound as being derived from the ^2H,^3H-labeled 15-keto-PGE_0. That the metabolite contains two ^2H per mole rather than three is expected, for a significant loss of deuterium (and tritium) must result from the metabolic transformation.

Taub et al. [12] recently reported the synthesis of I, and this work has been extended to the preparation of the ^2H,^3H-labeled I [13]. GLC—MS analysis of this compound as its bis-methyloxime,bis-methyl ester, TMS ether (III) gave the results presented in Figs. 4 (GLC) and 5 (MS). The two major GLC peaks are the *syn* and *anti* isomers, and the partial mass spectrum of the major isomer indicates that it contains seven ^2H per mole, with no signal at *m/e* 365. GLRC analysis of the trimethylsilylated TLC zone eluate from a urine sample spiked with this internal standard and carried through the assay procedure gave the results shown in Fig. 6b; the radioactive component possesses, as required, the same retention behaviour as the C_{24} fatty acid methyl ester. The isotope clusters of this isolated internal standard were the same as those shown in Fig. 5, demonstrating that no isotopic exchange occurred during the assay procedure.

A 20-ml aliquot of urine from a normal adult male was spiked with ^2H,^3H-I just prior to initiation of the assay procedure [9]; partway through the assay the bis-($^2H_3$-methyloxime),bis-methyl ester of synthetically prepared I [12] was also introduced as per the approach of Seyberth et al. [9]. Calculations based on the ^2H,^3H-I internal standard [monitoring of ions of *m/e* 365 (endogenous I) and 372 (internal standard)] gave a value of 0.18 μg I per 20 ml, whereas the value obtained based on the other internal standard [monitoring of ions of *m/e* 365 and 368 (internal standard)] was 0.13 μg I per 20 ml.

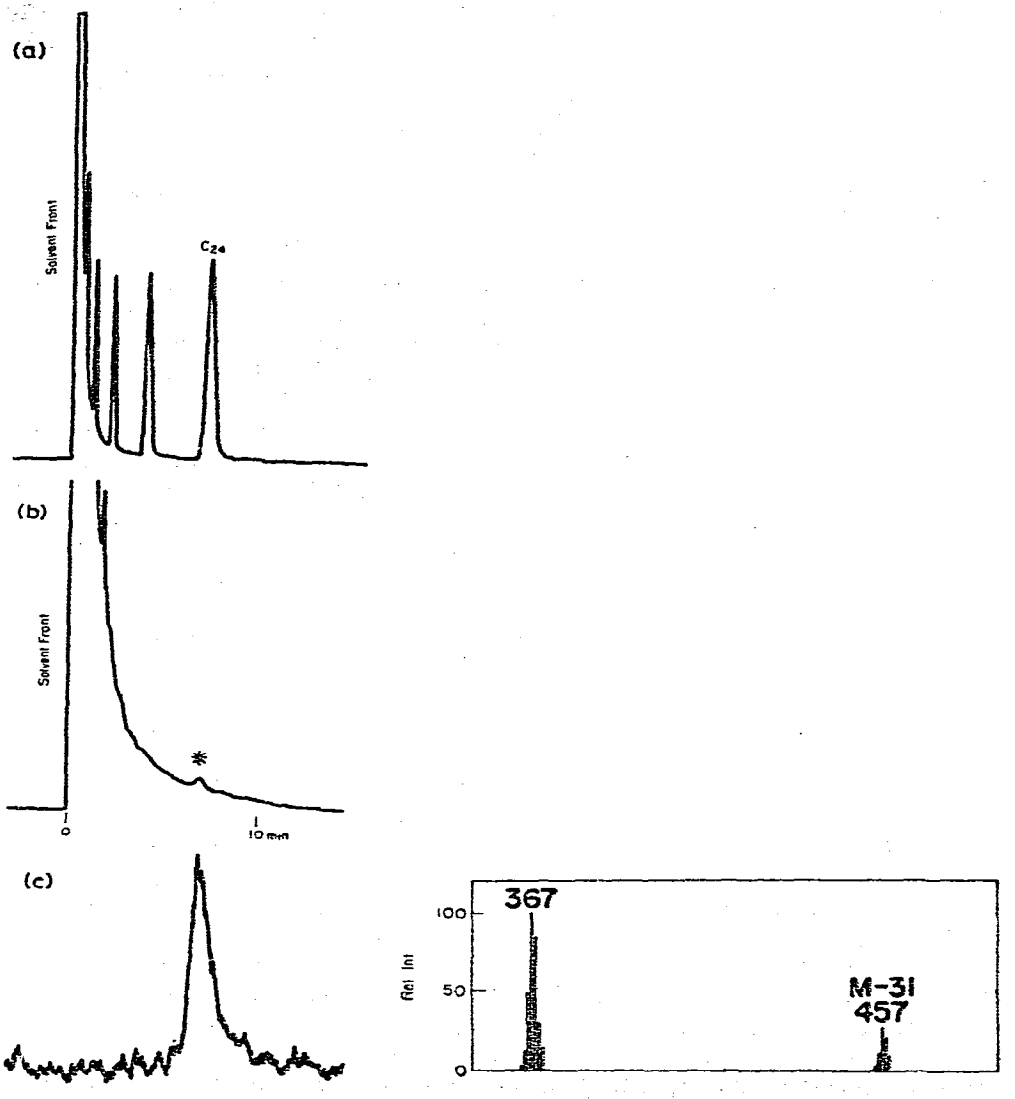


Fig. 2. (a) Gas chromatogram resulting from analysis of mixture of long-chain fatty acid methyl esters (C_{16}—C_{24}). Column conditions: 6 ft. × 4 mm I.D. glass U-tube packed with 1.0% OV-1 dimethylpolysiloxane stationary phase on 80—100 mesh acid-washed and silanized Gas-Chrom P, 220°; carrier gas flow-rate, 60 ml/min. (b) and (c) Simultaneous flame ionization and radioacitivity monitor detection records resulting from analysis of derivatized (bis-methyloxime,bis-methyl ester, TMS ether) rabbit metabolite of $^{2}H_{x}{}^{3}H$-labeled 15-keto-PGE_{2}. Column conditions as for (a).

Fig. 3. Partial mass spectrum of the radioactive compound described in Fig. 2c.

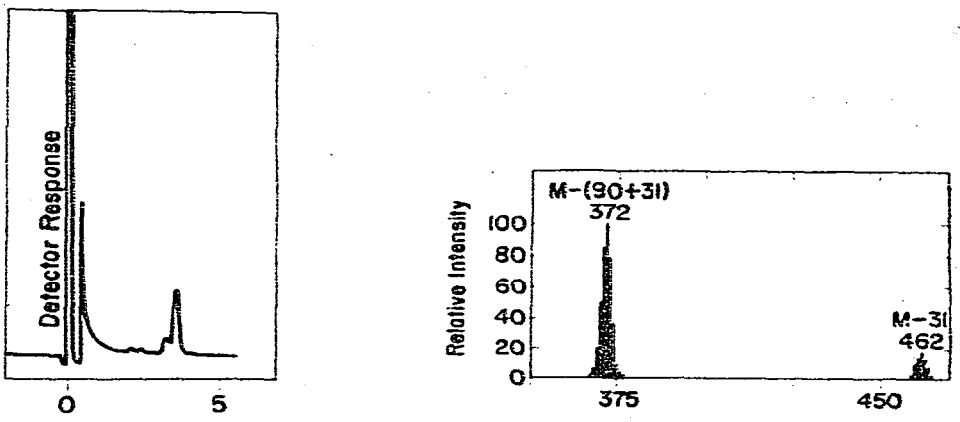


Fig. 4. Gas chromatogram resulting from GLC—MS analysis of synthetic ^{2}H, ^{3}H-labeled I (bis-methyloxime,bis-methyl ester, TMS ether). Column conditions: 5 ft. × 3 mm I.D. spiral glass tube packed with 1.5% SE-30 dimethyl polysiloxane stationary phase on acid-washed and silanized Gas-Chrom P, 218°; carrier gas flow-rate, 30 ml/min.

Fig. 5. Partial mass spectrum of the major component in the sample described in Fig. 4.

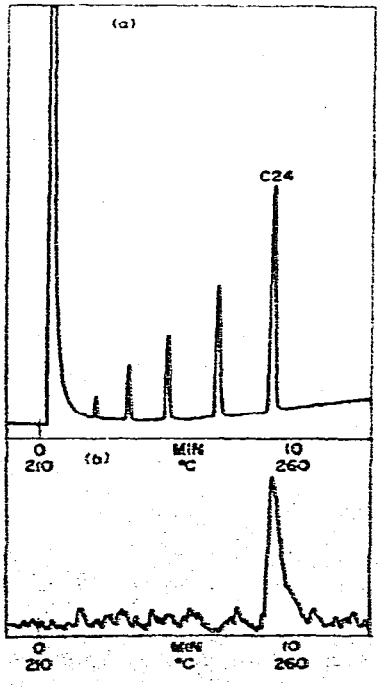


Fig. 6. (a) Gas chromatogram (flame ionization detection) resulting from analysis of mixture of long-chain fatty acid methyl esters (C_{16}—C_{24}). Column as described in Fig. 2; temperature programmed from 210° to 260° at 5°/min. (b) Radioactivity monitoring detection record resulting from analysis of a trimethylsilylated TLC zone eluate from a urine sample spiked with the synthetic ^{2}H,^{3}H-labeled I and carried through the assay procedure.

TABLE I

URINARY OUTPUT OF 7α-HYDROXY-5,11-DIKETOTETRANORPROSTANE-1,16-DIOIC ACID IN SUBJECTS TREATED WITH DIFLUNISAL*

Subject	Urinary output (μg per 24 h)		
	Control	2nd Day on run	5th Day on run
A	6.5	3.0	3.9
B	21.2	4.7	2.9
C	12.6	3.6	3.5

*375 mg twice a day.

The synthetically prepared $^{2}H,^{3}H$-I internal standard has been employed in a study to demonstrate the effect of the new analgesic diflunisal (2′,4′-difluoro-4-hydroxy-1′,1-diphenyl-3-carboxylic acid) upon the urinary excretion of I in normal male subjects [14]. The data in Table I demonstrate that administration of this drug resulted in a marked reduction in urinary output of the PG metabolite, presumably reflecting inhibition of PGE_1 and PGE_2 biosynthesis.

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