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Note

11-Dehydrothromboxane B_2: convenient preparation of its stable isotope-labelled analogue for use in quantitative gas chromatography–mass spectrometry

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11-Dehydrothromboxane B_2 is one of the major enzymic metabolites of thromboxane B_2 (TXB_2), the hydrolysis product of its biologically active precursor, thromboxane A_2 [1–3]. Although the half-life of TXB_2 would be sufficient to reflect thromboxane production in vivo, sampling-related artefacts produced by platelet activation make this metabolite inappropriate for use as an index of systemic thromboxane biosynthesis [4]. Measurement of enzymic metabolites minimizes this problem; therefore, 11-dehydro-TXB_2 was found to be a useful analytical target to detect phasic release of TXA_2 in the human circulation, as might occur in syndromes of platelet activation. Radioimmunoassay [5] and negative-ion chemical ionization (NICI) gas chromatography–mass spectrometry (GC–MS) [2,6–8] have been employed to detect the extremely low concentrations of 11-dehydro-TXB_2 (ca. 1–2 pg/ml plasma) in plasma and urine. A crucial requirement for GC–MS assays is the availability of a stable isotope-labelled analogue of the target compound. Recent papers [2,6–8] describe chemical synthesis of a deuterated analogue by oxidation of deuterated TXB_2. The expensive starting material and relatively harsh conditions of preparation make a convenient access to a stable isotope-labelled standard desirable. This paper describes the preparation of ^{18}O-labelled 11-dehydrothromboxane, based on previously elaborated methods for the labelling of prostanoids and drugs [9–14].

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EXPERIMENTAL

Reagents

11-Dehydrothromboxane B_2 was purchased from Cayman (Ann Arbor, MI, U.S.A.). $H_2^{18}O$ was obtained from Ventron (Karlsruhe, F.R.G.). Pentafluorobenzyl (PFB) bromide was supplied by Suppelco (Crans, Switzerland). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and all solvents of silylation grade were purchased from Pierce (Rockford, IL, U.S.A.). All other reagents and solvents of analytical grade were obtained from Merck (Darmstadt, F.R.G.).

Gas chromatography–mass spectrometry

A Finnigan 9610 gas chromatograph coupled to a Finnigan 4500 mass spectrometer was used. The chromatograph was equipped with a DB-5 fused-silica capillary column (25 m×0.25 mm I.D.) from J&W Scientific (Rancho Cordova, CA, U.S.A.), which was directly connected to the ion source of the mass spectrometer. The splitless Grob injector was kept at 280°C. The column was kept at 150°C for 1 min, then programmed to 300°C at increase of 25°C/min and maintained at this temperature until elution was complete. Helium was used as a carrier gas. NICI spectra were recorded with an electron energy of 120 eV and an emission current of 0.2 A. Methane was used as a moderating gas at an ion source pressure of $2.5 \cdot 10^{-5}$ Torr.

^{18}O Labelling of 11-dehydro-TXB_2

11-Dehydro-TXB_2 (300 μg) was converted into its methyl ester by reaction with ethereal diazomethane for 20 min at room temperature. After evaporation of the solvent, the dry residue was treated with 50 μl of $Li^{18}OH$ in $H_2^{18}O$ (0.2 *M*, prepared by dissolving the appropriate amount of lithium in $H_2^{18}O$). After vigorous shaking for 1 h, the mixture was acidified to pH 3.2 by addition of 0.5% formic acid and extracted twice with 3 ml of ethyl acetate–diethyl ether (1:1, v/v). The solvent was removed under a stream of nitrogen and the residue again esterified with diazomethane. Cycles of esterification and hydrolysis were repeated, until no unlabelled species was detectable by NICI GC–MS.

Derivatization

11-Dehydro-TXB_2 was converted into its PFB ester by reaction with 50 μl of a solution of PFB bromide in acetonitrile (7%) and 10 μl of diisopropylethylamine at room temperature for 10 min. Silylation was accomplished by addition of 50 μl BSTFA–pyridine (2:1) and heating to 60°C for 20 min. After removal of solvent and excess reagent under a stream of nitrogen, the derivative was reconstituted in 50 μl of *n*-hexane and an aliquot was injected into the GC–MS system.

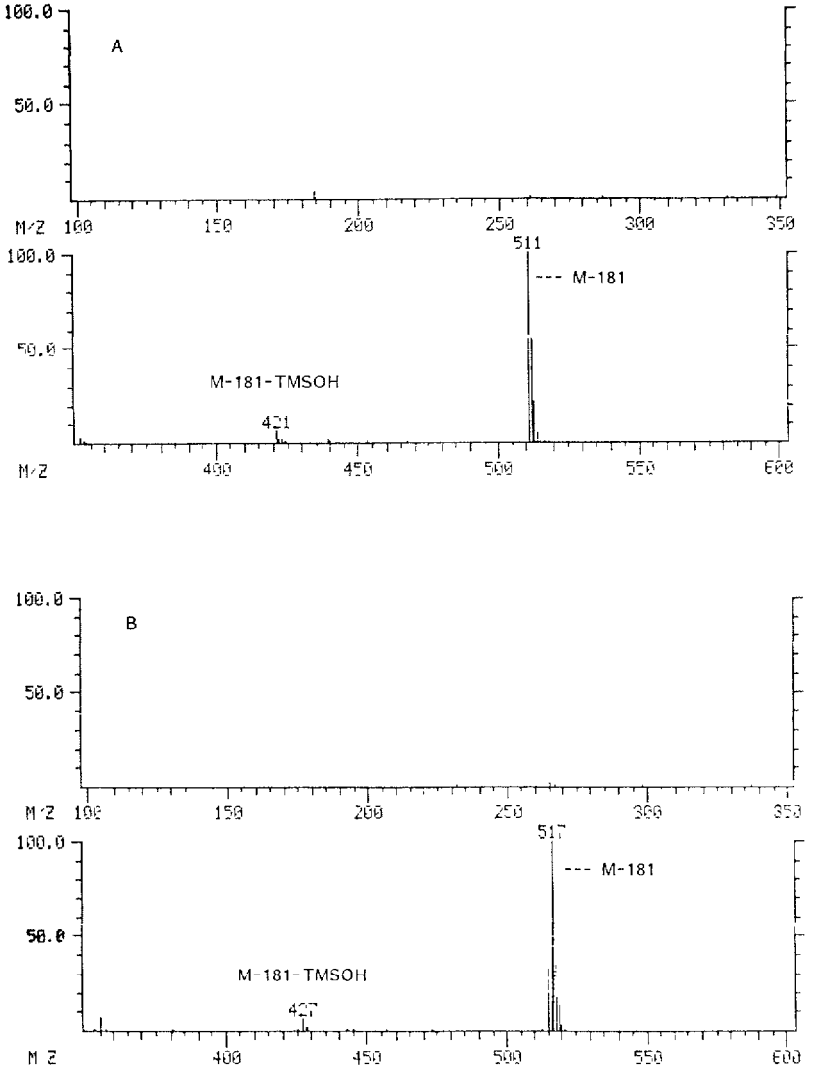

Fig. 1. NICI mass spectra of (a) native and (b) ^{18}O-labelled 11-dehydrothromboxane B_2 pentafluorobenzyl ester trimethylsilyl ether.

RESULTS AND DISCUSSION

The NICI mass spectra of 11-dehydro-TXB_2 in its native form and after five cycles of esterification and hydrolysis in $H_2^{18}O$ are shown in Fig. 1. Owing to the lactone structure, a maximum incorporation of three ^{18}O atoms can theo-

retically be achieved. Two of the labelled oxygen atoms should be positioned in the carboxylic acid group, since repetitive esterification and hydrolysis of the methyl ester must result in exchange of both oxygen atoms. Repetitive base-catalysed hydrolysis of the lactone ring yields a doubly labelled carboxylate anion in the same manner, but during acid-catalysed lactonization one of the labelled oxygen atoms is eliminated by the non-labelled alcoholic group on mechanistic grounds. The third labelled oxygen must therefore be the carbonyl oxygen of the lactone ring. These findings are in agreement with the electron-impact mass spectra of the methyl ester derivatives of both the labelled and the native compound (data not shown).

Only insignificant amounts (0.03%) of unlabelled species were detectable at this time, as indicated in the mass chromatogram in Fig. 2. The percentage of $m+6$ from the total amounts of isotopes formed was estimated at 39%. This is highly advantageous in trace level determinations, since the actual amount of internal standard is considerably higher than the amount of quantitation isotope. Thus it can act as a carrier throughout the assay procedure, thereby enhancing both the sensitivity and the product recovery.

To assess the usefulness of the labelled analogue for quantitative determination in biological media, back-exchange studies have been performed to verify the stability of the isotopic label in aqueous media. The labelled standard was added to 1 ml each of 0.5% formic acid, plasma and plasma containing 0.5% formic acid. Aliquots were taken after 1, 2, 3 and 4 h at room temperature and analysed after derivatization. It was observed that virtually no exchange occurred in formic acid and acidified plasma, whereas in authentic plasma the

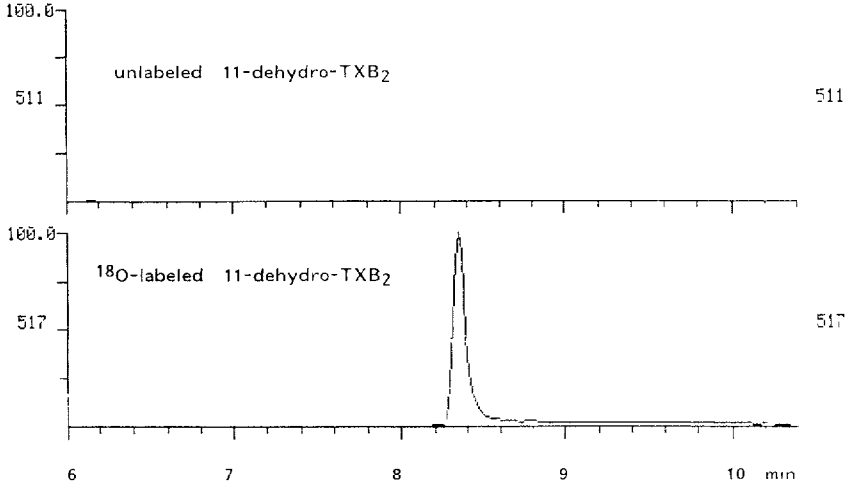

Fig. 2. Partial mass chromatogram obtained after analysis of 11-dehydrothromboxane B_2 pentafluorobenzyl ester trimethylsilyl ether after five cycles of esterification and hydrolysis in $H_2^{18}O$.

proportion of label decreased rapidly. This can be attributed to the action of esterase enzymes, which are denaturated by acidification. As mentioned above, there is a pH-dependent equilibrium between the lactone and acidic forms of 11-dehydro-TXB_2. At physiological pH, the compound exists in the acidic form, whereas the isotope-labelled standard is added as the lactone. Therefore, biological samples are left for 1 h at room temperature after acidification and addition of the standard to assure equilibration [6]. This does not affect the use of the labelled analogue for quantitative measurements, since there is no back-exchange within the equilibration time. These results confirm the applicability of the labelled standard for measurements in biological media, as long as the compound is added after acidification of the sample.

The procedure described in this paper provides a rapid and convenient route to a stable isotope-labelled standard of 11-dehydro-TXB_2 with a high degree of label, which should be useful in determination of the compound.

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