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Note

Simultaneous analysis of methyl methacrylate and methacrylic acid in blood by double isotope derivative dilution analysis

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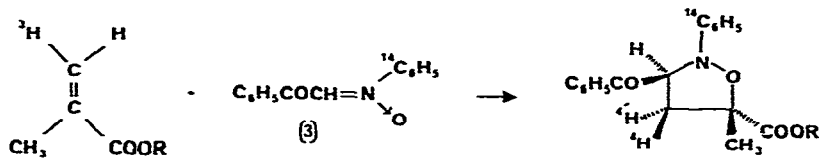
Polymethylmethacrylate is widely used in orthopaedic surgery for the fixation of prostheses [1—4]. It has been used with particular success in total hip replacement, an operation that is being performed on an ever-increasing scale. Suggestions that the entry of methyl methacrylate into the blood circulation was responsible for the frequent and occasionally irreversible hypotensive episodes during operations for total hip replacement [3, 5—9] made it imperative that a method for the analysis of methyl methacrylate should be developed. We had shown that in blood in vitro, methyl methacrylate is hydrolysed enzymatically to methacrylic acid [10]. Since methacrylic acid has been reported in animal experiments to exert cardiovascular effects similar to those of methyl methacrylate [11] it was necessary to be able to determine levels of methacrylic acid as well as those of the methyl ester.

The rapid enzymatic hydrolysis of methyl methacrylate explains the wide discrepancies in published estimations of methyl methacrylate levels in blood samples from patients undergoing total hip replacement, when analysis was carried out by gas chromatography at unspecified times after withdrawal of the blood sample from the patient [12—16]. In order to circumvent this problem a procedure was devised whereby a mixture of methyl-(*E*)-[3-^{3}H]methacrylate (1) or the corresponding acid (2) was added to the blood sample immediately



(1) R = CH_3
(2) R = H

(4) R = CH_3
(5) R = H

eqn.1

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after withdrawal from the patient. The methacrylate, diluted by endogenous material, was re-extracted into halothane and treated with N-[U-^{14}C]phenyl-C-benzoylnitrone (3) to give the stable isoxazolidines (4) and (5) (eqn. 1). The reaction mixture was separated by high-performance liquid chromatography (HPLC) on a column of μBondapak C_{18} (Fig. 1), the individual derivatives (4) and (5) were collected and their $^{3}H/^{14}C$ ratios were determined by liquid scintillation counting. Concentrations of methyl methacrylate and methacrylic acid were calculated by comparing these isotope ratios with those in the adducts derived from reaction between undiluted methyl-(*E*)-[3-^{3}H]methacrylate (1) or (*E*)-[3-^{3}H]methacrylic acid (2) and the ^{14}C-labelled nitrone (3).

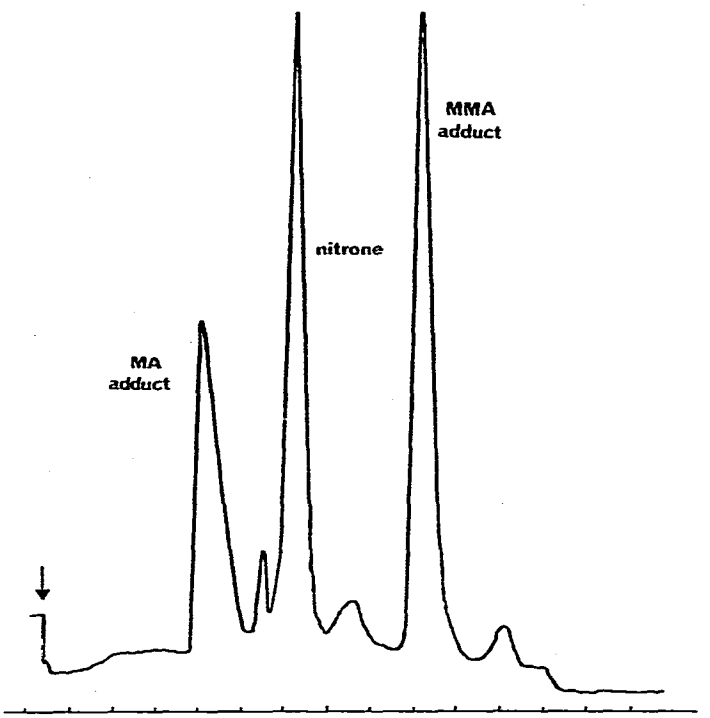

Fig. 1. High-performance liquid chromatogram of the product obtained by reacting methyl-methacrylate and methacrylic acid with N-phenyl-C-benzoylnitrone. For conditions, see under Experimental. MA adduct: derivative formed with methacrylic acid; MMA adduct: derivative formed with methyl methacrylate. The arrow indicates the point of injection. Detection of the adducts was made by monitoring their absorbance at 254 nm.

The required labelled methacrylic acid (2) was obtained using a procedure we have developed for the stereospecific synthesis of tritiated acrylates [17]. Conversion into the methyl ester was carried out using the method of Shaw et al. [18]. The ^{14}C-labelled nitrone was obtained by a modification of published procedures [19].

The results obtained by the application of these methods to the analysis of blood samples from patients undergoing total hip replacement have been described [20].

EXPERIMENTAL

Proton nuclear magnetic resonance (nmr) spectra were determined with a JEOL MH100 spectrometer and infrared spectra with a Hilger H900 Infrascan or Perkin-Elmer 357 Grating infrared spectrometer using KBr discs. HPLC was performed on a Waters Assoc. liquid chromatograph equipped with a Model 6000 solvent delivery system, fixed-wavelength (254 nm) UV detector and septum injector. Radio-activity was measured using a Packard Tri-Carb 2002 liquid scintillation counter, using solutions in Aquasol (New England Nuclear, Boston, MA, U.S.A.) or NEN 260 (Nuclear Enterprises Ltd., Edinburgh, Great Britain). All radiochemicals were purchased from The Radiochemical Centre, Amersham, Great Britain.

N-Phenyl-C-benzoylnitrone [as (3)]

The unlabelled material was prepared according to the published procedure [19].

N-[U-^{14}C]Phenyl-C-benzoylnitrone (3)

[U-^{14}C]Aniline. [U-^{14}C]Anilinium hydrogen sulphate (0.17 mg, 0.89 μmol, 50 μCi) and anilinium hydrogen sulphate (2.1 mg, 11 μmol) were dissolved in dilute ammonia (0.02 *M*, 5 cm^3). The solution was extracted with halothane (2 × 3 cm^3). The solvent was removed on a rotary evaporator at ambient temperature and the residue was dissolved in ethanol (1 cm^3).

[U-^{14}C]Nitrosobenzene. The ethanolic solution of [U-^{14}C]aniline was cooled to 6°C and treated with *m*-chloroperbenzoic acid (4.4 mg, 22 μmol). The mixture was stirred for 10 min, then transferred to a cooled (0°C) column (250 × 6 mm I.D.) containing basic alumina (10 g). The nitrosobenzene was eluted with ethanol (2 cm^3) and to the solution was added a solution of inactive nitrosobenzene (1 mg, 10 μmol) in ice-cooled ethanol (0.14 cm^3).

N-[U-^{14}C]Phenyl-C-benzoylnitrone (3). The ethanolic solution of [U-^{14}C]-nitrosobenzene was cooled to −5°C and added to a solution of N-phenacylpyridinium bromide (5 mg, 18 μmol) in water (0.5 cm^3) at 0°C. The mixture was cooled to −5°C and treated with aliquots (10 μl) of sodium hydroxide solution (0.125 *M*) every 2 min (total: 120 μl). The solution was purified by HPLC on a column of phenyl Porasil B (37—75 μm) (102 cm × 6 mm I.D.) in methanol–water (1:1). The fractions containing the labelled nitrone were combined and the solution (32 cm^3) was treated with inactive nitrone (20 mg, 86 μmol) in methanol (10 cm^3). The solution was extracted with halothane (3 × 10 cm^3), then the combined extracts were concentrated to 8 cm^3 under reduced pressure and dried ($MgSO_4$) at 0°C. The solution was filtered and evaporated, and the residue was dried over P_2O_5 under reduced pressure. The residue was dissolved in dry methanol (3 cm^3) and stored in nine equal aliquots in sealed ampoules at liquid nitrogen temperature until required. The purity of one aliquot was confirmed by HPLC as described above. Each aliquot contained 2.08 mg (estimated by UV) of the [^{14}C]nitrone (3), specific activity 57 μCi mmol^{-1}.

5-Methyl-2-phenyl-3-benzoylisoxazolidine-5-carboxylic acid (5)

Methacrylic acid [as (2)] (0.86 g, 10 mmol) was added to a solution of

N-phenyl-C-benzoylnitrone [as (3)] (2.23 g, 10 mmol) in ethanol (25 cm^3). The mixture was heated at 50°C for 90 min. The solution was cooled and the crystalline product was recrystallised (ethanol) to give the derivative [as (5)] (2.88 g, 93%) m.p. 131—132°C. ν_{max} 1702 (COOH) and 1680 (CO) cm^{-1}. τ ($C^2H_3O^2H/C^2H_3COC^2H_3$) 4.55 (dd, 1H, *J* 6 Hz, 8 Hz, H-3), 6.72 (dd, 1H, *J* 8 Hz*, 10 Hz*, H-4), 7.44 (dd, 1H, *J* 6 Hz, 10 Hz, H-4'), 1.96–3.4 (m, 10H, C_6H_5N, C_6H_5CO), 8.39 (s, 3H, CH_3C). Found: C, 69.85; H, 5.5: N, 4.5. $C_{18}H_{17}NO_4$ requires: C, 69.45; H, 5.5; N, 4.5%.

(E)-[3-^{3}H] Methacrylic acid (2)

To a slowly stirred solution of (*E*)-3-bromo-2-methylpropenoic acid (0.27 g) in ^3H_2O (1 g, 5 Ci g^{-1}) at 5°C was added, in aliquots over a period of 48 h, sodium amalgam (2.5%, 5.5 g). Following the final addition of sodium amalgam, the mixture was stirred for a further 25 h at 0—5°C. The aqueous solution was decanted, the mercury was washed with water (3 × 1 cm^3) and the combined aqueous solutions were acidified (congo red) with dilute hydrochloric acid. The solution was extracted with ether (3 × 3 cm^3); the ethereal solution was dried ($MgSO_4$), filtered and evaporated. The residue was dissolved in ethanol and the yield was determined by UV (24 mg, 20%, specific activity approximately 1.5 Ci g^{-1}).

Methyl [3-$^3H_1$] methacrylate (methyl-(E)-[3-^{3}H]-2-methylpropenoate) (1)

[3-^{3}H] Methacrylic acid (0.17 g, 975 μCi g^{-1}) in hexamethylphosphoramide (5 cm^3) was treated with sodium hydroxide solution (25%, 0.35 cm^3). The mixture was stirred for 90 min, treated with iodomethane (1.14 g), stirred for 2 h at 25°C, treated with dilute hydrochloric acid (5%, 10 cm^3) and extracted with diethyl ether (2 × 7.5 cm^3). Hydroquinone (1 mg) was added to the combined ethereal extracts which were washed with water (2 × 2.5 cm^3), boiled under reflux with mercury (1 cm^3) and anhydrous magnesium sulphate for 1 h and allowed to stand for 12 h. The ethereal solution was decanted, filtered and evaporated to give methyl-(*E*)-[3-^{3}H] methacrylate (0.1 g, 50%), pure by gas—liquid chromatography (15% SE-30 on Chromosorb W, 70°C).

Simultaneous analysis of methyl methacrylate and methacrylic acid in blood

Blood samples (2 cm^3) were added to centrifuge tubes containing known amounts of tritiated methyl methacrylate and methacrylic acid. The mixture was homogenised using a vortex mixer, treated with citrate buffer (pH 3.0, 1 *M*, pH 3.0) re-homogenised and extracted with halothane (2 cm^3) on the vortex mixer. The mixture was centrifuged (4000 *g* for 10 min), and the serum and erythrocytes were aspirated off from the halothane solution. To the halothane solution was added a solution of N-[U-^{14}C] phenyl-C-benzoylnitrone (3) (20 μg) in ethanol (40 μl) and the mixture was heated at 50°C for 1 h. The solvent was removed by a stream of nitrogen at room temperature; the residue was dissolved in ethanol (100 μl) and injected on to a column of μBondapak C_{18} (10 μm) (Waters Assoc., Milford, MA, U.S.A.) (300 mm × 4 mm I.D.). The

*Presumed coupling constants. The signal due to H-4 was partly obscured by the signal due to solvent C^2H_3OH.

derivatives were separated (Fig. 1) by elution with acetonitrile—water (43.5: 56.5). The derivatives were collected in 2 cm^3 of eluent and added to 12 cm^3 of liquid scintillation fluid for radioactivity determination. Amounts (m) of unlabelled material present in the blood sample were calculated from the expression

$$m = \frac{m'(r_u - r_d)}{r_d}$$

where m' = amount of labelled methacrylate added, r_u = $^3H/^{14}C$ ratio in the isoxazolidine derived from undiluted [^{3}H]methacrylate and [^{14}C]nitrone, and r_d = $^3H/^{14}C$ ratio in the isoxazolidine formed between the diluted methacrylate from the blood sample and ^{14}C-labelled nitrone.

The sensitivity of this method is limited by the specific activities of the labelled compounds used in the analysis. In this study, the methacrylate used had a specific activity of $1.5 \cdot 10^5$ μCi $mmol^{-1}$ and the N-phenyl-C-benzoylnitrone had a specific activity of 57 μCi $mmol^{-1}$. The latter therefore limited the sensitivity of the method to 0.1 μg of methacrylate. With 100% recovery, this would give a total ^{14}C activity in the final sample of 126 disintegrations per minute (dpm), but since the recoveries obtained were less than this, final counts were of the order of 50 dpm (40 counts per min) which, being approximately twice the background count rate, were taken as the lowest acceptable level. Since 2-cm^3 blood samples were used in the clinical studies, the specific activity of the tritiated methacrylate used put an effective limit of sensitivity on the procedure of 0.05 μg (per cm^3 of blood) for both methyl methacrylate and methacrylic acid. The size of the aliquots of tritiated methacrylate also limited the sensitivity to about the same value. Aliquots of approximately 0.75 $\mu g/cm^3$ of blood were used. If this were diluted with 0.05 μg of endogenous methacrylate, the change in specific activity of the labelled methacrylate would be 7%, which was considered to be the level of accuracy of the final counting procedures (± 5%, approximately).

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