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Note

Quantitation of ibuprofen in biological fluids by gas chromatography—mass spectrometry

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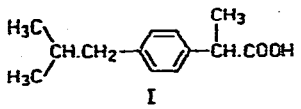
The binding of drugs to serum and tissue proteins is known to be an important determinant of their disposition kinetics and pharmacodynamics [1—5]. Consequently, there is an increasing awareness of the need to measure free drug concentrations in patient serum. This provides a more reliable approach to dosage adjustment and subsequent modulation of clinical effect [6—8]. If a drug is strongly and extensively (say >99%) bound to serum proteins and the total drug concentration achieved is in the low μg ml^{-1} range, then the assay of the free drug requires extremely sensitive and precise techniques. This is particularly so where the amount of biological material available for assay is small, for example, serum ultrafiltrate and synovial fluid.



I

Ibuprofen (I), reported in 1967 [9], is widely used in the treatment of rheumatoid arthritis and osteoarthroses. In vitro binding of the drug to whole human plasma [10] and purified albumins [11] has been determined to be in excess of 99% by scintillation counting of [^{14}C]ibuprofen, a technique unsuitable for in vivo estimations in man.

Kaiser and Vangiessen [12] have reported a gas—liquid chromatographic (GLC) determination of the drug in plasma, by extraction into benzene and subsequent analysis of the methyl ester derivative (MeIb). Also, Hoffman [13] has developed a simpler GLC assay for the underivatized drug utilizing a 5% FFAP stationary phase on Gas-Chrom W HP, 80—100 mesh. Whilst both assays show equal and adequate sensitivity (lower limit of detection, 0.5 μg

ml^{-1} plasma) for the determination of total serum ibuprofen in the normal therapeutic concentration range (approx. 20 μg ml^{-1}) they are grossly inadequate for the measurement of the much lower corresponding free drug concentrations.

We have developed and report here a method for the assay of ibuprofen in biological fluids which provides a 500-fold improvement in sensitivity with greater precision than previously published assays. This assay has been used successfully to determine free concentrations of ibuprofen in whole serum and synovial fluid samples, taken simultaneously from rheumatoid patients, as part of a study to examine distribution of the drug between these two regions. Details of this clinical study will be published elsewhere.

EXPERIMENTAL

Materials

Ibuprofen, 2-(4-isobutylphenyl)propionic acid, was a gift from The Boots Pure Drug Co. (Nottingham, Great Britain). Fresh batches of distilled ethereal diazomethane were prepared immediately before use from N-methyl-N-nitroso-*p*-toluene sulphonamide (Merck-Schuchardt, Munich, G.F.R.). Deuterated ibuprofen for use as the internal standard was synthesized as described below. All solvents were redistilled before use.

Internal standard

To a vigorously stirred mixture of ibuprofen (20 mg), dichloromethane (5 ml) and aluminium chloride (60 mg), ^2H_2O (0.04 ml) was added dropwise over 5 min. The reaction mixture was stirred for 30 min at room temperature and then poured onto ice (^2H_2O). The product was extracted with diethyl ether (2 × 5 ml) and the combined extracts washed with water (5 ml), dried (Na_2SO_4) and evaporated to yield pale brown crystals of deuterated ibuprofen. This material was redissolved in dichloromethane and the aluminium chloride/^2H_2O exchange reaction was repeated.

A portion of the product from the second exchange reaction was methylated with diazomethane and analysis by charge exchange mass spectrometry showed the deuterium content of the ibuprofen to be: d_0 (0.5%), d_1 (6.5%), d_2 (26.4%) d_3 (38.5%), d_4 (25.2%) and d_5 (2.9%). The mass spectrum also showed that deuterium incorporation had taken place almost exclusively on the aromatic ring.

Gas chromatographic—mass spectrometric (GC—MS) analysis of the methylated product, under the conditions given below, showed the presence of two isomeric compounds. The major isomer (retention time 1.75 min) was shown to be the methyl ester of deuterated ibuprofen and the minor isomer (retention time 1.4 min) was the methyl ester of 2-(4-*sec*.-butylphenyl)-propionic acid. These two isomers may be easily distinguished by their charge exchange mass spectra as the characteristic benzylic cleavage of the alkyl side-chain produces a different product in each case (Fig. 1). Since the two isomers were well resolved by the GC column no attempt was made to remove the minor isomeric impurity.

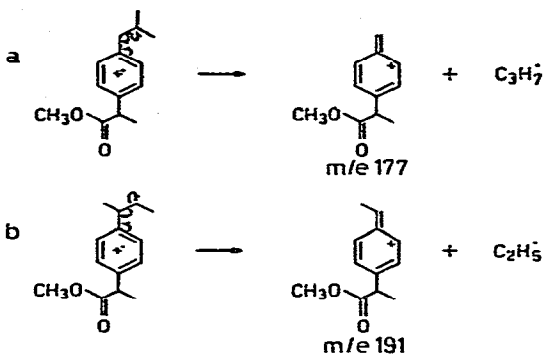


Fig. 1. Charge exchange-induced benzylic cleavage of the methyl esters of (a) 2-(4-isobutylphenyl)propionic acid and (b) 2-(4-*sec.*-butylphenyl)propionic acid.

Gas chromatography—mass spectrometry

GC—MS analyses were carried out on a Finnigan 3200 chemical ionization mass spectrometer interfaced to a Finnigan 9500 gas chromatograph. The glass GLC column (0.91 m × 2 mm I.D.) was packed with 3% OV-17 on Chromosorb W AW DMCS, 120—140 mesh. The column was operated at 150° and the injection port and GC—MS interface were maintained at 260°. Methane was used as the GC carrier gas and chemical ionization reactant gas. A flow-rate of 20 ml min^{-1} generated an ion source pressure of 130 Pa. Charge exchange mass spectra were generated by using helium in place of methane (flow-rate 20 ml min^{-1}, source pressure 55 Pa). An electron beam energy of 110 eV was used to generate chemical ionization mass spectra. The ion source and analyser regions of the mass spectrometer were operated at 60—100°. A Finnigan 6110 interactive data system was used to control the mass spectrometer during selected ion monitoring and to calculate the heights of peaks in selected ion chromatograms.

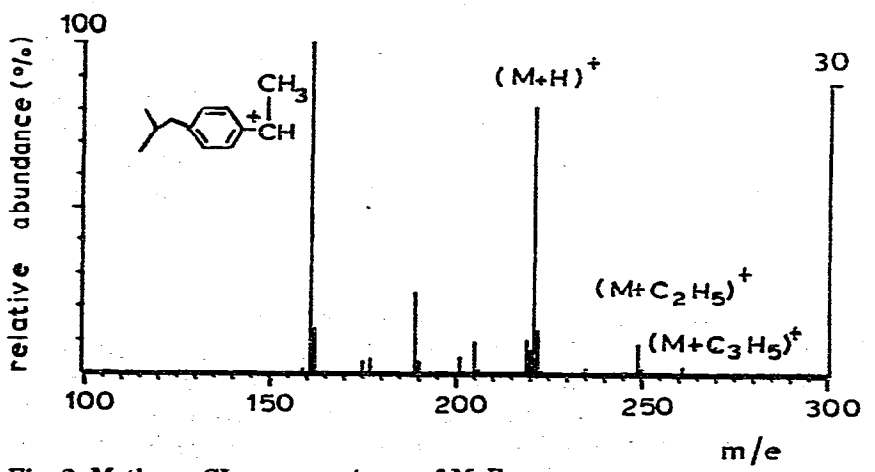


Fig. 2. Methane CI mass spectrum of MeIb.

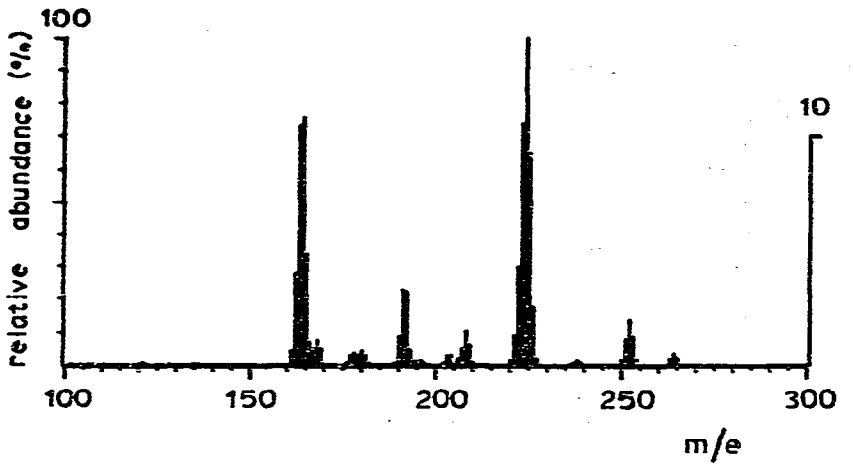


Fig. 3. Methane CI mass spectrum of deuterated MeIb.

Ibuprofen was analysed by monitoring the major (M+1) ion peak of its methyl ester derivative at *m/e* 221 (Fig. 2) and the (M+1) ion peak of the [$^2H_3$]ibuprofen internal standard at *m/e* 224 (Fig. 3).

Extraction procedure

Blood and synovial fluid, from rheumatoid patients on steady state ibuprofen therapy, were collected into 10-ml plain tubes. Clotted blood was centrifuged at 1000 *g* and ambient temperature for 15 min. An aliquot of recovered serum was equilibrated at 37° and ultrafiltered through a Pellicon PTGC series membrane, retention molecular weight 10,000 (Millipore, Bedford, Mass, U.S.A.), and approx. 0.2 ml of ultrafiltrate collected for subsequent free drug determination. Whole serum, serum ultrafiltrate and synovial fluid were stored at —20° until required for assay. The biological fluid (0.2 ml, made up to this volume if necessary with water) to be assayed was placed in a centrifuge tube and 0.2 ml of deuterated ibuprofen in 0.01 *M* NaOH (equivalent to either 50 or 1000 ng [$^2H_3$]ibuprofen) was added. The solution was acidified with 1 *M* HCl (0.5 ml), mixed thoroughly and extracted with diethyl ether (3 × 5 ml). The organic phase was concentrated on a water bath at 40° to approximately 10 μl. The extract was methylated with freshly prepared diazomethane (0.4 ml) in diethyl ether and the reaction allowed to go to completion (30 min). The sample volume was reduced to <10 μl on the water bath and the whole sample injected into the GC—MS system.

Calibration standards

Two standard curves were constructed to cover the drug concentration ranges expected in the samples (1—250 ng and 250—2000 ng). Aliquots of methanol containing known amounts of ibuprofen were evaporated to dryness in centrifuge tubes using nitrogen gas and 0.2 ml blank serum added with thorough mixing. These were extracted as described above, together with a blank serum sample to detect any interfering peaks. New standard curves were

obtained on each day samples were to be assayed. Estimates of precision at high and low drug concentration levels were obtained by replicate extraction and assay of both 500 and 1 ng calibration standards.

RESULTS AND DISCUSSION

Peak shapes in the selected ion chromatograms were good with little tailing (Fig. 4a) and therefore the more easily measured peak height ratio of MeIb/[$^2H_3$]MeIb was used for calibration. It is generally considered that the preparation of standard solutions is associated with much greater error than the actual mass spectrometric determinations [14]. For this reason the regression lines were calculated by minimization of the sum of squared deviations of the standards from the fitted line rather than the peak height ratios.

Calibration curve data for the lower range (1—250 ng) show excellent linearity (equation of regression line $y = mx + c$, where $m = 54.19$, $c = -7.41$ and $r = 0.9999$) and 1 ng of MeIb could be detected with a coefficient of variation (C.V.) of 6.8% ($n = 6$). Data for the upper range (250—2000 ng) were equally good ($m = 1055$, $c = -86$ and $r = 0.9998$), and 500 ng of MeIb were detected with a C.V. of 1.43% ($n = 6$).

The non-zero intercepts are a result of the presence of an ion of low abundance at *m/e* 221 in the chemical ionization (CI) mass spectrum of deuterated MeIb (Fig. 3). In this spectrum *m/e* 221 accounts for approximately 3% of the total abundance of the isotopic cluster of ions between *m/e* 221 and 227, a figure significantly higher than the abundance of the d_0-ibuprofen calculated from the charge exchange mass spectrum. The difference arises because the CI mass spectrum contains $(M - H)^+$ and $M^{+\cdot}$ ions in addition to the $(M+H)^+$ ion (Fig. 2). Therefore, in the CI spectrum of deuterated MeIb the

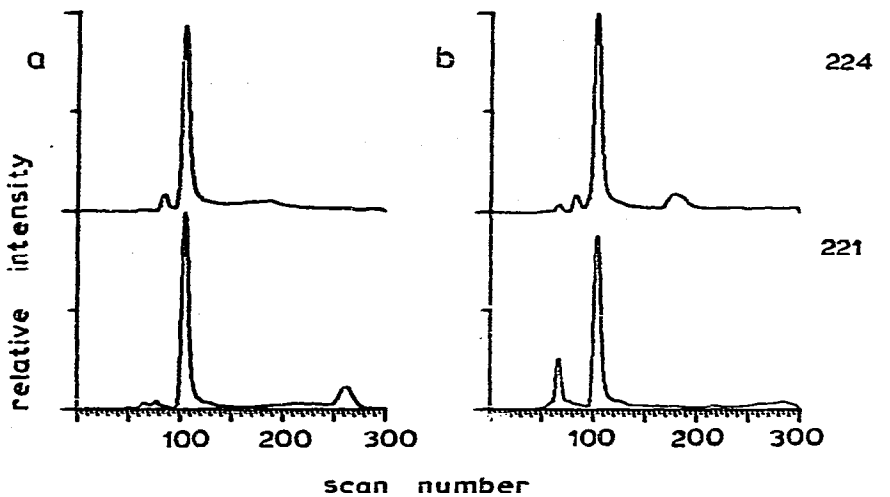


Fig. 4. Selected ion chromatograms *m/e* 221 (MeIb) and 224 ([$^2H_3$]MeIb). (a) Calibration standard (52.6 ng, ibuprofen) extracted from serum; (b) patient (B.C.) administered 400 mg ibuprofen three times daily, for two days prior to sample collection. Sample calculated to contain 42 ng ibuprofen in 130 μl serum ultrafiltrate.

$(M+H)^+$ ion at *m/e* 223 will be accompanied by a $(M-H)^+$ ion at *m/e* 221 and the $(M+H)^+$ ion at *m/e* 222 will be accompanied by an $M^{+\cdot}$ ion also at *m/e* 221. The situation is further complicated since the molecule contains both hydrogen and deuterium atoms, there is the possibility that the $(M+H)^+$ ion at *m/e* 224 will be accompanied by an $(M-D)^+$ ion again at *m/e* 221. However, as cleavage of C—H bonds is considerably more facile than cleavage of C—D bonds, $(M-D)^+$ ions will probably be of much lower abundance than $(M-H)^+$ ions.

This method has been successfully used to assay free concentrations of ibuprofen in whole serum and synovial fluid. Fig. 4b is a typical selected ion chromatogram obtained for unbound ibuprofen in whole serum, obtained from a rheumatoid patient (B.C.) on steady state therapy, and represents 42 ng of drug in 130 μl of serum ultrafiltrate.

The estimated detection limit for this assay is in the region of 200 pg and results from increasing interference of foreign peaks rather than from lack of GC—MS sensitivity. Experiments with standard aqueous solutions indicated that ibuprofen can be detected at the 50-pg level (signal-to-noise ratio >10:1) and a more sophisticated extraction technique may make detection at this level possible for serum extracts.

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