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QUANTITATIVE GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF INDOMETHACIN IN BIOLOGICAL FLUIDS

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SUMMARY

A sensitive and specific gas chromatographic method for the analysis of indomethacin has been developed. After extraction of the drug from blood or urine with ethylene dichloride a derivative is formed with pentafluorobenzyl bromide. 5-Fluoro-indomethacin is used as an internal standard. The overall recovery of indomethacin is 85.1 $\pm$ 2.3% and the method can detect 10 ng indomethacin in a 1 ml plasma or urine sample. No interference in the method is seen with some commonly used drugs. The method has been used to measure plasma indomethacin concentrations in 20 patients with rheumatoid arthritis, being treated with 25 mg indomethacin three times daily. The plasma concentration ranged from 168–596 ng/ml.

INTRODUCTION

Indomethacin is an anti-inflammatory drug that is widely used for the treatment of arthritis. A number of quantitative methods for the determination of indomethacin in biological fluids have been described but none have been ideal. The spectrofluorometric technique (Hucker *et al.*[1] and Hvidberg *et al.*[2]) lacks specificity and sensitivity. The radio-isotope methods (Skeith *et al.*[3], Duggan *et al.*[4]) while sensitive, involve the administration of radioactivity to patients and are not suitable for everyday use. Gas–liquid chromatographic (GLC) methods have been previously devised (Ferry *et al.*[5], Helleberg[6]) but accurate analysis is difficult because of the lack of an internal standard. A sensitive and specific mass fragmentographic method has been described[7] but this method demands ready access to a gas chromatograph–mass spectrometer. We have developed a specific and sensitive GLC assay for indomethacin based on the method of assay for warfarin in biological fluids devised by Kaiser and Martin[8], but using the same internal standard as Palmer *et al.*[7].

MATERIALS AND METHODS

A Pye series GCV chromatograph fitted with a nickel 63 electron-capture detector, and a Philips PM8100 potentiometric recorder were used. The detector was

operated at a temperature of 330°, with a purge flow-rate of 11.0 ml/min. The attenuation range used was 128 and the chart speed 5 mm/min. A 1.5 m × 4 mm I.D. coiled glass column was used, packed with 2% Dexsil 300 coated on Chromosorb W HP AW, 100–120 mesh (Perkin-Elmer, Beaconsfield, Great Britain) and conditioned at 360° for 48 h before use. The injector temperature was 360° and the oven temperature 305°. Oxygen-free nitrogen was used as carrier gas at a flow-rate of 45 ml/min.

The identity of the derivatives of indomethacin was checked by injection into a Pye 104 gas chromatograph linked to a VG 7070 mass spectrometer.

Chemicals

The following chemicals were used: ethylene dichloride, acetone, methanol and hexane (all analytical grade reagents), citric acid and sodium hydroxide for preparing Sorensen buffer to pH 5.0 all obtained from BDH, Poole, Great Britain. All organic solvents used were re-distilled twice. Pentafluorobenzyl bromide (Pierce, Rockford, Ill., U.S.A.) stock solution for use was prepared by diluting the supplied material 1:1000 (v/v) in acetone (Aristar grade). The internal standard, 5-fluoro-indomethacin; pure indomethacin, [^{14}C]indomethacin (39 μCi per mg) and O-desmethyl indomethacin were all a kind gift from Merck Sharp & Dohme, Rahway, N.J., U.S.A.).

Procedure

Duplicate samples of 1.0 ml of plasma or urine were pipetted into 20-ml glass test tubes to which had been added 50 μl of internal standard at a concentration of 10 μg/ml (500 ng). The tubes were each vortex mixed for 10 sec and 1 ml of Sorensen buffer pH 5.0 was added, followed by 10 ml of ethylene dichloride. The tubes were then vortex mixed for 2 min followed by centrifugation at 1500 *g* for 30 min.

The top aqueous phase was carefully removed by aspiration and 8 ml of the remaining organic phase transferred to a clean 10-ml screw-capped tube and evaporated to dryness at 45° under oxygen-free nitrogen. The walls of the tubes were then carefully rinsed down using 1 ml of acetone which was then evaporated to dryness. To each tube was added 25 mg of potassium carbonate and 0.5 ml of the stock solution of pentafluorobenzyl bromide and reaction carried out at 60° for 30 min using a water bath. The reaction tubes were carefully agitated every ten minutes during this period. The derivatising agent was then evaporated off under a fine jet of oxygen-free nitrogen at room temperature, and to each tube was added 0.5 ml distilled water and 0.5 ml hexane. The tubes were then mixed on a rotary mixer at a speed of 40 rpm for 10 min. GC was then carried out by injecting 0.5–1.0 μl of the upper hexane layer, into the column.

Standard solutions of indomethacin in plasma or urine were prepared by adding a solution of indomethacin in methanol (0.1 mg/ml) to indomethacin-free plasma or urine, to yield concentrations between 100 and 1000 ng/ml. The recovery of indomethacin from plasma or urine was checked using [^{14}C]indomethacin. The amount of indomethacin was calculated by comparison of the peak heights of indomethacin and 5-fluoro-indomethacin for both samples and standards. Reproducibility was checked by performing an analysis on plasma containing indomethacin at concentrations of 100 and 600 ng/ml. Each analysis was performed ten times. Blood samples were taken from 20 patients (8 men, 12 women) with rheumatoid arthritis who were being treated with 25 mg indomethacin three times daily. The blood samples were taken 4 h after the morning dose of indomethacin.

RESULTS AND DISCUSSION

Fig. 1 shows a typical GLC trace of indomethacin and 5-fluoro-indomethacin extracted from both plasma and urine. Under the conditions described the retention time of 5-fluoro-indomethacin was 2.4 min and of indomethacin 4.2 min. These were the only peaks seen in both extracts.

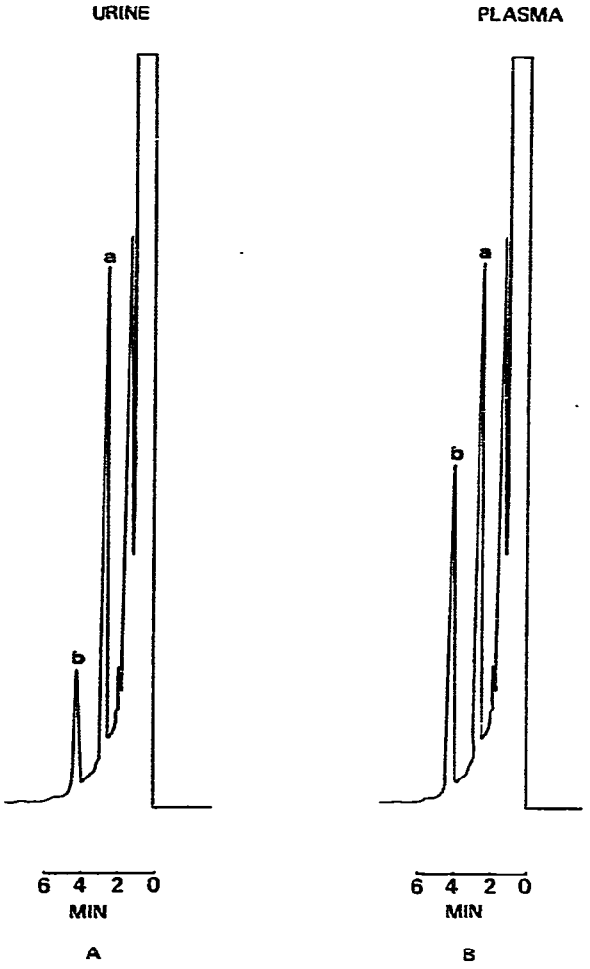

Fig. 1. Chromatographs of 5-fluoro-indomethacin (a) and indomethacin (b). A = Indomethacin, 200 ng/ml in urine; B = indomethacin, 500 ng/ml in plasma.

The mass spectra of these derivatives confirm the introduction of one pentafluorobenzyl group. Both derivatives show a molecular ion ($M^+ = 537$ for the indomethacin derivative and 525 for the internal standard) and the base peak in both spectra is an ion at *m/e* 139 corresponding to cleavage of the *p*-chlorobenzyl group. This fragment is also the base peak of the methyl ester derivatives[7]. The molecular ions of the pentafluorobenzyl derivatives are, however, more intense than those

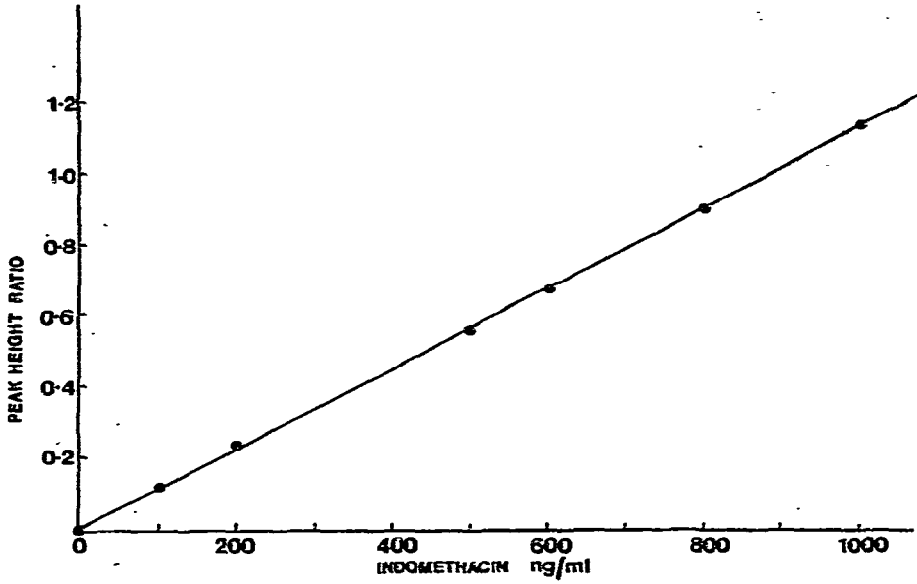

Fig. 2. Calibration curve for the determination of indomethacin in plasma. Each point is the mean of two determinations.

reported for the methyl ester, each being approximately 20% of the base peak intensity.

No other drugs were found to interfere with the assay of indomethacin and the method does not detect O-desmethyl indomethacin. This metabolite of indomethacin was added to plasma in concentrations up to 1000 ng/ml and subsequent analysis by GLC did not produce peaks on the trace obtained.

The following drugs did not produce peaks: paracetamol at a concentration

TABLE I

REPRODUCIBILITY AND ACCURACY

Concentration of indomethacin added to Plasma		*Concentration of indomethacin recovered*	
1	*2*	*1*	*2*
100	600	103	621
100	600	110	586
100	600	105	525
100	600	101	605
100	600	94	616
100	600	99	577
100	600	102	592
100	600	95	618
100	600	98	608
100	600	104	588
	Mean ± S.D.	101.1 ± 4.82	600.6 ± 15.1

of 250 μg/ml, salicylate at concentrations up to 50 μg/ml, nitrazepam, diazepam and chlordiazepoxide at concentrations of 200 ng/ml and hydrochlorothiazide at a concentration of 250 ng/ml.

Probenecid when added to plasma or urine to produce a concentration of 500 μg/ml did produce a peak which appeared just before the peak corresponding to 5-fluoro-indomethacin, however the two compounds were well resolved using a 1.5-m column.

2% Dexsil 300 was chosen as the column phase because of its thermal stability at the high temperatures employed. 3% OV-17, 2% OV-17 and SE-30 were all tried, but the detector suffered a gradual loss of sensitivity, exhibited by a fall in the measurable standing current, caused by deposition of these phases on the detector probe. 2% Dexsil 300 gave no such loss of sensitivity at temperatures up to 360°.

Optimal formation of the pentafluorobenzyl bromide derivatives was found to occur at a reaction temperature of 60° for a period of 30 min.

Experiments were performed at temperatures of 20°, 37°, 50° and 60° with reaction times varying from 5–60 min before the final choice of conditions was made.

Plasma indomethacin concentrations were measured in 20 patients, in the middle of the dosage interval and were found to be between 125 and 620 ng/ml, with a mean value of 359.9 ± 149.5 ng/ml (mean ± S.D.). These concentrations are very similar to those found by Alvan *et al.*[9] in both patients and volunteers taking the same dose of indomethacin.

This method provides a relatively simple, specific and sensitive assay for the measurement of indomethacin concentrations in biological fluids. In contrast to previous gas–liquid chromatographic assays[5,6] the use of an internal standard enables a much easier and more accurate quantitation to be made. The method is as sensitive as the mass fragmentographic method described by Palmer *et al.*[7], is performed on less sophisticated equipment and can handle a greater daily number of samples. Previously described methods, such as the spectro–fluorometric assay[1] lack sensitivity, whilst the method devised by Duggan *et al.*[4] involves the administration of radioactive drug to the patient.

Two recently described methods also have some potential disadvantages. The high-speed liquid chromatographic assay described by Skellern and Salole[10] has a limit of sensitivity of 100 ng/ml. This is close to plasma concentrations seen at the mid-dosage interval in some patients, and would therefore not allow prolonged pharmacokinetic measurements to be undertaken.

The recently described[11] radioimmunoassay is both simple and sensitive, but the antibody is however, more reactive to the glucuronide metabolite of indomethacin, than to indomethacin itself, and in consequence the method lacks specificity.

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