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THREE ALKYLATION METHODS FOR THE DETERMINATION OF INDOMETACIN IN PLASMA BY ELECTRON CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

The following alkylation methods for the determination of indometacin in plasma by electron capture gas chromatography are compared: 1, alkylation with diazopropane; 2, extractive alkylation; 3, alkylation by a solid–liquid phase transfer catalysed process.

The drug in plasma at pH 4.0 is initially extracted with heptane containing 5% *n*-pentanol. The methyl ester of indometacin is used as internal standard. After alkylation to the propyl ester according to one of the three alkylation methods, indometacin can be determined down to 5 ng per sample by electron capture gas chromatography. The relative standard deviations ($n = 10$) at the 200 ng level are 5.1% for the alkylation with diazopropane, 7.5% for the extractive alkylation technique and 3.5% for the alkylation by the solid–liquid phase transfer catalysed process. The comparatively low value obtained by the last method indicates that decomposition of indometacin can be avoided under such mild conditions.

INTRODUCTION

Quantitative determinations of the anti-inflammatory drug indometacin in biological material have been carried out by fluorimetry[1–4], radioisotope techniques[2,5–9], liquid chromatography[9,10], mass fragmentography[11] and gas chromatography (GC)[12–14].

Indometacin is metabolized to O-desmethylindometacin, N-deschlorobenzoylindometacin and O-desmethyl-N-deschlorobenzoylindometacin and their glucuronides.

Previously published GC methods make use of either diazomethane for the preparation of the methyl ester[12] or diazoethane for the preparation of the ethyl ester[13,14] of indometacin. In the first case there is a risk of codetermination of the O-desmethyl metabolite.

This paper describes three alkylation methods for a selective and sensitive GC determination of unmetabolized indometacin in plasma. The propyl ester derivative

was chosen to achieve good separation from both the O-desmethyl metabolite and the methyl ester, which was used as internal standard. The investigation was undertaken to find alternative methods to the diazoalkane techniques, which involve carcinogenicity, toxicity and explosion hazards.

EXPERIMENTAL

Gas chromatography

The reaction conditions were evaluated with a Varian 1400 gas chromatograph equipped with a flame ionization detector. The glass column (180 × 0.2 cm) was filled with 3% OV-1 on 80–100 mesh Gas-Chrom Q. The column temperature was 240° and the injector and detector temperatures were 250° and 300°, respectively. Nitrogen was used as carrier gas at a flow-rate of 30 ml/min.

Studies in the nanogram range were carried out with a Hewlett-Packard 5730 gas chromatograph. This apparatus was equipped with a frequency-modulated ^{63}Ni electron capture detector, and the carrier gas was argon containing 5% methane with a flow-rate of 30 ml/min. The glass column (120 × 0.2 cm) was filled with 3% XE-60 on 80–100 mesh Gas-Chrom Q. The column temperature was 230° and the injector and detector temperatures were 220° and 300°, respectively.

Reagents and chemicals

Sodium dihydrogen phosphate (Merck, Darmstadt, G.F.R.), 1 *M* solution, N-propyl-N-nitroso-N′-nitroguanidine (E.G.A. Chemie, Steinhem, G.F.R.), potassium hydroxide (Merck), tetrapentylammonium (TPeA) phosphate, 0.1 *M*, pH 7.0, [tetrapentylammonium hydroxide was prepared from the iodide (Fluka, Buchs, Switzerland) by shaking with silver oxide[15], and neutralized with phosphoric acid to pH 7.0], tetrabutylammonium (TBA) hydrogen sulphate (Fluka), 0.01 *M* in methylene chloride, sodium bicarbonate (Merck), alkyliodides (methyl, ethyl, propyl, butyl, pentyl, isopropyl and isobutyl iodide) (Fluka), heptane, *n*-pentanol, diethyl ether, ethylene glycol monoethyl ether, methylene chloride (Merck, spectranalysed quality), and indometacin (Polfa, Lodz, Poland) were used.

The methyl ester of indometacin prepared in milligram amounts by the solid–liquid phase transfer catalysed process, for use as internal standard, was dissolved in heptane containing 5% *n*-pentanol.

METHODS

Evaluation of reaction conditions

Extractive alkylation. To 1.0 ml of TBA or TPeA phosphate (0.1 *M*, pH 7) was added 1.0 ml of methylene chloride containing indometacin (10^{-3} *M*) and hexacosane ($0.5 \cdot 10^{-3}$ *M*, internal standard). Then 50 μl of alkyl iodide was added and the tube shaken (see Table I). The reaction was stopped with 5 ml of sulphuric acid (10^{-2} *M*) and the mixture was shaken for 5 min before centrifugation. Then 1 μl of the methylene chloride phase was injected into the gas chromatograph.

Alkylation by a solid–liquid phase transfer catalysed process. To 1.0 ml of methylene chloride containing TBA hydrogen sulphate (10^{-2} *M*), indometacin (10^{-3} *M*) and hexacosane ($0.5 \cdot 10^{-3}$ *M*) were added 0.3 g of sodium bicarbonate and

TABLE I

EXTRACTIVE ALKYLATION. YIELD OF ALKYLATED INDOMETACIN (%)

Conditions as in Experimental.

Reaction time (min)	*TBA 0.1 M, pH 7*			*TPeA 0.1 M, pH 7*
	Methyl derivative	*Propyl derivative*	*Pentyl derivative*	*Propyl derivative*
2	24	2	1	93
5	43	3	2	99
10	71	3	3	95
15	88	4	4	100
20	91	7	6	99
30	100	9	7	98

50 μl of alkyl iodide. The mixture was shaken (see Table II) and the reaction was stopped as described above. The peak area ratio was calculated.

The yields of the methyl and propyl derivatives were calculated against the derivatives prepared with diazomethane and diazopropane. The yields of the other derivatives were calculated after correction for the increasing carbon content compared to the methyl and propyl derivatives.

Procedures for the determination of indometacin in plasma

A sample of 0.5–1.0 ml of plasma, 2.0 ml of sodium dihydrogen phosphate (1 *M*) and 0.1 ml of internal standard solution were extracted with 5.0 ml of heptane containing 5% *n*-pentanol.

Alkylation with diazopropane. Diazopropane was generated from N-propyl-N-nitroso-N'-nitroguanidine and potassium hydroxide[16,17] and distilled over into the test tube containing the heptane extract of indometacin. Propylation was complete after 30 sec; the solution was then evaporated to dryness under flowing nitrogen at 55°. The residue was dissolved in an appropriate volume of heptane in an ultrasonic bath, and 1–2 μl were injected into the gas chromatograph.

Extractive alkylation. The heptane extract was evaporated to dryness and 1.0 ml of TPeA phosphate (0.1 *M*, pH 7), 1.0 ml of methylene chloride and 50 μl of

TABLE II

ALKYLATION BY A SOLID–LIQUID PHASE TRANSFER CATALYSED PROCESS. YIELD OF ALKYLATED INDOMETACIN (%)

Conditions as in Experimental.

Reaction time (min)	*Alkyl derivative*						
	Methyl	*Ethyl*	*Propyl*	*Butyl*	*Pentyl*	*Isopropyl*	*Isobutyl*
2	100	88	90	96	81	71	56
5	99	96	91	99	87	68	65
10	99	100	87	88	90	72	66
15	100	100	90	99	93	72	67
20	99	104	98	102	99	73	70
30	98	107	100	99	98	72	53

propyl iodide were added. The mixture was shaken for 30 min. The organic phase was transferred to another test tube and shaken with 5.0 ml of water. After centrifugation the organic phase was evaporated to dryness. The indometacin derivatives were dissolved in 0.2–2 ml of heptane in an ultrasonic bath, and 1–2 μl were injected into the gas chromatograph.

Alkylation by a solid–liquid phase transfer catalysed process. The heptane extract was evaporated and 1.0 ml of TBA hydrogen sulphate (10^{-2} *M* in methylene chloride), 0.3 g of sodium bicarbonate and 50 μl of propyl iodide were added. The mixture was shaken for 30 min, and then 5.0 ml of water were added. After shaking for 5 min and centrifugation, the organic phase was transferred to another test tube and evaporated to dryness. The derivatives were dissolved in heptane as described above.

RESULTS AND DISCUSSION

Extraction conditions

Indometacin was completely extracted from the biological material with heptane containing 5% *n*-pentanol, at pH 4. The partition ratio (equal phase volumes) as a function of pH is shown in Fig. 1 with heptane containing 2% and 5% *n*-pentanol as organic phases. Pure heptane gave a partition ratio of 2.8 at pH 4, and addition of 5% *n*-pentanol was necessary to obtain quantitative extraction (partition ratio $D > 100$). Methylene chloride gave a higher partition ratio, but was not chosen as it gave a great deal more background disturbance from the biological material. The low degree of extraction at low pH values reported by Helleberg[14] was not observed. At pH values above the pK_{HA} for indometacin (K_{HA} = acid dissociation constant) the degree of extraction will decrease because the acid is partly ionized. The partition studies shown in Fig. 1 indicate pK_{HA} *ca.* 5 for indometacin.

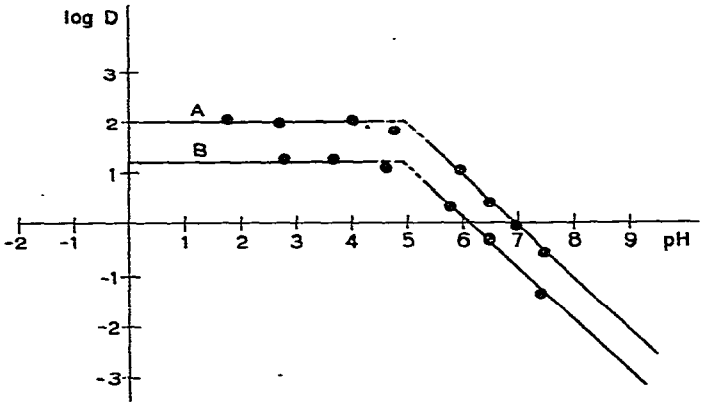

Fig. 1. Relation between the partition ratio (*D*) of indometacin and pH of the aqueous phase. Organic phase: A, heptane containing 5% *n*-pentanol; B, heptane containing 2% *n*-pentanol.

Preparation of derivatives

Alkylation with diazopropane. Diazopropane was freshly prepared from N-propyl-N-nitroso-N'-nitroguanidine and potassium hydroxide[16,17]. The propylation of indometacin was quantitative in 30 sec.

Extractive alkylation. This technique has been used by Brändström and Junggren[18] for organic synthesis. It has also been applied to the GC determination of ionizable compounds such as carboxylic acids[19], phenols[20], xanthine derivatives[21], sulphonamides[22,23] and barbiturates[24,25].

The extractive alkylation of indometacin was performed at pH 7, as indometacin was decomposed in a more alkaline medium, as noticed previously[9,14]. Table I shows that quantitative derivatization to the propyl ester was obtained with TPeA (0.1 *M*) as counter-ion. The reaction rate was influenced by both the concentration and the lipophilicity of the counter-ion[19,21,24]. When the less lipophilic ion TBA (0.1 *M*) was used the yield of the propyl derivative was only 9% after 30 min.

Alkylation by a solid–liquid phase transfer catalysed process. Since indometacin is sensitive to hydrolytic decomposition, alkylation in an anhydrous system was investigated to avoid the risk of decomposition in an aqueous phase. Greeley[26] has prepared some alkyl esters using quaternary ammonium hydroxides in a polar solvent system of N,N-dimethylacetamide and methanol with different alkyl iodides. However, this system could not be used in our investigation as it resulted in rapid decomposition of indometacin, as previously noticed[14]. Instead we used a liquid system of TBA hydrogen sulphate dissolved in methylene chloride and a solid phase of sodium bicarbonate. Indometacin could be quantitatively derivatized with methyl, ethyl, propyl, butyl and pentyl iodides (Table II), but not with isopropyl and isobutyl iodides. Sodium bicarbonate and the quaternary ammonium compound were both necessary for the reaction. To achieve quantitative derivatization the amount of quaternary ammonium salt had to be at least equivalent to the amount of indometacin. The bicarbonate is probably extracted into the organic phase as an ion pair with TBA. Evidence that the bicarbonate is transferred to the organic phase was provided by the fact that derivatization was also complete if the procedure was changed as follows. A solid phase of sodium bicarbonate was shaken for 30 min with methylene chloride containing TBA hydrogen sulphate. After centrifugation the methylene chloride phase was transferred to another test tube containing a solution of indometacin in methylene chloride. Methyl iodide was added and the tube was shaken for 30 min.

Solid–liquid phase transfer catalysed processes have been used by Durst and Liebeskind in the acetoacetic ester condensation[27] and by Zubrick *et al.* in the preparation of nitriles and cyanides[28]. Analytical applications have been worked out by Durst *et al.*[29] and Grushka *et al.*[30] for the preparation of phenacyl and benzyl derivatives of carboxylic acids. In the last three studies crown ethers were used instead of quaternary ammonium compounds.

Internal standard

The methyl ester of indometacin used as internal standard could be prepared by any of the three alkylation methods, and was stable for several months in heptane. The stability during the analytical procedures was checked by analysing 10 samples, without indometacin containing the methyl ester, according to the three alkylation methods. The methyl ester was neither decomposed nor changed under the conditions described for the determination of indometacin in plasma.

Gas chromatography

The separation of the methyl and propyl esters of indometacin was satisfactory

TABLE III

RELATIVE RETENTION OF ALKYL DERIVATIVES OF INDOMETACIN ON OV-1 AND XE-60 COLUMNS

The methyl derivative has a retention time of 7.3 min on OV-1 and 8.6 min on XE-60. Conditions as in Experimental.

Alkyl derivative	*Relative retention*	
	OV-1	*XE-60*
Methyl	1.00	1.00
Ethyl	1.17	1.06
Propyl	1.46	1.29
Butyl	1.80	1.50
Pentyl	2.26	1.81
Isopropyl	1.15	0.96
Isobutyl	1.58	1.39

with both OV-1 and XE-60 as stationary phase. The peak symmetry was much better on XE-60, and therefore this phase was chosen for the determination of indometacin in the low concentration range. Table III shows the relative retention of the different esters of indometacin. The minimum detectable quantities[31] were $1.1 \cdot 10^{-16}$ mol/sec for the methyl ester and $1.4 \cdot 10^{-16}$ mol/sec for the propyl ester of indometacin. The electron capture response was independent of the detector temperature in the range 210–360°, which indicates that the electron capture mechanism is of the dissociative type[32].

Application to biological samples

The three alkylation methods were tested by analysing 10 plasma samples containing 200 ng indometacin. The relative standard deviations are listed in Table IV. The lower value for the solid–liquid phase transfer catalysed process indicates that decomposition of indometacin can be avoided more successfully by this method than by extractive alkylation. Fig. 2 shows chromatograms from (a) blank plasma and (b) plasma sample containing 200 ng indometacin analysed by the solid–liquid phase transfer process. The other two methods gave similar chromatograms. The negative peak in the chromatograms is probably from cholesterol in the plasma samples. Other investigators have shown that cholesterol gives a negative peak under similar chro-

TABLE IV

RELATIVE STANDARD DEVIATIONS OF THE THREE DIFFERENT ALKYLATION METHODS AT THE 200 ng LEVEL ($n = 10$)

Method	*Relative standard deviation (%)*
Alkylation with diazopropane	5.1
Extractive alkylation	7.5
Alkylation by a solid–liquid phase transfer catalysed process	3.5

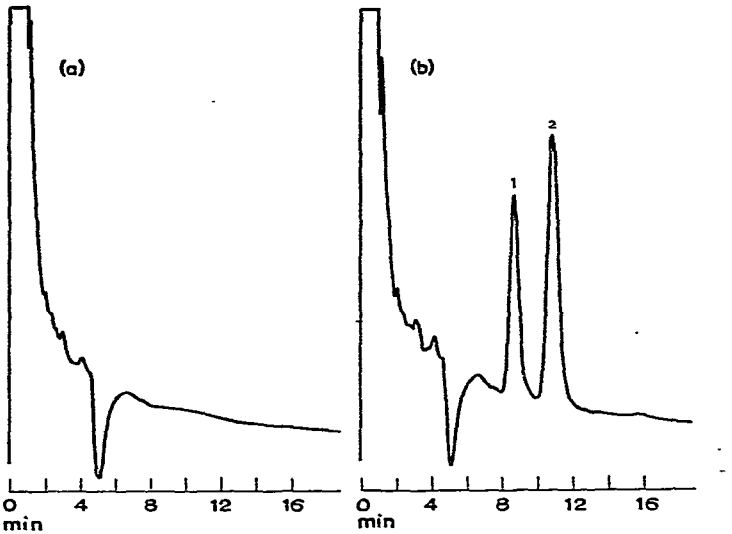

Fig. 2. Gas chromatograms from (a) blank plasma and (b) plasma sample containing 200 ng indometacin. 1, Internal standard: methyl ester of indometacin; 2, propyl ester of indometacin.

matographic conditions[33]. The lowest concentration measureable with acceptable accuracy was 5 ng per sample.

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