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PREPARATION, GAS CHROMATOGRAPHY AND MASS SPECTROMETRY OF METHYL AND TRIMETHYLSILYL ESTERS OF INDOMETHACIN

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SUMMARY

The preparation and gas chromatographic–mass spectrometric behavior of the methyl and trimethylsilyl esters of indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, are described. Reaction of this anti-inflammatory drug with diazomethane or bis(trimethylsilyl)acetamide forms the expected esters. Derivatization with dimethylformamide dimethylacetal yields two compounds, the methyl ester (major product) and a methyl ester–dimethylaminomethylene condensation (at the α-carbon of the side chain) product (minor). Experiments with 5-O-desmethylindomethacin have demonstrated that using the described diazomethane methylation conditions no alkylation of the phenolic group occurs. Esterification combined with an isolation procedure allows the determination of indomethacin levels in plasma and aqueous humor of rabbits, the 4-fluorobenzoyl analog serving as internal standard. The derivatives exhibit excellent electron capture properties allowing quantitative assay of the drug at the submicrogram level. Precision and accuracy for plasma samples varied from 92 ± 19% (5 ng/ml) to 96 ± 1.5% (1000 ng/ml). The analogous values for aqueous humor are superior: 97 ± 5.6% and 99 ± 2.2%, respectively.

INTRODUCTION

Indomethacin (Indocin®), 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, is an extensively used anti-inflammatory drug[1–3]. Biolevels have been studied by spectrofluorimetric procedures[4,5] and by radiometric methods after administration of ^{14}C-labeled compound[6]. Krasowska *et al.*[7] have indicated that in the spectrofluorimetric methods for indomethacin, its N-deschlorobenzoyl and 5-O-desmethyl metabolites interfere as they absorb and fluoresce at the same wavelength as the parent drug. Duggan *et al.*[8] have stated that the results of earlier clinical studies based upon non-specific isotope or fluorescence methodology are subject to considerable re-interpretation, and these authors have developed an isotope dilution assay procedure. Hucker *et al.*[9–11] have demonstrated that several indomethacin-

related structures undergo gas chromatography (GC) successfully, and more recently several investigators have described various methodologies for the assay of indomethacin in serum[12–18]. The present paper deals with the preparation, gas chromatographic properties and mass spectrometric (MS) characterization of two indomethacin esters, *i.e.*, methyl and trimethylsilyl (TMS). Procedures to quantitate the drug after extraction are briefly described.

EXPERIMENTAL

A Packard 7401 dual-column gas chromatograph equipped with flame ionization and 20-mCi ^{63}Ni electron capture detectors (FID and ECD, respectively) was employed in this work. The column packing was 3% SE-52 (Applied Science Labs., State College, Pa., U.S.A.) coated on 100–120 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) by the filtration method[19], as is the standard in this laboratory. The carrier gases were nitrogen, 70 ml/min (FID), and argon–methane (90:10), 80 ml/min (ECD), both purified by a 3-Å molecular sieve and Oxysorb (Regis, Morton Grove, Ill., U.S.A.). Column conditions: 4 ft. × 3 mm I.D. glass tubes; inlet temperature 270°; column temperature 260°. The FID was operated at 280° with hydrogen and air flow-rates of 40 and 400 ml/min, respectively, and a voltage of 200 V d.c. The ECD was operated at 290° with 50-V pulsed voltage (pulse width: 1 μsec; pulse interval: 100 μsec). A Hewlett-Packard 3380A recording integrator was used for quantitative analysis.

An LKB Model 9000 instrument was employed for gas chromatography–mass spectrometry. Column conditions: 4 ft. × 3 mm I.D. glass column; 3% OV-1 on 80–100 mesh Supelcoport; 245°; flow-rate 30 ml/min helium. Spectrometer conditions: 70 eV ionizing potential; 50 μA filament current; 3.5 kV accelerating potential: 250° source temperature.

Methyl esters were prepared by two methods. (A) From diazomethane: the requisite aliquot (generally 0.1 ml) of a stock solution of indomethacin in methanol (1 mg/ml) is evaporated under nitrogen in a conical vial. One-tenth milliliter of chilled diethyl ether–methanol (9:1) and 0.2 ml of a solution of ethereal diazomethane prepared from N-methyl-N′-nitro-N-nitrosoguanidine (Fluka, Buchs, Switzerland) as described by MacKay[20] are added. Following 15 min at 0°, the solution is evaporated to dryness under nitrogen and the residue is dissolved in the requisite volume of ethyl acetate to obtain the suitable concentration for FID, ECD or MS studies. (B) From dimethylformamide dimethylacetal (DMF-DMA, Fluka or Pierce, Rockford, Ill., U.S.A.)[21]: the requisite aliquot of indomethacin (*ca.* 0.1 mg) is obtained as above and heated at 60° for 20 min in 0.2 ml of DMF-DMA. The vial is cooled to room temperature, the contents are evaporated under nitrogen and the residue is dissolved in the appropriate volume of ethyl acetate.

TMS esters were prepared as indicated below.

Milligram scale: 0.1 ml of bis(trimethylsilyl)acetamide (BSA, Pierce) and 0.1 ml of ethyl acetate are added to 1 mg of indomethacin in a conical vial. The vial is sealed and heated for 15 min at 60°, then cooled to room temperature and the contents are diluted to 1 ml with ethyl acetate for FID and MS analyses.

Microgram and submicrogram scale: 0.1 to 1 ml of a 1 μg/ml solution of indomethacin in methanol is evaporated under nitrogen in a conical vial. The residue

is dissolved in 90 μl of ethyl acetate and 10 μl of BSA are added. The vial is treated as above, and the contents are diluted to 1 ml using 3% BSA in ethyl acetate.

To avoid adsorption losses during sample collecting, extraction procedures and derivatization, vials and volumetric glassware are cleaned and silanized as follows. 10% Mucasol (Merz & Co., Frankfurt/M., G.F.R.) in deionized water is used as cleaning agent. Vials are boiled for 1 h in this solution, rinsed extensively with tap and then distilled water. They are thoroughly dried in a 120° oven and immersed while still warm in a 5% dimethyldichlorosilane solution in ethyl acetate, rinsed with anhydrous methanol, and then dried.

Methanol (E. Merck, Darmstadt, G.F.R.) and ethyl acetate and methylene chloride (Carlo Erba, Milan, Italy) were pesticide grade.

The extraction of the drug from biological fluids is performed as follows. A suitable amount (*e.g.*, 250 ng) of internal standard [1-(*p*-fluorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] is added to samples. Plasma is delipidated by extraction with an hexane–ethyl acetate (4:1) mixture at pH 7.2. Drug and standard are then extracted by the same solvent after buffering at pH 4.5–5. The residue obtained after evaporation of the organic phase is treated with the appropriate derivatization reagent as described. The retention times of the two esters of the internal standard relative to those of the corresponding derivatives of indomethacin are 0.60. Calibration curves are obtained by plotting peak height or area ratio indomethacin/internal standard *vs.* known amounts of indomethacin. Precision and accuracy of results obtained on samples spiked with indomethacin, carried through the entire procedure and methylated, are described in Table I. Minimal detectable amounts were *ca.* 1 ng from aqueous humor and *ca.* 2 ng from plasma samples, *i.e.* injected amounts *ca.* 50 pg.

RESULTS AND DISCUSSION

Reaction of indomethacin with BSA gave a single product with a retention time of 4 min, whereas that for the methyl ester was 3.5 min (Fig. 1). Derivatization with DMF-DMA yields two peaks. The major one possesses the same retention time as the diazomethane derivative, whereas the retention time of the minor peak is more than twice that of the methyl ester, suggesting a significantly greater molecular weight. Identification of the components has been achieved by the use of GC–MS (see below).

The ECD ionization current is *ca.* 5×10^{-9} a.f.s./1 mV with an uncontaminated detector. The peak height under these conditions of the 2 ng standard was *ca.* 70% f.s.d. for the TMS ester and *ca.* 90% f.s.d. for the methyl ester (from diazomethane) at an attenuation of 32×10^{-10} a.f.s./1 mV. When the background current has dropped to *ca.* 2×10^{-9} a.f.s./1 mV, the response is reduced for both derivatives. Ionization values are *ca.* 7330 C/mole for the methyl ester and 5925 C/mole for the TMS ester with a background current close to 4×10^{-9} a.f.s./1 mV. The electron capture properties of the derivatives can be attributed to the conjugated *p*-chlorobenzoyl group, even though chlorobenzoyl derivatives are not described as displaying high affinity for thermal electrons[22]. It can be assumed the electron-capturing region in these esters is the carbonyl group in resonance with an imide structure as suggested by Matin and Rowland[23], with the two aromatic ring systems enhancing charge delocalization (Fig. 2).

Using the trimethylsilylation or diazomethane methylation procedure and the

TABLE I

PRECISION AND ACCURACY OF THE GC–ECD ASSAY OF INDOMETHACIN METHYL ESTER*

Relative standard deviation on peaks ratios and recovery percentage.

Amount (ng)	*Standards*** *(%)*	*Aqueous humor (%)*	*Plasma (%)*
5	±7.8	97.6 ± 5.6	92.4 ± 18.9***
25	±1.3	101.3 ± 1.0	100.8 ± 4.5
100	±1.6	100.9 ± 3.6	104.0 ± 2.3
250	±0.1	100.8 ± 0.5	101.4 ± 4.2
500	±1.7	99.9 ± 0.9	96.4 ± 3.8
1000	±1.4	99.3 ± 2.2	96.2 ± 1.4

* Internal standard amount: 250 ng.

** Standards are prepared after evaporation of aliquots of an indomethacin solution and reaction with diazomethane solution.

*** The assay of the 5 ng sample in plasma must be corrected by the use of a standard prepared in the presence of material extracted from control plasma.

GC–ECD conditions described in Experimental, known amounts of ester were injected and peak heights plotted *vs.* nanograms indomethacin (Fig. 3). Up to 2 ng the correlation coefficients are $r = 0.9993$ and $r = 0.9997$, respectively, for methyl ester and TMS ester. Within this upper limit, the linearity appears to be excellent with $P' < 0.001$ (refs. 24 and 25).

No formation of 5-O-methyl ether from 5-O-desmethylindomethacin (an indomethacin metabolite) was detected by thin-layer chromatography (TLC) and MS when using the described methylation procedure. TLC was performed on silica gel F_{254} (chloroform–acetic acid, 95:5). The mass spectrum of the reaction product between diazomethane and 5-O-desmethylindomethacin is represented in Fig. 4. The methyl ester of indomethacin appeared to be stable and would be the preferred derivative when amounts of drugs are smaller than 50 ng in the sample. TMS esters

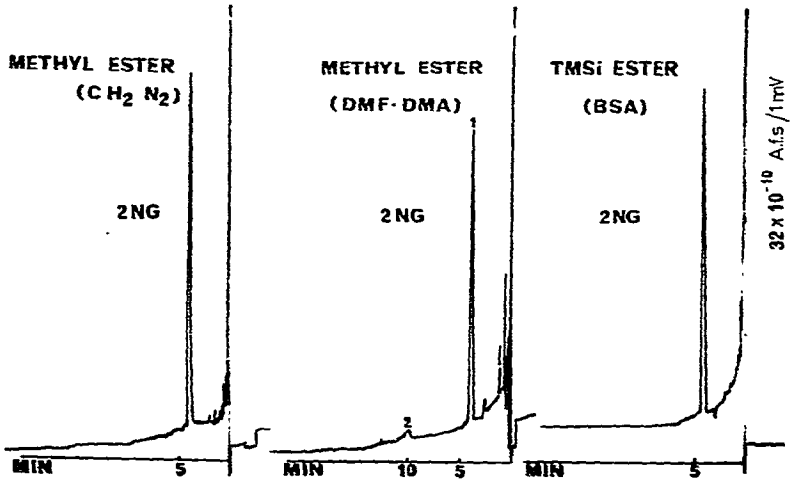


Fig. 1. Gas chromatograms resulting from indomethacin derivatized with diazomethane, DMF-DMA and BSA. See experimental section for derivatization and GC conditions.

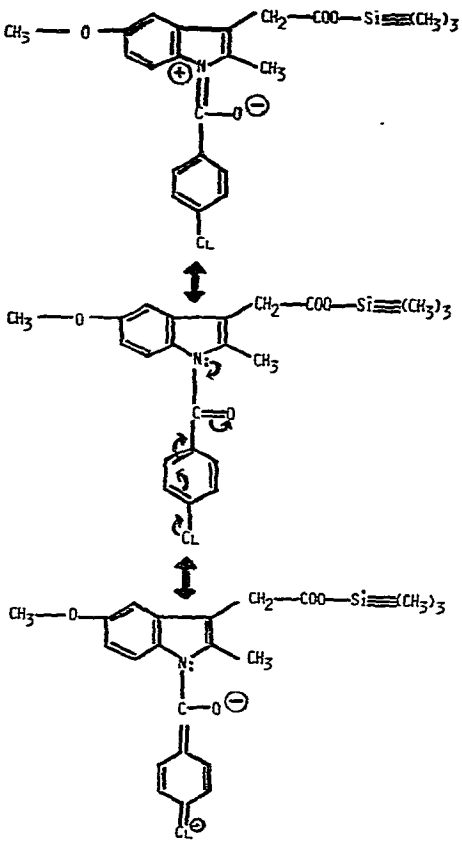


Fig. 2. Possible resonance forms of indomethacin TMS ester.

are reported to be less stable toward spontaneous hydrolysis than TMS ethers[26]. In order to preclude rapid hydrolysis, the solutions of low concentration are taken up in a solvent containing an excess of BSA (3% in acetic acid-free ethyl acetate). However, at higher levels the TMS derivative is convenient to use and would be preferred if interfering peaks are present in vicinity of the methyl ester peak.

The mass spectrum of indomethacin TMS ester (Fig. 5) is dominated by the ions of *m/e* 139–141, arising from the *p*-chlorobenzoyl fragment. The ion resulting from the related *p*-chlorophenyl fragment is noted at *m/e* 111–113. Other ions of moderate intensity are the molecular ions (*m/e* 429–431) and M — 117 (loss of COOTMS) at *m/e* 312–314; loss of methyl (*m/e* 414–416) is a minor pathway. That the later fragmentation involves loss of a TMS methyl rather than a methyl from indomethacin is demonstrated by MS of the TMS-d_9 ester, for M — 15 (CH_3) shifts to M — 18 (C^2H_3). The M — 117 ion shifts to M — 126 confirming that this fragmentation is loss of the carbotrimethylsilyloxy group. The low intensity ion of *m/e* 370–372 (M — 59) in the TMS ester shifts to M — 62 with the TMS-d_9 derivative and thus appears to be M — (CO_2 + CH_3).

Reaction of indomethacin with DMF-DMA yields two products, as demonstrated by GC. The major component possesses the same retention time (see Fig. 1)

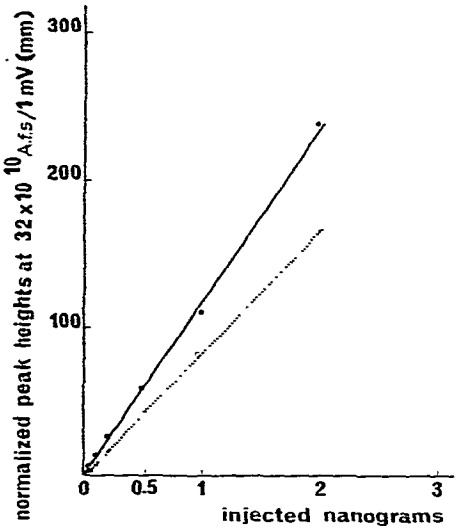


Fig. 3. Peak heights of methyl ester (continuous line) and TMS ester (dotted line). Standing current was 4.3×10^{-9} a.f.s./1 mV. Peak heights were measured at $4 \times$, $8 \times$, $16 \times$ and 32×01^{-10} a.f.s./1 mV and the corresponding heights were normalized as if obtained at 32×10^{-10}.

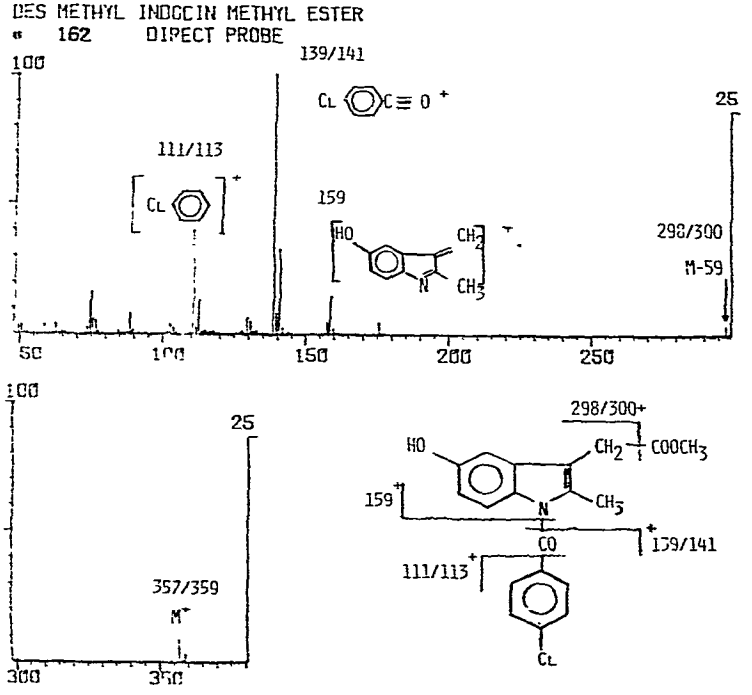


Fig. 4. Mass spectrum of the product obtained after reaction of diazomethane with 5-O-desmethyl-indomethacin (Finnigan Model 3200; 70 eV ionizing potential).

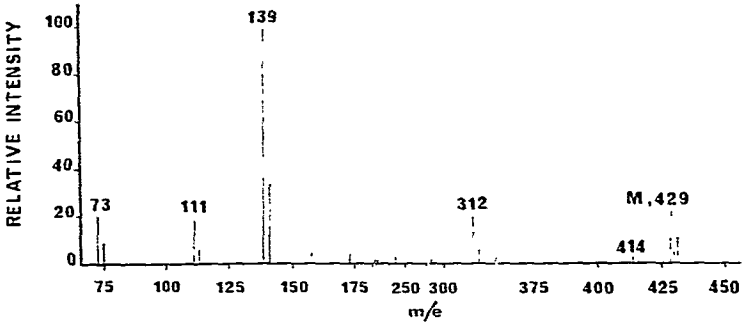


Fig. 5. Mass spectrum of TMS ester of indomethacin.

and mass spectrum as the diazomethane product. The base peak is again *m/e* 139–141 the *p*-chlorobenzoyl fragment. The molecular ion (*m/e* 371–373) is of moderate intensity, as is the *p*-chlorophenyl fragment ion, *m/e* 111–113. The ion of *m/e* 232, M — 139, arises from loss of the *p*-chlorobenzoyl group. M — 59, loss of carbomethoxy, gives an ion of moderate intensity. Reaction of indomethacin with DMF diethylacetal (DMF-DEA) also yields two GC peaks. The major component, of shorter retention time, is the ethyl ester, as demonstrated by the 14 a.m.u. shifts observed in the M and M — *p*-chlorobenzoyl ions: 371 → 385; 232 → 246. The *m/e* value of the M — carboalkoxy ion does not change.

The mass spectrum of the component of longer retention time from the DMF

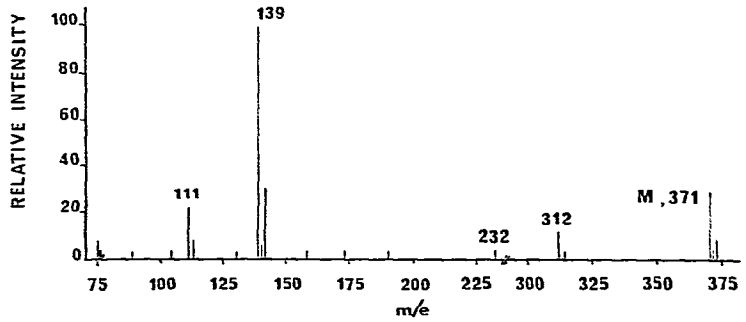


Fig. 6. Mass spectrum of methyl ester of indomethacin.

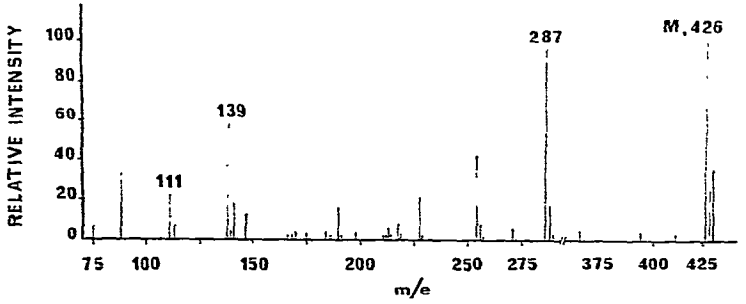
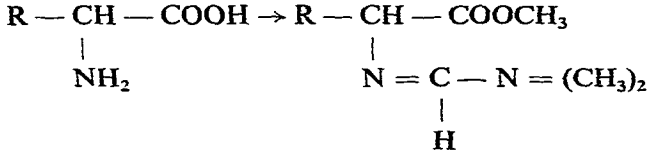
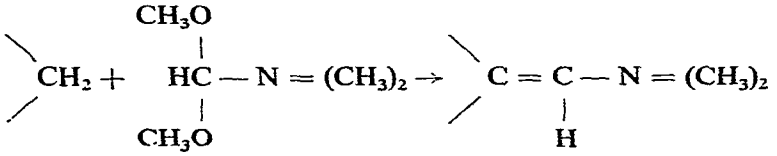


Fig. 7. Mass spectrum of minor component resulting from derivatization of indomethacin with DMF-DMA.

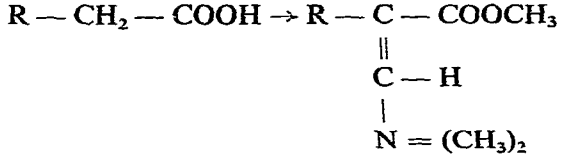
DMA reaction is presented in Fig. 7. It is strikingly different from the spectrum of the methyl ester (compare Figs. 6 and 7). The base peak for the minor component is the molecular ion, *m/e* 426–428, and the second most intense signal is *m/e* 287, M—139. The intensity of the *p*-chlorobenzoyl fragment ion is much diminished, as is that of the M — 59 ion. The difference in molecular weights between the simple methyl ester and the unknown is 55 a.m.u.; the same difference is observed between the ethyl ester and the DMF-DEA unknown (*M*, 440–442). Treatment of indomethacin with DMF-DMA thus forms the expected methyl ester (an increase in molecular weight of 14 a.m.u.) plus an unknown side product possessing a molecular weight 69 a.m.u. greater than that of indomethacin. This increase of 69 a.m.u. is the same as that observed by Thenot and Horning[27] for the reaction of simple amino acids with DMF-DMA:



The greatly enhanced intensities of the molecular and the M — 139 ions in the spectrum of the unknown suggest a change in the side-chain ester which stabilizes this moiety. As active methylene groups have been reported by Meerwein *et al.*[28] to react with DMF-DMA, *i.e.*



a likely structural alteration involving the indomethacin side chain is condensation of the methylene group with the reagent as depicted below:



This suggestion is strengthened by the observation that α-methylindomethacin, R — CH — COOH, yields only one product, the simple ester.
|
CH_3

The considerable intensities of the methyl and TMSi ester molecular ions (see Figs. 5 and 6) suggest that these ions could be monitored in a highly specific multiple ion detection GC–MS assay for indomethacin. As the *m/e* of the molecular ion of the TMS ester is the same as that of a background signal from column bleed, the TMS-d_9

ester (M, *m/e* 438) can be employed. Preliminary studies using the TMS-d_9 of indomethacin (M, 438) and indomethacin-d_5 (M, 443; internal standard) indicated a sensitivity level of *ca.* 2 ng/ml plasma. Of course, the intense *m/e* 139 ion could also be monitored (see, for example, Palmer *et al.*[15]), but would be less specific than the molecular ion for parent drug.

Application of the present methodology has been presented elsewhere[29]. The derivatives allow one to assay specifically for indomethacin in several biological fluids after administration of regular pharmacological doses with the required sensitivity. A typical chromatogram from a 0.5-ml plasma extract spiked with 500 ng of internal standard and methylated according to the desired procedure is presented in Fig. 8. It corresponds to a rabbit (*ca.* 2.7 kg) which was dosed in each eye with 50 μl of a 1% indomethacin ophthalmic suspension 30 min prior to blood sampling. When high dosages and levels are involved (*i.e.*, in the microgram per milliliter range) quantitation by electron capture detection may appear unnecessary. However, by diluting samples the background can be dramatically reduced and the specificity and sensitivity of this detection mode employed to the fullest.

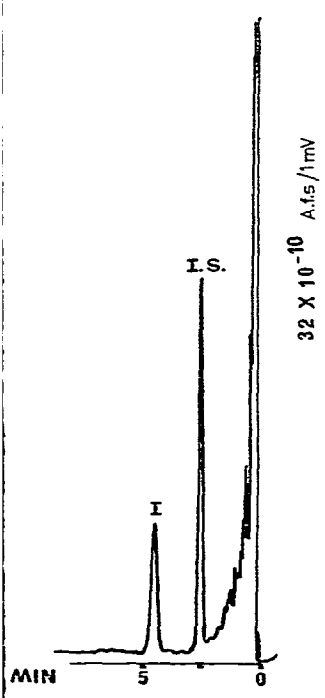


Fig. 8. Chromatogram of the methylated product from a 0.5 ml plasma (rabbit dosed in eyes with indomethacin) extract. Final volume was 1 ml and injected volume was 2 μl. I.S. = internal standard methyl ester peak (*ca.* 0.9 ng); I = indomethacin methyl ester peak (*ca.* 0.4 ng). For other details see text.

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