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DETERMINATION OF INDOMETHACIN IN SERUM AND URINE BY ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A specific and sensitive method for the quantitative determination of indomethacin in serum and urine is described. The drug is extracted at pH 5.0 with 1,2-dichloroethane and a portion of the organic extract is concentrated and made to react with diazoethane in diethyl ether. The ethyl ester derivative is analyzed by electron-capture gas–liquid chromatography, quantitation being achieved by comparison of peak areas for samples and standards, which are prepared in serum or urine and treated in the same manner as the samples. The limit of sensitivity is 50 ng/ml and the relative standard derivation for repeat determinations on the same sample is about 3%.

INTRODUCTION

Quantitative determinations of indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid] in biological fluids have in the past utilized either radioactive[1–4] or spectrofluorimetric techniques[1,5]. Except for the isotope dilution method of Duggan *et al.*[4], all of these methods suffer from lack of specificity, causing interference not only from the unconjugated metabolites of indomethacin, but also from co-extractives such as salicylates[5].

In man and all laboratory animals so far examined, indomethacin has been found to undergo extensive biotransformation to O-desmethylindomethacin, N-deschlorobenzoylindomethacin, O-desmethyl-N-deschlorobenzoylindomethacin and their glucuronides[1,4,6]. All of these metabolites are devoid of anti-inflammatory activity[7] and are present in significant amounts in the circulating plasma[4].

Yesair and Coutinho[8] separated indomethacin, O-desmethylindomethacin and N-deschlorobenzoylindomethacin by anion-exchange chromatography using spectrophotometry for quantitation. However, this method lacks the sensitivity needed for clinical pharmacological studies.

This paper describes a method in which electron-capture gas–liquid chromatography (GLC) permits the specific determination of serum and urine levels of indomethacin derived from therapeutic doses.

MATERIALS AND METHODS

Instrumentation

A Pye Series 104, Model 64, gas chromatograph with a nickel-63 electron-capture detector and a Philips PM 8000 strip-chart recorder was used. The detector was operated at 350° in the pulsed voltage mode with a pulse interval of 500 μsec. The attenuation factor was 500 and the chart speed 10 mm/min. The 1.5 m × 4 mm I.D. coiled glass column packed with 2% (w/w) SE-52 (Perkin-Elmer, Norwalk, Conn., U.S.A.) on Chromosorb AW DMCS, 100–120 mesh (Perkin-Elmer) was conditioned at 300° for 48 h and then "loaded" with repeated injections of indomethacin ethyl ester until the peak areas were constant. The injection port temperature was about 290° and the column temperature 245°. Nitrogen filtered through a 5-Å molecular sieve was used as carrier gas at the flow-rate 60 ml/min. Samples were injected with a 1-μl syringe (Scientific Glass Engineering, Melbourne, Australia).

Chemicals

The following chemicals were used: benzene (analytical-reagent grade), citric acid (analytical-reagent grade), 1,2-dichloroethane (extra pure) from Merck (Darmstadt, G.F.R.); diethyl ether, absolute over sodium (purissima), 1-ethyl-1-nitrosourea (purissima) from Fluka (Buchs, Switzerland); potassium hydroxide (analytical-reagent grade), sodium hydroxide (analytical-reagent grade) from Elektrokemiska AB (Bohus, Sweden); indomethacin from Dumex A/S (Copenhagen, Denmark); and O-desmethylindomethacin from Alfred Benzon A/S (Copenhagen, Denmark).

Procedures

Samples of 0.5 ml of serum or urine were pipetted into 15-ml glass-stoppered centrifuge tubes and diluted with 0.5 ml of Sorensens citrate buffer (pH 5.0). Using a Brand Dispensette (Wertheim-Glashütte, Wertheim, G.F.R.), 7.0 ml 1,2-dichloroethane were added and the tubes were shaken for 15 min in the horizontal position at a rate of approximately 75 strokes/min. Following centrifugation for 10 min at 1800 *g*, the aqueous phase was removed by aspiration. Occasional emulsions were broken by repeated centrifugation. A 6.0-ml volume of the organic phase was transferred into another centrifuge tube and evaporated to dryness under a gentle stream of dry nitrogen at 37°. The sides of the tubes were rinsed with 0.5 ml of diethyl ether, then 0.5 ml of a solution of diazoethane in diethyl ether (see below) was added and the tubes were incubated for 30 min at 30°. The diethyl ether and excess of diazoethane were removed under a gentle stream of dry nitrogen. The residue was dissolved in an appropriate volume of benzene and 1 μl of the solution was injected on to the column.

The solution of diazoethane in diethyl ether was prepared from 1-ethyl-1-nitrosourea by using the method for the synthesis of diazomethane described by Arndt[9]. The ether solution was dried over pellets of potassium hydroxide and stored at −18° in a desiccator for a maximum of 2 weeks.

Calculations

The amount of indomethacin was calculated by comparison of the peak areas, expressed as peak height × width at half-height, for samples and standards. Standards with known concentrations of indomethacin were prepared from indomethacin-

free serum and urine using a stock solution of indomethacin in methanol (0.1 mg/ml) stored at −18°. The standards were assayed in the same manner and run with each batch of samples.

RESULTS AND DISCUSSION

Sample preparation

The extraction of indomethacin with 1,2-dichloroethane was pH dependent. As shown in Fig. 1, there was a marked decrease in recovery when the pH was either higher or lower than 5.0. Identical curves were found for the extraction of indomethacin from serum and an aqueous phase without protein.

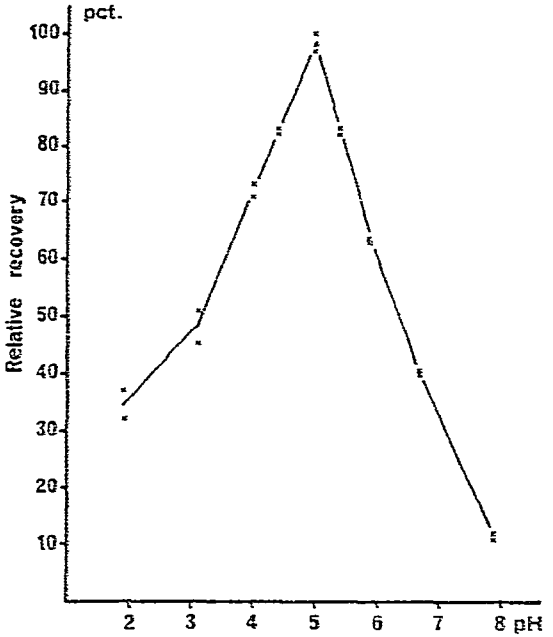

Fig. 1. Effect of pH on the extraction of indomethacin from human serum. The pH of the serum was adjusted by the addition of different Sorensen buffers. Indomethacin was quantitated spectrofluorimetrically[5].

Recoveries in the extraction of mixtures of equal volumes of serum and citrate buffer (pH 5.0) with a seven-fold greater volume of different organic solvents are given in Table I. It can be seen that the recovery increases with increasing polarity of the solvent. The recovery could be increased by repeating the extraction, but this increase was not sufficient to justify the additional labour. A solution of 3–5% of amyl alcohol in heptane has been used by other workers[1,5], but the low volatility of amyl alcohol made the use of this solvent impractical.

Indomethacin does not have a sufficient vapour pressure to permit its direct GLC at temperatures below 300°. The GLC of indoles which are aromatic acids following methylation with diazomethane has been described[10]. This procedure was

TABLE I

RECOVERIES IN THE EXTRACTION OF INDOMETHACIN FROM HUMAN SERUM

Sera were diluted with an equal volume of citrate buffer (pH 5.0) and extracted with a seven-fold greater volume of organic solvent. Indomethacin was quantitated spectofluorimetrically[5].

Solvent	*Recovery (%)*
Heptane	0
Ethyl acetate	62
Benzene	69
Amyl alcohol, 5% in heptane	87
1,2-Dichloroethane	95

not feasible, however, because O-desmethylindomethacin would be converted into the indomethacin methyl ester derivative.

The boron trifluoride–methanol complex has been used successfully to form the methyl ester of fatty acids[11] and acetylsalicylic acid[12], but even refluxing for 1 h did not result in a useful derivative of indomethacin. Esterification using an alkyl iodide in an alkaline polar solvent system has been described[13], but this principle could not be used here as indomethacin is decomposed in basic solutions[7,8].

The reaction between indomethacin and diazoethane appeared to be quantitative when diethyl ether was used as the solvent, as there was no increase in peak areas when the incubation of the reaction mixture was prolonged for 2 h, and as thin-layer chromatography using custom-made silica gel plates and chloroform–benzene–formic acid (100:20:2) as the solvent revealed no trace of underivatized indomethacin. The use of 1-ethyl-1-nitrosourea and diazoethane involves carcinogenicity, toxicity and explosion hazards. However, when proper precautions are taken, the small amounts involved minimize these problems.

Gas chromatography

A single symmetrical peak of the indomethacin ethyl ester derivative was obtained on Apiezon L, SE-30, SE-52 and QF-1 columns. However, the separation of the indomethacin and O-desmethylindomethacin derivative peaks was achieved only with the SE-52 column. Trace A in Fig. 2 shows the chromatogram of a mixture of 0.35 ng of indomethacin and about 1 ng of O-desmethylindomethacin derivatized with diazoethane. Under the chromatographic conditions described, the retention times for the indomethacin and the O-desmethylindomethacin derivatives were 9.4 and 10.6 min, respectively. A third peak with a retention time of about 14 min indicates that two derivatives of O-desmethylindomethacin were formed. The ethyl esters of N-deschlorobenzoylindomethacin and O-desmethyldeschlorobenzoylindomethacin were not recorded by the electron-capture detector.

The detector was calibrated by injections of known amounts of indomethacin ethyl ester derivative in 1 μl of benzene. As shown in Fig. 3, the detector response was linear over the range 0.05–0.40 ng of indomethacin injected.

A large number of compounds were screened as internal standards, but none was found suitable.

Chromatograms of human sera assayed as described are shown in Fig. 2. The sera were either indomethacin-free (C) or estimated to contain 2.6 μg/ml of indomethacin (B).

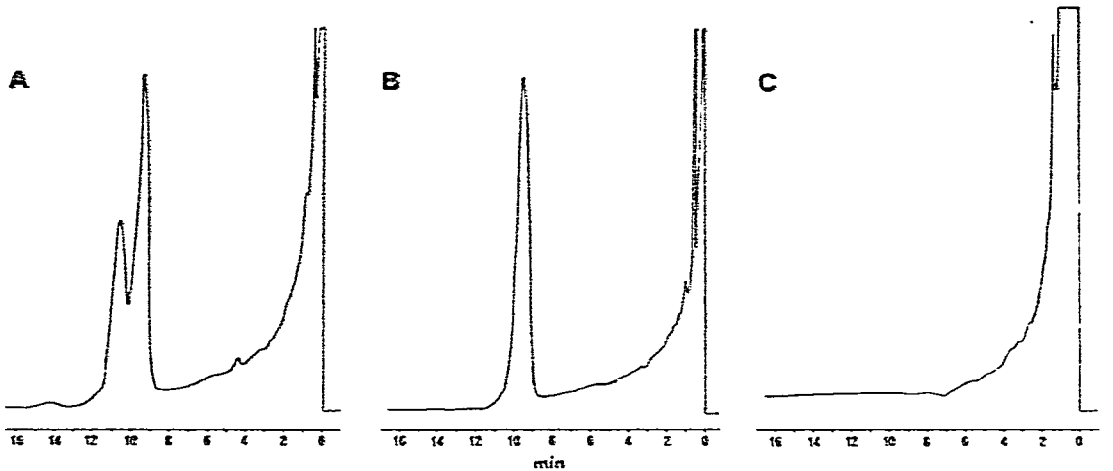

Fig. 2. Chromatograms of indomethacin. A, Mixture of 0.35 ng of indomethacin and about 1 ng of O-desmethylindomethacin reacted with diazoethane; B, extract of human serum estimated to contain 2.6 μg/ml of indomethacin; C, extract of indomethacin-free human serum.

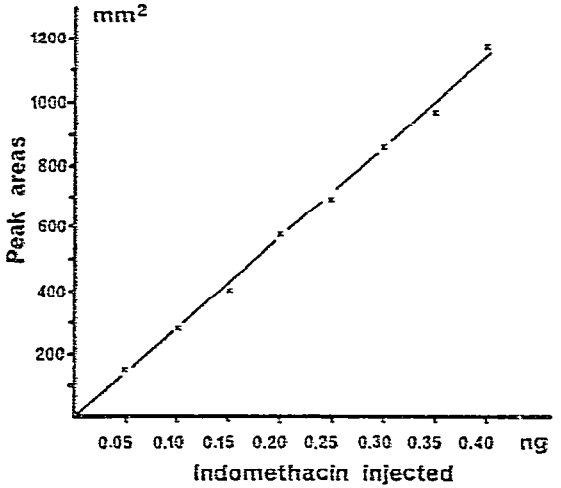

Fig. 3. Detector response to the indomethacin ethyl ester derivative. Peak areas were calculated as peak height × width at half-height.

Specificity, sensitivity and reproducibility

Serum and urine from several subjects who had not ingested indomethacin were analyzed, and no interfering peaks were observed. Among a large number of drugs that possess electron-capturing properties, only griseofulvin and chlorcyclizin disturbed the analysis, whereas commonly used drugs such as benzodiazepines and chlorpromazine did not interfere. The lower limit for the reliable quantitation of indomethacin using 0.5 ml of serum was 50 ng/ml. As the sensitivity was limited by the presence of impurities in the diazoethane reagent, the sensitivity could be enhanced by increasing the amount of serum used in the analysis.

Reproducibility studies were performed on serum with indomethacin added at concentrations of 0.1 and 5.0 μg/ml. For 10 samples analyzed at each concentration, the relative standard deviation was 3% in each instance.

Application

Fig. 4 shows the serum concentrations of indomethacin in a human subject following the intravenous administration of 25 mg of indomethacin.

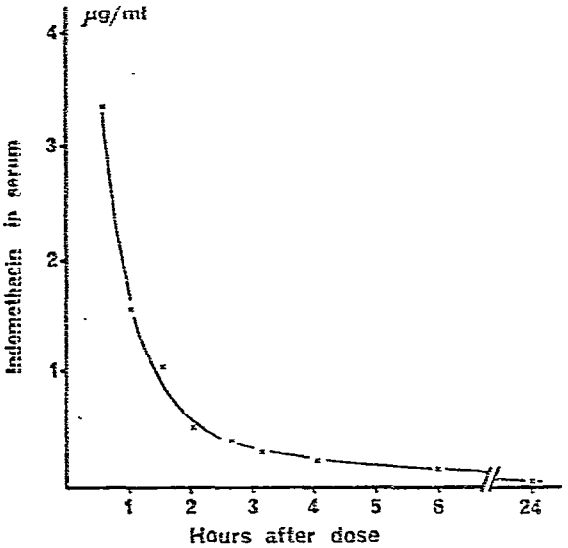

Fig. 4. Serum concentrations of indomethacin in a human subject following a rapid intravenous injection of 25 mg of indomethacin. The line represents the concentrations predicted by a two-compartment open model.

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