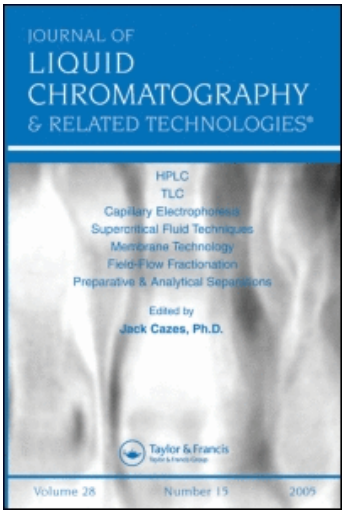

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The Rapid Determination of Indomethacin in 50 µ1 Blood Samples

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THE RAPID DETERMINATION OF INDOMETHACIN IN 50 µl BLOOD SAMPLES

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ABSTRACT

A method for the determination of indomethacin in 50 µl samples of rat blood by high-pressure liquid chromatography has been developed. After addition of sodium acetate buffer (pH= 5.4) and an internal standard (glutethimide), the blood was extracted twice with heptane containing 1.5% (v/v) isoamyl alcohol. The organic solvent was evaporated, the residue dissolved in methanol, and aliquots (5 µl) injected automatically into the chromatograph. The separation and quantification of indomethacin was achieved on a µBondapak C_{18} column with 66% (v/v) methanol in water solution at a flow rate of 2 ml/min and detected at 254 nm wavelength. The analysis was linear for concentrations ranging from 0.50 to 10 µg/ml indomethacin solutions. The method was tested to determine the concentration of indomethacin in blood of rats receiving orally 2 mg/kg of indomethacin.

INTRODUCTION

Indomethacin [1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid], a potent inhibitor of prostaglandin synthesis, is clinically used as an anti-inflammatory agent as well as a promoter of constriction of the patent ductus arteriosus in

premature infants (1,2). In biological research, it is widely used as a tool to clarify the involvement of prostaglandins in a number of physiological functions (3).

Several methods for the detection of indomethacin in biological samples have been described using gas-liquid chromatography (4-7), liquid chromatography (8), and mass fragmentography (9). These methods are sensitive and specific; however, they have some shortcomings such as the need for high-volume sample size, use of hazardous solvents, derivatization steps, or expensive detectors.

In our research program, we have the need for a fast, accurate, and economical method for quantification of indomethacin in small-size blood samples. This report presents such a method using glutethimide as the internal standard and reverse-phase high-pressure liquid chromatography under isocratic conditions with methanol as the eluant and automatic sample injection.

METHODS

Apparatus

The analyses were performed in a Waters Associated Liquid Chromatograph equipped with a Model 440 Absorbance Detector (254 nm wavelength), a WISP Model 710A Autosampler, and a Perkin-Elmer Sigma I Data System. The separation of indomethacin and the internal standard was achieved using a 4 mm i.d. x 30 cm μBondapak C_{18} column (Waters Associates) with a 66% (v/v) methanol in water solution at a flow rate of 2 ml/min.

Reagents

All chemicals used were reagent grade. The drug standards were obtained from Applied Science Laboratories.

Sensitivity and Linearity

Standard solutions of indomethacin in methanol were prepared in concentrations of 0, 1, 2, 4, 6, 8, or 10 µg/ml and containing 50 µg/ml of glutethimide (internal standard). Aliquots (5 µl) of these solutions were injected into the chromatograph.

Recovery

Blood standards were prepared by adding to 50 µl of drug-free blood samples 50 µl of 100 µg/ml glutethimide solution and 50 µl solution containing 0, 1, 2, 4, 6, 8, or 10 µg/ml indomethacin. These standards were extracted as described below. The recovery rates were calculated by comparison with a standard solution of the compounds in methanol.

Extraction Procedure

The 50 µl blood sample, collected in a capillary tube, was added to a screw cap test tube containing 50 µl (100 µg/ml) glutethimide solution and 125 µl sodium acetate buffer (pH=5.4), and the solution was vortexed for 30 seconds. A 5 ml solution of heptane containing 1.5% (v/v) isoamyl alcohol was added; the mixture was vortexed for 2 minutes and centrifuged on a clinical bench centrifuge for 15 minutes. The organic layer was removed to a second test tube. The sample was extracted a second time using another 5 ml of heptane-isoamyl solution. Both organic layers were combined and evaporated to dryness under a stream of nitrogen. The dried extract was reconstituted with 50 µl of methanol, vortexed for 30 seconds, and the solution transferred to the sample vials of the WISP automatic sampler. Each sample (5 µl) was injected automatically into the chromatograph twice at 10-minute intervals to obtain duplicate runs.

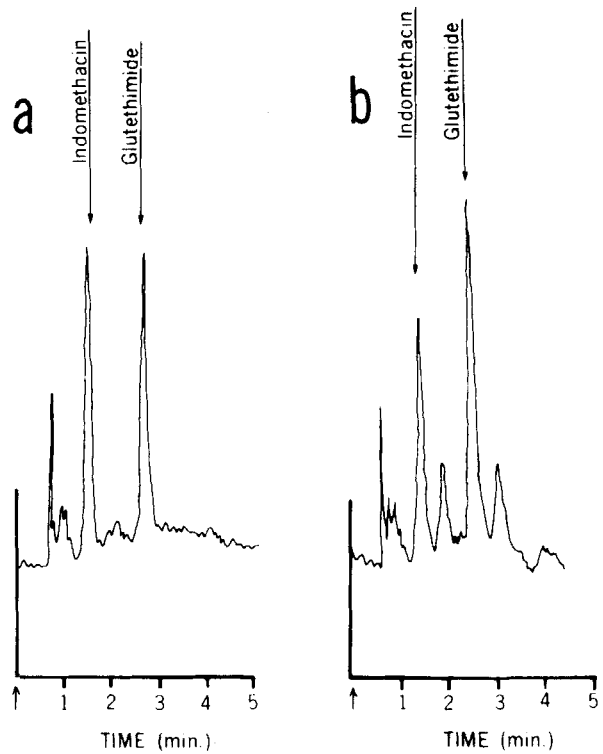

FIGURE 1. Chromatogram of the extract of (a) rat blood with indomethacin and glutethimide added and (b) blood from a rat injected with indomethacin and added glutethimide.

Indomethacin and Blood Levels in Rats

A group of 6 male Wistar rats 250-300 g in weight was administered by gastric intubation with 2 mg/kg indomethacin suspension in peanut oil. Tail vein samples (50 µl) were collected from each rat at 1.0, 1.5, 2.5, 3.5, and 4.5 hours after drug administration. A parallel set of 2 blood standards per 10 blood samples was prepared by adding to drug-free rat blood 2 µl (100 µg/ml) of indomethacin and 50 µl (100 µg/ml) of gluthethimide. The blood standards and samples were extracted simultaneously. The concentration of indomethacin in the blood samples

was determined using the blood standards as the calibrating solutions. This procedure eliminated the need for correction due to recovery losses.

Calculations

The peaks on the chromatogram were identified by their retention times relative to the internal standard. The concentration of indomethacin was calculated on the basis of the ratio of its peak height to that of glutethimide internal standard.

RESULTS AND DISCUSSION

Sensitivity and Linearity

The detection limit was 12.5 nanograms of indomethacin in 5 µl methanol injected into the chromatograph.

The detector response curve was linear in the range of 0.50 to 10 µg/ml for indomethacin solutions ($y = 0.13 + 0.56x$; corr. coeff. 0.993).

Recovery

The recovery rates after extraction from blank blood of indomethacin and glutethimide were 96±1 and 93±3%, respectively.

Indomethacin Blood Levels in Rats

A characteristic chromatogram of a blood sample from a rat administered 2 mg/kg indomethacin is shown in Figure 1. The run was completed in 10 minutes, and interfering peaks were not observed in the blood samples. The concentrations of indomethacin in blood of rats receiving the drug by gavage at a dose of 2 mg/kg are shown in Figure 2.

The simple extraction and the short time required for analyses of blood samples make this method very economical and fast for the analyses of a great number of samples. Furthermore,

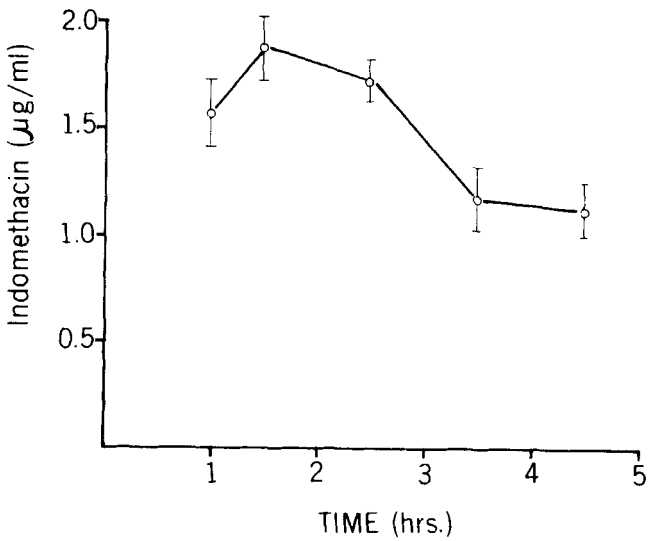

FIGURE 2. Indomethacin concentration in blood of rats receiving 2 mg/kg indomethacin orally. Each point represents the mean value of 6 samples ± S.E.M.

the small volume of sample needed allows for the determination of complete time-blood level curves for indomethacin in individual rats at reasonable times without significant risks to the animals.

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