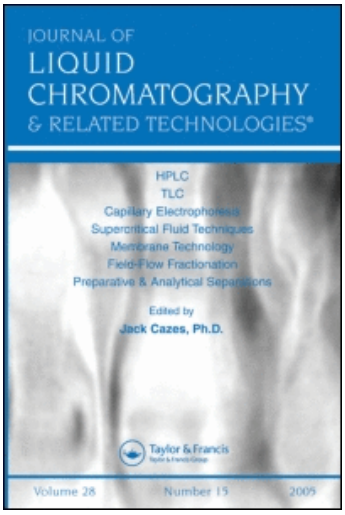

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QUANTITATION OF INDOMETHACIN IN SERUM BY HPLC USING FLUORESCENCE DETECTION

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ABSTRACT

An improved, sensitive and accurate HPLC procedure using fluorescence detection for quantitation of indomethacin in serum has been developed. After addition of an equal volume of phosphate buffer, pH 6.6 to serum along with the internal standard, the samples were extracted with methylene chloride. Prior to chromatography, the extracted indomethacin was deacylated to its fluorescent product (DBI) in 0.01 N-NaOH. The mobile phase consisted of methanol and pH 4 acetate buffer (3:7 V/V) and the separation was achieved on a C18 reversed phase column. The retention times of DBI and the internal standard were 7.5 and 16.0 min. respectively. The fluorometric excitation and emission wavelengths were 278 and 358 nm, respectively. The sensitivity of the assay was 1 ng/ml of serum and the CV at this concentration was 2.46%. The standard plot was linear ($r > 0.999$) for indomethacin concentrations between 1 and 500 ng/ml. The

inter- and intra-day studies showed high reproducibility (CV = 2.8%, F = 0.89, p >0.05). The method was used to determine the complete serum level vs. time profiles of indomethacin in animals.

INTRODUCTION

Indomethacin, 1-(p-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetic acid, a potent inhibitor of prostaglandin synthesis, is widely used as an antiinflammatory, antipyretic and analgesic agent.

Plasma concentration time profiles of indomethacin were found to vary considerably after equal oral doses in different patients which may account for the wide range of pharmacologic responses and an increased incidence of untoward side effects in certain individuals (1). In this regard, further pharmacokinetic studies of this drug are needed to elucidate the possible causes of large individual variability in drug absorption and blood levels using a highly sensitive and specific method of quantitation of this drug in biological fluids. Various procedures have been reported for the analysis of indomethacin in biological samples including gas-liquid chromatography (GLC) with electron-capture detection after derivatization (2-4), GLC with selective-ion monitoring (5), radioimmuno-assay (6), spectrofluorometric methods (7,8) and high-pressure liquid chromatography (9-13).

A radioimmuno-assay (6) was reported that can quantitate indomethacin in plasma as low as 50 ng/ml, but the method is nonspecific since the assay is highly cross reactive to the glucouronide conjugate of indomethacin and its other known metabolites such as desmethyl indomethacin.

Most GLC methods employed a derivatization of the drug as an integral part of the assay. More recently, several HPLC methods with either UV (9-11) or fluorescence detection (12,13) have been developed as an alternative to GLC. UV detection methods appeared to offer the sensitivity limits down to near 20 ng per ml of sample used. An HPLC method using alkaline hydrolysis of indomethacin to a fluorophore and its subsequent quantitation using fluorescence detection was first developed by Bayne et al. (12) to increase the assay sensitivity compared to UV detection. Although these procedures achieved higher sensitivity, they also have certain shortcomings such as the presence of interfering peaks from serum extracts (12,13).

The present study describes an improved HPLC method for quantitating indomethacin in serum after converting the extracted indomethacin into its fluorescent compound in an alkaline medium. The amount of serum required for analysis was 200 ul or less and the total time needed to complete the assay of ten samples was relatively short (about three hrs). The sensitivity of the assay was 1 ng/ml of indomethacin per ml of serum, which is about 25 times more sensitive than those previously reported by Bernstein

and Evans (13). In addition, the limit of detection found in this study was near 0.2 ng which is about 7.5 times higher than that shown by Bayne et al. (12).

EXPERIMENTAL

Materials

The following chemicals were used in this assay as received from their suppliers without further purification: indomethacin (Sigma Chemical Co., St. Louis, MO), α-methyl indomethacin (Merck Sharp & Dohme Co., Rahway, NJ), methanol HPLC, methylene chloride HPLC, acetic acid HPLC (J.T. Baker, Chemical Co., Phillipsburg, NJ) and water (HPLC grade). The drugs and chemicals used were of U.S.P. or A.C.S. quality.

Instrumentation

An isocratic HPLC equipped with a single pump (Model M-45, Waters, Milford, Mass.), a fixed volume injector with a 50 ul sample loop and a fluorescence detector (Model RF-530, Shimadzu, Kyoto, Japan) was used and operated at ambient temperature. The excitation wavelength was set at 278 nm and the emission wavelength at 358 nm. A stainless steel reversed-phase NOVA-PAK C18 column, 3.9 mm x 150 mm (Waters, Milford, Mass.) containing 4 um spherical silica was used with a C18 uptight precolumn, 30-40 um pellicular packing (Upchurch Scientific, Winter Park, Florida). Chromatograms were recorded on a strip chart recorder

(Esterline Anug, Esterline Co., Rainin, Woburn, Mass.) at a speed of 150 mm/hr.

Mobile Phase

The mobile phase consisted of 0.05 M acetate buffer (pH 4) and methanol (7:3 V/V). The mixture was filtered through a micro-filtration system with a type HA 0.45 um millipore filter paper and degassed by pumping helium gas for 5 min prior to use. Column equilibration with the mobile phase was maintained by pumping the mobile phase at a rate of 0.2 ml/min for overnight. The flow rate during the assay was set at 1.5 ml/min.

Selection of the Internal Standard

Because of the use of fluorescence detection for the quantitation of deschlorobenzoyl indomethacin (DBI), the hydrolyzed product of indomethacin, the choice of an internal standard exhibiting desirable chromatographic properties was limited. Among the compounds tested, α-methyl indomethacin was chosen as the internal standard for the assay from the considerations of its high similarity to indomethacin with respect to the solvent extractability, high peak resolution and adequate retention time. The absence of any interfering peaks at its retention time from the serum extract was also considered to be important for the choice of the internal standard.

Selection of the Extraction Solvent

Among different solvents (methylene chloride, methyl t-butyl ether, ethyl acetate, n-heptane, n-hexane and cyclo-hexane)

tried, methylene chloride was chosen with the considerations of highest recovery and absence of interfering peaks at the retention times of both the drug and its internal standard.

Stock Solution

A stock solution containing 10 ug/ml of α-methyl indomethacin was freshly prepared by dissolving 1 mg in 5 ml of methanol and further diluting the volume to 100 ml with HPLC water. The stock solution of indomethacin (10 ug/ml) was prepared by dissolving 1 mg of indomethacin in 5 ml of methanol and further diluting the volume to 100 ml with HPLC water, and was used within no longer than 7 days. Standard solutions of indomethacin in the concentrations between 1 and 500 ng/ml and containing 100 ng of α-methyl indomethacin were freshly prepared by diluting appropriate quantities of the stock solutions with HPLC water for the construction of standard calibration plots. The stock solutions were also used to prepare the samples for the recovery of the drug from spiked serum samples.

Sample Preparation and Analysis

Aliquots of blank serum (0.2 ml) placed in a 15 ml glass centrifuge tubes with teflon lined caps were spiked with varying amounts of the indomethacin stock solution to prepare the final drug concentration between 1 and 500 ng/ml. A stock solution containing 100 ng of α-methyl indomethacin in 0.2 ml of phosphate buffer (pH 6.6) was added to each of the standard solutions prepared with gentle shaking. These samples were mixed with 10

ml methylene chloride and shaken for 20 min using a mechanical shaker at high speed before being centrifuged at 2500 rpm for 5 min. After aspirating the upper aqueous layer, the remaining organic phase was slowly evaporated to dryness under nitrogen at 60°C. The residue was reconstituted in 1 ml of 0.01 N sodium hydroxide to deacylate indomethacin to its fluorescent product, DBI. The samples were gently shaken for 10 min on a mechanical shaker to complete the reaction. Fifty ul of the resulting solutions was directly injected onto the chromatograph for quantitation.

Effect of NaOH Concentrations on DBI Fluorescence

Three different concentrations of sodium hydroxide (0.01, 0.05 and 0.1 N) were used to test the effect of alkalinity on the intensity of fluorescence developed after deacylation of indomethacin. The fluorescence of DBI formed was measured by comparing peak height ratios of DBI to the internal standard obtained after dissolving 100 ng of indomethacin and 100 ng of the internal standard in 1 ml of 0.01, 0.05 and 0.1 N sodium hydroxide solutions. Six replicate samples for each of the three NaOH concentrations were similarly processed for statistical evaluation.

Effect of Buffer pH on Drug Recovery

The extraction efficiency of indomethacin from spiked serum samples was determined by comparing peak height ratios of DBI to the internal standard obtained after direct injection of the

known amounts of indomethacin (100 ng) and the internal standard (100 ng) dissolved in 1 ml of 0.01 N NaOH with those obtained after extracting the spiked serum samples containing the same amounts of indomethacin and internal standard. Three phosphate buffers (pH 5.0, 6.6 and 7.2) were mixed with the equal volume of the serum samples to study the effect of buffer pH on the extraction of indomethacin. Six replicate samples were prepared and processed for each pH.

Standard Plots

Standard plots were prepared for the spiked serum samples (0.2 ml) containing 1, 3, 10, 20, 40, 80, 100, 200 and 500 ng indomethacin and 100 ng of the internal standard per 1 ml of serum followed by the addition of 0.2 ml pH 6.6 phosphate buffer. Peak height ratios of DBI to the internal standard for the standard samples were plotted as a function of indomethacin concentrations.

Reproducibility

Reproducibility of the assay was tested by repeating the standard plot at three different days over one week period. Three replicate samples for each standard concentration were employed for statistical analysis of the intraday variation.

Quantitation

After subjecting spiked or dosed serum samples to the described extraction and chromatographic procedures, the amount of indomethacin was determined by comparing the peak height

ratios of DBI formed to the internal standard obtained from unknown samples using the standard calibration plots.

Animal Study

To demonstrate the applicability of the assay to the quantitation of indomethacin in serum, a single bolus dose of 2 mg/kg in an aqueous propylene glycol solution (1:1 V/V) was administered into the cephalic vein of three beagle dogs with an injection volume of approximately 2 ml and injection time less than one minute. Blood samples (3 ml) were withdrawn from the jugular vein using vacutainer tube at the time intervals of 0, 0.17, 0.33, 0.5, 0.66, 1, 2, 4, 6, 8 and 12 hrs after dose. Serum was obtained after centrifugation and stored in a screw-capped plastic vial at -20°C until assay, usually within a week after serum collection.

RESULTS

Chromatograms obtained at the highest sensitivity setting used in the study for drug free serum extracts showed no interfering peaks at the retention times of DBI and the internal standard, α-methyl indomethacin. Representative chromatograms for the samples prepared from blank dog serum (A), serum spiked with 100 ng/ml of indomethacin (B), and sample containing the drug (100 ng) and α-methyl indomethacin (100 ng/ml) (C) are shown in Figure 1. Using the chromatographic conditions described, the

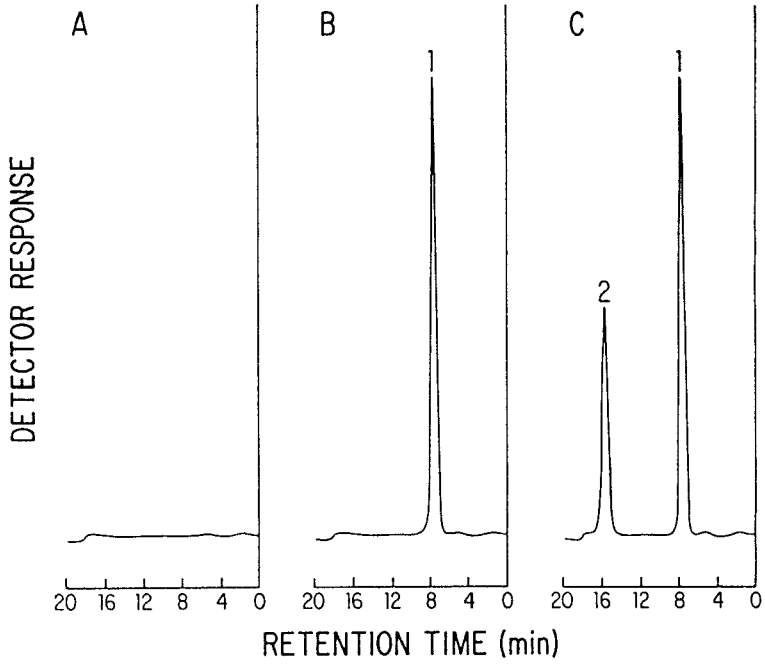

Figure 1. Chromatograms of blank dog serum (A), serum spiked with indomethacin (B), and serum spike with indomethacin and internal standard (C). Key: 1, DBI; 2, Internal Standard

internal standard and DBI were well separated and their respective retention times were 7.5 and 16.0 min, respectively. Both peaks were sharp and symmetrical with good baseline resolution, thus facilitating accurate measurement of the peak height ratios. No extraneous peaks due to unknown metabolites or other constituents of serum were observed.

A standard plot obtained for serum samples was highly linear in the concentration range studied (1-500 ng/ml) as shown in

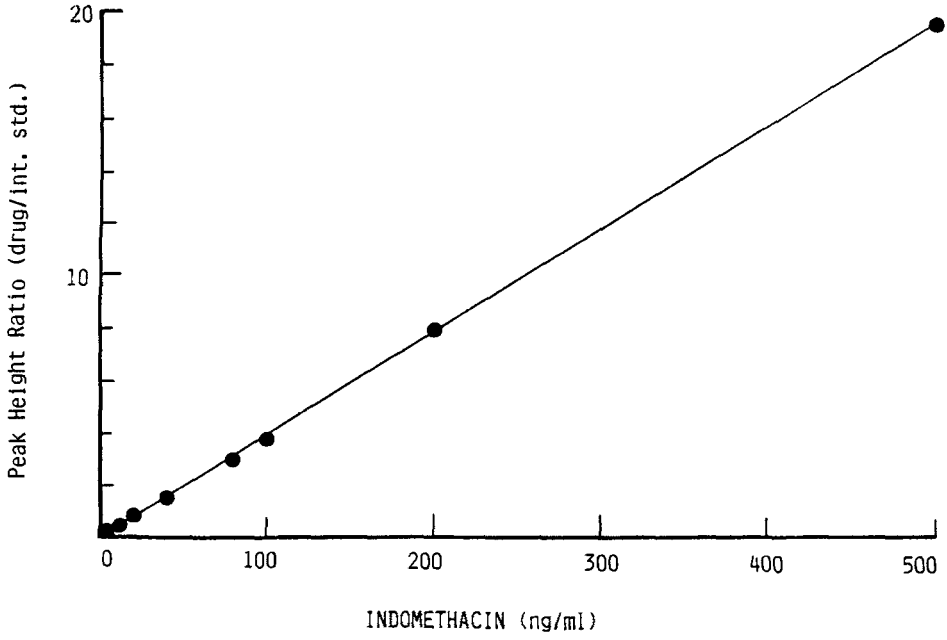

Figure 2. Typical calibration plot of indomethacin in dog serum.

Figure 2. Linear regression analyses of the standard calibration plots obtained on three different days are represented in Table 1. The intercepts of the calibration lines showed no significant deviation from zero, indicating that blank serum has negligible interference for the analyte. The correlation coefficient of the plots was all greater than 0.999.

The day-to-day reproducibility of the assay was evaluated by comparing the linear regression analyses of the three standard plots obtained on three different days over a one week period. The average correlation coefficient was greater than 0.999 and

TABLE 1

Interday Reproducibility of the Standard Plots

Standard Plot[a]	Slope	Intercept	r[b]
Day 1	0.0397	0.0039	0.9997
Day 2	0.0386	0.0247	0.9996
Day 3	0.0392	-0.0090	0.9998

[a] Mean of 3 determinations

[b] Coefficient of variation

the coefficient of variation of the slopes of the three lines was near 2.8%. Analysis of variance of these data indicated no detectable difference in the slopes of these calibration plots obtained on three different days during a 7-day period ($F=0.89$, $P > 0.05$). These results confirm the linearity of the calibration plot and the excellent reproducibility of the assay method with little variation in the slopes of the calibration line from day to day. The method is thus considered accurate and precise within the assay day as well as between days.

The results shown in Table 2 indicate that the average recovery of the drug from spiked serum samples after diluting with an equal volume of pH 5.0, 6.6 and 7.2 phosphate buffer was

TABLE 2

Effect of Buffer pH on Indomethacin Recovery

pH	Drug Conc. ng/ml	n[a]	Recovery (X ± SD)	CV %
5	100	6	85.9 ± 8.2	5.03
6.6	100	6	102.2 ± 7.5	5.51
7.2	100	6	93.9 ± 4.2	3.68

[a] Number of replicates

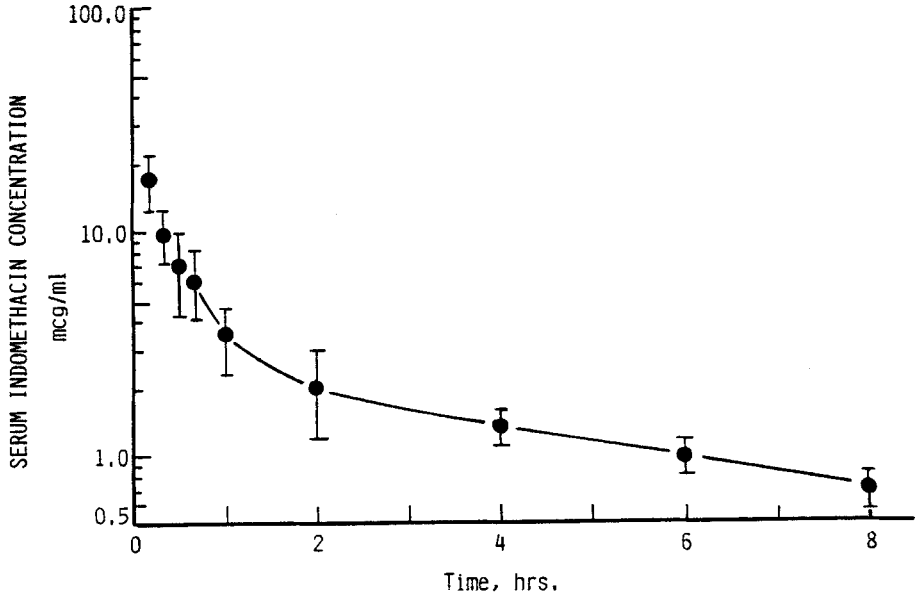

Figure 3. Average serum concentration of indomethacin in three dogs after IV bolus dose (2 mg/kg) of indomethacin.

approximately 85, 102 and 93%, respectively, with their respective coefficient of variation equal to 5.0, 5.5, and 3.7%. It was concluded that the highest recovery was obtained when the drug was extracted from serum diluted with the pH 6.6 buffer.

The minimum detectable amount defined as the amount in nanograms that gives peak height equal to twice the back ground noise was 0.2 ng of indomethacin. The sensitivity of the assay defined as the minimum concentration that can be quantitated with a statistically acceptable coefficient of variation in the peak height ratio was 1 ng/ml with CV equal to 2.5%.

The effect of sodium hydroxide concentration on the fluorescnece of the deacylated indomethacin was evaluated by comparing the peak height ratios of DBI with that of α-methyl indomethacin which were obtained after deacylating indomethacin

TABLE 3

Relative Fluorescence Intensity of DBI Formed[a] in Different Sodium Hydroxide Concentrations Used

NaOH Conc,N	n[b]	Mean Relative Intensity	CV %
0.01	6	2.1	2.9
0.05	6	1.2	8.3
0.10	6	1.0	6.6

a Indomethacin concentration, 100 ng/ml
b Number of replicate

in three different sodium hydroxide solutions (0.01, 0.05 and 0.1 N). Six replicate samples were measured at each concentration and the results are shown in Table 3. It was found that 0.01 N sodium hydroxide gave the highest mean peak height ratio which is at least twice higher than the values obtained using .05 and 0.1 N NaOH solutions, respectively.

DISCUSSION

The sensitivity of the assay for indomethacin in serum developed in this study (1 ng/ml) is approximately 25 times higher than that in the assay described by earlier reports using fluorometric detection (12,13). In the chromatograms shown by Bernstein and Evans (13), extraneous interfering peaks were present near the retention time of the internal standard used in the assay. Although Bayne et al. (12) reported that the lower limit of detection of indomethacin using a fluorometric detector was 1.5 ng/ml, nothing was mentioned about the limit of sensitivity. The lowest standard concentration shown in the report was 30 ng/ml. Bernstein and Evans (13) measured the fluorescence of hydrolyzed indomethacin after neutralizing the reconstituted 0.1 N sodium hydroxide with 0.1 N HCl solution. According to the preliminary data found in this study, the addition of 0.1 N HCl appeared to reduce the fluorescence intensity of DBI formed.

It was also found in this study that the fluorescence of DBI developed from the same molar concentration of indomethacin was lower at the higher alkalinity of the reacting solutions used as shown in Table 3. These results are in agreement with those reported by Hvidberg et al. (8). In their investigation on hydrolytic conditions for a spectrofluorometric assay of indomethacin, the authors showed that the fluorescence was developed slower but to a greater intensity in less alkaline solutions than 0.1 N NaOH. Therefore, in this study, 0.01 N NaOH was used to hydrolyze the extracted indomethacin from serum samples since the fluorescence developed in this NaOH solution in 30 min was approximately twice as high as that obtained in 0.1 N NaOH solution. Although the DBI fluorescence formed remained stable for at least 8 hrs at the ambient temperature the measurement of fluorescence was performed immediately after the completion of the hydrolytic reaction of indomethacin in the extracted samples. The finding that indomethacin was completely extracted into methylene chloride from serum samples diluted with equal volume of pH 6.6 buffer where almost complete ionization of indomethacin was expected is in close agreement with the results of Upton et al (14) who showed that other acidic drugs such as ketoprofen and naproxen were quantitatively extracted into ether from the medium of pH near 7.

The sensitivity of the assay for indomethacin in serum samples using this method is 1 ng/ml using 1 ml sample volume

which is approximately 25 times more sensitive than that reported by previous authors using fluorescence detection (12,13). The high sensitivity of the method could be attributed to the improved recovery of the drug using a less acidic serum pH and increased fluorescence intensity of DBI by reacting indomethacin in a less alkaline condition than the previous methods. The sensitivity of this method should allow the use of small sample volume (less than 0.1 ml) and quantitation of tissue drug levels in small experimental animals. In addition, the current assay is less time consuming than other HPLC methods requiring tedious sample clean-up procedures.

The applicability of the assay was demonstrated by monitoring the plasma level versus time profile of indomethacin in three beagle dogs weighing 9.7-11.0 kg after receiving a bolus IV dose of 2 mg/kg. All three dogs showed a biexponential blood level profile indicating that the disposition of the drug in these dogs could best be described by the two-compartment pharmacokinetic model as represented by:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p is the plasma indomethacin concentration, A and B are the intercepts on the concentration axis, and α and β are the first order hybrid rate constants for the rapid and slow disposition phases, respectively (15). The apparent half-life for the β phase was very short (~ 10 min) and the half-life for the terminal elimination phase was 5 hr. From 0 to 12 hr post-

dose, the plasma levels of indomethacin in the dogs were approximately in the range of 1.0-20.0 ug/ml. The volume of distribution at the phase (Vd) was ~10 l indicating that the drug was mostly distributed into the body fluid (16).

In conclusion, an improved reversed-phase HPLC method developed in this study is simple and very sensitive and could be used for pharmacokinetic and bioavailability studies of indomethacin in man and tissue distribution studies in small laboratory animals.

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