High-Pressure Liquid Chromatographic Method with Postcolumn, In-Line Hydrolysis and Fluorometric Detection for Indomethacin in Biological Fluids

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Abstract □ A rapid and sensitive method for the analysis of indomethacin in plasma and urine was developed using high-pressure liquid chromatography, postcolumn, in-line hydrolysis of indomethacin to a fluorophore, and detection of the fluorophore with a fluorometer. The lower limit of detection was 1.5 ng/ml of plasma. The coefficient of variation at 30 ng/ml of plasma was 4.5% (n = 5). The nonconjugated metabolites of indomethacin, aspirin, and salicylate were resolved from indomethacin and the internal standard, α-methylindomethacin.

Keyphrases □ Indomethacin—high-pressure liquid chromatographic determination using fluorometric detection, human plasma and urine □ High-pressure liquid chromatography—analysis, indomethacin, fluorometric detection following hydrolysis of indomethacin to fluorophore □ Anti-inflammatory agents—indomethacin, high-pressure liquid chromatographic determination using fluorescence detection

Indomethacin, a widely used anti-inflammatory, has been assayed in biological fluids and tissues by spectrofluorometry (1–6), radiometric methods (7, 8), GLC with electron-capture detection (9–12), GLC with selective-ion monitoring (13), high-pressure liquid chromatography (HPLC) (14–16), and TLC (17).

BACKGROUND

The original spectrofluorometric methods (1–5) were modified recently (6) to eliminate errors in the quantification of the drug caused by its metabolites, *N*-deschlorobenzoylindomethacin, *O*-desmethylindomethacin, and *N*-deschlorobenzoyl-*O*-desmethylindomethacin (7). The 5-*O*-desmethyl metabolites were destroyed by oxidation with cupric ion, and the *N*-deschlorobenzoyl metabolite resulting from alkaline hydrolysis of indomethacin was accounted for by a differential technique (hydrolysis *versus* no hydrolysis). The method was reported to be capable of quantifying 100 ng of indomethacin in 3 ml of plasma.

Isotope dilution analyses have been developed for indomethacin, its metabolites, and their conjugates in biological fluids (7). Although capable of quantifying the parent drug and its metabolites, the method requires administration of radiolabeled drug. Its principal use is in ascertaining the metabolic fate of the drug.

A radioimmunoassay for indomethacin was reported that can determine concentrations as low as 50 ng/ml (8). The assay is highly cross-reactive to the glucuronide conjugates of indomethacin and desmethylindomethacin and is slightly cross-reactive to desmethylindomethacin.

The GLC methods include derivatization of the drug to facilitate chromatography as an integral part of the assay. Esters of the drug have been prepared successfully by alkylation with diazoalkanes (diazomethane, diazoethane, and diazopropane) (9–12), by extractive alkylation with alkyl iodides (11), by alkylation in an organic solvent with alkyl iodides in contrast to extractive alkylations (11), and by silylation with bis(trimethylsilyl)acetamide (12). Diazomethane could be employed to esterify indomethacin without the concomitant methylation of the phenolic group of 5-*O*-desmethylindomethacin (12), which, if it occurred, could render the assay nonspecific.

To eliminate the necessity for derivatization and the potential hazards with the diazoalkanes, HPLC methods recently were developed as an alternative to GLC (14–16). The UV absorbance of the drug is used as a means of detection. Detection limits of 100 ng/ml of serum were reported for two of the HPLC methods, with absorbance of the drug being measured at 200 (15) and 235 (16) nm.

An HPLC method with postcolumn, in-line, alkaline hydrolysis of indomethacin to a fluorophore and measurement of the generated fluorophore with a fluorometric detector was developed to: (*a*) increase the sensitivity above that achievable with a UV absorbance detector and (*b*) reduce the biological background relative to indomethacin in extracted samples by increasing the specificity in detection. Furthermore, since hydrolysis was performed in-line, sample preparation and workup were considerably less intensive than with conventional spectrofluorometric methods.

EXPERIMENTAL

Apparatus—A liquid chromatographic system was assembled from two high-pressure reciprocating pumps[1]; a stainless steel chromatographic column (25 cm × 4 mm i.d.) packed with microparticulate material[2]; an automatic injector[3]; coiled polytef tubing (4.8 m × 0.7 cm i.d.), which served as a postcolumn, in-line reactor; a water bath[4] (64°) in which the polytef tubing was immersed; a T-fitting[5], which served to connect the reactor, one pump, and the column; and a fluorescence detector[6]. Fluorescence was quantified with an integrator-calculator[7].

Samples were chromatographed in the reversed-phase mode. An alkaline solution was introduced into the mobile phase exiting the column to hydrolyze indomethacin and the internal standard (α-methylindomethacin) to fluorophores. The fluorophores were excited at 295 nm with the detector, and fluorescence of <340 nm was excluded with a cutoff filter.

Solutions—The mobile phase was 76% methanol in 0.025 *M* phosphate buffer (pH 4.0), prepared from monobasic potassium phosphate and phosphoric acid. The volumetric flow rate of the mobile phase was 1.0 ml/min.

The alkaline solution introduced into the mobile phase, postcolumn, was 0.1 *N* NaOH at a flow rate of 0.1 ml/min.

β-Glucuronidase[8] (0.5 mg/ml) was dissolved in 0.5 *M* citrate buffer (pH 5) prepared from citric acid monohydrate and sodium hydroxide.

Indomethacin[9] and α-methylindomethacin[9] were weighed accurately and dissolved in methanol to prepare working solutions of 1.4 and 7 μg/ml, respectively. Methanolic solutions (5 μg/ml) of *N*-deschlorobenzoyl-*O*-desmethylindomethacin[9], *N*-deschlorobenzoylindomethacin[9], and *O*-desmethylindomethacin[9] were prepared and used to ascertain the resolution of metabolites from indomethacin and the internal standard.

Plasma Extraction—Extraction of plasma samples was carried out in silanized glass, 40-ml, stoppered centrifuge tubes. Citrate buffer (1 ml of a 0.5 *M* solution, pH 5.0) and ethylene dichloride (10 ml) were added to the plasma samples (1 ml). The centrifuge tubes were sealed with polytef stoppers and shaken gently with a horizontal shaker for 20 min. They then were centrifuged at 1000×*g* for 5 min, and the upper aqueous layer was aspirated. The remaining organic phase was carefully poured into a conical test tube (15 ml) and evaporated to dryness under nitrogen in a 50° water bath. The residues were dissolved in methanol (100 μl), and the solution was mixed with a vortex mixer.

Extraction of Urine—Urine samples were prepared for analysis in a manner similar to that described for the plasma samples. In addition, prior to extraction with ethylene dichloride, β-glucuronidase (0.3 ml of a 5000-units/ml solution) was added to the buffered urine sample to hydrolyze the glucuronide conjugate. The contents were incubated at 37° for 30 min. These hydrolytic conditions (incubation time and amount

[1] Model 6000 A solvent delivery system, Waters Associates, Milford, Mass.
[2] ODS-Hypersil, Shandon Southern.
[3] WISP automatic sample processor, Waters Associates, Milford, Mass.
[4] Model 1420 Thermomix, B. Braun.
[5] Altech, Arlington Heights, Ill.
[6] Model FS970 fluorometer, Schoeffel Instrument Corp., Westwood, N.J.
[7] Model 4100, Spectra-Physics, Santa Clara, Calif.
[8] Type B-10 (10,000 units/mg), Sigma Chemical Co., St. Louis, Mo.
[9] Merck Sharp and Dohme Research Laboratories, West Point, Pa.

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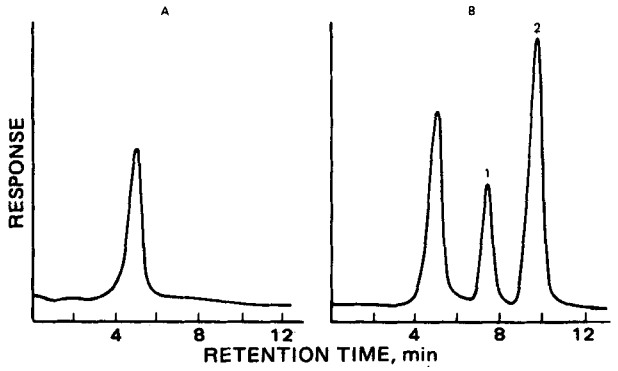

Figure 1—*Chromatograms from blank plasma (1 ml) (A) and from plasma (1 ml) containing 89.4 ng of indomethacin/ml (1) and 298 ng of α-methylindomethacin/ml (2) (B).*

of enzyme) produced the maximum amount of free indomethacin in urine samples from subjects previously administered the drug.

Standard Curves—Standard curves were generated by spiking blank plasma and urine samples (1-ml volumes) with varying amounts of indomethacin and a constant amount of internal standard. Microliter aliquots of the working solutions or a 1:10 dilution of the working solution were added to the biological samples. The internal standard concentrations in the plasma and urine samples were 0.25 and 2.5 μg/ml, respectively. The indomethacin concentration ranges in the plasma and urine samples were 0.03–3.0 and 0.3–30 μg/ml, respectively.

The samples were prepared according to the extraction procedures described. Plots of the peak area ratios (indomethacin to internal standard) against the respective indomethacin concentrations were linear. Typical standard curves for indomethacin in plasma and urine had a correlation coefficient of 0.9999.

The coefficients of variation for five replicate assays at 0.03, 0.09, 0.3, 0.9, and 3.0 μg/ml of plasma were 4.5, 1.8, 1.2, 0.6, and 0.9%, respectively. The coefficients of variation for five replicate assays at 0.3, 0.9, 3.0, 8.9, and 29.8 μg/ml of urine were 2.8, 0.9, 0.9, 1.2, and 0.8%, respectively.

A typical chromatogram for a plasma extract is presented in Fig. 1. The retention times for indomethacin and the internal standard were 7.5 and 9.5 min, respectively. Chromatograms obtained from urine extracts over the concentration range studied were similar to those obtained from the plasma extracts; only the background peak at 5 min was discernible.

RESULTS AND DISCUSSION

The achievable detection limit for indomethacin in plasma using the method described is 1.5 ng/ml (1 ml of plasma volume). The method is as sensitive as GLC methods utilizing electron-capture detection [2 (12) and 5 (11) ng/sample] but does not require derivatization. The high sensitivity of the method is due in part to the detection specificity that results from a low biological background relative to indomethacin. The high sensitivity permits the use of biological samples of <1 ml.

The recoveries of indomethacin (400 ng/ml) and the internal standard (400 ng/ml) were >99% when the compounds were extracted from plasma using the described procedure. Helleberg (10) showed that the recovery of indomethacin was nearly quantitative (95%) when serum was buffered at pH 5.0 and extracted with ethylene dichloride. The recoveries decreased markedly when the pH was greater or less than pH 5.0

The maximum responses (peak area) for indomethacin and α-methylindomethacin were achieved by delivering 0.1 *N* NaOH at a volumetric flow rate of 0.1 ml/min, postcolumn, into the mobile phase. As the sodium hydroxide concentration or the volumetric flow rate was increased, the responses decreased. At optimal conditions, the pH of the mixed streams leaving the reactor coil was 9.0. Hvidberg *et al.* (4), in investigating hydrolytic conditions for a spectrofluorometric method, stated that the fluorescence develops slower but to a greater intensity in buffers that are less alkaline than 0.1 *N* NaOH.

The reactor coil was heated in a water bath to facilitate rapid, in-line conversion of indomethacin to the fluorophore. Under the hydrolytic conditions described, the response per mole of indomethacin was greater than that for deschlorobenzoylindomethacin, the presumed hydrolysis product. Hence, the hydrolysis product apparently is converted to a nonfluorescent species as it proceeds through the reactor coil. However, although processes secondary to the hydrolysis of indomethacin are likely to occur in the reaction coil, the method is sensitive and reproducible.

The peak shape and resolution also were improved markedly by heating the reaction coil. It is possible that the flow characteristics approached that of plug flow at the elevated temperature.

The three major nonconjugated metabolites of indomethacin, *N*-deschlorobenzoylindomethacin, *O*-desmethylindomethacin, and *O*-desmethyl-*N*-deschlorobenzoylindomethacin, all had retention times less than the retention time of indomethacin. Aspirin and salicylate also had shorter retention times. Therefore, these compounds did not constitute a source of error in indomethacin quantification.

By utilizing a solvent gradient (methanol and 0.01 *M* phosphate buffer, pH 4.0), the drug and metabolites could be resolved from each other within 15 min. Thus, with fluorometric detection, this method has the potential to quantify the metabolites in addition to indomethacin, as is the case with a recently published HPLC method using spectrophotometric detection (16).

The method has been used to analyze indomethacin in over 1000 biological samples collected for a multiple-dose bioavailability study[10]. No deterioration of column performance or of the assay as a whole was observed.

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