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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—FLUORESCENCE ANALYSIS FOR INDOMETHACIN AND METABOLITES IN BIOLOGICAL FLUIDS

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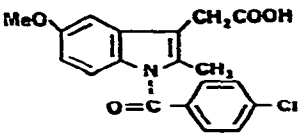
SUMMARY

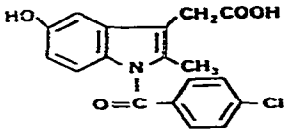
A rapid and sensitive high-performance liquid chromatography—fluorescence method is described for the quantitative analysis of indomethacin and its metabolites in urine. A modified version is also shown for the detection of indomethacin in plasma. The method consists of a single extraction of an acid-buffered plasma sample or single extractions of two buffered aliquots in parallel of urine using ethyl acetate, followed by evaporation of the organic phase. Indomethacin (I) and the metabolite desmethylindomethacin (DMI) were deacylated to their fluorescent products, deschlorobenzoylindomethacin (DBI) and desmethyldeschlorobenzoylindomethacin (DMBI), respectively, prior to chromatography. The chromatographic phase utilized a reversed-phase C_{18}-bonded column with a solvent system comprised of either 22.5% or 26% acetonitrile in 0.25% acetic acid. The elution times for indomethacin metabolites ranged from 12—26 min. The total DBI (including deacylated I) and DMBI (including deacylated DMI) in the extract were each determined using fluorometric detection, with excitation at 288 nm and emission at 390 nm (370 nm cutoff filter). An internal standard of indole-3-propionic acid was used for quantitation. The lower limit of sensitivity for I in plasma was 25 ng/ml.

INTRODUCTION

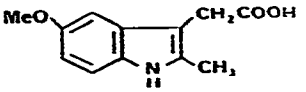
Indomethacin (I) is an anti-inflammatory drug widely used in the treatment of arthritic disease [1, 2]. Recent clinical studies have demonstrated that I may also be effective as a pharmacologic alternative to surgery in closure of the patent ductus arteriosus (PDA) occurring in premature infants [3, 4]. Closure of the fetal pulmonary-aortic shunt is believed to be prevented by the dilatory effects of prostaglandin E on the smooth muscle lining the ductus [5, 6]. Indomethacin blocks the initial step in the conversion of arachidonic acid to prostaglandins through inhibition of the cyclo-oxygenase enzyme [7].



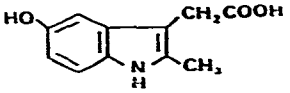
Indomethacin



DMI



DBI



DMBI

Fig. 1. The structural formulas of indomethacin and its metabolites: indomethacin (I) is 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid, M.W. 358; deschlorobenzoylindomethacin (DBI), M.W. 220; desmethylindomethacin (DMI), M.W. 344; desmethyldeschlorobenzoylindomethacin (DMBI), M.W. 206.

Methods for the quantitation of I in physiologic fluids have included spectrofluorometry [8, 9], gas chromatography (GC) [10—12], GC—mass spectrometry (GC—MS) [13], high-performance liquid chromatography (HPLC) [14, 15] and radioisotope dilution [16]. The HPLC—fluorescence assay to be described provides for a sensitive determination of not only I but also its three major metabolites, deschlorobenzoylindomethacin (DBI), desmethylindomethacin (DMI) and desmethyldeschlorobenzoylindomethacin (DMBI) (Fig. 1), in urine. In addition, this technique provides a level of sensitivity approaching that of GC but without the attendant difficulties. The utility of this sensitive assay is evident when considering the restricted therapeutic range of I in neonates [17, 18]. The correlation of I levels in plasma and I plus its metabolites in urine, with administered dose and therapeutic efficacy in PDA treatment, is of current interest [19].

EXPERIMENTAL

Reagents

Indomethacin and indole-3-propionic acid (I3P, internal standard) were

purchased from Sigma (St. Louis, MO, U.S.A.). Desmethylindomethacin was a gift from Merck (West Point, PA, U.S.A.). Spectrograde ethyl acetate was supplied by Pierce (Rockford, IL, U.S.A.). Acetonitrile (UV-grade) was from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All solutions were prepared using double-distilled water.

Protocol

(A) Urine. Analysis of I urine metabolites was based upon parallel extraction of two urine samples. One sample was used for determination of the total (I + DBI) and the total (DMI + DMBI) in the extract following alkaline hydrolysis, while the other sample extract was maintained in 100% ethanol for quantitation of directly extractable DBI and DMBI. Subtraction of the DBI found in the unhydrolyzed from that observed in the hydrolyzed sample extract provided individual values for both I and DBI. An analogous procedure was applied for quantitation of DMI and DMBI in urine.

(B) Plasma. I concentration in plasma was determined by alkaline hydrolysis of the solvent extract and quantitation of the observed DBI chromatographic peak.

Procedure

(A) Urine. Parallel 0.5-ml urine samples were buffered to pH 5.0 using 0.45 ml of 0.2 *M* sodium acetate buffer and extracted with 4.4 ml of ethyl acetate. A 50-μl aliquot of 10 μg/ml I3P (dissolved in pure ethanol) was added to the sample prior to extraction. The solvent extract in both samples was removed and evaporated to dryness using a vacuum centrifuge. One extract was redissolved in 50 μl of 0.1 *N* sodium hydroxide and allowed to stand for 15 min, then diluted with 0.4 ml of water and neutralized with 50 μl of 0.1 *N* hydrochloric acid. This sample was used for determination of total (I + DBI) and total (DMI + DMBI). The extract from the second sample was redissolved in 100 μl of pure ethanol and diluted with 0.4 ml water. This sample was used for directly extractable DBI and DMBI determinations. Approximately 50 μl from each sample were injected onto the column. Conjugated metabolites may be determined by overnight treatment with β-glucuronidase prior to extraction [16, 20].

(B) Plasma. A 100-μl aliquot of plasma was mixed with 100 μl acetate buffer (0.2 *M* sodium acetate, pH 5.0) and 50 μl of I3P (0.4 μg/ml). The mixture was extracted with 1.0 ml of ethyl acetate and the organic phase evaporated using a vacuum centrifuge. The residue was redissolved for 15 min in 40 μl of 0.1 *N* sodium hydroxide and then neutralized with 40 μl of 0.1 *N* hydrochloric acid. Approximately 60 μl from each sample was injected onto the column.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump coupled with a Waters Model U6K injector was utilized for the solvent delivery system. Separation of the individual metabolites was performed using a Whatman (Clifton, NJ, U.S.A.) 15% C_{18}-bonded reversed-phase column (25 cm × 4.6 mm) with 10-μm particles, together with a mobile phase of either 22.5% or 26% acetonitrile—0.25% acetic acid.

Fluorescence was measured with a Schoeffel (Westwood, NJ, U.S.A.) Model FS 970 fluorometer, using sensitivities in the range 0.02—0.5 μA. The excitation wavelength was 288 nm with emission monitored at 390 nm; a 370-nm cutoff filter was used in emission detection. A Hitachi (Tokyo, Japan) Model 100-30 variable-wavelength spectrophotometer was used to measure ultraviolet absorption at 285 nm.

Mass spectrometry

A Varian MAT (Dayton, OH, U.S.A.) Model 112S mass spectrometer in electron ionization mode, with an interactive spectral data system, was used to obtain mass spectra.

Calibration

Urine samples. Peak height ratios (PHR's) were computed for a series of I and DMI standards in drug-free urine, ranging from 0.25—25.0 μg/ml, using a chromatographic solvent system comprised of 22.5% acetonitrile—0.25% acetic acid with a flow-rate of 1.5 ml/min. A DBI and DMBI series of standards were also prepared, with a range of 0.15—12.0 μg/ml, and analyzed using a 26% acetonitrile—0.25% acetic acid solvent system with 2.0 ml/min flow-rate. In both cases the internal standard (I3P) concentration was 1.0 μg/ml in the sample injected onto the column.

Plasma samples. A standard series in the range 25—200 ng/ml of I in drug-free plasma was prepared and analyzed using a 22.5% acetonitrile—0.25% acetic acid mobile phase with a flow-rate of 2.0 ml/min. Peak height ratios were calculated and plotted against I concentration based upon an I3P concentration of 250 ng/ml in the injection sample (Table II).

RESULTS AND DISCUSSION

Both DBI and DMBI are capable of fluorescing while I is devoid of such activity [8]. DMI has fluorescent properties, however, it did not appear in HPLC—fluorescence chromatograms providing separation of DBI and DMBI despite the allowance of a 30-min elution interval [8]. Therefore, it was decided to measure DMI in its deacylated state, that is, as DMBI. Deacylation of I and DMI was accomplished by exposure to dilute alkali [20, 21]. Initial studies showed that I and DMI were completely converted by 0.1 *N* sodium hydroxide to the strongly fluorescing metabolites, DBI and DMBI, respectively, within 15 min [21]. The criteria for completeness of transformation were based upon (1) HPLC with UV detection and (2) mass spectrometry.

HPLC—UV

I and DMI were dissolved in pure ethanol, evaporated to dryness, treated with either 0.1, 0.25 or 0.5 *N* sodium hydroxide for intervals varying from 2.5 to 60 min, followed by dilution with water and neutralization with equally concentrated hydrochloric acid. Samples were then introduced onto the chromatographic column, utilizing a 25% acetonitrile—0.25% acetic acid mobile phase and detected using UV absorption at 285 nm. Peak heights were observed to increase under alkali treatment for time points up to 15

min, reaching the same maximum regardless of alkali concentration. The concentration of I and DMI used was 100 μg/ml, significantly higher than levels anticipated in biologic fluids. The internal standard peak height was unaffected by sodium hydroxide.

Mass spectrometry

Samples of DBI and DMBI were prepared by dissolving I and DMI, respec-

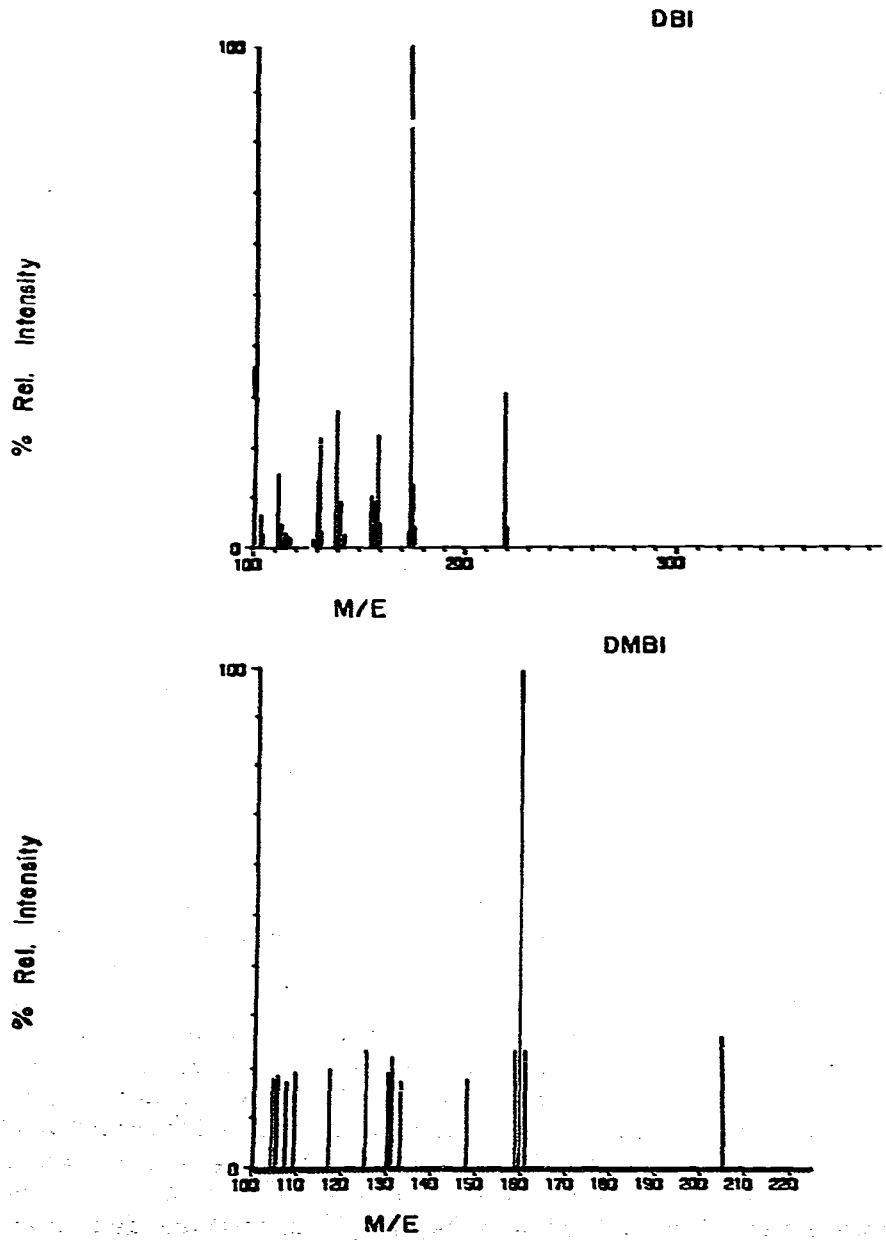


Fig. 2. Direct-probe electron ionization mass spectra of DBI and DMBI. DBI and DMBI were prepared by deacylation of I and DMI, respectively (see text).

tively, in pure ethanol and then evaporated to dryness. The residue was treated with 0.1 *N* sodium hydroxide for 15 min, diluted with water, neutralized with 0.1 *N* hydrochloric acid and extracted with ethyl acetate. The organic extract was dried and the residue placed into a crucible suitable for direct-probe MS. The alkaline-treated sample of I showed a molecular ion peak of *m/e* 219 with a base peak at *m/e* 174, corresponding to the molecular ion of DBI and its decarboxylated fragment, respectively. The alkaline-treated DMI sample exhibited a molecular ion peak of *m/e* 205 with a base peak at *m/e* 160, corresponding to the molecular ion of DMBI and its decarboxylated fragment, respectively. The mass spectra of unhydrolyzed I and DMI agreed with previously reported work [13, 22]. Mass spectra of DBI and DMBI are shown in Fig. 2.

Extraction efficiencies were determined by a comparison of peak heights, derived from HPLC—UV, of extracted versus non-extracted moieties. I and DMI were measured as DBI and DMBI, respectively. Extraction with ethyl acetate from the acetate buffer yielded efficiencies of 1.0, 1.0, 0.96, and 0.84 for I, DBI, DMI, and DMBI, respectively. Urine extractions showed somewhat lower efficiencies as summarized in Table I.

TABLE I

EXTRACTION EFFICIENCIES

I, DBI, DMI or DMBI (100 mg) was added to the sample medium, buffered to pH 5.0, extracted with ethyl acetate, dried and redissolved either in sodium hydroxide (I or DMI) or in ethanol (DBI or DMBI). An equal amount of the compound was dissolved either in sodium hydroxide or in ethanol for comparative purposes. Utilizing an HPLC solvent system of 25% acetonitrile—0.25% acetic acid with UV detection (285 nm), the mean peak height ratio of extracted/non-extracted sample for each compound was determined for extraction efficiency. Values reported are the mean of three separate extractions.

Medium	Metabolite			
	I	DBI	DMI	DMBI
0.2 *M* sodium acetate	1.0	1.0	0.96	0.84
Urine	0.94	0.92	0.92	0.75

Linear regression analyses of peak height ratios versus the concentration of compounds of interest yielded linear calibration curves. Eight concentrations each of I and DMI, in duplicate samples (with one exception) ranging from 0.25 to 25.0 μg/ml (with an internal standard of 10 μg/ml I3P), extracted from urine, provided curves with $r^2 \geq 0.99$. Similar analyses of DBI and DMBI, ranging from 0.15 to 12.3 μg/ml and extracted from urine, resulted in curves with $r^2 > 0.99$. Six concentrations of I, taken in triplicate or quadruplicate ($n = 21$), ranging from 25 to 200 ng/ml and extracted from plasma, yielded a calibration curve with $r^2 > 0.97$. A listing of these data is provided in Table II.

A given chromatogram (Fig. 3a) of a urine sample extract yielded three peaks of interest: (A) DMBI, (B) DBI and (C) I3P (in order of elution times). The concentrations of I and its metabolites in their unconjugated states were

TABLE II

CALIBRATION CURVE PARAMETERS

Linear regression analyses of the given data sets were carried out with Y-value being PHR (peak height of compound ÷ peak height of internal standard) and X-value being concentration (I, DMI, DBI or DMBI). n samples (including duplicate or triplicate determinations) were utilized for each calibration curve with r^2 (correlation coefficient) being a measure of linearity, b representing the Y-intercept and m indicating the slope of the regression. The consistency of data points was assessed with the mean coefficient of variation, $\overline{C.V.}$ as follows:

$$C.V._{x_j} = \frac{\sum_{i=1}^{n} \left(\frac{Y_i - \overline{Y_{x_j}}}{n} \right)}{\overline{Y_{x_j}}} \quad (100)$$

Y_i = the PHR's corresponding to concentration x_j from which the mean PHR, $\overline{Y_{x_j}}$, has been calculated.

$$\overline{C.V.} = \frac{1}{k} \left[\sum_{j=1}^{k} C.V._{x_j} \right]$$

Medium	Compound	Range (μg/ml)	n	b	m	r^2	$\overline{C.V.}$
Urine	I	0.25 —25.0	15	0.007	0.43	0.999	3.95
	DMI	0.25 —25.0	15	—0.06	0.32	0.999	6.19
Urine	DBI	0.15 —12.3	16	0.11	0.42	0.990	2.04
	DMBI	0.30 —12.0	14	—0.11	0.20	0.998	1.68
Plasma	I	0.025—0.20	21	—0.03	2.43	0.976	6.00

determined as follows:

(a) DBI and DMBI fluoresce and thus, were quantitated directly using peak height ratios.

(b) In a parallel sample, I and DMI were treated with sodium hydroxide, thereby converting the pair to DBI and DMBI, respectively; thus, the DBI and DMBI peaks observed in the chromatogram from hydrolyzed samples are a measure of (I + DBI) and of (DMI + DMBI), respectively.

(c) The apparent sample concentrations of I and DMI were determined by subtraction of the molar values obtained in the non-hydrolyzed samples from those of the sodium hydroxide-treated sample. However, the relative extraction efficiencies of DMI and DMBI must be considered upon calculation of the desmethyl metabolite levels; a simple subtraction is allowed only when extraction efficiencies are equal, as is the case for I and DBI.

Initial studies with plasma samples from infants receiving I failed to demonstrate the presence of metabolites. Thus, plasma samples were analyzed only for I (Fig. 3b).

The reason for the use of two different percentages of acetonitrile in the mobile phase was due to the unpredictable presence of endogenous compounds with peaks at the DMBI position of chromatographed urine samples. Similarly, a lengthy elution time is required for plasma samples due to endogenous interference with the DBI chromatographic peak. In spite of the 20 min

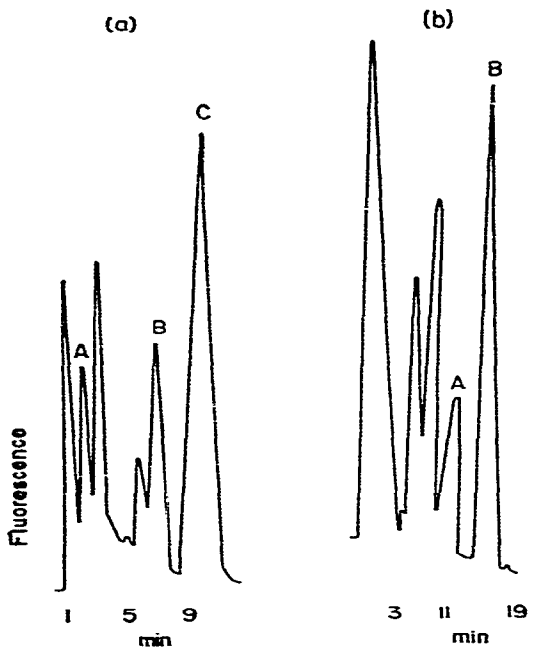


Fig. 3. HPLC chromatograms of indomethacin and two metabolites: (a) 0.60 μg/ml DMBI (A), 0.62 μg/ml DBI (B) and an internal standard, I3P (C), extracted from urine; a C_{18}-bonded reversed-phase column (25 cm × 4.6 mm) with a solvent system of 26% acetonitrile—0.25% acetic acid at 2.0 ml/min flow-rate and 0.1 μA fluorescence ($\lambda_{ex.}$ = 288 nm, $\lambda_{em.}$ = 390 nm) sensitivity was employed. (b) 100 ng/ml indomethacin (A), which has been converted to DBI (see text), with an internal standard, I3P (B), extracted from plasma: column as in (a) with a solvent system of 22.5% acetonitrile—0.25% acetic acid at 2.0 ml/min flow-rate and 0.02 μA fluorescence ($\lambda_{ex.}$ = 288 nm, $\lambda_{em.}$ = 390 nm) sensitivity was used.

allowed for plasma sample elution, endogenous plasma moieties would sometimes produce peaks coinciding with that of the internal standard. In these cases I concentration can be calculated using comparison of peak height with a standard curve. The present assay is currently being used in this laboratory for analyses of I and I metabolites in biological fluids.

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