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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF INDOMETHACIN AND ITS TWO PRIMARY METABOLITES IN URINE

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SUMMARY

Quantitation of total amounts (i.e., free compound plus glucuronide conjugate) of indomethacin (INDO) and its deschlorobenzoyl (DBI) and desmethyl (DMI) metabolites in human urine is described. An aliquot (0.4 ml) of urine is incubated with glucuronidase (1000 U, 2 h, 37°C) and extracted with 5 ml of dichloromethane containing the internal standards: the fluoro analogue of INDO, F-INDO, and indole-3-propionic acid (IPA). The organic phase is concentrated, dissolved in mobile phase and aliquots are injected onto the high-performance liquid chromatograph. INDO and DMI are measured with UV detection at 254 nm over a linear range of 0.25—125 μg/ml. Retention times for DMI, F-INDO and INDO are 4.0, 6.8 and 12.1 min, respectively, using a C_8 reversed-phase column with an acetonitrile—0.1 *M* acetate, pH 5.0 (30:70) mobile phase at a 2.5 ml/min flow-rate. DBI is measured using fluorescence detection (excitation = 305 nm, emission = 370 nm) over a linear range of 0.25—12.5 μg/ml. Retention times for DBI and IPA are 4.5 and 7.8 min, respectively, on the same C_8 column with an acetonitrile—0.025 *M* acetate, pH 4.0 (22:78) mobile phase at a 2.0 ml/min flow-rate. Inter- and intra-day precision were smaller than 10% for INDO, DMI and DBI over the concentration ranges indicated.

INTRODUCTION

Total urinary excretion of a drug and its primary metabolites is one measure of bioavailability and/or drug absorption. These measurements are currently recommended to aid in the assessment of indomethacin, [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid, INDO] absorption.

Although there have been numerous reports concerning the determination of INDO in urine, only a few have included an analysis of metabolites other than the glucuronic acid conjugate. INDO exhibits a significant amount of phase I

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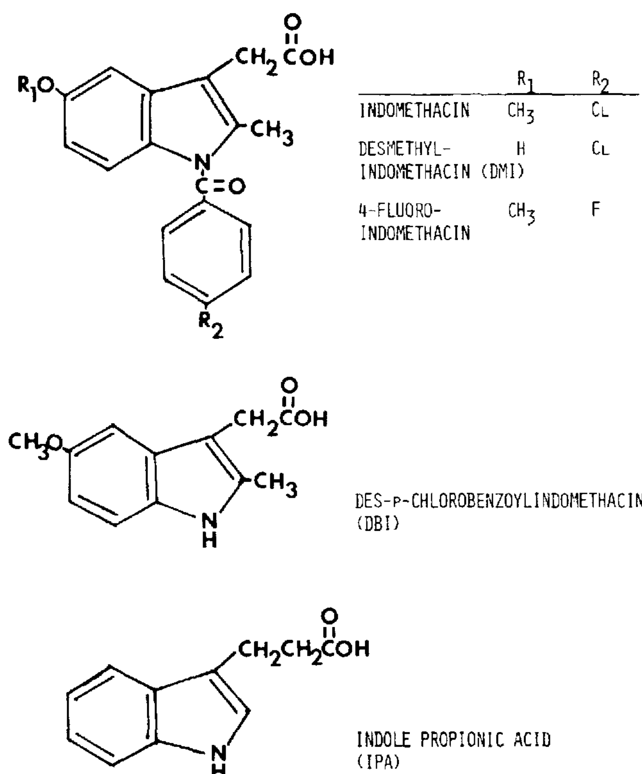

Fig. 1. Structures of indomethacin, its metabolites and the internal standards used in the analysis.

metabolism [1] to deschlorobenzoylindomethacin (DBI) and desmethylindomethacin (DMI). Both of these metabolites can also be subsequently conjugated with glucuronic acid or further metabolized to deschlorobenzoyldesmethylindomethacin (DMBI) which is a minor metabolite. INDO and its metabolites are depicted in Fig. 1. Two earlier reports on methods of analysis for INDO and its metabolites utilized radioactive [^{14}C]INDO with isotope dilution [1, 2]. Lack of labelled drug together with the difficulties of obtaining approval for radioisotope administration to man made this approach prohibitive. Bernstein and Evans [3] recently described a high-performance liquid chromatographic (HPLC) method for the analysis of INDO and its metabolites in both plasma and urine [3]. After alkaline hydrolysis INDO and DMI were measured using a difference method where the fluorescent products of hydrolysis, DBI and DMBI, were determined prior to and after alkaline hydrolysis. Besides the errors inherent in the difference method, the product DMBI is unstable in base [4, 5] which may lead to errors, unless the base hydrolysis is carried out under an inert atmosphere. Our appraoch was to measure the parent and metabolites in urine directly, after cleaving the glucuronic acid conjugates with β-glucuronidase. DMBI was not measured, as this was previously found to be a minor metabolite in the urine of man [1].

MATERIALS AND METHODS

Chemicals

Indomethacin, indole-3-propionic acid (IPA), and bovine liver, β-glucuronidase (Type B-10) were obtained from Sigma (St. Louis, MO, U.S.A.). DBI was commercially available from Aldrich (Milwaukee, WI, U.S.A.). DMI was initially synthesized by demethylation with pyridine hydrochloride as described [6]. A later report [7] mentioned DMI synthesis using boron bromide [4] which proved to be superior to the earlier method. 4-Fluoroindomethacin (F-INDO) (Fig. 1) was generously donated by Merck, Sharp and Dohme (West Point, PA, U.S.A.). Dichloromethane, acetonitrile and acetic acid were HPLC grade, obtained from Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were reagent grade.

Instrumentation

INDO and DMI were analyzed by UV detection. An HPLC pump, Series II, Perkin-Elmer (Norwalk, CT, U.S.A.) was combined with a Perkin-Elmer UV detector, LC-15. The wavelength used was 254 nm with an O.D. of 0.004 and a time constant of 1 sec. Injection of 50—100 μl was by a WISP® autosampler, Waters Assoc. (Milford, MA, U.S.A.). Output was recorded as peak area integration using a 3380A integrator, Hewlett-Packard (Palo Alto, CA, U.S.A.) set at an attenuation of 64 and slope sensitivity of 3. A mobile phase of 30% acetonitrile in 0.1 *M* sodium acetate, pH 5.0 was run at 2.5 ml/min at room temperature through a C_8 reversed-phase, 5 μm particle size, 150 mm × 4 mm I.D., Ultrasphere column, Altex (Berkeley, CA, U.S.A.). For fluorescence analysis of DBI a Perkin-Elmer Model 204A detector was set at an excitation wavelength of 305 nm and a slit of 10 nm, with an emission wavelength of 370 nm and a slit of 20 nm. PM gain was at 2 and sensitivity of 10 with a 10-mV output to a dual-pen recorder (Pedersen, Walnut Creek, CA, U.S.A.). For fluorescence analysis, sample sizes of only 3—5 μl were injected. The mobile phase used was acetonitrile—0.025 *M* sodium acetate, pH 4.0 (22:78) with a flow-rate of 2.0 ml/min at room temperature.

Procedure

The analysis was performed by the extraction of drug, metabolites and internal standard from 0.4 ml of urine after incubation with β-glucuronidase. To a 10-ml screw top tube was added 0.4 ml urine, then 0.4 ml of 0.5 *M* citrate buffer, pH 5.0 containing 1000 U of β-glucuronidase. The solution was mixed, then incubated with shaking at 37°C for 2 h. After incubation the solution was extracted with 5.0 ml of dichloromethane which had been spiked with the internal standards F-INDO and IPA at concentrations of 2.0 and 1.0 μg per 5.0 ml, respectively. The tube was then shaken (60 strokes per min) on a flatbed shaker of 15 min and any emulsion which had formed was broken by centrifugation in a clinical centrifuge (2250 *g*) for 10 min. The lower dichloromethane phase was removed, evaporated to dryness under a stream of nitrogen at 30—40°C, and the residue was dissolved in 0.4 ml of the mobile phase used for the UV assay. The solution was then transferred to vials to be injected by the autosampler. A single incubation and extraction was used for

both the UV analysis of INDO and DMI with F-INDO as the internal standard, and for the fluorescence analysis of DBI with IPA as the internal standard.

Standard curves were prepared by spiking blank urine samples with INDO, DMI and DBI at ten concentrations from 0.25 to 125 μg/ml for INDO and DMI and from 0.25 to 12.5 μg/ml for DBI. These concentrations covered the range found in urine after a 50-mg single oral dose of INDO. Stock solutions of 1.0 mg/ml were prepared for INDO in dichloromethane and for DMI and DBI in methanol. All three compounds were combined in methanol at 50 ng/ml to serve as the dilute solution needed daily for the preparation of the lower concentrations of the standard curve. Spiked urine samples were incubated with β-glucuronidase and extracted as described above.

Peak area ratios for INDO and DMI relative to the UV internal standard were used for quantitation. Because of the large range covered by the UV assay the standard curve was fitted with a weighted (1/concentration) least-squares regression. As the concentration range for DBI was smaller, it was better fitted using unweighted least squares. Since the linearity for the fluorescence assay was limited to a smaller concentration range, samples above 5 μg/ml necessitated injection of smaller sample volumes. Extraction efficiency was determined by comparing extracted spiked samples to unextracted samples using an external standard added prior to injection.

RESULTS

The extraction step produced much cleaner chromatography than was found when direct precipitation of proteins was tried. This allowed quantitation of

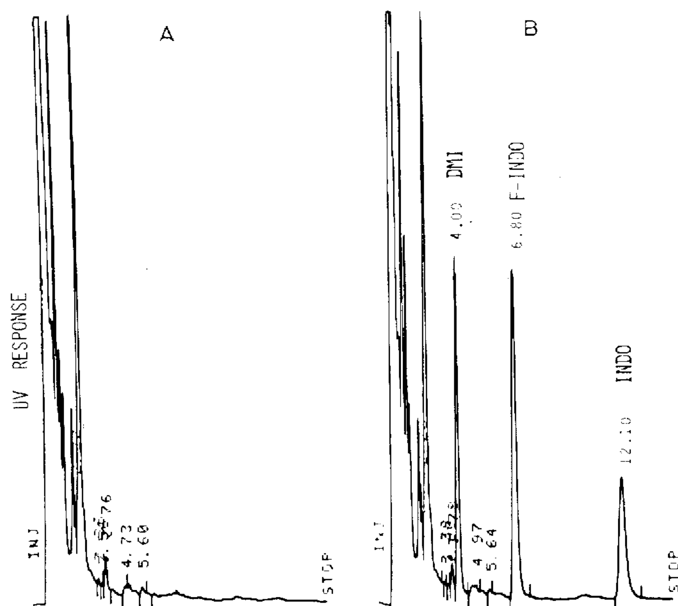

Fig. 2. UV chromatograms of (A) blank urine extract; (B) urine extracted after being spiked with 2.5 μg/ml of DMI and INDO. Conditions are described in the text.

DMI and INDO simultaneously. Retention times were 4.0, 6.8 and 12.1 min for DMI, F-INDO, and INDO, respectively as shown in Fig. 2. The intra-day variability through the range of 0.25 to 50.0 μg/ml, as listed in Table I, exhibit coefficients of variation (C.V.) which are less than 10% at the lowest concentration, 0.25 μg/ml. Between-day variability and extraction efficiencies are listed in Tables II and III, respectively.

Fluorescence provided selectivity for DBI analysis which could not be attained by UV analysis. Chromatograms for a blank sample and spiked urine are shown in Fig. 3. Retention times were 4.5 and 7.8 min for DBI and IPA, respectively. Within-day and between-day variability for the fluorescence assay are listed in Tables I and II.

Hydrolysis with β-glucuronidase was optimized by varying time and enzyme

TABLE I

WITHIN-DAY VARIABILITY FOR INDOMETHACIN AND ITS METABOLITES (n = 10)

Sample concn. (μg/ml)	INDO			DMI			DBI		
	Concn. found (μg/ml)	C.V. (%)	Error (%)	Concn. found (μg/ml)	C.V. (%)	Error (%)	Concn. found (μg/ml)	C.V. (%)	Error (%)
0.250	0.245	5.0	−2.0	0.240	8.2	−4.0	0.281	5.6	12.4
5.00	5.10	1.9	2.0	5.21	2.3	4.2	4.77	4.5	−4.6
50.0	52.3	2.9	4.6	51.8	0.8	3.6	—	—	—

TABLE II

BETWEEN-DAY VARIABILITY FOR INDOMETHACIN AND ITS METABOLITES (n = 10)

	INDO	DMI	DBI
Sample concn. (μg/ml)	5.00	5.00	5.00
Concn. found (μg/ml)	4.91	5.15	4.72
C.V. (%)	5.4	8.6	5.6
Error (%)	−1.8	−3.0	−5.6

TABLE III

EXTRACTION EFFICIENCY FOR INDOMETHACIN AND ITS METABOLITES (n = 7)

Concentration used was 5.0 μg/ml.

Compound	Extraction (%, ± S.D.)
INDO	99 ± 2.1
DMI	83 ± 2.9
F-INDO	99 ± 3.3
DBI	78 ± 6.4
IPA	80 ± 3.5

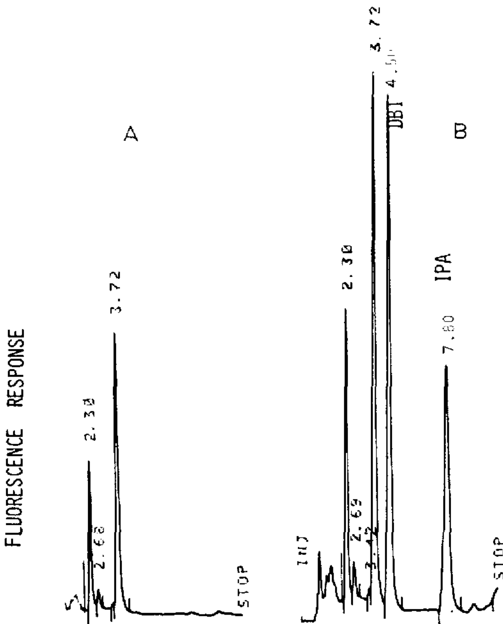

Fig. 3. Fluorescence chromatograms of (A) blank urine extract; (B) urine extracted after being spiked with 1.25 μg/ml DBI and the internal standard, IPA. Conditions are described in the text.

concentrations on several urine samples which had high levels of indomethacin. Incubation for 2 h, at 37°C with 1000 U provided maximum INDO concentrations, so these conditions were used for all subsequent samples. This assay has been applied to urine samples obtained after a single oral dose of 50 mg INDO. The concentration range of the standard curves cover normal urine concentrations found after this single oral dose. The sensitivity of the assay permitted detection of INDO and its metabolites in urine as long as 24—48 h after an oral dose. Chromatograms of patient samples are similar to those of the spiked samples presented in Figs. 2 and 3.

The extraction procedure above was also applicable to plasma samples, however, only INDO was able to be measured by a different reversed-phase HPLC system (data not presented). Interference of endogenous compounds and the lower concentrations in plasma as compared to urine prevented the application of this method to the measurement of DMI or DBI concentrations in plasma.

DISCUSSION

The method described provides an accurate determination of indomethacin and its principle metabolites in urine after glucuronide hydrolysis and has been used successfully for bioavailability studies. Although two separate HPLC systems were employed for the analysis, INDO, DMI and DBI could be extracted simultaneously into dichloromethane which simplified sample preparation. The use of pH 5.0 buffer and dichloromethane for the extraction yielded cleaner blank samples than could be obtained with lower pH solutions.

This was important when analyzing for DMI which, because of its polarity when compared to INDO, was not well separated from endogenous substances.

Because this method does not require radiolabelled drug or measurements of drug and metabolites by difference, it can be more easily implemented when studies on the excretion of INDO in urine need to be done. The advantages of the proposed method are: (a) direct measurement of all compounds, without determination by the difference method where variances are additive; (b) the chromatography has a more improved resolution than that provided by the method of Bernstein and Evans [3]; (c) there are no potential problems of DMBI degradation with base and; (d) glucuronic acid conjugates are cleaved prior to the extraction in our method, whereas Bernstein and Evans [3] suggest measuring unconjugated compounds in urine by extraction into ethyl acetate, but do not consider the likely possibility that conjugates are also extracted.

The availability of the internal standard, F-INDO, can be a problem. We have found, since the development of this method, that the acylation product of DMI with acetic anhydride yields a suitable potential internal standard with retention on the reversed-phase system described above between F-INDO and INDO. This acylation product has been utilized successfully as an internal standard for the analysis of indomethacin in plasma and should work equally well if used with urine.

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