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Note

Improved method for the determination of indomethacin in plasma and urine by reversed-phase high-performance liquid chromatography

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Indomethacin (I) is a widely used anti-inflammatory drug which is extensively metabolized and excreted in the urine. The drug itself is excreted intact and as the ester-linked glucuronide metabolite. Other known metabolites are O-desmethylindomethacin (II), N-deschlorobenzoylindomethacin (III), and N-deschlorobenzoyl-O-desmethylindomethacin (IV), which are also excreted intact and as their glucuronide conjugates.

Numerous methods have been reported in the literature using gas chromatography with electron-capture detection to measure indomethacin in biological fluids [1–6]. These methods all required solvent extraction and derivatization prior to analysis. In most reports the fate of the metabolites was not documented during these procedures. Combined gas chromatography–mass spectrometry [7,8] has also been reported, but required derivatization and sophisticated instrumentation. A radioimmunoassay [9] has also been developed, but indomethacin glucuronide cross-reacted and the assay was not, therefore, specific.

High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection has been the most commonly reported assay procedure for indomethacin in biological fluids [10–15]. These methods all involved solvent extraction of the drug from the biological matrix and often lacked high sensitivity. A novel HPLC method was described using post-column base hydrolysis and fluorescence detection [16]. The sample preparation step still involved solvent extraction, however, and the chromatography showed broad peaks for both the drug and the internal standard.

The following procedure also uses post-column hydrolysis and fluorescence detection. The sample preparation step for plasma involves protein precipitation with acetonitrile and direct injection of the supernatant. No solvent extraction

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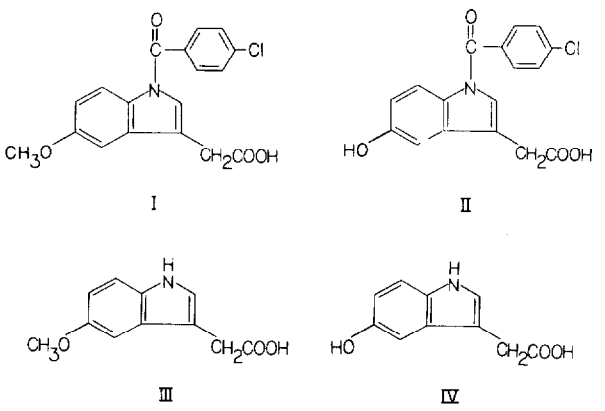

steps are necessary. Urine samples are hydrolyzed with Glusulase and then injected directly. For both plasma and urine there is no endogenous interference and the metabolites do not interfere with the quantification of indomethacin. The drug and internal standard both give sharp peaks and are baseline resolved in under 4 min, making this a particularly suitable method for large numbers of clinical samples. The limit of detection using this method is 10 ng/ml using a 250-μl aliquot of plasma and 50 ng/ml using a 50-μl aliquot of urine. The sensitivity can be increased if necessary by taking a larger sample volume or by increasing the injection volume.

EXPERIMENTAL

Materials

HPLC-grade methanol and acetonitrile were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium dihydrogen phosphate and phosphoric acid (85%) were HPLC grade and were supplied by Fisher (Pittsburgh, PA, U.S.A.). Sodium hydroxide was obtained from Mallinckrodt (Paris, KY, U.S.A.). Glusulase enzyme solution was supplied by DuPont (Wilmington, DE, U.S.A.) and sodium acetate was obtained from Fisher. Indomethacin and α-methylindomethacin, the internal standard, were obtained from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). Blank human plasma was supplied by Sera-Tec Biologicals (North Brunswick, NJ, U.S.A.).

Equipment

A Varian 5000 high-performance liquid chromatograph equipped with a built-in column heater was used for this work. A Waters WISP 710B autosampler was connected to the chromatograph and a Perkin-Elmer 650-10S fluorometer was used as the detector. A Spectra-Physics 4270 computing integrator was used to measure peak areas. The analytical HPLC column was 5 cm$\times$4.6 mm packed with 3-μm Sepralyte C_{18} (a covalently bonded silica gel material) and was obtained from Analytichem International (Harbor City, CA, U.S.A.). The effluent from the HPLC column was mixed with 0.1 *M* sodium hydroxide via a mixing tee and delivered by a Milton-Roy HPLC pump equipped with an SSI pulse dampener.

The combined effluent was passed through a reaction coil (Kratos, 2 ml total internal volume, nominal internal diameter 10 μm) maintained at 50°C in a Waters column heater. The effluent was then passed into the fluorometer.

Chromatographic conditions

The aqueous part of the mobile phase was 0.05 *M* ammonium dihydrogen phosphate and 0.01 *M* phosphoric acid, giving a pH of 3.5. The organic modifier was methanol and the ratio of aqueous buffer–organic was 42:58. The analytical column was maintained at 50°C at a flow-rate of 1.5 ml/min. The post-column pump used to deliver the 0.1 *M* sodium hydroxide was run at a flow-rate of 0.5 ml/min. The injection volumes used were 30 μl for plasma and 25 μl for urine. The fluorometer excitation and emission wavelengths were set at 295 and 372 nm, respectively. The slits were set at 15 nm and the sensitivity range was 0.1.

Standard and internal standard solutions

A stock standard solution of indomethacin (100 μg/ml) was prepared in acetonitrile–water (80:20). This solution was further diluted in the same solvent to give a series of indomethacin working standards. The concentrations were 40, 20, 10, 5, 2.5, 1.25 and 0.5 μg/ml for plasma and 500, 250, 100, 50, 25 and 10 μg/ml for urine. This produced equivalent plasma concentrations of 8000, 4000, 2000, 1000, 500, 250 and 100 ng/ml and equivalent urine concentrations of 50, 25, 10, 5, 2.5 and 1 μg/ml.

The internal standard, α-methylindomethacin, was also prepared as a stock solution (1 mg/ml) in the same solvent as indomethacin. A working internal standard of 1.25 μg/ml was used for plasma analysis and 5 μg/ml for urine analysis.

Plasma samples

The plasma standard line was prepared by combining blank human plasma (250 μl), working internal standard solution (50 μl), respective aliquots of indomethacin working standards (50 μl) and acetonitrile (500 μl). After vortex mixing (15 s), the tube was centrifuged (10 min at 2000 *g*) and the supernatant decanted into an autosampler vial. An aliquot (30 μl) of this solution was injected for HPLC analysis.

Plasma samples were prepared in the same way as standards. An aliquot (250 μl) of patients' plasma was substituted for blank plasma and acetonitrile–water (80:20, 50 μl) for the indomethacin working standards.

Urine samples

The Glusulase solution had an activity of approximately 90 000 U/ml of β-glucuronidase and 10 000 U/ml of sulfatase. A Glusulase working solution was prepared from Glusulase (200 μl), 1 *M* sodium acetate buffer, pH 5.2 (400 μl), stock internal standard solution (32 μl) and water (q.s. to 20 ml). The urine standard line was prepared from blank human urine (50 μl), Glusulase working solution (250 μl) and respective aliquots of indomethacin working standards (50 μl). After vortex mixing, these solutions were incubated (1 h at 37°C). Acetonitrile–water (80:20, 500 μl) was added to each and the contents mixed. After

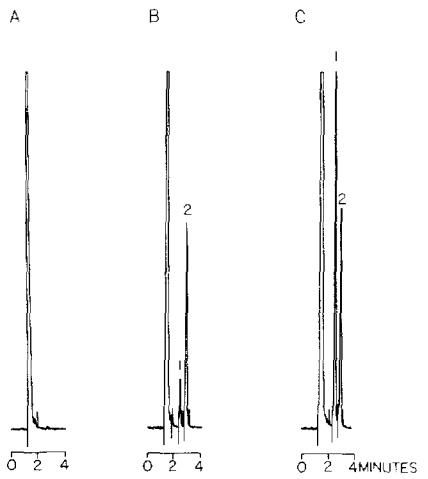

Fig. 1. Typical chromatograms for indomethacin (1) and internal standard, α-methylindomethacin (2) in plasma. (A) Blank plasma; (B) blank plasma containing 50 ng/ml of indomethacin and 250 ng/ml of internal standard; (C) patient sample containing 440 ng/ml of indomethacin and 250 ng/ml of internal standard.

centrifugation (10 min at 2000 *g*) the supernatant was decanted into an autosampler vial and an aliquot (25 μl) injected for analysis.

Urine samples were prepared in the same way as the standards, substituting patients' urine (50 μl) for blank urine and acetonitrile–water (80:20, 50 μl) for indomethacin working standard.

RESULTS AND DISCUSSION

The basis of this method was the formation of a fluorescent product by post-column base hydrolysis. The hydrolysis product of indomethacin was thought to be N-deschlorobenzoylindomethacin. Indomethacin itself is not fluorescent but does possess a good chromophore for UV detection. The sensitivity and selectivity obtained with a UV detector, however, are not as good as those obtained with the fluorescence detector described in the present method. There were several key factors that affected the fluorescent yield in this procedure. Methanol had to be used as the organic modifier in the mobile phase. No product was formed with acetonitrile, although acetonitrile would produce satisfactory chromatography. This was checked using a UV detector. The methanol concentration in the mobile phase also affected the fluorescent yield. A minimum of 50% methanol was required. If an adequate supply of methanol was not available from the mobile phase, it could be added to the 0.1 *M* sodium hydroxide that was introduced via the post-column pump.

The methods described for plasma and urine analysis gave baseline resolution of indomethacin and its internal standard with no endogenous interference. A typical chromatogram for plasma is shown in Fig. 1. Indomethacin eluted at 2.4 min and the internal standard at 2.9 min. Similar chromatograms were obtained

TABLE I

INTERDAY VARIATION FOR INDOMETHACIN IN PLASMA AND URINE

For both plasma and urine $n = 5$.

Concentration	Mean ratio drug/internal standard	S.D.	R.S.D. (%)
Plasma (ng/ml)			
100	0.100	0.002	1.9
250	0.262	0.002	0.5
500	0.532	0.008	1.5
1000	1.090	0.025	2.3
2000	2.233	0.023	1.0
4000	4.536	0.036	0.8
8000	9.284	0.118	1.3
Urine (μg/ml)			
1	0.136	0.006	4.6
2.5	0.353	0.009	2.5
5	0.716	0.016	1.5
10	1.492	0.035	2.3
25	3.764	0.103	2.7
50	7.739	0.126	1.6

for urine. The known metabolites of indomethacin (II–IV) did not interfere with either indomethacin or the internal standard.

This method has been further modified to study conjugated metabolites directly in urine samples. Using gradient rather than isocratic elution gave resolution of more polar peaks as well as of the metabolite peaks. Some of these polar peaks disappeared after hydrolysis with enzyme, and a corresponding increase in the peak areas of non-conjugated species occurred. The lack of authentic reference materials, however, has prevented conclusive identification of conjugated metabolites and further work is in progress.

The preparation of plasma samples was extremely simple. Protein precipitation with acetonitrile followed by injection of the supernatant was the only sample clean-up required. The linearity of the standard line from 100 to 8000 ng/ml was confirmed by plotting the drug concentration against the ratio of peak areas (drug/internal standard). Correlation coefficients were consistently higher than 0.9997. In the study for which this assay was being used, 100–8000 ng/ml was found to be the desired range for the standard line. As can be seen from Fig. 1, 50 ng/ml was easily detected and the limit of detection was found to be around 10 ng/ml (based on a signal-to-noise ratio of 5:1). This could easily be increased by injecting a larger sample volume or by using a larger sample size, as there was no endogenous interference. The reproducibility of the assay was assessed by measuring the interday variability of each point on the standard line over a five-day period. The results are given in Table I. All points on the standard line had relative standard deviations of 2.3% or less. The accuracy of the method was checked by preparing quality control samples in blank plasma. These were run throughout

TABLE II

QUALITY CONTROL SAMPLES FOR INDOMETHACIN IN PLASMA AND URINE

Sample	Mean ± S.D.	R.S.D. (%)
Plasma (n = 12)		
Low target (425 ng/ml)	428 ± 15.6	3.6
High target (4000 ng/ml)	3871 ± 102.4	2.7
Urine (n = 10)		
Low target (2.5 μg/ml)	2.48 ± 0.0962	3.9
High target (25 μg/ml)	24.4 ± 0.7486	3.1

the assay of patients' samples and the results are given in Table II. The results are in good agreement with their target values and show relative standard deviations of 3.6 and 2.7%, respectively, for the low and high values.

Urine samples were first hydrolyzed with Glusulase and then injected directly. Chemical hydrolysis could not be used because of the lability of indomethacin. The linearity and reproducibility were checked in the same way as for plasma. The interday variability results for urine are given in Table I. All points had relative standard deviations of 4.6% or less. Results from the analysis of quality control samples are given in Table II. Again there was good agreement between target values and measured values. The relative standard deviations were 3.9 and 3.1%, respectively, for the low and high values.

In summary, this method provides a very simple assay for indomethacin in plasma and urine. It has been successfully used to assay many clinical samples and has proved to be very rugged. Very few problems have been encountered with the sample preparation procedures or with the chromatography.

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