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Highly sensitive measurement of indomethacin using a high-performance liquid chromatographic technique combined with post-column in-line hydrolysis

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The measurement of indomethacin in biological fluids has been a topic of research for the last two decades. With the introduction of high-performance liquid chromatographic (HPLC) techniques, the emphasis shifted from sensitivity to specificity and rapidity. The need for high degrees of sensitivity, however, remains of importance, especially with the recognition that this drug as well as others in its family are highly protein-bound, and that the concentration of interest is that which is unbound and pharmacologically active. Since as much as 99% [1—4] of indomethacin may be bound to serum proteins, large sample volumes are usually needed to obtain accurate measurements of free drug concentration. Using such methods, assay sensitivities ranging from 0.2 to 0.02 μg/ml [5—11] have been reported. Use of fluorescence detection of hydrolysed indomethacin has resulted in an additional fifteen-fold improvement [12].

We developed a method using the new HPLC technique of micro HPLC in combination with post-column alkaline hydrolysis of indomethacin, thereby enabling fluorescence detection. This method increases assay sensitivity another 300 times, reaching to the low picogram range. We demonstrated the utility of this method in measuring indomethacin concentrations in small sample volumes collected from microperfusion of an isolated segment of renal proximal tubule.

EXPERIMENTAL

Apparatus
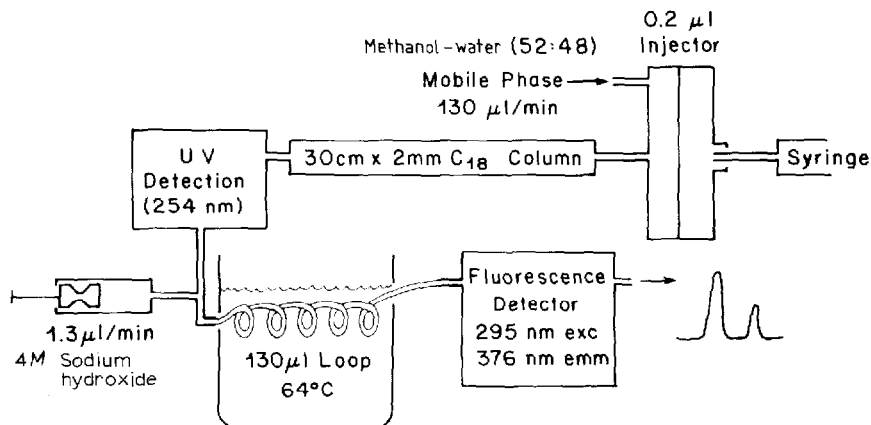A schematic drawing of the HPLC system is shown in Fig. 1. The solvent

Fig. 1. Schematic drawing of the microbore HPLC system and post-column derivatization. For details, see text.

delivery system consisted of two reciprocating high-pressure pumps (Model 6000A, Waters Assoc.). The electrical system was modified by changing a capacitor to enable flow-rates of μl/min. Since the ultimate purpose for development of this method was to measure indomethacin in extremely small sample volumes (in the nanoliter range), the syringe-loading injector had a sample volume chamber of 0.2 μl (Model 7520, Rheodyne). The connecting tubing was 1.59 mm $\times$ 0.25 mm I.D. stainless steel and was kept as short as possible. A small-volume precolumn filter was used (Model 7315, Rheodyne). The microbore column, 30 cm $\times$ 2 mm I.D., was packed with 10-μm μBondapak$^{®}$ C_{18} (Waters Assoc.). The UV absorbance detector (Model 440, Waters Assoc.) operated at 254 nm; a microbore cell (1.9 μl) replaced the original flow cell. Derivatization was effected post-column and post-UV detection by alkaline hydrolysis of indomethacin using sodium hydroxide: 4 M sodium hydroxide was introduced at a speed of 1.3 μl/min via a low-volume TEE connector using a syringe pump (Model 255-3, Sage Instruments). The reaction loop (130 μl) of Polytef tubing ran through a water bath kept at 64°C. This then connected to a fluorescence spectrophotometer (Model 650-10S, Perkin-Elmer) set at an excitation wavelength of 295 nm and a slit width of 20 nm, with an emission wavelength of 376 nm and a slit width of 20 nm. Prior studies showed these settings to be optimal for measurement of the indomethacin hydrolysis product. The original flow cell was used in the fluorescence measurements. The pH of the mobile phase was measured in-line directly after the fluorescence detector.

Solutions

Indomethacin was a gift from Merck Sharp and Dohme Labs. (West Point, PA, U.S.A.). Standards were made using methanol as a solvent. Either phenylbutazone or carprofen could serve as internal standards. The first is readily detected by its 254-nm UV absorption and shows no fluorescence at the mentioned settings. This allowed assessment of the pre-derivatization status of the injected sample. Carprofen has a slightly longer retention time than indomethacin and appears to be a good fluorophore at the spectrophotometer

settings used. The mobile phase for the HPLC separation consisted of methanol—water (52:48, v/v).

Isolated perfused tubule

Indomethacin transport across the isolated perfused renal proximal straight tubule (PST) was measured with the HPLC technique described above. Segments of rabbit PST were hand-dissected and perfused as previously described [13]. The tubule was bathed in and perfused with ultrafiltrate-like solution containing 104 mM sodium chloride, 25 mM sodium bicarbonate, 2.3 mM sodium biphosphate, 10 mM sodium acetate, 1.2 mM calcium chloride, 1 mM magnesium sulphate, 5 mM potassium chloride, 5 mM glucose and 5 mM alanine. Fetal bovine serum (5%, v/v) (Gibco Lab., New York, NY, U.S.A.) and indomethacin to a final concentration of 10^{-6} M were added to the bathing solution. The perfusion rate was between 10 and 15 nl/min and timed collections of the perfused fluid were made for indomethacin measurement. Aliquots of 100 nl of collected fluid were transferred to 600 nl of fluid containing the internal standard and injected into the above-described HPLC system.

RESULTS

The reaction speed of the alkaline hydrolysis at different pH values and at different temperatures was first tested in the above-mentioned spectrophotometer using cuvettes. The reaction (0.04 µg/ml indomethacin in methanol—water) was monitored by interval measurements to prevent the decaying effect of the exciting light on the reaction product [1]. The pH in the cuvette was changed using 4 M sodium hydroxide and ranged from 11 to 13 by steps of 0.4 pH units. At 20°C and at pH 13, the reaction was 91% complete within 1 min, whereas at pH 11 it was 55% complete in 1 min. On the other hand, if the reaction was allowed to proceed to completion (which
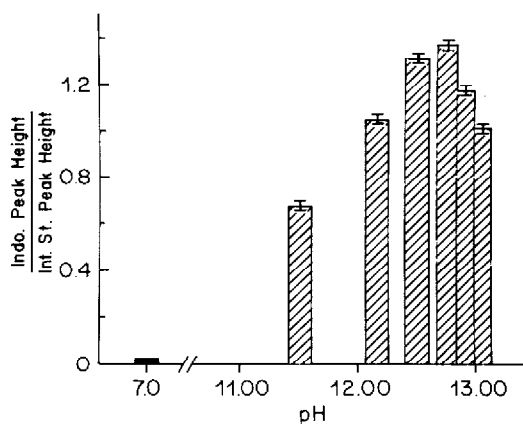

Fig. 2. Relation between the degree of in-line alkaline hydrolysis at different pH (*x*-axis) and the measured fluorescence of the hydrolysis product of indomethacin (220 pg). Phenylbutazone was used as the internal standard. The hatched bars represent the mean and standard error of the mean of four different indomethacin samples at the indicated pH in the reaction coil.

required 20 min), twice the fluorescence yield was observed at pH 11 compared to pH 13. However, a reaction loop of 20 min would cause too much band-spreading. Increasing the temperature in the cuvette to 64°C increased the reaction speed, but the fluorescence yield at 1 min did not differ between pH 11 and 13. Because of these characteristics, a reaction coil with a delay time of 1 min was chosen for post-column derivatization. In this in-line system, the optimum pH was tested. Fig. 2 shows that a pH of 12.75 results in an optimal fluorescence yield at 64°C.

Actual measurements of indomethacin standards (0.2 µl) ranging from 0.25 to 50 µg/ml were performed with both internal standards, carprofen and phenylbutazone, present in the same sample. The measured internal standards correlated well with each other (r^2 = 0.999). Using phenylbutazone as a
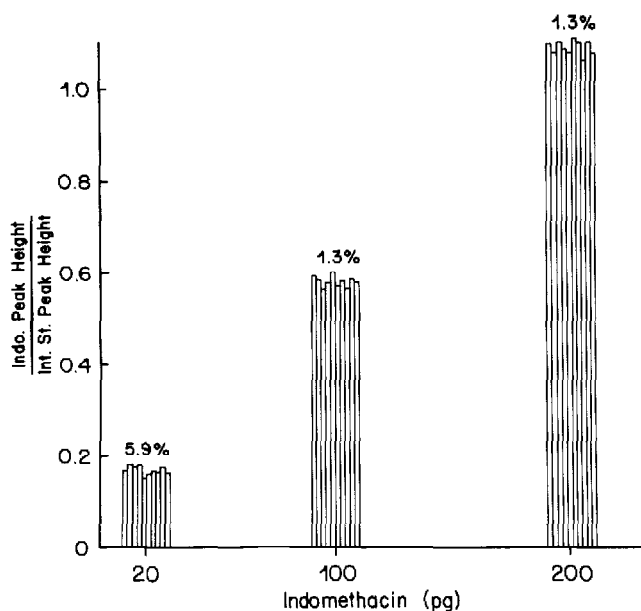

Fig. 3. Reproducibility of measuring three indomethacin standards (20, 100 and 200 pg) on ten consecutive workdays. Phenylbutazone was used as the internal standard. The coefficients of variation are shown.

TABLE I

INDOMETHACIN MEASUREMENT IN 10^{-6} M STANDARD SOLUTION, BATHING SOLUTION AND COLLECTED FLUID PERFUSED THROUGH ISOLATED SEGMENTS OF RABBIT PROXIMAL STRAIGHT TUBULES

Experiment	Indomethacin/internal standard peak-height ratio		
	10^{-6} M Standard	Bath	Lumen
1	0.206	0.215	1.012
2	0.195	0.185	0.402
3	0.221	0.243	1.091
4	0.210	0.198	1.174

standard, the standard curve for indomethacin (range 5–200 pg) showed a good linear correlation ($n = 8$; $r^2 = 0.999$). The detection limit was 5 pg of indomethacin. Intra-assay variation was within 5% at 20, 100 and 200 pg. Day-to-day variation was tested on ten consecutive workdays and the results are shown in Fig. 3.

Table I shows the results of indomethacin measurements in the bathing solution and in the collected perfusate from four different PST segments. These data show the feasibility of measuring indomethacin at physiological concentrations in these small collected volumes. All four tubule segments transported indomethacin from bath to lumen, resulting in a two- to six-fold increase in indomethacin concentration in the lumen.

DISCUSSION

The present study demonstrates the feasibility of combining micro HPLC techniques and post-column derivatization. The result is a greatly improved sensitivity for detection of indomethacin, reaching levels in the low picogram range.

Micro HPLC is advocated for increased sensitivity compared to conventional HPLC systems [14, 15]. With the latter, the lower limit of indomethacin detection is reported to be approximately 0.02 μg/ml, as we also found in our laboratory. Using a microbore system, the sensitivity theoretically increases about eight-fold [16]. Indeed, we found a twelve-fold increase in sensitivity measuring indomethacin standards with UV detection with the microbore system.

The combination of a microsystem with post-column derivatization allows an even more sensitive tool for indomethacin measurement. Hydrolysis of indomethacin was observed as early as 1965 as a possible approach to lowering the detection level of indomethacin [17]. This technique, however, had problems for it included hydrolysis of all metabolites of the drug as well as the parent compound. Therefore, post-column derivatization has the distinct advantage of allowing detection of the individual hydrolysis products. Bayne et al. [12] demonstrated the ability to measure indomethacin with standard HPLC techniques and in-line hydrolysis and, by doing so, increased sensitivity about fifteen-fold. Using the microbore system, we observe an additional 300-fold increase in sensitivity. We do not believe all of this improvement can be accounted for by the microbore system itself, and feel that differences in the post-column hydrolysis must be operative. Prior investigators noted the pH dependence of the hydrolysis reaction [2, 17], and we have confirmed that an optimal pH and temperature exist. In our system, adding 1% (v/v) of 4 M sodium hydroxide to the unbuffered mobile phase resulted in a pH of 12.7. Bayne et al. [12] used a different setting for their in-line hydrolysis. They added 10 vol.% of 0.1 M sodium hydroxide to a buffered mobile phase and observed a pH of 9. We have found that a pH of 9 in the reaction coil (at 64°C) reduces our fluorescence yield by 80% compared to a pH of 12.75. This difference may further explain the considerably improved sensitivity in our method compared to that of Bayne et al. [12].

The goal of this project was to measure indomethacin in very small sample

volumes. In most studies, when either low drug concentrations need to be detected or when the collected biological sample is small, radiolabeled drugs are used. However, when one wishes to measure pharmacological concentrations of drugs when only nanoliters of sample are available, the low specific activity of most radiolabeled drugs (including indomethacin) prevents tracer use. The present study demonstrates the feasibility of employing a highly sensitive direct assay for non-labeled drug. The HPLC separation excludes confounding by possible metabolites. Our results also demonstrate the feasibility of measuring picogram amounts of indomethacin in other biological fluids (when using larger injection volumes). Further optimization of the fluorescence detection and adaptation to the microsystem may well increase the sensitivity even further.

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