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Liquid-Chromatographic Determination of Indomethacin in Blood from Newborns with Patent Ductus Arteriosus

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This rapid, accurate “high-performance” liquid-chromatographic procedure is intended for measuring indomethacin in serum from neonates who are receiving indomethacin for symptomatic patent ductus arteriosus. Indomethacin and an internal standard (flufenamic acid) are extracted from serum or plasma with chloroform or diethyl ether at pH 5.0. For the chromatography we used a Waters’ Radial Compression Separation System (Radial-NOVA PÅK C18 reversed-phase column) and a mobile phase of methanol/sodium acetate buffer (10 mmol/L, pH 3.6), 70/30 by vol. The column effluent is monitored at 254 nm. Both indomethacin and flufenamic acid are eluted within 7 min. Indomethacin can be detected in concentrations as low as 50 μg/L in 100-μL samples. Response varies linearly with indomethacin concentration to at least 2 mg/L. Analytical recovery is 75%; relative recovery is 100%. Precision is excellent. Using this method, we were able to improve the success rate for pharmacological management of symptomatic patent ductus arteriosus, especially in neonates with fast clearance rates for the drug.

Additional Keyphrases: drug assay  ·  neonates  ·  patent ductus arteriosus

In patent ductus arteriosus (PDA), a congenital heart defect, a small duct between the aorta and the pulmonary artery, which normally closes soon after birth, instead remains open. Symptomatic patent ductus arteriosus is frequently observed in premature infants and can present life-threatening complications. Management of symptomatic PDA included digitalization, diuretics, and eventual surgical ligation until 1976, when Friedman et al. (1) and Heymann et al. (2) reported indomethacin to be effective in the closure of PDA in premature infants in the first postnatal month. Since then, some studies have supported the original observations and others have reported less-impressive responses to indomethacin administration. To identify factors affecting the efficacy of indomethacin in closing the ductus, Brash et al. (3) studied the pharmacokinetics of intravenous indomethacin in 35 premature infants with PDA. They concluded that successful use of indomethacin was associated with a concentration of the drug exceeding 250 μg per liter of plasma 24 h after its infusion. However, they also found a 20-fold variation in these values. Such variation and the relation between concentrations of the drug in plasma and ductus constriction make important the rapid measurement of indomethacin during the 6 to 24 h after the drug is first administered, so that the second dose can be correctly scheduled.

The various methods for determination of indomethacin include spectrophotometry (4, 5), gas–liquid chromatography (6, 7), radioimmunoassay (RIA) (8, 9), and “high-performance” liquid chromatography (HPLC) (10–16). Spectrophotometry is nonspecific, time-consuming, and requires a large volume of sample. Gas–liquid chromatography is more specific but is time-consuming and requires derivatization. RIA, although highly sensitive, is impractical for urgent determinations. Several reported HPLC methods either lack the necessary sensitivity or are subject to endogenous interference. Recently, Mehta and Calvert (17) reported an improved HPLC procedure for monitoring indomethacin in neonates, involving a 10-μm Radial-PAK C18 reversed-phase column. However, this method, a modification of the Skellern and Salole procedure (10), is subject to endogenous interferences, especially for plasma samples.

In 1981, we developed a liquid-chromatographic method for rapidly determining indomethacin in neonates. We have now tested the method over a two-year period and modified it to improve its accuracy and precision. Here, we report in detail this improved procedure.

**Materials and Methods**

**Apparatus.** We used a Model 110A HPLC pump (Beckman Instruments, Inc., Berkeley, CA 92634) equipped with a Model 7125 injector (Rhodeyne, Inc., Cotati, CA 94928), a Model 440 dual-beam ultraviolet–visible absorbance detector (Waters Associates, Milford, MA 01757), and a radially

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compressed C<sub>18</sub> reversed-phase column (NOVA PAK C<sub>18</sub> Radial-PAK, 8 × 100 mm, 5-µm particle size; Waters Associates). A chart recorder (Omni Scribe recorder, Model D5267; Houston Instruments, Austin, TX 78753) monitored the separation profile at a chart speed of 0.5 cm/min. The column was eluted with mobile phase at the rate of 4 mL/min at ambient temperature, and the column effluent was monitored at 254 nm with the detector range of 0.005 A full-scale.

**Reagents.** Indomethacin, desbenzoylindomethacin, and O-desmethylinmethacin were generous gifts from Dr. W. C. Vinek (Merck, Sharp, and Dohme, Research Laboratories, Westpoint, PA 19486). Flufenamic acid was from Aldrich Chemical Co., Milwaukee, WI 53201. All solvents were "reagent" or "HPLC" grade.

The working standards of indomethacin (200, 400, and 600 µg/L) were prepared by adding the stock solution (1 g/L, in methanol) to drug-free plasma. Aliquots of the standard solution were stable for at least a year at −70 °C. The working solution of the internal standard, flufenamic acid (2.5 mg/L), was prepared by diluting the stock solution (1 g/L, in methanol) with acetonitrile.

**Mobile phase.** Sodium acetate (10 mmol/L) was dissolved in doubly distilled water, adjusted to pH 3.6 with HCl, and filtered through a 0.45-µm (av pore size) filter (Millipore Corp., Bedford, MA 01730). The mobile phase consisted of 700 mL of methanol added to 300 mL of this buffer.

**Procedure.** Add 200 µL of serum, standard, or control to 200 µL of internal standard and 200 µL of phosphate buffer (0.1 mol/L, pH 5.0). Vortex-mix and extract with 5 mL of chloroform or diethyl ether by vortex-mixing for 90 s Centrifugae (3000 × g, 10 min). Transfer the chloroform (lower) layer or ether (upper) layer to a clean 1.3 × 10 cm disposable glass tube and evaporate under a stream of air. Redissolve the residue in 50 µL of methanol, and inject 20-µL aliquots of this solution onto the column.

**Recovery.** To assess analytical recovery, we compared the peak height measured for a drug-added serum sample (after extraction) with that of an aqueous solution containing the same concentration of indomethacin (direct injection without extraction). Relative recovery was calculated by comparing the quantities measured for drug-added serum with the actual quantities added. To assess interference, we analyzed serum from more than 30 patients who were not receiving indomethacin, but some of whom were being treated with other drugs.

**Results**

Figure 1 shows the relation between pH and the extraction of indomethacin. Analytical recovery was greatest at pH 5, which is consistent with the results of Helleberg (6) except that we noted no marked decrease in recovery when the pH was either higher or lower than 5.0. In our hands, with use of the phosphate buffer the recovery at pH 4.0 was 96% of that at pH 5.0.

Figure 2 depicts the separation of the indomethacin metabolites desbenzoylindomethacin and O-desmethylinmethacin from indomethacin and internal standard. Elution of these metabolites overlaps with that of endogenous contaminants in serum or plasma, so their quantification may not be feasible.

Typical chromatograms of serum and plasma specimens are shown in Figure 3. Note the extraneous peaks between indomethacin and internal standard in the chromatogram of plasma specimens. The major peak does not appear in the chromatogram of serum specimens. We do not know the nature of this peak, but it occurs only in plasma samples. The pH of the mobile phase is very important in resolving this extraneous peak from either indomethacin or flufenamic acid. If the pH of the mobile phase exceeds 3.6, the retention times of indomethacin and internal standard shorten but the peak for the endogenous plasma component

**Fig. 1. Effect of pH on the extraction of indomethacin**

**Fig. 2. Chromatogram of standard mixture of desbenzoyl indomethacin (DBl), O-desmethylinmethacin (DMI), indomethacin (INDO), and flufenamic acid (FA)**
Fig. 3. Chromatograms of extract of (left) plasma containing 600 μg of indomethacin (INDO) and 2.5 mg of flufenamic acid (FA) per liter, and (right) patient's serum containing 650 μg of indomethacin and 2.5 mg of flufenamic acid per liter

Discussion

Treatment of symptomatic PDA with indomethacin has become a popular alternative to surgical ligation since 1976, although its effectiveness remains controversial. Indomethacin inhibits synthesis of prostaglandin E in many neonates, but we do not yet know the price of this elegant pharmacological maneuver, i.e., the drug’s effect on intraventricular hemorrhage, necrotizing enterocolitis, or the many other possible complications involving the systems affected by prostaglandins. Nor do we know the effects of the complete or partial inhibition of the synthesis of all prostaglandins in the first few postnatal days. Ideally, development of a drug to inhibit specifically the synthesis of prostaglandin E₂, the one presumed to be responsible for dilating the ductus (18, 19), may reduce the risks of pharmacological management of PDA. Until then, the rapid, accurate assay of indomethacin will be very useful for establishing an optimal dosage schedule for the permanent closure of ductus without adverse side effects.

The chromatographic conditions we report give excellent resolution, including resolution of endogenous components from indomethacin and the internal standard. Turnaround time for a result is only 7 min, and we can analyze six to eight samples per hour. The mobile phase can be recycled as described elsewhere [20]. After each day’s use the NOVA-PAK column can be stored in a screw-capped tube containing the same mobile phase. This shortens the equilibration time for the next use of the column and the column can be used for more than 300 injections.

During the last two years, we evaluated three Waters’ "Radial PAK" reversed-phase columns and five regular stainless-steel C₁₈ columns for this purpose. We found that the Radial PAK columns perform better than the other columns we tested and that, of the various Radial PAK columns, the NOVA-PAK C₁₈ reversed-phase columns give the best resolution, completely resolving a small contaminant peak from the peak for indomethacin. If the Radial PAK C₁₈ 5-μm particle-size column is used, this peak appears as a “shoulder” and may be inappropriately integrated. Using peak-height ratios (indomethacin/internal standard) as the method of calculation can improve the accuracy with this column. The 10-μm Radial PAK C₁₈ column does not resolve this peak from indomethacin.

We now have used this method routinely for more than two years, during which the success rate of the pharmacological management of PDA in our neonatal population has increased quite drastically, especially in the case of those neonates who metabolize indomethacin unusually rapidly. The minimum effective concentration of indomethacin and how long it should be maintained for maximum success with minimal adverse effects are still under investigation.

References

Automated Kinetic Determination of Angiotensin-Converting Enzyme in Serum

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In this automated kinetic modification of a previous method (Anal Biochem 95: 540–548, 1979) for determining angiotensin-converting enzyme (ACE, EC 3.4.15.1), 3-(2-furylacryloyl)-L-phenylalanylglycylglycine (FAPGG) is used as the substrate. The change in absorbance at 340 nm is used to monitor hydrolysis of the substrate. The rate of hydrolysis is roughly threefold greater than with previously reported substrates, so assay time and sensitivity are improved.

Additional Keyphrases: enzyme activity • reference interval

Requests for determination of angiotensin-converting enzyme (ACE, EC 3.4.15.1) in serum, for differential diagnosis of lung sarcoidosis and its treatment with corticosteroids, have been increasing in recent years.¹ Of the several published methods for assay of this enzyme, most either require special equipment or are very laborious. Moreover, none is adaptable to automated analysis.

I describe here an automated kinetic modification of the ACE method of Holmquist et al. (1), later adapted to serum by Ronan-Testoni (2), in which a furanacryloyl-blocked tripeptide, 3-(2-furylacryloyl)-L-phenylalanylglycylglycine (FAPGG) is used as substrate. FAPGG absorbs strongly in the low-wavelength visible and high-wavelength ultraviolet region, with a maximum at 305 nm. Measurement of ACE activity is based on the change in the absorption spectrum when the substrate undergoes hydrolysis to furylacryloyl-L-phenylalanine (FAP) and glycylglycine. The absorbance change after this reaction is greatest at 328 nm but the difference is well measurable up to 352 nm. This method can be applied to automated enzyme analyzers that allow measurements to be made at 340 nm. ACE activity can be measured kinetically in either equilibrium mode or two-point mode.

Materials and Methods

Reagent and buffer solution. FAPGG was purchased from Sigma Chemical Co., St. Louis, MO. I prepared 80 mmol/L borate buffer containing 0.3 mol of NaCl per liter from sodium tetraborate decahydrate and NaCl and adjusted the pH to 8.2 (at 37 °C) with 1 mol/L HCl. The solution was filtered and stored refrigerated. The 0.8 mmol/L substrate solution, prepared by dissolving 32 mg of FAPGG in 100 mL of the buffer, was stored in a brown glass bottle in the refrigerator; it can be used for several weeks. Just before use in an assay, the substrate solution was allowed to come to room temperature.

Samples. Serum was separated from patients’ blood without delay and stored at −20 °C, a temperature at which the analyte seems to be stable for several months. Even if samples are thawed and then stored in the refrigerator, the ACE activity is essentially unchanged if the measurements are done within one or two weeks.

Apparatus. I used an Oli C Compact Clinical Analyzer (Kone Oy, Espoo, Finland) with program revision 8.1, which allows a kinetic measurement of enzymes in the two-point mode. ACE assays were made at 340 nm and 37 °C, with a measuring time of 10 or 15 min, depending on the mode used. The two-point mode is preferred, owing to the easy,

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¹ Nonstandard abbreviations: ACE, angiotensin-converting enzyme; FAPGG, 3-(2-furylacryloyl)-L-phenylalanylglycylglycine; FAP, furylacryloyl-L-phenylalanine.

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