David W. Seccombe,[1] *M.D.; Morris R. Pudek,*[2] *Ph.D.; and Karin H. Humphries,*[3] *M.Sc.*

Minimizing Analytical Interferences from Digoxin-Like Immunoreactive Substances (DLIS) in Cases of Digoxin Toxicity

REFERENCE: Seccombe, D. W., Pudek, M. R., and Humphries, K. H., **"Minimizing Analytical Interferences from Digoxin-Like Immunoreactive Substances (DLIS) in Cases of Digoxin Toxicity,"** *Journal of Forensic Sciences*, JFSCA, Vol. 32, No. 3, May 1987, pp. 650-657.

ABSTRACT: Recently, the value of therapeutic drug monitoring for digoxin has been called into question by the finding of endogenous digoxin-like immunoreactive substances (DLIS) in the serum of individuals, especially premature and full-term neonates, not being treated with digoxin. In some cases, values have been as high as 10 μg/L. Levels as high as 20 μg/L and 80 μg/g can be found in bile and meconium. Because of the magnitude of this interference, it is essential that methods be developed for measuring digoxin in the presence of DLIS. This is particularly important when such analyses are required in forensic science cases of suspected digoxin toxicity. This report outlines the high performance liquid chromatographic (HPLC) and radioimmunoassay (RIA) methods that we used in assessing the relative contribution made by digoxin, its metabolites, and DLIS to serum and tissue digoxin concentrations obtained by RIA in a forensic pediatric case of suspected digoxin toxicity.

KEYWORDS: pathology and biology, digoxin, chromatographic analysis, radioimmunoassay

Digoxin is the most frequently prescribed cardiac glycoside in North America. Because of its narrow therapeutic range (0.8 to 2.0 μg/L), the use of digoxin in the clinical setting is not without significant risk of toxicity. Clinicians have increasingly relied upon therapeutic drug monitoring of serum digoxin levels by radioimmunoassay (RIA) as a means of reducing the risks of toxicity.

Recently, the value of therapeutic drug monitoring for digoxin has been called into question by the finding of endogenous digoxin-like immunoreactive substances (DLIS) in the serum of individuals, especially premature and full-term neonates, not being treated with digoxin. In some cases, values have been as high as 10 μg/L.[4] Levels as high as 20 μg/L and 80 μg/g can be found in bile and meconium [*1*]. Because of the magnitude of this interference, it is essential that methods be developed for measuring digoxin in the presence of

Received for publication 9 June 1986; accepted for publication 15 July 1986.

[1]Assistant professor, Department of Pathology, University of British Columbia; medical biochemist, Lipid Research Group, Shaughnessy Hospital, Vancouver; and Department of Pathology, Division of Clinical Chemistry, Vancouver General Hospital, Vancouver, B.C., Canada.

[2]Clinical chemist, Department of Pathology, Division of Clinical Chemistry, Vancouver General Hospital, Vancouver, B.C., Canada.

[3]Department of Pathology, University of British Columbia and research assistant, Lipid Research Group, Shaughnessy Hospital, Vancouver, B.C., Canada.

[4]D. W. Seccombe and M. R. Pudek, unpublished data.

DLIS. This is particularly important when such analyses are required in forensic science cases of suspected digoxin toxicity. This report outlines the methods that we used in assessing the relative contribution being made by DLIS to serum and tissue digoxin levels obtained by RIA in a forensic pediatric case of suspected digoxin toxicity.

Case History

A three-month-old female child was admitted to the hospital with clinical signs of cardiac failure. A decision was made to digitalize the child at a dose of 0.04-mg/kg body weight. One half of the dose was given as a loading dose. The balance of the dose was given in equal amounts 6 and 12 h later. Immediately after the third dose, the child cried and vomited. A significant bradycardia soon developed together with second-degree atrioventricular (AV) block. Shortly thereafter, asystole occurred. The child did not respond to resuscitative procedures and was declared dead 2 h after the third dose of digoxin. An autopsy was performed 23 h after death. Blood was withdrawn from the left ventricle of the heart, the serum was separated by centrifugation and stored at −70°C until analyzed. A portion of the left ventricle of the heart (1 g) was placed into liquid nitrogen and stored at −70°C until analyzed. A serum digoxin value of 70 μg/L was obtained by standard RIA procedures in the referring laboratory. Subsequently, both the serum and cardiac tissues were forwarded to our laboratory with a request that the digoxin levels be confirmed.

Methods

An aliquot of the serum was diluted 35-fold with the 0 standard utilized in the Nuclear Medical Laboratories (NML, Irving Texas 75015) Digoxin RIA assay. This diluted sera was used throughout for all RIA digoxin assays.

RIA Method 1

Previous studies in our laboratory [*2*] have indicated that Nuclear Medical Laboratories digoxin antisera Lot DB-157 has very high cross-reactivity with digoxin-like immunoreactive substances found in the perinatal period. Our studies have also demonstrated that, when this antibody is used, DLIS in the specimen is additive to any digoxin that may be present [*2*]. Therefore, the DB-157 antibody would be expected to give a maximum value for digoxin plus DLIS when both are present in the same specimen. Analyses were performed in duplicate according to the manufacturer's instructions. Separation of bound from free antigen was achieved by charcoal precipitation.

RIA Method 2

This method involved a sequential modification of the standard NML RIA procedure using the digoxin specific antisera Lot DB-157 [*3*]. In this modification, antisera and unlabelled antigen are incubated for 18 h before the addition of labelled ^{125}I-digoxin. After the addition of ^{125}I-digoxin, the tubes are incubated for an additional 30 min, after which time the charcoal absorbent tablet is added. Studies using this procedure have indicated that this modification reduces the cross-reactivity with DLIS relative to RIA Method 1 by 50% [*3*]. The cross-reactivity to digoxin itself is unaffected by this modification. When the same specimen is analyzed for digoxin by Methods 1 and 2 and simultaneous equations are solved, the actual level of digoxin can be determined even though significant levels of DLIS may be present in the same specimen.

RIA Method 3

The digoxin RIA assay of Diagnostic Products Corp. (Los Angeles, CA 90045) was used for this method. This method, which uses a coated tube technology, reportedly has low cross-reactivity to DLIS [*4*]. The manufacturer's protocol was followed throughout.

RIA Method 4

This method for the determination of digoxin utilized digoxin specific anti-sera Lot 04-233 which was obtained from Nuclear Medical Laboratories. This antibody was found to have 50% the cross-reactivity with DLIS as compared to anti-sera DB-157 which was used in RIA Methods 1 and 2. The standard RIA protocol as suggested by the manufacturer was followed.

Extraction of Digoxin from Heart Tissue

Heart tissue (347 mg) was cubed and refrozen in liquid nitrogen. Subsequently, the frozen cubed tissue was pulverized in a Braun Mikrodismembrator (Melsungen, West Germany). The pulverized tissue was suspended in potassium phosphate buffer (0.1*M*, pH 7.2) and mixed to homogeneity. An aliquot of this homogenate was kept for noncollagenous protein determination [*5*]. The remaining homogenate was made up to 70% ethanol, centrifuged, the supernatant removed, and the pellet reextracted with 70% ethanol. Subsequently, the ethanol extracts were pooled, dried down under nitrogen, and reconstituted in 2.0 mL of a serum pool containing no digoxin immunoreactivity. The digoxin content was determined by RIA Methods 1 and 2 on a diluted aliquot and the tissue content of digoxin expressed relative to weight and noncollagenous protein.

Sep-Pak® Extraction of Digoxin from Serum

Serum (0.1 mL) was applied to the top of a disposable Sep-Pak C-18 cartridge (Water's Scientific). The cartridge was washed once with distilled water and digoxin eluted with 100% methanol (2.0 mL). The eluate was dried under nitrogen, resuspended in a known volume of methanol, and an aliquot injected into our high performance liquid chromatograph (HPLC). A similar procedure was followed for a pool of adult serum containing digoxin (2.3 ng/mL).

HPLC Fractionation of Sep-Pak Eluates

Reconstituted methanol eluates from the Sep-Paks were injected into a Varian Model 5000 HPLC equipped with a Whatman ODS-3 25-cm column and subsequently eluted by means of a linear acetonitrile-water (10 to 100%) gradient at a flow rate of 1.5 mL/min. Fractions (30 s) were collected by means of an LKB fraction collector, dried under nitrogen, and redissolved in a zero serum pool overnight on a tube rotator. All fractions were analyzed by RIA using the standard NML procedure (RIA Method 4).

The retention times of digoxin and its metabolites were determined by applying purified standards (digoxin, digoxigenin, digoxigenin monodigitoxoside, digoxigen bisdiditoxiside) to the HPLC and monitoring elution by ultraviolet (UV) absorbance at 220 nm. Following the chromatography of these standards, the column was washed and a blank linear gradient elution run was carried out and fractions analyzed by RIA for digoxin to confirm that no digoxin carryover had occurred.

Results

The results of the serum digoxin determinations are given in Table 1. All four methods gave virtually identical results. If significant levels of DLIS were present in the sample then the sequential assay results would have been decreased relative to the standard assay results [3].

Because the cross-reactivity of DLIS with digoxin antisera is both a function of antisera as well as assay conditions, specimens containing DLIS give different digoxin results when different digoxin methods are used [2]. Such variation is not seen with digoxin itself. Methods 1, 3, and 4 all used different antisera to measure digoxin by standard procedure protocol, and all three methods gave the same result for digoxin given the coefficient of variation of the methods involved. This finding is consistent with the conclusion that the specimen does not contain significant levels of DLIS [3].

When DLIS extracts from mixed cord blood are fractionated with the HPLC analytical procedure outlined in this paper, many different fractions are found to contain digoxin-like immunoreactive substances (see Fig. 1). Of particular note are the large number of fractions containing digoxin-like immunoreactive material eluting between 15 and 19 min. The parent drug digoxin and its metabolites elute within this same time interval (see Fig. 2).

The HPLC elution profile of the digoxin immunoreactivity extracted from the patient's serum specimen is shown in Fig. 3. Following the extraction and HPLC fractionation procedure outlined previously, the recovery of digoxin from serum was 99.5% by RIA and 99.6% by ^{3}H-digoxin. Fractionation of the serum extract demonstrated two peaks of digoxin immunoreactivity. The major peak eluted between 18.0 and 18.5 min and accounted for 91.5% of the total immunoreactivity. The second peak eluted between 15.5 and 16 min and accounted for 8.5% of the total immunoreactivity. The elution time of the major peak (18.0 to 18.5 min) coincides exactly with that for ^{3}H-digoxin and pure digoxin standard as monitored by UV absorbance (220 nm). Digoxin levels within this fraction were determined by RIA Methods 1 and 2. Both of these methods gave the same value for digoxin indicating that the fraction did not contain significant levels of DLIS [3]. The smaller peak eluting from 15.5 to 16.0 min coincided with the retention time of the major metabolite of digoxin, digoxigenin. No other digoxin-like immunoreactivity was found in any of the remaining fractions.

TABLE 1—*Serum digoxin levels as determined by four different RIA methods.*

RIA Method[a]	1:35 Dilution of Serum Sample, μg/L	Final Result, μg/L
1. NML: Antisera Lot DB-157	2.06	72.1
Standard method	2.00	70.0
2. NML: Antisera Lot DB-157	2.16	75.6
Sequential method	2.19	76.7
3. Diagnostic Products Corp.	2.10	73.5
Lot TD 12048	1.98	67.6
4. NML: Antisera Lot 04-233	2.01	70.3
Standard method	1.91	66.5
	Average overall:	71.5

[a]See **Methods** section for details.

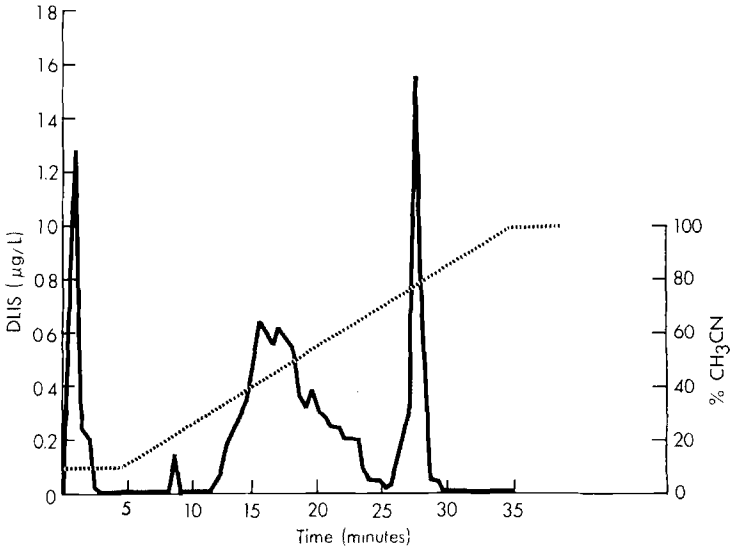

FIG. 1—*Elution profile of digoxin-like immunoreactive substances (DLIS) from mixed cord blood fractionated on an RP-18 analytical HPLC column. Acetonitrile linear gradient elution is indicated by the dashed line.*

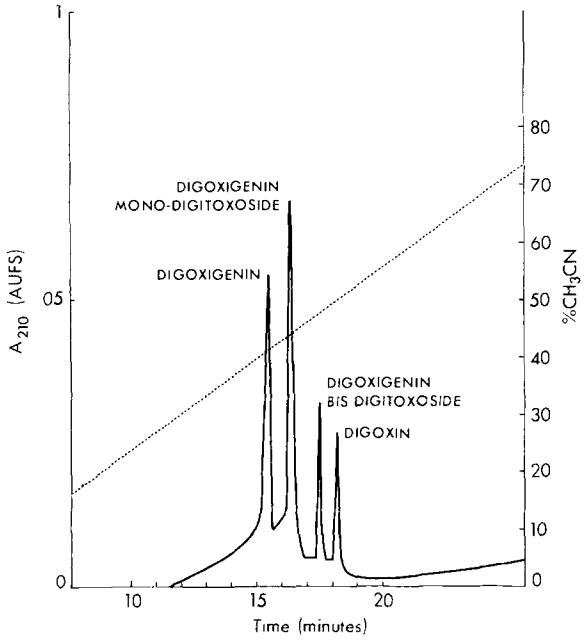

FIG. 2—*Elution profile of digoxin and its metabolites fractionated on an RP-18 analytical HPLC column. Acetonitrile linear gradient elution is indicated by the dashed line.*

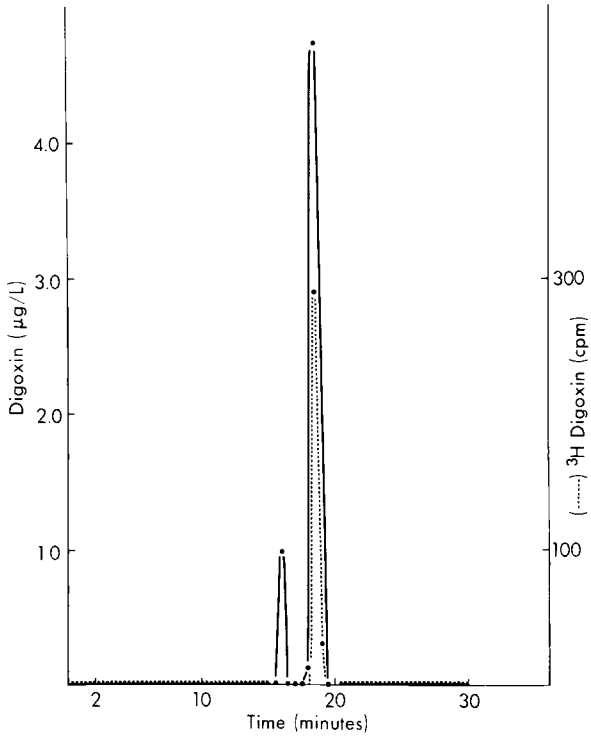

FIG. 3—*Elution profile of digoxin and metabolites as extracted from serum and fractionated on an RP-18 analytical HPLC column. Acetonitrile linear gradient elution was used.*

Digoxin-like immunoreactivity extracted from heart tissue was fractionated by HPLC. In tissue taken from the perinatal period, the heart is found to contain DLIS, albeit at low levels. Much higher levels are found in adrenal, liver, and bile [*1*]. Heart extracts from this patient contained a single peak of digoxin immunoreactivity which eluted between 18.0 and 18.5 min. This elution interval coincides with that for digoxin. Digoxin analysis of this fraction by RIA Methods 1 and 2 gave similar answers indicating that this fraction did not contain significant levels of digoxin-like immunoreactive substances [*3*]. Based on this method, the tissue levels of digoxin were 386-ng/g wet weight and 4401-ng/g noncollagenous protein. This tissue value for digoxin can be compared to that obtained for heart tissue removed at autopsy from a full-term infant which had been treated for two days with digoxin in which the digoxin content was 121-ng/g wet weight. The serum level of digoxin in this same infant was 4.0 ng/mL.

Discussion

Recent studies into the nature and significance of digoxin-like immunoreactive substances in the perinatal period have indicated that RIA methods for determining serum and tissue digoxin levels are unreliable within this age group [*6, 7*]. In some cases, the magnitude of this interference is sufficient to suggest digoxin toxicity where, indeed, none exists [*7*]. Studies have demonstrated that the parent drug digoxin is additive to endogenous serum levels of DLIS when standard RIA procedures are used for monitoring blood levels of digoxin [*2*]. Recent studies by Koren et al. [*8*] into excessive serum concentrations of digoxin in children

concluded that digoxin-like immunoreactive substances need to be considered in toxicological studies in this age group. Unfortunately, much of the early toxicological work involving digoxin used RIA and did not take into consideration digoxin-like immunoreactive substances [*9–14*].

Hastreiter et al. [*12*] in their workup of a pediatric case of accidental digoxin overdose reported finding very high levels of digoxin in the adrenal cortex, small intestine, and bile. They suggested that the high levels in the small intestine may have been due to oral dosing with digoxin or to the drug entering the intestinal tract directly from the intestinal wall or both, or from bile. They did not report on the relative degree of cross-reactivity of their RIA digoxin anti-sera with digoxin-like immunoreactive substances. We [*1*] and others [*15,16*] have reported finding significant levels of DLIS in the adrenal, liver, bile, and small intestine. The levels in some of these tissues are of sufficient magnitude to compromise significantly digoxin determinations by RIA resulting in erroneously high values. Our studies would indicate that tissue levels of DLIS are appreciably lower in myocardium and skeletal muscle in the perinatal age group. Hastreiter et al. [*12*] have suggested that, in the absence of blood, the tissues of choice for toxicological analysis of digoxin should include myocardium, liver, and skeletal muscle. Unless methods are used that eliminate interferences from DLIS, then we would not include liver in this recommendation.

Serum levels of DLIS in the perinatal period can, in some cases, be as high as 10 μg/L in digoxin equivalents.[4] The more premature the infant the higher levels of DLIS tend to be [*6*]. Because DLIS is excreted by the kidneys, the high serum levels in premature infants may be due, in part, to the low glomerular filtration rate that is characteristically found within this age group [*17*].

It is of interest that tissue digoxin levels are reported to be higher in premature and full-term newborns than in mature infants [*11*]. Our studies indicate that mature infants with normal renal function have serum levels of DLIS well below the detection limit of standard digoxin RIA methods [*7*]. This is in contrast to the premature and full-term infant [*6*]. It remains to be determined whether or not premature and full-term infants tend to concentrate digoxin in their tissues to a greater extent than mature infants. These early studies will have to be confirmed using methods that are free of interference from digoxin-like immunoreactive substances.

In the present case, the serum contained 71.5 $\pm$ 3.6 μg/L digoxin immunoreactivity. This material was extracted with greater than 99% recovery and fractionated on reverse phase HPLC. HPLC analysis coupled with sequential RIA analysis indicated that 91.5% of this immunoreactivity was due to digoxin, whereas 8.5% was due to the major digoxin metabolite, digoxigenin. There was no evidence, following HPLC separation and sequential RIA analysis, to indicate the presence of significant levels of digoxin-like immunoreactive substances in the serum specimen.

The left ventricle contained 386 ng of digoxin immunoreactivity per gram wet weight or 4401-ng/g noncollagenous protein. HPLC separation coupled with normal versus sequential RIA digoxin determinations demonstrated that DLIS was not present in significant amounts in this tissue extract.

Tissue levels of digoxin, as determined by RIA, are dependent upon many different factors including dose, age, time of dosing, elapsed time from death to sample acquisition, the specimen collected, digoxin extraction method, and RIA/antisera method used. The problem of obtaining an accurate result is now further complicated by the presence of digoxin-like immunoreactive substances which may give rise to falsely elevated digoxin values.

The relative importance of DLIS interference in cases of suspected digoxin toxicity is unknown. Further studies are required to identify the factors and conditions leading to elevation of DLIS levels in both pre- and post-mortem tissues. It is recommended that appropriate precautions be taken to eliminate DLIS interference in toxicological analyses relating to suspected digoxin toxicity until such studies are completed.

Acknowledgments

We thank Nuclear Medical Laboratories for their kind gift of antiserum Lot DB-157.

This work was funded by grants from the British Columbia Health Care Research Foundation and the British Columbia Heart Foundation.

References

[1] Seccombe, D. W., Pudek, M. R., Humphries, K. H., Matheson, B., Taylor, G., Jacobson, B. E., and Whitfield, M. P., "Further Studies Into the Nature and Organ Source of Digoxin-Like Immunoreactive Substances in the Perinatal Period" (submitted for publication to *Clinical Chemistry*).

[2] Pudek, M. R., Seccombe, D. W., Jacobson, B. E., and Whitfield, M. F., "Seven Different Digoxin Immunoassay Kits Compared With Respect to Interference by a Digoxin-Like Immunoreactive Substance in Serum From Premature and Full-Term Infants," *Clinical Chemistry*, Vol. 29, No. 11, Nov. 1983, pp. 1972-1974.

[3] Pudek, M. R., Seccombe, D. W., Jacobsen, B. E., and Humphries, K. H., "Effect of Assay Conditions on Cross-Reactivity of Digoxin-Like Immunoreactive Substance(s) with Radioimmunoassay Kits," *Clinical Chemistry*, Vol. 31, No. 11, Nov. 1985, pp. 1806-1810.

[4] Gortner, L. and Hellenbrecht, D., "An RIA Kit That is More Specific for Digoxin in Serum of Newborns," *Clinical Chemistry*, Vol. 31, No. 1, Jan. 1985, pp. 155-156.

[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., "Protein Measurement With the Folin Phenol Reagent," *Journal of Biological Chemistry*, Vol. 193, No. 1, 1951, pp. 265-275.

[6] Seccombe, D. W., Pudek, M. R., Whitfield, M. F., Jacobson, B. E., Wittmann, B. K., and King, J. F., "Perinatal Changes in a Digoxin-Like Immunoreactive Substance," *Pediatric Research*, Vol. 18, No. 11, 1984, pp. 1097-1099.

[7] Pudek, M. R., Seccombe, D. W., Whitfield, M. F., and Ling, E., "Digoxin-Like Immunoreactivity in Premature and Full-Term Infants Not Receiving Digoxin Therapy," *New England Journal of Medicine*, Vol. 308, No. 15, 14 April 1983, pp. 904-905.

[8] Koren, G. and Parker, R., "Interpretation of Excessive Serum Concentrations of Digoxin in Children," *American Journal of Cardiology*, Vol. 55, April 1985, pp. 1210-1214.

[9] Kim, P. W., Krasula, R. W., Soyka, L. F., and Hastreiter, A. R., "Postmortem Tissue Concentrations in Infants and Children," *Circulation*, Vol. 52, No. 4-6, Dec. 1975, pp. 1128-1131.

[10] Hastreiter, A. R. and Van Der Horst, R. L., "Postmortem Tissue Concentrations and Organ Content in Infancy and Childhood," *American Journal of Cardiology*, Vol. 52, Aug. 1983, pp. 330-335.

[11] Lang, D., Hofstetter, R., and Van Bermuth, G., "Postmortem Tissue and Plasma Concentrations of Digoxin in Newborns and Infants," *European Journal of Pediatrics*, Vol. 128, 1978, pp. 151-161.

[12] Hastreiter, A. R., Kim, P. W., and Van Der Horst, R., "Accidental Digoxin Overdose in an Infant: Postmortem Tissue Concentrations," *Journal of Forensic Sciences*, Vol. 28, No. 2, April 1983, pp. 482-488.

[13] Steentoft, A., "Fatal Digitalis Poisoning," *Acta Pharmacologica et Toxicologica Supplementum*, Vol. 32, No. 5, April 1973, pp. 353-357.

[14] Andersson, K. E., Bertler, A., and Wettrell, G., "Postmortem Distribution and Tissue Concentrations of Digoxin in Infants and Adults," *Acta Paediatrica Scandinavica*, Vol. 64, No. 3, May 1975, pp. 497-504.

[15] Spiehler, V. R., Fischer, W. R., and Richards, R. G., "Digoxin-Like Immunoreactive Substances in Postmortem Blood of Infants and Children," *Journal of Forensic Sciences*, Vol. 30, No. 1, Jan. 1985, pp. 86-91.

[16] Vasdev, S., Longerich, L., Janes, M., Ittel, T., and Gault, M. H., "Serum Digitalis-Like Factors in Combined Hepatic and Renal Failure and in Bile," *Clinical and Investigative Medicine*, Vol. 7, Suppl. 2, 1984, p. 52.

[17] Siegel, S. R. and Oh, W., "Renal Function as a Marker of Human Fetal Maturation," *Acta Paediatrica Scandinavica*, Vol. 65, No. 4, 1976, pp. 481-485.

Address requests for reprints or additional information to
David W. Seccombe, M.D.
Department of Pathology
Shaughnessy Hospital
L322-4500 Oak St.
Vancouver, B.C., V6H 3N1 Canada